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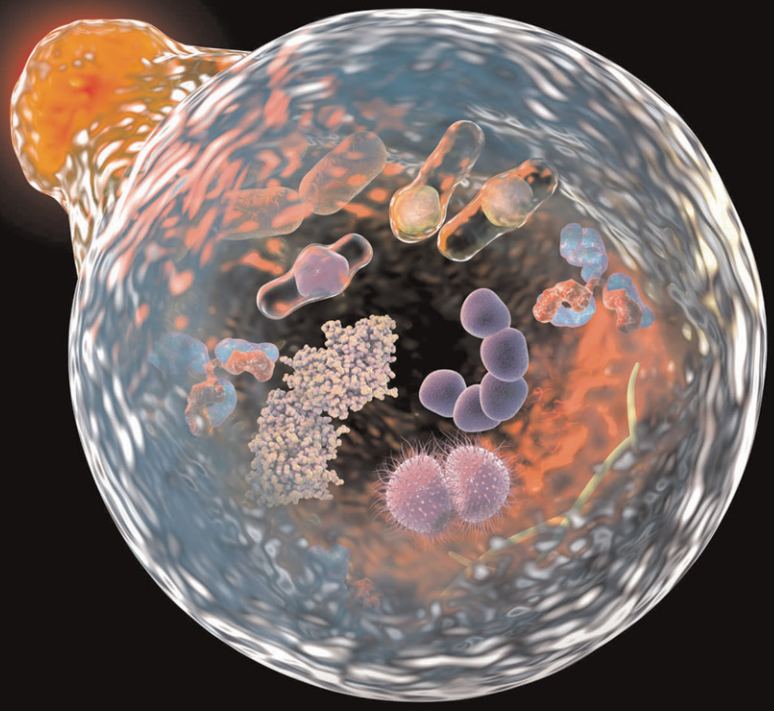
Ilpo Huhtaniemi and Luciano Martini



ENCYCLOPEDIA OF ENDOCRINE DISEASES

SECOND EDITION

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EDITORS IN CHIEF

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VOLUME 1

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DEDICATION

Professor Luciano Martini, 1927–2017

The other Editor in Chief of the Encyclopedia, Professor Luciano Martini, passed away on July 13th, 2017. He was an internationally acclaimed authority in the field of endocrinology, in particular neuroendocrinology, a brilliant and imaginative scientist, and an impressive and erudite scholar.

Luciano achieved the venerable age of 90, and his long career was full of outstanding scientific achievements, leadership positions in academia and in scientific societies, academies, and committees.

Luciano received his MD degree from the University of Milan in 1950. He then rapidly progressed through junior academic ranks up to the position of Professor and Chairman of the Department of Pharmacology at the University of Perugia in 1968, and subsequently, in 1972, he returned to his *alma mater*, the University of Milan, as full Professor and Chairman of the Department of Endocrinology, a post he held until 2001. He served in Milan as director of the training and research program entitled Physiology of Reproduction for nearly 20 years and attracted to his team top-class Italian and foreign scientists to address his main research interests of neuroendocrine regulation of reproductive functions.

Scientific severity, ethical integrity, fine perception, and deep farsightedness describe best Luciano's character as a scientist. He created in his institute a scientific research group devoted to experimental endocrinology, which grew over the years in size and visibility and became widely recognized internationally. Luciano published more than 400 peer-reviewed and highly cited papers mainly in the fields of neuroendocrinology, endocrine oncology, physiology of reproduction, and steroid and energy metabolisms.

Luciano was a prolific editor of scientific books and journals, which include the two volumes of *Neuroendocrinology* and the nine biennial volumes of *Frontiers in Neuroendocrinology*. He was Editor in Chief of *Comprehensive Endocrinology* published in 12 volumes and the first Edition of *Encyclopedia of Endocrine Diseases*. He served as President in many national and international scientific societies including the International Society of Neuroendocrinology, the Italian Society of Endocrinology, the International Society of Endocrinology, and the European Federation of Endocrine Societies. For his scientific achievements Luciano received honorary doctorates in the universities of Liège, Santiago de Compostela, Pécs, and Milan, and he was the recipient of numerous scientific awards and invited academy memberships.

Luciano's portrait could not be complete if one forgets to mention his life-time passion for music. He was a well-trained and accomplished pianist, a passionate music listener, and an enthusiastic connoisseur of all types of music. He also was an amateur in visual arts and deeply interested in history.

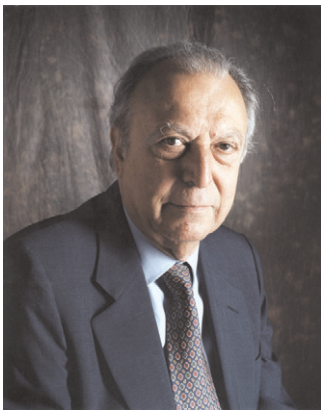
All of us who knew Professor Luciano Martini deeply mourn the loss of a great scientist and friend, the real "Il Maestro", teacher, colleague, and pioneer of modern neuroendocrinology. I trust Luciano would have been proud of this new edition of the Encyclopedia of Endocrine Diseases, and all of us having worked on its production would like to dedicate it to his memory.

Ilpo Huhtaniemi

Editor in Chief
Encyclopedia of Endocrine Diseases, 2nd edition

EDITORS IN CHIEF

Ilpo Huhtaniemi received his MD and PhD at University of Helsinki, Finland, did postdoctoral training in United States (UC San Francisco and NIH, Bethesda), and has been on sabbatical leave in Germany, United States and Scotland. In 1986–2002 he held the post of Professor and Chairman of Physiology at University of Turku, Finland. He moved in 2002 to UK to a Chair in Reproductive Endocrinology at Imperial College London, from which position he retired in 2015. He has received several national and international honors, amongst them a fellowship of The Academy of Medical Sciences, United Kingdom, and a Doctor Honoris Causa at the Medical University Łódź, Poland, and University of Szeged, Hungary. He was the Chief Managing Editor of *Molecular and Cellular Endocrinology* 1999-2017, has served in the Editorial Board of *Endocrinology and Endocrine Reviews* and is/has been the Editor or Editorial Board Member of several other scientific journals (e.g., *European Journal of Endocrinology*, *Clinical Endocrinology*, *Human Reproduction Update*, *Journal of Endocrinology*, *Molecular Human Reproduction*, *Reproduction*, *Asian Journal of Andrology*). He has extensive experience as Official of international scientific organizations (e.g., Past President of International Society of Andrology). His research interests include clinical and basic reproductive endocrinology, in particular the function of gonadotrophins and male reproductive endocrinology. He also has long-term interests in development of male contraception, hormone-dependent cancer, and the endocrinology of aging. He has authored about 700 peer-reviewed research articles and reviews, and his H-factor is 78.



Luciano Martini was born on May 14, 1927, in Milan, Italy. He obtained the degree of Medical Doctor "summa cum laude" on November 24, 1950, from the Faculty of Medicine of the University of Milan, Italy. He was Emeritus Professor of Pharmacology of the University of Perugia, Italy, and Emeritus Professor of Endocrinology of the University of Milan, Italy. He was Doctor Honoris Causa in Medicine of the Universities of Liège, Belgium, Santiago de Compostela, Spain, and Pécs, Hungary, and Doctor Honoris Causa in Biotechnological Sciences of the University of Milan, Italy. He was an author of more than 400 peer-reviewed scientific publications in the fields of endocrinology, neuroendocrinology, pharmacology, physiology of reproduction, steroid biochemistry, and basic oncology. He was elected member of the Accademia Nazionale dei Lincei (Italian National Academy) and of the American Academy of Arts and Sciences (Honorary Foreign Member).

Luciano Martini acted as Editor in Chief of the journal *Frontiers in Neuroendocrinology* from 1990 to 2001, and was a Member of the Editorial Board of *Endocrinology* (Foreign Consulting Editor, 1961–65), as well as of several other speciality journals, such as *Experimental and Clinical Endocrinology*, *Biochemistry*, and *Steroids*. He has acted as Editor of several textbooks (e.g., *Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1966–67, and *Clinical Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1977–82) as well of a series of books under the name *Comprehensive Endocrinology* (13 volumes), Raven Press, New York, 1979–84. He acted as Editor in Chief for the first edition of *Encyclopedia of Endocrine Diseases* (4 volumes), Academic Press-Elsevier, San Diego, 2004.

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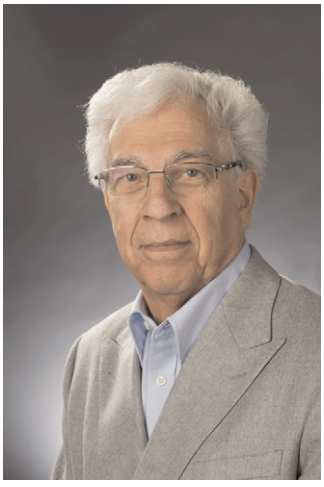
Professor **Jean-Jacques Body** has been trained as an endocrinologist and a medical oncologist. He was Head of the Department of Medicine at University Hospital Brugmann in Brussels and Full Professor of Medicine (Internal Medicine) at the Free University of Brussels, (ULB), Brussels, Belgium. He was previously Head of the Internal Medicine Clinic at Institute J. Bordet (Cancer Center of ULB). He has also developed the "Supportive Care Dept" at the same Institute. His particular research interests are osteoporosis and bone metastases. He has a long-standing interest for bone metabolism and turnover in osteoporosis and tumor bone diseases. He has authored or co-authored more than 250 international peer-reviewed papers and he counts more than 200 invited lectures for international meetings.

Felipe F. Casanueva is Professor of Medicine at University of Santiago de Compostela and Head of Department of Endocrinology and Nutrition at University Hospital Santiago. He has been President of the scientific societes, such as: European Federation of Endocrine Societies (EFES), The Pituitary Society, International Society of Endocrinology (ISE) and, Sociedad Española para el Estudio de la Obesidad (SEEDO). Has written more than 50 chapters in international books and published more than 700 papers in international journals. He has received several awards for research at national and international level, such as: Rey Jaime I to the Medical Research, Geoffrey Harris Prize in Neuroendocrinology, Fundación Lilly of Bio-medical Research Clinic, Fundacion Danone – Professional Career – Dr Carlos Martí Hennberg, European Hormone Medal by the European Society for Endocrinology (ESE); he has been named Honorary Doctorate in Medicine of the University of Łódź, Erciyes, and Belgrade, and Honorary Member of the European Society of Endocrinology.



Dr. **Jean-Louis Chiasson** is currently Full Professor of Medicine at the University of Montreal. He is Head of the Research Group on Diabetes and Metabolic Regulation at the Research Center of the Centre hospitalier de l'Université de Montréal (CRCHUM).

Dr. Chiasson obtained his MD at Laval University in Quebec City in 1967. He did his specialty training in Internal Medicine at Laval Univesity and in Endocrinology at McGill University. He then did a research Fellowship in Diabetes at Vanderbilt University in Nashville, Tennessee. In 1974–76 and 1978–80, he was appointed Assistant Professor in the Department of Medicine and Physiology respectively at Vanderbilt University. In 1980, he returned to Montreal as Assistant Professor in the Department of Medicine at the University of Montreal and as Endocrinologist at Hotel-Dieu Hospital, now merged into the Centre hospitalier de l'Université de Montréal.

Dr. Chiasson's research interests include the regulation of carbohydrate metabolism in health and diabetes, as well as the development and evaluation of new strategies for the treatment and prevention of diabetes and its vascular complications. He has contributed over 250 scientific publications and lectures nationally and internationally on various topics on diabetes mellitus, its pathogenesis, its treatment, and its prevention. His scientific contribution puts him in the prestigious club of the 100 most cited publications in the world in the field of diabetes.

Sophie Christin-Maitre received her MD at University of Paris XI and her PhD at University Paris VI, Pierre and Marie Curie, France. She did a postdoctoral training in United States (Massachusetts General Hospital, Harvard University, Boston); she specialized in reproductive medicine. She holds the post of Professor of Endocrinology at University of Sorbonne, Paris, France. She has been the head of the Adult Endocrine Unit, in Hôpital Saint-Antoine, Assistance-Publique Hôpitaux de Paris, since 2011. She is a member of the INSERM research unit UMR S_933, specialized in identifying new genes in reproductive disorders. Her interests include clinical and basic reproductive endocrinology, in particular the management of patients with Turner syndrome, patients with primary ovarian insufficiency, patients with hypogonadisms, and patients with abnormalities of sex development. She has authored approximately 150 peer-reviewed research articles and reviews.

Ulla Feldt-Rasmussen is Professor at Copenhagen University and Chief of Medical Endocrinology, National University Hospital. Her research interests involve the thyroid gland and autoimmunity, as well as pituitary and adrenal dysfunction.

She has published more than 410 papers in peer-reviewed journals on e.g., thyroid hormones and body composition, thyroid autoimmunity and cancer, cytokines as regulators of endocrine cells, influence of thyroid disrupting chemicals on thyroid cells, growth hormone deficiency related to body composition, bone metabolism and other pituitary axes, and transition from adolescent to adult care, as well as several aspects of Fabry disease. In recent years her group has embarked on studies on pituitary function after traumatic brain injury in a nationwide setting, and focusing on diagnostic accuracy of pituitary testing procedures. She has further authored numerous proceedings, textbook chapters, and other publications; as well as organized numerous international meetings and postgraduate courses, and has led several European projects and other collaborations within many areas of endocrinology.

Professor Feldt-Rasmussen reviews for international journals, and is an editorial board member of several endocrine journals. She belongs to many international professional organizations, including the Endocrine Society, ETA, ATA, ENEA, and GRS; she has served as Secretary-Treasurer of ETA and as President of the ETA Cancer Research Network.

Professor Feldt-Rasmussen serves on the advisory boards of several ad hoc endocrine committees, and has received many prestigious prizes including the Mayo Clinic's Haynes Lecturer's Award and ETA's Pinchera Research Prize.

Wouter W. de Herder M.D. Ph.D. (1960) is Professor of Endocrine Oncology at the Erasmus MC in Rotterdam, the Netherlands. In this University Hospital he is chairman of a multidisciplinary group for endocrine oncology (tumorwerkgroep endocriene tumoren) and he is head of the ENETS centre of excellence for neuroendocrine tumors. His major research interests are neuroendocrine and endocrine tumors.

Professor de Herder received his M.D. in 1985 and his Ph.D. in 1990 from the Erasmus University in Rotterdam, the Netherlands.

He is a member of several international and Dutch national societies, such as the Dutch Society for Endocrinology (NVE), the Endocrine Society (USA), the European Society of Endocrinology (ESE), European Neuroendocrine Tumor Society (ENETS) and the North American Neuroendocrine Tumor Society (NANETS). He served as a board member of the Dutch Society for Endocrinology (NVE) (2009–14). He served as chairman (2006–08) and vice-chairman of ENETS (2008–10) (European Neuroendocrine Tumour Society). He is member of the advisory boards of ENETS and NANETS.

Professor de Herder has (co-)published over 400 peer-reviewed papers and book chapters and is a reviewer for many international journals.

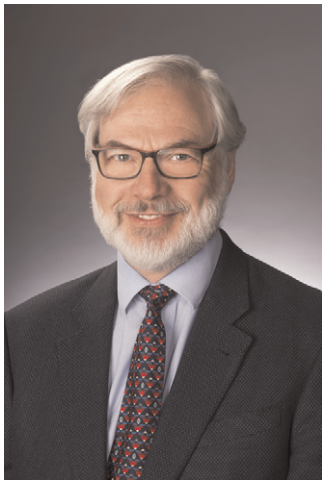
He is a member of the editorial boards of *Neuroendocrinology; Endocrinology, Diabetes & Metabolism Case Reports; Clinical Endocrinology*, and *Endocrine-Related Cancer*.

Professor de Herder has given over 200 invited presentations at Dutch national and international meetings.

Ieuan Hughes is currently Emeritus Professor of Pediatrics at the University of Cambridge and Honorary Consultant Pediatrician at Cambridge University Hospitals NHS Foundation Trust and Cambridge Biomedical Campus. He is the author of more than 300 papers and chapters across the whole range of paediatric endocrinology. His particular expertise is in disorders of sex development for which he coordinated the International Consensus on the approach to the investigation and management of this broad topic. Research interests focus on steroid enzyme deficiencies and molecular mechanisms of androgen action.
Professor Hughes has served on the editorial boards of several journals, including *Clinical Endocrinology*, *Journal of Clinical Endocrinology*, and *Metabolism* and *Archives of Disease in Childhood* where he was also the Associate Editor. He is Past-Secretary and President of the European Society for Pediatric Endocrinology and a recipient of the highest award of the Society, the Andrea Prader Prize. Professor Hughes is a James Spence Medallist of the Royal College of Pediatrics and Child Health for outstanding contributions to paediatric knowledge. He is a Fellow of the Academy of Medical Sciences, a Council Member of the Learned Society of Wales and a Trustee of two charities. The chapter on Disorders of Sex Development in *Williams Textbook of Endocrinology* (now in its 14e) by Hughes and co-authors is considered to be a definitive and up to date regular review of this topic, specific and key to pediatric endocrinology.

Dr. **Gregory Kaltsas** MD FRCP (Lon) is Professor in General Medicine and Endocrinology at the National and Kapodistrian University of Athens, Greece. He was trained in General Medicine in Athens, Greece and London, UK, and in Endocrinology at the Middlesex and St Bartholomew's Hospital, London, UK. He developed a particular interest in neuroendocrinology (pituitary and neuroendocrine tumors) and adrenal physiology and diseases. Upon returning to Greece he established a neuroendocrine network and he is currently running the European Neuroendocrine Tumor Society (ENETS) Center of Excellence at Laiko Hospital in Athens, Greece. He has served as a member of the advisory board of ENETS and of the Executive Committee of the European Neuroendocrine Association (ENEA) and he has been elected in the Executive Committee of the International Society of Endocrinology. He has recently been elected as a representative of the European Society of Endocrinology in the ExCo of the International Society of Endocrinology. He has published more than 300 original papers, reviews, and chapters and serves on editorial boards and as associate editor in several endocrine journals.

Jean-Marc Kaufman obtained his MD and PhD degrees at the Ghent University, Belgium. He was a Senior Postdoctoral Research Fellow (1982–84) in reproductive physiology at the University of Texas Medical School at Houston. He is board certified in Endocrinology and in Nuclear Medicine. In 1985 he joined the staff of the Ghent University Hospital; he headed the department of Endocrinology from 2003 to 2014 and the Laboratory for Hormonology from 1995 to 2014. He was appointed in 1993 Professor of Medicine at the Ghent University (1993) and is past Chair of the Department of Internal Medicine at the Ghent University (2010–14). From October 1st 2014 he is Professor Emeritus at the Ghent University where he is pursuing clinical and research activities. Main research interests are in the assessment, regulation, and action of sex steroids with focus on their role in health, disease, and aging in men, and in osteoporosis in men. He is (co)author of over 300 publications in international peer-reviewed journals.



André Lacroix, MD FCAHS is Professor of Medicine, Division of Endocrinology at Centre hospitalier de l'Université de Montréal (CHUM). His areas of interest include the mechanisms of adrenal Cushing syndrome, primary aldosteronism, adrenal tumorigenesis, the role of aberrant adrenal hormone receptors in adrenal overfunction, as well as new drugs in the therapy of Cushing disease, primary aldosteronism and adrenocortical cancer.
He was trained at the University of Montreal followed by fellowships in Endocrinology and research at Vanderebilt University and National Institutes of Health, USA. He was Chairman of Medicine and Director of Academic Affairs at CHUM. Former President of the Canadian Society of Endocrinology and Metabolism, he is currently chairperson of the International Society of Endocrinology (2016–20), Editor, Adrenal Section of UpToDate and *Encyclopedia of Endocrinology*, Senior Editor of the *European Journal of Endocrinology*. Fellow of the Canadian Academy of Health Sciences since 2008 and Foreign member of the National Academy of Medicine of France since 2016.

Franco Mantero received his MD at the University of Padua, Italy, did postdoctoral training in Switzerland (Clinique Medicale Therapeutique, Hopital Cantonal, University of Geneva) and in United States (University of California, San Francisco), and has been on sabbatical leave in United Kingdom, United States, and France. He held a post of Associate Professor in Medicine at the Institute of Semeiotica Medica, University of Padua (1981–86). In 1986 he moved to the University of Catania to the Chair of Andrology and Endocrinology, in 1992 to the University of Ancona, and in 2000 to the University of Padua to the Chair of Endocrinology and Chief of the Endocrinology Unit of the Department of Medicine. He has received national and international honors, including a Doctor Honoris Causa at the Semmelweis University, Budapest, Hungary.
He has been Editorial Board Member of several scientific journals (e.g., *Clinical Endocrinology*, *Endocrinology*, *Journal of Hypertension*, *Journal of Endocrinology Investigation Steroids*)
He has served as Member of the Council of several international scientific societies (including International Society of Endocrinology, International Aldosterone Conference, Journee Klotz d'Endocrinologie Clinique, ENS@T) and one of the founders of the European Network for the Study of Adrenal Tumors. His research interests include clinical and basic endocrinology of the adrenal gland and endocrinology of hypertension, in particular pathophysiology of mineralocorticoids and primary aldosteronism. He has authored approximately 500 peer–reviewed articles and edited several books and proceedings.

Jorma Toppari, MD, PhD, is Professor of Physiology at the University of Turku and Chief Physician of Pediatric Endocrinology at Turku University Hospital, Turku, Finland. He is also Adjunct Professor in the Department of Growth and Reproduction at the University of Copenhagen, Denmark. He has served as chief editor of International Journal of Andrology (2001–09), and has been on editorial boards of several endocrinological journals, including currently *Endocrinology* and *Journal of Clinical Endocrinology and Metabolism*. He is the past President of the European Academy of Andrology. He has made numerous contributions to the studies on endocrine disruption in the past 20 years. He has published approximately 400 articles on endocrinology.

Jacquetta Trasler is a James McGill Professor in the Departments of Pediatrics, Human Genetics, and Pharmacology and Therapeutics at McGill University and a Senior Scientist at the Research Institute of the McGill University Health Centre (RI-MUHC). She received her MD and PhD degrees from McGill University followed by postdoctoral training in reproductive molecular biology at Tufts University in Boston. She has served as Director of the McGill University MD-PhD Program, Scientific Director of the Montreal Children's Hospital Research Institute (and simultaneously as Deputy Director/CSO of the RI-MUHC), President of the Canadian Fertility and Andrology Society, Member of the Institute Advisory Board for the Canadian Institutes of Health Research (CIHR) Institute of Genetics and currently serves on the CIHR Stem Cell Oversight Committee and College of Reviewers. Her research focuses on epigenetics and epigenomics to better understand the molecular and cellular targets for drug effects on germ cells with implications for the resulting offspring. She has been involved in scientific program organization for numerous meetings in the field of reproductive biology and medicine and is collaborating with national and international colleagues in clinical studies to examine how assisted reproductive technologies, infertility, drug treatment, and folate deficiency and supplementation impact the human epigenome including that of future generations.

Christina Wang, MD is Professor of Medicine, Assistant Dean at the David Geffen School of Medicine at UCLA, and Associate Director for Clinical and Translational Science Institute and a faculty member of the Division of Endocrinology, Department of Medicine, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, California.

Dr. Wang has been involved in many funded basic and clinical research studies. Her current clinical research studies include androgen replacement therapy, hormonal male contraceptive development, late onset hypogonadism, accurate assessment of serum androgens, and diet and androgen metabolism. Her basic research studies focus on the regulation of spermatogenesis and mitochondrial derived peptides in spermatogenesis.

She has authored over 300 peer-reviewed publications, 67 chapters and reviews mainly on male reproductive biology including characterization of the pharmacokinetics and efficacy of androgens in men, trials of hormonal male contraceptive, regulation of germ cell apoptosis, and reproductive aging. Dr. Wang served on the Executive Council, several committees and was the President of the American Society of Andrology (2006–07). She also served the International Society of Andrology as Secretary (2001–05) and Chair of the Program Organizing Committee (2005–09). She was President of the International Society of Andrology (2009–13). She is a member of the Research Group on Methods for the Regulation of Male Fertility of the World Health Organization since 1984 and Chairperson (1991–2002).

She has mentored many physician and scientist and is an advocate of young investigators. Dr. Wang has been invited speaker and distinguished lecturer at many national and international endocrinology, reproductive endocrinology, and andrology conferences.

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PREFACE

The first Edition of the *Encyclopedia of Endocrine Diseases* was published in 2004. Because of the enormous development in the field it was found important to produce a completely revised and updated Second Edition of the Encyclopedia. The new Edition is a must-have one-stop reference covering every aspect of the physiological background, pathogenesis, clinical diagnostics, and therapeutic aspects of the wide array of endocrine and related metabolic diseases.

The functional balance of the body (homeostasis) is maintained by two regulatory circuits, i.e., the nervous and the endocrine systems. Among the most complex constructs in the body, the endocrine system comprises a group of glands that secrete hormones directly into the bloodstream, where they reach their specific receptors in other parts of the body, evoking specific intracellular signaling pathways leading to their biological effect. Many classically non-endocrine organs (e.g., the heart) have also turned out to have endocrine functions. The endocrine system maintains and regulates the body's homeostasis by using hormones to control metabolism, temperature, biological cycles, internal fluid volume, reproduction, growth, body composition, and development. The system is a marvel when functioning optimally, i.e., maintaining the body homeostasis. Unfortunately, there is a myriad of ways these processes, actions, and functions can go awry, resulting in various endocrine and metabolic diseases, which form the over-arching theme of the Encyclopedia.

The Encyclopedia is not meant as a primer on the subject of endocrinology, but instead intended to provide a comprehensive reference work on the extensive spectrum of diseases and disorders that can occur within the endocrine and metabolic system. The updated version of this groundbreaking encyclopedia is especially timely, as it covers the dramatic discoveries in the field of endocrinology and metabolism over the past 10 years, particularly with respect to novel diagnostic techniques and treatment approaches. In particular, there have been tremendous advancements in our understanding of the molecular basis of endocrine and metabolic diseases (mutations, epigenetics, signaling), as well as pathogenesis and therapy of the common forms of these diseases (e.g., diabetes, obesity, and endocrine malignancies).

The Encyclopedia offers a unique source of up-to-date information for the physicians and basic scientists working in the field. It is an essential resource for every clinician diagnosing and treating endocrine patients. The Encyclopedia also offers the prime source of information for students of medicine and science around the world, as well as basic research workers in academia, the pharma industry, and in other areas in need of information on endocrinology and metabolism. It also offers useful information for the lay public about normal and abnormal functions of hormones.

The Encyclopedia is intended to serve as a useful and comprehensive source of information spanning the many and varied aspects of the endocrine and metabolic system. The chapters have been written to be accessible to both clinical and nonclinical readers. The articles have been formatted in similar fashion and each is intended as a stand-alone presentation. Each article begins with a glossary list defining key terms that may be unfamiliar to the reader and are important for understanding the article. The body of the article begins with a brief introduction to the subject under discussion, bold headings lead the reader through the text, and figures and tables explain and illuminate most articles. The main text is followed by referenced citations to provide the reader with access to additional information on the topic, and cross-references lead the reader to related entries in the encyclopedia. The relatively short stand-alone articles have allowed us to recruit the best experts available for each topic.

Unlike the first Edition, where the articles were arranged in alphabetical order, the 2nd Edition is arranged in organ-based thematic order, where each organ-based group of diseases is presented as cluster of articles in the first four volumes. The fifth volume is a stand-alone compilation of all articles on pediatric endocrinology. The thematic organization gives the reader a better general view of the coverage of articles on a specific endocrine organ or disease type.

The Second Edition of the Encyclopedia builds of the first edition. Nevertheless, to bring a major reference work with such a broad scope from initial conception to final publication involved a great deal of planning and organization, together with the efforts of innumerable individuals. The authors of the first edition were invited to update their earlier texts. If this was not possible, the Section Editors invited another expert in the topic either to update the previous text or to write a de novo text; the latter happened in most of these cases. Hence, the Second Edition contains to a large extent totally new information, or at least the fluency of all texts has been scrutinized. Furthermore, all manuscripts have undergone peer-review arranged by the Section Editors.

Assembling a large volume of articles with the purpose to cover all essential topics of endocrine diseases posed multiple challenges. Coverage was a significant problem: on one hand some redundancy of the topics was almost impossible to avoid in places while, on the other, there were inevitable gaps. Some of these arose from late cancellations; others from oversights on our part. We can only promise to fill these gaps in future editions. We also note that as can be expected for a large multi-author compilation the individual articles do differ in detail and approach. We considered it more important to allow our experts substantial latitude in deciding how to present their topics than to apply rigid guidelines.

Most of the editing work of the Encyclopedia has been carried out by a highly competent board of 16 Section Editors, each of them internationally renowned experts in their respective field within clinical endocrinology. First, the broadest possible list of topics was compiled, aiming at the best possible coverage. Throughout the editorial process, the Section Editors supervised their subject area of expertise, recommended and corresponded with fellow editors and article contributors, reviewed the manuscripts, and continuously helped to refine the final list of topics. This has made the task of the Editor in Chief easy, mainly entailing the supervision of smooth progress of the project.

The Section Editors and their fields deserve being listed here: *Jean-Jacques Body* (Belgium, bone endocrinology), *Felipe F. Casanueva* (Spain, metabolism and obesity), *Richard N. Clayton* (United Kingdom, pituitary gland), *Jean-Louis Chiasson* (Canada, diabetes), *Sophie Christin-Maitre* (France, female reproduction), *Wouter W. de Herder* (The Netherlands, neuroendocrinology), *Ulla Feldt-Rasmussen* (Denmark, thyroid gland), *Ieuan Hughes* (United Kingdom, pediatric endocrinology), *Gregory Kaltsas*, Greece, and *Martin O. Weickert*, United Kingdom, (gastrointestinal hormones), *Jean-Marc Kaufman* (Belgium, endocrinology of aging), *André Lacroix* (Canada, adrenal cortex), *Franco Mantero* (Italy, adrenal medulla and endocrine hypertension), *Jorma Toppari* (Finland, endocrine disruptors), *Jacquetta Trasler* (Canada, endocrine epigenetics) and *Christina Wang* (United Kingdom, male reproduction).

The Elsevier editorial staff, *Will Smaldon*, *Laura Escalante Santos*, and *Kate Miklaszewska-Gorczyca*, have been of enormous help to the editors at every step during this long project. I admire the professionalism of everyone and am deeply indebted to all for their dedication and hard work to make the Encyclopedia the leading reference book of clinical endocrinology.

The authors of the individual chapters, more than 450 in total, were specifically selected by the Section Editors to represent the best available knowledge on the topic available. They all should be thanked for their dedication and the excellent quality of their contributions.

Ilpo T. Huhtaniemi
Editor in Chief

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History of Endocrinology

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The term "endocrinology" is a synthesis of the classical Greek words *endon* (within), *krinein/ekkrinein* (sift), and *logos* (speech, word, telling), denoting logy (a subject of study). Both "endocrine" and "endocrinology" were first used by the French physiologist Maurice-Adolphe Limon in 1904 (Limon, 1904).

In 1905, Ernest H. Starling (**Fig. 1**) first used the term "hormone." He defined hormones as chemical messengers, which are "carried from the organ where they are produced to the organ which they affect by means of the blood stream and the continually recurring physiological needs of the organism must determine their repeated production and circulation from the body" (Starling, 1905). Before that time, the term "internal secretion" had been introduced by Claude Bernard (**Fig. 2**) in 1855 (Bernard, 1879).

As early as 200 BC, the Chinese were already trying to isolate sex and pituitary hormones from the urine and using them for medicinal purposes. Around 655, they identified symptoms of diabetes mellitus, correctly associating it with sugar in the urine and around 1000, they used thyroid extracts to treat goiter (Temple, 2007). Ancient Greek and Roman scientists such as Aristotle, Hippocrates, Lucretius, Celsus, and Galen introduced the humoral approach to understanding biological function and disease. Early anatomists identified most relevant (endocrine) glands and tissues.

In the 19th century, two important endocrine diseases were first described: Robert Graves (**Fig. 3**) reported on a case of goiter with exophthalmos in 1835 (Graves, 1835). In 1840, Karl von Basedow (**Fig. 4**) independently reported on the same constellation of symptoms (Basedow, 1840). Thomas Addison (**Fig. 5**) described Addison disease in 1849 (Addison, 1849).

Already in 1792, John Hunter noted that castration of cockerels caused atrophy of their combs and wattles, but that this could be prevented if the testes were replaced in the abdominal cavity (Forbes, 1947). These experiments were repeated and confirmed in

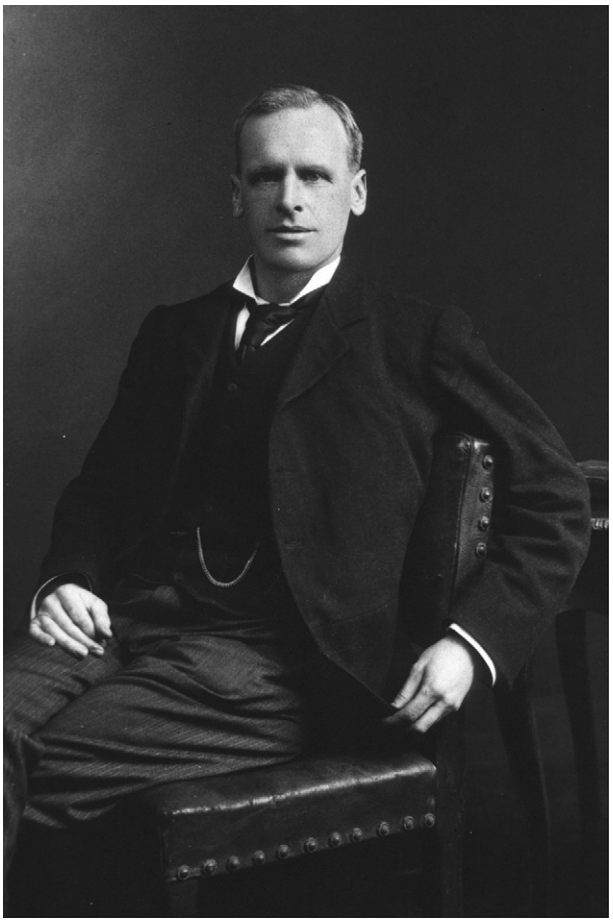

Fig. 1 Ernest H. Starling (1866–1927). From Wikimedia Commons, the free media repository.

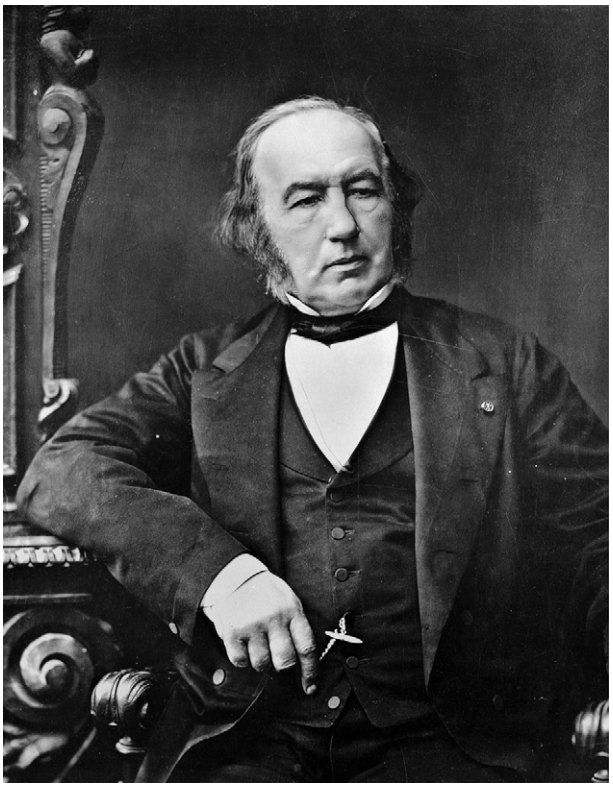

Fig. 2 Claude Bernard (1813–1878). From Wikimedia Commons, the free media repository.

Fig. 3 Robert J. Graves (1796–1853). From Wikimedia Commons, the free media repository.

Fig. 4 Karl A. von Basedow (1799–1854). From Wikimedia Commons, the free media repository.

Fig. 5 Thomas Addison (1793–1860). From Wikimedia Commons, the free media repository.

1849 by Arnold Berthold (Berthold, 1849). It took until 1935 for crystalline testosterone to be isolated (David *et al.*, 1935). At the end of the 19th century, the concept of endocrinology was formally tested in two human experiments: the 72-year-old Charles-Édouard Brown-Séquard (**Fig. 6**) repeatedly injected himself with a watery mixture of blood from the spermatic vein, semen, and the extracted testicles of freshly killed healthy young guinea pigs and dogs. He stated that he had "rejuvenated" himself. He claimed that this therapy increased his physical strength and mental abilities, relieved his constipation, and lengthened the arc of his urine! Brown-Séquard's claim prompted many researchers around the world to pursue this new field of "organotherapy," and for a short period of time it was believed that the "fountain of youth" could be found (Brown-Séquard, 1889a, b). In the same era, George Murray introduced the successful treatment of myxedema with injections of sheep thyroid extract (Murray, 1891).

William Bayliss (**Fig. 7**) and Ernest Starling (his brother-in-law) discovered the first hormone, secretin, in 1902 (Bayliss and Starling, 1902). The most famous hormone, insulin, was discovered in 1921 in parallel by Nicolae Paulescu (**Fig. 8**) and the group of James MacLeod, Frederick Banting, Charles Best (**Fig. 9**), and James Collip (Paulescu, 1921; Banting and Best, 1922; Bliss, 1984). Already in 1889, Joseph von Mering and Oskar Minkowski made the observation that removing the pancreas from a dog resulted in severe and fatal diabetes mellitus (von Mering and Minkowski, 1890).

We know now that the human body has about 79 organs and that approximately 75 hormones circulate in the human body. In 1967, it was discovered that certain hormones could be derived from pro-hormones (Chretien and Mbikay, 2016). The most recently discovered hormone is Asprosin, a fasting-induced glucogenic protein hormone, which is secreted by white adipose tissue and is recruited to the liver. It was discovered in 2016 by the research group of Atul Chopra (Romere *et al.*, 2016). It is impossible

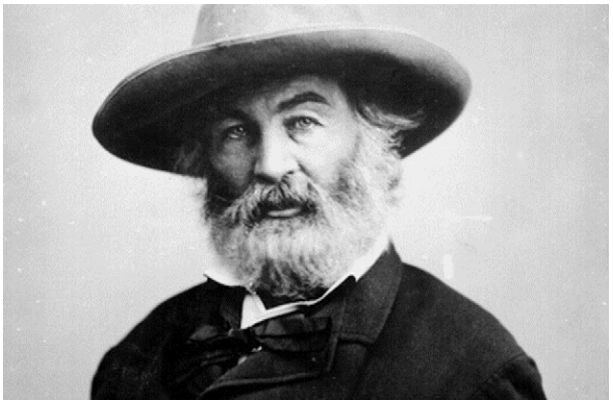

Fig. 6 Charles-Édouard Brown-Séquard (1817–1894). From Wikimedia Commons, the free media repository.

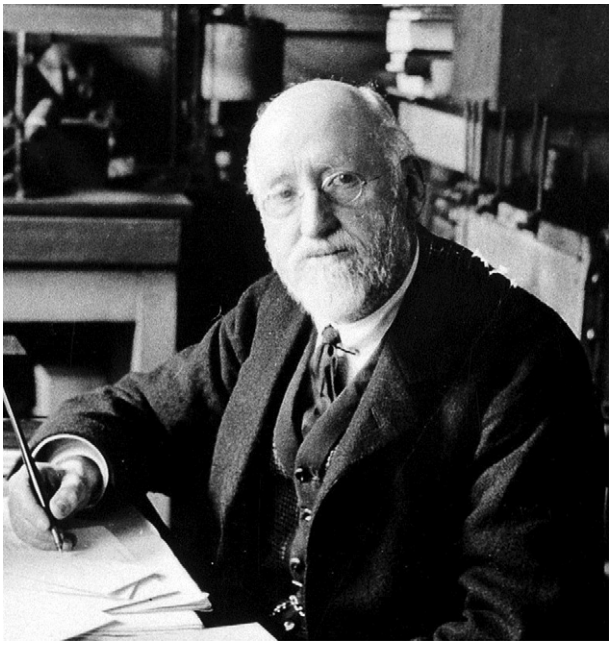

Fig. 7 William M. Bayliss (1860–1924). From Wikimedia Commons, the free media repository.

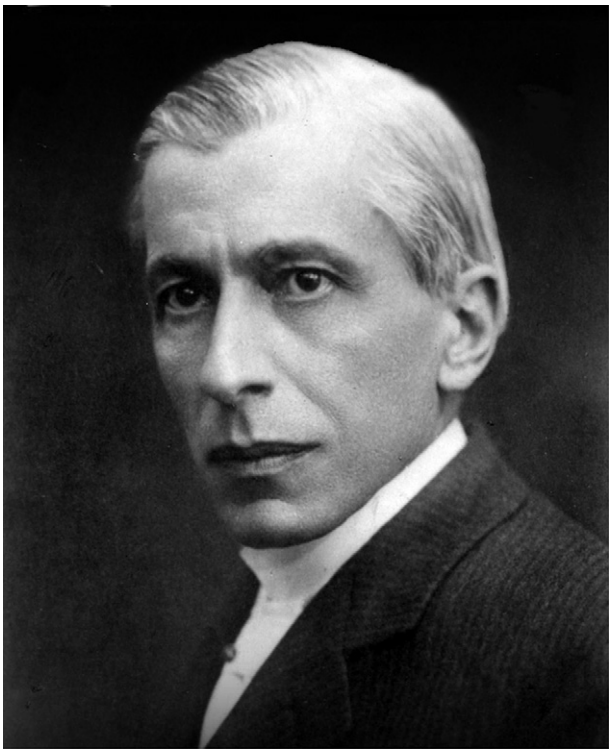

Fig. 8 Nicolae C. Paulescu (1869–1931). From Wikimedia Commons, the free media repository.

Fig. 9 Charles H. Best (1899–1978) and Frederick G. Banting (1891–1941). From Wikimedia Commons, the free media repository.

to review the discoveries of all the 75 known hormones, or all endocrine mechanisms in this historical overview. Therefore, only the main discoveries of hormones and/or endocrine mechanisms which led to Nobel Prize awards will be discussed further.

Not surprisingly, the Nobel Prize in Physiology or Medicine 1923 was awarded to Frederick Banting and John Macleod "for the discovery of insulin." Surprisingly, the other team members were not involved. Frederick Banting decided to share his prize money with Charles Best, and John Macleod gave half his award to James Collip (Bliss, 1984). The Nobel Prize in Physiology or Medicine 1936 was awarded jointly to Henry Dale and Otto Loewi "for their discoveries relating to chemical transmission of nerve impulses." Henry Dale and colleagues first identified acetylcholine in 1914 as a possible neurotransmitter (neurohormone), and Otto Loewi showed its importance in the nervous system (Loewi, 1921; Dale, 1914). The Nobel Prize in Chemistry 1939 was divided equally between Adolf Butenandt "for his work on sex hormones" and Leopold Ruzicka "for his work on polymethylenes and higher terpenes." In 1929, Adolf Butenandt isolated oestrone almost at the same time as the winner of the 1943 Nobel Prize for Physiology or Medicine, Edward Doisy (Butenandt, 1931). In 1931, Butenandt isolated androsterone (Butenandt, 1931). Adolf Butenandt, as well as Leopold Ruzicka, and independently of each other, obtained testosterone in 1939, a compound which had been purified from the testes by Ernst Laqueur in 1935 (Butenandt *et al.*, 1939; Ruzicka and Wettstein, 1935). Progesterone was isolated by Adolf Butenandt from the corpus luteum in 1934. The Nobel Prize in Physiology or Medicine 1947 was awarded to Carl Cori and (Gerty) Theresa Cori Radnitz "for their discovery of the course of the catalytic conversion of glycogen," and to Bernardo Houssay "for his discovery of the part played by the hormone of the anterior pituitary lobe in the metabolism of sugar." Bernardo Houssay's main contribution was on the experimental investigation of the role of the anterior pituitary in the metabolism of carbohydrates, particularly in diabetes mellitus. Houssay demonstrated the diabetogenic effect of anterior pituitary extracts and showed that the severity of diabetes decreased after anterior hypophysectomy (Houssay, 1942a,b). These discoveries were instrumental in initiating research into the mechanistic basis of hormonal feedback mechanisms. The Nobel Prize in Physiology or Medicine 1950 was awarded jointly to Edward Kendall, Tadeus Reichstein, and Philip Hench (**Figs. 10–12**) "for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects." Kendall was mostly recognized for the isolation, identification, and purification of several adrenal steroids. One of these isolated steroids was designated "Compound E," which subsequently became better known as "cortisone" (Mason *et al.*, 1936; Kendall, 1960). Tadeus Reichstein collaborated from 1953 to 1954 with James Tait, Sylvia Simpson Tait, Albert Wettstein, Robert Neher, and Marius Tausk in the isolation and characterization of aldosterone (Tait and Tait, 1998; Shampo *et al.*, 2013). Philip Hench collaborated with Edward Kendall in studies of the effect of Compound E (cortisone) on patients afflicted by rheumatoid arthritis (Hench *et al.*, 1949; Hench and Kendall, 1949). The Nobel Prize in Physiology or Medicine 1977 was divided between Rosalyn Yalow "for the development of radioimmunoassays of peptide hormones" and the other half was given jointly to Roger Guillemin and Andrew Schally "for their

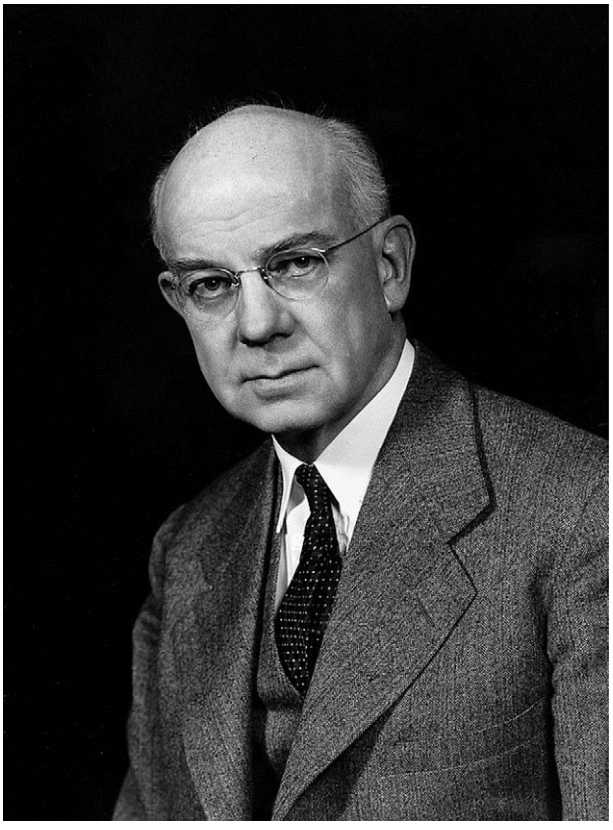

Fig. 10 Edward C. Kendall (1886–1972). From Wikimedia Commons, the free media repository.

Fig. 11 Tadeusz Reichstein (1897–1996). From Wikimedia Commons, the free media repository.

Fig. 12 Philip S. Hench (1896–1965). From Wikimedia Commons, the free media repository.

discoveries concerning the peptide hormone production of the brain" (**Figs. 13** and **14**). Roger Guillemin and his research team focused on unraveling the hypothalamic biochemical regulation of anterior pituitary function and secretion. Independent from but also in competition with the research group of Andrew Schally, Roger Guillemin and his coworkers discovered thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), and somatostatin (Guillemin, 1978a,b, 2008; Guillemin *et al.*, 1984; Brazeau *et al.*, 1973; Burgus *et al.*, 1970). In 1955, Andrew Schally and his research group studied corticotrophin-releasing hormone, TRH, luteinizing hormone-releasing hormone, GHRH, bombesin/gastrin-releasing peptide, and somatostatin (Saffran and Schally, 1955; Gual *et al.*, 1972; Kastin *et al.*, 1972; O'Byrne *et al.*, 1994). The Nobel Prize in Physiology or Medicine 1970 was awarded jointly to Bernard Katz, Ulf von Euler, and Julius Axelrod "for their discoveries concerning the humoral transmitters in the nerve terminals and the mechanism for their storage, release and inactivation." Katz and colleagues studied neurotransmitter release; von Euler and his group studied the production and storage of noradrenaline (von Euler, 1933); and Axelrod and coworkers studied the release, reuptake, and storage of adrenaline and noradrenaline (Hertting and Axelrod, 1961; Hertting *et al.*, 1961). Axelrod and his group also discovered and characterized the enzyme catechol-*O*-methyl transferase, which is involved in the breakdown of catecholamines, and studied the body's circadian rhythm, melatonin, and the pineal gland (Wurtman *et al.*, 1963, 1964). The Nobel Prize in Physiology or Medicine 1982 was awarded jointly to Sune Bergström, Bengt Samuelsson, and John Vane "for their discoveries concerning prostaglandins and related biologically active substances" (Raju, 1999). The Nobel Prize in Physiology or Medicine 2010 was awarded to Robert Edwards "for the development of in vitro fertilization." Edwards began his fundamental research on the biology of fertilization in the 1950s. In 1969, a human oocyte was fertilized for the first time in a test tube. Research in the IVF field was continued in collaboration with Patrick Steptoe, and on 25 July 1978, a healthy baby named Louise Brown was born through a Caesarean section after a full-term pregnancy, following

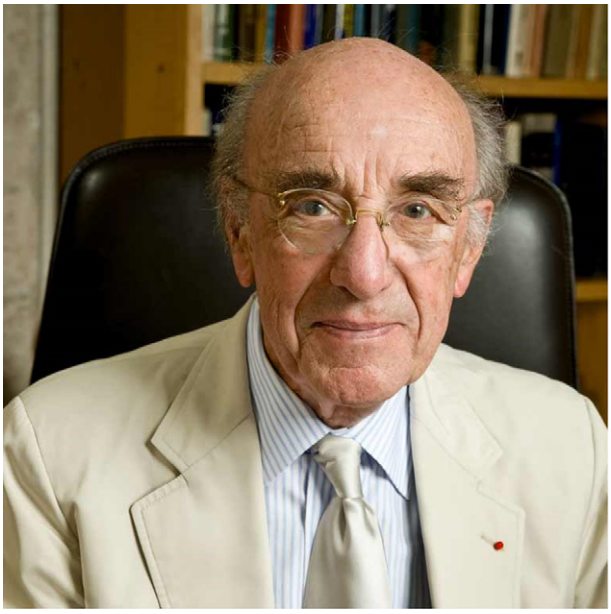

Fig. 13 Roger C.L. Guillemin. Roger (1924). From Wikimedia Commons, the free media repository.

Fig. 14 Andrew V. Schally (1926). From Wikimedia Commons, the free media repository.


BRITAIN'S BIGGEST EVENING SALE

Evening News

LONDON: THURSDAY JULY 27 1978 8p

LATE SPECIAL CITY PRICES

Meet Louise, the world's first test-tube arrival

SUPERBABE

Wide-eyed Louise Brown pictured in hospital 18 hours after she was born. Today she's doing well. See Page Three

Fig. 15 Newspaper front-page (27 July 1978) announcing the birth of Louise J. Brown, the first IVF baby, born on 25 July 1978. From *Evening News*.

successful IVF (Edwards, 1965; Steptoe and Edwards, 1978) (**Fig. 15**). The Nobel Prize in Chemistry 2012 was awarded to Robert Lefkowitz and Brian Kobilka "for studies of G-protein–coupled receptors." In 1968, Lefkowitz and his team started to use radiolabeled tracers to try to identify cell surface receptors. He and his team managed to unveil several receptors, among which were the ACTH receptor and the β-adrenergic receptor (Lefkowitz *et al.*, 1970a,b; Dixon *et al.*, 1986). In 1980, Kobilka joined the team. He succeeded in isolating the gene encoding for the β-adrenergic receptor and, furthermore, capturing an image of the crystalline structure of the β-adrenergic receptor (Rasmussen *et al.*, 2007; Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007).

As demonstrated above, the Nobel Prize committee has awarded their prestigious award several times to endocrinologists over almost 100 years. Endocrinology, thereby, has shown that it has always been a front-runner in the field of medicine. Let us hope it will keep this privileged position in the future and will keep on continuously rejuvenating itself. In line with this, it is reassuring that Vladimir Mironov and colleagues have succeeded in producing the first functional 3D bioprinted thyroid for implantation in mice in 2015.

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Statistics in Endocrinology: Meta-Analysis Advantages and Pitfalls

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Introduction

In 2004, the Lancet published a meta-analysis on the potentially increased risk for cardiovascular events in patients using Rofecoxib (Juni *et al.*, 2004). The study showed that Rofecoxib was associated with a more than twofold-increased risk for myocardial infarction compared to other painkillers. In a cumulative meta-analysis, the authors also showed that the evidence for an increased cardiovascular risk could have been known 3 years before the publication if the meta-analysis had been performed earlier.

This Rofecoxib example undoubtedly shows the potential of a meta-analysis: by systematically searching and assessing all the literature published on a specific topic, a robust answer to a research question can be provided. In the Rofecoxib case, all single randomized studies were not designed to show or rule out an effect of Rofecoxib on cardiovascular events. A major advantage of a meta-analysis is that combining estimates increases the precision, which enables the showing of associations that go unnoticed in single (small) studies. This is especially important in the context of side effects, where single randomized studies are by design not designed to detect rare outcomes.

A similar example was a meta-analysis on mortality risk in acromegaly. Although individual studies did not show a statistically increased risk, the combined estimate clearly showed that acromegaly was associated with an increased mortality risk (Dekkers *et al.*, 2008).

Meta-analyses are generally seen to provide high-level evidence. However, it is important to notice that the mere term "meta-analysis" is not a guarantee of high-quality evidence. The main reason is that any meta-analysis ultimately depends on the quality and validity of included studies; and if a meta-analysis is based on low-quality studies with limited internal validity, then the answer provided by the meta-analysis is still not valid; put simply, it is "garbage in, garbage out."

Formally, a distinction can be made between a systematic review and a meta-analysis. A meta-analysis is the mere statistical combining of studies deemed by the authors to be combinable. A systematic review consists of all other important elements of the review such as writing a protocol, a systematic search of the studies, and analysis of bias risk. This means that a systematic review can occur without a meta-analysis, if the authors decide not to combine study effects statistically for any reason. However, a meta-analysis should always be accompanied by a systematic review, as this includes crucial elements such as the systemic search and assessment of bias risk. Yet the distinction between a systematic review and a meta-analysis is not consistently made in literature.

The Research Question

As with other all research, a systematic review starts with a research question. In contrast to randomized trials, for example, research questions for systematic reviews are generally broader. Think of a randomized controlled trial on the treatment of subclinical hypothyroidism. Researchers can decide upfront whether to include only patients with TSH levels >10 mU/L, whether to apply treatment for a period of 1 year or 2 years, and what the target TSH should be. Also, with regard to endpoints, researchers can decide to focus on quality of life, clinical or biochemical parameters, or cardiovascular endpoints.

This is clearly different from a systematic review, where the researcher is ultimately dependent on what is "out there." It is therefore often not meaningful to start with a research question that is narrow or even extremely specified. A systematic review on the effect of 8 weeks of levothyroxine treatment on the quality of life in women with a TSH of 8–15 mU/L will almost certainly reveal no studies on the topic. One approach, and also opportunity, is to define the population under study broadly and not to be restrictive with regard to endpoints considered. This has the advantage that the systematic review provides a broad picture of a treatment effect of a drug (e.g., levothyroxine for subclinical hypothyroidism) or the effect of a risk factor (e.g., Cushing's disease) on a range of outcomes. The downside of such an inclusive approach is that much heterogeneity between studies can be expected a priori, which may hamper firm conclusions.

For systematic reviews on observational studies, the definition of exposure (i.e., the risk factor under study) requires careful consideration. Think of a systematic review on the effect of overweight on cortisol levels. As the researcher is not in control of the data, different ways of classifying body weight may have been used in different studies. Studies may have used BMI, but also waist-hip ratio to classify overweight. In addition, studies may be published using more advanced techniques to measure body fat (MR liver) and researchers should consider first whether it is meaningful to combine studies with different definitions of an exposure in one review. It should be borne in mind that if it is decided at an early stage to be inclusive in the approach, this does not mean that researchers assume a priori that all studies are combinable in one overall effect estimate.

These considerations show that defining a research question for a systematic review is a balancing act: if the question is too narrow, no studies may be eligible; if the question is too broad, between-study heterogeneity may prohibit a meaningful overview.

The Protocol

It is helpful to write a protocol that contains the strategy for the different elements of the systematic review and meta-analysis. Guidance has been published on how to write such a protocol (Shamseer *et al.*, 2015). Publication of the protocol is generally advised, although publication of protocols is still rare (Page *et al.*, 2016).

Although the protocol can be written without knowing what has been published on a specific topic, the researchers doing a systematic review will generally have an idea on what information is available. This prior knowledge should not completely guide the protocol for two reasons: (1) such prior knowledge is fallible, and it might be that important papers only show up after a systematic search; and (2) a negative statement can be an important message from a systematic review. Think again of a systematic review on the effect of levothyroxine treatment in subclinical hypothyroidism based on data from randomized trials. The researchers may consider not including mortality in the protocol, as they assume there are no trials studying this endpoint. It can be argued that mortality as an endpoint should be included in the protocol as, although unlikely, there may be a trial with data on mortality (point 1). However, even if no individual study addresses this important endpoint, the systematic review can come up with an important negative statement that points towards a gap in current knowledge (point 2).

Researchers should consider carefully how much detail to include in the protocol, as some decisions cannot be spelled out in advance in full detail. This may be mainly the case for systematic reviews of observational studies. The statistical analysis of a meta-analysis comparing two different treatment options (A and B) is fairly straightforward, and such an analysis can easily be pre-specified in a protocol. But for other topics, the statistical analysis will depend on what the data structure of included studies will look like. An example is a meta-analysis on the association between IFG1 and mortality risk (Burgers *et al.*, 2011). As different studies provided mortality risks for different IGF1 cut-offs (e.g., tertiles or quintiles), dose response modeling was performed to quantify the IGF1-mortality association. The statistical methods used will depend on how data are reported and whether unadjusted or adjusted relative risks are considered to be more valid. In such a case, the protocol may contain general remarks about dose-response analysis, with details omitted.

Searching and Including Studies

It is generally advisable to search in different electronic databases for eligible studies, since for most systematic reviews, no single database has full coverage of relevant papers (Lemeshow *et al.*, 2005). Commonly, three databases are searched (Page *et al.*, 2016). In addition to using electronic databases, checking key articles for relevant references can be of added value. An additional strategy is to track down important papers by looking into other papers that cite the former.

Searching electronic databases should be done in close collaboration with a trained librarian, for the reason that writing a search string requires knowledge of database-specific terminology and indexing. It is very helpful to define a set of key articles that should be detected by the search strategy for two reasons. Firstly, in this way the sensitivity of the search can be checked, and secondly, the librarian can use the articles to see how a specific topic is indexed in different databases.

In addition to the sensitivity of the search (does the search detect the important articles?), the specificity of the search also requires consideration (how many non-relevant papers are retrieved?). Especially for broadly formulated research questions the specificity of the search can be low, which means that many articles are retrieved, thereby increasing the workload considerably. For example, the search from a systematic review on the association between corticosteroid use and adrenal insufficiency identified 3616 papers, of which finally only 74 were eligible (Broersen *et al.*, 2015). It could be important to consider restrictions in the search to optimize its specificity. For example, if only studies with newer operation or imaging techniques are considered to provide relevant information for the research question, restrictions in calendar time may reduce the workload significantly.

Having two researchers to decide on eligibility may be relevant, because not all decisions may follow automatically from the eligibility criteria and some decisions may require discussion. If researchers restrict the review to studies in adult populations, it may turn out that some studies have included a small number of patients below adult age. If data are presented separately for adults and non-adults, only the data on adults can be extracted. If this is not the case, the researchers should make a decision regarding what proportion of non-adult patients is considered acceptable for a paper to be eligible.

Risk of Bias

A risk of bias analysis is central for every meta-analysis. It provides a judgment on how likely studies included are valid. This is crucial, as individual studies of limited internal validity will pose a direct threat to the validity of the meta-analysis.

Risk of bias is not synonymous with study quality. A high-quality study can be defined as a study that is optimally performed given the research question. But that does not mean that the study can assumed to be at low risk of bias by default. Consider a study on quality of life after two types of operation for an adrenal tumor: laparoscopic versus open surgery. Even in an optimally designed trial, the patients can never be blinded, which places the study at a higher risk of bias. On the other hand, if the sample size of a study is not well calculated, the risk of bias will not automatically be high. Incorrect sample size calculation will often translate into wide confidence intervals, and thus lower weight in the meta-analysis of a study; however, it will not affect risk of bias. In short: there is no direct translation from study quality in risk of bias.

Bear in mind that for a risk of bias analysis, reviewers have to deal with how a study is reported, and the reporting of the study may not always be a perfect measure for how that study is actually conducted. For example, it has been shown that treatment concealment is often well implemented, but not adequately reported (Soares *et al.*, 2004). Although reporting guidelines have improved reporting of studies, empirical studies have shown that reporting is still far from perfect (Poorolajal *et al.*, 2011).

For systematic reviews of randomized trials, a standardized risk of bias approach is generally accepted. This so-called Cochrane tool to assess the risk of bias in randomized trials consists of the following key domains (Higgins *et al.*, 2011).The key elements to be judged for each included study are:

- randomization;
- concealment of allocation;
- blinding of patients and care takers;
- blinding of the outcome assessment;
- incomplete outcome data; and
- selective reporting.

Whereas some elements of trial conduct may introduce bias for a study as a whole (adequate concealment of allocation, for example), other elements (blinding) may apply to specific outcomes only. For example, blinded outcome assessment is crucial for a valid assessment of radiological images, but not for mortality.

Researchers should judge the risk of bias for every study and preferably report the judgment in a transparent way at the level of individual studies. It should be kept in mind that risk of bias focusses on the internal validity, and should not include a judgment about generalizability. The reason is that risk of bias should facilitate the judgment of the study's effect estimate being valid, which is clearly different from a question about how well the results apply to other populations (generalizability) (Dekkers *et al.*, 2010).

For systematic reviews of observational studies on interventions, progress has been made in terms of how to perform a risk of bias analysis (Sterne *et al.*, 2016). However, the application of the published tool requires in-depth understanding of advanced epidemiological concepts. For systematic reviews about etiology, no widely accepted way to judge risk of bias is available; this was underlined by a review identifying 86 different tools (Sanderson *et al.*, 2007). A widely used tool, the Newcastle Ottawa scale, is not well validated (Stang, 2010).

A sensible approach for systematic reviews on etiology is to apply a risk of bias tool that is tailored to the research question under study. Researchers should thus think carefully which design elements can introduce bias in the included studies. Clearly, assessment of confounding is crucial for such a review. In case of confounding baseline imbalances in prognostic variables potentially bias estimated effects, and such confounding is not simply remedied by statistical adjustment. (Bosco *et al.*, 2010).

A specific type of bias that goes beyond individual studies is publication bias. This is the bias that arises when studies with significant results are more likely to get published, which distorts the full picture of the review. A study comparing published to non-published data for antidepressants, for example, showed an overestimation of the positive effect up to 50% when only the published data were taken into account (Turner *et al.*, 2008). Although publication bias is a significant threat to reviews in particular, this form of bias is a problem that can invalidate any overview of the medical literature, whether it is intended to inform guideline policy makers, regulatory instances, or doctors, or whether it is meant for scientific purposes only.

Clinical and Statistical Heterogeneity

For most systematic reviews, some diversity between included studies can be expected a priori. Studies will have included slightly different populations, will have different definitions of exposure or outcome, or may have investigated patients in different settings (primary care versus hospital care, for example). Such differences are an important result of the systematic reviews, and as such should be displayed (Blair *et al.*, 1995). As heterogeneity is an important feature of the review, such heterogeneity can be used for further exploration. Why and in what respect do studies differ? Do these differences translate into differences in effects?

Statistically, there are two measures of heterogeneity: the I^2 statistics and the Cochrane's Q-test. The I^2 statistics (formally not a test) expresses the percentage of variability between studies that cannot be attributed to chance (Higgins *et al.*, 2003). The Q-test tests whether there is evidence for between study heterogeneity; a *P*-value <0.05 is generally considered as evidence for heterogeneity. The problem with the Q-test is its low power to detect heterogeneity in reviews with few studies included (<10 as a rule of thumb); given that almost half of the reviews include fewer studies (Page *et al.*, 2016), this low power should be considered, in which case a non-significant *P*-value provides no evidence for homogeneity.

It is important to note that the absence of obvious heterogeneity is not an argument for the validity of the meta-analysis. The reason is obvious: all studies may for the same reason be biased. This was shown in a meta-analysis on the effect of beta-carotene on cardiovascular events (Egger *et al.*, 1998). Five out of six observational studies showed a consistent protective effect of beta-carotene; only the smallest study showed a null effect. As expected, the meta-analysis of these six studies showed a protective effect. However, as all randomized trials failed to show such a positive effect, the observational studies were likely all biased (healthy user effect).

There is unfortunately no straightforward answer to the question of how much heterogeneity is acceptable when considering a formal meta-analysis. Clearly, too much diversity between included studies can be an argument, and not give an overall pooled

effect. Out of all systematic reviews, a formal meta-analysis was performed in approximately 70% (Page *et al.*, 2016). However, if heterogeneity is considered important, this might also be an argument for subgroup analyses only, or for assessing heterogeneity in a meta-regression framework. Conceptually, a random effects model (see below) does not assume complete homogeneity when combining studies statistically, which can be a reason to use a random effects model in the case of heterogeneity. It should, however, be kept in mind that, although allowing for heterogeneity, the random effects model does not actually explain heterogeneity.

Statistical Analysis

A formal meta-analysis is nothing more complicated than the weighted average of study specific effects. The combined estimate can thus in no way fall outside the range of estimates of studies included in the meta-analysis. Mostly, weighting is performed according to the inverse of the standard error, and as standard errors are closely related to study size, larger studies receive more weight in a meta-analysis.

The forest plot is a graphical display of the effect estimates of individual studies, the weights that individual studies are given in the analysis (often displayed as either a percentage or as a box, with larger boxes representing larger weights), and the weighed combined estimate.

There are two statistical approaches that can be used for a meta-analysis: a fixed effect and a random effects model. The basic difference between these two models relates to the underlying assumption of the model. A fixed effects model assumes that the underlying true effect is the same for all studies, and that differences between studies are due only to chance. This assumption is rather strong, as mostly some heterogeneity will exist, due to either design or patient characteristics. In contrast, a random effects model relaxes this assumption, and assumes that underlying true effects between studies may differ. Especially in the context of observational studies, the random effects assumption may be more realistic (Egger *et al.*, 1997). Statistically, the estimates from the two models are identical if there is no between-study heterogeneity, whereas in the presence of heterogeneity, fixed and random effects models will give different answers. Generally, the confidence intervals of random effects estimates are wider. A second characteristic of the difference is that in a random effects model, the weights become more similar, meaning that smaller studies receive relatively more weight.

There is no ultimate answer to the question of which model to use. However, it seems reasonable to start with a random effects model if heterogeneity is expected.

Network Meta-Analysis

Standard meta-analytic techniques deal with a set of studies that compare two different treatments—for example, levothyroxine and placebo—and the meta-analysis provides a weighted average of study-specific effects. However, these standard techniques cannot account for a situation where more exposures should be compared (e.g., three or more drugs for the same indication). In such occasions a network meta-analysis may be a useful technique.

The basic idea behind network meta-analyses is that if you have the results of a comparison for drug A versus B and also some studies with data on the comparison of A versus C, these data allow the estimation of the effect of B versus C. If the evidence for the B versus C comparison only comes from studies on A versus B and A versus C, than we call the evidence for B versus C "indirect." It might also be that some data also come from studies for B versus C ("direct evidence"), and in this case the network meta-analysis will use both direct and indirect data, combined in "mixed effects." The main assumptions for the validity of a network approach is that results from direct and indirect evidence do not contradict each other, and the studies included are sufficiently similar with regard to clinical and methodological characteristics (Cipriani *et al.*, 2013).

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Evolution of Hormonal Mechanisms

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Abbreviations

3,5-T2	3,5-Diiodothyronine
AR	Androgen receptor
CO	Carbon monoxide
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
FXR	Farnesoid X receptor
GR	Glucocorticoid receptor
LH	Luteinizing hormone
LXR	Liver xenobiotic receptor
MR	Mineralocorticoid receptor
NO	Nitric oxide
PR	Progesterone receptor
Rev-erb, E75	Nuclear heme-binding receptors
TH	Thyroid hormones
TRIAC	Triiodothyroacetic acid
TSH	Thyroid-stimulating hormone

Glossary

Endocrine signaling Distant intercellular signaling through a body fluid.

Exocrine secretion Secretion of molecules (independent of their physiological role) outside the body cavity, mainly through the digestive system.

Paracrine signaling Local intercellular signaling that does not involves a transport by a body fluid.

Introduction: The Evolution of Hormonal Systems in the Context of Animal Evolution

Hormones are biologically active molecules that are secreted to the blood-stream by a gland, acting remotely and transported to target cells that express specific receptors. Hormones are therefore key messengers of chemical information. The endocrine system, with a wide range of hormones, is the body's second-largest regulatory system, and it works in synergy with the nervous system, which acts very quickly, in a few milliseconds, and whose action does not last much longer. Typically, the endocrine system is much slower, it can take a few seconds or even days to react, but its action can be felt also over a period of several days or even weeks.

The endocrine system fulfills the need of cells and organs to communicate with each other within the body to organize growth, differentiation and cell division in the adult but also during development. The term "hormone" (from a Greek term meaning "set in motion") was adopted by the British physiologist Ernest Starling in 1905 to designate the substances that provide the link between various organs (Sta rling, 1905). They are effectively the key in the coordination of physiological responses in different organs.

There are thousands of different hormones that control virtually all biological processes. Some cause emergency reactions (i.e., adrenaline), others play a regulating role (i.e., insulin, which regulates the glucose level in our blood), still others play a role in the management energy, adaptation to the external environment (temperature for example), the amount of water in the body, salt and ion concentrations, growth, reproduction (the estrogens and androgens that determine female and male sexual characters respectively), blood pressure, digestion etc. Also, some hormones like ecdysone that controls molting in insects or thyroid hormones that control the metamorphosis of a tadpole into a frog, are key regulators of life cycle transitions in animals. Very often, a hormone has several simultaneous effects, and interacts with other substances or hormones and it is more and more important to realize that hormone action is exquisitely regulated at the tissue level by extraordinarily complex and finely tuned mechanisms (Tata, 2005). It is now well demonstrated that controlling the amount of hormone or hormone signaling levels is of critical importance, hence the worry about the widespread presence in the environment of hormonally active pollutants acting as endocrine disruptors (Denver *et al.*, 2009).

Given the variety of hormones and their modes of action, discussing their evolution in general is a challenge. There are, however, a number of general principles that seem valid for all hormones. The first is that at the heart of hormonal systems and their evolution is the hormone/receptor couple. To understand the evolution of this couple we must separate protein or peptide hormones from hormones that are small molecules produced through complex biosynthetic pathways. The second is that the peripheral mechanisms that control the availability of ligands and their activation are extraordinarily important and have been too far neglected. But before we get to the heart of the matter, we need to look at the history of animals in order to have a framework in which we can discuss the evolution of hormonal systems.

More than a century ago, biologists were interested in the origin of animals, which in zoological terms are called the metazoans. They first asked when and how multicellular animals appeared. Paleontology provides some answers and suggests that

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animals are at least 650 million years old. But it is impossible to know how the first metazoans were built, because the oldest fossils attest a rather long earlier evolution giving almost no clues on the very first metazoans. These fossils are remains of already diversified animals, presenting with several modes of organization. They belong to the so-called Ediacara fauna, named after the Ediacara hills in Australia where they were initially exhumed, before they were also found on other continents. Their burial goes back to 600 million years ago. Therefore there is a deep and partly hidden history of metazoans and we mostly rely on molecular phylogeny, the reconstruction of the history of living Metazoans, to obtain some information on what could have been the first metazoans (Budd and Jensen, 2017).

The formation of metazoans required many innovations. It was necessary to join together cells, allow them to communicate with each other and to specialize. It was also necessary to build with each generation a new organism from a single cell; therefore to invent embryogenesis. Given the major role of hormonal systems in controlling cell differentiation, embryonic development, homeostasis and long-range communications in the body, it is evident that the establishment of the very first hormonal systems were somehow concomitant with the establishment of the first metazoans.

This is why it would be interesting to study in more detail the mechanisms of cellular communication in choanoflagellates, the sister group of metazoans. These are flagellated unicellular organisms that sometimes exhibit a colonial way of life and live either free-swimming in open water or fixed on a substrate. There are more than 125 widely distributed and often abundant species and the inventory is certainly not exhaustive. As, they are considered to be the closest living relatives of early animals they are used by evolutionary biologists as a model for reconstructions of the last ancestor of metazoans (Brunet and King, 2017).

It would be important also to have information about the very first phyla of metazoans. However, this question is very controversial because the phylogeny of basal metazoans is not completely resolved (Simion *et al.*, 2017; Whelan *et al.*, 2017). Depending whether sponges (sessile multicellular organisms with a simple body without obvious symmetry) or ctenophora, also known as comb jellies, (ciliated transparent carnivorous marine organisms with a rotational symmetry) are considered as the most basal metazoan group, the most ancient metazoan organism inferred would be very different. In one case it would be a relatively simple sponge-like inactive animal whereas in the other it would be more active and organized, with a primitive symmetry. We cannot discuss here further the implications of these two visions that are still unresolved but we can however insist on the fact that the resolution of this question will have implication for the level of complexity of global communication devices existing in the first metazoans. Clearly, in both cases, because they lack an internal circulating body fluid, these animals cannot have a classical hormonal system in which hormones are transported in the blood. However this does not mean that there is no intercellular signaling in those animals. Indeed the classical view of hormones defined as internal circulating molecules is changing and the frontier between hormones and growth factors and other paracrine signaling molecules is becoming more and more blurry with the acknowledgment of many short-range functional roles of hormones.

The Evolution of the Hormone/Receptor Couple

At the heart of the hormonal systems is the hormone/receptor couple. According to the classical vision, highlighted in numerous textbooks, hormones specifically turn on their receptors by fitting into a hormone-binding site (also called ligand binding pocket or LBP) as a key fills a lock. However, it is becoming increasingly clear that this view is by far too schematic and that in reality the situation is much more plastic. This should be emphasized as it may have implications at the evolutionary level. This plasticity is well exemplified in the case of nuclear receptors that are ligand-activated transcription factors mediating the effects of numerous hydrophobic hormones such as steroids or thyroid hormones. First, this family of well-conserved receptors illustrates very well that the strong difference we often make between hormones (signaling molecules acting at distance from their origin and transported by blood) and growth factors (signaling molecule acting close to its origin and even sometimes on neighboring cells) do not correspond to the biological reality. Indeed, the nuclear receptors cluster receptors for hormones (e.g., estrogens) but also to growth factors (e.g., retinoic acid) and even active molecules directly derived from food (e.g., fatty acids), and some compounds, such as steroids, can act both as distant hormones and local paracrine signaling factors. All these different classes of compounds act in a very similar fashion by regulating a gene regulatory cascade through a well-conserved superfamily of receptors. Second, the structural variety of ligands acting through nuclear receptors is really impressive and questions the distinction between ligands, that is molecules capable of binding and activating a receptor, and prosthetic group defined as organic molecule maintained permanently in a protein structure and essential for its activity. This is shown by the Rev-erb/E75 receptors that are able to bind heme, the well-known prosthetic group of hemoglobin. In Rev-erb, heme, a large molecule, is also permanently bound in the structure and its redox status controls the activity of the receptor. It is a gas, nitric oxide or carbon monoxide, that controls the redox status and heme and therefore the activity of the receptor. Therefore E75 in *Drosophila*, but also Rev-erbs in vertebrates, are biological sensors for diatomic gasses such as NO and CO (Marvin *et al.*, 2009). This example illustrates very well that hormone receptors can have a much diverse set of ligands than anticipated. Third, and lastly, it is now clear that a given receptor can bind, sometimes with different affinities, a diverse set of ligands that all have clear biological activities. This is well exemplified by the case of vitamin D receptor whose natural ligand is 1,25-dihydroxycholecalciferol regulates calcium metabolism, in particular in the bones. But the same receptor can also be activated in the liver and intestine by a bile acid, lithocholic acid that in fact controls its own degradation as it is a carcinogenic compound (Makishima *et al.*, 2002). Many other nuclear receptors are no longer viewed as highly specific for a small set of ligands that they bind with high affinity but are rather sensors that can be activated by many different compounds on which they bind with a much lower affinity (Sladek, 2010). This is the case for many nuclear receptors

implicated in the control of metabolism (e.g., LXR, FXR etc.…). Therefore the key-lock model although not wrong is an over-simplification and we should keep it in mind when discussing the evolution of hormone-receptor couple.

Being either nuclear receptors that activate a set of target genes or membrane receptors acting through second messengers, the hormonal receptors should of course control a specific response in order to fulfill their physiological need. Insulin must promote the absorption of glucose present in the blood, whereas glucagon, on the contrary causes an increase of the amount of glucose in the blood. Therefore, even if more complex than the key-lock model, specificity is a key concept for the hormone-receptor couple. And this specificity could rely on quite tiny differences. Let's compare the structure of 17β-estradiol and 5α-dihydrotestosterone, the respective ligands of estrogen and androgen receptors responsible of female, and male sex features respectively: one carbon and six hydrogen atoms differ between the two molecules that both harbor the classical steroid skeleton. Therefore, even if somewhat plastic, the receptors must recognize their ligands and not others. This question of specificity is the key in our understanding of the evolution of hormone-receptor couples.

For discussing how the hormone-receptor couple can evolve it is necessary to clearly separate two cases: the receptors for peptide or protein hormones in which both the hormone and the receptors are encoded by genes and the case of receptors for small molecules which are derived from a metabolic pathway that is a series of chemical reactions modifying a precursor compound.

When both hormone and receptor are encoded by genes, the situation is conceptually simple: when a mutation occurs on one of the partners, let say the ligand, a compensatory reaction must occur in the other partner, here the receptor, to allow the binding to be maintained. In case of gene duplication it is therefore quite simple to see how a ligand and a receptor couple can diverge from one another. This has been beautifully illustrated in the 90's by the case of FSH and LH and their respective receptors (Moyle *et al.*, 1994). It has been shown in that case that precise regions of the ligands act as determinants that restrict ligand-receptor interaction, therefore enabling selectivity and explaining binding specificities in a family of homologous ligands and their receptors. This model applies easily to cases where the respective members of the couple belong to the same families and render more easily tractable the structural basis of the selectivity (see Jiang *et al.*, 2014 for glycoprotein hormones). But this is not always the case and in other ligand–receptor pairs (e.g., cytokines) the receptors belong to the same family but their ligands belong to distinct families (Liongue *et al.*, 2016). However this model has well resisted the time (Szkudlinski, 2015) and has been extended to some other ligand-receptor couples such as ghrelin (Tine *et al.*, 2016), oxytocin (Yamashita and Kitano, 2013; Ren *et al.*, 2015) and even outside the endocrine field in the context of protein–protein interactions (Lovell and Robertson, 2010).

The situation is different when the ligand is not encoded by a gene but is a small molecule whose synthesis is controlled by a series of enzymatically-controlled biochemical reactions. This aspect has been studied in more details in the case of steroid receptors. The case is effectively interesting as the five steroid hormone receptors and their respective ligands in humans and other mammals (ER/estrogens, PR/progesterone, AR/androgen, as well as MR and GR/corticoids) arise from the same common ancestor. This raises the question: how did these receptors acquire their specificity with respect to their hormone molecule? As a simple coevolution mechanism obviously cannot operate, alternative models such as the ligand exploitation model (Thornton, 2001) have been proposed to explain how changes of specificity can take place during evolution. According to this model, the terminal ligand in a metabolic pathway has to be the primordial ligand for which a receptor should exist. The existence of this ligand implies the existence of intermediate molecules in the metabolic pathway since however the terminal ligand will not be maintained. In case of duplication of the gene encoding the receptor some duplicated copies can therefore exploit (hence the name of the model) these intermediate compounds. This may explain how novel hormone-receptor pairs can be created.

The ligand exploitation model has been proposed in the case of the estrogen receptors that was considered with its ligand 17β-estradiol to be the primordial steroid-receptor couple (Thornton, 2001). However this model has a fundamental limitation as it considers that the receptors are evolving but that the ligand remains the same across time. We know that this cannot be true and indeed in the case of estrogens this is not true. Detailed comparative genomics have shown that estrogens are a vertebrate specific compound and that the estrogen receptor in invertebrates should therefore have a different endogenous ligand. The fact that some invertebrate estrogen receptors effectively bind 17β-estradiol is therefore a pharmacological effect, unrelated to their endogenous ligand and physiological function that remains unclear.

Even if one cannot speak about direct coevolution, when the receptor is considered, the evolution of the ligand-binding pocket can occur by the slow accumulation of mutations that gradually change the structure of the pocket and its specificity. This has been exquisitely shown for the steroid receptors (Bridgham *et al.*, 2009), but also for retinoid receptors (Gutierrez-Mazariegos *et al.*, 2016) as well as for membrane receptors, in the case of cannabinoid receptors. Here the enzyme implicated in the metabolism of anandamide and 2-arachidonyl glycerol, the two endogenous ligands of cannabinoid receptors, have been shown to follow the evolution of the receptor suggesting some sort of coevolution (McPartland *et al.*, 2007).

Clearly the evolution of small molecules and their receptor should now be scrutinized in greater detail and this is becoming possible. Indeed by scrutinizing on steroidogenesis it has been possible to reconstruct an ancestral receptor-ligand couple (Markov *et al.*, 2017). For this the authors have retraced the history of steroidogenesis using an approach developed for the analysis of universal metabolism (Cunchillos and Lecointre, 2007). The originality of the approach was to use comparative anatomy methods to study evolution at a biochemical level. It is a simple principle: as with animal anatomical features, metabolic pathways are the product of evolution and are modified over the course of time. Using phylogenetic tree construction methods it is therefore possible to infer the evolution of the steroidogenic pathways and to show that, as suggested by comparative genomics approach the five steroid hormones share a unique common ancestor at the basis of vertebrates and are therefore specific to vertebrates. Interestingly, such an evolutionary tree makes it possible to predict the molecules that were present at each evolutionary stage, so it

was possible to determine the structure of the ancestral steroid. This molecule called "paraestrol A" was chemically synthesized and shown to bind and activate an ancestral receptor that was also predicted by molecular phylogeny. This interaction was confirmed by structural modeling, allowing to explain how the receptor specificity to its ligand was reinforced over the course of evolution. This "resurrection" of an ancestral hormone/receptor couple opens up the way to the study of small molecule evolution and is therefore opening a new era, as it will undoubtedly be reproduced in the case of other receptor-ligand couples.

Evolution of Peripheral Control of Hormone Response

Whatever the receptor system, the hormonal response must be carefully regulated both in time and space. The transport of hormones, their entry into target cells when needed, their activation in target tissues, or their degradation after use allows to precisely controlling the duration and magnitude of hormone response. Every step is regulated and therefore every step can be used by natural selection in order to allow the organisms to be in register with their environment, ultimately leading these organisms to be adapted.

The importance of a tight regulation of peripheral hormonal regulation is well exemplified by the case of the early metamorphosis of desert frogs (Buchholz and Hayes, 2005). Living in ephemeral desert pools, the spadefoot toad *Scaphiopus couchii* has the shortest larval period known among frogs, as it must complete its metamorphosis before complete drying of the pool. In this species, differences in TH physiology correlating with larval period differences were identified and are consistent with the notion that selection favoring rapid metamorphosis to avoid desiccation acted, at least in part, through TH physiology that occurred on a tissue-by-tissue level. This suggests that an increase in tissue thyroid hormone content and/or increased tissue sensitivity occurred in the ancestors of *Scaphiopus couchii*. Such a higher tissue response to higher TH levels led to more rapid transformation of metamorphic tissues and therefore to the adaptation to the ephemeral nature of the pond. This example shows very well how an important, and even vital change in larval period duration can be obtained by altered endocrine signaling and beautifully illustrates how change in hormonal response can play an important role. Other recent similar examples, among others also include the central regulation of TH level by TSH in freshwater versus marine sticklebacks (Kitano *et al.*, 2010) or the variation in tooth number controlled by the local production of retinoic acid in cypriniform fishes (Gibert *et al.*, 2015).

Even if given the wide diversity of hormonal systems it is difficult to generalize, it is interesting to note that the enzymes used to regulate in space and time the level of hormone signaling are effectively variable during evolution. Clearly the various enzymes controlling hormone availability vary often in number in different species and are therefore likely contribute to evolutionary innovations in the control of hormone response. For example the 3β-hydroxysteroid dehydrogenase, an essential enzyme involved in the biosynthesis of all classes of steroid hormones, is present in at least six genes in mouse, four in rats, three in hamster and two in humans, all coming from species-specific gene duplications (Simard *et al.*, 2005). Similarly, but in a different scale, the number of deiodinases, essential enzymes that control the tissue availability of triiodothyronine, the active thyroid hormone, is also changing in chordates: there are three deiodinase genes in most vertebrates, but at least five in the cephalochordate amphioxus and only one in sea squirt (Paris *et al.*, 2008). We could multiply the examples: the proteins controlling hormone production and availability in tissues are much more variable than those of the receptors. In addition detailed analysis of their sequence evolution often reveal strong evolutionary pressures in action.

Given these observations, the notion that the ligands are evolving should not come as a surprise. Of course this has been widely accepted since a long time for protein hormones but for obscure reasons this has remained virtually unexplored for small molecules. Strikingly, this was known since a long time for specific cases but never generalized. Indeed rodents and primate do not have the same major corticosteroid: cortisol is the major corticoid in human as well as most other mammals whereas corticosterone is the active compound in rodents. Similarly, in teleost fish the active androgen is 11keto-testosterone a derivative of testosterone. These cases clearly show that hormones are evolving in different species but still; the generalization of this observation is barely recognized by comparative endocrinologists. Because triiodothyronine is the bona fide ligand of thyroid hormone receptors in mammals it should the same in all animals: but this is not the case. In invertebrates, all suggests that the active ligand is not triiodothyronine but TRIAC, a deaminated derivative that is known since a long time but have not been seriously investigated, and the physiological importance of 3,5-T2 in teleost fishes is also gaining increasing recognition (Orozco *et al.*, 2017). The same is true for retinoids or, as discussed above, for estrogens. This is most probably the case in much other systems: it is not because a hormone is present in human that it must be also active in other animals. If there is an area where it is necessary to avoid any anthropomorphism it is indeed the one of hormonal ligands and their receptors.

The Origins of Hormones

If there is one mysterious question, it is the question of the origin of hormonal systems. That the very early metazoans need a system to allow efficient transfer of information from organs to organs in order to synchronize central and peripheral response is accepted since a long time. But how does such a system emerge? This vexing question has not yet received sufficient attention. One reason is that because of anthropomorphism, many people have tried to trace back the origin of human compounds rather to consider animal evolution in an unbiased manner. There is a strong need to biochemically characterize the hormones present in diverging metazoans. The effort that has been made in some systems (e.g., the nematode *Caenorhabditis elegans*) has revealed a

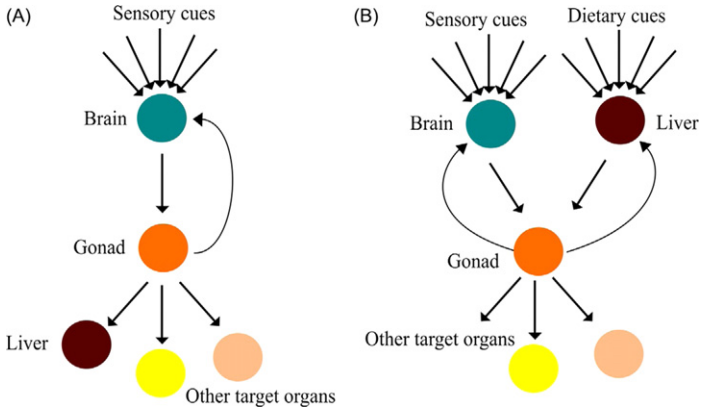

Fig. 1 *For a reappraisal of the integrative role of the digestive system.* (A) A classical textbook hierarchical regulatory model, where the signal goes upside-down -even if retroactions are acknowledged—from the nervous centers to the target organs, among which is the liver. (B) A "pluralistic view," where not only the brain, but also the liver, and possibly the gonad too, are all partial and complementary integration centers.

wide variety of compounds, some related to steroids, but none of them being simplified versions of human hormones, as many would have anticipated. Complexity and diversity are everywhere even in the apparently simplest animals. The analysis of complete genomes has shown that even the most basal metazoans may have surprising sophistication in signaling systems. Clearly, "basal" does not means "primitive" and even morphologically simple animals such as a placozoan or a hydra have the same amount of years of evolution as humans and therefore can be quite elaborate in other aspects of their biology. The unbiased characterization of active compounds (steroids, leukotrienes, prostaglandines etc.…) present in early metazoans will undoubtedly shed light on this important question.

However even in the present paucity of information one can make some interesting observations. Among the many types of hormones some, as the steroids but also the numerous derivatives of fatty acids, are particularly interesting because their chemical structures are conserved outside metazoans (Kushiro *et al.*, 2003). The fact that in such divergent systems similar active molecules are used may be revealing of a deep analogy between the hormonal systems. One reason could be that the biochemical pathways allowing the metabolism of these molecules predates the divergence between, say, animals, fungi and plants (Baker, 1995). This would therefore suggest that evolution has simply used again and again similar enzymatic systems and pathways to diversify an existing class of active molecules. This may explain for example the existence of vertebrate steroids, ecdysteroids of insects and brassinosteroids in plants. Another poorly explored, but quite exciting idea, could be that some structural or chemical properties render these molecules particularly apt to fulfill a hormonal function (Kushiro *et al.*, 2003). Being their hydrophobicity, size, ability to cross membranes, to be buried in protein cores or to be transported in biological fluids, steroids and fatty acid derivatives are apparently more suitable than others to serve as efficient molecules to transport, at long range, specific information. This must be further explored.

Lastly, there is now accumulating evidence than hormones may derive from food-related compounds. This is obvious for leukotrienes and prostaglandins that are fatty-acid derivatives but this is probably also true for steroids or thyroid hormones. The cladistic analysis of steroid hormones discussed above reveals that sex steroids are in fact, biochemically speaking, degradation products of cholesterol, a molecule directly linked to diet. In addition, the phylogenetic analysis of the four main families of steroidogeneic enzymes has revealed that many of these enzymes originate from detoxifying enzymes that hydroxylate toxic compounds targeting them towards degradation pathways. Thyroid hormones are clearly derived from iodinated molecules that were produced in the pharynx of filter-feeding organisms. Indeed many marine organisms filter planktonic particles by gluing them into mucus and it is known that in close vertebrate relatives such as amphioxus and sea squirt, the mucus contains iodinated proteins that help in this process (Holzer *et al.*, 2017). From this an endocrine secretion of iodinated thyroid hormones evolved and it came therefore as no surprise that thyroid hormones promote energy expenditure as their basal exocrine function was probably linked to the signaling of the presence of food. Clearly all these indications reveal a deep, ancient, relationship between nutrition and hormonal signaling. As many hormones are regulating reproduction, one can posit that hormone signaling derived from signaling pathways that were primitively involved in the regulation of the reproductive maturation according to nutritional conditions (Della Torre *et al.*, 2014). This has some important implications on the way we have to look at endocrinology. Classically, as we pointed out in the introduction, hormonal signaling is viewed as a bottom-up cascade from the nervous centers to the target cells, and hormones such as steroids, occupy an intermediary position in the middle of this cascade (see **Fig. 1**A). But our quick journey through metazoan signaling physiology reminds that a significant part of the environmental input is integrated not through the nervous system, but through the digestive system. This is consistent with the recent proposal that the liver may be act as an integrative center complementary to the brain (Della Torre *et al.*, 2011), and with the now well-acknowledged importance of the gut microbiome on the nervous system (Vuong *et al.*, 2017). Therefore we should perhaps now conceptualize a more "pluralistic view," in which not only the brain, but also the liver, the intestine and possibly the gonad too, are all partial and complementary integration centers (**Fig. 1**B). Probably due to the special status of the brain as a "noble organ," being considered

as the supreme integrator of body signals, the nervous system has benefitted from many studies from evolutionary biologists, with many ramifications even out of the strict biological field. Let us hope that the interest in hormones origins will stimulate next generations of scientists to dedicate the same attention to the digestive apparatus and to its functional connections with hormonal signaling.

Take-Home Message for Clinicians

An evolutionary perspective tells us that whatever their chemical nature, both partners in hormone-receptor couples are constantly evolving, as do the fine-tuned mechanisms controlling their availability in time and space. This explains why those systems are so plastic and so difficult to classify. The origin of the hormonal system is deeply rooted into the perception of external nutritional cues, which also explains why it is so sensitive to disruption by environmental chemicals.

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Relevant Websites

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General Principles of Endocrine Genetics

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Nomenclature

Lambda S (λ_S) The sibling relative risk. The risk of disease in a sibling of a case, divided by the risk in the general population.

LD Linkage diseqilibrium
MAF Minor allele frequency

Glossary

Aneuploidy An abnormality where the number of copies of genetic material is different from the normal two (or, in the case of sex chromosomes in males, one). Mostly used for whole chromosomes or parts thereof large enough to be seen with the light microscope (approximately >5 Mb). Smaller instances are referred to as CNV (copy-number variation) or indel (insertion-deletion) variants.

Haplotype A string of alleles at adjacent polymorphisms, on one of the two chromosomes of an individual. Example, aBCdeF for adjacent polymorphisms Aa, Bb, Cc, Dd, Ee, Ff. Contrasted to diplotype, that would be a string of two alleles, for example, AA, Bb, cc, Ee, FF in the same example.

Linkage disequilibrium The property of variants located close to each other on the chromosome, to give the same information. Consider a mutational event that created allele *B* in the place of the ancestral allele *b*. This mutation happened on an ancestral chromosome that has, say, allele *A* at the neighboring SNP *A*/*a*. In the immediate progeny of that ancestor, knowing the genotype at *A*/*a* would allow us to confidently infer the genotype at *B*/*b*. With the passage of generations, however, meiotic recombinations occur between the two loci, so that the correspondence no longer applies to 100% of chromosomes and the prediction is no longer absolute but becomes probabilistic. After a sufficiently large number of recombinations, the two loci equilibrate completely and the ability to predict one from the other is lost. In contemporary humans, SNPs in chromosomal proximity may be found in any stage of this process, from complete lack of equilibration (highly correlated, $r^2 = 1$) to fully equilibrated (no correlation, $r^2 = 0$). Meiotic recombinations do not occur uniformly in the genome but tend to concentrate on "hot spots," between which blocks of highly correlated SNPs can be found. The length of these blocks may range from a few kb to a Mb or more.

Mutation A single event that happened in the past and created a DNA sequence variant. Its use as a synonym for "pathogenic variant for a monogenic disease" has been established by usage and will be used in this chapter for convenience.

Locus (plural, Loci) A specific location in the genome usually (but not necessarily) linked to a disease phenotype. Specified as ChrN:p1:p2, where N is the chromosome number, p1 the beginning and p2 the end of the locus, in number of nucleotides counting from the tip of the short arm.

ROC (receiver-operator characteristic) curve Originally developed for the statistical evaluation of enemy-airplane sightings on radar, this curve plots the sensitivity (proportion of cases that score at or above the cut-off) against 1-specificity (proportion of healthy subjects who score likewise). The area under the curve, ranges from 0.5 (test no better than flipping a coin) to 1 (sensitivity and specificity both 100%).

Variant Any DNA sequence difference from the reference human genome. Although in the past it has been used to mean a harmless polymorphism, its modern usage has no connotation of pathogenicity. Phenotypic effects need to be separately determined and stated.

Redefining the Concept of "Genetic Disease"

The importance of genetics in many aspects of endocrinology has been well known for a long time. However, over the past 10 years or so, important breakthroughs in the methodologies of genetic analysis have brought about a redefinition of our understanding of how genetic variance determines endocrine disease, making a new discourse on endocrine genetics timely and topical.

In current usage, the term "genetic disease" is treated as a synonym for what one could more specifically refer to as "Mendelian" or "monogenic" disease: a DNA variant seriously compromising the function of a single gene, suffices to cause the disease. This results in a specific and recognizable pattern of familial inheritance following the patterns established by Mendel. Endocrinology encompasses a large number of such diseases which, although individually rare, collectively account for a nonnegligible part of endocrine practice. However, the bulk of a typical endocrinologist's time is spent caring for diseases not covered by this definition. A correspondingly large part of this discussion will, therefore, be devoted to demonstrating the relevance of genetics in these more complex, multifactorial phenotypes, especially as highlighted by recent discoveries in the past decade. It is not the purpose of this

brief overview to describe individual diseases in any detail but rather to cover the basic principles of how genetic variation can cause (or affect the risk for) endocrine diseases.

Chromosomal Anomalies

Whole-chromosome aneuploidies are compatible with postnatal survival only when they involve the sex chromosomes or Chr21, the smallest human chromosome with the smallest number of genes.

Trisomy of Chr21 causes the distinctive phenotype of Down syndrome which, at the molecular level, must be due to expression of genes on the chromosome at 150% of their normal level (Antonarakis, 2017). The endocrine interest in trisomy 21 is its association with greatly increased risk for autoimmune thyroid disease (Pueschel and Pezzullo, 1985), both thyroiditis and Grave's. Risk of Type 1 diabetes is also increased by as much as an order of magnitude (Anwar *et al.*, 1998). Which specific gene(s) are responsible for this is not currently known but *UBASH3A* (ubiquitin-associated and SH3 domain-containing protein A) is a good candidate for at least part of the effect. A risk variant for type 1 diabetes (T1D) (Grant *et al.*, 2009) and other autoimmune diseases (Zhernakova *et al.*, 2011), is associated with increased *UBASH3A* expression (Dixon *et al.*, 2007) (paralleling the expected effect of the trisomy).

Losses or gains of the X chromosome result in, respectively, the Turner (TS) or Klinefelter (KS) syndromes, both associated with gonadal pathology and infertility.

TS patients are born without gonads and may be thought of as females missing one X (if they carry only the paternal X) or males missing the Y (carriers of a maternal X). In either case, absence of sex hormones results in normal female external and internal genitalia, the hormone-independent default state. Mosaicism and/or partial deletion of ChrX can result in intermediate phenotypes but remarkably short stature is a consistent feature. It is current practice to treat it with growth hormone (GH), which results in an average gain of only 5 cm of adult height (Stephure and Canadian Growth Hormone Advisory, 2005), as usually seen in most conditions involving shortness not due to GH deficiency (Grimberg *et al.*, 2016).

KS due to supernumerary X chromosome(s) in a male, affects mostly the seminiferous tubules and Sertoli cells of the testis, with relatively intact Leydig cells, despite elevated gonadotropin levels. Testosterone treatment is not needed to induce full puberty in most boys with KS and the potential for psychological harm from this intervention has not been investigated. Cognitive improvement from testosterone treatment has been claimed but there is no biological plausibility or credible evidence base to support it.

Since normally one X is inactivated in all cells, both TS and KS must be due to under- or overexpression, respectively, of genes that escape X inactivation. Seven such genes have been discovered to completely escape inactivation, while partial escape is seen in as many as 23% of X-linked genes (Tukiainen *et al.*, 2017).

Monogenic Endocrinopathies

Given the complexity of the endocrine system, it is not surprising that mutations in any one of a very large number of genes cause endocrinopathies, most of them rather rare. General mechanistic principles connecting DNA sequence variation to disease phenotype will be discussed. Individual disorders will be mentioned only as examples to illustrate mechanisms.

Loss of Function of Hormones and Their Receptors and Mediators

Mutations in the actual sequence of peptide hormones themselves is the mechanistically most straightforward cause of monogenic endocrinopathy. They are all transmitted as recessive traits; both copies of the gene must carry loss-of-function variants for the disease to occur. As most of these hormones are subject to feedback regulation, one normal copy of the gene can be upregulated to produce twice the amount of hormone in healthy carriers. The phenotype is as one might predict. Homozygous or compound heterozygous mutations in growth hormone cause growth failure with normal or elevated levels of (bioinactive) hormone but low IGF1 (Phillips and Cogan, 1994). Biallelic loss of function of the beta subunit of either FSH (Matthews *et al.*, 1993) or LH (Weiss *et al.*, 1992), cause hypogonadotropic hypogonadism without anosmia and mutations in the PTH (parathyroid hormone) congenital hypoparathyroidism (Arnold *et al.*, 1990). Such mutations of the hormone itself are, for some reason, rare. Diabetes due to recessive loss-of-function variants of the insulin peptide has not, to my knowledge, been reported.

Deficiency of nonpeptide hormones can result from loss-of-function in the enzymes needed for their synthesis. Classical examples are the various types of thyroid dyshormonogenesis, involving any one of a number of enzymes and transporters needed for thyroxine production (Park and Chatterjee, 2005) and corticosteroid deficiency from loss of function of one of several steroidogenesis enzymes. By far the most common of the latter is deficiency of *CYP21A2* (encoding 21-hydroxylase), required for the production of hydrocortisone and aldosterone. A pseudogene in tandem with the active gene, mapping on Chr6p21.33, invites nonhomologous recombination and explains the high prevalence of this particular problem (White *et al.*, 1984; Higashi *et al.*, 1986). A result of the ensuing hormone deficiency is elevation of the corresponding trophic hormones (TSH and ACTH, respectively, in the above examples). In the case of the adrenal, this results in an additional pathogenetic mechanism, the accumulation of high levels of intermediate products upstream of the block. Their androgenic activity causes virilization of female embryos and premature sexual development. These effects are often the main medical issue, even when the loss of enzymatic function is incomplete and the adrenal can be stimulated enough to produce normal levels of the physiological hormones (simple virilizing congenital adrenal hyperplasia with no manifestations of deficiency).

Mutations in hormone receptors have less predictable phenotypes. Mutations of the TSH receptor do cause the expected simple congenital hypothyroidism (Paschke and Ludgate, 1997) and those of ACTH receptor isolated glucocorticoid deficiency (Tsigos *et al.*, 1993) but mutations in the PTH receptor 1 cause, in addition to hypocalcemia, Bloomstrand chondrodysplasia (Jobert *et al.*, 1998), likely because this receptor also transmits the action of PTHRP (PTH-related peptide).

Finally, a unique and interesting mechanism whereby loss-of-function mutations in a single gene can cause multiple endocrine diseases is seen in the recessively inherited syndrome of autoimmune polyendocrinopathy type 1. It is due to diallelic mutations of *AIRE* (AutoImmune REgulator) (Finnish-German, 1997), the gene that orchestrates the expression of tissue-restricted antigens (including endocrine-gland specific proteins) in the thymus epithelium so that T-cells that react to them can be eliminated through negative selection. In the absence of functional *AIRE*, these proteins are not recognized as self. The parathyroid gland is typically affected but thyroiditis, Addison's disease and type 1 diabetes can also be present.

Dominantly Inherited Endocrinopathies

While recessive mutations always represent loss of function of the gene involved, endocrinopathies can arise through diverse additional mechanisms when only one copy of the gene is mutated.

The simplest of these mechanisms is haploinsufficiency: again loss of function, in situations where 50% expression is not sufficient to maintain normal physiology and (unlike the case with genes encoding peptide hormones) the gene involved is not subject to negative feedback that can upregulate expression from the intact copy. The typical example of a group of such endocrinopathies are the different types of MODY (maturity-onset diabetes in the young), caused by haploinsufficiency of one of a group of dosage-sensitive genes of crucial importance in the development and/or function of the insulin-producing β-cells. The most common forms, MODY1 and MODY3, are due to heterozygous mutations of, respectively, HNF1α and 4α (hepatic nuclear factor 1-alpha and 4-alpha), transcription factors ubiquitously expressed but, apparently, dosage-sensitive only in β-cells (Yamagata *et al.*, 1996). They are important to differentiate from the common forms of diabetes (especially type 1) because they can be very effectively treated with oral sulfonylurea drugs. *IPF1* (insulin promoter factor 1), a transcription factor involved in both pancreatic development and insulin secretion, Glucokinase, the glucose sensor of the β-cells and *RFX6* (Patel *et al.*, 2017), encoding a transcription factor necessary for the specification of the islet endocrine phenotype (Smith *et al.*, 2010), are other examples of genes with obvious functional importance and mutated in, respectively, MODY 5, 2, and 14.

Dominant familial endocrine tumor syndromes are mediated by a special kind of heterozygous loss of function that does not involve haploinsufficiency. In multiple endocrine neoplasia syndromes type 1 and 2, the single intact copy of the mutated gene (respectively *MEN1* and *RET*) (Concolino *et al.*, 2016) is sufficient to maintain normal cellular function. However, somatic mutations are bound to happen during the astronomical number of mitoses that it takes to form each of the affected endocrine glands. If such a mutation destroys the intact copy of the gene in a single cell, this cell and its progeny acquire a proliferative advantage that forms the tumor, according to the Knudson two-hit hypothesis.

Heterozygous mutations may also cause disease through gain of function. This may be an increase in the normal function of a gene or gain of a novel, abnormal function. A typical example of the former is neonatal diabetes due to mutations that increase the function of Kir6.2 or SUR1, the K^+ channel that regulates insulin secretion in β-cells or its controller unit, respectively encoded by the *KCNJ11* and *ABCC8* genes. Such mutations in either gene keep the channel open, preventing the sequence of events that leads to insulin release (Nakhla and Polychronakos, 2005). They also respond to sulfonylureas, which act by binding to and inhibiting SUR1. A large proportion of these mutations are de novo, as might be expected in dominant disease that severely limits survival and reproductive fitness. The exact same mutation often independently recurs in different families, at specific "hot spots"—unlike loss of function, which can be the result of many different mutational events (missense, nonsense, frameshift, splicing), gain of function requires a very specific change. Interestingly, homozygous loss-of-function mutations of either gene cause neonatal hyperinsulinism and severe hypoglycemia.

Another type of neonatal diabetes, due to mutations in the insulin gene, is a good example of gain of abnormal function. Proinsulin peptide, misfolded as a result of a point mutation, accumulates in β-cells and causes apoptosis via an endoplasmic reticulum stress response (Colombo *et al.*, 2008). Through a similar mechanism, heterozygous mutants in Neurophysin II, the precursor that is cleaved into vasopressin and oxytocin, cause familial diabetes insipidus (vasopressin deficiency) by accumulating in and destroying the hypothalamic neurons (Ito *et al.*, 1997) whose projections form the posterior pituitary.

Complex Genetic Traits in Endocrinology; Diabetes as a Paradigm

Recent discoveries have confirmed previous epidemiology pointing to an important role for genetics in such conditions as type 1 or type 2 diabetes (T1D, T2D), Hashimoto's thyroiditis and Grave's disease, short stature, delayed puberty or osteoporosis. More importantly, these studies have come a long way in defining loci in the human genome with variants that measurably increase or decrease risk for each of these diseases. Most of this knowledge was acquired in the past 10 years, thanks to dramatic breakthroughs in human genetics methodology.

Absence of a Mendelian mode of inheritance does not mean that the phenotype is not dependent on genetics. It simply means that the genetic effect is mediated by the cumulative influence of a large number of genetic variants, each of which increases or

decreases disease risk. Most of these variants are single-nucleotide polymorphisms (SNP). Epidemiological evidence indicates that virtually every human phenotype that has been examined has a heritable component.

Genetic Epidemiology

The proportion of disease risk driven by genetic predisposition varies by disease and represents the heritability of the phenotype, defined as the fraction of risk explained by inherited genetic variants. It is assumed that the remaining represents environment but stochastic events may also play a role, as demonstrated in cancer. Heritability is not directly measurable and is context-dependent: uniformity in environmental exposure decreases variance, but a larger proportion of it is explained by genetics, while in the presence of large variation in environmental exposure genetics will explain a smaller fraction of disease risk.

A useful indicator of how much genes contribute to a disease is familial clustering, the simplest measure of which is the sibling recurrence risk (λ_S), the probability of disease in the sibling of a disease case over that in the general population (Risch, 1990). It can vary from less than double to many-fold. How much the λ_S may reflect shared environment, can be determined by twin and adoption studies.

Type 1 Diabetes (T1D)

Among complex traits, T1D (due to autoimmune destruction of the pancreatic β-cells) has one of the highest rates of familial clustering ($\lambda_S = 15$) (Risch, 1990). Concordance in monozygotic twins is >50%) (Redondo *et al.*, 2008), an order of magnitude higher than in dizygotic twins, who are no different from ordinary siblings (Redondo *et al.*, 2004) at about 5%. Therefore little, if any, of the λ_S can be accounted for by shared environment, including intrauterine environment. The term "shared" must be stressed here, to distinguish from population-level exposures which must play an important role: The several-fold increase in T1D incidence over a few generations in most countries studied (Onkamo *et al.*, 1999) can only be explained by environmental changes —but these must be at the population level, thus having no effect on the λ_S. Although cow's milk, viruses, vitamin D sufficiency and many other factors have been proposed, there is no conclusive evidence for any specific one of them.

Even prior to modern molecular genetics, T1D came up as one of the diseases most strongly associated with serological HLA types at the major histocompatibility complex (MHC) locus (Cudworth and Woodrow, 1974). This family of proteins, encoded by a cluster of genes on Chr6p, is associated with most autoimmune diseases. The protein products have a crucial function in adaptive immunity, including self-tolerance development, by presenting antigen to T-lymphocytes. More importantly, they are by far the most polymorphic human proteins and the main reason for allograft rejection. Each gene of class I (HLA − A, − B, − C) or class II (HLA − DP, − DQ, − DR) has hundreds of protein alleles, differing at multiple amino acids. Although not as specific as antibodies or T-cell receptors, the various alleles of each protein do have some selectivity for binding antigenic peptides. By increasing the range of antigenic peptides that can be presented, new mutations at the antigen-binding parts of these proteins have a selective advantage and are tolerated because of the absence of specific function, other than binding arbitrarily different peptides. Studies of large cohorts have mapped T1D risk mostly to alleles on the highly polymorphic exons 2 of DQB and DRB that encode the β-chains of DR and DQ. Because of the tight linkage disequilibrium (LD) between the two genes it is not possible to map T1D risk to one of them but probably both are important. Specific haplotypes of alleles, characterized by a mutation of the highly conserved 57Asp to another amino acid at DQB, confer a relative risk (RR) of 10-fold or more (Erlich *et al.*, 2008), an order of magnitude stronger than most effects in complex-trait genetics, especially given the frequency of these alleles (~15% of Europeans carry at least one). By contrast, the highly protective DQB*0602 (allele index follows asterisk) has a RR <0.1 (Erlich *et al.*, 2008).

The genetic locus conferring the second strongest effect after HLA is at the insulin gene itself at Chr11. A single allele at a common promoter polymorphism (40% of Europeans carry at least one allele) decreases T1D risk to half ($RR = 0.5$) by enhancing AIRE-activated insulin expression in the thymus (Vafiadis *et al.*, 1997), assuring robust self-tolerance to the main T1D autoantigen. Almost equally strong is the effect of the relatively common (9% in Caucasian chromosomes) 620Arg > Trp variant of *PTPN22*, the gene encoding the immune-cell specific LYP phosphatase (Polychronakos and Li, 2011) that modulates T-cell receptor signaling but also affects antigen-presenting cells via less-well understood mechanisms (Li *et al.*, 2017).

In the past decade, technical breakthroughs enabled genome-wide association studies (GWAS) that compare allele frequencies at all common polymorphisms in cases versus controls. This has raised the number of known T1D loci to over 50, but the effect on risk is much lower than described above, the RR ranging from 1.1 to 1.3 (**Fig. 1**). Nevertheless, collective examination of all these genetic effects allows the calculation of a genetic risk score (GRS) (Clayton, 2009) on the basis of which a diagnostic-test ROC (receiver operator characteristic) curve can be constructed (**Fig. 2**). This curve allows us to trade sensitivity versus specificity by choosing a cut-off along the GRS continuum and indicates important potential utility in T1D prediction/prevention. The discovery of an effective immune intervention to prevent T1 is currently pursued by a multitude of clinical trials. If one is found, selection of subjects to treat will be of extreme importance. Autoantibody testing allows quite reliable T1D prediction (Sosenko *et al.*, 2015) but it is logistically not feasible to apply to the entire population with yearly repeats of negative results. Genetic pre-screening can confine the testing to 18% of the population and still capture 80% of future T1D cases (**Fig. 2**).

Risk prediction may not be the most important benefit from these genetic studies. Genetic association loci for complex traits could be a rich source of pathophysiological insights not easily obtainable by conventional research. Examples are the genetics-driven discovery that insulin is expressed in the thymus and modulates risk, which has led to efforts to discover ways of enhancing

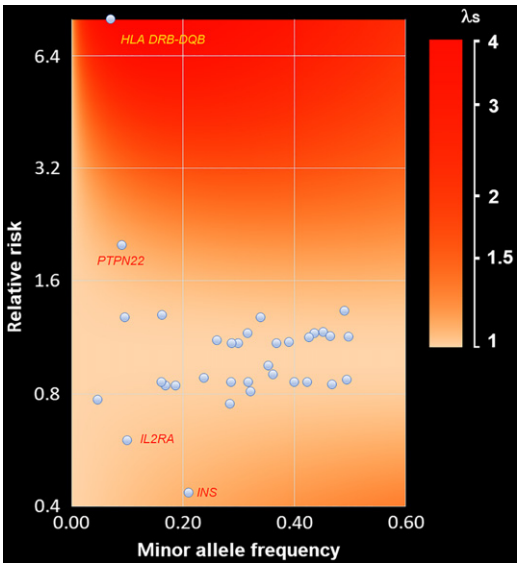

Fig. 1 Genetic loci predisposing to T1D, plotted by minor allele frequency (MAF) and relative risk, the two determinants of their contribution to the sibling recurrence rate (λ_S). The relative risk is based on the odds ratio. The background heat map indicates a locus contribution to the λ_S for any combination of MAF and relative risk.

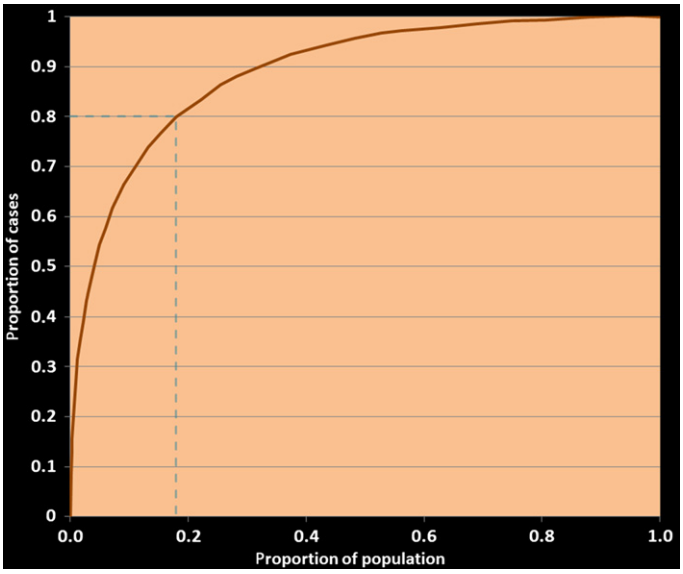

Fig. 2 Receiver operator-characteristic curve for genetic risk score (calculated in Clayton, 2009) based on known T1D loci. It plots the frequency of each risk score value in cases as a function of its frequency in the general population, that is, the sensitivity versus 1-specificity if that value is used as the cut-off. As an illustration of possible practical application, 80% of future diabetics can be predicted from within 18% of the general population.

its thymic expression (Yang *et al.*, 2012), or the discovery that the 620Trp risk allele of LYP confers gain-of-function, leading to a search for inhibitors (He *et al.*, 2013).

Type 2 Diabetes

Concordance in siblings for T2D is much higher than the 5% seen in T1D but because T2D is an order of magnitude more common, the λ_S is actually lower, estimated at two- to threefold over the general population (Hemminki *et al.*, 2010). As might be expected from this, the genetic effects of the well over 100 risk loci discovered by GWAS for T2D are small. The strongest allelic relative risk by a common variant is 1.5 and maps to an intronic SNP in *TCF7L2* (transcription factor 7-like 2) (Grant *et al.*, 2006).

TCF7L2 is a widely expressed transcription factor that regulates the expression of many genes. Its own expression is modulated by the T2D-associated variant but how it determines T2D risk is still unclear and controversial. A large variety of mechanisms have been proposed (reviewed in Florez, 2017) but which of these are true findings, remains to be seen.

Given the weak effects and the much stronger role of the environment in T2D, genetic prediction is not nearly as powerful as that of T1D. A ROC curve based on genetic score only marginally improves prediction based on clinical information (family history, adiposity, physical examination).

Nevertheless, the use the genetic findings to gain valuable functional insights into T2D pathophysiology and advance more specific and effective treatments is an active research field worldwide, stimulated by the high prevalence and devastating long-term morbidity of the disease. The first step toward such effort is to identify the gene and the variant involved in the risk locus. Contrary to the prevailing narrative, GWASs do not discover "disease genes." Most risk variants discovered in these studies are not inside the coding part of a gene and must affect function through effects on transcription. Even if the risk has been mapped to a single variant (a daunting task in itself, because of LD, arising from the historical coinheritance of neighboring variants), it could be affecting the expression of any one of several candidate genes that happen to have expression-regulating elements within the recombination interval (sometimes even if its protein-coding region is outside it). Recent progress in mapping tissue-specific epigenetic changes in the different human cell types by the Encode (Encyclopedia of DNA Elements) consortium and other efforts (Kellis *et al.*, 2014), have produced maps of methylation, histone modification and specific transcription binding sites that can be superimposed on the genetic mapping to drastically narrow down the possible gene-variant candidate pairs (Polychronakos and Alriyami, 2015). Positive identification of the gene involved is the first step towards developing therapeutics to target it. At least two GWAS loci map to genes (DIAbetes Genetics Replication and Meta-analysis (DIAGRAM) Consortium *et al.*, 2014), *PPARγ* and *KCNJ11*, targeted by existing drugs (thiazolidinediones and sulfonylureas) developed by conventional research. This justifies the expectation that many other such drug-gene pairs exist to be discovered starting from genetic rather than functional evidence. This is possible even starting from very weak effects; a weak genetic effect does not necessarily mean that the gene is unimportant in the disease process. It may rather be due to a very weak effect of the polymorphism on a gene extremely important in the disease and an excellent drug target.

The Missing Heritability

Typically, each variant conferring risk to T1D, T2D, or any other complex disease, explains a small part of the heritability. Even collectively, all known variants for each disease leave a substantial part of the heritability unexplained, a situation referred to as the "missing heritability." In T1D, a rare "success story" among complex traits, only about 20%–30% of the heritability remains to be explained. For T2D, this proportion is at best 50%. The missing heritability maps to a very large number of undiscovered genetic loci, each of which confers risk too small to give a signal in genetic studies (Lee *et al.*, 2011). This does not diminish the importance of genetics, it simply highlights the complexity.

Prospects for the Future

It is expected that studies building upon genetic findings in both monogenic and polygenic disease will continue to generate insights that will eventually lead to improved therapies. Although most of these therapies need not include correction of the actual disease-causing genetic variant, gene therapy is a possibility for the future. Like gene therapy for most other types of diseases it must, somehow, overcome the problem of applying it on a very large number of fully differentiated somatic cells and of reversing developmental defects that have already occurred in utero. In the foreseeable future, efforts to develop such approaches are likely to be confined to endocrine diseases for which no satisfactory conventional treatment exists.

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Relevant Websites

http://omim.org/—OMIM (online Mendelian Inheritance in Man). Full catalogue of genetic diseases.
http://genome.ucsc.edu/cgi-bin/hgGateway—The UCSC Genome browser.
https://www.cancer.gov/types/thyroid/hp/medullary-thyroid-genetics-pdq—Professional version of review of endocrine neoplasias, NIH.

Endocrine Epigenetics, Epigenetic Profiling and Biomarker Identification

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Introduction

The field of endocrinology investigates the secretion of hormones into the circulation by endocrine glands in response to a stimulus, the signaling of these hormones through the different axes in the body, how hormones influence the body's homeostasis and how abnormalities in endocrine function can lead to diseases. A growing body of evidence now suggests that alterations to the epigenome may be implicated in the pathophysiology or serve as biomarkers of endocrine diseases. Epigenetics is an emerging frontier of science that has provided quantum leap advances in our understanding of mechanisms playing prominent roles in normal developmental processes or in disease etiology. Epigenetics, as a simplified definition, refers to heritable but reversible modifications to the genome that control gene expression without changing the DNA sequence. However, to define how perturbations to the epigenome during one's life could be implicated to the onset of endocrine disorders, we must better understand the complex interactions that exist between genetics, epigenetics, and environmental factors. The scientific interest for epigenetics research has increased over the past decade, in large part because of recent advances in next-generation sequencing technologies that now make high-throughput and quantitative experiments possible. In this introductory article, we will define some of the terms and concepts related to epigenetic modifications, review some next-generation sequencing-based approaches to establish genome-wide epigenetic profiles, as well as provide examples of how dysregulations of epigenetic marks can be used as biomarkers in endocrine disorders.

The Many Faces of the Epigenome

Epigenetic Information and Chromatin Structure

In its original sense, Waddington referred to epigenetics as all molecular pathways modulating the expression of a genotype into a particular phenotype (Waddington, 1968). Over the years, the definition of the word epigenetics gained in complexity as it evolved in parallel with the discovery of additional key players and epigenetic mechanisms. Today, epigenetics can be described as a dynamic machinery that regulates gene expression by shaping the chromatin conformation without modifying the DNA sequence itself. This dynamic machinery comprises the addition of chemical modifications directly on the DNA sequence (e.g., DNA methylation) or on proteins (e.g., histone modifications), or interactions with noncoding RNAs. These epigenetic modifications can either act independently or act in a cooperative manner to regulate gene expression patterns.

To fully grasp the concept of epigenetics requires an understanding of chromatin organization (reviewed in Gilbert *et al.*, 2005; Dillon, 2006; Woodcock and Ghosh, 2010; Hubner *et al.*, 2013). The basic repeating structural and functional unit of the chromatin is the nucleosome, which is formed by the wrapping of 146 bp of DNA around an octamer of histone proteins (2 × H2A, H2B, H3, and H4). These core histone proteins have N-terminal tails, characterized by high lysine content protruding from the nucleosome, that give rise to an overall positive charge and contrasts with the surrounding negative DNA charge. Following nucleosome formation, the chromatin fiber will further compact into higher-order chromatin structures in order to fit inside the nucleus. Chromatin accessibility, nucleosome occupancy and 3D conformation of the genome are also important components of epigenetic mechanisms. Here, we will focus on the chemical modifications that are directly added to the residues of DNA or histone tails, as well as the implication of non-coding RNAs (ncRNAs) in post-transcriptional gene regulation.

DNA Methylation

In mammals, the majority of DNA methylation occurs on cytosines that precede a guanine nucleotide (CpG). Approximatively 30 million CpGs are found across the human genome, with 60%–80% being methylated in somatic cells. The presence of DNA methylation on the genome can directly influence the structure and compaction of the chromatin, thus promoting either activation or repression of transcription depending on the genomic location, background and factor occupancy (reviewed in Zhu *et al.*, 2016). In CpG rich promoters, the presence of DNA methylation correlates with transcriptional repression, whereas gene body DNA methylation was recently linked with active expression (Yang *et al.*, 2014). Methyl groups are added and maintained by enzymes from the DNA methyltransferase family (DNMT) and can be actively removed by ten eleven translocation (TET) enzymes (Bestor, 1988; Okano *et al.*, 1998; Tahiliani *et al.*, 2009; Ito *et al.*, 2010; reviewed in Messerschmidt *et al.*, 2014; Yin and Xu, 2016). DNMT1 is responsible for DNA methylation maintenance through cellular divisions, DNMT2 specifically methylates residues of transfer RNAs (tRNAs), DNMT3A and B catalyze de novo methylation, and DNMT3L, which lacks methyltransferase activity, stimulates de novo methylation and acts as a regulator of maternal imprint establishment (Bourc'his *et al.*, 2001; Reik *et al.*, 2001; Hata *et al.*, 2002; Kaneda *et al.*, 2004; reviewed in Messerschmidt *et al.*, 2014). DNA methylation patterns are established in the first

days of embryonic development during a fundamental reprogramming wave that erases and re-establishes most methylation marks across the genome, except for those at imprinted genes and other specific sequences that are protected from reprogramming (reviewed in Seisenberger *et al.*, 2013; Messerschmidt *et al.*, 2014). An absence of DNMT1 or DNMT3A/B during embryo development is lethal, as methylation profiles will not be established or maintained properly (Okano *et al.*, 1999; Ruchirawat *et al.*, 1987; Kurihara *et al.*, 2008; Arand *et al.*, 2012; McGraw *et al.*, 2013, 2015). The adequate establishment of DNA methylation patterns in early fetal development is of great importance; early perturbations could affect the developing endocrine system leading to a disease later in adulthood, such as obesity and type 2 diabetes (Law *et al.*, 1992; Stocker *et al.*, 2005).

Histone Modifications

Posttranslational modifications (e.g., methylation, acetylation, phosphorylation) on the histone tails can occur to varying degrees and are predominantly added on lysines (K) by histone modifying enzymes, although other residues such as arginine and serine can also be modified. Histone modifications are reversible, for instance, histone acetyltransferases (HATs) add acetyl groups onto lysines whereas histone deacetylases (HDACs) are responsible for their removal. Genome-wide chromatin analyses in mice and humans have shown that commonly repressed regions of the genome overlap with specific histone modifications (e.g., H3K27me3) and DNA methylation marks, whereas active regions containing H3K4me3 and H3K27ac are essentially depleted of DNA methylation (Balasubramanian *et al.*, 2012; Reddington *et al.*, 2013; Cao *et al.*, 2002). Transcriptional activity can also precede the deposition of histone modifications (i.e., H3K4me3 and H3K27me3) as recently observed in mouse embryonic stem cells (Galonska *et al.*, 2015). Although specific histone modifications have been correlated with permissive or repressive chromatin structure (Jenuwein and Allis, 2001; Strahl and Allis, 2000), the dynamic addition and removal of histone marks by histone modifying enzymes is more complex and context-specific than originally thought (reviewed in Henikoff and Shilatifard, 2011).

Non-Coding RNAs

ncRNAs are functional RNA molecules that regulate gene expression at the transcriptional and posttranscriptional level. Their implication in the control of epigenetic pathways goes beyond the classical portrait of epigenetic regulation (reviewed in Peschansky and Wahlestedt, 2014), with many ncRNAs essential for accurate targeting of histone modifying complexes, chromatin remodeling and DNA methylation processes for instance. Epigenetic associated ncRNAs consist of short (<200 nt: microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs)) as well as long (>200 nt: long ncRNAs (lncRNAs)) untranscribed RNA molecules. Many of these ncRNAs exhibit cell-specific, tissue-specific and/or developmental stage-specific expression and have functional roles in promoting or maintaining tissue identity.

Epigenetic Profiling

With the emergence of next-generation sequencing technologies (NGS) combined with in depth bioinformatics analyses, unprecedented advances in genome-wide epigenetic profiling have been accomplished. However, many factors (e.g., amount and quality of sample, required sensitivity and specificity, bioinformatic resources, cost) have to be considered when choosing the most appropriate method in order to produce unbiased results. Recent advances now allow for the processing of low input samples for many of these genome-wide epigenetic approaches, an essential feature when profiling limited quantities of patient material. Furthermore, with the emergence of single-cell technology, many genome-wide epigenetic profiling assays now give single-cell resolution of profiles. Such improvements will further refine our understanding of intercellular heterogeneity at a cell-type specific level in resistant versus non-resistant cells following drug treatment (Kim *et al.*, 2015; Schmidt and Efferth, 2016) or help monitor disease progression (reviewed in Clark *et al.*, 2016). A brief overview of some of these high-throughput NGS techniques will be presented in this section.

DNA Methylation

Sodium bisulfite conversion is still considered to be the gold standard for establishing methylation differences at a single-nucleotide-based resolution. This chemical treatment converts non-methylated cytosines into uracils while methylated cytosines are protected. Amongst the most widespread techniques using bisulfite conversion are Reduced Representation Bisulfite Sequencing (RRBS), Whole Genome Bisulfite Sequencing (WGBS) and Methyl-Seq. As its name suggests, RRBS reduces the portion of the genome that is sequenced by selecting candidate regions of the genome based on methylation-insensitive restriction enzymes to enrich for CpG-containing fragments. The advantages of RRBS include the reduction of genome complexity hence reducing sequencing costs, as well as allowing the profiling of approximately one million CpG dinucleotides located across the genome (e.g., gene promoters, CpG islands, gene bodies, repetitive elements, intergenic regions) (Boyle *et al.*, 2012; Meissner *et al.*, 2008; McGraw *et al.*, 2015). On the other hand, WGBS allows genome-wide DNA methylation profiling of all CpGs, which provides the best alternative to identify all possible differentially methylated regions (DMRs). However, since only a small portion of the genome has the potential to be differentially methylated, sequencing it at a single-base resolution is normally not required.

The associated high sequencing costs still limit the widespread application of WGBS. The Methyl-Seq approach circumvents some of the caveats of RRBS and WGBS by enriching for fractions of the genome that contain only regions of interest by using bait sequences (Ivanov *et al.*, 2013; Wang *et al.*, 2011; Hing *et al.*, 2015). These targeted enrichment approaches (i.e., Methyl-Seq) are fully customizable and can be designed for any reference genome. For now, readily commercially available enrichment kits are limited to humans, mice and rats, and can investigate up to 3.7 million CpGs across the genome (e.g., promoters, CpG islands, shores and shelves, DNaseI hypersensitive sites, RefGenes).

The main disadvantage of sodium bisulfite-based approaches is that they do not discriminate between methylated cytosines (5mC) and hydroxymethylated cytosines (5hmC). To overcome this problem, it is necessary to separate the two modifications and combine classic bisulfite sequencing with oxidative bisulfite sequencing (oxB-Seq) (Booth *et al.*, 2013) or Tet-assisted bisulfite sequencing (TAB-Seq) (Yu *et al.*, 2012) in order to differentiate 5mC from 5hmC. Worth mentioning are the Illumina human methylation arrays, a widely used nonsequencing-based method to detect DNA methylation profiles.

Histone Modifications

For most, the method of choice to define the exact location of histone modifications at a genomic scale is chromatin immunoprecipitation (ChIP) coupled to NGS. Using native or cross-linked chromatin and specific antibodies for the modification of interest (e.g., H3K4me3), chromatin fragments bearing the modification are immunoprecipitated, isolated and used for sequencing library preparation. The genomic localization of the proteins is deduced from the identification of the accompanying DNA molecule, which is achieved by next-generation sequencing (ChIP-seq). This approach can also be used to study the presence and localization of histone variants (Zhang *et al.*, 2005; Barski *et al.*, 2007). With these techniques, the potential for introducing methodological biases is not negligible. The quality/specificity of the antibody directly influences the specificity of the results, while neighboring proteins that could mask the epitope will impact histone/chromatin preparation methods, such as fixation and sample fragmentation (reviewed in Bernstein *et al.*, 2007; Schones and Zhao, 2008).

Non-Coding RNAs

The investigation of ncRNAs is relatively straightforward thanks to reliable whole transcriptome amplification procedures. The sensitivity and specificity of NGS-based approaches are well beyond microarray ('t Hoen *et al.*, 2008; Wang *et al.*, 2009) as they do not rely on target probe hybridization, thus allowing for the identification of variations in length or composition (Jung *et al.*, 2010) of transcripts at a single nucleotide resolution (reviewed in Zhou *et al.*, 2011). Standard RNA isolation methods for transcriptome studies are applied to identify lncRNA transcripts. However, for short ncRNAs, adapted kits for sample processing (e.g., extraction, amplification) need to be used to either capture or enrich. Further steps are usually needed to add length and to introduce a known sequence used for whole transcriptome amplification. It is noteworthy that lncRNA molecules are most often spliced and that some but not all bear a poly(A) tail of unknown length. RNA isolation based on the presence of a poly(A) tail will fraction the population of lncRNAs, as will a downstream reverse transcription primer using an oligo-dT.

Epigenetic Biomarkers

Extensive efforts have been made to identify epigenetic fingerprints that can be used for predictive risk assessment, diagnosis or prognosis/staging for endocrine diseases. Earlier studies relied on targeted approaches using individual genomic regions known or suspected to be implicated in the etiology of the disease of interest. However, such a strategy remained very challenging since it requires prior knowledge about the selection of loci used as candidate regions. The improvement of microarray-based technologies to investigate DNA methylation (e.g., Illumina's Infinium® HumanMethylation450 BeadChip: >450,000 CpGs) provided a more straightforward tool with increased genomic coverage to determine if discrepancies were observable in larger cohorts of patient samples. Using such methodology, a recent study investigated how epigenetic status correlated with aging and alterations in pancreatic islet function. They found an age-related increase in DNA methylation at 241 sites in the genome, with many of these loci previously associated with type 2 diabetes (T2D) (Bacos *et al.*, 2016). Furthermore, these age-related methylation changes partially overlapped with DNA methylation alterations observed in blood samples from prospective cohorts associated with higher insulin secretion and lower risk of T2D. Illumina has now released a new version of this array with enhanced genomic coverage (MethylationEPIC BeadChip; >850,000 CpGs). Still, to investigate all or most CpGs of the genome, WGBS remains the only available tool. Using WGBS to compare islets from diabetic versus control donors revealed 25,820 differentially methylated regions (Volkov *et al.*, 2017). In this comprehensive study, the authors also integrated RNA-seq experiments to further identify novel diabetes-related changes that contribute to islet dysfunction and impaired insulin secretion in T2D.

The potential implication of histone modifications in biomarker discovery in endocrine-related diseases is still a rather unexplored field. In one example, due to the association of endocrine dysfunction and impaired male fertility, it was suggested that alterations in sperm chromatin packaging and abnormal histone modifications could be used as putative markers for the diagnosis and prognosis of idiopathic male infertility (reviewed in Castillo *et al.*, 2015). Using Chip-Seq to investigate genome-wide histone modification profiles, Hammoud *et al.* showed a reduction of H3K4me3 and H3K27me3 on developmental gene

promoters in most infertile men, although the localization of modified histones was unaltered (Hammoud *et al.*, 2011). Furthermore, they showed that five of seven infertile men had randomly distributed histone retention in their sperm. It will be interesting to see if these observations can be extended to a larger cohort of infertile men and if these findings can be related with specific types of male infertility.

Perhaps one of the most promising areas of research regarding epigenetic biomarkers is related to ncRNAs, as many subtypes can be secreted by cells and detected in biological fluids (e.g., serum, plasma, urine) (reviewed in Butz *et al.*, 2016; Amri and Scheideler, 2017; Van Roosbroeck *et al.*, 2013). For example, miRNAs were shown to have an emerging role in the effect of endocrine disruptors (Derghal *et al.*, 2016) and are highly stable and abundant in peripheral blood. Long ncRNAs may possibly serve as non-invasive biomarkers. A recent study demonstrated that lncRNAs are abnormally expressed between sera and tissues of endometriosis patients (Wang *et al.*, 2016). The authors also found a unique set of lncRNAs that was associated with disease severity and progression. Altogether, this subset of observations point to the value of epigenetic modifications as potential new biomarkers for a wide array of endocrine associated diseases and disorders.

Conclusions

Epigenetics is one of the fastest growing fields of scientific research. It provides advanced understanding of the mechanisms at play in various diseases. Examples of potential predictive biomarkers for endocrine-associated diseases can be found throughout the literature. With the considerable improvement in genome-wide epigenetic profiling technologies and the rapid increase in publicly available genome-wide epigenetic data sets, many more of these epigenetic biomarkers will be put forward. However, many challenges will need to be resolved before they can truly be implemented clinically (Garcia-Gimenez *et al.*, 2017a,b). One of the most important and difficult task will be to validate some of these biomarkers in larger cohorts. The future of research on epigenetic biomarkers is very promising, as it will allow for new opportunities in prevention and treatment of endocrine disorders.

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Protein and Peptide Hormone Synthesis

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Introduction

Based on their molecular structure hormones can be categorized into groups of amino acid derivatives, peptide and protein hormones and steroids. Hormone production occurs both in specialized endocrine organs containing differentiated hormone-secreting cells, such as the anterior pituitary gland and pancreatic islets of Langerhans, but also in numerous other tissues spread throughout the human body, for example, fat, muscle, and intestine. This significantly widens the definition of an endocrine gland.

Hormone synthesis is controlled by various biochemical and neural signals that induce (sub-)acute fluctuations or periodic rhythms, leading to hourly, circadian, monthly or seasonal variations. After production, peptide and protein hormones are stored in intracellular secretory vesicles for release upon stimulation. These polypeptides generally interact with cell surface receptors in target organs, producing a wide variety of physiological effects. A key feature of polypeptide hormones is that many affect secondary hormonal systems which can exert negative or positive feedback on synthesis or secretion of the polypeptide hormone itself.

Although there is no official delineation peptides are generally considered to consist of 2–50 amino acids, whereas proteins are comprised of 50 or more amino acids and consequently have more complex three-dimensional structures. With regard to hormones, the peptides and proteins can range from the three amino acid structure of thyrotrophin releasing hormone (TRH) to the 140 kDa homodimeric anti-Müllerian hormone (AMH).

Transcription

Deoxyribonucleic acid, better known as DNA, contains the genetic code of an organism and serves as a molecular template for the construction of the working horses of the cell, the proteins. In order to produce proteins (or peptides) messenger ribonucleic acid (mRNA) precursor is first copied from the DNA by the RNA polymerase II complex; this process is termed transcription. This mRNA is a copy of a specific section of the DNA that is termed a gene and will be used for translation into a protein. In contrast to DNA, RNA is composed of a single string of ribose nucleotides. Its bases are adenine (A), guanine (G), cytosine (C), and uracil (U). The latter is the counterpart of the thymine nucleotide (T) present in DNA. By reading the DNA template strand the RNA polymerase ensures the production of a complementary RNA strand through specific Watson–Crick base-pairing: A–U, C–G, G–C, and T–A (DNA–RNA). As such the RNA bases are identical to the complementary or "sense" DNA strand that is not transcribed, with the exception of the T to U conversion. Bases within the RNA molecule are divided into triplets as these represent specific amino acid sequences that will be incorporated into the ultimate protein structure at translation.

Transcription is initiated after recognition of a conserved start region on the DNA termed promoter by a protein complex containing the RNA polymerase II (Cheung and Cramer, 2012) (**Fig. 1**). Several consensus sequences involved in recognition by

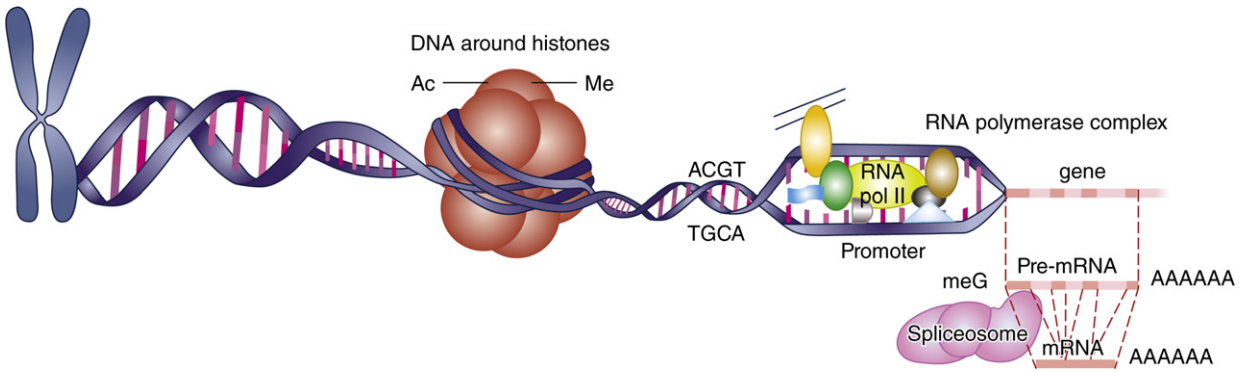

Fig. 1 Transcription. Nuclear DNA is condensed within nucleosomes around histone proteins. In decondensated regions transcription factors can bind to the DNA and guide the RNA polymerase II (RNA pol II) to gene promoter regions. The cumulative interaction between these proteins in the RNA pol II complex leads to induction of transcription and the formation of an RNA molecule that is a copy of the template DNA. The produced pre-mRNA is further processed to include a 5′ methylated guanine cap (meG) and a 3′ poly-adenosine tail (AAAAAA). Large sections of non-coding RNA or introns (white) are removed from the molecule by the spliceosome complex, leading to the mature mRNA molecule.

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the transcription complex have been described, including the TATA box and the initiator element. The TATA box sequence contains T and A nucleotides and is typically located 25 base pairs (bp) upstream from the transcription start site. Specific proteins termed transcription factors recognize and bind to the TATA box or adjacent DNA sequences. These proteins subsequently recruit other proteins including the RNA polymerase II allowing the transcription initiation complex to be formed. After phosphorylation of the C-terminal domain of the RNA polymerase II an open complex is formed and transcription is started with the unwinding of the double helix of DNA (Grunberg and Hahn, 2013). Individual genes can harbor multiple promoter sites allowing for different start sites for the RNA polymerase II. Consequently a single gene can lead to several different RNA molecules with diversity in the 5′ end. The presence of different cell-specific transcription factors can control the creation of various mRNA molecules from the same gene in different cells.

The elaborate nature of DNA requires resourceful packaging into a compact structure. This is accomplished in chromatin where DNA is tightly coiled around histone proteins. Functional units in which DNA is packaged around a histone are called nucleosomes. Promoter regions are generally not located within nucleosomes, as the presence of DNA-binding proteins prevents DNA coiling around histones in these regions. This enables easier access of the transcription complex to the DNA. Areas of the DNA that are transcriptionally inactive are generally tightly bound around histones; this is visible as dense chromatin. Histone protein modifications, including acetylation and methylation, alter the histone–DNA interaction allowing for (de-)condensation of chromatin. Through these specific histone modifications, particular DNA regions can be made more or less accessible to transcription factors and the transcription complex (Kulaeva *et al.*, 2013).

Despite the presence of the same DNA molecules in every cell in the human body, different proteins or peptides are formed in the panoply of cell types. For instance, the TSH β-subunit is only produced in the thyrotrophic cells in the pituitary gland whereas the dimeric hormone inhibin is only secreted from Sertoli and granulosa cells in the testes and ovaries, respectively. Transcriptional control is the main effector by which individual proteins or peptides are produced within a specific cell type (Reiter *et al.*, 2017). This form of regulation of expression is due to a highly complex interaction between a variety of transcription factors and the transcription complex. The DNA sequences bound by transcription factors can be located far away from the transcription start site and are thought to interact with the RNA polymerase II through looping of the DNA. Depending on whether the transcription factor stimulates or inhibits gene expression at a specific site it is called a transcriptional activator or repressor. Their corresponding DNA sequences or *cis*-regulatory elements are enhancer or silencer regions, respectively. The presence of tissue-specific transcription factors is responsible for the meticulous RNA transcription process and as such the production of specific proteins within the cells.

The activity of transcription factors can be manipulated by external signals, such as hormones, and by intracellular pathways. For instance, steroid hormones bind and activate nuclear receptors that act directly on the DNA as transcription factors in order to stimulate or repress gene expression. Alternatively, protein and peptide hormones induce intracellular signaling cascades by binding to cell-surface receptors. These pathways, frequently involving kinases and phosphatases, regulate the activity and DNA binding of transcription factors in a cell-specific manner.

Another form of transcriptional control is through covalent modification of DNA by methylation. Methylation of the cytosine nucleotide in CG residues is a common mechanism to impair gene transcription, that is, to switch a gene off (Antequera, 2003). Within the genome these residues are overrepresented in promoter regions, which highlights their importance in the regulation of gene expression. CpG island recognition and envelopment by proteins blocks the transcription complex from binding to the transcription start site.

From the double-stranded complementary DNA a single RNA molecule will be transcribed from 5′ to 3′ with the "anti-sense" strand as template in a 3′–5′ direction. The DNA double helix is transiently unwound for about 10 bp at a time to expose the DNA nucleotides to the transcription complex in order to generate RNA. After the initial pairing of the first two ribonucleoside triphosphates the transcription complex migrates along the DNA while unwinding the helix and adding new nucleotides to the growing RNA molecule. This process is termed elongation and is brought about by a series of elongation factors (Kwak and Lis, 2013). Several RNA polymerases can simultaneously bind a single gene allowing the generation of multiple RNA copies at the same time. A specific stop sequence (AAUAAA) termed termination site leads to cessation of RNA transcription by the polymerase at which point the mRNA and transcription complex are released from the DNA (Porrua and Libri, 2015). The DNA region from initiation to termination site is termed the transcriptional unit.

The pre-mRNA product undergoes further processing in the nucleus to prevent degradation and aid transportation and translation. This is accomplished with the help of the phosphorylated C-terminal domain of the RNA polymerase II that once released from the promoter guides proteins towards the transcript in order to facilitate its processing. During transcription the mRNA guanylyltransferase enzyme modifies the 5′ end of the molecule to add a methylated guanine nucleotide. The second modification is the addition of a poly-A tail. First, a polymerase cleaves the 3′ end of the pre-mRNA at approximately 20 nucleotides downstream of the termination site. This is followed by the addition of a poly-adenosine tail to the newly formed 3′ end.

A protein and small nuclear RNA (snRNA) complex termed the spliceosome removes large parts of pre-mRNA that are not used for the production of proteins (Bertram *et al.*, 2017). These non-coding intervening regions constituting the larger part of most mRNA precursors are spliced out and are known as introns. The mature mRNA molecule is constituted by the remaining protein-coding sequences, the exons. Specific coding sequences denote exon–intron boundaries and are recognized by the spliceosome. Precursor mRNA can be processed into different mature mRNA splice variants or isoforms depending on the location of the splice sites. This is not a random process, but is also regulated in a cell-specific manner. Alternative splicing thus adds to protein diversity

starting from a single pre-mRNA molecule. Only mature mRNAs that have undergone 5′ and 3′ modifications and splicing are allowed to enter the cytoplasm.

Translation

Following transport across the nuclear pores mRNA enters the cytoplasm and is free to interact with ribosomes. This intracellular complex with protein production as its main function is composed of proteins and ribosomal RNA (rRNA) molecules (Hinnebusch, 2017). The ribosome binds to the 5′ cap of a cytoplasmic mRNA molecule (**Fig. 2**). It "reads" the base code of the mRNA molecule and travels in 5′–3′ direction until the start triplet or codon (AUG) is encountered. Protein diversity is increased through the use of alternative start codons. Loose scanning by the ribosomal complex can allow skipping of unfavorable AUG codons and subsequent use of an alternative start codon located downstream.

Subsequently, the ribosome facilitates the formation of a protein commencing with the amino acid methionine that is delivered by a transfer RNA (tRNA). Following the start codon the ribosome continues to read the mRNA molecule whilst adding amino acids on the C-terminal end of the protein string as indicated by the codons. The 64 different triplet codons of RNA bases represent the codes for the 20 different amino acids or 3 stop signals. With the exception of methionine and tryptophan, amino acids are encoded by multiple triplets. Variation in codons representing the same amino acids is mostly encountered in the third or wobble base of the triplet. Specific tRNA molecules deliver the amino acids encoded by the triplets through interaction between the codon and complementary anti-codon. Using an ATP-consuming process the carboxyl group of one amino acid is covalently linked to the amino group of the next amino acid by the enzyme peptidyl transferase. The polymerization of amino acids into the elongating polypeptide is continued until a stop codon (UAA, UAG or UGA) is encountered on the mRNA at which point the

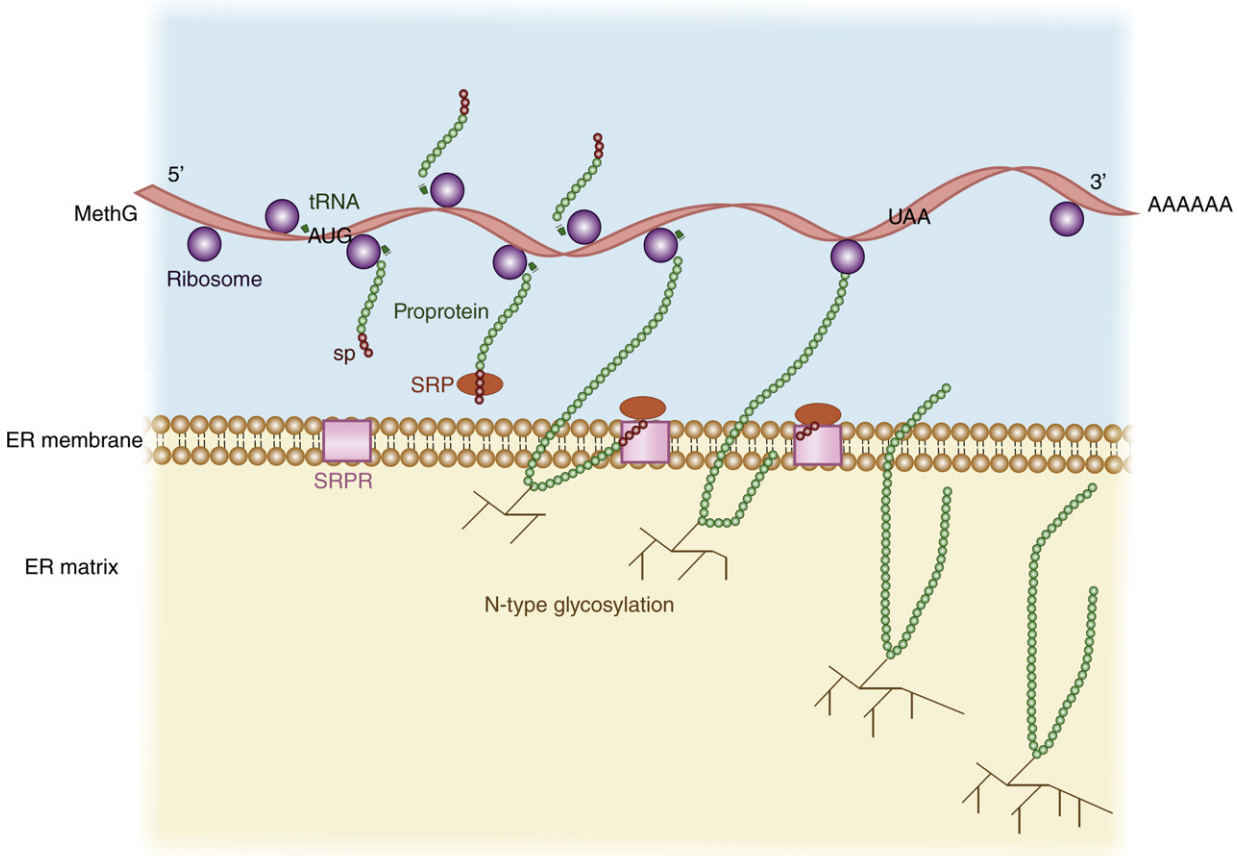

Fig. 2 Translation. Overview of a polysome containing a mRNA strand bound by several ribosomes that travel from 5′ to 3′ and its interaction with the endoplasmatic reticulum (ER). The start codon (AUG) signals the initiation of protein synthesis in which amino acids are successively incorporated into an elongating peptide chain. The emerging signal peptide (sp) is detected by a signal recognition particle (SRP) that guides the protein towards the ER. During translation the protein enters the ER matrix and the signal peptide is cleaved off. Simultaneously, glycosylation occurs at asparagine residues in the protein. Once the ribosome reaches a stop signal (UAA) translation is discontinued and the ribosome dissociates from the mRNA. The complete proprotein translocates into the ER lumen.

protein is released from the ribosome. This leaves the 3′ untranslated region and poly-A tail which are not read for the purpose of protein fabrication. Several ribosomal complexes can simultaneously translate a single mRNA strand, allowing for many copies of protein being produced at the same time in a so-called polysome. The translation process ultimately produces an unprocessed protein called the preprohormone.

Posttranscriptional regulation of protein production is controlled at an additional level through small 21–25 bp RNA molecules that silence translation (Iwakawa and Tomari, 2015). Although previously considered transcriptional noise or non-functioning RNAs, these microRNAs (miRNAs) exert critical, targeted control of up to 30% of protein expression. miRNAs form part of the cytoplasmatic RNA-induced silencing complex (RISC) that pairs to the 3′ untranslated region of mRNA molecules, resulting in their degradation or inhibition of translation.

Given their extracellular communication function protein and peptide hormones need to be secreted from endocrine cells. To that end, newly formed peptide and protein hormones in their prepro-form are prepared for storage in secretory vesicles. The initiation of this pathway towards regulated secretion is accomplished by a 5–10 hydrophobic amino acid signal peptide that is part of the N-terminus of the preproprotein (Nyathi *et al.*, 2013). During ongoing translation this N-terminal specific signal peptide is bound by a cytoplasmic signal recognition particle (SRP). This protein can guide the ribosome with its emerging polypeptide that is being translated towards the SRP receptor on the endoplasmatic reticulum (ER). The ER is a major membrane-enclosed organelle of the cell, which is instrumental in the production of secreted, organelle-bound or trans-membrane proteins and lipids. This guiding process allows for numerous ribosomes being connected to the ER giving it its granular appearance. Following ribosome–ER interaction, the protein will pass the ER membrane through a translocator termed the Sec61 complex and enter the ER. The signal peptide is concurrently cleaved on the luminal side of the membrane by signal peptidase leaving the N-terminal tail of the resulting proprotein inside the ER matrix. Following this co-translational process the production of the proprotein is continued by the ribosome while it protrudes through the ER membrane. After completion of translation the prohormone is released by the ribosome and unlike membrane-bound proteins is completely translocated into the ER lumen. It has now entered the start of the secretory pathway that is required for hormones to function as circulating cell–cell communicators. Meanwhile, the mRNA molecule remains attached to the granular ER as other ribosomes as parts of the polysome remain attached to the ER and continuously use it as a template for translation of new polypeptide chains.

During translation and entrance into the ER and before folding of the protein a cotranslational modification can take place. Many proteins including hormones undergo glycosylation at asparagine moieties, leading to the formation of glycoproteins (Aebi, 2013). A complex oligosaccharide including mannose, glucose and *N*-acetylgalactosamine is transferred from the ER membrane-bound dolichol phosphate to the protein while it is still being synthesized. After transfer further processing of the oligosaccharide chain takes place by glycosidase and glycosyltransferase enzymes. Like other modifications the addition of oligosaccharides has a significant effect on the molecular structure of the protein and its recognition by receptors and other proteins. Also, non-covalent interaction between hormone subunits can arise within the ER and lead to the formation of dimers. For instance, the three anterior pituitary hormones TSH, LH, and FSH all are heterodimers composed of a common glycoprotein hormone α-subunit linked non-covalently to a hormone-specific β-subunit.

Posttranslational Modifications

Once entered into the matrix of the ER prohormones must be transported towards the Golgi apparatus for further processing. In order to execute its main function of protein sorting factory in the cell the Golgi apparatus is composed of several membrane-enclosed compartments called Golgi cisternae (**Fig. 3**). Some peptides or proteins are already matured into end-products within the ER, while others will undergo modification after leaving the ER in downstream sections of the secretory pathway. Transport is accomplished in specialized membrane-enclosed vesicles termed transition elements (Gorelick and Shugrue, 2001). These vesicles bud off from the ER and fuse with the Golgi cisternae. The passage of proteins from the ER to the Golgi apparatus is the rate-limiting step in intracellular transport. Between organelles a highly regulated pathway ensures selective transport of proteins and other luminal molecules. Coat protein complex II (COPII)-coated transition elements make use of surface and target markers, such as the complex Rab protein family and their effectors, to recognize and fuse with their target organelle. Within the budding process of vesicles COPII interacts with acceptor proteins on the outer membrane surface. These acceptor proteins are linked to membrane-bound cargo receptors that bind luminal proteins, like hormones. In this way the transition elements enable specific transport to the Golgi apparatus.

Although the ER exit signals have been elucidated incompletely, the N-type glycosylation can serve as a protein-bound signal for transfer to the Golgi complex. Within the Golgi complex mature hormones are formed. Due to the polarity of the Golgi complex with proteins entering the *cis*-side and leaving through the *trans*-Golgi it is believed that different enzymes work at subsequent sections within the apparatus. In this manner consecutive protein alterations can take place in the functionally distinct compartments of the Golgi complex.

As many peptide and protein hormones are parts of much larger prohormones they need to undergo proteolytic processing. Amino acid residues within the polypeptide, like for instance lysine–arginine or arginine–arginine, mark cleavage sites for the production of active hormones by trypsin-like endopeptidases known as prohormone-converting enzymes (Muller and Lindberg, 1999). Exopeptidases further digest the terminal amino acids of the cleaved products, thereby producing the final length peptide or protein. Within this latter group of enzymes carboxypeptidase E appears crucial in the processing of several peptide hormones,

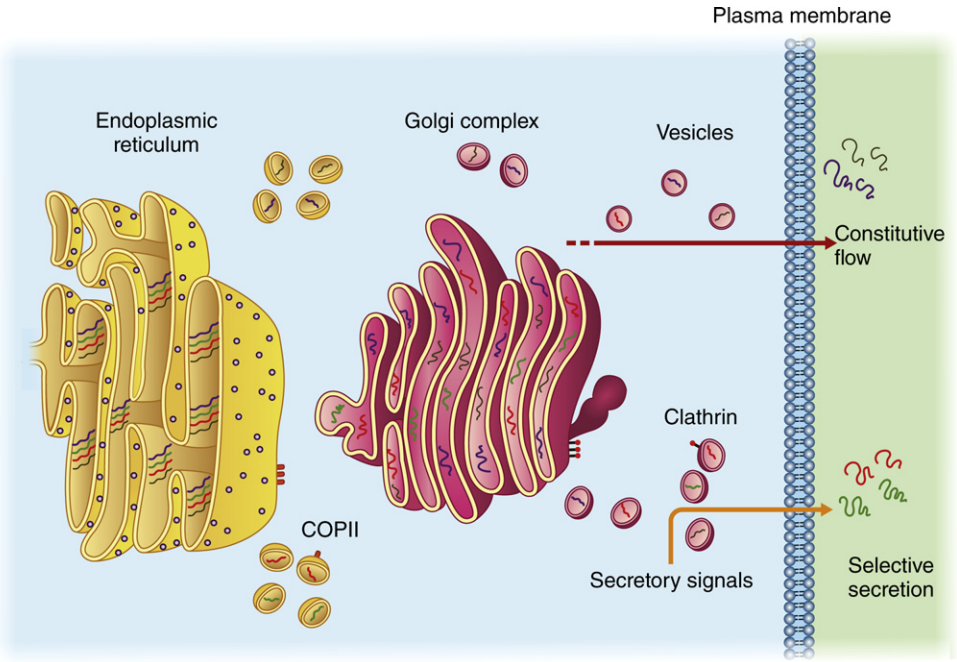

Fig. 3 Posttranslational transport. Proteins destined for secretion are sorted within the endoplasmatic reticulum for transport to the Golgi complex. The hormones are packaged in Coat protein complex II (COPII)-coated vesicles that travel to the Golgi complex and fuse with its membrane. During transport through the Golgi proteins undergo many posttranslational modifications that lead to their final active form. From the trans-Golgi network clathrin-coated vesicles bud off and undergo direct secretion at the plasma membrane in a constitutive flow or alternatively await a secretory signal near the plasma membrane for regulated, selective exocystosis.

such as proinsulin and growth hormone (Ji *et al.*, 2017). In many cases prohormones will give rise to the production of a single peptide or protein product. However, single prohormones can also contain multiple bioactive peptides, leading to the term polyprotein. A prime example of this is the pro-opiomelanocortin (POMC) precursor that contains several peptides, namely adrenocorticotrophic hormone (ACTH), α-, β-, and γ-melanocyte-stimulating hormone (MSH), corticotropin-like intermediate peptide (CLIP), β-endorphin and β-, and γ-lipotropin. For POMC this proteolytic processing occurs quite late in trans-Golgi elements and secretory vesicles. There is a tissue selectivity when it comes to specific cleavage of active peptides from the larger precursor. This is for instance illustrated by proglucagon, which is primarily cleaved into glucagon in the pancreatic α-cell or alternatively into glucagon-like peptide-1 (GLP-1) in the intestinal L cell. Several proteolytic steps might be necessary to convert complex precursors. Interestingly, proteolysis can also produce by-products that can be utilized for stabilization of the end-product. The arginine-vasopressin (AVP) prohormone contains the mature hormone and a protein known as neurophysin II that binds and stabilizes its endocrine counterpart. In the case of the cerebral and adrenomedullary hormone enkephalin a single peptide can also be encoded several times within one gene. Processing of its prohormone releases five copies of the methionine isoform and one copy of the leucine isoform of enkephalin.

During Golgi transport two cysteine residues in a protein can be linked to form a disulfide bridge. This reaction is accomplished by disulfide isomerase and leads to stabilization of the three-dimensional molecular structure. This occurs even in small peptides like the 9 amino acid-containing AVP. For the larger insulin precursor molecule this also constitutes a vital part of processing as this connects the alpha- and beta-chains. The interlinking C-peptide can subsequently be removed at a later stage in the secretory vesicle by proteases that cleave at two sites within the molecule. Disulfide bridges can also be formed at this stage between peptide or protein subunits that are encoded by the same or different genes, ultimately forming homo- or heterodimers or multimers. For instance, linkage of an inhibin βA-subunit to another βA-subunit produces the homodimer activin A, whereas connection of the inhibin βA-subunit to an inhibin α-subunit produces the heterodimer inhibin A.

Besides continued processing of the N-type oligosaccharide chain described earlier, the Golgi apparatus hosts O-type glycosylation of serine and threonine residues. In contrast to N-type glycosylation this only takes place after folding of the protein molecule. Surface-exposed serine and threonine moieties are subject to the addition of *N*-acetylgalactosamine followed by formation of highly diverse branched oligosaccharides by glycosyltransferases. Various *cis*-to-*trans* sections of the Golgi complex contain different membrane-bound enzymes involved in these processes leading to compartmentalization of glycoprotein processing. The oligosaccharides can function as signals for recognition molecules in the Golgi like the lectins. This interaction facilitates selective outbound transport in secretory granules towards the plasma membrane for exocytosis.

Together these posttranslational derivatizations result in significant conformational changes in folding of the polypeptide into a three-dimensional structure (Castro-Fernandez *et al.*, 2005). As such these alterations reveal another level of protein diversity

beyond the code that is encrypted into the DNA. Proteins assume their structure according to their amino acid sequence, known as the primary structure, through noncovalent interactions. The resulting polypeptide will spontaneously fold into the secondary structure that is energetically most favorable under the conditions of the solvent to a state called native conformation. Such rotations in backbone structure can be accomplished among others by the formation of α-helices and β-strands leading to complex motifs. Through a myriad of atomic interactions of the amino acid backbone together with the side chains a three-dimensional or tertiary protein structure is accomplished. Chaperone and chaperonin proteins facilitate this process of folding and limit the assembly of ineffective protein conformations. Quaternary structure only applies to a complex comprised of two or more polypeptides that are non-covalently bound.

Secretion

The trans-Golgi network (TGN) compacts the peptide and protein hormones into small membrane-encapsulated vesicles (Tooze, 1998). These clathrin-coated vesicles secure selective transport of relevant soluble proteins in their lumen through interaction with adaptor proteins and membrane-bound cargo receptors. The vesicles bud off from the Golgi and are transferred to the luminal side of the cellular membrane where they await the action of specific secretagogues triggering exocytosis. The secretory pathway can also be non-selective, as a consequence of bulk flow or constitutive movement. The constitutive exocytic pathway occurs in all cell types and directly targets vesicles to the plasma membrane. Molecules entering this pathway generally lack specific signals that allow them to enter other transport pathways.

Endocrine cells have a high dependence on a regulated exocytic pathway that leads to targeted exocytosis and secretion of hormones. To this end the TGN packages products as secretory vesicles that reside near the plasma membrane and wait for signals to start membrane fusion to release their contents extracellularly (Michael *et al.*, 2006). The formation of the secretory vesicles with their accumulation of specific proteins occurs through unknown mechanisms. Although these vesicles contain unique membrane-bound proteins, the process of targeting proteins for this pathway has not been extensively elucidated. Two proteins, secretogranin III and the above-mentioned carboxypeptidase E, are likely contributors to secretory vesicle formation for polypeptides. For hormones like proinsulin and POMC the membrane-bound exopeptidase can act as a sorting receptor: the pro-form of the hormone binds the carboxypeptidase E in the TGN which is followed by packaging into the secretory vesicles. The enzyme subsequently cleaves the prohormone into the final peptide or protein product during transport. Knockout of this enzyme leads to misdirected flow of several hormones via the constitutive pathway (Ji *et al.*, 2017). After budding from the TGN the content of the vesicle is concentrated up to 200-fold and following microtubule-guided transport to the plasma membrane these dense vesicles await a chemical signal for secretion. This often follows after stimulation of intracellular Ca^{2+} or cyclic AMP due to activation of plasma membrane ion-channels or cell-surface receptors, respectively (Seino and Shibasaki, 2005; Pang and Sudhof, 2010). Vesicles also contain the cleavage products, such as C-peptide in the case of insulin, that are co-secreted together with the hormone at the time of exocytosis.

Conclusion

The production and secretion of peptide and protein hormones is a highly evolved pathway on which many intracellular signals exert their influence. Transcriptional control remains the most critical pathway for regulating protein production, but translation and transport from the ER to the plasma membrane of individual hormones are also dependent on a plethora of stimulatory or inhibitory mechanisms. Aberrations in these physiological mechanisms can lead to changes in peptide or protein hormone levels or function and can ultimately result in clinical disease.

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Protein and Peptide Hormone Action

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Introduction

The functioning of cells is largely regulated by external factors such as hormones and neurotransmitters. These external factors may be able to enter the cells by diffusion or specialized transport systems, followed by binding to specific intracellular receptors which can then interact with the genome to stimulate or suppress the expression of specific genes. Signaling molecules which are unable to cross the cell membrane can influence cellular function by binding to receptors that directly affect ion channels. Alternatively, the signal of an extracellular protein or peptide hormone can be transferred to the intracellular milieu as the result of binding of the hormone to its specific receptor, which is characterized by a transmembrane domain. This hormone–receptor interaction results in the activation of a large variety of intracellular signal-response cascades, depending on the signal transduction pathway cognate to the receptor. Many of these pathways have been deciphered in terms of the reactions—mostly phosphorylation—and their substrates, which in turn affect specific aspects of cellular function. It is important to realize that the specificity of the binding of a hormone to a receptor is not always absolute; sometimes different hormones can interact with one receptor (**Fig. 1**). Furthermore, specific intracellular pathways may be stimulated or suppressed by more than one hormone–receptor combination.

After binding of the hormone, the hormone–receptor complex will be internalized into the cell, leading to separation of the complex and degradation of part of the proteins, while part of the receptors may also be recycled to the plasma membrane.

The aim of this article is the description of some examples of these pathways, focusing on the diversity of the underlying mechanisms.

G-protein Coupled Receptors

The family of G-protein coupled receptors (GPCRs) is the largest group of receptors known (Fredriksson *et al.*, 2003). It consists of more than a thousand transmembrane proteins, involved in the transduction of signals varying from the detection of light in the retina to interaction with odorous substances in the nasal mucosa and with hormones and neurotransmitters in many organs. These receptors share a structure formed by seven transmembrane α-helices, connected by three intra- and three extracellular loops with an extracellular NH_2-terminus and an intracellular COOH-terminus. The transmembrane helices have a high degree of sequence conservation, whereas the structure and extracellular part of the protein, which interacts with its ligand, the "first messenger," can show a high variety of structure and complexity. This interaction leads to conformational changes in the structure of the GPCR, which are transmitted to the intracellular part of the protein, making interaction of the receptor with one of different guanine nucleotide-binding proteins (G-proteins) possible. These G-proteins convey the signal of the first messenger to the intracellular machinery as "transducers." Until recently, it was supposed that single GPCR molecules combined with G-proteins only after combination with their ligands. However, more recently it was found that GPCRs can exist as homodimers or even heterotetramers in combination with their cognate G-proteins in an inactive state, to be activated after binding of the relevant hormone (Kasai and Kusumi, 2014; Ferré, 2015).

G-proteins

G-proteins are heterotrimers of α-, β-, and γ-subunits, of which the α-subunit can bind guanosine diphosphate (GDP). Upon binding to the intracellular part of the ligand-activated GPCR the GDP molecule will be exchanged for a molecule of guanosine triphosphate (GTP), leading to dissociation of the α-subunit from the $\beta\gamma$-heterodimer (Morris and Malbon, 1999) (**Fig. 2**). After dissociation, both the α-subunit-GTP complex and the $\beta\gamma$-heterodimer may affect the functioning of the cell. The effects depend on the type of subunit: for the α-subunit at least 23 subtypes are known, encoded by 17 genes. These subtypes can be divided in four categories: stimulating adenylyl cyclase ($G\alpha_s$), inhibiting adenylyl cyclase ($G\alpha_{i/0}$), activating phospholipase C ($G\alpha_{q/11}$) and activating Rho guanine exchange factors ($G\alpha_{12/13}$). Interestingly, the subtype of Gα-subunit activated does not need to be specific: multiple coupling of GPCRs to more than one type of Gα-subunit is possible (Hermans, 2003). After the stimulation, the intrinsic GTPase activity of the α-subunit converts the bound GTP back to GDP, enabling recombination with the $\beta\gamma$-heterodimer and ending the actions of the signal transducers.

The β- and γ-subunit are present in at least 6 and 12 different subtypes, respectively. The $\beta\gamma$-dimers can also affect cellular processes, for example, G protein-regulated inward rectifying potassium channels, and may act as coregulators of Gα-effectors such as adenylyl cyclases or phospholipase C (Birnbaumer, 2007).

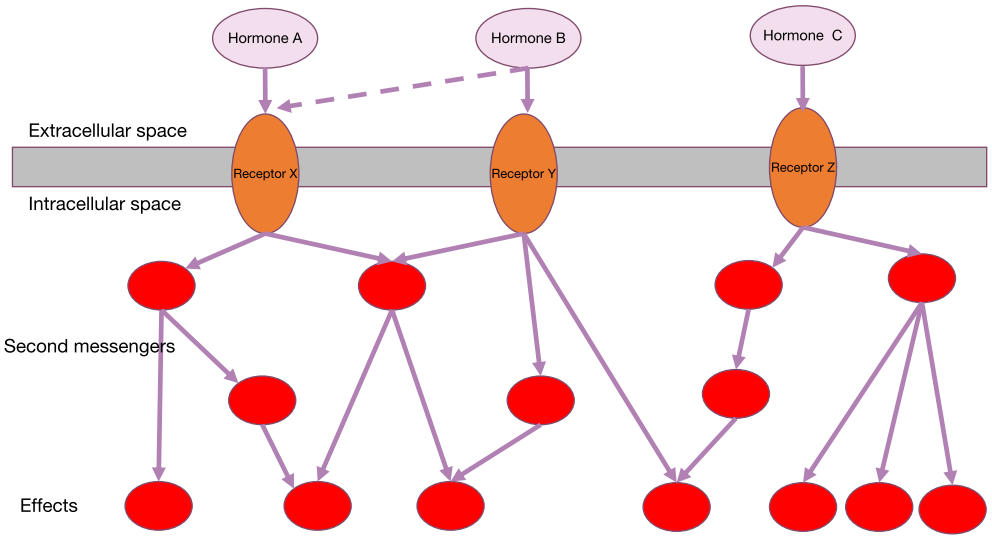

Fig. 1 General scheme for transduction of the signals of protein and peptide hormones into the cell. Hormones can bind to different transmembrane receptors that upon activation induce intracellular signaling cascades. There is a wide variety of second messengers in the cell as well as a multitude of physiological effects that can be regulated by hormones. Often activation of a single receptor or second messenger is transmitted to several downstream targets, thereby amplifying the signal. The large degree of overlap between individual hormonal cascades is caused by the use of the identical receptors or second messenger systems.

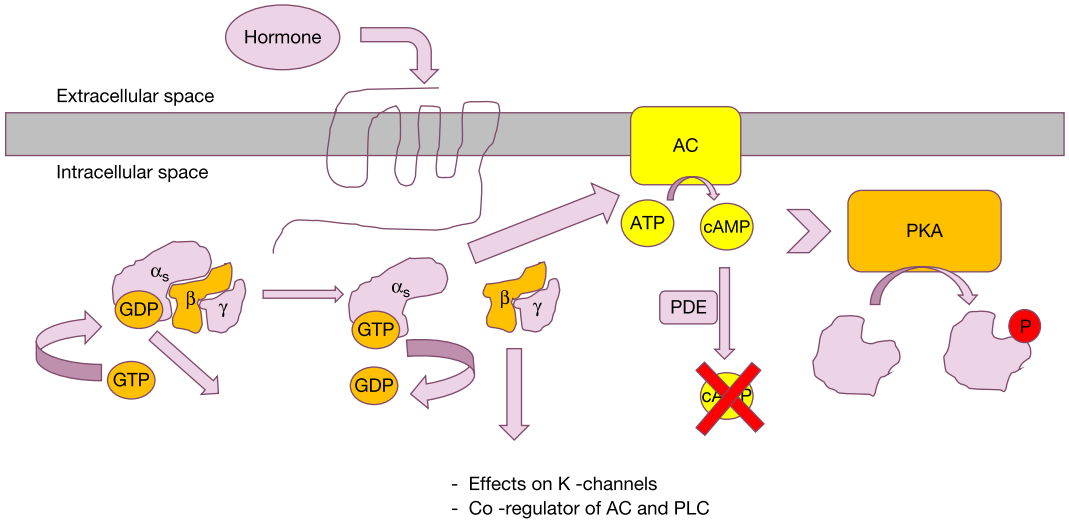

Fig. 2 Signal transduction from GPCR through action on G-protein's α_s- and $\beta\gamma$-subunits to adenylyl cyclase (AC) to production of cAMP to protein kinase A (PKA) to phosphorylation of proteins and effects on potassium channels, respectively.

Effectors of the GPCR-Generated Signals

As mentioned above, membrane-bound adenylyl cyclases can be activated or inhibited by $G\alpha_s$ or $G\alpha_{i/o}$, respectively, stimulating or inhibiting the conversion of ATP to cyclic AMP (cAMP). Nine different subtypes of membrane-bound adenylyl cyclase (ACs 1–9) have been described, each with its own tissue distribution, although many cell types express more than one subtype (Dessauer *et al.*, 2017). In addition, a soluble adenylyl cyclase (AC10) was found mainly in the spermatogenic cells in the testis. Compartmentalization of the different subtypes, in combination with differences in affinity for the various G-protein α-subunits can lead to a specific intracellular localization of the effect of activation of a specific GPCR. cAMP will interact with cAMP-dependent protein kinase (PKA), leading to phosphorylation of specific proteins at specific sites. cAMP response element-binding protein (CREB), a PKA target, can act as a transcription factor upon phosphorylation, controlling the expression of a multitude of target genes. Finally, the effect of phosphodiesterases (PDEs) on cAMP brings the stimulatory action to a halt by breaking the diester bonds between the phosphate and adenylyl groups. The receptor for adrenocorticotrophic hormone (ACTH) is an example of a $G\alpha_s$-protein coupled receptor. Binding of ACTH to this receptor on adrenocortical cells activates the GPCR leading to intracellular

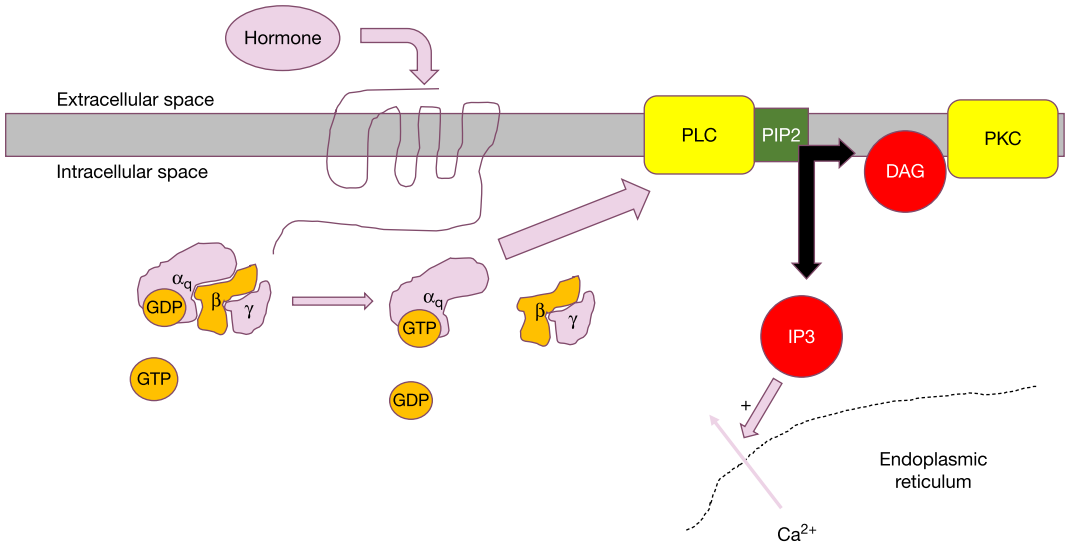

Fig. 3 Signal transduction from GPCR through action on G-protein's α_q-subunit to phospholipase C (PLC), leading to production of diacylglycerol (DAG), stimulating protein kinase C (PKC) and of inositol 1,4,5-trisphosphate (IP_3).

cAMP production. PKA subsequently stimulates adrenocortical steroid synthesis by phosphorylating the steroid acute regulatory protein, which delivers the steroid precursor cholesterol to the mitochondria, and by a CREB-mediated rise in gene expression of multiple steroidogenic enzymes.

Under the influence of $G\alpha_{q/11}$ phospholipase C (PLC) can be activated to cut the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) on the glycerol side of the phosphodiester bond resulting in the formation of 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (**Fig. 3**). IP_3 will promote the release of Ca^{2+} ions from the smooth endoplasmic reticulum, leading to increased intracellular calcium concentrations, while DAG can stimulate protein kinase C (Kadamur and Ross, 2013). The α1-adrenergic receptor is an example of a $G\alpha_{q/11}$-coupled receptor. Activation by its ligands adrenalin and noradrenalin induces smooth muscle contraction through a PLC-dependent mechanism.

Finally, under the influence of $G\alpha_{12/13}$ Rho guanine exchange factors can be activated. Guanine exchange factors (GEFs) are involved in the activation of small GTPases, which act as molecular switches in intracellular signaling pathways and have many downstream targets (Aittaleb *et al.*, 2010).

Recycling and Break-Down of GPCRs

The endocytic pathway tightly controls the activity of GPCRs. Ligand-induced endocytosis can drive receptors into divergent lysosomal and recycling pathways, producing essentially opposite effects on the strength and duration of cellular signaling via G proteins, and may also promote distinct signaling events from intracellular membranes. This ligand-induced endocytosis is mediated primarily by clathrin-coated pits. For the TSH receptor G-protein activation can continue upon internalization, inducing spatially confined signaling pathways within the cytoplasm (Godbole *et al.*, 2017). Lysosomal sorting of a number of GPCRs occurs via a highly conserved mechanism requiring covalent tagging of receptors with ubiquitin. There is increasing evidence that additional, noncovalent mechanisms control the sorting of endocytosed GPCRs to lysosomes in mammalian cells. Recycling of several GPCRs to the plasma membrane is also specifically sorted, via a mechanism requiring both receptor-specific and shared sorting proteins. The current data reveal an unprecedented degree of specificity and plasticity in the cellular regulation of mammalian GPCRs by endocytic membrane trafficking (Hanyaloglu and von Zastrow, 2008).

Kinase-Linked and Related Receptors

The second group of peptide and hormone receptors constitutes the kinase-linked receptors. These divergent and often multimeric receptors are characterized by an extracellular domain capable of binding the ligand, a single transmembrane domain and an intracellular domain with enzymatic activity. This intrinsic kinase activity is stimulated by binding of the hormone to the extracellular part of the receptor and results in phosphorylation of the receptor itself and of downstream messengers. Depending on the enzymatic activity, receptors are classified into tyrosine kinase or serine/threonine kinase families.

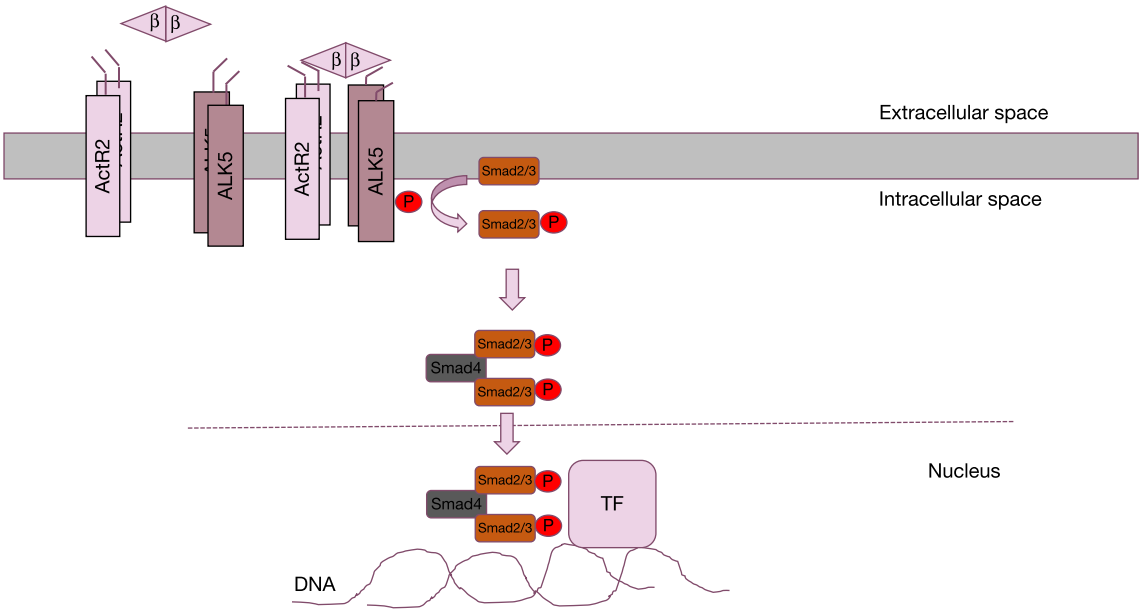

Fig. 4 Mechanism of action of activin on the activity of the activin receptors. Phosphorylation of the type 1 receptor ALK5 leads to phosphorylation of Smad2 or Smad3, which after combination with Smad4 is transported to the nucleus, where it combines with other transcription factors (TF) and regulates transcription of specific genes.

Serine/Threonine Kinase Receptors

Receptors for the TGFβ Superfamily of Growth and Differentiation Factors

The human transforming growth factor β (TGFβ) superfamily of growth and differentiation factors consists of 33 members, most of which are disulfide-bridge linked homodimeric proteins that control developmental processes and adult tissue homeostasis. In addition to TGFβs, this family includes the bone morphogenetic proteins (BMPs), the more restricted subfamily of growth and differentiation factors (GDFs), activins and nodal. TGFβ family members regulate cell growth, differentiation, migration and death, in a developmental context-dependent and cell type-specific manner. For instance, the TGFβs more often inhibit, but sometimes also stimulate, cell proliferation. As TGFβ ligands act multifunctionally in numerous tissue types, they also play complex roles in various human diseases, ranging from autoimmune to cardiovascular diseases and cancer.

All members of this family signal through a comparable sequence of mechanisms (Massagué, 2012) (**Fig. 4**). First, the dimeric growth factor binds to two copies of one of the five type II receptors, all transmembrane proteins with a single membrane spanning domain and a cytoplasmic kinase domain that has serine/threonine kinase activity. Upon binding of the ligand to the type II receptors a heterotetrameric complex is formed, by the additional recruitment of two copies of the appropriate type I receptor; there are 7 known type I receptors. Type I receptors are also known as activin receptor-like kinases (ALKs). Subsequently, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates serine residues in Ser-X-Ser motifs in one of the five specific stimulating (Smad1, -2, -3, -5, or -8) or one of the two inhibiting (Smad6 or -7) receptor-regulated Smad proteins (R-Smads), depending on the type I receptor involved. Two copies of the phosphorylated R-Smad can subsequently be bound to the common Smad4 to form a trimeric complex. Finally, the $(\text{R-Smad})_2$–Smad4 combination enters the nucleus, where it interacts with the chromatin and causes the final signal within the cell by binding to specific sequences in the DNA and enhancing or inhibiting transcription of specific genes.

There are a number of factors, which influence the possible combinations of ligands with the limited number of type II and type I receptors, subsequently leading to specific cellular responses. Finally, the stimulatory effects of the members of the TGFβ-family members ends by dephosphorylation, ubiquitination and proteasome-mediated degradation of the activated R-Smads and activated type II receptors and ALKs (Shi and Massagué, 2003).

TGFβ Signaling

Three highly homologous forms of TGFβ are produced in various cells: TGFβ1, -2 and -3. The mature dimers are secreted noncovalently attached to their pro-peptide (latency-associated peptide, LAP) and are only able to interact with their type II receptor (TGFBR2) after being freed by contacts with cell surface integrins (Shi *et al.*, 2011) provided the TGFβ-coreceptor betaglycan is present in the cell membrane to present the TGFβ-dimer in the right configuration to the receptor (Esparza-López *et al.*, 2001). The TGFβs1, -2 and -3 all signal through binding to the TGFBR2, but the affinities of the three members of the

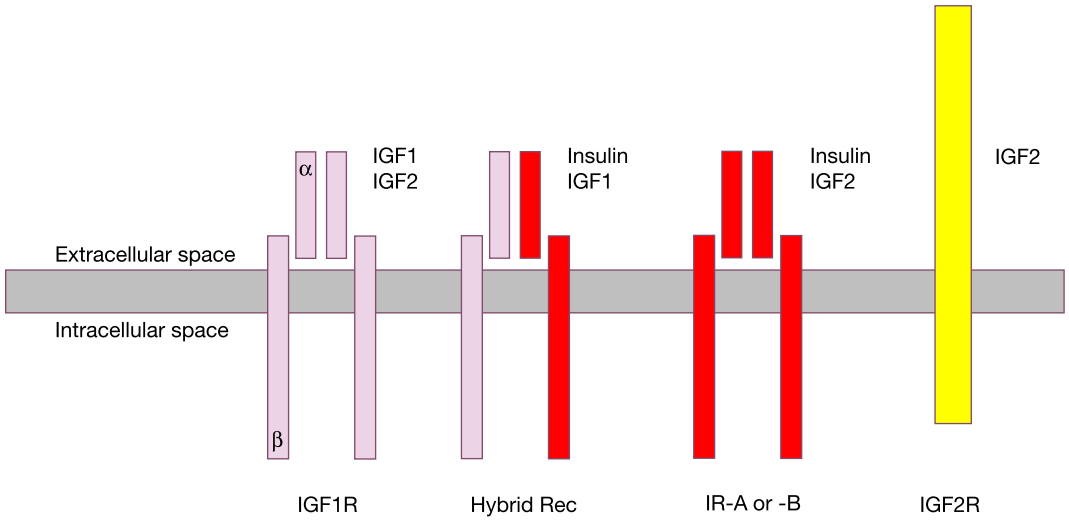

Fig. 5 Structure and combinations of receptors for insulin, IGF1 and IGF2.

subfamily differ. This interaction leads to recruitment of ALK5 into the complex, followed by phosphorylation of R-Smads2 or -3, their combination with Smad4 and the effects on transcription.

In addition to this pathway which is generally used for the signal transduction of members of the TGFβ superfamily, the TGFs can also signal through non-Smad pathways (Moustakas and Heldin, 2009). The TGFBR2 can directly phosphorylate the polarity protein PAR6, which in the end leads to breaking of the tight junctions between mammalian cells, leading to the disintegration of epithelial structure and dedifferentiation of the epithelial cells. An alternative pathway starts by the phosphorylation of proteins by activated ALK5, leading to activation of the mitogen-activated protein kinase (MAPK) signaling cascade, which can regulate cell proliferation or migration.

Signaling by Activin, Inhibin, Nodal, and Myostatin

Activins are combinations of two inhibin β-subunits, βA or βB, connected by disulfide bridges (activin A, activin AB, or activin B). The same β-subunits are part of the inhibin molecule, in which one of the β-subunits is connected to an inhibin α-subunit (inhibin A or inhibin B). Nodal and myostatin (also known as GDF8) are structurally related to activin. Activins, Nodal and myostatin use the Activin-receptors type 2 or type 2B (ActR2 or ActR2B) in combination with ALK4 or ALK5, followed by phosphorylation of Smad2 or -3, which can bind to Smad4 and convey the signal to the nucleus, in the same way as happens with TGFβ. Myostatin is also able to convey information through noncanonical pathways (Walker *et al.*, 2016).

Activin and myostatin can be bound to follistatin in the circulation; this complex is not biologically active. Similarly, Lefty1/2 and Cerberus inactivate Nodal. At the level of the receptor, the inhibins can counteract activin's actions by binding to the ActR2 or ActR2B, but only in the presence of betaglycan, which, apart from acting as a coreceptor for TGFβ, fulfills a similar role for inhibin (Gray *et al.*, 2002).

Signaling by BMPs, GDFs, and AMH

BMPs and the majority of the members of the subfamily of GDFs signal through the activin receptors ActR2 or -2B and the type 2 BMP-receptor BMPR2, combined with ALK1, -2, -3, or -6. GDF1, -3, and -9 signal also through ActR2 or -2B or BMPR2, with ALK4, -5, and -7 as type I receptors. AMH signals by addressing the AMH-receptor type 2 (AMHR2) in combination with ALK2, -3 or -6. Most of these factors use Smad1, -5 and -8 in combination with Smad4 to convey their signal to the nucleus, but GDF1, -3, and -9 signal through Smad2 and -3.

In the circulation, BMPs are bound to the binding proteins noggin and chordin.

Tyrosine Kinase Receptors

The Insulin Receptor Family

Insulin and the insulin-related growth factors (IGFs)1 and -2 signal through the activation of a limited number of receptors: the insulin-receptors (IRs)-A and -B, the IGF type 1 receptor (IGF1R) or the combination of these receptors (**Fig. 5**). An orphan receptor named insulin receptor related receptor (IRR) has also been found. They form a family of highly homologous proteins,

the structure of which is characterized by the presence of an extracellular α-domain, linked by disulphide bridges to a transmembrane β-domain. Both domains are originally synthesized as a single protein, which is cleaved after the formation of the disulphide bridges and subsequently incorporated in the plasma membrane, where they form dimers. IR-A and IR-B are formed from the same gene, where exon 11 can be skipped from its messenger RNA to form IR-A. This leads to the absence of a 12 amino acid sequence from the c-terminal end of the α-domain (Belfiore *et al.*, 2009).

Due to the high homology between the IRs and the IGF1R, they can bind insulin and the IGFs. However, the affinities of the ligands strongly depend on the composition of the receptor dimer: IR-A can bind insulin and IGF2 with high affinity, but IR-B and the hybrid IB-A/IR-B show high affinity binding only to insulin. The IGF1R binds to IGF1 and IGF2, but the hybrid receptor IR/IGF-IR binds both insulin and IGF1. Finally, an unrelated receptor for IGF2, IGF2R, or cation independent mannose-6-phosphate receptor has been described. This receptor consists of a single primarily extracytoplasmic polypeptide chain (Harris and Westwood, 2012) and acts via a G-protein coupled receptor in intracellular transport of lysosomal enzymes. In addition, it sequesters IGF2 from potential receptor activation by internalization and degradation.

Insulin Receptor Signal Transduction

After binding of the ligand to the α-subunit of one of the ligands IR or IGF1R undergo a conformational change inducing activation the kinase activity in the β-domains resulting in transphosphorylation among these subunits and further activation of phosphorylation at tyrosine moieties (Boucher *et al.*, 2014) (**Fig. 6**). This leads to binding and phosphorylation of insulin receptor substrates (IRSs) 1 to 6, which after phosphorylation at multiple tyrosines can bind to intracellular molecules and in turn stimulate their phosphorylation. Subsequently, two main pathways of signal transduction can be activated: one involved in the metabolic actions of insulin (the PI3-kinase/Akt pathway) and the other in cellular proliferation and differentiation (the RAS-MAPK pathway).

Phosphorylated IRS interacts with Src-homology 2 (SH2) docking sites in the regulatory subunit of phosphatidylinositol-(4,5)-bisphosphate 3-kinase (PI3-kinase), activating the catalytic subunit to convert PIP2 to phosphatidylinositol-(3, 4, 5)-trisphosphate (PIP3). PIP3 production leads to activation of several downstream enzymes, including the serine-threonine kinase Akt. Akt activation regulates metabolic enzymes, such as glycogen synthase kinase 3 and 6-phosphofructo-2-kinase, and it is involved in

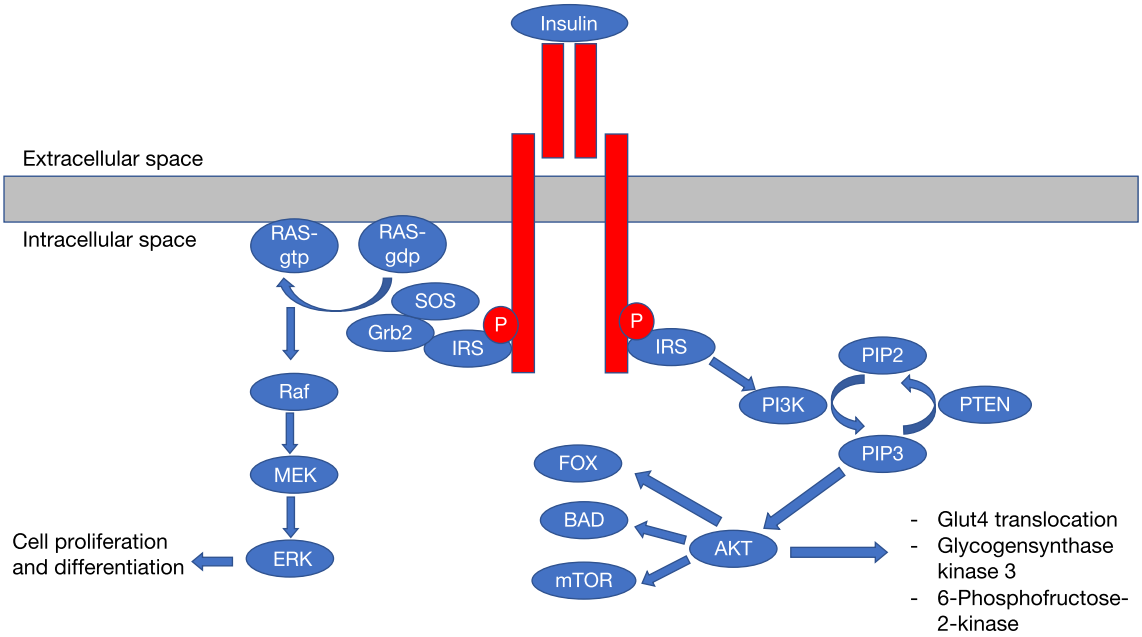

Fig. 6 Mechanism of action of the insulin- and IGF-stimulated receptors. After binding of the ligand, β-subunits are phosphorylated, leading to phosphorylation of insulin receptor substrate (IRS). Phosphorylated IRS stimulates phosphatidylinositol-(4,5)-bisphosphate 3-kinase (PI3-K), activating the catalytic subunit to convert phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phosphatidylinositol-(3, 4, 5)-trisphosphate (PIP3). PIP3 activates Act, which can stimulate a number of other proteins due to its serine-threonine kinase activity. Alternatively, phosphorylated IRS can activate growth factor receptor-bound protein 2 (Grb2). Grb2 activates the Ras guanine nucleotide exchange factor Son of Sevenless (Sos), which is a guanine nucleotide exchange factor for Ras, catalyzing the switch of Ras from the GDP-bound form (Ras-GDP) to the active, GTP-bound form (Ras-GTP). Ras-GTP interacts with and stimulates downstream effectors, such as the serine/threonine kinase Raf, which stimulates its downstream targets MEK1 and 2 that phosphorylate and activate the MAP kinases ERK1 and 2.

glucose metabolism by such activities as induction of glucose transporter (Glut4) translocation from intracellular storage compartments to the plasma membrane. Akt can also phosphorylate a number of other proteins, including BAD and forkhead box protein (FOX), which are involved in inhibition of apoptosis and cell growth. Furthermore, activated Akt can stimulate the mTOR pathway, which is involved in the regulation of mRNA translation into proteins.

The RAS-MAPK pathway is stimulated by IR after binding of growth factor receptor-bound protein 2 (Grb2) to phosphorylated IRS. Grb2 activates the Ras guanine nucleotide exchange factor Sos (Son of Sevenless), which is a guanine nucleotide exchange factor for Ras, catalyzing the switch of membrane-bound Ras from an inactive, GDP-bound form (Ras-GDP) to an active, GTP-bound form (Ras-GTP). Ras-GTP then interacts with and stimulates downstream effectors, such as the serine/threonine kinase Raf, which stimulates its downstream target MEK1 and -2 that phosphorylate and activate the MAP kinases ERK1 and -2. Stimulated ERK1 and -2 play a direct role in cell proliferation or differentiation, regulating gene expression or extranuclear events, such as cytoskeletal reorganization, through phosphorylation and activation of targets in the cytosol and nucleus.

A number of mechanisms play a role in obliteration of stimulated insulin signaling pathways. Since these pathways are stimulated after phosphorylation, phosphatases play an important role at a number of crucial points, starting at dephosphorylation at the level of the receptors and IRSs. Phosphatases like PTEN and SH2 domain-containing inositol 5-phosphatase 2 (SHIP2) dephosphorylate PIP3 at positions 3 and 5. Furthermore, in a number of conditions IR and IGF1R and IRS can be phosphorylated by serine-threonine kinases, leading to downregulation of the activity of the receptors.

The Growth Hormone Receptor Family

The GH receptor (GHR) is part of the larger family of cytokine receptor signal transducers also containing receptors for prolactin, erythropoietin, leptin, thrombopoietin, leukemia inhibiting factor, ciliary neurotrophic factor, oncostatin-M, carditropin-1 and most of the interleukins, together with many of the hematopoietic colony-stimulating factors (Waters, 2016). Members of this family exist in the form of homo- or heterodimers, consisting of proteins with a single transmembrane region. They act upon binding of the ligand by phosphorylation of both the intracellular part of the receptor and other effector proteins. GRHs are preformed homodimers, interacting with two binding sites on the GH molecule, first with a region called site 1, followed by binding of the other monomer with site 2. This binding causes a change in the configuration of the dimer, leading to transphosphorylation of associated Janus kinase 2 (JAK2) molecules on tyrosine residues, resulting in turn in tyrosine phosphorylation of intracellular tyrosines in the GHR providing docking sites or enabling binding of adaptor proteins such as members of the family of Signal transducer and activator of transcription (STATs) (Carter-Su *et al.*, 2016). STAT5 is involved in the stimulation of expression of IGF1 after binding to the promotor regions of these genes. Apart from the phosphorylation of STATs, JAK2 also phosphorylates tyrosine residues on the above discussed insulin receptor substrates (IRSs), causing effects similar to those caused by this branch of insulin signal transduction: the PI3-kinase/Akt pathway and the RAS-MAPK pathway. This explains the metabolic and proliferation/differentiation effects of GH.

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Roles of Plasma Binding Proteins in Modulation of Hormone Action and Metabolism

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Abbreviations

SERPIN	Serine proteinase inhibitor	**CBG**	Corticosteroid-binding globulin
DBP	Vitamin D-binding protein	**TBG**	Thyroxine-binding globulin
		SHBG	Sex hormone-binding globulin

Introduction

Small molecular weight lipophylic hormones, steroids and thyroid hormones, are shuttled by plasma proteins from their sites of synthesis to peripheral tissues where they enter cells to act via interactions with intracellular hormone receptors directly or after enzymatic activation. The major plasma hormone-binding proteins (**Table 1**) include albumin that is present in high concentrations and transports a variety of hormones and drugs with μM affinities and limited ligand-binding specificity, the albumin-related vitamin D binding protein that binds vitamin D secosterols with nM affinity, and several low abundance plasma proteins, i.e., corticosteroid-binding globulin, thyroxine-binding globulin and sex hormone-binding globulin, which also bind their ligands specifically with nM affinities. The unique physiochemical properties of these hormone-binding proteins and differences in their plasma concentrations determine the plasma distribution of their ligands between the protein-bound fraction and the "free" fraction that is accessible to tissues and able to enter target cells.

Albumin

Albumin is the most abundant plasma protein. It exhibits a diurnal rhythm, during which the plasma concentrations are lowest in the early hours of the morning (Jones *et al.*, 2017). Albumin is not glycosylated enzymatically, and has a remarkably long plasma half-life of ~19 days (Peters, 1996). Most of the albumin in the body leaves the blood circulation and resides in the interstitial compartments of tissues, apart from the brain, and this is especially pronounced in skin and muscle, which is the major site of albumin catabolism. Albumin is also recycled back into the blood from the interstitial compartments of tissues, multiple times, via the lymph (Peters, 1996), and this dynamic compartmentalization of albumin likely influences the access of its hormonal ligands to cells within tissues.

Albumin binds the thyroid hormones, triiodothyronine (T3) and thyroxine (T4), and steroid hormones with μM affinity. The binding affinity of albumin for T4 is ~4-fold greater than for T3 (Refetoff, 2000), and it displays a preference for estradiol followed by testosterone, progesterone and cortisol (Westphal, 1986). The high concentrations of albumin in plasma provide an unlimited capacity for these hormones, and albumin acts as a reservoir that buffers fluctuations in their plasma levels.

The tertiary structure of albumin comprises three internally similar domains (I-III) each with at least two (A and B) subdomains (He and Carter, 1992). Four binding sites for thyroxine are distributed across subdomains IIA, IIIA and IIIB, which also contain

Table 1 Common names, gene symbols, and properties of the major hormone-binding proteins in human blood

Common names	*Gene Symbol*	*M_r*[a]	*Modifications*	*Levels (M)*	*Affinity Kd*	*Specificity*
Albumin	*ALB*	66.5	Glycation, Cys-34 modifications[b]	500–600 (μM)	1–8 μM	Low
Vitamin D-binding globulin (DBP)	*GC*	52–58	*O-glycosylated* (1–2 sites)	7.0–8.5 (μM)	1–10 nM	High (25-D3 > 1,25-D3)
Corticosteroid-binding globulin (CBG)	*SERPINA6*	60–62	*N*-glycosylated (5 sites)	400–800[c] (nM)	1-10 nM	High (cortisol ≈ corticosterone > progesterone)
Thyroxine-binding globulin (TBG)	*SERPINA7*	54	*N*-glycosylated (4 sites)	180–350[c] (nM)	0.1–1.0 nM	High (T4 > T3)
Sex hormone-binding globulin (SHBG)	*SHBG*	48–50[b]	*N*-glycosylated (2 sites) O-glycosylated (1 site)	10–250[c] (nM)	1–10 nM	High (DHT > testosterone > estradiol)

[a]Relative molecular mass of protein in plasma.
[b]Homodimer subunit.
[c]High levels in pregnant women or women taking synthetic estrogens.

binding sites for many drugs and fatty acids (Curry *et al.*, 1998), and fatty acids induce conformational changes in albumin that create an additional thyroxine-binding site between domains I and III (Petitpas *et al.*, 2003). However, each molecule of albumin normally binds less than two fatty acids, and the ability of fatty acids to displace thyroid hormones or steroids is therefore limited. The testosterone and progesterone binding sites partially overlap within subdomains IIA and IIB, respectively, and glucocorticoids and other C21 steroids also bind within subdomain IIA (Abboud *et al.*, 2017). In addition, albumin is the main plasma binding protein for steroid hormone precursors and metabolites, as well as conjugated steroids, and it binds sulfated steroids with greater affinity than glucuronidated steroids (Westphal, 1986). Binding of sulfated steroids to albumin is determined primarily by their sulfate moieties, and sulfated steroids are not displaced from albumin by unconjugated steroids, implying that their binding sites are distinct (Westphal, 1986).

Production and Regulation of Plasma Levels

Plasma albumin production by hepatocytes is maintained at the transcriptional level by several liver enriched nuclear factors (Ogawa *et al.*, 1997) including HNF1 (Lichtsteiner and Schibler, 1989), C/EBP-α (Friedman *et al.*, 1989) and C/EBP-β (Descombes *et al.*, 1990). Decreases in plasma albumin levels during acute inflammation are mediated by the pro-inflammatory cytokines, IL-6 (Heinrich *et al.*, 1990) and TNF-α (Buck *et al.*, 2001) that act at the transcriptional level through signaling mechanisms involving C/EBP family members (Poli, 1998). Glucocorticoids enhance the production of albumin by isolated rat heptocytes at the transcriptional level (Nawa *et al.*, 1986), and increase plasma albumin levels in rats (Marinkovic *et al.*, 1989). They also reverse the fall in plasma albumin levels observed after IL-6 treatment (Marinkovic *et al.*, 1989).

Genetic Variants

More than 100 nonsynonymous mutations in the human *ALB* gene are listed in human single nucleotide polymorphism (SNP) databases (http://www.internationalgenome.org). Numerous naturally occurring mutations cause hypoalbuminemia, due to defects in albumin production, and are associated with adverse reactions to drugs (Minchiotti *et al.*, 2008), but there is no evidence that low plasma levels of albumin influence the activities of endogenous ligands like thyroid or steroid hormones. The same is true for individuals with rare cases of analbuminemia who may present with fatigue, edema and hyperlipidemia, but otherwise live and function normally. By contrast, albumin variants with increased affinity for thyroid hormones have been identified in patients with familial dysalbuminemic hyperthyroxinemia (Minchiotti *et al.*, 2008; Greenberg *et al.*, 2014), and the responsible amino acid substitutions have shed light on the main albumin-binding site for thyroxine within domain IIA (Petitpas *et al.*, 2003). Albumin variants with abnormal steroid-binding properties do not influence the levels or activities of steroid hormones. Structural analyses of these variants indicate that key contact points for testosterone are in sub-domain IIA, while progesterone interacts with residues within the helix connecting domains IIA and B (Kragh-Hansen *et al.*, 1990).

Vitamin D-Binding Protein

After the group-specific component of human serum, known as the GC-globulin, was implicated in the antiricketic effects of vitamin D (Thomas *et al.*, 1959), it was renamed as the vitamin D-binding protein (DBP) to reflect its role in the plasma transport of biologically active forms of vitamin D, but *GC* is still currently used as the gene symbol for DBP in genome databases.

Plasma DBP is structurally related to albumin, but domains I-III of DBP adopt a distinct overall shape (Verboven *et al.*, 2002) because a coil fold replaces helix 7 in domain II and its domain III is truncated C-terminally (Cooke and David, 1985). In crystal structures of human DBP, 25-hydroxy D3 hydrogen bonds with amino acids in a hydrophobic cleft between helices 1–6 within domain I. These structures also explain why the additional hydroxyl group of 1,25-hydroxy D3 reduces its binding affinity (Verboven *et al.*, 2002). Plasma DBP also binds the globular form of actin shed by cells after injury, facilitating its removal from the circulation (Mc Leod *et al.*, 1989). Although the actin and vitamin D binding sites overlap, actin and vitamin D bind independently (Otterbein *et al.*, 2002).

The plasma concentrations of DBP are normally at least 100 times lower than those of albumin, but its affinities for vitamin D metabolites exceed those of albumin by 2–3 orders of magnitude (**Table 1**). Importantly, the binding capacity of DBP for both forms of vitamin D exceeds their combined plasma concentrations by at least 20 times, and it exerts a major influence on their plasma distribution, protecting against vitamin D toxicity and acting as a reservoir for 25-hydroxy D3. In human blood, 88% of the 25-hydroxy D3 and 85% of 1,25-dihydroxy D3 is bound to DBP, with only $\sim$0.04 and 0.4%, respectively, being in the free fraction, and the remainder bound mainly to albumin (Delanghe *et al.*, 2015). Although the free concentrations of 1,25-dihydroxy D3 are $\sim$3 orders of magnitude lower than required to saturate the intracellular vitamin D receptor, plasma 25-hydroxy D3 concentrations are normally three orders of magnitude greater than those of 1,25-dihydroxy D3. This means that the amounts of 25-hydroxy D3 that enter cells exceeds those of 1,25-dihydroxy D3 by $\sim$2 orders of magnitude, and this is especially important in cells where 25-hydroxy D3 is converted into the preferred vitamin D receptor ligand, 1,25-dihydroxy D3.

Production and Regulation of Plasma Levels

Plasma DBP is produced by hepatocytes and has a plasma half-life of 2.5 days (Speeckaert *et al.*, 2006). Plasma levels of DBP (**Table 1**), like those of albumin, exhibit a diurnal rhythm, and are maintained within a relatively narrow range throughout life (Jones *et al.*, 2017; Speeckaert *et al.*, 2006). During human pregnancy, it is assumed that estrogens increase maternal plasma 1,25-dihydroxy D3 and DBP levels because similar increases occur after the administration of oral contraceptives (Bouillon *et al.*, 1981). Three HNF1-binding sites within the promoter of the rat gene encoding DBP are key regulators of its basal transcriptional activity. In the liver, HNF1α homodimers interact with these sites in conjunction with the dimerization co-factor of HNF1 to enhance transcription, whereas in the kidney, these sites appear to be occupied by heterodimers of HNF1α and HNF1β (Song *et al.*, 1998). The transcriptional response in kidney cells is much lower than in the liver and it has been attributed to HNF1β acting as a *trans*-dominant inhibitor of HNF1α-mediated enhancer activity. The production of DBP by human liver cells is decreased by TGFβ, while glucocorticoids and IL-6 have the opposite effects, and this explains why plasma DBP levels do not show an acute phase negative response during acute inflammation (Guha *et al.*, 1995).

Genetic Variants

Human DBP is highly polymorphic, with over 120 rare variants identified in different ethnic groups, but three common *GC* allelic variants defined by the nonsynonymous SNPs, rs7041 and rs4588, encode DBP variants that are distinguished by their electrophoretic mobility and *O*-glycosylation status (Borges *et al.*, 2008; Speeckaert *et al.*, 2006). Glycosylation of DBP does not influence its plasma half-life or its vitamin D binding properties, but the common *GC* alleles are linked to differences in plasma DBP and vitamin D levels (Wang *et al.*, 2010) and are associated with susceptibility or resistance to a spectrum of diseases (Speeckaert *et al.*, 2006).

Humans with no plasma DBP have never been identified. Mice that lack plasma DBP because of a gene deletion have undetectable 1,25-dihydroxy D3 in their blood, but they are normocalcemic when maintained on a diet containing vitamin D (Safadi *et al.*, 1999) because their tissue concentrations of 1,25-dihydroxy D are normal (Zella *et al.*, 2008). However, mice without DBP develop secondary hyperparathyroidism and bone abnormalities when fed a vitamin D deficient diet (Safadi *et al.*, 1999). A similar phenotype is observed in megalin deficient mice, and this was attributed to the megalin-mediated endocytosis of DBP in renal tubules, which serves as a means of recovering vitamin D excreted by the kidneys (Nykjaer *et al.*, 1999). Patients with a megalin deficiency have increased DBP in their urine but they are not vitamin D deficient, and megalin does not appear to be functionally important in recovering vitamin D from urine in humans (Storm *et al.*, 2013).

Corticosteroid-Binding Globulin

Plasma corticosteroid-binding globulin (CBG, also known as transcortin or SERPINA6), binds glucocorticoids and progesterone with nM affinity (Westphal, 1986). In human blood, $\sim$85% of cortisol is normally bound by CBG and $\sim$10% is bound by albumin, which together maintain the amount of unbound cortisol in plasma at $\sim$5% (Siiteri *et al.*, 1982; Dunn *et al.*, 1981). In addition to buffering against oscillations in cortisol levels, proteolysis of CBG provides a mechanism for the targeted delivery of its ligands to sites of inflammation or tissue remodeling (Perogamvros *et al.*, 2012; Hammond, 2016).

The primary structure of CBG identifies it as a clade A serine protease inhibitor (SERPINA) family member (Hammond *et al.*, 1987). Most *SERPINA* genes, including *SERPINA6*, are positioned in a syntenic cluster on chromosome 14q32.1. In humans, the CBG precursor includes a signal polypeptide of 22 residues that is removed prior to secretion (Hammond *et al.*, 1987). The mature polypeptide of 383 amino acids contains a single steroid-binding site and six potential *N*-glycosylation sites that are differentially utilized (Sumer-Bayraktar *et al.*, 2011), and glycosylation of N238 in human CBG influences its steroid-binding activity (Avvakumov *et al.*, 1993).

The SERPINs share a common tertiary structure comprising three β-sheets and seven or more α-helices, as well as a functionally important reactive center loop (RCL) that is specifically cleaved by proteases, such as neutrophil elastase that is produced during inflammation (Hammond *et al.*, 1990). Crystallographic analyses have identified structurally and functionally important amino acids within CBG, provided insight into how RCL positioning and integrity influences its steroid-binding activity, and explain how natural mutations effect the biological activities of CBG (Lin *et al.*, 2010).

Production and Regulation of Plasma Levels

Plasma CBG is produced by the liver and its half-life is $\sim$13 h (Seralini *et al.*, 1989). In animal models, *Serpina6* expression in the liver fluctuates in a biphasic manner during fetal life, and also occurs transiently in fetal exocrine pancreas and the developing kidney (Scrocchi *et al.*, 1993a,b; Berdusco *et al.*, 1995). These spatial and temporal changes in CBG production are expected to influence both the systemic and local actions of glucocorticoids at critical developmental stages.

Glucocorticoids influence *SerpinA6* expression differently at specific developmental stages. They increase hepatic CBG production in fetal and early postnatal life but reduce it in adults (Zhao *et al.*, 1997; Berdusco *et al.*, 1995; Pepe *et al.*, 1996), which explains the low plasma CBG levels in patients with Cushing syndrome or after systemic glucocorticoid administration (Schlechte

and Hamilton, 1987). The *SERPINA6* promoter lacks a glucocorticoid response element, and the effects of glucocorticoids on its transcriptional activity may be mediated by interactions between the glucocorticoid receptor and other transcription factors (Verhoog *et al.*, 2014). Cytokines regulate plasma CBG levels in different ways: IL-6 down regulates *SERPINA6* expression in human liver cells, while IL-1β stimulates CBG secretion at a post-translational level (Emptoz-Bonneton *et al.*, 1997).

Plasma Levels in Health and Disease

In humans, plasma CBG levels are similar between sexes (Dunn *et al.*, 1981; Brien, 1981; Ho *et al.*, 2007). Changes in plasma CBG levels occur normally during pregnancy and early development, as well as in patients suffering from acute inflammation caused by infection or tissue trauma, and likely alter the access of glucocorticoids to their target cells (Perogamvros *et al.*, 2012; Hammond, 2016).

In mammals, plasma CBG is transiently produced by fetal hepatocytes after mid-gestation, but declines to low levels shortly before (Scrocchi *et al.*, 1993b; Pepe *et al.*, 1996) or after (Berdusco *et al.*, 1995) birth. The developmental fluctuations in the hepatic production of plasma CBG in the human fetus are unknown but likely reflect those in the baboon (Pepe *et al.*, 1996). Plasma CBG levels in human neonates are low during the first week of life, and gradually rise to adult levels by 12 months of age (Brien, 1981). Low plasma CBG levels in neonates will increase the availability of cortisol to organs such as the lung, the maturation of which is glucocorticoid dependent.

In women, plasma CBG levels increase 2- to 3-fold during the second and third trimesters of pregnancy, along with increased concentrations of its major ligands, cortisol and progesterone (Dunn *et al.*, 1981; Brien, 1981; Ho *et al.*, 2007). Similar increases in plasma CBG occur in women taking synthetic estrogens (Moore *et al.*, 1978), and are presumably mediated by the estrogen receptor. During the final weeks of pregnancy, maternal plasma CBG levels decrease, together with a corresponding increase in free cortisol levels (Ho *et al.*, 2007). Since human CBG binds progesterone almost as well as cortisol, the large amounts of progesterone produced by trophoblasts compete with cortisol for CBG binding at the maternal-fetal interface, and CBG acts as the major determinant of progesterone concentrations in this location (Lei *et al.*, 2015; Hammond, 2016). Low maternal plasma CBG levels are observed in pregnancy-associated disease states, including preeclampsia and gestational diabetes, as well as in gamete recipient pregnancies (Ho *et al.*, 2007).

During inflammation, CBG responds as an acute phase "negative" protein, and reductions in its plasma levels occur in various pathologies involving inflammation and tissue trauma (Zouaghi *et al.*, 1984), including burn injury (Bernier *et al.*, 1998), acute pancreatitis (Muller *et al.*, 2007), sepsis, septic shock and multitrauma (Pugeat *et al.*, 1989; Beishuizen *et al.*, 2001). Pre-symptomatic decreases in plasma CBG have been observed in a rat model of inflammation, demonstrating that CBG is a biomarker of the onset and severity of an inflammatory response (Hill *et al.*, 2016). These reductions in plasma CBG during acute inflammation are attributed to proteolysis of its RCL, as well as a down-regulation of its production by the liver. In this paradigm, proteolysis of CBG occurs early during inflammatory reactions, rendering CBG nonfunctional and promoting the localized release of CBG-bound glucocorticoids at sites of inflammation. Enhanced exposure of tissues to glucocorticoids represses cytokine production and activity, limiting cytokine-mediated tissue damage. At the same time, plasma glucocorticoid levels increase acutely as a result of hypothalamic-pituitary-adrenal activation in response to stress, and synergize with elevations in inflammatory cytokines, such as IL-6, to further reduce plasma CBG production, thereby amplifying free glucocorticoid exposures. During recovery from inflammation, normalization of CBG levels likely plays a role in determining when, and to what extent, glucocorticoids act to restore normal homeostasis (Perogamvros *et al.*, 2012; Hammond, 2016).

Genetic Variants

In a recent genome-wide association study (GWAS), common SNPs in *SERPINA6*, linked to the plasma levels or steroid-binding properties of CBG, were identified as the main determinants of plasma cortisol levels (Bolton *et al.*, 2014). Most CBG variants occur at a low frequency in the general population, but some are enriched in specific ethnic groups. For example, the secretion deficient CBG A51V (rs146744332) occurs at a frequency of ~1:35 in Han Chinese (Lin *et al.*, 2012), Japanese and Kinh Vietnamese (http://www.internationalgenome.org/). In addition, the CBG L93H (rs113418909) and CBG D367N (rs28929488) variants are characterized by reduced affinities for cortisol, and are enriched in individuals of European (Bolton *et al.*, 2014) and Mediterranean/Middle Eastern decent (Emptoz-Bonneton *et al.*, 2000; Cizza *et al.*, 2011; Hill *et al.*, 2012), respectively. Other mutations identified in patients, such as CBG W371S (rs267607282) and CBG G237V (rs754814260), have no detectable cortisol-binding activity (Hill *et al.*, 2012; Perogamvros *et al.*, 2010), while mutations that introduce premature stop codons in the *SERPINA6* coding sequence (e.g., rs777245398) have no CBG in their blood (Torpy *et al.*, 2001; Torpy *et al.*, 2012). Numerous less common nonsynonymous *SERPINA6* SNP have been linked to abnormalities in CBG cortisol-binding activity, production, sensitivity of the RCL to proteolysis, or their immunochemical recognition (Simard *et al.*, 2015).

Many *SERPINA6* mutations that alter the production or function of plasma CBG were discovered in patients presenting with a variety of clinical conditions, including chronic pain, fatigue, depression, hypotension, and excess body weight (Emptoz-Bonneton *et al.*, 2000; Perogamvros *et al.*, 2010b; Hill *et al.*, 2012; Torpy *et al.*, 2001, 2012). However, not all individuals with CBG variants display these clinical features (Hill *et al.*, 2012; Perogamvros *et al.*, 2010b). Patients with CBG deficiencies generally present with low to undetectable serum cortisol levels but normal ACTH levels, and this is because their free cortisol levels in plasma or urine

are normal (Perogamvros *et al.*, 2012; Hammond, 2016). Studies of CBG deficiencies in animal models support a link between lower CBG levels and inflammation susceptibility (Petersen *et al.*, 2006), but most CBG variants that are produced or function abnormally in humans occur rarely, and their clinical consequences have been difficult to establish. Clinical studies of subjects with the secretion-deficient CBG A51V variant that is more common in some ethnic groups may help resolve this. In the only such study to date, pregnancy outcomes and the health of neonates of Chinese mothers with CBG A51V were normal, but a female skewed sex ratio of the offspring was observed (Lei *et al.*, 2015). The biological basis for this is unknown, but CBG deficiency may result in inappropriate cortisol or progesterone exposures during gestation and lead to male fetal demise (Lei *et al.*, 2015).

Thyroxine-Binding Globulin

Plasma thyroxine-binding globulin (TBG, also known as SERPINA7), binds T3 and T4 with nM affinities, but has a preference for T4 (**Table 1**). In human plasma, ~75% of T4 and T3 is bound by TBG. Albumin binds thyroid hormones with ~4 orders of magnitude lower affinity than TBG, and only ~5% of T4 and ~20% of T3 in plasma is bound by albumin. The latter difference reflects the fact that transthyretin also binds T4 with an ~100 fold greater affinity than albumin and has a 10-fold preference for T4 over T3, and as a result transthyretin binds ~20% and ~5% of the T4 and T3 in plasma, leaving <1% of these iodothyronines free in healthy individuals (Pappa *et al.*, 2015).

Unlike most other *SERPINA* members, the human TBG (*SERPINA7*) gene is located on the X chromosome. Human TBG is produced as a 415 amino acid precursor that includes a 20 amino acid signal sequence and four *N*-glycosylation sites (Flink *et al.*, 1986). Plasma TBG and CBG share 42% sequence identity (Hammond *et al.*, 1987), and their tertiary structures are very similar (Zhou *et al.*, 2006). The crystal structure of TBG has identified residues important for ligand binding (Zhou *et al.*, 2006), and provided insight into the functional consequences of TBG variants (Pappa *et al.*, 2015. The RCL of TBG shares little sequence similarity with that of CBG, and appears to move in and out of the main β-sheet A triggering an allosteric mechanism that influences its ligand-binding (Zhou *et al.*, 2006).

Production and Regulation of Plasma Levels

Plasma TBG is produced by hepatocytes and has a half-life of ~5 days (Refetoff, 2000). Several hepatic nuclear factor-binding sites (HNF1α, HNF3α and HNF3β) within the *SERPINA7* promoter, determine its transcriptional activity in hepatocytes (Hayashi *et al.*, 1993). An enhancer located 20 kb downstream of the *SERPINA7* coding sequence also appears to control its expression, because a genetic polymorphism within this region is associated with decreased plasma TBG levels (Ferrara *et al.*, 2015).

Exogenous administration of glucocorticoids reduces TBG levels in humans (Gamstedt *et al.*, 1979) and animal models (Emerson *et al.*, 1993). By contrast, estrogen-dependent increases in TBG in women taking synthetic estrogens or during pregnancy have been associated with increases in sialylation of its *N*-glycans (Ain *et al.*, 1987), and a reduced plasma clearance (Krassas *et al.*, 2010).

Plasma Levels in Health and Disease

A 50% increase in plasma TBG levels in women during pregnancy, accounts for corresponding increases in T3 and T4 levels. In the human fetus, plasma TBG levels gradually increase from 12 weeks of gestation to levels in neonates that are higher than in adults (Williams *et al.*, 2004).

Low plasma TBG levels are found in patients with inflammatory conditions, such as sepsis (Jirasakuldech *et al.*, 2000; Afandi *et al.*, 2000b) or cardiopulmonary bypass surgery (Afandi *et al.*, 2000a). As with CBG, these reductions in TBG levels have been attributed to neutrophil elastase mediated RCL proteolysis that induces a conformational change in TBG and reduces its capacity to bind thyroid hormones at sites of inflammation or tissue trauma (Janssen *et al.*, 2002).

Genetic Variants

Numerous (>50) TBG variants cause a spectrum of TBG deficiencies, from partial to complete, as well as abnormally high plasma TBG levels (Pappa *et al.*, 2015). Biochemically, these TBG deficiencies are caused by nonsense mutations that encode nonfunctional truncated proteins, or missense mutations that alter binding properties and protein expression. By contrast, *SERPINA7* duplication or triplication results in TBG excess (Mori *et al.*, 1995).

Given its chromosomal location, abnormal *SERPINA7* genes are expressed in an X-linked manner. In males, TBG deficits are fully expressed, whereas in heterozygous females, the effects are only partial, although selective X-inactivation in females may result in a complete TBG deficiency (Pappa *et al.*, 2015). The incidence of complete TBG deficiencies varies with ethnicity (Reutrakul *et al.*, 2002; Mandel *et al.*, 1993), for example their incidence in Japanese is 3–10 times higher than in Caucasians. Partial TBG deficiencies occur more frequently in some populations; for example the TBG-A (A191T, rs2234036) variant that has a reduced affinity for thyroid hormones, and susceptibility to inactivation at increased temperature, is present in 40% of Indigenous Australians (Takeda *et al.*, 1989).

Individuals with TBG deficiencies have lower plasma T3 and T4 levels, while the opposite occurs in those with TBG excess, but they are all generally euthyroid because their free T4/T3 levels are within normal ranges, and they do not suffer from major metabolic disturbances (Pappa *et al.*, 2015).

Sex Hormone-Binding Globulin

In humans, sex hormone-binding globulin (SHBG) is the major binding protein for androgens and estrogens in the blood. After removal of a signal polypeptide of 29 residues, SHBG is secreted by hepatocytes as a glycosylated homodimer of 373 residue subunits that comprise tandem laminin G-like domains. Each subunit contains independent steroid-binding sites that bind 5α-dihydrotestosterone (DHT) with the highest affinity, followed by testosterone and estradiol (**Table 1**).

Crystallographic structures have revealed how specific residues interact with steroids. Androgens and estrogens bind in opposite orientations in the binding pocket, and there are steroid-ligand dependent differences in the orientation of a loop region over an entrance to the binding pocket, with estrogens producing a more ordered loop structure (Grishkovskaya *et al.*, 2002). The positioning of this loop is altered by occupancy of a zinc-binding site, which specifically lowers the binding affinity for estrogens (Avvakumov *et al.*, 2000). Crystal structures also identified the binding site for Ca^{2+} that maintains the quaternary structure of the SHBG homodimer and its steroid-binding properties (Grishkovskaya *et al.*, 2000), both of which are adversely affected in frozen and thawed EDTA-plasma (Bocchinfuso and Hammond, 1994).

Production and Regulation of Plasma Levels

Human *SHBG* expression in hepatocytes is under hormonal, nutritional, and metabolic regulation. A nuclear hormone receptor response element positioned near the transcription start site in the *SHBG* promoter binds both HNF4α and COUP-TF, and functions as a transcriptional "on-off" switch, so that HNF4α binding promotes transcription, while COUP-TF binding represses it (Jänne and Hammond, 1998). This competitive relationship between HNF4α and COUP-TF binding at the *SHBG* promoter accounts for most responses observed in relation to the nutritional and metabolic mediators of plasma SHBG levels (Hammond *et al.*, 2012). For instance, hepatic HNF4α levels are increased after thyroid hormone treatment, weight loss or fasting, and this increases hepatic *SHBG* expression and plasma SHBG levels. Conversely, there is evidence that steatosis, pro-inflammatory cytokines and adiponectin all contribute to reductions in hepatic HNF4α levels allowing the "on-off" switch to be occupied by COUP-TF, thereby reducing plasma SHBG production in overweight individuals. A second nuclear hormone receptor-binding site in the *SHBG* promoter interacts with PPARγ to negatively regulate transcription (Selva *et al.*, 2002) or with CAR to increase its activity (Saez-Lopez *et al.*, 2017).

Plasma Levels in Health and Disease

Plasma SHBG levels fluctuate normally throughout life and in response to physiological changes (Hammond *et al.*, 2012), and the plasma half-life of ~1.5 days is influenced by its glycosylation status. During fetal life, changes in plasma SHBG levels likely control the activities of sex steroids during development. Plasma SHBG levels are low at birth and increase during the first few months of postnatal life, presumably in response to the maturation of thyroid hormone actions (Leger *et al.*, 1990). They are maintained at high levels until the onset of puberty, when plasma SHBG levels decrease in both sexes, but to a greater extent in males than females (Aydin and Winters, 2016). Plasma SHBG levels do not fluctuate through the menstrual cycle or change significantly after menopause when anthropometric parameters are taken into account (Pasquali *et al.*, 1997), but increase by as much as 10-fold during pregnancy and in women taking synthetic estrogens (Anderson, 1974), and these increases in plasma SHBG are assumed to be estrogen-dependent. The significance of increased maternal plasma SHBG during late pregnancy is unclear, but it may protect the mother from fetal adrenal androgens (Hogeveen *et al.*, 2002). In healthy normal weight individuals, plasma levels of SHBG in men are about two times lower than in women, a trend that continues through adulthood until old age, when there is an age dependent increase in men (Wu *et al.*, 2008).

Occupancy of the SHBG steroid-binding site varies considerably in relation to these sex and life cycle differences in its plasma levels (Hammond *et al.*, 2012). In young children, SHBG is predominantly unoccupied and this limits tissue exposure to sex steroids until puberty (Hammond, 2011). Steroid occupancy of SHBG also differs markedly between men and women, with SHBG in women being largely unoccupied, allowing it to more effectively limit the access of androgens to target cells (Dunn *et al.*, 1981). This effect is exaggerated in women taking oral contraceptives that increase SHBG levels, and has been exploited pharmaceutically to reduce symptoms of androgen excess (Dewis *et al.*, 1985). By contrast, in men, SHBG is primarily occupied by testosterone and lower plasma levels of SHBG result in proportionally higher amounts of available androgens in men than women (Dunn *et al.*, 1981).

Plasma levels of SHBG are used as a biomarker of a variety of endocrine and metabolic diseases (Hammond *et al.*, 2012). In women, low SHBG levels are commonly associated with clinical manifestations of androgen excess, especially in those who are overweight or present with polycystic ovarian syndrome (Botwood *et al.*, 1995). Plasma SHBG levels are abnormal in patients with thyroid hormone-dependent diseases and can be used to identify patients with thyrotoxicosis due to excessive thyroid hormone

production or availability (Krassas *et al.*, 2010), as well as those with thyroid hormone resistance (Sarne *et al.*, 1988; Krassas *et al.*, 2010). Inverse correlations exist between SHBG levels and body weight, with high levels in patients with anorexia and low levels in obese individuals. These body weight extremes and their associated plasma SHBG levels are linked to increased risk for osteoporosis in lean individuals, and the metabolic syndrome, diabetes and cardiovascular disease in those who are overweight (Hammond, 2017).

Genetic Variants

Polymorphism in *SHBG* influences both the production and function of plasma SHBG (Hammond *et al.*, 2012), and a GWAS has confirmed that SHBG is the major determinant of testosterone levels in men (Ohlsson *et al.*, 2011). A $(TAAAA)_n$ microsatellite located upstream of the *SHBG* promoter influences its transcriptional activity (Hogeveen *et al.*, 2001), as well as plasma SHBG levels (Cousin *et al.*, 2004), and has been associated with age of menarche, and several sex-hormone dependent diseases, including polycystic ovarian syndrome, atherosclerosis and cardiovascular risk, the metabolic syndrome, bone mineral density, and male infertility (Xita *et al.*, 2011; Saltiki *et al.*, 2011; Safarinejad *et al.*, 2011; Xita and Tsatsoulis, 2010; Eriksson *et al.*, 2006). A common SNP (rs1799941) near the *SHBG* transcription start site, as well as others positioned further upstream (rs858518) or downstream of the SHBG coding region (rs727428), are in strong linkage disequilibrium with each other, and are associated with SHBG levels (Thompson *et al.*, 2008). Another SNP located in intron 1 (rs6257) has been associated with low serum SHBG levels and increased risk of type 2 diabetes (Ding *et al.*, 2009) and breast cancer (Thompson *et al.*, 2008).

A nonsynonymous SNP that encodes SHBG D327N (rs6259) introduces a new *N*-glycosylation site (Power *et al.*, 1992), and a reduction in its plasma clearance (Cousin *et al.*, 1999) explains the slightly higher SHBG levels in carriers of this variant, which has been linked to a reduction in hip bone mineral density (Napoli *et al.*, 2009), and a reduced risk for type-2 diabetes (Ding *et al.*, 2009). An association between SHBG P156L (rs6258) and low plasma testosterone levels has been attributed to its reduced affinity for testosterone (Ohlsson *et al.*, 2011). The clinical significance of SHBG P156L and other nonsynonymous SNPs encoding rare SHBG variants with abnormalities in their production, glycosylation, steroid binding, interaction with fibulin family members, or antibody recognition (Wu and Hammond, 2014) is unknown, and may be difficult to determine if they escape detection in routine assays of plasma SHBG (Laurent and Vanderschueren, 2014).

Other Plasma Hormone Binding Proteins

The major plasma hormone binding proteins (**Table 1**) regulate the plasma concentrations of thyroid and steroid hormones, as well as their precursors and metabolites, and influence their metabolic clearance rates and access to target tissues and cells in various ways. However, these lipophylic molecules also bind several other plasma proteins including transthyretin, orosomucoid and apolipoproteins. Transthyretin has only a modest impact on the plasma distribution of thyroid hormones and this is not altered by changes in its plasma concentrations under pathological conditions because only a small fraction (0.5%) of its bindings sites are occupied. Likewise, transthyretin variants with reduced affinity for T3 and T4 have little effect on their plasma distribution, but variants with increased affinity for these iodothyronines are found in a small subgroup of subjects with euthyroid hyperthyroxinemia (Pappa *et al.*, 2015). In addition, the contribution of transthyretin or apolipoproteins to the plasma distribution of thyroid hormones is expected to be greater in individuals with deficiencies in albumin or TBG. Orosomucoid and apolipoproteins (Westphal, 1986) also bind steroids with limited specificity and relatively low affinity, and their impact on the plasma distribution of steroids is limited under normal conditions (Dunn *et al.*, 1981). However, because of their abundance, their effects on the plasma distribution of steroids may be much more significant in individuals with deficiencies in albumin or the plasma proteins that bind steroids specifically with high affinity.

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Dynamic Endocrine Rhythms

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Glossary

Circadian From Latin, circa "about" + dies "day." Refers to biological processes that recur approximately every 24 h. Circadian rhythms are endogenous, entrainable, and stable over a range of physiological temperatures.

Diurnal A general term for a rhythm or process with daily recurrence. The rhythm may or may not be endogenously generated.

Glucocorticoids Steroid hormones, synthesized in the adrenal cortex and responsible for a wide range of physiological effects including metabolic, immune, cognitive and homeostatic. The principal endogenous glucocorticoid in humans is cortisol.

HPA The hypothalamic–pituitary–adrenal axis is defined by complex interactions between the hypothalamus, pituitary and adrenal glands. A highly dynamic system, it regulates circulating glucocorticoid concentrations and plays a crucial role in the neuroendocrine response to stress.

Ultradian Rhythms with a period of less than 1 day but greater than 1 h.

Introduction

The metabolism of all living things is characterized by rhythmic patterns. Fundamental processes such as sleep, temperature regulation, feeding, nutrient digestion, and hormone secretion all exhibit biological rhythms. The period of these rhythms fluctuates from as short as a few minutes, as in the case of insulin secretion, to weeks-long infradian rhythms in case of the human menstrual cycle (Henley *et al.*, 2009a,b; Feng and Lazar, 2012; Waite *et al.*, 2012).

Biological rhythms can be described in terms of their pattern of activity. Diurnal rhythms generally refer to patterns that follow cycles of day and night. Humans and many other species of animal are diurnal creatures as the main period of activity is during daylight hours, with sleep (or other inactivity) at night. Many hormones express diurnal rhythms including thyroid hormones (Russell *et al.*, 2008), testosterone (Walton *et al.*, 2007; Waite *et al.*, 2009; Walton *et al.*, 2007) and prolactin (Van Kerkhof *et al.*, 2015).

Circadian rhythms are more precisely defined. Occurring with a period of approximately 1 day, they are both endogenous and self-sustained. Following these criteria, circadian rhythms may also be diurnal rhythms but not all diurnal rhythms are circadian. Examples of common circadian rhythms include body temperature (Buhr *et al.*, 2010), feeding (Eckel-Mahan and Sassone-Corsi, 2013) and the secretion of hormones, including melatonin (Benloucif *et al.*, 2005) and glucocorticoids (Keller-Wood and Dallman, 1984).

Circadian rhythms are entrained, or synchronized, to external cues. The most powerful environmental stimuli (often called *Zeitgebers*, from the German "time giver") are light and the timing of food (Albrecht, 2012; Asher and Sassone-Corsi, 2015). However, to be true circadian rhythms they must also continue to oscillate even in the absence of synchronizing input.

To accurately measure the endogenous properties of in vivo circadian rhythms, experiments must be conducted under conditions where light and other potential external synchronizers are carefully controlled. In humans, a commonly used protocol, known as "forced desynchrony," requires participants to be admitted to a specialized sleep unit. During this time, bedtime is moved progressively later, eventually resulting in a sleep–wake cycle that occurs within a 28-h "day." Rhythms driven by circadian pacemakers are unable to entrain to this extended day length, and become uncoupled from the sleep-wake cycle. This allows researchers to assess the endogenous "free running" period of the rhythms of interest. Experiments using this protocol indicate that the intrinsic period of cortisol, melatonin, and body temperature ranges between approximately 23.5 and 24.5 h (Czeisler *et al.*, 1999; Wyatt *et al.*, 1999; Gronfier *et al.*, 2007).

Rhythms with a period of less than 1 day but greater than 1 h are termed ultradian. Ultradian rhythms characterize many endocrine rhythms including insulin secretion (Simon and Brandenberger, 2002), growth hormone (Tannenbaum and Martin, 1976), and the reproductive axis (Choe *et al.*, 2013). Of special interest is the ultradian rhythm of glucocorticoids, which will be described in further detail later in this article. In the unstressed state, pulses of cortisol (corticosterone in rats) are secreted every 60–90 min (Veldhuis *et al.*, 1989; Jasper and Engeland, 1991; Windle *et al.*, 1998) on a background circadian rhythm. The complex interplay between the hypothalamic–pituitary pulse generator, the adrenal glands, and environmental stressors creates a dynamic system that if disturbed has important consequences for health and disease.

Intracellular Clocks and the Origin of Circadian Rhythms

Circadian rhythms are generated by the oscillation of intracellular "clocks." Clocks are present in almost all living things including plants, animals, and even single-cell organisms (Roenneberg and Merrow, 2005). In humans and other mammals, a specialized

cluster of cells known as the suprachiasmatic nucleus (SCN) acts as a circadian master clock. The SCN is located in anteroventral hypothalamus, above the optic chiasm and on either side of the third ventricle (Gillette, 1991). Time of day information is transmitted from the eye and the outside world via specialized photosensitive retinal ganglion cells contained within the retinohypothalamic tract. These neurons synapse directly with SCN neurons (Welsh *et al.*, 2010) and entrain the central circadian clock to the timing of light stimulus as well as having direct effects on adrenocortical secretion (Pilorz *et al.*, 2016). However, the SCN continues to function autonomously even when completed isolated from external stimuli. Circadian electrical activity continues within the SCN even when surgically isolated (Inouye and Kawamura, 1979) and in freely moving animals kept in constant darkness (Ono *et al.*, 2015).

The SCN functions as a master clock that coordinates peripheral rhythms. This has been demonstrated in numerous rodent experiments. For example, lesioning the SCN in rats abolishes circadian rhythms of temperature, feeding, movement, and pituitary hormone secretion (Stephan and Zucker, 1972; Abe *et al.*, 1979).

Over the past two decades there have been a number of breakthroughs in the understanding of circadian biology and clock function in mammals. In the late 1990s it was discovered that exposing cultured rat fibroblasts to serum shock induced circadian gene expression (Balsalobre *et al.*, 1998). Further experiments including the demonstration that individual fibroblast cells could function autonomously as circadian oscillators (Welsh *et al.*, 2004) led to the realization that the circadian clock apparatus was present in essentially all body organs and tissues (Hastings *et al.*, 2008; Albrecht, 2012).

These remarkable discoveries have led to a new era in circadian biology. The previous dogma asserting the SCN as the sole circadian pacemaker has been overthrown, replaced by a new model of the SCN as a "master clock" that coordinates and synchronizes rhythms in peripheral tissues (Yamazaki, 2000). Outputs from the SCN project to other brain structures including to the paraventricular nucleus (PVN) of the hypothalamus (Dibner *et al.*, 2010). Connections from the PVN synchronize peripheral clocks to the light/dark cycle via autonomic (parasympathetic and sympathetic) pathways and via hormone signaling (such as cortisol and melatonin) (Yamazaki, 2000; Dibner *et al.*, 2010; Welsh *et al.*, 2010).

The cell clock machinery present both in the master pacemaker neurons of the SCN and in peripheral tissues consists of transcriptional–translational feedback loops. These loops oscillate with a period of approximately 24 h, thereby generating the circadian rhythm. The principal loop is made up of two transcription activators: brain and muscle ARNTL-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK). The BMAL1/CLOCK dimer complex activates numerous so-called clock-controlled genes as well as inducing transcription of *Period* (Per) and *Cryptochrome* (CRY). Increasing levels of PER and CRY act via negative feedback to inhibit CLOCK and BMAL transcription. PER and CRY are degraded and the cycle begins once more (see Asher and Sassone-Corsi, 2015; Feng and Lazar, 2012 for excellent reviews). Feedback is also regulated by a number of post-translational mechanisms including phosphorylation and glycosylation of clock proteins (Crane and Young, 2014). When the clock mechanisms are disrupted, so too are circadian rhythms. Deactivating mutations of PER and CRY result in the complete dysregulation of glucocorticoid rhythms and clock gene expression in mice (Dallmann *et al.*, 2006; Oster *et al.*, 2006; Yang *et al.*, 2009).

The Hypothalamic–Pituitary–Adrenal Axis as a Model Dynamic Endocrine Rhythm

Glucocorticoids are synthesized and secreted from the adrenal cortex under control of the hypothalamic–pituitary–adrenal (HPA) axis. Acting through glucocorticoid and mineralocorticoid receptors which are nearly ubiquitously expressed, they regulate growth, metabolism and the response to stress. Glucocorticoids are intrinsically linked to the circadian system through their ability to influence the clock rhythms of multiple tissues (Oishi *et al.*, 2005; Pezük *et al.*, 2012). The potent synthetic corticosteroid dexamethasone alters the circadian phase of gene expression in liver, kidney, and heart but not the SCN (Balsalobre *et al.*, 2000), presumably because glucocorticoid receptors (GR) are not expressed there (Rosenfeld *et al.*, 1993).

The HPA Axis Circadian Rhythm

Basal secretion of cortisol in humans (and corticosterone in rats) is circadian with peak concentrations on waking (Lightman and Conway-Campbell, 2010), anticipating the onset of daily activity. The rhythm is modulated by both stimulatory and inhibitory outputs from the SCN whose axons project into the PVN of the hypothalamus (Kalsbeek *et al.*, 2012). The PVN contains corticotrophin-releasing hormone (CRH) neuroendocrine neurons that in term stimulate adrenocorticotrophic (ACTH) synthesis from the anterior pituitary (Spiga *et al.*, 2014). ACTH released into the peripheral circulation acts at the adrenal cortex to stimulate glucocorticoid synthesis and release (**Fig. 1**).

The adrenal response to ACTH, and thus cortisol secretion, is influenced by inputs from the central nervous system and by intrinsic clock mechanisms within the adrenal itself. Outputs from the SCN connect via a multisynaptic pathway to the autonomic nervous system (Buijs *et al.*, 1999). Branches of the thoracic splanchnic nerve provide extensive sympathetic autonomic innervation to the adrenal glands (Engeland, 1998). Sympathetic input appears to modulate the adrenal-ACTH response in different phases of the sleep/wake cycle. During the awake phase (nighttime in rats) sympathetic drive is highest, increasing ACTH sensitivity and promoting adrenal steroidogenesis (Ulrich-Lai *et al.*, 2006). Conversely, using the same rat model, transecting the

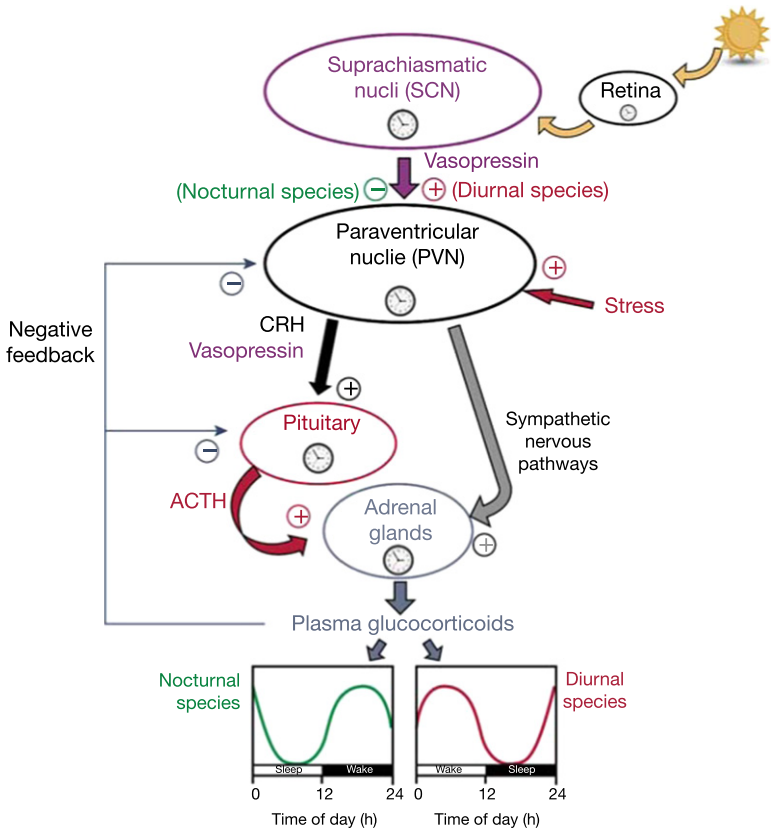

Fig. 1 Representation of the control of circadian glucocorticoid release in mammals. The circadian secretion of GC is dependent on the rhythmic release of ACTH and a gating process by the adrenal clock. *Clock symbols* represent self-sustained oscillators. GC secretion is also modulated by nervous signals coming from the PVN of the hypothalamus via sympathetic nervous pathways. ACTH release is controlled by the rhythmic release of CRH and vasopressin from the PVN. Rhythmic activity of the HPA axis is under the control of the master clock in the SCN, reset by ambient light via the retina. Reproduced from The Functional and Clinical Significance of the 24-Hour Rhythm of Circulating Glucocorticoids, Endocr Rev. 2016;38(1):3–45. doi:https://doi.org/10.1210/er.2015-1080. Copyright © 2017 Endocrine Society. This article was published under the terms of the Creative Commons Attribution License (CC BY; https://creativecommons.org/licenses/by/4.0/).

splanchnic nerve disrupts the normal diurnal variation in CORT levels by attenuating ACTH responsiveness and reducing CORT production at night (Jasper and Engeland, 1994).

Intrinsic properties of the adrenal cell also provide a "gating" mechanism for sensitivity to ACTH. In mouse adrenal slices, which are isolated from external synchronizing inputs, the response to ACTH varies depending on the phase of the circadian cycle (Oster *et al.*, 2006). In vivo experiments in wild-type mice show that ACTH stimulation at the time of zenith CORT results in significantly higher CORT secretion than the same stimulus given at the CORT nadir. However, in mice with mutated non-functioning clock genes, this variation is abolished—the ACTH response at both time points is similar to the "CORT nadir" time point in wild-type animals (Oster *et al.*, 2006).

In summary, glucocorticoids are secreted with a diurnal, circadian rhythm that is entrained by the SCN. The rhythm is modulated at the level of the adrenal by sympathetic nerve inputs from the CNS and by intrinsic adrenal clock mechanisms that "gate" sensitivity to ACTH stimulus depending on the phase of the circadian cycle.

The Ultradian Rhythm and the HPA Axis: Mechanisms and Clinical Significance

Pituitary hormones are not released continuously—rather they are secreted in pulses that vary in amplitude and/or frequency over a 24 h period (Weitzman *et al.*, 1971; Veldhuis *et al.*, 1990). High resolution sampling of blood in both animals (Jasper and Engeland, 1991) and humans (Weitzman *et al.*, 1971; Henley *et al.*, 2009a,b) demonstrate that glucocorticoids are secreted in pulses every 60–90 min (Veldhuis *et al.*, 2008; Spiga *et al.*, 2014). The origin of this ultradian rhythm was previously assumed to be hypothalamic. However, while lesions to the SCN produce the expected abolishment of circadian CORT secretion, they do not disrupt the pulsatile secretion of CORT, which remains ultradian (Waite *et al.*, 2012). This led to the suggestion that the ultradian CORT rhythm is generated outside of the hypothalamus and the convincing hypothesis that the rhythm is intrinsic to the properties of the HPA axis. Mathematical modeling predicts that ultradian oscillations in ACTH and cortisol secretion are a consequence of the dynamic interaction between positive feed-forward from hypothalamic CRH drive and pituitary ACTH

secretion, and negative feedback generated by adrenal cortex secretion of cortisol (Walker *et al.*, 2010). In vivo experiments using rats infused with constant levels of CRH generated ultradian oscillations in both ACTH and CORT (Walker *et al.*, 2012). This supports the premise that pulsatile CRH secretion from the hypothalamus is not required for the generation of the ultradian rhythm. Further, rapid negative feedback by glucocorticoid inhibition on the anterior pituitary must be the crucial factor regulating the dynamic rhythm (**Fig. 2**).

The concept of the pulsatile ultradian hormone rhythm, rather than simply a circadian biphasic rhythm, has become increasingly recognized as physiologically important. Pulsatile exposure to glucocorticoids affects expression of genes and gene transcripts in the liver (Stavreva *et al.*, 2009), brain (Conway-Campbell *et al.*, 2010; George *et al.*, 2017), and many other tissues. In contrast to rats exposed to constant levels of glucocorticoid, CORT given in ultradian pulses induced cyclical GR-mediated gene transcription, or "gene pulsing" (Stavreva *et al.*, 2009). On a behavioral level, responses to stress are blunted in adrenalectomized rats exposed to continuous compared to the normal response when given pulsed CORT replacement (Sarabdjitsingh *et al.*, 2010). These animal studies indicate that pulsatile exposure to glucocorticoids is necessary for normal gene transcription and behavior responses. However, to date very little is known about the implication of these findings in humans. It is proposed that there are likely to be significant biological consequences of altered glucocorticoid pulsatility, alluded to in a few studies of depression (Young *et al.*, 2007), obstructive sleep apnea (Henley *et al.*, 2009a,b), and major cardiac surgery (Gibbison *et al.*, 2013).

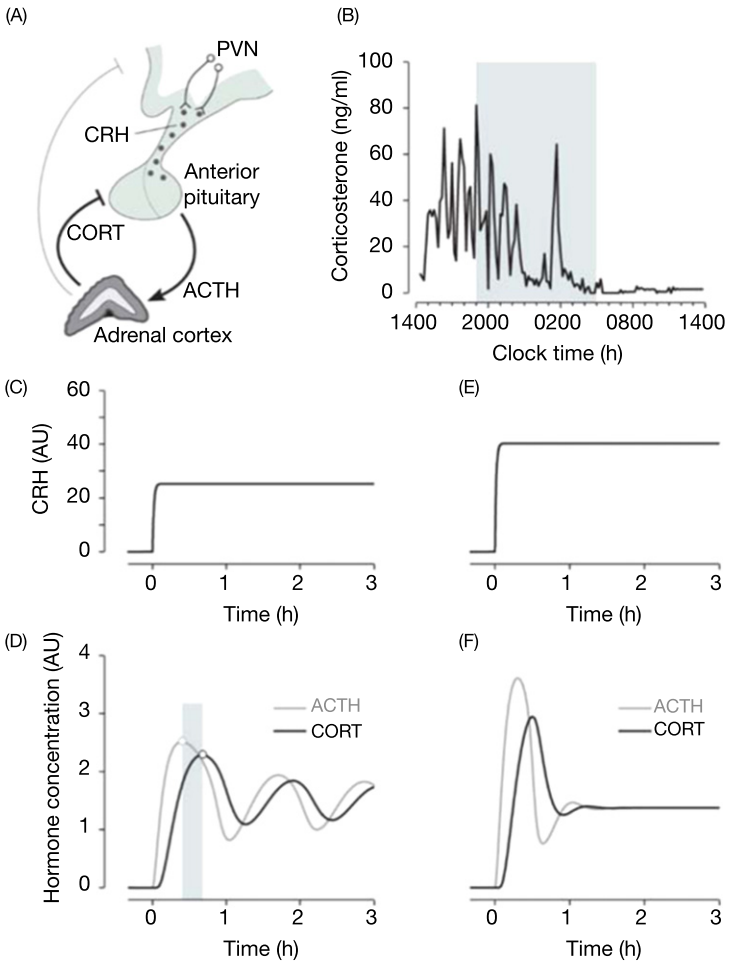

Fig. 2 Regulation of glucocorticoid secretion. (A) Negative feedback in the HPA axis plays a key role in regulating glucocorticoid (CORT) secretion. (B) Ultradian corticosterone (the main glucocorticoid in rodents) oscillations in a freely behaving male Sprague-Dawley rat. Shaded region indicates the dark phase. (C–F) Mathematical modeling predicts that ultradian ACTH and glucocorticoid (CORT) oscillations are regulated by a systems-level negative feedback mechanism in the pituitary-adrenal network, independent of pulsatile hypothalamic activity. Numerical simulations show that the pituitary-adrenal network can oscillate under conditions of constant CRH drive to the pituitary (C–D). Oscillations in ACTH and CORT are characterized by a small phase shift (*shaded region* indicates phase difference between oscillation peaks). For higher levels of CRH drive, the oscillations are rapidly damped to steady-state levels of hormone (E–F). *AU*, arbitrary units. Reproduced from The Origin of Glucocorticoid Hormone Oscillations. PLoS Biol 10(6): e1001341. https://doi.org/10.1371/journal.pbio.1001341.

Rhythms and Endocrine Disease

Endocrinological disorders affect tens of millions of people worldwide with a broad spectrum of manifestations and long-term health effects. Cushing syndrome broadly describes the condition of excess cortisol and may originate from ACTH secreting tumors, typically located in the anterior pituitary, or from excess production by adrenal tumors. When iatrogenic forms (caused by exogenous steroid administration) are excluded, incidence is approximately 0.2–5.0 per million per year, with a population prevalence estimated between 39 and 79 per million (Lacroix *et al.*, 2015). Cushing syndrome causes widespread disease manifestations with increased mortality principally a result of cardiovascular and cerebrovascular disease (Sharma *et al.*, 2015).

The converse of Cushing's, primary adrenal insufficiency, results from the loss or destruction of adrenal tissue and the consequent life-threatening deficiency in adrenal corticosteroid production. Prevalence is around 100–140 per million, and incidence is approximately 1–4:1,000,000/year in Western countries (Bornstein *et al.*, 2016). Even with so-called physiological glucocorticoid replacement, mortality remains twice that of the general population (Bergthorsdottir *et al.*, 2006) and quality of life as measured by subject health scores remains significantly lower than controls (Bleicken *et al.*, 2008).

The diagnostic approach for endocrine disorders is typically to use single-time point measures to demonstrate lack of hormone suppression (in diseases such as Cushing's (Nieman *et al.*, 2008), acromegaly (Katznelson *et al.*, 2014), and primary aldosteronism (PA) (Funder *et al.*, 2016)) or failure to provoke adequate response to stimulation (primary adrenal insufficiency (Bornstein *et al.*, 2016), growth hormone deficiency (Ho, 2007)). Results are often extremely difficult to interpret as the superimposition of circadian and ultradian rhythms results in a wide interindividual variation in the "normal" range (Spiga and Lightman, 2015). Further, variation in measurement methodology makes standardization of results very challenging. Using cortisol as an example, most assays measure total hormone concentrations rather than the free (active) fractions, apart from that measured in the urine and saliva. Salivary cortisol is a noninvasive technique but factors influencing results such as gender, age, and coexisting illness have not been fully characterized (Nieman *et al.*, 2008) and false positive rates may be 20%–40% (Liu *et al.*, 2005). Likewise 24-h urine cortisol collections are cumbersome, often unreliable and subject to multiple extrinsic factors that can falsely affect results (Petersenn *et al.*, 2014).

Studies of circadian or ultradian hormone patterns in humans with endocrine disease are generally limited as the investigative methods are laborious and invasive, requiring inpatient admission and blood sampling typically every 10–20 min. In a study of a small group of patients with primary adrenal insufficiency, blood was sampled every 30–60 min over 24 h. These patients, treated with twice daily oral glucocorticoid replacement, had markedly different profiles of ACTH and cortisol compared with healthy controls (Scott *et al.*, 1978). Now, almost 40 years after that study was published, very little has changed with respect to the regimen of glucocorticoid hormone replacement. So-called physiological doses of glucocorticoid replacement, typically prescribed two to three times per day, do not mimic circadian or ultradian secretion patterns of hormone release. It is therefore perhaps not surprising that quality of life and a wide spectrum of health outcomes remain poor among patients with primary adrenal insufficiency (Andela *et al.*, 2016; Bensing *et al.*, 2016).

Rhythms and Treatment in Hormone-Deficient States

Recent attempts to produce glucocorticoid replacement regimens that mimic endogenous conditions have primarily focused on replicating diurnal CORT rhythms. Once daily dose-modified release preparations of hydrocortisone intend to reproduce "physiological" cortisol profiles (Johannsson *et al.*, 2009, 2016; Mallappa *et al.*, 2015) but do not account for ultradian rhythms. Despite early evidence of some benefit (Quinkler *et al.*, 2015; Giordano *et al.*, 2016), the only prospective long-term study using these medications published to date has reported no improvement in quality of life at 5 years of follow-up (Nilsson *et al.*, 2017). Continuous subcutaneous infusions of cortisol can also mimic diurnal patterns more accurately than oral dosing, but again does not seem to improve quality of life (Gagliardi *et al.*, 2014). Together, these findings support the hypothesis that the ultradian pulsatile rhythm is extremely important and needs to be considered when prescribing replacement glucocorticoids.

Rhythms in Hormone Excess States

High frequency blood sampling in patients with secretory pituitary tumors predictably shows that hormone secretion patterns are manifestly abnormal with marked increases in total hormone secretion compared with controls (Roelfsema *et al.*, 2014). However, it is interesting to observe that secretion remains pulsatile in these conditions. Pulsatile secretion of ACTH and cortisol can be observed in 24-h profiles of patients with pituitary Cushing's disease, although the pattern is disordered with increased pulse frequency and amplitude (Roelfsema *et al.*, 1998). Following successful surgery, secretion profiles returned to normal, matching those of healthy controls (Roelfsema *et al.*, 2011). In a study of 12 patients with adrenal-based Cushing syndrome (where ACTH is suppressed), a similar pattern of abnormal cortisol secretion was observed, with increased pulse frequency and augmented pulsatile secretion (Van Aken *et al.*, 2005). A diurnal rhythm of secretion was maintained, although phase shifted compared with controls. It is likely that alternative outputs from the SCN contributed to this rhythm in the absence of oscillatory ACTH secretion, in addition to gated peripheral ACTH sensitivity mediated by adrenal cell circadian clock rhythms.

Aldosterone secretion is also rhythmic (Wolfe *et al.*, 1966). PA, the inappropriate secretion of aldosterone, is characterized by the development of pathological hypertension (Funder *et al.*, 2016). In a small cohort study comparing 10 patients with PA with

healthy controls, Siragy *et al.* used high frequency blood sampling to observe both circadian and ultradian rhythms of aldosterone secretion. Basal rates and the amplitude of pulsed aldosterone secretion were significantly augmented in PA compared with healthy controls but there was no difference in pulse frequency between the groups (Siragy *et al.*, 1995).

Together, these two conditions provide examples of how rhythms are intrinsic to endocrine processes and the important implications for diagnosis and management.

Ultradian Rhythms: Future Opportunities for Diagnosis and Treatment?

In the setting of adrenal insufficiency, administration of pulsatile subcutaneous glucocorticoid to adrenally suppressed healthy volunteers reproduces a more accurate ultradian rhythm than seen in any other studies in humans (Russell *et al.* 2014). Studies investigating the impact of pulsatile versus twice daily or continuous administration of CORT in patients with adrenal insufficiency are currently being conducted (see http://www.isrctn.com/ISRCTN67193733). A major multicenter clinical trial using ambulatory high frequency hormone sampling (https://clinicaltrials.gov/ct2/show/NCT02934399) aims to provide new information about ultradian endocrine rhythms in both health and disease, with implications for the diagnosis and treatment of these conditions. Paying heed to the ultradian as well as the circadian physiology of conditions such as PA, Cushing's and adrenal insufficiency will ultimately aide clinicians who are limited by the nonspecific, nondynamic set of investigations currently available.

Conclusions

In summary, endocrine hormones are typically secreted in rhythmic patterns with circadian and ultradian pulsatile secretion. Circadian rhythms are established and maintained by cellular clocks, both centrally and in peripheral tissues. Ultradian rhythms modulate processes on shorter time scales influencing gene transcription at the cell level and neurocognitive processes at the behavioral level. Current diagnosis and monitoring typically relies on single time point measurements using unreliable methods, and therefore cannot accurately reflect the true dynamic nature of the disease being studied. Approaches that take into account ultradian as well as circadian physiology promise to provide new insight into the diagnosis and management of endocrine conditions.

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Growth Factors

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Adipose Tissue as an Endocrine Organ

Adipose tissue is not simply an organ that only accumulates and releases lipids, but it is an important endocrine organ that secretes a great variety of substances including steroid hormones, cytokines and growth factors, regulators of lipid metabolism, etc. (Fischer-Posovszky *et al.*, 2007). These factors are called adipokines, and function as part of a complex set of physiological control systems that regulate the local tissue and the physiology of the whole organism. Therefore, adipokines contribute to the regulation of various biological processes including inflammation and immune function, vascular biology, hematopoiesis, cell proliferation and angiogenesis (Radin *et al.*, 2009). Herein, we highlight the adipokines more directly involved in the regulation of energy balance and metabolism.

Leptin

The term leptin comes from Greek (leptos: lean). It is a hormone secreted by the white adipose tissue discovered after the characterization of the effect of the absence of the gene of leptin and its receptor in mice (ob/ob and db/db respectively). These recessive mutations of the obese (ob) gene and the diabetes (db) gene produce in these mice obesity and type 2 diabetes, generating a syndrome similar to morbid obesity in humans (Coleman, 1978; Friedman and Leibel, 1992). The ob/ob and db/db mice have identical phenotypes, weighing three times more than normal mice, showing an increase of up to five times the body fat content. They are hyperphagic, have little locomotive activity and are sexually inactive. Leptin works within a long-term regulatory system, modulating the amount of food consumed in relation to the amount of energy expended (Friedman and Halaas, 1998). It is considered an efferent hormone with a negative feedback function that keeps the adipose tissue mass constant. Leptin is secreted by adipocytes either as a 16 KDa peptide as bound to a soluble form of its receptor (Ob-R). The amount of secreted leptin correlates positively with the amount of fat mass, suggesting that its regulation is linked to a lipid-sensitive mechanism (Maffei *et al.*, 1995). An increase in plasma levels generates a negative energy balance, while the reduction of circulating leptin levels produces a positive energy balance (intake > energy expenditure). Leptin reaches the CNS through the circumventricular organ, crossing the blood-brain barrier in a process dependent on tanycytes (specialized glial cells), which translocate leptin from the blood to the third ventricle, and from there accrete the hypothalamic nuclei (Balland *et al.*, 2014; Langlet *et al.*, 2013). In the hypothalamus, leptin exerts its action on neurons that express their receptor (Ob-R) through a signaling pathway dependent on STAT3 (Ghilardi *et al.*, 1996; Matsuoka *et al.*, 1999). Already in the ARC, leptin depolarizes the POMC neurons, whose anorexigenic response produces a decrease in intake and an increase in energy expenditure (Cowley *et al.*, 2001). On the other hand and simultaneously, leptin inhibits in the arcuate nucleus the neurons with orexigenic effect AgRP/NPY, enhancing the satiating effect of the hormone (van den Top *et al.*, 2004).

Adiponectin

It is a protein secreted and produced exclusively by adipocytes, being one of the most abundant plasma proteins in the bloodstream (Ouchi *et al.*, 2003). It is considered an anti-inflammatory factor, presenting a negative correlation in its plasma concentration with respect to the amount of adipose tissue (Ryo *et al.*, 2004). Therefore, the production of adiponectin is inhibited by pro-inflammatory factors such as tumor necrosis factor alpha (TNFα) and interleukin 6, as well as stress and hypoxia (Hosogai *et al.*, 2007). Numerous clinical studies show a relationship between adiponectin and obesity: adiponectin is reduced in patients with type 2 diabetes, the high concentration of this protein in plasma being a protective factor against diabetes. At the metabolic level, adiponectin increases the oxidation of fatty acids in the muscle and reduces plasma levels of glucose, free fatty acids and triglycerides via the AMP kinase pathway (Fruebis *et al.*, 2001).

Resistin

Resistin is an adipokine belonging to a group of proteins associated with inflammatory processes. Despite being an adipokine, it has been described that this protein is also produced by other tissues such as brown adipose tissue, stomach, intestine, and muscle (Nogueiras, 2003). Its name comes from its effect on the insulin resistance it produces in mice (Steppan *et al.*, 2001); knock-out mice for resistin have lower post-pandrial glucose levels and lower hepatic gluconeogenesis than normal mice (Banerjee *et al.*,

2004). The ability of resistin to modulate insulin resistance is associated with the activation of the suppressor of cytokine 3 signaling (SOCS3), an inhibitor of insulin signaling in adipocytes (Steppan *et al.*, 2005). In rodents, resistin is regulated by metabolic and nutritional status, decreasing in fasting and being increased in states of obesity and type 2 diabetes (Lazar, 2007). Like leptin, the main effects of resistin on energy metabolism are mediated by the hypothalamus; the central administration of resistin reduces the intake and regulates the lipid metabolism of peripheral tissues (Tovar *et al.*, 2005; Vazquez *et al.*, 2008).

Function of White Adipose Tissue on Obesity-Related Diseases

The hypertrophy of adipose tissue in obese organisms also contributes to other disease as type 2 diabetes or the generation of non-alcoholic fatty liver disease (NAFLD). There is a close relationship between hypertrophic adipocytes resistant to insulin, dysregulated immunity in both fat and liver and NAFLD induced by lipotoxicity. The adipocytes and the vascular fraction of the fatty tissue (stroma) secrete many hormones, complement factors, cytokines (TNF-α, interleukins), chemokines and adipokines functioning as an endocrine tissue. As mentioned previously, adiponectin is one of these adipokines, whose reduced secretion of it in obesity alters lipid metabolism and insulin sensitivity in the liver; The administration of adiponectin in obese mice fed a high-fat diet dramatically alleviates hepatomegaly, steatosis and inflammation (Xu *et al.*, 2003). In steatohepatitis, decreased secretion of adiponectin by dysfunctional adipocytes contributes to the formation of NASH (Polyzos *et al.*, 2010).

The macrophages of adipose tissue are an important fraction of the stroma (Halberg *et al.*, 2008; Fuentes *et al.*, 2010). Activation of adipose tissue macrophages plays an important role in fat dysfunction generating insulin resistance, free fatty acid release into the bloodstream and ectopic fatty deposition in the liver (Cusi, 2012). In the stroma there are two types of macrophages: (1) M1 macrophages or classical activation macrophages that play a key role in humoral immunity and in response to pathogens; secrete large amounts of pro-inflammatory cytokines (TNF-α, IL-6, IL-12, …) and low levels of anti-inflammatory cytokines (IL-10); and (2) M2 macrophages or alternative activation with anti-inflammatory function (Lumeng *et al.*, 2007). An increase in M1 macrophages relative to M2 is characteristic of rodents fed a high-fat diet (Lumeng *et al.*, 2007; Odegaard *et al.*, 2008) and obese humans (Lumeng and Saltiel, 2011).

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Normal Glucose Physiology☆

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Glossary

Gluconeogenesis Synthesis of new glucose molecules from substrates such as lactate, pyruvate, alanine, glutamine, and other amino acids by reversal of glycolysis.
Glycogen Large intracellular granules made up of polymers of glucose used for storage of carbohydrate mainly in liver and skeletal muscle.
Glycogenolysis Breakdown of glycogen molecules to glucose.
Glycolysis Anaerobic metabolic breakdown of glucose to lactate and pyruvate.
Lipolysis Breakdown of triglyceride molecules, yielding one molecule of glycerol and three free fatty acid molecules.
Triglycerides Fat droplets composed of molecules of three free fatty acids esterified to one molecule of glycerol.

Glucose: From Origins to Fates

Glucose in plasma either comes from dietary sources or can be the product of the breakdown of glycogen in liver (glycogenolysis) or the formation of glucose in liver and kidney from other carbons compounds (precursors) such as lactate, pyruvate, amino acids, and glycerol (gluconeogenesis).

In humans glucose removed from plasma may have different fates in different tissues and under different conditions(e.g., postabsorptive vs. postprandial), but the pathways for its disposal are relatively limited. It may be: (1) immediately stored as glycogen or (2) undergo glycolysis which can be *non-oxidative* producing pyruvate (which can be reduced to lactate or transaminated to form alanine) or *oxidative* through conversion to acetyl CoA which is further oxidized through the tricarboxylic acid cycle to form carbon dioxide and water. Non-oxidative glycolysis carbons undergo gluconeogenesis and the newly formed glucose is either stored as glycogen or released back into plasma (Fig. 1).

Importance of Glucose Homeostasis

Although free fatty acids (FFA) are the main fuel for most organs, glucose is the obligate metabolic fuel for the brain under physiologic conditions. This occurs because of low circulating concentrations of other possible alternative substrates (e.g.,

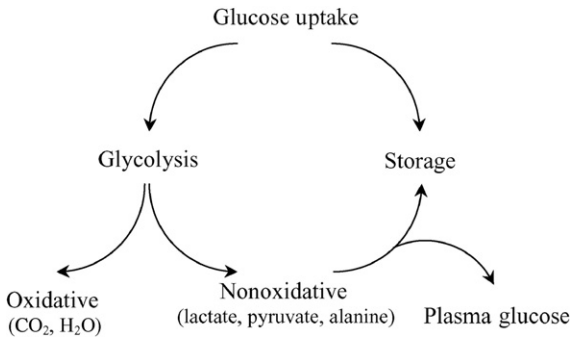

Fig. 1 Routes of post-prandial glucose disposal. From Woerle, H.J., Meyer, C., Dostou, J.M., Gosmanov, N.R., Islam, N., Popa, E., Wittlin, S.D., Welle, S.L. and Gerich, J.E. (2003). Pathways for glucose disposal after meal ingestion in humans. *American Journal of Physiology. Endocrinology and Metabolism* **284**, E716–E725, with permission. Copyright© 2003 The American Physiological Society.

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ketone bodies) or because of limitations of transport across the blood-brain barriers (e.g., FFA) (Siesjo, 1988). Brain cannot synthesize glucose or store it as glycogen more than few minutes supply. Thus brain is dependent on a continuous supply of glucose from plasma. Glucose plasma concentrations below 55 mg/dL (3 mmol/L) impair cerebral function (Mitrakou *et al.*, 1991a) whereas more severe and prolonged hypoglycemia cause convulsions, permanent brain damage and even death. Hypoglycemia is associated with multiple other complications. On the other hand, hyperglycemia or diabetes mellitus has its own risks to health.

General Considerations

Relative Changes in Glucose Fluxes

Plasma glucose concentrations are determined by the relative rates at which glucose enters and leaves the circulation. Thus the plasma glucose will increase only if the rate of entry exceeds its rate of exit and, conversely, plasma glucose level will decrease only if rates of exit exceeded the rates of entry. To maintain relatively stable plasma glucose concentrations, increases in rates of glucose delivery into the systemic circulation (e.g., when meal is ingested) require a comparable increase in rates of glucose removal from the circulation (Kelley *et al.*, 1988). For example, during vigorous exercise, fever or trauma when the body's utilization of glucose increases, there is normally a compensatory increase in glucose delivery (Wahren *et al.*, 1978).

Changes in glucose clearance, an index of efficiency of glucose removal from the circulation by itself do not affect plasma glucose concentrations independent of changes in rates of glucose entry and exit.

Factors Influencing Glucose Fluxes

The most important factors on a moment to moment basis are hormones (insulin, glucagon and catecholamines), the sympathetic nervous system activity as well the concentration of other substrates (FFA). On a more prolonged time basis (hours-days), other hormones (cortisol and growth hormone), nutritional factors (e.g., diet composition), exercise and physical fitness, along with concomitant changes in the sensitivity to hormones become important (Gerich, 1993). Cortisol, growth hormone and catecholamines affect glucose homeostasis by altering insulin sensitivity and also by changes in the availability of alternative substrates.

Fasting vs. Postprandial States

The mechanisms delivering glucose into the circulation (i.e., glycogenolysis vs. gluconeogenesis) and the sites for glucose disposal will vary depending on duration of fasting. For example, as fasting is prolonged, the proportion of gluconeogenesis increases and the contribution of hepatic glycogen stores decreases. Moreover, the relative contribution of the kidney increases. In regard to utilization, after an overnight fast, there is no net storage of glucose and all glucose taken up by tissues is either completely oxidized or converted to lactate.

Glucose Transport Pathways

Due to its hydrophilic nature, glucose diffuses slowly across the lipid bilayer of the cell membrane, and needs specific transporter proteins to facilitate its entry into cells. There are two distinct families of transport proteins (Bouche *et al.*, 2004). (1) *Facilitative GLUT family*: These transporters promote facilitated diffusion of glucose, a process that is not energy dependent and that follows Michaelis–Menten kinetics (Gottesman *et al.*, 1984). The high-affinity transporters (GLUT 1, 3, 4) have a Michaelis–Menten constant (K_m) below the normal range of blood glucose concentrations and are capable of providing glucose transport under basal conditions for many cells (Bouche *et al.*, 2004). GLUT1 is present in pancreatic α cells in which glucose entry is the major regulator of their glucagon secretion. GLUT3 is the major neuronal transporter (lowest K_m) whereas GLUT4 mediates insulin-stimulated glucose uptake by skeletal muscle, heart, and adipose tissues. Insulin and exercise promote GLUT3 expression on cell surface (Bouche *et al.*, 2004; Rodnick *et al.*, 1992). The low-affinity transporters (GLUT2) are present on ß cells and in tissues exposed to large glucose fluxes, such as intestine, liver, and kidney (Bouche *et al.*, 2004). (2) *SGLT family*: These transporters utilize the electrochemical sodium gradient to transport glucose against concentration gradients (Bouche *et al.*, 2004; Wright, 2001). SGLT1 is responsible for the dietary uptake of glucose from the small intestine lumen. Both SGLT1 and SGLT2 are present in the renal proximal convoluted tubulae and are responsible for reabsorption of glucose from glomerular filtrate as discussed in detail below (Bouche *et al.*, 2004; Wright, 2001; Bonner *et al.*, 2015; Mueckler, 1990; Cushman and Wardzala, 1980). SGLT2 inhibitors have recently been approved for treatment in patients with T2DM. They have significant glucose-lowering effect by decreasing reabsorption of glucose from glomerular filtrate and inducing glucosuria.

In the kidney, chronic hyperglycemia upregulates SGLT2 expression and activity (Mogensen, 1971; Dominguez *et al.*, 1994). The pathway for this increased expression involves protein kinase A and protein kinase C (Rorsman *et al.*, 2014; Komala *et al.*, 2013) with insulin being the physiologic agonist for this effect (Ghezzi and Wright, 2012). Multiple other factors have also been found to alter the expression of SGLT2 including hepatocyte nuclear factor 1α (HNF1α), serum and glucocorticoid-regulated kinase 1 (SGK1), transforming growth factor ß (TGFß), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Komala *et al.*, 2013).

Additionally, treatment with losartan (an angiotensin receptor blocker) reduced renal SGLT2 expression in diabetic rats suggesting that angiotensin II is involved in regulating SGLT2 expression and that changes in renal transporters expression could be important in the development of hypertension in diabetes (Osorio *et al.*, 2009).

Actions of Key Regulatory Factors (Table 1)

Insulin

Insulin regulates glucose metabolism by direct and indirect actions. Through binding to its receptors in the liver, kidney, muscle and adipose tissue, insulin activates its signaling pathway which involves a complex cascade of protein kinases and regulatory proteins of which IRS-1 and IRS-2 are the most important. This causes: (1) suppression of glucose release from liver and kidney (Meyer *et al.*, 1998a), (2) translocation of glucose transporters in muscle and adipose tissue to increase their glucose uptake (Oster-Jorgensen *et al.*, 1990), and (3) inhibition of release of FFA into the circulation due to suppression of the activity of *hormone-sensitive lipase* and a simultaneous increase in their clearance from the circulation (Meyer *et al.*, 1997). Although insulin does not increase glucose transport into liver, it promotes glycogen accumulation by inhibiting *glucose-6-phosphatase*② and *phosphorylase*① (glycogenolysis enzymes) while stimulating *glycogen synthase*③ (Gerich, 2000a).

The effect of insulin on circulating FFA levels indirectly reduces glucose release into circulation and promote glucose removal since FFA stimulate gluconeogenesis and reduce glucose transport into cells (Meyer *et al.*, 1997).

Metabolic processes vary in their sensitivity to insulin and their dose-response characteristics. At basal levels observed in the postabsorptive state ($\sim$5–10 μU/mL), insulin is already inhibiting glucose and FFA release 30%–50% (counteracting the effect of glucagon and the sympathetic nervous system) while having a trivial effect on tissue glucose uptake. Maximal suppression of glucose and FFA release normally is observed with plasma insulin concentrations seen postprandially ($\sim$40–50 μU/mL) whereas maximal stimulation of tissue glucose uptake requires plasma insulin concentrations $>$300 μU/mL levels not seen under normal physiological conditions except in extremely insulin resistant individuals in whom, of course, such level would not produce maximal effect (Gerich, 1993; Stumvoll *et al.*, 2000; Woerle *et al.*, 2003).

The leading regulator of insulin secretion is the plasma glucose concentration: increased plasma glucose after meal ingestion results in 3–4 folds increase in plasma insulin within 30–60 min whereas a decrease plasma glucose below 50 mg/dL ($\sim$2.7 mmol/L) will result in 80%–90% reduction in plasma insulin levels. The main pathway for insulin release is through ATP-regulated potassium (K_{ATP}) channels (Jones *et al.*, 1998). The pathway starts with glucose entry into ß cells. The intracellular glucose is then metabolized in a process that increases intracellular ATP which in turn triggers closure of K_{ATP} channels. The channel closure prevents potassium from leaving ß cells and this causes depolarization of cell membrane and subsequently calcium entry into ß cells through L-type voltage-gated calcium channels. The increase in intracellular calcium activates protein kinases and consequently exocytosis of insulin secretory granules/insulin release (Jones *et al.*, 1998).

Acute increases in amino acids, and to a lesser extent, FFA also increase insulin secretion (Gerich, 1993, 2000a; Stumvoll *et al.*, 2000; Woerle *et al.*, 2003). After meal ingestion, intestinal factors called incretins (e.g., gastrointestinal-inhibitory peptide [GIP], glucagon-like peptide [GLP-1]) augment insulin secretion. This is why plasma insulin concentrations increase to a greater extent after oral glucose load than after intravenous glucose despite identical plasma glucose concentrations (Woerle *et al.*, 2003; Gosmanov *et al.*, 2005).

Glucagon

Glucagon, a hormone secreted from the α cells of the endocrine pancreas, is the major counterpart to insulin in the moment to moment regulation of plasma glucose. Glucagon acts exclusively on the liver where it binds to its receptors and activates *adenylate cyclase*. As a result, intracellular cAMP level increases, enhancing glycogenolysis as a result of phosphorylase stimulation (Magnusson *et al.*, 1995; Gerich, 1981). This response wanes after several hours and is followed by an increase in gluconeogenesis due to a complex process involving both increased substrate uptake and enzyme activation (Gerich, 1993; Gosmanov *et al.*, 2005; Bolli *et al.*, 1984; Gromada *et al.*, 2007; Lecavalier *et al.*, 1989). Thus the main immediate action of glucagon to increase plasma glucose level is through stimulation of hepatic glycogenolysis (Lecavalier *et al.*, 1989).

Table 1 Mechanism of action of key metabolic regulators

	Glucose production	*Glucose utilization*	*Lipolysis*
Insulin	↓	↑	↓
Glucagon	↑	—	—
Epinephrine	↑	↓	↑
Cortisol	↑	↓	↑
Growth hormone	↑	↓	↑
FFA	↑	↓	—

Similar to insulin secretion from ß cells, glucagon secretion is influenced mainly by plasma glucose whereby its secretion is inhibited by hyperglycemia and stimulated by hypoglycemia. In humans, substrates other than glucose (e.g., FFA and amino acids) play less important role. The pathway for glucagon secretions also starts with glucose entry into α cells but the transporters appear to be different than those found in ß cells. The facilitative transporter in α cell is GLUT1 whereas it is GLUT2 in ß cells (Rorsman *et al.*, 2014; Heimberg *et al.*, 1995). More importantly, SGLT1 and SGLT2 transporters were recently also found to be present in α cells whereas they are absent in ß cells and are thought to play a key role in regulating glucagon secretion (Bonner *et al.*, 2015). For example, inhibiting SGLT2 via the SGLT2 inhibitors dapagliflozin (Ferrannini *et al.*, 2014; Merovci *et al.*, 2014) or SLC5A2 (the gene that encodes SGLT2) gene silencing triggers glucagon secretion (Bonner *et al.*, 2015). The exact intracellular pathway that leads to glucagon secretion following glucose entry into α cells is not completely understood. It is established however that the pathway involves ATP-regulated potassium (K_{ATP}) channels and that glucose-induced closure of these channels leads to inhibition of glucagon release (Rorsman *et al.*, 2014; Munoz *et al.*, 2005).

Catecholamines

Catecholamine release is mediated through changes in sympathetic nervous system, being increased during stress and hypoglycemia. Catecholamines inhibit insulin secretion while decreasing insulin action. Acting as both hormones (epinephrine) and neurotransmitters (norepinephrine), they are potent hyperglycemic factors whose actions, unlike those of glucagon, are sustained and affect both glucose release and glucose removal (Bolli *et al.*, 1984; Gerich *et al.*, 1980; Rizza *et al.*, 1980).

For the most part, catecholamines metabolic actions are mediated by beta 2 adrenergic receptors: at the liver they directly increase glycogenolysis via cAMP activation of *phosphorylase* and, to a lesser extent, augment gluconeogenesis indirectly through increasing gluconeogenic substrate availability and plasma FFA (Lecavalier *et al.*, 1989; Rizza *et al.*, 1980). At the kidney level, they are potent stimulators of gluconeogenesis both directly and indirectly as on the liver and are actually more potent stimulators of renal glucose release than hepatic glucose release (Stumvoll *et al.*, 1995). In skeletal muscles, they reduce glucose uptake and stimulate glycogenolysis which results in an increase in release of lactate- the major gluconeogenic precursor. In adipose tissue, catecholamines stimulate lipolysis via activation of *hormone-sensitive lipase* which results in an increase in the release of FFA and glycerol, another key gluconeogenic precursor (Lecavalier *et al.*, 1989; Gerich *et al.*, 1980; Rizza *et al.*, 1980; DeFeo *et al.*, 1991).

Growth Hormone and Cortisol

In contrast to glucagon and catecholamines which act almost immediately, the metabolic actions of growth hormone and cortisol generally take several hours to become evident. These can be summarized as being antagonistic to the action of insulin (i.e., they reduce the ability of insulin to suppress glucose release, stimulate glucose uptake and inhibit lipolysis) (Gerich *et al.*, 1980; Rizza *et al.*, 1982). Both hormones increase the synthesis of gluconeogenic enzymes and reduce glucose transport (Rizza *et al.*, 1982; DeFeo *et al.*, 1989a,b). In addition, cortisol can impair insulin secretion (DeFeo *et al.*, 1989b). Accordingly, the mechanisms for deterioration in glucose tolerance during immunosuppressive glucocorticoid treatment involve induction of insulin resistance and prevention of an appropriate compensatory increase in insulin secretion (DeFeo *et al.*, 1989b).

It is important to note that all of the counterregulatory hormones work via different intracellular mechanisms which reinforce/synergize with one another. Simultaneously small increases in their plasma levels will have greater effect than large increase in plasma level of only one hormone (Mitrakou *et al.*, 1991a).

FFA

FFA are the predominant fuel used by most tissues of the body, the major exceptions being the brain, renal medulla, and blood cells (Fanelli *et al.*, 1993; Cahill, 1970; Havel, 1972). Increases in plasma FFA have many potentially important metabolic consequences (Boden, 1997; McGarry, 1998): stimulation of hepatic and renal gluconeogenesis; inhibition of muscle glucose transport and competition with glucose as an oxidative fuel. The major regulators of circulating FFA levels are the sympathetic nervous system and growth hormone (Fanelli *et al.*, 1993) (which increase plasma FFA levels), insulin (which reduces plasma FFA levels by suppressing lipolysis and increasing FFA clearance) and hyperglycemia. There is evidence for heterogeneity of adipose depots with visceral fat being more metabolically active than subcutaneous fat (Fanelli *et al.*, 1993; McGarry, 1998).

Incretins: (Table 2)

The concept that certain factors secreted from the intestinal mucosa in response to nutrients can stimulate the pancreas to release insulin was first introduced to explain the phenomenon of greater increase in plasma insulin levels in response to oral glucose load compared with the same load of glucose given intravenously. The term *incretin* was used to denote these factors (Baggio and Drucker, 2007). Two main incretins are *gastric inhibitory polypeptide* (GIP) and *glucagon-like peptide-1* (GLP-1) (Baggio and Drucker, 2007; Brown *et al.*, 1975). Both peptides are secreted from intestinal endocrine mucosa (L and K cells) within minutes of nutrient ingestion and have short half-life (minutes) due to the rapid inactivation by a proteolytic enzyme called dipeptidyl peptidase-4 (DPP-4).

GLP-1 and GIP inhibit glucagon secretion (Nauck *et al.*, 2002); only GLP-1 delays gastric emptying and only GLP-1, possibly through a neural mechanism, promotes satiety, decreasing food intake and leads to weight loss (Baggio and Drucker, 2007).

Table 2 Effects of GLP1 and GIP on different tissues

	GLP1	*GIP*
Pancreas	↑ Insulin secretion ↓ Glucagon secretion	↑ Insulin secretion
Peripheral	↓ Hepatic glucose release ↑ Muscle glucose uptake	—
Gastric	Delays gastric emptying	↓ Gastric acid secretion only at supraphysiologic level
CNS	↑ Satiety, ↓ appetite, ↓ weight	—

Upper Gastrointestinal Function and Glycemic Homeostasis

Studies indicate that gastric emptying is a major physiologic determinant of nutrient delivery into the small intestine to the circulation and postprandial glycemia: it accounts for ~35% of the variance in peak blood glucose concentrations after ingestion of oral glucose in healthy volunteers (Rayner *et al.*, 2001; Horowitz *et al.*, 1993) or patients with type 2 diabetes (Rayner *et al.*, 2001; Jones *et al.*, 1996). It is delayed in acute hyperglycemia (Oster-Jorgensen *et al.*, 1990; MacGregor *et al.*, 1976), and accelerated during hypoglycemia (Bjornsson *et al.*, 1994).

Effect of Meal Composition

In healthy humans, adding protein or fat to oral glucose was found to lower postprandial glucose concentrations by slowing the gastric emptying and stimulating incretins. Protein also enhances non-glucose-dependent insulin release (Gentilcore *et al.*, 2006; Gerich *et al.*, 2001).

Gut Microbiota

Gut microbes affect glucose homeostasis through metabolic and immune interactions with the human host (Boulangé *et al.*, 2016; Greer *et al.*, 2016). They increase the density of small intestine villi capillaries and gut motility and also help digest dietary polysaccharides into absorbable sugars and short chain fatty acids (Boulangé *et al.*, 2016; Abrams and Bishop, 1967; Musso *et al.*, 2011; Gibson *et al.*, 2004). Short chain fatty acids in turn are ligands for G protein-coupled receptors including GPR43, the activation of which has been shown in animal studies to reduce fat accumulation, suppress insulin sensitivity in adipose tissues and increase insulin sensitivity in liver and muscle (Boulangé *et al.*, 2016; Backhed *et al.*, 2004). More recently, it has been shown in mice that *Akkermansia muciniphila*, a gut microbe also present in humans, mediate negative effects of interferon gamma (IFNγ) on glucose tolerance (Greer *et al.*, 2016). In IFNγ-deficient mice, *A. muciniphila* is increased and restoration of IFNγ levels reduces *A. muciniphila* in the microbiota. IFNγ-knockout mice whose microbiota has no *A. muciniphila* do not show improvement in glucose tolerance and adding back *A. muciniphila* increased glucose tolerance (Greer *et al.*, 2016). Preliminary data in humans showed that *A. muciniphila* is also linked to IFNγ-regulated gene expression in the human intestine and that individuals without diabetes had higher *A. muciniphila* population within their gut microbiota compared with individuals with type 2 diabetes or with pre-diabetes (Greer *et al.*, 2016).

The Role of Kidney

The kidney is involved in the regulation of glucose homeostasis via three different mechanisms: release of glucose into the circulation (gluconeogenesis), uptake of glucose from the circulation for its energy needs, and most importantly, glucose reabsorption from glomerular filtrate.

In humans, only the liver and kidney contain significant amounts of the enzyme glucose-6-phosphatase and therefore are the only organs that are able to perform gluconeogenesis. Approximately 20% of total glucose output in the normal postabsorptive state can be attributed to the kidney (Gerich *et al.*, 2001). Renal glucose output increases once liver glycogen stores become depleted during fasting (Bjorkman *et al.*, 1980; Ekberg *et al.*, 1999) and in response to hypoglycemia (~100% increase) (Meyer *et al.*, 1999a; Cersosimo *et al.*, 1999a).

The kidney also utilizes circulating glucose for its own energy needs. In the post-absorptive setting after an overnight fast, the kidneys utilize approximately 10% of all glucose utilized by the body (Meyer *et al.*, 2002). Postprandially, renal glucose uptake increases threefold; however, the proportion of systemic glucose disposal to the kidney changes very little as a result of alterations in whole-body glucose disposal (Meyer *et al.*, 2002).

Normally, approximately 180 L of plasma are filtered by the kidneys each day. As the average plasma glucose concentration throughout a 24-h period is ~100 mg/dL (~5.5 mmol/L), ~180 g of glucose is filtered by the kidneys each day. In healthy individuals, virtually all of this is reabsorbed into the circulation and the urine is essentially free from glucose. To put this into perspective, in a typical day, the kidneys produce 15–55 g glucose via gluconeogenesis and metabolize 25–35 g glucose (Gerich, 2010). Therefore, in terms of glucose economy, it is clear that renal reabsorption is the primary mechanism by which the kidney influences glucose homeostasis.

Reabsorption of glucose from glomerular filtrate occurs by means of sodium–glucose co-transporters (SGLT1 and SGLT2) in the proximal convoluted tubulae (Wright *et al.*, 2007). In animal models, approximately 90% of glucose is reabsorbed by SGLT2 and the remaining approximately 10% is mediated by SGLT1 (Wright, 2001; Brown, 2000). Reabsorbed glucose is then released into the circulation through the action of facilitative glucose transporters (GLUTs) at the basolateral membrane of the epithelial cells lining the proximal tubules (Hediger and Rhoads, 1994).

Glucose is freely filtered in the glomerulus, so that, as plasma glucose levels increase, the amount of glucose in the glomerular filtrate increases linearly. Reabsorption of filtered glucose also increases linearly until the maximal reabsorptive capacity is exceeded. This is often referred to as the renal threshold and equates to a filtration rate of 260–350 mg/min per 1.73 m^2 (Zelikovic, 2004), which occurs at plasma glucose concentrations of ~200 mg/dL (~11.0 mmol/L) in healthy adults (Moe *et al.*, 2008). Above this plasma glucose concentration, the percentage of filtered glucose that is reabsorbed decreases and the percentage of the filtered load of glucose that is excreted in the urine increases, resulting in glucosuria.

Glucose Production and Hepatorenal Glucose Reciprocity

A considerable body of evidence indicates that somehow release of glucose by the liver and kidney are interrelated so that a reduction in release by one organ is associated by an increase by the other to further maintain optimal glucose homeostasis. This relationship is referred to as hepatorenal glucose reciprocity (Gerich, 2002).

The kidney is responsible on average for about 20% of glucose released into the circulation in overnight fasted normal human volunteers. Moreover, a number of studies have shown that kidney increased its glucose release (gluconeogenesis) to compensate for restricted (physiologic), or impaired (pathologic) hepatic glucose release (Gerich, 2002; Meyer *et al.*, 1998b; Stumvoll *et al.*, 1998a,b; Cersosimo *et al.*, 1999a,b, 2000a,b; Ekberg *et al.*, 1999; Moller *et al.*, 2001).

Physiologic examples are: postprandially and after prolonged fasting. After meal ingestion, the hepatic glucose release is suppressed ~80% while renal glucose release increases and actually exceeds hepatic glucose release (HGR) for several hours (Meyer *et al.*, 2002) to allow for hepatic glycogen repletion (Gerich, 2002). Also after prolonged fasting (60 h), renal glucose release increases fourfold while hepatic glucose release decreases by ~45% (Ekberg *et al.*, 1999). Examples of renal compensation with pathologic process are: (1) *Hepatic diseases*: Hypoglycemia is extremely uncommon in patients with severe liver disease in the absence of other factors (infection, heart failure). Studies using an animal model for liver failure have demonstrated that there is a compensatory increase in renal glucose release to compensate for the reduced hepatic glucose release (Gerich, 2002; Garcia-Ruiz *et al.*, 1973; Bergman and Drury, 1938; Drury *et al.*, 1950). In humans, during the period of hepatic transplantation when patients have no functioning liver hypoglycemia does not occur; overall glucose release into the circulation either decreases minimally or not at all, and there is an increase in renal glucose release (Joseph *et al.*, 2000; Battezzati *et al.*, 1999). (2) *Acidosis*: Acidosis stimulates renal glucose release (Schoolwerth *et al.*, 1988) while inhibiting hepatic glucose release (Iles *et al.*, 1977). In patients with respiratory acidosis, an increase in net renal glucose release has been demonstrated inversely proportional to blood pH (Aber *et al.*, 1966). (3) *Glucose counterregulation in diabetes*: Patients with type 1 (Gerich, 1988), and prolonged type 2 (Gerich, 2000b) diabetes lose their glucagon response and become dependent on catecholamine responses. Catecholamines are the major hormonal factor responsible for the increase in renal glucose release during hypoglycemia (Meyer *et al.*, 1999b). Consequently, type 1 diabetic patients with both reduced glucagon and epinephrine responses have decreases in both hepatic and renal glucose release during hypoglycemia (Cersosimo *et al.*, 2001). In patients with type 2 diabetes who have reduced plasma glucagon responses, compensatory increases in hepatic glucose release during recovery from hypoglycemia are reduced whereas renal glucose release is increased (Woerle *et al.*, 2001).

The Postabsorptive State

The period after 14–16 h overnight fast is commonly referred to as the postabsorptive state. During this time plasma glucose concentration averages around 70–100 mg/dL (~3.8–5.5 mmol/L) and is relatively stable since rates of glucose release into the circulation approximate the rates of glucose exit from the circulation (~10 μg/kg/min) (Gerich, 1993).

Glucose Production (Table 3)

The liver is responsible for approximately 80% of glucose release into the circulation in the postabsorptive state (Stumvoll *et al.*, 1997). Under these conditions, ~50% of the glucose entering the circulation is due to glycogenolysis and the remainder (~5.0 μmol/kg/min) to gluconeogenesis (Landau *et al.*, 1996). The proportion owing to gluconeogenesis rapidly increases with the duration of fasting, as glycogen stores become depleted; by 24 h from the last meal, gluconeogenesis accounts for about 70% of all glucose released into the circulation, and by 48 h, it accounts for over 90% of all glucose released into the circulation (Landau *et al.*, 1996; Consoli *et al.*, 1987).

The kidney normally contains little glycogen, and renal cells that could make glycogen lack glucose-6-phosphatase. Consequently, virtually all the glucose released by the kidney is the results of gluconeogenesis (Stumvoll *et al.*, 1997). Although the liver releases about four times as much as the kidney under postabsorptive conditions, both organs release about the same amount (2.5–3.0 μmol/kg/min) from gluconeogenesis and the proportion of overall glucose release owing to renal gluconeogenesis increases even further with prolonged fasting (Cersosimo *et al.*, 2000a).

The liver releases glucose both by glycogenolysis and gluconeogenesis and can be considered to be the sole source of glucose due to glycogenolysis. In overnight fasted people, the liver contains about 75 g of glycogen (Nilsson and Hultman, 1973). Thus if it releases glycogen at a rate of 63 mg/min (5 μmol/kg/min), glycogen stores would be totally depleted in about 20 h and the sole source of glucose released into the circulation at this point would be gluconeogenesis (Gerich, 1993).

Regulation of glucose production: Hepatic vs. renal

Glucose release by the liver and kidney are regulated differently. Insulin suppresses glucose release by both organs; (1) directly by affecting enzyme activation/deactivation and (2) indirectly through actions such as limitations of gluconeogenic substrate availability and gluconeogenic activators (e.g., suppression of FFA and glucagon) (Meyer *et al.*, 1998a).

Glucagon, which increases both glycogenolysis and gluconeogenesis in the liver, however, has no effect on the kidney (Stumvoll *et al.*, 1998a). Epinephrine, which can directly activate hepatic glycogenolysis, appears to increase glucose release from the kidney, predominantly by directly stimulating renal gluconeogenesis and, to a lesser extent, by increasing availability of gluconeogenic precursors/activators (e.g., glycerol and FFA) (Stumvoll *et al.*, 1995, 1998b).

Glucose Utilization (Fig. 2)

Although the postabsorptive state is often considered to represent a steady-state, it is actually a pseudo-steady state since rates of glucose removal slightly, and undetectably, exceed rates of glucose release so that if fasting is prolonged, plasma glucose levels gradually decrease; by 20–24 h of fasting they may be 15%–20% lower. However, even after 72 h of fasting, they are usually maintained above 50 mg/dL (~2.8 mmol/L) (Consoli *et al.*, 1987).

In the postabsorptive state, there is no net storage of glucose; consequently, glucose taken up by tissues is either completely oxidized to CO_2 or released back into the circulation as lactate, alanine and glutamine (Perriello *et al.*, 1995) for re-incorporation into glucose via gluconeogenesis (**Table 4**).

Most glucose used by the body can be accounted for by five tissue: the brain (45%–60%), skeletal muscle (15%–20%), kidney (10%–15%), blood cells (5%–10%), splanchnic organs (3%–6%), and adipose tissue (2%–4%) (Gerich, 1993).

Table 3 Summary of postabsorptive glucose release

	Rate (μmol/kg/min)	*% of total*
Overall	10.0	100
A. Hepatic	8.0	80
1. Glycogenolysis	5.0	50
2. Gluconeogenesis	3.0	30
Lactate	1.3	13
Alanine	0.8	8
Other amino acids	0.2	2
Glycerol	0.4	4
Glutamine	0.3	3
B. Renal	2.0	20
1. Glycogenolysis	0	0
2. Gluconeogenesis	2.0	20
Lactate	1.2	12
Glutamine	0.4	4
Glycerol	0.2	2
Other amino acids	0.1	1
Alanine	0.1	1

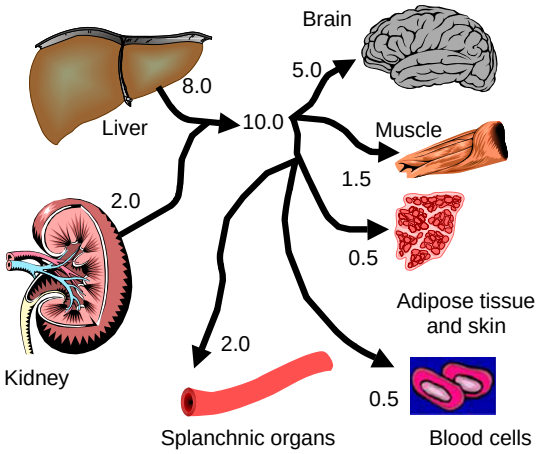

Fig. 2 Glucose utilization and production in the postabsorptive state. The liver and kidney contribute approximately 8.0 and 2.0 μmol/kg/min respectively top the total release of glucose into the circulation (10 μmol/kg/min); the brain, splanchnic tissue, muscle, adipose tissue, and blood cells account for approximately 5.0, 2.0, 1.5, 0.5, and 0.5 μmol/kg/min, respectively. From Gerich, J. (2001). Hypoglycemia. In: DeGroot, L.J. and Jameson, J.L. (eds.) *Endocrinology* (1 vol.), p. 923, with permission. Copyright© Elsevier 2001.

Table 4 Glucose disposal in the postabsorptive state

	Rate (μmol/kg/min)	*% of total*
Overall	10	100
A. Oxidation	~7	~70
B. Glycolysis	~3	~30
Tissues		
Brain	5	~50
Skeletal muscle	2	~20
Splanchnic organs	1	~10
Kidney	1	~10
Adipose tissue	0.5	~5
Blood cells	0.5	~5

Glucose taken up by the brain is completely oxidized whereas that taken up by the kidney, blood cell, splanchnic tissues and muscle mainly undergoes glycolysis. Recall that most of the body energy requirements are met by oxidation of FFA which compete with glucose as the fuel of choice in certain organs (e.g., skeletal muscles, heart and possibly kidney) (Boden, 1997).

Glucose uptake by brain, blood cells, renal medulla, and splanchnic tissue occurs largely independent of insulin, and plasma insulin is low in the postabsorptive state (<10 μU/mL). Under these conditions, amount of glucose removed from the circulation is determined almost exclusively by tissue demands, the mass action effect of the plasma glucose concentration per se, and the number and characteristics of the glucose transporters in specific tissue rather than by insulin. Insulin may be reviewed as playing a permissive role, while counterregulatory hormones that antagonize the action of insulin (e.g., cortisol, growth hormone, epinephrine, and thyroid hormones) can be viewed as modulating the sensitivity of tissue to the effect of insulin on tissue glucose uptake and utilization (Mitrakou *et al.*, 1991a; Gerich, 1993).

Prolonged Fasting (Table 5)

With prolongation of fasting, plasma insulin levels decrease while those of glucagon, catecholamines, growth hormone and cortisol increase. Consequently, plasma FFA, glycerol and the ketone bodies, products of FFA oxidation (beta hydroxybutyrate and acetoacetate) increase. Since hepatic glycogen stores become depleted by 60 h, virtually all of the glucose release at this time is due to gluconeogenesis. Initially hepatic gluconeogenesis decreases while renal gluconeogenesis increases, with an overall result of a decrease in overall glucose release and a slight increase in gluconeogenesis. With more prolonged fasting there is a further decrease in glucose release as gluconeogenesis decreases (Ekberg *et al.*, 1999).

Table 5 Glucose release and disposal after prolonged fasting (~60 h)

	Glucose disposal (μmol/kg/min)	*Glucose release (μmol/kg/min)*	
Overall	6.0	Overall	6.0
Oxidation	4.8	Gluconeogenesis	5.5
Glycolysis	1.2	Glycogenolysis	0.5
Tissues		Tissues	
Brain	3.5	Liver	2.7
Skeletal muscle	1.0	Kidney	2.8
Splanchnic organs	0.5		
Kidney	0.4		
Adipose tissue	0.2		
Blood cells	0.4		

Although more glycerol is available for gluconeogenesis, less lactate is available due to less being produced by glycolysis, and less amino acids are available because of muscle proteolysis decreases. These changes limit gluconeogenesis despite increase in plasma FFA and counterregulatory hormones which promote gluconeogenesis.

Initially during the course of the fast, decreases in glucose release are slightly greater than deceases in glucose uptake so that plasma glucose levels decrease slowly. However, eventually, the rates of uptake and release approximate one another so that a new pseudosteady state is established after 60–70 h with plasma glucose levels usually averaging 55–65 mg/dL (~3–3.6 mmol/L) (Ekberg *et al.*, 1999).

These changes during prolonged fasting are relevant to changes seen in chronically ill patients who often are anorexic, malnourished, and miss meals in hospital because of diagnostic or therapeutic procedures. Because of the limitations on gluconeogenesis, such patients (e.g., those with chronic renal failure, severe liver disease, or heart failure) are prone to develop hypoglycemia during infections or other situations which increase the body's glucose utilization (Gerich, 1993; Ekberg *et al.*, 1999).

The Postprandial State

Complete assimilation of the constituents of a mixed meal containing fat, protein and carbohydrate and restoration of the postabsorptive state takes at least 6 h (McMahon *et al.*, 1989a). Whereas assimilation of a pure carbohydrate load is generally complete within 4–5 h. Despite these time differences, there is little evidence that the fate of ingested carbohydrate differs markedly under the two conditions (Dinneen *et al.*, 1992). Because people usually eat at least three times a day, the majority of the day is spent in the postprandial state.

Various factors can affect the extent of circulating glucose excursions after meal ingestion. These include the time and the degree of physical activity since the last meal, the composition and form (liquid vs. solid), rate of gastric emptying, digestion within the lumen of the small intestine, absorption into the portal vein, extraction by the liver, suppression of endogenous glucose release and finally the uptake, storage, oxidation and glycolysis of glucose in posthepatic tissues (Marin *et al.*, 1992).

From a practical point of view, however, the major factors influencing postprandial glucose homeostasis are those that affect suppression of endogenous glucose release and those that affect hepatic and posthepatic tissue glucose uptake.

Glucose taken up by tissues postprandially can be considered either to be immediately stored or to undergo glycolysis. Therefore, initial direct storage of glucose (glucose to glucose-6-phosphate to glycogen) can be calculated as the difference between whole body glucose uptake and whole body glycolysis. Since postprandial de novo lipogenesis and adipose tissue glucose storage are negligible in humans, virtually all of this storage should represent glycogen formation (McMahon *et al.*, 1989a; Marin *et al.*, 1992).

Of the glucose undergoing glycolysis, some will be oxidized; the remainder will undergo nonoxidative glycolysis leading to the formation of pyruvate, lactate and alanine. These 3-carbon compounds will then be available to undergo gluconeogenesis and either be stored in glycogen via the indirect pathway or be released into plasma as glucose (Woerle *et al.*, 2003).

Fig. 3 depicts the pathways for disposal of a mixed meal containing 78 g of glucose (Woerle *et al.*, 2003). During the 6-h postprandial period, a total of ~98 g of glucose were disposed of. This was more than the glucose contained in the meal due to persistent endogenous glucose release (~21 g): splanchnic tissues initially took up ~23 g, and an additional ~75 g were removed from the systemic circulation. Direct glucose storage accounted for ~32 g and glycolysis ~66 g (oxidative ~43 g and non-oxidative ~23 g). About 11 g of glucose appeared in plasma as a result of gluconeogenesis. This indicates that glycolysis is the main initial postprandial fate of glucose, accounting for ~66% of overall disposal. Oxidation and storage each account for about 45%. The majority of glycogen is formed via the direct pathway (~73%).

Changes in Plasma Hormone and Substrate Concentration (Fig. 4)

After ingestion of 75 g glucose, plasma glucose levels increase to a peak in 30–60 min, usually not exceeding 160 mg/dL (~8.8 mmol/L) and gradually return to or slightly below post absorptive values by 3–4 h. Although plasma glucose levels have

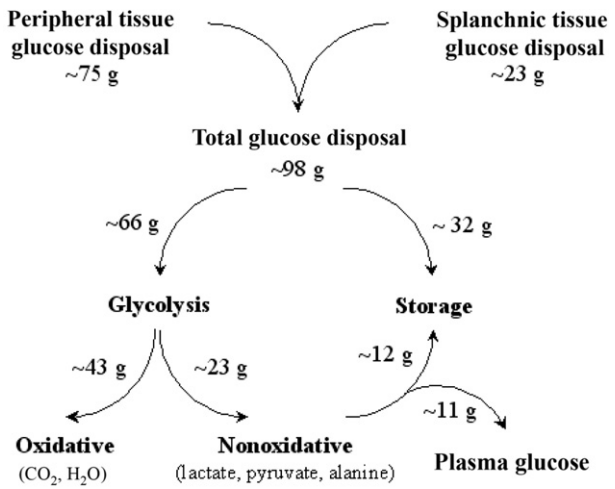

Fig. 3 Summary of sites and routes of postprandial glucose disposal. From Woerle, H.J., Meyer, C., Dostou, J.M., Gosmanov, N.R., Islam, N., Popa, E., Wittlin, S.D., Welle, S.L. and Gerich, J.E. (2003). Pathways for glucose disposal after meal ingestion in humans. *American Journal of Physiology. Endocrinology and Metabolism* **284**, E716–E725, with permission. Copyright© 2003 The American Physiological Society.

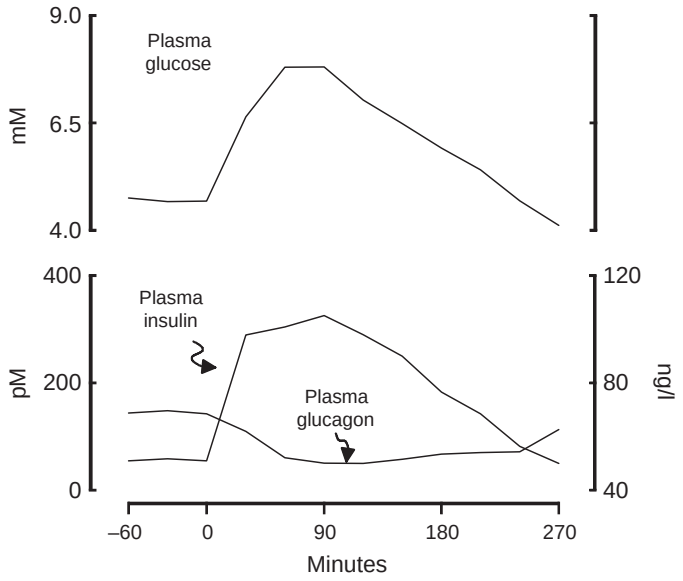

Fig. 4 Changes in plasma glucose, insulin and glucagon after ingestion of a 75 g oral glucose load in normal volunteers.

returned to postabsorptive levels, glucose fluxes and organ glucose exchange have not. Plasma insulin concentrations follow a similar profile to those of plasma glucose and average only about three to fourfold basal values during this period.

Plasma glucagon concentrations change reciprocally to those of insulin and are generally suppressed about 50%. Early insulin release (i.e., that accruing within 30–60 min) plays a critical role in maintaining normal postprandial glucose homeostasis (Dinneen *et al.*, 1992).

Plasma FFA and glycerol levels decrease due to inhibition of lipolysis while plasma lactate concentration increase as a result of increased glycolysis in liver, muscle, adipose tissue, and kidney. After ingestion of a mixed meal containing protein the circulating concentrations of several amino acids increase (Woerle *et al.*, 2003).

Changes in Rates of Glucose Entry Into and Exit From Plasma (Figs. 5 and 6)

Rates of glucose appearance in plasma represent the sum of orally ingested glucose escaping first pass splanchnic (hepatic) extraction and the residual release of endogenous glucose by liver and kidney. Appearance of ingested glucose in the systemic circulation is detected as early as 15 min, reaches a peak at 60–80 min, and gradually decrease thereafter (Woerle *et al.*, 2003).

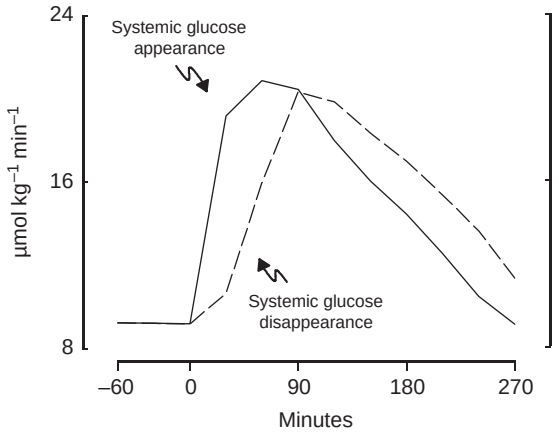

Fig. 5 Changes in rates of glucose entry into and removal from plasma after ingestion of a 75 g oral glucose load in normal volunteers.

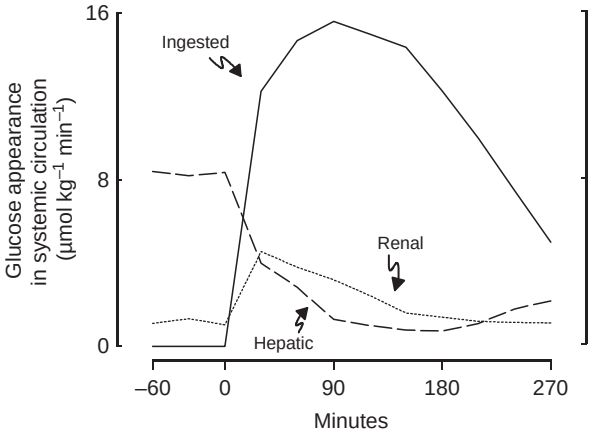

Fig. 6 Changes in rates of entry of glucose into the circulation from ingested glucose, liver and kidney.

On average during a 4–5 h postprandial period about 75% of the glucose molecules in plasma represent those from the meal. Endogenous glucose release by the liver decreases rapidly and is suppressed nearly 80% during the 5-h postprandial period. As a result, nearly 25 g less glucose due to endogenous production reaches the systemic circulation during this interval. In contrast to the liver, recent studies indicate that endogenous renal glucose release in not suppressed and actually increases during this period so that it exceeds hepatic glucose release (Meyer *et al.*, 1999b). This increase in renal glucose release would permit more complete suppression of hepatic glucose release and facilitate more efficient hepatic glycogen replenishment (Meyer *et al.*, 1999b).

Tissues Responsible for Disposal of Ingested Glucose

Based on a survey of published studies, a consensus view of the disposal of a hypothetical meal containing 100 g carbohydrate is depicted in **Fig. 7**. About 30% of the ingested glucose (~33 g) is initially extracted by splanchnic tissues (Kelley *et al.*, 1988, 1994; McMahon *et al.*, 1989a,b; Butler *et al.*, 1991; Ferrannini *et al.*, 1985; Jackson *et al.*, 1986; Mitrakou *et al.*, 1992). Most is taken up by the liver and immediately incorporated into glycogen via "direct pathway" to hepatic glycogen (Beckmann *et al.*, 1993; Petersen *et al.*, 2001). A significant portion of glucose taken up by the liver probably undergoes glycolysis and is released as lactate which is eventually taken up by the liver where it undergoes gluconeogenesis and is subsequently incorporated into glycogen via "indirect pathway" (Kelley *et al.*, 1988; Petersen *et al.*, 2001; Taylor *et al.*, 1996; Mitrakou *et al.*, 1991b). Inhibition of glucose-6-phosphatase causes the glucose-6-phosphate made from this lactate to enter glycogen rather than being released into the circulation as free glucose.

Of the remaining 70 g glucose, which enters the systemic circulation, 25–30 g is taken up by skeletal muscle (Kelley *et al.*, 1988, 1994; McMahon *et al.*, 1989a; Butler *et al.*, 1991; Jackson *et al.*, 1986; Mitrakou *et al.*, 1992; Firth *et al.*, 1986), initially to be oxidized in place of FFA and later (after 2–3 h) to be stored as glycogen (Marin *et al.*, 1992; Taylor *et al.*, 1993). Relatively little of the glucose taken up by muscle is released into the circulation as lactate and alanine (Kelley *et al.*, 1988; Radziuk and Inculet, 1983).

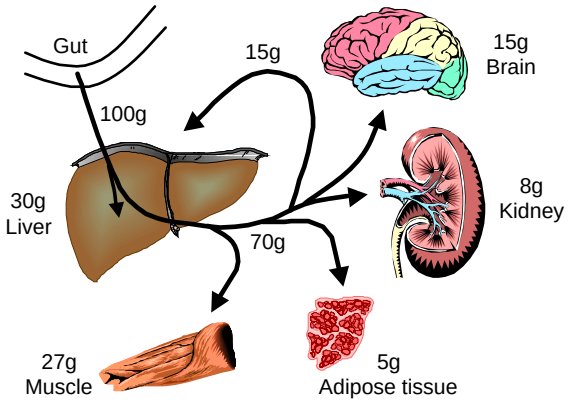

Fig. 7 Postprandial glucose disposal. Of 100 g glucose ingested 30% is taken up by the liver and 70% is released into the systemic circulation. Of this 70 g, 15 g (~20%) is extracted by the liver, 15 g (~20%) is taken up by the brain, 27 g (~40%) is taken up by skeletal muscle, and the remaining 20% is taken up by kidney, adipose tissue, skin, and blood cells.

About 15 g (~20% of the ingested glucose entering the circulation) is taken up by brain as a substitute for the endogenously produced glucose that normally would have been taken up during this period. Recall that endogenous release of glucose from the liver is markedly reduced postprandially.

Another 15 g is extracted from the systemic circulation by the liver either as intact glucose (direct pathway) or as lactate, alanine, and glutamine, whose carbon backbone originated from ingested glucose, for further glycogen formation (indirect pathway) (Taylor *et al.*, 1996). Thus, ultimately splanchnic tissues dispose of nearly half of the ingested glucose (Ferrannini *et al.*, 1980).

The kidney may take up as much as 8 g (~10% of the ingested glucose entering the circulation) (Meyer *et al.*, 1999b). This would leave 5–10 g (7%–15% of the ingested glucose) reaching the systemic circulation) to be taken up by adipose and other tissues (Marin *et al.*, 1992).

Summary

Human plasma glucose concentrations are maintained within a relatively narrow range throughout the day despite wide fluctuations in the delivery (e.g., meals) and removal (e.g., exercise) of glucose from the circulation. This is accomplished by a tightly linked balance between glucose production and glucose utilization regulated by complex mechanisms influenced by the nervous system, several hormones from different types of endocrine cells, and substrates (i.e., free fatty acid concentration and availability of gluconeogenic precursors).

In the postabsorptive stage gluconeogenesis and glycogenolysis contribute equally to glucose release. The liver is responsible for all of glycogenolysis and half of gluconeogenesis. In the postprandial stage almost all endogenous glucose release is via gluconeogenesis. The kidney is involved in the regulation of glucose homeostasis through gluconeogenesis, uptake of glucose from the circulation for its energy needs, and glucose reabsorption. Under a variety of conditions, reciprocal changes occur in hepatic and renal glucose release so as to maintain optimal glucose homeostasis.

See also: Glucose Metabolism and Hormonal Regulation

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Glucose Metabolism and Hormonal Regulation☆

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Carbohydrates [named for their empirical formula, $(CH_2O)_n$] include simple sugars (monosaccharides), disaccharides consisting of two linked sugars, and polysaccharides such as the glucose storage polymer glycogen.

Introduction

By definition, simple sugars are aldehydes or ketones with two or more hydroxyl groups; thus, the smallest sugars are the trioses (three-carbon sugars) glyceraldehyde and dihydroxyacetone. Sugars with three to seven carbons are commonly involved in cellular metabolism. Glucose is a hexose (six-carbon sugar) with an aldehyde group at one end; by convention, this is carbon-1. Fructose is also a hexose, but it has a keto group at carbon-2. Sucrose (common table sugar) is a disaccharide of glucose and fructose.

Glucose is the most important of the biological sugars. It is the essential fuel of the brain and thus control of its blood concentration is critical. It is also important in powering intense muscular work. A major metabolic function of the liver is to maintain blood glucose through appropriate release of the sugar from the storage polymer glycogen and through resynthesis of glucose from nonsugar precursors (gluconeogenesis). The blood glucose concentration is under hormonal control, with insulin promoting glucose uptake and storage in muscle and liver and the counterregulatory hormones, such as glucagon and epinephrine, promoting glycogen breakdown as well as gluconeogenesis in liver. Lack of or insufficient insulin is the cause of diabetes, and the resulting chronic elevated glucose levels lead to vascular damage and much of the cases of blindness and kidney disease and increased risk of heart disease. This article discusses the metabolism of glucose in the glycolytic pathway and the production of energy; the storage of glucose in glycogen and its breakdown; the resynthesis of glucose through the pathway of gluconeogenesis; the branch to the pentose phosphate pathway that produces ribose 5-phosphate, which is necessary for DNA and RNA synthesis; and how the metabolism of glucose is linked to the secretion of insulin in the β cells of the pancreatic islets. More details can be found in a basic textbook (Berg *et al.*, 2002) and in the intermediary metabolism book chapter (Ruderman *et al.*, 2001).

Glycolysis

The chemical logic of the glycolytic pathway is the generation of high-energy phosphate compounds that can be used to phosphorylate adenosine diphosphate (ADP) to adenosine triphosphate (ATP). ATP is the energy currency of the cell. The breakdown of ATP is used to power energy-consuming reactions of the cell, from biochemical syntheses to ion pumping to muscular contraction. The early steps in glycolysis actually involve the consumption of two ATPs, but in the later reactions four ATPs are produced, giving a net production of two ATPs per glucose molecule.

Reactions

The reactions of glycolysis are shown in **Fig. 1**. First, glucose is phosphorylated by the enzyme hexokinase ("kinase" means a phosphorylating enzyme) to yield glucose 6-phosphate. Glucose 6-phosphate is isomerized from the aldo sugar to the keto sugar fructose 6-phosphate, which is then phosphorylated at the other end by the key enzyme phosphofructokinase to generate fructose 1,6-bisphosphate. (By current convention, "bis" indicates that the phosphates are on separate carbons, not attached together as in adenosine diphosphate.) Fructose 1,6-bisphosphate is cleaved in the aldolase reaction to two triose phosphates, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Since the rest of glycolysis proceeds from the latter, dihydroxyacetone phosphate is converted to glyceraldehyde 3-phosphate by triose phosphate isomerase so that both halves of the glucose molecule can be metabolized. In the glyceraldehyde 3-phosphate dehydrogenase reaction, the aldehyde of glyceraldehyde 3-phosphate is oxidized to a carboxylic acid, but the energy of oxidation is used in part for the uptake of inorganic phosphate (P_i) into a phosphate anhydride in the product 1,3-bisphosphoglycerate. In this reaction, the acceptor of the reducing equivalents is the cofactor nicotinamide adenine dinucleotide (NAD), which becomes NADH with the addition of the hydride (hydrogen with both electrons), whereas a second hydrogen is released simply as a proton in solution. The phosphate anhydride generated at carbon-1 is high energy and thus can be used to phosphorylate ADP to ATP in the phosphoglycerate kinase reaction (so named from the reverse reaction). 3-Phosphoglycerate is then converted to the second high-energy intermediate phosphoenolpyruvate in two steps:

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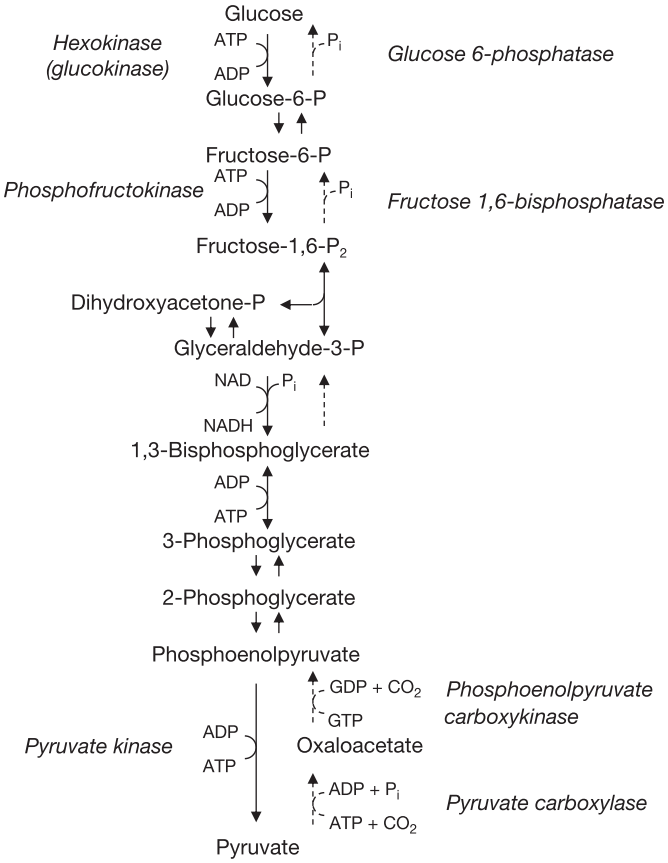

Fig. 1 The glycolytic (glucose breakdown) and gluconeogenic (glucose synthesis) pathways. The purely glycolytic enzymes are listed on the left. All tissues have hexokinase, but liver and pancreatic β cells have the high K_m isoform glucokinase that makes them sensitive to the blood glucose concentration. The purely gluconeogenic enzymes are listed on the right and their reactions are shown with dashed arrows. Liver and kidney have glucose 6-phosphatase, which allows them to produce glucose. All other enzyme reactions shown are used in both glycolysis and gluconeogenesis.

The phosphate is moved from the 3 to the 2 position by phosphoglycerate mutase and then water is removed in the enolase reaction. Phosphoenolpyruvate is then used to phosphorylate ADP to ATP by pyruvate kinase. It is the highly favored conversion of the initial product enolpyruvate to the normal keto form of pyruvate that pulls the reaction forward and causes phosphoenolpyruvate to be a high-energy phosphate donor.

Fate of Pyruvate

The fate of pyruvate depends on the oxidative state of the tissue. For glycolysis to proceed, the NADH produced in the glyceraldehyde 3-phosphate dehydrogenase reaction must be reoxidized back to NAD. Under aerobic conditions, the reducing equivalents from NADH may be transferred to the mitochondrial electron transport chain and ultimately to molecular oxygen. However, if there is insufficient oxygen or insufficient activity of the electron transport chain, then the pyruvate must be used to oxidize NADH and regenerate NAD in the lactate dehydrogenase reaction. This is the reason why strong muscular exercise produces lactate (lactic acid). Yeast lack lactate dehydrogenase and so instead under anaerobic conditions the pyruvate is first decarboxylated to acetaldehyde, which is then used in the alcohol dehydrogenase reaction, generating ethanol in the reoxidation of NADH to NAD. This is why fermentation to produce wine or other alcoholic beverages must be done anaerobically in sealed containers; otherwise, the acetaldehyde would be oxidized to acetic acid (vinegar), making a less palatable drink.

If pyruvate is not needed to be converted to lactate in order to reoxidize NADH, then the pyruvate can enter the mitochondria and be converted to acetyl-coenzyme A (CoA) in the pyruvate dehydrogenase reaction. Acetyl-CoA can be completely oxidized to CO_2 in the citric acid cycle. ATP production by oxidative phosphorylation from the two molecules of pyruvate, together with the reducing equivalents transferred from the two NADHs from the glyceraldehyde 3-phosphate dehydrogenase reaction, is on the order of 15 times greater than the two ATPs produced in glycolysis alone. The details of the citric acid cycle and oxidative phosphorylation are not considered here. If there is excess use of glucose beyond what would be necessary for energy production, then acetyl-CoA can also be used for synthesis of fatty acids.

Regulation of Glycolysis

Phosphofructokinase

The key regulatory enzyme of glycolysis is phosphofructokinase. It is inhibited by ATP and citrate and activated by AMP (and ADP), P_i, and fructose 2,6-bisphosphate. Although ATP is a substrate of the enzyme, it is also an important product of glycolysis, and the inhibition at a regulatory site is an example of classic feedback inhibition. Since ATP usage produces AMP, ADP, and P_i, these activators also signal a need for more ATP production, as would a decrease in ATP. Citrate may serve as an indicator of the sufficiency of alternative fuel, particularly fatty acids that are broken down to acetyl-CoA, which may then increase citrate so that glucose may be preserved for the brain. The importance of fructose 2,6-bisphosphate as an activator has principally been established in liver; it is discussed later in the context of reciprocal regulation of gluconeogenesis and glycolysis. (It is made by phosphorylation of fructose 6-phosphate on the 2 position by a phosphofructokinase-2, distinct from the glycolytic phosphofructokinase.)

Hexokinase and glucose transport

Hexokinase is nominally the first step in glycolysis, but it is not the major point of regulation because glucose 6-phosphate is a branch point leading to glycogen and to the pentose phosphate pathway. Importantly, glucose 6-phosphate is an inhibitor of hexokinase, so if the other pathways are slow and if phosphofructokinase is inhibited, then glucose 6-phosphate will increase and inhibit hexokinase. Conversely, if phosphofructokinase is activated, such as by a decrease in ATP and increase in AMP, then fructose 6-phosphate and glucose 6-phosphate will decrease and hexokinase will be disinhibited. Glucose transport into the cell is also stimulated by insulin in muscle and fat cells, which are tissues that have the insulin-regulated transporter Glut4. In contrast, glucose transport in and out of liver is rapid, catalyzed by Glut2.

Pyruvate Kinase—PKM2 and Cancer

The muscle isoform of pyruvate kinase is essentially unregulated, with weak product inhibition by ATP. The liver isoform is strongly regulated, by allosteric effectors and by phosphorylation, so that it can be inhibited for gluconeogenesis to proceed (see below). Recently there has been increasing interest in PKM2, an alternative transcript of the muscle gene that gives a fetal form of the enzyme but which has also been found in various cancer cell types. PKM2 is also inhibitable, which should allow the accumulation of upstream glycolytic intermediates, such that glucose carbon can be diverted into biosynthetic pathways to make phospholipids for membranes and certain amino acids for protein synthesis, as well as the ribose 5-phosphate for nucleotide synthesis, all needed by these rapidly growing and dividing cells.

Regulation of Pyruvate Dehydrogenase

Regulation of the conversion of pyruvate to acetyl-CoA is of great importance because although pyruvate can be converted back to glucose in the process of gluconeogenesis, there cannot be a net production of glucose from acetyl moieties in animal cells. Pyruvate dehydrogenase is inhibited by its products acetyl-CoA and NADH, and this is countered by the corresponding substrates CoA and NAD; thus, it can be considered that the enzyme is inhibited by high acetyl-CoA/CoA and NADH/NAD ratios. In addition, the enzyme is inactivated by phosphorylation by a specific protein kinase, which is itself activated by high acetyl-CoA/CoA, NADH/NAD, and ATP/ADP ratios. The enzyme can be reactivated by removal of the phosphate by a specific protein phosphatase that is stimulated by high pyruvate levels and by insulin. The pyruvate dehydrogenase reaction is carried out by a multienzyme complex, to which the specific kinase and phosphatase are also bound.

Glycogen Metabolism

Reactions of Glycogen Breakdown

Liver and muscle can contain large amounts of glycogen, a polymer that is a storage form of glucose. It is a branched polymer, with glucose residues largely linked 1–4 but with branches approximately every 10 residues with a 1–6 linkage. The enzyme mainly responsible for breaking down glycogen is phosphorylase (which is short for glycogen phosphorylase because it was the first of its class discovered); it breaks 1–4 linkages by the addition of P_i, releasing a residue as glucose 1-phosphate. However, it can only come within 4 residues of a branch point. Then a "transferase" transfers the branch, except for the penultimate residue, to the end of the main chain; α-1,6-glycosidase cleaves off the final branched residue as free glucose. With the branch removed, phosphorylase can continue.

Interestingly, the phosphorylase reaction can go in both directions, glycogen(n) + P_i ↔ glucose 1-phosphate + glycogen (n − 1), with the equilibrium at [P_i]/[glucose 1-phosphate] = 3.7. Carl and Gerty Cori, the discoverers of phosphorylase, received

the Nobel prize in part for finding the first enzyme that can synthesize a biological polymer since glycogen can be formed in the test tube in the presence of high glucose 1-phosphate. However, in vivo the $[P_i]/[\text{glucose 1-phosphate}]$ ratio is approximately 100, and the enzyme catalyzes glycogen breakdown. This was clearly demonstrated in individuals lacking phosphorylase who have a glycogen storage disease with excess glycogen, not low levels.

Since glucose 1-phosphate is in equilibrium with glucose 6-phosphate through the phosphoglucomutase reaction, the sugar residues released from glycogen can serve as fuel in the glycolytic pathway in muscle. In this context, the fact that glucose 6-phosphate is derived from glycogen phosphorolysis rather than hydrolysis means that there is a net three ATPs produced in glycolysis per glucose residue. In liver, the glucose 6-phosphate produced is generally dephosphorylated to provide blood glucose as needed, along with gluconeogenesis.

Reactions of Glycogen Synthesis

In the synthesis of glycogen by the enzyme glycogen synthase, the glucosyl residue donor is UDP-glucose. UDP-glucose in turn is synthesized from glucose 1-phosphate and UTP, with the release of pyrophosphate (which can be cleaved by pyrophosphatase to pull the reaction). Glycogen synthase resembles phosphorylase in that it makes only 1–4 linkages. The branches in glycogen are made by branching enzyme, which takes a chain of seven residues from the end of a growing chain and attaches it in 1–6 linkage to a glucosyl residue four positions further on.

Regulation of Glycogen Metabolism

The controlling enzymes of glycogen breakdown and synthesis are phosphorylase and glycogen synthase, respectively (Bollen *et al.*, 1998). Their regulation involves allosteric actions by activators and inhibitors as well as phosphorylation and dephosphorylation. The phosphorylation state affects the sensitivity to the allosteric effectors, and the effectors can modulate the phosphorylation state. Hormonal regulation is important to glycogen metabolism. Insulin, a 51-amino acid small protein secreted by the β cells of the pancreatic islets in response to high glucose, promotes glycogen storage. Glucagon, a 29-amino acid peptide secreted by the α cells of the pancreatic islets in response to low glucose, promotes glycogen breakdown in liver as well as gluconeogenesis. Glycogen breakdown in both liver and muscle is promoted by epinephrine, a catecholamine secreted by the adrenal medula in response to nerve impulses, such as when a rabbit sees a fox.

Phosphorylase was originally found to exist in two forms: a nonphosphorylated form (phosphorylase *b*) that required AMP for activity and a phosphorylated form (phosphorylase *a*, with a phosphate on a serine residue of each of its four subunits) that was active in the absence of AMP. Phosphorylase *b* is also inhibited by glucose 6-phosphate and ATP. Glucagon and epinephrine can activate phosphorylase by causing a sequence of reactions (the cascade; **Fig. 2**) that results in the conversion of phosphorylase *b* to *a*. First, these hormones bind to their specific receptors. (Glucagon is only effective on liver and not on muscle because muscle does not have glucagon receptors.) The binding of the hormone causes activation of adenylyl cyclase, which makes cyclic AMP from ATP. This in turn activates the cyclic AMP-dependent protein kinase (PKA), which can then phosphorylate and activate

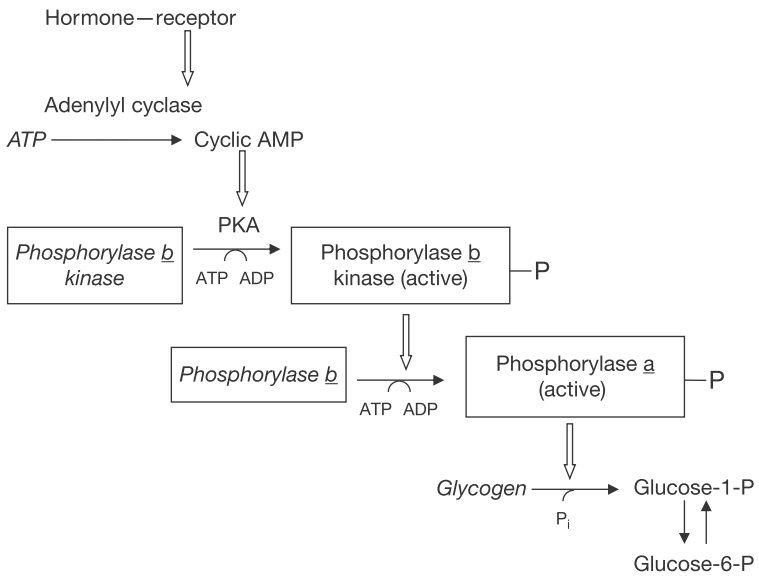

Fig. 2 Stimulation of glycogen breakdown by hormones such as epinephrine and glucagon—the cascade.

phosphorylase *b* kinase. The latter protein kinase then phosphorylates phosphorylase *b*, converting it to phosphorylase *a*, which is more active and breaks down glycogen.

In addition to phosphorylation, phosphorylase *b* kinase can be activated by an increase in intracellular free calcium ion. Thus, when muscular contraction is initiated by a release of calcium from the sarcoplasmic reticulum, the increase in calcium can also trigger glycogenolysis to help provide the needed fuel. Similarly, in liver, the action of epinephrine at low physiological levels to cause glycogen breakdown may be mediated by receptors linked to the release of calcium from the endoplasmic reticulum.

Glycogen synthase is also controlled by phosphorylation/dephosphorylation. In this case, the phosphorylated form is the less active one (originally called glycogen synthase D because it is dependent on glucose 6-phosphate, but now it is called synthase *b* in correspondence with the less active phosphorylase *b*; synthase *a* was originally called synthase I because it is independent of glucose 6-phosphate). The hormonal cascade described previously can cause the phosphorylation of glycogen synthase by PKA, thus inhibiting glycogen synthesis as well as activating glycogen breakdown. However, the situation for glycogen synthase is more complex because there are at least 10 phosphorylation sites that can be phosphorylated by at least nine different protein kinases. The importance or hierarchy of these sites and their kinases remains to be clarified. Insulin's effect in promoting glycogen storage appears to be in part due to inhibition of the action of glycogen synthase kinase 3 (Lawrence and Roach, 1997). Furthermore, insulin can promote the degradation of cyclic AMP via activation of cyclic nucleotide phosphodiesterase. In muscle, insulin's activation of glucose transport (Glut4) and perhaps phosphorylation is a major factor in promoting glycogen synthesis, and deficiency at this step is seen in type 2 diabetic patients and their near relatives.

The protein phosphatases that convert phosphorylase *a* to *b* and glycogen synthase *b* to *a*, particularly the protein phosphatase 1 that has a glycogen-binding subunit, are subject to regulation in several ways. First, binding of allosteric regulators to the protein substrate affects the activity of phosphatases and kinases. Thus, AMP binding to phosphorylase *a* makes it a poor substrate for the phosphatase because the phosphate is tucked in so that it cannot be hydrolyzed, whereas binding of glucose or glucose 6-phosphate causes the phosphate to become accessible. Binding of glucose 6-phosphate to phosphorylase *b* also makes it a poorer substrate for the kinase as well as inhibiting phosphorylase activity. Second, inhibitory proteins can bind to the phosphatase, and the action of these proteins is regulated by their phosphorylation and dephosphorylation. Third, the fact that the same phosphatase works on both phosphorylase *a* and synthase *b* means that when the phosphatase is bound to phosphorylase *a*—and cannot hydrolyze it because the phosphate is tucked in—the phosphatase is prevented from working on glycogen synthase.

Glycogen Storage Diseases

Genetic deficiency of enzymes of the liver related to glycogen breakdown can lead to the engorgement of the liver with glycogen. Thus, patients with Hers disease (type VI) lack liver phosphorylase and must therefore rely on gluconeogenesis to maintain blood glucose. Patients with Von Gierke disease (type I) lack glucose 6-phosphatase, cannot get glucose from either glycogenolysis or gluconeogenesis, and therefore can withstand only limited starvation. Presumably, they have increased glycogen because increased levels of glucose 6-phosphate promote glycogen synthesis and inhibit glycogen breakdown, as described previously. In McArdle disease (type V), muscle phosphorylase is absent (indicating that there are separate muscle and liver isoforms from separate genes) and exercise capacity is limited.

Gluconeogenesis

Gluconeogenesis is the synthesis of glucose from nonsugar precursors, such as lactate, pyruvate, and the carbon skeleton of glucogenic amino acids. This is a major metabolic function of the liver since the brain, in particular, is dependent on glucose as a fuel.

Reactions

In a sense, gluconeogenesis is a reversal of glycolysis, and many of the enzymes are the same for the reactions that are readily reversible. However, specifically gluconeogenic enzymes are needed to reverse three steps in glycolysis that have a large free energy drop. Thus, to reverse the pyruvate kinase reaction, first pyruvate is converted to oxaloacetate by pyruvate carboxylase, a reaction driven by ATP hydrolysis; then the oxaloacetate is decarboxylated and phosphorylated to generate phosphoenolpyruvate by the enzyme phosphoenolpyruvate carboxykinase using GTP (**Fig. 1**). Note that two high-energy phosphate bonds (from ATP and GTP) are needed to produce the very high-energy intermediate phosphoenolpyruvate. The reactions from there to fructose 1,6-bisphosphate occur via reversal of the glycolytic reactions. Then, to generate fructose 6-phosphate, the 1-phosphate is simply cleaved off by the gluconeogenic enzyme fructose 1,6-bisphosphatase (opposing the glycolytic enzyme phosphofructokinase). Fructose 6-phosphate equilibrates with glucose 6-phosphate in the phosphoglucose isomerase reaction as in glycolysis. Finally, glucose 6-phosphate is hydrolyzed to free glucose by glucose 6-phosphatase (opposing the hexokinase isoform glucokinase). Note that synthesis of a molecule of glucose requires the input of six ATP equivalents: the ATP at pyruvate carboxylase, the GTP at

phosphoenolpyruvate carboxykinase, and the ATP at the phosphoglycerate kinase reaction for each of the two halves of the glucose molecule, plus two equivalents of NADH for reversing the glyceraldehyde 3-phosphate dehydrogenase reaction.

Regulation of Gluconeogenesis

Control of net gluconeogenesis involves regulation of the opposing glycolytic enzymes and the corresponding specifically gluconeogenic enzymes. The glucose phosphorylating activity in liver is largely glucokinase, a high K_m (10 mm) isoform of hexokinase that is responsive to changes in the blood glucose concentration in the physiological range. Glucokinase is not inhibited by glucose 6-phosphate because it lacks the regulatory binding domain present in the other hexokinases. On the other hand, glucokinase has a specific inhibitory protein (GKIP) in the nucleus, whose effect is prevented by fructose 1-phosphate and increased by fructose 6-phosphate (Van Schaftingen *et al.*, 1997). (The disinhibition by fructose 1-phosphate explains the stimulation of glucose metabolism by fructose, which in the liver is largely phosphorylated to fructose 1-phosphate by fructokinase.) The liver isoform of pyruvate kinase has several regulatory properties designed to inhibit it so that gluconeogenesis can proceed: (i) strong allosteric inhibition by ATP; (ii) inhibition by alanine, an important gluconeogenic substrate; (iii) dependence on a high level of the activator fructose 1,6-bisphosphate (in order to have the same low K_m for phosphoenolpyruvate that the muscle isoform has with or without fructose 1,6-bisphosphate); and (iv) inhibition by phosphorylation by PKA in response to glucagon. It was originally thought that phosphofructokinase was similarly inhibited by phosphorylation by PKA, but in fact although phosphorylation of phosphofructokinase occurs, it appears to have little effect on the activity of the mammalian enzymes. Instead, PKA phosphorylation of liver phosphofructokinase-2/fructose 2,6-bisphosphatase (PFK2), the bifunctional enzyme that both makes and degrades fructose 2,6-bisphosphate, inhibits the kinase and activates the phosphatase activities, thus reducing the level of fructose 2,6-bisphosphate (Pilkis *et al.*, 1995). Since fructose 2,6-bisphosphate is an activator of the glycolytic phosphofructokinase (sometimes called phosphofructokinase-1 for clarity) as well as an inhibitor of fructose 1,6-bisphosphatase, glucagon can thus reduce glycolytic flux and promote gluconeogenesis at this step as well as at the pyruvate kinase step, in addition to its effect of stimulating glycogen breakdown and inhibiting glycogen synthesis. (Note: In muscle, one would not want epinephrine stimulation of PKA to cause inhibition of PFK2 and hence phosphofructokinase and glycolysis; thus the muscle isoform of PFK2 is an alternative transcript that lacks the PKA phosphorylation site. The cardiac isoform of PFK2, in contrast, has an activating phosphorylation site that is the substrate for AMP-activated protein kinase, and this may be important for increased glycolysis in ischemia.) Fructose 1,6-bisphosphatase is also inhibited by AMP, in contrast to the AMP activation of phosphofructokinase. Recently it has been found that liver PFK2 can have an additional regulatory role: When unphosphorylated, it can bind glucokinase to keep it in the cytosol and hence active. Phosphorylated PFK2 no longer binds glucokinase, which is then released to be bound and inhibited by GKIP (Agius, 2016). Hence, PKA directly or indirectly inhibits all of the key glycolytic enzymes. Pyruvate carboxylase has an absolute requirement for the activator acetyl-CoA; the rationale for this is that gluconeogenesis is quite energy consuming, the required ATP certainly cannot come from glucose metabolism, and the presence of acetyl-CoA would be indicative of sufficient alternative fuel, such as from fatty acids. The fact that phosphoenolpyruvate carboxykinase uses GTP (rather than ATP) may also provide a regulatory link, in that it is using GTP generated by the liver isoform of succinyl-CoA synthetase in the citric acid cycle, so that if there is insufficient citric acid cycle flux, then energy-consuming gluconeogenesis will not be attempted (Stark *et al.*, 2014). Finally, these enzymes are adaptive in that the amount of the glycolytic enzymes glucokinase, phosphofructokinase, and pyruvate kinase is increased by a high-carbohydrate diet, whereas the specifically gluconeogenic enzymes are increased by starvation or a low-carbohydrate diet. Interruption of insulin's normal repression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in part explains the inappropriate high glucose output from the liver in diabetic patients, contributing to their elevated blood glucose levels; lack of insulin's normal restraining of adipose lipolysis and fatty acid release to power gluconeogenesis may also be important.

Substrate Cycling

The opposing gluconeogenic and glycolytic enzymes form energy-consuming substrate cycles—that is, if glucokinase and glucose 6-phosphatase were active simultaneously so that glucose were converted to glucose 6-phosphate and back to glucose, the net reaction would be the hydrolysis of ATP to ADP and P_i, and similarly for phosphofructokinase and fructose 1,6-bisphosphatase. If phosphoenolpyruvate were converted to pyruvate and back, there would be production of one ATP but utilization of two equivalents, for a net consumption of one ATP. It was originally thought that such substrate cycling would be a waste of energy (hence the original term futile cycle) and that there should be tight regulation to prevent simultaneous operation of the opposing enzymes. However, sophisticated radiolabeling experiments have shown the occurrence of such substrate cycling. For example, if liver cells undergoing gluconeogenesis are provided with glucose with tritium (a radioactive isotope of hydrogen) attached to carbon-5, then after the glucose is metabolized past the phosphofructokinase reaction, the tritium will be released into water on equilibration of glyceraldehyde 3-phosphate with dihydroxyacetone phosphate in the triose phosphate isomerase reaction. Such experiments have shown a substantial glycolytic rate that is largely reduced by glucagon treatment in order to increase the net gluconeogenic rate. Such substrate cycling has the advantage that there is no "dead" range in which neither glycolysis nor gluconeogenesis is operative and thus there is no control. Furthermore, substrate cycling amplifies cellular signals because changes in activators/inhibitors of glycolysis and/or gluconeogenesis lead to much greater percentage changes in the net rate. Finally, as a

special case, bumblebees have unregulated fructose 1,6-bisphosphatase in their flight muscles so that substrate cycling with phosphofructokinase generates heat to warm up the muscles on cold days to allow flight, much as an electric heater is used to heat a gasoline engine preparatory to start an automobile in very cold climates.

Cori Cycle and Alanine Cycle

There are two important interorgan cycles involving glycolysis in muscle and gluconeogenesis in liver. In the Cori cycle, glucose is metabolized to pyruvate and then to lactate in muscle, the lactate is released into the blood and carried to the liver, where it is reconverted to pyruvate and used for gluconeogenesis, and the resulting glucose is released and travels back to muscle. Lactate is a particularly good gluconeogenic substrate because the reoxidation of lactate to pyruvate in the lactate dehydrogenase reaction also provides the NADH equivalents needed for gluconeogenesis. The alanine cycle is similar to the Cori cycle, except that muscle pyruvate is converted to the amino acid alanine rather than lactate by transamination in the glutamate–pyruvate transamination reaction. A very large proportion of the amino acid put out by muscle is alanine, much more so than its composition in muscle protein, due to the fact that the carbon skeleton derives from glycolytically generated pyruvate. In the liver, alanine is transaminated back to regenerate pyruvate, and the excess amino groups can be disposed of as urea since the urea cycle is also localized to the liver. The importance of alanine as a gluconeogenic substrate in this regard explains why it is a potent allosteric inhibitor of liver pyruvate kinase.

Pentose Phosphate Pathway

The pentose phosphate pathway also branches off from glycolysis at glucose 6-phosphate. It has two important products: ribose 5-phosphate, which is needed for synthesis of nucleotides and nucleic acids (DNA and RNA), and NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate), which provides the reducing equivalents for synthetic reactions such as fatty acid biosynthesis. The first reaction of the pathway, catalyzed by glucose 6-phosphate dehydrogenase, generates one molecule of NADPH from NADP, and the sugar aldehyde group is oxidized to a carboxylic acid. However, since the glucose 6-phosphate substrate is in the pyranose (hemiacetal) ring form with the carbon-5 oxygen linked to carbon-1, the initial product is an internal ester, 6-phosphoglucono δ-lactone, that is then cleaved by lactonase to yield 6-phosphogluconate. 6-Phosphogluconate dehydrogenase produces a second molecule of NADPH from NADP and also releases the carbon-1 carboxyl group as CO_2, leaving the 5-carbon compound ribulose 5-phosphate. The latter (a keto sugar) is converted to ribose 5-phosphate (an aldo sugar) by phosphopentose isomerase. This so-called oxidative branch of the pentose phosphate pathway thus produces two NADPHs and one pentose phosphate. If, however, this gives more pentose phosphate than is needed for nucleotide synthesis, the excess can be converted back to glycolytic intermediates by the non-oxidative branch. Three pentose phosphates (15 carbons total) are converted to two molecules of fructose 6-phosphate and one glyceraldehyde 3-phosphate as follows: First, transketolase transfers 2 carbons from xylulose 5-phosphate (an epimer from ribulose 5-phosphate made by switching the orientation of the carbon-3 hydroxyl by phosphopentose epimerase) to ribose 5-phosphate, thereby producing the 7-carbon sugar sedoheptulose 7-phosphate plus glyceraldehyde 3-phosphate. Second, transaldolase transfers 3 carbons back from sedoheptulase 7-phosphate to glyceraldehyde 3-phosphate, producing fructose 6-phosphate and the 4-carbon sugar erythrose 4-phosphate. Finally, transketolase transfers 2 carbons to the erythrose 4-phosphate from another molecule of xylulose 5-phosphate, producing fructose 6-phosphate and glyceraldehyde 3-phosphate.

Glucose Metabolism and Insulin Secretion

Not only does insulin regulate glucose metabolism but also glucose metabolism in the pancreatic β cells regulates insulin secretion (Prentki *et al.*, 1997). The β cell is responsive to glucose in the physiological range because it has a high K_m glucokinase like liver. In the β cell, the increased glucose metabolism causes an increase in the ATP/ADP ratio, which closes ATP-sensitive K^+ channels in the plasma membrane, causing membrane depolarization and an influx of Ca^{2+} through voltage-sensitive calcium channels that triggers secretion. There are also other glucose concentration-dependent enhancements of secretion that may involve lipid metabolism and signaling pathways, such as via protein kinase C. Interestingly, normal insulin secretion is pulsatile, and it has been proposed that this may be due to underlying oscillations in glucose metabolism.

See also: Normal Glucose Physiology

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Insulin Secretion: Functional Biochemical Aspects☆

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Glossary

cyclic AMP (cAMP) Adenosine-3′,5′-cyclic monophosphate

To maintain normoglycemia, the mammalian organism is dependent on a continuous and continuously adapted supply of pancreatic insulin. A dramatic illustration of such a permanent need for insulin is given by the hyperglycemia seen in normal animals injected with insulin-neutralizing guinea pig anti-insulin serum. In these animals, the rate of endogenous insulin secretion can be calculated from the progressive reduction in the pool of circulating unneutralized antibodies.

Introduction

The insulin-producing B cells are located in small groups of endocrine pancreatic cells known as the islets of Langerhans. Each islet contains up to approximately 2000 cells, with the larger islets having a diameter not exceeding close to 0.2 mm. In addition to insulin-producing cells, the islets of Langerhans contain other endocrine cell types secreting hormones such as glucagon, somatostatin, and pancreatic polypeptide.

Considerable progress in our understanding of the regulation of insulin release was made possible over the past four decades through (1) the development of an immunoassay procedure for the measurement of the low concentration of insulin in plasma (i.e., approximately 0.1 nM), and (2) a technique for the isolation of islets from the pancreatic gland.

This article aims at reviewing briefly the current knowledge on insulin secretion with an emphasis on the following themes: the regulation of insulin release under physiological conditions; the process by which insulin secretagogues, especially D-glucose, are recognized by the islet B cells as insulinotropic agents; the mechanism of coupling between the recognition of secretagogues and more distal steps in the secretory sequence; the participation of a mechanical effector system in the exocytosis of secretory granules; the perturbation of insulin secretion in certain pathological conditions; and the pharmacological tools used to modify insulin secretion in situations of hypo- or hyperinsulinemia.

Physiological Aspects

Under physiological conditions, the release of insulin by the endocrine pancreas is regulated in an immediate and direct manner by a series of circulating nutrients, hormones, and neurotransmitters. It is also modulated in a delayed fashion by dietary, hormonal, and ontogenic factors.

Circulating Nutrients

Circulating nutrients that stimulate insulin release include mainly D-glucose and other monosaccharides, pyruvic and lactic acid, amino acids, unesterified fatty acids, and ketone bodies. Among these nutrients, D-glucose plays an essential role. It is indeed the sole nutrient able to stimulate insulin secretion at its physiological extracellular concentration in the absence of any other exogenous nutrient. The process of glucose-induced insulin secretion is characterized by its sensitivity and rapidity. A sigmoidal curve relates the output of insulin to the concentration of D-glucose in the extracellular fluid. Thus, one may define a threshold concentration for the insulinotropic action of D-glucose (close to 5 mM), a concentration of the hexose yielding a half-maximal secretory response (close to 10 mM), and a maximal velocity of insulin release that is observed at high D-glucose concentrations (approximately 20 mM) and represents about 20 times the basal insulin output.

In response to a rapid increase in D-glucose concentration, the release of insulin exhibits a multiphasic pattern. An almost immediate peak of insulin release is followed by a lower, but higher than basal, insulin output. Thereafter, a progressive rise in the rate of insulin secretion is observed. A high rate of hormonal release is then eventually achieved.

As emphasized in what follows, the insulinotropic action of D-glucose is causally linked to the capacity of the hexose to act as a nutrient and to increase the rate of adenosine 5′-triphosphate (ATP) generation in insulin-producing cells. This fuel concept also

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applies to the insulin-releasing action of other nutrients mentioned previously. In the case of amino acids, however, the following more nuanced situation prevails. Certain amino acids (e.g., L-leucine, L-glutamine, L-asparagine) also owe their insulinotropic capacity to their role as nutrients in the islet B cells. However, they may also act as allosteric activator of mitochondrial glutamate dehydrogenase and, as a result, facilitate the catabolism of endogenous amino acids in the islet cells. For instance, such is the case for a transported but nonmetabolized analogue of L-leucine. Moreover, other amino acids, which are potent insulin secretagogues, are poor nutrients but stimulate insulin release as a result of their accumulation in the islet cells. Such is the case for cationic amino acids (e.g., L-arginine, L-ornithine), which, through their accumulation inside the islet cells, provoke a depolarization of the plasma membrane and a resulting gating of voltage-sensitive calcium channels.

Immediate and Direct Effects of Hormones and Neurotransmitters

Two examples of the immediate and direct effects of hormones on insulin release are mentioned here. First, adrenaline inhibits insulin secretion evoked by D-glucose or other insulin secretagogues. This effect is suppressed by α-adrenergic blocking agents and coincides with a lowering of the cyclic AMP (cAMP) content in islet cells. It was proposed that, by inhibiting insulin release under conditions of stress and exercise, adrenaline would be better able to mobilize D-glucose from the liver and to mobilize free fatty acids and glycerol from adipose tissue.

Second, certain gastrointestinal hormones, such as glucagon-like peptide 1, potentiates insulin release evoked by D-glucose and other nutrient secretagogues. This is attributable to activation of adenylate cyclase, with a resulting increase in the cAMP content of islet cells. The term 'hormonal enteroinsular axis' was proposed to indicate that the enhancement of insulin secretion by hormones released from the intestinal tract may account for the fact that hyperinsulinemia caused by D-glucose is more marked when the sugar is given orally rather than intravenously.

Likewise, vagal stimulation of insulin release could be a significant feature in the process of food intake and digestion. Cholinergic agents indeed augment insulin secretion evoked by D-glucose. This process involves occupancy of islet cells' muscarinic receptors, activation of phospholipase C, hydrolysis of polyphosphoinositides, and liberation of diacylglycerol and inositol 1,4,5-triphosphate with resulting activation of protein kinase C and mobilization of intracellular Ca^{2+}.

Long-Term Regulation of Insulin Release

Insulin release evoked by D-glucose is decreased in islets prepared from starved animals. It occurs at an abnormally high rate in islets from obese animals. Several hormones also affect the secretory responsiveness of the endocrine pancreas in a delayed manner. This may account for alteration of insulin release in situations such as pregnancy and lactation, acromegaly, hypo- or hypercorticism, and hypo- or hyperthyroidism. Finally, the long-term regulation of insulin release also involves ontogenic factors, as documented by the changes in islet secretory behavior found in fetal and neonatal life or aging.

Recognition of Secretagogues

As alluded to previously, the B cell is equipped with a number of receptors involved in the recognition of adrenergic factors, cholinergic neurotransmitters, and gastrointestinal hormones. By analogy, it had been proposed that each nutrient secretagogue may bind to a specific receptor possibly located at the B-cell plasma membrane. For instance, a previously widespread view postulated the presence of a stereospecific glucoreceptor in islet cells. However, a more pedestrian metabolic hypothesis eventually prevailed, postulating that the insulinotropic capacity of D-glucose is causally linked to its role as a nutrient in islet cells. This was then extended to other nutrient secretagogues and defined as the fuel concept for insulin release.

The key argument in support of this metabolic hypothesis for glucose-stimulated insulin secretion resides in the finding that the α-anomer of D-glucose, which is a more potent insulin secretagogue than ß-D-glucose, is also more efficiently metabolized in pancreatic islets. As a matter of fact, the metabolism of D-glucose in isolated pancreatic cells or in purified islet B cells displays a number of uncommon features, all of which optimize the increase in ATP generation rate resulting from the stimulation of islet cells by this hexose. A few examples of such features follow.

First, the concentration of D-glucose rapidly equilibrates across the plasma membrane of the B cell thanks to the efficiency of the glucose carrier GLUT2 (and GLUT1 in human B cells). Second, the phosphorylation of D-glucose in islet B cells is catalyzed not solely by an ubiquitous low-K_m hexokinase but also by a high-K_m glucokinase. As a result of these two features, the rate of D-glucose phosphorylation increases rapidly and sensibly in response to a rise in the extracellular concentration of the hexose. The regulation of D-glucose phosphorylation in the B cell also involves a number of other mechanisms such as a sequential synarchistic regulation by ATP, induction and repression of glucokinase, feedback inhibition of hexokinase by glucose 6-phosphate, intracellular translocation of glucokinase, intervention of a glucokinase regulatory protein, balance between glucose phosphorylation and dephosphorylation (by glucose-6-phosphatase), and binding of hexokinase isoenzymes to mitochondrial porin, with this process modifying kinetic properties (e.g., sensitivity to glucose 6-phosphate), coinciding with a preferential use of mitochondrial ATP for phosphorylation of D-glucose, and allowing a direct coupling between hexose phosphorylation and mitochondrial respiration.

Second, for the rate of glycolysis to keep pace with the rate of D-glucose phosphorylation, activation of key glycolytic enzymes takes place in glucose-stimulated B cells. For instance, such is the case for the activation of phosphofructokinase by fructose 2,6-bisphosphate. Incidentally, the regulation of phosphofructokinase activity in islet cells also displays the specific feature of an apparent resistance of fructose 6-phosphate,2-kinase to cAMP.

Finally, a preferential stimulation of mitochondrial oxidative events in either isolated islets or purified B cells exposed to increasing concentrations of D-glucose represents an essential feature of the B-cell glucose-sensing device. This phenomenon is attributable mainly to a Ca^{2+}-induced activation of key mitochondrial dehydrogenases such as the FAD-linked glycerophosphate dehydrogenase and 2-ketoglutarate dehydrogenase complex. Therefore, it should be considered as a secondary phenomenon caused by the accumulation of Ca^{2+} in the B cells. It optimizes the yield of ATP generated through the catabolism of D-glucose given that the mitochondrial oxidation of pyruvate provides the major fraction of such an ATP generation and is necessarily coupled with the oxidative modality of glycolysis. It should not be ignored, however, that it was recently documented that insulin-producing cells are equipped with a true sweet-tasting receptor and that activation of this receptor by artificial sweeteners as well as by D-glucose itself results in the stimulation of insulin release mediated by an array of coupling mechanisms.

Coupling Mechanisms

In the process of nutrient-stimulated insulin release, the coupling between metabolic events and more distal events in the secretory sequence appears to be multifactorial. Emphasis is currently placed on the closing of ATP-sensitive K^+ channels caused by the increase in the cytosolic ATP/adenosine diphosphate (ADP) ratio in nutrient-stimulated B cells. The closing of these channels then leads to depolarization of the plasma membrane and the subsequent opening of voltage-sensitive Ca^{2+} channels. The facilitated entry of Ca^{2+} into the B cell and the cytosolic accumulation of this divalent cation then acts as a trigger for the release of secretory granules.

A stimulatory effect of D-glucose on insulin release can still be documented, however, in cells exposed to both diazoxide (to maintain the K^+ channels in their open configuration) and a high extracellular K^+ concentration (to nevertheless cause depolarization of the plasma membrane). This finding points to the participation of other mechanisms in the stimulus–secretion coupling for glucose-induced insulin release. In this perspective, the following considerations should be underlined.

First, an increase in the ATP generation rate might not represent the sole factor coupling the catabolism of nutrient secretagogues to the remodeling of ionic fluxes in the islet cells. For instance, nutrient-induced changes in the redox state of islet cells and their intracellular pH may also participate in nutrient-stimulated insulin release.

Second, the cytosolic accumulation of Ca^{2+} may lead, via calmodulin, to the activation of adenylate cyclase in pancreatic islets. Pancreatic islets indeed contain calmodulin (about 0.1 pmol per islet). This protein binds to a particulate fraction derived from the islets and stimulates adenylate cyclase activity in this subcellular fraction, with both phenomena being activated by ionized calcium. Thus, a calcium-dependent stimulation of adenylate cyclase by endogenous calmodulin may contribute to the accumulation of cAMP evoked by insulin-releasing agents in the islet cells. cAMP should be considered as a modulator of the insulin secretory response to nutrient secretagogues. Its synthesis and breakdown are catalyzed by adenylate cyclase and phosphodiesterase, respectively. cAMP activates a protein kinase, leading to an apparent increase in the responsiveness of the effector system for insulin release to cytosolic Ca^{2+}.

Third, in nutrient-stimulated B cells, as in response to cholinergic agents, breakdown or otherwise accelerated turnover of inositol phospholipids takes place. The accelerated generation of polyphosphoinositides in nutrient-stimulated islet cells may result from a rise in cytosolic ATP production, whereas the stimulation by nutrients of the hydrolysis of inositol-containing phospholipids is currently ascribed to activation of phospholipase C through an increase in the cytosolic Ca^{2+} concentration. Enhanced phospholipid metabolism may then lead to the mobilization of calcium from nonmitochondrial intracellular stores by inositol 1,4,5-triphosphate and activation of protein kinase C by diacylglycerol.

Last, a second key component of the process of nutrient-stimulated insulin release consists in the gating of volume-sensitive anion channels. This concept is mainly based on the following findings. Whislt a rise in D-glucose concentration up to about 6-8 mM decreases the outflow of radioactive rubidium from prelabelled islets, indicating a decrease in potassium conductance, no further decrease in the outflow of radioactive rubidium and, on the contrary, a modest but significant increase is observed at higher D-glucose concentrations, namely in the range of glucidic concentrations provoking the most marked stimulation of insulin release. It seems likely, therefore, that another series of cellular events is responsible for enhancing insulin secretion at these high concentrations of the hexose. The anion channel hypothesis postulates that the entry of D-glucose in insulin-producing cells and the subsequent acceleration of its catabolism results in the intracellular accumulation of metabolites such as lactate and bicarbonate anions. The resulting increase of intracellular osmolarity may then provoke, through increased water uptake, an increase in cell volume and the subsequent gating of volume-sensitive anion channels. In the insulin-producing cells, the gating of these channels may allow the exit of chloride, orthophosphate, lactate and bicarbonate anions and, hence, further depolarization of the plasma membrane and gating of voltage-sensitive calcium channels. This novel concept is supported by a number of findings concerning for instance the stimulation by D-glucose of chloride anion efflux, the nutrient-induced transient increase of inorganic phosphate efflux, a phenomenon referred to as a phosphate flush, and the likely increase in bicarbonate anion outflow during the second and sustained phase of insulin secretion evoked by D-glucose and othet nutrient secretagogues. The latter proposal takes into account the finding that the carbonic anhydrase-catalyzed generation of radioactive bicarbonate from radioactive carbon dioxide itself produced through the oxidative catabolism of the hexose in islets exposed to glucose uniformly labelled with carbon

14 accounts for the majority of the latter production. The anion channel hypothesis also led to investigations concerning the expression in pancreatic islets of the sodium-bicarbonate-cotransporter NBCe1-A and aquaglyceroporin 7.

Effector System

The increase in the cytosolic concentration of ionized calcium caused by D-glucose or other insulin secretagogues triggers the exocytosis of secretory granules by causing the activation of a microtubular–microfilamentous system. The participation of this effector microtubular–microfilamentous system in the process of insulin release has been documented by a number of ultrastructural, biochemical, functional, and cinematographical studies.

Microtubules (21–25 nm in diameter) are considered to represent the cytoskeleton of B cells. They are scattered in the cytoplasm. They can be found between rows of aligned secretory granules. They are thought to provide oriented pathways for back-and-forth saltatory movements of secretory granules. They are also prominent in the ectoplasmic area. At that level, the cell web occupies cytoplasmic areas of variable thickness (50–300 nm) just beneath the plasma membrane and extends into the core of microvillous processes. It consists of a network of actin-like microfilaments (4–7 nm in diameter) generally disposed to form irregularly shaped polygons. This contractile cell web acts as a sphincter, either restricting or favoring the access of secretory granules to their exocytotic site at the plasma membrane.

The tools most commonly used to interfere experimentally with the function of the microtubular–microfilamentous system include mitotic spindle inhibitors (e.g., colchicine, vincristine), microtubule stabilizers (e.g., D_2O), and the microfilamentous modifier cytochalasin B.

At the exocytotic site, the fusion between the limiting membrane of insulin secretory granules and plasma membrane and subsequent fission of the fused membrane seem to involve an anion–osmotic process in which the insertion at the plasma membrane of an anion transport system derived from the limiting membrane of the secretory granules allows for the entry of anions (e.g., Cl^-, OH^-) into the lumen of these granules, followed by their osmotic fission. This process also accounts for the release of several granules aligned along an oriented microtubular pathway, a phenomenon known as the chain release of secretory granules or compound exocytosis.

Pathological Aspects

Defective insulin release is a typical feature of diabetes mellitus. In insulin-dependent diabetes, it results from an autoimmune destruction of insulin-producing cells. In non-insulin-dependent diabetes, the deficiency of insulin release may be caused by a number of distinct site-specific anomalies. Indeed, virtually each step in the process of insulin secretion may be affected by inherited or acquired factors. For instance, a defect in the conversion of proinsulin to insulin and C-peptide may result in the release of large amounts of proinsulin, which is virtually devoid of hypoglycemic action. On the most distal step of the secretory sequence, a defect of the microtubular apparatus may be responsible for a sluggish secretory response to D-glucose in certain animal models of type 2 diabetes.

In most but not all patients with type 2 diabetes, the endocrine pancreas displays a preferential impairment of its secretory responsiveness to D-glucose, as distinct from other nutrient or nonnutrient insulin secretagogues. Site-specific defects responsible for such a situation include (1) a decrease in the number of B cells suitably equipped with sufficient GLUT2 carriers, (2) a nonsense mutation of the glucokinase gene, (3) excessive activity of glucose-6-phosphatase leading to an ATP-wasting futile cycle in the reaction catalyzed by hexokinase isoenzymes and glucose-6-phosphatase, and (4) a deficient activity of the mitochondrial FAD-linked glycerophosphate dehydrogenase, the key enzyme of the glycerol phosphate shuttle. Moreover, glycogen accumulation takes place in the B cell in situations of chronic hyperglycemia. It is held responsible for the secondary process of so-called B-cell glucotoxicity or incompetence. Two typical phenomenological aspects of this process consist in a paradoxical early and transient decrease in insulin release and the perturbation of its anomeric specificity in response to the rapid intravenous administration of D-glucose to patients with type 2 diabetes.

Excessive secretion of insulin can also occur in certain pathological conditions. Such is the case, for instance, in individuals bearing an insulinoma or in patients with persistent hyperinsulinemia during childhood.

Pharmacological Aspects

The therapeutic agents currently used to modify insulin secretion belong to two classes. First, to enhance insulin release in non-insulin-dependent diabetic individuals, either hypoglycemic sulfonylureas (e.g., tolbutamide, glibenclamide) or meglitinide analogues (e.g., nateglinide, repaglinide) are given orally. These antidiabetic agents display a common configuration and act by causing a direct closing of ATP-sensitive K^+ channels without affecting the ATP content of insulin-producing cells.

Second, to prevent excessive insulin secretion in certain pathological conditions, diazoxide and related drugs are used. They cause a direct opening of ATP-sensitive K^+ channels and, hence, inhibit insulin release caused by D-glucose and other secretagogues. This effect of diazoxide is abolished in the presence of hypoglycemic sulfonylureas or meglitinide analogues.

The destruction of insulin-producing cells (e.g., in patients bearing an insulinoma) can be achieved by selective ß-cytotoxic agents, especially streptozotocin.

See also: Insulin Action; Post-Receptor Mechanisms

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Insulin Action; Post-Receptor Mechanisms☆

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Insulin is a polypeptide hormone that is secreted from beta cells of the pancreas. It is the primary hormone responsible for regulating glucose uptake and utilization in cells. Insulin is also known as an anabolic hormone and is involved in the synthesis of glycogen, proteins and lipids. A defect in insulin secretion or action leads to aberrant glucose homeostasis and diabetes. Because of its role in the regulation of glucose concentration in the blood and in the pathogenesis of diabetes, an enormous amount of effort has been directed to understand the molecular mechanism of insulin action. In this article we aim to provide an overview on the postreceptor mechanisms which contribute to the key glucoregulatory responses of insulin in its target tissues.

Insulin Receptor and Insulin Receptor Substrates

The first event in insulin action involves the binding of insulin to its receptor. The insulin receptor (IR) is composed of two extracellular α-subunits and two transmembrane β-subunits linked to each other by disulfide bonds. The α-subunit is mostly extracellular and contains the insulin-binding site, while the β-subunit has an intracellular component and possesses an intrinsic insulin-regulated tyrosine kinase activity. Binding of insulin to the α-subunit of IR causes a conformational change, leading to an enhanced protein tyrosine kinase (PTK) activity of the β-subunit by multisite tyrosine phosphorylation (Tyr − 1158, Tyr − 1162, Tyr-1163) (White and Kahn, 1994) (**Fig. 1**). Activated IR-PTK phosphorylates its downstream substrates in tyrosine residues (White, 2003; White, 2006). These substrates include insulin receptor substrates (IRSs), Shc (Src homology and collagen domain) proteins and Grb2 associated binding protein 1 (Gab1). IRSs have multiple tyrosine phosphorylation sites that, once phosphorylated, serve as docking sites for Src homology 2 (SH2) domain-containing signaling molecules (White, 2003; White, 2006). In addition, IRSs have several serine/threonine residues that are phosphorylated by serine/threonine kinases and reduce the binding of these signaling molecules to IRSs. The IRS family has several members (IRS1-IRS6), however, among these, the role of IRS1 and IRS2 has been studied in some detail with regards to their involvement in mediating insulin action and insulin resistance. IRS gene knockout studies in mice have demonstrated that deletion of the IRS1 gene results in normal glucose tolerance despite mild insulin resistance in muscle tissues, whereas deletion of IRS2 was associated with attenuated insulin signaling, insulin resistance and diabetes (Araki et al., 1994; Kubota *et al.*, 2000; Kido *et al.*, 2000; Withers *et al.*, 1998; White, 2006). Consistent with a role of IRS1 in insulin resistance, some mutations in IRS1 have been associated with type 2 diabetes in human subjects (Whitehead *et al.*, 1998; Almind *et al.*, 1996; Burguete-Garcia *et al.*, 2010; Martinez-Gomez *et al.*, 2011), however, a strong association between these mutations and diabetes remains to be established.

Phosphatidylinositol-3-Kinase (PI3K)

As stated earlier, the tyrosine phosphorylated and activated forms of IRSs serve as docking sites for binding with key signaling molecules. These signaling molecules invariably possess SH2 domains and bind to specific phosphotyrosine residues on the IRS proteins. PI3K is one such signaling molecule and is a key mediator of the glucoregulatory and metabolic responses of insulin. PI3K belongs to a family of lipid kinases which catalyze the formation of phosphatidylinositol 3, 4, 5-triphosphate (PIP3) by adding a phosphate group at position 3 of the inositol ring of phosphatidylinositol 4, 5-bisphosphate (PIP2). PI3K family is composed of 3 different members among which class 1A is a heterodimeric protein consisting of a regulatory subunit, p85 and a catalytic subunit p110α. Binding of the regulatory subunit of PI3K to IRS activates the catalytic properties of the p110α subunit resulting in the conversion of PIP2 to PIP3 (Myers *et al.*, 1992). PIP3 then serves as a lipid second messenger for the activation of pleckstrin (PH) domain-containing protein kinases such as three phosphoinositide-dependent–kinase 1(PDK1). PDK1 plays a critical role in activating protein kinase B (PKB) also known as AKT, a downstream effector of PI3K and a critical component of insulin signaling pathway (**Fig. 1**).

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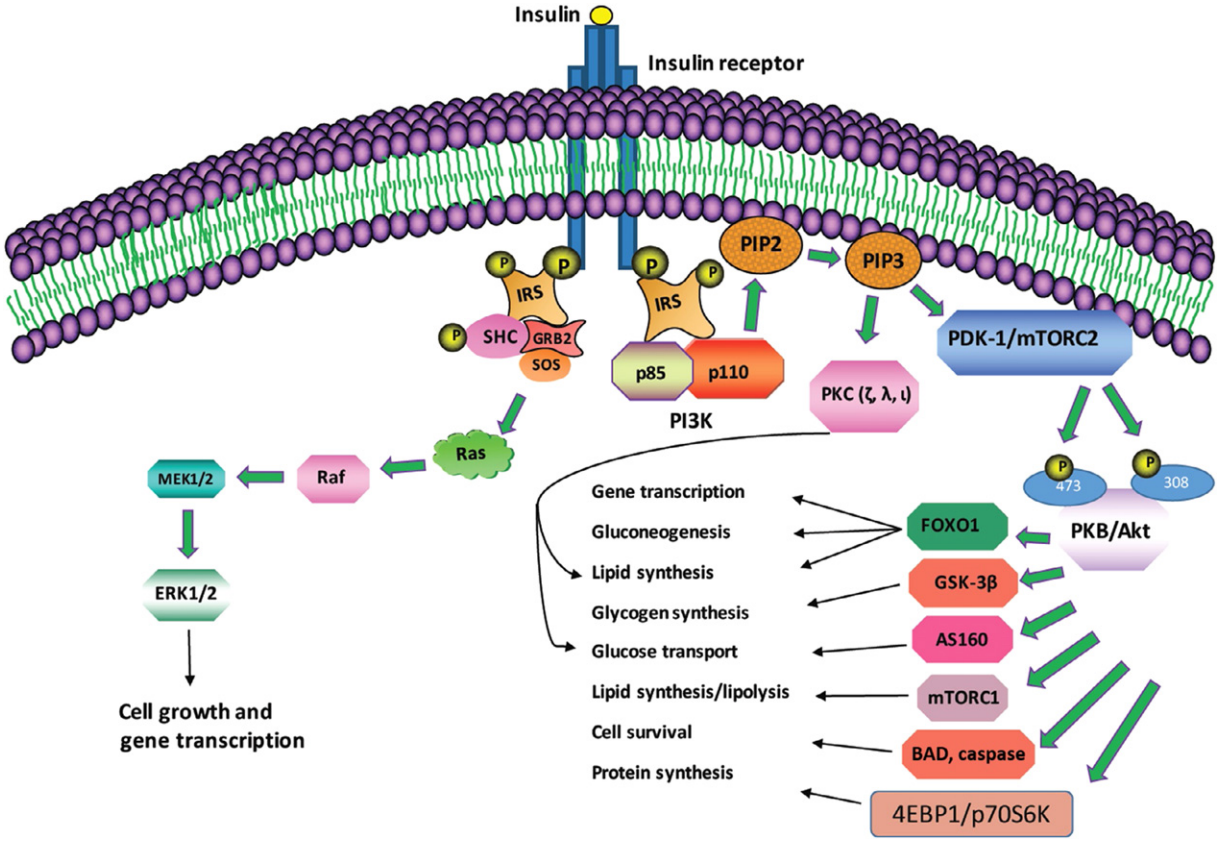

Fig. 1 *Schematic model showing key elements of the insulin signaling pathway.* Insulin-induced tyrosine phosphorylation and activation of IRSs by the PTK of β-subunits lead to the recruitment of SH-2 domain-containing signaling proteins such as Shc, Grb-2-SOS and the p85 regulatory subunit of PI3-K. The IRS-associated complex initiates two main signaling pathways. One pathway, known as the MAPK pathway, consists of Ras/Raf/MEK/ERK1/2. ERK1/2 can be translocated to the nucleus where they can activate transcription by inducing phosphorylation of several transcription factors. The second pathway involves PI3-K activation, which results in the generation of PIP3. PIP3 activates a variety of downstream signaling components, including PDKs, PKB, mTORC1/2, ribosomal protein S-6-kinase (p70s6k), PKCs, GSK-3, and FoxO. PKB and PKC are involved in glucose transport/GLUT4 translocation while mTORC1 regulates protein synthesis via p70s6k. Glycogen synthesis is regulated by PKB via GSK-3. PKB-mediated phosphorylation of FoxO regulates gluconeogenesis by inhibits transcription of PEPCK and G6Pase. mTORC1 controls lipogenesis and lipolysis.

Pkb/Akt and Insulin Action

PKB/AKT is a serine/threonine protein kinase that was originally discovered as a cellular homolog of v-AKT, a retroviral oncogene (Manning and Toker, 2017; Staal, 1987). It has been termed as PKB because of its structural similarities with protein kinase A and protein kinase C (Coffer and Woodgett, 1991). Subsequent studies have led to the identification and characterization of three different homologous isoforms of PKB: PKBα/Akt1; PKBβ/Akt2 and PKBγ/Akt3 (Brazil and Hemmings, 2001; Manning and Toker, 2017). Of these isoforms, PKBβ/Akt2 is highly expressed in insulin responsive tissues and is believed to mediate most of glucoregulatory and metabolic effects of insulin. All isoforms of PKB/AKT, in addition to having a catalytic protein kinase domain, also possess a lipid binding regulatory PH domain which binds to PIP3 generated by PI3K. This binding results in its translocation to the cell membrane where it is activated by its phosphorylation on threonine 308 and serine 473 residues catalyzed by 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and mammalian target of rapamycin complex 2 (m-TORC-2) respectively (**Fig. 1**) (Manning and Toker, 2017).

Glucose Transport

Activated PKB phosphorylates a multitude of downstream substrates that mediate the glucoregulatory responses in insulin target tissues. These substrates include the Akt substrate of 160 kDa (AS160) which is a GTPase activating protein (GAP) and has been suggested to play an important role in insulin-stimulated glucose transport by promoting the translocation of insulin-dependent glucose transporter protein, GLUT4, from the intracellular sites to the plasma membrane (Klip *et al.*, 2014). Although the precise steps that AS160 regulates in GLUT4 trafficking pathway remain elusive, phosphorylation-induced reduction in its GAP activity

appears to be essential for this response because expression of a nonphosphorylatable mutant of AS160 was shown to inhibit insulin-induced GLUT4 translocation (Randhawa *et al.*, 2008; Foley *et al.*, 2011; Klip *et al.*, 2014). Moreover, the results showing that the knock down of AS160 leads to a redistribution GLUT4 from the intracellular sites to the plasma membrane have provided additional support for an implication of AS160 in insulin-induced GLUT4 translocation and glucose transport (**Fig. 1**) (Eguez *et al.*, 2005).

Glycogen Synthesis

Another key physiological response of insulin is the storage of glucose in the form of glycogen in target tissues. Glycogen synthesis in cells is catalyzed by glycogen synthase (GS) and its activity is regulated by a reversible phosphorylation/dephosphorylation pathway (Srivastava and Pandey, 1998). The phosphorylation of GS is achieved by a variety of serine/threonine protein kinases whereas it is dephosphorylated by protein phosphatases. The dephospho-GS is an active form and phospho-GS is inactive, and unable to fully induce glycogen synthesis in response to insulin (Srivastava and Pandey, 1998). One of the protein kinases that phosphorylates and inactivates GS is glycogen synthase kinase-3 (GSK-3) (Srivastava and Pandey, 1998; Roach, 1991). Two isoforms of GSK-3 have been identified in mammalian cells, termed GSK-3α and GSK-3β. In the basal state, GSK-3 remains constitutively active however, PKB/AKT-induced phosphorylation on serine21 in GSK-3α and serine 9 in GSK-3β renders it inactive. Inactivation of GSK-3 results in decreased phosphorylation of GS and thereby enhances its ability to catalyze the synthesis of glycogen in response to insulin (**Fig. 1**). Gene knock out studies as well as use of pharmacological inhibitors have demonstrated that GSK-3 is a critical mediator of insulin-induced glycogen synthesis in both muscle and liver tissues (MacAulay and Woodgett, 2008). In addition, the activation of protein phosphatases can also decrease the phosphorylation of GS and induce glycogen synthesis in response to insulin.

Gluconeogenesis

In addition to stimulating glucose transport and glycogen synthesis, insulin also regulates glucose homeostasis by suppressing hepatic glucose output. This is achieved by the inhibition of gluconeogenesis. In the absence of insulin, the activity of phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two rate limiting enzymes of gluconeogenic pathway, remains elevated allowing increased production of glucose through this pathway. Insulin inhibits the expression of PEPCK and G6Pase in the liver via PKB-dependent phosphorylation of the forkhead box O (FoxO) family of transcription factors. In the basal state, the FoxOs reside in the nucleus and induce the expression of PEPCK and G6Pase, however, PKB-induced phosphorylation of FoxO results in their binding to 14-3-3 adaptor proteins and nuclear export leading to the downregulation of PEPCK and G6Pase gene expression (**Fig. 1**). Consistent with a critical role of FoxOs in the regulation of gluconeogenesis, genetic deletion of FoxO1 has been reported to inhibit gluconeogenesis in mouse models (Pajvani and Accili, 2015).

Lipogenesis and Lipolysis

Being an anabolic hormone, insulin stimulates lipogenesis and inhibits lipolysis. Sterol-regulatory element binding protein-1C (SREBP-1C), which is a key transcription factor that activates the expression of lipogenic gene transcription program, plays a key role in insulin—induced de novo lipid synthesis. Both mTORC1-dependent and independent pathways regulate the processing and expression of SREBP-1C. A potential role of early growth response-1, a zinc finger transcription factor, in the suppression of insulin-induced lipolysis by inhibiting the expression of adipose triglyceride lipase (ATGL) via mTORC 1 pathway has been suggested (Chakrabarti *et al.*, 2013). While Akt/mTORC1 signaling activates SREB-1C–induced lipogenic program, it suppresses lipolysis (Wang *et al.*, 2015; Kandror, 2017) (**Fig. 1**). Phosphodiesterase 3B (PDE3B) has been shown to play a role in the suppression of lipolysis because adipocytes from PDE3 knockout animals show a reduction in the antilipolytic effect of insulin (Choi *et al.*, 2006).

Ras/Raf/Erk Pathway

In addition to the PI3K/PKB/AKT pathway, insulin also triggers another pathway downstream of IR and IRS-1. In this pathway, SH2 domain-containing proteins Shc (SH2 domain and collagen homologous region) and growth factor receptor binder-2 (Grb-2)/mammalian son of sevenless (SOS) complex results in the activation of Ras-mitogen activated protein kinase (MAPK) signaling. In this cascade, SOS serves as guanine exchange factor for Ras and stimulates the formation of active RAS-GTP complex and activation of a serine/threonine kinase Raf. Raf then phosphorylates MEK (MAPK kinase), which in turn phosphorylates extracellular signal-regulated kinase (ERK1/2) on Thr and Tyr residues located in the activation loop of the kinase (**Fig. 1**). ERKs, via MAPK interacting proteins (MINK1/MINK2), participate in protein synthesis and/or get translocated to the nucleus where they

phosphorylate transcription factors such as c-Jun, CHOP, CREB and MEF-2 to induce gene transcription and contribute to the mitogenic and growth-promoting effects of insulin (Fig. 1).

Calcium and Protein Kinase C in Insulin Signaling

Insulin has been shown to trigger Ca^{2+} influx as well as mediate insulin-induced MAPK and PI3K/AKT signaling cascades in hepatocytes and 3T3-adipocytes (Benzeroual *et al.*, 2000; Benzeroual *et al.*, 1997; Whitehead *et al.*, 2001). In these studies, calcium depletion/chelation, despite blocking PI3K/AKT activation, failed to reduce insulin-stimulated tyrosine phosphorylation of both IR and IRS1, suggesting that Ca^{2+} exerted its effect downstream of IR/IRS. Other cell types, such as L6-myotubes and cardiomyocytes, also exhibit an increase in cytosolic Ca^{2+} in response to insulin via inositol-3-phosphate receptor and ryanodine receptor–mediated events which participate in insulin stimulation of GLUT4 translocation and glucose uptake (Contreras-Ferrat *et al.*, 2014a; Contreras-Ferrat *et al.*, 2014b). An implication of Ca^{2+} in insulin-stimulated AKT phosphorylation and glucose uptake in 3T3-L1 adipocytes has also been reported (Worrall and Olefsky, 2002), establishing a clear role of Ca^{2+} in this process.

The atypical class of PKC family members (PKC ζ, λ and ι) are also important components of the insulin signaling cascade. Atypical PKCs are activated by phospholipids such as PIP3 generated by PI3K activation and require the phosphorylation of a threonine residue in the activation loop for stimulation of their catalytic activity. Once activated, these PKCs participate in insulin-induced glucose transport, GLUT4 translocation as well as lipogenesis by regulating the transcription of SREBP-1C transcription (Fig. 1) (Farese *et al.*, 2014; Farese and Sajan, 2010).

Defects in Postreceptor Insulin Signaling

An aberrant insulin action in insulin resistant states and diabetes has been attributed to defective insulin signaling. This defect can occur at various steps in the insulin signaling pathway and many components of this pathway are subject to negative regulation by serine/threonine protein kinase and protein phosphatases. A series of serine/threonine protein kinases that are activated by free fatty acids, reactive oxygen species and inflammatory cytokines such as TNF-α, that are elevated in insulin resistant sates, catalyze the phosphorylation of IRS-1 on serine residues-301, 307 and 632, dissociate the IRS-1-PI3K complex and reduce PI3K activity, which in turn inhibits insulin action. Protein tyrosine phosphatases (PTPases) also regulate insulin action by dephosphorylating phospho-tyrosines in activated forms of IR and IRS and disabling the downstream signaling at the level of PI3K and PKB/AKT. Ablation of PTB1B, a PTPase with high expression in insulin target tissues, confers protection against high fat diet–induced insulin resistance and improves insulin signaling (Boura-Halfon and Zick, 2009; Morino *et al.*, 2008; Saini, 2010; Boucher *et al.*, 2014).

Summary

Decades of research in many laboratories has helped unravel the signaling pathway induced by IR activation and has identified various components of this pathway that mediate the physiological effects of insulin. As a result, two main pathways have been implicated in mediating the key physiological responses of insulin in insulin target tissues. In one pathway, IRS-associated Shc-Grb2-Sos complex activates the Ras/Raf/MEK/ERK signaling pathway which plays a key role in inducing the mitogenic and hypertrophic responses by initiating protein synthesis and gene transcription. The second pathway that radiates from IRSs involves PI3K activation that generates PIP3. PIP3 recruits PKB/AKT and PDK1 to the membrane where PKB becomes phosphorylated and activated. Activated PKB phosphorylates several downstream substrates such as GSK-3, AS160, FoxO which mediate the effect of insulin in enhancing glucose uptake, GLUT4 translocation, glycogen synthesis and lipogenesis, in the inhibition of lipolysis and gluconeogenesis. Atypical PKCs that are activated by PIP3 also participate in the regulation of GLUT 4 translocation and lipogenesis. PI3K-independent pathways have also been suggested to contribute to insulin–induced responses.

See also: Insulin Secretion: Functional Biochemical Aspects

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Diagnosis and Classification of Diabetes Mellitus

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Glossary

Beta cells Cells found in the pancreatic islets of the pancreas that produce insulin.
Glycated hemoglobin Biochemical marker of blood glucose control.
Hyperglycemia High blood sugar level main characteristic of diabetes mellitus.
Insulin Hormone produced by beta cells to control blood glucose levels.
Ketoacidosis Serious complication of diabetes when the body produces high levels of blood acids called ketones due to lack of insulin.
Macrovascular disease Large vessel disease.
Microvascular disease Small vessel disease.
Polydipsia Excessive drinking due to excessive thirst.
Polyphagia Excessive eating.
Polyuria Excessive and frequent production of urine.
Prediabetes Clinical stage preceding diabetes (see below).

Introduction

In the ancient Egyptian papyrus discovered by George Ebers, polyuric states resembling diabetes mellitus were described as early as 1550 BC. The association of polyuria with a sweet-tasting urine was rapidly noted. The term diabetes mellitus, an allusion to the honeyed taste of the urine was used to distinguish it from other polyuric states in which the urine was tasteless.

Diabetes mellitus is now defined as a state of chronic hyperglycemia which may result from many environmental and genetic factors often acting jointly.

Definition of Diabetes and Prediabetes

Diabetes

The usual clinical symptoms of diabetes, polyuria and polydipsia, are the direct consequences of the high blood glucose concentration. Weight loss in spite of polyphagia, ketoacidosis, visual changes, skin and urogenital infections, sepsis and pruritus may be present as well. Mild hyperglycemia is usually symptomless. Almost 50% of persons with diabetes do not present these diabetic symptoms. It is therefore quite clear why diagnosis based solely on symptoms bears a very low incidence.

To study the natural history and pathogenesis of diabetes as a whole, the biochemical definition is the only common and stable factor. Hyperglycemia remains therefore the most important factor required for the diagnosis of diabetes.

The chronic hyperglycemia is due to a defective insulin secretion (relative insulin deficiency) and/or insulin resistance (defective insulin action). Chronic exposure to high blood glucose levels induces specific long-term microvascular complications affecting the nerves, the kidneys and the eyes, as well as an increased risk for macrovascular disease, specifically cardiovascular disease (CVD). The diagnostic criteria for diabetes are based on thresholds of glycemia that are associated with microvascular disease, especially retinopathy.

Prediabetes

Prediabetes is a term referring to impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or a glycated hemoglobin (A1C) of 6.0%–6.4%, each of which places individuals at high risk of developing diabetes and its complications. IFG, IGT, A1c are measured from blood samples at a fasting state (IFG) or 2 h after an oral absorption of a standardized amount of 75 g of glucose, that is, oral glucose tolerance test (OGTT) or at random (A1C).

Diagnostic Criteria

Diabetes

The diagnostic criteria for diabetes are summarized in **Table 1**. They are based on venous samples and laboratory methods. A fasting plasma glucose (FPG) level of 7.0 mmol/L correlates closely with a 2-h plasma (2hPG) value of 11.1 mmol/L or more in a 75 g OGTT, and each predicts the development of retinopathy.

Table 1 Diagnosis of diabetes

FPG $\geq$ 7.0 mmol/L
Fasting = no caloric intake for at least 8 h
or
A1C $\geq$ 6.5% (in adults)
Using a standardized, validated assay, in the absence of factors that affect the accuracy of the A1C and not for suspected type 1 diabetes
or
2hPG in a 75-g OGTT $\geq$ 11.1 mmol/L
or
Random PG $\geq$ 11.1 mmol/L
Random = any time of the day, without regard to the interval since the last meal

2hPG = 2-h Plasma glucose; FPG = fasting plasma glucose; OGTT = oral glucose tolerance test; PG = plasma glucose.

The relationship between A1C and retinopathy is similar to that of FPG or 2hPG with a threshold at around 6.5%. A1C is also a continuous cardiovascular (cv) risk factor and a better predictor of macrovascular events than FPG or 2hPG. Although many people identified by A1C as having diabetes will not have diabetes by traditional glucose criteria and vice versa, there are several advantages to using A1C for diabetes diagnosis. A1C is more convenient than FPG or 2hPG In a 75 g OGTT since it can be measured at any time of day. A1c testing also avoids the problem of day-to-day variability of glucose values as it reflects the average PG over the previous 3 months. In individuals with various hemoglobinopathies, iron deficiency, hemolytic anemias, and severe hepatic and renal disease, A1c may be misleading. Studies of various ethnicities indicate that Africans, African Americans, American Indians, Hispanics and Asians have A1C values that are up to 0.4% higher than those of Caucasian individuals. May be more studies will help to determine if age- or ethnic-specific adjusted A1C thresholds are required for diabetes diagnosis. A1c is not recommended for diagnostic purposes in children, adolescents, pregnant women or those with suspected type 1 diabetes (see classification).

The decision of which test to use for diabetes diagnosis (**Table 1**) is left to clinical judgment.

FPG is a well established standard, fast, easy to perform and only one sample is needed. FPG is also a good predictor of microvascular complications (eye, kidney and nerve disease) Some of its disadvantages to mention are the unstability of the sample, a high day-to-day variability and the fact that it reflects glucose homeostasis at a single point in time.

2hPG is also a well established standard and an excellent predictor of microvascular complications (eye, kidney and nerve disease). The main disadvantages are the unstability of the sample, the high day-to-day variability, its inconveniency and unpalatability as well as its cost.

In the absence of symptomatic hyperglycemia, if a single laboratory test is in the diabetes range, a repeat confirmatory laboratory test must be done on another day.

The use of OGTT as a diagnostic tool has considerably been reduced. OGTT is still or may be used in the following clinical conditions: a random blood glucose is equivocal, as part of special clinical investigation, in pregnancy (diagnosis of gestational diabetes), for experimental and epidemiological purposes and finally to exclude or make the diagnosis of diabetes and impaired glucose tolerance.

In the case of symptomatic hyperglycemia, the diagnostic has been made and a confirmatory test is not required before treatment is initiated. If results of two different tests are available and both are above the diagnostic cutpoints, the diagnosis of diabetes is confirmed. When the results of more than 1 test are available (among FPG, A1c, 2hPG, in a 75 g OGTT) and the results are discordant, the test whose result is above the diagnostic cutpoint should be repeated and the diagnosis made on the basis of the repeat test.

Prediabetes

Table 2 summarizes the diagnostic criteria for prediabetes. The term "prediabetes" refers to IFG, IGT or an A1C of 6.0%–6.4%, each of which places individuals at high risk of developing diabetes and its complications.

IFG is defined as a FPG value of 5.6–6.9 mmol/L. IGT is defined as a 2hPG in a 75 g OGTT plasma glucose value between 7.8 and 11.0 mmol/L. A1C levels between 6.0% and 6.4% are associated with a higher risk for diabetes than lower levels. They are therefore indicative of prediabetes. The cutoffpoints may slightly vary according to different diabetic associations worldwide.

Not all individuals with prediabetes will necessarily progress to diabetes. A significant proportion of them will revert to normoglycaemia probably due to life style modification (increased physical activity and changes in dietary habits).

Classification of Diabetes

There are four main types of diabetes: type 1 diabetes mellitus (DM1), type 2 diabetes mellitus (DM2), gestational diabetes (GDM) and other types of diabetes.

Table 2 Diagnosis of prediabetes[a]

Test	*Result*	*Prediabetes category*
Fasting plasma glucose (mmol/L)	6.1–6.9	Impaired fasting glucose (IFG)
2-h Plasma glucose in a 75-g oral glucose tolerance test (mmol/L)	7.8–11.0	Impaired glucose tolerance (IGT)
Glycated hemoglobin (A1C) (%)	6.0–6.4	Prediabetes

[a]Prediabetes = IFG, IGT or A1C 6.0–6.4% → high risk of developing T2DM.

Type 1 Diabetes

Type 1 diabetes (DM 1) indicates the processes of beta cell destruction that may ultimately lead to diabetes in which insulin is required for survival in order to prevent the development of ketoacidosis, coma and death. This form of diabetes includes cases due to an autoimmune process and those for which the etiology of beta cell destruction is unknown. DM1, previously encompassed by the terms insulin-dependent diabetes, type 1 diabetes, or juvenile-onset diabetes, represents 5%–10% of all cases of diabetes, appears mostly in childhood and adolescence but may be present in adult and elderly individuals. Patients are rarely obese when they present with this type of diabetes. However, the presence of obesity is not incompatible with this form of diabetes. It is strongly associated with certain components of the histocompatibility system, human leucocyte antigens (HLA). The rate of beta cell destruction is quite variable. It can be rapid in children, but a slowly progressive, previously known as latent autoimmune diabetes in adults (LADA), is well described in adults. What triggers the destruction of the beta cells that normally produce insulin has not yet fully been understood. Environmental factors, such as viruses, may play a role in some genetically susceptible individuals.

There are some forms of DM1 which have no known etiologies. Some of these patients have permanent lack of insulin and are prone to ketoacidosis but have no evidence of autoimmunity. This form, known as an idiopathic DM1, is more common among individuals of African and Asian origin. In another form found in Africans, African Americans and Caribbeans and in some other individuals in Latin America, an absolute requirement for insulin replacement therapy in affected patients may fluctuate with time, and come and go, and patients periodically develop ketoacidosis.

Type 2 Diabetes

Type 2 diabetes (DM2) is the commonest form of diabetes (90% of all cases of diabetes). It is a complex metabolic disorder ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with or without insulin resistance. In summary, this type of diabetes previously known as noninsulin-dependent diabetes (NIDDM) or adult onset diabetes, is characterized by a relative insulin deficiency rather than absolute. People with DM2 frequently are resistant to the action of insulin. At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive.

Unfortunately DM2 is frequently undiagnosed for many years (lack of symptoms) because the hyperglycemia is not severe enough to provoke noticeable symptoms of diabetes. Nevertheless such patients are at increased risk of developing complications related to diabetes such as eye, kidney, nerves and macrovascular complications. Although the specific etiology of this form of diabetes is not known, autoimmune destruction of the pancreas does not occur. Patients with DM2 are obese and those not obese by traditional criteria, body mass index (BMI) for example, may have an increased percentage of body fat distributed predominantly in the abdominal region. Obesity itself causes insulin resistance. Ketoacidosis seldom occurs in DM2, and when seen it usually arises in association with the stress of another illness such as infection.

The risk of developing DM2 increases with age, obesity and physical inactivity. More and more cases are detected in childhood and adolescence. It occurs more frequently in women with prior gestational diabetes (GDM) the third type of diabetes, in those with hypertension or dyslipidemia, and its frequency in different ethnic groups.

DM2 is often associated with strong familial, likely genetic, predisposition. The genetics of DM2 are quite complex and not yet clearly defined.

Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) refers to glucose intolerance with onset or first recognition during pregnancy. The definition applies irrespective of whether or not insulin is used for treatment or the condition persists after pregnancy. Women who become pregnant and who are known to have diabetes mellitus which antedates pregnancy do not have gestational diabetes but have diabetes mellitus and pregnancy and should be treated accordingly before, during and the pregnancy.

Individuals at high risk for gestational diabetes include older women for pregnancy (more than 25 years of age), those with abnormal body weight, those with previous history of prediabetes (IFG and/or IGT) or family history of diabetes, those with a history of large for gestational-age babies, women from certain high-risk ethnic groups such as Hispanic American, African American, Native American, Asian American, Pacific Islander and others and any pregnant woman who has elevated fasting or

casual blood glucose levels. It may be therefore appropriate to screen pregnant women belonging to high-risk populations during the first trimester of pregnancy in order to detect previously undiagnosed diabetes mellitus. If they are found to have GDM at that initial screening, they should be retested between 24 and 28 weeks of gestation.

Formal systematic testing for gestational is usually between 24 and 28 weeks of gestation.

The risk of elevated glucose levels can have adverse effects on the fetus in the first trimester. They can induce fetal malformations. In the second and third trimester there is a high risk of macrosomia and metabolic complications in the presence of elevated glucose levels. It is therefore imperative to make the diagnosis of diabetes during pregnancy.

Diagnosis of Gestational Diabetes

To determine if gestational diabetes is present in pregnant women, a standard OGTT should be performed after overnight fasting (8–14 h). There are two different approaches (**Table 3**). *One step approach*: Perform a diagnostic OGTT without prior plasma or serum glucose screening. This approach may be cost-effective in high-risk patients or populations.

Two-step approach: Perform an initial screening by measuring the plasma or serum glucose concentration 1 h after a 50 g oral glucose load (glucose challenge test: GCT) and perform a diagnostic OGTT on that subset of women exceeding the glucose threshold value on the GCT. With either approach, the diagnosis of GDM is based on OGTT.

Other Types of Diabetes

In this category is included a wide variety of relatively uncommon conditions.

Genetic defects of beta cell function

The diabetic state may be associated with monogenic defects in beta cell function. These forms are characterized by onset of mild hyperglycemia at early age. Patients with these forms of diabetes, formerly referred to as maturity-onset diabetes of the young (MODY), have impaired insulin secretion with minimal or no defect in insulin action.

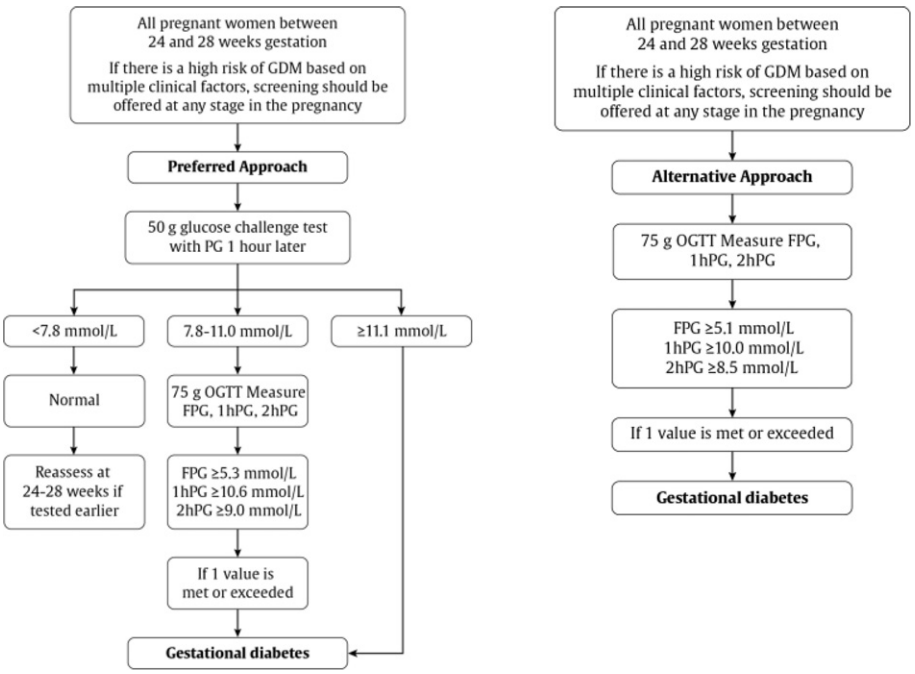
Genetic defects in insulin action

These causes of diabetes are unusual and result from genetically determined abnormalities of insulin action (i.e., mutations of the insulin receptor).

Diseases of the exocrine pancreas

Pancreatitis, trauma, infection, pancreatic cancer and pancreatectomy are some of the acquired processes that can cause diabetes. Any process that diffusely injures the pancreas can cause diabetes.

Table 3 GDM Diagnosis: Two approaches



Endocrinopathies

Insulin action can be antagonized by several hormones. Diseases associated with excess secretion of these hormones can cause diabetes such as acromegaly (growth hormone) or Cushing syndrome (cortisol) etc.

Drug-or chemical-induced diabetes

Insulin secretion may be impaired by many drugs. They may not, by themselves, cause diabetes, but they may precipitate diabetes in persons with insulin resistance.

Infections

Certain viruses have been associated with beta cell destruction. Diabetes occurs for instance in some patients with rubella.

Conclusion

Diabetes is a very common chronic disease with an increasing prevalence and incidence worldwide. There are four main types of diabetes, Type 1, Type 2, gestational diabetes and a final group composed of other types of diabetes. Type 2 is the most common form followed by Type 1, gestational diabetes and other forms most of the time secondary to other clinical situations.

When the symptoms are present the diagnosis is confirmed by elevated blood glucose levels regardless of the time of the day. When symptoms are lacking, measurements made after fasting or after a glucose load may be necessary to confirm or refute the diagnosis of diabetes. An entire investigation is needed if symptoms are questionable. In the case of a medical, obstetrical or family history of diabetes, a single elevated blood glucose measurement may or may not be decisive. An OGTT is indicated in this situation. The OGTT also helps for the diagnosis of prediabetes (IGT). The recent recognition of A1c as a diagnostic tool for prediabetes (IGT) as well as for the diagnosis of diabetes has considerably help to reduce the number of individuals who, otherwise, might have had an OGTT.

Finally, it is of a paramount importance to make the diagnosis of diabetes as early as possible in order to start the treatment without any delay. Early glycemic control is strongly associated with a considerable reduction of acute and long term complications of diabetes.

Further Reading

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Type 1 Diabetes☆

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Glossary

Diabetes Diabetes mellitus is a descriptive term for a heterogeneous group of disorders that are characterized by chronic hyperglycemia due to defects in insulin secretion, insulin action, or both, associated with the development of long-term vascular and neuropathic complications.

Insulin An anabolic hormone produced by the β-cells of pancreatic islets that stimulates glucose uptake by muscle and adipose tissue, glycolysis (glucose metabolism for energy), lipogenesis (triglyceride synthesis), protein synthesis, glycogen synthesis and suppresses gluconeogenesis, glycogenolysis (glycogen breakdown), and lipolysis (triglyceride breakdown). Insulin replacement is necessary for life in type 1 diabetes.

Prediabetes The asymptomatic prodromal period which precedes the clinical manifestation of type 1 diabetes. It may last a few months to several years, during which the autoimmune destruction of the insulin-producing β-cells in the pancreas can be identified.

Introduction

Diabetes mellitus refers to a group of metabolic diseases characterized by chronic hyperglycemia secondary to decreased insulin secretion, insulin action, or both. It is associated with the development of long-term microvascular, macrovascular and neuropathic complications. An expert committee convened by the American Diabetes Association on the diagnosis and classification of diabetes mellitus classified diabetes into four etiologic types. Type 1 diabetes results from either autoimmune mediated or idiopathic destruction of the insulin-producing β-cells of the pancreas, and is characterized by absolute insulin deficiency. Type 2 diabetes is characterized by a combination of insulin resistance and inadequate compensatory insulin secretory response. However, there are some patients that cannot be clearly categorized as type 1 or type 2 diabetes. "Other specific types of diabetes" include genetic defects in β-cell function or insulin action, diseases of the exocrine pancreas, endocrinopathies, drug or chemically induced insulin deficiency, uncommon forms of immune-mediated diabetes, infections and genetic syndromes associated with diabetes. The last form of diabetes is gestational diabetes mellitus.

Type 1 diabetes is most commonly caused by autoimmune-mediated destruction of the pancreatic islet cells. It accounts for 10% of all cases of diabetes mellitus. The incidence of type 1 diabetes tends to be seasonal, with more cases occurring during the autumn and winter months. There is also a worldwide variability in the incidence of the disease with the highest incidence reported in Finland (60–64 cases per 100,000 per year), and the lowest incidences in China, India, and Venezuela (0.1/100000 per year). In the United States, the incidence is estimated at $\sim$21/100,000 per year.

Type 1 diabetes (formerly known as insulin-dependent diabetes or juvenile diabetes) may present at any age. However, approximately 75% of known cases present between the ages of 3 and 18 years with two peaks—one between 5 and 7 years of age, and the other occurring at or near puberty. It is suspected that a similar number of cases actually occur in adults. These cases are often misdiagnosed as type 2 diabetes, and are referred to as latent autoimmune diabetes of adults (LADA). LADA accounts for almost 5%–15% of those diagnosed as type 2 patients. Type 1 diabetes is considered to be a multifactorial autoimmune disease involving both genetic and environmental factors interacting with the immune system, resulting in a T-lymphocyte-mediated destruction of the insulin-producing β-cells in the pancreatic islets. The autoimmune destructive process appears to be more rapid in infants and younger children, and may evolve over several years in older children and adults. There is also an increased recognition of the heterogeneity of type 1 diabetes. An earlier model of the natural history of type 1 diabetes suggested that $<10\%$ of β-cell mass remains at the onset of clinical disease, necessitating the need for lifelong exogenous insulin administration. However, recent studies have shown that almost 40%–50% β-cell function may be present at disease onset and this may be influenced by other factors such as age, body mass index, or physical activity. Thus, functional islets may persist for decades after diagnosis.

☆*Change History*: March 2018. Bimota Nambam William Winter, and Desmond Schatz updated the results of the TRIGR and TEDDY study under "environmental agents," pathogenesis of diabetic ketoacidosis, and sections of prevention studies where results of recent preventive studies have been discussed. There is also an update on current prevention and interventional studies in type 1 diabetes.

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Genetics of Type 1 Diabetes

The inheritance pattern of type 1 diabetes is complex and does not fit a Mendelian or single gene pattern. In the United States, the risk of developing type 1 diabetes in the general population is 1 in 300. The risk increases to 1 in 20 when individuals have a first degree relative with type 1 diabetes. Children of type 1 diabetes mothers have only a 2% risk of developing the disease; the risk increases to 7% when the father has the disease. The risk is 6%–10% in dizygotic twins while monozygotic twins have a disease concordance rate of approximately 65%, when followed over several decades. However, it never reaches 100%. This discordance suggests that environmental factors, in addition to genetic susceptibility, play a major role in the development of this disease. Additionally, 85% of new cases of type 1 diabetes have no family history.

Approximately 50 genetic loci have been associated with susceptibility to type 1 diabetes. The human leukocyte antigen (HLA) region, located in the major histocompatibility complex (MHC) on chromosome 6p21, accounts for up to 60% of genetic susceptibility. The MHC complex spans a 3.5-megabase region of chromosome 6 and is divided into three regions (Class I–III). HLA class II MHC genes have the strongest association with type 1 diabetes. The majority of patients with type 1 diabetes carry either the HLA-DR3 or HLA-DR4 class II MHC alleles, with approximately 40% being DR3/DR4 heterozygous. Heterozygosity for the DR3/DR4 genotype confers the highest risk for development of type 1 diabetes in Caucasians, followed by DR4 and DR3 homozygosity, respectively. Protective alleles for type 1 diabetes have also been described. One such allele is the class II MHC allele, DQB10602, and has been reported in $<1\%$ of patients with type 1 diabetes. It has been proposed that the degree of susceptibility or protection from the HLA genotype correlates with specific amino acid positions of the DQB1 gene. Position 57 is thought to play a crucial role in determining susceptibility. The amino acid residue at position 57 contributes to the shaping of the antigen-presenting pocket of the molecule, suggesting that the development of diabetes may depend on events surrounding antigen presentation. However, class II MHC genes do not explain all of the HLA association with type 1 diabetes. Class I genes also have been postulated to influence susceptibility and clinical aspects of the disease, such as age of onset and the rate of beta-cell destruction. Other non-HLA susceptibility genes have been mapped, including a region located upstream of the insulin gene that includes variable number of tandem repeats (VNTR). Fewer repeats confer a higher risk while higher numbers of repeats confer a lower risk. However, the overall contribution is low (odds ratio of 1.5). Another non-HLA gene associated with susceptibility to type 1 diabetes is cytotoxic T-lymphocyte associated-4 (*CTLA-4*). This gene encodes a protein that is important for the regulation of T-cell function and immune response to self. Again, the overall risk for development of type 1 diabetes is low. *PTPN22* and *CD25* genes have also been associated with type 1 diabetes risk. The majority of these genes that have been associated with type 1 diabetes risk are involved in immune responsiveness.

Environmental Factors

Due to variability in geographic prevalence rates, an increase in global incidence even in genetically low incidence regions, high disease discordance rates in twins, and rapid assimilation of local disease incidence rates when individuals migrate from low- to high-incidence countries, environmental factors are considered to play a major role in the development of type 1 diabetes.

Traditionally, infectious agents such as rubella have been thought to influence the initiation of the autoimmune process in type 1 diabetes. Approximately 30% of children with congenital rubella develop autoimmune diabetes. Viruses could trigger an autoimmune response either by directly damaging the pancreatic β-cells or by invoking molecular mimicry. At least 14 different viruses have been reported to be associated with the development of type 1 diabetes in human or animal models. Viruses suspected to induce autoimmunity to the β-cells via molecular mimicry include retrovirus, mumps, rubella, cytomegalovirus, and Epstein–Barr virus, whereas viruses that are suspected to induce autoimmunity via direct cytolytic damage to the β-cells include coxsackie-B and other enteroviruses. Enteroviruses as triggers for type 1 diabetes have received much attention. This association has been based on observations of increased enteroviral antibodies in individuals before disease onset, detection of enteroviral particles by immunohistochemistry studies, or viral RNA in autopsy studies of the pancreas in organ donors with type 1 diabetes.

Immunizations have also been implicated in the pathogenesis of type 1 diabetes, although there is little supporting data. An increase in the incidence of type 1 diabetes in Finland was correlated with the initiation of a mandatory immunization program against diphtheria–pertussis–tetanus. There were also reports of an association between vaccination for Hemophilus influenza and an increased incidence of type 1 diabetes. However, in 1998 an expert committee from the National Institutes of Health found no evidence to support these assertions.

Dietary influences on type 1 diabetes autoimmunity, including the protective effects of breast feeding and increased risk of the disease with early exposure to cow's milk have also been debated. Complex proteins in breast or cow's milk such as casein, bovine albumin, or bovine insulin have been implicated in type 1 diabetes development. However, findings from large scale studies such as from "The Environmental Determinants of Diabetes in the Young (TEDDY)" and "Trial to Reduce IDDM in the Genetically at Risk (TRIGR)" study groups do not support a role of complex proteins such as bovine insulin in influencing autoimmunity in those genetically at risk for type 1 diabetes. In fact, infants fed hydrolyzed formula were associated with a higher progression to autoimmunity in both studies.

The role of the gut microbiome and associated changes during early life, in influencing autoimmunity has also been studied over the past few years. Another recent study from the TEDDY group reported a decreased risk of progression to autoimmunity in

infants with the highest risk of type 1 diabetes (HLADR3/4) when fed probiotics in the first 27 days of life. Researchers have also looked into the association between antibiotic use in early life and increased risk for autoimmunity with conflicting results.

Autoimmunity

The detection of islet specific autoantibodies such as islet cell cytoplasmic autoantibodies (ICA), autoantibodies to glutamic acid decarboxylase (GADA), insulin autoantibodies (IAA), insulinoma-associated antigen-2 (IA-2) autoantibodies, and zinc transporter 8 (ZnT8) autoantibodies distinguishes type 1 diabetes from other types of diabetes. Combinations of these autoantibodies have also been found to determine risk for the subsequent development of clinical type 1 diabetes, both in unaffected relatives of individuals with type 1 diabetes and in the general population.

Autoreactive T-cells have been traditionally thought to be mainly responsible for β-cell destruction leading to clinical type 1 diabetes, with islet autoantibodies being formed during the process and having no pathological role in the disease process. This concept has been challenged by recent findings in animal studies, where islet autoantibodies were reported to enhance accumulation of islet-reactive CD4 + T-cells via FcγR mediated pathways. This highlights a possible role of autoantibodies in providing a positive feedback on the dysregulation of autoreactive helper T-cells. Also, short term benefits of rituximab, an anti-CD20 monoclonal antibody that targets B-cells, in preserving β-cell function in new-onset type 1 diabetes patients may also support this concept. Alternatively the benefit of rituximab may result from impairment in B-cell function as antigen-presenting cells to autoreactive T-cells.

Identification of At-Risk Subjects

Identification of individuals susceptible to the development of type 1 diabetes prior to clinical manifestation of the disease can be achieved by genetic screening of newborns with high-risk HLA alleles, and quantification of risk by further autoantibody, genetic, and metabolic testing. Antibody testing is based on the finding that $>90\%$ of patients with new-onset type 1 diabetes have islet autoantibodies. In high-risk individuals, antibodies often develop during childhood with IAA being the first to be detected at around 1 year of age followed by other antibodies. The presence of a single autoantibody imparts an approximate 10% 5-year risk of subsequently progressing to disease, 50%–60% for subjects with two autoantibodies, 70% for those with three autoantibodies, and almost 80% for those with four autoantibodies (without including ICA). The presence of metabolic derangements in addition to serologic markers increases the predictability of screening. Persistent loss of first-phase insulin response during an intravenous glucose tolerance test carries a 50%–70% 5-year risk in autoantibody-positive individuals. Changes in glucose tolerance measured during an oral glucose tolerance test generally occur later in the disease process, indicating significant β-cell destruction. Identification of at-risk individuals has led to a multitude of clinical trials in an attempt to prevent the development of this disease.

Diagnosis

The diagnosis of diabetes is made if any of the following features are present (1) the presence of the classical symptoms of diabetes (polyuria, polydipsia, and unexplained weight loss) with unequivocal hyperglycemia (random plasma glucose 200 mg/dL or 11 mmol/L); (2) 2-h plasma glucose $\geq$200 mg/dL or 11 mmol/L following a 75-g oral glucose challenge in adults (or 1.75 g/kg in children) on two occasions; (3) fasting plasma glucose $\geq$126 mg/dL (7 mmol/L) on two occasions; (4) HbA1c $\geq$ 6.5% on two occasions performed using a validated assay. If two different tests are both above the diagnostic threshold, this also confirms the diagnosis.

Between 20% and 40% of patients with new-onset type 1 diabetes have ketoacidosis at presentation. Unfortunately, the rate of diabetic ketoacidosis at disease onset has not decreased in recent years. Patients with type 1 diabetes have been traditionally viewed as being lean and frankly symptomatic (DKA). However, with increase in obesity rates in patients with type 1 diabetes and ketoacidosis in those with type 2 diabetes, BMI and the presence or absence of ketoacidosis are no longer reliable in distinguishing between the two especially in children and adolescents. When the diagnosis is in doubt, type 1 diabetes can be confirmed by the finding of islet-related autoantibodies. GADA and ICA are found in approximately 70%–80% of new-onset cases, ZnT8A in 63%, IA-2A in 68%, and IAA in 55%. Using all five autoantibodies, $>90\%$ of new-onset type 1 diabetes patients are positive for islet specific antibodies. IAA assays are not valid after 10 days of insulin therapy. Also, following the onset of T1DM, ZnT8A concentrations decline rapidly.

Management

In 1921, Frederick Banting and Charles Best discovered what was thought to be the cure for diabetes—insulin. Prior to the discovery of insulin, type 1 diabetes was a slow but sure death sentence, for which the only treatment was a diet low in

carbohydrates/sugar and high in fats and protein. Of course, insulin is not the cure for diabetes, but rather the vital tool for managing this disease and controlling complications.

The 1993 Diabetes Control and Complications Trial (DCCT) demonstrated a strong relationship between intensive management with good metabolic control and decreased rate of progression of complications. However, intensive therapy should be tailored according to the age of the patient, psycho-social factors including cognitive impairment (especially in the elderly population), presence or absence of frequent hypoglycemia etc. Improved glycemic control should be sought at all ages while avoiding severe hypoglycemia, weight gain, or emotional problems for the patient and the family.

Type 1 diabetes management, both in children and adults is complex, requiring a multidisciplinary medical team including a diabetologist, certified diabetes educator, nutritionist, psychologist, and where needed, a social worker and exercise physiologist. The disease should be approached as affecting the whole family unit and not just the individual with diabetes. The team works together to teach family members (especially for pediatric patients) and involve them in the decision-making process for the patient. Patient empowerment (if done appropriately) is crucial to successful management. A successful management program must be consistent and flexible. A balance must be sought between the needs of the medical establishment (insulin, nutrition, exercise, blood glucose testing, HbA1c tests, lipids, urine microalbumin testing, and complication surveillance) and the lifestyle demands and desires of the patient. Each patient needs individualized therapy based on age, schedule, social environment, and capabilities.

The following are the overall goals of diabetes management:

- Set realistic goals for each child and family: Consider the patient's age, family involvement and social situation, economic factors, and history of hypoglycemia.
- Near normalization of blood glucose levels and HbA1c measurements or, when not possible, improvement on subsequent follow-up.
- Prevention of diabetic ketoacidosis.
- Avoidance of severe hypoglycemia.
- Maintenance of normal quality of life.
- Achievement of normal growth, development, and maturation in children.
- Multidisciplinary support, including nutritional education and psychological support.
- Close surveillance and prevention of microvascular and macrovascular complications.

A variety of insulin regimens are available for use that must be individualized depending on patient compliance, the patient's level of social support, patient's preference based on their lifestyle including dietary and exercise regimens. Today, most patients are placed on basal bolus therapies employing subcutaneous injections of basal (long-acting) insulin with bolus insulin therapy before meals, or insulin pumps. The increased availability of continuous glucose monitoring as well as newer insulin pumps, recently introduced in the market that has the ability to combine data from CGM to automatically adjust basal insulin delivery will not only improve glycemic control, but also the overall quality of life of patients with type 1 diabetes.

Diabetic Ketoacidosis (DKA): Pathogenesis, Definition, and Management

DKA is defined biochemically by the presence of hyperglycemia (blood glucose $>$ 200 mg/dL or 11 mmol/L), venous pH $<$7.3 or bicarbonate $<$15 mmol/L, and ketonemia and ketonuria. Based on the degree of acidosis, DKA can be mild (venous pH $<$7.3 or bicarbonate $<$15 mmol/L), moderate (pH $<$7.2, bicarbonate $<$10 mmol/L), or severe (pH $<$7.1, bicarbonate $<$5 mmol/L).

DKA occurs when there is absolute or relative insulin deficiency combined with the presence of infection, stress, trauma, etc. In addition, there is an increase in circulating counter regulatory hormones such as glucagon, cortisol, growth hormone, and catecholamines. This leads to decreased peripheral glucose utilization, increased glycogenolysis and gluconeogenesis with subsequent hyperglycemia, glycosuria with osmotic diuresis, loss of electrolytes and free water, dehydration, and hyperosmolarity. Hence, patients in DKA are often dehydrated and the degree of dehydration is assumed to be around 10% of total body weight. Although, electrolyte levels in laboratory studies at the time of presentation of DKA are often normal, there is total body depletion of potassium, phosphate, and magnesium. There is also increased lipolysis which increases circulating free fatty acids (FFA) and flux of FFA toward the pathway of hepatic beta oxidation. This leads to increased ketogenesis to form ketone bodies (acetone, acetoacetate, and beta-hydroxybutyrate) which are used as alternative energy sources. Of the three, beta-hydroxybutyrate contributes almost 70% of total circulating ketone bodies during DKA. Ketone bodies are acidic and deplete the alkali reserve in the body. Once the accumulated ketone bodies overwhelm the homeostatic acid–base buffering system, there is ketonuria, and patients develop metabolic acidosis (DKA) which is a life threatening condition. Increased circulating ketone bodies also triggers nausea, abdominal pain and vomiting; this can compound the already existing fluid and electrolyte depleted state. Patients are also tachypneic (Kussmaul respiration) secondary to respiratory compensation of the metabolic acidosis. DKA is a life threatening condition requiring emergency treatment with intravenous fluids and insulin; in severe cases where treatment is delayed, patients can progress to coma and death.

DKA is often seen in patients with new-onset diabetes or when patients with established type 1 diabetes fail to take insulin. New-onset diabetes with DKA is more common in children younger than 2 years of age due to delayed diagnosis and treatment. In

patients with known type 1 diabetes, some of the common risk factors for DKA include noncompliance with insulin regimen, poor family structure and support, lower socio-economic status and limited access to health care.

Many institutions and hospitals have individualized DKA management protocols. Most patients are assumed to have dehydration of 10% and should initially receive a 0.9% NaCl solution intravenous bolus of 10–20 mL/kg given over 1–2 h. The fluid deficit plus maintenance fluids should be administered over 48 h. Normal saline should be used as the initial fluid for the first 4–6 h and if required, switch to a fluid with a tonicity equal to or $>0.45\%$ saline. One of the concerns of using hypotonic fluids in pediatric DKA management is the development of cerebral edema. Hence, many pediatric providers use isotonic fluids for all phases of DKA management. However, this traditional view is being challenged; the ongoing Pediatric Emergency Care Applied Research Network (PECARN) DKA FLUID study is investigating the neurological outcomes of using 0.45% NaCl versus 0.9% NaCl at two different rates in pediatric DKA.

Intravenous insulin should be commenced as soon as fluid replacement has been started at a dose of 0.05–0.1 units/kg per hour, with lower doses used in very young children or in those with marked sensitivity to insulin. Initial intravenous insulin boluses are not recommended since they may increase the risk of cerebral edema (in children) and hypokalemia. Intravenous bicarbonate infusion to correct DKA is also not recommended, as many studies have not found faster resolution of DKA with this method, and there is an increased risk of cerebral edema especially in children. It is only in life threatening cardiovascular instability secondary to severe acidosis that IV bicarbonate should be used cautiously.

Hourly bedside blood glucose checks with 2–4 hourly monitoring of pH, bicarbonate, and electrolytes (sodium, potassium, calcium, and phosphorus) should also be monitored to assess for improvement of acidosis and correction of electrolyte imbalances. Intravenous insulin therapy drives potassium and phosphate into cells, and this can precipitate hypokalemia and hypophosphatemia. Hence, replacement fluids should contain potassium (after patient has had a urine output) and phosphate. Once acidosis has resolved and patient indicates ability to tolerate oral intake, he/she should be transitioned to subcutaneous insulin therapy.

Hyperglycemic Hyperosmolar State (HHS)

Hyperglycemia to >600 mg/dL with hyperosmolarity (>330 mOSm/kg) and minimal ketosis should alert the physician to hyperglycemic hyperosmolar state (HHS). HHS is more common in patients with type 2 diabetes but can also be seen in type 1 diabetes. The distinction between DKA and HHS is important as patients with HHS often are profoundly dehydrated (almost 15% dehydration should be assumed) and need aggressive fluid replacement. The initial normal saline fluid bolus should be ~ 20 mL/kg and if required, additional boluses should be given followed by administration of maintenance and replacement fluid (0.45%–0.75% NaCl) over 48 h. Hyperglycemia improves rapidly with aggressive initial fluid hydration, and low dose insulin at 0.025–0.05 units/kg/h should be initiated when there is no further reduction in blood glucose levels (<50–75 mg/dL/h or 3–4 mmol/L/h) with fluid therapy. Insulin doses must be titrated based on improvement of blood glucose and ketosis. Due to severe hyperglycemia and hypertonicity at presentation, initial intravascular blood volume is often maintained. Once there is reduction in blood glucose and osmolality due to hydration, insulin action, and increased glycosuria secondary to improvement in renal perfusion, there is movement of water from intravascular sites, and this can precipitate a sudden decrease in blood volume leading to hypotensive shock and cardiovascular collapse. If there is nonimprovement or worsening of hemodynamic status, normal saline should be used for fluid replacement even after the first 4–6 h of presentation. The risk of mortality in HHS is much higher compared to DKA.

Search for a Cure and Prevention of Type 1 Diabetes

Type 1 diabetes places a considerable burden on the individual and society with approximately 15 billion dollars spent annually. The ability to identify individuals at high risk of developing the disease, newer understanding of the disease process, together with the tremendous and increasing burden of the disease, has led to the development of multiple studies aimed at the prevention of type 1 diabetes or halting disease progression. Some of the studies are discussed briefly.

A recently completed study by TrialNet investigated the effect of oral insulin (7.5 mg daily) in preventing clinical type 1 diabetes in autoantibody positive, high-risk individuals. This intervention is based on the concept of "inverse vaccination" where repeated exposure of the immune system to exogenous autoantigens leads to long-term tolerance to the specific antigen. The intervention failed to prevent development of type 1 diabetes. As immune responses to antigens vary according to dose, frequency of administration, route of administration, as well as background genetics, it is unclear if the failure was related to these variables. However, a subset of patients with lower insulin secretion had delay in diabetes development by 31 months compared to controls. TrialNet is further investigating immune changes associated with oral insulin intake in another study but with a higher dose (67.5 mg daily vs. 500 mg weekly for 6 months). Of note, the Pre-POINT study recently demonstrated that 67.5 mg oral insulin daily administered for 8 months in children (2–7 years) with high risk HLA-genes, but negative for islet autoimmunity induced a tolerogenic response. This is being further investigated in younger children (6 months–2 years). Another recently completed study (DiAPREV-IT) using GAD-alum vaccine also failed to halt progression of autoimmune process in children with multiple islet autoantibodies. The same study group is conducting another study to evaluate if GAD-alum vaccine in combination with high

dose vitamin D3 (DiAPREV-IT2) can stop disease progression in multiple autoantibody positive children. There are also ongoing/recently completed studies using antigen based therapies such as the MultiPepT1De and use of proinsulin peptide to halt disease progression in new-onset type 1 diabetes.

TrialNet is also conducting two separate studies to evaluate if teplizumab and abatacept can prevent progression of stage 2 of type 1 diabetes (abnormal blood glucose but asymptomatic) to clinical type 1 diabetes. Teplizumab is a humanized anti-CD3 monoclonal antibody which decreases autoreactive T-cells involved in the immune destruction of pancreatic beta-cell while abatacept (CTLA4-Ig), an immunoglobulin that binds to the Cytotoxic T-Lymphocyte Antigen 4 receptor, interferes with the early phases of T-lymphocyte activation, proliferation, and survival which are required for the progression of the disease process of type 1 diabetes.

There are also several studies in newly diagnosed type 1 diabetes subjects which have evaluated the use of immunomodulators and immunosuppressive agents to prolong endogenous insulin secretion after diagnosis. Advances in pancreatic and islet cell transplantation have led to enhanced patient and graft survival. Unfortunately, there are not enough donors to support the vast number of patients with type 1 diabetes. In addition, patients require lifelong immunosuppression to prevent rejection (alloimmunity) and possibly prevent recurrence (autoimmunity). Other promising therapies include stem cell therapy based approaches which are currently being studied in many centers.

Conclusion

The incidence of type 1 diabetes continues to increase placing a heavy economic burden both on individuals and the society worldwide. Despite therapeutic advances and an increased understanding of the disease process, much remains to be uncovered and the quest for prevention and cure still remains a priority.

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Type 2 Diabetes☆

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Glossary

Beta cell A cell present in the pancreas islets that synthesizes and secretes insulin.

Glucagon like peptide-1 An incretin hormone released by the intestine after a meal which augments insulin secretion from the beta cell.

Glycated hemoglobin Also called Hemoglobin A1C. A per cent of glycation of hemoglobin as a surrogate measure of average blood glucose.

Insulin A hormone that is secreted from the pancreas in response to glucose levels. Insulin binds its receptor on the cell surface of tissues such as the muscle to promote glucose uptake. Insulin also decreases hepatic glucose production and promotes storage of fatty acids as triglycerides in the adipose tissue.

Macrovascular disease Pertaining to diabetes induced damage to the cardiovascular system that predisposes patients to heart attacks and strokes.

Metabolic syndrome A cluster of physical characteristics that predisposes to the development of type 2 diabetes and cardiovascular disease. Metabolic syndrome includes dyslipidemia, central obesity and glucose intolerance.

Microvascular disease Pertaining to diabetes induced damage to the small blood vessels including capillaries which leads to retinopathy, nephropathy, and neuropathy.

Type 2 diabetes (T2D) results when the pancreatic beta cell is unable to meet increased insulin demands caused by insulin resistance which is usually a result of obesity. With decreased glucose utilization and inappropriate glucose production, hyperglycemia ensues which causes both macrovascular and microvascular complications.

Diagnosis

The diagnostic criteria for T2D originally were based on the glycemic threshold at which retinal complications are manifest. Current criteria include elevated fasting plasma glucose (FPG) ($\geq$126 mg/dL or 7.0 mmoL/L) and/or glucose intolerance with a 2-h plasma glucose level $\geq$200 mg/dL (11.1 mmoL/L) after a standard oral dose of 75 g of glucose (an oral glucose tolerance test or OGTT) (American Diabetes Association, 2018a). A random plasma glucose of $\geq$200 mg/dL (11.1 mmoL/L) also is diagnostic in a patient with symptoms such as thirst, excess urination and/or weight loss.

The National Glycohemoglobin Standardization Program (NGSP) was implemented to align laboratory assays for percent glycated hemoglobin (hemoglobin A1C) with those used in the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS). In 2009, an international expert committee with representatives from the American Diabetes Association (ADA), the International Diabetes Federation (IDF) and the European Association for the Study of Diabetes (EASD) jointly advocated the incorporation of the A1C threshold of $\geq$6.5% into the diagnostic criteria for T2D (International Expert Committee, 2009). The ADA added A1C criterion in 2010 and the EASD added it in 2013 (the first revision of the guidelines since 2007). However, A1C is inaccurate in certain disease states and it must be used judiciously as a diagnostic tool (Genuth *et al.*, 2017) (**Table 1**). A1C is affected by changes in erythrocyte production or destruction. Levels of glycation also can be increased or decreased in patients with hemoglobinopathies, alcoholism and chronic kidney insufficiency. A1C assay interference occurs with carbamylated hemoglobin from chronic kidney disease, hyperbilirubinemia, alcoholism, and hypertriglyceridemia.

"Pre-diabetes" is a term applied to patients with glucose levels intermediate between normoglycemia and T2D due to the presence of impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) by OGTT (American Diabetes Association, 2018a). Many organizations agree that the criterion for IFG is 100–125 mg/dL (5.6–6.9 mmol/L). IGT is defined as a plasma glucose of 140–199 mg/dL (7.8–11.0 mmol/L) during an OGTT, or an A1C of $\geq$5.7% but $<$6.5%. The American Association of Clinical Endocrinologists (AACE) pre-diabetes definition also includes patients who meet metabolic syndrome (MetS) criteria with $\geq$3 of 5 of elevated blood pressure, elevated low density lipoprotein-C (LDL-C), elevated triglycerides, increased waist circumference, and low high density lipoprotein-C (HDL) because of the fivefold risk of developing T2D compared to age and sex matched controls (Samson and Garber, 2014). The rate of progression from the pre-diabetic state to T2D varies widely among different ethnic populations but a crude estimate from published studies is in the range of 4%–9% per year, with body mass index as a strong predictor of progression.

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Table 1 Factors affecting the accuracy of Hemoglobin A1C for glycemic status

Increased A1C	
Decreased erythropoiesis	Iron or B12 deficiency
Decreased erythrocyte destruction	Splenectomy
Increased glycation	Alcoholism
	Chronic kidney disease (glucose dialysate)
Assay interference	Chronic kidney disease (carbamylation)
	Hemoglobinopathies
	Hyperbilirubinemia
	Alcoholism
	High dose aspirin
	Chronic opiate use
Decreased A1C	
Increased erythropoiesis	Treatment of iron or B12 deficiency
	Erythropoietin
	Reticulocytosis
Increased erythrocyte destruction	Hemoglobinopathies
	Splenomegaly
	Hemolytic anemias (autoimmune, drugs, mechanical)
	Inherited disorders of the erythrocyte membrane
	Inherited disorders of erythrocyte glycolysis
	Increased disorders that increase erythrocyte oxidative stress (Glucose-6-phosphate dehydrogenase deficiency, glutathione synthetase deficiency)
	Paroxysmal nocturnal hemoglobinuria
Decreased glycation	Vitamin E
	Vitamin C
	Aspirin
	Hemoglobinopathies
Assay interference	Hypertriglyceridemia

Table 2 Diabetes prevalence among ethnic groups in the United States

	Men (%)	*Women (%)*
American Indian/Alaskan Natives	14.9	15.3
Asian	9.0	7.3
Black, non-Hispanic	12.2	13.2
Hispanic	12.6	11.7
White, non-Hispanic	8.1	6.8

Data from 2013 to 2015 National Health Interview Survey and 2015 Indian Health Services National Data Warehouse.

Epidemiology

The prevalence of T2D is increasing at an alarming rate. The worldwide prevalence of adult (age 20–79 years) diabetes, of which T2D comprises >90% of cases, is estimated at 425 million in 2017 with a projection to 629 million by 2045 (IDF, 2017). For Europe and North America the increase in prevalence is predicted to be 16% and 35%, respectively. Even more extreme are 2045 projections for increased diabetes prevalence in Central and South America (+62%), the Middle East and North Africa (+72%), Africa (+156%), and Southeast Asia (+84%). Estimates of prevalence also are complicated by the knowledge that T2D is markedly underdiagnosed, with one-third to one-half of people unaware they have T2D. The prevalence of T2D varies widely among peoples of different ethnicities (**Table 2**), underscoring that the risk for T2D is a combination of epigenetic and genetic factors combined with contributions from lifestyle and environment.

Pathogenesis

The increased prevalence of T2D has followed the trajectory of the prevalence of obesity. Positive energy balance, from excess calories and a sedentary lifestyle, is stored as fat. Visceral fat, in comparison to subcutaneous fat, is associated with higher insulin resistance. If the beta cell cannot compensate, there is relative hypoinsulinemia allowing for lipolysis from visceral fat depots with

Table 3 Available pharmacologic therapies for type 2 diabetes

Drug class	*Available formulations (worldwide)*	*Mechanism*	*Benefits*	*Adverse effects and cautions*
Biguanides	Metformin	Inhibition of hepatic glucose production and promotion of skeletal muscle glucose uptake	Weight loss or weight neutral Decreased progression from prediabetes to diabetes Low cost	Gastrointestinal upset (diarrhea) Lactic acidosis (higher risk with chronic kidney failure) B12 or folate deficiency
Sulfonylureas and meglitinides	Glipizide Gliclazide Glyburide Glimepiride Nateglinide Repaglinide	Depolarization of the beta cell membrane to increase insulin secretion	Low cost	Hypoglycemia Weight gain Acceleration of loss of beta cell function
Thiazolidinediones	Pioglitazone Rosiglitazone	Activation of nuclear receptor peroxisome proliferator activated receptor gamma (PPAR) to increase adiponectin and improve insulin resistance	Decreased progression from prediabetes to diabetes Redistribution of visceral adipose tissue to subcutaneous	Weight gain (fluid and adipose) Fluid retention Chronic heart failure exacerbation Osteoporosis Bladder cancer Suppressed hematopoiesis
Alpha-glucosidase inhibitors	Acarbose Miglitol Voglibose	Inhibition of hydrolysis of starches and carbohydrates in the gut to decrease absorption	Weight loss (decreased absorption of calories) Decreased progression from prediabetes to diabetes (acarbose, voglibose)	Flatulence Abdominal pain Diarrhea
Glucagon like peptide-1 (GLP-1) receptor agonists	Exenatide (twice daily and weekly) Liraglutide (daily) Lixisenatide (daily) Dulaglutide (weekly) Albiglutide (weekly) Semaglutide (weekly)	Activation of the GLP-1 receptor to augment insulin secretion and inhibit glucagon secretion	Weight loss (decreased meal size) Decreased cardiovascular events (liraglutide, semaglutide)	Nausea Subcutaneous injection Contraindicated for patients with a history of pancreatitis, medullary thyroid carcinoma, or multiple endocrine neoplasia type 2 Possible retinopathy progression (semaglutide)
Dipeptidyl peptidase 4 (DPP4) inhibitors	Sitagliptin Vildagliptin Saxagliptin Linagliptin Gemigliptin Anagliptin Teneligliptin Alogliptin Trelagliptin Omarigliptin Evogliptin Gosogliptin	Inhibition of endogenous GLP-1 inactivation to augment insulin secretion and inhibit glucagon secretion	Weight neutral No cardiovascular harm Oral formulation compared to GLP-1 receptor agonists	Nausea Contraindicated for patients with a history of pancreatitis, medullary thyroid carcinoma, or multiple endocrine neoplasia type 2
Amylin analogue	Pramlintide	Slowing of gastric emptying and inhibition of glucagon secretion	Weight loss (decreased meal size)	Nausea Subcutaneous injection
Sodium-glucose cotransporter 2 (SGLT-2) inhibitors	Canagliflozin Dapagliflozin Empagliflozin Ipragliflozin	Inhibition of renal glucose resorption	Weight loss (loss of calories in the urine) Decreased cardiovascular events (empagliflozin, canagliflozin)	Urinary tract infections Genital yeast infections Normoglycemic diabetic ketoacidosis
Dopamine agonists	Bromocriptine quick release	Increased central nervous system sympathetic and dopaminergic tone to decreased hepatic glucose output, lipolysis and insulin resistance	Weight loss	Nausea Asthenia Dizziness Postural hypotension Constipation Rhinitis Exacerbation of psychotic disorders Inhibition of lactation Avoid use with other ergots Effects on CYP3A4 metabolism

(*Continued*)

Table 3 Continued

Drug class	*Available formulations (worldwide)*	*Mechanism*	*Benefits*	*Adverse effects and cautions*
Bile acid sequestrants	Colesevelam	Activation of nuclear receptor activity in the liver and intestines (Farnesoid X receptor, liver X receptor) to improve glucose metabolism and increase GLP-1	Reduction of low density lipoprotein-C levels	Increased triglycerides Flatulence Constipation Dyspepsia Vitamin K deficiency reduced bioavailability of other drugs (oral contraceptives, levothyroxine, phenytoin)

Table 4 Available insulins and insulin analogues with estimated pharmacokinetics

	Insulin products	*Onset*	*Peak*	*Duration of action*
Bolus (Prandial)				
Rapid acting	Lispro[a] Aspart[a] Glulisine[a]	5-15 min	0.5–1.5 h	3–5 h
Short-acting	Regular	0.5–1 h	2–4 h	6–8 h
Basal				
Intermediate acting	Neutral protamine Hagedorn (NPH) Neutral protamine Lispro[a,b] (NPL) Regular U-500	2–4 h 0.5 h	7–8 h 1–3 h	10–12 h 8–24 h
Long acting	Glargine[a] Detemir[a] (>0.8 units/kg)	1–2 h 1–2 h	No peak 6–8 h	24 h 18–24 h[c]
Very long acting	Degludec[a]	2 h	No peak	>40 h

[a]Insulin analogues.
[b]Only as pre-mix with Lispro.
[c]Dose dependent >0.4 units/kg to increase duration.

excess free fatty acid (FFA) production. The increased FFA flux to peripheral tissues, including the liver and skeletal muscle, inhibits insulin signaling. With hepatic insulin resistance and an abundance of FFA substrate, gluconeogenesis is increased, further contributing to hyperglycemia. Skeletal muscle insulin resistance results in decreased glucose disposal peripherally. Over time, the pancreatic beta cell can no longer compensate for the increased insulin demands, and T2D is the unfortunate consequence. The role of beta cell failure in the development of T2D is highlighted by the discovery that many of the genes found to influence T2D risk are linked to beta cell function or maintenance, rather than obesity or insulin resistance.

Treatment

An important pillar of T2D prevention and treatment is lifestyle change, with increased exercise, decreased caloric intake, nutritional modifications and weight loss. The Diabetes Prevention Program (DPP) showed that progression to T2D for patients with IGT was reduced 58% with intensive lifestyle modification during the 2.8 year follow-up (Knowler *et al.*, 2002). Weight loss of 5% can improve glycemia and reduce the need for anti-diabetic medications in patients with T2D. A 500–700 kcal deficit per day is recommended to achieve weight loss (American Diabetes Association, 2018b). Patients with T2D should be counseled on reduction in refined carbohydrates and limitation of dietary fat to 20%–30% of calories with an emphasis on monounsaturated fats. Exercise recommendations from the ADA are 150 min of moderate to vigorous intensity exercise spread over the week, similar to the DPP design. Motivation for continued lifestyle changes can wane and effective programs, such as with the DPP, incorporate "coaches" and continued counseling and encouragement to achieve success.

There is a plethora of non-insulin therapies approved for the treatment of T2D with diverse mechanisms of action (**Table 3**). Often, a combination of medications is required to attain glycemic control initially or with time, as a patient progresses toward complete beta cell failure. In considering a combination, the clinician should choose therapies that will target different defects present in T2D for an additive or synergistic effect on glycemic control. Patient specific factors also need to be weighed—age, weight, hypoglycemia risk, cardiovascular disease, etc.—to derive the maximum benefit and minimize the risks of each medication or combination.

When glycemic control cannot be attained with non-insulin therapies, insulin can be initiated (**Table 4**), as a basal injection of a long-acting or intermediate-acting insulin. Further addition of prandial (bolus) insulin for one to three meals per day may be necessary. The commencement of insulin therapy does not necessitate the discontinuation of all other T2D therapies as there can be continued benefit. For example, the combination of metformin or a GLP-1 receptor agonist with insulin can be insulin sparing.

Complications

Complications are the major cause of morbidity and mortality in T2D and are categorized as microvascular (kidney disease, neuropathy, retinopathy) and macrovascular (cardiovascular disease or CVD). Data from the DCCT (DCCT research Group, 1993), the long-term follow-up study the Epidemiology of Diabetes Interventions and Complications (Nathan *et al.*, 2005), and the UKPDS (UKPDS Study Group, 1998a) provided the primary evidence that glycemic control is the key factor to reduce the progression of diabetic microvascular complications. This has been confirmed by data from more recent trials, Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Action in Diabetes and Vascular Disease (ADVANCE) (Cefalu, 2008). Although A1C is a predictor of CVD risk, the impact of glycemic control on cardiovascular disease prevention for T2D is less clear, although there was a trend to decreased non-fatal and fatal myocardial infarction, but not stroke, in the UKPDS intensive control arm (Kooy *et al.*, 2009; United Kingdom Prospective Diabetes Study (UKPDS) Group, 1998b). Data from the ACCORD trial revealed that intensive glycemic control has to used judiciously with regard to CVD outcomes if implemented in patients with history of hypoglycemia, known CVD, advanced age and a long duration of T2D (ACCORD Study Group, 2008).

The pathogenesis of diabetes complications is complex and not fully elucidated, but several pathologic effects of hyperglycemia are proposed (Forbes and Cooper, 2013). Posttranslational modifications to cell proteins include glycosylation, peroxidation, phosphorylation, nitrosylation and prenylation, with ensuing adverse effects on cellular function and integrity. With excess glucose and fatty acid substrates, there are alterations in cellular respiration leading to mitochondrial dysfunction and respiratory chain uncoupling and, ultimately, a decrease in ATP production for the cell. The presence of excess glucose also activates the pentose phosphate (PP) pathway resulting increased production of UDP *N*-acetylglucosamine which leads to protein glycosylation and alterations in signal transduction and gene expression which go on to promote cell damage. The NAD(P)H produced from the PP pathway further facilitates the conversion of glucose to sorbitol by aldose reductase, increasing oxidative and osmotic stress.

Diabetic retinopathy is the leading cause of blindness in adults (Tan and Wong, 2015). In the early, non-proliferative phase, blood vessel wall pericyte death leads to increased permeability and degeneration. Capillary microaneurysms form and lipid and proteins leak from the vessels to form hard exudates. Cotton-wool spots are evidence of infarcts in the retinal nerve fiber layer. With local hypoxia, angiogenic factors promote neovascularization of the retina during the proliferative stage of retinopathy. The development of fibrovascular changes predispose the patient to retinal detachment and fluid accumulation leads to macular edema. Beyond glycemic control for prevention, management of blood pressure and dyslipidemia are key. In the late stages, retinal photocoagulation treatment of proliferative retinopathy is required and intravitreal injections of anti-Vascular Endothelial Growth Factor (anti-VEGF) are used to treat vision threatening macular edema.

Diabetic nephropathy is the leading cause of kidney failure in developed nations (Forbes and Cooper, 2013). Early in the disease, the kidney undergoes hypertrophy and hyperfiltration is observed. Pathologic changes occur in the glomeruli, where there is expansion of the mesangium and thickening of the basement membrane. There is loss of podocytes which allows protein leakage into the urine. Extracellular matrix is deposited in the tubules leading to fibrosis and loss of glomerular function (glomerulosclerosis). Clinically, nephropathy is diagnosed by the presence of microalbuminuria which can progress to overt proteinuria with nephrotic syndrome. Blood pressure and lipid control along with angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARB) can slow the progression of nephropathy.

Peripheral diabetic neuropathy can affect both somatic and autonomic nerves (Marathe *et al.*, 2015). The microvascular contribution to neuropathy is thought to involve local hypoxia due to thickening of the capillary basement membranes (Forbes and Harcourt, 2015). However, there also may be effects on neuron health independent of vascular changes, with direct toxicity of hyperglycemia to glial cells causing nerve fiber degeneration (Forbes and Cooper, 2013). Symptoms of sensory neuropathy include paresthesias, allodynia and loss of proprioception, temperature discrimination and vibration sense. Decreased sensation to touch progresses from the extremities in a "glove and stocking" pattern. Over time, injuries to the skin and joints can abound leading to non-healing skin ulcerations and joint deformities, with increased risk of amputation. Effects on autonomic nerves can cause gastroparesis and other gastrointestinal difficulties, erectile dysfunction, orthostasis, and bladder incontinence.

T2D is a cardiovascular risk equivalent and half of patients with diabetes will die of a CVD event (myocardial infarction or stroke). Premature atherosclerosis develops with immune cell infiltration of vessel walls, proliferation of smooth muscle cells, and accumulation of low-density lipoproteins in macrophages leading to plaque formation (Forbes and Cooper, 2013). Although hyperglycemia contributes to increased inflammation and oxidative stress, atherogenesis is further driven by concomitant dyslipidemia and hypertension as co-components of MetS (Samson and Garber, 2014). Therefore, primary and secondary prevention must include management of LDL-C (HMG-CoA reductase inhibitors or statins), triglycerides (niacin, fibric acid derivatives, omega-3-fatty acids), blood pressure, and aspirin therapy as indicated by age and risk (American Diabetes Association, 2018c). Evidence that available T2D medical therapies have benefit for CVD, beyond glycemic control, has been sparse. However, from UKPDS data, subjects in the intensive arm treated with metformin showed a 36% reduction in all-cause mortality and stroke and a reduction in risk for myocardial infarction (UKPDS, 1998b). The LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of

Cardiovascular Outcome Results) trial enrolled patients with T2D who were high risk for CVD events (>50 years old with known CVD, chronic kidney disease, chronic heart failure or >60 years old with CVD risk factors). After nearly 4 years of follow-up, liraglutide treated subjects had a 20% reduction in major adverse CVD events compared to usual care without liraglutide (Marso *et al.*, 2016b). The long acting GLP-1 receptor agonist, semaglutide, also may have benefit in high risk patients. From the SUSTAIN-6 trial (semaglutide and cardiovascular outcomes in patients with type 2 diabetes), there was a 26% reduction in the primary composite endpoint which was comprised of death from cardiovascular causes, nonfatal MI, and nonfatal stroke (Marso *et al.*, 2016a).

Conclusion

T2D prevalence is increasing throughout the world. It is a significant cause of morbidity and mortality, and pharmacologic treatment and management of complications consumes a large portion of health care costs. Treatment of T2D requires lifestyle and pharmacologic intervention to control glycemia and reduce microvascular and macrovascular complications.

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Diabetes Mellitus and Pregnancy

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Introduction

Significance

Normal pregnancy is characterized by multiple metabolic adaptations which enable the fetus to have an ample supply of fuel and nutrients throughout development in utero. Pregnancy is a diabetogenic state; the progressive insulin resistance which develops during pregnancy as a result of placental elaboration of diabetogenic hormones serves to decrease glucose entry into maternal cells and divert fuel to the developing fetus. Gestational diabetes mellitus (GDM) develops during pregnancy in some women whose pancreatic beta-cell function is insufficient to overcome the insulin resistance that occurs during pregnancy. In women who have preexisting diabetes mellitus (DM) who become pregnant, the physiologic metabolic alterations of pregnancy create unique therapeutic challenges, including large changes in insulin requirements over the course of the pregnancy.

The presence of hyperglycemia during pregnancy has wide-ranging health implications for both fetus and mother, beginning at conception and extending many years and decades beyond the pregnancy. Several adverse perinatal outcomes have been associated with diabetes in pregnancy, and treatment reduces risk of many complications for both women and their infants. Hyperglycemia increases risk of excessive fetal growth and neonatal hypoglycemia, and excessive fetal growth in turn can lead to operative delivery, birth trauma, and rarely perinatal mortality. For women with preexisting diabetes that antedates pregnancy, the risk of congenital malformations also increases progressively with increasing severity of periconceptional hyperglycemia, as a result of fetal exposure to maternal hyperglycemia in the early weeks of pregnancy. Furthermore, the hyperglycemia and adverse pregnancy outcome (HAPO) Study has demonstrated that maternal glycemia, even at levels below the range diagnostic of diabetes, is strongly associated with fetal growth, fetal hyperinsulinemia, and fetal adiposity (Metzger *et al.*, 2008; Hapo Study Cooperative Research Group, 2009). Good glycemic control during pregnancy is associated with decreased maternal, fetal, and neonatal complications among women with preexisting DM (Kitzmiller *et al.*, 2008), and two large randomized controlled trials have shown that treatment of GDM decreases birth weight and the frequency of hypertensive disorders of pregnancy (Crowther *et al.*, 2005; Landon *et al.*, 2009). Pregnancy is an opportune time for healthcare providers to counsel, educate and heighten awareness regarding diabetes management strategies.

In addition to the well-known perinatal consequences of hyperglycemia during pregnancy, the altered intrauterine environment carries lifelong health implications for mother and infant which extend well beyond pregnancy. Women with GDM are at significantly increased risk for the future development of type 2 diabetes later in life, even when maternal glucose metabolism returns to normal immediately following pregnancy. In this way, pregnancy may be viewed as a physiologic "stress test" that unmasks a woman's underlying predisposition to chronic disease, and can serve as a valuable window to a woman's future health at a time in her life when targeted preventive strategies may be most effective. Alterations in maternal glucose metabolism in utero can also influence the offspring's long-term risk for obesity and type 2 diabetes later in his or her life.

Categories of Hyperglycemia During Pregnancy

Any type of preexisting DM in a woman of reproductive age may complicate pregnancy. The most common types of preexisting DM are type 1 DM and type 2 DM. Type 1 DM is caused by destruction of the pancreatic insulin-producing beta cells (usually autoimmune in nature), resulting in absolute insulin deficiency. The pathogenesis of type 2 DM is complex; the hallmark pathophysiologic defects are insulin resistance and relative insulin deficiency. GDM is classically defined as "carbohydrate intolerance of varying degrees of severity, with onset or first recognition during pregnancy" (Metzger and Coustan, 1998). However, women with severe hyperglycemia characteristic of previously undiagnosed overt type 2 or (less commonly) type 1 DM who are first diagnosed during pregnancy would be included in this classic definition of GDM. Newer definitions of GDM (International Association of Diabetes Pregnancy Study Groups Consensus Panel *et al.*, 2010; American Diabetes Association, 2017), therefore, distinguish previously-unrecognized overt DM from GDM by excluding those women who meet standard diagnostic criteria for DM. Rarer forms of preexisting DM include glucocorticoid-induced or other drug-induced DM (such as occurs in association with immunosuppressant medications used after organ transplantation or with medications used to treat HIV), DM associated with pancreatic insufficiency (e.g., pancreatitis, cystic fibrosis), and monogenic DM (also known as maturity-onset diabetes of the young; MODY).

GDM is currently defined as diabetes that is first diagnosed during pregnancy that was not clearly overt diabetes prior to gestation (American Diabetes Association, 2017), and refers to the hyperglycemia that transiently results from the pregnancy-induced increase in insulin resistance during the second half of pregnancy.

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Prevalence

In parallel with the increasing prevalence of obesity and type 2 diabetes in the population as a whole (Shaw *et al.*, 2010; Ogden *et al.*, 2015), the prevalence of pregnancies affected by diabetes has also increased over time and is expected to continue to increase (Hartling *et al.*, 2013). GDM accounts for the vast majority (approximately 85%) of cases, with preexisting DM accounting for the remainder and affecting approximately 1% of all pregnancies (Albrecht *et al.*, 2010). Depending on the population studied and the diagnostic criteria employed, estimates of the prevalence of GDM vary, ranging from 1% to 25% (Moyer and Force, 2014). The number of pregnancies complicated by DM has increased steadily over time; between 1994 and 2004, the relative increase was greatest for pregnancies complicated by T2DM, though pregnancies complicated by all types of DM increased (Albrecht *et al.*, 2010). Multiple factors likely contribute to the increased prevalence observed, including changes in screening and diagnostic criteria, as well as increases in obesity and other diabetes risk factors (such as inactivity and poor diet) that predispose women to both T2DM and GDM.

Metabolic Alterations During Pregnancy

During normal pregnancy, several metabolic adaptations occur to enable a continuous supply of nutrients from the mother to the growing fetus. Glucose is the primary source of energy for the fetus, and diffuses down a concentration gradient from mother to fetus. In addition, amino acids are transported across the placenta from mother to fetus to enable essential protein accretion. Early pregnancy is an anabolic state—maternal fat accumulation results from increased synthesis and deposition of triglycerides in adipose tissues and inhibited lipolysis. During early pregnancy, insulin sensitivity can be increased. Later in pregnancy, the anabolic state gives way to a catabolic state characterized by increased lipolysis. The major metabolic adaptations during later pregnancy have been classically described as a state of "accelerated starvation and facilitated anabolism" (Freinkel, 1980). "Accelerated starvation" refers to maternal adaptive mechanisms during the fasting state that enable continued shunting of fuels such as glucose and amino acids to the fetus even during times of fasting. In addition to the more rapid decrease in plasma glucose which occurs during the fasting among pregnant women, maternal metabolism is more readily diverted toward fat breakdown (in association with increases in free fatty acids and ketones), thus sparing glucose and amino acids for use by the fetus. "Facilitated anabolism" describes the fed state during late pregnancy. Postprandially, there are greater increases in glucose and triglycerides after a glucose load. Glucose is diverted in a concentration-dependent manner toward the fetus and the increased triglycerides provide fuel that can be utilized by the mother and thus further spares glucose for the fetus. The progressive increase in insulin resistance which parallels the growth of the fetoplacental unit is the primary driver of this potential for carbohydrate intolerance, such that by the third trimester insulin sensitivity is 50%–60% lower than in the nonpregnant state (Catalano *et al.*, 1999).

The progressive increase in insulin resistance and insulin secretion that characterizes late pregnancy is thought to be mediated by a number of factors of placental origin, including progesterone, human chorionic somatomammotropin (hCS), placental growth hormone variant, cytokines (Desoye and Hauguel-de Mouzon, 2007), and others; the precise mechanisms remain elusive. In the postpartum period, the marked insulin resistance dissipates immediately.

Pathogenesis of GDM

GDM develops in women who are unable to maintain euglycemia in the face of the diabetogenic changes that occur during normal pregnancy. The relative decrease in insulin sensitivity during late pregnancy is similar among women with and without GDM (approximately 50%–60%), but the starting point differs (Catalano *et al.*, 1999). The initial absolute degree of insulin sensitivity tends to be lower among women with GDM compared to those without; when these women become even more insulin resistant during pregnancy and pancreatic β-cell function is insufficient to compensate for the increased insulin resistance, hyperglycemia occurs. Baseline pregravid metabolic status, therefore, is a major determinant of whether GDM will develop in the face of the more universal relative decrease in insulin sensitivity which occurs during pregnancy (**Fig. 1**). In this way, GDM is a quintessential example of the concept of pregnancy complications as a "window" into a woman's future health (Rich-Edwards *et al.*, 2010). The metabolic stress of pregnancy enables an underlying predisposition to disease (in this case future risk of developing diabetes) to become clinically manifest at a young age, a time when a woman may otherwise be unaware of her future health risks but in many ways may be most poised to enact the lifestyle modifications which could stave off future disease.

Consequences of Hyperglycemia in Pregnancy

The hyperglycemia that occurs as a result of diabetes in pregnancy (regardless of the etiology of the diabetes) can lead to fetal and neonatal consequences, as well as metabolic abnormalities later in life. According to the Pedersen–Freinkel hypotheses (**Fig. 2**), excess maternal fuels (glucose as well as other insulin-dependent fuels such as amino acids and lipids) cross the placenta in a concentration-dependent manner and stimulate the production of excessive insulin and other growth factors in the developing fetus (Pedersen and Osler, 1961; Freinkel, 1980). Increased fetal insulin secretion via increased fetal beta-cell mass (which can be

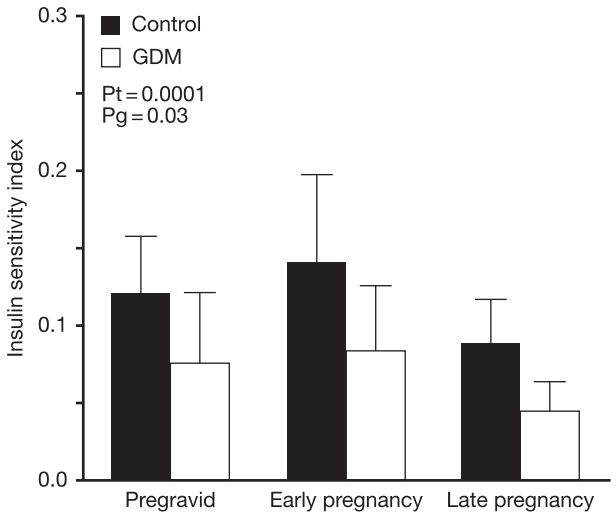

Fig. 1 Longitudinal changes in insulin sensitivity index during 40 mU m^{-2} min^{-1} insulin infusion (mean ± SD). GDM, Gestational diabetes mellitus; Pt, individual longitudinal changes with time; Pg, difference between groups (Catalano *et al.*, 1999).

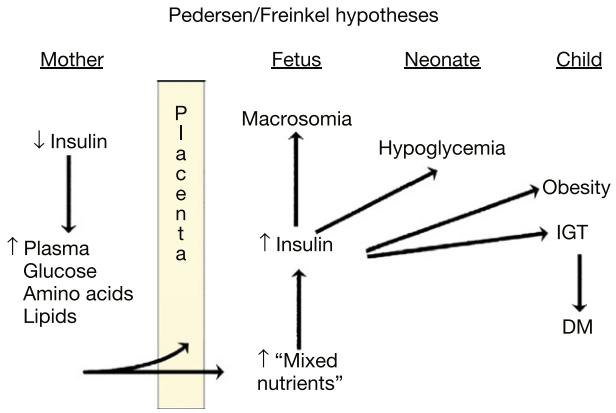

Fig. 2 Effect of maternal fuels on fetal development. The classic hyperglycemia-hyperinsulinemia hypothesis of Pedersen has been modified to show the contribution of other insulin-responsive maternal fuels in addition to glucose. Altered fetal nutrients and fetal hyperinsulinemia are associated with consequences that extend beyond the neonatal period (Silverman *et al.*, 1996).

identified as early as the second trimester) stimulates excessive fetal growth and increased fetal adiposity. The excessive fetal adiposity tends to be disproportionately distributed in the chest and shoulder areas, thereby increasing risks of shoulder dystocia, birth trauma, and operative delivery. In the neonatal period, risk of neonatal hypoglycemia increases as a result of hyperinsulinemia that persists even after cessation of the excess supply of glucose and other nutrients from the mother. Later in life, increased risk of obesity, impaired glucose metabolism, and diabetes result from the altered intrauterine environment (see below).

Preexisting DM

Fetal Implications

In early pregnancy, maternal hyperglycemia significantly increases risk of congenital malformations that develop during the first few weeks of pregnancy when the mother may not yet be aware that she is pregnant. The risk of congenital malformations is estimated to be two- to sixfold elevated among pregnancies complicated by preexisting type 1 or type 2 DM (Landon *et al.*, 2017; Bell *et al.*, 2012; Yang *et al.*, 2006). This risk has been shown to increase linearly with increasing periconceptional A1c above the normal range (Bell *et al.*, 2012; Guerin *et al.*, 2007), though others have observed the increased risk only at higher A1c thresholds (Jensen *et al.*, 2009). Since a large number of pregnancies are unintended (nearly half of pregnancies in the U.S. (Finer and Zolna, 2011)), preconception counseling and optimal glycemic control in women of reproductive age with preexisting DM are of paramount importance in reducing this risk. Optimizing glycemic control as close to normal as is safely possible is the single most

effective strategy to improve pregnancy outcome, and prepregnancy care programs may reduce risk of adverse pregnancy outcomes among women with type 1 and type 2 DM (Murphy *et al.*, 2010). Additional preconception testing is summarized in **Table 1**.

Later in pregnancy, hyperglycemia induces fetal hyperinsulinemia, excess fetal growth and its attendant consequences, and increased future risk of obesity and abnormal glucose metabolism among the offspring (as discussed below under GDM). Risk of perinatal mortality, while low when metabolic control is good, is significantly increased among pregnancies complicated by preexisting type 1 and type 2 DM (Tennant *et al.*, 2014).

Obstetric Complications

Hyperglycemia in early pregnancy increases risk of spontaneous abortion, and the risk increases linearly with increasing periconceptional A1c (Mills *et al.*, 1988). Conversely, early glycemic control achieved through a preconception management program led to significantly decreased rates of early fetal loss (Rosenn *et al.*, 1991). Pregnant women with preexisting DM are also at higher risk for preeclampsia/gestational hypertension, polyhydramnios, preterm delivery, and operative delivery.

Maternal Implications

Women with preexisting DM are at increased risk of hypoglycemia during early pregnancy (Nielsen *et al.*, 2008). Contributors to the increased hypoglycemia risk include the more intensive treatment targets employed during pregnancy, the physiologic decline in fasting glucose, nausea and resulting irregular food intake, difficulty in recognition related to the overlap between symptoms of hypoglycemia and the common symptoms of pregnancy itself (such as nausea, palpitations, sweating, warmth, hunger, inability to concentrate), and most importantly, diminished subjective symptoms of hypoglycemia (Bjorklund *et al.*, 1998).

Risk of diabetic ketoacidosis (DKA) is also increased during pregnancy among women with preexisting DM as a result of the increased insulin resistance and resulting augmentation of lipolysis and ketogenesis which occurs during pregnancy. While fortunately an infrequent complication of pregnancy, a high index of suspicion and low threshold for hospitalization must be maintained for pregnant women who experience symptoms suggestive of DKA (such as nausea, vomiting, abdominal pain, fever, or decreased oral intake) even at moderately increased glucose levels, due to the potentially life-threatening consequences of DKA to the mother and fetus (Sibai and Viteri, 2014). Potential precipitating factors of DKA include intractable vomiting/starvation, infection, insulin deficiency resulting from poor compliance with treatment or insulin pump failure, use of β-sympathomimetic agents for tocolysis, glucocorticoid administration for fetal lung maturation or other medical indications, and gastroparesis (Sibai and Viteri, 2014). Pregnant women should be instructed to test for urine ketones when ill or when blood glucose levels persistently exceed 200 mg/dL (11.1 mmol/L) and to notify the healthcare team when positive values occur.

In general, pregnancy is not considered to increase independently the risk for long-term progression of microvascular complications (Verier-Mine *et al.*, 2005; Diabetes and Complications Trial Research, 2000). Although short-term worsening of retinopathy may occur during and for 1 year after pregnancy, this transient increase in risk did not persist long-term in the Diabetes Control and Complications Trial (DCCT) (Diabetes and Complications Trial Research, 2000). Women without baseline retinopathy are very unlikely to develop severe retinopathy during pregnancy. Among women with moderate or severe retinopathy at

Table 1 Diabetes preconception health checklist

Address family planning and contraception
Optimize glycemic control prior to pregnancy, including A1c $<6.5\%$
Understand meal plan guidelines, recommendations for weight gain, and physical activity during pregnancy
Eat a nutritious meal plan with adequate distribution of carbohydrate, protein, and fat
Evaluation for microvascular complications, including comprehensive eye exam, serum creatinine, and urinary albumin–to–creatinine ratio
Measure thyroid-stimulating hormone (TSH)
Take 400 micrograms of folic acid daily
Discontinue potentially teratogenic medications, including ACE inhibitors, angiotensin receptor blockers, and statins
Smoking cessation
Limit alcohol consumption to less than one drink per day
Stop all alcohol use when actively trying to become pregnant
Gynecological exam and screening for communicable disease
Weight management
Daily physical activity
Seek intervention to address mood disturbances such as depression or anxiety

conception, however, the risk of progression is significant (Chew *et al.*, 1995). Poor glycemic control at conception also increases risk of worsening retinopathy (Chew *et al.*, 1995). Since the transient effect of pregnancy on retinopathy risk persisted for 1 year postpartum in the DCCT, continued frequent ophthalmologic monitoring is warranted for 1 year postpartum (Diabetes and Complications Trial Research, 2000).

Mirroring the patterns noted above for diabetic retinopathy, risk of nephropathy progression also correlates with the degree of baseline disease. Women with no or mild diabetic nephropathy prior to pregnancy are at low risk for durable progression of kidney disease, while those with significant baseline kidney disease are at significant risk of disease progression during pregnancy. While a transient increase in albumin excretion may occur during pregnancy among those with albuminuria at baseline, these changes typically normalize following delivery (Young *et al.*, 2011). In contrast, the minority of women with moderate-to-severe renal insufficiency at baseline are at risk for progression of renal impairment and even development of end-stage renal disease during pregnancy (Purdy *et al.*, 1996). In addition to the potential impact of pregnancy on the progression of diabetic nephropathy, impaired renal function or severe proteinuria at baseline also increases risk of adverse pregnancy outcomes, including preterm delivery, preeclampsia, and fetal growth restriction (Landon, 2007).

Treatment Principles

Good glycemic control throughout pregnancy is associated with the lowest rates of complications for the fetus, pregnancy, and mother (Kitzmiller *et al.*, 2008). While no randomized trials have or will be done to formally evaluate the independent effects of periconceptional and antenatal glycemic control on adverse pregnancy outcomes, multiple observational studies provide strong support for the benefits of excellent glycemic control in reducing these risks. The risk of serious adverse pregnancy outcome (congenital malformations and/or perinatal mortality) was elevated among pregnant women with type 1 DM and periconceptional A1c above 6.9% (Jensen *et al.*, 2009). Similarly, A1c above 6.5% measured in late pregnancy among women with type 1 DM correlated with increased risk of multiple adverse outcomes including preterm delivery, preeclampsia, and neonatal hypoglycemia; A1c above even 6% was associated with increased risk of large for gestational age (Maresh *et al.*, 2015). The risks increased progressively with higher A1c.

Insulin requirements change dramatically over the course of gestation, in parallel with the growth of the placenta and fetus (Garcia-Patterson *et al.*, 2010). In early pregnancy, insulin sensitivity tends to increase, leading to a heightened risk of hypoglycemia at the end of the first and beginning of the second trimester. Subsequently, a progressive increase in insulin resistance occurs, starting at approximately 16 weeks gestational age and peaking in late third trimester (**Fig. 3**). Over the course of the second and third trimesters, a two- to threefold increase in daily insulin requirement typically occurs (Garcia-Patterson *et al.*, 2010), with a disproportionate increase in prandial versus basal insulin (Roeder *et al.*, 2012). Following delivery, the marked insulin resistance disappears immediately, and insulin requirements decrease markedly.

Insulin therapy can be accomplished via multiple daily injections of insulin or insulin pump therapy. Insulin pump therapy offers several advantages, including the ability to provide varying basal rates over the course of the day, to alter the duration of mealtime boluses for foods with varying absorption rates, and to precisely deliver small insulin doses. To date, systematic analysis has not demonstrated significant differences in glycemic control or pregnancy outcomes with insulin pump use compared to

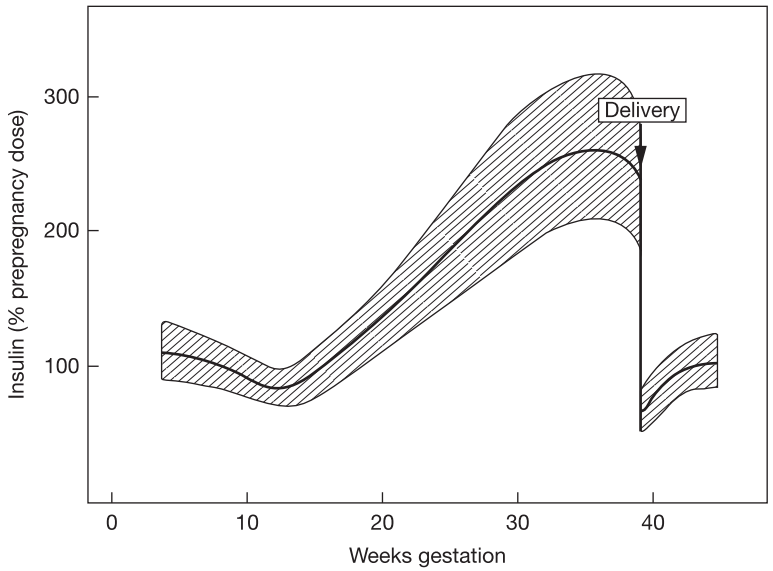

Fig. 3 Schematic representation of varying insulin requirements over the course of pregnancy and after delivery in pregestational diabetes mellitus (Phelps et al., 1988).

traditional multiple daily injections, but many women prefer the flexibility offered by insulin pump therapy in helping them to manage the large and rapidly changing insulin requirements that typically occur over the course of gestation.

Blood glucose targets are fasting glucose ≤90–95 mg/dL (5–5.3 mmol/L) and either 1-h postprandial glucose ≤140 mg/dL (7.8 mmol/L) or 2-h postprandial glucose ≤120 mg/dL (6.7 mmol/L), similar to the targets for GDM discussed below (Blumer *et al.*, 2013; ACOG Committee on Practice Bulletins, 2005; American Diabetes Association, 2017). The recommended A1C target in pregnancy is 6%–6.5%, noting that <6% may be optimal if it can be achieved without significant hypoglycemia, and that <7% may be used if needed to prevent hypoglycemia (American Diabetes Association, 2017).

Continuous glucose monitoring (CGM) systems measure repeated glucose levels in interstitial fluid (which parallel plasma glucose levels) using an electrochemical enzymatic sensor. Many women derive significant benefit from real-time CGM data, which can provide insight into glycemic patterns across the day and night, the degree and duration of postprandial glucose excursion in response to different foods, and the impact of lifestyle factors such as exercise and dietary manipulations on glycemia. This data can be harnessed to inform the frequent insulin dose adjustments which become necessary in the face of continually changing insulin requirements across gestation. CGM systems can also deliver alerts that can warn of hypoglycemia (a valuable safety feature), hyperglycemia, as well as impending hypo- and hyperglycemia. Outside of pregnancy, CGM use has documented benefit in producing glycemic improvement and reduction in hypoglycemia (Beck *et al.*, 2017). Studies evaluating the impact of CGM use during pregnancy on glycemic control and pregnancy outcomes, however, have produced conflicting results to date (Secher *et al.*, 2013; Voormolen *et al.*, 2013; Murphy *et al.*, 2008). One trial demonstrated that improved glycemic control resulted from overnight use of a partially-automated "closed-loop" system that adjusts basal insulin rates in response to glucose levels measured via real-time continuous glucose monitoring (Stewart *et al.*, 2016).

Gestational DM

Diagnosis

The original diagnostic criteria for diagnosis of GDM proposed by O'Sullivan and Mahan were developed more than five decades ago (O'Sullivan and Mahan, 1964). These criteria were developed with the goal of identifying women at increased risk for future development of type 2 DM, rather than those at increased risk for adverse perinatal outcomes. With extrapolation to account for current use of plasma rather than whole blood samples and contemporary laboratory methodology, these criteria remain in use today (**Table 2**). In 2010, the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) proposed a new set of criteria based on data obtained from the landmark HAPO study. The IADPSG diagnostic criteria defined GDM based on the fasting, 1-h, and 2-h plasma glucose levels at which the odds for adverse perinatal outcomes was 1.75 times the corresponding odds of these outcomes at the study population's mean levels (International Association of Diabetes Pregnancy Study Groups Consensus Panel *et al.*, 2010). Therefore, these criteria are unique in that they were the first derived from risk of adverse perinatal outcomes, rather than maternal risk of future metabolic derangement.

Detection of diabetes in early pregnancy (for example, at the first prenatal visit) is recommended among women with elevated risk of type 2 diabetes, including those with a past history of GDM, obesity, or known abnormal glucose metabolism (American Diabetes Association, 2017). The current goal of early screening is to identify women with undiagnosed preexisting (or inter-pregnancy) type 2 DM and its well-established associated risks during pregnancy. How to manage—or label—women with lesser degrees of hyperglycemia in early pregnancy (above normal, but below the diagnostic threshold for overt DM) remains controversial at this time, as the currently-established diagnostic criteria for GDM were not derived from data obtained in early pregnancy, and trials demonstrating incremental benefit of identifying and treating GDM prior to the standard time of diagnosis in late pregnancy are lacking. Women who have negative early testing and all women who have not undergone early testing should

Table 2 Glucose criteria for detection and diagnosis of GDM

	O'Sullivan–Mahan[a,c,e] *(mg/dL)*	*NDDG*[a,c,f] *(mg/dL)*	*Carpenter–Coustan*[a,c,f] *(mg/dL)*	*IADPSG*[b,d,f] *(mg/dL)*
Fasting	90	105	95	92
1 h	165	190	180	180
2 h	145	165	155	153
3 h	125	145	140	–

[a]Uses 100-g oral glucose load.
[b]Uses 75-g oral glucose load.
[c]Positive result requires that at least two levels must be greater than or equal to the thresholds listed.
[d]Positive result requires that at least one level must be greater than or equal to the thresholds listed.
[e]Based on whole-blood sample.
[f]Based on plasma blood sample.
GTT should be performed in the morning after an overnight fast of 8–14 h and after at least 3 days of unrestricted diet (≥150 g carbohydrate daily). To convert mg/dL to mmol/L, multiply by 0.0555.
NDDG: National Diabetes Data Group; IADPSG: International Association of Diabetes Pregnancy Study Groups.

undergo universal testing at 24–28 weeks gestational age (American Diabetes Association, 2017; Committee on Practice Bulletins-Obstetrics, 2017).

Experts continue to debate the optimal method for GDM diagnosis. In the United States, detection and diagnosis of GDM is most commonly performed using a two-step approach. First, a "glucose challenge test" is administered fasting or non-fasting; a glucose value >130–140 mg/dL (7.2–7.8 mmol/L) 1 h after ingestion of 50 g of glucose is considered a positive screen (with different threshold values within that range imparting varying sensitivities and specificities). Screen-positive women then undergo a 100-g, 3-h oral glucose tolerance test (**Table 1**). More recently, a one-step 75-g, 2-h glucose tolerance test has been proposed by the IADPSG as an alternative approach to detection and diagnosis of GDM, and has been endorsed by some (American Diabetes Association, 2017; International Association of Diabetes Pregnancy Study Groups Consensus Panel *et al.*, 2010; Blumer *et al.*, 2013) but not all (Vandorsten *et al.*, 2013; Committee on Practice Bulletins-Obstetrics, 2017) organizations. Primarily because the IADPSG criteria define a positive diagnostic test as meeting or exceeding one rather than two thresholds, adoption of these criteria leads to a significant increase in GDM prevalence.

While it has been argued that this increased prevalence would have unwanted consequences such as constraints on limited healthcare resources, the higher prevalence of GDM as defined by the outcome-based IADPSG criteria more closely mirrors the prevalence of abnormal glucose metabolism among U.S. women of reproductive age (approximately 30% of U.S. women aged 18–44 years have diabetes or prediabetes based on NHANES data (The International Association of Diabetes & Pregnancy Study Groups (IADPSG) Consensus Panel Writing Group and the Hyperglycemia & Adverse Pregnancy Outcome (HAPO) Study Steering Committee *et al.*, 2012)) and therefore may more accurately reflect the prevalence of pregnancies affected by hyperglycemia and its attendant consequences.

Fetal Implications

The major impact of maternal hyperglycemia on the fetus derives from the basic postulates of the Pedersen–Freinkel hypotheses. Fetal overnutrition and resulting fetal hyperinsulinemia leads to excessive fetal growth (macrosomia), which in turn increases risk of shoulder dystocia and infant birth trauma. Postnatal fetal hyperinsulinemia which persists after cessation of the maternal glucose supply at delivery increases risk of neonatal hypoglycemia and its associated consequences. Other adverse fetal outcomes associated with GDM include neonatal metabolic complications (including hyperbilirubinemia, hypocalcemia, and polycythemia) and very rarely perinatal mortality. Notably, the HAPO study demonstrated that risks of multiple outcomes (macrosomia, fetal hyperinsulinemia, neonatal hypoglycemia, shoulder dystocia or birth injury, preeclampsia and preterm delivery) increased progressively in a continuous manner with increasing fasting, 1-h, and 2-h glucose levels, even within a range currently considered normal (Metzger *et al.*, 2008). In contrast to preexisting DM, GDM is generally not associated with increased risk of congenital malformations, as disease onset is late in pregnancy after organogenesis is complete.

In addition to perinatal consequences, multiple long-term observational studies have demonstrated that maternal history of GDM predicts increased future risk of obesity (Silverman *et al.*, 1991) and impaired glucose tolerance (Silverman *et al.*, 1995) in the offspring. In one large observational study, childhood obesity correlated with amniotic fluid insulin measured during the third trimester of pregnancy (Silverman *et al.*, 1991), bolstering the fundamental hypotheses posited by Pedersen and Freinkel that excess maternal fuel is the key driver of subsequent metabolic derangement. In the Pima Indian population, the prevalence of type 2 DM was dramatically higher among offspring of women who had diabetes during pregnancy, compared to offspring of women without diabetes, even after adjusting for paternal diabetes and offspring weight (Pettitt *et al.*, 1988). Perhaps the strongest line of evidence supporting independent effects of intrauterine exposure to hyperglycemia comes from studies examining discordant sibships. One study examining data from the Pima Indian community found that the risk of diabetes was significantly increased among siblings born after the mother developed diabetes compared to siblings born before maternal DM diagnosis (Dabelea *et al.*, 2000). Since multiple potential confounding factors remain constant between sibling pairs (genetic and most environmental factors), these findings provide strong support for the specific metabolic effects directly attributable to an altered intrauterine environment.

The above relationships set the stage for a vicious transgenerational cycle of diabetes and obesity (Dabelea and Crume, 2011). The fetal overnutrition that results from maternal diabetes increases future risk in the offspring of obesity and abnormal glucose metabolism; female offspring who develop type 2 diabetes or impaired glucose metabolism and become pregnant then impart to their own offspring an increased risk of obesity and diabetes, and the cycle continues.

Obstetric Complications

GDM has been associated with increased risk of multiple adverse obstetric outcomes including preeclampsia, polyhydramnios, operative delivery, maternal birth trauma, and associated morbidities.

Maternal Implications

Women with GDM are at markedly increased risk for future development of type 2 diabetes (Bellamy *et al.*, 2009). Estimates of the cumulative incidence of type 2 diabetes in the postpartum years have varied in different populations and with different lengths of follow-up, ranging 20%–60% or higher at 5–10 years postpartum (Kim *et al.*, 2002; Feig *et al.*, 2008). Since GDM stems from chronic beta-cell

insufficiency, it is not surprising that these women are at high risk for future development of overt diabetes with increasing age and exposure to other risk factors for type 2 DM such as weight gain and physical inactivity. The diagnosis of GDM during a woman's reproductive years, therefore, serves as a crucial window into a woman's future health, and bestows a woman with actionable information at a young age when she might not otherwise be as keenly aware of the need for lifestyle measures aimed at diabetes prevention.

Treatment Principles

The mainstay of treatment for all women with GDM is medical nutrition therapy (MNT), and MNT is sufficient as sole therapy for approximately 80%–90% of women (Crowther *et al.*, 2005; Landon *et al.*, 2009). Lifestyle interventions have been associated with decreased risk of macrosomia and decreased neonatal adiposity, as well as decreased risk of postpartum depression and increased likelihood of achieving postpartum weight goals (Brown *et al.*, 2017; Crowther *et al.*, 2005; Landon *et al.*, 2009). Women are advised to eat three small- to moderate-sized meals and two to four snacks that are balanced in whole-grain carbohydrates, protein, and unsaturated fats. Breakfast is typically smaller than lunch and dinner (for example, 30 g compared to 45–60 g of carbohydrate), as carbohydrate intolerance is commonly more pronounced at the morning meal. Pairing protein with carbohydrate at all meals and snacks is emphasized, not only to increase satiety but also to blunt postprandial carbohydrate-induced glycemic excursion. A bedtime snack is often necessary counteract the tendency toward accelerated starvation (and resulting ketosis) that accompanies the overnight fasting state. When glycemic goals are not met with MNT, insulin therapy is added. Women are asked to monitor capillary blood glucose fasting and 1 h after meals to guide insulin dose adjustments, and to monitor urine ketone levels 1–2 times daily to assess for excessive restriction of carbohydrate intake. Glucose targets are fasting $\leq$95 mg/dL (5.3 mmol/L) and either 1-h postprandial glucose $\leq$140 mg/dL (7.8 mmol/L) or 2-h postprandial glucose $\leq$120 mg/dL (6.7 mmol/L) (American Diabetes Association, 2017; Committee on Practice Bulletins-Obstetrics, 2017).

Treatment of GDM, via MNT with or without insulin therapy, has been shown in two large randomized controlled trials to reduce risk of adverse neonatal outcomes (Crowther *et al.*, 2005; Landon *et al.*, 2009). Specifically, treatment was shown to reduce rates of macrosomia (Crowther *et al.*, 2005; Landon *et al.*, 2009), shoulder dystocia (Landon *et al.*, 2009), neonatal fat mass (Landon *et al.*, 2009), cesarean section (Landon *et al.*, 2009), preeclampsia (Landon *et al.*, 2009, Crowther *et al.*, 2005), and maternal weight gain (Crowther *et al.*, 2005; Landon *et al.*, 2009).

Postpartum Care

Postpartum care for women with diabetes is the springboard to long-term diabetes care, prevention of long-term diabetes complications, and planning for subsequent pregnancies. Postpartum care should be treated as a transition from obstetric to general medical healthcare providers.

Preexisting DM

After delivery, immediate postpartum insulin requirements decrease dramatically among women with preexisting DM due the rapid decrease in placental hormone levels (**Fig. 3**). Maternal insulin requirements for women with type 1 DM usually return to pre-pregnancy levels or lower (Kjos, 2007). Medication requirements vary among women with type 2 DM, depending on the degree of hyperglycemia and the pre-pregnancy treatment regimen. Some may not require medical therapy following delivery and may monitor capillary glucose levels. When medical therapy is indicated, women may resume insulin therapy at reduced doses or oral agents (see breastfeeding considerations below).

Table 3 Metabolic assessments after GDM

Time	*Test*	*Purpose*
Post-delivery (1–3 days)	Fasting or random plasma glucose	Detect persistent, overt diabetes
Early postpartum (around the time of postpartum visit)	75 g 2-h OGTT	Postpartum classification of glucose metabolism[a]
1 year postpartum	75 g 2-h OGTT	Assess glucose metabolism
Annually	Fasting plasma glucose	Assess glucose metabolism
Tri-annually	75 g 2-h OGTT	Assess glucose metabolism
Prepregnancy	75 g 2-h OGTT	Classify glucose metabolism

[a]Classification of glucose metabolism by criteria recommended by the American Diabetes Association. OGTT, oral glucose tolerance test.

Metzger, B. E., Buchanan, T. A., Coustan, D. R., *et al.* (2007). Summary and recommendations of the fifth international workshop-conference on gestational diabetes mellitus. Diabetes Care 30(Suppl 2), S251–260.

GDM

The majority of women with GDM, even those treated with insulin during pregnancy, will have normal glucose levels immediately postpartum. Blood glucose monitoring is required postpartum, however, to exclude the small number of women with unrecognized preexisting type 2 DM (Kjos, 2007). Short- and long-term metabolic assessment after GDM is needed (**Table 3**).

Women should be counseled during both the antepartum and postpartum phases regarding the increased future risk of developing type 2 DM (see "GDM and Maternal Implications" sections). Pharmacologic therapy and lifestyle interventions have been shown to reduce the risk of type 2 DM. In the Diabetes Prevention Program (DPP)/DPP Outcomes Study, metformin and lifestyle changes were equally effective among women with a history of GDM in decreasing progression to diabetes over 10 years of follow-up (Aroda *et al.*, 2015; Nathan *et al.*, 2017). GDM frequently recurs in subsequent pregnancies. Factors which increase risk of recurrence include increased maternal age and parity, and interpregnancy weight gain.

Breastfeeding

Women with both preexisting DM and GDM should be encouraged to breastfeed. Among women with GDM, breastfeeding has been associated with reduced risk of developing type 2 DM in the future (Gunderson *et al.*, 2015). Women should continue a nutrient-dense meal plan, with caloric and nutritional requirements adjusted to meet the additional demands of lactation. Women should be counseled prior to delivery about potential barriers to breastfeeding, including possible post-operative recovery, the possibility of a neonate who requires intensive medical care, and maternal hypoglycemia. There are pros and cons regarding the use of oral medications for treatment of hyperglycemia during breastfeeding. Thus, a decision regarding use of oral agents versus insulin should be made jointly between caregivers and mothers.

Contraception

Ideally, a discussion regarding contraception should take place in the antepartum and immediate postpartum phase. Health care providers should counsel women regarding the need for prevention of unplanned pregnancy with suboptimal glycemic control, metabolic and cardiovascular impact, and individual lifestyle factors. Barrier methods such as condoms, diaphragms, cervical caps, and contraceptive sponges have no adverse systemic effects. Yet couples need to be extremely motivated to ensure proper use due to the high failure rate. Intrauterine devices are commonly recommended for women with DM. Neither the copper IUD nor the levonorgestrel-releasing IUD significantly impact glucose tolerance or lipids. Progestin-only oral contraceptive preparations are not thought to significantly impact diabetes control or blood pressure. Injectable progestin-only contraceptives (depo-medroxyprogesterone acetate, DMPA) may adversely affect lipids, insulin resistance, and weight gain, and is therefore not a first-line choice in this setting (Damm *et al.*, 2007; Xiang *et al.*, 2007). If future pregnancy is not desired, tubal ligation or vasectomy are options.

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Diabetes Mellitus; Diagnosis and Treatment in the Elderly☆

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Glossary

Body mass index (BMI) The measurement of the body mass divided by the square of the stature (total height) of an adult person. BMI is commonly used to assess the nutritional status of adults as low, normal, overweight or obese.

Diabetes mellitus A group of chronic diseases characterized by a deficiency in the production of insulin by the pancreas or by lack of efficiency of the produced insulin, which results in elevated concentrations of glucose concentrations in blood and urine.

Elderly An imprecise term, most often related to the age of retirement, which varies widely across countries. In the United States, older adults or elders are usually considered those aged 65 or more years of age.

Glucose A simple sugar, also known as dextrose, found in blood. Glucose is the main source of energy for the human body.

Glycemia Glucose concentration in the bloodstream. Commonly expressed in the United States as milligrams per deciliter (mg/dL).

Glycosylated hemoglobin Usually referred to as hemoglobin (Hb) AIC, it is a measure of the average glycemic control during a 3- or 4-month period.

Gestational diabetes mellitus (GDM) Diabetes diagnosed in the second or third trimester of pregnancy, without a previous history.

Glycated hemoglobin or hemoglobin A1C (HbA1c or A1c) It reveals the average plasma glucose concentration during the previous 3–4 months. Measurements of A1c are commonly used for screening, diagnosis, and monitoring of diabetes mellitus.

Hyperglycemia Elevated concentrations of glucose in the blood. Fasting hyperglycemia is blood glucose above a desirable level after a person has fasted for at least 8 h.

Hypoglycemia Abnormally low concentrations of glucose in the blood.

Insulin An anabolic hormone produced in the β cells of the islets of Langerhans in the pancreas. It is required for proper glucose, fat, and protein metabolism.

Milligrams per deciliter (mg/dL) It is the measurement unit used to describe the concentration of blood plasma glucose regarding its weight dimension.

Millimole per deciliter (mmol/dL) It is the measurement unit used to describe the concentration of blood plasma glucose regarding its molarity.

Type 1 diabetes (T1D) Autoimmune b-cell destruction, which leads to complete insulin deficiency.

Type 2 diabetes (T2D) Insulin resistance, which leads to a progressive loss of b-cell insulin secretion.

Introduction

The world population is living longer, and this fast-increasing number of older adults is occurring in all world regions, particularly in Asian and African Regions as seen in **Fig. 1**. By the year 2050, it is projected that about one in four of the global population will be an elderly individual of 60 or more years of age (United Nations, Department of Economic and Social Affairs and Population Division, 2017). And, as age is a nonmodifiable risk factor for type 2 diabetes, the number of cases of older adults with diabetes will also increase rapidly. Currently, approximately one of every two cases of diabetes occurs in people older than 55 years of age, and, for example, more than 25% of the United States population aged 65 or older have diabetes, with diabetes-related mortality being higher in this age group (Centers for Disease Control and Prevention, 2017).

This article focuses on the magnitude of diabetes mellitus in the elderly and the relevant epidemiological and clinical aspects associated with this disease, including the identification of risk factors, signs, and symptoms of the disease as well as complications associated with inadequate glycemic control of older people with diabetes. Management therapies, including diet, exercise, and medications that may be used with the special considerations of the geriatric patients in mind, are also discussed.

The aging process is characterized by a gradual decline in function and overall well-being (Lopez-Otin *et al.*, 2013). This aging decline occurs across several organ systems, with a progressive deterioration of physiological functions (de Almeida *et al.*, 2017). Therefore, aging is a risk factor for several diseases including type 2 diabetes, cardiovascular disease, osteoarthritis, dementia, and more. The rapid increases in the size and age structure of the worldwide elderly population, in addition to the extended longevity of this population group, is contributing to increasing numbers of older adults who are affected by chronic diseases, particularly type 2 diabetes and associated comorbidities.

☆*Change History*: March 2018. Odilia I. Bermudez of the first article was involved in the updated chapter. Tables 1, 2 and 7 are new for this chapter. Tables 3, 4, 5, and 6 were in the first chapter and update for this new chapter. The figures 1, 2 and 3 are new additions for the new chapter.

This article is an update of Odilia I Bermudez, Diabetes Mellitus, Diagnosis, and Treatment in the Elderly, In Encyclopedia of Endocrine Diseases, edited by L Martini, Elsevier, New York, 2004, Pages 661–665.

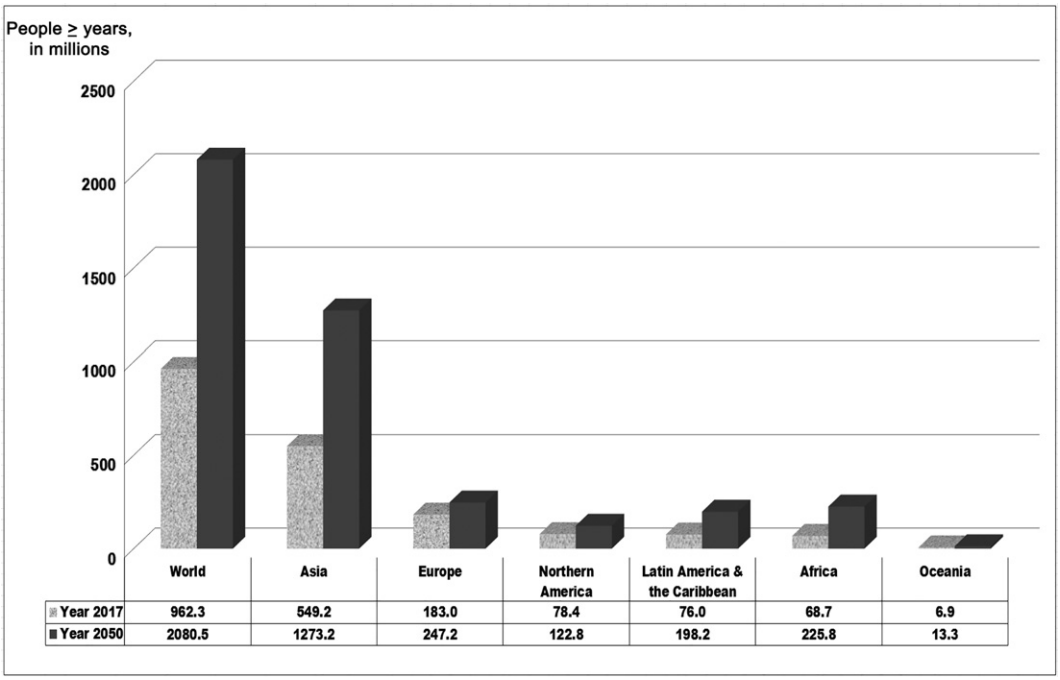

Fig. 1 World population, aged 60 and plus years, by Regions, for the years 2017 and 2050. United Nations, Department of Economic and Social Affairs and Population Division (2017). *World Population Ageing* [Highlights]. New York: United Nations.

Type 2 diabetes, also known as adult-onset diabetes, is the predominant form in older adults. In fact, more than 90% of all cases of diabetes among older adults are type 2. However, evidence shows that up to 12% of individuals older than age 65 with diabetes present islet cell autoimmunity, more similar to type 1 diabetes, and with an elevated risk for hyperglycemia (Pietropaolo *et al.*, 2000). Elders with type 2 diabetes also present at least one or more other major cardiovascular disease risk factors, particularly hypertension, abdominal obesity, and dyslipidemia. The clustering of these conditions has been called the metabolic syndrome, also known as the insulin-resistance syndrome (Oda, 2018).

As mentioned previously, type 2 diabetes is the most common type of the disease in the elderly. Therefore, the remaining sections focus on type 2 diabetes in the elderly.

Prevalence and Incidence of Diabetes Mellitus in the Elderly

According to the International Diabetes Federation (2017), the numbers of the global population between 20 and 79 years of age, affected with type 2 diabetes, is expected to increase from 425 million in 2017 to 629 million in 2045, an overall increase of 148% (see **Table 1**). The largest increase (195%) will occur in the elderly group (65–79 years).

The global burden of diabetes is significant, with large increases in the number of adults living with diabetes. Based on reports of the World Health Organization (WHO), 108 million adults living with diabetes in 1980 and by the year 2014, the number of adults living with diabetes increased to 422 million, which represents an increment of 391% (World Health Organization, 2016).

Similar to the global trends, the prevalence of diabetes among the US population is also increasing across age groups, particularly those in the older groups. As observed in **Fig. 2**, by the year 2015, the US elderly group (>65 years of age) had higher proportions of people with undiagnosed and diagnosed diabetes, as compared to their younger counterparts of 18–44 and 45–64 years of age (Centers for Disease Control and Prevention, 2017). Due to the aging of the US populations, it is expected that the fast growth of the numbers of people with diabetes will continue to grow higher and faster in the elderly groups (**Table 2**). And along with this fast growing, the associated costs of medical care for people with diabetes will also grow at very high levels (Caspersen *et al.*, 2015).

Incidence of Diabetes in the American Adult Population

For the year 2015, the CDC estimated 1.5 million of new cases of diabetes among US adults 18 years or older (Centers for Disease Control and Prevention 2017), which represented seven new cases per 1000 people. From those incident cases, more than half were among middle-aged adults (45–64 years), with equal numbers for men and women. Also, it was reported that incidence of diabetes varied with ethnicity, with higher rates for Hispanics (rate of 8.4 per 1000) and Blacks non-Hispanics (rate of 9.0 per 1000) (Centers for Disease Control and Prevention, 2017). Less than high school education was also identified as a risk factor when compared to lower rates for those with high school or beyond a high school education (**Fig. 3**).

Table 1 Global prevalence of type 2 diabetes and changes (%) between the years 2017 and 2045

Age groups (years)	*2017*	*2045*	*Increases (%)*
20–64	327	438	134
65–79	98	191	195
Total (20–79)	425	629	148

International Diabetes Federation (2017). *IDF diabetes atlas*, 8th edn. Brussels: International Diabetes Federation, 50p. ISBN: 978-2-930229-87-4.

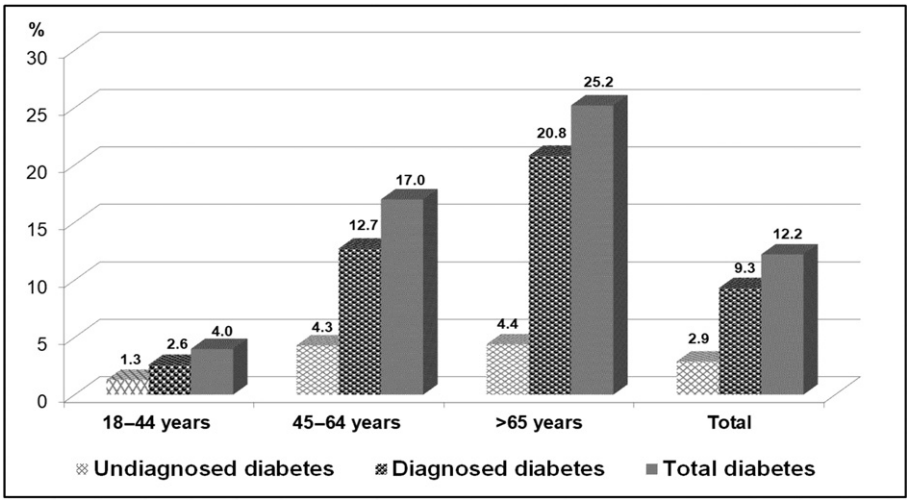

Fig. 2 Percentages of undiagnosed and diagnosed diabetes in US adults for the year 2015. Centers for Disease Control and Prevention (2017). National Diabetes Statistics Report, 2017. Atlanta, GA: Centers for Disease Control and Prevention, U.S. Department of Health and Human Services: p. 20.

Table 2 Numbers (in millions) of US adults (>18 years of age) with undiagnosed or diagnosed diabetes, as estimated for the year 2015

Age groups (years)	*Undiagnosed diabetes (millions)*	*Diagnosed diabetes (millions)*	*Total diabetes (millions)*
18–44	1.6	3.0	4.6
45–64	3.6	10.7	14.3
>65	2.1	9.9	12.0
Total	7.2	23.0	30.2

Centers for Disease Control and Prevention (2017). National Diabetes Statistics Report, 2017. Atlanta, GA: Centers for Disease Control and Prevention, U.S. Department of Health and Human Services: 20.

Risk Factors Associated With Diabetes

There is uncertainty about the specific factors that trigger the development of type 2 diabetes. For example, ethnicity is an important risk factor in the United States. It has been reported that some US ethnic and racial groups are at a higher risk for diabetes than the general population. There it is noted that Asian and Black non-Hispanic adults, as well as Hispanics, have a higher prevalence of diabetes as compared to non-Hispanic whites (Centers for Disease Control and Prevention, 2017).

Growing old is another important risk factor for developing type 2 diabetes (Menke *et al.*, 2015; American Diabetes Association, 2017). It has been estimated that by the year 2050, the number of US older adults (>65 years of age) will grow to 27 million, the equivalent of 55% of all cases of diabetes by that year (Caspersen *et al.*, 2012). There is a positive association between age and developing diabetes beginning after age 30. In fact, being older than 45 years of age is one of the risk factors listed in **Table 3**. And diabetes prevalence will be higher among non-Hispanic black, non-Hispanic Asian, and Hispanic adults as compared to other ethnic groups (Menke *et al.*, 2015).

Evidence also shows that approximately 80% of adults with type 2 diabetes are overweight and physically inactive (American Diabetes Association, 2017). Those with a family history of diabetes are also at increased risk for the disease (American Diabetes Association, 2017). Women who have had gestational diabetes, or who have given birth to a baby weighing more than 9 pounds, are also at higher risk for developing diabetes later in their lives (Daly *et al.*, 2018). Furthermore, there is an elevated risk for diabetes among women with polycystic ovary syndrome (Jaliseh *et al.*, 2017). The risk is also high for those known to have impaired glucose tolerance

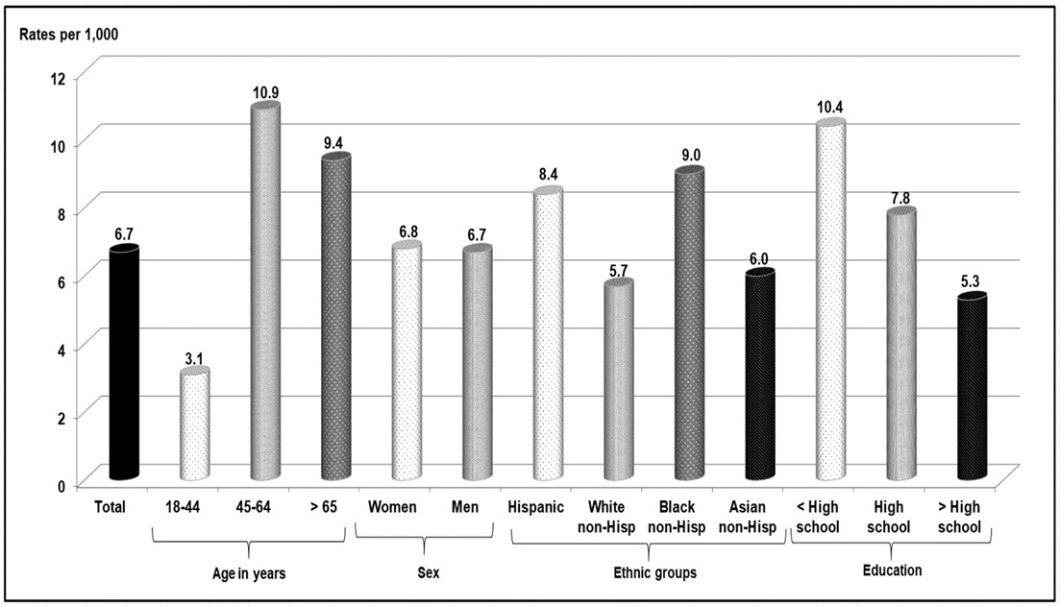

Fig. 3 The incidence of diabetes in the American adult population (>18 years), by age group, sex, ethnicity, and education. Centers for Disease Control and Prevention (2017). National Diabetes Statistics Report, 2017. Atlanta, GA: Centers for Disease Control and Prevention, U.S. Department of Health and Human Services: p. 20.

Table 3 Risk factors for type 2 diabetes

Risk factors
Older than 45 years of age
Ethnicity
Family history of diabetes
Impaired fasting glucose
Impaired glucose tolerance
History of gestational diabetes
Obesity
Hypertension
Dyslipidemia

American Diabetes Association (2017). Standards of medical Care in Diabetes. *Diabetes Care* 40(Suppl. 1), S1–S135.

(IGT) or impaired fasting glycemia (IFG) (American Diabetes Association, 2017), as well as for those who have high blood pressure or signs of heart disease or poor circulation as detailed in **Table 3** (American Diabetes Association, 2017).

Symptoms of Diabetes in the Elderly

Type 2 diabetes has a gradual onset, with signs of the disease developing over the years (Kirkman *et al.*, 2012). The classic symptoms, listed in **Table 4**, are usually subdued or even absent in older individuals, and the disease is often diagnosed several years after its onset when complications are already present. Regular screening for the disease after age 45 is usually the best way to detect early type 2 diabetes and avoid complications associated with the disease later during the senescence period (American Diabetes Association, 2017). Also, diabetes is associated with increased healthcare expenditure in managing diabetes-related hospital admissions and the associated vascular complications.

Diagnosis of Diabetes in the Elderly

Detection of diabetes among older individuals occurs late. It is estimated that onset of type 2 diabetes precedes clinical diagnosis by several years (Kirkman *et al.*, 2012). Compared to younger adults with diabetes, those in the older group have higher rates of myocardial infarction, visual impairment, end-stage renal disease, and lower-extremity amputation (Kirkman *et al.*, 2012). They

Table 4 Symptoms of type 2 diabetes presented in the elderly population

Symptoms of type 2 diabetes
Polyuria (frequent urination)
Polydipsia (excessive thirst)
Weight loss
Severe fatigue and/or nausea
Blurred vision
Frequent and recurrent infections
Slow healing of wounds or sores

American Diabetes Association (2017). Standards of medical Care in Diabetes. *Diabetes Care* 40(*Suppl. 1*), S1–S135.

Table 5 Criteria for the diagnosis of diabetes mellitus

Criteria for diagnosis	*Details*
Fasting plasma glucose (FPG) >126 mg/dL (7.0 mmol/L)	Fasting is defined as no caloric intake for at least 8 h
2-h plasma glucose >200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT)	The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water
Hemoglobin A1c >6.5% (48 mmol/mol)	The test should be performed in a laboratory using a method that is certified by the National *Glycohemoglobin Standardization* Program (NGSP) and standardized to the Diabetes Control and Complications Trial (*DCCT*) assay
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose >200 mg/dL (11.1 mmol/L)	

American Diabetes Association (2017). Standards of medical Care in Diabetes. *Diabetes Care* 40(Suppl. 1), S1–S135.

are also more likely to present conditions such as depression, cognitive impairment, muscle weakness (sarcopenia), falls and fractures, and physical frailty (Kim *et al.*, 2012).

It has been reported that among people with undiagnosed type 2 diabetes, 10%–29% have retinopathy, 10%–37% have proteinuria, and 9% have neuropathy at the time of diagnosis (Caspersen *et al.*, 2012). These complications, associated with uncontrolled diabetes, take 10 or more years to develop in the presence of the disease. These considerations are very strong justification for the need to look for early symptoms of type 2 diabetes in older adults as early as possible.

The current criteria for the diagnosis of diabetes mellitus are based on the 2017 recommendations of an international expert committee from the American Diabetes Association (2017) and also from the World Health Organization (2016). Based on these recommendations, and according to the 2017 guidelines from the American Diabetes Association (2017), three diagnosis criteria may be used, with confirmation on a subsequent day (see **Table 5**).

Also, and based on the recommendations from ADA, when testing for prediabetes, it is equally appropriate to do fasting plasma glucose or the oral glucose tolerance test or the A1C test, as detailed in **Table 5**. In patients with prediabetes, particularly older adults, the testing or identification of other risk factors for cardiovascular disease is also important to take into consideration (American Diabetes Association, 2017).

Complications of Type 2 Diabetes

Since the metabolic problems associated with diabetes affect the entire body, older adults with this condition face multiple potential complications affecting many different organ systems. If the management of the disease is poorly controlled, if the disease is left untreated, or if the diagnosis is made late, serious complications can occur. Moreover, poor glycemic control among people with diabetes is a major risk factor for end-stage complications of this condition (American Diabetes Association, 2017).

Cardiovascular disease (e.g., coronary heart disease and stroke) is the leading cause of morbidity and mortality among persons with diabetes (Qazi and Malik, 2013; American Diabetes Association, 2017). And cardiovascular disease is two to four times more common in adults with diabetes (Fox, 2010). Also, a chronic kidney disease associated with diabetes is present in about 20%–40% of people with diabetes. This health condition is identified by the elevated excretion of urinary albumin, or by the identification of low rates of glomerular filtration, or by other indicators of kidney impairment (American Diabetes Association, 2017).

Diabetes is also the leading cause of blindness and visual impairment in adults (Danet-Lamasou *et al.*, 2018). Also, nerve damage, combined with peripheral vascular disease, make diabetes the most common cause of lower extremity amputation (Hoffstad *et al.*, 2015). Between 60% and 70% of persons with diabetes have mild to severe forms of diabetic nerve damage, and more than half of lower limb amputations in the United States occur among those people with diabetes (National Institute of Diabetes and Digestive and Kidney Diseases, 2018).

As the evidence indicates, and for all types of diabetes, the risk of developing complications and the progression of the disease, is considerably reduced with tight control of blood glucose levels. Results from the Diabetes Control and Complications Trial Research on type 1 diabetes (Diabetes Control and Complications Trial Research Group *et al.*, 1993), the United Kingdom Prospective Diabetes Study on type 2 diabetes (Ismail-Beigi *et al.*, 2010), and the Kumamoto study (Ohkubo *et al.*, 1995), confirmed that better glycemic control is associated with reductions in both the incidence and the progression of retinopathy and nephropathy in subjects with type 1 and type 2 diabetes mellitus. Available evidence also links macrovascular disease (cardiovascular, peripheral vascular and cerebrovascular disease) to glycemic status (Qazi and Malik, 2013; American Diabetes Association, 2017). Furthermore, subjects with good long-term glycemic control have better survival rates than those with elevated fasting blood glucose (Diabetes Control and Complications Trial Research Group *et al.*, 1993; American Diabetes Association, 2017).

Clinical Management of Diabetes in the Elderly

Older adults with diabetes require careful management of their diabetes condition, with a focus on minimizing long-term vascular complications. Therefore, this should be an important focus in defining glycemic targets.

Careful planning and adherence to the clinical management designed for the elderly with diabetes can provide good glycemic control. The initial clinical management of diabetes in the elderly usually consists of medical nutrition therapy and increases in physical activity. However, few older adults can adhere strictly to the required changes in diet and physical activity, and controlling hyperglycemia becomes a challenge. Therefore, in the vast majority of cases, drug therapy is needed soon after the disease is detected. Drug options include second-generation oral sulfonylureas, metformin, acarbose, insulin, insulin analogs, and combinations of these drugs (American Diabetes Association, 2017). These drugs have demonstrated efficacy in increasing insulin availability, decreasing insulin requirements, or both. Additionally, a combination therapy with dipeptidyl peptidase-4 inhibitor (DPP4i) and sodium-glucose cotransporter type 2 inhibitor (SGLT2i) has been reported to be safe and effective in treating type 2 diabetes (Cho *et al.*, 2018).

Self-monitoring of blood glucose by the elder diabetic, along with clinical diabetes monitoring of hemoglobin AIC are good parameters for monitoring the disease, as it informs the medical team about glucose control during the past 3 or 4 months. A1C lower than 7% is usually the goal of achieving good control (American Diabetes Association, 2017).

The evidence is lacking about the benefits of tight glycemic control in older adults (older than 65 years of age). However, elderly diabetic patients with a prognosis for extended life and who can manage their disease should have the same goals for glycemic control as younger adults, as seen in **Table 6**. For elders with advanced diabetes, disease complications, advanced cognitive or physical impairment, or comorbidities that may reduce their life expectancy, less intensive goals can be pursued. In any case, it is advisable to individualize the clinical management of diabetes among elderly individuals (American Diabetes Association, 2017). In particular, factors like duration of diabetes, age, life expectancy, comorbidities, including CVD or microvascular complications plus other individually-identified considerations, must be taken into consideration (American Diabetes Association, 2017).

Proper clinical and self-management of older adults with diabetes is often difficult and costly due to circumstances associated with aging and comorbidities. Physical ailments, affective or cognitive disorders, psychosocial problems such as depression and living situation can all interfere with the proper management of diabetes in the elderly and potentially exacerbate problems associated with noncompliance with medications, the absence of glucose monitoring, and lack of adequate physical activity (American Diabetes Association, 2017). Additional comorbidities and adverse complications are listed in **Table 7**.

Furthermore, the impact of cardiovascular risk factors is significant among older adults with diabetes. Therefore, the management of diabetes requires tight control of cardiovascular risk factors, including hyperglycemia, hypertension, and dyslipidemia.

Table 6 Goals for glycemic and cardiovascular control of adults

Goal	*Criteria*
For glycemic control	
Hemoglobin A1c	<7% (53 mmol/mol)
Preprandial plasma glucose	80–130 mg/dL (4.4–7.2 mmol/L)
Postprandial plasma glucose	<180 mg/dL (10.0 mmol/L)
For cardiovascular health control	
Blood pressure	Systolic <130 mmHg and/or diastolic <80 mmHg
Lipid profile	
High-density lipoprotein cholesterol	Men: >40 mg/dL (1.0 mmol/L) Women: >50 mg/dL (1.3 mmol/L)
Low-density lipoprotein cholesterol	<100 mg/dL (2.6 mmol/L)
Triglycerides	<150 mg/dL (1.7 mmol/L)

American Diabetes Association (2017). Standards of medical Care in Diabetes. *Diabetes Care* 40(Suppl. 1), S1–S135.

Table 7 Comorbidities and adverse events in older adults with diabetes

Co-existing illnesses
Coronary heart disease
Hypertension
Stroke
Geriatric syndromes
Cognitive impairment
Urinary incontinence
Injurious falls
Polypharmacy
Persistent pain
Adverse health events
Functional disability
Premature death

American Diabetes Association (2017). Standards of medical Care in Diabetes. *Diabetes Care* 40(*Suppl. 1*), S1–S135.

Diabetes needs to be viewed and treated as part of the metabolic syndrome, keeping in mind that the main goals of diabetes management, whenever the circumstances apply, are tight glycemic control and more aggressive management of risk factors associated with cardiovascular disease.

Conclusion

The rapidly increasing rates of diabetes among older adults and the tremendous social and economic impact of the disease require that it be treated as a high-priority public health problem. Prevention of the disease among the growing elderly population is the best strategy to reduce the burden of diabetes. Lifestyle strategies (healthy eating and increased physical activity) are more effective than drugs for preventing the development of diabetes, as indicated results from the Diabetes Control and Complications Trial Research Group *et al.* (1993). These lifestyle changes were particularly successful for people older than age 60.

From this evidence, a strong argument can be made for focusing on the prevention of diabetes in older adults through improvement of the favorable factors, particularly lifestyle factors, associated with diabetes control.

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Carbohydrate Metabolism: Diabetes Mellitus, Genomic Aberrations☆

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Glossary

Acanthosis nigricans A velvety and pigmented skin rash, usually on the back of the neck, armpits or other flexures.
Autoimmune A condition in which the immune system attacks certain cells, tissues, or organs, resulting in their being damaged or destroyed.
Autosomal dominant The pattern of inheritance of a disease resulting from the inheritance of a single mutated gene that is passed down from one parent, with each child of an affected parent having a 50% chance of inheriting the condition.
Autosomal recessive The pattern of inheritance of a disease resulting from the inheritance of two copies of a mutated gene, one from each parent; there is a 25% chance of conceiving an affected child with each pregnancy from unaffected parents each with a single copy of the mutated gene.
Heterozygous The presence of two different copies of a given gene.
Homozygous The presence of two of the same copies (normal or mutated) of a given gene.
Hyperandrogenism Abnormally high levels of male sex hormones, which in woman can result in abnormal facial hair growth, an enlarged clitoris, and/or irregular or absent menses.
Hypertriglyceridemia Abnormally high levels of triglycerides (fat) in the blood.
Insulin A hormone secreted by beta-cells of the pancreas that promotes the uptake of glucose from the bloodstream into cells where the glucose is used as energy or is stored for future use.
Insulin resistance Decreased ability of insulin to lower blood glucose.
Ketoacidosis A condition in which toxic ketones and acids accumulate in the bloodstream and organs of the body.
Penetrance The likelihood that a mutated gene, when inherited, will result in a disease.
Polygenic The involvement of multiple genes in the development of a disease or condition.

Introduction

Diabetes mellitus is simply defined by an abnormally high blood glucose concentration. The arbitrary diagnostic levels of blood glucose were assigned based on natural history studies establishing the glucose threshold at which hyperglycaemia-related clinical complications start to occur. All forms of diabetes require pancreatic beta cell failure to secrete adequate amounts of insulin. This beta cell failure ranges from the absolute, as in type 1 diabetes, to the relative – that is, insulin secretion persists but is insufficient to maintain normal blood glucose in the face of insulin resistance. In the case of severe insulin resistance insulin secretion may actually be elevated to one or two orders of magnitude above normal, but is nevertheless unable to compensate for the degree of tissue insulin resistance present. The long term complications of hyperglycemia, and those used in setting diagnostic criteria, are eye disease (retinopathy), kidney disease (nephropathy), and nerve disease (neuropathy). Diabetes is also strongly associated with premature atherosclerotic vascular disease (heart attack, stroke, and lower limb amputation), however this is less directly linked to hyperglycemia *per se*, and more directly attributable to other aspects of the metabolic derangement seen in diabetes such as an altered lipoprotein profile.

Diabetes mellitus is divided into many subtypes depending on the underlying aetiology, where known, or on non specific biochemical characteristics such as degree of beta cell failure. Forms of diabetes due to mutations in single genes represent at most a few per cent of all diabetes, but have a high penetrance. At least in the case of monogenic beta cell failure, environmental factors have less influence than in the more common forms of diabetes which are heterogeneous and caused by variation in multiple genes (Fig. 1).

Single-Gene Causes of Diabetes

Traditional family-based genetic studies, or studies of "candidate genes" in patients with rare familial forms of diabetes, particularly in the 1990s, had great success in identifying single gene defects causing diabetes. Since the application of next generation sequencing technologies over the past 10 years, the number of single gene forms of diabetes has increased enormously, although in some cases only a handful of families, or even a single individual have been reported. The genetic basis and characteristic features of a selection of currently known monogenic diabetes syndromes, including all those that are less rare, are summarised in Tables 1–3.

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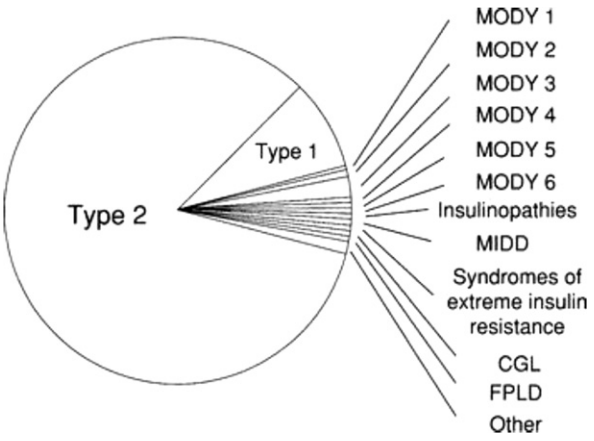

Fig. 1 Schematic illustrating approximate prevalence of different subtypes of diabetes. MODY, maturity onset diabetes of the young; MIDD, maternally inherited diabetes and deafness; CGL, congenital generalized lipoatrophic diabetes mellitus; FPLD, familial partial lipoatrophic diabetes mellitus.

Table 1 Selected forms of monogenic insulin-deficient diabetes

Syndrome	*Mode of inheritance*	*Gene*	*Gene product*	*Distinguishing features/Comment*
Permanent neonatal diabetes (c.50% neonatal DM)	Sporadic or AD	*KCNJ11*	Together form the ATP-sensitive potassium channel in beta cells	c. 40% PNDM
	Sporadic or AR	*ABCC8*		
	AR	*INS*	Proinsulin	c. 15% PNDM
	AR	*GCK*	Glucokinase	
Transient neonatal diabetes (c.50% neonatal DM)	Sporadic or AD with parent of origin effect	*PLAGL1 (ZAC)*	ZAC tumour repressor	Both genes are expressed only from the paternal allele. In TNDM expression is increased due to altered methylation, or due to an extra copy of the paternal allele. Accounts for c. 70% of TNDM.
		HYMAI	HYMAI	
	Sporadic or AD	*KCNJ11*	See above	Mutations generally distinct from those causing PNDM
	Sporadic or AR	*ABCC8*		
	AR	*INS*	Proinsulin	
Maturity onset diabetes of the young (MODY) 1	AD	*HNF4A*	Hepatic Nuclear Factor 4α	Low HDL and high LDL cholesterol common. Sensitive to sulphonylureas
MODY 2	AD	*GCK*	Glucokinase	Mild, non progressive hyperglycaemia only
MODY 3	AD	*HNF1A*	Hepatic Nuclear Factor 1α	Commonest form of MODY; Very sensitive to sulphonylureas; Glycosuria an early feaure
MODY 4	AD	*PDX1*	pancreatic and duodenal homeobox 1	Extremely rare
MODY 5	AD	*HNF1B*	Hepatic Nuclear Factor 1β	Developmental anomalies in kidneys, pancreas and genital trac common. Renal cysts most common
MODY 6	AD	*NEUROD1*	Neuro D1 transcription factor	Extremely rare
Maternally inherited diabetes and deafness (MIDD)	Maternal	*$tRNA^{Leu}$, deletions*	$tRNA^{Leu}$, deletions	Diabetes manifest mostly by decreased insulin secretion and also accompanied by deafness or milder degree of hearing loss

**KCNJ11*, *ABCC8* and *INS* cause up to 1% of MODY cases, but they are far better known for their role in neonatal diabetes (see below). Other genes have also been implicated in MODY including *BLK*, *KLF11* and *CEL1*.

Maturity-Onset Diabetes of the Young (MODY)

Maturity-onset diabetes of the young (MODY) was classically described as autosomal dominantly transmitted diabetes with onset during childhood or early adulthood, lack of obesity (in most cases), and impaired insulin secretion without evidence of antibodies against beta cells. Classical criteria suggested to identify MODY since the 1970s are diagnosis during the first to third decades of life of diabetes in two or more consecutive generations of the same family, with at least one member diagnosed under 25 years of age. More recent screening using modern genetic technologies has shown that some patients with sporadic diabetes also have MODY, and has also allowed a wider view of the phenotypic spectrum. This has lead to the suggestion that the term

Table 2 Selected monogenic forms of insulin resistant diabetes

Syndrome	*Mode of inheritance*	*Gene*	*Gene product*	*Distinguishing features/comment*
Primary Disorders of Insulin Signalling				
Donohue Syndrome (formerly leprechaunism)	AR	*INSR*	Insulin receptor	Extreme insulin resistance, impaired linear growth, paucity of fat and muscle but overgrowth of many other soft tissues. Death usually in infancy.
Rabson–Mendenhall syndrome	AR	*INSR*	Insulin receptor	Milder than Donohue Syndrome, also featuring impaired linear growth, soft tissue overgrowth and extreme insulin resistance. Abnormal dentition and nails, protuberant abdomen, and thick rapidly growing hair usual
Type A Insulin Resistance	Usually AD (may be AR or sporadic)	*INSR*	Insulin receptor	Severe insulin resistance with a normal lipid profile, no fatty liver, and preserved or elevated plasma adiponectin. Acanthosis nigricans, hyperandrogenism,
SHORT Syndrome	AD or sporadic	*PIK3R1*	p85α regulatory subunit of phosphatidylinositol-3-kinase	Short stature, Hyperextensibility or hernias, Ocular depression, Rieger's anomaly, delayed Teething. Lipodystrophy and severe insulin resistance common.
Severe insulin resistance	AD	*AKT2*	Serine threonine kinase AKT2 (protein kinase B)	Severe insulin resistance, found in one family only to date
Lipodystrophies				
Familial partial lipodystrophy (FPLD)	AD	*LMNA*	Lamin A/C	Loss of subcutaneous fat from whole body except head and neck and labia majora
	AD	*PPARG*	Peroxisome proliferator-activated receptor γ (PPARγ)	Lack of fat usually limited to the limbs, and may be subtle; hypertension common
	AD	*PLIN*	Perilipin	Limb lipodystrophy
Congenital Generalised Lipodytrophy (CGLD)	AD	*BSCL2*	Seipin	Mutations in these genes account for 95% of cases of congenital generalised lipodystrophy.
	AR	*AGPAT2*	1-acylglycerol-3-phosphate D-acyltransferase 2	
	AR	*PTRF*		Myopathy. Bone marrow fat may be preserved
	AR	*PCYT1A*	phosphate cytidylyltransferase 1 alpha (PCYT1A)	Also associated with spondylometaphyseal dysplasia + cone-rod dystrophy.

"MODY" be abandoned in favor of precise labeling with the relevant gene name, however the label "MODY" continues to be widely used. There are more than six known forms of MODY defined by the underlying genetic defects.

MODY2 is caused by a mutation in glucokinase, an enzyme produced in pancreatic beta-cells and important for glucose sensing and insulin secretion. Heterozygous loss-of-function mutations in glucokinase lead to an elevated setpoint for blood glucose, with glucose homeostasis otherwise preserved. Hyperglycemia in patients with MODY2 is typically mild, non progressive, and can be treated by diet. Chronic diabetic complications are very rare. MODY1, 3,4,5 and 6, in contrast, are caused by mutations in transcription factors that are expressed in the pancreas and are thought to play a role in beta-cell development and function. These include, most importantly, hepatocyte nuclear factors 4α (MODY2), 1α (MODY3), and 1β (MODY4). HNF1α is the most frequent gene mutated in MODY. Patients with MODY due to mutations in these transcription factors typically have a progressive decrease in insulin secretion with increasing endogenous insulin requirements. Long-term diabetes complications occur with similar frequency to more typical forms of diabetes. In addition, renal cystic disease is a common feature of MODY4 caused by HNF1β mutations.

One of the major challenges in population-wide management of MODY is now to ensure that those affected have access to diagnostic testing, which is particularly important as many patients with transcription factor MODY can safely be managed in the long term on oral sulphonylurea therapy, sometimes even managing to transition from to oral therapy after several years of injected insulin. A variety of different biochemical tests have been employed to aid calculation of MODY risk prior to genetic testing, which has greatly increased rates of diagnosis.

Neonatal Diabetes Mellitus (NDM)

Diabetes presenting in the first 6 months of life is almost invariably non-autoimmune in origin. It is rare and affects 1 in 90000–260000 births. Two subgroups exist, namely Transient Neonatal Diabetes (TNDM), which usually remits by 12 weeks but may relapse after some years and Permanent Neonatal Diabetes (PNDM). Mutations in >20 genes have now been associated with NDM.

Table 3 Selected complex diabetes syndromes

Syndrome	*Mode of inheritance*	*Gene*	*Gene product*	*Distinguishing features/comment*
Insulin-Deficient Diabetes				
Wolfram Syndrome	AR	*WFS1*	WFS1	Optic atrophy, diabetes insipidus, and deafness. Additional features may include renal abnormalities, ataxia, dementia or mental retardation.
Wolcott–Rallison syndrome	AR	*EIF2AK3*	Eukaryotic translation initiation factor 2-alpha kinase 3	Short stature, skeletal dysplasia
Thiamine-responsive megaoloblastic anemia syndrome	AR	*SLC19A2*	Thiamine transporter SLC19A2	Classic triad is megaloblastic anemia, diabetes, and deafness
Histiocytosis-lymphadenopathy plus syndrome (encompassing H syndrome)	AR	*SLC29A3*	Nucleoside transporter SLC29A3	Complex disease spectrum that may include short stature, organomegaly, hypertrichosis, exocrine pancreatic insufficiency, and multisystem inflammation.
Insulin Resistant Diabetes				
Alström syndrome	AR	*ALMS1*	ALMS1	Early onset blindness due to rod-cone dystrophy; sensorineural deafness; cardiomyopathy; obesity; renal failure
MOPDII	AR	*PCNT*	Pericentrin	Primordial dwarfism with skeletal dysplasia
Werner Syndrome	AR	*WRN*	Werner DNA helicase	Loss of peripheral fat and contractures, cataracts, greying hair, atherosclerosis and cancers in the 3rd decade
Bloom Syndrome	AR	*BLM*	Bloom DNA helicase	Short stature, cutaneous photosensitivity, hypogonadism
Primordial dwarfism with severe insulin resistance	AR	*NSMCE2*	NSE2 or MMS21 SUMO ligase	Primordial dwarfism, hypogonadism
Mandibular hypoplasia, Deafness and Progeroid features (MDP) syndrome	AR	*POLD1*	DNA polymerase delta, catalytic subunit	Selective loss of subcutaneous adipose tissue with contractures, sensorineural deafness, male hypogonadism

40% of PNDM is caused by activating mutations in the KCNJ11 and ABCC8 genes, encoding subunits of the pancreatic β-cell potassium-sensitive ATP channel. These mutations lead to severely reduced or absent insulin secretion, but, critically, this may be treated successfully with high doses of sulfonylurea. Some channel mutations also cause psychomotor developmental delay, epilepsy and diabetes, known as DEND syndrome. Insulin (INS) gene mutations cause around 15% of PNDM, leading to ketoacidosis and requiring lifelong insulin treatment. Biallelic inactivating mutations of GCK are a rare cause of insulin-dependent PNDM.

TNDM usually presents in the 1st week of life but disappears by the 12th week, although it recurs later in childhood in around 50% of cases. Insulin treatment is needed but can gradually be weaned. Although TNDM may be caused by mutations in KCNJ11, ABCC8 or INS, in most cases it is caused by abnormally increased expression of the PLAGL1 (also known as ZAC) or HYMAI genes on chromosome 6q24. This region is imprinted, with only the allele inherited from the father being expressed. Diabetes results when an extra copy of the paternal allele is inherited, when two paternal copies but no maternal copies of the locus are found, or when abnormal methylation and thus expression of the maternal allele is seen.

Primary Disorders of Insulin Signalling

Genetic syndromes of extreme insulin resistance may result from mutations in genes encoding key elements of the insulin signaling pathway that transmits the insulin signal into responsive tissues. Mutations in the insulin receptor gene itself, INSR, have been known since the late 1980s, and more than 150 mutations are now known. Mutations impair the ability of insulin to stimulate glucose uptake and use. Characteristically, glucose levels are normal to elevated and insulin levels are very high (due to compensatory increases in beta-cell insulin secretion). At least three distinct clinical syndromes of extreme insulin resistance exist: type A syndrome, Donohue syndrome (formerly known as leprechaunism), and Rabson–Mendenhall syndrome. Donohue and Rabson–Mendenhall syndrome are extremely rare, featuring mutations in both alleles of the receptor gene, and are inherited as an autosomal recessive disorder. Both conditions, as well as extreme insulin-resistant diabetes, feature failure to thrive and impaired linear growth, with soft tissue overgrowth including skin, hair, and viscera. They form a phenoypic spectrum broadly determined by the degree of loss of receptor function. Type A syndrome is considerably more common, and is usually autosomal dominant, being caused by mutations that have the ability to interfere with the function of the co-expressed wild type allele. Type A syndrome most commonly presents as a severe form of "polycystic ovary syndrome" in a lean adolescent girl with acanthosis nigricans, with diabetes and insulin resistance often only discovered during subsequent investigation. Males present much later, and indeed may commonly remain undetected, diagnosed simply as "Type 2 diabetes" in midlife.

The only other form of diabetes caused by a mutation of an insulin signaling gene, and known to affect more than a handful of individuals, is SHORT syndrome. SHORT denotes Short stature, Hyperextensibility or hernias, Ocular depression, Rieger's anomaly (a developmental abnormality of the iris), and delayed Teething. Although not featuring in the acronym, lipodystrophy and severely insulin resistance diabetes are common, although uniquely among lipodystrophic forms of diabetes, lipid profiles are characteristically benign. SHORT syndrome is caused by mutations affecting a regulatory subunit of the enzyme phosphaidylinositol-3-kinase, which is activated in cells by stimulation of the INSR.

Congenital Generalized Lipodystrophy

Congenital generalized lipoatrophic diabetes (CGL), or Berardinelli–Seip syndrome, is a rare, autosomal recessive disorder characterized by the near absence of adipose tissue from birth. This leads to severe insulin resistance with attendant clinical features (glucose intolerance or overt diabetes, hyperandrogenism and early puberty, acanthosis nigricans) as well as hypertriglyceridemia with enlarged, severely fatty liver, and prominent muscles. Mutations in either the BSCL2 gene, encoding an endoplasmic reticulum-associated protein called seipin, or in the AGPAT2 gene, encoding1-acylglycerol-3-phosphate-D-acetyltransferase 2, which plays a key role in triglyceride synthesis, account for around 95% of cases. CGL provide one of the stronges pieces of evidence for the critical importance of fat tissue in metabolic health: without adipose tissue in which to store excess ingested calories safely, lipid and other toxic metabolites accumulates in liver, muscle and elsewhere, blocking insulin action. Severe calorie restriction is an effective though very challenging treatment. Subcutaneous injections of the satiety hormone leptin, which is extremely low or absent in CGL, can blunt he increased appetite often seen in CGL and thereby facilitate dietary restraint, and is now licensed for therapeutic use in some parts of the world including the USA.

Familial Partial Lipodystrophy

Partial lipodystrophy refers to failed or abnormal development of adipose tissue in only some regions of the body. This is often inherited as an autosomal dominant disorder, and is the commonest inherited form of severe insulin resistance with diabetes. The prototypic and commonest form is known as Familial partial lipoatrophic diabetes (FPLD) type 2 (FPLD2), also referred to as Dunnigan-Köbberling familial partial lipoatrophy. It is an autosomal dominant disorder that results from mutations in the lamin A/C gene. Affected patients have apparently normal fat distribution until puberty, when failure of normal pubertal subcutaneous fat tissue accretion in the arms, trunk, and lower extremities unmasks the underlying disorder. The face is typically spared from fat loss, and fat may actually accumulate abnormally in the head and neck, labia majora, and intra-abdominal region. As in CGL, insulin resistant diabetes, dyslipidaemia (often leading to pancreatitis) and severe fatty liver are the norm, and are seen together with features of severe insulin resistance described above.

The second most common form of familial partial lipodystrophy, sometimes known as FPLD4, is caused by mutations in the PPARG gene, which encodes the nuclear receptor that is the target of the thiazolidinedione class of anti-diabetic pharmaceuticals. The degree of subcutaneous fat loss in this condition is often less striking than in FPLD2, with truncal fat often relatively preserved, and indeed in some cases the disorder features only an unusually centripetal pattern of fat mass rather than frank lipodystrophy. Early hypertension and pre-eclampsia appear to be particularly common in this subset of lipodystrophy.

Other Single-Gene Diabetes Syndromes

Many other rare single gene forms of diabetes exist, some of which feature the metabolic disorder only in the context of a much more complex, multisystem disorder. Detailed treatment of these is beyond the scope of this article, however a selection of the disorders is shown in Table 3.

Genetics of Common Forms of Diabetes

The single-gene forms of diabetes described so far constitute no more than 2–5% of cases of diabetes. The more common forms of diabetes are known to have important genetic underpinnings but are not inherited in a predictable pattern. This observation suggests that the common forms of diabetes are likely to be due to several (many) mutant genes that are relatively common in the population. Each gene variant individually is likely to have a modest effect, but together the mutant genes act additively or synergistically. Furthermore, environmental provocations are thought to have important effects on the likelihood that a given gene variant or group of gene variants will express themselves. Thus, the common forms of diabetes are classified genetically as being heterogeneous and complex.

Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) occurs predominantly in children and adolescents. In the United States, T1DM accounts for approximately 5–10% of all diabetic cases diagnosed. The incidence of T1DM is inexorably rising, but varies greatly worldwide, with the highest reported incidence in Northern Europe and the lowest rates in China, Korea, and Mexico. T1DM is characterized by autoimmune-mediated dysfunction and destruction of the beta-cells of the endocrine pancreas, resulting in insulin deficiency with potential for ketoacidosis,

coma, and death if untreated. The beta-cell loss begins months to years before the actual clinical onset of insulin dependency. Environmental factors such as viral infections and toxins have been suggested to play a key role in the pathophysiology of this disorder. These environmental provocations may activate cells of the immune system (T lymphocytes, B lymphocytes, and natural killer cells), initiating and perpetuating a chronic inflammatory reaction that ultimately leads to beta-cell loss and insulin deficiency.

The greatest genetic risk for T1DM lies in a cluster of genes on chromosome 6, termed the human leukocyte antigen (HLA) locus, which is known to modulate immune function. The presence of certain HLA haplotypes has long been known to predispose individuals to T1DM, whereas other HLA haplotypes appear to be protective. Even before the advent of "next generation" sequencing in the last decade, other genes or chromosomal loci had also been implicated in defining the risk of developing T1DM, including variants near the proinsulin gene and the cytotoxic T-cell lymphocyte associated-4 (CTLA4) gene. Nevertheless it is only with the development of technologies to analyze massive amounts of gene sequence data in parallel, and to compare these between patients with and without T1DM, that major inroads have been made into understanding the nature of the immunological perturbation underlying T1DM. Continuing work is uncovering the precise autoantigens that play a key role in initiation of beta cell autoimmunity, and is gradually elucidating the key role played by cytokines such as IL-2 in the propagation of the immune insult. As this work progresses, so further complementary advances are expected in both risk estimation for individuals, and in development of targeted immunotherapy to delay or halt the disease process.

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, accounting for at least 90% of all cases. Its prevalence has reached pandemic proportions, with 350 million people estimated to be affected worldwide. Hyperglycemia and overt diabetes result from underlying insulin resistance and impaired insulin secretion secondary to beta-cell dysfunction and failure. Unlike T1DM, the beta cell failure in T2DM is not autoimmune in origin. Both environmental factors (caloric excess and physical inactivity) and genetic predisposition have been implicated in the pathogenesis of T2DM. The strongest evidence for genetic predisposition to T2DM comes from identical twin studies (with 60–90% concordance rates) and studies that show clustering of the disease in families. Current estimates of heritability – that is, the proportion of the prevalence attributable to genetic risk – have ranged from 30–70%. However the underlying genetic risk factors are thought to be heterogeneous.

The first phase of research into the genetic basis of T2DM focused on genetic linkage studies within families, or on detailed study and comparison of candidate genes between affected and unaffected groups. However although such studies led to the identification of several putative T2DM susceptibility genes, not all of these stood up to replication studies, and in retrospect this research approach, limited by the genetic technologies of the time, appears very inefficient. The sequencing of the human genome, and the development of techniques to study common genetic variations across the whole genome in large populations using the so-called genome-wide association study (GWAS) approach, however, has transformed efforts to identify the genes underlying T2DM risk. GWAS-based studies have now identified more than 80 genetic variants associated with T2DM risk. A large minority of these play roles in pancreatic beta cell function or growth, and a smaller number are believed to be involved in insulin action in tissues. The roles played by many of the associations remain to be explained, however.

Despite the excitement around GWAS, only a small amount of the heritability of T2DM has so far been explained, and even the most comprehensive gene-based risk scores perform little better than panels of conventional clinical risk markers. Nevertheless, as the power of next generation sequencing is used in following up these GWAS studies, it is very likely that sufficient insights will be gained into the nature of T2DM susceptibility to make major differences to our understanding of its pathogenesis, and o development of possible novel treatment approaches.

Conclusion

Diabetes mellitus is a heterogeneous group of disorders defined by hyperglycemia. An increasing number of forms of diabetes are known to be caused by mutations in single genes. Although these single-gene forms of diabetes are rare (approximately 2–5% of cases), they have provided important insights into glucose regulation and into the underlying disease process. In some cases discovery of the genetic defect has led to dramatic, mechanism-based changes in the medical care of affected patients, as seen in the use of sulfonylureas in neonatal diabetes and MODY, and the use of leptin in CGL. By contrast, T1DM and T2DM are far more common and are due to the interaction of multiple genes with the environment. Identification of the genes responsible for susceptibility to these common forms of diabetes has been slower, but use of modern genetic technologies in large populations has recently dramatically accelerated elucidation of their genetic "architecture". As this work continues, personalized risk assessments and diabetes treatments based on individual genetic profiles will becomes increasingly practical aims in routine clinical care.

Further Reading

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Lifestyle Diabetes Prevention

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Introduction

Diabetes is a known major cause of premature mortality with high economic costs. The future predictions are also gloomy given that 1 in 10 people worldwide are expected to have type 2 diabetes (T2DM) by 2030 (IDF, 2015). The global prevalence of diabetes especially T2DM, is no longer a problem solely for the developed western world such as the UK and US, but there is an emerging trend of increasing prevalence in developing countries, especially in Asia. On the one hand, T2DM continues to rise in most populations around the world. For example, there was a 60% increase in diagnoses in the last 10 years and a 10% rise in National Health Service spend on drugs to manage this condition (Diabetes UK, 2015). On the other hand, Asia is set to become the 'epicenter' of this epidemic with noted proportionally higher levels of young to middle-aged people being diagnosed with T2DM (Shaw *et al.*, 2010). The consequences of diabetes epidemic are alarming, and a diabetes-related mortality event is recorded every 7 s, and unfortunately it is thought that current statistics underestimate the true prevalence, with almost 50% of cases undiagnosed (IDF, 2015).

Common diabetes determinants include excess body fat, poor nutritional habits, physical inactivity, high blood pressure, and family history of diabetes (IDF, 2015). Obesity in particular, is considered a significant contributory factor in the development of T2DM, especially that both obesity and T2DM cases continue to grow in parallel at an alarming rate (WHO, 2016). Despite major steps taken to reverse the trend by public health strategies, there has been increased growth in patient numbers and Diabetes, especially T2DM remains a significant cause of premature mortality in many countries, along with other major non-communicable diseases (NCDs) such as cardiovascular disease (CVD) and cancer (WHO, 2016).

Based on such determinants, appropriate prevention strategies have primarily focused on lifestyle interventions involving physical activity and diet. Diet and exercise prevention strategies focusing on pre-diabetes and high-risk individuals have conclusively shown significant reduction in T2DM incidence rate around the world, ranging from 28% to 58% (Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002; Pan *et al.*, 1997; Ramachandran *et al.*, 2006). Such evidence made essential for more countries to adopt community-based interventions to reduce obesity and T2DM (Diabetes UK, 2015), and emphasizes the role of lifestyle prevention using physical activity and healthy nutritional strategies. However, lifestyle prevention of diabetes also requires a personalized approach and an understanding of the inter- and intra-individual variability in prescribing and promoting a dietary or an exercise intervention. A personalized approach may also play an adjunctive role when applying pharmaceutical therapies in combination with lifestyle to prevent and manage diabetes.

This article discusses the latest research advances in lifestyle prevention of diabetes, with a particular focus on the role of physical activity, innovative nutritional and exercise-based interventions and their single or combined effectiveness. For example, certain functional foods have been scientifically proven to have unequivocal health benefits in preventing T2DM and associated NCDs. The health protective effectiveness of functional foods can be enhanced by a variety of physical activity patterns, including moderate intensity exercise, high-intensity training and strength training. However, the effectiveness of a multi-component lifestyle intervention needs to consider behavioral barriers and associated models to ensure long term adherence. The article will shed some light on how such lifestyle preventative benefits can fit within individualized and localized multi-component T2DM prevention and management models.

Understanding the Lifestyle Determinants of Type 2 Diabetes

Sedentary Lifestyle and Physical Inactivity

Physical activity is defined as any bodily movement produced by the skeletal muscles that use energy. This includes any daily activity such as walking, doing household responsibilities or gardening, or regular physical activities defined as "exercise" and organized physical activities as "sports" such as soccer. Sedentariness has been defined as any waking behavior characterized by an energy expenditure $\leq$1.5 METs (metabolic equivalent of task) while in a sitting or reclining posture such as seated computer use, driving, reading, watching TV or lying down (Sedentary Behavior Research Network, 2012).

Sedentary lifestyle is a major cause of obesity, overweight, and is directly and indirectly linked with major chronic diseases including diabetes and cardiovascular disease (WHO, 2016). The highest prevalence of sedentary behavior exists within the Americas and the Eastern Mediterranean region. Given that 31% of the world's population is physically inactive, amounting for 6% of mortality rates, and that 27% of diabetes cases are attributed to low activity levels (WHO, 2016), it is essential to understand the determinants of both sedentariness and physical inactivity in diabetes prevention.

Sedentary behaviors such as watching TV and prolonged sitting hours are now known causes of diabetes and cardiovascular disease (Hu *et al.*, 2004a,b; Alkhatib, 2016a). Approximately 55% of the waking day is spent in sedentary activity, and individuals

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who spend >10 h a day sitting have a 34% higher all-cause mortality risk than those who spend just one hour of their waking day sitting (Matthews *et al.*, 2008) which could be either a leisure-based or workplace-based sedentariness (Alkhatib, 2016b; Chau *et al.*, 2012). Epidemiological evidence reported that sitting activities such as one hour of watching TV is associated with 21.8 min reduction in life expectancy (Veerman *et al.*, 2012) whilst TV watching is associated with the risk of T2DM, cardiovascular disease and all-cause mortality, independently of physical activity levels (Grøntved and Hu, 2011). More alarmingly, the sedentary-related health risks, including CVD and mortality risk, may not be reversed by only meeting physical activity guidelines (Biswas *et al.*, 2015). Recent meta-analysis of data from >1 million men and women suggested that physical activity does not attenuate or eliminate the detrimental association of sitting time with mortality (Ekelund *et al.*, 2016), which suggests that sedentary behavior needs to be addressed independently of physical inactivity in T2DM prevention.

Sedentary lifestyle is even more dangerous for those with T2DM, who have been noted to take on average 2000 less steps per day compared to their non-diabetic counterparts (Liese *et al.*, 2013). A dose–response relationship exists between the volume of time of uninterrupted sitting and poor metabolic health (Owen *et al.*, 2010). Measures of glycemic control [insulin resistance, fasting glucose and 2-h postprandial glucose] were all associated with sedentary time and patterns in individuals with T2DM (Sardinha *et al.*, 2017). Recent objective assessment of sedentary and physical activity levels using accelerometers, showed that adults with T2DM were not sufficiently active (<30 min day^{-1} of moderate activity), were highly sedentary (over 9 h day^{-1}), and with their BMI positively correlated with time spent in sedentariness (Mathe *et al.*, 2017). Similar sedentary associations have been reported for hyperglycemia and insulin-resistance levels over 1 year of using concurrent wrist accelerometry and continuous glucose monitoring sensor monitoring T2DM patients (Fritschi *et al.*, 2016). Therefore, reducing sedentary behavior requires further action that is additional to that of promoting physical activity.

Physical activity levels of and exercise capacity are known to be associated with reduced risk of mortality and chronic diseases including diabetes (Myers *et al.*, 2002). Physical activity reduces the risk of several T2DM risk factors especially obesity, and physically active obese appear to have a lower risk of CVD and mortality than lean sedentary individuals (Blair and Brodney, 1999). Several prospective observational studies have reported an association between physical activity and incident diabetes in established cohorts of men and women from different countries (Hu *et al.*, 2004a; Hsia *et al.*, 2005; Wannamethee *et al.*, 2000).

Prospective associations have also been established between physical activity levels in people with T2DM and reduced mortality risk (Church *et al.*, 2005; Hu *et al.*, 2004b), suggesting physical activity levels as a vital prognosis. Evidence from prospective cohort studies and meta analyses involving individuals with diabetes from the EPIC study (European Prospective Investigation Into Cancer and Nutrition) has demonstrated that the lowest mortality risk for people with diabetes was observed in moderately active persons (hazard ratios were 0.60 for all-cause mortality) compared with the physically inactive (Sluik *et al.*, 2012). This suggests that even small amounts of physical activity are beneficial in both prevention and management of diabetes.

Effects of Exercise and Physical Activity on Preventing and Managing T2DM

The role of physical activity training in preventing and managing diabetes is well established through global prospective studies have shown that moderate-to-vigorous physical activity is associated with a 28%–58% reduction in the risk of developing T2DM (Pan *et al.*, 1997; Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002). Both the Finnish Diabetes Study (Tuomilehto *et al.*, 2001) and the US Diabetes Prevention Program (Knowler *et al.*, 2002) have remarkably demonstrated a 58% reduction in T2DM incidence. One showed that moderate exercise, even without achieving target weight-loss induces significant risk-reduction reaching 70% (Tuomilehto *et al.*, 2001; Laaksonen *et al.*, 2005), and the other showed 58% reduced incidence rate compared with taking Metformin (Knowler *et al.*, 2002). More recently the Look Ahead Multicenter Study concluded that enhancements in moderate to vigorous exercise significantly improves the management of cardiovascular diseases risk factors in T2DM participants, and thereby reduces medication use and associated treatment cost (Redmon *et al.*, 2010).

Physical activity levels are often expressed by its main determinant, which is aerobic power or cardiorespiratory fitness, among other environmental and genetic determinants such as age, sex, health status and genetics (LaMonte *et al.*, 2005). Aerobic endurance exercise has been the traditional exercise prescription for those with T2DM, but this have evolved to include resistance training for its added cardio-protective benefits for people with T2DM (Yavari *et al.*, 2012). Current exercise recommendations for adults advocate doing aerobic exercise at a moderate intensity for 30 min on minimally 5 days (but preferably most days) of the week. This should be supplemented with strength-based exercise for the large muscle groups on at least 2 days of the week (IDF, 2015), while an alternative adult exercise prescription of 75 min of vigorous aerobic activity (coupled with two days of strength training) also exists (ACSM, 2009).

Several physiological mechanisms have been found to explain the T2DM adaptations associated with such preventative interventions (Sardinha *et al.*, 2017). These include exercise-stimulated signal transduction, which can restore glucose metabolism in insulin-resistant muscle through both acute activation of glucose transport and by improving insulin sensitivity for up to 48 h after exercise (Sylow *et al.*, 2017), partial or complete remission of T2DM in 11.5% of T2DM diagnosed participants within the first year of intervention and an additional 7% had partial or complete remission of T2DM after 4 years of exercise intervention (Gregg *et al.*, 2012), and potential epigenetic adaptations associated with transient changes in DNA methylation in adult skeletal muscle following acute exercise (Barrès *et al.*, 2012; Hargreaves, 2015), that may improve glucose homeostasis.

There are recent suggestions about similar or superior metabolic health benefits from a shorter duration, more intense physical activity types, and the term "high intensity interval training (HIIT)" has been recently proposed, as a time-efficient strategy for

T2DM prevention (Francois and Little, 2015; Rynders and Weltman, 2014; Gibala, 2009). HIIT training consists of repeated short bouts of intense exercise (usually above 80% of maximal oxygen uptake or age-predicted heart rate) lasting for about 1–4 min in duration, followed by approximately equal periods of low-intensity exercise (Gibala, 2009). Evidence is emerging of HIIT effectiveness across different T2DM patient cohorts including enhanced postprandial glycemic control, hepatic and improved muscle insulin resistance (Francois and Little, 2015). For example, reported effects included, a reduction in 24 h glucose levels and a reduced 3-h postprandial glucose, and increased muscle mitochondrial capacity (citrate synthase activity, protein content) in obese T2DM patients (Little *et al.*, 2011). Others reported a reduced insulin resistance after each session of a 4xHIIT sessions in 40-years old T2DM patients (Shaban *et al.*, 2014). Conversely, others reported no additional benefits for increasing the exercise training intensity when participants with T2DM are matched for their energy cost, suggesting that either intensity is equally effective in lowering blood glycated hemoglobin and increasing whole body and skeletal muscle oxidative capacity in obese T2DM patients (Hansen *et al.*, 1999). Interesting comparison was made between HIIT and resistance-type exercise training for 12-weeks, showing that although HIIT is superior in terms of enhancing mitochondrial capacity (protein synthesis), either HIIT or resistance training improves insulin sensitivity, with better combined effects on both hypertrophy and aerobic and mitochondrial capacity in groups of young and older adults (Robinson *et al.*, 2017), suggesting a combined HIIT and strength training approach. However, research is still needed to demonstrate how to define and implement such a strategy as part of a lifestyle prevention and achieve long-term adherence (Biddle and Batterham, 2015). The generalizability of the outcomes from most of the HIIT studies are often hampered by small sample sizes, acute effects nature, training supervision requirement, participants' adherence and injury, and presumed low levels of fitness in people with T2DM, all factors to be still considered before promoting HIIT compared with lower intensity exercise regimes as a diabetes prevention strategy.

Effective physical activity strategies focus on long-term adherence and behavior management using professional behavioral support with the emphasis on the individual and their needs, utilizing their support networks, and are community-based (Daly *et al.*, 2002; Cooper *et al.*, 1999). This is especially important in the absence of an instructor-led exercise intervention, those who are returning to leisure-time exercise following diagnosis can lack confidence and knowledge regarding starting a routine and lifestyle behavior change has been noted to be challenging for those with T2DM (Simonavice and Wiggins, 2008; Ibrahim *et al.*, 2002).

For high-risk individuals who have been physically inactive or sedentary for a long time, conditioning to physical activity in a safe and progressive exercise prescription is important to adopt and adhere to physical activity (Cooper *et al.*, 1999; Daly *et al.*, 2002). For example, adding on 5 min per day of moderate aerobic exercise each week, till the target goal of 150 min week^{-1} is reached, or simply increasing the volume of walking and reducing the volume of sitting (IDF, 2015). Pedometers are noted to be effective motivational and cost-free tools to advancing volumes of walking-based activity, and a baseline of 3000 steps day^{-1} can be used to build upon, adding a little more within the daily routine until the target 10,000 steps day^{-1} is reached (Minsoo *et al.*, 2009; Yates *et al.*, 2014). Home-based exercise could allow a combined aerobic and resistance-based physical activity routines in a safe environment. For example, resistance-band exercise routines can recruit large muscle groups and positively influence lower limb blood flow, and functional strength in people with T2DM (Egaña *et al.*, 2010). Self-managed home-based routines may be able to demonstrate exercise adherence and strength gains with dropout reduced if there is supported instruction and guidance initially (Chan and Ried, 2013). Furthermore, increased compliance can be achieved partners, friends or relatives can also participate with such exercise interventions (Hammond *et al.*, 1997). There is a merit for home-based exercise for the prevention of T2DM additionally to other efforts within the community and local healthcare settings, especially in terms of adherence and overcoming psycho-social barriers and the individually-adapted approach (Cameron and Alkhatib, 2016).

Role for Nutrition in Diabetes

Poor nutritional habits, including reduced calorific expenditure coupled with excess energy intake (calorie-dense, high sugar, high fat diets) has led to increased proportions of the adult population with reduced insulin sensitivity, most of whom are obese (Edelman, 1998; Lieberman, 2003). Truncal (abdominal) obesity, has been linked to raised levels of plasma leptin, fatty acids and tumor necrosis factor-alpha (TNF-α), all are thought to contribute to insulin resistance. The obesity related peptides or adipocytokines such as resistin, adiponectin and ghrelin can lead to higher baseline levels of *E*-selectin, intra-cellular and extra-cellular adhesion molecules, which increase the likelihood of endothelial cell adhesion, promoting the progression of insulin resistance to type 2 diabetes and endothelial dysfunction to atherosclerosis (Vendrell *et al.*, 2004). There can also be a release of protein kinase, heightening production of basement membrane materials and increasing capillary permeability. This pathophysiology coupled with raised levels of free fatty acids, post-prandial hyperlipidaemia and increased production of free radicals contributes to atherosclerosis and cardiovascular disease (Skilton *et al.*, 2005).

Effective Diets and Nutrition in the Prevention and Management of T2DM

While it is established that dietary intake is a key modifiable factor in the prevention and management of T2DM, variety of food patterns comprising healthy food components have been proposed to have health protective benefits. Those diets include Mediterranean style diet, Nordic diet, vegetarian diet, traditional Korean diet, Japanese diet, Dietary Approaches to Stop Hypertension (DASH) diet, and low-glycemic-index diet, and all have shown various degrees of effectiveness in

preventing T2DM or reducing CVD risk in people with diabetes (Archundia Herrera *et al.*, 2017). Common healthy food ingredients within such diets rely on higher intake of plant-based fruits (e.g. berries), vegetables (leafy green vegetables), legumes (including nuts and seeds), wholegrain (e.g. oat), and seafood-based (fish and shellfish), olive oil and rapeseed oil, and lower intake from dairy and meat products.

The Mediterranean diet (MD) has long been known to be one of the healthiest diets associated with longevity and prevention of metabolic and cardiovascular disease (Willett *et al.*, 1995), and is now an established prevention of mortality (Estruch *et al.*, 2013; Salas-Salvado *et al.*, 2014; Salas-Salvado *et al.*, 2008). A typical MD comprise of high intake of plant-based food items such as fruit, vegetables and legumes, a high intake of olive oil, tree nuts and oily fish, moderate intake dairy products and red wine, and low intake of red wine (Martinez-Gonzalez *et al.*, 2012). It has been suggested that MD components provide a model for their joint effectiveness in prevention and management of T2DM (ADA, 2017). Collectively MD components contain a significant amount of polyphenol compounds from both plant based foods (fruits, vegetables, olive oil, tree nuts), and seafood (e.g. oily fish), which makes some researchers attribute MD health protective benefits to polyphenols (Perona *et al.*, 2006; Urpi-Sarda *et al.*, 2012; Alkhatib *et al.*, 2017). Herbs and spices are included sometimes instead of salt are also rich in polyphenols, among other compounds (Esposito *et al.*, 2017).

Healthy MD components are traditionally consumed in regions bordering the Mediterranean region, where MD was associated with longevity (Willett *et al.*, 1995). However, Adherence to MD is dwindling with the spread of westernized diets in such regions (Sofi *et al.*, 2005; Kontogianni *et al.*, 2008; Jimenez-Redondo *et al.*, 2016). Conversely, MD components are not exclusive to any geographical region, and there are promising findings about the implementation of MD in non-Mediterranean regions (Alkhatib and Klonizakis, 2014; Middleton *et al.*, 2015).

Epidemiological studies have long shown an inverse relationship between MD consumption and incidence of T2DM (Panagiotakos *et al.*, 2005) and more recently a reduced risk of gestational diabetes (Karamanos *et al.*, 2014). Additionally, several recent systematic reviews and randomized controlled trials have demonstrated better T2DM management, and enhanced metabolic state with high-risk individuals, including impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and metabolic syndrome, associated with MD consumption (Salas-Salvado *et al.*, 2014; Esposito *et al.*, 2015; Ley *et al.*, 2014).

Compared with control diets, MD adherence has been shown to reduce glycosylated hemoglobin A1c (HbA1c) levels by 0.30%–0.47% in T2DM patients (Esposito *et al.*, 2017), and longitudinally is associated with 14.7% and 5% reduced reliance on medication 1 and 5 years post-diagnosis, respectively compared with low-fat diet (Esposito *et al.*, 2014). Prospective analysis of the PERIMED study (Prevención con Dieta Mediterránea) results of 1–5 years have also shown an inverse T2DM incidence rate associated with MD consumption compared with low fat diet (Salas-Salvado *et al.*, 2014; Salas-Salvado *et al.*, 2008). Recent meta-analyses have demonstrated that adherence to MD components of fruit, vegetables and legumes (measured by MD 1–9 adherence score, and 136-item food frequency questionnaire) (Martinez-Gonzalez *et al.*, 2012) reduces incidence rates irrespectively of obesity changes (indicated by Body Mass Index; BMI) during 9.5-year follow-up, and suggesting that MD may attenuate the adverse effects of obesity on the risk of T2DM (Eguaras *et al.*, 2017). MD is not necessarily based on caloric restriction or aimed at reducing weight as an outcome, since some of its central elements are high in energy, but rather an effective and easy to adopt method for diabetes prevention and management.

Other healthy diets have also been recommended based on locality, availability of healthy of variety of healthy food components, such as those forming" Nordic diet". Nordic diet is described by higher consumption of locally available plant-based vegetables, fruits such as oat, wholegrain, berries, rapeseed oil, and sea foods such as fish, shellfish from, and reduced intake of sweetened beverages and salt (Bere and Brug, 2009; Olsen *et al.*, 2011). Such foods have been shown to promote decreased improved several biomarkers associated with positive effects on metabolic profile and insulin sensitivity indicated by changes in LDL, HDL, apolipoproteins, and IL-1 Ra levels (Uusitupa *et al.*, 2013). Nonetheless, large diabetes prevention studies have focused more on an overall lifestyle modification, where a hypocaloric or isocaloric intake of common healthy ingredients was the focus (Knowler *et al.*, 2002; Tuomilehto *et al.*, 2001). For example, the Finnish Diabetes Risk Score considers daily consumption of fruits, berries, or vegetables as categorical variables, embedded with other lifestyle determinants such as age, BMI, waist circumference, history of antihypertensive drug treatment and high blood glucose and physical activity (Lindström and Tuomilehto, 2003).

Functional Foods in the Prevention and Management of Diabetes

Irrespective of the dietary approach or diet name, there are common foods with biologically active ingredients that are considered "functional" based on their association with physiological health benefits related to the prevention and management of T2DM (Alkhatib *et al.*, 2017). Regular consumption of functional foods has been linked with numerous positive effects on glycemic control, blood pressure regulation, activation of antioxidant enzymes, gut microbiota and suppression of over-production of pro-inflammatory cytokines during diabetes, suggesting better prevention and management of diabetes and associated complications (Mirmiran *et al.*, 2014).

For example, the components of the MD can be used a model for how various combinations of functional foods induce health protective benefits. Functional foods present within the MD contain polyphenols, terpenoids, flavonoids, alkaloids, sterols, pigments and unsaturated fatty acids play an important role in maintaining wellness, and contribute to preventing T2DM and associated disease including cancer, obesity, asthma, and cognitive decline (Vasto *et al.*, 2014; Alkhatib, 2015). Preventative actions such foods include an enhanced anti-oxidant, anti-inflammatory, insulin resistance and sensitivity and cholesterol lowering properties (Georgoulis *et al.*, 2014).

The benefits of MD components in T2DM have been attributed to specific nutraceuticals within MD food components including monounsaturated fatty acids (MUFA) such as oleic acid in olive oil, polyunsaturated fatty acids omega-3 (alpha-linolenic acid) found in tree nuts such as walnuts (Salas-Salvado *et al.*, 2008), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in oily fish, high amounts of flavonoids and antioxidants found in fruits and vegetables (Muraki *et al.*, 2013), and high amount of fiber found mainly in cereal and whole-grain foods with a low Glycemic Index (GI) (Gil *et al.*, 2011; Tighe *et al.*, 2010). For example, some studies have underlined the importance of olive oil fatty acids, including oleic acid, phytosterols (Beta-sitosterol), antioxidants (alpha-tocopherol), and plant polyphenols in reducing inflammation, oxidation and determining improvements in the endothelial micro- and macro-vascular function (Archundia Herrera *et al.*, 2017, Bahorun *et al.*, 2010). Such effects are known to have preventative roles in both T2DM and CVD. Others highlighted the importance of fruit and vegetable intake to reduce T2DM risk (Muraki *et al.*, 2013, and conversely low intake of such nutrients is linked with and increased disease risk and even mortality (Ezzati and Riboli, 2013).

It is not possible to attribute diabetes risk-reduction benefits to a single functional food or a nutraceutical in MD. Epidemiological studies attempting to link specific MD components to T2DM risk-reduction have found conflicting associations (Ley *et al.*, 2014). For example, omega-3 fatty acids, obtained from fish and seafood were only associated with reduced T2DM risk in Asian populations, but not in European or North American populations (Wu *et al.*, 2012; Ley *et al.*, 2014). Others have also shown that longitudinal adherence to low fat diets did not lower T2DM or CVD risk in postmenopausal women (Tinker *et al.*, 2008). There may be some key functional MD components such as extra-virgin olive oil and tree nuts that have been associated with metabolic mechanistic protective effects such as reducing serum C-Reactive Protein (CRP), interleukin-6 (IL-6) and endothelial and monocyte adhesion molecules in high-risk men and women (Peyrol *et al.*, 2017). Others have also attributed the protective benefits of MD in T2DM (improved insulin resistance, glucose control, and other cardiometabolic risk factors) to the polyphenolic content, especially flavan-3-ols and their food sources (fruits, vegetables, whole grains, and legumes, and also tea, coffee, red wine and cocoa) (Guasch-Ferre *et al.*, 2017).

The latter reviewed a sporadic evidence about the effectiveness of polyphenol lignans-rich foods (such as flaxseeds) in reducing insulin, glucose and CRP levels and improving HOMA-IR in selected patient groups, and a contrasting epidemiological evidence for total flavonoid intake association with T2DM risk was reported (Guasch-Ferre *et al.*, 2017). Polyphenol-rich olive products, including olive leaves, their crude extract, and extra virgin olive oil, were also reviewed elsewhere for their partial effective on aspects of the metabolic syndrome (Saibandith *et al.*, 2017). Whereas, non-flavonoid polyphenolic compound hydroxytyrosol, the main polyphenol of olive oil, has been shown to improve the lipid profile, glycaemia, and insulin sensitivity, and counteract oxidative and inflammatory processes (Peyrol *et al.*, 2017), and resveratrol (found in grapes, grape products) has been shown to increase intra-cellular transport of glucose and reduce insulin secretion, using various animal and tissue models (Szkudelski and Szkudelska, 2011), conferring several benefits for prevention and management of T2DM. Nevertheless, the largest amounts of polyphenols in diets in many populations are derived from coffee, and in Asia from tea, and both of them are inversely associated with reduced risk of T2DM (Matusheski *et al.*, 2012) and reduce low-grade inflammation (Kempf *et al.*, 2010). While each functional food has its own unique characteristics and protective benefits, we recommend following a holistic approach to implement functional foods into variety of dietary approaches within diabetes lifestyle prevention.

Herbal Ingestions and their Protective Role

Herbal ingestions are common among many cultures for centuries. Numerous medicinal plants and natural products have been reviewed for its anti-diabetic functions showing various degrees of effectiveness in preventing and managing T2DM. Examples include fukugetin, palmatine, berberine, honokiol, amorfrutins, trigonelline, gymnemic acids, gurmarin, phlorizin, aloe, banaba, bitter melon, caper, cinnamon, cocoa, coffee, fenugreek, garlic, guava, gymnema, nettle, sage, soybean, green and black tea, turmeric, walnut, and yerba maté (Rios *et al.*, 2015). Reported functions of such ingestions include for instance the inhibition of α-glucosidase and α-amylase enzymes that regulate glucose absorption from the gut, effects on glucose uptake and glucose transporters, modification of mechanisms mediated by the peroxisome proliferator-activated receptor, inhibition of protein tyrosine phosphatase 1B activity, modification of gene expression, and activities of hormones involved in glucose homeostasis such as adiponectin, resistin, and incretin, and reduction of oxidative stress (Rios *et al.*, 2015).

Whilst taking a multi-component to diabetes prevention, it is important to highlight some of the literature around some of the common herbal ingestions, in particular, herbal tea ingestions to prevent and manage T2DM. Several local teas have become popular worldwide because of their popularized health and disease prevention benefits. For example, black and green teas primarily native to south Asian countries are now consumed worldwide, and yerba maté, native to South America is now consumed by millions of people in North America, and parts of Europe and the Levant (Heck and de Mejia, 2007, Zheng *et al.*, 2013).

Tea specific phenolics, particularly green tea includes (+)-catechin and epigallocatechin gallate (ECGC), which suppress oxidative stress, inflammation and cell death, via activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, leading to the upregulation of antioxidant response element (ARE) gene expression, and enhanced protective enzymes and free-radical scavengers (Zheng *et al.*, 2013). Nrf2 pathway is also influenced by coffee drinking (Carstensen *et al.*, 2013). Antioxidant compounds in green and black tea have been demonstrated to interact with reactive oxygen species and redox active transition metal ions using a multi-antioxidant assay system (Bahorun *et al.*, 2010; Bahorun *et al.*, 2012). In number of randomized and

clinical trials, it was shown that Mauritian black and green tea intake reduces levels of fasting blood glucose (− 18.4%), triglyceride (− 35.8%), LDL/HDL plasma cholesterol ratio (− 16.6%), and C-reactive proteins, and increases plasma antioxidant propensity (Ferric Reducing Antioxidant Power (FRAP): + 418%) in healthy adults (Bahorun *et al.*, 2010; Bahorun *et al.*, 2012). Black and green teas have also been shown to suppress advanced glycation end products formation and their induced oxidative stress in 3T3-L1 preadipocytes, and that consumption of both herbs induces comparable cellular protection against glycation and antidiabetic potential (Ramlagan *et al.*, 2017).

Beyond their significant antioxidant capacity, polyphenols present within cocoa, coffee and yerba maté, include phenolic compounds with different functions. These include caffeoyl derivatives, procyanidins and chlorogenic acid, all have demonstrated ability to influence insulin sensitivity, vascular endothelial function, fat and carbohydrate metabolism, and inflammatory mediators (Mao *et al.*, 2000; Johnston *et al.*, 2003; Rodriguez-Ramiro *et al.*, 2011; Alkhatib and Atcheson, 2017).

For example, several animal studies have suggested that yerba maté can be effective for preventing and treating metabolic disorders including diabetes (Heck and de Mejia, 2007). Only recently, human studies have shown such potential in healthy (Alkhatib, 2014; Alkhatib and Atcheson, 2017), and obese individuals (Kim *et al.*, 2015). The acute yerba maté effects includes 24% augmented fatty acids oxidation, and energy expenditure during exercise compared with exercise alone (Alkhatib, 2014). This was combined by significant positive effects on mood state (focus, energy, and concentration), and appetite and satiety measures (hunger, prospective eating, and desire to eat) in both resting exercise conditions following yerba maté ingestion (Alkhatib and Atcheson, 2017). Chronic ingestion of yerba maté have been shown to enhance weight-loss outcomes after 12 weeks of ingestion in obese individuals (decrease body fat mass, percent body fat and waist to hip ratio) (Kim *et al.*, 2015). Such effects have been explained by thermogenic gene mRNA expression in white adipose tissue (WAT) and decreased fatty acid synthase mRNA expression in WAT, which may be linked to observed decreases in body weight, WAT weight, epididymal adipocyte size, and plasma leptin level (Choi *et al.*, 2017).

These studies suggest that the use of popular herbal teas (e.g. green tea, back tea and yerba maté) have direct and indirect protective outcomes for T2DM. Positive metabolic and behavior adaptations related to herbal intake, especially when combined with physical activity are essential outcomes for designing an optimized lifestyle prevention for metabolic health and diabetes prevention. However, more human studies are needed, and findings need to be integrated into lifestyle intervention studies, involving behavioral components, especially exercise, and to test different the effectiveness and safety of different doses among high-risk populations.

Effects of Combined Physical Activity and Nutritional Interventions, and Multi-Component Models

A multi-component approach which encompasses behavioral and physical aspects is likely to be more effective than a single component diabetes prevention program. Even based on observational studies, it has been found that among older adults (60–80 years), those who consumed MD are more likely to have an active lifestyle compared with those who consume a western diet (Bibiloni *et al.*, 2017). For the prevention of T2DM incidence, it is established that lifestyle interventions combining variety of physical activity patterns with different dietary regimes are more effective than either exercise, diet or medication alone (Knowler *et al.*, 2002; Tuomilehto *et al.*, 2001). The US Diabetes Prevention Program reported significantly better risk reduction benefits (58%) when exercise and diet approach was used compared with 31% when insulin-sensitizing drug Metformin was used (Knowler *et al.*, 2002), and a 15 year follow up still showed that medication did not elicit a better risk-reduction benefit (15 year follow up on DPP).

When a healthy diet, such as the MD, was added to exercise training intervention, combining physical activity with healthy diets such as the MD, is likely to trigger or augment additional protective functions including reduced lipid peroxidation and anti-inflammatory functions, which reflect a better microvascular and macrovascular function and an improved cardiorespiratory capacity in high-risk and older populations (Alkhatib and Klonizakis, 2014; Alkhatib, 2015; Klonizakis *et al.*, 2013). On the one hand, MD added to exercise training as part of short and long term interventions combining MD with moderate-to-heavy exercise, produced a better increase in endothelial vascular activity and cardiorespiratory capacity than exercise alone (Alkhatib and Klonizakis, 2014; Klonizakis *et al.*, 2014). On the other hand, adding a weight-loss program (strength training) to a MD adherence program, doubled the protective immunological protective functions in high-risk postmenopausal women (Richard *et al.*, 2013). A 26% reduction in C-reactive protein (CRP) concentrations and a 10% reduction in an arbitrary inflammatory score that included, interleukin-6 (IL-6), IL-18, and Tumor necrosis factor (TNF)-α when the group followed MD only, while when a weight loss program was combined with MD, the reduction in inflammatory markers was almost doubled for plasma IL-6 (− 21%) and IL-18 (− 15.6%) compared with the control diet, and with no significant impact on CRP concentration (Richard *et al.*, 2013). Another study in people with metabolic syndrome, has also shown that a combined hypocaloric MD with 12 weeks of moderate-to-heavy exercise training, is more effective than MD alone in enhancing physical aspects (weight loss, physical fitness and improvement of metabolic syndrome risk factors) and mental domains of health-related quality of life measures (vitality, general physical health, emotional role, and self-perception of health) (Landaeta-Diaz *et al.*, 2013).

The role of pharmacological therapies may also play an adjunctive role in combination with lifestyle to prevent diabetes. Glucose-lowering agents such as metformin, sulphonylureas, thiazolidinediones, insulin, newer incretin-based therapies and sodium glucose co-transporter 2 inhibitors have been commonly reported in diabetes prevention trials with varying success (Chatterjee *et al.*, 2018). Non-glucose-lowering therapies such as orlistat and renin angiotensin system blockers can also prevent

diabetes, whereas statins are associated with slightly increased risk, but can be effective in personalized patient circumstances and phenotypic profile (Chatterjee *et al.*, 2018).

Personalized Lifestyle Diabetes Prevention

Designing an individualized and locally-tailored lifestyle and physical activity recommendations to prevent, treat and manage diabetes, considering innate and environmental factors. The personalized profiling of individuals' molecular characteristics such as genetics (DNA sequence), epigenetics (DNA modification), transcriptomics (gene expression), proteomics (protein products of coding genes), metabolomics (metabolite products of metabolic pathways) and microbiome (bacteria species interacting with host) at different levels has been coined as "OMICS" or "Personalized Medicine" (Chen and Snyder, 2013; de Toro-Martin *et al.*, 2017), also called as "precision medicine". Utilizing the personalized approach in a comprehensive lifestyle diabetes prevention strategy, requires a wider understanding of the behavioral as well as the biochemical and genetic factors governing diabetes, as recently proposed (Alkhatib *et al.*, 2017), and updated (**Fig. 1**).

For example, determining the responders vs. non-responders to diabetes intervention can rely on lifestyle factors determining long-term adherence such as exercise enjoyment (self-selected exercise), physical activity training intensity (individualized prescription based on individuals' cardiorespiratory and metabolic response during exercise), and age and gender-specific risks (e.g. post-menopause related risks) (Alkhatib and Klonizakis, 2014). It can also rely on cellular metabolomics and transcriptomics mediators (e.g. IL-6, TNF-α, GRP78) and genes expression (e.g. DUSP1), which have been linked to differential responses to exercise intervention in obese individuals (Tiss *et al.*, 2014; Khadir *et al.*, 2015). The latter found that IL-6, TNF-α, and DUSP1 were all decreased in some but not all obese individuals who followed a 12-week exercise intervention, and similarly only some subjects displayed an improvement in the profile of lipids (LDL, HDL, TG, cholesterol) and glucose (HbA1c and fasting blood glucose), despite no BMI overall change.

Integrating a personalized approach for preventing and managing T2DM should consider both biological and behavioral models, and embed nutrition education and counseling as part of lifestyle diabetes prevention studies. For example, understanding the barriers to changing sedentary behavior, increasing physical activity or diet requires studying the barriers and facilitators to changing behavior and considering the psycho-social determinants such as the importance of purpose, motivation, and attitudes associated with changing behavior, which meets the needs of the individual, family and local community (Aitaoto *et al.*, 2017). Such approach can be effective in enhancing specific outcomes associated with nutritional interventions, such as assisting individuals in adopting new dietary approaches, such as adherence to non-geographical dietary style (Alkhatib and

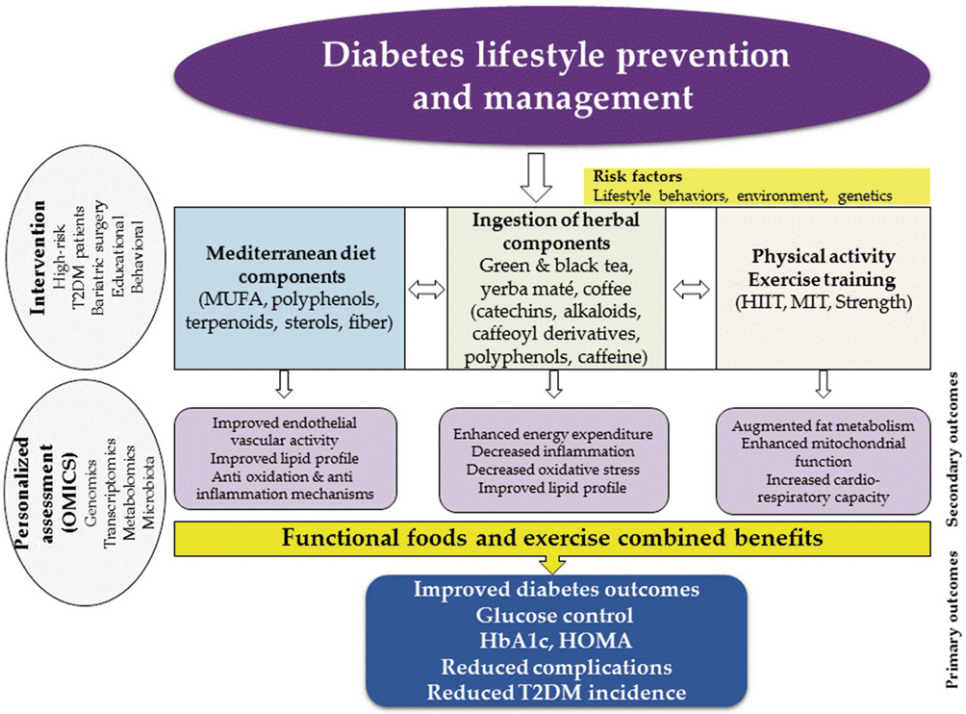

Fig. 1 Integration model of diabetes lifestyle prevention and management to understand biological processes and improve clinical outcomes. MUFA (monounsaturated fatty acids), HIIT (high intensity interval training), MIT (moderate intensity training), HbA1c (glycosylated hemoglobin A1c) HOMA (homeostatic model assessment), T2DM (type 2 diabetes mellitus).

Klonizakis, 2014; Middleton *et al.*, 2015, Martinez-Gonzalez, 2016; ADA, 2017). It can also be effective with those who follow an invasive metabolic and bariatric surgeries (Moize *et al.*, 2016; Eickhoff, 2017). Nonetheless, adapting individualized tools to make combined behavioral and biological changes as part of lifestyle diabetes prevention requires further research.

Overweight and Obesity

Obesity is a complex, adiposity-based chronic disorder, and the major risk indicator for T2DM.

The global prevalence of both overweight and obesity is now widely recognized as the major epidemic of the 21st century. Assessment of overweight and obesity in the clinical setting is done by calculating the body mass index (BMI): overweight is defined as a BMI of 25–29.9 kg m^{-2} and 22–27.9 kg m^{-2} in individuals with an Asian ethnic background whereas obesity is defined as a BMI >30 and $\geq$ 28 kg m^{-2} in individuals with an Asian ethnic background. It has been suggested that the BMI cutoff of $\geq$23 kg m^{-2} is an optimal criterion for screening in East and South Asian ethnicities based on correlations with cardiometabolic risk (Jensen *et al.*, 2014).

Obesity usually arises from the interactions between genetic and environmental factors and is associated with weight-related complications which cause significant rates of morbidity and mortality. People can easily monitor their weight, but it is also important to record weight at health service encounters for people considered overweight or obese.

Meta-analyses have assessed the association between obesity and diabetes (Abdullah *et al.*, 2010, NCD Risk Factor Collaboration, 2017). Vazquez *et al.* (2007) examined 32 studies conducted in multiple countries between 1985 and 2004 and found that the relative risk for incident diabetes was 1.87 (95% CI, 1.67–2.10) for every standard deviation increase in BMI. In addition to baseline BMI, several studies have shown that weight gain represents an important risk factor for diabetes in adult populations. Both, the Nurses' Health Study (Colditz *et al.*, 1997) and the Health Professionals Follow-up Study reported that individuals who gained 5.0–9.9 kg when compared to those who maintained their weight within 2 kg of their weight as young adults had 1.5–3 fold increased risks of diabetes, coronary heart disease, and hypertension. These increases in risk were greater in adults with greatest weight gain. In the Finnish Diabetes Prevention Study (DPS) in overweight people with IGT, weight reduction was associated with prevention of T2D in a linear fashion; a 5% weight reduction resulted in a 66% risk reduction in T2D incidence (Lindström *et al.*, 2005). One should however remember that weight reduction is a result from changes in diet and/or physical activity.

Central Obesity

Intra-abdominal fat is recognized as a driver for the progression of metabolic risk factors independently of BMI. We also know that ectopic visceral, intra-abdominal fat distributed around organs in the peritoneal cavity and retroperitoneal around the kidneys are not necessarily correlated with BMI. Increased waist circumference in the presence other metabolic risk factors is not the perfect measure but identifies the presence of intra-abdominal fat and is an important parameter in assessing cardiovascular risk in routine clinical practice (Chalasani *et al.*, 2012).

A recent analysis comprising individual-level data from 21 studies with 155,000 participants and over 9000 compared data on BMI, waist circumference (WC), waist-hip and waist-height ratio (WHtR) for risk of incident diabetes (Lee *et al.*, 2017). Each of the measures had a positive association with incident diabetes. A one standard deviation increment in each of the measures was associated with 64%–80% higher diabetes risk. WC and WHtR were more strongly associated with risk than BMI, but there was no appreciable difference between the four measures in the predictive accuracy for diabetes at five years. Thus, any of these measures would suffice to assist in primary diabetes prevention efforts.

Conclusion

Diabetes is a major cause of morbidity and is associated with increased risk of major non-communicable disease and mortality, and placing a significant burden on the healthcare system and economy. Preventing diabetes especially T2DM is possible through understanding and addressing lifestyle determinants, especially physical activity and diet. The increased prevalence of sedentary lifestyle is linked with several NCDs including diabetes and is independently associated with mortality risk. This makes it important to consider prevention strategies which focus on interrupting sitting times in addition to increasing work and leisure-time physical activity levels. Appropriate physical activity patterns increase the effectiveness whether moderate, vigorous aerobic training or strength-type exercise have all been shown various degree of effectiveness in preventing and managing T2DM. Emerging evidence about the benefits of HIIT is encouraging but longitudinal and prospective cohort studies are required before such approach is recommended as a public health strategy. There is also a significant emerging evidence about T2DM risk-reduction benefits associated with functional foods such as components of the MD and popular herbal ingestions because of their role in enhancing immunological and cardio-metabolic functions. Innovative laboratory and field-based approaches have made it possible to objectively assess the various risks associated with T2DM and to individualize the prevention strategy at different genetic, molecular, tissue and whole-body levels. Nonetheless,

it is essential that any lifestyle prevention to consider combined approaches integrating physical activity, diet, medication and education for sustainable risk-reduction outcomes.

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Hyperglycemic Hyperosmolar State☆

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Definition and Epidemiology

Hyperglycemic hyperosmolar state used to be named hyperglycemic hyperosmolar nonketotic coma, but this term was not applicable to all patients as it was found that it frequently presents without coma. It was also named hyperglycemic hyperosmolar nonketotic state, but findings of moderate ketonemia in some patients lead to the acceptance of its updated term hyperglycemic hyperosmolar state (HHS). This is a potentially fatal hyperglycemic crisis that occurs as acute complication of uncontrolled diabetes mellitus.

HHS is most frequently encountered in middle-aged or elderly subjects with type 2 diabetes however it has also been reported in type 1 diabetes as a simultaneous occurrence with diabetic ketoacidosis (DeFronzo *et al.*, 1994; Rosenbloom, 2010). The mortality rate from HHS is still high and approaches 20% (Wachtel *et al.*, 1991). On the other hand, the incidence of HHS is less than 1 case per 1000 person-years.

Pathophysiology

The syndrome is characterized by severe hyperglycemia with glucose concentrations frequently greater than 600 mg dl^{-1}, dehydration and invariably elevated osmolality to above 320 mOsm kg^{-1}. By convention, significant hyperketonemia and metabolic acidosis are absent but the syndrome is considered to overlap with diabetic ketoacidosis in about one-third of cases (Wachtel, 1990). Patients with previously undiagnosed diabetes account for approximately 20% of cases.

Hyperosmolar state develops as a result of osmotic diuresis caused by hyperglycemia, which then creates severe fluid loss. The total body deficit of water is usually about 7–12 l in HHS, which represents a loss of about 10%–15% of body weight. Although mild ketosis can be seen with HHS, it is generally absent in this state. It is considered that patients with HHS with type 2 diabetes still do have enough insulin to be protected from exaggerated lipolysis and the consequent abundance of FFA (Kitabchi *et al.*, 2009). They do not, however, have enough insulin to prevent hyperglycemia (Smiley *et al.*, 2011).

Insulin Deficiency

There is relative, rather than absolute, insulin deficiency in patients with type 2 diabetes. This permits acceleration of hepatic glycogenolysis and gluconeogenesis. The net result is an increase in hepatic glucose production that, in the presence of elevated counterregulatory hormones, leads to a progressive rise in blood glucose concentration.

Insulin Resistance

Intercurrent illness (e.g., severe sepsis, acute myocardial infarction) is associated with elevations of circulating counterregulatory hormone concentrations. Hyperglycemia is exacerbated via direct and indirect tissue actions of these hormones, i.e., exacerbation of accelerated hepatic glucose production and antagonism of insulin-mediated glucose disposal in muscle. High levels of catecholamines (epinephrine and norepinephrine) inhibit endogenous insulin secretion, thereby compounding the preexisting defect in patients with type 2 diabetes. As hyperglycemia develops, the renal threshold for reabsorption of glucose – approximately 180 mg dl^{-1} but often higher in the elderly - is exceeded. This results in renal losses of glucose that provide temporary partial protection against more severe hyperglycemia. Renal losses of electrolytes (sodium, potassium, phosphate, and magnesium) accompany the osmotic diuresis. Subsequent contraction of intravascular volume causes a fall in renal perfusion. The absence of significant ketosis may reflect the presence of endogenous insulin secretion sufficient to restrain lipolysis, the latter process being suppressed at lower insulin concentrations than those required to stimulate glucose disposal.

Clinical Features

Symptoms of progressively intense thirst and polyuria reflecting hyperglycemia and dehydration develop over several days or even weeks. Many patients are in a moribund state of severe dehydration by the time they are admitted to hospital. Elderly or infirm patients who have been unable to maintain adequate oral fluid intake are at particular risk. Most patients with HHS will have some

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Table 1 Guide to initial treatment of hyperglycemic hyperosmolar state in adults

Fluids and electrolytes

Volumes
- 1 l per hour × 2–3, thereafter adjusted according to the degree of hydration and taking continuing polyuria into account. N.B. Use caution in patients with known renal disease or cardiovascular insufficiency; consider central venous pressure monitoring.

Fluids
- Isotonic saline (0.9%, 150 mEq l^{-1} sodium chloride) initially.
- Hypotonic saline (0.45%, 75 mEq l^{-1} sodium chloride) if serum sodium exceeds 150 mEq l^{-1} (restrict to 1–2 l in total and consider use of 5% dextrose with increased insulin if marked or worsening hypernatremia).
- 5% Dextrose at 1 l every 4–6 h replaces saline when blood glucose has fallen to 250 mg dl^{-1} until patient is eating and drinking again.

Potassium replacement
- No potassium chloride added to the first liter of fluid, unless initial plasma potassium <3.5 mEq l^{-1}.
- Measure serum potassium every 2 h initially (see below: other measures)
- Thereafter, add dosages below to each liter of fluid:

If plasma K^+:
- <3.5 mEq l^{-1}, add 40 mEq potassium chloride (severe hypokalemia may require more aggressive replacement with careful monitoring of serum potassium concentration).
- 3.5–5.5 mEq l^{-1}, add 20 mEq potassium chloride.
- >5.5 mEq l^{-1}, add no potassium chloride to the infusion.

Insulin
- 5–10 U h^{-1} (average 6 U h^{-1} in adults) by continuous intravenous infusion initially until blood glucose has fallen to 250 mg dl^{-1}. Thereafter, adjust rate (usually 1–4 U h^{-1} required in the absence of severe infection) together with dextrose infusion to maintain serum glucose between 100 and 200 mg dl^{-1} until patient is eating and drinking again.
- Thereafter, change to an appropriate subcutaneous insulin regimen.
- Review continuing need for insulin therapy 2–3 months after full recovery.

Other measures
- Search for and treat precipitating cause (e.g., infection, myocardial infarction).
- Systemic hypotension usually responds to adequate fluid replacement with crystalloids.
- Pass bladder catheter if level of consciousness is impaired or no urine is passed within 2 h of the start of therapy.
- Continuous electrocardiographic monitoring may warn of hyperkalemia or hypokalemia (serum potassium should be measured hourly if <3.5 or >5.5 mEq l^{-1}).
- Consider cranial computed tomography imaging to exclude other pathology (e.g., cerebral hemorrhage, venous sinus thrombosis) if the level of consciousness remains impaired following correction of hyperosmolar state.
- Treat acute thrombo-embolic complications with anticoagulant doses of heparin.
- Update clinical and biochemical progress using a purpose-designed flowchart.

degree of neurologic disturbance. If osmolality is normal and patients show severe neurologic deficit, further workup is indicated to rule out underlying neurologic pathologic condition. Elderly patients are particularly susceptible to these disturbances. Some neurologic presentations include irritability, restlessness, stupor, muscular twitching, hyperreflexia, spasticity, seizures, and coma (Arieff, 1984; Butts, 1987). The clinical signs and symptoms reflect the severity of the hyperosmolality (Palevsky, 1998).

Precipitating Factors

Respiratory and urinary tract infections are common precipitants; others include myocardial infarction, pulmonary embolism, and acute pancreatitis. Patients can present with symptoms of the preceding illness, such as infection or myocardial ischemia. Certain antihypertensive drugs, including high-dose diuretics and beta-blockers, have been implicated; diuretics may exacerbate dehydration and potassium depletion. Other drugs include phenytoin, cimetidine, and chlorpromazine. High-dose corticosteroids have potent effects on carbohydrate and lipid metabolism, readily precipitating metabolic decompensation in predisposed individuals.

Diagnosis

Evaluation of volume status is one of the initial assessments of patients with HHS.

Urinalysis reveals marked glycosuria with a "negative" or "trace" ketone reaction using semiquantitative nitroprusside-based test strips. Blood glucose concentration is markedly elevated, i.e., >500 mg dl^{-1}. Blood urea nitrogen is elevated and hematocrit is raised. The serum osmolality is determined by the concentrations of the different solutes in the plasma. In normal subjects, sodium salts, glucose, and urea are the primary circulating solutes. Increased serum osmolality to more than 320 mOsmol kg^{-1} is seen in patients with neurologic abnormalities and is typical for HHS. Rarely, serum osmolality can be more than 400 mOsm

kg^{-1}. The formula for the calculation of serum osmolality is given below (Rasouli and Kalantari, 2005; Worthley *et al.*, 1987).

$$\text{Serum osmolality} = (2 \times \text{serum [Na]}) + (\text{glucose, in mg dl}^{-1})/18 + (\text{BUN in mg dl}^{-1})/2.8$$

(where BUN is serum urea nitrogen)

The formula with all units in millimoles per liter is the following:

$$\text{Serum osmolality} = (2 \times \text{serum [Na]}) + (\text{glucose}) + (\text{urea})$$

Most patients will present with hyponatremia. (Each 100 mg dl^{-1} of the glucose level more than normal lowers the serum sodium level by about 1.6 mEq L^{-1}.) An additional decrease in serum sodium is present with confounding pseudohyponatremia that occurs with hyperlipidemia or hyperproteinemia with some laboratory assays (Dhatt *et al.*, 2012; Weisberg, 1989). In some cases of HHS, patients may present with hypernatremia secondary to osmotic diuresis and more severe dehydration (Liamis *et al.*, 2000; Milionis *et al.*, 2001). Hypernatremia indicates a profound degree of water loss. Arterial plasma pH is >7.30 and bicarbonate is >15 mEq l^{-1}. However, a degree of acidosis may result from impaired renal excretion of H^+ ions allied to tissue hypoperfusion. Serum potassium is frequently paradoxically elevated despite the total body deficit in HHS. Treatment with insulin shifts potassium into the cell and causes a rapid decrease of the serum potassium levels. Hypokalemia is frequently encountered after starting insulin treatment (Maletkovic and Drexler, 2013).

Treatment and Complications

The management of HHS consists of fluid and electrolyte repletion, insulin administration, and the treatment of the precipitating cause if one can be identified. Patients should be admitted to a monitored unit where close observation of mental status, blood pressure, heart rate and rhythm, and urine output can be done (**Table 1**).

Complications and Outcomes

Nontraumatic rhabdomyolysis in patients with greater degrees of hyperosmolarity may precipitate acute renal failure. There is a high frequency of thrombo-embolic complications. The average mortality rate is approximately 15%, higher than for patients with diabetic ketoacidosis. The acute outcome tends to be worse for individuals of advanced age or with serious comorbidity.

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Hypoglycemia☆

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Glossary

Hypoglycemia Mild: blood glucose levels between 50 and 70 mg dL^{-1}. Severe: blood glucose levels <50 mg dL^{-1} that requires resuscitation.

Hypoglycemia associated autonomic failure (HAAF) A situation in which previous episodes of hypoglycemia (or exercise) blunt autonomic, neuroendocrine, metabolic and symptomatic response to subsequent episodes of hypoglycemia.

Type 1 diabetes mellitus An autoimmune disorder in which the patient's body cannot make insulin, and consequently requires exogenous insulin administration.

Type 2 diabetes mellitus A disorder of insulin resistance and progressive beta-cell dysfunction resulting in hyperglycemia, and consequently requiring oral and/or injection therapy including exogenous insulin.

Introduction

Typically, blood glucose levels are tightly regulated between 70 and 150 mg dL^{-1} in healthy man. Hypoglycemia (blood glucose levels of <70 mg dL^{-1}) occurs as a consequence of fasting (>24 h) or different disease conditions including diabetes mellitus, insulinomas, inborn errors of metabolism such as galactosemia, persistent hyperinsulinemic hypoglycemia of infancy and hereditary fructose intolerance, postbariatric surgery, and severe prolonged exercise. While the incidence of hypoglycemia has historically been considered to be associated with type 1 diabetes mellitus (T1DM), the prevalence of hypoglycemia in type 2 diabetes mellitus (T2DM) also increases greatly as endogenous insulin secretion fails and treatment is shifted to insulin and/or insulin secretagogues. Imbalances of exogenous insulin with food intake and physical activity create challenges in the maintenance of glucose homeostasis in T1DM and T2DM.

Maintenance of Blood Glucose

Blood glucose homeostasis is maintained by the complex interplay of factors regulating glucose absorption from the gut, glucose production by the liver (glycogenolysis and gluconeogenesis), and glucose uptake in tissues such as muscle, liver and brain. Once the gut absorbs glucose it enters the portal circulation where it is first taken up by the liver. In the postabsorptive state, the liver will release glucose into the circulation via the process of glycogenolysis. After an overnight fast, gluconeogenesis becomes the primary source of endogenous glucose production. The fate of glucose taken up by the tissues (stored or metabolized) depends on the type of tissue and energy status. The majority of glucose uptake in the fasting state is by the central nervous system as glucose is the brain's primary fuel. The brain has limited glucose stores and thus, is dependent on endogenous (primarily hepatic) glucose output for its glucose supply. Glucose is transported into the tissues by a specific family of proteins called glucose transporters. Different tissues usually have distinct isoforms of these transporters. For example, GLUT-1 and GLUT-3 isoforms are located in the brain, GLUT-2 is located in the liver and pancreas, GLUT-4 is located in muscle and adipocytes and GLUT-5 is located in the epithelial cells of the jejunum. Both the autonomic nervous system (ANS) and the endocrine system regulate glucose homeostasis. Insulin lowers plasma glucose by (1) suppressing endogenous glucose production (both by direct action on the liver and indirect actions to reduce substrate flux such as lactate, pyruvate, glycerol, and free fatty acids to the liver) and (2) increasing glucose uptake into peripheral tissues (predominantly muscle). Glucagon increases plasma glucose via direct regulation of gluconeogenesis and glycogenolysis to increase hepatic glucose production.

☆*Change History*: March 2018. Davis et al. made the following changes: Hypoglycemia and Diabetes has been updated to include data on type 2 diabetes. Negative Cardiovascular Effects of Hypoglycemia has been added. Strategies to Reduce Hypoglycemia has been added. The glossary has been updated. Additional reading citations have been updated and added. M Hedrington and S Davis made the following changes: The abstract has been revised. Hypoglycemia and Diabetes has been updated. Effects of Hypoglycemia on the Quality of Life has been added. Hypoglycemia Risk Reduction and Patient-Centered Considerations has been added. Additional reading citations have been added.

This article is an update of Darleen A. Sandoval and Stephen N. Davis, Hypoglycemia, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 636-640.

Physiological Response to Hypoglycemia

The physiological response to hypoglycemia involves the ANS and many neuroendocrine and metabolic processes (Tables 1 and 2). The initial defense is a reduction in endogenous insulin secretion, which occurs when plasma glucose is in the low normal range (80–70 mg dL^{-1}). Glucagon and epinephrine secretion are activated at glucose levels just below normal (70 mg dL^{-1}), and, together with inhibition of endogenous insulin secretion, are the primary defenders against hypoglycemia. Glucagon is a powerful and quick acting stimulus for hepatic glucose production. The increase in hepatic glucose production is primarily through glycogenolysis but gluconeogenesis becomes more important as hypoglycemia is prolonged. Epinephrine increases heart rate, cardiac contractility, and lipolysis and inhibits glucose uptake, while also increasing endogenous glucose production, primarily from the liver but also from the kidney. Glucagon responses are absent within 5 years duration of T1DM and in long-duration T2DM, increasing reliance on epinephrine for defense against hypoglycemia. Growth hormone, from the pituitary gland, cortisol, from the adrenal cortex, and norepinephrine spillover from the sympathetic nervous system also increase in response to moderate hypoglycemia but have modest metabolic effects. Pancreatic polypeptide, an index of parasympathetic nervous system activity, oxytocin, vasopressin, thyroid hormone, aldosterone and adrenocorticotropic hormone are also released during hypoglycemia but do not appear to have discernable acute metabolic effects.

Symptoms of Hypoglycemia

The symptoms of hypoglycemia can be separated into those that are neurogenic (autonomic) and those that are neuroglycopenic. Examples of autonomic symptoms experienced when glucose levels fall below 60 mg dL^{-1} are tremulousness, palpitations, anxiety, sweating, and dry mouth. These symptoms are autonomic in origin and stem from increased sympathetic drive. For example sweating is cholinergically mediated via sympathetic nerve fibers and tremor is correlated with increased circulating levels of epinephrine. Symptoms that are neuroglycopenic generally occur at glucose levels of 50 mg dL^{-1} or less, result directly from brain glucose deprivation, and include difficulty in thinking, confusion, weakness, fatigue, paresthesias, seizures, coma and death. Impairment of cognitive function occurs at glucose levels of about 45 mg dL^{-1} and is implicated as a cause for fatal accidents occurring in patients.

Hypoglycemia and Diabetes

Type 1 Diabetes Mellitus

Persistent hyperglycemia has been determined to be the cause of long-term microvascular complications associated with T1DM. The Diabetes Control and Complications Trial (DCCT), a landmark multicenter randomized clinical trial, was developed to determine the risks and benefits of tight glucose control. The results showed that tight glucose control, defined as an HbA_{1c}

Table 1 Effects of a 120-min hyperinsulinemic hypoglycemic clamp on neuroendocrine and autonomic responses in overnight fasted men

	Basal	*Final 30 min of the clamp*
Norepinephrine (pg mL^{-1})		
Euglycemic clamp	163 ± 15	225 ± 24[a]
Hypoglycemic clamp	165 ± 10	328 ± 20[a,b]
Epinephrine (pg mL^{-1})		
Euglycemic clamp	38 ± 6	35 ± 5
Hypoglycemic clamp	36 ± 5	934 ± 110[a,b]
Pancreatic polypeptide (pg mL^{-1})		
Euglycemic clamp	168 ± 15	86 ± 15
Hypoglycemic clamp	132 ± 25	1209 ± 134[a,b]
Glucagon (pg mL^{-1})		
Euglycemic clamp	78 ± 10	59 ± 3
Hypoglycemic clamp	71 ± 12	253 ± 35[a,b]
Cortisol (μg dL^{-1})		
Euglycemic clamp	9 ± 1	10 ± 1
Hypoglycemic clamp	9 ± 1	25 ± 2[a,b]
Growth hormone (ng mL^{-1})		
Euglycemic clamp	2 ± 1	1 ± 1
Hypoglycemic clamp	2 ± 1	53 ± 8[a,b]
MSNA (bursts min^{-1})		
Euglycemic clamp	28 ± 3	32 ± 3[a]
Hypoglycemic clamp	25 ± 3	43 ± 4[b]

[a]Values are significantly increased versus basal period.
[b]Values are significantly different versus euglycemic clamp. MSNA, muscle sympathetic nerve activity.
Data are means ± SE.

Table 2 Effects of a120 min hyperinsulinemic hypoglycemic clamp on metabolic and cardiovascular responses in overnight fasted men

	Basal	*Final 30 min of the clamp*
Endogenous glucose production (mg kg^{-1} min^{-1})		
Euglycemic clamp	2 ± 1	0 ± 1[a]
Hypoglycemic clamp	2 ± 1	2 ± 1[b]
Glucose infusion rate (mg kg^{-1} min^{-1})		
Euglycemic clamp	0 ± 0	8 ± 1[a]
Hypoglycemic clamp	0 ± 0	0.3 ± 0.1[b]
Glycerol (μmol L^{-1})		
Euglycemic clamp	33 ± 3	14 ± 2[a]
Hypoglycemic clamp	38 ± 2	37 ± 4[b]
Plasma free fatty acids (μmol L^{-1})		
Euglycemic clamp	440 ± 70	119 ± 24[a]
Hypoglycemic clamp	590 ± 70	228 ± 30[ab,]
Lactate (μmol L^{-1})		
Euglycemic clamp	705 ± 128	1059 ± 58[a]
Hypoglycemic clamp	661 ± 50	1195 ± 115[a,b]
Heart rate (bpm)		
Euglycemic clamp	60 ± 3	62 ± 3
Hypoglycemic clamp	59 ± 3	70 ± 4[a,b]
Mean arterial pressure (mmHg)		
Euglycemic clamp	83 ± 3	81 ± 4
Hypoglycemic clamp	81 ± 3	81 ± 3

[a]Values are significantly different versus basal period.
[b]Values are significantly different versus euglycemic clamp.
Data are means ± SE.

≤ 7.2%, obtained by using either multiple insulin injections or continuous subcutaneous insulin infusion prevented or delayed the progression of diabetes complications as compared to conventional treatment (HbA_{1c} ≥ 9.0%). However, patients with tight glucose control also suffered a threefold increase in the incidence of severe hypoglycemia (requiring outside assistance to recover) and coma. In fact, 90% of all patients with T1DM experienced mild to moderate hypoglycemia.

Type 2 Diabetes Mellitus

Similar to findings from the DCCT, the UK Prospective Diabetes Study found that in patients with T2DM, intensive blood glucose control (HbA_{1c} 7.0%) reduced microvascular complications by 25%, compared to conventional blood glucose control (HbA_{1c} 7.9%). Again though, improved glucose control, primarily in patients who use insulin, is associated with a higher incidence of hypoglycemia in T2DM, occurring in 70% of participants in recent trials. Moreover, in addition to tight glucose control (HbA_{1c} < 6.0%), poor glucose control (HbA_{1c} ≥ 9.0%) is also associated with an increased risk of hypoglycemia in T2DM. In large trials, patients with higher HbA_{1c} levels that have been randomized to conventional glucose control have poorer outcomes in association with severe hypoglycemic episodes than patients randomized to intensive control. Overall, nearly equivalent rates of severe hypoglycemia have been reported in T1DM and insulin-treated T2DM (11.5 and 11.8 events per 100 patient years, respectively). Recently, three large randomized controlled trials (ACCORD (Action to Control Cardiovascular Risk in Diabetes), ADVANCE (Action in Diabetes and Vascular Disease), and VADT (Veterans Affairs Diabetes Trial)) have demonstrated that individuals with T2DM are particularly susceptible to the adverse consequences of hypoglycemia. In all three trials, episodes of severe hypoglycemia were associated with serious adverse cardiovascular events and increased mortality.

In diabetes, hypoglycemia is initially caused by insulin excess but can be potentiated by lack of food intake, physical activity, and autonomic dysfunction. Hypoglycemia associated autonomic failure (HAAF) is an acute failure of the autonomic response to hypoglycemia that is induced by prior episodes of hypoglycemia in both T1DM and T2DM. This creates a situation where the patient has reduced capability to defend against impending hypoglycemia. Unfortunately hypoglycemia is the complication of diabetes most feared by insulin-requiring diabetics. Consequently, the increased prevalence of hypoglycemia is the major roadblock preventing patients from realizing the benefits of tight glucose control.

Hypoglycemic Unawareness

One component of HAAF is hypoglycemic unawareness. Hypoglycemic unawareness occurs when an individual has a reduced or complete loss of symptoms that indicates the presence of hypoglycemia. The pathogenesis of hypoglycemic unawareness is multifactorial but includes a shifting of the glycemic threshold. That is, symptoms only occur at progressively lower glucose levels. If this threshold occurs

below a critical level, hypoglycemic symptoms may not be activated and a patient may slip into a coma with little or no warning. Importantly, hypoglycemia unawareness can be reversed by meticulous avoidance of iatrogenic hypoglycemia.

Role of Antecedent Stress in Incidence of Hypoglycemia

Many studies have demonstrated the importance of antecedent hypoglycemia in the pathogenesis of HAAF. Patients with tight glucose control typically have low glycemic thresholds due to the repeated exposure to prior hypoglycemia. Prior episodes of hypoglycemia cause a shift in the glycemic threshold, which results in a progressive diminution of autonomic and neuroendocrine responses to subsequent episodes of hypoglycemia. As stated above, the autonomic and neuroendocrine responses to hypoglycemia are necessary to stimulate the liver to release more glucose and inhibit peripheral uptake of glucose in an attempt to defend against the falling glycemia. With these defenses reduced, combined with the absence of glucagon, the T1DM patient is left particularly vulnerable to repeated hypoglycemia. Tables 3 and 4 contain data from healthy subjects exposed to either day 1 clamped euglycemia or hypoglycemia followed by a subsequent bout of hypoglycemia on day 2. These data clearly demonstrate the blunted day 2 neuroendocrine and autonomic nervous system responses after day 1 hypoglycemia and reveal the considerably greater amounts of glucose that had to be infused during the day 2 experiments to maintain the desired level of hypoglycemia. The phenomenon of blunted counterregulatory responses to recurring hypoglycemia has been extended to T2DM as well. Mild antecedent hypoglycemia (60 mg dL^{-1}) blunted autonomic nervous system responses to subsequent hypoglycemia in participants with T2DM that were randomized to either suboptimal (HbA_{1c} ~10.0%) or intensive glycemic control (HbA_{1c} ~6.7%).

Exercise is important in the maintenance and well-being of individuals with diabetes. Physical activity improves insulin sensitivity, helps in body weight maintenance and can reduce postprandial hyperglycemia. Unfortunately, exercise is also associated with increased hypoglycemia in T1DM patients. This may be due in part to the fact that prior episodes of hypoglycemia also reduce the autonomic and neuroendocrine response to a subsequent bout of prolonged exercise. The reverse is also true, prior prolonged exercise blunts autonomic and neuroendocrine responses to next day hypoglycemia. Also, one bout of exercise in the morning blunts neuroendocrine and metabolic counterregulatory response to a second bout of exercise in the afternoon. Thus insulin delivery (reduced) and carbohydrate intake (increased) during exercise following a hypoglycemic episode may have to be modified in order to prevent further hypoglycemia.

The mechanism(s) responsible for the blunting effect of prior episodes of hypoglycemia are not fully understood but likely involve several interactive pathways. Cortisol is known to blunt sympathetic nervous system (SNS) responses to stress in both animal and human experimental models. Therefore prior increases in cortisol during hypoglycemia or exercise could be one factor that causes blunted SNS responses to subsequent episodes of hypoglycemia or bouts of exercise. Additionally, within the CNS, several studies found decreases in expression and activity of glucokinase (a rate-limiting enzyme of glycolysis) and AMP-activated kinase (a highly conserved fuel-sensing enzyme), as well as changes in neurotransmitter level or activity. For example,

Table 3 Effects of Day 1 hypoglycemia on neuroendocrine and autonomic responses to Day 2 hypoglycemia in overnight fasted men and women

	Basal	*Final 30 min of Day 2 hypoglycemic clamp*
Norepinephrine (pg mL^{-1})		
Day 1 Euglycemia	178 ± 12	346 ± 37[a]
Day 1 Hypoglycemic	172 ± 18	293 ± 21[ab,]
Epinephrine (pg mL^{-1})		
Day 1 Euglycemia	42 ± 3	950 ± 4[a]
Day 1 Hypoglycemic	33 ± 3	421 ± 83[ab,]
Pancreatic polypeptide (pg mL^{-1})		
Day 1 Euglycemia	163 ± 38	1174 ± 129[a]
Day 1 Hypoglycemic	125 ± 17	862 ± 151[ab,]
Glucagon (pg mL^{-1})		
Day 1 Euglycemia	102 ± 12	375 ± 28[a]
Day 1 Hypoglycemic	72 ± 12	171 ± 25[a,b]
Cortisol (μg dL^{-1})		
Day 1 Euglycemia	8 ± 1	22 ± 1[a]
Day 1 Hypoglycemic	5 ± 1	17 ± 2[a,b]
Growth hormone (ng mL^{-1})		
Day 1 Euglycemia	2 ± 1	46 ± 6[a]
Day 1 Hypoglycemic	2 ± 1	32 ± 5[a,b]
MSNA (bursts min^{-1})		
Day 1 Euglycemia	30 ± 7	44 ± 3[a]
Day 1 Hypoglycemic	28 ± 9	33 ± 8[a,b]

[a]Values are significantly increased versus basal period ($P < 0.05$).

[b]Values are significantly reduced versus Day 1 Euglycemia ($P < 0.05$). MSNA, muscle sympathetic nerve activity. Data are means ± SE.

Table 4 Effects of Day 1 hypoglycemic on metabolic and cardiovascular responses to Day 2 hypoglycemia in overnight fasted men and women

	Basal	*Final 30 min of Day 2 hypoglycemic clamp*
Endogenous glucose production		
Day 1 Euglycemia	2 ± 1	2 ± 1
Day 1 Hypoglycemic	2 ± 1	1 ± 2[a,b]
Glucose infusion rate (mg kg^{-1} min^{-1})		
Day 1 Euglycemia	0 ± 0	0 ± 0
Day 1 Hypoglycemic	0 ± 0	4.3 ± 2.2[a,b]
Glycerol (μmol L^{-1})		
Day 1 Euglycemia	32 ± 4	29 ± 4
Day 1 Hypoglycemic	35 ± 2	24 ± 2[a,b]
Plasma free fatty acids (μmol L^{-1})		
Day 1 Euglycemia	384 ± 100	178 ± 32[a]
Day 1 Hypoglycemic	492 ± 43	199 ± 20[a,b]
Lactate (μmol L^{-1})		
Day 1 Euglycemia	924 ± 101	1747 ± 14[a]
Day 1 Hypoglycemic	945 ± 96	1535 ± 73[a,b]
Heart rate (bpm)		
Day 1 Euglycemia	63 ± 4	78 ± 5[a]
Day 1 Hypoglycemic	61 ± 3	72 ± 5[a]
Mean arterial pressure (mmHg)		
Day 1 Euglycemia	83 ± 3	84 ± 4
Day 1 Hypoglycemic	82 ± 2	82 ± 3

[a]Values are significantly different versus basal period ($P < 0.05$).
[b]Values are significantly different versus Day 1 Euglycemia ($P < 0.05$).
Data are means ± SE.

γ-aminobutyric acid (an inhibitory neurotransmitter), corticotrophin-releasing factor, and urocortin are all reduced in level or activity and these changes are associated with blunted responses to hypoglycemia. Insulin, lipid, and neuropeptide signaling, as well as alterations in brain fuel selection (i.e., monocarboxylic acid utilization), uptake and/or metabolism may also be involved in modulation of counterregulation during repeated episodes of hypoglycemia.

Negative Cardiovascular Effects of Hypoglycemia

In several large-scale clinical trials, improved management of hyperglycemia has not led to improved cardiovascular outcomes in patients with T2DM. The reason for this is unclear, but the increased rate of hypoglycemia with increased glycemic control may be one confounding factor. Limited evidence exists to suggest that hypoglycemia can induce acute EKG abnormalities, such as atrial and ventricular ectopic beats, ST-segment depression, QT-interval prolongation, and P- and T-wave abnormalities, which could lead to dangerous arrhythmias. However, the more ubiquitous mechanisms by which hypoglycemia exerts long-term damaging cardiovascular effects likely involve promotion of inflammation, oxidative stress, leukocytosis, endothelial dysfunction, and prothrombogenic and atherogenic pathways. Patients with a history of recurring hypoglycemia have been found to have increased inflammation and intima-media thickness and reduced flow-mediated arterial dilation.

Effects of Hypoglycemia on the Quality of Life

Even a single bout of hypoglycemia has a considerable impact on the quality of life in type 1 as well as T2DM. Studies have shown that a hypoglycemic episode of only 1 h duration the night before markedly affected the individual's sense of well-being the next day. Various trials have also demonstrated that hypoglycemia is associated with increased anxiety, symptoms of depression, reduced alertness during driving and diminished ability to function at work.

Strategies to Reduce Hypoglycemia

Special attention to an individuals' regimen, age and comorbidities allows the practitioner to individualize treatment plans and glycemic goals. The dosing and timing of insulin and insulin-secretagogue therapy to meals and exercise should be reviewed. Antihyperglycemic agents that are not routinely associated with hypoglycemia (i.e., GLP-1 agonists, DPP-4 inhibitors, acarbose, metformin), may result in unanticipated hypoglycemic events when combined with insulin. Strict avoidance of hypoglycemia by tolerating a higher average glucose for 2–3 weeks can improve hypoglycemic symptom awareness.

Newer, basal insulin analogs have also been demonstrated to lower rates of hypoglycemia, particularly during the night. Glycemic variability is reduced with the addition of sensor-augmented insulin pump therapy compared to multidaily insulin injections. And, glycemic goals are more likely to be achieved without increasing the rate of hypoglycemia. A continuous glucose monitor can improve glucose monitoring and alert individuals to a rapidly falling glucose. These devices when used in combination with insulin pumps equipped with features to suspend insulin delivery can reduce nocturnal hypoglycemia without increasing HbA_{1c} values.

Hypoglycemia Risk Reduction and Patient-Centered Considerations

The degree of importance for reducing the risk of hypoglycemia and associated difficulties vary among people with diabetes. Thus, each patient should be approached with individualized treatment strategies and glycemic targets. Factors that need to be taken into consideration are patient's age and life expectancy, any existing comorbidities and likely consequences of hypoglycemia on the patient's life. For example, an aggressive target might be appropriate for an active, healthy individual with diabetes who has preserved awareness of hypoglycemia and uses real-time CGM with sounding alarms.

Adverse consequences of hypoglycemia often outweigh the benefits of tight glycemic control in children, older individuals, patients with comorbidities and/or shorter life expectancy. Children with T1DM have high risk of hypoglycemia since eating and activity patterns are usually hard to predict, which makes insulin-dosing challenging. In addition, children with T1DM often lack ability to recognize symptoms of hypoglycemia.

Severe hypoglycemia is common in older adults with diabetes. Factors that contribute include geriatric syndrome, comorbidities, polypharmacy, decline in renal and hepatic function, impaired counterregulatory responses to hypoglycemia, hypoglycemia unawareness. Even mild hypoglycemia can cause dizziness and blurry vision, which in older adults dramatically increases the likelihood of falls. The latter, according to the CDC is the leading cause of injury and death in older Americans. Additionally, elderly patients with diabetes are particularly vulnerable to hypoglycemia associated malignant ventricular arrhythmias that can be life threatening. Thus, American Diabetes Association and American Geriatric Society recommend avoiding hypoglycemia in older adults by targeting HbA1c $\sim$7.3% for individuals with good functional status and few comorbidities and 8%–9% for ailing patients with multiple comorbidities and limited life expectancy. It remains undecided whether hypoglycemia per se increases the risk of dementia.

Real-time CGM allows people with diabetes to not only view their glucose level almost immediately, but also identify direction and speed of change. The studies have shown that real-time CGM use can reduce number and duration of hypoglycemic episodes, improve HbA1c and reverse hypoglycemia unawareness. Current CGM models (RT-CGM) also offer an integrated alarm system that is triggered when glucose level reaches a preset value and the direction of change projects hypoglycemia.

Hypoglycemia is of course always a subject of concern when diabetes is treated with insulin. Analog insulins have been designed to more closely mimic normal insulin profiles. Besides improved HbA1c and fasting plasma glucose, insulin analogs are also associated with markedly reduced risk of hypoglycemia. And each subsequent generation seems to have even better safety profile than the one preceding it. One current example is insulin degludec, which has been demonstrated to be noninferior to insulin glargine in trials studying type 1 as well as type 2 diabetic patients. Degludec has also been associated with significantly lower rates of nocturnal hypoglycemia compared to insulin glargine and insulin detemir.

Medtronic's MiniMed 670G—a hybrid closed loop insulin delivery system is the first FDA approved artificial pancreas. The closed loop system combines a CGM, an insulin pump and sophisticated predictive algorithms embedded on an external processor (e.g., on a smartphone). The processor receives information from the CGM and after performing algorithmic calculations, sends insulin-dosing signals to the pump. The studies have demonstrated that using this system in a controlled as well as in unmonitored (in-home) environment considerably increases the time, in which glycemia is in the target range, improves HbA1c and reduces hyper and hypoglycemia. Several companies are working on adding a glucagon pump to this system, which would further reduce hypoglycemia.

Conclusions

A vicious cycle is generated for T1DM patients when intensive control results in iatrogenic hypoglycemia. An increased frequency of hypoglycemia leads to lower glycemic thresholds, blunted neuroendocrine and autonomic nervous system responses, hypoglycemia unawareness and unfortunately further episodes of hypoglycemia. Patient-centered treatment that focus on preventing hypoglycemia but allow maintenance of optimal control of blood glucose levels can add significantly to the quality of life for the T1DM and T2DM patient.

See also: Insulin Pumps and Artificial Pancreas

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Glycation- and/or Polyol Pathway-Inducing Complications☆

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Glossary

Advanced glycation endproduct (AGE) A stable, endstage product formed in glycation reactions.
Early-stage glycation adducts Schiff's bases and fructosamines.
Glycation The nonenzymatic reaction of glucose, α-oxoaldehydes and other saccharide derivatives with proteins, nucleotides and lipids.
Glycoxidation Glycation processes involving oxidation.
Polyol pathway A metabolic pathway converting glucose to fructose via sorbitol.

Early-Stage Glycation—Formation of Fructosamine: Glycated Hemoglobin and Glycated Albumin

Glycation of lysyl side chain and N-terminal amino groups of proteins by glucose was the first major glycation process studied. Glucose reacts with these amino groups initially to form a Schiff's base which undergoes an Amadori rearrangement to form *N*-(1-deoxy-D-fructos-1-yl)-amino acid or fructosamine, on lysine residues forming fructosyl-lysine (FL) and N-terminal amino acids to form N-terminal fructosyl-amino acid residues (Rabbani and Thornalley, 2012b)—**Fig. 1**A. The major glycation adduct of glycated hemoglobin HbA1c (A1C) is the *β*-chain N-terminal fructosyl-valine residue with a secondary important glycation site at α-chain lys-61 (Delpierre *et al.*, 2006; Zhang *et al.*, 2001) and the major sites of glycation by glucose in human serum albumin (HSA) are, in order of reactivity: N-terminal aspartate and lys-525, lys-199 and lys-439 residues (Barnaby *et al.*, 2011). FL and related fructosamines are early-stage glycation adducts which are repaired and removed from cellular proteins enzymatically by fructosamine-3-phosphokinase (F3PK) (Delpierre *et al.*, 2000)—**Fig. 1**B. Gene deletion of F3PK produces marked increases in FL without evidence of pathogenesis or decreased lifespan (Veiga-Da-Cunha *et al.*, 2006), suggesting that glycation by glucose to fructosamine does not, per se, contribute to pathology. Indeed, studies with high dose thiamine therapy in experimental diabetes showed that diabetic nephropathy, retinopathy and neuropathy could be prevented without correction of increased FL content of the kidney, retina and nerve (Karachalias *et al.*, 2010; Thornalley, 2005). The likely explanation is that formation of FL and N-terminal amino acid fructosamine residues do not markedly impair protein function—fructosamine formation does produce loss of charge of the glycated residue, for example, and lysine and N-terminal amino acid residues have a relatively low probability of location in functional domains of proteins, compared to arginine residues – the major site of location of AGEs (Gallet *et al.*, 2000; Rabbani and Thornalley, 2014). Levels of early-stage glycation adducts are rather directly linked to extent and duration of hyperglycemia and therefore their measurement finds use as biomarkers of glycemic control—including, recently, for A1C, a criterion for diagnosis of diabetes (Bonora and Tuomilehto, 2011).

Protein Glycation in Monitoring Glycemic Control

Fructosamine concentrations change in response to hyperglycemia; they respond strongly to persistent hyperglycemia and weakly to short-term postprandial hyperglycemia. The assay of fructosamine residues is a diagnostic marker of medium term glycemic control in diabetes mellitus. Approximately 6%–15% of the HSA is glycated with a fructosamine in vivo. Glycated HSA reflects glycemic control in the 2–3 weeks preceding analysis. Glycated hemoglobin accounts for c. 7.5% of total hemoglobin in normal healthy human subjects. The different forms are designated HbA_{1a1}, HbA_{1a2}, HbA_{1b} and HbA_{1c} (A1C). A1C is the most abundant of the minor components in human red blood cells in vivo, accounting for approximately 5% of total hemoglobin. It too is a mixture of mostly the fructosamine adducts of the *β*-val-1 (60%) and α-lys-61 (40%)—see above. HbA_{1a1} and HbA_{1a2} are the *β*-chain N-terminal adducts of fructose-1,6-bisphosphate and glucose-6-phosphate, respectively. HbA_{1b} is thought to result from a deamidation in the *β*-chain of HbA_{1c}. The measurement of A1C reflects glycemic control over the 6–8 weeks preceding analysis. In diabetes mellitus, the concentration of fructosamine of serum proteins is typically increased 2-fold, relative to those of normal human subjects (c. 5 vs. 2 nmol/mg protein). A1C has become an important clinical chemistry marker of glycemic control in diabetes: 6.0%–6.9% good control, 7.0%–7.9% moderate control, and ≥8.0%, poor control. A1C is also a screening marker for diagnosis of diabetes (AIC ≥6.5%) and prediabetes state of impaired glucose tolerance (IGT; A1C 5.7%—6.4%)

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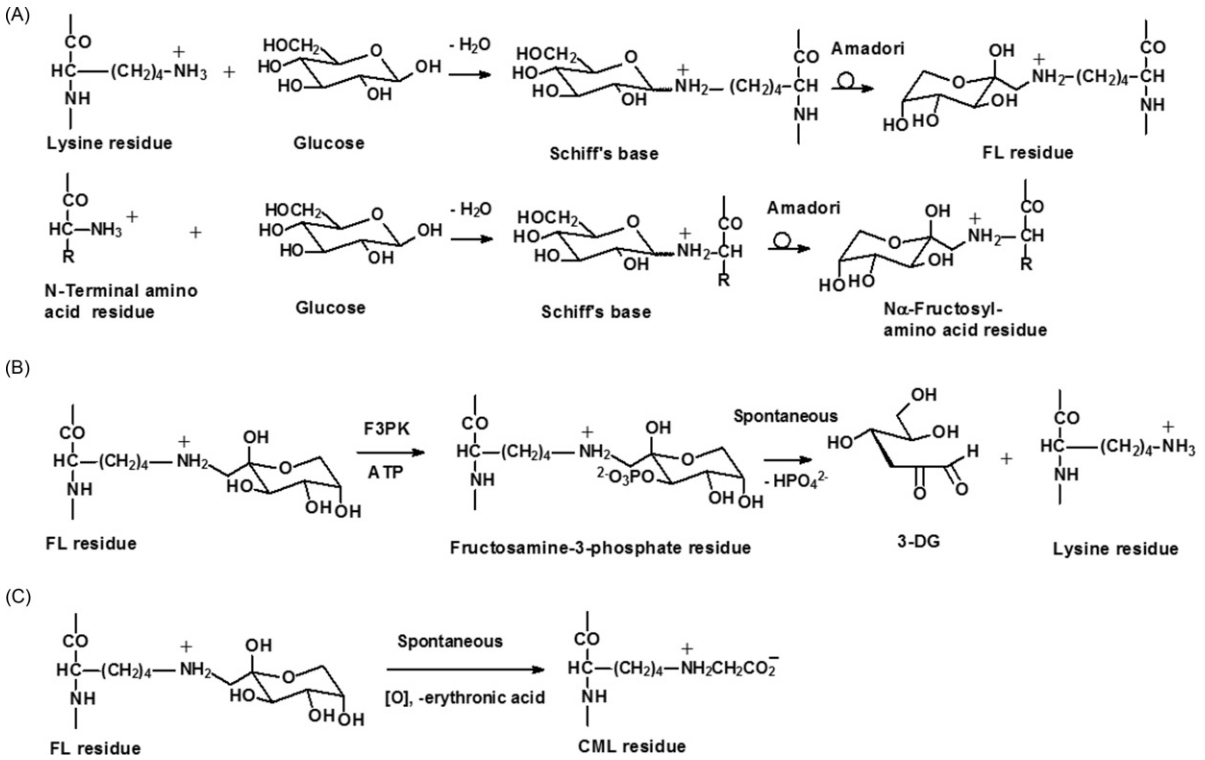

Fig. 1 *Glycation of proteins on lysine and N-terminal residues—formation and metabolism of fructosamines by glycation of protein with glucose.* (A) Reaction of glucose with lysine residues to form a Schiff's bases with Amadori rearrangement to a fructosamine, fructosyl-lysine (FL). Similar reactions occur with N-terminal amino acid residues, for example, β-val-1 of hemoglobin in A1C. (B) Deglycation of fructosamine residues by fructosamine-3-phosphokinase (F3PK). (C) Oxidative degradation of fructosyl-lysine to form the AGE, N_ε-carboxymethyl-lysine (CML). FL and CML adduct residues are shown with the peptide backbone –CO-CHR-NH- shown on the left. For the corresponding free adducts at physiological pH, the N-terminal amino group is protonated $-NH_3^+$ and the C-terminal carbonyl is a carboxylate $-CO_2^-$ moiety.

(American-Diabetes-Association, 2014; Bonora and Tuomilehto, 2011). A1C is routinely measured by cation exchange chromatography (Sacks, 2012).

Formation of Advanced Glycation Endproducts

FL and other fructosamine residues also degrade to form AGEs. The major AGEs formed from FL degradation are N_ε-carboxymethyl-lysine (CML) and glucosepane—the latter producing a crosslink with a neighboring arginine reside (**Fig. 1**C). Reactive dicarbonyl metabolites, glyoxal, methylglyoxal (MG), 3-deoxyglucosone (3-DG) are also important precursors of AGEs in vivo—**Fig. 2**A. Glyoxal is formed by lipid peroxidation and the nonenzymatic oxidative degradation of glucose and proteins glycated by glucose, MG is formed mainly by the degradation of triosephosphates, and 3-DG is formed by degradation of fructosamine-3-phosphate in the repair of early glycated proteins, the degradation of fructose-3-phosphate and the slow, non-enzymatic oxidative degradation of glucose and proteins glycated by glucose (Rabbani and Thornalley, 2012a). Unlike glucose, glycation by dicarbonyls is directed to arginine residues—for example, glycation of arginine residues by MG forms a stable hydroimidazolone, N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine (MG-H1)—**Fig. 2**B. MG and glyoxal are metabolized mainly by the glyoxalase 1 (Glo1) of the cytoplasmic glyoxalase system—**Fig. 3**A. Clinical homozygous frameshift mutation of the GLO1 gene is embryonically lethal, indicating that glycation by dicarbonyl substrates of Glo1—particularly MG—is a highly physiologically damaging form of glycation (Rabbani and Thornalley, 2014; Rabbani *et al.*, 2014b).

Quantitative assessments of AGEs show that major protein AGEs are: MG-H1, CML and the crosslink glucosepane (Ahmed *et al.*, 1986; Monnier *et al.*, 2013; Rabbani and Thornalley, 2012c). Other important AGEs in vivo are: glyoxal-derived hydroimidazolone G-H1 and 3-DG-derived hydroimidazolone isomers 3DG-H, pyrraline; and the pentose-derived fluorophore, pentosidine—**Fig. 3**C–F. Pyrraline is an AGE formed at high temperatures of culinary processing and mostly originates from ingested food (Rabbani *et al.*, 2014a). AGE residues are released from proteins by cellular proteolysis. The AGE free adducts thereby formed are released into plasma and excreted in urine. MG-H1 and CML are the major quantitative glycation free adducts excreted in human urine. AGE free adducts were detected in plasma, urine, cerebrospinal fluid, synovial fluid and haemodialysis and peritoneal dialysate. Loss of renal clearance in diabetic and nondiabetic chronic kidney disease leads to accumulation of AGE free

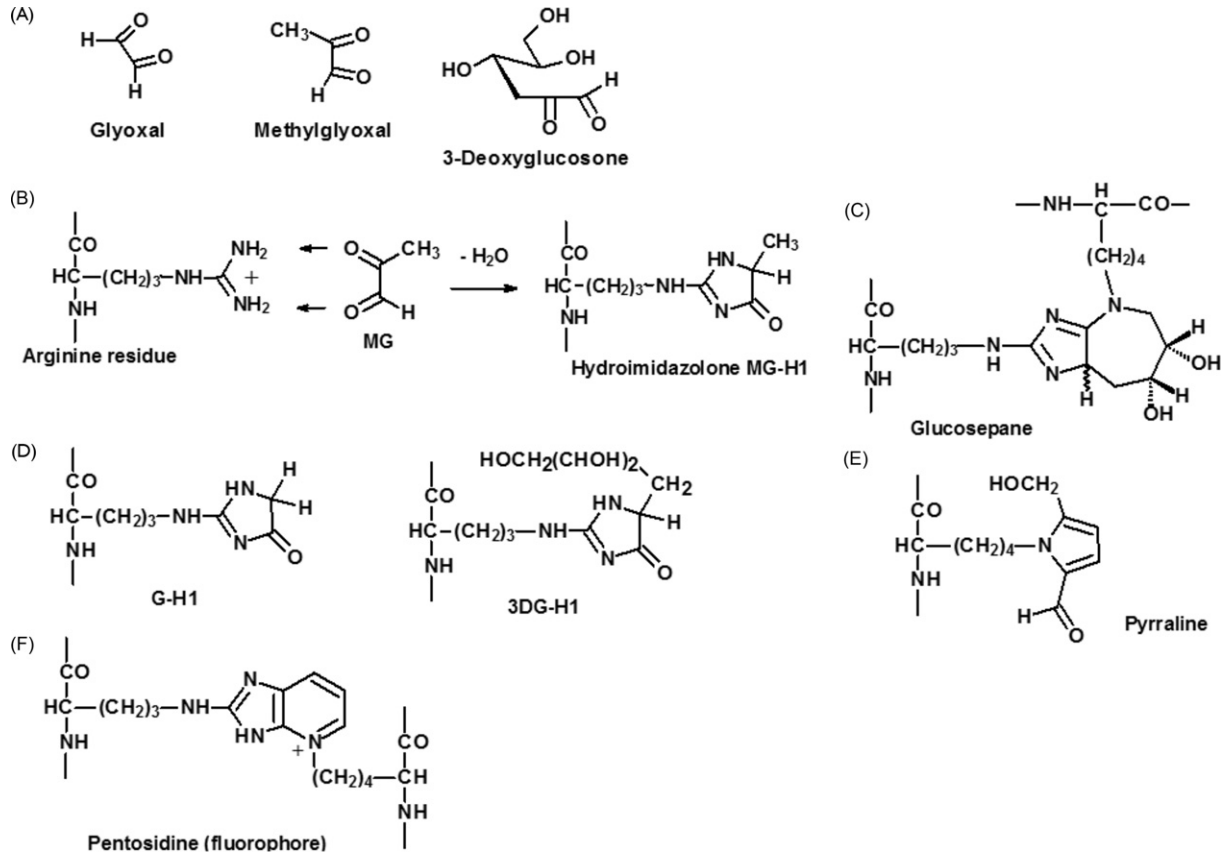

Fig. 2 *Glycation of proteins to form advanced glycation endproducts.* (A) Reactive α-oxoaldehydes in physiological glycation process. Molecular structures of AGEs. (B) Reaction of methylglyoxal with arginine residues to form hydroimidazolone N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl) ornithine (MG-H1). Other AGEs: (C) Glucosepane; (D) Hydroimidazolones derived from glyoxal, G-H1, and 3-deoxyglucosone, 3DG-H1, formed with related structural isomers; (E) Pyrraline; and (F) Pentosidine. AGE adduct residues are shown with the peptide backbone –CO-CHR-NH- on the left. For the corresponding free adducts at physiological pH, the N-terminal amino group is protonated $-NH_3^+$ and the C-terminal carbonyl is a carboxylate $-CO_2^-$ moiety.

adducts in plasma. In patients with renal failure on peritoneal dialysis and hemodialysis, plasma MG-H1 free adduct increased 18- and 40-fold.

Glyoxal and MG are also important precursors of nucleotide AGEs. The quantitatively major nucleotide AGE is formed by MG —deoxyguanosine-derived imidazopurinone, 3-(2′-deoxyribosyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purine-9(8)one isomers (MGdG) (Thornalley *et al.*, 2010).

AGEs are quantified by stable isotopic dilution analysis liquid chromatography–tandem mass spectrometry (LC-MS/MS). There is limited correlation between amounts of different AGEs as they have different metabolic precursors, stabilities and in situ enzymatic activities preventing their formation. Estimates of individual AGEs are therefore required. AGE residues in protein and DNA are determined after prior exhaustive enzymatic digestion. AGE free adducts are determined directly in ultrafiltration of plasma, urine or other physiological fluids. Immunochemical techniques may be used for assay of AGEs. They perform best when corroborated by LC-MS/MS as interferences and lack of robust quantitation can give misleading estimates. Fluorescence of the trace AGE, pentosidine, in skin contributes to skin autofluorescence (SAF). The many other non-AGE fluorophores contributing to SAF and lack of responsiveness to major non-fluorescent AGEs make SAF an unreliable measure of AGEs (Rabbani *et al.*, 2014a; Thornalley and Rabbani, 2014; Thornalley *et al.*, 2010).

AGEs have different stabilities: some AGEs are highly stable (half-lives of years)—such as CML, whereas others have only moderate stability (half-lives of 2–3 weeks)—such as hydroimidazolones G-H1, MG-H1 and 3DG-H. There are at least four processes involved in the formation of AGEs in physiological systems: (i) monosaccharide autoxidation (autoxidative glycosylation)—the degradation of saccharide unattached to a protein, (ii) Schiff's base fragmentation, (iii) fructosamine degradation, and (iv) direct reaction of dicarbonyls formed from the degradation of glycolytic intermediates and lipid peroxidation with proteins (Rabbani and Thornalley, 2014; Thornalley *et al.*, 1999). Glycation is usually a minor posttranslational modification of protein and DNA. In proteins, the extent of glycation depends on concentration, reactivity and half-life of the protein and the concentration and reactivity of the glycating agent. The range of extent of glycation is typically 0.1%–1% of lysine and arginine

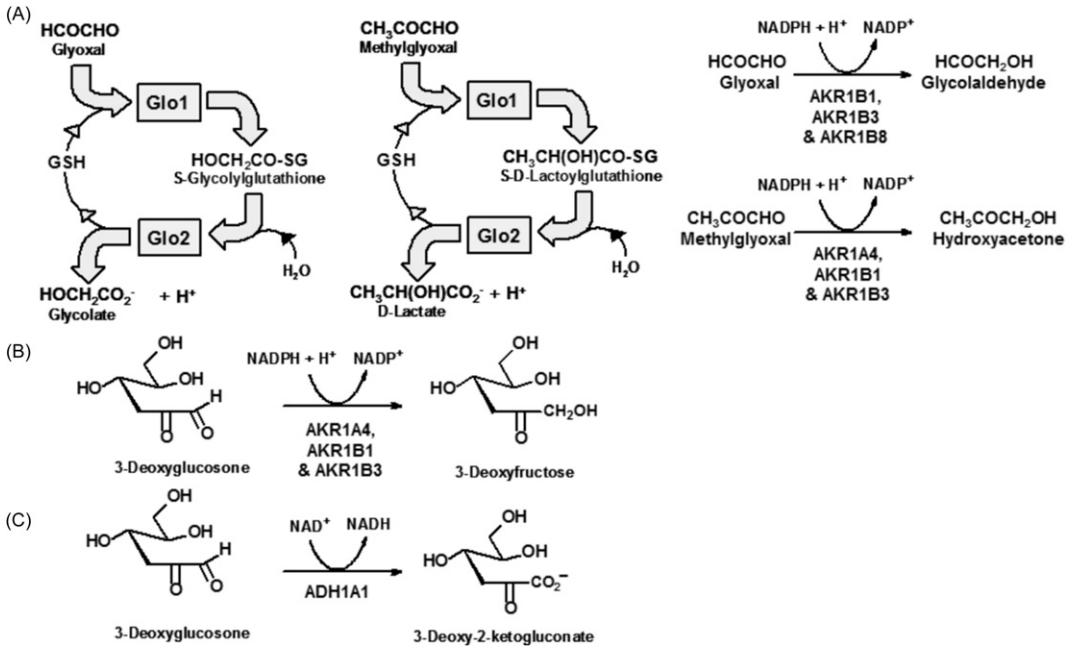

Fig. 3 *Metabolism of dicarbonyl metabolites—the enzymatic defense against glycation.* (A) Metabolism of glyoxal and methylglyoxal by the glyoxalase system and aldoketo reductases. (B) Metabolism of 3-deoxyglucosone by 3-DG reductase (AKRs 1A4, 1B1 and 1B3). (C) Metabolism of 3-deoxyglucosone by 3-DG dehydrogenase reductase (ADH 1A1).

residues in proteins and up to 1 in 10^5 nucleotides in DNA (Rabbani and Thornalley, 2012c; Thornalley *et al.*, 2010). For HSA, the concentration ranges (mol/mol albumin) of Schiff's base adduct, fructosamine and AGEs are c. 1%–5%, 6%–15% and 0.01%–7%.

Metabolism of dicarbonyls provides a physiological defense against AGE formation. Glyoxal and MG are detoxified to glycolate and D-lactate, respectively, by the glutathione-dependent glyoxalase system. When the glyoxalase system is impaired, AKR isozymes 1B1 (aldose reductase), 1B3 and 1B8 metabolize glyoxal to glycolaldehyde and AKR isozymes 1A4, 1B1 and 1B3 metabolize MG to mainly hydroxyacetone—**Fig. 3**A. The rate controlling step is catalyzed by Glo1 which has expression regulated by the transcription factor Nrf2. 3-DG is metabolized to 3-deoxyfructose by AKRs 1A4, 1B1 and 1B3 (3-DG reductase activity) and to 3-deoxy-2-ketogluconate by aldehyde dehydrogenase ADH1A1 (3-DG dehydrogenase activity)—**Fig. 3**B and C. All genes encoding these enzymes have expression regulated by transcription factor Nrf2. Additionally, there is a high cysteinyl thiol pool that binds dicarbonyls reversibly and thereby suppresses their irreversible reactions to form AGEs (Rabbani and Thornalley, 2012a).

Contribution of Food AGEs to Clinical AGE Exposure and Health Impairment

Thermally processed food is a rich source of AGEs which may be absorbed as free adducts after digestion of ingested protein. Dietary AGEs have poor bioavailability. There is no evidence that the AGE free adducts absorbed therefrom are incorporated into endogenous proteins. Rather, AGE free adducts have high renal clearances and are efficiently excreted in urine. So that unless renal function is impaired they are rapidly cleared and excreted (Agalou *et al.*, 2005; Rabbani and Thornalley, 2014). The proportions of urinary AGE free adducts sourced from food and endogenous glycation may be deduced by plotting urinary AGE free adduct excretion against the urinary excretion of pyrraline. Urinary excretion of AGE free adducts provides an estimate of total body AGE exposure and urinary exertion of pyrraline reflects absorption of AGEs from food. Correlation of urinary excretion of a particular AGE with urinary exertion of pyrraline reflects a significant contribution of the AGE from food. Linear regression of urinary AGE excretion on urinary exertion of pyrraline and extrapolation to zero pyrraline excretion gives an estimate of the flux of AGE formed endogenously in the body. AGE absorbed from food then equals the total urinary AGE flux minus this deduced endogenous AGE flux. This analysis was performed recently for MG-H1. In healthy overweight and obese subjects, the flux of endogenous formation of MG-H1 at baseline was c. 13 nmol/mg creatinine, representing 68% of total MG-H1 exposure; the mean contribution to total MG-H1 exposure from the diet was 32% but highly variable (Xue *et al.*, 2016). The diet is likely often a minor source of MG-H1 exposure.

Several clinical studies with interventions to decrease exposure to dietary AGEs for improved vascular health in chronic kidney disease have been performed (Harcourt *et al.*, 2011; Vlassara *et al.*, 2009; Yacoub *et al.*, 2017). The outcomes are difficult to interpret as unblinded study designs were used. These and other studies were evaluated by meta-analysis for overall assessment of evidence of health benefits (Kellow *et al.*, 2014). It was concluded that there is insufficient evidence, at present, to recommend dietary AGE restriction for health benefit (Kellow *et al.*, 2014). There is a need for long-term, high-quality randomized controlled

trials with large sample size and masking of the AGE intervention to provide more robust evidence on the health impact of dietary AGEs—also taking advantage of recent advances in improved quantitation of AGEs in food (Scheijen *et al.*, 2016).

Functional Consequences of Glycation

Glycation occurs inside and outside of cells. Glycation of basic phospholipids may induce changes in membrane structure and lipid peroxidation. Glycation of nucleotides in DNA normally induces DNA repair but may induce apoptosis or senescence if glycation is excessive. Glycation of proteins may also induce apoptosis by modification of mitochondrial proteins and activation of mitochondrial apoptotic pathway and glycation of extracellular proteins leading to cell detachment-stimulated apoptosis. Glycation of cellular and extracellular proteins produces loss of function or acquisition of new pathogenic function. These effects are countered by protein degradation and renewal. Glycation by MG to form MG-H1 is particularly damaging because: (i) loss of positive charge of arginine residues with MG-H1 formation produces loss of all electrostatic interactions involved in neighboring group and ligand binding interactions; (ii) arginine residues have the highest probability of any amino acid residue for location at functional sites of proteins; and (iii) MG-H1 is one of the most quantitatively important, spontaneous irreversible modifications of the proteome in health and disease (Rabbani and Thornalley, 2014). The extent of modification of proteins is only minor but increases in this low level of modification may have profound physiological effects. For example, detachment of vascular endothelial cells by glycation of collagen-IV of the extracellular matrix and atherogenic transformation of LDL by glycation of apolipoprotein B100 with MG—both contributory factors to increased cardiovascular disease, and MG modification of mitochondrial proteins linked to mitochondrial dysfunction and increased formation of reactive oxygen species (ROS) (Thornalley and Rabbani, 2010).

Protein Glycation and Risk of Developing Diabetic Complications

A1C is a risk factor for the development of chronic clinical complications as a surrogate indicator of hyperglycemia (Stratton *et al.*, 2000; The Diabetes *et al.*, 2000). Skin collagen content of stable AGEs such as CML, pentosidine, glucosepane and others are linked to risk of developing and microvascular complications of diabetes and their progression, probably as surrogate markers of long-term metabolic control (McCance *et al.*, 1993; Monnier *et al.*, 2013). In recent studies of AGEs and related analysis of skin collagen in patients with type 1 diabetes mellitus (T1DM), an analyte panel of glucosepane, MG-H1, CML, N_{ϵ}(1-carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), pentosidine, furosine, collagen fluorescence, skin collagen acid solubility and pepsin digestibility was linked to risk of progression of diabetic microvascular complications. The FL-linked analyte, furosine, was a strong predictor—likley as a surrogate indicator of hyperglycemia (Genuth *et al.*, 2015). In contrast, CML residue content of plasma protein alone was not linked to the risk of developing diabetic nephropathy (Klein *et al.*, 2017). Plasma MG-H1 free adduct concentration was an independent risk predictor for progression of diabetic kidney disease (Beisswenger *et al.*, 2013).

Dicarbonyl stress and related arginine directed glycation appears to be a driver for development of vascular complication of diabetes. There was a 5–6-fold increase of MG concentration in blood samples of patients with T1DM and 3–4-fold increase of whole blood MG in patients with type 2 diabetes mellitus (T2DM). This was disproportionately high compared to increase in glucose and D-lactate concentration—both increased 2–3-fold (McLellan *et al.*, 1994). The disproportionate increase in MG concentration likely reflects decrease in Glo1 activity at peripheral tissue sites – including kidney, peripheral nerve, retina and vascular endothelial cells (Bierhaus *et al.*, 2012; Miller *et al.*, 2010; Yao and Brownlee, 2009). Decreased Glo1 activity may reflect decreased Glo1 expression in response to inflammatory and/or hypoxia signaling and increased proteolysis (Bierhaus *et al.*, 2012; Yao and Brownlee, 2009; Zhang *et al.*, 2012). Increased MG has emerged as a factor linked to the development of chronic microvascular complications of diabetes (nephropathy, retinopathy and neuropathy) (Fosmark *et al.*, 2006; Hammes *et al.*, 1999; Kilhovd *et al.*, 1999; McLellan *et al.*, 1994). Functional genomics studies with Glo1 deficient and Glo1 overexpressing transgenic mice support this (Berner *et al.*, 2012; Bierhaus *et al.*, 2012; Giacco *et al.*, 2014).

The contribution of dicarbonyl glycation to diabetic macrovascular disease (coronary artery disease, peripheral arterial disease and stroke) is less certain. MG modification of LDL induced atherogenic transformation to small, dense LDL and MG modification of HDL induced destabilization and increase plasma clearance (Rabbani and Thornalley, 2014; Rabbani *et al.*, 2014b). In a large genome-wide integrated transcriptomic study, Glo1 deficiency emerged as a previously unrecognized driver of coronary heart disease (Mäkinen *et al.*, 2014). Treatment of apolipoprotein E deficient mice with a cell permeable Glo1 inhibitor increased vascular inflammation and atherogenesis in normoglycemia similar in extent to that found in diabetic mice (Tikellis *et al.*, 2014). Dicarbonyl glycation, therefore, likely contributes to dyslipidemia risk factors of cardiovascular disease and vascular cell dysfunction driving vascular inflammation and atherogenesis which is exacerbated in the diabetic state.

Accumulation of AGE crosslinks in proteins have been proposed as a mechanism contributing to basement membrane thickening in diabetes through increased resistance to proteolysis. This may be an oversimplification, however, as there are non-sulfhydryl crosslink of the extracellular matrix of markedly higher quantitative content that AGEs—such as transglutaminase-formed $N\epsilon$-(γ-glutamyl)lysine and dual oxidase-formed dityrosine and metabolic dysfunction in diabetes produced increased extracellular matrix deposition.

Alleviation of the damaging effects of glycation may be achieved through improvement of glycemic control but this is likely insufficient alone. Meta-analysis indicated that clinically achievable glycemic control addresses only 22% of risk of diabetic nephropathy (Hemmingsen *et al.*, 2011). Scavengers of dicarbonyls have been proposed as therapeutic agents but the high reactivity of them required to compete effectively with tissue proteins is often associated with toxicity and instability. Some current treatments have secondary pharmacological activities that decrease dicarbonyl glycation; for example, metformin and angiotensin receptor blocker Irbesartan (Rabbani *et al.*, 2011, 2010). Increasing Glo1 expression and activity would likely be more effective. A strategy to produce this is to develop Glo1 inducers (Xue *et al.*, 2012)—reviewed in (Rabbani and Thornalley, 2018).

Dietary bioactive compounds were screened as activators of transcription factor Nrf2 and inducers of Glo1 expression via a functional antioxidant response element and the best inducer of Glo1 expression or "Glo1 inducer" found to be a combination of *trans*-resveratrol and hesperetin (tRES-HESP). In a double blind, randomized, placebo-controlled crossover study evaluation of tRES-HESP, expression and activity of Glo1 in peripheral blood mononuclear cells was increased, plasma MG decreased and total body MG-protein glycation decreased. There was related decreased fasting plasma glucose (FPG) and postprandial plasma glucose (PPG), increased insulin sensitivity, improved arterial dilatation and decreased vascular inflammation marker, soluble intracellular vascular cell adhesion molecule-1 (sICAM-1). There was also minor weight loss and improvement in renal function (Xue *et al.*, 2016). Clinical therapeutic response of the tRES-HESP combination was profoundly stronger than either compound alone. This is likely due to HESP improving bioavailability of tRES by inhibition of intestinal glucuronosyltransferases—reviewed in (Rabbani and Thornalley, 2018).

Comparison of the effect of tRES-HESP in overweight and obese subjects to that of metformin and Olristat on similar subjects groups in similar intervention trials suggested the effect of tRES-HESP on glycaemic control exceeds that of metformin and matches that of Olristat (Mancini and Halpern, 2008; Park *et al.*, 2009). Decrease in FPG in the normal range may help reduce the risk of developing T2DM (Tirosh *et al.*, 2005) and improvement in insulin resistance was comparable to that achieved with extreme weight loss with gastric band surgery in morbid obesity (Hanusch-Enserer *et al.*, 2004). tRES-HESP could be a suitable treatment for improved metabolic and vascular health in overweight and obese populations. Glo1 inducer development appears to be particularly appropriate and timely for treatment of high prevalence complications of obesity such as nonalcoholic fatty liver disease (NAFLD) and vascular complications of diabetes. Increased AGEs have been associated with the development of T1DM (Beyan *et al.*, 2012) and increased Glo1 protein was found to be protective of experimental chemically-induced pancreatic beta-cell toxicity (Kim *et al.*, 2013). The Glo1 inducer formulation may also be considered for prevention of T1DM, particularly as it is a synergistic binary combination of active agents involving tRES. tRES has previously been found to be effective in prevention of diabetes in experimental mouse model of non-obese type 1 diabetes, albeit at doses higher than tolerated clinically (Lee *et al.*, 2011).

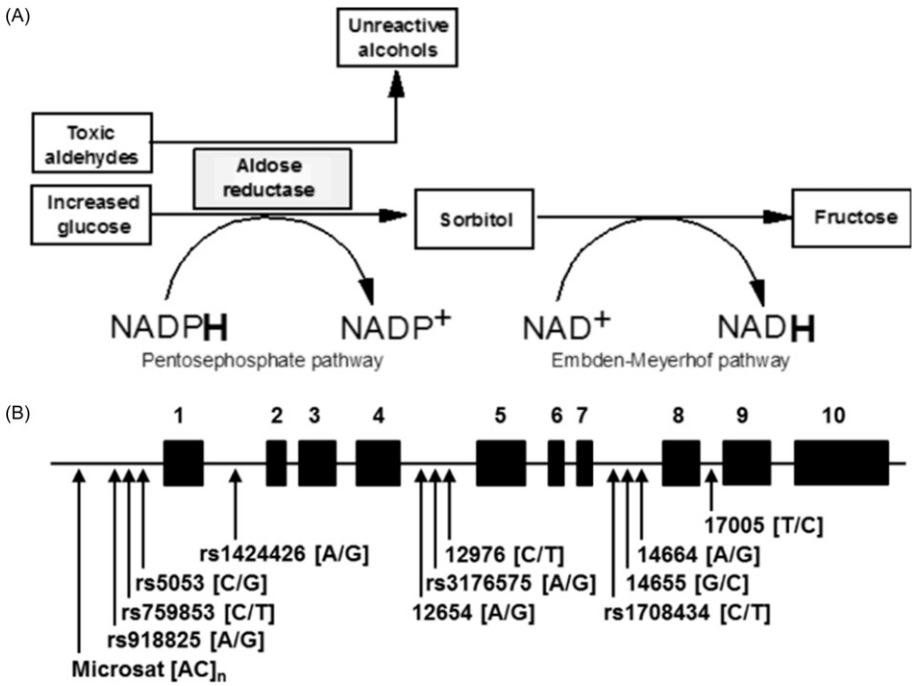

Fig. 4 *The polyol pathway.* (A) Metabolic pathway and cofactors. (B) AK1B1 Gene structure showing location of polymorphisms. The position of each variant within AKR1B1 is designated by an arrow. Exons are numbered and are represented by filled rectangles. Single nucleotide polymorphism (SNPs) are identified by the dbSNP identifier when available or by the nucleotide position within the aldose reductase genomic sequence (AF032455); the polymorphic dinucleotide repeat is designated "microsat." The locus size is approximately 17 kb (Wolford et al., 2006).

Polyol Pathway and Diabetic Complications

Aldose reductase (AR; AKR1B1) is the first enzyme in the polyol pathway. It catalyzes the NADPH-dependent reduction of glucose and many other carbonyl compounds to sorbitol and the corresponding alcohol derivatives—**Fig. 4**A. AR also catalyzes the formation of prostaglandin F2α from prostaglandin H2 and thereby influencing the production of prostaglandin E2 (PGE2) (Lacroix Pépin *et al.*, 2013) and catalyzes the reduction of MG to hydroxyacetone—although this is only a significant fate for MG metabolism in the renal medulla where expression of AR is high (Rabbani *et al.*, 2016). Indeed, AR has an extraordinarily high expression in the human renal medulla where it constitutes c. 2% total protein and a cellular concentration of c. 100 μM (Nishimura *et al.*, 1993). Its role therein is to reduce glucose to sorbitol and produce intracellular osmotic pressure to counter extracellular hypertonicity of increasing sodium chloride and urea concentrations produced during antidiuresis (Moriyama *et al.*, 1989). Sorbitol content of the renal medulla is relatedly very high—c. 100 mM, as expected for a role in osmoregulation (Grunewald *et al.*, 1995). In comparison, vascular endothelial cell content of AR is 0.08% total protein and sorbitol concentration is much lower, c. 0.6 mM, were there is limited effect on osmotic pressure of AR activity (Hawthorne *et al.*, 1989; Nakamura *et al.*, 2000).

AR has a low affinity (high K_M) for glucose. Hence, there is an increased flux of glucose metabolism via the polyol pathway in hyperglycemia. In the renal medulla of streptozotocin (STZ)-induced diabetic rats, sorbitol synthesis was increased and tissue content of sorbitol increased twofold (Willi and Kinne, 1989). In human red blood cells, glucose metabolism increases from 3% of total glucose metabolism in normoglycemia to 6% in moderate hyperglycemia (Morrison *et al.*, 1970). Red blood cell content of sorbitol in healthy subjects is c. 3 μM and increased threefold in patients with type 1 diabetes (Malone *et al.*, 1984). In vascular endothelial cells the flux of glucose metabolized by the polyol pathway, as judged by pentosephosphate pathway activity, was relatively low in normoglycemia and increased little in model hyperglycemia in vitro (Kashiwagi *et al.*, 1997) with an increase in sorbitol content to c. 1.1 mM (Hawthorne *et al.*, 1989). Estimates of sorbitol concentration in sural nerve of patients with diabetes were c. 0.1 mM (Greene *et al.*, 1999). Sorbitol was increased in retina of STZ-induced diabetic rats from c. 0.06 mM in healthy controls to c. 0.66 mM in diabetes (Poulsom *et al.*, 1983). This suggests that AR activity may not contribute significantly to osmotic pressure in tissues other than the renal medulla in diabetes. The effects of activation of the polyol pathway in diabetes have also been attributed to decreased Na^+K^+ ATPase activity, in situ inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by change in NADH/NAD^+ ratio, in situ inhibition of glutathione reductase by depletion of NADPH—exacerbating intracellular oxidative stress. These effects have been linked to depletion of myo-inositol with decreased incorporation into cell phosphoinositides, chronic elevation of diacylglycerol and abnormal activation of protein kinase C (PKC) and decreased activity of NAD^+-dependent deacetylase sirtuin-1 and protein hyperacetylation (Vedantham *et al.*, 2014; Xia *et al.*, 1994). More recently, hyperglycemia-induced sorbitol accumulation in Schwann cells of peripheral nerve has been linked to their dedifferentiation to immature cells and peripheral nerve de-myelination (Hao *et al.*, 2015). This was prevented by treatment with an AR inhibitor (ARI).

Sorbitol produced in the first step of the polyol pathway is oxidized by NAD^+-dependent sorbitol dehydrogenase (SDH) to fructose in the second step. The complete traverse of the polyol pathway involves oxidation of NADPH to $NADP^+$ and reduction of NAD^+ to NADH. This represents a net hydride transfer from NADPH in the pentosephosphate pathway to NAD^+ in Embden-Meyerhof pathway and thereby a net reduction of NAD^+ to NADH in Embden-Meyerhof pathway and an increase in cytosolic NADH/NAD^+ ratio.

Mice with AR gene deletion had defective urine concentrating ability with a phenotype resembling nephrogenic diabetes insipidus (Ho *et al.*, 2000), reflecting the role of AR in osmoregulation and antidiuresis. Diabetes induced in homozygous AR knockout mice protected against decreased GSH content of sciatic nerve, decreased motor nerve conduction velocity, in contrast to wild-type mice and degeneration of retinal capillaries (Liu *et al.*, 2011; Tang *et al.*, 2013). AR deficiency prevented renal extracellular matrix accumulation and collagen IV overproduction, activation of renal cortical PKC, transforming growth factor-beta 1 (TGF-β1) and glomerular hypertrophy in diabetic mice and decreased urinary albumin excretion (Liu *et al.*, 2011). In contrast, AR deficiency increased development of atherosclerosis in diabetic apolipoprotein E deficient mice (Srivastava *et al.*, 2009)—exemplifying the complex involvement of AR in pathogenesis in diabetes.

The human AR gene—**Fig. 4**A,B, AKR1B1 (also known as ALR2), is located at chromosome 7q35. In meta-analysis, AKR1B1 polymorphisms were associated with diabetic nephropathy: the $Z-2$ allele was associated with increased risk whereas the $Z+2$ allele was associated with decreased risk of diabetic nephropathy. The T allele in SNP rs759853 was associated with increased risk of diabetic nephropathy. AKR1B1 polymorphisms were also associated with diabetic retinopathy: the $z-2$ microsatellite increased risk in type 1 and type 2 diabetes and $z+2$ conferred protection against diabetic retinopathy in type 2 diabetes. The T allele of the AKR1B1 promoter rs759853 variant was protective against diabetic retinopathy in type 1 diabetes (Abhary *et al.*, 2009; Mooyaart *et al.*, 2011).

Studies of the inhibition of the polyol pathway in vivo with specific inhibitors have given inconsistent results. Several clinical trials have been performed for treatment of diabetic neuropathy with negative outcomes. Recent meta-analysis of clinical trials of ARIs indicate there are beneficial effects in diabetic cardiovascular autonomic neuropathy (Hu *et al.*, 2014). A potential problem for ARIs is the adverse effect of decreasing the antidiuretic activity of AR in the kidney.

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Hypertension and Diabetes☆

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Glossary

Diastolic blood pressure Pressure as measured during the rhythmic relaxation of the muscles of the heart chambers during the time they fill with blood.

Hyperglycemia An abnormally high concentration of sugar in the blood.

Nephropathy Any disease of the kidney.

Macrovascular disease Disease involving large blood vessels

Microvascular disease Disease involving small blood vessels.

Systolic blood pressure SBP Pressure as measured during contraction of the heart chambers.

Introduction

The management of the hypertension so frequently seen in diabetic patients has now become one of the most critical challenges of health care. However, numerous controversies over the appropriate way in which to treat hypertension in diabetic patients have arisen, including, the appropriate level of blood pressure at which antihypertensive therapy should be started, the blood pressure goals of such therapy, and the best choices of antihypertensive drugs. Here, we aim to review the evidence and make recommendations for management of hypertension in patients with type 2 diabetes mellitus.

Evidence for Intensive Treatment of Blood Pressure

Randomized controlled trials (RCTs) have provided conclusive evidence of the impressive benefits of reducing the blood pressure at least to below 150/85 mmHg (**Table 1**). In the diabetes subgroups of the systolic hypertension in the elderly program (SHEP, $n = 583$) and the systolic hypertension in Europe (Syst-Eur, $n = 492$) trials, both of which treated patients age ≥ 60 years with isolated systolic hypertension, treatment to a systolic blood pressure goal <150 mmHg with therapy based on the thiazide-type diuretic chlorthalidone or the dihydropyridine calcium channel blocker (CCB) nitrendipine, respectively, reduced major cardiovascular disease events by 34% and 62%, respectively (Curb *et al.*, 1996; Tuomilehto *et al.*, 1999). The mean achieved blood pressure in the active treatment arms of the diabetic subgroups in SHEP and SYS-EUR trial were 146/68 and 153/78, respectively.

In the United Kingdom Prospective Diabetes Study (UKPDS), control of blood pressure to a target $<150/85$ mmHg versus a target of $<180/105$ mmHg significantly reduced microvascular disease, stroke, heart failure, and deaths related to diabetes (UK Prospective Diabetes Study Group, 1998). The attained mean blood pressure was 144/82 mmHg in the "tight" control arm and 154/87 mmHg in the less tight control arm.

In a post hoc analysis of the hypertension optimal treatment (HOT) trial, 1501 diabetic hypertensives were randomly allocated to achieve diastolic levels of less than 90, 85, or 80 mmHg with therapy based on a CCB but often requiring addition of one or two other drugs. Those assigned to the 80-mmHg goal reached a mean level of 81 mmHg, only 4 mmHg lower than those assigned to the 90-mmHg goal. Despite this relatively small difference in achieved pressure, there was a very impressive reduction in all events. However, some guideline committees discount this analysis since it was not prespecified.

The hypertension component of the ABCD (appropriate blood pressure control in diabetes) trial, compared intensive control of blood pressure (diastolic blood pressure of 75 mmHg) with moderate control of blood pressure (target diastolic blood pressure of 80–89): achieved blood pressure was 132/78 mmHg and 138/86 mmHg, respectively.

While there was no reduction in the primary microvascular or secondary cardiovascular disease, the total mortality was significantly reduced (Schrier *et al.*, 1996).

In the action in diabetes and vascular disease: Preterax and diamicron modified-release controlled evaluation (ADVANCE) trial, treatment with an angiotensin converting enzyme (ACE) inhibitor and a thiazide-type diuretic in patients with type 2 diabetes reduced deaths from all causes significantly (Patel *et al.*, 2007). The ADVANCE trial had no specified blood pressure goals but the mean achieved systolic blood pressure in the more intensive arm was 134 mmHg.

The action to control cardiovascular disease in diabetes—blood pressure (ACCORD—BP) trial is the only randomized controlled trial that has examined the effect of a systolic blood pressure goal of lower than 140 mmHg on major cardiovascular disease

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Table 1 Randomized controlled trials assessing blood pressure among diabetic patients

	n	*Duration (years)*	*Systolic BP goal (mmhg)*	*Diastolic BP goal (mmhg)*	*Mean BP achieved-less intense (mmhg)*	*Mean BP achieved-more intense (mmhg)*	*Outcome (statistically significant risk reduction)*
SHEP	585	5	<148	None	155/72	146/68	CV events (34%), CHD (56%)
SYS-EUR	492	2	<150	None	162/82	153/78	Stroke (69%), CVD events (62%)
HOT	1501	3	None	<80	148/85	144/81	CV events (51%), MI (50%), CV mortality (67%)
UKPDS	1148	8.4	<150	<85	154/87	144/82	Diabetes endpoints (34%), deaths (32%), Stroke (44%), microvascular events (37%)
ABCD	470	5.3	None	<75	138/86	132/78	Total mortality (49%)
ACCORD-BP	4733	4.7	<120	None	134	119	Stroke (41%)
ADVANCE	11140	4.3	None	None	140/77	134/75	All-cause mortality (14%), CVD mortality (18%) combined macro and microvascular mortality (9%)

Modified Ferrannini, E. and Cushman, W.C. (2012). Diabetes and hypertension: The bad companions. The Lancet 380(9841), 601–610.

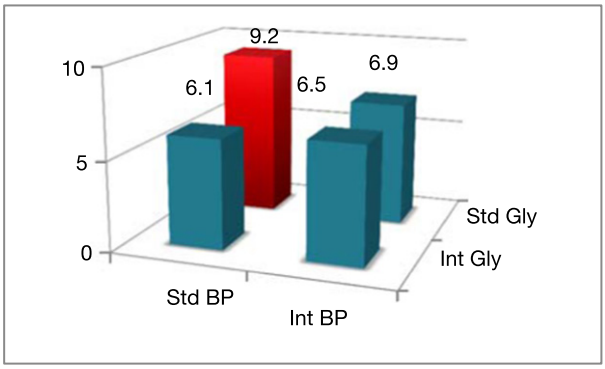

Fig. 1 ACCORD-BP: Primary 5 year CVD event rates by glycemia and BP randomized subgroups.

outcomes among diabetics. This trial compared intensive treatment (systolic blood pressure goal of <120 mmHg) with standard treatment (goal of <140 mmHg) in 4733 patients with type 2 diabetes with entry systolic blood pressure of 130–180 mmHg (The ACCORD Study Group, 2010). The 12% reduction in mortality or overall primary cardiovascular outcomes in the intensive treatment group compared with the standard goal group was not statistically significant. However, intensive treatment was associated with a significant reduction in the occurrence of stroke (risk reduction 41%; $P = .01$), which was a prespecified secondary outcome. In addition, in the standard glycemia subgroup (approximately ½ of the BP study population, the primary outcome was reduced by 26% with intensive treatment of blood pressure compared with standard blood pressure treatment (Margolis *et al.*, 2014). Standard glycemic/standard BP subgroup had significantly higher CVD event rate than other subgroups (**Fig. 1**).

In 2015, the findings from the systolic blood pressure intervention trial (SPRINT) brought the discussion of target blood pressure goals in diabetic patients to light again. SPRINT randomized 9361 persons with systolic BP >130 mmHg and increased CV risk, but without type 2 DM, to a systolic BP target <120 mmHg (intensive treatment) or a target of <140 mmHg (standard treatment). At 1 year, the mean systolic BP was 121.4 mmHg in the intensive treatment group and 136.2 mmHg in the standard-treatment group (The SPRINT Reseach Group, 2015). The study was stopped early after a median follow-up of 3.26 years due to a 25% lower rate of the primary composite outcome in the intensive-treatment group than in the standard-treatment group ($P < .001$). All-cause mortality was also lower by 27% in the intensive treatment group ($P = .003$). SPRINT findings support intensive BP lowering in nondiabetic patients with increased CV risk. Diabetic patients were not included in the SPRINT but are known to be at increased risk for CV events and one could speculate that SPRINT results could apply to diabetics. In fact, it was recently reported from SPRINT that the beneficial effects of intensive systolic blood pressure treatment were similar among those with prediabetes (fasting glucose ≥100 mg/dL) and fasting normoglycemia (Bress *et al.*, 2017).

Several reasons can explain the discrepancy of the findings of ACCORD and SPRINT (Margolis *et al.*, 2014). Participants in ACCORD were younger compared to SPRINT (mean age of 62 vs. 68), and had lower event rates than initially predicted. Participants in the BP intervention of ACCORD had healthier cardiovascular risk profile than expected because patients with dyslipidemia were assigned to the lipid arm and were excluded from the BP arm and those with chronic kidney disease (creatinine >1.5 mg/dL) were excluded. The complex factorial study design in ACCORD could have made it harder to detect a statistically

significant finding. Because event rates were 50% lower than was expected in the standard-treatment group, ACCORD—BP could be viewed as underpowered.

A large network meta-analyses of 42 randomized clinical trials, including 144,220 patients with multiple comorbidities including diabetes, compared multiple levels of achieved systolic blood pressure on the risk of CVD and all-cause mortality. The lowest risk of cardiovascular disease and mortality was present for randomized groups with a mean achieved SBP range of 120 to 124 mmHg (Bundy *et al.*, 2017). This group had a hazard ratio (HR) for major cardiovascular disease of 0.71 (95% CI, 0.60–0.83) compared with randomized groups with a mean achieved SBP of 130–134 mmHg. A sensitivity analysis was also conducted excluding SPRINT from the meta-analyses, HRs and 95% CIs were consistent with results from the main analyses for major CVD, CHD, and all-cause mortality, and most of the trials included significant numbers of participants with diabetes mellitus.

The totality of evidence suggests that a target of BP $<$130/80–85 mmHg is reasonable in most diabetic patients and it is possible that if diabetic patients were included in SPRINT, they could have similarly benefited from intensive systolic BP lowering to about 120 mmHg. Based on the currently available evidence, the 2017 Canadian Hypertension guidelines and a 2017 American Diabetes Association position paper have both recommended advising patients with diabetes (and high cardiovascular risk in the ADA statement) to be treated to attain a blood pressure of $<$130/80 mmHg (Leung *et al.*, 2016; American Diabetic Association, 2017).

Evidence That Antihypertensive Drugs Reduce Risk

There have been a number of major outcome trials demonstrating that diuretics, CCBs, angiotensin-converting enzyme inhibitors (ACEIs), and angiotensin II receptor blockers (ARBs) reduce cardiovascular morbidity and mortality or progression of renal damage in patients with diabetes mellitus. Therefore, the initial several drug classes for treatment for hypertension in diabetes should include these classes. For patients with albuminuria (urine albumin-to-creatinine ratio [UACR] $\geq$30 mg/g), and possibly patients with chronic kidney disease without albuminuria, initial treatment should include an ACEI or ARB in order to reduce the risk of progressive kidney disease. ACEIs and ARBs have not been shown to be superior in reducing cardiovascular events when compared with other antihypertensive agents, and thiazide-type diuretics and CCBs are superior to ACEIs and ARBs in reducing cardiovascular events in blacks with diabetes. β-blockers may be used for the treatment of coronary disease or heart failure in diabetes but have not been shown to reduce cardiovascular events more than these other agents in treatment of hypertension in the absence of these conditions.

Comparison of Major Drug Classes

The overall data from comparative trials of diabetic hypertensives provide no clear proof that one class of drug is better than another among diabetic without kidney disease. However, the current recommendations vary among different guidelines about the role of RAS blockade for treatment of hypertension among diabetics without involvement of kidneys.

A prospective meta-analysis by the Blood Pressure Lowering Treatment Trialists' Collaboration showed that major cardiovascular disease events were reduced to a similar extent in individuals with or without diabetes regardless of the drug class used to reduce blood pressure (Turnbull *et al.*, 2005). However, this meta-analysis lumped diuretics and β-blockers together and treated them as one class. However, these drug classes and their results in trials are quite different from each other.

The antihypertensive and lipid-lowering treatment to prevent heart attack trial (ALLHAT) studied various antihypertensive agents in a large number (13,101) of diabetic patients. The trial failed to show superiority with use of CCBs or ACEIs compared with a thiazide-type diuretic, when used as the first-step antihypertensive agent (Whelton *et al.*, 2005). A significantly higher incidence of heart failure was present among those assigned to amlodipine compared with chlorthalidone in the diabetic patients (RR, 1.39 [95% CI, 1.22–1.59]) (**Fig. 2**).

An overview of eight systematic reviews regarding choice of antihypertensive agents among diabetic patient with hypertension, failed to show any difference between ACEIs, ARBs, β-blockers, CCBs, and diuretics in preventing all-cause or cardiovascular mortality, combined cardiovascular disease, coronary heart disease, and end-stage renal disease (Brunström, *et al.*, 2017).

A meta-analysis of 19 randomized controlled trials (including 25,414 patients with diabetes and elevated blood pressure), studied use of RAS blockers versus other antihypertensive agents. It concluded that RAS blockers did not have any advantages compared with commonly used antihypertensive agents such as β-blockers, CCBs, and thiazide diuretics with respect to death, myocardial infarction, heart failure, stroke, or end stage renal disease (Bangalore *et al.*, 2016).

The use of β-blockers among diabetic patients as an initial antihypertensive agent have been discouraged by several current guidelines. This is supported by the findings from the diabetes subgroups of the LIFE (Losartan Intervention for Endpoint reduction in hypertension) and ASCOT—BPLA (Anglo-Scandinavian Cardiac Outcomes Trial—Blood Pressure Lowering Arm) trials. In the LIFE study, losartan significantly reduced cardiovascular disease mortality, total mortality, heart failure, and composite cardiovascular disease outcomes compared with the β-blocker atenolol among the diabetic subgroup (Lindholm *et al.*, 2002). Amlodipine improved total cardiovascular disease events and procedures, and fatal and nonfatal stroke, compared with atenolol among diabetics enrolled in ASCOT (Ostergren *et al.*, 2008).

All of the above findings are in support of the 2014 United States evidence-based guidelines from the panel members of the eighth Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure who also

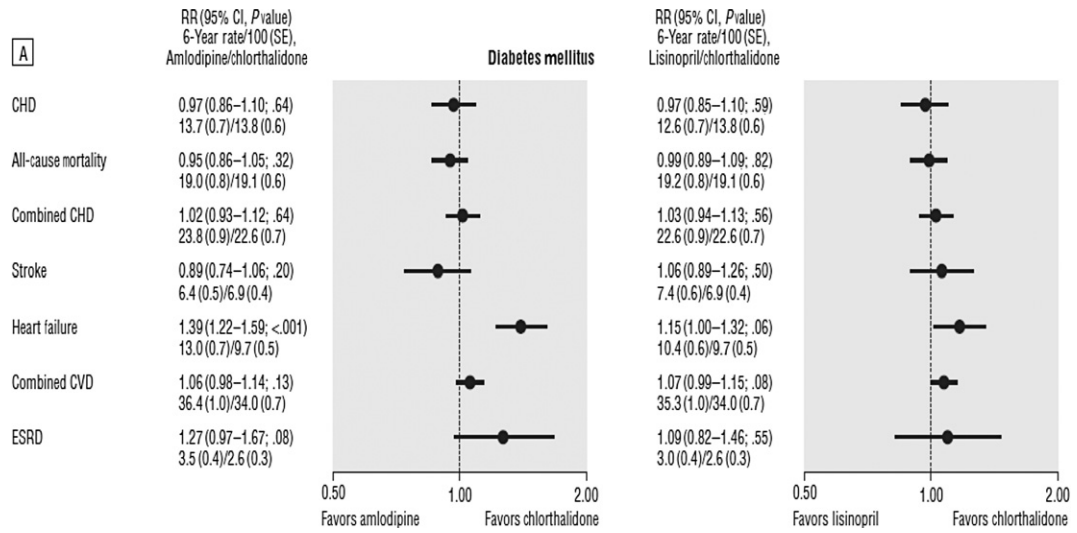

Fig. 2 Relative risks (RRs), 95% confidence intervals (CIs), *P* values, and 6-year rates per 100 and standard error (SE) for nondiuretic treatment compared with diuretic treatment for participants with diabetes mellitus at baseline, for coronary heart disease (CHD), all-cause mortality, combined CHD (includes CHD, coronary revascularization, or hospitalized angina), stroke, heart failure, combined cardiovascular disease (CVD) (includes combined CHD, stroke, other treated angina, heart failure, or peripheral arterial disease), and end-stage renal disease (ESRD) in the Antihypertensive and Lipid-Lowering to Prevent Heart Attack Trial (ALLHAT) Whelton, P.K., Barzilay, J., Cushman, W.C., Davis, B.R., Ilamathi, E., Kostis, J.B., Leenen, F.H., Louis, G.T., Margolis, K.L., Mathis, D.E. and Moloo, J. (2005). Clinical outcomes in antihypertensive treatment of type 2 diabetes, impaired fasting glucose concentration, and normoglycemia: Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). *Archives of Internal Medicine* 165(12), 1401–1409.

recommends use of thiazide-type diuretics, ACEIs, ARBs, and CCBs as initial antihypertensive drug classes for patients with diabetes without proteinuria or microalbuminuria (James *et al.*, 2014).

It is known that presence of albuminuria or reduced eGFR is associated with significant risk of CVD among diabetic patients (Solini *et al.*, 2012). There is considerable evidence that RAS blockers are protective against one of the most common and serious complications seen in diabetic hypertensives: progressive renal damage leading to end stage renal disease. In the Irbesartan Type II Diabetic Nephropathy Trial (1715 patients with diabetic nephropathy) the RAS blocker irbesartan compared with the calcium channel blocker amlodipine was associated with a significant reduction in the risk of the primary composite end point (32.6% vs. 41.1%; $P = .006$) of a doubling of serum creatinine concentration, the development of end stage renal disease, or death from any cause. Benefits of angiotensin receptor blockers were also demonstrated in the Microalbuminuria, Cardiovascular, and Renal Outcomes substudy of the Heart Outcomes Prevention Evaluation study (MICRO-HOPE) and Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan study (RENAAL) (Bakris *et al.*, 2003) These studies demonstrated that RAS blockers when compared to placebo were associated with a reduction in doubling of serum creatinine concentration and in the rate of progression to end stage renal disease or death from any cause (Heart Outcomes Prevention Evaluation (HOPE) Study Investigators, 2000; Bakris *et al.*, 2003).

In a meta-analysis of trials that included patients with diabetes and kidney disease, end stage renal disease and a doubling of creatinine concentration was less likely and regression of albuminuria was more likely with ACE inhibitors or ARBs compared with placebo.

The Need for Multiple Drugs

To achieve the desired reduction in blood pressure to a level of 130/80 mmHg or lower, most diabetic hypertensives will require two, three, or four antihypertensive drugs. As noted in the position statement by American Diabetic Association (2017):

> Multiple-drug therapy is often required to achieve blood pressure targets, particularly in the setting of diabetic kidney disease. However, the use of both ACE inhibitors and ARBs in combination is not recommended given the lack of added ASCVD benefit and increased rate of adverse events namely, hyperkalemia, syncope, and acute kidney injury (71–73). Titration of and/or addition of further blood pressure medications should be made in a timely fashion to overcome clinical inertia in achieving blood pressure targets.

The burden of such multiple drugs, both financial and beyond, can be lessened by intensive attention to lifestyle modifications, including weight reduction, physical activity, and moderation of sodium, protein, and alcohol intake. It will helpful to attempt to simplify the regimen by using combination antihypertensive medications and once-daily long-acting medications as much as

possible. These maneuvers may also improve control of hyperglycemia and dyslipidemia, which also demand attention. Despite the need for such intensive therapy, the benefits may well exceed the miseries and costs of uncontrolled diabetes and hypertension.

Conclusion

The following guidelines seem to be appropriate for the management of hypertension in diabetic patients:

- The target of blood pressure reduction should be lower than 130/85 mmHg.
- The initial several drug classes for treatment of hypertension in diabetes should include classes with the best evidence for benefit in outcome trials: ACEIs, ARBs, thiazide-type diuretics, and CCBs.
- Multiple drugs will usually be needed to achieve the target.
- The choice of drugs should usually include an ACEI or an ARB (but not both) and a diuretic and/or a CCB. Other classes, including a β-blocker, or an α-blocker may be used.
- Attention should be given to lifestyle changes (e.g., weight reduction; regular exercise; moderation of sodium and alcohol) as well as control of hyperglycemia, dyslipidemia, and proteinuria.

As the population grows older and more obese, diabetes and hypertension will become increasingly common. It is hoped that such an approach can reduce their serious consequences.

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Dyslipidemia in Diabetes

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Abbreviations

C	Cholesterol
CAD	Coronary artery disease
CVD	Cardiovascular disease
FFA	Free fatty acids
HDL	High density lipoprotein
HTG	Hypertriglyceridemia
LDL	Low-density lipoprotein
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
TG	Triglyceride
VLDL	Very low-density lipoproteins

Introduction

Worldwide over 400 million people have diabetes, a chronic disease characterized by hyperglycemia and associated complications. Type 2 diabetes (T2D), the most common form, accounts for 90%–95% of cases. Atherosclerotic cardiovascular disease (CVD) is the leading cause of morbidity and mortality in T2D, where the risk of death from CVD is increased two- to threefold (Stamler *et al.*, 1993). Individuals with diabetes have a worse prognosis after an acute coronary event than those without diabetes, with a higher frequency of congestive heart failure and higher mortality rate (Gaede *et al.*, 2003; Gu *et al.*, 1998; Kannel and McGee, 1979). Individuals with T2D often have features of the metabolic syndrome, a clustering of risk factors that significantly increase CVD risk. Development of these features, including abnormal fasting glucose, lipid abnormalities and central adiposity, often predates the development of T2D (Adiels *et al.*, 2006a).

People with autoimmune or type 1 diabetes (T1D) also have a two- to eightfold increased risk of CVD and death (Harjutsalo *et al.*, 2011). Atherosclerotic CVD is more aggressive in individuals with T1D than in individuals without diabetes.

Hyperglycemia alone cannot account for the increased CVD risk in diabetes (Skyler *et al.*, 2009). It is well established that dyslipidemia is a major risk factor for CVD. Dyslipidemia is a characteristic feature of the metabolic syndrome and T2D; the presence of features of the metabolic syndrome is associated with significantly increased CVD risk (Dekker *et al.*, 2005; Malik *et al.*, 2004). In T1D, the dyslipidemia that occurs with untreated or poorly controlled T1D corrects with tight glycemic control. However, correction of insulin deficiency can bring out features of genetic dyslipidemias or the metabolic syndrome (Purnell *et al.*, 2013). In this article, we will review the pathophysiology of dyslipidemia in diabetes, including rarer causes, and discuss management strategies.

Description of Diabetic Dyslipidemia

Dyslipidemia in Type 2 Diabetes

Lipid abnormalities, which can be quantitative, qualitative, and kinetic, commonly occur in T2D even with good glycemic control (Verges, 2015). The hallmark of dyslipidemia in diabetes is high plasma triglycerides (TGs) and low high density lipoprotein cholesterol (HDL-C). Low-density lipoprotein cholesterol (LDL-C) concentrations are normal or only slightly elevated, but the LDL particles are characteristically small and dense with TG-enrichment (Verges, 2015). The decrease in HDL-C is associated with qualitatively altered HDL particles with TG-enrichment and decreased phospholipids and surface apoproteins such as apoE. Plasma apoA-I and apo-II levels are decreased (Syvanne *et al.*, 1995). Reduced HDL levels in individuals with T2D are manifest as decreased HDL_{2b} subspecies and increase in smaller and denser HDL_3 particles (Gordon *et al.*, 2013; Hopkins and Barter, 1986). ApoC-III levels are increased in insulin resistance and T2D (Brahimaj *et al.*, 2017; Juntti-Berggren and Berggren, 2017). Postprandial lipemia also is accentuated (Sondergaard *et al.*, 2017). Poor glycemic control can further increase TG levels, cause modest increases in LDL-C, and affect HDL functionality (Gomez Rosso *et al.*, 2017). It is important to note that similar lipid changes (especially elevated TGs and low HDL-C) also occur in the metabolic syndrome; these changes often are present years before development of overt diabetes (Adiels *et al.*, 2006a). Reduced adiponectin levels in T2D inversely correlate with plasma TGs and positively with HDL-C (Schulze *et al.*, 2004).

Dyslipidemia in Type 1 Diabetes

Dyslipidemia in T1D primarily occurs in the setting of dysglycemia. With diabetic ketoacidosis, absolute insulin deficiency results in hypertriglyceridemia (HTG) with low HDL-C and LDL-C levels (Verges, 2009), which improve with treatment. HTG with increased LDL-C levels are observed with suboptimal glycemic control (Marcovecchio *et al.*, 2009). Elevated apoB levels and increased small, dense LDL particles have been described in young adults with T1D, regardless of glycemic control (Guy *et al.*,

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2009). In the presence of diabetic kidney disease (DKD), total cholesterol, TG and LDL-C are elevated, and HDL-C is decreased (Mattock *et al.*, 2001; Haaber *et al.*, 1993). Elevated LDL-C levels also are independently associated with progression of nephropathy in T1D (Thomas *et al.*, 2006).

Individuals with well-controlled T1D often have better lipid profiles than their counterparts without diabetes (Wadwa *et al.*, 2005), with average total and LDL cholesterol levels that are lower and HDL-C levels that are higher than individuals without diabetes (Guy *et al.*, 2009; Wadwa *et al.*, 2005; Eckel *et al.*, 1981). The increase in HDL-C levels may relate to the HDL_2 fraction and is associated with an increase in apoA-I (Eckel *et al.*, 1981; Kahri *et al.*, 1993; Winocour *et al.*, 1986).

Lipoprotein (a)

Lipoprotein (a) (Lp(a)) is an atherogenic lipoprotein, which is an independent risk factor for CVD (Bennet *et al.*, 2008; Kamstrup *et al.*, 2009). Lp(a) levels usually are within the normal range in T2D and are not affected by glycemic control. However, the presence of DKD is associated with an increase in Lp(a). High Lp(a) also is associated with development of retinopathy (Tu *et al.*, 2017). In small cohort studies, elevated Lp(a) was an independent cardiovascular risk factor in T2D (Lim *et al.*, 2016). Lp(a) levels are frequently elevated in T1D and improve with glycemic control (Haffner *et al.*, 1991; Purnell *et al.*, 1995). The development of microalbuminuria and the onset of DKD also are associated with an increase in Lp(a) levels in T1D (Groop *et al.*, 1994; Winocour *et al.*, 1991).

Prevalence of Dyslipidemia in Diabetes

The prevalence of dyslipidemia in T2D ranges between 70% and 80% (Doucet *et al.*, 2012) and is associated with increased CVD risk (Anon, 1998). Of patients with diabetes, 30%–40% have TG levels >200 mg/dL and 10% >400 mg/dL (Taskinen and Boren, 2015). In NHANES III, 62% of T2D subjects >50 years old had TG levels >150 mg/dL, and 60% had low HDL-C levels (Carroll *et al.*, 2012). In the United Kingdom Prospective Diabetes Study (UKPDS), baseline HDL-C levels were 9% lower in men with diabetes and 23% lower in women with diabetes compared with individuals without diabetes (Anon, 1998). TG levels were 50% higher and LDL-C values were similar in men and higher in women with T2D compared with their counterparts without diabetes; 38% of recruited subjects had both high TGs (>230 mg/dL) and low HDL-C in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study (Keech *et al.*, 2005). Thus, despite treatment with statins, the high prevalence of dyslipidemia in T2D subjects leaves considerable residual risk. The prevalence of lipid disorders in well-controlled T1D is generally thought to be similar to that of the general population (Verges, 2009).

Pathophysiology of Dyslipidemia in Diabetes

Type 2 Diabetes

Type 2 diabetes is characterized by hyperinsulinemia, insulin resistance, and gradual pancreatic β-cell failure. Factors that contribute to the dyslipidemia in diabetes are complex.

Overproduction of TG-rich lipoproteins (TRLs)

In the fasted state, the liver constitutively synthesizes apoB-100, the primary structural apolipoprotein of very low-density lipoprotein (VLDL). Newly synthesized apoB-100 undergoes lipidation by microsomal transfer protein (MTP) to form pre-VLDL. Additional lipidation results in its further maturation of the particle, transport to the Golgi apparatus, and after more TGs are added, secretion as mature VLDL (Adiels *et al.*, 2006b). ApoB-100 synthesized in excess of TG available for VLDL secretion undergoes mis-folding and degradation (Olofsson *et al.*, 2012).

A key abnormality contributing to dyslipidemia in T2D is overproduction of VLDL. The rate of synthesis and secretion of VLDL is highly dependent on TG availability, which is abundant in diabetes. As a result, excess lipidation of apoB-100 leads to increased VLDL formation and secretion. In the absence of diabetes, insulin inhibits apoB-100 secretion by increasing intrahepatic apoB-100 degradation (Fisher, 2012). However, because of insulin resistance in T2D and the metabolic syndrome, the ability of insulin to inhibit apoB-100 secretion is blunted, resulting in overproduction of apoB-100 and VLDL (Taskinen and Boren, 2015). Peripheral insulin resistance also increases availability of circulating free fatty acids (FFAs), which are a substrate for TG synthesis, thus increasing TG availability.

Visceral obesity, commonly present in the metabolic syndrome and T2D, is accompanied by increased cytokine production by adipose tissue macrophages. Proinflammatory cytokines such as TNF-α and IL-1β stimulate lipolysis in adipocytes (Hardardottir *et al.*, 1992) and increase circulating FFA levels, providing additional substrate for hepatic TG synthesis (Grunfeld and Feingold, 1996). In the liver, these cytokines also can stimulate de novo fatty acid and TG synthesis (Grunfeld and Feingold, 1996).

Decreased catabolism of TRLs

While overproduction of TRLs is the major cause of HTG in T2D, reduced TG clearance also can contribute. The activity of lipoprotein lipase (LPL), the rate limiting enzyme in TG catabolism, is decreased in diabetes. Since insulin stimulates LPL activity,

abnormal insulin action in T2D decreases LPL activity (Taskinen *et al.*, 1982), thereby decreasing hydrolysis of the TGs in chylomicrons and VLDL. Patients with T2D also have increased levels of apoC-III (Hiukka *et al.*, 2005), an inhibitor of LPL. ApoC-III plays a critical role in the hepatic clearance of VLDL remnants (Chan *et al.*, 2008). Therefore, excess apoC-III may result in impaired removal of these particles and a prolonged residence time in the circulation, as shown by delayed catabolism of VLDL-TG and apoB in T2D subjects with increased plasma levels of apoC-III (Chan *et al.*, 2008).

Intestinal lipoprotein overproduction and delayed clearance of chylomicrons

Increased chylomicron production in T2D contributes to postprandial hyperlipidemia (Hogue *et al.*, 2007). Individuals with T2D have increased intestinal apoB-48 secretion and augmented intestinal expression of MTP. The normal acute suppression of postprandial chylomicron secretion by insulin also is reduced (Nogueira *et al.*, 2012). Chylomicron clearance is impaired (Adiels *et al.*, 2012) due to reduced LPL activity, and increased plasma levels of apoC-III.

Production of small dense LDL and HDL

The increase in TRLs affects other circulating lipoproteins. Cholesteryl ester transfer protein (CETP) mediates exchange of TGs from TRLs to LDL and HDL, leading to TG-enrichment of LDL and HDL. Hydrolysis of LDL and HDL-TG by hepatic lipase and LPL leads to the production of small dense LDL and HDL (Garvey *et al.*, 2003). Notably hepatic lipase activity is increased in patients with T2D (Laakso *et al.*, 1987). The atherogenicity of small, dense LDL particles has been attributed to their increased vulnerability to oxidative modification (Chait *et al.*, 1993; Tribble *et al.*, 2001), increased binding to vascular proteoglycans (Olin-Lewis *et al.*, 2002) and increased uptake by the arterial wall (Bjornheden *et al.*, 1996). Poor glycemic control in T2D enhances compositional changes in small, dense HDL, resulting in defective functionality of these HDL particles (Gomez Rosso *et al.*, 2017). Insulin stimulates apoA-I expression (Mooradian *et al.*, 2004). Therefore, a reduction in insulin actin due to insulin resistance or decreased insulin levels may decrease apoA-I expression. High glucose levels also can activate carbohydrate response element binding protein, a transcription factor that inhibits apoA-I expression (Murao *et al.*, 1998). Therefore, production of apoA-I also can be reduced in diabetes (Mooradian *et al.*, 2004).

Proinflammatory cytokines can affect HDL metabolism by decreasing apoA-I production and reverse cholesterol transport. HDL from people with T2D stimulate tumor necrosis factor-α (TNF-α) secretion by human peripheral blood mononuclear cells than HDL from control subjects (Sun *et al.*, 2016). This proinflammatory property of HDL predicted CAD in these patients. In addition, HDL particle number predicted CHD and CVD in patients with the metabolic syndrome and diabetes, whereas in the metabolic syndrome in the absence of diabetes, only LDL particle number positively associated with CVD (Tehrani *et al.*, 2016).

Type 1 Diabetes

Significant dyslipidemia in T1D usually occurs in the setting of poor glycemic control, nephropathy, central adiposity, or the coexistence of genetic forms of HTG. Since insulin stimulates LPL activity, severe insulin deficiency impairs TRL catabolism in diabetic ketoacidosis (Taskinen, 1987), in which there is a concomitant drop in LDL-C and HDL-C (Weidman *et al.*, 1982), changes that all reverse with insulin therapy (Verges, 2009). Moderate HTG due to increased VLDL production results from increased FFA availability associated with insulin deficiency (Nikkila and Kekki, 1973). LPL activity also can be impaired in T1D patients with poor or suboptimal glycemic control (Verges, 2009). In T1D patients optimally controlled with intensive insulin therapy, plasma TG and LDL-C are normal or slightly decreased (Verges, 2009).

Qualitative changes in lipoproteins also have been observed in individuals with T1D. VLDL can be enriched in esterified cholesterol at the expense of TGs, likely due to increased cholesteryl ester transfer between lipoproteins (Bagdade *et al.*, 1991a). LDL can be TG-enriched, leading to the formation of small, dense LDL particles despite normal LDL-C levels. HDL particles have been shown to have higher TG content, increased sphingomyelin, as well as glycation of apoA-I (Denimal *et al.*, 2015). Functional changes in HDL include reduction in the activity of the antioxidant enzyme paraoxonase (Karabina *et al.*, 2005), and increased susceptibility to oxidative stress (Ganjali *et al.*, 2017). Some of these compositional and functional changes in HDL are independent of glycemic control (Manjunatha *et al.*, 2016).

Role of Glycemic Control

Poor glycemic control can adversely affect lipid and lipoprotein metabolism in both T1D and T2D. In T2D, deterioration of glycemic control will exacerbate underlying dyslipidemia resulting in greater increases in plasma TG levels. This is due to decreased catabolism of TRLs as well as increased FFA flux to the liver, leading to increased VLDL synthesis. Improved glycemic control can lower plasma TG and increase HDL-C levels. However, subcutaneous insulin therapy does not appear to alter lipoprotein composition despite good glycemic control (Bagdade *et al.*, 1991b). Uncontrolled T1D results in HTG due to insulin deficiency and decreased LPL activity (see above).

Table 1 summarizes the changes in the various lipoprotein classes in both types of diabetes.

Diabetic Kidney Disease (DKD)

Renal complications of diabetes, including microalbuminuria, overt proteinuria, and nephrotic syndrome, manifests with HTG and low HDL-C. Plasma levels of apoB and apoC-III often are elevated (Thomas *et al.*, 2015). Hyperglycemia, hyperlipidemia, abdominal obesity, hypertension, and smoking are risk factors for microalbuminuria in patients with diabetes (Rutledge *et al.*, 2010). Dyslipidemia promotes progression of renal insufficiency through glomerular and tubulointerstitial injury with concomitant accelerated atherosclerosis (Shoji *et al.*, 2001). CVD risk factors such as elevated levels of circulating cell-adhesion molecules and systemic inflammatory markers are found in the early stages of both DKD and nondiabetic chronic kidney disease (Thomas *et al.*, 2015). Macroalbuminuria has been associated with an increase in oxidized LDL levels (Jandeleit-Dahm *et al.*, 1999), and HDL may lose antiinflammatory and antioxidant properties in DKD (Thomas *et al.*, 2015). HDL_3-C, sphingolipid, and TG-enrichment of HDL have been suggested as potential contributors to DKD (Thomas *et al.*, 2015).

Role of Genetics

Human genetic studies have attempted to identify variants that associate with lipid traits and CAD risk. Genome wide association studies (GWAS) have identified over 40 genetic variants associated with CHD, several of which also associate with T2D (Strawbridge *et al.*, 2011; Willer *et al.*, 2013). Single nucleotide polymorphisms significantly associated with CHD have been identified in patients with T2D (Qi *et al.*, 2012). Some genetic data link diabetes and CAD to dyslipidemia. A common genetic basis for regulation of glucose and lipid homeostasis has been reported (Albert *et al.*, 2014), as well as polygenic overlap between T1D, T2D, LDL, and CAD (LeBlanc *et al.*, 2016; Zhao *et al.*, 2017). Genetic loci exclusively associated with TG levels also have been associated with CAD risk, suggesting a causal role for TRLs in CVD (Do *et al.*, 2013). There also is evidence that genetic predisposition to low HDL-C or high TGs significantly increases T2D risk (Qi *et al.*, 2012). Data concerning the role of genetics in CVD in T1D is more limited. Associations of certain polymorphisms with CAD in T1D have been identified, possibly acting through TGs (Pettersson-Fernholm *et al.*, 2003, 2004). With advances in knowledge of the genetics of human lipid biology, important insights into mechanisms of CAD in diabetes can be expected.

Special Considerations

Genetic disorders of lipid metabolism

Genetic disorders of lipid metabolism can be present in individuals with diabetes. While the prevalence of these disorders in individuals with diabetes is unknown, underlying lipid disorders often compound CVD risk and are hence important to identify and treat. Genetic lipid disorders that can coexist in individuals with diabetes include disorders of cholesterol (familial hypercholesterolemia), accumulation of LDL and VLDL (familial combined hyperlipidemia), disorders of TG metabolism (e.g., familial HTG), and accumulation of remnants (remnant removal disease or dysbetalipoproteinemia). Familial hypercholesterolemia is characterized by mutations in the LDL receptor pathway and typically results in elevations of LDL-C only, unless features of the metabolic syndrome coexist. All of the other genetic disorders usually result in mild-to-moderate HTG. Importantly, coexistence of diabetes with these genetic disorders can lead to marked elevations of TG levels and the multifactorial chylomicronemia syndrome (MFCS). Very severe HTG in association with diabetes also can be seen in lipodystrophic syndromes.

Familial partial lipodystrophies (FPLD)

The lipodystrophies are a group of heterogeneous inherited or acquired disorders that are characterized by selective loss of body fat. Congenital forms that manifest at birth with generalized fat loss and profound metabolic dysfunction are beyond the scope of this discussion. In FPLD, where fat loss is localized to the extremities, HTG and diabetes with severe insulin resistance is common, the extent of fat loss being a major determinant of the severity of metabolic complications. The two most common forms of FPLD are Kobberling's and Dunnigan's lipodystrophy, which are genetically distinct. Both appear to have autosomal-dominant inheritance, with variable fat loss from extremities and truncal regions. Onset is typically around puberty or early adulthood (Lightbourne and Brown, 2017). Patients with the Kobberling variety lose subcutaneous fat on their limbs, with excessive fat accumulation in the dorso-cervical, supraclavicular and submental regions, sometimes leading to a Cushingoid appearance (Guillin-Amarelle *et al.*, 2016; Herbst *et al.*, 2003; Kobberling *et al.*, 1975). The Dunnigan variety involves fat loss from both the extremities and the trunk. HTG is common in both forms, potential mechanisms being decreased storage capacity of fat in adipose tissue, with increased hepatic VLDL synthesis and delayed clearance. Individuals with both types of FPLD can develop TG-induced pancreatitis, especially in the presence of marked insulin resistance and hyperglycemia.

Diabetes in adolescents and youths

Lipid abnormalities occur in youth with both T1D and T2D (Kershnar *et al.*, 2006). T2D in children and adolescents has increased in prevalence, especially in minority populations (Mayer-Davis *et al.*, 2017). Dyslipidemia in adolescent and youth with diabetes is similar to that seen in adults, with mild-to-moderate TG elevations and reduced HDL-C. Pathology and imaging studies have demonstrated progression of atherosclerotic lesions in children and adolescents with T1D (Dahl-Jorgensen *et al.*, 2005). Lipid levels in childhood are predictive of adult lipoprotein profiles in T1D (Guy *et al.*, 2009).

Few longitudinal studies exist of changes in lipids in youth with T1D. However, in a 7 year study of youth with T1D, progression of dyslipidemia occurred in ~25%, predictors of progression being waist to hip ratio and A1C burden over time, particularly in males (Shah *et al.*, 2017).

Management of Dyslipidemia in Diabetes

Brief Overview of Current Guidelines for Adults With Diabetes

Current guidelines from the American Diabetes Association (ADA) recommends measuring a lipid profile at the time of diagnosis of diabetes, at initial evaluation, or prior to starting therapy (American Diabetes Association, 2018a). In people under the age of 40, lipid testing every 5 years is recommended, with consideration for more frequent testing for those with longer duration of diabetes. Monitoring of the lipid profile is recommended 4–12 weeks after initiation of statin therapy, after any change in dosing, or as needed to assess for efficacy and adherence. Recommendations for lipid-lowering therapy in diabetes are similar to those by the American Heart Association/American College of Cardiology (Stone *et al.*, 2013). Statin treatment is recommended to reduce CVD risk in adults with T1D or T2D. High-intensity statins in addition to lifestyle therapy should be prescribed for individuals of all ages with diabetes and CVD, with a goal of LDL-C < 70 mg/dL. Moderate intensity statins are recommended for primary prevention in individuals aged 40–75 years without CVD. Clinical trial evidence is limited for individuals >75 years, but since CVD risk increases with age, therapy with moderate intensity statins may offer benefit. If LDL-C remains ≥70 mg/dL on maximally tolerated statin dose, additional LDL-lowering therapy (such as ezetimibe or a proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitor) may further reduce CVD risk. Guidelines from the Canadian Diabetes Association, albeit similar to the US guidelines, recommend statins for individuals younger than 40 with diabetes if they have microvascular complications, have had diabetes for more than 15 years, and for those younger than 30 years with other CVD risk factors such as hypertension or renal disease (Anderson *et al.*, 2016; Canadian Diabetes Association Clinical Practice Guidelines Expert Committee *et al.*, 2013). The National Lipid Association recommends goals for LDL-C < 100 mg/dL and non-HDL-C < 130 mg/dL, with lower targets in those at very high risk. The European Society of Cardiology and the European Association for the Study of Diabetes recommend LDL-C levels of <70 mg/dL, or a reduction of >50% from baseline LDL-C values if target cannot be reached in T1D and T2D at very high risk (known CVD, severe chronic kidney disease (CKD) with end-organ damage). The common feature of all guidelines is use of statins in all individuals older than 40 with diabetes, with more aggressive LDL-C lowering in those at very high risk. Non-HDL-C targets also are recommended in Canadian and European guidelines.

Lifestyle Intervention

Guidelines for the management of people with diabetes always include a section on nutrition therapy and physical activity. In addition, weight loss is recommended for the many overweight people with the metabolic syndrome and T2D. Weight loss results in a mild-to-moderate decrease in TG levels and a modest increase in HDL-C. In addition, small, dense LDL particles decrease (Purnell *et al.*, 2000). Short of surgical intervention, maintenance of weight loss has proved to be difficult, but can be aided by regular physical activity (Knowler *et al.*, 2002). Nonetheless, maintaining even modest amounts of weight loss can beneficially affect plasma lipids and lipoproteins (Knowler *et al.*, 2002; Tuomilehto *et al.*, 2001). Frequent moderate intensity aerobic exercise increases HDL-C, and leads to a small decrease in TGs in association with a decrease in intraabdominal fat (Leon and Sanchez, 2001; Ross *et al.*, 2000).

Nutrition therapy should be individualized for all patients with diabetes, since one size does not fit for all. Nutrition therapy in patients with T1D and those with T2D on a flexible insulin regimen typically involves carbohydrate counting to determine mealtime insulin dosing for postprandial glucose control. Some controversy still exists as to the best macronutrient composition for individuals with T2D. While the mainstay for glycemic control is carbohydrate restriction, replacement of carbohydrate with saturated fats can increase LDL-C levels (American Diabetes Association, 2018b), which increase the risk of developing CVD. Moreover, dyslipidemia in the metabolic syndrome and T2D is characterized by HTG, which can respond to both weight loss and carbohydrate restriction, including consumption of a low glycemic index diet with a high fiber content (Jung and Choi, 2017).

Effect of Glucose Lowering Agents on Lipids

Initiation of insulin treatment has long been known to reduce plasma TG levels in people with diabetes (Lewis *et al.*, 1972). As TG levels fall, HDL-C levels increase (Brunzell, 2007). Some studies have shown that insulin also lowers LDL-C levels (Chaudhuri *et al.*, 2012). Moreover, initiation of therapy in previously undiagnosed or untreated diabetes also can improve lipid and lipoprotein levels, independent of the modality of pharmacological therapy (Pfeifer *et al.*, 1983). How much of these effects are due to their effects on glycemic control versus specific effects of the drugs is difficult to ascertain, since several drugs used for the treatment of hyperglycemia may independently affect lipid and lipoprotein levels. These include metformin, the glucagon-like peptide 1 (GLP1)-receptor agonists, thiazolidinediones (TZDs), and the sodium-glucose cotransporter 2 (SGLT2) inhibitors, as well as insulin. Several studies have shown that metformin modestly lowers plasma TGs and increases HDL-C (Bolen *et al.*, 2007; Buse *et al.*, 2004; Stumvoll *et al.*, 1995), although some of this effect might be due to the modest weight loss commonly seen with its

use. Although metformin has been associated with some protection against CVD (Anon, 1998), including in patients with T1D (Livingstone *et al.*, 2017), this data is not robust. Fasting as well as postprandial TG levels are reduced and HDL-C levels modestly increased by GLP1 receptor analogs (Bandsma and Lewis, 2010; Chaudhuri and Dandona, 2011; Hermansen *et al.*, 2013), which also may in part relate to the weight loss commonly seen with this class of drugs. In addition, liraglutide led to a decrease in CVD outcomes in the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial (Marso *et al.*, 2016). Cardiovascular benefit also has been demonstrated with semaglutide (Marso *et al.*, 2016), but not with exenatide (Holman *et al.*, 2017). While TZDs also reduce plasma TG levels, LDL-C levels increase with the use of this class of drugs (Buse *et al.*, 2004; Chaudhuri and Dandona, 2011), accompanied by no change in apoB levels and by the presence of larger, more buoyant LDL particles (Freed *et al.*, 2002). One TZD, pioglitazone, improved CVD outcomes in the PROspective pioglitAzone Clinical Trial In macroVascular Events (PROactive) trial (Erdmann *et al.*, 2007), as well as reducing recurrent strokes in patients with metabolic syndrome (Kernan *et al.*, 2016). The effect of rosiglitazone on CVD outcomes is more controversial, with early studies suggesting that it actually increased CVD (Nissen and Wolski, 2007), and effect that was reversed with later trials and analyses (Home *et al.*, 2007; Stafylas *et al.*, 2009). The SGLT2 inhibitors adversely affect both LDL-C and apoB levels (Briand *et al.*, 2016; Fulcher *et al.*, 2016; Ptaszynska *et al.*, 2013; Zaccardi *et al.*, 2016); however, this effect is likely to be abrogated by the widespread use of statins in people with diabetes. Two SGLT2 inhibitors, empagliflozin and canagliflozin, have been shown to reduce both CVD events and deaths (Neal *et al.*, 2017; Zinman *et al.*, 2015) by mechanisms that are not well understood.

Although some studies have suggested mild beneficial changes in lipids and lipoproteins, sulfonylureas, dipeptidyl peptidase 4 inhibitors, meglitinides and alpha-glucosidase inhibitors (Buse *et al.*, 2004; Monami *et al.*, 2012) are generally considered lipid neutral (Chaudhuri and Dandona, 2011).

Effects of Bariatric Surgery on Lipids

The most commonly performed bariatric procedures are Roux-en Y gastric bypass and sleeve gastrectomy, both of which improve dyslipidemia on patients with and without diabetes, Although HDL-C levels tend to fall during the first 6 months of rapid postsurgical weight loss, both procedures result in a decrease in LDL-C, non-HDL-C, apoB and TG levels, and an eventual increase in HDL-C (Bays *et al.*, 2016). Lp(a) levels do not change (Woodard *et al.*, 2010).

Drugs for Lipid Lowering

Statins

Of the various therapies to prevent or treat CVD in diabetes, particularly T2D, the most successful to date has been statins, which inhibit cholesterol synthesis, thereby increasing the levels of hepatic LDL receptors and clearance of LDL from plasma. Statins reduce CVD events in diabetes, despite elevation of LDL-C not being characteristic of either T2D or the metabolic syndrome (see earlier). The first study to show a benefit of statin therapy on CVD endpoints specifically in subjects with T2D was the Collaborative Atorvastatin Diabetes Study (CARDS) (Colhoun *et al.*, 2004). These benefits have been confirmed and extended in subsequent studies, including meta-analyses that have included several thousand subjects with T2D. Meta-analyses show that statin-induced reduction of LDL-C by approximately 40 mg/dL was associated with an ~20% reduction in CVD events and an ~10% decrease in mortality (Cholesterol Treatment Trialists Collaborators *et al.*, 2008). Very limited clinical trial evidence exists for statins and CVD risk reduction in individuals with T1D. In the Heart Protection Study, which included 615 patients with T1D, statin therapy resulted in a proportionately similar (though not statistically significant) reduction in CVD risk as in T2D (Collins *et al.*, 2003). Similarly, meta-analysis also has shown a reduction in CVD events in mortality in subjects with T1D (Cholesterol Treatment Trialists Collaborators *et al.*, 2008). As a result, statin use has become the mainstay of CVD prevention, and are included in all guidelines for CVD prevention in diabetes (see earlier). There is less information on the role of statins in the metabolic syndrome without overt diabetes, although similar outcomes for CVD prevention have been observed as for diabetes (Matikainen and Taskinen, 2012).

A downside of statin use of is an increased risk of developing diabetes. It is estimated that there is an ~10% increased risk of developing diabetes for every 40 mg/dL reduction of LDL-C by statins, and that this risk is greatest in individuals at high risk of developing diabetes, including those with the metabolic syndrome (Preiss and Sattar, 2011). However, best estimates strongly suggest that the benefit of statin use on CVD prevention far outweighs the risk of developing diabetes, the biggest consequence of which is developing premature CVD (Preiss and Sattar, 2011). Hence all guidelines still advise that all patients with T2D and adult patients with T1D be treated with statins (see earlier), unless contraindicated for various reasons.

Fibrates

Fibrates are PPARα agonists, that are effective in lowering plasma TGs and modestly increase HDL-C levels. Fenofibrate also modestly reduces LDL-C. Since HTG and low levels of HDL-C are hallmarks of T2D and the metabolic syndrome, the use of TG-lowering drugs is logical. Despite fibrates having been around for several decades, their value in the prevention of CVD in diabetes remains uncertain. Early studies such as The Diabetes Atherosclerosis Intervention Study (DAIS), in which progression of angiographically assessed CAD in T2D was reduced with fenofibrate use (Anon, 2001), gave confidence that fibrates might also reduce CVD endpoints in diabetes. Moreover, post hoc analysis of several earlier trials (The Helsinki Heart study, the Veterans

Affairs HDL Intervention Trial and the Bezafibrate Infarction Prevention study) all found that the reduction in CAD events was confined to patients with high TG and low HDL-C levels (Bezafibrate Infarction Prevention Study, 2000; Koskinen *et al.*, 1992). In the FIELD study, the greatest benefit of fenofibrate treatment also was in patients with the combination of elevated TGs and low HDL-C, with 27% relative reduction in CVD events (Keech *et al.*, 2005). Nonetheless, the Action to Control Cardiovascular Risk in Diabetes (ACCORD)-LIPID trial (ACCORD Study Group *et al.*, 2010), included diabetic subjects that were not hypertriglyceridemic. Perhaps not surprisingly there was no CVD benefit of fenofibrate in the entire cohort, but those diabetic subjects with baseline HTG once again demonstrated reduced CVD events. Although no fibrate studies to date has focused solely on subjects with HTG, evidence from post hoc analysis of these various clinical trials suggest that they might well be of use, either alone or in combination with a statin if used in the appropriate patient. Prominent, an ongoing clinical trial that is testing a novel selective peroxisome proliferator-activated receptor α modulator with unique PPARα activity and selectivity in individuals with HTG and diabetes (Camejo, 2017) might for once and for all provide a definitive answer to whether the addition of a fibrate to a statin will further reduce CVD risk in people with diabetes.

Ezetimibe

The cholesterol absorption inhibitor, ezetimibe, also lowers LDL-C, although to a lesser extent than statins. It often is used as monotherapy in individual who are statin intolerant, as well as in combination with statins in those unable to reach LDL goals on statins alone. Little data exists as to the value of ezetimibe in persons with diabetes. However, in the Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) secondary prevention study, in which statin plus ezetimibe had a beneficial effect on CVD outcomes compared with statin alone, the strongest benefit of ezetimibe addition was in subjects with diabetes (Cannon *et al.*, 2015; Giugliano *et al.*, 2017). Thus, ezetimibe is being increasingly used in statin-intolerant patients with diabetes, or as an adjunct to statin therapy.

Bile Acid Sequestrants

This class of drugs works by binding bile acids in the intestine, thereby interrupting the enterohepatic circulation of bile acids. As a result, hepatic LDL receptors are induced and plasma LDL levels fall, although not nearly as markedly as with statins, averaging around 15% (Hou and Goldberg, 2009; Out *et al.*, 2012). Although these agents have been around for many years and were the first to demonstrate a beneficial effect on clinical outcomes (Anon, 1984), it was only more recently appreciated that they also were able to modestly improve glycemic control and to lower HbA1C levels by about 0.5% (Brunetti and DeSantis, 2015). The newest agent in this class is colesevelam, with which compliance is much improved compared to the older bile acid sequestrants. Use of bile acid sequestrants, either as monotherapy in statin-intolerant subject, or in combination with a statin, is somewhat limited since they all raise plasma TGs (Denke and Grundy, 1988).

Proprotein convertase subtilisin/kexin type 9 (PCSK9) antibodies

These injectable drugs have been available since late 2015 and are indicated in most countries for individuals with familial hypercholesterolemia or with established CVD unable to achieve target LDL-C levels with other lipid-lowering therapies. These antibodies trap PCSK9 in plasma, thereby preventing PCSK-9 from binding to LDL receptors at the hepatocyte surface. As a result, LDL receptors are targeted for recycling to the cell surface rather than for lysosomal degradation. PCSK9 antibodies lower LDL-C levels well beyond that achievable with statins with or without ezetimibe (Farnier *et al.*, 2016; Sabatine *et al.*, 2017a, 2015). There is little data on their use in people with diabetes, although the first clinical trial to show benefit of these inhibitors, the Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk (FOURIER) trial (Sabatine *et al.*, 2017b), did include some patient with diabetes, who appeared to benefit in a similar to those without diabetes. Interestingly, that trial did not show an increase in diabetes associated with the use of a PCSK9 antibody.

Niacin

Niacin, which has been in use for many years, reduces plasma TGs in addition to lowering LDL-C and apoB, and increases HDL-C. Thus niacin seems ideal for treating the dyslipidemia of diabetes. However, it also increases insulin resistance, thus limiting its use in people with diabetes. Studies in the prestatin era showed a beneficial effect of niacin in reducing both CVD events and mortality in hypercholesterolemic subjects (Canner *et al.*, 1986). However, negative outcomes in two recent clinical trials in which niacin, when added to statin therapy in nondiabetic subjects failed to show a beneficial effect on CVD endpoint (HPS2-THRIVE Collaborative Group *et al.*, 2014; AIM-HIGH Investigators, 2011), has dampened enthusiasm for its use, especially in diabetic dyslipidemia, where it increases insulin resistance and worsens glycemic control.

Omega 3-fatty acids

Omega-3 fatty acids lower plasma TG levels. Although one clinical trial showed a reduction in the cumulative incidence of major coronary events in a subgroup of subjects with HTG and low HDL-C (Saito *et al.*, 2008), benefits of omega-3 fatty acids appears to relate mainly to a reduction in sudden death (Rice *et al.*, 2016). Most meta-analyses show little if any beneficial effect on CVD events, which may be a result of inadequate doses of omega-3 fatty acids being used (Rice *et al.*, 2016). Several CVD outcomes trials with omega-3 fatty acids are in progress.

Management in Special Situations

Multifactorial chylomicronemia syndrome (MFCS) and familial partial lipodystrophy (FPLD)

Most, if not all, patients with MFCS have a genetic form of HTG coexisting with one or more secondary forms of HTG (Chait and Brunzell, 1983). TG levels can increase markedly with suboptimal glycemic control. Following correction of treatable secondary forms of HTG in the MFCS, TG levels usually decrease to the moderately elevated levels seen in their affected relatives (Chait and Brunzell, 1983). Individuals with FPLD present with severe insulin resistance and difficult to control diabetes and marked HTG (Herbst *et al.*, 2003). Recurrent pancreatitis can occur (Herbst *et al.*, 2003) and may lead to exocrine pancreatic insufficiency.

In both MCFS and FPLD, the primary goal of therapy is to lower TGs sufficiently to prevent pancreatitis. From a practical standpoint, it is best to maintain levels <1000 mg/dL, ideally lower if possible. This requires reversal of any secondary cause of HTG, including aggressive glycemic control. Acute pancreatitis is managed similar to non-TG-induced pancreatitis, other than for avoidance of TG emulsions for parenteral feeding. TGs fall rapidly with cessation of oral intake and intravenous hydration. Once stable, fibrate therapy is indicated to prevent recurrent pancreatitis.

Diabetic kidney disease (DKD)

While LDL-C is an established CVD risk factor in the general population, its prognostic value appears to be less in those with CKD due to DKD or other causes (Tonelli *et al.*, 2013), despite the magnitude of LDL-C reduction with statin therapy in CKD and end-stage renal disease (ESRD) being similar to that seen in individuals with preserved kidney function. However, CVD events and mortality were reduced with statins and statins/ezetimibe in a study with mild-to-moderate CKD (Sarnak *et al.*, 2015).

ESRD is associated with very high risk for CVD events. Randomized clinical trials of lipid therapies have not consistently shown a reduction in CVD mortality or survival benefit in this population. Moreover, intensive lipid-lowering to decrease cardiovascular risk in these very ill patients may not be practical (Palmer *et al.*, 2013). There are no clear recommendations for lipid lowering therapy in ESRD and should be individualized based on clinical judgment.

Management of adolescent and youth with diabetes

In children and adolescents with T1D, the ADA recommends a screening nonfasting lipid profile at the age of 10 years. Data from the SEARCH for Diabetes in Youth (SEARCH) study show that improved glucose control over a 2-year period improved the lipid profile; however, improved glycemic control alone will not normalize lipids in dyslipidemic youth with T1D (Guy *et al.*, 2009). Assessment of other risk factors including smoking, obesity, physical activity, age-appropriate blood pressure, and family history of CVD should be routinely sought (Springer *et al.*, 2013). Lifestyle management should be initiated to improve lipid levels (Kavey *et al.*, 2006). Statins should be considered after the age of 10 for LDL-C $>$ 160 mg/dL after lifestyle intervention, or for LDL-C $>$ 130 mg/dL with one or more additional CVD risk factors (American Diabetes Association, 2018c). The goal of LDL-lowering in childhood and adolescence is to reduce LDL-C to below the 95th percentile or <130 mg/dL (American Diabetes Association, 2018c).

A fasting lipid panel and risk factor assessment is recommended at the time of diagnosis of T2D in youth, since comorbidities often already exist (American Diabetes Association, 2018c; Nadeau *et al.*, 2016). The coexistence of obesity with the polycystic ovarian syndrome can contribute to dyslipidemia. Treatment recommendations are similar to youth with T1D. Lifestyle modification is an essential part of management of these patients. As in adults, weight loss results in TG reduction. Reduction of simple carbohydrate intake with an increase in complex carbohydrates and an increase in physical activity are important in management of dyslipidemia in these young patients. Recommendations for statin therapy is similar for youth with T1D.

Conclusions

The constellation of lipid and lipoprotein abnormalities that comprise diabetic dyslipidemia differs between T1D and T2D. Dyslipidemia in T1D often disappears with good glycemic control, unless concomitant features of the metabolic syndrome, nephropathy or a genetic lipid disorder is present. Dyslipidemia in T2D resembles that seen in the metabolic syndrome, which often precedes the onset of hyperglycemia. Treatment of diabetic dyslipidemia is a mainstay of preventing the onset or recurrence of CVD, the risk of which is increased in both types of diabetes. Current data indicates that statins confer the most benefit, despite elevations of LDL-C not being a characteristic hallmark of diabetic dyslipidemia. While HTG is a major feature of diabetic dyslipidemia, uncertainty still exists as to whether lowering of the TRLs with fibrates or other means will reduce the incidence of CVD. Severe HTG can occur in diabetes, especially in the presence of a concomitant genetic form of hypertriglyceridemia or familial forms of partial lipodystrophy. When TGs are markedly elevated, the risk of pancreatitis is increased, in addition to the risk of CVD.

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Ocular Manifestations Associated With Diabetes☆

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Introduction

Diabetes mellitus (DM) has deleterious effects on every organ system in the body, and the eye is no exception. DM impacts every ocular structure, from the cornea to the optic nerve. However, no part of the eye suffers more greatly from the effects of DM than the retina. Diabetic retinopathy (DR) continues to be the primary cause of vision loss and blindness among working age people in the United States. Vision loss due to DR can occur as a result of many mechanisms, such as retinal detachment, macular ischemia, vitreous hemorrhage, and macular edema. This chapter describes the current classification and treatment of DR, in addition to other adverse sequelae such as neurotrophic keratitis, neovascular glaucoma, and cataract, that may arise as a result of DM.

Diabetic Retinopathy

DR affects roughly a third of people with diabetes above 40 years old, and the prevalence increases with disease duration (Zhang *et al.*, 2010). The Diabetes Control and Complication Trial (DCCT) provided definitive evidence that intensive glycemic control reduces the likelihood of developing vision-threatening complications from type 1 DM (The Diabetes Control and Complications Trial Research Group, 1993), and the United Kingdom Prospective Diabetes Study (UKPDS) provided similar evidence for those with type 2 DM (UK Prospective Diabetes Study (UKPDS) Group, 1998). In addition to sustained hyperglycemia, uncontrolled hypertension and pregnancy are among the main risk factors for DR progression.

DR has classically been characterized as a microvascular complication of DM due to the histological and clinically evident effects that DM has on the retinal vasculature. For example, DM leads to loss of pericytes, the modified smooth muscle cells that surround capillary walls, early in the course of the disease. Loss of pericytes is associated with the formation of microaneurysms, which are focal dilations in capillaries. Prolonged hyperglycemia is also associated with "capillary dropout" in the diabetic retina, which can lead to retinal ischemia. The early effects of ischemia, or decreased blood flow, manifest in the retina as hemorrhages or cotton wool spots (CWS), which are focal infarctions taking place mostly in the retinal nerve fiber layer. Progressive retinal ischemia contributes to the vision-threatening complications of DR such as proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME).

Traditionally, DR is clinically classified as either non-proliferative or proliferative, referring to the absence or presence of new blood vessels in the retina, respectively. Non-proliferative diabetic retinopathy (NPDR) exists along a spectrum ranging from mild to very severe disease. Microaneurysms are the only retinal finding in mild NPDR, whereas severe NPDR is characterized by extensive hemorrhages, venous beading, or intraretinal microvascular abnormalities (IRMA), which may represent dilated pre-existing capillary networks.

About 50% of patients with severe or very severe NPDR will develop PDR within one year (Royle *et al.*, 2015). The hallmark of PDR is retinal neovascularization. With prolonged exposure to the diabetic milieu, oxygen deprivation of the retinal tissue results in the release of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) (Aiello *et al.*, 1994). Molecules like VEGF promote the development of new blood vessels within the retina. However, the newly formed blood vessels are fragile and ineffective in restoring retinal perfusion. The vessels are prone to bleeding and fibrosis, which can result in the vision-threatening complications of DR such as vitreous hemorrhage and tractional retinal detachment.

Another vision-threatening complication of DR is DME, which is characterized by accumulation of fluid and lipids within the neuroretina, thought to mostly originate from the retinal capillaries. As with PDR, molecules such as VEGF may disrupt endothelial cell tight junctions, which increases capillary permeability and results in leakage of substances from the intravascular compartment into the retinal tissue. There are two patterns of DME: diffuse and focal. Diffuse DME involves widespread and poorly defined leakage in the macula, while focal macular edema is typically characterized by a "circinate ring" of exudates around a leaky microaneurysm.

Eye-specific treatments for DR are aimed at halting the progression of PDR or reducing macular swelling in DME. Pan retinal photocoagulation (PRP) has been the mainstay of treatment for PDR for decades (The Diabetic Retinopathy Study Research Group, 1981). Typical PRP involves the use of an argon laser to destroy the peripheral outer retina — photoreceptors and RPE — thus sacrificing peripheral vision in an attempt to save central vision or macular function. The exact mechanism through which PRP promotes regression of new blood vessels in PDR is unclear, though one hypothesis is that destruction of the peripheral outer retina decreases the oxygen demand and overall metabolic needs of the retina as a whole, thus reducing the liberation of pro-

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angiogenic factors and the stimulus for neovascularization. A current exciting new treatment for PDR that is being actively studied is the injection of anti-VEGF agents directly into the eye (Writing Committee for the Diabetic Retinopathy Clinical Research Network *et al.*, 2015).

While anti-VEGF treatments are currently under investigation for the treatment of PDR, they have become the gold standard for the treatment of DME. Before the development of anti-VEGF medications such as bevacizumab, ranibizumab, and alfibercept, the treatment for DME consisted mainly of either focal or grid laser onto the retina. This was moderately effective in preventing severe vision loss in patients with DME (Early Treatment Diabetic Retinopathy Study research group, 1985). Anti-VEGF therapy revolutionized the treatment of DME because such treatment can prevent subsequent vision loss, or in some cases even reverse it (Diabetic Retinopathy Clinical Research Network *et al.*, 2015). Nevertheless, anti-VEGF therapy is only effective in ~50% of patients with DME (Nguyen *et al.*, 2012) and requires frequent injections (as many as one per month) in order to prevent vision loss. Intravitreal delivery of steroids is another treatment option for DME patients, especially in those non-responsive to anti-VEGF therapy. The anti-inflammatory and antiangiogenic properties of steroids have been shown to stabilize the blood-retinal barrier and reduce intraocular inflammation (Sarao *et al.*, 2014). However, like anti-VEGF agents, corticosteroids are only effective in a subset of patients and can lead to negative side effects such as glaucoma and cataract. Thus, newer and more sustainable treatments for DME are still needed.

Neurotrophic Keratitis

Trigeminal nerve damage caused by DM results in the breakdown of corneal epithelial cells and reduced corneal healing and sensitivity (Lockwood *et al.*, 2006). If left untreated, this degenerative disease, known as neurotrophic keratitis (NK), can lead to corneal ulceration, infection, and perforation. NK is commonly classified by three progressive stages (Bonini *et al.*, 2003). Stage 1 includes corneal epithelial changes, such as punctate epithelial erosions or epithelial irregularities. Stage 2 consists of a persistent epithelial defect that is usually oval in shape and surrounded by an area of loose epithelium that can spontaneously detach. Stromal melting, corneal ulcer, and potential perforation characterize stage 3. Early diagnosis is essential for the treatment of NK, as progression of the disease can lead to blindness. Current treatments aim to halt the progression of corneal damage and differ according to the stage of the disease. In mild cases of NK, the application of preservative-free artificial tears can help to preserve the corneal surface, thus preventing epithelial breakdown. In severe cases, surgical methods such as amniotic membrane grafting may be used to prevent corneal ulceration and perforation.

Neovascular Glaucoma

Progressive retinal ischemia in patients with DM causes an increase in the synthesis and release of VEGF in the retina, which can diffuse into the vitreous and aqueous humor. It has been suggested that this spread of VEGF can trigger abnormal blood vessel growth on the iris, just as it can promote abnormal vascular growth in the retina. When fibrovascular proliferation on the iris spreads to the trabecular meshwork, it can result in a severe rise in intraocular pressure known as neovascular glaucoma (NVG). Normally, aqueous humor is produced by the ciliary body and drains through the trabecular meshwork, which is located near the junction of the iris, cornea, ciliary body, and scleral spur in the "angle" of the eye. In patients, with NVG, the rate of aqueous production exceeds the capacity of the trabecular meshwork to drain fluid. The sustained increase in intraocular pressure can result in irreversible vision loss as a result of damage to the optic nerve. Treatment options for patients with NVG are similar to those of patients with PDR and are aimed at reducing the stimulus for VEGF production by sacrificing the peripheral retina. While PRP remains the primary therapeutic option, anti-VEGF agents can also be used to treat patients with NVG. However, despite these treatments, patients with NVG often require adjunct medical therapy to lower intraocular pressure and in many cases surgical intervention with glaucoma drainage devices is also necessary to restore normal intraocular pressure.

Cataract and Lens Changes

A cataract is defined as a clouding or whitening of the lens inside the eye and is a natural consequence of aging. However, there is an increased prevalence of nuclear, cortical, and especially posterior subcapsular cataract in patients with DM. Furthermore, cataract occurs earlier and progresses more rapidly in diabetic individuals. Currently, cataract removal with surgery is the only available method to restore vision in patients with severe cataracts. However, cataract surgery in patients with DR may promote progression of retinopathy, and thus the risks and benefits of cataract surgery must be carefully considered prior to proceeding with cataract removal.

Diabetes can produce transient refractive changes and blurred vision in individuals due to the effects of hyper or hypoglycemia on lens hydration and refractive index. Severe hyperglycemia can lead to myopic or hyperopic shifts in a patient's refraction, but these effects are typically transient and reverse upon restoration of normoglycemia.

Conclusion

Diabetes has devastating effects on ocular structure and function, but these effects can be mitigated with intense glycemic control and regulation of blood pressure. However, even patients with DM and superb metabolic control can develop ocular complications from DM such as DR, NK, NVG, and cataract. Eye-specific treatments for DR are aimed at reducing the stimulus for neovascularization or capillary leakage and include PRP, focal laser, steroids or anti-VEGF therapy. As in other parts of the body, DM seems to exert its deleterious effects on the eye through a combination of dysregulated glucose metabolism, alteration of trophic factor signaling, and ischemia. Preventive, eye-specific treatments for diabetic eye disease are needed to help reduce the burden of patients with vision-threatening complications of DM.

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Kidney Disease in Diabetes☆

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Driven by the obesity epidemic, the prevalence of diabetes mellitus (DM) has become a worldwide epidemic that continues to escalate. Its health sequela that comprises micro- and macrovascular complications is undeniably pressuring all health care systems. Apart from its associated risk with coronary heart disease, stroke, and visual impairment, DM is the leading cause of end stage kidney disease in Western countries. Renal involvement in DM is manifested in a variety of ways that can be collectively called diabetic kidney disease (DKD). These syndromes include diabetic nephropathy (DN), ischemic nephropathy, cholesterol embolic disease, and renal interstitial fibrosis. In DN, microalbuminuria (urine albumin 30–300 mg per 24 h) was once considered to define early or incipient nephropathy despite not always being a predictive marker of the disease or its progression. In patients with DM, microalbuminuria predicts progression in only 25%–40% and even in those with proven renal injury, many have diagnoses other than DN. Regression of microalbuminuria has been recognized in patients with either type 1 or type 2 DM though appears to be irreversible in African Americans. More importantly, only 45% of patients with an estimated glomerular filtration rate (eGFR) <60 mL/min and DM have microalbuminuria. Consequently, the presence or absence of microalbuminuria in patients with DM should not be solely used as a prerequisite for the diagnosis or exclusion of DN.

Similar histological and clinical courses in all diabetic individuals suggest that the pathogenesis of DN is similar in patients with both types and is closely related to longstanding metabolic and hemodynamic derangements. However, the fact that DN develops in only a subset of diabetic patients implies that other modulating factors such as inflammatory (infectious and non-infectious), environmental, and/or genetic exposures may also be involved in determining disease onset and severity. To date, glycemic control, blood pressure control, lipid control, and renin–angiotensin–aldosterone system (RAAS) inhibition remain the mainstays of therapy in patients with DKD.

Histological Changes

All patients with diabetes mellitus develop some histological changes within the kidney. Kidney hypertrophy is usually the earliest structural change in DKD although this is not usually associated with any specific findings on light microscopy. The first changes seen on light microscopy are cellular enlargement and an increased production of extracellular matrix (ECM), the latter resulting in mesangial expansion. Areas of severe mesangial expansion occur in some patients and result in nodular glomerulosclerosis classically known as Kimmelstiel-Wilson nodules. Glomerular vessels develop intimal hyaline thickening resulting in arteriolar hyalinosis, efferent arteriolar narrowing leading to glomerular hyperfiltration. Other ultrastructural changes seen on electron microscopy include a thickening of the glomerular basement membrane and podocyte hypertrophy. Podocytes later become effaced, leading to functional changes such as albumin excretion. With time, continued mesangial expansion results in external compression of the glomerular capillaries resulting in reduced blood flow to the kidney and ultimately a decline in glomerular filtration rate.

Clinical Course

DN is clinically characterized by glomerular hyperfiltration with increased filtration fraction in 50% of patients with type 2 DM. This is followed by a reduction in glomerular filtration rate (GFR) with or without albuminuria, ultimately leading to end stage kidney disease. In patients who develop macroalbuminuria (urinary albumin excretion >300 mg/day), progression of disease is likely and higher quantities of urinary albumin excretion correlate with the decline in glomerular filtration rate. Hence, treatment often focuses on reducing albuminuria to slow progression of DN.

Pathogenesis

The development of DN occurs as a consequence of multiple factors and pathways. The hemodynamic pathway is attributing to RAAS and endothelin-1 (ET-1) activation which occurs in the metabolic milieu of diabetes. Both increased angiotensin II levels and ET-1 are key mediators of efferent arteriole vasoconstriction resulting in glomerular hyperfiltration. With time, this causes

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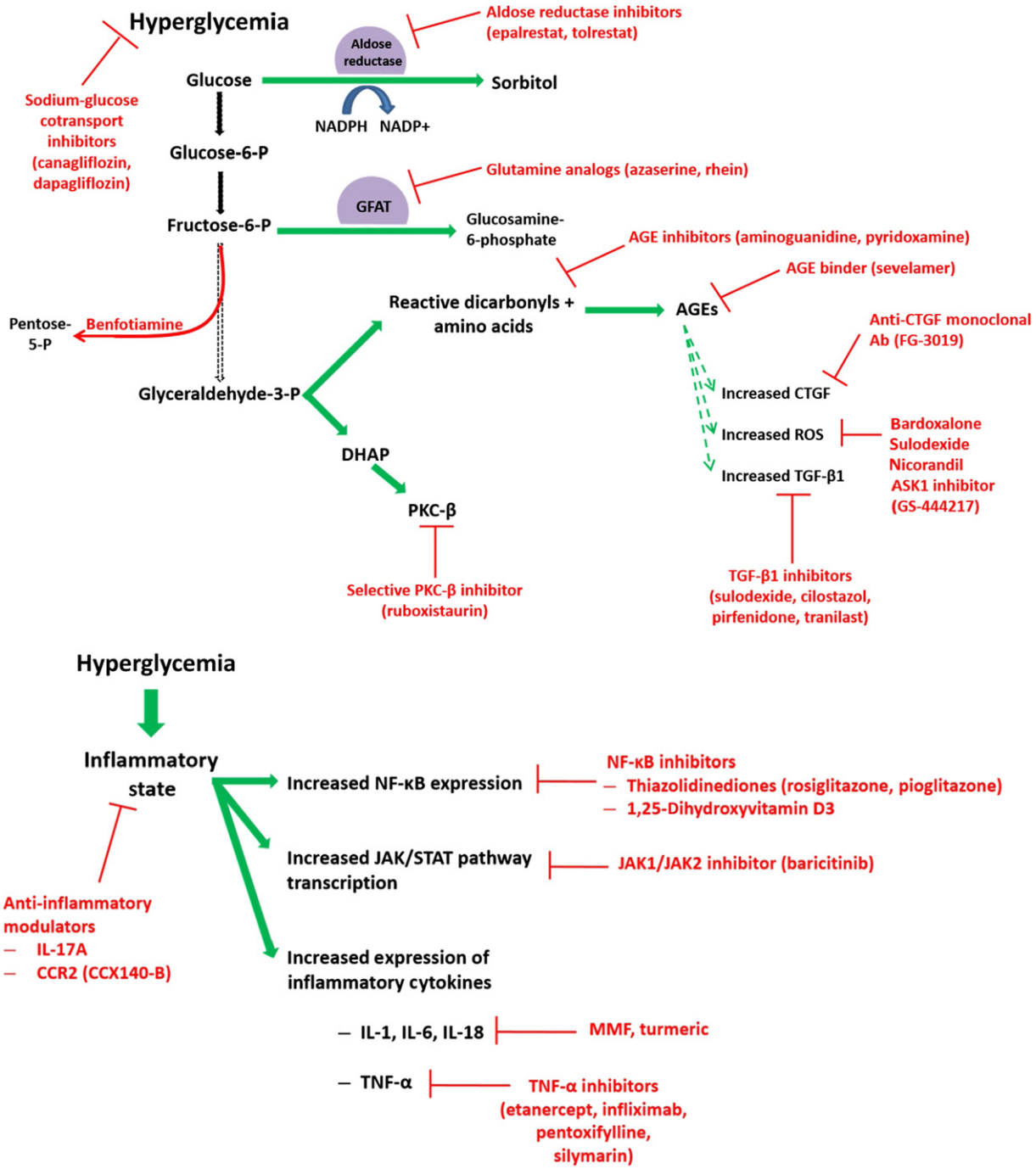

Fig. 1 Summary of the various pathways involved in the pathogenesis of DN and potential therapeutic interventions. Reprinted with permission from Toth-Manikowski, S. M. and Atta, M. G. (2015). Diabetic kidney disease: Pathophysiology and therapeutic targets. *Journal of Diabetes Research* 697010. Epub 2015 Apr 30.

endothelial dysfunction, inflammation, and fibrosis within the glomerulus ultimately leading to worsening albuminuria and progression of DN. RAAS activation is also involved in the pathogenesis of mesangial cell hypertrophy and ECM expansion.

The metabolic pathway (Fig. 1) of DN is driven by the consequences of longstanding hyperglycemia. In a non-diabetic kidney, normoglycemia predominates and glucose is normally metabolized via glycolysis. However, in patients with diabetes, a hyperglycemic state predominates leading to the activation of excess superoxide generation. The latter inhibits the enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) which is paramount to allowing glycolysis to occur in a normal manner. When glycolysis is inhibited, glucose is metabolized into precursors that are not normally present in the bloodstream such as: fructose, glucosamine-6-phosphate, advanced glycation end products (AGEs), and protein kinase C (PKC). These precursors activate a

cascade of events in the diabetic glomerulus such as ECM expansion and production of reactive oxygen species, cytokines, and growth factors. There is also upregulation of transforming growth factor-β (TGF-β), collagen, laminin, and fibronectin which in turn lead to tubular hypertrophy, glomerular hypertrophy, and ultimately glomerular sclerosis.

The inflammatory pathway (Fig. 1) is a distinct pathway that supports the notion that DN does not occur solely as a result of perturbed hemodynamics and hyperglycemia. It proposes that patients with DM have a chronically activated low-grade inflammatory state with subsequent increased expression of NF-κB and other inflammatory cytokines such as interleukins 1, 6, and 18 (IL-1, IL-6, IL-18, respectively) as well as tumor necrosis factor-α (TNF-α). Together, these inflammatory cytokines paly key role in the development of mesangial hypertrophy, glomerular hypercellularity, GBM thickening, albuminuria, and overt proteinuria.

Lastly, there are additional or "alternate" pathways, although perhaps not as clearly delineated, that have also been suggested to have a role in the pathogenesis of DN. For example, patients with diabetes are believed to have alterations in evolutionary protective mechanisms that are otherwise well conserved in a normoglycemic phenotype. Autophagy is an example of a highly conserved protective mechanism that usually allow cells to degrade cytotoxic proteins whenever a cell is experiencing stress. Podocytes are cells that are particularly sensitive to hyperglycemic conditions and defective autophagy ultimately results in podocyte injury within the kidney. Another highly conserved evolutionary mechanism is the sodium-glucose transporter 2 (SGLT2), a transporter in the renal proximal tubule responsible for $>90\%$ of glucose reabsorption in the proximal tubules. In normoglycemic conditions, this transporter helps conserve energy which benefits both the body and brain. In hyperglycemic conditions, SGLT2 counterproductively absorbs excess glucose which contributes further to a hyperglycemic state.

Finally, genetic susceptibility appears to play a key role in both development and progression of DN as suggested by cross-sectional studies demonstrating a concordance for DN in both type 1 and 2 diabetic sibling pairs.

Treatment

Despite multiple advances in drug therapy, the mainstay of DKD management remains glycemic control, blood pressure control, lipid control, and RAAS inhibition. This strategy is centered on targeting aspects of the metabolic and hemodynamic pathways. The diabetes control and complications trial (DCCT) demonstrated a reduction in micro- and macroalbuminuria in patients with type 1 diabetes that were randomized to intensive glucose control. Long-term follow-up was notable for a reduction in the rate of decline in GFR. The United Kingdom prospective diabetes study (UKPDS) demonstrated a reduction in microvascular complications in patients with type 2 diabetes randomized to intensive glycemic control. Long-term follow-up of this study was notable for a decrease in diabetes-related death, which included death from macrovascular complications and renal disease in the group randomized to intensive glycemic control. Similarly, RAAS inhibition with angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) have consistently demonstrated a reduction in albuminuria and progression to DN and ESRD. Their benefit is largely attributed to reduced vasoconstriction of the efferent arteriole which in turn reduces glomerular hyperfiltration. The addition of a nonsteroidal mineralocorticoid receptor antagonist (MRA) was recently shown to augment the effects of an ACEI or ARB when used together in patients with type 2 DM and DN. Finerenone is a more selective MRA than its predecessors, spironolactone and eplerenone, and was found to reduce albuminuria in a dose-dependent fashion without incurring any adverse events such as hyperkalemia or worsened kidney function. Its efficacy and safety on cardiovascular morbidity are being tested in the FIGARO-DKD phase III multicenter trial. Other therapeutic investigative options that target the hemodynamic pathway include ET-1 antagonists that are developed to counteract the glomerular hyperfiltration that occurs as a result of efferent arteriolar vasoconstriction. The combination of the ET-1 antagonist, atrasentan, with a RAAS inhibitor demonstrated a dose-dependent decrease in albuminuria and is currently in the final stages of clinical assessment.

Because the metabolic pathway plays such an important role in the pathogenesis of DN, multiple therapeutic options have been developed to target various aspects of this pathway. For example, data are now emerging on the favorable renal outcome in patients with DKD with the use of the sodium-glucose co-transporter 2 (SGLT2) inhibitors; empagliflozin, canagliflozin, dapagliflozin, and ipragliflozin, ertugliflozin, and luseogliflozin (the latter three agents have not been approved for use in the United States). They work by decreasing the amount of glucose that is reabsorbed from the renal proximal tubules. The most impressive evidence to date in support of SGLT2 inhibitors for DN comes from the EMPA-REG OUTCOME trial, a study that randomized patients with type 2 diabetes and an eGFR ≥ 30 mL/min to receive either empagliflozin (at an oral dose of 10 mg or 25 mg daily) or placebo in addition to standard care. In addition to a lower risk for composite cardiovascular outcome, those receiving empagliflozin were less likely to experience worsening of their DN (as defined by progression of macroalbuminuria), doubling of their creatinine, and were less likely to be initiated on renal replacement therapy. A study of canagliflozin (at an oral dose of 100 or 300 mg daily) compared to glimepiride at a dose of 6–8 mg demonstrated a significant reduction in the rate of eGFR decline compared to glimepiride. Additionally, macroalbuminuric participants receiving canagliflozin had a more pronounced decrease in urinary albumin-to-creatinine ratio compared to those on glimepiride alone. The latter study did not evaluate harder clinical endpoints such as doubling of serum creatinine or time to renal replacement therapy. A pooled analysis of 11 phase 3 clinical trials of dapagliflozin demonstrated that while the effect of HbA1c decline decreases with lower levels of eGFR, placebo-adjusted reductions in urinary albumin-to-creatinine ratio were actually largest in the lowest eGFR subgroup. The final results of the CANVAS and DECLARE-TIMI-58 trials, the two clinical trials studying canagliflozin and dapagliflozin, respectively, are still pending. Of note, while all SGLT2 inhibitors carry an FDA warning about the increase in risk for severe urinary tract infections and

Table 1 Effects of empagliflozin, canagliflozin, dapagliflozin, and ipragliflozin on renal-relevant endpoints

Drug name	*Dose*	*Comparator*	*Study characteristics*	*Summary of renal-relevant endpoints*
Empagliflozin	10 or 25 mg daily	Placebo	• Double blind, randomized controlled trial • $N = 7020$	• Empagliflozin was associated with a decreased risk of incident or worsening nephropathy; HR 0.61, 95%CI (0.53–0.70) • Progression to macroalbuminuria occurred in 11.2% of empagliflozin group and 16.2% in placebo group, a significant relative risk reduction of 38%; HR 0.61, 95% CI (0.54–0.72) • Doubling of serum creatinine level occurred in 1.5% of empagliflozin group and 2.6% in placebo group, a significant relative risk reduction of 44%; HR 0.56, 95% CI (0.39–0.79) • Initiation of RRT occurred in 0.3% of empagliflozin group and 0.6% in placebo group, a significant relative risk reduction of 55%; HR 0.45, 95% CI (0.21–0.97) • Doubling of serum creatinine, initiation of RRT, or death from renal disease occurred in 1.7% of empagliflozin group and 3.1% in placebo group; HR 0.54, 95% CI (0.40–0.75)
Canagliflozin	100 or 300 mg daily	Glimepiride	• Double blind, randomized controlled trial • $N = 1450$	• Annual decline in eGFR was 3.3 mL/min with glimepiride, 0.5 mL/min with canagliflozin 100 mg (compared to glimepiride, $P < 0.001$), and 0.9 mL/min with canagliflozin 300 mg (compared to glimepiride, $P = 0.002$) • Relative to glimepiride, canagliflozin 300 mg decreased UACR by 11.2%, 95% CI (3.6–18.3) • Among patients with UACR $\geq$30 mg/g, canagliflozin 100 and 300 mg decreased UACR by 31.7% and 59.3%, respectively, when compared to glimepiride; 95% CI (8.6–48.9) and (31.9–62.2), respectively
Dapagliflozin	10 mg daily	Placebo	• Pooled analysis of 11 phase 3 clinical trials • $N = 4404$	• Among patients with UACR >30 mg/g, after 24 weeks of dapagliflozin therapy, UACR was decreased by 16.1% (95% CI: -32.3, 3.8), 23.3% (95% CI: -35.5, -8.7), and 38.3% (95% CI: -54.5, -16.6) in participants with eGFR of $\geq$90 mL/min, $\geq$60- < 90 mL/min, $\geq$45- < 60 mL/min, respectively
Ipragliflozin[a,b]	50 mg daily	None	• Multicenter, open-label study • $N = 50$	• After 24 weeks of ipragliflozin therapy, median UACR decreased from 15.5 to 12.9 mg/g Cr ($P = 0.007$). In subgroup analyses, the significant drop in UACR was only noted in those with eGFR $\geq$90 mL/min ($n = 20$)

[a]This drug is not yet approved by the FDA for use in the United States.

[b]Phase 4 study of ipragliflozin and its effects on UACR, efficacy and safety in patients with type 2 diabetes is currently underway.

RCT, randomized controlled trial; *RRT*, renal replacement therapy; *UACR*, urine albumin-to-creatinine ratio.

Note: Ertugliflozin, luseogliflozin, and tofogliflozin have not yet undergone studies to evaluate effects on renal-relevant endpoints and were not been included in this table for this reason.

ketoacidosis and have not been approved for use in patients with type 1 diabetes, canagliflozin carries a drug-specific FDA warning for increased risk of leg and foot amputations. The effects of gliflozins on renal-relevant endpoints are summarized in **Table 1**.

Aldose reductase inhibitors such as epalrestat and tolrestat were first developed to decrease conversion of glucose to sorbitol and fructose. Tolrestat was later removed from the market when it was found to have an association with hepatic necrosis. Azaserine and rhein were both developed to inhibit an enzyme which would otherwise lead to increased transcription of TNF-α and TGF-β1. While neither of these have yet been tested in humans, in vivo studies have shown promise. AGEs are another product of the metabolic pathway that when unhindered, lead to ECM expansion, increased reactive oxygen species (ROS), cytokine production, and eventually DKD progression. Drugs such as benfotiamine, aminoguanidine, pyridoxamine have all attempted to diminish AGE production. While a large trial evaluating aminoguanidine was stopped due to lack of efficacy and safety concerns, pyridoxamine is currently undergoing a phase 3 study to evaluate its effect on time to ESRD in patients with DN. There are also multiple therapeutic agents that target the downstream products of AGEs. These include nicorandil, bardoxolone, sulodexide, pirfenidone, sevelamer, cilostazol, tranilast, and FG-3019 and they target downstream products such as connective tissue growth factor (CTGF), ROS, and TGF-β1. Of these agents, nicorandil has shown promise in diabetic mouse models whereas pirfenidone,

sevelamer, cilostazol, tranilast, and FG-3019 have all shown promise in patients with DN. The latter two agents are currently undergoing testing in patients with DN. Lastly, ruboxistaurin was developed as a protein kinase C-β inhibitor, one of the final endpoints in the metabolic pathway. Although this agent was sufficiently promising to initiate a phase 3 trial, it was halted for business considerations.

Treatment options have also targeted the inflammatory markers and cytokines that are prevalent as part of the inflammatory pathway. These drugs include NF-κB inhibitors such as thiazolidinediones and 1, 25-dihydroxyvitamin D3 and JAK1/STAT pathway inhibitors such as baricitinib. The latter is currently undergoing a phase 2 study in patients with DN whereas thiazolidinediones are undergoing a phase 4 study to evaluate the long-term effects of these agents on patients with DM type 2 and DN. Apoptosis signal-regulating kinase 1 (ASK1) mediates the downstream inflammatory signals of ROS. Despite early stage potential of AKS1 inhibition on this pathway, it failed in phase II clinical trial to demonstrate clinical benefit in in patients with DN. Existing or novel agents that have been developed to target the more ubiquitous inflammatory pathway include mycophenolate mofetil, drugs derived from turmeric, and TNF-α inhibitors such as etanercept, infliximab, pentoxifylline, and selective CCR2 inhibitors (CCX140-B). While some of these agents have far-reaching effects in the body which unfortunately limit their use solely for DN, pentoxifylline, CCX140-B, silymarin, and curcumin (the latter two being derived from turmeric) have all shown promise in patients with DN. Conversely, cytokines that favorably modulate inflammation such as IL-17A have been shown to treat, reverse, and even prevent established nephropathy in diabetic mouse models and may become a therapeutic option beyond animal models.

In the end, it is conceivable that management of DN will require first a good and reproducible marker, other than microalbuminuria, that accurately predicts disease onset and progression; and second a multi-targeted approach that works in concert with currently existing therapeutic options in order to modify the diverse pathways involved in the pathogenesis of the disease. Key to success of these new approaches is not only to prove their efficacy in halting or reversing progression of DN but also avoidance of their potentially off-target effects.

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Diabetic Nerve Disease, Neuropathy☆

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Glossary

Antiarrhythmic A drug that restores normal heartbeat regularity.
Arrhythmia An irregularity of the heartbeat.
Axon A nerve cell process responsible for conducting electrochemical impulses.
Glycation The addition of sugars to other molecules.
Hyperglycemia Elevated blood glucose levels.
Hypoxia Decreased levels of oxygen in air, blood, or tissue.
Ischemia Reduced blood flow.
Polyol pathway Polyhydroxy alcohol, specifically the metabolites and enzymes that create sugar alcohols and inositols.
Proprioception Information concerning movement and position of body parts.

Distal Symmetric Sensorimotor Polyneuropathy

Distal symmetric sensorimotor polyneuropathy (DPN) first appears as a sensory disorder of the feet, reflecting distal axonal damage. As the disease progresses, loss of sensation ascends, and by the time DPN reaches midcalf, many patients notice a sensory loss in their hands. This slow progression results in the classical "stocking-glove" sensory loss. Vibration and proprioception are transmitted along large myelinated fibers, whereas small myelinated and unmyelinated nerve fibers transmit information concerning pain, light touch, and temperature. Patients with DPN have symptoms of decreased vibration and light touch, altered temperature sensation, and poor pain perception. This indicates damage to or loss of all three fiber types. Patients may have "positive" symptoms and complain of pain, paresthesias, and/or dysesthesias. More often, patients demonstrate "negative" symptoms, with sensory loss only identifiable following an examination.

Weakness of the toes and ankles is a late manifestation of DPN. Sensory loss combined with weakness predisposes the foot to ulcer formation. Ulceration is classified into two categories: acute and chronic. Patients with insensate feet are vulnerable to acute foot ulcers caused by improper shoeware. Chronic ulceration is likely to have many causes, including autonomic neuropathy and compromised vascular supply.

Estimates of the prevalence of DPN vary greatly according to the diagnostic criteria and population studied. Large epidemiological studies have shown that DNP will develop within 10 years of the onset of diabetes in 40%–50% of both types of diabetes. Up to 50% of DPN may be asymptomatic.

Pathogenesis of DPN

Despite intense research, the exact pathogenesis of DPN remains unknown. The Diabetes Control and Complications Trial (DCCT) proved that hyperglycemia is essential for the development of DPN. Commonly proposed theories for DPN include alterations in the polyol pathway, vascular insufficiency, abnormal glycation of proteins and lipids, increased oxidative stress, altered nitric oxide synthesis, impaired axonal transport, and reduced neurotrophism. Classically, these theories are categorized as metabolic or vascular. It is likely that these are related and overlapping factors and all contribute to the loss of peripheral nerve function and subsequent symptoms of DPN.

The metabolic theory is based on the conversion of excess glucose to sorbitol by aldose reductase as part of the polyol pathway. Elevated sorbitol results in reciprocal decreases of myoinositol and taurine. Depletion of these metabolites becomes rate limiting for essential intracellular metabolism. The vascular theory was once considered separate from the metabolic theory. It states that reduced nerve blood flow results in hypoxia/ischemia and causes the development of DPN. It is now clear that the metabolic and vascular theories are linked on many levels. Glucose flux through the polyol pathway causes the depletion of NADPH and NAD^+. Without sufficient NADPH, cells cannot regenerate the glutathionine required to neutralize reactive oxygen species. Consequently, the peripheral nerve sustains oxidative damage that decreases endoneurial blood flow and promotes ischemia. The activity of the potent vasodilator, nitric oxide (NO), is also tightly linked to NADPH. NO, synthesized by NO synthase, is a NADPH-dependent reaction. Therefore, depletion of NADPH limits NO synthesis and in turn causes vasoconstriction and ischemia, which contribute to nerve conduction slowing.

☆*Change History*: February 2018. Ékoé involved in preparing the update. Section on distal symmetric sensorimotor polyneuropathy has been updated as well as section on treatment.

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Diagnosis and Treatment

Three treatment strategies are clinically available. Of most importance is the early diagnosis of DPN. Early diagnosis allows for the implementation of the second strategy: good glycemic control and foot care. The third approach is focused on the treatment of painful DPN and is added to good glycemic control and foot care.

The early diagnosis of DPN is imperative. It makes early intervention possible, thereby significantly decreasing patient morbidity. It is important that other causes of neuropathy are excluded prior to DPN being related to diabetes. This is especially important if there are unusual features of the neuropathy, such as rapid progression, marked asymmetry, or more motor than sensory deficits.

In 1988, the San Antonio Consensus Panel recommended five quantifiable measures for the accurate diagnosis of DPN: a symptom questionnaire, a standardized clinical examination, quantitative sensory testing, nerve conduction studies, and autonomic function testing. Patients are classified as having stage I (no symptoms) or stage II (symptoms) neuropathy. Each stage is further divided into grades from A to C depending on the number of positive test results and the severity of clinical impairment. These criteria are being used to study the Rochester Diabetic Cohort. Modified criteria have been implemented in several clinical trials, including the DCCT.

Simpler screening instruments for DPN are also available. These instruments were developed because patient and physician resources are frequently limited, making completion of the San Antonio criteria difficult. In the United Kingdom, DPN is diagnosed if patients present with mild signs and moderate symptoms or moderate signs alone, in the absence of symptoms. The Michigan Neuropathy Screening Instrument includes inspecting the feet for dry skin, callus, fissure, or ulceration; assessment of vibratory sensation in the great toes; and testing for ankle reflexes. A score of >2 indicates neuropathy with a high sensitivity (80%) and specificity (95%). Because of its simplicity, the Michigan Neuropathy Screening Instrument is also highly reproducible. Another simple method for screening patients for the presence of DPN is the use of a 10-g nylon monofilament. The filament is pressed against the sole of the foot until the filament buckles, indicating a known force has been applied. If a patient is unable to perceive the filament, he or she is at increased risk for complications of DPN.

Glycemic Control and Foot Care

The DCCT clearly demonstrates that improved glycemic control decreases the frequency of DPN in patients with type I diabetes mellitus. Glycemic control requires thorough patient education. Patients are instructed on the importance of diet and regular glucose monitoring. This is achieved with a team consisting of the patient, a diabetes nurse educator, a dietician, and a physician.

Good foot care is also important. Patients are instructed to inspect their feet every night for evidence of dry skin, cracking, or fissuring of the skin. The importance of shoeware is also well documented. Shoes must cushion the points of contact between the foot and the shoe and must accommodate any inherent or acquired foot deformities. For patients with mild neuropathy, cushioned socks and high-quality athletic shoes with room for the forefoot and toes are helpful. In severe cases, patients may require customized inserts or molded shoes.

Acute and Chronic Painful DPN

Multiple treatments are available for the management of neuropathic pain. It has been shown that few people have complete relief of painful symptoms with any treatment and that a 30%–50% reduction in baseline pain is considered to be a clinically meaningful response. The lack of comparative studies makes it difficult to recommend which oral medication should be used first line. Opioids have shown good efficacy for neuropathic pain but they are not recommended due to the high risk of dependency, tolerance, and dose escalation.

A stepwise treatment protocol is used for patients with painful DPN. Nonsteroidal anti-inflammatory drugs provide relief in many patients with chronic painful DPN. In a double-blind, placebo-controlled trial, both ibuprofen (600 mg four times per day) and sulindac (200 mg two times per day) effectively decreased pain associated with DPN. However, this class of drugs cannot be used in patients with renal impairment.

Anticonvulsants (gabapentin, pregabalin) and antidepressants are most commonly used as first-line therapy.

The tricyclic antidepressants are the best studied class of drugs for the treatment of painful DPN. In double-blind, placebo-controlled trials, amytriptyline, nortriptyline, and imipramine were each effective in the treatment of painful DPN. Duloxetine and venlafaxine are newer antidepressants that may also be useful for the treatment of painful neuropathy. If a patient continues to experience disabling pain, a second drug, *pregabalin* or *gabapentin*, is added to the therapeutic regimen. Patients may also be started on gabapentin as a first-line therapy, with a tricyclic added if a second medication is needed. Carbamazepine is also a good drug to add to either a tricyclic antidepressant or a serotonin uptake inhibitor. Double-blind, placebo-controlled trials have found that it provides symptomatic relief to a large percentage of afflicted patients.

For patients who are on two medications and still experiencing significant discomfort, capsaicin cream may be used. Capsaicin cream is a topical therapy that inhibits the uptake of substance P at sensory endings. Patients are instructed to apply capsaicin cream (0.075%) four times per day, the regimen that was successful in a double-blind, placebo-controlled trial. Topical nitrate sprays are another therapeutic option.

If these therapeutic strategies fail, the second drug is discontinued and a new drug is instituted. The first choice is the cardiac antiarrhythmic drug mexiletine, which is effective in patients who otherwise are refractory to treatment. The antiepileptic topiramate can also be used in refractory patients. If a patient remains resistant to these treatment strategies, he or she should be referred to a comprehensive pain clinic. Here, patients frequently receive local nerve blocks, a transcutaneous electrical nerve stimulation (TENS) unit, or, in certain cases, acupuncture. Unfortunately, the prognosis for good pain relief in patients who require a pain clinic referral is low.

In summary, either pregabalin or duloxetine are recommended today as initial pharmacologic treatments for neuropathic pain in diabetes. They have received regulatory approval by the US Food and Drug Administration (FDA), Health Canada, and the European Medicines Agency (EMA) for the treatment of neuropathic pain in diabetes.

Conclusion

A systematic, stepwise approach to patients with DPN ensures optimal patient care and decreases the risks for short- and long-term disability (American Diabetes Association, 2018). Future therapies hold promise, particularly those targeted toward ameliorating the metabolic and vascular abnormalities that develop in the peripheral nervous system as a result of continued hyperglycemia.

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Neurological Disease and Diabetes, Autonomic☆

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Glossary

Autonomic nervous system The portion of the nervous system that regulates individual organ function and homeostasis not under voluntary control. The autonomic nervous system is also responsible for conveying visceral sensation. The autonomic nervous system is typically divided into two divisions, the parasympathetic system and the sympathetic system, based on anatomical and functional differences.

Diabetic neuropathy A demonstrable disorder, either clinically evident or subclinical, that occurs in the setting of diabetes mellitus without other causes of peripheral neuropathy. The neuropathic disorder associated with diabetes includes manifestations in the somatic and/or autonomic parts of the peripheral nervous system.

Parasympathetic nervous system The portion of the autonomic nervous system concerned with the conservation and restoration of energy. It causes a reduction in heart rate and blood pressure, facilitates the digestion and absorption of nutrients, and facilitates the excretion of waste products from the body.

Sympathetic nervous system The portion of the autonomic nervous system that enables the body to be prepared for fear, flight, or fight. Sympathetic responses include increases in heart rate, blood pressure, and cardiac output and the diversion of blood flow from the skin and splanchnic vessels to those vessels supplying the skeletal muscle.

Introduction

Individuals are not aware of their autonomic nervous system when it functions normally; only when there is dysfunction can the manifestations be diverse and devastating. Diabetes mellitus can cause dysfunction of any or every part of the autonomic nervous system, leading to a wide range of disorders. If an individual knows the autonomic nervous system and its disorders, he or she will know the whole of medicine. The organ systems that most often exhibit prominent clinical autonomic signs and symptoms in diabetes include the ocular pupil, sweat glands, genitourinary system, gastrointestinal tract system, adrenal medullary system, and cardiovascular system. Clinical symptoms of autonomic neuropathy generally do not occur until long after the onset of diabetes. Subclinical autonomic dysfunction can, however, occur within 1 year of diagnosis in type 2 diabetes patients and within 2 years in type 1 diabetes patients. In fact, impaired autonomic function may be associated with early glucose dysmetabolism (impaired glucose tolerance and impaired fasting glucose) and be involved in the pathogenic pathway leading to the development of diabetes.

Epidemiology of Diabetic Autonomic Neuropathy

The reported prevalence of diabetic autonomic neuropathy varies, with community-based studies finding lower rates than clinic-based and hospital-based studies, in which the prevalence may be as high as 90%. The variance among prevalence studies also reflects the type and number of tests performed and the presence or absence of signs and symptoms of autonomic neuropathy. Another important factor that accounts for the marked variability in reported prevalence rates is the lack of a standard accepted definition of diabetic autonomic neuropathy. Ziegler found that 25.3% of patients with type 1 diabetes and 34.3% of patients with type 2 diabetes had abnormal findings in more than two of six autonomic function tests.

Pathogenesis of Diabetic Autonomic Neuropathy

Hypotheses concerning the multiple etiologies of diabetic autonomic neuropathy include a metabolic insult to nerve fibers, neurovascular insufficiency, autoimmune damage, and neurohormonal growth factor deficiency. The most important clinical factors include poor glycemic control, diabetes duration, age, female sex, and higher body mass index (BMI). Several different

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pathogenic processes have been described. Hyperglycemic activation of the polyol pathway leading to the accumulation of sorbitol and potential changes in the NAD:NADH ratio may cause direct neuronal damage and/or decreased nerve blood flow. Activation of protein kinase C induces vasoconstriction and reduces neuronal blood flow. Increased oxidative stress, with increased free radical production, causes vascular endothelium damage and reduces nitric oxide bioavailability. Alternatively, excess nitric oxide production may result in the formation of peroxynitrite and damage to the endothelium and neurons, a process referred to as nitrosative stress. In a subpopulation of individuals with neuropathy, immune system mechanisms may also be involved. Reduction in neurotrophic growth factors, deficiency of essential fatty acids, and formation of advanced glycation end products (AGEs) also result in reduced endoneural blood flow and nerve hypoxia with altered nerve function. The result of this multifactorial process may be the activation of poly(ADP) ribosylation depletion of ATP, resulting in cell necrosis and the activation of genes involved in neuronal damage.

Clinical Manifestations of Diabetic Autonomic Neuropathy

The metabolic disorders of diabetes lead to diffuse and widespread damage of nerves and small vessels. Clinical manifestations of autonomic dysfunction frequently occur concurrently but in inconsistent patterns (Table 1). Diabetic autonomic neuropathy is typically assessed by focusing on symptoms or dysfunction attributable to a specific organ system. Cardiac autonomic neuropathy is the most prominent focus because of the life-threatening consequences of this complication and the availability of direct tests of cardiovascular autonomic function. However, neuropathies involving other organ systems should also be considered in the optimal care of patients with diabetes.

Cardiac Autonomic Neuropathy

Cardiac autonomic neuropathy results from damage to the autonomic nerve fibers that innervate the heart and blood vessels and results in abnormalities in heart-rate control and vascular dynamics. The 5-year mortality rate from this serious complication is five times higher for individuals with cardiac autonomic neuropathy than for individuals without cardiovascular autonomic involvement. Autonomic dysfunction can impair exercise tolerance. The severity of cardiac autonomic neuropathy has been shown to correlate inversely with an increase in heart rate at any time during exercise and with the maximal increase in heart rate. Cardiac autonomic neuropathy also leads to a reduced cardiac ejection fraction, systolic dysfunction, and decreased diastolic filling. In fact,

Table 1 Clinical Manifestations of Autonomic Neuropathy

Cardiovascular
Resting tachycardia
Exercise intolerance
Orthostatic hypotension, orthostatic tachycardia and orthostatic bradycardia
Silent myocardial ischemia
Sudden death
Gastrointestinal
Esophageal dysmotility
Gastroparesis diabeticorum
Constipation
Diarrhea
Fecal incontinence
Genitourinary
Neurogenic bladder
Erectile dysfunction
Retrograde ejaculation
Female sexual dysfunction (loss of vaginal lubrication)
Metabolic
Hypoglycemia unawareness
Hypoglycemia-associated autonomic failure
Sudomotor
Anhidrosis
Heat intolerance and hyperhidrosis
Gustatory sweating
Dry skin
Pupillary
Argyll-Robertson pupil
Decreased diameter of dark-adapted pupil
Failure of pupillary constriction with light exposure

diastolic dysfunction is considered the earliest manifestation of diabetic cardiomyopathy. There is a consistent association between cardiac autonomic neuropathy and the presence of silent myocardial ischemia (MI). The perception of angina is severely impaired in diabetic patients, allowing these individuals to exercise longer after the onset of MI with dire consequences. Chest pain in any location in a patient with diabetes should be considered to be of myocardial origin until proven otherwise; however, of equal importance, unexplained fatigue, confusion, tiredness, edema, hemoptysis, nausea and vomiting, diaphoresis, arrhythmias, cough, or dyspnea should alert the clinician to the possibility of silent MI. Mortality rates after a MI are also higher for diabetic patients than for nondiabetic patients, and higher still if autonomic dysfunction is present. This may be due to regional differences in autonomic nerve damage within the heart, increasing the tendency for the development of ventricular arrhythmia and cardiovascular events after infarction. Cardiac failure is also more frequent following a MI in patients with autonomic neuropathy.

Orthostatic hypotension, another sign of autonomic neuropathy, is a fall in systolic blood pressure of greater than 30 mm Hg on standing. It is aggravated by peripheral vascular pooling of blood such as occurs with hot baths or drugs used to treat hypertension. Patients with orthostatic hypotension typically present with lightheadedness and presyncopal symptoms. Symptoms such as dizziness, weakness, fatigue, visual blurring, and neck pain also may be due to orthostatic hypotension. It is now recognized that a similar syndrome can occur with orthostatic tachycardia and/or bradycardia without a drop in blood pressure simply because there is a reduction in cardiac output.

Gastrointestinal Autonomic Neuropathy

Gastrointestinal symptoms are relatively common among patients with diabetes and often reflect diabetic gastrointestinal autonomic neuropathy. Esophageal dysfunction results at least in part from vagal neuropathy; symptoms include heartburn and dysphagia for solids. Gastroparesis in diabetes is usually clinically silent and may be the most important cause of "brittle diabetes." Severe diabetic gastroparesis is one of the most debilitating of all diabetic gastrointestinal complications. Via the use of radioisotopic techniques that quantify gastric emptying, it appears that 30–50% of patients with longstanding diabetes have delayed gastric emptying. Major clinical features of this disorder are early satiety, anorexia, nausea, vomiting, epigastric discomfort, and bloating. Episodes of nausea or vomiting may last for days to months or occur in cycles. Even with mild symptoms, gastroparesis interferes with nutrient delivery to the small bowel and therefore disrupts the relationship between glucose absorption and exogenous insulin administration. These changes may result in wide swings in glucose levels, unexpected episodes of postprandial hypoglycemia and apparent brittle diabetes. Gastroparesis should therefore always be suspected in patients with erratic glucose control. Diarrhea is evident in 20% of diabetic patients, particularly those with known diabetic autonomic neuropathy. Diarrhea is typically intermittent, but bowel movements may occur 20 or more times per day with urgency, and the stools are often watery. Constipation is the most common gastrointestinal complication, affecting nearly 25% of diabetic patients. Individuals with constipation may have less than three bowel movements per week and these may alternate with diarrhea. Fecal incontinence due to poor sphincter tone is common in individuals with diabetes and may be associated with severe paroxysmal diarrhea or constitute an independent disorder of anorectal dysfunction.

Genitourinary Autonomic Neuropathy

Neurogenic bladder

The earliest bladder dysfunction is a sensory abnormality that results in impaired bladder sensation, an elevated threshold for initiating the micturition reflex, and an asymptomatic increase in bladder capacity with urinary retention. Overflow incontinence occurs because of denervation of the external and internal sphincters. Individuals with bladder dysfunction are predisposed to the development of urinary tract infections, including pyelonephritis, which may accelerate or exacerbate renal failure.

Erectile dysfunction

Erectile dysfunction is the most common form of organic sexual dysfunction in males with diabetes, with an incidence estimated to be between 35% and 75%. It is defined as the consistent inability to attain and maintain an erection adequate for sexual intercourse. Erectile dysfunction may be the presenting symptom of diabetes and more than 50% of men with diabetes notice the onset of erectile dysfunction within 10 years of onset of the diabetes. Erectile dysfunction is a marker for the presence and development of generalized vascular disease and for premature demise from a myocardial infarct. Penile failure may portend an upcoming and possibly preventable, cardiovascular event. Erectile dysfunction should alert physicians to perform cardiovascular evaluations for these patients. Retrograde ejaculation into the bladder also occurs in diabetic males.

Hypoglycemia Unawareness

The counterregulatory hormone responses and awareness of hypoglycemia are reduced in patients with diabetes mellitus. Unawareness of hypoglycemia and unresponsiveness to it are serious problems that hamper the patient's ability to manage his or her diabetes. In most diabetic patients, catecholamine release, triggered by low glucose levels, produces noticeable symptoms, such as tremulousness and cold sweat, that alert the patients to eat and take other measures to prevent coma. Diabetic autonomic neuropathy impairs catecholamine release and prevents the warning signs of hypoglycemia, leaving the patient unaware of it. The

related problem of hypoglycemic unresponsiveness occurs when impaired autonomic responses derange glucose counter-regulation during fasting or periods of increased insulin activity. In healthy people and in patients with early stage diabetes, these autonomic responses result in the release of glucagon and epinephrine for short-term glucose counterregulation and the release of growth hormone and cortisol for long-term regulation. Failure in glucose counter-regulation can be confirmed by the absence of glucagon and epinephrine response to hypoglycemia induced by administration of a controlled dose of insulin. The glucagon response becomes impaired after 1–5 years of type 1 diabetes. After 15–30 years, the glucagon response is almost undetectable, and it is absent in patients with autonomic neuropathy. The difficulty is in distinguishing the loss of counterregulation due to autonomic neuropathy from that due to the dampening effects of intensive glycemic control and recurrent hypoglycemia, called "hypoglycemia-associated autonomic failure."

Neurovascular Disturbances

Skin blood flow is important in maintaining nutrition, maintaining regional and whole body temperature, and healing skin trauma. The apical (glabrous) skin is present in the palmar surface of the hand, the plantar surface of the foot, and the face. It contains a large number of arteriovenous anastomoses or shunts and functions in thermoregulation. In contrast, nonapical (hairy) skin is present over most of the body surface. There are relatively few arteriovenous shunt vessels and blood flow is primarily nutritive in function. Microvascular skin flow is under the control of the autonomic nervous system and is regulated by both the central and peripheral components. In diabetes, the rhythmic contraction of arterioles and small arteries is disordered. Microvascular blood flow can be accurately measured noninvasively using laser Doppler flowmetry. Defective blood flow in the small capillary circulation is found with decreased responsiveness to mental arithmetic, cold pressor, handgrip, and heating. The defect is associated with a reduction in the amplitude of vasomotion and resembles premature aging. There are differences in the glabrous and hairy skin circulations. In hairy skin, a functional defect is found before the development of neuropathy; it occurs in family members and it may precede the development of diabetes. The clinical impact is dry skin, loss of sweating, and the development of fissures and cracks that are portals of entry for microorganisms leading to infectious ulcers and ultimately gangrene. The effect of autonomic neuropathy on the risk of developing a foot ulcer is independent of other measures of sensory neuropathy. Autonomic neuropathy may also lead to hyperperfusion of bone with increased osteoclastic activity, resulting in reduced bone density. Thus, a hot foot should alert the physician to impending Charcot's neuroarthropathy.

Diagnostic Tests of Diabetic Autonomic Neuropathy

Assessing Cardiovascular Autonomic Function

Tests of cardiovascular reflexes are sensitive, reproducible, simple, and noninvasive; they allow extensive evaluation of diabetic cardiovascular autonomic neuropathy and are regarded as the gold standard for clinical autonomic testing. These include measurements of the resting heart rate, beat-to-beat heart rate variation, blood pressure response to the Valsalva maneuver, changes in heart rate and systolic blood pressure in response to sustained exercise, and the QT interval. An increased resting heart rate and loss of heart rate variation in response to deep breathing are primary indicators of parasympathetic dysfunction. Tests for sympathetic dysfunction include measurements of changes in heart rate and blood pressure in response to standing, exercise, and handgrip. Heart rate variability (HRV) can be evaluated in the time and frequency domain derived from ECG recordings, ideally under paced breathing. Reduced HRV is the earliest clinical indicator of cardiac autonomic neuropathy and a strong independent predictor of mortality after acute myocardial infarction. A 24 h recording of HRV can reveal abnormal circadian rhythms regulated by sympathovagal activity. In vagal dysfunction, the high-frequency (HF) component of HRV is reduced; in sympathetic dysfunction, the low-frequency (LF) and very low-frequency components are reduced. Furthermore, in advanced cardiac autonomic neuropathy, all three components are reduced, as is the LF:HF ratio, which represents sympathovagal balance. Recently it has been recognized that the frequency of oscillations may yield important objective measures even with normal traditional changes in HRV. A reduction in the total spectral power and/or a reduction in the root mean square of the difference of successive RR intervals (rmsSD) is a measure of both sympathetic and parasympathetic activity while the standard deviation of normal RR intervals (SDNN) reflects predominantly parasympathetic nervous system function.

Both time and frequency analyses of HRV should be assessed during deep breathing, the Valsalva maneuver, and standing. They may be used to diagnose the cause, for example, of gastroparesis or erectile dysfunction, to give an incentive for intensive glycemic control, to quantitate the risk of macrovascular events and guide the use of a more conservative approach to medical management, and to monitor the response to therapy. Abnormalities in two or more of these tests suggest a diagnosis of autonomic neuropathy. Sympathetic/parasympathetic imbalance may produce significant symptoms and even herald the development of autonomic neuropathy and its consequences. The sympathetic innervation of the heart can also be visualized and quantified by single-photon emission computed tomography with metaiodobenzylguanidine in a research setting.

Thermoregulatory control of sweating occurs via sympathetic C fibers of the autonomic nervous system. Sweating disturbances are prevalent very early in diabetes and its precursors. Quantification of sweating using a sudomotor function test may be useful as an index of diagnosis of somatic and, probably, autonomic dysfunction.

The American Diabetes Association (ADA) and the American Association of Clinical Endocrinologists now recommend that patients with type 1 diabetes be screened for signs and symptoms of cardiac autonomic neuropathy 5 years after diagnosis and yearly thereafter and that patients with type 2 diabetes be tested at diagnosis and yearly thereafter.

Assessing Gastrointestinal Autonomic Function

The finding of retained food in the stomach after an 8–12 h fast in the absence of obstruction is diagnostic of gastroparesis. Basic diagnostic tests include upper gastrointestinal (GI) endoscopy or barium series to rule out structural or mucosal abnormalities of the GI tract. Gastric emptying can be visualized by scintigraphic imaging after the patient consumes radionuclide-labeled food, but the scintigraphic results do not always correlate with the severity of the symptoms. The blood glucose should be normal at the time of testing because hyperglycemia decreases gastric motility. Gastroduodenal manometry may be helpful in patients with symptoms but apparently normal emptying because it can help identify pylorospasm or incoordinate gastric and duodenal motility. Tachygastria and bradygastria, abnormal frequencies and propagation of gastric myoelectric activity, result in abnormalities in gastric emptying and can produce symptoms traditionally associated with gastroparesis.

Before attributing constipation to diabetic autonomic neuropathy, the clinician should rule out other causes of constipation such as hypothyroidism, side effects of drugs such as amitriptyline or calcium channel blockers, and colonic carcinoma. All patients should have a careful digital rectal examination and women should have a bimanual pelvic examination. Three stool specimens should be tested for occult blood. Anorectal manometry may be used to assess the rectal anal inhibitory reflex, which can distinguish rectosigmoid dysfunction and outlet-obstructive symptoms from colonic hypomotility.

Assessing Genitourinary Autonomic Function

A thorough workup for erectile dysfunction in men should include a medical and sexual history, physical and psychological evaluations, blood tests for testosterone, prolactin, and thyroid hormones, a test for nocturnal erections, tests to assess penile, pelvic, and spinal nerve functions, and tests to assess penile blood supply and blood pressure. Physical examination must include an evaluation of the autonomic nervous system, vascular supply, and hypothalamic–pituitary–gonadal axis. Autonomic neuropathy that causes erectile dysfunction is usually accompanied by loss of the HRV, ankle jerk reflex, and absent or reduced vibration sense over the large toes. To determine the integrity of sacral parasympathetic divisions, the physician should assess perianal sensation, sphincter tone, and the bulbocavernous reflex. Stenosis of the internal pudental artery is another potential cause of impotence. A penile/brachial index of less than 0.7 indicates diminished blood supply. A venous leak manifests as unresponsiveness to vasodilators and must be evaluated by penile Doppler sonography. In simple terms, unresponsiveness to intracavernosal injection of a direct vasodilator means arterial or venous insufficiency and requires inflatable devices, prostheses, or vascular reconstruction. A response to the vasodilator indicates autonomic insufficiency or a psychogenic cause that can be further defined using the HRV.

Treatment of Autonomic Neuropathy

Intensive glycemic control is critical to prevent the onset of diabetic autonomic neuropathy and slow its progression. The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) provided extensive clinical evidence that good metabolic control reduces diabetic complications. Specifically, DCCT/EDIC showed that intensive glycemic control reduced cardiovascular autonomic neuropathy by 45% in individuals with type 1 diabetes, and the incidence and prevalence of CAN remained lower in the former intensively treated group despite similar levels of glycemic control at 13–14 years of follow-up. The Steno Memorial Study emphasized a multifactorial approach including blood pressure, lipid, and glucose control and the use of vitamins and antioxidants and the researchers were able to reduce the likelihood of development of autonomic neuropathy by 68%. Early identification of cardiac autonomic neuropathy permits the timely initiation of therapy with the antioxidant α-lipoic acid, which appears to slow or reverse the progression of neuropathies in some studies. The use of cardioselective (e.g., atenolol) or lipophilic (e.g., propranolol) beta-blockers may also modulate the effects of autonomic dysfunction (**Table 2**). By opposing the sympathetic stimulus, they may restore the

Table 2 Diagnosis and management of autonomic dysfunction

Symptoms	*Assessment modalities*	*Management*
Resting tachycardia, exercise intolerance, early fatigue and weakness with exercise	HRV, respiratory HRV, MUGA thallium scan, 1231 MIBG scan	Graded supervised exercise, beta blockers, ACE-inhibitors
Postural hypotension, lightheadedness, weakness, fatigue, syncope, tachycardia/ bradycardia Hyperhidrosis	HRV, blood pressure measurement lying and standing Sympathetic/ parasympathetic balance	Mechanical measures, clonidine, midodrine, octreotide, erythropoietin, pyridostigmine Clonidine, amitryptyline, trihexphenidyle, propantheline, or scopolamine, botox, glycopyrrolate

ACE, angiotensin–converting enzyme; HRV, heart rate variability, MIBG, metaiodobenzylguanidine; MUGA, multi-gated acquisition.
Reproduced from vinik *et al.*[66].

parasympathetic–sympathetic balance. Studies using graded exercise, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, C-peptide, digoxin, and verapamil as a means to improve HRV have resulted in suggestive results in small populations in research studies. Cardiovascular evaluation should include macrovascular risk factors and their elimination.

Orthostasis is best treated by avoiding precipitating factors such as hot baths and drugs that accentuate the fall in blood pressure. Patients should stand slowly and useful drugs include clonidine, midodrine, and somatostatin. The use of fludrosterone and the liberalization of salt intake usually result in weight gain, edema and inadequate control of the orthostatic symptoms. Northera (droxidopa), a dopamine agonist, has just received approval by the FDA for the treatment of neurogenic orthostatic hypotension.

Neurovascular dysfunction requires the use of emollient creams, scrupulous attention to foot care, vasodilators, bisphosphonates and TNF-α antagonists for impending Charcot's neuroarthropathy.

Treatment of gastroparesis should stress improvement of glycemic control and correction of other metabolic abnormalities. It also includes dietary modification, gastric suction, and prokinetic agents such as metoclopramide, domperidone, erythromycin, Amitiza (lubiprostone) and Linzess (linaclotide). In some severe cases, jejunostomy may be needed to provide for nutrient intake and to allow the stomach to rest until such time that it recovers its function.

The severe and intermittent nature of diabetic diarrhea makes treatment difficult. Because afferent denervation may contribute to the problem, a bowel program that includes restriction of soluble fiber and regular effort to move the bowels is indicated. In addition, trials of lactose restriction or of a gluten-free diet, cholestyramine, clonidine, somatostatin analogue, pancreatic enzyme supplements, and antibiotics, such as metronidazole, may be indicated. Treatment of constipation should begin with an emphasis on good bowel habits, including regular exercise and maintenance of adequate hydration and fiber consumption. Sorbitol and lactulose may be helpful.

A grossly overdistended bladder should be drained by catheter to improve contractility and the patient should be instructed to void by the clock rather than waiting for the sensation of bladder distension. Cholinergic agents or clean intermittent self-catheterization may also be used to facilitate emptying.

Treatment of erectile dysfunction may include withdrawal from offending medications coupled with psychological counseling, medical treatment, or surgery. Medical treatment may include the 5′-phosphodiesterase inhibitors, such as sildenafil, tandalophil, or vardenofil. A lower dosage is needed for individuals with renal failure or liver dysfunction. It may, however, take several doses to achieve an effect in patients with diabetes. These drugs should not be taken by individuals with unstable ischemic heart disease or those using nitroglycerin or other nitrate-containing medications. Alternative treatments include suction devices with or without a constriction ring, injections of vasodilators into the corpus cavernosum, and ultimately prostheses.

Prospects for the Future

With improved understanding of the pathogenesis of autonomic neuropathy, agents that have the potential for treating the underlying biologic defect rather than purely symptomatic therapy are being investigated. The situation is much less gloomy than the 1994 Lancet editorial that stated, “all we can do is make the diagnosis and commiserate with the patient.”

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Foot Disease in Diabetes

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Glossary

Amputation Removal of an anatomic segment of the lower extremity, most often below the knee.
Diabetic foot Foot problems related to the metabolic and physiologic complications of diabetes mellitus.
Foot ulcer Full-thickness ulceration of the skin, usually at a site of high pressure in an insensate foot.
Neuropathy Damage to the sensory, motor, or autonomic pathways of the peripheral nerves of the foot.

Because of the metabolic and physiological complications of their disease, diabetic patients are prone to various foot disorders that cause substantial morbidity, the most dreaded of which is amputation.

Introduction

Foot lesions are a common, complex, and costly problem among patients with diabetes mellitus. Caring for diabetes-related foot wounds is often looked on as a thankless task, where much effort yields few rewards. However, increasing research in recent years has resulted in a better understanding of the pathophysiology and proper management of this problem. Diabetes-related peripheral neuropathy of the foot has emerged as the key factor leading to skin ulceration and its attendant complications. Pain insensitivity and motor-neuropathy-induced deformities are the principal disorders leading to skin ulceration. Other factors (e.g., autonomic nerve dysfunction, immunologic deficiencies, and metabolic perturbations) also contribute to the problem. Gaining more complete knowledge of the causes of these problems is a first step to reducing their consequences, but it will take a coordinated effort by interested providers or multidisciplinary teams to properly apply this knowledge. Data from the United States Department of Veterans Affairs Health System show that amputation rates are falling in all classes of patients *except* those with diabetes, emphasizing the need to improve efforts in this arena.

Epidemiology of Diabetic Foot Lesions

Incidence and Prevalence of Ulcers

Approximately 5%–10% of diabetic persons have had or currently have a foot ulceration. Foot problems are the major cause of hospitalization in patients with diabetes-related complications. One longitudinal study found that the incidence of ulceration over a 4-year period was 9.5%, with the risk increasing with the duration of diabetes. A study of mostly type 1 diabetic patients under age 50 in Sweden found that the prevalence of active ulcers was 2%, whereas 10% had a past history of ulceration. In an American investigation of a large health maintenance organization diabetic population, the cumulative incidence of ulceration was 5.8% over 3 years. In this study, 15% of patients with an ulcer required amputation.

Amputation Related to Foot Ulcers

Lower limb amputation can be life-saving in some patients and may improve the quality of life for those with recalcitrant or recurring foot ulcers. Improvements in prosthetic devices have enabled patients who require an amputation to enjoy a reasonable quality of life. In most instances, however, amputation is an outcome to be avoided. Diabetes is the commonest cause of nontraumatic lower limb amputation in the developed world. Persons with diabetes are at 15 to 46 times greater risk for lower extremity amputation than persons without diabetes. Foot wounds are the proximate cause of 90% of these nontraumatic amputations. In addition to the physical and emotional problems they cause, amputations have been associated with an increased mortality in the subsequent 3 years. By 2025, there will be more than 300 million persons with diabetes worldwide, emphasizing the urgency of this problem.

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Financial Cost

As with the clinical morbidity, the financial cost of treating diabetic foot ulcers is substantial. One database of approximately 7 million patients followed over a 2-year period revealed that the total direct cost for treated diabetic foot ulcers was $16 million or an average of $4595 per ulcer episode. Another study identified attributable costs for a 40- to 65-year-old male with a new foot ulcer as $27,987 for the 2 years after its occurrence. Studies of diabetes-related lower extremity amputation in Europe and the United States over the past decade have reported direct related costs ranging from $20,000 to $27,000. Indirect costs, such as loss of productivity and long-term care at skilled facilities or by family members, may double the total cost to society. The recent National Diabetes Statistics Report (2017) about the estimates of diabetes and its burden in the United States shows striking figures. Diabetes foot complications cost more than the five deadly forms of cancer. A total amount of 245 billion dollars has been spent in 2012 for diabetes including 15 billion dollars for diabetic foot health. An amputation cost on average over 70,000 dollars.

Pathways to Ulceration and Amputation

Risk Factors for Foot Problems

Many factors, especially poor foot care and inappropriate footwear, increase the risk of foot ulceration. Most studies have shown that foot lesions generally arise from a combination of three factors. Peripheral neuropathy (the insensate foot) is the most important and can be identified by the patient's inability to feel 10 g of pressure applied with a Semmes-Weinstein monofilament. Loss of sensation to the 10 g Semmes-Weinstein monofilament is a significant and independent predictor of future foot ulcer and lower-extremity amputation. The other two factors are foot deformity, largely related to motor neuropathy, and repetitive moderate pressure or stress. In a case-control model assessing 225 diabetic patients, Lavery and coworkers noted that the presence of neuropathy alone increased the risk of ulceration 1.7-fold, neuropathy and deformity increased the risk 12.1-fold, and neuropathy, deformity, and a history of previous ulcer or amputation increased the risk of developing another ulcer approximately 36-fold. The major precipitating cause of foot amputation is an ulceration that fails to heal. An additional contributing factor is the presence of arterial vasculopathy, most frequently involving the infrageniculate blood vessels. Accelerated atherosclerosis associated with diabetes can lead to critical foot ischemia that impairs wound healing and infection resolution.

The Role of Infection

All open wounds are colonized by microorganisms, but when the lesion is complicated by clinical signs and symptoms of infection (which occurs in perhaps half the cases), the likelihood of a poor outcome greatly increases. Diabetic foot ulcers with concomitant infection and ischemia are up to 90 times more likely to result in a high-level amputation than wounds without those two additional risk factors. Infection is usually caused by aerobic gram-positive cocci, particularly staphylococci; chronic wounds, especially those previously treated with antibiotics, may become infected with aerobic gram-negative rods and anaerobes. Deep infections, which often involve the underlying bone, can be both limb- and life-threatening. Diagnosing bone infection can be difficult and may require imaging procedures (preferably magnetic resonance studies) or bone biopsy.

Treating Foot Wounds

Once an ulcer develops, it is critical to get it healed as quickly as possible. This requires attention to local, systemic, and social or psychological issues. The wound must be carefully assessed for neurologic, vascular, and infectious complications. Some patients will need to be hospitalized, whereas others can be safely treated at home. Several wounds classifications have been developed to provide objective assessment of foot ulcer severity. The simple Wagner classification is commonly used: Wagner Grade 0, skin intact; Grade 1, superficial ulcer; Grade 2, ulcer extending to tendon, capsule or bone; Grade 3, deep ulcer with osteomyelitis or abcess; Grade 4, gangrene of toes or forefoot; Grade 5, gangrene of midfoot or hindfoot. The University of Texas Diabetic Wound Classification System has been validated as a predictor of serious outcomes in people with diabetes who have foot ulcers. Almost all wounds will require some debridement of necrotic material and callus; some may require further surgical intervention (e.g., incision and drainage, or bone resection). Infected wounds should be cultured (preferably by sending a sample of tissue, not a swab) and empiric antibiotic therapy should carefully be selected. If the limb is ischemic, a vascular evaluation may be needed. Appropriate dressings must be selected, based on the type of wound, and the patient or caregiver must be instructed on how to properly change them. Limb edema and dry skin should be treated. Any systemic metabolic problems (e.g., poor glycemic control, malnutrition) should be addressed. The patient should never walk out of the office in the same shoe that caused an ulceration; proper footwear and methods or devices to offload the involved site are crucial to healing the wound. Foot lesions present the provider with a teachable moment to instruct the patient on the causes of foot problems and how they may be prevented.

How Ulcers and Amputations Can Be Prevented

Intervention at any point in the critical pathway to ulceration may prevent the serious foot complications of diabetes. For instance, reducing foot pressure points with appropriate footwear or orthoses and modulating activity should help avoid ulceration. Treating infected wounds with appropriate (and not excessive) antimicrobial therapy should prevent deeper infections. Improving blood flow (e.g., with a distal bypass procedure) to a critically ischemic wound should lessen the likelihood of amputation. Other key preventative factors include the following: regular foot inspection by the patient and health care providers; for patients with risk factors for foot ulcerations, regular foot examination and care by primary care and podiatric providers; wearing appropriate shoe gear; and, in some instances, undergoing judicious surgical interventions to reduce deformity may be helpful. Lower extremity ulcerations and amputations in persons with diabetes take a terrible toll on the individual and on the world's health care systems, but the good news is that they are almost entirely preventable with appropriate care and education.

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Cardiovascular Disease in Diabetes☆

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Cardiovascular Burden of Diabetes

Diabetes mellitus (DM), a chronic metabolic condition characterized by hyperglycemia, represent a major risk factors for microvascular and macrovascular diseases (Goldenberg and Punthakee, 2013). Cardiovascular disease (CVD) is the most prevalent cause of morbidity and mortality in diabetic populations. Approximately 75% of patients with type 2 DM (T2DM) will die from CVD (Leiter and Fitchett, 2006; Klein and Gheorghiade, 2004; Emerging Risk Factors Collaboration *et al.*, 2011). The United Kingdom Prospective DM Study (UKPDS) showed a progressive increase in risk for nonfatal and fatal myocardial infarction, nonfatal and fatal stroke, amputation from peripheral vascular disease, and heart failure (HF) with increasing hemoglobin A1c (A1C) levels (Stratton *et al.*, 2000b). Atherosclerotic CVD remains the principal cause of disability and death among patients with DM. Type 2 DM is associated with a two- to fourfold increased risk for coronary artery disease (CAD) and a fourfold increase in mortality especially in women (Emerging Risk Factors Collaboration *et al.*, 2011; Abi Khalil *et al.*, 2012; Lloyd-Jones *et al.*, 2006). Diabetes mellitus poses the greatest life-time risk of any traditional cardiovascular (CV) risk factor for the development of CAD (Lloyd-Jones *et al.*, 2006). Myocardial infarction occurs 15 years earlier in patients with DM, with greater severity, and with more diffuse distribution than in those without (Booth *et al.*, 2006). Type 1 DM (T1DM) also markedly increases the risk of premature CVD; in patients aged <40 years the risk of CAD was >10-fold higher than that of the general population (Dorman *et al.*, 1984; Retnakaran and Zinman, 2008).

Although the relationship between A1C levels and microvascular outcomes is much stronger than that observed with risk of myocardial infarction (Stratton *et al.*, 2000b), close to 50% of patients with CAD have co-existing DM; CAD is frequently observed in patients at the time of diagnosis of T2DM, and even at the time of presentation of CVD, many with DM are undiagnosed (Hu *et al.*, 2002; Standl, 2015; Bartnik *et al.*, 2004; Gholap *et al.*, 2012). In CAD patients without known DM, a considerable proportion have evidence of either impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) or undiagnosed DM with the latter affecting 14%–22% of them (Bartnik *et al.*, 2004). Both undiagnosed T2DM and other disorders of glucose metabolism are risk factors for CVD. Diabetes mellitus is also a major risk factor for HF (Schocken *et al.*, 2008) and is found in 24%–40% of patients with HF (Dei Cas *et al.*, 2015). The presence of HF in DM patients is associated with a 3 year mortality of 40%, which is 10 times higher than individuals with DM and no HF (Cubbon *et al.*, 2013) (**Table 1**).

Mechanisms of Increased Cardiovascular Risk and Mortality in Diabetes

Both T1DM and T2DM increase the risk of CVD and associated mortality, largely because of increased atherosclerosis. Patients with DM have greater atherosclerotic plaque burden, higher atheroma volume, and smaller coronary artery lumen diameter than persons without DM (Nicholls *et al.*, 2008). Multiple cellular and molecular pathophysiologic factors participate in the diabetic atherosclerosis burden (Ross, 1993; Tabas *et al.*, 2015) (**Fig. 1**). Pathophysiology of CVD in DM is complex and is affected by the type of diabetes (T1DM and T2DM). Type 1 DM, generally of young onset, reflects largely a hyperglycemia-driven disease caused by insulin deficiency, whereas in T2DM, generally of adult onset, reflects a hyperglycemia-driven disease that is modified by

Table 1 Cardiovascular burden of diabetes

- Patients with diabetes mellitus have a reduced life expectancy largely secondary to CVD
- Diabetes mellitus increases risk of CVD two- to fourfold and mortality fourfold especially in women
- Diabetes mellitus poses the greatest life-time risk of any traditional CV risk factor and enlarge impact of other CV risk factors
- Diabetes mellitus increases the morbidity and mortality of all CVD including HF
- Diabetes mellitus is common in patients with CVD and is frequently undiagnosed even at the time of presentation of CVD

CVD, Cardiovascular disease; CV, Cardiovascular; HF, Heart failure.

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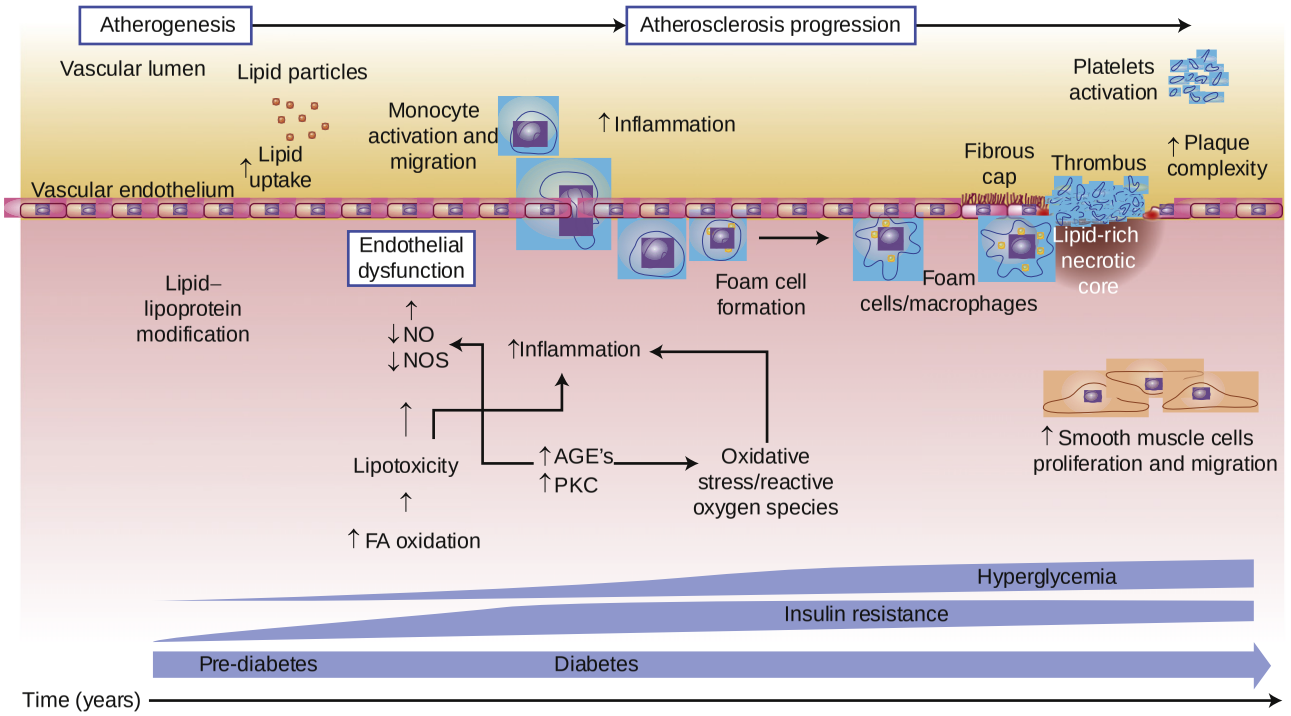

Fig. 1 Atherosclerosis development and progression in diabetes mellitus. Insulin resistance is present before the onset of prediabetes or diabetes mellitus, and increases progressively over time, whereas hyperglycemia develops in prediabetes and worsens with development of diabetes mellitus. Insulin resistance with impairment of insulin signaling and hyperglycemia contribute both to multiple processes of atherosclerosis. NO, Nitric oxide; NOS, Nitric oxide synthase; FA, Fatty acids; AGE, Advanced glycation end-product; PKC, Protein kinase C.

multiple other mechanisms including insulin resistance, abnormal fatty acid metabolism and CV co-morbidities leading to accelerated atherosclerosis (Armstrong *et al.*, 2013; Bornfeldt and Tabas, 2011; Ferreiro and Angiolillo, 2011; Catrina, 2014; Fiorentino *et al.*, 2013; Kayama *et al.*, 2015; Kozakova and Palombo, 2016; Laakso and Kuusisto, 2014; Loader *et al.*, 2015; Mapanga and Essop, 2016; Tkac, 2013; Zeadin *et al.*, 2013).

Hyperglycemia

Abundant epidemiological data support the association between hyperglycemia and increased CV risk (Gerstein *et al.*, 2005; Stratton *et al.*, 2000a). There is strong evidence demonstrating greater risk for CAD with increasing dysglycemia (Emerging Risk Factors Collaboration *et al.*, 2010, 2011; Coutinho *et al.*, 1999) with an estimated 11%–16% increase in CV events for every 1% increase in A1C (Stratton *et al.*, 2000b; Rodriguez *et al.*, 1999). In vitro studies and in vivo models in which hyperglycemia is induced in the absence of elevated lipids are consistent with a direct effect of hyperglycemia on endothelial dysfunction (Perkins *et al.*, 2015; Ceriello *et al.*, 2002), atherosclerotic lesion severity and complexity (Suzuki *et al.*, 2001) and plaque burden (Bornfeldt, 2014) (**Fig. 1**).

Hypoglycemia

Hypoglycemia, a consequence of therapy, also has deleterious CV effects mediated by multiple mechanisms (Dandona *et al.*, 2010; Bedenis *et al.*, 2014; Ziegler *et al.*, 2008; Stahn *et al.*, 2014; Sommerfield *et al.*, 2007; Odeh *et al.*, 1990; Desouza *et al.*, 2010; Chow and Heller, 2012; Sanon *et al.*, 2014; Yang *et al.*, 2016). Risk factors for hypoglycemia include age, cognitive impairment, use of insulin or insulin-secretagogues, renal impairment, presence of diabetic neuropathy and prior episodes of hypoglycemia (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee *et al.*, 2015). A higher annual rate of severe hypoglycemia in the Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease (ADVANCE) and Veterans Affairs Diabetes Trial (VADT) in the intensive vs. the standard treatment groups was reported (Bonds *et al.*, 2010; Advance Collaborative Group *et al.*, 2008; Duckworth *et al.*, 2009a) and was associated with >4-fold higher CV mortality in VADT and a >2.8% mortality rate per year in ACCORD. While specific mechanisms mediating adverse CV effects are unique to either hyperglycemia or hypoglycemia, there are both many similarities. They both impair endothelial function, augment thrombotic and platelet aggregation, compromise cardiac function and disrupt the renin-angiotensin-aldosterone and autonomic nervous systems. Thus, from a CV perspective, it should be emphasized to avoid acute hyper- and hypoglycemia on chronic vascular insults and this principle should be reflected in therapeutic choices (Goto *et al.*, 2013).

Insulin Resistance

Insulin resistance plays an important role in the pathophysiology of DM and CVD, and both genetic and environmental factors facilitate its development. Insulin resistance is a characteristic feature of T2DM but is also a consistent finding in patients with T1DM (Yki-Jarvinen and Koivisto, 1986; Cleland *et al.*, 2013). Epidemiological evidence strongly associates insulin resistance with CV risk in humans (Rodriguez *et al.*, 1999; Howard *et al.*, 1996; Ferrannini *et al.*, 2007; Wilson *et al.*, 1998). Insulin resistance is a general term meaning that insulin does not exert its normal effects in insulin-sensitive target tissues, such as skeletal muscle, adipose tissue, liver, heart and pancreas, the major target tissues for insulin action in glucose metabolism. Insulin resistance, measured using the clamp technique, is associated with asymptomatic atherosclerosis (Laakso *et al.*, 1991) and CAD (Bressler *et al.*, 1996) in individuals without DM. Fasting insulin levels, a marker of insulin resistance, have also been associated with CV events in nondiabetic individuals, independent of other risk factors (Laakso, 2001, 1996). Insulin resistance can promote atherogenesis and plaque progression via multiple mechanisms, including changes in insulin signaling pathways (Bornfeldt and Tabas, 2011), and through their effects on CV risk factors. Impairment of insulin signaling at multiple points in the insulin signaling pathway in endothelial cells (Muniyappa *et al.*, 2007; Potenza *et al.*, 2009), vascular smooth muscle cells (Wang *et al.*, 2004; Suzuki *et al.*, 2001), and macrophages (Bornfeldt, 2014) promotes development and progression of atherosclerosis, as does the pro-inflammatory state induced in insulin resistance (**Fig. 1**). Insulin resistance alone or combined with hyperglycemia promotes adverse changes in CV risk factors, including pro-atherogenic dyslipidemia and blood pressure elevation (Ferrannini *et al.*, 2007; Golden *et al.*, 2002; Eckel *et al.*, 2005), which contribute to the development, progression, and complexity of atherosclerosis.

Cardiovascular Risk Factors in Diabetes

CV risk factors such as obesity, hypertension and dyslipidemia are common in patients with T2DM, placing them at increased risk for the development of early CVD (**Table 2**). These result in metabolic changes which, coupled with a sedentary lifestyle and smoking, enhance the deleterious effects of hyperglycemia and accelerate atherosclerotic disease in the vasculature (**Fig. 2**). In the United Kingdom Prospective Diabetes Study (UKPDS), the major risk factors for myocardial infarction (MI) in patients with T2DM were high LDL cholesterol levels, low HDL cholesterol, elevated diastolic blood pressure, smoking, and A1C levels (Turner

Table 2 Common cardiovascular risk factors in diabetes

Cardiac risk factors
Age
Hypertension
Obesity
Insulin resistance
Dyslipidemia
Family history of premature CAD
Cigarette smoking
Sedentary lifestyle
Diabetes-specific risk factors
Duration of diabetes
Poor glycemic control
Presence of microvascular complications

CAD, Coronary artery disease.

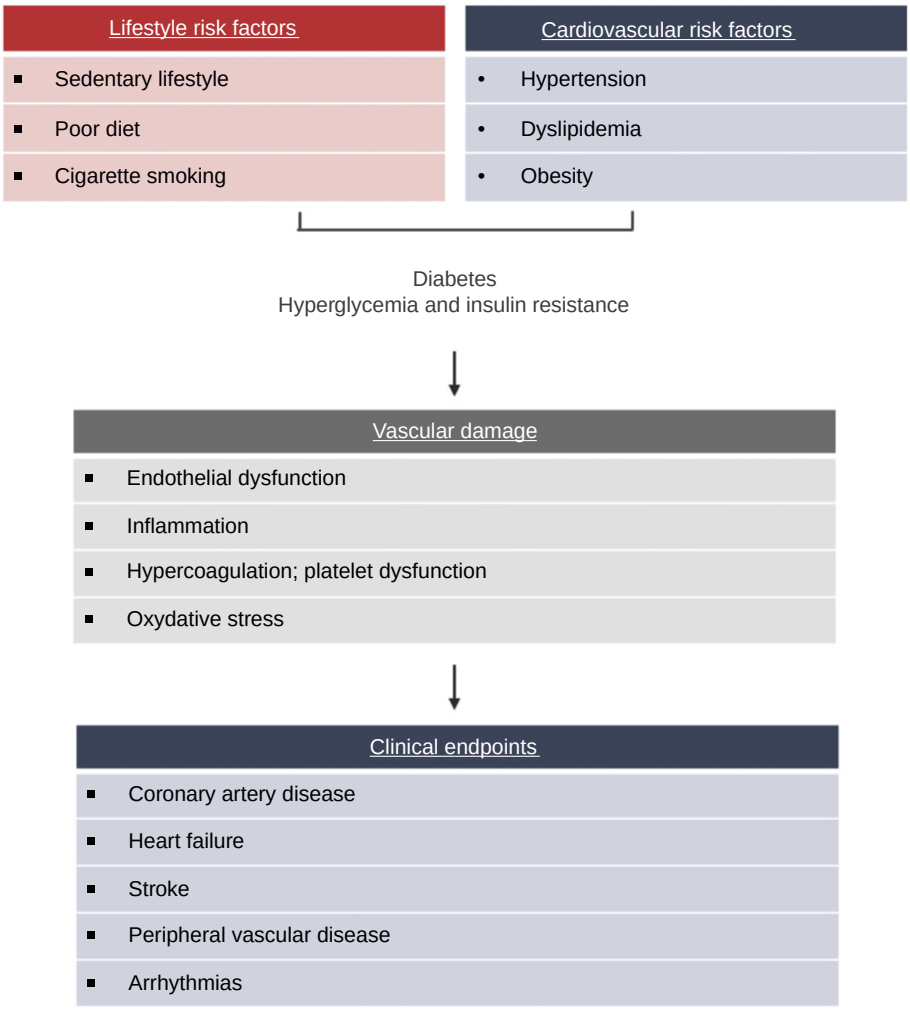

Fig. 2 Pathways of cardiovascular risk in diabetes.

et al., 1998). These factors were similar to findings from a 7-year follow-up study of 1059 patients with T2DM in Finland (Lehto *et al.*, 1997), and suggest that traditional CV risk factors are major predictors of CV events in patients with T2DM (**Table 3**).

DM and dyslipidemia commonly occur together, with lipid abnormalities affecting 60%–70% of T2DM (Parhofer, 2015), and hyperglycemia accelerates atheroma formation in the setting of diabetic dyslipidemia (Renard *et al.*, 2004). LDL cholesterol

Table 3 Association between cardiovascular risk factors and cardiovascular events in T1DM and T2DM

	T1DM	*T2DM*
Hypertension	+ + +	+ +
Cigarette smoking	+ +	+ +
Inflammation	+	+ +
High LDL-cholesterol	+	+ +
Low HDL-cholesterol	0, +	+ +
Triglycerides	No data	+ +
Microalbuminuria	+ + +	+ + +
Insulin resistance	+	+ + +
Poor glycemic control	+ +	+ + +

+, strong association; + + stronger association; + + +, strongest association.

T1DM, Type 1 diabetes mellitus; T2DM, Type 2 diabetes mellitus; LDL, Low-density lipoprotein; HDL, High-density lipoprotein.

Table 4 Characteristics of dyslipidemia in type 2 diabetes

- Elevated triglycerides
- Low HDL cholesterol
- Small dense LDL particles
- Higher concentrations of apolipoprotein B-containing particles

HDL, High-density lipoprotein; LDL, Low-density lipoprotein.

particles are more atherogenic in DM even in the absence of overt increased LDL concentrations (Carmena *et al.*, 2004), with small dense particles that are particularly prone to modification and oxidation (Rabbani *et al.*, 2011). Diabetic dyslipidemia is also characterized by elevated triglycerides, low HDL cholesterol, and higher concentrations of apolipoprotein B-containing particles (Taskinen, 2003; Kannel, 1985) (**Table 4**). Lipid changes are observe in insulin resistant individuals with normal glucose tolerance and in those with metabolic syndrome years before clinical diagnosis of T2DM (Lorenzo *et al.*, 2013), suggesting either co-associations of independent disorders or a pathophysiologic role for insulin resistance, rather than hyperglycemia, in the development of DM dyslipidemia. About two-thirds of patients with T2DM have elevated blood pressure (Ferrannini and Cushman, 2012). In addition to sympathetic nervous system overactivity, the effects of insulin resistance on the nitric oxide pathway, smooth muscle growth, sodium and fluid retention, and the effect of hyperglycemia on the renin–angiotensin–aldosterone system are plausible mechanisms that increase blood pressure in patients with T2DM (Ferrannini and Cushman, 2012). Obesity is a typical CV risk factor in individuals with T2DM. The effects of weight gain (define as a change in body mass index (BMI)) over the 6.5-year follow-up period on CV risk factors was investigated in 1168 participants in the Diabetes Control and Complications Trial (DCCT) (Purnell *et al.*, 2013). Excess weight gain (an increase in BMI of ≥ 4.39 kg/m^2 vs. a BMI increase < 4.39 kg/m^2) was associated with sustained increases in central obesity, insulin dose, dyslipidemia and systolic and diastolic blood pressure, as well as more extensive atherosclerosis (evaluated by carotid intima–media thickness) (Purnell *et al.*, 2013).

Targeting individual CV risk factors reduces CV risk in DM, but addressing multiple risk factors simultaneously may synergistically reduce CV event risk even further. This hypothesis is supported by the Steno-2 study, in which participant with T2DM and albuminuria were randomized to intensive vs. conventional control of glycemia, blood pressure, and lipids, and followed for a mean of 7.8 years (Gaede *et al.*, 2003). The trial showed a statistically and clinically significant 53% reduction for the primary composite CV event end point. CV risk differences between intensive and conventional therapy separated after 1 year. The number needed-to-treat was 5 over 7.8 years to achieve this magnitude of CV risk reduction. The long-term 21 year follow-up data from the Steno-2 study have demonstrated the sustainability of intensified multifactorial intervention strategy in T2DM and microalbuminuria, in terms of gained years of life and years free from incident CVD over conventional DM treatment (Gaede *et al.*, 2016). Metabolic changes specific to DM very likely contribute to the excess risk of CVD in patients with T2DM. A secondary analysis of the Bypass Angioplasty Revascularization Investigation in Type 2 Diabetes (BARI-2D) trial evaluating multiple interventions also supports concurrent risk factor control lowers total mortality and the composite of death, MI, and stroke in patients with T2DM and established coronary heart disease (Bittner *et al.*, 2015). While achievement of multiple treatment goals in DM care has improved over time, currently only 14.3% of US adults with T2DM are at recommended goals for A1C, blood pressure, and LDL cholesterol (Ali *et al.*, 2013).

Diabetes and Heart Failure

Risk for HF in DM increases 2.4-fold in men and fivefold in women in comparison with those without DM (Kannel and McGee, 1979). Conversely, DM is an important predictor of HF, independent of concomitant hypertension or CAD. Heart failure is the second most common manifestation of CVD after peripheral artery disease, as demonstrate in the largest cohort study of almost

1.9 million patients with T2DM followed for a median of 5.5 years (Shah *et al.*, 2015). Multiple factors, including age, ischemic heart disease, and peripheral artery disease, as well as DM-specific risk factors, such as poor glycemic control (higher A1C) and insulin resistance, have been associated with HF in patients with DM (Nichols *et al.*, 2001; Iribarren *et al.*, 2001). The pathogenesis of HF in DM is complex and multifactorial (**Fig. 3**). Although hyperglycemia and insulin resistance are the main physiological disturbances in DM, multiple mechanisms, such as derangements in cellular metabolism, function and structure, autonomic neuropathy, and neurohormonal dysregulation are believed to play a role in the associated HF (Chong *et al.*, 2017; Levelt *et al.*, 2016). Accelerated atherosclerosis of DM may be diffuse and severe. Consequently, ischemic cardiomyopathy is a major cause of HF in the DM population. Even in the absence of CAD, microvascular disease, characterized by arterial thickening and fibrosis, as well as endothelial and vasomotor dysfunction, can increase risk of HF in DM. Hypertension is another common comorbid condition in DM, causing left ventricular hypertrophy and contributing to the development of HF (Adler *et al.*, 2000). Furthermore, although DM is now accepted as an independent cause of HF, severity of DM-related cardiac abnormalities may be amplified by comorbid conditions which impact the CV system, ultimately increasing the risk for HF.

Assessment of Cardiovascular Risk in Diabetes

Compared to subjects without DM, patients with T1DM and T2DM (especially women) are at higher risk of developing CVD, at an earlier age. Furthermore, people with DM have a high prevalence of silent myocardial ischemia, and almost one-third of MI occur without recognized or typical symptoms (silent MI) (Cohn *et al.*, 2003). Silent ischemia is most likely to occur in individuals with DM who are older (mean age 65 years), and is more frequent in those with DM who also have microalbuminuria (Rutter *et al.*, 1999). People with DM and silent ischemia have an annual event rate for CAD of 6.2% (50% of events were new-onset angina and 50% were cardiac death or MI) (Rutter *et al.*, 2002). The 2013 Joint European Society guidelines on CVD prevention recommended that patients with DM, and at least one other CV risk factor or target organ damage, should be considered to be at very high risk and all other patients with DM to be at high risk (Authors/Task Force *et al.*, 2013).

The goals of screening for CVD in DM are to improve quality of life and life expectancy by preventing CV events and HF through early detection of significant CAD. There is some evidence that early screening and intervention in people with T2DM and with silent ischemia is beneficial and may improve long-term survival (Inoguchi *et al.*, 2000; Sorajja *et al.*, 2005). In the absence of data to the contrary, one approach to identifying CVD in peoples with T1DM is to apply the same CV risk assessment and diagnostic strategies used in T2DM or in the general population (Greenland *et al.*, 2010). Identification of asymptomatic CAD may allow the opportunity for more aggressive lifestyle or pharmacological interventions to prevent clinical events or, when disease is advanced, the pursuit of cardiac revascularization.

The most predictive clinical observation for CAD in patients with DM is a history of chest pain or discomfort, but these features will be absent in a significant number (20%–50%) of patients with DM (Zellweger *et al.*, 2004; Wackers *et al.*, 2004). Clinical

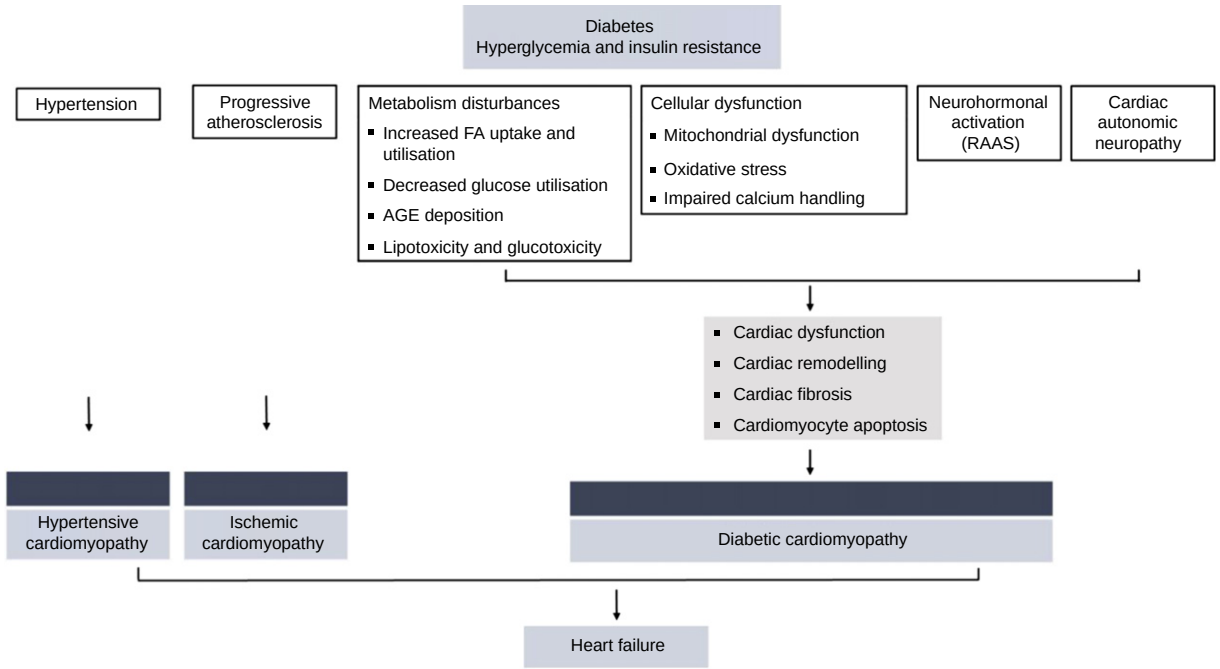

Fig. 3 Mechanisms of heart failure in diabetes. FA, Fatty acids; AGE, Advanced glycation end-products; RAAS, Renin angiotensin aldosterone system.

findings, such as resting electrocardiogram (ECG) abnormalities or multiple risk factors for atherosclerosis, may also suggest the presence of CAD. Successful CV risk prevention depends on a comprehensive detection and management of all modifiable CV risk factors. This includes evaluation of CV risk factors (e.g., lifestyle habits including smoking, hypertension and dyslipidemia), microvascular and macrovascular diseases, cardiac co-morbidities (e.g., HF and arrhythmias) and inducible myocardial ischemia by means of exercise testing, or cardiac imaging.

Cardiovascular Screening Tests

Although it is important to individualize clinical decision making, widespread screening for silent CAD in DM cannot be recommended at this time (Upchurch and Barrett, 2012). Screening for silent myocardial ischemia may be considered in selected high risk patient with DM (**Fig. 4**).

Resting Electrocardiogram

The presence of CV risk factors and resting ECG abnormalities identify patients with DM at increased risk of important CAD burden. Several prior studies have demonstrated that baseline ECG abnormalities are common in asymptomatic patients with DM and no history of CAD. In the UKPDS, 1 in 6 patients with newly diagnosed T2DM had evidence of silent myocardial ischemia on baseline ECG (Davis *et al.*, 2013). An ECG with ST-T abnormalities at rest has been shown to be most predictive for silent ischemia and was the only significant predictor of silent ischemia in women (Milan Study on Atherosclerosis and Diabetes (MiSAD) Group, 1997). The relevance of ST-T abnormalities as a predictive factor for silent ischemia emphasizes the importance of recording a resting ECG in most individuals with T2DM. An abnormal resting ECG may indicate the need for further investigations and result in the earlier detection and treatment/revascularization of CAD.

Exercise Stress Testing

Exercise stress testing is useful in patients at high risk of CAD for the assessment of prognosis and the identification of individuals who may benefit from coronary artery revascularization to improve long-term survival. An abnormal exercise

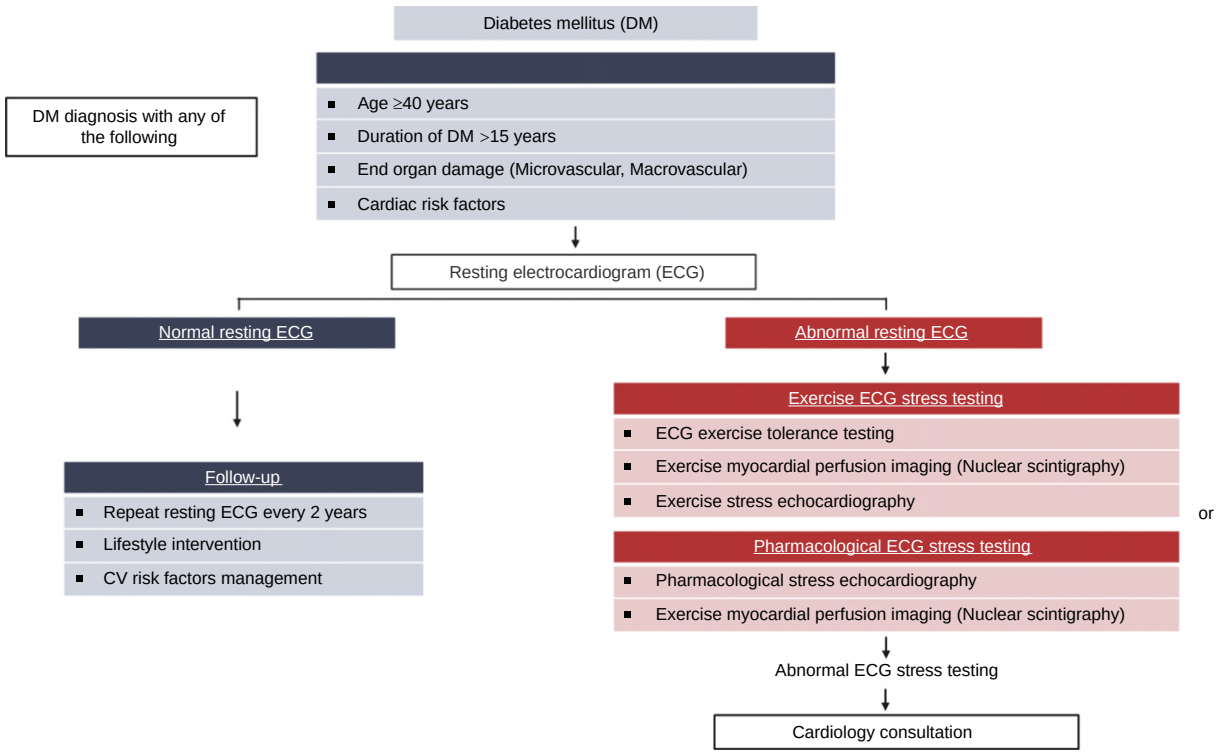

Fig. 4 Investigational algorithm and cardiovascular disease management in diabetes. DM, Diabetes mellitus; ECG, Electrocardiogram; CV, Cardiovascular.

ECG is associated with an annual CAD event rate of 2.1%, compared with 0.97% in subjects with normal exercise ECG (Faglia *et al.*, 2005). Myocardial ischemia (whether silent or symptomatic) detected during exercise stress testing in individuals with DM is associated with poorer long-term survival compared to individuals without DM (Inoguchi *et al.*, 2000). The strongest and most consistent prognostic marker identified during exercise ECG stress testing is the person's maximum exercise capacity (Zellweger *et al.*, 2004). Although exercise capacity is decreased in individuals with DM (Poirier *et al.*, 2000), it is still of prognostic importance.

The choice of initial stress test should be based on evaluation of the resting ECG, the individual's ability to exercise, and local expertise and technology. Screening tests include ECG exercise tolerance testing, exercise (or pharmacological) myocardial perfusion imaging (nuclear scintigraphy), and exercise (or pharmacological) stress echocardiography. The ankle-brachial index and coronary artery calcium scoring by computed tomography (CT) are used to detect evidence of atherosclerosis, although these methods cannot assess for active or inducible ischemia. Emerging techniques include CT angiography, cardiac magnetic resonance imaging, and cardiac positron emission tomography, but none have had wide spread application in asymptomatic patients. The utility of these newer CAD diagnostic modalities, is currently unknown in terms of guiding management decisions in patients with DM. The reliability of exercise testing, stress echocardiography, or myocardial scintigraphy is of a particular concern in the detection of ischemia in DM. Confounders are a high threshold for pain due to autonomic dysfunction, the multivessel nature of CAD, baseline ECG abnormalities, co-existence of peripheral artery disease and use of multiple medications.

Exercise capacity is frequently impaired in people with DM due to the high prevalence of obesity, sedentary lifestyle, peripheral neuropathy (both sensory and motor), and vascular disease in this population. Individuals who cannot adequately exercise on a stress test have a poorer prognosis than those who can, regardless of the reason for this incapacity (Zellweger *et al.*, 2004). Perfusion imaging also provides important prognostic information. Myocardial perfusion imaging has similar predictive value for cardiac death and nonfatal MI in individuals with DM as in those without DM (Shaw and Iskandrian, 2004). For those unable to perform an exercise ECG stress test, pharmacologic stress imaging, using dipyridamole, adenosine, or dobutamine testing, is required. Stress echocardiography and stress nuclear imaging have similar values for cardiac events in the general population (Schinkel *et al.*, 2004), but no comparative data are available for patients with DM.

Future large, randomized trials are needed to determine whether screening for subclinical CAD, particularly with newer modalities that may improve detection of functional CAD, can reduce CV event rates in patients with DM. Such studies would need to be adequately powered to assess the potential of additive impact of screening results and subsequent interventions on actual patient outcomes.

Establishing a Diagnosis of Diabetes in Cardiovascular Disease

Screening for DM using a fasting blood glucose and/or A1C is recommended every 3 years in individuals $\geq$40 years of age or at high risk for developing DM based on a DM risk score (Ekoe *et al.*, 2013; Robinson *et al.*, 2011; Abbasi *et al.*, 2012; Lindstrom and Tuomilehto, 2003). More frequent and/or earlier testing should be considered in those at very high risk or in people with additional risk factors for DM, including the presence of coronary, cerebrovascular and peripheral vascular disease (Robinson *et al.*, 2011; Ekoe *et al.*, 2013; Gyberg *et al.*, 2015). A 75 g Oral Glucose Tolerance Test (OGTT) should be strongly considered in patients with impaired fasting glucose or with A1C in the prediabetes range. Diagnosis in patients with CVD is especially important. A cross sectional survey of 4004 patients with stable CAD demonstrated that 29% had undetected DM and screening with an OGTT detected 96% of cases, while combining the fasting plasma glucose and A1C test identified 81% (Gyberg *et al.*, 2015; Norhammar *et al.*, 2002).

Fasting plasma glucose can be transiently elevated in acute coronary syndrome (ACS) and is unreliable for establishing a diagnosis in the first 2 days of an ACS (Hage *et al.*, 2010). While impaired glucose tolerance (IGT) is the predominant glucose abnormality in ACS (Bartnik *et al.*, 2004), an OGTT is rarely performed in that setting and may also be unreliable if done within 4–5 days of the event (Hage *et al.*, 2010). An in-hospital A1C test has been proposed as a practical method for screening patients with ACS. Those with A1C $\geq$6.5% should undergo a confirmatory measurement 4–8 weeks post-discharge, and those with A1C between 6.0% and 6.4% should have an OGTT 6–8 weeks post discharge (Gholap *et al.*, 2012).

Cardiovascular Risk Reduction in Diabetes

Targeting individual CV risk factors reduces CVD risk in DM, but addressing multiple risk factors concurrently may synergistically reduce CV event risk even further (**Table 5**). DM therapies should otherwise be selected on an individual basis, with special caution used when treating older patients or specific population. CV protective measures in patients with DM include health behavior interventions (diet, weight modification, increased physical activity, smoking cessation) and pharmacological therapies [anti-platelet agents, statins, angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs), blood glucose and blood pressure control]. A systematic approach to all CV protective measures has been proven to reduce the risk of CV events (Gaede *et al.*, 2008; Anselmino *et al.*, 2008).

Table 5 Cardiovascular risk reduction in diabetes

- Glucose control alone has not been clearly shown to reduce CV risk in diabetes mellitus
- Multiple risk factors contribute to CV risk in T1DM and T2DM
- Addressing multiple risk factors simultaneously may synergistically reduce CV event risk
- Effective CV risk management in diabetes mellitus requires lifestyle intervention, management of glucose control and CV risk factors

CV, Cardiovascular; T1DM, Type 1 diabetes mellitus; T2DM, Type 2 diabetes mellitus.

Lifestyle Management

A joint scientific statement from the ADA and EASD advocates lifestyle management (including healthy eating, physical activity and cessation of smoking) as a first measure for the prevention and/or management of T2DM, with targets of weight loss and reduction of CV risk (Inzucchi *et al.*, 2012). An intensive program including counseling about medical nutrition therapy, physical activity, and behavior changes, with ongoing support and frequent follow-up are needed for long term lifestyle management of body weight, CV risk factors and glycemia itself in DM. Weight loss, a healthy eating pattern, and increasing physical activity are also effective for reducing CV risk factors in individuals without DM. Lifestyle interventions should be recommended in all patients with DM, patients at risk for CVD, and patients with known CVD. The Look AHEAD trial is a large multicentre randomized clinical trial on the effects of long-term lifestyle intervention-associated weight loss on glycemia and prevention of CVD events in T2DM. One year results in the intensive lifestyle intervention group have showed an average 8.6% weight loss, a significant reduction in A1C, an improvement in fitness and a reduction in several CVD risk factors, benefits that were sustained after 4 years (Pi-Sunyer *et al.*, 2007; Look and Wing, 2010). Despite greater weight loss, reduction in A1C, and improvements in fitness and CV risk factors in the intervention group, there was no difference in the incident of CV events.

Weight Loss Bariatric Surgery

Most individuals with T2DM are obese, and weight control has been considered a central component of lifestyle intervention. Weight reduction or at least stabilization in overweight or moderately obese patients will still be an important component in a lifestyle programme and may have pleiotropic effects. In severely obese individuals, bariatric surgery causes long-term weight loss and reduces the rate of incident T2DM and mortality (Sjöström *et al.*, 2004). Bariatric surgery induces remission or improvement in T2DM in 40%–85% of patients (Sjöström *et al.*, 2004; Puzziferri *et al.*, 2014). It is not known whether the remission in T2DM seen after bariatric surgery will lead to a reduction in CV events.

Glucose Control

Myocardial infarction and stroke accounts for up to 80% of mortality in T2DM (Morrish *et al.*, 2001). Medications taken to manage hyperglycemia must not adversely impact CV risk factors or increase CVD, and ideally would reduce CV risk. However, many of the medications available to treat hyperglycemia in DM have no effect on CV risk or may have a theoretical concern for exacerbating CV risk factors or CVD itself. Although lowering glucose concentrations will ameliorate immediate symptoms of hyperglycemia (polyuria, polydipsia, and weight loss) and longer term microvascular complications of DM (Ross, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998a,b), there is less evidence for targeting A1C to lower CV risk (Nathan *et al.*, 2005; Holman *et al.*, 2008a). The reasons, of which there are several, include the presence of multiple co-morbidities in long-standing T2DM and the complex risk phenotype generated in the presence of insulin resistance.

Intensive glycemic control reduces relative risk of nonfatal myocardial infarction and coronary heart disease events by about 15%, although there is no effect of intensive glycemic control on all-cause mortality in meta-analysis of the multiple randomized clinical trials targeting different intensity of glycemic control (Ray *et al.*, 2009). Most studies are of relatively short duration, and long-term follow-up of both the Diabetes Control and Complications Trial (DCCT) (Nathan *et al.*, 2005) and United Kingdom Prospective Diabetes Study (UKPDS) (Holman *et al.*, 2008a) show reduced risk for CV events emerge over time in those with recently diagnosed T1DM and T2DM, respectively, who were previously randomized to intensive glycemic control. However, these effects take years to manifest and few studies extend observations over the extended time frame which may be necessary to provide benefit of glucose lowering per se.

Glucose Targets

An A1C target of 7.0% to reduce microvascular disease is a generally accepted level (The Diabetes Control and Complications Trial Research Group, 1993; Holman *et al.*, 2008a). The evidence for an A1C target in relation to macrovascular risk is less compelling, in part due to the complexities surrounding the chronic, progressive nature of DM and the effects of "metabolic memory."

Consensus indicates that an A1C of ≤7% should be targeted, this can be achieved through balanced assessment of efficacy, risk of hypoglycemia, effect on body weight, and costs of available glucose-lowering agents and their combination (Inzucchi *et al.*, 2015). Ideally, tight control should be instigated early in the course of the disorder in younger patients and without attendant co-morbidities. Fasting plasma glucose should be 7.2 mmol/L (120 mg/dL) and post-prandial 9–10 mmol/L (160–180 mg/dL) on an individualized basis (Inzucchi *et al.*, 2015). Successful glucose lowering therapy is assisted by self-monitoring of blood glucose, most notably in patients with insulin-treated DM.

This "universal" glycemic target (A1C value ≤7%), however, has been challenged by the results of subsequent intervention trials (Gerstein *et al.*, 2008; Advance Collaborative Group *et al.*, 2008; Duckworth *et al.*, 2009b). Given the critical analysis of the results of these trials, the American Diabetes Association (ADA) and the American Heart Association suggested that an individualized A1C target should be identified based on the duration of DM, life expectancy, presence and severity of DM complications, and propensity for hypoglycemia (Skyler *et al.*, 2009). This suggestion has been endorsed by several international guidelines. Although the concept of treatment individualization is appealing, how to pursue it in the clinical setting remains highly debated. On the basis of the patient's individual phenotypic and genetic profiles, precision medicine aims at predicting which patient is more likely to benefit and which one is more likely to experience side effects in response to therapeutic modalities (Lyssenko *et al.*, 2016). These advances open attractive opportunity to determine the utility of phenotypic and genetic profiles to predict treatment response. However, interpretation is not always clear and translation into clinical guidance remain uncertain and insufficiently investigated.

More stringent targets (e.g., A1C 6.0%–6.5%) might be considered in selected patients with short disease duration, long life expectancy and no significant CVD, if it can be achieved without hypoglycemia or other adverse effects. As discussed above, the accumulated results from T2DM CV trials suggest that not everyone benefits from aggressive glucose management. It follows that it is important to individualize treatment targets guidelines support the concept of targeting A1C and individualization of A1C goals in order to minimize microvascular and macrovascular complications (Imran *et al.*, 2013, American Diabetes Association, 2016, The Diabetes Control and Complications Trial Research Group, 1993, UK Prospective Diabetes Study (UKPDS) Group, 1998b, Advance Collaborative Group *et al.*, 2008). For the vast majority, the A1C goal is 7% or less (Imran *et al.*, 2013). In T2DM, this has been shown to reduce microvascular complications, and over time, macrovascular complications when implemented early in the disease course (UK Prospective Diabetes Study (UKPDS) Group, 1998b, Holman *et al.*, 2008b). For those who have had longer duration of T2DM, intensive glycemic control also reduces microvascular complications (Advance Collaborative Group *et al.*, 2008; Ismail-Beigi *et al.*, 2010) and the risk of nonfatal MI (Control Group *et al.*, 2009). However, mortality was slightly increased when targeting an aggressive A1C of 6% or less in high risk patients (Action to Control Cardiovascular Risk in Diabetes Study Group *et al.*, 2008; Duckworth *et al.*, 2009a). Therefore, for some patients a higher A1C target is more appropriate. This issue is particularly important in patients with existing CVD (Imran *et al.*, 2013).

Patients with a shorter life expectancy may not live long enough to procure the benefits of tight glycemic control and the frail patient will be at higher risk of hypoglycemia, less tolerant of many side effects and prone to the drawbacks of polypharmacy. In addition, some patients may be at very high risk of ischemic events secondary to the negative impact of hypoglycemia. Therefore, in these scenarios, a target A1C of 7.1%–8.5% is reasonable. However, a CV patient who does not have these limitations would still benefit from an A1C target of ≤7% (Advance Collaborative Group *et al.*, 2008; Ismail-Beigi *et al.*, 2010; Control Group *et al.*, 2009; Action to Control Cardiovascular Risk in Diabetes Study Group *et al.*, 2008; Duckworth *et al.*, 2009a; Hayward *et al.*, 2015).

Glucose Lowering Agents

In T1DM, the pharmacologic therapy is insulin due to complete insulin deficiency, and insulin is also used in the metabolically compromised patient with T2DM requiring urgent therapy. In stable T2DM, there are a number of noninsulin options that counteract the diverse mechanisms contributing to hyperglycemia (Harper *et al.*, 2013, 2015; Liu *et al.*, 2012) (**Fig. 5**).

Multiple factors must be considered in the selection of diabetes medications for patients with CVD. The strong data supporting the effect of glucose-lowering to reduce microvascular disease which has a direct impact on CV risk and eventual long-term reduction in CVD over 20 years (from the UKPDS follow-up study) must be balanced against potential acute effects of hypoglycemic episodes, increased risk of HF (thiazolidinediones and potentially some DPP-4 inhibitors) and weight gain (sulfonylureas, insulin), other side effects, and cost. Excluding contraindications, metformin is the traditional first choice followed by add-on agents selected on the basis of key patient characteristics and preferences despite low evidence for CV protection (Harper *et al.*, 2015), among which documentation of CV safety (or superiority) and a low risk of hypoglycemia should be weighted. To date, there are no convincing data to suggest that any single type of antihyperglycemic therapy in T2DM has a CV advantage over another other than perhaps metformin (Joffe *et al.*, 2010). There are limited data on the comparative effectiveness of the many other effective antihyperglycemic drugs; most studies are of short duration and focus on glycemic lowering and side effects rather than CV outcomes.

A number of new approaches for glucose lowering are under development and may provide agents with dual benefit for DM and the diabetic heart. Additionally, drugs that are developed for CV risk reduction may also lower glycemia. In the meantime, CV outcome trials will provide useful information on CV safety of use of DM drugs in patients with established heart disease or at high risk for CV events and additional exposure information that may reveal previously unrecognized positive or negative effects. These trials may help to better address the question of the impact of specific glucose-lowering drugs and pharmacologic class agents on the diabetic heart.

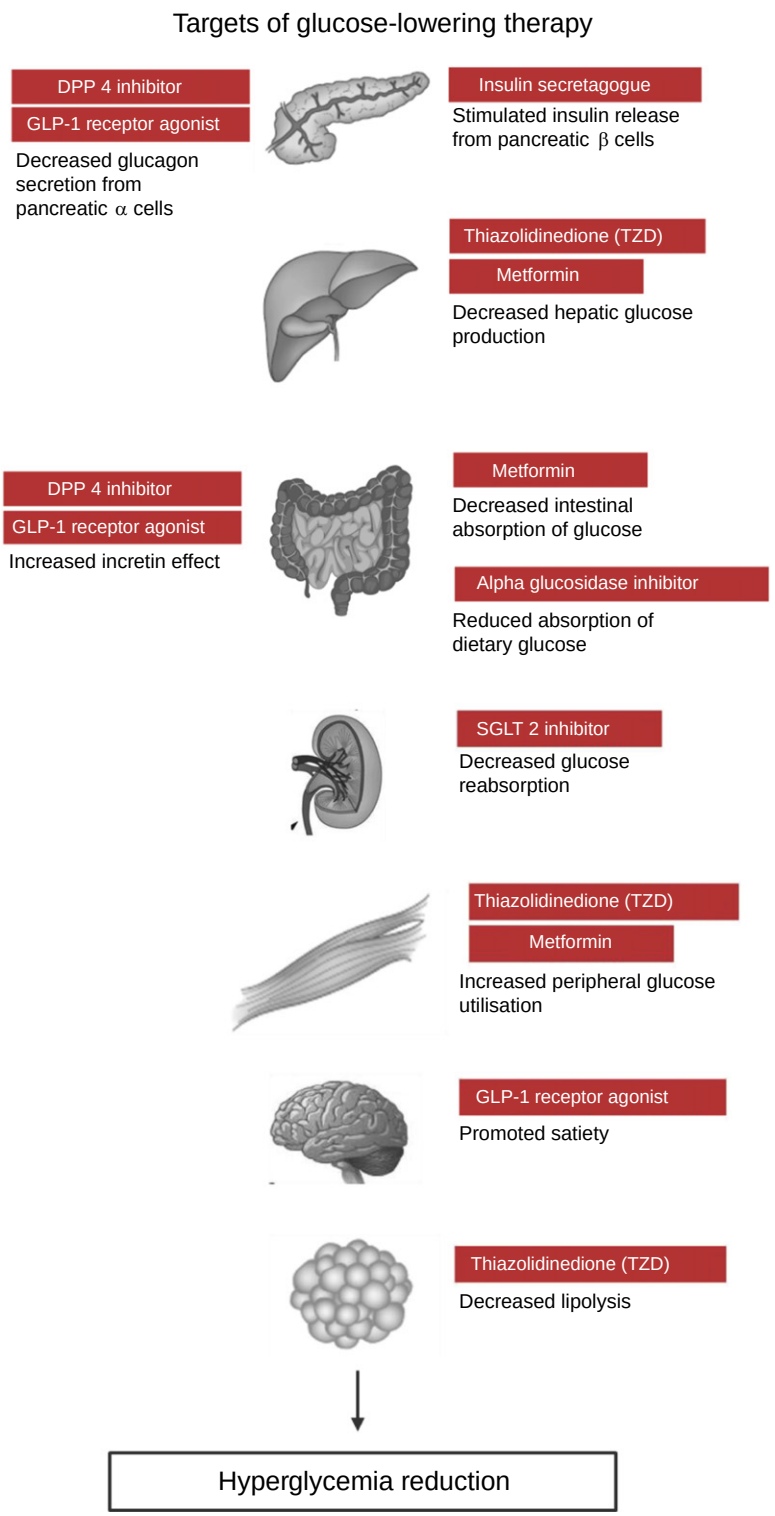

Fig. 5 Mechanism of action of antihyperglycemic medication classes. DPP-4, Dipeptidyl peptidase 4; GLP-1, Glucagon-like peptide 1; SGLT2, Sodium-glucose cotransporter 2.

CV Safety of Glucose Lowering Agents

CV safety has been demonstrated for insulin glargine (Origin Trial Investigators *et al.*, 2012; Marso *et al.*, 2017), thiazolidinediones (TZDs) (rosiglitazone, Home *et al.*, 2009), pioglitazone (Dormandy *et al.*, 2005; Kernan *et al.*, 2016), DPP-4 inhibitors (alogliptin,

White *et al.*, 2013), saxagliptin (Scirica *et al.*, 2013), sitaglipitin (Green *et al.*, 2015) and GLP-1 receptor agonists (lixisenatide, Pfeffer *et al.*, 2015). There was an unexpected finding of increased hospitalization for HF noted with saxagliptin that was not seen with the other CV trials of DPP-4 inhibitors and the explanation remains elusive (Scirica *et al.*, 2013). Moreover, based on diverse and large registries of clinical use, the class as a whole appears to be quite safe regarding HF (Filion *et al.*, 2016). The SGLT2 inhibitor CV outcome trial using empagliflozin (Zinman *et al.*, 2015; Neal *et al.*, 2017) demonstrated superiority with reduction in CV death, hospitalization for HF and all-cause mortality compared to placebo (Zinman *et al.*, 2015). Mechanisms of benefit remains speculative (DeFronzo, 2016; Kalra, 2015) but the Canadian Diabetes Association clinical practice guidelines committee has recommended the use of an SGLT2 inhibitor with proven CV benefit, in patients with T2DM and clinical CVD on top of existing antihyperglycemic therapy (Harper *et al.*, 2015; Goldenberg *et al.*, 2016). Also, the recently published results from the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial and the Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes (SUSTAIN-6) show not only CV safety but also CV superiority (Marso *et al.*, 2013, 2016).

Special Circumstances

Several complicated circumstances will often require a multidisciplinary team approach to ensure optimal management that can be enhanced (Leiter and Fitchett, 2006; Tardif *et al.*, 2013). These include management of women with CVD and DM in the peripregnancy period, hyperglycemia management in the setting of ACS, and management during CV procedures such as diagnostic catheterization or cardiac surgery including coronary artery bypass grafting.

The Peripregnancy Period

In addition to macrosomia, observational studies show an increased risk of congenital heart disease and other DM-related embryopathies that increase directly with elevations in A1C. Therefore, glucose control must be optimized before conception so that it is optimal in the first 12 weeks of pregnancy. All potentially teratogenic CV medications (e.g. ACE inhibitors, statins) should be avoided (American Diabetes Association, 2015) as should antihyperglycemic agents (DPP-4 inhibitors, TZDs, GLP-1 RAs, SGLT-2 inhibitors, alpha-glucosidase inhibitors) for which safety during pregnancy has not been established. Additionally, patients planning to conceive should have any indicated investigations completed well in advance. Because most women of conceiving age who have DM have T1DM, the management is generally performed in high risk pregnancy clinics and the focus is on insulin optimization. In the minority of women with T2DM who are not post-menopausal, metformin and sulfonylureas have been shown to be safe in terms of birth defects, but may not provide as good glycemic control and have been associated with higher perinatal mortality perhaps because of that. This is why insulin is always the therapy of choice. If warranted, lipid management with resins can be considered and BP control can be achieved with methyldopa, diltiazem, labetolol, clonidine, or prazosin. If angina is present, beta-blockers can be used cautiously.

Management of Patients With Diabetes Mellitus and/or Hyperglycemia During ACS

Approximately 15%–35% of patients admitted with an ACS have known DM; an additional 15%–25% have dysglycemia (Tardif *et al.*, 2013; Deedwania *et al.*, 2008). Elevated plasma glucose during an ACS is associated with a more serious prognosis in patients with DM than without, including a >2-fold increased incidence of short- and long-term recurrent ischemic events, HF, cardiogenic shock, and death (Klein and Gheorghiade, 2004; Tardif *et al.*, 2013; Deedwania *et al.*, 2008). The worse outcome in ACS in DM patients is independent of other known prognostic risk factors (Leiter and Fitchett, 2006; Klein and Gheorghiade, 2004; Deedwania *et al.*, 2008). Hyperglycemia may relate to previously undetected glucose perturbations, but also to stress-induced catecholamine release increasing free fatty acid concentrations, decreased insulin production and increasing insulin resistance and glycogenolysis (Opie, 2008), with a negative impact on cardiac metabolism and function.

The possible benefits of glucose management have been studied in ACS in randomized controlled trials Diabetes and Insulin–Glucose Infusion in Acute Myocardial Infarction (DIGAMI) 1 and 2 and Hyperglycemia: Intensive Insulin Infusion in Infarction (HI-5). The Diabetes Mellitus, Insulin-Glucose infusion in Acute Myocardial Infarction (DIGAMI 1) trial (Malmberg *et al.*, 1995, 1996) randomized patients with an acute MI and a mean glucose of 15.6 mmol/L and A1C 8.1% at entry to treatment with IV insulin followed by 3 months of intensive insulin therapy or standard treatment. Intensive treatment had a significantly lower 1 (8.6% vs. 18%) and 3 (33% vs. 44%) year mortality (Malmberg *et al.*, 1999; Amsterdam *et al.*, 2014). But these results were not confirmed in the subsequent DIGAMI 2 (Malmberg *et al.*, 2005) which compared 3 methods of achieving glucose control: IV insulin infusion followed by long-term subcutaneous insulin, IV insulin then standard treatment and a third group treated by local standards. The overall mortality was 18.4% and there was no difference among groups. The most plausible reason for this discrepancy is that, in DIGAMI 1, admission A1C decreased more (1.5%), from a higher level (9.1%), compared with 0.5% from 8.3% in DIGAMI 2. In addition, the use of beta-blockers, statins and revascularization was more extensive in DIGAMI 2. The difference in glucose levels between the control and insulin groups in the HI-5 study was small and there was no reduction in

mortality among patients treated with insulin (Cheung *et al.*, 2006). Pooled data from the three studies confirmed that insulin–glucose infusion did not reduce mortality in the absence of glucose control in patients with ACS and DM (Zhao *et al.*, 2010). Finally, the BIOMarker Study to Identify the Acute Risk of a Coronary Syndrome-2 (BIOMArCS-2) trial (de Mulder *et al.*, 2013) compared very tight control (target 4.7–6.1 mmol/L) with standard management in patients with ACS. Glucose levels were lower in the intensive arm but averaged <10 mmol/L in both groups. There was no difference in the primary endpoint of infarct size but the intensively treated group had more hypoglycemia and a higher incidence of death or a second infarction before discharge. In summary, regarding patients with ACS, the evidence supports avoidance of hypoglycemia and achieving glucose levels <10 mmol/L during admission. This is more important than the agents used to achieve this level of glucose control. Longer term A1C goals should adhere to the principles already outlined for patients with CVD.

Management of Patients With Diabetes During Cardiac Procedures

Although hypoglycemic medications may influence the safety of coronary angiography, as well as early and late outcomes of revascularization with percutaneous coronary intervention or coronary artery bypass grafting, few trials have addressed interactions with myocardial revascularization in DM.

Percutaneous Coronary Intervention

Metformin is the most commonly used oral antihyperglycemic medication; concerns about metformin-associated lactic acidosis have previously led to the practice of metformin discontinuation prior to diagnostic coronary angiography and percutaneous coronary intervention (Baerlocher *et al.*, 2013; Benko *et al.*, 2007). However, more recent randomized trials and observational studies have not identified cases of nonfatal or fatal lactic acidosis (Maznyczka *et al.*, 2012; Goergen *et al.*, 2010). Most guidelines have now relaxed their recommendations around the holding of metformin, ranging from 24 to 48 h prior to the day of the procedure if renal dysfunction is present, to no stopping at all (Benko *et al.*, 2007; Miller and Richman, 2016). And when stopped, metformin is generally restarted ≥ 48 h after contrast administration if renal function has not deteriorated (Baerlocher *et al.*, 2013, Benko *et al.*, 2007). Rather than stopping metformin treatment in all patients, a reasonable approach is to carefully monitor renal function after the procedure and to withhold metformin for 48 h if it deteriorates and until renal function has resumed its previous level. Observational data reported concern over the use of sulphonylureas in patients treated with primary percutaneous coronary intervention for ACS: this has not been confirmed by a post hoc analysis of the DIGAMI 2 trial, although the number of patients undergoing primary percutaneous coronary intervention in this trial was low (5%) (Mellbin *et al.*, 2008). Arrhythmias and ischemic complications were reported less frequent in patients receiving gliclazide/glimepiride (Zeller *et al.*, 2010). Thiazolidinediones might be associated with lower re-stenosis rates after percutaneous coronary intervention with bare metal stents (Takagi *et al.*, 2009), but carry an increased risk of HF. No trial has demonstrated that the administration of insulin or insulin infusion improves percutaneous coronary intervention outcome after STEMI.

Cardiac Surgery

Diabetes mellitus is also common in patients undergoing cardiac surgery and is associated with increased rates of early and late mortality as well as renal failure, deep sternal infections and stroke (Herlitz *et al.*, 1996). In surgical studies, a dose-response relationship has been reported with each 1.1 mmol/L increase in glucose >5.5 mmol/L associated with a 34% increased risk of complications (Gandhi *et al.*, 2007). The Portland Diabetic Project demonstrated that using IV insulin infusions rather than subcutaneous sliding scale resulted in decreased mortality, reduced infections and shorter hospital stay (Furnary *et al.*, 1999). Lazar *et al.* in a randomized trial found that maintaining glucose levels in the 6.7–9.9 mmol/L range resulted in fewer infections and in-hospital cardiac complications as well as less recurrent ischemia and a significantly higher 5-year survival (Lazar *et al.*, 2004). However, studies have also shown that extremely tight glucose control is not beneficial in cardiac surgery patients (Lazar *et al.*, 2011; Umpierrez *et al.*, 2015). The current consensus is that moderate glucose control, generally assumed to mean target glucose levels in the 7.8–10 mmol/L range, provides the best outcomes.

Conclusions

The long-term treatment of DM is challenging because of diverse goals: to address metabolic derangements and to reduce risks for both microvascular and macrovascular adverse outcomes. Diabetes mellitus clearly exacerbates mechanisms of atherosclerosis and HF. Unfortunately, these mechanisms are not adequately fully modulated or addressed by focusing solely on optimal glycemic control. Aggressive global management of CV risk factors, along with glycemic management, provides substantial improvements in CV outcomes.

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Treatment: Alpha Glucosidase Inhibitors

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Carbohydrate Digestion and Glucosidases

Carbohydrate intake from whole grains, vegetables, fruits, bread, rice, and legumes is an essential part of nutrition therapy in diabetes and should provide 40%–50% of the patient's daily energy consumption in diabetes therapy (American Diabetes Association, 2016). Carbohydrates are digested by enzymes of the brush border of the small intestine with alpha glucosidases playing a key role for the release of monosaccharides which are then absorbed by complex cellular transport mechanisms. Thus, alpha glucosidase enzyme activity regulates availability of glucose for intestinal absorption as well as speed and extent of postprandial hyperglycemia (Puls, 1996). Alpha glucosidase inhibitors (AGIs) reversibly reduce or abolish the hydrolytic activity of alpha glucosidases. These inhibitors were developed in the 1980s when the importance of postprandial hyperglycemia for better diabetes control and cardiovascular risk, respectively, was realized. Postprandial hyperglycemia accounts for about 70% of the daily blood glucose load. Using continuous glucose measurement (CGMS) of diurnal glucose profiles, it could be shown that the relative contribution of postprandial hyperglycemia to HbA1c in patients with type 2 diabetes is high and varies depending on the quality of diabetes control between 30% and up to 70% in well controlled individuals (Monnier *et al.*, 2003). CGMS measurements furthermore revealed that uncontrolled postprandial hyperglycemia is a strong predictor of chronic progression of diabetes (Monnier *et al.*, 2007). Moreover, postprandial hyperglycemia was identified as an independent risk factor of cardiovascular diseases in individuals with prediabetes and early diabetes (Hanefeld *et al.*, 1996; Temelkova-Kurktschiev *et al.*, 2000; Qiao *et al.*, 2002). The disappointing cardiovascular outcome results of the University Group Diabetes Program (UGDP) evaluating efficacy of intensified glucose control with biguanide (phenoformin), sulfonylurea (tolbutamide), and insulin in type 2 diabetes (Goldner *et al.*, 1971) were another reason for the development of an alternative therapeutic approach. Thus, from the viewpoint of the pathophysiology of glucose homeostasis and with a key role of postprandial hyperglycemia for diabetes control, AGIs were the first oral antidiabetic drugs to act via a therapeutic modification of carbohydrate digestion and gastrointestinal transport on nutrients with pleiotropic effects on insulin resistance and the metabolic syndrome.

Clinical Pharmacology of Alpha Glucosidase Inhibitors

Fig. 1 shows the molecular structures of the three AGIs in therapeutic use: acarbose, miglitol, and voglibose, while **Figs. 2** and **3** show their mechanism of action.

Acarbose was the first AGI with now >25 years in clinical use, and has the most data on mode of action, therapeutic benefits, and cardiovascular outcome trials. It is a pseudo tetrasaccharide and has a 10^4–10^5 higher affinity to alpha glucosidases of the brush border than oligosaccharides. Acarbose is derived from bacterial cultures of *Actinoplanes utahensis*, thus representing a highly purified, standardized natural product. It is a potent reversible inhibitor of glucoamylase, sucrose, maltase, and isomaltase with moderate effect on amylase. Under adequate dosing it delays carbohydrate digestion to lower parts of the small intestine but causes no malabsorption (Truscheit *et al.*, 1988). After administration as a tablet with the first bite of a meal, acarbose is rapidly bound to the microvilli of the brush border with the consequence of an antihyperglycemic effect and reduced postprandial glucose excursion resulting in lower demand on insulin secretion. It is excreted to >95% as degradation products, ~51% of these via the feces and 30%–40% renally (**Table 1**). Only <2% are absorbed as unchanged drug and excreted renally (no systemic metabolization). Voglibose is also a nonsystemic AGI and is mainly used in Japan. After oral administration, it is nearly completely excreted unchanged in the feces (Maeshiba *et al.*, 1991) (**Table 1**).

Miglitol by contrast is synthesized by biotechnological and chemical procedures. >70% is absorbed after oral administration; >95% of the absorbed compound is excreted unchanged by the kidneys (**Table 1**). Miglitol and voglibose have no effect on amylase but specifically act on disaccharides.

Effects on Hormonal Regulation and the Metabolic Syndrome

AGIs primarily act on postprandial hyperglycemia by an antihyperglycemic effect. They exert no direct effects on insulin secretion. Data of clinical investigations show a ~30% lower postprandial insulin excursion (Hanefeld *et al.*, 1991). However, due to the subsequently lower glucotoxicity, insulin resistance is improved. In a placebo-controlled, hyperglycemic

Retired.

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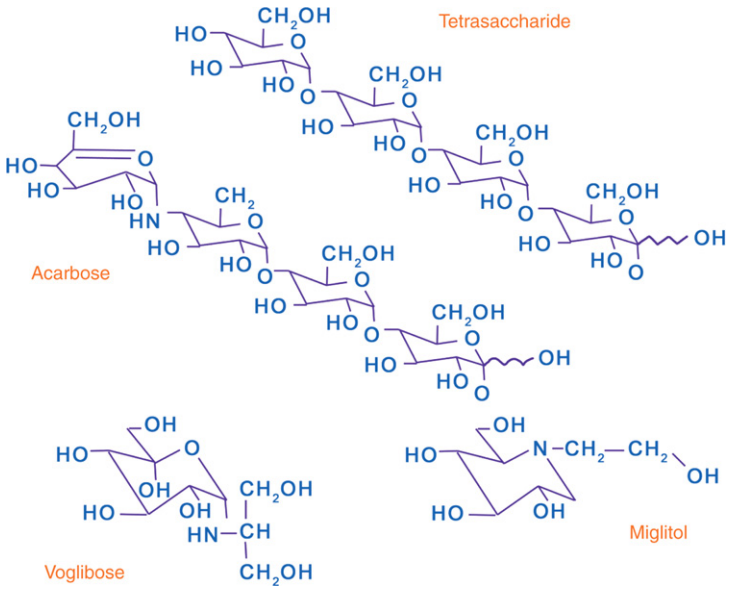

Fig. 1 Molecular structure of alpha glucosidase inhibitors. Reprinted from Joshi, S.R., Standl, E., Tong, N., *et al.*, (2015). Therapeutic potential of α-glucosidase inhibitors in type 2 diabetes mellitus: An evidence-based review. *Expert Opinion on Pharmacotherapy* 16, 1959 – 1981 with permission from Taylor & Francis Ltd. (http://www.tandfonline.com).

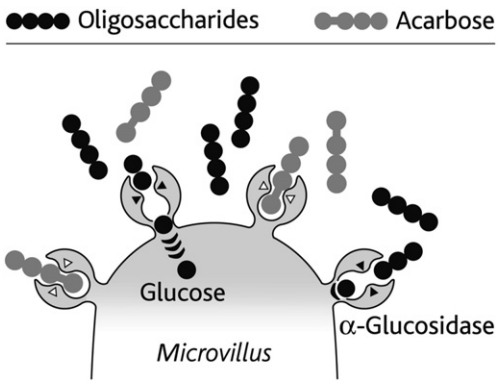

Fig. 2 Mechanism of action of acarbose: competitive inhibition of intestinal enzymatic hydrolysis of oligosaccharides. Adapted from Bischoff, H. (1991). Effect of acarbose on diabetic late complications and risk factors—New animal experimental results. *Aktuelle Endokrinologie und Stoffwechsel* 12, 25 – 32 with permission from Thieme Publishers.

CLAMP study, acarbose intake significantly improved insulin resistance in elderly patients but had no effect on insulin secretion after one treatment year (Meneilly *et al.*, 2000). The same effect was observed in subjects with impaired glucose tolerance (IGT) (Chiasson *et al.*, 1996).

Since more complex carbohydrates reach the lower parts of the small intestine, the release of glucagon like peptide 1 (GLP_1) is induced (Qualmann *et al.*, 1995; Enc *et al.*, 2001; Zheng *et al.*, 2013). GLP_1 increase was associated with enhanced nitric oxide synthase activity (Zheng *et al.*, 2013). Gastric emptying is delayed, and glucagon release reduced. The unique postprandial antihyperglycemic mechanism leads to several pleiotropic effects associated with downregulation of insulin resistance (Chiasson *et al.*, 1996; Meneilly *et al.*, 2000); Delgado *et al.*, 2002), protection (also of ß-cells) from oxidative stress (Ceriello *et al.*, 1996; Meneilly *et al.*, 2000; Hanefeld *et al.*, 2004a; Wang *et al.*, 2011; Sun *et al.*, 2016), decrease of low grade inflammation (Wang *et al.*, 2003; Li *et al.*, 2016), downregulation of proinflammatory transcription factor NFκB (Rudofski *et al.*, 2004), reduction of post-prandial leucocyte increase (Hanefeld *et al.*, 2009a), and improvement of prodiabetic microbioma (Weaver *et al.*, 1997; Yin *et al.*, 2014; Su *et al.*, 2015; Zhang *et al.*, 2017) (**Table 2**). A reduction in hypertriglyceridemia, in particular postprandial, was observed (Kado *et al.*, 1998; Ogawa *et al.*, 2004). Acarbose also modulated vasoregulatory peptide levels in type 2 diabetic patients (Rudovich *et al.*, 2016).

Acarbose extended lifespan in mice, preferentially in male animals (Harrison *et al.*, 2014). AGIs can reduce bodyweight by 1–3 kg or help maintain weight if combined with insulinotropic antidiabetic drugs (van de Laar *et al.*, 2005; Schnell *et al.*, 2016; Nakhaee and Sanjari, 2013). The beneficial effect on body weight appears to be more pronounced in Asian than

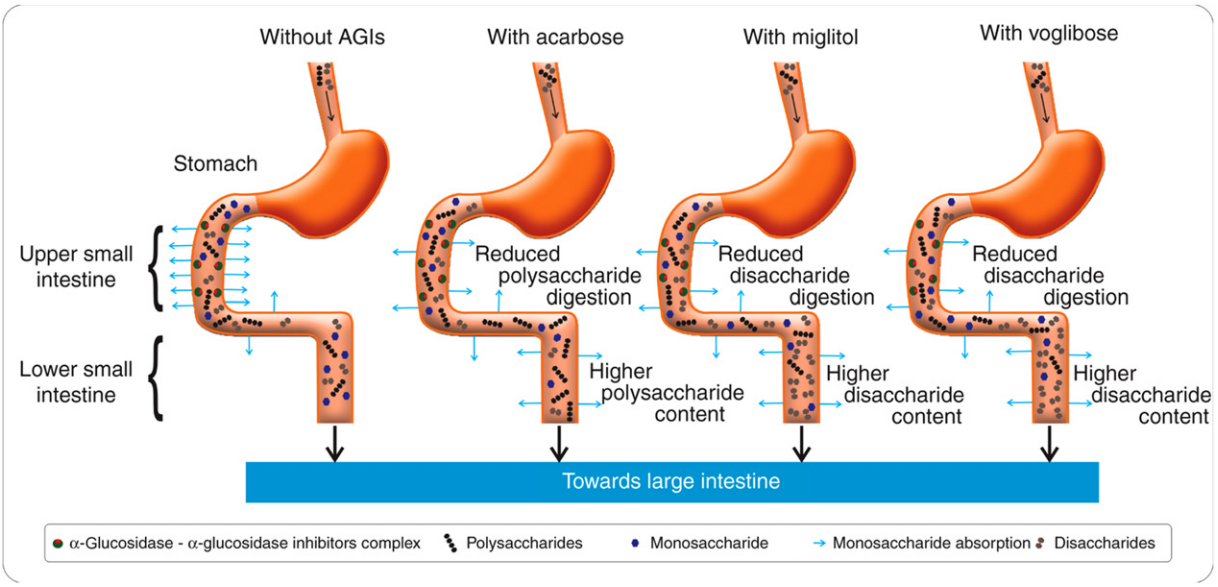

Fig. 3 Mechanism of action of alpha glucosidase inhibitors. Reprinted from Joshi, S.R., Standl, E., Tong, N., *et al.*, (2015). Therapeutic potential of α-glucosidase inhibitors in type 2 diabetes mellitus: An evidence-based review. *Expert Opinion on Pharmacotherapy* 16, 1959 – 1981 with permission from Taylor & Francis Ltd. (http://www.tandfonline.com).

Table 1 Pharmacokinetic properties of alpha glucosidase inhibitors in therapeutic use

Properties	*Acarbose*	*Miglitol*	*Voglibose*
Global launch	1990	1996	1994
Doses			
Initial	25 mg t.i.d., orally at the start of each meal	25 mg t.i.d., orally at the start of each meal	0.2 mg t.i.d., orally before/with each meal
Maintenance	25 → 50 mg t.i.d. after 4–8 weeks intervals 50 → 100 mg t.i.d.	25 → 50 mg t.i.d. after 4–8 weeks intervals 50 → 100 mg t.i.d.	May be increased up to 0.3 mg t.i.d.
Maximum	Up to 100 mg t.i.d.	100 mg t.i.d.	Not mentioned
Extent of absorption	Poorly absorbed (<2% as unchanged drug), bioavailability is low	25 mg completely absorbed; 100 mg 50%–70% absorption	Poorly absorbed (<6%); absorption is dose dependent
Distribution	Extracellular fluid	Extracellular fluid	Lumen of gastrointestinal tract
Metabolism	Gastrointestinal tract principally by intestinal bacteria, also by digestive enzymes	None, metabolites not detected in plasma, urine/feces	Intestinal enzymes and microbial flora
Excretion			
Fecal	51%	—	98%
Renal	34% (metabolites)	>95%	5%
Plasma elimination half-life	2 h	2 h	Not available
Therapeutic indications	Adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus Adjunct to diet and insulin in type 1 diabetes mellitus Prevention of onset of type 2 diabetes mellitus in individuals with impaired glucose tolerance in combination with diet and exercise	Adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus	Adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus

Western patients and more marked than under gliptins or metformin (He *et al.*, 2014). Furthermore, it has beneficial effects on major components of the metabolic syndrome as shown in the Study to Prevent Non-Insulin-Dependent Diabetes Mellitus (STOP-NIDDM) (Chiasson, *et al.*, 2003) (**Table 2**). Vice versa, in subjects with IGT and the metabolic syndrome, the number needed to treat to prevent one case of newly diagnosed diabetes was 5.8 versus 16.5 in IGT subjects without the metabolic syndrome (Hanefeld *et al.*, 2009b).

Table 2 Pleiotropic effects of alpha glucosidase inhibitors on cardiovascular risk factors and traits of the metabolic syndrome

Factor	*Effect*
Body weight	1–3 kg ↓ (neutral in combinations)
Blood pressure	Newly diagnosed hypertension in impaired glucose tolerance ↓ Clinical hypertension in overt diabetes ↓
Lipids	Triglycerides (postprandial) ↓ Free fatty acids ↓
Biomarkers of inflammation	Nuclear factor kappa B ↓ High sensitive C-reactive protein ↓ Leucocytes (postprandial) ↓
Endothelium	Flow mediated vasodilation ↑ Nitric oxide synthase activity ↑
Blood coagulation	Plasminogen activator inhibitor ↓ Prothrombin fragments 1 + 2 ↓ Di-dimers ↓
Microbiota	Improved Lactobacillus ↑

Therapeutic Indications and Clinical Use

AGIs are used in patients with type 2 diabetes as monotherapy (first line) or in combination with all other antidiabetic drugs. Because of their special antihyperglycemic mode of action, they can be well combined with all hypoglycemic substances. Acarbose is also used for the treatment of excessive postprandial hyperglycemia and rapid fluctuations in type 1 diabetes. It has complex beneficial effects in elderly patients with type 2 diabetes. As the efficacy of acarbose has been documented in a huge number of clinical studies and has evidence from cardiovascular outcome trials, the focus in this article will be on this drug. A systematic Cochrane review of double-blind, controlled clinical studies found a placebo-subtracted mean HbA1c decrease of 0.8% in type 2 diabetes patients treated with acarbose (van de Laar *et al.*, 2005). In comparative studies, acarbose shows the same effect on HbA1c as other established oral antidiabetic drugs (Joshi *et al.*, 2015). Several double-blind, controlled studies reveal a comparable efficacy of acarbose with the first-line drug metformin. Acarbose is also recommended as first-line drug in China (Yang *et al.*, 2001). It should be emphasized again that by contrast to other antidiabetic drugs, the efficacy is mainly due to better postprandial glucose control.

Under acarbose, efficacy depends on the composition of diet and carbohydrate intake. In a comparative trial of Western versus Asian food, acarbose showed larger HbA1c reductions with Chinese food (by 1.54% vs. 0.52% with Western food) (Zhu *et al.*, 2013). Studies in Chinese patients often observe higher HbA1c reductions (He *et al.*, 2014; Hu *et al.*, 2015) than Western studies (which were primarily enclosed in the Cochrane analysis). Long-term observations show a sustained effect on type 2 diabetes control under real world conditions (Mertes, 2001; Li *et al.*, 2011). In a recent review of efficacy of diabetes treatment in Eastern populations, acarbose was equipotent with metformin in the reduction of HbA1c (Gu *et al.*, 2015).

To ensure maximum efficacy, AGIs must be taken directly with the first bite of a meal or during meals. A study with acarbose showed a 50% reduction in efficacy when taken 15 min before a meal (Rosak *et al.*, 1995).

AGIs for IGT Treatment and Diabetes Prevention

With a global tsunami of newly diagnosed type 2 diabetes primary prevention by treatment of IGT is of the utmost importance. Worldwide prevalence of IGT was estimated at 318 million adults aged 20–79 years (6.7% of adults) in 2015 (IDF Diabetes Atlas, 2015). Numbers are high for Asian populations; a 2013 survey found that more than one-third of Chinese adults suffer from prediabetes (Wang *et al.*, 2017). Furthermore, IGT is a significant coronary risk factor (Qiao *et al.*, 2003; Grundy, 2012). Acarbose is the only oral antidiabetic drug besides metformin that is approved in 84 countries for the treatment of prediabetes. In the STOP-NIDDM trial, acarbose reduced incidence in people with IGT by 25% and was associated with a significant conversion to normal glucose tolerance (Chiasson *et al.*, 2002). Recently published data of the Acarbose Cardiovascular Evaluation (ACE) study in patients with coronary heart disease and IGT has shown a decrease in the incidence of newly diagnosed diabetes by 18% after 5 years follow up (Holman *et al.*, 2017). In a 4-arm prospective controlled trial in Chinese IGT subjects comparing regular life style advice, intensified diet and exercise education, metformin and acarbose, a significant reduction of the incidence of newly diagnosed diabetes could be achieved with pharmacological intervention (Yang *et al.*, 2001). Acarbose was numerically more effective than metformin. Furthermore, a 3-year placebo-controlled study with voglibose in high-risk Japanese IGT subjects revealed a 40% reduction of diabetes incidence (Kawamori *et al.*, 2009).

Cardiovascular Results and Possible Pleiotropic Effects on Comorbidities

Pathophysiological studies with acarbose reveal its potential for beneficial effects on cardiovascular diseases. Besides advantageous effects on inflammation and oxidative stress, several acarbose studies and one miglitol study found an improvement in endothelial function (Wascher, *et al.*, 2005; Shimabukuro *et al.*, 2006; Pistrosch *et al.*, 2009; Kato *et al.*, 2010; Kitano *et al.*, 2013). Several studies showed a reduced progression in intima media thickness (Hanefeld *et al.*, 2004a; Yamasaki *et al.*, 2005; Geng *et al.*, 2011; Patel *et al.*, 2013).

In IGT subjects, the STOP-NIDDM trial investigated the efficacy of acarbose on cardiovascular events over 3 years as secondary objective (Chiasson, *et al.*, 2003). The incidence of myocardial infarction and cardiovascular events was significantly reduced. Accordingly, a meta-analysis of seven double-blind, controlled trials in patients with type 2 diabetes calculated a 64% risk reduction of myocardial infarction and 35% risk reduction of any cardiovascular event in the acarbose treatment group (Hanefeld, *et al.*, 2004b).

The large cardiovascular outcome ACE trial including patients with preexisting coronary heart disease and IGT over 6 years in China ended with a neutral result regarding cardiovascular diseases and mortality (Holman *et al.*, 2017). Although the incidence of newly diagnosed diabetes was significantly reduced in this study, no significant beneficial effects on cardiovascular events were observed in the sensitivity analysis. The different outcome regarding cardiovascular events in the STOP-NIDDM and the ACE trial might be explained by the differences between the trials in acarbose dosing regimen, study population characteristics, and cardiovascular therapy. Patients in the ACE trial received a lower acarbose dose (50 mg t.i.d. vs. 100 mg t.i.d. in the STOP-NIDDM trial), were older (median 64.3 years vs. 54.5 years) and of a different ethnicity (Chinese vs. Caucasian), and all presented with established coronary heart disease (inclusion criterion vs. low cardiovascular risk in the STOP-NIDDM trial). Recommended secondary cardiovascular prevention measures had to be consistent with internationally accepted treatment guidelines and were more aggressive than treatment in the 1990s when the STOP-NIDDM trial was carried out.

It must be emphasized that acarbose is at least safe for individuals with preexisting cardiovascular disease. No conclusive data are available on the effect of AGIs on microvascular disease.

As acarbose acts in the intestine, its relationship with intestinal diseases has been discussed since its approval for type 2 diabetes. Patients with type 2 diabetes and the metabolic syndrome exhibit an increased risk of cancer with colon cancer as a leading cause of death from neoplasia. A nationwide prospective register with newly diagnosed type 2 diabetes in Taiwan has revealed that patients treated with acarbose as first-line drug had a significantly (27%) lower dose-dependent incidence of colon cancer versus standard therapy including metformin and other antidiabetics (Tseng *et al.*, 2015). This finding might theoretically be a consequence of the beneficial effects of acarbose on the microbiota and the intestinal environment.

Favorable effects on microbiota and improvement in hepatic encephalopathy were found in a randomized controlled trial of acarbose in patients with liver cirrhosis (Gentile *et al.*, 2005). Similar to positive results in a mouse model (Nozaki *et al.*, 2009), a combination of acarbose and ezetimibe was reported to have therapeutic effects on the development of nonalcoholic fatty liver in a preliminary study (Rudovich *et al.*, 2010). The sample size was, however, very small.

Safety, Adverse Effects, and Contraindications

The side effect profile is similar for all AGIs. Based on >25 years of therapeutic use, a large number of controlled clinical trials, follow up and postmarketing studies, AGIs do not show any signs of toxicity or possible relationships with subsequent comorbidities. Consistently, no serious or fatal systemic complications related to acarbose have been reported so far. Monotherapy was not associated with clinical hypoglycemia. Hypoglycemia in combination therapy with hypoglycemic agents is a rare event. If it occurs, it must be treated with glucose, not with starch or table sugar. Transient gastrointestinal symptoms are the main adverse effects under AGI therapy, because meteorism, flatulence, soft stool, and diarrhea may occur at the beginning of treatment. The reported incidences vary dramatically between $<10\%$ and up to 50% depending on the study site and the geographic areas with their different dietary habits (Rosak and Mertes, 2012). The lowest rates are reported in Asian trials. These initial side effects can be minimized by a diet of complex carbohydrates prior to therapy avoiding table sugar or fructose and sorbitol and by appropriate application: start low, go slow. Slow titration from low to medium dose, depending on the individual patient's tolerability, is recommended.

A recent reappraisal of the clinical drug–drug interaction potential of AGIs did not find a meaningful influence on absorption and bioavailability of many co-administered medications (Dash *et al.*, 2018). Labeled contraindications are related to conditions that might deteriorate as a result of possibly increased gas formation in the intestine as pregnancy, lactation, inflammatory bowel disease, and partial intestinal obstruction. Due to lack of experience AGIs should also not be used in patients younger than 18 years, in pregnant women, and in cases of renal insufficiency.

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Further Reading

Bischoff, H., 1991. Effect of acarbose on diabetic late complications and risk factors—New animal experimental results. Aktuelle Endokrinologie und Stoffwechsel 12, 25–32.

Insulin Pumps and Artificial Pancreas

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Glossary

Basal insulin Insulin that controls blood glucose in the fasting state.
Beta cells Insulin-producing cells located in the islets of Langerhans of the endocrine pancreas.
Bolus insulin Insulin administered to cover requirements for meals or used on occasional basis to correct high glucose levels.
Carbohydrate counting Meal planning method where the patients need to estimate the carbohydrate content of their meals and match their insulin bolus accordingly. See also insulin-to-carbohydrate ratio.
Continuous subcutaneous insulin infusion Insulin infusion through the subcutaneous depot via an infusion device, also called insulin pump.
Continuous glucose monitoring system Device measuring glucose levels in the subcutaneous interstitial fluid every 1–5 min via a small subcutaneous probe.
Glycated hemoglobin (HbA1c) A biomarker of glucose control which represents the average glycemia over the past 2–3 months.
Gestational diabetes Diabetes that develops during pregnancy and which in most cases resolve after delivery.
Glucometer Device that measures capillary blood glucose.
Hyperglycemia High blood glucose, possibly accompanied with symptoms (e.g., thirst, headache). Generally defined as blood glucose >10.0 mmol/L.
Hypoglycemia Low blood glucose, typically accompanied with symptoms (e.g., trembling, sweating, hunger). Generally defined as blood glucose <4.0 mmol/L.
Hypoglycemia unawareness A condition where hypoglycemia symptoms recognition is attenuated or absent.
Insulin pump A device that infuses insulin through the subcutaneous depot, also called continuous subcutaneous insulin infusion.
Insulin-to-carbohydrate ratio A method to determine how much insulin is needed proportionally to the carbohydrate content of the meal, generally expressed in units of insulin per 10 g of carbohydrates. See also carbohydrate counting.
Ketosis Excess formation of ketones in the blood seen in cases of hyperglycemia in the context of low insulin levels and metabolism predominantly based on lipid substrates.
Self-monitoring of blood glucose Measurement of blood glucose with glucometers.

Introduction

The two most common types of diabetes are distinguished by their main pathophysiology. Type 1 diabetes (T1DM) is characterized by an autoimmune destruction of the endocrine beta cells of the pancreas resulting in complete insulin deficiency (Acharjee *et al.*, 2013). It thus necessitates life-long insulin replacement from the time of diagnosis, which can occur at any age starting in childhood. Type 2 diabetes (T2DM) is approximately 10 times more frequent, it occurs mainly in adulthood and is due, in its early stages, to a state of insulin resistance coupled with relative insulin deficiency (Fonseca, 2009). T2DM is treated with a large array of oral or injectable hypoglycemic agents; however, insulin deficiency worsens over the years requiring insulin replacement for many patients at some point in the course of their disease. Both types of diabetes (T1DM and T2DM) face a rising prevalence. An optimal control of blood glucose levels is essential to prevent diabetes-related microvascular complications (retinopathy, nephropathy, and neuropathy) and, on the long-term, also possibly contribute to macrovascular risk reduction (coronary artery disease, strokes, etc.) (Diabetes Control and Complications Trial Research Group *et al.*, 1993; Nathan *et al.*, 2005).

Insulin replacement in diabetes is achieved with subcutaneous injections using multiple daily injections (MDI) or continuous subcutaneous insulin infusion (CSII) systems also called insulin pumps (CADTH, 2015; Gururaj Setty *et al.*, 2016). The main goal of CSII is to provide a more flexible insulin delivery. To mimic insulin action in subjects without diabetes, exogenous human insulins or analogs are designed as long or rapid release formulations from the subcutaneous depot; long release insulins are used to replace daily basal insulin needs while rapid acting boluses cover prandial insulin requirements (**Fig. 1**) (Gururaj Setty *et al.*, 2016). Only rapid acting insulins are used in pumps because of their continuous infusion mode of action.

Despite significant improvements in insulin formulations, delivery systems and treatment modalities, many insulin-dependent patients do not reach the recommended glycemic target (represented by a glycated hemoglobin [HbA1C] level that is $<6.5\%$ or $<7\%$, depending on various diabetes associations recommendations as well as patient's age and conditions) (Miller *et al.*, 2015; Hayward *et al.*, 2015). Intensifying insulin replacement to decrease hyperglycemia events is often challenged by an increased risk of iatrogenic hypoglycemia complicating diabetes management. Moreover, increased physical activity, illnesses, missed meals or

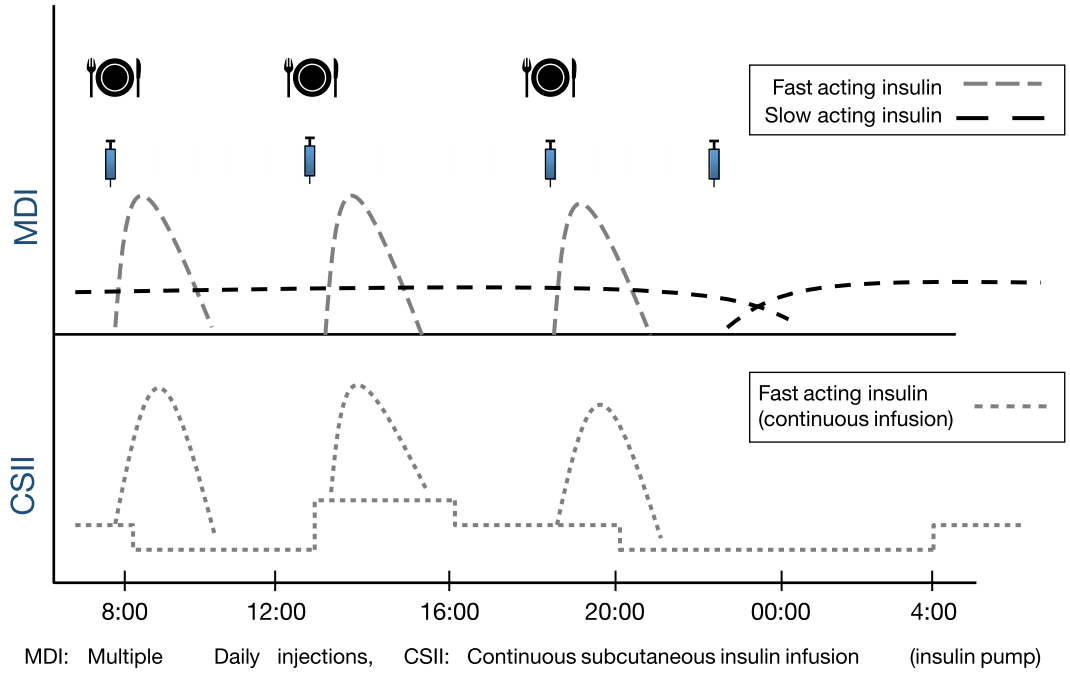

Fig. 1 Comparison of insulin use in multiple daily injection (MDI) regimens and in continuous subcutaneous insulin infusion (CSII) systems.

erroneous carbohydrate counting, stress, low reproducibility in insulin absorption, infrequent blood glucose level monitoring via capillary testing (glucometers), etc., adds-up and can lead to unpredictable changes in daily glucose levels.

Mimicking hormonal secretion by a normal pancreas with a technology that dynamically controls insulin infusion in a timely manner according to changing glucose levels has haunted researchers in the diabetes field for ages. In the years 2000s, continuous glucose monitoring systems (CGMS) have been implemented paving the way to further technological advances (Klonoff, 2005). CGMS measures glucose levels in the interstitial fluid every 5–15 min via a small subcutaneous probe providing an indirect measurement of blood glucose levels in the surrounding capillaries (Klonoff, 2005). With CGMS and better performing insulin pumps, the development of the external artificial pancreas, also called closed-loop automated insulin delivery system (CLS), was made possible (Thabit and Hovorka, 2016a). An artificial pancreas consists of three components that communicate remotely including a CGMS, a hormone infusion pump and a dosing algorithm implanted in an external device (e.g., smart phone) or in the pump (Thabit and Hovorka, 2016a). The CGMS sends its glucose readings in real-time to the dosing algorithm that commands dynamic changes in hormonal infusion closing the loop of glucose control (**Fig. 2**) (Thabit and Hovorka, 2016a). The number of studies on the artificial pancreas has grown exponentially in the past years leading to the commercialization of the first system in the United States in 2017 (Voelker, 2016).

This article aims to explore the use of technology in diabetes management focusing on the uses, benefits and limitations of the insulin pumps, CGMS and artificial pancreas systems. We are also addressing challenges that need to be overcome in the future with these technologies.

Insulin Pumps (CSII)

Description

Since the first therapeutic use of insulin in 1921, an important shift towards the use of technology has been observed starting in the 1970s with the introduction of the insulin pumps (CSII). The first pumps were not very popular at the time, mainly because of their inconvenient size and multiple technical issues (Lenhard and Reeves, 2001). The new-generation pumps are smaller and come in two main designs: most available pumps have the reservoir connecting via a tubule to the subcutaneous catheter that infuses insulin; other systems called patch or tubeless pumps are directly attached to the skin with an underneath subcutaneous catheter (**Fig. 3**) (Thabit and Hovorka, 2016b). Based on the typical variation of insulin requirements across the day, patients can program their pump to deliver various basal rates and boluses of insulin. The pump is also used to administer meal boluses according to individualized insulin-to-carbohydrate ratios. The programming of pumps has become more sophisticated over the years. For example, nowadays insulin pumps offer several delivery profile selections (e.g., for days with and without physical activity), an estimation of the insulin on board (estimation of insulin already injected but not yet absorbed) and integrated meal as well as hyperglycemia correcting bolus calculator (Thabit and Hovorka, 2016b). Insulin pumps are mostly used by patients with T1DM and very few with T2DM. Rates of use in T1DM

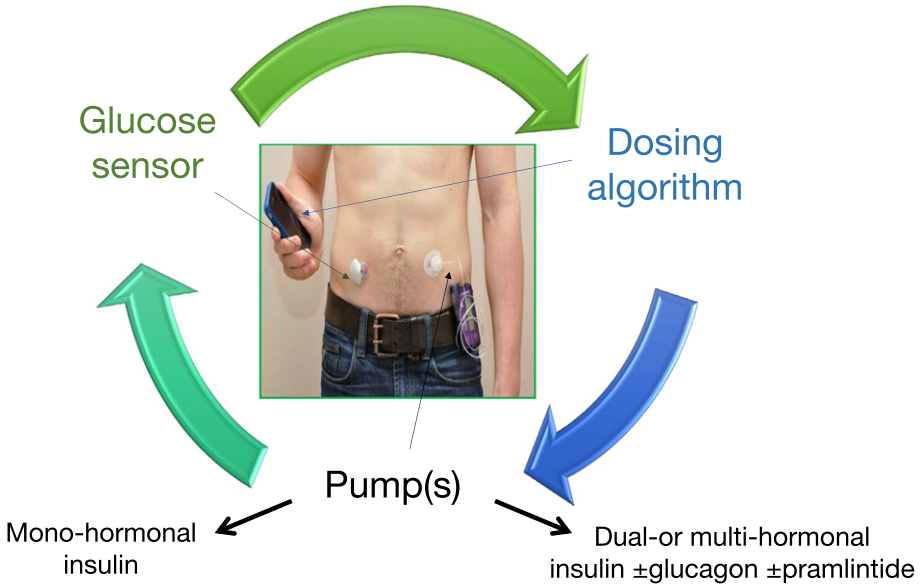

Fig. 2 Artificial pancreas or closed-loop insulin infusion system (CLS) components.

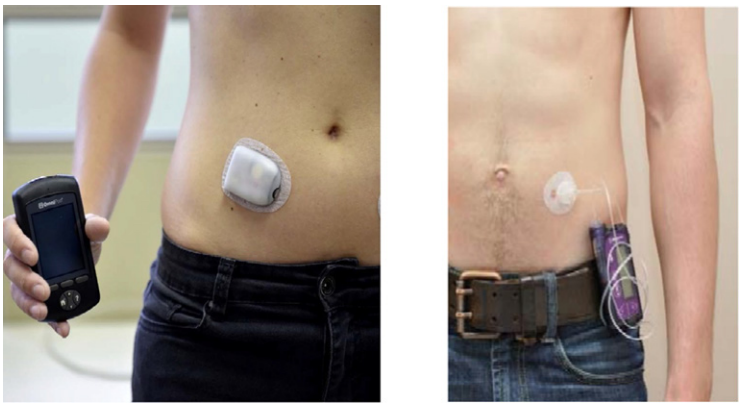

Fig. 3 Examples of the two main insulin pump designs available on the market.

vary widely across different countries (e.g., 20%–25% in the United States and Norway and 6% in the United Kingdom) (Thabit and Hovorka, 2016b). According to the T1DM exchange registry in the United States, the rate of CSII use varies also by age (up to 50% in children and adolescents) and ethnicity (22.1% in minorities vs. 34.55% in nonminorities) (Miller *et al.*, 2015). CSII use rates can also be influenced by other factors such as diabetes duration, therapeutic objectives, general health, interest and motivation as well as reimbursement policy (e.g., private insurance or public drug coverage).

Clinical Benefits

The clinical efficacy of CSII has long been established while its superiority (lower HbA1c with less hypoglycemia) in comparison to MDI has been proven in most patient populations; however, some authors and healthcare authorities challenge the magnitude of the cost required to achieve such benefits (Lenhard and Reeves, 2001). A Cochrane systematic review and a recent meta-analysis in adult patients with T1DM revealed a decrease in HbA1C of 0.3% with CSII as opposed to MDI (Misso *et al.*, 2010; Yeh *et al.*, 2012). The incidence of severe hypoglycemia (requiring third-party assistance) is also decreased with CSII versus MDI especially for patients who are at increased risk for hypoglycemia (hypoglycemia unaware; low perception of hypoglycemia symptoms) (Misso *et al.*, 2010; Pickup and Sutton, 2008). The comparative evidence is less clear and harder to interpret in the case of mild and moderate hypoglycemia due to discrepancies in reporting and defining thresholds for these episodes. In the pediatric population, the evidence comes mostly from registries and prospective cohorts suggesting a better glycemic control with CSII. In the T1DM Registry, CSII users had lower mean HbA1c and were more likely to achieve levels lower than 7.5% while the difference was not significant in a European registry with younger participants (Maahs *et al.*, 2014). In a large population based case-control European

cohort, children using CSII maintained average HbA1c values that are 0.6% lower than in MDI users and had a decrease in the incidence of severe hypoglycemia over a mean follow-up of 7 years (Johnson *et al.*, 2013). In general, positive effects on quality of life and personal satisfaction, partly due to the greater treatment flexibility, were reported with CSII use across all age groups (Life EQSG–EoQo, Costs in Diabetes Type 1, *et al.*, 2008; Muller-Godeffroy *et al.*, 2009; Linkeschova *et al.*, 2002).

Few patients with advanced T2DM who are treated with basal-bolus insulin regimens use pumps. However, the number of users is expected to rise as more trials show a better glycemic outcome with CSII therapy. Patients with poorly controlled diabetes on MDI are the ones to benefit the most from shifting to CSII. In the OpT2mise trial undertaken in patients with poorly controlled T2DM despite using MDI, patients who switched to CSII required less total insulin doses and had a 0.7% additional HbA1c reduction, in comparison with patients kept on MDI, without increasing their risk of hypoglycemia (Reznik *et al.*, 2014). In a recent meta-analysis, a 26% reduction of insulin requirements without weight change and a reduction in HbA1c by 0.4% were observed in patients with T2DM after shifting to CSII therapy (Pickup *et al.*, 2017).

In the specific case of pregnant women with preexisting diabetes or with gestational diabetes, the current evidence does not support CSII treatment over MDI. The available trials are small and do not always assess similar outcomes according to the latest Cochrane review including only five trials (total of 154 pregnancies) that are adequate for analysis (Farrar *et al.*, 2007). No clear differences between CSII and MDI could be detected for some of the reported primary outcomes including caesarian section risk, large-for-gestational-age birth and perinatal mortality nor for secondary outcomes such as maternal weight gain, 24-h mean blood glucose, mean maternal HbA1c and maternal hypoglycemia or hyperglycemia (Farrar *et al.*, 2007). Larger trials with adequate reporting of outcomes are still needed to compare CSII with MDI for pregnant women.

CSII Related Adverse Events

The use of CSII is associated with a number of adverse events despite the progress in this technology and can be categorized into perfusion site or catheter problems, cutaneous reactions, metabolic adverse events, and pump software problems. These adverse events are common and can occur in 40% of users per year but they necessitate hospital admissions only in very rare cases (Ross *et al.*, 2015). The most serious adverse events are related to metabolic control, particularly diabetic ketoacidosis that can result from perfusion site/catheter failure and prolonged insulin infusion interruption (Ross *et al.*, 2015). In a recent online survey including adult patients and examining their perceptions about CSII use, the majority of participants perceived it positively, reporting improvements in glucose control without compromising their quality of life. However, technical issues related to perfusion site, catheter and cutaneous problems were quite common with only 3% of patients reporting no problems of any kind in the past year of use (Nadine Taleb *et al.*, 2017a). Other major issues with CSII are the need to carry the pump at all times as well as associated costs (significantly higher than MDI) leading to limited access and coverage in a lot of countries.

Glucose Sensors, Sensor-Augmented and Threshold-Suspend Pump Therapy

Glucose Sensors

Glucose concentration monitoring is an essential part of diabetes management, especially for T1DM. Self-monitoring of blood glucose (SMBG) with capillary glucometers is cumbersome and is usually limited to few measurements per day. On the other hand, continuous glucose monitoring (CGMS) allows 24-h glucose profiling in real-time with glycemic trends to foresee hyper or hypoglycemic episodes to which, in most of the cases, the patient can also be alerted by preset sensor alarms (Klonoff, 2005). CGMS includes a subcutaneous probe connected to the outer scan by a transmitter that sends glucose measurements to the pump or to a separate receiver or, for a new type of sensors, it requires a scanning with a dedicated receiver (**Fig. 4**).

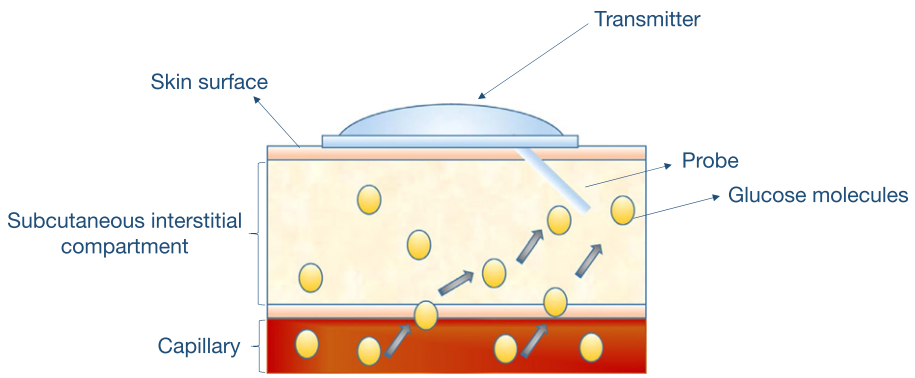

Fig. 4 Schematisation of the subcutaneous probe and the transmitter which are two main components of the continuous glucose monitoring system (CGMS).

Most CGMS still require at least two calibrations per day with capillary glucose levels obtained from a glucometer but some of the newer generations do not need calibration anymore (Bailey *et al.*, 2015). Interstitial glucose is an estimation of capillary glucose and not a direct measure. A certain delay is expected to equilibrate glucose between the interstitial and capillary compartments especially when blood glucose is rapidly changing such as during meals or physical activity; for example, the sensor may lag behind resulting in an underestimation of the rising postprandial blood glucose or, on the opposite, may result in a more problematic situation with an overestimation of the blood glucose while it's declining during exercise (Taleb *et al.*, 2016a). Accuracy and performance of new-generation CGMS have improved significantly with a margin of error in comparison to blood glucose that decreased from $\pm 20\%$ with the first generation to $\pm 10\%$ with the third ones (Rodbard, 2016). Due to these improvements, United States health authorities have recently approved one CGMS for making diabetes treatment decisions without the need for confirmation of sensor readings using a capillary blood glucose measurement (Shapiro, 2017). Clinical data supports the importance of CGMS in improving glucose control. A study conducted by the Juvenile Diabetes Research Foundation (JDRF) showed a reduction in HbA1c with regular use of CGMS in children and adolescents. This was similarly confirmed in adults with T1DM or T2DM (Rodbard, 2016; Ehrhardt *et al.*, 2011; Garg *et al.*, 2009). A recent meta-analysis of 11 randomized controlled trials similarly found a decrease in HbA1c (-0.3%) for patients with T1DM using CGMS and a decrease in time spent in hypoglycemia that did not, however, reach statistical significance. These benefits are observed in patients with insulin pumps as well as those treated with multiple daily injections (Garg *et al.*, 2011); yet, less than 10% of patients with T1DM use CGMS on a regular basis, with a particularly low use of the devices in the pediatric population (Wong *et al.*, 2014). This is mainly related to cost and low public or private coverage, but also to possible difficulties for some patients with the use of technology, the amount of data it generates and the need to have an additional device attached to the body.

Sensor-Augmented, Suspend and Predictive Suspend Pump Therapy

The development of CGMS allowed the progression of CSII into sensor-augmented pump (SAP) therapy. SAP potentiates insulin therapy with real-time glucose levels to help patients in adjusting pump's insulin infusion accordingly (Bergenstal *et al.*, 2013; Ly *et al.*, 2012). Improvement in glucose control, in comparison with MDI, have been observed in most studies including a meta-analysis of four randomized controlled trial (HbA1C decreased by an average of 0.7% with SAP) (Yeh *et al.*, 2012). Improvement in HbA1c and a decrease in hypoglycemia were also seen with SAP in comparison with conventional CSII (Soupal *et al.*, 2016).

Recently, two new systems, suspend and predictive-suspend, have evolved adding advanced features to SAP with the objective of reducing hypoglycemia. In the suspend pump system (also referred to as threshold-suspend), low glucose sensor values can trigger an automatic transient (up to 2 h) insulin infusion suspension. Suspend systems have shown the ability to reduce the frequency and duration of nonsevere hypoglycemic episodes by one third without increasing the risk of ketosis or worsening HbA1c levels (Bergenstal *et al.*, 2013; Ly *et al.*, 2012). Further progress has been achieved with the predictive—suspend systems which predict the risk of reaching the defined hypoglycemia threshold and allow an earlier insulin suspension. A trial addressing the risk of severe hypoglycemia has shown that such insulin suspension systems can probably eliminate this major acute complication (Ly *et al.*, 2012).

External Artificial Pancreas or Closed-Loop System (CLS)

Definition and Components

The artificial pancreas (AP) or closed-loop automated insulin delivery system (CLS) brought the combination of CGMS and pump therapy a step further with automation of hormone delivery to improve glucose control and reduce the burden of self-management. It is one of the most promising therapies for T1DM. In this system, insulin only (single-hormone artificial pancreas) or insulin and a second drug, typically glucagon (dual-hormone artificial pancreas), infusion rates are regulated in a dynamic fashion based on algorithm-generated recommendations which rely on continuous glucose monitoring systems readings. In dual-hormone artificial pancreas, glucagon is administered as intermittent mini-boluses to further prevent and treat hypoglycemia in a preemptive way and/or to allow more aggressive insulin therapy without increasing hypoglycemic risk. The *JDRF* recently published a pathway towards fully automated artificial pancreas starting from currently available systems and the future steps that need to be achieved (Kowalski, 2015) (**Fig. 5**).

A key step in recent years was the ability to move to outpatient studies with automated glucose control. Kovatchev *et al.* conducted, in 2014, the first automated single-hormone artificial pancreas study using the DiAs platform (Kovatchev *et al.*, 2014) and since then, many research groups have developed and studied automated systems (Bally *et al.*, 2017; El-Khatib *et al.*, 2017; Russell *et al.*, 2014; Garg *et al.*, 2017; Grosman *et al.*, 2016). The first hybrid artificial pancreas (MiniMed 670G; Medtronic) has been introduced to the United States market in April 2017. This system is called hybrid because it still requires interventions to be applied to the artificial pancreas system by the patient in order to announce and to initiate required adjustments during exercise (e.g., suspension or reduction of insulin infusion) and during meals (e.g., bolus based on carbohydrate counting).

In an artificial pancreas, the key addition to the pump and CGMS is the predictive dosing algorithm used with the system. The general goal of the algorithm is to maintain glucose levels within a predefined target range minimizing hypo and hyperglycemia episodes. Two control models, frequently used in the current artificial pancreas studies, are the Proportional-Integral-Derivative

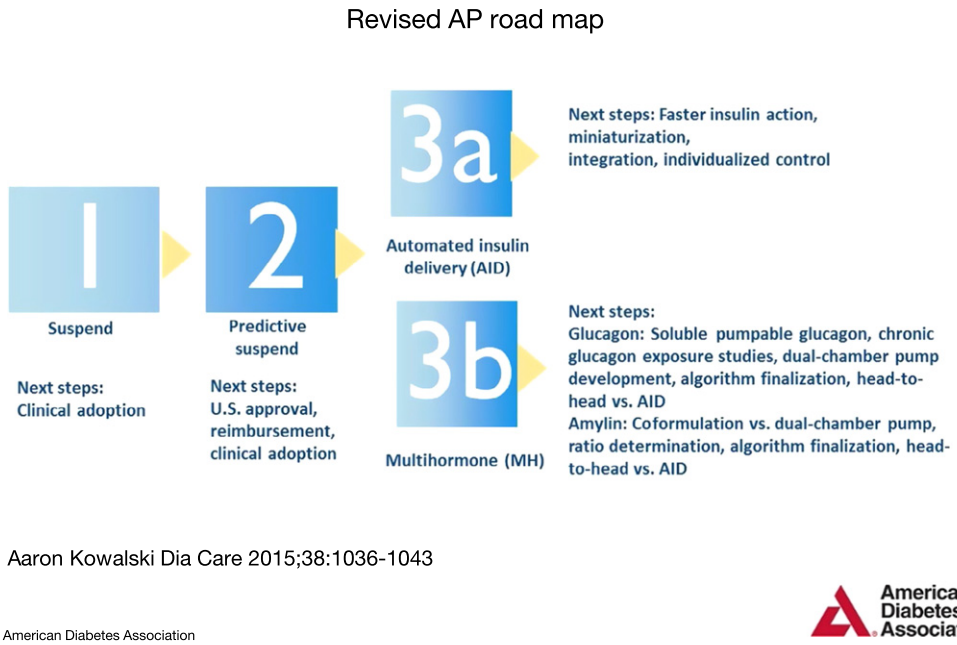

Fig. 5 Revised artificial pancreas road-map from the JDRF. From Kowalski, A. (2015). Pathway to artificial pancreas systems revisited: Moving downstream. *Diabetes Care* **38**(*6*), 1036–1043.

(PID) and the Model Predictive Control (MPC). The PID model is a reactive strategy that takes into account the actual glycemia and the baseline glucose level (proportional component), the area under the curve between measured and desired target glucose level (integral component) and rate of change of glucose levels (derivative component) (Thabit and Hovorka, 2016a). Adaptations to this model have been proposed to reduce the risk of early postprandial hypoglycemia but resulted in higher mean glucose levels (Gondhalekar *et al.*, 2013; Palerm, 2011). MPC has been favored by most research groups over the past few years. This model integrates subcutaneous insulin and interstitial glucose kinetics through compartments to calculate insulin dosing. MPC allows adjustments in response to meals and manual insulin boluses or corrections by adding a meal compartment (Thabit and Hovorka, 2016a). A compartment for glucagon kinetics is also incorporated in dual-hormone artificial pancreas systems. Importantly, MPC is based on repeated optimisation steps at finite times. Cobelli *et al.* compare the strategies of MPC to those applied in a chess game (Cobelli *et al.*, 2014). Previous glucose levels constitute the history of the game and are used to plan several moves-ahead (insulin infusion rates) but only the first move is applied (ex. insulin infusion rate over the next 10 min) followed by a re-evaluation of the adversary response (new glucose data) to re-plan the subsequent moves-ahead and so on, repeatedly. This strategy can thus adjust for unexpected events such as new information entered into the meal compartment. Some controllers include the fuzzy logic approach in their models (Haidar *et al.*, 2014; Mauseth *et al.*, 2013) which relies on estimated rather than accurate values and thus allows integrating the empirical knowledge of glucose control. Several algorithms also have safety checks in their models to limit the maximum rate of insulin infusion or to suspend it in the case of rapidly decreasing glucose levels (Thabit and Hovorka, 2016a).

Clinical Studies and Benefits

The artificial pancreas could help reduce the burden associated with day-to-day self-management while improving glucose control (Thabit and Hovorka, 2016a). Its efficacy over conventional insulin pump therapy for glycemic control in patients with T1DM was demonstrated in several studies (Thabit and Hovorka, 2016a; Hovorka, 2011; Haidar *et al.*, 2013). The main benefits associated with the artificial pancreas are: improved glucose time in target range and reduced time in hypoglycemia. Both benefits being more pronounced at night-time than during daytime (Phillip *et al.*, 2013; Haidar *et al.*, 2015a, Del Favero *et al.*, 2015; Nimri *et al.*, 2014; Kropff *et al.*, 2015; Russell *et al.*, 2014; Wolpert *et al.*, 2016).

Studies comparing the artificial pancreas to conventional pump therapy have shown improvements from 10% to 15% of the time spent in target glucose range. The largest outpatient study so far included 33 adults and 25 children, and lasted 12 weeks. In this study, Thabit *et al.* (AP@home Consortium) compared the suspend-threshold pump system to an automated single-hormone artificial pancreas over a 12-week period in children and adults with T1DM (Thabit *et al.*, 2015a). Children were using the system overnight only while adults were using it all day and night. Time in target range was significantly improved by 11% in adults and 9% in children using the artificial pancreas while reducing mean glycemia (− 0.8 mmol/L), HbA1c (− 0.4%) and time spent in hypoglycemia (Thabit *et al.*, 2015a). More recently, with the 12-week use of the MiniMed 670G (Medtronic, first hybrid artificial pancreas system on the market), a 7% improvement of time in target range was observed in adolescents, and a 5% improvement

was observed in adults, to note that this study is a safety nonrandomized trial (Garg *et al.*, 2017). Improvements in time in target range vary based on study length, type of artificial pancreas used (single or dual-hormone), time of its use (night only or all day) and treatment comparators (conventional insulin pump or sensor-augmented pump therapy) (Weisman *et al.*, 2017). A meta-analysis showed that, overall, the artificial pancreas improves time in target range by 12.59% [9.02%–16.16%] (equivalent to 172 min/day) compared to conventional treatment, and that dual-hormone systems are associated with a greater improvement (19.52%, 281 min/day) compared to single-hormone systems (11.06%, 159 min/day) (Weisman *et al.*, 2017). The different artificial pancreas systems have been particularly efficient at night. A combined analysis of the studies conducted by the Cambridge research team showed that the single-hormone artificial pancreas, as compared to conventional pump therapy, was associated with an 18.5% increased time in target range and a 0.9% lower time spent in hypoglycemia (Thabit *et al.*, 2015b). A 2-month study with a single-hormone system worn only at night showed the same benefits along with a reduced glycated hemoglobin (Kropff *et al.*, 2015).

An important reduction of the time spent in hypoglycemia, in the order of four to eight times, has also been observed with the artificial pancreas in comparison to conventional pump therapy depending on study conditions (Nimri *et al.*, 2014; Russell *et al.*, 2016; Kovatchev *et al.*, 2017; Haidar *et al.*, 2015b). This reduction is one of the most consistent benefits of artificial pancreas systems and it has been particularly important at night. According to the latest meta-analysis, time spent in hypoglycemia (glucose <3.9 mmol/L) was reduced by an average of 35 min per day overall with the artificial pancreas (27 min with single-hormone systems and 54 min in dual-hormone systems) (Weisman *et al.*, 2017).

No significant safety issues have emerged from the longest available trials (3 months) with single-hormone artificial pancreas systems (Thabit *et al.*, 2015a; Bergenstal *et al.*, 2016). For example, in the longest outpatient trial that was conducted over 3 months, loss of connectivity between the algorithm and the pump occurred in two instances and once the artificial pancreas system was not turned on by mistake. In the case of loss of connectivity, the pump operates with a preset insulin infusion as a safety measure (Thabit *et al.*, 2015a).

Multihormonal Approach With the Artificial Pancreas

Achieving fully automated glucose control with a single-hormone approach may prove to be impractical especially that diabetes is not limited to insulin deficiency but involves other hormonal defects as well. Thus, the multihormonal approach with artificial pancreas systems is of significant interest. The hormone most used is glucagon through dual-hormone studies; glucagon addition mainly aims to reduce the risk of hypoglycemia but it could also allow more aggressive insulin infusion aiming for lower mean blood glucose without increasing hypoglycemic risk (Thabit *et al.*, 2015a). The Bionic Pancreas group (Boston) examined the efficacy of the dual-hormone artificial pancreas in several automated outpatient studies (El-Khatib *et al.*, 2017; Russell *et al.*, 2014, 2016). Their longest study to date extended over 11 days and showed, in adults, reduced means of glycemia (-1.1 mmol/L) and time spent in hypoglycemia (-1.3%) compared to conventional or sensor augmented pump therapies (El-Khatib *et al.*, 2017). In children (6—11 years old), the dual-hormone artificial pancreas was also associated with a lower mean glycemia compared with conventional therapy (7.6 vs. 9.3 mmol/L) while reducing hypoglycemia frequency (Russell *et al.*, 2016). The CLASS (Closed Loop Assessment Study) Program (Montreal) is the first to have developed in parallel single- and dual-hormone systems, allowing a direct comparison between the two strategies in different contexts (Haidar *et al.*, 2013, 2015a,b, 2016; Gingras *et al.*, 2016a; Taleb *et al.*, 2016b). As observed in previous studies, the dual-hormone artificial pancreas improved time in target range while reducing time spent in hypoglycemia compared to conventional therapy as well as to single-hormone artificial pancreas (Haidar *et al.*, 2013); the difference being more pronounced at night than for the entire day (Haidar *et al.*, 2015b, 2017). The longest published face to face comparison of single and dual-hormone artificial pancreas systems (60 h) demonstrated benefit for dual-hormone over single-home artificial pancreas for glucose time below 3.5 mmol/L while both systems were superior to sensor augmented pump therapy for all hyperglycemic thresholds (Haidar *et al.*, 2017).

The addition of pramlintide to the artificial pancreas has recently been suggested and is currently being investigated to further improve glucose control (Weinzimer *et al.*, 2012; Sherr *et al.*, 2016). Pramlintide is a stable analog of amylin, another defective hormone in diabetes, and is known to reduce postmeal glycemic excursion mainly by slowing gastric emptying and suppressing glucagon release (Hay *et al.*, 2015). Meal pramlintide injections in the context of artificial pancreas have been associated with improved postprandial blood glucose excursions in the small studies conducted to date (Weinzimer *et al.*, 2012;Edelman *et al.*, 2006). The use of pramlintide in an infusion pump in the artificial pancreas, before meals or as a continuous infusion with insulin, is being proposed and tested by some research groups. Short-term studies have also explored the addition of other adjunct therapies such as incretins (DPP4 inhibitors and GLP-1 analogs) (Renukuntla *et al.*, 2014; Underland *et al.*, 2017).

Awaiting further studies, it is expected that multihormone artificial pancreas systems would be beneficial for certain patients or in certain situations such as in patients failing to achieve optimal glucose control with single-hormone systems, those with impaired awareness of hypoglycemia or with brittle diabetes, or during exercise (Taleb *et al.*, 2016b). Adding other hormones to the artificial pancreas imposes increased complexity (additional material, pumps, reservoirs, tubules, and catheters) and costs. Another challenge is to prove the safety of such chronic daily use of additional hormones or their analogs. The safety of chronic glucagon use has not yet been established and considering the multiple systemic effects of glucagon, its long-term chronic use has to be monitored (Taleb *et al.*, 2016c). No side effects have been reported in most dual-hormone systems, but an increased nausea perception was observed with the longest study of 11 days (El-Khatib *et al.*, 2017). Finding a stable glucagon formulation that

withstands the fibrillation and degradation in an infusion pump is also a pressing need. The currently used formulation of the hypoglycemia emergency kits require reconstitution and immediate use. Available evidence support possible use in pumps for up to 24-h thus requiring daily change with freshly reconstituted glucagon (Taleb *et al.*, 2017). Thus, despite its potential benefits (lower hypoglycemic risk, lower mean glucose, better postprandial glucose control), multihormone artificial pancreas systems still have a lot of challenges to overcome and warrant further clinical testing to confirm their feasibility before appearing on the market.

Artificial Pancreas Strategies in Challenging Conditions and Use in Specific Patient Populations

Besides the multiple benefits associated with artificial pancreas use, some challenges remain to be tackled such as optimal strategies for glucose control during and postmeals, and exercise. Its potential use and efficacy for specific patient populations also needs to be studied such as in children, pregnant women, and patients with T2DM.

Meal strategies with the artificial pancreas

Controlling postprandial glucose excursions is identified as a key component to achieve recommended HbA1c targets (Ceriello *et al.*, 2004; Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2013) and meal carbohydrate content is the main determinant of this excursion (Scavone *et al.*, 2010; Rabasa-Lhoret *et al.*, 1999). Consequently, in intensive insulin therapy, prandial insulin dosage depends on the carbohydrate content of each ingested meal (carbohydrate counting) (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2013). Precision of carbohydrate counting is associated with better glycemic control (Mehta *et al.*, 2009) but it remains a challenging task for patients (Fortin *et al.*, 2017), with an average error in carbohydrate counting of approximately 20% (Brazeau *et al.*, 2013). Any technology or strategy that would alleviate the burden of carbohydrate counting could not only improve overall glycemic control but patients' quality of life as well. Several strategies have been proposed to control postmeal glucose excursions with the artificial pancreas: (1) a fully automated artificial pancreas (no meal announcement); (2) hybrid artificial pancreas with a meal bolus announcement implemented by the patient based on the carbohydrate content of the meal; (3) hybrid artificial pancreas with a meal announcement strategy that is completely or partially independent of the carbohydrate meal content (simplified meal bolus) (Gingras *et al.*, 2018).

Fully automated artificial pancreas (no meal insulin bolus)

A fully automated artificial pancreas is entirely reactive to real-time glucose values and no meal information is manually announced to the algorithm. While it offers the advantage of relieving the patient from frequent interventions, such as carbohydrate counting; it should not be at the expense of good glucose control. Few groups are investigating the potential of a fully automated artificial pancreas in patients with T1DM (Steil *et al.*, 2006; Ruiz *et al.*, 2012; El-Khatib *et al.*, 2010; Blauw *et al.*, 2016), but these groups have, so far, all faced significant postprandial hyperglycemic excursions and late postprandial hypoglycemia (Gingras *et al.*, 2018). Some meal detection algorithms (Dassau *et al.*, 2008) or the addition of pramlintide (Weinzimer *et al.*, 2012) could potentially improve the performance of the fully automated system, but the strategies have not yet been tested in large randomized controlled studies.

Hybrid artificial pancreas (accompanied with input from patients for meal bolus based on carbohydrate content of meals)

Hybrid artificial pancreas, or artificial pancreas with carbohydrate-matched meal bolus that is manually fed to the algorithm has been well studied (Hovorka, 2006; Clarke *et al.*, 2009). These studies typically demonstrate that hybrid systems, as compared to CSII, increase overall time in target range, reduces time in hypoglycemia, and improves glucose control overnight. However, even if preprandial glucose values are improved, postprandial glucose control remains suboptimal. Glucose levels are kept in-target for 70%–75% of the time with hybrid artificial pancreas with the remaining time spent outside target mostly due to postmeal hyperglycemia (Thabit and Hovorka, 2016a). Limitations of a hybrid system include the cumbersome carbohydrate counting and a potential increased risk of late postprandial hypoglycemia (Steil *et al.*, 2006). A partial prandial bolus could thus be interesting but the optimal strategy remains to be determined. Administration of a prandial bolus equivalent to 30%–50% of a carbohydrate-matched bolus with the rest of insulin being administered based on early postprandial glucose profile has been associated with an improved postprandial glucose profile compared to the fully-automated system; yet, this strategy still requires carbohydrate counting (Weinzimer *et al.*, 2008).

Hybrid artificial pancreas (accompanied with simplified patient input for meal bolus)

Additional strategies have been tested to reduce or avoid the need for carbohydrate counting. In a pilot project, a prandial bolus based on body weight (0.047 U of insulin per kg) was compared with a carbohydrate-matched bolus within the context of dual-hormone artificial pancreas. The simplified approach resulted in more prolonged glycemic excursion (Haidar *et al.*, 2014). The efficacy of a meal bolus based on semiquantitative carbohydrate content assessment: patient's current insulin-to-carbohydrate ratio and meal category (e.g., snack, regular meal, large meal or very large meal) has also been investigated (Gingras *et al.*, 2016a). This strategy was compared to carbohydrate-matched boluses in the context of single and dual-hormone artificial pancreas demonstrating its ability to achieve similar glucose control (Gingras *et al.*, 2016a,b). Adaptive meal-priming boluses have been developed and used in a first study by El-Khatib *et al.* (2014). With this strategy, patients have to select a meal (breakfast, lunch or dinner), and then select a meal size: typical, more than usual, less than typical or a small bite, eliminating the need for exact carbohydrate

counting. Outpatient studies have demonstrated the merits of this approach to improve glucose control in both adults and children (Russell *et al.*, 2016; El-Khatib *et al.*, 2014). Other teams using a similar approach based on semiquantitative carbohydrate meal content rather than precise carbohydrate counting have shown comparable results (Gingras *et al.*, 2016a,b, 2018). However, the simpler meal classification, the way to educate patients, risk associated with meal misclassification, impact of other macronutrients, possible risk of hypo and hyperglycemia, etc., still need to be explored in larger groups of patients with less controlled conditions.

In the current context, informing the algorithm of a pending meal consumption is probably a needed compromise so that the system is prepared to manage rapid prandial changes in blood glucose. Simplified meal priming approaches still require some carbohydrate content or meal size assessment, but are simpler and more convenient to patients than exact carbohydrate counting.

Postprandial glucose control in the context of the artificial pancreas is mainly challenged by CGMS lag time in the context of rapidly varying blood glucose, current pharmacokinetic profiles (delayed action, variability, etc.) of subcutaneously injected insulin, the impact of other nutrients (lipids, proteins, etc.) and the inter and intra-individual variability in insulin sensitivity (Gingras *et al.*, 2018). Some advances could compensate or resolve some of these issues, including: faster acting insulins such as the faster Aspart (FiAsp) in which modified excipients allow faster absorption which improves postprandial glucose profile (Russell-Jones *et al.*, 2017; Fath *et al.*, 2017; Heise *et al.*, 2016), intraperitoneal insulin infusion allowing a more physiologic hepatic action (Renard, 2008), and the use of other adjunctive therapies alongside advanced algorithms such as pramlintide, glucagon-like peptide-1 (GLP-1) analogs and sodium-glucose co-transporter-2 inhibitors (SGLT2-i).

Artificial pancreas strategies with exercise

Despite the established beneficial health effects of exercise, the majority of patients with T1DM lead a sedentary lifestyle (Brazeau *et al.*, 2012; Chimen *et al.*, 2012). This is partly due to the loss of metabolic control and the increased risk of hypoglycemia during and up to 18 h postexercise (Riddell and Sigal, 2013; Brazeau *et al.*, 2008). While in healthy subjects a suppression of insulin secretion and an increase of counter-regulatory hormones (catecholamines, glucagon, etc.) occurs in a timely manner in response to increased uptake of glucose by muscles during exercise, patients with T1DM are challenged by the inability to adjust absorption of previously injected insulin and defective counter-regulatory hormones increasing their risk of hypoglycemia with most types of exercise (Riddell and Sigal, 2013; Colberg *et al.*, 2015; Richter and Hargreaves, 2013). Several strategies have been studied to manage glucose during exercise such as the addition of snacks and/or reduction of basal or insulin boluses, but none of these strategies allow a reasonable hypoglycemia prevention and all require significant anticipation (Taleb and Rabasa-Lhoret, 2016). The first artificial pancreas trials included exercise sessions but were not specifically designed or powered to examine its efficacy with exercise. Analysis of recent studies that specifically address exercise is also limited by the large variability in terms of type, duration, and intensity of exercise as well as snack consumption and time to preceding meals (Taleb *et al.*, 2016b; Breton *et al.*, 2017; Jayawardene *et al.*, 2017). Artificial pancreas control during exercise has shown potential for better glucose control than strategies applied with conventional therapy (Breton *et al.*, 2017; Dovc *et al.*, 2017; Huyett *et al.*, 2017). For example, glucose control was improved with artificial pancreas compared to sensor augmented pump during high intensity prolonged exercise (skiing) in adolescents mainly with improved time in target that was particularly observed late at night (Riddell *et al.*, 2015). Nevertheless, important challenges still need to be overcome with artificial pancreas systems during exercise. This is due to the sharp changes in glucose levels and subsequent delays in glucose sensing which impedes insulin infusion adjustment by the algorithm in a timely manner. Several strategies with the artificial pancreas are being tested such as manual entry of pending exercise to the algorithm (ahead or at its start) or exercise detection by heart rate monitors, accelerometers or other sensors (Taleb *et al.*, 2016b; DeBoer *et al.*, 2016; Jacobs *et al.*, 2015). Another promising strategy is the addition of glucagon in dual-hormone systems; in comparison to single-hormone artificial pancreas the number of exercise-induced hypoglycemic events was significantly reduced (3 vs. 15 episodes out of 34 interventions) with glucagon addition during continuous and interval exercise sessions lasting 60 min (Taleb *et al.*, 2016b). Therefore, the artificial pancreas has a good potential to improve glucose control with physical activity but larger studies are still needed to determine the best strategies that allow a maximum benefit of this technology during exercise.

Particularities of the artificial pancreas in children

The use of the artificial pancreas in children is more recent; yet, studies have shown that its use in children and adolescents is feasible and improves glucose control, especially at night (Weinzimer *et al.*, 2008; Elleri *et al.*, 2013). Many studies have investigated overnight glycemic control in children and these studies showed a substantial reduction of hypoglycemia frequency and duration (Haidar *et al.*, 2015a; Elleri *et al.*, 2014; Hovorka *et al.*, 2014). Fewer studies have investigated the benefits of the artificial pancreas during the day, but a recent study with children aged 5–8 years showed a reduced mean glycemia without increasing time in hypoglycemia using a single-hormone system compared to pump therapy over a 68-h period (DeBoer *et al.*, 2017). Young children are more vulnerable to hypoglycemia and this subgroup of patients presents specific challenges such as unpredictable appetite and food intake and variable physical activity patterns, and thus, the artificial pancreas could be particularly beneficial to address these challenges.

Artificial pancreas in pregnant women with diabetes

Diabetes in pregnancy could be related to a preexisting diagnosis of T1DM or T2DM or could be diagnosed during pregnancy, also called gestational diabetes (Berger *et al.*, 2016). A tight glucose control is required throughout gestation to reduce the multiple

diabetes-related complications for the fetus (macrosomia, neonatal hypoglycemia, respiratory difficulties, etc.) and the mother (infections, preeclampsia, preterm delivery, C-section, etc.) (Berger *et al.*, 2016). Insulin is the mainstay treatment for diabetes in pregnancy through basal-bolus insulin regimen or insulin pumps. The first artificial pancreas study in pregnancy undertaken with a single-hormone system in 12 women with preexisting T1DM showed that thigh glucose control was feasible and safe (less time spent in hypoglycemia) (Murphy *et al.*, 2011). A more recent study compared overnight artificial pancreas control with sensor augmented pumps over a 4-week period in 16 women showing a 15.2% increase in time in target with the artificial pancreas without differences in hypoglycemia (Stewart *et al.*, 2016). The same women were then continued on artificial pancreas control during day and night for up to 14.6 weeks (including hospitalization, labor and delivery); glucose was in target for 68.7% of the time with a mean value of 7 mmol/L (Stewart *et al.*, 2016). The artificial pancreas in pregnancy seems to be safe and promising, thus larger studies are expected in the future.

Artificial pancreas in type 2 diabetes

Many patients with T2DM progress over the years to a state of insulin requirement on top of their insulin resistance (Fonseca, 2009). The use of basal insulin therapy and then intensive basal-bolus insulin regimen are thus needed for a large proportion of patients over the course of their disease. But even under insulin therapy, only one third of these patients reach glycemic targets. It is therefore proposed that the artificial pancreas could potentially improve glucose management in T2DM. So far, only two published studies have tested single-hormone artificial pancreas in T2DM. In the first trial, patients who were treated with oral hypoglycemic agents had 20% improvement in time spent in target glucose levels when the artificial pancreas was used over 24 h in a controlled laboratory setting (Kumareswaran *et al.*, 2014). The second trial was conducted in patients admitted to the emergency ward who were treated with insulin to control their glucose levels; time in target was increased from 38% to 60% with artificial pancreas therapy during their hospital stay (Thabit *et al.*, 2017). An ongoing randomized cross-over study is currently comparing artificial pancreas with MDI over 24-h in patients with T2DM on basal-bolus insulin regimen in a controlled laboratory setting (NCT02490085). Preliminary results showed more time spent within target glucose levels with artificial pancreas (+ 10%) which was particularly significant in the overnight period (+ 28%) without increasing hypoglycemic risk (Nadine Taleb *et al.*, 2017b). Clinical testing of the artificial pancreas is thus warranted in a broader population of patients with T2DM through larger and longer trials including outpatient settings.

Patients' Expectations and Perceptions With Artificial Pancreas Use

Most importantly, for an optimal use of the artificial pancreas, patients' expectations and perceptions need to be considered and prioritized. A study in parents of children with T1DM showed they were interested in the use of the artificial pancreas for nocturnal glycemic control and that they would trust such a system for their children (Elleri *et al.*, 2010). Similarly, a high interest in artificial pancreas use has been observed in adults (van Bon *et al.*, 2011). What patients expect from this technology is an improvement in glucose control with a system that is easy to use and that is reliable (Gonder-Frederick *et al.*, 2011). Recently, a focus group study following artificial pancreas use showed that patients did perceive benefits including improved glycemic control, long-term reduced risk of complications, improved quality of life and a reduced diabetes-associated psychological burden (Iturralde *et al.*, 2017). However, tasks that required their intervention (boluses, calibrations, etc.), technical issues with wearing the system, and a perception of elevated hyperglycemia frequency were reported as negative aspects of this technology (Iturralde *et al.*, 2017). Artificial pancreas development and implementation need to account for patients' experiences and in some cases, some unrealistic expectations must be realigned.

Conclusion

Significant technological advances to manage diabetes have marked the last two decades and continue to move at a fast pace. Insulin pumps are offering a more flexible insulin delivery than multiple daily injections and can be an option for insulin-treated patients. Glucose sensors have taken glucose monitoring to a high level of detailed profiling. Combining these two technologies in sensor augmented pump therapy is currently the gold standard for intensive insulin therapy allowing the lowest achievable HbA1c concurrently to a reduced hypoglycemic risk. Despite the advantages of these advanced features, many barriers still need to be faced to make these costly therapies accessible to patients. Other unmet needs include the way to select patients that would most-benefit from incorporating technology in their treatment as well as optimal educational strategies allowing patients to take advantage of all the features of various devices.

These technologies also allowed the development of the artificial pancreas that has proved its efficacy in further improving blood glucose control and reducing hypoglycemic risk. Several challenges still need to be tackled with the artificial pancreas and larger and longer trials are needed. The addition of other hormones (glucagon and pramlintide) offers an interesting perspective (e.g., achieving fully automated glucose control) but still requires additional work specially to demonstrate incremental benefit and safety. Due to its increased complexity and cost, this multihormonal approach might be only justified in specific patients or conditions. However, on the long-term, a multihormonal approach is viewed as the way to reach fully automated glucose control. The first hybrid system has already been approved in the United States and other systems such as the iLet (Beta Bionics) and

Diabeloop (in France) will reach the market in the coming 2–3 years. The artificial pancreas is considered by most experts as the most promising therapy to revolutionize diabetes management making it safer and simpler.

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See also: Hypoglycemia

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Transplantation: Pancreatic and Islet Cells

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Nomenclature

β cells Beta cell
HbA_{1c} Hemoglobin A1c
MSC Mesenchymal stem cells
T1DM Type 1 diabetes mellitus
Tregs T regulatory cells

Glossary

Type 1 diabetes Type 1 diabetes is a chronic autoimmune disorder that attacks beta cells within islets of Langerhans in the pancreas. Insulin injections are life-sustaining but imperfect, with imprecise glycemia, risk of hypoglycemia and accelerated secondary diabetic complications.

Islet cell transplant Islet cell transplant is the clinical application of isolating islets from a donor pancreas with intraportal transplantation into patients with unstable type 1 diabetes. Once transplanted, endogenous regulated insulin secretion is restored to variable degrees dependent upon engrafted islet mass.

Introduction

Diabetes currently affects over 382 million people throughout the world and is expected to rise to 592 million by 2035 (Guariguata *et al.*, 2014). Type 1 Diabetes Mellitus (T1DM) is treated with exogenous insulin. This is typically given in multiple boluses by subcutaneous injection of short and long-acting forms. Tight glycemic control mitigates secondary complications and end-organ failure, but increases the risk of severe and recurrent hypoglycemic reactions. Continuous glucose monitoring systems (CGMs) may improve monitoring and control, and closed loop technology that infuses insulin and/or glucagon in response to glycemic thresholds may further optimize control. These systems are bulky however, must be worn externally, and often still fail to provide perfect glycemic control.

T1DM is an autoimmune disease with complex genetic and environmental overlay. Controlling autoimmunity in new onset diabetes remains challenging due to the complexity of the multicomponent and heterogeneous immunologic response. The potential to intervene and potentially reverse beta (β) cell destruction after clinical diagnosis of diabetes could be accomplished by identifying potential risk markers and quantified risk projection (Skyler and Ricordi, 2011; Skyler *et al.*, 2008). Intensive pilot trials conducted through TrialNET have demonstrated sustained honeymoon periods but do not routinely restore normoglycemia. One exceptional clinical trial (NCT00315133) conducted in Brazil by Voltarelli *et al.* attempted to immune reset newly diagnosed T1DM patients. The trial utilized peripheral blood autologous bone marrow-derived hematopoietic stem cells after myeloablative conditioning with cyclophosphamide and rescue with granulocyte-colony stimulating factor. Approximately two to 3 weeks post myeloablative conditioning, autologous stem cells were thawed and administered intravenously. The majority of subjects (20 of 23) demonstrated reversal of diabetes for sustained periods. However the immune reset caused dysregulation of T and B cell responses, subjects frequently developed cyclophosphamide-related oligospermia and other adverse endocrine dysfunctions, polyendocrine syndrome and side effects such as pneumonia and rashs (Malmegrim *et al.*, 2017; Couri *et al.*, 2009; Voltarelli *et al.*, 2007). The future direction to prevent or decrease the progression of T1DM needs to evaluate the memory T and B cell subsets in an effort to abolish recurrent autoimmune destruction.

An alternate means to restore euglycemia in patients suffering from T1D is to replace β cell mass through transplantation of a whole pancreas or an islet transplant. Whole pancreas transplantation is an invasive surgical procedure that carries risk of complications and potential death, but is currently the most reliable means to restore insulin independence with glycemic reserve. Islets of Langerhans are clusters of cells composed of four main cellular components: glucagon-producing α cells, insulin-producing β cells, somatostatin-producing δ cells, and pancreatic polypeptide-producing cells (Halban, 2004). Islets range in size from <50 to ~800 μm of diameter, and only constitute $\sim1\%$–2% of the total pancreatic tissue (Bonner-Weir *et al.*, 1993; Halban, 2004; Vanikar *et al.*, 2016). As highly vascularized cells, islets rely on complex cell–cell interactions between different cell subsets in order to maintain glucose homeostasis. Islet transplantation (IT) is generally considered a safer approach as it does not require invasive surgery, and only the islets contained in the pancreas are transplanted without the challenges associated with exocrine drainage. Islet cells are obtained from deceased organ donor pancreatectomy, ABO compatible with the recipient, and infused into the recipient portal vein under the cover of anticoagulation, antiinflammatory medications and inductive and maintenance immunosuppression. While insulin independence is often achieved at early time points, maintaining a completely insulin free state is challenging due to early and late islet cell loss. Islet loss may occur due to an instant blood-mediated inflammatory reaction (IBMIR) triggered by tissue factor expressed on the islet surface, inflammation, alloimmune rejection, recurrence of autoimmunity, or toxicity to β cells from immunosuppressive medications. To alleviate islet cell loss, alternative transplantation sites, stem cell-derived cellular products, cotransplantation with alternative cells or immune protective agents are the focus of active study. **Table 1**

Table 1 Comparison of islet versus whole pancreas transplantation

	Islet	*Pancreas*
Reduce or eliminate hypoglycemia	Yes	Yes
Insulin independence	Yes—but often transient	Yes—more durable reserve than islet
Improves glycemia and HbA_{1c}	Yes	Yes
Improves diabetic-related complication	Not Known	Improved
Procedural risks	Minor	Major
Number of procedure	Multiple infusions	One transplant

Both islet and pancreas transplantation can render insulin independence and normoglycemia. Pancreas transplantation is associated with multiple risks, including graft thrombosis, hemorrhage, pancreatitis, wound infection, etc. Whereas islet transplantation has minimal procedural risks such as posttransplant lymphoma, thrombosis posttransplant.

compares the relative benefits and disadvantages of islet over whole pancreas transplantation. While islet transplantation (IT) is safer than pancreas transplantation, it is associated with lower insulin reserve and lower rates of sustained insulin independence over time. Both require potent immunosuppression to sustain allograft function.

Pancreas Transplantation

Whole pancreas transplantation is an alternative procedure that can restore normoglycemia for patients without β cell function. The first pancreas transplant was carried out at the University of Minnesota by Kelly and Lillehei in 1966 (Kelly *et al.*, 1967). The rationale for the transplant was to prevent the recurrence of diabetic nephropathy in the previous transplanted kidney. Early outcomes were dismal, due to the need for high dose corticosteroid therapy. Introduction of more potent immunosuppressive agents such as cyclosporine and tacrolimus, and more potent inductive T-cell depletion therapies improved short and long-term outcomes of this surgical therapy in diabetes. By 1980, first and third year patient survival was 91% and 72%, respectively and pancreas graft survival was 84% and 62%, respectively (Gruessner and Sutherland, 1996). Currently, the International Pancreas Transplant Registry (IPTR) reports >42,000 pancreas transplants have been performed worldwide (>27,000 in the US and >15,000 elsewhere). Pancreas transplantation can be subcategorized as pancreas after kidney transplantation (PAK), islet after kidney transplantation (IAK) or simultaneous islet-kidney transplantation (SIK). The patient survival postpancreas-transplant can be as high as 95%, with up to 90% graft survival postprocedure (Sutherland *et al.*, 2001). Whole pancreas transplantation is a high-risk procedure that is currently the most reliable means to restore and maintain insulin independence.

Islet Transplantation

History

The earliest evidence of IT can be traced back to 1893 when Watson Williams and Harsant subcutaneously implanted fragments of sheep pancreases into a 13-year-old boy (Williams, 1894). The patient died within 3 days of the procedure, with the xenograft cells being rapidly rejected. Paul Lacy was the first to demonstrate in 1972 that chemically diabetic rats could be cured with islet transplantation (Ballinger and Lacy, 1972). In 1980, Najarian and colleagues from Minnesota performed a successful islet autotransplant, whereby the patient's own islets were transplanted into the portal vein in the setting of chronic pancreatitis (Najarian *et al.*, 1980). Ricordi developed a unique chamber and semiautomated method in 1988 to digest the human pancreas and liberate islets that could then be purified and transplanted (Ricordi *et al.*, 1989). The ability to obtain large quantities of islets was important for the clinical application of IT in 1990 when the first case of transient insulin independence was achieved post IT by Scharp *et al.* (1990). Almost 300 clinical attempts at islet allotransplantation were carried out in patients with T1DM between 1980 and 1999, but fewer than 8% resulted in anything more than transient insulin independence. In 2000, Shapiro and colleagues developed the Edmonton Protocol, the results of which transformed the field. The Edmonton Protocol was groundbreaking through use of: (i) a corticosteroid-free immunosuppressive protocol using the combination of potent drugs sirolimus and tacrolimus, combined with an anti-CD25 antibody to protect against rejection and recurrent autoimmunity; and (ii) augmenting the islet mass (>13,000 islet equivalents (IE) kg^{-1} recipient body weight) through transplantation of islets prepared from two or more fresh donor pancreatic islet preparations (Shapiro *et al.*, 2000). As a consequence of this refined approach, seven consecutive patients receiving an islet transplant achieved insulin independence up to 1 year posttransplant, a feat unprecedented at the time. Since the inception of the Edmonton Protocol, approximately 300 patients have received islet transplants at the University of Alberta with one-year insulin independence rates achieved in 80% of transplant recipients. The success of the Edmonton Protocol rejuvenated global interest in clinical islet transplantation, with numerous programs developed globally. Since 2000, >1000 patients have been transplanted using recent variants of the Edmonton Protocol in almost 50 centers worldwide (2015). At the University of Alberta, insulin independence is maintained in approximately 25% of patients at 5 years' post-transplant. Perhaps more importantly, these rates double to 50% in such patients receiving newer immunosuppression and

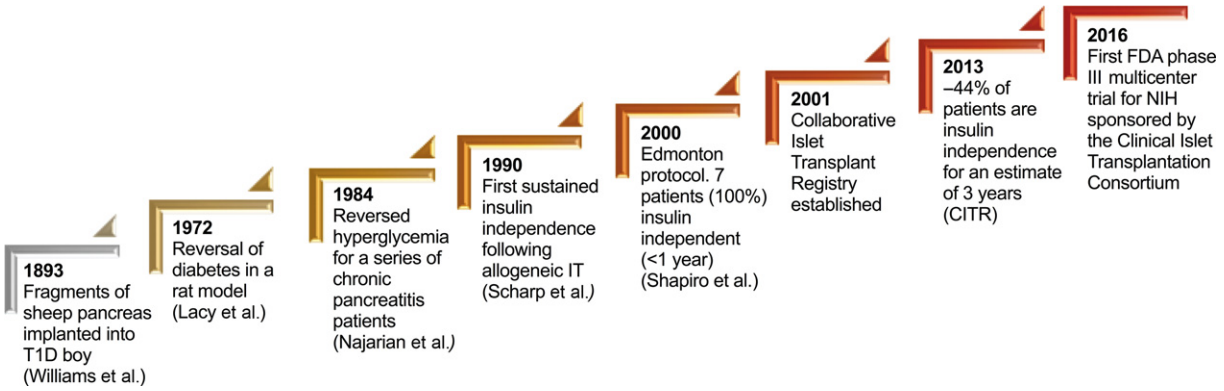

Fig. 1 Islet cell transplantation timeline chart. Throughout the past few decades, islet cell transplantation has evolved into a feasible clinical treatment for patients with brittle diabetes.

antiinflammatory induction protocols. The discrepancy between the prevalence of maintained C-peptide secretion relative to long-term insulin independence rates remains elusive, although it is accepted that multiple culprits may be responsible for hindering graft function and survival over time. To achieve insulin independence, most patients require two islet transplant procedures, and in some cases, three infusions. The achievement of insulin independence after single-donor islet infusion has occurred in few transplant centers globally, with the most success reported from the University of Minnesota (Hering *et al.*, 2004, 2005). At the University of Minnesota, they investigated donor characteristics of young donors (<50 years) with a high BMI (>27 kg m^{-2}) can achieve insulin independence via a single donor (Shapiro *et al.*, 2006). Moving forward, the field of IT has nonetheless continued to grow from an experimental procedure into a well-tolerated and effective clinical procedure. According to the Collaborative Islet Transplant Registry (CITR), over 1584 IT infusions in 819 recipients have been performed from 1999 to 2013, and currently 27 active registered centers are open (Collaborative Islet Transplant Registry, 2016). IT has drastically improved over the past few decades due to technology, improved islet quality and redefined immunosuppression regimens (**Fig. 1**). However, IT has yet to be a feasible clinical procedure for all spectrums of diabetes, and a breakthrough in the IT community is bound to emerge.

Islet Isolation, Purification and Culture

The islet cell isolation and transplant procedure can be foreseen as harmful to the islets, because it strips the islets from their native vascularization and environment. The automated method is part of the 5–7 h multistep process that carefully isolates, purifies and processes donor pancreatic tissue into singular clusters of islet cells (**Fig. 2**). The process commences with pancreas procurement from a deceased donor. The donor characteristics are important for acquiring an optimal islet cell yield. Characteristics such as age (between 20 and 50 years), BMI (>30 kg m^{-2}) and normalized HbA_{1c} levels are critical (Shapiro *et al.*, 2017). The donor pancreas is "cleaned," by the removal of the spleen, duodenum, surrounding fat, lymph nodes and vessels. Intravenous catheters are inserted into the main pancreatic ducts, and the pancreas is perfused with cooled collagenase and serine–protease inhibitor that serves to initiate enzymatic digestion. Once distended, the pancreas is cut into several small pieces and placed into the Ricordi chamber. The Ricordi® chamber is contains a superior and an inferior component that is seperated by a 700 μm filter. Within the chamber are stainless steel balls and is filled with digestion solution. The chamber is agitated to mechanically disrupts the tissue, and a peristaltic pump connected to the system creates a flow throughout the closed circuit. The digestion is terminated when most of the islets are free from the surrounding exocrine tissue by the addition of cold media (10°C). It is important to note, the time of terminating the digestion is critical because over digestion can lead to a decrease in islet cell yield and compromised function. The islets undergo a purification step, where a COBE 2991 cell processor utilizes a continuous density gradient of Biochrom Ficoll that separates the islets and acinar tissue. At the end of this step, the islets are collected and counted based on their morphology by dithizone (DTZ) staining. DTZ bonds to the zinc in the insulin granules and gives a visual red appearance to the islets, differentiating islets from exocrine tissue. The islets undergo a regimen of safety testing that analyzes the cells' viability, purity, insulin content, cell numbers and insulin secretory response. Once analyzed, the final enriched islet cell product must have the adequate islet purity ($>50\%$), dose (>5000 IEQ kg^{-1}), and have a volume less than 5cm^3 in size, in order to be safely infused into the recipient by a intraportal infusion (Kawahara *et al.*, 2011). Depending on the center preference, the purified islet cells can be infused within a few hours after the end of the isolation or cultured at 20°C or 37°C for a period up to 72 h (Yamamoto *et al.*, 2009). The culture period can be foreseen as beneficial for the islets because it may help reduce the immunogenicity of the islets and allow time for the recipient to commence immunosuppressive treatment, but despite considerable research efforts aimed to delineate optimal culture conditions, protocols have yet to be standardized (Ichii *et al.*, 2007).

Islet Transplant Procedure

Clinical islet transplantation is routinely carried out by intrahepatic infusion via the portal vein. The portal vein is located by interventional radiology that uses ultrasonographic and fluoroscopic guidance to make the hepatic infusion. The advent of the

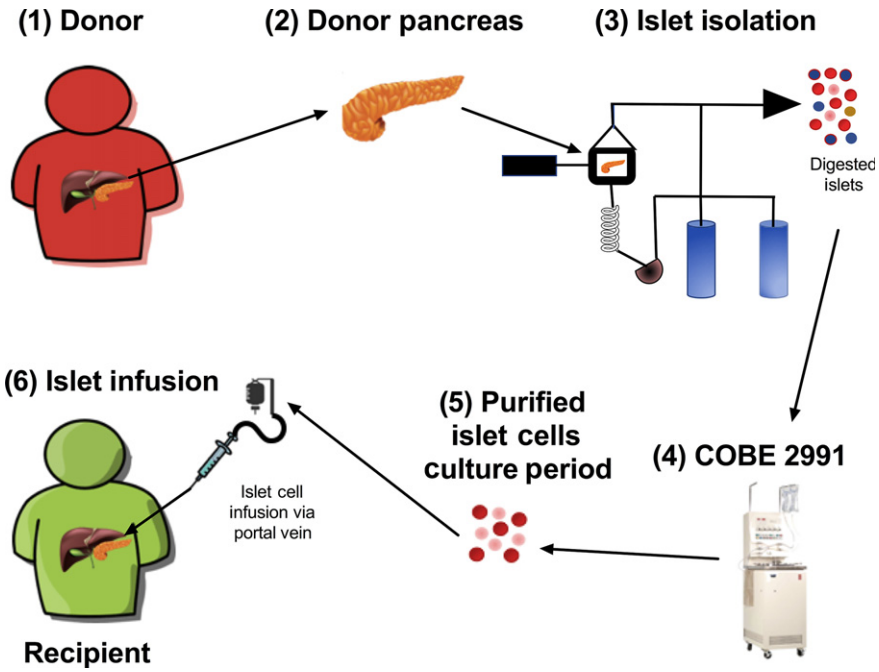

Fig. 2 Islet cell procedure. (1) A deceased human donor pancreas is isolated. (2) The pancreas is "cleaned" from surrounding tissue and is perfused with collagenase enzyme. (3) The schematic of digestion through the Ricordi chamber. (4) COBE 2991 cell purifies the islets utilizing a continuous Biochrom osmotic gradients. (5) After purification, viability and other islet assessments, the islets are cultured for a period up to 72 h. (6) The highly purified human islets are infused into the portal vein of a recipient with type 1 diabetes.

percutaneous method has made the procedure relatively noninvasive because it does not require general anesthesia or surgical incision. During the procedure, a potential complication is the risk of thrombosis and bleeding; however, sealing the catheter upon abstraction with a thrombostatic paste and administering heparin reduces the potential of thrombosis and hemorrhage (Kawahara *et al.*, 2011). Throughout the 15 to 60-min procedure, the portal pressure is well documented to be below 20 mmHg and the patient status is monitored. Even with minimal complications, the intrahepatic site may not be an optimal environment for islet engraftment. It is estimated that 50%–70% of islets are acutely destroyed following an intrahepatic infusion (Shapiro *et al.*, 2001). Alternative implantation sites have been explored in the clinical setting like the bone marrow (Maffi *et al.*, 2013), striated muscle (Christoffersson *et al.*, 2010), and omentum (Baidal *et al.*, 2017), but no site has yet to provide regulated glycemic control as efficiently as the intrahepatic infusion.

Islet Engraftment

Subsequent to organ retrieval, islet isolation and culture, the islet engraftment as a result of transplantation may present with some obstacles that may impede successful outcomes. To survive and function posttransplantation, islets must adequately engraft by developing new capillaries and vessels with the native islet vasculature. The engraftment process initiates 1–3 days posttransplant, whereby the new network develops from the recipient's blood vessels in concert with the remnants of the donor islet endothelium (Emamaullee and Shapiro, 2007). During this time, islets are subjected to considerable environmental stresses within the first days of transplant that can contribute to a reduction in the original islet mass by 50%–70% (Emamaullee and Shapiro, 2007; Merani *et al.*, 2008; Wang *et al.*, 2011).

The IBMIR reaction accounts for considerable islet loss in the acute transplant period. Extensive work by Korsgren *et al.* have elucidated that IBMIR is initiated upon infusion of islets into the portal vasculature (Ozmen *et al.*, 2002). IBMIR negatively influences islet engraftment through a cascade initiated by the expression of tissue factor, resulting in platelet adherence, activation, clot formation and lymphocyte recruitment (Plesner and Verchere, 2011; Emamaullee and Shapiro, 2006). These results suggest that despite being the current best clinical site for islet transplantation, the portal vein still has substantial limitations that need to be further optimized if long term outcomes of islet transplantation are to continue to improve.

Immunosuppression for Islet Transplantation—Risks and Side Effects

Islet transplant recipients require chronic maintenance immunosuppression to prevent graft rejection and to mitigate risk of recurrence of diabetic autoimmunity. The most potent antirejection treatments (tacrolimus and cyclosporine calcineurin inhibitors (CNI) have direct toxicity to beta cells (Nir *et al.*, 2007; Chand *et al.*, 2001; Ekberg *et al.*, 2009). Further, they inhibit insulin section

of the human islets (Rangel, 2014). Alternative CNI-free immunosuppression regimens have been investigated for IT, but no protocol has yet to provide significant evidence that avails long-term islet survival and renal protection (Posselt *et al.*, 2010). Notably, Efalizumab (Turgeon *et al.*, 2010) and Belatacept (Vincenti *et al.*, 2010) demonstrated the ability to become insulin independent for sustained periods without CNIs. Although a CNI-free regimen is desirable for islet engraftment, all medications that suppress the immune surveillance system pose increased risk of both cancer and infections for recipients (Penn, 2000). Immunological tolerance seeks to induce a stable, nonresponsive state to an islet or other organ allograft without need for chronic immunosuppression, and remains a much sought after goal in IT (Shapiro *et al.*, 2016). Moving forward, the use of encapsulation devices or therapeutic agents could potentially facilitate this, but are inherently challenged due to failure of engraftment and low cell survival, mainly due to lack of early rapid neovascularization.

Potential Islet Engraftment Improvements

Cotransplantation with mesenchymal stem cells

Mesenchymal stem cells (MSCs) are nonhematopoietic precursor cells that can differentiate into mesoderm lineages: adipocytes, osteocytes, chondrocytes, and myocytes (Brusko, 2009; Majumdar *et al.*, 2000). MSCs can be isolated from a multiple tissues sources such as amniotic fluid (Loukogeorgakis and De Coppi, 2016), skeletal muscle (Williams *et al.*, 1999), adipose tissue (Mahmoudifar and Doran, 2015), or umbilical cord (Lazarus *et al.*, 2005). Currently, inefficient evidence of MCSs' ability to differentiate into insulin producing cells is available, but the utilization of cotransplanting islets with MSCs can support islet engraftment (English and Wood, 2013).

The employment of MSCs in the IT field is an exciting endeavor because MSCs' can ameliorate islet engraftment by their secretion of trophic factors and assist the immune regulation (Zhang *et al.*, 2004b). Trophic factors secreted by MSCs include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Angiopoetin-1 (Ang-1), and transforming growth factor-β (TGF-β) have been shown to enhance islet engraftment (Kinnaird *et al.*, 2004; Liu and Han, 2008). In doing so, the trophic factors assist in the reduction of the migration and proliferation of immune cells, and promote vascularization in the islet engraftment (Sordi *et al.*, 2005; Caplan and Dennis, 2006; Chen *et al.*, 2008a). Carrying over, MSCs have demonstrated its ability to downregulate the immune response by interfering with different pathways of the immune response, via direct and indirect cell-to-cell interactions in both in vitro and in vivo (Vija *et al.*, 2009) (Le Blanc *et al.*, 2003; Liu and Han, 2008; Ramasamy *et al.*, 2007). MSCs can suppress T and B cell proliferation, interfere with dendritic cell maturation, modulate natural killer cells cytotoxicity, and decrease immunoglobulin production to a certain extent that promotes islet cell engraftment survival (Healy *et al.*, 2015; Kim *et al.*, 2011; English, 2016). Evidently, the cotransplantation of human islets with MSCs has the potential to help islet engraftment in the clinical setting (Yeung *et al.*, 2012).

T regulatory cells infusion

T regulatory cells (Tregs) are immune subpopulations of lymphocytes that can promote the tolerance of foreign particles and maintain immune homeostasis (Krzystyniak *et al.*, 2014). Regarding T1DM, Bluestone and colleagues demonstrated the ability to isolate and expand Tregs into newly diagnosed T1DM patients in an effort to repair or replace the native Tregs from autoimmune β cell destruction. (Gitelman and Bluestone, 2016; Bluestone *et al.*, 2015). Specifically for IT, Tregs cotransplanted with islets have been shown to help prevent autoimmunity and rejection of the islet engraftment (Marek *et al.*, 2011; Wu *et al.*, 2013). In the animal model, a localized cotransplantation with Tregs and islets within a kidney graft can significantly protract islet function and survival (Krzystyniak *et al.*, 2014). In the clinical setting, a ratio of 5:1, Tregs ($\sim 53 \times 10^9$ Tregs) to islets, can potentially induce the immunological tolerance during an IT (Tang and Bluestone, 2013). Tregs capability to regulate both the alloresponse and the autoimmunity make it an exciting addition in the employment of IT.

The Subcutaneous Space as an Alternative to Portal Vein Infusion

Intraportal islet infusion currently serves as the site of transplantation in the clinical realm. As previously noted, percutaneous infusion of islets into the portal vein has demonstrated the ability to regulate glycemic control in patients with brittle diabetes (Rajab, 2010). While the procedure itself is minimally invasive, it is not void of procedural risks and complications, including bleeding to portal vein thrombosis (Barshes *et al.*, 2005). Moreover, the initiation of inflammatory cascades through platelet activation and coagulation can confer significant islet loss. These events have propagated considerable experimental and clinical interest in identifying an alternative anatomical site for clinical islet transplantation. It is proposed that the ideal site for islet transplantation and subsequent engraftment should be in close proximity to vascular networks that supply physiologically sufficient oxygen and nutrients, as well as provide ample space to accommodate the necessary transplant volume. Furthermore, a site that can be easily monitored and is retrievable is also desirable, particularly if insulin-producing stem cells are to be transferred into clinical practice (Veriter *et al.*, 2013).

The translation of alternative transplant sites from experimental models to clinical practice has been met with considerable limitations. For example, the kidney capsule has demonstrated the ability accommodate a smaller islet mass capable of restoring euglycemia in rodent models, however when translated to humans, this site is clinically unfeasible, namely in that a greater transplant mass is required to restore glycemia in contrast to portal vein infusion. The subcapsular space is also considerably much more invasive and as such would present as less ideal from this perspective (Jindal *et al.*, 1998). Numerous additional transplant

sites have been explored experimentally, including the spleen, muscle and immune privileged sites, however, these sites have not conferred significant benefit. Within recent years, the subcutaneous space has garnered significant attention as an ideal site for islet transplantation for multiple reasons, including the ability to accommodate large transplant volumes, is easily accessible, and retrievable, if required. A primary limitation to the subcutaneous space is that it presents with poor blood supply, relative to the portal site, of which could compromise islet viability in the acute transplant period. As such, the utility of subcutaneous devices to promote neovascularization prior to islet infusion have been explored, and in some cases have been met with limited clinical success (Gala-Lopez *et al.*, 2016). Pepper *et al.* explores an effective means to exploit the foreign-body response, by creating a vascularized network in the subcutaneous space by the temporary transplantation of a medically approved vascular access catheter up to 4 weeks postimplantation. Removal of the catheter extinguishes the foreign body response, and the resulting void space is capable of accommodating the survival of human islets as well insulin-producing stem cells (Pepper *et al.*, 2015; Pepper *et al.*, 2017). This modified approach eliminates the need for long-term implantation of a subcutaneous device, while sufficiently correcting hyperglycemia in rodent models. Strategies aimed to utilize the subcutaneous space as an alternative site for islet engraftment holds significant promise for current and future clinical applications.

Alternative Cellular Sources to Ameliorate T1DM

Xenotransplantation

Since numerous islet infusions are often required to correct the consequences associated with T1DM, the identification of a xenogeneic supply of islets is an attractive alternative option to circumvent the need for human donor pancreases. This approach could provide an unlimited source of tissue to treat as many diabetic patients as required. One such xenogeneic option is the pig, a widely available source capable of producing insulin that is functional in humans. In 1994, Groth and colleagues presented the first clinical case where fetal porcine islet cell clusters transplanted under the renal capsule of a T1DM patient. This case demonstrated that these clusters were capable of surviving in patients as evidenced by sustained release of porcine C-peptide for many months, though unable to restore euglycemia and insulin-independence (Groth *et al.*, 1994).

Despite limited clinical success within the last decade, preclinical porcine islet xenotransplantation continues to garner enthusiasm. Of note, considerable progress using porcine tissue has been made in nonhuman primate models that utilize genetic donor manipulation and immunosuppressive protocols (Cardona *et al.*, 2006; Hering and Walawalkar, 2009; Hering *et al.*, 2006; van der Windt *et al.*, 2009; van der Windt *et al.*, 2012). For example, in immunosuppressed diabetic nonhuman primate studies where wild-type or genetically engineered porcine islets have been transplanted, the achievement of normoglycemia has been observed for more than 1 year posttransplant (Ekser *et al.*, 2016; Park *et al.*, 2015). Indeed, considerable efforts are currently being undertaken to establish highly monitored clean pig colonies to eliminate the potential for the transmission of zoonotic infections or endogenous retroviruses (Bottino and Trucco, 2015; Fishman and Patience, 2004; van der Windt *et al.*, 2012).

Efforts to transplant encapsulated neonatal porcine islets in nonimmunosuppressed diabetic patients are currently underway in New Zealand. Results from this clinical trial revealed only modest clinical outcomes, as a marginal reduction in hypoglycemic unawareness was reported (Matsumoto *et al.*, 2014). A second nationally regulated clinical trial in Argentina demonstrated improvements in HbA_{1c} with reduced hypoglycemic unawareness events up to 2 years posttransplant, though again only marginal reduction in insulin dose was observed (Matsumoto *et al.*, 2016). No risk of porcine endogenous retroviruses (PERV) was observed (Ekser *et al.*, 2016; Matsumoto *et al.*, 2016; Morozov *et al.*, 2017). Furthermore, the utilization of CRISPR-Cas9 can inactivate 62 copies of the PERV *pol* gene in a porcine kidney cell line and results in a >1000-fold reduction in PERV transmission to human cells (Yang *et al.*, 2015). Recently, PERV-inactivated pigs were successfully generated by Nui and colleagues and sheds light on the safety concern of the clinical application of porcine-to-human xenotransplantation (Niu *et al.*, 2017) Indeed, the results from this clinical trial made inroads in clinical islet xenotransplantation, however, further refinements to porcine islet xenotransplantation are necessary to advance large-scale clinical trials (van der Windt *et al.*, 2012).

Progress in gene therapy

Gene therapy is being explored both locally within transplanted islets to make them more robust, or systemically to transmute the native liver hepatocytes to secrete insulin. The possibility of genetically modifying islets to obviate immune rejection is an attractive approach, but current adenoviral vector strategies carry potential risk, and typically only affect the mantle islet cells and fail to reach the core. Application of adeno-associated viral vectors, herpes simplex viruses, retroviral vectors and other associated vectors have shown promise in rodent models (Wang *et al.*, 2011). To improve islet function, several factors must be considered, but decreasing the period to revascularization is beneficial for islet engraftment. To improve vascularization, an adenoviral transduced with VEGF-A has exhibited superior microvascular density and long-term survival in a mouse model (Zhang *et al.*, 2004a; Yancopoulos *et al.*, 2000). Likewise, Olerud and colleauges revealed that the utilization of a Thrombospondin 1 (TSP-1) knockout mouse model by RNA silencing can improve islet vascularizing (Olerud *et al.*, 2008). To help alleviate the occurrence of apoptosis prior to and postisolation can be foreseen by three different mechanisms; blocking the inducer, modulation of key proteins, and inhibiting effector caspases (Wang *et al.*, 2011). Key inhibitors for apoptosis proteins (IAPs), such as X-linked IAP has demonstrated its ability to restrain apoptotic cell death (Hui *et al.*, 2005).

Recent advances in genetic engineering and immunobiology have opened up the possibility of abrogating immune rejection through genome editing. An alternative approach to restore endogenous insulin production may be facilitated by viral vectors with transcriptional factors specific to β cell development, but transduced within non-β cells. Target transcriptional factors include

PDX1, NeuroD1, Neurog3, Nkx2.2, and Mafa. The ability to transfect alternative cell sources has been demonstrated in pancreatic exocrine cells (Li *et al.*, 2014), keratinocytes (Mauda-Havakuk *et al.*, 2011), adipose-derived stem cells (Lin *et al.*, 2009), and hepatocytes. Hepatocyte transduction represents a strong possibility as hepatocyte development shares similar embryological pathway development with the pancreas. The liver and pancreas both arise from the foregut endoderm with similar time in embryonic development and have similar glucose-sensing mechanism, such as glucokinase (GK) and glucose transporters 2 (Glut2) (Burcelin *et al.*, 2000). Diabetes has been reversed successfully in both rodent and large animal models using viral transfection (Dong *et al.*, 2001; Auricchio *et al.*, 2002; Calne, 2005; Alam and Sollinger, 2002; Chen *et al.*, 2008b; Gan *et al.*, 2016; Fodor *et al.*, 2007). Notable investigations from Calne *et al.* evaluate adeno-viruses, such as single stranded serotype 8 pseudotyped adeno-associated virus (ssAAV2/8) vectors, that encode human proinsulin in hepatocytes and demonstrate the ability to secrete human C-peptide and both improve and maintain euglycemia in an animal model (Gan *et al.*, 2016). These advances will escalate rapidly if a regulated glucose-sensitive insulin promotor and on/off control mechanisms could be built into the constructs.

An alternative approach is the application of a lentiviral transduction of the insulin gene into MSCs, such as primary canine MSCs, that demonstrate the ability to secreting insulin immediately and long term in vitro (Gautam *et al.*, 2016). Auto-transplantation could alleviate the necessity for immunosuppressive drugs since the cellular source is derived from the transplanted host, but factors such as unregulated insulin gene expression, surgical removal for biopsies, autoimmune destruction and possible teratoma limits its applicability, as do the possibility of autoimmune destruction from recurrent T1DM (Lee *et al.*, 2012). The ability to improve and utilize gene therapy may have important implications for the progression of diabetes management, but the lack of investigation to date may deprive its clinical potential.

Stem cell transplantation

Inroads in developmental biology and regenerative medicine have significantly contributed to research efforts currently underway to identify suitable islet precursor cells. Given that donor criteria, pancreas digestion and islet isolation can all contribute to compromised islet engraftment outcomes, the identification precursors with the potential to differentiate into an unlimited source of insulin-producing β-cells is an attractive approach to negate the need for pancreas donors. History has proven, however, that such a feat has been challenging to achieve; the ability to produce such cells capable of secreting insulin in a physiologically responsive manner whilst proliferating in a controlled manner (Bonner-Weir and Weir, 2005; Otonkoski *et al.*, 2005).

Considerable efforts have been undertaken to establish a renewable source of insulin-producing cells. For example, some approaches have been undertaken to exploit the pancreas's native ability to reestablish its β-cell population in response to injury by utilizing various transcription factors (Bouwens *et al.*, 2013; Zhou *et al.*, 2008). Others have sought to utilize cells derived from the hematopoietic lineage, including bone marrow-derived cells, as well as umbilical cord blood, as precursors to insulin-producing cells. Umbilical cord blood as a precursor is advantageous in that it can be easily obtained and would avoid some of the ethical implications associated with the use of stem cells. However, some animal studies utilizing hematopoietic lineages as a means to augment endogenous β-cell levels were inconclusive (Ianus *et al.*, 2003; Suri *et al.*, 2006). However, in a clinical study conducted by Haller and colleagues whereby newly diagnosed patients receiving autologous umbilical cord blood revealed lower HbA_{1c} and reduced insulin requirements (Haller *et al.*, 2008). Moreover, a study by Couri *et al.* revealed that newly diagnosed patients with T1DM infused with hematopoietic stem cells achieved insulin independence and exhibited elevated C-peptide levels (Couri *et al.*, 2009).

The self-renewing capacity and pluripotent properties of embryonic stem cells (ESCs) have warranted considerable research attention in recent years. Strategies to differentiate ESCs into insulin-producing cells have been underway since the early 2000s, and while initially promising, were met by impediments including low numbers of insulin-positive cells and a lack of glucose sensitivity (Assady *et al.*, 2001; Hori *et al.*, 2002; Lumelsky *et al.*, 2001; Soria *et al.*, 2000). Pivotal work in 2004 and 2006 laid the foundation to the development of effective strategies to differentiate human ESCs into pancreatic endoderm cells (PEC), containing both insulin and C-peptide (D'Amour *et al.*, 2006; Kubo *et al.*, 2004). Subsequent improvements resulted in the development of glucose-sensitive cells capable of restoring euglycemia in diabetic rodent models (Kroon *et al.*, 2008). Novel approaches have also been undertaken to differentiate human ESCs into pancreatic progenitor cells in vitro with final stages of differentiation into glucose-responsive, insulin-producing β cells in vivo (Bruin *et al.*, 2013; D'Amour *et al.*, 2006; Kelly *et al.*, 2011; Kroon *et al.*, 2008; Rezania *et al.*, 2012; Schulz *et al.*, 2012). Strategies to consistently produce PEC in sufficient quantities for clinical trials have also been developed (Schulz, 2015) with the capacity to restore normoglycemia (Pagliuca *et al.*, 2014; Rezania *et al.*, 2014).

Many of these seminal studies were paramount in the establishment of a US Food and Drug Administration- and Health Canada-approved, first-in-human pilot phase 1/2 clinical trial to be conducted by ViaCyte Inc., a commercial leader in regenerative medicine technologies. Approved in 2014, this trial sought to test the VC-01 combination product, which combines CyT49 hESC-derived PEC contained within an immune-protective, macroencapsulated device transplanted subcutaneously in a small cohort of patients with T1DM (NCT02239354). The results of this pilot study lead to further approval of a safety and tolerability study of Viacyte's PEC-Direct VC-02 combination product in patients with T1DM (NCT03162926). Further studies utilizing pancreatic progenitor cells transplanted into extrahepatic, subcutaneous devices will undoubtedly require further optimization and refinements to improve engraftment, oxygen delivery and metabolic exchange.

The prospect of establishing a limitless supply of glucose-responsive, insulin-producing cells as means for β-cell replacement therapy is undeniably attractive. Concerns pertaining to the risk of teratogenicity, as well as ethical and religious considerations over the use of ESCs warrant notable caution to proceed (Liu *et al.*, 2013; Werbowetski-Ogilvie *et al.*, 2009; Vanikar *et al.*, 2016). However, if such a product can be ethically derived from a donated, discarded human embryonic blastocyst from an in vitro

fertilization clinic, then the possibility to transition islet transplantation as a therapy for a select population of patients suffering from T1DM, to a β-cell replacement therapy for all.

Concluding Remarks

The main goal of IT is to stabilize glycemic control in patients at high risk of severe, recurrent hypoglycemia, that cannot be stabilized by other more standard means. Insulin independence is a desirable by-product of that process, but is currently often not sustainable long-term. IT has been shown to improve quality of life, and markedly reduce risk of hypoglycemia. Remarkable progress has occurred in this field, as documented in the recent reports of the Collaborative Islet Transplant Registry (CITR). Today, IT is an advantageous clinical procedure for patients with brittle T1DM and is covered by public or private health care services in countries like Canada, Australia, the UK, Switzerland, Italy, France, and other parts of Europe (Shapiro *et al.*, 2017). Recently, the NIH funded phase III multicenter trial in North America has confirmed IT as a safe and effective method that lead to the FDA approval for USA. Compared to whole pancreas transplantation, IT is a safer procedure to render insulin independence and glycemic control. Optimistically, IT has the future potential to serve as a feasible procedure for all patients with diabetes.

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http://www.islet.ca—Clinical Islet Transplant Program.
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Hypoglycemic State, Nondiabetic☆

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Glossary

C peptide The peptide connecting the A and B chains of insulin in the proinsulin molecule; measured in the plasma C peptide is an index of endogenous insulin secretion.

Counterregulatory hormones Hormones released in response to hypoglycemia and contributing to raise blood glucose levels.

Factitious hypoglycemia Clandestine self-administration of insulin, a situation more frequently encountered in the relatives of diabetic patients, in the medical or paramedical profession, and sometimes in diabetic patients themselves.

Insulin The hormone released from the B cells of the islets of Langerhans; diabetes caused by lack of insulin; hypoglycemia caused by excess insulin.

Insulinoma Tumor developed from the B cells of the islets of Langerhans.

Islets of Langerhans Small group of endocrine cells making clusters (islets) in the pancreatic gland; represent about 1% of the mass of the pancreas.

Neuroglycopenia Neurological signs and symptoms associated with hypoglycemia.

Nesidioblastosis Neoformation of islets of Langerhans from pancreatic duct epithelium.

Introduction

Under physiological conditions, blood glucose is maintained within a given range, "normoglycemia," by remarkable regulatory mechanisms. In 30 healthy volunteers, ages 25 to 55 years, who were investigated by Marks, capillary blood glucose levels were determined 17 or 18 times a day during ordinary everyday life. The mean of all 498 blood samples was 4.2 $\pm$ 0.8 mmol/L. The lowest mean blood glucose of the day was 3.9 $\pm$ 0.6 mmol/L and was in samples collected at 17:00 h. The highest level was found in samples collected at 14:00 h and averaged only 4.9 $\pm$ 1.0 mmol/L. Blood glucose values of 3.0 mmol/L or less were recorded in 24 samples from 10 participants. In 14 samples (2.8%) collected in five participants, the concentration was 2.8 mmol/L (50 mg/dL) or less. Fully 95% of all blood glucose values were more than 3 mmol/L (54 mg/dL). These figures were recently confirmed by 24-h ambulant continuous glucose monitoring in young and middle-age individuals from the general population by Wijsman et al. In young individuals, minimum blood glucose was 3.45 mmol/L (95% C.I.: 3.10–3.70) and in middle-age individuals 3.59 mmol/L (95% C.I.: 3.40–3.78).

Hypoglycemia occurs when the blood glucose level is lower than the lowest limit of normal fluctuations (i.e., 2.8–3.0 mmol/L or 50–54 mg/dL). Such values have been confirmed in numerous experiments performed in healthy volunteers where mild hypoglycemia was induced using an insulin infusion. The threshold for symptoms in five different studies was precisely 3 mmol/L; the symptoms included weakness, difficulty in thinking and concentrating, diaphoresis, palpitations, and tremor. Neurophysiological testing, including reaction times, suggested a threshold between 2.2 and 3.5 mmol/L. Objective electrophysiological testing showed alterations between 2.4 and 3.0 mmol/L, with the exception of one study in which a response was already seen at 4 mmol/L. The remarkable study of Mitrakou and colleagues permitted identification of the various thresholds in the same 10 healthy volunteers submitted to progressively increasing insulin infusion rates. Activation of counterregulatory hormones (glucagon, epinephrine, norepinephrine, and growth hormone) began at arterialized venous plasma glucose levels of about 3.7 mmol/L. Autonomic symptoms (anxiety, palpitations, sweating, irritability, and tremor) began at 3.2 mmol/L, which was significantly lower. Neuroglycopenic symptoms (hunger, dizziness, tingling, blurred vision, difficulty in thinking, and faintness) and deterioration in cognitive function tests began at 2.8 and 2.7 mmol/L, respectively. There is a precise hierarchy of hormonal responses that remarkably maximizes the opportunity to avoid hypoglycemia. As depicted above, blood glucose values similar to those giving autonomic symptoms (3.2 mmol/L) and symptoms of neuroglycopenia or impairment of cognitive function (2.5–3.0 mmol/L) are sometimes observed in everyday life conditions in some individuals.

Various Forms of Nondiabetic Hypoglycemia, Diagnosis, and Treatment

There are two principal forms of hypoglycemia: (1) exogenous hypoglycemia attributable to the administration (injection or ingestion) of a hypoglycemic compound and (2) endogenous hypoglycemia.

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Exogenous Hypoglycemia

Insulin is by far the most frequent cause of hypoglycemia. In nondiabetic as well as diabetic patients, insulin has been used for homicidal or suicidal purposes. Severe unexplained hypoglycemia in a nondiabetic individual should always raise the possibility of an exogenous insulin administration, either suicidal or criminal. Such cases are encountered more frequently in the medical milieu or in the families or neighborhoods of diabetic patients. Inadvertent insulin administration to a hospitalized nondiabetic patient has also been reported. Diagnosis of these conditions is often difficult. It requires the simultaneous demonstration of hypoglycemia, high plasma circulating insulin levels, and undetectable plasma C-peptide concentrations. In psychiatric patients, purposely induced insulin shock therapy sometimes leads to prolonged hypoglycemia and irreversible brain damage. Factitious hypoglycemia due to clandestine self-administration of insulin must always be considered in the differential diagnosis of hypoglycemia; again, this situation is encountered more frequently in the relatives of diabetic patients, in the medical or paramedical profession, or in diabetic patients themselves. Similarly, oral antidiabetic agents, mainly sulfonylureas, can be involved in the pathogenesis of hypoglycemia in nondiabetic individuals: inadvertent administration, accidental ingestion (mainly in children), suicide attempt, and clandestine ingestion (a variety of factitious hypoglycemia). Alcohol ingestion may lead to hypoglycemia if it occurs in fasting conditions while accidental intake of alcohol in children can also induce severe hypoglycemia. Sometimes, alcohol favors the "reactive hypoglycemia" following sugar ingestion (see below). Numerous other agents or drugs may induce hypoglycemia. In a systematic review of the literature, Murad et al. found 448 eligible studies that described 2696 cases of hypoglycemia associated with 164 different drugs. The quality of evidence supporting associations between drugs and hypoglycemia was mostly very low due to methodological limitations and imprecision. The most commonly reported hypoglycemia-associated drugs include salicylates, quinine, β-receptor blocking agents, pentamidine, mebendazole, isoproterenol, disopyramide, tranylcypramine, haloperidol, clofibrate, and, particularly, angiotensin-converting enzyme inhibitors (see **Table 1** for the most probable underlying mechanisms). Also note that all drugs that interfere with the metabolism of sulfonylureas could also induce hypoglycemia (not considered in the present section).

Endogenous Hypoglycemia

Endogenous hypoglycemia may be organic or functional.

Organic hypoglycemia

Insulinomas

Insulinomas are uncommon neoplasms, most often benign, that derive from the B-cells of the islets of Langerhans of the pancreas. An insulinoma should be suspected in any patient presenting with the triad described by Whipple: symptoms precipitated by fasting or exercise, proven hypoglycemia associated with symptoms, and relief of symptoms by glucose. The demonstration of endogenous plasma insulin (and C-peptide) levels inappropriate to the prevailing blood glucose level is the cornerstone of the diagnosis. Evidence of high circulating levels of proinsulin is confirmatory. Simultaneous determination of blood glucose and plasma insulin (and C-peptide) levels after an overnight fast and, mainly, during a 24- to 48-h fast is probably the best procedure to demonstrate relative hyperinsulinism. Before concluding on the presence of an insulinoma in case of low blood glucose associated with high plasma and C-peptide levels, factitious intake of sulfonylurea should be excluded with a sulfonylurea screen to avoid inappropriate imaging procedures and surgery. When one is convinced of the diagnosis, one attempts to localize the tumor before sending the patient to surgery. Preoperative localization procedures include tomodensitometry, conventional ultrasonography, selective arteriography, magnetic resonance imaging, ^{18}F-FDOPA PET/CT imaging, and (the most useful) endoscopic transduodenal ultrasonography. Surgical removal of the tumor is the first and obvious choice of treatment. It must always be accompanied by intraoperative ultrasensitive pancreas echotomography, particularly because there may be more than one tumor. In recent years, endoscopic ultrasound-guided ethanol ablation of insulinomas has emerged as a new therapeutic

Table 1 Most commonly cited drugs to be associated with hypoglycemia (outside antidiabetic agents) and most probable underlying mechanism

Drugs	*Increased insulin secretion*	*Increased insulin sensitivity*	*Miscellaneous effects*
Antibacterial sulfonamides	*		
Quinine/quinidine	*		
Pentamidine	*		
Disopyramide	*		
Fluoroquinolones (mainly gatifloxacin)	*		
Salicylates	*	*	
Beta-blockers (mainly nonselective)		*	
Angiotensin converting enzyme inhibitors		*	
Monoamine oxidase inhibitors		*	
Indomethacin			*

option, especially for elderly patients and candidates unfit for surgery, but the long-term effects of this procedure still need to be validated.

Medical management, mainly using diazoxide or somatostatin analogs, is reserved for patients who do not accept surgery or in whom major contraindications for the operation exist. Streptozotocin, in association with fluorouracil or doxorubicin, was considered to be the most effective antitumor agent for treating the rare metastatic malignant insulinomas, possibly after surgical reduction of the tumor mass and/or removal of liver metastasis. Other more modern therapeutic options may include targeted medical therapies using either mTOR inhibitors (everolimus) or small molecule inhibitors of the tyrosine kinase activity (sunitinib) (see review in Kinova, 2015).

Extrapancreatic neoplasms

Extrapancreatic neoplasms secreting insulin-like growth factor-2 (IGF-2), or a large form of it known as "big IGF-2," are usually large tumors present as masses in the mediastinum or the retroperitoneal space. They often have a mesenchymal origin but can originate from the liver, gastrointestinal tract, or pancreas or can be associated with lymphomas and leukemias. In these patients, hypoglycemia coexists with low or undetectable insulin and C-peptide levels, whereas high circulating levels of IGF-2 and related peptides are found, hence the recent proposal to label these neoplasms "IFG-2-omas." When feasible, surgery is the treatment of choice. When hypoglycemia persists after surgical interventions have reached their limits, several modalities have been employed and include glucocorticoids at increasing doses, and, experimentally antibodies against IGF-2 and specific IGF-2 antagonists (review in Dynkevich *et al.*).

Neonatal and infancy hypoglycemia

Numerous inborn errors of metabolism can induce hypoglycemia in neonates and young infants. They include hereditary fructose intolerance, fructose-1, 6-diphosphatase deficiency, phosphoenolpyruvate carboxykinase deficiency, some cases of galactosemia, and some of the 11 varieties of glycogen storage disease. Nesidioblastosis, now called "persistent hyperinsulinemic hypoglycemia of infancy" (PHHI), is a rare disease leading to persistent hypoglycemia of infancy. It is basically histologically characterized by the budding off from duct epithelium of endocrine cells and by the presence of microadenomas in the pancreas. The onset of symptoms of beta cell hyperplasia may occur during the first days of life but most commonly within the first 6 months. A few cases beginning with symptoms beyond 1 year of age have been reported. The group of Saudubray in Paris reported the features of 52 neonates with hyperinsulinism. Of these, 30 had diffuse B-cell hyperfunction and 22 had focal adenomatous islet cell hyperplasia. Among the latter, the lesions were in the head of the pancreas in nine, in the isthmus in three, in the body in eight, and in the tail in two neonates. Partial pancreatectomy has been successful in curing 19 of the 22 neonates in whom this procedure has been proposed. Recent studies have shown that congenital hyperinsulinism with focal or diffuse nesidioblastosis can be associated with several mutations affecting the beta cell such as the genes encoding for the sulfonylurea receptor, the glucokinase enzyme, the intramitochondrial enzyme glutamate dehydrogenase, the hepatocyte nuclear factor 4α (HNF-4α)… (see Review in Arya *et al.*). Medical treatment includes frequent feeding, diazoxide, and octreotide. Near-total pancreatectomy is often required.

Other causes of hypoglycemia during infancy (more functional in nature) include erythroblastosis fetalis, infants of diabetic mothers, leucine-induced hypoglycemia, ketotic or ketogenic hypoglycemia, maple sugar urine disease, and adrenal hyporesponsiveness.

Functional hypoglycemia

Alimentary hypoglycemia

Alimentary hypoglycemia can occur 1 to 2 h after a carbohydrate-rich meal in individuals who have had a gastrectomy or who, for other reasons, have rapid gastric emptying. It is believed that the rapid dumping of carbohydrates in the upper small intestine elicits an excessive insulin release mediated by both the release of intestinal gut factors (e.g., GLP-1, GIP) and a rapid rise in blood glucose. The treatment is identical to that of spontaneous reactive hypoglycemia.

Hypoglycemia complicating bariatric surgery

Consequent to the current obesity epidemics, bariatric surgery is worldwide performed with ever increasing frequency. Severe late postprandial hypoglycemia has been described in some patients after Roux-en-Y gastric bypass or duodenal switch. Continuous blood glucose monitoring in patients having undergone these procedures has shown frequent, but mainly, unnoticed, hypoglycemic episodes (Abrahamsson *et al.*). In a minority of patients (around 1%), hypoglycemia is severe. In some patients, postbariatric surgery hypoglycemia is associated with nesidioblastosis or even insulinoma. Better understanding of the mechanisms involved would make it possible to identify preoperatively those patients at greater risk of this complication. The recent study of Craig et al. has identified a critical role for GLP-1 in postbariatric hypoglycemia. In that study, GLP-1 receptor blockade with Exendin (9–39) prevented hypoglycemia in 100% of individuals studied, normalized β-cell function and reversed neuroglycopenic symptoms. Competitive antagonism at the GLP-1 receptor merits consideration as a therapeutic strategy in severe postbariatric surgery.

Spontaneous reactive hypoglycemia

Spontaneous reactive hypoglycemia is a poorly defined entity. The term is usually applied to a syndrome with the following features: (1) symptoms that resemble those seen in insulin-induced hypoglycemia (e.g., diaphoresis, tachycardia, tremulousness,

headache) but that often are accompanied by other symptoms less typical of hypoglycemia (e.g., fatigue, drowsiness, feelings of incipient syncope, depersonalization, irritability, lack of motivation); (2) symptoms that may be episodic, sometimes aggravated by carbohydrate-rich meals; and (3) plasma glucose concentrations that drop to 45 mg/dL (2.5 mmol/L) or less at one or more of the half-hourly samples taken in a 5- to 6-h glucose tolerance test. Abnormal insulin secretory patterns have been reported in some of these patients.

This entity has had a widespread vogue, particularly in the United States, over the past 40 years but has been said to be diagnosed more rarely elsewhere in the world. The American Diabetes Association and the Endocrine Society issued a joint statement to the effect that this entity is probably overdiagnosed. Indeed, the very existence of this condition has been called into question following several studies demonstrating that 25%–30% of apparently healthy individuals without any hypoglycemic symptoms may exhibit low plasma glucose values on being given a glucose load. Furthermore, the similarity of the symptoms to those of hyperventilation, and indeed to those of other functional syndromes, emphasizes the need to reevaluate the whole matter of so-called functional or reactive hypoglycemia. The question of cause and effect has not been settled. It would be reasonable at the current time to restrict the diagnosis of reactive hypoglycemia to individuals in whom hypoglycemic blood glucose levels are demonstrated in samples taken after the sort of meals that are said to induce their symptoms. Continuous blood glucose monitoring for a few days with precise recording of the allegated symptoms may be necessary. Furthermore, and as has been discussed by Lefèbvre, some patients have adrenergic responses after a meal or during oral glucose tolerance test without hypoglycemia. Such "adrenergic hormone postprandial syndrome" probably results from an altered glycemic threshold (a higher glucose level) for generating an adrenergic response. This results in confusion. A critical analysis of the reactive hypoglycemia syndrome can be found in the proceedings of an international symposium held in Rome in September 1986.

Diet is the first treatment of alimentary and reactive hypoglycemia. Simple sugars should be omitted and replaced by complex carbohydrates. If symptoms persist, small but frequent high-protein, low-carbohydrate meals should be tried. The pharmacological treatment of choice is α-glucosidase inhibitors such as acarbose and miglitol.

Alcohol-promoted reactive hypoglycemia

Alcohol-promoted reactive hypoglycemia can occur when insulinotropic sugars (e.g., glucose, saccharose) are ingested together with alcohol (e.g., beer, gin and tonic, rum and cola, whisky and ginger ale). Such mixtures should be avoided in susceptible individuals.

Other causes of functional hypoglycemia

Other causes of functional hypoglycemia include discontinuation of total parenteral nutrition, an endocrine deficiency state (glucocorticoid, growth hormone, or glucagon deficiency), severe liver disease, profound malnutrition, prolonged muscular exercise, the autoimmune insulin syndrome (where hypoglycemia is considered to be the consequence of inappropriate release of insulin from insulin–antibody complexes), and the rare syndrome of antibodies directed against the insulin receptor (where hypoglycemia is attributed to an insulinomimetic action of the antibody).

Conclusion

Hypoglycemia in nondiabetic individuals is not a rare condition. It is diagnosed when the blood glucose level is lower than the lowest limit of normal, that is, lower than about 3 mmol/L (or 54 mg/dL), a value also corresponding to the threshold for symptoms in various experimental studies performed in healthy volunteers in whom mild hypoglycemia was induced using graded insulin infusion. Hypoglycemia can result from the administration (injection or ingestion) of a hypoglycemic compound (e.g., insulin, oral antidiabetic agents, alcohol, various drugs). It can also be endogenous in nature. Organic endogenous hypoglycemia can be due to an insulin-producing tumor (insulinoma) or an extrapancreatic neoplasm. In neonates and young infants, hypoglycemia results mainly from various inborn errors of metabolism or of nesidioblastosis, now known as the syndrome of persistent hyperinsulinemic hypoglycemia of infancy, a condition in which several mutations in genes affecting the B-cell of the islets of Langerhans have been described. Functional hypoglycemia is called alimentary if it is due to gastrectomy or a too rapid gastric emptying or as a result of bariatric surgery. It is recognized as spontaneous reactive hypoglycemia if it occurs without any identified cause. Caution must be exerted in the diagnosis of this type of hypoglycemia. However, the diagnosis of reactive hypoglycemia can be made on the basis of a careful clinical and biochemical strategy. In such a case, simple therapeutic measures can be applied and the patient's quality of life can potentially be markedly improved.

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Classification of Hyperlipidemias and Dyslipidemias

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Dyslipidemias are the main disorders of lipid metabolism and are typified by quantitative or qualitative changes in plasma lipoproteins. On the one hand, quantitative alterations are referred to increases or decreases in lipoproteins plasma concentrations (hyperlipidemias or hypolipidemias respectively). On the other hand, qualitative changes, which sometimes occur in isolation but are mainly associated with quantitative disorders, affect the composition of plasma lipoproteins as a consequence of modifications in their metabolisms (Ramasamy, 2016).

In most cases, both hyperlipidemias and hypolipidemias or qualitative changes in the composition of lipoproteins can be studied considering that the lipoproteins mainly affected contain cholesterol or triglycerides. As a consequence, cholesterol and triglycerides are the main biological markers of dyslipidemia and the frequent starting point for their studies.

The enormous importance of dyslipidemias with changes in the plasma concentrations of cholesterol and/or triglycerides is due to its potential association with cardiovascular diseases. In fact, it is considered that without the causal role of cholesterol it would be difficult to understand the vascular systemic affectation, origin of cardiovascular diseases, as the main cause of morbidity and mortality in our environment (Catapano *et al.*, 2016; Chapman *et al.*, 2011). Although the role of triglycerides has less impact on cardiovascular diseases, its role as an independent risk factor is out of doubt.

Furthermore, the study and analysis of the different lipoprotein fractions allows us to offer a clinical panorama that facilitates us the diagnosis and treatment of dyslipidemias. While chylomicrons (QL), very low-density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) are families of lipoproteins rich in triglycerides, low-density lipoproteins (LDL) are rich in cholesterol.

In this way, the dyslipidemias most frequently associated with cardiovascular diseases are characterized by any of the following features:

- increase of cholesterol-rich LDL or its small and dense LDL particles phenotype;
- increase of triglycerides-rich VLDL;
- combined increase of LDL and VLDL (mixed dyslipidemia);
- decrease of apoAI-rich high density lipoproteins (HDL), responsible for the reverse cholesterol transport; and
- increase of apoB-rich lipoproteins (LDL, VLDL and remnant lipoproteins or IDL).

Classification of Dyslipidemias

Considering the combination between cholesterol and triglycerides plasma concentrations and different involved lipoproteins, the alterations in lipid metabolism has classified since almost 50 years ago (classification of phenotypes by Frederickson in 1970) as follows:

Lipoprotein increase	*Fundamental increase*	*Phenotype*
QL	Triglycerides	I
LDL	Cholesterol	IIA
LDL & VLDL	Cholesterol & Triglycerides	IIB
IDL	Triglycerides	III
VLDL	Triglycerides	IV
QL and VLDL	Triglycerides	V

QL, Chylomicrons; *LDL*, low density lipoproteins; *VLDL*, very low density lipoproteins; *IDL*, intermediate density lipoproteins.

However, a very accurate classification to assess an initial diagnostic with clear repercussions in the treatment is to combine the main affected fraction (cholesterol and/or triglycerides) with the origin of the alteration (primary or secondary).

Thus, the main primary hyperlipidemias to consider are the following:

1. Hypercholesterolemias
   a. Monogenic familial hypercholesterolemia
      i. Autosomal dominant
         1. Homozygous
         2. Heterozygous
      ii. Autosomal recessive

   b. Polygenic familial hypercholesterolemia
   c. Hyperlipoproteinemia (a)
   d. Hyperalphalipoproteinemia
   e. Sitosterolemia
   f. Cholesterol-7-alpha-hydroxylase deficiency
2. Hypertriglyceridemia
   a. Primary hypertriglyceridemia due to lipoprotein lipase (LPL), apoCII, apoAV, glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1) and lipase maturation factor (LMF1) genetic mutations.
3. Mixed hyperlipidemia
   a. Combined familial hyperlipidemia
   b. Familial dysbetalipoproteinemia
   c. Lysosomal acid lipase deficiency

In respect to secondary hyperlipemias, the main types are:

1. Hypercholesterolemias
   a. Hypothyroidism
   b. Nephrotic syndrome
   c. Cholestasis
   d. Drugs (cyclosporine, progestins, anti-HIV protease inhibitors, corticosteroids, thiazides)
   e. Acute intermittent porphyria
2. Hypertriglyceridemia
   a. Diabetes mellitus
   b. Obesity
   c. Metabolic syndrome
   d. Alcohol
   e. Chronic kidney disease
   f. Drugs (corticosteroids, beta-blockers, estrogens, thiazides, tamoxifen, antipsychotics, cyclophosphamide)
   g. Glycogenosis
   h. Lipodystrophies
   i. AIDS and treatment with antiretrovirals
   j. Cushing syndrome
   k. Pregnancy
   l. Acute hepatitis
   m. Connective tissue diseases (systemic lupus erythematosus)
   n. Stress, sepsis

Finally, we have to contemplate the small group of dyslipidemias clinically characterized by a decrease of cholesterol or triglycerides levels. These hypolipidemias are usually caused by specific mutations:

1. Hypocholesterolemias
   a. ApoCII deficiency
   b. ApoAI deficiency (hypoalphalipoproteinemia)
   c. Lecithin-cholesterol-acyl-transferase deficiency (LCAT)
   d. Lipoprotein lipase deficiency
   e. Glucocerebrosidase deficiency
   f. Tangier's disease
2. Hypotriglyceridemias
   a. ApoCIII deficiency
   b. Disfunction in lipoprotein lipase activity due to ANGPTL 3 and 4 genetic mutations

Most Common Primary Dyslipidemias

Familial Autosomal Dominant Hypercholesterolemia

As a consequence of its autosomal dominant hereditary pattern, it is transmitted to 50% of the offspring. The two clinical forms, homozygous and heterozygous, are composed by marked increases in total cholesterol and LDL cholesterol (LDL-C). Specifically, the homozygous form, which occurs in 1/300,000–600,000 individuals according to different populations, presents LDL-C ranges between 500 and 1000 mg/dL; whereas the heterozygous form, whose prevalence is 1/250–500 individuals, shows LDL-C figures between 190 and 500 mg/dL. In primary care, the clinical history may help us to detect first degree relatives with very high LDL-C levels. Moreover, familial hypercholesterolemia stigmas can be found in a clinical examination, being very characteristical their

clinical signs (tuberous and tendinous xanthomas in Achilles tendon, elbows, interphalangeal joints and knees; as well as corneal arcus at early ages) (Hopkins *et al.*, 2011; Civeira, 2004).

The incidence of cardiovascular disease, mainly coronary heart disease either in the affections or in first-degree relatives, is very high at an early age because the early deposition of cholesterol in the vascular tree. This can be recognized in a rigorous analysis of the family tree (Wiegman, 2015).

The cause of the disease is placed in a gene mutation, having detected in more than 90% of cases this alteration in the gene that codes for the LDL receptor located on chromosome 19, which means that its number and/or activity is greatly diminished and consequently the patient has an inability for plasma clearance of LDL particles (Hobbs, 1992; Goldstein, 2001; Rader *et al.*, 2003). While heterozygous cases may have a 50% clearance reduction, homozygous forms can express frequently less than 20% and even to very small activities (1%–2%) (Defesche, 2000; Raal and Santos, 2012).

Other less frequent cases are due to mutations in the apolipoprotein B or the PCSK9 (proprotein-convertase subtilisin-kexin type 9) genes (Innerarity *et al.*, 1987; Myant, 1993; Boren *et al.*, 2001; Abifadel *et al.*, 2003; Maxwell *et al.*, 2005). In the first case, the LDL particle cannot bind to the receptor even if it is undamaged. In the second, the PCSK9 activity, which intervens in the recycling of this LDL receptor, conditions its increased catabolism (Cohen *et al.*, 2006).

In all cases, the final result is the same. The LDL plasma concentration triking increase promotes the consequent rise of LDL vascular depositions, triggering the high incidence of cardiovascular diseases.

Autosomal Recessive Familial Hypercholesterolemia

Very infrequent recessive form of monogenic familial hypercholesterolemia (1/5 million inhabitants). It is due to a gene mutation that encodes the protein that facilitates the LDL receptor internalization after having bounded to the LDL particle (Soutar and Naoumova, 2004).

Polygenic Hypercholesterolemia

It is the most common primary hypercholesterolemia and shows moderately or frankly high levels of total cholesterol (not usually exceeding 300 mg/dL) or LDL (frequently less than 190 mg/dL).

It is a typical case of interaction between genetic factors and environmental factors, mainly those related to lifestyle. There are combined hypocatabolism elements (genetic) with an increase in cholesterol intake or synthesis (environmental).

Hyperlipoproteinemia (a)

The particular lipoprotein (a) has a specific apoprotein, apo (a), which, like LDL particles, is attached to apoB. This apo (a) molecule bears a strong resemblance to the plasminogen molecule, being able to compete for plasminogen activation and to cause hypofibrinolysis.

It is an inherited independent risk factor in a codominant manner, having been described up to different 30 isoforms, and it is associated with a high incidence of cardiovascular disease (Nordestgaard *et al.*, 2010).

Hyperalphalipoproteinemia

There is a mutation in the gene for the cholesterol ester transfer protein (CETP) or an increase in the synthesis of apoAI, the HDL fundamental apoprotein, causes an HDL cholesterol growth in plasma. In both cases, there is an evident rise in the HDL synthesis and its concentration, which reaches 100–200 mg/dL with regular levels in the LDL and VLDL fractions.

Although the cardiovascular risk in these patients is diminished, there is a controversy about the true protective role of very high HDL levels because, more than the quantity, it is basic to ensure a quality molecule in order to allow an adequate cholesterol reverse transport.

Sitosterolemia

It coexits an excessive intestinal absorption of cholesterol and plant sterols (sitosterol and campesterol), and a decrease in bile secretion. These cholesterol and sterol accumulations in different tissues motivate remarkable deposits, clinically represented by stigmas (tendon and corneal arc xantomas). Patients consequently have an increasing incidence of early cardiovascular disease (Escola Gil *et al.*, 2014).

The cause are located in genetical mutations of membrane transporters in the intestinal cells, whose modification produces an increasing cholesterol absorption, and in the hepatocytes, which entails a decreasing export to the biliary secretion. Nevertheless, the expression of the LDL receptor is secondarily modified.

Deficit of Cholesterol-7-Alpha-Hydroxylase

It is transmitted by an autosomal codominant manner, causing a reduction in the bile acids synthesis in the liver from cholesterol. As a consequence, there is an increase in the cholesterol hepatic deposits, the LDL receptor expression is frankly diminished and the LDL particles plasma clearance is finally reduced. According to this background, these patients logically present a higher cardiovascular risk and a higher incidence of cholelithiasis.

Primary Hypertriglyceridemia

The incidence of monogenic primary hypertriglyceridemia is approximately 1/1,000,000 individuals, manifesting triglyceride levels above 880 mg/dL (Chait *et al.*, 2000; Hegele *et al.*, 2014). The most severe hypertriglyceridemias are related to a pancreatitis elevated risk, while the more moderate hypertriglyceridemias (150–880 mg/dL) are accompanied by an increased cardiovascular risk (Valdivielso *et al.*, 2014) There are homozygous and heterozygous forms of different genetical alterations that determine diverse deficiencies in the triglycerides-rich lipoproteins metabolism (Lewis *et al.*, 2015).

Lipoprotein lipase (LPL) deficiency causes a hyperchylomicronemia, being affected the enzyme function of hydrolyzing the triglycerides in chylomicrons and VLDL; so that the organism disposes free fatty acids as an energy source. It is the most frequent cause of primary hypertriglyceridemia (Martin Campos *et al.*, 2014, Brahm and Hegele, 2015). There are discovered more than 100 mutations and, due to the severe hypertriglyceridemia, abdominal pain and pancreatitis episodes are very frequent. In the heterozygous forms, in which LPL activity is reduced approximately by half, moderate or even severe hypertriglyceridemia are developed when external factors coexist (alcohol, obesity, diabetes, high-fat diet) (Wang and Eckel, 2009).

ApoCII deficiency causes a partial or almost complete LPL activity decrease, bearing in mind it is its natural activator. Patients present severe hyperchylomicronemia and hypertriglyceridemia with pancreatitis and eruptive xanthomas episodes.

ApoAV deficiency is associated with IV and V hyperlipidemias phenotypes. The apoAV gene is linked to apoAI, apoAIV and apoCIII, a genes cluster with decisive influence on the triglycerides metabolism.

Glycosylphosphatidylinositol-anchored high density lipoprotein–binding protein 1 (GPIHBP1) deficiency causes a LPL transport protein defect towards the endothelial cell surface in the capillarie lights, difficulting its action on lipoproteins rich in triglycerides (Fong *et al.*, 2016, Ariza *et al.*, 2016). The homozygous forms present severe hyperchylomicronemia and hypertriglyceridemia, and pancreatitis and deposit stigmata episodes (lipemia retinalis and eruptive xanthomas), whereas the heterozygous forms may not show clinical manifestations.

Hepatic triglyceride lipase deficiency. Hepatic lipase (HL) is an important enzyme in the metabolism of triglyceride-rich lipoproteins and high density lipoproteins (Kobayashi *et al.*, 2015). Complete HL deficiency is a rare genetic disorder, inherited as an autosomal recessive or codominant trait. The absence of HL leads to pathologic levels of circulating lipoproteins and an increased risk of cardiovascular risk. HL deficiency is characterized by increased levels of triglycerides and cholesterol in the blood; and affected people may also have increased levels of HDL (Hegele *et al.*, 1993; Connely and Hegele, 1998).

Lipase maturation factor 1 (LMF1) deficiency, which is due to diverse mutations, affects the LPL activity being it an essential enzyme for its maturation.

Combined Family Hyperlipidemia

In this case, both different genes (polygenic primary hyperlipidemia) and environmental factors take part in its physiopathology. It is one of the most frequent hyperlipidemias, having a very variable phenotype and predominating hypercholesterolemia, hypertriglyceridemia or both (Brouwers *et al.*, 2012).

There is an increase in the lipoproteins rich in apoB and rich in triglycerides (VLDL) synthesis (Castro Cabezas *et al.*, 1993). These VLDL have a reducing plasma clearance motivated by a LPL decreasing activity. Its cardiovascular risk is very high because the apoB is characteristically increased, so that the LDL particles rate is boosted although its small size (small and dense LDL).

Familial Dysbetalipoproteinemia

It is consequence of an apoE genotype alteration, which has three possible isoforms (apoE2, apoE3 and apoE4) (Utermann *et al.*, 1975; Hopkins *et al.*, 2014). The specific apoE2/E2 modificated genotype shows a lower affinity for the LDL receptor to remaining particles, representing an infrequent 1% of apoE2 homozygous forms. As a result, VLDL and intermediate density lipoproteins (IDL) levels in plasma are very high, provoking that their cholesterol and triglycerides elevated concentrations are accompanied by an extraordinary elevation of cardiovascular risk (Marais *et al.*, 2014).

Lysosomal Acid Lipase (LAL) Deficiency

Not only is this enzyme responsible for hydrolyzing triglycerides to generate free fatty acids, but it does also for the colesterol esters in order to release cholesterol. Thanks to both constituent elements, fatty acids and cholesterol, the body manages to store energy and synthesize diverse structural (membranes) and functional (hormones and steroids) elements (Reiner *et al.*, 2014).

A LAL enzyme deficiency causes triglycerides and cholesterol esters lysosomal deposits (Fouchier and Defesche, 2013). As a result, the LDL receptors synthesis is upwardly regulated (up-regulation) and the colesterol intracellular deposits exponentially and perpetually increase.

The LAL gene mutations are autosomal recessive manner transmitted and its prevalence is low (1/200,000–300,000 individuals).

Most Frequent Secondary Dyslipidemias

Hypothyroidism

The hypercholesterolemia and hypothyroidism association is widely admitted. The mechanisms through which dyslipidemia can develop in the hypothyroidism are diverse (Shin and Osborne, 2003; Pearce, 2012):

- Thyroid hormone induces the cholesterol intracellular synthesis (by expression of the HMGCoAR enzyme), so in its absence the cholesterol hepatic synthesis is reduced.
- The LDL receptors expression is increased by thyroid hormone. Consequently, a low hormone level decreases the LDL plasmatic clearance.
- In hypothyroidism, there is a greater cholesterol intestinal absorption because the thyroid hormone modulates the intestinal expression of the protein Niemann-Pick C1-like 1.
- In these patients, the concentration and plasma activity of the cholesterol ester transfer protein are reduced.
- Hepatic lipase concentration and plasma activity are also dropped.
- Cholesterol reverse transport is declined due to a low ATP binding cassette A1 (ABCA1) activity.
- Lipoprotein lipase activity is diminished in hypothyroidism.
- Hepatic synthesis of bile acids from cholesterol is lessened.

Dyslipidemia associated with hypothyroidism is consistently global, affecting all lipoprotein families and having to highlight:

- Hypercholesterolemia with LDL-C increase.
- Hypertriglyceridemia with lipoproteins rich in apoB increase.
- Cholesterol efflux reduction due to dysfunctional HDL-C.

From the practical view and the high prevalence, it is always necessary to rule out hypothyroidism as secondary cause in the diagnosis of hypercholesterolemia. In addition, the previous correction of the thyroid disorder, regardless of the possible statin uses to reduce the cholesterol plasma concentrations, it will always be necessary.

Nephrotic Syndrome

Even though there is a global dyslipidemia, the most characteristic alteration is a total cholesterol and LDL-C increase. This is presented mainly for the increased hepatic synthesis and decreased plasma clearance confluence (Vaziri, 2003). Some of the involved mechanisms in the lipid metabolism disorder in nephrotic syndrome are:

- Acquired deficiency in the LDL receptor with the consequent increase in the cholesterol intracellular synthesis due to an increase in the HMGCoAR enzyme activity.
- Increase in the PCSK9 enzyme with a LDL receptor catabolism ensuing increase.
- Increased acyl-CoA-cholesterol acyl-transferase (ACAT-2) hepatic activity catalyzes the cholesterol esters formation from free cholesterol, thus increasing the LDL lipoproteins rich in cholesterol.
- Urinary loss in the lecithin-cholesterol acyl-transferase (LCAT) accompanying proteinuria.
- Decrease in the lipoprotein lipase (LPL) activity.
- Reduction in the 7-alpha-hydroxylase enzyme and the bile acids production, accumulating cholesterol secondary deposits.
- Increase in the Lp (a) synthesis.

The dyslipidemia plasmatic profile in nephrotic syndrome is consequently very varied (de Sain-van der Velden *et al.*, 1998):

- Hypercholesterolemia with increased LDL-C.
- Hypertriglyceridemia with increased VLDL.
- Increase in remnant lipoproteins with increased IDL.
- Increase in lipoprotein (a).
- Frequent decline in HDL-C with cholesterol inversed efflux reduction.

Diabetes Mellitus (DM)

Diabetes mellitus is a clinical model of accelerated atherosclerosis. In general, its frequent connection with risk factors, such as obesity, hypertension and dyslipidemia have to be considered, being very prevalent the association of some of them constituting the metabolic syndrome (Ascaso *et al.*, 1997).

Diabetic dyslipidemia is mainly characterized by increased triglyceride-rich lipoproteins (fasting and postprandial), decreased HDL-C and regularly or slightly increased small and dense LDL-C particles (Taskinen, 2002). The atherogenic triad, constituited by hypertriglyceridemia, low HDL and small and dense LDL is known as atherogenic dyslipidemia.

The fasting hypertriglyceridemia is explained because, in the mechanism of triglyceride production, the insulin resistance roles a determining factor conditioning the lipase activity in adipose tissue. This generates two facts, an increased supply of free fatty acids to the liver, which, once transformed into triglycerides, are heavily deposited (hepatic steatosis); and a VLDL release in excess from the hepatocytes. Otherwise, the postprandial hypertriglyceridemia is explicated because the triglycerides lipolysis, partly lipoprotein lipasa via, occurs thanks to its low action over the chylomicrons (Taskinen, 2003).

The increase of lipoproteins rich in circulating triglycerides is accompanied by an increased activity of the cholesterol esters transfer protein (CETP), whose mission is to exchange triglycerides for cholesterol between different lipoproteins. It means that LDL and HDL particles reduce their cholesterol contents by generating smaller and denser particles because their protein contents remain constant (Taskinen and Boren, 2015).

In diabetes mellitus type 2 individuals, given that their cardiovascular risk is high or very high, depending on whether or not there are previous cardiovascular diseases, the central interest is the diabetic dyslipidemia management (Valdivielso *et al.*, 2009). The primary therapeutic objective is to reach a LDL-C control and, secondarily, a triglycerides and HDL-C management. The LDL-C objective, when the characteristical atherogenic dyslipidemia is present, can be frequently replaced by the non-HDL cholesterol target (total cholesterol minus HDL-C) because it represents the authentic dimension of the atherogenic cholesterol set in all different lipoprotein families (LDL, IDL, chylomicrons, remnant particles). As in other mixed dyslipidemia cases occurs, treatment with hypocholesterolemic and hypotriglyceridemic drugs is usually a very effective therapeutic alternative.

Alcohol

The peculiar dyslipidemia associated with moderate or excessive alcohol intake is an hypertriglyceridemia with an increased triglyceride-rich lipoproteins (VLDL) (Bessembinders *et al.*, 2011). There is an involved combination of external factors, alcohol, and endogenous factors, genetic polymorphisms that condition the alcohol metabolism (Klop *et al.*, 2013).

The production mechanism is based on an increase in the fatty acids supply to the liver from adipose tissue due to a decreased peripheral lipolysis. This contributes to an increase in the triglycerides hepatic synthesis, to which is added a mitochondrial limitation for the oxidation of such fatty acids.

HIV Infection and Treatment With Antiretrovirals

In infected patients with the human immunodeficiency virus (HIV), hypertriglyceridemia is frequently found over and above decreases in LDL-C and HDL-C (Palacios *et al.*, 2003).

Although HIV infection itself is responsible for this dyslipidemia, the antiretroviral effect of treatments is much more determining. In practice, protease inhibitors treatments are proved to be commonly accompanied by atherogenic dyslipidemia, despite that the new generation drugs cause less metabolic changes.

When these circumstances appear, once the lifestyle measures have been optimized, treatment with drugs is required in order to reduce the cardiovascular risk associated with antiretroviral treatments (Santos *et al.*, 2005).

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Atherosclerosis

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Glossary

Atherosclerotic plaque Deposit of lipids and cellular and fibrous elements in the artery wall.
Foam cells Lipid-engorged cells, mostly macrophages.
Infarction Tissue necrosis caused by hypoxia or anoxia resulting from insufficient or absent blood perfusion.
Ischemia Insufficient blood flow to satisfy the tissue metabolic needs.
Vascular thrombosis Thrombus formation frequently occurring at the level of an unstable atherosclerotic plaque.

Atherosclerosis is a chronic inflammatory disease characterized by the progressive accumulation of lipids and cellular and fibrous elements in large and medium-sized elastic and muscular arteries.

Introduction

Atherosclerosis is a vascular syndrome that is the primary cause of ischemic heart disease and stroke. In Western societies, it is the underlying cause of approximately 50% of total mortality. For many decades, it was considered a degenerative disease with no major potential for intervention, but it is better understood as a very dynamic, chronic inflammatory condition that is converted to an acute clinical event by the induction of plaque rupture leading to thrombosis, with a great potential for intervention. Epidemiological studies have revealed several important environmental and genetic risk factors. Work with genetically modified mice has made it feasible to experimentally examine mechanistic hypotheses, resulting in a clearer understanding of the cellular and molecular processes involved. Important tools for the management of the various clinical entities have been developed for diagnosis, risk stratification, and therapeutics. Risk factors can be classified as those with a strong genetic component and those that are mostly environmental (Table 1). However, approximately 50% of the casualties related to ischemic vascular disease are not explained by known pathogenic factors, posing a challenge to achieve a more complete understanding.

Pathogenesis

Progression of the Atherosclerotic Lesions

Stary described the microscopical evolution of atherosclerosis. The earliest stage (Stary I lesion), which is not apparent macroscopically, consists of subendothelial accumulations of lipid-loaded macrophages, called foam cells, and is found in 45% of infants up to 8 months of age. By puberty, it is common to see the classical "fatty streak" (Stary II lesions), visualized macroscopically with Sudan IV stain as a flat or slightly raised streak, consisting of lipid-loaded macrophages and smooth muscle cells containing lipid droplets and minimal, scattered, extracellular lipid. More advanced lesions include those with multiple extracellular lipid cores (Stary III lesion) that appear as a raised fatty streak or an atheroma (Stary IV lesion), characterized by a single confluent extracellular lipid core. By the third decade of life, fibrous lesions (Stary V lesions) appear when Stary III or Stary IV lesions are surrounded and/or caped by smooth muscle cells and collagen. These plaques can harbor calcification and areas of microthrombi in different stages of fibrotic organization. Different components of an advanced atherosclerotic lesion are shown in Fig. 1. The majority of acute coronary ischemic events occur as a result of plaque fissuring/ rupture, with exposure of the subendothelial matrix and the subsequent development of an occluding thrombus on the surface of the plaque.

Initial Events: Foam Cell Formation and Generation of the Fatty Streak

The events of atherosclerosis have been greatly clarified by studies in animal models, including rabbits, pigs, nonhuman primates, and rodents. Mice deficient in apolipoprotein E or the low-density lipoprotein (LDL) receptor develop advanced lesions and are the models most used in genetic and physiological studies. Fig. 2 depicts a model of the main events that occur in atherosclerotic plaque formation. The first observable change in the artery wall following the feeding of a high-fat, high-cholesterol diet is the accumulation of lipoprotein particles and their aggregates in the intima at sites of lesion predilection. LDL diffuses passively through endothelial cell (EC) junctions, and it is retained in the vessel wall, which seems to involve interactions between the LDL

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Table 1 Genetic and environmental factors associated with atherosclerosis and CAD[a]

Factor	*Evidence*
Strong genetic component	
Elevated levels of LDL/VLDL	Epidemiologic studies, studies of genetic disorders, and animal models; Small, dense LDL particles are particularly strongly associated with CAD. Clinical trials have shown benefits of cholesterol reduction.
Reduced levels of HDL	Epidemiological studies, studies of genetic diseases, and studies of animal models.
Elevated levels of Lp(a)	Epidemiological studies: Animal studies have yielded both positive and negative results.
Elevated blood pressure	Epidemiological studies: Clinical trials have demonstrated benefits of blood pressure reduction, with a particularly strong effect on stroke.
Elevated levels of homocysteine	Epidemiological studies: Animal studies have been inconclusive.
Family history	Independent factor in nearly all studies.
Diabetes and obesity	Epidemiological and animal studies.
Elevated levels of hemostatic factors	Associations have been observed with elevated levels of fibrinogen, plasminogen activator inhibitor type 1, and platelet reactivity.
Depression and other behavioral traits	Associations have been observed in several studies. The results are complicated by associated traits.
Gender (male)	Men younger than 60 years of age develop CAD at more than twice the rate as women. Recent trials has raised concerns about the benefits of postmenopausal estrogen replacement.
Systemic inflammation	Elevated levels of inflammatory molecules, such as $sPLA_2$, or inflammation markers, such as CRP, are associated with CAD.
The metabolic syndrome	This cluster of metabolic disturbances, with insulin resistance as a central feature, is strongly linked to CAD.
Environmental factors	
High-fat diet	Population migration studies have revealed very strong associations with lifestyle, and diet appears to be the most significant factor. High-fat, high-cholesterol diets are usually required for development of atherosclerosis in experimental animals.
Smoking	Epidemiological studies: It has been estimated that approximately 30% of CAD deaths are due to smoking. Clinical trials have demonstrated benefits of cessation of smoking.
Low antioxidant levels	Antioxidants protect against atherosclerosis in experimental animals. However, clinical trials with antioxidants are mixed and are not conclusive.
Lack of exercise	A significant independent relationship has been. observed.
Infectious agents	Animal studies: Epidemiological studies have shown associations with various agents, such as Chlamydia pneumoniae. Clinical trials have been inconclusive.

[a]Abbreviations used: CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

constituent apolipoprotein B (apoB) and matrix proteoglycans. In addition to LDL, other apoB-containing lipoproteins, namely lipoprotein(a) and remnants, can accumulate in the intima and promote atherosclerosis.

The buildup of LDL in the subendothelial space is determined by the rate of retention as well as the rate of influx. Trapped LDL is modified by oxidation, lipolysis, proteolysis, and aggregation. Lipid oxidation as a result of exposure to oxidative waste of vascular cells is the most significant modification. Such modifications initially give rise to "minimally oxidized" LDL species, which can stimulate the overlying ECs to produce a number of proinflammatory molecules, including adhesion molecules and growth factors such as macrophage colony-stimulating factor (M-CSF). The retention of LDL and the biological effects of the trapped LDL depend on the extent that they are oxidatively modified, which is related to the balance of pro-oxidant and antioxidant elements. Two important factors in the antioxidant repertoire are heme oxygenase-1 (HO-1) expression and high-density lipoprotein (HDL). HO-1 seems to be both an anti-inflammatory and an antioxidant gene. HDL is strongly protective against atherosclerosis, presumably because of its capacity to remove cholesterol from the vascular tissue and also to inhibit lipoprotein oxidation. The antioxidant properties of HDL derive in part from serum paraoxonase, an esterase carried on HDL that can degrade certain biologically active oxidized phospholipids.

Within days or weeks of diet initiation, monocytes can be observed adhering to the surface of the endothelium. The first step in adhesion, the "rolling" of leukocytes along the endothelial surface, is mediated by selectins that bind to carbohydrate ligands on leukocytes. The firm adhesion of monocytes to endothelium can be mediated by the integrin VLA-4 on these cells, which interacts with both VCAM-1 on the endothelium and the CS-1 splice variant of fibronectin. Monocyte chemotactic protein-1 and its receptor, CCR2, are also important in monocyte recruitment. Once present in the subendothelial space, monocytes are stimulated to proliferate and differentiate into macrophages, a process partly mediated by M-CSF.

LDL is extensively modified to generate the highly oxidized LDL by the action of reactive oxygen species and by several enzymes, such as lipoxygenases, myeloperoxidase, sphingomyelinase, and secretory phospholipase produced by the vascular cells. Oxidized LDL is then rapidly taken up by macrophages through their scavenger receptors, especially SR-A and CD36, to form foam cells. Macrophage scavenger receptor expression is regulated by peroxisome proliferator-activated receptor-g, an important transcription factor whose ligands include oxidized fatty acids, and by cytokines such as tumor necrosis factor-a, interferon-g (IFN-g), and M-CSF. Oxidized LDL can also inhibit the production of nitric oxide, a chemical mediator with multiple antiatherogenic

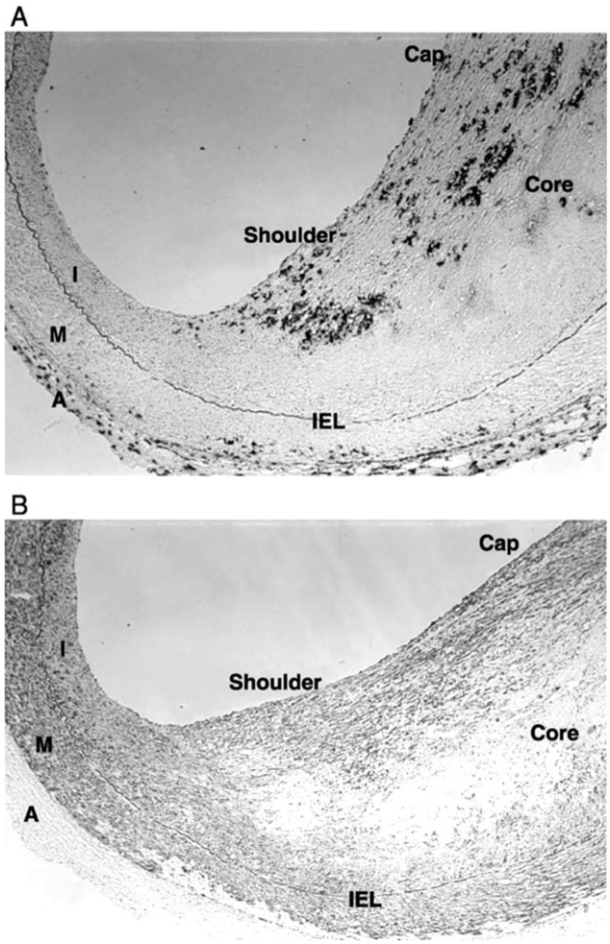

Fig. 1 Human coronary artery lesion. (A) All three main layers of the arterial wall are seen: Intima (I), media (M), and adventitia (A). The internal elastica lamina (IEL) clearly separates the intima from the media. The fibrous plaque core is rich in extracellular lipids and foam cells. Staining with mAb to macrophages (HAM56) allows visualization as dark infiltrates in the atherosclerotic plaque, prominent at the shoulder of the lesion. Notice the remarkable difference in the intima thickness between the neighbor normal arterial wall and the affected wall by the atherosclerotic plaque. (B) Staining with mAb to muscle actin (HHF35) allows visualization of the smooth muscle cells on top of the lesion core.

properties, including vasorelaxation. When sufficient foam cells accumulate in addition to extracellular lipid, a fatty streak lesion can be seen macroscopically by staining with Sudan IV.

Advanced Lesions: Progression to Fibrous Plaques and Complicated Plaques

The increasing number of macrophages and T lymphocytes secrete cytokines and growth factors (e.g., platelet-derived growth factor) that are responsible for smooth muscle cell (SMC) migration, proliferation, and extracellular matrix production. In addition to the growing mass of extracellular lipid, this leads to the formation of fibrous plaques.

Various factors contribute to the development of advanced lesions. The engagement of CD40–CD40L between all major vascular cell types results in the production of inflammatory cytokines, matrix-degrading proteases, and adhesion molecules. Elevated homocysteine levels appear to injure ECs and to stimulate proliferation of vascular SMCs. Activation of the renin–angiotensin system also stimulates SMC growth and the production of extracellular matrix. A vicious cycle of inflammation ensues as inflammatory conditions promote the retention and modification of LDL and increasingly more modified LDL stimulates inflammation.

As the atherosclerotic plaque grows, active intimal calcification, development of new vessels from the media, and rupture of vessels with resulting intramural hemorrhage or thrombosis may occur. The progressive and insidious growth of plaque burden into the lumen of the vessel leads to narrowing of the arterial lumen. When this reaches a critical level, ischemia occurs, causing various symptoms depending on the affected tissue.

However, it is the unstable plaque that ruptures and exposes its subendothelial components with subsequent thrombus formation that has the most significance, at least in the coronary arteries, and is responsible for the majority of myocardial infarctions. Plaque vulnerability is related to the fibrous cap thickness, which reflects the dynamics of matrix production and

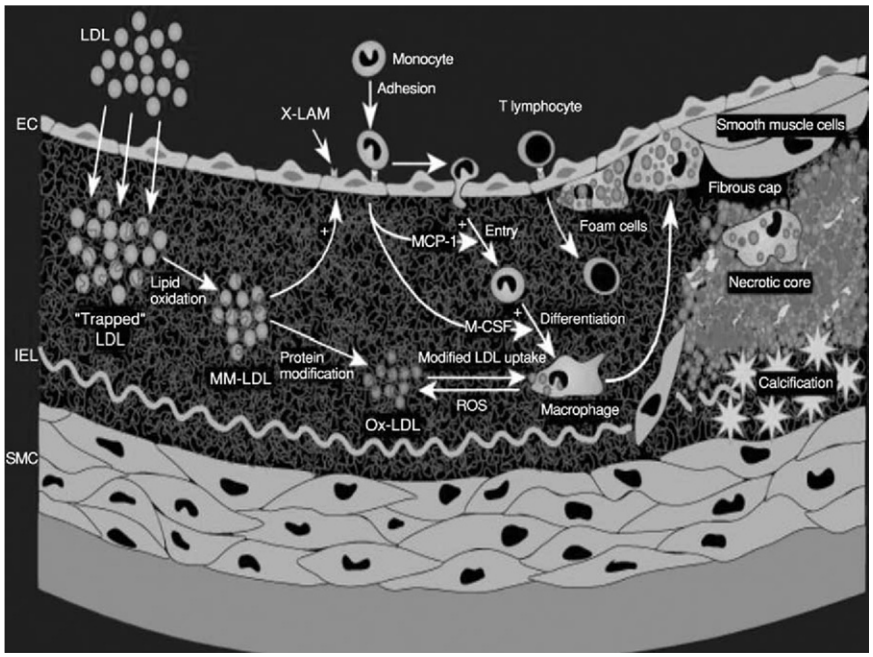

Fig. 2 Atherosclerosis model. The artery wall architecture has three main segments: The intima, from the endothelial cell (EC) monolayer to internal elastica lamina membrane (IEL); the media, consisting of smooth muscle cells (SMC); and the adventitia, which is the outer layer. Low-density lipoproteins (LDL) or other atherogenic lipoproteins enter and accumulate in the subendothelial space. Trapped LDL particles become oxidized probably as a result of interactions with reactive oxygen species (ROS) produced by vascular cells. Such minimally modified LDLs (MM-LDL) induce endothelial cells to express adhesion molecules for monocytes (X-LAM), monocyte chemotactic protein (MCP-1), and macrophage colony-stimulating factor (M-CSF), resulting in more recruitment of blood monocytes and subsequent differentiation into macrophages. With time, LDL particles become highly oxidized (Ox-LDL) and are then recognized by scavenger receptors on macrophages that endocytose them, giving rise to cholesterol-engorged foam cells. Such foam cells, the hallmark of fatty streaks, contribute to the development of advanced lesions by the production of various cytokines and growth factors that stimulate smooth muscle proliferation and fibrous cap formation. Calcification, hemorrhage, and thrombosis complicate plaques at advanced stages.

Table 2 Clinical manifestations due to atherosclerosis

Organ/system	*Clinical entity*
Heart	Coronary artery disease (stable and unstable angina and myocardial infarction) Congestive heart failure.
Central nervous system	Cerebrovascular disease (transient ischemic attacks and strokes).
Peripheral vasculature	Peripheral vascular disease (intermittent claudication, vascular insufficiency, and gangrene).

degradation. Vulnerable plaques generally have thin fibrous caps, increased numbers of inflammatory cells, and frequently rupture at the lesion edges where macrophages produce proteases that degrade extracellular matrix, including collagenases, gelatinases, and stromolysin. At the same time, matrix production by SMCs may be inhibited by IFN-g produced by T lymphocytes.

Clinical Manifestations

Atherosclerotic plaques may compromise the lumen of the vessels and obstruct blood flow to the respective tissue. Ischemia and/or infarction occur depending on the magnitude of the obstruction (partial, subtotal, or total), the duration of the obstruction, the time course (acute versus chronic), the collateral circulation, the degree of reversibility, and/or spontaneous reperfusion of the affected vascular territory. The most common clinical entities are shown in **Table 2**.

Atherosclerosis is a long and silent disease. By the time the patient presents with chest pain due to coronary artery disease or intermittent claudication due to arterial insufficiency, years and decades of lipid deposition and plaque progression have occurred. Atherosclerosis is a debilitating disease. Stroke and congestive heart failure due to ischemic cardiomyopathy incapacitate a large number of individuals, imposing an incredible burden on the patients, their families, and society in general. It is of extreme concern that the first manifestation is frequently sudden death, eliminating opportunities for intervention. Symptomatic patients may be fortunate in the sense that there is an opportunity for diagnosis, risk factor modification, and therapeutic intervention. It is clear that we cannot rely on the clinical manifestations for the diagnosis of these entities since it may be too late by then.

Assessment

In animals, assessment has been performed mostly by postmortem evaluation of atherosclerotic lesions that originate at different levels in the arterial vasculature (aorta, carotid, coronary or femoral arteries, etc.) under the defined experimental variables employed. The evaluation can be quantitative or qualitative and either at the macroscopic or at the microscopic level.

In humans, the situation is much more complex given the necessity of achieving a phenotypic characterization in vivo. Postmortem correlations can also be established and are useful, but they provide only a narrow view of the very dynamic and multifactorial process in question. When present, the specific clinical manifestations in the corresponding clinical scenario help in deciding which diagnostic modality to use. It is even more difficult to approach the asymptomatic individual. The same strategies used with clinical atherosclerosis have been used here, but often these miss the earlier stages of the disease, when preventive actions are desirable. Diagnostic modalities can be classified as invasive and noninvasive techniques. Invasive techniques, such as angiography (the gold standard) and intravascular ultrasound, offer the greatest degree of accuracy available, limited by cost and potential complications. Angioscopy allows direct visualization of the arterial wall, revealing plaque surface traits that are not representative of the internal heterogeneity of the plaque.

Noninvasive techniques can be classified as anatomic methods aimed at detecting the atherosclerotic plaque per se and physiologic methods aimed at detecting a physiologic abnormality created by impairment of blood flow in an arterial bed or impairment of vasodilation. Among the former, transesophageal echocardiography, electron-beam computed tomography, magnetic resonance angiography, and magnetic resonance imaging stand out. Among the latter, stress testing, coronary positron emission tomography, ultrasonic brachial vasodilatation, and the ankle–arm systolic blood pressure index techniques are commonly used. There is no one single and optimal method for phenotypic assessment, and the choice must be based on the specific indication.

Therapeutics

Table 3 lists various treatment strategies for atherosclerosis-related disorders, mainly coronary artery disease. These include drugs that improve symptoms or modify risk factors, invasive medical procedures, and surgical procedures. Some agents improve both symptoms and survival; for example, beta-blockers decrease cardiac work and myocardial oxygen demand by decreasing heart rate

Table 3 Strategies for Treatment of Symptoms or Improvement of Life Expectancy for Atherosclerosis-Related Disorders

Therapy	*Intervention*	*Mechanism*
Lipid-modifying drugs	Statins	Decrease LDL cholesterol, and/or increase HDL cholesterol, and/or decrease triglycerides (varies with the specific drug).
	Gemfibrozil	
	Niacin	
Antiplatelet drugs	ASA	Decrease platelet reactivity.
	Clopidogrel, ticlopidine	Decrease thrombus formation.
	Glycoprotein IIB–IIIA inhibitors	
Antithrombotic drugs	Thrombolytic agents	Lysis of thrombus.
	Unfractioned heparin	Decrease thrombus formation.
	Low-molecular-weight heparin	
Antiangina drugs	Beta blockers	Improvement of myocardial oxygen offer–demand balance.
	Nitrates	
	Calcium channel blockers	
Antihypertensive drugs	Diuretics	Blood pressure control.
	ACE inhibitors	
	Beta blockers	
	Calcium channel blockers	
	Alpha blockers	
	Direct vasodilators	
Antioxidant drugs	Vitamin E	Increase antioxidant reserve.
Interventional techniques	Angioplasty	Mechanical resolution of coronary and peripheral artery blockages.
	Stents	
	Catheter-based endarterectomy	
Surgical techniques	Coronary artery bypass graft surgery	Surgical bypass or repair of coronary, carotid, or peripheral artery blockages.
	Peripheral artery bypass surgery	
	Endarterectomy for carotid and peripheral arteries	
	Aortic aneurysm repair	

and cardiac contractility. Some agents improve symptoms without affecting mortality; thus, nitrates decrease cardiac work and oxygen demand by increasing venous capacitance, decreasing venous return, and decreasing cardiac volume preload. Also, some agents improve symptoms but may have harmful effects (e.g., short-acting calcium channel blockers).

The development of interventional cardiology in recent years has revolutionized the treatment of coronary disease. Angioplasty with or without stent placement is the customary choice of one- and twovessel coronary disease. It is particularly useful after medical therapy has been optimized without resolution of symptoms. With the better understanding of this disease and the development of better agents, surgical cases are decreasing in frequency.

However, cardiovascular disease is still the most common cause of death in the Western world. The known risk factors explain only about 50% of susceptibility to the disease. A better understanding of how to optimize therapy for the individual patient is required.

Genetic Dissection

Atherosclerosis is a multifactorial and complex disease. Risk factors can be classified as those with an important genetic component and those that are largely environmental (**Table 1**). Common forms of coronary artery disease result from the combination of genetic susceptibility and an unhealthy environment.

Rare mendelian forms, such as familial hypercholesterolemia and Tangier disease, have provided important insights into the disease. Studies of candidate genes associated with predisposing conditions, such as hyperlipidemia, low HDL levels, diabetes, hypertension, and pro-coagulant disorders, have revealed a number of genes with significant or suggestive association or linkage with traits relevant to atherosclerosis.

As a result of the genome project and large-scale sequencing, thousands of single-nucleotide polymorphisms are being identified and a catalogue of all common variations in humans will be generated during the next few years. It can be anticipated that profiles of genetic factors, perhaps in the form of risk haplotypes, can be generated and that individuals in the future may be screened for these and therapies may be tailored based on the particular risks for each individual.

New Venues

Active and intense research is being conducted at all levels, from the basic aspects of genetics and molecular and cellular biology to the development of better diagnostic resources and risk-stratifying tools and the formulation of better therapeutic strategies. In the middle of the great genomic revolution, as our understanding of the genetics of the disease grows, genetic diagnosis will become increasingly important. It is hoped that this will allow genetic screening for diagnostic and risk-stratification purposes, which will enable better assignment of management resources and adequate and tailored pharmacological therapy.

Conclusion

Atherosclerosis is a chronic inflammatory disease characterized by progressive accumulation of lipids and cellular and fibrous elements in large and medium-sized elastic and muscular arteries. Elevated cholesterol levels, especially in the LDL fraction, are a principal risk factor. Lipoprotein retention and oxidation in the vascular wall leads to endothelium activation, monocyte infiltration, foam cell formation, inflammation, and proliferation of smooth muscle cells. Subsequently, a necrotic core of lipid and cell debris develops and vulnerable plaques rupture, leading to thrombosis. Clinical manifestations result from ischemia and vascular thrombosis in the respective vessels, leading to myocardial infarction, stroke, vascular insufficiency, and gangrene of the extremities.

Various resources are available for diagnosis and risk stratification, but all are limited and far from ideal. Despite an array of therapeutic options tailored for the different clinical manifestations, better strategies are needed to stop or reverse the disease and to be able to adjust pharmacological agents to each individual. It is hoped that the genetic dissection of this syndrome will allow genetic diagnosis to support screening and tailored therapy.

Acknowledgments

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Atherogenesis☆

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Introduction

The American Heart Association classified the various stages of lesion progression into six phases and lesion types (**Fig. 1**). The first stage has been called arterial intimal thickening (AIT) (Type I) and starts with the migration of vascular smooth muscle cells (VSMCs) to the intima and the small accumulation of low density lipoprotein (LDL) and macrophages in the subendothelial–intimal space. Modified LDL is a chemoattractant for monocytes and VSMCs; these cells, by taking up modified LDL in a noncholesterol down-regulated manner, become foam cells initiating the fatty streak formation (Type II). The extracellular lipid accumulation coming from death cells besides the foam cells is characteristic of lesion Type III and lesion Type IV typical of the third decade of life. In more advanced lesions (Types V, VI, and VII), pathological changes occur in VSMCs of the media layer that contain a significant number of foam cells. In Type V lesions, several collagen layers cover the lipid core. These lesions may have a cap of VSMCs and extracellular matrix (ECM) covering the lipid core that modulates their susceptibility to rupture and thrombus formation. The successive accumulation of collagen fibers leads to a progressive lumen narrowing. During the fourth decade of life, lesions can rupture and trigger thrombotic depositions (Type VI lesions). During this stage, the formation of an occlusive thrombus can have dramatic effects. During the fifth decade of life, most of the lesions show an advanced stage and are composed mainly of calcifications (Type VII lesions) or fibrous tissue (Type VIII lesions).

Still not much is known about the disease initiation and progression, and the major risk factors can explain only approximately 50% of the coronary events found in humans. A major mystery in human atherogenesis is the well-known variation in lesion progression among individuals with similar plasma lipid profiles or risk factors. Furthermore, other factors affecting the molecular and cellular mechanisms in the arterial wall may be critical for the pathogenesis of the disease. Various findings suggest a diversity of agents that can induce endothelial injury, leading to a vascular response in a harmful chronic state. Besides LDL, cytokines and growth factors are important regulators of the inflammatory response, cellular growth, and lipid deposition. All of these processes are responsible for atherosclerotic plaque progression. During the final stages of the disease, arterial plaques become complex aggregations of lipids, ECM, and necrotic and apoptotic cells covered by a fibrous cap consisting primarily of smooth muscle cells surrounded by collagens and proteoglycans (PGs). The vulnerable plaques contain a lipid-rich core and a soft fibrous cap.

Role of Inflammation

It has been postulated that inflammation is involved in atherogenesis due to the observation that elevated levels of cytokines can be measured during acute coronary syndrome (ACS) presentation. In fact, one of the hypotheses for the

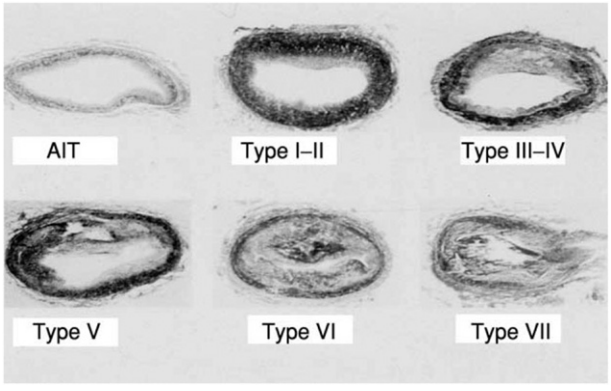

Fig. 1 Classification of the atherosclerotic lesions according to the American Heart Association. AIT, arterial intimal thickening; Type I–II; Type III–IV; Type V; Type VI; Type VII. (Original preparations.).

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This article is an update of Lina Badimon, Vicenta Llorente-Cortés, Atherogenesis, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 278-287.

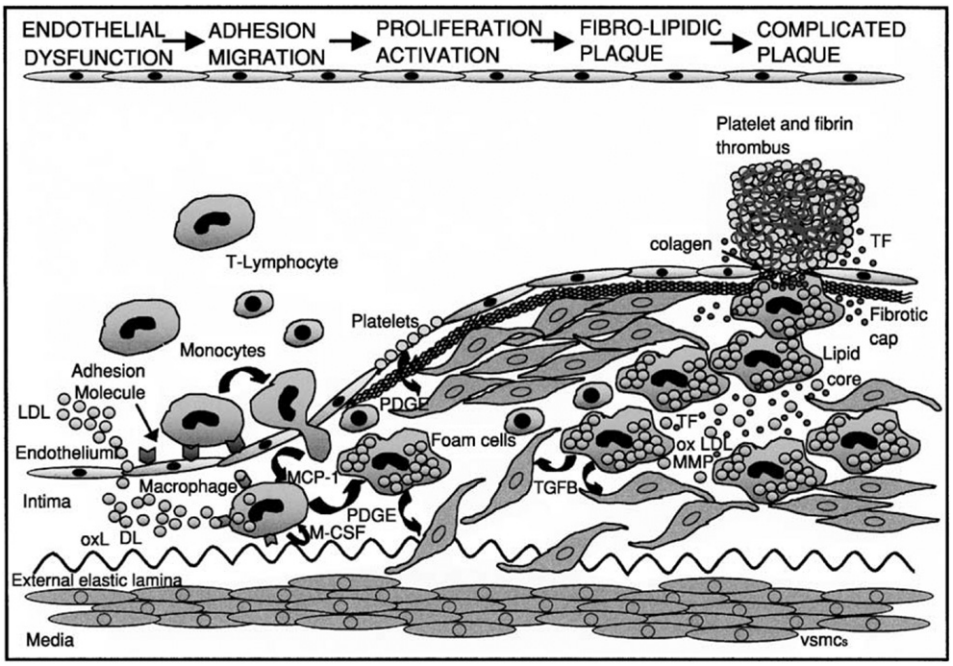

Fig. 2 Illustration showing the molecular mechanisms that lead to initiation and progression of the atherosclerotic lesions. PDGF, platelet-derived growth factor; M-CSF, macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; oxLDL, oxidized LDL; TF, tissue factor.

initiation of atherosclerosis is the so called "response to injury hypothesis" involving the response of the innate immune system to the accumulation and modifications of lipoproteins in the arterial intima. Various constituents of the modified lipoproteins trigger the production of mediators of innate immunity. There are also nonlipid mediators involved in inflammation such as homocysteine, angiotensin II, and microbial products that can induce the elaboration of cytokines from atheroma-associated cells. In normal circumstances, the endothelial monolayer in contact with flowing blood is inert to the adhesion of leukocytes. The situation changes in dysfunctional endothelium. One of the endothelial–leukocyte adhesion molecules involved mainly in the early adhesion of mononuclear leukocytes to arterial endothelium is the vascular cell adhesion molecule-1 (VCAM-1), which binds particularly those classes of leukocytes found in nascent atheroma: the monocyte and the T lymphocyte. In addition to VCAM-1, P-and E-selectin and intercellular adhesion molecule-1 (ICAM-1) also seem to contribute to leukocyte recruitment. As shown in **Fig. 2**, once the leukocyte adheres to the endothelium, it enters into the intima by diapedesis, a process that is facilitated by various chemokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8), and a trio of CXC chemokines induced by interferon-gamma. Myeloperoxidase (MPO) has emerged as a potential participant in the promotion and/or propagation of atherosclerosis and other CVD. This enzyme present in activated neutrophils, monocytes and tissue macrophages, catalyzes the formation of reactive oxygen species (ROS). MPO and ROS are abundant in human atherosclerotic plaques and show increased expression levels in the infarct area after an acute myocardial infarction. High plasmatic MPO levels predict endothelial dysfunction and coronary artery disease while low plasmatic levels and certain specific MPO polymorphisms have been described as cardioprotective.

Macrophages are very versatile and, depending on the local microenvironment, can assume different phenotypes and functional characteristics, which is a reversible process termed "polarization". Distinct macrophage subtypes (M2 and M1) have been detected depending on the stage of atherosclerosis development. One differentiated, macrophages exhibit high levels of surface pattern recognition receptors which have the ability to take up modified LDLs, become lipid-laden and convert into foam cells. Many of these lipid-laden macrophages undergo apoptosis at early stages of atherosclerosis development and are promptly removed by M2 macrophages in a process referred to as efferocytosis. Nevertheless, excessive intake of apoptotic cells by macrophages may eventually stress the endoplasmic reticulum leading to the deterioration of efferocytosis, subsequent macrophage death and release of lipids, and pro-inflammatory/pro-thrombotic mediators.

One of the most important clinical markers of inflammation is the C-reactive protein (CRP), a marker of inflammation that has been shown in multiple epidemiological studies to predict incidencia of myocardial infarction, stroke, peripheral arterial disease, and sudden cardiac death. In terms of clinical application, some data indicate that CRP seems to be a strong predictor of cardiovascular events and adds prognostic information at all levels of calculated Framingham risk and at all levels of metabolic syndrome. The feature that distinguishes CRP from LDL cholesterol is the fact that inflammation (but

not elevated cholesterol LDL) plays a major role in nearly all processes associated with metabolic syndrome.

Acquired immunity, mainly dependent on T cells [T helper (Th) 1 and Th2] and antibodies, is also critically involved in the progression of atherosclerosis. In this regard, the cytokine IL-8 has been shown to orchestrate the immunological link between the innate and adaptative immune responses, enhancing atherosclerosis activity and progression.

Recently, the epigenetic regulation has been regarded as a plausible mechanism by which risk factors such as diet, environment of life style contribute to the atherogenic inflammatory response. DNA hypermethylation in the inflammatory mononuclear cells seems to be linked to predisposition to atherosclerosis. However, which genes are directly affected by DNA methylation remains to be determined. Other new players in the link between inflammation and atherosclerosis are microRNAs (miRNAs). miRNAs are endogenous nucleotides that bind to mRNA and induce translation repression. As an example, miR-143 and miR-145 play a crucial role regulating VSMC phenotypes and controlling neointima formation.

Role of Lipoproteins

Cholesterol is transported into the vessel well as a component of the lipoproteins. LDLs are considered the most atherogenic lipoproteins because they accumulate in the intima and carry large amounts of plasmatic cholesterol (up to 70%). The coronary risk lipid profile that uses the total cholesterol/high density lipoprotein (HDL) ratio (or the LDL/HDL ratio) predicts risk of vascular disease and is used as a tool for therapeutic management of patients at risk for vascular disease. Although these methods are predictive of coronary artery disease (CAD) in general, it is also well known that the extent of occlusive disease and CAD varies greatly among individuals with similar cholesterol and HDL lipid profiles. For this reason, the National Cholesterol Education Program Expert Panel revised these guidelines and recommends monitoring LDL and HDL in the context of coronary heart disease (CHD) risk factors and "risk equivalents."

High levels of circulating LDL have shown to be predictive markers of high risk of cardiovascular disease (CVD) in individual persons. It appears that discrete LDL subclasses carry various levels of atherogenicity. The "atherogenic lipoprotein phenotype" describes a combination of moderate hypertriglyceridemia, low HDL cholesterol, and a predominance of small dense LDL particles. This dyslipemia is prevalent in patients with metabolic syndrome, in those with Type 2 diabetes, and in postmenopausal women. In particular, small dense LDL particles have increased affinity by the proteoglycans of extracellular matrix, exacerbating LDL retention and modification. Besides small dense LDL particles, oxidized LDL glycated LDL, and electronegative LDL are increased in diabetes.

Table 1 Main dietary fatty acids

Saturated fatty acids	*Monounsaturated fatty acids*	*Polyunsaturated fatty acids*
Lauric acid (12:0)	Oleic acid (18:1n-9)	Linoleic acid (18:2n-6)
Myristic acid (14:0)	*Trans* (16:1 + 18:1)	α-Linolenic acid (18:3n-3)
Palmitic acid (16:0)		Eicosapentenoic acid (20:5n-3)
Stearic acid (18:0)		Docosahexenoic acid (22:6n-3)

Table 2 Mechanisms by which diet potentially influences risk of CHD

- Lipid levels
  - LDL cholesterol
  - HDL cholesterol
  - Triglycerides
  - Lipoprotein
- Blood pressure
- Thrombotic tendency
- Cardiac rhythm
- Endothelial function
- Systemic inflammation
- Insulin sensitivity
- Oxidative stress
- Homocysteine level

Table 3 Modified forms of LDL

LDL modification	*Taken up by*	*Reference(s)*
Interaction with PGs	MØ, VSMCs	Camejo *et al.* (1993)
Glycosylation	MØ, VSMCs	Witztum *et al.* (1989) Parlinski *et al.* (1995)
(4-HNE)-LDL	MØ	Jürgens *et al.* (1987) Hoff *et al.* (1989)
MDA-LDL	MØ	Fogelman *et al.* (1980) Haberland *et al.* (1988)
Self-aggregatio	MØ, VSMCs	Khoo *et al.* (1988)
Carbamylation and maleylation	MØ	Weisgraber *et al.* (1978)
Desialylation	MØ, VSMCs	Orekhov *et al.* (1989) Tertov *et al.* (1992)
Oxidation	MØ	Witztum *et al.* (1994)

Oils, Lipoproteins, and Coronary Risk

Dietary fats and oils differ in the chain lengths of their constituent fatty acids and the number and geometry of their double bands. These differences markedly affect concentrations of lipids in plasma, and differences in the amount and type of fat in the diet can induce differences of 30 to 40% in serum LDL concentrations. **Table 1** shows the main saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). When SFAs are replaced by unsaturated fats, total plasma cholesterol is lowered. A review of metabolic studies, prospective cohort studies, and clinical trials indicates that there are multiple mechanisms by which diet potentially influences risk of CHD, and there are dietary strategies that are effective in preventing CHD (**Table 2**). As such, the, substitution of nonhydrogenated unsaturated fats for saturated and trans fats; the increase in fruits, vegetables, nuts, and whole grains and the decrease in refined grain products are good strategies to prevent CHD. It has been recently reported that among persons at high cardiovascular risk, a Mediterranean diet supplemented with extra-virgin olive oil or nuts reduced the incidence of major cardiovascular events. These results are consistent with epidemiological studies reporting an inverse association between the Mediterranean diet or olive-oil consumption and incident stroke. It has been proposed that nutrients of the Mediterranean diet promote favorable changes in pathways involved in cardiometabolic risk, blood lipids, insulin sensitivity, resistance to oxidation, inflammation and vasoreactivity. Moreover, in an study focused on gene-diet interactions determining obesity, a genetic score including the *MC4R*rs17782313 and *FTO*rs9939609 variants was associated with body mass index (BMI), higher adherence to traditional Mediterranean diet was associated with lower BMI in genetically susceptible individuals. Mediterranean diet reduced genetic risk of these individuals. Both Mediterranean diets of PREDIMED trial were able to reduce the adverse events of the *TCF7L2*rs7903146 (C>T) polymorphism on intermediate cardiovascular risk factors and mainly on stroke incidence.

Free fatty acids (FFA) play a role in early steps of atherogenesis and have implications on the prevention and treatment of CVD. FFA increase the generation of ROS by the production of cytokines in mononuclear cells. FFA can also induced the activation of pro-inflammatory NF-kB pathway in human endothelial cells. To prove the clinical relevance of FFA, a group of healthy subjects received a 48-h infusion of low-dose lipid to increase plasma FFA to levels of obesity and diabetes. Plasmatic levels of endothelial activation biomarkers (ICAM and VCAM), systemic inflammation biomarkers (MPO) and thrombosis biomarkers (tPAI-1) were increased. These results provide direct evidence in human that mild short-term lipid-oversupply is sufficient to initiate early vascular abnormalities that may lead to atherosclerosis and CVD.

Modified Forms of LDL

Extracellular accumulation of lipids in the arterial intima occurs very early in response to increased plasma lipoprotein level. PGs and protein-bound lipoprotein particles, perhaps in a microenvironment shielded from plasma antioxidants, can undergo modifications. As shown in **Table 3**, such modifications include oxidation, aggregation, and enzymatic and nonenzymatic modifications of LDL. Modified forms of LDL are associated with increased atherogenicity because the physicochemical properties of the lipoprotein become altered. These changes in the biological properties of the LDL increase LDL susceptibility to other types of modifications.

Cellular Effects of Modified LDL

Endothelial dysfunction

Hypercholesterolemia induces an increase of leukocyte recruitment by a higher expression of adhesion molecules, a decreased endothelium-dependent vasodilatation, and alterations in the thrombosis/fibrinolysis balance. Our group has demonstrated that

systemic hypercholesterolemia can induce endothelial dysfunction by altering the expression of genes that are regulated through the down-regulation of sterol regulatory element-binding proteins (SREBPs) in endothelial cells

Synthesis and degradation of extracellular matrix

Modified LDL can modulate the synthesis of PGs in various cell types. The increase of PG synthesis induced by LDL might have important consequences for the intimal LDL retention. In addition, the exposure of endothelial cells to apolipoprotein E (apoE)-containing HDL has been shown to stimulate the production of heparan sulfate proteoglycans (HS-PGs) that have increased sulfation. Intracellular lipids derived from modified lipoprotein uptake reportedly inhibits Src-dependent assembly of fibronectin and Type I collagen in vascular smooth muscle cells. VSMC-intracellular lipids also provoke crucial alterations in VSMC-tropoelastin levels, mechanical properties and elastin capacity to breakdown. Taken together, these results point to lipid-loaded VSMC as crucial players in extracellular matrix alterations during atherosclerosis.

Lipoproteins also modulate the expression of metalloproteinases (MMPs), enzymes that digest various connective tissue components. An increase in metalloproteinase expression and activity is associated with the disruption of the fibrous cap of lesions and plaque rupture. In addition, the ECM break-down products may be biologically active and might increase processes that are fundamental for the pathogenesis of the atherosclerosis. By modulating metalloproteinase activity, LDL may modulate VSMC migration. Atherogenic concentrations of both native and modified LDL have been shown to significantly reduce the migratory capacity of human VSMC.

Mononuclear Cell Recruitment

Modified LDL particles induce endothelial secretion of chemotactic substances and the expression of adhesion receptors including integrins and selectins that favour leukocyte (monocyte and T-cell) recruitment, adhesion, and transmigration into the arterial wall (**Fig. 2**). This is caused by simultaneous expression of different chemotactic and adhesion molecules suggest through a common transcription factor, nuclear factor-kb (NF-kb). For instance, the chemokine monocyte chemoattractant protein (MCP-1) interacts with monocyte receptor CCR2 recruiting the monocytes tohe endothelial layer favouring their entry by diapedesis. Transmigration of monocytes preferably occurs in areas where the basal lamina is enriched with modified LDL particles and takes place mainly through the junctions between endothelial cells. Junction adhesion molecule (JAM)-A and C have been shown to be involved in the control of vascular permeability and leucocyte transmigration across endothelial-cell surfaces.

Foam Cell Formation

Modified lipoproteins are taken up through mechanisms not regulated by cholesterol, leading to high intracellular cholesteryl ester accumulation and foam cell formation. The accumulation of lipid-laden foam cells is one of the earliest steps in the progression of the atherosclerotic plaque. In macrophages, the SRs are mainly responsible for modified LDL uptake. One monocytes reach the intimal space, colony-stimulatinf factor (CSF) induces monocytes to phenotypically transform into macrophages and begin the uptake of modified lipoproteins. Macrophage-related mechanisms and receptors include scavenger receptor class A (SRA)-I and SRA-II, CD36, LOX-1, or CXCL16 for oxidized LDL. For aggregated LDL, the main receptor involved in its uptake by macrophages is low density lipopoprotein receptor-related protein (LRP1). Macrophages become foam cells through the uptake of diversely modified LDLs, whereas the aggregation of LDLs (agLDL) seems to be a key condition for lipid accumulation in VSMCs. AgLDLs obtained by vortexing LDL in vitro share structural characteristics with LDL aggregates present in atherosclerotic lesions. Our previous results demonstrate that hypercholesterolemia might increase the capacity of VSMCs to take up LDL from the intima by regulating cellular low density lipoprotein receptor-related protein 1 (LRP1), the receptor for agLDL uptake in human VSMC.

Most of the modified lipoproteins could lead to a progressive cholesteryl ester accumulation, not only by being taken up through non-down-regulated receptors but also by up-regulating their own receptors. Besides hypercholesterolemia, other cardiovascular risk factors such as hypertension or hypoxia strongly upregulate LRP1 expression in cultured VSMC and in the vascular wall of in vivo models. Transcription factors including sterol regulatory element binding proteins (SREBPs) and hypoxia-inducible factor-1 (HIF-1a) mediate the up-regulatory effects of hypercholesterolemia and hypoxia on LRP1 expression, respectively.

Lipoproteins and Metabolic Syndrome

Metabolic syndrome was defined by the National Institutes of Health in 2001 as a cluster of disorders that include abdominal obesity, insulin resistance, diabetes, endothelial dysfunction, elevated blood pressure, and impaired fibrinolysis. The risk factors that constitute metabolic syndrome consist of atherogenic dyslipemia, elevated blood pressure, elevated

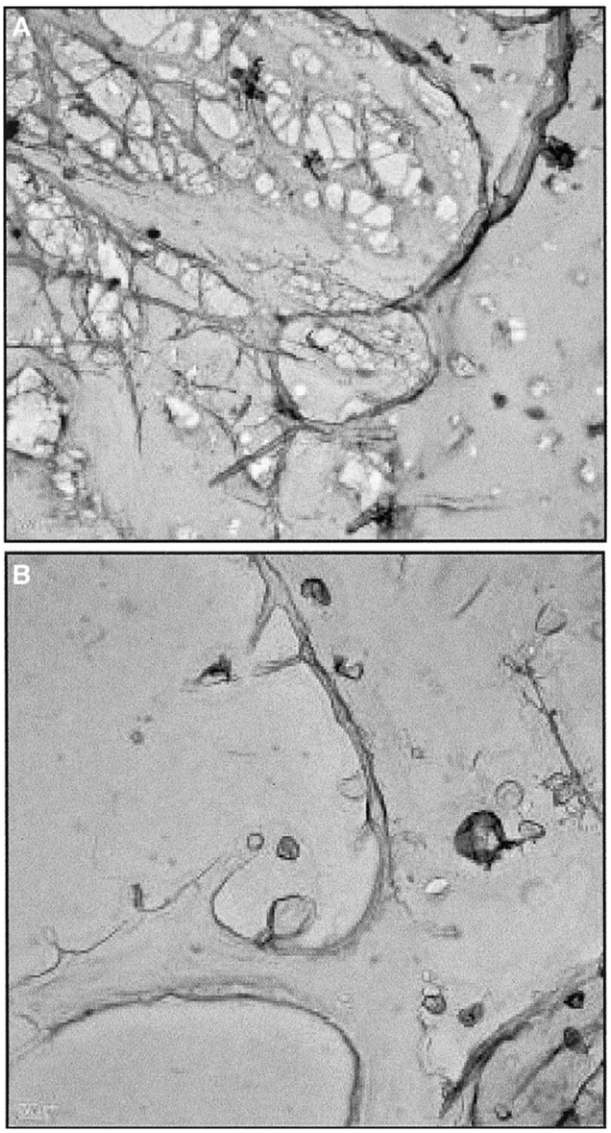

Fig. 3 Extracellular matrix ultrastructure of VSMCs. Cells were washed, fixed, and processed for freeze-drying and replication in electron microscopy. (A) Nonenzymatically treated human VSMCs. (B) Heparinase-treated human VSMCs. Modified from Llorente-Cortés, V. *et al.*, 2002. Arterioscler. Thromb. Vasc. Biol. 22, 1905–1911.

plasma glucose, and a prothrombotic state. Metabolic syndrome is closely linked to the metabolic derangement called insulin resistance. This condition is characterized by a generalized defect in the insulin-signaling pathway. Because insulin induces a myriad of metabolic responses, a defect in insulin signaling results in several metabolic changes. The presence of insulin resistance predisposes to the development of Type 2 diabetes. There are four major causes of insulin resistance: genetics, obesity, lack of exercise, and diet composition. Insulin resistance and metabolic syndrome are related to the atherogenic dyslipemia also called "atherogenic lipoprotein phenotype," which is characterized by increased triglyceride-rich lipoproteins, increased small LDL particles, and reduced HDL levels. These changes in lipoprotein and fatty acid profile observed in insulin resistance may influence various proatherosclerotic mechanisms such as membrane lipid composition, metabolism, and signal–transduction pathways. In insulin-resistance patients, there is an increase in plasma small, dense, electronegative LDL [LDL(−)]. LDL(−) has been defined as a pool of LDLs modified by different mechanisms presenting pro-inflammatory activity, reduced affinity to the LDL receptor and increased binding to arterial proteoglycans, among other properties. LDL(−) induces a plethora of deleterious effects on several cell types involved in atherogenesis including the release of cytokines by endothelial cells, monocytes and lymphocytes, the impairment of endothelial progenitor cell differentiation, endothelial apoptosis and platelet activation. Under normoxic conditions, LDL(−) raises intracellular NEFA and TG levels, and upregulates Perilipin5 (Plin5) expression in cardiomyocytes. However, under hypoxia, LDL(−) delivers

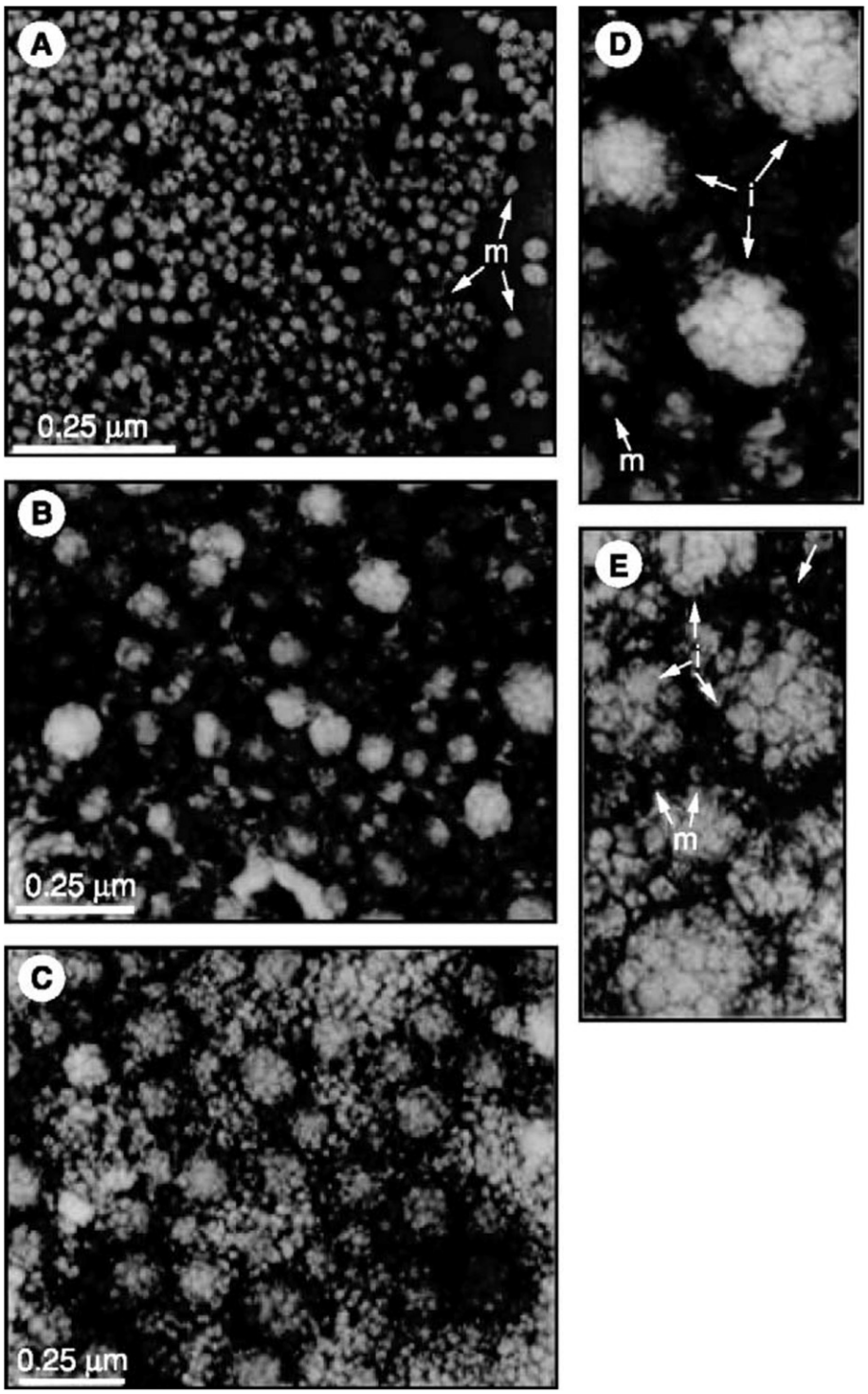

Fig. 4 Electron microscopy of nLDL, agLDL, and versicanmodified LDL. (A) nLDL. (B) Precipitable fraction from vortexed LDL. (C) Precipitable fraction from versican LDL. (D) Doubly magnified section from panel B. (E) Doubly magnified section from panel C. Samples were negatively stained and observed by electron microscopy. m, monomeric; f, fused; a, aggregated. Modified from Llorente-Cortés, V. et al., 2002. Arterioscler. Thromb. Vasc. Biol. 22, 387–393).

increased amount of NEFAs to cardiomyocytes and induces higher intracellular TG accumulation but, in contrast, fails to induce Plin5 expression. As a consequence, combined LDL(–) and hypoxia favours a high intracellular NEFA content and promotes ROS production in cardiomyocytes.

Role of the Various Components of the Vascular Wall

Extracellular matrix

The ECM of the arterial intima is a relatively large compartment made of collagen, elastin, complex PGs, hyaluronate, and multidomain proteins such as fibronectin, laminin, and tenascin (**Fig. 3**). The ECM occupies 60% of the arterial intima and regulates numerous cellular functions. The main PGs structuring the ECM are chondroitin sulfate (CS)-PGs, such as versican and biglycan, which have the longest negatively charged glycosaminoglycan (GAG) chains and are synthesized mainly by VSMCs. HS-PGs, such as perlecan, are constituents of the basement membrane and are synthesized mainly by endothelial cells and VSMCs. In addition, other HS-PGs, such as syndecan and glypican, are found in the cell membranes of the vascular cells. Whereas CS-PGs play a major role in LDL retention in the arterial intima, cell surface HS-PGs are dynamic molecules that mediate ligand catabolism. Collagens play a central role not only in maintaining the integrity and stability of the wall but also in many cellular functions.

Heparan sulfate proteoglycans

HS-PGs consist of a protein core and at least one heparan sulfate side chain. HS-PGs can be secreted (perlecan) or shed from the cell surface (perlecan, syndecan, and glypican). Syndecan and perlecan can function as receptors for several growth factors and lipoproteins. HS-PGs act as a receptors for basic fibroblast growth factor (bFGF), which stimulates VSMC proliferation. They may act as potential receptors for atherogenic lipoproteins, such as apoE–triglyceride rich lipoprotein particles and lipoprotein lipase, or may facilitate the uptake of ligands by a process called ligand transfer to lipoprotein receptors, such as the LRP1.

Chondroitin sulfate proteoglycans

CS-PGs are considered to be atherogenic due to their ability to trap and aggregate cholesterol-rich lipoproteins. Because one of the key initiation events in early atherogenesis is the subendothelial retention of atherogenic lipoproteins, CS-PGs play a main role in the initiation of atherogenesis ("response to retention" hypothesis). Apoproteins have been found to colocalize with specific PGs in human atherosclerotic plaques, providing support to the "response to retention" hypothesis. Several studies demonstrate that CS-PGs act as sites for apoB-100 lipoprotein retention by the interaction between positively charged heparin-binding domains on apoB-100 or apoE and negatively charged GAG chains of the PGs. The GAGs of versican induce alterations in the LDL particles that facilitate oxidative enzymatic modification, fusion, and aggregation of LDL particles. **Fig. 4** shows an electron microscopy image of versican modified LDL in comparison with nLDL and agLDL. Versican clearly induces the aggregation of LDL particles. Aggregation substantially increases its binding to arterial PGs due to the creation of a multimeric ligand with better exposure of key positive residues on apolipoprotein B. Modified LDL is avidly taken up by macrophages and VSMCs, leading to foam cell formation. In addition, some CS-PGs such as decorin, a small DS/CS PG, regulate cell growth and growth factor activity.

Collagen

Collagen is the major component of the ECM of the vessel wall and has a critical impact in atherogenesis. Collagen is essential for the maintenance of vessel wall integrity and elasticity. Collagen is involved in the process of cell differentiation, adhesion, migration, proliferation, and apoptosis. It has been reported that the bulk of vascular collagen is produced by smooth muscle cells.

Table 4 Factors synthesized by the endothelium

Factors	*Process*	*Contribution*
PDGF	Growth	Stimulator
TGF-β		Inhibitor
Heparin-like GAG		Inhibitor
NO	Vasodilatation	Stimulator
Endothelin		Inhibitor
Angiotensin II		Inhibitor
TF	Thrombosis	Stimulator
Trombomodulin		Stimulator
vWF		Inhibitor
t-PA	Fibrinolysis	Stimulator
PAI-1		Inhibitor
Prostacyclin	Others	
E-selectin, P-selectin, ICAM-1, VCAM-1. IL-1, IL-6, IL-8, MCP-1, GM-CSF		

However, collagens can also be produced by endothelial cells, adventicial fibroblasts, and macrophages. Among the 19 collagens described, 13 are found in the vessel wall or are expressed by cells of the vessel wall in vitro. The most abundant are Type I and Type III collagen, which form an interconnected network of cross-banded fibers along with smaller amounts of Type VI and Type VIII collagen, associated with the fibers and the elastic lamellae in the blood vessel wall. Type I and Type III collagen seems to play an important role in arterial wall remodeling in response to hypertension. It has also been reported that Type I collagen expression was localized mainly in the adventicia, outer media, and intima and that Type III collagen expression was uniformly localized across the arterial wall in response to an elevation of blood pressure. Type III collagen accumulates in atherosclerotic lesions, and in various in vitro studies in cultured endothelial cells. The involvement of Type VIII collagen in processes of endothelial differentiation and organization and in vitro angiogenesis has been demonstrated.

Cellular Components

Endothelium

In health, the vascular endothelium forms a multifunctional interface between circulating blood and the various tissues and organs in the body. It constitutes a selectively permeable barrier for macromolecules as well as a nonthrombogenic surface that actively maintains the fluidity of blood. The endothelium is a complex and dynamic organ that responds to environmental stimuli and activates vasoactive substances, including vasoconstrictors such as angiotensin II and vasodilators such as nitric oxide (**Table 4**). These vasoactive substances mediate vascular tone, structure, and function influencing

Table 5 Cell surface receptors that mediate the uptake of modified LDL

Receptors	*Ligands*	*Cellular type*	*Atherosclerotic plaque*	*References*
LDL receptor	Unmodified LDL LDL interacting with PGs	EC, VSMCs and MØ	±	Goldstein (1979) Kodama (1990)
SRs-AI, SR-AII	OxLDL			Acton *et al.* (1994)
CD36	OxLDL,HDL	MØ	+	Rigotti *et al.* (1997) Endeman *et al.* (1993)
CD68	OxLDL	MØ	++	Ramprasad *et al.* (1995)
FcRII-B2	OxLDL aggregates	MØ	++	Stanton (1992) Sawamura *et al.* (1997)
LOX-1	OxLDL	EC, VSMCs, and MØ	++	
SRs-PSOX	OxLDL	MØ	++	
LRP	AgLDL Versican-fused LDL ApoE–lipid complexes	VSMCs and MØ	+++	Llorente *et al.* (2000) Luoma (1994)

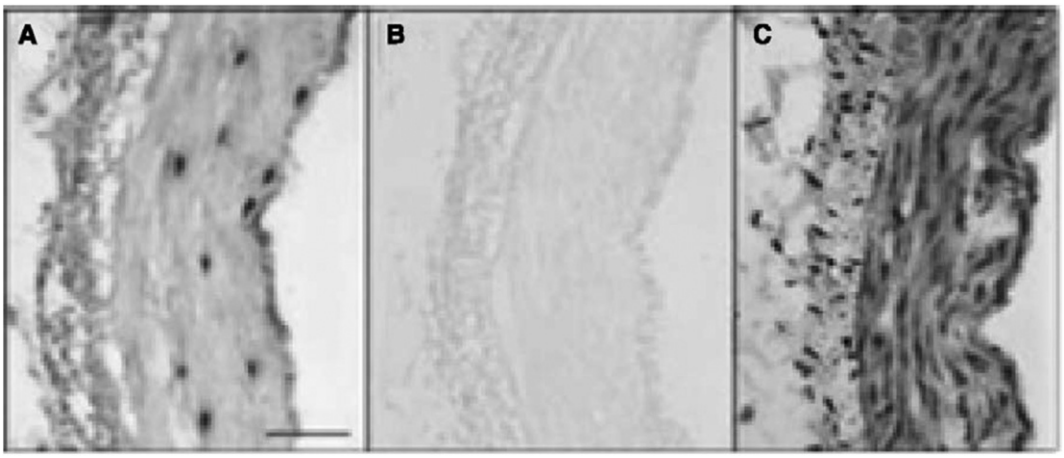

Fig. 5 NOR-1 overexpression in angioplasty. In situ reverse transcription polymerase chain reaction (RT-PCR) shows the induction of NOR-1 in medial VSMCs after PTCA, with serial sections from a dilated artery. (A) Vessel showing NOR-1 expression detected by antisense oligonucleotide anti-NOR-1. (B) Negative RT-PCR controls. (C) Hematoxylin- and eosinstained vessel. Modified from Martìnez-González, J. *et al.*, 2003. Circ. Res. 92, 96–103.

VSMC growth, apoptosis, platelet aggregation, monocyte and leukocyte adhesion, and thrombosis. The homeostasis of vasoactive substances is disrupted by endothelial dysfunction, leading to changes in vascular structure and function. Hypertension and other risk factors for CVD are associated with endothelial dysfunction and vascular remodeling. Elevated angiotensin II activity, which is strongly correlated with hypertension, is a major trigger of endothelial dysfunction in hypertensive patients. Angiotensin II stimulates nicotinamide adenine dinucleotide phosphate (NADPH)/ nicotinamide adenine dinucleotide (NADH) oxidase in the endothelium, VSMCs, and adventicia of blood vessels to generate reactive oxygen species, leading to endothelial dysfunction, cell growth, and inflammation. These changes result in up-regulation of endothelin-1, adhesion molecules, nuclear factor-kB (NF-kB), and other inflammatory mediators as well as increased breakdown of nitric oxide and uncoupling of nitric oxide synthase. Thus, the balance of vasoconstriction and vasodilation

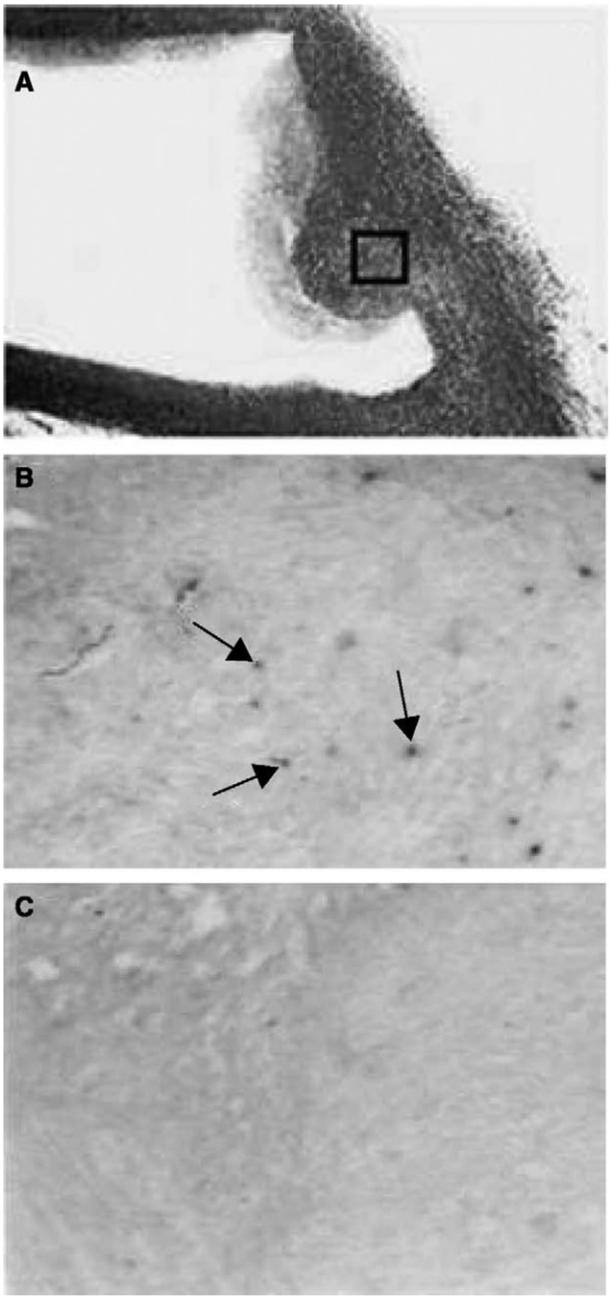

Fig. 6 In situ hybridization analysis of LRP expression in a hypercholesterolemic pig. (A) Masson trichromic stain of samples from abdominal aorta. (B) In situ hybridization with LRP antisense riboprobe. (C) Controls with LRP sense riboprobe. Arrows indicate LRP mRNA expression. Magnification × 100. Modified from Llorente-Cortés, J. *et al.*, 2002. Circulation 106, 3104–3110.

is disrupted, with resultant vascular remodeling and injury. Through these processes, endothelial dysfunction aggravates existing hypertension, accelerating the progression of atherosclerosis. Endothelial dysfunction is now recognized as a key early risk factor for cardiovascular morbidity and mortality. Certain consequences of endothelial dysfunction are directly related to the pathogenesis of atherosclerosis and its complications. Pathophysiological stimuli of arterial endothelial dysfunction that are especially relevant to atherogenesis include activation by cytokines and bacterial endotoxin, infection by viruses, advanced glycosylation end products that are generated in diabetes and with aging, hyperhomocysteinemia, and hypercholesterolemia and oxidized LDL. In addition to these humoral stimuli, it is clear that biochemical forces generated by flowing blood can also influence endothelial cell structure and function, modulating the expression of pathophysiologically relevant genes. The possibility that hemodynamic forces can act as pathophysiological stimuli for endothelial dysfunction provides a conceptual rationale for the long-standing observation that the earliest lesions of atherosclerosis characteristically develop in a nonrandom pattern, the geometry of which correlates with branch points and other regions of altered blood flow. There have been documented a variety of changes in the metabolic and synthetic activities of endothelial cells in response to defined biomechanical forces. These include the production of arachidonate metabolites, growth factors, coagulation and fibrinolytic components, ECM components, and vasoactive mediators. Certain of these more acute shear-induced changes appear to involve regulation at the level of rate-limiting enzymes and/or substrate availability. However, especially in the case of delayed responses in which de novo synthesis occurs, up-regulation of gene expression appears to occur as a direct consequence of exposure to fluid mechanical forces. There are genes, such as platelet-derived growth factor-B (PDGF-B), MCP-1, VCAM-1, and endothelin-1, that have a "shear stress response element" in the promoter.

Monocytes/Macrophages

The state and function of the macrophages in the atherosclerotic lesions may be critical for the development of atherosclerosis. Macrophages play an important role in innate immune responses, cellular adhesion, phagocytosis of apoptotic cells, and lipid uptake. Most of these macrophage functions are mediated by SRs. Since the cloning of the first two macrophage SRs (now called SRA-I and SRA-II), the broad SR family has grown considerably (**Table 5**). On the basis of functional studies and expression in the arterial intima, only some of the SRs are good candidates to contribute to atherosclerotic foam cell formation.

Besides their role in lipid accumulation, macrophages may contribute to atherosclerosis through secretory inflammatory products. In fact, atherosclerosis can be considered a chronic inflammatory process. Activated lymphocytes and macrophages with a wide expression of class II histocompatibility antigen have been found at every stage of atherosclerotic lesions, indicating that macrophages may participate in local immune responses.

Vascular smooth muscle cells

VSMCs represent an average of 50% of the cellular component in advanced atherosclerotic plaque and may reach 90 to 95% in early lesions. In response to multiple stimuli, VSMCs from the arterial tunica media are activated and migrate to the intima, where they proliferate. These seem to be early steps in the onset of the atherosclerotic process. The proliferation and migration of VSMCs from the media to the intima is one of the key events in early atherosclerosis. The understanding of the molecular mechanisms involved in VSMC activation and differentiation requires an accurate mapping of the cascade of transcription factors induced by atherogenic stimuli. Recently, various nuclear receptors, including peroxisome proliferator-activated receptors (PPARs), retinoid receptors, both retinoid X receptors (RXRs) and retinoic acid receptors (RARs), and retinoid-related orphan receptors (RORs), have been identified in VSMC activation/ proliferation and, consequently, have been implicated in atherogenic processes. Our group has identified neuron-derived organ receptor-1 (NOR-1) as a new early-response gene in VSMCs. NOR-1 is strongly increased by growth factors and thrombin. NOR-1 is overexpressed in atherosclerotic lesions from patients with CAD (**Fig. 5**), and balloon angioplasty transiently induces NOR-1 in porcine coronary arteries. These results suggest that NOR-1 may play a role in the molecular mechanisms underlying both spontaneous and accelerated atherosclerosis.

VSMCs also contribute to the lesion by synthesizing ECM. In fact, proliferative VSMCs have a high capacity to synthesize sulfated PGs, and it is well established that PGs in the arterial wall are involved in the focal deposition of cholesterol-rich particles during the early phases of atherogenesis.

Finally, VSMCs also have great importance in foam cell formation. Our group demonstrated that agLDL can cause high intracellular cholesteryl ester accumulation in VSMCs. We demonstrated, for the first time, that in VSMCs, LRP1 mediates the binding and internalization of agLDL. We also demonstrated that in the absence of LRP1 function, VSMCs are unable to accumulate cholesterol. In addition, this receptor is up-regulated by agLDL in cultured human VSMC and by hypercholesterolemia in vivo. Hypoxic conditions cause a strong increase in HIF-1α and LRP1 in VSMC and that HIF-1α and LRP1 protein staining coincides with VSMC in human advanced atherosclerotic lesions. Hypoxic VSMC may thus contribute to the high levels of HIF-1α associated with foam cells in human advanced atherosclerotic lesions. **Fig. 6** shows the consequences of human VSMC lipid loading for VSMC functionality. These foam cells synthesize and release increased amounts of tissue factor, a key element in the prothrombotic transformation of the vascular wall and thus in the progression of atherosclerosis to thrombosis. It has been recently reported that at least 50% of the foam cells previously

regarded as monocyte-derived macrophages in human atherosclerotic plaques originate in fact from VSMCs. Taken together, these findings suggest that generation of hVSMC-derived foam cells through LRP1-mediated AgLDL uptake is a key mechanism for vascular lipid deposition in atherosclerosis.

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Epigenetics, the Vascular Wall, and Atherosclerosis

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Introduction

The ability to identify genes that are associated with disease phenotypes represents a cornerstone in molecular medicine. Firstly, it leads to increased knowledge of the underlying mechanisms leading to disease, and secondly may lead to useful markers or therapeutic targets of disease. A representative example is the autosomal recessive pattern of inheritance of the cystic fibrosis transmembrane conductance regulator (CFTR) gene discovered in the late 80s (Riordan *et al.*, 1989). Such findings fuelled genome-wide sequencing efforts and genome-wide association studies (GWAS) that have successfully identified genes causative of ~50% of diseases with Mendelian patterns of inheritance (autosomal recessive, autosomal dominant, co-dominant or sex-linked; Online Mendelian Inheritance in Man, www.ncbi.nlm.nih.gov/omim). Conversely, these approaches have been of limited success with respect to non-communicable diseases, which currently represent the primary causes of death worldwide. In particular, there is a gap between disease risk heritability as inferred by twin-based epidemiological studies and the extent of heritability attributable to DNA sequence variants identified by GWAS. For example, twin-based evidence that cardiovascular disease has a significant genetic component dates back nearly four decades ago (Liljefors, 1970) and more recently, heritability of up2 to 60% has been shown for coronary artery disease, a prominent form of cardiovascular disease (Zdravkovic *et al.*, 2002). In turn, GWAS has been successful in identifying a number of genetic variants that collectively explain only 28% or less of cardiovascular disease risk (Stylianou *et al.*, 2012; McPherson and Tybjaerg-Hansen, 2016). The occurrence of missing heritability suggests that non-communicable diseases show complex genetic inheritance (multi gene, somatic mutations) and/or result from the interplay between genetic and epigenetic mechanisms.

Epigenetic mechanisms refer to chromatin modifications that are linked to changes in gene expression and are independent of mutational events. Such modifications are known to be heritable, reversible and respond to external signals. At the molecular level there are two major types of known epigenetic modifications; i.e. modifications of DNA and histones (the proteinaceous component of chromatin), that both have well established roles in development, X-chromosome inactivation, genomic imprinting and the repression of transposable elements. The most important epigenetic DNA modification is the addition of a methyl group, mostly to a cytosine base (5-methylcytosine, 5mC) in a CpG (cytosine-phosphate-guanine) dinucleotide context. In turn, the N-terminal tails of histones carry specific modifications such as acetylation, methylation, ubiquitination and sumoylation (Bannister and Kouzarides, 2011; Verdin and Ott, 2014). These epigenetic modifications act in a combinatorial fashion that are linked to different transcriptional states (Cedar and Bergman, 2009). As an example, DNA methylation generally coexists with trimethylation of lysine 36 on histone H3 (H3K36me3), a modification that is typically associated with active transcription, while H3K4me3 marks active promoters and is antagonistic to DNA methylation (Sun *et al.*, 2005; Mikkelsen *et al.*, 2007; Hodges *et al.*, 2009).

The following paragraphs include a general introduction to the dynamic changes in DNA methylation that occur during normal development and its potential impact on transcription, followed by a section specifically related to DNA methylation changes in vascular disease.

DNA Methylation Dynamics

Methylating and Demethylating Enzymes

DNA methylation was first discovered in 1948 in higher eukaryotes (Hotchkiss, 1948) and results from the transfer of a methyl group from the donor *S*-adenosylmethionine (AdoMet or SAM) to the 5′ position of the cytosine pyrimidine ring. The enzymes that catalyze this reaction are known as DNA methyltransferases (DNMT). DNMTs are capable of introducing methyl groups to previously unmethylated substrates or maintaining DNA methylation profiles following DNA replication. These distinct activities are performed by the de nov*o* methyltransferases DNMT3A, DNMT3B, DNMT3C, the catalytically inactive DNMT3L and the major maintenance methyltransferase DNMT1, respectively (Bestor, 2000; Goll and Bestor, 2005; Barau *et al.*, 2016). Indeed, the importance of DNA methylation in mammalian development was evidenced by the aberrant phenotypes associated with knockout or insertional mutagenesis of both types of DNA methyltransferases (Li *et al.*, 1992; Okano *et al.*, 1999; Bourc'his *et al.*, 2001; Howell *et al.*, 2001; Barau *et al.*, 2016). Specific domains in the N-terminal regions of DNMTs play an important role in guiding these enzymes to specific genomic locations. The DNMT1 replication focus targeting domain (RFTD) targets DNMT1 to replication foci where it interacts with UHRF1 (ubiquitin-like with PHD and ring finger domains 1), a protein that preferentially binds to hemimethylated DNA (Leonhardt *et al.*, 1992; Bostick *et al.*,

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2007; Sharif *et al.*, 2007; Bashtrykov *et al.*, 2014; Berkyurek *et al.*, 2014). In this manner, DNMT1 faithfully copies the methylation pattern from the parental DNA strand onto the newly synthesized daughter strand. Similarly the N-terminal PWWP motif of DNMT3A and DNMT3B specifically recognizes H3K36me3 (Otani *et al.*, 2009; Dhayalan *et al.*, 2010; Baubec *et al.*, 2015; Rondelet *et al.*, 2016), while DNMT3L is targeted to unmethylated H3K4 via the ADD domain (Ooi *et al.*, 2007).

Conversely, the removal of DNA methylation can occur either passively by limiting DNMT activity, or actively via the modification of 5mC (deamination or oxidation) followed by glycosylation and base excision repair. Deamination is catalyzed by AID (activation induced cytosine deaminase) or APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1), generating thymidine, while the family of ten-eleven translocation (TET) dioxigenases (TET1, TET2 and TET3) convert 5mC to 5-hydroxymethycytosine (5hmC), 5 formylcytosine (5fC) and 5 carboxylcytosine (5caC) (Tahiliani *et al.*, 2009; Iqbal *et al.*, 2011; Wossidlo *et al.*, 2011; Wu and Zhang, 2011). In either case, these 5mC intermediates—excluding 5hmC—are removed by known glycosylases such as TDG (thymine DNA glycosylase) and MBD4 (methyl-CpG-binding domain protein 4) and are replaced with cytosine by base excision repair (Morgan *et al.*, 2004). With respect to the intermediate 5hmC, given that this modification is a poor substrate of DNMT1, DNA methylation is successively diluted during DNA replication (Valinluck and Sowers, 2007; Hashimoto *et al.*, 2012).

Taken together, the relative activities of DNMTs and TETs are important factors in determining the methylation state of a given substrate (**Fig. 1**). For additional and more detailed information on the targeting and function of DNMTs and TETs the readers are referred to the articles under "Further Reading" (Jeltsch and Jurkowska, 2014; Wu and Zhang, 2014; Jurkowska and Jeltsch, 2016; Rasmussen and Helin, 2016).

Genome-Wide Patterns of DNA Methylation and Its Role in Transcription

Looking at the distribution of DNA methylation from a bird's-eye view, most methylation is concentrated in repetitive transposon-derived sequences (LINEs, LTRs, SINEs), in addition to the gene body, defined as the region from the transcription start to transcription termination sites (Ball *et al.*, 2009; Lister *et al.*, 2009; Zemach *et al.*, 2010). With respect to regulatory regions, the majority of promoters are devoid of DNA methylation (Ioshikhes and Zhang, 2000; Lister *et al.*, 2009; Edwards *et al.*, 2010), notwithstanding the fact that these regions are characterized by a high density of CpG dinucleotides (known as CpG islands, CGI) (Cooper *et al.*, 1983; Bird *et al.*, 1985). CGIs are also found within gene bodies or between genes (orphan CGI) where they can function as intragenic or distal promoters, respectively (Illingworth *et al.*, 2010; Maunakea *et al.*, 2010; Sarda *et al.*, 2017). However, in contrast to promoter CGI, intragenic and distal promoters

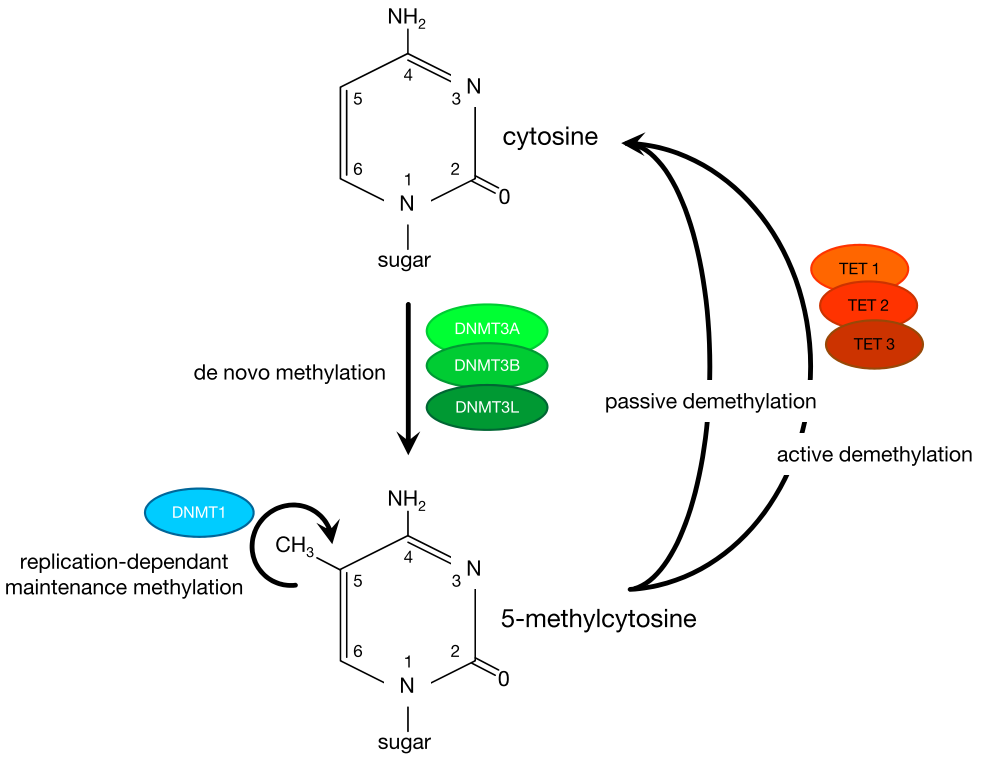

Fig. 1 DNA methylation and demethylation.

show both tissue-specific and developmental specific differences in DNA methylation (Illingworth *et al.*, 2008, 2010; Lister *et al.*, 2009; Rauch *et al.*, 2009). Similar variable methylation has also been documented within DNA regions of lower CpG density, such as CpG shores (2 kb regions flanking CGIs) and CpG-poor promoter regions (Irizarry *et al.*, 2009; Stadler *et al.*, 2011; Long *et al.*, 2013).

The effect of DNA methylation on transcription is complex, as exemplified by observations that promoter CGI are usually unmethylated irrespective of transcriptional activity (Meissner *et al.*, 2008; Illingworth and Bird, 2009; Jones, 2012). Likewise, methylated promoters can also be associated with active transcription (Irizarry *et al.*, 2009). However, such inconsistencies can in part be explained by alternative promoter usage (Rauch *et al.*, 2009; Walter *et al.*, 2016; Sarda *et al.*, 2017) (**Fig. 2**), or by the presence of additional chromatin modifications that are linked to alternative states of gene expression. Despite these complexities, the general consensus is that DNA methylation within regulatory regions is linked to lack of transcriptional activity, while gene body methylation shows the opposite association (Klose and Bird, 2006; Rebhan *et al.*, 2007; Jones, 2012). Accordingly, most transcription factor (TF) families preferentially bind to unmethylated promoters, exceptions being TFs belonging to homeodomain and NFAT families that preferentially bind methylated DNA (Yin *et al.*, 2017). However, whether promoter hypomethylation precedes, or is the consequence of, TF binding is still under debate (Zhu *et al.*, 2016). Conversely, methylation of promoter regions and gene bodies is likely to be the consequence of transcriptional repression and activation, respectively (Adalsteinsson and Ferguson-Smith, 2014). Examples of the latter include Oct3/4 and Liz (long isoform of Zdbf2) that are downregulated prior to promoter de novo methylation (Greenberg *et al.*, 2016; Epsztejn-Litman *et al.*, 2008) and transcription-dependent gene body methylation (Rauch *et al.*, 2009; Baubec *et al.*, 2015).

In essence, these data point to a functional role of methylation in stabilizing gene silencing by inhibiting TF binding, both within canonical and intragenic promoters. For additional and more detailed information on the relationship between TF-binding and methylation states, the readers are referred to the article under "Further Reading" (Schübeler, 2015).

DNA Methylation in Development and Disease

Most somatic methylation patterns are established early in embryogenesis by the concerted actions of DNMT3A, DNMT3B and DNMT3L and are subsequently maintained following DNA replication by DNMT1 (Okano *et al.*, 1999). Specifically, the mammalian genome undergoes two waves of demethylation/methylation: the first wave of demethylation occurs during the migration of proliferating primordial germ cells (PGC), with remethylation occurring in non-dividing prospermatogonia and growing oocytes, respectively, while the second demethylation/methylation takes place between cleavage stage embryos and the blastocyst stage (Monk *et al.*, 1987; Kafri *et al.*, 1992; Smith *et al.*, 2012). During these stages, the transient loss of DNA methylation is prominent for repetitive gene regions. However young CpG-rich transposons and CGI—that remain methylated and unmethylated, respectively—are immune to this methylation reprogramming (Hajkova *et al.*, 2002; Seisenberger *et al.*, 2012; Smith *et al.*, 2012; Ficz and Reik, 2013). In addition, complex regulatory DNA sequences that determine imprinted gene expression (i.e. imprinted control regions, ICRs) only undergo loss of DNA methylation in PGC (Bourc'his *et al.*, 2001; Smith *et al.*, 2012). This implies that exposure to adverse conditions early in development could lead to aberrant programming of DNA methylation that in turn affects disease probability in adulthood. An example of transcription-induced germline programming of DNA methylation that impacts on adult phenotype has recently been described (Greenberg *et al.*, 2016).

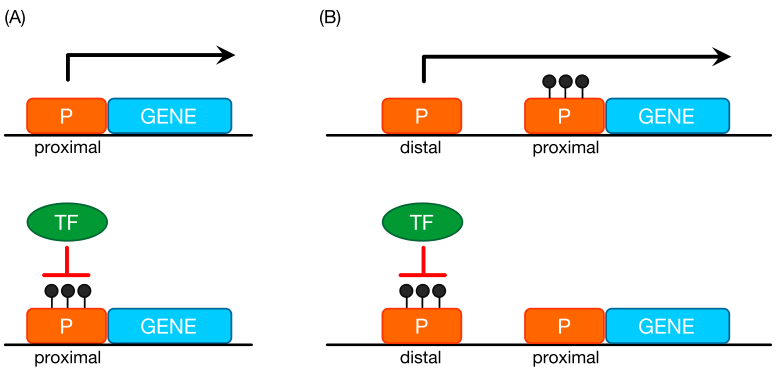

Fig. 2 Associations between DNA methylation and expression. (A) General consensus showing the repressive nature of promoter methylation on transcription factor binding and gene expression. (B) Transcription from an alternative upstream distal promoter (that gives rise to a transcript that overlaps with the gene downstream of the proximal promoter) can in some instances explain the lack of association between proximal promoter DNA methylation and transcription. *P*, promoter; *TF*, transcription factor.

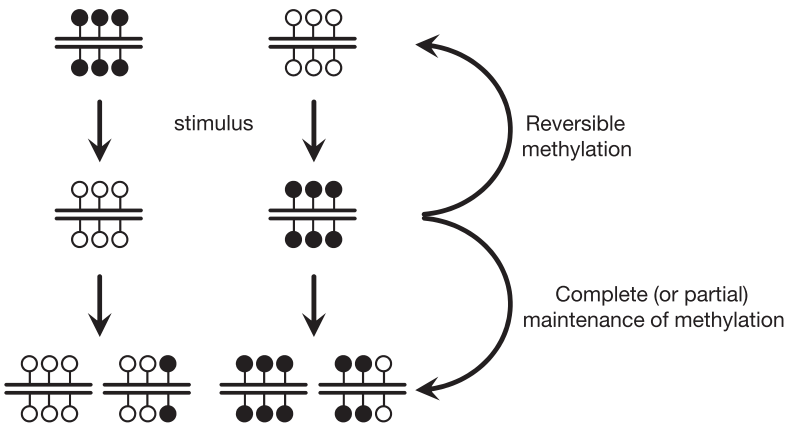

Fig. 3 Transient and stable DNA methylation in response to a given external stimulus. *Filled* or *empty lollipops* represent methylated and unmethylated CpG residues.

The genome also undergoes changes in methylation as a result of aging, disease and/or in response to specific external stimuli. Most complex human diseases such as cancer, diabetes type 1 and 2, atherosclerosis (Rakyan *et al.*, 2011; Dayeh *et al.*, 2014; Zaina *et al.*, 2014; Valencia-Morales Mdel *et al.*, 2015; Feinberg *et al.*, 2016), in addition to known risk factors of these diseases (smoking, obesity, high fat diet) are associated with genome-wide alterations in DNA methylation, some of which are transient in nature (Wang *et al.*, 2010; Jacobsen *et al.*, 2012; Zeilinger *et al.*, 2013; Benton *et al.*, 2015). As an example of the latter, a recent study of past, present and non-smoking individuals found that most smoking-related DNA methylation profiles were reversible in past smokers; i.e. only 7% of smoker-specific DNA methylation was detectable up to 35 years following smoking cessation (Ambatipudi *et al.*, 2016; Joehanes *et al.*, 2016). Such studies underline the largely reversible nature of smoking-related DNA methylation that may be associated with favorable health outcomes following smoking cessation. In addition, these studies illustrate that selected methylation profiles serve as historical footprint of past exposure (**Fig. 3**). That latter may be of particular interest in epidemiological research of environmentally related chronic diseases and for identifying early biomarkers for disease-risk.

Factors that drive disease-associated DNA methylation include, not surprisingly, mutations in both TETs and DNMTs (Xu *et al.*, 1999; Yan *et al.*, 2011; Chan and Majeti, 2013). Furthermore, cellular concentrations of metabolites of Krebs cycle and methionine and folate metabolism, which are known regulators of TETs and DNMT activity, also impact DNA methylation levels (Wellen *et al.*, 2009; Crider *et al.*, 2012; Donohoe and Bultman, 2012; Kaelin and McKnight, 2013; Salminen *et al.*, 2014). Thus, modification of these metabolite levels by dietary intervention may be a promising strategy to reverse disease-related DNA methylation. For additional and more detailed information on the relationship between metabolism, nutrition and DNA methylation, the readers are referred to the articles under "Further Reading" (Sharma and Rando, 2017; Zeisel, 2017).

Atherosclerosis

Atherosclerosis is a chronic-degenerative disease of large and medium size arteries. The anatomical manifestation of atherosclerosis is the development of a lipid-rich, fibrocellular lesion—i.e. composed of living cells and extracellular matrix (ECM) material—that alters the normal histological structure of the intima—one of the layers of the arterial wall—and protrudes into the lumen. The lesion is commonly referred to as atheroma or plaque. The word atheroma was first used in a cardiovascular context by the Swiss scientist Albrecht von Haller in the 18th century and derives from the Greek "gruel-like tumor," which alludes to the loss of the orderly morphology of the healthy arterial wall (Fye, 1995; Pajukanta *et al.*, 2007). The progression of the atheroma is mostly asymptomatic and spans decades in humans. During this phase, the atheroma is contained below the innermost single cell layer of the artery—the endothelium. In a minority of atherosclerotic subjects, the atheroma undergoes structural weakening which exposes its interior to the blood flow (Tabas, 2017). The resulting thrombosis causes impaired blood flow downstream to the site of the ruptured atheroma and is the primary cause of the clinical complications of atherosclerosis: myocardial infarction, cerebrovascular events (stroke) and peripheral artery disease.

Epidemiologically, atherosclerosis is multifactorial. Besides the four risk factors consistently identified by epidemiological studies—cholesterol, blood pressure, glucose and smoking—a plethora of environmental, life style-related and endocrine-related (obesity, diabetes) factors have been identified (Tzoulaki *et al.*, 2016). This complexity is reflected by the intricate molecular and cellular phenomena that accompany the progression of the disease. The current dominant view is

that atherosclerosis is an inflammatory disease (Ross, 1999). The infiltration of small size lipoproteins into the arterial wall accompanied by endothelial dysfunction—i.e. loss of selective barrier function—is an early event in atherosclerosis. Subsequently, circulating monocytes are recruited by specific endothelial receptors and migrate into the arterial wall where they differentiate to macrophages. Macrophages perform their natural duty of scavenging lipoproteins, thus converting into lipid-loaded cells known as foam cells. That initial inflammatory response is self-sustaining rather than resolved. A corollary phenomenon to macrophage invasion is a dramatic change in vascular smooth muscle cell (VSMC) phenotype (Stavenow, 1984). In a healthy vessel, VSMCs are differentiated, quiescent cells specialized in contracting to regulate blood pressure. In atherosclerosis, VSMCs dedifferentiate and recover the ability to migrate and proliferate. As a result, VSMCs migrate from the underlying arterial wall to the macrophage-rich space, where they synthesize and deposit ECM. This latter function identifies the atherosclerosis-associated VSMC phenotype as "synthetic" as opposed to the "contractile" physiological phenotype. The resulting ECM-rich structure is relatively stable until destabilizing factors prevail. Although the cellular/molecular details of atheroma weakening are not fully understood, macrophage-derived proteases seem to play a pivotal role (Tabas and Bornfeldt, 2016; Tabas, 2017).

Atherosclerosis and DNA Methylation

Seminal experiments in animal models showed that the diet can change DNA methylation profiles and that such changes can result in stable phenotypes (Waterland and Jirtle, 2003). A flurry of evidence followed, which demonstrated DNA methylation-modulating activity for a large number of dietary, environmental and life style factors (Waterland *et al.*, 2006; Baccarelli *et al.*, 2009; Breitling *et al.*, 2011). These findings were concomitant with mounting evidence for the existence of a significant missing heritability for cardiovascular disease (see above) (Stylianou *et al.*, 2012). The missing heritability problem prompted researchers to explore other avenues than genetics. Epigenetics is regarded as a promising alternative (Ordovás and Smith, 2010; Zaina, 2014).

Cardiovascular epigenetics research has taken two distinct directions. On the one hand, the GWAS approach has been replicated in epigenome-wide association studies (EWAS) to seek DNA methylation profiles mostly in peripheral blood, which associate with disease risk. This approach focuses on heritability. Examples are EWAS that have successfully demonstrated significant associations with glucose and lipids for a number of CpG loci (Hidalgo *et al.*, 2014; Irvin *et al.*, 2014). This genetic approach to cardiovascular epigenetics is supported by the notion that up to 60% of DNA methylation is DNA sequence-dependent, with effects both *in cis* and *in trans* (Kerkel *et al.*, 2008; Bonder *et al.*, 2017). Yet, this same rationale implies that like GWAS, EWAS might yield epigenetic variants with small effects. This has been so far indeed the case (Zaina and Lund, 2014). Another important issue is that peripheral blood-based EWAS might yield valuable disease markers, but due to inter-tissue DNA methylome heterogeneity, do not necessarily identify vascular tissue-specific pathological DNA methylation profiles of potential therapeutic relevance. This is particularly true in the light of the reported uniqueness of the peripheral blood DNA methylome (Lowe *et al.*, 2015; Silva-Martínez *et al.*, 2016).

The second approach to cardiovascular epigenetics seeks differential DNA methylation between normal and diseased blood vessels. This approach is mechanistic and focuses on seeking DNA methylation profiles that account for pro- or antiatherogenic gene expression and help to identify the effects of risk factors at the genome level in the disease target tissue (Zaina and Lund, 2012). In the following paragraphs, we will review this latter mechanistic approach. For an excellent discussion of the strengths and limitations of the "genetic-like" EWAS approach, the reader is referred to the work by Aslibekyan and coworkers (Aslibekyan *et al.*, 2014).

Participation of Specific DNMTs and TETs in Atherosclerosis

Increased DNMT activity is likely involved in VSMC switch to a synthetic phenotype, as forced hypermethylation induced by expression of the bacterial DNMT HpaII methylase induces smooth muscle proliferation in mice (Carpinteyro-Espín *et al.*, 2011). Also, biochemical inhibition of DNMTs in vivo revealed that DNA hypermethylation plays a causal role in atherosclerosis (Cao *et al.*, 2014; Dunn *et al.*, 2014). Notably, accumulating independent evidence assigns de novo methylation activity to DNMT1 in atherosclerosis, although that DNMT has been traditionally considered a specialized maintenance enzyme (Hermann *et al.*, 2004). Knock-down experiments showed that DNMT1 is responsible for lipoprotein-induced DNA hypermethylation in cultured human macrophages (Rangel-Salazar *et al.*, 2011). In the same study, kinomics identified protein kinase zeta as a mediator of lipoprotein signaling in accordance with an independent study (Lavoie *et al.*, 2011). Furthermore, in vivo studies reached the same conclusions: disturbed blood flow, a pivotal inducer of endothelial dysfunction and atherosclerosis, induces DNA hypermethylation through DNMT1 activation (Zhou *et al.*, 2014). Additionally, DNMT1 overexpression enhances inflammation in a mouse atherosclerosis model by downregulating peroxisome proliferator activating receptor gamma and estrogen receptor alpha, two nuclear receptors that maintain an antiatherogenic transcriptional program (Wang *et al.*, 2012; Yu *et al.*, 2016). DNMT1 is also involved in disturbance of lipid homeostasis by homocysteine, an independent cardiovascular risk factor (Yang *et al.*, 2015).

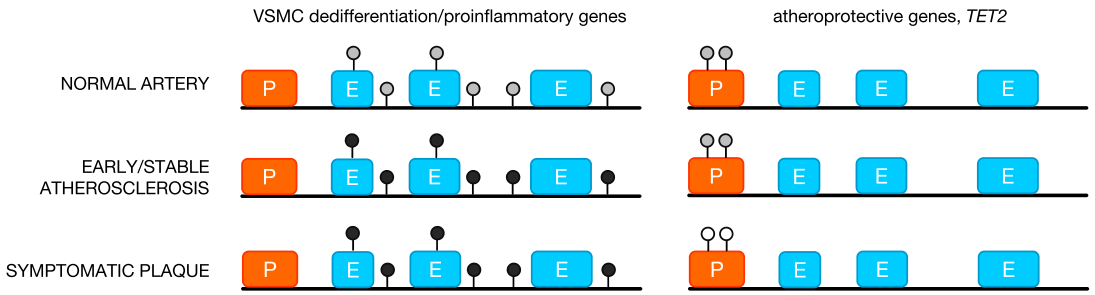

Fig. 4 Main changes in gene DNA methylation at various stages of atherosclerosis. Only profiles in genic regions with significant differential methylation are shown. In proatherogenic genes, changes are mainly in the gene body, i.e. in exons and introns (*blue boxes* and *black line*, respectively), during aorta and carotid stable plaque evolution. In atheroprotective genes, changes are mainly in promoters (P, *orange boxes*) in symptomatic carotid lesions. *White*, *gray* and *black lollipops*, nearly unmethylated, low methylation and high methylation state, respectively.

As for TETs, an atheroprotective role for TET2 has been suggested by a number of studies. TET2 is downregulated in mouse atherosclerotic lesions compared to controls, and its overexpression or knock-down coordinately decreased or decreased DNA methylation and lesion size, respectively (Peng *et al.*, 2016). The study identified monocyte recruitment and proinflammatory genes as specific TET2 targets. Similar data were obtained by TET2 expression manipulation in a cell culture model of oxidized lipoprotein-induced endothelial damage (Peng *et al.*, 2017). Accordingly, TET2 expression decreases as VSMCs shift from the contractile to the synthetic undifferentiated phenotype (Liu *et al.*, 2013). These studies identify TET2 activation as a potential therapeutic strategy.

Dynamics of the Atherosclerotic DNA Methylome

Gene candidate-based searches for differentially methylated loci in atherosclerosis date back to the late 90s (Laukkanen *et al.*, 1999). These early studies showed that altered DNA methylation profiles are present in the arterial tissue before any histologically detectable lesions appear in mice (Lund *et al.*, 2004). More recently, the availability of next generation sequencing and affordable high-density DNA methylation microarrays has allowed epigenome-wide profiling of atherosclerotic vessels (Sandoval *et al.*, 2011). Collectively, those experiments revealed that an increase in DNA methylation—hypermethylation—characterizes the stable, AHA grade III-VII atherosclerotic aorta compared to atheroma-free donor-matched control aortas (Zaina *et al.*, 2014; Valencia-Morales Mdel *et al.*, 2015). Hypermethylation at a portion of those loci is lesion grade-independent and therefore may arise at early phases of atheroma progression or even before any detectable histological change. Hypermethylation targeted mostly the gene body and regions outside of CGIs (**Fig. 4**). Global DNA hypermethylation was also documented in peripheral blood of coronary artery disease patients (Sharma *et al.*, 2008, 2014).

Following the drift towards DNA hypermethylation in stable atheromas, symptomatic (post-cerebrovascular event) carotid lesions undergo a significant loss of methylation (Zaina *et al.*, 2015). This shift does not involve reversal of gene body methylation acquired during stable lesion progression, but rather targets CGIs (**Fig. 4**). A substantial subset of hypomethylated CGIs map to promoters of atheroprotective genes involved in cholesterol efflux and oxidative stress control. Parallel RNAseq—a transcriptome-wide next generation sequencing technology—experiments revealed that CGI demethylation was accompanied by increased expression. Although counterintuitive, the data echo evidence that post-cerebrovascular event lesions undergo compensatory changes towards a relatively stable structure (Peeters *et al.*, 2009). Interestingly, one of the upregulated genes was TET2, an enzyme involved in active DNA demethylation (see above).

Collectively, the epigenomics data presented here and the outcome of DNMT and TET molecular manipulation reviewed in paragraph 3.2 converge to support a causal role of DNA hypermethylation in atherosclerosis. Yet, a number of studies seem to contradict that notion. Early rabbit and mouse atherosclerosis model studies detected genomic DNA hypomethylation (Laukkanen *et al.*, 1999; Hiltunen *et al.*, 2002; Lund *et al.*, 2004). Studies in humans reached the same conclusions (Castillo-Díaz *et al.*, 2010; Aavik *et al.*, 2014; Greißel *et al.*, 2015). These studies have in common that diseased and control vessels were obtained from different individuals or animals, and in some cases from entirely different anatomic locations—for example femoral *versus* mammary arteries. It is therefore possible that inter-individual and inter-tissue differential methylation masks genuine atherosclerosis-specific profiles. This is an important issue that limits our understanding of the atherosclerosis epigenome and therefore warrants further research.

Atherosclerosis Risk Factors and DNA Methylation

One of the early approaches to identify any effects of proatherogenic factors on DNA methylation was to stimulate cultured cells with lipids and lipoproteins. These efforts showed that triglyceride-rich lipoproteins induce DNA hypermethylation in

cultured cells (Lund *et al.*, 2004; Rangel-Salazar *et al.*, 2011). Fatty acids are among the mediators of lipoprotein-induced DNA hypermethylation, as arachidonic acid-induced DNA methylation profiles in cultured macrophages significantly overlap with human atherosclerotic aorta-specific profiles (Silva-Martínez *et al.*, 2016; Silva-Martínez *et al.*, 2017). Furthermore, supplementation of trans fatty acid elaidate in utero induces DNA hypermethylation in the progeny that persists after the stimulus (Flores-Sierra *et al.*, 2016), thus offering another example of a stable epigenetic modification (**Fig. 3**). Also, high-fat diet, a predisposing factor for cardiovascular disease, induces hypermethylation of repeated elements, a surrogate of the whole genome (Yoo *et al.*, 2012). Collectively, these results are supported by evidence that lipids shape the DNA methylome, not *vice versa* (Dekkers *et al.*, 2016).

Atherosclerosis is not regarded as an endocrine disease *per se*, but altered hormone signaling or availability contribute to cardiovascular risk. DNA methylation studies have provided insights into the endocrine component of atherosclerosis at the molecular level. As mentioned above, estrogens have a recognized atheroprotective role. Accordingly, estrogen receptor alpha promoter methylation and expression are increased and decreased, respectively, in atherosclerotic compared with donor-matched and artery type-matched normal controls (Post *et al.*, 1999; Valencia-Morales Mdel *et al.*, 2015).

Diabetes is one of the cardiovascular risk factors most clearly identified by epidemiological studies. Yet, the comparison of DNA methylation profiles yields little overlap between the two diseases. Among differentially methylated genes in atherosclerotic and normal human aorta, the insulin signaling function is enriched with only marginal significance (Zaina *et al.*, 2014). Accordingly, no overlap was observed between stable aortic atherosclerosis-specific DNA methylation profiles and type 1 or type 2 diabetes mellitus-specific peripheral blood counterparts (Rakyan *et al.*, 2011; Toperoff *et al.*, 2012). A pancreatic islet-based DNA methylation analysis of type 2 diabetes patients and healthy controls yielded 276 differentially methylated CpG loci of which only five overlapped with human atherosclerotic aorta counterparts, and all those CpG changed in opposite directions in the two diseases—i.e. hypomethylation and hypermethylation in diabetes and atherosclerosis, respectively (Volkmar *et al.*, 2012). A comparative analysis with a DNA methylation microarray-based profiling of human pancreatic cells revealed similar results (Dayeh *et al.*, 2014; Silva-Martínez *et al.*, 2016; Silva-Martínez *et al.*, 2017). Interestingly, the latter studies also indicate a surprising lack of overlap between diabetes-specific and proinflammatory free fatty acid (arachidonate, palmitate)-induced DNA methylation profiles. Taken together, the data suggest that epigenetic events occurring in the diabetic pancreas and in the atherosclerotic vascular tissue are causally related but distinct. A yet untested fascinating implication of these data is that the epigenetic divergence between the pancreatic islet and the atherosclerotic vessels is increased in comorbid subjects.

As for obesity, the comparative methylome survey described in the previous paragraph indicates a close clustering between differentially methylated profiles in the adipose tissue of obese subjects and human atherosclerotic aortas, with a marked DNA hypermethylation in both conditions (Benton *et al.*, 2015; Silva-Martínez *et al.*, 2016).

See also: Low HDL and High HDL Syndromes. Primary Mixed Dyslipidemias. Hypercholesterolemia. Classification of Hyperlipidemias and Dyslipidemias. Cardiovascular Disease in Diabetes

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Primary Mixed Dyslipidemias

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Abbreviations

Apo B	Apolipoprotein B
Apo E	Apolipoprotein E
CHD	Coronary heart disease
CVD	Cardiovascular disease
FCH	Familial combined hyperlipidemia
FDBL	Familial dysbetalipoproteinemia
FFA	Free fatty acids
FH	Familial hypercholesterolemia
GWAS	Genome-wide association studies
HDL-C	High density lipoprotein cholesterol
HL	Hepatic lipase
IDL	Intermediate density lipoprotein
IR	Insulin resistance
LDL-C	Low density lipoprotein cholesterol
LPL	Lipoprotein lipase
MTTP	Microsomal triglyceride transfer protein
Non-HDL-C	Total cholesterol minus HDL-C values
SNP	Single nucleotide polymorphism
PCSK9	Proprotein convertase subtilisin/kexin type 9
VLDL	Very low density lipoprotein

Introduction

Mixed dyslipidemias are defined as alterations in lipid metabolism characterized by a complex phenotype with plasma triglycerides and low density lipoprotein-cholesterol (LDL-C) elevations, either in isolation or in combination; these are usually associated with low levels of high density lipoprotein-cholesterol (HDL-C) as well as elevated levels of plasma apolipoprotein B (Apo B) and non-HDL cholesterol. Mixed dyslipidemias are clinically important because of their frequent association with premature cardiovascular disease (CVD). In this article we will review the main primary mixed dyslipidemias of genetic origin, including familial combined hyperlipidemia (FCH), familial dysbetalipoproteinemia (FDBL), and hepatic lipase (HL) deficiency. Secondary forms of mixed dyslipidemias, as seen in diabetes mellitus, insulin resistance (IR) states, and metabolic syndrome, are described in the Diabetes Section of this Encyclopedia.

Familial Combined Hyperlipidemia

FCH is a mixed hyperlipidemia which is frequently encountered clinically and is associated with a five-fold increased risk of premature coronary heart disease (CHD). FCH is the most frequent genetic cause of dyslipidemia, with a 1%–2% prevalence in the general population, exceeding that of familial hypercholesterolemia (FH). FCH is also considered the most frequent metabolic cause of premature atherosclerosis; about 20% of patients who present CHD before the age 60—and 38% of those younger than 40 years—have FCH (Goldstein *et al.*, 1973; Ripatti *et al.*, 2016).

Definition

The phenotypic definition of FCH is challenging and the shifting nature of the phenotype in the same individual patients over time has hindered its classification. Affected subjects usually present one of three possible phenotypes: (1) elevations above the 90th or 95th percentile for total cholesterol or LDL-C (including small dense LDL particles), (2) elevated very low density lipoprotein (VLDL) triglycerides, or (3) elevated plasma levels of cholesterol and triglycerides; in all cases, HDL-C levels are usually reduced. These altered lipid profiles shift over time, depending on exogenous factors such as diet, body weight, and exercise. Moreover, FCH is observed within family clusters or in kindreds exhibiting premature CHD (Brahm and Hegele, 2016).

In addition, Apo B is consistently elevated in FCH and so many consider it to be a biochemical marker of the disease (Sniderman *et al.*, 2002). A consensus of opinion acknowledges that the most consistent lipoprotein abnormalities that present over time, within and between different affected individuals, is the combination of both elevated Apo B and triglycerides. This has led to the current proposed definition of FCH: the presentation of Apo B higher than 1.2 g/L and triglycerides exceeding 1.5 mmol/L, both adjusted for age and sex, in at least two family members (Sniderman *et al.*, 2002).

Genetics

As with its definition, the genetic basis of FCH still remains poorly understood. The disorder is neither homogeneous nor dominantly inherited. Genetic susceptibility probably results from the accumulation of multiple variants within the genome of affected family members. These seem to include a combination of rare large-effect variants, plus a high burden of common

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polymorphisms (van Greevenbroek *et al.*, 2014; Brahm and Hegele, 2016). Two different genetic defects could be involved: rare large-effect variants in genes coding for the LDL receptor and/or hepatic Apo B synthesis, and multiple common small-effect lipid-altering variants which together raise LDL-C and/or triglycerides via multiple mechanisms. In addition, a cluster of other factors, such as poor diet, obesity, fatty liver, or diabetes further modulate the shifting expression of the biochemically defined FCH phenotype. The adjective "familial" may be misleading since FCH seems to be polygenic rather than monogenic and its heredity is almost always non-Mendelian. In fact, after more than 40 years of study, no single monogenic cause has been found to explain the phenotype, even in a subset of patients with FCH (Brahm and Hegele, 2016). However, using the term "familial" to designate this disorder is inserted in clinical practice, is meaningful, and helps to remind clinicians of potential family links, and thus, it should not be abandoned, although a more appropriate term might be polygenic combined hyperlipidemia (Brahm and Hegele, 2016; Rader and Hobbs, 2015).

Genome-wide association studies (GWAS) in kindreds affected by FCH have identified up to 40 genes of interest, some of which are implicated in the various metabolic pathways which are disrupted in this disease (Brouwers *et al.*, 2012; van Greevenbroek *et al.*, 2014; Ripatti *et al.*, 2016). Among these, the genes most frequently found encode lipoprotein lipase (LPL), HL, lecithin cholesterol acyl transferase, the cluster apo-$A_I/C_{III}/A_{IV}/A_V$, and the transcription factor upstream stimulatory factor 1 (*USF1* gene). The latter controls numerous genes involved in lipid and glucose metabolism. Some USF1 variants may affect hepatic triglyceride metabolism by modulating the Forkhead Box protein A2 (FOXA2)-induced microsomal triglycerides transfer protein expression (Auer *et al.*, 2012). Large-scale GWAS have shown that several of these loci—in particular the APOA1-C3-A4-A5 gene cluster, LPL and proprotein convertase subtilisin/kexin type 9 (PCSK9)—are also significant, if modest, determinants of plasma lipid traits in large populations. Recent GWAS analyses have also identified a genetic overlap between type 2 diabetes mellitus and FCH, although the nature of the pathogenic connection between these two conditions is still not well understood (Auer *et al.*, 2012).

To date, these combined findings suggest that the FCH phenotype is genetically very complex and we can only speculate about the mechanism of its inheritance. Attempts to replicate family studies of FCH inheritance employing linkage analysis to find susceptibility loci under the assumption of Mendelian inheritance patterns have generally failed. In contrast with FH, which is largely monogenic, FCH lacks a monogenic component, and it does not have any specific genetic markers, making its diagnosis more difficult.

Nonetheless, the availability of single nucleotide polymorphism (SNP) chips for GWAS has improved our understanding of the genetic architecture of hyperlipidemic disorders. In the case of FCH, it is most likely that its inheritance is polygenic, with the proband inheriting a higher than average dose of triglyceride-raising SNPs (i.e., an elevated triglyceride phenotype) or that they inherit more LDL-C-raising SNPs than average, leading to an elevated LDL-C phenotype (Talmud *et al.*, 2014). Moreover, interaction with secondary factors, such as obesity or diabetes, also modulates individuals' underlying genetic susceptibility to FCH and the expression of its biochemically defined phenotype.

Pathogenesis

The primary defect in FCH appears to be increased hepatic secretion of Apo B, presumably because of decreased hepatic degradation of Apo B-containing lipoproteins. Moreover, the increase in Apo B is not linked to the *Apo B* gene. IR is frequently found in patients with FCH, both in obese and normoweight individuals (Martínez-Hervás *et al.*, 2008). The plasma insulin concentrations in these patients are positively correlated with plasma Apo B and triglyceride but inversely correlated with HDL-C concentrations (Castro-Cabezas *et al.*, 1993; Carmena *et al.*, 1996; Purnell *et al.*, 2001). The adipose tissue in FCH patients is characterized by reduced triglyceride removal and decreased storage rates, compared with healthy controls (Arner *et al.*, 2011); this dysfunction leads to an increased flux of free fatty acids (FFA) into the liver, resulting in hepatic fat accumulation and VLDL and Apo B overproduction. Finally, the clustering of cardiovascular risk factors associated with IR in FCH indicates a common metabolic basis for both the FCH phenotype and IR, which is probably mediated by impaired FFA metabolism (Castro-Cabezas *et al.*, 1993; Carmena *et al.*, 1996). In summary, FCH can be envisaged as a crossroad between lipid and carbohydrate metabolism which generates a dyslipidemic and dysglycemic syndrome with hyper-Apo B status.

Diagnosis

In the absence of a genetic marker, FCH is diagnosed using clinical criteria, including the elevations in Apo B, cholesterol, and triglycerides mentioned in the definition, as well as a shifting lipid phenotype, and a family history of mixed dyslipidemia and premature CHD. Moreover, the disorder is often associated with type 2 diabetes mellitus, arterial hypertension, central obesity, IR, and metabolic syndrome (Ascaso *et al.*, 1999; Aguilar-Salinas *et al.*, 2004). Plasma Apo B levels are also elevated in the condition known as hyperbetalipoproteinemia but in that case, in contrast to the presentation in FCH, the LDL-C remains within the normal range (Durrington, 2007a).

Clinical Manifestations

As previously mentioned, FCH has a complex phenotype whose expression shifts over time in genetically predisposed individuals. It usually presents in the second decade of life, although the increasing prevalence of childhood obesity has recently led to the

appearance of FCH in children and adolescents. About 50% of first-degree relatives show at least one of the abnormal phenotypes (i.e., high LDL-C, and/or triglycerides, along with low HDL-C and elevated Apo B). The lipid phenotype within individual families changes with time and shows high inter- and intraindividual variability which especially depends on exogenous factors including diet, alcohol intake, body weight, and exercise (Ascaso *et al.*, 1999; Durrington, 2007a).

FCH is frequently associated with abdominal obesity, metabolic syndrome, IR, hypertension, type 2 diabetes, gout, non-alcoholic hepatic steatosis, low adiponectin levels, and an increased concentration of plasminogen activator inhibitor-1 (Rader and Hobbs, 2015). The elevated plasma levels of Apo B are disproportionately high relative to the LDL-C concentrations, revealing the presence of small, dense, LDL particles, another characteristic of FCH. The size and composition of VLDL and HDL are also abnormal, resulting in particles with greater atherogenic capacity. Together, these changes contribute to the elevated CVD risk characteristic of FCH. Moreover, increased plasma levels of PCSK9 have recently been identified in FCH patients (Brouwers *et al.*, 2011) and could contribute to the atherogenic potential of this disease.

CHD usually manifests during the fourth decade in males, and a decade later in women. Contrary to other dyslipidemias, patients with FCH do not present tendon xanthomas; xanthelasmas or corneal arcus may occur, but their presence is not helpful in making the diagnosis (Durrington, 2007a).

Treatment

Due to the significantly increased risk of premature CHD in individuals with FCH, these patients should be treated aggressively. Decreased dietary intake of saturated fat and simple carbohydrates, aerobic exercise, and weight loss should all have beneficial effects on the patient lipid profile. Lipid-lowering drug therapy is required in most patients. Statins are effective and should be used as the first-line drug therapy, although many patients will require a second drug (ezetimibe, fibrates, ω-3 supplements, or fish oils) for optimal control of the dyslipidemia. In FCH patients with a very high CVD risk, and in view of their elevated plasma PCSK9 levels, treatment with monoclonal antibodies against PCSK9 can be an important adjuvant to statins to further lower the LDL-C concentration. New selective PPAR-α modulators are currently under study in mixed dyslipidemias with promising early results (Camejo, 2017). In patients at very high risk of CHD events, the target LDL-C level should be $\leq$1.8 mmol/L (Catapano *et al.*, 2016). Identification and management of other CVD risk factors, such as type 2 diabetes, smoking, and hypertension are fundamental to reduce the CVD risk burden.

Familial Dysbetalipoproteinemia

Definition

FDBL is also known as broad beta disease, xanthoma tuberosum, remnant removal disease, or type III hyperlipoproteinemia in the classic Fredickson's classification (Fredrickson *et al.*, 1967). It is a rare autosomal-recessive inherited lipid metabolism disorder characterized by elevated cholesterol and triglyceride plasma concentrations caused by the accumulation of remnant lipoprotein particles (chylomicron remnants and VLDL remnants or intermediate density lipoprotein (IDL), also called β-VLDL). The disease becomes fully expressed in adulthood and is more common in men. FDBL is associated with an increased risk of premature coronary, peripheral vascular (intermittent claudication), and cerebrovascular disease (Carmena *et al.*, 2000; Durrington, 2007b; Stalenhoef, 2011).

Genetics

In the majority of cases, FDBL is an autosomal-recessive disorder resulting from genetic variations in apolipoprotein E (Apo E), most commonly Apo E2 (Davignon *et al.*, 1988). Multiple copies of Apo E are present in chylomicron remnants and IDL and are able to bind to the LDL receptor and the heparan sulfate proteoglycan receptors with high affinity, promoting the uptake and clearance of their remnants by the liver. The Apo E gene is polymorphic, and encodes three common *APOE* alleles (ε2, ε3, and ε4) of which ε3 is the most common in the general population (82%), followed by ε4 (11%) and ε2 (7%) (Mahley and Rall, 1995; Bennet *et al.*, 2007). Together, these alleles make up for six *APOE* genotypes: ε2ε2, ε2ε3, ε2ε4, ε3ε3, ε3ϵ4, and ε4ε4. The Apo E2 protein (associated with the ε2ε2 genotype) has a very low LDL receptor-binding affinity which impairs remnant removal and clearance, thus leading to elevated plasma concentrations of cholesterol and triglycerides. In most cases, FDBL is associated with the ε2ε2 genotype and its inheritance is autosomal recessive. However, approximately 10% of cases are caused by other rare variants of Apo E or a complete absence of Apo E (Apo E 0/0), and the inheritance in these is autosomal-dominant (Blum and Type, 2016; Koopal *et al.*, 2017; Zannis, 1986). Importantly, only about 15% of individuals with an ε2ε2 genotype develop FDBL, and that an additional metabolic disturbance such as obesity, diabetes mellitus, IR, untreated hypothyroidism, pregnancy, or alcoholism are frequently required to trigger it.

Pathogenesis

The pathogenesis of FDBL is explained by the accumulation of remnant lipoprotein particles—which are abnormally rich in cholesterol—in plasma, and is caused by homozygosity for a particular isoform of Apo E, E2. Apo E2, the mutant apolipoprotein,

differs from the wild-type ApoE (E3) by a single amino acid. The most common Apo E2 variant bears the arginine^{158} → cysteine mutation, and accounts for over 90% of FDBL cases (Mahley and Rall, 1995; Blum and Type, 2016). Remnant lipoproteins carrying this Apo E mutation are poorly recognized by its receptors in the liver and so they accumulate in the plasma. Furthermore, they are stripped of the majority of their triglycerides by LPL-mediated hydrolysis, therefore making them cholesterol rich. The inheritance of the homozygous mutation is autosomal recessive and has a low penetrance (about 1% of the general population). However, FDBL only occurs in 5% of these homozygotes and requires the previously mentioned additional disturbances in order to manifest itself. Heterozygotes with the arginine^{158} → cysteine mutation and one normal allele coding for Apo E3 do not develop FDBL.

On the other hand, approximately 10% of patients with FDHL have other, rarer Apo E variants or lack it altogether, but these show high-penetrance autosomal-dominant inheritance and cause FDBL in heterozygotes carrying only one mutant Apo E allele (Blum and Type, 2016).

Diagnosis

FDBL occurs at a frequency of around 1 in 5000 individuals in the general population and is often missed in clinical practice. Many physicians are unaware of the disease, and diagnosis via its lipid profiles is not straightforward. One striking lipid abnormality that should lead to a suspicion of FDBL is elevation of both total cholesterol and triglyceride concentrations to a similar degree if measured in mg/dL, usually between 300 and 600 mg/dL (7.75–15.50 mmol/L for cholesterol; 3.38–6.77 mmol/L for triglycerides) although triglycerides may occasionally rise up to 1000 mg/dL (11.30 mmol/L). Plasma levels of LDL-C are low due to defective metabolism of VLDL to LDL, while HDL-C levels are normal or reduced; the predominant remnant particles in FDBL are β-VLDLs and these are particularly rich in cholesterol. In fact, the name "dysbetalipoproteinemia" stems from the abnormal gel electrophoresis migration pattern of VLDL, identified as a broad-β band, which is continuous from the β to pre-β levels (Fredrickson *et al.*, 1967; Mahley and Rall, 1995).

The gold standard for FDBL diagnosis was a ratio of VLDL-cholesterol to VLDL-triglycerides >0.30, as determined by ultracentrifugation, but this time-consuming method is no longer used in clinical chemistry laboratories (Blum and Type, 2016).

Instead, FDBL can be diagnosed if the Apo B to total cholesterol ratio is <0.15 g/mmol. Another alternative is the use of an Apo B algorithm that defines FDBL as: Apo B <1.2 g/L, triglycerides exceeding 1.5 mmol/L, a ratio of triglycerides to Apo B <10 and a total cholesterol to Apo B ratio of at least 6.2. *APOE* genotyping, determination of the presence of ε2ε2 by DNA analysis, or isoelectric focusing, as carried out in lipid laboratories, will suffice in the presence of mixed hyperlipidemia (Koopal *et al.*, 2017); in autosomal-dominant FDBL, identification of the rare Apo E variants requires full *APOE* gene sequencing.

Clinical Manifestations

Atherosclerotic CVD and xanthomatosis are the main clinical manifestations of FDBL. Cholesterol-rich chylomicron remnants and IDL or β-VLDL are associated with low-grade inflammation and are highly atherogenic. Moreover, remnant cholesterol appears to confer a greater risk than a similar concentration of LDL-C (Carmena *et al.*, 2004; Nordestgaard, 2016). Recent studies have provided more evidence for cholesterol-rich remnant lipoproteins as important contributors to CVD and to the so-called residual cardiovascular risk that is, the risk remaining after adequate pharmacological LDL-C lowering (Varbo and Nordestgaard, 2017).

FDBL patients have a high prevalence of premature CVD, which is 5–10-fold higher than gender and aged matched control subjects (Hopkins *et al.*, 2005). The prevalence of peripheral vascular disease is notable and is almost the same as that of CHD in some series. This is in sharp contrast to observations in patients with in FH, in whom peripheral vascular disease is uncommon. Associated metabolic disorders (type 2 diabetes, obesity, etc.) are infrequent in FDBL patients.

Palmar planar xanthomas are considered to be virtually pathognomonic for FDBL and present as orange-yellow nodular deposits in the palmar creases (Davignon and Dufour, 2008). Tuberous xanthomas (pedunculated nodules) and tuberoeruptive xanthomas (aggregation of eruptive xanthomas) are common in untreated patients. They tend to occur over the tibial tuberosity, the elbows, knees, and the buttocks, and to decrease or disappear with treatment. The occurrence of proteinuria has been described in Apo E2 homozygotes and in these cases renal biopsy displays the presence of foam cells in the glomerulus, that is, so-called lipoprotein glomerulopathy. These alterations are largely resolved upon treatment of the dyslipidemia (Durrington, 2007a).

The coexistence of FDBL and FH (Carmena *et al.*, 2000) results in a combination of physical findings typical of both disorders: arcus corneae, Achilles and extensor tendon xanthomas, tuberous xanthomas, palmar orange-colored striae, and a tendency toward high body mass index values. The prevalence of CHD is lower than in FH alone, but the prevalence of peripheral and carotid artery disease is high in both genders in this mixed disease.

Treatment

FDBL patients are generally very responsive to lifestyle changes including dietary intervention; a reduction in saturated fat, replacing it with unsaturated fat or long chain *n*-6 (omega-6) polyunsaturated fatty acids, can be of benefit. Where FDBL is associated with diabetes, carbohydrate intake must be reduced, and the intake of daily calories should be restricted if the patient is

overweight or obese. The primary lipid treatment target in FDBL should be non-HDL-C, because LDL-C is usually absent or low (Koopal *et al.*, 2017). The levels for non-HDL-C should be set 0.8 mmol/L higher than the LDL-C target, which means $\leq$2.6 mmol/L in very high-risk patients and $\leq$3.4 mmol/L in high-risk patients (Catapano et al., 2016; Verbeek *et al.*, 2015). Pharmacological treatment includes the use of statins, usually also associated with fenofibrate or bezafibrate, especially when triglycerides are high. Unfortunately, there is a lack of randomized clinical trials evaluating the effect of lipid-lowering drugs on clinical endpoints in these patients.

HL Deficiency

HL is a key liver enzyme involved in catalyzing the hydrolysis of triglycerides and phospholipids in remnant lipoproteins and HDL particles, which thus plays an important role in the conversion of IDL to LDL. HL lipid hydrolysis in remnant particles contributes to their hepatic uptake via an Apo *E*-mediated process. In addition, HL is involved in remodeling remnant particles, HDL, and LDL, as well as in the production of small, dense LDL (Carmena *et al.*, 2004; Kobayashi *et al.*, 2015).

HL deficiency is a rare autosomal-recessive disease resulting in mixed hyperlipidemia, characterized by cholesterol and triglyceride elevations caused by the accumulation of lipoprotein remnants, and this may be accompanied by normal or elevated levels of HDL-C (Hegele *et al.*, 1993). Different mutations in the HL gene (*LIPC*) have been shown to give rise to loss of circulating HL activity, causing an increase in plasma remnants and triglyceride-rich HDL, which produces an increased CHD risk (Chatterjee and Sparks, 2011). The phenotype is similar to that found in FDBL, with elevated levels of total cholesterol and triglycerides, premature arcus cornea, palmar and tubero-eruptive xanthomas, and premature CVD (Semenkovich *et al.*, 2016).

The diagnosis requires the demonstration of HL deficiency with in vitro assays of HL activity in postheparin plasma samples or DNA analysis to identify a mutation. As indicated in the treatment of FDBL, statin therapy is recommended to reduce remnant lipoproteins and CVD risk in this disease.

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Hypercholesterolemia

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Definition

Hypercholesterolemia can be defined as the presence of high plasma cholesterol levels, with normal plasma triglycerides, as a consequence of the rise of cholesterol and apolipoprotein B (apoB)-rich lipoproteins, called low-density lipoprotein (LDL). According to the WHO definition (1970), hypercholesterolemia would be included in IIa phenotype (Ramasamy, 2016).

The limits to define hypercholesterolemia can be established according to plasma levels of total and LDL cholesterol (LDL-C) above the 95th percentile corrected for age and gender in each population.

Classification of Hypercholesterolemia

From an etiological perspective, hypercholesterolemia can be classified in (Ramasamy, 2016; Sniderman *et al.*, 2014):

1. Primary: lipid metabolism disturbances with genetic cause, familial (many cases in the same family) or in isolated cases, without known genetic mutation or without family aggregation, when secondary causes have been discarded.
   This group includes:
   - Familial hypercholesterolemia (FH), which includes:
     - Autosomal dominant hypercholesterolemia (ADH)
       FH by mutations of receptor of the LDL (LDLR)
       FH by familial defective apoB 100
       FH by mutations of proprotein convertase subtilisin/kexin type 9 (PCSK9) or FH3
     - Autosomal recessive hypercholesterolemia (ARH or FH2)
   - Polygenic hypercholesterolemia (PH)
     The distinguishing characteristics between these entities are shown in **Table 1**.
   - Hyperlipoproteinemia(a)
2. Secondary: basically, as a consequence of environmental factors or other disease causing dyslipidemia: hypothyroidism, nephrotic syndrome, cholestasis, asymptomatic acute porphyria, nervous anorexia, hepatoma, different drugs: cyclosporine, progestogens, thiazide diuretics, etc.

Familial Hypercholesterolemia

The FH are a group of inherited genetic defects resulting in severely elevated serum cholesterol concentrations and increased risk of premature cardiovascular disease.

Table 1 Distinguishing characteristics between primary hypercholesterolemia

	ADH	*ARH*	*PH*
Beginning	From birth	From birth	From 20 years old
Frequency	0.5%	Rare	>10%
Xanthomas	Yes	Yes	No
Corneal arcus	+++	+++	+
CVD	+++	+++	++
Phenotype	IIa	IIa	IIa
Heredity	Autosomal dominant	Autosomal recessive	Polygenic
Etiology	LDLR ApoB PCSK9	ARH protein	Various polymorphisms and exogenous factors
Gen	LDLR: Chromosome 19 ApoB: Chromosome 2 PCSK9: Chromosome 1	Chromosome 1	Various polymorphisms

ADH, autosomal dominant hypercholesterolemia; *ApoB*, apolipoprotein B; *ARH*, autosomal recessive hypercholesterolemia; *CVD*, cardiovascular disease; *LDLR*, low-density lipoprotein receptor; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PH*, polygenic hypercholesterolemia.

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There are two types of FH: autosomal dominant and recessive. The most common form of FH, the heterozygous state (HeFH) of ADH, is the most frequent monogenic disorder of human metabolism (Nordestgaard *et al.*, 2013). Recent genetic studies suggest that the prevalence of HeFH is twofold higher than previously reported (Patterson and Slack, 1972), being 1:200 to 250 individuals (Nordestgaard *et al.*, 2013). Even in certain populations the disease is more prevalent (Raal and Santos, 2012). On the contrary, homozygous ADH (HoFH) affects approximately 1:1,000,000 individuals (Austin *et al.*, 2004), although recent data suggest that the prevalence could be quite higher (Sjouke *et al.*, 2015; Sanchez-Hernandez *et al.*, 2016).

Pathophysiology

The pathophysiology of FH is due to decreased clearance of LDL from the plasma. Under physiological conditions, the synthesized LDLR is transported to the cell surface. Upon binding to the LDL are internalized into clathrin-coated vesicles that form endosomes in the cytosol where the dissociation of the LDLR and LDL particles occurs. The LDL is catabolized in the lysosomes, and the LDLR, depending on the cholesterol concentration and of diverse regulatory proteins, is catabolized (degraded into amino acids), or on the contrary it can be recycled returning to the cell surface. All these steps can be altered in the FH depending on the class mutations.

ADH can be caused by mutations in three different genes:

- Defects in the LDLR. LDLR gene is located in chromosome 19 (19p13.1–13.3) and codifies a membrane glycoprotein which binds to apoB and apolipoprotein E (apoE) contained in the lipoproteins, through its extracellular domain, that is implicated in the uptake of LDL particles removing cholesterol from plasma. Mutations in this gene are the most frequent (>90%) of ADH. More than 1600 mutations have been described (Calandra *et al.*, 2011). The defects in the LDLR have been classified into five categories: defective ligand binding, defective transport, defective internalization, recycling, and complete lack of receptor (Soutar and Naoumova, 2007).

  Due to their physiological function and clinical repercussion, the mutations are divided into null and defective receptor.

  The LDLR mutations can be divided into five classes (De Castro-Orós *et al.*, 2010):

  Class 1 mutations or null alleles. They occur by deletion of the LDLR promoter, by nonsense changes, or by large rearrangements, all of them leading to an absence or abnormal messenger ribonucleic acid (mRNA).

  Class 2 mutations. They are produced by defective alleles involved in the intracellular transport of the LDLR. This defect is usually caused by small deletions or missense mutations.

  Class 3 mutations. In this group, the LDLR is synthesized and transported to the cell surface. However, the LDLR is not able to bind LDL particles. The binding is variable, and usually is lower than 30% of normality.

  Class 4 mutations. These mutations generate proteins that are not able to group into clathrin-coated pits because there is a defect in the internalization of LDLR.

  Class 5 mutations. These are missense mutations causing a defect in the recycling of the LDLR, eluding the receptor return to cell surface.
- Familial defective apolipoproteinB-100 (FDB). The cause is a mutation affecting a critical region of apoB-100. ApoB is essential to bind LDL to the LDLR. It supposes ~3% of mutations of FH (Ejarque *et al.*, 2008). It is an alteration of ligand lipoproteins with normal receptors of LDL.

  The most frequent mutation detected is R3500Q (Arg3500Gln), located in codon 3500 in chromosome 2 (2p23–24). Other mutations responsible of FDB have been described as the change of arginine by tryptophan in the same position R3500W. New mutations described with low frequency in various populations related to this hypercholesterolemia are R3531C R3480W, and H3543Y (Whitfield *et al.*, 2004).

  In FDB, the cholesterol levels and clinical severity are milder than in FH caused by LDLR mutations, due to the normality of the receptor, capable of binding lipoproteins with apoE.
- The PCSK9 gene mutations in chromosome 1 were described studying a family with ADH. PCSK9 is responsible for the degradation of LDLR. Its presence makes possible the association with LDLR and LDL. The LDLR-PCSK9-LDL complex does not dissociate in the endosomes and is degraded into amino acids and individual lipid components, which are released into cytosol (Zhang, 2017).

  Gain-of-function PCSK9 gene mutations increase the LDLR degradation. Thus, the availability of LDLR on the cell surface is reduced and plasma LDL-C is increased. The FH by PCSK9 mutations supposes a very rare mutation (0.1%) (Blesa *et al.*, 2008).

ARH is a rare autosomal recessive form of FH first described in a Lebanese family with clinical manifestations of HoFH with a recessive pattern of inheritance (Kachadurian and Uthman, 1973). ARH is caused by a rare loss-of-function mutation in the LDLR adaptor protein 1 (LDLRAP1) which encodes a protein required for clathrin-mediated internalization of the LDLR (Tada *et al.*, 2015). AHR is a very rare form of FH and most reported cases have come from Lebanon and Sardinia, and from consanguineous marriages.

Thus, any defect in the previous described genes leads to accumulation of LDL-C, in a moderate way if there is a single copy of the defective gene or in an extensive way in the case of two copies of the same defective gene (homozygous) or two coexisting mutations (compound heterozygote) (Bouhairie and Godlberg, 2016).

Clinical Characteristics

FH is a genetic disease with autosomal dominant transmission pattern (except for ARH) that clinically characterizes by high plasma LDL-C levels, xanthomas, corneal arcus, coronary atheroma plaques, and premature cardiovascular disease.

FH is a disorder with a gene dosage effect. Independently of the genetic defect background the severity of the FH phenotype depends on the residual activity of the LDLR (**Table 2**). Subjects with null mutation in the LDLR gene show higher levels of LDL-C and more frequent cardiovascular disease than those with defective mutation.

HoFH

In individuals with true HoFH the LDLR pathway is markedly defective or nonfunctional. As a consequence, the plasma LDL-C levels rise four to eight times above average (>500 mg/dL) and the patients suffer from severe cutaneous and tuberous xanthomas. The presence of xanthomas in children is highly suggestive of HoFH, especially interdigital xanthomas. In the more severe cases, cholesterol can be deposited in the tendons and joints leading to tendinitis and joint pain, requiring surgical removal. In rare cases, patients could present giant ectopic cholesterol xanthomas in the brain, mediastinum, and gluteus muscles.

Other important manifestation is cardiovascular disease, characterized by accelerated atherosclerosis, that affects the aortic root, coronaries arteries, and other territories, such as the carotid, descending aorta, and ileofemoral and renal arteries. These patients show clinical manifestations as acute myocardial infarction, need for revascularization or sudden death, occurring in the first decade of life. Furthermore, the presence of aortic stenosis due to lipid deposition (Cuchel *et al.*, 2014) is also frequent.

HeFH

Subjects with HeFH show plasma LDL-C levels two to three times above average. Tendinous xanthomas, especially in the extensor tendons of the hand, knees, and Achilles tendon, corneal arcus, and cardiovascular disease appear belatedly. There is more variability in clinical manifestations depending on the age, type of LDLR gene mutation (null or defective), interaction with other genes, and the presence of additional risk factors (hypertension, smoking, etc.) (Nordestgaard *et al.*, 2013).

ARH

Overall the clinical features of ARH patients are similar to those found in HoFH with defective receptor mutations. Compared to negative receptor HoFH, although ARH patients have similar prevalence of xanthomas, these subjects show lower levels of LDL-C, lower prevalence of coronary heart disease, and lower prevalence of aortic valve disease (Fellin *et al.*, 2015).

Diagnosis

Many times, clinicians miss FH. Once hypercholesterolemia is detected, secondary causes should be ruled out. FH diagnosis is based on clinical, biochemical, and genetic criteria. FH must be suspected in the presence of high plasma LDL-C levels, first-degree relatives with hypercholesterolemia, premature coronary disease or sudden death, and physical findings. The presence at an early age of corneal arcus (under the age of 45) or xanthomas should prompt suspicion for this genetic dyslipidemia. However, not all the patients with FH show physical findings (Hovingh *et al.*, 2013).

Certainty diagnosis is given by the finding of causative mutations, even in the absence of other criteria. However, the diagnosis of FH does not require genetic testing. There are validated algorithms which are useful for clinical diagnosis of FH. There is no single internationally accepted set of criteria. The most commonly used are the US MEDPED (Make Early Diagnosis to Prevent

Table 2 Phenotypic variability of primary hypercholesterolemia according to genetic background

	Type of mutation	*LDL-C (mg/dL)*	*Clinical severity*
HoFH	Homozygous null LDLR	500–1000	HoFH phenotype (+++++)
	Homozygous defective LDLR	500–750	HoFH phenotype (++++)
	Homozygous ARH		
	Compound heterozygous LDL/ApoB/PCSK9	400–750	HeFH/HoFH phenotype (+++/+++++)
	Homozygous PCSK9	300–450	HeFH phenotype (+++)
	Homozygous ApoB		
HeFH	Heterozygous LDLR	190–450	HeFH phenotype (++)
	Heterozygous ApoB		LDLR = PCSK9 > ApoB
	Heterozygous PCSK9		
PH	Polygenic	160–190	+
Hyperlipoproteinemia(a)	LPA gene	160–300[a]	+/++

[a]As a consequence of Lp(a) rise.

ApoB, apolipoprotein B; *ARH*, autosomal recessive hypercholesterolemia; *HeFH*, heterozygous familial hypercholesterolemia; *HoFH*, homozygous familial hypercholesterolemia; *LDL-C*, low density lipoprotein cholesterol; *LDLR*, low density lipoprotein receptor; *LPA*, lipoprotein (a); *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PH*, polygenic hypercholesterolemia.

Clinical severity is expressed as + to +++++.

Early Death), the Dutch Lipid Clinic Criteria, and the UK criteria (The Simon Broom register). The US criteria only use lipid levels. However, the criteria developed by the groups in The Netherlands and in the United Kingdom include in addition to cholesterol levels, family and personal history, physical signs and genetic studies, classifying the diagnosis as definite, probable, possible, and unlikely (**Tables 3** and **4**; Hovingh *et al.*, 2013). In the case of absence of familial history of dyslipidemia, after discarding ADH, the LDLRAP1 gene should be studied.

When FH is diagnosed, it is recommended a cascade screening in the family of the index patient to identify people carrying a genetic condition. If genetic study is not available, the screening could be carried out by determination of lipid profiles of close relatives of the index patient (Sniderman *et al.*, 2014).

Treatment

FH is characterized by high levels of LDL-C and high risk of cardiovascular disease, higher than 20%, being considered as a high cardiovascular risk by different guidelines and Scientific Societies (Versmissen *et al.*, 2008; Catapano *et al.*, 2016).

The main objective of the treatment is the reduction of cardiovascular disease events. Thus, it is fundamental non-pharmacological and pharmacological therapy for hypercholesterolemia and other risk factors.

It is of great importance the early initiation of the treatment because of the cumulative cholesterol burden. It has been demonstrated that early treatment and long-term drug therapy can reduce or eliminate the risk of coronary heart disease up to similar incidence than general population (Versmissen *et al.*, 2008).

Lifestyle and other cardiovascular risk factors are an important part of the treatment. All the patients should be advised on lifestyle changes, such as dietary modifications (low in trans and saturated fats, cholesterol, and refined sugars), regular physical activity, avoidance of weight gain, and cessation of smoking. Furthermore, other associated cardiovascular risk factors (hypertension, diabetes, etc.) must be treated.

Lipid-lowering drugs should be initiated as soon as possible after the diagnosis of FH. Lowering LDL-C is the principal objective of the treatment. High-dose statins are first-line therapy. Statin monotherapy allows to decrease LDL-C up to 50%–60%. However, many patients with FH will require adding other LDL-lowering agents depending on the baseline LDL-C levels and the responsiveness to therapy. Drugs that can be added to statins in second line are ezetimibe, bile-acid sequestrants, and niacin. These lipid-lowering drugs are also options in the case of intolerance to statins. The most usual approach is the addition of ezetimibe.

Even, some patients will need three or more drugs to lower LDL-C to achieve the goal. In fact, the achievement rate of LDL-C goal in subjects with FH is just 11% according to previous reports. Thus, most of the patients will not reach the target.

Recently, there are available other medications for lowering LDL-C such as PCSK9 inhibitors. Alirocumab and evolocumab are monoclonal antibodies administered by subcutaneous injection. These drugs have shown reductions of LDL-C up to 60%, being expected that most FH patients will get LDL-C goals.

Additional options include lipoprotein apheresis. This procedure involves extracorporeal filtering of lipoproteins from blood, normally performed biweekly. It is used when drug therapy is ineffective or not tolerated. LDL apheresis achieve up to 60%–70% reductions in LDL-C.

Table 3 Criteria for the clinical diagnosis of FH: Dutch lipid clinic network diagnostic criteria

Dutch lipid clinic network diagnostic criteria for FH	
Family history	
First-degree relative with known premature[a] coronary and vascular disease	1
First-degree relative with known LDL-C level above the 95th percentile	1
First-degree relative with tendinous xanthomata and/or corneal arcus	2
Children aged <18 years with LDL-C above the 95th percentile	2
Clinical history	
Patient with premature[a] coronary artery disease	2
Patient with premature[a] cerebral or peripheral vascular disease	1
Physical examination	
Tendinous xanthomata	6
Corneal arcus prior to age 45 years	4
Cholesterol levels mg/dL (mmol/L)	
LDL-C ≥330 mg/dL (≥8.5)	8
LDL-C 250–329 mg/dL (6.5–8.4)	5
LDL-C 190–249 mg/dL (5.0–6.4)	3
LDL-C 155–189 mg/dL (4.0–4.9)	1
DNA analysis	
Functional mutation in LDLR, apo B or PCSK9 gene	8
Diagnosis (based on the total number of points obtained)	
Definite ≥8, Probable 6–7, Possible 3–5, Unlikely <3	

[a]Premature ≤55 years in men; <60 years in women.

Table 4 Criteria for the clinical diagnosis of FH: Simon Broome diagnostic criteria

Simon Broome diagnostic criteria for FH	
Point	*Criteria*
1	Total cholesterol levels >290 mg/dL (7.5 mmol/L) or LDL-C > 190 mg/dL (4.9 mmoL/L) in adults Total cholesterol levels >260 mg/dL (6.7 mmol/L) or LDL-C > 155 mg/dL (4.0 mmol/L)
2	Tendon xanthomas in the patient or tendon xanthomas in a first- or second-degree relative
3	DNA-based evidence of an LDLR mutation, familial defective apoB-100, or a PCSK9 mutation
4	Family history of myocardial infarction before age 50 years in a second-degree relative or before age 60 years in a first-degree relative
5	Family history of elevated total cholesterol >290 mg/dL (7.5 mmol/L) in an adult first- or second-degree relative Family history of elevated total cholesterol >260 mg/dL (6.7 mmol/L) in a child, brother, or sister 16 years or younger
Diagnosis	
Definite FH = 1 + 2 or 3	
Possible FH = 1 + 4 or 5	

The use of LDL apheresis in subjects with HeFH has been reduced after the appearance of PCSK9 inhibitors for HeFH, currently indicated after adequate pharmacologic treatment when LDL-C is above 300 mg/dL or above 200 mg/dL for those with coronary artery disease. In the case of HoFH it is an essential treatment which must be considered in all the cases (McGowan, 2013).

Recently, two lipid-lowering drugs have been approved for HoFH, lomitapide, and mipomersen. Lomitapide is an oral inhibitor of the microsomal triglyceride transport protein, which is responsible for transferring triglycerides and phospholipids onto chylomicron and VLDL during their assembly in the intestine and the liver, respectively. Lomitapide reduces the secretion of these lipoproteins into the circulation, lowering plasma LDL-C levels by 50%. Mipomersen is an antisense oligonucleotide, administered by subcutaneous injection, that targets the mRNA of apoB. Mipomersen reduces translation of apoB mRNA and the synthesis of apoB by the ribosome, leading to reduced secretion of VLDL, lowering plasma LDL-C levels by 20%–30%.

Currently, the liver transplantation is a therapeutic possibility just indicated in the case of failure of pharmacological therapy and LDL apheresis.

Polygenic Hypercholesterolemia

PH is the most frequent primary hypercholesterolemia with IIa phenotype. It is characterized by plasma levels of LDL-C above the 95th percentile corrected for age and gender in each population, with no monogenic inheritance.

The pathogenic mechanisms are not completely known. PH has been associated with an additive effect of common alleles that have minimal effect on the rise of LDL-C when are present isolated. However, the accumulation of different single nucleotide variations with hypercholesterolemic effect can cause phenotype clinically similar to FH, although autosomal dominant pattern cannot be demonstrated. The presence of different genetic variations could explain why approximately 50% of subjects clinically diagnosed of HeFH do not show mutations. In **Table 5** are shown different loci associated with total cholesterol and LDL-C levels (Willer *et al.*, 2013). Furthermore, exogenous factors, especially lifestyle, may increase the hypercholesterolemic effect of single nucleotide variations.

Clinical manifestations are characterized by high levels of LDL-C when subjects are over 20 years old and cardiovascular disease over 50 years old. Tendinous xanthomas and corneal arcus are rare (**Table 1**). Only 10% of the subjects have family history of hypercholesterolemia (Talmud *et al.*, 2013).

Hyperlipoproteinemia(a)

Significant increases in plasma levels of lipoprotein(a) or Lp(a) are another genetic cause of primary hypercholesterolemia. Lp(a) is a lipoprotein constituted by an LDL particle with an apo(a) molecule linked by a disulfide bridge.

The concentration of Lp (a) in plasma is very variable, from almost nonexistent up to above 300 mg/dL, due to variations in the *LPA* gene encoding the apo(a) protein. It is a monogenic alteration transmitted with autosomal dominant pattern.

Although the physiological role of Lp(a) is not well known, it seems to play a protective role for macrophages and platelets, leading to fibrin activation and healing of lesions. On the other hand, the increase in plasma levels of Lp(a) seems to be involved in the development of atherosclerosis, cardiovascular disease, and aortic stenosis. Lp(a) has been found in several studies to be an additional independent risk marker for cardiovascular diseases, especially when Lp(a) is above the 80th percentile (50 mg/dL).

A usual and reasonable option for patients at risk with high Lp(a) is an intensive treatment of the modifiable risk factors, including LDL-C. Lp(a) reduction can only be achieved by few lipid-lowering drugs. PCSK9 inhibitors and nicotinic acid lower Lp (a) up to 30%. Other option with more intense reduction of circulating levels of Lp(a) up to 80% is antisense drugs targeting the LPA gene.

Table 5 Loci associated with total cholesterol and LDL-C levels

	Total cholesterol (25 loci)		*LDL-C (10 loci)*	*Total cholesterol and LDL-C (47 loci)*	
Loci	*ABCA1*	*ABCB11*	*ANXA9-CERS2*	*ABCG5/8*	*ABO*
	ASAP3	*C6orf106*	*APOH-PRXCA*	*ANGPTL3*	*APOA1*
	ERGIC3	*EVI5*	*BRCA2*	*APOB*	*APOE*
	FAM117B	*GPR146*	*EHBP1*	*BRAP*	*CETP*
	HBS1L	*HNF4A*	*FN1*	*CILP*	*CMTM6*
	KCNK17	*LIPC*	*MTMR3*	*CSNK1G3*	*CYP7A1*
	LIPG	*MAMSTR*	*NYNRIN*	*DLG4*	*DNAH11*
	MARCH8-ALOX5	*PHC1-A2ML1*	*PIGV-NR0B2*	*FADS1-2-3*	*FRK*
	PHLDB1	*PXK*	*SNX5*	*GPAM*	*HFE*
	RAB3GAP1	*RAF1*	*SPTLC3*	*HLA*	*HMGCR*
	SPTY2D1	*TOM1*		*HNF1A*	*HPR*
	TTC39B	*UBASH3B*		*INSIG2*	*IRF2BP2*
	VIM-CUBN			*LDLR*	*LDLRAP1*
				LOC84931	*LPA*
				LRPAP1	*MAFB*
				MIR148A	*MOSC1*
				MYLIP	*NPC1L1*
				OSBPL7	*PCSK9*
				PLEC1	*PPARA*
				PPP1R3B	*SORT1*
				SOX17	*ST3GAL4*
				TIMD4	*TOP1*
				TRIB1	*UGT1A1*
				VLDLR	

Based on Willer, C. J., Schmidt, E. M., Sengupta, S., et al. (2013). Discovery and refinement of loci associated with lipid levels. Nature Genetics 45, 1274–1283.

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https://thefoundation.org/—FH Foundation: Familial hypercholesterolemia.

Low HDL and High HDL Syndromes☆

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Glossary

Apolipoprotein Lipoprotein associated proteins that play a critical role in the structure, solubility, antigenicity, transport, enzyme activation, and cellular uptake of lipoproteins.

Atherosclerosis A common form of arteriosclerosis in which deposits of yellow plaques containing cholesteryl esters, and lipid-laden macrophages (foam cells) are formed with large and medium-sized arteries.

Hepatic Relating to or affecting the liver.

Lecithin A phosphoglyceride that is the major component of cell membranes, consisting of esters of glycerol with two molecules of long-chain aliphatic acids and one of phosphoric acid.

Introduction

Plasma high-density lipoprotein cholesterol (HDL-C) level was shown to correlate negatively with the incidence of coronary heart disease (CHD). Low HDL syndrome is a condition in which plasma HDL-C is low, is a dyslipidemia frequently observed in patients with premature CHD. The anti-atherogenic functions of HDL have been demonstrated by a number of experiments in vitro and in vivo. The injection of HDL and its major apolipoprotein (apo) constituent, apoA-I, was shown to attenuate atherosclerosis in some animal models. Lipid-poor apoA-I and HDL remove cholesterol from lipid-laden macrophages (foam cells) via ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), respectively. The cholesterol is esterified by lecithin: cholesterol acyltransferase (LCAT) to form cholesteryl ester (CE). The CE of HDL is subsequently transferred by plasma cholesteryl ester transfer protein (CETP) to very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and low-density lipoprotein (LDL) in exchange for triglycerides (TG). The TG in HDL is hydrolyzed by hepatic lipase (HL) and HDL becomes smaller to take up more cholesterol from foam cells. Furthermore, the IDL and LDL are taken up by hepatic LDL receptor. The CE of HDL particle is taken up by the liver via scavenger receptor class B type I (SR-BI). Thus, excess cholesterol in the foam cells of arteries is transported back to the liver, a process called "reverse cholesterol transport (RCT)" (Fig. 1). In the liver, cholesterol is catabolized into bile acid and excreted into the bile.

The plasma levels of HDL-C as well as the biochemical composition and functions of HDL particles are regulated by apolipoproteins, lipolytic enzymes, lipid transfer proteins, receptors, and cellular transporters such as ABCA1 and ABCG1. Furthermore, transcription factors such as liver X receptor (LXR) and farnesoid X receptor (FXR) that regulate the expression of ABCA1 and ABCG1 are also involved in the regulation of plasma HDL-C levels. Low HDL syndrome is caused by abnormalities of a variety of molecules involved in RCT.

The etiology of high HDL syndrome is also based upon abnormalities in the molecules involved in RCT. In contrast to low HDL syndrome, the story of high HDL syndrome is somewhat complicated. Although high HDL syndrome was previously believed to be associated with a longevity due to a reduced incidence of CHD, Matsuzawa et al. reported two cases of hyper-alphalipoproteinemia (HALP) with premature corneal opacity and in one case angina pectoris, and proposed that high HDL syndrome is heterogeneous, not always beneficial, and sometimes rather atherogenic. Recently, evidence supporting this proposal has been accumulated from many experimental and epidemiological studies.

In the current review, we summarize the clinical characteristics and pathophysiology of low HDL syndrome and high HDL syndrome and the dynamics and efficiency of RCT in these conditions are discussed. Due to limitations of space, many data on genetically engineered mice with altered levels of plasma HDL-C are not discussed.

Factors Regulating Plasma HDL-Cholesterol Levels

HDL particles consist of heterogeneous subclasses. They have a density range of 1.063–1.21 g mL^{-1} and are small in size (Stokes' diameter: 5–17 nm). They consist of approximately 50% lipids and 50% proteins. HDL can be classified by density (HDL_2 or HDL_3 fraction), electrophoretic mobility (α- and pre-β-electrophoretic mobility), and apolipoprotein composition (Lp A-I or Lp A-I/A-II). The quantity and quality of HDL particles are regulated by many factors, such as plasma enzymes, lipid transfer proteins, and cell surface receptors as well as transporters.

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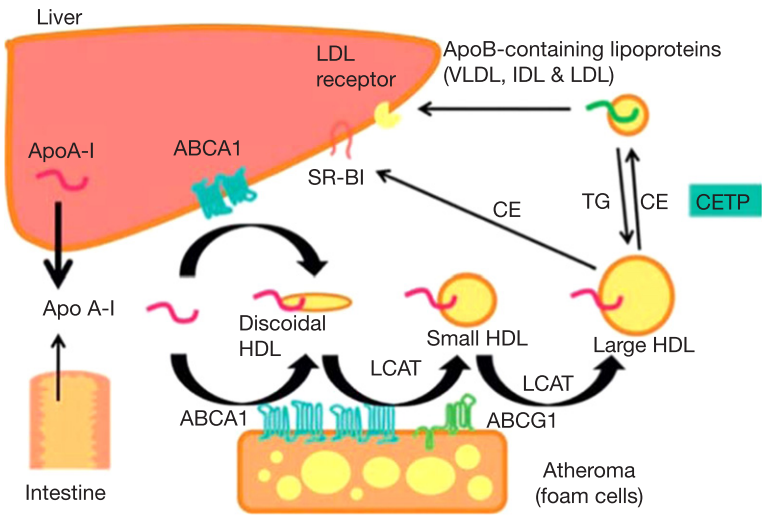

Fig. 1 Metabolic map of apoA-I and HDL-mediated reverse cholesterol transport in humans. *Arrows* denote cholesterol transport in the human body mediated by lipoproteins, plasma lipid transfer proteins, and cell surface receptors and transporters. Cholesterol efflux from peripheral cells is thought to be very diverse. There are some candidates for cell surface receptors such as ABCA1 and ABCG1.

Synthesis and Secretion of HDL

The major constituent of HDL, apoA-I, is synthesized in the liver (~80%) and small intestines (~20%). ABCA1 interacts with apoA-I and is involved in efflux of cholesterol and phospholipids on the plasma membranes, generating "discoidal nascent HDL" particles. The genetic deficiency of ABCA1 causes Tangier disease characterized by enhanced atherosclerosis, hepatosplenomegaly and orange tonsils due to massive deposition of CE in tissues. Transporters such as ABCG1 and other molecules may be involved in HDL-mediated cholesterol efflux, although ABCA1 is requisite for the apoA-I-mediated production of HDL particles. Free cholesterol on nascent HDL particles is esterified to form CE by the action of LCAT. LCAT plays a pivotal role in making HDL particles spherical and mature.

Modification of HDL Particles in Plasma

Several enzymes are involved in modifying the quality and quantity of HDL particles in plasma. The movement of lipids and apoliporoteins during lipolysis is one of the important sources for HDL particles. Two lipases, lipoprotein lipase (LPL) and hepatic lipase (HL), are involved in the lipolysis of chylomicrons/chylomicron remnants and VLDL/IDL, respectively. The hydrolysis of TG-rich lipoproteins by these lipases releases some apolipoproteins, cholesterol and phospholipids into plasma, and these constituents can be used for the formation of new HDL particles. Plasma phospholipid transfer protein (PLTP) transfers phospholipids and cholesterol from apoB-containing lipoproteins to HDL. HL, located on the liver sinusoid, is thought to remodel large and TG-rich HDL particles into smaller less TG-rich ones.

As mentioned earlier, plasma CETP facilitates the transfer of neutral lipids between lipoproteins. CETP transfers CE from HDL to apoB-containing lipoproteins in exchange for TG from apoB-containing lipoproteins to HDL. Plasma CETP deficiency causes a marked elevation of plasma HDL-C levels.

Endothelial lipase (EL) is a phospholipase belonging to the LPL family, which includes LPL and HL. In contrast to LPL and HL, EL mainly regulates HDL metabolism and HDL-C levels in humans and mice. The inhibition of EL activity may elevate the HDL-C level. EL hydrolyzes the phospholipids in HDL and is involved in the remodeling of HDL particles. Although the inhibition of EL activity leads to elevation of HDL-C levels, it has not been established in humans whether EL inhibition may attenuate atherosclerosis.

Hepatic Uptake of HDL and HDL Lipids

As the last step of RCT, at least two distinct pathways are involved in taking up cholesterol from plasma. One is the LDL receptor-mediated pathway and the other is the HDL receptor(s)-mediated pathway. Although the impact and significance of this pathway are not completely understood in humans, SR-BI is the physiologically relevant hepatic HDL receptor established in mice. SR-BI mediates the selective uptake of CE in HDL. There may be other possible pathways, in which whole particles of HDL are taken up and catabolized.

Degradation of ApoA-I or HDL Particles

In the kidney, apoA-I is catabolized by a size-dependent filtration process. Cubilin is thought to mediate the reabsorption of apoA-I from the renal proximal tubule lumen.

Multiple Functions of HDL and ApoA-I

Recently, HDL and apoA-I have been demonstrated to exert a variety of functions, including anti-oxidative, anti-inflammatory, anti- thrombotic, anti-apoptotic, anti-diabetic, anti-infectious, endothelial cell-repairing, vascular endothelial function-improving effects in addition to cellular cholesterol efflux and RCT. These effects may be comprehensively involved in the prevention of atherosclerosis progression.

Recent proteomic analysis of HDL by mass spectrometry identified that HDL is associated with >85 proteins. In addition to proteins consistent with traditionally accepted roles in lipid transport, HDL carries unique constituents such as protease inhibitors involved in hemostasis, acute-phase response proteins, immune function mediators, complement pathway members and metal-binding proteins. This compositional diversity of HDL may suggest the pleiotropic functions of HDL, including roles in lipid transport, oxidation, inflammation, hemostasis, and immune system.

Functional HDL and Dysfunctional HDL

As mentioned earlier, HDL is known to modulate systemic inflammation and is thus anti-inflammatory (functional HDL). In the absence of inflammation, HDL has a complement of antioxidant enzymes that work to maintain an anti-inflammatory state. Conversely, in the presence of systemic inflammation, antioxidant enzymes can be inactivated, resulting in the accumulation of oxidized lipids and proteins that may make HDL proinflammatory (dysfunctional HDL) and increase vascular inflammation. Under these conditions, the apoA-I of HDL particles can be modified by reactive oxygen species. This oxidative modification impairs the ability of HDL to promote cholesterol efflux via ABCA1 and ABCG1. HDL may be a shuttle that can be either anti-inflammatory or proinflammatory, depending on its cargo of proteins, enzymes, and lipids. Under some conditions, HDL can become dysfunctional and even proinflammatory, but this characterization can change with resolution of systemic inflammation or use of certain treatments.

Animal and human studies suggested that measures of the quality and novel functions of HDL might provide an improved means of identifying subjects at increased risk for atherosclerotic events, compared with the current practice of only measuring HDL-C levels. Therapeutic approaches that reduce coronary risk, such as statins and lifestyle changes, can favorably moderate the characteristics of proinflammatory HDL. Furthermore, apoA-I mimetic peptides, delipidated HDL and other compounds that target functional aspects of HDL may provide novel approaches to reduce cardiovascular risk.

Human Genetic Studies as a Tool for Identifying Serum HDL-C Levels and Their Association to CHD

Environmental factors may affect serum HDL-C levels and HDL function, however approximately 40%–60% of the variability of HDL-C can be explained by inherited genetic variations. The study of monogenic diseases showed the impact of selected genes on a certain phenotype of dyslipidemia. The loss of function mutations in the genes *APOA1*, *ABCA1*, and *LCAT* caused conditions of extremely low HDL-C levels. In contrast, the loss of function mutations in *CETP*, *LIPG*, and *SCRB1* underlie extremely high HDL-C levels. Mendelian variants demonstrate a good tool to explore the extreme phenotypes, however they are extremely rare and do not usually explain the great inter-individual population-level variabilities in serum HDL-C levels. Thus, additional tools have been developed to detect the missing heritability of HDL-C levels, including (1) genome-wide association studies (GWAS), (2) whole exome sequencing and target resequencing, and (3) exome-wide genotyping arrays and targeted genotyping panels.

Low HDL Syndrome

Primary Low HDL Syndrome

The etiology of low HDL syndrome can be divided into two; primary low HDL syndrome and secondary low HDL syndrome. The primary or secondary abnormalities in molecules involved in RCT may cause low HDL syndrome. **Table 1** shows the comparison of clinical and biochemical characteristics of primary low HDL syndrome.

Tangier disease

Tangier disease is a familial HDL deficiency characterized by enlarged orange tonsils, mild hepatosplenomegaly, corneal opacification, and a marked reduction of serum HDL-C and LDL-C as well as mild hypertriglyceridemia. Mutations in the *ABCA1* gene

Table 1 Comparison of clinical and biochemical characteristics of primary low HDL syndrome

Affected gene	*Tangier disease*	*Familial HDL deficiency without Tangier disease phenotype*	*Familial LCAT deficiency*	*Fish eye disease*	*ApoA-I deficiency*
	ABCA1	ABCA1 *(some cases)*	*LCAT*	*LCAT*	*apoA-I*
Clinical signs and symptoms					
Typical Tangier phenotype (orange tonsils, hepatosplenomegaly, neuropathy)	+	−	−	−	−
Corneal opacity	+	+	+ + +	+ + +	+ +
Nephropathy	−	−	+	−	−
Risk for coronary heart disease	Moderately increased	Moderately increased	Normal/ increased	Normal/ increased	Increased
Biochemical data					
Plasma total cholesterol	Low	Low	Low	Low	Normal
LDL-cholesterol	Low	Normal	Normal	Normal	Normal
HDL-cholesterol	None	None	Very low	Very low	None
Plasma triglycerides	Increased	Normal	Increased	Increased	Normal
% Cholesteryl ester	Normal	Normal	Low	Low	Normal
Apolipoprotein (apo) A-I	Very low	Very low	Very low	Very low	None
Relative increase in apoA-I precursor	+	+	−	−	−
α-LCAT activity	Normal	Normal	Very low	Very low	Normal
Cell biological data					
Cholesterol efflux from cells (apo A-I-mediated)	Absent	Absent	Normal	Normal	Normal

was identified in patients with Tangier disease. ABCA1 is expressed throughout the body and mediates apoA-I-mediated cholesterol efflux at the plasma membrane. In Tangier disease, cholesterol-laden macrophages are found in various tissues such as liver, spleen, bone marrow, tonsils, nerves, smooth muscle cells, arterial wall, and β cells. The heterozygous parents have about half normal levels of HDL-C. Homozygotes of Tangier disease show a marked increase in the fractional catabolic rate of HDL proteins such as apoA-I, whereas heterozygotes also demonstrate enhanced clearance. Homozygotes have only preβ-1 HDL present in their plasma, while heterozygotes lack large α-1 and α-2 HDL particles, normal preβ-1 HDL, and only 50% of normal cellular cholesterol efflux. It is still controversial whether patients with homozygous Tangier disease develop premature CHD. We previously reported severely calcified coronary arteries visualized by coronary intravascular ultrasonography and impaired insulin secretion in *ABCA1* deficiency. In the Danish general population, two SNPs (V771M and V825I) of *ABCA1* gene were associated with increases in HDL-C, one (R1587K) with decreases in HDL-C, whereas three (R219K, I883M, and E1172D) did not affect HDL-C levels. However, five out of six SNPs (V771M, V825I, I883M, E1172D, R1587K) predicted increased risk of CHD. A stepwise regression analysis showed V771M, I883M, and E1172D as the most important predictors of CHD. Thus, three of six nonsynonymous SNPs in *ABCA1* gene predict risk of CHD in the general population.

Familial LCAT deficiency (FLD) and fish eye disease (FED)

FLD is a very rare autosomal inherited disorder, which includes two distinct clinical syndromes: FLD and FED. Both diseases are caused by the mutations in the *LCAT* gene, leading to a marked reduction in plasma HDL-C. LCAT catalyzes the esterification of free cholesterol on HDL, therefore its deficiency results in accumulation of free cholesterol, but not CE, in a variety of tissues, including cornea and kidneys. Thus, the plasma CE ratio (CE divided by total cholesterol) is markedly reduced. The lipoproteins of familial LCAT deficiency are morphologically abnormal, with the appearance of multilamellar vesicles, rouleaux, LpX-like particles. The major clinical symptoms in FLD are corneal opacities, anemia (red blood cells appear as acanthocytes), and proteinuria, which may eventually progress to renal failure in cases with total LCAT deficiency. Foam cells accumulate in a variety of tissues, including cornea, kidney, liver, spleen, and arteries. Interestingly, patients with FLD are usually not accompanied by premature CHD.

Patients with FED or partial LCAT deficiency present with no clinical signs such as nephropathy except for the characteristic dense age-dependent corneal opacities. The patients are characterized by HDL deficiency and elevated TG levels. They demonstrate an apparently normal activity of LCAT enzyme to esterify cholesterol in plasma, a partial reduction in LCAT concentration, and a nearly normal cholesterol esterification rate. Although the CE content of HDL is very low, the relative CE content of VLDL and LDL is normal. It was postulated that LCAT exhibits two activities: α-LCAT activity specific for HDL that migrate with α-mobility upon gel electrophoresis, and β-LCAT activity specific for pre-β and β-migrating lipoproteins (VLDL and LDL, respectively). Thus, FED was classified as α-LCAT deficiency whereas FLD was due to the lack of both α- and β-LCAT activities. However, after cloning of the LCAT gene, α- and β-LCAT activity represented two functional aspects of the same protein in humans. Total cholesterol and CE levels are relatively higher in FED patients compared with those FLD, despite very low serum HDL-C levels. It is not yet conclusive whether LCAT deficiency is associated with increased risk of CHD. It is possible that other factors such as LDL-C and TG levels may affect the susceptibility to atherosclerosis in patients with FLD and FED.

Apo A-I *genetic mutations and polymorphisms affecting HDL-cholesterol levels*

The human *APOA1* gene is located on chromosome 11 in a cluster with two other apolipoprotein genes, *APOC3* and *APOA4*. Various disruptions and mutations of the *APOA1* gene have been reported. Some *APOA1* gene mutations caused a marked deficiency of plasma HDL-C levels as observed in patients with a genetic deficiency of *ABCA1* or *LCAT* and are associated with premature CHD. However, HDL-deficient patients with *APOA1* mutation do not usually show anemia, proteinuria, orange tonsils, hepatosplenomegaly and premature CHD. Some mutations or polymorphisms in the *APOA1* gene are related to the phenotypic expression of amyloidosis and neuropathy.

Secondary Low HDL Syndrome

Smoking

Low HDL-C syndrome is often observed in subjects who smoke. The reduction of plasma HDL-C by smoking is supposed to be due to the reduced activity of LCAT. Cessation of smoking results in an elevation of plasma HDL-C levels.

Physical inactivity

Low HDL-C syndrome is also observed in subjects who are physically inactive. The mechanism for the decrease of plasma HDL-C by physical inactivity is supposed to be related to the reduced activity of LPL. Aerobic exercise is known to increase HDL-C levels.

Hypertriglyceridemia

Patients with hypertriglyceridemia are often associated with the reduction of plasma HDL-C, but hypertriglyceridemic subjects do not always show the reduction of HDL-C. Hypertriglyceridemia appears to stimulate the lipid exchange and accelerate the catabolism of HDL protein. Reduced activity of plasma lipolytic enzymes causes the impaired catabolism of TG-rich lipoproteins, leading to a reduction of the source of lipids for generating new HDL particles. Patients with low HDL-C have a higher risk for CHD, but a combination of hypertriglyceridemia and low HDL-C is highly atherogenic and markedly increases the CHD risk. The treatment of hypertriglyceridemia by fibrates and nicotinic acids usually increases plasma HDL-C levels.

Visceral fat obesity and metabolic syndrome (insulin resistance syndrome)

Low HDL-C syndrome is often observed in patients with visceral fat obesity. Even in non-obese patients with metabolic syndrome due to increased visceral adiposity, plasma HDL-C level is usually low. The reduction of plasma HDL-C in these subjects may be partly attributed to hypertriglyceridemia and reduction of adiponectin that was shown to increase the hepatic synthesis of HDL via induction of hepatic ABCA1 and apoA-I protein and enhance the cholesterol efflux from macrophages via induction of macrophage ABCA1 protein. After reduction of body weight in patients with visceral fat obesity or metabolic syndrome, plasma HDL-C is usually increased.

Patients with a genetic deficiency of long-chain fatty acid transporter CD36 are often associated with insulin resistance and a clustering of multiple risk factors such as hypertriglyceridemia and low HDL-C levels.

Inflammation

Plasma HDL-C level may be a negative marker for systemic or local inflammation. Some pathological conditions with chronic or acute systemic inflammation (e.g., severe infection and hematological malignancy) are associated with a reduction of HDL-C along with increased serum amyloid A proteins. Several studies have indicated that low HDL-C may be a marker for cardiac events during short-term follow-up in association with an increase in high-sensitivity C-reactive protein (hsCRP).

Cholestatic disorders

In obstructive liver diseases such as end-stage liver cirrhosis, plasma HDL-C levels may be markedly reduced to the same extent as in primary HDL deficiency. The reduction of HDL-C is based upon the decrease in hepatic protein synthesis including LCAT.

Drugs

Some drugs, such as androgens, progesterone, anti-hypertensive drugs (thiazides and beta blockers) or an anti-hyperlipidemic drug, probucol, are known to decrease plasma HDL-C levels. Probucol is a potent antioxidant and anti-hyperlipidemic drug that attenuates xanthelasma and xanthoma formation even in patients homozygous for familial hypercholesterolemia and in Watanabe heritable hyperlipidemic rabbits. Although probucol reduces HDL-C, probucol has a variety of anti-atherogenic functions, which will be discussed later.

High HDL Syndrome

Primary High HDL Syndrome

Etiologies for high HDL syndrome are listed in **Table 2**.

Table 2 Primary and secondary high HDL syndrome

Primary high HDL syndrome
Familial cholesteryl ester transfer protein (CETP) deficiency
Familial hepatic triglyceride lipase (HL) deficiency
Familial hyperalphalipoproteinemia with premature corneal opacity (combined deficiency of CETP and HL activity)
Familial hyperalphalipoproteinemia with genetic abnormalities in scavenger receptor class B type I (SR-BI) gene (*SCARB1*)
Familial hyperalphalipoproteinemia with genetic abnormalities in endothelial lipase (EL) gene (*LIPG*)
Familial hyperalphalipoproteinemia with increased production of apoA-I (*APOA1*)
Familial hyperalphalipoproteinemia with reduced uptake of HDL by lymphocytes
Other genes implicated for high HDL syndrome
Secondary high HDL
Syndrome chronic heavy
Alcohol consumption
Primary biliary cirrhosis
Inhibitors of CETP in plasma
Multiple symmetric lipomatosis
Chronic obstructive pulmonary disease (COPD)
Aerobic exercise
Drugs
Insulin
Estrogen and derivatives
Glucocorticoids
HMG-CoA reductase inhibitor (statins)
Intestinal cholesterol transporter inhibitor (ezetimibe)
Fibrates
Nicotinic acid and its derivative
PCSK9 inhibitors
Cyclosporin etc.

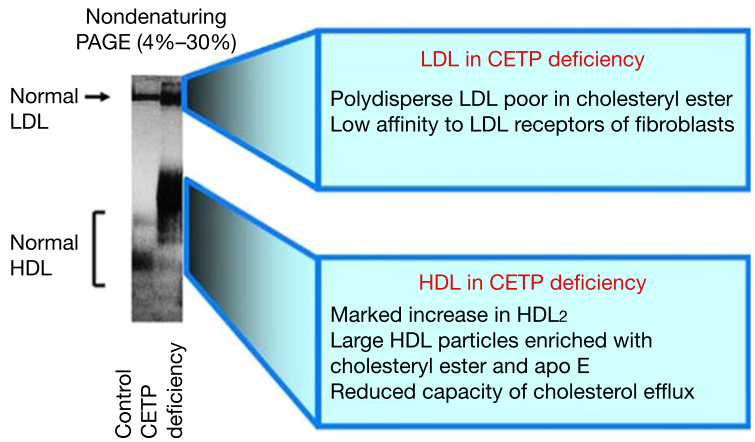

Fig. 2 Lipoprotein abnormalities of CETP deficiency. The functions and composition of both LDL and HDL are abnormal in CETP deficiency. Thus, hyperalphalipoproteinemia due to CETP deficiency is a disorder of RCT.

Familial CETP deficiency and the development of CETP inhibitors

The most important and frequent cause of primary high HDL syndrome is CETP deficiency. In Japan, many patients with HALP due to CETP deficiency have been identified. Patients with homozygous CETP deficiency present with extremely high plasma HDL-C levels (three to six times normal levels). Plasma levels of apoA-I, apoA-II, apoC-III, and apoE are also increased whereas plasma apoB and apoB-containing lipoproteins are a little decreased. As illustrated in Fig. 2, both LDL and HDL from CETP-deficient homozygotes are markedly abnormal in terms of their biochemical compositions and biological functions. Their HDL particles are very large and enriched with CE and apoE. Their LDL particles are small, polydisperse, and enriched with TG and apoB. From the point of biological function, their LDL demonstrates a reduced affinity for LDL receptors of fibroblasts whereas their HDL has a reduced ability to mediate cholesterol efflux from lipid-laden macrophages, suggesting the atherogenicity of both LDL and HDL. HDL isolated from homozygous and heterozygous carriers of *CETP* null mutations were evaluated for their ability to promote NO production in cultured endothelial cells. When compared at the same protein concentration, HDL and HDL fractions from carriers of CETP deficiency were significantly less effective than control HDL and HDL fractions in stimulating NO production, due to a reduced eNOS activating capacity, likely because of a reduced S1P content.

The first and major *CETP* gene mutation identified was an intron 14 splicing defect (IN14), which is a null mutation with a dominant effect on plasma CETP activity/mass and HDL-C levels. The second major *CETP* gene mutation was the missense mutation in exon 15 (D442:G). These mutations are very common in the Japanese population. The effect of D442:G mutation on plasma lipoproteins is less severe than that of IN14. D442:G homozygotes show moderately increased HDL-C levels. In other countries, some genetic variations have also been reported to affect CETP mass and HDL-C levels.

There have been controversies on the atherogenicity of CETP deficiency. We found a unique area (Omagari, Akita Prefecture, Japan) where the subjects with a marked HALP (HDL-C $\geq 100\ \mathrm{mg\ dL^{-1}}$) due to the IN14 mutation are very frequent. A population-based study in this area indicated a U-shaped relationship between plasma HDL-C level and the incidence of ischemic electrocardiographic changes. In subjects with HDL-C $< 1.81\ \mathrm{mmol\ L^{-1}}$ ($70\ \mathrm{mg\ dL^{-1}}$), the incidence increased in proportion to the HDL-C levels. The frequency of the IN14 *CETP* gene mutation was higher in patients with CHD than in control subjects. In subjects aged >80 years, the prevalence of both marked HALP and the IN14 splicing defect was significantly lower than in the younger generation. Thus, a marked HALP caused by IN14 *CETP* gene mutation does not represent a longevity syndrome, suggesting the importance of re-evaluation of the clinical significance and pathophysiology of a marked HALP.

The relationship between *CETP* gene mutations and CHD in Japanese-American men in the Honolulu Heart Program cohort was evaluated. Men, most of whom had a D442:G mutation, showed an increased risk of CHD, but the atherogenic effect of CETP deficiency was observed in subjects with moderately increased HDL-C levels but not in subjects with a marked HALP. In the Framingham Heart Study, plasma CETP activity was measured in 1978 participants and on the average follow-up of 15 years. In multivariable analyses, plasma CETP activity was inversely related to the incidence of cardiovascular disease events. These prospective data suggested that lower plasma CETP activity was associated with a greater cardiovascular disease risk. Similar data were reported from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study in Europe, in which low plasma CETP mass levels were associated with increased cardiovascular and all-cause mortality. In LURIC study, decreased cholesterol efflux capacity was shown in patients with low plasma CETP mass levels. Furthermore, the post hoc analyses of KAROLA study demonstrated a similar tendency in relations between low plasma CETP mass levels and cardiovascular and all-cause mortality. The Ile405Val mutation in the *CETP* gene increases HDL-C levels. Women not treated with hormone replacement therapy who were heterozygous or homozygous for Val405 had a 1.4 to 2.1-fold increase in the risk of CHD, however no significant associations were found in men. Increased HDL-C levels caused by *CETP* gene mutations were associated with an increased risk of CHD in white women.

Regarding the effect of CETP genetic variants on intracerebral hemorrhage (ICH), 12 variants in *CETP* gene showed nominal association with ICH, with the strongest association at the rs173539 locus (OR1.25, $P = 6.0 \times 10^{-4}$). A genetic score of CETP variants found to increase HDL-C by $\sim 2.85\ \mathrm{mg\ dL^{-1}}$ in the Global Lipids Genetics Consortium was strongly associated with ICH risk (OR1.86, $P = 1.39 \times 10^{-6}$). Thus, genetic variants in CETP associated with increased HDL-C raise the risk of ICH. These studies challenge the rationale of pharmacological CETP inhibition.

Inhibiting CETP activity may raise HDL-C and decrease LDL-C levels. Thus, a number of CETP inhibitors have been developed. The initial clinical trial (ILLUMINATE Study) with a CETP inhibitor, torcetrapib, in combination with atorvastatin was prematurely terminated because of an increased cardiovascular event rate and mortality in torcetrapib-treated patients. RADIANCE1 and RADIANCE2 studies examining the effect of torcetrapib on carotid intima-media thickness in patients with familial hypercholesterolemia and mixed dyslipidemia, respectively, were also terminated. No beneficial effect of torcetrapib was observed on carotid intima-media thickness. ILLUSTRATE study also showed a small favorable effect for torcetrapib in the change in normalized atheroma volume, however there was no significant difference in the change in atheroma volume for the most diseased vessel segment, suggesting that torcetrapib may have no significant effect on the progression of coronary atherosclerosis. The lack of efficacy of torcetrapib may be related to the mechanism of action of this drug class or to molecule-specific adverse effects. Torcetrapib significantly increased blood pressure because of the increase in aldosterone levels.

Dalcetrapib is a CETP inhibitor which raises plasma HDL-C levels, but does not decrease plasma LDL-C. Dal-OUTCOMES study investigated the effect of dalcetrapib, at a dose of 600 mg daily, or placebo, in addition to the best available evidence-based care on the risk of recurrent cardiovascular events who had a recent acute coronary syndrome. The study was also terminated because dalcetrapib increased HDL-C levels, but did not reduce the risk of recurrent cardiovascular events. Later, the effect of dalcetrapib on recurrent cardiovascular events in the dal-OUTCOMES study and dal-PLAQUE-2 imaging trial was pharmacogenomically evaluated, using a genome-wide approach. A single-nucleotide polymorphism (SNP) in the adenylate cyclase type 9 (*ADCY9*) gene on chromosome 16 (rs1967309) was identified to be associated with cardiovascular events in the dalcetrapib arm. Patients with genotype AA at rs1967309 showed a significant 39% reduction in the composite cardiovascular endpoint with dalcetrapib compared with placebo. In patients with genotype GG, events rate was increased by 27% with dalcetrapib versus placebo. Thus, the effects of dalcetrapib on atherosclerotic outcomes were determined by correlated SNPs in the *ADCY9* gene.

Studies on another CETP inhibitor evacetrapib also failed because of the absence of cardiovascular event reduction. ACCELERATE trial enrolled 12,092 patients with at least one of the following high-risk conditions: an acute coronary syndrome, cerebrovascular atherosclerotic disease, peripheral artery disease, or diabetes mellitus with CHD. Patients were randomly assigned to receive either evacetrapib at a dose of 130 mg daily or placebo, in addition to standard medical therapy. The primary efficacy end point was the first occurrence of any component of the composite of death from cardiovascular causes, myocardial infarction, stroke, coronary revascularization, or hospitalization for unstable angina. At 3 months, evacetrapib decreased the mean LDL-C level by 31.1% compared with a 6.0% increase with placebo. The mean HDL-C level was increased by 133.2% with evacetrapib versus 1.6% increase with placebo. Although evacetrapib had favorable effects on LDL-C and HDL-C levels, it did not reduce cardiovascular events than placebo among patients with high-risk vascular disease.

A controversial result on another CETP inhibitor anacetrapib has recently been reported. REVEAL trial included 30,449 adults with atherosclerotic vascular disease who were receiving intensive atorvastatin therapy with a mean LDL-C level of 61 mg dL^{-1} and a mean HDL-C level of 40 mg dL^{-1}. The patients were randomly assigned to receive either 100 mg of anacetrapib once daily or placebo. The primary outcome was the first major coronary event, a composite of coronary death, myocardial infarction, or coronary revascularization. During the median follow-up period of 4.1 years, the primary outcome occurred in significantly fewer patients in the anacetrapib group than in the placebo group (Hazard ratio: 0.91; 95% confidence interval, 0.85–0.97; $P = 0.004$). At the trial midpoint, the mean HDL-C level was higher by 43 mg dL^{-1} in the anacetrapib group than in the placebo group (104% increase). Anacetrapib lowered the incidence of major coronary events than placebo, however the development of anacetrapib was finally terminated by the developer Merck. A recent evidence suggests that CETP inhibitors form a complex between themselves, CETP and HDL particles, which might interfere with many physiological functions of HDL. Therefore, CETP inhibition is not a good target of HDL-modifying therapy. We predicted the failure of development of CETP inhibitors based upon the lipoprotein abnormalities and clinical manifestations of patients with CETP deficiency. The details why CETP inhibitors failed are summarized in other reviews.

In contrast to CETP inhibitors, an enhancement of RCT may be a promising strategy. A lipid-lowering drug, probucol, has a long history of clinical application with established efficacy and safety profiles. It is a potent antioxidant drug that has been in clinical use during the past few decades for the treatment and prevention of cardiovascular diseases. As mentioned above, probucol is a unique drug because it reduces plasma HDL-C levels, but attenuates xanthomas. Its mechanism of pharmacologic actions at the molecular level has recently been elucidated. HDL-C reduction by probucol is based upon the increase in plasma CETP activity and hepatic SR-BI expression, and may not be a "side effect" but it most likely might reflect an acceleration of RCT. Probucol inhibits the oxidation of LDL and enhances the cholesterol efflux capacity and anti-oxidative function of HDL particles because it transforms HDL particles to cholesterol-poor small ones and it increases the expression of PON-1 on HDL. The molecular bases of the various anti-atherogenic effects of probucol are illustrated in Fig. 3. Probucol could be reconsidered as an option at least in case statins, which are known to be effective for lowering LDL-C levels and CHD risk, are not effective. A marked CHD risk reduction has recently been reported in long-term probucol treatment of patients with heterozygous familial hypercholesterolemia FH as well as those after coronary revascularization. Therefore, there is more than enough reason to believe that this old drug has much more to offer than known so far.

HL deficiency

HL plays a role in the conversion of IDL into LDL by its TG lipase activity. HL also has the function for the remodeling of large, TG-rich HDL particles into smaller ones by hydrolyzing TG of HDL. HL also enhances the hepatic uptake of HDL lipids. Several mutations have been reported in the human HL gene. Human HL deficiency is characterized by increased IDL-cholesterol levels as well as large and TG-rich HDL particles. Some patients with HL deficiency were reported to have premature atherosclerosis. The subjects from the Copenhagen City Heart Study were genotyped for the -216, -480, and -729 SNPs in the HL promoter. Compared with wild-type, HDL-C levels were 4% and 10% higher in heterozygotes and homozygotes. In prospective and case-control studies, mutation homozygotes versus wild-type had odds ratio for CHD of 1.5 (95%CI: 1.0–2.2) and 1.4 (95%CI, 1.1–1.9) when adjusted for age, gender, and HDL-C. Thus, HL promoter SNPs were associated with increased HDL-C, but an increased risk of CHD.

Familial HALP with premature corneal opacity (combined deficiency of CETP and HL activity)

Several patients with a combined reduction in CETP and HL activity were reported to present with corneal arcus and suffer from CHD. The impact of the combined reduction on atherosclerosis appears to be stronger than that of CETP deficiency alone. One of

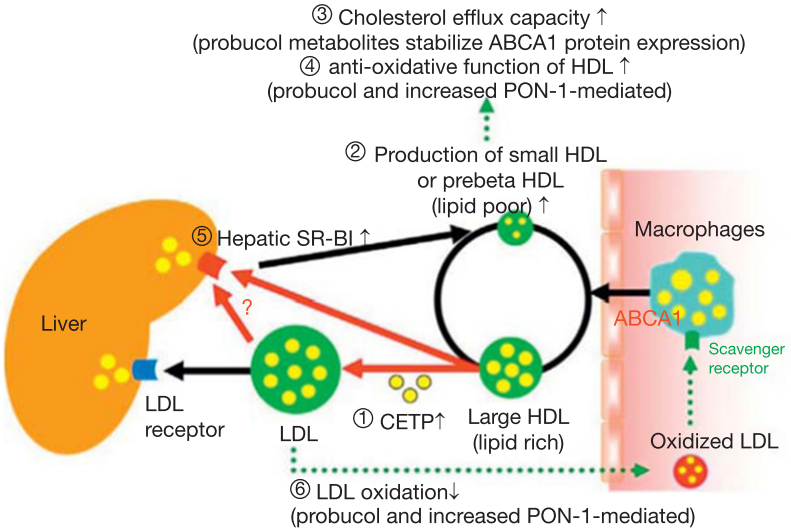

Fig. 3 Molecular mechanisms for anti-atherogenic effects of probucol.

the possible mechanisms is that both CETP and HL play important roles in the remodeling of HDL particles from large to small particles, which are relatively more active for cholesterol efflux. The combined reduction of CETP and HL leads to the marked elevation of HDL-C, with the appearance of very large HDL particles, which are not active for cholesterol efflux.

Familial HALP with genetic abnormalities in SR-BI *(*SCARB1*) gene*

Hepatic SR-BI takes up CE from HDL particles. Adenovirus-mediated hepatic overexpression of *SR-BI (SCARB1)* gene in mice resulted in virtual disappearance of plasma HDL and a substantial increase in biliary cholesterol. In contrast, the deletion of hepatic SR-BI increased plasma HDL-C, but enhanced the development and progression of atherosclerosis in mice. From subjects with elevated HDL-C levels, a family with a missense mutation (P297S) in the *SCARB1* gene was identified. Cholesterol uptake from HDL by primary murine hepatocytes that expressed mutant *SR-BI* was reduced to half of that of hepatocytes expressing wild-type *SR-BI*. The P297S carriers showed increased HDL-C levels and a reduced capacity for cholesterol efflux from macrophages. However, the carotid artery intima-media thickness was similar in carriers and in family noncarriers. Platelets from carriers had increased unesterified cholesterol content and impaired function. In carriers, adrenal steroidogenesis was attenuated. Two point mutations in human *Scarb1* gene, S112F or T175A, were also identified in subjects with high HDL-C levels. More recently, a rare variant in *Scarb1* gene was shown to raise HDL-C and increase the risk of CHD. Targeted sequencing of coding regions of lipid-modifying genes in individuals with extremely high HDL-C levels identified a homozygote for a loss-of-function variant P376L in *Scarb1* gene. The P376L variant impairs post-translational processing of SR-BI and attenuates the selective HDL cholesterol uptake in transfected cells, in hepatocyte-like cells derived from induced pluripotent stem cells from the homozygous subject, and in mice. Subjects heterozygous carriers of the P376L variant showed significantly increased levels of plasma HDL-C and a significantly increased risk of CHD (OR 1.79).

*Familial HALP with genetic abnormalities in EL gene (*LIPG*)*

LIPG encodes EL, a member of triglyceride lipase family. EL mediates HDL catabolism through hydrolyzing the phospholipids of HDL particles, generating smaller HDL particles and enhancing the clearance of HDL in vivo. Targeted sequencing of *LIPG* gene in individuals with extremely high HDL-C showed 17 rare nonsynonymous coding or noncoding variants in the proximal promoter of *LIPG* gene. Similar data were reported which show the association of *LIPG* variants with high HDL-C. Recent a Mendelian randomization study revealed that carriers of the *LIPG* Asn396Ser allele had higher HDL-C levels across the cohorts. However, the 396Ser allele was not associated with a reduced risk of myocardial infarction, suggesting that lifetime elevated HDL-C via *LIPG* loss-of-function does not protect from the risk of myocardial infarction in humans. These data challenge the concept that raising of plasma HDL-C by inhibition of EL to reduce the risk of myocardial infarction.

Familial HALP with increased production of ApoA-I

A family with a marked HALP was reported to have an overproduction of apoA-I. The primary cause(s) is not known. We reported a HALP family with predominant increase in HDL3. The cause of this HALP is speculated to be an increased production of apoA-I. This condition may be protected from atherosclerosis.

Familial HALP with reduced uptake of HDL by lymphocytes

A case of a marked HALP due to a reduced uptake of HDL by lymphocytes was reported. The molecular mechanisms for the reduction of HDL uptake by lymphocytes are unknown.

Other genes implicated for high HDL syndrome

GWAS techniques have identified the causal genes and mechanisms at newly implicated loci associated with HDL-C. Among them, *TTC39B*, *GALNT2*, and *KLF14* will be described as below.

TTC39B

TTC39B denotes tetratricopeptide repeat domain protein 39B. A common variant in the first intron of *TTC49B* was reported to be linked to HDL-C in a large GWAS study. Alleles associated with increased HDL-C were also associated with reduced expression of *TTC39B* in human liver. The product of *TTC39B* gene was shown to be a scaffolding protein, regulating the degradation of the transcription factor LXR through ubiquitination. Chow-fed mice lacking *TTC39B* displayed increased HDL-C levels in association with increased enterocyte *ABCA1* expression and increased LXR protein without change in LXR mRNA. When loaded with a high fat/high cholesterol/bile salt diet, *TTC39B*-deficient mice or mice with hepatocyte-specific *TTC39B* deficiency demonstrate increased hepatic LXR protein and target gene expression, but protection from steatohepatitis and death. Mice fed a Western-type diet and lacking both *LDLR* (LDL receptor) and *TTC39B* showed decreased fatty liver, increased HDL-C, decreased LDL-C, and reduced atherosclerosis. Although this locus was not associated with CHD in GWAS, further studies are necessary to prove *TTC39B* in the development of atherosclerosis and non-alcoholic steatohepatitis.

GALNT2

The *GALNT2* locus was reported to be associated with blood lipid levels in 2008. The *GALNT2* locus was later shown associated with both HDL-C and TG levels. The *GALNT2* encodes an enzyme GalNAc-T2 which exerts the *O*-glycosylation of specific protein

substrates. However, the specific targets and physiological functions of glycosylation by GalNAc-T2 has not been clarified yet. The heterozygous variant of *GALNT2* gene, D314A, was identified in two subjects with HALP. This variant was shown to lead to modest loss of GalNAc-T2 enzyme function and improve postprandial TG clearance in addition to changes in apoC-III glycosylation. However, recent studies indicate contrasting results. Loss-of-function of GalNAc-T2 was associated with lower HDL-C in humans and animal models. PLTP was shown to be one of the targets of GalNAc-T2 glycosylation in humans and deletion of *GALNT2* in humans results in reduction of plasma PLTP activity. The *GALNT2* SNP alleles associated with lower HDL-C were linked to reduced GALNT2 expression human liver. Thus, *GALNT2* is strongly involved in regulation of HDL metabolism, however the possible role of *GALNT2* in atherogenesis remains to be investigated.

KLF14

GWAS studies demonstrated that SNPs about 14 kb upstream of *KLF14* on human chromosome 7 was associated with HDL-C, type 2 diabetes mellitus, additional metabolic traits and CHD. Overexpression of *KLF14* in vivo increased HDL-C and apoA-I levels, whereas deletion of hepatic *KLF14* reduced serum HDL-C and apoA-I levels. *KLF14* mRNA is expressed abundantly in human adipose tissues, however the roles of KLF14 in human liver and adipose tissues and contribution to HDL metabolism remain to be investigated.

Secondary High HDL Syndrome

Chronic heavy alcohol consumption

Chronic heavy alcohol consumption is known to increase plasma HDL-C levels. Some enzymes and transfer proteins, such as CETP and HL, are altered in chronic heavy alcohol drinkers. CETP activity is reduced in these drinkers, but is normalized after cessation of alcohol. The association between alcohol intake and mortality is U-shaped, suggesting that the beneficial effect of alcohol intake is only observed in mild to moderate drinkers.

Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is a primary cholestatic liver disease, but its primary defect is unknown. In the end-stage of PBC, patients have very low HDL-C with the appearance of Lp-X, similar to other obstructive or cholestatic liver diseases. In the early stage of PBC, patients often demonstrate high HDL syndrome. In some cases, plasma HDL-C is markedly increased to the same extent as that in patients with CETP deficiency. In contrast to CETP deficiency, both activities and protein mass of CETP are markedly increased, whereas HL is reduced in patients with PBC.

Inhibitors of CETP in plasma

Defect in cholesteryl ester transport in serum of patients with uremia receiving maintenance hemodialysis was reported, suggesting an increased inhibitor activity against CETP in these patients.

Other factors or disease states accompanied by HALP

Aerobic exercise increases plasma HDL-C levels. Thus, HALP is sometimes observed in subjects who are continuously doing aerobic exercises. Patients with multiple symmetric lipomatosis, chronic obstructive pulmonary disease (COPD) and hypothyroidism are sometimes accompanied by HALP, but its mechanism is unknown except that HL activity is reduced in hypothyroidism.

Drugs

Some drugs, such as insulin, glucocorticoids, estrogen derivatives, fibrates, HMG-CoA reductase inhibitors (statins), nicotinic acids and their derivatives, intestinal cholesterol transporter inhibitor (ezetimibe), PCSK9 inhibitor and cyclosporin were reported to increase plasma HDL-C levels. Prospective studies using a fibrate, gemfibrozil, demonstrated that increases in HDL-C during treatment were correlated with the prevention of cardiac events.

Recent Epidemiological Observations on the Clinical Implications of High HDL Syndrome

Recently, a marked HALP has been shown to be paradoxically associated with high mortality. In the combination of two prospective population-based studies, the Copenhagen City Heart Study and the Copenhagen General Population Study, the association between HDL-C concentrations and all-cause mortality was U-shaped for both men and women, with both extreme high and low concentrations being associated with high all-cause mortality. The concentration of HDL-C associated with the lowest all-cause mortality was 1.9 mmol L^{-1} (73 mg dL^{-1}) in men and 2.4 mmol L^{-1} (93 mg dL^{-1}) in women. When compared with the groups with the lowest risk, the multifactorially adjusted hazard ratios for all-cause mortality were 1.36 for men with HDL-C of 2.5–2.99 mmol L^{-1} (97–115 mg dL^{-1}) and 2.06 for men with HDL-C $\geq$3.0 mmol L^{-1} (116 mg dL^{-1}). For women, corresponding hazard ratios were 1.10 for HDL-C of 3.0–3.49 mmol L^{-1} (116–134 mg dL^{-1}) and 1.68 for HDL-C $\geq$3.5 mmol L^{-1} (135 mg dL^{-1}). Similar U-shaped association between HDL-C and cardiovascular mortality was demonstrated for both men

and women. Thus, men and women in the general population with extreme high HDL-C paradoxically have high all-cause mortality.

Similarly, a pooled analysis of six cohort studies examined the relation of HDL-C level with CHD and total mortality across a broad range of HDL-C, including values in excess of 80 mg dL^{-1}. In men, the association between HDL-C and CHD events was inverse and linear across most HDL-C values; however, there was a plateau effect in the pattern of association at HDL-C values >90 mg dL^{-1}. In women, the association between HDL-C and CHD events was inverse and linear across lower values of HDL-C, however at HDL-C values >75 mg dL^{-1} there were no further reductions in the hazard ratio for CHD. In unadjusted models, there were increased total mortality risks in men with very high HDL-C, however mortality risks observed in participants with very high HDL-C were attenuated after adjustment for traditional risk factors. Thus, further reductions in CHD risk were not observed with HDL-C values higher than 90 mg dL^{-1} in men and 75 mg dL^{-1} in women, respectively.

In Japan, NIPPON DATA90 followed up 7019 individuals from the Japanese general population and examined the association between HDL-C and all-cause mortality or stroke. HDL-C levels were defined as follows: low (HDL-C <1.04 mmol L^{-1}), reference (1.04–1.55 mmol L^{-1}), high (1.56–2.06 mmol L^{-1}), very high (≥ 2.07 mmol L^{-1}). No significant association was observed between HDL-C and all-cause mortality. Serum HDL-C also showed no association with stroke. The risk for CHD among high HDL-C was lower than reference, however very high HDL-C did not show significant association with CHD and other cause-specific mortality.

An observational cohort study using The CANHEART (Cardiovascular Health in Ambulatory Care Research Team) data set examined the association of HDL-C level with cardiovascular and non-cardiovascular mortality by a "big data" approach. Individuals with lower HDL-C levels were independently associated with higher risk of CV, cancer, and other mortality compared with individuals in the reference ranges of HDL-C levels. Individuals with higher HDL levels (>70 mg dL^{-1} in men, >90 mg dL^{-1} in women) had increased hazard of non-cardiovascular mortality. Previous data suggested that elevations in serum HDL-C may increase the risk of spontaneous ICH. Moreover, as described above, genetic variants in *CETP* gene associated with increased HDL-C raise the risk of ICH.

The mechanisms for the increased atherogenicity in a marked HALP subjects with CHD were investigated. HDL phospholipid composition was significantly lower in HALP patients with CHD than in controls. HDL cholesterol efflux capacity was also significantly lower in HALP patients with CHD than in controls. Therefore, people with very high HDL-C, reduced HDL phospholipid content and cholesterol efflux capacity are associated with the enhanced development of CHD.

Conclusion

Various factors are involved in the etiology of low HDL syndrome and high HDL syndrome. A marked HALP caused by genetic CETP deficiency may not be protected from atherosclerotic cardiovascular diseases, therefore strategies to raise plasma HDL-C levels by CETP inhibitors have been challenged without a success. The enhancement of RCT by drugs such as probucol may have a greater potential as an anti-atherosclerotic treatment despite reduction of plasma HDL-C. The pleiotropic functions of HDL and efficiency of HDL-mediated RCT should be tested when we develop an HDL-targeted drug therapy for prevention of atherosclerosis.

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Hypertriglyceridemias and Their Treatment

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Definition and Clinical Consequences of Hypertriglyceridemia

Hypertriglyceridemia (HTG) is a hyperlipemia characterized by increased triglycerides plasma or serum values (TG). In the case, when this increase is accompanied by high levels of cholesterol, we consider mixed dyslipidemia. In the case that the TG increase is associated with low HDL cholesterol plasma levels (HDL-C), we discuss atherogenic dyslipidemia (NCEP Panel, 2002). In this article, we focus on pure HTG.

Normal TG plasma values vary among populations, gender, and age. It is difficult to define cut points for pathological high TG plasma values (NCEP Panel, 2002). We usually accept as a pathological cut point value the 90 percentile for the TG plasma distribution observed in a defined population. For Western populations, different guidelines and recommendations accept a level below 200 mg dL^{-1} as adequate TG plasma values, and TG values below 150 mg dL^{-1} as an ideal recommended level (Jacobson *et al.*, 2015).

HTG is frequently detected in individuals who have had a lipid profile as part of their cardiovascular risk assessment (NCEP Panel, 2002). The prevalence of HTG varies among populations. In the NHANES study (American population), 33% of the subjects presented values >150 mg dL^{-1}, 18% >200 mg dL^{-1}, and 1.7% higher than 500 mg dL^{-1} (Ford *et al.*, 2009). The incidence of HTG is particularly high in men aged 40–50 and is calculated to be around 35% (Carroll *et al.*, 2015). This incidence also varies by race, being highest in Hispanic populations.

Several studies have shown a relation between elevated plasma TG values and atherosclerosis and cardiovascular events (Miller *et al.*, 2011, Sarwar *et al.*, 2007). However, the TG increase is usually associated with other cardiovascular risk factors (abdominal obesity, insulin-resistance, metabolic syndrome, or diabetes) and low levels of HDL-C. In such situations, HTG is associated with qualitative alterations of LDL, remnants of VLDL and chylomicrons, hypercoagulability, blood hyperviscosity, and insulin-resistance, all factors related to endothelial dysfunction, atherosclerosis, and cardiovascular disease (Hargreaves *et al.*, 1992; Fukushima *et al.*, 2001).

High TG plasma values are independently associated with increased risk for cardiovascular disease (CVD) events (Miller *et al.*, 2011). This causal relationship has been supported by several observational studies. Mendelian randomization studies supported this causal effect, with odds ratios for CVD events of about 1.6 (Rosenson *et al.*, 2014). Classical prospective studies in large populations have also found this association, when comparing upper to lower tertiles o quintiles (Sarwar *et al.*, 2007). In addition, HTG is associated with increased mortality in patients with established atherosclerotic coronary heart disease and reduced event-free survival after cardiac bypass surgery (Sprecher *et al.*, 2000).Some authors, assuming this risk, suggest four categories to classify individuals according to fasting TG values (NCEP Panel, 2002), although there are slight differences according to different guidelines in the definition of mild, moderate, and severe HTG. We can accept this classification, which is as follows:

- normal (or ideal): TG $<$ 150 mg dL^{-1};
- borderline high: 150–199 mg dL^{-1};
- high: 200–499 mg dL^{-1};
- very high (or severe): $\geq$500 mg dL^{-1}.

On the other hand, very high TG plasma values ($\geq$500 mg dL^{-1}) have been considered as a risk factor for acute pancreatitis (Pedersen *et al.*, 2016). It is assumed that in a postprandial state, subjects with such fasting values can obtain, after diet intake, values of $>$2000–3000 mg dL^{-1}. Acute pancreatitis is associated with very high TG values caused by elevation of chylomicrons. The exact mechanism of how HTG produces pancreatitis is not known. This disease is a life-treating condition and generates elevated morbidity. The risk of repeated acute pancreatitis is particularly high in individuals with genetic forms of severe hyperchylomicronemia (see below) (Brahm and Hegele, 2013). About 1%–4% of cases of acute pancreatitis are thought to be caused by HTG.

Physiopathology

TG elevations may be caused by an increase in the synthesis of TG-rich particles such as chylomicrons, VLDL or its remnants, or by reduced catabolism of them. The reduced catabolism can be produced by a decrease in the lipolitic action of enzymes such as lipoprotein lipase (LPL), hepatic lipase, or activators of these enzymes (apoCIII), or decreased action of receptors that catabolized these lipoproteins (Brahm and Hegele, 2013).

Causes of Hypertriglyceridemias

The causes of HTG are secondary to other diseases, to the intake of ethanol or drugs (**Table 1**), or primarily genetically based (**Table 2**).

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Table 1 Causes of hypertriglyceridemia

Lifestyle	*Disease*	*Drugs*	*Genes*
Alcohol intake	Diabetes	Atypical anthipycotics	LPL
Sedentarism	Obesity (abdominal obesity)	Bile acids sequestrans	APO C2
High sugar intake	Polycystic ovary syndrome	Corticoids	APO A5
Reduce physical activity	Metabolic syndrome	Ciclosporine	LMF-1
Weight gain	Chronic kidney disease	Immunosuppressive agents	GIPHBP-1
	Renal insufficiency	Oral estrogens	
	Hypothyroidism	Protease inhibitors	
	HIV infection	Beta blockers	
		Interferon	
		Cyclophosphamida	
		Tamoxifeno	
		Rosiglitazone	
		Retinoids	
Menopause, pregnancy, elderly			

Abbreviations: *LPL*, Lipoprotein lipase; *APO*, apolipoprotein; *LMF-1*, Lipoprotein maturation factor 1; *GIPHBP-1*, Glycosylphosphatidylinositol-anchored HDL binding protein 1.

Table 2 Principal characteristics of primary monogenic hypertriglyceridemias

	Family history	*Lipid phenotype*	*Eruptive xantomas*	*Genetic mutations*	*CVD*	*Pancreatitis*
Hyperchylomicronemias	No	↑TC y ↑↑↑TG	Yes/No	LPL, APOC2, APO A5	No	Yes
Familial hypertriglyceridemia	Yes	N TC y ↑TG	No	LPL	Yes	No
Hepatic lipase deficiency	No	↑TC y ↑↑TG	Yes	HL	Yes/No	No

Abbreviations: *TC*: Total cholesterol; *TG*, Triglycerides; *LPL*, Lipoprotein lipase; *APO*, Apolipoprotein; *HL*, Hepatic lipase; *CVD*, cardiovascular disease.

Secondary Hypertriglyceridemias

Secondary HTG is the most frequent cause of increased TG values. It is often secondary to other diseases or exogenous factors capable of altering TG metabolism (Hegele *et al.*, 2014) (**Table 1**). Some causes are very prevalent in our population, such as diabetes (8%–12% in the adult population).

Lifestyle has become the major cause of TG elevation. A sedentary lifestyle with a diet containing high saturated fat and refined carbohydrates is the principal cause of obesity increase, which is associated with HTG. Particularly interesting is the fact that visceral fat accumulation in obese patients is strongly associated with HTG and atherogenic dyslipidemia. Visceral adiposity is accompanied by insulin-resistance that generates increased free fatty acids due to acquired deficiency of LPL action. In this situation, the liver secretion of VLDL increases, and cannot be suppressed by insulin, thus generating high VLDL values and HTG.

About one-third of diabetic patients have TG plasma values $>200\ \mathrm{mg\,dL^{-1}}$, as a result of visceral adiposity and insulin-resistance. In diabetic patients, HTG is associated with qualitative alterations of LDL and low HDL-C values, known as atherogenic dyslipidemia. This atherogenic dyslipidemia is in part responsible for the high cardiovascular risk of type 2 diabetic patients. The quantitative and qualitative lipid alteration found in these patients is associated with insulin-resistance, poor glycemic control, and obesity. Insulin-resistance facilitates a decrease of TG rich lipoprotein catabolism via interaction with LPL and other lipolitic enzymes. In these patients, an increase in VLDL secretion is also found. The elevation in TG plasma levels results in increases in non-HDL-C lipoproteins such as chylomicrons, VLDL, and their remnants.

A secondary cause of HTG is obesity and being overweight, which have a prevalence in the adult population of >30% and 17%, respectively. Abdominal obesity with metabolic syndrome is a very common cause of HTG with low HDL-C values. Other situations that can raise the TG are pregnancy, hypothyroidism, and renal failure.

Lipid abnormalities occur in 40%–60% of patients with chronic kidney disease and are responsible in part of the high CVD risk observed in these patients. HTG occurs in the first stages of renal insufficiency.

The most frequent factor related to HTG is the consumption of alcohol, which must always be dismissed in all cases. Drugs associated with HTG are hormone replacement therapy with estrogens, tamoxifen, glucocorticoids, cyclosporine, and antiretrovirals (**Table 1**).

Genetically Based Hypertriglyceridemia

Primary hypertriglyceridemia includes genetically based HTG due to mutations in the principal genes implicated in TG metabolism (monogenic HTG). There are also polygenic HTGs that include polymorphism or mutations in several genes implicated in TG metabolism and their interaction with exogenous factors or conditions related to HTG (Hegele *et al.*, 2014).

Monogenic HTG

Hyperchylomicronemias

Hyperchylomicronemia courses show very high values of TG, usually >1000 mg dL^{-1} at fasting. Clinically, they can be accompanied by occasional eruptive xanthomas, lipemia retinalis, hepatosplenomegaly, abdominal pain, or acute pancreatitis (Brunzell and Deeb, 2001). Most patients with hyperchylomicronemia have a secondary form, in which other dyslipemia (partial lipoprotein lipase deficiency) is exacerbated by secondary causes of HTG, such as drugs-related causes (**Table 1**) or poor diabetic glycemic control in type 2 diabetic patients.

We differentiated two phenotypes of hyperchylomicronemias: type I, which occurs in pediatric age, and the increase of TG, which is related to an increase of fasting and postprandial chylomicrons. These forms are autosomal recessive and cause mutations of the lipoprotein-lipase (LPL) gene (complete absence of LPL activity, type Ia), apo C-II gene, lipoprotein maturation factor 1 (LMF-1), apolipoprotein 5, and glycosylphosphatidylinositol-anchored HDL binding protein 1 (GPIHBP-1). These proteins are necessary for the adequate lipolitic function of LPL.

These mutations produce an absence of LPL activity. LPL is an enzyme found in cell surface of capillaries that hydrolyzes TG transported by chylomicrons and VLDL into free fatty acids.

"Classical" type Ia hyperchylomicronemia is a rare genetic disorder characterized by a complete deficiency of LPL activity. Mutations in LPL gene or apo CII, its regulation protein, due to homozygous heritability are responsible for this complete deficiency. Patients are detected in pediatric age with clinical signs such as eruptive xanthomas, lipemia retinalis, recurrent acute pancreatitis, and extremely high TG values with an increase of chylomicrons factions. For this condition there is an approved genetic therapy using LPL (S447X) gene variant in an adeno-associated viral vector that is administered intramuscularly (Gaudet *et al.*, 2010).

In type V forms, HTG is due to an increase of chylomicrons and VLDL, appear in patient carriers of LPL gene mutations that causes partial LPL deficiency, and is diagnosed in the context of other dyslipemias (familial combined hyperlipidemia), in which the presence of exogenous factors, such as those previously discussed (**Table 1**), rise very significantly in TG values and generate this lipoprotein phenotype.

Familial hypertriglyceridemia

Familial hypertriglyceridemia is a rare autosomal dominant disease. Affected subjects presented moderate to severe TG with normal total cholesterol values (Brunzell and Deeb, 2001, Hegele *et al.*, 2014). This disease is often associated with resistance to insulin and high blood pressure. Some studies have demonstrated that affected subjects are carriers in heterozygosis of inactivating mutations in the LPL. These subjects have elevations ranging from 20% to 80% of TG, accompanied by low levels of HDL-C.

Hepatic lipase deficiency

This dyslipidemia originated as a result of a deficiency action of hepatic lipase (LH) (Brunzell and Deeb, 2001). The disease has an autosomal recessive transmission pattern. Numerous studies suggest that LH is significantly involved in the metabolism of remnant lipoproteins and HDL. Patients with LH deficiency have an increase of different particles such as VLDL, chylomicrons, and their remnants. Therefore, the plasma lipoprotein pattern is usually characterized by the presence of moderate HTG. An increase in the incidence of premature coronary heart disease (Kobayashi *et al.*, 2015) has been described in patients with a complete deficiency of LH.

Polygenic HTG

These forms are more prevalent than the previously described classical monogenic HTG. Approximately 26% of pure HTG has as its base some mutations or variants that affect different genes implicated in TG metabolism (Hegele *et al.*, 2014). Some authors have demonstrated in large populations using different molecular analysis methods that 32 loci are associated with HTG (Hegele *et al.*, 2014).

In these subjects, fasting TG values ranged from 200 to 800 mg dL^{-1}, and some of them have a high risk of cardiovascular disease.

Diagnosis of Hypertriglyceridemia

Many patients with HTG have no symptoms; the detection will be carried out by a lipid profile in the context of global cardiovascular risk assessment. Sometimes the lipid alteration is discovered after studying the lipid profile in the evaluation of other diseases that are secondary causes of HTG (Brunzell, 2007). For example, in a patient with diabetes, it is mandatory to perform a lipid profile for evaluating cardiovascular risk, and then the HTG may be discovered.

In patients with primary forms, we can observe clinical signs of HTG: eruptive xanthomas, lipemia retinalis, xanthelasmas, and abdominal pain (Brunzell and Deeb, 2001). In the hyperchylomicronemia syndrome, acute pancreatitis can be the presentation form of the HTG.

In all patients with hypertriglyceridemia, we try to identify the cause (**Tables 1** and **2**) and evaluate other cardiovascular risk factors. In patients with HTG, anamnesis is very important to discover alcohol taking, a diet rich in saturated fat, a history of

diabetes and obesity, and taking drugs involved in TG metabolism such as estrogen, cyclosporine, and corticosteroids (Hegele *et al.*, 2014).

Physical examination of patients with HTG is required to measure their weight, height, BMI, waist circumference, and blood pressure. We also can find xanthelasmas and eruptive xanthomas, and explore the presence of goiter (Brunzell, 2007).

In subjects with HTG, it is necessary to study blood glucose, kidney function, thyroid function, and albuminuria to rule out frequent secondary causes. In patients with a history of abdominal pain and severe HTG, we should suspect acute pancreatitis and look for a cause of hyperchylomicronemia.

When a monogenic form of HTG is suspected, it will be necessary to draw up a family tree and study first-degree relatives in forms that involve dominant inheritance patterns. With the diagnosis of primary HTG, we can study the mutations of genes implicated in its etiology, such as the LPL, apo CII, and apolipoprotein 5 (**Tables 1** and **2**).

Treatment of Hypertriglyceridemia

There are few intervention studies that answer the question about which patients with HTG should be treated. On the other hand, it is not clear if the treatment of HTG may modify CV disease risk, prevent CV events, or prevent acute pancreatitis (Dallinga-Thie *et al.*, 2016).

The treatment of HTG is based on nonpharmacological interventions (lifestyle changes) and pharmacological treatment.

Goal

Most guidelines recommended a desirable TG level of <150 mg dL^{-1}, but unless HTG is severe, the primary treatment goal is LDL-C or non-HDL-C for CVD prevention (Chapman *et al.*, 2011). In patients with severe HTG the primary goal is to reduce TG plasma values to <500 mg dL^{-1} in order to prevent acute pancreatitis.

Nonpharmacological Interventions

In general, we recommend that all patients with mild-to-moderate HTG should avoid taking alcohol and trans fatty acids, and reduce their intake of saturated fat ($<7\%$ of total caloric daily intake), fructose, and simple sugars (Dallinga-Thie *et al.*, 2016). Ingestion of polyunsaturated fat (omega 3–6) is accepted in the form of fish and nuts, since these foods decrease plasma TG levels. Guidelines also recommend increasing complex carbohydrates intake. Diet modifications can obtain up to a 50% decrease in plasma TG.

Obese or overweight subjects must follow a low-calorie diet looking initially for weight loss and then for obtaining a normal weight as a medium-long goal. Reducing weight by 5%–10% can decrease TG plasma values by 20%.

In patients with severe hyperchylomicronemia, their diet should be very low in all kinds of fats (20–40 g per day), whether unsaturated or not. In these cases, to improve the tolerability of the diet, medium chain fatty acids can be useful, since these fatty acids do not raise the chylomicrons.

Aerobic exercise practiced on a regular basis obtains declines of up to 30% of plasma TG. Therefore, we recommend that all patients with HTG perform moderate-to-intense aerobic physical activity for at least 30 min, three times per week.

In secondary HTG, it is necessary to prevent or control the responsible factor for HTG—for example, optimizing glycemic control in a diabetic patient or avoiding drugs related to HTG in subjects who take them (Chapman *et al.*, 2011).

Pharmacological Treatment

Indications of pharmacological treatment

Pharmacological treatment is indicated for the prevention of CVD and pancreatitis (Abourbih *et al.*, 2009).

In subjects with TG $>$ 500 mg dL^{-1}, the risk of acute pancreatitis is high, so we shall indicate, in addition to the discussed changes in lifestyle, the taking of drugs. In patients with a personal history of acute pancreatitis and TG $>$ 500 mg dL^{-1}, pharmacological treatment is also indicated (Chapman *et al.*, 2011).

On the other hand, there is no clear evidence that the treatment of HTG improves CVD risk. Some studies found cardiovascular prevention using drugs that specifically decrease total cholesterol and also TG (statins) or the association of statins with fibrates in selected subgroups of patients (type 2 diabetes, metabolic syndrome).

In all patients with high CV disease risk, the primary aim for CVD prevention is to obtain a decrease in LDL-C or in non-HDL-C with statins (Martin *et al.*, 2012). In subjects with very high cardiovascular risk such as diabetics, patients with multiple cardiovascular risk factors, or patients in secondary prevention, the aim of LDL-C is <70 and <100 mg dL^{-1} in non-HDL-C values (Martin *et al.*, 2012).

In some cases, high-dose statins can decrease TG up to 35% (Martin *et al.*, 2012). In some subgroups of patients, intervention studies suggest that they may benefit from combination therapy of statins with fibrates for cardiovascular prevention. This has

Table 3 Pharmacological treatment of hypertriglyceridemias

Drug	*LDL-C%*	*HDL-C%*	*TG%*	*Mechanism*
Fibrate	↓5–20	↑10–15	↓20–50	Enhance catabolism of TG rich lipoproteins via activating LPL Reduce secretion of hepatic VLDL
Omega 3 fatty acids	↓10	↑5–10	↓20–50	Activation of LPL Inhibition of hepatic dyacilglycerol acyltransferase-1 and 2
Nicotinic acid	↓5–25	↑15–35	↓20–40	Inhibition of hepatic dyacilglycerol acyltransferase-2
Statins	↓30–50	↑5–10	↓10–30	Enhance expression of LDL-R Decrease in hepatic VLDL secretion Inhibition of APO B100 hepatic synthesis

Abbreviations: *LDL-C*, Low density lipoprotein cholesterol; *HDL-C*, High density lipoprotein cholesterol; *TG*, Triglycerides; *LPL*, Lipoprotein lipase; *VLDL*, Very low density lipoprotein; *LDL-R*, Low density lipoprotein receptor; *APO*, Apolipoprotein.

been demonstrated in subjects with TG $> 200\ mg\ dL^{-1}$ and low HDL-C (HDL-C $< 40\ mg\ dL^{-1}$ in men and $50\ mg\ dL^{-1}$ in women), especially in type 2 diabetic subjects and subjects with metabolic syndrome (Abourbih *et al.*, 2009).

In the Helsinki Heart Study, a benefit using gemfibrozil was confirmed in a high risk group with TG levels $>200\ mg\ dL^{-1}$ and LDL-C/HDL-C ratio > 5. In the ACCORD Lipid trial in type 2 diabetic patients, fenofibrate showed no benefit when adding it to a statin in CVD prevention; however, it tended to improve outcomes in a subgroup of patients showing TG $> 204\ mg\ dL^{-1}$ and low HDL-C (ACCORD Study Group *et al.*, 2010).

Drugs

Fibrates

Fibrates (gemfibrozil, fenofibrate) reduce TG 30%–50% (**Table 3**), reaching the maximum reduction between 4 and 6 weeks of treatment (Abourbih *et al.*, 2009). These drugs activate LPL, via the PPAR gamma, which activates the lipolysis of TG-rich particles (VLDL and chylomicrons) (Staels *et al.*, 1998).

In general, fibrates are well-tolerated drugs, although they can cause muscular alterations, especially if they are associated with statins. The best option if you opt for a combination therapy will be to associate fenofibrate with pravastatin, fluvastatin, or pitavastatin (Abourbih *et al.*, 2009).

The risks in terms of side effects of statins increase steeply when used in combination with gemfibrozil. Gemfibrozil interferes with the renal elimination of statins (glucoronization) and reduces the statin catabolism via CYP3A4.

Nicotinic acid

Nicotinic acid in doses of 1.5–2 g per day can reduce total cholesterol and TG (15%–25%). We rarely use this drug in monotherapy to reduce TG levels. Fibrates are more potent and have a better side effect profile. Nicotinic acid induces flush and worsened glycemic control in type 2 diabetic patients (Grundy *et al.*, 2002).

Fish oil

Eicosapentaenoic acid, docosahexaenoic acid, and omega 3–6-rich fish oils can reduce TG plasma values up to 50%. Omega 3–6 can be used in severe forms of HTG associated with fibrates or in patients with moderate HTG and fibrates intolerance (Harris *et al.*, 1997).

Other nonconventional therapies

In cases of severe HTG, severe hyperchylomicronemias with acute pancreatitis or abdominal pain, we use plasma exchange/plasmapheresis if plasma TG values are $>1000\ mg\ dL^{-1}$ and refractory to fibrates or association therapy with fish oil supplements (Chapman *et al.*, 2011).

Genetic therapy with an adenovirus vector modified LPL variant has been approved in adult patients with LPL deficiency and repeated pancreatitis (Gaudet *et al.*, 2010). Emerging therapies are APOC3 messenger RNA inhibitors and ANGLPLT3 inhibitors.

Conclusions

In the general population, hypertriglyceridemia is a very common hyperlipemia and is secondary to very prevalent causes such as diabetes, metabolic syndrome, and obesity.

These lipid alterations, according to the presented phenotype, may be associated with increased risk of cardiovascular events (phenotypes IIb, III, and IV with low HDL-C) or acute pancreatitis (phenotypes I and V).

The diagnosis of the hypertriglyceridemia must focus on the search for the cause.

The treatment of hypertriglyceridemia is based on lifestyle changes (low fat diet, avoiding ingestion of alcohol, and performing regular aerobic physical activity) and pharmacological treatment, usually with fibrates in selected patients.

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Relevant Website

https://www.eas-society.org—European Atherosclerosis Society.

Genetic Basis of Obesity

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Glossary

Central dogma of molecular biology The central dogma of molecular biology explains the flow of genetic information, from DNA to RNA, to make a functional product, a protein. It was first proposed in 1958 by F. Crick. However, many exceptions to this dogma are now known.

Copy number variations (CNV) Refers to the duplication or deletion of stretches of a chromosomal region. These can be as large as megabases or smaller than 1000 base pairs.

Genome-wide association study (GWAs) A GWAs is a non-targeted approach that involves rapidly scanning markers across the whole genome, of many people to find genetic variants associated with a particular disease.

Gene–environment interaction Refers to a different effect of a genotype on disease risk in persons with different environmental exposures.

Linkage disequilibrium Describes the association of two alleles more frequently than would occur by chance.

Next generation sequencing (NGS) Is the catch-all term used to describe a number of different modern sequencing technologies that allow us to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing.

Omics integration Is the integration of omics data obtained for different biological molecules in order to understand their interrelation and the functioning of larger systems.

Single nucleotide polymorphism (SNP) Is a variation at a single position in a DNA sequence among individuals.

Introduction

Knowledge on the genetic basis of obesity has undergone significant development in recent years as a result of optimization, greater speed and reduction in the costs of omics technologies. However, despite this development, many question marks still remain on which genes are the most important in increasing obesity risk and what the contribution of environmental factors is in increasing or decreasing genetic susceptibility. Research into the environmental factors that may promote or neutralize genetic susceptibility to obesity is also increasing in complexity, given that every day more potential contributors are revealed. Hence, we have moved on from investigating the main classic environmental factors (the environmental factor being understood as all that which is not genetic), mainly focusing on diet physical activity, to now consider a wider range of environmental factors, outstanding among which are socio-economic status, educational level, drug consumption, stress, tobacco, sleep habits, meal times or microbiota, among others. Obesity, therefore, even though it may have a strong genetic basis in some individuals, is, for the majority of the population, the result of the interaction between genetic and environmental factors, and so greater genetic susceptibility to obesity can be modulated by environmental factors and vice versa. Despite several initial studies on twins providing percentages of environmental and genetic contributions to obesity (e.g., 40% genetic and 60% environmental), it must be said that these estimates in percentages vary considerably and change a great deal depending on the population analyzed and the methodology employed. For that reason, it is now accepted that, apart from in very few cases of monogenic obesity in which a very strong genetic component exists alongside strong heredity, under most circumstances the obesity phenotype is the result of the interaction between the genetic and the environmental contribution in a balance that appears to vary over time. This introduces a dynamic factor to the genetics of obesity which is not fully understood yet and is still being investigated. It is known that several genetic variants have a greater influence on obesity phenotypes in the early stages of life, whereas others seem to have a greater influence in later life. Nevertheless, more longitudinal studies are required that include individuals from birth to old age in order study the variability of the genetic contribution more accurately from a more dynamic point of view, as well as to study the main interactions with environmental factors during different stages of life. Apart from age, gender may also be another of the factors that have an additional influence on contributing to genetic susceptibility. The additional modulations of gene–environment interactions depending on gender have not been widely studied, so a greater number of studies are required. Recently there has been more interest shown in investigating the genetic and environmental contribution to obesity from a gender perspective, which will provide important data in the not too distant future. Likewise, the possible differences between ethnic groups for some of the genetic variants related with obesity and their environmental modulation need to be researched in greater detail. It is clear from all this that, quite apart from the great genetic heterogeneity that exists in obesity, there are also other factors such as age, gender and ethnicity that together with multiple environmental factors may be capable of modulating genetic susceptibility, so giving rise to a wide range of phenotypes and interactions related with obesity, and that these are different in each individual. Understanding these individual characteristics in detail is essential in the new framework of so-called Precision

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Medicine. In this article we shall review the most recent knowledge on the genetic bases of obesity, also taking into account that these genetic variants cannot be studied in isolation, but have to be integrated into the study of environmental factors.

Genetic Variants Associated With Obesity

The human genome has approximately 6 billion base pairs (adenine, thymine, guanine and cytosine) and it is organized into 23 pairs of chromosomes. It is estimated that there are about 20,000 genes in the human genome, many fewer than those originally planned, due to the fact that the same gene can give rise to several proteins, as we will detail later. Genes constitute the transcriptionally active part of chromosomes. In the structure of a gene we basically distinguish introns and exons. Introns are non-coding sequences, whereas exons are coding. In the DNA sequence, variations can occur that can be of different types (insertions, deletions, duplications, etc.). The best known are the changes of a single nucleotide, so-called SNPs (single nucleotide polymorphisms). The human genome counts >10 million of SNPs. These SNPs are distributed throughout the genome and their effects depend on the biological role (e.g., exon or regulatory) and state (e.g., silent or active) of the genomic regions where they occur. Interestingly, some of the SNPs are not independent and the linkage disequilibrium (LD) parameters are relevant indicators in the genetic studies. LD, defined as the nonrandom association of alleles at different loci is commonly used to indicate that two SNPs are associated. Thus, sometimes, in a study the researchers show statistically significant associations between one SNP and the disease phenotype, however the polymorphism tested is not directly casual but is in LD with an adjacent polymorphism that is. The non-random association of alleles at more than two loci constitutes an LD block (haplotype) and can predict the allele of adjacent (not genotyped) polymorphism. A commonly used measure of association between alleles, r^2, may be used to represent LD. Large values of r^2 indicate stronger association between alleles. A threshold based on r^2 ($r^2 \geq 0.8$) can be used to determine whether two SNPs are associated. A practical advantage of the LD in epidemiological studies is that because of LD in many regions of a gene, every SNP in a genome does not need to be genotyped. Instead, LD-tagging SNPs can be selected that cover the major LD blocks. Likewise non-determined genotypes can be imputed so increasing the genotyping density. Aside from the SNPs, there are other types common polymorphisms that affect more than a pair of bases. These polymorphisms may be insertions of several bases, deletions of several bases or also other polymorphisms known as "copy number variations" (CNV). The CNVs are DNA segments of one kilobase or larger that is present at a variable copy number in comparison with a reference genome. The CNV have been associated with different diseases, among them obesity. Thus, Kutalik and co-workers published in 2017 a large-scale CNV association meta-analysis on anthropometric traits in up to 191,161 adults from 26 cohorts. The study revealed five CNV associations at 1q21.1, 3q29, 7q11.23, 11p14.2, and 18q21.32 with anthropometric traits. In addition the authors confirmed findings previously shown at 16p11.2 and 22q11.21, for at least on obesity-phenotype.

Although there are several types of obesity that present a clear genetic influence through variations in a single gene or a small set of genes, most cases of obesity seem to have a multi-genic influence with variations in multiple genes and whose effects can be additive. Hence two types of obesity have been established the so-called monogenic type that follows the former pattern and the polygenic type in which, there would also be a modulation by environmental factors. Observed in monogenic obesity is a high level of penetrance and at times a SNP in the DNA may give rise to a functional variation whose effects are enough for obesity to develop. There are several well-documented types of SNPs related with monogenic obesity, such as those found in the leptin gene (LEP), the leptin receptor (RLEP), in the melanocortin 4 receptor (MC4R), etc. These SNPs, characterized by low prevalence are associated with very early signs of monogenic obesity and require early and highly personalized treatment. On other occasions, instead of a SNP being involved, there are alterations of larger pieces of the DNA, as, for example, the microdeletion in the segment 15q11–q13 in Prader–Willi syndrome, which affects one in every 25,000 births and leads to well characterized monogenic obesity. There are other well-characterized types of monogenic obesity that have been presented in detail in other articles. However, there are also other types of monogenic obesity that either because they are less frequent or are clinically more heterogeneous, are more difficult to characterize. In these cases, the possibility of undertaking direct DNA sequencing of the patients at low cost and quickly is very useful and in the near future we will be able to gather more knowledge on new types of monogenic obesity.

Nonetheless, despite the advances in technology for analyzing the genome, most cases of obesity respond to a polygenic pattern that is more difficult to investigate. Initially, the first studies on the genetics of obesity focused on the analysis of so-called candidate genes, that is, those that either physiologically or biochemically were known to codify for known proteins that were well-characterized and implicated in the pathways related with obesity. However, the emergence of the genome-wide association studies (GWAS) has led to further advances in gene identification. Interestingly, there is some partially overlapping continuum between monogenic and polygenic forms of obesity with several genes involved in the two conditions. Some loci that were found to be associated with polygenic obesity include a gene that is mutated in a monogenic form of obesity. The MC4R gene is a well-studied example. Several rare loss-of-function coding mutations in the MC4R have been associated with monogenic obesity. In addition, common polymorphisms at the MC4R gene (e.g., SNP rs17782313 near MC4R) have been associated with an increased risk of common obesity.

Obesity Phenotypes

When investigating genetic influence in obesity risk, both for the candidate gene or for the GWAs approaches, the definition of obesity is very important. Most studies have analyzed the obesity phenotype following the World Health Organization (WHO)

definition, that is, a body mass index (BMI) $\geq$ 30 kg/m^2. However, other studies have focused on abdominal obesity, based on the measurement of waist circumference. Also, the use of BMI as continuous variable without categorizing it as presence or absence of obesity, has been frequently used as phenotype. Less frequent have been studies that use the more complex measurements of adiposity, fat distribution, etc. Although various genetic variants are associated at the same time with a number of these phenotypes, others may be more specific for the phenotype analyzed. Additionally, the study design is important as it is known that genetic variants associated with prevalent obesity may be distinct from those associated with incident obesity and it seems there may be still ore difference in the variants associated with greater or lower weight loss in obese individuals following a lifestyle intervention.

Candidate Genes Associated With Obesity

Although the candidate genes approach was employed at the beginning of research into the genetic bases of obesity, this was later replaced by other strategies employing the screening of the whole genome. However, the candidate gene approach is now beginning to resurface as more data on new genes and the metabolic pathways related to obesity become known. Thus several classifications have been used to define the candidate genes related to obesity. Among them, we can consider a big group those genes involved in: (A) genes encoding factors regulating food intake; (B) genes encoding factors implicated in energy expenditure and (C) genes encoding factors implicated in adipogenesis. Greater than 300 polymorphisms in these candidate genes have been associated with several obesity phenotypes. Some of them have been consistently replicated in more than four studies.

Among the genes encoding factors regulating food/energy intake (A), although there are still many unidentified features concerning energy homoeostasis and energy balance, it is accepted that hypothalamic and brain stem centers are involved in the regulation of these processes. Thus, variations in the LEP and LEPR genes were extensively investigated. It is well known that monogenic human leptin deficiency has been identified in children showing severe early-onset obesity with intense hyperphagia and undetectable levels of serum leptin due functional mutations resulting in a premature codon stop. In the general population, common polymorphism in the LEP or RLEP gene have been associated with a small or moderated increased risk of obesity or higher BMI. Similarly, mutations in proopiomelanocortin (POMC) gene are associated with hyperphagia and early-onset obesity due to the deficient activation of the MC4R gene. Common SNPs in the POMC gene have also been associated with higher BMI or obesity risk in subjects from the general population. Following the same pattern, mutations in the proprotein convertase 1 (PC1) gene (associated with hypocortisolaemia); neuropeptide Y (NPY) gene or the ghrelin receptor gene have been reported to be associated both with early-forms of obesity as well as with a relatively small increased risk of common obesity phenotypes. The list of candidate genes encoding factors regulating food intake is increasing and even novel genes recently discovered by GWAs are being included once the potential mechanisms have been investigated. Also in this group are included the genes related to taste perception and food preferences. Taste perception has been suggested as a key component of food preferences and choice. Five taste modalities (bitter, sweet, umami, salty, and sour) are generally accepted as basic tastes. In recent years, a sixth taste-that of fat taste-has also been proposed. Taste perception has a strong genetic component, this being greater for the bitter taste. Functional variants in the TAS2R38 gene have been strongly associated to bitterness and the common SNPs in the TAS2R16 gene, to astringency. Less strong associations but still statistically significant have been reported between the TAS1R2 and TAS1R3 genes and sweet taste. Similarly, variation in umami taste depends on TAS1R1 and TAS1R3 polymorphism. Genes determining salty and sour tastes are less investigated. However, the fat taste seems consistently associated with SNPs in the CD36 gene. Overall several studies have associated SNPs in the taste genes with obesity phenotypes using a Mendelian randomization approach. Some of them have found statistically significant associations, however there is a great heterogeneity and more research has to be done.

Regarding variations in genes encoding factors implicated in energy expenditure (B), several candidate genes have been consistently associates with obesity phenotypes taking into account that adaptive thermogenesis in related to the mobilization of lipids form fat tissues. Polymorphisms in the B-adrenoceptor genes (ADRB1, ADRB2, ADRB3) have been massively studied, and dozens of statistically significant associations among ADRBs SNPs and obesity phenotypes, reported. Likewise, uncoupling proteins (UCPs) gene polymorphisms, mainly in the UCP1 and UCP2, mediating mitochondrial proton leak releasing energy stores as heat (energy metabolism efficiency) have been associated with obesity phenotypes in several studies. Regarding candidate genes encoding factors implicated in adipogenesis (C) this is a dynamic area and the list of these candidate genes is increasing. There are several transcription factors involved, the PPARs, mainly the PPARG2 gene having a key role in determining obesity phenotypes and related diseases.

Recently, genes related with circadian rhythms (CLOCK, BMAL1, PER1, PER2, RORA, etc.) have been other emerging candidate genes associated with obesity being involved in several of the main functional groups previously described.

Genome-Wide Approach

Unlike the earlier approach in which specific variants in certain candidate genes are investigated with a specific hypothesis, in the genome-wide approach there is no specific hypothesis but an analysis of polymorphisms in all the chromosomes to see which of them are more significantly associated with obesity or related phenotypes. In order to carry out genotyping, high-density arrays are used. In recent years technology has allowed us to determine a greater number of SNPs at an increasingly faster and cheaper rate.

While the first arrays to be called complete genome arrays provided an analysis of 10.000 SNPs distributed in all the chromosomes, later this density increased up to the point to now when we can quite easily determine 2 million or more SNPs for each person. Basically what one does in these studies is genotyping using high-density arrays a group of cases of obesity and compare the frequency with that which each SNPs presents in a non-obese control group. These studies are known as GWAs, as previously mentioned. Sample sizes have to be large in order to achieve a sufficient statistical power and often meta-analyses from various GWAs studies are used to increase statistical power. In statistical analyses, besides cases and controls, anthropometric measurements can be analyzed as continuous variables. Manhattan Plots are often used to show the results. The higher a SNP ($-\log10$ of the association *P*-value), the more associated it is the corresponding SNP with the obesity phenotype (its *P* value of association is smaller). To consider an association as statistically significant, the nominal value of statistical significance ($P < 0.05$) is not used but rather that value is corrected by the number of comparisons made in order to minimize false positives. The commonly accepted value as the threshold for considering an association as being statistically significant on the GWAs level is $P < 5 \times 10^{-8}$. To date hundreds of GWAs and meta-analysis of GWAs (by several International Consortia) have been undertaken with different obesity phenotypes, and dozens of genes associated with obesity have been identified which were not revealed through the candidate gene approach. Although initial GWAs reported promising associations between common variants in the INGIG2, GAD2 and ENPP1 genes and BMI, the results have not been widely replicated. Hence, the first gene consistently associated with common obesity was identified by Frayling and co-workers in 2007 by a GWAs undertaken in the U.K. using a small sample size and a relatively low density array (compared to present metrics), which allowed us to analyze approximately 500.000 SNPs. The SNP that was most significantly associated with common obesity was the famous rs9939609, situated in intron 1 of the fat mass and obesity (FTO) gene. Although this SNP was not apparently functional, given that it is in an intron, on replicating the association in another 13 cohorts (with 38,759 participants), in the meta-analysis estimations, the risk allele was significantly associated with a 1.67-fold increased odds of obesity when compared with those not inheriting a risk allele. Later GWAs have found new genetic variants associated with obesity phenotypes (some of the main genes initially identified were: TMEM18, MC4R, GNPDA2, BDNF, NEGR1, SH2B1, ETV5, MTCH2, KCTD15, SEC16B, TFAP2B, FAIM2, NRXN3, RBJ, GPRC5B, MAP2K5, QPCTL, TNNI3K, SLC39A8, FLJ35779, LRRN6C, TMEM160, FANCL, CADM2, RKD1, LRP1B, PTBP2, MTIF3, RPL27A and NUDT3, among others). This number increased as the sample size increases in new meta-analyses that now include hundreds of thousands individuals. Moreover, these meta-analyses of GWAs have been extending from the initial ones carried out mainly on Caucasian populations to other less well studied populations, such as Asian, Afro-American, etc. so that we now have more specific information. A few GWAs have also analyzed possible gene-sex and gene-age interactions at the genome-wide level and have detected some heterogeneous effects that require deeper study of their mechanisms. Thus, Winkler and co-workers, in 2015 carried out a meta-analysis of 114 studies (up to 320,485 individuals) with GWAs data in determining BMI in waist-to-hip ratio (adjusted for BMI, WHRadjBMI). Four strata (men $\leq$50years, men $>$50years, women $\leq$50years, women $>$50years) were considered. For BMI they identified 15 loci (11 previously established for main effects, 4 novel) that showed age-specific effects. For WHRadjBMI, they identified 44 loci (17 novel) with sex-specific effects (28 showing large effects in women).

Beyond GWAs: Genetic Risk Scores, Next Generation Sequencing and More

GWAs allow us to discover the main SNPs associated with the phenotype of interest in a separate way. To discover the joint contribution of various SNPs to obesity risk or related phenotypes, so called GRS (genetic risk scores) are used. **Fig. 1** presents the calculation of the GRS in their two main modalities: (a) unweighted and (b) weighted. In the unweighted, the score calculation is done by adding up the number of risk alleles that the individual has, whereas in weighted GRS, one also has to take into account the effect (magnitude of the association with the obesity phenotype: regression coefficient, etc.) of each genetic variant and that not all genetic variants have the same association with obesity traits, some being more relevant than others. Multiple studies have analyzed and quantified the influence of various GRS with obesity in different populations. Among them, Song and co-workers recently focused on a GRS including 96 SNPs associated with adult BMI. The authors analyzed the trajectories of body fatness from age 5 years up to 65 for women (Nurses' Health Study) and men (Health Professionals). They concluded that individuals with higher GRS at baseline were more likely to maintain a heavy BMI and gain weight throughout life. This GRS has been used in other studies and the SNP included for Caucasian are as follows: AGBL4(rs657452), ATP2A1(rs3888190), BCDIN3D(rs7138803), BDNF(rs11030104), C18orf8(rs1808579), C6orf106(rs205262), C9orf93(rs4740619), CADM1(rs12286929), CADM2 (rs13078960), CLIP1(rs11057405), DMXL2(rs3736485), EHBP1(rs11688816), ELAVL4(rs11583200), EPB41L4B(rs6477694), ERBB4(rs7599312), ETV5(rs1516725), FHIT(rs2365389), FLJ30838(rs1016287), FOXO3(rs9400239), FPGT-TNNI3K (rs12566985), FTO(rs1558902), FUBP1(rs12401738), GBE1(rs3849570), GNAT2(rs17024393), GNPDA2(rs10938397), GPRC5B(rs12446632), GRID1(rs7899106), GRP(rs7243357), HHIP(rs11727676), HIF1AN (rs17094222), HIP1(rs1167827), HNF4G(rs17405819), HSD17B12(rs2176598), KAT8(rs9925964), KCNK3(rs11126666), KCTD15(rs29941), LINGO2 (rs10968576), LMX1B(rs10733682), LRP1B(rs2121279), MAP2K5(rs16951275), MC4R(rs6567160), MTCH2(rs3817334), MTIF3(rs12016871), NAV1(rs2820292), NEGR1(rs3101336), NLRC3(rs758747), NRXN3(rs7141420), NT5C2(rs11191560), OLFM4(rs12429545), PARK2(rs13191362), PGPEP1(rs17724992), PMS2L11(rs2245368), POC5(rs2112347), PRKD1 (rs11847697), PRKD1(rs12885454), PTBP2(rs11165643), QPCTL(rs2287019), RABEP1(rs1000940), RALYL(rs2033732), RARB (rs6804842), RASA2(rs16851483), RPTOR(rs12940622), SBK1(rs2650492), SCARB2(rs17001654), SEC16B(rs543874), SLC39A8

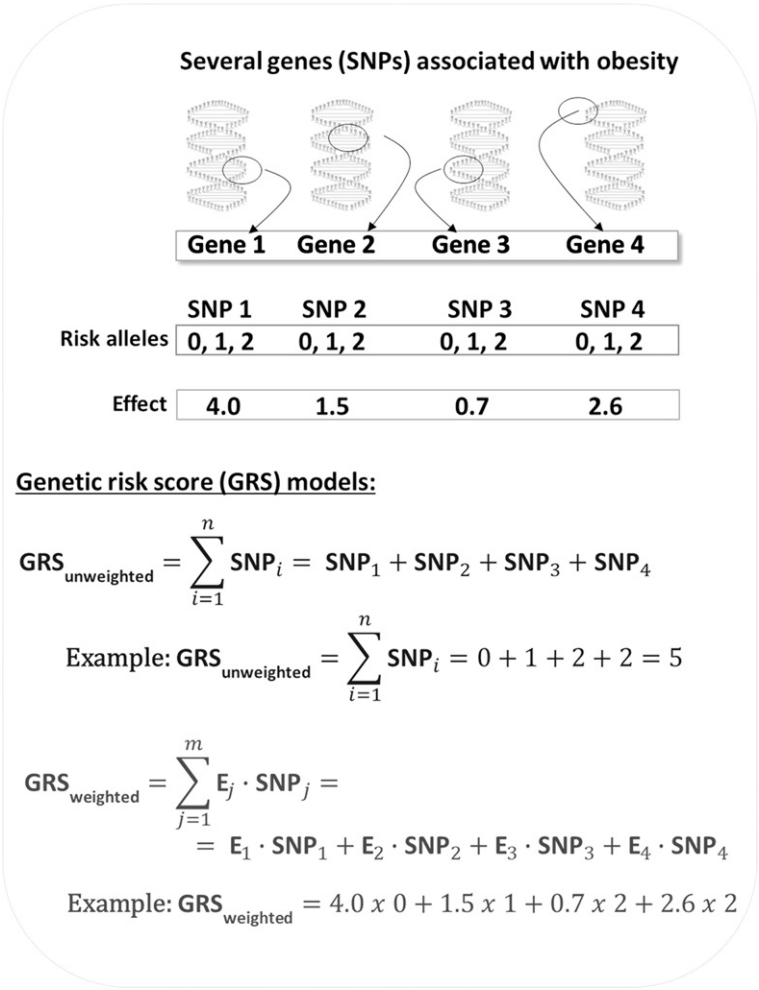

Fig. 1 Genetic risk scores.

(rs13107325), STXBP6(rs10132280), TCF7L2(rs7903146), TDRG1(rs2033529), TFAP2B(rs2207139), TLR4(rs1928295), TMEM18(rs13021737), TOMM40(rs2075650), TRIM66(rs4256980), UBE2E3(rs1528435), ZC3H4(rs3810291).

Currently, the SNPs selected to be included in a GRS are mainly based on the *P*-value obtained in the GWAs (top-ranked). However, this is a limitation. Other analytical strategies such as pathways and networks will be ideally employed to integrate data from several omics. Briefly, in pathway analysis, gene sets corresponding to biological pathways are tested for significant relationships with a phenotype. Networks can also collect genes and other biological elements for quantitative and visual assessment of relationships. These approaches can improve the GRS construction and validation and more work in needed. However, a limitation of the pathway-based studies is the absence of guidelines, leading lack of optimal methods, high variability in results, and barriers to further application. At the same time, we also have also enjoyed a great technological improvement in direct sequencing, now called NGS (next generation sequencing), as instead of being based on the rudimentary techniques applied in the Human Genome Project (traditional Sanger method of sequencing capillary electrophoresis, considered as first generation) NGS technologies provide a greater yield of data at a lower cost. At present, the sequencing of exomes is already widespread in genetic studies of obesity and various individual and meta-analysis studies have been undertaken to identify various low-frequency genetic variants associated with obesity. Moreover, with the emergence of NGS, this is a covenant moment for drawing targets on gene enrichment and pathways analysis for future development in the obesity field.

Genetic Modulation

Despite the great strides taken in sequencing the genome, pathways analysis, etc., knowledge of gene variants is insufficient to predict the risk of obesity, as here are other more dynamic regulatory elements known as the epigenome that are in turn capable of regulating the expression of DNA sequences. Today, therefore, it is essential to jointly study the genome and epigenome for a better understanding of the molecular bases in cardiovascular risk phenotypes. The existence of these regulations helps us to understand

why the fundamental dogma of biology (Fig. 2) is not fulfilled. Panel A shows the classic process in which a DNA is transcribed to a RNA and the latter results in a protein, while panel B shows the now recognized more complex situation through which a single DNA may give rise to different proteins due to the influence of differing regulatory processes. Epigenomics is the study of the key functional elements that regulate the genetic expression of a cell. Unlike the genome that is the same in all somatic cells, the epigenome is specific for each type of cell, so adding complexity to the study and making the origin of the sample that has been taken for analysis more important. There are many types of epigenetic modifications. The most studied ones are methylations and regulations by non-codifying RNAs, among which are microRNAs, but also other types of RNAs. Modifications of histones (acetylations, phosphorylations, glycations, etc.), another type of epigenetic regulation, have a still higher level of complexity and have been little studied in epidemiological studies of obesity in humans. The huge interest in deepening our knowledge of epigenetic regulators lies in the fact that they are dynamic. Unlike SNPs in DNA, epigenetic markers can be modified and the knowledge of the factors that intervene in that modification may be crucial for prevention and/or treatment of obesity. It is at this point where environmental factors seem to be decisive by exercising epigenetic influences and, therefore, modulating genetic susceptibilities. Although not well-known, many of the effects of diet, physical activity, drug consumption, sleep, stress, etc. on modulating genetic expression appear to act through epigenetic mechanisms and so it is necessary to study both genetic and environmental factors jointly by employing an integrated omic approach.

Although their mechanisms are unknown, for more than a decade various gene–environment interactions have been described in the main genes associated with obesity. Outstanding, therefore, are the gene-diet interactions with the FTO gene (and other obesity genes in form of GRS) with total fat intake, saturated fat, proteins, etc. that can modulate the genetic effect. All these interactions have been widely reported in the nutrigenetic filed. Also the gene–environment interaction of the FTO gene with physical activity is also very consistent and relevant. According to this interaction, subjects with the FTO-risk allele do not present the corresponding obesity phenotype.

Summary and Conclusions

Developments in technology, with the possibility of increasing the sample sizes in association studies of the complete genome as well as the number of polymorphisms analyzed, will allow us to identify new genetic variants associated with the different phenotypes of obesity. These genetic variants may be incorporated into already existing GRS and contribute to increasing the

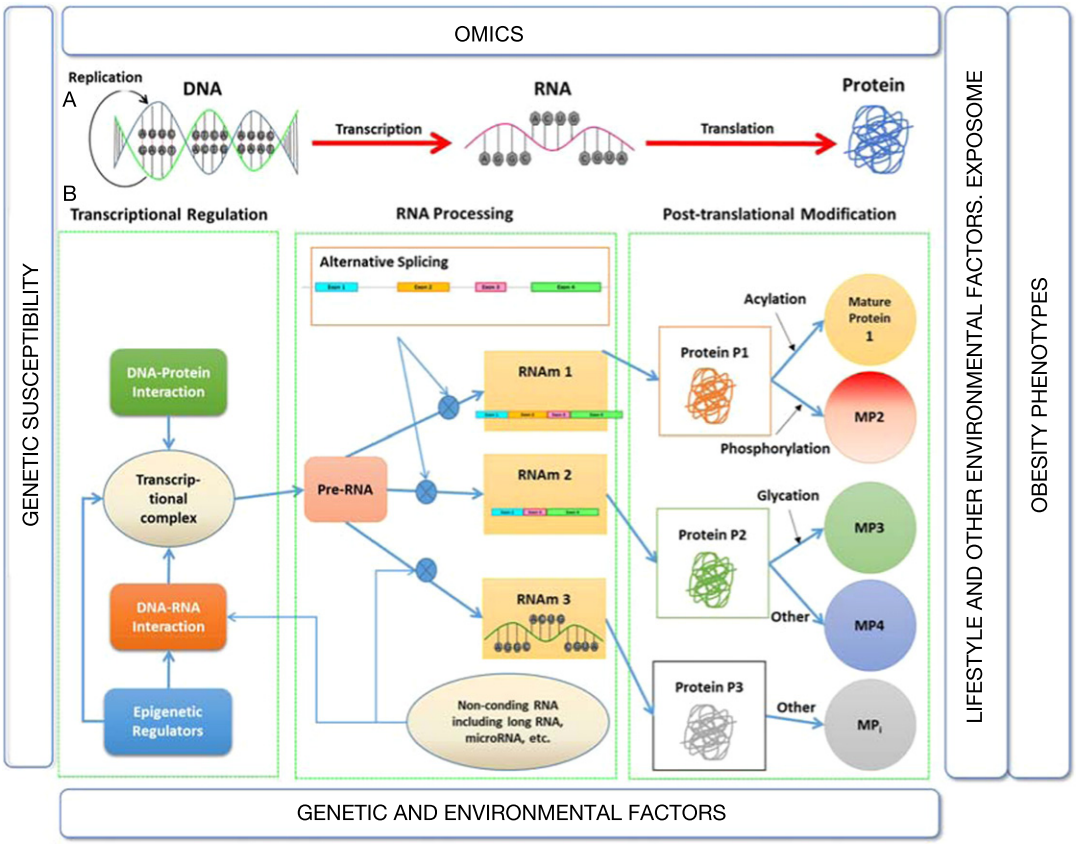

Fig. 2 The central dogma of biology and the current approach.

percentage of explained variability. Nevertheless, this, we know, only constitutes a partial approach and new strategies are required, as, in common obesity the percentage of explained variability continues to be very low. Among these new strategies, besides having to take into account interactions with environmental factors, it is also essential to go deeper into the analysis of pathways and undertake more specific and directed statistical analyses, whilst at the same time bearing in mind other additional variables such as gender, age, health-disease phenotypes and the dynamic approach over time. In addition the integration of omics, also considering epigenetic factors in genetic scores, may be of use in better understanding the genetic bases of obesity.

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Gut Microbiota; Its Importance in Obesity

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Introduction

The term microbiota, gut bacteria, refers to the community of microorganisms living gathered in a particular ecological niche. This ecosystem includes native species that permanently colonize the gastrointestinal tract and a variable group of microorganisms that transit temporarily through the digestive tube. Gut microbiota is heterogeneous. It is composed predominantly of bacteria, with a minority of viruses, fungi and eukaryotic cells. The number of microbial cells in the lumen is 10 times greater than the number of eukaryotic cells in the entire organism. They represent about 1.5 kg of total body weight. The gut microbiome, set of microbial genes, is 100 times larger than the human genome. The acquisition of gut microbiota at childbirth from the mother's microbiota can be considered as the inheritance of a parallel genome (Lozupone *et al.*, 2012; Eckburg *et al.*, 2005).

The gut microbiota plays an important role both locally and systemically. There is a symbiotic relationship between microbiota and its host. Under normal conditions, gut microbiota contributes to the anatomical and physiological gut structure. It improves the absorption surface, promotes the renewal of villi cells, increases the intraluminal content and accelerates intestinal transit. In addition, these microorganisms constitute an enormous enzymatic potential in the gut with a wide variety of metabolic functions. They participate in digestion and obtaining energy through the hydrolysis of diet components (carbohydrates, proteins, and lipids), vitamin synthesis (K, B12, biotin, folic acid, and pantothenic acid), and favor the absorption of minerals (calcium, phosphorus, magnesium, and iron) (Bik, 2009). The gut microbiota is also involved in immunomodulation. It interacts with the immune system and favors immune cell maturation and toxin and carcinogen destruction. It also prevents our gut from being colonized by pathogenic bacteria. Thus, the gut can be considered a metabolically adaptable, flexible and quickly renewable virtual organ that plays an important role in obtaining energy and nutrients from the diet (Chow *et al.*, 2010).

Composition, Estructure and Major Characteristics of Human Gut Microbiota

Microbes colonize all surfaces of the human body exposed to the environment. Most of them reside in the intestinal tract, although they also live in the mouth, vagina and skin. There is more similarity between the oral bacterial communities of different individuals than between the bacterial communities of the skin and mouth of a single individual (Costello *et al.*, 2009). However, there is also considerable inter-individual variability (Costello *et al.*, 2009; Robinson *et al.*, 2010). Gut microbiota varies along different gastrointestinal portions. These microorganisms increase in quantity and complexity the gastrointestinal tract. See **Table 1**.

The greatest number of bacteria in the human gastrointestinal tract resides in the large intestine. There are several factors that facilitate bacterial development: the elevation of pH close to neutrality, a decrease in bile salt concentration and remnants of pancreatic secretion. Moreover, transit time is slow in the colon. It gives microorganisms the opportunity to proliferate by fermenting the available substrates derived from the diet or endogenous secretions (Guamer, 2007).

The bacterial component of the microbiota has been deeply studied over the past years, with large-scale projects such as the Human Microbiome Project (Peterson *et al.*, 2009; Turnbaugh *et al.*, 2007) and MetaHIT (Qin *et al.*, 2010). During the last decade, a lot of information on gut ecosystem diversity has been obtained with the introduction of 16S ribosomal RNA and whole genome (WGS) sequencing (Turnbaugh *et al.*, 2009a,b). They provide an overview of the commensal microbial communities and their functional capacity. They have shown a great variability in the microbiota composition in healthy individuals. Even twins share less than 50% of their bacterial taxa at the species level (Turnbaugh *et al.*, 2010). However, this does not mean that genetics do not play a role in the establishment and conformation of the gut microbiota. It has been shown that the composition of the bacterial community is influenced by specific genomic loci in the host (Benson *et al.*, 2010). Metagenomic studies have established that, in spite of extensive interpersonal variability, there are groups of bacteria that share functionalities (Turnbaugh *et al.*, 2009a,b; Burke *et al.*, 2011; Guirro *et al.*, 2018).

The main bacteria correspond to three major phyla: *Firmicutes* (gram-positive), *Bacteroidetes* (gram-negative) and *Actinobacteria* (gram-positive). *Firmicutes* are the most common phylum (60%), including about 200 genera. The most important are Mycoplasma, Bacillus, and Clostridium. Each genus has different species. *Bacteroidetes* and *Actinobacteria* represent 10% of gut microbiota. The rest of the microorganisms belong to 10 minority families.

The establishment of microbial populations that colonize the gut accompanies the development of the human from childbirth. The most drastic changes in composition occur during early childhood. Although it has been traditionally argued that the acquisition of the intestinal microbiota begins at birth (Adlerberth and Wold, 2009) and is consolidated throughout the life of the individual, some evidence shows that the fetal gastrointestinal tract may be inhabited by some microorganisms (Jiménez *et al.*, 2008). The mother probably represents the most important influence for the development of the infant microbiome due to

Table 1 Predominant microorganisms in the human gastrointestinal tract

Stomach (10^4 UFC/g of gut content)	*Helicobacter pylori* (phyla: *Proteobacteria*) *Lactobacillus* (phyla: *Firmicutes*) *Streptococcus* (phyla: *Firmicutes*)
Duodenum (10^3–10^4 UFC/g of gut content)	*Bacteroides* (phyla: *Bacteroidetes*) *Lactobacillus* (phyla: *Firmicutes*) *Streptococcus* (phyla: *Firmicutes*) *Staphylococcus* (phyla: *Firmicutes*)
Jejunum (10^5–10^7 UFC/g of gut content)	*Bacteroides* (phyla: *Bacteroidetes*) *Lactobacillus* (phyla: *Firmicutes*) *Streptococcus* (phyla: *Firmicutes*) *Bacillus* (phyla: *Firmicutes*)
Ileum (10^7–10^8 UFC/g of gut content)	*Bacteroides* (phyla: *Bacteroidetes*) *Clostridium* (phyla: *Firmicutes*) *Enterobacteriaceae* (phyla: *Proteobacteria)* *Enterococcus* (phyla: *Firmicutes*) *Lactobacillus*(phyla: *Firmicutes*) *Veillonella* (phyla: *Firmicutes*)
Colon (10^{10}–10^{11} UFC/g of gut content)	*Bacteroides* (phyla: *Bacteroidetes*) *Bacillus* (phyla: *Firmicutes*) *Bifidobacterium* (phyla: *Actinobacteria*) *Clostridium* (phyla: *Firmicutes*) *Enterococcus* (phyla: *Firmicutes*) *Eubacterium* (phyla: *Firmicutes*) *Fusobacterium* (phyla: *Fusobacteria*) *Peptostreptococcus* (phyla: *Firmicutes*) *Ruminococcus* (phyla: *Firmicutes*) *Streptococcus* (phyla: *Firmicutes*)

intimate contact during childbirth and early feeding (Palmer *et al.*, 2007; Dominguez-Bello *et al.*, 2010; Matamoros *et al.*, 2013). The constitution of gut microbiota is affected by many variables including type of delivery (vaginal or cesarean), diet (breast-feeding or formula feeding), environment, cultural, geographical factors, and family environment (Palmer *et al.*, 2007; Dominguez-Bello *et al.*, 2010; Matamoros *et al.*, 2013). Infants born vaginally have communities similar to those found in their mother's vaginal microbiota. In contrast, those born by cesarean have a similar microbiota to the skin and predominated by taxa such as Staphylococcus and Propionibacterium spp. (Dominguez-Bello *et al.*, 2010). Some results hypothesize that type of delivery influences immune function during the first year of life through the development of gut microbiota. Breastfeeding is one of the key factors in the development of the neonatal gut microbiota, ensuring a continuous supply of bacteria throughout the lactation period (Matamoros *et al.*, 2013; Vaishampayan *et al.*, 2010; Le Huërou-Luron *et al.*, 2010).

The following major changes in gut microbiota composition occur after introduction of solid food and weaning, when a microbiota of greater richness and diversity is developed. At the same time, the immune system "learns" to differentiate between commensal and pathogenic bacteria. The bacterial composition begins to converge towards an adult microbiota profile at the end of the first year of life and completely resembles the adult microbiota at two and a half years (Palmer *et al.*, 2007). *Firmicutes* and *Bacteroidetes* predominate from this stage, whereas in the first days after childbirth the *Proteobacteria* and the *Actinobacterias* are dominant. Once the microbiota reaches maturity, it remains largely stable until old age, when this stability is reduced (Claesson *et al.*, 2011).

Dietary changes have important effects on the microbiota. Diet can enhance, inhibit, or even change the composition and functions of the gut microbiota. These changes in microbiota composition in response to dietary intake could be related to the fact that different bacterial species are genetically better equipped to use different substrates. Many studies have shown that an increased fat intake leads to a higher gram-negative to gram-positive bacteria rate in gut microbiota. The association between type of diet and different microbial groups is still unclear (De Filippo *et al.*, 2010). The microbiota of western communities, characterized by higher fat and animal protein intake and lower fiber content, appear to contain reduced *Bacteroidetes* and increased *Firmicutes* (especially Mollicutes), compared to those of Eastern communities (Harakeh *et al.*, 2016). Specifically, a high-fat diet is associated with a decrease in the *Lactobacillus* genus, and an increase in microbial populations that secrete pro-inflammatory products and alter the gut barrier. A high-fat diet favors an increase in systemic endotoxemia and inflammation, and improves energy harvest, with a positive energy balance, and tends to induce insulin resistance and obesity (Shen *et al.*, 2014; Guirro *et al.*, 2018).

De Filippo *et al.* (2010) found important differences in the composition of gut microbiota in rural African children compared to European children. There was a different proportion of *Firmicutes* to *Bacteroidetes* and of gram-negative to gram-positive bacteria. This was attributed to a low dietary fiber intake in Europeans. African children had greater microbial richness with a higher proportion of *Bacteroidetes* in their gut, whereas the Western lifestyle led to increased *Firmicutes*.

Another study demonstrated how the Bacteroides and Prevotella enterotypes were strongly associated with dietary habits. In particular, diets rich in animal proteins and fats were highly related to the Bacteroides enterotype and those rich in plant fiber with Prevotella (Wu *et al.*, 2011). Both authors (De Filippo *et al.*, 2010; Wu *et al.*, 2011) postulated that gut microbiota develops with a vegetarian diet maximizes energy extraction from dietary fiber and, at the same time, protects against inflammation. Certain diets predispose to acquiring diseases, and this relationship between diet and disease could be mediated by microbiota (Cani and Delzenne, 2009; Cani *et al.*, 2007). Therefore, diet is a key factor that maintains or not a healthy commensal relationship (Zhang *et al.*, 2018).

Dietary interventions, nutritional modulation strategies and the consumption of probiotics and prebiotics can change gut microbiota, improving the integrity of the gut barrier and decreasing metabolic endotoxemia and inflammatory markers (Requena *et al.*, 2018). These factors are important because of their relationship with insulin resistance, systemic inflammation and increase in fat mass, key aspects in the development of diseases such as obesity or diabetes. The modifications in the microbial populations after dietary intervention with probiotics suppose a greater bacterial abundance of gut barrier function modulators such as *Lactobacillus* and *Bifidobacterium*, as live micro-organism or fermented products, achieve proper gut environment and an important reduction in opportunistic pathogens such as the Enterobacteriaceae, Desulfovibrionaceae, and Streptococcaceae families (Xiao and Zhao, 2014). The species *Akkermansia muciniphila* has also been associated with improved metabolic profiles. In addition, its daily oral administration or its increase after nutritional intervention through the use of prebiotics reverses metabolic disorders induced by a high-fat diet (Everard and Cani, 2013). In addition, administration of prebiotics along with probiotics improves the body weight, abdominal fat and intestinal barrier function (Pothuraju and Sharma, 2018).

The proposed mechanisms by which prebiotics improve intestinal health and reduce metabolic complications of obesity include: (1) increased *Bifidobacterium* population, correlated positively with gut health and negatively with obesity and diabetes. (2) Reduced metabolic endotoxemia through improved gut barrier function. (3) Increased intestinal satiety signals through YY peptide or ghrelin reduction. (4) Improved insulin action increasing GLP-1 secretion, and (5) reduced adipogenesis by synthesis of short chain fatty acids (SCFA) (Shen *et al.*, 2014).

Pre-, pro-, and synbiotics could prove useful, but further research is needed to clarify their clinical relevance for the prevention and management of metabolic disease. (Barengolts, 2016).

Loss of Gut Microbiota Biodiversity

Over the last few decades, existing evidence has shown a significant change in gut microbiota with an increase in certain species and a decrease in all the rest. The most surprising finding is the loss of microbial diversity observed in developed countries. Factors that have influenced this change in our microbiota are: water sanitation, increased cesarean sections, higher use of pre-term antibiotics, reduced breastfeeding, smaller family sizes, increased use of antibiotics and toilet and antibacterial soaps.

Available evidence suggests that there are significant changes in the microbiota after antibiotic treatment. We found a decrease in phylogenetic diversity and changes in the proportions of different families, which may alter the development of the immune system and predispose the host to developing bacterial or viral infections, or allergies (Ubeda and Pamer, 2012). Overuse of antibiotics is linked to an increase in antibiotic-resistant pathogens (Dethlefsen *et al.*, 2008; Sullivan *et al.*, 2001). Although the particular taxon affected varies among individuals, some taxa do not recover even after months of treatment, and in general there is a long-term decline in the biodiversity of bacteria after use.

Many studies have been published in this regard; Neyrinck and Delzenne (2010) observed how after administration of a broad-spectrum antibiotic in obese rodents there was an improvement in metabolic alterations and changes in phyla such as *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. Other experiments (Hansen *et al.*, 2012) have shown that the use of broad-spectrum antibiotic treatments such as vancomycin may increase the abundance of *A. muciniphila* in rodents, reducing the incidence of diabetes. Cani *et al.* support that broad spectrum antibiotic therapy significantly reduces plasma levels of lipopolysaccharide (LPS) in animal models (Cani *et al.*, 2008). Membrez *et al.* (2008) observed how genetically obese rodents with insulin resistance and on a high-fat diet improved glucose and weight tolerance upon antibiotic treatment. The use of antibiotic therapy reduced the degree of inflammation, infiltration of macrophages and oxidative stress in the adipose tissue.

On the other hand, some studies have associated the exposure to antibiotics with increases in body mass index in different circumstances, such as the first months of life, childhood, in malnourished subjects, or *Helicobacter pylori* infection in individuals with cystic fibrosis. It is difficult to discern whether this weight gain is due to the beneficial effects of antibiotic treatment in the prevention or treatment of bacterial infections, or through their effects on the microbiota. A possible explanation could be a mixture of these two mechanisms in many of the scenarios described (Million *et al.*, 2013; Trasande *et al.*, 2013; Garly *et al.*, 2006).

Epidemiological studies have supported the increased risk of overweight in late childhood after exposure to antibiotic therapy in early childhood (Murphy *et al.*, 2014). They have even linked the progressive increase of obesity in the population with higher use of antibiotics (Luoto *et al.*, 2010; Dao *et al.*, 2016). For decades, subtherapeutic doses of antibiotics have been used to promote growth in farm animals, with resulting increased fat mass. This relationship shows that increasing antibiotics use promotes obesity (Trehan *et al.*, 2013). In contrast, antibiotic exposure during the first years of life in children of overweight mothers has been associated with a lower risk of overweight in childhood (Ajslev *et al.*, 2011). Overall, these studies suggest that exposure to

antibiotics in the early stages of life may disrupt healthy gut microbiota, but conversely, it also has the potential to modify disturbed microbiota to a healthier state (Allin *et al.*, 2015).

Microbiota and Metabolic Diseases

Transcendent publications analyze trends in body mass index in 200 countries and estimate that the global prevalence of obesity has increased from 105 million people to 641 million in just four decades (NCD Risk Factor Collaboration (NCD-RisC), 2016). In Spain, over the last 20 years the rate of obese subjects has doubled from close to 12% in the 1990s to far exceed 20% at the present time. The determinants of this exponential growth of obesity prevalence cannot be just a consequence of increasing caloric intake and decreasing physical activity. Other environmental factors may explain this trend.

The origin of obesity is a complicated challenge due to the great genetic diversity and social differences between humans. Despite the tremendous efforts and the identification of some point mutations in the genome, an overall view of the exact molecular mechanisms involved in the development of diabetes and obesity has not been developed. Indeed, the discovery of candidate genes in genome-wide association studies (GWAS) has helped to identify novel genes for sensitivity/resistance to diabetes and extreme metabolic phenotypes (Jacquemont *et al.*, 2011). However, these steps forward cannot yet explain the overall diversity in the natural histories of metabolic diseases (Beck-Nielsen *et al.*, 2003). Epigenetic and environmental factors such as drastic change in dietary habits, less fiber and a high-fat diet contribute to the origin of metabolic diseases. Further investigations are trying to find a new paradigm that takes into account genetic diversity, the impact of environmental factors, the rapid development of metabolic diseases, and individual behavior in the development of diabetes and obesity.

Numerous published papers suggest that gut microbiota, and more specifically its variations in composition and diversity, play an important role in the development of metabolic disorders, such as diabetes and obesity. Perturbations of the gut microbiota, termed gut dysbiosis, affect the interplay between the gut microbiota and host cells, resulting in dysregulation of inflammation that contributes to the pathogenesis of chronic inflammatory diseases. Many studies demonstrate profound changes in the composition and metabolic function of the microbiota in obese subjects (Andersson *et al.*, 2008; Klindworth *et al.*, 2013). Gut microbiota is increasingly recognized as a fundamental element connecting genes, environment, and immune system, being involved in the regulation of metabolic function, development of low-grade inflammation and regulation of energy balance (Barengolts, 2016).

Pathophysiological Proposed Mechanisms Influencing Microbiota and Energy Homeostasis

Several mechanisms have been proposed as a link between gut microbiota and obesity. We summarize them in this list:

1. Generation of active metabolites such as short chain fatty acids (SCFA) or bile acids with well known antiinflammatory effects and regulatory properties on carbohydrates and lipid metabolism (Li *et al.*, 2017; Harakeh *et al.*, 2016; Moran and Shanahan, 2014; Tilg, 2010).

   Butyrate, propionate and acetate are SCFA produced by gut bacteria when digesting dietary fiber. These SCFA are absorbed in the intestine where butyrate, in particular, provides energy to the epithelial cells of the colon and contributes to decreasing intestinal permeability.

   Lipopolysaccharides (LPS) are a pro-inflammatory component of the cell wall of gram-negative bacteria. Metabolic endotoxemia and increased systemic LPS levels are related to an increase in gut permeability and the establishment of a chronic inflammatory state. Both animal and human studies have documented its importance in the development of obesity in (Moran and Shanahan, 2014).

   Obesity and diabetes have a low-grade inflammatory component accurately described in tissues directly involved in the regulation of metabolism, such as liver, adipose tissue and muscle. Animal experiments have shown how changes in microbiota are capable of altering the degree of inflammation in adipose tissue.

   Other studies have shown a relationship between dietary fiber intake or prebiotic substrate for the production of SCFA by microbiota and the release of gastrointestinal hormones such as GLP-1, YY intestinal peptide, ghrelin and other peptide hormones in both humans (Freeland *et al.*, 2010; Parnell and Reimer, 2009) and rodents (Zhou *et al.*, 2008). This phenomenon helps increasing satiety after a meal and decreases food intake. Also, SCFA have been linked to leptin expression through the GPR41 receptor in murine models (Xiong *et al.*, 2004). Likewise, adipocytes detect increased levels of SCFA produced by intestinal microbial fermentation and induce leptin production regulating host energy balance. Data obtained with experimental obesity models (Neyrinck and Delzenne, 2010; Dewulf *et al.*, 2011) on dietary supplementation with nondigestible carbohydrates demonstrate beneficial effects for the host. They reduced adiposity by counteracting G protein-coupled receptor 43 (GPR43) expression in adipose tissue inducing a decrease in the processes activated by the PPAR-gamma receptor (Li *et al.*, 2017). Similarly, ghrelin levels have shown negative correlations with *Bifidobacterium*, *Lactobacillus*, *Blautia coccoides*, and *Eubacterium rectale* and positive correlations with Bacteroides and Prevotella, suggesting the role of gut microbiota in the control of satiety and its association with the food-intake regulating hormones leptin and ghrelin.

   Interestingly, butyrate production is also linked to serotonin levels in animal models. Serotonin can regulate intestinal

permeability and is an important neurotransmitter in the gut and brain, involved in regulating body weight and satiety control (Simansky, 1996; Zhu *et al.*, 2009).

Another theory is related to primary bile acids. Five percent of these primary bile acids escape from enterohepatic circulation reaching the large intestine and transforming into the secondary bile acids through the action of gut microbiota. In overweight and diabetic subjects, fewer bile acids were observed in comparison to healthy subjects. Secondary bile acids appear to play an insulin-sensitizing role. They act as signaling-mediator molecules in brown adipose tissue and muscle and increase mitochondrial activity. They appear to improve carbohydrate metabolism stimulating the production of peptides such as GLP-1 by intestinal L cells (Allin *et al.*, 2015; Palau-Rodriguez *et al.*, 2015).

2. Increased endocannabinoid system tone (Moran and Shanahan, 2014; Cani *et al.*, 2016).

   The endocannabinoid system (ES) is a mediator involved in the communication between gut microbiota and adipose tissue, playing an important role in energy homeostasis through the regulation of appetite and metabolism. Moreover, the ES is involved in gut physiology through modulation of gastric emptying, gastrointestinal motility and inflammation. In animal models, the ES has been shown to control gut barrier function and adipogenesis by facilitating adipogenesis and expansion of adipose tissue, and regulating inflammation (Muccioli *et al.*, 2010). These effects of the ES and related bioactive lipids on gut physiology might be involved in the regulation of fat intake, satiety and postprandial glycaemia. The role of the ES in the regulation of brown adipose tissue metabolism and thermogenesis has been proposed to be dependent on cannabinoid receptor B1.

   These results provide strong support for crosstalk between adipose tissue and the gut microbiota, with the endocannabinoid system functioning as a potent mediator (Cani *et al.*, 2016). In humans, a direct relationship between changes in the gut microbiota and endocannabinoid system tone has yet to be conclusively demonstrated.

3. Decreased gut gene expression of fasting-induced adipocyte factor (FIAF) responsible for inhibiting lipoprotein lipase activity in relation to hepatic and fat adipose storage (Tilg, 2010; Burcelin *et al.*, 2012).

   Gut microbiota could decrease the production of FIAF carried out by the intestinal cells, which inhibit lipoprotein lipase activity. This enzyme promotes the release of non-esterified fatty acids into tissues such as liver and adipose cells (Bäckhed *et al.*, 2007).

4. Gut modulation derived from peptide secretion (GLP-1, GLP-2, YY peptide…) (Musso *et al.*, 2010).

   Yadav *et al.* (2013) demonstrated that the modulation of gut microbiota composition through the use of probiotics stimulates the production of butyrate. It favors the secretion of GLP-1 in L cells, improving the inflammatory state and resistance to insulin. Most of the pathways underlying the effects of SCFA remain unknown. Several studies have suggested that the effects of SCFA are mediated by members of the newly-identified G protein-coupled receptor family. These are G protein-coupled receptors 43 and 41 (GPR43 and GPR41, respectively). The binding of SCFA to GPR43 and GPR41 receptors increases plasma levels of GLP-1 and YY peptide, leading to improvements in glucose homeostasis and reduced appetite (Kuwahara, 2014; Everard and Cani, 2014; Li *et al.*, 2017).

   The GPR41 and GPR43 receptors are also found in the enteroendocrine L cells in the colon. The mode of action GLP-1 and YY peptide release in the colon is different from the mode of action in the small intestine. SCFA from the colonic lumen could be involved in regulating long-term energy homeostasis by inducing GLP-1 secretion through their action on GPR41 and GPR43 receptors. In the small intestine they would be involved in appetite control, ingestion, and short-term energy homeostasis (Kuwahara, 2014; Li *et al.*, 2017).

   Ingestion of fructoolisaccharides in the diet influences the density of L cells expressing GPR43 and GPR41 receptors (Ley *et al.*, 2005). Increases in the GLP-1 and YY intestinal peptide production have been related to the increase in *Bifidobacterium* generated by the use of some prebiotics. Higher *Bifidobacterium* modulates inflammation in obese mice by increasing incretin production and reducing intestinal permeability (Cani and Delzenne, 2009).

Evidence Link Between Gut Microbiota and Obesity

The first evidence of the relationship between gut microbiota in energy homeostasis regulation and adiposity comes from Ley *et al.* (2005) and Bäckhed *et al.* (2007). They observed how after microbial colonization of germ-free mice (axenic mice) there was an increase in weight and body fat. They found that axenic mice had 40% less total body fat than conventional mice, although the calorie consumption of the conventional animals was 29% higher. However, after microbial colonization, the total body fat of germ-free mice increased by 57% in 2 weeks (Bäckhed *et al.*, 2004).

There are many publications and discoveries that have emerged microbiota to be considered as an important factor in the genesis of metabolic diseases. In animal models, it has been shown that mice with a leptin gene mutation, and therefore genetically obese, had a different microbiota compared to mice without this mutation. In these obese animal models, the interaction between the dominant intestinal phyla, *Bacteroidetes* and *Firmicutes*, is modified with a significant reduction in the first and a corresponding increase in the second. The same trend was observed in humans. Ley *et al.* (2006) reported an altered composition in the microbiota of obese humans with a marked decrease in bacterial diversity, and similar characteristics to obese mice. Other authors (Turnbaugh *et al.*, 2009a,b; Schwiertz *et al.*, 2010; Kallus and Brandt, 2012; Ley, 2010) have confirmed this disturbance of the microbiota in relation to these two main phyla. In most of the published studies, an increase in the *Firmicutes* to *Bacteroidetes* ratio in the microbiome of obese subjects has been associated with an increased low-grade inflammatory state and a

higher capacity to capture the energy coming from food through the edge *Firmicutes* has over *Bacteroidetes*. Although within the edge held by Bacteroidetes, Bacteroides thetaiotaomicron (Hooper *et al.*, 2001) has shown to improve the absorption and processing of host nutrients. In addition, all these studies agree on the existence of gut microbiota variations with reduced microbial diversity. It is associated with greater adiposity, insulin resistance and a more pronounced inflammatory phenotype.

Specifically, the increase in *Firmicutes* observed in obese animals and subjects could be associated with an increase in the capacity to digest some indigestible polysaccharides and then produce monosaccharides and SCFA that are absorbed by the host and obtain energy from substances that would otherwise be eliminated by the feces. Therefore, there is a specific microbiota that is able to obtain more energy from the same daily caloric intake (Turnbaugh *et al.*, 2009a,b). Zhang *et al.* suggest that a higher energy harvest in obese individuals is related to the transfer of hydrogen between taxa. They observed a simultaneous increase in Prevotella that produces hydrogen and in methanogenic archaea that uses hydrogen (Zhang *et al.*, 2009). These findings agree with the observation that germ-free mice fed a high-fat diet gained less weight than their conventional counterparts (Bäckhed *et al.*, 2004).

However, not all studies found interaction between the dominant intestinal phyla *Bacteroidetes* and *Firmicutes* (Wu *et al.*, 2011; Jumpertz *et al.*, 2011; Duncan *et al.*, 2008; Hold *et al.*, 2014). A important study concludes that obesity is probably related to an increase in the phyla *Firmicutes* and *Actinobacteria*, with a reduction in *Bacteroidetes*, *Verrucomicrobia*, and *Faecalibacterium prausnitzii* species (Chakraborti, 2015). In this regard, authors such as Turnbaugh *et al.*, (2009a,b) and Furet *et al.*, (2010) propose a variable microbial pattern composition in obese individuals with a smaller representation of *Bacteroidetes* and greater representation of *Actinobacteria* (Monira *et al.*, 2011), with no apparent differences in *Firmicutes* compared to normal-weight subjects. This has also been associated with a decrease in the amounts of *Bifidobacterium* and Ruminococcus (Moran and Shanahan, 2014; Martín *et al.*, 2003).

A large study (Turnbaugh *et al.*, 2009a,b) describes how 75% of the microbial genes associated with obesity belonged to *Actinobacteria* and 25% to *Firmicutes*, while 42% of the genes associated with thinness belonged to *Bacteroidetes* (El Kaoutari *et al.*, 2013). The absorption of nutrients in relation to the different microbial patterns varies according to the study population, with the same microbial population responding differently to determined diets in normal weight versus obese individuals (Jumpertz *et al.*, 2011).

Significant studies have demonstrated modifications in the communities that are part of the gut microbiota of individuals who achieve weight loss with an increase in certain species of the genus *Bacteroides* (*Bacteroides fragilis*), Prevotella and *Lactobacillus* and a decrease in species of the genus *Clostridium* (*C. coccoides*, *C. histolyticum*), *Bifidobacterium* (*B. longum*), *Eubacterium* (*E. rectale*), and *Akkermansia* (Santacruz *et al.*, 2009; Nadal *et al.*, 2009). In addition, the relative proportion of *Bacteroidetes* and *Firmicutes* increased in obese subjects after weight loss indicates that modulation of microbiota can be an effective means for weight management. Caloric restriction in obese or overweight people has also shown changes in the fetal microbiome with increased microbial richness, correlating with improved metabolic parameters (Voreades *et al.*, 2014).

However, the experiment that has given the most solidity to the causality between microbiota and obesity was performed by Turnbaugh *et al.* in (2006). They demonstrated how microbiota transplantation from genetically obese mice to germ-free mice caused a significant increase in weight compared to germ-free mice transplanted with the microbes of thin mice, reinforcing the theory of the existence of a causal relationship between microbiota and obesity. Thus, surprisingly, the energy-harvesting phenotype is transmissible simply by the transfer of "obese microbiota" to healthy and lean individuals (Turnbaugh *et al.*, 2006, 2008). But not all genera and species play the same role within a given phylum. Different bacterial species present different characteristics, which may be related to beneficial or harmful traits. For example, *Lactobacillus* representation is greater in obese compared to thin individuals. However paradoxically, overweight adolescents who lose weight due to calorie restriction and increased physical activity have amplified *Lactobacillus* count. Thus, the probiotic approach with specific strains of *Lactobacillus* does not escalate, but even reduces the metabolic alterations that occur in obesity. A positive impact on insulin sensitivity has even been shown. The administration of specific probiotic strains such as *Lactobacillus rhamnosus* and *Lactobacillus gasseri* have shown beneficial effects on obesity with decreased body mass index and visceral and subcutaneous fat mass in overweight and obese subjects (Delzenne *et al.*, 2011). In addition, other species of *Lactobacillus* such as *Lactobacillus plantarum* and paracasei have been associated with thinness, compared to species such as Lactobacillus reuteri that have been associated with obesity. This suggests that the physiological effects of probiotics are highly dependent on the strain (Luoto *et al.*, 2010; Million *et al.*, 2012).

At the genus level, the presence of Acinetobacter, Aliivibrio, Marinomonas, Pseudoalteromonas, Shewanella, Lachnospira, Citrobacter and Shigella, as well as Parabacteroides distasonis and *Escherichia coli* have been positively associated with obesity. However, the presence of Bacteroides, Prevotella, Sutterella, and Methanobrevibacter has shown a negative association (Million *et al.*, 2013). Reduced levels of *A. muciniphila*, a mucin-degrading bacterium, have been associated with obesity and diabetes. Normalization of its levels using prebiotics and feeding type is associated with an improved metabolic profile in rodents (Walsh *et al.*, 2014; Everard *et al.*, 2013).

New findings indicate that an increased *A. muciniphila* abundance in obese or overweight subjects exposed to a calorie-restricted diet is associated with a healthier metabolic state, including improved lipid and carbohydrates metabolism and body fat distribution with decreased waist circumference (Dao *et al.*, 2016). Further investigation is needed building upon current data to determine the potential therapeutic applicability of *A. muciniphila*. This also indicates the importance of certain genera or species that exist in small proportions in the pathogenesis of obesity.

In addition, alterations in the microbiota seem to precede alterations in weight. The study by Kalliomäki *et al.* (2008) showed a greater proportion of *Bifidobacterium* and smaller number of *Staphylococcus aureus* in children with normal weight compared to those who were overweight over the years, proposing that *S. aureus* may act as a trigger for low-grade inflammation.

On the other hand, bariatric surgery is the most effective treatment nowadays for patients with morbid obesity. But the beneficial effects of bariatric surgery on carbohydrate homeostasis, energy expenditure and increased satiety are not completely explained by the ingestion restriction and malabsorption generated. Emerging data suggest changes in hormone levels and gut microbial profiles could be an added influence (Jain *et al.*, 2018). Certain surgical interventions such as Roux-en-Y gastric bypass have shown alterations in gut microbiota, which may contribute to some of the favorable effects of these surgeries. Zhang *et al.* (2009) published changes in the microbiota of subjects after Roux-en-Y gastric bypass with large increases in Gammaproteobacteria and proportional decreases in *Firmicutes*. A French study (Kong *et al.*, 2013) supports these findings and also describes an increase in the richness of the microbiome after surgery.

In animal models, (Liou *et al.*, 2013) germ-free mice received Roux-en-Y gastric bypass-microbiota, which induced a more significant weight loss and fat mass decrease compared to germ-free mice that received microbiota from mice undergoing other surgery.

Final Considerations

The obesity epidemic is increasing in recent decades so the traditional theories of weight gain as an imbalance between energy expenditure and consumption results too simplistic. Other elements such as gut microbiota must be involved as possible etiopathogenic factors.

Obesity is associated with profound changes in the composition and metabolic function of gut microbiota. The "obese microbiota" obtains energy from the diet more efficiently through greater absorption and deposition of nutrients. This is due to an improved capacity to decompose indigestible polysaccharides that cannot be digested by the host, which leads to increased monosaccharide and SCFA production. In addition, certain bacterial phyla are associated with greater energy utilization of food consumed. However, this increase in fat mass is not only related to a more efficient energy harvest, but also to the participation of microbiota in changes in the hormonal environment, the expression of genes regulating lipogenesis and insulin resistance.

Gut microbiota disturbances contribute to the development of metabolic disorders. In particular, these changes in the microbial community seem to precede the weight gain and metabolic consequences of obesity. The composition of the obese subjects microbiota is different from that of thin controls. The mechanisms by which the microbiota may influence the pathogenesis of obesity include modifications in FIAF expression, increases in the endocannabinoid system tone, changes in incretin secretion and active metabolites such as SCFA and secondary bile acids. Microbial transplants have been able to alter weight in animal models.

Overall, going deeper into strategies to integrate this emerging knowledge to understand the complex relationships between the host, gut microbiota and diet is necessary for the prevention and management of metabolic disease such us obesity.

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Regulation of Food Intake

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Abbreviations

AgRP	Agouti-related peptide
Alpha-MSH	Alpha-melanocyte stimulating hormone
BBB	Blood–brain barrier
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CNS	Central nervous system
GABA	Gamma amino butyric acid
GHS	Growth hormone secretagogue receptor
GLP1	Glucagon-like peptide 1
LHA	Lateral hypothalamic area
MC4R	Melanocortin 4 receptor
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarious
PYY	Peptide YY

Introduction

In the healthy subject, many signals of internal and external signals of nutrient availability, such as behavior (i.e., food intake), the endocrine system, and the autonomic nervous system, translate information to the brain. All this information will be engaged in the central nervous system (CNS) to provide a synchronized response to fluctuations in energy balance and body weight that include the volitional control of intake and the energy expenditure (Broberger, 2005; Farr *et al.*, 2016). Under healthy conditions, there is a close correspondence between the intake and the expenditure of energy, and body weight is relatively constant: an excess of intake over expenditure of only 1% would lead to an increase in weight of more than 1 kg per year.

Traditional accounts of brain regulation of food intake describe two large systems that balance each other: the hypothalamus, receiving signals from the periphery that notify the decrease in energy stores, and the brainstem, receiving oral and gastrointestinal information as an online signal of the quantities and qualities of the food that has been ingested. This procedure would allow the hypothalamus to function as a long-term control orchestrating meal initiation and the brainstem would serve as a short-term control for meal termination (Broberger, 2005).

Peripheral signals from the gastrointestinal tract and from currently available metabolic fuels are both humoral and neural. Neural inputs are mainly transmitted via afferent (sensory) fibers on the vagus nerve (i.e., gastric distension is sensed and transmitted to the brain by vagal afferents in the stomach wall). Humoral factors comprise hormones (i.e., leptin and insulin), gut peptides (i.e., ghrelin), and circulating metabolites (i.e., fatty acids and glucose).

Although control of energy homeostasis includes a short-term (homeostatic) and a longer-term (hedonic) control of appetite and food intake, clearly the two pathways are interrelated and dependent (Saper *et al.*, 2002). Despite continuous improvement in both basic and clinical research, the control of normal eating remains only partly understood. In this article, part of this knowledge will be highlighted.

Peripheral Signals From the Gastrointestinal Tract

The gastrointestinal tract is one of several organs that contribute to the peripheral signaling of food intake and satiety. In fact, eating is affected by a wide variety of peptides emerging from the alimentary tract that communicate with the brain through both hormonal and neural signals, acting as a feedback controls (Badman and Flier, 2005) (**Fig. 1**).

Four are the main gastrointestinal peptides [cholecystokinin (CCK), peptide YY (PYY), ghrelin, and glucagon-like peptide 1 (GLP1)] that bind to their respective receptors located at the vagus nerve fiber terminals, distributed through the digestive mucosal layer. When gut peptide information is converted to electrical signals, it reaches the nucleous tractus solitarious (NTS) in the brainstem. Ghrelin is the only that enhances appetite. The other three peptides play a role in appetite suppression (Crawley and Corwin, 1994).

Cholecystokinin

CCK is secreted by the neuroendocrine I cell in the duodenal and jejunal mucosa in response to the presence of high intraluminal concentrations of proteins and long-chain fatty acids derived from the digestion of triglycerides. So that, CCK is involved in the short-term regulation of energy intake, with an important role as a satiety factor (Gibbs *et al.*, 1973). This satiating effect of CCK is enhanced by the administration of central leptin, suggesting the presence of physiologic interactions between short-term (CCK) and long-term (leptin) regulators of energy homeostasis (Emond *et al.*, 1999).

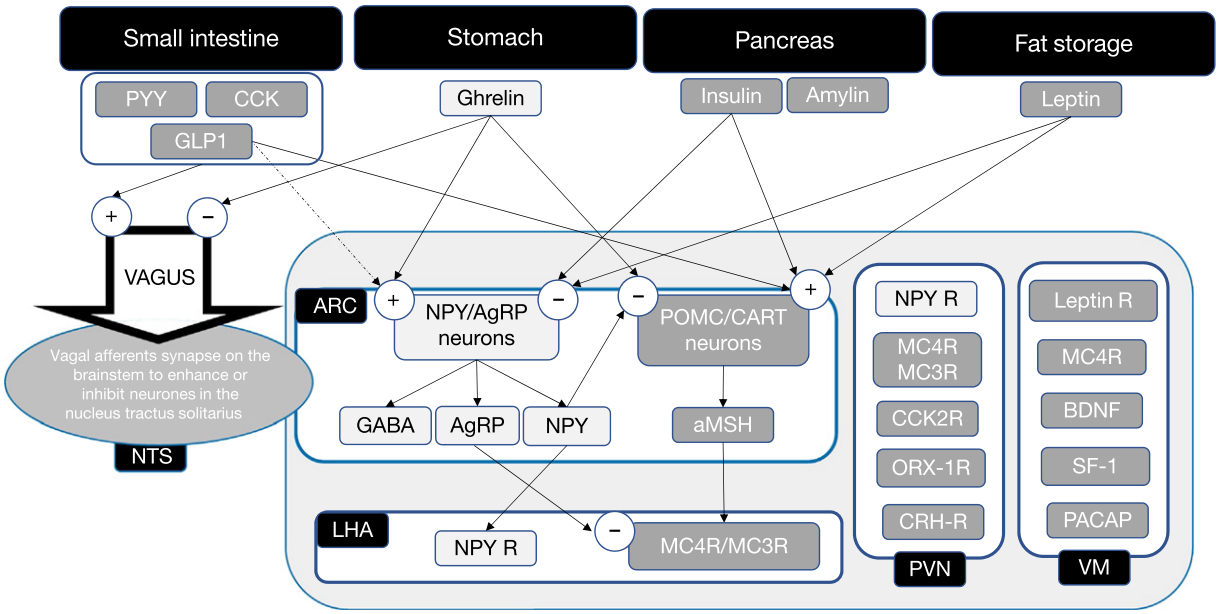

Fig. 1 Summary of the main functions of the key peptides and neurotransmitters that regulate food intake. *Black boxes* reflect anatomical places. *Dark gray* boxes indicate peptides that reduce food intake. *Light gray* boxes indicate appetite stimulators. AgRP, Agouti-related peptide; aMSH, alpha-melanocyte stimulating hormone; ARC, arcuatus nucleus; BDNF, brain-derived neurotropic factor; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; GABA, gamma amino butyric acid; GLP1, glucagon-like peptide 1; LHA, lateral hypothalamic area; MC4R, melanocortin 4 receptor; MC3R, melanocortin 3 receptor; NPY, neuropeptide Y; NTS, nucleous tractus solitarious; orexin A (ORX-1R); PYY, peptide YY; PVN, paraventricular nucleus; PACAP, Pituitary adenylate cyclase-activating polypeptide; SF-1, steroidogenic factor 1; VHA, ventromedial hypothalamic area.

CCK acts on the CCK-1 (originally named CCK-A) receptors on vagal sensory terminals in the upper gut, which project to the NTS in the brainstem (Kopin *et al.*, 1999). Mutations of this receptor produce severe hyperphagia and morbid obesity. In addition, CCK is not only a neurotransmitter; it also acts as an intestinal peptide hormone with actions that include contraction of the gallbladder.

Ghrelin

Ghrelin is a gut peptide described in 1999 as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), a G-coupled receptor broadly expressed in the CNS and in peripheral tissues (Kojima *et al.*, 1999; Mihalache *et al.*, 2016). Composed by 28-amino-acid, it is secreted mainly by X/A-like gastric cells in the oxyntic glands of the gastric fundus mucosa, and also in the brain, including the hypothalamic neurons (Stengel and Tache, 2012; Shuto *et al.*, 2001). And ghrelin is present in human plasma in two forms: an inactive form known as deacylated ghrelin, and an active form, the acylated ghrelin synthesized under the action of ghrelin *O*-acyltransferase enzyme (GOAT).

Ghrelin contributes to the control of energy homeostasis by acting as an orexigenic (appetite-stimulating) signal by a mixed mechanism. Ghrelin promotes the electrical activity of vagal afferent pathways, and this information is transmitted to noradrenaline neurons at NTS in the brainstem. In addition, ghrelin also acts through the GHS-R, which is widely expressed in hypothalamic appetite centers, enhancing NPY/AgRP neurons and suppressing POMC neurons (Nakazato *et al.*, 2001; Date *et al.*, 2006). So, ghrelin is the only peripheral orexigenic hormone that enhances appetite actions through its receptors localized in the hypothalamus.

Plasma ghrelin concentrations are closely related to hunger. Ghrelin secretion and serum levels exhibit a preprandial rise and a postprandially fall, which strongly suggest that the peptide plays a physiological role in meal initiation (Cummings *et al.*, 2001, 2005; Cummings and Overduin, 2007). In addition, besides the stimulation of food intake, ghrelin is also an adiposity signal that decreases its serum concentration with the increase of body fat (Tschöp *et al.*, 2001). Therefore, ghrelin also brings about a decrease in energy expenditure and promotes the storage of fatty acids in adipocytes. Ghrelin mutations have been associated with obesity (Ukkola *et al.*, 2001).

Glucagon Like Peptide 1

GLP1 is a hormone secreted predominantly by the L cells of the ileum in response to nutrient intake (Baggio and Drucker, 2007). Its main action is directed to stimulate the secretion of insulin by pancreatic beta cells and the suppression of glucagon secretion by pancreatic alpha cells.

The effect of GLP1 on food intake is exerted both indirectly and directly (Ban *et al.*, 2008; van Bloemendaal *et al.*, 2014). A portion of the GLP1 that reaches the portal circulation will contact the receptors present in the afferent roots of the vagus nerve, which will project its satiating effect on the NST in the brainstem. In turn, the NTS neurons are projected into several areas of regulation of intake, most of which also contain GLP1 receptors, including the ventral tegmental area and the nucleus accumbens.

Another part of the intestinal GLP1 will reach the HPT through the systemic circulation and permeable areas of the blood–brain barrier (BBB). In the arcuate nucleus, the neurons that produce POMC/CART have a higher density of GLP-1 receptors than the NPY/AgRP neurons, enhancing its satiating effect (Secher *et al.*, 2014).

Peptide YY

The peptide YY (PYY), also known as peptide tyrosine tyrosine is a peptide encoded by the gene PYY in humans. PYY is a short peptide of 36 amino-acid released from enteroendocrine cells in the ileum and colon in response to feeding (Adrian *et al.*, 1985). PYY, under the presence of lipids and carbohydrates, enhances a comparatively modest effect on satiety (Batterham *et al.*, 2002).

Peripheral Signals From Currently Available Metabolic Fuels

In human adults, changes in body weight are mainly attributable to fluctuations in adipose tissue. The regulation of this variability is partly explained by four hormones that circulate at levels proportional to fat mass, and participate in the control of food intake and energy expenditure. Leptin is the only one secreted by the adipocytes themselves; the other three are insulin, ghrelin and amylin.

Leptin

Leptin is a 16 kD hormone containing 146 amino acids that is encoded by the ob gene. Leptin is synthesized in the periphery mainly by white adipose tissue. In well-nourished humans, plasma leptin concentration is proportional to body fat mass (Ahima *et al.*, 2000). Therefore, its main role is to signal the amount of stored energy as adipose tissue, and consequently, to regulate energy intake and expenditure to maintain body weight (Stieg *et al.*, 2015; Farr *et al.*, 2015). At higher levels of body fat, increased circulating levels of leptin will limit food intake and increase energy outflow. This effect is facilitated by a saturable leptin transport system through the BBB. Once leptin has passed the BBB, it binds to its receptors (Ob-Rb) in several brain regions, mainly in the arcuate nucleus, where suppresses appetite by enhancing POMC/CART neurons and inhibiting NPY/AgRP neurons (Malik and Young, 1996; Zhang *et al.*, 1994; Pinto *et al.*, 2004). Similarly, in addition to its long-term lipostatic function, short-term famine results in severe decreases of leptin levels (Chan *et al.*, 2003). So, at lower levels of body fat, leptin circulates at lower levels increasing food intake and decreasing energy expenditure. Another known effect of leptin is also that enhances the satiating effect of CCK.

An inconsistent finding in the relationship between leptin and it strong regulatory effect in energy homeostasis occurs in obese patients, that show increased serum leptin concentrations. It has been suggested that, analogous to the reduced effects of insulin in people with insulin resistance and type 2 diabetes who overcome hyperinsulinemic, obese people develop a leptin resistance that impedes its homeostatic effects.

Mutations in the leptin gene cause an insatiable appetite, morbid obesity and numerous clinical abnormalities, all of which are corrected by leptin administration.

Insulin

The role of insulin is not limited to act as a key glucostatic regulator controlling peripheral glucose uptake. Insulin is also a circulating afferent signal, whose concentrations correlates with body fat mass, and shows many anabolic central effects, in synergism with leptin (Moehlecke *et al.*, 2016). Insulin enters by receptor-mediated processes in the BBB in the CNS, where its receptors are expressed in the hypothalamus and elsewhere (Banks and Kastin, 1998). When insulin activates its receptors in the arcuate nucleus stimulates the POMC/CART and inhibits the NPY/AgRP neurons to finally decrease food intake. In fact, mice without insulin receptors in the CNS develop hyperphagia and fat deposition (Obici *et al.*, 2002). However, obese individuals become resistant to insulin, similar to leptin, and remain hyperphagic despite high circulating levels of both hormones (Heymsfield *et al.*, 1999).

Amylin

Amylin is co-secreted with insulin from the pancreatic β-cells in response to nutrient stimuli during a meal, and suppresses postprandial blood glucose levels (Mietlicki-Baase, 2016; Scherbaum, 1998; Betsholtz *et al.*, 1989). However, amylin also has potent effects on energy balance, and acts within the brain to reduce food intake and body weight, promoting negative energy balance (Mietlicki-Baase and Hayes, 2014; Lutz, 2005). These effects are mediated via direct action in the central nervous system

(Lutz *et al.*, 1995) as amylin cross the blood–brain barrier to suppress food intake (Banks and Kastin, 1998) to activate its receptors. Amylin binding sites are distributed widely throughout the brain, mainly in the area postrema but also in hypothalamic nucleus and caudal brainstem (Lutz, 2012).

Integration of Peripheral Signals Through the Vagus Nerve

The vagus nerve is the 10th cranial nerve, is composed of both afferent (visceral sensory nerve) and efferent (motor nerve) fibers, and is an essential pathway in the regulation of food intake and energy metabolism (Berthoud and Neuhuber, 2000; Ueno and Nakazato, 2016). The vagus afferent nerve is a bipolar neuron with the cell body located in the nodose ganglion, exhibiting a projection to one to various peripheral organs and another to the NTS. That means that peripheral nerve terminals of the vagal afferent fibers distributed along the mucosal and submucosal layers of the digestive tract will exchange integrated nutritional information with the CNS. In addition, mucosal afferents terminals are most abundant in the upper small intestine, near to mucosal enteroendocrine cells (Powley *et al.*, 2011). Finally, it should be remembered that vagal afferent neurones signal in the NTS via glutamate and the neuropeptide cocaine- and amphetamine-regulated transcript (CART).

Multiple receptors are co-expressed on one cell body in the vagus nerve, and affect food intake in an additive, synergistic or antagonistic manner. Therefore, vagal afferents are broadly sensitive in a dose-dependent manner to gastrointestinal signals, including peptides produced by endocrine cells in the gut wall, the presence of chemically distinct nutrients within the gastrointestinal tract as well as gastroduodenal distension. Any abnormality in this essential pathway between the gastrointestinal tract and the CNS will lead to disrupted transmission of signals for appetite regulation.

Most GI peptides activate their receptors on vagal afferent nerve endings in a mostly paracrine manner increasing vagal afferent trigger. This vagal activation causes satiation and meal termination (Raybould, 2007). Ghrelin, in contrast, is released from the stomach and inhibits vagal afferent firing (Date, 2012). In addition, gastrointestinal peptides like CCK and GLP1 also enter the bloodstream and act as a neurotransmitter within the brainstem to affect vagal afferent and efferent responses. Although the contribution of paracrine versus non-paracrine modulation of vagal neurocircuits is not clear, gastrointestinal peptides seem to be able to exercise coordinated but temporally dissimilar actions to regulate vagally dependent functions.

The Neuroanatomy of Eating Regulation

Many brain areas are involved in a hierarchical mode in the neural regulation that regulates body weight, appetite, and energy homeostasis. In humans, the last integrator of metabolic information and hedonic drives that regulates food consumption is the hypothalamus, which contains many microscopically defined nuclei and interconnected areas, that are involved in the control of eating (Münzberg *et al.*, 2016; Berthoud *et al.*, 2017).

Brainstem and the Nucleus Tractus Solitarius

The brainstem is the second main port from vagal and other neural sensory signals that carry information pertaining to energy balance from the alimentary organs to the brain. Vagal afferents synapse on the brainstem to enhance or inhibit neurones in the NTS (Broberger, 2005). From the brainstem, projections fan further into the brain to involve other regions in the initiation and organization of food intake.

The Arcuate Nucleus

First, hormonal signals reflecting the availability and demand for metabolic fuel is relayed via neurones in the hypothalamus. The receptors for these signals are primarily expressed on two neurochemically distinct sets of neurones located in the arcuate nucleus (Arc) in the mediobasal hypothalamus, alongside the third ventricle [40]. One neurone group expresses neuropeptide Y (NPY); increasing NPY release or activation of these neurones by a variety of approaches results in increased food intake and decreased energy expenditure. The other group expresses the neuropeptide precursor pro-opiomelanocortin (POMC), which is processed to melanocortin peptides; activation of these neurons has the opposite effect of triggering the NPY cells, that is, decreased food intake and increased energy expenditure.

From the NTS signal reflecting the availability and demand for metabolic fuel will go to the arcuate nucleus, that lies around of the base of the third ventricle in the mediobasal hypothalamus. The ARC nucleus contains two distinct set of neurons that are crucial for controlling food intake and regulating body weight. The formers are the “hunger” neurons NPY/AgRP. They express two orexigenic peptides, mainly the neuropeptide Y (NPY) and also the Agouti-related protein (AgRP). Neuropeptide Y is one of the most potent feeding stimulators known, and translates the low nutrient availability into motivated behavioral action: increase appetite and decrease energy expenditure (Schwartz, 2005).

The second are the appetite-suppressing POMC/CART neurons that expresses the neuropeptides pro-opiomelanocortin (POMC) and CART. The triggering of these neurons transforms POMC, a large precursor protein, in several bioactive peptides,

mainly the alpha-melanocyte stimulating hormone (alpha-MSH). The alpha-MSH produced by POMC neurons suppresses appetite by acting as an agonist of the melanocortin 3 and 4 receptors (MC3R and MC4R) (Ollmann *et al.*, 1997). In fact, humans carrying the MCR4 mutation present with hyperphagia and obesity (Farooqi *et al.*, 2003).

In the arcuate nucleus, the AgRP/NPY neurons are able to control the activity of the POMC/CART cells via two mechanisms. First, the AgRP is an endogenous melanocortin antagonist in the MC4R. Thus, at the axon terminal, melanocortin action can be blocked by simultaneous release of AgRP. Secondly, at the cell body level, POMC neurones are innervated by NPY-ergic terminals and express the Y1 receptor, through which NPY causes a powerful inhibitory effect. Surprisingly, no reciprocal innervation has yet been described.

In addition, both groups of neurons are modulated by peripheral hormonal signals (insulin, leptin, ghrelin, and GLP1) that are transported by the specialized BBB of the arcuate nucleus to receptors on one or both populations: the AgRP/NPY population express receptors for the three peptides, whilst the POMC/CART neurons express receptors for leptin, insulin and GLP1. Insulin and leptin receptors serve to inhibit the transcription of NPY and increase POMC mRNA levels through differential second messenger systems. Furthermore, the NPY/AgRP neurons also release gamma amino butyric acid (GABA) and suppress POMC neurons through the GABA receptor (Wu *et al.*, 2009).

Finally, the axons of POMC/CART neurons and NPY/AgRP neurons have extensive projections to other nuclei of the hypothalamus, involved in eating as the lateral hypothalamic area (LHA) and the paraventricular nucleus, the ventromedial nucleus, the lateral nucleus and the dorsomedial nucleus. In additions, there are also projections to other non-hypothalamic brain regions like the corticolimbic system and the brainstem.

The Lateral Hypothalamic Area

The effects of POMC/CART and AgRP/NPY on food intake depend on subsequent excitatory effects in the lateral hypothalamic area (LHA) (Stratford and Kelley, 1999). This is an anatomically diffuse area that contains independent sets of neurons that express NPY and alpha-MSH receptors (MCR4). In response to these inputs, the LHA set of neurons secrete two eating-stimulating peptides: orexin A (also called hypocretin) and melanin-concentrating hormone (MCH) (Broberger *et al.*, 1998). In rodents, intracerebroventricular injection of MCH induces feeding. While fasting upregulates MCH mRNA expression (Qu *et al.*, 1996). MCH knockout mice are hypophagic and lean (Shimada *et al.*, 1998) whereas mice that overexpress MCH are obese and hyperleptinemic (Ludwig *et al.*, 2001). Moreover, the LHA also contains neurons that may sense serum long-chain fatty acids and glucose. On the other hand, the orexin neurons also project back to the ARC nucleus.

The Paraventricular Nucleus

The paraventricular nucleus (PVN) receives neuronal inputs from the arcuate nucleus and the HLA (Maejima *et al.*, 2009; Arletti *et al.*, 1989). Its neurons express receptors for NPY and alpha-MSH, as well as for other neurotransmitters that affect eating, mainly peptides suppressors of appetite such as CCK (CCK-R2), orexin A (ORX-1R), oxytocin, corticotrophin releasing hormone (CRH), nesfatin, thyrotropin releasing hormone (TRH), and serotonine. Neuronal projections to histamine neurons that suppress appetite are also observed in the PVN.

The Ventromedial Hypothalamic Area

The ventromedial nucleus (VMN) of the hypothalamus is considered a "satiety center." In concordance, the VMN expresses high levels of the leptin receptor and MC4R, together with the CCK-2 receptor. In addition, in the VMN there are also neurons containing other eating-inhibitory peptides, such as the brain-derived neurotrophic factors (BDNF), the steroidogenic factor 1 (that up-regulates leptin receptors expression), and the pituitary adenylate cyclase-activating peptide (that activates the POMC/CART neuron in the ARC nucleus (Mounien *et al.*, 2009; Kim *et al.*, 2011; Matsuda *et al.*, 2013; Choi *et al.*, 2013).

The Dorsomedial Hypothalamus Area (DMH)

The role of the dorsomedial hypothalamus (DMH) area is less known. Neurons producing NPY have been described in this hypothalamic area, with projections to the PVN. This PNY neurons contains CCK-1 receptors, and are tonically inhibited from the CCK released from neurons rather than the CCK derived from the periphery. Sustained exercise seems to be another cause of tonic inhibition of these neurons (Moran and Bi, 2006).

The Corticolimbic System

The corticolimbic system consist of large cortical areas, basal ganglia, hippocampus, and amygdala. This system is intimately connected to the hypothalamus and brainstem and provides the emotional, cognitive, and executive support for ingestion behavior (Kelley *et al.*, 2005; Murray and Rudebeck, 2013).

Food Reward

Feeding has an important motivational component. Food cues (visual and olfactory stimuli) and food consumption (gustatory stimuli, gastrointestinal signals) are naturally pleasant for humans, and typically acts on the reward pathways in the brain (Flier, 2004). These pathways are based in the dopaminergic neurons which originate in the ventral tegmental area (VTA) and substantia nigra in the midbrain (Burger and Stice, 2011). These neurons expand through the human brain to the main areas involved in the reception and integration of these dopaminergic inputs: the nucleus accumbens, striatum, and orbitofrontal cortex (Blum *et al.*, 2014). In fact, PET studies have consistently reported lower availability of dopamine receptors in the striatum of obese subjects as compared to normal weight individuals. These data suggest that lower dopaminergic signaling may lead to the search for highly rewarding foods (high calorie or high fat) and this in turn, leads to obesity.

A Last Remark

Few tasks hold greater survival value than keeping us fed and in adequate nutritional state. Most importantly, control of eating in human brain not only includes the homeostatic brain systems (hypothalamus), but also attention systems (including the parietal and visual cortices), emotion and memory systems (such as the amygdala and hippocampus), cognitive control (including the prefrontal cortex), and the reward network (including the VTA and striatum). It must be remembered that, much of neural processing within this core processor occurs outside awareness, rendering it relatively inaccessible to conscious manipulation.

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Adipose Tissue

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Introduction

A leading reason for the success of the evolution of humans is our adaptability. Humans have favorably evolved to survive in almost all environments and circumstances. Even if in some occasions the lack of food has been substantial, the human body has always built-in different defences against starvation like our own storage of fat. Traditionally, the adipose tissue (AT) was simply defined as a mere storage organ for excess calories in the fed state from which free fatty acids (FFA) are released during fasting to fuel the energy demands of the organism (Scherer, 2006; Rosen and Spiegelman, 2014). Research over the past two decades has revealed AT as one of the largest endocrine organs in the body as well as an active tissue for cellular reactions with a key role in energy homeostasis secreting and expressing a wide range of biologically active molecules, which are known as adipokines (Fain *et al.*, 2004; Rodríguez *et al.*, 2007; Ibrahim, 2010). Evidence further shows that AT is also an important site expressing different receptors involved in adipokine-related metabolic processes exerting autocrine and paracrine actions. Adipokines may be also released into the systemic circulation showing further endocrine effects (Vona-Davis and Rose, 2007; Dunmore and Brown, 2013).

Obesity, defined as an excess of adiposity due to a prolonged status of positive energy balance, leads not only to changes in AT distribution but also to alterations in the metabolism as well as in the adipokines and lipid secretion profiles (Gesta *et al.*, 2007). The factors that determine AT mass and volume in adult humans are not totally understood, however, the increased lipid storage in the adipocytes due to an unhealthy lifestyle is thought to be an important reason (Björntorp, 1974; Hirsch and Batchelor, 1976; Frühbeck, 2012). The distribution of the different fat depots inside the human body is also an important factor influencing the development of obesity-associated comorbidities (Rodríguez *et al.*, 2007; Ibrahim, 2010). In this regard, a relationship between the distribution of central and upper body fat storages with metabolic diseases such as insulin resistance (IR), high blood pressure, dyslipidemia, fatty liver, cardiovascular diseases (CVD) or even cancer has been demonstrated in multiple studies, leading to a decreased life expectancy (Mokdad *et al.*, 2003; Angulo, 2006; Moore, 2010; Trayhurn, 2013; Van Gaal *et al.*, 2006; Landsberg *et al.*, 2013; Calle and Kaaks, 2004; Picon-Ruiz *et al.*, 2017).

Characteristics of Adipose Tissue

In mammals, three types of AT can be distinguished: white, brown and beige or "brite" (brown in white) that differ in their origin, morphology, location, and function (Frühbeck *et al.*, 2009a). White adipose tissue (WAT) is a heterogeneous tissue and its different anatomical locations determine its metabolic identity and core functions (Kusminski *et al.*, 2016; Frayn *et al.*, 2003; Lee *et al.*, 2013a). In humans, WAT mainly consists in a central intraabdominal component (visceral adipose tissue [VAT]), which is associated with metabolic disease risk and a peripheral subcutaneous AT depot (SAT), associated with protective effects on energy homeostasis (Rodríguez *et al.*, 2007; Fox *et al.*, 2007; Koster *et al.*, 2010; Björntorp, 1988). The major role of WAT is related to maintaining energy homeostasis by storing triglycerides and releasing fatty acids for energy synthesis. However, it is well-established that WAT also controls a wide variety of functions including immune and inflammatory regulation, glucose and lipid homeostasis, food intake control or metabolism by secreting a great number of adipokines (Catalán *et al.*, 2009a; Falcão-Pires *et al.*, 2012). Energy-storing white adipocytes exhibited a variable size (25–200 μm) and contain a single, large and unilocular lipid droplet surrounded by a layer of cytoplasm. Within the adipocyte, the nucleus is flattened and located on the periphery and few mitochondria with a low oxidative rate are found (Luo and Liu, 2016; Jeanson *et al.*, 2015; Frühbeck *et al.*, 2009b). Furthermore, these developed and spherical white adipocytes are well-vascularized by anatomically surrounding vessels. Cells are densely distributed and are subdivided into small lobules that are innervated by, both, the sympathetic and parasympathetic nervous systems.

Brown adipose tissue (BAT) is the main site of nonshivering thermogenesis and energy expenditure in mammals (Feng *et al.*, 2013; Cannon and Nedergaard, 2004; Villarroya *et al.*, 2017). BAT is a remarkably plastic tissue and its depots enlarge via hypertrophic and hyperplasic processes when thermogenesis is activated (Cannon and Nedergaard, 2004). Contrary to the initial concept that BAT is restricted in humans to neonates and young children, recent studies using positron emission tomography imaging techniques have documented the presence of functional BAT in adult humans, being mainly located in the ventral neck, the supraclavicular area as well as in mediastinum, paravertebral and suprarenal fat (Frühbeck *et al.*, 2009b; Virtanen *et al.*, 2009; Schulz *et al.*, 2011; Cypess *et al.*, 2009). Reportedly, BAT has been inversely correlated with BMI (Cypess *et al.*, 2009). The specific secretor profile of BAT is quite distinct from that of WAT, probably due to the different and largely opposite, physiological roles in

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energy metabolism (Villarroya *et al.*, 2017). The brown adipocytes are characterized by expressing uncoupling protein 1 (UCP-1), which through the oxidative phosphorylation for ATP synthesis dissipates energy as heat, constituting a key regulator of thermogenesis (Wu *et al.*, 2012). Energy-expending brown adipocytes are cells with small lipid droplets in a multilocular pattern and contain many mitochondria with high oxidative capacity (Lynes and Tseng, 2017; Ricquier *et al.*, 1982). The higher amount of mitochondria compared to WAT favors its brownish color. BAT is highly vascularized and innervated by the sympathetic nervous system, promoting the dissipation of heat via blood vessels (Frühbeck *et al.*, 2009b; Park, 2014).

Recently, the existence of a third kind of AT has been proposed, the beige adipose tissue (Wu *et al.*, 2013). Sustained thermogenic activation induced by prolonged cold exposure leads to the "browning" of WAT, with the emergence of brown adipocyte-like cells in WAT depots (Wu *et al.*, 2012; Siersbæk *et al.*, 2012). The browning process is regulated by a complex hormonal interplay and many environmental factors such as chronic cold exposure, exercise, and environmental enrichment (Schulz *et al.*, 2013; Bartelt and Heeren, 2013; Jankovic *et al.*, 2015; Sidossis and Kajimura, 2015). Beige adipocytes are most abundant in the inguinal WAT, which is a major subcutaneous depot in rodents (Vitali *et al.*, 2012). The amount of beige adipocytes in different human WAT depots has not been carefully determined (Harms and Seale, 2013; Elabd *et al.*, 2009). These brite or beige cells exhibit different gene expression patterns compared to those of white or brown adipocytes (Svensson *et al.*, 2011; Shabalina *et al.*, 2013). Importantly, the thermogenic profile of beige adipocytes is reversible. On warm adaptation, former brite adipocytes change their morphology and their gene expression profile to that of a white adipocyte (Rosenwald *et al.*, 2013).

Cellular Heterogeneity of Adipose Tissue

Although adipocytes are the main cells of the AT as well as the responsible for energy homeostasis control and adipokine release, this connective tissue is formed by other different cellular types including preadipocytes, immune cells, nerve cells, and vascular cells that interplay among them contributing to the main functions of each AT depots (Lynes and Tseng, 2017).

During normal AT turnover, mature adipocytes are replaced by newly differentiated and mature adipocyte progenitors or preadipocytes in a process called adipogenesis (Shan *et al.*, 2017). In adult humans, an adipocyte turnover of approximately 10% is observed (Spalding *et al.*, 2008). Preadipocyte functions in fat depots comprise the regulation of extracellular matrix (ECM) remodeling and the secretion of endocrine factors. Immune cells are also important cellular components present within the varied AT cellularity. Adipose tissue-resident macrophages (ATM) were the first immune cells described in AT showing a close association with obesity (Weisberg *et al.*, 2003). AT is innervated by a neuronal mesh that regulates processes such as lipolysis or thermogenesis. In cold environments, nerve cells comprising the neuronal mesh activate the sympathetic neural system in the parenchymal of WAT, leading to the release of FAA via lipolysis and also triggering BAT thermogenesis (Locke *et al.*, 2015; Garretson *et al.*, 2016). Equally, WAT is connected to other organs, including the brain, by sensory nerve afferent networks (Bamshad *et al.*, 1998; Bartness *et al.*, 2014). Vascularization of fat depots relies on endothelial and smooth muscle cells. Interestingly, high adipocyte precursor areas are highly vascularized compared to other sections of the AT, predicting the existence of a relationship between mature adipocyte turnover and vasculature. Adipocyte metabolic processes are also dependent on the adequate uptake or release of nutrients, with blood vessels being fundamental for this process (Sotornik *et al.*, 2012; Abreu-Vieira *et al.*, 2015).

Origin of Adipose Tissue

Each AT depot has a unique developmental origin with a different gene signature, lipid storage capacity and adipokine profile (Frühbeck *et al.*, 2009b; Tchkonia *et al.*, 2013). Although white and brown adipocytes derive from the same mesenchymal stem cells (Enerbäck, 2009), during gastrulation, the mesenchymal stem cells of the paraxial mesoderm express the myogenic transcription factor Myf5 and become brown adipocytes or myocytes. However, the mesenchymal stem cells of the lateral mesoderm do not express Myf5 and differentiate into white adipocytes or blood vessel-associated pericytes (Rodríguez *et al.*, 2015).

White adipogenesis is a tightly regulated two-step process involving multiple factors. Firstly, a pluripotent stem cell is differentiated into a unipotent adipocyte precursor or preadipocyte, which subsequently is differentiated into a phenotype of mature adipocytes achieving their corresponding functional properties. Peroxisome proliferator-activated receptor (PPAR)-γ, a member of the nuclear-receptor superfamily, has been identified as a master regulator of white adipogenesis (Rosen *et al.*, 2000). Whereas the deficiency of PPAR-γ, fails to promote adipogenesis, its overexpression is sufficient to induce adipocyte differentiation in fibroblasts (Tontonoz *et al.*, 1994; Koutnikova *et al.*, 2003). Other factors or pathways including pro-adipogenic factors such as C/EBPs and Krüppel-like factors (KLFs) regulate adipogenesis via PPARγ-dependent mechanisms (Luo and Liu, 2016). Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β superfamily, also play an important role in this transformation due to their regulatory function by boosting the differentiation of pluripotent stem cells to chondrocytes, osteoblasts or adipocytes (Bandyopadhyay *et al.*, 2013). BMP-2 and BMP-4 through their binding to two distinct serine/threonine kinase receptors induce the phosphorylation of SMAD-1, -5 and -8 that form complexes with SMAD-4 that move into the nucleus, regulating the transcription of target genes involved in white adipogenesis (Miyazono *et al.*, 2005). BMPs also mediate their signals through SMAD-independent pathways including the MKK3/p38 MAPK and TAK1 signaling pathways, regulating the expression of BMP target genes. Nonetheless, other transcription factors repress adipogenesis such as the antiadipogenic GATA transcription factor.

Adipogenesis of brown adipocytes is totally different from that of white adipocytes. The activation of both, BMP-7 and PR domain 16 (PRDM16), is necessary to promote BAT adipogenesis (Harms *et al.*, 2014). PRDM16 selectively initiates the switch of myoblasts to brown adipocytes by forming a transcriptional complex with C/EBPβ and by suppressing white adipocyte-specific genes forming complexes with C-terminal binding proteins CTBP1 and CTBP2 (Kajimura *et al.*, 2008) and also by the upregulation of brown fat-specific markers, such as PRDM16, PPAR coactivator 1 (PGC-1) α/β and UCP1 (Rodríguez *et al.*, 2015).

Dysfunctional White Adipose Tissue in Obesity

Obesity has reached epidemic proportions constituting one of the most important diseases of this century (Bray *et al.*, 2016; NCD Risk Factor Collaboration (NCD-RisC), 2017; GBD 2015 Obesity Collaborators, 2017; Anon, 2006). Despite body mass index (BMI) being the most widely used method to diagnose and define obesity, recent studies have proposed that BMI is only a surrogate for body fatness and does not provide an accurate measurement of body composition, misclassifying subjects with increased cardiovascular risk factors (Blundell *et al.*, 2014). In this regard, other indicators to determine the prevalence of obesity have been proposed with body fat percentage being one of the most useful due to its superior ability to stratify patients according to their metabolic and cardiovascular risks (Gómez-Ambrosi *et al.*, 2011, 2012). Thus, in adults, normal body fat levels are considered to be between 10%–20% and 20%–30% in males and females, respectively (Gómez-Ambrosi *et al.*, 2011).

During obesity, AT becomes severely dysfunctional and fails to appropriately expand to store the surplus energy being associated with disrupted metabolic homeostasis and promoting the obesity-associated metabolic alterations including type 2 diabetes mellitus (T2D) (Mokdad *et al.*, 2003), nonalcoholic fatty liver disease (NAFLD) and other chronic liver diseases such as cirrhosis (Angulo, 2006; Moore, 2010), obstructive sleep apnoea (OSA) (Trayhurn, 2013) as well as cardiovascular diseases (CVD) including cerebrovascular disorders and hypertension (Van Gaal *et al.*, 2006; Landsberg *et al.*, 2013). Furthermore, emerging evidence relates obesity with several types of cancer (Calle and Kaaks, 2004; Picon-Ruiz *et al.*, 2017).

Adipose Tissue Expansion: Hyperplasia and Hypertrophy

Adipocyte hyperplasia (increase in cell number) and hypertrophy (increase in cell size) can both contribute to AT expansion (Sun *et al.*, 2011). Small variations in the adipocyte cell number during adulthood has been postulated, suggesting that the number of adipocytes for lean and obese individuals is set during childhood and adolescence and that any alterations in fat mass during adulthood are due to alterations in adipocyte hypertrophy (Spalding *et al.*, 2008; Arner *et al.*, 2011). The total number of adipocytes in AT appears to be tightly controlled with the rate of newly emerging adipocytes being regulated by adipocyte death. Alterations in two forms of programmed cell death, apoptosis and autophagy, are closely related to AT dysfunction. Autophagy is a complex evolutionarily conserved cellular process that targets intracellular components of lysosomal degradation involving many autophagy-related genes (ATGs) (Maixner *et al.*, 2016; Ezquerro *et al.*, 2017). Autophagy is thought to be upregulated in human AT in obesity particularly in patients with insulin resistance and/or T2D, being also higher in the visceral depot (Maixner *et al.*, 2012; Kosacka *et al.*, 2015). However, controversial results about the topic with other studies showing obesity-related attenuated autophagy in adipocytes have been reported (Soussi *et al.*, 2015; Yoshizaki *et al.*, 2012).

Adipose Tissue Inflammation

Obesity is characterized by a chronic state of low-grade inflammation (Wellen and Hotamisligil, 2003). The interaction between immune cells and adipocytes leads to inflammation and subsequent AT dysfunction accompanied by changes in adipokine production profile (Nishimura *et al.*, 2009).

Immunometabolism and adipocyte-derived cytokines

AT contains different types of immune cells including macrophages, lymphocytes, mast cells, eosinophils, neutrophils, and foam cells (Rodríguez *et al.*, 2015; Olefsky and Glass, 2010; Shapiro *et al.*, 2013) that establish complex interactions with adipocytes leading to the development of inflammation and subsequent AT dysfunction. Immune activated cells infiltrate the AT in a chronological sequence regulated by both, the innate and the adaptive immune systems (Catalán *et al.*, 2013a).

– *The innate immune system* provides plenty of different pro-inflammatory cells with macrophages and monocytes being the most representative immune cells (McFarlin *et al.*, 2012; Dey *et al.*, 2015; Dalmas *et al.*, 2011). Growing evidence reveals that AT from obese subjects is markedly infiltrated by macrophages that participate in inflammatory pathways with important roles in obesity-associated comorbidities (Weisberg *et al.*, 2003). Reportedly, infiltrated macrophages in AT depots from obese patients account for almost 50% of the fat depot, as opposed to the 5% observed in lean individuals (Weisberg *et al.*, 2003; Xu *et al.*, 2003; Kanneganti and Dixit, 2012). Depending on the secretion profile of cytokines and markers present on their surface, two phenotypes of macrophages can be differentiated: M1 and M2 macrophages (Bai and Sun, 2015). The proliferation of M1 cells might be stimulated in an intrinsic manner by the secretion of internal proteins such as interferon (IFN)-γ or tumor necrosis factor (TNF)-α and in an extrinsic manner, by lipopolysaccharides (LPS) from bacterial walls, constituting one of the most potent inducers of M1 differentiation (Rodríguez *et al.*, 2015; Festuccia *et al.*, 2014). Oppositely, the switching to the

antiinflammatory phenotype M2 is influenced by the production of specific immunosuppresive agents including arginase or different interleukins (IL) [IL-10, IL-4, IL-13, IL-33, and IL-1Ra] (Bai and Sun, 2015). A phenotype switch from M2 to M1 macrophages in the AT of obese subjects has been described, leading to increased levels of inflammatory markers (Lumeng *et al.*, 2007). Mast cells, eosinophils and neutrophils are other cells from the innate immune system (Ferrante, 2013; Hotamisligil, 2006). The role of these types of cells during inflammation in dysfunctional AT has not been totally determined. Mast cells, predominantly localized in fibrotic fat depots, have been proposed to secrete IL-6 and IFN-γ favoring inflammation and strengthening their link with the pathophysiology of obese AT (Ferrante, 2013; Divoux *et al.*, 2012). Eosinophils could exert an important protection against metabolic disorders via the secretion of the antiinflammatory cytokines IL-4 and IL-13 (Nakamura *et al.*, 2014). Finally, neutrophils act as precursors of inflammatory processes by secreting chemokines and cytokines that help the infiltration of macrophages in the AT.

- *The adaptive immune system*. On the other hand, B- and T-lymphocytes constitute the most important immune cells of the adaptive immune system. The vast majority of T lymphocytes can be classified in CD4+ and CD8+ cells with important functions in the regulation of inflammation of AT by regulating not only effective immune responses to pathogens but also macrophage differentiation, activation and migration. Important subgroups of obesity-related CD4+ T-lymphocytes have been recently identified in AT (Couturier *et al.*, 2015; Bornstein *et al.*, 2000). T-helper 1 cells (Th1), with a pro-inflammatory profile, are responsible for the secretion of IFN-γ, stimulating macrophage differentiation and increasing their infiltration into the AT in the obese state. Oppositely, T-helper 2 cells (Th2) are activated to exert an antiinflammatory role by the expression of IL-4 and IL-13. T regulatory cells (Treg) have been recently described in AT regulating the activity of macrophages and adipocytes by secreting IL-10. In the case of obesity, a decrease in the number of Treg cells has been described, promoting the infiltration of macrophages into the AT and increasing the production of pro-inflammatory cytokines (McLaughlin *et al.*, 2014; Lee and Lee, 2014; Rocha *et al.*, 2008; Hansson and Libby, 2006). The presence of CD8+ cells in hypoxic areas of the AT reinforces the hypothesis about their involvement in the recruitment and activation of macrophages. In this line, the removal of CD8+ cells in genetic knock-out animal models was associated with an improvement of the inflammatory state and the insulin sensitivity of the AT (Nishimura *et al.*, 2009; Khan *et al.*, 2014). B lymphocytes have been characterized as the precursors of metabolic abnormalities due to their accumulation into inflamed areas of AT before the arrival of T lymphocytes. B cells promote T cell activation and the production of pro-inflammatory cytokines, that also favors the polarization of M1 macrophages (DeFuria *et al.*, 2013; Winer *et al.*, 2014). Natural killer T (NKT) cells have been also related to the development of obesity-associated diseases. NKT cells are mainly located in AT and liver being of special interest to disentangle their activation sequence in order to get insight into the mechanisms involved in the crosstalk between the AT and the liver (Catalán *et al.*, 2013a; Ferrante, 2013; Satoh *et al.*, 2012).

Dysregulated adipokine profile in obesity

Adipose tissue acts as an important endocrine organ via the synthesis of several adipokines, which regulate insulin sensitivity, control of energy intake or inflammatory processes (**Fig. 1**) (Frühbeck and Gómez-Ambrosi, 2001; Frühbeck, 2006a). Obesity affects the secretion profile of adipokines leading to alteration in multiple physiological processes (Jung and Choi, 2014; Ahima and Osei, 2008). The identification of leptin and adiponectin were the first indications that AT is an endocrine organ with control over systemic energy homeostasis (Stern *et al.*, 2016). In addition to the well-known role of these classic adipokines, new adipokines involved in the regulation of multiple physiological processes have emerged in recent years (**Table 1**).

- *Adiponectin*, also known as Acrp30, Adipoq, apM1 or GBP28, is a 30-kDa protein mainly expressed in AT, with considerably high circulating concentrations, accounting for approximately 0.01% of total serum protein (Scherer *et al.*, 1995; Kadowaki and Yamauchi, 2005). It was originally identified independently by four groups using different approaches (Scherer *et al.*, 1995; Hu *et al.*, 1996; Maeda *et al.*, 2012; Nakano *et al.*, 1996) while its insulin-sensitizing effect was first identified by three independent groups in 2001 (Yamauchi *et al.*, 2001; Berg *et al.*, 2001; Fruebis *et al.*, 2001). Adiponectin exerts a wide variety of biological effects mediated by its binding of at least three different transmembrane receptors, AdipoR1, AdipoR2, and T-cadherin, which are expressed in many tissues and activate the downstream targets AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor (PPAR)-α and p38 MAPK (Kadowaki and Yamauchi, 2005). Plasma adiponectin levels have been reported to be reduced in humans with obesity, particularly those with visceral obesity (Arita *et al.*, 2012; Weyer *et al.*, 2001). Hypoadiponectinaemia has been also associated with insulin resistance and patients with T2D are also reported to have decreased concentrations of adiponectin (Weyer *et al.*, 2001; Hotta *et al.*, 2000; Spranger *et al.*, 2003). On the contrary, adiponectin levels increase with weight loss (Yang *et al.*, 2001). Recently, circulating concentrations of adiponectin have been shown to be positively regulated by leptin ameliorating obesity-associated oxidative stress and inflammation in mice (Frühbeck *et al.*, 2017).
- *Leptin*, the *OB* gene product, is a16 kDa-secreted protein mainly produced by adipocytes (Zhang *et al.*, 1994) and directly correlated with adipose tissue mass (Beltowski, 2006). In AT, the secretion levels of leptin are strictly controlled to ensure an adequate regulation of food intake, energy expenditure, and, hence, body weight by its binding to specific hypothalamic receptors (Zhang *et al.*, 1994; Friedman and Halaas, 1998; Halaas *et al.*, 1995). Since its receptor is expressed in almost all tissues (Tartaglia *et al.*, 1995; Lee *et al.*, 1996; Frühbeck *et al.*, 1999), leptin shows a high functional pleiotropism involving glucose metabolism, energy homeostasis, angiogenesis, wound healing, immunity, reproduction, gastrointestinal function, bone remodeling, and cardiovascular function (Frühbeck *et al.*, 1998; Frühbeck and Salvador, 2000; Muruzábal *et al.*, 2002;

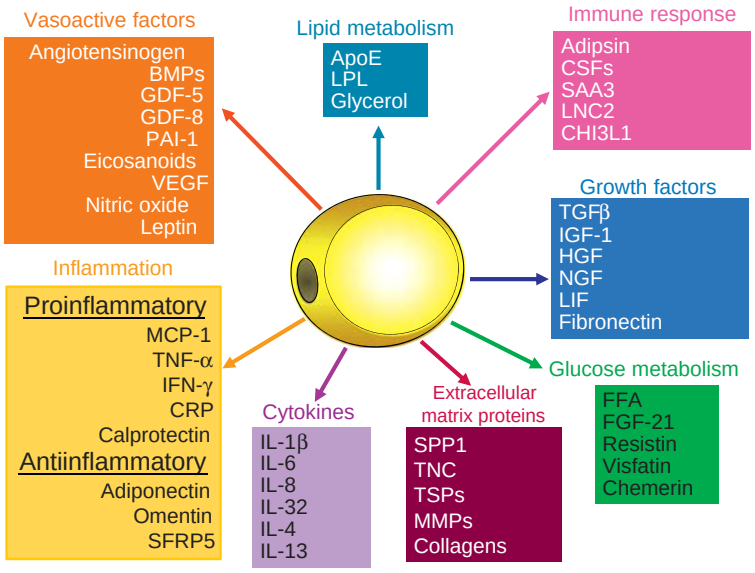

Fig. 1 Adipose tissue (AT) plays a crucial role in the regulation of whole-body homeostasis via the synthesis and release of adipocyte-specific factors known as adipokines. Much of the research in this area has focused on the study of classical cytokines and chemokines, vasoactive and coagulation factors, regulators of lipid and glucose metabolism and proteins specifically secreted by the adipocytes, such as leptin or adiponectin. However, the number of adipokines has notably increased in the last years with new molecules including visfatin, tenascin C, osteopontin, omentin or IL-32, demonstrating the complexity of the human adipokinome. *ApoE*, apolipoprotein E; *BMPs*, bone morphogenic proteins; *CHI3L1*, chitinase 3 like 1; *CRP*, C-Reactive protein; *CSFs*, colony stimulating factors; *FGF-21*, fibroblast growth factor 21; *FFA*, free fatty acid; *GDF-5*, growth/differentiation factor 5; *GDF8*, growth/differentiation factor 8; *HGF*, hepatocyte growth factor; *IGF-1*, insulin growth factor; *IFN-γ*, interferon γ; *IL-1β*, interleukin 1β; *IL-6*, interleukin 6; *IL-8*, interleukin 8; *IL-32*, interleukin 32; *IL-4*, interleukin 4; *IL-13*, interleukin 13; *LIF*, leukemia inhibitory factor; *LNC2*, lipocalin 2; *LPL*, lipoprotein lipase; *MCP-1*, monocyte chemoattractant protein 1; *MMPs*, metalloproteinases; *NGF*, nerve growth factor; *PAI-1*, plasminogen activator inhibitor 1; *SFRP5*, secreted frizzled-related protein 5; *SAA3*, serum amyloid A3; *SPP1*, osteopontin; *TNC*, tenascin C; *TGF-β*, tumor growth factor β; *TNF-α*, tumor necrosis factor α; *TSPs*, thrombospondins; *VEGF*, vascular endothelial growth factor.

Myers *et al.*, 2008; Gautron and Elmquist, 2011). Contrary to adiponectin, leptin levels are increased in obese individuals (Ryan *et al.*, 2003), being strongly correlated with BMI and the percentage of body fat, as well as with leptin mRNA expression in AT (Frühbeck *et al.*, 2001; Frühbeck, 2006b). Mice knockout for leptin and its receptor exhibit an early-onset obesity, diabetes and reduced energy expenditure (Lindström, 2007). Leptin replacement induces a dramatic loss of adipose mass in rodents (Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995) and a reduction in body weight mainly at the expense of the fat compartment in humans (Farooqi et al., 1999, 2002; Paz-Filho *et al.*, 2011). Furthermore, a low adiponectin/leptin ratio, hallmark of a dysfunctional AT, has been suggested to contribute to an increased oxidative stress and inflammation in humans (Frühbeck *et al.*, 2017).

- *Tumor necrosis factor (TNF)-α* is a recognized multifunctional regulatory cytokine that can regulate many cellular and biological processes such as inflammation, cell apoptosis, survival, and proliferation as well as the induction of insulin resistance and energy metabolism (Cawthorn and Sethi, 2008; Ruan and Lodish, 2003). TNF-α represents the first evidence that inflammatory proteins can mediate insulin resistance. Hotamisligil et al. reported that TNF-α was highly induced in the AT of obese animals (Hotamisligil *et al.*, 1993) and human subjects (Hotamisligil *et al.*, 1995) with adipocytes being the predominant source of TNF-α and also expressing both types of TNF-α receptors (Hotamisligil *et al.*, 1993; Montague *et al.*, 1998; Kern *et al.*, 1995). Further investigations have revealed that long-term exposure of cultured cells to TNF-α induces insulin resistance (Hotamisligil *et al.*, 1994a), whereas the blockade of TNF-α action in *fa/fa* Zucker rats can restore insulin sensitivity (Hotamisligil *et al.*, 1994b). Interestingly, TNF-α action on AT can modify the expression of different adipokines with relevant systemic effects of TNF-α on insulin sensitivity and whole body energy homeostasis. In this sense, TNF-α can regulate the production of other pro-inflammatory cytokines (IL-6 and IL-1) (Cawthorn and Sethi, 2008) as well as suppress the production of adiponectin (Ruan *et al.*, 2002); indeed, plasma levels of adiponectin are negatively associated with circulating levels of TNF-α (Bruun *et al.*, 2003). A main role of TNF-α on lipid metabolism in adipocytes by inhibiting the uptake of FFA and lipogenesis as well as by stimulating FAA release has been also described (Cawthorn and Sethi, 2008).
- *Interleukin-6* has been strongly associated with chronic inflammatory states, including the low-grade inflammation associated with obesity and T2D (Pradhan *et al.*, 2001; Bastard *et al.*, 2000, 2002; Wellen and Hotamisligil, 2005). However, increasing evidence points to a beneficial role of IL-6 in a variety of pathophysiological conditions, including functions related to obesity and insulin resistance (Mauer *et al.*, 2015). In support of this, *Il6* knockout mice develop systemic insulin resistance and late-onset obesity (Matthews *et al.*, 2010). Furthermore, an acute IL-6 treatment results in an increase in insulin-stimulated whole-body glucose disposal in humans (Carey and Febbraio, 2004; Carey *et al.*, 2006). Reportedly, IL-6 is also produced by

Table 1 Adipokines secreted by white adipose tissue (WAT)

Symbol	*Name*	*Metabolic function*
ADIPOQ	Adiponectin	Hormone with antidiabetic, antiatherogenic, and antiinflammatory properties
AGT	Angiotensinogen	Precursor molecule of angiotensin II, that causes vasoconstriction, increased BP and release of aldosterone from the adrenal cortex
APL	Apelin	Peptide implicated in the control of BP and one of the most potent stimulators of cardiac contractility; inhibits insulin secretion; involved in lipolysis
CCL2	C–C Motif chemokine ligand 2	Chemokines that promotes inflammation and monocyte chemotaxis
CFD	Adipsin	Protein involved in the activation of the alternative complement pathway
CHI3L1	YKL-40 (Chitinase 3 like 1)	Proinflammatory factor that stimulates innate immune system, extracellular matrix remodeling, and angiogenesis
CLU	Clusterin	Lipoprotein that promotes tumor progression, angiogenesis, and is involved in metabolic and cardiovascular diseases
CRP	C-reactive protein	Acute-phase reactant involved in inflammatory processes
CTF-1	Cardiotrophin-1	Cytokine involved in the hypertrophy of cardiomyocytes
CTS	Cathepsins	Cystein proteases that promote adipogenesis and extracellular matrix remodeling
CXCL8	Interleukin 8	Chemokine involved in the pathogenesis of atherosclerosis and cardiovascular diseases
CXCL10	C–X–C motif chemokine 10	Chemokine produced by T cells
FGF-21	Fibroblast growth factor 21	Hormone that stimulates glucose uptake into adipocytes, increases thermogenesis, energy expenditure and fat utilization
FIAF/ ANGPTL4	Angiopoietin like 4	Protein induced by fasting and hypoxia
FNDC5	Irisin	Myokine/adipokine involved in the promotion of myogenenesis and fat browning
GHRL	Ghrelin	Orexigenic and adipogenic hormone that exerts a depressor effect on BP control and acts as a cardioprotective agent
GRN	Progranulin	Chemoattractant protein involved in adipose tissue inflammation and neurodegenerative diseases
HAMP	Hepcidin	Pro-inflammatory cytokine that activates MMP-9
HGF	Hepatocyte growth factor	Factor that stimulates proliferation and development in adipocytes and antiinflamatory effects
HMGB1	High mobility group box 1	Alarmin involved in DNA repair and the secretion of insulin in pancreatic β cells
HP	Haptoglobin	Acute-phase reactant with angiogenic and chemotactic properties
HSPA1A	Heat shock protein 1A1	Damage-activated protein that induces neutrophil and natural killer cell recruitment
IGF-1	Insulin growth factor	Factor that stimulates proliferation and differentiation in adipocytes
IL-1 β	Interleukin 1 β	Proinflammatory cytokine involved in a paracrine inflammatory pathway in adipose tissue
IL-6	Interleukin 6	Proinflammatory cytokine implicated in acute-phase responses
IL-32	Interleukin 32	Proinflammatory cytokine involved in adipose tissue inflammation and remodeling
ITLN1	Omentin	Novel adipokine that modulates insulin sensitivity and exerts antiinflammatory properties
LEP	Leptin	Anorexigenic hormone with lipolytic and vasoactive effects in addition to other pleiotropic activities
MIF	Macrophage migration inhibitory factor	Factor involved in proinflammatory processes and immunoregulation
MMPs	Metalloproteinases	Proteins implicated in adipogenesis
NAMPT	Visfatin	NAD+ biosynthetic enzyme involved in the regulation of β pancreatic cells
NGAL/LNC2	Lipocalin-2	Adipokine showing antiinflammatory properties
NGF	Nerve growth factor	Neurotropin involved in the development and survival of sympathetic neurons
NUCB2	Nesfatin-1	Anorexigenic peptide also involved in the inflammatory response
PAI-1	Plasminogen activator inhibitor 1	Potent inhibitor of fibrinolysis that is implicated in the development of atherosclerotic plaques
PEDF	Pigment epithelium derived factor	Secreted glycoprotein that belongs to the noninhibitory serpin group with antiangiogenic, antioxidant, antiinflammatory, and lipolytic effects
RARRES2	Chemerin	Chemoattractant protein involved in adaptive and innate immunity as well as in adipogenesis
RBP4	Retinol binding protein 4	Factor involved in the development of insulin resistance, visceral fat distribution, and dyslipidemia
RETN	Resistin	Hormone involved in the development of insulin resistance, which participates in the proinflammatory response
S100A8, S100A9	Calprotectin	Proinflammatory factor involved in cell adhesion, chemotaxis, and antimicrobial activity
SAA	Serum amyloid A	Acute-phase reactant produced in response to injury, infection or inflammation
SERPINA12	Vaspin	Adipokine of the serine protease inhibitor family showing insulin-sensitizing effects
SPP1	Osteopontin	Proinflammatory factor involved in vascular and myocardial remodeling; cytokine implicated in insulin resistance, and cancer
SRFP5	Secreted frizzled related protein 5	Inhibitor of WNT5A signaling with antiinflammatory properties
STEAP4	Six transmembrane protate protein 2	Metalloreductase that plays a role in cellular import of copper and iron as well as in the reduction of macrophage recruitment and polarization towards the M1 phenotype

Table 1 Continued

Symbol	*Name*	*Metabolic function*
TIMP1	Tissue inhibitor of metalloproteinase 1	Inhibitor that decreases adipogenesis and impairs glucose tolerance
TNC	Tenascin C	Damage-activated protein that induces immune responses and extracellular matrix remodeling
TGF-β	Tumor growth factor β	Regulatory factor of preadipocyte proliferation, differentiation and apoptosis
TNF-α	Tumor necrosis factor α	Proinflammatory cytokine involved in systemic inflammation and the development of insulin resistance in obesity
VEGF	Vascular endothelial growth factor	Factor involved in angiogenesis stimulation in adipose tissue
WNT5A	WNT family member 5A	Secreted glycoprotein of the WNT family with antiadipogenic and proinflammatory actions

ADIPOQ, adiponectin; *AGT*, angiotensinogen; *APL*, apelin; *CCL2*, C–C motif chemokine ligand 2; *CFD*, adipsin; *CHI3L1*, chitinase 3 like 1; *CLU*, clusterin; *CRP*, C-reactive protein; *CTF-1*, cardiotrophin-1; *CTS*, cathepsins; *CXCL8*, interleukin 8; *CXCL10*, C–X–C motif chemokine 10; *FDF-21*, fibroblast growth factor 21; *ANGPTL4*, angiopoietin like 4; *FNDC5*, irisin; *GHRL*, ghrelin; *GRN*, progranulin; *HAMP*, hepcidin; *HGF*, hepatocyte growth factor; *HMGB1*, high mobility group box 1; *HP*, haptoglobin; *HSPA1A*, heat shock protein 1A1; *IGF-1*, insulin growth factor; *IL-1β*, inteleukin 1 β; *IL-6*, interleukin 6; *IL-32*, interleukin 32; *ITLN1*, omentin; *LEP*, leptin; *MIF*, macrophage migration inhibitory factor; *MMPs*, metalloproteinases; *NAMPT*, visfatin; *NGAL*, lipocalin-2; *NGF*, nerve growth factor; *NUCB2*, nesfatin-1; *PAI-1*, plasminogen activator inhibitor 1; *PEDF*, pigment epithelium derived factor; *RARRES2*, chemerin; *RBP4*, retinol binding protein 4; *RETN*, resistin; *S100A8-S100A9*, calprotectin; *SAA*, serum amyloid A; *SERPINA12*, vaspin; *SPP1*, osteopontin; *SRFP5*, secreted frizzled related protein 5; *STEAP4*, six transmembrane prostate protein 2; *TIMP1*, tissue inhibitor of metalloproteinase 1; *TNC*, tenascin C; *TGF-β*, tumor growth factor β; *TNF-α*, tumor necrosis factor α; *VEGF*, vascular endothelial growth factor; *WNT5A*, WNT family member 5A.

M2-polarized ATMs in obesity, promoting M2 polarization, probably by the upregulation of IL-4Rα expression (Braune *et al.*, 2017). These beneficial metabolic effects seem to be mediated by an IL-6 alternative signaling pathway known as *trans*-signaling, in which IL-6 binds to a soluble form of the IL-6R (Timper *et al.*, 2017). Therefore, the role of IL-6 as a pro-inflammatory cytokine and its prognostic value in obesity need to be reconsidered.

- *Resistin* is a member of the resistin-like molecule (RELM) hormone family. It is a 12-kDa cysteine-rich protein initially proposed as a link between increased adiposity and the development of insulin resistance and obesity in rodents (Steppan *et al.*, 2001). The expression patterns of resistin in rodents and humans differ considerably and while AT constitutes the main source of resistin in mice, this molecule is mainly secreted by monocytes and macrophages in humans (Dupont *et al.*, 2012). The precise role of resistin in human physiology and whether or not it is involved in the development of insulin resistance is not completely elucidated (McTernan *et al.*, 2006; Kusminski *et al.*, 2005; Schwartz and Lazar, 2011). Human resistin plays a major regulatory role in the inflammatory response (Filková *et al.*, 2009) upregulating the expression of proinflammatory cytokines such as TNF-α, IL-6, IL-12, and monocyte chemoattractant protein 1 (MCP-1) (Bokarewa *et al.*, 2005). Resistin expression has also been identified in the stromal vascular fraction of WAT, fibrotic livers, and atherosclerotic lesions. Indeed, resistin has been closely implicated in the pathogenesis of atherosclerosis by promoting smooth muscle cell proliferation and migration, ICAM-1, and VCAM-1 expression, as well as by regulating patterns of adhesion and inflammation in atherosclerotic plaques (Filková *et al.*, 2009).
- *Monocyte chemoattractant protein-1 (MCP-1)* is a proinflammatory cytokine involved in the infiltration of macrophages into the AT (Kanda *et al.*, 2006; Linton and Fazio, 2003) as well as in their in situ proliferation (Amano *et al.*, 2014). The gene expression levels of MCP-1 are significantly upregulated in adipocytes (Amano *et al.*, 2014) whereas its receptor, chemokine (C-C motif) receptor 2 (CCR2), has been shown to be mostly expressed in ATM (Lumeng *et al.*, 2008). Indeed, the gene expression levels of MCP-1 correlate with measures of adiposity in human AT (Christiansen *et al.*, 2005) and higher levels of MCP-1 can be found in obese subjects compared with lean subjects (Catalán *et al.*, 2007). Similar results have been reported in animal models with the expression of MCP-1 being increased in obese mice as compared with wild type ones (Takahashi *et al.*, 2003). MCP-1 is also involved in the regulation of insulin sensitivity in 3T3-L1 adipocytes via downregulation of genes such as *SLC2A4* (the gene encoding GLUT-4), lipoprotein lipase, and peroxisome proliferator-activated receptor-γ (Sartipy and Loskutoff, 2003). In this regard, MCP-1 is elevated in patients with T2D (Nomura *et al.*, 2000).
- *Visfatin* also known as pre-B cell colony enhancing factor (PBEF) or nicotinamide phosphoribosyltransferase (NAMPT), is a 52-kDa protein originally identified as a modulator of B cell differentiation expressed in lymphocytes, bone marrow, skeletal muscle, and liver (Ouchi *et al.*, 2011). The name of visfatin is because it is highly secreted by visceral fat of both mice and humans and its expression levels in serum increase with obesity and T2D (Ouchi *et al.*, 2011; Fukuhara *et al.*, 2005). Visfatin was reported to have insulin-mimetic actions (Fukuhara *et al.*, 2005). However, these findings are currently controversial with Fukuhara and colleagues being forced to retract some of their original findings (Fukuhara *et al.*, 2007). However, visfatin has been shown to stimulate glucose-induced insulin secretion in pancreatic β-cells (Revollo *et al.*, 2007) and appears to have an important role in different inflammatory conditions (Moschen *et al.*, 2007).
- *Lipocalin 2 (LNC2)* is a 25 kDa glycoprotein member of the highly heterogeneous family of lipocalins. LCN2 is a component of the innate immune system with a significant role in the induction of apoptosis as well as in the acute-phase response to infection (Flo *et al.*, 2004; Devireddy *et al.*, 2005). LCN2 is expressed in many tissues including AT and liver (Liu and Nilsen-Hamilton, 1995). LCN2 has been also identified as an adipokine associated to obesity and insulin resistance with increased

expression levels in *db/db* obese diabetic mice (Yan *et al.*, 2007). Furthermore, higher circulating levels of LCN2 have been described in obese humans being associated with anthropometric variables, measures of insulin resistance and inflammatory markers (Wang *et al.*, 2007; Catalán et al., 2009b).

- *Osteopontin (OPN)*, also known as secreted phosphoprotein-1 (SPP1), early T lymphocyte activation (Eta-1) and bone sialoprotein-1, is a matrix phosphoprotein expressed by a wide variety of cell types including osteoclasts, macrophages hepatocytes, and adipocytes (Gómez-Ambrosi *et al.*, 2008). As reported, OPN gene expression is extensively upregulated upon obesity in human and murine AT with OPN plasma concentrations being elevated in morbidly obese patients (Kiefer *et al.*, 2008; Nomiyama *et al.*, 2007). Moreover, OPN has been suggested to play a pivotal role linking obesity to insulin resistance development by promoting inflammation and the infiltration of macrophages into the AT (Nomiyama *et al.*, 2007). In murine models and cell cultures, OPN expression was required for the development of insulin resistance, being prevented by the deletion of OPN (Chapman *et al.*, 2010; Zeyda *et al.*, 2011; Kiefer *et al.*, 2010). Higher levels of expression of osteopontin have been shown to be associated with liver steatosis in morbidly obese subjects (Bertola *et al.*, 2009) and in diet-induced obese mice (Lancha *et al.*, 2014), by the promotion of inflammation, fibrosis, and oxidative stress (Lancha *et al.*, 2014; Syn *et al.*, 2011).
- *Chitinase-3-like protein 1 (CHI3L1)* also known as YKL-40 or human cartilage glucoprotein-39, is a member of mammalian chitinase-like proteins involved in the activation of the innate immune system and in the extracellular matrix remodeling. Increased circulating and AT gene expression levels of YKL-40 in obesity and T2D have been reported (Catalán *et al.*, 2011; El-Mesallamy *et al.*, 2011), with YKL-40 being proposed as a new inflammatory marker related to insulin resistance (Rathcke and Vestergaard, 2006).
- *Tenascin C* is an extracellular matrix glycoprotein that belongs to the damage-associated molecular patterns (DAMPs) family (Midwood *et al.*, 2011). The expression of TNC is specifically induced and tightly controlled during acute inflammation and persistently expressed in chronic inflammation (Chiquet-Ehrismann and Chiquet, 2003; Goh *et al.*, 2010). In this line, TNC has been identified as the endogenous activator of TLR4, whose activation promotes innate and adaptive immune responses as well as the expression of procollagen type 1 and integrin-1β (Midwood *et al.*, 2009; Lu *et al.*, 2008). Interestingly, increased levels of *TNC* in the VAT of obese subjects with or without T2D as well as in obese volunteers with NAFLD have been reported (Catalán *et al.*, 2011). Consistently, the AT gene expression levels of *Tnc* are also increased in murine models of obesity (Catalán *et al.*, 2011).
- *Chemerin* or retinoic acid receptor responder 2 (RARRES2) is an adipokine with important roles in the regulation of adipogenesis and glucose metabolism (Bozaoglu *et al.*, 2007; Goralski *et al.*, 2007; Roman *et al.*, 2012). Chemerin and its receptor, the chemokine-like receptor 1 (CMKLR1) are highly expressed in AT (Catalán *et al.*, 2013b.) and different studies have demonstrated an association of chemerin with markers of the metabolic syndrome and inflammation including TNF-α, IL-6 and C-reactive protein (Bozaoglu *et al.*, 2009; Weigert *et al.*, 2010; Lehrke *et al.*, 2009). In particular, chemerin has been considered as a possible link between obesity and the development of T2D (Ernst and Sinal, 2010). Weight loss induced by both, caloric restriction or bariatric surgery, is accompanied by reduced serum chemerin levels, supporting the relationship between adiposity and chemerin (Chakaroun *et al.*, 2012; Sell *et al.*, 2010).
- *Interleukin-32* is a recently described cytokine produced by epithelial cells, blood monocytes, T lymphocytes and natural killer cells that acts as an important regulator of inflammation (Dahl *et al.*, 1992; Dinarello and Kim, 2006; Joosten *et al.*, 2006). Increased IL-32 expression has been found in VAT from patients with obesity promoting inflammation and ECM remodeling. Reportedly, the increased circulating levels of IL-32 in human obesity and obesity-associated T2D decrease after weight loss (Catalán *et al.*, 2016).

The adipocyte as a key regulator of the extracellular matrix components

Adipocytes and the stromovascular fraction cells are surrounded by a specific extracellular matrix (ECM). The ECM is a complex nest of collagens, fibrillins, proteoglycans and nonproteoglycan polysaccharides (Lin *et al.*, 2016; Mariman and Wang, 2010) characterized by a constant remodeling that not only serve as a mechanical support but also regulate adipocyte functions including nutritional demand adaptation, responses to different signaling factors and secretion of adipokines (Yoshizaki *et al.*, 2012; Sun *et al.*, 2013). The ECM is maintained through secretion of ECM components by both resident adipocytes and stromal cells, particularly fibroblasts (Crewe *et al.*, 2017). Maintaining a high degree of flexibility of the ECM allows AT to expand in a healthy manner, without adverse metabolic consequences (Virtue and Vidal-Puig, 2010).

- *Collagen* accumulation in WAT is considered an important pathological alteration related to obesity-associated comorbidities (Sun *et al.*, 2013; Divoux *et al.*, 2010). Compared to other ECM components, collagen I, V, and VI are the major ECM components to support adipocyte structure and functions, including cell adhesion, migration, and differentiation (Sun *et al.*, 2013). Among them, collagen VI is highly enriched in adipocytes and evidences of its contribution to the pathology of obesity-related diseases exist (Pasarica *et al.*, 2009; Spencer *et al.*, 2010). For a stable collagen VI formation, functionality of the three subunits is needed: α1, α2, and α3. A high upregulation of collagen VI in AT from obese and diabetic *db/db* mice has been reported (Khan *et al.*, 2009). In this line, its expression increases with BMI independently of the presence of diabetes and is also accompanied by AT inflammation in humans (Pasarica *et al.*, 2009). Collagen V expression is also upregulated in obesity being localized close to fibrotic areas (Henegar *et al.*, 2008; Spencer *et al.*, 2011). Reportedly, SAT displayed increased collagen V degradation after bariatric surgery-induced weight loss and related metabolic improvements (Liu *et al.*, 2016). Studies

performed in obese animal models by genetic engineering or induced by a high-fat diet have reported an increase in myocardial collagen types I and III (Toblli *et al.*, 2005; Carroll *et al.*, 2006). In contrast, no effect was found on myocardial collagen type III expression in diet-induced obese rats after 30 weeks (da Silva *et al.*, 2013).

- *Fibronectin* is an adhesion protein with chemotactic activity for inflammatory cell recruitment, including macrophages and monocytes (Adair-Kirk and Senior, 2008). An important decrease in fibronectin synthesis during adipose conversion of 3T3-F442A cells has been reported (Antras *et al.*, 1989; Spiegelman and Ginty, 1983). Additionally, depot-specific fibronectin expression in AT as well as significant associations of fibronectin expressions in both VAT and SAT with obesity parameters and serum leptin concentrations have been shown, suggesting an important function of fibronectin in the pathophysiology of obesity-related diseases (Lee et al., 2013b).
- *Hyaluronan acid (HA)* is increasingly recognized as an active player in AT fibrosis and metabolic dysfunction (Zhu *et al.*, 2016). It is a linear nonsulfated glycosaminoglycan with repeated units of D-glucuronic acid and *N*-acetyl-D-glucosamine (Dicker *et al.*, 2014; Fraser *et al.*, 1997). Despite its simple structure, hyaluronan has diverse biological activities depending on its molecular size and its cellular localization (Cyphert *et al.*, 2015). Hyaluronan synthesis is stimulated by the induction of the differentiation of the preadipocytes 3T3-L1 (Allingham *et al.*, 2006; Calvo *et al.*, 1991) and its downregulation in the ECM has been reported to inhibit in vitro and in vivo adipogenesis (Park *et al.*, 2015; Ji *et al.*, 2014). Furthermore, hyaluronan fragments can stimulate inflammation or induce the loss of the ECM components (Park *et al.*, 2015). The blocking of hyaluronan has been proposed as a possible therapeutic option in obesity and its related metabolic diseases (Ji *et al.*, 2014).
- *Thrombospondins (TSPs)* constitute a family of five extracellular matrix secreted proteins with different expression profiles, structures and, therefore, functions (Calabro *et al.*, 2014). Whereas TSP-1 and TSP-2 are of particular interest due to their antiangiogenic activity and therefore, their roles in wound repair and tissue remodeling, the function of TSP-3, TSP-4, and TSP-5 remains unclear. Pro-fibrotic properties have been also associated to TSP-1 by the activation of the tumor growth factor (TGF)-β (Calabro *et al.*, 2014; Stenina-Adognravi, 2013; Belmadani *et al.*, 2007). Recently, the usefulness of the determination of circulating levels of TSP-1 as a mediator of insulin resistance and AT inflammation has been demonstrated (Matsuo *et al.*, 2015).
- *Matrix metalloproteinases (MMPs)* are also important enzymes secreted by the adipocytes that affect directly or indirectly the regulation of the ECM. MMPs constitute a family of zinc-dependent endopeptidases involved in degrading the ECM components (Lin *et al.*, 2016; Kawatani *et al.*, 2015). At least 25 types of MMPs have been discovered in humans and classified depending on their function and substrate specificity in collagenases, gelatinases, stromelysin, matrilysin, membrane-type MMPs, and elastases (Lin *et al.*, 2016). Since their initial identification, the functions and activation pathways of these types of molecules in relation to metabolic diseases has been characterized in more depth. In this line, MMP-2 and MMP-9 are of great interest due to their role in AT remodeling that occurs during the development of obesity with their mRNA expression levels being upregulated in metabolic disorders (Catalán *et al.*, 2009b; Stamenkovic, 2003).

Brown Adipose Tissue As a Secretory Organ

Our understanding about the potential roles of BAT has been transformed with the recognition that it is also present in adulthood in humans (Nedergaard *et al.*, 2007; Cypess *et al.*, 2013). The traditional concept of BAT just as a site of metabolic energy consumption is changing. Although the exact endocrine roles of BAT remain to be fully understood, accumulating evidence indicates that BAT releases multiple bioactive molecules that act in a paracrine or autocrine manner, strengthening the concept of BAT as a secretory organ (Villarroya et al., 2013, 2017) (**Table 2**). Members of the bone morphogenetic proteins (BMPs) have a significant impact on the differentiation of brown and beige adipocytes (Tseng *et al.*, 2008). In this sense, an early expression in brown adipogenesis of BMP-7 is necessary for the formation of classic BAT depots (Villarroya *et al.*, 2017). Moreover, mice lacking Bmp7 showed a reduction in the total mass of BAT (Tseng *et al.*, 2008). Another member of the family, BMP-8b, is induced in response to nutritional and thermogenic factors in mature BAT (Whittle *et al.*, 2012). Recent evidences suggest that BMPs not only has autocrine effects in BAT but also acts in differentiated mature adipocytes and neurons (Whittle *et al.*, 2012). Another interesting batokine is the fibroblast growth factor 21 (FGF21), mainly synthesized in the liver but also secreted by BAT when it is thermogenically activated (Chartoumpekis *et al.*, 2011; Hondares *et al.*, 2011; Izaguirre *et al.*, 2017). FGF21 was identified by Kharitonenkov et al. as a potent enhancer of glucose uptake through the upregulation of glucose transporter 1 (GLUT-1) expression levels (Kharitonenkov *et al.*, 2005). Furthermore, FGF21 enhances thermogenic pathways in BAT and also induces the browning of WAT (Hondares *et al.*, 2011). Nevertheless, recent findings have shown that FGF21 also induces the expression and secretion of adiponectin, an insulin-sensitizing adipokine (Holland *et al.*, 2013). Wang et al. demonstrated that BAT protects against diet-induced insulin resistance and hepatic steatosis through attenuating de novo liver lipid synthesis via neuregulin 4 (Nrg4), suggesting a potential therapeutic role for the treatment of obesity-associated disorders for this brown fat-enriched endocrine factor (Wang *et al.*, 2014; Pfeifer, 2015). Recent studies have shown a positive correlation between BAT and bone mineral density (Bredella *et al.*, 2014) with several factors secreted in vivo by beige fat tissue such as the insulin-like growth factor binding protein 2 (IGFBP-2) exhibiting an important role in this process (Rahman *et al.*, 2013). The expression of betatrophin or angiopoietin-like 8 is induced in BAT in response to cold (Fu *et al.*, 2013) but its biological significance remains unclear. Brown adipocytes have been also shown to express the vascular endothelial growth factor A (VEGFA) (Asano *et al.*, 2001). VEGFA not only targets endothelial cells to induce vascularization of BAT (Fredriksson *et al.*, 2005) but also promotes direct beneficial

Table 2 Batokines secreted by brown adipose tissue (BAT)

Symbol	*Name*	*Function*
ADIPOQ	Adiponectin	Induces M2 macrophages proliferation
BMP8b	Bone morphogenic protein 8b	Enhances local response of BAT to β3-adrenergic stimulation Increases activation of BAT thermogenesis by inducing the activity of the hypothalamus
ET-1	Endothelin-1	Inhibit thermogenic processes
FGF-21	Fibroblast growth factor 21	Enhances glucose waste in AT, promoting glycaemia and lipidaemia
FGF-2	Fibroblast growth factor	Controls sympathetic innervation and number of preadipocytes in BAT
FST	Follistatin	Antagonizes GDF-8 effects
GDF5	Growth/differentiation factor 5	Controls brown and beige adipogenesis and boost energy expenditure
GDF8	Growth/differentiation factor 8	Inhibits BAT thermogenesis, WAT browning and metabolic behavior
IL-1α	Interleukin 1α	Enhances BAT thermogenesis
IL-6	Interleukin 6	Enhances BAT thermogenesis
METRNL	Meteorin-like protein	Induces the release of IL-4 release by activating eosinophils
NGF	Nerve growth factor	Controls sympathetic innervation and the number of preadipocytes in BAT
NO	Nitric oxygen	Increases blood flow Regulates trophic adaptation of BAT after thermogenesis
RARRES2	Chemerin	Promotes lipid deposition Recruits immune cells into BAT
sLR11	Soluble form of low-density lipoprotein receptor relative 11	Suppresses thermogenesis Prevents excessive energy waste
T_3	Triiodothyronine	Induces the expression UCP-1
VEGFA	Vascular endothelial growth factor A	Promotes BAT vascularization leading to higher perfusion Increases thermogenesis by upregulating UCP-1 expression
	Adenosine	Enhances thermogenic activation.
	Endocannabinoids	Control of β3-adrenoreceptor-induced BAT

ADIPOQ, adiponectin; *BMP8b*, bone morphogenic protein 8b; *ET-1*, endothelin-1; *FGF-2*, fibroblast growth factor; *FGF-21*; fibroblast growth factor 21; *FST*, follistatin; *GDF5*, growth/differentiation factor 5; *GDF8*, growth/differentiation factor 8; *IL-1α*, interleukin 1α; *IL-6*, interleukin 6; *METRNL*, meteorin-like protein; *NGF*, nerve growth factor; *NO*, nitric oxygen; *RARRES2*, chemerin; *sLR11*, soluble form of low-density lipoprotein receptor relative 11; *T3*, triiodothyronine; *VEGFA*, vascular endothelial growth factor A.

effects including their survival, proliferation, and maintenance of mitochondria (Bagchi *et al.*, 2013; Mahdaviani *et al.*, 2016). We are currently in a new era of BAT research aimed to exploit its thermogenic capacity for the management of obesity and metabolic diseases.

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Comorbidities of Obesity

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Introduction

Obesity meets all accepted criteria of a medical disease, including a known etiology, recognized signs and symptoms, and a range of structural and functional changes that culminate in pathologic consequences. It represents major public health concern in many countries from the dual aspects of prevalence and consequences. Obesity is associated with a number of important chronic diseases such as type 2 diabetes (T2D), hypertension (HTN), dyslipidemia, coronary heart disease, stroke, several cancers, disability, and increased mortality (**Fig. 1**). Subjects suffering from obesity have also a higher risk of liver disorders, such as nonalcoholic fatty liver (NAFLD) and nonalcoholic steatohepatitis (NASH). Moreover obstructive sleep apnea (OSA) and osteoarthritis further affect the quality of life of these patients. Therefore additional goal of obesity treatment is the prevention of these complications.

Type 2 Diabetes

Obesity is a major risk factor for T2D, and trends in prevalence and incidence of T2D have closely mirrored those of obesity. When trends were examined by body mass index (BMI), diabetes mellitus only increased among those who were obese, suggesting that much of the increase in the prevalence of diabetes mellitus is because of the increasing prevalence of obesity. In fact, more than 80% of people with T2D are overweight or obese (Guh *et al.*, 2009). As obesity is a primary risk factor for T2D and because both diseases share common causes, the term "diabesity" is currently used when the two abnormalities are present simultaneously (Eckel *et al.*, 2011). However, not all patients with obesity develop T2D, and not all the patients with T2D are overweight or obese. Furthermore, genetic studies have failed to demonstrate a shared heritability between the two diseases. Therefore, the

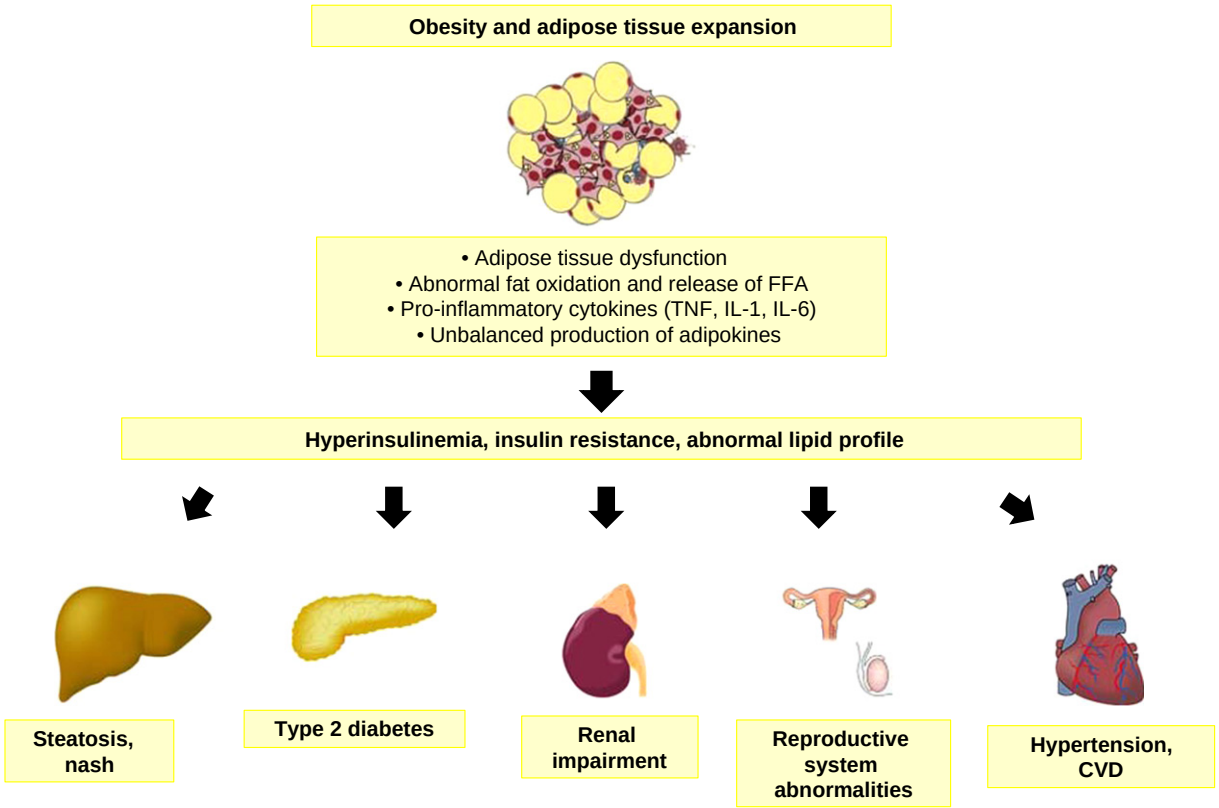

Fig. 1 Comorbidities of obesity. Dysfunction of adipose tissue is involved in several tissue abnormalities.

pathophysiological mechanisms underlying this association are probably more complex and far to be clearly identified, resulting from an interconnection between genetics, environmental factors and the presence of concomitant diseases.

As already mentioned above, obesity is a condition tightly associated with insulin resistance (IR) and hyperinsulinemia, and the development of diabetes is correlated with chronic impaired insulin signaling cascade and a progressive reduction of pancreatic β-cells function (Grundy, 2004). Several hypothesis have been proposed to explain the connection between obesity and T2DM and, at present, the relationship between body fat accumulation and the development of the related metabolic diseases has been clearly recognized as a cause-effect phenomenon. Obesity-related IR due to insulin-signaling cascade defects in the target organs, such as liver, adipose tissue and skeletal muscle is probably the major determinant in the progression from obesity to T2D in particular when it combines with insufficient insulin secretion from the pancreatic β-cells.

Severity of obesity is defined according to BMI-based categories. However, more than excessive fat deposition, adipose tissue architectural and functional changes have a prominent role in the genesis of IR and diabetes. The positive energetic balance induced by excessive calorie intake in genetically predisposed subjects promotes a pathological enlargement of adipocytes, with a concomitant reduction of capillary density and expansion of stromal extracellular matrix. These anatomic abnormalities result in functional alterations of fat depots, in particular visceral adipose tissue (VAT), which is assessed by abdominal circumference or waist-hip ratio. Respect to subcutaneous adipose tissue (SAT), VAT present a higher metabolic activity, with a strong production of adipokines, pro-inflammatory citokines as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) which contribute to the metabolic and vascular diseases (Bays, 2011).

When the adipose storage capacity of intra-abdominal adipose tissue is exceeded, an increased splanchnic FFAs overflow is observed. Acute and chronic exposure to high levels of FFAs beside to reduce insulin sensitivity of visceral organs is involved in the altered production of insulin by the Langherans islets. NAFLD typically correlates with a reduced insulin response, leading to an increase in hepatic gluconeogenesis. On the other hand, the insulin resistant status of adipose cell fails to inhibit properly the lipolysis process thereby enhancing the adipose FFAs leakage. Lipotoxicity due to their deposition in different tissue contributes to a further impairement of muscle glucose uptake and to pancreatic β-cell exhaustion. The low-grade inflammation of adipose tissue and the production of cytokines induce an impaired expression of genes encoding for transcription factors involved in insulin signaling and adipocyte differentiation.

Above the abnormal fat metabolism and the releasing of pro-inflammatory cytokines, also intracellular modification can be responsible for the risk of T2DM in obese subjects. Mitocondrial dysfunction and endoplasmic reticulum stress are issues of adipocyte dysfunction and these abnormalities are commonly observed also in the β-cells.

There is consistent evidence that obesity treatment can delay progression from prediabetes to T2D and may be beneficial in the treatment of T2D. In obese patients with T2D, even a modest but sustained weight loss has been shown to improve glycemic control and to reduce the need for glucose-lowering medications. Weight loss–induced improvements in glycemia are most likely to occur early in the natural history of T2D when obesity-associated IR has caused reversible β-cell dysfunction but insulin secretory capacity remains relatively preserved. The significant weight loss induced by GLP-1 receptor agonist treatment, and in particular liraglutide, has extended its indication to weight loss in obese subjects (BMI >30 or >27 with comorbidities) independently of the presence of diabetes, and it has also shown a clearly positive effect in the prevention of T2D development (Pi-Sunyer *et al.*, 2015). The amelioration, and sometimes, the remission of diabetes after bariatric surgery have been extensively demonstrated, with a greater effect in comparison to other glucose-lowering medications in morbidly obese patients with T2D (Schauer *et al.*, 2017). Obesity and T2D are two quantitatively defined diseases sharing a common epidemiology, pathophysiology, natural history, and complications. They clearly represent a continuum of the same disease with a progressive clinical worsening giving rise to deadly complications. The dysfunctional adipose organ probably accounts for the large proportion of all the detrimental phenomena which induce and accelerate the progression of obesity toward T2D and its complications.

Hypertension

Obesity-associated hypertension is characterized by activation of the sympathetic nervous system (SNS), activation of the renin-angiotensin system (RAS), and sodium retention, among other abnormalities; however, mechanisms linking obesity and arterial hypertension are much more complex and not yet fully clarified.

Blood pressure levels are tightly linked with BMI. Obesity represent a major cause of HTN, and subjects with a BMI greater than 26 kg/m^2 have a two- to threefold increased risk of HTN compared with those with normal weight, and the risk increases further with the severity of obesity (Huang *et al.*, 1998). The Framingham Heart Study has shown that 78% essential hypertension in men is related to excess weight gain, and weight reduction is in fact considered as one of the most relevant strategy for the prevention of hypertension in normotensive subjects or blood pressure reduction in hypertensive patients.

IR has been demonstrated to be associated with hypertension because of a complex action at the level of the kidney. Chronic hyperinsulinemia, as could be seen in obesity, seems to induce the production of angiotensinogen and aldosterone, unbalancing the RAS. Furthermore, hyperinsulinemia and hyperglycemia increase renal sodium reabsorption directly. The increased tubular sodium retention results in an increased cardiac output and vascular stress, with arterial remodeling and vasoconstriction leading to HTN. Renal anatomical and structural changes have also a great impact on the development of HTN in obese subjects. An increased blood pressure can partially results from external renal compression by increased retroperitoneal fat surrounding the kidneys, together with increased abdominal pressure secondary to central obesity (Hall *et al.*, 2015). This mechanical stress is

responsible for increased intraparenchymal pressure, glomerular hyperfiltration, and reduced natriuresis, which further contributed to increase blood pressure levels.

Obesity causes renal vasodilatation and glomerular hyperfiltration as compensatory mechanisms to maintain sodium balance despite the increased tubular reabsorption that, along with increased arterial blood pressure and metabolic abnormalities as well as other factors such as inflammation, oxidative stress and lipotoxicity, may contribute to the exacerbation of renal injury or dysfunction through a vicious cycle.

However, obesity per se can induce hypertension, independently from hormone and metabolic changes.

Obesity is in fact associated with elevated cardiac output due to the increased adipose accumulation and to an even greater extent fat-free mass, resulting in an increased total and central blood volume, which in turn leads to hemodynamic modifications such as augmentation in left ventricular (LV) stroke volume, LV hypertrophy and imbalanced production of cardiac natriuretic peptides.

These modifications, in absence of a reduction of systemic resistance, are responsible for the increased blood pressure levels; this condition is defined as obesity-associated hypertension (Hall, 2000). The dysfunctional adipose tissue induces deep changes in the pattern of secretion of several adipokines among them leptin, which is involved not only in the control of energy balance by regulating satiety and energy intake, but also in the activation of SNS with consequences for both central and peripheral regulation of blood pressure.

Dyslipidemia

Metabolic syndrome is characterized by the presence of atherogenic dyslipidemia, characterized by elevated levels of triglycerides (>150 mg/dL) with increased VLDL particle, increased small LDL, and/or low HDL-C (<40 mg/dL in men and <50 mg/dL in women). These abnormalities are common in obese subjects (60%–70% of obese subjects suffer for dyslipidemia), and they are vary associated with insulin resistance and adipose tissue expansion (Bays *et al.*, 2013). Insulin acts on adipose tissue (1) by stimulating glucose uptake and triglyceride synthesis and (2) by suppressing triglyceride hydrolysis and release of FFA and glycerol into the circulation. Adipose tissue insulin resistance, that is, the impaired suppression of lipolysis, has been associated with elevated plasma FFA levels with consequent peripheral deposition in nonadipose tissue organs. In particular, increased FFA afflux in the liver circulation promotes hepatic synthesis of TGs and, consequently, of VLDL particles. The increased levels of TGs influence the synthesis of the other lipoproteins. In particular, TGs accumulation modulate cholesterol ester transfer protein (CETP) activity: this enzymes increases the triglyceride content of LDL particles, which are subsequently hydrolyzed in the liver, with the production of small dense LDL.

Remnants of chylomicrons and VLDL are involved in the development of atherosclerosis and postprandial hyperlipidemia, and accumulation of atherogenic remnants is especially linked to visceral obesity. It has been shown that diurnal triglyceridemia in obese subjects correlates better to waist circumference than to BMI, which is in agreement with the hypothesis that the distribution of adipose tissue modulates postprandial lipemia.

Inflammation and adipokines have a further role for predisposition to dyslipidemia in obese subjects. TNF and IL-1 seems further promote the triglyceride production through an inhibition of lipoprotein lipase via expression of angiopoietin-like protein 4 (ANGPTL-4) (Khovidhunkit *et al.*, 2004). In addition, they also reduce the synthesis of HDL lipoproteins. Leptin and adiponectin seem involved in these abnormalities: leptin increase enzymatic lipolytic activity of adipose tissue resulting in higher production of FFA, whether on the contrary adiponectin, which levels are low in obese subjects, reduce TGs levels and increase the activity of lipoprotein lipase.

Adipose tissue in man is a major site for cholesterol storage. In obesity over half of total body cholesterol may reside within this tissue; however the uptake, synthesis, and mobilization of adipose tissue cholesterol appears to be mediated and/or regulated, as in other tissues, by the plasma lipoproteins. Recent studies suggest that dietary cholesterol contributes to increased adipocyte cholesterol. However, there is an emerging role of dietary cholesterol in AT cholesterol balance, inflammation, and systemic energy metabolism. It is now becoming obvious that cholesterol can no longer be considered only as a structural component of plasma membranes, but it also actively participates in the regulation of cell physiology. Reducing excessive dietary cholesterol intake is suggested as a simple, but novel, way to attenuate obesity-associated metabolic diseases (Chung and Parks, 2016).

Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver abnormalities that ranges from simple liver steatosis to steatohepatitis and cirrhosis, which actually represents the most common chronic liver disorder worldwide and is predicted to became the main indication for liver transplantation in the future (Charlton *et al.*, 2011).

NAFLD present a strong epidemiological and physiopathological correlation with obesity, T2DM, and dyslipidemia, and it is in fact considered as an additional feature of metabolic syndrome. Obesity is the major phenotype and risk condition for NAFLD.

BMI and waist circumference are positively related to the presence of NAFLD and predict the progression of the disease. The association is supported by high rates of NASH, advanced fibrosis and cirrhosis in morbidly obese patients undergoing bariatric surgery. Hepatic steatosis on ultrasound ("bright liver") is common in obesity, and the prevalence of NAFLD varies, according to the different studies, up to 80% in diabetic subjects (Ortiz-Lopez *et al.*, 2012), and from 80% to 90% in severe obese subjects (Bellentani *et al.*, 2010). According with international guidelines, in presence of NAFLD a comprehensive evaluation of associated

metabolic conditions is suggested in patients at risk (Marchesini *et al.*, 2016). Overweight patients with visceral fat accumulation or a dysfunctional adipose tissue can exhibit NAFLD with/without abnormal liver tests. Therefore the currently used concept of "metabolically healthy obese" individuals should be considered with caution, given that these individuals may have altered liver tests and adverse health outcomes when longitudinally examined.

A "multiple-hit process" model has been put forth to understand the molecular events about NAFLD induced liver injury. It is believed that the initial "hit" is an ectopic accumulation in liver fat, resulting in hepatic lipotoxicity, which eventually triggers the inflammatory response in the microenvironment of liver and causes other "hits." Obesity is characterized by overeating and excessive calorie intake, exceeding the storage capacity of adipose tissue and leading to ectopic deposition of fat. This condition leads to dysfunctional adipocytes an increased plasma levels of FFAs because of increased triglycerides hydrolysis and consequent increased uptake of circulating FFAs by the liver. In addition with abnormal glucose homeostasis, these metabolic disarrangements promote the accumulation of intra-hepatic triglycerides (IHTG).

In NAFLD patients, excess of FFA, IHTG, and consequent lipotoxicity have crucial role for the progression of metabolic alteration and the fibrosis, increasing the production of diacylglicerol, activating protein kinase C-delta (PKC-δ), nuclear factor kinase-B (NF-κB), and leading IR and inflammation (Fabbrini *et al.*, 2010).

The liver-adipocyte axis well represent the multisystem consequences of obesity: adipose tissue dysfunction (with increased lipolysis), adipose-derived cytokines and hormones, along with the low-grade inflammation promote insulin resistance and increase circulating levels of FFA in the portal vein; insulin resistance favor increased hepatic lipogenesis and concomitant reduced fatty acid oxidation, then lipid accumulation induced liver steatosis and IHTGs induce hepatic inflammation and reduce hepatic insulin sensibility (Lomonaco *et al.*, 2012). Inflammation and hepatocyte injury and death are the hallmarks of non-alcoholic steatohepatitis (NASH), the progressive form of NAFLD. Recent observations have also shown that in subjects with NAFLD, more than liver damage, cardiovascular complications represent the leading cause of mortality (Targher *et al.*, 2010). So, NAFLD represents a new challenge for treatment of patient with obesity and/or metabolic syndrome.

Cardiovascular Complications

The most important consequences of obesity are the long term effects of obesity-related metabolic disease on cardiovascular risk associated with greater rates of atherosclerosis, HTN, vascular disease and cardiovascular mortality. The complications associated with obesity, and in particular when T2D develops, include peripheral vascular disease, coronary artery disease, heart failure (HF), and atrial fibrillation (AF).

Vascular markers of inflammation such as plasminogen activation inhibitor (PAI-1), fibrinogen and C-reactive protein (CRP) are also positively correlated with obesity thus explaining the higher prevalence of deep venous thrombosis and pulmonary embolism in obesity (Head, 2015; Campello *et al.*, 2015).

As already mentioned, obesity leads to cardiac ventricular abnormalities, both of right and left ventricle: increased LV stroke volume, LV hypertrophy, and LV diastolic dysfunction are in fact commonly observed in patients with obesity. These morphological and functional changes explains why obesity represents an important risk of heart failure (Kenchaiah *et al.*, 2002; Horwich and Fonarow, 2010). However, patients with HF and higher BMI either with reduced ejection fraction or with preserved ejection fraction had a lower mortality rate than those with a lower BMI suggesting a protective effect of obesity status called "obesity paradox." It has been described that mortality was highest in the low body fat (BF)/low lean mass index (LMI) group (15%), followed by the high BF/low LMI group (5.7%), low BF/high LMI group (4.5%), and high BF/high LMI group (2.2%) (Lavie *et al.*, 2012).

Moreover, left atrial enlargement is a common feature in patients who are obese, particularly those with class III obesity and comorbid hypertension; abnormal left atrial strain has been reported in such individuals predisposing to AF (Lavie *et al.*, 2018).

Alterations in microvascular function could contribute to the increased cardiovascular morbidity and mortality in obesity and the influence of obesity on coronary microvessels has been investigated only recently by assessing the relationship between coronary microvascular dysfunction, inflammation, and obesity. Coronary flow reserve (CFR) by transthoracic Doppler echocardiography is reduced in obese subjects without clinical evidence of heart disease, suggesting a coronary microvascular impairment. This microvascular dysfunction seems to be related to a chronic inflammation mediated by adipocytokines rather than to the increased adiposity (Tona *et al.*, 2014).

Obstructive Sleep Apnea

In obese subjects the most serious impact upon respiration comes during recumbent sleep when increased fat and soft tissue in the throat obstruct the airway which is known as obstructive sleep apnoea (OSA) strongly linked to an increased risk of developing hypertension because of an increased sympathetic activity.

OSA is a condition characterized by repeated episodes of upper airway collapse which happen during sleep. These phenomena usually occur during the REM phase, and patients go awakening to interrupt apnea episodes. This condition presents a relevant social burden because of its association with clinical entities affecting different organs.

OSA is in fact associated with several diseases, such as arterial hypertension, pulmonary hypertension, atrial fibrillation, but also T2DM, cardiovascular diseases, and all causes mortality.

Obesity may promote or aggravate OSA, and in obese patients prevalence of OSA is about 45% (Young *et al.*, 1993), with higher percentages with a more severe grade of obesity. Weight gain in patients with OSA is associated with a worsening of the disease; on the contrary, weight loss can achieve an improvement of OSA.

The connection between obesity and OSA lies in the anatomical and metabolic basis of the disease. In fact, sleep fragmentation has been demonstrated to affect several metabolic pathways and, in this sense, a bidirectional interconnection more than a putative mechanism can explain the association between these two conditions. Subjects with obesity have a larger neck circumference because of excessive fat deposition on upper airways, but they suffer also for restrictive pattern because of truncal and abdominal obesity. These abnormalities present adverse mechanical effects on lung function responsible for a condition of chronic hypoxia.

OSA is independently associated with insulin resistance, inflammatory cytokines, higher levels of C-reactive protein (CRP) and systemic inflammatory state. On the other hand, sleep deprivation (and night shift) is a recognized risk factor for weight gain. Sleep disorders are associated with activation of neuronal centers responsible for energy intake, and seems influence leptin production with consequent loss of satiety, overeating and hypercaloric diet preference (Romero-Corral *et al.*, 2010).

However, whether CPAP treatment seems to have positive effects on some metabolic complications of OSA, there is no data about the effects of this therapy on weight reduction. On the contrary, surgical weight loss has shown an amelioration also on apnea-hypopnea index (AHI) in patients with OSA (Greenburg *et al.*, 2009).

Reproductive Function Abnormalities

Sexual dysfunctions and reproductive system abnormalities are common in subjects suffering for obesity. Obese women suffer for menstrual irregularity, reduced fertility and also polycystic ovary syndrome (PCOS), whether obese men show signs of testosterone deficiency. Obesity represent also a risk factor for gestational diabetes, gravidic hypertension and risk of obesity for children.

Prevalence of PCOS in reproductive women is 4%–7%, but this increase in obese subjects, ranging from 6% to 100% (Lim *et al.*, 2012). Despite the common association, the role of obesity in the development of PCOS is not entirely known. Hyperinsulinemia seems affect the peripheral synthesis of sexual hormones, and in women it may stimulate ovarian androgen production inducing phenotype modifications typical of hyperandrogenemia, such as hirsutism and infertility. Treatment with metformin reduces circulating androgen levels increasing insulin sensitivity.

Male-obesity secondary hypogonadism (MOSH) represents a relevant endocrine dysfunction related to obesity, with a reported prevalence about 40%–50%, but it is often unrecognized in clinical practice. MOSH is characterized by decreased lean body mass, sexual dysfunction, and depression. VAT alter sex hormones levels by aromatization of circulating testosterone to estradiol, whether low testosterone levels promote abdominal fat deposition. Furthermore, obesity may affect gonadal function and testosterone levels through its related metabolic comorbidities, in particular T2D and metabolic syndrome. Several studies have demonstrated an inverse relationship between BMI, waist circumference and testosterone levels. In addition, low testosterone levels was been associated with the development of T2D (Stellato *et al.*, 2000), and low circulating levels of total and free testosterone are frequently observed in subjects with metabolic syndrome, whether testosterone therapy seems have positive effect on manifestations of metabolic syndrome.

Body weight loss appears to revert hypogonadism in patients suffering for obesity, and extensive literature has shown that weight loss after bariatric surgery is correlated with increase in testosterone levels greater than those obtained with lifestyle interventions. However, the effects of bariatric surgery on gonadal function in obese male remain not entirely known.

Osteoarthritis

Osteoarthritis (OA) represents one of the most relevant cause of disability for obese subjects. Mechanical stress associated with higher BMI is responsible of a chronic damage of weight-bearing joints, increasing the risk of knee or hip replacement (Liu *et al.*, 2007). The excessive body weight and the consequent articular overload give reason for a predilection of the lower extremities (knee and hip), but osteoarthropathy is associated with high disability also when are involved different joints, such as the carpometacarpal of the hand.

Pathophysiology of OA in obesity in fact seems go beyond the simple weight-induced mechanical damage. In fact, metabolic abnormalities seems to affects articular function, with a possible tissutal effects of adipose-derived cytokines and adipokines. This mechanisms can explain the OA of nonweight bearing joints which are usually found in obese subjects. In this sense, OA represents another systemic manifestation of obesity-related inflammatory state (Yusuf *et al.*, 2010).

In addition, also an in situ production of cytokines and adipokines has been demonstrated by synovial components, chondrocytes and adipose tissue near joints as infrapatellar fat pad, with release of matrix metalloproteinases, nitric-oxide (NO), and further pro-inflammatory mediators.

Weight loss seems associated to a modification in proteoglycan component and reduction of pro-inflammatory cytokines (Messier *et al.*, 2000), and bariatric surgery can promote articular remodeling not only reducing joint overload, but also decreasing systemic and local inflammation.

Gout

Gout represents an important disorder affecting obese patients, and the impact of gout in this population is likely to increase.

The prevalence of metabolic syndrome in patient with gout is in fact about 62% (Roddy and Choi, 2014), and the risk of gout is higher in presence of hypertension, abdominal obesity, dyslipidemia or diabetes.

However, the pathophysiological mechanisms explaining this association still remain unclear. Diet represent most relevant modifiable risk factors, but the other metabolic diseases seems further influence this risk. Dysfunctional adipose tissue and adipocytokines can influence the metabolism of uric acid (UA): IR and hyperinsulinemia affect the renal metabolism of UA because insulin enhances urate tubular reabsorption, resulting in increased serum levels of UA responsible for the higher risk of gout.

Weight loss and dietary changes represent the first line treatment for patients with gout (Khanna *et al.*, 2012), but there is limited evidence about the effects of surgical weight loss: bariatric surgery is associated with reduction of hyperuricemia in the long-term, despite a higher frequency of acute gouty attacks is observed in the early postoperative period.

Rheumatologic and Immune-Mediated Diseases

Secretory activity of adipose tissue, with production of inflammatory cytokines seems influence also the immune system, and this mechanism is considered responsible for emerging association between obesity and several autoimmune disorders.

Obesity is in fact is vary associated with psoriasis and psoriatic arthritis, asthma, rheumatoid arthritis, and systemic lupus erythematosus (Versini *et al.*, 2014). Hashimoto thyroiditis is often observed in overweight and obese subjects, but adipose tissue expansion seems to play a role also in development of type 1 diabetes, Crohn's disease, inflammatory bowel disease, and also multiple sclerosis.

The dysfunction of adipose tissue is responsible for the development of a chronic low-grade inflammation, which is partially the results of a in situ modulation of regulating T-cells present in the extracellular matrix. It is possible that adipose tissue can modulate the immune response of the whole body, and in this sense the disregulation of immune systems observed in obesity can lead to autoimmune disorders.

However, at this moment, epidemiological studies have described only a mild association between obesity and immune-mediated diseases.

Cancer

Increasing evidence has shown the link between body mass and cancer, with excessive BMI which seems associated with an increased risk for cancer mortality whereas weight loss is linked with a reduction of this risk (**Fig. 2**).

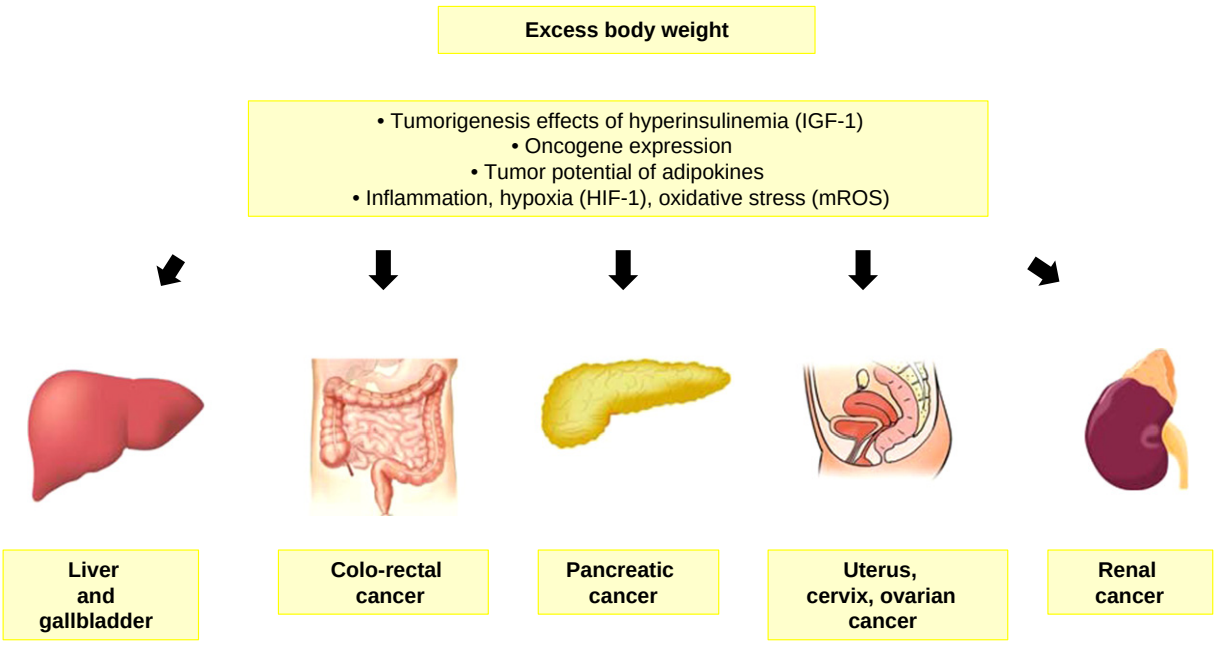

Fig. 2 Association between obesity and some forms of cancer.

In a prospectively studied population of more than 900,000 US adults who were free of cancer at enrollment in 1982, there were 57,145 deaths from cancer during 16 years of follow-up. The heaviest members of this cohort had death rates from all cancers combined that were 52% higher (for men) and 62% higher (for women) than the rates in men and women of normal weight. For men, the relative risk of death was 1.52; for women, the relative risk was 1.62. In both men and women, BMI was also significantly associated with higher rates of death due to cancer of the esophagus, colon and rectum, liver, gallbladder, pancreas, and kidney; the same was true for death due to non-Hodgkin's lymphoma and multiple myeloma. Significant trends of increasing risk with higher BMI values were observed for death from cancers of the stomach and prostate in men and for death from cancers of the breast, uterus, cervix, and ovary in women. On the basis of associations observed in this study, we estimate that current patterns of overweight and obesity in the United States could account for 14% of all deaths from cancer in men and 20% of those in women (Calle *et al.*, 2003).

In a recent UK absolute estimates of population effect emphasized the importance of BMI in driving the incidence of several cancers (Bhaskaran *et al.*, 2014).

The swedish obese subjects (SOS) study was the first intervention trial in the obese population to provide prospective, controlled cancer-incidence data and the effect of intentional weight loss. Weight loss induced by bariatric surgery was associated with reduced cancer incidence in obese women but not in obese men (Sjöström *et al.*, 2009).

There is paucity of studies evaluating hypothetical mechanisms to explain this associations. One of the most considered theory is the tumorigenesis effects of hyperinsulinemia, which promotes the activation of insulin-like growth factor 1 (IGF-1) pathway. In vitro studies have shown the effects of this metabolic via on promotion of apoptosis and cell proliferation. Several lines of evidence suggest that obesity-associated adipose tissue and neoplastic cells share key pathophysiological features in terms of diabetogenic, atherogenic, prothrombotic, and proinflammatory abnormalities. For example, low-grade chronic inflammation is both a hallmark of adiposopathy and a trigger for oncogene expression. Also adipokines are been evaluated for the development of cancer, and elevated levels of leptin has been found in some forms of cancer, such as colon and breast cancer, but its tumoral potential is unknown. Other mechanisms are inflammation, oxidative stress, and obesity-related hypoxia (Calle and Kaaks, 2004). Oxidative stress can promote cancer development because of excess of free radicals and reactive'oxygen species (ROS), whether inflammation and activation of NF-κB system are associated with induction of apoptosis. An important player is therefore represented by mitochondrial reactive oxygen species (mROS), which on one side are induced by altered lipid metabolism, and on the other promote neoplastic transformation and cancer progression. In addition hypoxia-inducible factor 1-α (HIF1-α), a master regulator of cancer progression and metastasis formation, contributes to obesity-induced inflammation and insulin resistance. Thus, a yet unexplored two- way direct communication may exist between these two tissues, which influence *the fate of both*.

Conclusions

There is convincing evidence in order to consider obesity a "disease" which increases the risk of developing a number of serious health conditions that, as for the other diseases, we can call complications. The severity of obesity, together with the presence and severity of complications and age, are important to grade interventions. The so-called EOSS (Edmonton Obesity Staging System), which takes into consideration all these variables, is able to identify patients at increased mortality risk who therefore deserve more clinical and therapeutic attention (Sharma and Kushner, 2009).

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Hypothalamic Control of Food Intake and Energy Homeostasis

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Introduction

Increasing prevalence of obesity constitutes now a prime importance problem for public health (Farooqi and O'Rahilly, 2005; Flier, 2004; Friedman, 2003; Medina-Gomez and Vidal-Puig, 2005). The reasons for the rising prevalence of obesity are complex, but social and environmental factors in combination with genetic predisposition are first to overall positive energy balance. Nowadays, obesity has become a pandemic disease around the world (Tobias *et al.*, 2014). In addition, increased prevalence was found either in developed countries as in developing countries (Hossain *et al.*, 2007; Zheng *et al.*, 2011).

Obesity is a consequence of an imbalance between energy intake and energy expenditure which leads to an increase in body weight gain and adiposity (Popkin and Gordon-Larsen, 2004; Simopoulos, 1999). In addition, the altered energy homeostasis in obesity is related with the metabolic syndrome characterized by insulin resistance and type 2 diabetes, fatty liver (Cota *et al.*, 2007; Farooqi and O'Rahilly, 2005; Flier, 2004; Medina-Gomez and Vidal-Puig, 2005; Plum *et al.*, 2006; Sarafidis and Nilsson, 2006), and other pathologies such as different types of cancer (Calle and Kaaks, 2004).

In the last years several metabolic pathways have been revealed as crucial actors in the regulation of energy balance. In fact, lipid metabolism is directly involved in metabolic homeostasis, since an excess of lipids may result in an ectopic storage of lipids in organs such as liver or skeletal muscle, causing lipotoxicity (Astrup *et al.*, 2008; Astrup and Raben, 1992; Kahn *et al.*, 2006).

The interaction between central nervous system (CNS) and peripheral organs can regulate energy balance (Carling *et al.*, 2008; Kahn *et al.*, 2005; Lage *et al.*, 2008; Ruderman *et al.*, 2003). This was revealed almost 80 years ago when a study focus in obesity and homeostasis control showed that animals with hypothalamic lesions present an increase in adiposity, diabetes, and obesity. Under this context several central mechanisms, specially located at hypothalamic level, constitute the main regulators of energy homeostasis and have been shown involved in the physiological process regulating food intake, body weight but also lipid metabolism and glucose homeostasis.

Hypothalamic Control of Food Intake

In the past, it was described that specific hypothalamic nuclei modulate food intake (Anand and Brobeck, 1951; Hervey, 1959; Teitelbaum and Stellar, 1954). This relation was supported by the finding showing that lesions in the hypothalamic nuclei modulate orexigenic or anorexigenic responses. Accordingly, it was described that lesions in ventromedial nucleus of hypothalamus (VMH) leads to hyperphagia, while lesions located in the lateral hypothalamic area (LHA) induce anorexia and loss of weight (Miller, 1957).

Energy balance is coordinated into the CNS, and the most studied area in this regard is the hypothalamus, which regulates food intake and energy expenditure in response to neural, hormonal, and nutrient-related signals. The hypothalamus is situated under the thalamus, including the major portion of the ventral diencephalon. This brain region is involved in a wide range of homeostatic functions such as the regulation of endocrine axes and energy balance. The hypothalamus is organized in neuronal clusters, known as nuclei, which constituted interconnected neuronal circuits via axonal projections (Elmquist *et al.*, 2005; Gao and Horvath, 2007; Lopez *et al.*, 2007a,b; Morton *et al.*, 2006). The different hypothalamic nuclei including arcuate nucleus (ARC), the paraventricular nucleus (PVH), the LHA, the dorsomedial nucleus (DMH), and the VMH (Adam and Mercer, 2004; Kalra *et al.*, 1999; Morton *et al.*, 2006; Schneeberger *et al.*, 2014; Schwartz *et al.*, 2000).

In response to changes in energy status, hypothalamic nuclei modulate the expression of neurotransmitters/neuromodulators that lead adaptations in energy intake and expenditure (Elmquist *et al.*, 2005; Gao and Horvath, 2007; Lopez *et al.*, 2007a,b; Morton *et al.*, 2006). In response to peripheral metabolic signals such as glucose, amino acids, and lipids and also to hormones, such as leptin, ghrelin, adiponectin (ADPN), resistin (RSTN), glucagon-like peptide-1 and insulin the hypothalamic neurons regulate the expression and synthesis of neuropeptides (Elmquist *et al.*, 2005; Gao and Horvath, 2007; Lopez *et al.*, 2007a,b; Morton *et al.*, 2006).

The arcuate nucleus (ARC) is a hypothalamic area known as the *"master hypothalamic center"* because it is mainly involved in the control of feeding by different neural populations. Different nutritional/feeding factors from the periphery are integrated in different neuronal populations in the ARC. A group of neurons in the ARC expresses the orexigenic (increasing appetite) neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY) which promote feeding and project to other hypothalamic nuclei. On the other way, in the ARC a population of neurons expresses the anorexigenic (inhibiting food intake) products of proopiomelanocortin (POMC), the precursor of alpha-melanocyte stimulating hormone (α-MSH) and the cocaine and amphetamine regulated transcript (CART), the two of them inhibitors of feeding. Neurons expressing AgRP and NPY project to other

second order neurons located in different hypothalamic nuclei, mainly to PVH; differently neurons expressing anorexigenic neuropeptides (POMC, α-MSH, and CART), affects to secondary hypothalamic nuclei such as the DMH, LHA, PVH, and the perifornical area (PFA). The VMH receives projections from AgRP/NPY and CART/POMC neurons in the ARC. Moreover, the VMH neurons direct their axons to the ARC, DMH, LHA, and other brainstem regions including the nucleus of the solitary tract (NTS). When energy intake is higher than energy expenditure, the expression of orexigenic neuropeptides, such as AgRP and NPY, are decreased. Opposed, when energy expenditure exceeds food intake, anorexigenic neuropeptides POMC and CART are expressed (Farooqi and O'Rahilly, 2005; Flier, 2004; Friedman, 2003; Lage *et al.*, 2008; Lopez *et al.*, 2008, 2007a,b; Martinez de Morentin *et al.*, 2010; Morton *et al.*, 2006). In addition to the mentioned hypothalamic nuclei and neuropeptides, the LHA is also involved in the regulation of energy balance by expressing orexins and melanin-concentrating hormone (MCH), neuropeptides which play an important role in orexigenic responses.

Hypothalamic Control of Energy Homeostasis

Energy homeostasis is regulated in addition to food intake by lipid metabolism and glucose homeostasis regulation. The CNS, especially at hypothalamic level, has a relevant role on energy homeostasis regulation mainly through the regulation of lipid metabolism and glucose homeostasis in peripheral organs.

Hypothalamic Control of Energy Expenditure

In addition to the regulation of food intake, the hypothalamus also controls energy expenditure. In this sense, one of the most attractive facts is the regulation of the activity of the brown adipose tissue (BAT), since it was found to be active un human adults (Cypess *et al.*, 2009; Mueez *et al.*, 2017; van Marken Lichtenbelt *et al.*, 2009). The hypothalamus requires the sympathetic nervous system to control BAT activity.

For a long time, the preoptic area (POA) has been considered the only region in the brain involved in thermoregulation (Boulant, 2000; Fuller *et al.*, 1975; Imai-Matsumura *et al.*, 1984; Satinoff *et al.*, 1976). However, we now know that other hypothalamic areas are also relevant. For instance, electrical stimulation of the VMH increased interscapular BAT temperature (Holt *et al.*, 1987; Kelly and Bielajew, 1991; Saito *et al.*, 1987; Yoshida *et al.*, 1984), being AMPK a key player in this area (Lopez *et al.*, 2016). Also the activation of neurons in the DMH and PVH stimulates body temperature (Bewick *et al.*, 2005; Cao *et al.*, 2004; Zaretskaia *et al.*, 2002). The ARC regulates also BAT thermogenesis, since the ARC orexigenic population inhibits thermogenesis and that partial loss of AgRP/NPY neurons leads to a lean, hypophagic phenotype, also characterized by sympathetic activation of BAT (Bewick *et al.*, 2005; Shi *et al.*, 2013).

Hypothalamic Control of Lipid Metabolism

The hypothalamus does not only regulate food intake, but also other components of the balance such as lipid metabolism. In the last years, a central mechanism in charge of lipid sensing has been revealed as clue in energy homeostasis regulation. Nutrients in circulation derived from the diet or from hepatic glucose synthesis and adipocyte lipolysis are sense at central levels. Alterations in this lipid sensing mechanism have been associated to obesity and comorbidities. Moreover it has been proposed that in obesity the characteristic hyperphagia is a state of resistance to the satiety effects of the lipids (Pocai *et al.*, 2005b). Based on a recent series of reports, specific circuits in the CNS are directly connected to white adipose tissue (WAT) and liver thereby directly influencing adipocyte and hepatic metabolism. Among the main circulating factors that act a central level and regulate peripheral lipid metabolism leptin and ghrelin should be mentioned.

Leptin and lipid metabolism

The anorexigenic hormone leptin acts at central level regulating peripheral lipid and glucose metabolism through neuronal pathways (Fruhbeck *et al.*, 1997; Kamohara *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997). Leptin receptor is expressed in the hypothalamus, and the arcuate nuclei is the main target for leptin action. Leptin induces opposite effects on two neuronal subpopulations in the arcuate: inhibits the orexigenic NPY/AgRP-containing neurons and it stimulates the anorexigenic POMC neurons (Flier, 2004; Kalra *et al.*, 1999; Spiegelman and Flier, 2001). The activation of leptin receptor (LepR) in ARC activates the signaling cascade involving Janus kinase and signal transducer and activator of transcription 3 (Jak-STAT3) (Bates and Myers, 2004) as well as the IRS-PI3K pathway (Niswender *et al.*, 2003).

In addition to the anorexigenic action of leptin, a role in the regulation of peripheral lipid and glucose metabolism it was also shown (Fruhbeck *et al.*, 1997; Kamohara *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997). The administration of leptin to experimentation animals induce lipolysis in adipose tissue (Fruhbeck *et al.*, 1998). Different studies in the last years have shown that multiple mechanisms are involved in the peripheral effect in WAT. Among the mechanisms involved in this effect central leptin affects the different pathways involved in the de novo conversion of saturated fatty acids to the monounsaturated fats in WAT (Lin *et al.*, 2003) and WAT lipogenesis (Clauson *et al.*, 2005). A decrease in stearoyl-CoA desaturase 1 (SCD-1), fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) gene expression in WAT and liver (Gallardo *et al.*, 2007) might be related to this effect as well as the

modulation the main indicator of lipolysis in WAT, the hormone-sensitive lipase (HSL) (Tajima *et al.*, 2005). Under this context, leptin, decreases fat tissue mass and lipid accumulation through this action at central level.

Ghrelin and lipid metabolism

Opposed actions of ghrelin and leptin regarding the regulation of energy homeostasis has been described. Ghrelin hormone mainly produced in stomach stimulates food intake and increase adiposity (Billington *et al.*, 1994; Nakazato *et al.*, 2001; Tschop *et al.*, 2000). In the CNS ghrelin activates hypothalamic neurons co-expressing NPY/AgRP (Wren *et al.*, 2000) in an opposite way that leptin. In addition to its actions on appetite, central administered ghrelin regulates BAT sympathetic activity (van der Lely *et al.*, 2004). The involvement of the autonomous nervous system on central ghrelin actions in the periphery was showed to be carried out by the vagus nerve (Yasuda *et al.*, 2003). In fact, chronic central ghrelin infusion increases the expression in WAT of the main enzyme with lipogenic action (FAS, SCD-1, ACC, and LPL); while it decreases the expression of carnitoyl-palmitoyl transferase 1 (CPT-1) the main marker of fat oxidation. These central effects of ghrelin on WAT metabolism were shown to be independent of food intake (Theander-Carrillo *et al.*, 2006). It was probed that the effects of central ghrelin on WAT metabolism are leaded by the sympathetic nervous system (Theander-Carrillo *et al.*, 2006). In addition to the central actions of peripheral hormones the hypothalamic neuropeptides have a key role in lipid metabolism regulation.

Hypothalamic NPY and melanocortin system and lipid metabolism

NPY expression is regulated in an opposite way by leptin and ghrelin. The orexigenic NPY is mainly produced in the ARC. Besides the effect of central NPY on feeding and energy expenditure, it was also described a potent effect inducing de novo lipogenesis in both, liver and adipose tissue (Zarjevski *et al.*, 1994). In addition to the NPY neurons, another set of hypothalamic adjacent neurons are directly involved in lipid metabolism regulation, the neurons expressing the anorexigenic peptide POMC (Flier, 2004; Kalra *et al.*, 1999; Spiegelman and Flier, 2001). Related to this, the family of peptides derived from the posttranslational processing of POMC named melanocortins, act through a family of five members of the family of G-protein-coupled receptors (MCRs) (Cone 2005, 2006). Among those receptors, MC3Rs and MC4Rs are the most important regarding energy homeostasis. The activation of these receptors at hypothalamic level decreases food intake, body weight, and fat mass (Fan *et al.*, 1997). In addition to its effect in food intake, a role in lipid metabolism was also attributed to the melanocortin system in the CNS. The administration of melanocortin system derivates at central level has shown to stimulate lipolysis (Nogueiras *et al.*, 2007; Song *et al.*, 2005). Moreover, the activation of the CNS melanocortin system increased lipid mobilization in WAT and its blockade elicit lipid uptake and triglyceride synthesis (Nogueiras *et al.*, 2007). The sympathetic nervous system seems to be mediating the effects of CNS melanocortin system on lipid metabolism in adipose tissue (Song *et al.*, 2005) since the central activation of melanocortin system increase sympathetic nervous activity in WAT (Nogueiras *et al.*, 2007).

Hypothalamic Control of Glucose Homeostasis

Hypothalamic leptin and glucose homeostasis

The effects of leptin on energy homeostasis regulation are not only related to this action on food intake and lipid metabolism, it was also probed that leptin is directly related to glucose homeostasis. Among the effects of leptin centrally administered an improvement in insulin sensitivity was reported in animal studies (Cusin *et al.*, 1998; Rouru *et al.*, 1999; Shi *et al.*, 1998). In addition, an increase in glucose uptake in several tissues such as heart, brown adipose tissue (BAT), and muscle was found after leptin administration (Minokoshi *et al.*, 1999). The actions of leptin on glucose homeostasis might be elicited through the activation of the PI3K pathway (Morton *et al.*, 2005).

Hypothalamic melanocortin system and glucose homeostasis

The mentioned effects of leptin increasing insulin sensitivity and decreasing adiposity might be mediated by the central melanocortin system (Barzilai *et al.*, 1997). Possibly, the effects of the melanocortin on glucose homeostasis might be secondary to the mentioned effects of this system decreasing adiposity since visceral adiposity is a well-recognized risk factor for insulin resistance and diabetes. Accordingly, the central blockade of melanocortin system reduces insulin sensitivity (Obici *et al.*, 2001).

Hypothalamic NPY and glucose homeostasis

The actions of central NPY on glucose homeostasis have been revealed to be tissue specific. Supporting this it was found that central infusion of NPY increasing glucose uptake in adipose tissue probably mediated by an increase in the expression of the main glucose transporter GLUT4. Opposed, in muscle tissue the central infusion of NPY decrease glucose uptake and GLUT4 expression (Zarjevski *et al.*, 1994).

Hypothalamic insulin and glucose homeostasis

A relevant factor modulating glucose homeostasis at central level is insulin. At central level, specifically in the arcuate nuclei of the hypothalamus (ARC) the insulin receptors lead the central effects of insulin on glucoses metabolism. Immediately after food intake, insulin is secreted from the pancreatic β-cells blocking the endogenous glucose synthesis and increasing glucose uptake. When the circulating levels of insulin are decreased the central activation of insulin pathway in the hypothalamus is able for decrease

endogenous glucose production and thus regulate glucose homeostasis (Obici *et al.*, 2002). At hypothalamic level the channels ATP-sensitive potassium (KATP) are involved in the action of insulin since it was found that the activation of his channels by insulin have the capacity of decrease blood glucose levels as a consequence of an inhibition of the gluconeogenesis (Obici *et al.*, 2002; Pocai *et al.*, 2005a,b). These facts highlight the crucial role of hypothalamic insulin signaling in the maintenance of glucose homeostasis.

See also: ACTH, Melanocortin Receptors, MRAP Accessory Proteins

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Gastrointestinal Hormones and Their Regulation of Food Intake

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Introduction

Energy balance, the equilibrium between food intake and energy expenditure, is regulated tightly in humans at the level of the central nervous system (CNS), mostly in areas pertaining to the hypothalamus and the brainstem. Orexigenic and anorectic neurons located in the arcuate nucleus of the hypothalamus receive both central and peripheral signals, integrate this complex array of data, and regulate their activity in order to provide an appropriate coordinate response in term of eating behavior, drive for feeding and energy expenditure (Schwartz *et al.*, 2000). Peripheral signals can be divided in long-term and short-term signals (Schwartz *et al.*, 2000). Long-term signals communicate to the hypothalamus information regarding body's energy stores, endocrine status, and general health. Many of them are represented by circulating hormones, like the adipose tissue hormone leptin and the pancreatic hormone insulin. Short-term signals are believed to regulate meal initiation and termination, and mostly of them are originated in the gastrointestinal (GI) system. The GI tract can communicate with the hypothalamus both through vagal afferent nerves that innervate brain regions involved in the immediate need for food intake, and through a large number of humoral messengers produced at the GI level and sensed in the CNS, globally named as GI hormones or gut hormones (Schwartz *et al.*, 2000).

Many peptide hormones generated in the gastrointestinal (GI) tract modulate appetite in both animal and human studies (**Fig. 1** and **Table 1**) (Lean and Malkova, 2016; Steinert *et al.*, 2017). The site of production, mechanism of action and effects of the most relevant of these GI hormones will be revised in this article. Moreover, for each of these relevant hormones, information will be provided on the variations in their levels induced by obesity and by the changes induced by dietary restriction, physical exercise, and bariatric surgery (**Tables 2** and **3**). The physiology, effects and regulation of insulin and glucagon, which most important actions relate to glucose homeostasis, will be not included.

Glucagon-Like Peptide 1 (GLP-1)

GLP-1 physiology. GLP-1 is secreted primarily from entero-endocrine L cells located in the distal jejunum and ileum, and its release is stimulated by carbohydrate and fat intake (Madsbad, 2014). Secretion of GLP-1 in response to food intake occurs rapidly after food ingestion (within 10–15 min) with a second peak of secretion at 30–60 min. After release, GLP-1 is rapidly inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4) with a plasma half-life of less than 2 min (Madsbad, 2014). GLP-1 receptors are widely distributed in the brain, in peripheral organs such as the pancreatic islets, and in the GI tract. GLP-1 has an important role on post-prandial glucose homeostasis stimulating glucose-induced insulin release and inhibiting glucagon release at the pancreatic level ("incretin" effect) (Willms *et al.*, 1996). At the GI level, GLP-1 delays gastric emptying with a slow nutrient absorption, that may contribute to the reduction of postprandial glycaemia, and an enhanced satiety, that may contribute to food intake reduction ("ileal brake" effect) (Madsbad, 2014). However, the most important site of action for the effects of GLP-1 on appetite is the CNS. In the brain, GLP-1 receptors are found in areas that are implicated in the control of food intake and energy balance. The central administration of GLP-1 in animals has been shown to significantly reduce food intake (Lean and Malkova, 2016) (**Table 1**).

GLP-1 in obesity. Obesity has been associated with an attenuated post-prandial GLP-1 response to test meals in several studies. Adam and Westerterp-Plantenga (2005) analyzed GLP-1 release after a standard breakfast in 30 normal-weight subjects and in 28 patients with overweight/obesity. Pre-prandial GLP-1 levels were similar in the two groups, but the post-prandial GLP-1 response was significantly blunted in patients with obesity. A flat GLP-1 post-prandial curve was also observed by Carroll *et al.* (2007) in 20 patients with obesity as compared with 19 normal-weight controls. A reduced post-prandial GLP-1 response in patients with severe obesity was also confirmed by Verdich *et al.* (2001) in 19 patients and 12 lean controls. The attenuated GLP-1 post-prandial response observed in patients with obesity has been linked to increased appetite (Adam and Westerterp-Plantenga, 2005) and increased food intake during an ad libitum lunch (Verdich *et al.*, 2001). Therefore, the defective GLP-1 regulation could be involved in the pathophysiology of obesity or in its maintenance (**Table 2**).

GLP-1 and calorie restriction. Unfortunately, the majority of the studies that examined the effects of dietary intervention with calorie restriction on GLP-1 levels demonstrated a further reduction in GLP-1 levels, accompanied by a reduced satiety and a greater appetite. Sloth *et al.* (2009) observed a reduction of fasting GLP-1 levels and greater appetite after an 8-week low-energy diet. Sumithran *et al.* (2011) monitored the circulating levels of several peripheral hormones involved in the homeostatic regulation of body weight, including post-prandial GLP-1 levels, in 50 patients with overweight or obesity treated by an 8-week very-low calorie diet followed by a 2-week weight stabilization period and a 52-week weight maintenance phase. Mean weight loss was 14% of initial body weight after weight stabilization (week 10) and 8% at the end of the maintenance phase (week 62). Mean levels of GLP-1 did not change significantly between baseline and week 10, but the GLP-1 levels at week 62 were slightly but

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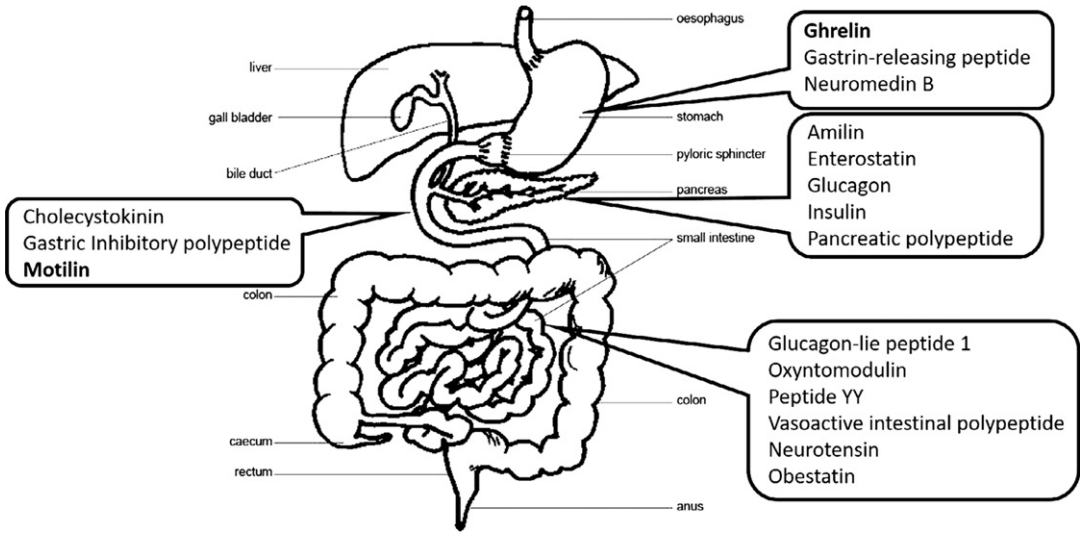

Fig. 1 Sites of production of the gastrointestinal hormones having a role in the regulation of energy balance. Most of the hormones have an anorectic activity. The few orexigenic hormones are evidenced in bold.

significantly lower than baseline levels. These studies support the view that diet-induced weight loss can result in long-term reductions in GLP-1, postulated to favor increased appetite and weight regain, thus at least in part explaining why weight loss through caloric restriction is often so difficult to achieve and/or maintain (Lean and Malkova, 2016) (**Table 2**).

GLP-1 and exercise. The effects of physical exercise on GLP-1 levels seems to go in opposite way respective to those observed after calorie restriction. A recent meta-analysis on the effects of acute exercise on the hormones related to appetite regulation reported a small acute post-exercise increase in GLP-1 levels in normal-weight individuals (Schubert *et al.*, 2014). More interestingly, Martins *et al.* (2010) examined the effect of a more prolonged 12-week aerobic exercise training (500 kcal of treadmill walking or running 5 days per week) on fasting and postprandial levels of GLP-1 in 22 sedentary patients with obesity and observed a tendency toward an increase in the delayed release of GLP-1 90–180 min after exercise. These beneficial changes may explain why long-term exercise training typically prevent weight regain (**Table 2**).

GLP-1 after bariatric surgery. The available evidences about the changes observed in GI hormones levels after bariatric surgery have been recently reviewed by Dimitriadis *et al.* (2017). Whereas laparoscopic adjustable gastric banding (LAGB) has none or very few effects on post-prandial GLP-1 levels, both sleeve gastrectomy (LSG) and Roux-en-Y gastric bypass (RYGB) in particular are followed by a substantial increase in post-prandial GLP-1 concentrations (Evans *et al.*, 2012; Tsoli *et al.*, 2013; Dimitriadis *et al.*, 2017). Reasons for GLP-1 food hyper-stimulation after surgery are not fully understood. Both the passage of more intact nutrients to the ileum through anatomical changes or increased intestinal transit (Nausheen *et al.*, 2013) and the bypassing of the upper small intestine (Rubino and Marescaux, 2004) have been advocated as potential mechanisms in different procedures. Whatever the mechanisms, taking into account the multiple effects of GLP-1 on glucose homeostasis and energy balance, the persistence of post-bariatric GLP-1 elevation is seen as one of the potential mechanisms explaining the favorable and durable effect that bariatric procedures exert on both glucose metabolism and body weight (Dimitriadis *et al.*, 2017). Indeed, weight regain occurring in some patients after bariatric surgery, RYGB in particular, may be associated to a tendency to GLP-1 levels to return to pre-operative levels (Santo *et al.*, 2016) (**Table 3**).

Gastric Inhibitory Polypeptide (GIP)

GIP physiology. GIP is released from duodenal endocrine K cells immediately upon the absorption of fat or glucose (Falko *et al.*, 1975). In mice, high-fat diet is associated to hypersecretion of GIP and extreme fat deposition with insulin resistance. In contrast, mice lacking the GIP receptor are protected from diet-induced obesity and insulin resistance (Miyawaki *et al.*, 2002). These effects are believed to be driven mostly by the peripheral effects of GIP: GIP promotes a more efficient storage of ingested fat in the adipocytes with secondary insulin resistance and hyperinsulinemia. On the contrary, the intra-cerebroventricular administration of GIP does not affect food intake (Woods *et al.*, 1981). These results indicate that there is little if any direct effect of GIP on feeding behavior.

Cholecystokinin (CKK)

CKK physiology. CCK is secreted by the duodenal and jejunal L cells in response to luminal nutrient (particularly fat) content and it was probably the first GI for which an action on food intake regulation has been discovered (Fried *et al.*, 1991; Matzinger *et al.*, 1999). CKK has multiple effects on GI system that tend to facilitate nutrient absorption, including stimulation of gall bladder

Table 1 Gastrointestinal hormones involved in appetite regulation

Hormone	*Primary source for appetite regulation*	*Propose mechanism of action on appetite*
Anorexigenic factors		
Amylin	Pancreatic β cells (co-secreted with insulin)	Delays gastric emptying; targets the hindbrain and hypothalamus
Cholecystokinin (CCK)	Duodenal/jejunal I cells	Delays gastric emptying; targets the vagus nerve, hindbrain, and hypothalamus
Enterostatin	Exocrine pancreas	Inhibits fat intake, targets the hypothalamus
Gastrin-releasing peptide (GRP)	Gastric myenteric neurons	Contributes to meal termination
Glucagon	Pancreatic α cells	Modulates gastric emptying and vagal tone
Glucagon-like peptide 1 (GLP-1)	Intestinal L cells (co-secreted with PYY, OXM)	Delays gastric emptying, promotes insulin secretion, suppresses glucagon secretion; targets the vagus nerve, hindbrain, and hypothalamus
Insulin	Pancreatic β cells	Targets the hypothalamus
Neuromedin B (NMB)	Gastric myenteric neurons	Contributes to meal termination
Neurotensin	Gastrointestinal enteroendocrine cells	Decrease in food intake is acute only
Obestatin	Stomach, intestine	Decreases food intake, slows gastric emptying
Oxyntomodulin (OXM)	Intestinal L cells (co-secreted with GLP-1, PYY)	Delays gastric emptying; targets the hypothalamus
Pancreatic polypeptide (PP)	Pancreatic F cells	Delays gastric emptying; targets the vagus nerve and hindbrain
Peptide YY (PYY)	Intestinal L cells (co-secreted with GLP-1, OXM)	Delays gastric emptying; targets the vagus nerve and hypothalamus
Vasoactive intestinal polypeptide (VIP)	Intestine, pancreas	Targets hypothalamus
Orexigenic factors		
Ghrelin	Gastric antrum and fundus	Increases gastric emptying, decreases insulin secretion; targets the vagus nerve, hindbrain and hypothalamus
Motilin	Small intestine	Increases gastric motility, targets vagus nerve

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contraction, enhanced pancreatic enzyme secretion and delayed gastric emptying (Lean and Malkova, 2016). Delayed gastric emptying has been considered in the past as the most important mechanism by which CCK suppresses appetite (De Graaf *et al.*, 2004). However, CCK is also present in different parts of the brain involved in the regulation of energy balance and food intake (Lean and Malkova, 2016). (**Table 1**).

CCK in obesity. Studies regarding CCK secretion in obesity reported controversial results (Steinert *et al.*, 2017). Baranowska *et al.* (2000) reported reduced fasting CCK levels in patients with obesity, but this observation was not confirmed by others (Stewart *et al.*, 2011; Brennan *et al.*, 2012). Stewart *et al.* (2011) reported delayed and reduced CCK response to an intra-duodenal oleic acid infusion in patients with overweight or obesity compared with normal weight subjects, but CCK responses to high-fat, high-carbohydrate, and high-protein meals were found to be comparable in subjects with or without obesity by Brennan *et al.* (2012). Finally, French *et al.* (1993) reported a higher CCK response to a high-fat meal in patients with obesity than in normal-weight controls (**Table 2**).

CCK and calorie restriction. In its study on the levels of GI hormones in patients with overweight or obesity treated by a very-low calorie diet followed by a weight maintenance phase, Sumithran *et al.* (2011) demonstrated a rapid reduction of post-prandial CKK levels after the first phase of rapid weight loss and the persistence of this reduction until the end of the weight maintenance period. Chearskul *et al.* (2008) confirmed a significant reduction in post-prandial CCK concentrations after a phase of rapid weight loss obtained by a very-low-calorie diet intervention in patients with obesity. These studies suggest that, beside GLP-1 reduction, also CCK lowering may be involved in increasing appetite and stimulating weight regain after diet-induced weight loss (**Table 2**).

*CCK and exercise. E*vidence regarding the effect of exercise on CCK is very limited (Lean and Malkova, 2016). Studies regarding the impact of acute exercise on CCK levels have been conducted in normal weight individuals and reported increased CCK responses immediately after exercise (Schubert *et al.*, 2014). However, more long-term studies in patients with obesity losing weight through an exercise training program failed to demonstrated any variation on either fasting or post-prandial concentrations of CCK measured at least 48 h after the last exercise session (Martins *et al.*, 2010) (**Table 2**).

CCK after bariatric surgery. Few studies examined the changes in CCK occurring after bariatric procedures (Dimitriadis *et al.*, 2017). However, a rise in the post-prandial CCK levels have been reported both after RYGB (Jacobsen *et al.*, 2012) and LSG (Mans *et al.*, 2015). Peterli *et al.* (2012) directly compared in a randomized trial hormonal changes after RYGB and LSG, and described a larger CCK increase in the LSG than in the RYGB group (**Table 3**).

Table 2 Variations in the levels of gastrointestinal hormones induced by obesity and their changes after dietary restriction or physical exercise

Hormone	*Levels in obesity*	*After diet*	*After exercise*
Anorexigenic factors			
Cholecystokinin (CCK)	=?	↓	=↑
Glucagon-like peptide 1 (GLP-1)	↓	↓↓	↑
Oxyntomodulin (OXM)	?	?	?
Pancreatic polypeptide (PP)	=↓	↑	↑
Peptide YY (PYY)	↓	↓↓	=↑
Orexigenic factors			
Ghrelin	=↑	↑↑	?

Table 3 Variations in the levels of gastrointestinal hormones after bariatric surgery procedures

Hormone	*LAGB*	*LSG*	*RYGB*
Anorexigenic factors			
Cholecystokinin (CCK)	?	↑↑	↑
Glucagon-like peptide 1 (GLP-1)	=↑	↑	↑↑
Oxyntomodulin (OXM)	?	?	↑
Pancreatic polypeptide (PP)	=	?	?
Peptide YY (PYY)	↑	↑	↑
Orexigenic factors			
Ghrelin	=	↓↓	↓

LAGB: Laparoscopic adjustable gastric banding; LSG: sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass.

Peptide YY (PYY)

PYY physiology. Peptide YY (PYY) is a satiety hormone co-secreted with GLP-1 by L cells in the lower intestine (Adrian *et al.*, 1985). Fat intake is considered the strongest stimulant of PYY secretion, whereas carbohydrate intake seems to have a limited effect (Essah *et al.*, 2007). Plasma PYY is a mix of PYY(1–36), the secreted form, and PYY(3–36), the active form, which results from cleavage of PYY(1–36) by DPP-4 (Steinert *et al.*, 2017). The anorectic effect of PYY is mostly attributed to delayed gastric emptying and vagal nerve stimulation that are dose-related and dependent on the amount of fat in the meal ("ileal brake" effect) (Lean and Malkova, 2016). However animal studies showing that intra-CNS injections of PYY(3–36) reduce food intake and that peripherally administered PYY(3–36) inhibits eating even in vagotomized mice seems to suggest also the possibility of a central action (Steinert *et al.*, 2017) (**Table 1**).

PYY in obesity. Several studies documented an attenuation of post-prandial PYY response in obesity (Lean and Malkova, 2016). For example, le Roux *et al.* (2006) demonstrated attenuated post-prandial PYY release and reduced satiety in 20 patients with obesity compared with 20 normal-weight subjects. Zwirska-Korczala *et al.* (2007) also observed blunted post-prandial PYY responses in obese and morbidly obese women compared with lean control subjects. (**Table 2**).

PYY and calorie restriction. Similar to GLP-1 levels, Sloth *et al.* (2009) observed a reduction of PYY levels and greater appetite after an 8-week low-energy diet in patients with obesity. Reduction of PYY levels during energy restriction has been confirmed in patients with overweight or obesity by Sumithran *et al.* (2011). This blunted PYY response was found to be persistent over a 12-month weight maintenance phase (Sumithran *et al.*, 2011) (**Table 2**).

PYY and exercise. A small acute increase of PYY immediately after a single exercise session has been consistently reported (Schubert *et al.*, 2014). However, the results regarding more long-term effects of exercise on PYY levels are more contrasting. A sustained increase in fasting plasma PYY levels after a prolonged period of exercise was found in two pediatric (Jones *et al.*, 2009; Roth *et al.*, 2005) and one adult intervention studies (Martins *et al.*, 2010). On the contrary, Guelfi *et al.* (2013) did not found modifications in fasting or postprandial PYY levels in overweight and obese men doing endurance or resistance training (**Table 2**).

PYY after bariatric surgery. Post-prandial PYY levels appear to increase following bariatric surgery (Dimitriadis *et al.*, 2017), regardless of the type of bariatric procedure (Korner *et al.*, 2009; Tsoli *et al.*, 2013) (**Table 3**).

Oxyntomodulin (OXM)

OXM physiology. OXM is an anorectic hormone co-secreted with PYY and GLP-1 from the intestinal L cells. Its anorectic effect is mostly attributed to binding to GLP-1 and glucagon receptors (Choudhury *et al.*, 2016). OXM peripheral administration can stop weight gain in rats and cause weight loss in humans (Murphy *et al.*, 2006). However, no clear data are available about OXM levels in obesity and the effects of energy restriction or exercise (**Table 2**).

OXM after bariatric surgery. Very few studies have looked at the effects of bariatric surgery on OXM (Dimitriadis *et al.*, 2017). However, at least two small studies reported an increase of OXM levels after gastric bypass (Laferrère *et al.*, 2010; Falkén *et al.*, 2011) (**Table 3**).

Pancreatic Polypeptide (PP)

PP physiology. PP is produced in response to a meal by the F cells of the endocrine pancreas, and to a lesser extent by the exocrine pancreas, colon and rectum (Lean and Malkova, 2016). The anorectic effect of PP is believed to in part centrally mediated, and in part related to the effects of the hormone on gastric emptying (**Table 1**).

PP in obesity. Studies of PP in patients with obesity reported conflicting results, with some study showing no differences (Adamska *et al.*, 2014) and some studies demonstrating lower fasting PP levels in patients with obesity as compared to normal-weight subjects (Reinehr *et al.*, 2006) (**Table 2**).

PP and calorie restriction. An increase in PP concentrations after dietary induced weight loss has been demonstrated both in adults (Sumithran *et al.*, 2011) and in children with obesity (Reinehr *et al.*, 2006) (**Table 2**). These observations seem to suggest that PP behaves in a different manner in respect to other anorectic GI hormones that tend to reduce during energy restriction (Lean and Malkova, 2016). However, the clinical relevance of this observation may be questioned, giving that in both the above studies the appetite scores were increased and not reduced after weight loss.

PP and exercise. An acute increase of PYY immediately after a single exercise session has been reported (Schubert *et al.*, 2014). No data are available for more time-sustained effects of exercise on PP levels (**Table 2**).

PP after bariatric surgery. Information about the effects of bariatric procedures on PP levels are very scarce. No significant changes in post-prandial PP levels were observed after gastric banding (Dixon *et al.*, 2011). No information are available about the changes of PP levels after other bariatric procedures (**Table 3**).

Amylin

Amylin physiology. Amylin is co-secreted with insulin by the pancreatic β-cells and therefore meal-associated fluctuations of circulating amylin levels directly reflect changes in β-cell secretion (Lutz, 2016). Chronic administration of amylin in rats reduces body weight by reducing food intake and increasing energy expenditure (Lutz, 2016). Amylin is sensed by specific receptors located in areas of the SNC typically involved in the regulation of energy intake and energy expenditure (area postrema) and the anorectic effect of amylin is therefore believed to be mostly centrally mediated (Lutz, 2016). Moreover, amylin seems to interact with leptin at the CNS level. Interestingly, amylin may be able to reduce the leptin resistance that is commonly associated with obesity and the anorectic effect of amylin is potentiated by its co-administration with leptin (Lutz, 2016). Initial clinical studies showed that co-administration of amylin and leptin analogs pramlintide and metreleptin, was effective in lowering body weight in humans (Roth *et al.*, 2008). More information are needed about amylin levels and regulation in obesity and after weight loss.

Ghrelin

Ghrelin physiology. Ghrelin has been originally identified as the endogenous ligand of the growth hormone secretagogue receptor 1a (GHRS-1a), but its fundamental physiological role is indeed related to eating behavior: ghrelin is the most relevant orexigenic GI hormone yet discovered (Lean and Malkova, 2016). Ghrelin is secreted by specific cells in the GI tract, primarily at the gastric antrum and fundus, and its orexigenic action has been mostly attributed to the interaction of its acylated form with GHRS-1a at the hypothalamic level (Delhanty and van der Lely, 2011). Moreover, ghrelin accelerates gastric emptying (Tack *et al.*, 2006). In normal-weight individuals, ghrelin levels raise in response to fasting and decline after eating (Cummings *et al.*, 2001). Therefore, ghrelin seems to fluctuate and act in a way that is opposite to the anorectic GI hormones mentioned above (Lean and Malkova, 2016) (**Table 1**). However, the physiologic relevance of ghrelin as a stimulus for meal initiation and food intake regulator has been recently questioned by the fact that ghrelin infusion was not able to modulate appetite or spontaneous meal request in normal-weight males (Lippl *et al.*, 2012).

Ghrelin in obesity. Fasting ghrelin levels were reported to be lower in patients with obesity than in normal-weight subjects in one study (Carlson *et al.*, 2009), but this observation was not confirmed by others (Lean and Malkova, 2016). However, compared with lean subjects, patients with obesity consistently demonstrated less suppression of ghrelin levels following meals (Yang *et al.*, 2009; English *et al.*, 2002). This attenuated response to ghrelin to meals, with persistently higher flat values, has been linked to the tendency to continuous eating or "grazing" observed in some patients with obesity (Lean and Malkova, 2016) (**Table 2**).

Ghrelin and calorie restriction. Diet-induced weight loss was accompanied by alterations in ghrelin levels in several studies (Lean and Malkova, 2016). Fasting ghrelin levels increased by 17% in a group of seventy premenopausal women with overweight or obesity who had a 4.5% weight loss after a 10-week intervention program including an energy-restricted diet and moderate physical activity (Ata *et al.*, 2010). Cummings *et al.* (2002) demonstrated an elevation of 24-h ghrelin profiles and meal-related ghrelin fluctuations in 13 patients with obesity after a 6-month dietary intervention. Finally, Sumithran *et al.* (2011) described

increased post-prandial ghrelin levels in 50 patients with overweight or obesity both at the end of an 8-week very-low calorie diet and after a 52-week weight maintenance phase (**Table 2**).

Ghrelin and exercise. An acute suppression of acylated ghrelin levels in the hours immediately following a single exercise burst has been consistently reported (Schubert *et al.*, 2014). The effects of chronic exercise on ghrelin levels are more complex and less clear (Lean and Malkova, 2016). Chronic exercise was associated to increased fasting ghrelin levels, but to increased suppression of post-prandial ghrelin levels in patients with overweight and obesity (Martins *et al.*, 2010). However, no significant changes in fasting or post-prandial ghrelin levels have been observed in adults with severe obesity treated by an integrated program including exercise (Morpurgo *et al.*, 2003) or in exercising children with obesity (Kim *et al.*, 2008) (**Table 2**).

Ghrelin after bariatric surgery. Several studies demonstrated a reduction of ghrelin levels after the most commonly performed bariatric procedures (Dimitriadis *et al.*, 2017). Sleeve gastrectomy, that includes the removal of the part of the stomach where ghrelin is mostly produced, is associated to a reduction of ghrelin levels not observed after gastric banding (Yousseif *et al.*, 2014). A differential effect on ghrelin levels in comparison to gastric banding has been observed also after RYGB, with no changes after gastric banding and reduction after RYGB (Frühbeck *et al.*, 2004). Cummings *et al.* (2002) demonstrated a nearly complete suppression of 24-h ghrelin profiles and meal-related ghrelin fluctuations in five patients treated with RYGB (**Table 3**).

Conclusion

Accumulating evidences clearly demonstrated that GI hormones play an important physiologic role in regulating food intake, meals initiation and termination, and eating behavior in humans. A large number of anorectic and a few orexigenic hormones interact in a coordinate network to play this role. The balance between anorectic and orexigenic hormones seems altered in patients with obesity, where a tendency toward reduced anorectic pathways and increased orexigenic signals have been reported. Attempts to reduce body weight trough dietary restriction tend to accentuate this alteration, with a further reduction in anorectic hormones and an increase of the orexigenic ones. These diet-induced changes in GI hormones are considered one of the most important factor causing weight regain and obesity recidivism after diet-induced weight loss. A different picture may be observed after bariatric surgery, particularly after sleeve gastrectomy and gastric bypass, where anorectic hormones are stimulated and orexigenic signals suppressed. These changes may partly explain the superiority of bariatric surgery over lifestyle modifications in maintaining weight loss over time. Therefore, GI hormones regulation seem important both in physiology and in the obese state. Pharmacologic targeting GI hormone are increasingly recognized as an important tool for improving obesity management in the long term.

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Body Composition

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Abbreviations

2C-model	2 compartment model
4C-model	4 compartment model
AT	Adipose tissue
ADP	Air displacement plethysmography
BIA	Bioelectrical impedance analysis
BIS	Bioelectrical impedance spectroscopy
BCM	Body cell mass
BMD	Bone mineral density
BMI	Body mass index
BNP	Brain natriuretic peptide
BCA	Body composition analysis
BAT	Brown adipose tissue
BIVA	Bioelectrical impedance vector analysis
CT	Computer tomography
DHEAS	Dehydroepiandrosteronesulfate
D_2O	Deuterium
DXA	Dual-energy-absorptiometry
ECW	Extracellular water
FM	Fat mass
FFM	Fat free mass
FT3	Free tri-iodo-thyronine
FT4	Free thyroxine
ICW	Intracellular water
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
LST	Lean soft tissue
LBM	Lean body mass
MRS	Magnetic resonance spectroscopy
MRI	Magnetic resonance imaging
NA	Neutron activation
NaBr	Sodium bromide
PET	Positron emission tomography
PA	Phase angle
QMR	Quantitative magnetic resonance
R	Resistance
SAT	Subcutaneous adipose tissue
SMM	Skeletal muscle mass
SHBG	Sex hormone binding protein
TBK	Total body potassium
TBW	Total body water
TAT	Total = whole body adipose tissue
TSH	Thyroid stimulating hormone
TBN	Total body nitrogen
TAG	Tri-acyl-glycerol
VAT	Visceral adipose tissue
WC	Waist circumference
WAT	White adipose tissue
Xc	Reactance

Body composition analysis (BCA) differentiates body weight into different body components, organs and tissues. Body composition reflects body responses to internal (i.e., hormones, cytokines, myokines) and external (e.g., diet, physical activity, infection) determinants, it characterizes over- and malnutrition. BCA applies concepts of cellular/molecular physiology, biochemistry and experimental approaches to understand the masses and functions at different biological levels, that is, the whole body or its individual organs, tissues, cells, and molecules (Forbes, 1987; Heymsfield *et al.*, 2005, 2015). Suitable applications of BCA are (i) interpretation of body functions (e.g., energy expenditure, glucose turnover and protein synthesis in relation to muscle mass) and their disturbances in the context of individual body components and their associations and vice versa (ii) interpretation of the meaning of individual body components in the context of their functional consequences (e.g., fat mass in relation to the secretion of adipokines, inflammation, and insulin resistance) (Müller, 2013).

In clinical practice, BCA is used to describe energy stores (fat mass), "functional" body mass (i.e., the sum of oxygen consuming cells), hydration status (total body water, TBW, intracellular water, ICW, extracellular water, ECW), fracture risk (bone mineral content and density) and cardio-metabolic risks (e.g., visceral adipose tissue, VAT, and liver fat related to insulin resistance) (Müller, 2013). BCA addresses changes in energy and fluid balances (e.g., during weight gain or weight loss or in response to fluid overload or dehydration) and adds to identify clinical issues such as sarcopenia (e.g., a reduced skeletal muscle mass in the elderly), cachexia (e.g., involuntary weight loss in cancer patients) and fluid disturbances (e.g., fluid overload in patients with heart failure, liver cirrhosis or impaired renal function).

BCA is an essential part of clinical investigation and cardio-metabolic risk assessment, it characterizes overweight and malnutrition. Since individual body components add to variances of plasma hormone levels (e.g., there is a close correlation between fat mass and plasma leptin levels) and metabolism (e.g., fat free mass, FFM, is a major determinant of resting energy expenditure, REE) body components are used to explain their inter-individual variances and for adjustment of either plasma hormone concentrations or metabolism (Heymsfield *et al.*, 2012; Bosy-Westphal *et al.*, 2013a). BCA adds to characterize development and growth, aging as well as physical performance. Thus, detailed body composition data provide a sound basis for differentiated nutritional assessment, understanding endocrine and metabolic functions, defining personalized medical treatment strategies and *in depth* phenotyping in biomedical research (Müller, 2013; Prado and Heymsfield 2014; Heymsfield *et al.*, 2015).

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Contrary to detailed BCA, primitive and crude estimates of the nutritional status such as BMI and waist circumference (WC) have a limited accuracy to characterize energy balance and physical, endocrine and metabolic functions related to weight status (Müller *et al.*, 2016a). In addition, these crude estimates have limited value in estimating individual health risks and morbidity. They may be used at the population level. In clinical practice BMI and WC are reserved for a first categorization of patients, that is, categorizing overweight and obese patients. At the population level they are used to estimate cardio-metabolic risks.

From Anatomy to Compartment Models (Fig. 1)

Human macroscopic anatomy is about appearance, position and structures of organs and tissues of the body. This knowledge is mainly based on post-mortem autopsy data (Snyder *et al.*, 1975; ICPR, 2002). By contrast, modern in vivo BCA is about models and so-called compartments. A "two compartment model" (or 2C-model) divides the body into two compartments, fat mass (FM), and FFM (Forbes 1987; Withers *et al.*, 1999; Heymsfield *et al.*, 2005; **Fig. 1**). While FFM includes cellular water, protein and minerals within adipocytes FM refers to chemical fat only, that is, energy stores with tri-acyl-glycerol (TAG) accounting for about 60%–90% of adipose tissue. FFM refers to TBW, minerals and body protein. When compared with FFM, lean soft tissue (LST) is the sum of all lean compartments, organs and tissues except bone, and includes non-adipose tissue lipids. When compared with LST lean body mass (LBM) includes bone mass.

Extending the "2C-model" refers to different levels (Heymsfield *et al.*, 2005, 2015; Müller, 2013; Müller *et al.*, 2016b). The "atomic" level includes the eleven major elements, H, O, N, C, Na, K, Cl, P, Ca, Mg, S), whereas the "molecular" relates to six components, lipid, water, protein, carbohydrates, bone minerals, and soft tissue minerals. At the "cellular" level three or four components, body cell mass (BCM), ICW, ECW, extracellular solids are described. The "tissue-organ level" comprises major tissues, adipose tissue, skeletal muscle, visceral organs, bone with further organ level components such as brain, liver, kidneys, heart, spleen. Finally, the "whole body level" divides the body into body regions, that is, head, trunk, upper and lower limbs. When compared with the "2C-model" all these models are considered as "multi-component" or "multi-compartment models" (Heymsfield *et al.*, 2005, 2015; **Fig. 1**).

Methods and Models Used for BCA (Table 1)

Methodological aspects of BCA have been extensively described (Heymsfield *et al.*, 2005; Preedy, 2012). Anthropometric methods are non-invasive, they are still used in population studies to assess for example, skinfold thickness (as an estimate of subcutaneous FM) and midarm or thigh circumference (which can be used as a measures of skeletal muscle mass after correction for subcutaneous fat). Strictly spoken, these are local or at least regional assessments of body composition resulting in a "2C-model," because they have been validated against so-called "2C-reference methods" (see below). Age-and sex-specific estimates of whole body FM are based on algorithms generated from the statistical associations between anthropometric measurements and the data obtained by the reference or gold standard method.

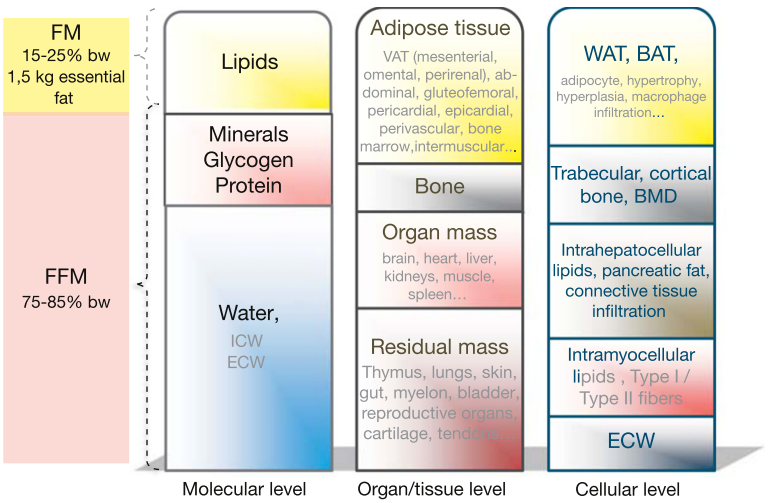

Fig. 1 From a 2-compartment model to different multi-compartment models (see text for further explanations). Abbreviations used: *VAT*, visceral adipose tissue; *WAT*, white adipose tissue; *BAT*, brown adipose tissue; *BMD*, bone mineral density; *ICW*, intra-cellular water; *ECW*, extracellular water. Adapted from Muller, M.J. (2013). From BMI to functional body composition. *European Journal of Clinical Nutrition* **67**, 1119–1121.

Table 1 Characteristics of individual methods used for body composition analysis

	Methods	*Outcomes*	*MDC, kg*	*Precision, %*
Gold standards	MRI/CT whole body, regional	AT, SAT, VAT, BAT?,MM, OM (brain, heart, liver, kidneys), ectopic fat in liver, skeletal muscle, pancreas	0.2	1,1
	4C model	FM, FFM, hydration of FFM	1	
Individual reference methods	DXA whole body, regional	lean body mass, FM, bone mass and bone mineral density	1	2
	Dilution methods D_2O, NaBr	Total body water, extra- + intra-cellular water, tissue hydration	2	1–2 (for TBW)
	Densitometry ADP, underwater weighing	Body volume and density, FM	2	2
	QMR	FM, lean tissue, free + total water	0,2	0,7
Field methods	BIA	Resistance, reactance, phase angle, BIVA	1.5	1
	Skinfolds	SAT	2–3	>5
	Ultrasound	SAT, MM thickness, OM, liver fat	?	?

MDC, minimal detectable change (fat mass, kg); prec', precision (Fat mass, %); *MRI*, magnetic resonance imaging; *CT*, computer tomography; *DXA*, dual X-ray absorptiometry; *ADP*, air displacement plethysmography; *QMR*, quantitative magnetic resonance; *BIA*, bioelectrical impedance analysis; *TBW*, total body water; *AT*, adipose tissue; *SAT*, subcutaneous adipose tissue; *VAT*, visceral adipose tissue; *BAT*, brown adipose tissue; *MM*, muscle mass; *OM*, organ mass; *FM*, fat mass; *FFM*, fat free mass.

There are a number of non-invasive and accurate methods for BCA. Different reference methods are used which differ in techniques, concepts and outcomes. At the whole body level reliable and valid measurements of body volume and thus body density (calculated as the ratio of body mass and body volume) by either underwater weighing or air displacement plethysmography (ADP) aim to assess FM and FFM (Forbes, 1987). Dilution techniques measure hydration status, that is, D_2O-dilution to assess TBW and NaBr-dilution to measure ECW. ICW is then calculated from the difference between TBW and ECW (Heymsfield *et al.*, 2015). Dual X-ray-absorptiometry (DXA) assesses bone mineral content and density (BMD), LST, and FM. LST of leg and arms (or LST of limbs) can be taken as a measure of skeletal muscle mass (so-called appendicular skeletal muscle mass; Heymsfield *et al.*, 1990; Gallagher *et al.*, 1997; Kim *et al.*, 2002). Major body elements (e.g., total body K, N, Ca, etc.) are quantified by whole body counting (e.g., in a total body K counter as total body potassium, TBK, or by neutron activation analysis, NAA, of different elements, e.g., total body nitrogen, TBN) (Heymsfield *et al.*, 2005). Today the latter two methods are of very limited use because of specialized equipment, requirements of high technical skills, high costs and a very limited availability. Quantitative magnetic resonance (QMR) technology is non-imaging and based on nuclear magnetic resonance (NMR) (Bosy-Westphal and Müller, 2015). QMR requires a low magnetic field of 67 G (or 0.0067 T). QMR measures FM, lean mass (with the exclusion of solid components mainly in the bone) and free water. Today, QMR is the most precise method for BCA (see **Table 1**). QMR estimates FM independently of FFM hydration.

Models used in BCA rely on certain assumptions, which are considered as fixed (e.g., 73.2% water content of FFM or measurements at a body temperature of 36°C or 37°C). In addition, it is usually assumed that an individual body component has a homogenous composition. These assumptions may be questioned in daily practice, for example, tissue hydration differs between children and the elderly and also between obese and normal weight patients. In addition, FFM hydration changes with weight loss and throughout the course of a clinical condition, for example, with inflammation and sepsis and in patients with liver cirrhosis. To minimize the shortcomings of individual methods, the results of different methods are combined, that is, DXA + ADP + D_2O-dilution resulting in a so-called "4-compartment-" or "4C-model" (Fuller *et al.*, 1992; Withers *et al.*, 1999; Shen *et al.*, 2005; Heymsfield *et al.*, 2015). Thus the "4C-model" avoids assumptions of a fixed composition of FFM. It is considered as a gold standard for BCA.

Detailed body composition can be assessed by imaging technologies, that is, whole-body magnetic resonance imaging (MRI) or computer tomography (CT) (Müller *et al.*, 2002; Heymsfield *et al.*, 2005; Prado and Heymsfield, 2014). MRI allows reconstruction of all organs and tissues of the body. Transversal images are taken at different distances (e.g., a slice thickness of 7–10 mm for abdominal organs). Cross-sectional organ areas are segmented by hand or automatically using a validated software. Calculation of organ and tissue volumes is based on the sum of areas multiplied by slice thickness and the distance between the scans. The accuracy of volume determination is improved by using contiguously obtained images. Organ and tissue volumes are then converted into masses by taking into account their specific densities (i.e., 0.916 for adipose tissue, 1.0414 for skeletal muscle, 1.0298 for heart, 1.05 for liver, kidneys and spleen, 1.030 for brain, 1.99 for bone corticalis adding up to a whole body density of 1.07 for males and 1.04 for females). Using CT, attenuation intervals (Hounsfield units; HU) can be used for detailed BCA at the organ/tissue level. The interval between −1001 and 191 HU covers air, gas and lung, −190 and −30 HU reflects adipose tissue and yellow bone mass, −29 and 151 HU covers soft tissue whereas cortical bone and spongiosa are defined by an interval of +151 and 2001 HU. To assess whole body subcutaneous and visceral adipose tissue (SAT, VAT) and skeletal muscle mass (SMM)

a whole body-protocol can be reduced to measurements at a single slice at lumbar vertebra 3 (L3; Schweitzer *et al.*, 2015, 2016). This site has been validated for healthy adults and the elderly. However as for longitudinal observations (e.g., during weight loss or weight gain) the variance in changes of regional fat depots limits the value of a single slice estimate (Schweitzer *et al.*, 2015).

Besides the 4C-model, MRI and CT are also considered as Gold standard methods of BCA (Müller *et al.*, 2002; Prado and Heymsfield, 2014). Comparing 4C-model data with MRI- or CT-derived estimates of body composition some differences become evident, that is, chemically defined fat mass as assessed by the 4C-model does not closely resemble the volume of adipose tissue as measured by MRI. There is a considerable inter-individual variance in these data with a fat content of adipose tissue volume varying between 60% and 90%. Thus, strictly spoken the results obtained by imaging technologies cannot be directly compared with the results obtained by the use of either a single reference methods or the 4C-model.

Magnetic resonance spectroscopy (MRS) can be used to assess fat infiltrations in liver, pancreas and skeletal muscle. Liver fat can also be measured by MRI using the 2-point Dixon method that calculates "fat-only" and "water-only" images from "in-phase" and "opposed-phase" images (Ma, 2008).

When compared to reference methods and the gold standard methods, bioelectrical impedance analysis (BIA) has become a widely applied field method for BCA (Lukaski, 2013). Individual BIA devices have been validated against the different reference methods, the "4C-model" and whole body MRI too (Bosy-Westphal *et al.*, 2013b, 2017). These validations are specific for the individual devices, the reference populations and the individual reference or gold standard methods. In a standard approach impedance is measured with a current of 100 mA at a single frequency of 50 kHz. Using multifrequency BIA or bioelectrical impedance spectroscopy (BIS) frequencies between 1 and 1000 kHz body composition is calculated from the impedance to the flow of an electric current through total body fluid. The conductive volume (V, which represents TBW or FFM) is proportional to the square length of the conductor (Ht^2) and inversely correlated to resistance (R) of the cross-sectional area ($V = \rho \times Ht^2/R$, where ρ is the specific resistance of the conductor). TBW can be further differentiated into ICW and ECW. It distinguishes excess fluid from the hydration of major body tissues (Chamney *et al.*, 2007).

Whole-body impedance is mainly based on the impedance of the distal parts of the limbs near the electrodes. Algorithms used to calculate body composition from BIA measurements are based on statistical relationships between impedance and either TBW or FFM or muscle mass. FM is then calculated from the difference between body weight and FFM. Population specificity, the reference method used to generate the BIA algorithm and the BIA device add to a nearly endless list of varying BIA algorithms published so far. Thus, using a BIA device in a clinical setting, population specificity and validation and the device used for generation of a specific BIA algorithm has to be scrutinized.

Alternatively, the use of BIA raw data has gained popularity in body composition research (Bosy-Westphal *et al.*, 2005, 2006). Resistance (R) and reactance (Xc) are standardized by body height in a bioelectrical impedance vector analysis (BIVA) to characterize hydration status and body cell mass (BCM). In a clinical setting, BIVA can be used to follow changes in hydration and BCM and thus malnutrition (e.g., in tumor patients undergoing treatment) (Norman *et al.*, 2015). In addition, the phase angle (PA) can be directly calculated from R and Xc as arc-tangent (Xc/R) $180^\circ/\pi$. PA is associated with body cell mass (BCM), changes in cellular membrane integrity and alterations in fluid balance. A low PA is used for the diagnosis of malnutrition and clinical prognosis. For device-specific BIVA and PA, reference values from different populations stratified according to ethnic, age, and body mass index (BMI) groups are available. Modern BIA techniques are valid tools to estimate body composition in healthy and euvolemic adults. In the clinical setting the use of BIA raw data has value. By contrast, using standard BIA algorithms generated in healthy subjects for BCA in patients has obvious limitations.

The accuracy and outcomes of different methods used for BCA are presented in **Table 1**.

Clinical Applications of BCA (Tables 2 and 3)

The use of appropriate models and methods in BCA depends on both, the question of interest as well as the accuracy needed to address that question (see **Tables 2** and **3**; Müller *et al.*, 2016b). As far as energy balance is concerned, FM and FFM (or LST, LBM) are suitable outcomes. While FM is a measure of energy stores FFM is the major determinant of REE. FM and FFM can be assessed appropriately by either ADP, underwater weighing or DXA. However, faced with the limited precision of either reference methods or the "4C-model," an assessment of changes in body components as an estimate of energy balance is sound at weight changes of >2 kg. By contrast, and within short-term and during controlled over- and under-feeding, QMR allows an accurate assessment of changes in energy stores (i.e., within ± 150 g changes in FM). Quantifying VAT and liver fat as part of cardio-metabolic risk assessment is brought about by MRI, CT, and magnetic resonance spectroscopy (MRS). Whole body and regional fat masses are interpreted in the context of adipokines (e.g., plasma leptin levels) and its inflammatory activity adding up to cardio-metabolic risk assessment.

The clinical phenotypes of low muscle mass and impaired muscle function, that is, sarcopenia (i.e., sarcopenia occurs at under-, normal-, overweight and in obese subjects and may also be associated with osteopenia) are characterized by skeletal muscle mass SMM, with or without increases in VAT and/or alterations in SAT and BMD together with a measure of muscle strength (e.g., as obtained by isokinetic hand-held dynamometry). This phenotype can be assessed by whole body MRI or CT together with DXA. In the case of malnutrition, FFM (or LST, LBM) and muscle mass are measured by either DXA (LST), ADP (FFM), D_2O-dilution (FFM), BIA (FFM) and/or MRI/CT (skeletal muscle mass). In malnourished patients, a PA as assessed by BIA is an estimate of poor prognosis (Bosy-Westphal *et al.*, 2006; Norman *et al.*, 2010, 2015). Osteopenia and osteoporosis are characterized by a low BMD

Table 2 Application of anthropometrics and body composition analysis in clinical problem solving

Characteristics	*Clinical problem*	*Clinical application*	*Detailed phenotyping*	*Method of choice*
1. BMI	• Overweight • Obesity • Malnutrition	• Risk assessment • Treatment (weight loss, weight gain)	Ø	Ø
2. Waist circumference	• Overweight • Obesity	Risk assessment	VAT, SAT, liver fat	MRI; CT
3. Fat mass	• Energy storage • Energy balance	Diet	Total and regional AT, SAT, VAT, BAT	MRI; CT; PET; thermo-graphy;DXA; ADP
4. Muscle mass (FFM, BCM)	• Malnutrition • Growth development • Sarcopenia • Rehabilitation	Treatment	Skeletal mus-cle (total, regional), heart muscle	DXA; MRI
5. Body water (ICW, ECW)	• De-& hyperhydration • Heart failure • Impaired renal function • Liver cirrhosis	Treatment	Edema, ascites, liver, kidney, heart	D_2O; NaBr, BIA/BIS
6. Bone mineral content	Osteoporosis	Treatment	Bone mineral density	DXA

Abbreviations used: *AT*, adipose tissue; *VAT*, visceral adipose tissue; *SAT*, subcutaneous adipose tissue; *BAT*, brown adipose tissue; *MRI*, magnetic resonance imaging; *CT*, computer tomography; *PET*, positron emission tomography; *DXA*, dual energy X-ray absorptiometry; *ADP*, air displacement plethysmography; *NaBr*, sodium bromide; *BIA*, bioelectrical impedance analysis; *BIS*, bioelectrical impedance spectroscopy.

Table 3 Body composition analysis follows the question of interest: What do we want know?

Questions	*Characteristic*	*Methods*
Energy stores?	Fat mass (FFM)	ADP, HD, DXA, BIA, QMR
Metabolic risk?	VAT, ectopic fat	MRI,MRS, CT, (DXA)
Sarkopenia?	Muscle mass	DXA, MRI, NA, BIA
Osteoporosis?	Bone density	DXA, (CT), NA
Hydration/edema?	TBW/ECW	D_2O-, NaBr-dilution, BIA
Prognosis?	Phase angle	BIA
Partitioning?	Fat mass, FFM	QMR

Abbreviations used: *FFM*, fat free mass; *TBW*, total body water; *ECW*, extracellular water; *ADP*, air displacement plethysmography; *HD*, hydrodensitometry = underwater weighing; *DXA*, dual energy X-ray absorptiometry; *BIA*, bioelectrical impedance analysis; *QMR*, quantitative magnetic resonance; *MRI*, magnetic resonance imaging; *MRS*, magnetic resonance spectroscopy; *CT*, computer tomography, *NA*, neutron activation; *naBr*, sodium bromide.

and trabecular structure as measured by either DXA or CT. Overhydration (e.g., in cardiac failure, chronic kidney or liver diseases) and dehydration (e.g., in the elderly) can be assessed by either dilution techniques (D_2O, NaBr) or multifrequency BIA/BIS.

Endocrine Correlates of Detailed Body Composition (Table 4)

Several hormones are correlated with masses and volumes of individual body components (**Table 4**). Since body components are again inter-correlated some of the associations between plasma hormone levels and body composition have to be interpreted with caution. The strongest correlations were seen between plasma leptin levels and SAT as well as between plasma testosterone and skeletal muscle mass. Thus, to explain the inter-individual variances in hormone concentrations there is need of an adjustment for body composition.

About a Reference Man and a Reference Women (Fig. 2)

The so-called *Reference Man* is a medical model used for calculation of radiation doses. Further applications relate to assessment of pharmacokinetics of drugs and modeling substrate metabolism (e.g., related to insulin resistance). The *Reference Man* is not a reference in epidemiological studies. The *1975 Reference Man* was based on characteristics of body composition which were mainly

Table 4 Endocrine correlates of individual organs and body components

Parameter	*FM*	*FFM*	*VAT*	*SAT*	*TAT*	*SMM*	*Brain*	*Liver*	*Heart*	*Kidneys*	*Spleen*	*TBW*	*ECW*	*ICW*
Leptin	+ +	+	+	+ +	+ +	n.s	+	+ +	+	+ +	+	+ +	+	+
Adiponectin	+	+	+	+	+	n.s.	+	+	+	+	+	+	+	+
Insulin	+	+	+	+	+	n.s.	n.s.	+	+	+	+	+	+	+
IGF 1	n.s.	+ +	n.s.	n.s.	n.s.	+ +	+ +	+ +	n.s.	n.s.	n.s.	+ +	n.d.	n.d.
IGFBP3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.	n.s.	n.s.	n.d.	n.d.
TSH	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
FT4	+	+	n.s.	+	+	n.s.	n.s.	+	n.s.	n.s.	n.s.	+	+	+
FT3	+	+	n.s.	n.s	n.s	n.s	n.s	+	+	+	n.s.	n.s.	n.s.	n.s.
Adrenaline	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.s.	n.d.	n.d.
Noradrenaline	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.s.	n.d.	n.d.
Testosterone	+	+ +	n.s.	+	+	+ +	+ +	+ +	n.s.	+	+	+ +	n.d.	n.d.
DHEAS	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.d.
SHBG	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.s.	n.d.	n.d.
Myostatin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.d.
Aldosterone	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.s.	n.d.	n.d.
BNP	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.s.	n.d.	n.d.

+ + = correlation coefficient >0.500; + = correlation coefficient <0.500; ns. = not significant; n.d. = no data. Abbreviations used: *FT3*, free tri-iodo-thyronine; *FT4*, free thyroxine, *TSH*, thyroid stimulating hormone; *BNP*, brain natriuretic peptide; *IGF*, insulin-like growth factor; *IGFBP*, insulin-like growth factor binding protein; *SHBG*, sex hormone binding protein; *FM*, fat mass; *FFM*, fat free mass; *VAT*, visceral adipose tissue; *SAT*, subcutaneous adipose tissue; *TAT*, total = whole body adipose tissue; *SMM*, skeletal muscle mass; *TBW*, total body water; *ECW*, extracellular water; *ICW*, intracellular water. Unpublished data from the Kiel Reference Center of Body Composition. The study population includes body composition data sets obtained in 1152 healthy subjects (mean age of 30.9 years.; age range 6.1–82.0 years.; BMI range 12.3–52.7 kg/m^2; 52.3% females, 47.7% males) using reference and gold standard methods.

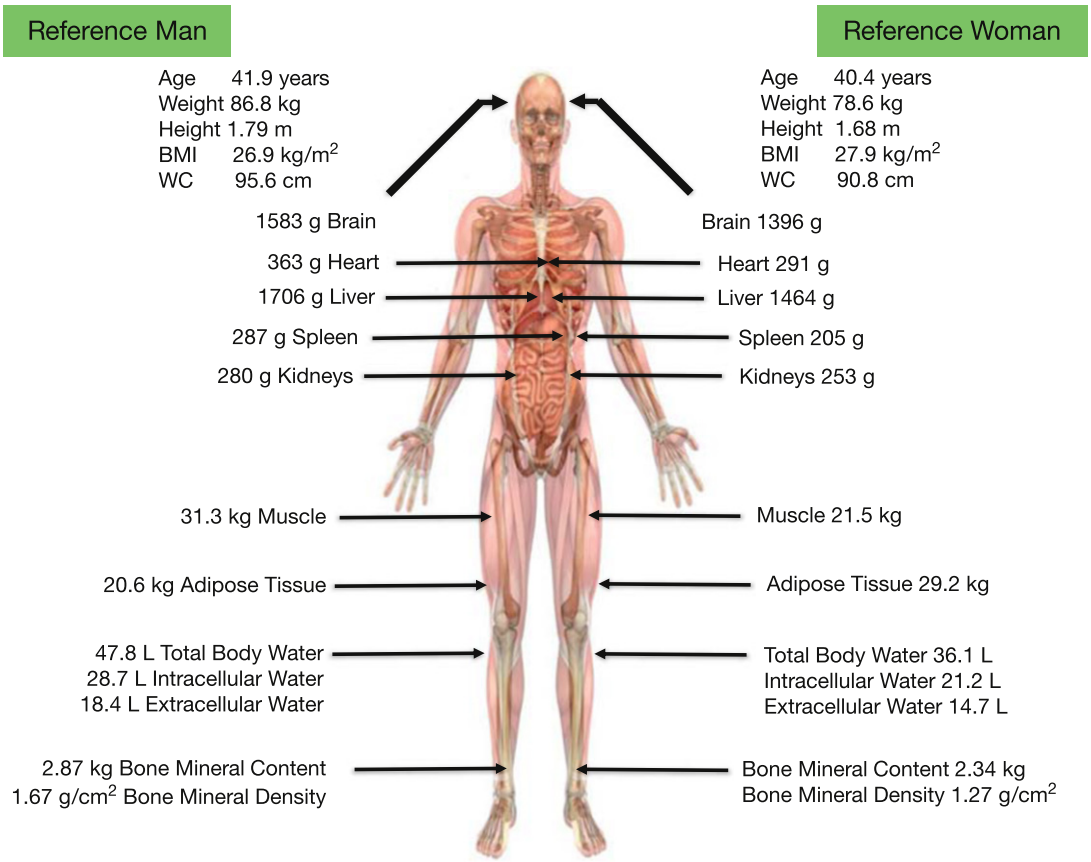

Fig. 2 The Kiel Reference Man + Reference Woman. Unpublished data from the Kiel Reference Center of Body Composition (Later *et al.*, 2010; Müller *et al.*, 2011; Bosy-Westphal *et al.*, 2013a,b). The study population includes body composition data sets obtained in 731 healthy adult subjects (mean age of 41.1 years; age range 18–82.0 years; BMI range 16.8–47.7 kg/m^2; 52.3% females, 47.7% males) using reference and gold standard methods. See text for further details.

autopsy data (Snyder *et al.*, 1975; ICPR, 2002). Faced with technological advances of in vivo BCA, new mathematical models and socio-demographic changes characterized by an increase in elderly and overweight subjects the development of a timely *Reference Man* was developed (see Later *et al.*, 2010; Müller *et al.*, 2011; Bosy-Westphal *et al.*, 2013b). To do this, the worldwide largest in vivo human body composition database in Kiel was analyzed using *in depth* body composition as assessed by gold standard methods, that is, whole body MRI and the 4-C model. **Fig. 2** represents a detailed characterized of a timely Reference Man and Reference Woman.

Summary

Body composition analysis is an integral part the assessment of patients. The structures of the body are closely related to endocrine activities, metabolism, inflammation and cardio-metabolic risks as well as nutrient, fluid and mineral balances. In addition, body composition provides the contexts to understand endocrine and metabolic variables and their changes with weight changes.

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Obesity and Food Addiction

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Introduction

Over the past 25 years, the prevalence of overweight and obesity has undergone a considerable increase worldwide. A study exploring overweight and obesity between 1990 and 2015 found that high BMI accounted for 4.0 million deaths globally, nearly 40% of which occurred in persons who were not obese (Collaborators, 2017). Owing to the similarities between the overconsumption of palatable foods and addictive drugs, a growing number of clinicians and scientists have suggested that excess weight may be a form "food addiction" (Davis *et al.*, 2011; Gearhardt *et al.*, 2011; Volkow *et al.*, 2017). The recent popularity of the food addiction model is reflected in the substantial increase in the number of scientific publications on food addiction (Meule, 2015) (**Fig. 1**).

Proponents of the food addiction model contend that there is sufficient neuroscience evidence to demonstrate that the intake certain energy-dense food and addictive drugs bring about similar neural responses and some argue that the identification of the neurobiological drivers of overconsumption could potentially lead to more effective treatments for obesity (Schulte *et al.*, 2016a,b). Preclinical and clinical studies using a variety of sophisticated techniques have found both drugs of abuse and hyperpalatable foods act on neural reward pathways and therefore, to some extent, support the claim that some types of overeating and obesity constitute a food addiction (Smith and Robbins, 2013).

In this article, we will critically review the applicability of the addiction model to the context of obesity. Additionally, we will provide an overview of the differences and similarities between obesity and other addictive behaviors by expounding upon the comorbidity of both conditions and their common risk factors.

Addiction Model

One possible factor that may be contributing to the rising rates of obesity and the difficulty maintaining long-term weight loss is the potential role of an addictive process in problematic eating behavior. The model of addiction that is traditionally applied to substances like, alcohol, suggests that these substances can become so reinforcing that systems involved in reward, motivation, and memory can become compulsively focused on acquiring and using the substance of abuse and

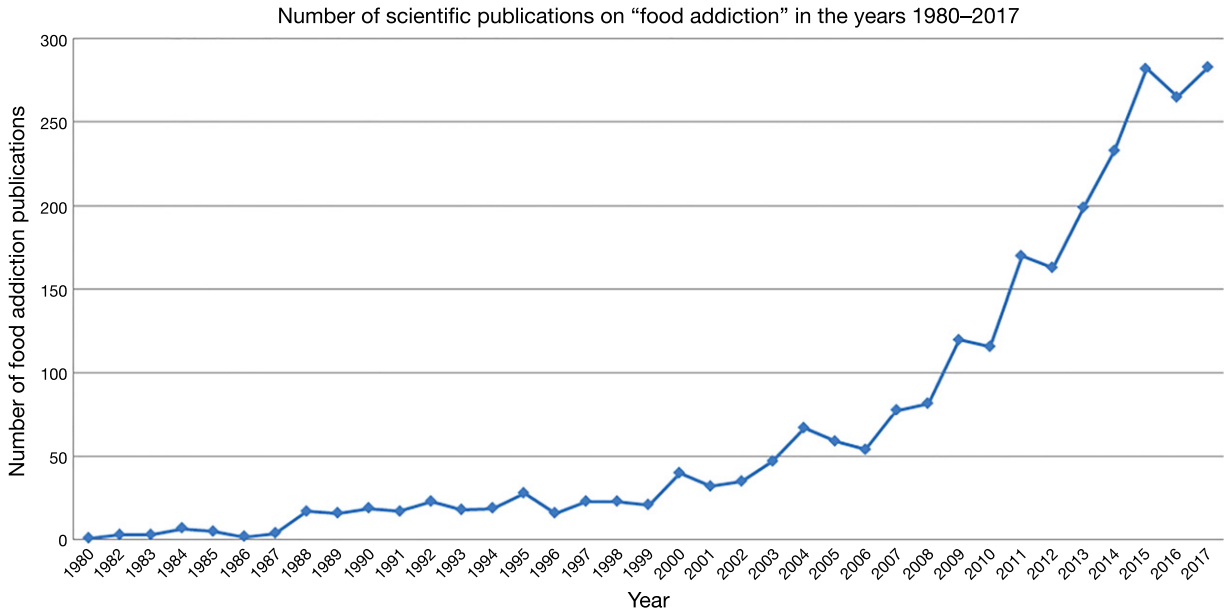

Fig. 1 Values represent the "results by year" using the search term "food addiction" in the NCBI PubMed database. Values for 2017 were extracted in November of that year.

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addiction is the outcome (Volkow and Wise, 2005). The substance may initially be used because the individual likes the experience of consuming the substance (i.e., pleasure), but as the addiction progresses the cues that signal the availability of the substance become powerful triggers of desire. These cues can start to trigger wanting so intensely that even if the individual stops liking the experience of the substance, he/she will still compulsively consume it (Robinson and Berridge, 2001). A strong body of research suggests that certain behaviors (like gambling) can also be reinforcing enough to cause similar addiction-like changes (Potenza, 2008). Similar behavioral criteria are used to diagnose both substance and behavioral addictions, including an inability to cut down despite a desire to do so, intense cravings, and continued use despite negative consequences (APA, 2013). Withdrawal (i.e., negative affective or physical symptoms) in response to cutting down or stopping use/engagement and increased tolerance (i.e., needing more and more to get the same effects) are symptoms assessed for both substance and behavioral addictions, but these are not necessary for a diagnosis of an addiction (**Table 1**).

The modern food environment has changed drastically in the last 40 years. Highly processed foods that have high levels of intensely rewarding ingredients, such as sugar and fat, have become widely available, heavily advertised, and very affordable. One possibility is that these highly processed foods have become so intensely reinforcing, that like alcohol, cigarettes, and gambling, these foods are able to trigger an addictive process in at least some individuals. There is evidence to support this concept. First, as these highly processed foods spread around the world the rates of compulsive overeating, obesity, and health-related disease spread with them (Gearhardt *et al.*, 2011). Second, in animal models, highly processed foods are effective at engaging neural circuitry associated with addiction and to even cause addictive-like changes in the brain more rapidly than traditional drugs of abuse (Ahmed *et al.*, 2013; Oginsky *et al.*, 2016). Third, in humans, the behavioral indicators of addiction are also demonstrated in eating behavior, including an inability to cut down on the consumption of food, intense cravings for food, and continued use despite both physical and psychological consequences (Meule and Gearhardt, 2014). Humans also self-report behaviors consistent with withdrawal and tolerance, although experimental research in this domain is lacking (Schulte and Gearhardt, 2017). Importantly, these behaviors are far more likely to occur regarding highly processed foods (e.g., chocolate, pizza) than naturally occurring foods (e.g., fruits, vegetables) (Schulte *et al.*, 2015).

There are notable implications of the addiction model when applied to food. The addiction model highlights the importance of the reinforcing properties of the food and (the role of the food industry in creating highly processed foods) in compulsive overeating and the associated health consequences. This may highlight the application of the substance-focused policies that have been effective in reducing the public health consequences of addictive substances (like cigarettes) to change the environment surrounding highly processed foods. For example, restricting advertising, using zoning to reduce accessibility, and applying taxation to increase prices are all options that have been used to address cigarettes that could be considered to reduce the impact of highly processed foods (Gearhardt *et al.*, 2011). There are also treatment implications. The development of thoughtful treatment approaches that acknowledge the reinforcing nature of the highly processed foods while not unintentionally increasing rigid dietary rules and disordered eating behavior is needed. Harm reduction treatment approaches employed in substance use disorders teach people to use lower risk substances (e.g., light beer rather than liquor) in lower risk contexts (e.g., in a emotionally calm state rather than when feeling sad or angry) might be useful in the context of addictive-like eating (Schulte *et al.*, 2016b). In this case, strategies to help people consume lower risk foods that they still find reinforcing (e.g., strawberries, nuts) rather than higher risk foods (e.g., ice cream, potato chips) in lower (e.g., with supportive friends) rather than higher risk contexts (e.g., alone late at night) is a potential future direction.

Table 1 Presence of DSM-5 substance use disorders criteria across disorders

DSM-5 substance use disorders criteria	*Obesity*	*Binge eating disorder*	*Behavioral addictions*	*Healthy controls*
Substance taken in larger amount and for longer period than intended	+ +	+ + +	+ + +	+
Persistent desire or repeated unsuccessful attempts to quit	+ + +	+ + +	+ + +	—
Much time/activity to obtain, use, recover	+	+ +	+	—
Important social, occupational or recreational activities given up or reduced	+	+ +	+ + +	—
Use continues despite knowledge of adverse consequences	+ + +	+ + +	+	+
Tolerance	+	+ +	+ +	—
Characteristic withdrawal symptoms; substance taken to relieve withdrawal	+	+	+ +	—
Continued use despite social or interpersonal problems	+ +	+ + +	+ + +	—
Failure to fulfill major role obligations	—	+	+ + +	—
Use in physically hazardous situations	—	—	+	—
Craving, or a strong desire or urge to use	+ + +	+ + +	+ + +	—
Use causes clinically significant impairment	+	+ +	+ + +	—
Use causes clinically significant distress	+	+ +	+ + +	—

+ + +: very frequent; + +: frequent; +: occasional; —: not typically present.

However, there are important questions that remain to be answer regarding the addiction perspective on eating. There are questions about whether addictive-like eating more closely resembles a substance or a behavioral addiction (Hebebranda *et al.*, 2014). The behavioral addiction perspective suggests that the act of eating (rather than the food itself) may be addictive. Proponents of the behavioral addiction perspective highlight that a specific addictive ingredient in food has not been identified and the indicators of addictive-like eating are behavioral in nature. However, proponents of the substance-focused approach highlight that addictive-like eating is far more likely to occur with highly processed foods and that the research into identifying what might increase the addictive potential of a food is just beginning. Additionally, behavioral indicators are also used to identify substance addictions, thus that does not discriminate between substance and behavioral addictions. Further, eating is necessary for survival, whereas the consumption of highly processed foods is not. Thus, substance-addiction relative to behavioral addiction perspectives highlight the nature of the specific foods being consumed (Schulte *et al.*, 2017a,b). Given that substance and behavioral addictions are the result of the same mechanisms, whether addictive-like eating is seen as more of a behavioral or substance addiction may be more of a matter of semantics. Related to this question there are also debates about the best way to assess addictive-like eating. The Yale Food Addiction Scale (YFAS) applies the substance addiction criteria to the consumption of highly processed food and is the most widely used validated measure to assess this construction (Granero *et al.*, 2014; Schulte and Gearhardt, 2017). However, there are critics of the YFAS that suggest that alternative approaches would be more appropriate, including those inspired by behavioral addictions (Schulte *et al.*, 2017a,b; Ruddock *et al.*, 2017). Future research will be needed to evaluate what is the most valid approach to assessing addictive-like eating.

Comorbidity

Obesity, understood as a multifactorial and complex chronic disease, is not considered a mental disorder by the Diagnostic and Statistical Manual (DSM-5; APA, 2013). However, current scientific literature has proven its robust association with different psychiatric conditions, mostly addictions, eating disorders and mood disorders (Maremmani *et al.*, 2017). These comorbidities are understood to interfere significantly with the quality of life of people with obesity (Ivezaj *et al.*, 2017).

Substance Use Disorders

At present, the available operational information about neurobiology of addictive behaviors suggest that obesity and substance use disorders share neuronal mechanisms implicated in loss of control and overconsumption (Volkow *et al.*, 2013). It is not unexpected, therefore, that several studies have demonstrated greater rates of substance use disorders in people with obesity in comparison to healthy weight controls (Barry and Petry, 2009; Michaud *et al.*, 2017). In a recent study by Donnadieu-Rigole *et al.* (2016), the authors found a greater prevalence of cocaine, amphetamine, and cannabis use in patients with obesity in comparison to the general population.

With regards to alcohol consumption, investigators have noted that people with a family history of alcoholism, especially women, presented a 49% greater likelihood of obesity than those without this antecedent, highlighting the etiologic associations between both disorders (Grucza *et al.*, 2010). The concurrence of alcohol with obesity after bariatric surgery has also been reported in the literature (Li and Wu, 2016), and some authors suggest a possible behavioral change within the pattern of abuse that shifts from excessive food intake to the overconsumption of alcohol (Donnadieu-Rigole *et al.*, 2016).

Food Addiction and Other Behavioral Addictions

Due to the complex clinical similarity between obesity and addictions, the food addiction model has emerged as a relevant construct in the obesity field (Maremmani *et al.*, 2017). Food addiction includes representative clinical features of both, addiction to substances and behavioral addictions, chiefly a recurrent and uncontrollable involvement in behaviors in spite of the possible negative consequences (Jiménez-Murcia *et al.*, 2017). In this vein, Pursey *et al.* (2014) reported that the prevalence of food addiction in overweight and obese people ranged between 7.7% and 56.8% whereas other studies have obtained rates between 34% and 40% (Meule *et al.*, 2014; Ceccarini *et al.*, 2015). Food addiction is also found to be more prevalent in women than in men (Meule, 2012). Finally, taking personality traits into account, Jiménez-Murcia *et al.* (2017) found, in a clinical sample with gambling disorder, that those who had comorbid food addiction obtained low self-directedness levels and high scores in self-transcendence. Individuals with this personality profile tend to experience difficulties in defining vital goals and directing their behavior toward them (Cloninger *et al.*, 1998).

Regarding adolescents, Burrows *et al.* (2017) studied food addiction from an intergenerational perspective, finding a positive correlation between food addiction levels in parents and children. Moreover, the authors observed that, in the case of children, there was a significant association between food addiction and body mass index (BMI).

Taking other behavioral addictions into account, some studies suggest an association between buying disorder and BMI (Sansone *et al.*, 2013) and eating disorders, especially binge eating disorder (Müller *et al.*, 2013). In this vein, Leppink *et al.* (2016) studied the association between BMI and symptom change in patients with gambling disorder, detecting less improvements in

gambling symptomatology in participants with obesity. Despite these findings, there is a lack of empirical evidence taking the comorbidity between obesity and behavioral addictions into account.

Eating Disorders

Studies investigating eating disorder comorbidity indicate that binge-eating disorder is the most prevalent disorder in people with obesity (Kessler *et al.*, 2013). Suitable reasons as to why these two disorders are strongly linked can be explained by taking the clinical features of binge eating disorder into account. Binge eating disorder is characterized by recurrent uncontrollable overeating episodes followed by an intense perception of loss of control and in the absence of compensatory behaviors, such as purging (APA, 2013). These dysfunctional eating behaviors can, therefore, imply a significant weight increase and ultimately lead to obesity (Aviram-Friedman *et al.*, 2017; Goldschmidt *et al.*, 2018). Specifically, people with binge eating disorder are approximately 3–6 times more likely to present obesity, in comparison with people without an eating disorder diagnosis (McCuen-Wurst *et al.*, 2017). Although the clinical aspects of both disorders are becoming better explored and understood, some authors suggest that a clear distinction between obesity with and without BED needs to be formulated (Villarejo *et al.*, 2012; Aviram-Friedman *et al.*, 2017).

When examining comorbidity between food addiction and eating disorders, the prevalence of patients with eating disorders who met the criteria for food addiction, in a study by Granero *et al.* (2014), was 72.8%. Within this sample, lower food addiction prevalence was found in anorexia nervosa-restricting subtype, whereas bulimic-spectrum disorders (bulimia nervosa, binge eating disorder and anorexia nervosa-bingeing/purging subtype) showed greater comorbidity. In general, these patients presented higher dependence symptoms and a tendency to consume more high-fat/high-sugar foods than planned.

Other Psychiatric Comorbidities

In addition to the comorbidities mentioned above and the health complications associated with obesity, obesity is often closely related to high levels of psychological distress and, especially, to anxious and depressive symptomatology (Yoder *et al.*, 2017). In this line, the National Co-morbidity Survey Replication stated a prevalence rate of 20.8% for mood disorders in people with a clinical diagnosis of obesity (Kessler *et al.*, 2005). In a recent study by Donnadieu-Rigole *et al.* (2016), 18% of the participants with obesity reported one or more depressive episodes in their lifetime. In the case of adolescents with obesity, Lanza *et al.* (2015) identified a higher chance of presenting greater deviant peer affiliation and depression levels than in normal-weight adolescents. Other researchers have made the case that high scores on the Yale Food Addiction Scale may be used as a proxy measure for interrelated clinical features, including greater eating disorder severity, greater obesity severity, greater posttraumatic stress disorder (PTSD) symptoms, greater psychiatric comorbidity, as well as greater medical morbidity and mortality (Brewerton, 2017).

Shared neurocognitive deficits and personality traits in individuals with obesity and in addictive behaviors.

Research indicates a number of personality traits and neurocognitive impairments in individuals with obesity and excess food intake that are present in individuals with addictive behaviors (Volkow *et al.*, 2011). These deficits encompass cognitive domains such as decision-making, set shifting and delay discounting. Studies evaluating decision-making using behavioral measures such as the Iowa Gambling Task have linked increased BMI to poorer performance compared to healthy weight controls (Fitzpatrick *et al.*, 2013). One recent study compared Iowa Gambling Task performance in a large sample of adults with substance-use disorder, obesity, and gambling disorder, and in healthy controls, and found that all three clinical groups presented similar deficits in decision-making, suggesting the presence a shared underlying deficiency in individuals with addictive behaviors (Mallorquí-Bagué *et al.*, 2016). Similarly, the compulsive eating patterns that are characteristic of binge eating disorders have been associated with a tendency to focus on immediate gratification at the expense of larger, future rewards (Steward *et al.*, 2017b; Stojek and MacKillop, 2017). Similar heightened delay discounting attributes have also been found in individuals with gambling disorder and substance use disorders (Amlung *et al.*, 2017; Steward *et al.*, 2017a).

Personality traits play an important role both as risk and protective factors in the development and maintenance of obesity and food addiction. A recent systematic review of 70 empirical studies of obesity and personality found that neuroticism, impulsivity and sensitivity to reward appeared to be risk factors in relation to weight gain, whereas conscientiousness and self-control have a protective function (Gerlach *et al.*, 2015). Personality traits have also been shown to be a predictor of response to bariatric surgery, with one study finding that individuals with higher scores in novelty seeking and lower scores in persistence lost significantly less weight after surgery than a more successful comparison group (García-Ruiz-de-Gordejuela *et al.*, 2017). Likewise, higher food addiction severity has also been associated with poorer treatment outcomes in the case of eating disorders marked by binge eating episodes (Hilker *et al.*, 2016). Such results suggest that obesity patients with impulsive or addictive traits could potentially benefit from targeted psychological interventions (Carter *et al.*, 2016). Post-surgical interventions are especially important given that postoperative, but not preoperative, problem eating behaviors are predictors of poorer weight loss trajectories after surgery (Conceição *et al.*, 2017).

Recent studies on the etiology underlying obesity have shed more light on the factors contributing to its development and maintenance. Altered emotion regulation stands out as a common risk factor in food addiction and other addictive behaviors (Sloan *et al.*, 2017; Hardy *et al.*, 2018). An inability to regulate negative affect via emotion regulation strategies is thought to cause some individuals to find relief from emotional distress in highly palatable food (Fernandes *et al.*, 2018). Dysfunctional emotion regulation also hinders adherence to long-term goals and is believed to be interfere with weight-loss interventions (Forman and

Butryn, 2015). One functional resonance magnetic imaging (fMRI) found that young people with overweight and obesity had decreased negative functional coupling between the right anterior insula and regions in the prefrontal cortex during cognitive reappraisal, suggesting a reduced ability to regulate negative affective states (Steward *et al.*, 2016). This position is supported by the behavioral findings of another study pointing to a double vulnerability in bariatric surgery patients with food addiction. Patients with food addiction endorsed greater emotion dysregulation than those without and also presented lower levels of the personality trait self-directedness (Ouellette *et al.*, 2017).

Conclusions

Evidence from animal and human research is supportive of the classification of specific food and eating behaviors as being potentially addictive. Nonetheless, this classification remains underdeveloped and more empirical studies are required to test the validity of the food addiction phenotype. A shift in public health policy toward the food addiction model would have a major impact on how obesity is perceived by the lay public and potentially lead to increased support for measures that reduce the overconsumption of harmful foods in the general population, akin to the public health campaigns to reduce tobacco use. In this review, we have highlighted the similarities found in individuals with obesity and in addictive behaviors. However, taking into consideration the vast reach of the obesity epidemic, researchers and clinicians should be careful not to assume that the presence of obesity implies the presence of food addiction. Resources should be dedicated to identifying which individuals best fit the food addiction model and how weight-loss interventions can be tailored to their specific needs. It has yet to be tested whether individuals with food addiction benefit to greater extent from pharmacological approaches targeting the dopamine or opioid systems or whether these individuals respond better to psychological treatments focusing on the cognitive processes involved in addiction (Carter *et al.*, 2016).

See also: Appetite and Weight. Endocrine Disruptors and Obesity. Hypertension and the Renin–Angiotensin–Aldosterone System. Management of Obesity in Children and Adolescents: Lifestyle and Exercise Options. Obesity and Reproduction. Obesity, Childhood, and Adolescence. Type 2 Diabetes

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Leptin☆

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Historical Perspective

The obese *(ob)* mutation was first described in 1950 as causing early onset (4–6 weeks) obesity and insulin resistance in mice (Ingalls *et al.*, 1950). Subsequent cross circulation experiments between mice with the *ob* mutation and those of normal weight suggested that the obese mice lacked a circulating factor that suppressed food intake (Coleman, 1978). Using positional cloning techniques, the *ob* gene and its human homolog were identified in 1994 as an adipose tissue specific gene (Zhang *et al.*, 1994). Shortly thereafter, recombinant ob protein was shown to induce weight loss in mice (Pelleymounter *et al.*, 1995; Halaas *et al.*, 1995; Campfield *et al.*, 1995), and the protein was named leptin, from the Greek root leptos meaning thin (Halaas *et al.*, 1995).

Within 1 year following the discovery of leptin and the demonstration that leptin could reduce food intake and body weight in mice, the leptin receptor was cloned (Tartaglia *et al.*, 1995). Very quickly it was proven that the leptin receptor was encoded by the *db* gene (Lee *et al.*, 1996; Chen *et al.*, 1996). It had been previously hypothesized that the *db* gene, which resulted in obesity in mice, encoded a receptor for the circulating factor regulating food intake that was missing in *ob*/*ob* mice (Coleman, 1978).

The discovery of leptin, its receptor, and actions within the brain opened novel areas of research into the central neural pathways regulating energy intake and expenditure. The cloning of leptin also replaced the perception of the adipocyte as merely a site of triglyceride storage with that of adipose tissue as a dynamic participant in the regulation of whole body metabolism.

Adipose Tissue Leptin Synthesis

The gene for leptin in both humans (*LEP*) and rodents (*Lep* replacing *ob*) encodes a 167-amino-acid protein with an amino-terminal secretory signal sequence of 21 amino acids (Zhang *et al.*, 1994). Leptin circulates in the blood as a 146-amino-acid, 16 kDa protein. Release of leptin from the adipocyte occurs primarily via a constitutive secretory pathway, although insulin may acutely increase the movement of leptin from the endoplasmic reticulum to the plasma membrane for release. Increased or decreased leptin release from the adipocyte is generally associated with a parallel change in *Lep* gene expression; thus, leptin synthesis and release appear primarily regulated by transcriptional mechanisms (Considine, 2001).

Fat Mass and Gender Determine Serum Leptin Concentration

The two major factors that determine serum leptin concentrations in humans under conditions of consistent food intake are fat mass and gender. Leptin positively correlates with fat mass in adults, children, and newborns. *LEP* gene expression is greater in the larger adipocytes present in subjects with obesity, and leptin secretion is strongly correlated with fat cell volume. Thus, the elevation in serum leptin in obesity is the result of secretion by a greater number of fat cells and increased leptin synthesis in the larger adipocytes of characteristic of these subjects (Bell and Considine, 2006).

Serum leptin is significantly greater in women than in men with the same amount of body fat (Rosenbaum and Leibel, 1999). One explanation for this finding is that women have a significantly greater subcutaneous adipose tissue mass relative to visceral adipose mass than men. *LEP* gene expression/leptin production is greater in subcutaneous than visceral adipocytes. Sex steroids also regulate leptin production. Testosterone replacement reduces serum leptin in hypogonadal men and leptin levels decrease as serum testosterone increases during pubertal development in boys. Estrogen, in combination with anti-androgens, increases leptin in male-to-female transsexuals and testosterone decreases leptin in female-to-male transsexuals, independent of changes in adipose tissue mass. In vitro, estradiol stimulates, and dihydrotestosterone inhibits, leptin production in human visceral adipose tissue pieces in culture for 48 h.

Energy Intake Regulates Serum Leptin Levels

Energy intake can regulate serum leptin concentrations independent of effects on adipose tissue mass. With standard timing for food intake (three meals per day in humans), leptin levels are reasonably constant from day to day, exhibiting a maximal daily variation of approximately 30%. However, serum leptin falls with short-term fasting (24 h) and increases within 4–5 h of

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refeeding. Maintenance of euglycemia with dextrose infusion prevents the fasting-induced drop in leptin, implicating insulin and/or glucose as the nutritional signal recognized by the adipocyte for leptin synthesis (Kolaczynski *et al.*, 1996; Boden *et al.*, 1996). In a prolonged study of energy restriction (moderate and severe), changes in serum leptin were best correlated with changes in glycemia (Wisse *et al.*, 1999).

Serum leptin exhibits a diurnal profile entrained to food intake (Sinha *et al.*, 1996, Schoeller *et al.*, 1997). The peak in serum leptin occurs at ~0200 h, in both lean and obese subjects under standard living conditions. Day/night reversal shifts the peak in serum leptin by 12 h. A shift of 6.5 h in meal timing without a change in light or sleep cycles shifts the leptin peak 5–7 h, suggesting that the nocturnal peak in serum leptin is a delayed postprandial response induced by after-meal excursions in glucose and insulin. The macronutrient content of meals also regulates serum leptin levels. High-fat/low-carbohydrate meals (60%/20%) over the course of 1 day reduce leptin levels (Havel *et al.*, 1999). High-fat/low-carbohydrate meals induce smaller insulin and glucose excursions than meals of standard fat/carbohydrate content, again implicating insulin and glucose in the nutritional regulation of leptin production.

The regulation of leptin synthesis by glucose and insulin has been directly demonstrated using the euglycemic–hyperinsulinemic clamp. Serum leptin is elevated by the end of prolonged clamps (9 h) at physiologic insulin or within 4–8 h with supraphysiologic insulin concentrations in humans (Saad *et al.*, 1998; Utriainen *et al.*, 1996). The synthesis of hexosamines within the adipose tissue is one link between insulin-stimulated glucose uptake/metabolism and leptin production. In rodents, infusion of glucosamine, uridine, or free fatty acids during a 3 h euglycemic–hyperinsulinemic clamp increased muscle uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), muscle *Lep* gene expression, and serum leptin, compared to saline-infused controls clamped under identical conditions (Wang *et al.*, 1998). In transgenic mice overexpressing glutamine: fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme in hexosamine biosynthesis, serum leptin and adipose tissue *Lep* gene expression is significantly increased (McClain *et al.*, 2000). In subcutaneous adipose tissue of obese humans, UDP-GlcNAc is increased 3.2-fold compared to lean subjects and there is a significant positive relationship between adipose tissue UDP-GlcNAc and body mass index (Considine *et al.*, 2000). An increase in hexosamine biosynthesis in cultured human subcutaneous adipocytes increases leptin release and inhibition of GFAT activity reduces glucose-stimulated leptin release.

Other Regulation of Leptin Synthesis in Adipocytes

Sympathetic nervous system

Leptin-induced activation of the sympathetic nervous system is postulated to be a negative feedback signal for leptin synthesis (Haynes *et al.*, 1997; Trayhurn *et al.*, 1998). Infusion of isoproterenol in humans (to mimic sympathetic activation) significantly reduces serum leptin (Donahoo *et al.*, 1997; Pinkney *et al.*, 1998; Stumvoll *et al.*, 2000), in agreement with preclinical data showing catecholamines inhibit leptin production in cultured cells and rodent models (Considine, 2001).

PPARγ agonists

Thiazolidinediones inhibit *Lep* gene expression and leptin release in rodents, isolated adipocytes and cultured cell lines (Considine and Caro, 1997). In cultured human adipocytes, troglitazone alone had little effect on leptin production but inhibited dexamethasone-induced leptin release (Williams *et al.*, 2000). PPARγ activation resulting in inhibition of *LEP* gene expression is counterintuitive since this highly expressed transcription factor is central to the differentiation of preadipocytes to adipocytes (Cornelius *et al.*, 1994). It has been proposed that PPARγ does not bind the *LEP* promoter but instead antagonizes binding of C/EBPα to down regulate expression (Hollenberg *et al.*, 1997).

Glucocorticoids

Dexamethasone is a potent stimulus for leptin secretion in vivo and in vitro and several studies suggest that glucocorticoids synergize with insulin to increase leptin production (Fried *et al.*, 2000). However, cortisol is not an absolute requirement for leptin synthesis and it is not clear how glucocorticoids increase *LEP* mRNA as the glucocorticoid receptor does not bind the *LEP* promoter.

Leptin Signaling in the Brain

The Leptin Receptor

The hypothalamic leptin receptor (Ob-Rb, using mouse nomenclature) is a member of the class I cytokine receptor family. As with other cytokine receptors, binding of leptin to its receptor activates an associated janus kinase (JAK), which phosphorylates tyrosines within the leptin receptor itself and signal transducer and activator of transcription (STAT) proteins associated with the receptor. Phosphorylated STAT proteins dimerize and translocate to the nucleus to initiate gene transcription. STAT-3 is the major STAT protein activated by the hypothalamic leptin receptor in mice (Tartaglia, 1997; Friedman, 1998). Binding of leptin to its receptor has also been reported to activate phosphatidylinositol-3-kinase, phosphodiesterase-3B, and members of the mitogen-activated protein kinase superfamily. Low-level expression of Ob-Rb has been detected in cells other than those in the hypothalamus and it has been shown that leptin activates AMP-activated protein kinase in skeletal muscle (Bjørbaek and Kahn, 2004).

In addition to the hypothalamic leptin receptor Ob-Rb (long receptor isoform) described above, five other leptin receptors mRNAs have been identified in mice, all encoded by alternative splicing of the *Lep* gene. The extracellular domain of all leptin receptors is identical; however, the intracellular domains terminate at different lengths. Due to the truncated intracellular domain, the short leptin receptors lack the box 2 motif necessary for STAT protein interaction and these leptin receptors do not phosphorylate STATs. Ob-Ra is the most abundant and widely distributed of the short receptor isoforms, and is found on most cell types in the body. Ob-Ra is highly expressed in the choroid plexus where it functions to transport leptin across the blood–cerebrospinal fluid barrier. The Ob-Re receptor isoform, lacking an intracellular domain and a transmembrane domain, circulates in the blood of rodents as a leptin-binding protein, prolonging the half-life of the hormone. In humans, the soluble leptin receptor binding protein is generated by proteolytic cleavage of the extracellular domain of membrane bound leptin receptor (Ahima and Flier, 2000).

Leptin-Activated Hypothalamic Pathways

Leptin receptor is detectable in many areas of the CNS but is highly expressed in the arcuate, paraventricular, ventromedial, and dorsomedial nuclei of the hypothalamus, which is responsible for integration of the numerous neural signals regulating energy intake. Leptin is detectable in cerebrospinal fluid but leptin likely accesses the arcuate nucleus directly from the blood within the median eminence, which lacks the tight junctions characteristic of the blood–brain barrier. In the arcuate nucleus, leptin reduces the expression of neuropeptides that stimulate food intake and increases expression of neuropeptides that inhibit feeding. As such, leptin suppresses neuropeptide Y and agouti-related peptide expression in neurons expressing these neurotransmitters and induces the expression of α-melanocyte-stimulating hormone in proopiomelanocortin neurons. These neurons innervate other nuclei within the hypothalamus and also communicate with other areas of the brain. Leptin-binding neurons in the arcuate nucleus mediate the effects of leptin on the sympathetic nervous system and hypothalamic pituitary axes. Leptin actions in the arcuate nucleus can amplify neuronal signals of food intake from the gastrointestinal tract to the nucleus of the solitary tract in the hindbrain (Elmquist *et al.*, 1999).

Leptin Binding in the Brain Reduces Food Intake and Body Weight in Mice

The role of leptin in regulation of body weight was first demonstrated in C57BL/6 J *ob*/*ob* mice (Pelleymounter *et al.*, 1995; Halaas *et al.*, 1995; Campfield *et al.*, 1995). The lack of leptin in *ob*/*ob* mice causes hyperphagia, cold intolerance, and morbid obesity. Daily intraperitoneal injection of recombinant leptin resulted in a dose- and time-dependent decrease in body weight of *ob*/*ob* mice. Weight loss was mediated by an increase in metabolic rate and locomotor activity, as well as a reduction in food intake. Leptin also reduced body weight in lean C57BL/6 J heterozygotes (+/?) and lean wild-type mice, although much higher doses of leptin were needed. In lean mice, leptin administration had no effect on body temperature or locomotor activity but did reduce food intake. Leptin treatment lowered insulin and glucose in *ob*/*ob* mice. Since these initial experiments, many investigators have observed that leptin, either peripherally or centrally administered, effectively reduces food intake to stimulate weight loss in most animal models of obesity, with the exception of those with inactivating leptin receptor mutations.

C57BL/KsJ *db*/*db* mice are hyperphagic, morbidly obese, and diabetic due to a mutation in the hypothalamic leptin receptor gene. A nucleotide substitution (G to T) generates a new splice donor within an exon of the short receptor (Ob-Ra). This new splice donor competes with the upstream splice donor of the long form of the receptor, resulting in alternative splicing and the insertion of a premature termination signal in the long form of the receptor. The truncated long receptor protein is unable to associate with STAT proteins, interrupting JAK/STAT signaling and resulting in obesity in *db*/*db* mice. Exogenous leptin administration to C57BL/6J *db*/*db* mice does not result in weight loss at any concentration tested. A different leptin receptor mutation was identified in Zucker *fatty* (*fa*/*fa*) rats. An A-to-C substitution changes glutamine-269 to a proline in the extracellular domain of the receptor, reducing the amount of receptor on the cell surface and its capacity to signal. In obese Koletsky rats, a third receptor mutation, T2349A in codon 763 of the leptin receptor gene, creates a premature stop codon just before the transmembrane domain. Leptin treatment does not induce weight loss in either Zucker *fatty* rats or Koletsky rats (Tartaglia, 1997; Friedman, 1998).

Leptin, Synaptic Plasticity and the Hippocampus

In addition to leptin's activity in the hypothalamus modulating energy balance, leptin signaling influences function in other brain areas. The hippocampus, part of the limbic system involved in learning and memory, exhibits leptin receptor on both neurons and glial cells. Leptin deficient rodent models have display impairments in synaptic long term potentiation (synaptic plasticity) and spatial learning. Leptin regulates hippocampal excitability by modulating calcium-activated potassium channels in vitro, and improves memory processing in mice (Harvey *et al.*, 2006).

Leptin and Body Weight Regulation in Humans

Leptin and Leptin Receptor Mutations Cause Obesity in Humans

Mutations in the human *LEP* gene were first identified in children of a consanguineous Pakastani family. In these subjects, deletion of a guanine nucleotide in codon 133 produces a frameshift resulting in truncated leptin protein that is degraded by the proteasome. There is no leptin in the circulation. A third child from an unrelated family (at least five generations back) was found to have the same *LEP* gene mutation. The lack of leptin in these children causes hyperphagia and extreme early onset morbid obesity. A second *LEP* gene mutation, a C-to-T substitution in the first base of codon 105, changing arginine to tryptophan, was identified in a Turkish family. In three subjects homozygous for the mutation, serum leptin was very low but detectable. The low leptin in these subjects resulted in hyperphagia, morbid obesity, and hypogonadism. One subject also exhibited low sympathetic tone (Farooqi and O'Rahilly, 2005).

As with mutations in the *LEP* gene, mutations in the leptin receptor in humans are rare. A G-to-A substitution in the splice donor site of exon 16 has been identified in three sisters of a Kabilian family. This mutation results in a truncated leptin receptor lacking the transmembrane and intracellular domains. The mutant extracellular domain is present at high concentrations in the blood, acting as a leptin-binding protein to greatly increase serum leptin concentrations. This leptin receptor mutation results in early onset morbid obesity, hyperphagia, and hypothalamic hypogonadism (Farooqi and O'Rahilly, 2005).

Leptin Induces Weight Loss in Humans With *LEP* Mutations

Farooqi and colleagues have demonstrated that subcutaneous administration of recombinant leptin is an effective long-term therapy for obesity in the three children lacking leptin due to *LEP* gene mutations. With treatment for up to 4 years (0.01 mg kg^{-1} lean body weight), leptin therapy induced significant weight loss via a reduction in caloric intake. Weight loss in these children was associated with a reduction in insulin, serum triglycerides, and cholesterol. Leptin treatment also improved T cell proliferation and increased $CD4^+$ T cell number and function, which was severely impaired in the absence of endogenous leptin production. Adults with congenital leptin deficiency or leptin receptor mutations are prepubertal. Leptin replacement was permissive for pubertal progression of the oldest child treated (therapy started at age 10 years), but did not induce precocious puberty when younger children (3 and 4.5 years old) were treated (Farooqi and O'Rahilly, 2005). With respect to effects of leptin on neuroplasticity discussed above, leptin replacement therapy improved the rate of cognitive development in one child tested (Paz-Filho *et al.*, 2008).

Leptin Resistance

Inactivating mutations in the leptin and leptin receptor gene in humans are rare and not the cause of obesity in the general population. Rather, it has been observed that serum leptin levels are elevated in obese subjects. This observation has led to the hypothesis that obese individuals are "leptin resistant"; i.e., these subjects do not respond to the weight-reducing actions of leptin (Considine *et al.*, 1996). Leptin resistance is dependent on the premise that the major function of leptin is to oppose increases in body weight. Flier and colleagues have noted that from an evolutionary perspective, it is difficult to conceive of a mechanism that would limit food intake in times of excess. Therefore, they hypothesize that the major function of leptin is to signal the reduction in energy intake associated with fasting rather than the excess storage of energy in times of food abundance. Evidence for this proposal is derived from the observation that the reduction in leptin with fasting is associated with metabolic, hormonal, and behavioral changes promoting energy conservation and increased energy intake. Leptin administration during fasting prevents these adaptations to food restriction (Ahima *et al.*, 1996). The concept of leptin resistance is compatible with the role of leptin as a signal of energy deprivation. It is conceivable that the central nervous system in obese individuals does not properly receive or process the leptin signal generated by the adipose tissue, despite elevated serum leptin levels. This would result in metabolic adaptations to conserve energy and increase energy intake. Several explanations for leptin resistance have been put forth.

High-fat feeding in rodents decreases the effectiveness of intraperitoneal or intravenously injected leptin to induce weight loss, although the response to leptin administered directly to the brain is maintained. Therefore, high-fat feeding appears to cause a defect in access of leptin to the central nervous system (Banks *et al.*, 2006). An alternative interpretation is that the palatability of the high-fat diet provides a neural signal for increased consumption that is stronger than the signal for decreased food intake provided by intraperitoneally or intravenously administered leptin. The fact that central leptin administration could overcome this palatability signal may be the result of activation of additional neural pathways that inhibit food intake with central injection.

Suppressors of cytokine signaling (SOCS) are a group of genes rapidly activated following STAT binding in the nucleus. SOCS proteins act in a negative feedback loop to limit intracellular cytokine signaling. SOCS-3 is a potent inhibitor of leptin receptor-initiated JAK/STAT signal transduction in cultured cell lines. Leptin also induces SOCS-3 mRNA expression in the hypothalamus. SOCS-3 message is increased in leptin-binding neurons in the hypothalamus suggesting that the increased SOCS-3 expression could cause leptin resistance. Protein tyrosine phosphatase 1B also attenuated leptin receptor signaling in vitro and in vivo (Münzberg and Myers, 2005).

Leptin Therapy in Obese Adults Without Leptin Deficiency

Modest leptin-induced weight loss was achieved in obese subjects with normal adipose tissue leptin production (Heymsfield *et al.*, 1999). In this study, 47 obese subjects underwent 24 weeks of twice-daily injections of leptin (0.1–0.3 mg kg^{-1} body weight) or placebo. There was statistically significant weight loss with the highest dose of leptin tested (mean $\pm$ SD 7.1 $\pm$ 8.5 kg, $n = 8$). It is important to note that the dose of leptin needed to induce weight loss in leptin-replete subjects is greater than 10 times that needed in the children with *LEP* gene defects. As discussed above, serum leptin concentrations increase in parallel with body fat in all obese individuals who do not have a *LEP* gene mutation. This apparent "resistance" to the weight-reducing effects of endogenously produced leptin suggests that exogenous leptin administration would be ineffective in decreasing body weight. The demonstration that leptin therapy can induce weight loss in leptin-replete subjects suggests that leptin resistance is relative, and can be overcome with high-dose treatment. However, whether these subjects would become resistant to the weight loss effects of the therapeutic dose of leptin over longer treatment periods is not known.

As discussed above, Flier and colleagues suggest that the major role of leptin is to decrease with prolonged fasting/starvation and initiate processes to conserve energy and maintain body weight. Therefore, the reduction in leptin with caloric restriction during a diet would be counterproductive to the attempt to lose weight. In a controlled study in which subjects lost 10% of body weight, circulating triiodothyronine and thyroxine levels and total daily energy expenditure were reduced. Administration of recombinant leptin to these subjects normalized thyroid hormone levels and increased total energy expenditure (Rosenbaum *et al.*, 2002). These observations support a role for leptin as opposing weight loss and suggest that leptin therapy may be a useful augmentation to weight loss induced by diet and exercise or other pharmacologic treatment.

Leptin Therapy in Lipodystrophy

Lipodystrophy is a condition in which adipose tissue is either partially or fully absent. Individuals with lipodystrophy generally exhibit hypertriglyceridemia, insulin resistance, and hyperglycemia due to deposition of lipid in liver and muscle. As a consequence of the lack of adipose tissue, leptin levels are low (Chan and Oral, 2010). Leptin replacement therapy has been FDA approved for generalized forms of lipodystrophy. Leptin therapy significantly decreased fasting glucose, hemoglobin A1c, triglycerides and liver volume. Subjects also report an increase in satiation. There is some weight loss at initiation of therapy but body weight stabilizes at approximately 6 months (Meehan *et al.*, 2016).

Effect of Leptin on Other Physiologic Processes

In addition to its role in body weight regulation, leptin has been implicated as a regulatory signal in a variety of other physiological processes. Many of these functions of leptin are mediated through the central nervous system, although the presence of leptin receptors on numerous nonneural cells suggests that leptin may have direct effects on these cells and tissues.

Glucose Homeostasis and Lipid Metabolism

Leptin improves insulin sensitivity directly and independently of its effects on food intake and body weight. In rodents, insulin and glucose are reduced shortly after the initiation of leptin treatment, before significant reductions in body fat content are observed. With long-term leptin administration resulting in weight loss, insulin and glucose are reduced to a greater extent than in pair-fed controls. Intracerebroventricular leptin acutely enhances whole body glucose uptake in mice, suggesting that leptin influences insulin sensitivity through central mechanisms. Leptin therapy also reverses insulin resistance and diabetes in two different transgenic models of lipodystrophy through mechanisms not related to reductions in food intake (Morgan and Considine, 2015).

Leptin increases lipolysis in white adipose tissue both in vivo and in vitro. This effect of leptin results from centrally mediated activation of the sympathetic nervous system as well as a direct effect of leptin on adipocytes. Hyperleptinemia in rats does not result in the release of free fatty acids into the blood but rather the oxidation of fatty acids within adipocytes. Leptin has also been observed to stimulate fatty acid oxidation in rodent muscle via activation of AMP-activated protein kinase (Morgan and Considine, 2015).

In rodent models of type 1 diabetes leptin lowers glucose, HbA1c and free fatty acids. Type 1 diabetes may be considered a "relatively" leptin deficient state due to loss of insulin-stimulated leptin synthesis in adipocytes. Leptin therapy in two patients with type 1 diabetes and lipodystrophy lowered glucose, lipids and insulin requirements (Park *et al.*, 2008). Thus, there is interest in use of leptin in the treatment of type 1 diabetes.

Reproduction

Successful reproduction requires an extensive investment of energy. Leptin serves as a permissive signal that energy stores are sufficient for the increased demand for energy during reproduction. *Ob*/*ob* mice lacking leptin as a signal of energy stores are infertile. Leptin treatment reverses infertility in *ob*/*ob* mice and accelerates the onset of puberty in normal mice. During starvation,

when endogenous leptin levels in mice are low, exogenous leptin maintains LH release and estrous cycle periodicity (Hileman *et al.*, 2000). In humans, the age of menarche is inversely related to serum leptin concentrations (Matkovic *et al.*, 1997). Humans with *LEP* gene defects have hypothalamic hypogonadism and remain prepubertal. Leptin therapy in these subjects increases gonadotropin secretion and the progression through puberty (Farooqi and O'Rahilly, 2005). Overall, these observations suggest that leptin is a coordinating signal to the central nervous system that energy stores are sufficient to support the higher energy needs associated with reproduction.

Maternal serum leptin increases during pregnancy in both rodents and humans and this increase is not due solely to the increase in fat mass. Human placenta synthesizes leptin, which contributes to the elevation in maternal leptin levels during pregnancy. In rodents, the placenta also secretes soluble leptin receptor binding protein, which increases the half-life of leptin in the maternal circulation. The role of increased leptin during pregnancy is not fully understood. It is possible that the increase in leptin might regulate maternal–fetal energy metabolism or that leptin may act in a paracrine/autocrine manner on placental or fetal growth (Henson and Castracane, 2006).

Immune Function

Nutritional deprivation suppresses immune function and malnutrition predisposes to death from infectious diseases. Leptin appears to be a coordinating signal for activation/inactivation of the immune response during times of energy excess or deprivation. Leptin administration prevents the starvation-induced reduction in immune function in mice. Leptin also stimulates the proliferation of T cells in vitro, an effect mediated by direct interaction of leptin with leptin receptor isoform Ob-Rb expressed on the T cell (Lord, 2002). The number of $CD4^+$ T cells and their function was severely impaired in children lacking endogenous leptin due to *LEP* gene mutations. Leptin treatment improved T cell proliferation and activity in these children (Farooqi and O'Rahilly, 2005).

Bone Metabolism

Obesity is in general protective against bone loss and fractures. A role for leptin as mediator of this protective effect is supported by the observations that leptin administration reduces ovariectomy-induced bone loss in rats and increases bone mineral content and bone density in *ob*/*ob* mice. That these effects are the result of a direct effect of leptin on bone is inferred from observations that leptin promotes differentiation of bone marrow stromal cells to osteoblasts and promotes mineralization of these cells. Leptin also inhibits osteoclast generation from blood mononuclear cells. In contrast to observations that leptin promotes bone formation, leptin-deficient *ob*/*ob* and leptin-resistant *db*/*db* mice have significantly greater bone mass than wild-type littermates. Intracerebroventricular administration of leptin to *ob*/*ob* mice results in bone loss in these animals. A unifying hypothesis for these disparate findings is that the central effect of leptin to decrease bone formation is offset by its peripheral effects to promote bone growth. The importance of this postulate is that during periods of starvation, low leptin levels result in suppression of the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes and bone would be lost. However, the low leptin levels in the brain then also activate pathways that protect the skeleton during starvation. The mechanisms by which the hypothalamus regulates bone formation have not been fully elucidated (Upadhyay *et al.*, 2015).

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See also: Endocrine Disruptors and Obesity. Functional Hypothalamic Amenorrhea. Hormonal Control of Puberty. Human Menstrual Cycle. Secular Changes in Puberty

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Lifestyle and Nutrition

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Glossary

ADA diets Dietary approach based on American Diabetes Association recommendations.
AHA/ACC/TOS American Heart Association/American College of Cardiology/The Obesity Society.
AHI Apnea-hypopnea index used to indicate severity of sleep apnea. AHI is calculated by dividing the number of pauses of breathing (apnea) by the number of hours of sleep.
BMI Body Mass Index—a measurement based on height and weight, an indicator of the degree of nutrition.
DASH Dietary approach for regulation of hypertension.
FTO gene Fat mass and obesity-associated gene, associated with reproducibly with human body mass.
HbA1c Glycated hemoglobin, an indicator of the quality of glycoregulation for the last 3 months.
HCLF High-carbohydrate, low-fat dietary regiment.
HOMA IR Homeostatic model assessment, mathematical model for insulin resistance calculation.
HRQOL Health related quality of life.
LDL Low density lipoprotein, proatherogenic cholesterol.
MET Unit equal with energy expenditure at rest.
MUFAs Monounsaturated fat acids.
NIH National Institute for Health.
OSA Obstructive sleep apnea, common disorders in obesity which compromise oxygenation during the sleep.
REM Rapid eye movement, a sleep phase.

Severe obesity as a serious public health concern requires efficacious treatment. Available drugs are not enough efficacious and bariatric surgery is effective but expensive, thus some other treatments are needed. Lifestyle interventions can help severely obese individuals to achieve long-term weight loss and to improve the risk factors for cardiovascular diseases (Unick *et al.*, 2013).

The aim of lifestyle modifications is to reduce the burden of obesity and obesity related comorbidities. Some people consider that small amount of weight loss is disappointing, but even a small amount of weight loss like 5%–10% from baseline is associated with significant benefits. Sedentary behavior is associated with a lot of metabolic and cardiovascular comorbidities like obesity, diabetes, metabolic syndrome, and cardiovascular disease (Chau *et al.*, 2015). Environmental factors such as sitting jobs, access to television and the Internet in households for which people sit for hours in front of the screen cause lower energy expenditure. U.S. population spent sitting approximately 7.7 h/day (Matthews *et al.*, 2012; Liao *et al.*, 2011). Sedentary lifestyle leading to reduction in energy expenditure although overeating of high calorie foods increases energy consumption. Patients with lower educational level and low income have several times greater chance to develop sedentary lifestyle (Konevic *et al.*, 2015). One study on large sample of older adults in United Kingdom with self-reposted TV viewing duration demonstrated that increasing in watching TV was associated with higher BMI besides other parameters for example, physical inactivity, lower socioeconomic status depression and being a smoker (Gardner *et al.*, 2014). Study conducted in United Kingdom on 3777 non obese adult participants analyzed association between TV viewing and obesity. Strong association was found between TV watching more than 6 h/day and central obesity but they did not find association between the time spent in front of TV screen and BMI > 30 kg/m^2. The possible explanation for isolated central but not overall obesity was that older people have predominantly central fat distribution.

Trying to collect the data regarding sedentary behavior a numerous authors used traditional questionnaire as the first-line tool. Disadvantage of this common method is that requiring long periods of recall. Some investigators use MET. 1 MET value is equal with energy expenditure at rest. MET has a disadvantage that cannot distinguish sitting from the standing position as well as a waking state of sleep (Owen *et al.*, 2011).

Different types of activity monitors, like accelerometry-based monitors have been used in order to measure sedentary time, or time spent in physical activity. Those accelerometer fixed to the hip could detect body position (sitting, standing, lying). Inclinometers with higher ability than accelerometer to detect change in posture had been used in another type of devices (Trost *et al.*, 2005; VAN Loo *et al.*, 2017).

In order to develop tool for objective monitoring with ability to correct errors in the self-report method physical activity measurement survey (PAMS) was conducted as NIH-funded project. 24PAR (physical activity recall) RC was acceptable to respondents because they were supposed to provide data regarding physical activity only from the previous day (Dunton *et al.*, 2009).

Specialist in behavioral epidemiology suggests six phases of research in order to advance approach to sedentary behavior. The first phase is identifying associations between sedentary behavior and health conditions, there are also adequate measures of sedentary behavior, defining prevalence of sedentary behavior across different populations, identifying the determinants of sedentary behavior, developing interventions to influence in sedentary behavior and using the relevant evidence for public health guidelines and policy (Owen *et al.*, 2010).

Change in lifestyle is the first step in management of obesity. Term "lifestyle" includes dietary habits and physical activity. In order to help patients to implement new lifestyle recommendations, physicians should be interested in patients' lifestyle history and possible contributors to weight gain. Information about previous weight reductions attempts, medical conditions and therapy and family history of obesity could be very useful in planning strategy for weight management for every patient in order to reach therapeutic goals. Desirable weight loss from baseline is 5%–10% within 6 months according the AHA/ACC/TOS guidelines (Jensen *et al.*, 2014).

The energy deficit of at least 500 kcal/day is necessary for weight loss. This deficit could be provided by calorie restriction or through physical activity. Best results achieving the patients who combine the both approach which are including in group sessions or in individual program with trained interventionist.

Usual components of weight loss program are reduced calorie-intake. At the same time or soon after the start of the dietary regime it is recommended to gradually intensify physical activity. Recommendations for childhood obesity treatment included also multi-disciplinary approach, integrated care model and family-based multicomponent behavioral therapy (Wilfley *et al.*, 2017).

Lifestyle intervention begins during the pregnancy. Pregnant women are the first in line for implementation a lifestyle intervention in order to prevent obesity. It was demonstrated that increased intake of refined-grain during pregnancy was significantly related to a greater BMI among children in 7 years of age (Zhu *et al.*, 2017).

Change in dietary habits and physical activity influences not just on metabolic obesity related disorders but also on of obstructive sleep apnea (OSA). One study analyzed OSA severity in REM (rapid eye movement) sleep, in overweight patients with diabetes type 2, during 4 years follow-up under the intensive lifestyle intervention. Lifestyle changes reduced severity of OSA, REM-AHI, and NONREM-AHI as well as HbA1c and other metabolic parameters. Weight loss was a better predictor of improving glycemic control than reductions in AHI **Fig. 1** (Shechter *et al.*, 2017).

Despite one meta-analysis from Arabic-speaking countries indicated that lifestyle modifications programs were no more effective than other treatments (Kreidieh *et al.*, 2017) the most of studies demonstrated tight connection between lifestyle intervention and improvement in metabolic and cardiovascular diseases (Shechter *et al.*, 2017; Stavropoulos *et al.*, 2017; Bloch, 2017; Davy and Melby, 2003; Gulati and Misra, 2017).

Dietary Treatment

Dietary treatment was probably the first and main therapeutic approach for obesity in the past. This treatment is based on negative energy balance caused by lower energy intake but also in change in composition of food and proper rhythm of meals. There are a numerous available commercial diets but fad or crush diets published in magazines could damage health and should be avoided. Diets have to be medically confirmed and, if it is possible, patient tailored. Long-term calorie reduction with appropriate ingestion of necessary nutrients reduces the risk for obesity comorbidities (Sofer *et al.*, 2015).

Common dietary recommendations are:

- Reducing fat, carbohydrates with high glycemic index, protein or alcohol consumption
- Smaller portion size
- Distribution of energy intake at various time of day with a largest amount of calorie was taken in the morning
- Low-calorie food
- Combining different foods

After successful diet the main issue is how to maintain the weight loss. Eating habits need to change for a long time.

During the time there were different dietary recommendations. The data indicate that improving in dietary ingredients influence on blood glucose, insulin, serum lipids, and inflammatory markers. In order to prevent vitamin and mineral deficiency

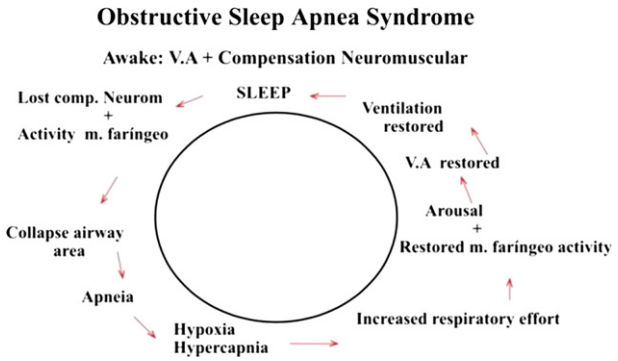

Fig. 1 Obstructive sleep apnea syndrome. From Maciel de Carvalho, T. (2015). Obstructive sleep apnea in Down syndrome children. *EC Dental Science* **2**(4), 321–327.

well balanced diet is recommended. Change in food ingredients implies more complex carbohydrates, more monounsaturated fatty acids and increasing in protein intake.

The data show that improving quality of carbohydrates (more complex carbohydrates), improving fat quality (more monounsaturated fatty acids and omega three polyunsaturated fatty acids) and increasing protein intake could improve blood glucose, serum insulin, lipids, inflammatory markers and hepatic fat, but more studies are needed (Stavropoulos *et al.*, 2017).

Traditional dietary recommendations for obesity and obesity related co morbidities include high-carbohydrate, low-fat (HCLF) regimens. Despite the positive experience with HCLF diet and scientific approval this diet for metabolic disease management, the rates of obesity and metabolic syndrome continue to rise. The Women's Health Initiative study demonstrated that the low-fat diet failed to prevent cardiovascular events, cancer and impaired glycemic control in patients with diabetes (Howard *et al.*, 2006). Dietary Guideline Advisory Committee in the United States in 2015 withdrawn recommendation for fat limitation and concluded that limiting total fat intake could not prevent obesity (Mozaffarian and Ludwig, 2015). In some populations, low-carbohydrate diet was accepted as dietary therapeutic approach for some diseases. In Japan, low-carbohydrate diet proves to be effective in patients with type 2 diabetes. NIPPON DATA 80 had shown that low-carbohydrate diet is safe, effective and strongly associated with lower mortality rate in Japanese population.

Ketogenic Diet

Ketogenic diet with extremely low carbohydrate intake, less than 50 g/day are associated with lipid profile impairment. The main concern in a low-carbohydrate diet is increase in lipid levels and cardiovascular risk (Kwiterovich *et al.*, 2003).

There is evidence that a high intake of carbohydrates may influence abnormalities associated with metabolic syndrome. A lot of studies emphasize the importance of dietary fiber intake.

Dietary fibers provide sense of fullness and prolonged satiety. Also fibers rich diet allows gradually degradation of carbohydrates and low serum glucose oscillations. Therefore, high fiber diets could delay further impairment of glucose toleration (**Table 1**) (Polovina and Micić, 2010). Eating carbohydrates last ("Carbo-last" diet) is based on facts that eating vegetables first (dietary fibers), than meat and staple food caused suppression of gluconeogenesis. Underlying mechanism of delay gastric empting in this diet is incretin effect (Kuwata *et al.*, 2016).

Mediterranean Diet

Mediterranean diet composed by olive oil, fruits, vegetables, whole grains, nuts, legumes, small amounts of diary (cheese and yogurt) and fish has proven effect in preventing diabetes (Sofer *et al.*, 2015). The same diet was not such effective in glycemic control in patients with diabetes. Possible explanation for unsatisfied glycemic control is greater fruit intake in Mediterranean diet that is recommendable for diabetic patients (Díez-Espino *et al.*, 2011). The main difference between the Mediterranean and ADA diets is in monounsaturated (MUFAs) content. MUFAs has impact on insulin sensitivity, postprandial glucose level, and lipid profile (Esposito *et al.*, 2010).

DASH

DASH trial demonstrated that an HCLF diets with 20–35 g/day dietary fiber intake (regarding recommendations of the American Dietetic Association), may be beneficial for patients with metabolic syndrome (Bloch, 2017). Another, most popular diet is low-carbohydrate, high fat diet regimens well known as Dr. Atkins' new diet revolution. Despite popularity, there is no enough scientific evidence in favor of safety and efficacy of this diet on cardiovascular health (Stevens Ohlson 2006; Davy and Melby, 2003).

Table 1 Metabolic parameters before and 12 weeks after fiber rich diet

Parameter	*Group A BD*	*Group A AD*	*P*	*Group B BD*	*Group B AD*	*P*
Waist circumference	123 ± 28.6	116 ± 23.2	<.001	129 ± 36.2	131 ± 34.6	NS
FPG	6.1 ± 0.7	5.4 ± 0.5	<.05	6.2 ± 1.1	6.2 ± 0.8	NS
Insulin	123 ± 36	86 ± 19.1	<.05	121 ± 24.2	124 ± 22.1	NS
HOMA IR	9.8 ± 3.2	5.03 ± 1.3	<.001	10.3 ± 3.1	10.8 ± 2.8	NS
HDL-c	0.96 ± 0.5	1.1 ± 0.3	<.05	0.92 ± 0.7	0.9 ± 0.1	NS
Triglycerides	2.9 ± 1.1	2.1 ± 0.6	<.001	2.7 ± 0.9	2.5	NS

BD, before diet; *AD*, after diet; *FPG*, fasting plasma glucose; *HOMA IR*, homeostatic model assessment for insulin resistance; *HDL-c*, high density lipoprotein cholesterol.
From Polovina, S. and Micic, D. (2010). The influence of diet with reduction in calorie intake on metabolic syndrome parameters in obese subjects with impaired glucose tolerance. Medicinski Pregled, 63(7–8), 465–469. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21446131 [Accessed January 11, 2017].

Vegetarian Diet

Vegetarian diet is presented in two principles: vegan (do not eat animal products), lacto-vegetarian (consuming dairy products along plants), ovo-vegetarian (using eggs along plants), and lactoovo-vegetarians (eat dairy and eggs besides plants). Previous studies demonstrated that vegan diet is more successful in total cholesterol and LDL-c lowering, HBA1c reduction and reduction in diabetes drugs than standard ADA treatment although weight reduction was similar. The overall conclusion was that vegetarian diets could have positive effect on glycemic control and noninferior effect on weight reduction (Ajala *et al.*, 2013).

A communities should engage their resources in order to address obesity-related behaviors and implementation healthy eating and activity across the lifespan (HEAL) integration model (Berge *et al.*, 2017). Finally, regulatory framework must be tightened to impose taxes on sugar-sweetened beverages, oils such as palmolein, and dietary fats and limit trans fats (Gulati and Misra, 2017).

In order to achieve greater weight loss "meal replacement programs" were developed. The main characteristic of these programs is replacing normal meals with prepared meal supplements like soups, bars or vitamin-rich shakes. The meal replacement formulas provide enough energy that not causes malnutrition. Underlying mechanism of this treatment is mild ketosis induced by limited carbohydrates intake. Ketosis is a consequence of break down fat storage for energy (Proietto and Baur, 2004).

Some authors underlined the importance of time for food intake. One study showed that carbohydrates eaten just at dinner could decrease abdominal obesity and parameters of metabolic syndrome. One animal study demonstrated that time-restricted feeding improved insulin resistance, inflammation, fat content in liver, obesity and obesity related comorbidities. This study also shows that timed high-fat diet provides better satiety and significant lower ghrelin concentrations. Late main meal is associated with less weight reduction despite nutrient composition, energy expenditure, and sleep duration. Greater weight loss and waist circumference reduction was in large breakfast group due to lower ghrelin level and high satiety scores. This group is also characterized with significant improvement in HOMA IR.

The most satiating nutrient is dietary protein. Proteins could increase feeling of fullness and reduce the hunger (Jiwa *et al.*, 2015). Addition amount of protein in breakfast might help to reduce hunger in the middle of the day (Ossolinski *et al.*, 2017). Some studies conducted during Ramadan demonstrated that concentrated carbohydrate intake at dinner time, but no during the day, could be helpful for obese people or for people with metabolic syndrome. One possible underlying mechanism is increase in serum leptin at midday and feeling of satiety and reduced level of ghrelin. Adiponectin, antiinflammatory hormone, which is low in obese people, become elevated after a whole day starvation. This antiinflammatory effect reduced risk factors for metabolic syndrome (Kreidieh *et al.*, 2017).

Physical Activity

Physical inactivity is associated with increased risk for several noncommunicable diseases and one of the main causes of mortality. Increased physical activity play very important role in long-term maintaining weight loss (Catenacci and Wyatt, 2007). Current recommendations are that adults should have at least 150 min of moderate-intensity physical activity during a week (Kohl *et al.*, 2012). The represent of moderate-intensity physical activity is walking. Walking provides psychological and physical benefits, requires minimal equipment, and risk for injury is low (Baker *et al.*, 2010).

Although lifestyle interventions including physical activity and change in dietary habits, weight regain and returning to sedentary behavior are common in high number of patients. Self-monitoring or group support could be very important in maintained the intense of physical activity over a long period of time. Results from SAVE (slow the adverse vascular effects) study demonstrated that, in comparison to patient who did not use any specific strategies to apply lifestyle interventions, patients who used self-monitoring or group support had greater increase in physical activity (Lott *et al.*, 2017).

Study conducted in order to analyze association of sedentary lifestyle and obesity in EU shown that obesity prevalence was greater in those who spent more than 35 h sitting during a leisure time per week in comparison with those people who spent this time in physical activity (Martínez-González *et al.*, 1999).

There was no definitive conclusion that physical activity could facilitate greater improvement in HRQOL than diet alone. Also, there was no existing evidence that an exercise prescription without diet for may improve HRQOL in obese adults (van den Hoek *et al.*, 2017).

One animal study had shown association between that high-intensity interval in Rev-erbα and CPT1 expression and prevention of diet-induced obesity. The authors concluded that increased caloric expenditure achieved with HI interval protocol might be useful to improve lipid metabolism (Shen *et al.*, 2015).

In order to maintain weight loss increased physical activity is essential beside dietary treatment. Exercise program should be gradually built up along with cardio respiratory fitness improvement. About 30 min of walking 3/5 days per week is recommended for as a good start point. The patients could start with walking to nearby places, climbing the stairs rather than choosing escalator or elevator. For extremely obese activities in water such swimming or walking could improve fitness on the beginning. Psychologist could help obese patients to produce a long term adherence to exercise program. Adherence to the diet and physical activity vary depend of age and motivation. One nationwide survey shown that structured program in school campus may help that children accept healthy lifestyle (Proietto and Baur, 2004).

In order to support increased physical activity, self-monitoring using pedometers could be effective. One investigation implemented pedometers as motivational tools to motivated males in maintained of physical activity. This weight management

program for overweight or obese men lasted 12 weeks. The results shown that in majority of participants pedometer provide proof of progress and enabled autonomy in monitoring the program effect. Those participants that accepted pedometer as allied felt increasing competence for change. The minority of men who not achieved the physical activity goals felt pedometer as controlling. Some man continued to use pedometers after the program because they found that pedometer helps them to maintain their physical activity. The men which no longer used the pedometer were less successful in weight loss (Donnachie *et al.*, 2017).

Motivation for Lifestyle Change

During past time, there was a lot attempts to established the best therapeutic approach to motivate obese patients to lose a weight. One approach was multidisciplinary obesity treatment program (MOTP) with the idea to achieving long-term outcome regarding weight reduction and emotional state. The second objectives of this program were to analyze the influence of psychological factors on weight change. The first, intensive treatment part of this program lasted 12 weeks, included dietary counseling, exercise and cognitive behavioral therapy in order to define realistic weight goals and dysfunctional eating habits if they are existed. They were trained in emotion regulation skills. Dietetic counseling included facts about balanced diet, combination of nutrients and reduction of some ingredients. The exercise program consisted of water gymnastics, Nordic walking or weight lifting. The program included session with psychotherapist which analyzed the progress of patients. Patients and psychotherapist also evaluated obstacles to their goals. The patients visited dietician once monthly and trained doctor every 3 months. Results showed that MOTP was associated with improved obesity related psychological factors like attention, emotions, somatization, depression, and shame. These patients also experienced significant weight loss (Pjanic *et al.*, 2017).

Behavioral therapy for obesity, based on behaviorism, was introduced in 1960s. Behavior (sedentary lifestyle and overeating) influenced in occurrence of obesity. Specific educational programs are designed in order to facilitate changes in eating habits and physical activity thru modification of environmental elements and reinforcements of food intake. In next years in behavioral therapy were integrated some procedures derived from cognitive therapy. Those procedures in combination with dietary changes and exercise were recognized as weight-loss style modification (Dalle Grave *et al.*, 2013).

Conscious cognitive processes play important role in weight reduction and maintaining weight. Specific cognitive factors are associated with unhealthy eating habits, dietary treatment discontinuation, and weight loss maintenance. More than half of patients on behavioral therapy experienced weight regain after 5 years. In the first decade of 21st century Cooper developed cognitive behavioral therapy for obesity (CBT-OB) in order to address the fail in behavioral treatments used in weight loss. CBT-OB analyzed factors responsible for failure to achieve desirable weight loss and weight maintained like unrealistic weight goals and unsatisfied training in weight maintained (Dalle Grave *et al.*, 2017).

Multistep cognitive behavioral therapy for obesity may be implemented at three levels of care: outpatient, day hospital, and residential. CBT-OB incorporates the physical activity and dietary recommendations and cognitive behavioral strategies. In study with multistep CBT-OB approach, outpatient CBT-OB was leading by single therapist in group or individually. The first step was to lose weight at least 10%, and the second one was to develop a mind-set conductive to weight maintenance. Each patient was given a low-calorie Mediterranean diet and functional rehabilitation program in order to improve cardio respiratory capacities. Patients were passing thru six modules: monitoring food intake, physical activity and body weight, changing eating with 500–750 kcal deficit per day, active lifestyle, impediments to weight loss, addressing to weight loss and addressing to weight loss maintained. Patients achieved more than 10% weigh loss with no weight regain in further 12 months. Patients with binge eating disorder before hospitalization, after 3 week of residential CBT-OB showed recovering from binge eating disorder (Calugi *et al.*, 2016). Authors concluded that CBT-OB is suitable for extreme obese patients and obesity related comorbidities including the patients referred for bariatric surgery (Dalle Grave *et al.*, 2017).

Visual effect is very important for majority of people. This appearance is also highly important for obese people who wish to reduce weight.

Change in dietary habits and physical activity requires initial motivation of obese person and further, continued effort to maintained new lifestyle. One study demonstrated that computerized future self-image may be useful in weight loss, especially in female population (Jiwa *et al.*, 2015; Ossolinski *et al.*, 2017). This pilot study used application "future me" which included dietary and exercise information in order to predict BMI. One self-image from the future time points has been chosen and was printed as well as current image. Patients were also chosen to receive future self-image on the beginning of the investigation or after 8 weeks after start. Greater weight reduction was in group with delayed self-image. The possible explanation for this unrespectable result was that future self-image stimulates additional effort for further weight loss after a few weeks of weight reduction attempt (Ossolinski *et al.*, 2017).

Barriers to Behavior Change

Poor motivation, social and environmental pressure, socioeconomic status, lack of knowledge or physical limitations could be barriers to lifestyle change. The predictors of behavior compliance are older age, male sex, lower baseline BMI, and weight loss in early phase of treatment. This data suggest that program for lifestyle change should be individualized. Unrealistic expectations regarding weight reduction and negative mood could implicate lack in adherence (Burgess *et al.*, 2017). Large trial Look AHEAD

investigated possible association of degree of weight reduction and fitness with cardiovascular events. This study included 5145 overweight or obese participants with type 2 diabetes in United States who were randomized to lifestyle intervention or to diabetes education and support. Results suggest tight association between degree of weight reduction and incidence of cardiovascular disease in patients with type 2 diabetes (Look AHEAD Research Group *et al.*, 2016). This findings are the challenge for community to incorporate intensive lifestyle intervention into public health programs. One of community-based lifestyle weight loss program adapted from Look AHEAD trial is Lifestyle Interventions for the Treatment of Diabetes study (LIFT Diabetes) with reduction in CVD risk as a primary goal (Katula *et al.*, 2017).

Therapeutic treatment could be slightly difficult in obese adolescents who have an aversion to usual obesity management. Using *E*-health lifestyle intervention might be useful to reach desirable aim in this population. One study demonstrated that adolescents who participated in the *E*-health lifestyle intervention had a greater decrease in BMI then those who did not participated in this program. This program with login on a weekly basis was supported by at least one parent of adolescent. Web-based intervention encouraged healthy eating, reduced screen time and physical activity during 8 months. *E*-health intervention delivered through the internet is cost-effective and acceptable method for adolescents (Tu *et al.*, 2017).

If outpatient treatment failed in initial weight loss, inpatient management could be useful. Study conducted in one inpatient clinic, showed that 4-week inpatient program in closed group, enabled satisfied weight reduction, and maintained further weight loss during the first year after inpatient stay. Program included dietary, exercise and behavior modules. Patients who reduced weight of $>10\%$ were offered to participate in 2-week stabilization program. After dismiss patients and the clinic team maintains contact by phone or by mail. This approach could prevent weight regain (Weinreich *et al.*, 2017).

Fat mass and obesity associated protein (FTO) is one of the most important gene with impact on energy balance and foot intake. Single nucleotide polymorphisms in the FTO gen causes desire for intake energy rich foods. The effects of FTO gene on effect of lifestyle intervention are controversial although some study demonstrated that improvement in environmental factors (lifestyle) can reduced effect of FTO polymorphisms on obesity. One study showed that reduced calorie intake could help in patients with FTO risk allele (Kalantari *et al.*, 2016).

In case that lifestyle change failed in weight loss, combination of calorie restriction and some drugs could be useful. Lorcaserin with behavioral weight loss facilitates the maintenance of losses of $\geq 5\%$ of initial weight (Tronieri *et al.*, 2017).

In spite everything that has been said, some authors suggest that simply treating of obesity with the "best diet" should not solve the problem with chronic noncommunicable metabolic diseases. They also warning that weight loss should not be only one problem when low-grade inflammatory processes may be related to serious metabolic disorders (Egger and Dixon, 2014).

Summary

Change in lifestyle is the first step in management of obesity. The term "lifestyle" includes dietary habits and physical activity. Lifestyle interventions can help severely obese individuals to achieve long-term weight loss and decrease risk for cardiovascular diseases. A small amount of weight loss is associated with significant health benefits. The energy deficit of at least 500 kcal/day is necessary for weight loss. This deficit could be provided by calorie restriction or through physical activity. Best results achieving the patients who combine the both approach which are including in group sessions or in individual program with trained interventionist. There are a numerous available commercial diets but fad or crush diets published in magazines could damage health and should be avoided. Diets have to be medically confirmed and, if it is possible, patient tailored. Long-term calorie reduction with appropriate ingestion of necessary nutrients reduces the risk for obesity comorbidities. In order to maintain weight loss increased physical activity is essential beside dietary treatment. Exercise program should be gradually built up along with cardio respiratory fitness improvement. Change in dietary habits and physical activity requires initial motivation of obese person and further, continued effort to maintained new lifestyle.

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Physical Activity and Exercise

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Introduction and Definitions

The WHO defines physical activity as any bodily movement produced by skeletal muscles that requires energy expenditure —including activities undertaken while working, playing, carrying out household chores, traveling, and engaging in recreational pursuits. Meanwhile exercise is a subcategory of physical activity that is planned, structured, repetitive, and aims to improve or maintain one or more components of physical fitness. Both modalities at moderate and vigorous intensity improve health.

As defined, physical activity is a structural component of life style, independently of including planned exercise or sport. Since obesity and being overweight are regarded as an energy imbalance between consumed and expended calories, physical activity is a key factor in the pathophysiology of this disease. In fact, the WHO attributes to physical inactivity and sedentarism a key role in the development of obesity. Physical inactivity in relation to the nature of work, transportation modes in the cities, and increasing urbanization of humanity has increased in the last few decades affecting all human populations. Recent statistics (WHO report 2010) show that at least 23% of adults over 18 were not active enough (men 20% and women 35%) globally, but that proportions increase to 26% in men and 35% in women in high-income countries (vs. 12% men and 24% women in low-income countries). Those figures closely correlate with current prevalence of obesity. The reduction in physical activity in modern lifestyle is particularly due to inaction during leisure time and sedentary jobs, as predominant behaviors. In addition, this is negatively affected by the predominant passive modes of transportation inside cities and their surroundings. Much more worrying are the conditions in adolescents aged under 18, as 81% of them are insufficiently physically active (84% girls and 78% boys), not reaching the WHO recommendations. Undoubtedly, physical inactivity is a key factor that causes obesity, a disease increasing in prevalence in last decades.

Relevance of Exercise for Health

Recent reports by the WHO states that insufficient physical activity is 1 of the 10 leading risk factors for death worldwide and a key risk factor for noncommunicable diseases (NCDs) such as diabetes, cardiovascular complications and cancer. Conversely, physical activity and exercise have significant health benefits preventing NCDs. There is solid evidence demonstrating that active adults compared to inactive ones, have lower rates of all causes of mortality, coronary heart diseases, stroke, blood pressure, type-2 diabetes, risk of falls and bone fractures, breast and colon cancer, and depression. In addition, they have higher levels of muscular and cardiovascular fitness and, finally, a higher probability of maintaining normal weight and better body composition, preventing obesity and abdominal adiposity.

Worldwide, one in four adults is not active enough and more that 80% of the adolescent population are not physically active and exercise not enough. Therefore, WHO member states agreed to introduce policies to reduce insufficient physical activity by 10% by 2025, which seems a very modest objective. All those benefits are achieved irrespective of the age of the subject, however the emphasis should be on the younger, children and adolescents, as they will have the largest benefit for life (WHO report 2016).

Physical activity is the major factor for energy expenditure in physiological conditions, and a key determinant of energy needs of the body. Under exercise, there are principal changes in fuel fluxes from the body stores (adipose tissue and liver) to expending tissues (muscles, heart and brain) in a typical catabolic condition.

Beyond metabolic changes, there are physiological adaptations to the increased tissue demand. Those changes consist in redistribution of blood flow to the brain and muscles but reduced blood flow in abdominal organs and skin, increased heart expenditure, increased pulmonary ventilation, activation of sympathetic nervous system and inhibition of the parasympathetic branch, and switch of hormonal systems: reduction of insulin levels (inhibited by sympathetic nervous system), increased adrenaline, glucocorticoids, GH and blockade of the gonadotrophic axis.

Increase in heart activity inherent to exercise is one of the major factors in preserving health, keeping this muscular organ fit. Increase in pulmonary ventilation and circulation of respiratory gases, and oxygenation of tissues, also greatly contribute to maintaining trophic functions of all the organs including the brain. The brain, being the central regulatory organ of the body function, and as such having a predominant role in human health, benefits immensely from regular exercise. Exercise improves general function of the brain, mainly superior and cognitive capacities. As exercise dramatically contributes to keeping the brain and the heart in good condition, it is a warranty of having a healthy life. Mobilization of muscles and joints during exercise also have direct healthy effects on the body, improving general capacities and abilities to adapt to everyday challenges. In addition, exercise is the most important factor in maintaining bone density by avoiding the calcium loss linked to inactivity, and thus preventing bone pathology in aging. Overall, exercise is the most efficient way to prevent disease and improve health.

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Metabolism at Exercise: Physiology of Exercise, Energy Expenditure, and Metabolism

Despite common belief lipids are the preferred fuel for muscles, and just at the point of intense effort carbohydrates (CHO, carbs) are more important. Different classic studies have shown at least four preferred fuels to supply muscle metabolism: intramuscular lipids, intramuscular carbs (glycogen), lipids accumulated in adipose tissue, and circulating glucose (van Loon, 2004). They are all used in moderate and vigorous exercise, although in different proportions, depending on duration and intensity of the effort. There are four physiological energy resources used during exercise: intramuscular fat, circulating triglycerides extracted from adipose tissue, muscular glycogen, and circulating glucose release from the liver on demand to supply increasing needs during exercise. The liver produces glucose from hepatic stored glycogen (about 5% of liver weight) and from de novo synthesis from other energy sources, such as as glycerol (from triglycerides), aminoacids (especially alanine), and circulating lactate. Circulating lactate is released from exercising muscles through the MCT-2 membrane transporter (Mono-Carboxylate Transporter-2) which appeared in muscle membranes during exercise. Lactate is produced in muscles by glycolysis of monosaccharides and glucose, and is then released from muscle glycogen. The liver resynthesizes glucose from lactate in a chemical cycle discovered by the Cory couple. This "lactate shuttle" is a refined process allowing the organism to keep glycemia homeostasis by using muscle glycogen during exercise, and therefore warranting the glucose supply to critical organs as the brain. Several tissues might also use lactate as primary carb surge instead of glucose, such as the heart, kidneys, gut cells and the glia and neurons of the central nervous system (Hui *et al.*, 2017). Therefore, hyperlactatemia has preserving functions in metabolic control during strenuous exercise.

A classic study (Romijn *et al.*, 1993) showed that the proportion of energy supply to the muscle by each of the four main sources deeply depends on the exercise intensity. At low intense exercise (25% of the maximum oxygen consumption VO_2max) the major energy source is plasma FFA with minor inputs from muscle triglycerides and plasma glucose. At medium intensities of exercise (65% VO_2max) muscle triglycerides (especially glycogen) are the major energy resource in the active muscles. And finally, at very high intense exercise ($>85\%$ VO_2max) the energy input from glycogen is critical, plasma glucose is also a relevant input, and in general the major source to maintain the effort are carbs in detriment of lipids. However, these high intensity exercises cannot be maintained for long periods of time unless in very well-trained athletes; most exercise in common people, and especially in overweight and obese subjects are in the range of low-to-medium intensities. Thus, and in contrary to common beliefs, lipids are the major source of energy for muscle metabolism in most of the range of exercise for humans, except very short efforts and among sportsmen and athletes. It has been previously described that lipids are the major source for exercise between 45% and 75% of VO_2max or maximal heart rate (MHR), to the misconception that outside those limits muscles use other energy substrates. All along the range of exercise intensities muscle metabolism uses lipids as a preferred resource, however, at very high intensities muscle uses glycogen or glucose to provide energy bursts for very short extreme efforts. In addition, there is a large variability in the capacity of fat oxidation between individuals, and it also depends on the type of exercise. Following this rationale, low-to-medium intense prolonged exercise will demand high amounts of lipids, and by the contrary strength training is more depending on carb supply. Combined exercises, as most team sports are, and depending on their duration, they will demand different proportions of lipids and carbs in a quite individual manner.

In fact, one of the major adaptations to training exercise includes the increase of lipid depots in the working muscular fibers. This is very important, as it allows more efficient energy production, longer duration of exercise, and better performance. In such a way, the muscles of obese subjects have lower amounts of intramuscular fat with respect to athletes. Interestingly, the increase in intramuscular fat also increases insulin sensitivity, which is characteristic in trained muscles of sportsmen.

Benefits of Exercise in Obesity and Metabolic Syndrome

Exercise alone promotes multiple changes that improve health in obese subjects. Recent studies have demonstrated that even very light exercise in heavily obese subjects greatly improves cardiovascular condition and reduces the risk of cardiovascular events to a greater extent than losing weight. Furthermore, the reduction in cardiovascular risk is proportional to the intensity and duration of exercise: vigorous exercise is better than moderate; and moderate exercise is better than light physical activity. In summary, exercise is the most reducing cardiovascular risk of those studied yet, including reduction in BMI-body weight, waist circumference or biochemical parameters (total cholesterol, LDL, etc.)

As described above, muscle incorporation of glucose becomes independent of insulin action, and that condition sustains at least several hours after finishing the exercise. It has been extensively described that the expression of GLUT-4 in the membrane surface of muscular fibers is greatly increased during exercise even with very low circulating levels of insulin.

Similarly, transmembrane transport of lipids is also increased in exercising muscles. In this case the expression of CD36/FABP4, the most important membrane lipid transporter, is greatly augmented during exercise, facilitating the lipid incorporation into muscle metabolism.

Exercise increases energy expenditure during execution but also enhances resting energy expenditure and prevents the deposit of visceral fat, increases the fat oxidation in muscle, reduces the depot of adipose tissue, and greatly increases the tissue perfusion and oxygenation. Trained subjects use a higher proportion of lipids as mitochondrial fuel in muscle during exercise performance. Exercise increases brown fat development from white adipose tissue, likely by the activation of the sympathetic nervous system.

In addition, exercise reduces insulin needs during execution and several hours afterwards, and complementary increases IGF-1 circulating levels by increasing GH secretion. All those ameliorate multiple functions in different tissues including muscles (Ana Fernandez) and brain (I torres-alemán).

Chronic exercise decreases food intake and modifies food preferences to healthy foods. In addition, exercise promotes brown fat development which is highly dependent on sympathetic nervous system activation, and enhances resting energy expenditure, reducing adiposity, especially in the abdominal organs.

Exercise improves fitness, cardiovascular function, reduces cardiovascular risk and it has a direct effect on increasing survival and life expectancy.

Exercise in Weight Loss and Maintenance

Given that physical exercise is the part of metabolic energy that may be modulated and increased by will, it should be regarded in terms of energy magnitudes as Kcal or kJ in order to control body weight. If we see the alterations in body weight in terms of energy imbalance with respect to the amount of calories eaten versus the calories expended, exercise should play a key role in strategies of weight loss and body weight maintenance. However, the real influence of exercise in both processes is typically different.

In fact, although exercise is the best way to expend calories, current exercise at medium or low intensity consumes modest amounts of energy and so we need to perform the exercise for longer times. Physical activity without caloric restriction very often promotes modest weight loss. And in fact, it is not strange that some obese even gain weight during exercise programs by an increase in muscular mass. It also takes into account that in many subjects light exercise programs increase food appetite, which may interfere with dietary restrictions, especially immediately after finishing the exercise.

On the other hand, exercise is a very efficient tool in maintaining body weight. There are several reasons to justify the effectiveness of exercise in maintenance of body weight. Firstly, certainly doing exercise by routine will balance the energy equilibria of income/expenditure in the body. Secondly, doing exercise is clearly associated with other healthy habits, reduced alcohol intake, longer times and better sleep quality, reduced smoke consumption, more equilibrated diet, and hydrating strategies. Thirdly, exercise has a direct effect on improving the physiological function and responses of many organs including heart and vessels, lungs, kidneys, gastrointestinal system, central and peripheral nervous system, muscle and skeletal system and general metabolism.

In addition, it must be emphasized that independently of the effect in weight body balance, any amount of exercise will promote some improvement in healthy parameters in overweight and obese subjects. Moreover, those obese subjects obtained larger benefits from even very low amounts of exercise, independently of final mark in body reduction or even with negligible losses. In fact, increasing physical activity is the major factor that reduces cardiovascular risk, which much more potent than weight loss or even reduction in waist circumference as a marker of abdominal obesity.

Exercise Recommendations: Modifying Factors (Age, Physiological Condition, Physical Handicaps, etc.)

Any amount of exercise and physical activity will provide some kind of benefit for health or for specific organs. However, it is not easy to give a general recommendation with respect to the most adequate exercise to reach the optimal benefits. Including exercise in the treatment of obesity would regard proposals of changes of life habits and transport and programmed exercise.

Exercise must be defined in relation to at least the following parameters: frequency, duration, intensity, type and groups of muscles intervening. Exercise restricted to upper or lower limbs, or even individual extremities might be prolonged for large times to obtain a general benefit. On the other hand, simple and current exercise (walking, swimming, etc.) involving the whole body will potentially promote great improvements in health.

In subjects over 50 years old with a previous habit of large sedentarism for long periods, there must be detailed consideration of their physical and health condition. Concurrence of comorbidities or even frank pathology is very frequent. In such a case, obesity is one of the multiple factors conditioning health of individuals. This patient might be reluctant to do any exercise, as sedentarism has always been their way of life. Recent studies have shown that they are susceptible of the highest health benefits with the lowest increase of physical activity and exercise.

WHO stratifies the population into three age groups: below 18, from 18 to 64, and above 65. In all of them exercise will bring great benefits to health, but the amounts of exercise needed are different at various ages, and since there are different recommendations to reach optimum efficiency. A general rule is to do as much exercise as tolerable at each age, and then more duration and intensity in youngsters that may be reduced with age, although there is a clear conditioning individual factor.

Preparing for Exercise: Medical Exam, Incompatibilities, Physiotherapy Reconditioning, Progressive Guided Intensity

The American College of Sport Medicine establishes that low-impact physical activities (walking, cycling, swimming, etc.) at low intensities might be initiated by any obese subjects without further evaluation. Patients with high risk or previous history of

cardiovascular events, respiratory diseases and likely morbidly obese with poor fitness and/or musculoskeletal alterations should be evaluated first as initiating exercise of moderate or vigorous intensity from almost complete sedentarism is a risky practice. Several precautions should be taken in order to get the maximum benefit of practicing exercise and sports with the lowest risk for health. In such a way, the exercise programs for the obese must be safe, effective, and motivating. Frequently, starting a physical exercise protocol needs to recondition at least several organic systems. As described above, the cardiovascular system rapidly adapts its functional parameters to actual needs, and an increased demand needs an adapting period. The same will happen with pulmonary capacities, which must increase in order to supply the exercise demand of oxygen. This might be a relevant point as pathology affecting lung functionally is rather common in obese subjects, and eventually might be a limiting factor. Obesity is associated with long periods of physical inactivity, and then the reduction of functional capacities of many muscular groups and joints. It is common to suffer muscular or joint injuries at the beginning of exercise in obese and overweight subjects, that require a sudden and disturbing interruption of the activity, a necessary period of inactivity follows a discouraging incentive that may drive to the failure of the program.

These risks are greatest at the very beginning and wane once training is a routine habit, and most will be significantly prevented by a preexercise evaluation.

A complete physical examination is mandatory, including cardiovascular explorations and complementary test (ECG, ECOCG) and spirometry. In obese subjects, especially those most sedentary or aged above 40, a cardiovascular stress test is highly recommended, and must be performed by expert professionals with experience in interpreting this kind of data.

A full skeletal examination, characterizing functional capacities of major joitns and muscular groups, will greatly contribute to the prevention of injuries. The role, not just that of a medical specialist, but also of experienced physiotherapy professionals is of extraordinary value at the beginning of the physical exercise, to identify motility problems, to supervise early stages and to recommend appropriate scaled protocols according to the physical condition and fitness of every obese subject.

Although, almost all subjects will benefit from increased physical activity adjusted to any specific clinical condition, it must be regarded to exclude those with valvular heart disease, dangerous arrhythmias, and malignant hypertension. In addition, others should be carefully supervised during the exercise program, such as those with hemoglobinopathies, exercise-induced asthma and diabetics, and those initially morbidly obese.

Table 1 displays a complete collection of pathologies that indicate a partial restriction of exercise or need specific therapeutic exercise programs.

Prescribing Exercise for Obese Subjects. Protocol for Beginners. Uncharged in Water. Progression. Fitting Objectives Looking to Health

The WHO recommends a general not specific rule of being engaged in regular physical activity for a minimum of 60 min per day for children and 150 min per week in adults. Although this protocol will bring relevant health benefits for all people, it might not be enough to reach significant reductions in body weight in overweight and obese subjects, as explained above. In fact 60 min of moderate to vigorous intensity exercise every day is the minimal proposed for children to keep healthy. And the same should be said for 150 min/week of moderate-intensity, or at least 75 min of vigorous-intensity, physical activity in adults. In fact, additional benefits will be obtained with 300 min/week of moderate intensity or 150 min/week of vigorous-intensity. In adults, it is also recommended to include muscle-strengthening activities involving two major muscular groups at least 2 days a week or more.

In terms of energy expenditure, it is a good practice to start with tolerable exercise to increase the cardio-respiratory functional capacity prior to any other objective. An increase of about 1300 kcal/week of aerobic exercise greatly improves the cardio-metabolic functional capacity and reduced cardiovascular risk in any condition. It has been also reported that an increase of more than 1500 kcal/week by resistance exercise may reduce the progression of atherosclerotic lesions, and more than 2000 kcal/week might promote the regression of coronary atherosclerotic plaques in patient with myocardium isquemia.

After a health evaluation of the subject the next step is to calculate the whole energy expenditure, in order to plan a personalized program. To that purpose it is recommended to carry out a complete survey about habits including resting time, leisure time activities, work and transportation, sleep duration and quality, and schedules for each activity and meals. Calculations may be inferred based on accepted formulas, such as Harris-Benedict and others.

Therefore, a complete plan to increase physical activity in all common life events should be created, which should also include an exercise/sport program as appropriate. WHO emphasizes that reduction of physical activity in life common actions, including work, commute, and leisure time are determinants in the whole energy expenditure, and modifying these habits would greatly contribute to the prevention of being overweight and obesity.

As seen above initiating an exercise program needs some preparation in order to achieve at least three objectives: to warrant a safe activity avoiding exercise risk, provide the best conditions to obtain an optimum health benefit, and to encourage the follow-up of the program in the long-term avoiding premature withdrawal.

As obese individuals have been usually completely sedentary for long periods, it is a good practice to start with exercise that reduces weight load as much as possible. Some options may include swimming, cycling (although perhaps not in the very first moments), and gym seated/lying activities. All those should be always supervised by a qualified exercise monitor who must understand that obese subjects are unfit, with limited joint mobility and usually low motor coordination capacities. Group

Table 1 Some therapeutic exercise routines for specific patient populations

Department	*Disease*	*Therapeutic exercise*
Cardiac (Romijn et al., 1993; van Loon, 2004)	Ischemic heart disease (CAD) Post-MI Stable angina Stable chronic heart failure (CHF) with sinus rhythm and ejection fraction of 40%	AROM; endurance training (e.g., after a 5 min warm-up, exercise until the heart rate reaches that attained at 50% of VO_2max) Training muscle strength by resistance training, which must be carried out with great caution and adjusted to each patient's physical fitness level
Pulmonary	Pneumonia Chronic bronchitis Bronchiectasis	Postural drainage exercises
	Asthma Emphysema	Breathing techniques Relaxation techniques
	Respiratory insufficiency Restrictive lung disease	Stretching exercises to mobilize respiratory muscles *Note:* The level of physical effort should be limited because exercise may provoke bronchospasm
Orthopedics	Fractures Osteoarthritis Amputations	Preoperative and postoperative exercises Isometric exercises for joints with minimal ROM ROM exercises to prevent contractures and heterotopic ossification
Burns		Passive and active exercises assisted by therapists to prevent contractures
Rehabilitation	Cervical, thoracic, and lumbar problems	Training of the Swedish Back School[a] Treatment of muscle contractures Myofascial release Flexibility training (stretching) to mobilize joints Resistance (which may include isometric exercises) and PNF training of muscle strength in muscles that have become weakened, as well as in the back extensors and abdominal muscles Graded fitness training
	Ankylosing spondylitis	Mobilization of spinal vertebrae Extension exercises
Rheumatoid arthritis		Flexibility training Gentle fitness training
Gynecology and obstetrics	Pregnancy and postdelivery	Prenatal and postnatal exercises Relaxation techniques
	After mastectomy	Training to reduce lymphedema
	Urinary incontinence	Isometric exercises to pelvic muscles

[a]The Swedish Back School derived from pioneering Swedish studies in the 1970s that measured intradiscal pressure in normal nuclei pulposi at the L3 level. The pressure at L3, measured with a subject standing erect, was found to be 100 kp/cm^2 in a male weighing 75 kg. The pressure increased to 250 kp/cm^2 when an individual was sitting bent forward and diminished to 50 kp/cm^2 when he was lying prone.
ROM, range of motion; AROM, Active range of motion.

activities may be a good initiating strategy for example: gym, dancing or trekking. This group exercise promotes additional motivation to continue the exercise program. In recent years, social networks have contributed to sharing exercise and sport goals, which in many cases is a very good reinforcement to keep doing it. Other important recommendations are to avoid very high-intensity exercise in obese subjects, emphasizing the duration and exercise frequency in sessions per week as tolerable, and to include modifications in the nutrition plan accordingly to the exercise program (meal preexercise, hydratation habits and post-exercise dieting).

Sport clothing must always be comfortable and allowing wide range mobility; appropriate shoes and socks are also very important. Using hydrating creams before and after exercise will prevent uncomfortable galls and skin inflammation especially around hips, groin, knees, and armpits.

The objectives should be initially centered in getting a better fitting with significant improvements in respiratory and cardiovascular responses, which will yield the highest health benefits. Afterwards programed exercise will be a good adjuvant of the weight-loss strategy, but never alone and always contextualized in an integrated therapeutic plan, including a diet regime, resting and sleeping recommendations, psychological support and any other therapeutic tool that may be needed.

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Treatment of Obesity with Bariatric Surgery

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Management of Patients with Obesity: Different Therapies and Clinical Use

The global pandemic of obesity is increasing over the past decades, becoming a major public health challenge (NCD Risk Factor Collaboration (NCD-RisC), 2017; GBD 2015 Obesity Collaborators, 2017). Obesity is directly linked to a number of different diseases including type 2 diabetes (T2D), hypertension, hyperlipidaemia, obstructive sleep apnoea (OSA), cancer as well as psychological and psychiatric morbidities (Allott and Hursting, 2015; DeMarco *et al.*, 2014; Nedeltcheva and Scheer, 2014). According to different projections, the cost of overweight and obesity to society could increase to $50 billion in 2050 if obesity rates continue to rise (National Clinical Guideline Centre, 2014). Treatment options for obesity may include non-surgical treatments and bariatric surgery (Bray *et al.*, 2016). Non-surgical treatments involve lifestyle interventions including caloric restriction, reduction of sedentary behaviors and increased physical activity as well as pharmacotherapy. However, diet therapy may have little effect on weight loss (WL) and may be relatively ineffective in treating obesity in the long-term (Bray *et al.*, 2016). Furthermore, several pharmaceutical agents approved for reducing excess adiposity (Dixon, 2016) have been unfortunately associated with low efficacy and diverse side effects (Rucker *et al.*, 2007). By contrast, surgical procedures have expanded quickly in the last decade, due to their ability to produce long-term WL, ameliorating obesity-related comorbidities and reducing long-term mortality (Sjöström *et al.*, 2004; Adams *et al.*, 2007; Frühbeck, 2015). In this sense, bariatric surgery is considered the most effective treatment for individuals with severe obesity [body mass index (BMI) $> 40.0\ kg/m^2$] or those with a BMI $> 35\ kg/m^2$ who also have important comorbidities, such as T2D, hypertension or OSA (Pi-Sunyer *et al.*, 1998; Fried et al., 2013a). In this regard, different guidelines for treating obesity and its associated comorbidities in an effective way have been developed (Fried et al., 2013b, 2014; De Luca *et al.*, 2016).

Different Techniques of Bariatric Surgery

The term bariatric surgery refers to all surgical procedures with the aim to reduce excess weight (Frühbeck, 2015). Bariatric surgery techniques began with the observation that patients with short bowel syndrome (undergoing extensive resection of a large portion of the intestinal tract) or patients after gastrectomy experienced a significant WL. The first surgeries to achieve WL were performed >50 years ago by Drs. Mason and Ito (Kremen *et al.*, 1954). However, in the past 20 years, the dramatic increase in the prevalence of severe obesity combined with relevant improvements in the efficacy and safety of bariatric surgical techniques have led to an important increase in the number of procedures annually performed. In fact, in 2013, nearly half a million procedures were performed worldwide (Angrisani *et al.*, 2015). A range of procedures is now well established with different degrees of WL achieved after surgery. Traditionally, surgeries are classified in three main groups: restrictive, malabsortive or mixed techniques (**Table 1**). Nowadays all bariatric surgery techniques are carried out by a laparoscopic approach. The selection of the type of bariatric surgery needs to be carefully considered based on the patient's characteristics and the surgeon's preferences evaluating the risks and benefits of each procedure.

Restrictive Techniques

Restrictive procedures promote a reduction in food intake by limiting the size of the stomach. Different types of restrictive WL surgery procedures include vertical banded gastroplasty (VBG), adjustable gastric banding (AGB), sleeve gastrectomy (SG) and gastric plication (GP).

Table 1 Classification of the main bariatric surgery procedures

Restrictive techniques	*Malabsortive techniques*	*Mixed techniques*
Vertical banded gastroplasty (VBG)	Jejuno-ileal bypass	Roux-en-Y gastric bypass (RYGB)
Adjustable gastric banding (AGB)	Duodenal-jejunal bypass	Biliopancreatic diversion with duodenal switch (BPD-DS)
Sleeve gastrectomy (SG)		Biliopancreatic diversion (BPD)
Gastric plication (GP)		

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- **Vertical banded gastroplasty (VBG)** was developed by Dr. Edward E. Mason (Mason *et al.*, 1998). This purely restrictive procedure stapled vertically the upper stomach creating a small pouch along the inner curve of the stomach. The passage of food through the rest of the stomach is restricted by a plastic band, which delays the emptying of food from the pouch, causing a feeling of fullness. It was popular in the 1980s, but it has been progressively abandoned due to intractable reflux and vomiting problems after surgery (Goldberg *et al.*, 2000).
- **Adjustable gastric banding (AGB)** is one of the most commonly performed bariatric surgery in the world (Buchwald and Oien, 2009). The original technique was performed as an open procedure in Sweden, but nowadays it has been modified to become a laparoscopical technique (Miller and Hell, 2003). An adjustable silicone band is bucked around the upper stomach in a restrictive procedure. The lumen of the band is connected via a tube to a subcutaneous reservoir and injection of saline allows the band to be adjusted. Injection or withdrawal of fluid from the system via this port allows the surgeon to tighten or loosen the band depending on the patient's symptoms and the desirable degree of WL to be achieved.
- **Sleeve gastrectomy (SG)** was introduced in the early 1990s and its popularity is increasing because it is considered a secure technique with low complication rates and minor long-term nutritional deficiencies (Nguyen *et al.*, 2013). This restrictive procedure involves an 80% vertical resection of the stomach following the major curve, creating a tubular stomach. Currently, SG is considered as an acceptable option as a primary bariatric procedure with a suitable risk/benefit profile, placed between RYGB and AGB. In fact, this procedure climbed from up to 37% of total procedures between 2003 and 2013 worldwide and, in some regions, such as USA/Canada and Asia Pacific, has become one of the most frequently performed procedures (Angrisani *et al.*, 2015).
- **Gastric plication (GP)**. The current model of laparoscopic gastric placation was described by Talebpour and Amoli (Talebpour and Amoli, 2007). This procedure is based on the reduction of the size and capacity of the stomach approximately 75% by folding the stomach's lining inside itself. As compared to SG, gastric plication does not involve any gastric resection without leaving a staple line behind (Pujol Gebelli *et al.*, 2011). Moreover, no foreign body placement is included alleviating the associated problems. In addition, GP is a reversible procedure that allows revision to other bariatric surgeries (Skrekas *et al.*, 2011; Tsang and Jain, 2011). However, it must take into account that gastric plication has been recently described and further studies are needed to prove its effectiveness in long-term WL and its role in the improvement of obesity-associatedcomorbidities.

Malabsortive Techniques

Malabsortive techniques are based on the removal of small intestine portions and the diversion of biliopancreatic secretions, limiting the absorption of nutrients in the intestine. Duodeno-jejunal bypass and jejuno-ileal bypass are classified as pure malabsortive methods.

- **Jejuno-ileal bypass (JIB)** became popular in the late 1960s and early 1970s (Griffen Jr. *et al.*, 1983). The technique involves an intestinal bypass in which the proximal jejunum is anastomosed to the distal ileum, being a purely malabsortive procedure (Griffen Jr. *et al.*, 1977). This type of procedure results in extreme WL and its practice was abandoned due to the development of serious complications, such as renal failure, liver disease, fat-soluble vitamin deficiencies and malnutrition, among others (Requarth *et al.*, 1995).

Mixed Techniques

Biliopancreatic diversion, with (BPD/DS) or without (BPD) duodenal switch, and Roux-en-Y gastric bypass (RYGB) are defined as mixed procedures, being a combination of restrictive and malabsortive techniques (DeMaria, 2007).

- **Biliopancreatic diversion (BPD) and biliopancreatic diversion with duodenal switch (BPD/DS).** Biliopancreatic diversion was described by Scopinaro in 1979 (Scopinaro *et al.*, 1979). The surgery consists of a horizontal distal gastrectomy that leaves behind a functional upper stomach. This remnant stomach is anastomosed to the distal small intestine (alimentary limb). The excluded small intestine (including the duodenum, the jejunum, and part of the proximal limb) carries bile acids and pancreatic secretions (biliopancreatic limb) and it is connected to the alimentary limb at a distal ileal level. The "common limb" is the only segment of small bowel where digestive secretions and nutrients mix, leading to a delayed mixing of food with biliopancreatic secretions.

  In the biliopancreatic diversion with duodenal switch (BPD-DS) instead of distal gastrectomy, a sleeve type gastrectomy is performed and Roux limb is anastomosed not to the stomach but to the duodenum (duodenal switch), thus preserving the pylorus (Crookes, 2006) and theoretically regulating gastric emptying and reducing dumping syndrome. The BPD-DS was popularized by Marceau et al. (Marceau *et al.*, 1993) and Hess & Hess (Hess and Hess, 1998) in 1993. However, its technical complexity and long-term significant nutrient deficiencies has led to a decrease in the number of this type of surgery representing only 2% of total bariatric surgery procedures worldwide (Buchwald and Oien, 2009).
- **Roux-en-Y Gastric Bypass**. The gastric bypass was originally introduced in 1969 by Mason and Ito (Mason and Ito, 1969) and it has undergone different modifications. The most common version, the laparoscopic Roux-en-Y-gastric bypass (RYGB), represents one of the most performed bariatric operation throughout the world (Angrisani *et al.*, 2015). This includes a small

and vertically oriented proximal gastric pouch of 15–20 mL that is anastomosed to the jejunum through a gastro-jejunal anastomosis in a Roux-en-Y manner. As in BPD and in BPD-DS, three limbs appear: an alimentary limb (jejunal Roux-en-Y limb anastomosed to the stomach), a biliopancreatic limb (which transmits bile and pancreatic juices to the jejuno-jejunostomy) and a common channel limb (from enteroenterostomy and ileocecal valve).

Currently, SG and RYGB are the most commonly performed bariatric surgery techniques. However, the mechanisms of action of bariatric surgery are complex and involve multiple neuroendocrine signals that exert effects at the central nervous system as well as in peripheral organs, contributing to overall benefits of these procedures (Frühbeck, 2015).

Health Benefits of Bariatric Surgery

Important studies initiated almost three decades ago have analyzed the effect of bariatric surgery to achieve WL and the resolution of obesity-associated comorbidities (Buchwald and Oien, 2009; Courcoulas *et al.*, 2013; Schauer *et al.*, 2012; Buchwald *et al.*, 2009; Arterburn and Courcoulas, 2014; Sjöström *et al.*, 2012). A body of evidence exists showing that bariatric surgical procedures contribute to an increased body WL, an improvement in the control of comorbidities, and a better quality of life in patients with obesity compared to non-surgical treatments (Colquitt *et al.*, 2009). Although most results from different studies are limited to the first two or three years of follow-up after bariatric surgery, immense efforts have been made to evaluate the long-term effects of these surgeries (**Table 2**). Our main current knowledge about the long-term results of bariatric surgery comes from the Swedish Obese Subjects (SOS) study (Sjöström et al., 2004, 2007). This study was started in 1987 and it is the largest and longest prospective, non-randomized and interventional trial examining the effects of bariatric surgery in over 4000 subjects. Other long-term observational studies comparing bariatric procedures with non-surgical management of obesity, such as the Utah obesity study and the Longitudinal Assessment of Bariatric Surgery (LABS-2) have also demonstrated that bariatric procedures are more effective than medical or lifestyle interventions in achieving WL and the resolution of obesity-related comorbidities (Courcoulas *et al.*, 2014; Karlsson *et al.*, 2007; Kolotkin *et al.*, 2012).

Table 2 Long-term effects of bariatric surgery compared with non-surgical management

Study	*Location*	*Study Details*	*Population*	*Weight change*	*T2D remission*	*T2D incidence*
Swedish Obese Study (SOS)	Sweden	The largest and longest prospective, non-randomized study with matched controls. Started in 1987	$n = 2010$ underwent bariatric surgery: 68% VBG, 19% AGB, 13% RYGB; $n = 2037$ matched control group	The mean changes in body weight after 2, 10, 15 and 20 years were: −23%, −17%, −16% and −18% in the surgery group and 0%, 1%, −1% and −1% in the control group, respectively	After 2 years: 72% of patients with T2D were in remission. After 10 years: 50% of patients with remission of T2D had relapsed	Bariatric surgery reduced risk of developing T2D by 96%, 84% and 78% after 2, 10 and 15 years, respectively
Utah Obesity Study	Utah, USA	Prospective, observational study with matched controls. Started in 2000	RYGB: $n = 418$ Control 1: $n = 417$ bariatric surgery seekers who did not undergo surgery; Control 2: $n = 321$ population-based severely obese matched controls	Six years after surgery: −27.7%, +0.2% and 0% of their body weight in RYGB, control 1 and control 2, respectively	Six years after surgery: 62% in RYGB, 8% and 6% in control 1 and control 2, respectively	Six years after surgery incidence decreased to 2%, 17% and 15% for bariatric surgery, control 1 and control 2, respectively
Longitudinal Assessment of Bariatric Surgery (LABS)	USA	Multicenter observational cohort study. Started in 2006	$n = 2458$: 70.7% RYGB, 24.8% AGB and 4.5% other procedures	Three years after surgery: −31.5% and −15.9% of their body weight in RYGB and AGB, respectively	Three years after bariatric surgery: 67.5% of RYGB and 28.6% of LAGB experienced partial remission of T2D	Incidence of diabetes was 0.9% after RYGB and 3.2% after AGB

VBG: vertical banded gastroplasty, RYGB: Roux-en-Y gastric bypass, AGB: adjustable gastric banding.

Effects of Bariatric Surgery on Weight Loss

Long-term effect on body weight is one of the most important points to determine success after bariatric surgery. Sustained total body WL improves obesity-associated comorbidities and to date, bariatric surgery is the most effective treatment to achieve a substantial and durable WL. However, changes in body composition towards a reduced adiposity are also of great importance after bariatric surgery (Frühbeck, 2015; Gómez-Ambrosi *et al.*, 2017). A series of randomized controlled trials have provided evidence that patients lose more weight up to 5 years after bariatric surgery compared to non-surgical interventions (Gloy *et al.*, 2013).

The effectiveness of bariatric surgery was first described in a meta-analysis in which the main surgical procedures were studied. The mean percentage of excess weight loss (EWL) was 47.5% for gastric banding, 61.6% for gastric bypass, 68.2% for gastroplasty and 70.7% for biliopancreatic diversion or duodenal switch. In most cases, the WL outcomes did not differ significantly at 2 years or less compared with those at over 2 years (Buchwald *et al.*, 2004). In an additional long-term study comparing AGB and RYGB after a 10-year follow-up, patients who underwent RYGB experienced a greater percentage of mean EWL (69%) than the AGB (46%) patients. However, RYGB lead to a higher risk of developing early and long-term surgical complication rates than AGB (Angrisani *et al.*, 2013). In the same line, a recent meta-analysis of randomized controlled trials including 11 studies and 796 individuals, has demonstrated that patients lost more weight (mean difference − 26 kg) after bariatric surgery than individuals after non-surgical treatment (Gloy *et al.*, 2013). On the other hand, Camerini et al. reported a good short-term WL after AGB (BMI was 79% of preoperative value at 1 year) but BMI tended to increase over time, by approximately 0.42 BMI units per year and, at 13 years postoperatively, 60% of bands had to be removed (Camerini *et al.*, 2004).

Weight loss outcomes up to 20 years after bariatric surgery have been reported from the SOS study (**Table 2**) (Sjöström, 2013). However, in most cases a weight regain (WR) in all the surgery subgroups was observed (from the first year), although this tendency stopped after 8–10 years. In the same line, Freire et al. reported that WR increased significantly with time after surgery, specifically 14.7% after 2 years, 69.7% from 2 to 5 years, and 84.8% over 5 years (Freire *et al.*, 2012).

Another important ongoing prospective study performed in Utah, found that six years after surgery, patients who underwent RYGB experienced a significant WL compared to two different control groups (Adams *et al.*, 2012). In the LABS multicenter observational cohort, including 2458 patients, the percentage of WL for participants who underwent RYGB mostly occurred during the first year (Courcoulas *et al.*, 2013). Furthermore, a recent study has concluded that the measurement of body composition and body fat after the first and the second year after RYGB may yield clinically more relevant information about cardiometabolic risk factors than body weight, BMI and EWL (Gómez-Ambrosi *et al.*, 2017).

Thus, bariatric surgery is markedly more efficient than non-surgical treatments to achieve WL, improving quality of life and reducing mortality associated to obesity (Sjöström, 2008). However, WR and failure to sustain WL have been observed in 20–30% of patients and should be also contemplated (Meguid *et al.*, 2008). Individuals who present WR are difficult to predict pre-operatively and, consequently, diverse studies are trying to identify predictors of WR (Livhits *et al.*, 2011). The main etiological factors responsible for weight recidivism are discussed below (Karmali *et al.*, 2013; Maleckas *et al.*, 2016).

Factors responsible for weight recidivism following bariatric surgery

- *Nutritional non-compliance.* Poor diet quality, characterized by excessive calorie intake in the form of snacks, sweets and fatty foods, has been reported as one of the main factors that influence WR (Freire *et al.*, 2012). In addition, eating disturbances and the loss of control when eating are of great importance after gastric bypass, constituting risk factors for decreased WL outcomes (Kofman *et al.*, 2010). For this reason, an appropriate education and diet counseling by a multidisciplinary team are crucial.
- *Hormonal/metabolic imbalance.* Early findings have suggested that WR is associated with a hormonal imbalance. In this regard, post-prandial suppression of plasma ghrelin has been correlated with the maintenance of WL (Engstrom *et al.*, 2007), whereas high levels of this hormone in plasma after surgery have been associated with WR (Bohdjalian *et al.*, 2010). Conversely, no influence of ghrelin secretion on WL or WR has been recently described while the decreased levels of the glucose-dependent insulinotropic polypeptide (GIP) and the glucagon like peptide (GLP-1) may be important factors promoting the WR process (Santo *et al.*, 2015). Reportedly, WR is attributed to a failure to sustain elevated plasma peptide YY (PYY) concentrations (Meguid *et al.*, 2008). The hormone imbalance effect on WL has been demonstrated and diverse studies are currently developing therapeutic compounds in order to regulate this gut hormones misbalance (Bhat, 2017; Monteiro, 2011).

On the other hand, reactive hypoglycaemia caused by changes in intestinal anatomy after bariatric surgery has been described as another cause of WR, inducing an early and important insulin surge by the hypersecretion of GIP and GLP-1 (Roslin *et al.*, 2011; Salehi *et al.*, 2011). These episodes of low glucose levels may stimulate appetite, leading to a snacking or grazing eating behavior. Management of this problem is focused on changing dietary regimen in order to minimize glucose level variations and subsequent hunger (Roslin *et al.*, 2011).

- *Mental health.* Unrecognized and untreated eating and psychiatric disorders may cause WR in some patients after bariatric surgery because eating disturbances tend to persist after surgery. The frequency of grazing, which is defined as uncontrolled eating of small amounts of food (Saunders, 2004), has been positively correlated with WR (Kofman *et al.*, 2010). In this regard, patients classified as binge eaters showed an increase in their BMI compared to non-binge eaters after RYGB (Kalarchian *et al.*, 2002). Furthermore, patients with two or more psychiatric diagnoses, showed an increase in WR after RYGB (six times) relative

to those without or with 1 psychiatric diagnosis (Rutledge *et al.*, 2011). Hence, a psychological evaluation by a qualified mental health team of all patients before and after surgery is of great importance.

- *Physical inactivity.* Physical activity is closely related to WL and patients who perform physical exercise have the lowest WR incidence (Freire *et al.*, 2012). Although patients undergoing bariatric surgery are not very physically active, a recent meta-analysis has demonstrated that from a total of seventeen studies, seven have reported an increase in activity at 3–6 months, and at 11–12 months (Herring *et al.*, 2016). However, further studies are needed to clearly define the mode, frequency, duration and intensity of physical activity to provide health benefits after bariatric surgery (Coen and Goodpaster, 2015)
- *Anatomic/surgical factors.* Pouch dilatation, increase in stoma size and gastro-gastric fistula are identified as common causes of WR after gastric bypass (Heneghan *et al.*, 2012; O'Brien *et al.*, 2013) and each specific complication appears according to the type of bariatric surgery. In AGB, as the stomach is a distensible organ, able to expand to accommodate food boluses, gastric pouch may enlarge in up to 50% of patients at 4 years (Kuzmak and Burak, 1993). In RYGB, in addition to enlargement of gastric pouch, stomal dilation and the presence of a gastro-gastric fistula are important anatomic changes that reduce the effectiveness of the RYGB (Yimcharoen *et al.*, 2011; Cucchi *et al.*, 1995). Finally, some authors have suggested that gastric capacity increases after SG, with the size of the gastric sleeve being linearly correlated with post-operative BMI (Braghetto *et al.*, 2009; Weiner *et al.*, 2007). In this sense, several surgical interventions with varying efficiency have been proposed in order to reduce its adverse effects.

Bariatric Surgery in Type 2 Diabetes Remission

In the 1990s, Pories et al. reported that among 146 morbidly obese patients with T2D who underwent gastric bypass, 121 (83%) experienced a rapid and prolonged post-operative normalization of plasma glucose levels without the need for glucose-lowering medication (Pories *et al.*, 1995). The improvement or remission of T2D after bariatric surgery has been later confirmed in other studies (Schauer et al., 2012, 2014, 2017; Adams *et al.*, 2012; Carlsson *et al.*, 2012; Kashyap *et al.*, 2013). However, depending on the type of surgery performed, some differences in patient outcomes may be observed. In fact, restrictive interventions have generally demonstrated a smaller effect on T2D resolution, hypertension and dyslipidemia than mixed techniques (Frühbeck, 2015).

The SOS study described 28.4 cases per 1000 person-year in the control group whereas 6.8 cases per 1000 person-years in the bariatric surgery group diagnosed with T2D (Carlsson *et al.*, 2012). In the LABS study, among participants who had diabetes at baseline, 67.5% that underwent RYGB and 28.6% of patients undergoing AGB experienced partial remission of T2D at 3 years, achieving levels of haemoglobinA1c (HbA1c) $<6.5\%$ or fasting plasma glucose values <126 mg/dL without pharmacological therapy (Courcoulas *et al.*, 2013; Schauer *et al.*, 2014). Different randomized controlled trials have studied patients with suboptimal controlled T2D and BMI ranging from 30.0 to 39.9 kg/m^2, showing that 49% of patients who underwent RYGB achieved defined T2D management goals (HbA1c $< 7.0\%$, LDL- cholesterol <100 mg/dL and systolic blood pressure < 130 mmHg) compared with 19% of patients in the lifestyle-medical management group (Ikramuddin *et al.*, 2013).

However, despite the recognized efficacy of bariatric surgery in improving T2D, not all patients respond in the same way and not all of them experience resolution of the disease (Davies *et al.*, 2014). For this reason, the identification of risk prediction models of T2D remission after bariatric surgery constitutes a topic of great interest for clinicians and patients. Diverse studies have evaluated different parameters associated with the improvement of T2D after bariatric surgery. In 2003, Schauer et al. described that the preoperative duration of T2D, the use of insulin, as well as a high percentage of HbA1c and lower WL after surgery, were factors that influence the remission of T2D (Schauer *et al.*, 2003). Few years ago, the percentage of excess weight loss (% EWL) was suggested as the only predictor for diabetes remission (Hamza *et al.*, 2011). Young age and lower BMI have been also proposed as predictors of long-term glycaemic control (Huang *et al.*, 2011). Although several studies are in line with these findings, a reproducible risk prediction model has not been validated until now (Zhang *et al.*, 2016). Among the available systems, only two have undergone external validation, the Diabetes Surgery score (ABCD score) and the Diabetes Remission (DiaRem) score (Lee *et al.*, 2013; Still *et al.*, 2014). The ABCD score includes four categorical variables to predict the success in the remission of T2D: age, body mass index (BMI), C-peptide levels, and T2D duration. The ABCD score is a multidimensional 10-point scale, constructed with these parameters, in which greater scores indicate a better chance of T2D remission. The model has been validated in three independent cohorts (Lee et al., 2013, 2015a, b). The DiaRem score also includes four preoperative clinical variables to predict remission of T2D after RYGB: age, HbA1c levels, type of antidiabetic treatment and the use of insulin. DiaRem ranges from 0 to 22 points and it is divided into five groups corresponding to five probability-ranges for T2D remission: 0–2 points (88%–99% remission), 3–7 points (64%–88% remission), 8–12 points (23%–49% remission), 13–17 points (11%–33% remission), 18–22 points (2%–16% remission). This model has been validated in two independent cohorts (Still *et al.*, 2014).

Interestingly, the improvements in glycaemic control after surgery often occur immediately within days of the operation, independently of WL (Dirksen *et al.*, 2012; Madsbad *et al.*, 2014). In fact, due to the beneficial effects of bariatric surgery on the resolution of diabetes and other metabolic alterations, these procedures are now also considered as metabolic surgery (Frühbeck, 2015). For this reason, the eligibility criteria for metabolic surgery have been changed and new guidelines consider metabolic surgery in patients with a BMI ranging from 30.0 to 34.9 kg/m^2 with associated T2D (Moncada *et al.*, 2016; Rubino *et al.*, 2016).

Metabolic surgery

Although the mechanisms by which bariatric surgeries improve glycaemic levels remain poorly understood, different explanations have been proposed (Ashrafian *et al.*, 2010). There is evidence that a lower caloric intake significantly influences glucose metabolism (Gumbs *et al.*, 2005). Changes in secretion of gut derived hormones, such as GLP-1, PYY, oxyntomodulin, GIP and ghrelin have been also suggested as potential mechanisms (Dirksen *et al.*, 2012). Moreover, an increase in insulin secretion and sensitivity in addition to changes in neural connection between the intestine and the brain, especially the vagus nerve, might play an important role in T2D remission (Joshua *et al.*, 2009). Finally, postoperative alterations in bile acid recirculation, as well as intestinal rearrangement and changes in gut microbiota composition have been linked to improvements in the glucose metabolism (Seeley *et al.*, 2015; Vitek and Haluzik, 2016).

Taken into account the multiple mechanisms contributing to the remission of diabetes after gastric bypass, 3 main hypotheses have been proposed to understand the early, weight-independent effects of metabolic surgery: the foregut, the midgut and the hindgut hypothesis. None of these theories necessarily excludes the others, so the three hypotheses are possible and may occur at the same time (**Fig. 1**).

- *Foregut exclusion hypothesis:* This theory contends that the exclusion of a short segment of the proximal small intestine (primarily the duodenum) and the alteration of the way of ingested food after gastric bypass, results in the release of a signal or signals with anti-diabetic properties (Guidone *et al.*, 2006; Hickey *et al.*, 1998; Pories and Albrecht, 2001). Evidence for this hypothesis was provided in a non-obese diabetic rodent model (Goto-Kakizaki) where rats underwent a duodenal-jejunal bypass (DJB) (which is a stomach-preserving RYGB that excludes the proximal intestine) or a gastrojejunostomy (GJ) (without bypassing any intestine). Rats undergoing DJB showed an improved glucose tolerance, whereas rats undergoing GJ did not exhibit any improvements. When the procedures were surgically reversed, diabetes returned when the DJB was converted to a GJ and resolved when the GJ was converted to a DJB (Rubino *et al.*, 2006).
- *Midgut hypothesis:* When glucose is present in the portal vein, portal afferents communicate with the vagus nerve decreasing hunger through hypothalamic centers. Using a gastric bypass model in mice, Troy et al. concluded that this procedure promoted intestinal gluconeogenesis and stimulated the hepatoportal glucose sensor (Troy *et al.*, 2008). Consequently, gastric bypass modifies the insulin sensitivity of hepatic glucose production and food intake independently of body WL and gut hormone actions.
- *Hindgut hypothesis:* This hypothesis suggests that the rapid delivery of ingested nutrients to the distal bowel accentuates secretion of gut hormones, which are delivered from the hindgut (terminal ileum, colon and rectum) (Rubino *et al.*, 2004; Cummings *et al.*, 2007). Among these hormones are GLP-1, PYY and oxyntomodulin. These peptides can reduce food intake and their contribution to diabetes resolution has been increasingly recognized (Ashrafian and le Roux, 2009).

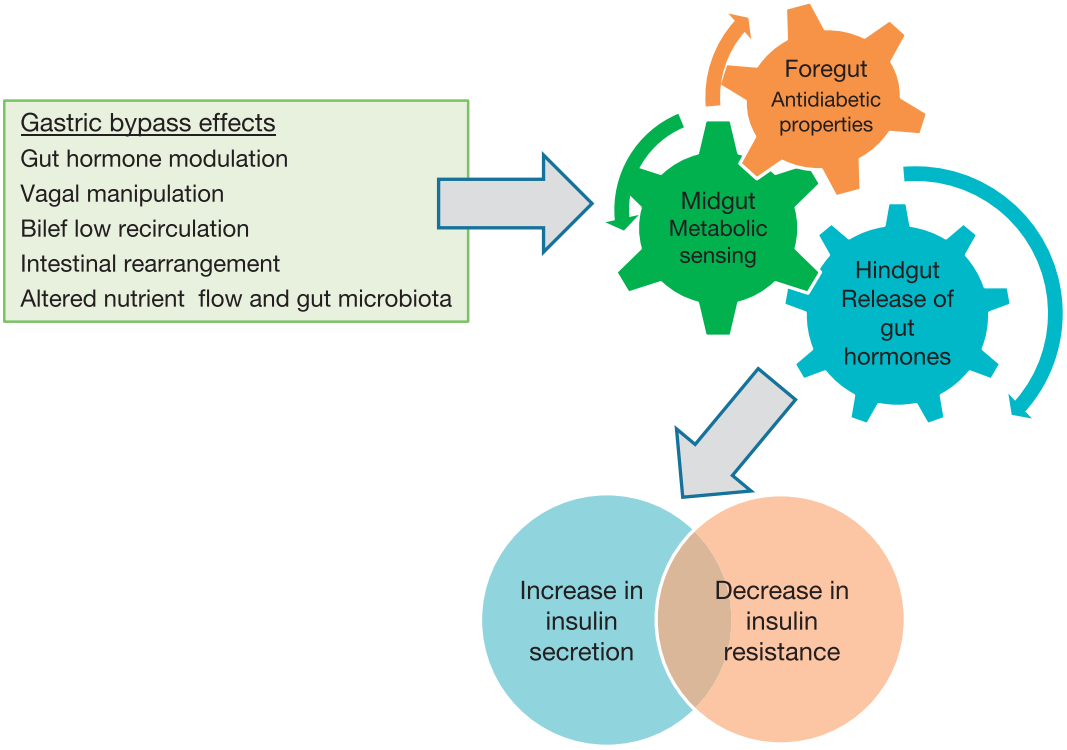

Fig. 1 Hypotheses proposed in the resolution of type 2 diabetes after gastric bypass.Diabetes resolution and hyperinsulinemia after metabolic Roux-en-Y gastric bypass.

In conclusion, weight-dependent and weight-independent mechanisms are responsible for the improvements in glycaemic control, and probably additional effects, which are still unknown, may be also participating. For this reason, further studies are required to determine the precise mechanisms of diabetes resolution after surgery.

Long-Term Cardiovascular Events after Surgery

In comparison with pharmacotherapy, bariatric surgery was associated with lower long-term cardiovascular (CV) death and lower incidence of CV events in obese adults (Sjöström *et al.*, 2012; Kwok *et al.*, 2014; Beamish *et al.*, 2016). Interestingly, bariatric surgery was also associated with a reduced myocardial infarction (MI) incidence in obese individuals with T2D, while no effect was observed on stroke incidence. Moreover, the effect of surgery in reducing MI incidence was stronger in individuals with higher serum total cholesterol and triglycerides at baseline, whereas BMI was not related with surgical outcomes (Romeo *et al.*, 2012).

Together with WL, there are different possible mechanisms that may explain the role of bariatric surgery in the reduction of CV events (Boido *et al.*, 2015). After surgery, WL is associated with reduction of visceral fat and a decreased number of adipocytes, directly modulating the levels of adipokines secretion, specifically adiponectin, leptin and C-reactive protein (CRP), which are intimately involved in the reduction of CV diseases and CV mortality (Gómez-Ambrosi *et al.*, 2017; Appachi and Kashyap, 2013). On the other hand, surgery decreases serum levels of molecules mediating endothelial dysfunction. Among them, oxidized low density lipoprotein (LDL), intracellular adhesion molecule-1 (ICAM1), endothelin-1, *E*-selectin, P-selectin and vascular endothelial growth factor A (VEGFA) have been described as markers reflecting the CV risk level (Uzun *et al.*, 2004; Pontiroli *et al.*, 2004; Garcia de la Torre *et al.*, 2008; Nijhuis *et al.*, 2007). The intervention also reduce systemic inflammation, oxidative stress, erythrocyte sedimentation rate, known to predict coronary mortality (Bacci *et al.*, 2002), as well as levels of sialic acid, PAI-1, malondialdehyde and von Willebrand factor, which have important roles in atherosclerosis development (Uzun *et al.*, 2004; Vazquez *et al.*, 2005). Recently a significant increase in HDL-cholesterol and apolipoprotein ApoA4 levels after gastric bypass has been reported being associated with a relevant improvement in hepatic insulin sensitivity (Raffaelli *et al.*, 2014). Finally, several trials have shown that liver steatosis, which is an emerging risk factor for CV disease (Calori *et al.*, 2011), decreases after bariatric surgery (Dixon *et al.*, 2006; Folini *et al.*, 2014).

In this sense, pleiotropic effects of WL achieved by surgery mediate improvements in CV events and the prevention of CV disease may depend, among other factors, on the baseline conditions of the patients. However, further research should be conducted to evaluate more effects of bariatric surgery at physiological and cellular levels, as well as the efficacy of different surgical techniques.

Other Health Benefits

Obstructive sleep apnoea (OSA)

Obesity is frequently associated with OSA, a common disorder of repetitive pharyngeal collapse during sleep, leading to oxygen desaturation and disrupted sleep (Young *et al.*, 2002; Jordan *et al.*, 2014). Bariatric surgery considerably improves or resolves the symptoms of OSA in most morbidly obese individuals (Ashrafian *et al.*, 2012). The beneficial effects of the operation on OSA may include mechanical weight-dependent and metabolic weight-independent mechanisms. These include adipokine effects, cytokine actions, altered gut hormonal release and the improvements of insulin resistance (Ashrafian *et al.*, 2012).

In the SOS study, patients with sustained WL after bariatric surgery display less severe sleep apnoea, reduced inflammatory activity, and enhanced cardiac function. In addition, persisting sleep apnoea appears to limit the beneficial effects of WL on inflammation and cardiac performance (Kardassis *et al.*, 2013). Further research is required to evaluate differences between surgical and non-surgical treatment of obesity and to determine the mechanisms underlying resolution of OSA.

Osteoarthritis and joint problems

Osteoarthritis is a very common problem in weight-bearing joints, especially in aged population and obesity has been identified as the main modifiable risk factor (Blagojevic *et al.*, 2010). The excess adipose tissue in obese individuals leads to the release of pro-inflammatory mediators, which may damage the cartilage within joints (Messier, 2008). WL reduces the risk of incident knee OA, and, in established disease, reduces symptoms, improves function and is likely to reduce disease progression. However, osteoarthritis also has a multifactorial etiology with its systemic effects being related to the adipokine profile and metainflammation (Wluka *et al.*, 2013). Recent studies have demonstrated that bariatric surgery may benefit obese patients with hip or knee osteoarthritis. However, more randomized controlled trials are needed to clarify the role and indications for bariatric surgery in these type of diseases (Gill *et al.*, 2011).

Kidney function

Obesity itself has been known to be an independent risk factor for the development of chronic kidney disease (CKD) (de Jong *et al.*, 2002). Moreover, hyperglycemia induces the production of vasodilatory prostaglandins, angiotensin II, inflammatory cytokines and reactive oxygen species, which damage the glomerulus (Caramori and Mauer, 2003). For this reason, in patients with T2D, obesity amplifies the risk for CKD (Ziyadeh, 2004).

Several studies have reported that bariatric surgery improves kidney function decreasing albuminuria proportionally to WL (Agrawal *et al.*, 2009; Iaconelli *et al.*, 2011). Moreover, the resolution of diabetes chronic disease (DKD) following RYGB in obese patients has been also described in different case report studies (Izzedine *et al.*, 2005; Perez *et al.*, 2006). It is true that to date, few analyses have examined the effects of bariatric surgery beyond the first post-operative year, but a recent 5-year prospective study has demonstrated that the improvement in glomerular filtration is maintained for up to 5 years (Neff *et al.*, 2017).

However, it should be noted that mixed procedures, such as RYGB, increase the risk for oxalate nephropathy, which could rapidly progress to end stage kidney disease (ESKD), especially in patients with long-standing diabetes and pre-existing renal insufficiency. In this sense, more controlled studies are warranted to provide with more details the effects and mechanisms of action of bariatric surgery on kidneys function (Rao *et al.*, 2014).

Factors Involved in the Metabolic Effects of Bariatric Surgery

The exact molecular, cellular and physiological mechanisms for metabolic improvements in patients who undergo bariatric surgery remain unclear. Although these procedures cause a reduction of the gastric size and a decrease in calorie absorption, these changes in the digestive tract cannot fully explain all the metabolic improvements that occur after bariatric surgery. For this reason, immense efforts are being performed to identify the mechanisms that occur across different biological systems after bariatric surgery (Dirksen *et al.*, 2012; Miras and le Roux, 2013; Abdeen and le Roux, 2015; Kohli and Diabetes, 2013; Kohli *et al.*, 2011; Ionut *et al.*, 2013).

Changes in Adipocyte-Derived Factors

Several studies have investigated the potential role of adipokines after bariatric surgery. It was clearly demonstrated that the plasma levels of adiponectin increase post-operatively (Trakhtenbroit *et al.*, 2009; Korner *et al.*, 2009) being associated with a lower risk of T2D (Li *et al.*, 2009). In contrast, other adipose-derived factors such as leptin and insulin decrease in proportion to WL achieved by bariatric surgery (Garcia de la Torre *et al.*, 2008; Trakhtenbroit *et al.*, 2009). On the other hand, inflammation in adipose tissue is known to contribute to local and peripheral insulin resistance (Reilly and Saltiel, 2017). The infiltrated macrophages of the adipose tissue constitute an important source of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, plasminogen activator 1 (PAI-1) and interleukin-6 (IL-6) (Galic *et al.*, 2010). A link between the resolution of the inflammatory state and metabolic improvements in obese subjects after bariatric surgery has been described, reducing pro-inflammatory cytokine levels and ameliorating glucose metabolism (Zhang *et al.*, 2011; Forsythe *et al.*, 2008; Hu *et al.*, 2013).

Changes in Gut-Derived Hormones

Changes in gastrointestinal hormones have been proposed as key candidates in the context of metabolic improvements after bariatric surgery. GLP-1 is an incretin secreted by L-cells in the small intestine in response to food intake (Suzuki *et al.*, 2012). GLP-1 stimulates insulin release and inhibits glucagon secretion, gastric emptying as well as food intake, leading to reduction of glycaemia and weight loss (Drucker, 2001). Moreover, PYY is also secreted by L-cells together with GLP-1 after food intake, and by acting at the arcuate nucleus of the hypothalamus decreases appetite (Batterham *et al.*, 2002). In addition, gastric bypass also modulates the secretion of other glucose-regulatory gut hormones such as oxyntomodulin and GIP. Although an increase in the secretion of these hormones improves glucose homeostasis, most of the studies reveal a non-significant modulation of their levels (Ashrafian *et al.*, 2010). Furthermore, reduced ghrelin is another of the proposed hormonal mechanisms to explain WL after surgery. Plasma ghrelin is notably reduced after SG and RYGB being this decrease dependent on the excluded gastric fundus in the surgery (Frühbeck et al., 2004a, b).

Bile Acids and Fibroblast Growth Factor 19 (FGF19)

Increased serum levels of bile acids after RYGB have been reported by different studies (Ahmad *et al.*, 2013; Nakatani *et al.*, 2009). Patti et al. demonstrated that post bile acid concentrations correlate with key metabolic parameters after RYGB, including an inverse association with 2-h post-meal glucose, triglycerides, and thyroid-stimulating hormone (TSH) levels, as well as a positive correlation with adiponectin and with the post-meal peak of GLP-1 levels (Patti *et al.*, 2009).

Gerhard et al. confirmed previous results showing that bile acids in addition to fibroblast growth factor 19 (FGF19), were higher in patients with sustained remission of T2D after RYGB (Gerhard *et al.*, 2013). In agreement with this finding, a recent study has established that FGF19 levels in obese patients increase after bariatric surgery-induced WL independently of the type of surgical procedure, but not after diet-induced WL (Gómez-Ambrosi *et al.*, 2016). FGF19 is expressed in ileal enterocytes of small intestine and colon (Fon Tacer *et al.*, 2010; Izaguirre *et al.*, 2017) and acts principally on the liver regulating negatively bile acid synthesis (Holt *et al.*, 2003). The increased serum levels of both, bile acids and FGF-19, is explained by the hepatic insulin resistance present in patients with T2D, exhibiting a decreased uptake of bile acids by the liver cells (Kohli and Diabetes, 2013).

The Role of the Gut Microbiota

The role of the gut microbiota in the context of obesity has currently attracted considerable interest to explain the beneficial effects of bariatric surgery. The gut microbiota may play a role in obesity by increasing the host's energy-harvesting efficiency (Turnbaugh *et al.*, 2006). Reportedly, RYGB restructures the gut microbiota, prompting the hypothesis that these changes may lead to WL after surgery (Furet *et al.*, 2010; Zhang *et al.*, 2009). In fact, Liou et al. have demonstrated that transfecting the gut microbiota from RYGB-treated mice to non-operated ones, resulted in WL and reduced fat mass in the recipient animals (Liou *et al.*, 2013). These results highlight the important roles of the gut microbiota after bariatric surgery and further studies are warranted to determine exactly their potential action to reduce the weight and adiposity of the host after RYGB.

In conclusion, although new studies are helping to understand the molecular mechanisms behind the bariatric surgery, more controlled studies are needed to improve the use of these procedures. Thus, bariatric surgery remains an inspiration in the search for new drug targets for WL.

Assessing the Long-Term Outcomes of Bariatric Surgery in Adolescents

The prevalence of obesity in adolescents has markedly increased worldwide across the past three decades (Ng *et al.*, 2014; Skinner and Skelton, 2014) and, as in the adult population, it is associated with preventable chronic comorbidities including hypertension, T2D, OSA, polycystic ovarian syndrome, dyslipidaemia, fatty liver disease and different musculoskeletal disorders (Rocchini, 2011; Juonala *et al.*, 2011; Shah *et al.*, 2015). Furthermore, obese children and adolescent loose self-esteem, suffer psychologically and perceive social exclusion (Russell-Mayhew *et al.*, 2012). Thus, effective treatment strategies are urgently needed.

Nowadays, obesity in adolescents is mostly managed by behavioral (diet, exercise and lifestyle modifications) and pharmacological approaches, but they are frequently limited to the short-term (Danielsson *et al.*, 2012). As a result, bariatric procedures have begun to be considered as a potential treatment option and consequently, the number of adolescents undergoing bariatric surgery is increasing worldwide (Lennerz *et al.*, 2014; Kelleher *et al.*, 2013). The guidelines from the *International Paediatric Endosurgery Group (IPEG)* state that adolescents with a BMI >40 kg/m^2 or a BMI $>$ 35 kg/m^2 combined with severe comorbidities should be considered for surgical intervention, if they have (nearly) achieved adult stature (International Pediatric Endosurgery Group (IPEG), 2009).

Inge et al. from USA (Inge *et al.*, 2017) and Olbers and colleagues from Sweden (Olbers *et al.*, 2017) have recently published two novel and important studies showing the long-term effects of bariatric surgery in adolescents. Inge et al. *(Inge* et al., *2017)* showed a substantial and durable body weight reduction as well as cardiometabolic benefits for young adults after bariatric surgery in a long-term longitudinal study including 58 adolescents (aged 13–21 years at baseline). However, some of the patients included in the study presented mild anemia, hyperparathyroidism and low amounts of vitamin B12. In this regard, they proposed that long-term health maintenance after RYGB should be focused on adherence to dietary supplements and the management of micronutrient deficiencies (Inge *et al.*, 2017). Olbers and colleagues (Olbers *et al.*, 2017) included 81 adolescents (aged 13–18 years at baseline) in their *Adolescent Morbid Obesity Surgery (AMOS)* prospective study. In the same line, adolescents with severe obesity undergoing RYGB showed a substantial WL over 5 years together with improvements in comorbidities and cardiovascular risk factors. However, gastric bypass was associated with additional surgical interventions and nutritional deficiencies. In contrast, conventional non-surgical treatment was associated with WR and a quarter of patients underwent bariatric surgery within 5 years (Olbers *et al.*, 2017).

Nevertheless, the available literature describing long-term outcomes in teenagers is still limited and it is necessary to consider some concerns related to adolescent bariatric surgery (Beamish and Reinehr, 2017). Firstly, it has to be highlighted that different side effects after bariatric surgery in adolescents have been outlined in different studies including the *Teen-Longitudinal Assessment of Bariatric Surgery (Teen-LABS)* study from the United States (Inge et al., 2014, 2016), the *AMOS* study in Sweden (Olbers *et al.*, 2012) and the *Germany Obesity Registry*, among others (Lennerz *et al.*, 2014). One of the main side effects is the dumping syndrome, which is a well-known complication of upper gastrointestinal surgery (Laurenius *et al.*, 2013) that comprises diverse symptoms such as abdominal pain, diarrhea, nausea, bloating, palpitations as well as hypotension and syncope. Secondly, all bariatric surgery techniques implicate a risk of vitamin and micronutrient deficiency that may lead to growth retardation (Becker *et al.*, 2012). Finally, the refeeding syndrome, which is an important complication after resumption of nutrition, might produce severe electrolyte imbalance and, hence, its early recognition is vital to reduce morbidity and mortality after bariatric surgery (Mehanna *et al.*, 2008). On the other hand, eating disorders and other psychiatric diseases among adolescents with severe obesity may be considered as exclusion criteria for bariatric surgery due to the suboptimal WL or even continued weight gain after bariatric procedure in these patients. Importantly, two cases of attempted suicide in the *AMOS* study and different cases of drug abusers have been reported after bariatric surgery (Jarvholm *et al.*, 2012).

Therefore, a multidisciplinary team should discuss bariatric surgery on an individual-case to assess the potential deterioration in the quality of life after the bariatric procedure. Moreover, the patients and their families must be educated regarding the benefits and risks, including the irreversibility of some procedures, potential side effects, expected changes in eating behavior and the lifelong requirement for regular medical follow-up after surgery (Beamish and Reinehr, 2017).

Complications of Bariatric Surgery

Although bariatric surgical procedures confer effective WL with a reduction in comorbid conditions, they carry substantial consequences that physicians should be aware of and consider in a pre- and post-operative assessment.

Dumping Syndrome

The dumping syndrome refers to symptoms and signs that occur when food reaches the small bowel too rapidly as a result of a defect in grinding or sieving within the stomach (Tack *et al.*, 2009). After bariatric surgical procedures that involve partial gastrectomy, such as RYGB and SG, up to 40% of patients have dumping syndrome symptoms (Banerjee *et al.*, 2013; Tzovaras *et al.*, 2012).

Symptoms normally start with food intake and they are divided into "early" and "late" symptoms (Tack *et al.*, 2009; Arts *et al.*, 2009) (**Table 3**). Early symptoms occur in response to the rapid passage of hyperosmolar contents to the duodenum, causing the move of fluid from the intravascular compartment to the intestinal lumen (Johnson *et al.*, 1962). Late dumping occurs 1 or 3 h after meal ingestion and it is caused by a reactive hypoglycaemia secondary to a hyperinsulinemia (Eloy *et al.*, 1975). Recently, the gut hormone GLP-1 has been shown to play a key role in the pathogenesis of late hypoglycaemia after gastric bypass (Salehi *et al.*, 2014).

Initial therapy for dumping consists of different dietary guidelines such as: 1) eat small and frequent meals; 2) avoid ingestion of liquids within 30 min of a solid-food meal; 3) avoid simple sugars and increase the intake of fiber and complex carbohydrates; and 4) increase protein intake (Heber *et al.*, 2010). An adequate response to the dumping syndrome is observed in 95% of cases after nutritional counseling (Zurita Mv *et al.*, 2013). However, different pharmacological therapies to slow gastric emptying and to inhibit the release of several gastrointestinal peptides involved in the development of the dumping syndrome such as somatostatin analogues, may be considered if dietary guidelines fail (Arts *et al.*, 2009; Deloose *et al.*, 2014). Finally, in difficult-to-manage patients ($<1\%$), a surgical re-intervention may be considered to reconstruct the gastric reservoir or to insert a short anti-peristaltic loop (Zurita Mv *et al.*, 2013).

Nutritional Deficiencies after Bariatric Surgery

Nutrient deficiencies constitute the most important long-term complications of bariatric surgery leading to the development of hematological, metabolic and especially neurological disorders that are not always reversible.

Unfortunately, the more effective surgical options for inducing WL are more likely to develop nutritional deficiencies. Restrictive techniques such as AGB and SG produce less frequently nutritional deficits, whereas RYGB and BPD, with a malabsortive component, are associated with higher prevalence of nutritional deficiencies. The preoperative nutritional status of the patient is another variable mainly related with nutritional deficiencies after bariatric surgery (Hammer, 2012). In addition, a common factor to all bariatric surgery techniques contributing to the occurrence of deficiencies is the reduced overall nutrient intake in the early post-operative phase (Saltzman and Karl, 2013). Specific deficiencies may require precise approaches and supplements, as described below:

Vitamin deficiencies after bariatric surgery

- Vitamin B1 (thiamine) deficiency is present in up to 49% of patients after RYGB, being the major nutritional complication (Lakhani *et al.*, 2008). Vomiting after bariatric intervention together with poor food and supplements are the principal risk

Table 3 Symptoms in dumping syndrome

Early dumping	*Late dumping*
Gastrointestinal symptoms:	**Hypoglycemia**:
Abdominal pain	Perspiration
Diarrhea	Palpitations
Bloating	Hunger
Nausea	Weakness
	Confusion
	Tremor
	Syncope
Vasomotor symptoms	

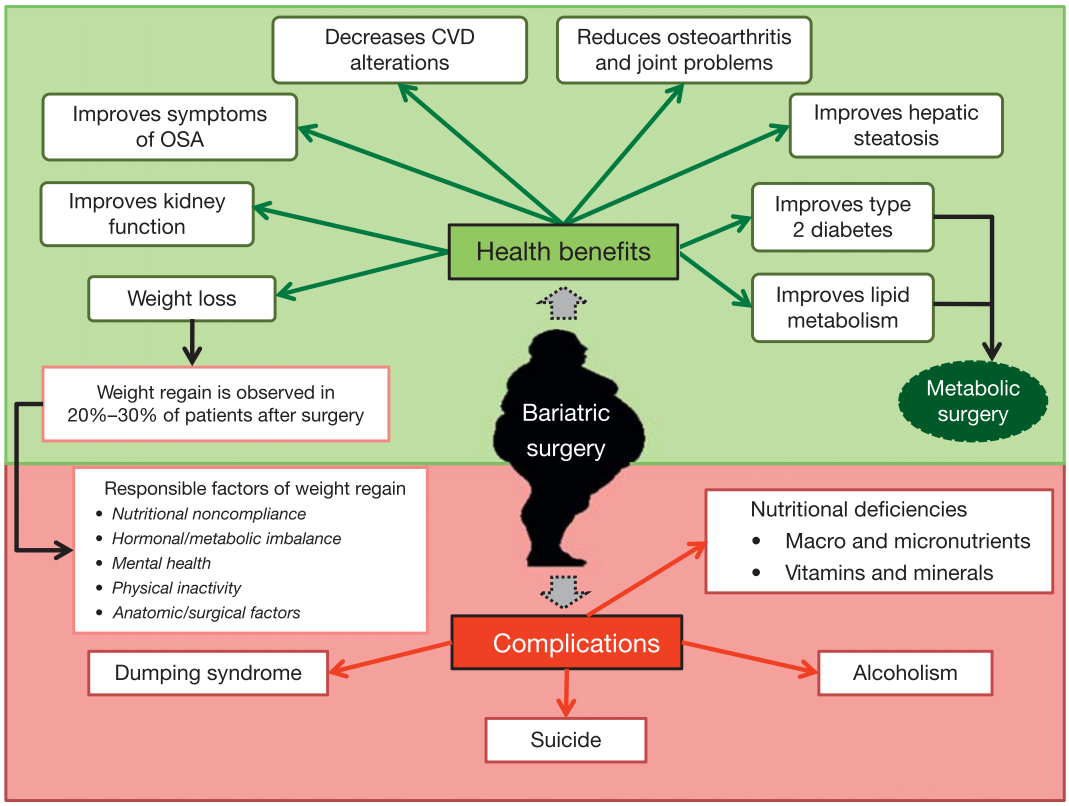

Fig. 2 The main health benefits and complications of bariatric surgery.

factors for developing thiamine deficiency (Galvin *et al.*, 2010). Clinical manifestations of thiamine lack include Wernicke's encephalopathy and Korsakoff syndrome, among others (Landais, 2014). Since thiamine deficiency may lead to irreversible neurological damage, the *European Federation of Neurological Societies* recommends a follow up of thiamine status at least 6 months post-operatively as well as a parenteral thiamine supplementation (beriberi factor) (Galvin *et al.*, 2010). In particular cases, oral thiamine supplementation after bariatric surgery may not resolve thiamine deficiency, being associated with small intestinal bacterial overgrowth and antibiotic therapy may be required (Lakhani *et al.*, 2008).

- Vitamin B12 (cobalamin) deficiency. The prevalence of the lack of vitamin B12 is 3.6% 12-months after RYGB (Clements *et al.*, 2006), rising to 61.8% at 5-years after RYGB (Dalcanale *et al.*, 2010). The mechanisms underlying vitamin B12 deficiency are the reduced intake of meat, the diminished contact of food with gastric acid and the decreased secretion of intrinsic factor (Herrmann and Obeid, 2008). Clinical manifestations include megaloblastic anemia, potentially irreversible peripheral neuropathy, as well as depression and neuropsychiatric symptoms. Effective treatment for vitamin B12 deficiency include oral, intramuscular, nasal or sublingual vitamin B12 supplementation (Herrmann and Obeid, 2008).
- Vitamin B9 (folate) deficiency. Megaloblastic anemia, weakness, anorexia and WL are potential symptoms of folate deficiency. It is of special consideration in pregnant women because maternal folate deficiency is associated with neural tube defects in newborns (American College of Obstetricians and Gynecologists, 2005). For prevention, women who are trying to become pregnant after bariatric surgery should receive 1 mg of folic acid daily, as a routine supplement (Bal *et al.*, 2010).
- Vitamin D deficiency. It is described as a common cause of calcium metabolism disorders and metabolic bone disease leading to osteoporosis and possibly fractures after surgical procedures (Johnson *et al.*, 2005). Aarts et al. described that 39% of patients exhibited vitamin D deficiency despite daily multivitamin supplementation (Aarts *et al.*, 2011). Intake of vitamin D3 or D2 should be initiated with oral vitamin D once weekly and 8–12 weeks after the start of supplementation 25-hydroxyvitamin D levels should be measured to confirm repletion (Levinson *et al.*, 2013). Moreover, in order to avoid bone fractures after bariatric surgery, measurements of calcium, vitamin D and parathyroid hormone as well as preoperative and postoperative follow-up bone mineral density measurements is recommended after bariatric surgery (Heber *et al.*, 2010).
- Vitamin A deficiency. The origin of vitamin A deficiency includes a relative deficiency of bile acids in the bypassed duodenojejunal segment as well as desconjugation of bile acids by upper gut bacterial overgrowth because fat soluble vitamins require micelle formation with bile acids to be assimilated (Hatizifotis *et al.*, 2003). Clinical manifestations include a sensation of dry eyes, reduced night vision, itching and dry hair. In case of vitamin A deficiency, the initial treatment is oral supplementation but in women who want to become pregnant, supplements of vitamin A should be carefully used because hypervitaminosis A may induce teratogenic effects (Tack and Deloose, 2014).

Mineral deficiencies after bariatric surgery

- Iron deficiency. Anemia is common after bariatric surgery, specifically after RYGB (36% of patients 1 year after RYGB) (Cable *et al.*, 2011) compared to SG (1.5% of patients 1 year after SG) (Hakeam *et al.*, 2009). The responsible factors for iron deficiency are probably the reduced intake of meat, diminished contact of food with gastric acid and decreased intestinal absorption. The risk is increased in menstruating or pregnant females (Saltzman and Karl, 2013). Treatment of iron deficiency after bariatric surgery includes either 150–200 mg per day of oral elemental iron or ferrous salt-vitamin C combination. Non-responder patients to oral iron supplements are occasionally treated with parenteral therapies (Bal *et al.*, 2012).
- Zinc deficiency. A high risk for zinc deficiency after bariatric surgery has been reported, with higher prevalence 1 year after duodenal switch than after RYGB (Bal *et al.*, 2012). Symptoms of hypozincaemia include skin eruption, nail dystrophy, alopecia, hypoalbuminaemia (in patients with severe deficiency) and glossitis. In case of deficiency, doses of 60 mg of elemental zinc are recommended. However, administration of zinc may deplete copper stores and, hence, this should be considered when administering zinc supplements (Tack and Deloose, 2014).
- Copper deficiency has been reported in up to 18% of patients after bariatric surgery usually several months after the intervention (Levinson *et al.*, 2013). Low serum copper levels can lead to anemia, neutropenia and pancytopenia (Todd *et al.*, 2004; Fuhrman *et al.*, 2000). In addition, in the past decade, neurologists reported that some patients with hypocupraemia developed a new myeloneuropathy-like disorder with splastic gain and sensory ataxia (Kumar, 2006) and the symptoms were not fully reversed with cupper therapy. On the other hand, sudden bilateral blindness has been also reported in a patient with cupper deficiency after RYGB (Naismith *et al.*, 2009) and, unfortunately, the appropriate treatment for blindness after bariatric surgery is uncertain.

Conclusions

Bariatric surgery is the most effective treatment in producing sustainable WL and improvements in obesity-related comorbidities. The positive effects obtained regarding ameliorations in T2D after bariatric surgery, have expanded the eligibility criteria for metabolic surgery. However, operated patients do not respond in the same way, with a subset of subjects showing WR, failure of sustained WL and even T2D recidivism. Although it is true that some potential responsible factors have already been described, additional studies are needed to examine plausible undiscovered effects in an effort to enhance patients' benefits. On the other hand, bariatric procedures are not without risk with nutritional deficiencies being the most important long-term complication after bariatric surgery leading sometimes to metabolic and neurological disorders, which are not always reversible. Given the uncertainties between risks and benefits of bariatric surgery, the decision to undergo surgery should be based on a high quality multidisciplinary team in order to optimize patient selection criteria and outcomes (**Fig. 2**).

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Metabolic Surgery; Indications and Outcomes

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Introduction

Obesity prevalence and incidence are increasing not only in developed countries but also in developing countries. Obesity is an independent risk factor for cardiovascular diseases and diabetes and it is associated with a significant increase in all-cause mortality. Lifestyle modifications are indeed effective, but the number of patients responding well to the lifestyle interventions is limited (Look AHEAD Research Group *et al.*, 2016). The response rate to bariatric surgery is good also in the long term and thus these surgical interventions are becoming more popular.

In addition to weight loss, bariatric surgery has a large number of benefits, including improved control of glycaemia, hypertension, dyslipidemia with reduced cardiovascular diseases and female cancer mortality, often independent of body weight reduction. For these reasons bariatric surgery was renamed as metabolic surgery.

In this review we report the effects of metabolic surgery on obesity and its metabolic complications.

Mortality Reduction

The Swedish Obesity Study (SOS) showed that the mortality hazard ratio adjusted for gender-, age- and risk factors was 0.71 ($P = 0.01$) for obese subjects after bariatric surgery as compared with medical treatment (Sjöström *et al.*, 2007). The mortality reduction was driven by a substantial reduction in myocardial infarctions and female cancers, while insulin resistance rather than body mass index appeared to predict which patients would benefit most (Sjöström *et al.*, 2007).

In a retrospective cohort study with an average follow-up of 7.1 years the adjusted mortality from any cause in the Roux-en-Y gastric Bypass (RYGB) group decreased by 40%, as compared with that in the control group ($P < 0.001$) (Adams *et al.*, 2007). Cause-specific mortality was in particular lower for coronary artery disease, diabetes and female cancers. However, rates of death not caused by disease, such as accidents and suicide, were 58% higher in the surgery compared to the control group ($P = 0.04$).

Type 2 Diabetes and Its Vascular Complications

Metabolic surgery can place type 2 diabetes (T2D) in remission in several patients while almost all have a substantial improvement in glycemic control. Remission of T2D is common within the short term, that is, 1–2 years after surgery, but relapse occurs in many in the longer term, although glycemic control remains much improved (Schauer *et al.*, 2012, 2017; Mingrone *et al.*, 2012, 2015; Sjöström *et al.*, 2004, 2014). Importantly, the cumulative incidence of microvascular complications at 15 years follow-up (Sjöström *et al.*, 2014) was 41.8 per 1000 person-years (95% CI, 35.3–49.5) for patients not receiving surgery and 20.6 per 1000 person-years (95% CI, 17.0–24.9) in the surgery group (hazard ratio [HR], 0.44; 95% CI, 0.34–0.56; $P < 0.001$). Macrovascular complications (Sjöström *et al.*, 2014) were observed in 44.2 per 1000 person-years (95% CI, 37.5–52.1) in patients not receiving surgery and 31.7 per 1000 person-years (95% CI, 27.0–37.2) for the surgical group (HR, 0.68; 95% CI, 0.54–0.85; $P = 0.001$).

The benefits of surgery on microvascular disease are inversely proportional to diabetes duration. Thus the longer the length of the disease the lower is the reduction of diabetic vascular complications for the group (Sjöström *et al.*, 2014), this does however not mean that patients with long standing diabetes do not benefit. The numbers needed to treat (NNT) to achieve benefit may indeed be lower when surgery is used in those with established microvascular disease secondary to T2D as their disease may be controlled and even put into remission (Iaconelli *et al.*, 2011).

At the moment only two randomized controlled trials (RCT) report data 5 years after surgical or medical interventions. Mingrone *et al.* (2015) showed 5 year diabetes remission rates (defined as a fasting glucose concentration of 5.6 mmol/L or HbA1c $<6.5\%$ [≤ 47.5 mmol/mol] without active pharmacological treatment) of 37% after Roux en Y gastric bypass (RYGB) and of 63% after biliopancreatic diversion (BPD) while none of the patients in the medical arm achieved the criteria.

Schauer *et al.* (2017) found that 28.6% in the RYGB and 23.4% in the sleeve gastrectomy (SG) arms had HbA1c $<6\%$ with or without medication, while the end point was achieved in only 5.3% of the patients in the medical arm with the use of medication. However, while the difference with the medical arm was statistically significant ($P = 0.03$) after adjustment for multiple comparisons for RYGB it did not reach a statistical significance ($P = 0.07$) for SG patients. Importantly, if we focus on glycemic control rather than remission then metabolic surgery showed substantially better improvements in comparison with medical treatment (HbA1c 6.5 ± 1.3 vs. $7.6\% \pm 1.7\%$) (Schauer *et al.*, 2017).

The results on glycemic control after metabolic surgery as average between short and long term follow-up in RCTs is summarized in **Fig. 1** (Schauer *et al.*, 2012, 2014, 2017; Mingrone *et al.*, 2012, 2015; Cummings *et al.*, 2016; Ding *et al.*, 2015; Dixon

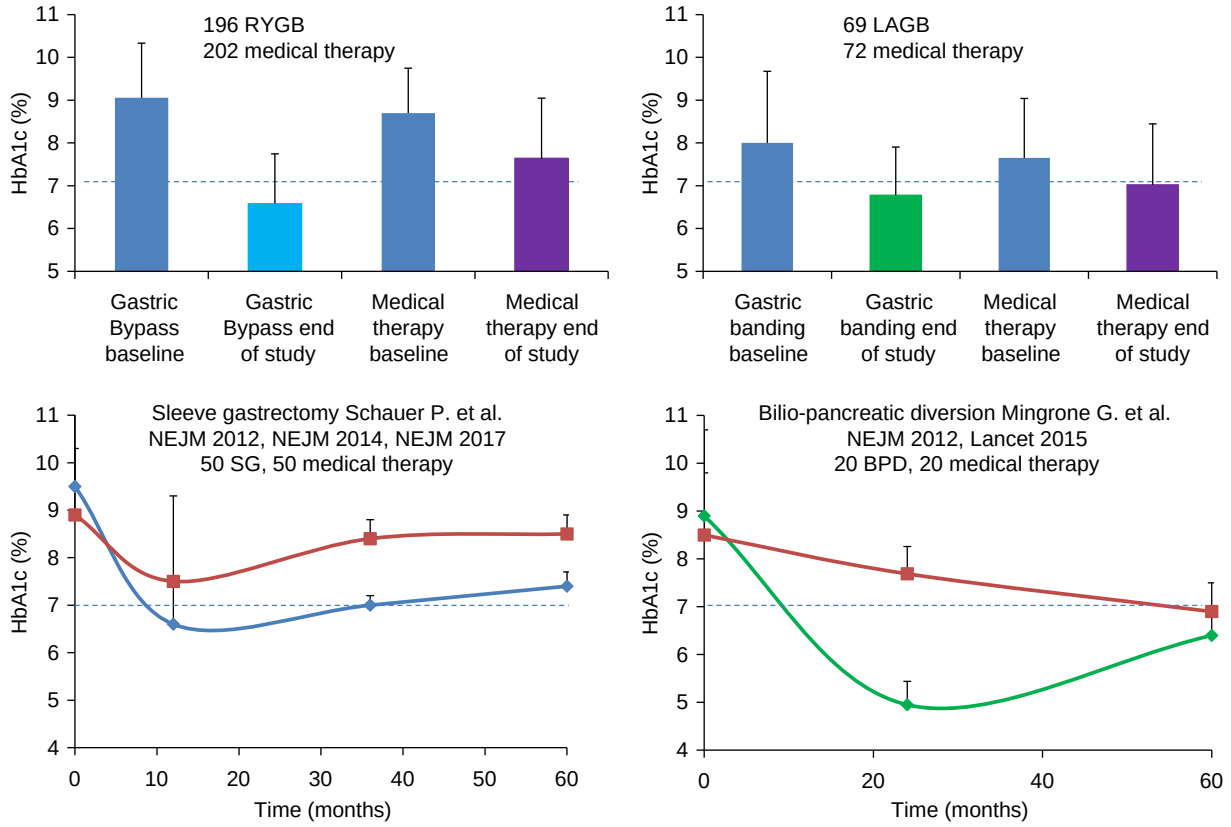

Fig. 1 End-study average glycated hemoglobin (mean ± SD) in type 2 diabetic subjects undergone bariatric/metabolic surgery ($n = 698$) or medical treatment plus lifestyle modifications ($n = 503$) from randomized controlled trials. Reference numbers are provided into brackets.

et al., 2008; Courcoulas *et al.*, 2014, 2015; Halperin *et al.*, 2014; Ikramuddin *et al.*, 2013, 2015; Liang *et al.*, 2013; Parikh *et al.*, 2014; Wentworth *et al.*, 2014).

Hypertension

Few clinical trials had hypertension improvement after bariatric surgery as primary outcome.

The Diabetes Surgery Study (Ikramuddin *et al.*, 2013, 2015) with achievement of the composite treatment goal of HbA_{1c} <7.0% (53 mmol/mol), LDL cholesterol <2.59 mmol/L, and systolic blood pressure <130 mmHg at 12 months as primary endpoint had 76% and 69% SBP reduction in the lifestyle and medical management and 82% and 80% in the RYGB group at 1 year and at 2 years respectively ($P = 0.2$). RYGB also had similar improvements ($P = 0.79$) than a program of intensive lifestyle intervention on nocturnal hypertension (Nordstrand *et al.*, 2012). The remission rate for hypertension was 19% after bariatric surgery as compared to 11% after medical treatment in the 10 years follow-up of the SOS study (Sjöström *et al.*, 2004).

Therefore, at first glance it seems that surgery isn't much better than anti-hypertensive drug treatment to improve blood pressure control, but it should be remembered that historically clinicians thought it a good idea to discontinue effective pharmacotherapy after bariatric surgery, while in more recent years surgery is now seen as an "add on therapy" and that effective pharmacotherapy should be continued to keep chronic diseases in control (Miras and le Roux, 2017). This later approach is likely to result in a reduction of dose of the medication but a substantial improvement in control of metabolic parameter (Miras and le Roux, 2014).

Hyperlipidemia

The Program on the Surgical Control of the Hyperlipidemias (POSCH) was the only RCT using a surgical approach with the primary endpoint of overall mortality linked to hyperlipidemia/atherosclerosis. Partial ileal bypass was compared with medical treatment and showed that the urgical approach normalized lipid profiles and reduced overall mortality at 18 years follow-up (Cottam *et al.*, 2006).

Ikramuddin *et al.* (2013) demonstrated in their RCT with as composite end-point HbA1c <7.0%, low-density lipoprotein cholesterol <100 mg/dL, and systolic blood pressure <130 mmHg, that 49% of patients reached the goal in the RYGB and 19% in the lifestyle-medical management arm, respectively.

We showed a highly significant ($P < 0.0001$) increase in HDL-cholesterol concentrations after RYGB (from 41.9 ± 7.2 to 56.6 ± 9.0 mg/dL) but not after dieting (Raffaelli *et al.*, 2014). Many prospective non randomized (Sjöström *et al.*, 2004; Fløl*o* *et al.*, 2017; Kothari *et al.*, 2017) and randomized (Schauer *et al.*, 2012, 2017; Mingrone *et al.*, 2012, 2015; Cummings *et al.*, 2016; Ding *et al.*, 2015; Dixon *et al.*, 2008; Courcoulas *et al.*, 2014, 2015; Halperin *et al.*, 2014; Ikramuddin *et al.*, 2013, 2015; Liang *et al.*, 2013; Parikh *et al.*, 2014; Schauer *et al.*, 2014; Wentworth *et al.*, 2014) studies have demonstrated a net improvement of plasma lipid profile after metabolic surgery, but most of these studies reduced the doses of effective statin therapies after surgery. The beneficial effect of combining surgery with lower doses statins may result in the benefits of high dose statins without the side effects (Nissen *et al.*, 2006).

Obstructive Sleep Apnea

Obstructive sleep apnea (OSA) is characterized by recurrent episodes of collapse of the upper airways during sleep due to reduced airway dilator muscle tone, with consequent desaturation and sleep fragmentation leading to daytime fatigue. It is a frequent complication of overweight and obesity occurring in 41% of adults with a BMI >28 kg/m^2 (Vgontzas *et al.*, 1994) and in 78% of patients referred for bariatric surgery (Lopez *et al.*, 2008).

Although obese patients are recommended to lose weight the results of clinical trials are controversial. A very low calorie diet (VLCD) for 9 weeks in obese subjects with a BMI between 30 and 40 kg/m^2 reduced the apnoea–hypopnoea index (AHI) by 21 events/h and weight by 18 kg (Johansson *et al.*, 2011). These results seemed very promising in view of the great weight loss obtained with bariatric surgery.

Two metaanalysis of surgical interventions published in 2004 and in 2009 showed opposite results. The first demonstrated remission of OSA in 85% of patients who underwent bariatric surgery (Buchwald *et al.*, 2004), the second demonstrated that bariatric surgery reduces AHI but that most continued to need medical treatment (Greenburg *et al.*, 2009). The differences can be explained by the various definitions that were used as surgery reduces AHI substantially, but often not below the threshold where CPAP would no longer be beneficial.

A randomized controlled study comparing low energy diet (including if necessary VLCD) with gastric banding confirmed this ascertain as weight loss was 5.1 kg with dieting and 27.8 kg with LAGB and AHI decreased by 14.0 events/h (95% CI, 3.3–24.6 events/h) with dieting and 25.5 events/h (95% CI, 14.2–36.7 events/h) with surgery, but there was no statistical difference in the number of subjects where the AHI decreased below the threshold where CPAP was not indicated (Dixon *et al.*, 2012).

These results were confirmed by another RCT with primary endpoint of the number of patients fulfilling the criteria for weaning from non-invasive ventilation (NIV) after either dieting or LAGB. Again there was no significant difference between the two arms (Feigel-Guiller *et al.*, 2015) spite of a much greater weight reduction after surgery than after lifestyle modification both at 1 and at 3 years.

Sexual Dysfunction

Obesity is frequently associated with sexual dysfunction. In women early onset of obesity is often associated with menstrual irregularities, oligo- to an-ovulation and subfertility. Obesity also increases the risk of miscarriages. Polycystic ovary syndrome (PCOS) is present in 36% of women with morbid obesity while male obesity-associated secondary hypogonadism (MOSH) is observed in 64% of severely obese men (Escobar-Morreale *et al.*, 2017).

A recent metaanalysis showed that PCOS resolved in 96% of cases and that resolution of MOSH occurred in 87% of affected men after bariatric surgery (Escobar-Morreale *et al.*, 2017). Sexual function improved in 28% ($P = 0.02$) of 29 obese women in reproductive age 12 months after gastric bypass (Legro *et al.*, 2012).

The Swedish Medical Birth Register compared 670 singleton pregnancies that occurred from 2006 through 2011 in women after bariatric surgery (Johansson *et al.*, 2015) with matched control pregnancies. Post-surgery pregnancies were associated with an increased risk of small-for-gestational-age infants (15.6% vs. 7.6%; odds ratio, 2.20; 95% CI, 1.64–2.95; $P < 0.001$) and shorter gestation (273.0 vs. 277.5 days; mean difference −4.5 days; 95% CI, −2.9 to −6.0; $P < 0.001$). The authors concluded that bariatric surgery was safe but pointed to a non-significant trend to increased still births after bariatric surgery (Johansson *et al.*, 2015).

Appetite Reduction and Food Preferences

Many patients after bariatric surgery point to the most beneficial effect of the interventions being the increased satiety and reduction in hunger which allows them to lose weight and maintain weight loss (le Roux *et al.*, 2011). The profound improvements in eating behaviour are the consequences of multiple visceral signals (le Roux *et al.*, 2011) which appears to fundamentally

alter subcortical brain centres involved with appetite and reward. Whether patients eat fewer calories and shift their preferences to lower calorie dense foods remain controversial (Goldstone *et al.*, 2016).

Conclusions

A recent metaanalysis including both RCTs and observational studies showed that mortality associated with metabolic surgery is low, ranging from 0.08% to 0.35% (Chang *et al.*, 2014). Nonetheless, surgical complications are relatively frequent, 10%–17% (Chang *et al.*, 2014). In addition, long term nutritional complications are common, in fact anemia is observed in 33%–49% of patients within 2 years from surgery and calcium/vitamin D malabsorption with secondary hyperparathyroidism with bone loss is present in 8%–13% of the cases (Lupoli *et al.*, 2017).

Although globally the long term benefits of metabolic surgery exceed surgical side effects, it is desirable that less invasive approaches to the obesity epidemics can be found in the future.

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Treatment: New Drugs

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Introduction

Obesity, globally representing one of the most preventable causes of death, remains a worldwide health concern as its incidence has dramatically grown in the last 40 years. Although, our knowledge in the understanding of pathophysiological mechanisms driving to obesity has constantly advanced in the recent period, the effectiveness of interventions to limit obesity epidemic progression has been highly disappointing. As confirmed by the most recent guidelines of international societies of obesity and human nutrition, lifestyle intervention (changes in dietary pattern and physical exercise) remains the cornerstone in weight loss strategies (Williamson, 2017). However, changes in lifestyle are unanimously considered the most difficult action to be maintained lifelong by patients and consequently their effect on body weight (BW) are limited by the high attrition rate. For most people, a nutritional program to reduce BW is generally considered a prescription to be followed for a limited period, with a start and an end, rather than a road to achieving healthier nutritional habits. This misleading idea also comes from the widespread availability of "dietary" restrictive protocols (based on the exclusion of food categories, i.e., sugars, carbohydrates), being on one hand potentially dangerous and on the other, for sure, not applicable for a long time in everyday life. Furthermore, BW loss is followed by the activation of preservative biological mechanisms (e.g., ghrelin increase, reduction of energy expenditure) inducing weight regain which are the reason for failure of diet-behavioral therapy in the long-term maintenance of weight loss (Apovian *et al.*, 2015).

It is nowadays commonly well acknowledged that in selected patients, especially when changes in lifestyle become a constant failure and an irrepressible source of frustration, the surgical approach to reduce BW is strongly indicated. Indeed, to date, bariatric surgery represents the most effective and potentially definitive treatment for severe obesity and its metabolic correlates (primarily type 2 diabetes). This procedure, however, involves a certain risk and often high costs, therefore it is applicable only for patients who have a totally favorable risk/benefit ratio.

The devastating effects of obesity on health and the scarce impact of pressure on the general population to ameliorate the lifestyles paved the way, especially in the second half of the last century, to alternative therapeutic strategies to tackle obesity, increasing scientific interest in the development of antiobesity drugs. Research in the field of pharmacologic therapy of obesity has gone hand in hand with the understanding of the complex network between central and peripheral structures involved in the regulation of food intake. Hypothalamus is known to be pivotal in the regulation of homeostatic mechanisms, as it receives inputs from other areas of the brain and from the periphery in order to modulate feeding and energy metabolism (Berthoud *et al.*, 2017; Camilleri, 2015; Suzuki *et al.*, 2010). The arcuate nucleus of the hypothalamus, in particular, is the key area involved in these mechanisms, containing the orexigenic neuropeptide Y (NPY) and Agouti-related protein (AgRP) neurons and the anorexigenic melanocortin system derived from propiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) producing neurons (Berthoud *et al.*, 2017). Many hormones (ghrelin, leptin, insulin, cholecystokinin, peptide YY, oxyntomodulin, and GLP-1) also have a role in the modulation of hypothalamus nuclei activity and, by influencing the AgRP/NPY pathway or the melanocortin system, they eventually control appetite (Berthoud *et al.*, 2017). To date, all approved drugs for weight management, except from orlistat, target appetite regulation mechanisms and hedonic/rewarding role of food (Apovian *et al.*, 2015).

Prescriptive Guidelines and Regulatory Authorities Point of View

In 1998 the National Institute of Health Guidelines for treatment of overweight and obesity in adults proposed that pharmacological treatment for obesity was indicated for patients with Body Mass Index (BMI) $\geq 30\ kg/m^2$ or BMI $\geq 27\ kg/m^2$ with comorbidities (type 2 diabetes, hypertension, dyslipidemia, coronary heart disease, and sleep apnea) (Anon, 1998). These criteria have been recently confirmed by both American and European Obesity Societies guidelines, that also suggested that pharmacologic therapy should be considered only after at least one attempt of nutritional intervention has proved insufficient and must be associated to continuous behavioral counseling. Interaction between these two strategies may enhance adherence to nutritional changes and physical activity, thus improving weight loss and increasing the probability of maintaining a lower BW. Moreover, as for any pharmacological treatment, an issue has been raised concerning the potential side effects produced by antiobesity drugs. A further encouragement to constantly perform periodic re-evaluation of both efficacy and side effects for each patient undergoing pharmacological antiobesity treatment has been conclusively expressed (U.S. Department of Health and Human Services—Food and Drug Administration—Center for Drug Evaluation and Research (CDER), 2007; European Medicines Agency (EMA)—Committee for Medicinal Products for Human Use (CHMP), 2016). This last recommendation, in particular, has achieved increasing importance in the last decade, after the withdrawal from the market of most antiobesity drugs for the onset of serious

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side effects (Rodgers *et al.*, 2012). At present, United States and European regulatory drug agencies have further defined specific guidelines and standards to be followed in the development of antiobesity pharmacologic treatments. According to the American Food and Drug Administration (FDA) draft guidance, the efficacy of an antiobesity drug should be primarily defined if after 1 year/treatment the difference in weight loss between the tested drug and placebo is at least 5%, or in the same timeframe of treatment, the proportion of subjects who lose $\geq 5\%$ of baseline BW with the drug is at least 35% or approximately double the proportion in the placebo. FDA also requires that medications show evidence of improvement in metabolic biomarkers, including blood pressure, lipid levels, and glycemic control (U.S. Department of Health and Human Services—Food and Drug Administration—Center for Drug Evaluation and Research (CDER), 2007). On the other hand, the European Medicines Agency (EMA) required that after 1 year/treatment the difference in weight loss between drug and baseline BW should be at least 10% and should also be at least 5% higher than that achieved on placebo. As an alternative, EMA guideline stated that proportions of responders could be considered as an alternative primary efficacy criterion where response is more than 10% weight loss at the end of a 12-month period (European Medicines Agency (EMA)—Committee for Medicinal Products for Human Use (CHMP), 2016). As secondary endpoint, the improvement of parameters affecting cardiovascular risk (blood pressure, lipid profile, blood glucose) must be documented prior to authorization. To define the impact of the drug on glycemic control, authorities have highly encouraged studies focusing on the delay in the time of onset of diabetes from a prediabetic state as well as specific studies in diabetic cohorts. Concomitant evaluation of obesity comorbidities (sleep apnea, urinary incontinence, mood disorders, impaired fertility, functional limitation) and quality of life by distinct studies or subanalyses should be included as secondary, but not less important endpoints (U.S. Department of Health and Human Services—Food and Drug Administration—Center for Drug Evaluation and Research (CDER), 2007; European Medicines Agency (EMA)—Committee for Medicinal Products for Human Use (CHMP), 2016). Pharmacologic safety has to be taken into account in the study design of phase II–III trials. Especially, cardiovascular safety (in terms of incidence of cardiovascular events/mortality) is a pivotal requirement both in preauthorization studies (which must include a relevant proportion of patients with coexisting cardiovascular profile) and in postmarketing extended trials (U.S. Department of Health and Human Services—Food and Drug Administration—Center for Drug Evaluation and Research (CDER), 2007; European Medicines Agency (EMA)—Committee for Medicinal Products for Human Use (CHMP), 2016). The efficacy of antiobesity drugs should be evaluated after the first 3 months of therapy. If weight loss achieved $>5\%$ loss in nondiabetic and $>3\%$ in diabetic patients treatment should be continued, otherwise the drug should be discontinued and the patients should be defined as nonresponders.

Central Sympathomimetic Agents for Obesity Management

Amphetamines and their congeners were the first antiobesity drug approved by FDA at the end of the 1940s. This heterogeneous group includes norepinephrine (and possibly dopamine) releasing molecules (desoxyephedrine, phenmetrazine, diethylpropion phentermine, phendimetrazine, benzphetamine), and serotonin releasing agents (fenfluramine, dexfenfluramine). All these agents are known to increase central nervous system (CNS) activity and resting energy expenditure, and decrease appetite and food intake, thus inducing weight loss. The use of amphetamine-based drugs spread widely in United States between the 1950s and 1970s, but along with their diffusion, the report of adverse events, as well as concerns about abuse potential and transient efficacy, rapidly increased. In 1977 the FDA limited of the use of phentermine, diethylpropion, phendimetrazine, benzphetamine only for short-term treatment (from a few weeks, up to 3 months) leading to the decline in their use (Haslam, 2016; Colman, 2012, 2005; Gomez and Stanford, 2017). Interest in amphetamine congeners reemerged in 1990s after authorization of dexfenfluramine (an isomer of fenfluramine) and of the association phentermine–fenfluramine ("Phen–Fen") for long-term treatment of obesity. Unfortunately, after a few years, emerging data on pulmonary hypertension and left-sided heart-valve fibrosis and degeneration (due to the interaction with receptor 5-HT(2B) (Connolly *et al.*, 1997; Rothman *et al.*, 2000), led to the withdrawal in United States of both Phen–Fen and dexfenfluramine. Since 1999, the EMA recommended the withdrawal of all amphetamine-derived drugs (Onakpoya *et al.*, 2016). On the other hand, in the United States phentermine is nowadays the most prescribed antiobesity drug for short-term use (Hampp *et al.*, 2013). Its efficacy has been described in a short-term outcome meta-analysis of six studies (ranging from 2 to 24 weeks), which reported a mean BW loss of 6.3 kg in patients treated with 15–30 mg/day phentermine (3.6 kg over the placebo group) (Haddock *et al.*, 2002). Only one randomized, double blind, placebo-controlled trial evaluated the effects of a longer treatment with phentermine, comparing continuous (37.5 mg/day for 36 weeks) versus discontinuous (alternating phentermine and placebo every 4 weeks) therapy. Mean weight loss was -12.2% in the phentermine continuous-group, -13% in the discontinuous-group, and -4.8 in the placebo group (Munro *et al.*, 1968). The more frequent side effects are palpitation, blood pressure elevation, dizziness, insomnia, dryness of the mouth, therefore it is contraindicated in patients with cardiovascular disease, moderate/severe hypertension, hyperthyroidism, known hypersensitivity to the sympathomimetic amines, glaucoma.

Orlistat

Orlistat was the first developed and approved antiobesity agent with a completely different mechanism of action (not central but peripheral) compared to previous drugs. It is a gastric and pancreatic lipase-inhibitor which reduces the absorption of about the

30% of dietary fats (Food and Drug Administration (FDA), 2015). It is available both in the United States and Europe in 120 mg tablets (prescription only) and 60 mg tablets (as over-the-counter medicine), and the daily recommended dose is up to 120 mg thrice daily. The Xendos study compared obese prediabetic/diabetic patients treated with orlistat 120 mg or placebo: mean BW loss was significantly higher in treated patients than in placebo group both at mid- and long-term follow-up (at 1 year: −10.6 vs. −6.2 kg; at 4 years: −5.8 vs. −3 kg). Furthermore, the number patients achieving a BW loss ≥5% and ≥10% were significantly higher in orlistat treated patients than placebo both at 1 and 4 years treatment (Torgerson *et al.*, 2004). The results at 1 year were confirmed in a meta-analysis of 16 double blind randomized placebo-controlled clinical studies, reporting a mean weight loss in orlistat-treated patients of 2.9% more than placebo. In four studies of 2 years duration, both orlistat and placebo showed similar weight-regain therefore preserving the differential in weight reduction (Rucker *et al.*, 2007). Within secondary endpoints, orlistat was proven to be effective, at least in the short/mid term, in improving glycemic control (blood glucose, HbA1c) (Rucker *et al.*, 2007), insulin sensitivity (Lucas *et al.*, 2003), lipid profile (total cholesterol, high density lipoprotein—HDL cholesterol, low density lipoprotein—LDL cholesterol, triglycerides) (Hutton and Fergusson, 2004; Sahebkar *et al.*, 2017), and systolic/diastolic blood pressure (Rucker *et al.*, 2007; Siebenhofer *et al.*, 2016). Moreover, it significantly reduces the cumulative incidence rate of type 2 diabetes after 4 years treatment in patients with impaired glucose tolerance at baseline (2.9% with orlistat vs. 4.2% for placebo), corresponding to a 41% risk reduction (hazard ratio 0.593) (Torgerson *et al.*, 2004). A meta-analysis of 11 studies reported a significant reduction in the risk of developing diabetes in patients treated with orlistat alone or orlistat + diet + exercise versus standard care (hazard ratio 0.38 and 0.31, 95% CrI, respectively) (Stevens *et al.*, 2015). Treatment with orlistat is typically associated with gastrointestinal side effects: abdominal pain and discomfort, steatorrhea/oily stool, oily spotting, fecal urgency (Xenical, 2009). Due to its mechanism of action, orlistat reduces the absorption of fat-soluble vitamins: although true deficiency states have not been reported, patients receiving orlistat must be advised to take oral multivitamins (Food and Drug Administration (FDA), 2015).

Phentermine/Topiramate

Phentermine/Extended-Release Topiramate (PHEN/ER TOP), Qnexa®, was the first combination of central acting drugs approved by FDA in 2012 for long-term treatment of overweight/obesity in adults (Yanovski and Yanovski, 2014). Topiramate is an anticonvulsant drug, used also for bipolar disease and in migraine-prophylaxis (Food and Drug Administration (FDA), 2012). It is known to inhibit carbonic anhydrase activity, block voltage-dependent sodium channel, and antagonize kinate subtype glutamate receptors (Kramer *et al.*, 2011). The exact mechanisms associated with topiramate-induced BW loss are still unclear, although an anorectic action has been postulated following carbonic-anhydrase inhibition on taste and the activation of γ-aminobutyric acid (GABA)-A receptor in the lateral hypothalamus known to interact with leptin pathway (Turenius *et al.*, 2009). The observation of BW loss following administration of topiramate, led to its evaluation as a possible antiobesity agent (Bray *et al.*, 2003; Astrup *et al.*, 2004). A meta-analysis of 10 RCTs reported a significant weight loss in overweight-obese patients treated with topiramate versus placebo, furthermore patients losing ≥5% and ≥10% were significantly more in the topiramate group; interestingly, the weight-losing effect of topiramate increased with dosage and duration of treatment, while it resulted independent of the presence of diabetes (Kramer *et al.*, 2011). In diabetic subjects, topiramate monotherapy 96–192 mg compared to placebo (alone or in combination with lifestyle interventions and/or metformin or sulfonylurea), showed a significantly higher BW and BMI reduction associated to a significant reduction of HbA1c (Paravattil *et al.*, 2016). Weight-loss treatment with topiramate resulted associated to a twofold incidence of side effects (paresthesia, taste perversion, psychomotor disturbances, and hypoesthesia, memory/attentional difficulties, dizziness), especially with higher doses (greater than 96 mg/day) (Kramer *et al.*, 2011; Paravattil *et al.*, 2016). Both topiramate and phentermine were proved to reduce BW, but their use was limited by the activation of biological compensatory mechanisms and the high incidence of side effects and adverse events reported at efficient doses. Clinical studies comparing the efficacy of the two single molecules versus their combination highlighted the superiority of the association on weight reduction (Singh and Kumar, 2015). The latter achieved results at lower doses of each component (because of their complementary and synergic effect on appetite regulation and energy balance) (Bays, 2010). The authorization of PHEN/ER TOP followed a RCT conducted on 2487 overweight-obese patients with BMI 27–45 kg/m^2 and at least two metabolic comorbidities (CONQUER) (Gadde *et al.*, 2011). Diabetic subjects were included, without lower BMI limit, treated with lifestyle or metformin monotherapy. Participants were randomized at PHEN/ER TOP 15/92 mg once daily, PHEN/ER TOP 7.5/46 mg once daily or placebo, in addition to standardized counseling on diet (−500 kcal/day) and lifestyle modifications. After 56 weeks both PHEN/ER TOP groups showed significantly greater weight loss (−12.4% for PHEN/ER TOP 15/92, −9.6% for PHEN/ER TOP 7.5/46 mg, −1.6% or placebo) with a significantly higher proportion of patients achieving ≥5% and ≥10% weight reduction compared to placebo (70% in PHEN/ER TOP 15/92 mg, 62% in PHEN/ER TOP 7.5/46 mg, 21% in placebo group); a subanalysis restricted to diabetic patients showed even greater results on weight loss (Garvey *et al.*, 2014). Results on BW after 56 weeks, were confirmed in the EQUIP study, conducted on higher-BMI subjects (BMI ≥35 kg/m^2, mean BMI 42.0 kg/m^2), in whom the administration of PHEN/ER TOP 15/92 mg once daily induced significantly higher body reduction compared to PHEN/ER TOP 3.75/23 mg once daily and placebo (PHEN/ER TOP 15/92 > PHEN/ER TOP 3.75/23 mg > placebo). Furthermore, results were independent by baseline BMI (Allison *et al.*, 2012). In the CONQUER trial, the superiority of PHEN/ER TOP versus placebo concerned also significant improvements in cardiovascular risk factors (blood pressure/hypertensive treatments, waist circumference, lipid profile/lipid lowering medications, blood glucose, inflammatory biomarkers) (Gadde *et al.*, 2011); this difference was confirmed in

higher-BMI subjects between PHEN/ER TOP 15/92 mg and placebo, but not always between PHEN/ER TOP 3.75/23 mg and placebo (Allison *et al.*, 2012). Both the RCTs reported a slight improve in heart rate in PHEN/ER TOP patients compared to placebo (Gadde *et al.*, 2011; Garvey *et al.*, 2014; Allison *et al.*, 2012; Vorsanger *et al.*, 2016). Phentermine/ER Topiramate has been proved to improve glycemic control in diabetic patients: after 56 weeks, patients taking PHEN/ER TOP 15/92 mg daily exhibited greater reduction in HbA1c and greater reduction in antidiabetic medications compared to placebo; furthermore a greater portion of PHEN/ER TOP-treated subjects achieved HbA1c targets (Gadde *et al.*, 2011; Allison *et al.*, 2012; Davidson *et al.*, 2013). Concerning OSA, a RCT was conducted on 45 subjects with BMI 30–40 kg/m^2 diagnosed with moderate/severe OSA but unable or unwilling to comply with CPAP treatment, treated with PHEN/ER TOP or placebo: PHEN/ER TOP patients experienced a significant improve in apnea/hypopnea index (AHI) already at 8 weeks and further at 28 weeks, as well as in additional OSA parameters and self-assessed quality of sleep (Winslow *et al.*, 2012). Durability of Phentermine/ER Topiramate was analyzed in the SEQUEL study (an extension of the Conquer trial): after 108 weeks, the significant difference between placebo and both PHEN/ER TOP groups with respect to weight loss and cardiovascular risk factors was sustained (− 1.8% for placebo, − 9.3% for 7.5/46 PHEN/ER TOP, and − 10.5% for 15/92 mg PHEN/ER TOP). A higher percentage of subjects treated with both doses of PHEN/ER TOP experienced weight losses of 5%, 10%, 15%, and 20% when compared with placebo-treated subjects. As seen at 56 weeks, PHEN/ER TOP resulted effective in all BMI categories; interestingly, both the 7.5/46 and 15/92 mg doses were statistically similar in their effectiveness in the lower baseline BMI, whereas, for BMI 40–45 kg/m^2, the 15/92 mg group showed a significantly greater weight loss than the 7.5/46 mg group (Garvey *et al.*, 2012). Among cardiovascular risk factors: blood pressure reduction was similar between treatment arms, but PHEN/ER TOP experienced a reduction in hypertensive medications; fasting glucose, insulin concentrations, incidence of diabetes and diabetes progression rate were significantly reduced in PHEN/ER TOP groups; also, triglycerides decreased and HDL cholesterol increased more in PHEN/ER TOP groups than placebo. Phentermine/ER Topiramate is currently approved at doses of 3.75/23, 7.5/46, and 15 mg/92 mg (recommended dose) for long-term overweight/obesity management. The side effects are due to both components of the drug. The most frequently reported are dry mouth, paraesthesia (sometimes causing drug discontinuation), constipation, upper respiratory tract infection/nasopharyngitis, dysgeusia (at higher doses), depression (Gadde *et al.*, 2011; Garvey *et al.*, 2014, 2012; Allison *et al.*, 2012). Phentermine/ER Topiramate is contraindicated in pregnancy (for fetal toxicity by topiramate) (Hernández-Díaz *et al.*, 2017), glaucoma, hyperthyroidism, during or within 14 days of taking monoamine oxidase inhibitors. Heart rate, mood disorders, and suicidal ideation must be monitored during treatment (Food and Drug Administration (FDA), 2017). With regards to cardiovascular safety, the RCTs conducted so far have shown a reduction in cardiovascular risk factors with no cardiovascular events reported in treated patients. Given the previous reports of palpitation, blood pressure elevation, and cardiac injuries due to phentermine (and its consequent limitation), data on blood pressure after Phentermine/ER Topiramate indicate that the metabolic benefit overcomes the sympathomimetic effect (Vorsanger *et al.*, 2016).

Naltrexone/Bupropion

In ARC of hypothalamus, POMC neurons release anorexigenic αMSH and β-endorphin. The latter inhibits POMC neurons and decrease αMSH concentration in a negative feedback loop, thus ensuring energy homeostasis (RBS, 2017). The μ-opioid receptor subtype, bind by β-endorphin, has a key role in this mechanism, but is known to modulate hedonic control of eating too, being expressed in critical area such as nucleus accumbens and other brain regions part of the reward system (RBS, 2017). Naltrexone is a high affinity μ-opioid receptor antagonist, also used to treat alcoholism and opioid addiction. Naltrexone increases POMC neurons activity blocking β-endorphin negative feedback on the μ-receptor. Moreover, it decreases dopamine levels in the nucleus accumbens, leading to a reduction in food seeking, binge eating and preference and consumption of highly palatable, sugary and fat foods (Giuliano *et al.*, 2012). Bupropion is an antidepressant drug inhibitor of dopamine and norepinephrine reuptake (Stahl *et al.*, 2004). Through this mechanism, it stimulates POMC neurons to release αMSH, thus inducing an anorexigenic effect (RBS, 2017; Stahl *et al.*, 2004). *CONTRAVE*® (Orexigen Therapeutics, USA) is an extended release formula containing naltrexone (8 mg) and bupropion (90 mg). The drug is orally administered with an initial 4-week titration to achieve the total dose of 32 mg of naltrexone and 360 mg of bupropion (Caixas *et al.*, 2014; Anon, 2010). It should not be used in patients with uncontrolled hypertension, seizure disorders, a history of drug addiction, bulimia, anorexia nervosa (Srivastava and Apovian, 2017; Gadde *et al.*, 2017), in individuals receiving chronic treatment with opioids and in those who abruptly stopped alcohol, benzodiazepines, barbiturates, and antiepileptic drugs (Srivastava and Apovian, 2017; Gadde *et al.*, 2017). The efficacy and safety of Contrave® were established by the Contrave Obesity Research Program (COR), which included four 56-week, randomized, double-blind, placebo-controlled, phase III trials: COR-I, COR-II, COR-Diabetes, and COR-behavioral modification (BMOD) (Greenway *et al.*, 2010; Apovian *et al.*, 2013; Wadden *et al.*, 2011; Hollander *et al.*, 2013). The COR-I study involved patients with either a BMI of 30–45 or a BMI of 27 kg/m^2 and comorbidities such as controlled hypertension, dyslipidemia, or both. They were randomly assigned to be treated with either 32 mg naltrexone + 360 mg bupropion per day (NB32), 16 mg naltrexone + 360 mg bupropion per day (NB16) or placebo. Weight loss in the NB groups began early (by the 4th week) and continued for the whole trial duration. The mean percentage of weight loss was 6.1%, 5%, and 1.3% in the three groups respectively and more patients in the NB groups compared to placebo group lost 5% or more, 10% or more, and 15% or more of their BW. Better results regarding insulin resistance, fasting insulin, and glucose concentration were observed in the NB groups than in placebo, as well as a better lipid metabolism control. Participants assigned to the NB groups also showed improvements in their quality of life, as stated by higher

scores in Impact of Weight on Quality of Life-Lite (IWQOL-Lite) Questionnaire, and in their feeding behavior and relationship with food, estimated by the Control of Eating Questionnaire (COEQ) (Greenway *et al.*, 2010). The COR-II study, which had the same design and confronted patients receiving 32 mg naltrexone + 360 mg bupropion per day or placebo, confirmed the previous results, showing a significantly greater weight loss and superior improvement in glucose and lipid metabolism in treated subjects than in placebo group (Apovian *et al.*, 2013). A comparable favorable outcome was demonstrated in COR-BMOD, which added an intense group lifestyle modification program to the administered drug or placebo. Again, NB receiving patients exhibited a better weight loss and a better improvement of cardio-metabolic risk parameters than placebo treated subjects (Wadden *et al.*, 2011). The COR-Diabetes trial finally examined the safety and efficacy of the two molecules in obese patients with Type 2 Diabetes Mellitus. Confirming former results, participants treated with NB lost more weight than placebo-treated individuals, and more patients in the NB group achieved $\geq 5\%$ and $\geq 10\%$ reduction in BW. In addition, NB group showed a greater improvement in HbA1c and a higher percentage of patients achieving an HbA1c $< 7\%$ and $< 6.5\%$, in a weight loss-correlated manner. Furthermore, higher baseline HbA1c values demonstrated the greater reduction, while fasting glucose and HOMA-IR did not significantly differ between the two groups (Hollander *et al.*, 2013). The main adverse events in the NB groups were gastrointestinal, above all nausea, which tended to disappear once the total dose was reached. Headache and constipation were also reported (Greenway *et al.*, 2010; Apovian *et al.*, 2013; Wadden *et al.*, 2011; Hollander *et al.*, 2013). Neurological and psychiatric adverse events did not differ between NB and placebo groups (Gadde and Pritham Raj, 2017). Despite that, as a single case of seizure and a case of passive suicide ideation were reported, a careful screening for depression, suicidal thoughts, and personal history of seizures is necessary to exclude these conditions before drug administration (Gadde and Pritham Raj, 2017). NB may raise heart rate and blood pressure and should not be used in patients with uncontrolled hypertension. The clinical significance of the slight increase in these parameters is unclear, especially for patients with heart-related and cerebrovascular disease, since patients at high risk for cardiovascular diseases were excluded from the clinical trials above mentioned. FDA mandated a placebo-controlled, non-inferiority cardiovascular outcomes trial, however because of the unanticipated early termination of the trial, it was not possible to assess non inferiority of NB versus placebo. Accordingly, cardiovascular safety of this treatment remained uncertain and will require evaluation in a new adequately powered outcome trial (Nissen *et al.*, 2016).

Liraglutide

Liraglutide is an acylated glucagon-like peptide-1 (GLP-1) analog with a 97% homology to endogenous human GLP-1. GLP-1 is secreted by L-cells in the gut and by neurons in the nucleus tractus solitarius (Merchenthaler *et al.*, 1999; Baggio and Drucker, 2007; Drucker and Nauck, 2006) and it is a physiological regulator of glucose metabolism, as it increases insulin secretion and reduces glucagon release after meals. Furthermore, it inhibits gastric emptying and promotes satiation by acting on afferent vagal fibers. The metabolic role of GLP-1 has been exploited in the treatment of type 2 diabetes, by developing GLP-1 receptor agonists resistant to degradation by dipeptidyl peptidase-4 (DPP-4). Animal and human studies have shown that GLP-1 receptor agonists produce also significant weight loss when compared to controls, due to a direct stimulation of POMC/CART neurons in the arcuate nucleus of the hypothalamus (Baggio and Drucker, 2014). There are six GLP-1 based drugs currently approved by the agencies: albiglutide, dulaglutide, exenatide, liraglutide, lixisenatide, semaglutide. Each of these is able, by a distinct modification, to extend the duration of action of GLP-1. Once-day liraglutide 3.0 mg has been tested in obese patients with or without comorbidities and according to the results has been licensed for the treatment of obesity. The SCALE Obesity and Prediabetes study, 3731 patients with BMI ≥ 30 kg/m^2 or BMI ≥ 27 kg/m^2 associated to dyslipidemia and/or hypertension, were stratified according to prediabetes status at screening and BMI at baseline (< 30 kg/m^2 or ≥ 30 kg/m^2). After 56 weeks, patients in the liraglutide group showed a significantly greater weight loss compared to placebo (-8.0% vs. -2.6%) as well as a greater proportion of patients losing at least 5%, 10%, and 15% of baseline BW (63.2%, 33.1%, and 14.4% in lira-group vs. 27.1%, 10.6%, and 3.5% in placebo group) (Pi-Sunyer *et al.*, 2015). Interestingly liraglutide appeared less effective in patients with a BMI ≥ 40 kg/m^2 (Pi-Sunyer *et al.*, 2015), but a recent post hoc analysis showed the same results (in BW loss) between patients having BMI 27–35 kg/m^2 or ≥ 35 kg/m^2 (le Roux *et al.*, 2017). Lipid profile, systolic and diastolic blood pressure improved significantly more in the liraglutide group than in placebo, as well as the prevalence of prediabetes and the development of diabetes were significantly lower in the liraglutide group than in the placebo group (Pi-Sunyer *et al.*, 2015). The superiority of liraglutide versus placebo on BW loss as well as on glucose metabolism and diabetes incidence/development has been confirmed in the 3-year extension trial (SCALE maintenance) (Davies *et al.*, 2015); furthermore, after 160 weeks, the liraglutide group showed significantly higher regression rate from prediabetes to euglycemia (Davies *et al.*, 2015). Effects on fasting lipids and cardiovascular markers were modest with the exception of high-sensitivity C-reactive protein (Davies *et al.*, 2015). Liraglutide was also associated with improvement in health-related quality of life questionnaires score (Pi-Sunyer *et al.*, 2015; Davies *et al.*, 2015). The SCALE Diabetes study involved overweight/obese diabetic patients with poor glycemic control (HbA1c range 7%–10%), treated with diet and physical exercise alone or in combination with one to three oral hypoglycemic agents (Wadden *et al.*, 2013). The study compared treatment with liraglutide 3.0 mg, 1.8 mg and placebo for 56 weeks: the results showed a mean weight loss of 60%, 47%, and 20% respectively ($P < .001$ for any liraglutide dose vs. placebo) as well as greater reduction of BMI and waist circumference for liraglutide patients. Moreover, liraglutide was associated with significantly improved glycemic control, glucagon and proinsulin levels, proinsulin-to-insulin ratio, reduction of oral hypoglycemic agents after 56 weeks compared to the placebo group. Liraglutide 3.0 mg, but not 1.8 mg, improved systolic and diastolic blood pressure, levels of total cholesterol, very low LDL cholesterol (VLDL-C), HDL-cholesterol, and triglycerides, but no

effects were observed on LDL cholesterol or free fatty acids. Liraglutide 3.0 significantly improved quality of life in a dose dependent way (Wadden *et al.*, 2013). These results support the use of liraglutide 3 mg in patients with prediabetes or diabetes who find it difficult to achieve their HbA1c goal (Golden, 2017). Moreover, liraglutide proved to be effective in improving apnea–hypopnea index and sleeping saturation parameters in obese nondiabetic patients, after 32 weeks (Blackman *et al.*, 2016). Liraglutide has been approved for treatment of overweight/obesity in 2014 by FDA and in 2015 by EMA. It is administered subcutaneously at a 3.0 mg daily dose, achieved within 5 weeks with a weekly increase of 0.6 mg to improve gastrointestinal tolerability. The most common side effects, reported as mild or moderate in 94% of cases, involve the gastrointestinal tract (nausea, diarrhea, constipation, vomiting, dyspepsia, and abdominal pain) and in all RCTs were one of the main causes for drug discontinuation. Nausea and vomiting occurred mostly within the first 4–8 weeks of treatment, worsening as the dose increased. Gallbladder-related events (cholelithiasis and cholecystitis) were reported more frequently in liraglutide patients and led to elective cholecystectomy, followed by treatment restoration (Pi-Sunyer *et al.*, 2015; Davies *et al.*, 2015; Wadden *et al.*, 2013). Few cases of pancreatitis were reported but many patients showed increases in serum levels of lipase (only a few were greater than three times the upper limit of normal range) and pancreatic amylase (not greater than three times the upper limit of normal range). Further studies have shown that the association between increased levels of these two markers and incidence of pancreatitis and pancreatic cancer was not statistically significant (Chalmer *et al.*, 2015; Funch *et al.*, 2014). Cardiovascular safety of liraglutide 3 mg is being studied. A recent post hoc analysis on cardiovascular events reported in SCALE trials, suggested a protective cardiovascular role of liraglutide 3 mg (Davies *et al.*, 2017): in the pooled analysis, 8 cardiovascular events (both fatal or nonfatal) were reported in the liraglutide group versus 10 in the placebo group, with a hazard ratio of 0.45 (95% confidence interval (CI), 0.17–1.08). The same analysis confirmed a slight but significant increase in pulse rate associated to liraglutide treatment (Davies *et al.*, 2017). These preliminary data, together with results of the LEADER study with lower doses of liraglutide (Marso *et al.*, 2016), may suggest an even greater protective role of liraglutide 3 mg on both cardiovascular profile and events. Nevertheless, further postmarketing data on a wider population are due. Recently, semaglutide, a once-weekly GLP-1 analog has been tested in a 12 week treatment trial in obese women versus placebo showing a significant reduction in ad libitum energy intake with a corresponding loss of body weight. In addition, semaglutide in the same group of women showed reduced appetite and food cravings. Obviously, more prolonged studies are needed to derive definitive conclusions about this novel compound (Blundell *et al.*, 2017).

Lorcaserin

Serotonin (5-hydroxytryptamine, 5HT) is synthesized both peripherally and centrally, and affects food intake through different pathways. Peripherally, enterochromaffin cells and mast cells produce 5HT which binds to $5HT_3$-receptors and activates ascending vagal afferent fibers, thus increasing satiety. Centrally, 5HT is produced by neurons of the raphe nuclei and by mast cells, it binds to the $5HT_{2C}$ receptors (located on POMC neurons) and the $5HT_{1B}$ receptors (located on AgRP neurons, with inhibitory effect), inducing a synergic stimulation on POMC neuron signals and, consequently, an increase in α-MSH release and hypophagia (RBS, 2017). Lorcaserin is a selective serotonin $5HT_{2C}$ receptor agonist with minimal affinity for $5HT_{2B}$ receptors (known to be responsible of valvular heart disease and fibrosis in patients treated with fenfluramine and dexfenfluramine) (Food and Drug Administration (FDA), 2017). Lorcaserin hydrochloride is available in two oral formulations: BELVIQ, containing lorcaserin 10 mg, administered orally twice daily; BELVIQ XR, an extended release tablet containing lorcaserin 20 mg, administered once daily (Blundell *et al.*, 2017). The BLOSSOM study assessed the efficacy and safety of lorcaserin administered 10 mg twice daily (BID) or 10 mg once daily (QD) versus placebo, in patients with BMI ≥ 30 kg/m^2 or 27 $\leq$BMI > 30 kg/m^2 with comorbidities (with the exception of type 2 diabetes). After 52 weeks, the results showed a significantly greater weight loss as well as greater proportion of patients losing at least 5% of baseline BW, in both lorcaserin groups compared to placebo. In the lorcaserin groups, weight loss results were dose-dependently increased. Similarly, BMI, waist circumference and body fat significantly decreased in lorcaserin-treated subjects compared to placebo. Concerning lipid profile, lorcaserin groups did not show any significant improvements other than those secondary to weight loss. Quality of life (assessed by the Impact of Weight on Quality of Life-Lite Questionnaire score) significantly improved in patients taking lorcaserin (Fidler *et al.*, 2011). When administered to patients with type 2 diabetes for 52 weeks (BLOOM-DM study), lorcaserin significantly improved HbA1c, fasting plasma glucose and insulin resistance (assessed by HOMA-IR) as compared to placebo. Moreover, lorcaserin BID and QD groups showed a higher rate of diabetic patients achieving glycemic target (HbA1c $\leq 7\%$) and a significant reduction in the overall use of oral antidiabetic agents, as compared to placebo. Lorcaserin was associated to a higher incidence of hypoglycemia (7.4% in BID group, 10.5% QD group, and 6.3% in placebo), but no severe episode has been reported (O'Neil *et al.*, 2012). The efficacy of lorcaserin has been confirmed in the 2-years extension trial (BLOOM trial): patients treated with lorcaserin 10 mg twice daily, experienced a significantly greater weight loss compared to placebo within the first year, which sustained in the second year in a significantly higher proportion. Weight loss was associated with BMI and waist circumference reduction as well as improvement in blood pressure, fasting glucose, insulin, glycated hemoglobin, total cholesterol, LDL cholesterol, triglyceride levels and reduction of high-sensitivity C-reactive and fibrinogen levels. In the second year, both groups showed a partial weight regain, associated to an increase in glycemic parameters and lipids levels. Systolic and diastolic blood pressure, at the end of the second year, decreased slightly but significantly between baseline and the end of year 2 in lorcaserin group as compared to placebo (Smith *et al.*, 2010). The most common adverse events are headache, upper respiratory infection, nausea, dizziness, dry mouth, fatigue and back pain; the incidence of side effects increases along with the dose, and has been reported among the main causes of drug discontinuation (Fidler *et al.*, 2011; O'Neil

et al., 2012; Smith *et al.*, 2010). Lorcaserin treatment was not associated to an increase in the incidence of FDA-defined valvulopathies (Fidler *et al.*, 2011; O'Neil *et al.*, 2012; Smith *et al.*, 2010); furthermore, in the BLOSSOM study, subjects with preexisting valvulopathies and treated with lorcaserin, showed a lower increase of mitral or aortic regurgitation as compared to placebo (Fidler *et al.*, 2011). The incidence of depression and suicidal ideation was not significantly affected by lorcaserin administration (Fidler *et al.*, 2011; O'Neil *et al.*, 2012; Smith *et al.*, 2010). Serotonin syndrome or neuroleptic malignant syndrome-like reactions can occur with concurrent use of two serotonergic agents, therefore selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs) and other drugs affecting the serotonergic system, should not be prescribed to patients taking lorcaserine (Kim *et al.*, 2013). Among the five agents taken into consideration, lorcaserin is the one associated with the least incidence of adverse events, whereas naltrexone-bupropion and liraglutide are the most likely to being discontinued due to adverse events (Khera *et al.*, 2016).

Conclusions

The history of pharmacotherapy for obesity has been marked by severe restrictions from the EMA and FDA regulatory committees. However, recently, at least two novel antiobesity medications are available in Europe and the United States, together with another two still marketed in the United States with some restrictions. Today, pharmacological treatment should definitively be considered part of a comprehensive strategy of disease management, helping patients maintain compliance, reducing cardiovascular risk factors, and improving quality of life. All the currently approved drugs help patients limit food intake and achieve better compliance with the diet. Antiobesity drug mode of action has not always been completely clarified, but in general we can summarize that all prescribable drugs are able to determine a reduction in food intake by limiting hunger and food craving, as well as enhancing the sense of satiety. For the future however, it is strongly recommended that patients should be carefully examined before starting any specific antiobesity drug therapy, so that this is tailored to respond to their specific needs and conditions.

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Binge-Eating, Bulimia, and Other Eating Disorders

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Abbreviations

BED	Binge eating disorder
BN	Bulimia nervosa
BSED	Bulimic-spectrum eating disorders
BWL	Behavioral weight loss treatment
CBT	Cognitive behavior therapy
CBT-BN	Bulimia-nervosa-focused cognitive behavior therapy
CBT-E	Enhance version of cognitive behavior therapy
CR	Cognitive remediation
CR-OB	Cognitive remediation for obesity
DBS	Deep brain stimulation
DBT	Dialectical behavioral therapy
DLPFC	Dorso lateral prefrontal cortex
DMPFC	Dorso medial prefrontal cortex
DSM-5	Diagnostic and statistical manual of mental disorders, fifth edition
ED	Eating disorders
EEG	Electroencephalographic measure
IGT	Iowa gambling task
IPT	Interpersonal psychotherapy
LDX	Lisdexamfetamine
NICE	National Institute of clinical excellence guidelines
SSRIs	Selective serotonin reuptake inhibitors
TDCS	Transcranial direct current stimulation
TMS	Transcranial magnetic stimulation
WHO	World Health Organization

Definition of Bulimic-Spectrum Eating Disorders

The bulimic-spectrum eating disorders (BSED) include serious psychiatric nosological entities, such as bulimia nervosa (BN) and binge eating disorder (BED), which are characterized by recurrent binge eating episodes as main symptom. An episode of binge eating is defined as excessive food consumption, in a discrete period of time, accompanied by a sense of loss of control over eating.

According to the *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-5; American Psychiatric Association, 2013), BN is defined as an eating disorders (ED) characterized by recurrent episodes of binge eating followed by compensatory behaviors, such as fasting, dieting, excessive exercise and/or purging behaviors (e.g., self-induced vomiting, misuse of laxatives, diuretics, or enemas) to prevent weight gain. In addition, individuals with BN place a great emphasis on body shape or weight that influence their self-evaluation. Likewise, BED is also characterized by recurrent episodes of binge eating, but without inappropriate compensatory behaviors. For both disorders, binge eating episodes have to be present at least once a week for 3 months, and are also accompanied by marked distress.

Due to the caloric overconsumption, BSED are strongly associated with (or lead to) obesity (Razzoli *et al.*, 2017; Villarejo *et al.*, 2012). Obesity is considered a public health problem with worldwide epidemic consequences. It is defined by the World Health Organization (WHO) as a complex and multifactorial disease that occurs when an individual's food consumption exceeds his or her energy expenditure. Therefore, obesity is characterized by abnormal and excessive fat accumulation which, in turn, may impair health and be life-threatening.

Furthermore, BSED frequently suffer from comorbid psychiatric disorders (e.g., mood, anxiety, substance use disorders). BSED and obesity have also been associated with social and psychopathological impairments (e.g., emotional distress and negative affect), as well as with increased risk for medical comorbidities such as type II diabetes, hypertension, dyslipidemia, coronary heart disease, congestive heart failure, and various types of cancer (Mitchell, 2016). Thus, BSED are associated with increased healthcare costs in addition to reduced quality of life and increased morbidity and mortality (Ágh *et al.*, 2016).

Etiology and Prevalence

The prevalence of BSED, namely BN and BED, has increased substantially throughout the past few decades, especially in women who live in developed countries. The increasing prevalence of BSED appears to be especially related to a maladaptive, population-wide exposure to excessive cultural inducements to diet as well as an increasing idealization of thinness. Social pressure to be thin and personal internalization of this unrealistic appearance ideal contribute to the development of body dissatisfaction, which leads to increased negative affect and also reduced dietary intake in order to better conform to the appearance ideal. Finally,

negative affect and elevated dietary restriction may act as risk factors increasing the odds of developing binge eating and unhealthy compensatory behaviors (Stice, 2001).

Both BN and BED are prevalent mental disorders framed in BSED. When exploring community samples, around 1% of population suffers from lifetime BN, whereas BED lifetime prevalence ranges from 1.9% to 2.8% (Kessler *et al.*, 2013; Hudson *et al.*, 2007). Nevertheless, when the lifetime occurrence of BSED is examined in clinical samples comprising individuals with obesity, the estimated rates obtained are notably higher. In patients with obesity, BN and BED lifetime prevalence reaches up to 4.9% and 9.3% respectively (Herpertz *et al.*, 2006). These results suggest an association between BSED prevalence and BMI, being BSED prevalence higher at the same time that BMI increases. Furthermore, BSED lifetime prevalence also varies depending on the gender, appearing to be higher in women (1.5%–3.5%) compared with men (0.5%–2%) (Cossrow *et al.*, 2016; Hudson *et al.*, 2007).

Given that evidence points to a strong association between BSED and BMI, in some cases obesity could be understood as a consequence of the binge eating behaviors observed in BSED. In this regard, the percentage of obesity in people who suffer BSED is higher than in nonclinical population. In this line, BED individuals display the highest percentage of obesity (87.8%) followed by BN (33.2%) (Villarejo *et al.*, 2012), probably due to the absence of compensatory behaviors after binge episodes.

Endocrine and Medical Complications

Both BN and BED are associated with a multitude of medical complications and comorbidities. These should be kept in mind as they greatly impact on the morbidity and also mortality of these patients.

In patients with BN, several complications are directly related to the purging behavior and depend on the mode and frequency of this behavior. These encompass hypokalaemia and metabolic alkalosis. Moreover, purging leads to a loss of fluid and often results in a compensatory increase in aldosterone secretion in order to maintain blood pressure in a normal range. In combination, these changes were termed pseudo-Barter syndrome (Bahia *et al.*, 2012) and may increase the risk for edema formation after ceasing of vomiting and during volume therapy (Trent *et al.*, 2013).

When related to vomiting, these changes are due to a loss in gastric acid and potassium and often associated with gastric acid reflux (Denholm and Jankowski, 2011) and subsequent dyspepsia (Santonicola *et al.*, 2012). Local acid complications encompass alterations of the vocal cords (Ferreira *et al.*, 2010) as well as perimolysis, an erosion of dentin and enamel (Uhlen *et al.*, 2014). Moreover, acute hematemesis may occur due to a Mallory-Weiss lesion. Chronic vomiting is often associated with an enlargement of the parotid glands (Coleman *et al.*, 1998), a condition with the exact pathomechanism yet to be unraveled.

When related to laxative abuse hypokalaemia is often associated with early hyperchloraemic metabolic acidosis, whereas the metabolic alkalosis develops later due to a subsequent stimulation of aldosterone secretion. Long-term laxative abuse may lead to cathartic colon syndrome (Müller-Lissner, 1996), a motility disorder recently challenged (Müller-Lissner *et al.*, 2005). Moreover, local complications of excessive laxative use encompass diarrhea, hematochezia (Xing and Soffer, 2001) and rectal prolapse (Malik *et al.*, 1997). Lastly, when local enemas are used severe hyperphosphataemia may occur due to respective ingredients of the enema (Ori *et al.*, 2012).

Hypokalaemia and metabolic alkalosis increase the risk for cardiac arrhythmias and thereby considerably contribute to the mortality of BN (Crow *et al.*, 2009). It is important to note that when syrup of ipecac is used to induce vomiting also irreversible congestive heart failure has to be considered (Ho *et al.*, 1998) as the alkaloid-containing syrup has cumulative toxicity.

Patients with BED display a higher risk to develop obesity (Kessler *et al.*, 2013) and obesity-associated diseases including hypertension, dyslipidaemia or diabetes (Hudson *et al.*, 2010). Interestingly, the risk of developing these diseases was greater than the risk solely attributable to obesity. Moreover, binge eating disorder was also associated with chronic pain disorders (Javaras *et al.*, 2008) sleep disorders (Trace *et al.*, 2012), asthma (Alonso *et al.*, 2014), gastrointestinal symptoms (Cremonini *et al.*, 2009), reduced cardiac output (Lelli *et al.*, 2015) as well as gynecological diseases such as menstrual dysfunction (Algars *et al.*, 2014), polycystic ovary syndrome (Hollinrake *et al.*, 2007), and complications during pregnancy (Linna *et al.*, 2014). Also these associations remained visible after controlling for body mass index. Therefore, more studies are needed in order to better understand the pathomechanisms underlying these associations.

Clinical and Personality Features

Eating/Weight-Related Symptoms

Body dissatisfaction is one of the main characteristics of BSED. Individuals with BSED place an excessive emphasis on body shape or weight in their self-evaluation. Body image is continuously assessed by BSED individuals taking as reference the ideal body type established by their culture. The result of their self-evaluation frequently leads to high levels of body dissatisfaction and it negatively influences their self-esteem. In this line, an overvaluation of shape and weight seems to be associated with eating pathology severity; as well as body dissatisfaction could be considered a predictor for worsening of eating symptoms and negative affect displayed by BSED individuals (Rikani *et al.*, 2013; Kornstein *et al.*, 2016).

Another essential feature of BSED is recurrent episodes of binge eating. Although these episodes are commonly defined as a large consumption of calories in a brief period of time, BSED individuals usually tend to define binge episodes depending on the type of food consumed and their mood state while consuming, not necessarily by the actual among of

calories ingested. Some types of food, especially hypercaloric foods, are considered as forbidden to those patients and, when consumed, they constitute a binge regardless of the overall amount or caloric content consumed. For instance, a large quantity of fruits and vegetables of the same caloric amount as a candy bar could not be considered as a binge because fruits and vegetables are "good" or "safe" foods (Rushing *et al.*, 2003). Despite binge episodes are not characterized by a craving for a specific nutrient, patients frequently tend to consume hypercaloric foods that they would otherwise avoid, forbidden foods for them; however, the type of food consumed during binges may vary depending on both the patient and the moment of intake. The most frequent triggers of binge eating episodes are negative affect, interpersonal stressors, dietary restraint, boredom, and negative feelings related to body and food. Additionally, binge episodes are normally planned in order to not be interrupted by any setback such as lack of the desired food at home, and they continue until the individual feels uncomfortably and painfully full. Individuals with BSED are typically ashamed of their eating problems and attempt to conceal their symptoms. For this reason, binge eating episodes generally occurs in secrecy or as inconspicuously as possible. BSED patients also avoid some situations in which they are likely to be exposed to food or will find it difficult to control their eating, such as when eating out with others. This avoidance behavior may result in a decline of social relationships (Kornstein *et al.*, 2016; NICE, 2004; American Psychiatric Association, 2013).

Subjective feelings of loss of control during binge eating episodes and marked distress over binge eating are also part of the typical symptomatology of patients with BSED. Individuals report not being able to refrain from eating or to stop eating once started, displaying an increased reward sensitivity and impulsivity in response to food-related stimuli. In most cases, binge eating episodes are characterized by a more generalized pattern of uncontrolled eating rather than a specific feeling of loss of control. Furthermore, this lack of control might not be complete; individuals can interrupt the binge immediately in the presence of certain events, for example, if someone enters in the room. In addition, the lack of control and the marked distress that these individuals suffer from during binge eating episodes appears to increase eating disorder pathology (Kornstein *et al.*, 2016; American Psychiatric Association, 2013).

In the end, another symptom only present in patients with BN is inappropriate compensatory behaviors in order to prevent weight gain. These compensatory behaviors are commonly associated with extreme feelings of guilt and shame. Nevertheless, BN patients do not suppress their compensatory behaviors by the fear to face the consequences of their binge eating such as excessive weight gain. Individuals with BN employ several methods to compensate (vomiting, fasting, exercising, etc.), collectively referred to as purge behaviors or purging. The most common compensatory behavior is vomiting. Patients usually vomit to relieve physical discomfort and reduce the fear of gaining weight after a binge eating episode. However, sometimes they vomit after eating a small amount of food in order to loss more weight. As they repeat vomiting behaviors numerous times, individuals generally learn to induce the vomit without use any method, they finally can vomit at will. Other purging behaviors include the misuse of laxatives, diuretics and enemas, as well as fasting and excessive exercise. BN patients may abuse of exercise and it could become excessive when it significantly interferes with important daily activities, when individuals exercise at inappropriate times or in unsuitable settings, or when they exercise despite injury or other medical complications. Finally, in some cases, BN patients may take thyroid hormone or, in the case of diabetic patients, they may omit or reduce insulin doses in an attempt to avoid weight gain (NICE, 2004; American Psychiatric Association, 2013) (**Table 1**).

Related-Psychopathological Symptoms and Comorbid Psychiatric Disorders

A large body of work suggests that BSED usually co-occur with other psychopathological symptoms and mental disorders; with the most common comorbid conditions being anxiety, mood and substance use disorders as well as other eating disorders (Kaye *et al.*, 2004; Hudson *et al.*, 2007; Welch *et al.*, 2016). Previous studies also report that in some occasions these symptoms (e.g., anxiety conditions) can have their onset before the development of the BSED therefore they can also be considered vulnerability factors.

Table 1 Clinical characteristics

	BSED			
Symptomatology	BN	BED	OB	HC
Binge eating episodes	+ + +	+ + +	−	−
Purging behaviors	+ + +	−	−	−
BMI	+ +	+ + +	+ + +	+
EDI-2:drive of thinness	+ + +	+ + +	+ +	+
EDI-2: body dissatisfaction	+ + +	+ + +	+ + +	+
EDI-2: bulimia	+ + +	+ +	−	−

BSED, Bulimic-spectrum eating disorders; *BN*, bulimia nervosa; *BED*, binge eating disorder; *OB*, obesity; *HC*, healthy controls; *BMI*, body mass index; *EDI-2*, eating disorder inventory-2.

Sources: Villarejo, C. *et al.* (2014). Loss of control over eating: A description of the eating disorder/obesity spectrum in women. European Eating Disorders Review 22(1), 25–31; Agüera, Z. et al. (2013). Cognitive behaviour therapy response and dropout rate across purging and nonpurging bulimia nervosa and binge eating disorder: DSM-5 implications. BMC Psychiatry 13(1), 285; Núñez-Navarro, A. et al. (2011). Differentiating purging and nonpurging bulimia nervosa and binge eating disorder. International Journal of Eating Disorders 44 (6), 488–496.

Another particular clinical concern is the elevated risk for suicide in individuals with BSED (Crow *et al.*, 2009; Franko and Keel, 2006), which is described to be high even when controlling for the presence of depressive or comorbid symptoms (Welch *et al.*, 2016).

As previously mentioned, obesity is highly associated with BSED thus it is important to explore both conditions commonalities and differences. Like BSED, obesity also co-occurs with high level of psychopathology, mainly with anxiety and mood disorders as well as stress related conditions (Erermis *et al.*, 2004). However, despite the prevalence of the specified disorders is higher in individuals with obesity than normo-weight individuals from nonpsychiatric population, when comparing patients with BSED and individuals with obesity, the latter presents fewer comorbid psychopathological symptoms (Tanofsky-Kraff *et al.*, 2004). It is also important to note that studies reveal that suicide risk and mood disorder rates are elevated regardless of the presence of comorbid obesity in BSED (Welch *et al.*, 2016).

The substantial comorbidity rates observed in BSED and obesity highlight the clinical importance of assessing and addressing the full array of symptoms that these patients present. However, more studies are required to establish the role of the described symptoms as vulnerability factors for both BSED and obesity. Additionally more controlled treatment effectiveness studies are required determine long-term effects of these conditions on secondary disorders.

Personality

Previous studies and etiologic models have highlighted the role of personality traits in the development, symptomatic expression, and maintenance of the BSED, as well as its relevance in treatment outcomes. Therefore, most studies assessing personality in these pathologies are based on the Cloninger's psychobiological model of personality, which includes seven dimensions divided into two major components of personality, temperament, and character (Cloninger *et al.*, 1993).

Temperament is defined as an inherited neuropsychological mechanism, relatively stable throughout life, and mediated by neurotransmitter functioning in the central nervous system. It is identified by four dimensions named: novelty seeking, harm avoidance, reward dependence and persistence. Novelty seeking is associated with exploratory activity and impulsive decision making. High levels of novelty seeking have been related to impulsive, excitable, dramatic, and intolerant of routine individuals. Thus, it is also related to low dopaminergic activity. Harm avoidance refers the tendency to avoid potentially threatening situations. It is related to high serotonergic activity, and is characteristic of pessimistic, fearful, insecure, frustration-prone and overly concerned individuals. Reward dependence has been associated with social approval, seeking social and emotional support from others, and emotional expression. It has been suggested that is influenced by the noradrenergic system. Finally, persistence is defined as perseverance in spite of fatigue or frustration.

Character includes those personality traits acquired through experience, related to learning, and the environment. The three character dimensions are: self-directedness, cooperativeness, and self-transcendence. Self-directedness is described as the ability of planning, control, make decisions, and adapt the behavior in accordance with goals and values. It is conceptually related to locus of control. Cooperativeness is associated with tolerance, empathy, altruism and compassion as opposed to intolerance, insensitivity or selfishness. Self-transcendence is a personality trait associated with experiencing spiritual ideas, acceptance, identification or spiritual union with nature and its origin.

The temperament and character dimensions present different combinations, being responsible of the functional organization that underlies the personality of the individuals. The personality dimensions are influenced by interaction between genetic and environmental variables, so personality develops as a complex adaptive system.

Notably, BSED patients have been frequently associated with specific personality traits such as high levels of both novelty seeking/impulsiveness and harm avoidance, low levels of self-directedness, and lack of persistence (Farstad *et al.*, 2016; Villarejo *et al.*, 2014). A recent systematic review consolidates the evidence that patients with binge eating symptoms represent a distinct phenotype within the obesity spectrum that is characterized by increased impulsivity (Giel *et al.*, 2017). This personality profile characterized by high both impulsivity and harm avoidance (correlated with neuroticism) has been conceptualized as *negative urgency*, defined as the tendency to engage in impulsive and risky behaviors when experiencing strong negative emotions or emotional distress (Racine *et al.*, 2013). Negative urgency has been significantly associated with vulnerability to stress, difficulties in problem solving and coping strategies of avoidance and, therefore, with increased lifetime prevalence of binge eating behaviors (Lee-Winn *et al.*, 2016). Likewise, low levels of self-directedness and emotion dysregulation have been also related to greater binge eating symptomatology. In addition, it has been documented that those individuals with poor self-directedness and high negative urgency are at risk for developing addictive eating patterns associated with BSED, such as food addiction (Wolz *et al.*, 2017).

Neuropsychological Features and Brain Activity

Cognitive Profiles

Cognitive functions are frequently divided into the domains of memory, attention, processing speed, language, visuospatial abilities, and executive functions. In BSED neuropsychological difficulties have specifically been identified in attention, memory, verbal function, and executive function (including inhibitory control, cognitive flexibility, decision-making, and working memory) (Lena *et al.*, 2004; Manasse *et al.*, 2015).

However, among all these cognitive difficulties, executive function impairments (with an special emphasis in decision making, response inhibition and cognitive flexibility) are the ones most broadly acknowledged for its core role in the development and maintenance of BSED (Degortes *et al.*, 2016; Lena *et al.*, 2004; Fagundo *et al.*, 2012; Smith *et al.*, 2011). Hence, current literature in BSED supports the hypothesis of an alteration on the inhibitory control-emotional regulation-executive function circuit. With this regards, different studies exploring decision making in BSED by means of the Iowa Gambling Task (IGT; a well-established measure for assessing decision making patterns) report difficulties not only in the final outcome of the task but also in the learning process when comparing it with healthy controls and general population. Additionally, response inhibition has been shown to be crucial for impulse control related to food consumption and recently linked to attentional impulsivity (Hege *et al.*, 2015). It is important to note that not only BED and BN present difficulties in response inhibition but also individuals with obesity in absence of any comorbid eating disorder. Finally, cognitive/mental flexibility has been linked to different forms of psychopathology and BSED are no exception. In this case, studies report that when the eating disorder is present together with obesity the impairment would be heightened (Mobbs *et al.*, 2011).

Consistent with this theory, it is not surprising that patients with BED or BN as well as some individuals with obesity often display cognitive problems in terms of impulsivity or regulating behavior (Schag *et al.*, 2013) which usually lead to inadequate self-control and maladaptive eating patterns. This research evidence has also contributed to the "food addiction" model which is still inconclusive but considers obesity in terms of a dysfunctional brain reward circuitry. Particularly, it has been demonstrated that individuals with obesity display similar decision making patterns to substance use disorders if assessed by the IGT (Mallorquí-Bagué *et al.*, 2016).

The cognitive impairments mentioned above have been associated with BSED and often proposed as risk and maintenance factors. However, it is essential to emphasized that in some circumstances they are also considered a consequence of the eating condition. For instance patients with obesity, in addition to their metabolic disturbances, also experience a progressive cognitive decline in certain domains. This decline could be a result of their metabolic problems or due to a direct effect of chronic inflammation in the brain which is caused by obesity in itself (Morris *et al.*, 2015; Spyridaki *et al.*, 2016; Restivo *et al.*, 2016).

Electrophysiological and Neuroimaging Techniques

Recent research has delved into neuropsychological mechanisms that could be involved in the development and maintenance of BSED. Growing evidence suggests alterations in the brain functional connectivity of patients who suffer from BSED. Several studies have employed electroencephalographic (EEG) measures to identify neuronal activity underlying attentional processing of stimuli with high temporal resolution. Results show that patients with BSED display a consistent attentional bias towards food stimuli. Specifically, BN patients show an increased visual attention to high-caloric and low-caloric food pictures during early sensory processing, which is related to attentional orienting and selection processes. Abnormal eating behaviors could be associated with this enlarged early orienting to food stimuli. On the other hand, BED patients only display an enhanced visual attention to pictures with high-caloric content during late sensory processing, which corresponds to motivated attention. These results indicate their difficulty to regulate motivated attention toward food stimuli (Wolz *et al.*, 2015). Furthermore, other results obtained in BED patients reflect an impairment of frontal control network and visual processing networks, which lead patients to be more vulnerable to food cues and lack of control with regards to over eating (Imperatori *et al.*, 2015).

Additionally, studies have also investigated neural basis of BSED employing neuroimaging techniques, which allow for identifying brain areas involved in processing of stimuli with high spatial accuracy. The evidence from neuroimaging reports structural abnormalities and neural vulnerability factors related to BSED. Findings suggest that BN patients present increased reward sensitivity to food stimuli in striatal regions; moreover, hypothalamic inputs could be overridden by top-down emotional-cognitive control regions. Both BN and BED patients show altered neural responses to food and a diminished recruitment of prefrontal cognitive control circuitry, which may contribute to the binge eating of palatable foods (Steward *et al.*, 2017). Moreover, BED patients also present corticostriatal circuitry alterations similar to those observed in individuals with substance abuse disorders; these abnormalities include altered function of prefrontal, insular, and orbitofrontal cortices and the striatum (Kessler *et al.*, 2016).

Treatment Approaches

Current treatment options for BN and BED include psychological, behavioral and pharmacological modalities. Outpatient treatment is the first-line treatment setting for these disorders (Hilbert *et al.*, 2017). The National Institute of Clinical Excellence (NICE) guideline recommends the Cognitive Behavior Therapy (CBT) as the primary first-line treatment approach for BSED. Other recommended treatment modalities include CBT guided self-help, interpersonal psychotherapy (IPT), dialectical behavioral therapy (DBT), and behavioral weight loss, as well as pharmacotherapy.

Roughly, treatment approaches for BSED should address several aspects, such as the disordered eating (reduced binge-eating), the associated psychopathology (especially depression and anxiety), the coping strategies, and the psychosocial functioning, but also the excess weight and metabolic problems for guarantying the effectiveness of the treatment (Grilo, 2017).

Psychological First-Line Treatments

NICE Guideline (Eating disorders: recognition and treatment. 23 May 2017. Retrieved from: https://www.nice.org.uk/guidance/ng69) supports the efficacy of therapist-led CBT as first-line treatment for BN and BED. The modalities of bulimia-nervosa-focused CBT (CBT-BN) and the enhance version (CBT-E) have been consistently declared as standard effective therapies. The CBT for both BN and BED adults typically consists of 16 weekly 90-min group sessions, but individual sessions are also possible if group therapy is not available or the person declines it. This therapeutic program includes psychoeducation, weekly monitoring of binge eating and compensatory behaviors, daily food intake dietaries, and identifying binge eating cues. It directly targets the normalization of eating patterns and promotes behavioral and cognitive changes in order to: reduce or remove the episodes of binge eating and purging behaviors; minimize food restriction; address the clinical characteristics and the problematic areas that underlie the disorder (e.g., body image concerns and self-esteem); promote coping styles and problem-solving strategies. In addition, it helps with relapses prevention and coping with current and future risks and triggers. For children and young people with BSED is recommended to offer the same CBT-ED treatment than for adults with the same diagnostic. As primary outcomes, the CBT model presents high short and long-term rates of remission/recovery with reductions in behavioral and cognitive ED symptoms (mainly for binge eating and purging symptomatology), but also improvements in comorbid psychopathology (Linardon and Brennan, 2017).

Evidence-based clinical guidelines and recent systematic reviews further recommend other psychological treatments, such as guided self-help treatment, interpersonal psychotherapy and dialectical behavioral therapy for BN and BED (Hilbert *et al.*, 2017). CBT self-help has also demonstrated to be efficacious (Wilson *et al.*, 2010); though overall there is less evidence for benefit. CBT guided self-help is recommended as an alternative to therapist-led CBT by most guidelines, likely for economic reasons (Hilbert *et al.*, 2017). The IPT model is considered a strong empirically-supported alternative to CBT for bulimic disorders (Linardon and Brennan, 2017). IPT is well suited for helping patients to address interpersonal difficulties which appear to be maintaining the eating disorder and therefore, facilitating its recovery (Murphy *et al.*, 2012). IPT has shown slower acting than CBT, but as effective as in the longer term (Hilbert *et al.*, 2017). Other therapeutic approaches, to a lesser extent, such as DBT (mainly focused in emotion regulation, distress tolerance and interpersonal effectiveness) has also shown promising results in reducing binge eating and associated psychopathology in BED patients, but further researches are needed to elucidate its long-term effectiveness (Iacovino *et al.*, 2012). Finally, family-based therapy was in particular recommended mostly for younger patients with BN (Hilbert *et al.*, 2017).

However, although psychological therapies have a sustained effect on bulimic symptomatology, and stopping binge eating can have effect on slowing down the course of weight gain, they fail to produce body weight loss in these patients who frequently present with obesity (Grilo, 2017).

Behavioral Treatments

Behavioral therapies based on caloric reduction and promoting physical activity, namely behavioral weight loss (BWL), are recommended for BN and BED patients with overweight/obesity. BWL is widely used to treat obesity. However, although BWL appears to be useful for weight loss, it is less effective than CBT or IPT in maintaining binge eating reduction during follow-ups (Wilson *et al.*, 2010).

Furthermore, adjuvant treatment to usual therapy, such as mindfulness, has shown preliminary usefulness and effectiveness for improving overall health, well-being, and sustainability of outcomes (Iacovino *et al.*, 2012). In other words, both mindfulness and emotion regulation strategies might be effective to address negative urgency (a key feature involved in the development of BSED) (Racine *et al.*, 2017).

On the other hand, some studies converge on the benefits of adding exercise to CBT for ED, showing both a reduction in binge-eating symptomatology and an improvement in comorbid depression and anxiety (Vancampfort *et al.*, 2013). BSED patients often report physical complications due to comorbid obesity and the sedentary lifestyle, the majority being extremely inactive (Hrabosky *et al.*, 2007; Vancampfort *et al.*, 2014b). Recent findings indicate that BED patients, when compared to obese non-BED patients and normal weight controls, are those with the greatest limitations in physical activity and self-perception. In addition, the positive effect of enhancing physical activity/exercise in these patients has been shown to be associated with improved quality of life and physical self-perception (Vancampfort *et al.*, 2014a).

Pharmacological Treatments

Pharmacotherapy cannot be indicated as the sole treatment for BSED. However, although medication is not the primary choice of treatment for BSED, is often prescribed as adjuvant to other psychological therapies. Several randomized placebo-controlled studies have demonstrated that combining CBT with some medications improves the effectiveness of treatment, especially for managing comorbid symptoms or improving treatment outcomes in certain individuals (Davis and Attia, 2017).

Antidepressant medications, especially selective serotonin reuptake inhibitors (SSRIs), are often indicated as adjuvant treatment for BSED. Particularly, fluoxetine is the most commonly prescribed drug for the treatment of BSED. Evidence-based results have shown that fluoxetine yield clinically significant results by reducing binge eating and purge episodes, even in the absence of depressive symptoms. In addition, other SSRIs have been useful in the treatment of BSED, such as citalopram, sertraline, and

fluvoxamine. Antidepressants are generally safe and well-tolerated, but they do not have the desired effect on weight loss in these patients, who are often in overweight/obesity (Davis and Attia, 2017).

The combination of CBT and topiramate (an antiepileptic medication) has also demonstrated efficacy for both BED and BN with severe symptoms, improving the efficacy of the psychotherapy, reducing binge eating and purging frequency, and producing weight changes (Appolinario and McElroy, 2004; Claudino *et al.*, 2007). However, topiramate has limited tolerability and should occasionally be withdrawn because of its adverse effects such as headache, paresthesias, dry mouth or sedation (Appolinario and McElroy, 2004).

Recently, some studies have focused on lisdexamfetamine (LDX), a stimulant of the central nervous system, as effective medication for reducing binge eating behaviors and treating comorbid symptoms (McElroy *et al.*, 2016; Bello and Yeomans, 2017). LDX has also been associated with a decreased weight in BED patients. Therefore, LDX has proved to be specially indicated for treating patients with BED. However, although LDX is generally safe and well-tolerated, it can have emergent adverse effects (e.g., dry mouth, headache or elevations in heart rate and blood pressure), so that physician should closely monitor these parameters, particularly heart rate and blood pressure, during the treatment with LDX.

On the other hand, antiobesity or weight management medications, such as Orlistat (a lipase inhibitor used to treat obesity), has been shown to be useful for achieving weight loss. However, Orlistat has revealed ineffective in reducing bulimic symptomatology (Grilo and White, 2013).

Cognitive Stimulation Interventions

Given the cognitive impairments observed in BSED, a set of new treatment techniques for improving attention, memory, language and or/executive functions have been created as to complement the effect of first line treatments (i.e., psychotherapeutic treatments and pharmacological treatments) (Treasure *et al.*, 2015). With illness (i.e., BN, BED) and obesity progression, cognitive secondary changes underlying rigid eating habits and addictive-like changes and brain abnormalities develop and in turn reinforce the illness; thus it is important to work on new treatment approaches that target these changes (**Fig. 1**).

Different cognitive stimulation interventions have been proposed for disorders that present cognitive impairments, such as cognitive remediation (CR) or the use of brain simulation (deep brain stimulation (DBS), transcranial magnetic stimulation (TMS), transcranial direct current stimulation (TDCS). However, these treatment approaches are still in their early stages when it comes to BSED and more research is needed before they can be consistently validated and established as treatment. Still, a brief summary of the current knowledge on the field of BSED and cognitive stimulation interventions is provided bellow.

Cognitive remediation

CR is a type of rehabilitation treatment that offers exercises with the aim of reducing cognitive deficits by improving attention, memory, language and executive functions. Notably, two randomized controlled trials report significant improvements with a version of CR for obesity (CR-OB) that specially addresses executive function impairments (Raman *et al.*, 2014; Smith and Whittingham, 2017). Thus, CR-OB has been suggested as a good therapeutic approach for enhanced executive function and weight loss in obese adults. Finally, although CR also has the potential to be a good approach to complement first line treatments in BN and BED, more studies are needed before it can robustly be endorsed.

Brain stimulation (internal and external)

Deep brain stimulation (DBS; a neurosurgical technique to target dysfunctional brain circuits using electrical impulses through the implantation of a brain pacemaker into the cortex), repetitive transcranial magnetic stimulation (rTMS; via the generation of an electromagnetic field from a coil it generates magnetic stimulation of the cerebral cortex) and transcranial direct current stimulation

Fig. 1 Cognitive stimulation interventions.

(tDCS; via the placement of electrodes onto the scalp, applies low current to a target area of the cortex in order to modulate neural excitability) have emerged as a valid neuromodulation form with reversible means of altering brain circuitry in different mental health conditions. In eating disorders the target circuits involve reward, inhibition and mood regulation (Treasure *et al.*, 2015).

With regards to rTMS, promising findings have been found when targeting the dorso medial prefrontal cortex (DMPFC) in patients with treatment resistant BN (Downar *et al.*, 2012) and some other studies point toward lowers cue-induced food craving after high-frequency rTMS of the left dorso lateral prefrontal cortex (DLPFC) in people with a bulimic eating disorder and may reduce binge eating (Van den Eynde *et al.*, 2010). Some significant results are also observed with patients with no eating disorder but food craving (Lowe *et al.*, 2017). Unfortunately, research in BSED and tDCS is still lucking evidence to report conclusive results. Finally despite noninvasive techniques (i.e., rTMS, tDCS) need further investigation before they can be considered for clinical practice, there is sufficient available data suggesting that DBS may become an acceptable therapy for patients with treatment-resistant obesity (Göbel *et al.*, 2017) as well as promising results for neuromodulation and BN and BED (Lutter, 2017).

See also: Appetite and Weight

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Lipodystrophy

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Introduction

Lipodystrophy is defined as a clinical entity characterized by an abnormal distribution of adipose tissue in the body. The use of the term lipodystrophy in clinics is associated with alterations such as total or partial lipoatrophy, although in some patients lipohypertrophy occurs at some anatomical sites in association with lipoatrophy at other sites. Obesity or lipomatosis, which involve altered distribution of hypertrophied adipose tissue only, are not included in the term "lipodystrophy" as commonly used in clinics. Apart from human immunodeficiency virus (HIV)-associated lipodystrophy, lipodystrophies occur very infrequently. Because of its rarity, the prevalence of these syndromes is very difficult to determine; a recent study (Chiquette *et al.*, 2017), based on the evaluation of several electronic databases of medical records and the review of Literature published from 1946 to 2012 in Europe, has estimated that the prevalence of the main lipodystrophy subtypes is approximately 1.3–4.7 cases/million, with partial lipodystrophies occurring more frequently (2.84 cases/million) than generalized lipodystrophies (less than 1 case/million). Lipodystrophies are classified as congenital (mostly monogenic diseases) and acquired, although ongoing research tends to identify sometimes a genetic component in lipodystrophies originally classified as acquired forms. **Table 1** shows the classifications of lipodystrophies (modified from Brown *et al.*, 2016).

Congenital Lipodystrophies

Since 1996, more than 15 genes have been found to be related to different subtypes of lipodystrophies. The most widely reported are those causing type 1 and type 2 congenital generalized lipodystrophy (CGL) and type 2 familial partial lipodystrophy (FPLD), with approximately 500 cases reported in the literature, while the other subtypes are rarer.

CGL (Berardinelli–Seip Syndrome)

CGL (**Fig. 1**) is a recessive entity characterized by a near-complete lack of fat, starting at birth or childhood depending on the causative gene (*AGPAT2*, *BSCL2*, *CAV1*, or *PTRF*) (Agarwal et al., 2003a; Garg, 2004). These children are born with a triangular and coarse face. During the first years of life, the musculature is very well-defined, which later appears as muscular hypertrophy, mainly affecting the calves. Additionally, during the first few years, hypertrichosis frequently observed, which can affect the whole body. Severe acanthosis nigricans, achocordons, acromegaloid features, and flebomegaly are common characteristics, as are a voracious appetite and accelerated growth. At onset, patients present hepatomegaly with liver steatosis, which over time can evolve into cirrhosis, and sometimes to splenomegaly and lymphadenopathy. Most patients have umbilical hernia or umbilical prominence. The practical absence of leptin leads to a voracious appetite. The growth rate is accelerated, although the final size is expected for parental stature, and these individuals exhibit a higher bone age. Basal energy expenditure is increased. Patients also have hypertriglyceridemia, which can lead to episodes of acute pancreatitis early in life. As a result of extreme insulin resistance, nonketotic diabetes mellitus may appear at puberty or later and is often very difficult to control. Hyperinsulinemia, hypoleptinemia, and hypoadiponectinemia are common laboratory findings. High-density lipoprotein-cholesterol (HDLc) concentrations are typically low. Hepatomegaly, which results from liver steatosis, leads to abdominal distention, and increased liver enzymes.

Women may present with clitoromegaly, polycystic ovarian syndrome (PCOS), hirsutism, and menstrual disorders, being gestation exceptional. Sexual function in men is normal and they are fertile, although some cases of hypogonadism have been reported (Jiang *et al.*, 2014). In some subtypes, fat is also absence from the bone marrow. The marked absence of fat, acromegaloid traits, and acanthosis nigricans cause significant social stigmatization (Garg, 2011; Rochford, 2010; Vantyghem *et al.*, 2012).

According to the causative gene, there are specific conditions which can be useful for diagnosis. The lack of fat is more severe in type 2 CGL, which also affects mechanical adipose tissue (Simha and Garg, 2003). Mental retardation has been associated with type 2 CGL (Van Maldergem *et al.*, 2002), and a variant associated with the *BSCL2* gene causes a lethal neurodegenerative condition in the second childhood has been reported (Guillén-Navarro *et al.*, 2013). Twenty-five percent of type 1 and type 2 CLG presents hypertrophic cardiomyopathy. Lytic cystic lesions in appendicular bones have been associated with type 1 CGL (Fleckenstein *et al.*, 1992), although the risk of fractures is not increased, while myopathy, skeletal abnormalities, cardiac arrhythmia, and esophageal dysmotility are the hallmarks of type 4 CGL (Rajab *et al.*, 2010). Without adequate treatment, the life span of these patients is reduced, and death is typically related to diabetes complications or hepatic cirrhosis.

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Table 1 Classification of lipodystrophies

Congenital
- Generalized
  - Type 1 congenital generalized lipodystrophy (*AGPAT2*)
  - Type 2 congenital generalized lipodystrophy (*BSCL2*)
  - Type 3 congenital generalized lipodystrophy (*CAV1*)
  - Type 4 congenital generalized lipodystrophy (*PTRF*)
  - *PPARG*-associated congenital generalized lipodystrophy
  - Leprechaunism (Donoue syndrome)
- Partial
  - Type 1 familial partial lipodystrophy (unknown genes)
  - Type 2 familial partial lipodystrophy (*LMNA*)
  - Type 3 familial partial lipodystrophy (*PPARG*)
  - Type 4 familial partial lipodystrophy (*PLIN1*)
  - Type 5 familial partial lipodystrophy (*CIDEC*)
  - Type 6 familial partial lipodystrophy (*LIPE*)
  - *AKT2*-linked lipodystrophy
  - Partial lipodystrophy, congenital cataracts, and neurodegeneration syndrome (*CAV1*)
- Systemic
  - Progeria syndromes
    - Hutchinson–Gilford progeria syndrome (*LMNA*)
    - Nestor–Guillermo progeria syndrome (*BANF1*)
    - Type A mandibuloacral dysplasia (*LMNA*)
    - Type B mandibuloacral dysplasia (*ZMPSTE24*)
    - MDPL syndrome (*POLD1)*
    - Werner syndrome (*RECQL2*)
    - Atypical Werner syndrome and Atypical Progeroid syndrome (*LMNA*)
    - Bloom syndrome (*RECQL3*)
    - *PCYT1A* lipodystrophy
    - SHORT syndrome (*PIK3R1*)
    - Marfan syndrome with neonatal progeroid syndrome-like lipodystrophy (*FBN1*)
    - Cockayne syndrome (*ERCC6, ERCC8*)
    - Neonatal progeroid syndrome
    - Keppen–Lubinsky syndrome (*KCNJ6*)
    - Ruijs–Aalfs syndrome (*SPRTN*)
    - *CAV1*-associated neonatal onset lipodystrophy syndrome
    - Neonatal progeroid syndrome
  - Autoinflammatory syndromes
    - Nakajo–Nishimura syndrome (*PSMB8*)
    - JMP syndrome (*PSMB8*)
    - CANDLE syndrome (*PSMB8*)

Acquired
- Generalized
  - Acquired generalized lipodystrophy (Lawrence syndrome)
- Partial
  - Acquired partial lipodystrophy (Barraquer–Simons syndrome)
  - HIV-associated lipodystrophy
  - Lipodystrophy associated with total body radiation and hematopoietic stem cell transplantation
- Localized
  - Caused by drug injections
  - Lipodystrophy semicircularis
  - Centrifugal lipodystrophy
  - Panniculitis-associated lipodystrophy

Modified from Brown, R. J., Araujo-Vilar, D., Cheung, P. T., et al. (2016). The diagnosis and management of lipodystrophy syndromes: a multi-society practice guideline. Journal of Clinical Endocrinology and Metabolism 101, 4500–4511.

The pathogenetic mechanisms leading to adipose tissue loss are not well-known. In cases with mutations in *AGPAT2*, it is thought neutral lipids cannot be stored in the adipocyte lipid vacuole, as this gene encodes 1-acylglycerol-3-phosfate *O*-acyl-transferase 2, an enzyme that catalyzes the passage of lysophosphatidic acid to phosphatidic acid. The *BSCL2* gene encodes for seipin, an endoplasmic reticulum resident protein believed to play an important role in the formation of lipid droplets, in adipogenesis, and in the synthesis of membrane phospholipids (Cartwright and Goodman, 2012; Salo *et al.*, 2016; Wang

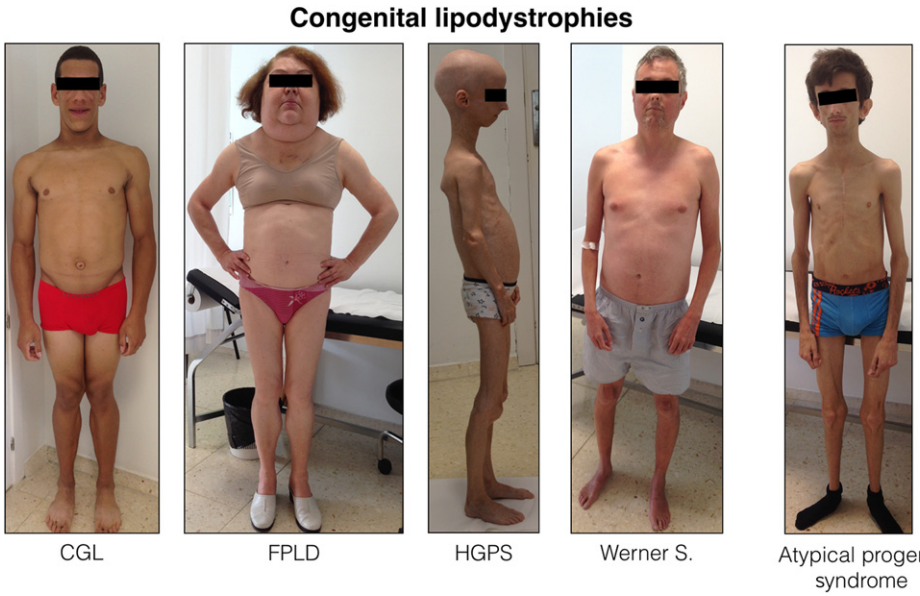

Fig. 1 Examples of congenital lipodystrophies. CGL: Congenital generalized lipodystrophy. Male, 16 years old, shown a generalized absence of body fat from birth, herculean aspect, acromegaloid facies, acanthosis nigricans, and umbilical protrusion. FPLD: A 55-year-old female with familial partial lipodystrophy due to mutation in *LMNA*, showing loss of fat in limbs and hips, and an accumulation of adipose tissue on face and neck. HGPS: A 16-year-old female with the classic phenotype of Hutchinson-Gilford progeria. Werner S: A 36-year-old male with Werner syndrome due to a mutation in *RECQL2*, with a short stature, aging appearance, early graying, and loss of adipose tissue in extremities. Atypical Progeria Syndrome: A 16-year-old male with a de novo mutation in *LMNA*. He shows short stature, aged facies, generalized lipoatrophy, and articular contractures. This patient had severe dilated cardiomyopathy requiring cardiac transplantation at 12 years of age.

et al., 2016). Both CAV1 and PTRF encode proteins resident in the caveola of the plasma membrane. Caveolin 1 binds to fatty acids and moves them to lipid droplets, which are necessary for the transport of triglycerides into adipocytes. PTRF, also known as cavin, is involved in the biogenesis of caveolae and regulates the expression of caveolin 1 and 3. Caveolas are formed from the lipid rafts of the cell membrane and consist of phospholipids, glycosphingolipids, and caveolin-1. Endocytosis of the caveolae forms caveolin vesicles that can fuse with the lipid droplets to translocate fatty acids to them (Garg, 2011; Rochford, 2010).

Familial Partial Lipodystrophy

This disorder was described by Dunnigan et al. (1974), and is a group of Mendelian disorders characterized by a loss of subcutaneous adipose tissue, affecting the limbs, buttocks, and hips (Garg, 2004). The loss of subcutaneous fat in the abdomen is variable. In general, fat accumulates on the face, buttocks, underarms, and labia majora, while visceral fat is preserved. The phenotype appears characteristically in women at the onset of puberty, while in men, because of the characteristic distribution of adipose tissue, it may go unnoticed for many years. Patients present muscular hypertrophy, which is generally more pronounced in calves, and phlebomegaly. Insulin resistance appears early (Araújo-Vilar *et al.*, 2003), leading to the onset of acanthosis nigricans. Leptin levels are relatively low, but not undetectable. These patients have hypertriglyceridemia with low HDLc and a higher risk of developing insulin-resistant diabetes mellitus. Hepatomegaly and high liver enzymes are also common in relation to hepatic steatosis or nonalcoholic steatohepatitis. Both metabolic and cardiovascular complications (ischemic heart disease and stroke) occur during adulthood, and are more common in women than in men (Hegele, 2001). The "classical" phenotype is characteristically associated with mutations in codon 482 of the *LMNA* gene, in which lipodystrophy is more severe and other clinical manifestations are more obvious. In other cases with mutations in other codons of the *LMNA* gene or in other genes (vide infra), lipodystrophy is less obvious and is referred to as "atypical forms" (Garg *et al.*, 2001). Additionally, higher rates of PCOS, hirsutism, miscarriages and stillbirths (Vantyghem *et al.*, 2008), gestational diabetes, hypoplastic breasts, and rough hands with short fingers have been reported. The presence of encapsulated lipomas even in lipoatrophic areas (Araújo-Vilar *et al.*, 2012) and muscular pain have been reported. In some cases, episodes of acute pancreatitis and eruptive xanthomas have been reported to be associated with severe hypertriglyceridemia (Garg, 2011; Vantyghem *et al.*, 2012). The prognosis is better than for other lipodystrophies, and it is conditioned by the metabolic and cardiac complications.

To date, six loci have been identified, although in more than 50% of the cases the responsible gene is unknown. These genes are: LMNA, PPARG, AKT2, CIDEC, PLIN1, and LIPE (Brown *et al.*, 2016). Except for mutations in CIDEC and LIPE, in which the disorder is autosomal recessive, other FPLD forms follow an autosomal dominant pattern of inheritance (or co-dominant in the case of *LMNA* mutations).

Type 1 FPLD

Notably, the type 1 FPLD or Köbberling variety, the most common subtype, has not been associated with mutations in any gene to date (Guillín-Amarelle *et al.*, 2016; Lotta *et al.*, 2017). Although some pedigrees shown a clear autosomal dominant pattern of inheritance, the disease is likely oligogenic or polygenic (Lotta *et al.*, 2017). This subtype is characterized by a loss of adipose tissue that mainly affects the buttocks and extremities, with characteristic accumulation of both subcutaneous and visceral fat in the abdomen (Guillín-Amarelle *et al.*, 2016). It is important to note that the absence of fat in the limbs and hips manifests in childhood or adolescence. Because of this particular fat distribution (typical male pattern), only women are diagnosed. They are typically obese and have a waist/hip ratio greater than 1, often present with diabetes mellitus, hypertriglyceridemia and, in some cases, acanthosis nigricans. Episodes of acute pancreatitis related to hypertriglyceridemia are not uncommon, and the prevalence of cardiovascular disease is high. Plasma levels of leptin are in line with body mass index (BMI).

Type 2 FPLD

Mutations in *LMNA* give rise to the "classical" form of type 2 FPLD (FPLD2) or Dunnigan disease (**Fig. 1**) when located at codon 482. Although mutations have been described in nearly all exons of this gene, most mutations occur in exons 8, 9, 10, and 11, which encode for the carboxy-terminal globular end of lamin A. FPLD2 is the most frequent form of genetic lipodystrophy with a molecular diagnosis and has been reported in more than 300 patients. Cardiac alterations have also been described, ranging from disorders in the conduction system to valvulopathies. Mutations in LMNA may cause other conditions such as Emery–Dreifuss muscular dystrophy, familial dilated cardiomyopathy, Charcot–Marie–Tooth disease, or atypical Werner syndrome, among others. Overlapping syndromes of lipodystrophy and some of these disorders have also been reported (Worman *et al.*, 2009).

The mechanisms by which a mutation in *LMNA* causes a loss of adipose tissue are not fully understood. It is unclear which of the multiple roles attributed to the nuclear lamina is involved in the selective alteration of adipogenesis, such as the maintenance of the nucleus form and structure, transcriptional regulation, function and positioning of nuclear pores, or organization of heterochromatin (Paulsen *et al.*, 2017). The predicted pathogenetic mechanisms include those related to a dysfunction of the transcription factors involved in adipogenesis. Some authors have proposed that, as in Hutchinson–Gilford progeria (vide infra), mutations in LMNA causing FPLD would result in an accumulation of prelamin A, the immature form of lamin A; excess of this immature lamin A may "kidnap" certain essential proteins in the adipocyte maturation process such as SREBP1c. Another proposed mechanisms are malfunction of the Wnt-beta-catenin pathway in relation to peroxisome proliferator-activated receptor-γ function. Finally, given the involvement of the nuclear lamina in the nucleus reordering process during mitosis, mutations in this gene lead to premature senescence of mesenchymal stem cells. Why adipose tissue is affected rather than other tissues remains unclear (Garg, 2011; Rochford, 2010; Vantyghem *et al.*, 2012; Worman *et al.*, 2009; Handelsman *et al.*, 2013; Hegele *et al.*, 2007; Araújo-Vilar *et al.*, 2009).

Type 3 FPLD

Approximately 30 cases of type 3 FPLD have been reported. This type is caused by mutations in the *PPARG* gene, and differs from the classical form of FPLD2 in that in the former the lipodystrophic condition is less severe and can appear at any age; however, insulin resistance with conditions such as hyperinsulinemia, diabetes mellitus, acanthosis nigricans, PCOS, and hirsutism are more severe or appear earlier, including hypertension, which is typically severe. Ischemic heart disease occurs earlier than in FPLD2. Lipodystrophy in these patients affects more to the distal regions of the limbs than proximal regions. Unlike FPLD2, this form of partial lipodystrophy does not always show accumulation of fat in the face. There may also be excess abdominal fat, both visceral and subcutaneous. As a monogenic form of metabolic syndrome, hypertriglyceridemia, low HDLc levels, and hepatic steatosis are common. In some cases, a pseudo-acromegaloid appearance has been described, as well as episodes of acute pancreatitis. Muscle enzymes may be elevated without a clear explanation. Plasma levels of leptin are in the normal-low range for BMI, while adiponectin levels are significantly low. Responsible mutations are often missense in heterozygosis, and mutations in the promoter and de novo have been described. The mechanisms responsible for lipodystrophy-related mutations in the *PPARG* gene are easier to understand, as this gene encodes a key transcriptional factor in adipogenesis. The mutated forms of the receptor function either by a "dominant negative" mechanism or by haploinsufficiency (Garg, 2011; Rochford, 2010; Vantyghem *et al.*, 2012).

Type 4 FPLD

Type 4 FPLD, which has been reported in five families (Gandotra *et al.*, 2011; Kozusko *et al.*, 2015), is a disorder due to mutations in the *PLIN1* gene that codes for perilipin 1, a component of lipid droplets. This protein is essential in the uptake and release of lipids. Affected patients present fat loss from infancy, which mainly affects the limbs and buttocks, without accumulation of fat in the neck. Compared with other forms of FPLD, lipodystrophy is more homogeneous. It is frequently associated with hypertrophy of the calves, acanthosis nigricans, insulin resistance, alterations in glucose metabolism, hypertriglyceridemia, tendency to have hypoleptinemia, hypoadiponectinemia, hepatic steatosis, and PCOS. Adipose tissue contains smaller adipocytes and shows macrophages infiltration and fibrosis. In adipocytes, lipid droplets are smaller, with less accumulation of triglycerides and increased basal lipolysis. The mutations identified in *PLIN1* give rise to an aberrant protein and its pathogenetic mechanism is associated with haploinsufficiency (Garg, 2011; Vantyghem *et al.*, 2012).

Type 5 FPLD

Type 5 FPLD is due to a missense mutation in the CIDEC gene, which encodes a member of the cell death-inducing DNA fragmentation factor-like effector family. This subtype is an autosomal recessive disorder with only one case has been described in an Ecuadorian woman of 19 years of age. She presents with insulin resistance, diabetes mellitus, acanthosis nigricans, hypertriglyceridemia with secondary pancreatitis, hypertension, hepatic steatosis, and partial lipodystrophy. Lipodystrophy was confined only to the buttocks and lower limbs, remaining unaltered the rest of the adipose depots (Rubio-Cabezas *et al.*, 2009). CIDEC encodes for a protein that is a component of lipid droplets, and its deficiency reduces fat mass, playing important roles in apoptosis, and giving rise to adipocytes with multiloculated lipid vacuoles (Garg, 2011; Vantyghem *et al.*, 2012).

Type 6 FPLD

Type 6 FPLD is a recessive disorder due to mutations in the LIPE gene, which encodes the hormone-sensitive lipase E. Mutations in this gene give rise to a late-onset FPLD. Two pedigrees have been reported so far, one of which was associated with multiple symmetric lipomatosis (Farhan *et al.*, 2014; Zolotov *et al.*, 2017). The phenotype becomes evident at up to 30 years of age with a progressive reduction of subcutaneous fat in the lower limbs and fat accumulation in the neck, abdomen, clavicular regions, axillae, labia majora, back, and area below the triceps. These patients can develop insulin-resistant diabetes, hepatic steatosis, hypertriglyceridemia, hypercholesterolemia, hyperadiponectinemia, and high levels of creatine kinase. One patient showed mild proximal muscle weakness and dystrophic changes in muscular biopsy.

To date, only a single pedigree bearing a missense mutation in the AKT2 gene has been reported to be associated with partial lipodystrophy (Tan *et al.*, 2007). These patients also have diabetes mellitus and severe insulin resistance, and the lack of AKT in adipocytes causes severe lipodystrophy in mice (Shearin *et al.*, 2016). However, because the lipodystrophic phenotype was not described in detail, no differences from other types of FPLD can be established. The AKT2 gene (murine thymoma-oncogene homolog-2) encodes a protein belonging to a subfamily of serine/threonine kinases containing SH2-like (Src homology 2-like) domains involved in glucose metabolism, postreceptor signaling of insulin and adipocyte differentiation (Garg, 2011; Rochford, 2010; Vantyghem *et al.*, 2012).

Progeria Syndromes

Hutchinson–Gilford progeria syndrome

This is the classic form of premature aging and is associated with de novo mutations in the *LMNA* gene (**Fig. 1**). The disease characteristically begins to manifest after the first year of life. Patients present growth retardation, low stature, alopecia, osteolysis, facial aging characteristics, disproportionately large head, "pinched" nose, joint stiffness, dentition abnormalities, whistle voice, osteoporosis, and generalized lipodystrophy affecting the limbs, face, and trunk, while preserving intraabdominal depots. Other features are prolonged prothrombin times, elevated platelet counts and serum phosphorus levels, low-frequency conductive hearing loss, and functional oral deficits. Cardiovascular studies have revealed diminishing vascular function with age, including elevated blood pressure, reduced vascular compliance, decreased ankle-brachial indices, and adventitial thickening. Patients typically die before age 15 years because of myocardial infarction or stroke (Merideth *et al.*, 2008). The most frequent mutation causing Hutchinson–Gilford progeria syndrome (HGPS) is a de novo C-to-T transition resulting in a silent gly-to-gly change at codon 608 within exon 11 (G608G) (De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003). This mutation was shown to activate of a cryptic splice site within exon 11 of *LMNA*, causing production of a protein product with 50 amino acids deleted near the C terminus (named as LaminΔ50 or progerin). The cellular hallmarks of HGPS include nuclear blebbing, loss of peripheral heterochromatin, defective epigenetic inheritance, altered gene expression, and senescence (Goldman *et al.*, 2004). It has been proposed that abnormal farnesylation of progerin plays a role in the cellular phenotype in HGPS cells. HGPS-induced pluripotent stem cells (iPSCs) showed the absence of progerin, and lacked the nuclear envelope and epigenetic alterations; however, upon differentiation of HGPS iPSCs, progerin and nuclear abnormalities were restored. These studies identified DNA-dependent protein kinase catalytic subunit (PRKDC) as a downstream target of progerin. The absence of nuclear PRKDC holoenzyme was correlated with premature aging (Liu *et al.*, 2011). In contrast, HGPS skin fibroblasts show a loss chromatin compartmentalization at later passages because of the loss of H3K27 trimethylation in gene-poor regions, gain of H3K27 trimethylation in gene-rich regions, and detachment of chromatin from the lamina (McCord *et al.*, 2013). Another pathogenetic mechanism involved in lipodystrophy-associated HGPS is disruption of the mechanistic target of rapamycin pathway, which leads to abnormal autophagy, altered proliferative rate, and downregulation of genes that regulate adipogenesis (Charar and Gruenbaum, 2017).

Mandibuloacral dysplasia

Mandibuloacral dysplasia (MAD) is an extremely rare autosomal recessive disorder that occurs in early childhood (2–4 years) and is characterized by multiple musculoskeletal abnormalities (progressive osteolysis of the distal phalanges and clavicles and mandibular hypoplasia, which cause retrognatia and stepped shoulders), progeroid features (cutaneous atrophy with marked superficial vasculature and mottled hyperpigmentation, thin "pinched" nose and hair loss), delayed teething and enclosure of the cranial sutures, dental crowding, joint stiffness, lipodystrophy with insulin resistance, hypertriglyceridemia, low HDLc, and impaired glucose metabolism. There are two types of lipodystrophy: A (partial loss of fat in limbs preserving that of the neck and trunk) and B (generalized). MAD may be due to mutations in *LMNA* (type A) (Novelli *et al.*, 2002) or in *ZMPSTE24* (type B) genes

(Agarwal et al., 2003b). The latter gene encodes for a zinc-metalloprotease involved in the posttranslational processing of prelamin A. A deficiency in *ZMPSTE24* will result in the accumulation of toxic farnesylated prelamin A in cells. MAD due to mutations in *LMNA* has been reported in approximately 30 patients, whereas less than a dozen of patients have been reported with mutations in *ZMPSTE24* (Garg, 2011; Vantyghem *et al.*, 2012; Worman *et al.*, 2009).

Werner syndrome

This recessive syndrome (**Fig. 1**) is due to mutations in the RECQL2 gene (Yu *et al.*, 1996), which codes for a DNA helicase. Patients present short stature, late-onset progeroid features (second childhood-adolescence), such as premature graying, cataracts, cutaneous signs of scleroderma and osteoporosis. In addition, these patients present lipodystrophy affecting the trunk, face, and extremities associated with insulin resistance and diabetes, acute voice, bird face with beaky nose, hypogonadism, limb muscle atrophy, calcification of blood vessels and premature death (3rd–4th decade) related to cardiovascular disease or cancer. Malignancy is frequent in these patients (10%), with nonepithelial cancers (osteosarcoma, soft tissue sarcoma, melanoma) more frequent than in the general population (Goto *et al.*, 1996). The characteristic features are short stature, aged appearance, thin limbs, and robust trunk (Hegele *et al.*, 2007). The mutant *RECQL2* gene triggers a sequence of premature expression of inhibitors of DNA synthesis and other genes, with resulting in inhibition of DNA synthesis and early cellular senescence (Thweatt and Goldstein, 1993).

Atypical Werner syndrome and atypical progeroid syndrome

Some missense mutations in heterozygosis and, generally de novo, in the *LMNA* gene give rise to other subtypes of premature aging different than the classic HGPS. In all cases, lipodystrophy is present, albeit to varying degrees, from generalized forms to partial forms affecting only the distal extremities of the limbs. The clinical features of these disorders in some cases coincide with other entities related to *LMNA* mutations, such as HGPS, LDPF2, MAD, Emery–Dreiffus muscular dystrophy, or familial-dilated cardiomyopathy, suggesting that more than independent clinical entities are different forms of presentation of the same disorder, modulated by unknown factors (endogenous and/or exogenous). Additionally, the same mutation can cause different clinical features.

The so-called atypical Werner syndrome shows similar clinic features as the classic form of this syndrome, although there are some differences. In general, the onset of early signs (early graying) typically occurs at earlier ages and disease progression is often more accelerated in atypical forms than in the classical form. Insulin resistance is typically present and patients may develop diabetes. Some cases associated with dyslipidemia, hepato-splenomegaly, cardiomyopathy, joint contractures, and leucomelanodermic papules have been reported; however, there is high phenotypic heterogeneity. Generally, these patients commonly present with short stature, premature graying, lipodystrophy, and aged appearance.

Atypical progeroid syndrome (APS) (**Fig. 1**) has been linked to the following mutations in *LMNA*: T10I, A57P, L59R, R133L, L140R, S143F, E145K, V169fsX176, D300N, E578V, and R644C. Although most are de novo mutations in heterozygosis, in some cases mutations were transmitted in an autosomal dominant manner affecting two generations. These cases present progeroid signs, such as short stature, beaked nose, premature graying, partial alopecia, nasal voice, and cutaneous lesions (spotted hypopigmentation and sclerodermatous changes, especially on the dorsum of the hands and feet), but not acanthosis nigricans. Other features that may appear are: mandibular hypoplasia, mild clavicular resorption, acro-osteolysis, dental crowding, and ojival palate. Some patients experience contractures affecting the elbows, wrists, knees, and ankles. Valvulopathies and dilated cardiomyopathy have also been reported in over 50% of cases. Patients with greater lipodystrophy develop diabetes mellitus with severe insulin resistance, hypertriglyceridemia, and hepatic steatosis. All women of childbearing age show poor breast development, but their menses were regular, although premature ovarian failure has been reported in some cases. Marrow fat is always preserved. Although the phenotype is heterogeneous, it can be distinguished from HGPS and MAD. In contrast to these two syndromes, APS shows no acro-osteolysis or minimal involvement of the distal phalanges, and mandibular hypoplasia is rare and mild. Alopecia is minor. The onset of clinical manifestations is later than in HGPS or MAD, and survival is greater compared to HGPS. However, it is often extremely difficult to discriminate between APS and atypical Werner syndrome (Garg *et al.*, 2009; Guillín-Amarelle *et al.*, 2015).

Nestor–Guillermo progeria syndrome

This entity is an autosomal recessive disorder caused by mutations in the *BANF1* gene; to date, only two cases have been reported (Cabanillas *et al.*, 2011). Nestor–Guillermo progeria syndrome presents numerous similarities with LMNA-associated progeria syndrome, but is considered a chronic progeria. This disorder begins to manifest from 2 years of age, with growth retardation, short stature, partial alopecia but preserving eyebrows and eyelashes, ocular protrusion, generalized lipodystrophy, severe osteolysis affecting the jaws, clavicles, ribs and phalanges, dental crowding, joint stiffness, delayed closure of fontanelles, loss of nasal bridge, aging appearance with dry and wrinkled skin, spotted pigmentation, severe scoliosis, and valvulopathies. Analytical studies revealed only vitamin D2 deficiency and severe hypoleptinemia. Unlike HGPS and MAD, these patients did not present metabolic alterations, hepatic steatosis, or atherosclerosis.

The *BANF1* gene encodes for barrier-to-autointegration factor 1 (BAF), which is involved in nuclear envelope assembly. BAF interacts with DNA and different proteins, including lamin A. Immunofluorescence studies of primary fibroblasts from these patients revealed abnormalities in nuclear morphology (herniations), which were similar to those observed in other laminopathies. Particularly, the location of lamina A was unaffected, but that of emerine was not (Cabanillas *et al.*, 2011). In conclusion,

mutations in this gene, which forms part of the nuclear membrane protein framework, cause abnormal distribution of nuclear lamina components in a manner similar to that in other progeroid disorders associated with *LMNA* mutations.

Neonatal progeroid syndrome

This disorder, also known as Wiedemann–Rautenstrauch syndrome, follows a pattern of autosomal recessive inheritance. Its molecular basis is unknown, although it has been suggested that the syndrome can be caused by disturbed RNA polymerase III subunit A (POLR3A) functioning (Paolacci *et al.*, 2017). Affected children are characterized by intrauterine growth retardation, failure to thrive, short stature, progeroid appearance with cranial deformities, hypotonia, variable mental impairment, and death in childhood (Hegele *et al.*, 2007). Lipodystrophy is almost generalized, and in some cases paradoxical accumulation of fat around the buttocks and anogenital area and on the flanks have been reported (O'Neill *et al.*, 2007; Arboleda *et al.*, 1997). Recently (Garg *et al.*, 2015), two patients with some features resembling neonatal progeroid syndrome but with longer survival were reported with mutations in the *CAV1* gene.

MPLD syndrome

MPLD is an autosomal dominant disorder due to de novo mutations in the *POLD1* gene characterized by mandibuloacral hypoplasia, deafness, progeroid manifestations, nondescent tests and male hypogonadism, metabolic abnormalities including insulin resistance and diabetes mellitus and generalized lipodystrophy (Weedon *et al.*, 2013). The disease appears in early childhood with poor growth and thin limbs caused by loss of subcutaneous adipose tissue. Sensorineural deafness occurred later. Facial features included beaked nose, prominent eyes, crowded teeth, small mouth and uvula, and long eyelashes in some cases.

PCYT1A lipodystrophy

Only two subjects have been reported to suffer this rare recessive condition (Payne *et al.*, 2014), which is due to mutation in *PCYT1A* encoding choline phosphate cytidylyltransferase-1α. Lipodystrophy is generalized and observed mostly in the limbs. Patients show insulin resistance, diabetes mellitus, hypertriglyceridemia, low HDLc, and liver steatosis during childhood. Characteristically, patients have growth failure and short stature.

SHORT syndrome

SHORT is the acronym of S = stature; H = hyperextensibility of joints or hernia (inguinal) or both; O = ocular depression; R = Rieger anomaly; T = teething delay. In this autosomal dominant syndrome, nonprogressive lipodystrophy is manifested mostly by a lack of subcutaneous fat in the face, chest, and upper extremities, relatively sparing the legs, generalized thin stature, and sometimes by local loss of fat resulting in small cutaneous pits on the elbows or buttocks (Koenig *et al.*, 2003). All patients presented with relatively short stature compared with their families. Other features are triangular face, prominent forehead, deep-set eyes, hypoplastic or thin alae nasi, small chin, and large ears. Mild midfacial hypoplasia gives the patients the impression of apparent prognathism despite micrognathia. The thin, wrinkled skin and readily visible veins intensify the progeroid impression (Koenig *et al.*, 2003). Rieger anomaly may manifest at birth with congenital glaucoma and cloudy cornea (Brodsky *et al.*, 1996) or total absence of the iris stroma. Despite speech delays in childhood, mental status appears to be normal or only slightly subnormal (Gorlin *et al.*, 1975). The disorder is caused by heterozygous mutation in the *PIK3R1* gene (Thauvin-Robinet *et al.*, 2013), which encodes for phosphoinositide-3-kinase regulatory subunit-1, a lipid kinase that phosphorylates the inositol ring of phosphatidylinositol and related compounds.

Bloom syndrome

Bloom syndrome is an autosomal recessive pediatric disorder caused by mutations in *RECQL3*, which encodes RecQ helicase (Ellis *et al.*, 1995). This syndrome is characterized by growth deficiency, telangiectasia, altered skin pigment, photo-sensitivity, hypertrichosis, polydactyly, predisposition to malignancy, and chromosomal instability. Lipodystrophy affects fat accumulation in the limbs and abdomen. Diabetes may be present.

Other progeroid syndromes

Other progeroid syndromes such as Cockayne syndrome (CS) and Keppen–Lubinsky syndrome present with severe mental retardation, seizures, and generalized lipodystrophy. CS is a recessive disease characterized by a marked failure to thrive, cachectic dwarfism, microcephaly, ataxia, mandible prognathism, and deep-set eyes with congenital cataracts, progressive pigmentary retinopathy, and sensorineural hearing loss among other features; the patients typically die in the second decade of life. CS is a genetically heterogeneous disorder, and certain types show some overlap with specific forms of xeroderma pigmentosum, although these patients show no significant increase in skin cancer. CS is caused by mutations in *ERCC8* or ERCC6 genes, which are part of the nucleotide excision repair pathway, a complex system that eliminates structural DNA lesions (Troelstra *et al.*, 1992). In Keppen–Lubinsky syndrome, the patients show poor postnatal growth, microcephaly, and aged facial appearance because of their tightly adherent facial skin, with open mouth and large prominent eyes (Masotti *et al.*, 2015).

Autoinflammatory Syndromes

This term encompasses Nakajo–Nishimura syndrome (Kitamura *et al.*, 2011), JMP syndrome (joint contractures, muscular atrophy, microcytic anemia, and panniculitis-induced lipodystrophy) (Garg *et al.*, 2010) and CANDLE (chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature) syndrome (Torrelo *et al.*, 2010). All these disorders are recessive disorders related to mutations in *PSMB8*, which encodes for proteasome subunit beta 8. Immunoproteasome-mediated proteolysis generates immunogenic epitopes presented by major histocompatibility complex class I macromolecules. Mutations in *PSMB8* may trigger auto-inflammatory responses, leading to infiltration of lymphocytes and other immune cell to adipose tissue, and consequently, adipocyte loss (Garg, 2011; Vantyghem *et al.*, 2012).

These recessive disorders begin in childhood and lipodystrophy can be generalized or partial, affecting the face and limbs. JMP is characterized by joint contractures, muscular atrophy, microcytic anemia, and panniculitis-induced lipodystrophy. Other features include intermittent fever, hypergammaglobulinemia, increased sedimentation rate, hepato-splenomegaly, and calcification of the basal ganglia. For CANDLE syndrome, five patients have been reported to present childhood onset with recurrent fevers and violaceous annular plaques on the eyelids and lips, evolving through childhood to a loss of subcutaneous fat in the face and upper limbs. The cases also had edematous purplish eyelids, hepato-splenomegaly, arthralgias, microcytic anemia, increased sedimentation rate, and calcifications in the basal ganglia. These features likely represent the initial forms of presentation of JMP syndrome (Garg, 2011).

Acquired Lipodystrophies

Acquired Generalized Lipodystrophy (Lawrence Syndrome)

This is a rare disorder (**Fig. 2**) characterized by the disappearance of adipose tissue throughout the body and is associated with diabetes mellitus, hypertriglyceridemia, and hepatomegaly (Lawrence, 1946). Unlike CGL, it does not manifest from birth but develops after childhood or adolescence. This is a rare disease that affects women more frequently than men. Fat loss occurs throughout the body, including characteristically the sole of the feet and the palm of the hands. Loss of adipose tissue is typically gradual and slow, beginning in particular parts (often face and extremities) of the body before

Fig. 2 Example of acquired generalized lipodystrophy (Lawrence syndrome). Woman at 3 and 13 years of age. Fat loss started at 6 years. There is generalized loss of fat with well-defined musculature and abdominal prominence caused by severe hepatomegaly due to hepatic steatosis.

generalizing to other parts over months or years, although sometimes the process may last only a few weeks. In addition to adipose tissue involvement, severe hypertriglyceridemia is frequent, which is sometimes responsible for episodes of pancreatitis and eruptive xanthomas. Other metabolic disorders include diabetes mellitus, insulin resistance, hyperinsulinemia, and hypoleptinemia (Misra and Garg, 2003). Reproductive capacity is preserved. Hepatomegaly, sometimes associated with cirrhosis, is a consequence of triglyceride deposition. In some patients, kidney tubular lipidosis and focal glomerular sclerosis have been reported. Etiology and pathophysiology of the disease is poorly understood; panniculitis as well as concomitant autoimmune diseases have been associated with several cases.

Acquired Partial Lipodystrophies

Barraquer–Simons syndrome

Accquired partial lipodystrophy (APL) (**Fig. 3**), also named Barraquer–Simons syndrome or cephalothoracic lipodystrophy, was described at the beginning of the 20th century by Barraquer and Simons (Misra *et al.*, 2004). Since then, approximately 200 cases have been reported. Although it is considered an acquired form of lipodystrophy, in 2006, APL was suggested to be associated with mutations in the *LMNB2* gene (Hegele *et al.*, 2006).

APL is a disorder that begins in childhood, although it may also appear later in life, sometimes after a viral infection. It affects females more frequently than males. Fat loss occurs in the head, neck, shoulders, upper limbs, thorax, and upper abdomen. On the face, loss of fat predominately affects the temples and cheeks. In the upper limbs, superficial veins are prominent and the loss of adipose tissue gives an appearance of extreme muscles. In women, after puberty, fat is deposited on the hips and thighs. Fat deposits have also been described in the breasts and scattered areas of the body. Fat in the gluteal region, bone marrow, orbits, and mediastinal region is not affected. Intermuscular, intraperitoneal, and perirenal fat is also normal. Fat loss may occur over a limited period (1–2 years), although it can also occur periodically for several years. Unlike other types of lipodystrophy, APL is rarely associated with insulin resistance, dyslipidemia, diabetes mellitus, or menstrual disorders. Patients with Barraquer–Simons syndrome tend to have low serum complement three levels, and in some cases presence of C3 nephritic factor. A characteristic feature is its association with mesangiocapillar glomerulonephritis, which affects approximately one-third of patients (Misra *et al.*, 2004).These patients typically do not present clinical evidence of renal disease or anomalies in renal function up to 10 years after the loss of adipose tissue begins.

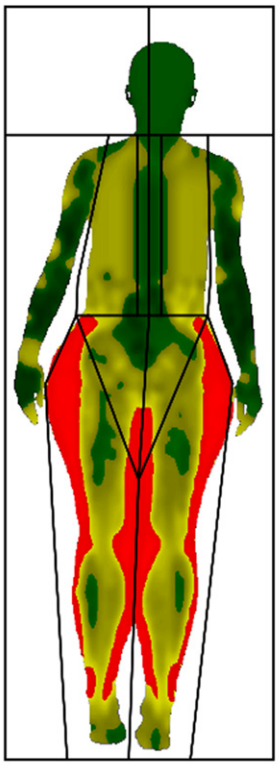

Fig. 3 Example of acquired partial lipodystrophy (Barraquer–Simons syndrome). Dual-energy X-ray absorptiometry (DEXA) image of a 38-year-old woman with cephalon-caudal acquired partial lipodystrophy. Loss of fat in the upper body and accumulation of adipose tissue in hips and lower extremities (in *red*) is observed.

Autoimmune diseases and antinuclear and anti-DNA antibodies have been detected in several patients. The morbidity and mortality of this disorder is fundamentally related to kidney involvement and autoimmune diseases with which it is often associated.

Lipodystrophy associated with HIV infection

Lipodystrophy in human immunodeficiency virus type 1 (HIV-1)-infected patients is an adipose tissue redistribution disorder commonly associated with comorbidities such as systemic metabolic alterations, dyslipidemia, and cardiovascular disease (Grinspoon and Carr, 2005; Villarroya *et al.*, 2005; Caron-Debarle *et al.*, 2010). Morphological changes and metabolic disturbances among HIV-1-infected patients were first described in the mid-1990s with the introduction of combination antiretroviral therapy (Carr and Cooper, 2000). Since then, HIV-1-associated lipodystrophy syndrome (HALS) has described as a syndrome characterized by loss and/or accumulation of fat in different adipose tissue depots. Mainly, lipoatrophy occurs in the peripheral area (loss of subcutaneous fat from the face, arms, buttocks, and legs) (**Fig. 4**), may be mixed with lipohypertrophy in the visceral area (fat accumulation on the abdominal area), and/or is located in specific lipoma areas, that is, accumulation of dorsocervical fat ("buffalo humps") (Milinkovic and Martinez, 2005).

HALS is primarily considered to be an adverse effect of a chronic inflammatory state due to HIV-1 virus infection and therapy treatment. Antiretroviral drug toxicity is not limited to a specific drug or a class of drugs, although new antiretroviral drugs are thought to be less prone to induce lipodystrophy (Srinivasa and Grinspoon, 2014). In early reports, HALS was reported to affect up to half of HIV-infected patients receiving antiretroviral therapy (Carr *et al.*, 1998). According to the UNAIDS report on the global AIDS epidemic (UNAIDS.org, 2016), the prevalence of people living with HIV globally was estimated as 36.7 million in 2016. The number of HIV-infected patients on antiretroviral therapy has been continuously increasing, reaching 17.0 million people in 2016. Thus, acquired lipodystrophy in HIV-infected patients is by far the most prevalent type of lipodystrophy (Garg, 2004). The progressive substitution of some antiretroviral drugs, mainly nucleoside analog inhibitors of viral reverse transcriptase (typically thymidine analogs such as stavudine), for other less toxic drugs has substantially reduced the development of overt lipoatrophy (i.e., facial lipoatrophy) in HIV-infected patients, and, in the short-term, the appearance of new lipodystrophy cases is less frequent when last-generation drugs are used for antiretroviral therapy.

The pathophysiology of HALS appears to be multifactorial and several key pathophysiological factors associated with HALS have been identified. These include mitochondrial dysfunction, adipocyte differentiation disturbances, high adipocyte lipolysis, proinflammatory milieu, and adipocyte apoptosis in lipoatrophic adipose tissue (Giralt *et al.*, 2011). However, adipose tissue disturbances vary by anatomical location and lipodystrophy status. Thus, visceral adipose tissue from HIV patients on antiretroviral treatment show unaltered adipogenesis and milder induction of proinflammatory markers (Gallego-Escuredo *et al.*, 2013). In contrast, acquisition of a distorted white-to-brown adipose phenotype with mitochondrial alterations but absence of local inflammatory status was reported to occur in enlarged dorsocervical subcutaneous adipose tissue ("buffalo humps") (Guallar *et al.*, 2008; Bérériat *et al.*, 2011; Torriani *et al.*, 2012). Additionally, adipose tissue dysfunction expands to involve systemic

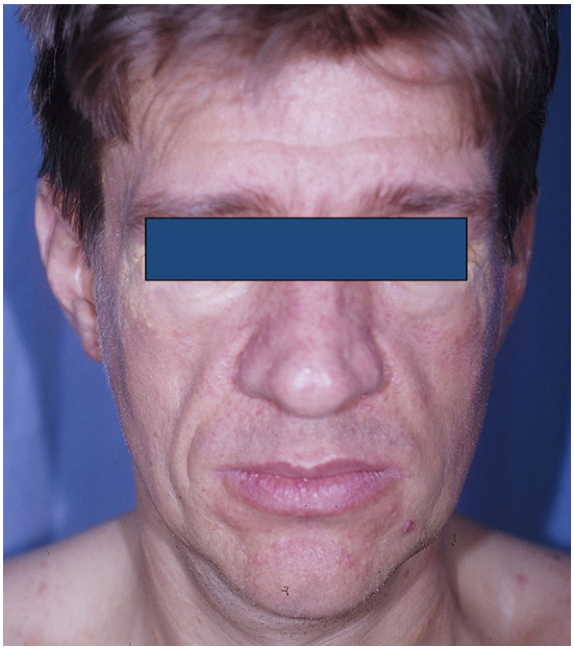

Fig. 4 Example of facial lipoatrophy in lipodystrophy associated with HIV infection. Facial lipoatrophy in a HIV-infected patient after 5 years receiving highly active antiretroviral therapy of first generation (stavudine + lamivudine + indinavir). Courtesy of Pere Domingo, MD PhD, Department of Infectious Diseases, Hospital Universitari Arnau de Vilanova, Lleida, Spain.

metabolic homeostasis through alterations in endocrine functions of adipose tissue (i.e., frequent low levels of circulating leptin and adiponectin), enhanced production of proinflammatory cytokines, and excess free fatty acid release due to lipolysis (Lagathu *et al.*, 2005; Giralt *et al.*, 2011; Paruthi *et al.*, 2013). Several research groups have identified the deleterious action of diverse antiretroviral drugs as important factors in eliciting these alterations in adipose tissue (Villarroya *et al.*, 2005; Caron-Debarle *et al.*, 2010; Erlandson and Lake, 2016). Moreover, HIV-1 infection-related events (HIV-1-encoded proteins, local inflammation in adipose tissue) contribute directly to the complex development of HALS by affecting adipocyte biology (Giralt *et al.*, 2006).

While the prevention of HALS may be addressed by choosing drugs with poor toxicity, treatment options for HALS once established remain scarce. Switching the antiretroviral drug regimen is a potential mechanism for preventing or minimizing lipodystrophy, but peripheral lipoatrophy is difficult to reverse (Lake and Currier, 2013). A few small studies of leptin replacement therapy in HIV patients with lipoatrophy demonstrated improvements in visceral adiposity and metabolic parameters, but did not observe improved peripheral lipoatrophy (Paruthi *et al.*, 2013). Reduced visceral adipose mass without loss of insulin sensitivity or exacerbation of lipoatrophy has been reported in HIV patients treated with rosiglitazone and/or growth hormone analogs (Falutz, 2011; Glesby *et al.*, 2013). Mediterranean diet may have protective effects on both HALS lipodystrophy and its metabolic complications (Tsiodras *et al.*, 2009). Finally, autologous fat transplant and subdermal fillers are recognized treatment options for facial lipoatrophy (Lake and Currier, 2013).

Lipodystrophy in adults associated with total body radiation and hematopoietic stem cell transplantation in childhood

Several reports have described a pattern of higher visceral and lower subcutaneous fat among patients who have undergone radiotherapy, including childhood cancer survivors and those who received hematopoietic stem cell transplantation (Adachi *et al.*, 2017; Wei *et al.*, 2015; Adachi *et al.*, 2013). Such lipodystrophy adds to the high risk of developing endocrinopathies and metabolic derangements as late complications after hematopoietic stem cell transplantation. Because of the lower lean mass of patients, this syndrome is referred to as "lipodystrophic and sarcopenic" (Adachi *et al.*, 2013). In these patients, lipoatrophy is remarkable in the gluteal regions and extremities, whereas fat is preserved in the cheeks, neck, and abdomen. This is associated with increased deposition of visceral fat, insulin resistance, and hypertriglyceridemia. These features resemble those of FPL2.

Localized lipodystrophies

These forms of lipodystrophy are characterized by subcutaneous fat loss in a small area of the body, in contrast with the generalized or partial (but not local) forms of lipodystrophy described earlier.

Localized lipodystrophy caused by drugs

Abnormal reactions in subcutaneous fat to drugs, mainly insulin injection in patients with diabetes, have been reported (Radermecker *et al.*, 2007). Injected insulin may cause lipohypertrophy (a lipomatous development secondary to lipogenic effect of insulin) or lipoatrophy, which is considered an adverse immunological side effect of insulin action (Ma *et al.*, 2012). Insulin injection-induced lipoatrophy typically occurs in children and young type 1 diabetic patients. Lipoatrophy is becoming progressively uncommon with the availability of newer insulin analogs, whereas lipohypertrophy remains prevalent (Hussein *et al.*, 2007). Moreover, injected pegvisomant, a growth hormone antagonist used to treat acromegaly, has been reported to cause lipohypertrophy at the abdominal wall, at the site of injections, in some patients (Bonert *et al.*, 2008). These primary localized lipoatrophies usually resolve spontaneously and are not associated with systemic disorders. Education of patients about rotating injection sites and switching of injection area appears the most beneficial to avoid local lipodystrophy as a consequence of insulin or pegvisomant injections in affected patients. Localized subcutaneous lipoatrophy is also a common adverse effect of repeated intramuscular corticosteroid injection (Hamidou *et al.*, 1991; Avilés-Izquierdo *et al.*, 2006) and also resolves spontaneously, although cosmetic treatment using poly-L-lactic acid (Brodell and Marchese Johnson, 2014) or hyaluronic acid fillers (Di Gregorio and D'Arpa, 2016) have been reported.

Semicircular lipoatrophy

Semicircular lipoatrophy (also called "lipoatrophia semicircularis") is an infrequent condition characterized by semicircular depressions of subcutaneous adipose tissue in the anterolateral thighs (Hodak *et al.*, 1990). It mainly affects office workers and is considered an occupational disease. Skin and underlying muscles remain intact. The origin of this peculiar form of lipoatrophy is unknown, but repeated mechanical microtraumatisms and localized pressure on the affected thighs, and even electromagnetic fields have been proposed (Linares-García *et al.*, 2015). Reports of semicircular atrophy involve mainly women, and it has been proposed that the anatomical adipose constitution of women's thighs predisposes that a persistent mechanical pressure originates a relative impaired circulation on a tenuous perfused tissue, and induces the development of this type of lipoatrophy (Herane *et al.*, 2007). Recent studies claim that avoiding exposure to mechanical pressure (office table edges) improves the incidence of new cases as well as the recovery of affected individuals (Reinoso-Barbero *et al.*, 2013).

Centrifugal lipodystrophy

Centrifugal lipodystrophy ("lipodystrophia centrifugalis abdominalis infantilis") is a localized form of lipodystrophy affecting young children. Most of the patients are Japanese, Korean, or Chinese, although a few cases of Caucasian patients have been reported (Imamura, 2012). Patients usually develop depressed lesions, with loss of subcutaneous adipose tissue, during the first 3–4 years of life in the groin or axilla, often surrounded by slight erythematous appearance. The depressed lesions extend

centrifugally to involve the abdominal or chest walls. In most cases, such enlargement spontaneously ceases after a few years and most patients show spontaneous improvement after the cessation of enlargement before reaching adulthood. The etiopathology of this alteration is unknown.

Panniculitis-associated lipodystrophy

This is a rare condition, also called lipoatrophic panniculitis, lipophagic lipoatrophic panniculitis, and annular lipoatrophic panniculitis of the ankles (Corredera *et al.*, 2011; Shen *et al.*, 2010), in which permanent local lipoatrophy in children follows inflammatory panniculitis (Peters and Winkelmann, 1980). In patients, circumferential bands of lipoatrophy are often observed on the arms and legs. The causes of this disease are unknown, but it has been associated with autoimmune phenomena. It has been hypothesized that inflammatory signals arise locally from panniculitis target adipocytes and promote lipoatrophy (Levy *et al.*, 2017).

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GI Hormone Development (Families and Phylogeny)☆

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Glossary

Genome The complete gene complement of an organism.
Paleontology The scientific study of life in the geologic past; it involves the analysis of plant and animal fossils.
Phylogeny The evolutionary history of a species.

Introduction

Most peptide hormones identified in humans and other mammals have relatives in all major vertebrate classes. The assignment of such relationships is based primarily on structural similarities, but the site (organ) of production and functional characteristics may be also considered. However, caution is also warranted since, for example, function of structurally conserved hormones may have changed during evolution. In this article, only peptide hormones are described.

The phylogeny of species has been established by compilation of many kinds of information, mainly from paleontology and anatomy (**Fig. 1**). But important clues have also been obtained from comparison of the sequences of key proteins, such as a number of enzymes, with structures so crucial that only small variations are allowed (Feng *et al.*, 1997). Thus, the degree of dissimilarity of these structures can aid in the evaluation of their evolutionary distances. However, the peptide hormones are rather short, so variation of their structures cannot be used to evaluate evolution. In contrast, the hormone structures can be investigated in relation to the established phylogeny of the species to evaluate the constraints, or lack thereof, that have governed the changes in the sequences. Some hormone structures appear to be so conserved that they have hardly changed at all within vertebrates (e.g., pituitary adenylate cyclase-activating polypeptide (PACAP)), whereas others have changed to such a degree that the relationship is hardly recognizable from fish to human (e.g., growth hormone-releasing hormone (GHRH)). When amino acid sequences of proteins and peptides of different species are compared, it should also be remembered that the information is obtained from living representatives of the classes. These modern forms may have changed further since their divergence as separate species several hundred million years ago. Thus, comparing sequences provide only circumstantial evidence about the situation at the time when the divergence occurred (Holmgren and Jensen, 2001; Hoyle, 1998).

Peptide hormones and neuropeptides are not restricted to vertebrates. In fact, the number of regulatory peptides in some invertebrate species may far exceed that observed in mammals and other vertebrates. Nevertheless, there are surprisingly few genuine invertebrate relatives of the known vertebrate hormones. There have been many indications that homologues to vertebrate hormones have been detected in invertebrates. However, most of these reports were based on antibody recognition. Antibodies are powerful tools for detection, visualization, and quantification, as well as many other purposes, when the epitope of the binding partner is known to be identical to the original antigen or can be assumed with high probability to be so. But assuming that there is such a degree of identity between an epitope and antigen from different species is often not justified. Thus, many apparently positive reactions may be due to cross-reactivity of the antibody with unrelated peptides or proteins that by chance show structural similarities to the hormone in question. The opposite situation has also been observed: the antibody is so specific that single amino acid substitutions that may be without any functional consequences can abolish the reaction with the antibody in part or in total. Thus, observations in one species based on antibodies raised against peptides from another species must be evaluated with caution, since both false-positive and false-negative results can occur.

During evolution, duplication of genes and exons followed by substitutions, insertions, and deletions has been an important mechanism for the creation of new peptides that acquire new functions. Two major rounds of whole genome duplications (WGD) occurred during the evolution of vertebrates, one at the beginning of the "Cambrian explosion" (approximately 500 million years ago) and one in the early Devonian, 2R, (approximately 450 million years ago), to produce up to four copies of the original genome. In fish, a third duplication, 3R, appears to have occurred in the late Devonian (approximately 300 million years ago). In the salmonid lineage even a fourth duplication occurred around 100 million years ago (Berthelot *et al.*, 2014). Many of the duplicated gene products developed into new proteins with new functions, thus giving rise to distinct families of proteins (e.g., regulatory peptides), with family members having related or even very distant functions.

☆*Change History*: May 2018. The sections "The PP-fold Family," "The Cholecystokinin/Gastrin Family," "The Tachykinin Family," "The Gastrin-Releasing-Peptide/Bombesin/Neuromedin B Family," "Motilin and Ghrelin" have been updated with references. The remaining sections have been updated to varying degrees, including new references and the PACAP/Glucagon introduction has been transformed into a table.

This article is an update of Anders H. Johnsen, GI Hormone Development (Families and Phylogeny), In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 168–172.

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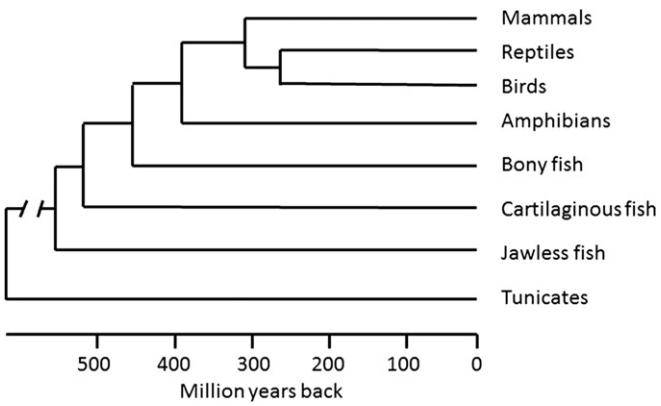

Fig. 1 Evolutionary relationship of the chordate classes. Apart from the tunicates (or urochordates), the remaining classes are vertebrate animals. The lengths of the horizontal lines indicate the period when the respective classes are assumed to have segregated.

Many of the gastrointestinal hormones can clearly be classified as belonging to families originating from a common ancestor. Others appear as singular peptides although they can be traced to most vertebrate classes. The following discussion of the relationships between specific peptides and peptide families considers mainly solid structural information and to some extent function and anatomical origin. Also, only hormones from the Chordata (including tunicates) are considered.

An interesting aspect of evolution is the coevolution of peptide ligands and their receptors (Jiang *et al.*, 2014), which however will not be further addressed in this article.

Hormone Families

The PACAP/Glucagon Superfamily

The PACAP/glucagon superfamily includes 10 structurally related hormones in human, encoded by six genes, see **Table 1**.

PACAP-like subfamily

The PACAP subfamily can be traced back to tunicates, in which PACAP and GHRH have been identified; actually, there are two different precursors in tunicates, each coding for both peptides. Most likely they segregated already during the first round of whole genome duplication. Interestingly, PACAP has been extremely well conserved from tunicate to human, whereas the structure of GHRH is highly variable and even between different mammals there is a low degree of similarity.

The glucagon family

Glucagon, GLP-1, and GLP-2 have been detected in all vertebrate classes, but not (yet) in tunicates. The three peptides are derived from a common precursor and this organization has been conserved from jawless fish to mammals. The differential expression of the three peptides is tissue specific. Glucagon is highly conserved in all vertebrates while both GLP-1 and GLP-2 appear to be considerably more variable (Irwin, 2001, 2009).

GIP, PHM/PHI, secretin, and VIP

GIP has been identified in mammals, birds and reptiles and secretin while PHM/PHI have been identified only in mammals and birds. Whether they are represented in the other vertebrate classes remains an open question. In contrast, VIP has been detected in all vertebrates except jawless fish. Furthermore, the structure of VIP is highly conserved with only minor variations allowed at nine positions of the 28-residue peptide (Cardoso *et al.*, 2010).

The PP-Fold Family

The PP-fold family of peptides derives its name from a structural characteristic of all the members: a hairpin-like fold with an extended proline helix containing three proline residues, a turn, and an α-helix with two tyrosine residues that fit in between the three proline residues. The family consists of neuropeptide Y (NPY) and peptide YY (PYY), present in all vertebrate classes, pancreatic polypeptide (PP), found only in tetrapods, and a pancreatic peptide Y (PY), found in some fish. NPY and PYY most likely originate from duplication of a common ancestor before the development of the jawless fish. NPY displays a high degree of sequence conservation, whereas PYY is more varied. The occurrence of PP only in tetrapods suggests that it is the result of yet another gene duplication that occurred before the emergence of the amphibians. The sequence variability of PP is quite high, with only 50% identity between mammals, birds, and amphibians. PY constitutes a special case and probably arose from a duplication of the PYY gene unrelated to the duplication event that generated PP (Larhammar *et al.*, 1993; Conlon, 2002).

Table 1 Members of the PACAP/glucagon superfamily

Name	*Short name*	*Gene (mammals)*
Pituitary adenylate cyclase-activating polypeptide	PACAP	A
PACAP-related peptide	PRP	A
Glucagon	–	B
Glucagon-like peptide-1	GLP-1	B
Glucagon-like peptide-2	GLP-2	B
Peptide histidine-methionine (or -isoleucine)	PHM (or PHI)	C
Vasoactive intestinal peptide	VIP	C
Growth hormone-releasing factor	GHRH	D
Secretin	–	E
Glucose-dependent insulinotropic peptide	GIP	F

The Cholecystokinin/Gastrin Family

Cholecystokinin (CCK) and gastrin constitute a small family comprising only these two members. They are characterized by a common amidated C-terminal tetrapeptide sequence, which also constitutes the minimal structure necessary for biological activity of both peptides. Hence, it appears most likely that CCK and gastrin have evolved from a common ancestor. Cionin, isolated from *Ciona intestinalis*, a representative of the tunicates, which occupy a key position at the transition to vertebrates, also contains the characteristic tetrapeptide sequence and thus represents the oldest genuine member of the CCK/gastrin family thus far known, dating the emergence of these peptides back to at least 500 million years ago. The CCK/gastrin family is represented throughout the entire chordate phylum, including cartilaginous and bony fish, amphibians, reptiles, birds, and mammals (Johnsen, 1998; Baldwin *et al.*, 2010). A duplication of the ancestral gene appears to have occurred at 2R giving rise to two peptides most likely homologous to CCK and gastrin and a third duplication occurred at the level of cartilaginous fish to produce two CCKs (Dupré and Tostivint, 2014). At the amphibian level, the two separate peptide systems have been shown to exert distinct physiological gastrin and CCK actions. Interestingly, while CCK is well conserved in all vertebrate species, the gastrins vary more and the mammalian gastrins at first sight appear as a distinct group with little similarity to the nonmammalian gastrins outside the invariant C-terminal tetrapeptide. However, closer examination reveals that even if a major structural change was introduced at the transition to mammals, there exists a clear evolutionary relationship between mammalian and nonmammalian gastrins (Johnsen, 1998).

The Tachykinin Family

The tachykinins share a C-terminally amidated pentapeptide, -Phe-Xaa-Gly-Leu-Met-amide, where Xaa can vary among a few possible amino acids. In mammals, the family comprises four peptides encoded by two genes: preprotachykinin A, encoding substance P (SP), neurokinin A (NKA), and neuropeptide γ (NPγ, which is an N-terminally extended form of neurokinin A); and preprotachykinin B, encoding neurokinin B. SP, NKA, and NPγ have been identified in most vertebrate classes, whereas NKB has been identified only in mammals and a frog (Amphibia). NKA (a decapeptide) has been highly conserved and SP (an undecapeptide) somewhat less so. Interestingly, SP from amphibians shows less identity to the mammalian form than SP from the phylogenetically more distant fish. However, the peptides are rather short and even if many tachykinins from nonmammalian vertebrates have been identified, almost no genes for these tachykinins have been discovered (Zhou *et al.*, 2012). Thus, solid conclusions regarding the evolution of the tachykinins are difficult to make, but there is no doubt that the tachykinins constitute a family, that they must have arisen from a common ancestor, and that their diversity must have been brought about by exon duplication, gene duplication, and point mutations.

The Somatostatin Family

In mammals, the somatostatin (SS) family contains two members, somatostatin (known since 1973) and the neuropeptide, cortistatin, which shows a high degree of similarity to somatostatin. The bioactive forms of somatostatin are somatostatin-14 and the N-terminally extended somatostatin-28. Somatostatin-14 is highly conserved, being identical in all vertebrates from jawless fish to mammals. In addition to genuine somatostatin (SS1), most, if not all, investigated vertebrate species express a second gene, SS2, very similar to preprosomatostatin-I. It has been suggested that the duplication of the original somatostatin gene occurred early in evolution, predating or concomitant with the development of the chordates. Subsequently, the third round of duplication in the stem lineage leading to bony fish after divergence from the line leading to tetrapods has resulted in the occurrence of no less than at total of six SS-related peptides, SS1–6 (Tostivint *et al.*, 2013).

The Gastrin-Releasing-Peptide/Bombesin/Neuromedin B Family

Gastrin-releasing peptide (GRP) has been identified in all major vertebrate classes except jawless fish. It consists of 22 (goldfish) to 29 (rat) amino acid residues and has an invariant C-terminal octapeptide sequence in all species. Neuromedin B, which shows similarity to GRP, has been identified only in mammals and a toad (Amphibia) and is encoded by a separate gene. The bombesins constitute an interesting subgroup, all being isolated from the skin of amphibians, where they may serve protective purposes, and also arising from independent genes. Since only GRP is known to exist in many vertebrate classes, it is not possible to further elaborate on the phylogeny of these peptides (Baldwin *et al.*, 2007).

Motilin and Ghrelin

Motilin is produced in endocrine cells of the upper intestine. Since the identification of porcine motilin in 1972, motilin has been identified in a number of mammals but in only one nonmammalian species, the chicken. The mature form of motilin consists of 22 amino acid residues and between human and chicken there are seven substitutions, although most are conservative. Thus, little can be said about the evolution of motilin. In 1999–2000, a new GHRH was identified independently by two groups and was designated ghrelin and prepromotilin-related peptide, respectively. Since then ghrelin has been identified in all vertebrate classes and there are ample evidence that motilin and ghrelin are phylogenitically related (He *et al.*, 2011).

Galanin

Galanin is a widespread neuropeptide and is also common in the gut. Since its discovery in 1983, galanin has been identified in a number of species representing all major vertebrate classes. It comprises 29 (or 30, in human) amino acid residues, of which the 14 N-terminal residues are identical in all species investigated. Despite repeated efforts to discover related peptides, none were found until 1999, when galanin-like peptide (GALP) coded by a separate gene (Lawrence and Fraley, 2011), was isolated from hypothalamus based on its stimulation of galanin type 2 receptors. Thirteen residues (positions 9–21) of GALP are identical to the N terminus of galanin. GALP has been identified only in mammals (human, pig, and rat). Recently, a mass spectrometry peptidomic analysis of the central nervous system of the ascidian, *Ciona intestinalis*, identified 33 peptides, 26 of which were novel (Kawada *et al.*, 2011). Among these a galanin/GALP-like peptide was identified by a highly conserved 12-amino acid sequence. Based on additional criteria the peptide was designated Ci-GALP, and thus it represents the first non-mammalian GALP and extends the galanin/GALP-family back the protochordates.

See also: Cholecystokinin (CCK). Gastrinomas. Gastrointestinal Hormones in Cancer. Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS)

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GI Tract: General Anatomy (Cells)☆

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Glossary

Endocrine cell Type of cell that produces a signal that reaches the target via the blood circulation.

Fluorescence Emission of light of longer wavelength (e.g., visible light) following the absorption of light of shorter wavelength (e.g., ultraviolet light).

Histochemistry Defined chemical reactions performed directly in tissue sections and visualized in the microscope.

Immunohistochemistry The process of selectively detection of antigens (e.g., proteins) in cells of a tissue section using antibodies specifically binding to these antigens.

Paracrine cell Type of cell that produces a signal that is aimed at reaching neighboring cells by direct contact or diffusion.

Reducing capacity Ability of a cell to convert certain metal ions to free metal precipitate that can be visualized in the microscope. Reducing substances include amines, such as catecholamines and serotonin.

Secretory granules Particles composed of peptide hormones, and also amines, that are stored in membrane-bound vesicles in the cytoplasm before release.

Introduction

Endocrine (neuroendocrine or enteroendocrine) cells of the gastrointestinal (GI) tract belong to the dispersed neuroendocrine system, composed of cells that are widely distributed in the body, regardless of their embryologic derivation (Tischler, 1989). They are found within the mucosa all along the GI tract. Taking into account the size of the GI tract, the endocrine cells together constitute a prominent endocrine organ. In fact, calculations have made it clear that the endocrine cells of the GI tract form the largest endocrine organ in the body, and furthermore, harbor the greatest number and variety of endocrine cell types and hormones (Ahlman and Nilsson, 2001). The different endocrine cell types along the GI tract although diverse, have several histochemical and ultrastructural features in common. They produce hormone peptides, biogenic amines or neuroregulatory substances. After their synthesis in the endoplasmic reticulum, the endocrine cells are capable of packaging and storing these products into dense core vesicles (also named secretory granules) in the Golgi apparatus. (Vazquez-Martinez and Gasman, 2014). Therefore, one of the ultrastructural characteristic is the presence of membrane-bound secretory granules, which vary in size, shape and electron density (Capella *et al.*, 1976; Buffa *et al.*, 1978). There are several histochemical and immunohistochemical methods distinguishing endocrine from non-endocrine cells.

Historical Annotation

The first descriptions of the cells known as endocrine cells in the GI tract were published more than a century ago. In 1879 chromaffin cells and osmiophilic cells and in 1897 acidophil cells with basally located granules were described. The term enterochromaffin (EC) cell was first used in 1907. In 1914 Masson introduced a silver impregnation technique to demonstrate argentaffin cells, based on their capacity to form silver precipitates from a silver nitrate solution. It was also suggested at that time that the cells were endocrine. During the following years, several other staining methods were developed. Friedrich Feyrter in his classical work on disseminated endocrine cells, published in 1938, described these cells as "clear cells." In 1952 the endogenous substance ("enteramine") in the argentaffin/chromaffin cells capable of reducing ionic silver and chromium to the corresponding metallic precipitate was identified as 5-hydroxyptamine (5-HT or serotonin), (**Table 1**).

Interestingly, in 1912 it had already been observed that the GI tract was rich in cells with morphology similar to that of the EC cells, but lacking endogenous reducing substances. For several years, these cells were thought to be progenitors of EC cells. The presence of such non-EC endocrine cells was confirmed in several studies during the 1940s and 1950s. These studies have shown that endocrine cells even of non-EC type could be argyrophil, capable of forming silver precipitate, when an exogenous reducing agent was used. In fact, it turned out that the vast majority of endocrine cells in the GI tract were argyrophil. A similar to Bodian stain originally described in 1936 in neurons, was introduced by Grimelius, to detect argyrophil cells in 1968 and modified in 1980 (Grimelius, 2008). An important histochemical advancement in the early 1960s was the possibility of demonstrating monoamines, such as catecholamines and 5-HT (and their immediate amino acid precursors), by fluorescence microscopy taking advantage of the ability of these amines to

☆*Change History*: December 2017. G. Kontogeorgos updated the text and figures.
This chapter is an update of F. Sundler, GI Tract, General Anatomy (Cells), In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 208–215.

Table 1 History of gut endocrine cells

Heidenhein (1870)	Chromaffin cells described
Nussbaum (1879)	Osmiophilic cells described
Gritzner and Menzel (1879)	
Kultschitzky (1897)	Basigranular acidophil cells described
Ciaccio (1907)	Term enterochromaffin cells coined
Kull (1913)	Nonargentaffin endocrine-like cells described
Masson (1914)	Argentaffin cells described
Vialli and Erspamer (1937)	Enteramine in EC cells described
Feyrter (1938)	Clear cells described
Erspamer (1939)	Argyrophilic cells (interpreted as EC cell progenitors) described
Dawson (1948)	Argentophilic (argyrophil) cells in the stomach described
Erspamer and Asero (1952)	Enteramine determined to be identical to 5-HT (serotonin)

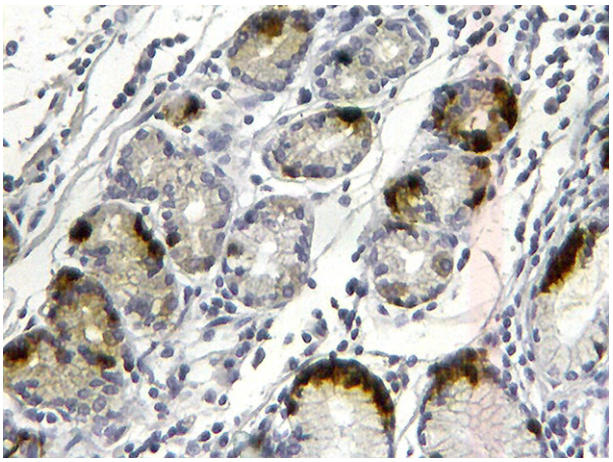

Fig. 1 Distribution of endocrine cells within the acini in the gastric mucosa, detected by chromogranin A. The endocrine cells are localized among non-endocrine gastric cells (20 ×).

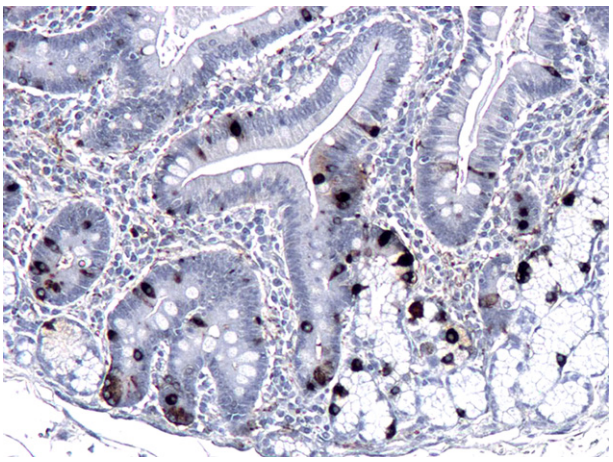

Fig. 2 Localization of endocrine cells in the ampulla, detected by synaptophysin. The endocrine cells are randomly distributed within the acini, including Brunner nests (15 ×).

form fluorescent conjugates with formaldehyde vapors under certain conditions (the Falck-Hillarp technique). Using this technique, it was found that the endocrine cells in the GI tract had the capacity to form fluorogenic monoamines, such as dopamine and 5-HT, on administration of the corresponding amine precursor (L-DOPA and 5-hydroxytryptophan, respectively) and to store large amounts of them in their secretory granules. Based on these properties the cells have been referred to as APUD (Amine Precursor Uptake and

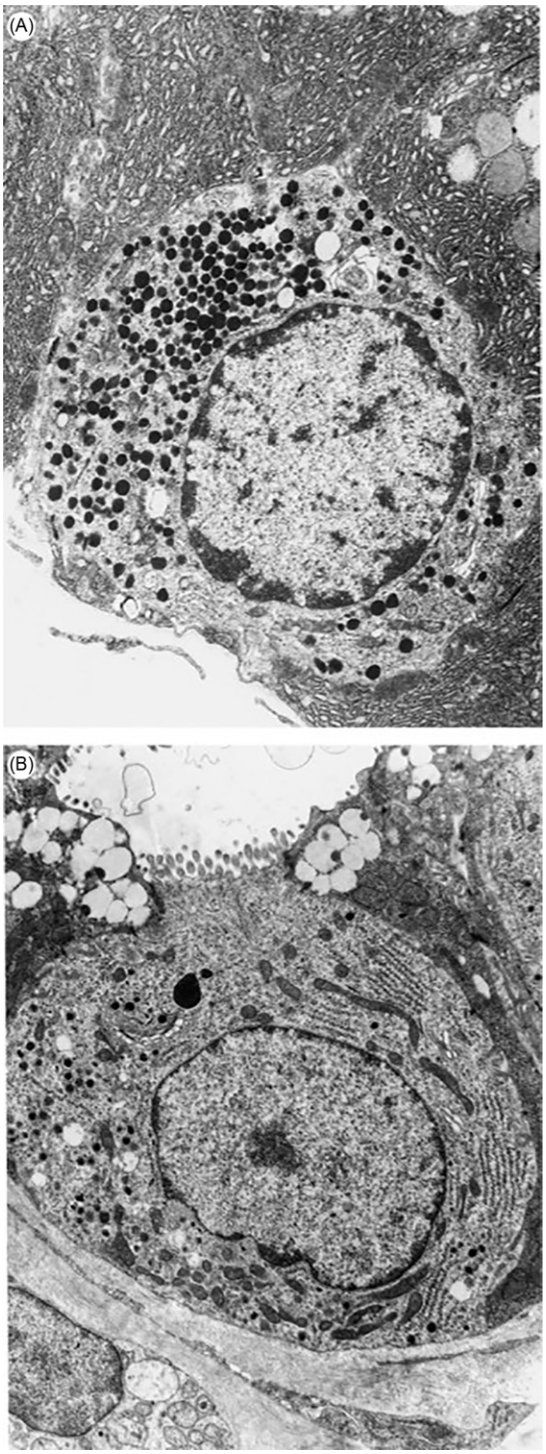

Fig. 3 Electron micrographs illustrating (A) an endocrine cell of closed type (A-like cell) in the acid-producing mucosa and (B) an open endocrine cell in the antral mucosa (G cell) of the rat stomach. Note numerous microvilli at the lumenal surface.

Decarboxylation). Given that amine-handling properties, as well as some other histochemical features are similar to those of neurons it was long thought that the GI endocrine cells have an embryonic neuroectodermal origin, deriving from neural crest (Pearse, 1969). Currently, the experimental evidence strongly supports their endodermal origin (Lloyd, 1990).

During the past few decades, immunocytochemistry, with the use of antibodies against individual hormones or other specific neuroendocrine cell markers, has become the most important technique for distinguishing the individual endocrine cell populations with respect to their cellular morphology, their regional and topographic distribution, and their function. Conversely, there

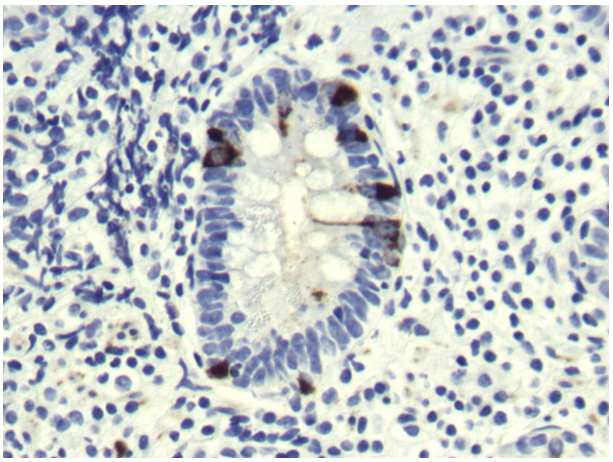

Fig. 4 Gastric gland harboring randomly distributed endocrine cells. Immunohistochemistry for chromogranin A highlights the attenuated thin cell processes towards the lumen surface (25 ×).

are cell populations that differ in their hormone content, but nevertheless share both light microscopic features and secretory granule appearance. Several broad-spectrum immunohistochemical markers have been introduced to detect and localize endocrine cells. Chromogranin A and synaptophysin represent the most important for diagnostic pathology (**Fig. 1**). Chromogranin A, a matrix-soluble glycoprotein, is member of the granin family of secretory proteins that are ubiquitous to the endocrine cells and neurons (Feldman and Eiden, 2003). Synaptophysin, a 38 kDa, is matrix-soluble glycoprotein molecule, component of the membrane presynaptic vesicle membrane, is also found diffusely in the cytoplasm of endocrine cells (**Fig. 2**). Neuron specific enolase (NSE) is a very sensitive, but not very specific marker for neuroendocrine cells (Lloyd, 1990). Other general markers, although nonspecific, include the protein cell product 9.5 (PGP-9.5), a soluble protein originally isolated from the brain, the Leu 7 (CD57), the 7B2 protein, the synaptic proteins (SNAP-23, SNAP-25, Rab3A) and the neural cell adhesion molecule (NCAM/CD56). Specific immunohistochemical markers are designated to reveal the exact type of hormonal product in endocrine cells. Holgate *et al.* (1983) introduced in 1983 the immunogold'silver technique for light microscopy and later on, the method was modified for application to electron microscopy ultrathin sections (Holgate *et al.*, 1983; Lackie *et al.*, 1985). Using specific antibodies conjugated with colloidal gold particles (from 5 to 15 nm), this technique can specifically detect the hormone peptide products concentrated within the secretory granules of endocrine cells.

The in situ hybridization technique allows demonstrating the presence of mRNA sequences specific for a peptide hormone or the biogenic amine, using isotopic or nonisotopic labeled complementary mRNA probes. However, besides the intensive research made during the last decades using histochemical, immunohistochemical and molecular techniques, and electron microscopy, some populations of endocrine cells remain yet undefined in terms to which hormone are linked.

General Morphological Features

Endocrine cells of the GI tract are tall columnar cells within the crypts and their apical cytoplasmic process forming microvilli that extend towards the luminal surface (**Fig. 3**). They are often found isolated from one another interspersed by non-endocrine epithelial cells. They are located within the deeper half, of mucosa and comprise only a small minority (< 1%) of the overall epithelial cell population. Some endocrine cells have a very characteristic appearance. They display a flask-shaped with a narrow, often quite elongated neck reaching the lumen and thus of open type. By electron microscope, microvilli represent a specialized sensory apparatus that detects changes in the lumen and respond to luminal stimuli by releasing their hormonal product thus, modulating endocrine cell secretion. (El-Salhy *et al.*, 2017). The cytoplasmic process often seems to be directed towards the base of the crypt. Interestingly, there are several indications that such widely distributed cells may serve to influence cells in their vicinity, in addition to the role of delivering their products by exocytosis to the blood circulation. Such cell-to-cell interaction refers to as paracrine function. Under confocal laser scanning microscopy they also show alone pseudopod-like basal process approximately 50–70 μm long, extending from these cells and form an interface between the mucosal epithelium and the lamina propria. These processes resembling a synapse appear to interact with neighboring cells and might be involved in their paracrine function (Sundler *et al.*, 1989; Bohórquez *et al.*, 2011). A general morphological characteristic of the endocrine cells in the upper, acid-producing part of the stomach (i.e., the corpus or fundus) is that they are "closed" in that the apical end of the cell does not reach the gland lumen. In the antrum, on the other hand, the vast majority of cells do reach the lumen via a narrow neck (**Figs. 3** and **4**). One possible explanation for this morphological difference is that open cells respond to lumenal stimuli (e.g., nutrients, pH changes), whereas closed cells are sensitive to other types of stimuli (e.g., distension, temperature changes, neuronal, and hormonal messengers). Therefore, it is conceivable that these cells serve both endocrine and paracrine functions (Sundler *et al.*, 1989). With respect to genuinely endocrine cells, it has been argued that each such population should have a restricted distribution along

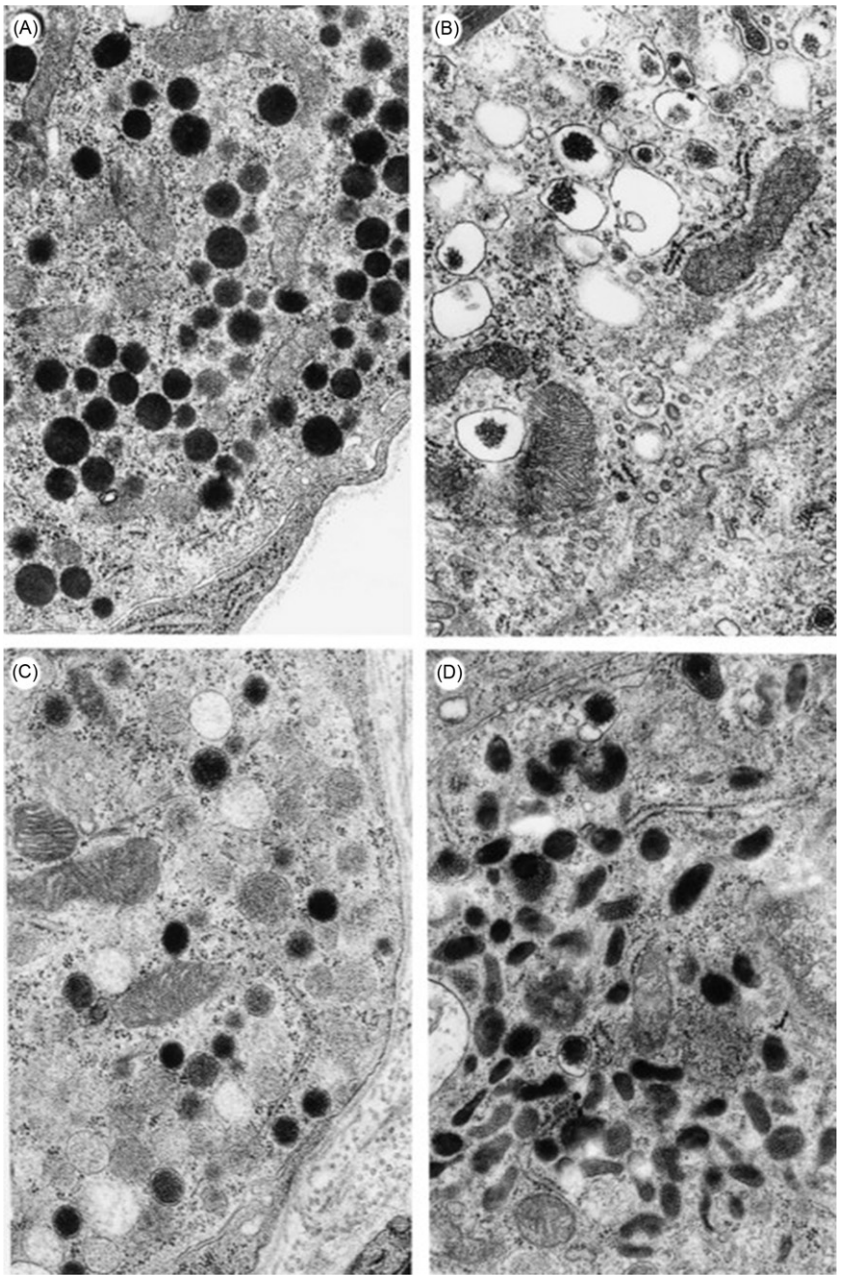

Fig. 5 Electron micrographs of secretory granules of endocrine/paracrine cells in the rat stomach. (A) A-like cell. The granules are round and highly electron-dense. (B) ECL cell. The granules are of the vesicular type with a flocculent electron-dense core surrounded by a wide electron-lucent halo. (C) G cell. Note the varying electron density of the granules. (D) EC cell. Note granule pleomorphism.

the GI tract in order for the target cells to be able to respond to a circulating messenger in a meaningful way. Apparently, endocrine cells have a multi-messenger capacity, considerably more powerful as transmitters of information than might be anticipated by the traditional and now discarded one-cell, one-messenger hypothesis (Tischler, 1989). By electron microscope, the endocrine cells show similarities and differences regarding the size, electron density, distribution, morphology and pleomorphism of secretory granules (exemplified in **Fig. 5**). Typically, the secretory granules accumulate at the base of the cell, but may sometimes be more diffusely distributed, although they are always confined to the cytoplasm and never in the nucleus.

Interaction to Nervous System

The endocrine cells of the GI tract exhibit both endocrine and neuron-like characteristics. In fact, several biogenic amines or hormone peptides are fount to both endocrine cells and neurons. In addition, the endocrine cells have a basal cytoplasmic process

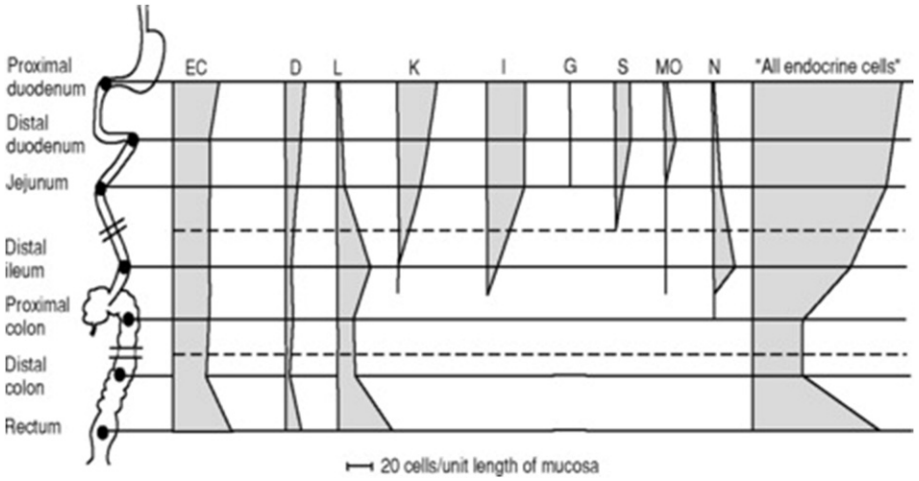

Fig. 6 Schematic diagram illustrating the distribution and relative frequency (number of cells/unit length of mucosa) of several different populations of endocrine cells in the human intestine. Data are based on immunocytochemical observations using hormone antibodies. (*Left*) *Black dots* on the outline of the intestine indicate tissue sampling sites. (*Right*) A compilation of the data. Note that some cell types are distributed throughout the intestine, whereas other cell types have a more restricted distribution. Note also that the rectum is almost as rich in endocrine cells as the duodenum.

Table 2 Endocrine cells in the mammalian gastrointestinal tract

Cell type	*Stomach*		*Small intestine*		*Large intestine*	*Hormone*
	Oxyntic	*Pyloric*	*Upper*	*Lower*		
A	+[a]	–	–	–	–	Glucagon
D	+	+	+	+	+	Somatostatin
D_1	+	+	+	+	+	VIP
EC	+	+	+	+	+	Serotonin + various peptides
ECL	+	–	–	–	–	Histamine
G	–	+	+	–	–	Gastrin
I	–	–	+	+	–	Cholecystokinin
K	–	–	+	+	–	GIP
L	–	–	+	+	+	GLP-1, GLP-2, PYY, glicentin
M or MO	–	–	+	+	–	Motilin
N	–	–	–	+	–	Neurotensin
P	+	+	+	–	–	Leptin
P/D_1 (X/A-like cells)[b]	+	+	+	+	+	Ghrelin
S	–	–	+	+	–	Secretin

[a]In certain species, e.g., carnivores.
[b]In other species.

that refers to us neuropods, indicating that these cells have the necessary elements for synaptic transmission. Therefore, the GI hormones released in the lamina propria could act locally by paracrine mode on neighboring cells or neurons, by endocrine mode through the circulating blood, or by afferent and efferent synaptic transmission (El-Salhy *et al.*, 2017).

Distribution, Types and Nomenclature

The endocrine cells arise in all segments of the GI tract except for the esophagus. As a rule their regional distribution and frequency is characteristic almost for each cell type (Sundler *et al.*, 1989). Thus, certain cell types are restricted to, or greatly predominate, in the stomach. Others are confined to the upper small intestine and still others predominate in the distal small intestine and/or the large intestine (**Fig. 6** and **Table 2**). Intestinal endocrine cells are predominately located within its deeper half, and comprise approximately 1% of the entire gut epithelial population. They often lie isolated from one another interspersed by non-endocrine epithelial cells, facing the GI lumen (Buffa *et al.*, 1978; Sternini *et al.*, 2008). A few cell types are distributed all along the GI tract. Traditionally, endocrine cells of the GI tract are named with an alphabetical letter, according to their origin. However, due to a huge amount of accumulated information provided by the extensive use of immunohistochemistry as well as to the discovery of

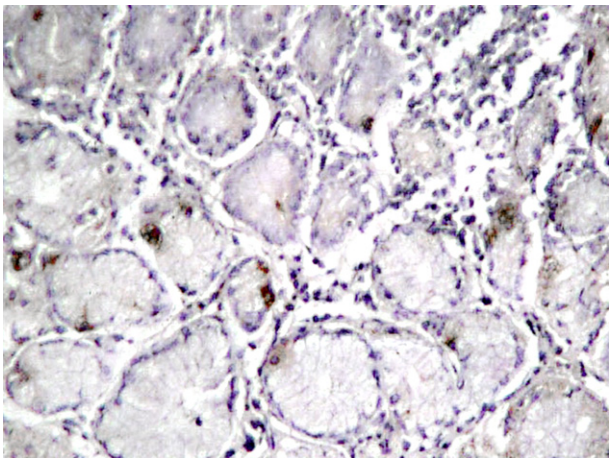

Fig. 7 Immunohistochemistry reveals scattered gastrin-producing cells distributed among non-endocrine cells within the glands in the gastric mucosa (10 ×).

new peptides, it is better to characterize a certain endocrine cell after its principal secretory product. The GI endocrine cells are divided into at least 15 different types depending on their morphology, specific regional distribution and hormonal peptide or amine substances they produce and secrete. These include a variety of about 50 hormone peptides or signaling molecules, including somatostatin (D cells), gastrin (G cells), serotonin (EC cells), histamine (ECL cells), vasoactive intestinal peptide (VIP) (D_1 cells), cholecystokinin (CCK) (I cells), glucose-dependent insulinotropic peptide (GIP) (K cells), glucagon-like peptides (GLPs) and peptide YY (PYY) (L cells), and ghrelin (P/D_1 cells). Upon stimulation, the hormone products, which are stored in secretory granules, are secreted by exocytosis at the basolateral membrane into the interstitial space. They exert their functional activity locally or on distant targets after release to bloodstream (Sternini *et al.*, 2008). Two or more hormones can be detected in the same type of endocrine cell and may be co-localized in the same secretory granule as well (Egerod *et al.*, 2012).

Stomach

Based on various histochemical techniques, immunohistochemistry, and electron microscopy, with special emphasis on the morphological features of the secretory granules (**Fig. 5**), at least seven endocrine cell types have been described in the stomach. They comprise G cells, D cells, EC cells, enterochromaffin-like (ECL) cells, and two types of cells with small granules and designated P cells and D_1 (P/D_1 or X/A like) cells (**Table 2**). Additionally, in certain species, notably carnivores such as dogs and cats, "true" glucagon-producing A cells, being well-known constituents of the pancreatic islets, are also present in substantial numbers in the stomach. The gastrin-producing G cells are exclusively found in the antrum of the gastric mucosa, where they are located within a zone at the upper part of the glands (**Fig. 7**). Ultrastructurally, these cells contain secretory granules 150–300 nm in diameter, distributed mostly in the basal portions of the G-cell cytoplasm and the apical portion of the cell reaches the glandular lumen (Lloyd, 1990). The somatostatin producing D cells, are found throughout the GI tract but contrastingly are found in much lower numbers, making up around 3%–5% of the endocrine cell population in the lower GI tract. The D cells in the stomach are diffusely distributed all over the stomach in all species. These spindle shaped cells, very often have a slender apical cytoplasmic process that can have a shorter wider basal extension of nerve fiber-like appearance, ending with a knob-like swelling filled with granules (**Fig. 8**). The secretory granules range from 300 to 400 nm in diameter (Lloyd, 1990). The D cells have been found to often make direct contact with G cells in the antrum and with parietal cells in the acid-producing part of the gastric mucosa. This contact is usually established via the cytoplasmic processes of the D cells. These features, together with the wide distribution of the cells both within the GI tract and outside of it (the pancreatic islets in particular), have contributed to the designation of the D cells as paracrine cells. Cells containing small granules (P and D_1 cells), like the D cells, seem to occur all over the stomach. VIP producing D_1 cells are widely distributed throughout the length of the gut mucosa including the stomach, never being numerous at any site (Bloom *et al.*, 1975; Said, 1975; Buffa *et al.*, 1977; Bryant and Bloom, 1979). Leptin-secreting P cells are localized in the stomach. Leptin directs the central nervous system to adjust food intake (Kelesidis *et al.*, 2010). The endocrine cells greatly predominate in the antrum in certain mammals, such as rodents, whereas in the stomach of human and many larger mammals they also occur in the acid-producing part of the stomach in quite high numbers. The ECL histamine-producing cells are the largest endocrine cell population in the stomach. They constitute one—and possibly the only—gastric endocrine cell type that is confined to the acidic gastric mucosa, upper part (corpus fundus or body), (**Table 3**). This seems to be the case in all vertebrate species examined, from fish to human, but in different percentages. The secretory granules have a very characteristic ultrastructure, which at least in part, seem to be related to the histamine production by these cells.

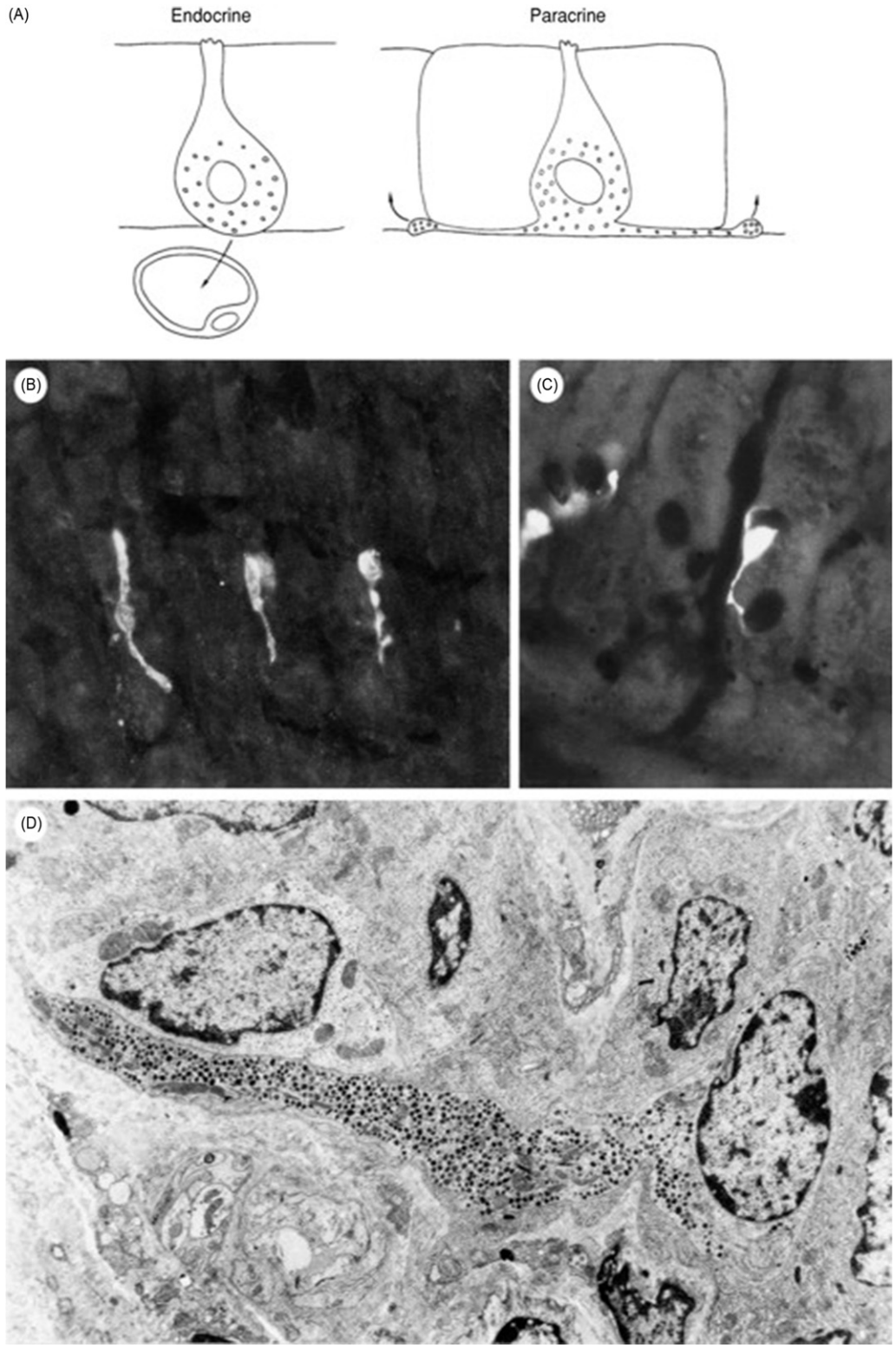

Fig. 8 (A) Schematic morphological distinction between endocrine and paracrine cells. (B and C) D cells of rat gastric mucosa (somatostatin immunofluorescence). (B) The cells issue long, slender cytoplasmic processes, sometimes ending in a knob-like swelling. (C) D cell process ending on a G cell (stained black by gastrin immunoperoxidase). (D) Electron micrograph showing a D cell process filled with secretory granules. The process runs along the base of the gland epithelium near the top of the figure.

Table 3 Relative frequency of different endocrine cells in gastric oxyntic mucosa of human and rat

Cell type	*Human (%)*	*Rat (%)*
ECL cells	35	65
D_1 + A-like (X) + P + cells	14	24
Somatostatin cells	26	10
EC cells	25	0

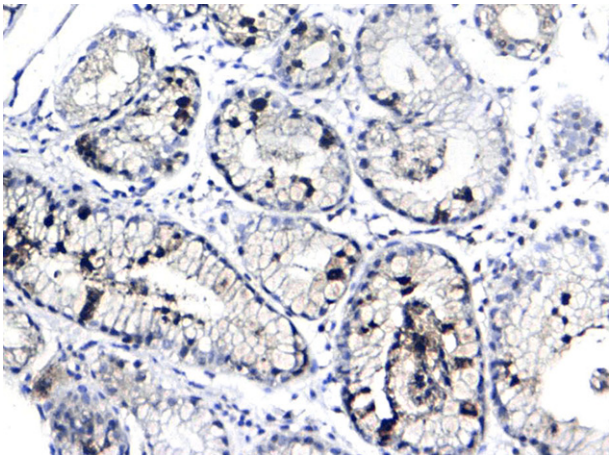

Fig. 9 Ghrelin-producing cells localized by immunohistochemistry within the glands of the gastric mucosa (10 ×).

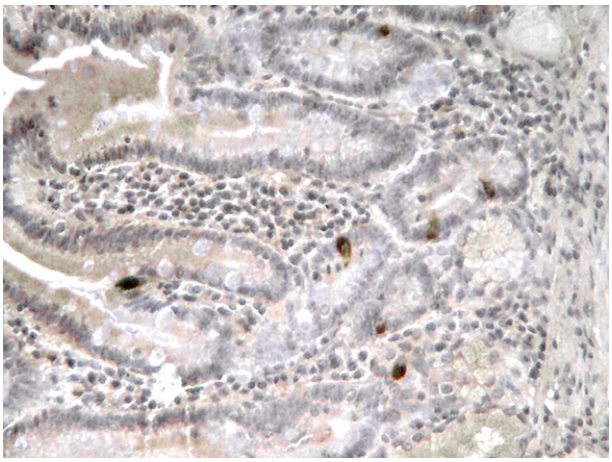

Fig. 10 Immunohistochemical detection and distribution of somatostatin-producing cells within normal cell of the small intestinal mucosa. They are mostly lying isolated from one another, interspersed in non-endocrine epithelial cells (10 ×).

The peptide ghrelin is produced by well-defined as P/D_1 cells in humans, which in the past had been labeled differently in various mammals, as A-like cells in rats and X-like type in dogs. X/A-like cells are primarily found in the oxyntic part of the gastric mucosa, accounting for 20%–30% of the oxyntic endocrine cells and therefore represent the second most abundant gastric endocrine cell type (Rindi *et al.*, 2004). They may also occur in smaller numbers in the distal non-acid-producing part (antrum, pylorus) of the stomach. The ghrelin immunoreactive cells in the antrum are distinct from the gastrin cells, the serotonin-containing EC cells and the D cells. Ghrelin producing cells represent the main source of circulating ghrelin as demonstrated by the sharp decline of ghrelin levels following gastrectomy (Jeon *et al.*, 2004) (**Fig. 9**). Ghrelin, among its numerous functions, stimulates appetite and increases food intake by stimulating the production of neuropeptide Y (NPY) and promotes fat storage (Wren *et al.*, 2001). For this reason, it is termed the "hunger hormone." Ghrelin, P/D_1 type cells contain characteristic round, compact, electron-dense secretory granules with a mean diameter 147 ± 30 nm (Rindi *et al.*, 2002).

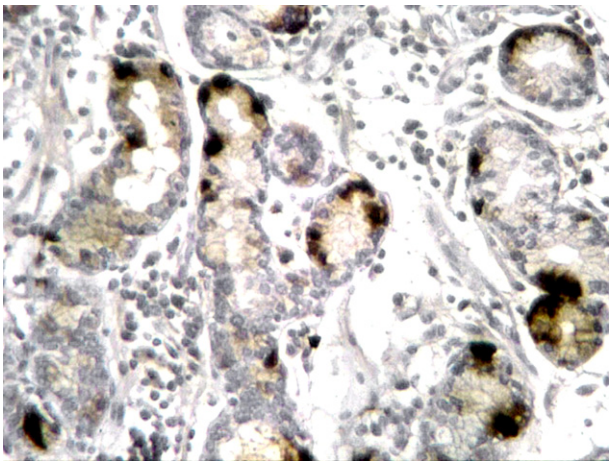

Fig. 11 Endocrine cells in the glands of the small intestine immunopositive for cholocystokinin (10 ×).

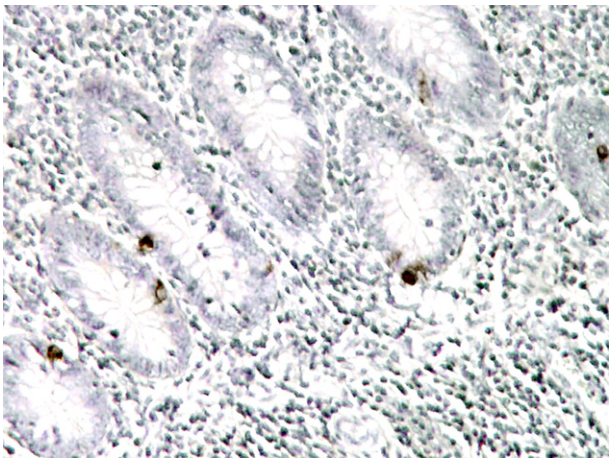

Fig. 12 Scattered serotonin-producing cells are detected by immunohistochemistry among mucin producing columnar cells in the glands of the large bowel mucosa (10 ×).

Small Intestine

The endocrine cells are characteristically flask-shaped with a narrow, often quite elongated neck reaching the lumen and thus of open type. The secretory granules accumulate at the base of the cell, but may sometimes be more diffusely distributed, although they are always confined to the cytoplasm. The classification and naming of the intestinal endocrine cells were for a long time based primarily on the size and morphology of the secretory granules. Accordingly, the cells were categorized as, for example, S (small granules), I (intermediate-size granules), and L (large granules) cells (**Table 2** and **Fig. 6**). Subsequent immunohistochemical observations about the cellular localization of various hormones complicated matters and resulted in attempts to retain the letter classification, the inevitable renaming of some cells, and the addition of hormonally classified cells that did not fit into previous classifications. Thus, the classification of endocrine cells in the small intestine is often a mixture of letter naming and naming based on the hormone content of the cells. The EC cells are distributed throughout the small intestine with maximal numbers in the duodenum. The somatostatin producing D cells also occur throughout the small intestine, with the highest frequency in the duodenum including Brunner's glands, and gradually in smaller numbers distally (**Fig. 10**). The gastrin producing G cells, in addition to gastric mucosa, are also found in the duodenal mucosa and in Brunner's glands of the duodenal bulb (Lloyd, 1990). Some cell types are more restricted in their regional distribution. Thus, S cells, I cells, K cells, and M cells predominate in the duodenum and upper jejunum and are only rarely found in the ileum. The opposite is true for L cells and N cells, which predominate in the ileum, but are scarce in the duodenum (Rindi *et al.*, 2004; Egerod *et al.*, 2012). According to ultrastructural criteria, P cells are also present in the small intestine, with a distribution mainly in the upper part. One cell type with very large granules has been reported to occur throughout the human small intestine. The secretin-producing S cells are present in the duodenum and jejunum of most mammalian species. These cells have granules 180–220 nm in diameter and show positive immunoreactivity for secretin. The I cells, are also located in the duodenum and jejunum. They secrete CCK in response to the

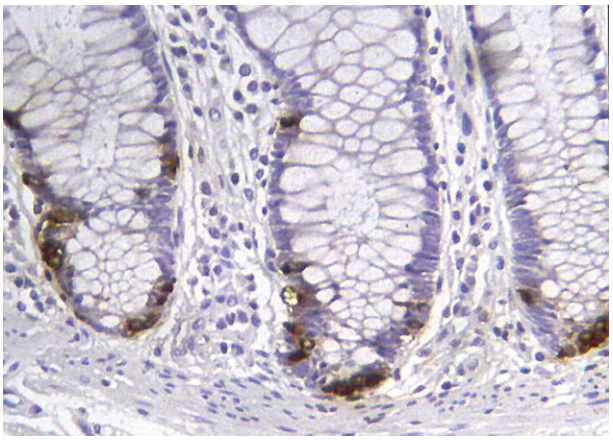

Fig. 13 Immunohistochemical localization and distribution of endocrine cells within the mucosal glands in the large intestine, detected by chromogranin A (25 ×).

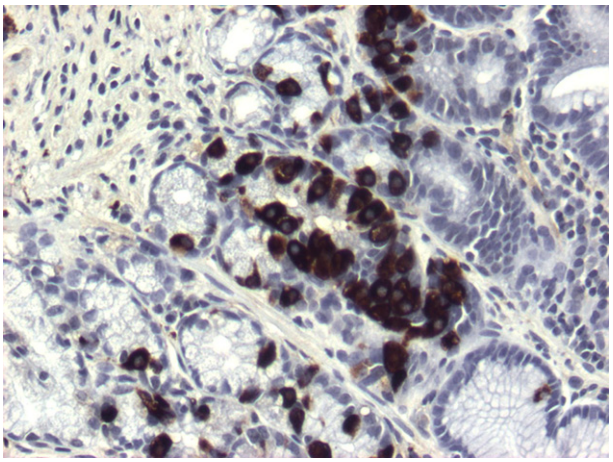

Fig. 14 Chromogranin A immunohistochemistry reveals hyperplasia of endocrine cells, characterized by occupation of a large portion of glands in the gastric mucosa. More than three endocrine cells accumulate in a continuous lining and are packed together (40 ×).

emptying of the stomach contents into the duodenum (**Fig. 11**). The secretory granules of these cells measure from 250 to 300 nm (Lloyd, 1990). Secretin is co-expressed with CCK in endocrine cells of the small intestine. K cells are predominantly found in the duodenum and jejunum. The K cells secrete gastric inhibitory peptide (GIP) and L cells glucagon-like peptide-1 (GLP-1). A population of K/L cells has been found to secrete both GIP and GLP-2. The L cells secrete GLP-1, GLP-2, and PYY, which delay gastric emptying and oxyntomodulin, which inhibits gastric emptying. In the intestine, L cells occur especially in the duodenum and are rare proximal to the terminal ileum. They are found in all parts of the crypts, but with some predominance in the basal portion. Glicentin and enteroglucagon immunoreactivity has also been identified in these cells. They have secretory granules of 250–300 nm in diameter (Lloyd, 1990). M (or MO) cells occur in both upper and low parts of the small intestine and secrete molitin. The N cells are found in the jejunum. They release neurotensin and control smooth muscle contraction. In addition to oxyntic gastric mucosa, ghrelin is also produced by P/D_1 type endocrine cells of the jejunum and low part of the small intestine. The secretin-producing S cells are present in the duodenum and jejunum. They contain secretory granules 180–220 nm in diameter (Lloyd, 1990).

Large Intestine

The large intestine also harbors endocrine cells of several different kinds, although it seems that the number and the variety of different populations are less diverse than in the small intestine. Interestingly, the endocrine cells are collectively more numerous in the rectum than in the colon, as calculated in the human gut (**Fig. 6**). At least five different endocrine cell populations can be distinguished in the large intestine. It is notable that there is a marked overlap in the distribution of certain endocrine cell populations between the distal small intestine and the large intestine (**Table 2**). Thus, EC cells and L cells are quite numerous also

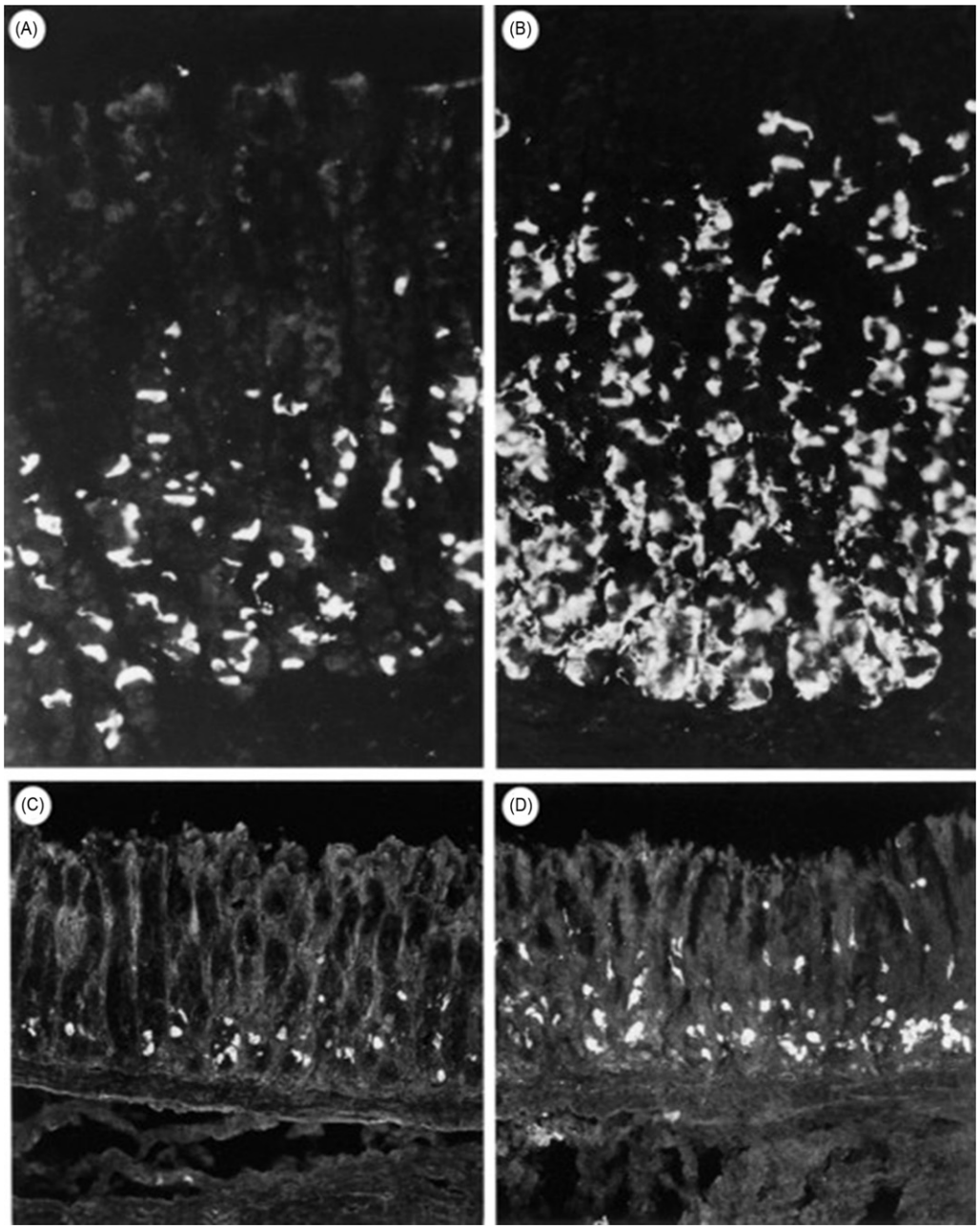

Fig. 15 Endocrine cell plasticity illustrated by the marked hyperplasia of rat ECL cells (B) and G cells (D) on pharmacological blockade of acid secretion for 8 weeks. Acid blockade causes G cell activation and hyperplasia with hypergastrinemia, which in turn brings about activation of the ECL cells with increased histamine secretion and, with time, proliferation. ECL cells are visualized with histidine decarboxylase immunofluorescence and G cells are visualized with gastrin immunofluorescence.

in the large intestine. N cells, on the other hand, are only rarely found in the large intestine and D cells are also clearly fewer in number here than in the small intestine. Overall, 95% of the body's serotonin content is found within the GI tract. The serotonin secreting EC cells are abundant, distributed in all parts of the GI tract, populating the gastric antrum, duodenum, jejunum, ileum and appendix, the colon and rectum (**Fig. 12**). They account for the largest population making up over 70% of the endocrine cells in the proximal large bowel, which falls to around 40% in the rectum (Cristina *et al.*, 1978; Sjölund *et al.*, 1983). By electron microscopy the EC cells are approximately 8 μm in size with triangular or pyramidal shape and display a slender apical process often extending to the luminal surface. They secretory granules measure approximately 150–500 nm in diameter (Buffa *et al.*, 1978; Spiller, 2008). Findings from experimental studies suggest that serotonin released by endocrine cells is, at least in part, neurally regulated. D cells are also clearly found, but fewer in number than in the small intestine making-up around 3%–5% of the endocrine cell population (Buffa *et al.*, 1978; Cristina *et al.*, 1978; Sjölund *et al.*, 1983). They represent the least common endocrine

cell type in colon and rectum (Fig. 13). The ultrastructure of D cells is relatively uniform in the large intestine with one elongated apical extension and one shorter, wider basal extension. This differs from the overall appearance of D cells in the stomach. The secretory vesicles of D cells are approximately 200–400 nm in diameter. Ghrelin is also produced, although in much lower amounts, in large bowel. The L cells constitute the second largest population of large intestine, their frequency rising from proximal to distal, reaching approximately 14% of the endocrine cell numbers in the rectum (Cristina *et al.*, 1978; Sjölund *et al.*, 1983). They secrete glicentin, which inhibits gastric emptying, oxyntomodulin and GLP-1, GLP-2 (Gunawardene *et al.*, 2011). L cells have characteristic morphology with a bottle or flask shaped configuration. They often display an open morphology with a beaded process running along the base of the epithelium and an apical protrusion of cytoplasm extending to the luminal surface and forming microvilli (Capella *et al.*, 1976; Sjölund *et al.*, 1983). Under electron microscopy, the secretory vesicles occur adjacent to the basolateral membranes and are approximately 150–300 nm in diameter. Secretin-producing S cells, motilin-secreting M cells and neurotensin-producing N cells are absent from the large intestine (Rindi *et al.*, 2004).

Concluding Remarks

As is obvious from the data presented here, there is some confusion and remaining uncertainties as to the classification and naming of endocrine cells in the GI tract. This is due to the fact that endocrine cells sharing staining characteristics with cells occurring outside the GI tract, notably, the pancreatic islets, which are known by the "islet" name. This applies to islet A cells and D cells. Another problem arose when an ultrastructurally defined cell type, for example, the L cell, was later found to comprise at least two functionally distinct cell populations, one producing proglucagon-derived peptides (retaining the L cell designation) and another producing neurotensin (renamed N cells).

Another problem is related to the I cell. Obviously, several functionally distinct endocrine cell populations fall into this somewhat vague category. Thus, CCK cells, K cells, and M cells are all "I" cells, but with the current possibilities of a more detailed functional classification, the hormone name, or its short name, is gradually replacing the original letter name. Furthermore, EC cells are distributed widely throughout the GI track. They are probably not one single cell type, but rather several functionally distinct populations, unified merely because they produce large amounts of serotonin. This view is favored by available ultrastructural data showing different types of granules in EC cells in different regions of the gut, and sometimes even in different EC cells within the same region, together with the fact that certain regulatory peptides are restricted to subpopulations of EC cells only.

Finally, it should be mentioned that there is a remarkable potential for plasticity in gut endocrine cell systems. This is perhaps most readily observed in the stomach, where each of the endocrine cell populations has a restricted regional distribution and the cells are more frequent per unit area. Thus, marked hyperplasia of G cells and ECL cells (within their normal regional boundaries) evolves within some time (days to a few weeks) after, for example, profound pharmacological blockade of acid secretion (Figs. 14 and 15) and ECL cell hypoplasia after, for example, removal of the G cells (antrectomy).

See also: GI Hormone Development (Families and Phylogeny)

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GI Hormones Outside the Gut: Central and Peripheral Nervous Systems☆

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Glossary

Classical neurotransmitters Neurotransmitters that are released by calcium-dependent exocytosis of small clear synaptic vesicles and act at postsynaptic membranes often at receptors that are ion channels, for example, nicotinic acetylcholine, γ-aminobutyric acid type A receptor, serotonin type 3, and *N*-methyl-D-aspartate-type glutamate receptors.

Cotransmission Production of multiple transmitters by a single neuron and their interaction at a common cellular target; may include classical transmitters and neuropeptides that are also gut hormones.

Neuropeptides Biologically active peptides synthesized in neurons, sequestered into secretory vesicles, and released on neuronal stimulation. Their actions are mediated by G protein coupled-receptors, which may be on cells distant to the release site.

Regulatory peptides A generic term to cover biologically active peptides that may function as hormones, paracrine or autocrine agents, and neurotransmitters. Typically, peptides are produced from larger precursors, are stored in secretory vesicles, are released by exocytosis, and act at G protein-coupled receptors.

Introduction

The three main gut hormones discovered in the early 20th century, gastrin, secretin, and cholecystokinin (CCK), were thought for many years to be exclusively concerned with the regulation of digestion. However, the availability of rapid and specific methods for the identification, localization, and estimation of these peptides in the 1970s prompted rigorous studies of their distribution outside the gut (Dockray, 2014). These studies came at a time when it was becoming clear that the main gut hormones belonged in families of structurally related peptides and that different family members may in any case be expressed outside the gastrointestinal tract. Three of the earliest relevant findings were that (1) CCK occurred in high concentrations in brain; (2) a secretin-related peptide, vasoactive intestinal polypeptide (VIP), was widely distributed in CNS and PNS neurons; and (3) the hypothalamic peptide, somatostatin, was found in many endocrine cells of the gut and pancreas. Since then, more peptides have been characterized as gut hormones and several of these have been shown to occur in CNS or PNS neurons. Conversely, some peptides first identified as neurotransmitters have subsequently been identified as putative gut hormones. Although there are many examples of gut hormones in CNS or PNS neurons, there are also several important groups of neuropeptides whose presence in gut endocrine cells, or roles as gut hormones, is unlikely or unclear; these include the opioid peptides (e.g., enkephalins), the tachykinins (e.g., substance P), the super-family of peptides terminating in –Arg-Phe-amide, and calcitonin gene-related peptide.

Evolutionary Relationships

Regulatory Peptides

It is thought that the role of peptides as intercellular molecular messengers emerged early in evolution (Hokfelt *et al.*, 2000). Subsequently, the genes encoding regulatory peptides duplicated and diverged to produce families of structurally related peptides. Different members of the same family may be expressed in neurons, endocrine cells, glial cells and other cells or tissues including amphibian skin glands, myofibroblasts and tumors. The gene encoding a specific regulatory peptide may also be expressed by multiple cell types. These patterns may themselves change during evolution, giving rise to species differences in cellular expression.

Receptors

Hormonal and neurotransmitter peptides typically act at G protein-coupled receptors (GPCRs). GPCRs constitute a large family of structurally related proteins that are thought to have also evolved by the duplication and divergence of ancestral genes. Multiple GPCRs may exhibit differential affinity for the various members of regulatory peptide families. The receptors may themselves be expressed by multiple cell types including CNS or PNS neurons and nonneuronal cells, for example, secretory cells or smooth muscle cells.

☆*Change History*: March 2018. Graham Dockray updated reference.

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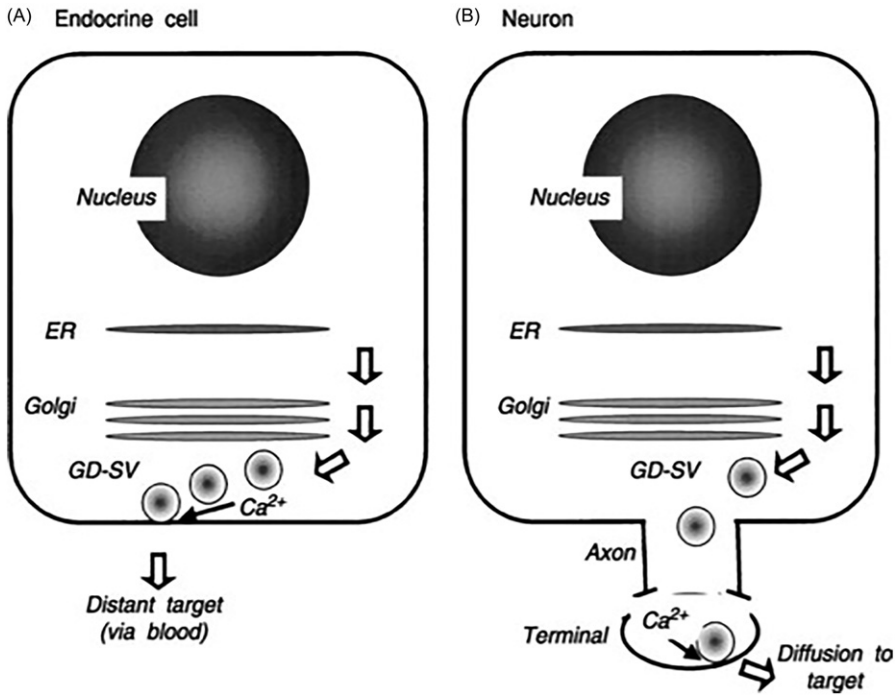

Fig. 1 Schematic representation of the intracellular progression of hormonal peptides produced by gut endocrine cells (A) and by central or peripheral neurons (B). In both cases, mRNA is translated at the endoplasmic reticulum (ER) and precursor peptides progress through the Golgi complex; mature peptides are stored in Golgi-derived secretory vesicles (GD-SV) and released by calcium-dependent exocytosis. Peptide released at the basolateral membrane of endocrine cells is conveyed to target cells via the circulation. In neurons, secretory vesicles progress along the axon to nerve terminals for release; in this case, the secreted peptide typically diffuses to its target cells, although peptides released into the hypothalamo-hypophyseal portal vessels are an exception.

Criteria for the Identification of Gut Hormones in PNS or CNS Neurons

Evidence for the presence of gut hormones in PNS and CNS neurons should be based on the following lines of evidence: (1) expression of the gene identified by techniques such as reverse transcription-polymerase chain reaction, Northern blot analysis, and in situ hybridization and confirmation by cloning and sequencing of the relevant cDNA; (2) the presence of the relevant peptide determined by radioimmunoassay or ELISA, preferably using antibodies to several epitopes, coupled with high-performance liquid chromatography and confirmation by bioassay, chemical isolation, and amino acid sequencing; and (3) demonstration of cellular origins by immunocytochemistry, preferably using multiple antibodies to several epitopes. Localization of neuropeptides may be a useful way of distinguishing subsets of neurons already identified by the presence of classical transmitters and for this purpose double-labeling immunocytochemistry is useful. There may be problems with the specificity of particular antibodies. For these reasons, the interpretation of studies based on the use of a single experimental method, for example, immunohistochemistry, or using a single primary antibody, should be approached with caution.

Cellular Aspects

Regulatory peptides produced in gut endocrine cells, CNS or PNS neurons are, in each case, generated by mRNA translation at the rough endoplasmic reticulum (Fig. 1). The product (prepropeptide) is usually biologically inactive and is rapidly converted to a precursor peptide (propeptide) that progresses through the Golgi complex and is sequestered in secretory vesicles that bud from the *trans*-Golgi network. During transit through the Golgi complex, or in secretory vesicles, the propeptide is subject to any of a variety of different posttranslational modifications. These may include glycosylation, Tyr sulfation, and Ser phosphorylation (all of which occur in the Golgi complex), cleavage of the peptide chain, or COOH-terminal α-amino amidation (which occurs in secretory vesicles). The patterns of posttranslational modification of a specific peptide may differ in neurons and endocrine cells. The Golgi-derived secretory vesicles often possess electron-dense cores and their morphology is characteristic of particular cell types. These vesicles are distinct from the small, clear, synaptic vesicles that recycle at nerve terminals and contain classical transmitters such as acetylcholine, monoamines, γ-aminobutyric acid (GABA), and glutamate. Expression of the genes encoding gut hormones is physiologically regulated, but different mechanisms may regulate the expression of these genes in PNS or CNS neurons.

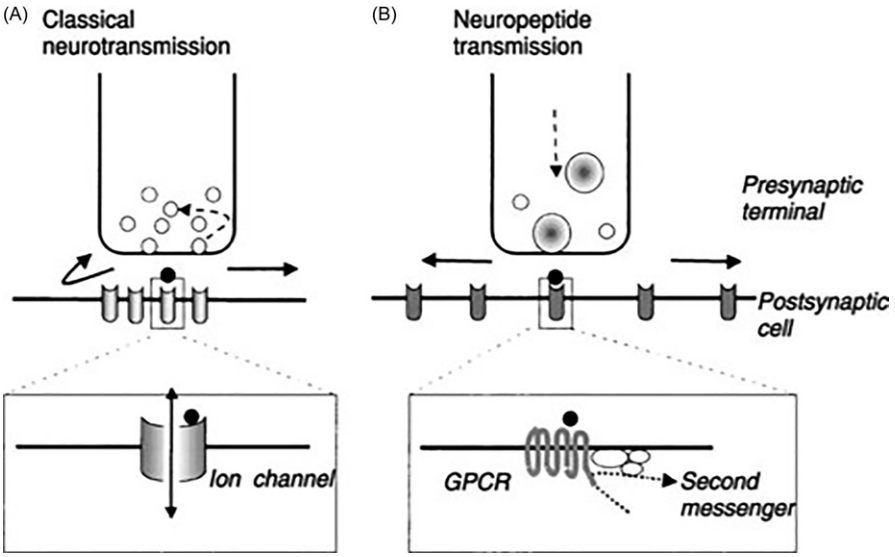

Fig. 2 Schematic representation of some events involving neurotransmission by classical (A) and neuropeptide (B) transmitters. Classical transmitters are stored in small, clear synaptic vesicles that are recycled at the nerve terminal. There are reuptake mechanisms for the neurotransmitter, which is resequestered in these vesicles. Receptors for classical transmitters are concentrated in the postsynaptic membrane and are often ion channels. In contrast, neuropeptides are located in Golgi-derived dense-cored vesicles and after secretion they are degraded by proteolysis either locally or elsewhere after diffusion away from the release site. Neuropeptides typically act at G protein-coupled receptors (GPCR) to trigger second-messenger responses; the receptors may be diffusely distributed on postsynaptic cells and may be some distance from the release site. (●) Putative transmitter.

Cotransmission

Increased intracellular Ca^{2+} leads to exocytosis of both Golgi-derived (neuropeptide-containing) and synaptic vesicle populations (Fig. 2). The actions of neuropeptides are exerted at GPCRs, which are not exclusively localized to postsynaptic membranes or even to neurons. As a consequence, peptides may act over longer distances and for longer times than classical neurotransmitters. Where both neuropeptide and classical transmitters act on the same postsynaptic cell, there may be interactions between them (Hokfelt *et al.*, 2000). For example, increased or decreased sensitivity to the classical transmitter may be mediated by neuropeptides.

Overview of Gut Hormones Expressed by PNS Neurons

Representatives of the gut hormone families are commonly expressed in the major divisions of the autonomic nervous system. In postganglionic parasympathetic neurons, the secretin-like peptides VIP, peptide histidine isoleucine amide (PHI), and pituitary adenylate cyclase-activating peptide (PACAP) are commonly expressed, whereas in postganglionic sympathetic neurons, neuropeptide tyrosine (NPY) is commonly expressed. The enteric nervous system, which is sometimes considered to be a third division of the autonomic nervous system, is an abundant source of neuropeptides, including VIP, CCK, and NPY (Furness, 2000). Many primary afferent neurons express neuropeptides; some of those belonging to the main families of gut hormones (VIP, NPY) are up-regulated after nerve damage. Gut hormonal peptides are not generally found in somatic efferent neurons.

Overview of Gut Hormones Expressed by CNS Neurons

The expression of neuropeptides is a property of very many CNS neurons. Gut hormones or related peptides, for example, somatostatin, CCK, and NPY, are expressed in hypothalamic neurons, where they may act in local circuits or in the control of pituitary hormone release after secretion into hypothalamo-hypophyseal portal vessels. In addition, these and other gut hormones are found in many other CNS regions, including cerebral cortex, hippocampus, brainstem, and striatum.

Major Gut Hormone Families and Their Expression in CNS and PNS Neurons

The following brief sketches are grouped on the basis of peptide families that include at least one member that is produced and released by gut endocrine cells and at least one that is produced and released by CNS or PNS neurons (for more detailed accounts see Walsh and Dockray, 1994).

The Cholecystokinin/Gastrin Family

The *CCK* gene is expressed in intestinal I-type endocrine cells and in many CNS neurons and some PNS neurons. There are differences in posttranslational processing that account for the reported variation between neurons and endocrine cells in the major form of CCK present. In cerebral cortical neurons, CCK may occur together with GABA and in some nigrostriatal neurons it occurs with dopamine. Gastrin originates from pyloric antral G cells and may be found in small amounts in hypothalamus. The CCK1 (also called CCK-A) receptor has high affinity for CCK and low affinity for gastrin; it is expressed in pancreas, gallbladder, and some CNS and PNS neurons. The CCK2 (also called gastrin-CCK-B) receptor has high affinity for both gastrin and CCK and is found on parietal and enterochromaffin-like cells in the stomach and many CNS neurons. The main function that has been ascribed to CCK in the brain is inhibition of food intake. In addition, CCK is thought by some to be associated with anxiety or panic attacks and it may modulate noxious sensations.

The Secretin/Glucagon/VIP/PHI/PACAP/GIP Superfamily

The superfamily of structurally related peptides that includes the classical gut hormones secretin and glucose-dependent insulinotropic peptide (GIP) and the pancreatic hormone glucagon also includes major neuropeptide transmitters such as VIP, PHI, and PACAP. In the case of glucagon, there is good evidence that a single precursor molecule may yield several different products through alternative posttranslational processing. In the pancreatic islet alpha cells, glucagon itself is a primary product; in intestinal L cells, glucagon-like peptide-1 (GLP-1) and GLP-2 are primary products (but not glucagon). CNS neurons resemble intestine more closely than pancreas in the processing of the glucagon precursor; one possible role for the CNS GLPs is in the control of food intake. Two members of the family that seem to be predominantly expressed in neurons, and not endocrine cells, are VIP (the precursor of which also gives rise to the related peptide PHI) and the closely related peptide PACAP.

Somatostatin

Somatostatin-producing endocrine (D) cells are found throughout the gastrointestinal tract and in the islets of Langerhans. In these systems, somatostatin acts locally as a paracrine inhibitor of secretion from nearby cells, for example, G cells in the pyloric antrum and beta cells in the pancreas. In addition, somatostatin is produced in hypothalamic neurons and functions as an inhibitor of growth hormone secretion from the anterior pituitary. It is also found in enteric neurons, sympathetic neurons, and some somatic afferent neurons.

Neurotensin

Neurotensin was first isolated from the hypothalamus and then discovered to be produced in N-type endocrine cells of the ileum and colon, where it is a putative mediator of the ileal brake in the gut. Neurotensin has been identified in multiple CNS neurons; interactions with dopamine have been identified and possible actions as an anti-psychotic agent reported.

The Peptide YY/NPY/Pancreatic Polypeptide Family

Peptide YY is named for the presence of tyrosine residues (Y in the single-letter notation) at both the COOH– and NH_2-terminal positions. It is normally produced in endocrine cells of the ileum and colon. Putative hormonal actions include the inhibition of pancreatic secretion, intestinal transit, and food intake. There is little evidence for neurotransmitter functions, although the 3-36 fragment of PYY acts at Y2 receptors expressed by vagal afferent neurons to inhibit gastric emptying and food intake. The closely related NPY is widely expressed in CNS and peripheral neurons. One of its most striking actions is the stimulation of food intake on injection into the hypothalamus. In addition, it is present in sympathetic postganglionic neurons and may modulate responses to noradrenaline. Another member of the family is pancreatic polypeptide, produced in the islets of Langerhans; its functions as either a hormone or a neurotransmitter remain uncertain.

Ghrelin and Motilin

The related peptides ghrelin and motilin are produced in gastric and small intestinal endocrine cells, respectively. They are distinct from other gut hormones in that they are released from these cells during fasting. Ghrelin is a naturally occurring ligand for an orphan receptor (GHS) originally identified as a putative mediator of growth hormone secretion. Small amounts of ghrelin are

thought to be produced in the hypothalamus. It is a powerful stimulant of food intake; the peptide released from the stomach may stimulate food intake by delivery to the hypothalamus through the circulation or by acting on vagal afferent neurons to suppress the action of satiety factors such as CCK and PYY3-36.

See also: GI Hormone Development (Families and Phylogeny)

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GI Tract: General Pathology of Neuroendocrine Growths☆

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Glossary

Carcinoma Malignant epithelial neoplasm; when composed by neuroendocrine tumor cells is defined as neuroendocrine carcinoma and implies poorly differentiated morphology (NEC) and a rapidly progressive clinical behavior.

Dysplasia Disordered epithelial growth; when for neuroendocrine cells in the gut mucosa it refers to clustered moderately atypical neuroendocrine cells above the basal membrane.

Hyperplasia Increased cell numbers; when for neuroendocrine cells in the gut mucosa it refers to increased number of typical neuroendocrine cells above the basal membrane.

Neoplasia Abnormal mass (tumor) of transformed cells with uncontrolled proliferation and death, either clinically benign and malignant; when composed by neuroendocrine tumor cells is defined as neuroendocrine neoplasm (NEN) and implies malignant clinical behavior.

Neuroendocrine cell Gut epithelial cell specialized in producing hormones and other bioactive substances.

Tumor Epithelial neoplasm with well differentiated morphology; when composed by neuroendocrine tumor cells is defined as neuroendocrine tumor (NET) and implies slowly progressive clinical behavior.

Definition of the Subject

Nonneoplastic and neoplastic growths of the neuroendocrine cells of the gastrointestinal tract are lesions made by abnormal accumulation of neuroendocrine cells and are classified as hyperplastic, dysplastic or neoplastic epithelial cell changes.

History and Histogenesis

Cells belonging to the so-called diffuse neuroendocrine system (DNES) of the gut (**Table 1**) (Solcia *et al.*, 1998) attracted the attention of scientists in the second half of the 19th and early 20th centuries since their ability to interact with chromium salts (chromaffinity) and to reduce silver ions in the absence (argentaffinity) or in presence (argyrophilia) of reducing agents. In parallel, a nonconventional, epithelial tumor of the intestine with slow-growing attitude, the "carcinoid" (carcinoma-like), was described and soon was proven to be composed by argentaffin cells. Gut neuroendocrine cells express common antigens like chromogranin A, neuron specific enolase (NSE) and synaptophysin (defined as general markers of neuroendocrine differentiation) as well as specific hormones (defined as cell-specific markers of neuroendocrine differentiation) (**Table 1**). In the small intestine neuroendocrine cells may express more than hormone, like intestinal secretin cells coexpressing serotonin and GIP cells coexpressing enteroglucagon. In addition, they produce a number of trophic factors and abundantly express the somatostatin receptor subtype 2 (SSR2).

Gut neuroendocrine cells may undergo cell-cycle deregulation resulting in proliferation and tumor growth, as demonstrated in hereditary conditions like multiple endocrine neoplasia syndromes type 1 and 2 (MEN1 and 2) in man and after genetic manipulation in mice. Proliferating or transformed gut neuroendocrine cells may variably express the same antigens as their normal counterpart depending on their differentiation status. Expression of the somatostatin receptor subtype 2 (SSR2) in neuroendocrine tumor cells is important for both diagnostic and therapeutic applications.

Nonneoplastic Growths of Gut Euroendocrine Cells

Nonneoplastic growths of gut neuroendocrine cells have been reported in the stomach, the duodenum and occasionally in the ileum and colon (Rindi *et al.*, 2010b).

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Table 1 Types, hormonal product and distribution of the neuroendocrine cells of the human gastrointestinal tract

Cell	*Main product*	*Stomach*		*Intestine*					
				Small			*Ap*	*Large*	
		CF	*An*	*D*	*J*	*I*		*C*	*R*
P/D1	Ghrelin	+	r	r	r	r			
EC	Serotonin*	+	+	+	+	+	+	+	+
D	Somatostatin	+	+	+	+	r	r	r	r
L	GLI/pYY			r	+	+	+	+	+
A	Glucagon	f							
PP	PP			f					
ECL	Histamine	+							
G	Gastrin		+	+					
CCK	Cholecystokinin			+	+	r			
S	Secretin			+	+				
GIP	GIP			+	+		r		
M	Motilin			+	+		r		
N	Neurotensin			r	+		+		

CF: Corpus-fundus; An: antrum; D: duodenum; J: jejunum; I: ileum; Ap: appendix; C: colon; R: rectum; EC: enterochromaffin; ECL: enterochromaffin-like cell; GIP: gastric inhibitory polypeptide; GLI: preproglucagon fragments; pYY: peptide Tyrosine Tyrosine; PP: pancreatic polypeptide; *: substance P, neurokinins, opioids, guanylin and other peptides. +: presence of cells; r: presence of rare cells; f: presence of cells in fetus and newborn.
Modified from Solcia, E., Capella, C., Fiocca, R., Sessa, F., Larosa, S. & Rindi, G. 1998. Disorders of the endocrine system. In: Pathology of the gastrointestinal tract. In: Ming SC, G. H.(ed.) Pathology of the gastrointestinal tract. 2 ed. Philadelphia: Williams and Wilkins.

Stomach

Nonneoplastic growths are restricted to proliferation of histamine-producing enterochromaffin-like (ECL) cells in the corpus/fundus and of gastrin-producing (G) and somatostatin-producing (D) cells in the antrum/pylorus. Hypergastrinemia-promoted ECL cell hyperplasia is categorized as *diffuse* (increase in neuroendocrine cells of more than twice the standard deviation as compared to age/sex-matched controls), *linear* (sequences of five or more cells inside the basement membrane of the gastric gland), *micronodular* (clusters of five or more neuroendocrine cells up to 150 μm in diameter) and *adenomatoid* (collection of five or more micronodules adherent to each other but with interposition of basement membranes and thin strands of *lamina propria*). Dysplasia is characterized by 150–500 μm lesions formed by moderately atypical neuroendocrine cells and defined as *enlarged micronodules* (nodules of $\geq$150 μm), *adenomatous micronodules* (collections of at least five micronodules), *fused micronodules* (disappearance of the intervening basal membrane between adjacent micronodules) and *microinfiltrative lesions* (microinfiltration of the *lamina propria* by neuroendocrine cells filling the space in between glands).

G cell hyperplasia is defined as an increase of gastrin cell number (above 140) when counted per linear millimeter of mucosa in 5 μm thick histological sections. It may be associated with reduced somatostatin (D) cell count resulting in elevated G/D cell ratio.

Long-standing hyperchlorhydria associated with duodenal G cell tumor may result in increased D cells of the antrum and reduced G/D cell ratio.

Small intestine

Similar to the stomach hyperplastic changes classified as diffuse (>2 times SD), linear ($\geq$5 cells; 2 chains/mm) and micronodular ($\geq$5 cells; size 30–90 μm) are identified in the duodenum as composed by either G od D cells (Anlauf *et al.*, 2005, 2007). On the same line, lesions between 90–120 μm in size are defined as dysplasia.

Increased numbers of somatostatin D cells are observed in the duodenum/ileum of patients with coeliac disease, as well as increased neuroendocrine cells in idiopathic inflammatory bowel conditions.

Neoplasia

General

Neuroendocrine neoplasms of the gut are found at any level of the gastrointestinal tract (**Table 2**). According to recent epidemiological data, in western countries neuroendocrine neoplasms more commonly display an age-standardized rate of about 1/100,000 per anatomical site of the gut, more frequently occur in the colorectum, followed by the small intestine and stomach of females at increasing incidence with age.

The diagnosis of neuroendocrine tumors is based on the identification of their characteristic morphology, the expression of markers of neuroendocrine differentiation and grading. Grading is defined by the World Health Organization (WHO) (Rindi *et al.*, 2010a; Kloppel *et al.*, 2017) and the American Joint Committee on Cancer (AJCC) (Woltering *et al.*, 2017) in three tiers by the mitotic index per 10 high power fields (G1, <2 mitosis; G2, 2–20; G3, >20) and Ki67 proliferation index in percent of tumor

Table 2 Tumor type, distribution and cell features of the neuroendocrine neoplasms of the gastrointestinal tract

Tumor type	*Preferred site*	*Main cell type*	*Hormonal products*
NET	Stomach body/fundus	ECL	Histamine 5HT/5HTP
"	Antrum, duodenum, jejunum	G	Gastrin
"	Duodenum, jejunum	D	Somatostatin
"	Jejunum, ileum, coecum, appendix	EC	Serotonin*
"	Colon, rectum	L	Glicentin, PP, PYY
Gangliocytic paraganglioma	Duodenum	PP, D	PP, Somatostatin
NEC	Stomach, intestine	proto-endocrine	none

ECL: Enterochromaffin-like cell; EC: enterochromaffin; 5HT: 5-hydroxytryptamine (serotonin); 5HTP: 5-hydroxytryptophan; PP: pancreatic polypeptide; PYY: peptide Tyrosine Tyrosine; *: substance P, tachykinins and other peptides.

cells (G1, <3%; G2, 3%–20%; G3 > 20%). According to the current WHO and AJCC classifications, gut neuroendocrine neoplasms are classified as:

- neuroendocrine tumors, NET G1-G3, displaying well differentiated morphology;
- neuroendocrine carcinomas, NEC, by default G3, displaying poorly differentiated morphology.

Staging tools are also provided for each anatomical gut site by both WHO and AJCC.

Neuroendocrine Tumor, NET

Neuroendocrine tumors, NETs are generally characterized by bland histological and cytological features, low cytological atypia, low mitotic index and diffuse expression of general markers of neuroendocrine differentiation. Cell specific markers are in general observed in tumor cell subpopulations. The degree of atypia and the occasional presence of spotty necrosis progresses with the grade progression.

The clinical behavior of NETs correlates directly with grade and stage, the higher the worse.

Stomach

ECL cell NETs

By far the most frequent neuroendocrine tumor of the stomach, ECL cell tumors display strong argyrophilia by silver impregnation techniques, immunoreactivity for chromogranin A and vesicular monoamine transporter 2 (VMAT2), and no or focal immunoreactivity for ghrelin, serotonin, gastrin and somatostatin. The largest fraction of gastric NENs are NET G1-G2, usually at low stage (stage I and II) at diagnosis in more than 60% of surgical series (Solcia *et al.*, 1986; La Rosa *et al.*, 2011).

Three clinico-pathologic subtypes (Rindi *et al.*, 1993, 1999) are defined as: type I, associated with diffuse chronic atrophic gastritis of autoimmune or A type (A-CAG); type II, associated with hypertrophic gastropathy (HG), usually in conjunction with MEN1 and Zollinger–Ellison syndrome (ZES) and type III or sporadic, not associated with specific gastric pathology. Of the three subtypes, type I tumors are the most frequent (70%–80% of published series), occur in elderly female patients and, though multiple and multicentric, are small and limited to mucosa and submucosa and display an excellent survival. Type II tumors are rare (~6% of published series), multiple and multicentric usually display a good prognosis, though metastases may be present and rare cases with aggressive course have been described. By converse the solitary type III tumors are less frequent (~15% of published series), raise in male patients, are larger and, in general, display a more aggressive behavior with frequent metastases and malignant course. Of note, type III tumors may associate with the so-called "atypical carcinoid syndrome" due to histamine and/or 5-hydroxytryptophan hypersecretion.

Other tumor types

Exceedingly rare gastrin G cell NETs have been reported in the antrum similar to rare D cell tumors of both oxyntic and antral mucosa. None of such tumors usually associate with any hyperfunctional syndrome.

Intestine

Gastrin G cell NETs

Gastrin G cell tumors raise in the duodenum or upper jejunum of male patients. When "functioning" they can be defined as gastrinomas, are cause of the ulcerogenic ZE Syndrome and frequently associate with MEN1. Though small in size, G cell tumors, especially when functioning, frequently metastasize especially to local lymph nodes. G cell NETs are usually G1 or, rarely, G2 and at low stage (stage I or II) at diagnosis in more than 50% of surgical series (Vanoli *et al.*, 2017).

D cell NETs

Somatostatin D cell tumors are rare tumors of the duodenum (preferentially at the ampulla of Vater) and upper jejunum in patients of the fifth decade. Often associating with type 1 neurofibromatosis and only rarely with diabetes and/or gallstones, may

be defined as "functioning," though the complete somatostatinoma syndrome (diabetes mellitus, diarrhea, steatorrhea, hypo- or achlorhydria, anemia and gallstones) has been described for pancreatic D cell tumors only. Intestinal D cell tumors are often malignant and may metastasize to both local lymph nodes and the liver. D cell NETs are usually G1 or, rarely, G2 and usually at low stage (stage I or II) at diagnosis in more than 50% of surgical series (Vanoli *et al.*, 2017).

Ganglioneuromatous paraganglioma

Rare tumors composed by neuroendocrine cells, mature ganglion cells and Schwann-like spindle cells, develop in the submucosa of the periampullary duodenal region in middle aged patients. In general, ganglioneuromatous paragangliomas display benign behavior, though local lymph node metastases of the neuroendocrine component have been reported in occasional cases (Vanoli *et al.*, 2017).

Serotonin EC cell NETs

Most serotonin EC cell tumors develop in the intestine, though with decreasing frequency in the ileum, coecum, appendix, jejunum, duodenum, distal colon and rectum. Intestinal EC cell NETs may run a relatively aggressive behavior since often multiple (small intestine), of large size (colon) and deeply infiltrating the intestinal wall, with frequent lymph-node metastases. A typical carcinoid syndrome due to unregulated release of serotonin and other active substances by tumor EC cells may associate, though depending on the establishment of liver metastases. By converse, appendix EC cell tumors, in spite of the deep wall invasion often observed, in general run a benign course. EC cell NETs are usually G1 or, rarely, G2, invariably at high stage (stage III or IV) at diagnosis in more than 90% of surgical series (Jann *et al.*, 2011; Norlen *et al.*, 2012).

Glicentin/PYY L cell NETs

L cell tumors most frequently develop in the colon and rectum and rarely in other sites of the intestine and appendix. The majority of L cell tumors are small, do not associate with any hyperfunctional syndrome and run a benign course (Fiocca *et al.*, 1980). Usually L cell NETS are G1 or, rarely, G2, and at low stage (stage I or II) at diagnosis (Weinstock *et al.*, 2013).

Neuroendocrine carcinoma, NEC

Neuroendocrine carcinomas, NECs are clinically aggressive carcinomas, poorly differentiated in morphology, reported to occur at any site of the gut of patients of the seventh decade. Neuroendocrine carcinomas display solid structure, abundant necrosis, composed by small to intermediate cells with severe cellular atypia, high mitotic index, faint but diffuse chromogranin A expression and intense and diffuse expression of synaptophysin and NSE. Additional features include high Ki67 index and p53 accumulation at immunohistochemistry, while cell-specific neuroendocrine markers are absent. The majority of NEN are high staged (stage III or IV) at diagnosis. Similar to undifferentiated carcinomas, neuroendocrine carcinomas run a very aggressive course.

See also: GI Hormone Development (Families and Phylogeny). Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS). Somatostatin Receptor Expression in Gastrointestinal Tumors

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Gastrin☆

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Glossary

Gastric acid secretion Hydrochloric acid produced by parietal cells in the stomach in concentrations up to 130 mM; secretion is stimulated by gastrin, histamine, and acetylcholine and is inhibited by somatostatin.

Gastric endocrine cells Cells of the gastric epithelium specialized for secretion at their basolateral membrane of regulatory peptides and amines; various populations of cells producing gastrin, somatostatin, or serotonin occur in the pyloric antral part of the stomach, and cells producing histamine, somatostatin, or ghrelin occur in the body of the stomach.

Gastric epithelial cell proliferation Division of stem cells located in the isthmus region of gastric glands; proliferation is stimulated by growth factors and gastrin; cells leaving the cell cycle migrate up or down gastric glands and differentiate into the major gastric epithelial cell types.

Gastric innervation Either nerve fibers running in the vagal or splanchnic nerve trunks to the stomach or neurons with cell bodies in the myenteric or submucous plexus of the gastric wall; various neuronal populations may have sensory, motor, or interneuron functions.

Histamine Released by enterochromaffin-like cells in the body of the stomach and stimulates acid secretion; synthesis, storage, and release of histamine all are regulated by gastrin.

Gastrin is a hormone produced mainly by specialized endocrine (G) cells of the pyloric antral part of the stomach and is secreted in response to food in the stomach. It acts on histamine-secreting enterochromaffin-like cells and acid-secreting parietal cells in the body of the stomach to increase acid secretion. It may also regulate proliferation of epithelial cells in the stomach and other parts of the gastrointestinal tract. Gastrin secretion is inhibited by gastric acid, probably via the paracrine mediator somatostatin. When acid secretion is reduced (in patients with pernicious anemia or individuals treated with proton pump inhibitors), plasma gastrin is elevated. There is also increased plasma gastrin in patients with gastrinoma (Zollinger–Ellison syndrome). The main receptor for gastrin is the cholecystokinin (CCK)-2 (or gastrin–CCK_B) receptor.

Introduction

The idea that gastric acid secretion might be regulated by a hormone was suggested by John Sidney Edkins in 1905. He showed that extracts of the stomach stimulated acid secretion and named the active substance "gastrin." Edkins was influenced in his thinking by the work of William M Bayliss and Ernest Starling, who had shown earlier that an active factor from the small intestine, secretin, stimulated pancreatic secretion. For many years, it was unclear whether gastrin was identical to histamine given that histamine is also abundant in the stomach and stimulates acid secretion. The relationship was clarified by the chemical characterization of gastrin by Rod Gregory and Hilda Tracy and by the demonstration that gastrin releases histamine from enterochromaffin-like (ECL) cells of the gastric corpus.

The Gastrin Gene and Progastrin

A single gastrin gene encodes a precursor peptide of 101 residues in humans. *Gastrin* gene expression is increased by food in the stomach and is inhibited by gastric acid. The main product of mRNA translation, preprogastrin, is rapidly converted to progastrin in the endoplasmic reticulum. Further conversion of progastrin occurs during passage along the secretory pathway.

Progastrin and Its Products

Progastrin may be sulfated at a tyrosine residue in position 86, and phosphorylated at a serine residue in position 96, during passage through the trans-Golgi network (Fig. 1). Progastrin is then sequestered in secretory vesicles, where it may be cleaved by

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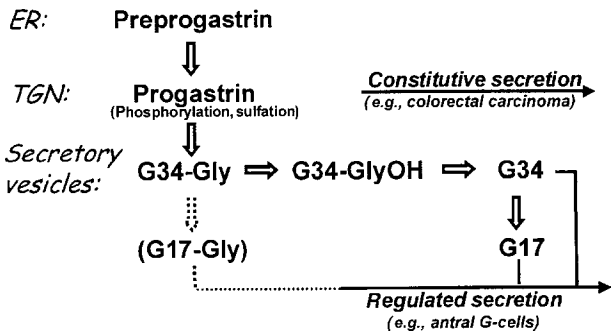

Fig. 1 Schematic representation of the biosynthesis of gastrin. Preprogastrin is translated in the endoplasmic reticulum (ER) and is converted there to progastrin, which translocates through the Golgi complex to secretory vesicles. In the trans-Golgi network (TGN), progastrin may be phosphorylated on Ser-96, sulfated on Tyr-86, or both. In secretory vesicles, it is cleaved to yield a COOH-terminal Gly peptide of 34 residues (G34-Gly), which may then be converted by COOH-terminal α-amidation to G34 or cleaved to G17-Gly. In pyloric antral G cells, G17 and (to a lesser extent) G34 are the main products of regulated secretion. In some tumor cells, progastrin may be secreted by the constitutive pathway direct from the TGN to the cell surface.

enzymes of the prohormone convertase family. Biosynthetic intermediates with a COOH-terminal glycine residue (Gly-gastrins) provide substrates for the enzyme peptidyl α-amino mono'oxygenase, which yields COOH-terminally amidated gastrins (Fig. 1) (Dockray *et al.*, 2001). The amidated gastrins are typically peptides of 17 and 34 amino acid residues (G17 and G34), both of which occur as tyrosine-sulfated and unsulfated peptides. G34 corresponds to an NH_2-terminal extended form of G17. Human gastrin (G) cells normally secrete mainly amidated gastrins, but small amounts of both progastrin and Gly-gastrins may also be released. The COOH-terminal pentapeptide amide of G17 (–Gly–Trp–Met–Asp–Phe–NH_2) is identical to that in the related hormone cholecystokinin (CCK).

Circulating Forms and Metabolism

The concentrations of amidated gastrins in the plasma of normal fasting humans are generally less than 30 pM. A mixed meal increases gastrin concentrations up to about threefold. The major circulating form of gastrin in humans is G34; however, this form is typically $<10\%$ of gastrin stored in G cells. In humans, it is cleared with a half-life of approximately 35 min, compared with approximately 7 min for G17. The difference in clearance accounts in part for the tendency of G34 to accumulate in plasma.

The G Cell and Its Control

G cells are found in the pyloric antral mucosa of all mammalian species. In humans, there are also some G cells in the duodenum. After a meal, gastrin is released in response to gastric luminal stimuli (mainly protein, peptides, and amino acids) and in response to nervous stimuli (Fig. 2). One candidate neurotransmitter regulating the G cell is gastrin-releasing peptide (GRP), which is a mammalian relative of the amphibian skin peptide bombesin. Gastric acid inhibits gastrin release, producing a negative feedback loop. Therefore, decreases in gastric acid secretion, either through disease (e.g., gastritis) or by drug treatment (e.g., proton pump inhibitors), tend to increase gastrin release. The effects of acid are mediated by release of somatostatin from D cells in the antrum, which then suppresses the G cell by a paracrine mechanism (Fig. 2). In patients with *Helicobacter pylori*, there is a tendency for

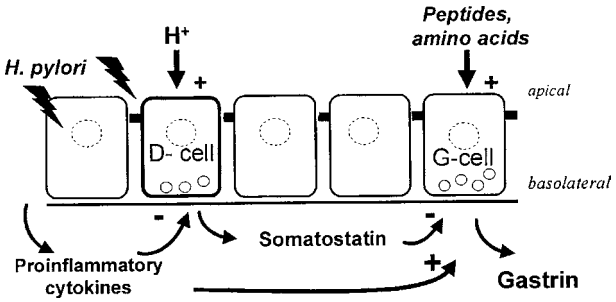

Fig. 2 Schematic representation of cellular relationships in the pyloric antral epithelium. The release of gastrin from G cells is stimulated by peptides and amino acids in the gastric lumen and by neurotransmitters (not shown). Acid releases somatostatin from D cells, and this inhibits gastrin release. In the presence of the gastric bacterium *Helicobacter pylori*, proinflammatory cytokines may inhibit D cells and stimulate G cells and so enhance gastrin release indirectly and directly.

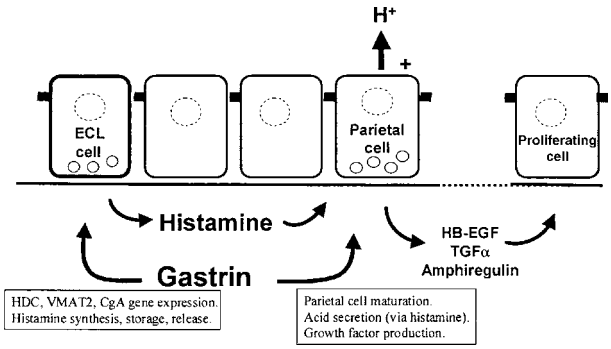

Fig. 3 Schematic representation of some effects of gastrin in the gastric corpus. Gastrin stimulates histamine release from enterochromaffin-like (ECL) cells, and this in turn stimulates acid secretion from parietal cells. In ECL cells, gastrin also increases the production of histidine decarboxylase (HDC), which makes histamine from histidine; vesicular monoamine transporter type 2 (VMAT2), which sequesters histamine in secretory vesicles; and chromogranin A (CgA), which is co-stored with histamine. ECL cell numbers are regulated by gastrin. Parietal cells may also respond directly to gastrin. In addition to acute effects on acid secretion, gastrin controls the maturation of parietal cells and stimulates the production of growth factors, particularly members of the epidermal growth factor (EGF) family, including heparin-binding EGF, transforming growth factor (TGF)-α, and amphiregulin, which increase proliferation of other cells in the epithelium.

increased gastrin release. This is attributable to proinflammatory cytokines that are thought to act partly by inhibiting D-cell function and partly by directly stimulating G cells (**Fig. 2**).

Gastrin Receptors

The effects of gastrin are exerted at CCK2 receptors (also known as gastrin–CCK_B receptors). These are found on gastric parietal and ECL cells, some smooth muscle cells, and some central nervous system (CNS) neurons. This receptor has high affinity for peptides with the C-terminal sequence –Try–Met–Asp–Phe–NH_2; the C-terminal amide is essential for activity. Both gastrin and CCK therefore exhibit high affinity at CCK2 receptors. However, gastrin is the main ligand for receptors on parietal and ECL cells because its plasma concentrations are roughly 10 times higher than those of CCK. However, CCK is the main ligand for CNS receptors because there is abundant CCK, but little or no gastrin, in the brain. The CCK2 receptor belongs to the family of G protein-coupled receptors, characterized by seven transmembrane domains. It acts via the $G\alpha_{q/11}$ group of G proteins to increase intracellular calcium concentrations and protein kinase C activity. Several antagonists have been generated, including YF476 (netazeptide) and L-740,093.

The Physiology of Gastrin

Acid Secretion

The primary action of gastrin is the stimulation of acid secretion. The question of whether gastrin acts directly on the acid-producing parietal cells or indirectly via histamine release from ECL cells has been the subject of intense debate. It is now generally thought that gastrin acts mainly via histamine release from ECL cells (**Fig. 3**). Immunoneutralization of circulating gastrin, or administration of CCK2 receptor antagonists, inhibits gastric acid responses to food. In mice in which the gastrin or CCK2 receptor genes have been deleted by homologous recombination, there is a loss of acid secretion that is refractory to acute stimulation by gastrin, histamine, or cholinergic agonists (Hinkle and Samuelson, 1999). However, acid secretory capacity can be restored by infusion of gastrin, indicating a role for this hormone in maturation of the parietal cell as well as in acute secretory responses after a meal.

ECL Cells

Gastrin increases the expression of several genes in ECL cells that are important for histamine synthesis and storage; these include histidine decarboxylase (HDC), which makes histamine from histidine; vesicular monoamine transporter type 2 (VMAT2), which transports histamine into secretory vesicles; and chromogranin A, which is co-stored with histamine in secretory vesicles (**Fig. 3**). In addition, gastrin increases ECL cell number. In patients with prolonged increases in plasma gastrin, there is a tendency for ECL cell hyperplasia.

Proliferation

Gastrin increases the proliferation of epithelial cells in the stomach. Apart from ECL cells, it is not clear whether proliferating cells express the CCK2 receptor. Therefore, proliferative responses are thought to be due to release of growth factors, including those of the epidermal growth factor (EGF) family (**Fig. 3**) (Dockray, 1999).

Motility

Gastrin contracts smooth muscle and may regulate gastrointestinal motility, but the physiological significance of these effects is still uncertain.

Central Nervous System

There are abundant CCK2 receptors on CNS neurons, but it is thought that the related peptide CCK is the main ligand for these receptors. There is little evidence to suggest that circulating gastrin released from antral G cells in mammals influences CNS function.

Gastrin in Gastrointestinal Disease

Elevated plasma gastrin concentrations occur in patients with gastrin-secreting tumors of the pancreas (Zollinger–Ellison syndrome, gastrinoma) and are associated with increased acid secretion and intractable peptic ulcer. There are also high plasma gastrin concentrations in patients with pernicious anemia, which is attributable to autoimmune destruction of parietal cells and loss of acid inhibition of the G cell. In these patients, and also in patients with mutations of the *menin* gene (i.e., multiple endocrine neoplasia type 1), hypgastrinemia is associated with ECL cell neuroendocrine (carcinoid) tumors. In patients with pernicious anemia, these may resolve after removal of gastrin by antrectomy.

Moderately elevated plasma gastrin concentrations also occur in some patients infected with *H. pylori* (**Fig. 2**). When the infection is limited to the antrum, increased gastrin release may lead to increased acid secretion and duodenal ulcer.

The *gastrin* gene is also expressed in colorectal carcinoma, but the main products of expression are progastrin and Gly-gastrin (**Fig. 1**). There is some evidence that these are growth factors for colon tumor cells. They do not act at CCK2 receptors, and the relevant receptor is still uncertain. There is also evidence that gastrin may play a role in tumors of the pancreas, stomach and esophagus (Rozengurt and Walsh, 2001; Hayakawa *et al.*, 2016).

Two strategies are presently being explored for inhibiting the actions of gastrin. A CCK2 receptor antagonist (netazepide) has been shown to be useful for treatment of gastric neuroendocrine tumors in patients with hypergastrinemia (Boyce *et al.*, 2016). An immunoneutralization strategy using a vaccine promoting antibodies directly to the N-terminus of G17 and G17Gly is being explored for treatment of patients with pancreatic cancer.

See also: Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS). Pancreatic Islet Cell Tumors. Somatostatin Receptor Expression in Gastrointestinal Tumors

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Cholecystokinin (CCK)☆

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Glossary

Gastrin A gastrointestinal polypeptide hormone produced by enteroendocrine cells of the gastric antrum that stimulates gastric acid secretion.

G Protein-coupled receptor A family of cell surface receptor proteins consisting of seven transmembrane regions; the intracellular region interacts with GTP-binding proteins that, on ligand binding, transduce signals within the cell, leading to a cellular response (e.g., secretion, motility, growth).

Lamina propria The intestinal lamina propria is the layer separating the intestine's epithelial-cell layer and muscle-cell layers; it is composed extra-cellular fluid, connective tissue, capillaries, lymphatic vessels, and myofibroblasts that support the villi, and it contains numerous nerve endings and immune cells; enteroendocrine cells secrete directly into the lamina propria.

Microvilli Finger-like extensions along the apical surface of intestinal mucosal cells; enterocyte microvilli increase the absorptive surface of the cell; enteroendocrine cell microvilli allow potential intra-luminal stimuli greater exposure to receptors.

Myenteric plexus Also known as Auerbach's plexus, a system of nerves and ganglia lying within the longitudinal and circular muscle layers of the intestine; its nerves innervate numerous targets, including the myenteric externa, mucosa, and sympathetic prevertebral ganglia.

Pylorus The region of circular smooth muscle surrounding at the junction of the stomach and duodenum; pyloric contractions are critical regulators of gastric emptying.

Sphincter of Oddi The region of circular smooth muscle at the distal end of the common bile duct and pancreatic duct as they enter into the duodenum; when constricted, this sphincter prevents flow of bile and pancreatic juice into the duodenum and restricts reflux of duodenal contents back into the bile and pancreatic ducts.

Submucosal plexus A network of nerves and small ganglia found in the submucosa of the intestine; comprised of outer and inner layers; controls intestinal secretion, motility and vasodilation; primary sensory nerves contained in this plexus, which also communicates with the myenteric plexus.

Cholecystokinin (CCK) is a peptide produced and secreted by enteroendocrine cells of the upper small intestine and by neurons in the gastrointestinal tracts and brain. It has several functions. Gastrointestinal CCK, the subject of this article, is the major hormone responsible for stimulating pancreatic enzyme secretion and gallbladder contraction, contributes to meal-ending satiation, slows gastric emptying, and affects intestinal motility.

Introduction

Cholecystokinin (CCK) is a peptide hormone produced by enteroendocrine cells of the upper small intestine. It is secreted during and after ingestion of meals. It was discovered in 1928 as the third gastrointestinal hormone (after secretin and gastrin) by Ivy and Oldberg, who found that intestinal extracts could stimulate gallbladder contraction in dogs and named the substance cholecystokinin ("cholecyst" [gallbladder] and "kinin" [to move]). In 1943, Harper and Raper noted that a similar extract, which they named "pancreozymin," stimulated pancreatic enzyme secretion. Not until CCK was purified and its amino acid sequence was determined by Jorpes and Mutt in 1968 was it clear that CCK and pancreozymin were the same hormone. The original name, cholecystokinin, is now used.

Molecular Forms

Hormonal CCK circulates predominantly in a 58 amino-acid form (CCK-58) (Rehfeld *et al.*, 2007; Stengel *et al.*, 2009). In contrast, neuronal CCK is an eight amino acid peptide, CCK-8. The biologically active region of CCK resides in its carboxyl terminus, which is identical in all forms of CCK: -Gly-Trp-Asp-Met-Phe-NH2. This terminal sequence is identical to that of gastrin, so that gastrin

☆*Change History*: April 2018. Nori Geary updated the text of the entire chapter, added the section "Biological Actions of CCK," removed figures, and updated references.

This article is an update of Rodger A. Liddle, CCK (Cholecystokinin), In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2003, Pages 485–489.

*Retired.

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has some weak CCK-like activity, and CCK has some weak gastrin-like activity. CCK is produced from a single gene that encodes a 115 amino acid preprohormone. In humans, the CCK gene is located on chromosome 3, NC_000003.12. CCK expression developmentally regulated in a tissue-specific fashion. In the intestine, the CCK gene is expressed prenatally and is regulated postnatally primarily by the frequent exposure of CCK cells to nutrients.

Distribution

Cholecystokinin cells are gastrointestinal endocrine cells (enteroendocrine cells) that are scattered throughout the mucosa of the small intestines, with the density greatest in the duodenum and proximal jejunum and least in the ileum. Early electron microscopy results suggested that CCK was synthesized and released by a unique enteroendocrine cell type, called I-cells (Polak *et al.*, 1975). Recent data, however, indicate that many enteroendocrine cells that express and secrete CCK also express and secrete other peptides, including ghrelin, GLP-1, PYY, GIP, neurotensin, and secretin.

The apical surface of enteroendocrine CCK cells is open to the lumen of the intestine. Like enterocytes, these enteroendocrine cells possess microvilli that increase the exposed surface area, thereby increasing exposure to intra-luminal stimuli. The apical surface of CCK cells express a variety of nutrient receptors that control CCK secretion. CCK is released through the basal aspect of the CCK cells into the intestinal lamina propria, where it may act on nearby cells or diffuse into intestinal capillaries and be carried via the hepatic portal vein and liver into the systemic circulation.

CCK is also present in vagal (cranial nerve 10) efferent neurons in the abdomen. In the intestine, vagal efferent CCK neurons synapse upon postganglionic cholinergic neurons that stimulate smooth muscle contraction. Vagal efferent CCK neurons also innervate the islets of Langerhans of the endocrine pancreas. In addition, CCK is present in neurons of the myenteric and submucosal plexi of enteric neurons within the gastrointestinal tract.

CCK is expressed by many neurons in the central nervous system (CNS), including the cerebral cortex, olfactory bulb, several hypothalamic nuclei, the thalamus, hippocampus, amygdala, ventral tegmental area, substantia nigra, nucleus of the solitary tract, and more. CNS CCK, which is mainly CCK-8, may have roles in learning and memory, anxiety, analgesia, and other functions. For a review of CNS CCK and its functions, see Beinfeld (2013).

CCK Receptors

CCK exerts its biological actions by binding to cell-surface receptors on its target tissues. Gastrointestinal CCK receptors are expressed widely in the pancreas, gallbladder, stomach, pylorus, lower esophageal sphincter, intestines, vagal afferent neurons, enteric and peripheral nerves and the central nervous system. Two types of CCK receptors have been identified. CCKAR (CCK "alimentary" receptors; sometimes called CCK-1 receptors) predominate in the periphery and appear to mediate most of intestinal CCK's effects. Full activation of the CCKAR requires the seven carboxyl terminal amino acids and sulfation of the tyrosine located seven amino acids from the terminus. CCKBR (CCK "brain" receptors; sometimes called CCK-2 receptors) are identical to the gastrin receptor and predominate in the central nervous system. Both receptors are G-protein-coupled, seven-membrane-spanning proteins (Wank *et al.*, 1994; Kopin *et al.*, 1992). The human CCKAR gene is located on chromosome 4, NC_000004.12, and the human CCKBR gene, on chromosome 11, NC_000011.10.

CCK Release

Intestinal CCK cells release CCK in response to ingestion of proteins, lipids and carbohydrates (Steinert *et al.*, 2017). On a calorie basis, lipids are the most potent secretagogues, proteins intermediate, and carbohydrates least. Secretion is stimulated by the detection of the products of digestive hydrolysis of proteins and triglycerides by specialized receptor molecules on the apical surface of intestinal CCK cells (**Table 1**). Fatty acids with chain length $\geq$ 12C stimulate CCK secretion much more than fatty acids $<$12C; less saturated long-chain fatty acids may be more potent secretagogues than more saturated fatty acids. Carbohydrate digestion may not be required, as inhibition of carbohydrate hydrolysis did not affect CCK secretion. In addition to these direct nutrient effects, CCK is secreted indirectly by CCK-releasing factors such as "pancreatic monitor peptide" and "intestinal luminal CCK-releasing factor" (Miyasaka *et al.*, 1989; Wang *et al.*, 2002), at least in rodents. Such releasing factors are ordinarily degraded by pancreatic proteases. After a meal, however, the proteases bind preferentially to ingested proteins, allowing more releasing factors to reach the CCK cells and stimulate secretion. There may also be neural inputs to CCK cells that affect CCK secretion. As a result of all these controls of secretion, plasma levels of CCK increase from basal levels ($\sim$1–3 pMol) within about 10 min of the onset of a mixed-nutrient meal, reach a peak of $\sim$5–8 pMol 30–60 min after meal onset, and return to basal after 3–5 h (Eysselein *et al.*, 1990; Rehfeld, 1998).

The table is based on the evidence of receptor expression in mice, rats or humans; see Steinert *et al.* (2017) for details. Abbreviations: CASR, calcium-sensing receptor; CD36, thrombospondin receptor; FFAR, free-fatty acid receptor; GNAT3, guanine nucleotide-binding protein, alpha transducing 3; LPAR5, lysophosphatidic acid receptor 5; SLC15A1, solute carrier family 15 (oligopeptide transporter), member 1; TAS1R1, taste receptor, member 1; TAS1R2, taste receptor, member 2; TAS1R3, taste

Table 1 Nutrient receptors expressed by enteroendocrine CCK cells

Receptor	*Nutrient sensed*
CASR	Calcium
CD36	Fatty acids
FFAR1	Fatty acids
FFAR4	Fatty acids
LPAR5	Lipids
GNAT3	Sugars, bitter
LPAR5	Lipids
SLC15A1	Peptides, amino acids
TAS1R1/TAS1R3	Glutamate, sugars
TAS1R2/TAS1R3	Bitter, sugars

receptor, member 3.; T1R1/T1R3; note that abbreviations are for the human genes, although many of the receptors indicated have been identified on the respective enteroendocrine cells so far only in mice or rats. Names and abbreviations are current recommendations of the National Library of Medicine of the United States (www.ncbi.nlm.nih.gov/gene/).

Modes of Action

Neural CCK appears to act as in a neurocrine mode; that is, it is a neurotransmitter that is released from a presynaptic CCK neuron into a narrow synaptic cleft and diffuses the short distance to receptors on the post-synaptic neuron. Intestinal CCK was long assumed to act in a classical endocrine mode; that is., secreted CCK reached distant target tissues via the circulation. Animal research indicates that this is only part of the story, and that intestinal CCK probably has three additional, local modes of action that do not involve transport via the blood. First, it appears to act in a paracrine mode. This involves diffusion of CCK released into the intestinal lamina propria to CCK receptors on neighboring nonneural cells. Second, CCK act in a mixed neuroendocrine-paracrine mode, in which it diffuses to reach CCK receptors on vagal and other nerve endings in the lamina propria. Third, recent discoveries by Liddle and his colleague indicate that CCK may act in an additional neurocrine-like mode following release from axon-like cytoplasmic extensions of the enteroendocrine cells, called neuropods (Bohorquez *et al.*, 2015). These neuropods end mainly in close apposition to glial cells of the enteric nervous system. Ultra-structural data suggests that these appositions have a synapse-like function. The neuropod mode of action presumably provides for more specific and rapid cell-to-cell signaling than other local modes or the endocrine mode. The convention is to continue to identify signaling molecules that have local as well as endocrine modes of action hormones.

Biological Actions of CCK

Potential biological functions of hormones and neurotransmitters are identified by administration of hormones in pharmacological doses or patterns, by transgenic gene-deletion ("knock-out") models, or other similarly gross methods. Proof of endogenous function is more difficult and requires fulfillment of several exacting criteria. The most difficult of these involve measurement and mimicry of endogenous levels and patterns of hormone or neurotransmitter levels at their sites of action and reversal of the action of the hormone or neurotransmitter by antagonism of receptor function within its dynamic range. When the receptors are freely accessible to the blood, blood levels of hormones are considered adequate to assess hormone levels at the site of actions. This would not be the case, for example, for assessing the actions of peripheral hormones within the brain, which is functionally separated from the circulating hormones by the blood-brain barrier.

A classic series of experiments by Liddle and his colleagues (Liddle *et al.*, 1985, 1989) demonstrated conclusively that Ivy and Oldberg's (1928) hypothesis that CCK stimulates gallbladder contraction was correct. These investigators, first, measured plasma CCK after a mixed-nutrient meal and found a close relationship between the increase in plasma CCK and gall bladder contraction, which was visualized ultrasonically; second, found a CCK infusion dose that mimicked these plasma levels; third, found that such CCK infusions were sufficient to stimulate gall bladder contraction in the absence of food intake; and, fourth, found that pretreatment with a selective and potent CCKAR antagonist completely blocked the meal-stimulated gall bladder contractions. Thus, CCK is a physiological controller of human meal-related gall bladder contraction.

Similar experiments involving tests of infusions of "physiological" CCK doses or CCK receptor antagonists in healthy humans indicate that intestinal CCK is also a physiological controller of pancreatic exocrine secretion, relaxation of the sphincter of Oddi, pyloric contraction, gastric and duodenal motility, gastric emptying, at least of liquid foods, and meal-ending satiation (Hildebrand *et al.*, 1990; Konturek *et al.*, 1990; Steinert *et al.*, 2017). As a result of its effect to slow gastric emptying, CCK also contributes to the physiological regulation of meal-related plasma glucose levels (Steinert *et al.*, 2017). Most of the functions

mentioned are mainly or wholly CCKAR-mediated, except CCK's pancreatic actions, which are predominately CCKBR-mediated. Other effects remain under investigation in animals and humans.

CCK's satiation effect is especially interesting. Although many peripheral hormones are hypothesized to contribute to the physiologic control of eating, at present only CCK has fulfilled both the physiological dose and the antagonist criteria (Steinert *et al.*, 2017). Studies in rodents indicate that CCK satiation is mediated by vagal afferents (Smith *et al.*, 1985), that it physiologically modulates the potent eating-stimulatory effect of hypothalamic AgRP neurons (Beutler *et al.*, 2017), and that in females, CCK satiation is modulated by estrogens (Asarian and Geary, 2013). In a sample of obese patients, allelic variations in CCK were related to increased predisposition to eat overly large meals (de Krom *et al.*, 2007). The potential of several CCKAR agonists as anti-obesity therapies is currently under investigation in preclinical studies.

Clinical Aspects

CCK has several diagnostic uses, including to stimulation of gallbladder contraction for radiographic tests of the gallbladder function and, together with secretin, as a challenge test of pancreatic exocrine insufficiency.

CCK injections are sometimes used to stimulate gallbladder contraction in order to reduce gallbladder sludge and prevent gallstone formation in patients undergoing parental alimentation.

Meal-related secretion of CCK is markedly blunted in patients with and bulimia nervosa (Geracioti and Liddle, 1988), but the causes of this and its potential significance to the pathophysiology of bulimia nervosa are unknown. Low blood levels of CCK sometimes occur in patients with celiac disease or other conditions that delay gastric emptying. The defect in CCK secretion in celiac disease is likely due to loss of function of small intestinal CCK cells that are affected by the disease.

It is not known whether CCK deficiency has pathophysiological consequences, nor are there known diseases of cholecystokinin excess.

See also: GI Hormone Development (Families and Phylogeny). Gastrin. GI Tract: General Anatomy (Cells)

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Pancreatic Polypeptide (PP)☆

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Glossary

Diabetes mellitus A metabolic disease in which there is a deficiency or absence of insulin secretion by the pancreas.
Gastric antrum A dilated portion of the lower end of the stomach, between the body of the stomach and the pyloric canal.
Hypoglycemia A condition in which the concentration of glucose in the blood is abnormally low.
Pancreatogenic diabetes A form of secondary diabetes due to disease, injury, or absence of the exocrine pancreas.

Introduction

After early studies in animals suggested a role of PP as a late-phase anti-digestive hormone with inhibitory effects on exocrine secretion and choleresis, subsequent studies in animals and man have demonstrated that PP is a gluco-regulatory peptide. Since our previous review of this subject (Kono *et al.*, 2004), new research has further supported the possible therapeutic role of PP. This article summarizes the properties of PP and the studies that support its potentially important clinical role in glucose homeostasis. Therapeutic opportunities that may result from an improved understanding of the physiology of PP in diabetes and other clinical conditions are also highlighted.

Structure, Synthesis, and Distribution

PP is a 36-amino-acid straight-chain peptide with a free NH_2 terminus and a COOH-terminal tyrosine amide with a characteristic 'hairpin fold' (**Fig. 1**). In 1968, J. R. Kimmel and colleagues discovered PP during purification of insulin from the pancreatic extracts of chickens. The peptide, first named avian PP, was later extracted from bovine, porcine, and human pancreatic tissue by R. E. Chance and co-workers. The peptides isolated from different mammalian species differ by only one or two residues (Lin and Chance, 1974). In man, the hormone is secreted from specialized cells called F cells or PP cells that are predominantly located in the ventral (uncinate) process of the pancreas (Gates and Lazarus, 1977) (**Fig. 2**). As shown by L. Orci (1976), PP cells account for 10%–15% of the total islet cell population, and outnumber both alpha and delta cells. They are found in the islets and in clusters throughout the exocrine pancreatic tissue. In some species, such as the dog, a few PP cells are found in the gastric antrum (Gersell *et al.*, 1979). In addition, antibodies to PP react with a population of neurons in both the central and peripheral nervous systems.

PP is a member of the 'PP family' of structurally related regulatory peptide hormones. Other peptides include peptide YY (PYY) and neuropeptide Y (NPY). As their names suggest, the peptide members share considerable amino acid sequence homology, but are found in

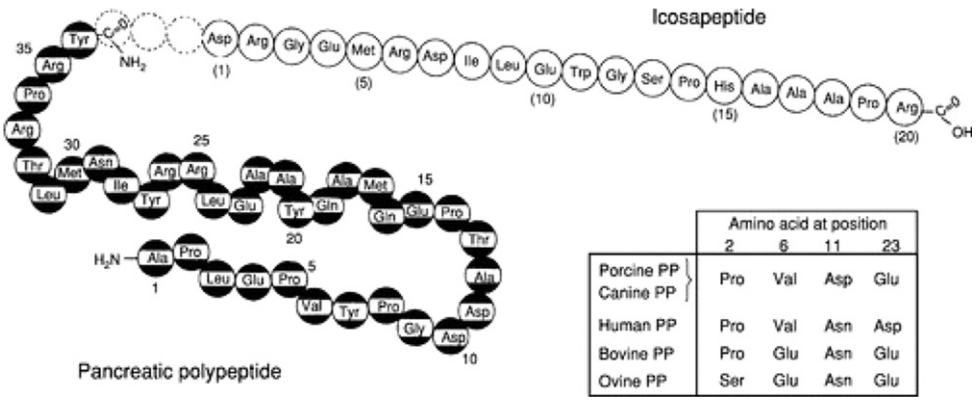

Fig. 1 Pancreatic polypeptide is a 36-amino-acid peptide that is secreted by the F cells of the islet. There is significant homology across species.

☆*Change History:* July 2014. Andersen and Brunicardi extensively revised the text.
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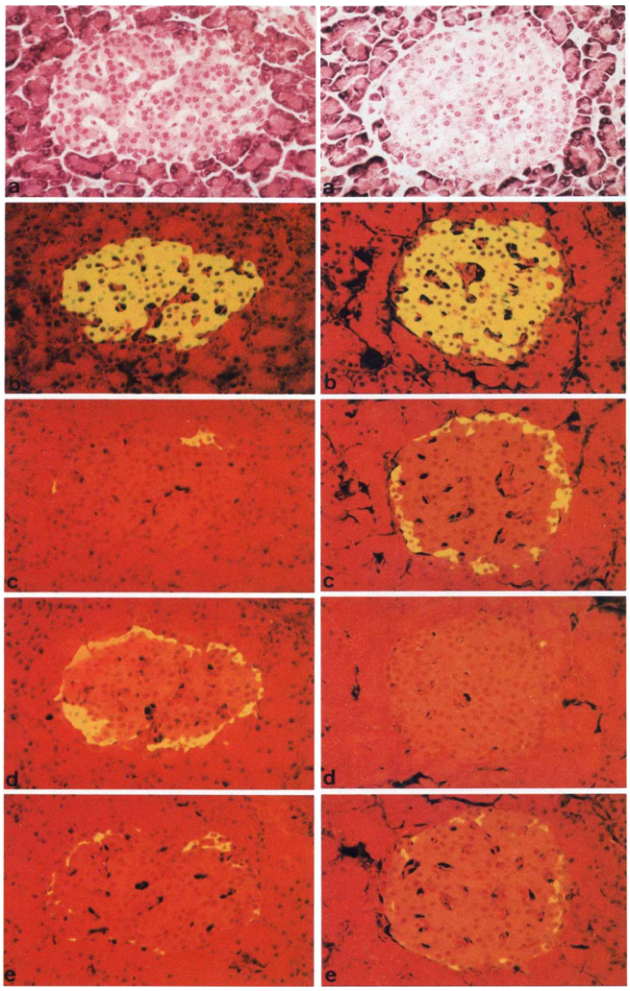

Fig. 2 Histologic anatomy of the islet. Serial sections of a representative islet found in the head or ventral (left panel) and tail or dorsal (right panel) portions of the pancreas. (a) cells stained with hematoxylin and eosin. (b) Beta-cells immunohistochemically stained with anti-insulin antiserum. (c) Alpha-cells stained with anti-glucagon antiserum. (d) Pancreatic polypeptide cells stained with anti-pancreatic polypeptide antiserum. (e) Delta-cells stained with anti-somatostatin antiserum. Reprinted from Orci, L. (1982). Macro- and micro-domains in the endocrine pancreas. *Diabetes* **31**, 538, with permission.

Table 1 Alterations in PP levels

Elevated levels in:	Normal aging
	Type 2 diabetes (Obesity accompanied by glucose intolerance)
	Early stage type 1 diabetes
	Neuroendocrine (islet cell) tumors
Deficient levels in:	Type 3c diabetes due to chronic pancreatitis, cystic fibrosis, proximal or total pancreatectomy
	Late stage Type 1 diabetes
	Diabetic autonomic neuropathy
	Vagal denervation

widely disparate locations, including the pancreas (PP), the distal gut (PYY), and the central nervous system (NPY). The circulating levels of PP have been found to vary with a number of normal and pathologic conditions in humans (**Table 1**). The presence of a family of G protein-coupled receptors specific for the PP family called Y receptors has been confirmed and the population of high affinity hepatic Y4 receptors has been shown to vary inversely with the circulating levels of PP (Seymour *et al.*, 1998).

In addition to the liver, the Y receptor family has been found to be expressed in the intestine, spinal cord, adrenal gland, in ganglia of the midbrain and brain stem, and in the vas deferens. At least 5 sub-types of Y receptors have been identified, and the PP family of peptides cross-react with different members of the Y receptor family with variable affinity (Berglund *et al.*, 2003). PP has the greatest affinity for the Y4 receptor sub-group, and Y4 receptors have been found in the gastro-entero-hepatic system, the brain, and the cardiovascular system (Bard *et al.*, 1995).

Pharmacokinetics and Metabolism

The half-life of exogenously administered PP in the vascular system is 5–7 min (Floyd *et al.*, 1978). The metabolic clearance rate and volume of distribution do not change significantly when blood concentrations of the peptide are increased. Sustained elevations of PP after a meal cannot be accounted for by a low metabolic clearance rate, but rather indicate that the peptide is continuously released in the postprandial period to maintain the high level.

PP levels are elevated in patients with chronic renal failure. A close inverse correlation exists between the glomerular filtration rate and serum PP levels, which suggests that the kidney may play a dominant role in the clearance of PP from the circulation.

Regulation of Secretion

PP is promptly released into the blood following a meal. The response to food is generally biphasic, with plasma concentrations rising four- to sixfold above basal levels beginning within 5 min after food ingestion. The initial release lasts 30–60 min and is followed by a secondary response lasting up to 5 h. Protein is the most potent enteral stimulator of PP release, closely followed by fat, whereas glucose has a lesser effect (Floyd *et al.*, 1978).

Vagal mechanisms are not only the most powerful stimulators of PP secretion, but also are required for nearly all other stimuli of the PP cell. Central vagal activity can be increased either by sham-feeding, a rather specific but mild stimulation, or by hypoglycemia, a less specific but stronger stimulus. The PP response is eliminated by vagotomy and can be abolished by atropine, even at very small doses. The presence of an intact PP response to ingested nutrients has therefore been taken as an indication of vagal integrity, or as a test for incomplete vagotomy (Glaser *et al.*, 1980).

After sham-feeding, PP concentrations remain elevated for hours, even though the stimulus may last for only 15 min. Cessation of electrical vagal stimulation is followed by a rapid decline of plasma PP concentrations with a half-life of 5.5 min, similar to the half-life of exogenously administered PP. Thus, hypoglycemia or sham-feeding causes prolonged activation of the 'vagal center' responsible for PP secretion. This phenomenon may be an important aspect of the prolonged PP secretion detected after a meal.

The isolated perfused human pancreas model has been used to investigate the neural regulatory mechanism of the endocrine pancreas. In this model, human pancreata are procured from heart-beating organ donors. Following organ procurement, the pancreas is perfused via the splenic artery and the effluent is collected from the splenic vein. Electrical stimulation of the neural plexus along the splenic artery can be performed in the presence or absence of selective neural blockers and hormonal responses in the venous effluent can be examined. In this model, cholinergic stimulation with combined perfusion of phentolamine and propranolol (α- and β-adrenergic antagonists) during splanchnic nerve stimulation causes a marked stimulation of PP release, consistent with the vagal dependency of *in vivo* PP release (Brunicardi *et al.*, 1988). Activation of α-adrenergic fibers causes strong suppression of PP secretion and activation of β-adrenergic fibers causes mild stimulation.

PP release may also be mediated by enteric hormonal agents such as cholecystokinin (CCK), secretin, bombesin, and gastric inhibitory polypeptide (GIP). Intravenous infusion of cerulein, an analogue of CCK, stimulates an increase in plasma PP levels in humans and infusions of 20% pure CCK in humans achieve similar results. The most potent stimulus for PP release on a molar basis in dogs is pure CCK, followed by octapeptide of CCK and synthetic human gastrin. Phenylalanine and tryptophan, instilled into the duodenum, cause a significant increase in PP levels. Duodenal acidification also releases PP, but the response is significantly less than that obtained by infusion of the two amino acids. The mechanism of PP release by phenylalanine and tryptophan appears likely to involve the intermediate release of CCK, which in turn stimulates the release of PP. Oleate and liver extract, both strong releasers of CCK, also stimulate the release of significant amounts of PP when infused into the intestine of dogs. These findings suggest that endogenously released CCK plays an important role in the prolonged or so-called intestinal phase of PP release.

Gastro-Entero-Hepatic Actions of PP

The basal secretion of pancreatic fluid and protein is modestly inhibited by physiologic doses of PP in dogs and in humans. Therefore PP has been proposed as a post-digestive hormone which serves as a 'brake' or reversal agent for meal-stimulated secretion (Hazelwood *et al.*, 1973). At physiologic doses, PP inhibits secretin- and cerulein-stimulated pancreatic protein and bicarbonate secretion in dogs. Secretin- and CCK-stimulated pancreatic secretion of trypsin was markedly inhibited and bicarbonate output was less markedly, but significantly, inhibited by PP. In humans, PP inhibited secretin-stimulated pancreatic secretory volume and enzyme concentration, but had no effect on pancreatic fluid bicarbonate concentration.

PP infusion does not affect gastric acid or pepsin secretion or the plasma levels of insulin, glucagon, secretin, gastrin, glucose, or lipids in normal man (Bloom *et al.*, 1978). Additionally, there are no signs or symptoms of cardiovascular or gastrointestinal alterations in healthy persons receiving intravenous infusion of PP. In dogs, pharmacologic doses of PP had no effect on steady-state bile flow but did cause choledochal resistance and relaxation of the gallbladder. Basal and stimulated output of bilirubin is inhibited significantly by PP infusion at physiologic levels.

Satiety, Obesity, and Hyperphagia

A number of studies suggest that PP may play a role in feeding and obesity and, therefore, in glucose metabolism. R. L. Gingerich and co-workers observed that the amount of PP in the pancreas is elevated in hyperglycemic, obese mice. Subsequently, R. J. Gates and N. R. Lazarus (1977) reported reversal of hyperphagia, obesity, and glucose intolerance after administration of PP in obese, hyperphagic mice and concluded that the abnormalities seen were due to a deficiency of PP release.

B. Glaser and colleagues (1988) studied the relationship of PP levels with both obesity and glucose intolerance in humans. Obese subjects with normal oral glucose intolerance demonstrated low basal and low stimulated levels of PP compared to age-matched controls. Obese subjects with abnormal oral glucose tolerance demonstrated elevated basal levels of PP, however, and an exaggerated PP response to stimulation.

W. B. Zipf and colleagues (1990) observed that obese children with Prader–Willi syndrome were PP-deficient and that their hyperphagia was reduced by PP administration. PP administration to normal dogs has also been shown to induce weight loss. A 14-day continuous subcutaneous infusion of PP resulted in a 3.5% loss of body weight in 20 kg dogs allowed normal access to food. The weight loss was reversed within 4 weeks after cessation of the PP infusions (YS Sun *et al.*, 1986). Other investigators have found elevated levels of PP in patients with bulimia. A satiety effect of NPY has also been observed by several investigators and laboratory and clinical studies continue to explore the possible role of PP and NPY as satiety-inducing agents. Despite positive results of experiments in rodents and non-human mammals, PP has yet to be shown as an anorexigenic agent in man. The role of PP is also being investigated in patients undergoing bariatric surgery.

Regulation of Insulin Action

The liver occupies a central role in the regulation of glucose metabolism. The ability of the liver to produce glucose during times of fasting or stress, and to take up glucose after feeding, is essential for metabolic homeostasis and PP appears to play an important role in the hepatic response to insulin. A study by Y. S. Sun and colleagues (1986) examined pancreatogenic diabetes in a canine model of chronic pancreatitis created by pancreatic duct ligation. The hyperinsulinemic–euglycemic glucose clamp technique and radioisotopic methods were used to quantify hepatic and nonhepatic insulin-stimulated glucose turnover. Hepatic glucose production in normal animals was suppressed by insulin, but dogs with chronic pancreatitis demonstrated far less insulin-induced suppression of hepatic glucose production. This insulin resistance could be demonstrated only in the liver and not in nonhepatic tissue. Furthermore, it occurred only in animals with impaired meal-stimulated PP secretory activity. Thus, PP deficiency was uniformly associated with profound hepatic insulin resistance.

In subsequent experiments, the same animals were restudied at varying time points during continuous subcutaneous infusion of bovine PP. The hepatic insulin resistance previously observed in PP-deficient animals was corrected after PP administration (**Fig. 3**). Serial glucose tolerance tests, performed over 28 weeks after the PP infusion period, demonstrated that glucose tolerance was significantly improved posttreatment, as compared to pretreatment values.

Seymour *et al.* (1995) studied hepatocyte insulin-binding characteristics to determine whether insulin receptor function or expression is altered by PP. Maximal insulin-binding capacity was significantly lower in hepatic tissue from animals with chronic pancreatitis than from control, sham-operated animals (**Fig. 4**). This loss of high-affinity binding sites was observed in the liver but not in skeletal muscle. PP administration in animals with chronic pancreatitis resulted in significantly greater hepatic insulin-binding capacity than was observed after vehicle administration alone and approximated that of the control (nonpancreatitic) group. During glucose tolerance tests performed immediately prior to the procurement of tissues, the magnitude and duration of the serum glucose elevation following an intraduodenal dextrose challenge were reduced by PP administration in animals with pancreatitis.

Serum immunoreactive insulin levels were not altered by PP administration, but the restoration of high-affinity insulin-binding sites produced by exogenously administered PP suggested alterations in the expression of hepatic insulin receptors. Subsequent studies by this group showed that diminished hepatic insulin receptor gene expression seen in PP-deficient animals is reversed by exogenous PP administration (Spector *et al.*, 1997).

The glucoregulatory effect of PP therefore appears to reside in its role in the expression of the hepatic insulin receptor protein. Diminished PP levels are associated with a loss of high-affinity insulin receptor sites on hepatocytes, which creates a state of hepatic insulin resistance. Although other factors may play a role in the hepatic insulin resistance that characterizes pancreatogenic diabetes, a loss of insulin-binding sites due to the reduced expression of hepatic insulin receptor protein appears to be the primary mechanism of PP's glucoregulatory role. Replacement of PP by exogenous administration reverses the hepatic insulin resistance and at least partially corrects the abnormal hepatic glucose production (Cui and Andersen, 2011).

Evidence for the Role of PP as an Adjunct to Insulin Therapy

The artificial endocrine pancreas, used clinically, is simple in its concept. A sensor electrode measures the level of blood glucose. This information is fed into a small computer that energizes an infusion pump, and from a small reservoir, controlled amounts of insulin enter the patient's circulation. K. Hanazaki and colleagues (2001) developed a new artificial pancreas with a multiple-chamber injection pump to facilitate infusion of not only insulin, but also PP. The addition of PP decreased insulin requirements

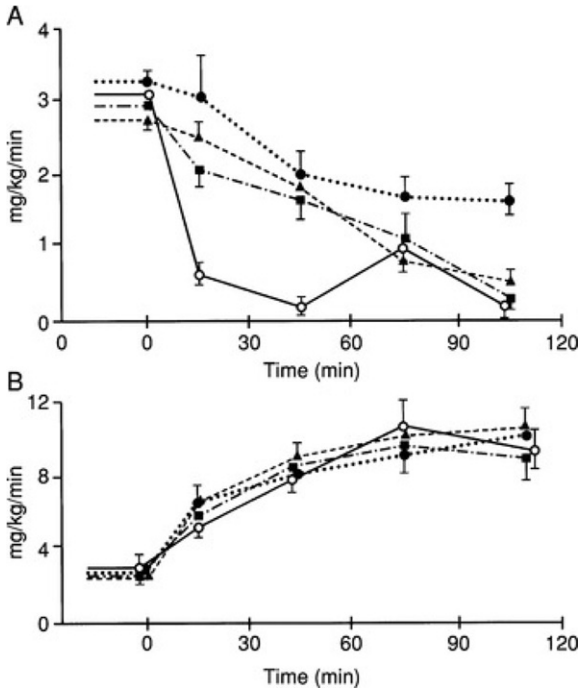

Fig. 3 (A) Hepatic glucose production rates. The responses of dogs with chronic pancreatitis at days 6 (▲) and 11 (■) of continuous subcutaneous PP infusion were significantly lower than the pretreatment response (•) by paired analysis compared to control, sham operated animals (○) ($P<0.05$). (B) The overall glucose disposal rates achieved by insulin infusion in these studies. Reprinted from Sun *et al.* (1986), with permission.

in pancreatectomized dogs during a 72 h trial period. This observation suggested that PP, or a PP-receptor agonist, may become a useful adjunct in the treatment of diabetes.

To determine whether the addition of PP would reduce the insulin requirements in Type 1 (T1D) and Type 3c (T3cD) diabetics who were using an insulin pump, Rabiee *et al.* (2011) conducted a pilot study in which a randomized, blinded 72 h infusion of either PP or saline was administered subcutaneously in 7 T1D and 3 T3cD subjects. The average blood glucose levels, measured by a blinded continuous glucose monitor, were not different during the PP and saline infusions, as the subjects followed their usual algorithm for adjustment of their insulin infusions. The amount of insulin required for the same degree of glucose control was significantly less during the second and third day of the PP infusion (**Fig. 5**). Insulin sensitivity, calculated from the amount of insulin infused corrected for the mean glucose levels, increased 34–45% during the PP infusion period compared to the saline infusion period. Furthermore, the improvement in insulin sensitivity was seen not only in all of the T3cD subjects, but in 5 of the 7 T1D subjects as well. Further studies of the potential role of PP as an adjunct to insulin therapy are in progress.

The Role of PP in Pancreatogenic Diabetes

Pancreatogenic diabetes, or T3cD, is defined by the American Diabetes Association and the European Association for the Study of Diabetes as a form of secondary diabetes due to exocrine disease of the pancreas. This includes chronic pancreatitis, proximal or total pancreatectomy, cystic fibrosis, hemochromatosis, 'tropical' fibrocalcific pancreatitis, pancreatic cancer, pancreatic trauma, and agenesis of the pancreas. Chronic pancreatitis accounts for 75–80% of T3cD, according to a study by Hardt *et al.* (2008) of almost 2000 diabetic patients referred to an academic medical center in Germany. In their population, T3cD accounted for 8% of the entire population of diabetics, and half of the T3cD patients has been previously misdiagnosed as T2D or, less commonly, as T1D (**Fig. 6**).

Patients with severe chronic pancreatitis have a profound meal-stimulated PP secretory deficiency compared to normal subjects (**Fig. 7**) (Sive *et al.*, 1978, Valenzuela *et al.*, 1979). During an initial, baseline hyperinsulinemic–euglycemic clamp study, F.C. Brunicardi *et al.* (1996) found a pattern of profound hepatic insulin resistance similar to that seen in the canine studies in PP-deficient chronic pancreatitis patients (**Fig. 8**). Glucose clamp studies were repeated during the final 2 h of an 8 h intravenous infusion of bovine PP, which replicated physiologic serum concentrations of postcibal immunoreactive PP (750–1000 pg ml^{-1}). Hepatic responses to insulin in PP-deficient patients were restored to normal by PP (**Fig. 8**). However, insulin-stimulated glucose disposal in normal control subjects was not altered by the PP infusion. There was no PP-induced alteration in serum insulin or

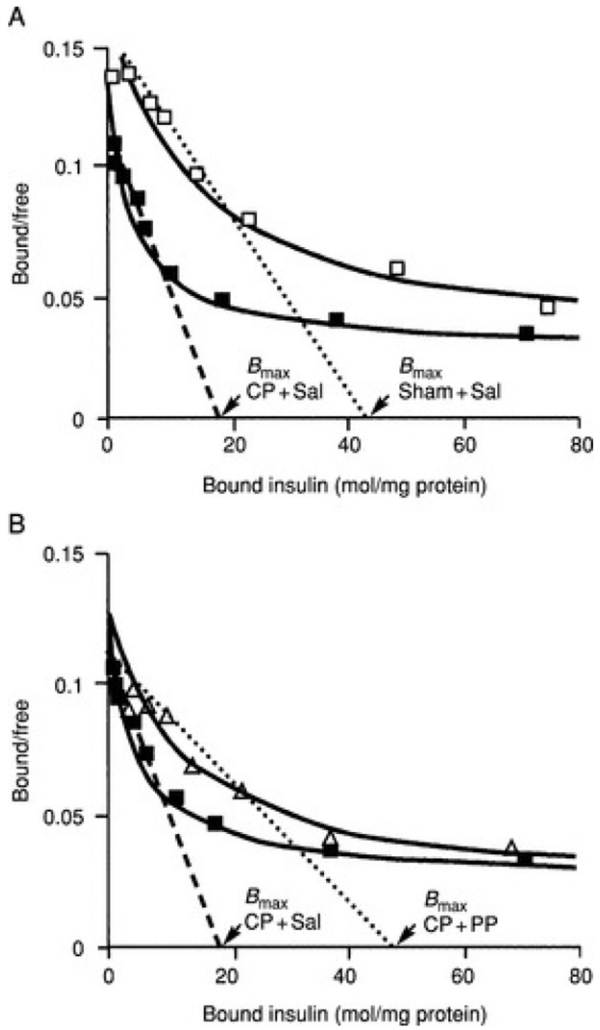

Fig. 4 (A) Effect of chronic pancreatitis on hepatic insulin binding: Scatchard plot of insulin binding in solubilized hepatic membranes from saline-administered, sham-operated rats (sham, open squares) and saline-administered, chronic pancreatitic rats (CP, filled squares). (B) Effects of PP administration (PP, 200 $\mu g\ kg^{-1}$ per day) on hepatic insulin binding in chronic pancreatitis: Scatchard plot of insulin binding in solubilized hepatic membranes from saline-administered chronic pancreatitic rats (CP + Sal, filled squares) and PP-treated rats (CP + PP, open triangles). Reprinted from Seymour *et al.* (1995). Alteration in hepatocyte insulin binding in chronic pancreatitis: Effect of pancreatic polypeptide. *Am. J. Surg.* **169**, 105, with permission.

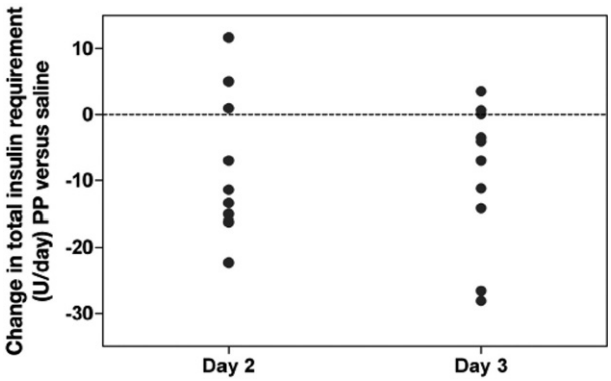

Fig. 5 Change in total daily insulin infusion requirement during PP infusion compared to saline infusion on day 2 and day 3 of infusions in 7 T1D and 3 T3cD patients on insulin pump therapy. The reduction in daily insulin requirements averaged 8.4 ± 3.44 Units (U) on day 2 ($p < 0.05$) and 9.0 ± 3.49 U on day 3 ($p < 0.05$). Reprinted from A. Rabiee *et al.* (2011) with permission.

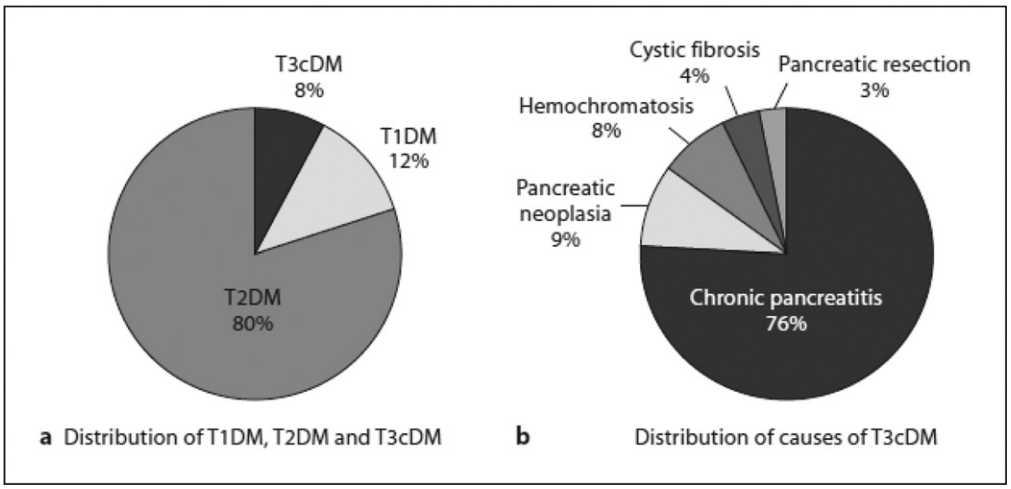

Fig. 6 Distribution of types of diabetes (a) and causes of type 3c (pancreatogenic) diabetes (b) based on studies of 1922 diabetic patients by P. D. Hardt *et al.* (2008). Reprinted from Y. F. Cui and D. K. Andersen (2011) with permission.

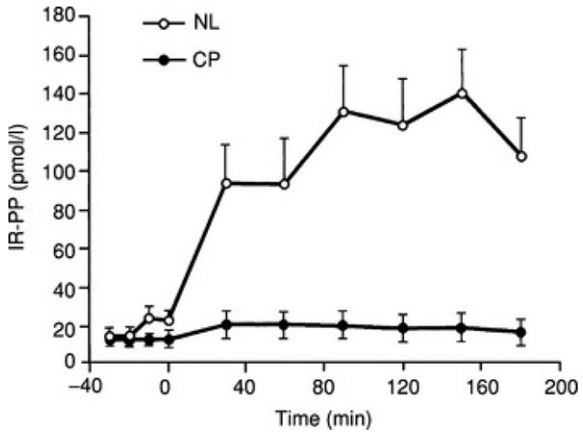

Fig. 7 PP response to test meal. Immunoreactive PP (IR-PP) responses in normal control subjects (NL, $n=6$) and patients with chronic pancreatitis (CP, $n=5$) accompanied by PP deficiency. Test meal was administered at 0 min. Reprinted from Brunicardi *et al.* (1996), with permission.

glucagon concentrations in either of the experimental groups. These data indicate that PP replacement was successful in reestablishing the normal hepatic response to insulin. A third set of glucose clamp studies was performed 1–2 months after the PP administration. Hepatic glucose output in response to insulin in PP-deficient patients had returned to near-baseline values, which confirmed the etiologic role of PP deficiency in the hepatic resistance to insulin.

An oral glucose tolerance test (OGTT) was administered to the subjects in Brunicardi's study on the day after the clamp experiment, and the mean plasma glucose levels from the OGTT performed before, 1 day after, and 1 month after the PP infusion are shown in **Fig. 9**. In every subject in whom mean plasma glucose levels were abnormally high in the baseline study, glucose levels were lower immediately after the 8 h PP infusion. These findings indicate that the enhancement of hepatic insulin sensitivity by PP in PP-deficient subjects contributed to an improvement in their glycemic response to oral glucose.

Seymour *et al.* (1988) examined glucose turnover in patients with a remote history of pancreatectomy. In these subjects, proximal pancreatectomy and distal pancreatectomy were distinguished from one another, since only the former was associated with a loss of PP secretory activity and low circulating PP levels (**Fig. 10**). Three hyperinsulinemic–eugycemic clamp studies were performed in each subject, separated by at least 1 month. An 8 h intravenous PP infusion was administered before the second study. PP-deficient, proximal pancreatectomy subjects demonstrated an impairment in hepatic insulin responses in the first study and required significantly smaller glucose infusions than normal control subjects to maintain euglycemia during insulin infusion (**Fig. 11**). This insulin resistance was completely reversed by PP administration during the second clamp study, but was evident again during the final infusion study in the absence of PP. No alteration in peripheral glucose disposal was observed with either pancreatectomy or PP administration.

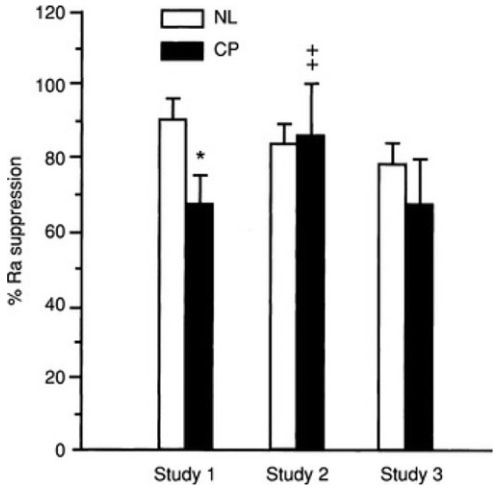

Fig. 8 Percentage suppression of RA (rate of glucose appearance) during hyperinsulinemic–euglycemic clamps for studies 1, 2, and 3 for normal subjects (NL, $n=6$) and CP patients (CP, $n=5$). Study 1 (baseline) showed significant ($*p<0.05$)resistance to insulin-mediated suppression of hepatic glucose production. Study 2 was performed during the last 2 hr of an 8 hr infusion of bovine PP; the suppression of hepatic glucose production by insulin was significantly increased from baseline ($+p<0.05$). Study 3 was performed one month later. Reprinted from Brunicardi *et al.* (1996), with permission.

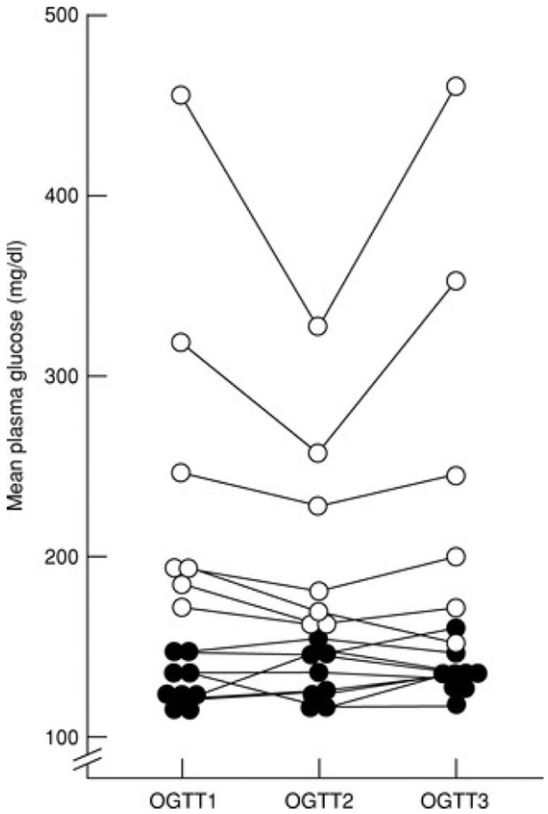

Fig. 9 Mean plasma glucose concentration during 180 min after 40 g m^{-2} oral glucose tolerance test (OGTT) in chronic pancreatitis patients ($n=10$) and controls ($n=6$). Open circles indicate impaired or diabetic status during the initial OGTT. Filled circles indicate normal or nondiagnostic status during the initial OGTT. OGTT 2 was performed 18 h after an 8 h infusion of bovine PP (2 pmol kg^{-1} min^{-1}). OGTT 3 was performed 1 month later. Eighteen hours after PP infusion, every patient with an impaired or diabetic OGTT showed a lower mean plasma glucose response. Reprinted from Andersen (1990). The role of pancreatic polypeptide in glucose metabolism. *In* 'Gastrointestinal Endocrinology: Receptor and Post-receptor Mechanisms,' (J. C. Thompson, ed.), pp. 333–357. Academic Press, San Diego, CA, with permission.

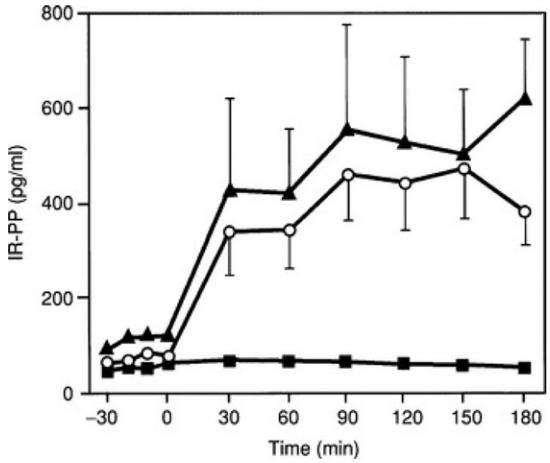

Fig. 10 Immunoreactive PP (IR-PP) response to a test meal for eight control subjects (open circles), four non-PP-deficient resection patients (filled triangles), and six PP-deficient resection patients (filled squares). Reprinted from Seymour *et al.* (1988), with permission.

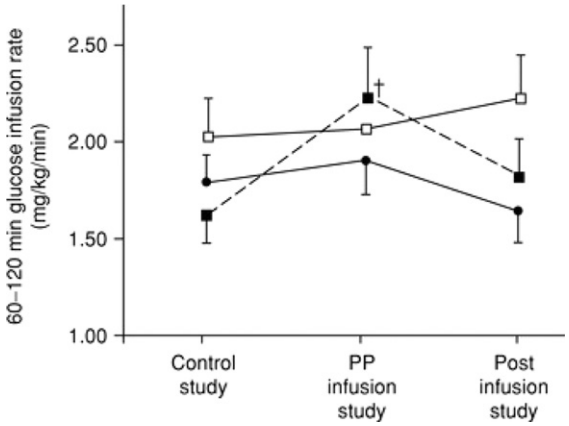

Fig. 11 Glucose infusion rate (mg kg^{-1} min^{-1}) during the final hours of a 2 h insulin infusion in normal control (open squares), non-PP-deficient resection (filled circles), and PP-deficient resection (filled squares) subjects. The glucose infusion requirement for PP-deficient subjects was significantly greater during the PP infusion study than during the control study ($P<0.03$). Reprinted from Seymour *et al.* (1988), with permission.

These studies provide strong evidence for a metabolic role of PP in the regulation of hepatic glucose metabolism, and suggest that PP might have benefit as a therapeutic agent in T3cD. Administration of PP requires continuous infusion either intravenously or subcutaneously due to the short half-life of the peptide, however. Therefore, efforts have been explored to find a method to protect PP from rapid degradation to prolong its bioavailability. K. Bellman-Sickert and colleagues (2011) showed that acetylation with fatty acids (lipidation) or polyethylene glycol (PEGylation) of PP results in a prolonged circulation half-life due to prevention of renal degradation. A. Banerjee and H. Onyuksel (2013) incorporated human PP in PEGylated phospholipid micelles and showed improved insulin sensitivity and glucose tolerance in a rat model of T3cD due to chronic pancreatitis. Further efforts are underway to create a modified peptide, a delivery vehicle for preventing degradation of the peptide, or a durable Y4 receptor agonist, for therapeutic trials.

Clinical Implications of Alterations in PP Secretion

Diabetes mellitus

Mean basal concentrations of plasma PP are elevated in normal aging and in T2D (**Fig. 12**). PP levels are particularly elevated in type 2 (adult onset, non-insulin-dependent) diabetic patients, which may represent a compensatory response to a loss of sufficient insulin synthesis or the development of insulin resistance. Recent studies by C. W. Chia *et al.* (2014) indicate that the elevation in PP levels in T2D is secondary to increased levels of GIP, which is tropic to PP secretion. Early type 1 diabetic patients may also exhibit high levels of PP before they experience loss of islet responsiveness due to the progression of disease.

Pancreatogenic or type 3c diabetes differs from both T1D and T2D in several respects (**Table 2**). T3cD rarely, if ever, results in ketoacidosis, but can be associated with labile blood glucose levels and frequent episodes of iatrogenic hypoglycemia. This specific pattern of abnormal glucose homeostasis is likely caused by the simultaneous deficiencies in insulin, glucagon, and PP secretion.

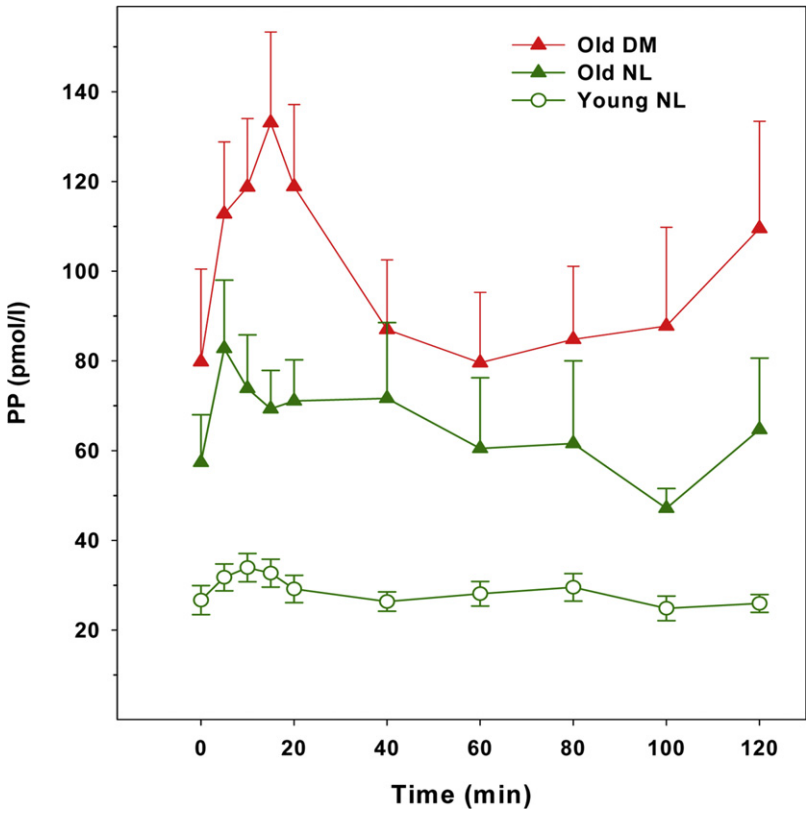

Fig. 12 Serum PP responses to 75 g of glucose ingested at time = 0 in 10 healthy young (age younger than 40 years; NL Young), 19 healthy elderly (age greater than 65 years) with normal glucose tolerance (NL Old), and 21 elderly subjects with T2DM (DM Old). Data are shown as mean ± SE. Reprinted from J. T. Magruder, D. Elahi, and D. K. Andersen. Pancreas **40** (2011) 339-351, with permission.

Table 2 Types of diabetes mellitus

Parameter	*Type 1 IDDM Autoimmune*	*Type 2 NIDDM Obesity-related*	*Type 3c pancreatogenic Prior pancreatic disease*
Ketoacidosis	Common	Rare	Rare
Hyperglycemia	Severe	Usually mild	Mild
Hypoglycemia	Common	Rare	Common
Peripheral insulin sensitivity	Normal or increased	Decreased	Increased
Hepatic insulin sensitivity	Normal	Normal or decreased	Decreased
Insulin levels	Low	High	Low
Glucagon levels	Normal or high	Normal or high	Low
PP levels	High early, Low late	High	Low
Typical age of onset	Childhood or adolescence	Adulthood	Any

Note. IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; PP, pancreatic polypeptide. (From Slezak and Andersen 2001)

Therefore, a loss of PP responsiveness to ingested nutrients discriminates T3cD from T2D, and is a marker for pancreatogenic diabetes. As described by Y. F. Cui and D. K. Andersen (2011), this form of diabetes requires special attention in its management.

Chronic pancreatitis

Chronic pancreatitis is a complex pathologic state consisting of lifelong exocrine and endocrine derangement. Endocrine dysfunction manifests itself as diabetes mellitus, but multiple hormone deficiencies have been demonstrated. Meal-stimulated levels of circulating immunoreactive PP are especially affected by advanced chronic pancreatitis, so much so that the measurement of PP levels has been suggested as the best endocrine screening procedure for the diagnosis of chronic pancreatitis by Rickels *et al.* (2013). In PP-deficient states such as chronic pancreatitis, there is demonstrable hepatic resistance to insulin, which can be reversed by the administration of PP (**Fig. 8**).

Pancreatic surgery

K. Inoue and colleagues confirmed the abolishment of PP responses to ingested nutrients in patients after pancreaticoduodenectomy. In the study by Seymour *et al.* (1988), healthy, young (age less than 30 years) male patients who had undergone pancreatic resection for trauma and who had a deficient PP response as a consequence of their pancreatic resection had a measurable impairment of hepatic insulin sensitivity (**Fig. 11**). This effect was not associated with impaired glucose tolerance, however, when insulin and glucagon secretory function was present. Although 100% of patients are PP deficient after pancreaticoduodenectomy, only 50% are diabetic. Therefore PP deficiency, per se, in the setting of adequate beta-cell function does not cause overt disease.

The risk of pancreatogenic diabetes is increased when proximal pancreatectomy is performed in older patients, however, or when near-total or total pancreatectomy is performed. The development of duodenum-preserving pancreatic resection techniques by H. G. Beger and colleagues, and by C. F. Frey, has been shown to be associated with improved glucose tolerance postoperatively. The preservation of glucose tolerance with operative techniques that spare part or all of the PP-rich region of the pancreas further supports the role of PP in glucose homeostasis (Slezak and Andersen, 2001).

Islet-cell transplantation, either by allo-transplantation or by auto-transplantation after total pancreatectomy, is also associated with an impaired PP response and PP deficiency. Disruption of the cholinergic innervation of the islet is thought to be involved in the mechanism.

Summary

PP release is both hormonally and neurally mediated and both exaggerated or diminished PP responses are valuable indicators of the integrity of the neuro-entero-pancreatic system. Although subtle effects on pancreatic exocrine function and choleresis are associated with PP, its major role appears to be that of a glucoregulatory hormone. It remains to be determined whether PP plays a role in food ingestion as a satiety mediator in man, but its most consistent function appears to be that of a regulator of hepatic insulin action, through its mediation of hepatic insulin receptor protein synthesis. A deficient PP response to ingested nutrients appears to be a useful marker to distinguish T3cD from T2D, and replacement of PP in PP deficient patients improves hepatic insulin sensitivity and glucose homeostasis. Therapeutic opportunities are being explored with the use of methods to extend the half-life of PP. In the future, PP analogues, as well as PP-receptor agonists or antagonists, are likely to become useful clinical tools.

See also: GI Hormone Development (Families and Phylogeny). GI Tract: General Anatomy (Cells). Pancreatic Islet Cell Tumors

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Peptide YY (PYY)

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Glossary

Bariatric surgery Surgical intervention to reduce food intake and induce weight loss in obese people, involving a variety of procedures to diminish the size of the stomach or bypass the stomach.

Biologically active peptide A chain of amino acids joined by peptide bonds, the sequence of amino acids and the tertiary structure of the molecule determining the biological properties and function of the peptide.

Enteroendocrine cells Hormone-producing endocrine cells interspersed between epithelial cells of the gastrointestinal mucosa.

Pancreatic α-cells Endocrine cells in the pancreatic islets of Langerhans expressing glucagon, peptide YY, glucagon-like peptide 1 and glucagon-like peptide 2.

Y receptors Receptors located in the cell membrane, which when activated by peptide YY, pancreatic polypeptide or neuropeptide Y elicit a cellular response.

PYY and Y Receptors

Peptide YY (PYY) is an amidated peptide that was identified by Tatemoto and Mutt (1980) and named after the tyrosine (Y) residues constituting each end of its amino acid chain (Field *et al.*, 2010). It belongs to a family of biologically active peptides that also includes pancreatic polypeptide (PP) and neuropeptide Y (NPY). As these peptides share the PP fold (hairpin fold) as common tertiary structural motif, they are also referred to as PP-related peptides. PYY, NPY, and PP are all composed of 36 amino acids and exhibit high sequence homology, 70% of the amino acid residues being identical in PYY and NPY and 50% in PYY and PP (Pedragosa-Badia *et al.*, 2013). The N-terminus of PYY (like that of NPY) is readily truncated by dipeptidyl peptidase 4 (DPP-4) (EC 3.4.14.5), yielding the fragment PYY(3–36) which is the major postprandial circulating form of PYY (Mentlein *et al.*, 1993; Medeiros and Turner, 1994). Other enzymes such as aminopeptidase-P and endopeptidase-24.11 also contribute to the degradation of the peptide.

The structural commonalities between the PP-related peptides explain why their biological actions are brought about by binding to a group of rhodopsin-like (class A) G protein-coupled receptors (GPRs) termed NPY or, in brief, Y receptors. In mammals, 5 Y receptor subtypes have been identified: Y1, Y2, Y4, Y5, and y6. The y6 receptor is commonly written in lower case because it is a pseudogene in humans, but functional in mouse, rabbit and some other mammals (Alexander *et al.*, 2017). The receptor originally named Y3 receptor has later been identified as the CXCR4 chemokine receptor (Alexander *et al.*, 2017). The Y receptor subtypes display differential affinities for the different members of the PP-related peptide family and their fragments (Alexander *et al.*, 2017). While PYY and NPY do not markedly differ in their selectivity for the Y1, Y2 and Y5 receptor subtypes, PYY (3–36) is a preferred agonist at Y2 receptors and PP a preferred agonist at Y4 receptors (Alexander *et al.*, 2017).

The differential affinities of PYY, NPY and PP to the Y receptor subtypes are not only the result of different structural homologies between the ligands but also of grossly varying sequence identities of the receptors (Pedragosa-Badia *et al.*, 2013). Once activated, the Y receptors are typically coupled to pertussis toxin-sensitive $G_{i/o}$ proteins, transduction through which leads to inhibition of adenylate cyclase, diminution of cAMP formation, and modulation of Ca^{2+} and K^{+} channels (Pedragosa-Badia *et al.*, 2013). In addition it has been reported that coupling of Y2 and Y4 receptors to G_q proteins can increase inositol 1,4,5-phosphate production via activation of phospholipase C-β (PLC) (Pedragosa-Badia *et al.*, 2013).

Expression of PYY in Gut, Pancreas and Brain

PYY was originally isolated from the porcine intestine, and the gastrointestinal tract (GIT) is the prime source of this peptide in the body although appreciable amounts of PYY are also expressed in the pancreas and brain. In the GIT, enteroendocrine L cells are the predominant source of PYY, these cells appearing very early during ontogeny, which suggests that this hormone is relevant to the development of the GIT (Boey *et al.*, 2007; El-Salhy *et al.*, 2013). The topographic distribution of PYY in the gut differs markedly between different vertebrate species. While in lower vertebrates the peptide is concentrated in the upper GIT, PYY-expressing enteroendocrine cells occur in all parts of the small and large intestine of the rat. This pattern is again different in primates in which low levels of PYY are present in the stomach and its concentration increases along the alimentary canal such that the highest density of PYY-immunoreactive enteroendocrine cells is found in the rectum (Field *et al.*, 2010; El-Salhy *et al.*, 2013; Persaud and Bewick, 2014). In mice and humans about 50% of the colorectal L-cells contain PYY which in these cells is co-expressed with pro-glucagon products such as glicentin, glucagon-like peptide 1 (GLP-1) and GLP- 2 and copackaged in the same secretory granules (Cox, 2007; Persaud and Bewick, 2014). Apart from its predominant expression by enteroendocrine cells, PYY

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has also been localized to enteric neurons in the stomach of several mammalian species including rat, cat, ferret and pig, which are different from those expressing NPY (Boey *et al.*, 2007; Cox, 2007).

Since the gut and the pancreas share a common embryological origin, it is not surprising that PYY is also expressed in pancreatic endocrine cells in which the peptide appears during the earliest stage of pancreatic islet cell precursor differentiation (Persaud and Bewick, 2014). A large proportion of the early PYY-positive cells in the islets of Langerhans also contain glucagon (α-cells) and insulin (β-cells) and, later in development, PYY is also found in cells that express somatostatin (δ-cells) and PP (F cells) (Persaud and Bewick, 2014). Although this co-expression pattern is altered in adulthood, about 40% of the pancreatic glucagon cells (α-cells) are descendants of PYY-positive cells, with PYY being in addition expressed by subpopulations of PP cells and the other islet cell types (Boey *et al.*, 2007; Persaud and Bewick, 2014). As is true for the intestinal L-cells, the pancreatic α-cells containing glucagon and PYY also co-express GLP-1 and GLP-2. These observations are consistent with the view that PYY plays a role in the differentiation, growth, development and regulation of islet endocrine cells (Boey *et al.*, 2007; Persaud and Bewick, 2014). The expression of PYY in cells of the exocrine pancreas has not yet been well characterized.

Although PYY is primarily an endocrine peptide, it is also expressed by neurons of the central nervous system (CNS), apart from enteric neurons in the stomach of some mammalian species. In contrast, the expression of PYY mRNA in sympathetic neurons has not been confirmed in a study using in situ hybridization (Pieribone *et al.*, 1992). In the CNS, PYY has been localized to neurons of the spinal cord, brainstem, pons and hypothalamus (Boey *et al.*, 2007; Shi *et al.*, 2015). Specifically, PYY is found in neurons of the gigantocellular reticular nucleus of the rostral medulla wherefrom they project to the lateral parabrachial nucleus, the nucleus of the solitary tract, the dorsal vagal complex and the hypoglossal nucleus (Glavas *et al.*, 2008; Gelegen *et al.*, 2012; Alhadeff *et al.* 2015). Unlike in the circulation, the predominant form of PYY found in the brain is full-length PYY(1–36) (Gelegen *et al.*, 2012).

Circulating PYY and PYY(3–36)

PYY released from endocrine cells in the GIT and pancreas can, on the one hand, have a local paracrine effect on neighboring cells and, on the other hand, exert endocrine effects on remote cells which it reaches via the blood stream. The role of intestinal PYY in the temporal and spatial coordination of postprandial digestion and in the regulation of food intake, energy homeostasis and behavior requires the peptide to be released into, and distributed by, the circulation. Fasting plasma levels of PYY are under the influence of several factors including exercise, body weight status, adiposity level and ethnicity, whereas sex and age have little effect (Cooper, 2014).

The plasma levels of PYY are lowest in the absence of food intake and start to rise within 15 min of a meal through release from the intestinal L cells. PYY entering the circulation is relatively rapidly attacked by DPP-4 and in part hydrolysed to PYY(3–36), the predominant postprandial form of PYY in plasma, which potently stimulates Y2 receptors and, to some extent, Y5 receptors in the periphery and brain (Cox, 2007; Alexander *et al.*, 2017). The initial increase in circulating PYY concentration occurs long before food has reached the L cells in the lower GIT, which indicates that a neuronal or endocrine mechanism operating during the cephalic phase of digestion stimulates the L cells (Cox, 2007; Cooper, 2014). Maximal plasma concentrations of PYY in humans are observed 1–2 h postprandially when the nutrients have arrived at the L cells, and elevated levels are maintained for up to 6 h (Cooper, 2014; Persaud and Bewick, 2014).

The postprandial release of PYY is proportional to energy intake but also modified by the macronutrient composition of the diet, as intraluminal lipid hydrolysis as well as a high-protein diet enhance the effect of food intake on PYY release (Cox, 2007; Field *et al.*, 2010; Cooper, 2014). In addition, the composition of fatty acids from dietary fat has an impact, given that both the size ($\geq$C12) and nature of fatty acids are of relevance: polyunsaturated and saturated fatty acids evoke a greater PYY response than monounsaturated fatty acids (Cox, 2007; Cooper, 2014). PYY-releasing enteroendocrine cells sense the presence of digested nutrients in the lumen of the GIT by a variety of nutrient-selective receptors. Particular GPRs expressed by L cells are involved in the effect of free fatty acids and certain bacterial fermentation products to release PYY as well as GLP-1 and GLP-2 (Cox *et al.*, 2010; Spreckley and Murphy, 2015). Short chain fatty acids (SCFAs) generated from indigestible carbohydrate fibers by the intestinal microbiota, such as butyric and propionic acid, bind to free fatty acid receptor 1 (FFA1, previously known as GPR40), FFA2 (previously known as GPR43), FFA3 (previously known as GPR41), FFA4 (previously known as GPR120) and hydroxycarboxylic acid receptor 2 (previously known as GPR109A) and in this way stimulate enteroendocrine L cells in the lower GIT (Samuel *et al.*, 2008; Bindels *et al.*, 2013; Tan *et al.*, 2014; Psichas *et al.*, 2015; Spreckley and Murphy, 2015; Brooks *et al.*, 2016; Moodaley *et al.*, 2017).

While the majority of circulating PYY is derived from the intestinal L cells and pancreatic endocrine cells, there may also be some contribution from leukocytes. Thus, PYY can be expressed by human peripheral blood mononuclear cells as shown by PYY mRNA and peptide expression (Holler *et al.*, 2014). Once these cells have differentiated into macrophages in vitro, treatment with lipopolysaccharide causes down-regulation of peptide YY mRNA levels (Holler *et al.*, 2014). Any functional implication of this finding awaits to be explored.

Effects of PYY on Digestive Activity

PYY subserves several roles in the regulation of digestion, which it exerts both via a paracrine and endocrine route, depending on the expression of different Y receptor subtypes by the gastrointestinal effector systems. The effects of both PYY and PYY(3–36) on

gastrointestinal motility are of an inhibitory nature, these actions being mediated by 3 major Y receptor subtypes: Y1 receptors on enterocytes, myenteric and submucosal neurons and endothelial cells, Y2 receptors on myenteric and submucosal neurons, extrinsic primary afferent nerve fibers, and Y4 receptors on enterocytes (Cox, 2007; Wang *et al.*, 2010; Holzer *et al.*, 2012). Plasma concentrations approximately equivalent to postprandial levels inhibit gastric acid secretion, delay gastric emptying, inhibit the cephalic phase of gallbladder contraction as well as pancreatic exocrine secretion, promote intestinal fluid and electrolyte secretion by inhibition of their secretion, and prolong the mouth-to-cecum transit time (Field *et al.*, 2010). The inhibition of gastric acid secretion involves Y1 and Y2 receptors and is mediated both by an action in the brainstem and stomach, while the effect of PYY (3–36) to increase gastric pressure via stimulation of Y2 receptors does not involve the vagus nerve (Holzer *et al.*, 2012). PYY released by SCFAs (administered exogenously or produced by the intestinal microbiota) inhibits gastrointestinal motility and electrolyte and water secretion by neural and non-neural mechanisms (Cox, 2007; Holzer *et al.*, 2012).

In physiological terms, PYY is thought to be a major mediator of ileal and colonic brakes which are set into operation when macronutrients able to release PYY from L cells reach the lower gut: the brakes slow gastric emptying and intestinal transit, which in conjunction with the antisecretory action of PYY facilitates nutrient absorption (Cox, 2007; Holzer *et al.*, 2012). The inhibitory effect of PYY and PYY(3–36) on colonic transit in the mouse is brought about by Y2 receptors on the enteric nervous system (Wang *et al.*, 2010; Tough *et al.*, 2011), and there is evidence that PYY and related peptides exert a tonic influence on gastrointestinal motor and secretory activity (Tough *et al.*, 2011; Holzer *et al.*, 2012). Colonic transit is tonically accelerated by Y1 receptor stimulation and tonically inhibited by Y2 receptor stimulation, while secretory activity in the human and mouse colonic mucosa is blunted by epithelial Y1 and neuronal Y2 receptors (Tough *et al.*, 2011; Holzer *et al.*, 2012).

Effects of PYY on Pancreatic Function

PYY shares some of the incretin-like effects of GLP-1, a gut hormone frequently co-expressed by enteroendocrine L cells (Cox *et al.*, 2010). These cells sense the presence of luminal nutrients by the use of various nutrient-selective GPRs, which in turn leads to the release of PYY and GLP-1. Activation of one of these nutrient-selective GPRs, the acylethanolamine receptor GPR119, inhibits epithelial electrolyte secretion in the human and mouse colon in a glucose-sensitive fashion, these effects being mediated by PYY and Y1 receptor stimulation. At the same time, GPR119 agonism improves glucose tolerance, an effect that is absent in PYY knockout mice (Cox *et al.*, 2010).

These findings of an incretin-like effect of enteroendocrine PYY are in keeping with ample evidence for a role of pancreatic PYY in insulin regulation and glucose homeostasis. Such an antidiabetic function of endogenous PYY has been deduced from the glucose intolerance and hyperinsulinemia observed in PYY knockout mice. This observation is consistent with an inhibitory effect of PYY on insulin secretion both in vitro and in vivo (Bertrand *et al.*, 1992; Boey *et al.*, 2007; Loh *et al.*, 2017). The ability of intravenously administered PYY to attenuate glucose-stimulated insulin secretion in rodents is primarily mediated by Y1 receptors on β-cells and δ-cells in the islets of Langerhans but may also involve parasympathetic vagal output from the brainstem (Boey *et al.*, 2007; Loh *et al.*, 2017). A crucial role of the Y1 receptor in β-cell regulation has been confirmed by the finding that inhibition of Y1 receptor signaling improves islet function and delays the onset of chemically induced diabetes in mice (Loh *et al.*, 2017). In this context it is important to consider, however, that Y receptor signaling mediates not only the effect of PYY, but also that of PP, on pancreatic cell function.

In contrast, PYY(3–36) is little active in inhibiting insulin secretion, which is attributed to the absence of Y2 receptors in the pancreas (Boey *et al.*, 2007; Persaud and Bewick, 2014). Inhibition of Y1 receptor function in pancreatic islets, both genetic and pharmacological, accelerates the normalization of hyperglycemia in mice with chemically induced diabetes (Loh *et al.*, 2017). Manipulation of Y1 receptor function in pancreatic islets thus emerges as a potential therapeutic target in treating metabolic diseases. While, on the one hand, impaired Y1 receptor signaling may play a role in the development of metabolic diseases such as obesity and hyperinsulinemia, inhibition of Y1 receptor signaling may prove to be a useful approach to boost β-cell function under conditions where insulin secretion is limited (Loh *et al.*, 2017).

There is some evidence for additional effects of PYY in regulating insulin secretion and activity. Peripheral administration of PYY(3–36) or a Y2 receptor-selective agonist to mice improves glucose tolerance by increasing plasma insulin levels, an effect that is associated with increased GLP-1 secretion from enteroendocrine L cells (Chandarana *et al.*, 2013). PYY(3–36) may in addition regulate glucose homeostasis by improving insulin sensitivity (Persaud and Bewick, 2014). Taken all data together, PYY(1–36) produced locally in the pancreas appears to provide a paracrine brake to the various stimulant inputs that β-cells receive, thus ensuring sensitive regulation of insulin release. In contrast, circulating PYY(3–36) improves glucose tolerance through an incretin-like effect mediated by GLP-1 release from enteroendocrine L cells (Persaud and Bewick, 2014).

Apart from regulating insulin secretion and sensitivity, PYY emerges to have a beneficial impact on pancreatic endocrine function by enhancing β-cell mass in the islets of Langerhans (Persaud and Bewick, 2014; Shi *et al.*, 2015). Transgenic mice overexpressing PYY in islet β-cells exhibit enhanced insulin levels and improved glucose tolerance, which are due to increased β-cell mass and function (Shi *et al.*, 2015). This effect is unlikely to involve Y1 receptors because prolonged treatment of mice with a Y1 receptor antagonist likewise increases islet size and β-cell proliferation (Loh *et al.*, 2017). In addition, PYY is able to protect islet β-cells from apoptosis. Thus, ablation of PYY-expressing cells in adult mice disrupts islet architecture and causes β-cell destruction, followed by the development of insulin-dependent diabetes (Sam *et al.*, 2012). A role of PYY in providing trophic support to β-cells is corroborated by the observation that β-cell loss following PYY cell ablation or chemically induced diabetes is prevented

by stimulation of Y1, but not Y2, receptors (Sam *et al.*, 2012). The protective influence of PYY on islet β-cells may be of clinical potential, given that replenishment of β-cell mass is a key aim of novel therapeutic interventions in diabetes (Persaud and Bewick, 2014).

Inhibitory Effect of PYY(3–36) on Food Intake

The postprandial release of PYY from enteroendocrine L cells together with its inhibitory effects on gastrointestinal transit and food intake has attracted considerable interest because of the potential of this peptide to arrest weight gain and attenuate obesity. The pertinent action is thought to take place preferentially in the CNS, which attributes the major postprandial form of PYY in the circulation, PYY(3–36), an important messenger role between the gut and brain. As a result, many studies have addressed the role of PYY(3–36) and the Y2 receptor, which PYY(3–36) prefers, in modulating feeding behavior and initiating satiety. The initial observation that systemic administration of PYY(3–36) diminishes food intake and weight gain in rodents (Batterham *et al.*, 2002) was contested by other investigators (Tschöp *et al.*, 2004) who were unable to replicate this finding. The discrepancy was in part attributed to different experimental stress levels. Although human obesity is not associated with abnormal plasma levels of PYY and PYY(3–36) (Pfluger *et al.*, 2007), later studies have affirmed that PYY(3–36) diminishes food intake in both rodents and humans (Koda *et al.*, 2005; Field *et al.*, 2010; Steinert *et al.*, 2010; Stadlbauer *et al.*, 2013a; Shi *et al.*, 2017). The anorectic effect of PYY(3–36) is seen in both lean and obese subjects and in higher doses is associated with nausea and vomiting (Field *et al.*, 2010). This aversive reaction to systemic PYY(3–36), which appears to be brought about by stimulation of the chemotrigger zone in the area postrema, can be avoided by local administration of PYY(3–36) into the oral cavity, which in mice activates only brain areas known to regulate food intake (Hurtado *et al.*, 2013).

Food intake is inhibited by PYY(3–36) both via a stimulant effect on Y2 receptors directly on vagal afferent neurons (Koda *et al.*, 2005; Iwasaki *et al.*, 2013) and an interaction with Y2 receptors in the hypothalamus (Holzer *et al.*, 2012). This is consistent with the ability of PYY(3–36) to gain access to the brain via circumventricular organs such as the area postrema and subfornical organ (Dumont *et al.*, 2007) but also to permeate the blood-brain barrier to a certain extent (Nonaka *et al.*, 2003). In addition, PYY (3–36) can activate Y2 receptors in the oral cavity, presumably on afferent cranial nerve fibers, which signal via the brainstem to hypothalamic satiety centers where they inhibit food intake without causing taste aversion, an adverse reaction seen following systemic administration of PYY(3–36) (Hurtado *et al.*, 2013).

With the emergence of the gut microbiota as a relevant factor in gut-brain communication, PYY and other hormones of the enteroendocrine cells have also been recognized as messengers of gut microbiota-brain interaction. Microbial metabolites and fermentation products such as SCFAs have a direct impact on enteroendocrine cells that express various fatty acid-sensitive GPRs (Holzer *et al.*, 2012; Clarke *et al.*, 2014). SCFAs are known to promote satiety and to lower hunger and energy intake, an effect that is associated with enhanced release of GLP-1 and PYY from the gut. Through their biological activities, PYY and GLP-1 transform microbial signals into satietogenic and metabolic effects (Cani *et al.*, 2009; Delzenne *et al.*, 2011; Brooks *et al.*, 2016). In addition to SCFAs, bacterial proteins such *E. coli* stationary phase proteins are able to increase plasma PYY levels and to suppress food intake when injected systemically (Breton *et al.* 2016). The implication of microbial factors in gut hormone regulation of food intake and satiety is further corroborated by the finding that the expression of FFA2 (GPR43), FFA3 (GPR41), PYY, GLP-1 and cholecystokinin, another intestinal satiety-inducing peptide, is decreased in germ-free mice (Duca *et al.*, 2012). In the hypothalamus of germ-free mice, the expression of orexigenic factors such as NPY is increased, while anorexigenic neuropeptides are down-regulated (Schéle *et al.*, 2013).

Within the brain, PYY(3–36) reduces food intake primarily via activation of Y2 receptors in the arcuate nucleus of the hypothalamus, which is an important center for integrating peripheral and central signals in the control of appetite and energy homeostasis (Chee and Colmers, 2008). The arcuate nucleus contains at least two populations of neurons that are relevant in this respect: orexigenic neurons expressing NPY and agouti-related protein (AgRP) and anorexigenic neurons expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Chee and Colmers, 2008; Holzer *et al.*, 2012; Lau and Herzog, 2014). These neurons issue projections to various areas of the hypothalamus (including the paraventricular nucleus) and reciprocally inhibit the orexigenic/anorexigenic tone exerted by NPY/AgRP neurons and POMC/CART neurons, respectively (Chee and Colmers, 2008). The anorectic effect of PYY(3–36) was originally thought to be mediated by presynaptic Y2 receptors on NPY/AgRP neurons in the arcuate nucleus, inhibiting their orexigenic action, and by stimulating/disinhibiting POMC/CART neurons (Batterham *et al.*, 2002). Later investigations showed, however, that PYY(3–36) inhibits POMC/CART neurons through activation of postsynaptic Y2 receptors and that POMC/CART neurons are not essential for transducing the anorectic effect of PYY(3–36) (Ghamari-Langroudi *et al.*, 2005).

Further analysis has identified additional neuronal pathways in the brain that may be relevant to the satiation caused by PYY (3–36). Infusion of PYY(3–36) into the hepatic portal vein of rats has been found to activate neurons not only in the hypothalamic arcuate and paraventricular nuclei but also in the nucleus tractus solitarii, the central area of the amygdala and the nucleus accumbens but not in the area postrema and parabrachial nucleus (Stadlbauer *et al.*, 2013a). Functional magnetic resonance imaging shows that infusion of PYY(3–36) to human subjects has a differential effect on neuronal activity within hypothalamic, corticolimbic and higher cortical brain regions, depending on the plasma concentrations of the peptide (Batterham *et al.*, 2007). In particular, the study results show that PYY(3–36) switches food intake regulation from a homeostatic (hypothalamic) to a hedonic reward-related state reflected by activity in corticolimbic brain areas (Batterham *et al.*, 2007).

Emerging evidence indicates that not only exogenous PYY(3–36) but also endogenous PYY subserves a role in the regulation of food intake, appetite and satiety. Co-administration of PYY(3–36) and GLP-1(17–36) amide to fasted human subjects has an anorectic effect similar to that observed following a meal (De Silva *et al.*, 2011). This modification of energy intake is associated with activation of brain regions implicated in appetite control (e.g., nucleus accumbens, orbitofrontal cortex and insula) as visualized by functional magnetic resonance imaging (De Silva *et al.*, 2011). It follows that postprandial satiety is brought about by a combined action of the two anorectic gut hormones PYY and GLP-1 (De Silva *et al.*, 2011), a finding that also implies that combined activation of PYY and GLP-1 signaling may be a more efficacious anti-obesity treatment regimen than either hormone alone. This inference is supported by another study in which co-infusion of GLP-1 and PYY(3–36) was found to synergize in reducing energy intake by healthy male subjects (Schmidt *et al.*, 2014). Co-infusion was accompanied by slightly increased nausea and a decrease in plasma ghrelin, but neither of these factors could explain the reduction in energy intake (Schmidt *et al.*, 2014).

It is relevant to note in this context that attenuation of food intake is not the only effect which PYY(3–36) can induce in the brain because, owing to its limited selectivity for Y2 receptors, it can also stimulate other Y receptor subtypes. This is true for the action of PYY in the lateral parabrachial nucleus of the brainstem in which PYY-expressing neurons originating from the gigantocellular reticular nucleus play a role in the regulation of energy homeostasis (Alhadeff *et al.* 2015). Microinjection of PYY(1–36) or PYY(3–36) into this nucleus increases food intake, an effect that is primarily mediated by Y1 receptors, although Y2 and Y5 receptors are also present in this region (Alhadeff *et al.* 2015). Y5 receptor signaling has in fact been shown to counteract the anorectic effect of PYY(3–36) (Shi *et al.*, 2017).

The effect of PYY(3–36) to reduce appetite and food intake in rodents and humans and to attenuate weight gain in rodents attributes this peptide a potential role in treating obesity. Its presumed benefit as an anti-obesity drug, however, is challenged by the narrow therapeutic index, given that nausea and vomiting may occur when administered at higher doses. The ability of PYY (3–36) to activate Y receptors other than Y2 receptors is also a drawback, given that stimulation of Y1 receptors in the lateral parabrachial nucleus stimulates food intake (Alhadeff *et al.* 2015). Although systemic administration of PYY(3–36) to obese human subjects causes an acute reduction of food intake, no significant body weight loss was achieved following a 12-week treatment with the peptide administered intranasally (Gantz *et al.*, 2007). Chronic subcutaneous infusion of PYY(3–36) to mice leads in fact to a Y5 receptor-dependent increase in food intake and body weight (Shi *et al.*, 2017). In humans there is limited information on the long-term efficacy and safety of PYY(3–36) administration. Several options have been considered to overcome the limitations in pharmacokinetic properties, receptor selectivity, efficacy and adverse effect profile of PYY(3–36). Nausea and vomiting seem to be related to rapid absorption and access of the peptide to its effector sites following systemic (subcutaneous, intravenous) or intranasal administration, while oral formulations enabling the peptide to survive the gastric passage seem to be less prone to cause sickness (Steinert *et al.*, 2010; De Silva and Bloom, 2012). The design of highly Y2 receptor-selective and long-acting derivatives of PYY, the refinement of the administration routes, and the combination of PYY preparations with GLP-1 analogues (Steinert *et al.*, 2010) may be further approaches to novel anti-obesity therapies.

PYY as a Mediator of Weight Loss Caused by Bariatric Surgery

Alterations in the secretion of PYY along with other gut hormones are associated with the weight loss caused by bariatric surgery, which provides further evidence for a physiological role of this gut hormone in the regulation of food intake and energy homeostasis. Bariatric surgery is able to achieve substantial and permanent weight loss, especially if it includes an element of gastrointestinal bypass, such as Roux-en-Y gastric bypass (Field *et al.*, 2010). Observations that postprandial PYY(3–36) levels appear to be reduced in obese patients compared to healthy volunteers suggest that obesity is a state of PYY(3–36) deficiency (Meek *et al.*, 2016). Following bariatric surgery, both fasting and postprandial levels of PYY increase, an effect that may persist for a long period (Meek *et al.*, 2016). Weight loss after Roux-en-Y gastric bypass is most prominent in patients who exhibit the greatest postprandial rise of PYY and other L cell-derived hormones (Field *et al.*, 2010). These observations have been confirmed and extended in mice suffering from diet-induced obesity and subjected to bariatric surgery. After surgical intervention, the obese mice lost weight and exhibited enhanced expression of PYY in the colon (Chandarana *et al.*, 2011). In addition, both fasting PYY and postprandial PYY and GLP-1 levels in the circulation increased after bariatric surgery. Since the effect of bariatric surgery to cause body weight loss was absent in PYY knockout mice, it appears as if PYY is an important contributor to the weight loss following surgical intervention (Chandarana *et al.*, 2011).

Implications of PYY in Mental Processes and Pain

Apart from regulating ingestion and energy homeostasis, gut hormones are increasingly emerging to have an impact on emotional-affective behavior and mental homeostasis (Holzer *et al.*, 2012). Seen from an evolutionary point of view, co-regulation of appetite and emotional state is an important strategy for survival, given that anxiety and/or depression would be an adverse condition when there is a need to seek food (Holzer *et al.*, 2012). This argument is supported by the activity profile of ghrelin which is released from the upper GIT under conditions of hunger and reduces both anxiety-like and depression-related behavior (Lutter *et al.*, 2008). The activity profile of PYY, which is released under fed conditions, is different, given that the mental status of human subjects treated with PYY(3–36) after a meal is changed to a hedonic state (Batterham *et al.*, 2007). This ability of PYY to promote

hedonic behavior is in keeping with the observation that knockout of PYY increases depression-like behavior but does not alter anxiety (Painsipp *et al.*, 2011). The site where the antidepressant effect of PYY takes place and the Y receptor subtypes that mediate this effect await to be elucidated.

Experimental and clinical studies have shown that peripheral administration of PYY(3–36) alters not only the activity of hypothalamic centers involved in feeding control but has also an impact on neuronal activity in the brainstem, in corticolimbic and higher cortical brain regions (Batterham *et al.*, 2007; De Silva *et al.*, 2011; Stadlbauer *et al.*, 2013a). In line with these findings, PYY(3–36) influences a wide spectrum of behavioral and cognitive functions that are pivotal for basic processes of perception and judgment, including central information processing, working memory, and behavioral responding to novelty (Stadlbauer *et al.*, 2015). Specifically, systemic administration of PYY(3–36) inhibits social interaction without affecting innate anxiety, impairs performance in particular learning and memory tasks and, at a high dose, disrupts sensorimotor gating in the form of a prepulse inhibition deficit (Stadlbauer *et al.*, 2013b). Some of these behavioral disturbances are prevented by dopamine D2 receptor antagonism (Stadlbauer *et al.*, 2013b). In addition, intraperitoneally injected PYY(3–36) increases novel object exploration and enhances the motor responses to indirect and direct dopamine receptor agonists (Stadlbauer *et al.*, 2014). These behavioral alterations are associated with activation of neurons producing γ-aminobutyric acid in the dorsal and ventral striatum (nucleus accumbens and caudate putamen) (Stadlbauer *et al.*, 2014). Taken all findings together, PYY-mediated signaling from the gut to the brain may affect a variety of mental processes, but the physiological and pathophysiological relevance remains yet to be delineated.

While NPY has been implicated in nociception, a potential role of PYY in pain sensitivity has remained largely unexplored, although the expression of this gut hormone is altered in functional and inflammatory bowel disorders. It is hence worth noting that visceral pain-related behaviors in response to chemical irritation of the colon are significantly exaggerated in PYY knockout mice (Hassan *et al.*, 2017). This observation has been confirmed with a peripherally active Y2 receptor antagonist. In addition, PYY knockout mice are more sensitive to somatic thermal pain compared to wild-type animals (Hassan *et al.*, 2017). These results indicate that endogenous PYY has the potential to diminish visceral chemical pain, an effect that seems to be mediated by peripheral Y2 receptors.

Pathophysiological Implications of PYY

While any implication of PYY in neuropsychiatric disorders awaits to be explored, the peripheral PYY system has been found to be disturbed in a number of gastroenterological and metabolic diseases. In most cases, changes in PYY expression and release appear to be secondary disturbances that take place in response to the pathophysiological conditions caused by the disease (El-Salhy *et al.*, 2013). This applies to gastrointestinal diseases/disorders such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), celiac disease, systemic sclerosis, and post-intestinal resection state. In contrast, the changes in the PYY system seen in chronic idiopathic slow transit constipation may be one etiological factor of the disease (El-Salhy *et al.*, 2013). In this condition, the number of PYY cells and tissue concentrations of PYY in the colon are increased in some patients in whom the excess of PYY might decrease secretion of water and electrolytes and strengthen the ileal brake (El-Salhy *et al.*, 2013). In general, the pathophysiological aberrations of the PYY system may, on the one hand, contribute to the development of symptoms present in IBS, IBD, gastroenteropathy in long-standing diabetes and slow transit constipation and, on the other hand, reflect attempts to compensate for the primary disturbances associated, e.g., with celiac disease, systemic sclerosis and post-intestinal resection state (El-Salhy *et al.*, 2013).

The density of PYY cells in the colon of both IBS patients with constipation and IBS patients with diarrhea is down-regulated, and this change is proposed to be secondary to alterations in the serotonin and cholecystokinin systems (El-Salhy *et al.*, 2013). In contrast, the number of PYY-positive cells is reported to be increased in the large intestine of post-infectious IBS patients who have a history of gastroenteritis or other infections (El-Salhy *et al.*, 2013). In ulcerative colitis and Crohn's disease, the two major forms of IBD, the expression of PYY in the colon is decreased, a change that extends to the ileum of patients with Crohn's disease (El-Salhy *et al.*, 2013). Down-regulation of colonic PYY is also observed in two rodent models of IBD, that is, in the colitis associated with interleukin-2 knockout and in dextran sulfate sodium-induced colitis (El-Salhy *et al.*, 2013).

In celiac disease, both basal and postprandial plasma levels of PYY are elevated, a perturbation that normalizes within 8 months on a gluten-free diet. The changes in the endocrine cell system in the small intestine of patients with celiac disease are thought to be reactions to the marked decrease in intestinal absorptive area, resulting in diarrhea and steatorrhea (El-Salhy *et al.*, 2013). The elevated levels of circulating PYY may represent an attempt to slow down the intestinal transit and increase intestinal absorption. The finding that patients with diarrhea due to other causes, such as chronic destructive pancreatitis and infectious gastroenteritis, also have high plasma levels of PYY support this assumption (El-Salhy *et al.*, 2013). A similar relationship may explain why the plasma levels of PYY are increased in patients with systemic sclerosis that suffer from fat malabsorption and diarrhea (El-Salhy *et al.*, 2013).

The gastroenteropathy in long-standing diabetes manifests itself in manifold symptoms including nausea, vomiting, diarrhea, constipation and abdominal pain and is thought to result from gastrointestinal dysmotility and a disturbance of secretion/absorption. In patients with long-standing diabetes type 1 the number of rectal PYY cells is significantly higher than in healthy volunteers (El-Salhy *et al.*, 2013). A similar increase in the number of colonic PYY cells and the colonic tissue concentration of PYY has been found in a murine model of diabetes type 2 (db/db mice). However, in a murine model of diabetes type 1 (non-obese

diabetic mice) and another model of diabetes type 2 (ob/ob mice) the number of colonic PYY cells and the tissue level of PYY are diminished (El-Salhy *et al.*, 2013). The discrepancy in these findings is explained by differences in the duration of the diabetic state, given that the alterations in the PYY system may reflect secondary and compensatory reactions to the gastrointestinal disturbances that manifest themselves during the course of disease (El-Salhy *et al.*, 2013).

Surgical resection of the stomach results in elevated levels of circulating PYY, which may reflect an adaptive attempt to slow down gastric transit (El-Salhy *et al.*, 2013). Basal and postprandial levels of PYY are increased after a massive small bowel resection in the dog, an intervention which in piglets results in an increase in colonic PYY cells. A rise of basal and postprandial PYY concentrations has likewise been observed in patients subjected to small bowel resection, mainly due to Crohn's disease, or resection of the colon. Further analysis has shown that, following resection of a considerable part of either the small or large intestine, PYY synthesis and release increase as an adaptive response to slow the rapid gastrointestinal transit and to facilitate nutrient and electrolyte absorption (El-Salhy *et al.*, 2013).

This selection of pathophysiological implications illustrates the potential of the PYY system as a target for therapeutic intervention. Selective agonists or antagonists for the Y receptors operated by PYY would be of therapeutic benefit in those conditions in which changes in PYY activity contribute to the manifestation of disease symptoms. These indications also include attempts to enhance β-cell mass in diabetes and to prevent/reduce obesity. Given the complex endocrine and neuronal regulation of those physiological processes in which PYY plays a role, it will be a challenge to develop Y receptor ligands with sufficient bioavailability, selectivity, efficacy and safety.

See also: Endocrine Disruptors and Obesity. Gastro-Intestinal Hormones and Their Regulation of Food Intake. Management of Obesity in Children and Adolescents: Lifestyle and Exercise Options. Obesity and Food Addiction. Obesity and Reproduction. Obesity, Childhood, and Adolescence

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https://www.ncbi.nlm.nih.gov/gene/18167–The National Center for Biotechnology Information (NCBI). Npy2r neuropeptide Y receptor Y2.
http://www.genecards.org/cgi-bin/carddisp.pl?gene=NPY1R–GeneCards® Human Gene Database (Weizmann Institute of Science). *NPY1R* Gene.
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http://www.guidetopharmacology.org/–IUPHAR/BPS Guide to Pharmacology.
http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=305–IUPHAR/BPS Guide to Pharmacology. Y_1 receptor.
http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=306–IUPHAR/BPS Guide to Pharmacology. Y_2 receptor.

Glucagon and Glucagon-Like Peptides☆

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Glossary

Apoptosis A highly regulated intracellular process resulting in the programmed death, destruction, and clearance of a cell.

Differentiation The process by which cells develop into a particular phenotype or functional end-point by specific alterations in their gene expression profiles.

Hyperglycemia A physiological state of abnormally elevated levels of plasma glucose.

Insulinotropic The ability to potentiate insulin expression and secretion.

Introduction

The biologic activity of the hormone glucagon was first encountered in the 1920s in crude preparations of insulin derived from pancreas extracts and was considered to be a hyperglycemia-producing contaminant in the extract (Lefèbvre, 2011). Glucagon was later isolated from pancreatic extracts, purified, and characterized and its physiological role in glucose regulation was established. Since that time, the development of improved techniques and experimental tools has allowed researchers to further understand the biologic properties of glucagon and of its closely related peptide family members, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) (Kieffer and Habener, 1999). As is the case in all fields of biological research, the advent of modern molecular and cellular biology has allowed for a precise resolution of the biosynthesis and biological activities of these peptides. This article discusses the biology of glucagon and the glucagon-like peptides.

Genetic Evolution

Glucagon, GLP-1, and GLP-2 are distinct peptide hormones closely related in their amino acid sequences, as shown in **Fig. 1** (Kieffer and Habener, 1999). However, each of these peptide hormones plays diverse roles in the regulation of energy homeostasis. They are encoded by the transcription of a single preproglucagon gene, which in humans consists of six exons and five introns, spans 9.4 kb, and is located on chromosome 2 (Drucker *et al.*, 2017). The preproglucagon mRNA is translated into a proglucagon precursor protein that is then differentially cleaved into functional peptide hormones in a tissue-specific manner. As each hormone is encoded by a separate exon within the gene, it is likely that these three peptides initially evolved from a triplication of one exon that encoded an original glucagon-like peptide. This replicative step within the proglucagon gene occurred prior to the diversification of vertebrates more than 500 million years ago and the amino acid sequences of these peptides appear to have independently evolved in an episodic manner. These events probably reflect an evolutionary change in the functions of the hormones, such that in humans, glucagon, GLP-1, and GLP-2 display distinct tissue-specific expression patterns and biological functions.

Regulation of Expression

Not only are glucagon, GLP-1, and GLP-2 encoded by a single gene, but each peptide is derived from a single mRNA sequence and, furthermore, from a single peptide precursor (proglucagon) (Kieffer and Habener, 1999; Drucker *et al.*, 2017). Consistent with their divergent biological functions, however, the biosynthesis and secretion of these hormones are tissue-specific. To achieve selective expression, a distinct set of prohormone convertase enzymes directs the posttranslational modification of proglucagon to generate glucagon, GLP-1, or GLP-2 in a cell-type-dependent manner. Whereas the main product of posttranslational processing in alpha cells of the endocrine pancreas is glucagon, in enteroendocrine L cells of the gut GLP-1 and GLP-2 are the main gene

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Fig. 1 Similarities of amino acid sequences of glucagon and the glucagon-like peptides.

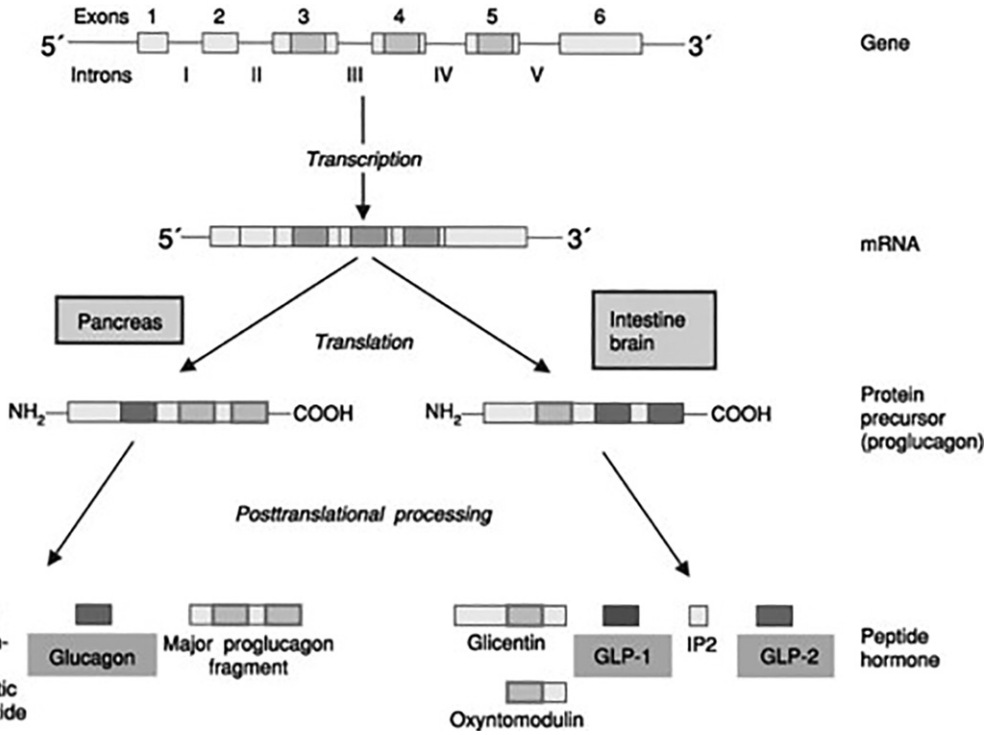

Fig. 2 Tissue-specific processing of the preproglucagon gene.

products. The steps in the expression of the preproglucagon gene are outlined in Fig. 2. Several other potentially bioactive peptides are generated from proglucagon such as oxyntomodulin and IP2 (intervening peptide 2) (as shown in Fig. 2). In addition, two small peptides (five and nine amino acids) with potential anti-oxidant activities and that increase insulin sensitivity, are derived from the C-terminus of GLP-1 (Tomas and Habener, 2010).

Intra-islet Paracrine Cross-talk Signaling Between Alpha and Beta Cells

The close anatomical physical proximity of alpha and beta cells in the islets bespeak local intra-islet paracrine interactions between these two cell types (Bosco *et al.*, 2010). Indeed, recent studies have clearly shown that chemical injury to insulin-producing beta cells sends signals to glucagon-producing alpha cells resulting in the cleavage of proglucagon into GLP-1. The GLP-1 so produced acts locally on beta cells to promote their regeneration and to inhibit apoptosis (Stanojevic and Habener, 2015; Habener and Stanojevic, 2013). Impaired glucagon receptor signaling in beta cells results in a marked hyperplasia of the alpha cells. Further, complete loss of beta cells induces the trans-differentiation of alpha cells into beta cells (Collombat *et al.*, 2010; Stanojevic and Habener, 2015; Habener and Stanojevic, 2013).

Hormone Receptors and Cell Signaling

The functional effects of glucagon and the GLPs are mediated by specific receptors located within the plasma membrane of target cells (Campbell and Drucker, 2013; Gromada *et al.*, 2007; Kieffer and Habener, 1999). Like the peptide hormones, the hormone-specific receptors share extensive amino acid sequence homology and form part of a closely related subfamily of receptors as part of the larger superfamily of G protein-coupled receptors. This superfamily is distinguished by the tertiary structure of its members, comprising seven transmembrane domains, and the coupling to trimeric G proteins. The coupling to the stimulatory G protein G_s, the activation of adenylyl cyclase, and the subsequent increase in the intracellular production of cyclic AMP (cAMP) constitute the most established functional responses elicited by all the members of the glucagon receptor subfamily. This uniform response in

intracellular function suggests that the divergent physiological effects of glucagon, GLP-1, and GLP-2 are due primarily to tissue-specific expression of the receptors, whereby cAMP imparts distinct cellular functions due to the equally diverse array of gene expression patterns in different cell types. Furthermore, temporal and tissue-specific patterns of secretion of the hormones themselves play a critical role in initiating the appropriate functional response. Notably, in the pancreatic beta cell, in which GLP-1 and glucagon receptors are expressed, both glucagon and GLP-1 stimulate insulin secretion, mediated at least in part by cAMP. Paradoxically, however, insulin gene expression is inhibited by glucagon and stimulated by GLP-1. This contradiction suggests that more subtle distinctions in receptor signaling exist, leading to differential intracellular responses to the two hormones, glucagon and GLP-1.

Physiology

Glucagon

The glucagon receptor is widely expressed and can be found in the liver, adipose tissue, heart, kidney, pancreatic islets, stomach, small intestine, thyroid, and skeletal muscle (Campbell and Drucker, 2013; Gromada *et al.*, 2007). Although contradictions in the specific expression pattern exist, likely due to the sensitivity of detection techniques between laboratories, the less ambiguous targets with high receptor levels correspond to the most well-established physiological actions of glucagon. The hepatocyte is a primary target cell of glucagon to which it is exposed when the hormone is released into the portal vein following secretion from the pancreatic alpha cells. Glucagon increases glucose output from the liver, an effect that results from inhibition of glycogen synthesis and stimulation of both glycogenolysis and gluconeogenesis (**Table 1**). Evidence suggests that these effects of glucagon are mediated by cAMP, although the glycogenolytic effect of glucagon may occur via a cAMP-independent mechanism. It is not surprising, therefore, that glucagon helps to maintain hepatic glucose output and thereby to sustain blood glucose levels during fasting. There exists a general consensus that the hyperglycemic activity of glucagon represents the first line of defense against hypoglycemia. Accordingly, stimulants of glucagon secretion from alpha cells include the presence and actions of factors at times when the body requires glucose for sustenance (during fasting) or fuel (during exercise).

The interplay between insulin and glucagon provides a tightly controlled equilibrium in blood glucose concentration. Insulin stimulates glucose uptake into fat and muscle cells and also inhibits glucagon expression and secretion from alpha cells. Thus, during periods when plasma insulin levels are low, such as during periods of fasting and exercising, plasma glucagon levels are elevated. The anatomical structure of the pancreatic islets of Langerhans further reflects this mechanistic action of insulin on glucagon secretion. Morphological studies have shown that in a given islet, the microcirculation goes from the core to the mantle. This circumstance suggests that in vivo, peripheral alpha cells are exposed to high concentrations of insulin released from the more centrally located beta cells in the islets. Thus, insulin and glucagon function inversely to regulate blood glucose levels.

Dysregulation of glucagon by a breakdown in the intra-islet insulin–glucagon relationship appears to be a substantial component in the pathogenesis of diabetes. As a result of insufficient insulin secretion, plasma glucagon levels are elevated in type 1 (insulin-dependent) diabetes and also to a lesser extent in type 2 (non-insulin-dependent) diabetes, thereby exacerbating the hyperglycemic state. This effect is further heightened when plasma glucagon levels fail to be suppressed following a meal and postprandial hyperglycemia ensues. Both excessive glucagon production and failure of insulin production and action are believed to contribute to the hyperglycemia of diabetes (Unger and Cherrington, 2012). It is compelling to speculate, therefore, that agents that are able to antagonize glucagon action or secretion may be of value in the treatment of patients with diabetes.

Paradoxically, a major feature of long-term diabetes is the impaired glucagon response to insulin-induced hypoglycemia, a condition of impairment that is almost universally present after 5 years of duration of type 2 diabetes. Studies have shown that hyperinsulinemia, which may be caused by insulin therapy and insulin resistance, can impair glucagon release resulting in a loss of counter-regulation and the induction of hypoglycemia (Mellman *et al.*, 1992). These examples serve to emphasize the finely balanced hormonal balance that characterizes the normal physiological state of glucose homeostasis.

Consistent with the role of glucagon in providing the body with usable energy, additional established targets of glucagon action are the adipocyte and skeletal myocytes (**Table 1**). Although studies of the effects of glucagon on adipose tissue and other tissues are difficult to evaluate owing to the rapid breakdown of the peptide by proteolytic activity associated with these cells, substantial evidence demonstrates that glucagon induces lipolysis, stimulates the release of glycerol and free fatty acids in adipocytes, and

Table 1 Actions of Glucagon

↑ Hyperglycemia
↑ Glycogenolysis
↑ Gluconeogenesis
↑ Glycerol and free fatty acid release
↑ Lypolysis
↓ Glycogen synthesis

Table 2 Actions of GLP-1

↑ Insulin biosynthesis and secretion
↑ Islet proliferation and neogenesis
↑ Islet cell differentiation
↑ Glucose uptake
↓ Gastric emptying
↓ Acid secretion
↓ Food intake (appetite)
↓ Glucagon secretion

stimulates glycogenolysis in myocytes. Therefore, glucagon could qualify as the "hormone of fuel need" rather than just a "hormone of glucose need."

GLP-1

The major secreted and bioactive form of GLP-1 from entero-endocrine L cells is a truncated isopeptide cleaved from the full 37-amino-acid peptide. Cleavage of the six N-terminal amino acids of GLP-1(1–37) to form GLP-1 (7–37) and removal of the glycine residue at position 37 followed by amidation of the exposed arginine at position 36 generate GLP-1(7–36)amide (Kieffer and Habener, 1999; Campbell and Drucker, 2013). The most significant physiological function of GLP-1 is the stimulation of the synthesis and secretion of insulin from pancreatic islet beta cells (**Table 2**). The mechanism of action of GLP-1 is a potentiation of glucose-induced insulin secretion. In the absence of glucose, GLP-1 fails to stimulate insulin secretion, whereas at elevated glucose concentrations, GLP-1 stimulates secretion at a rate that exceeds that induced by glucose alone and thus potentiates secretion in a glucose-dependent manner. Notably, the secretion of GLP-1 from L cells is stimulated by glucose (and other nutrients) following oral ingestion. Thus, the temporal release of GLP-1, coincident with enhanced blood glucose levels, cooperates synergistically to provide a highly sensitive insulin-releasing mechanism. GLP-1 is thus regarded as a "glucose competence factor." (Holz *et al.*, 1993) As described above, insulin suppresses plasma glucagon levels and activates glucose uptake and metabolism, thus returning blood glucose levels to normal following a meal and maintaining glucose homeostasis in general. In this regard, insulin and GLP-1 play an important role in preventing hyperglycemia.

Evidence for additional mechanisms of action of GLP-1 in lowering blood glucose levels have emerged; GLP-1 has been shown to augment insulin-mediated glucose uptake in patients with diabetes. Extra-pancreatic targets of GLP-1 actions, particularly those of adipose and skeletal muscle tissues, are under investigation. It seems possible that a novel GLP-1 receptor mediates these effects of GLP-1 on adipose tissue and skeletal muscle, as well as liver. There is also evidence that small peptides of 5 and 9 amino acids, cleaved from the C-terminal end of GLP-1, exert anti-oxidant actions on peripheral tissues and thereby increase their insulin sensitivity (Tomas and Habener, 2010).

Additional functions of GLP-1 include the suppression of glucagon secretion and the inhibition of gastric emptying (by inhibiting intestinal motor activity in response to nutrients in the distal gut) and of meal-stimulated gastric acid secretion. A role for GLP-1 in feeding behavior has also been identified. Centrally administered GLP-1 inhibited both feeding and drinking activity in rats, suggesting that GLP-1 may be a central neurotransmitter that modulates visceral functions. Finally, in addition to the detection of pluripotent stem cells within the pancreas, some evidence suggests that GLP-1 stimulates the proliferation and differentiation of these stem/progenitor cells into beta cells (Habener, 2001; Cho *et al.*, 2014). Accordingly, GLP-1 may have a role in the embryonic development of the endocrine pancreas.

Because of its multifunctional profile GLP-1 is in use as for the treatment of diabetes mellitus (Ahrén, 2013; Sandoval and D'Alessio, 2015). By enhancing both the means to secrete insulin (by incretin action and differentiation of new beta cells), the functional ability of insulin to induce glucose disposal, and to reduce appetite, GLP-1 receptor agonists have proven to be effective in controlling blood sugar levels in diabetic patients (Drucker *et al.*, 2017; Sandoval and D'Alessio, 2015).

GLP-2

GLP-2 is synthesized in the enteroendocrine L cells (along with GLP-1) and in the brainstem and hypothalamus of the central nervous system (Drucker and Yusta, 2014). GLP-2 may act on the hypothalamus to curtail appetite and food intake. However, the regulation and function of GLP-2 in the intestinal system are better understood than those of GLP-2 in the central nervous system.

Initially, several lines of evidence suggested the importance of GLP-2 in maintaining the functional integrity of the gastrointestinal epithelium (Drucker and Yusta, 2014). Examples included the coincidence of intestinal injury and inflammatory bowel disease with elevated levels of circulating GLP-2 and patients with glucagon-secreting tumors who presented with small bowel villus hyperplasia (**Table 3**). It was subsequently shown that administration of GLP-2 to mice leads to induction of intestinal epithelial proliferation. Furthermore, following treatment of mice and rats with exogenous GLP-2 for 7–10 days, a marked increase in villus height and small bowel mass and a smaller increment in small bowel length are observed. GLP-2 also appears to slow the

Table 3 Actions of GLP-2

↑ Nutrient absorption
↑ Crypt cell proliferation
↑ Small and large bowel growth
↑ Villus height
↓ Food intake (appetite)
↓ Gastric motility
↓ Enterocyte and crypt apoptosis

ingestion and transit of food through the gastrointestinal tract, while increasing the absorption of nutrients from the small intestine.

As with glucagon and GLP-1, the tissue-specific effects of GLP-2 action are closely reflected by the expression profile of the GLP-2 receptor, which is predominantly found in the stomach, jejunum, ileum, and colon (Drucker and Yusta, 2014). Although GLP-2 presumably exerts direct effects on cells expressing its putative receptor, some physiological effects of GLP-2 in the intestine are believed to be exerted indirectly in an autocrine, paracrine, or endocrine manner by stimulating the release of as yet unknown factors from GLP-2 receptor-positive cells. This hypothesis arose due to conflicting data regarding the exact tissue distribution of the GLP-2 receptor. Whereas one group observed that binding of labeled GLP-2 localized diffusely along the villous epithelium, others have reported the absence of the receptor among several intestinal epithelial cell lines. Thus, because the localization profile of the GLP-2 receptor is not known with certainty, the mechanism of the pleiotropic biological actions of GLP-2 in the gut, such as modulation of gastric motility, small bowel permeability, and both crypt cell proliferation and apoptosis, is speculative. However, GLP-2 has proven to be effective in the clinical treatment of short bowel syndrome by allowing for a reduction in the frequency of parental nutrition (Jeppesen, 2012).

Apart from its intestinal functions, GLP-2 is also expressed and received in the brain. Receptors were located in the hypothalamus and in certain cell groups in the cerebellum, medulla, amygdala, and hippocampus. Although studies of GLP-2 action in the brain are just beginning, a functional role of GLP-2 appears to be in the regulation of food intake. Pharmacological and behavioral studies indicate that GLP-2 acts as a specific transmitter that may inhibit feeding behavior and has potential long-term effects on body weight homeostasis.

Inactivation of GLPs

The active half-life of both GLP-1 and GLP-2 is regulated by dipeptidyl peptidase IV (DPP IV/CD26), an extracellular soluble or membrane-anchored protease, which cleaves the two N-terminal amino acids and subsequently inactivates the hormones, with respect to their known functions (Mentlein, 1999). This potent inactivation restricts the circulating bioactive half-lives to 2–3 min in vivo. Particularly high levels of expression of DPP IV are found in the kidney, lung, liver, and jejunum. The expression of DPP IV in endothelial cells of the blood vessels, and the presence of small amounts of DPP IV as a soluble enzyme in the blood, results in close contact with circulating substrate and added rapidity of peptide inactivation. Consequently, specific DPP IV inhibitors are of special interest for physiological investigations and are in use as a treatment of type 2 diabetes and improvement of mucosal regeneration. Studies have shown that prolonged activation of GLP-1 by DPP IV inhibitors results in a potentiation of the insulinotropic effect of GLP-1 in vivo and administration of GLP-2 to DPP IV-deficient rats markedly increased the bioactivity of GLP-2, resulting in a significant increase in small bowel weight. A second approach to enhance the bioactive half-life of the GLPs is the synthesis of DPP IV-resistant analogues of the GLPs for pharmacological administration. Several of these analogues are currently in clinical use and others are late stage development for use in the treatment of type 2 diabetes.

See also: Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS). Pancreatic Islet Cell Tumors

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Glucagon Like Peptide 2 (GLP-2)☆

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Introduction

Glucagon-like peptide 2 (GLP-2) is a 33-amino acid peptide created by specific posttranslational proteolytic cleavage of proglucagon in a process that also liberates the related Glucagon-like peptide 1 (GLP-1). GLP-2 is produced by the intestinal endocrine L cell and by various neurones in the central nervous system. Intestinal GLP-2 is cosecreted along with GLP-1 upon nutrient ingestion. GLP-2 appears to have a role in stimulating gut hypertrophy by ileal cell hyperplasia and reducing apoptosis and has been used therapeutically in patients with short bowel syndrome (Estall and Drucker, 2003; Martin *et al.*, 2005). The actions of GLP-2 are transduced by the GLP-2 receptor (GLP2R), which is localized within the gastrointestinal GI) tract to enteroendocrine cells, sub-epithelial myofibroblast cells, and in the neurons of the enteric nervous system. The main site of action for the hormone peptide is the GI tract (where the peptide exerts trophic properties). In addition to promoting expansion of the GI mucosal surface area, GLP-2 has been shown to affect gastrointestinal motility in humans and rodents. In fact, it inhibits gastric emptying (Nagell *et al.*, 2004), decreases gastric fundic tone leading to enhanced gastric capacity (Amato *et al.*, 2009). Besides reducing intestinal transit in vivo (McDonagh *et al.*, 2007), GLP-2 additionally reduces small and large intestinal motility in vitro (Amato *et al.* 2010; Cinci *et al.*, 2011).

Physiology

GLP-2 is a pleiotropic hormone with a wide range of actions and interactions with other systems. It exerts its main biological effects in energy homeostasis, intestinal mucosal adaptation to enteral refeeding, small-intestine susceptibility to mucosal injury and intestinal host–microbiota interaction (Drucker and Yusta, 2014). It is released in response to stimulation by intake of luminal nutrients, such as glucose, fatty acids and dietary fiber (Brubaker, 2006). GLP-2 however, circulates at low basal levels in the fasting period too, and plasma levels rise rapidly after food ingestion. Renal clearance and enzymatic inactivation, control the elimination of bioactive GLP-2. GLP-2 is cleaved to inactive GLP-2 (3-33) by DPP-IV; consequently, the half-life of intravenous GLP-2 is very short, about 7 min in healthy humans (Hartmann *et al.*, 2000). Although, GLP-2 (3-33) may act as a weak agonist at pharmacological concentrations (Shin *et al.*, 2005); it is however able to act as a competitive antagonist of the GLP-2 receptor (GLP2R) in rodents (Thulesen *et al.*, 2002; Shin *et al.*, 2005; Baldassano *et al.*, 2009, 2013). Dipeptidyl-peptidase (DPP)-IV-resistant GLP-2 analogues, such as [Gly2]-GLP2 (teduglutide), exhibit greater bioactivity relative to the native molecule, due to their longer circulating half-lives (Tavares *et al.*, 2000; Baldassano and Amato, 2014).

GLP-2 promotes energy absorption within the GI tract through nonspecific and specific adaptation. In fact, it induces crypt cell proliferation and inhibition of apoptosis, resulting in an increase of villous height and in the expansion of the absorptive mucosal surface in the small and large intestine (Drucker and Yusta, 2014). Evidence from studies on humans and animal models suggests that GLP-2 also plays a role in lipid absorption. Indeed, GLP-2 facilitates intestinal absorption of lipids (Meier *et al.*, 2006; Hsieh *et al.*, 2009) and enhances and regulates chylomicron secretion from the intestine (Hsieh *et al.*, 2009). The GI responses to GLP-2 are mediated via GLP-2R, a member of the glucagon/secretin G protein coupled receptor superfamily that is located on enteric (Bjerknes and Cheng, 2001; Baldassano *et al.*, 2009) and vagal nerves (Nelson *et al.*, 2007), sub-epithelial myofibroblasts (Ørskov *et al.*, 2005) and a subset of intestinal epithelial cells (Thulesen *et al.*, 2000).

GLP-2 is cleaved to inactive GLP-2 (3-33) by DPP-IV; consequently, the half-life of intravenous GLP-2 is very short, about 7 min in healthy humans (Hartmann *et al.*, 2000). Although, GLP-2 (3-33) may act as a weak agonist at pharmacological concentrations (Shin *et al.*, 2005); it is however able to act as a competitive antagonist of the GLP-2 receptor (GLP-2R) in rodents (Thulesen *et al.*, 2002; Shin *et al.*, 2005; Baldassano *et al.*, 2009, 2013). Then DPP-IV-resistant GLP-2 analogues, such as [Gly2]-GLP2 (teduglutide), exhibit greater bioactivity relative to native molecule, due to their longer circulating half-lives (Tavares *et al.*, 2000; Baldassano and Amato, 2014). Teduglutide is currently licensed and available for the management of adult patients with short bowel syndrome under specialist follow-up.

☆*Change History*: April 2018. Dr Georgios K Dimitriadis and Dr Alexander D Miras prepared the updated manuscript and the new manuscript is entirely different than the original by Lovshin and Drucker.

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GLP-2 and Glucose Homeostasis

To date, the role of GLP-2 in glucose homeostasis has not been fully elucidated. However, evidence from studies in GLP-2R KO mice have shown that global deficiency is not crucial for glucose equilibrium in healthy or lean mice with diabetes and is not associated with changes in fasting glucose, glucose tolerance or plasma glucagon (Bahrami *et al.*, 2010). Furthermore, chronic exposure to a GLP-2R antagonist (3-33), used to study the physiological actions of GLP-2 did not affect glucose or insulin levels and β-cell mass in rodents. This has led us to believe that endogenous GLP-2 has no role in glucose homeostasis in the normal state. In addition, changes in glucagon levels or glycaemia have not been reported following acute or chronic GLP-2 or teduglutide administration in patients with short bowel syndrome (Drucker and Yusta, 2014). It is well established that GLP-2 does not exhibit any insulin-releasing properties (Schmidt *et al.*, 1985; Ørskov *et al.*, 1988). On the contrary, intravenous GLP-2 infusion has been demonstrated to increase glucagon secretion in healthy, nonobese human subjects both in physiological and pharmacological plasma concentration (Meier *et al.*, 2006) or in patients with T2DM (Lund *et al.*, 2011). GLP-2R has been localized to α-cells in both human and rat islets by means of immunohistochemistry as well as real-time qPCR (de Heer *et al.*, 2007).

In contrast, full-length GLP-2R mRNA transcripts have not been detected in RNA from murine islets, and GLP-2 does not increase plasma glucagon levels in mice (Bahrami *et al.*, 2010). The discrepancy could be attributed to different species; however, caution should be taken in ruling out a glucagonotrophic role of GLP-2 in mice. In fact, recent research have demonstrated that GLP-2R is more widely expressed than it was estimated (Angelone *et al.*, 2012; El-Jamal *et al.*, 2014). Therefore, it is necessary to explore the presence and localization of GLP-2R in the pancreatic islets using validated GLP-2R antibody and the opportune positive and negative controls to yield to conclusive results. In any case, GLP-2 glucagonotrophic properties could suggest that the endogenous or exogenous peptide exacerbates hyperglycemia conditions related to diabetes. Nevertheless, glucagon hypersecretion induced by oral glucose, which is typical of patients with T2D (Knop *et al.*, 2007), is not a consequence of exaggerated secretion or effect of GLP-2 (Lund *et al.*, 2011), and GLP-2R global absence in genetically obese mice increases glucagon secretion and hyperglycemia (Bahrami *et al.*, 2010). Therefore, the importance of GLP-2 in the control of glucagon secretion in different species and in diverse pathological conditions related to glucose impairment requires further elucidation.

New evidence obtained from GLP-2R tissue-specific KO mice indicates that GLP-2R in POMC neurons is essential for suppressing hepatic glucose production (Shi *et al.*, 2013). Indeed, mice lacking GLP-2R selectively in POMC neurons display impaired postprandial glucose tolerance and hepatic insulin resistance (by increased gluconeogenesis), suggesting a physiological significance of GLP-2 neural action in glycaemic control. Moreover, intracerebroventricular infusion of GLP-2 increases glucose tolerance and insulin sensitivity and suppresses basal hepatic glucose production through GLP-2R activation in POMC neurons (Shi *et al.*, 2013). Therefore, GLP-2 has been proposed as a crucial neuroendocrine signal for glucose homeostasis (Guan, 2014). It will be crucial to determine whether CNS GLP-2R is a key contributor to glycaemic control and insulin sensitivity also in humans.

GLP-2 and Energy Homeostasis

Because no changes in body weight or in the daily food intake have been observed in GLP2 (3-33)- or Gly2-GLP-2-treated mice in comparison with untreated animals, an indirect effect mediated by an anti-obesity action has not been supported (Baldassano *et al.*, 2016). Reduced adiposity could contribute to the preservation of insulin sensitivity because the adipose tissue has a considerable influence on systemic glucose homeostasis through secretion of adipocytokines (McArdle *et al.*, 2013). Interestingly, GLP-2R mRNA expression has been detected in mouse mesenteric adipose tissue (El-Jamal *et al.*, 2014); however, its functional significance is still unknown. It is important to characterize whether GLP-2 increases glucose incorporation in adipocytes when their response to insulin is impaired by obesity conditions. In fact, although there are conflicting reports regarding the alterations in the basal glucose uptake, most reports provide evidence that insulin-induced glucose incorporation is suppressed in the obese state (Talior *et al.*, 2003; Sancho *et al.*, 2006; Crowe *et al.*, 2008), and as GLP-2 rapidly increases intestinal hexose absorption (Cheeseman, 1997; Au *et al.*, 2002), suggesting that it could contribute to clearance of plasma glucose through an action on adipocytes. In addition, or alternatively, GLP-2 might influence and modulate the quality and quantity of secreted adipokines and the inflammatory state of adipose tissue.

There are various studies assessing the effects of Roux-en-Y Gastric Bypass (RYGB) on GLP-2 regulation. Taqi *et al.* in an experimental study, demonstrated a significant increase in GLP-2 levels after gastric bypass in rats (Taqi *et al.*, 2010). LeRoux *et al.*, in a human prospective study, demonstrated a significant increase in the postprandial levels of GLP-2 after RYGB, with a secretion peak observed 6 months after the procedure (LeRoux *et al.*, 2010) Cazzo *et al.*, in a human prospective study, observed a significant increase in the GLP-2 levels 12 months after surgery and demonstrated that this increase was significantly correlated with aspects of satiety regulation (Cazzo *et al.*, 2017). Comparing individuals who underwent RYGB and vertical sleeve gastrectomy (VSG), Romero *et al.* observed in a prospective study that both procedures led to a significant increase in the postprandial levels of GLP-2 6 weeks after surgery, without significant difference between the two evaluated procedures (Romero *et al.*, 2012). Cummings *et al.*, in an experimental study, demonstrated a significant increase in the GLP-2 levels in rats after VSG (Cummings *et al.*, 2012). Evidence suggests that GLP-2 postoperatively increases, and this change may be potentially related to weight loss stabilization, late

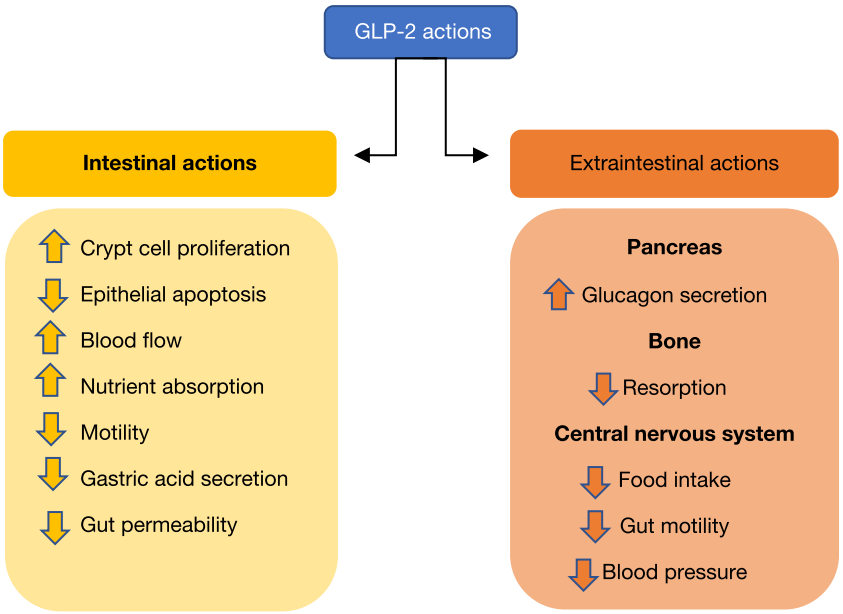

Fig. 1 Intestinal and extraintestinal actions of GLP-2.

reduction of diarrhea and malabsorption, partial compensation of effects on bone mineral metabolism, minimization of the consequences of bacterial overgrowth and satiety regulation (Fig. 1).

Conclusion and Perspective

Recent advances in our understanding of gut biology following bariatric surgery highlight the role of GLP-2 in promoting control and insulin sensitivity in the animal model, particularly in conditions associated with energy excess such is obesity. Results from human studies so far remain inconclusive; therefore, future research in this area is crucial to underpin the role of GLP-2 in preventing human insulin resistance and, in turn, the development of T2DM. The potential mechanisms driving the beneficial effects of GLP-2 receptor activation remain elusive. More work is necessary to understand the role of GLP-2 and its potential synergistic effect with other anorexigenic peptides in T2DM and obesity management.

See also: Glucagon and Glucagon-Like Peptides

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Enterochromaffin-Like Cells

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Anatomy and Histology of the Stomach

The stomach is a dilated portion of the gastrointestinal tract in which the bolus of the food received from esophagus undergoes mechanical and chemical reduction to form chyme. Anatomically the stomach is divided into four regions: the cardia, fundus, corpus (body), and pylorus. The functional units of the stomach are the gastric glands, three to seven of which empty into the bottom of a single gastric pit. The mucosa of the fundus and corpus comprises the major histological compartment of the stomach. It contains numerous parietal cells that produce hydrochloric acid (oxyntic mucosa) and intrinsic factor. Furthermore, it consists of chief cells that produce pepsinogen, mucus secreting cells, as well as endocrine cells. A fraction of the latter cells are responsible for histamine production. The pylorus has a different glandular conformation. Its cells besides producing and secreting mucus can also synthesize, store and secrete gastrin.

Gastric Endocrine Cells

The history of gastric endocrine cells started with Heidenhain in 1870 when he observed cells in the gastric mucosa of dogs and rabbits that stained with dichromate solutions (Heidenhain, 1870). In 1906, Ciaccio introduced the term "enterchromaffin" (EC) cells as he noticed that their staining properties were similar to those of the adrenal chromaffin cells (Ciaccio, 1906). In 1914, Masson showed that the aforementioned cells had the ability to reduce ammoniacal silver salts without adding reducing agents and termed them "argentaffin" to describe this feature (Masson, 1914). An indoleamine called enteramine was initially purified from rabbit gastric mucosa by Vialli and Erspamer and later characterized as 5-hydroxytryptamine (5-HT) by Erspamer and Asero that showed that it is the argentaffin/chromaffin product of the EC cells (Erspamer and Asero, 1952). The last authors recognized that 5-HT is identical to serotonin, which had been isolated, in 1948, by Rapport *et al.* (1948). Gastrin was the first peptide hormone to be detected in epithelial cells of the gastric mucosa (McGuigan, 1968) termed as "G" cells, a type of cells shown to be characteristic of the antral-pyloric glands in the gastric mucosa (Sasagawa *et al.*, 1970). Håkanson and Owman were the first to describe a large population of dopamine containing, green fluorescent epithelial cells in the gastric oxyntic mucosa of rats, by means of fluorescence microscopy. The cells were morphologically similar to yellow fluorescent, serotonin containing EC cells of the pyloric mucosa, and the term "enterochromaffin-like" (ECL) was introduced. A portion of these cells was found to contain histamine by using the fluorescence histochemical *o*-phthalaldehyde technique (Hakanson and Owman, 1967). Further ultrastructural studies of the murine oxyntic mucosa revealed that the predominant population of endocrine cells in the oxyntic mucosa consists of ECL cells (Capella *et al.*, 1971). G and ECL cells comprise the largest endocrine cell population of the oxyntic mucosa, followed by ghrelin cells which are ultrastructurally similar with the previously described P/D_1 cells (Kojima *et al.*, 1999; Rindi *et al.*, 2002). Somatostatin-producing (D cells) and serotonin producing (EC cells) are present in the entire gastric mucosa (D'Adda *et al.*, 1989). The volume density of all endocrine cells in the normal human oxyntic mucosa accounts 1.2% $\pm$ 0.4% of the total mucosal epithelial volume (D'Adda *et al.*, 1989).

In the human gastric mucosa, the above five different endocrine cell types have been identified and characterized (**Table 1**) (Rindi *et al.*, 2002; Solcia *et al.*, 1975; Simonsson *et al.*, 1988). They are divided in "closed" and "open" type cells (Fujita and Kobayashi, 1977; Kobayashi *et al.*, 1971). The "closed" type pattern consists of cells thought to be involved in paracrine and/or endocrine regulatory mechanisms (Solcia *et al.*, 1975; Bordi *et al.*, 1983). On the contrary, the "open" type of endocrine cell is provided with apical cytoplasmatic extensions that project into the glandular lumen with short microvilli. These structures represent the anatomical cornerstone for the gastric endocrine cell response to stimuli from the gastric gland content (Solcia *et al.*, 1975).

Table 1 Characteristics of the human gastric endocrine cells

Cell type	*Main product*	*Distribution*	*Relative incidence (%)*	*Size of secretory granule (nm)*	*Neoplasia*
ECL	Histamine	Fundus/corpus	40–50	160–300	ECL-CCs
P/D_1	Ghrelin	Whole stomach	20–30	110–180	Ghrelinoma[a]
D	Somatostatin	Whole stomach	10–20	200–400	Somatostatinoma[a]
EC	Serotonin	Whole stomach	10–20	150–500	EC cell carcinoid[a]
G	Gastrin	Pylorus	40–60	150–350	Gastrinoma[a]

[a]Very rare cases have been reported in the gastric mucosa.

ECL, enterochromaffin-like; *EC*, enterochromaffin; *ECL-CC*, enterochromaffin-like cell carcinoid.

ECL Cell

The ECL cells are located in the basal part of the gastric pits in the vicinity of parietal cells (Fujiwara *et al.*, 1999) and comprise approximately 40%–50% of all human gastric endocrine cells (Solcia *et al.*, 2000). They are argyrophilic and can be identified by light microscopy using silver impregnation techniques such as Grimelius (Lundqvist *et al.*, 1990) and Sevier-Munger (Solcia *et al.*, 1975), and also by chromogranin A (CgA) immunostaining (Hakanson *et al.*, 1986). Ultrastractural studies have shown that this spherical cell type structures, which is 8–10 μm in diameter, contains numerous secretory granules (Capella *et al.*, 1971; Hakanson *et al.*, 1971a, 1993; Rubin and Schwartz, 1979; Helander, 1981; Delwaide *et al.*, 1991; D'Adda *et al.*, 1991; Andersson *et al.*, 1992; Chen *et al.*, 1996). At present, there is no commercial antibody against histamine for immunohistochemical identification of ECL cells in routine formalin-fixed sections, because its preservation requires a specific fixation procedure. Histamine can be demonstrated in Carnoy-fixed paraffin sections by *o*-phthalaldehyde (Hopwood, 2002). However, these cells can be visualized indirectly by an antibody raised versus a protein involved in the transport of histamine within the granules, vesicular monoamine transporter-2 (VMAT-2) (Erickson *et al.*, 1996; De Giorgio *et al.*, 1996). Accumulation of histamine in the granules of ECL cell is dependent on V-type ATPase and VMAT-2 (Merickel and Edwards, 1995; Dimaline, 1996). The granular H^+ gradient is established by V-type ATPase, which is pumping H^+ into the granules (Forgac, 1999; Nishi and Forgac, 2002). To maintain electro neutrality VMAT-2, which is localized at the vesicular membrane, transfers H^+ outside the granules counter transporting histamine.

The ECL cell expresses subtype somatostatin receptor-2 (Reubi *et al.*, 1992; Prinz *et al.*, 1999), cholecystokinin-B/Gastrin receptor (CCK-B/gastrin receptor) (Wank *et al.*, 1992) and pituitary adenylate cyclase-1 (PAC-1) receptor (Zeng *et al.*, 1998a, 1999a), which represent targets for the relevant hormones and play an important role in gastric acid release. In addition, gastrin has trophic effects on ECL cells in humans and experimental models (Solcia *et al.*, 1993; Bordi *et al.*, 1995; Nilsson *et al.*, 1993). Besides gastrin, helicobacter pylori infection and consequent inflammation have been suggested to induce ECL cell growth. Helicobacter pylori lipopolysaccharide may stimulate ECL cell histamine release and proliferation (Kidd *et al.*, 1997).

ECL Cell and Gastric Acid Release

It is well described, that gastrin is released in response to aromatic amino acids in the lumen of the antrum (DelValle and Yamada, 1990) and reaches the ECL cell via the vascular system. GRP (bombesin) (DuVal *et al.*, 1981) and acetylcholine (Zeng *et al.*, 1996) are two other factors that stimulate gastrin release. Gastrin influences, mainly, gastric acid secretion by stimulating ECL cells via CCK-B/Gastrin receptor, which results to calcium mobilization (Zeng *et al.*, 1999b). When activated by gastrin, the ECL cell responds with increased production and release of histamine, as well as with activation of histidine decarboxylase (HDC) and increase of its mRNA expression (Dartsch *et al.*, 1999; Bjorkqvist *et al.*, 1999; Kitano *et al.*, 2000). The stimulation of HDC transcription by gastrin is mediated by protein kinase C, indirect activation of c-fos and c-jun and stimulation of MAP kinase (Zhang *et al.*, 1996; Hocker *et al.*, 1997a,b). Simultaneously, the gene expression of VMAT-2 is stimulated (Watson *et al.*, 2001) and the CgA promoter is activated (Hocker *et al.*, 1998). Histamine is synthesized by enzymatic decarboxylation of the amino acid histidine by HDC (Viguera *et al.*, 1994), and then stored in secretory granules until its release (Hakanson *et al.*, 1971a,b; Prinz *et al.*, 1999). Once released, histamine interacts with H_2 receptors on parietal cells (Hakanson *et al.*, 1978). Thus, histamine serves as the most important direct stimulus for parietal cells to secrete gastric acid. Then histamine is degraded in various ways. In man, the major pathway includes methylation and oxidative deamination producing 1, 4-methyl imidazole acetic acid (MeImAA) that is excreted into the urine. In this way approximately 40%–60% of histamine is degraded. Around 4%–8% of histamine can undergo methylation and be excreted as methylhistamine. Another substantial fraction (around 20%–40%) of histamine is not methylated and produces the metabolite imidazole acetic acid, which is mainly conjugated to ribose. Finally, a small portion (1%–3%) of histamine is excreted unmetabolized (Granerus *et al.*, 1966).

It has also been proved that pituitary adenylate cyclase activating polypeptide-27 (PACAP-27), which is a neural sympathetic mediator and mediates the cephalic phase of acid secretion (Zeng *et al.*, 1999a; Pisegna *et al.*, 2000; Sandvik *et al.*, 2001; Lindstrom *et al.*, 2001), also plays an important role to gastric acid secretion. PACAP-27 binds to the PAC-1 receptor subtype, which is expressed in ECL cells and induces histamine release through a calcium dependent pathway (Zeng *et al.*, 1999a,b; Pisegna *et al.*, 2000; Lindstrom *et al.*, 2001). Other stimulants proved to evoke histamine secretion from ECL cells are acetylcholine (Zeng *et al.*, 1996), epinephrine and VIP (Prinz *et al.*, 1993; Hakanson *et al.*, 2001). Ghrelin has also been suggested to increase gastric acid secretion (Masuda *et al.*, 2000; Date *et al.*, 2001), whereas galanin, somatostatin, PYY and PGE_2 exhibit inhibitory action on the ECL cell (Zeng *et al.*, 1998a, b; Prinz *et al.*, 1993, 1994; Lindstrom and Hakanson, 2001).

Hyperplasia and Dysplasia of the Gastric Endocrine Cells With Focus on ECL Cell

G-, ECL-, Ghrelin-, and D-cell hyperplasia have been described in the gastric mucosa (Solcia *et al.*, 1988, 1991; Tsolakis *et al.*, 2008). The first three cell types are the most common to undergo hyperplasia which occurs in patients with type A chronic atrophic gastritis (A-CAG). In this autoimmune type of gastritis the body generates antibodies against the parietal cells causing megaloblastic anemia. *Helicobacter pylori* infection has also been described to cause ECL-cell hyperplasia (Solcia *et al.*, 2000). Hypergastrinemia under the concept of Multiple Endocrine Neoplasia 1/Zollinger–Ellison syndrome (MEN1/ZES) can induce both ECL

and ghrelin cell hyperplasia (Tsolakis *et al.*, 2008; Solcia *et al.*, 2010). Four patterns of endocrine cell hyperplasia have been described: simple or diffuse, linear, nodular and adenomatoid (Solcia *et al.*, 2000). Simple or diffuse pattern is termed as increased density of endocrine cells, more than twice the standard deviation as compared to age and sex matched controls. The linear pattern includes linear sequences of five or more endocrine cells along the basement membrane of the gastric glands. Nodular pattern is defined as clusters of five or more endocrine cells forming a nodule. The adenomatoid hyperplasia pattern is the less common and is termed as the collection of five or more nodules of endocrine cells adherent to each other with interposition of basement membrane and thin strands of lamina propria.

Dysplastic or precarcinoid ECL cell lesions are growths between 150 and 500 nm in size that escape endoscopic observation and are scattered in the mucosa. The cells are relatively atypical with features of enlarging or fusing micronodules, or newly formed stroma (Solcia *et al.*, 2010). Despite the fact that these are considered precursor lesions of enterochromaffin-like cell carcinoids (ECL-CCs) they do not infiltrate beyond the muscularis mucosa (Solcia *et al.*, 1986, 1988, 1991, 2010). Nodules that become bigger than 0.5 mm are classified as microcarcinoids (Solcia *et al.*, 2010).

Gastric Neuroendocrine Tumors and Enterochromaffin-Like Cell Carcinoids

According to the WHO 2010 classification the well differentiated gastric neuroendocrine tumors (GNETs) are divided in type I, II, III ECL-CCs, as well as in non-ECL-CC type (Solcia *et al.*, 2010). **Table 2** summarizes the characteristics of ECL-CCs.

Well Differentiated Gastric Neuroendocrine Tumors

Type I ECL-CCs

Type I ECL-CCs have been reported to represent the majority (approximately 75–80%) of GNETs with a female preponderance (M:F ratio, 1:2.5) (Solcia *et al.*, 2010). The incidence of type I ECL-CCs is rising which can be explained by the improved diagnosis of neuroendocrine tumors, doctors' awareness and the increased number of endoscopies performed. However, their overall incidence remains low at around 0.3 cases per 100,000 population, being more common in elderly women (Lawrence *et al.*, 2011; Tsikitis *et al.*, 2012). These tumors develop in $<1\%$ of patients with A-CAG involving the corpus-fundus mucosa. The destruction of parietal cells results to achlorhydria which in its turn causes hypergastrinemia as a secondary response of pyloric G cells (Solcia *et al.*, 2010; Rappel *et al.*, 1995). The long standing hypergastrinemia exerts a trophic effect to ECL cells that ends to ECL-CCs under a hyperplasia–dysplasia–neoplasia sequence. Patients with type I ECL-CCs may also be accompanied with pernicious anemia and other autoimmune diseases (**Table 3**) (Thomas *et al.*, 2013; Modlin *et al.*, 2003). These tumors are usually small, often multiple with relatively uniform cells arranged in small clusters or trabecules (Solcia *et al.*, 2010; Rappel *et al.*, 1995). Most lesions are located in the mucosa, sometimes also in the submucosa, but only rarely with a deeper invasion into the gastric wall (Solcia *et al.*, 2010; Modlin *et al.*, 2003; Rindi *et al.*, 1999). Endocrine cell hyperplasia (ECL- and G-cell) is a constant feature and dysplastic growths are frequently observed (Solcia *et al.*, 1988, 2010; Rindi *et al.*, 1996). The patients are usually asymptomatic, and the diagnosis is often accidental during gastroscopy performed for dyspepsia or megaloblastic anemia. Genetically, these lesions are monoclonal, when assessed by the pattern of X-chromosome inactivation. This fact indicates that type I ECL-CCs are true neoplasms in spite of their indolent nature (D'Adda *et al.*, 1999). However, angioinvasion, lymph node- and distant metastases have also been described (Rindi *et al.*, 1999; Grozinsky-Glasberg *et al.*, 2013).

Type II ECL-CCs

Type II ECL-CCs (M:F ratio, 1:1) account 2%–10% of all GNETs and develop in patients with hypergastrinemia due to gastrin-producing tumor(s) under the ZES/MEN-1 (Solcia *et al.*, 2010; Modlin *et al.*, 2003; Rindi *et al.*, 1999; Feurle, 1994; Peghini *et al.*, 2002). Hypertrophic, hypersecretory gastropathy represent characteristic diagnostic findings (Solcia *et al.*, 2010). The tumors, usually multiple, are associated with ECL cell hyperplasia and/or dysplasia (Rindi *et al.*, 1996). In contrast to the type I ECL-CCs,

Table 2 Characteristics of ECL-CCs

	Type 1 ECL-CCs	*Type II ECL-CCs*	*Type III ECL-CCs*
Percentage of GNETs	75%–80%	2%–10%	10%–15%
Sex preponderance	Female	Equal	Male
Associations	Autoimmune gastritis	MEN1/ZES	None
Localization	Fundus/corpus	Fundus/corpus/(antrum)	Fundus/corpus/antrum
Tumor size	<1 cm	<1 cm	>2 cm
Histopathology	Hyperplasia/dysplasia/neoplasia	Hyperplasia/dysplasia/neoplasia	Normal adjacent mucosa
Biological behavior	Slow growth, rarely metastasizes	Slow growth, may metastasize	Aggressive
Gastrin levels	↑–↑↑	↑–↑↑–↑↑↑	Normal
Gastric acid output	Low or absent	High	Normal
Prognosis	Excellent	Very good	Poor

Table 3 Related autoimmune diseases in a cohort of 111 patients with type I ECL-CCs as presented in a multicenter study (Thomas *et al.*, 2013)

Disease	n	*Percentage (%)*
B12 deficiency	47	42.3
Hashimoto's thyroiditis	41	36.9
Diabetes mellitus type 1	7	6.3
Multinodular goiter	6	5.4
Rheumatoid arthritis	4	3.6
Sjögren's	3	2.7
Systemic lupus erythematosus	3	2.7
Addisson's disease	1	0.9
Vitiligo	1	0.9
Grave's disease	1	0.9

the surrounding of the ECL-CC mucosa has no signs of A-CAG (Solcia *et al.*, 2010). Type II ECL-CCs are mainly located in the corpus-fundus but they can also be identified in the antrum (Bordi *et al.*, 2001). Taking in consideration the lesions' malignant potential type II ECL-CCs are intermediately placed in this respect, between these of type I and type III (Solcia *et al.*, 2010; Modlin *et al.*, 2003; Rindi *et al.*, 1999; Modlin and Tang, 1996).

Type III ECL-CCs

The type III ECL-CCs are also called "sporadic" due to the absence of any specific gastric pathology (no association to hypergastrinemia or A-CAG) (Solcia *et al.*, 2010). They constitute approximately 10%–15% of all GNETs and are observed mainly in males (M:F ratio, 2.8:1) (Solcia *et al.*, 2010; Modlin *et al.*, 2003). They are usually single but occasional multiple lesions have also been described (Solcia *et al.*, 2010; Modlin *et al.*, 2003; Rindi *et al.*, 1996; Moriyama *et al.*, 2003). Most of the tumors are large, and have already metastasized at diagnosis; on that basis they are considered to represent the most malignant type of ECL-CCs (Solcia *et al.*, 2010; Modlin *et al.*, 2003; Rindi *et al.*, 1999). Minute carcinoids of this type are reported to give regional lymph node metastasis (Shinohara *et al.*, 2003).

Non-ECL-CCs

The non-ECL-CCs constitute a group of uncommon tumors that may present with symptoms related to the substance they produce. Serotonin- (Rindi *et al.*, 1999; Quinonez *et al.*, 1988), gastrin- (Soule *et al.*, 1976), ACTH-producing (Hirata *et al.*, 1976; Tsuchiya *et al.*, 2005), and ossifying lesions (Yamagishi *et al.*, 2004) are some examples that have been described in the literature. Recently a new entity of GNET has been described and termed ghrelinoma (Tsolakis *et al.*, 2004).

See also: Carcinoid Syndrome

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Substance P☆

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Glossary

Aprepitant (Emend, MK-869, L-754,030) A nonpeptide NK_1 receptor antagonist used in clinical practice for the prevention of postoperative nausea and vomiting and chemotherapy-induced nausea and vomiting. In addition to the antiemetic action, aprepitant exerts antipruritic effects and it is a broad-spectrum anticancer compound.

Fosaprepitant (Ivemend) It is a prodrug of aprepitant that exerts an antitumor action. Fosaprepitant (intravenous use), via the action of ubiquitous phosphatases, is rapidly converted to aprepitant.

Nonpeptide NK_1 receptor antagonists They are brain-penetrant and are not degraded by peptidases. Aprepitant, fosaprepitant and rolapitant are the only NK_1 receptor antagonists used in clinical practice. Nonpeptide NK_1 receptor antagonists are safe and well tolerated.

Peptide NK_1 receptor antagonists (SP analogue antagonists) In the SP molecule, L-amino acids are replaced by D-amino acids. They are not brain-penetrant, are rapidly degraded by peptidases, have a toxic action and show a poor potency.

Introduction

Substance P (SP) is an 11-amino-acid peptide with the primary structure Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met, which contains a C-terminally α-amidated methionine residue. SP is a member of the biosynthetically and evolutionarily related tachykinin family of regulatory peptides that are characterized by common structural features and a shared spectrum of biological activities. Neurokinin A (NKA), neurokinin B (NKB), and hemokinin-1 (HK-1) peptides also belong to this family. SP is derived from the *TAC1* gene. This gene contains seven exons and the sequence encoding SP is contained in exon 3. The primary RNA transcript of *TAC1* is spliced to produce four types of mRNA (α-TAC1, β-TAC1, γ-TAC1, and δ-TAC1) each of them encode a single-copy of the peptide; β-TAC1 and γ-TAC1 also encode NKA; β-TAC1 the neuropeptide K and γ-TAC1 the neuropeptide γ. After the posttranslational proteolytic processing of the protachykinin protein precursor, SP can be further processed, producing SP fragments (SP (1–4), SP (1–7)) with biological activities.

Tachykinins exert their biological actions through three metabotropic receptors belonging to the G-protein-coupled receptor family: neurokinin-1 (NK_1), NK_2, and NK_3. These receptors are encoded respectively by the *TACR1*, *TACR2*, and *TACR3* genes. In humans, the *TACR1* gene has been located on chromosome 2 and spans 45–60 kb, and it is contained in five exons. SP, NKA, and NKB bind to the three NK receptors. The affinity of the NK_1 receptor for NKB and NKA is, respectively, 500- and 100-fold lower than for SP. Thus, the NK_1 receptor binds the peptide with the highest affinity, so this receptor is often termed the SP receptor. This receptor also shows a high affinity for HK-1 (in nonneural tissues, HK-1 is a potent ligand for the NK_1 receptor). HK-1 induces the proliferation of cells (e.g., B cells, T-cell precursors) and angiogenesis, decreases blood pressure and exerts an antiapoptotic effect.

Historical Introduction

The presence of a vasodilator compound in extracts of horse brain and intestine that also stimulated the motility of the rabbit intestine in the presence of atropine was first reported by von Euler and Gaddum in 1931. A crude preparation of this material was obtained and the active component was shown to be peptidic in nature and was referred to as substance P (P for powder). SP resisted all attempts at purification and structural characterization until 1971, when Chang and Leeman demonstrated that an 11-amino-acid peptide isolated from an extract of bovine hypothalamus on the basis of its potent sialagogic effect in rats was identical to the factor of von Euler and Gaddum. Since then the NK_1 receptor antagonists aprepitant (oral administration), fosaprepitant (prodrug of aprepitant, intravenous use) and rolapitant (oral administration/intravenous use) are used for the prevention of chemotherapy-induced nausea and vomiting (CINV) and postoperative nausea and vomiting (PONV). To date, these drugs are the only NK_1 receptor antagonists used in clinical practice.

☆*Change History*: May 2018. Miguel Muñoz and Rafael Coveñas updated the abstract and the entire text. Table 1 was updated by adding new biological activities and disease correlations. The references appearing in "See Also the Following Articles" and in "Further Readings" were deleted: updated references were added in both sections. The terms appearing in the Glossary were deleted and new terms were added.

This article is an update of J Michael Conlon, Substance P, In Encyclopedia of Endocrine Diseases, edited by L Martini, Elsevier, New York, 2004, Pages 339–342.

Distribution of Substance P and the NK_1 Receptor

Substance P

In the higher mammals, SP is localized in neurons of the central and peripheral nervous systems. SP-containing cell bodies and fiber networks are widely distributed in the human brain, with the highest concentrations being in the substantia nigra, striatum, hypothalamus, globus pallidus, and medulla oblongata. SP is highly concentrated in the superficial layers (laminae I–III) of the dorsal horn of the spinal cord, where most primary afferent fibers terminate. Colocalization of the peptide with neurokinin A and calcitonin gene-related peptide in unmyelinated primary afferent C-fibers has been demonstrated.

Within the gastrointestinal tract, most of the SP-containing neurons are of intrinsic origin with cell bodies occurring in both myenteric and submucous nerve plexi. In the gut, the coexistence of SP and NKA occurs in fibers of the afferent neurons, in neurons of the enteric nervous system and in immune and enterochromaffin cells of the mucosa. SP-containing neurons originating in the myenteric plexus project within the plexus itself and to the underlying circular smooth muscle layer and to the submucous ganglia and mucosa. Colocalization of SP and enkephalins in such nerve fibers in the human stomach and intestine has been described. Neurons in the submucous plexus project predominantly to the mucosal layer and SP is frequently colocalized with acetylcholine. SP fibers in the mucosa are particularly numerous in humans. The gastrointestinal tract is also innervated by extrinsic SP-containing fibers that originate primarily from dorsal root ganglia.

In the pancreas, a sparse distribution of SP fibers, which are probably of extrinsic origin, innervate blood vessels and acini and such fibers are also found surrounding acini and along blood vessels in the salivary glands. In the hepatobiliary system, immunoreactive fibers are found in the parenchyma of the liver and hepatic vasculature and are localized to the ganglionated and mucosal plexi of the gallbladder. In the trachea and bronchi, extrinsic SP-containing nerve fibers are found within the smooth muscle layer and around local ganglion cells and, in the nasal mucosa, within and under the epithelium and around arterioles, venules, and exocrine glands. In the human heart, SP fibers are found in close proximity to arterioles and are localized to the adventitia and to the border between the adventitia and media in a wide range of blood vessels. Within the urogenital system, immunoreactive nerve fibers of extrinsic origin are present in the urinary bladder throughout the ureter close to smooth muscle cells and around blood vessels in the kidney cortex often close to renal tubules and glomeruli. The female (uterus, oviduct, and vagina) and male (seminal vesicle, testis, epididymis, and vas deferens) genital organs are also innervated by extrinsic SP fibers.

SP is also located in cells that do not belong to the nervous system (e.g., endothelial, smooth muscle and Leydig cells, fibroblasts, platelets, and placenta cells) and in the body fluids (e.g., breast milk, cerebrospinal fluid, and blood). The synthesis and release of SP by cells of the immune system (monocytes, lymphocytes, eosinophils, and macrophages) have been reported and SP fibers innervate thymus, spleen, lymph nodes, and bone marrow. In malignant cells, the expression of both SP and the NK_1 receptor is increased. Human tumor cells (e.g., breast and lung carcinoma, melanoma) express the peptide, which is located in both the cytoplasm and nucleus. It seems that SP is released into the blood vessels from the tumor mass. In human gastric tumor samples, an increased nuclear localization of SP has been reported in cancer cells when compared to human gastric normal cells. Consistent with the rapid rate of clearance from the circulation (half-life <1 min), the plasma concentration of SP in healthy subjects is very low (<10 fmol/mL). However, elevated concentrations of circulating SP are often seen in patients with carcinoid tumors, particularly metastatic tumors of the midgut region.

NK_1 Receptor

The NK_1 receptor (also named tachykinin 1 receptor) is widely distributed in the central and peripheral nervous systems of mammals (e.g., spinal cord, medulla oblongata, striatum, hippocampus, and cerebral cortex). In peripheral tissues, NK_1 receptors are present on human pulmonary arterial blood vessels, on circular and longitudinal smooth muscle throughout the human gastrointestinal tract, and over ganglia of the myenteric plexus. These receptors have been also located in the placenta, thyroid gland, endothelial cells, immune cells (e.g., dendritic cells, macrophages, monocytes, and lymphocytes) and in platelets. The occurrence of NK_1 receptors in spleen, in thymus, on arterioles and venules of the lymph nodes, and on T lymphocytes provides further evidence for an involvement of SP in immunoregulation. The potent vasodilator action of SP is mediated primarily by binding to NK_1 receptors on the endothelium of peripheral arterial blood vessels. The potent sialagogic effect of SP is consistent with the high concentration on NK_1 receptors on rat submaxillary gland. In the urogenital system, NK_1 sites are expressed on blood vessels in the urinary bladder, uterus, and ureter; in the skin, NK_1 sites are present at high density in postcapillary venules in the dermis and in keratinocytes (SP controls the production of proinflammatory cytokines by these cells). In the gut of rodents, NK_1 receptors were observed in the smooth muscle, interstitial cells of Cajal, inhibitory, excitatory and secretomotor neurons and in epithelial and immune cells. The activation of the NK_1 receptors located on the intrinsic primary afferent neurons of the submucosal and myenteric plexus, interstitial cells of Cajal and on the muscle cells, facilitated the gastrointestinal motor activity. In the case of the interstitial cells of Cajal this stimulation enforced motility by prolonging the duration of the slow waves originated in these cells. In the enteric nervous system, NK_1 receptors located on the inhibitory motor pathways are involved in the depression of the motor activity via release of adenosine triphosphate and nitric oxide.

The NK_1 receptor shows different active conformations and each of them has a different affinity for distinct agonists/antagonists. This receptor is highly conserved along the species and contains seven putative hydrophobic α-helical transmembrane domains (showing three extracellular and three intracellular loops with the possibility for a four intracellular loop due to the

palmitoylation of cysteine); its C-terminus (intracellular) contains putative threonine/serine phosphorylation sites (once phosphorylated induces the desensitization of the receptor when it is repeatedly activated by SP), whereas its N-terminus (extracellular) contains potential asparagine glycosylation sites. SP (the conserved COOH-terminal sequence is required for the activation of the receptor) binds to residues 178–183 (Val-Val-Cys-Met-Ile-Glu) which are located in the middle of the second extracellular loop, whereas the third intracellular loop binds to the G protein. NK_1 receptor antagonists bind between the third and sixth transmembrane domains.

The glycosylation/phosphorylation of the NK_1 receptor influences the NK_1 receptor signaling. SP generates second messengers and affects many signaling pathways controlling the cell function: activation of phospholipases A_2/C, protein kinases A/C and adenylyl cyclase, synthesis of diacylglicerol/inositol triphosphate/arachidonic acid, mobilization of intracellular Ca^{++}, generation of thromboxane/leukotrienes, phosphorylation of myosin regulatory light chain, and activation of Rho-associated protein-kinase (ROCK). SP, via the NK_1 receptor, transactivates the epidermal growth factor receptor (EGFR) leading to the activation of mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK) 1 and 2, DNA synthesis and proliferation. SP exerts and antiapoptotic effect involving the Janus kinase 2 (JAK-2) and phosphoinositide 3-kinase (PI3K)-mediated activation of the antiapoptotic molecule Akt (protein kinase B). SP activates p38, promotes the synthesis of proinflammatory cytokines (e.g., interleukin-6, interleukin-8) and activates proinflammatory transcription factors (e.g., nuclear factor kappa B (NF-κB) by mechanisms in which the activation of the Rho family kinases is involved).

The NK_1 receptor shows two isoforms: the full-length (407 amino acids, 46 kDa molecular mass; present mainly in the brain) and the truncated isoform (311 amino acids without its C-terminus tail; it is mainly located in peripheral tissues, although in the nucleus accumbens, cerebellum, locus coeruleus and cingulated cortex the truncated isoform was observed). Both isoforms show a different functional significance due to the different signaling capability. The full-length induces the slow growth of cancer cells, whereas the truncated ones (the oncogenic isoform of the NK_1 receptor) mediates the malignancy/growth of tumor cells and stimulates the synthesis of cytokines, which upregulate the truncated form. The full-length isoform is phosphorylated and interacts with β-arrestins, which are involved in desensitization/internalization/endocytosis of the receptor. This receptor activates ERK1/2 and NF-κB and promotes the expression of interleukin-8. However, after stimulation of the truncated form, the expression of interleukin-8 was inhibited. SP, via cytokines, promotes the activation of NF-κB, which slightly increases the full-length isoform but upregulates the truncated ones. An NF-κB binding site has been located in the promoter of the *TACR1* gene and this promoter contains binding sites for other transcription factors (e.g., Oct2, Sp1, and AP1), which regulate many inflammatory genes. In airway epithelial cells, via MAPK/ERK and JAK/STAT pathways, the leukemia inhibitory factor favors the expression of the NK_1 receptor. In pancreatitis, the NK_1 receptor is upregulated in acinar cells and this is mediated by JNK and, in colonic epithelial cells, SP activated the JAK/STAT pathway and promoted the release of prostaglandins.

SP induces a clathrin-dependent internalization of the NK_1 receptor, the SP/NK_1 receptor complex is internalized into endosomes and then SP is degraded and the NK_1 receptor returns to the cell surface (this occurs during a brief exposure to low concentrations of SP). After a maintained exposure with a high concentration of SP (e.g., this occurs during the inflammatory processes), the NK_1 receptor is ubiquitinated and degraded. The loss of the C-terminal threonine/serine residues is crucial for G protein-coupled receptor kinase interaction and β-arrestin recruitment for receptor internalization. The truncated isoform is able to prolong the responses after the binding of SP, because its internalization and desensitization are altered (the truncated form has a reduced efficiency regarding both processes because is not phosphorylated and does not interact with β-arrestins). The truncated form increases its tumorigenic potential due the diminished endocytosis/desensitization of this form. In colitis-associated cancer the expression of the truncated isoform is related to the progression from quiescent colitis to dysplasia and cancer and it is increased in colonic epithelial cells from patients suffering colitis-associated cancer. However, the full-length is not affected. The truncated isoform was overexpressed in human hepatoblastoma cells but negligible levels of the truncated isoform were found in nonmalignant cells. The overexpression of the truncated form induced the transformation of breast cancer cells, but not the full-length.

Physiological Roles

The principal biological actions of SP are summarized in **Table 1**. The undecapeptide controls cell function via neuroendocrine, endocrine, paracrine and/or autocrine processes. It is well established that SP acts as a neurotransmitter in primary afferent sensory neurons and is involved in the perception of pain. The release of SP from small-diameter sensory "pain" fibers into the dorsal horn of the spinal cord following intense peripheral stimulation promotes central hyperexcitability and increases sensitivity to pain. Intrathecal injection of SP in mice elicits behaviors suggestive of pain sensation. However, in rats, intracerebroventricularly administered SP displays a clear-cut antinociceptive activity in the hot plate test, which is abolished by treatment with the opioid antagonist, naloxone. It has been suggested that proteolytic conversion of SP to its (1–7) fragment is a prerequisite for development of the analgesic action. This fragment also inhibited grooming and aggressive behavior. Knockout mice lacking expression of the *TAC1* gene exhibit a surprisingly healthy phenotype and a normal response to mildly painful stimuli, for example, the tail flick assay, but the response to moderate to intense pain was significantly reduced. Similarly, mice with a targeted deletion of the NK_1 receptor for SP were healthy and fertile and responded normally to acute painful stimuli but were unable to develop fully the stress-induced analgesia of control animals.

Table 1 A summary of the principal biological actions of substance P with correlations to human diseases

Organ system	*Biological activity*	*Disease correlation*
Central nervous system	Neurotransmitter/neuromodulator function	Migraine
		Riley–Day syndrome
		Emesis
		Alcohol addiction
		Depression/anxiety
		Seizures
	Nociception	Neuropathic pain
	↑ Neurogenic inflammation	Parkinson's disease
		Huntington's disease
		Alzheimer's disease
	↑ Permeability of blood-brain barrier	Traumatic brain injury
	Bacterial infection	Bacterial meningitis
	Viral infection	Viral encephalitis
	Parasitic infection	Neurocysticercosis
	Mitogenesis of tumor cells	Astrocytoma/glioma
Cardiovascular system	Vasodilation	Carcinoid flush
	Plasma extravasation	
	Tachycardia	
	↑ Cardiac output	
		Pulmonary arterial hypertension
		Vascular anomalies
	↑ Neurogenic inflammation	Viral-myocarditis
	Viral infection	
Blood	Platelet aggregation	Thrombosis
	↑ Neurogenic inflammation	Sepsis
	Bacterial infection	
	HIV	AIDS
	Mitogenesis of tumor cells	Acute lymphoblastic leukaemia B and T cells
	Immunoregulation	
Respiratory system	Bronchoconstriction	Asthma
	Vasodilation	
	↑ Vascular permeability	
	↑ Mucus secretion	
	↑ Neurogenic inflammation	Bronchiolitis
	Viral infection	Respiratory syncytial virus
		Pulmonary arterial hypertension
	Mitogenesis of tumor cells	Small and nonsmall lung cancer, larynx cancer
Salivary glands	↑ Salivation	
Gastrointestinal tract	↑ Peristalsis	Hirschprung's disease
	↑ Water and electrolyte secretion	Ulcerative colitis
	↑ Blood flow	Crohn's disease
	↑ Neurogenic inflammation	
	Mitogenesis of tumor cells	Gastric and colon cancer
Pancreas	↑ Exocrine secretion	
	Mitogenesis of tumor cells	Pancreatic cancer
Hepatobiliary system	↓ Bile secretion	Cholestasis
	↑ Gallbladder motility	
	↑ Neurogenic inflammation	Hepatitis
		Hepatotoxicity
	Mitogenesis of tumor cells	Hepatoblastoma, cholangiocarcinoma
Urogenital system	↑ Motility of ureter and bladder	Urinary incontinence
	↑ Blood flow and motility of uterus	
	Renal vasodilatation	
	Placenta function	
	↑ Neurogenic inflammation	Abortus
	Mitogenesis of tumor cells	Endometrial adenocarcinoma
Musculoskeletal system	↑ Neurogenic inflammation	Arthritis
	↑ Blood flow to skeletal muscle	
	Mitogenesis of tumor cells	Osteosarcoma
Skin	↑ Neurogenic inflammation	Dermatitis, pruritus
	Mitogenesis of tumor cells	Melanoma

(*Continued*)

Table 1 Continued

Organ system	*Biological activity*	*Disease correlation*
Ocular system	↑ Neurogenic inflammation	Keratitis
	Mitogenesis of tumor cells	Retinoblastoma
Oral system	Nocicepcion	Dental pain
	↑ Neurogenic inflammation	Pulpitis
		Oral liquen planus
	Mitogenesis of tumor cells	Oral squamous cell carcinoma, keratocystic odontogenic tumor

SP increases the firing of striatal neurons and promotes the release of methionine-enkephalin, dopamine and acetylcholine; the peptide also controls the nigrostriatal dopamine release and, after infusion of SP into the substantia nigra, an increase in rearing, sniffing and grooming was reported. The administration of SP into the ventral tegmental area increased rearing and locomotion and its infusion into the nucleus accumbens exerted memory-promoting effects. In the amygdala, the level of SP was higher in males than in females and after castration the level of SP decreased. SP controls the release of anterior pituitary hormones (e.g., growth hormone, prolactin) and in the hypothalamus acts as an orexigenic substance, promoting gain.

Intra-cerebroventricular injections of SP in rats and mice induce a wide range of behavioral responses, including increased grooming and scratching (behavioral defense), increased hindlimb rearing, decreased aggressive behavior, an enhancement of inhibitory avoidance learning, anxiety and respiratory and cardiovascular responses (e.g., increase in blood pressure, heart rate, hindlimb vasodilation, and visceral vasoconstriction). Intra-arterial infusions of SP in humans result in a fall in diastolic blood pressure, an increase in heart rate, and an increase in cardiac output, due mostly to a greater stroke volume. The peptide is a powerful vasodilator in humans with high-dose (70 pmol/kg/min) infusions, producing a bright red flushing of the skin, particularly in the neck and face, with a subjective feeling of warmth. Intra-dermal injections of SP in humans produce flare, wheal, and itching that are related to the release of histamine and an increase in capillary permeability. An antipruritic effect has been reported when the NK_1 receptor antagonist aprepitant was administered.

In macrophages and eosinophils, the expression of both NK_1 receptors and SP is upregulated during the inflammatory processes. In these processes, SP is involved in the recruitment of leukocytes (e.g., eosinophils) and stimulates the synthesis of inflammatory cytokines by monocytes. In the gastrointestinal tract, the peptide facilitates the activation and recruitment of granulocytes and mast cells. SP promotes white cell endothelial adhesion and platelet aggregation (thrombus formation), vasodilation and increases capillary permeability and plasma extravasation. The peptide, acting on the NK_1 receptors located in the endothelial cells, augments in the intestine the blood vessel permeability, facilitating the extravasation of leukocytes and plasma proteins. Lymphocyte T cells express SP and the NK_1 receptor during infection/inflammation and the peptide controls the proliferation of these cells and the release of cytokines. SP, via the NK_1 receptor, promotes the release of cytokines and increases the cytotoxicity of the natural killer cells. SP also regulates the functional activity of neutrophils (e.g., release of cytokines, bacterial phagocytosis, and ROS generation).

In macrophages of the lung, hypoxia processes upregulated the expression of NK_1 receptors. This has been also observed in the same cells of smokers. In the rat, SP promotes the release of the endothelial growth factor/tumor necrosis factor-alpha by mast cells and the release of histamine from these cells after a stressful stimulus. In this rodent, immobilization stress promoted the release of SP in the medial nucleus of the amygdala, but the central administration of an NK_1 receptor antagonist blocked the stress-induced anxiogenic behavior.

SP and NKA coexist in extrinsic afferent neurons and in intrinsic enteric neurons. In the enteric nervous system, tachykinins are involved in the slow postsynaptic excitation, contributing to the communication between intrinsic primary afferent neurons and inhibitory motoneurons. The precise actions of SP on the motility of the gastrointestinal tract are species-dependent, but in vitro the peptide generally evokes a contractile response on tissues from all regions and in vivo is a strong stimulator of peristalsis by a mechanism that involves both a direct action on smooth muscle and a release of acetylcholine from cholinergic neurons. SP stimulates the secretion of water and electrolytes from the mucosa of the small intestine and colon of various mammals and produces an increase in short-circuit current in the guinea pig colon that is indicative of net ion transport across the tissue. In the human colon, the stimulation of the NK_1 receptor increased the mucosal ion transport. SP, released from the intrinsic primary afferent neurons of the myenteric and submucosal plexus, facilitated the secretion of chloride. In the jejunal mucosa of monkeys infected with the protozoan parasite *Cryptosporidium parvum* (induces a diarrheal disorder), the upregulation of both SP and the NK_1 receptor has been reported and this system is also involved in glucose malabsorption and increased secretion of Cl^-. The release of SP from afferent fibers innervating the stomach and gut and from intrinsic fibers of the submucous ganglia supplying submucosal arteries produces vasodilation, leading to an increase in blood flow in the mesenteric artery. A cytoprotective role for the gastric mucosa has been proposed for the SP released from primary afferent terminals.

Intra-arterial infusions of SP in dogs weakly stimulate basal output of pancreatic juice, amylase, and bicarbonate, whereas in the isolated rat pancreas, the peptide inhibits cholecystokinin-induced amylase release and secretin-induced flow and, at high concentrations, weakly stimulates the release of insulin, glucagon, and somatostatin. SP is also expressed in human sperm and, via

the NK_1 receptor, the peptide increased sperm motility. SP is also observed in the epididymis, stimulating the motility of the sperm. Prostate contraction is also mediated by the NK_1 receptor.

Pathophysiology and Disease

The involvement of SP in human disease is summarized in **Table 1**. A link between SP and nociception has been clearly established. Cerebral and pial arteries are richly innervated by SP fibers and it has been suggested that neurogenic inflammation within the cerebral tissues leading to the release of SP may be involved in the pathogenesis of acute migraine and cluster headaches. Both SP and the NK_1 receptor are upregulated in the inflammatory processes. Similarly, the inflammatory response to trauma in the eye may be due to a release of SP. Patients with reduced sensitivity to pain and temperature, as in Riley–Day syndrome, are associated with a marked depletion in the SP content of the medulla oblongata and substantia gelatinosa of the spinal cord. In mice, the genetic deletion of NK_1 receptors prevented mechanical hyperalgesia in the colon and NK_1 receptor antagonists reduced in the jejunum/colon of rodents the induced hypersensitivity to distension. In rodents, these antagonists also blunted the pancreatitis-evoked abdominal hyperalgesia. In rats, after an experimental cystitis or colitis, the expression of the NK_1 receptor was upregulated in the dorsal horn of the spinal cord. This also occurred in the pancreas (acute pancreatitis), in neurons of the spinal cord (chronic stress), and in the cingulate cortex (HIV-positive patients). In the latter patients, the level of SP was also increased in the blood and this peptide increased HIV replication in macrophages/mononuclear phagocytes; this mechanism being mediated by the NK_1 receptor.

An involvement of SP in the pathophysiology of neurodegenerative diseases is indicated by the significant decrease in the concentration of the peptide in the substantia nigra and globus pallidus of patients with Huntington's disease and Parkinson's disease. A similar decrease in the SP content in the cerebral cortex and hippocampus has been found in patients with Alzheimer's disease.

The deletion of the NK_1 receptor blocked stress symptoms induced by pain. In rodents, SP antagonists suppressed anxiety-related behaviors and mice with selective deletion of the gene encoding SP or the NK_1 receptor showed a decrease in such behaviors. The NK_1 receptor antagonists AV608 decreased pain ratings and anxiety in women with irritable bowel syndrome. In this syndrome, a decrease in the binding of an NK_1 receptor PET agonist was observed in the brain. Changes in the levels of SP and NK_1 receptors have been reported in schizophrenia and bipolar disorders. SP is also involved in acute brain injury; the peptide promoted the death of neurons and the development of vasogenic edema. SP also increased the immune response of glial cells.

In the periphery, a role for SP in the pathogenesis of rheumatoid arthritis is suggested by the observations that joints that develop severe arthritis are densely innervated by SP-containing fibers and concentrations of the peptide in synovial fluid are increased. NK_1 receptors are also found on synovial endothelial cells in patients with rheumatoid arthritis. In the serum, these patients also showed an increased level of SP. Thus, tachykinins derived from hyperactive afferent nerve endings in the arthritic joints may promote the chronic inflammatory processes.

In the gastrointestinal tract, SP-containing fibers are almost absent from the aganglionic colons of patients with Hirschprung's disease. In conditions of chronic inflammatory bowel disease, such as ulcerative colitis and Crohn's disease, there is an increase in the concentration of SP in the inflamed mucosa together with a marked increase in the expression of SP-selective NK_1 receptor sites in the arterioles and lymphatic tissue. In the mucosa of patients suffering from ulcerative colitis/Crohn's disease, the secretory response mediated by SP was blunted when NK_1 receptor antagonists were used. This action was also observed in rodents suffering from oesophagitis, colitis or pancreatitis when an NK_1 receptor antagonist was administered. The intestinal inflammation mediated by *Clostridium difficile* toxin A was also blocked when NK_1 receptor antagonists were used. NK_1 receptors are expressed in fibroblasts of patients with Crohn's disease and, in these cells, SP promoted the synthesis of collagen (fibrosis). In the intestinal inflammation, SP plays a dual action: promotes inflammation but also mediates repair (e.g., fibrosis, protects colonocytes from apoptosis). The increase in vascular permeability mediated by SP is amplified in inflamed tissue because the inflammation downregulated the activity of the neutral endopeptidase that control the level of the extracellular SP (in normal tissues, this endopeptidase maintains low the level of the extracellular SP, limiting its proinflammatory effect).

SP is a potent stimulator of histamine release from mast cells in the intestines of nematode-infected rats. In the pseudo-membranous colitis due to the infection with *Clostridium difficile* an overexpression of the NK_1 receptor has been reported. A decrease in the SP-innervation of the colonic circular muscle was observed in children suffering a slow transit constipation. In adult patients suffering from the same problem, the response of the colonic circular muscle to NK_1 receptor agonists was enhanced. NK_1 receptor antagonists inhibited stress-induced defecation and corrected the muscular hyperresponsiveness and hypomovility produced by pain, inflammation and anaphylaxis and also exerted antiobesity effects. It seems that SP, via the NK_1 receptor located in the adipose tissue, mediates obesity. Stress activates NK_1 receptors located in the intestine and spinal cord, leading to motility disorders, and hyperalgesia. After surgical manipulation of the intestine, fibrous adhesions occurred in the abdominal cavity. During this manipulation, SP, via the NK_1 receptor, promoted the formation of these adhesions, whereas NK_1 receptor antagonists decreased them.

In the airways, the SP/NK1 receptor system has been involved in allergic hyperreactivity. In the nasal secretion of coughing subjects, a high level of SP has been reported. In patients with cystic fibrosis, airway seromucous glands did not respond to SP, whereas in normal patients SP promoted an increased secretion.

SP, via the NK_1 receptor, is a mitogen in normal/cancer cells. The synthesis of SP in neoplastic tissue has been described for a range of neuroendocrine tumors. The peptide has been detected at relatively low concentrations in pheochromocytomas, medullary carcinomas of the thyroid, and small-cell lung carcinomas. Much higher concentrations are found in carcinoid tumors, particularly those arising in the midgut region and their metastases. It is generally accepted that the etiology of the marked cutaneous vasodilation and tachycardia seen in the carcinoid flush is multifactorial but SP (and other tachykinins) released by tumor tissue into the circulation has been implicated in some, but not all, patients. SP facilitates colitis-associated cancer (in which the NK_1 receptor is overexpressed) and the mitogenesis of cancer cell lines derived from human colonic and gastric adenocarcinomas. These actions were inhibited by NK_1 receptor antagonists. In a rat model of colitis-associated colon cancer, these antagonists decreased microscopic/macroscopic damages. An overexpression of the NK_1 receptor occurs in tumor cells in comparison with normal cells. In malignant tissues, the expression of the mRNA NK_1 receptor was higher than that found in benign tissues. It seems that the number of NK_1 receptors is related to the degree of malignancy. This means that a specific treatment (using NK_1 receptor antagonists, for example aprepitant which is a broad-spectrum anticancer compound) against cancer cells is possible. The safety of aprepitant is known: the IC_{100} for cancer cells is 60 μM but the IC_{50} for noncancer cells is 90 μM. In human trials, 300 mg/day of aprepitant was well tolerated. The NK_1 receptor has been observed in many human cancer cell lines (e.g., oral, gastric, colon and pancreatic carcinomas, neuroblastoma, glioma, melanoma, retinoblastoma, osteosarcoma, hepatoblastoma, and acute lymphoblastic leukemia). In these cells, the NK_1 receptor was observed in the plasma membrane and in both nucleus and cytoplasm. In tumor cells, SP via the NK_1 receptor, promotes (1) mitogenesis (the mitogen-activated protein kinase signaling (MAPK) pathway is activated (including p38MAPK and ERK1/2); upon activation, ERK1/2 is translocated into the nucleus inducing the proliferation of cells and promoting an anti-apoptotic effect; this requires the presence of the epidermal growth factor receptor); (2) cell migration (changes in cellular shape, including blebbing, which is crucial for cancer cell infiltration and metastasis); (3) angiogenesis (by increasing the mitogenesis of endothelial cells, the tumor blood flow is increased and this mechanism helps to the development of the tumor; in most tumors, a high density of SP and NK_1 receptors has been located in intra- and peri-tumor blood vessels); (4) the breakdown of glycogen (Warburg effect: tumor cells show a glycolytic rate up to 200 times higher than that of normal tissues or origin. SP facilitates the glycogen breakdown and the glucose obtained augments the metabolisms of tumor cells), and (5) an antiapoptotic effect (mediates by the formation of a β-arrestin-dependent scaffolding complex; NK_1 receptor antagonists increase apoptosis). All the above effects are blocked by NK_1 receptor antagonists. SP, via the NK_1 receptor, promotes the release of interleukins, taurine and glutamate from astrocytoma cells and increases the phosphorylation/activity of Akt (protein kinase B); this activation suppresses the apoptotic mechanisms. NK_1 receptor antagonists block the activity of Akt, inducing apoptosis. The immuno-blockade of SP induced cancer cell death and decreased the levels of both epidermal growth factor receptor 2 (HER-2) and EGFR. NK_1 receptor antagonists, in a concentration-dependent manner, induced the apoptosis of tumor cells and a synergic action against tumor cells has been reported for the combination of cytostatic drugs and NK_1 receptor antagonists. A synergy also occurs between ritonavir (blocks the HIV protease activity) but not acyclovir, cidofovir and maraviroc, and the NK_1 receptor antagonist aprepitant against human glioma cells. NK_1 receptor antagonists decrease the side-effects of both radiation therapy and cytostatics. In cancer cells, the NK_1 receptor is involved in the viability of these cells (tumor cells need the NK_1 receptor for their own survival and depend of the potent mitotic signal mediated by SP. The overexpression of NK_1 receptors in tumor cells neutralize their own pathways leading to cell death).

A clear relationship occurs between chronic inflammation and the risk to develop cancer. In both cancer and chronic inflammation, the SP/NK_1 receptor system is overexpressed. Cancer originates from chronically inflamed tissue (e.g., gastrointestinal tract) and patients suffering pancreatitis (in which the mRNA NK_1 receptor expression is upregulated) showed a high risk to develop pancreatic cancer. SP induces the proliferation, migration and invasion of gastric cancer cells, whereas NK_1 receptor antagonists exert an antitumor action against colon cancer stem cells and human pancreatic, gastric, colon, and hepatoblastoma cells.

SP is involved in viral infection, replication and proliferation (e.g., herpes virus, measles virus, respiratory syncytial virus, encephalomyocarditis virus, and human immunodeficiency virus) and NK_1 receptor antagonists exerted antiviral effects. The SP/NK_1 receptor system is also involved in severe bacterial infection (sepsis, meningitis), in colostasis/pruritus, in the control of alcohol intake (in the mouse, the deletion of the NK_1 receptor blocked alcohol consumption; this deletion also diminished the rewarding effect of opioids), in smoking (inhaled cigarette smoke promoted the release of SP leading to the neurogenic inflammation and an increase in the generation of ROS; the activation of the NK_1 receptor promoted the formation of ROS which was decreased by NK_1 receptor antagonists), in seizure and in neurodegeneration (NK_1 receptor antagonists may be a novel neuroprotective strategy).

In the bronchi of patients with asthma an increase in the expression of NK_1 receptor has been reported and these patients were hyperresponsive to SP. Airway epithelial cells expressed SP, the airway SP release is controlled by the nerve growth factor and corticosteroids downregulated the expression of the NK_1 receptor in airway myocytes. Increased levels of SP were reported in people attempting suicide, major depression, fibromyalgia, and schizophrenia. In the urinary bladder of patients with multiple sclerosis, an increased density of SP-immunoreactive fibers has been reported and it has been involved to the detrusor overactivity observed in these patients.

A wide range of antagonists (peptide and nonpeptide) for the NK_1 receptor have become available for potential use as therapeutic agents. In humans, NK_1 receptor antagonists (e.g., aprepitant, fosaprepitant) exert an antiemetic action by blocking the emetic reflex at the brainstem. Both antagonists are currently used in clinical practice for the prevention of postoperative vomiting and nausea and chemotherapy-induced vomiting and nausea. Moreover, in vitro and in vivo, both antagonists (in a concentration-

dependent manner) showed an antitumor action. In general, NK_1 receptor antagonists are selective, potent, well tolerated, and safe. In irritable bowel syndrome patients, the administration of mono-tachykinin receptor antagonists did not inhibit gastrointestinal pain, but the use of an antagonist (DNK333) showing affinity by the three tachykinin receptors increased the rate of satisfactory relief from abdominal pain and discomfort in women suffering diarrhea-predominant irritable bowel syndrome. The SP/NK_1 receptor system is involved in many pathologies in which this system is upregulated and hence NK_1 receptor antagonists could be used for the treatment of these pathologies. NK_1 receptor antagonists have different chemical compositions but they share their affinity for the NK_1 receptor. Peptides (e.g., SP) are released when cells are strongly activated and/or under pathological conditions. It seems that NK_1 receptor antagonists have not effect in normal conditions and they will only act on deranged systems with increased SP release. Pharmacological therapy may be exploited due to the many pathologies mediated by the SP/NK_1 receptor system.

See also: GI Hormone Development (Families and Phylogeny). GI Hormones Outside the Gut: Central and Peripheral Nervous Systems. GI Tract: General Anatomy (Cells)

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Gastrointestinal Hormones in Cancer☆

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Gastrin and Cancer

Gastrin is synthesized from its precursor, preprogastrin, processed into progastrin and gastrin peptide fragments by enzymatic cleavage. There are several forms of gastrin, the two major ones being G34 and G17. The final modification forms the mature gastrin peptide, or amidated-gastrin. Gastrin is synthesized and secreted mainly from gastrin-containing cells (G-cells) in the antral mucosa, and to a lesser degree from endocrine cells of the nonantral stomach, duodenum, jejunum, ileum, pancreas and outside the gastrointestinal tract (GIT) (e.g., in the brain, adrenal glands, respiratory tract, and reproductive organs). Gastric pH is a major determinant of gastrin secretion. Increased gastric acid in the stomach (during fasting) inhibits its release, whereas a high gastric pH (or meal digestion) provides a strong stimulus for its secretion. The G-cells are also regulated by two counterbalancing hormones, the stimulatory gastrin-releasing peptide, and the inhibitory somatostatin (Edkins, 1905; Rehfeld, 1998).

The receptors for gastrin and cholecystokinin (CCK) are related and constitute the "CCK/Gastrin receptor family.". Two classic types of receptors have been characterized: CCK-A receptor (previously CCK-1R), having affinity mainly for CCK, and CCK-B receptor (previously CCR-2R) which is the main receptor for gastrin. Both are classical G protein-coupled receptors. Gastrin activation of the CCK-B receptor, via the mitogen activated protein kinases (MAPK) pathways (including the ERK1/2 pathway), mediates cellular processes, including cell proliferation (partly through EGFR transactivation), and gastrin dependent genes transcription (Smith *et al.*, 2016). CCK-B receptors are highly expressed in different cancer tissues, including gastric, pancreatic, colon, and lung carcinomas.

Gastrin is the major regulator of gastric acid secretion. The acid-secreting parietal cell possesses receptors for acetylcholine, histamine (secreted from ECL cells), and gastrin, all of which being able to stimulate acid secretion. The ECL cell also receives stimulatory signals from gastrin, to secrete histamine, which in turn also stimulates the parietal cell (Hersey and Sachs, 1995; Sachs and Prinz, 1996).

Gastrin is an important growth factor for the GIT development (Smith *et al.*, 2017). Studies in the past decades have focused on evaluating the proliferative effect of gastrin (and its precursors), and their role in cancer development. Hypergastrinemia is observed in patients with atrophic gastritis, pernicious anemia, Zollinger–Ellison syndrome (and gastrinoma), and in patients treated with proton pump inhibitors (Hayakawa *et al.*, 2016).

In the stomach, elevated gastrin levels are known to increase the risk for ECL cell hyperplasia, and rarely the development of neuroendocrine tumors (NETs) (mainly in patients with genetic background) (Hayakawa *et al.*, 2016). Gastrin was also associated with increased risk of gastric adenocarcinoma localized in the corpus of the stomach, and with a worse prognosis (Fossmark *et al.*, 2015). Hypergastrinemia has been associated with increased risk for colon cancer and pancreatic cancer, however studies in both animals and humans suggest that gastrin is not mutagenic by itself, but rather increases the risk in subjects with precancerous lesions or high risk genetic mutations, by its pro-proliferative effect. With the wide use of gastric acid suppressing proton pump inhibitors (PPIs), it has been speculated that the elevation of serum gastrin caused by these medications could increase the risk of pancreatic cancer and other cancers arising from tissues with CCK receptors. In large population studies of patients with *Helicobacter pylori* infection, those with elevated gastrin levels had a fourfold increase in colorectal cancers (Thorburn *et al.*, 1998), but other conditions characterized by elevated gastrin concentrations levels such as atrophic gastritis, pernicious anemia and Zollinger–Ellison syndrome did not result in gastrointestinal adenocarcinomas, implying the need of an additional carcinogenic factor such as APC gene or precancerous colonic polyps to trigger carcinogenesis (Smith *et al.*, 2016; Thomas *et al.*, 2003).

In the fetal pancreas, gastrin is thought to have a role in the growth and differentiation of the GIT. It becomes silenced after birth, but reappears in premalignant pancreatic lesions, and in pancreatic cancer cells stimulating growth in an autocrine mechanism, while inducing its own transcription by activating the CCK receptor. This process of re-expression is thought to be mediated by micro-RNAs. There is also a significant increase in CCK-B receptor expression in the cancer cells, compared to the normal pancreas. Several mutations of the receptor have been described. One variant of mutated receptor, with alternative splicing of the forth intron, inducing the addition of 69 amino acids in the receptor site involved in cell proliferation signaling, has been termed CCK-C receptor (CCK-cancer). Downregulation of the mutant receptors was shown to inhibit growth (Smith *et al.*, 2004). It is estimated that the CCK-C receptor is present in about 40% of patients with pancreatic carcinoma, and is probably associated with worse prognosis, even without elevated gastrin levels (Smith *et al.*, 2015). The germline mutation of the specific receptor is

☆*Change History:* April 2018. Inbal Uri, Kristallenia Alexandraki, Simona Grozinsky-Glasberg updated the literature on each hormone, and added for each hormone the possible therapeutic implications.

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found in other cancers. On the other hand, when gastrin expression is downregulated with RNA interference in human pancreatic cancer cells, growth of the primary cancer is inhibited and metastases do not occur.

The CCK receptors have been described also outside the GIT, for example, in small cell lung cancer, with CCK-B receptor having the main involvement in malignant cell proliferation, partially via an EGFR dependent manner (Smith *et al.*, 2016).

Since gastrin was shown to stimulate the growth of several malignancies, there have been attempts to decrease gastrin's proliferative activity, by targeting the gastrin molecule, or blocking the interaction with the CCK-BR (Smith *et al.*, 2017).

Therapeutic Implications

- *Blockade of CCK receptors*—Several antagonists for the CCK-B receptor have been developed, with high potency in preclinical studies for reversal of tumor cell growth. Netazepide (YF476) is evaluated in patients with type 1 gastric NETs (Boyce *et al.*, 2017), Gastrazole (JB5008) and Z-360 have been used in patients with pancreatic cancer (Chau *et al.*, 2006; Meyer *et al.*, 2010), both with preliminarily good results, but further studies are needed. Ceclazepide (TR2-A) is a new benzodiazepine derived CCK-B receptor antagonist, in preclinical development (Boyce *et al.*, 2016).
- *Polyclonal antibody stimulator (PAS)*—An immunogen containing 9-amino acid epitope from the aminoterminal sequence of gastrin-17, in conjugation with diphtheria toxoid, formerly called G17DT or gastrimmune, elicits the production of antibodies directed to gastrin-17 and gly-gastrin. In animal models it was shown to decrease tumor growth and metastases. Several clinical trials have been conducted, in patients with gastric carcinoma, pancreatic carcinoma, colorectal cancer, and type 1 gastric NET, with survival benefit in patients demonstrating good antibody titer response (Smith *et al.*, 2017; Rocha-Lima *et al.*, 2014; Gilliam *et al.*, 2012; Tieppo *et al.*, 2011).

Gastrin-Releasing Peptide (GRP)/Bombesin and Cancer

Bombesin is a 14-amino acid peptide, isolated from the skin of the frog *Bombina bombina*. Two mammalian bombesin-related peptides and their receptors were described, involved in physiological and pathophysiological processes. Gastrin-releasing peptide (GRP) is a 27-amino acid peptide, processed from preproGRP, a 148-amino acid peptide; neuromedin-B (NMB), is a decapeptide, processed from preproNMB, a 76-amino acid precursor.

There are three receptors in the bombesin-receptor family; NMB-receptor (NMBR)-bombesin-receptor subtype 1 (BRS-1), GRP-receptor (GRPR)-bombesin-receptor subtype 2 (BRS-2), and bombesin-receptor subtype 3 (BRS-3), with its natural ligand yet unknown.

The bombesin-related peptides are secreted from many tumors, and function as autocrine growth factors, activating their own receptors on the tumor cells. They were found to stimulate the growth and differentiation of tumor cells, mediated by transactivation of tumor EGFR or HER2. GRPR is overexpressed by tumors of the prostate, breast, colon, small cell and nonsmall cell lung carcinoma, head and neck squamous cell tumors, pancreas, gliomas, meningiomas, and neuroblastomas. NMBR is overexpressed by tumors of the lung, pancreas, colon, bronchial and intestinal NETs. BRS-3 is also expressed by tumors of the lung, pancreas, pituitary, ovary, prostate and neuroendocrine tumors (NETs) (Moreno *et al.*, 2016).

There is great interest in using bombesin analogues for imaging of tumors expressing BnRs, similarly to what is widely used today with somatostatin analogues in NETs expressing somatostatin receptors. In the past, only BnR agonists were used, assuming there is major role for the internalization if the ligand, for optimal response. However, recent studies showed similarly good results using BnR antagonists (Strosberg *et al.*, 2017; Sancho *et al.*, 2011).

Therapeutic Implications

- *Bombesin-receptor (BnR) antagonists*, mainly for GRPR, have been studied and showed effect on the growth of several tumors. However, a phase 1 trial of GRPR antagonist (RC-3095) in 25 patients with solid tumors, failed to show any objective response (Schwartsmann *et al.*, 2006; Kelley *et al.*, 1997).
- A novel approach is the combination of BnR antagonists with other cytotoxic agents, with promising results, however with increased toxicity. For example, the combination of BnR antagonist with EGFR antagonist have potentiating effect on tumor growth in head and neck squamous cell cancers, medulloblastoma and lung cancer (Moody *et al.*, 2016a); the combination of GRPR antagonist with 5-FU in colon cancer produce greater inhibition of growth (Rick *et al.*, 2012).
- BnR ligands have also been coupled to several compounds and cytotoxic agents, used together to deliver the drugs to the BnR overexpressing tumor cells. These include coupling of BnR agonists or antagonists to the radioisotopes Y90 or Lu177 (Reynolds *et al.*, 2016), coupling to chemotherapeutic agents like doxorubicin (Wang *et al.*, 2016), coupling to gold nanoparticles (Hosta-Rigau *et al.*, 2010; Chanda *et al.*, 2010), cytotoxic toxins (Moody *et al.*, 2008), agents that activate the immune system (Zhou *et al.*, 2006), and other agents.

- Another approach receiving increasing attention is silencing of the BnR using small interfering (si) RNA, which may be delivered to the tumor cells by conjugation to BnR ligands, showing promising results in ovarian cancer cells, neuroblastomas, prostate cancer cell lines, and others (Rellinger *et al.*, 2015; Langer *et al.*, 2002; Fang *et al.*, 2009).

Somatostatin

Somatostatin is a tetradecapeptide (somatostatin-14) acting through five different somatostatin receptor (SSTR) subtypes 1,2,3,4 and 5. Different receptor subtypes are coupled to different intracellular transmission cascades in a cell type-dependent manner. Somatostatin has been detected in the nerves and cell bodies of the central and peripheral nervous systems, including the autonomous nervous system of the GIT and the D cells (the majority) of the pancreas and mucosa of the stomach and intestine. Somatostatin is an inhibitory regulator peptide, and considered as the universal endocrine switch off-hormone (Thomas *et al.*, 2003). Somatostatin inhibits the release of GH and somatomedin C and all known GI hormones as well as the gastric acid secretion and gut's motility, intestinal absorption, and pancreatic bicarbonate and enzyme secretion, and selectively decreases splanchnic and portal blood flow. Somatostatin may exert cytostatic (G_1-phase cell arrest) or cytotoxic (apoptosis induction) effects, depending on the receptor subtype expressed on the target cell, inhibiting the growth of normal and neoplastic tissues. Hence, somatostatin inhibits the growth of the GI mucosa and normal pancreas indirectly by inhibiting trophic hormones or directly through interaction with the SSTR2. Because of the short half-life of somatostatin and the need of continuous intravenous infusion, long-acting somatostatin analogues (SSAs) were developed such as octreotide long-acting release (LAR) and lanreotide autogel targeting mainly SSTR2 and less SSTR5 and SSTR3 receptor subtypes, while pasireotide targets mainly SSTR5 and less SSTR1, SSTR3 and SSTR2. SSAs can be used as first line antiproliferative treatment in patients with slow growing gastro-entero-pancreatic (GEP)-NETs (Alexandraki *et al.*, 2017). Octreotide is useful to ameliorate symptoms associated with hormonal-over-producing tumors such as intractable diarrhea in the case of carcinoid syndrome, in the management of inoperable bowel obstruction, pancreatic and enterocutaneous fistulas. Side effects of these agents consist mainly of gastrointestinal complaints, cholelithiasis, and effects on glucose metabolism (Grozinsky-Glasberg *et al.*, 2008).

Moreover, radiolabeled SSAs have been employed for the localization of primary and metastatic tumors expressing SSTR, as a diagnostic tool but its use as treatment with radiopharmaceuticals known as peptide receptor radionuclide therapy (PRRT) is a promising therapy in tumors expressing SSTR.

Therapeutic Implications

- Somatostatin may inhibit cell proliferation in the mucosa of the normal GIT and antagonize the trophic activity of gastrin, particularly in the fundus and antrum of the stomach. An inhibitory effect of somatostatin in the jejunum and ileum has been also suggested, and apparently blocking endogenous somatostatin allowed increased proliferation of the normal pancreas (Thomas *et al.*, 2003).
- Clinical trials have been performed to assess SSAs effects in the growth of endocrine and nonendocrine cancers. Despite their beneficial role for the former tumors (on both hormonal hypersecretion and tumor growth), their role on the latter cancers is equivocal with rather negative results. However, the discovery of new SSAs may suggest encouraging results for a somatostatin-directed therapy (medical or radiopeptide) when the presence of SSTR is documented (e.g., in prostate or breast cancer, with neuroendocrine features) (Wachter *et al.*, 2014; Surcel *et al.*, 2015).

Cholecystokinin (CCK)

Cholecystokinin (CCK) is a peptide related to gastrin, normally produced in the I-cells of the duodenum. Its physiologic activities include inducing gallbladder contraction, enhancing the secretion of pancreatic enzymes, increasing bowel motility, and inhibiting gastric emptying, as well as trophic effects on the pancreas and GI mucosa; both gastrin and CCK are the ligand of the CCK receptor (Smith *et al.*, 2016; Thomas *et al.*, 2003).

As mentioned above, two classic types of CCK receptors have been cloned and characterized, CCK-A receptor predominant type in the normal murine pancreas (previously known as CCK-1R) and CCK-B receptor (previously known as CCK-2R) predominant type in the normal human pancreas (Smith *et al.*, 2016).

CCK receptor is markedly overexpressed in pancreatic cancer. As it was mentioned earlier for gastrin, similarly CCK is not mutagenic, but both may increase cancer risk when they display high concentrations in a subject with a precancerous lesion such as a pancreatic intraepithelial neoplasia and a KRAS mutation (Carriere *et al.*, 2009). Moreover, high CCK blood levels from dietary fat have been shown to promote the growth of pancreatic cancer in animal models; subjects with chronic pancreatitis have elevated CCK blood levels and increased risk for pancreatic and other cancers that possess CCK receptors. In 74% of surgical specimens from human pancreatic cancer high levels of α-amidated gastrin and its precursor were detected while CCK was not (Goetze *et al.*, 2000). At the contrary, CCK immunoreactivity was reported in some pancreatic cancer surgical specimens but tumor production of

CCK did not influence growth of the tumor. Summarizing, both CCK and gastrin peptides may be present in malignant tissue but only the re-expression of endogenous gastrin stimulates pancreatic cancer growth by an autocrine mechanism (Smith *et al.*, 2016).

Gastrin or CCK-stimulated cancer growth was found to be mediated only through CCK-B receptor. CCK-C receptor as mentioned before has been reported in up to 40% of patients with pancreatic cancer and its presence was associated with a more aggressive course with shortened survival. Downregulation of the CCK-B receptor in pancreatic cancer cells results in apoptosis and halts cell proliferation by interference with intracellular signaling. CCK receptors have been described in other GI malignancies including gastric cancer and colon cancers as well as outside the GIT, with abundant expression of both CCK-A and CCK-B receptors in lung cancer (Staley *et al.*, 1990, 1989).

Therapeutic Implications

- Before the documentation that CCK-B receptor isotype is the main mediator of cancer growth a CCK receptor antagonist failed to demonstrate a clinical benefit for advanced pancreatic cancer, but the specific factor was a CCK-A receptor antagonist (Abbruzzese *et al.*, 1992). Hence, netazepide (YF476) has been developed and used to treat patients with type 1 gastric NENs. Moreover, a nonselective CCK receptor antagonist, proglumide, arrested the progression of pancreatic intraepithelial neoplasias in an animal model implying that selective CCK receptor antagonists may prevent pancreatic cancer development in high risk subjects (Smith *et al.*, 2016, 2014).

Neurotensin (NT)

NT is a tridecapeptide localized in the central nervous system (predominantly hypothalamus and pituitary, involved in the regulation of luteinizing hormone and connected to the dopaminergic system), and in endocrine cells of the jejunal and ileal mucosa (Thomas *et al.*, 2003; Galoian and Patel, 2017). In hypothalamus it induces multiple effects, such as hypothermal and increased locomotor activity, whereas in the intestine it supports smooth muscle contraction in the small intestine.

NT act through NT receptor (NTR) and is released in response to increased intraluminal fats, with numerous functions in the GIT, including stimulation of pancreatic and biliary secretion, inhibition of gastric and small bowel motility, facilitation of fatty acid translocation from the intestinal lumen, and growth of the gastric antrum (minimal effect), small bowel (pronounced effect), colon (medium effect), and pancreas (Smith *et al.*, 2016). NT is produced from preproneurotensin that contains both NT 1–13 and the related peptideneuromedin N. Neuromedin N comprises the C-terminal portion of the proneurotensin peptide after NT is removed. NT 1–13, the major product of proneurotensin in the ileum and brain, is cleaved in the inactive NT 1–8 and the biologically active NT 9–13. On the other hand, substance P, muscarinic agonists (carbachol), catecholamines, and BBS/GRP stimulate NT release.

NT has been shown to contribute to carcinogen-induced gastric cancer in animal models (Thomas *et al.*, 2003). NT stimulates pancreatic cancer proliferation as shown in human pancreatic cell lines while 75%–90% of pancreatic adenocarcinomas expressing NTRs. NT is considered a cancer promoting mitogen in colon cancer, stimulating growth of certain human colon cancer cell lines expressing high levels of NTRs both in vitro and in vivo. Since intraluminal fat is a potent stimulus for NT release, an etiologic association between fat and colon carcinogenesis may imply a role for NT, which increases colon cancer proliferation.

Lastly, NTS/NTSR also shows potential of being utilized as a diagnostic biomarker for cancers as well as targets for functional imaging.

Therapeutic Implications

Blockade and modulation of the NTS/NTSR signaling pathways appears to reduce colorectal cancer growth in cell cultures and animal studies (Qiu *et al.*, 2017).

- A possible role for NT was suggested for lung carcinomas, as seen in small cell lung carcinomas (SCLC) cell lines and confirmed by a beneficial effect of a NTR antagonist.
- NT stimulates also the proliferation of prostate cancer cells, particularly through the NTR subtype NTR 3, during conditions of prostate cancer androgen insensitivity, providing support for the potential use of NTR antagonists as potential therapy for a subset of prostate cancers that continue to grow after androgen withdrawal.

Ghrelin

Ghrelin, a 28-amino-acid peptide, is the natural ligand for the growth hormone secretagogue receptor (GHS-R). It is secreted mainly from the stomach and duodenum, however lower amounts are found in the pancreas, pituitary, kidney, and placenta. A

limited region of the arcuate nucleus of the hypothalamus contains small amounts of ghrelin (Kojima *et al.*, 1999; Korbonits *et al.*, 2004).

The ghrelin receptor (GHS-R) is a member of a G protein-coupled receptor. Two forms of the receptor exist, GHS-R1a that modulated most physiologic actions of ghrelin, and GHS-R1b that has been regarded as a nonfunctional receptor. In the brain, GHS-R1a expression is highest in the hypothalamus, but it is also found in the dorsal motor nucleus of the vagus and parasympathetic preganglionic neurons. In peripheral tissues, GHS-R1a is found in the anterior pituitary, islet cells of the pancreas, thyroid, adrenal glands, and heart (Müller *et al.*, 2015; Muccioli *et al.*, 2007; Leung *et al.*, 2007).

Ghrelin has many physiological functions, including hormonal secretion regulation (of GH, ACTH, prolactin, gonadal hormones), energy balance, motility and secretion of the GIT and the pancreas, etc.

Ghrelin and its receptors were found to be expressed in malignant tumors of the kidney, pancreas, thyroid, lung, breast, prostate, ovary, stomach, colorectal cancer, and pituitary adenoma, and in distant metastases (Lin and Hsiao, 2017). On one hand, ghrelin was found to promote tumor proliferation through several signaling pathways, including the PI3K/AKT/mTOR (through phosphorylation of AKT and mTOR), Ras/RAF/ERK, JAK/STAT, and Src kinase (Zhu *et al.*, 2017). On the other hand, also antiproliferative effects have been reported.

- The PI3K/AKT/mTOR signaling pathway activation is associated with the development and progression of GISTs, suggesting ghrelin has a role in the pathogenesis of these tumors (Zhu *et al.*, 2017).
- Ghrelin was shown to promote pancreatic cancer cell migration and invasion via the GHRS/PI3K/Akt pathway activation, which is blocked by ghrelin receptor antagonist.
- Similar influence is observed with colorectal cancer cells.
- In renal cell carcinoma patients, ghrelin was found to be an independent predictor in correlation with poor prognosis and metastatic disease (Lin and Hsiao, 2017). In breast-cancer cell lines ghrelin inhibits proliferation. There is increased ghrelin expression in well differentiated, compared with poorly differentiated breast cancer, and ghrelin expression was found to be correlated with better prognosis (Grönberg *et al.*, 2017).

The role of ghrelin as promoter or inhibitor of cancer is not fully clear, and it differs between cancers. Suggested theory is that some actions are mediated through other unknown receptor subtypes. Exploring the possible antiproliferative effect of ghrelin analogues or antagonists, will require characterization of individual tumors, and personalized approach.

Glucagon Like Peptide-2 (GLP-2)

GLP-2 belongs to the glucagon superfamily of peptides, with the shared precursor proglucagon. They are expressed in the nervous system (central and peripheral), pancreas and intestine. Proglucagon undergoes different posttranslational processing in different cell type, by tissue specific enzymes. While glucagon and GLP-1 have a role in glucose metabolism, GLP-2 mainly enhances cellular proliferation.

The GLP-2 receptor is a G-protein coupled receptor, expressed in the hypothalamus, brainstem, lung, and GIT. GLP-2 is secreted from the intestinal L-cells, and was shown to act in an autocrine mechanism, by binding to its receptor on the L-cells, and stimulating its own secretion. The receptor binding initiates the PI3K/AKT/mTOR pathway, and also ligands for ErbB and its related signaling pathway, enhancing intestinal growth. There is also an association with increased IGF-1 activity; IGF-1 increased GLP-2 receptor expression, and GLP-2 increased IGF-1 receptor expression.

GLP-2 was shown to improve intestinal wound healing through the transforming growth factor β (TGF-β) pathway, to stimulate proliferation of colon cancer cell lines, and to induce vascular endothelial growth factor (VEGF) and TGF-β1 (Kannen *et al.*, 2013).

A systematic review assessing the potential risk of intestinal neoplasia in patients receiving treatment with GLP-2, did not confer an increased risk of intestinal neoplasia in patients or animals, but a small number of patients was studied. However, GLP-2 treatment was shown to promote tumor growth, in animals with a preinduced cancer (Ring *et al.*, 2017).

Therapeutic Implications

- Therapies targeted at the tumor signaling pathways, like multiple tyrosine kinase inhibitors, increased survival of patients with colorectal carcinoma, when combined with chemotherapy. This is the rational for blocking the GLP-2 activity and its related signaling pathways, by using antibodies against GLP-2 or its receptor, or targeting the prohormone convertases involved in proglucagon cleavage.
- Another suggested use of GLP-2 in the treatment of colon cancer, is as a chemosensitizer, by increasing IGF-1 expression and promoting the cancer cells toward proliferation, making them more susceptible to chemotherapy (Kannen *et al.*, 2013).

Vasoactive Intestinal Peptide (VIP)

VIP is produced by various cells, but its primary location is within neurons; this peptide is expressed in the peripheral and central nervous systems, as well as in certain tumors. VIP shares 68% homology with pituitary adenylate cyclase activating polypeptide (PACAP) as a member of secretin-glucagon-VIP peptide family.

Its biologic actions are mediated through the members of the class II/class-B secretin-like receptors VPAC1, VPAC2, PAC1. VIP-receptors have been found in 60% of lung-carcinoma-cells, with VIP, pro-VIP and COOH-terminal-extended forms frequently found (Vona-Davis and McFadden, 2007). Both VIP- and PACAP-receptor antagonists inhibit the growth of SCLC-cells, while a VIP-analog acting as antagonist inhibited lung cancer growth and had synergistic inhibitory-effects with chemotherapeutic agents.

Breast-cancer cells have VIP/PACAP receptors in up to 100% of cases and have high densities of VPAC1 and its mRNA, and VPAC1, VPC2, and PAC1 are reported in breast-cancer tumors. Furthermore, the addition of a VPAC inhibitor to various breast-cancer cell lines, potentiated the ability of taxol to inhibit proliferation.

VIP/PACAP receptors are present in 88%–100% of prostate cancers, each of the three subtypes are found in different studies, however the predominant subtype is VPAC1 as opposed to normal prostate tissue in which for the PAC1-receptor predominates.

VIP/PACAP receptors are reported in 96% of colon adenocarcinoma and specifically, 35% of well-differentiated had VPAC1, 65% of moderately differentiated and 87% of poorly differentiated tumors.

PAC1 receptors were present in 81%–100% in various human gliomas, and in 20% of meningiomas VPAC1 and VPAC2 receptors were also found in glioma cell lines, with the VPAC1-receptor localized strongly to the nucleus, and there was a positive correlation between the degree of VPAC1 nuclear localization and glioma grade.

Almost all neuroblastoma tumor cells possess at least one of the VPAC/PAC-receptors, with VPAC1-receptors reported in 60%–100%, VPAC2 in 31% and PAC1 in 60%–100%. Both VIP/PACAP have an effect on the growth/differentiation of various neuroblastoma tumor cells along with the induction of the differentiation and neuritogenesis.

Pancreatic ductal cancer was found to express in approximately 65% VIP/PACAP receptors. VIP hypersecretion by neural tumors such as ganglioblastomas/neuroblastomas in children or by pancreatic-tumors (VIPomas), causes Verner–Morrison syndrome characterized by large volume watery diarrhea, hypokalemia and achlorhydria. VIPoma cells express both VPAC1 and VIP, raising the possibility of an autoregulatory effect on the tumor cells.

The overexpression of VPAC/PAC receptor has been studied in other tumors such as pheochromocytomas, neuroendocrine tumors, choriocarcinoma and gastric, cervical or renal cancers (Moody *et al.*, 2016b).

Peptide YY (PYY)

PYY is a 36-amino-acid peptide, synthesized as prepropeptide, homologous to pancreatic polypeptide (PP) and neuropeptide Y (NPY). PYY co-localizes with glucagon and glucagon-like products within endocrine L-cells of the intestinal mucosa and to a lesser extent in alpha cells of the pancreas. PYY inhibits small bowel fluid and electrolyte secretion, contraction of the gallbladder, gastric emptying and intestinal transit, regulating gastrointestinal motility, while it stimulates the proliferation of small bowel and colonic mucosa (Thomas *et al.*, 2003), acting on the gut and pancreas by regulating growth, digestion and absorption (Vona-Davis and McFadden, 2007).

Experiments in human pancreatic cancer cell lines with PYY analogs, showed that PYY, can inhibit pancreatic cancer growth but it was not determined whether the pancreatic cancers express receptors for PYY (Tseng and Liu, 2002).

Therapeutic Implications

- While decreased expression of PYY seems to be relevant to the development and progression of colon adenocarcinoma, the treatment with PYY decreases growth in pancreatic and breast tumors, most likely through a reduction in intracellular cAMP (Baldassano *et al.*, 2017).
- Synthetic analogs of PYY have been developed that are potent inhibitors of the proliferation of pancreatic cancer along with improving of the patients' nutritional status and have been studied as hormonal adjunct in the chemotherapy of pancreatic adenocarcinoma (Vona-Davis and McFadden, 2007).

See also: Carcinoid Syndrome. Gastrin. Pancreatic Islet Cell Tumors. Somatostatin Receptor Expression in Gastrointestinal Tumors. VIPoma, Glucagonoma, and Somatostatinoma

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Somatostatin Receptor Expression in Gastrointestinal Tumors

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Glossary

Cyclic AMP (cAMP) Adenosine-3′,5′-cyclic monophosphate, a derivative of adenosine triphosphate (ATP), is a second messenger important for intracellular signal transduction.

Immunohistochemistry The staining of cells in vitro with antibodies raised against unique antigens contained in or on the cell surfaces.

Neuroendocrine neoplasms (NENs) Tumors that originate from endocrine cells in endocrine organs (pituitary, thyroid, pancreas) or from neuroendocrine cells in disseminated endocrine tissues such as lung or gastrointestinal system. They often secrete high levels of biologically active hormones or neurotransmitters that produce characteristic symptoms and disease.

Reverse transcription polymerase chain reaction (RT-PCR) A variant of polymerase chain reaction (PCR) is a technique commonly used in molecular biology to detect RNA expression.

Introduction

Somatostatin (SST) was discovered almost half a century ago (Brazeau *et al.*, 1973), after being isolated from rat hypothalamic extracts and was named from its ability to inhibit pituitary growth hormone (GH) release (somatotropin release-inhibiting factor). Soon it was realized that this cyclic oligopeptide hormone is abundantly distributed, exhibiting various physiological actions, such as inhibition of hormone secretion by the pituitary gland (not only GH but also adrenocorticotropic hormone, prolactin, and thyroid stimulating hormone (TSH)), inhibition of insulin and glucagon secretion by the endocrine pancreas, inhibition of motility and exocrine secretion in the gastrointestinal tract, regulation of motor, cognitive, and sensory functions in the central nervous system and pain transmission in the peripheral nervous system (Reubi and Schonbrunn, 2013). Due to its broad antisecretory potency, SST was used early on to treat patients with hormone-producing tumors such as pituitary adenomas, neuroendocrine neoplasms (NENs) causing carcinoid syndrome, and islet-cell pancreatic tumors (insulinomas, gastrinomas, and glucagonomas), even before its mechanism of action was elucidated.

In humans, SST exists in two forms, 14 and 28 amino acids in length, generated by alternative proteolytic processing from a single precursor (pre-prosomatostatin) which is encoded by a gene on chromosome 3. It was also the first human peptide to be produced by bacterial recombination (Itakura *et al.*, 1977), but the short half-life (<3 min) of the native hormone was one of the main reasons limiting its routine clinical use. Production and commercial availability of somatostatin analogues (SSAs) resistant to degradation, with extended half-life (octreotide, lanreotide, and pasireotide) particularly in their long acting formulations that facilitated monthly administration (octreotide and pasireotide LAR, lanreotide autogel), considerably expanded their application in clinical practice (**Fig. 1**).

SST Receptors and Signaling

SST and its analogues exert their action by binding to somatostatin receptors (SSTRs) which belong to the seven-transmembrane domain G-protein-coupled receptors superfamily. Five distinct SST receptor genes, named sstr1 to sstr5, were cloned and located in chromosomes 14, 17, 22, 20, and 16, respectively (Hoyer *et al.*, 1995). The sstr2 gene protein product can be alternatively spliced to generate two receptor proteins, SSTR2A and SSTR2B, which differ in length (SSTR2B is shorter) and their cytoplasmic carboxy-terminal sequences (Vanetti *et al.*, 1992). Human tissues contain the SSTR2A variant almost exclusively, whereas both forms have been identified in rodents. SSTR5 also exists as truncated isoforms with four or five transmembrane domains (sst5TDM4 and sst5TDM5) (Duran-Prado *et al.*, 2009); distribution and physiological functions of truncated SSTR5 variants are not yet known, but they have been linked with pituitary tumor resistance to SSA treatment (Cordoba-Chacon *et al.*, 2011) and pancreatic NEN (pNEN) aggressiveness (Sampedro-Nunez *et al.*, 2016). Native SST has high affinity for all SSTR types; however, the available synthetic SSAs show restricted affinity profiles. Octreotide and lanreotide have high affinity for SSTR2 and low affinity for SSTR3 and SSTR5. Pasireotide (SOM230) displays a broader affinity spectrum with higher affinity for SSTR5 and to a lesser extent for SSTR1, SSTR2, and SSTR3, while multireceptor ligands with high affinity for all five known SSTRs have been produced such as KE108 (**Table 1**).

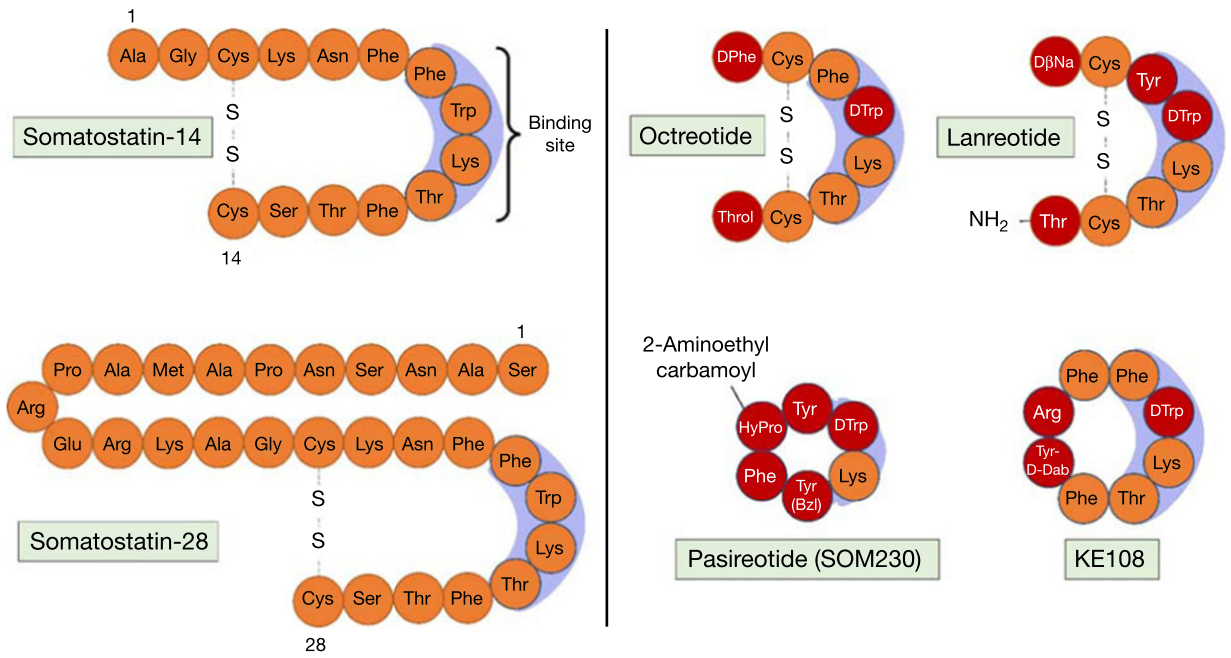

Fig. 1 Amino acid structures of native somatostatin (SS-14 and SS-28) and the somatostatin analogues discussed.

Table 1 Affinity of native somatostatin and its analogues for different SSTR subtypes

Ligand	*Binding affinity for each SSTR (IC_{50}, nM)*				
	SSTR1	*SSTR2*	*SSTR3*	*SSTR4*	*SSTR5*
Somatostatin 14	2.26	0.23	1.43	1.77	0.88
Somatostatin 28	1.85	0.31	1.3	ND	0.4
Octreotide	1140	0.56	34	7030	7
Lanreotide	2330	0.75	107	2100	5.2
Pasireotide	9.3	1	1.5	>100	0.16
KE108	2.6	0.9	1.5	1.6	0.65

Lower values represent higher affinity. Data were obtained from membrane-binding studies using cells transfected with individual receptor subtypes and may depend on the conditions of the assay used. *ND*, not determined.

Modified from Grozinsky-Glasberg, S.; Shimon, I.; Korbonits, M.; Grossman, A. B. Somatostatin Analogues in the Control of Neuroendocrine Tumours: Efficacy and Mechanisms. Endocr. Relat. Cancer 2008, 15, 701–720; Veenstra, M. J.; De Herder, W. W.; Feelders, R. A.; Hofland, L. J. Targeting the Somatostatin Receptor in Pituitary and Neuroendocrine Tumors. Expert Opin. Ther. Targets 2013, 17, 1329–1343.

Binding of a SST ligand to SSTRs leads to intracellular activation of different second messenger pathways resulting in inhibition of hormone secretion and tumor growth (Fig. 2). The antisecretory action of SSTRs is mediated by coupling to Gi/Go proteins, which subsequently leads to inhibition of adenylyl cyclase and reduction of cyclic AMP (cAMP). In addition, activation of the Gi/Go reduces intracellular calcium by inhibiting calcium channels directly and indirectly by opening potassium channels which results in membrane hyperpolarization and inhibition of calcium influx through voltage-dependent calcium channels (Patel, 1999). The simultaneous reduction of both second messengers (cAMP and calcium) by SST results in a synergistic inhibitory effect on hormone exocytosis. The antiproliferative effect of ligand-activated SSTRs results from activation of protein tyrosine phosphatases that induce growth arrest through inhibition of mitogen-activated protein kinase and apoptosis via induction of p53 and Bax (Pyronnet *et al.*, 2008; Sharma and Srikant, 1998). There are also indirect antiproliferative effects of SSTR activation, through inhibition of secretion of paracrine growth factors and trophic hormones, inhibition of angiogenesis, and modulation of the immune system (Woltering, 2003).

Following ligand activation, SSTRs are phosphorylated and β-arrestins are recruited leading to uncoupling of the receptor from the second messenger pathway, thereby interrupting signaling. Subsequently, the receptor–ligand complex can be internalized, and the receptor can then relocate to the cell membrane (recycling), or enter lysosomal degradation. For each receptor subtype, this trafficking process is different. The internalization of the SSTR2, SSTR3, and SSTR5 occurs to a much higher extent after SST exposure than that of SSTR1 or SSTR4. After endocytosis, SSTR2 and SSTR5 recycle to the plasma membrane, whereas SSTR3 is predominantly degraded by lysosomes and downregulated. It should also be noted that when different SSAs act as ligands the receptor trafficking process is not the same as when native SST activates the receptor. For example pasireotide internalizes SSTR3 more effectively than octreotide, but for SSTR2 it does so to a much lesser extent (Csaba *et al.*, 2012; Lesche *et al.*, 2009).

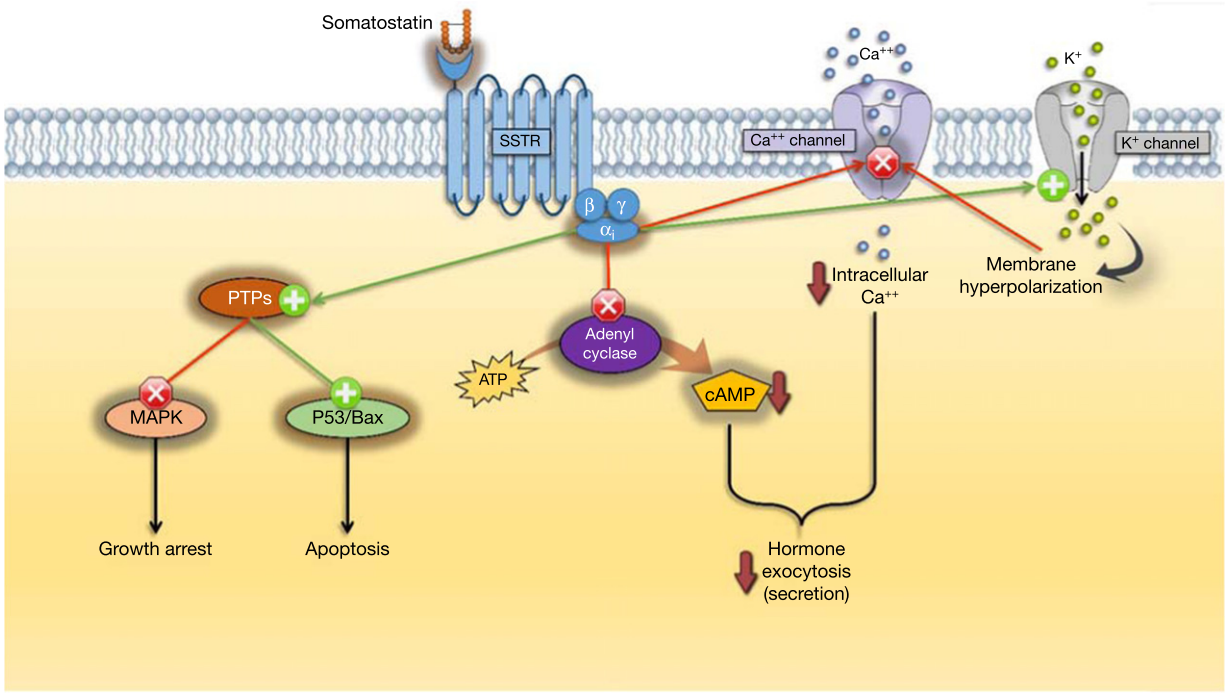

Fig. 2 Somatostatin receptor modulation of signaling cascades leading to changes in hormone secretion, apoptosis, and cell growth.

Methods of SSTR Detection In Vitro

Before discussing the current knowledge on SSTR expression in gastrointestinal tumors, a short review of the different methods available to determine SSTR at the tissue level is essential to critically appreciate the available research data. Several methods have been used to determine the expression of SSTR in vitro. All of them have intrinsic limitations and advantages, and therefore, the most robust data can be generated by the combination of two or more of them. **Table 2** lists a comparative representation of the most relevant detection methods.

Autoradiography

SSTR tissue localization had originally been demonstrated by means of autoradiography using radiolabeled SSTR ligands (i.e., ^{125}I–Tyr^{3}-octreotide) that is still considered the gold-standard method (Reubi *et al.*, 1987). This method has the unique property of identifying the presence of "functionally active" receptors; however, it is affected by low reproducibility, is only applicable to high-quality frozen material, and does not recognize the SSTR subtype expressed, unless using highly subtype-specific ligands.

SSTR mRNA Detection

Several other studies tried to identify SSTR expression in normal tissue and tumors by means of detecting mRNA expression by in situ hybridization, northern blot, or reverse transcription polymerase chain reaction (RT-PCR) (Papotti *et al.*, 2002). However, mRNA expression from tissue extracts (especially in RT-PCR) is variably affected by signals from SSTR-expressing cells different from those that represent the target of the measurement. Normal and nontumoral tissues (blood vessels, nerves, lymphocytes, as well as nontumoral endocrine cells) located adjacent to a receptor-negative tumor sample may erroneously suggest tumor positivity. Another inherent limitation of this method lies on the fact that receptor mRNA levels may not directly reflect levels of functional receptor protein, and the quality of the frozen tissue sample is again a relevant component for obtaining adequate results.

SSTR Protein Detection

In parallel with mRNA determination, several researchers aimed at detecting SSTRs by immunohistochemistry (IHC) using SSTR-specific antibodies against the N- or the C-terminus of the protein (Hofland *et al.*, 1999). Advantages of IHC include SSTR localization, recognition of the specific SSTR subtypes, a high cost/benefit ratio and reproducibility and lastly,

Table 2 Comparison between the different methods of SSTR detection on tissues

	Autoradiography	*mRNA expression*	*Immunohistochemistry*
SSTR localization	✓	–	✓
Identification of SSTR subtype	–[a]	✓	✓
Mapping of functional SSTRs	✓	–	–
Low cost	–	–	✓
Sensitivity	✓	✓	–[b]
Specificity	✓	–[c]	✓
Tissue material	Frozen only	Frozen only	Frozen and paraffin-embedded[d]
Applicability in clinical practice	–	–	✓

[a]Identification of SSTR subtypes is possible if SSTR-subtype specific ligands are used.
[b]Sensitive antibodies are inherently difficult to be produced and quantification of positivity is not standardized.
[c]Results may be contaminated by SSTR expression of adjacent normal cells.
[d]Can be applied on biopsy or FNA (fine-needle aspiration) material and not exclusively on resected tissues.

applicability on paraffin-embedded tissues facilitating investigation on retrospective archival material or even fine needle aspiration or biopsy samples of inoperable tumors. Conversely, the major caveat of IHC has been the inherent difficulty to develop highly sensitive and specific SSTR antibodies. Recently, the development of monoclonal antibodies (UMB-1) against SSTR2, the most predominantly expressed SSTR subtype, and against SSTR5 (UMB-4), have shown reliable correlation with the established autoradiography method, with only minor discrepancies (Korner *et al.*, 2012; Lupp *et al.*, 2011). However, since these antibodies only identify epitopes other than the SST binding domain of the receptor, they do not actually map the active SST-binding sites which represent the molecular targets for the clinical application of SSAs. In addition, lack of standardized quantification of positive staining is considered a relative disadvantage of IHC, although several scoring systems have been proposed (Volante *et al.*, 2007). In general, a fine membranous staining is considered the most specific, although a cytoplasmic staining may be a result of receptor internalization, especially in the case of patients treated with SSAs (Reubi *et al.*, 2010).

More recently, several studies have suggested a potential prognostic value of in vitro SSTR subtyping, associating SSTR2 expression with better outcomes in patients with NENs treated with SSAs (Asnacios *et al.*, 2008; Kim *et al.*, 2011; Corleto *et al.*, 2009; Okuwaki *et al.*, 2013). The same effect has also been observed for SSTR5 expression (Wang *et al.*, 2017; Song *et al.*, 2016). Recently, detection of SSTR2 and SSTR5 with IHC was demonstrated on circulating tumor cells from NEN patients, providing an intriguing biomarker for evaluating SSTR-targeted therapies (Childs *et al.*, 2016). Future studies are needed to validate SSTR determination at the tissue level, as a useful complementary approach for better definition of therapeutic strategies in patients affected by NENs.

Distribution of SSTRs

SSTR Expression in Normal Tissues

As implied earlier by the various SST actions in humans, SSTR expression has been confirmed in many normal tissues, including the brain, pituitary, pancreas, gastrointestinal system, thyroid, adrenal, kidney, and the immune system. All SSTR subtypes are found in different parts of the brain, while SSTR2 and SSTR5 are the main receptors found in the adenohypophysis with SSTR1 and SSTR3 being expressed at lower levels. SSTR1 is expressed in the jejunum and stomach and SSTR2 in the kidney. In the pancreas, α-cells express mainly SSTR2, β-cells SSTR1 and SSTR5, and δ-cells SSTR5. The adrenals express SSTR2 and SSTR5. In the immune system, lymphocytes express SSTR3 and thymus SSTR1, SSTR2 and SSTR3. Liver expresses SSTR1 and SSTR2. SSTR4 is present in the lung, pancreas, and heart. Although SSTR subtypes are expressed in a complex tissue-specific pattern, SSTR2A appears to be the most abundant receptor subtype (Reubi, 2003).

SSTR Expression in Tumors

A wide range of different tumors overexpress SSTRs, as compared to originating normal cells. The underlying stimuli that induce this overexpression as well as the functional meaning for the biology of tumor cells have not been conclusively explained. It is possible that the upregulation of SSTRs serves as homeostatic growth inhibitory response to the deregulated tumor cell proliferation in an autocrine/paracrine way (Msaouel *et al.*, 2009). These tumors include pituitary adenomas (in particular GH- and TSH-producing adenomas), gastroenteropancreatic NENs (GEP-NENs) and lung NENs, pheochromocytomas, and paragangliomas. Tumors of the nervous system, such as medulloblastomas, meningiomas, and neuroblastomas, also express SSTRs at high density. Furthermore, nonneural tumors and non-NENs can also express SSTRs, although clearly at a lower incidence and/or density than NENs. Such tumors include breast, prostate, hepatocellular and gastric carcinomas, lymphohematological malignancies, lung small cell, and renal cell carcinomas (Hasskarl *et al.*, 2011). Finally, it should also be mentioned that inflammatory

diseases such as sarcoidosis, rheumatoid arthritis, inflammatory bowel disease, thyrotoxic orbitopathy, and various retinal diseases are associated with increased SSTR expression.

Particularly for GEP-NENs, SSTR overexpression is considered a common histopathological feature offering unique opportunities for application of diagnostic and therapeutic modalities. Expression patterns in GEP-NENs display a wide heterogeneity between different NEN types and even between patients harboring the same tumor type, or even between cell populations within the same lesions. It has also been shown that metastatic sites can exhibit different SSTR expression profiles when compared with the primary tumor (Nasir *et al.*, 2006; Hofman and Hicks, 2012; Kaemmerer *et al.*, 2015). More than one SSTR subtype are usually present, but the most prominent subtype found in these tumors is SSTR2 followed by SSTR1 and SSTR5, whereas SSTR3 is less frequent and SSTR4 is poorly expressed. pNENs (including gastrinomas, glucagonomas, VIPomas) and intestinal NENs express SSTR2 in 80%–100% of cases, whereas insulinomas have a lower incidence of SSTR2 expression (50%–70%) (Reubi and Waser, 2003). By contrast, insulinomas often express SSTR1 or, alternatively, SSTR5 together with or instead of SSTR2. SSTR expression also correlates with the level of tumor differentiation, since well-differentiated GEP-NENs usually express a greater density and number of receptors than do poorly differentiated neoplasms, an observation which is useful for therapeutic decisions (Reubi *et al.*, 1990). **Table 3** summarizes the frequency of SSTR expression in various GEP-NENs reported by researchers using all three described detection methods.

Rarely some NENs arising primarily in the duodenum and pancreas can also secrete increased amounts of SST causing a constellation of symptoms related to the physiologic actions of SST (such as steatorrhea, cholelithiasis, hyperglycemia, and weight loss), known as the somatostatinoma syndrome (Soga and Yakuwa, 1999). Only about 10% of patients with somatostatinomas (tumors containing immunoreactive granules with SST) experience functional symptoms, and in those the primary tumor is usually located in the pancreas. On the contrary, somatostatinomas arising from the duodenum, particularly in the ampullary and periampullary area, rarely cause secretory symptoms and are frequently associated with neurofibromatosis type 1 (Von Recklinghausen's disease). In symptomatic patients, treatment with SSAs, although it may seem paradoxical, is effective in symptom relief, since these tumors also express SSTRs (Angeletti *et al.*, 1998).

SSTR Detection In Vivo

SSTR overexpression in GEP-NENs has constituted the basis for diagnostic imaging using scintigraphy with radio-labeled SSAs and has been applied in clinical practice for in vivo demonstration of SSTR presence in these tumors (somatostatin receptor imaging—SRI). Receptor internalization following binding with such a radiopeptide leads to retention and accumulation of radioactivity in tumor cells allowing macroscopic visualization with devices that capture emitted γ-radiation (γ-cameras). The same principle can be utilized to bind ligands with α- or β-emitting isotopes, permitting selective radiotherapy of the tumor-expressing SSTRs in vivo (peptide receptor radionuclide therapy).

The first commercially available agent for SRI, ^{111}In-DTPA-D-Phe^{1}-octreotide, was officially introduced in 1994 (Octreoscan), and its use to visualize various SSTR-positive tumors and tissues became the "gold standard" in the localization, staging, and follow-up in patients with NENs, especially in conjunction with single-photon emission computed tomography (SPECT). The sensitivity and specificity of Octreoscan, in the diagnosis of intestinal NENs, including primary foci and metastatic lesions, are 71%–96% and 76%–95%, respectively (Kwekkeboom *et al.*, 1993). In order to overcome the limitations of Octreoscan, such as the high cost of ^{111}In (a cyclotron-produced radionuclide) and the suboptimal physical features of this radioisotope (poor image resolution and high radiation dose to the patient), labeling SSAs with ^{99m}Tc led to development of a new radiopharmaceutical, ^{99m}Tc-EDDA/HYNIC-TOC (Tektrotyd), with superior imaging features (Hubalewska-Dydejczyk *et al.*, 2006).

Further development of SSAs for use in SRI, like DOTA-D-Phe^{1}-Tyr^{3}-octreotide (DOTATOC), DOTA-D-Phe^{1}-Tyr^{3}-Thr^{8}-octreotide (DOTATATE), and DOTA-1-Nal^{3}-octreotide (DOTANOC) opened the prospect for convenient radiolabeling with ^{68}Ga for PET/CT (positron emission tomography/computed tomography) imaging. DOTATOC and DOTATATE display a very high affinity for the SSTR2, low or negligible affinity for SSTR3 and SSTR5, and no significant affinity for SSTR1 and SSTR4. On the contrary, DOTANOC has not only a higher affinity to SSTR3 and SSTR5 but also binds more avidly to SSTR2. PET/CT using ^{68}Ga-labeled SSAs enables molecular imaging of NENs and their metastases with very high diagnostic sensitivity and specificity. The accuracy of ^{68}Ga-DOTATOC PET (96%) was found to be significantly higher than that of computerized tomography (CT) (75%) and ^{111}In-DOTATOC SPECT (58%) (Gabriel *et al.*, 2007). It could detect more lesions than SPECT and CT, but also was true positive where SPECT results were false negative, visualizing small tumors or tumors bearing only a low density of SSTRs and more metastatic foci in the lymph nodes, liver, and bones (Kowalski *et al.*, 2003). In addition, it provides superior resolution, low radiation burden (10–12 mSv), faster protocol times (60–90 min) and quantitative, reproducible data (by means of determining standardized uptake values). More recently, novel tracers (^{64}Cu-DOTATATE) using ^{64}Cu, an isotope characterized by better spatial resolution and longer half-life than ^{68}Ga, exhibited similar patient-based sensitivity compared to ^{68}Ga-DOTATOC but detected significantly more lesions (Johnbeck *et al.*, 2017).

The role of SST receptor antagonists has also been investigated recently with promising results. Potent SST receptor antagonists, known to poorly internalize after binding with SSTRs, can visualize tumors in vivo as well or even better than agonists. This is because agonists preferably recognize the active state of receptors, whereas antagonists bind with high affinity to both active and inactive receptor states. Because receptors are primarily in the inactive state in cells, agonist binding will represent a fraction of what can be labeled with an antagonist (Ginj *et al.*, 2006; Cescato *et al.*, 2008; Wild *et al.*, 2010).

Table 3 Expression profiles of somatostatin receptor subtypes by various NENs reported by researchers using immunohistochemistry (IHC), mRNA detection (RT-PCR, Northern blot, or in situ hybridization), and autoradiography with subtype-specific ligands

SSTR subtype	*Method of detection*	*Intestinal NENs*	*%*	*Insulinomas*	*%*	*Gastrinomas*	*%*	*Glucagonomas*	*%*	*VIPomas*	*%*	*NF-PNENs*	*%*	*References*
SSTR1	IHC	–	45.5	–	29.1	–	31.6	–	66.7	–	100	–	50.3	Papotti *et al.* (2002)
		13/35		11/36		10/33		–		–		–		Kulaksiz *et al.* (2002)
		–		4/16		–		–		–		–		Portela-Gomes *et al.* (2007)
		–		–		–		–		–		68/146		Song *et al.* (2016)
		37/75		–		–		–		–		–		Zamora *et al.* (2010)
		–		1/3		2/6		2/3		1/1		13/15		Fjällskog *et al.* (2003)
	mRNA	12/13	73.1	17/19	78.8	1/1	77.8	1/1	100	1/1	66.7	9/10	69.8	Papotti *et al.* (2002)
		5/6		2/4		7/11		–		0/1		8/12		Jais *et al.* (1997)
		5/11		7/10		9/9		–		–		1/7		Wulbrand *et al.* (1998)
		8/10		–		–		–		–		–		Nilsson *et al.* (1998)
		7/11		–		4/5		–		–		–		Schaer *et al.* (1997)
		12/16		–		0/1		–		1/1		12/14		Corleto *et al.* (2009)
	Autoradiography	14/27	51.9	16/26	61.5	1/10	10	2/3	66.7	1/4	25	–	–	Reubi and Waser (2003)
SSTR2	IHC	23/28	92	13/21	51.3	5/5	93.2	4/4	100	1/1	100	10/13	50.6	Papotti *et al.* (2002)
		30/35		21/36		33/33		–		–		–		Kulaksiz *et al.* (2002)
		–		2/16		–		–		–		–		Portela-Gomes *et al.* (2007)
		–		–		–		–		–		64/146		Song *et al.* (2016)
		67/75		–		–		–		–		–		Zamora *et al.* (2010)
		–		3/3		3/6		3/3		1/1		14/15		Fjällskog *et al.* (2003)
	mRNA	13/13	86.5	14/19	81.8	1/1	92.9	1/4	25	1/1	100	10/10	88.4	Papotti *et al.* (2002)
		6/6		4/4		10/11		–		1/1		11/12		Jais *et al.* (1997)
		8/11		9/10		9/9		–		–		4/7		Wulbrand *et al.* (1998)
		10/10		–		–		–		–		–		Nilsson *et al.* (1998)
		7/11		–		5/5		–		–		–		Schaer *et al.* (1997)
		14/16		–		1/1		–		1/1		13/14		Corleto *et al.* (2009)
	Autoradiography	26/27	96.3	18/26	69.2	10/10	100	2/3	66.7	4/4	100	–	–	Reubi and Waser (2003)
SSTR3	IHC	17/26	42.6	13/21	59.2	3/5	68.2	1/4	42.9	1/1	100	7/13	31.6	Papotti *et al.* (2002)
		25/35		28/36		26/33		–		–		–		Kulaksiz *et al.* (2002)
		–		3/16		–		–		–		–		Portela-Gomes *et al.* (2007)
		–		–		–		–		–		39/146		Song *et al.* (2016)
		16/75		–		–		–		–		–		Zamora *et al.* (2010)
		–		1/3		1/6		2/3		1/1		9/15		Fjällskog *et al.* (2003)
	mRNA	7/13	38.8	16/19	60.6	1/1	33.3	0/1	0	1/1	66.7	6/10	54.8	Papotti *et al.* (2002)
		1/6		3/4		4/11		–		0/1		7/12		Jais *et al.* (1997)
		0/11		1/10		2/9		–		–		0/7		Wulbrand *et al.* (1998)
		8/10		–		–		–		–		–		Nilsson *et al.* (1998)
		1/11		–		1/5		–		–		–		Schaer *et al.* (1997)
		9/16		–		1/1		–		1/1		10/13		Corleto *et al.* (2009)
	Autoradiography	4/27	3.7	9/26	34.6	2/10	20	1/3	33.3	1/4	25	–	–	Reubi and Waser (2003)
SSTR4	IHC		28		89.5		83.3		66.7		100		48.4	

(*Continued*)

		–		–		–		–		–		–	Papotti *et al.* (2002)
		–		–		–		–		–		–	Kulaksiz *et al.* (2002)
		–		14/16		–		–		–		–	Portela-Gomes *et al.* (2007)
		–		–		–		–		–		63/146	Song *et al.* (2016)
		21/75		–		–		–		–		–	Zamora *et al.* (2010)
		–		3/3		5/6		2/3		1/1		15/15	Fjällskog *et al.* (2003)
	mRNA	2/13	41.8	8/19	42.4	0/1	50	0/1	0	0/1	0	0/10	19	Papotti *et al.* (2002)
		6/6		4/4		9/11		–		0/1		8/12		Jais *et al.* (1997)
		4/11		2/10		2/9		–		–		0/7		Wulbrand *et al.* (1998)
		10/10		–		–		–		–		–		Nilsson *et al.* (1998)
		–		–		–		–		–		–		Schaer *et al.* (1997)
		1/15		–		0/1		–		0/1		0/13		Corleto *et al.* (2009)
	Autoradiography	1/27	3.7	1/26	3.8	0/10	0	0/3	0	0/4	0	–	–	Reubi and Waser (2003)
SSTR5	IHC	21/26	72	9/21	56	4/5	72.7	3/4	71.4	1/1	100	8/13	21.3	Papotti *et al.* (2002)
		29/35		28/36		25/33		–		–		–		Kulaksiz *et al.* (2002)
		–		3/15		–		–		–		–		Portela-Gomes *et al.* (2007)
		–		–		–		–		–		20/146		Song *et al.* (2016)
		48/75		–		–		–		–		–		Zamora *et al.* (2010)
		–		2/3		3/6		2/3		1/1		9/15		Fjällskog *et al.* (2003)
	mRNA	10/13	68.2	8/19	54.5	0/1	88.9	0/1	0	0/1	0	6/10	55.8	Papotti *et al.* (2002)
		6/6		4/4		9/11		–		0/1		9/12		Jais *et al.* (1997)
		7/11		6/10		9/9		–		–		0/7		Wulbrand *et al.* (1998)
		10/10		–		–		–		–		–		Nilsson *et al.* (1998)
		4/11		–		5/5		–		–		–		Schaer *et al.* (1997)
		8/15		–		1/1		–		0/1		9/14		Corleto *et al.* (2009)
	Autoradiography	13/27	48.1	5/25	20	3/9	33.3	0/3	0	1/4	25	–	–	Reubi and Waser (2003)

Several studies have confirmed that SRI correlates strongly with tissue expression of SSTRs (Diakatou *et al.*, 2015; Kaemmerer *et al.*, 2011; Miederer *et al.*, 2009); however, caution should be taken, since all aforementioned imaging modalities are not disease specific but receptor specific. Thus, in order to be able to interpret their results, the underlying tumor biology must be taken into account. A negative scan may indicate absence of the SSTR subtypes that the radiolabeled ligand recognizes, tumor regression or tumor dedifferentiation. Correlation with anatomical imaging is always mandatory. A negative Octreoscan should also not be an excluding factor for the decision to administer SSA treatment (Sevilla *et al.*, 2016). In fact, patients with negative Octreoscan have been shown to respond to SSAs (Hillman *et al.*, 1998), while in the PROMID study which evaluated the effect of Octreotide in the control of tumor growth in patients with metastatic midgut NENs, patients with negative Octreoscan were responsive to octreotide (Rinke *et al.*, 2009). After all, Octreoscan and other SRI modalities verify whether an uptake of the radiolabeled SSA is present and not whether SSTRs are expressed. Factors like the size of the lesion in relation with the spatial resolution of the imaging method, as well as the signal intensity measured in Krenning scale (lower than, equal to, or greater than normal liver tissue; or higher than normal spleen or kidney uptake: grades 1, 2, 3, and 4, respectively), suggest that results considered as negative may not represent the true SSTR expression on microscopic level.

Conclusion

SST and its receptors are broadly expressed in the human body where they exert many physiological actions. Moreover, they can be expressed in many pathological tissues and particularly with high density in GEP-NENs. As soon as the high expression level of SSTRs in NENs was documented, SSAs were developed to control hypersecretion and tumor growth, but it also brought upon a revolution in the role of nuclear medicine in the diagnosis and therapy of such patients. This was indeed paralleled by the development of various radiopharmaceuticals targeting not only SSTRs but also other peptide receptors and metabolic pathways unique for this type of tumor cells. In recent years, knowledge on the physiology of SST and its receptors has exploded, with the elucidation of many of the molecular details involved in SST receptor signaling and regulation. First-generation SSAs (octreotide and lanreotide) were developed at a time when far less was known about the SST receptor family and are now rediscovered by unveiling of their diverse and complex interactions with the various subtypes of SSTRs. Novel second-generation SSAs such as pasireotide only emphasize the need to develop more effective SST receptor-directed agents. Further elucidation of SST receptor pharmacology will provide new opportunities to design more sophisticated means to target these receptors in an individualized and effective way, both in terms of developing diagnostic tools and of manipulating receptor signaling as a therapeutic strategy.

See also: Enterochromaffin-Like Cells. Gastrinomas. Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS). Pancreatic Islet Cell Tumors

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Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS)☆

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Glossary

Carcinoid syndrome A clinical syndrome characterized by diarrhea, flushing, asthma, and heart disease caused by ectopic release of bioactive peptides/amines by a carcinoid tumor (almost always metastatic to liver).

Gastrointestinal neuroendocrine tumors (NETs) Composed of carcinoid tumors and pancreatic neuroendocrine tumors derived from the diffuse neuroendocrine system of the gastrointestinal (GI) tract. They share neuroendocrine cell markers and pathologic and biologic features, and they frequently ectopically secrete hormones/amines that cause a clinical syndrome.

Pancreatic neuroendocrine tumor (panNET) syndrome Clinical syndrome caused by ectopic secretion of bioactive peptides by a GI endocrine tumor, frequently located in the pancreas. Characteristically named after the hormone ectopically secreted (e.g., insulinoma, gastrinoma, glucagonoma, and VIPoma).

Somatostatin A naturally occurring neuropeptide/hormone that occurs as a 14- or 28-amino acid form in the GI tract and central nervous system and that frequently has inhibitory effects on biologic processes (i.e., growth, secretion, and hormone release). Synthetic analogues (octreotide and lanreotide) are frequently used to inhibit ectopic release of bioactive substances and growth of NETs. Radiolabeled analogues are used to localize NETs using somatostatin receptor scintigraphy and to treat widespread metastatic disease.

Gastrointestinal neuroendocrine tumor (GI NET) syndromes are clinical syndromes caused by the release of biologically active peptides/amines by GI neuroendocrine tumors (Ito et al., 2013a). NETs comprise carcinoid tumors and pancreatic neuroendocrine tumors (panNETs) and are derived from amine- and peptide-producing cells of the diffuse neuroendocrine system

Introduction

GI NETs are also classified as APUDomas (amine precursor uptake and decarboxylation), as are medullary thyroid carcinomas, melanomas, and pheochromocytomas, because they share various cytochemical, pathologic, and biologic features (**Table 1**) (Klimstra, 2016). These tumors were originally proposed to have a common embryonic origin from neural crest cells but subsequent studies showed that this was not the case. All NETs share a number of common features (Klimstra, 2016). However, the different clinical syndromes have important differences and there are a number of differences between carcinoids and panNETs.

Pathology/Tumor Biology of NETs

By light microscopy, NETs are generally composed of monotonous sheets of small round cells with uniform nuclei and only a few mitoses in most cases. Their presence is generally suspected by routine histology but established by specific immunohistological staining for common cellular proteins (**Table 1**). Historically, silver staining was used; currently, immunolocalization of the secretory protein chromogranin (A, B, C), the cytosolic protein neuron-specific enolase, or the membrane protein synaptophysin is generally used (Klimstra, 2016).

By electron microscopy, NETs have numerous electron-dense granules (>80 nm) and small clear vesicles that correspond to synaptic vesicles of neurons. NETs synthesize numerous growth factors, peptides, and bioactive amines that may be ectopically released and cause a clinical syndrome (**Table 2**). The diagnosis of a given NET syndrome is based on clinical findings and cannot be made from the immunocytochemical analysis. Furthermore, pathologists cannot generally distinguish between benign and malignant NETs unless metastases or invasion are present. NETs are now graded into three grades depending on the NETs differentiation (G1, G2 = well-differentiated vs. G3-G3-NET, well-differentiated, G3-NEC (neuroendocrine carcinoma)-poorly differentiated), and their preoperative rates assessed by Ki-67 or determining the mitotic index by counting mitoses (Klimstra, 2016; Singhi and Klimstra, 2018). Numerous recent studies demonstrate that prognosis is worse with increasing grade (G1 better G2 better than G3) as is also the case for increasing category in the TNM classification. These classifications/grading (ENETs, WHO,

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Table 1 General characteristics of gastrointestinal neuroendocrine tumors (NETs) (panNETs and carcinoids) (Klimstra, 2016;Singhi and Klimstra, 2018)

Share general neuroendocrine cell markers, including
Chromogranins (Cgs) (A, B, C) are acidic monomeric-soluble proteins found in the large secretory granules. CgA is most widely used
Neuron-specific enolase (NSE) is the γ–γ dimer of the enzyme enolase and is a cytosolic marker of neuroendocrine differentiation
Synaptophysin is an integral membrane glycoprotein (38 kDa) found in small vesicles of neurons and neuroendocrine tumors
Pathologic similarities
All NETs show amine precursor uptake and decarboxylation (APUDomas)
Ultrastructurally, they have dense-core secretory granules ($>$80 nm)
Histologically, they appear similar with most having few mitoses and uniform nuclei
Frequently, they synthesize multiple peptides/amines, which can be detected immunocytochemically but may not be secreted
The presence or absence of clinical syndrome or type cannot be predicted by immunocytochemical studies
Histological grading classifications predict biologic behavior. Only invasion or metastases establish malignancy
A common TNM classification and grading system is used for both carcinoids and panNETs NETs
Similarities of biologic behavior
They secrete biologically active peptides/amines, which can cause clinical symptoms
They generally have high densities of somatostatin receptors, which are used for both localization and treatment

UICC) are now essential for the proper management of all NET patients because they not only have prognostic significance, they also can affect the choice of therapy (Klimstra, 2016; Singhi and Klimstra, 2018).

panNETs are classified according to the specific clinical syndrome they cause or, if no clinical syndrome occurs, as nonfunctional (Ito *et al.*, 2013a; Metz and Jensen, 2008) (**Table 2**). Nonfunctional panNET in the strict sense is a misnomer because these tumors frequently secrete multiple peptides (pancreatic polypeptide, CgA, and neurotensin); however, they do not cause a specific clinical syndrome. The symptoms caused by nonfunctional panNETs are due to the tumor per se (Ito *et al.*, 2013a; Metz and Jensen, 2008).

Carcinoid tumors are frequently classified by location of origin (foregut, midgut, or hindgut) because they share histochemical, functional, and biologic characteristics within these areas (Jensen *et al.*, 2016) (**Table 3**). Foregut carcinoids generally have low serotonin (5-HT) content, are argentaffin negative on silver staining, synthesize several hormones, and occasionally secrete ACTH or 5-hydroxytryptophane (5-HTP) causing an atypical carcinoid syndrome. Midgut carcinoids are generally argentaffin positive, have high serotonin content, frequently multihormonal, rarely secrete 5-HTP or ACTH, and most frequently cause carcinoid syndrome when they metastasize (**Table 3**). Hindgut carcinoids (transverse colon to rectum) are argentaffin negative; rarely contain serotonin, 5-HTP, and ACTH or cause carcinoid syndrome; contain numerous peptides; and frequently metastasize to bone. Most carcinoids (70%) occur in one of four sites—bronchus, jejunum/ileum, rectum, or appendix—although they can occur in any tissue (Jensen *et al.*, 2016) (**Table 3**). Overall, the GI tract is the most common site for carcinoids (74% of cases), and the respiratory tract the second most common site (25% of cases).

The frequency of NETs varies according to whether they are symptomatic. The incidence of clinically significant panNETs is 10 cases/1 million population/year. Their relative incidence is insulinoma, gastrinoma, and nonfunctional (0.5–2.5 cases/1 million/year) $>$ VIPomas (2–8 times less common) $>$ glucagonomas (17–30 times less common) $>$ somatostatinomas. In autopsy studies, 0.5%–1.5% of all cases have panNETs, with $<$1 in 1000 symptomatic. In recent series of panNETs, the nonfunctional panNETs makeup as much as 66%–85% of cases because they are increasingly found when patients are asymptomatic detected when being investigated for nonspecific complaints (Ito *et al.*, 2013a; Metz and Jensen, 2008). Clinically significant carcinoids occur in 7–13 cases/1 million/year and malignant cases at autopsy in 21–84 cases/1 million/year.

Both panNETs and carcinoids can show malignant behavior. With panNETs, 50%–100% are malignant, except for insulinomas, in which $<$10% are malignant (Ito *et al.*, 2013a; Metz and Jensen, 2008). (**Table 2**). With carcinoid tumors the percentage that are malignant varies with different locations (**Table 3**). A number of factors are predictive of tumor aggressiveness and/or survival for NETs (**Table 4**). The presence of liver metastases is the most important prognostic factor. Primary tumor size is also an important predictor. The factors in **Table 4** need to be considered when determining the aggressiveness of treatment of NETs.

Until recently, the molecular pathogenesis of NETs was largely unknown because common oncogenes (*ras*, *fos*, etc.) and common tumor suppressor genes (retinoblastoma gene, *p53*) were usually not mutated. Recent studies provide evidence that various chromosomal gains and losses as well as alteration in the chromatin remodeling genes (ATRX/DAXX), *HER2/neu* oncogene, *MEN-1* gene, and *p16INK4a* and overexpression of growth factors (epidermal growth factor, vascular endothelial growth factor, insulin-like growth factor-1, and transforming growth factor-2) and their receptors may be important in the pathogenesis (Jensen *et al.*, 2016) (**Table 4**). An increased understanding of their molecular pathogenesis may lead to newer treatments as well as have prognostic significance.

Specific Pancreatic Neuroendocrine Tumor Syndromes

Functional panNETs almost always present with symptoms due to the specific hormone ectopically secreted. Nonfunctional panNETs present with symptoms due to the tumor per se and usually present late in the disease course with advanced metastatic disease. Treatment of functional panNETs requires strategies directed at controlling the hormone-excess state as well as at the

Table 2 Gastrointestinal NET syndromes

Syndrome	*Biologically active peptide (s) secreted*	*Incidence (new cases/10^6 population/year)*	*Tumor location*	*Malignant (%)*	*Main symptoms/signs*
Established specific functional syndrome					
panNET					
Zollinger–Ellison syndrome (ZES, gastrinoma)	Gastrin	0.5–1.5	Duodenum (70%) Pancreas (25%) Other sites (5%)	60–90	Pain (79%–100%) Diarrhea (30%–75%) Esophageal symptoms (31%–56%)
Insulinoma	Insulin	1–2	Pancreas (>99%)	<10	Hypoglycemic symptoms (100%)
VIPoma (Verner–Morrison syndrome, pancreatic cholera, WDHA)	Vasoactive intestinal peptide	0.05–0.2	Pancreas (90%; adult) Other (10%; neural, adrenal, periganglionic)	40–70	Diarrhea (90%–100%) Hypokalemic (80%–100%) Dehydration (83%)
Glucagonoma	Glucagon	0.01–0.1	Pancreas (100%)	50–80	Rash (67%–90%) Glucose intolerance (38%–87%) Weight loss (66%–96%)
Somatostatinoma	Somatostatin	Rare	Pancreas (55%) Duodenum/jejunum (44%)	>70	Diabetes mellitus (63%–90%) Cholelithiases (65%–90%) Diarrhea (35%–90%)
GRFoma	Growth hormone-releasing hormone	Unknown	Pancreas (30%) Lung (54%) Jejunum (7%) Other (13%)	>60	Acromegaly (100%)
ACTHoma	ACTH	Rare	Pancreas (4%–16% all ectopic Cushing's)	>95	Cushing's syndrome (100%)
[a]Causing carcinoid syndrome	Serotonin ?Tachykinins	Rare (43 cases)	Pancreas (<1% all carcinoids)	60–80	Same as carcinoid syndrome above
Carcinoid tumor					
Carcinoid syndrome	Serotonin, possibly tackykinins, motilin, prostaglandins	0.5–2	Midgut (75%–87%) Foregut (2%–33%) Hindgut (1%–8%) Unknown (2%–15%)	95–100	Diarrhea (32%–84%) Flushing (63%–75%) Pain (10%–34%) Asthma (4%–18%) Heart disease (11%–41%)
Possible specific functional panNET syndrome					
panNET secreting calcitonin	Calcitonin	Rare	Pancreas (rare cause of hypercalcitonemia)	>80%	Diarrhea (50%)
panNET secreting renin	Renin	Rare	Pancreas	Unknown	Hypertension
No functional syndrome					
PPoma/nonfunctional	None	1–2	Pancreas (100%)	>60	Weight loss (30%–90%) Abdominal mass (10%–30%) Pain (30%–95%)

[a]panNET, pancreatic neuroendocrine tumor.

panNETs per se because they are frequently malignant. Unfortunately, these tumors are not curable by surgery due to the extent of the disease; therefore, both treatment aspects cannot adequately be dealt with by curative resection.

Zollinger–Ellison Syndrome (Ito *et al.*, 2013a)

Zollinger–Ellison syndrome (ZES) is only briefly discussed here because it is included in a separate article in this encyclopedia.

Table 3 Carcinoid tumor location, frequency of metastases, and association with carcinoid syndrome

	Location (% of total)[a]	*Incidence of metastases*[a]	*Incidence of carcinoid syndrome*[b]
Foregut			
Esophagus	<1	67	—
Stomach	3.8	31	9.5
Duodenum	2.1	—	3.4
Pancreas	<1	76	20
Gallbladder	<1	56	5
Bronchus, lung	32.5	27	13
Midgut			
Jejunum	2.3	70	9
Ileum	17.6	70	9
Meckel's diverticulum	0.4	—	13
Appendix	7.6	35	<1
Colon	6.3	71	5
Liver	<1	29	—
Ovary	<1	32	50
Testis	<1	—	50
Cervix	<1	67	3
Hindgut			
Rectum	10	14	—

[a]From 5468 cases studied from 1973 to 1991 reported by I. M. Modlin (1997).
[b]From 4349 cases studied from 1950 to 1971 reported by J. D. Godwin (1975).

Clinical Features and Diagnosis (Ito *et al.*, 2012a; Roy *et al.*, 2000)

ZES is a clinical syndrome caused by ectopic secretion of gastrin by a panNET (gastrinoma) that results in gastric hypersecretion. The acid hypersecretion characteristically causes peptic ulcer disease (often severe and refractory), diarrhea, or esophageal reflux disease (Roy *et al.*, 2001) (**Table 2**). The diagnosis is made by demonstrating inappropriate secretion of gastrin by assessing fasting gastrin levels, gastric pH and in some cases by secretin testing.

Pathology/Pathogenesis

Almost all the presenting symptoms are due to the effects of gastric hypersecretion because they disappear when the acid secretion is treated. Sixty to ninenty percentage of gastrinomas are malignant. Most gastrinomas (60%–90%) are found in the duodenum, followed by the pancreas (10%–40%). Other intra-abdominal sites include mesentery, lymph nodes, liver and ovary. Approximately 20%–25% of ZES patients have multiple endocrine neoplasia type 1 (MEN-1) (Gibril *et al.*, 2004).

Treatment (Ito *et al.*, 2013b; Jensen and Norton, 2017)

Acid hypersecretion can be controlled medically in almost every patient. Proton pump inhibitors (PPIs) are the drugs of choice because of their long duration of action. Histamine H2 receptor antagonists are also effective but frequent (every 4–6 h) high doses are usually needed. Long-term surgical cure is possible in 30% of patients with ZES without MEN-1 but is rare (<1%) in patients with ZES with MEN-1.

Insulinomas (Falconi *et al.*, 2016; Jensen *et al.*, 2016; Mathur *et al.*, 2009; Metz and Jensen, 2008)

Insulinomas are neuroendocrine tumors of the pancreas thought to be derived from the β cells of the islets that ectopically secrete insulin, which results in hypoglycemia.

Clinical Features

The most common symptoms are due to the effects of hypoglycemia on the central nervous system (neuroglycemic symptoms), including confusion, headache, altered consciousness, and even seizure (**Table 5**). Many patients also have symptoms due to excess catecholamine release, including sweating or tremulousness. Attacks characteristically occur during fasting.

Table 4 Molecular Abnormalities and Prognostic Factors in NETs (Jensen et al., 2016)

I. Molecular abnormalities

panNETs

HER2/neu (*erbB-2*) expressed in 100% GAS

MEN1 gene LOH, 46%; mutation, 42% GAS/10% INS

$p16^{INK4a}$, 30% gene abnormality

TGF-α/EGFR, 72% IHC +

IGF-1/IGFR-1, 70% PCR +

Loss of 1p (21%), 3p (8%–47%), 3q (8%–41%), 11q (21%–62%), 6q (18%–68%), Y (45%). Gains at 17q (10%–55%), 7q (16%–68%), 4q (33%), 18 (up to 45%)

Alterations in the VHL gene [deletion, methylation]; presence of FGFR4-G388R single-nucleotide polymorphism

ATRX/DAXX mutations in 43%, MEN 1 mutations in 44%, mTor mutations (14%); uncommon in midgut GI-NETs (0%–2%)

Loss of ATRX/DAXX or positive for alternative lengthening of telomeres

Carcinoids

MEN-1 gene LOH, 36%; mutation, 10%

$p16^{INK4a}$, 33% gene abnormality

TGF-α/EGFR, 85% IHC +

IGF-1/IGFR-1, 40%–80% IHC +

VEGF, 79% IHC +

GI-NETs (carcinoids)—loss of 18q (38%–88%), >18p (33%–43%), >9p, 16q21 (21%–23%). Gains at 17q, 19p (57%), 4q (33%), 14q (20%), 5 (up to 36%)

Both carcinoids and panNETs

Alterations in common oncogenes (*ras*, *fos*, etc.) or common tumor suppressor (p53, Rb) uncommon in well-differentiated forms

II. Prognostic factors

panNETs

Ha-Ras oncogene or *p53* overexpression

Female gender

MEN-1 syndrome absent

Laboratory findings (increased chromogranin A in some studies; gastrinomas, increased gastrin level)

Chrom. changes: chr 1q, 3p, 3q, or 6q LOH [$P = 0.0004$], EGF receptor overexpression [$P = 0.034$], gains in chr 7q, 17q, 17p, 20q

Alterations in the VHL gene [deletion, methylation]; presence of FGFR4-G388R single-nucleotide polymorphism); loss of ATRX/DAXX or positive for alternative lengthening of telomeres($P < 0.001$); high nuclear survivin expression ($P < 0.01$)

PHLDA3 LOH; Altered miRNA expression (Inc miRNA-21,miRNA-196)

Carcinoid tumors

The presence of carcinoid syndrome

Laboratory results: urinary 5-HIAA level ($P < 0.01$), plasma neuropeptide K ($P < 0.05$), serum chromogranin A ($P < 0.01$)

The presence of a second malignancy

Male gender ($P < 0.001$)

Older age ($P < 0.01$)

Mode of discovery (incidental > symptomatic)

Molecular findings (TGF-α expression ($P < 0.05$), chr 16q LOH or gain chr 4p ($P < 0.05$), gain in chr 14, loss of 3p13, loss of succinate dehydrogenase expression (ileal carcinoid), upregulation of Hoxc6), molecular profiling category(mutations, epigenetic changes, copy number-Small intestinal NETs)

Both carcinoid tumors and panNETs

Performance status ($P < 0.04$)

Symptomatic presentation ($P < 0.05$)

The presence of liver metastases ($P < 0.001$)

Extent of liver metastases ($P < 0.001$)

The presence of lymph node metastases ($P < 0.001$)

Rapid rate of tumor growth

Elevated serum alkaline phosphatase levels ($P = 0.003$)

Depth of invasion ($P < 0.001$)

Primary tumor site ($P < 0.001$)

Primary tumor size ($P < 0.005$)

High serum chromogranin A level ($P < 0.01$)

Presence of one or more circulating tumor cells ($P < 0.001$)

Various histologic features

Tumor differentiation ($P < 0.001$)

High growth indices (high Ki-67 index, PCNA expression)

High mitotic counts ($P < 0.001$)

Vascular or perineural invasion

Flow cytometric features (i.e., aneuploidy)

Vessel density (low microvessel density, increased lymphatic density)

Table 4 Continued

High CD10 metalloproteinase expression (in series with all grades of NETs)
Flow cytometric features (i.e., aneuploidy)
High VEGF expression (in low-grade or well-differentiated NETs only)
Abnormal expression of p53, Rb, and SMADs
Loss of p27 expression(nuclear) ($P < 0.001$)
WHO, ENETS, AJCC/UICC stage, and grade

Abbreviations used: TGF-α, transforming growth factor-α; EGFR, receptor for epidermal growth factor; IGF/IGFR, insulin-like growth factor and its receptor; IHC, immunohistochemistry; MEN–1, multiple endocrine neoplasia type 1; PCR, polymerase chain reaction; panNET, pancreatic neuroendocrine tumor; VEGF, vascular endothelial growth factor; MEN, multiple endocrine neoplasia; NET, neuroendocrine tumor; PCNA, proliferating cell nuclear antigen; Ki-67, proliferation-associated nuclear antigen recognized by Ki-67 monoclonal antibody.

Table 5 Frequency of clinical symptoms, signs, and laboratory findings in patients with insulinoma

Clinical symptoms signs	*Frequency (%)*
Occurrence anytime during clinical course	
Neuropsychiatric (loss of consciousness, confusion, dizziness, diplopia)	92
Confusion or abnormal behavior	80
Obesity	52
Amnesia or coma	47
Convulsions (grand mal)	12
Cardiovascular symptoms, palpitations, tachycardia	17
Occurrence during first attack	
Neuroglycopenic	
Visual disturbances (diplopia, blurred vision)	59
Confusion	51
Altered consciousness	38
Weakness	32
Transient motor defects, hemiplegia	29
Dizziness	28
Fatigue	27
Inappropriate behavior	27
Speech difficulty	24
Headache	23
Seizure	23
Syncope	21
Adrenergic	
Sweating	43
Tremulousness	23
Hunger, nausea	12
Palpitations	10

Pathology/Pathophysiology

Almost all insulinomas (>99%) occur in the pancreas. Insulinomas are usually small (40% <1 cm), solitary, and distributed evenly throughout the pancreas. In adults with hyperinsulinism with islet disease, 86% have an insulinoma, 5%–15% have adenomatosis, nesioblastosis occurs in 4%, and hyperplasia occurs in 1%. Insulin is normally synthesized and stored in islet β cells. It is derived from proinsulin, which consists of a 21-amino acid α chain and a 30-amino acid β chain connected by a 33-amino acid connecting peptide (C-peptide). In normal subjects, <25% of serum insulin is proinsulin, whereas >90% of patients with an insulinoma have an elevated serum proinsulin.

Diagnosis/Differential Diagnosis

Whipple's triad, published in 1933, was long used as the diagnostic criteria for insulinoma and consists of the occurrence of hypoglycemic symptoms, hypoglycemia (blood glucose <50 mg/dL), and relief of symptoms following glucose injection.

Table 6 Causes of spontaneous hypoglycemia

Postprandial (reactive) hypoglycemia
Functional: recognizable anatomic lesion
Alimentary hyperinsulinoma, usually secondary to previous gastric surgery such as Billroth gastrectomy
Secondary to mild diabetes
Idiopathic
Due to specific hepatic enzyme deficiencies
Hereditary fructose intolerance (infants, children)
Galactosemia (infants, children)
Familial fructose and galactose intolerance (rare)
Fasting hypoglycemia
Organic hyperinsulinism: specific anatomic lesion present
Pancreatic islet disease
Insulinoma, single or multiple
Microadenomatosis with or without macroscopic adenomas
Carcinoma
Hyperplasia
Nesidioblastosis
Nonpancreatic tumors
Severe congestive heart failure
Severe renal insufficiency in noninsulin-dependent diabetes
Due to hepatic enzyme deficiencies or decreased hepatic glucose output (primarily in infants, children)
Glycogen storage diseases
Glycogen synthetase deficiencies
Other enzyme deficiencies (fructose-1,6-diphosphate deficiencies)
Endocrine hypofunction
Anterior pituitary (usually in infants, children)
Adrenocortical (Addison's disease)
Diffuse acquired liver disease
Ethanol
Severe malnutrition, sepsis
Due to exogenous agents (factitious)
Sulfonylureas, biguanides
Insulin administration
Ingestion of ackee fruits (hypoglycine)
Other drugs (aspirin, pentamidine)
Functional hypoglycemia with no persistent anatomical defect
Autoantibodies to insulin receptor
Spontaneous autoimmune anti-insulin antibody syndrome
Transient hypoglycemia on infancy

Unfortunately, this triad is not specific for insulinoma (Table 6). The diagnosis requires the establishment of hypoglycemia (<40 mg/dL) at the time of an elevated fasting plasma insulin level. The usual method of diagnosing insulinoma is to perform a fast of up to 72 h, with assessment of serum glucose, insulin, and C-peptide every 4–8 h. If at any time the patient becomes symptomatic, the test should be terminated by giving glucose after obtaining the previously mentioned serum measurements. During the first 24 h, 70%–80% of patients with insulinomas develop symptoms, and 98% of patients develop symptoms by 48 h. In nonobese normal subjects, blood insulin values should decrease to <6 μM/mL if blood decreases to ≤ 40 mg/dL and the ratio of insulin to glucose (mg/dL) is <0.3. Some investigators also require elevated serum C-peptide and proinsulin levels and/or insulin: glucose ratio >0.3 for an insulinoma diagnosis. A frequently used set of criteria to make the diagnosis of insulinoma include: blood glucose levels (≤ 2.2 mmol/L (≤ 40 mg/dL); concomitant insulin levels ≥ 6 U/L (≥ 36 pmol/L; ≥ 3 U/L by ICMA); C-peptide levels ≥ 200 pmol/L; proinsulin levels ≥ 5 pmol/L; β-hydroxybutyrate levels ≤ 2.7 mmol. There are numerous causes of hypoglycemia besides insulinoma, including pancreatic islet disease and factitious use of insulin or hypoglycemic agents (Table 6). Combinations of proinsulin levels, C-peptide levels, assessment of antibodies to insulin, and sulfonylurea levels may be required to make the correct diagnosis.

Treatment

Only 5%–15% of insulinomas are malignant; therefore, after tumor localization studies, surgery should be performed. Between 75% and 95% of patients are cured by surgery. Prior to surgery, hypoglycemia is usually controlled by frequent, small meals and the use of diazoxide (150–800 mg/day). Diazoxide controls symptoms in 50%–60% of patients. Other drugs used to control the

Table 7 Clinical characteristics of patients with VIPoma syndrome

	Frequency (%)
Symptoms/signs	
Secretory diarrhea	89–100
Dehydration	44–100
Weight loss	36–100
Abdominal cramps, colic	10–63
Flushing	14–28
Laboratory findings	
Hypokalemia	67–100
Hypochlorhydria	34–72
Hypercalcemia	41–50
Hyperglycemia	18–50

hypoglycemia include verapamil and diphenylhydantoin. Long-acting somatostatin analogues such as octreotide are effective in 40%–50% of patients. However, in some patient's octreotide may worsen the hypoglycemia. The preferred tumor localization studies include computed tomography (CT) scan to exclude liver metastases and endoscopic ultrasound. Somatostatin receptor scintigraphy (SRS) using ^{111}In-penetreotate, which is the preferred imaging modality in all other NETs, is frequently negative (40%–70%) in patients with insulinomas due to low numbers of somatostatin receptors. However, ^{68}Ga DOTATE PET/CT is increasingly being used more because it is more sensitive. Endoscopic ultrasound is particularly sensitive for locating insulinomas because they are almost all pancreatic in localization. A newer method which shows promising high sensitivity, however it is not yet approved or generally available, involves the use of radiolabeled GLP1 receptor ligands, a receptor that is frequently over-expressed by insulinomas.

VIPomas (Ito *et al.*, 2013a; Metz and Jensen, 2008)

VIPomas are neuroendocrine tumors that occur primarily in the pancreas, secrete excessive amounts of vasoactive intestinal peptide (VIP), and cause a distinct syndrome characterized by large-volume diarrhea, hypokalemia, and dehydration (**Tables 2** and **7**). This syndrome is also called the Verner–Morrison syndrome, pancreatic cholera, or WDHA syndrome (watery diarrhea, hypokalemia, and achlorhydria; **Table 2**).

Clinical Features

The mean age of occurrence is 49 years; however, the syndrome can also occur in children. The principal symptoms are large-volume diarrhea leading to dehydration, acidosis, and hypokalemia. Other symptoms/signs due to the actions of VIP include flushing, hypochlorhydria, hyperglycemia, and hypercalcemia. Steatorrhea is uncommon (16%). The diarrhea is characteristically secretory in nature (persists during fasting).

Pathology/Pathophysiology

VIP is a 28-amino acid neuropeptide normally present in the GI tract and central nervous system. It causes stimulation of small intestinal chloride secretion, has effects on smooth muscle contractility, inhibits acid secretion, and has vasodilatory effects, which explain many of the symptoms of the VIPoma syndrome. In adults, 80%–90% of VIPomas are pancreatic, whereas in children the syndrome is usually caused by ganglioneuromas/ganglioblastomas. VIPomas are usually solitary, with 50%–75% in the pancreatic tail, and 40%–70% of patients have liver metastases at diagnosis.

Diagnosis/Differential Diagnosis

The diagnosis requires the demonstration of an elevated plasma VIP level and the presence of large-volume diarrhea. Almost all patients have a stool volume >1 L/day (most have a volume >3 L/day), and it is proposed that the diagnosis be excluded if the volume is not >700 mL/day. A number of other causes of large-volume diarrhea can be excluded by fasting the patient. Other diseases that can cause fasting large volume diarrhea include Zollinger–Ellison syndrome, chronic laxative abuse, systemic mastocytosis, diabetic diarrhea, AIDS, carcinoid syndrome, and, rarely, medullary thyroid cancer.

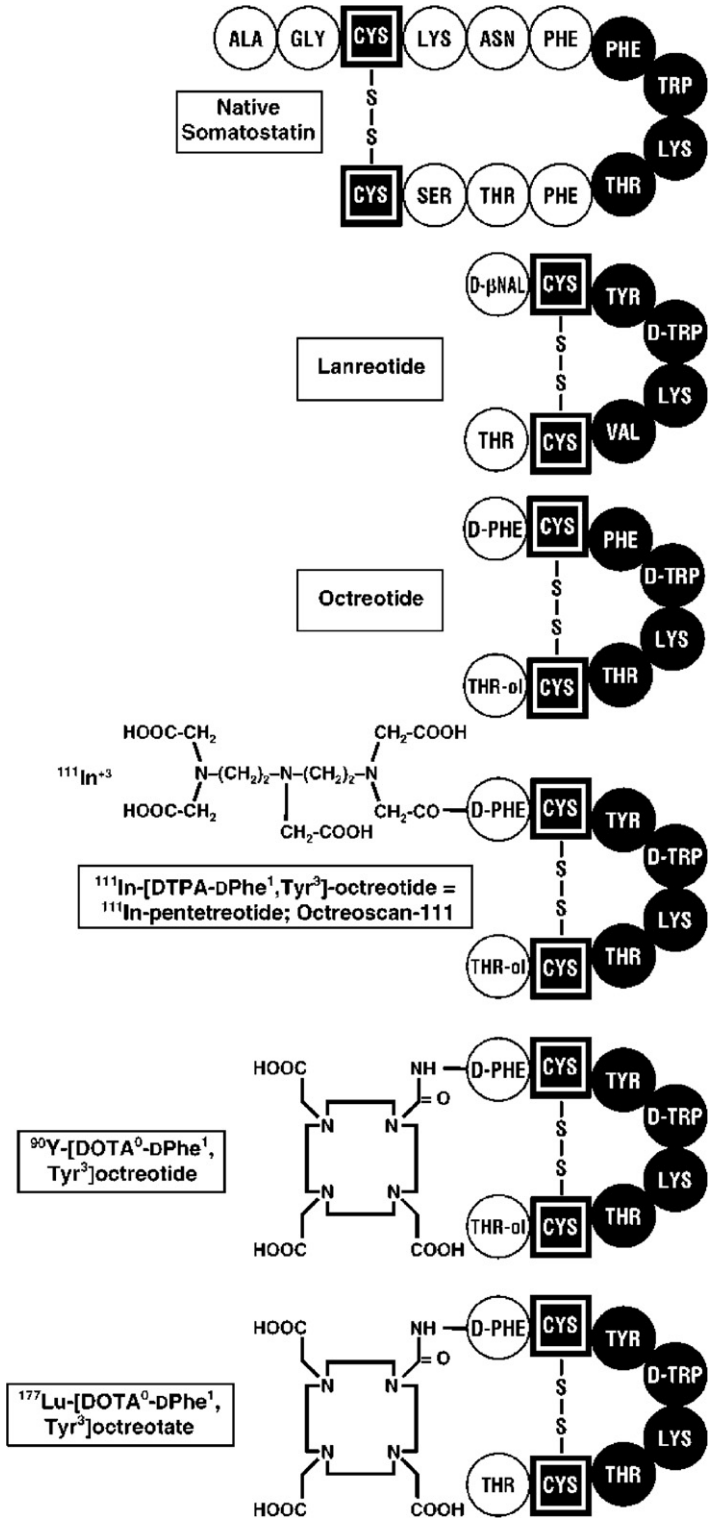

Fig. 1 Structure of somatostatin and synthetic analogues used for diagnostic (tumor localization) or therapeutic indications in patients with neuroendocrine tumors. The indium (^{111}In), yttrium (^{90}Y), or lutetium (^{177}Lu) compounds are used for therapeutic purposes. DOTA-1,4,7,10-tetra-azacylododecane-*N*,*N*′,*N*″, *N*‴-tetra-acetic acid.

Table 8 Frequency of clinical symptoms and laboratory findings in patients with glucagonoma

	Frequency (%)
Clinical symptoms	
Dermatitis	64–90
Diabetes/glucose intolerance	38–96
Weight loss	56–96
Glossitis/stomatitis/cheilitis	29–40
Diarrhea	14–15
Abdominal pain	12
Thromboembolic disease	12–35
Venous thrombosis	24
Pulmonary emboli	11
Psychiatric disturbance	0–17
Laboratory abnormality	
Anemia	33–90
Hypoaminoacidemia	26–100
Hypocholesterolemia	80

Treatment

The most important first step in treatment is to control the diarrhea and correct the dehydration, hypokalemia, and acidosis. Patients may require >5 L/day of fluid and >350 mm/day of potassium when the diarrhea is not controlled. Octreotide will control the diarrhea in 85%–90% of VIPoma patients. In nonresponders or patients who have become refractory, combinations of octreotide and glucocorticoids may be helpful. Other drugs that may help in individual patients include clonidine, indomethacin, lithium, phenothiazine, loperamide, lidamidine, propanolol, and metoclopramide.

In 40%–70% of adult patients with VIPoma, diffuse metastatic disease in the liver is present initially; therefore, surgical cure is not possible. For these patients, long-acting somatostatin analogues such as octreotide or lanreotide (**Fig. 1**) are the drugs of choice for treatment. If these fail or tumor growth continues, treatment with chemoembolization, hepatic embolization, or chemotherapy or radiolabeled somatostatin analogues (**Fig. 1**) may be helpful. If the majority of the metastatic disease in the liver can be safely resected, cytoreductive surgery may be of value in helping to control the symptoms of the hormone-excess state.

VIPomas similar to other NETs, overexpress somatostatin receptors in 80%–100% of well differentiated forms (comprise 80%–95% of all NETs). In addition to this over-expression being used to image these tumors by somatostatin receptor imaging (SRI), it is also being used to direct cytotoxic agents, particularly ^{177}Lu labeled somatostatin analogues (PRRT, peptide radioreceptor therapy). This approach is increasingly being used to treat NET patients with advanced, progressive disease and it has been reported to be particularly helpful in some functional NET syndromes, including VIPomas and carcinoid syndrome.

Glucagonomas (Jensen *et al.*, 2016; John and Schwartz, 2016; Metz and Jensen, 2008)

Glucagonomas are neuroendocrine tumors of the pancreas that ectopically secrete excessive amounts of glucagon and cause a syndrome characterized by a dermatitis (migratory necrolytic erythema), glucose intolerance or diabetes, and weight loss (**Table 8**).

Clinical Features

Glucagonomas usually occur in middle-aged and elderly populations. Migratory necrolytic erythema usually starts as an erythematous area typically at perifacial or intertriginous areas, such as the groin, buttocks, or thighs. It subsequently becomes raised and bullae form and break, resulting in eroded areas. The skin lesions may wax and wane and frequently precede the diagnosis of glucagonoma (mean, 6–8 years). Hypoaminoacidemia is a characteristic feature of glucagonomas, with plasma amino acid levels decreasing to $<25\%$ of normal, especially glycogenic amino acids such as alanine and glycine. Weight loss is another prominent feature of this syndrome and is not seen early in other panNETs unless malabsorption is present, suggesting that it is an intrinsic feature of this syndrome. Experimental studies support the conclusion that a novel anorectic substance, independent of glucagon, is released by these tumors and is responsible for the weight loss. Other prominent symptoms/clinical findings include glossitis or stomatitis, glucose intolerance or diabetes that may precede the diagnosis by years, predisposition to thromboembolism and anemia (**Table 8**).

Pathology/Pathophysiology

Glucagonomas are characteristically diagnosed late in their course and are usually large (average, 5–10 cm), and 50%–80% have evidence of metastatic spread at diagnosis, usually to the liver (43%–80%). Glucagonomas are usually single tumors and 50%–80% occur in the pancreatic tail.

Glucagon is a naturally occurring, 29-amino acid peptide characteristically released by the pancreatic A cells. Most of the findings of the syndrome are compatible with the known actions of glucagon stimulating glucogenolysis and gluconeogenesis and affecting gut secretion and motility. The exact pathogenesis of the migratory necrolytic erythema remains unclear and has been attributed by some to hypoaminoacidemia or to nutritional deficiencies such as zinc.

Diagnosis/Differential Diagnosis

The diagnosis is established by demonstrating an elevated plasma glucagon level (normal level is usually <150 ng/L). Plasma glucagon levels usually exceed 1000 ng/L in glucagonoma patients (90%), and in patients with symptoms/laboratory findings of glucagonoma (**Table 8**), a level >1000 ng/L is diagnostic. Other conditions can also cause elevated plasma glucagon levels, including pancreatitis, hepatic failure disease, renal failure, Cushing's syndrome, prolonged fast, or familial hyperglucagonemia. Except for cirrhosis, these disorders usually do not cause increases in plasma glucagon levels >500 ng/L. Recently new diseases are described that can cause hyperglucagonemia and can mimic glucagonomas. Mahvash disease is due to an inactivating mutation (homozygous P86S mutation) of the human glucagon receptor. It is associated with the development of α-cell hyperplasia, hyperglucagonemia, and the development of nonfunctioning pNETs. More recently, additional patients with other inactivating mutations of the human glucagon receptor have been described with similar findings, which has lead to the suggestion that a hepato-pancreatic feedback regulation of the cells, possibly involving amino acids, may exist in humans. A second disease called glucagon cell adenomatosis can also mimic glucagonoma syndrome and is characterized by the presence of hyperplastic islets staining positive for glucagon instead of a single glucagonoma.

Treatment

Diffuse hepatic metastases are usually present at diagnosis (in up to 80% of patients), so curative surgical resection is usually not possible. Surgical debulking or other antitumor treatments may be of palliative benefit. The drugs of choice are the long-acting somatostatin analogues (octreotide and lanreotide) (**Fig. 1**), which improve the rash in 75%–80% of patients and may improve the weight loss, pain, and diarrhea but usually do not improve the diabetes/glucose intolerance.

Somatostatinoma Syndrome (Jensen *et al.*, 2016; Metz and Jensen, 2008)

The somatostatinoma syndrome is caused by a neuroendocrine tumor of the GI tract that ectopically secretes excess amounts of somatostatin (**Fig. 1**), which frequently causes diabetes mellitus, gallbladder disease, diarrhea, and steatorrhea (**Table 9**). There is no general agreement on what constitutes a somatostatinoma, and there is no distinction in the literature regarding the presence of a somatostatinoma and/or the somatostatinoma syndrome. In the literature, the term somatostatinoma is generally used to mean a GI neuroendocrine tumor possessing somatostatin-like immunoreactivity, and it may (11%–45%) or may not (55%–89%) be associated with clinical symptoms due to ectopic release of somatostatin (**Table 9**). Because of this confusion, the term

Table 9 Clinical and laboratory findings in patients with somatostatinomas with or without somatostatinoma syndrome

	Somatostatinoma[a]		*Overall frequency in somatostatinoma syndrome (%)*[a]
	Pancreatic (%)	*Intestinal (%)*	
Clinical finding			
Diabetes mellitus	95	21	95
Gallbladder disease	94	43	68
Diarrhea	66–97	11–36	37
Weight loss	32–90	20–44	68
Laboratory finding			
Steatorrhea	83	12	47
Hypochlorhydria	86	17	26

[a]Somatostatinoma is the occurrence of a pancreatic neuroendocrine tumor containing somatostatin by immunocytochemistry that can occur with (11%) or without (89%) the somatostatinoma syndrome, which is due to ectopically released somatostatin.

Table 10 Clinical features in patients with carcinoid syndrome

	At presentation	*During course of disease*
Symptoms/signs		
Diarrhea (%)	32–73	68–84
Flushing (%)	23–65	63–74
Pain (%)	10	34
Asthma/wheezing (%)	4–8	3–18
Pellagra (%)	2	5
None (%)	12	22
Carcinoid heart disease (%)	11	14–41
Demographics		
Male (%)	46–59	46–61
Mean age, years (range)	57 (25–79)	53 (9–91)
Tumor location		
Foregut (%)	5–9	2–33
Midgut (%)	78–87	60–87
Hindgut (%)	1–5	1–8
Unknown (%)	2–11	2–15

somatostatinoma syndrome is used here to indicate a panNET-releasing somatostatinoma, which causes clinical symptoms, and the term somatostatinoma is used to indicate a panNET containing somatostatin immunoreactivity.

Clinical Features

The mean age of onset is 51–53 years. In one large review ($N = 173$), only 11% of all somatostatinomas in the literature were associated with the somatostatinoma syndrome. The principal clinical features of the somatostatinoma syndrome are gallbladder disease, diabetes mellitus, diarrhea, weight loss, and steatorrhea. The frequency of these symptoms depends on the location of the somatostatinoma (**Table 9**). Each symptom characteristic of the syndrome is reported more frequently with pancreatic than intestinal somatostatinomas. Recently the existence of whether a clinical somatostatinoma syndrome actually occurs has been called into question. This occurred because in one review of a large number of patients with patients with duodenal/pancreatic NETs, in the proportion of which showed predominant somatostatin expression, none had the somatostatinoma syndrome. However, in a number of other studies, a proportion of the patients with somatostatin positive pancreatic or duodenal NETs had number of the proposed features of the somatostatinoma syndrome, although the full constellation of symptoms was uncommon.

Pathology/Pathophysiology

Somatostatin is a naturally occurring tetradecapeptide (**Fig. 1**) found widely in the central nervous system and GI tract, where it functions as a neurotransmitter or has paracrine or autocrine actions. In general, it is a potent inhibitor of processes, including the release of numerous hormones, gastric and intestinal pancreatic secretion, and absorption. The hypochlorhydria, diabetes, steatorrhea, and gallbladder diseases that occur in somatostatinoma syndrome are caused by the known inhibitor effects of somatostatin.

Somatostatinomas occur in the pancreas in 56%–74% of cases and are mainly found in the pancreatic head. In most of the remaining cases, they are found in the intestine, with 90% in the duodenum, particularly in the periampullary area. They are usually solitary, large tumors (mean, 3.6–4.9 cm), and 53%–84% have metastatic spread at diagnosis (usually to the liver). They are frequently associated with neurofibromatosis I (von Recklinghausen's disease).

Diagnosis/Differential Diagnosis

In most cases in the literature, somatostatinomas have been found incidentally either at the time of cholecystectomy or during endoscopy. The presence of psammoma bodies in a duodenal neuroendocrine tumor should raise this possibility. Duodenal somatostatinomas are associated with von Recklinghausen's disease. Most of these patients do not develop the somatostatinoma syndrome and have normal plasma somatostatin levels. The diagnosis of the somatostatinoma syndrome requires the demonstration of an elevated plasma somatostatin level.

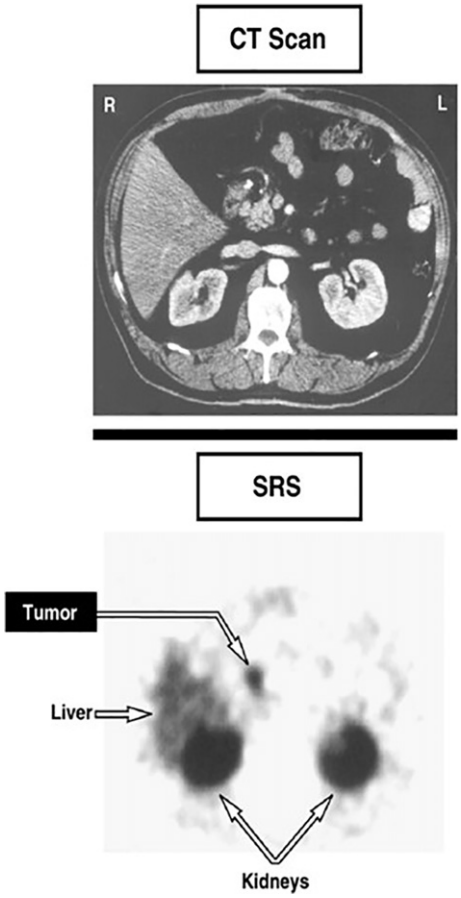

Fig. 2 Computed tomography (CT) scan (*top*) and somatostatin receptor scintigraphy (SRS) (*bottom*) of a patient with a pancreatic neuroendocrine tumor (gastrinoma). This patient previously had a duodenal tumor removed. The CT scan was negative, whereas the SRS showed a tumor medial and anterior to the right kidney. At surgery, a panNET was found in a metastatic lymph node in the periduodenal area. This case illustrates the greater sensitivity of SRS compared to conventional imaging studies.

Treatment

Symptoms of the somatostatinoma syndrome are improved by administration of long-acting synthetic somatostatin analogues such as octreotide/lanreotide (**Fig. 1**). Pancreatic tumors (70%–92%) and intestinal tumors (30%–69%) are associated with liver metastases at presentation and antitumor treatment is frequently needed.

GRFomas and Other Rarer Functional panNET Syndromes (Ito *et al.*, 2013a)

GRFomas are neuroendocrine tumors that ectopically secrete growth hormone-releasing factor (GRF). GRF is a 44-amino acid peptide that stimulates growth hormone release. GRFomas occur in lung (47%–54%), panNETs (29%–30%), small intestinal carcinoids (8%–10%), and other sites (12%). The symptoms are those of acromegaly and the mean age of patients is 38 years. The acromegaly is indistinguishable from classical acromegaly due to a pituitary adenoma. Pancreatic GRFomas are usually large (mean, >6 cm) and liver metastases are present in 39% of cases. They should be suspected in a patient with acromegaly with an abdominal tumor, MEN-1, or hyperprolactinemia, which occurs in 70% of GRFomas. The diagnosis is established by performing plasma assays for GRF and growth hormone. Surgery is the treatment of choice if possible. Symptoms can be controlled in $>75\%$ of patients by long-acting somatostatin analogues, such as octreotide or lanreotide (**Fig. 1**).

Cushing's syndrome due to a panNET occurs in 4%–16% of all patients with ectopic Cushing's syndrome. It occurs in 5% of patients with sporadic gastrinomas and is associated with hepatic metastases and a poor prognosis. Paraneoplastic hypercalcemia due to a panNET releasing PTH-related peptide is rare. These tumors are usually large and malignant. panNETs secreting calcitonin may cause a specific syndrome associated with diarrhea (**Table 2**). panNETs also cause the carcinoid syndrome (**Table 2**). These are characteristically large and malignant (68%–88%) and may cause an atypical carcinoid syndrome because they lack DOPA decarboxylase. A renin-producing panNET was described in a patient presenting with hypertension (**Table 2**). Ghrelin is a 28-amino acid peptide with a number of metabolic functions. Although expression has been demonstrated in most panNETs, in a one

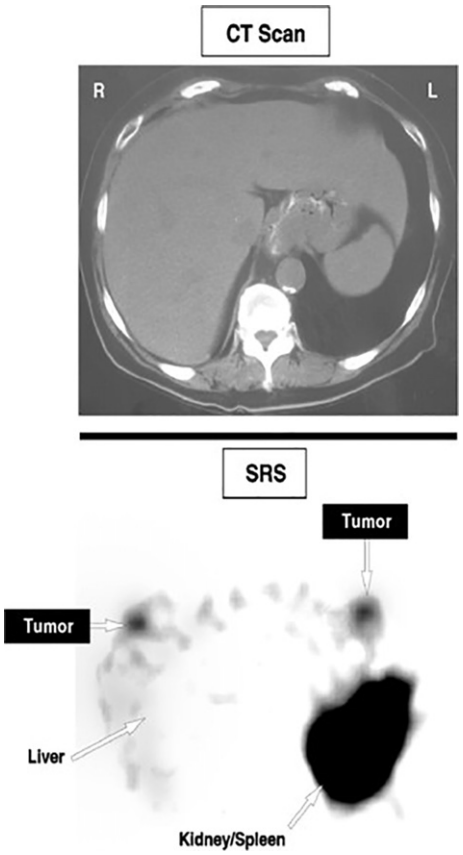

Fig. 3 Computed tomography (CT) scan and somatostatin receptor scintigraphy (SRS) of a patient with a metastatic panNET to the liver. The CT scan showed no liver lesions, whereas the SRS showed two hepatic metastases (one in the *right* lobe and one in the *left* lobe). This case illustrates the increased sensitivity of SRS compared to conventional imaging studies for localizing hepatic metastases.

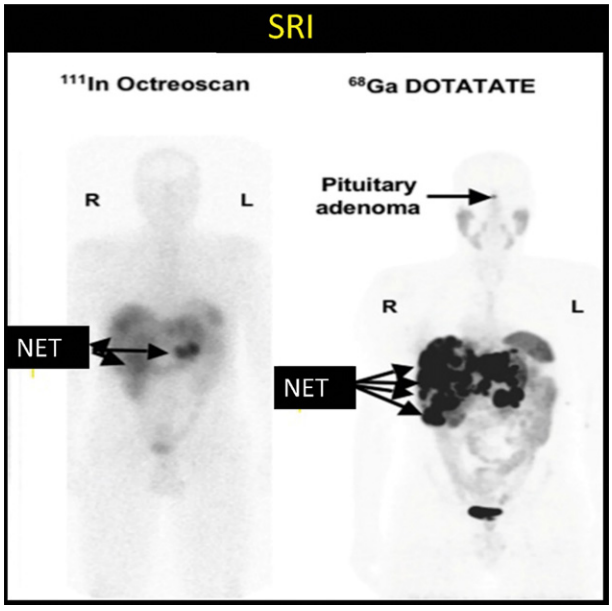

Fig. 4 Increased sensitivity of SRI with ^{68}Ga-DOTATATE PET/CT over SRS with [^{111}In-DTPA-DPhe^{1}] octreotide (Octreoscan) in a patient MEN1 with a pNET with liver metastases.

study only 1 of 24 patients (4%) with a panNET had an elevated plasma ghrelin level. This patient was asymptomatic, suggesting that no specific syndrome is associated with ectopic release of ghrelin by a panNET. A number of other very rare panNET syndromes involving a few cases (less than five) have been described; these include a panNET secreting erythropoietin, resulting in polycythemia; panNETs secreting IGF-II, causing hypoglycemia; a panNET secreting cholecystokinin (CCKoma) which can mimic ZES clinically with patients presenting with severe peptic ulcer disease, diarrhea, weight loss and gallstones, but with a normal fasting gastrin panNETs secreting enteroglucagon, causing small intestinal hypertrophy, colonic/SI stasis, and malabsorption.

Nonfunctional panNETs (Falconi *et al.*, 2012, 2016; Imamura *et al.*, 1992; Jensen *et al.*, 2012)

Nonfunctional PANNETs are neuroendocrine tumors of the pancreas that secrete no products or the secreted products do not cause a distinct functional syndrome. The symptoms from these tumors are therefore due entirely to the tumor per se (i.e., pain, jaundice, weight loss, etc.). Nonfunctional panNETs frequently secrete chromogranin A (CgA) (80%–100%), CgB (90%–100%), pancreatic polypeptide (PP) (58%), and the α-subunit of hCG (40%), and many secrete other hormones such as neurotensin, ghrelin or calcitonin. These tumors characteristically present late in the disease course. In a number of recent series an increasing proportion of NF-panNETs are asymptomatic (up to 50%) and are found at screening for various nonspecific symptoms. Characteristically, NF-panNETs are generally large (72% > 5 cm) and invasive, and hepatic metastases are usually present (64%–92%), although in the more recent series the asymptomatic ones are frequently small (<2 cm)and not metastatic. The most common symptoms are abdominal pain (30%–50%), jaundice (20%–35%), weight loss, fatigue, and bleeding. In 10%–15% of cases, they are found incidentally. The diagnosis is established by histology with appropriate neuroendocrine tumor immunohistochemistry and by assessing plasma hormone levels/clinical symptoms. Plasma PP is increased in 22%–71% of patients, CgA levels are increased in 80%–100%, and in patients with a pancreatic mass without a functional syndrome, this finding suggests that a nonfunctional panNET is present. In the older series where these patients generally presented late in the disease course curative surgical resection is rarely possible, and treatment needs to be directed against the malignant panNET. Currently the treatment of patients with asymptomatic NF-panNETs is controversial with some advocating a wait and watch with surveillance, while other propose resection.

Functional Syndromes Due to Carcinoid Tumors

Carcinoid tumors can cause a specific functional syndrome, the carcinoid syndrome, or occasionally can release biologically active peptides that cause the specific panNET syndromes discussed previously. Because carcinoids are also malignant (**Table 3**), specific treatments need to be directed at both the carcinoid tumor and the functional syndrome it produces.

Carcinoid Syndrome (Ito *et al.*, 2018)

The carcinoid syndrome is caused by a neuroendocrine tumor, usually present in the gastrointestinal tract in the midgut region, ectopically secreting bioactive amines/peptides, which results in a clinical syndrome characterized by diarrhea, flushing, asthma/wheezing, and, occasionally, heart disease (Ito *et al.*, 2018) (**Table 10**).

Clinical Features (Ito *et al.*, 2018)

The mean age at presentation is 57 years, but it occurs over a wide age range (9–91 years) (**Table 10**). The principal symptoms are diarrhea and flushing, which occur in up to 73% of cases initially and 84% during the course of the disease. The flush is usually of sudden onset, associated with a deep red to violaceous erythema of the upper body, and often associated with a feeling of warmth and occasionally with lacrimation, pruritis, or diarrhea. It may be precipitated by food, exercise, alcohol, or drugs, particularly serotonin reuptake inhibitors. Carcinoid syndrome is usually caused by metastatic midgut tumors. Flushing with midgut tumors and bronchial or gastric carcinoids may differ in duration, associated symptoms (salivation and lacrimation), and skin color. Diarrhea usually occurs with flushing (85% of cases) and is usually watery and of small volume (60% < 1 L/day). Steatorrhea is present in 67%, and in 50% of patients it is >15 g/day of fecal fat. Cardiac manifestations occur in 11% initially and 14%–41% during the disease course (**Table 10**), and they are due to endocardial fibrosis, primarily on the right side (tricuspid > pulmonary), but they can also occur on the left side. Fibrosis results in valve constriction and up to 80% of patients develop heart failure. Pellagra-like symptoms (2%–25%) and symptoms due to increased fibrotic tissue (i.e., retroperitoneal fibrosis, Peyronie's disease, and intraabdominal fibrosis) are unusual features of this disease.

A life-threatening complication of the carcinoid syndrome is the development of a carcinoid crisis (Ito *et al.*, 2018). This is most frequently seen in patients with high 5-hydroxyindoleacetic acid (5-HIAA) levels and may be provoked by anesthesia, endoscopy, stress, surgery, a radiological procedure, or a biopsy. Patients develop intense flushing, diarrhea, abdominal pain, hypotension, and cardiac abnormalities. If not adequately treated, it can be fatal.

Pathology/Pathobiology (Ito *et al.*, 2018; Klimstra, 2016)

Carcinoid symptoms occurred in 8% of 8876 patients with carcinoid tumors. Carcinoid syndrome occurs only when tumor-secreted products reach the systemic circulation in sufficient concentrations. In 91% of cases, this occurs due to liver metastases, and in the remainder it occurs due to retroperitoneal invasion by gut or pancreatic tumors or due to lung, testis or ovary carcinoids with direct access to the systemic circulation. Midgut carcinoids account for 60%–67% of cases of the carcinoid syndrome, foregut tumors account for 2%–33%, hindgut accounts for 1%–8%, and an unknown primary accounts for 2%–15%.

One of the main secretory products of carcinoid tumors is serotonin (5-HT), and overproduction occurs in 90%–100% of patients with carcinoid syndrome. Serotonin is thought to primarily mediate diarrhea by its effects on gut motility and intestinal secretion. However, prostaglandins and tachykinins (substance P, K; neuropeptide K) may also be important in causing diarrhea in some patients. Patients with the carcinoid syndrome have accelerated colonic motility with a shortened transit time as well as a possible secretory/absorptive alteration that is compatible with the known actions of serotonin in the gut mediated primarily through 5-HT_3 and, to a lesser degree, 5-HT_4 receptors. Serotonin receptor antagonists (especially 5-HT_3 antagonists) relieve the diarrhea in many, but not all, patients. A tryptophan 5-hydroxylase inhibitor, telotristat (LX-10310), which inhibits serotonin synthesis in peripheral tissues, caused a decrease in bowel movement frequency in 40%–50% of patients with the carcinoid syndrome (Ito *et al.*, 2018; Kulke *et al.*, 2014).

Flushing is not relieved by serotonin antagonists nor by the tryptophan 5-hydroxylase inhibitor, telotristat (LX-10310), which inhibits serotonin synthesis in peripheral tissues. In patients with gastric carcinoids flushing can be caused by histamine hypersecretion. Tachykinins are released during flushing and may be important in its mediation. Both histamine and serotonin may be responsible for the wheezing/asthma as well as the fibrotic reactions characteristic of this disease. The heart disease is likely due to the actions of serotonin because it is similar to that seen with appetite-suppressant drugs (e.g., fenfluramine) that have high affinity for serotonin receptors.

Patients may develop a typical or atypical carcinoid syndrome. The typical syndrome is due to a midgut tumor over-synthesizing serotonin from tryptophan. The atypical syndrome occurs when there is a deficiency in the enzyme required to convert 5-HTP to 5-HT and there is overproduction of 5-HTP. The atypical syndrome is more likely to occur with foregut carcinoids.

Diagnosis/Differential Diagnosis (Ito *et al.*, 2018)

The diagnosis of carcinoid syndrome requires measurement of urinary or plasma serotonin or its metabolites. The measurement of urinary 5-HIAA is most frequently performed. Generally, the urinary 24-h 5-HIAA excretion rate is assessed when this diagnosis is suspected; even though studies report an overnight urinary collection is just as accurate. Assessment of plasma and platelet serotonin levels and plasma 5-HIAA, if available, may provide additional information and/or substitute for the 24 h urinary 5-HIAA study. Platelet serotonin levels are more sensitive than urinary 5-HIAA but are not generally available. A single plasma 5-HIAA determination is recently reported to have similar sensitivity/specificity to that with the 24-h urinary 5-HIAA assessment and because it avoids the problem of incomplete or improper collections as seen with urinary collections, it may be generally useful. It however, could be affected by renal disease. False positives may occur if a patient eats serotonin-rich foods (bananas, walnuts, pecans, and pineapple) or takes certain medications (L-Dopa, cough syrups with guaniforesin, and salicylates). If an atypical carcinoid syndrome is suspected, urinary 5-HIAA may be only slightly elevated, and other metabolites of tryptophan such as 5-HTP should be considered. Serum CgA levels are elevated in $>50\%$ of patients with carcinoid tumors, and the level correlates with the tumor extent. However, serum CgA levels lack specificity for carcinoids because they can occur with other NETs, including panNETs. Furthermore, potent acid antisecretory drugs such as proton pump inhibitors (PPIs) (omeprazole and related drugs) can elevate serum CgA levels; the elevation occurs rapidly (3–5 days) with continued use, and the elevated levels overlap with the levels seen in many patients with NETs. Plasma neuron-specific enolase levels are also used as a marker of GI-NETs (carcinoids) but are less sensitive than CgA, being increased in only $<50\%$ of patients. Newer markers have been proposed including pancreastatin (a chromogranin A breakdown product), and activin A. Activin A is not affected by proton pump inhibitors; however, its sensitivity and specificity are not established. Plasma activin elevations are reported to correlate with the presence of cardiac disease with a sensitivity of 87% and specificity of 57%. Plasma levels of N-terminal pro Brain Natriuretic Peptide also correlate with carcinoid heart disease severity.

Flushing can be caused by other conditions, including systemic mastocytosis, reactions to alcohol or glutamate, effects of drugs, and menopause. These need to be excluded.

Treatment (Ito *et al.*, 2018)

Treatment consists of avoiding conditions that precipitate attacks and treatment of wheezing with bronchodilators, treatment of heart failure with diuretics, and treatment of diarrhea with antidiarrheal agents, such as loperamide or diphenoxylate. If symptoms persist, somatostatin analogues (octreotide and lanreotide) (**Fig. 1**) or serotonin receptor antagonists are the drugs of choice. Somatostatin analogues control symptoms in 80% of patients with flushing or diarrhea, and 70% show a $>50\%$ decrease in urinary 5-HIAA. Approximately 40% of patients show some resistance after 4–6 months and doses may have to be increased.

Sustained-release preparations, such as octreotide-LAR (long-acting release) and lanreotide-PR (prolonged release), are widely used because they can be given less frequently. Octreotide-LAR is usually given monthly and lanreotide-PR every 10–14 days as opposed to the usual preparation of octreotide/lanreotide that is given subcutaneously every 6–12 h. Somatostatin analogues can be given to both treat and prevent carcinoid crises. Prior to a possible precipitating event, such as surgery, anesthesia, or stress, it is recommended that octreotide be administered.

Side effects from the somatostatin analogues occur in 40%–60% of patients with subcutaneous analogues. Pain at the injection site and GI side effects (discomfort, nausea, diarrhea, and cramping) are the most common. They are usually short-lived and do not interrupt treatment. Long-term complications include an increased incidence of gallstones/biliary sludge (52%), steatorrhea, and worsening glucose tolerance. Interferon-α may also control symptoms in some patients.

A recent phase 3 prospective, double-blind study demonstrates that the peripheral tryptophan hydroxylase inhibitor, telotristat, can be effective at controlling the diarrhea in many of these patients.

Carcinoid heart disease is associated with a decreased mean survival and therefore, it should be sought for and carefully assessed in all patients with carcinoid syndrome. The use of transthoracic echocardiography is important in establishing the diagnosis of carcinoid heart disease and determining the extent and type of cardiac abnormalities. Treatment with diuretics and somatostatin analogues can reduce the negative hemodynamic effects and secondary heart failure. It currently is not established whether long-term treatment with these drugs or with the tryptophan hydroxylase inhibitor, telotristat, when it becomes available, will stop and/or reverse the progression of carcinoid heart disease.

Surgery should be performed if possible; however, almost all patients with carcinoid syndrome have metastatic disease in the liver and curative resection is not possible. Treatment directed against the tumor may be needed.

NET Tumor Localization (Dromain *et al.*, 2016; ElGuindy *et al.*, 2017; Ito and Jensen, 2016, 2017) (Figs. 1–4)

Tumor localization is needed for all management phases of both panNETs and carcinoid tumors (Falconi *et al.*, 2016; Ito and Jensen, 2016, 2017). Both the localization of the primary tumor and the determination of the location and extent of metastatic disease are required to appropriately manage these patients. Conventional imaging studies (CT, magnetic resonance imaging (MRI), ultrasound, and angiography) and somatostatin receptor imaging (SRI) are widely used (**Figs. 1–4**) (Ito and Jensen, 2017; Sundin *et al.*, 2015, 2017). For SRI, originally somatostatin receptor scintigraphy (SRS) was used with ^{111}In-penetreotide and SPECT/CT detection, but it is being rapidly replaced by ^{68}Ga DOTATATE PET/CT which has a greater resolution resulting in higher sensitivity and high specificity (Ito and Jensen, 2017; Sundin *et al.*, 2017) (**Fig. 4**). For panNETs, endoscopic ultrasound is also widely used. Bronchial carcinoids are usually detected by chest X-ray and assessed by CT. Rectal, duodenal, colonic, and gastric carcinoids are usually detected by GI endoscopy.

Both panNETs and carcinoids frequently (90%–100%) overexpress somatostatin receptors, which have a high affinity for radiolabeled somatostatin analogues (**Figs. 1–4**) that can be used to localize them. Because of its greater sensitivity compared to that of conventional imaging studies and its ability to localize a number of tumors throughout the body simultaneously, SRI is the imaging modality of choice for localizing all primary and metastatic NET tumors except insulinomas (Ito and Jensen, 2017; Sundin *et al.*, 2017) (**Figs. 2** and **3**). Insulinomas are usually small and have a low density of somatostatin receptors, with the result that SRI is positive in only 12%–50% of patients with insulinomas. GLP1 based radionuclide imaging has recently be shown to be both sensitive and specific in identifying insulinomas. In contrast, SRI is positive in 73%–100% of patients with carcinoids and 60%–100% of patients with panNETs other than insulinomas. SRI is positive in almost all patients with well-differentiated NETs, but in a lower percentage with poorly differentiated NETs (G3-NEC).

Figs. 2 and **3** show two examples of the ability of SRS to image a primary panNET and metastatic disease in the liver when conventional imaging studies were negative. For panNETs localized in the pancreas, endoscopic ultrasound is highly sensitive, localizing 73%–100% of insulinomas, which occur almost exclusively within the pancreas. SRS occasionally gives false positives (12% in one study) because normal and abnormal cells can have increased somatostatin receptors such as granulomas, thyroid disease, and activated lymphocytes (abscess and infection). Furthermore, SRS does not provide information on tumor size or the exact location of metastases, and a CT scan or MRI are frequently used to provide this information. **Fig. 4** shows the greater sensitivity of SRI with ^{68}Ga DOTATATE PET/CT over ^{111}In-penetreotide and SPECT/CT detection.

Treatment of Advanced Diffuse Metastatic Disease in Patients With Malignant NETs (Ito *et al.*, 2012b, 2016; Kunz *et al.*, 2013; Pavel *et al.*, 2016)

Of the numerous prognostic factors identified for NETs (**Table 4**), the presence and the extent of the hepatic metastases are the most important in almost every study. For patients with gastrinomas, 5-year survival is 98%–100% without liver metastases, 78% with limited metastases in one lobe, and 16% with diffuse metastases. For carcinoid tumors without liver metastases, 5-year survival is 80%–90%, and with diffuse metastases it is 50%. A number of treatments are reported to be effective, including various liver-directed therapies (embolization, chemoembolization, radioembolization); chemotherapy; cytoreductive surgery (removal of all visible tumor); treatment with biologics (somatostatin analogues, α-interferon); tyrosine kinase inhibitors (sunitinib); mTOR

inhibitors (everolimus); radiotherapy using radiolabeled somatostatin analogues to target the tumor (PRRT) (**Fig. 1**), and liver transplantation.

Specific Antitumor Treatments

The recent validation of the prognostic value of the different classification and grading systems (World Health Organization (WHO), European Neuroendocrine Tumor Society (ENETS), the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC)) are resulting in them playing an essential role to stratify patients into different risk groups and also in influencing the type of therapy (Klimstra, 2016; Singhi and Klimstra, 2018). A particular important prognostic factor is whether the NET is well differentiated (G1/G2) or poorly differentiated (<1% of all NETs) (G3). Well-differentiated NETs have a 5-year survival of 60%–80%, whereas poorly differentiated NETs have a 5-year survival of only 0%–15%.

Unfortunately, cytoreductive surgery is only possible in 10%–20% of patients who have limited hepatic metastases, allowing surgical removal of at least 90% of visible tumor. Although it is reported to provide palliative treatment, there are no control studies to support this conclusion. However, studies suggest that it may increase survival; therefore, cytoreductive surgery is recommended, if possible.

Chemotherapy for metastatic carcinoids is generally disappointing, with response rates of 0%–40% with various two to three drug combinations (Angelousi *et al.*, 2017; Metz and Jensen, 2008; Pavel *et al.*, 2016). With panNETs, chemotherapy has been more successful, with response rates of 30%–70%. The regimen of choice for many years for panNETs was streptozotocin and doxorubicin, however recent studies demonstrate the combination of temozolomide (TMZ) with capecitabine is effective in panNETs and thus it is receiving attention as a possible alternative to streptozotocin based therapy. An important distinction in patients with pNETs is whether the tumor is well differentiated (G1/G2) or poorly differentiated (G3). The chemotherapeutic approach is different for these two groups. Whereas the current regimen of choice for patients with well-differentiated pNETs is the combination of streptozotocin and doxorubicin with or without 5-fluorouracil, this is not the case for poorly differentiated NETs. The classification systems have therapeutic implications because it is proposed to treat the G3 NET tumors similar to treatment for well-differentiated G2 tumors, whereas for G3 NEC tumors (poorly differentiated G3), treatment with cisplatin-based regimens with etoposide or other agents (vincristine, paclitaxel) is recommended. The response rates with this protocol are 40%–70%; however, responses are generally short-lived (<12 months). This chemotherapy regimen can be associated with significant toxicity including GI toxicities (nausea, vomiting), myelosuppression.

Unfortunately, chemotherapy in almost never curative and has substantial toxicity. Therefore, it is generally reserved for patients with rapidly growing panNETs who fail other treatments.

Long-acting somatostatin analogues (**Fig. 1**) and α-interferon rarely decrease tumor size (i.e., 0%–15%); however, these drugs have prominent tumoristatic effects, stopping growth in 50%–90% of patients with NETs. Studies suggest that they are particularly effective in slower growing tumors. It has not been proven that these agents extend survival; however, because of the availability of long-acting formulations (e.g., one injection of somatostatin/month), their low toxicity, and their long-term effectiveness in some patients, they are the antitumor agents of choice. Recently large randomized double-blind phase 3 trials have supported the effectiveness of long-acting somatostatin analogues for extending progression free survival in patients with panNETs and carcinoids (Caplin *et al.*, 2014; Yao *et al.*, 2011).

Molecular targeted medical treatment with either an mTOR inhibitor (everolimus) or a tyrosine kinase inhibitor (sunitinib) is now approved treatment in the United States/Europe for patients with metastatic unresectable panNET, each supported by a phase 3, double-blind, prospective, placebo-controlled trial (Raymond *et al.*, 2011; Yao *et al.*, 2011). In addition, a recent phase 3 double -blind study (RADIANT-4) (Yao *et al.*, 2011) demonstrated the effectiveness of everolimus in in advanced, nonfunctional NETs of the lung or GI-Tract. In this study involving patients with advanced, progressive well differentiated, NF-lung/GI-NETs, everolimus significantly ($P < 0.000001$) improved progression-free survival and this has led to FDA approval for its use in patients with these tumors in the United States.

Hepatic embolization or chemoembolization (i.e., chemotherapy with embolization) can decrease tumor bulk and help control the symptoms of the hormone-excess state. This approach is usually reserved for patients who have disease largely confined to the liver, who have a patent portal vein, and who fail treatment with other modalities. Selective internal radiation therapy (SIRT) using yttrium-90 (^{90}Y) glass or resin microspheres is a relatively newer approach being evaluated in patients with unresectable NET liver metastases. The response rate varied from 50% to 70% (partial or complete), tumor stabilization occurred in 20%–40%, 60%–100% had symptomatic improvement, and overall survival varied from 25 to 70 months.

Somatostatin receptor-directed cytotoxicity using radiolabeled somatostatin analogues that are internalized by somatostatin receptors (Kwekkeboom and Krenning, 2016) (**Fig. 1**) overexpressed on the NET are being widely investigated. The following are being evaluated for treatment: ^{111}indium (^{111}Ln)-labeled compounds, which emit gamma rays, internal conversion, and Auger electrons; ^{90}yttrium-coupled analogues, which emit high-energy β particles; and ^{177}lutetium (^{177}Lu)-coupled analogues, which emit both. The ^{177}Lu and ^{111}In compounds have respectively been shown to stabilize disease in 41% and 40% and decrease tumor size in 38% and 30% of patients with advanced metastatic NETs. Recently results from a double-blind, prospective, randomized trial (NETTER-1 Study) demonstrate the efficacy and safety of PRRT in patients with advanced inoperable, progressive midgut GI-NETs (carcinoids). In this trial which included patients with metastatic, progressive midgut carcinoids, a marked increased in progressive-free survival ($P < 0.0001$) was seen with PRRT treatment with a ^{177}Lu-labeled-somatostatin-analog, with an

acceptable safety profile and with a suggestion of an improved survival (Strosberg *et al.*, 2017), although final survival analysis is not yet complete. This study is now under review at the FDA in the US and is not approved for general use at present. The ENETS 2016, NANETS, Nordic 2010, and European Society for Medical Oncology (ESMO) guidelines list PRRT as an experimental or investigational treatment at present.

Liver transplantation, although largely abandoned for most metastatic tumors, is still a consideration for patients with metastatic NETs because of their slower growth. In 103 cases of malignant NETs (43 carcinoid, 48 panNETs), liver transplantation achieved 2- and 5-year survival rates of 60% and 47%, respectively. Liver transplantation has been suggested for younger patients with metastatic NETs limited to the liver.

See also: Gastrinomas. Pancreatic Islet Cell Tumors. Somatostatin Receptor Expression in Gastrointestinal Tumors

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Gastrinomas☆

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Glossary

Gastrin A hormone of 17 or 34 amino acids that is produced primarily in G cells of the gastric antrum and regulates gastric acid secretion and proliferation of cells of gastric oxyntic mucosa.

Multiple endocrine neoplasia type 1 (MEN1) An inherited disorder characterized by hyperplasia/tumors of multiple endocrine organs (parathyroid > pancreatic endocrine tumors (PETs) > pituitary) and present in 20% to 25% of patients with Zollinger–Ellison syndrome; caused by abnormality in the MEN1 gene on chromosome 11q13, which encodes for a 610-amino acid nuclear protein, MENIN.

Zollinger–Ellison syndrome (ZES) Clinical syndrome caused by gastrinomas secreting excess gastrin (e.g., severe peptic disease, acid hypersecretion).

Introduction

In 1955, R. M. Zollinger and E. H. Ellison at Ohio State University described two patients with intractable peptic ulcer disease (PUD) due to massive gastric acid hypersecretion and non-beta islet cell tumors of the pancreas (Zollinger and Ellison, 1955). Zollinger and Ellison proposed that the tumors were secreting an ulcerogenic substance. In 1961, R. A. Gregory reported the extraction of a gastrin-like substance from a patient with Zollinger–Ellison syndrome (ZES), and in 1969, Gregory reported the acid secretagogue to be gastrin, a hormone released under normal physiological conditions (e.g., meal ingestion, low acid levels in the stomach) from gastric antral G cells (Gregory *et al.*, 1969). The main circulating form contains 17 amino acids (G-17) or an N-terminally extended form (G-34), although gastrinomas also have other precursor forms (e.g., progastrin, COOH glycine-extended forms). Patients with ZES have high serum levels of gastrin (hypergastrinemia), and this causes continuous secretion of acid from the parietal cells as well as inducing growth of the gastric mucosal cells, especially the parietal cells and gastric enterochromaffin-like (ECL) cells of the oxyntic mucosa. In addition to causing clinical symptoms due to the gastric acid hypersecretion, 60%–90% of gastrinomas are malignant (Metz and Jensen, 2008).

Clinical Features (Roy *et al.*, 2000)

The mean age at presentation is in the fifth decade of life, and the presenting symptoms are almost always due to the consequences of the gastric hypersecretion. Abdominal pain due to PUD and diarrhea due to the acid hypersecretion are the most common symptoms. Esophageal disease (e.g., heartburn, stricture), nausea, vomiting, and bleeding, either alone or in combination, are not infrequent (**Table 1**). Only late in the course of the disease does the tumor per se generally cause symptoms (e.g., weight loss, pain). The diagnosis is characteristically delayed 5–7 years from onset of symptoms because early ZES is usually mistaken for idiopathic PUD. Approximately one-fourth of patients have ZES as part of the familial syndrome, multiple endocrine neoplasia type 1 (MEN1), which is almost invariably associated with hyperparathyroidism, frequently with renal stones (Gibril *et al.*, 2004). In a certain proportion of patients, the ZES diagnosis is delayed sufficiently and the disease is severe enough to cause complications of PUD as important symptoms (e.g., bleeding, perforation, esophageal stricture).

Pathophysiology/Pathology

In almost all patients all of the initial symptoms listed in **Table 1** are due to the gastric acid hypersecretion. Usually, only late in the disease course with advanced disease are there any symptoms due to the growth of the gastrinoma per se.

Gastrinomas were originally reported to be non-beta cell tumors of the pancreas, but currently approximately 50%–90% are found in the duodenum, 10%–30% in the pancreas, and the remainder in other sites (**Table 1**). One controversial area is whether primary gastrinomas occur in abdominal lymph nodes. A long-term National Institutes of Health (NIH) study suggests that 10%–11% of all primary gastrinomas may occur in lymph nodes (Norton *et al.*, 2003). Other unusual intra-abdominal sites

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Table 1 Clinical features of patients with ZES

Feature	*NIH data (n = 261)*	*Literature data (range)*
Gender (percentage % male)	56	44–70
Mean age onset (years)	41	41–53
Initial symptom (percentage)		
Abdominal pain	75	26–98
Diarrhea	73	17–73
Heartburn	44	0–56
Nausea	30	8–37
Vomiting	25	26–51
Bleeding	24	8–75
Pain and bleeding	19	19–44
Pain and diarrhea	55	28–56
Mean duration of symptoms (years)	5.2	3.2–8.7
MEN1 present (percentage)	22	10–48
History-confirmed peptic ulcer (percentage)	71	71–93
Esophageal stricture (percentage)	4	4–6
Abdominal perforation (percentage)	5	5–18

Note: NIH data are from 261 patients with ZES. Literature data are from 10 series with 12–369 patients.

Table 2 Characteristics of gastrinomas (percentages)

Feature	*NIH data*	*Literature data (range)*
Location		
Pancreas	>20	21–70
Duodenum	>53	6–32
Lymph node	>11	<1
Other	>9	0–18
Unknown	>16	7–48
Duodenal location		
D-1	>57	ND
D-2	>31	ND
D-3	>6	ND
D-4	>3	ND
Extent of disease		
No tumor found	>13	7–48
Localized disease	>70	23–51
Metastatic disease to liver	>17	13–53
Extent metastases		
Primary only	>36	23–51
Primary and lymph nodes	>29	8–61
Primary and liver metastases	>23	15–38
Liver metastases only	>3	4–14
Lymph node metastases only	16	4–23

include the gastric antrum, biliary tract or liver, mesentery, ovary, and spleen. Extra-abdominal gastrinomas also occur rarely (<0.5%) in the cardiac intraventricular septum and due to a small cell lung cancer secreting gastrin. In the duodenum, 57% of the gastrinomas occur in the first portion and 31% occur in the second portion. Most patients have localized disease at presentation (83%) with no liver metastases, although 17% have or develop liver metastases (Jensen *et al.*, 2006). Approximately one-third of patients have a primary tumor only found at surgery, one-third have a primary tumor with lymph node metastases, one-fourth have a primary tumor with liver metastases, and one-fifth have either only liver metastases (3%) or only lymph node metastases (19%) (**Table 2**). At surgery, 60%–90% of gastrinomas occur within the "gastrinoma-triangle," which is an area formed by the junction of the cystic/common bile ducts posteriorly, the junction of the second/third parts of the duodenum inferiorly, and the junction of the pancreatic neck/body medially (Stabile *et al.*, 1984). This occurs because of the high frequency of duodenal gastrinomas now being detected which were missed previously.

All gastrinomas, like other pNETs and carcinoid tumors, are now classified as neuroendocrine tumors (NETs). A number of different classification systems have been proposed for staging and grading NETs and were developed by the World Health

Organization (WHO), European Neuroendocrine Tumor Society (ENETs) Union for International Cancer Control (UICC). These are now required to manage patients with NETs, because they have prognostic value and are can dictate different therapeutic approaches in some cases. These classification systems are based on the degree of tumoral differentiation (good vs. poor), tumor size, tumor invasion, and tumor extent. The tumor grading, reflects tumoral cell proliferation rate, is particularly important and involves establishing which of three different grades the tumor is in. The three grades vary in proliferative indices including mitotic rate and Ki 67. Currently for GI-NETs/pNETs low grade-G1 is <2 mitoses/10-HPF or Ki67 $< 3\%$; intermediate grade-G2 is 2–20 mitosis/10-HPF or Ki67 3%–20% and high grade-G3 is >20 mitosis/HPF or Ki67 $> 20\%$).

Molecular Pathogenesis of Gastrinomas

Similar to other gastrointestinal neuroendocrine tumors (NETs) (e.g., carcinoids, pancreatic endocrine tumors, PETS), mutations in common oncogenes (e.g., ras, c-Jun, 1-fos) are uncommon in gastrinomas, as are alterations in common tumor suppressor genes (e.g., retinoblastoma, p53). However, recent studies show that mutations in both the p53 pathway and the retinoblastoma (RB) pathway occur in pNETs. The Rb pathway can be inactivated in many pNETs (including gastrinomas) by amplification of genes encoding the cyclin-dependent kinases Cdk4/Cdk6. Another study found the p53 pathway was altered in 70% of pNETs through aberrant activation of its negative regulators-MDM2 (mouse double minute 2 homolog), MDM4, and WIPI (WD-repeat protein interacting with phosphoinosides).

In approximately 20%–25% of patients with ZES have Multiple Endocrine Neoplasia type 1 syndrome (Wermer syndrome) (MEN1/ZES). MEN1 is an autosomal dominant disorder due to mutations in the MEN1 gene on chromosome 11 (11q13) (Jensen *et al.*, 2008). The MEN1 gene has 10-exons encoding for a 610 amino acid protein, MENIN. MENIN is a nuclear protein that interacts with a large number of proteins such as JunD; nuclear factor-kB(NF-kB), FANCD2 (a DNA-repair-factor), NM23, SMAD3; RPA2 (a DNA-processing-factor); the AP1-transcription factor, nucleoside diphosphate kinase, cytoskeletal-associated proteins, and various histone-modifying enzymes. Mutations in the MEN1 gene occur in one-third of sporadic gastrinomas. A recent study reported results of exomic sequencing of 10 sporadic pNETs, and found the MEN1 gene was the most frequently altered gene. MEN1 gene mutations occurred in 44% followed in frequency by mutations in 43% in genes encoding for two subunits of a transcription/chromatin remodeling complex consisting of DAXX (death-domain associated-protein) and ATRX (a-thalassemia/mental retardation syndrome X-linked), followed by mutations in mToR pathway genes (15%).

Recent studies provide evidence for the importance in pNETs/gastrinomas of alterations in p16/MTS1 tumor suppressor gene (50%–90%), DPC4/Smad gene (20% in pNETs), amplification of the HER-2/neu proto-oncogene, mTor/Akt/PI3K pathway, as well as increased expression of a number of growth factors and/or their receptors. A number of recent studies provide evidence that the mToR/Akt/PI3K pathway is particularly important for mediating the growth of pNETs. This evidence includes the success of the mTOR inhibitor, everolimus, in extending disease-free survival in patients with advanced pNETs (Yao *et al.*, 2011).

Comparative genomic hybridization (CGH) and genomic-wide allelotyping studies report that chromosomal gains/losses occur frequently in most pNETs, including in gastrinomas. The frequency of these changes differs between GI-NETs (carcinoids) and pNETs, supporting the conclusion that they have a different pathogenesis. In pNETs, allelic losses occur most frequently at chromosomal locus 1p (20%–75%), 1q (20%–90%), 3p (40%–96%), 11p (30%–50%), 11q (30%–70%), and 22q (40%–95%). With pNETs, chromosomal gains occur most frequently at 17q (10%–55%), 7q (15%–70%), and 4 q (33%). A number of these alterations are associated with malignant behavior including deletions at chromosome 1, 3p, 6, 11q, 17p and 22p, and gains on chromosome 4, 7, 14q, Xp.

Results are reported from a number of studies in which pNETs were studied using microarrays to assess gene expression profiles. Results from eight studies in pNETs have been summarized and they demonstrate a wide variation in the number of genes upregulated or downregulated. It is not clear which specific gene changes are important in the molecular pathogenesis of the pNET.

Diagnosis/Differential Diagnosis

The diagnosis remains elusive, with a 4- to 7-year delay in diagnosis from onset of symptoms (**Table 1**). This delay occurs because ZES is uncommon (1–3 new cases/1 million population/year) and its presentation closely mimics common disorders: idiopathic PUD (2300 cases/1 million population/year), idiopathic gastroesophageal reflux disease (GERD), which occurs in 3%–4% of the population and drug-induced PUD. The widespread use of proton pump inhibitors (PPIs) is both complicating and delaying the diagnosis of ZES (Corleto *et al.*, 2001). This is occurring because PPIs are potent acid suppressants that, in contrast to histamine H2-receptor antagonists, can control symptoms in patients with ZES at doses usually used for GERD/PUD in non-ZES patients, thereby masking the diagnosis. Furthermore, PPIs can cause hypergastrinemia in patients without ZES, and this can complicate the diagnosis of ZES in these patients. The diagnosis of ZES should be particularly suspected in patients with PUD with diarrhea, endocrinopathies, family history of PUD or endocrinopathies, and/or recalcitrant PUD disease as well as in patients with PUD without *Helicobacter pylori* or with PUD complications. The first step in establishing the diagnosis is made by measuring fasting gastrin levels which are elevated in 90% to 98% of patients (Berna *et al.*, 2006b). If the fasting gastrin level is elevated, gastric fluid pH should be assessed and

the fasting gastrin should be repeated. If the gastric pH is <2.0 and the fasting serum gastrin is elevated, it is supports the diagnosis that ZES is present (Berna *et al.*, 2006b; Roy *et al.*, 2001). If the fasting gastrin is elevated >10-fold with a gastric pH <2, ZES is confirmed when there is no previous history of gastric resection. Unfortunately, two-thirds of patients have fasting gastrin levels increased <10-fold (101–999 pg/mL), and a number of other conditions can mimic ZES in this range (e.g., *H. pylori* infection, gastric outlet obstruction, antral G-cell hyperplasia/hyperfunction, chronic renal failure, short bowel syndrome). These can be excluded by both measuring a basal gastric secretory rate for 1 h and performing a secretin test (Berna *et al.*, 2006a). The secretin test yields no false positives except in patients with achlorhydria or taking PPIs, and it is positive in 94% of patients with ZES (≥120 pg/mL increase post secretin) in this fasting gastrin range. The calcium stimulation test is now rarely used with the recent availability of secretin again. One of the main difficulties in establishing the diagnosis of ZES is that in most patients the PPI has to be stopped or withheld (e.g., PPIs × 1 week, histamine H_2 antagonists × 1 day) to obtain a gastric pH <2. This can be dangerous in a true ZES patient (Poitras *et al.*, 2012) and therefore is best performed by referring the patient to gastrointestinal unit with expertise in the diagnosis and treatment of ZES (Ito *et al.*, 2012a; Metz, 2012).

Tumor Localization

Localizing the primary tumor, as well as determining the extent of the tumor, is essential for all management steps (Ito *et al.*, 2013a; Jensen *et al.*, 2006). It is necessary to determine whether surgical exploration should be undertaken, the extent of possible resection, and the location of the primary tumor (to enhance the possibility of cure), as well as to assess changes in tumor size with patients with advanced metastatic disease, either in response to antitumor treatment or in determining the need for it. The most sensitive imaging modality at present is to use somatostatin receptor imaging (SRI) (Ito and Jensen, 2017). This was developed when it was realized that almost all gastrinomas, as well as most well differentiated pNETs and carcinoids, overexpress somatostatin receptors and thus can be imaged using radiolabeled somatostatin analogues (Krenning *et al.*, 1993). Until recently this was generally performed using somatostatin receptor scintigraphy (SRS) with [^{111}In-DTPA-DPhe^1]-octreotide (**Figs. 1** and **2**) with detection with single photon emission computed tomography (SPECT). SRS is more sensitive than all conventional imaging modalities combined (e.g., computed tomography, CT, magnetic resonance imaging, MRI, ultrasound), but it does not give reliable information on tumor size (**Figs. 2** and **3**). Therefore, a CT scan with contrast is also recommended. Recently the use of ^{68}Ga-DOTATATE has been approved in the United States and most countries because it has greater sensitivity and specificity that SRS (**Fig. 4**) and is now the procedure of choice for SRI imaging (Ito *et al.*, 2013a; Ito and Jensen, 2017). Endoscopic ultrasound is recommended by some but rarely localizes duodenal tumors; therefore, it is helpful in a minority of the patients. For the rare patients with negative SRI, functional/localization studies measuring gastrin gradients with angiography are occasionally used.

Management

General

Treatment needs to be directed at controlling the gastric acid hypersecretion initially and long term as well as directed at the gastrinoma per se (Falconi *et al.*, 2016; Jensen *et al.*, 2006; Metz and Jensen, 2008). It is also important to determine whether the patient has ZES due to the presence of MEN1 because this requires additional treatment strategies. MEN1 is generally recognized by a careful family and personal history for endocrinopathies (especially hyperparathyroidism, pituitary disease, and other pancreatic endocrine tumors (PETs) and assessment for these endocrinopathies by appropriate hormonal assays. The hyperparathyroidism is usually present prior to ZES onset and is best diagnosed by measuring serum ionized calcium levels and plasma-intact PTH (**Table 3**).

Control of Acid Hypersecretion

Many patients with ZES have basal acid secretory rates elevated more than fivefold and can rapidly develop complications due to the gastric acid hypersecretion if it is not controlled both acutely and for the long term (Ito *et al.*, 2013b; Jensen *et al.*, 2006; Roy *et al.*, 2001). PPIs (e.g., omeprazole, lansoprazole, pantoprazole, rabeprazole, esomeprazole) are now the mainstay of treatment, starting at a dose equivalent to 60 mg per day of omeprazole. Patients with MEN1, severe GERD, or previous gastric acid-reducing surgery should be started on this dose given twice a day. With time, the dose can be decreased in most patients. During periods when parenteral antisecretory drugs are needed, intravenous PPIs (intravenous pantoprazole in the United States) are now the drugs of choice. Oral or intravenous histamine H_2 receptors can be used, but high, frequent oral dosing is needed and if needed parenterally, continuous intravenous infusions are recommended. Total gastrectomy is rarely used today and is reserved for the rare patient who cannot take oral medication (**Table 3**) (Ito *et al.*, 2013b; Jensen *et al.*, 2006; Roy *et al.*, 2001).

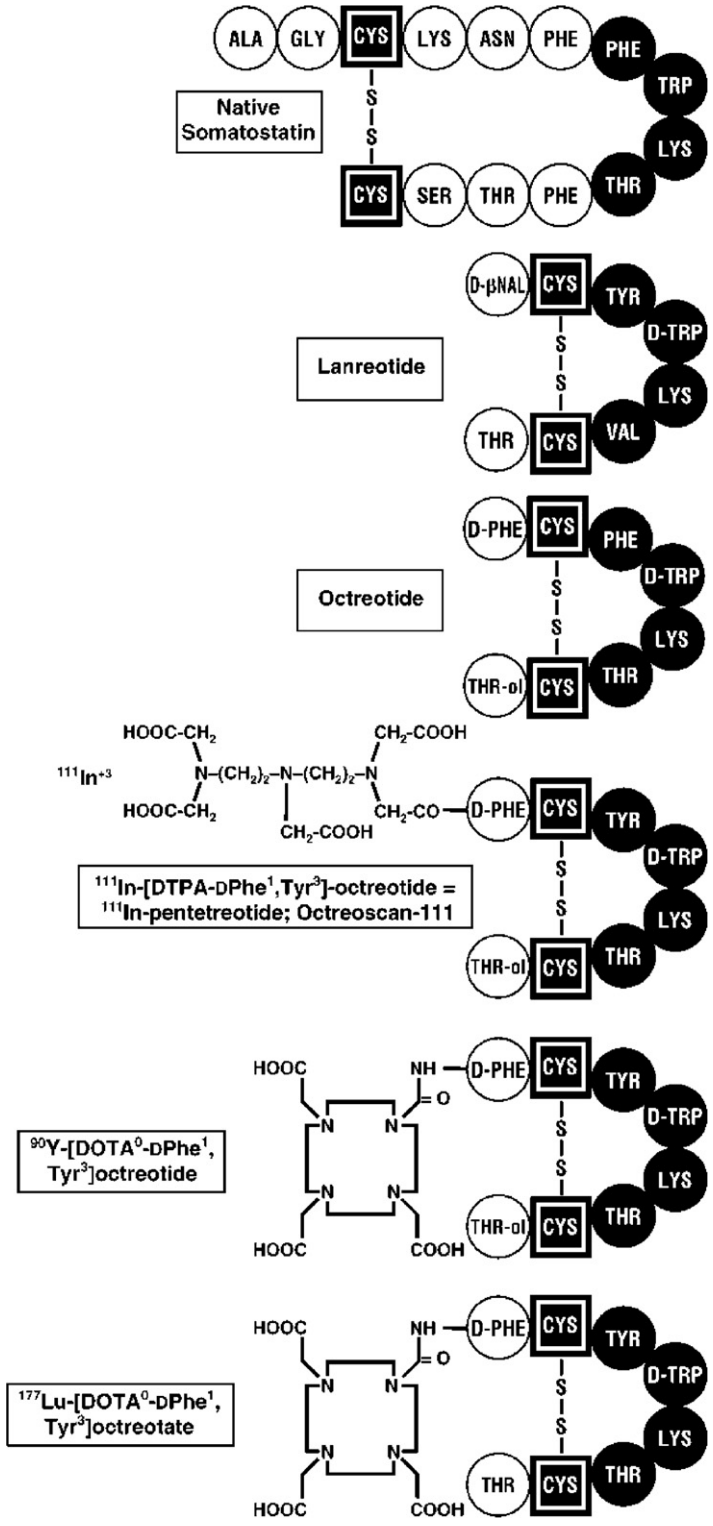

Fig. 1 Structure of somatostatin and synthetic analogues used for tumor localization or radiotherapy.

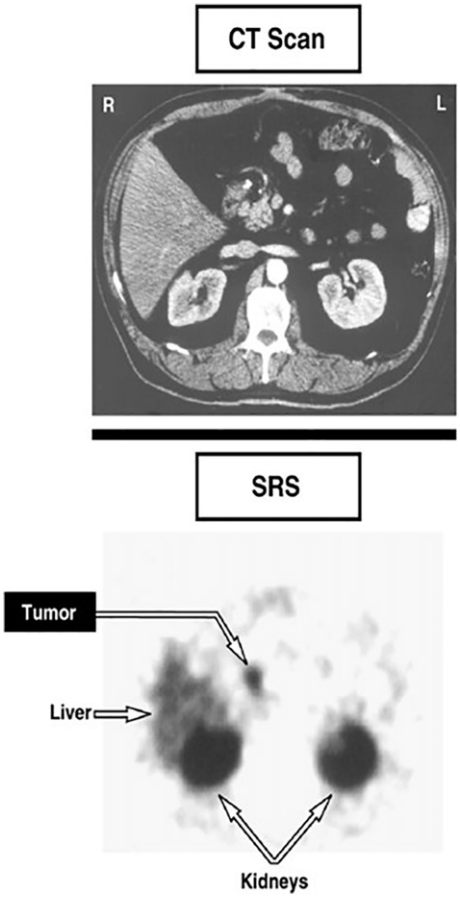

Fig. 2 Comparison of the ability of CT scan and SRS to localize a primary gastrinoma in a patient with ZES. Only the SRS is positive, showing its greater sensitivity.

Treatment of the Gastrinoma

Surgical

In all patients with sporadic ZES (without MEN1), without liver or distant metastatic disease, and without concomitant illnesses that shorten life expectancy or increase the risk of surgery, exploratory laparotomy by a surgeon experienced in the treatment of this disease should be performed (Falconi *et al.*, 2016; Jensen *et al.*, 2006). Enucleation of pancreatic head/body tumors should be performed. If needed, distal pancreatectomy for pancreatic tail lesions should be performed. Whipple resection is generally not recommended. In 5%–15% of patients with advanced disease with liver metastases, cytoreductive surgery should be considered if $>90\%$ of the visible tumor can be removed.

In patients with MEN1/ZES, the type of surgery and when it should be performed are controversial because these patients cannot be cured generally without aggressive resections such as Whipple resections, but still have an excellent long-term prognosis (Jensen and Norton, 2017). We recommend exploration when any lesion >2 cm in diameter is imaged because numerous studies demonstrate that tumor size is a strong prognostic factor for metastatic spread to the liver, which is associated with decreased survival. Lymph node metastases are frequent, even with small duodenal gastrinomas, but have not been shown to be associated with decreased survival.

Medical

Patients with metastatic gastrinoma in the liver have a decreased survival rate (Weber *et al.*, 1995; Yu *et al.*, 1999). Studies show that survival is especially decreased in the 40% of patients with a rapidly growing metastatic tumor. In this subset of patients, antitumor treatment is indicated. Long-acting somatostatin analogues, such as octreotide–LAR (**Fig. 1**) (given once monthly) is now the recommended initial treatments (Falconi *et al.*, 2016; Ito *et al.*, 2012b; Jensen *et al.*, 2006). Somatostatin analogues cause a decrease in tumor size in only 15%–20% of patients; however, they cause a cessation of growth in 50%–70% of patients and continue to remain effective for years in many patients. Chemotherapy (e.g., streptozotocin, doxorubicin $\pm$5-fluorouracil,

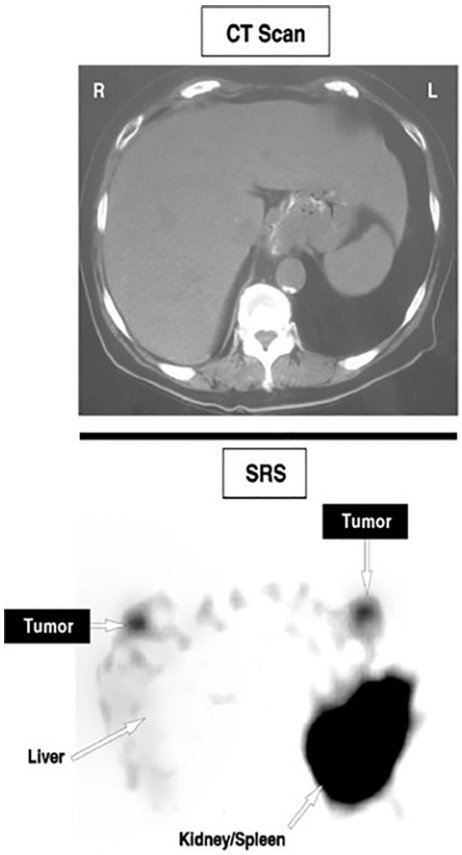

Fig. 3 Comparison of the ability of CT scan and SRS to localize metastatic disease in the liver in a patient with ZES. Only the SRS localizes a metastasis in the left lobe, showing its greater sensitivity.

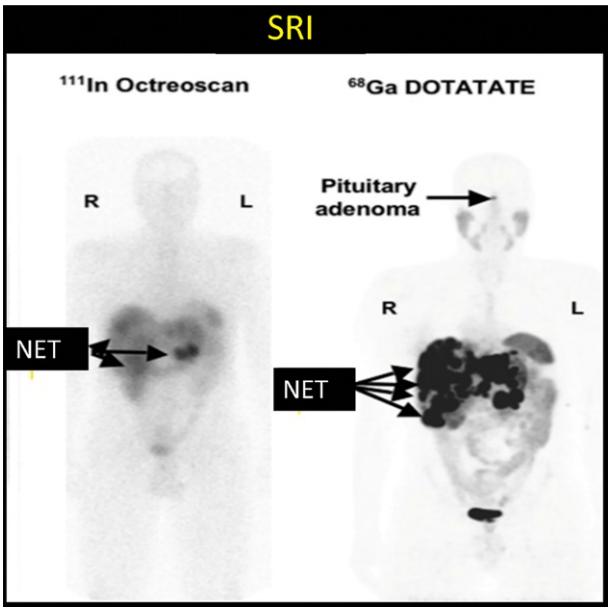

Fig. 4 Increased sensitivity of ^{68}Ga-DOTATATE PET/CT over SRS with [^{111}In-DTPA-DPhe^{1}] octreotide (Octreoscan) in a patient MEN1 with a pNET with liver metastases.

temozolomide with capecitabine) causes a decrease in tumor size in 5%–50% of patients but has significant toxicity and has not been shown to extend life in patients with gastrinomas. Both everolimus and sunitinib have been shown in large randomized trials to extend PFS (progression free survival) in patients with advanced pNETs and can also be used (Falconi *et al.*, 2016; Ito *et al.*,

Table 3 Management of ZES

Treatment of gastric acid hypersecretion
Drugs of choice: PPIs (e.g., omeprazole, lansoprazole, pantoprazole, rabeprazole, esomeprazole)
PPIs control acid secretion >99%
Histamine H_2 receptor antagonist less effective (frequent, high doses needed)
Total gastrectomy (needed only in rare patient who cannot take oral drugs)
Treatment of gastrinoma
In patients without MEN1 (75%) (sporadic ZES)
Surgery cures 60% in short term (<1 year); 30%–35% at 5 years postresection
At surgery, routine duodenotomy, routine removal of lymph nodes from gastrinoma triangle area and intraoperative ultrasound needed, with tumor enucleation used most frequently
Aggressive resection (Whipple procedure) rarely needed
In patients with MEN1/ZES (25%)
Tumor enucleation rarely (<1%) curative given that 60%–80% of gastrinomas are duodenal in location, multiple, and/or with lymph node metastases (50%–70%)
Surgery generally recommended if tumor is imaged ≥2–3 cm. otherwise observation

Note: PPIs, proton pump inhibitors.

2013a; Raymond *et al.*, 2011; Yao *et al.*, 2011). Peptide radioreceptor therapy (PRRT) is effective in patients with advanced NETs and is now approved for use in the United States and number of other countries.

Conclusion

Gastrinomas are an uncommon cause of PUD, but if diagnosed, the gastric acid hypersecretion can be controlled in every patient. The natural history of the gastrinoma is becoming an increasingly important determinant of long-term survival, so the typical 5-year delay in diagnosis remains a major challenge to instituting treatment early in the disease course in many patients. Furthermore, studies suggest that the widespread use of PPIs may further delay the diagnosis of ZES.

See also: Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS). Pancreatic Islet Cell Tumors. Somatostatin Receptor Expression in Gastrointestinal Tumors

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Pancreatic Islet Cell Tumors☆

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Introduction

Incidence

The incidence of pancreatic neuroendocrine neoplasms (pNENs) has increased from around 0.1/100.000 in 1988 to more than 0.5/100.000 in 2008 according to the SEER registry (Kuo and Salem, 2013). Small nonfunctioning pNENs (<1–2 cm) are more frequently discovered incidentally due to the widespread use of improved high-quality imaging techniques (Falconi *et al.*, 2012, 2016). The incidence of pancreatic neuroendocrine carcinomas (pNECs) is also increasing due to the increased awareness of pathologists applying specific stains for chromogranin A (CgA) and synaptophysin if a pNEC is suspected (Falconi *et al.* 2012, 2016; Ilett *et al.*, 2015; Jensen *et al.*, 2012). However, staining for CgA may be negative in pNEC.

WHO Classification

pNENs are as gastrointestinal NENs classified according to the WHO 2010 classification (**Table 1**). The classification was revised in the WHO 2017 classification for pNECs as they according to differentiation was divided into G3 neuroendocrine tumors (pNETs) for well differentiated pNENs and G3 pNECs for poorly differentiated pNENs.

Functional Classification of pNENs

pNENs are divided into functioning (20%–30%) and nonfunctioning tumors (70%–80%). The functioning pNENs release hormones that give rise to specific syndromes, whereas the nonfunctioning tumors do not cause hormone-related symptoms even though they may be immunohistochemically reactive for hormones or secrete inert hormones such as pancreatic polypeptide (PP). Most of the small incidentalomas as well as pNECs are nonfunctioning. Few nonfunctioning tumors may over time become functioning and give hormone specific symptoms (Jensen *et al.*, 2012).

Immunohistochemical Neuroendocrine Markers

In general, pNENs stain for CgA, synaptophysin, and pancreatic polypeptide (PP). In addition, tumors may stain for the cell specific hormone synthesized in the tumor cell for example, insulin or gastrin. However, a positive staining for a specific hormone does not imply that the tumor can be classified as a functioning pNEN unless it causes specific endocrine symptoms. pNECs may be negative for CgA and PP and rarely stain positive for tumor specific hormones.

Mutations in ATRX (alpha thalassemia mental retardation gene) and DAXX (death-domain-associated protein) are mutually exclusive and target genetic alterations in well differentiated pNENs whereas Rb (retinoblastoma) targets genetic alterations in pNECs.

Table 1 WHO 2010 classification

Grade	*NET G1*	*NET G2*	*NEC G3 (small/large cell)*
Mitotic count	<2	2–20	>20
Ki67 proliferation	≤2	3–20	>20
Distribution	35%	40%	25%

NETs, Neuroendocrine tumors; *NECs*, neuroendocrine carcinomas.

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The stroma of pNETs is usually a delicate fibrovascular or hyaline stroma. In insulinomas the stroma is often characterized by amyloid deposition. pNECs most often have a similar gross presentation as a ductal adenocarcinoma and microscopically they, in contrast to pNETs, often show cellular pleomorphism, solid growth pattern, necrosis, and an often desmoplastic stroma with large vessels.

Hereditary Tumor Syndromes

pNENs may in some patients be part of a hereditary syndrome (Jensen *et al.*, 2008). They are autosomal dominant diseases and present one to two decades earlier than sporadic pNENs. A family history should be obtained from patients with pNENs and a relevant hormonal screening should be performed. If indicated a mutation analysis should be performed following patient acceptance.

Multiple endocrine neoplasia type 1 (MEN-1) presents most frequently with parathyroid and pituitary adenomas and pancreatico-duodenal NETs and more rarely with broncho-pulmonary, adrenal, and thymic NETs. Nonfunctioning or functioning pancreatico-duodenal NETs are found in up to 80% of patients with MEN-1. They have a more indolent behavior than the sporadic pNENs.

von Hippel-Lindau Disease (VHL) comprises cerebral hemangioblastoma, retinal angioma, renal cell carcinoma, pNETs, and pheochromocytoma. The incidence of pNENs, which are solid or cystic nonfunctioning tumors, is around 15%.

Neurofibromatosis type 1 (NF-1; von Recklinghausen's disease) presents with café-au lait macules, neurofibromas, skin-fold freckling, iris Lisch nodules, and bone dysplasia. Nonfunctioning somatostatinomas located at the papilla of Vater are found in up to 10%. Gastrointestinal stromal tumors are reported in a few of the patients with NF-1 and somatostatinoma. Up to 50% occur as a sporadic disease.

Tuberous sclerosis complex (TSC) presents with hamartomas in several organs. Functioning and nonfunctioning pNENs are found in a minor percentage.

Functioning pNENs

Insulinomas account for 10%–15% of pNENs. They are usually <20 mm in size, evenly distributed in the pancreas, diagnosed in the fourth–fifth decade and slightly more common in females. Around 90% are benign. About 5% are associated with MEN-1. The malignant insulinomas are larger with a Ki67-index $>10\%$, sporadic and most have metastasized at diagnosis (Jensen *et al.*, 2012).

The hypersecretion of insulin causes attacks of hypoglycemia which again may cause symptoms as confusion, headaches, behavioral and visual disturbances, and may end in coma. The hypoglycemia induced stimulation of the adrenergic system causes sweating, tremor, palpitations, and irritability. Due to these symptoms patients may be referred to neurological or psychiatric clinics. To relieve symptoms the increased glucose consumption may cause a considerable increase in body weight. The diagnostic delay is 3–5 years from start of symptoms.

Gastrinomas account for 10% of pNENs. The vast majority are located within the "gastrinoma triangle" including the duodenum and the pancreas located to the right of the superior mesenteric vessels. About 75% are located in the duodenum. MEN-1 syndrome is seen in 20% of the patients and the gastrinomas are most frequently found in the duodenum, often as multiple tumors and may also be associated with nonfunctioning tumors in the pancreas. In patients with sporadic gastrinomas the age at diagnosis is 45–60 years and one to two decades younger in patients with MEN-1. More than 50% of the pancreatic gastrinomas are malignant with metastases to regional lymph nodes or the liver at diagnosis. The duodenal gastrinomas and gastrinomas in relation to MEN-1 have a more indolent behavior and only 15%–20% of the patients have regional lymph node metastases at diagnosis.

The Zollinger–Ellison syndrome (ZES) is caused by a gastrin-induced stimulation of gastric acid secretion, which results in recurrent gastro-duodenal peptic ulcers, gastritis, dyspepsia and gastro-esophageal reflux disease in 80%–90% of the patients. *Heliobacter pylori* negative patients with recurrent dyspepsia and peptic ulcers should be suspected of having a gastrinoma. Complications such as peptic ulcer bleeding or perforation are rarely seen today due to the frequent use of proton pump inhibitors (PPI), which also prevent the acid-induced diarrhea. Due to acidification of the duodenal contents, pancreatic enzymes are inactivated and bile deconjugation diarrhea is seen in around 50% of the gastrinoma patients. In 10% diarrhea is the only symptom. Even tumors <1 cm in size may cause the ZES (Jensen *et al.*, 2012).

Glucagonomas are rare, usually malignant tumors that are evenly distributed in the pancreas. At diagnosis most tumors are >2 cm and have often spread to regional lymph nodes and the liver. They may be related to MEN-1. pNETs may stain positive for glucagon without elevated plasma glucagon and the presence of the glucagonoma syndrome.

The glucagonoma syndrome presents with diabetes, necrolytic migratory erythema, stomatitis, glossitis, anemia, fatigue, weight loss, thrombosis, hypoaminoacidemia. All symptoms may not be present simultaneously. Plasma glucagon is elevated >5-times normal level (Jensen *et al.*, 2012).

VIPomas (Vasoactive intestinal peptide; Verner–Morrissons syndrome; WDHA) are rare and evenly distributed in the pancreas, but may also develop from paragangliomas (10%). Most are malignant with disseminated disease at diagnosis. Few VIPomas are related to MEN-1. Plasma VIP is increased.

Symptoms include watery diarrhea, hypokalemia, achlorhydria (50%), metabolic acidosis, flushing (20%), hypercalcaemia (50%), glucose intolerance (50%). Severe watery diarrhea and hypokalemia may require constant iv fluid infusion and electrolyte substitution (Jensen *et al.*, 2012).

Somatostatinomas are rare and occur in the pancreas (60%) or the duodenum (40%). The pancreatic somatostatinomas are malignant often with disseminated disease at diagnosis whereas the duodenal somatostatinomas are solitary and usually have a benign behavior. The pancreatic somatostatinomas may be related to MEN-1, while the duodenal somatostatinomas may be related to NF-1. Plasma somatostatin is elevated in pancreatic somatostatinomas but rarely in the duodenal tumors (Jensen *et al.*, 2012).

The pancreatic somatostatinomas may present with diabetes mellitus, steatorrhoea/diarrhea, weight loss, abdominal pain, and bile stones (somatostatinoma syndrome). However, all symptoms are never seen simultaneously.

The duodenal somatostatinomas have no endocrine symptoms but may cause symptoms due to occlusion of the common bile duct.

Other very rare functioning pNENs may secrete ACTH, GHRH, serotonin, PTH-RP, calcitonin cholecystokinin, LH, renin, erythropoietin. The increased secretion of ACTH, growth hormone releasing hormone (GHRH), serotonin and PTH-RP leads to Cushing syndrome, acromegaly, carcinoid syndrome and hypercalcemia, respectably (Jensen *et al.*, 2012).

Nonfunctioning pNENs

Nonfunctioning pNENs are evenly distributed in the gland and in most cases have a Ki67 index between 3% and 20%. Around 20% are G3 pNEC with a Ki67 $>$ 20%. They are generally large and have metastasized to regional nodes and the liver in more than 50% of the patients. Symptoms are with abdominal pain (35%–78%), weight loss (20%–35%), anorexia, nausea (45%), and jaundice (17%–50%) (Falconi *et al.*, 2012). About 20% of nonfunctioning pNETs are seen in relation to MEN-1 (Falconi *et al.*, 2012).

With the development of more sensitive imaging techniques, asymptomatic nonfunctioning localized pNETs $<$1–2 cm are more frequently found incidentally. Whether these small nonfunctioning tumors have a benign or malignant behavior is uncertain.

Biochemical Diagnosis

Chromogranin A

CgA is the best validated circulating general marker of well differentiated NENs including pNENs (Modlin *et al.*, 2010a). CgA is present in the large secretory granules that store peptide hormones throughout the neuroendocrine system. Increased plasma levels of CgA are observed in around 80% of functioning and nonfunctioning pNENs, with the exception of insulinomas where only a minority of patients have increased CgA levels. Day to day variation of CgA is around 25%. Level of CgA is related to tumor burden, and in case of a large tumor burden levels can increase to more than 100 times upper normal range (Modlin *et al.*, 2010a). Changes in CgA may reflect tumor progression or regression and thereby the effect of treatment. Poorly differentiated pNECs (Ki-67 $>$ 20%) can lose the secretory capacity with subsequent normal levels of CgA (Ilett *et al.*, 2015).

An elevated plasma CgA without the presence of a NEN is often found in patients with renal failure, hepatic failure and inflammatory diseases. Hypochlorhydria/achlorhydria in for example, chronic atrophic gastritis and treatment with proton pump inhibitors (PPI), is also associated with increased levels of CgA (Modlin *et al.*, 2010a).

Biochemical Diagnosis of Functioning pNENs

The diagnosis of functioning pNENs is based on relevant clinical symptoms and signs together with an inappropriate elevation of a specific pancreatic hormone.

Insulinomas

Plasma glucose below 2.2 mmol/L together with inappropriately high levels of proinsulin ($>$5 pmol/L), insulin ($>$36 pmol/L), and c-peptid ($>$200 pmol/L) suggests insulinoma. The final diagnosis is based on Whipples triad (1: symptoms of hypoglycemia, 2: plasma glucose $<$2.2 mmol/L, and 3: relief of symptoms after glucose infusion) during a 72 h fasting test (Jensen *et al.*, 2012).

Gastrinomas

Fasting serum gastrin is an excellent screening test for diagnosing gastrinomas with a very high sensitivity. However, elevated gastrin has a low specificity for gastrinoma since reduced gastric acid secretion for whatever reason will lead to hypergastrinemia. Thus, a diagnosis of gastrinoma is based on hypergastrinemia together with low gastric pH. Gastric pH $<$2 and gastrin above 10 times upper normal range is highly suggestive of a gastrinoma. Around 40% of gastrinoma patients fulfill these criteria (Berna *et al.*, 2006b). In the remaining patients with less elevated serum gastrin provocative tests are needed. The most common used test is the secretin test, in which the patients receive an iv bolus of secretin with subsequent repeated measurements of serum gastrin for 30 min (Berna *et al.*, 2006a). Secretin stimulates secretion of gastrin from gastrinomas but not from the antral G-cells. Hence, a

rise in serum gastrin indicates the presence of a gastrinoma. However, there is no specific diagnostic cut-offs, and numerous criteria have been proposed for a positive secretin provocative test.

VIPomas, glucagonomas, somatostatinomas, and other very rare functioning pNENs will present with elevated fasting plasma levels of the respective tumor hormone and related symptoms (Jensen *et al.*, 2012).

Circulating Tumor Cells, Circulating DNA, and Circulating mRNA

The diagnostic and prognostic role of circulating tumor cells, tumor DNA, and tumor mRNA in cancer has been increasingly investigated in recent years. There are some promising scientific data on the use of in particular circulating mRNA as a biomarker in patients with NENs. However, more data are needed before these new technics can be recommended for clinical use (Oberg *et al.*, 2016; Zatelli *et al.*, 2017).

Imaging

Information on localization and spread is always of importance for treatment planning and monitoring of therapy. Currently, the modalities used include functional imaging with somatostatin-receptor imaging (SRI) using nuclear medicine methods as well as morphological imaging using computed tomography (CT), magnetic resonance imaging (MRI) or endoscopic ultrasound.

SRI is based on the overexpression of somatostatin receptors, in particular subtype 2 (SST2), on NENs (Binderup *et al.*, 2008). In general >90% of NETs, including pNETs, express SST2 with the exception of insulinomas where only 70% are SST2 positive. Previously, SRI was performed using gamma camera imaging with SPECT and the tracer 111In-octreotide. However, PET tracers mostly labeled with ^{68}Ga are now implemented in most NET centers. The most commonly used PET tracers are ^{68}Ga-DOTATATE, ^{68}Ga-DOTATOC, and ^{68}Ga-DOTANOC (Johnbeck *et al.*, 2014), which when performed as a PET/CT have a sensitivity and specificity above 90% (Geijer and Breimer, 2013; Sundin *et al.*, 2017; Treglia *et al.*, 2012; Yang *et al.*, 2014). However, for insulinomas the sensitivity was only 26% (Sharma *et al.*, 2016). Accordingly, SRI PET/CT is therefore now considered the primary imaging modality in noninsulinoma pNETs and should be performed as preoperative staging (Falconi *et al.*, 2016). Occasionally, small pNETs may be overlooked using SRI PET/CT due to the limited spatial resolution on ^{68}Ga-PET. However, recently a new ^{64}Cu-labeled PET tracer, ^{64}Cu-DOTATATE, was evaluated for detection of NETs (Pfeifer *et al.*, 2012, 2015). ^{64}Cu has the advantage of a shorter positron range and thereby a better spatial resolution than ^{68}Ga and ^{64}Cu-DOTATATE seems to have a better lesion detection rate than ^{68}Ga labeled tracers (Johnbeck *et al.*, 2017). Accordingly, this tracer may prove valuable for lesions not found using ^{68}Ga-labeled tracers for SRI. In contrast, there is probably no need for ^{18}F-DOPA or ^{11}C-5-HTP PET as their superiority was based on comparison with SPECT SRI (Kjaer and Knigge, 2015).

SRI is often negative in pNECs and FDG-PET/CT may be used instead. FDG-PET/CT is positive in around 50% of NENs, but it is an excellent prognostic marker as NEN patients with a positive FDG-PET/CT have a poorer survival than patients with a negative FDG-PET/CT (Binderup *et al.*, 2010).

For insulinomas, specific PET tracers that bind to the glucagon-like peptide 1 (GLP-1) receptor may be of value (Eriksson *et al.*, 2014) and clinically applicable (Velikyan *et al.* 2017). However, more data are needed. Also, these tracers are only available in few centers.

The use of CT alone, which should preferentially be performed as a three-phase CT with iv contrast and bolus tracking, has a reported mean sensitivity of 82% and specificity of 96% (Sundin *et al.*, 2017).

Although MRI could be used in line with CT, the availability, cost and longer scan time is in most centers limited to challenging cases. MRI has a mean sensitivity of 79% and specificity of 100% (Sundin *et al.*, 2017). In addition to standard morphological imaging, MRI has the ability to perform diffusion-weighted imaging (DWI) which reflects the restricted interstitial space in cancer tissue limiting the diffusion of water. Parametric DWI with ADC values (apparent diffusion coefficient) has been shown to be a marker of malignancy and to correlate with Ki67 in pNETs (Wang *et al.*, 2011).

Finally, endoscopic ultrasound with its possibility of fine-needle aspiration has a role for detection and cytological confirmation of pNENs. The method is highly operator dependent and therefore exact numbers on performance are difficult to obtain (Falconi *et al.*, 2016).

Interventional Treatment

Radical surgery is the only treatment that may cure patients with pNENs. Major surgical complications are significantly lower in high volume centers and patients can usually be treated with a mortality rate close to nil. Common pancreatic operations include pancreaticoduodenectomy (Whipple procedure), distal pancreatectomy with resection of the gland to the left of the mesenteric vessels, and a total pancreatectomy. Total pancreatectomy has earlier been avoided due to difficult diabetic control (brittle diabetes), but with modern insulin treatment and substitution with pancreatic enzymes a total pancreatectomy is widely accepted. Even aggressive surgery with curative intent in locally advanced tumors including nearby organ involvement may lead to a 5-year survival of up to 80% (Fischer *et al.*, 2008).

Enucleation of tumor is an option in benign pNETs, preferably insulinomas, but the risk of postoperative fistulas is up to 30% (Brient *et al.*, 2012). However, enucleation of a benign pNET in the head of the gland may sometimes be preferred rather than a pancreaticoduodenectomy, if the tumor is less than 2 cm and if the two main pancreatic ducts can be left intact. Malignant tumors and tumors with uncertain behavior should never be enucleated due to uncertain radicality. A central pancreatic resection with anastomosis to the cephalic and caudal parts of the gland is favored by some centers to conserve glandular tissue.

Special consideration should be offered pNENs in patients with MEN1. Patients usually have multiple tumors in the gland, and in case of gastrinomas, in the duodenum as well, where they account for the major part. Long time follow up has shown, however, that even though metastases to local lymph nodes are present, liver metastases are seldom seen, if tumors are less than 2 cm. Therefore, operation is only advisable when tumors exceed 2 cm to avoid hepatic metastases (Falconi *et al.*, 2016). An even lower propensity to distant metastases has been observed in patients with Von Hippel-Lindau disease and pNENs, for which reason these tumors should be followed.

The occasional finding of small nonfunctioning pNENs (incidentalomas) has increased in line with the frequent use of improved CT and MRI scans. Most incidentalomas are probably benign and may be observed rather than resected. ENETS guidelines advise expectancy if tumor is found incidentally, is less than 2 cm and remains unaltered in size. A CT or MRI scan every 6 months is mandatory, EUL is not recommended due to inter- and intraobservation variability. If tumor increases in size more than 0.5 cm or exceeds 2 cm in diameter, operation is advised (Falconi *et al.*, 2012).

Other investigators have warned against this procedure, since metastases have been recorded in patients with incidentalomas less than 2 cm. Expectancy should only be offered elderly patients as well as patients with severe comorbidity, and close follow-ups with CT or MRI scan and a biopsy for estimation of Ki67 proliferation index is indicated.

pNETs with multiple metastases are seldom curable, but operation may still be an option, if metastases are few and resectable, or if debulking may relieve the patient from local and/or endocrine symptoms. Resection of the primary tumor with radical resection of liver metastases has survival rates of up to 76% in well-differentiated tumors (Ki67 $< 5\%$) compared to a 5-year survival of 30%–40% in untreated cases (Partelli *et al.*, 2015). However, tumor recurrence is up to 75% within 2 years after surgery.

The rate of cure is higher in patients with liver metastases from functioning pNENs than nonfunctioning (90% vs. 40%) (Bagante *et al.*, 2017). Resection of the primary together with major synchronous liver surgery should be avoided due to increased complications and mortality. Hence liver resection should be postponed to a second operation.

Resection of a primary pNET in the presence of nonresectable hepatic metastases is controversial. Contrary to small intestinal NENs, ENETS guidelines do not recommend palliative resection of pNETs. However, studies have shown that palliative resection of the primary in the setting of nonresectable metastatic disease may increase survival, but data are from retrospective and heterogeneous cohorts (Keutgen *et al.*, 2016). Only patients with localized pNECs (G3 tumors, Ki67 $> 20\%$) are candidates for surgery. However, most have distant metastases at diagnosis and should follow oncologic protocols.

Conventional ablative techniques such as radio-frequency ablation (RFA) and microwave ablation (MWA) can be used to treat liver metastases, but should be avoided in pNETs due to the risk of injury to the pancreatic duct, the superior mesenteric vessels, and duodenum. Ablative techniques for treatment of hepatic metastases from pNETs should only be used in palliation or in combination with liver resection to conserve liver tissue. RFA is restricted to metastases <4 cm in diameter and <8–10 metastases in total. The effect of RFA is reduced in metastases close to the major intrahepatic vessels due to heat loss. Microwave ablation is a nonthermal procedure and metastases up to 7 cm and close to the hepatic vessels can be treated (de Baere *et al.*, 2015; Pavel *et al.*, 2012).

Irreversible electroporation (IRE) is another nonthermal ablative therapy based on the delivery of high-voltage electric current through electrodes positioned adjacent to the tumor. This causes irreversible injury to tumor cells but with conservation of vessels, nerves, and ductal structures. IRE can be used in the palliative treatment of pNETs and liver metastases.

Liver metastases have their blood supply from the hepatic arteries. Liver embolization via the hepatic arteries results in tumor necrosis and can be performed as bland- or chemo-embolization in patients with numerous liver metastases not suited for surgical resection or ablation (Pavel *et al.*, 2012). Embolization can be repeated but is contra-indicated in patients with occlusion of the portal vein (liver failure), previous Whipple operation or biliary-intestinal anastomosis (infection and liver abscess) and relatively contra-indicated if the hepatic tumor burden is $>75\%$ of the total liver volume. Selective internal radiation therapy (SIRT) with 90Y-microspheres is another option for treatment of liver metastases. Radiological tumor response rates are seen in 60% (Pavel *et al.*, 2012).

Medical Treatment

Low-Grade pNETs

A somatostatin analog (SSA) is first-line therapy for symptom control and antitumor treatment in functioning and nonfunctioning pNETs. It may also be used as add-on therapy in combination with other antiproliferative therapies. Octreotide and lanreotide are considered equally effective for symptom control. In general, long-acting formulations (octreotide LAR 30 mg/4 weeks; lanreotide autogel 120 mg/4 weeks) are used over a medium- to long-term period. Symptomatic and biochemical response is seen in the majority of the patients (Modlin *et al.*, 2010b; Pavel *et al.*, 2016). Dose escalation may be required and can be achieved by shortening the injection interval from 4 to 3 weeks with long-acting SSA (Al-Efraij *et al.*, 2015).

In nonfunctioning pNETs, treatment with lanreotide autogel results in superior progression-free survival (PFS) compared to placebo (24 vs. 18 months) (Caplin *et al.*, 2014). Treatment with SSA is therefore indicated in functioning and nonfunctioning pNETs with Ki67-index $<10\%$. Side effects of SSA are usually mild, and may include abdominal pain, diarrhea, flatulence, nausea, injection site reactions, and development of bile stones (Modlin *et al.*, 2010b).

Interferon alpha (IFN) may reduce tumor growth and hormone-induced symptoms. The dose should be titrated individually ranging between 3 and 5 MU SC three to five times weekly for recombinant IFN and 50–150 μg SC weekly for pegylated IFN. IFN may be used as an add-on to SSA. Side-effects to IFN include flu-like symptoms and fatigue. Patients with autoimmune diseases or a history of mental disorder should not be treated with IFN (Pavel *et al.*, 2006).

Streptozocin (streptozotocin, STZ) in combination with 5-fluorouracil (5FU) is often used as first-line chemotherapy for advanced pNETs with Ki67 index above 10%. It is administrated as a 5-day induction course followed by 1-day infusions every third week. Response rates of up to 40% with PFS of up to 20 months and median survival of 2 years have been reported. STZ is nephrotoxic. 5FU may be replaced by doxorubicin. However, due to the risk of cumulative cardiotoxicity and the resulting limited possible treatment time, this option is rarely used (Janson *et al.*, 2014; Moertel *et al.*, 1980).

Temozolomide may also be considered in the treatment of advanced pNETs with Ki67 $>$ 10%, either as monotherapy or in combination with capecitabine. Retrospective data showed a response rate of 70% and PFS of 18 months with the combination of temozolomide and capecitabine (Strosberg *et al.*, 2011). In a retrospective analysis of patients who received various temozolomide-based treatments, one-third experienced partial response with a median PFS of 14 months (Kulke *et al.*, 2009). Toxicity is usually mild with nausea and hematological toxicity.

The targeted therapies, everolimus (mTor inhibitor), and sunitinib (multikinase VEGFR inhibitor) are both approved antiproliferative therapies in progressive pNETs, and thus represent different treatment options next to SSA and systemic chemotherapy. In two prospective, randomized trials, treatment with these agents doubled the PFS from approximately 6–12 months compared to placebo in patients with progressive well and moderately differentiated pNETs (Raymond *et al.*, 2011; Yao *et al.*, 2011).

The toxicity profile of the two agents are different and therefore may influence the choice of treatment: Common side effects associated with everolimus are stomatitis, rash, diarrhea, hyperglycaemia, and fatigue, whereas patients on sunitinib may experience rash, diarrhea, nausea, vomiting, asthenia, hypertension, and fatigue.

High-Grade pNECs

Untreated, patients with metastatic G3 pNECs (Ki67 $>$ 20%) have very short expected survival time. Treatment with cis- or carboplatin and etoposide has for long been the mainstay in advanced NECs, including pNECs. The treatment is associated with an objective response rate above 30%, disease control in two thirds of the patients with a median of 4–5 months, and an overall survival time of median 10–14 months. Side-effects include emesis, bone marrow suppression, nephro- and neurotoxicity and may be both dose limiting and serious. A Ki67 index below 50%–55% is usually associated with a lower objective response rate to platinum-based chemotherapy. Hence, other regimens could be considered, such as temozolomide- or 5FU-based therapies (Sorbye *et al.*, 2013).

There are no established second-line therapies for pNECs. Temozolomide-based therapy, irinotecan, topotecan, and 5FU-bases combinations (FOLFIRI/FOLFOX) have showed moderate efficacy and may thus be considered, taking performance status and organ function into consideration (Hentic *et al.*, 2012; Strosberg *et al.*, 2010).

The aggressive behavior of pNECs warrants consideration of adjuvant therapy after radical surgery. Although definite data are missing, four to six cycles of cis/carboplatin and etoposide should be considered (Sorbye *et al.*, 2013). Prophylactic cerebral irradiation is not recommended.

Specialized Symptomatic Treatment

Insulinomas

Diazoxide (50–600 mg/day) may control attacks of hypoglycemia by inhibition of insulin release from the beta-cells. However, diazoxide has side-effects such as edema and weight gain. Somatostatin analogs may prevent hypoglycemia, but may in some cases also worsen hypoglycemia. In selected patients, everolimus has been shown to prevent hypoglycemia. Glucocorticoid or growth hormone administration may reduce severe hypoglycemia. Some patients require continuous glucose infusion to keep blood glucose levels normal (Jensen *et al.*, 2012).

Gastrinomas

Proton pump inhibitors (PPI) are the first line of treatment in patients with the Zollinger–Ellison syndrome. Often high and frequent dosing is required to control gastric acid secretion. Treatment should not be paused as ulcer perforation or bleeding may occur. SSA may be added to the PPI treatment with significant symptomatic effect by inhibition of the gastrin secretion (Jensen *et al.*, 2012).

In rare functioning pNETs such as glucagonomas, VIPomas and somatostatinomas, treatment with somatostatin analogs may significantly diminish the hormone induced symptoms. Treatment with long acting SSA may be supplemented with short acting

octreotide or interferon alpha. Patients with uncontrollable symptoms from a VIPoma may require continuous electrolyte infusion (Jensen *et al.*, 2012).

Peptide Receptor Radionuclide Therapy

Somatostatin SST2 receptors are expressed in >90% of NETs. This is utilized in peptide receptor radionuclide therapy (PRRT) with the beta-emitter ^{90}Yttrium (^{90}Y -DOTATOC) or the beta- and gamma-emitter ^{177}Lutetium (^{177}Lu-DOTATATE). Prerequisites of PRRT are a higher up-take in tumors and metastases than physiologic liver up-take, a normal kidney function, limited amount of bone metastases and a Ki67 < 30%. PRRT with ^{90}Y are given in two to three cycles with 6–8 weeks interval and ^{177}Lu in 4 cycles with 8 weeks interval. Additional cycles depend on bone marrow and renal toxicity. Following PRRT complete response is seen in <5%, partial response in 10%–35%, minor response + stable disease in 50%–80% and progressive disease in 10%–20%. A tardive response may be seen 6–12 months after PRRT. The median PFS is 15–30 months and the median OS 30–50 months. Pancreatic NETs seem to respond better than small intestinal NETs (Kjaer and Knigge, 2015). Concomitant treatment with somatostatin analogs may have an additive effect on PFS and OS (Strosberg *et al.*, 2017).

Temporary or persistent renal toxicity is seen in about 35% of the patients. Risk factors of kidney damage are hypertension, diabetes mellitus, and previous chemotherapy. Bone marrow toxicity is seen in about 80%, less than 10% severe. Kidney and bone marrow toxicity is more rare in patients treated with ^{177}LU than with ^{90}Y. Development of myelodysplastic syndrome or leukemia is seen in less than 1% of patients (Kjaer and Knigge, 2015).

Follow-up

All patients with functioning and nonfunctioning pNENs should have life-long clinical follow-up at a specialized NET Center. An exception is radically resected benign insulinomas (Knigge *et al.*, 2017).

Imaging with thoraco-abdominal-pelvic CT or abdominal-pelvic MRI is performed every 6–12 months for G1/G2 pNETs. For G3 pNECs thoraco-abdominal-pelvic CT is performed every 3–6 months. In patients with curatively treated G3 pNECs CT/MRI is maximally performed for 5 years. If recurrence or progression occurs tumor biopsy is often required for a new Ki67 determination, as tumors may undergo a more malignant transformation. If positive on somatostatin receptor imaging (SRI) at diagnosis, SRI may be performed every other year, but the significance of SRI has not been evaluated.

Patients with radically resected solitary G1/G2 insulinomas only require one follow-up with measurement of insulin, C-peptide, pro-insulin, and chromogranin A after 3–6 months. Further follow-up is only required if symptoms recur. Patients with nonresected or metastatic G1/G2 insulinomas should have follow-up as described below.

Patients with other resected, nonresected or metastatic G1/G2 functioning tumors (e.g., gastrinomas, glucagonomas, VIPomas, somatostatinomas) should have clinical follow-up with relevant blood samples every 3–6 months. This includes tumor specific hormones and CgA. In patients with gastrinoma vitamin B12, ionized calcium, and PTH should also be measured. Gastric pH measurement or secretin test may be performed, if symptoms recur in resected patients.

Patients with nonfunctioning resected, nonresected or metastatic G1/G2 NETs should have clinical follow-up with CgA measurement every 3–6 months.

All patients with G3 NECs independent of resection should have clinical follow-up with CgA and relevant hormones every third month.

Prognosis

The prognosis for pancreatic NENs depends on dissemination and pathologic grade (Ki67). Other factors are advanced age, bone metastases and rapid progression of liver metastases. Furthermore, patients with a positive FDG-PET have a poorer survival.

Functioning pNETs

Patients with benign insulinomas are cured after radical resection. In malignant insulinomas with liver metastases the OS is less than 2 years. The OS of gastrinoma patients with local disease is almost 100% after resection but the presence of liver metastases reduces the 5-year survival to 45%.

In patients with rare functioning tumors survival is equivalent to survival in patients with nonfunctioning NETs (Jensen *et al.*, 2012).

Nonfunctioning pNETs

The 5-year survival rate is around 43% and the median OS is 38 months. In patients with localized disease, regional and disseminated disease the median OS is 124, 70, and 23 months, respectably (Falconi *et al.*, 2012; Yao *et al.*, 2008).

pNECs

For patients with pNECs the median OS is 10–20 months (Sorbye *et al.*, 2013).

See also: Enterochromaffin-Like Cells. Gastrointestinal Neuroendocrine Tumor Syndromes (GI NETS). Somatostatin Receptor Expression in Gastrointestinal Tumors

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Peptide Neurotransmitters and Smooth Muscle in the Gut☆

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Glossary

Circular muscle layer Innermost layer of smooth muscle in which individual muscle cells are oriented radially. Contraction of this muscle decreases the diameter of the gut and increases intraluminal pressure.

Enteric nervous system A network of two ganglionated neural plexuses contained wholly within the wall of the gut; also called the intrinsic nervous system.

Excitatory motor neuron Neuron of the enteric nervous system that projects into the muscle layer and releases a neurotransmitter that causes contraction of muscle.

Inhibitory motor neuron Neuron of the enteric nervous system that projects into the muscle layer and releases a neurotransmitter that causes relaxation of muscle.

Interneuron Neuron of the enteric nervous system that projects to other neurons of the enteric nervous system and releases a neurotransmitter that modulates the activity of other enteric neurons.

Longitudinal muscle layer Outermost layer of smooth muscle in which individual muscle cells are oriented along the oral–anal axis. Contraction of this muscle layer shortens the gut.

Myenteric plexus One of the two ganglionated plexuses of neurons that comprise the enteric nervous system. This plexus is located between the inner circular and outer longitudinal muscle layers.

Sensory neuron Neuron that is activated by a stimulus, such as stretching of the gut wall or chemical stimulation of the mucosa, and that in turn activates other neurons within the enteric nervous system.

Introduction

The debate initiated at the beginning of the 20th century regarding the relative importance of hormones and nerves in the gut was concluded at the end of the century with the recognition that peptide and nonpeptide neurotransmitters are the main regulators of function in the gut. Six peptide hormones [gastrin, cholecystokinin (CCK), secretin, motilin, and the glucose-dependent insulinotropic peptides (GIP) and glucagon-like peptide-1 (GLP-1)] are undoubtedly physiological, but their secretion is regulated, and their actions are usually mediated or modulated by enteric neurotransmitters. Two of these hormones, CCK and motilin, play physiological roles in smooth muscle. All other muscular functions are regulated by neurotransmitters, including tone and sphincteric activity, electrical and mechanical rhythmic activity, and the initiation and termination of intestinal peristaltic activity. Two neurally mediated functions have been attributed to circulating CCK: (i) postprandial stimulation of gall bladder contraction and sphincter of Oddi relaxation and (ii) activation of the sensory limb of a long reflex arc that inhibits gastric tone and slows emptying. Gall bladder contraction is largely mediated by CCK-induced release of acetylcholine (ACh) from cholinergic neurons, whereas sphincter relaxation is mediated by release of inhibitory neurotransmitters [vasoactive intestinal peptide (VIP) and nitric oxide (NO)] from noncholinergic neurons.

Motilin is the only hormonal or neural peptide whose main function is interdigestive. It is released in cycles that coincide with the start of the migrating myoelectric complex. Peaks of circulating motilin coincide with phase 3 of the cycle, a short period of intense motor activity that sweeps residual contents of the intestine caudad. Immunoneutralization of circulating motilin disrupts phase 3 activity; conversely, infusion of motilin in concentrations that mimic circulating levels triggers premature phase 3 activity. Neural activity can modulate motilin levels and motor activity, but the precise mechanisms are not known.

Peptide Neurotransmitters and Smooth Muscle Activity

Neural Organization

Neurons of the enteric nervous system, especially neurons of the myenteric plexus, modulate the intrinsic electrical and mechanical properties of smooth muscle. They constitute the final relay to smooth muscle cells and interstitial cells of Cajal; the latter appear to be interposed between motor nerve terminals and smooth muscle cells in some regions of the gut. Extrinsic adrenergic and peptidergic neurons in pre- and paravertebral ganglia synapse with and modulate the activity of enteric neurons.

Neurons of the myenteric plexus located between the circular and longitudinal smooth muscle layers fall into two broad categories. Approximately 25% contain VIP and/or its homologue, pituitary adenylate cyclase activating peptide (PACAP), often found together with neuronal nitric oxide synthase (nNOS). More than 60% contain ACh, often found together with the

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tachykinins, substance P (SP) and neurokinin A (NKA); the latter are derived from the same precursor, β-protachykinin. VIP neurons also contain a homologous peptide derived from the same precursor designated PHI in animals or PHM in humans. There is little or no overlap between neurons of these two categories. Acetylcholine and the tachykinins are the major excitatory motor neurotransmitters, whereas VIP and its homologs and NO are the major inhibitory neurotransmitters. The term inhibitory refers to their ability to inhibit (i.e., relax) smooth muscle tone and rhythmic electrical and contractile activity.

Pharmacology of Neurotransmitters

VIP, PACAP, and PHI/PHM

Receptors for these peptides are present on both smooth muscle cells and neurons. VIP and PACAP interact with $VPAC_2$ receptors on smooth muscle cells, which are coupled via G_s to stimulation of cAMP formation and inhibition of muscle tone and rhythmic activity. Neural VIP/PACAP receptors are present in two locations: on nerve terminals, where they act as autoinhibitory receptors to suppress further neurotransmitter release, and on cholinergic/tachykinin motor neurons that innervate the longitudinal muscle layer: activation of these receptors upon release of VIP and PACAP from a subpopulation of myenteric interneurons stimulates release of ACh and tachykinins and induces longitudinal muscle contraction.

The discovery of VIP (and related peptides) in myenteric motor neurons, and its release from these neurons by electrical field stimulation leading to relaxation of circular smooth muscle that was selectively suppressed by VIP antiserum or VIP antagonists entitled it to consideration as an inhibitory neurotransmitter. The subsequent discovery of nitric oxide synthase in the same or adjacent neurons, and the release of NO from these neurons by electrical field stimulation leading to muscle relaxation that was selectively inhibited by NOS inhibitors raised the possibility that VIP and NO act as functionally linked inhibitory (relaxant) co-transmitters. It soon became clear that the linkage between VIP and NO was more complex: NO produced in nerve terminals regulates: 1) ICC and smooth muscle cell activity, and 2) VIP release in the same or adjacent nerve terminals; 3) VIP, in turn, regenerates NO in target smooth muscle cells by activating a distinct constitutive, Ca^{2+}/calmodulin-dependent NOS. The signaling cascade that leads to this outcome is detailed below (**Fig. 1** and **Table 1**). Evidence for this dual source of NO was derived from studies in neuronal preparations (i.e., isolated myenteric plexus and enriched synaptosomes) devoid of smooth muscle cells, and in isolated smooth muscle preparations devoid of neural elements. Studies on whole muscle tissue preparations can lead to flawed interpretations unless the dual source of NO and the role of VIP are taken into consideration.

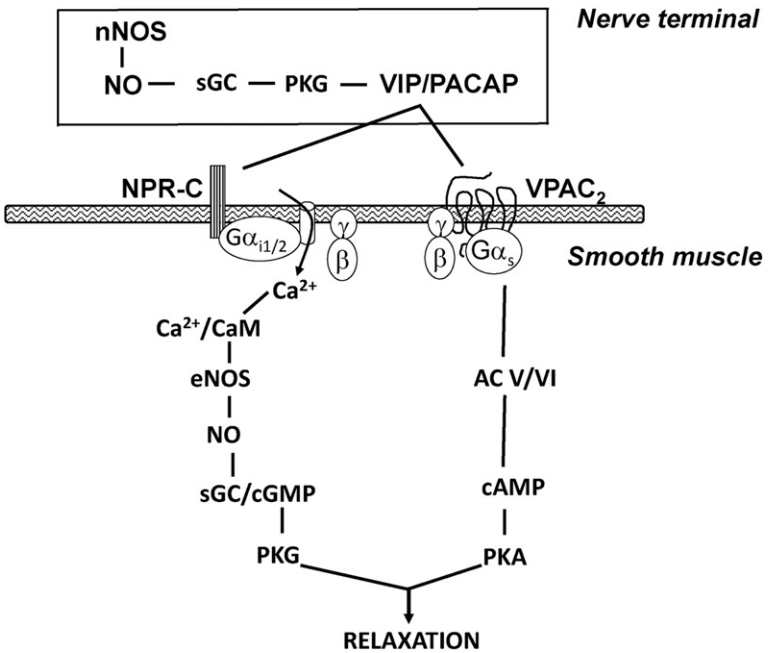

Fig. 1 Model depicting interplay of NO and VIP/PACAP in nerve terminals and smooth muscle cells. NO produced by nNOS in nerve terminals stimulates VIP/PACAP release in the same or adjacent nerve terminals via a pathway involving activation of soluble guanylate cyclase (sGC), formation of cGMP, and activation of cGMP-dependent protein kinase (PKG). VIP and PACAP release can occur independently of NO at higher intensities of nerve stimulation. In smooth muscle cells, VIP and PACAP interact with cognate $VPAC_2$ receptors and with the single-transmembrane natriuretic peptide receptor NPR-C, which is abundantly expressed in smooth muscle cells of the gut. Interaction with $VPAC_2$ receptors coupled to G_s leads to activation of adenylate cyclase V/VI, cAMP formation, and activation of PKA. Interaction of VIP and PACAP with NPR-C initiates a cascade involving $G_{i1/2}$-dependent Ca^{2+} influx, activation of a Ca^{2+}/calmodulin-dependent constitutive NOS, generation of NO, and activation of the sGC/cGMP/PKG pathway. PKA and PKG act together to cause relaxation and sustained hyperpolarization.

Table 1 Effect of various agents on signaling cascades activated by VIP and PACAP in isolated smooth muscle cells

	GDPβS	*PTx*	*Nifedipine*	*Calmida- zolium*	*L-NNA*	*LY83583*	*KT-5823*	*Myr-PKI*
Ca^{2+}	I	I	I	Nil	Nil	Nil	Nil	Nil
NO	I	I	I	I	I	Nil	Nil	Nil
cGMP	I	I	I	I	I	I	Nil	Nil
cAMP	I	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Relaxation	I	P/I	P/I	P/I	P/I	P/I	P/I	P/I

NO stimulates VIP release from myenteric neurons: Studies on the myenteric plexus isolated from guinea pig, rabbit, and opossum intestine showed that NO added exogenously or produced endogenously by myenteric neurons stimulates VIP release, whereas VIP added exogenously has no effect on NO production. VIP release was abolished by the NOS inhibitor, N^G-nitro-L-arginine (L-NNA), as well as by the soluble guanylyl cyclase (sGC) inhibitor, LY83583, and the cyclic GMP-dependent protein kinase (PKG) inhibitor, KT5823. A similar NO/sGC/PKG pathway triggered by NO donors or cGMP analogues was shown to elicit VIP release from enriched synaptosomes of rat intestine, whereas exogenous VIP had no effect on NO production.

VIP stimulates NO production in isolated smooth muscle cells: The ability of VIP to stimulate NO production has now been demonstrated in smooth muscle cells isolated from various regions of the gastrointestinal tract (gastric fundus, small intestine, colon, esophageal and internal anal sphincters) in several species including human, dog, rabbit, opossum, guinea pig, rat, and mouse. VIP-stimulated NO production is inhibited by NOS inhibitors (e.g., L-NNA) and the VIP antagonist VIP10-28, but is not affected by oxyhemoglobin, which neutralizes extracellular NO, clearly implying that VIP-stimulated NO production originates in smooth muscle cells.

The receptor with which VIP and PACAP interact to generate NO in smooth muscle cells has been identified as the natriuretic peptide receptor C (NPR-C), a single-transmembrane receptor which is widely expressed in smooth muscle cells of the gut and is coupled to G_{i1} and G_{i2}. Detailed cellular and molecular studies have shown that interaction of VIP or PACAP with NPR-C leads to $G_{i1/2}$-dependent activation of eNOS and stimulation of NO formation in smooth muscle cells. The signaling pathway depicted in **Fig. 1** and analyzed in **Table 1**, is initiated by $G_{i1/2}$-dependent Ca^{2+} influx, and Ca^{2+}/calmodulin-dependent activation of a constitutive NOS (eNOS) and NO formation. NO activates sGC leading to cGMP formation and activation of PKG. Interaction of VIP and PACAP with $VPAC_2$ receptors initiates a parallel pathway involving sequential activation of adenylyl cyclase, cAMP formation, and activation of PKA. Both pathways converge to regulate muscle relaxation. With few exceptions, all actions attributed to VIP in this summary review are applicable to PACAP: both peptides interact with equal affinity with $VPAC_2$ receptors and NPR-C. The G protein-activating domain of NPR-C has now been identified as a 17-amino acid sequence in the middle region of the intracellular domain and comprises two arginine residues at the N-terminal and a B-B-X-X-B motif, where B and X are basic and non-basic residues, at the C-terminal [R^{469}*RNHQEESNIGKHRELR*^{485}]. Recent reconstitution studies in COS-1 cells and Xenopus oocytes confirm the ability of VIP, ANP, and the selective NPR-C ligand, cANP(4-23) to compete for binding to NPR-C. In cells from kidney, aorta, and heart that normally express NPR-C, natriuretic peptides activate eNOS via NPR-C.

The table summarizes evidence for the signaling cascades depicted in **Fig 1**. As expected, GDPβS inhibits all responses mediated by G protein-coupled receptors, whereas pertussis toxin (PTX), nifedipine, the calmodulin inhibitor calmidazolium, and L-NNA inhibit responses mediated by G_i-coupled NPR-C. LY83583 (cGMP inhibitor), KT5823 (PKG inhibitor), myristoylated PKI (PKA inhibitor). All inhibitors block only one pathway and elicit only partial inhibition (p/I, 45–55% inhibition) of relaxation. Together PKA and PKG inhibitors abolish relaxation. Oxy-hemoglobin had no effect implying that NO was generated within the muscle cells.

A cautionary note: A study by Lefebvre and coworkers (J.M.C. Dick et al, Br. J. Pharmacol. 129: 751–763, 2000) appeared to show that relaxation elicited by VIP in isolated smooth muscle cells was inhibited by both L-NNA and 1400 W, an inhibitor of inducible NOS (iNOS). The effect of 1400 W in this study was probably non-specific, since 1400 W also inhibited relaxation elicited by a wide variety of unrelated agents including isoprenaline, forskolin, sodium nitroprusside, and ANP (see Table 2 of this reference). It is worth noting that smooth muscle cells isolated from inflamed organs often express iNOS; in order to avoid introducing artefacts, it is essential to study smooth muscle cells isolated only from uncontaminated tissues.

Linkage of VIP release and NO production in innervated muscle strips: The two properties outlined above (VIP-stimulated NO production in muscle cells and NO-stimulated VIP release from nerve terminals) are reflected when measurements are made in innervated muscle strips. In circular muscle strips of rabbit gastric fundus, NO stimulated VIP release and elicited relaxation in a concentration-dependent fashion (threshold: 0.1 nM NO). Conversely, VIP stimulated NO production and elicited relaxation in a concentration-dependent fashion (threshold: 10 pM VIP). NO production stimulated by VIP was abolished and relaxation was partly inhibited ($\sim$50%) by L-NNA; oxyhemoglobin had no effect implying that NO generated by the action of VIP in smooth muscle cells did not traverse the extracellular space. Residual relaxation reflected the ability of VIP to interact with $VPAC_2$ receptors and elicit relaxation via a cAMP/PKA pathway.

Role of VIP and NO in inhibitory neurotransmission: *Inhibitory junction potential* (*IJP*): Smooth muscle of the gut exhibits sustained tone on which are superposed rhythmic contractions determined by the amplitude and frequency of slow waves that originate in pacemaker cells (ICC), located at the boundaries of the circular muscle layer. Muscle tone is determined by excitatory and inhibitory neurotransmitters diffusing from nerve terminals (varicosities) to regulate ionic channel activity and initiate receptor-

mediated signaling cascades in smooth muscle cells. The precise location of some channels cannot be determined because smooth muscle exists in a syncytium coupled electrically to ICCs and fibroblast-like PDGFRα^+ cells. Inhibitory neural input predominates in some locations (e.g., murine colonic circular muscle) where it attenuates tone and phasic activity: these are unmasked in the presence of tetrodotoxin, NOS inhibitors, or VIP antagonists and antisera. Excitatory neural input in the form of an excitatory junction potential or EJP depolarizes membrane potential, and enhances Ca^{2+} influx and the amplitude or frequency of corresponding rhythmic contractions. Inhibitory neural input in the form of an inhibitory (i.e., hyperpolarizing) junction potential or IJP has the reverse effect.

Inhibitory junction potentials (IJPs) consist of three components: an initial fast, high amplitude hyperpolarization, a slower, lower amplitude hyperpolarization, followed by a sustained low amplitude hyperpolarization of long duration. The three phases appear to reflect the release of purinergic (ATP or a preferential $P2Y_1$ agonist), nitrergic (NO), and peptidergic (VIP/PACAP) neurotransmitters. Recent studies have shown that the initial fast, high amplitude component reflects $P2Y_1$-mediated activation of small-conductance, Ca^{2+}-activated K^+ channels (SK channels) located in PDGFRα^+ cells, which are electrically coupled via gap junctions to ICCs and smooth muscle cells. The second nitrergic component reflects NO release from nerve terminals and/or VIP-mediated NO release from smooth muscle cells; suppression of this component by NOS inhibitors does not distinguish between these two sources of NO. The last long-duration component (evident in esophageal and internal anal sphincter muscle) represents a direct effect of VIP on smooth muscle cells, since it can be reproduced by exogenous VIP, is suppressed by VIP antagonists, and is absent in $VIP^{-/-}$ animals. This component also is sensitive to NOS inhibitors suggesting that it may reflect in part the ability of VIP to stimulate NO production in smooth muscle cells. Recent studies suggest that neuronal NO activates preferentially ICCs, from where the electrical signal is relayed to smooth muscle cells. This challenging hypothesis remains controversial and appears to preclude signaling by NO and VIP in smooth muscle cells.

Interplay of VIP and NO during nerve stimulation: *relaxation of tone*: The transient nature of IJPs does not permit concurrent measurements of NO and VIP. Thus, while pharmacological analysis of IJPs demonstrates the participation of both NO and VIP, it does not offer decisive evidence for their functional linkage. The latter is easily demonstrable in preparations where relaxation of resting tone is induced by electrical field stimulation. Defective experimental techniques such as the use of contractile agents to raise muscle tone have led to conflicting results, mainly because these agents stimulate protein kinase C, which in turn inactivates smooth muscle NOS.

Exclusive reliance on NOS inhibitors to discern the source of NO, without direct measurement of NO and VIP, leads to flawed conclusions. To avoid this pitfall, Jin et al. made concurrent measurements of VIP release, NO production, and muscle relaxation in gastric muscle strips from rabbit and rat fundus, combined with judicious use of inhibitors to identify the dual source of NO and demonstrate the interplay of VIP and NO in mediating relaxation of resting tone induced by a wide range of electrical field stimulation (EFS; 0.025-16 Hz; 3–960 pulses). The results obtained in both species were similar and only those obtained in rabbit are outlined below. EFS caused frequency-dependent increase in VIP release, NO production, and muscle relaxation, all of which were significant at the lowest stimulus (0.025 Hz/3 pulses). L-NNA abolished NO production at all frequencies; abolished VIP release and relaxation at frequencies below 0.5–1 Hz, and partly inhibited VIP release ($\sim$50%) and relaxation ($\sim$70%) at the highest frequency (16 Hz). Thus, both VIP release and relaxation were entirely dependent on NO at lower frequencies, and only partly dependent on NO at higher intensities of stimulation. Residual relaxation in the presence of L-NNA represented the effect of VIP acting directly on muscle cells via NO-independent mechanisms. The VIP antagonist, VIP10-28, abolished NO production and muscle relaxation at frequencies below 0.5–1 Hz, and partly inhibited NO production ($\sim$80%) and muscle relaxation (44%) at 16 Hz. Oxyhemoglobin inhibited VIP release and muscle relaxation, especially at low frequencies implying that some of the NO mediating VIP release traversed the extracellular space to adjacent VIP-containing nerve terminals. Thus, recognizing the ability of NO to regulate VIP release and of VIP to stimulate NO production in muscle cells is essential in interpreting their respective roles in inhibitory neurotransmission. Inhibition of relaxation by NOS inhibitors cannot be attributed solely to suppression of neuronal NO production, since NOS inhibitors also suppress VIP release stimulated by NO in nerve terminals as well as NO production stimulated by VIP in smooth muscle cells. Similarly, inhibition of relaxation by VIP10-28 cannot be attributed solely to inhibition of VIP's action on muscle cells via a $VPAC_2$/cAMP/PKA pathway, since VIP10-28 also blocks the ability of VIP to cause relaxation in muscle cells by generating NO via interaction with NPR-C and activation of the cGMP/PKG pathway.

Analysis of the peristaltic reflex confirms the interplay of VIP/PACAP and NO under physiological conditions, that is where neural simulation is applied by radial stretch or mucosal stroking (see below). Both types of stimuli elicit caudad muscle relaxation accompanied by VIP and PACAP release and NO production. NOS inhibitors (L-NNA) abolish NO production and partly inhibit VIP and PACAP release and relaxation. VIP10-28 strongly inhibits NO production and relaxation. As discussed above, the pattern clearly illustrates the interplay of VIP/PACAP and NO and further underlines the caution required in interpreting the effect of inhibitors, particularly NOS inhibitors.

SP and NKA

SP and NKA interact preferentially with NK_1 and NK_2 receptors on smooth muscle cells and nerve terminals. NKB, the preferred ligand for NK_3 receptors, is the product of a precursor that is virtually absent from the gut. NK_1 and NK_2 receptors are coupled via G_q to IP_3-dependent Ca^{2+} release and contraction in circular muscle and to arachidonic acid-mediated Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release in longitudinal muscle. The receptors stimulate ACh release from cholinergic neurons and induce a contraction that is superimposed on the direct contraction.

Enkephalin and Dynorphin

Opioid neurons of the myenteric plexus release a variety of peptides derived from two precursors: proenkephalin, which yields [Met]enkephalin and C-terminally extended derivatives, and prodynorphin, which yields α- and β-endorphin and dynorphin-17; the latter is processed to smaller dynorphin fragments and eventually to [Leu]enkephalin. [Met]enkephalin and its derivatives are much more abundant than dynorphin and its derivatives in the gut. Three opioid receptor types (μ, δ, and κ) are present on circular smooth muscle cells only where they mediate contraction. In addition, μ receptors on cholinergic neurons mediate inhibition of ACh release, and δ receptors on inhibitory neurons mediate inhibition of NO, VIP, and related peptides. The neural receptors are most relevant functionally, particularly δ receptors on inhibitory neurons. When opioids are injected *in vivo* or added to smooth muscle strips *in vitro*, they produce a transient increase in muscle tone by direct action on smooth muscle cells and a sustained increase in rhythmic, nonpropulsive contractile activity that reflects the suppression of a predominant inhibitory neural input.

Somatostatin

Somatostatin is present exclusively in a small population of myenteric interneurons, where it serves as a relay in the peristaltic reflex. Although somatostatin receptors that mediate contraction are present on circular muscle cells, they lack functional relevance in the absence of muscle innervation by somatostatin neurons.

CGRP

In the stomach, CGRP-containing nerve fibers are extrinsic primary afferent fibers, whereas in the intestine and colon they are both extrinsic and intrinsic; the latter mediate the peristaltic reflex induced by mucosal stimuli. CGRP is present in other neurons, but its function is unknown. CGRP receptors on muscle cells are reported to cause direct relaxation.

In summary, neurotransmitters of functional importance are the excitatory motor neurotransmitters, ACh and tachykinins; the inhibitory motor neurotransmitters, NO, VIP and its homologues; the modulatory neurotransmitters, opioids and somatostatin; and the sensory neurotransmitter, CGRP. Excitatory motor neurotransmitters depolarize and inhibitory motor neurotransmitters hyperpolarize membrane potential. At an appropriate threshold, depolarization opens voltage-sensitive Ca^{2+} channels, resulting in Ca^{2+} influx, an increase in both tone and the amplitude or frequency of rhythmic contractions. Hyperpolarization affects mainly rhythmic activity, which is either decreased or suppressed, but can affect tone in regions where membrane potential is already depolarized.

The Peristaltic Reflex: Interplay of Motor, Modulatory, and Sensory Neurotransmitters

The functional role of peptide and nonpeptide neurotransmitters is best exemplified by their coordinated release during the intestinal peristaltic reflex (Fig. 2). The reflex can be evoked by mechanical or chemical stimulation of the mucosa (stroking or pH change) and by radial muscle stretch. The reflex consists of a descending phase, during which circular muscle relaxes and longitudinal muscle contracts caudad to the site of stimulation, and an ascending phase, during which circular muscle contracts and longitudinal muscle relaxes. Two types of *in vitro* preparations have been used to study the reflex that permit simultaneous measurement of mechanical response and neurotransmitter release. In one preparation, a hollow segment of intestine is stretched at the caudad or orad end, and mechanical response is measured as the medium is sampled for neurotransmitter release. In the other, the segment is opened and pinned as a flat sheet, separated into three compartments. The reflex is evoked by stimulation in the central compartment, while mechanical response and neurotransmitter release in each compartment is measured.

Neurotransmitter Release during the Ascending and Descending Phases of the Reflex

A precise pattern of neurotransmitter release occurs with each phase. During the descending phase, there is an increase in the release of VIP, PACAP, NO, and somatostatin and a decrease in the release of [Met]enkephalin. The pattern of release reflects the role of these neurotransmitters in mediating the descending phase. Thus, release of VIP, PACAP, and NO from the nerve terminals of motor neurons innervating circular muscle mediates descending (caudad) relaxation of this layer. Release of the same neurotransmitters from interneurons that synapse with and activate ACh/tachykinin motor neurons that innervate longitudinal muscle mediates reciprocal contraction of this layer. The increase in the activity of VIP/PACAP/NOS motor neurons and interneurons is caused by a decrease in the tonic inhibitory influence of opioid ([Met]enkephalin) interneurons, which in turn is caused by an increase in the activity of somatostatin interneurons. Thus, as the reflex is evoked sensory input is relayed to somatostatin interneurons coupled to opioid interneurons, which in turn are coupled to VIP/PACAP/NOS motor neurons and interneurons (Fig. 2).

A reverse pattern of neurotransmitter release accompanied by an increase in SP and NKA release occurs during the ascending phase and determines the contraction of circular muscle and relaxation of longitudinal muscle. There is little direct inhibitory input to longitudinal muscle, at least in small animals. Contraction and relaxation of this muscle reflect, respectively, the increase and decrease in ACh and tachykinin release from motor neurons (Fig. 2).

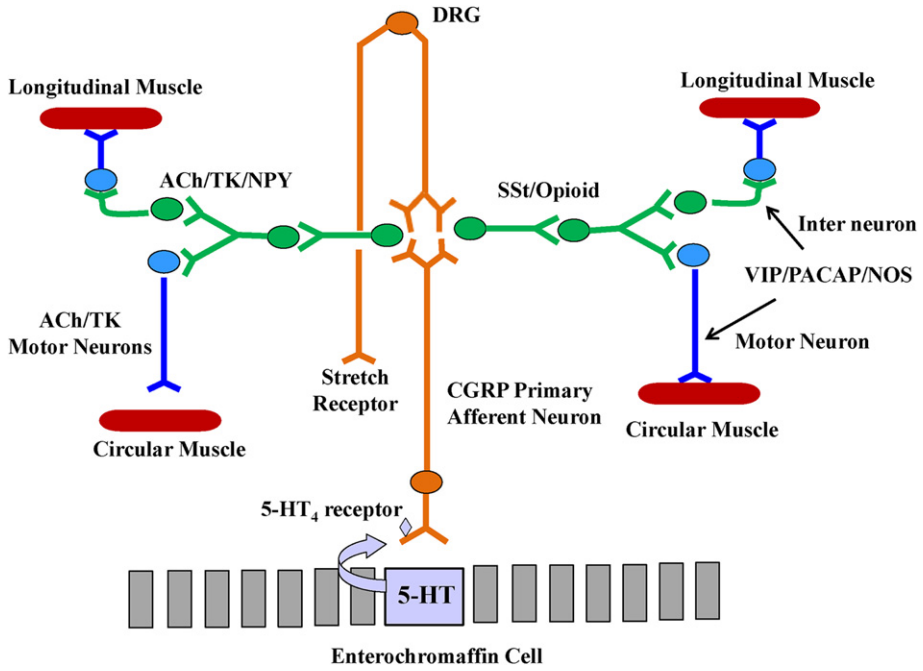

Fig. 2 Model illustrating the regulation of intestinal peristaltic reflex. Mucosal stimulation causes the release of 5-HT, which acts on a 5-HT_4 receptor on intrinsic primary afferent CGRP neurons. Mechanical distension of the gut wall activates extrinsic primary afferent CGRP neurons with cell bodies located in the dorsal root ganglion (DRG). Both afferent neurons activate the same reflex pathway. The descending or caudad pathway is mediated by somatostatin (SSt) and opioid peptide interneurons coupled in series to (i) VIP/PACAP/NOS motor neurons, which innervate the circular muscle layer and cause descending relaxation of circular muscle, and (ii) VIP interneurons coupled to ACh/tachykinin (TK) motor neurons, which innervate the longitudinal muscle layer and cause descending contraction of longitudinal muscle during circular muscle relaxation. The ascending or orad pathway is less well defined but involves cholinergic interneurons and results in activation of ACh/TK motor neurons, which innervate the circular muscle layer and cause ascending contraction of circular muscle, and inhibition of ACh/TK motor neurons, which innervate the longitudinal muscle layer and cause ascending relaxation of longitudinal muscle during circular muscle contraction.

The Sensory Limb of the Peristaltic Reflex

Two distinct populations of sensory neurons mediate the peristaltic reflex evoked by muscle stretch and mucosal stimulation. After nerve degeneration following surgical or chemical (using capsaicin) ganglionectomy, the reflex can be evoked by mucosal stimulation but not by muscle stretch. Conversely, after the mucosa is removed while maintaining intact extrinsic innervation, the reflex can still be evoked by muscle stretch. Thus, mucosal stimulation activates intrinsic sensory neurons with terminals in the mucosa and cell bodies in the myenteric plexus, whereas circular muscle stretch activates sensory neurons with nerve terminals in circular muscle and cell bodies in the dorsal root ganglia. The main sensory neurotransmitter in extrinsic and intrinsic neurons is CGRP, and its effect is relayed to the same circuits of interneurons and motor neurons that mediate the ascending and descending phases of the reflex.

The mechanism by which CGRP-containing intrinsic sensory neurons are activated is unique in that it involves release of 5-hydroxytryptamine (5-HT) from the mucosa. In both humans and experimental animals, mucosal stimulation releases 5-HT from enterochromaffin cells, one of two large stores of 5-HT in the body. 5-HT acts on 5-HT_4 receptors located on mucosal sensory nerve terminals, causing release of CGRP (Fig. 2). The addition of 5-HT or a selective 5-HT_4 agonist to the mucosal surface releases CGRP from capsaicin-sensitive sensory neurons and triggers the peristaltic reflex. Selective 5-HT_4 antagonists block CGRP release and the peristaltic reflex evoked by mucosal stimulation or by the addition of 5-HT to the mucosa. Both 5-HT_4 and CGRP antagonists suppress the ascending and descending phases of the reflex and the release of corresponding neurotransmitters. No release of 5-HT is seen when the peristatlic reflex is evoked by radial muscle stretch. Although the reflex has been traditionally viewed as a stereotypical response to intestinal distension (radial muscle stretch), it is unlikely that this represents the normal physiological modality. The mere passage and chemical composition of digesta appear to be the main physiological triggers of the reflex and propulsion of intestinal contents.

Propagation of the Reflex

The caudad propagation of the reflex leads to propulsion of intestinal contents. The velocity of propulsion is readily measured *in vitro* using synthetic fecal pellets inserted into the orad end of the segment without distending the lumen. 5-HT_4 agonists added to the lumen of the segment accelerate the velocity of propulsion, whereas 5-HT_4 antagonists have the opposite effect. Elimination

of the inhibitory influence of opioid neurons on VIP/PACAP/NOS neurons with opioid antagonists (particularly δ receptor antagonists) accelerates propulsion. The suppression of opioid activity greatly potentiates the ability of 5-HT to accelerate propulsion, such that a combination of near-threshold concentrations of a 5-HT_4 agonist and an opioid δ antagonist produces near-maximal stimulation of propulsion. The remarkable effect of this combination suggests that it may have therapeutic potential, and highlights the importance of understanding the physiological interplay of peptide neurotransmitters and their regulators.

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Pituitary Tumors Associated With Multiple Endocrine Neoplasia Syndromes

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Introduction

Pituitary adenomas are typically sporadic and the various subtypes include prolactinomas (50%–55%), clinically non-functioning pituitary adenomas (NFPA) (20%–25%) that primarily originate from gonadotrope cells, pure growth hormone (GH) secreting pituitary adenomas or mixed GH and prolactin secreting pituitary adenomas (15%–20%), corticotropinomas (5%) and TSH-secreting pituitary adenomas (1%). The prevalence of clinically relevant pituitary adenomas is about 1:1000 (Daly *et al.*, 2006b). Pituitary adenomas can occur in a familial context in 5%–6% of cases, mainly as familial isolated pituitary adenomas (FIPA), in which pituitary adenomas are the only tumoral presentation or as part of multiple endocrine neoplasia type 1 (MEN1) syndrome (Beckers *et al.*, 2013). Rare genetic forms of pituitary adenomas include Carney complex (CNC), MEN4, the "3PAs" syndrome (paraganglioma/pheochromocytoma/pituitary adenoma association) and X-Linked Acrogigantism (X-LAG) syndrome. Some characteristics of the inherited forms of pituitary adenomas can distinguish them from sporadic tumors; however, these characteristics can vary between individuals and within families. This heterogeneous clinical expression must be kept in mind during investigation and follow-up.

Recent progress in the genetics of endocrine tumors has resulted in an increased recognition of the genes that predispose to the development of pituitary adenomas in the context of multiple neoplasia syndromes (Beckers *et al.*, 2012) and as isolated familial forms (Daly *et al.*, 2006a; Trivellin *et al.*, 2014). In addition, some apparently sporadic forms of pituitary adenomas can also arise on a genetic basis. As a consequence, a better understanding of some molecular mechanisms of pituitary tumorigenesis can offer the possibility of meaningful genetic screening among the at-risk population (**Table 1**). Awareness of these complex syndromes can facilitate a more timely diagnosis, while helping to direct appropriate screening and follow-up of patients and clinically unaffected mutation carriers.

Multiple Endocrine Neoplasia Type 1 (MEN1)

MEN1 is the main multi-organ syndromic condition associated with pituitary adenomas. This is an autosomal dominant condition involving about 2.7% of all PAs in some series. MEN1 can present as a sporadic form or in a familial setting. In MEN1, pituitary adenomas usually occur in association with other endocrine and non-endocrine tumors. MEN1 related disease usually becomes clinically evident in adolescents and in young adults, and >95% of patients develop at least one manifestation of the disease by the age of 50 (Verges *et al.*, 2002).

Primary hyperparathyroidism (PHPT) is the most common (95%–98% of cases) and often the first manifestation. Gastro-enteropancreatic neuroendocrine tumors (NETs) (30%–70% of cases) are the second most frequently encountered tumor in MEN1 and include mostly gastrinomas, insulinomas, and non-functioning tumors (Wautot *et al.*, 2002; Thakker, 2014).

Table 1 Recognized genetic causes of pituitary adenomas occurring in a familial, syndromic or inherited context

Condition	*Gene*	*Physiopathology*	*Phenotype*
MEN1	*MEN1* (Chr11q13)	Decrease of menin function	All pituitary tumor types
MEN4	*CDKN1B* (Chr12p13)	Lack of tumor suppression	Only acromegaly and Cushing's disease to date
Carney complex	*PRKAR1A* (Chr17q22–24)	1A regulatory subunit of protein kinase A expression/function alteration	GH and GH/prolactin secreting adenomas
McCune–Albright syndrome	*GNAS1* (Chr20q13.3)	Activation of the stimulatory alpha-subunit (Gsα) of protein G	Pituitary hyperplasia and adenomas, acrogigantism or Cushing syndrome
3PAs	*SDHA*, *SDHB*, *SDHC*, *MEN1*, *MAX*	Decreased activity of SDH, MAX	Often aggressive macroadenomas secreting prolactin or GH
Pituitary blastoma	*DICER1* (Chr14q32.13)	Alteration of DICER1 activity	Potential for hypersecretion of ACTH and young onset Cushing disease
FIPA	*AIP* (Chr11q13.32) in 15%–20% of cases	Various	All pituitary adenoma subtypes involved. *AIP* mutation cases include somatotropinomas, prolactinomas, mixed GH/prolactin tumors, non-secreting adenomas, TSH-omas
X-LAG	*GPR101* (ChrXq26.3 microduplications and mosaicism)	Increased expression of GPR101, GHRH and GH secretion	Early onset acrogigantism, somatotropinomas/somatotrope hyperplasia with prolactin secretion

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Pituitary adenomas are diagnosed in 40% of MEN1 patients. Most of these tumors are prolactinomas (50%–60%), followed by other functioning pituitary adenomas (somatotropinomas in 10% and corticotropinomas in 5%) and NFPA (5%–15%) (Burgess *et al.*, 1996). PAs with mixed hormonal secretion and pituitary hyperplasia have been also reported in MEN1 (Thakker, 2014). In about 17% of cases the pituitary tumor is the initial manifestation of MEN1 (Verges *et al.*, 2002).

When compared with sporadic cases, MEN1-associated pituitary adenomas develop at a younger age and present with more aggressive features. They are more likely to manifest as large prolactinomas (85% are macroadenomas) with more frequent local compression symptoms, and resistance to treatment with dopamine agonists (Verges *et al.*, 2002; Wautot *et al.*, 2002; Beckers *et al.*, 2003). However, GH-secreting adenomas have also been reported in MEN1 from a very young age that could lead to abnormal growth acceleration and tall stature in these patients (Stratakis *et al.*, 2000). In some cases, MEN1 pituitary carcinomas have also been described (Philippon *et al.*, 2012; Vroonen *et al.*, 2012).

Other less frequent components of MEN1 syndrome include adrenal cortical lesions (25%–40%), carcinoids (10%), bronchopulmonary and thymic neuroendocrine tumors (2%) and pheochromocytomas (<1%), as well as non-endocrine lesions such as angiofibromas (80%), collagenomas (70%), and lipomas (20%–30%) (Thakker *et al.*, 2012; Thakker, 2014).

The *MEN1* gene consists of 10 exons, coding for the 610 amino acid nuclear protein menin (Chandrasekharappa *et al.*, 1997). Through protein–protein interaction, and also epigenetic modifications, it participates in cell cycle control, oxidative stress regulation and DNA repair. For example, it suppresses the transcription of prolactin and induces cell cycle inhibitors including p27 (Agarwal *et al.*, 2004; Thakker, 2014; Scacheri *et al.*, 2006). Inactivating germline *MEN1* mutations are present in 80% of cases, among which 10% are de novo mutations. >700 mutations in *MEN1* have been described (Thakker, 2010). In 75% of cases, these mutations are nonsense inactivating mutations (23%), frameshift (41%) or with splice site alterations. In 1%–5% of the cases with normal sequencing, large genomic rearrangements can be found by multiple ligation-dependent probe amplification (MLPA) and can be seen in familial MEN1 cases. In >90% of MEN1 tumors, the second allele is inactivated, as demonstrated by loss of heterozygosity (LOH) at chromosome 11q13 (Thakker, 2010). There is no genotype–phenotype correlation, except for rare forms with a high prevalence of prolactinomas, described in families from the Burin peninsula in Newfoundland, Canada (MEN1-Burin) (Thakker, 2010; Wautot *et al.*, 2002; Olufemi *et al.*, 1998).

MEN1 guidelines recommend genetic screening in patients with two or more typical MEN1 manifestations and in first-degree relatives of proven mutation carriers. Regular clinical and biochemical screening for potential MEN1-associated tumor development is advised starting from a young age, due to the observation of MEN1 manifestations in pediatric patients (Thakker *et al.*, 2012). A MEN1-associated pituitary adenoma has been reported in a boy as young as 5 years of age (Stratakis *et al.*, 2000). Genetic testing for *MEN1* and *AIP* gene mutations should be considered in sporadic pediatric or young adult cases with pituitary adenomas, as *AIP* mutations are frequent and *MEN1* mutations are detected in 8% in those with a young age at onset of pituitary disease (Cuny *et al.*, 2013; Tichomirowa *et al.*, 2011; Stratakis *et al.*, 2010).

Multiple Endocrine Neoplasia Type 4 (MEN4)

Among patients that present with a MEN1 phenotype, up to 20% have no *MEN1* mutation; these are usually sporadic cases. One alternate genetic cause of a MEN1-like presentation is MEN4 syndrome caused by an inactivating germline mutation of the *CDKN1B* gene coding for a 196 amino-acid cyclin-dependent kinase inhibitor (p27Kip1). Initially, Pellegata and collaborators described this condition as MENX in a rat model with autosomal recessive transmission, characterized by mixed clinical manifestations between MEN1 and MEN2 (Fritz *et al.*, 2002; Pellegata *et al.*, 2006). They also reported a pathogenic mutation in the human *CDKN1B* gene in a family with acromegaly, PHPT, renal angio-myolipoma, and a testicular tumor, which was named MEN4 (Lee and Pellegata, 2013). Both sporadic and familial forms of MEN4 can occur. MEN4 is a rare and heterogeneous condition, most frequently occurring with PHTP, although several cases of pituitary adenomas of various phenotypes (including acromegaly) have also been reported in the context of this syndrome (Lee and Pellegata, 2013). Other MEN4 manifestations include gastroenteropancreatic NETs, adrenal and thyroid tumors, carcinoids and other non-endocrine abnormalities. Mutations of *CDKN1B* are reported in 2%–3% of MEN1-negative cases (Lee and Pellegata, 2013). However, MEN4 appears to explain very few cases of syndromic pituitary adenomas (Stratakis *et al.*, 2010), and *CDKN1B* mutations should be considered as a very rare genetic predisposition to pituitary adenomas.

Carney Complex (CNC)

A complex condition was defined by Carney in 1985 as an association of myxomas, point-like pigmented lesions of the skin and endocrine hyperactivity. It is a rare autosomal dominant syndrome (<1000 cases described) characterized by over-activity of the cAMP pathway and development of lentigines (70%), myxomas of various locations including the heart (30%), schwannomas, primary pigmented nodular adrenal disease (PPNAD) (~60%), testicular (60%), and ovarian (30%) tumors and pituitary hypersecretion (Boikos and Stratakis, 2007). In 70% of cases Carney complex is familial (Stratakis *et al.*, 2001). Given the potential severity of cardiac myxomas, very early family screening is recommended.

Multifocal somatomammotrope hyperplasia and/or GH-prolactin secreting pituitary adenomas can cause acromegaly in about 10% of CNC cases. However subclinical hypersecretion of GH/IGF1 and/or prolactin can occur frequently (75%). Acromegaly in

CNC has a mean age at diagnosis of 35 years, and usually has a mild course (Correa *et al.*, 2015). Mixed PAs other than GH/prolactin secreting (with positive staining for TSH, LH or alpha subunit) are also found in CNC (Pack *et al.*, 2000).

The main gene found to be responsible in 60%–65% of CNC is *PRKAR1A* on chromosome 17q24, encoding the 1A regulatory subunit of protein kinase A (PKA) (Bertherat *et al.*, 2009; Horvath *et al.*, 2010). While another potential locus (CNC2) exists at 2p16, alterations in individual genes have not been yet characterized (Casey *et al.*, 1998; Stratakis *et al.*, 1996). A case of CNC-like phenotype presenting with acromegaly due to a pituitary tumor in association with pigmented spots and myxomas was identified recently and was associated with a triplication on chromosome 1p31.1, including the *PRKACB* gene which encodes the catalytic subunit of PKA (Forlino *et al.*, 2014).

3P Association (3PAs)

Cases of pituitary adenoma coexisting with pheochromocytoma were described as early as 1952 (Iversen, 1952). This association of pituitary tumor and pheochromocytomas/paragangliomas (termed "3P Association" or 3PAs) has recently been demonstrated to occur in patients with mutations in genes encoding the subunits of succinate-dehydrogenase (SDH), which are known pheochromocytoma/paraganglioma risk genes (Xekouki *et al.*, 2012; Dwight *et al.*, 2013; Denes *et al.*, 2015). Familial and sporadic 3PAs cases were reported. In families, each affected person can have both pituitary adenomas and pheochromocytoma/paraganglioma or have each tumor separately. Inactivating mutations in *SDHx* genes are detected in 40% of 3PAs (Denes *et al.*, 2015). The most common SDHx mutation- associated PAs are somatotropinomas, followed by prolactinomas and NFPA. These pituitary tumors almost all are macroadenomas and appear to be aggressive (Denes *et al.*, 2015). LOH at the corresponding locus was found in some 3PAs- associated pituitary adenoma samples. These findings demonstrated the pathogenic role of *SDHx* gene mutations in pituitary tumorigenesis (Denes *et al.*, 2015; Xekouki *et al.*, 2012). However, *SDHx*-related pituitary adenomas are very rare, accounting for only 0.3% of all pituitary tumors (Gill *et al.*, 2014). Based on these data, the genetic screening for *SDHx* mutations is recommended only in patients with both pituitary adenomas and pheochromocytoma/paraganglioma and in kindreds corresponding to familial 3PAs. Alterations in other genes involved in the onset of pheochromocytomas/paragangliomas seem to have only an exceptionally rare association with pituitary adenomas (O'Toole *et al.*, 2015). For instance, intragenic deletions of one or more exons in the *MAX* gene have recently been shown to occur in patients with aggressive pheochromocytomas in association with pituitary adenomas (Daly *et al.*, 2018).

DICER1 Syndrome and Pituitary Blastoma

Pituitary blastomas are very rare pituitary tumors of embryonic origin (Scheithauer *et al.*, 2012). They usually occur in pediatric patients, as ACTH-dependent hypercortisolism and young onset Cushing's disease (de Kock *et al.*, 2014). This rare condition was found to be associated with germinal or somatic mutations of the *DICER1* gene, coding for a ribonuclease involved in the maturation of microRNAs. Classically, mutations in *DICER1* were reported in other pediatric tumors (nephromas, testicular Leydig or Sertoli cell tumors, and sarcomas). The identification of pathogenic *DICER1* mutations requires screening for other DICER1-related tumors in these patients, although mosaicism for mutations in *DICER1* can be present, leading to a diverse phenotypic presentation (de Kock *et al.*, 2014).

McCune–Albright Syndrome (MAS)

MAS is non-familial genetic syndrome due to a mosaicism for an activating mutation of the α-subunit of the stimulatory guanine binding (Gs) protein gene (*GNAS1*), which induces constitutive activation of the cAMP pathway (Weinstein *et al.*, 1991). The clinical phenotype therefore depends on the type of cells affected by the mutation. Clinically it presents as a specific triad: an association of hyperpigmented "café au lait" spots, polyostotic fibrous dysplasia and various endocrine gland hypersecretion. The latter can lead to precocious puberty, hyperthyroidism, Cushing syndrome and GH/IGF1 hypersecretion (Dumitrescu and Collins, 2008).

GH hypersecretion is often due to pituitary hyperplasia and is frequently accompanied by hyperprolactinemia (Salenave *et al.*, 2014). Interestingly, the pituitary abnormalities in MAS can present also as a somatotropinoma surrounded by diffuse pituitary hyperplasia (Vasilev *et al.*, 2014). Acromegaly affects 20%–30% of MAS cases, with a young age of onset. Early exposure to GH excess can lead to pituitary gigantism (Vasilev *et al.*, 2014). In addition to MAS, the presence of pituitary somatotrope hyperplasia and adenoma in young patients with overgrowth can also occur in association with *AIP* mutations, Carney complex or in the setting of X-LAG syndrome (germline Xq26.3 microduplications or mosaicism), so this finding on histopathology should stimulate genetic investigations (Villa *et al.*, 2011; Trivellin *et al.*, 2014; Daly *et al.*, 2016).

Considering the absence of familial cases of MAS reported in humans and the low rate of the *GNAS1* mutation detection in the leukocyte DNA (no higher than 45% even in the patients with the complete MAS triad), the recommendations for genetic screening are ambiguous. Some new techniques like digital droplet PCR (ddPCR) for *GNAS1* somatic mosaicism can help to

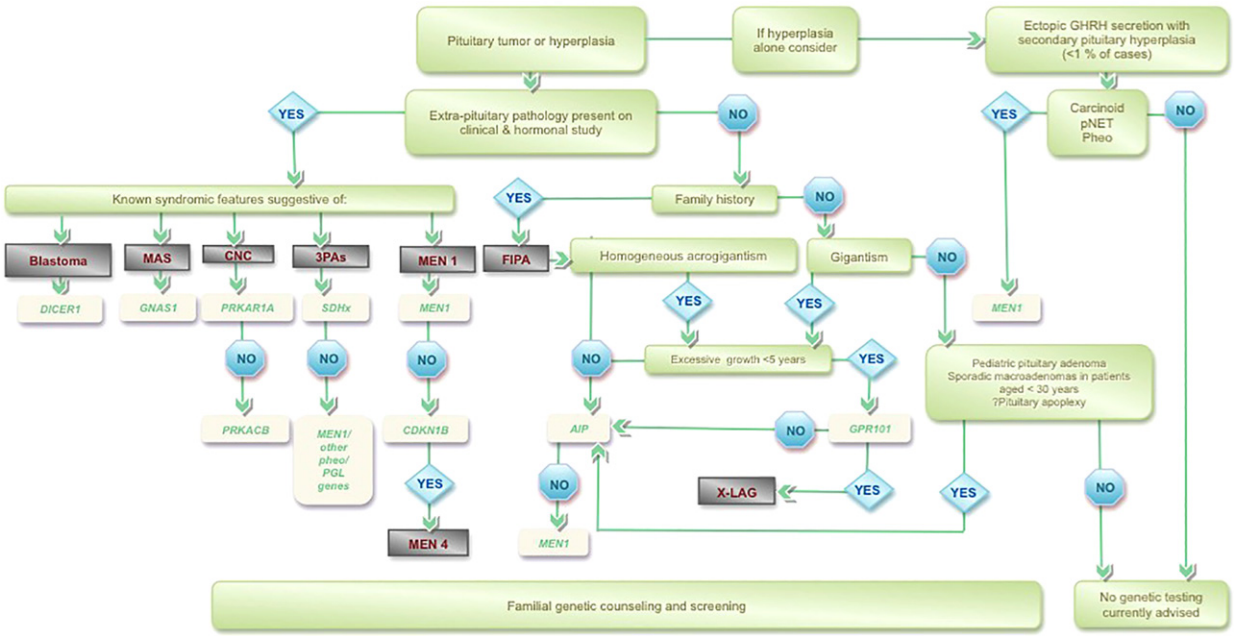

Fig. 1 Genetic screening algorithm in patients with pituitary adenomas. Adapted with permission by the authors from their own work in Rostomyan, L. and Beckers, A. (2016). Screening for genetic causes of growth hormone hypersecretion. *Growth Hormone & Igf Research*, **30–31**, 52–57.

increase the diagnostic yield (Vasilev *et al.*, 2014). Inheritance is not described in humans, although it has been established in transgenic mice (Saggio *et al.*, 2014).

Conclusions

Inherited and genetic forms of PAs can be present in association with various endocrine and non-endocrine abnormalities. Genetic alterations specific for each condition contribute to the clinical features of PA, as noted above. Most of PAs with a genetic predisposition have early onset and aggressive behavior, present frequently as large invasive tumors and some are resistant to conventional medical treatment options.

Genetic studies of the main genes of interest in inherited pituitary tumor syndromes, when informed by the clinical presentation can identify high-risk groups for the genetic predisposition to pituitary adenomas (**Fig. 1**). Consequently, adequate biochemical and clinical screening algorithms can lead to early detection of these tumors at an early stage.

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Aging and Longevity of Human Populations☆

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Glossary

Antioxidant A substance that chemically inhibits oxidation reactions.

Caloric restriction Eating fewer calories than *ad libitum* while achieving adequate or optimal nutrition. This has extended both mean and maximum life span in various animal models.

Cellular senescence A cell fate decision and stress response characterized by a permanent arrest of cell proliferation coupled to a complex secretory phenotype.

Mitochondria Any of various round or long cellular organelles of most eukaryotes that are found outside the nucleus, produce energy for the cell through oxidative processes, and are rich in fats, proteins, and enzymes.

Nuclear DNA DNA found within the nucleus of the cell.

Oxidative theory of aging The theory that declines in physiological function with age are due to damage accumulated from reactive oxygen species.

Superoxide disumutase (SOD) A potent intracellular antioxidant.

Telomere The natural end sequence of a eukaryotic chromosome.

Aging and longevity are fundamental characteristics of human populations. Over the last two centuries, human life expectancy has more than doubled, resulting in a significant increase in the number of older individuals worldwide. Aging typically means the loss with increased chronological age of the functional capability of different organs of the body resulting in an increased risk of disease and death. While aging has for long been regarded a nonmodifiable risk factor for major chronic diseases, such as cancer or cardiovascular diseases, discoveries in experimental model organisms have provided evidence that the rate of aging can be modulated by genetic and environmental interventions in a number of conserved mechanisms that seem to be the main drivers of aging. Often, aging has been defined at the cellular level, such as the replicative senescence of cells whose telomere reaches a critical length, or related to mitochondrial dysfunction in energy production due to the accumulated damage of reactive oxygen species. Longevity, the number of years lived, is generally viewed as being determined by senescent processes reaching their end stage. To capture the key biological mechanisms underlying aging and longevity and their complex interrelationships in a conceptual framework "the hallmarks of aging" have recently been proposed. In this conceptual framework, aging features that occur at different hierarchical levels are integrated, namely the subcellular or molecular level, the single cell level, and the tissue and whole-body level. Neuro-endocrine mechanisms have been identified as important regulators of aging and longevity by affecting several of the hallmarks of aging, in ways that are complex and always not well understood.

Cell-Based Senescence

Biological models of human aging and longevity have often focused on the senescence and death of the individual cell—the fundamental building block of the human organism. For example, one model of senescent-limited longevity is based on a model of genetic restrictions on the number of cell replications that can occur under the so-called Hayflick limit. The physical basis for this phenomenon called replicative senescence is believed to be the telomere, the end sequence of the chromosome that tends to decrease in length with each replication of the cell. These cell-based models tend to underplay the significance of endocrine and growth factors.

One problem with this perspective is that an experimental analysis of the decline in the number of replications that a cell can perform (often estimated at 50–60) per year of age in vitro was estimated to be 0.2/year in humans aged 30–80, implying that this mechanism limited the human life span to 250–300 years. The highest reliable age reported at death is currently that of Madame J. Calment, who died at age 122. A second problem is that although the Hayflick limit may explain longevity bounds, it does not explain loss of function with age.

Several studies observed that in healthy human subjects, and controlling for the biopsy site, there was no significant negative correlation between the cell's replicative capacity in a given tissue and subjects' age in vitro. As a consequence, questions are raised about the importance and exact role of the loss of cell replicative capacity as a limiting factor in human longevity. Additionally, studies have found the correlation of telomere length with replicative senescence to be complex because it varies across tissue types and because the telomere may have additional cellular functions, such as monitoring oxidative cell damage. Further complicating

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analysis of the mechanisms controlling longevity is the fact that most tissues, including the brain, are now thought to have a small proportion of stem cells from which new cells can generate.

While telomere shortening can induce replicative senescence, cellular senescence can be induced by different genomic, epigenomic, or metabolic stresses that put replication-competent cells at risk for neoplastic transformation. Cellular senescence is characterized by a permanent arrest of cell proliferation coupled to a complex pro-inflammatory senescence-associated secretory phenotype (SASP). The proliferative arrest is a potent anticancer mechanism, whereas the complex secretory response can promote wound healing and tissue repair by transiently providing the required proteases, cytokines and growth factors. With age, numbers of senescent cells increase in most mammalian tissues, and senescent cells are enriched at sites of age-related pathologies. It is unknown what causes senescent cells to accumulate with age, but age-related decreases in the efficiency of clearance by the immune system and via apoptosis are thought to contribute. When present in sufficiently high numbers, senescent cells can negatively influence tissue homeostasis. Their proliferative arrest can hamper tissue repair and regeneration by curtailing the proliferation of stem or progenitor cells. Likewise, the senescence-associated secretory phenotype can create a chronic inflammatory milieu that may drive or exacerbate several age-related diseases, including cancer. The development of therapies that pharmacologically target senescent cells may constitute a novel strategy for treatment of age-related diseases. In experimental animal models, removal of senescent cell by targeted induction of apoptosis has recently been shown to delay several age-related pathologies and increase healthy lifespan.

Oxidative Stress and Mitochondrial Influences on Aging

Another promising model of the cellular mechanisms limiting the human life span is the senescence of the human mitochondria. This is a specific form of the general theory of an oxidative basis for aging originally proposed by Harman. Mitochondrial DNA (mtDNA) has fewer error monitoring and correcting mechanisms than does nuclear DNA (nDNA). Furthermore, since the mitochondria is the primary center of energy production in cells, the organelle is thought to be especially vulnerable to the oxidative stress of reactive oxygen species (ROS), which are produced in the respiratory processes centered in the mitochondria.

Because the mutation rate of mtDNA is much greater (approximately 10-fold) than that of nDNA, in a variant of the oxidative theory of aging, "aging" of the mitochondria has been suggested to be the physiological process limiting human longevity to a maximum of 130 years.

Of interest is that the production of many mitochondrial proteins in humans has been taken over by nDNA, with 47 of 60 mitochondrial proteins produced in the nucleus, in which error monitoring and corrective mechanisms are more complete. An interesting corollary of this model is that as aging degrades the efficiency of energy production, the physical activity of the organism is degraded, inducing a correlation of functional loss and longevity. Cell-specific failure can be communicated systematically by cytokines such as interleukin-8, which is increased by stress due to ROS, and interleukin-6, which is a proinflammatory cytokine.

Exogenous Modulators of Mitochondrial Function

Problems arise with this model of longevity and senescence when one considers how "external" factors affect the process of mitochondrial senescence and functional degeneration. A common model for life-expectancy extension is caloric restriction. In this case, the lowering of caloric consumption without malnutrition is thought to decrease basal metabolism, reduce oxidative stress, and retard age-related degeneration. Since this involves energy production and metabolism, it is reasonable to consider the mitochondria as the focal point of these processes. Such an intervention has been shown to increase life expectancy in experimental models of rodents and other lower organisms. Recently, different studies have assessed the effects of caloric restriction in Rhesus monkeys. While a study by the University of Wisconsin study reported a positive impact of caloric restriction on survival, in a study by the National Institute on Aging study no significant survival effect was detected. While differences in study design, including differences in the age of onset of caloric restriction, the amount of food consumed by the control group and the composition of diet have likely contributed to the differences in the effect on survival between studies, both studies observed health benefits of caloric restriction in non-human primates. Additionally, several genetic mutations in *Caenorhabditis elegans* and *Drosophila* have shown that slowing metabolism, and reducing growth and reproduction, can significantly extend the life span. It is believed that longevity-extending mutations in these organisms either protect against ROS species (e.g., genes controlling SOD2 production) or alter insulin and insulin growth factor-1 pathways and signaling.

A significant counterexample to these experimental models is *Apis mellifera*, the common honeybee. Both bee workers and queens have identical genotypes. Their polyphenotypic differentiation occurs due to differences in larval nutrition whereby larva destined to become queens are fed royal jelly, which contains juvenile hormone that alters the developmental trajectory, increases body size and respiration/metabolic rate, increases life expectancy 15- to 30-fold over that of the worker bee, and increases the level of royal jelly produced in queens.

Juvenile hormone seems to affect basal metabolic rate by increasing the production of mitochondrial transcription factors and certain mitochondrial enzymes (Cox-1 and Cytc-C) involved in the respiratory process. Thus, juvenile hormone in bees appears to play roles similar to those of thyroid hormones in humans, which can also affect the developmental process as well as the

mitochondrial energy production function. Of interest is that the nuclear receptors for thyroid hormone T_3 also code for the type of mitochondrial translation proteins elevated in queen bees. One of the crucial findings of experimental studies of the queen bee was that the increases in respiration rate were due to enhanced mitochondrial function and not to increased numbers of mitochondrial organelles. The queen bee model raises the question of how thyroid hormones in humans might regulate senescence and affect longevity by controlling mitochondrial behavior and function (a process not extensively studied), especially regarding how nutritional and physical activity (energy expenditure) factors may affect mitochondrial function.

There is significant evidence that nutritional or dietary factors can alter mitochondrial function and, indeed, changes in mitochondrial functions with age in animal models. Improved mitochondrial efficiency would lead to the generation of less ROS and lower amounts of oxidative stress. It was recently observed that in humans, a two-year caloric restriction intervention reduced whole body oxidative stress based on measurement of urinary concentrations of F2-isoprostanes.

Tissue Interdependence and Hormonal Control in Longevity

Any model that attempts to explain human longevity and senescence solely at a cellular or molecular level is likely to fail as an oversimplified representation of the physiological and molecular mechanisms involved. The fact is that the molecular functioning of a cell is important not only for its internal maintenance but also for the functioning of the organism. Thus, part of the question of the relation of senescence and longevity concerns the biological complexity of the organism due to the functional differentiation of tissue and organs and the hormonal synchronization of the wide variety of heterogeneous physiological functions that need to be performed.

The hormone and signaling pathways studied most intensively for their effects on longevity and aging are insulin, insulin-like growth factor 1 (IGF-1), and growth hormone (GH) because of the experimental observation of increases in longevity conferred by single gene mutations affecting these hormonal pathways, as well as by nutritional interventions, such as caloric restriction. Among the experimental models most frequently used in such studies are *C. elegans*, *Drosophila melangocaster*, yeast, and rodents. IGF-1 and GH have been intensively studied because of the conceptual attractiveness of the oxidative theory of aging and because of the effects of caloric restriction on life span in experimental models (e.g., rodents), which are believed to be mediated, in part, by IGF-1, insulin, and/or GH. The logic of the relation is that caloric restriction slowed metabolism, the production of ROS, the accumulation of oxidative damage, and thus the aging rate. In laboratory mice, significant increases in longevity conferred by genetic mutations that result in GH resistance or deficiency are accompanied by increased production of antioxidants, such as catalase or SOD2, greater stress resistance, reduced inflammation and cellular senescence, in concert with shifts in mitochondrial function and metabolism, including enhanced insulin sensitivity and lower circulating levels of insulin. The ability of GH to activate nutrient sensing pathways, such as the mechanistic target of rapamycin (mTOR) signaling pathway has been put forward as an explanation for the negative correlation between GH signaling and longevity that is observed among laboratory mice. Activation of the mTOR pathway prevents cell death, promotes protein synthesis and growth and (over) stimulation of this pathway is thought to contribute to accumulation of unfolded proteins, increased ROS production, inhibition of autophagy and stimulation of cellular senescence.

It is unclear, however, whether the effect of such genetic mutations or nutritional interventions work in the same way in humans because of their greater histological (especially hormonal) complexity. Genetic mutations that altered GH resistance or deficiency, although associated in experimental models with increased longevity, have been associated in humans with a number of adverse conditions, such as obesity. However, despite increased obesity and lack of a consistent effect on longevity, GH resistance in the syndrome of Laron dwarfism has been observed to provide protection from cancer and diabetes.

An additional rationale for the focus on of research on associations of insulin, insulin-like growth factor 1 (IGF-1), and growth hormone (GH) and longevity, is reasoning based on evolutionary models that there is a trade-off of energy expended in growth and reproductive function and somatic maintenance. Periods of scarce food supplies causes metabolism in *C. elegans* to decrease and reduces the requirement for energy and may cause organisms to enter a quiescent, or dauer, state. This quiescent, low-energy consumption state is often associated with reduced oxidative stress and increased longevity but a lower rate of reproduction and less growth.

Hormonal control, however, is more complex in humans than in these experimental models. The IGF-1/insulin effect in the fly and the worm may favor localized hormonal actions. In worms and flies, there was a single receptor that favored local system actions of insulin and IGF peptides, permitting aging regulation to be dominated by a single tissue type (e.g., the neuroendocrine tissue of *C. elegans*). In tetrapods, in contrast, there are four candidate receptors—IGF-1, IGF-2, IR, and RR. This may have led to the developmental differentiation of insulin to control metabolic factors and IGF-1 to control mechanisms of growth in humans. As a consequence, in humans, caloric restriction may have become disassociated from factors controlling longevity, with factors linked to human longevity no longer simply associated with caloric restriction. Indeed, recent animal studies show that intermittent starvation, as opposed to caloric restriction, leads to preservation of growth while having even greater effects on insulin and insulin sensitivity and, consequently, on longevity. Another experiment in mice is also of interest. Mice were raised without a gene for the insulin receptor in adipose tissue. These mice were found to have greater longevity ($+18\%$) than control mice. In addition, the gene knockout mice were lighter and much leaner than the control mice, even though they consumed more food. This suggests that they had a higher rate of respiration along with their greater average and maximal longevity. Indeed, increasing the efficiency of mitochondrial function and energy expenditure appears to also be associated with increased longevity in rodents with profound

changes in thyroid hormone metabolism. Thus, both insulin regulation and thyroid function appear to be factors that can strongly regulate human longevity and perhaps senescence, suggesting systematic multiorgan regulation of longevity rather than simple, universal cellular "clocks."

Whereas insulin, IGF-1, and GH have been extensively studied, one endocrine system's effects on aging and longevity that has not been well studied is that of the thyroid. Thus, an interesting future focus of hormonal research on human longevity and aging is on the effects of thyroid hormone, T_3, on mitochondrial respiration. It is suggested that thyroid hormone has at least three effects. The first is up-regulation of mitochondrial respiration by altering their membrane structure and permeability. A second effect is the stimulation of mitochondrial biogenesis which increases mitochondrial respiratory capacity. A third effect is the promotion of mitochondrial uncoupling mechanisms to generate heat at the expense of ATP production, which is thought to result in a reduction of ROS production. Thus, there are fundamental interactions between mitochondrial performance and T_3 hormone. The action of T_3 also seems to be involved in stem cell function and differentiation so that there is a direct linkage of metabolic level and thyroid hormone availability and the energy demands of organism development. Tight local control of thyroid hormone action has been implicated in coordination of tissue development, growth, and maintenance and repair, with reduced thyroid hormone availability favoring cell proliferation and increased thyroid hormone availability favoring cell differentiation. It can be hypothesized that inappropriate local regulation of thyroid hormone action, either too much or too little, may exhaust the stem cell population precociously or contribute to pathological amplification. The relation of these factors in controlling human longevity and development/senescence needs extensive study.

Also significantly involved in senescence and longevity is the endocrine control of immune function during aging by the hypothalamic–pituitary–adrenal axis. Hormones play many roles in cytokine production, and cytokines perform a number of important tasks. It is thought that the level of interleukin-6 (IL-6) increases with age. IL-6 controls inflammatory processes in part by stimulating C-reactive protein production in the liver. However, this mechanism is controversial as a model of aging, disability, and longevity because IL-6 is not clearly elevated in centenarians. Possibly, successful aging into advanced ages is characterized by immunoremodeling that reflects adaptations to chronic immunological challenges and exposures.

A Conceptual Framework of Aging and Longevity

To capture the key biological mechanisms underlying aging and longevity and their complex interrelationships in a conceptual framework "the nine hallmarks of aging" have been proposed (in analogy with "the hallmarks of cancer"). In this conceptual framework, aging features that occur at different hierarchical levels are integrated, namely the subcellular or molecular level, the single cell level, and the tissue and whole-body level. Four primary hallmarks of aging (genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis) have been proposed as the main types of molecular damage that drive aging. Three antagonistic hallmarks (deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence) have been proposed as cellular stress responsive processes that confer beneficial and protective effects at low levels but become deleterious at high levels. Finally, two integrative hallmarks (stem cell exhaustion and altered intercellular communication) have been proposed as the ultimate causes of loss of physiological integrity that occur as a deleterious result of accumulated damage and stress responses. The interaction between endocrine functions and aging are many and complex. On the one hand, neuro-endocrine mechanisms have been identified as important regulators of aging and longevity by affecting several of the hallmarks of aging. On the other hand, with aging, changes occur in the levels and actions of several hormones. It is not always clear whether such changes are neutral, detrimental or adaptive, or possibly a combination of these. A well-known example is the decreased secretion of growth hormone with aging (known as the "somatopause"). While decreased secretion of growth hormone may negatively affect body composition and accelerate loss of muscle mass, it may at the same time represent an adaptive response aimed at directing available resources towards maintenance and repair so as to reduce risk of cancer.

Conclusion

As the biological complexity of organisms increases, hormonal factors increase in importance as determinants of the correlation of senescence and mortality. Since no single organ dominates survival, the coordination and communication between organ systems becomes more crucial to survival and successful functioning. Because of the increased biological complexity of humans, the linkage of GH, IGF-1, and insulin observed in many studies of simple organisms likely does not function in the same way in humans. The role of thyroid hormones, especially their roles in mitochondrial function and in tissue maintenance and repair, must be further studied as a fundamental factor in senescence and longevity. It is reasonable to assume hormonal factors significantly affect human longevity and related aging processes in a variety of complex ways, that these are altered by exogenous factors such as nutrition, physical activity, by genetic polymorphisms, and that, if understood, could indicate how to better control senescent loss of function and overall survival.

Further Reading

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Animal Models for Aging☆

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Glossary

DNA sequencing Technique used to determine the sequence of nucleotide bases present in a DNA molecule or fragment.
Epithelial Layers of cells forming the outer surface of the body and also lining its internal cavities.
Mutagenesis Causing a permanent heritable change in the nucleotide sequence of a chromosome.
Phenotype Observable characteristics of a cell or an organism.
Transgenesis The stable introduction of a cloned gene into a plant or an animal, allowing its transmission to future generations.

General Principles of Aging Research

As described by Weindruch, animal models have been used in different categories of aging studies. Many investigators have used cross-sectional and (less commonly) longitudinal study designs to examine the influence of normal aging on parameters that are thought to either cause aging or be the consequence of aging processes. One major pitfall of this type of study is attributing observed differences to normal aging without taking into account the presence of confounding disease processes. Other difficulties arise from an inappropriate choice of ages. For example, an investigator comparing very young (e.g., newborn) rats with older adult (e.g., middle-aged) rats may be studying maturational rather than aging processes, and a failure to include intermediate ages between young and old may cause the investigator to miss important changes that may herald the aging process. Interestingly, the absence of appropriate life tables for the determination of survival curves has been the limiting factor in the use of some potentially useful aging models.

Other, more comparative studies examine species with widely different longevity to identify factors associated with longevity and aging. Similarly, different strains within the same species (e.g., congenic mice) may possess major differences in longevity. Finally, animal models of accelerated aging (e.g., senescence accelerated mouse) and models of decelerated aging (e.g., Drosophila overexpressing catalase or superoxide dismutase) both have proved to be useful in aging studies.

The Laboratory Rat

NIA's decision to provide the Fischer 344 (F344) rat strain to the scientific community has played a major role in its great popularity for aging studies. Many investigators also welcomed this inbred strain because the commonly used outbred Sprague–Dawley strain tended to become grossly obese in old age and exhibited tremendous variability when purchased from different suppliers. Unfortunately, although inbred F344 rats have the advantage of being genetically identical, this also results in their frequent development of tumors (testicular interstitial cell and pituitary) as well as renal failure from nephropathy. Although these tumors are rarely metastatic or secretory, screening is important because both the tumors and renal failure could confound research data. Fortunately, the NIA also offers the inbred Brown Norway (BN) rat, as well as an F1 hybrid (F344/BN), through a contractual arrangement. Caloric restriction still remains the best validated and most robust means of extending longevity in rodents while also decreasing their burden of disease (including F344 nephropathy). Many investigators have argued for the use of rodents that have undergone modest caloric restriction because such animals tend to be healthier in old age, minimizing the confounding effect of illness and disability. Interestingly, some investigators have used the heterogeneity present in other outbred strains (e.g., Wistar, Long–Evans) to great advantage in aging studies. For example, Long–Evans rats remain relatively healthy into old age and exhibit great variability in their spatial memory performance on a Morris water maze test.

The rat is a popular model for examining endocrine and reproductive issues in old age. Although this has resulted in the availability of considerable information regarding this model, great caution must be exercised when extrapolating from these studies to human conditions. For example, unlike women whose ovaries cease to produce estrogens past menopause, rat ovaries appear to be

☆*Change History:* July 2014. E Lee, B Rogina, L Haynes and GA Kuchel includes authorship change with E Lee and L Haynes replacing Q Zhu and SH Clark who have retired; word "different" to replace "five" in the first sentence of "General Principles of Aging Research" section; Information was added on the Diversity Outbred and the Collaborative Cross added at the end of the section on "The Laboratory Mouse", a Section on "Other Rodents" added right after the section on "The Laboratory Mouse", a Discussion on "C Elegans" has been completely rewritten by a new co-author (E Lee) and a Discussion on "Drosophila Melanogaster" has been completely rewritten by a co-author (B Rogina).

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capable of normal or near-normal function throughout their life span. In contrast to human menopause, reproductive senescence in rats is associated with the development of irregular cycles. This is followed by a period constant estrus in nearly one-half of the animals, which then proceed to a state of persistent diestrus, whereas the other half proceeds immediately to persistent diestrus. As a result, approximately one-half of 2-year-old rats will be in a state of constant estrus with a minimum of three consecutive epithelial vaginal smear cycles, whereas the other half will proceed to a state of persistent diestrus with a minimum of three leucocytic smears. Interestingly, estrogen levels are only slightly higher in the former group. As discussed previously, a high prevalence of pituitary and testicular tumors, particularly in aged F344 rats, also needs to be considered when using this strain in aging studies.

The Laboratory Mouse

Being smaller than rats, mice provide investigators with smaller amounts of tissue, and systemic physiological studies can sometimes be difficult. However, mouse genetics are extremely well known, and the ability to create genetically modified mice has become an important tool for studying aging and age-related processes. The presentation of a phenotype in any organism is a composite of the interactions between its genetic complement and the environment in which it is placed. Although the process of aging is a complex phenotype, it is no exception to this generally accepted biological principle. In contemporary biomedical research, the laboratory mouse has become a premier model organism to study processes important to human biology. This surge in mouse-related research has been driven largely by the ability to develop genetically engineered mice both by transgenesis via pronuclear injection and with gene targeting using embryonic stem (ES) cells. In addition, large mutagenesis programs have been initiated to isolate new mutations associated with a wide variety of biological processes. These activities have been complemented with other technological advances, including the initiation of a DNA sequencing effort to completely sequence the mouse genome and the development of microarray technology. Although the promise of these efforts in contributing to the genetic dissection of complex biological processes is extraordinary, having the appropriate model to which one can apply these emerging technologies is paramount to a successful and productive outcome.

Although the mouse has many advantages, it is not without its disadvantages. It must be recognized that not all discoveries or observations made with the mouse will have direct parallels in the human. Furthermore, with a life span of many months as compared with days in a species such as Drosophila, another powerful genetic model, data collection is inherently slower. Finally, housing costs and the operation of a barrier facility is a major consideration in the selection of this model.

The mouse model provides an opportunity for investigators to control both the nutrition and housing environments for their experiment animals. It has been well documented that caloric restriction can substantially lengthen the life span of laboratory mice. This principle seems to be true not only for mice and other rodents but also for insects, worms, and nonhuman primates, with possible implications for humans. Clearly, continued work with genetic models such as Drosophila and the mouse will ultimately reveal the genetic basis for this phenomenon. In the design of an aging study, the program for housing animals should be evaluated carefully. Caging style, light–dark cycle, ambient temperature, and the number of mice housed per cage all are obvious concerns. Other factors for consideration include extraneous noise and consistency in caretaker handling of the animals. Careful training of caretakers for uniformity in handling of the mice during cage-changing activities should not be underestimated.

Infectious diseases have long been known to have a potential impact on experimental analyses in mice. It is important to be able to determine whether the onset of a disease state is a function of the aging process or the result of a pathogen infection in the colony. In this context, the development and maintenance of specific pathogen-free (SPF) colonies for aging studies is essential. A researcher is also aided in this area by the existence of a large literature base on diseases in mice and on variations in susceptibility to diseases in specific lines or strains. The SPF status of study animals should be defined and maintained by careful husbandry practices. Frequently, an SPF barrier facility equipped with micro-isolator caging is employed to minimize the risk of an unwanted pathogen entering the colony. Access to such barriers is limited, and strict protocols for entry and animal-handling practices are mandated for staff working in such areas. A carefully designed sentinel program is essential for monitoring the health status of the colony and should be associated with a careful plan to respond if a break in the SPF status should occur.

Many inbred strains of mice have been developed and maintained by brother–sister matings for numerous generations. One major advantage of an inbred strain is genetic uniformity from one generation to the next. This feature permits an experiment to be repeated in both time and space with an identical or nearly identical genetic environment. In addition, a wealth of information on individual strain characteristics has been accumulated through years of research with these lines. Several inbred strains have been used in aging studies and can be obtained from a variety of sources. Inbred mice currently available through the NIA include BALB/cBy, CBA, C57BL/6, and DBA/2. Hybrids and calorically restricted mice will be increasingly available in future years. However, genetic uniformity of the inbred strain does not come without a price in that certain lines come with their own particular set of problems that can affect an aging study. In the selection of a particular strain for analysis, previous work with that strain, the nature of the aging question being addressed, and potential strain idiosyncrasies must be evaluated. Fortunately, databases that allow rapid electronic access to much of this information have been developed to assist investigators with strain selection.

One approach to reduce the potential problems associated with the use of inbred strains is the production of an F1 hybrid mouse that is created by mating individuals from two different inbred strains. These F1 animals have the virtue of being genetically identical as well as being potentially heterozygous at most loci (F1 mice will be homozygous only for loci that happened to be identical in their inbred parents), reducing the impact of certain idiosyncrasies found in their inbred parents.

The number of variants at individual genetic loci has grown precipitously as a function of genetic engineering and focused mutagenesis programs that have been initiated relatively recently. Because designer variants and many spontaneous mutations are often isolated on different or mixed genetic backgrounds, breeding programs are frequently conducted to place each variant in a defined genetic background. This is accomplished by a series of back-crosses of the variant locus to a selected inbred strain such as C57BL/6. Lines established in this fashion are referred to as congenic strains. To avoid the complications of studying a variant in an inbred setting, a second congenic line in a different inbred strain can be created. By crossing these two congenic strains, a particular variant can be analyzed in an F1 hybrid and compared with the phenotype in the inbred strain.

Although the inbred strains and F1 hybrids allow rigid control of the genetic environment in a set of experimental animals, not all researchers agree with this approach. Concerns about the lack of genetic variance in these experimental paradigms (inbred lines and F1 hybrid) has led some researchers to favor experimental systems that provide more genetic heterogeneity. One experimental paradigm involves F2 hybrids. In this model, experimental mice are obtained by mating two F1 mice of the same type (e.g., F1 mice produced by crossing C57BL/6 mice with C3H mice). Alternatively, F2 animals can be developed using the four-way breeding scheme. This breeding program involves the mating of two different F1 parents. In this type of mating system, the resulting F2 animals have four different grandparents, whereas they have only two different grandparents if the F1 parents are of the same type. In either system, the F2 mice are genetically heterogeneous, yet the genetic makeup of the population is defined and reproducible from laboratory to laboratory.

More recently, aging mice generated through the Diversity Outbred and the Collaborative Cross Inbred strains have offered powerful insights into novel genetic linkages to specific mouse phenotypes and outcomes [1]

Other Rodents

Although mice and rats represent by far the most commonly rodents, several other rodents have found a unique place in aging research. Most important among these is the naked mole rat which at 28.3 years is the longest lived rodent known. Moreover, the naked mole rat's slower rate of aging makes it a far more relevant as a model of human aging processes. In fact, it has been argued that given an adjustment for its body size, the naked mole rat may represent a model of successful human aging.

Invertebrate Model Systems in Aging

Saccharomyces cerevisiae (yeast), *Caenorhabditis elegans* (nematode), and *Drosophila melanogaster* (fruit fly) are the leading invertebrate model systems used in aging research. Their great advantages include ease of maintenance, short life spans, advanced molecular and genetic tools, and availability of their complete genomic sequences. Genetic screens have already identified a number of genes that appear to be involved in the aging process. Genetic and molecular characterization of these genes has provided valuable information about the physiological pathways that may be important in the aging process. Furthermore, the molecular conservation that has been so widely observed among yeast, *C. elegans*, *Drosophila*, and mammalian model systems such as mice and rats suggests that information obtained in these "simpler" systems is certain to facilitate our understanding of aging.

The budding yeast, *S. cerevisiae* (a single-cell organism), has been used in aging research since 1959, when Mortimer and Johnson described an increase in volume and number of scars as an aging phenotype. Aging of the yeast is measured as the average number of cell divisions that a mother cell can undergo or as the survival of nondividing cells during the stationary phase. The asymmetric cell divisions result in a larger mother cell and smaller daughter cells that can be easily separated, leaving behind the scar. Average cell divisions can range from 15 to 30 generations, depending on the strain. During the past decade, more than 16 longevity genes have been discovered in yeast, many of which are now being found to be important in the aging process of other invertebrates and mammals.

C.elegans research has contributed immensely to our current understanding of longevity and aging. As an experimentally tractable, multicellular eukaryotic organism, this invertebrate nematode will continue to be an impactful model organism in aging research. *C.elegans* as a model organism offers a number of experimental benefits for research and for aging research specifically. Experimentally, *C. elegans* is easily cultured and manipulable, offering many practical aspects of research benefit. In either liquid or solid agar based media, *C. elegans* is grown at 20 °C (*i.e.*, close to room temperature) from fertilized egg to adult within ~3 days (isolatable life stages include egg, L1, L2, L3, and L4 larval stages, adult, and egg-producing, fully reproductive adult stage); the average median lifespan of wild-type *C. elegans* ranges from ~10 to 30 days (Gems and Riddle, 2000; Johnson and Wood, 1982) with the longest lived strains and single worms living still less than ~4 months. *C. elegans* are hermaphrodites, and a single wild-type worm may produce up to 300–400 eggs over their fertile lifespan. This allows for culture of thousands of worms within just a few days and both single worms and lysates of masses of worms can be assayed. *C. elegans* may also reproduce sexually for a more genetically controllable method of reproduction. The food source for culture is most often a non-pathogenic OP50 *E. coli* strain, but diet can be altered according to experimental needs. Finally, mutant strains of countless genetic *C. elegans* models are available for purchase at merely $7 per strain and the worm community offers endless diversity in available mutants and genetic tools. Needless to say, *C. elegans* is an inexpensive, rapid, and easy model organism to work with. The power of this easy model is represented by some of the fundamental work that has led to 5 Nobel Prize researchers being awarded for work in *C. elegans*.

To complement its ease of use as an animal model, *C. elegans* possesses many features that make it ideal for a discovery model in diverse types of controlled experiments in aging. The *C. elegans* genome is highly conserved, fully sequenced, and extensively annotated. Both forward and reverse genetics are easily possible. Reverse genetics can be performed in this model by feeding, soaking, injection and requires only engineered *E. coli* strains producing dsRNA for a particular gene of interest. By feeding, *C. elegans* may ingest dsRNA producing *E. coli* and dsRNA is taken up by all somatic cells through the gut. By soaking, introduction of silencing dsRNA is taken up by cells directly from the bathing media, and by injection, dsRNA can be injected into target tissues or eggs. The effects of gene knockdown by RNA interference (RNAi) can be immediate, within the experimental worm, and can be removed by removal of the dsRNA stimulus, and re-introduced within the same lifespan of a single worm. Prolonged RNAi over many generations can be introduced by continuous exposure to dsRNA producing bacterial food sources. Over 20,000 different strains of *E. coli* producing dsRNA for almost every gene in the C. elegans genome are available for easy purchase from commercial libraries (Fraser *et al.*, 2000; Kamath and Ahringer, 2003; Kamath *et al.*, 2003). Furthermore, the most recent developments in using CRISPR-caspase methods of easily introducing more permanent gene mutations in the *C. elegans* genome to create novel mutants, is already well-developed in this animal model and moving forward rapidly (Chen *et al.*, 2013; Chiu *et al.*, 2013).

C. elegans genetic tractability has made it a powerful tool to perform genome-wide screens for genes that can be implicated in lifespan extension, shortening, or even more functionally important, preservation of healthy signs of aging. Dozens of genome-wide screens have been performed in *C. elegans* and each genome wide screen of the effects of any of the given ~20,000 RNAi clones can take as little as 3–4 months to complete (Solis and Petrascheck, 2011; Wang *et al.*, 2013). Many of the fundamental aging research has stemmed from genome-wide screens in *C. elegans* that have identified hormonal, metabolic, insulin-signaling, proteostasis, mitochondrial, antioxidant, and stress-related roles in lifespan extension and prolonged healthy aging (Bennett *et al.*, 2014; Gonidakis *et al.*, 2010; Hamilton *et al.*, 2005; Hansen *et al.*, 2007; Kenyon, 2011; Lin *et al.*, 2001; McCormick *et al.*, 2012; Samuelson *et al.*, 2007a,b). The effects of multiple gene knockdown via RNAi in concert with multiplexed mutations in single worm strains also adds power to genetics questions of aging that we can ask with *C. elegans*. In similar ways, we are now able to screen *C. elegans* strains and genome-wide variants for the effects of pharmacological agents, supplements, and diets on aging (Ye *et al.*, 2014). Contextualizing or applying genome-wide RNAi or pharmacological screens within or to specific models of aging diseases such as Parkinson's or Alzheimer's adds power to C. elegans utility as an aging research model (Bennett *et al.*, 2014; Brignull *et al.*, 2006; Labbadia and Morimoto, 2014).

Additional features of *C. elegans* that have served the aging research community well, are the physiological, behavioral, and microscopic assays that are available to scientists. *C. elegans* is a transparent invertebrate animal that at its largest is 1mm in length. Developmentally, the cell fate/lineage of each of the 959 somatic cells has been fully characterized from embryo to adult. The development of every stage post-fertilization is visually observable as every egg in the reproductive tract is sequentially and linearly organized according to stage of development. The simple body plan also has allowed full characterization of each of the 302 neurons for example; researchers focused on aging induced spontaneous protein aggregation inn neurons, have been able to use *C. elegans* to better understand conserved mechanisms of neurodegeneration (David *et al.*, 2010; Kraemer *et al.*, 2003). Approaching aging research using the powerful genetic, microscopy, and other biochemical tools available in *C. elegans*, in concert with functional behavioral and physiological assays has allowed researchers to deeply probe key pathways in lifespan regulation, including highly conserved pathways such as insulin signaling and proteostasis.

Drosophila Melanogaster

Drosophila melanogaster (fruit fly) is one of the leading invertebrate model systems used in aging research. *Drosophila* and closely related species have been used to study aging since 1915. *Drosophila* show an age-related functional decline as well as a decline in behavioral performance similarly to other species. For example, *Drosophila*'s learning abilities, heart function, sensory function, fecundity, and locomotor activity declines with age. There are numerous advantages of using *Drosophila* as a model system to study aging, such as the fact that fruit flies are not expensive to breed, are easy to maintain, and have a relatively short life span. *Drosophila* live about 2 months at 25 °C, much shorter than mice who live 4 years or nonhuman primates, such as *Macaca mulatta* who live 29 years. There are a number of environmental manipulations that can alter the life span of *Drosophila*, such as temperature of the environment, reproductive status, addition of drugs, as well as changing the caloric or dietary content of the food. Flies, who are poikilothermic organisms, live about 3 months at 18 °C and live only 1 month at 29 °C. Another manipulation that affects *Drosophila* longevity is a change in reproduction status. Reproduction has a strong negative effect on longevity in fruit flies. Both virgin female and male flies live significantly longer than do fully mated ones. The costs of reproduction in flies include energy allocation for courtship and mating, egg production and egg laying, and direct toxic effects of mating. Dietary restriction (DR) without malnutrition is the most efficient manipulation that extends longevity of a variety of species, including *Drosophila*. DR causes many changes in fly physiology that are similar to changes found in DR mammals. Such changes include decrease in weight, increased spontaneous locomotor activity, decreased resistance to starvation, decrease in fecundity, and lower levels of carbohydrates and triglycerides. All of those changes contribute to longer and healthier fly life. The use of flies to determine effects of different drugs on health and longevity has been an important part of longevity studies. These experiments have discovered that addition of resveratrol, rapamycin, metformin or other nutraceuticals in fly food can modulate aging by promoting many health benefits. Also of interest is that a majority of the cells of adult fruit flies are postmitotic, with exceptions of intestinal stem cells in the fly gut, malphigian tubules (fly kidneys), salivary glands, gonads and brain. Therefore, most of the

tissues in *Drosophila* age synchronously. Examination of the role of intestinal stem cell homeostasis in fly health and longevity has been of interest in recent years.

The genetic tools that have been developed over the past 100 years or so of *Drosophila* research have reached a point where researchers have great control over the molecular genetics of the fly. Through a combination of various available techniques, it is possible to increase or decrease the level of expression of any gene in a tissue specific manner at any time in life, from development to old age. Genetic screens have identified a number of genes that appear to be involved in the aging process. Genetic and molecular characterization of these genes has provided valuable information about the physiological pathways that may be important in the aging process. Among the first genes identified to be involved in fly longevity were *Methuselah*, *Indy* (*I'm not dead yet*) and members of the insulin/insulin like pathway. A number of stocks necessary to perform research are easily available from several Stock Centers (Bloomington *Drosophila* Stock Center, The Exelixis Collection at the Harvard medical School, Vienna *Drosophila* RNAi Center). In addition, the complete genomic sequences are now available for *Drosophila* and related species. The molecular conservation in sequences that has been so widely observed between *Drosophila* and mammalian model systems such as mice and rats suggests that information obtained in fly systems is certain to greatly contribute to our understanding of aging. This is illustrated by a number of important physiological pathways that have been implicated in fly longevity, which have been also implicated in longevity of other species. For instance, decreases in the insulin/insulin-like growth factor-1 (IGF-1) and the Target of Rapamycin (TOR) signaling pathway have been shown to affect life span in flies as well as in yeast, worms and mice.

Nonhuman Primates

The high cost and small availability of aged nonhuman primates have limited their use to selected research centers. Further compounding the difficulties in conducting research with these animals is their long life span and a lack of good survival data. At the same time, the phylogenetic proximity of these primates to humans makes these highly attractive, and in some cases even indispensable, models for the study of human aging and disease. For example, true menstruation occurs only among some primate species, making some nonhuman primates invaluable models for the study of female human menopause. Another area is brain structure, where only nonhuman primates represent adequate models for the study of many aspects of higher cortical structure and physiology.

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Oxidative Stress and Aging☆

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Glossary

Antioxidants Proteins and non-protein molecules that remove or neutralize oxidants.

Environmental change Refers to organism-specific parameters rather than habitat and may include such things as the ability to adjust body temperature when the surroundings become either too hot or cold, increasing cardiac output during physical stress, or maintaining the capacity to heal injuries.

Free radical Atom, ion, or molecule that contains an unpaired electron.

Oxidant Substances that accept electrons.

Redox State The ratio of oxidizing to reducing equivalents; charge potential.

Reductant Substances that donate electrons.

Definition

Aging is a progressive, time-dependent deterioration in the capacity of an organism to respond adaptively to environmental change resulting in an increased vulnerability to death. Oxidative stress reflects the extent to which the concentration of oxidants in cells exceeds the cellular capacity to remove them. It can alter the cellular redox environment, damage cellular constituents, influence gene expression and cause cells to divert energy and other resources from growth to repair.

Introduction

Aging

The irreversible, time-dependent deterioration in the capacity of an organism to adapt to environmental changes and resulting in an increased vulnerability to death is generally referred to as aging. Although aging itself does not directly kill the organism, it results in a progressive increase in the probability of its death. The probability that certain diseases will occur increases with the passage of time; however, aging itself is distinct from any known disease pathology because it crosses virtually all species barriers and, unlike any known disease, aging affects all members of a species. The exact causes of aging are unknown, but there is much evidence to support a role for cellular oxidants as a primary causal factor in the phenomenon.

Oxidants and Oxidative Stress

There are many different types of cellular oxidants. In order to understand how they form it is important to recall that molecules must contain only paired electrons in order to be stable. Atoms and molecules that contained unpaired electrons quickly react with whatever surrounds them and share electrons to meet the paired electron stability rule imposed by nature. Most of the time molecules that contain unpaired electrons can be mass-produced only under very controlled laboratory conditions; they are almost always very unstable, meaning that they can exist only a few thousandths or millionths of a second after their creation. Molecules that contain unpaired electrons are referred to as free radicals. In organisms, enzyme molecules catalyze chemical reactions that sometimes pass through intermediate steps in which they contain unpaired electrons. During normal metabolism in mitochondria, dioxygen (O_2) molecules act as the terminal electron acceptor of oxidative phosphorylation. Each molecule of oxygen accepts four electrons to form water.

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

Most of the oxygen consumed by cells is used in this way, but a small amount (12%) escapes from the electron transport chain after receiving only one electron rather than the usual four. When this happens, superoxide radicals ($O_2^{\bullet -}$) are created. Of course, superoxide like other free radicals is very short-lived; its half-life is about one millionth of a second (s). $O_2^{\bullet -}$ radicals can react with the surrounding cellular structures and cause damage; however, they often simply react with each other to form hydrogen peroxide (H_2O_2). H_2O_2 is also produced directly by some enzymes such as glucose or amino acid oxidases. H_2O_2 is not a radical because it

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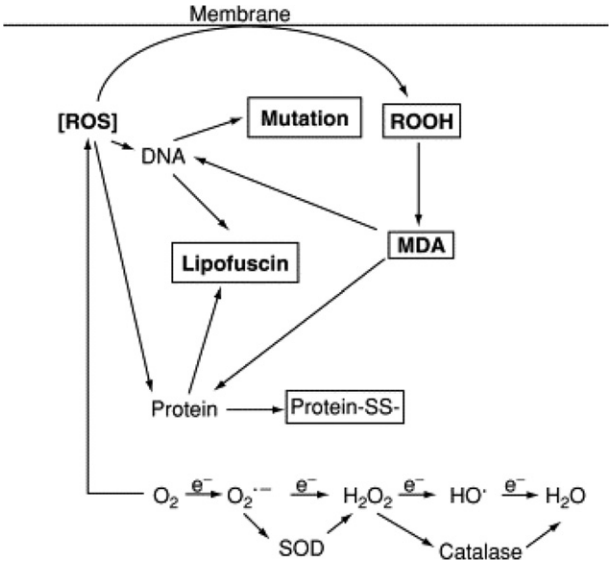

Fig. 1 Pathways of oxidant formation and reaction. ROS = reactive oxygen species; MDA = malondialdehyde; ROOH = organic peroxide; protein-SS- = protein disulfide. See text for discussion.

contains only paired electrons. It is therefore far more stable than free radicals and exhibits a half-life of many years in pure water. Nevertheless, H_2O_2 is still much more reactive than water particularly in the presence of metal ions and can damage many cellular components by reacting with them (see Fig. 1).

In some cases, H_2O_2 reacts with $O_2^{\bullet -}$ and forms the hydroxyl radical (HO•). This reaction is catalyzed by transition metals such as iron. HO• is much more reactive than either H_2O_2 or $O_2^{\bullet -}$; in fact, it can even break water molecules apart. Collectively these reactive forms of oxygen are called reactive oxygen species (ROS). There are other types of ROS. For example, ozone is composed of three oxygen atoms that share electrons. Oxygen molecules can also simply absorb energy to form reactive molecules that are not free radicals, but that are more reactive than ground state oxygen. These energized oxygen molecules are referred to as singlet oxygen. Normally, the production of oxidants by cells is in balance with oxidant removal (see next subsection). When the production of ROS exceeds the cellular capacity to remove them or when an external source of oxidation is added, the result is oxidative stress. The severity of the stress will depend on the relative rates of oxidant production/removal. Severe stress resulting from bombardment by uncontrolled oxidant generation is lethal. In addition to the oxygen centered radicals, nitrogen centered free radicals and reactive species also form in cells and may influence a number of biological processes.

Antioxidant Defenses

The production of ROS is fatal to unprotected cells; however, cells that use oxygen in their energy metabolism also contains proteins and other small molecules collectively referred to as antioxidant defenses that remove more reactive species and prevent cellular damage. These include superoxide dismutases (SOD) that remove $O_2^{\bullet -}$ and produce H_2O_2, and catalase and peroxidases that remove H_2O_2. Enzymic defenses that remove HO• radicals have never been identified, but non-enzymatic antioxidant defenses, such as tocopherol (Vitamin E), carotenoids (such as beta carotene), ascorbate (Vitamin C), uric acid, lipoic acid and glutathione (GSH), can remove free radicals including HO• by reacting directly with them. The non-enzyme antioxidants can also break chain oxidation reactions and dissipate the extra energy from the singlet oxygen. The equilibrium between oxidant generation and removal is ultimately manifested in the distribution of cellular charges. The ratio of oxidizing to reducing cellular components is often referred to as cellular redox state. Glutathione, a tripeptide consisting of glycine, cysteine and glutamic acid moieties, is the primary cellular component that affects redox state. It exists as both a reduced (GSH) and an oxidized form (GSSG). GSH contains more than 90% of the reducing equivalents present in most cells. Because of this, glutathione is often referred to as a cellular redox buffer.

Oxidative Stress and Aging

The idea that oxidative stress contributes to aging is relatively old although it has been repackaged in several different ways. The most encompassing theory that directly linked oxygen metabolism to aging was Denham Harman's Free Radical Theory of Aging.

Harman theorized that low levels of oxidants are produced in metabolic pathways resulting in an incessant bombardment of cellular components by ROS. Oxidation reactions resulted in accumulation of damage, cellular dysfunction and ultimately organ dysfunction. An implication of this theory is that free radicals would be expected to have an impact on all organisms that utilize

oxygen. Although the Free Radical Theory of Aging now enjoys great popularity, it was not immediately accepted. For more than a decade after it was proposed an intense controversy raged over whether free radicals were actually ever produced in living cells. Several studies demonstrated that various types of radicals existed in skin and in specific types of cells, but many scientists questioned whether these results were artifacts of the methods used to measure free radicals. Additionally, some enzymes such as xanthine oxidase were also observed to produce free radicals when added to a reaction mixture. However, it was unclear that radicals would be produced *in vivo*. It was not until 1969 when McCord and Fridovich identified superoxide dismutase as an enzyme that used superoxide radicals as a substrate that this issue was definitively resolved. Cell must produce free radicals if they contained enzymes to remove them.

Evidence Supporting Free Radicals and the Damage Hypothesis of Aging

Several studies have shown that the rate at which cells generate oxidants increases with aging. In most cases the antioxidant defenses remain unchanged or decline with increasing age. The net effect of these changes is that the cells of young individuals exhibit a level of oxidation (redox state) that differs from the one seen in older individuals. In general, the tissues of older individuals tend to become more oxidized.

Lipid peroxidation products accumulate with age

Some of the most compelling evidence supporting the Free Radical Theory of Aging is found in the fact that by-products of free radical reactions accumulate in animals as they age. For example, lipid peroxidation occurs in cells. This is the same process that causes fats to turn rancid. Once they form, lipid peroxides are either removed by defense enzymes or breakdown and rearrange to form malondialdehyde (MDA) an even more reactive chemical that can damage DNA and proteins. The reaction products of MDA and various cellular components are further rearranged to form a fluorescent pigment called lipofuscin (**Fig. 1**). It is essentially cellular trash that can be rearranged or processed no further.

Since cells have no mechanism to dump this by-product they simply accumulate it as more is formed. The pigment is observed mainly in postmitotic cells such as neurons and cardiac myocytes. Unlike its precursors, lipofuscin is stable. Although the concentration of lipid peroxide does not necessarily increase dramatically in older organisms, the more stable lipofuscin accumulates progressively with age in animals ranging in biological complexity from insects to humans.

Lipofuscin does not cause aging; however, older individuals typically have more of it than do young individuals. Because of this it has been used as a marker to predict remaining lifespan. For example, houseflies typically live 2 weeks. If placed in a small container that prevents them from flying (low activity) their lifespan is doubled to about 4 weeks. When lipofuscin is measured, it is found to accumulate more slowly in the longer-lived flies. The longest living flies in both control and low activity populations have accumulated the same amount of lipofuscin by the end of their life, but the longer-lived low activity group accumulates it at a proportionally slower rate.

Unfortunately, lipofuscin has not proven to be a reliable marker of physiological age, since factors that do not necessarily influence lifespan can markedly influence the accumulation of lipofuscin. For example, the ingestion of some dietary antioxidants can retard the formation of lipofuscin even when they fail to increase lifespan. Some oxidants, such as the herbicide paraquat, also impede lipofuscin formation, apparently by destroying precursors needed for its formation.

Some types of DNA Damage accumulate with age

In general, the sequence of genomic DNA (the DNA in the nucleus of the cell) is not greatly altered by age because repair mechanisms eliminate damage and more heavily damaged cells die. Nevertheless, DNA breaks accumulate during aging. Somatic mutations (mutations that occur in cells outside the reproductive system) also accumulate but at a slow rate. Longer-lived species exhibit greater DNA repair capacity than shorter-lived species. It has been proposed that accumulation of DNA damage contributes to the aging process; although, the existing evidence fails to directly support this hypothesis. Nevertheless, oxidatively modified DNA bases (8-OH dG) are excreted in urine and can be isolated from mitochondrial and nuclear DNA.

In contrast to the low rate of mutation seen in nuclei, the DNA found in cellular mitochondria rapidly accumulates damage with the passage of time. This is significant because most of the oxygen consumed by cells (and hence, many of the oxidants produced) is in mitochondria. Mitochondrial membranes also tend to become peroxidized more rapidly than cell membranes. Although damaged mitochondrial DNA cannot be repaired, there are many copies of the mitochondrial genome within each mitochondrion; this permits mitochondria to continue to replicate even if some copies of their DNA have been damaged. As compared to cells, which can live more than 90 years in humans, mitochondria have short lifespans of no more than a few weeks. Damaged mitochondria are usually replaced relatively quickly; however, the rate at which they are replaced declines with age. Thus, the cells of older individuals frequently exhibit greater numbers of dysfunctional mitochondria. Although most types of DNA damage are lethal to mitochondria, they are able to survive some types of deletions. Those that do survive can replicate, and may even become predominant in cells. The energy metabolism of those cells is compromised. The accumulation of defective mitochondria in cells has also been postulated to contribute to the aging process, but this hypothesis remains controversial because the number of defective mitochondria usually remains small and the number of cells in which defective mitochondria become predominant is also low. The central point here is that damage to mitochondrial DNA is much greater than damage to nuclear DNA, presumably because mitochondria are also the sites of oxidant generation.

Protein damage accumulates with age

It is important to note that the proteins made by older animals are exactly the same as those made by young animals. The cellular machinery that encodes and synthesizes proteins does not produce errors as a result of aging, albeit, the rate of synthesis may decline with age. After proteins have been synthesized, they are quickly removed (digested and the amino acids recycled into new proteins) from cells if they become damaged by oxidants. In older animals the rate of protein turnover decreases. As a result, the subpopulation of damaged protein molecules is greater than is observed in the cells of younger animals. It is noteworthy that cellular redox state tends to increase (become more oxidized) during aging. This promotes the formation of disulfide bridges between proteins, which also impairs or inactivates them completely. The effects of damaged protein accumulation can be significant. For example oxidative damage to lens proteins appears to contribute significantly to cataract formation.

Under certain conditions, antioxidants increase lifespan

A large number of studies have examined the effects of feeding various antioxidants to organisms. In many cases, the average lifespan of test organisms is increased by these treatments but the maximum lifespans reached by the oldest members of the population are usually unaffected. The reasons that maximum lifespans tend to be unaffected by antioxidant treatments appear to be many. The antioxidants used in these studies may not be distributed in cells evenly after they are ingested. In some cases, intestinal absorption of antioxidants decreases when larger amounts are ingested. Chemical antioxidants can have harmful side effects. Also, some of antioxidants become toxic after they react with oxidants.

For many years it was speculated that if antioxidant enzymes could be increased in cells, they would increase lifespan without the uncontrollable effects typically seen when treating with chemical antioxidants. Rats that overexpressed SOD were engineered, but showed no increase in lifespan. Similarly, insects that strongly overexpress SOD in all of their tissues failed to exhibit any increase in maximum lifespan. Flies that overexpressed SOD only in certain tissues (such as brain) or that only weakly over-expressed SOD in all their tissues exhibited some increases in lifespan. Conversely, it was shown that fruit flies that had only half the normal complement of SOD lived as long as normal controls. One reason for these apparent inconsistencies is that the product of SOD is H_2O_2, a toxic oxidant. Thus, increasing SOD also increases H_2O_2, which obviates any beneficial effect of superoxide radical removal. Similarly, increasing enzymes that remove H_2O_2 but not SOD fails to effect lifespan in insects or mammals.

Insects that over-expressed both SOD (to remove superoxide) and catalase (to remove H_2O_2) exhibit increased average and maximum lifespan. In fact, in some strains of flies the maximum lifespan is increased by as much as 50% with this dual treatment. It is also significant that amount of metabolic work (usually indicated by the total amount of oxygen consumed during life) was also increased by 50%. Many other treatments that increase maximum lifespan in cold-blooded animals tend to decrease the rate of metabolism and the total lifetime energy expenditure indicating that, although lifespan is longer, the quality of life is diminished. These results with dual overexpression of both SOD and catalase support the view that ROS contribute to the aging process and that removal of oxidants can retard aging in invertebrates.

Oxidative Stress and Gene Expression

The contribution of oxidants to the aging process has generally been inferred to stem from their propensity to inflict cellular damage. But is damage the only mechanism by which ROS influence the aging process? It has become apparent that ROS can stimulate changes in the expression of certain genes. Extremely large changes in oxidant production and in antioxidant defense levels are observed during embryonic development. These changes have been shown to promote differentiation of cells without inflicting large-scale cellular damage. This suggests that some of the genes critical to differentiation are sensitive to the changes in ROS. In fact, treatments that increase H_2O_2 or other ROS (including introduction of SOD into cells) stimulate differentiation in some types of cells, while treatment with chemical antioxidants can inhibit or slow some types of differentiation. It was recently demonstrated that the developmental fate of stem cells is influenced by experimental modulation of the level of oxidative stress.

ROS have been shown to play an important role in many cellular signaling pathways. For example, the addition of growth factors such as platelet-derived growth factor (PDGF) to cells causes them to produce small bursts of oxidants that activate signaling molecules in the cells. The activated signal transduction cascade stimulates genes associated with cell growth. At least some elements in each of the MAP kinase pathways are sensitive to changes in cellular redox balance. Stimulation of these pathways can lead to either activation or repression of genes, depending on the target.

The pathways that respond to redox changes during development remain sensitive to increases in ROS production in adult cells. The fact that aging is associated with a tendency for cells to become increasingly oxidized may ultimately be linked to some of the age-associated changes in gene expression. For example, if the cellular environment becomes more oxidized, it might be expected that pathways activated by oxidants would be less sensitive. This is not to say that the pathways will be inactive in older people; however, it does suggest that they may be less efficient. This type of change is consistent with the original Free Radical Theory of Aging although the target is different than the ones that were originally proposed.

In another type of study, it has been observed that inactivation of certain genes in fruit flies as well as in nematodes can increase lifespan. In depth analyses of these effects reveal that in most cases the increases in longevity are associated with either decreased rates of metabolism or increased mitochondrial density. These observations would suggest that changes in levels of oxidative stress plays a crucial role in the life lengthening effects of certain gene deletions.

Oxidative stress and cancer

The frequency of many types of cancer increases with age. Cellular accumulation of somatic mutations resulting from oxidative damage is almost certainly a decisive factor in the aging associated increase in malignancy. However, it should not be assumed that increased oxidation is always a factor in cancer formation. Paradoxically, antioxidation can also contribute to tumor formation. For example, basal cell carcinomas exhibit increased expression of the gene *Bcl-2*, which increases the enzyme antioxidant defenses. This increase in antioxidant defense tends to make the cells resistant to apoptosis. It has been observed that melatonin has antioxidant properties and can increase average lifespan when fed to rats; however, the treated group exhibits higher rates of cancer than the controls. Although the enzyme defenses are frequently maintained at low levels in tumor cells, GSH is often elevated in tumors. Of course, antioxidants are not a major cause of cancers, neither are they a panacea for all of the deleterious effects attributed to oxidants. Indeed, not all oxidation is bad. Increased cellular oxidation is an important step in killing defective (tumor) cells through apoptosis. A strict maintenance of cellular redox state and capacity to respond to stress appears to be far more important to the normal function of cells than complete elimination of oxidants.

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Immunology and Aging☆

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Glossary

Antigen A substance that can elicit an immune response following recognition by T-cells or B-cells. Antigens can be of many sorts: proteins, carbohydrates, nucleic acids, lipids, hormones. The immune system usually responds only against foreign antigens (e.g. from pathogens), but can also react against modified self-antigens (in case of cancer) or self-antigens (in case of autoimmune diseases).

B-cells A population of white blood lymphocytes found in the bone marrow, lymph nodes and blood circulation. B-cells are generated and mature in the bone marrow; their role is to produce antibodies to bind and neutralize pathogens and toxins. Each antibody is strictly specific to only one antigen.

Cytokines Proteins synthesized by cells of the immune system. Cytokines include an extensive range of mediators (interleukins, interferons, chemokines and growth factors) with various functions (e.g. immunostimulatory or immunosuppressive).

Immunosenescence Deterioration of the immune system or its function with time.

T-cells A population of white blood lymphocytes found in the thymus, lymph nodes and blood circulation. T-cell precursors are generated in the bone marrow; following their maturation in the thymus, naive T-cells are exposed to foreign antigens, and produce effector T-cells, as well as memory T-cells, which are long-lived antigen-experienced cells that rapidly respond to a subsequent exposure with the same antigen. T-cells have many functions including the elimination of foreign pathogens, infected cells and tumor cells, and the regulation of the immune response by assisting or suppressing other cells of the immune system.

Introduction

The study of our native immunity began in the late 18th century, and has led to incredible discoveries regarding the role of our immune system and the mechanisms through which pathogens attempt to penetrate our defences. Immunogerontology is a relatively new field in comparison, which is gradually becoming more relevant as the older population is increasing at an unprecedented rate. Owing to the progress in medicine and public health, life expectancy in developed countries has nearly doubled over the last century. Nowadays, it is common to live beyond 70 years old, and this trend is likely to continue, with an important impact on human demographics. However, advanced age is accompanied by an increase in chronic diseases, resulting in a significant decline in quality of life. The aging of the human population represents therefore a considerable challenge to public health authorities, raising both healthcare related and socio-economic issues.

Old people usually present increased severity and susceptibility to infectious, malignant and autoimmune diseases. The development of these pathologies is thought to be directly linked to the decline of the immune system with age. It is indeed well established that the functional capacity of the immune system gradually declines with age, so that the immune system cannot respond as quickly and as efficiently to stimuli, in particular new antigens (Dorshkind *et al.*, 2009). This deterioration, also referred to as immunosenescence, is reflected by a deregulation of the immune system, characterized by changes in cellular phenotype and function, and alterations of whole organs. The study of the aging immune system, or immunogerontology, is therefore a public health priority with crucial implications to optimize care of elderly people, as well as to develop better therapies and vaccines for this population.

Immune Alterations Associated with Aging

The Hayflick Limit

In 1961, Hayflick and Moorhead showed that cultures of fetal human fibroblasts could reach an irreversible state of growth arrest, hence reporting the first evidence for replicative senescence. Accumulating evidence shows that the so-called Hayflick limit is not restricted to cultured human fibroblasts, but applies also to the cells of the immune system, which may have a limited replicative lifespan *in vivo* (Effros and Pawelec, 1997). Although the notion of immunosenescence is not limited to cellular senescence, the concept of limited replicative lifespan is important in the context of immune aging. The occurrence of replicative senescence is primarily related to the number of cell divisions, varying according to cell type and cellular environment. A commonly used

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marker of replicative history is the length of the telomeres (DNA repeats found at the end of the chromosomes) which is reduced after each cell division. Cells which have reached a state of replicative senescence have short telomeres.

Hematopoiesis

All the cells which constitute our immune system originate from hematopoietic stem cells (found in the bone marrow) which differentiate and commit themselves to one specific cellular lineage (e.g. myeloid or lymphoid) to continuously generate new immunocompetent cells of the innate (e.g., neutrophils and monocytes) or adaptive (e.g., naïve lymphocytes) arms of immunity. Increasing evidence indicates that hematopoietic stem cells are affected with aging, resulting in a deregulation of hematopoiesis. Hematopoietic stem cells exhibit a reduced capacity to generate new immunocompetent cells with old age. Their telomeres appear to be shorter in adults than in the cord blood of newborns. Neutrophils and naïve T-cells show a shortening in telomere length associated with age, suggesting that this applies also to hematopoietic stem cells (Rufer *et al.*, 1999). Moreover, there is a clear bias towards fewer hematopoietic progenitors with lymphoid, versus myeloid, precursor capacity in elderly individuals (Kuranda *et al.*, 2011). Hematopoietic stem cells thus appear to have a limited replicative potential and survival capacity so that their ability to replenish the cells of the immune system is not infinite (Wang *et al.*, 2011). In addition to intrinsic defaults of old hematopoietic stem cells, extrinsic causes of altered hematopoiesis may also be related to decreased production of growth hormone and loss of bone marrow stromal cells in the elderly. Although there is still debate regarding whether altered hematopoiesis has a real consequence on the immune function in aging, it implies that the renewal capacity of the immune system is not unlimited and can reach exhaustion over time.

Adaptive Immunity

The adaptive system involves mounting of highly specific immune responses, with the establishment of immunological memory. There are two types of adaptive immune responses: humoral (mediated by antibodies produced by B-lymphocytes) and cellular (mediated by T-lymphocytes). Over the years, strong evidence has accumulated to show that aging results in important alterations of the T- and B-cell numbers and functions.

Thymic involution and homeostatic T-cell proliferation

Mounting of primary immune response is highly dependent on T-cell priming, when, encountering a new antigen, naïve T-cells become activated and differentiate into antigen-experienced T-cells, which exhibit direct effector functions and can expand greatly to eliminate foreign antigens from the body. Naïve T-cells represent therefore the primary source to mount an immune response against pathogens or tumors. The thymus is the primary lymphoid organ where lymphoid precursors mature into naïve T-cells. However, with aging, the thymus undergoes a progressive shrinking, characterized by changes in its architecture and decreased mass of its functional tissues, referred to as thymic involution (Gruver *et al.*, 2007). Historically, it has been accepted that the young thymus sets the T-lymphocyte repertoire during early life, whereupon atrophy begins until the elderly thymus is a non-functional residual trace. Despite the dramatic decline in thymic output from puberty to old age, humans have a remarkable ability to maintain relatively constant lymphocyte numbers across many decades. This phenomenon, called homeostasis, is achieved by matching the production, self-renewal, death, and rates of phenotype transition across varied lymphocyte subpopulations. Increased naive T-cell proliferation has been reported in people over 70 years old. However, while increased homeostatic proliferation can partially compensate reduced thymic output, this does not appear to be sufficient to maintain a constant number of peripheral naïve T-cells in elderly adults (Sauce *et al.*, 2012). Thymus output decline is associated with the gradual reduction in naive $CD4^+$ and $CD8^+$ T-cell numbers with age, and its deterioration is thought to be directly linked of the reduction in immunosurveillance in the elderly (Linton and Dorshkind, 2004).

Reduced TCR repertoire and intrinsic alterations of naïve T-cells

A central facet of the naïve T-cell pool resides in the large diversity of its T-cell receptor (TCR) repertoire, which permits the response to an infinite array of antigens, and is key for effective immunity. However, a progressive loss of naïve TCR repertoire diversity, associated with their reduced production and frequency, has been reported in aging murine models. Mouse infection models showed that contraction of the naive T-cell repertoire can result in impaired T-cell response to immunodominant epitopes (Yager *et al.*, 2008). A lower precursor frequency is likely to affect negatively the properties and efficacy of T-cell responses. In humans, the diversity of the naïve T-cell TCR repertoire (shown for $CD4^+$ T-cells) experiences also a sudden and profound decline around age 70 (Naylor *et al.*, 2005). In addition to quantitative alterations, evidence of intrinsic defects of old naïve T-cell responsiveness has accumulated in aged mice. Altered gene expression profiles in old naïve $CD8^+$ and $CD4^+$ T-cells have been observed (Mirza *et al.*, 2011). Upon stimulation, naïve T-cells from old mice present defects in T-cell synapses and early TCR signaling events (Sadighi Akha and Miller, 2005), and are characterized by altered expression of inhibitory molecules and cytokine secretion capacity (Decman *et al.*, 2012; Valkenburg *et al.*, 2012). Alterations of naive T-cell activation and differentiation (shown for $CD4^+$ T-cells) also occur in old humans, with age-associated defect in TCR-induced extracellular signal-regulated kinase phosphorylation (Li *et al.*, 2012). Eventually, both quantitative and qualitative alterations in the naïve T-cell compartments with

advanced age may result in impaired induction of *de novo* T-cell responses, and a reduction of the elderly immune competence against cancer and pathogens (Appay and Sauce, 2014).

Changes in the pool of memory T-cells

Historically, a primary hallmark of immunosenescence in humans has been the increasing proportion of terminally differentiated memory T-cells (CD28 negative), often considered as cells approaching senescence (Pawelec *et al.*, 1999). Aging is actually associated with a variety of changes in the characteristics and function of antigen-experienced T-cells: (1) changes in T-cell receptor signal transduction and in the expression of cell surface receptors including the loss of co-stimulatory receptors (e.g., CD28, CD27) involved in activation; (2) changes in the profile of cytokine expression, for example decreased production of IL-2 (necessary for proliferation); (3) reduced capacity to expand upon stimulation, associated with shorter telomere length, and increased susceptibility to undergo activation induced cell death; (4) restricted T-cell repertoire, so that T-cells recognize a narrower range of antigens (Nikolich-Zugich, 2008). All these functional changes have been postulated as playing a role in the decrease of immunocompetence associated with age. Interestingly these characteristics may reflect a change in the T-cell subset distribution, with a shift towards more differentiated (with short telomeres) and more oligoclonal memory T-cell populations, so that the lymphocyte repertoire becomes increasingly skewed towards previously encountered antigens (see **Figure 1**).

Alterations of the B-cell compartment with age

Interestingly, quantitative and qualitative impairments of the B-cell compartment also occur with aging (Siegrist and Aspinall, 2009), similarly to the changes observed for the T-cell compartment. B-cell precursor (pre-B and pro-B) numbers are decreased in the bone marrow of aged mice, as well as their ability to mature into naÿve B-cells. This is associated with a reduced production of naïve B-cells and altered B-cell repertoire in the periphery. The quantity and the quality of the production of antibodies by B-cells are directly affected with old age. The elderly is characterized by lower antibody production and ability to switch (with more secretion of IgM). The affinity of the antibodies produced during a humoral immune response, and therefore their protective nature, are decreased. Eventually this is associated with a reduction of the quantity, diversity, and quality of serum antibodies specific for foreign antigens and reduced duration of an effective humoral response. Deregulated humoral immunity in elderly is also characterized by a shift of the specificity towards autologous antigens (with production of autoantibodies).

Innate Immunity

The innate system consists of immunocompetent cells which are always present at the site of infection and have an immediate action against microbes. The main cellular components of the innate immune system are phagocytic leukocytes (i.e. neutrophils, macrophages and monocytes), dendritic cells (DCs) and natural killer (NK) cells. Several studies of the impact of age on innate immunity are ongoing, and increasing evidence highlight age related abnormalities. These concern qualitative, rather than

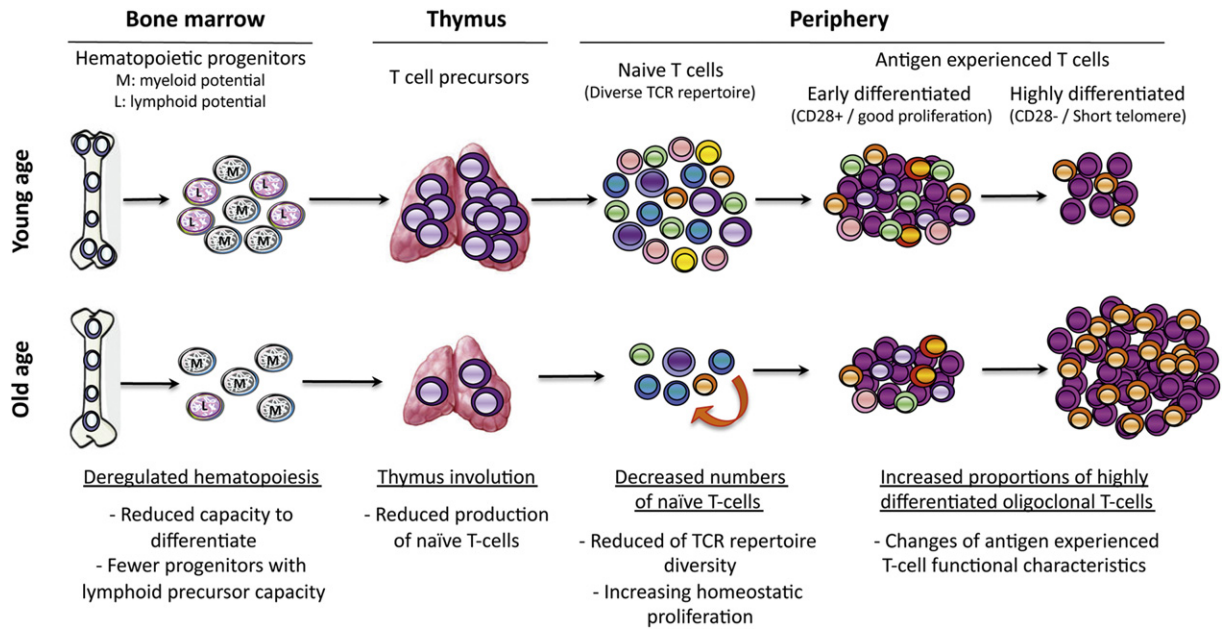

Fig. 1 Alterations of T-cell development associated with aging. Schematic representation of T-cell development from hematopoietic stem cells to highly differentiated memory T cells. The figure highlights the differences between young and old adults.

quantitative, attributes of innate immune cells. Migration and effector functions (e.g., phagocytosis, anti-bacterial activity, pro-inflammatory cytokine secretion) of neutrophils, monocytes, as well as NK cells appear to be altered with advanced age. Evidence suggests that aging compromises also the capacity of DCs to capture and process antigens, and that their response to stress molecules like ligands for toll like receptors and C-type lectin receptors is altered (Agrawal *et al.*, 2008). Since antigen presentation is at the heart of T- and B-cell response induction, impaired DC functions in the elderly is likely to have a significant impact on the mounting of effective adaptive immune responses.

Implications of Immunosenescence

The Causes of Immunosenescence

The precise mechanisms involved in the development of immunosenescence are still not completely understood, however the evidence accumulated so far converges towards the following hypothesis with regards to declining adaptive immunity. In adults, the ratio of naïve to antigen-experienced T- and B-cells is progressively reversed as naive cells are increasingly exposed to antigens. However with age, the naive lymphocyte pools are poorly replenished, a likely consequence of the gradual deterioration of the bone marrow and thymus capacity to produce mature T- and B-cells. The alteration of the hematopoietic stem cell compartment may play a role in the reduced production of naïve lymphocytes. Eventually highly differentiated oligoclonal antigen-experienced lymphocytes accumulate in the elderly, associated with a reduced T- and B-cell repertoire diversity. The very old individual can even become practically devoid of naïve T-cells. Immunosenescence may therefore be the result of the continuous challenge by a variety of antigens (e.g. derived from pathogens) to which the immune system is exposed throughout a life time. The maintenance of a protective immunity overtime may cause the eventual exhaustion of the finite renewal capacity of the adaptive immune system. Of note, chronic infections with persistent viruses, in particular cytomegalovirus (CMV), a prominent inducer of highly differentiated oligoclonal T-cell populations, have been shown to enhance the development of such 'immunosenescence profile' in humans (Koch *et al.*, 2006). In one study, the sum of certain parameters such as a high frequency of highly differentiated memory T-lymphocytes, a weak proliferative response of the T-lymphocyte pool and CMV seropositivity has even been associated to all cause early mortality in the elderly (Wikby *et al.*, 1998). The determinants of impaired innate immunity and inflammatory responses, and their link to the hyper-inflammatory status of the elderly remain to be clarified.

The Consequences of Immunosenescence

Overall, these manifestations are likely to affect the induction of effective cellular and humoral immune responses in the elderly. The age associated decline in immunocompetence results in an increase of the incidence and/or severity of many infectious diseases (e.g. influenza, pneumonia, meningitis, sepsis, varicella zoster virus, HIV, SARS …) in particular in developing countries where healthcare is not optimal. Similarly, this immune decline may also be related to the prevalence of cancers in the elderly. In addition deregulation of immune function may participate in the onset of non-infectious diseases such as autoimmune disorders (e.g. diabetes, multiple-sclerosis and even atherosclerosis) due to a decrease of the ability to discriminate between 'self' and 'non-self,' and possibly neurodegenerative disorders such as Alzheimer's disease. Interestingly, many of the immune characteristics associated with aging are also common to other conditions, independent of age, resulting also in a decline of immunocompetence (e.g., HIV infection and the genetic disorder ataxia telangectasia). These observations link the immunosenescence with the development of some degree of immunodeficiency. In the context of HIV infection, the virus is thought to cause elevated and chronic immune activation that may lead to a premature exhaustion or aging of the immune resources, which participates to the progression towards AIDS (Appay and Sauce, 2008).

Vaccination and Aging

It is also well-documented that the elderly do not respond to vaccination (and the adjuvants with which they are formulated) as well as young individuals. For example, the effectiveness of influenza vaccine is estimated to be twice lower in people greater than 65 years of age. In the elderly, T-cell and antibody responses to vaccines are slower and not as strong as in younger people (Weinberger *et al.*, 2008). Vaccines aim at inducing effective memory T- and B-cells from the pool of naive precursors present in vaccinees. Improving our knowledge of the exact causes and mechanisms underlying the decline in naïve T- and B-cell priming with age is necessary to improve vaccination efficacy for the elderly (Goronzy and Weyand, 2013). Although it is clear that detectable antigen-specific cells can be induced safely in humans by vaccination, the challenge is now to improve vaccine immunogenicity in the elderly populations. Several challenges must be faced to achieve this, including (i) the identification of the best vaccine formulation (e.g. antigen and adjuvant) to achieve priming of naive cell precursors in older individuals; and (ii) the development of personalized strategies that can be used to optimize vaccine procedures, knowing that variable responses will be due to a combined genetic difference and functional, age-related variance.

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Premature Aging Syndromes☆

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Introduction

Hutchinson–Gilford Progeria Syndrome (HGPS) and Werner syndrome (WS), also referred as to childhood- and adulthood-progeria respectively, represent two of the best-characterized premature aging disorders in humans. The identification of their genetic basis, paralleled by the creation of disease registers and databases, has allowed a better characterization of the phenotypes and a deeper understanding of associated symptoms, revealing the existence of a disease spectrum ranging from moderate and mild–severe to very aggressive forms, known as atypical progeroid syndromes.

Hutchinson–Gilford Progeria Syndrome

HGPS is an extremely rare genetic disorder named after the reports by doctors Hutchinson (1886) and Gilford (1904) who independently described the syndrome. The disease is a segmental progeroid syndrome involving multiple organs and tissues that mimic phenotypes associated with normal aging.

Epidemiology

HGPS has a reported birth prevalence of 1 in 8 million, but taking into consideration misdiagnosed or unreported cases, the estimated prevalence is of 1 in 4 million births. According to the Progeria Research Foundation database, which is the major online database of the disease (http://www.progeriaresearch.org), there is an estimate of between 350 and 400 children worldwide living with progeria, but only 132 of them have been identified as of October 1, 2015. The identified cases are from 46 different Countries, suggesting that the disease affects all ethnic groups and genders equally and there are no reported geographic clusters.

Clinical Manifestations

The affected children look normal and healthy at birth but the development of HGPS clinical features starts during the first year of their life. The author has recently reviewed the main HGPS clinical symptoms and their frequency (Coppedè, 2013) and an updated summary is provided below. The first signs in those infants are often a visible vein across the nasal bridge and skin lesions, followed by a profound failure to thrive resulting in the gain of only about ½ kg per year, and leading HGPS children to reach an average final weight of almost 14–15 kg. Most HGPS infants become bald by their second-third year of life due to progressive alopecia. Other symptoms that become apparent in the first years of life include a characteristic facies, loss of subcutaneous fat and abnormal tightness of the skin that with time becomes delicate, dry, translucent, and sometimes with hyperkeratosis. Stiffness of joints and bone changes also occur.

Facial abnormalities include a small face with a pinched nose and prominent eyes and scalp veins; a large cranium in relation to the facial size is seen in most of the patients, and other typical craniofacial anomalies are reported (Ullrich *et al.*, 2012). The subcutaneous fat in the face gradually disappears and the facial muscles decrease in size. HGPS infants also have a receding mandible, a high-pitched voice, protruding ears that lack lobules, and later develop low frequency conductive hearing loss. Dentition is delayed and crowded. HGPS children retain normal motor and mental development and have a normal social behaviour for their age. Also the cognitive functions are preserved, and there is no report of dementia or neurodegeneration (Ullrich and Gordon, 2015).

With time also the body shows a progressive loss of subcutaneous fat, musculoskeletal degeneration and joint protruding. At the bone level, small clavicles, coxa valga and acroosteolysis are seen in most of the patients. Other frequent skeletal abnormalities are hip dysplasia and thin ribs. Generalized osteopenia and other skeletal dysmorphisms are reported in less than 50% of the patients (Cleveland *et al.*, 2012).

HGPS individuals are not sexually mature and do not reproduce; with time their appearance becomes like that of an old person until death that usually occurs between ages 6 and 20 years. Cardiovascular and cerebrovascular complications culminate in mortality from myocardial infarction, stroke or congestive cardiac failure at an average age of 14.6 years. Autopsy data have shown widespread atherosclerosis in HGPS patients. Cerebrovascular arteriopathy, intracranial steno-occlusive arterial lesions, and early and clinically silent strokes have been also reported as a prevalent disease characteristic in HGPS (Silvera *et al.*, 2013).

☆*Change History:* January 2016. F. Coppedè has changed the entire text.

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Genetics

Progerin-producing LMNA mutations

In 2003 the *LMNA* gene was identified as the causative HGPS gene (De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003). The commonest HGPS causative mutation is a de novo dominant point mutation in exon 11 of the gene (c.1824C>T, p.G608G) resulting in the activation of a cryptic donor splice site and leading to the production of a lamin A isoform containing an internal deletion of 50 amino acids, known as progerin (Eriksson *et al.*, 2003). It was however clear from the beginning that also less common *LMNA* gene mutations could cause the disease (Eriksson *et al.*, 2003). As of October 1, 2015, 104 out of the 132 living children with progeria identified by the Progeria Research Foundation have a progerin-producing mutation in the *LMNA* gene, and 28 have mutations in the lamin pathway but don't produce progerin. The heterozygous *LMNA* c.1824C>T mutation is found in about 85–90% of progerin-producing HGPS cases, referred as to classic HGPS. Other progerin-producing mutations found in 1–5% of the cases each, include heterozygous exon 11 c.1822G>A (p.G608S), c.1821G>A (p.V607V), and c.1868C>G (T623S) mutations, as well as heterozygous mutations at the intronic border of exon 11, such as c.1968+1G>A, c.1968+1G>C, c.1968+5G>C (http://www.progeriaresearch.org) and others (Gordon *et al.*, 2015). Despite that all those mutations result in the production of progerin, the resulting phenotype can be less or more aggressive depending on the nature of the mutation itself and the resultant final amount of progerin protein that is produced. For example both c.1968+1G>A and c.1821G>A mutations lead to a strong activation of the aberrant splice site, with subsequent increased progerin expression and a more severe phenotype compared to classic HGPS cases. By contrast, the c.1868C>G mutation results in the deletion of only 35 amino acids in the protein leading to a slowly progressing progeria compared to classic HGPS (Gordon *et al.*, 2015). A list of *LMNA* mutations causing classic HGPS or progerin-producing progeroid laminopathies can be found at the UMD-LMNA Mutations Database (http://www.umd.be/LMNA/).

Other LMNA mutations

Several atypical progeroid syndromes, also called nonclassical progeria, atypical HGPS, or atypical Werner syndrome, are not caused by progerin-producing *LMNA* mutations, but result from over 20 different heterozygous, homozygous, or compound heterozygous mutations in the *LMNA* gene. The clinical features of atypical progeroid syndromes include growth retardation and involve the same body systems as in classical HGPS, but the patients differ in age at onset and severity of the symptoms, which can be similar, more or less severe than in classic HGPS. A detailed review of those mutations and their models of inheritance can be found in Coppedè (2013). Additional data can be found at the UMD-LMNA Mutations Database (http://www.umd.be/LMNA/).

ZMPSTE24 mutations

Lamin A proteins are synthesized as prelamin A proteins, which undergo farnesylation and other posttranslational modifications to become mature proteins. The zinc metalloprotease ZMPSTE24 is required in prelamin A processing to yield mature lamin A. Recessive mutations in *ZMPSTE24* impair lamin A maturation causing three distinct premature aging disorders with increasing disease severity: (1) mandibuloacral dysplasia, which is a mild progeroid disorder characterized by postnatal growth retardation and craniofacial, skeletal, and skin abnormalities, (2) atypical HGPS, a severe non-classical form of HGPS, and (3) restrictive dermopathy, a fatal neonatal disorder representing an "extreme form" of progeria resulting from intrauterine growth delay. Also for *ZMPSTE24*, several different mutations have been identified in homozygosis or compound heterozygosis in the patients, and disease severity is inversely correlated with the residual protein activity conferred by those alleles, so that complete loss-of-function alleles lead to restrictive dermopathy, whereas alleles resulting in residual protein activity are associated with less severe phenotypes. An updated list of premature aging-related *ZMPSTE24* mutations can be found in Navarro *et al.* (2014).

Molecular Mechanisms

Lamins are the major components of the nuclear lamina and are essential not only for the formation and maintenance of a proper nuclear shape and structure, but also for the organization of the chromatin structure and of several nuclear processes such as transcription, DNA replication, DNA repair, epigenetic mechanisms and cell division and differentiation, all impaired in HGPS and likely contributing to the premature aging phenotype. Cells obtained from HGPS individuals accumulate defects in nuclear structure and architecture with cell passaging, such as lobulation, clustering of the nuclear pores and loss of peripheral heterochromatin (Goldman *et al.*, 2004). It is therefore believed that the accumulation of a non-mature and persistently farnesylated lamin A, either resulting from progerin-producing *LMNA* or from *ZMPSTE24* mutations, causes impaired structural and mechanical properties of the nuclear lamina because of the retain of the farnesyl group that renders progerin, or other farnesylated lamin A isoforms, permanently intercalated into the inner nuclear membrane. By contrast, non-progerin producing *LMNA* alleles lead to the production of lamin A isoforms with variable structure and function but all able to induce nuclear damages that overlap with those of progerin-producing alleles (Gordon *et al.*, 2015).

Diagnosis

The diagnosis of classic or atypical HGPS is made in individuals with common clinical features (listed above) or with features resembling classic HGPS, confirmed by the genetic test for *LMNA* mutations searching for the heterozygous c.1824C>T mutation

in exon 11 in classic HGPS or for less frequent *LMNA* mutations by sequencing of the coding and flanking regions in atypical HGPS forms.

Management and Experimental Therapies

Unfortunately there is actually no cure to halt the development of the premature aging phenotype in HGPS infants, so that drugs, dietary changes, exercise and hydrotherapy are recommended for HGPS individuals to counteract atherosclerosis risk, body fat reduction, and muscular atrophy. The extraction of primary teeth may help to avoid crowding, and sunscreens, hearing aids, eye care and shoe pads are also recommended when necessary. Low-dose aspirin and an adequate oral hydration are recommended for the prevention of cardiovascular and cerebrovascular complications (Gordon *et al.*, 2015).

There are actually three agents under investigation in human clinical trials for HGPS: lonafarnib, pravastatin, and zoledronate, all acting as inhibitors of the post-translational farnesylation of progerin (Gordon *et al.*, 2015). Available data on lonafarnib clinical trials revealed that the drug improved the rate of weight gain, vascular distensibility, bone structure, neurosensory hearing, and lifespan (Gordon *et al.*, 2015).

Werner Syndrome

WS is a rare genetic disease named after the doctoral thesis by Doctor Werner (1904) that first described it. It is a segmental progeroid syndrome and the most studied disease model of premature aging in adulthood.

Epidemiology

WS is an autosomal recessive disorder with an estimated sex ratio of 1:1. In the Japanese population, due to a founder effect, the disease frequency ranges from about 1 in 20.000 to 1 in 40.000. Another genetic cluster is found in Sardinia, with an estimated frequency of 1 in 50.000. WS prevalence in other populations is unknown, but far less frequent than in Japan and likely ranging from 1 to a few cases per 1 million individuals (Oshima *et al.*, 2014). Indeed, it was recently observed that almost 1.500 WS cases have been reported worldwide from 1904 to 2008, 75% of them in Japan (Goto *et al.*, 2013).

Clinical Manifestations

The author has recently reviewed the main WS clinical symptoms and their frequency (Coppedè, 2013) and an updated summary is provided below. WS individuals develop normally during the first decade of life, and the lack of a pubertal growth spurt during their early teen years, leading to a final short stature and low bodyweight, is the first clinical sign. In their 20s WS individuals begin to manifest greying or loss of hair and scleroderma-like skin lesions followed, in the 30s, by bilateral cataracts, type 2 diabetes mellitus, hypogonadism, skin ulcers, and osteoporosis. A prematurely aged face with beaked nose is found in most of the patients and fertility declines in both sexes soon after sexual maturity. WS subjects usually die at a mean age of 53–54 years from cancer or myocardial infarction.

An unusual spectrum of cancers has been reported in WS individuals, and the most common cancers are thyroid neoplasms, melanoma, meningioma, soft tissue sarcomas, hematologic/lymphoid cancers, and osteosarcomas (Lauper *et al.*, 2013).

Concerning vascular diseases and their complications, several forms of arteriosclerosis have been observed in WS individuals, with coronary artery atherosclerosis often leading to myocardial infarction and death. Less frequent are cerebrovascular disorders, observed in 2–5% of the cases (Oshima *et al.*, 2014; Takemoto *et al.*, 2013).

Concerning the involvement of the nervous system in WS only a few cases of dementia or peripheral neuropathy have been reported, but there is no evidence of increased risk for Alzheimer's disease (Coppedè, 2012).

Genetics

WRN mutations

Typical WS is caused by mutations of the *WRN* gene that codes for the WRN protein, a member of the RecQ DNA helicase family with multiple nuclear functions (Yu *et al.*, 1996). The *WRN* gene consists of 35 exons and over 70 different mutations have been identified in WS patients, including missense, nonsense, ins/del, and splice mutations. Founder mutations are reported in Japanese (c.3139-1G > C, resulting in skipping of exon 26) and Sardinian patients (c.2089-3024A > G, creating a novel exon between exons 18 and 19 and introducing a premature stop codon), and common or potential founder mutations are reported in other populations, such as the common mutation in Caucasians (c.1105C > T, p.R369X) creating a stop codon in exon 9, and potential founder mutations in Moroccan (c.2179dupT, p.C727fs), Dutch (c.3590delA, p.N1197fs), and Turkish (c.3460-2A > G, exon 30 deletion) populations (Friedrich *et al.*, 2010; Oshima and Hisama, 2014). Most of those mutations are null mutations, resulting in protein truncation with loss of the nuclear localization signal and/or in decay of the mutant mRNAs. Also missense mutations are

Table 1 Main characteristics of classic HGPS and typical WS

	Classic HGPS	*Typical WS*
Disease	Premature aging disease of childhood — segmental progeroid syndrome	Premature aging disease of adulthood — segmental progeroid syndrome
Prevalence	1 in 4–8 million births	1:20.000–40.000 in Japan 1:50.000 in Sardinia Less frequent elsewhere
Onset of symptoms	1st year of life	2nd-3rd decade of life
Mean life expentancy	14.6 years	53–54 years
Main cause of death	Myocardial infarction or stroke resulting from vascular diseases	Cancer or myocardial infarction, the latter resulting from vascular disease
Causative gene	*LMNA*	*WRN*
Mendelian inheritance	Autosomal dominant	Autosomal recessive
Sex ratio	1:1	1:1
Ethnic differences	Not reported	Frequent in Japan and Sardinia, due to a founder effect. Less frequent elsewhere
Main symptoms	Severe failure to thrive in infancy. Skin lesions. Progressive alopecia leading to total alopecia. Characteristic facies. Loss of subcutaneous fat. Bone changes. Skeletal anomalies. Musculoskeletal degeneration. Hearing loss. High-pitched voice. Delayed and crowded dentition. Atherosclerosis. Cerebrovascular disease	Lack of the pubertal growth spurt during early teen years. Greying or loss of hair. Scleroderma-like skin lesions. Bilateral cataracts. Type 2 diabetes mellitus. Hypogonadism. Skin ulcers. Osteoporosis. Characteristic facies. Arteriosclerosis
Cancer risk	Not reported	Elevated risk of thyroid neoplasms, melanoma, meningioma, soft tissue sarcomas, hematologic/lymphoid cancers, osteosarcomas, and other less frequent cancers
Nervous system disorders	Normal cognitive and motor functions. Signs of neurodegeneration are not reported	Only a few reported cases of dementia or peripheral neuropathy, but no clear evidence of increased risk for neurodegenerative disorders
Fertility	Sexually immature-do not reproduce	Reduced fertility-can reproduce
Diagnosis	Presence of disease symptoms confirmed by detection of the common *LMNA* c.1824C>T mutation	Presence of disease cardinal signs and molecular detection of biallelic *WRN* pathogenic variants
Treatments	Symptomatic	Symptomatic

mainly regarded as null mutations causing protein instability, or a significant decreased protein activity (Oshima and Hisama, 2014). A complete list of *WRN* mutations can be found at the Werner Syndrome Mutational Database (http://www.pathology.washington.edu/research/werner/database/).

LMNA mutations in atypical WS

Recessive *WRN* mutations account for most of WS cases denoted as typical WS. However, atypical forms of the disease (atypical WS) are reported, with patients showing no *WRN* mutations and often an early onset of the symptoms and an accelerated disease progression. Bilateral cataracts and diabetes are not common features in atypical WS. The International Registry of Werner Syndrome (Department of Pathology, University of Washington, Seattle, WA, USA) (www.wernersyndrome.org) contains data on 11 atypical WS individuals with mutations in the *LMNA* gene, including missense mutations and others at the intronic border of exon 11 leading to a weak activation of the same cryptic splice site as in HGPS (Oshima and Hisama, 2014). Interestingly, atypical WS cases that have neither *WRN* nor *LMNA* mutations are also reported (Oshima and Hisama, 2014).

Molecular Mechanisms

WRN is a nuclear protein with multiple functional domains involved in the maintenance of genome stability by means of DNA dependent ATPase, $3'\rightarrow5'$ helicase, $3'\rightarrow5'$ exonuclease, and DNA strand annealing activities involved in DNA replication, recombination, repair and transcription as well as in telomere maintenance and apoptosis. As such it is regarded as a "caretaker of the genome" (Muftuoglu *et al.*, 2008). As discussed above, most of the WRN mutations found in WS patients are null mutations leading to absence of a functional WRN protein in nuclei, and cells isolated from WS individuals display increased chromosomal aberrations, accelerated telomere shortening, DNA replication impairments, and premature senescence in culture (Melcher *et al.*, 2000).

Diagnosis

The clinical diagnosis of WS is based on the presence of four cardinal signs (bilateral cataracts, skin changes, short stature, and greying or loss of hair), which are all simultaneously observable in almost 91% of the patients, and supported by the presence of additional signs (bird-like face, osteoporosis, voice change, type 2 diabetes mellitus, atherosclerosis, and so on). The diagnosis is then molecularly confirmed by the detection of *WRN* pathogenic variants (Friedrich *et al.*, 2010; Oshima and Hisama, 2014).

Management and treatments

Treatments for WS are only symptomatic. Indeed, cholesterol-lowering drugs in case of abnormal lipid profile, treatment of skin ulcers, surgical treatment of cataracts, control of type 2 diabetes mellitus, and standard treatment of malignancies are among the available medicaments, accompanied by smoking avoidance and dietary/physical regimens to reduce atherosclerosis risk (Coppedè, 2013).

Summary and Comparison of the Main Characteristics Between Classic HGPS and Typical WS

Table 1 provides a summary of the text featuring the main characteristics of classic HGPS and typical WS.

Web Resources

HGPS

The Progeria Research Foundation database (http://www.progeriaresearch.org) is a major online source of HGPS by the numbers, containing updated information on HGPS, an updated database of identified cases, and disease management strategies.

The UMD-LMNA mutations database (http://www.umd.be/LMNA/) provides up-to-date information about mutations of the *LMNA* gene and related diseases, including HGPS and atypical progeroid syndromes.

WS

The International Registry of Werner Syndrome (www.wernersyndrome.org) is located in the Department of Pathology, University of Washington, Seattle, Washington and contains information on the disease and a registry of submitted cases.

The Werner Syndrome Mutational Database (http://www.pathology.washington.edu/research/werner/database/) contains up to date list of *WRN* causative mutations.

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Hormonal Circadian Rhythms and Sleep in Aging

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Introduction

A prominent feature of endocrine secretion in man is its high degree of temporal organization. Hormonal variations may be observed in the ultradian range (i.e., once per 1–2 h), in the circadian range (i.e., approximately once per 24 h) and in the infradian range (i.e., periods longer than 24 h, for instance the menstrual cycle). This configuration provides the endocrine system with a high and complex degree of flexibility. The temporal regulation of hormonal secretions throughout the 24-h cycle results from the interaction, in the central nervous system, of two sophisticated time-keeping mechanisms: the so-called *sleep-wake homeostasis*, a mechanism linking the timing and the intensity of sleep to the duration of prior wakefulness, and the so-called *circadian pacemaker* (Copinschi and Challet, 2016).

The master circadian pacemaker—or circadian clock—is located in two bilaterally paired small nuclei in the anterior hypothalamus: the suprachiasmatic nucleus (SCN) (Rosenwasser and Turek, 2005). The generation and maintenance of circadian oscillations of the master circadian pacemaker involve a series of clock genes, which interact in complex feedback loops (Zhang and Kay, 2010; Partch *et al.*, 2014). Secondary circadian clocks are present in other brain areas (Abe *et al.*, 2002; Feillet *et al.*, 2008; Guilding *et al.*, 2009) and in almost all peripheral tissues, including endocrine glands (Yoo *et al.*, 2004; Balsalobre *et al.*, 1998; Oster *et al.*, 2006). These secondary circadian clocks are under the control of the SCN through the entrainment of the same circadian machinery at the local level. Together, the master and the secondary circadian clocks define a multioscillatory network generating circadian oscillations. The SCN controls and synchronizes peripheral endocrine clocks, either directly via neural signals transmitted through the autonomous nervous system, or indirectly via the sleep-wake cycle or the rhythm of feeding. In addition, circadian variations of corticosteroids and of melatonin release—two hormones tightly controlled by the SCN—probably contribute to the transmission of temporal signals throughout the body (Pevet and Challet, 2011; Balsalobre *et al.*, 2000; Segall *et al.*, 2006; Malek *et al.*, 2007). However, the endogenous circadian period is close to, but not exactly, 24 h. This implies that the circadian clock has to be entrained by external agents to avoid to fall out of synchrony with the environmental day. The primary synchronizing agent is the 24-h light-dark cycle (Meijer and Schwartz, 2003).

The SCN controls the timing of most circadian rhythms, including endocrine rhythms. In addition, SCN partly regulates the sleep-wake cycle. In turn, the sleep-wake cycle regulates the timing and the expression of many endocrine rhythms. Thus, the 24-h hormonal profiles frequently result from an interaction between the circadian pacemaker, the sleep-wake homeostasis, and an ultradian, or pulsatile, pattern of release, while the 24-h sleep-wake cycle is driven partly by the circadian clock and partly by the homeostatic regulation of sleep pressure.

Sleep and Aging

Sleep involves two distinct states of brain activity that are each generated in specific brain regions and are characterized by different patterns of cerebral and peripheral activity: the rapid eye movement (REM) stage and the non-REM stages. Throughout the sleep period, non-REM sleep and REM sleep occur in successive cycles lasting approximately 90 min. Each cycle is normally initiated by the lighter non-REM stages (stages 1 + 2), followed by a deeper non-REM stage (stage 3), then by REM sleep. During stage 3 sleep, the EEG is synchronized with low-frequency high-amplitude waveforms, referred to as slow-waves (SW). Therefore, stage 3 sleep is currently referred to as slow-wave sleep. The intensity of SW sleep, referred to as slow-wave activity (SWA), may be quantified by a spectral electroencephalogram (EEG) analysis. SW sleep is the most "restorative" sleep stage. During REM sleep, EEG is similar to that of active waking, muscle tone is inhibited and eye movements are present (Copinschi and Challet, 2016). Most dreams occur during REM sleep (Siegel, 2011).

In young healthy subjects (15–25 years), each night involves 4–6 successive sleep cycles. During the first cycle, the sequence normally includes an initial 10–20 min period of light non-REM sleep, followed by a nearly 60 min period of deeper non-REM sleep, then by a few minutes of REM sleep. As the night progresses, transient awakenings become longer and more frequent, non-REM sleep becomes shallower and the duration of REM sleep periods increases. In healthy young subjects, approximately 50% of a normal night is spent in light non-REM stages, 20% in SW sleep, 25% in REM sleep, and 5% in wake. A typical individual sleep pattern in a healthy young man is illustrated in **Fig. 1**.

During normal aging, this sleep-wake pattern undergoes major modifications, resulting in major alterations in sleep architecture. From early adulthood (16–25 years) to midlife (35–50 years), a spectacular decrease in SW sleep occurs, together with a compensatory increase in light non-REM sleep (stages 1 and 2), but there are no significant modifications in the duration of REM sleep, nor in the duration of transient nocturnal awakenings, and the total sleep time remains largely unchanged (Van Cauter *et al.*, 2000). From mid-life to old age (70–80 years), no additional decrease in SW sleep is observed, but both REM sleep and light non-

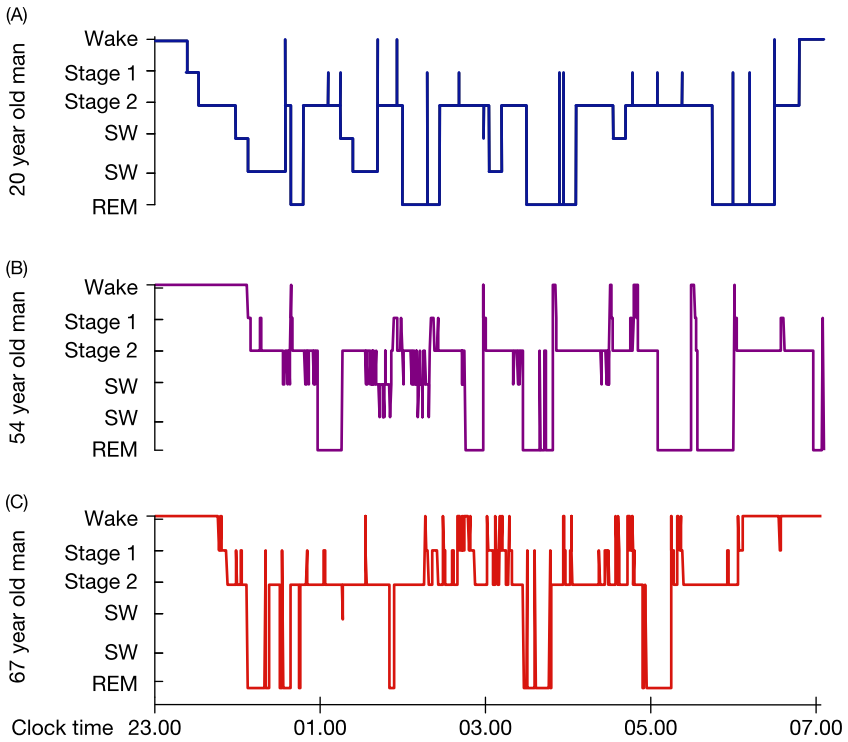

Fig. 1 Hypnograms recorded in healthy men aged 20 (A), 54 (B), and 67 (C) years. Unpublished data kindly provided by Rachel Leproult, Ph.D.

REM sleep progressively decline, associated with a mirror increase in wake time, resulting in sleep fragmentation and a decrease in total sleep time (Landolt *et al.*, 1996; Miles and Dement, 1980; Ohayon *et al.*, 2004; Van Cauter *et al.*, 2000). Moreover, there is a redistribution of REM stages across the sleep period, with a shift toward the initial part of the night. Typical individual sleep profiles in healthy 54- and 67-year old men are illustrated in **Fig. 1**. The time-course of age-related modifications of sleep architecture is illustrated in **Fig. 2**.

Those marked alterations in sleep architecture are frequently associated with major alterations in subjective sleep quality, which are more important and more frequent in older women than in older men (Groeger *et al.*, 2004; Middelkoop *et al.*, 1996; Vitiello *et al.*, 2004). During the night, healthy elderly subjects often complain of shallow sleep with frequent transient awakenings and an early final morning awakening, resulting in unwanted daytime naps (Prinz *et al.*, 1990; Bliwise, 1993; Prinz, 1995). Moreover, age-associated deficits in the quality of nocturnal sleep may be paralleled by decreased daytime alertness and performance, and attention and memory deficits (Ancoli-Israel, 2005).

Circadian System and Aging

Age-related alterations in hormonal circadian rhythms widely differ from one hormone to another. One of the most prominent alterations is a reduction in rhythm amplitude. Many hormonal circadian rhythms are also phase-advanced, so that phase-points of the rhythms occur earlier in the elderly than in young subjects (Copinschi and Challet, 2016). Those alterations are likely to result from both a reduction in the strength of the circadian signal and a reduction in the homeostatic drive to sleep (Dijk *et al.*, 1999). Reduced outdoor activity resulting in decreased exposure to bright light, reduced professional and social constraints and decreased mobility all also contribute to disruptions in circadian rhythmicity. Regular exposure to bright light and enhanced social activities may improve nighttime sleep and daytime alertness and reinforce circadian rhythms in the elderly (Copinschi and Challet, 2016).

The senescence process may be accelerated by conditions of circadian misalignment, such as occur in shift work or in jet lag, which induce a state of internal desynchrony between central and peripheral rhythms, with major disruptions of the internal temporal organization of a number of physiologic functions (Evans and Davidson, 2013; Leproult *et al.*, 2014; Scheer *et al.*, 2009). Not surprisingly, shift work is associated with increased risk for insomnia, infertility, weight gain, dyslipidemia, insulin resistance, diabetes, cardiovascular, and gastrointestinal disorders (Czeisler *et al.*, 1990; Knutsson *et al.*, 1986; Rosa, 1995; Van Amelsvoort *et al.*, 1999; Nagaya *et al.*, 2002; Pan *et al.*, 2011). In animals, prolonged circadian misalignment was found to reduce their lifetime (Penev *et al.*, 1998; Davidson *et al.*, 2006). Furthermore, a study performed in young grass rats has shown that circadian desynchronization triggers premature cellular aging (Grosbellet *et al.*, 2015).

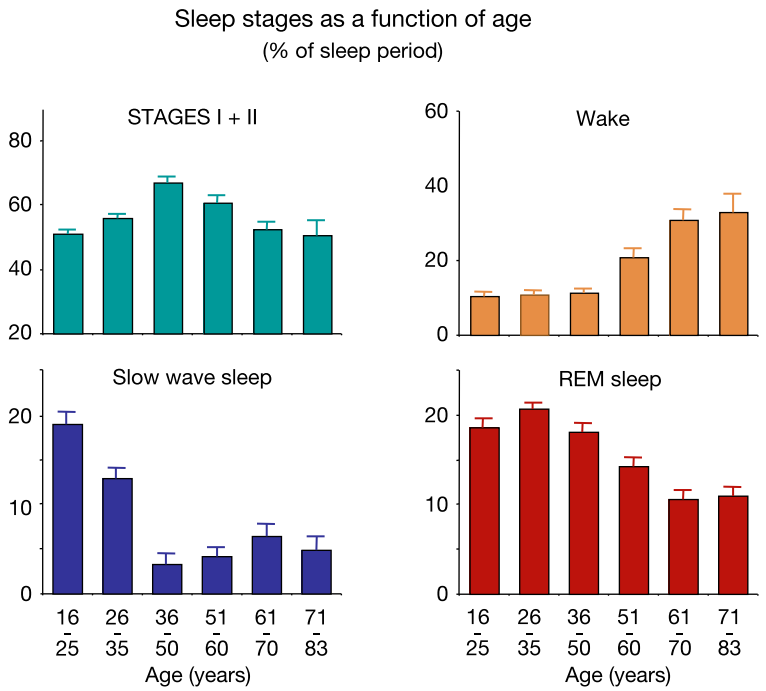

Fig. 2 Sleep stages as a function of age in 149 healthy men, aged 16–83 years. Values shown are mean (+SEM) for each age group. Data from Van Cauter, E., Leproult, R. and Plat, L. (2000). Age-related changes in slow wave sleep and REM sleep and relationship with growth hormone and cortisol levels in healthy men. *JAMA* **284**, 861–868.

The aging-related effects on melatonin, hormones of the hypothalamo–pituitary axis, glucose and insulin are reviewed in the following sections.

Melatonin

Melatonin rhythmicity is primarily dependent on the master circadian pacemaker (Rosenthal, 1991). In addition, a pineal molecular clockwork plays a stimulatory role on the amplitude of the major melatonin circadian rise, starting between 21.00 and 23.00 h, culminating around the middle of the night, followed by a progressive decline to return by 08.00 or 09.00 h to low levels that are maintained throughout daytime until the next evening circadian rise (Copinschi and Challet, 2016). Melatonin rhythmicity is highly sensitive to lighting conditions, as night exposure to light exerts an immediate dose-dependent inhibition of melatonin synthesis (Lewy *et al.*, 1980; Van Cauter et al., 1998a).

Daytime low melatonin levels are similar in young and old adults. But the evening circadian elevation is markedly dampened in most, but not all, older subjects (Van Coevorden *et al.*, 1991). This dampening may partly result from insufficient daytime environmental light since it may be reversed by midday exposure to bright light (Mishima *et al.*, 2001). In addition, the circadian rise is advanced by almost 1.5 h in the elderly, illustrating the advance of circadian phase (Van Coevorden *et al.*, 1991).

ACTH, Cortisol, Dehydroepiandrosterone

The circadian rhythms of hormones of the corticotropic axis are primarily controlled by central and adrenal circadian clocks, thanks to a flexible sophisticated machinery (Copinschi and Challet, 2016). Outputs from the SCN activate the circadian release of hypothalamic corticotropin-releasing hormone (CRH) that in turn stimulates circadian ACTH secretion. The 24-h profile of adrenocortical secretion primarily depends on ACTH diurnal pattern. In addition, neuronal signals generated by the SCN are also transmitted to the adrenal cortex by a multisynaptic neural pathway (Buijs *et al.*, 1999; Dickmeis *et al.*, 2013). The secondary adrenal circadian clock gates the adrenal response to ACTH, that is, modulates diurnal variations of the adrenal responsiveness to ACTH (Oster *et al.*, 2006).

In young adults, the 24-h profiles of ACTH and cortisol normally show an early morning peak, a progressive decrease toward minimal levels centered around midnight (referred to as the quiescent period), followed during the second part of the night by a sharp increase (referred to as the circadian rise) toward the early morning peak (**Fig. 3**). The diurnal pattern of cortisol secretion reflects a succession of secretory pulses, the magnitude of which is modulated by the circadian rhythm, without any evidence of tonic secretion (Van Cauter *et al.*, 1990). This pattern is largely unaffected by alterations of the usual sleep schedule, illustrating the

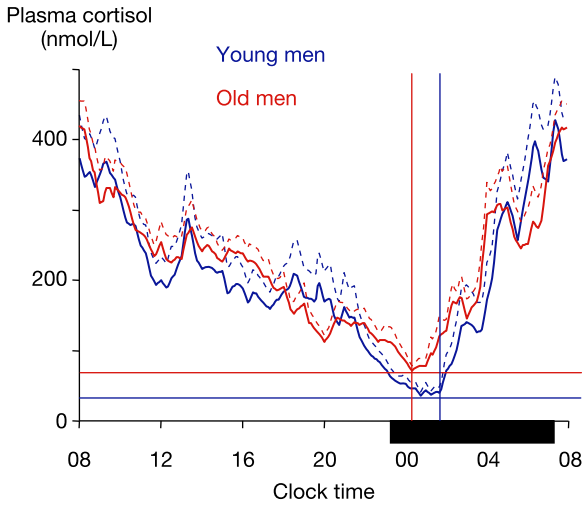

Fig. 3 Mean (+SEM) 24-h plasma cortisol profiles in eight healthy young men aged 17–24 years and in eight healthy old men aged 70–83 years matched for BMI. Dashed lines indicate SEM. The *black* bar represents the mean sleep period. *Horizontal* lines indicate the nocturnal minimum cortisol level in each group. *Vertical* lines indicate the timing of the circadian cortisol rise in each group. Note that in the older group, evening cortisol levels are increased and the timing of the circadian rise is advanced. Data from Van Cauter, E., Leproult, R. and Plat, L. (2000). Age-related changes in slow wave sleep and REM sleep and relationship with growth hormone and cortisol levels in healthy men. *JAMA* **284**, 861–868.

strength of the circadian control. Diurnal variations parallel to those of cortisol have been evidenced for other adrenal steroids, such as dehydroepiandrosterone (DHEA) (Copinschi and Challet, 2016).

However, cortisol release is also modulated by the sleep-wake homeostasis. Sleep onset (and more precisely SW sleep) is consistently associated with an immediate drop in circulating cortisol levels (Weitzman *et al.*, 1983; Van Cauter *et al.*, 1991; Weibel *et al.*, 1995; Caufriez *et al.*, 2002). This drop may be undetectable in early morning, when sleep onset coincides with a peak of corticotropic activity. Conversely, all awakenings at the end of the sleep period, as well as transient awakenings during this period, consistently trigger cortisol secretory pulses (Caufriez *et al.*, 2002; Follenius *et al.*, 1992; Spath-Schwalbe *et al.*, 1991; Van Cauter et al., 1990, 1991). In an analysis of nocturnal cortisol profiles performed in healthy young men and women, all transient awakenings that interrupted sleep during 10 min or longer were followed within 20 min by significant cortisol secretory pulses (Caufriez *et al.*, 2002). In normal sleepers, 24-h cortisol levels were found to be positively associated with the duration of nocturnal awakenings (Vgontzas *et al.*, 2003). Chronic insomnia (resulting in a reduction of total sleep time) was found to be associated with elevated nocturnal cortisol levels (Vgontzas et al., 2001a).

Furthermore, in rabbits and rats, intracerebral injections of CRH inhibit SW sleep (Ehlers *et al.*, 1986; Opp *et al.*, 1989), while blockade of CRH receptors reduce wakefulness (Chang and Opp, 1998). In humans, intravenous CRH injections may reduce sleep efficiency and SW sleep (Holsboer *et al.*, 1988; Tsuchiyama *et al.*, 1995; Vgontzas et al., 2001b), while cortisol administration may enhance SW sleep (Born *et al.*, 1991; Friess *et al.*, 1994; Bohlhalter *et al.*, 1997), presumably via a negative feedback on endogenous CRH secretion.

In healthy subjects, the circadian rhythm of cortisol persists throughout the aging process and is even present in the very elderly (Van Coevorden *et al.*, 1991; Van Cauter *et al.*, 1996), but, from 50 years of age onwards, evening cortisol levels progressively increase, the relative amplitude of the diurnal rhythmicity is dampened and the timing of the circadian rise is advanced (Van Cauter *et al.*, 1996), as illustrated in **Fig. 3**. After 70 years of age, late evening levels are markedly higher than in young adults (Van Cauter *et al.*, 1996). This elevation probably reflects a reduced resiliency of the corticotropic axis, resulting from age-associated alterations of hippocampal neurons (McEwen, 1998b). In older subjects, the quiescent period is markedly shortened, as it starts later, ends earlier and is fragmented. These alterations are more pronounced in women than in men: between 25 and 65 years of age, the quiescent period shortening averages approximately 4.5 h in women but less than 3 h in men (Van Cauter *et al.*, 1996). Not surprisingly, the circadian rhythm of dehydroepiandrosterone, though still present, is dampened in the elderly (Al-Turk and Al-Dujaili, 2016).

As illustrated in **Fig. 4**, the age-related elevation of evening cortisol levels and the age-related decrease of REM sleep occur in mirror image (Van Cauter *et al.*, 2000), suggesting that in the elderly, alterations of sleep quality could contribute to alterations of the corticotropic axis. This hypothesis is supported by a number of interventional studies. In young subjects, acute total or partial (4 h in bed) sleep deprivation results on the following day in significant elevations of cortisol levels in late afternoon and evening (Leproult *et al.*, 1997). Similar alterations occur if partial sleep restriction (4 h in bed per night) is enforced during six consecutive days (Spiegel *et al.*, 1999). These alterations are strikingly similar to those observed in healthy elderly with normal sleep schedules (**Fig. 5**). Furthermore, in a 4-year longitudinal study performed in 157 older adults, sleep duration was found to buffer long-term elevations of diurnal cortisol secretion, suggesting that long sleep protects to aging-associated increases in diurnal cortisol secretion

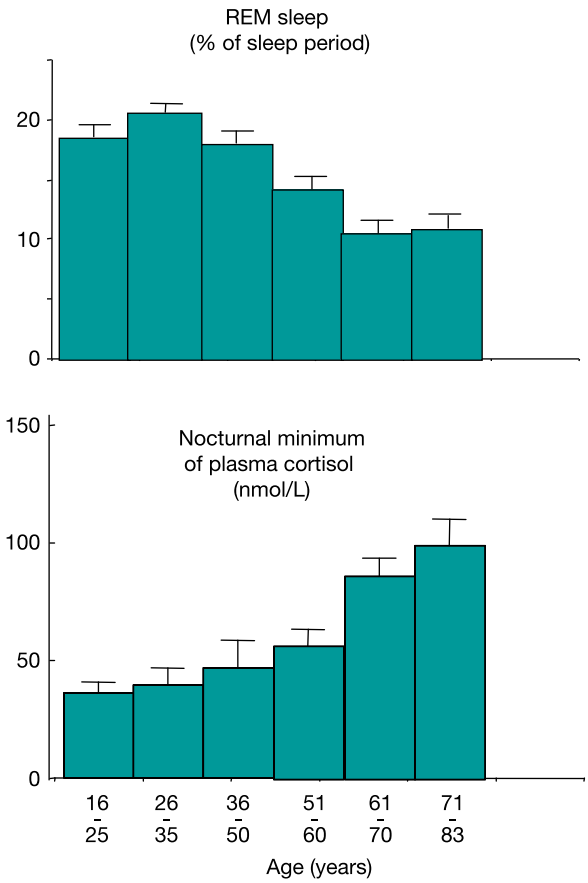

Fig. 4 REM sleep and nocturnal minimum of plasma cortisol levels as a function of age in 149 healthy men, aged 16–83 years. Values shown are mean (+SEM) for each age group. Note the temporal concomitance between the decline in REM sleep and the elevation in cortisol levels. Data from Van Cauter, E., Leproult, R. and Plat, L. (2000). Age-related changes in slow wave sleep and REM sleep and relationship with growth hormone and cortisol levels in healthy men. *JAMA* **284**, 861–868.

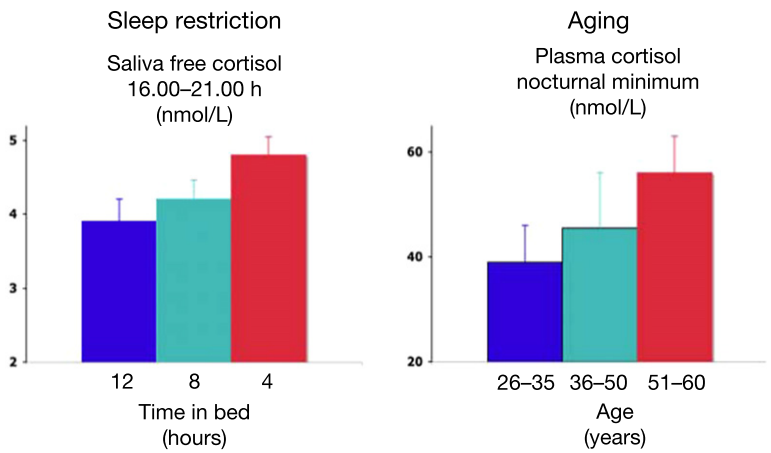

Fig. 5 *Left panel*: Afternoon-evening (16.00–21.00 h) saliva free cortisol levels in healthy young men as a function of time allowed to sleep (data from Spiegel, K., Leproult, R. and Van Cauter, E. (1999). Impact of sleep debt on metabolic and endocrine function. *Lancet* **354**, 1435–1439; Spiegel, K., Leproult, R., L'Hermite-Baleriaux, M. et al. (2004). Leptin levels are dependent on sleep duration: Relationships with sympathovagal balance, carbohydrate regulation, cortisol, and thyrotropin. *The Journal of Clinical Endocrinology and Metabolism* **89**, 5762–5771); *Right panel*: Nocturnal minimum plasma cortisol levels in healthy men as a function of age (data from Van Cauter, E., Leproult, R. and Plat, L. (2000). Age-related changes in slow wave sleep and REM sleep and relationship with growth hormone and cortisol levels in healthy men. *JAMA* **284**, 861–868). Values shown are mean (+SEM). Note the similarity between the effects of sleep restriction and the effects of aging.

(Rueggeberg *et al.*, 2012). Thus, recurrent sleep curtailment is very likely to accelerate the senescence process of the corticotropic axis. Glucocorticoid excess, especially in the evening when cortisol levels are normally low, could favor the development of central and peripheral disturbances such as insulin resistance, osteoporosis and impaired cognitive function (Dallman *et al.*, 1993; Dennison *et al.*, 1999; McEwen, 1998a; McEwen and Stellar, 1993; Plat *et al.*, 1999; Johar *et al.*, 2015). Moreover, offspring of nonagenarian siblings are marked by a lower activity of the corticotropic axis, as assessed by lower diurnal salivary cortisol levels (Noordam *et al.*, 2012).

TSH and Thyroid Hormones

In healthy young adults, TSH levels during daytime are low and fairly stable. As illustrated in **Fig. 6**, they begin to rise in late afternoon or early evening, well before sleep onset. Maximal levels are reached around sleep onset. Thereafter, TSH levels progressively decrease to return to daytime low levels shortly after final morning awakening (Brabant *et al.*, 1990). The evening elevation, referred to as the circadian rise, appears to be controlled by the circadian clock, while the subsequent decline throughout the sleep period reflects an inhibitory action of sleep on TSH secretion, as the nocturnal TSH decline is associated with SW sleep (Goichot *et al.*, 1992; Gronfier *et al.*, 1995). Conversely, transient nocturnal awakenings are frequently associated with TSH elevations (Hirschfeld *et al.*, 1996). The 24-h TSH profile is generated by amplitude and frequency modulation of TRH-driven TSH secretory pulses (Veldhuis *et al.*, 1990). Free triiodothyronine (fT3) shows low amplitude diurnal variations that parallel TSH variations (Russell *et al.*, 2008). It is of interest to note that in healthy subjects with normal and regular sleep schedules, elevated TSH levels may occasionally be observed in blood samples collected in early morning.

As illustrated in **Fig. 6**, aging is associated with an advance shifting of the timing of the circadian rise (Van Coevorden *et al.*, 1991; Roelfsema *et al.*, 2014) and with a progressive decrease of TSH secretion, in particular during the night, resulting in a dampening of the amplitude of the circadian elevation (Van Coevorden *et al.*, 1991; Barreca *et al.*, 1985). After 60 years of age, TSH levels in healthy subjects are lower than in young adults throughout the 24-h span (Van Coevorden *et al.*, 1991). This decrease might result from a diminished capacity of the hypothalamus to synthesize thyrotropin-releasing hormone (TRH) (Mariotti *et al.*, 1995). In the elderly, thyroid hormone secretion is reduced, but circulating thyroxin levels may remain fairly unchanged since its renal clearance is also altered (Mariotti *et al.*, 1995).

Prolactin

In healthy young subjects, minimal prolactin levels are observed around noon, followed by a modest increase in the afternoon, then by a major nocturnal elevation starting shortly after sleep onset (**Fig. 7**). Maximal levels are reached around mid-sleep, with an average elevation of 200% above minimum day levels (Spiegel *et al.*, 1994; Waldstreicher *et al.*, 1996). The 24-h prolactin profile is generated by diurnal variations both in tonic secretion and in amplitude of secretory pulses (Veldhuis *et al.*, 1990). Prolactin levels are higher in cycling women than in young men or in postmenopausal women (Katznelson *et al.*, 1998). Sleep onset (and more precisely SW sleep) is constantly associated with a rise in prolactin levels, irrespective of the time of the day, and prolonged transient awakenings during the sleep period are associated with drops in prolactin levels (Spiegel *et al.*, 1995). Moreover, shifts in the sleep-wake cycle are immediately followed by parallel shifts in the diurnal profile (Van Cauter et al., 1998b).

Therefore, diurnal prolactin variations appear to be primarily dependent on the sleep-wake homeostasis. However, the amplitude of diurnal prolactin elevations may be dampened when associated with shifted daytime sleep as compared with nocturnal sleep (Van Cauter and Refetoff, 1985), and modest prolactin rises may occur during waking periods around the usual time of sleep onset, particularly in women (Waldstreicher *et al.*, 1996). Thus, diurnal variations of prolactin secretion also appear

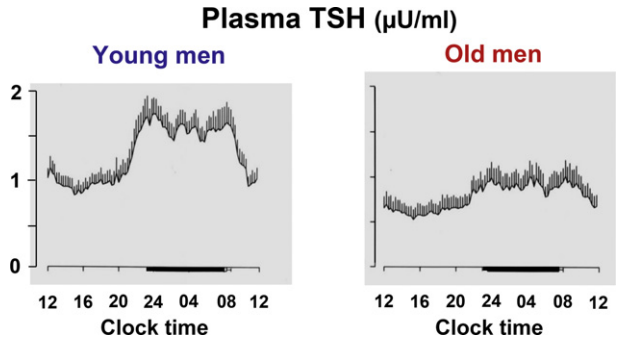

Fig. 6 Mean (+SEM) 24-h TSH profiles in eight young (20–27 years) and eight old (67–84 years) healthy men. Black bars represent the mean sleep periods. Note that in the older group, the onset of the circadian rise is advanced and its amplitude is reduced. Data from Van Coevorden, A., Mockel, J., Laurent, E., et al. (1991). Neuroendocrine rhythms and sleep in aging men. *American Journal of Physiology* **260**, E651–E661.

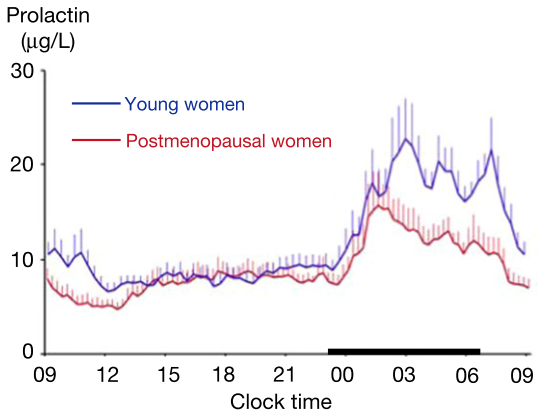

Fig. 7 Mean (+SEM) 24-h plasma prolactin profiles in 10 healthy cycling young women (21–36 years) at early-mid follicular phase and in eight healthy postmenopausal women (48–74 years). The black bar represents the mean sleep period. In both groups, the onset of the nocturnal rise is concomitant with the onset of the sleep period. Note the dampening of the nocturnal rise in the older group. Data from Caufriez, A., Leproult, R., L'Hermite-Baleriaux, M., Moreno-Reyes, R. and Copinschi, G. (2009). A potential role of endogenous progesterone in modulation of GH, prolactin and thyrotrophin secretion during normal menstrual cycle. *Clinical Endocrinology* **71**, 535–542; Caufriez, A., Leproult, R., L'Hermite-Baleriaux, M., Kerkhofs, M. and Copinschi, G. (2011). Progesterone prevents sleep disturbances and modulates GH, TSH, and melatonin secretion in postmenopausal women. *The Journal of Clinical Endocrinology and Metabolism* **96**, E614–E623.

to be partly modulated by circadian rhythmicity. Maximal diurnal elevations occur when the effects of sleep and of circadian rhythmicity are superimposed, that is at the usual bedtime (Spiegel *et al.*, 1994; Waldstreicher *et al.*, 1996; Désir *et al.*, 1982).

Shallow and fragmented sleep is usually associated with a dampening of the nocturnal prolactin elevation. This is indeed observed in the elderly who may have a nearly 50% dampening of the nocturnal prolactin elevation usually observed in young healthy individuals (Greenspan *et al.*, 1990; Van Coevorden *et al.*, 1991). As illustrated in **Fig. 7**, there is no advance shifting of the nocturnal elevation, reflecting the fact that this elevation is associated with sleep onset and is not dependent on the circadian clock.

Growth Hormone (GH)

Pituitary GH secretion is stimulated by hypothalamic GH-releasing hormone (GHRH) and by the acylated form of ghrelin (a peptide mainly produced by the stomach), and inhibited by somatostatin (Copinschi and Challet, 2016). In healthy young adults, the 24-h GH profile exhibits stable low levels abruptly interrupted by secretory pulses (**Fig. 8**). In baseline conditions, the most reproducible pulse occurs shortly after sleep onset, in temporal association with the first phase of SW sleep (Van Cauter et al., 1998b), but other secretory episodes may be observed in later sleep and during wakefulness. In young men, this sleep-onset GH pulse is consistently the largest secretory episode over the 24-h span (Van Cauter et al., 1998b), while in normally cycling women, larger daytime pulses are frequently observed, resulting in higher 24-h GH levels than in age-matched men (Ho *et al.*, 1987). Daytime GH secretion was found to be higher during the luteal phase than during the follicular phase in normally cycling women (Caufriez *et al.*, 2009).

Sleep onset is closely associated, temporally and quantitatively, with a GH secretory episode, even when sleep is delayed, advanced, interrupted or fragmented (Holl *et al.*, 1991; Van Cauter *et al.*, 1992; Gronfier *et al.*, 1996). Conversely, GH secretion is inhibited by transient awakenings (Holl *et al.*, 1991; Van Cauter *et al.*, 1992; Gronfier *et al.*, 1996). Thus, shifts of the sleep-wake cycle are immediately followed by concomitant shifts of the GH secretory profile (Van Cauter et al., 1998b).

The mechanisms underlying relationships between GH release and SW sleep probably involve synchronous activity of two or more different populations of GHRH neurons in the hypothalamus (Obal and Krueger, 2003). In animals, inhibition of endogenous GHRH secretion inhibits both sleep and GH secretion (Obal et al., 1988, 1996). In rodents, GHRH injections stimulate both GH secretion and non-REM sleep in intact as well as in hypophysectomized animals (Obal et al., 1988, 1996). In healthy young adults, GHRH injections during the sleep periods may stimulate both GH release and SW sleep (Kerkhofs *et al.*, 1993; Marshall *et al.*, 1996), while octreotide, a somatostatin analog, inhibits both GH release and SW sleep (Ziegenbein *et al.*, 2004). Furthermore, stimulation of SW sleep by oral administration of low doses of gamma-hydroxybutyrate (a natural metabolite of GABA used in the treatment of narcolepsy) or of ritanserin (a selective 5HT2 antagonist) results in concomitant and correlated elevations of nocturnal GH secretion (Gronfier *et al.*, 1996; Van Cauter *et al.*, 1997) and SW sleep.

Thus, the temporal organization of GH secretion is clearly primarily dependent on the sleep-wake cycle. However, there is also some evidence for a circadian modulation of the occurrence and the amplitude of GH secretory pulses. The amplitude of the sleep-onset GH pulse is maximal when the surge of hypothalamic GHRH is concomitant with a period of decreased somatostatin inhibitory activity, in the evening and during the night (Van Cauter et al., 1998b; Ocampo-Lim *et al.*, 1996).

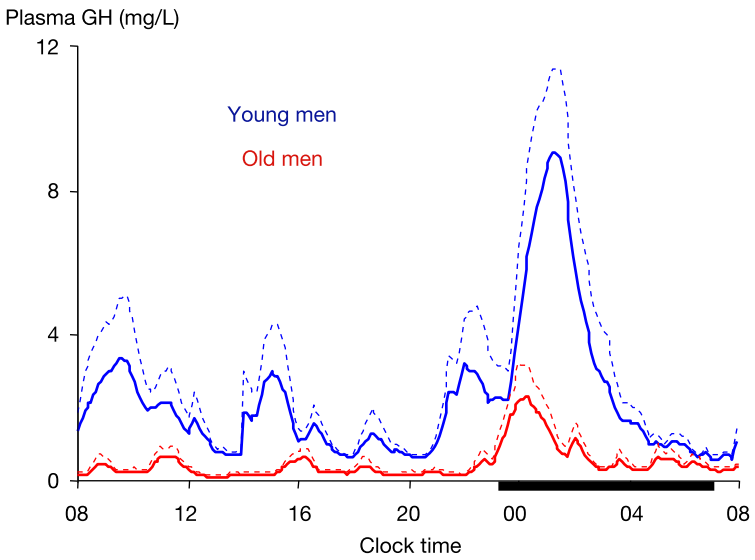

Fig. 8 Mean (+SEM) 24-h plasma GH profiles in eight healthy young men aged 17–24 years and in eight healthy old men aged 70–83 years matched for BMI. *Dashed lines* indicate SEM. The black bar represents the mean sleep period. Data from Van Cauter, E., Leproult, R. and Plat, L. (2000). Age-related changes in slow wave sleep and REM sleep and relationship with growth hormone and cortisol levels in healthy men. *JAMA* **284**, 861–868.

GH secretion is maximal during the pubertal spurt. Thereafter, a dramatic drop of GH production occurs in an exponential fashion between young adulthood and mid-age (Van Cauter *et al.*, 2000; Ho *et al.*, 1987). This decrease results from a reduction in amplitude, rather than in frequency, of secretory pulses (Van Coevorden *et al.*, 1991; Veldhuis *et al.*, 1995; Vermeulen, 1987). At mid-age (30–35 years), plasma GH levels fall to values below half the levels recorded in young adults despite the persistence of high levels of sex steroids. Twenty-four-hour plasma GH profiles in young and old healthy men are illustrated in **Fig. 8**. The age-related decline of GH secretion follows the same chronology as the decline of SW sleep (**Fig. 9**), and appears to be associated with these sleep alterations. Indeed, in a cross-sectional study performed to investigate the variance of sleep-associated GH secretion in relation to age and to SW sleep, it was shown that SW sleep and the interaction between age and SW sleep accounted for the largest part of the variance, while the effects of age per se were not significant (Van Cauter *et al.*, 2000). From midlife to old age, smaller progressive decrements of GH secretion occur (Van Cauter *et al.*, 2000), as shown in **Fig. 9**. In the elderly, 24-h GH profiles are largely similar in women and in men. IGF-I levels exhibit a progressive linear decrease from young adults to old age (Landin-Wilhelmsen and Wilhelmsen, 1994).

Gonadotropins and Sex Steroids

Men

Rhythms in the gonadotropic axis embrace a very wide range of frequencies, from ultradian pulsatile release to menstrual cycle. Interactions of these rhythms contribute to provide a sophisticated temporal system to organize the development of the reproductive axis and its operation at the different stages of maturation.

In young men, LH secretory pulses mainly reflect gonadotropin-releasing hormone (GnRH) pulsatility, and exhibit an important interindividual variability. During sleep, LH pulses appear to start preferentially during non-REM stages and to decrease during REM stages (Fehm *et al.*, 1991). Diurnal variations of LH levels over the 24-h span are of low amplitude or even undetectable. FSH profiles show occasional secretory pulses without any detectable diurnal variation. In contrast, a clear diurnal rhythm of testosterone levels is observed in healthy young men, with peaks in the early morning and troughs in late evening (Lejeune-Lenain *et al.*, 1987). Those diurnal testosterone variations are primarily dependent on the sleep-wake cycle, since daytime sleep, as well as nighttime sleep, is associated with significant elevations of circulating testosterone levels (Axelsson *et al.*, 2005). However, though dampened as compared with nocturnal sleep, a progressive increase in testosterone circulating levels persists during nocturnal wakefulness (Axelsson *et al.*, 2005), indicating the existence of a weak circadian component.

Aging is associated with a progressive decrease in circulating testosterone levels, by 1%–2% per year from 30 years of age onwards (Harman *et al.*, 2001; Kaufman and Vermeulen, 2005). This decline contrasts with a simultaneous increase in sex hormone binding globulin, so that the fall in bioavailable testosterone is more marked than the fall in total testosterone (Feldman *et al.*, 2002; Lejeune *et al.*, 2003; Yeap *et al.*, 2007; Lapauw *et al.*, 2008).

This decline in testosterone levels has a mixed etiology. On the one hand, primary functional alterations of testicular Leydig cells. On the other hand, altered neuroendocrine regulation of pituitary LH secretion (Kaufman and Vermeulen, 2005). In

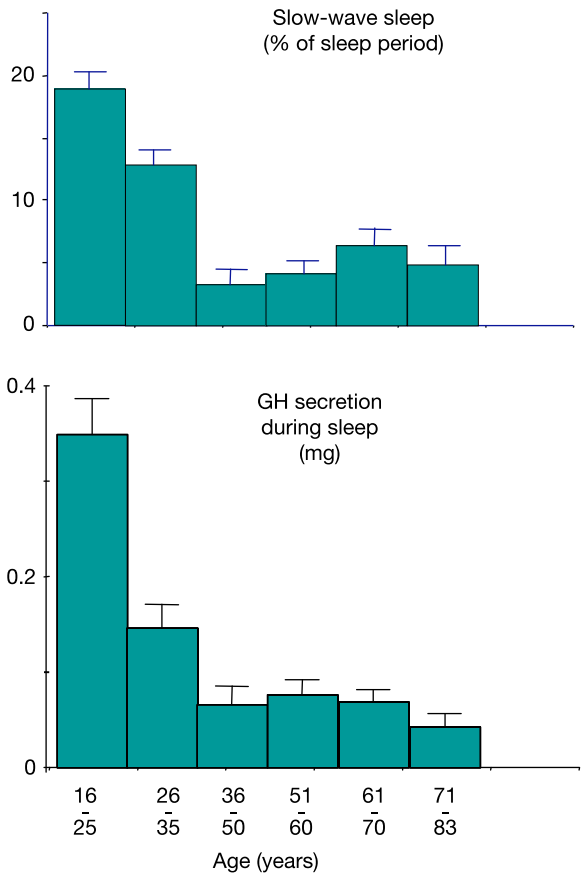

Fig. 9 SW sleep and GH secretion during sleep as a function of age in 149 healthy men, aged 16–83 years. Values shown are mean (+SEM) for each age group. Note the temporal concomitance between the decline in SW sleep and the decrease in GH secretion. Data from Van Cauter, E., Leproult, R. and Plat, L. (2000). Age-related changes in slow wave sleep and REM sleep and relationship with growth hormone and cortisol levels in healthy men. *JAMA* **284**, 861–868.

addition, the testosterone decline may also be modulated by age-related sleep disturbances. In older men, the duration of nocturnal sleep was found to be an independent predictor of morning free and total testosterone (Penev, 2007). Consistent with this finding, sleep restriction (5 h in bed per night for 1 week) was associated in young healthy men with significant decreases in daytime testosterone levels (Leproult and Van Cauter, 2011).

Animal studies indicate that the circadian rhythm of the Leydig cells endocrine function is attenuated in aged rats (Baburski *et al.*, 2016). Though dampened, the diurnal variation of circulating testosterone levels persists in older men (Bremner *et al.*, 1983; Tenover *et al.*, 1988), but LH diurnal variations are no longer detectable (Tenover *et al.*, 1988).

Women

In healthy adult menstruating women, no circadian rhythm of FSH and LH can be evidenced in constant routine conditions, indicating that the circadian pacemaker plays no role in their diurnal variations (Klingman *et al.*, 2011). Circulating FSH, LH, estradiol, and progesterone profiles show episodic pulses throughout the 24-h span (Backstrom *et al.*, 1982). Frequency and amplitude of gonadotropin pulses are modulated by estrogen levels and therefore vary markedly throughout the menstrual cycle (Filicori *et al.*, 1986; Caufriez *et al.*, 2011). In addition, they are also modulated by sleep. In early follicular phase, LH pulse frequency is diminished during nocturnal sleep as well as during daytime sleep, but not during nighttime wakefulness (Filicori *et al.*, 1986; Caufriez *et al.*, 2011; Hall *et al.*, 2005). During the sleep period, LH pulses preferentially occur in association with transient awakenings (Hall *et al.*, 2005). Therefore, sleep alterations (fragmentation and shortening of the sleep period) commonly observed in night and shift workers are likely to favor alterations of the menstrual function. Conversely, potential effects of the menstrual cycle on sleep architecture have also been evidenced. Awakenings are less frequent in the early luteal phase, when both estrogen and progesterone levels are increasing, and more frequent in late luteal phase when both hormones levels are decreasing.

Ovarian steroid secretions fall markedly at menopause. In postmenopausal women, estradiol, progesterone and testosterone circulating levels are very low, resulting in major gonadotropin elevations, principally of FSH. Gonadotropin levels and LH pulse frequency are already higher in normally cycling women beyond 40 years of age than in younger women (Reame *et al.*, 1996).

At the same time, postmenopausal women frequently complain of sleep problems, especially of more nocturnal transient awakenings. Awakenings may occur independently of vasomotor symptoms (hot flushes), but nocturnal flushes are consistently associated with concomitant awakenings (Freedman, 1998; Manber and Armitage, 1999; Moe, 2004). Hot flushes and concomitant sleep disruptions mostly occur around 04.00–05.00 h, that is, in temporal association with enhanced cortisol secretion, suggesting a possible involvement of the corticotropic axis. Both hot flushes and low inhibin B levels were found to be independent predictors of poor sleep quality (Pien *et al.*, 2008). Vasomotor symptoms were reported to be associated with increased cardiovascular risk (Gerber *et al.*, 2007; Gast *et al.*, 2008; Thurston et al., 2008, 2010, 2011; Bechlioulis *et al.*, 2010).

Studies in postmenopausal women concerning possible effects of hormonal replacement therapy on sleep quality have yielded inconsistent results, most likely because the protocol design varied widely from one study to another. In view of the fact that progesterone is also an active neurosteroid (Baulieu and Schumacher, 2000), it is of interest to note that oral administration of progesterone was found to restore normal sleep in postmenopausal women when sleep was disturbed by environmental conditions, improving both sleep duration and sleep quality with an increase in SW sleep (Caufriez *et al.*, 2011).

Glucose and Insulin

Glucose homeostasis is primarily dependent on the balance between glucose production by the liver and glucose consumption by insulin dependent tissues (such as muscle, adipose tissue) and noninsulin dependent tissues (such as brain). But in healthy subjects, glucose utilization varies throughout the 24-h span. In normal young subjects, fasting during daytime wakefulness is associated with a clear fall in circulating glucose levels. Conversely, during nocturnal sleep, glucose levels remain stable or decline only minimally despite relatively prolonged fasting. On the other hand, in case of prolonged fasting during nighttime wakefulness, glucose levels decrease, while they remain stable during daytime sleep, indicating that sleep associated mechanisms are operative to prevent inappropriate hypoglycemia (Van Cauter *et al.*, 1991).

Experimental investigations involving constant intravenous glucose infusion or constant enteral nutrition have shown that glucose tolerance deteriorates during the first part of nighttime sleep to reach a minimum around the middle of the night, and improves thereafter to progressively return to morning levels (Van Cauter *et al.*, 1991; Simon *et al.*, 1998; Scheen *et al.*, 1996). This sleep-associated decline in glucose tolerance results from a reduction in both peripheral and cerebral glucose utilization (Boyle *et al.*, 1994). Positron emission tomography studies have evidenced that during SW sleep (a dominant stage during the first half of the normal sleep period), cerebral glucose utilization is markedly lower than during REM sleep or during wake (Maquet, 1997, 2000). This is physiologically important since cerebral glucose utilization is estimated to represent at least 20% of total body glucose utilization (Defronzo, 2004; Magistretti, 2006). In addition, glucose uptake by insulin-dependent tissues is probably inhibited by the sleep-onset associated GH release (Moller *et al.*, 1990). It appears therefore that sleep-wake homeostasis exerts significant modulatory effects on glucose regulation.

Aging is associated with a decrease in both insulin secretion and insulin activity, resulting in a decrease in glucose tolerance (Chen *et al.*, 1985). Interestingly, following an intravenous glucose tolerance test (IVGTT), the magnitude of the reduction of glucose clearance rate in healthy older subjects beyond 65 years as compared with young controls, is fairly similar to the reduction

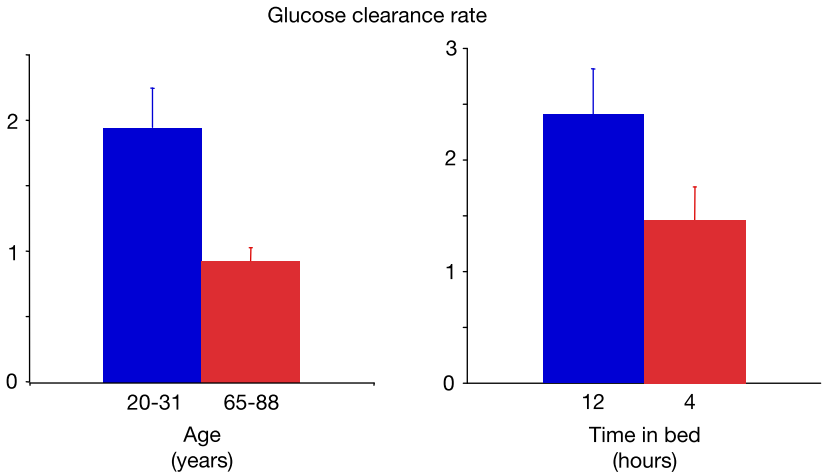

Fig. 10 *Left panel*: Glucose clearance rate following intravenous glucose injection (IVGTT) in 11 healthy old subjects (65–88 years) as compared to 11 healthy young subjects (20–31 years) (data from Palmer, J.P. and Ensinck, J.W. (1975). Acute-phase insulin secretion and glucose tolerance in young and aged normal men and diabetic patients. *The Journal of Clinical Endocrinology and Metabolism* **41**, 498–503); *right panel*: glucose clearance rate following intravenous glucose injection (IVGTT) in 11 healthy young subjects (18–27 years) in a sleep-debt condition as compared to fully rested condition (data from Spiegel, K., Leproult, R. and Van Cauter, E. (1999). Impact of sleep debt on metabolic and endocrine function. *Lancet* **354**, 1435–1439). Values shown are mean (+SEM). Note the similarity between the effects of aging and the effects of sleep restriction.

observed in healthy young subjects submitted to partial sleep restriction as compared to a fully rested condition (Spiegel *et al.*, 1999; Garcia *et al.*, 1997; Palmer and Ensinck, 1975), as illustrated in **Fig. 10**. Indeed, well-controlled laboratory studies have consistently shown that partial sleep deprivation for 1–7 days may result in increased insulin resistance (Spiegel et al., 1999, 2004, 2005; Buxton *et al.*, 2010). Prolonged (at least 6 months) voluntary sleep restriction was also shown to be associated with the development of insulin resistance (Mander *et al.*, 2001). Even alterations in sleep quality (all-night selective suppression of SW sleep), without reduction in total sleep duration, were found to induce a reduction in glucose tolerance (Tasali *et al.*, 2008). Moreover, these observations are consistent with data recorded in numerous epidemiologic studies showing that short sleep duration is significantly associated with an increased risk of developing diabetes (Knutson and Van Cauter, 2008). This bunch of data strongly suggests that sleep curtailment might accelerate age-associated alterations of glucose metabolism.

Conclusions

Though the respective roles of sleep and of the circadian clock in the regulation of endocrine function widely differ from one hormone to another, age-related sleep and endocrine-metabolic alterations frequently interact with each other. Most prominent age-related alterations of hormonal circadian rhythms are a phase-advance and a reduction in diurnal amplitude, which are likely to result from both a reduction in the strength of the circadian signal and a reduction in the homeostatic drive to sleep.

For a number of hormones, age-related alterations are strikingly similar to those observed in young healthy subjects submitted to sleep restriction. Thus, voluntary or not, recurrent sleep curtailment, currently experienced by an increasing number of children and adults, is likely to accelerate the senescence of endocrine and metabolic function.

See also: Adrenal Cortex; Physiology. Adrenocorticotropic Hormone (ACTH): Physiology and Its Involvement in Pathophysiology. Circadian Clock, Epigenetic Regulators (Sirtuins), and Metabolism. Growth Hormone (GH). Hypothalamic Control of Food Intake and Energy Homeostasis. Luteinizing Hormone (LH). Molecular Genetics of Cushing Disease. Regulation of POMC and ACTH Secretion. Stress and Endocrine Physiology. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Circadian Clock, Epigenetic Regulators (Sirtuins), and Metabolism

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Glossary

Amplitude Defines the magnitude of oscillation in a rhythmic process, which is often measured as differences between peak and trough values.

Chronobiology Research at the intersection of time and biology

Circadian entrainment Resetting process of biological rhythms to synchronize them to daily environmental cycles by external factors called zeitgebers (see below).

Circadian rhythm Rhythm of approximately 24 h in length (from Latin "circa" = about and "dies = day)

Free-running Endogenous period length that circadian rhythms assume in the absence of all zeitgebers.

Period length The time that passes between the same occurrences in a rhythmic process. For instance, if a patient's body temperature is lowest every day at 4:00 am, and highest every evening at 6:00 pm, then the period length is about 24 h.

Phase In a rhythm the phase is defined by timing of the recurrence of peak, trough (nadir), or onset of production (such as, e.g., melatonin).

Phase-shifting A change in timing of the circadian clock, which takes typically several days to complete, through entrainment by zeitgebers, such as during travel between time zones or when working night shifts.

Rhythm Biological measures or events that repeat themselves on a regular basis.

Zeitgeber: From German "time giver" Agents that provide circadian entrainment, such as daylight, feeding, physical activity or social cues.

Introduction

Probably based on the rotation of Earth, most lifeforms on our planet have developed daily rhythms that control many aspects of their lives. The "diurnal cycles" of the planet provide varying environmental conditions of light or temperature that may have led to the evolution of endogenous rhythms in cyanobacteria, plants and animals, probably as an adaptive advantage, that are called "circadian rhythms." These endogenous circadian rhythms are self-sustained and allow the animal to synchronize their daily routines to environmental cues such as a light/dark cycle of about 24 h. True circadian rhythms are formally defined by their endogenous nature, which makes them a special case among the other 24 h oscillations that occur in the lives of animals and plants and that are more dependent on environmental cues. Circadian rhythms therefore occur in an oscillating manner even in the absence of external stimuli, like an internal clockwork, but they are generally also adjustable. Environmental cues that occur repetitively, including, for example, light or temperature are called "zeitgeber(s)," which means "time giver" in German, because they are able to adjust ("entrain") circadian rhythms. The phenomenon can be appreciated when traveling between time zones and "jet lag" occurs until the personal "inner clock" is reset.

Much of our understanding of circadian clocks stems from research in Drosophila as a model organism. Because of the profound impact of circadian rhythms on human health, Drs. Michael W. Young, Jeffrey C. Hall and Michael Rosbash received the 2017 Nobel Prize in physiology or medicine for their groundbreaking discoveries in molecular chronobiology using fruit flies (Callaway and Ledford, 2017; Dibner and Schibler, 2017).

In mammals, the central circadian clock, which shares many similarities with the one in fruit flies at the molecular level, is located in the brain, where neurons of the hypothalamic suprachiasmatic nucleus (SCN) show synchronized oscillating transcription patterns that control behavior, as well as hormone levels and other central endocrine functions (Moore and Eichler, 1972; reviewed in Rusak and Zucker, 1979). Notably, many peripheral tissues such as the liver, kidney, and even individual cultured stem cells (Stringari *et al.*, 2015), also exhibit endogenous circadian rhythms that can continue entirely autonomously under isolated conditions. An important aspect of mammalian rhythmicity is that the central circadian clock in the SCN is able to entrain these peripheral clocks by providing endocrine cues, for example, in response to light/dark cycles that the SCN senses directly through the retinal-hypothalamic tract. Peripheral circadian clocks are also modulated by tissue-specific and environmental cues, like food intake, which may put them out of synchrony with the central circadian clock. Abnormal circadian rhythms in humans are known as "circadian rhythm disorders" (Bass, 2012; Vitaterna *et al.*, 2001), which will be discussed in the last section of this article.

Molecular Structure of the Core Mammalian Circadian Clock

At the molecular level, the clock machinery is present in almost all cell types where it regulates expression of at least 10% of all transcribed genes (Rosbash *et al.*, 2007). The central mechanism of the Circadian Clock system consists of positive and negative

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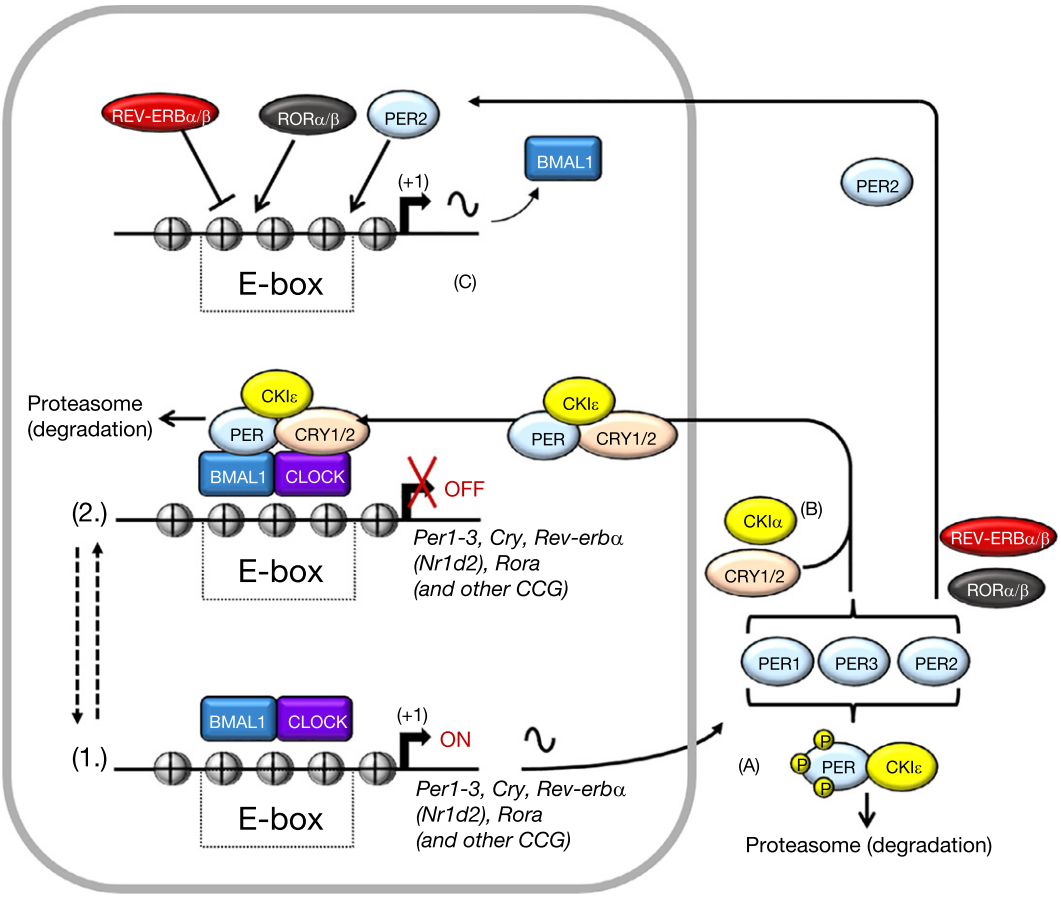

Fig. 1 Molecular architecture of the core circadian clock. Step 1. CLOCK and BMAL1 form a complex that binds to an *E*-box motif in a target gene, which activates it. Transcription and translation of the newly expressed proteins PER1–3 and CRY1/2 places them in the cytoplasm, where they are immediately phosphorylated by CKIε, which marks them for proteasomal degradation (A). Once their concentration becomes high enough, PER and CRY proteins form stable heterodimers, and enter the nucleus in a complex with CKIε (B). When PER/CRY complexes bind to BMAL1/CLOCK, the gene activating activity of BMAL1/CLOCK is inhibited (2.). Parallel pathways exist in the form of REV-ERBα/*β*-, PER2- and RORα/*γ*-dependent regulation of BMAL1 expression (C), which provides additional stability to the rhythmicity of this oscillating negative/positive transcriptional feedback cycle. Steps (1.) and (2.) together occur with a slightly longer interval of approximately 24 h. Note that in addition to these protein components of the core circadian clock listed in the figure many other genes, including many that are involved in metabolism, are (co-) regulated by these transcriptional oscillations due to the presence of E-box motives in their promoter regions.

transcriptional–translational feedback loops that are conserved across mammals (**Fig. 1**, reviewed in Song and Rogulja, 2017). The binding of two transcription factors, BMAL1 (brain and muscle ARNT-like protein 1, also known as ARNTL, aryl hydrocarbon receptor nuclear translocator like) and CLOCK (Circadian Locomotor Output Cycles Kaput), positively regulate expression of a large number of "circadian clock genes (CCG)" by binding to *E*-box elements in promoters of certain genes. Among the products of the CCG genes are repressor proteins that in turn suppress expression initiated by BMAL1 and CLOCK, thereby providing a negative feedback loop. These negative regulators include two cryptochromes (CRY1, CRY2) and three period proteins (PER1–3) in mammals. PER and CRY proteins form stable homo- and heterodimers in the cytoplasm in a concentration-dependent manner. If the concentration of PER protein is too low to form heterodimers with CRY, PERs are phosphorylated by the casein kinase CKI epsilon (CK1ε) (Eide *et al.*, 2005; Lowrey *et al.*, 2000), which triggers proteasomal degradation of PER. Once PER protein expression reaches sufficient levels and stable PER/CRY heterodimers can be formed, they enter the nucleus in a complex with CK1ε and bind to BMAL1 and CLOCK, which inhibits their gene activating function. By blocking their own expression, the concentration of the negative factor proteins declines over time due to their continuously ongoing degradation because no new copies of the proteins are expressed. This depletes PER and CRY levels to a point where BMAL1 and CLOCK are no longer inhibited (Rosbash and Hall, 1989). The degradation of PER and CRY proteins therefore sets the time interval until their transcription will commence again. The quintessential result is that PER and CRY protein levels oscillate in a circadian fashion, along with numerous other CCG output genes. In contrast to PER and CRY proteins, CLOCK levels are relatively constant. BMAL1 expression on the other hands oscillates as well because its expression is negatively regulated by REV-ERBα and REV-ERB*β* (Cho *et al.*, 2012; Preitner *et al.*, 2002) but positively regulated by PER2, RORα and ROR*γ* (RAR related orphan receptors *α*, *γ*). All of those are CCG products expressed under the control of CLOCK and BMAL1, which results in an overall oscillation of BMAL1 expression. RORα/*γ* bind to a

specific response element termed RORE that is present in many promoters that also have *E*-boxes (Sato *et al.*, 2004). REV-ERBα and REV-ERBβ also bind to RORE to antagonize RORα/γ-dependent gene activation. This results in a negative feedback loop that works in parallel with the PER/CRY negative feedback loop to regulate a large number of circadian clock genes along with regulating BMAL1 expression (Cho *et al.*, 2012; Stratmann and Schibler, 2012).

Molecular Mechanisms Linking Metabolism With the Circadian Clock (Food Entrainment)

The discovery that individual cultured fibroblasts exhibit serum-induced, self-sustaining circadian oscillations consistent with a circadian clock demonstrated that peripheral clocks can function autonomously (Balsalobre *et al.*, 1998). That begged the question to what extent the activities of the clocks in the brain and the peripheral tissues are coordinated and how much they are independent of each other. The central circadian clock located in the brain is regulated by light and controls the rhythmic neuroendocrine release of circulating factors known to be important general regulators of body functions, such as thyroid stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4) (Allan and Czeisler, 1994; Amir and Robinson, 2006), or glucocorticoids (Balsalobre *et al.*, 2000; Dickmeis *et al.*, 2013). While these and other secretory factors are important for entraining circadian rhythms in many peripheral clockworks, for many peripheral organs feeding-fasting rhythms are the dominant zeitgebers (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). Available evidence now shows that peripheral circadian oscillators like those of the liver may be coupled to the master circadian clock in the brain primarily through rhythmic behavior, such as feeding (Stokkan *et al.*, 2001). It is not yet completely understood how feeding cycles can entrain the liver and other peripheral organs independently of the master clock in the SCN and the light cycle. Several emerging mechanisms have been identified as discussed below.

Genetic Regulation of Metabolism by Circadian Clock-Controlled Genes

The repression of BMAL1 expression by the PER/CRY negative feedback loop is counteracted by RORα/γ and another nuclear receptor protein, PPARα (peroxisome proliferator-activated receptor alpha). Genome-wide analyses of RORα/γ, PPARα and REV-ERBα/β binding sites showed that these transcription factors cooperate to regulate many CCG, including many metabolic genes (Canaple *et al.*, 2006; Lemberger *et al.*, 1996; Sato *et al.*, 2004). Similarly, analyses of the binding activity of core clock components BMAL1, CLOCK, PER1/2, and CRY1/2 showed that these transcription factors are enriched in the promoters of genes involved in metabolism, which further supports the view that metabolism is in part directly controlled by the circadian clock (Bugge *et al.*, 2012; Cho *et al.*, 2012; Koike *et al.*, 2012; Rey *et al.*, 2011). In addition, the transcriptional coactivator PGC1α, a major metabolic regulator, stimulates transcription of BMAL1 and of the proteins REV-ERBα and β by promoting the activity of RORα/γ (Liu *et al.*, 2007). Another example is the regulation of cholesterol biosynthesis by the genetic regulation of the rate-limiting enzyme HMGCoA reductase (HMGCR) by BMAL1 and REV-ERBα, in concert with other transcription factors that are also involved in cellular sterol sensing (Cho *et al.*, 2012; Koike *et al.*, 2012; Sancar and Brunner, 2014). Expression of HMGCR follows a strict circadian rhythm and peaks at night (Edwards *et al.*, 1972).

Similar to cholesterol synthesis, gluconeogenesis follows a circadian rhythm through the clock-mediated regulation of behaviors like feeding and fasting (Kida *et al.*, 1980). During fasting, enhanced gluconeogenesis ensures the maintenance of blood glucose levels. Low food intake stimulates release of the hormone glucagon, which in turn activates a heterotrimeric G protein to increase cellular cAMP synthesis by adenylyl cyclase in target tissues. The elevated cAMP levels activate PKA (protein kinase A) to phosphorylate, and hence activate, the transcription factor CREB (cAMP regulatory element binding transcription factor). CREB positively regulates expression of genes that code key enzymes in gluconeogenesis, like PCK1 and others. CREB activity during fasting is modulated by CRY1 and CRY2, which are rhythmically expressed in the liver. Elevated CRY1 expression during the night–day transition reduced fasting gluconeogenic gene expression by blocking glucagon-mediated increases in intracellular cAMP concentrations and in the protein kinase A-mediated phosphorylation of CREB (Zhang *et al.*, 2010; reviewed in Jitrapakdee, 2012). In mice, a liver-specific variant, CREBH regulates circadian expression of the key genes involved in hepatic triglyceride (TG) and fatty acid (FA) metabolism and is required to maintain circadian amplitudes of blood TG and FA (Zheng *et al.*, 2016). In addition, CRY proteins interact with the glucocorticoid receptor and thus inhibit its transcriptional activation of target genes involved in gluconeogenesis during the day when CRY1/2 levels are high. Specifically, CRY1/2 inhibit the glucocorticoid-stimulated expression of PCK1, the rate-limiting enzyme of gluconeogenesis, which therefore results in the circadian oscillation of blood glucose levels (Kalsbeek *et al.*, 2014; Lamia *et al.*, 2011). In summary, there is substantial evidence that metabolic genes are at least in part controlled by oscillations of circadian clock genes. As an example supporting the theory that peripheral circadian clocks may also be involved in cell differentiation, studies in mice have shown that the peripheral circadian clock in adipose tissue regulates the differentiation of adipocyte precursor cells. The deletion of *Per3* promotes adipogenesis in vivo in which PER3 and BMAL1 directly regulate expression of the master transcription factor Krüppel-like factor 15 (KLF15), a developmentally relevant regulator of adipogenesis (Aggarwal *et al.*, 2017).

Food Entrainment: Epigenetic Regulation of Metabolic Genes Controlled by the Circadian Clock

The circadian oscillation of REV-ERBα expression, which in rats leads to high levels of REV-ERBα protein when the animals rest during the day, is coupled to the expression of genes involved in lipogenesis. When REV-ERBα levels are high, it recruits NCoR1 (nuclear receptor corepressor 1), which strongly interacts with the histone deacetylase HDAC3, to RORE to form a repressive protein complex that shuts down lipogenic gene expression by removing gene activating histone acetylation marks (Feng *et al.*, 2011). When the levels of REV-ERBα decline and the animals enter their daily phase of physical activity at night, the repressive NCOR1/HDAC3/REV-ERBα complex is no longer present and new histone acetylation activates transcription of genes needed for lipogenesis (Feng *et al.*, 2011).

AMP/ATP ratio

The nutrient-responsive adenosine monophosphate-activated protein kinase (AMPK) phosphorylates and destabilizes CRY1/2 proteins, which provides another mechanism how metabolic cycles in the liver interact with the circadian clock (Lamia *et al.*, 2009; Suter and Schibler, 2009). AMPK acts as a cellular energy sensor that is conserved in eukaryotic cells. Its kinase activity is activated by stimuli that increase the cellular AMP/ATP ratio. AMPK-mediated phosphorylation of CRY1 and CRY2 stimulates their interaction with the protein FBXL3 (F-Box and Leucine Rich Repeat Protein 3). FBXL3 targets Skp-Cullin-F-box (SCF) E3 ligases to them, which mediate the degradation of phosphorylated proteins, in this case phosphorylated CRY1 and CRY2 (Ho *et al.*, 2006; Jordan and Lamia, 2013; Lamia *et al.*, 2009). AMPK can also phosphorylate CK1ε, which enhances the activity of the casein kinase I to phosphorylate CRY1/2 proteins, which in turns triggers their degradation (Um *et al.*, 2007).

NAD^+/NADH

Nicotinamide adenine dinucleotide (NAD) could act as a signal of nutrition intake because humans depend on vitamin B3 as a source for NAD synthesis. The vitamin B3 group ("niacin") includes nicotinic acid, nicotinamide and nicotinamide riboside, which all serve as dietary precursors for NAD synthesis via the Preiss–Handler pathway (Meyer-Ficca and Kirkland, 2016). NAD^+ and the reduced form NADH are essential coenzymes for redox reactions in central metabolic processes, including the Krebs cycle, glycolysis, fatty acid oxidation, gluconeogenesis, and fat and steroid synthesis. In addition, NAD^+ also serves as substrate for various enzymes, including sirtuins and ADP-ribose transferases. Sirtuins are class III NAD-dependent histone deacetylases regulating metabolic function, longevity and aging (for recent reviews, see, e.g., Imai and Guarente, 2016; O'Callaghan and Vassilopoulos, 2017).

Cellular NAD levels oscillate in a circadian rhythm in the liver and body-wide, which is at least in part food intake dependent. NAD levels are also determined in part by the rhythmic expression of nicotinamide phosphoribosyltransferase (NAMPT) under control of the circadian clock (Ramsey *et al.*, 2009) (**Fig. 2**). NAMPT is the rate-limiting enzyme that converts nicotinamide to nicotinamide mononucleotide in the NAD biosynthetic pathway from nicotinamide in mammals (Revollo *et al.*, 2004, 2007). How exactly NAD provides cues to entrain the circadian system is still not clear. However, a number of molecular mechanisms have been identified that demonstrate a strong regulatory role of NAD in circadian clock-controlled expression of metabolic genes (Nakahata *et al.*, 2008; Tahara and Shibata, 2013).

First clues to this were provided by the discovery that CLOCK has histone acetyl transferase properties (Doi *et al.*, 2006). CLOCK directs acetylation of histone H3 and of its dimerization partner BMAL1 at Lys537, an event essential for circadian function (Nakahata *et al.*, 2008), as well as acetylating PER proteins (Asher *et al.*, 2008) (**Fig. 2**). These acetylation marks are removed by the histone deacetylase (HDAC) activity of the NAD^+-dependent SIRT1 enzyme in a circadian manner, resulting in a rhythmic acetylation and deacetylation of BMAL1 and H3 (at H3K9 and H3K14 lysine residues) at circadian promoters. SIRT1 binds in a timed fashion to the CLOCK/BMAL1 chromatin complex at circadian promoters where it deacetylates BMAL1 and PER, which leads to targeting of PER for proteasomal degradation (Asher *et al.*, 2008). Acetylated H3 provides an open chromatin conformation conducive to active transcription from a promoter site. Additional and concurrent methylation of H3K4 by the histone methyltransferase MLL1 enhances the open chromatin state, attracts binding of the CLOCK/BMAL1 complex and provides a submissive epigenetic state of circadian gene promoters (Aguilar-Arnal *et al.*, 2015; Katada and Sassone-Corsi, 2010). To reverse the periodic activation of clock promoters, SIRT1, and likely SIRT6 (Masri *et al.*, 2014) deacetylate MLL1, which needs to be acetylated by CLOCK in positions K1130 and K1133 to be active. SIRT1 and SIRT6 deacetylation of H3 also promotes a closed chromatin conformation and silencing of the promoters. According to this model, high concentrations of NAD therefore activate SIRT1 when energy (i.e., NAD) levels are high to shut down metabolic genes that are involved in the generation of energy. On the same note, increasing concentrations of nicotinamide, which is one of the two products of the NAD-dependent SIRT1 reaction and the substrate of NAMPT, is an indicator of low cellular energy status and a specific inhibitor of SIRT1. Taken together, the available data show that SIRT1 functions as an enzymatic rheostat of circadian function by transducing signals that originate from cellular metabolites to the circadian clock (Nakahata *et al.*, 2008).

Poly(ADP-ribosyl)ation by PARP1

Chromatin modulation by histone acetylation and methylation by CLOCK, SIRT1 and MLL1 obviously provides an important epigenetic mechanism of circadian clock transcriptional control. Similar to SIRT1, poly(ADP-ribose) polymerase (PARP) enzymes are another group of epigenetic modulators whose activity depends on cellular levels of NAD^+. PARPs cleave NAD^+ into nicotinamide, which is recycled through the NAD salvage pathway via NAMPT, and ADP-ribose, which is polymerized to poly

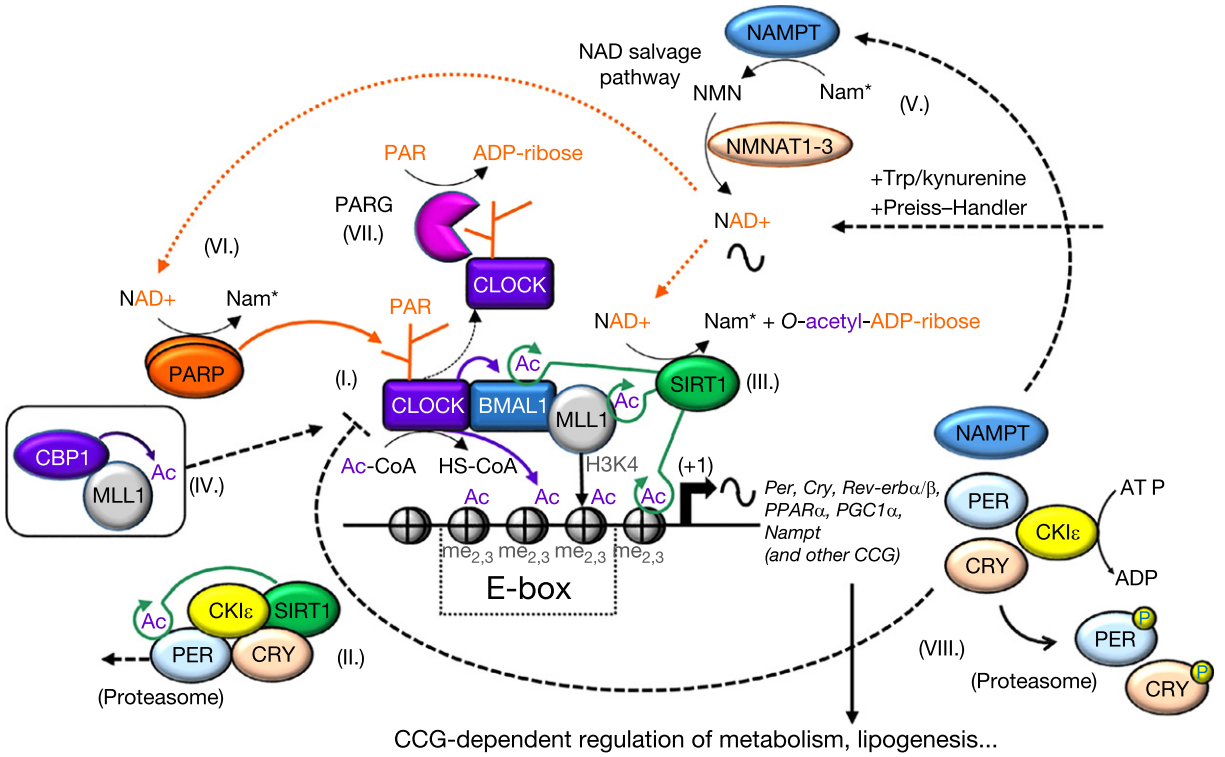

Fig. 2 NAD-dependent regulation and/or entrainment of SIRT1- and PARP1/2-mediated regulation of circadian genes involved in energy metabolism. (I.) CLOCK and BMAL1 bind to an E-box motif and CLOCK acetylates H3K9/K14 in the promoter region to activate transcription of target genes including Nampt, which encodes nicotinamide phosphotransferase (increases NAD synthesis). CLOCK also acetylates BMAL1, which later recruits PER and CRY proteins to form a repressive complex to inhibit promoter activity (see also **Fig. 1**), where PER proteins are stabilized by acetylation (II.). The NAD-dependent SIRT1 then deacetylates H3 (III.), which leads to deactivation of the promoter. SIRT1 also deacetylates BMAL1 and PER proteins (III.). This leads to degradation of PER proteins (II.). The promoter remains inactive until CLOCK/BMAL1 bind to the promoter and activate it again. The box to the left (IV.) shows MLL1 acetylation by CBP1, which activates the H3K4 di- and tri-methylating property of MLL1. H3K4me2/3 is a gene activating epigenetic mark. Collectively, if NAD concentrations are high due to feeding, NAMPT expression and resulting increased NAD levels (V.), SIRT1 activation leads to deacetylation and inactivation of MLL1, BMAL1 and H3K9/K14 (III.), which contributes collectively to silencing of the metabolic gene(s) in response to peaking cellular energy (i.e., NAD) levels. High NAD levels also activate PARP1 and PARP2 (VI.) to poly(ADP-ribosyl)ate (PAR) CLOCK, which in turn reduces its affinity to DNA and physically removes CLOCK from the promoter, stopping gene activation by the BMAL1/CLOCK complex. Poly(ADP-ribose) glycohydrolase (PARG) removes poly(ADP-ribose) from CLOCK, allowing the transcription factor to bind to the E-box motif again (VII.). The depicted CKIε-dependent phosphorylation of PER and CRY proteins (VIII.), which targets these proteins for degradation until a sufficient amount of PER and CRY proteins has been accumulated to enter the nucleus and inactivate the BMAL1/CLOCK complex, is ATP-dependent, potentially providing an additional link of circadian gene expression with cellular energy status.

(ADP-ribose) (PAR) and attached to target proteins as a large posttranslational modification ("PARylation," for reviews see Gupte *et al.*, 2017; Meyer-Ficca *et al.*, 2005). PAR carries a strong electronegative charge, which reduces the interaction of proteins with other peptides, DNA or RNA. PARylation of histones, particularly histone H1, results in an immediate and transient opening of chromatin (decondensation), which is an important requirement for DNA repair, DNA replication and transcription. PARP1 and PARP2, the two major nuclear PARP enzymes, are therefore important NAD-dependent enzymes with widespread functions in chromatin remodeling processes. PARP1/2 are enzymatically inactive until they become activated by either binding to DNA strand breaks, for example, during base excision repair, or by their posttranslational modification. PARP enzymes are, for example, phosphorylated, acetylated, methylated and ADP-ribosylated in accord with their diverse functions, which are mediated by several biochemical pathways (reviewed in Piao *et al.*, 2018). As seen for SIRT1, nicotinamide inhibits PARP enzymes and PARP activity is dependent on the availability of NAD^+. The finding that liver PARP1 activity oscillates with a circadian rhythm and is regulated by feeding suggested a role of the enzyme in regulating the circadian clock.

PARP1 binds to CLOCK-BMAL1 heterodimers and poly(ADP-ribosyl)ates CLOCK in a daily manner. Poly(ADP-ribosyl)ation of CLOCK modulates the DNA-binding activity of CLOCK/BMAL1 and its interaction with components of the negative limb, the PER and CRY proteins, which in turn alters circadian gene expression. PARylated CLOCK leaves the complex and no longer binds to the *E*-box sequence in promoters until a PAR glycohydrolase (PARG) degrades CLOCK's PAR modification to free ADP-ribose monomers. Free monomeric ADP-ribose is subsequently hydrolyzed by ADP-ribases, which are pyrophosphatase-type enzymes, to AMP and ribose-5-phosphate, which enters into sugar metabolism. Removal of PAR from CLOCK therefore quickly restores

CLOCK functions. Remarkably, the entrainment of liver clocks to inverted feeding, that is, feeding at a time when mice would normally be resting instead of feeding, is significantly delayed in the absence of PARP1 in PARP1 KO mice (Asher *et al.*, 2010) (see **Fig. 2**). The resetting of the circadian phase of the liver clock induced by restricted feeding is also delayed in PARP1 KO mice (Asher *et al.*, 2010). Hence, PARP1 might be involved in food entrainment of peripheral organs by transmitting the feeding signal to the circadian clock. Poly(ADP-ribose) modifications synthesized by PARPs are degraded by poly(ADP-ribose) glycohydrolases (PARG). In the plant *Arabidopsis thaliana*, a mutation in the tej gene encoding a poly(ADP-ribose) glycohydrolase lengthens the period of the circadian clock (Panda *et al.*, 2002). The long-period phenotype can be rescued by a PARP inhibitor, suggesting a role of the PARylation in the plant circadian clock although target protein(s) have not yet been identified.

In summary, PARP1-mediated modulation of the circadian clock is dependent on feeding and NAD^+ levels, thereby providing a circadian integration of metabolism and energetics (Bass and Takahashi, 2010; Nakahata and Bessho, 2016).

Pathologies Associated With CLOCK Desynchrony

Circadian rhythms have evolved to orchestrate metabolism with the energy requirements of animals, including humans, throughout the day when feeding and fasting cycles alternate during the daily routine. Circadian organization of metabolism likely offers the evolutionary advantage of being able to separate catabolic and anabolic processes in synchrony with activity and resting periods to optimize metabolic efficiency and maximize fitness (Gerhart-Hines and Lazar, 2015; Panda, 2016; Poggiogalle *et al.*, 2017). The underlying clockwork regulates thousands of genes, which makes it the largest regulatory network in normal physiology (Poggiogalle *et al.*, 2017). The central clock regulates circadian feeding and resting behavior in synchrony with light/dark cycles, but data from laboratory animal research have provided overwhelming evidence that the peripheral clocks are entrained by feeding and other cues to harmonize metabolism with the daily routine provided by the central master clock (reviewed in Tahara and Shibata, 2017). Of clinical interest is what happens when the balance of each clock is perturbed or when the central clock is not in synchrony with the peripheral ones.

When traveling between different time zones, one may experience the profound impact the central circadian clock has on human physiology in the form of "jet lag." The problem persists until our circadian clock has been reset to the local 24 h light/dark cycle, which speaks to the ability of all circadian clocks to be entrained by external stimuli. Unfortunately, modern human life is not necessarily still well suited for natural entrainment of circadian clocks to stay synchronized, as was the case when the fine-balanced system of circadian oscillations developed. There are artificial light, heating and cooling that keep environmental conditions constant, shift work, prevalence of a sedentary life style, as well as cheap, calorie-dense food that is available around the clock. Regular entrainment clues by the environment are upset or missing, which may have been contributing to the dramatic increase in "civilization diseases" we are currently experiencing.

The number of proteins and metabolites that are dependent on circadian rhythms is vast, and so is the number of pathological effects that can result from circadian misalignment. Forced desynchrony experimentation in rodents and humans has revealed cardiometabolic, immunological and neuronal dysfunction and other clinically potentially highly relevant effects, because physiological function, disease phenotypes and clinical pharmacokinetics and pharmacodynamics all vary by the time of day. The multitude of measurements and biochemical observations has recently led to the initiation of comprehensive approaches. These approaches intend to capture genetic and epigenetic effects of circadian clock misalignment on proteomics, metabolomics and cardiovascular physiology as well as other body functions in an attempt to characterize the human "chronobiome" (Castro *et al.*, 2015; Curtis *et al.*, 2007; Dallmann *et al.*, 2012; Feng and Lazar, 2012; Lowrey and Takahashi, 2004; Mauvoisin *et al.*, 2014; Skarke *et al.*, 2017).

Because circadian rhythms regulate so many rate-limiting steps in fatty acid and cholesterol synthesis (Feng *et al.*, 2011; Sitaula *et al.*, 2017) and hepatic gluconeogenesis (Zhang *et al.*, 2010), an array of metabolic processes – including insulin sensitivity, insulin secretion, cholesterol synthesis, fat oxidation, and energy expenditure – all follow a rhythm across the 24-h day (Li *et al.*, 2012). Data therefore increasingly suggest that disruption of the circadian system increases the risk of metabolic diseases (Baron and Reid, 2014; Gerhart-Hines and Lazar, 2015). In summary, when the external rhythms are out-of-sync with endogenous circadian rhythms, such as through exposure to bright light at night, sleeping during the daytime, or eating at night, several aspects of normal metabolism are expected to be impaired (Poggiogalle *et al.*, 2017).

The following section is intended to provide a nonexhaustive overview of the most robustly observed pathological effects of such circadian clock misalignments or defects. Current research continues to identify additional health risks of desynchronized circadian rhythms that are too numerous and preliminary to be discussed in detail here.

Obesity and Metabolic Syndrome

Time-of-day-dependent variations in the hormonal and metabolic responses to food are well established (Morgan *et al.*, 1998). Shift workers are at a higher risk of cardiovascular disease and of becoming obese and are more likely to present with other signs of metabolic syndrome, probably due to inappropriate timing of food consumption (reviewed in Guerrero-Vargas *et al.*, 2018). Studies of nighttime food consumption by simulated night-shift workers showed a postprandial persistence of relative lipid intolerance throughout the night (Al-Naimi *et al.*, 2004). Similarly, only four consecutive days of simulated shift work were sufficient to reduce insulin sensitivity and glucose metabolism in healthy subjects, which would be expected to increase the risk of

type 2 diabetes (Bescos *et al.*, 2018). These results and other available data provide consistent evidence that the consumption of food during the circadian evening and/or night, independent of more traditional risk factors such as amount or content of food intake and activity level, plays an important role in body composition (Albrecht, 2017; Engin, 2017; Hutchison *et al.*, 2017; McHill *et al.*, 2017; Plano *et al.*, 2017). Restriction of dietary food intake throughout the night may therefore be beneficial in night-shift workers who are not adapted to nighttime working (Al-Naimi *et al.*, 2004; Bescos *et al.*, 2018). A possible strategy to prevent the adverse health effects of night work may also include bright light phototherapy to facilitate rapid resetting of the peripheral clocks (Cuesta *et al.*, 2017; Danilenko *et al.*, 2013). In a similar vein, childhood obesity may be caused by social jetlag, that is, the discrepancy between an individual's circadian clock and social rhythms, and is measured as the difference in hours between the midpoint of sleep during work/school days and on free (weekend) days (Stoner *et al.*, 2018). Obesity is also a symptom of the night eating syndrome (NES), which is characterized by evening hyperphagia and frequent awakenings accompanied by food intake. Patients with NES display a delayed circadian pattern of food intake but retain a normal sleep–wake cycle. Phototherapy has also been suggested for the treatment of NES (Goel *et al.*, 2009).

Obesity and the metabolic syndrome entail numerous forms of detrimental impact on the cardiovascular system that are secondary to the misalignment of circadian clocks. In addition, the kidney, brain, nervous system, vasculature, and heart have all been identified through the study of mouse models and through clinical trials as peripheral clock regulators of blood pressure. The dysregulation of this circadian pattern of blood pressure, with or without hypertension, is associated with increased risk for cardiovascular disease (Douma and Gumz, 2017) and misalignment with the external light environment has been shown to potentially drive cardiac dysfunction (West *et al.*, 2017).

Sleep Disorders

Melatonin is a pineal hormone that is synthesized from serotonin and that is released in a circadian rhythm in response to the 24 h daylight rhythm. During the day, melatonin levels are negligible but at the onset of night, around 10:00 pm in normal individuals, melatonin is released and serves as a time cue to various organs. This includes the SCN itself and in the absence of light melatonin release may entrain the sleep–wake and neuroendocrine systems to the 24 h cycle (Zisapel, 2018). Melatonin levels peak between 2:00 am and 4:00 am before they return to very low daytime levels around 5:00 am. Melatonin levels in saliva and melatonin metabolites in urine have traditionally been used to monitor melatonin release in circadian rhythm research (reviewed in Pevet *et al.*, 2017). This daily synchronization process generally poses no problems for sighted individuals except when jetlag or working night shifts cause conditions of circadian desynchrony. The role of chronobiology in sleep disorders has long been known in this context (Monk and Welsh, 2003). In contrast, totally blind subjects with no light perception are more susceptible to periodical circadian desynchrony. These patients experience periodic or cyclical episodes of poor sleep and daytime dysfunction, which is a free-running disorder, also known as "Non-24 Sleep-Wake Rhythm Disorder" that can be confirmed clinically by measurements of circadian biomarkers such as urinary melatonin to demonstrate a circadian period outside the normal range. Management approaches include behavioral modification and pharmacological treatment using hypnotics, or alternatively, timed melatonin administration, which has shown promising results (Quera Salva *et al.*, 2017). There is a well-documented negative impact of light exposure during the night, even when the light was dim, on human circadian rhythm (reviewed in Monk and Welsh, 2003), which again emphasizes the power of environmental light as a zeitgeber. Along the same line, timed daytime exposure to bright light was found to help with realigning circadian peripheral clocks and to improve circadian melatonin release in individuals with sleep disorders and personnel working at Arctic and Antarctic latitudes (Mishima *et al.*, 2001; Park and Tokura, 1999; Takasu *et al.*, 2006; Arendt, 2012; Fukushige *et al.*, 2014; Albreiki *et al.*, 2017).

Cancer

There is overwhelming evidence that disruption of the circadian rhythm is associated with a 50%–100% higher risk of breast cancer in female night time workers such as nurses (reviewed in Touitou *et al.*, 2017). The precise mechanisms are unclear but the inhibited night time secretion of melatonin, which was shown to suppress breast cancer development, likely has an important role (Tamarkin *et al.*, 1981). In addition to its role for maintaining the 24-h circadian rhythms, melatonin also is a powerful antioxidant as a result of its ability to scavenge free radicals and to activate antioxidative enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase, explaining its cancer suppressing properties (for review, see, e.g., Mediavilla *et al.*, 2010; Zhou *et al.*, 2015). Other functions of melatonin in the prevention of breast cancer may include its interactions with the estrogen signaling pathways. These include (a) an indirect neuroendocrine mechanism where melatonin downregulates the hypothalamic–pituitary reproductive axis and consequently reduces levels of circulating gonadal estrogens and (b) direct melatonin actions at a tumor cell level where it directly interferes with the activation of the estrogen receptor (Cos *et al.*, 2006).

Links between other forms of cancer, including prostate cancer with desynchronization of circadian rhythms have been suggested (Lamia, 2017) but, although the concept is intriguing, available evidence is currently still too scarce to determine whether or not causal links exists (Touitou *et al.*, 2017; Wendeu-Foyet and Menegaux, 2017).

See also: Lifestyle and Nutrition. Hypothalamic Control of Food Intake and Energy Homeostasis. Obesity and Food Addiction. Adipose Tissue. Hormonal Circadian Rhythms and Sleep in Aging. Obesity, Childhood, and Adolescence

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Somatotropic Axis in Human Aging

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The Physiology of Somatotropic Axis in Human Aging

The Somatotropic Axis

Growth hormone (GH), insulin-like growth factor I (IGF-I), insulin and their receptors interacts with a major impact on aging and longevity (Bartke *et al.*, 2013). GH is secreted by the anterior pituitary gland and positively regulated by growth hormone releasing hormone (RHGH), ghrelin, and some proteins. Instead GH secretion is negative regulated by IGF-I (though a negative feedback), somatostatin and other neuroendocrine hormones, as insulin (Ceda *et al.*, 1985) (**Fig. 1**). At least, GH and insulin enhances hepatic IGF-I synthesis, expression and secretion (Ceda *et al.*, 1985).

The Aging

The average length of human life is currently from 75 to 78 years and may increase to 85 years during the following 10 years (Fries, 1980). Most data indicate a modest gain in the number of healthy years lived but, a far greater increase in years of compromised physical, mental and social function (Campion, 1994). In fact, throughout the adult life, all physiological function start to gradually decline (Rudman and Rao, 1992). According to a progressive reduced capacity for cellular protein synthesis, several

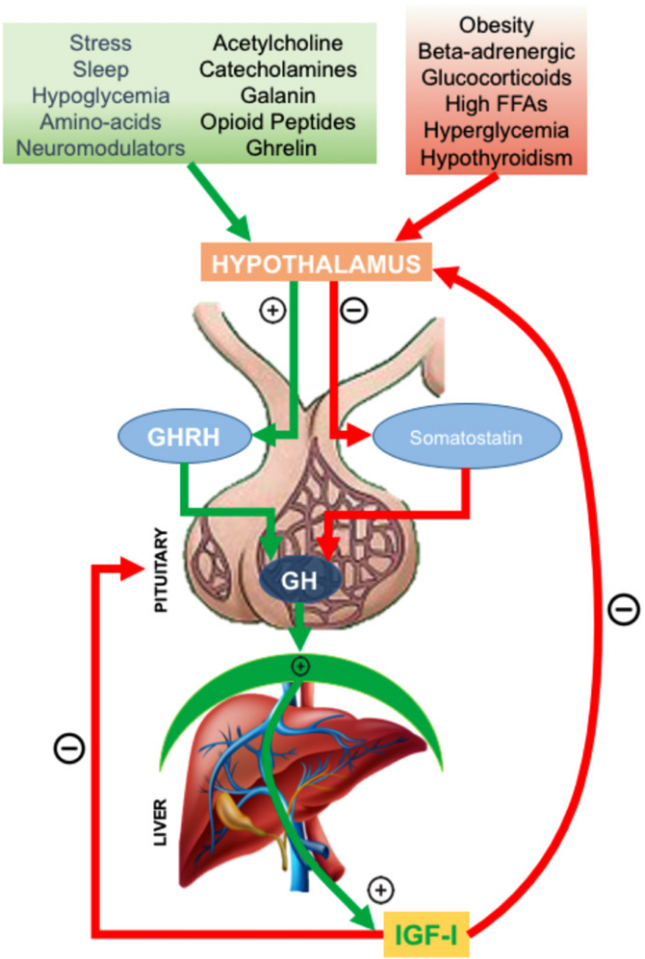

Fig. 1 Physiological mechanisms of GH secretion: positive and negative factors in the regulation of GH/IGF-I axis.

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mechanisms occurred as decline of the immune function, increase of the fat mass, loss of mass and strength of the muscle, decrease of the bone mineral density (BMD) (Rudman and Rao, 1992). In fact, age-related disability, also called "physical frailty," is characterized by generalized weakness, impaired mobility and balance and poor endurance. Clinical correlates of physical frailty include falls, fractures, impairment in activities of daily living and loss of independence. Part of the aging process involving body composition (as loss of muscle strength and reduction of bone mineral density and increase of fat mass) might also be related to changes in endocrine system.

Changes of Somatotropic Axis in Human Aging

Although the most important clinical changes, in endocrine activity, during aging, involve pancreas, thyroid and gonadal glands, also the axis GH/IGF-I gradually declines its activity (Rudman and Rao, 1992; Corpas *et al.*, 1992), in term of reduction of GH mean pulse amplitude and duration, without any change in pulse frequency. GH secretion declines around 14% per decade in normal individuals (Toogood *et al.*, 1996, 1998). In parallel, a progressive drop of circulating IGF-I levels occurred in both sexes (Corpas *et al.*, 1992; Blackman, 1987). In fact, serum IGF-I levels are from 20% to 80% lower in healthy elderly individuals as compared to healthy young individuals (Borst *et al.*, 1994). This physiological process, called *somatopause*, seem due to hypothalamic mechanisms, rather than pituitary or peripheral factors.

The effect of GH and IGF-I age-related decline on the health in humans is complex and remains not completely resolved. The physiological attenuation of GH/IGF-I signaling, during aging, may also play a protective role from age-related disease (Milman *et al.*, 2016). This evidence is more robust for site specific cancers but remains equivocal for cardiovascular disease (CVD), mellitus diabetes type II (T2DM), osteoporosis and neurodegenerative disease.

Epidemiological studies demonstrated an increased risk of cancer in humans in cases of elevated circulating IGF-I levels (Renehan *et al.*, 2004), as in acromegaly (Boguszewski and Ayuk, 2016), particularly at hormone sensitive organs as breast, prostate, thyroid, and colon (Cao *et al.*, 2015; Key *et al.*, 2010; Rinaldi *et al.*, 2010).

The somatotropic axis is intrinsically linked to insulin pathway and glucose homeostasis (Milman *et al.*, 2016): IGF-I and insulin in fact can each bind the IGF-I and insulin receptors (respectively IGF-IR and InsR), although with a different affinity (Novosyadlyy and Leroith, 2012). Consequently, IGF-I acts biologically as insulin both in the periphery and in the brain, for regulating glucose homeostasis. However, metabolic changes in the elderly are extremely complex and influenced by several factors (Barzilai *et al.*, 2012), as concomitant hypogonadism and hypothyroidism and secretion of adipokines, leptin, and other inflammatory markers (PAI-1, TNF-alpha, and IL-6), secreted also by visceral fat. Moreover, the concomitant age-dependent decline of GH and IGF-1 serum levels may promote the loss of muscle mass and strength, inducing a condition of sarcopenia, and the increased of visceral fat mass. Actually, data on GH and insulin in elderly remain controversial.

Similarly, several studies conducted on older population (Janssen *et al.*, 1998; Langlois *et al.*, 1998; Ohlsson *et al.*, 2011; van Varsseveld *et al.*, 2015; Yamaguchi *et al.*, 2006), have documented a positive correlation between IGF-I levels and BMD and increased risk of vertebral fractures, as it is well known that both GH and IGF-I acts on osteoblasts, though paracrine and endocrine effect. However, data on bone metabolism and GH8IGF-I axis in elderly are actually not-conclusive.

Instead, data regarding IGF-I concentration, cognitive function remain controversial. In a study, conducted on older individuals, higher circulating IGF-I levels seem positively correlated with cognitive function, only in females (Al-Delaimy *et al.*, 2009). However, as suggested by animal models, neurological effects of GH and IGF-I should be due to a paracrine effect of locally produced GH and IGF-I.

Growth Hormone Deficit in Elderly

As the attenuation of GH/IGF-I signaling, during aging, is a physiological process, growth hormone deficit (GHD) is widely recognized as a distinct pathological clinical syndrome with an increased morbidity and mortality, that consequently has to be distinguished, diagnosed, and properly managed. Body changes occurred in health elderly and in elderly GHD individuals are summarized in **Table 1**.

Epidemiology and Clinical Implication of GHD in Elderly

GHD in elderly may be of either adult or childhood onset (Doga *et al.*, 2006). In elderly, GHD usually results from damage to the pituitary gland or hypothalamus: in around 70% of cases due the presence or history of previous not-functioning or secreting pituitary adenomas (Elgzyri *et al.*, 2004). Although rarer, GHD in elderly can occur also in patients carried cystic pituitary lesions, craniopharyngiomas, primary or secondary empty sella syndrome, Sheehan syndrome (Elgzyri *et al.*, 2004; Clayton *et al.*, 2007; De Marinis *et al.*, 2005; Chiloiro *et al.*, 2016, 2017). GHD in elderly is a clinical syndrome, characterized by a wide array of symptoms, as decrease of lean body mass, exercise capacity, mass and strength of muscle, cardiac performance and BMD, increase of weight and body fat mass, poor sleep and impaired sense of well-being (Drake *et al.*, 2001).

Inherently, in elderly GHD patients, GHD is associated to multiple pituitary hormone deficits (Clayton *et al.*, 2007).

Table 1 Difference of changes in body composition in health elderly individual, elderly GHD and acromegaly patients

	Physiological aging	*Elderly GHD*	*Elderly acromegaly*
Lean body mass muscle strength	Reduced	Reduced	Increased
Aerobic capacity	Reduced	Reduced	Increased
Percentage of body fat	Increased	Several increased	Reduced
Total and LDL cholesterol	Increased	Increased	Increased
Insulin sensitivity	Reduced	Reduced	Reduced
Serum IGF-I value	Within normal Range adjusted for age and gender	Within or lower of normal range adjusted for age and gender	Higher of normal ranges adjusted for age and gender
GH Peak after stimulation test	Normal	Reduced	NA

NA, Not applicable.

All these conditions can implicate several and important chronic complications, such as cardiovascular disease, increased risk of fracture rates, which may be responsible for an increased mortality. Although similarities have been observed between the changes associated with physiological aging in the healthy individuals and the symptoms of GHD, as increased fat mass and decreased muscle mass, Toogood (2003) demonstrated that fat mass and waist: hip ratio were significantly increased in elderly GHD patients, as compared to elderly healthy subjects, with a prevalent fat mass distribution in the trunk, arms and legs. Hypopituitary elderly GHD patients have been shown to have an increased number of atheromatous plaques in carotid and femoral arteries, compared with control individuals. Markers of atheromatosis found in GHD patients include a greater intima-media thickness, which is recognized as an independent predictor of acute myocardial infarction in men, increased stiffness of carotid arteries and reduced aortic distensibility (Markussis *et al.*, 1992). Evidence proven from KIMS Database (Feldt-Rasmussen *et al.*, 2004) have suggested that waist/hip ratio, waist circumference, prevalence of diabetes mellitus, coronary heart disease, stroke, and hypertension are higher in elderly (higher than 65 years old) GHD patients and compared to younger ones. Similarly, quality of life was lower in older GHD patients as compared to younger ones, suggesting that older patients perceived a worse quality of life.

Data on BMD in elderly GHD patients are not conclusive, as some authors identified superimposable BMD at different sites in elderly GHD patients as compared to healthy age and sex-matched subjects (Fernholm *et al.*, 2000; Toogood, 2003). Although it is widely demonstrated that adult GHD patients suffer from secondary osteoporosis and skeletal fragility (Wuster *et al.*, 2001; Mazziotti *et al.*, 2006; Holmer *et al.*, 2007; Mormando *et al.*, 2016), a positive correlation between age and BMD value at lumbar spine, femoral neck and total hip was proved in GHD affected patients: young adults GHD were observed to have reduced bone mass, whereas the elderly GHD patients had normal *Z* scores (Murray *et al.*, 2004). These discrepancies may be justified by interferences demonstrated in BMD evaluation that can be influenced by several factors, such as gender, presence of concomitant hypogonadism, age-related abnormalities of bone structure. In fact, degenerative joint alterations (characterized by osteophyte formation and facet-joint hypertrophy) may lead to an overestimation of BMD measured at lumbar spine (Romijn, 2013). However, evaluation of markers of bone formation and resorption demonstrated a significantly reduction in the elderly GHD patients compared with elderly healthy subjects (Toogood, 2003).

The clinical implication of GHD in elderly patients is moreover indirectly underlines by the real benefits of rhGH replacement treatment in these patients (Clayton *et al.*, 2007): in ederly GHD patients, GH replacement therapy achieve beneficial effects equivalent to those seen in younger patients with lower doses of rhGH (Monson and Jönsson, 2003; Feldt-Rasmussen *et al.*, 2004; Fernholm *et al.*, 2000).

Improvements of serum lipoproteins (Elgzyri *et al.*, 2004), of body composition (Toogood and Shalet, 1999; Monson and Jönsson, 2003) and of systolic and diastolic arterial pressure (Monson and Jönsson, 2003) were documented in elderly patients on rhGH treatment. In fact, several knowledges of the relationship between GH, IGF-I and obesity derive from researches conducted on Laron syndrome (Bartke *et al.*, 2013; Laron *et al.*, 1968), showing that in these patients, long-term IGF-I replacement therapy improved subcutaneous and visceral adiposity and nonalcoholic fatty liver (Laron and Klinger, 1993) and suggesting, consequently, a direct action of IGF-I on adipose tissue storage, according to the typical GH receptor resistance condition.

Date on effects of rhGH therapy on bone metabolism in elderly patients are, still actually, controversial: as rhGH seem not to prove significant change on BMD (Kokshoorn *et al.*, 2011), several studies documented significant variation of markers for bone formation (bone-specific alkaline phosphatase activity, osteocalcin, and procollagen I carboxyl-terminal peptide in serum) and bone resorption (pyridinoline in urine) (Fernholm *et al.*, 2000; Franco *et al.*, 2006). These finding, in fact, suggest that, also in elderly, rhGH replacement treatment acts on bone remodeling with its well-known typically "biphasic model," inducing in the first 6 months of treatment an increment of bone resorption and in the following 12 months an increment of bone formation (Ohlsson *et al.*, 1998). Consequently, as in younger GHD patients, also in older ones, long-term study on elderly patients on rhGH are required to better understand this mechanism.

On large interest, are evidence regarding quality of life in older GHD patients treated with rhGH. Indeed, quality-of-life data from KIMS showed that older patients with hypopituitarism experienced a significant improvement of quality of life (similarly to younger patients), during rhGH replacement treatment, particularly in term of restored of asthenia and fatigue (Clayton *et al.*,

2007), that in GHD condition seem related to impaired aerobic capacity (e.g., oxygen consumption and ventilation threshold) rather than to physical changes in the performance of skeletal muscle fibers (Woodhouse *et al.*, 2006). Benefits were observed for total quality of life, as well as for specific domains such as memory, self-confidence, tiredness (i.e., items relating to fatigue and energy levels), tenseness, and socializing. GH role in the control of cognitive function remain not completely known. Actually, have been provided growing evidences that GH can cross the blood-brain barrier. In fact, an increased concentration of GH in the cerebrospinal fluid during rhGH replacement treatment in GHD affected patients has been described (Johansson *et al.*, 1995). Moreover, several studies suggested that GH can influence mood and cognitive, as well as learning and memory formation (Sonntag *et al.*, 2005) and that reduction of circulating IGF-I levels is associated to brain deterioration (Piriz *et al.*, 2011). Conversely, restored IGF-I levels was associated to an enhance of neurogenesis and amelioration of age-related cognitive malfunction in aged brain (Sonntag *et al.*, 1998).

Diagnosis of GHD in Elderly

The diagnosis of GHD requires particular consideration in elderly individuals, according to the identification of the correct clinical context of GHD, the potential fragility of the elderly patients and the lower sensitivity of the most commonly used diagnostic tests.

Particularly, GHD should be investigated only after replaced and balanced all other pituitary tropin deficit.

As GH is secreted in a pulsatile manner, the assessment of random serum GH concentrations is worthless for diagnosis (Giustina *et al.*, 2008). Similarly, IGF-I measurements are rarely of value in isolation (Mukherjee *et al.*, 2003). About one-third of patients with GHD (diagnosed by GH stimulation tests) has IGF-I levels in the normal sex and age-adjusted range (Giustina *et al.*, 2008). Particularly, IGF-I sensitivity as marker of GHD declines with age, despite adjustment according to age-specific IGF-I reference ranges (Thomas and Monson, 2009). Toogood *et al.* found that only 21% of elderly GHD patients had a serum IGF-I concentration lower than age-matched controls (Toogood *et al.*, 1996). Moreover, in elderly low IGF-I value can be also due to intercurrent illness, as poor nutritional status, hepatic disease, renal disease, hypothyroidis, and diabetes mellitus (Fusco *et al.*, 2012; Baum *et al.*, 1996).

In elderly, GHD diagnosis must be established biochemically by provocative test of GH secretory reserve (Giustina *et al.*, 2008), as during aging the hypothalamic stimulation signaling for pituitary secretion of GH declines, instead the releasable reserve of GH in the pituitary gland is not affected (Toogood, 2003). In fact, IGF-I values can decline in health elderly subject and in elderly GHD, but provocative test can distinguish between the elderly functional reduction of IGF-I value and the "true" elderly GHD affected subjects.

Although insulin tolerance test (ITT) has been considered the gold standard for GHD diagnosis by the most important scientific societies (Giustina *et al.*, 2008), it is associated with important disadvantages when used to diagnose GHD, particularly, in the elderly, resulting contraindicated in patients with epilepsy, ischemic heart disease or cerebrovascular disease (Clayton *et al.*, 2007). In fact, as the likelihood of cardiovascular or seizure-related contraindications to ITT increases with age, alternative tests of GH reserve have been applied and, particularly, GHRH plus arginine have been proposed as valuable alternative to ITT (Thomas and Monson, 2009). In fact, in the hypopituitary control and complications study (HypoCCS), it was demonstrated, between 1996 and 2005, a shift in clinical practice from isolated arginine, clonidine and L-dopa testing, towards an increased use of the GHRH–arginine test (Webb *et al.*, 2009). In fact, as the usefulness of isolated arginine is limited by marked variation in response according to body mass index (BMI) (Clayton *et al.*, 2007) and the usefulness of isolated GHRH is limited by marked variation in response according to individual age (Ghigo *et al.*, 1990), recent studies have proven that GHRH with arginine stimulates GH secretion more in elderly patients as in younger adults or children, showing a high degree of sensitivity of this test, for the diagnosis of GHD in older patients (Ghigo *et al.*, 1998). In fact, GHD elderly patients showed a lower mean GH pak after GHRH plus arginine stimulation, as compared to younger GHD patients (De Marinis *et al.*, 2002).

rhGH Treatment, Dosage, and Adverse Events

The rationale for GH replacement therapy in elderly GHD patients is rafforced by the beneficial effects on some clinical end-points. Over the recent years, guidelines on the use of rhGH as a substitution treatment in adult hypopituitarism have been issued by international (Growth hormone research society-GRS, Endocrine Society) and relevant national (National Institute of Clinical Excellence-UK, NICE) institutions. rhGH replacement therapy should be considered in elderly patients with severe GHD (GH peak after stimulation with RHGH plus arginin lower than 9 ng/mL), in the adequate clinical context: all comorbidities, with particularly interest in the screening of oncological disease, have to been checked and, moreover, all pituitary tropin deficits have to be corrected and balanced, before starting rhGH replacement treatment.

In the first clinical trials and studies, conducted on adult GHD patients, initial rhGH replacement doses were based on body weight or surface area, resulting in several side effects due to fluid retention, as carpel tunnel syndrome and arthralgia (Thomas and Monson, 2009; Fernholm *et al.*, 2000). Interestly, in these studies, frequency of reported adverse events was superimposable between elderly and young GHD patients, although youngers were more frequently affected by fluid retention-related adverse events (as headache, oedema, and arthralgias) and elderly patients by increased occurrence of disorders of glucose metabolism, cerebrovascular events and neoplasms (Feldt-Rasmussen *et al.*, 2004).

Moreover, over the time, according to the current clinical practice, rhGH treatment is started at low dosage (0.1–0.2 mg daily) and titrated on an individual basis, with the aim to reach and maintaining IGF-I levels within the normal age- and sex-related range or clinical improvements, also according to quality of life. Consequently, rhGH dosage results significantly lower in elderly GHD patients as compared youngers, without difference in absolute change in IGF-I levels (Clayton *et al.*, 2007; Monson and Jönsson, 2003).

This procedure results in good efficacy and excellent tolerability (Clayton *et al.*, 2005), avoiding overtreatment and related adverse events (Thomas and Monson, 2009).

Acromegaly in Elderly

Acromegaly is a rare condition characterized by an excess of autonomous GH secretion, in most of cases due to the presence of a pituitary GH secreting adenoma. Although biochemically active acromegaly disease is characterized by an increased mortality as compared to general population (Rajasoorya *et al.*, 1994), most recent evidences have proved that life expectancy is now close to that of the general population, in cured or biochemically controlled disease, probably for the better management of the GH/IGF-I excess and related comorbidities (Maione *et al.*, 2017; Capatina and Wass, 2015). However, until now, in acromegaly, prevalent cause of death is neoplastic disease, reported in around 10% of patients, with a standardized incidence ratio of 1.34 (95% CI: 0.94–1.87) in men and 1.24 (0.77–1.73) in women (Maione *et al.*, 2017).

Epidemiology, Clinical, and Diagnosis of Acromegaly in Elderly

Around 3% of acromegaly patients are considered elderly (Kazunori *et al.*, 2008). It is widely proposed that in younger patients, acromegaly is characterized a more aggressive biological behavior (Fusco *et al.*, 2008; Bianchi *et al.*, 2013), similarly as occurred in all types of pituitary adenomas (Chiloiro *et al.*, 2015).

Elderly acromegaly patients typically present headache as onset symptom (Puchner *et al.*, 1995): in fact in these patients, typical acromegaly clinical features may be milder than in younger patients (Turner and Wass, 1997), also according to the lower GH/IGF-I value reported in elderly acromegaly as compared to youngers (Dupuy *et al.*, 2009). Instead, systemic complications, as diabetes mellitus, hypertension and impaired cognitive functions are more frequent in older acromegaly patients (Puchner *et al.*, 1995; Minniti *et al.*, 2005; Hatipoglu *et al.*, 2015).

Diagnosis of acromegaly in elderly patients can represent a challenge: age-corrected insulin-like growth factor I (IGF-I) ranges must be considered because of the physiological decline of IGF-I secretion (Minniti *et al.*, 2005). Since overt diabetes mellitus may limit the feasibility or reliability of the oral glucose-tolerance test for GH suppression, alternative testing may be indicated.

Moreover, an accurate morphological evaluation, trough contrasted magnetic resonance, of the pituitary region and the pituitary adenoma is mandatory, in cases of biochemically diagnosis of acromegaly.

Treatment of Acromegaly in the Elderly

Neurosurgery

Neurosurgery is considered by most authors as the first therapeutic choice in acromegaly, as biochemical remission of acromegaly can be reached in around 50%–60% of patients (Giustina *et al.*, 2000).

Also, in elderly patients transsphenoidal pituitary neurosurgery (TSS) is considered safe and can be proposed, after a careful evaluation of anaesthesiological risk.

According to the high incidence of hypertension, left ventricular hypertrophy and glucose metabolism abnormalities, anaesthesiological risk in elderly acromegaly patients resulted increased in more than 70% of patients (Minniti *et al.*, 2005). Moreover, no mortality or major complications were observed in recent series of elderly patients operated on by TSS (Minniti *et al.*, 2005), suggesting that in a medical center devoted to pituitary disease, the adequate presurgery evaluation and management of elderly acromegaly comorbidities and the surgeon's experience can allow to achieve a high percentage of cured acromegaly, without major complications (Barker *et al.*, 2003). Actually, GH/IGF-I normalization was achieved in 68% of patients underwent pituitary neurosurgery (Minniti *et al.*, 2005), with a superimposable percentage of controlled disease 1 year after treatment (Dupuy *et al.*, 2009). Moreover, successful TSS was accompanied by a significant improvement in cardiovascular and metabolic abnormalities (Minniti *et al.*, 2005). Consequently, current data on TSS in elderly acromegalics are encouraging.

Medical therapy for acromegaly

Elderly acromegaly patients show an especially sensitive to somatostatin analogues treatments (SSA) (van der Lely *et al.*, 1992), that might be due to the less aggressive biochemical characteristics of GH-secreting adenomas in this age group. SSA are able to normalize GH/IGF-I levels in 60%–70% of acromegalic patients with acceptable side effects and a significant improvement in disease-related cardiovascular abnormalities (Freda, 2003). Moreover, pharmacological treatment with SSA can be proposed in different ways. Although preoperative SSA treatment has not show to improve significantly surgical outcome, it can reduce pneumological and anesthetic complications (Ben-Shlomo and Melmed, 2003), particularly in elderly.

Other medical options in acromegaly are the dopamine-agonist (bromocriptine or cabergoline), the GH receptor antagonist (pegvisomant) and the pan-ligands for the somatostatin receptor (pasireotide).

Dopamine agonist treatment achieves normalization of GH/IGF-I level in only 30% of patients (Freda, 2003) and it is proposed particularly in cases of GH-/PRL-secreting adenomas. It is proposed particularly in cases of GH-/PRL-secreting adenomas, although with dosage higher than those commonly employed for prolactinomas. Consequently, in elderly patients, according to the potential valvulopathy risk, a careful cardiological with echocardiography should be scheduled at least annually.

To our knowledge, there are no age-related peculiarities in the use of pegvisomant and pasireotide, which are currently reserved for otherwise uncontrolled acromegaly.

At least, in elderly patients, the efficacy of conventional radiotherapy is hampered by the interval required to achieve biochemical remission: GH/IGF-I levels reach normal values in about 50% of acromegalics over a period of 10–15 years (Barrande *et al.*, 2000). Thus, radiotherapy remains an important option in elderly patients unresponsive to medical treatment.

Conclusion

During the human aging, GH/IGF-I axis gradually and physiologically declines its activity, according to the reduction of hypothalamic signaling. This mechanism, also called somatopause, plays a protective role for age-related disease, particularly cardiovascular, metabolic, and neoplastic. Symptoms of body change during aging overlap with the symptoms of the elderly GHD, that has to be investigated in the correct clinical context. Also, elderly patients affected by GHD may benefit clinically for replacement treatment with rhGH, that has to be prescribed in medical center with a large experience on pituitary disease and that required a careful management at prescription and during follow-up. Similarly to GHD, also GH excess condition, as acromegaly, is associated with an increased morbidity and mortality, requiring a proper diagnosis and management.

See also: Growth Hormone (GH). Hormonal Circadian Rhythms and Sleep in Aging. Hypothalamic Disease. Introduction to Endocrine Toxicology and Endocrine Disruption

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Abnormalities in Water Homeostasis in the Elderly☆

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Introduction

Findley first proposed the presence of age-related dysfunction of the hypothalamic–neurohypophyseal–renal axis >50 years ago (Findley, 1949). Physiologic processes that occur with aging are associated with changes in both water metabolism and sodium balance, leading to alterations in plasma osmolality and body fluid compartment volumes (**Table 1**). As a result of these changes, the elderly have increased frequency and severity of hypo- and hyperosmolality, manifested by hypo- and hypernatremia, as well as hypo- and hypervolemia. These abnormalities have clinically significant implications with regard to cognition, gait instability, osteoporosis, bone fractures, and overall morbidity and mortality.

Clinical Overview of Hyponatremia

Hyponatremia is the most common electrolyte disorder encountered in clinical practice (Janicic and Verbalis, 2003). This hyponatremia becomes clinically significant when accompanied by plasma hypoosmolality. When hyponatremia is defined as a serum [Na^+] of <135 mmol/L, the inpatient incidence is reported to be between 15% and 22%. Studies that define hyponatremia as a serum [Na^+] < 130 mmol/L demonstrate a lower, but still significant, incidence of 1%–4% (Verbalis, 2001). Several excellent observational studies examining this issue have been published, but the literature has lacked a uniform threshold for defining hyponatremia. One such study suggests that the incidence of hyponatremia in elderly populations has been reported to vary widely between 0.2% and 29.8%, depending on the criteria used to define both "hyponatremia" and "elderly" (Hawkins, 2003). While the true incidence of hyponatremia in the elderly is difficult to define given differing diagnostic criteria across studies, it is clear that the problem is not uncommon.

The syndrome of inappropriate antidiuretic hormone secretion (SIADH) is a common cause of hyponatremia in elderly populations. SIADH was first described by Bartter and Schwartz in 1957 (Schwartz *et al.*, 2001). The defining criteria presented in this landmark publication remain valid and clinically relevant today (Verbalis, 2001). SIADH can be caused by many types of diseases and injuries common in the elderly, including central nervous system injury and degeneration, pulmonary diseases, paraneoplastic malignancy, nausea, and pain. An idiopathic form of SIADH associated with aging is also quite common. Several studies have demonstrated that SIADH accounts for approximately half (50%–58.7%) of the hyponatremia observed in some elderly populations (Miller *et al.*, 1996; Anpalahan, 2001; Hirshberg and Ben-Yehuda, 1997), and one-quarter to one-half (26%–60%) of elderly patients with SIADH appear to have the idiopathic form of this disorder (Miller et al., 1996; Anpalahan, 2001; Hirshberg and Ben-Yehuda, 1997).

Clinical Implications of Hyponatremia

Recent data has confirmed that hyponatremia in the elderly population has multiple clinically significant outcomes with regard to neurocognitive effects and falls (Munsch *et al.*, 2003), hospital readmission and need for long-term care (Wald *et al.*, 2010),

Table 1 Summary of the multiple factors that impair maintenance of normal body fluid homeostasis with aging

1. Effects due to altered body composition
   a. Reduced plasma volume
   b. Increased osmolal "flux"
2. Effects due to altered kidney function
   a. Impaired renal free water excretion
   b. Decreased urine concentrating ability
3. Effects due to altered brain function
   a. Decreased thirst perception
   b. Increased AVP secretion

From Cowen, L.E., Hodak, S.P., and Verbalis, J.G. (2013). Age-associated abnormalities of water homeostasis. *Endocrinology and Metabolism Clinics of North America* **42**(2), 349–370.

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incidence of bone fractures (Gankam *et al.*, 2008), and osteoporosis (Verbalis *et al.*, 2010). Hyponatremia is a strong independent predictor of mortality, reported to be as high as 60% in some series (Fried and Palevsky, 1997; Terzian *et al.*, 1994).

Terzian *et al.*, studied the occurrence of admission hyponatremia and its association with in-hospital mortality in a geriatric patient cohort over age 65 admitted to a community teaching hospital (Terzian *et al.*, 1994). Serum $[Na^+] < 130$ mmol/L within 24 h of admission was the cutoff for inclusion. The relative risk of in-hospital mortality associated with admission hyponatremia was significantly increased at 2.0. There was evidence of a linear association of in-hospital mortality with sodium level, with mortality increasing as sodium levels decreased. This study therefore suggested that the degree of hyponatremia may be an important prognostic indicator in older individuals (Terzian *et al.*, 1994).

In addition to an increase in inpatient mortality, studies have demonstrated an increase in outpatient mortality associated with hyponatremia as well. Hoorn *et al.*, examined the effect of hyponatremia on all-cause mortality within the framework of the Rotterdam Study, an ongoing prospective cohort study among outpatients older than 55 living in the Netherlands (Hoorn *et al.*, 2011a). All-cause mortality was higher in subjects with hyponatremia than those without hyponatremia (51.6% vs. 32.6%, $P < 0.001$) (Hoorn *et al.*, 2011a). Consequently, it should be clear that hyponatremia should no longer be considered a benign condition, particularly in the elderly population.

Renneboog *et al.*, conducted a case–control study to determine the functional significance of so-called "asymptomatic" mild chronic hyponatremia on falls and cognitive impairment (Renneboog *et al.*, 2006). In this study, 122 Belgian patients with $[Na^+]$ between 115 and 132 mmol/L, all judged to be asymptomatic at the time of emergency room presentation were compared with 244 age-, sex-, and disease-matched controls presenting during the same time period. Twenty-one percent of the hyponatremic patients presented because of a recent fall as compared to only 5% of controls, resulting in an adjusted odds ratio of 67 for hyponatremia. This study clearly demonstrated an increased incidence of falls in hyponatremic patients.

Renneboog *et al.*, also evaluated the clinical implications of asymptomatic hyponatremia on gait instability and attention deficits (Renneboog *et al.*, 2006). A subset of 12 patients with hyponatremia secondary to SIADH with $[Na^+]$ in the range of 124–130 mmol/L demonstrated significant gait instability that normalized with correction of hyponatremia. The patients were asked to walk a tandem gait on a computerized platform that measured the center of gravity on the ball of their foot. Deviation from the straight line was measured as "Total Traveled Way" (TTW). The hyponatremic patients wandered markedly off the tandem gait line in terms of their center of balance, but corrected significantly once their hyponatremia was corrected (**Fig. 1**). When performing a series of attention tests, patients in the hyponatremic subset (mean $[Na^+] = 128$ mmol/L) had prolonged response latencies compared with a group of patients after acute alcohol intake (blood alcohol concentration 0.6 g/L). These impairments suggested a global decrease of attentional capabilities that is more pronounced in hyponatremic patients (Renneboog *et al.*, 2006), which may contribute to gait instability and falls in the elderly.

Several international studies have demonstrated increased fracture rates in hyponatremic patients (Hoorn *et al.*, 2011b). The work by Kengne *et al.* investigated the association between bone fracture, falls, and clinically asymptomatic hyponatremia in an ambulatory elderly population older than 65 compared with controls (Gankam *et al.*, 2008). The prevalence of hyponatremia ($[Na^+] < 135$ mmol/L) was 13.06% and 3.9% in patients with bone fractures and control patients respectively. The adjusted odds ratio for bone fracture associated with hyponatremia was 4.16. The risk of bone fracture attributable to hyponatremia was 9.2% in all patients with bone fractures and attributable to the hyponatremia alone is up to 72.5% of cases.

Verbalis *et al.* explored the effect of hyponatremia and bone quality and demonstrated a link between chronic hyponatremia and metabolic bone loss (Verbalis *et al.*, 2010). In this study, Verbalis *et al.* used a rat model of SIADH to study the effects of hyponatremia on bone at the level of resorption and mineralization. Dual-energy X-ray absorptiometry (DXA) analysis of excised femurs established that hyponatremia for 3 months significantly reduced bone mineral density by approximately 30% compared with normonatremic control rats. The most striking histologic finding was an increase in the number of osteoclast numbers per bone area and osteoclast surface per bone surface in the hyponatremic rats. This study demonstrated that chronic hyponatremia causes a significant reduction of bone mass at the cellular level. Subsequent epidemiological analysis of 2.9 million patient records showed that chronic hyponatremia was associated with odds ratios of 3.99 for osteoporosis and 3.05 for fractures, thus confirming the translational significant of the animal studies (Usala *et al.*, 2015). Hyponatremia-induced bone resorption and osteoporosis are unique in that they represent attempts of the body to preserve sodium homeostasis at the expense of bone structural integrity (Barsony *et al.*, 2012).

Clinical Overview of Hypernatremia

Hypernatremia necessarily reflects an increase in plasma osmolality. Cross-sectional studies of both hospitalized elderly patients and elderly residents of long-term care facilities show incidences of hypernatremia that vary between 0.3% and 8.9% (Hawkins, 2003; Fried and Palevsky, 1997). While hypernatremia is a common presenting diagnosis in the elderly, 60%–80% of hypernatremia in elderly populations occurs after hospital admission (Fried and Palevsky, 1997). Similarly, up to 30% of elderly nursing home patients experience hypernatremia following hospital admission (Beck, 1998).

The clinical implications of hypernatremia in hospitalized elderly are significant. In a retrospective study, Snyder reviewed outcomes in 162 hypernatremic elderly patients, representing 1.1% of all elderly patients admitted for acute hospital care to a community teaching hospital (Snyder *et al.*, 1987). All patients were at least 60 years of age with a serum $[Na^+] > 148$ mmol/L. All-cause mortality in the hypernatremic elderly patients was 42%, which was seven times greater than age-matched

normonatremic patients. Furthermore, 38% of the hypernatremic patients who survived to discharge had a significantly decreased ability to provide self-care (Snyder *et al.*, 1987).

As hypernatremia develops, normal physiologic responses preserve water homeostasis through osmotically stimulated secretion of AVP to promote renal water conservation along with accompanying potent stimulation of thirst to restore body water deficits (Wong and Verbalis, 2002). Although renal water conservation can forestall the development of severe hyperosmolality, only appropriate stimulation of thirst with subsequent increase in water ingestion can replace body fluid deficits thereby reversing hyperosmolality (Palevsky, 1998). This entire physiologic response is impaired with aging: elderly patients have a decreased thirst perception (Phillips *et al.*, 1985), and blunted ability to maximally concentrate their urine in response to please AVP (Beck, 1998). Thus, the elderly have a greatly increased susceptibility to a variety of situations that can induce hypernatremia and hyperosmolality, with the attendant increases in morbidity and mortality that accompany this disorder (Snyder *et al.*, 1987; Beck, 2000).

Mechanisms Involved With Disturbances of Water Metabolism in the Elderly

Aging causes distinct changes that impact normal water homeostasis at several discreet locations along the neuro-renal axis responsible for maintaining normal water balance. The net result of these changes is that the elderly experience a loss of homeostatic reserve, and increased susceptibility to pathologic and iatrogenic causes of disturbed water homeostasis.

The sensation of thirst and the appropriate drinking response to thirst is compromised with aging. It is likely, as Phillips *et al.*, have suggested, that part of this defect is through loss of normal neural pathways that convey sensory input to the higher cortical

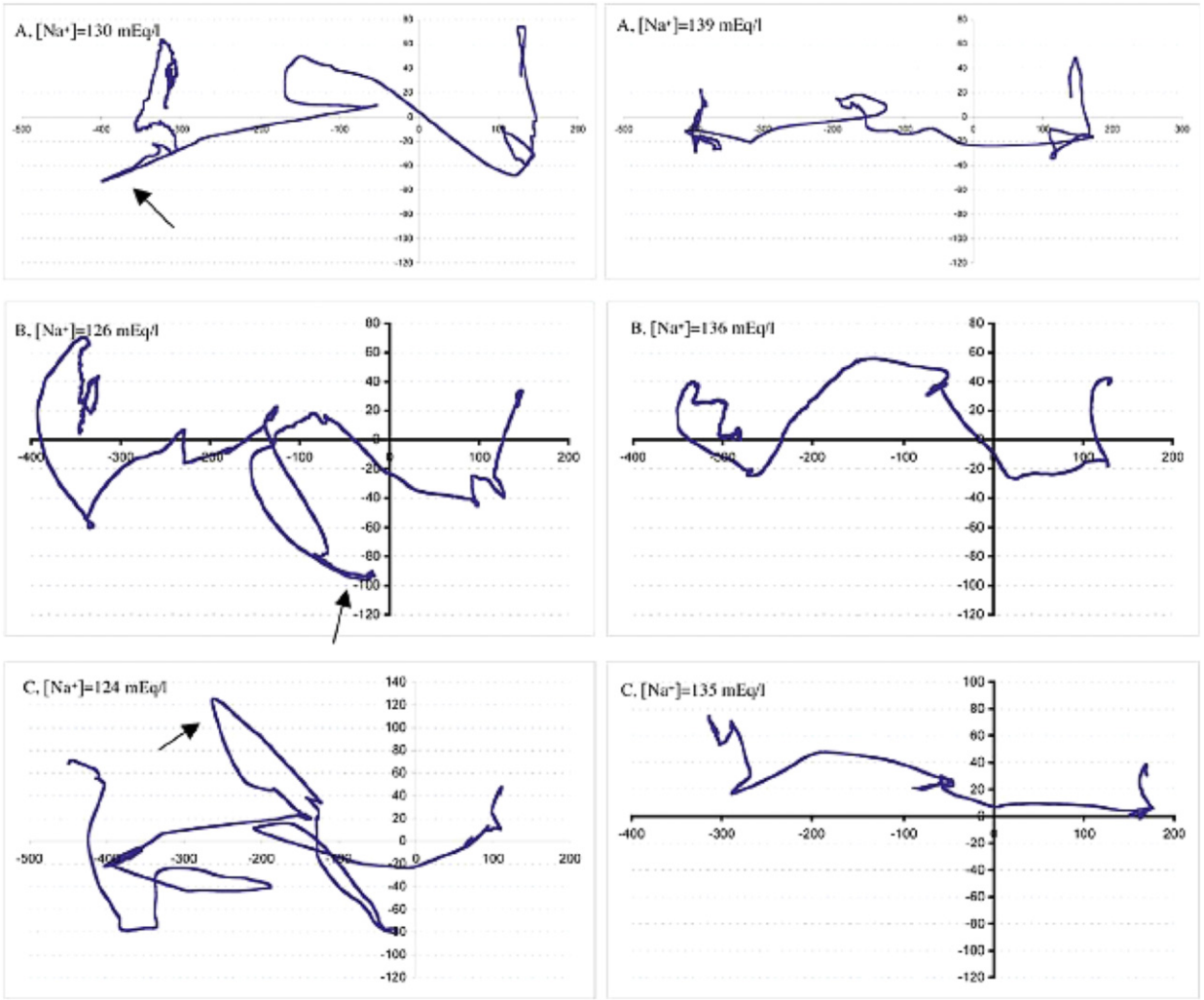

Fig. 1 Evolution of the "total traveled way" (TTW) by the center of pressure in the dynamic test to walk on the platform 3 stereotyped steps "in tandem," eyes open, in three patients (A, B, C) with mild asymptomatic hyponatremia and after correction. Patients are walking from right to left. Irregular paths of the center of pressure observed in the hyponatremia condition (*arrows*). From Renneboog, B., Musch, W., Vandemergel, X., Manto, M.U., and Decaux, G. (2006). Mild chronic hyponatremia is associated with falls, unsteadiness, and attention deficits. *The American Journal of Medicine* **119**(1), 71.

centers where thirst is perceived, and from which the thirst-activated drinking response emanates (Phillips *et al.*, 1993). Other studies have demonstrated that there is a change in baroreceptor-mediated control of thirst in the elderly. Stachenfeld's studies have clearly demonstrated that plasma volume expansion in elderly subjects does not generate the normal suppression of thirst found in the young (Stachenfeld *et al.*, 1997). A clear age-related deficit in the thirst response appears to arise from decreased sensitivity to osmolar stimulation. Studies by Mack *et al.*, suggest that this defect is due to a higher osmotic set point leading to a blunted thirst response in the elderly (Mack *et al.*, 1994). Most importantly, the loss of an appropriate thirst response compromises the critical compensatory mechanisms responsible for the drive to replace lost body fluid, and the only true physiologic means of correcting a hyperosmolar state.

Impaired GFR and resultant loss of maximal urinary concentrating ability appear a common, if not certain, consequence of aging (Lindeman, 1993; Lindeman *et al.*, 1985). The consequence of such a defect is clear: decreased GFR causes an inability to maximally conserve free water and favors development of inappropriate body water deficits. This can lead to clinically relevant hyperosmolality, and is also a likely cause of the observed increase in the frequency of hypernatremia in the elderly.

Paradoxically, a decrement in maximal water excretion also occurs in the elderly (Faull *et al.*, 1993; Clark *et al.*, 1994). The elderly are at a higher risk of developing diseases such as congestive heart failure that are associated with volume overload. So too, are the elderly at risk for inadvertent iatrogenic overhydration from intravenous and enteral hydration therapy. The inability to appropriately excrete an excessive fluid load would predispose to hypoosmolality in elderly individuals.

The secretion and end organ effects of AVP account for two of the most interesting, and perhaps least well understood aspects of water regulation in the elderly. Although a few exceptions exist, most agree that basal AVP secretion is at least maintained, and more likely increased, with normal aging (Wong and Verbalis, 2002). Further, the AVP secretory response, that is, the osmoreceptor sensitivity to osmolar stimuli, is also increased in normal aging (Davies *et al.*, 1995). Thus, AVP secretion represents one of the few endocrine stimulatory responses that increases rather than decreases with age. It is likely that enhanced secretion of AVP in the elderly and inability to appropriately suppress AVP secretion during fluid intake (Phillips *et al.*, 1993), combined with an intrinsic inability to maximally excrete free water (Faull *et al.*, 1993; Clark *et al.*, 1994), increase the likelihood that idiopathic SIADH will occur in this group of patients.

In conclusion, much has been learned in six decades since Findley's original reflections about the effects of aging on water homeostasis. Since then, clearly demonstrated deficits in renal function, thirst and responses to osmotic and volume stimulation have been repeatedly demonstrated in this population. The lessons learned serve to emphasize the fragile nature of water balance characteristic of aging. The elderly are at increased risk for disturbances of water homeostasis due to both intrinsic disease and iatrogenic causes. Recent studies have shown that these disturbances have real-life clinical implications in terms of neurocognitive effects, falls, hospital readmission and need for long-term care, and incidence of osteoporosis and bone fractures. It is therefore incumbent upon all those who care for the elderly to realize the more limited nature of the compensatory and regulatory mechanisms that occur with aging and to incorporate this understanding into the diagnoses and clinical interventions that are made in the care of this vulnerable group of patients.

See also: Aldosterone; Action and Function. Cushing Syndrome; Screening and Differential Diagnosis. Diuretics. Hypertension and the Renin–Angiotensin–Aldosterone System. Hyporeninemic Hypoaldosteronism. Regulation of Potassium Homeostasis

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Body Weight, Body Composition, and Aging☆

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Glossary

Body Mass Index (BMI) A practical measure of an individual's weight in relation to height, and is used to define overweight and obesity.

Fat-free Mass (FFM) Encompasses all of the body's nonfat tissues including the skeleton, water, muscle, connective tissue, and organ tissues.

Intramuscular Fat (IMAT) A fat depot found beneath the fascia and within the muscle.

Osteoporosis A condition characterized by low bone mass (density) and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture.

Sarcopenia A condition characterized by an involuntary age-related decline in lean body mass, primarily due to the loss of skeletal muscle, that affects functional capacity and strength of older adults.

Visceral fat (intra-abdominal fat) A portion of the internal fat in the abdominal cavity lining the intestinal tract

Introduction

One of the fastest growing segments of the population is individuals >65 years of age. This age group which currently accounts for $\sim$15% of the population is expected to grow to account for more than 20% of the population by 2030. Increased and changing distributions of body fat and loss of bone mineral density (BMD) and muscle mass are defining characteristics of the aging process. These changes in body composition occur as a result of normal aging, have a detrimental impact on health status, and substantial economic consequences on the health care system. Obesity is associated with an increased prevalence of co-morbidities including cardiovascular disease, type 2 diabetes mellitus, hypertension, dyslipidemia, and other metabolic diseases. The decline in skeletal muscle mass is associated with weakness, functional disability, frailty and morbidity, whereas the decrease in BMD increases the risk of bone fractures and ultimately results in high rates of disability, morbidity and mortality in the elderly. This chapter focuses first on the classification and prevalence of overweight and obesity. This is followed by a discussion of the changes that occur during the aging process with specific emphasis on body weight, fat mass and fat-free mass and its constituents of skeletal muscle mass, total body water and bone, and intramuscular fat (IMAT). These changes in body composition are also described in context with metabolic disease states.

Classification of Overweight and Obesity

Body Mass Index (BMI), a practical measure of an individual's weight in relation to height, is used to define overweight and obesity. A BMI between 25 and 29 kg m^{-2} defines overweight in adults, whereas obesity is defined as a BMI$\geq$30 kg m^{-2}. Obesity can be further classified into Class 1 (30.0–34.9 kg m^{-2}), Class 2 (35.0–39.9 kg m^{-2}), and Class 3 ($>$40 kg m^{-2}). These criteria for defining overweight and obesity are based on epidemiologic evidence that shows a strong association between BMI$>$25 kg m^{-2} and an increased incidence of cardiovascular disease, type 2 diabetes mellitus, hypertension, and dyslipidemia that affect mortality and morbidity. Although the determination of BMI is a simple index of obesity to obtain, it does not differentiate between fat mass and lean muscle mass. Therefore, it can overestimate body fat in persons who are very muscular and underestimate body fat in persons who have excess fat and reduced muscle mass but normal body weight. Thus, other methods such as dual-energy X-ray absorptiometry and bioelectric impedance are necessary to more accurately determine body composition and are frequently used in research settings.

Prevalence of Overweight and Obesity

The National Center for Health Statistics has examined the prevalence and trends of overweight U.S. adults from 1960 to 2000 via the National Health Examination Survey (NHES I) and National Health and Nutrition Examination Surveys (NHANES I, II, III, continuous). The prevalence of overweight and obesity increased significantly over the 40-year period from 43% in 1960–62 to 68.5% in 2011–12. Most of this increase is attributable to a dramatic rise in the prevalence of obesity (BMI$>$30 kg m^{-2}) that

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Table 1 Estimated data based on measured height and weight of a sample of the civilian noninstitutionalized population

		Males BMI (kg m^{-2})						*Females BMI (kg m^{-2})*					
		18.5–24.9	*≥25.0*	*≥30.0*	*30.0–34.9*	*35.0–39.9*	*≥40.0*	*18.5–24.9*	*≥25.0*	*≥30.0*	*30.0–34.9*	*35.0–39.9*	*≥40.0*
Age (years)	20–34	37.5	60.9	28.9	19.6	6.3	3.0	40.8	55.2	30.0	14.4	7.9	7.7
	35–44	21.0	78.9	38.1	22.7	9.7	5.6	35.2	62.4	36.0	19.3	8.4	8.3
	45–54	20.0	79.3	38.1	24.4	8.2	[a]	27.3	70.5	38.3	19.0	10.7	8.6
	55–64	21.9	77.4	38.1	25.6	7.1	[a]	23.8	75.1	42.9	22.7	11.1	9.1
	65–75	22.4	76.9	36.4	21.9	10.8	[a]	23.5	73.8	44.2	21.1	12.3	10.7
	75+	28.2	70.4	27.4	21.1	[a]	[a]	35.3	62.4	29.8	19.1	6.6	[a]

[a]No data included because estimates are unreliable.

Percent of population for each age and BMI category presented. Percents do not sum to 100 because the percentage of persons with BMI less than 18.5 is not shown. Obesity is a subset of the percentage with overweight.

Chart adapted from: National Center for Health Statistics. Health, United States, 2014: With Special Feature on Adults Aged 55–64. Hyattsville, MD. 2015.

remained stable at ~15% for the first three survey periods (1960–80), and then rose dramatically to 30.5% in 1999–2000. The increased prevalence of overweight and obesity in adults during this time period was evident in both genders, across all races and ethnicities, and across all age groups [Table 1]. The most recent survey (2011–12) estimates that 34.9% of the population is obese with even higher rates among Black (47.8%) and Latino (42.5%) adults.

Change in Body Weight and Fat Mass with Aging

In healthy people, body weight increases gradually from early adulthood until the fifth to sixth decade of life and then remains stable until age 65–70 years. During the period of weight gain, body weight increase occurs at a rate of 0.39–0.45 kg year^{-1} for women and 0.36–0.41 kg year^{-1} for men, irrespective of race. The slightly higher rate in women compared to men suggests that the transition from pre- to postmenopausal status affects the amount of weight gain. After age 60 years, body weight decreases slowly at a rate between 0.05 and 0.36 kg year^{-1} depending on race and gender. The rate of decrease is usually higher in women compared to men, regardless of ethnicity. Moreover, the rate of body weight decrease is highest in African-American women and lowest in African-American men. The variation in body weight regulation suggests that a nonlinear pattern of weight change occurs through the aging process.

The pattern of weight gain is generally consistent between cross-sectional and longitudinal studies and is characterized by a greater increase of fat than lean mass accounting for the increase in body weight. The relationship between age and body fat is curvilinear, with the greatest increase in body fat occurring in middle-aged persons and smaller increases in both the young and the elderly. That is, there is a pattern of increasing fat mass with age until 50–69 years, followed by a slow decline in fat mass in older age groups (>70 years). However, irrespective of the age group, 7–31% of the variation in the increase in fat mass (absolute or relative) in the adult population is accounted for by age.

The increase in weight with age is accompanied by an age-related redistribution of fat away from the subcutaneous fat depot beneath the skin, towards other more harmful locations such as in and around the abdominal organs and underneath the muscles fascia in and between muscle fibers. Sedentary women in their fifth decade of life have an average 1.5- to 3-fold higher visceral and subcutaneous abdominal fat area compared to their younger counterparts and athletes of similar age [Fig. 1]. Similarly, older women athletes in the fifth and sixth decade of life have a two- to threefold higher visceral fat area compared to younger athletes despite similar BMI and percent body fat [Fig. 1]. Therefore, there is an accumulation of fat in the abdominal region during the aging process irrespective of physical activity status. However, maintaining an active lifestyle partially negates the consequences of aging by dramatically reducing the amount of abdominal fat deposition. The ability to alter the accumulation of fat in the abdominal region is of clinical significance when one considers that intra-abdominal fat areas >100–110 cm^2 increase the risk for developing cardiovascular disease and other metabolic diseases. The age associated increase in visceral adiposity may contribute to the increase in triglyceride and total cholesterol concentrations, and glucose intolerance with age.

An increase in the infiltration of fat around and within skeletal muscle (IMAT) also occurs with aging. Similar to visceral adipose tissue it has been implied that IMAT increases with aging with estimates of increases in whole body IMAT ranging from 9 to 70 gyear^{-1}. In women aged ~18–70 years with a wide range of body fat and fitness, intramuscular fat increased with age and mid-thigh muscle area declined [Fig. 2]. Increases in IMAT adversely influences glucose and lipoprotein metabolism as well as muscle strength. CT-scans document a twofold increase in mid-thigh subcutaneous fat between pre- and postmenopausal sedentary women. Moreover, there is also a twofold increase in low-density lean tissue, a marker of intra-muscular fat. Approximately 27–33% of the variation in the increase in mid-thigh subcutaneous fat and low-density lean tissue areas in the mid-thigh region is accounted for by age. A 5 year longitudinal study of older adults age 70–79 found an increase in IMAT even after accounting for race, weight changes, and activity levels. However, some more recent work suggests that the increase in IMAT with

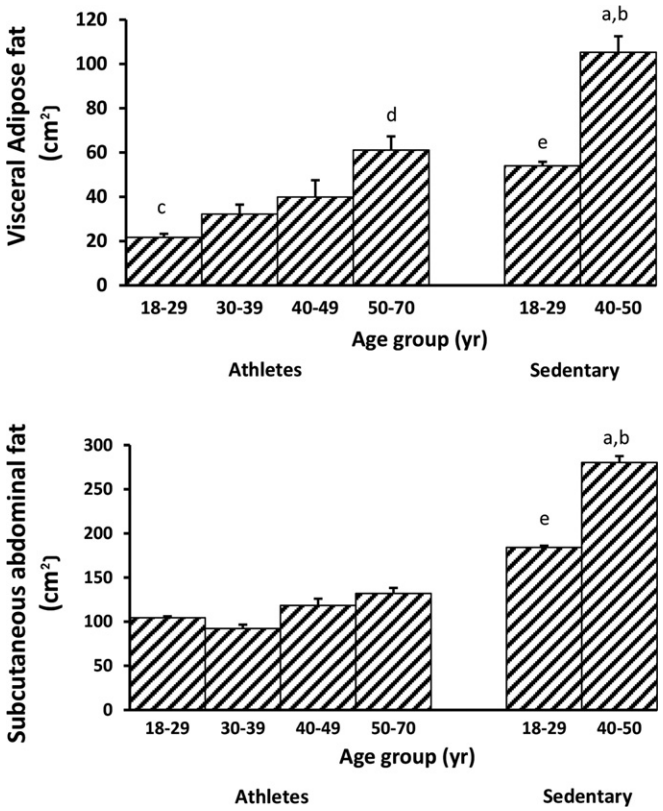

Fig. 1 Values of the visceral abdominal fat and subcutaneous abdominal fat are means ±SE of nos. of athletes and controls. It was determined by computed tomography. Significant differences ($P < 0.05$): [a] Sedentary Group 40–50 vs. Athletes Group 40–49; [b] Sedentary Group 40–50 vs. Sedentary Group 18–29; [c] Athletes Group 18–29 vs. Athletes Group 30–39; [d] Athletes Group 50–70 vs. Athletes Group 18–29; [e] Sedentary Group 18–29 vs. Athletes Group18–29.

aging may be at least partially due to increased inactivity often associated with aging. Longitudinal identical twin studies demonstrate that after 32 years of differing activity habits, IMAT was 54% higher in the inactive twin.

Higher amounts of IMAT may contribute to decreased muscle strength and metabolic dysfunction with aging. Multiple studies have reported a relationship between increased amounts of IMAT and decreased strength in the thigh and calf muscles of older adults. IMAT also appears to be a predictor of metabolic impairments. High levels of IMAT are strong predictors of fasting glucose and insulin impairments even when accounting for BMI. The contribution of intra-muscular fat to the metabolic dysfunction associated with the insulin resistance syndrome may help explain racial difference in glucose metabolism.

Change in FFM and It's Constituents with Aging

Even as body weight and fat mass increase with age, a decrease in skeletal muscle mass occurs even in healthy and active older adults. While lean mass accounts for 50% of the total body weight in young adults, by the age of 75, it accounts for less than 25% of total body weight. The loss of muscle mass may begin as soon as the fourth decade of life and by the age of 70 may occur at a rate of 1% per year in healthy individuals. This rate of loss may be even greater in chronically ill or inactive older adults. The loss of muscle mass with aging is not limited to just skeletal muscle mass but also occurs in total fat-free mass (FFM) and its constituents (all lipid-free chemicals and tissues including water, muscle, bone connective tissue and internal organs). In adults between 18–94 years, the overall change in FFM is 1.5 kg per decade lower in men and 0.8 kg per decade lower in women with greater losses in >60 year olds (men: 1.7 kg per decade and women: 1.1 kg per decade) suggesting that losses in FFM are higher as adults reach a certain older age. In adults over the age of 70 years, the ~5-year decline in body mass is -0.34 kg year^{-1} in men and -0.45 kg year^{-1} in women with the loss of FFM reported as -0.48 and -0.14 kg year^{-1} in men and women, respectively. In an examination of vastus lateralis muscle, muscle size begins to decrease at approximately age 30, decreasing 10% by age 50. Thereafter, muscle area declines more precipitously, largely from decreased total number of muscle fibers. Physical activity levels may attenuate the decline in body mass and changes in body composition with aging. Although energy expenditure from physical activity is associated with greater FFM, this does not change the rate of loss of FFM in older adults. Moreover, adults approximately 70 years of age who lose appendicular and leg FFM over a 5-year period are over twofold more likely to report increased disability.

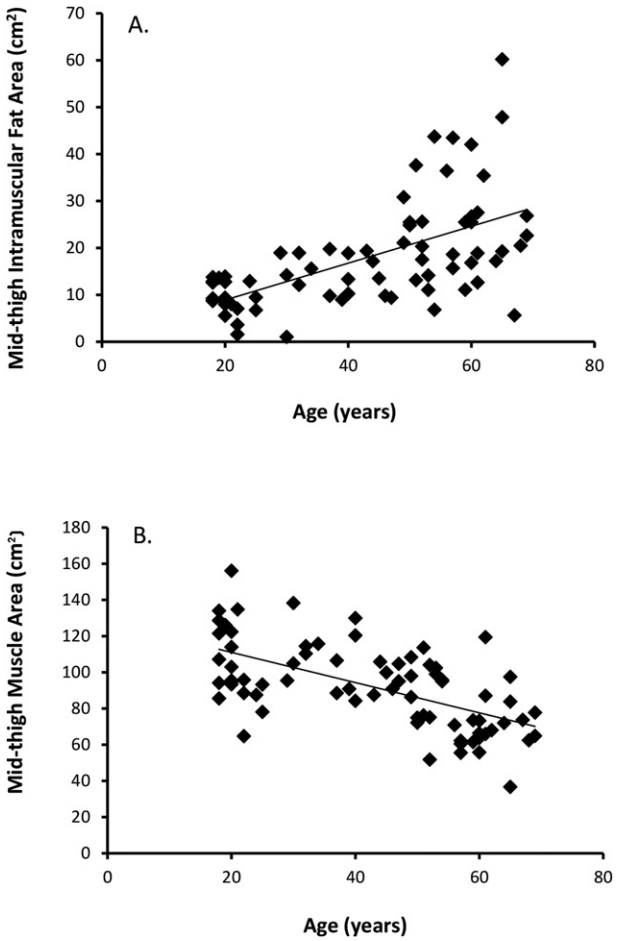

Fig. 2 Relationship between age and intramuscular fat area ($r=0.58$, $P<0.0001$) and muscle area ($r=-0.59$, $P<0.0001$).

The involuntary age-related decline in FFM and strength, primarily due to the loss of skeletal muscle, is termed "sarcopenia" and affects both the function and mobility of older adults. The prevalence of sarcopenia is estimated to be up to 33% of community dwelling elders. Sarcopenia is associated with a three- to fourfold increase in functional impairment, disabilities, and falls in the elderly and also increases the risk for hospital admissions, need for long term care, and death.

The loss of skeletal muscle mass is strongly associated with a loss of body water, because a large proportion of skeletal muscle (~75–80%) is water. Total body water accounts for approximately 80% of FFM at birth. In young adults, TBW comprises approximately 72% of FFM. Thus, a loss of body water occurs until maturity but remains relatively constant throughout adulthood and middle age. Total body water on average is lower in females than males. Losses of body water occur after age 70 in females and somewhat earlier in males with a nadir at this age (70–80 years). It is unclear whether the loss in TBW is due to a decrease in intracellular water, extracellular water, or a combination of the two, but most studies agree that TBW is decreased in elderly subjects and even more so in the very old. The decline in TBW suggests a change in the hydration of the fat-free compartment (increased with normal aging), although definitive conclusions are lacking.

In addition to the losses of FFM, skeletal muscle mass and TBW with age, the loss of bone mass is consistently documented. Peak bone mineral mass is reached at age 20–30 years followed by a progressive decline. Worldwide one out of three women and one out of five men will experience a fracture related to low bone mineral mass in their lifetime. By age 70, spinal and femoral neck bone mineral density (BMD) is diminished by approximately 20% and 25%, respectively. Furthermore, the rate of bone loss varies with site and may be greater in areas with more trabecular bone than those areas of predominantly compact bone. Total body mineral may decline at a slower rate than the decline observed in specific sites. In women, a more dramatic loss of bone mass occurs during menopause. The rate of BMD loss is greater among perimenopausal women compared to pre- and postmenopausal women and is site specific. Longitudinal studies estimate the rate of premenopausal BMD loss at 0.7–1.3% per year at the lumbar spine and 0.2–0.3% per year at the femoral neck. In contrast, the rate of BMD loss for perimenopausal women is 2–3% per year at the lumbar spine and 0.6–1% per year at the femoral neck. The estimated loss of BMD loss at the lumbar spine and the femoral neck is 1.3–1.5% per year and 1–1.4% per year in postmenopausal women, with the fastest rate of bone loss occurring immediately after menopause. The bone loss associated with menopause means women suffer a greater amount of bone loss at a younger age compared to men. Post-menopausal women experience a 2–5 time greater loss of bone resulting in a twofold decrease

in bone strength compared to men of the same age. Men experience a loss of BMD at two-thirds the rate of women in the spine, and one-half the rate of women in the femoral neck. The slower rate of bone loss result in a lower prevalence of osteoporosis in men (~4–6%) compared to women (~20%). The differences in bone loss and bone strength contribute to the increased risk of osteoporotic fracture risk in women compared to men. Overall women experience 61% of all osteoporotic fractures that occur. While women do experience a higher percentage of osteoporotic fractures, an age related increase in osteoporotic fractures is also evident in men who have an estimated 27% lifetime risk of experience a fracture related to low BMD.

Summary

The changes in body weight and composition associated with aging have major public health implications. The increase in total and abdominal obesity as well as increased IMAT within the muscle is associated with increased risk for developing cardiovascular disease, type 2 diabetes, hypertension, dyslipidemia and other metabolic diseases at an estimated cost of over $147 billion per year to the health care system. More importantly, approximately 112000 deaths a year may be attributable to obesity. Osteoporotic fractures generate substantial cost due to acute hospitalization and subsequent rehabilitation at an estimated cost of $17 billion per year with a projected increase to $22 billion per year as the population ages, and like obesity increase morbidity and mortality (e.g. 20% mortality rate during the first year after a hip fracture). However, structured programs that emphasize lifestyle behavior changes such as proper nutrition, increased regular physical activity, and intentional weight loss reduce the risk for developing metabolic and cardiovascular diseases. Moreover, caloric restriction to induce weight loss of 5–10%, aerobic exercise alone, and weight loss in combination with an aerobic exercise training program reduces the incidence of developing type 2 diabetes and improves the cardiovascular risk profiles in obese individuals. Aerobic and resistive exercise training programs >6 months duration maintain or increase muscle and bone mass and in effect may prevent the progression to sarcopenia and osteoporosis. With epidemiologic studies documenting the fastest growing segment of the population is individuals >65 years and an increase in the average life-span, structured behavioral programs that include nutrition and exercise should be recommended to alter the adverse body composition changes observed with aging.

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See also: Body Composition. Diabetes Mellitus; Diagnosis, and Treatment in the Elderly. Lifestyle and Nutrition. Lifestyle Diabetes Prevention. Type 2 Diabetes

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Lipid Disorders in the Elderly☆

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Glossary

Atherosclerotic cardiovascular disease A disorder affecting medium and large arteries in which subintimal deposits of lipids and connective tissue cause a decrease or an obstruction of blood flow through the arteries; includes coronary artery disease, peripheral arterial disease, carotid arterial disease, cerebrovascular disease, and abdominal aortic aneurysm

Coronary artery disease a disorder in which one or more coronary arteries are narrowed by atherosclerotic plaques or vascular spasm; includes stable and unstable angina pectoris, myocardial infarction, and sudden cardiac death

Dyslipidemia a lipid disorder consisting of hypercholesterolemia, increased serum low-density lipoprotein (LDL) cholesterol, decreased serum high-density lipoprotein (HDL) cholesterol, hypertriglyceridemia, or combinations there of. The incidence of atherosclerotic cardiovascular disease (ASCVD) is much higher in older men and women than in younger men and women, suggesting that an elevated serum low-density lipoprotein (LDL) cholesterol may contribute more to the burden of atherosclerotic vascular disease in the elderly than in the young.

Introduction

Cholesterol, cholesteryl esters, and triglycerides are fats or lipids. To circulate in blood, these lipids are combined with phospholipids and protein in particles called lipoproteins. Only three classes of lipoproteins–LDL, high-density liporotein (HDL), and very low-density lipoprotein (VLDL) –are found in the serum of fasting persons.

Following ingestion of a fat-containing meal, cholesterol and triglyceride are absorbed from the intestine and transported in chylomicrons via intestinal lacteals and the thoracic duct to the plasma compartment to produce the alimentary lipemia that can give rise to lactesence in postprandial plasma. Cholesterol and triglyceride of endogenous origin also enter the plasma compartment from the liver as VLDL. Both chylomicrons and VLDL are converted to somewhat smaller, denser, relatively cholesterol-enriched remnant lipoproteins following the selective extraction of triglyceride by the enzyme lipoprotein lipase. The remnant lipoproteins are removed from the plasma by hepatic chylomicron receptors that recognize surface apolipoprotein E and/or the structural apolipoprotein B100 or are further metabolized by hepatic triglyceride lipase to LDL and then removed by LDL receptors. HDL is composed principallyof apolipoproteins A-I and A-II and cholesterol and phospholipid. HDL is synthesized in both the intestine and the liver, and it acquires cholesterol by exchange from triglyceride-rich lipoproteins and in the process of facilitating reverse transport of cholesterol from the periphery to the liver, where it is excreted in the bile unchanged or after conversion to bile acids.

LDL is the major cholesterol-containing lipoprotein implicated in the development of atherosclerosis and is the primary target of therapeutic hypolipidemic interventions. LDL cholesterol may be elevated to undesirable levels because of excessive intake of dietary cholesterol and saturated fat (especially the latter), obesity (and increased hepatic synthesis), and/or genetic disorders that increase synthesis or impair LDL removal (e.g., LDL receptor deficiency in familiar hypercholesterolemia) or because of secondary causes such as hypothyroidism, nephrotic syndrome, renal failure, and biliary cirrhosis. LDL levels may be decreased in certain genetic disorders associated with hypocholesterolemia (e.g., familial hypobetalipoproteinemia, a so-called longevity syndrome) but are also seen in inflammatory syndromes commonly seen in frail persons, especially prevalent in elderly persons in hospitals or long-term care settings.

HDL is synthesized in both the liver and the intestine, and it exerts a protective effect on the development of atherosclerotic vascular disease. In addition to reducing arterial tissue cholesterol levels that contribute to such atherosclerosis by facilitating reverse cholesterol transport, HDL inhibits oxidation and aggregation of LDL in the arterial wall. Lower HDL cholesterol may be associated with genetic disorders, nutritional habits (high carbohydrate intake), obesity, hypertriglyceridemia, cigarette smoking, and lack of exercise, whereas HDL levels are increased with alcohol ingestion and estrogens or in familial hyperalphalipoproteinemia, another longevity syndrome.

☆*Change History:* WS Aronow August 2014. Abstract and keywords added. Further reading includes 9 references, 8 of which are new. The 2013 American College of Cardiology (ACC)/American Heart Association (AHA) lipid guidelines state that ASCVD includes CAD, stroke, transient ischemic attack, and PAD which includes carotid arterial disease and abdominal aortic aneurysm as well as PAD of the lower extremities. These guidelines use a Pooled Heart Equation to estimate the 10-year risk of developing ASCVD. This equation includes sex, age, race, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, diabetes mellitus, and smoking. High-dose statins and moderate-dose statins are discussed. New clinical trial data on use of statins for secondary prevention and for primary prevention are discussed. The 2013 American College of Cardiology/American Heart Association lipid guidelines are discussed extensively.

This article is an update of Wilbert S. Aronow, Lipid Disorders in the Elderly, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 178-181.

VLDL is a triglyceride-rich lipoprotein synthesized and secreted by the liver. Hypertriglyceridemia is associated with genetic disorders, obesity, diabetes mellitus, renal failure, heavy alcohol intake, and drugs such as estrogens.

The measurement of fasting levels of LDL cholesterol, HDL cholesterol, and triglycerides in the serum is obtained as part of comprehensive assessment of the risk of ASCVD that is commonly estimated according to the following formula: total cholesterol = LDL cholesterol + HDL cholesterol + triglycerides/5. Dyslipidemia includes hypercholesterolemia, an increased LDL cholesterol, a decreased HDL cholesterol, and hypertriglyceridemia. Hypercholesterolemia is diagnosed if the serum total cholesterol is 200 mg dl^{-1} (5.2 mmol l^{-1}) or higher. A low serum HDL cholesterol is less than 40 mg dl^{-1} (1.04 mmol l^{-1}) in men and less than 50 mg dl^{-1} (1.2 mmol l^{-1}) in women. Hypertriglyceridemia is a triglycerides level of 150 mg dl^{-1} (3.9 mmol l^{-1}) or higher. Patients with atherosclerotic vascular disease should have their LDL cholesterol reduced to less than 70 mg dl^{-1} (1.82 mmol l^{-1}). Increased LDL cholesterol and decreased HDL cholesterol are risk factors for coronary artery disease (CAD), peripheral arterial disease (PAD), ischemic stroke, transient ischemic attack, carotid arterial disease, heart failure, abdominal aortic aneurysm, valvular aortic stenosis, and mitral annular calcium in elderly persons.

The 2013 American College of Cardiology (ACC)/American Heart Association (AHA) lipid guidelines state that ASCVD includes CAD, stroke, transient ischemic attack, and PAD which includes carotid arterial disease and abdominal aortic aneurysm as well as PAD of the lower extremities. These guidelines use a Pooled Heart Equation to estimate the 10-year risk of developing ASCVD. This equation includes sex, age, race, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, diabetes mellitus, and smoking.

Lifestyle Measures

Persons with hypercholesterolemia with and without ASCVD should be treated with a Step II AHA diet. They should achieve and maintain an ideal body weight. Cholesterol intake should be less than 200 mg/day. Less than 30% of total caloric intake should be fatty acids. Saturated fatty acids should comprise less than 7% of total calories, polyunsaturated fatty acids should account for up to 10% of total calories, and monounsaturated fatty acids should comprise 10–15% of total calories. Protein intake should account for 10%–20% of total calories. Carbohydrates should comprise 50%–60% of total calories. In addition to loss of weight in obese persons, daily aerobic exercise, and dietary treatment of hypercholesterolemia, cigarette smoking should be stopped, hypertension should be treated, and diabetes mellitus controlled with the hemoglobin A1c level reduced to less than 7.0%.

The 2011 ACC/AHA guidelines on treatment of hypertension in the elderly recommend reducing the blood pressure to less than 140/90 mm Hg in elderly persons younger than age 80 years. In persons aged 80 years and older, these guidelines recommend reducing the systolic blood pressure to 140–145 mmHg if tolerated. The 2013 ACC/AHA guidelines on treatment of hypercholesterolemia state that lifestyle modification must be used both prior to cholesterol-lowering drug therapy and together with use of cholesterol-lowering drug therapy.

Metabolic Syndrome

The clustering of high serum triglycerides, small dense LDL particles, low serum HDL cholesterol levels, hypertension, insulin resistance (with or without glucose intolerance), and a prothrombotic state is diagnosed as the metabolic syndrome. This constellation is extremely common in persons with type 2 diabetes mellitus and is thought to be a precursor to diabetes mellitus in persons at increased risk for developing diabetes mellitus with aging. The metabolic syndrome should be treated with lifestyle modification and with statin therapy to treat the atherogenic dyslipidemia of the metabolic syndrome.

Statins

The most effective lipid-lowering drugs in reducing cardiovascular events and mortality in elderly and younger persons are the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statin drugs. Statins reduce serum total cholesterol, LDL cholesterol, and triglycerides, and they increase serum HDL cholesterol. They also cause many pleiotropic effects which contribute to a reduction in cardiovascular events and mortality. The statin drugs include rosuvastatin, atorvastatin, simvastatin, pravastatin, lovastatin, fluvastatin, and pitavastatin. Statin drugs suppress cholesterrol biosynthesis by competitively inhibiting HMG-CoA reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, a precursor of sterols (including cholesterol). This action induces upregulation of LDL receptors in the liver and increased clearance of LDL receptors in the liver and increased clearance of LDL from the plasma, thereby reducing plasma cholesterol levels.

High-dose statins (rosuvastatin 20–40 mg daily and atorvastatin (40–80 mg daily) reduce LDL cholesterol 50% or more. Moderate-dose statins (rosuvastatin 5–10 mg daily, atorvastatin 10–20 mg daily, simvastatin 20–40 mg daily, pravastatin 40–80 mg daily, lovastatin 40 mg daily, fluvastatin XL 80 mg daily, fluvastatin 40 mg twice daily, and pitavastatin 2–4 mg daily) reduce LDL cholesterol 30%–49%. Low-dose statins reduce LDL cholesterol less than 30%.

Numerous double-blind, randomized, placebo-controlled trials have shown that elderly persons with and without ASCVD or diabetes mellitus treated with statins have a reduction in cardiovascular events and in mortality. The lower the serum LDL

cholesterol reduced by statins, the greater the reduction in cardiovascular events and mortality. In the Heart Protection Study, where 5,806 of the 20,536 men and women at increased risk for cardiovascular events randomized to simvastatin or to double-blind placebo were 70–80 years of age at study entry and were 75–85 years of age at follow-up, 5 years of simvastatin therapy prevented myocardial infarction, stroke, and revascularization in 70–100 persons per 1,000 treated persons regardless of age, gender, or initial levels of serum lipids. In this study, reduction of serum LDL cholesterol in persons with a baseline serum LDL cholesterol of less than 100 mg dl^{-1} (2.6 mmol l^{-1}) was as effective in reducing cardiovascular events and mortality as reducing serum LDL cholesterol in persons with higher serum LDL cholesterol levels.

At 5-year follow-up, compared to placebo, simvastatin significantly decreased all-cause mortality by 13%, any cardiovascular death by 17%, major coronary events by 27%, any stroke by 25%, coronary or noncoronary revascularization by 24%, and any major cardiovascular event by 24%.

In the Study Assessing Goals in the Elderly (SAGE), 893 ambulatory CAD patients aged 65–85 years with at least 1 episode of myocardial ischemia lasting at least 3 min during 48-hour ambulatory electrocardiographic screening were randomized to atorvastatin 80 mg daily or to pravastatin 40 mg daily and followed for 12 months. Total duration of myocardial ischemia detected by 48-hour ambulatory electrocardiograms at month 3 and at month 12 after randomization was significantly reduced by both atorvastatin and pravastatin with no significant difference between the 2 treatment groups. Compared with pravastatin, atorvastatin significantly reduced serum LDL cholesterol levels, insignificantly reduced major acute cardiovascular events by 22%, and significantly reduced all-cause mortality by 67%.

In the Justification for the Use of Statins in Prevention: an Intervention Trial evaluating Rosuvastatin (JUPITER), 17,082 apparently healthy persons, median age 66 years, with a serum LDL cholesterol of less than 130 mg dl^{-1} (3.36 mmol l^{-1}) and high-sensitivity C-reactive protein levels of 2.0 mg l^{-1} or higher were randomized to rosuvastatin 20 mg daily or placebo [42]. At 1.9-year median follow-up, rosuvastatin significantly reduced serum LDL cholesterol levels by 50%, high-sensitivity C-reactive protein levels by 37%, and the primary end point of myocardial infarction, stroke, arterial revascularization, hospitalization for unstable angina pectoris, or death from cardiovascular causes by 44%.

A meta-analysis was performed in 9 randomized trials of statins for secondary prevention in 19, 569 patients aged 65–82 years Over 5 years, statins reduced all-cause mortality 22% (95% CI, 11–35%), CAD mortality 30% (95% CI, 17%–47%), nonfatal myocardial infarction 26% (95% CI, 11%–40%), need for revascularization 30% (95% CI, 17%–47%), and stroke 25% (95% CI, 6%–44%). The estimated number needed to treat to save 1 life was 28.

2013 ACC/AHA Lipid Guidelines

The 2013 ACC/AHA lipid guidelines recommend the use of high-dose statins to adults aged 75 years and younger with ASCVD unless contraindicated with a class I indication. Moderate-dose or high-dose statins are reasonable to administer to persons with ASCVD older than 75 years with a class IIa indication. Persons aged 21 years and older with a serum LDL cholesterol of 190 mg dl^{-1} (4.94 mmol l^{-1}) or higher should be treated with high-dose statins with a class I indication. For primary prevention in diabetics aged 40–75 years and a serum LDL cholesterol between 70 and 189 mg dl^{-1} (1.82–4.91 mmol l^{-1}), moderate-dose statins should be administered with a class I indication. For primary prevention in diabetics aged 40–75 years, a serum LDL cholesterol between 70 and 189 mg dl^{-1} (1.82–4.91 mmol l^{-1}), and a 10-year risk of ASCVD of 7.5% or higher calculated from the Pooled Heart Equation, high-dose statins should be administered with a class IIa indication. For primary prevention in diabetics aged 21–39 years or older than 75 years and a serum LDL cholesterol between 70 and 189 mg dl^{-1} (1.82–4.91 mmol l^{-1}), moderate-dose statins or high-dose statins should be administered with a class IIa indication. Adults aged 40–75 years of age without diabetes mellitus or ASCVD with a serum LDL cholesterol between 70 and 189 mg dl^{-1} (1.82–4.91 mmol l^{-1}) and a 10-year risk of ASCVD of 7.5% or higher calculated from the Pooled Heart Equation should be treated with high-dose statins or moderate-dose statins with a class I indication. Adults aged 40–75 years of age without diabetes mellitus or ASCVD with a serum LDL cholesterol between 70 and 189 mg dl^{-1} (1.82–4.91 mmol l^{-1}) and a 10-year risk of ASCVD of 5%–7.4% calculated from the Pooled Heart Equation should be treated with moderate-dose statins with a class IIa indication.

Besides the use of statins in these groups, other factors may be considered for use of statins. These factors include a primary serum LDL cholesterol of 160 mg dl^{-1} (4.16 mmol l^{-1}) or higher or other evidence of genetic hyperlipidemia, a family history of premature ASCVD with onset before age 55 years in a first-degree male relative, onset before age 55 years in a first-degree male relative or onset before age 65 years in a first-degree female relative, high-sensitivity C-reactive protein 2 mg l^{-1} and higher, a coronary calcium score of 300 Agaston units or higher or 75% and higher for age, sex, and ethnicity, an ankle-brachial index below 0.90, or an increased lifetime risk of ASCVD.

These guidelines also state that there is no additional ASCVD reduction from adding nonstatin therapy to further lower non-HDL cholesterol once an LDL cholesterol goal has been reached. Clinical trials have demonstrated no reduction in cardiovascular events or mortality in persons treated with statins by addition of nicotinic acid, fibric acid derivatives, ezetemibe, or drugs that raise HDL cholesterol.

Serum triglycerides 500 mg dl^{-1} (13.0 mmol l^{-1}) or higher should be treated to reduce the risk of pancreatitis. Gemfibrozil should not be given to patients on statins. Fenofibrate may be considered with low-dose or moderate dose statins with a class IIb indication, should not be used if the estimated glomerular filtration rate is less than 30 ml min/1.73 m^2, and should not exceed a dose of 54 mg daily if the estimated glomerular filtration rate is 30–59 ml min/1.73 m^2. If omega-3 fatty acids are used to treat

serum triglycerides 500 mg dl^{-1} (13.0 mmol l^{-1}) or higher, it is reasonable to evaluate the patient for gastrointestinal disturbances, skin changes, and bleeding.

In 18 randomized clinical trials (19 cohorts) for primary prevention of ASCVD by statins which included 56, 934 patients between 28 and 97 years of age, compared with placebo, statins significantly reduced LDL cholesterol 39 mg dl^{-1} (1.01 mmol l^{-1}), all-cause mortality 14%,fatal and nonfatal cardiovascular disease 25%, fatal and nonfatal CAD 27%, fatal and nonfatal stroke 22%, and coronary revascularization 38%. The incidence of cancers, myalgia, rhabdomyolysis, liver enzyme elevation, renal dysfunction, or arthritis did not differ between statins and placebo. The rates of adverse events (17%) and of stopping treatment (12%) did not differ between statins and placebo. One of 2 trials reported an increased risk of diabetes mellitus caused by statins (number needed to treat = 198 persons).

See also: Hypercholesterolemia. Hypertriglyceridemias and Their Treatment. Low HDL and High HDL Syndromes

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Gonadotropins and Testicular Function in Aging

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Glossary

Late Onset or "Functional" Hypogonadism A decline in testicular function with aging, resulting in decreased testosterone production that may manifest with clinical presentations, including sexual dysfunction, mood changes, decreased energy, frailty, and decreased bone and muscle mass.

Feedback control Process in which the target organ (testis) secretes substances (testosterone, inhibin) to regulate the secretion of hormones (gonadotropin-releasing hormone, follicle-stimulating hormone, and luteinizing hormone) by the regulating organs (hypothalamus and pituitary) operating at molecular, cellular, and glandular levels.

Introduction

The hypothalamic–pituitary–testicular (HPT) axis controls the levels of testosterone (T) minute by minute via signaling interactions among gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and T. GnRH neurons in the arcuate/infundibular nucleus of the hypothalamus, influenced by excitatory and inhibitory neuromodulators from the kisspeptin/neurokinin B/dynorphin (KNDy) neurons among others, secretes GnRH in pulses. GnRH stimulates the secretion of both FSH and LH from the anterior pituitary. LH is released in pulses, and stimulates Leydig cells to secrete T (Pinilla *et al.*, 2012). Unlike the menopause in women where there is more abrupt cessation of ovarian function, in men there is no distinct time point at which T production changes (Araujo and Wittert, 2011; Wu *et al.*, 2010). As men age, they have a gradual decline in circulating T levels which may be related with many co-morbidities occurring in aging men (Yeap, 2009; Yeap *et al.*, 2012; Wu *et al.*, 2010; Travison *et al.*, 2007a; Tajar *et al.*, 2012). However, this decline occurs even in men without co-morbidities and is also associated with multiple regulatory changes in the HPT axis which are present even in healthy men without known diseases. This suggests that some of these changes are age-related, rather than related to pathological processes that accumulate with age. Aging-related mechanisms that contribute to the decline in testosterone include (1) decreased hypothalamic GnRH outflow, (2) diminished effect of negative feedback by testosterone on the HPT axis, (3) impaired Leydig cell steroidogenesis in response to LH, and (4) increase in sex hormone binding globulin with age and decrease with obesity affecting both total and free T estimations.

Evidence Supporting Age-Related Testosterone Decline Men

In aging men, decreased T secretion from Leydig cell dysfunction should elevate LH and FSH due to the negative feedback mechanism, as seen in men with primary hypogonadism. However, serum FSH and LH levels are frequently not as elevated as would be expected given their serum levels of T. In older men, the increase in serum LH observed in longitudinal studies is approximately 0.9% per year, and the increase in serum FSH is approximately 3.1% per year (Wu *et al.*, 2008; Tajar *et al.*, 2010). LH concentrations may be normal when T concentrations are below the lower limit of normal. The Massachusetts Male Aging Study in community-dwelling men corroborated this decline in T with age, and noted that the existence of comorbidities increased the likelihood of hypogonadism. However, even healthy men had a decline in T at age 70 compared to age 40 (Travison *et al.*, 2007b; Araujo *et al.*, 2004a,b; Feldman *et al.*, 2002). Most available data show that the decline in T is due to both decline in Leydig cell function and in HPT axis dysfunction. There is also evidence that sex hormone binding globulin (SHBG) increases with age, independent of obesity, and free T declines more steeply than total T (Liu *et al.*, 2007). Recent studies of middle age and older men indicate that aging causes Leydig cell dysfunction manifested by low T and elevated LH, where abnormalities of the HPT axis is associated with comorbidities such as obesity (Wu *et al.*, 2008).

Decreased GnRH Release With Preservation of Pituitary Response to GnRH

There is indirect evidence that hypothalamic secretion of GnRH is reduced in older men. In rodent studies, there was diminished GnRH release by hypothalamic tissue (Gruenewald *et al.*, 1994) and decreased density of GnRH neuronal synapses (Witkin, 1987). Transplantation of fetal hypothalamic neurons to impotent aged male rats restored their reproductive function and improved LH

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and T levels (Huang *et al.*, 1987). Studies have also demonstrated that pituitary response to GnRH appears to be preserved. In rodent studies, LH response to exogenous GnRH was preserved (Gruenewald *et al.*, 2000). In humans, in comparisons of aged and young men, the aged men had the same mean serum LH concentrations as the young men, but their LH pulse frequency was significantly higher, and there was loss of high-amplitude LH secretory pulses (Mulligan *et al.*, 1995). However, when aged and young men were both treated with GnRH for 14 days, the 24 h serum LH concentrations were indistinguishable by cluster analysis to determine pulse frequency and amplitude and the orderliness was identical by approximate entropy (Mulligan *et al.*, 1999). This implies that the pituitary's ability to respond to GnRH is preserved.

Diminished Pituitary Negative Feedback From Androgens

How or whether androgen feedback is altered in older men is controversial. As seen in the European Male Aging Study (Wu *et al.*, 2008), the negative feedback mechanism of the HPT axis appears to be diminished in elderly men. LH levels may be normal despite lower levels of T. This impaired negative feedback mechanism has also been demonstrated in animal studies. In elderly rats, castration did not increase LH as much as it did in younger rats (Shaar *et al.*, 1975). Another study demonstrated reduced androgen receptor (AR) expression in the hypothalamus and pituitary, and also confirmed a decreased LH response to gonadectomy (Haji *et al.*, 1981). Altogether, these results suggest diminished responsiveness to negative feedback from androgens. In humans, several studies reported that suppression of LH concentrations in aged men after administration of high dose.

T was reduced (Gentili *et al.*, 2002), but could be increased with lower dose androgen therapy (Gentili *et al.*, 2002; Winters *et al.*, 1984; Winters and Atkinson, 1997). In studies using the antiestrogen clomiphene citrate, the increases in LH and FSH in aged men were less, compared to that of young men (Tenover *et al.*, 1987). These seemingly contradictory findings could be explained by differences in negative feedback at the hypothalamus compared with the pituitary. Mathematical constructs using graded competitive GnRH receptor antagonism and a calibrating mid-physiological dose of exogenous GnRH suggest that although negative feedback at the pituitary may be diminished by age, feedback suppression of androgens on hypothalamic GnRH drive may actually be augmented by age (Keenan *et al.*, 2011).

Impairment of LH Secretion

The result of these changes in the HPT axis is impairment of LH secretion. In elderly men, LH pulse amplitude decreases, pulse frequency increases, and the secretion patterns become more irregular/disorderly. Several studies have demonstrated this change in LH secretion (Mulligan *et al.*, 1999). The mean basal levels of LH remain the same. Deconvolution analysis of LH pulses predicts frequent and small LH secretory bursts with diminished T pulses (**Fig. 1**).

Testicular Function Declines With Aging

The decrease in serum T is partially due to defective Leydig cell steroidogenic capacity as well as decreased responsiveness of the Leydig cells to endogenous LH. Studies in our laboratory demonstrated that in aging male rats (Brown Norway rat), the lower serum T when compared to younger animals are due to both defective HPT axis (Bonavera *et al.*, 1997, 1998) and testicular dysfunction that may arise from inadequate blood flow, increased inflammation and chronic testicular stress resulting in increased reactive oxygen species (Wang *et al.*, 1999, 2002). In addition to low T levels, there was aged related reduction in Leydig cell volume per testis; the total number of Sertoli cell per testis; tubule diameter, length of tubules, and volume of tubules and their lumens. The testicular sperm concentration and total sperm production were significantly reduced in the 22- and 30-month-old rats (Wang *et al.*, 1993). Furthermore extensive studies of the Leydig cells in the same rat model demonstrated decreased steroidogenic capacity of Leydig cells both in vitro and in vivo in response to LH which is related to reduced cAMP production. Treatment of aged cells with dibutyryl cAMP or placing them in a younger rat testis environment resulted in restoration of LH stimulated Leydig cell steroidogenic capacity (Chen *et al.*, 1996, 2009; Beattie *et al.*, 2013).

In older men, there was less T secreted per LH burst (Mulligan *et al.*, 1995). Furthermore, pulsatile recombinant human LH administered under ganirelix blockade of endogenous LH secretion to mimic that typically observed in young men, results in lower mean T concentrations, reduced pulsatile secretion and reduced LH-T feedforward synchrony when applied to older, compared with younger, men (Liu *et al.*, 2005). Studies using this paradigm in 92 healthy men aged 18–75 years confirm these relationships (Veldhuis *et al.*, 2012). These data strongly implicate impaired Leydig cell responsiveness to LH stimulation with aging in men in the human.

Aging is associated with a progressive decline in serum total T as well as bioavailable and free T. In earlier studies serum total T levels decline by 1.6% and bioavailable T declines by 2.3% per year, while sex hormone binding globulin (SHBG) increases by 1.3% per year (Feldman *et al.*, 2002; Harman *et al.*, 2001). There is also a concomitant decline in androstenedione, dehydroepiandrosterone (DHEA), DHEA sulfate, and estrogen (Labrie *et al.*, 1997). Serum dihydrotestosterone (DHT) increases in

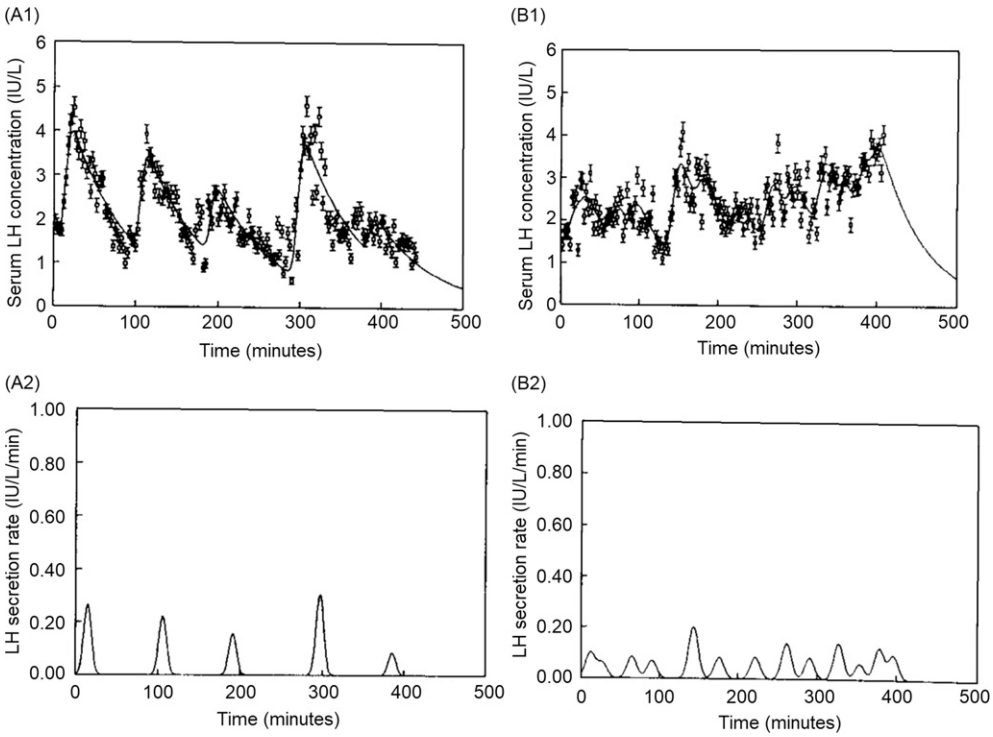

Fig. 1 Serum LH concentrations and LH secretion rate in a young man (A) and old man (B). The continuous lines are generated by deconvolution analysis. There is loss of high-amplitude LH and testosterone secretory pulses in older men with preservation of basal secretion rates. Entropy analyses showed irregularity of release of LH and testosterone in the older men. Reproduced from Mulligan, T., Iranmanesh, A., Gheorghiu, S., et al. (1995). Amplified nocturnal luteinizing hormone (LH) secretory burst frequency with selective attenuation of pulsatile (but not basal) testosterone secretion in healthy aged men: possible Leydig cell desensitization to endogenous LH signaling—A clinical research center study. *The Journal of Clinical Endocrinology and Metabolism* 80, 3025–3031 with permission from The Endocrine Society.

older men, possibly due to increased 5α-reductase activity in the liver, skin, or prostate. However, the levels of intraprostatic DHT remain stable (Geller *et al.*, 1976). In the European Male Aging study (EMAS, 3200 community dwelling men aged from 40 to 79 years in eight countries), serum T decrease was less than free T and serum LH and SHBG increased with aging. In addition to aging, higher body mass index was associated with lower T and free T and presence of comorbidities is also associated with lower total T without compensatory increases in LH levels (Wu *et al.*, 2008) (**Fig. 2**) The prevalence of symptomatic hypogonadism, that is, low T and specific symptoms, increases in age, from 0.1% for men 40–49 years old to 5.1% for men 70–79 years old (Wu *et al.*, 2010). The prevalence of hypogonadism increases with an increase in comorbidities (Wu *et al.*, 2010; Yeap *et al.*, 2012). Decrease in serum T was observed in men with decreased sexual desire, erectile dysfunction and decreased morning erection and ability to perform vigorous physical activity. This association of symptoms with decline in serum T does not appear to be linear; decrease in ability to perform vigorous activity appear to require a higher serum T threshold than sexual desire and erectile dysfunction (Zitzmann *et al.*, 2006; Wu *et al.*, 2010). Furthermore, men with low T and high LH (primary hypogonadism) are usually older and remained hypogonadal at 4 years follow up. Those with lower LH and low T are more obese (secondary hypogonadism) and 42% of them became eugonadal on follow up. The recovery from hypogonadism was predicted by nonobesity, weight loss, lower waist circumference and young age suggesting that the development of secondary hypogonadism can be reversed with weight loss (Tajar *et al.*, 2010; Rastrelli *et al.*, 2015).

The germinal epithelium continues to produce spermatozoa in elderly healthy men, despite the decline in T (Nieschlag *et al.*, 1982). In a larger study of over 1000 men aged 45–80 (mean 53) showed decline in sperm concentration, motility and morphology and only 46% of these men had semen quality that are considered within the adult male range (Hellstrom *et al.*, 2006). There are studies that showed the spermatozoa of older men may have more DNA fragmentation (Das *et al.*, 2013; Schmid *et al.*, 2013). Studies show that circulating inhibin B, a marker of Sertoli cell function, is maintained with age, though FSH rises and the inhibin B:FSH ratio decreases, indicating deficient Sertoli cell function (Mahmoud *et al.*, 2003). Aging is associated with decreased testis volume and testicular metabolism due to decreased volume of the seminiferous tubules (Yang *et al.*, 2011). On histology, there is a range of different seminiferous tubule abnormalities, with some tubules that appear normal, though with reduced spermatogenesis, to completely sclerosed tubules. Because the loss of germ cells is patchy, it is likely that other factors such as vascular ischemia, increased cytokines, reactive oxygen species, and other paracrine factors may play important roles in addition to decreased T production by Leydig cells (Sasano and Ichijo, 1969; Paniagua *et al.*, 1991).

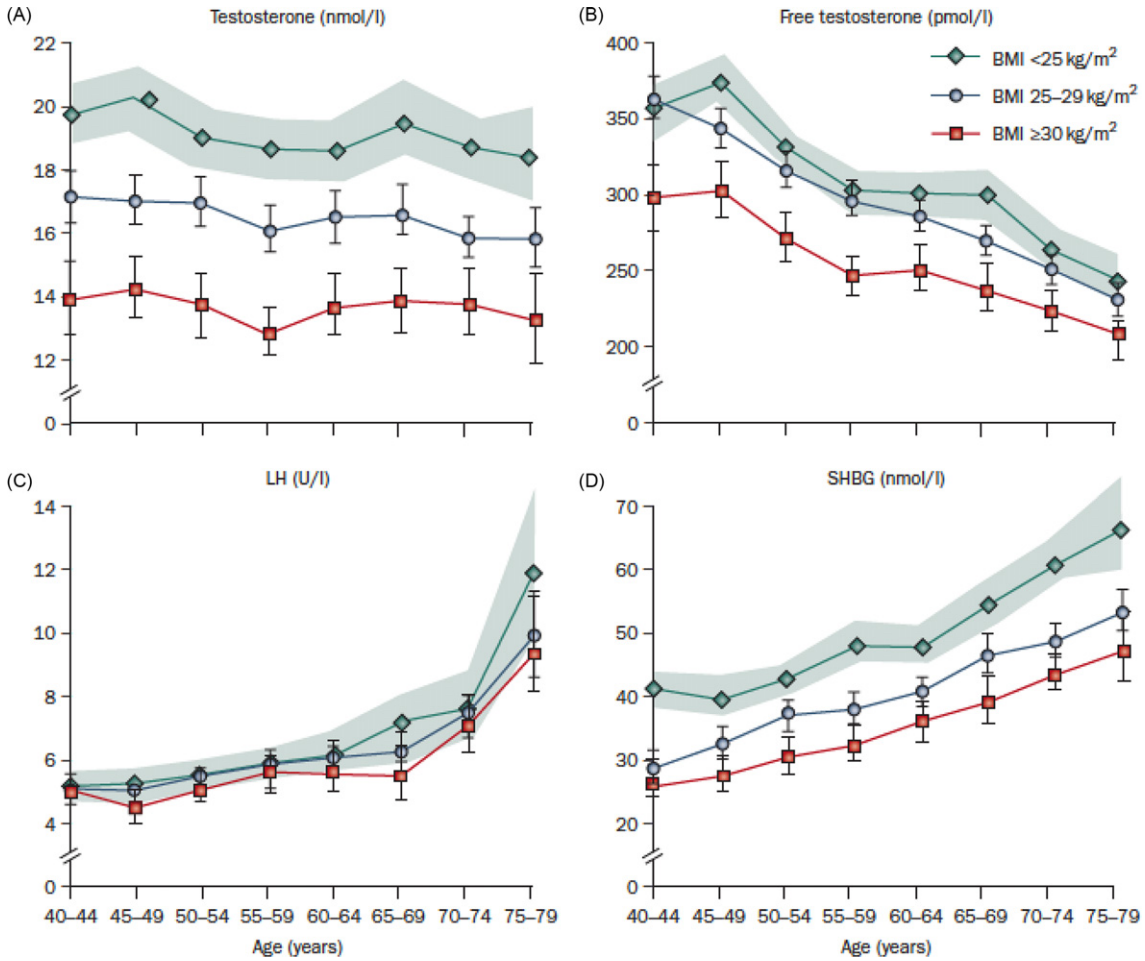

Fig. 2 Relationship between age, BMI, and hormones. The cohort was stratified according to BMI into three groups: nonobese (BMI <25 kg/m^2), overweight (BMI 25–30 kg/m^2), and obese (BMI >30 kg/m^2). Mean (95% CI in shaded area and vertical lines) total and free T (A, B) and SHBG (D) were significantly lower in the overweight and obese at all ages, compared with nonobese. The total T and SHBG age trends in the three BMI categories were similar, indicating no interaction between BMI and age. The free T age trend in the obese group was less steep than in the other two groups, indicating an interaction between BMI and age. Mean LH (C) was not significantly different among the three groups at the median age of 60 year. LH was higher in the older than 70 years nonobese group, compared with the overweight and obese groups, due to a negative BMI-age interaction. Reproduced from Wu, F. C., Tajar, A., Pye, S. R., et al. (2008). Hypothalamic–pituitary–testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: The European Male Aging Study. *Journal of Clinical Endocrinology Metabolism* **93**, 2737–2745 with permission from The Endocrine Society.

Conclusion

In summary, evidence from animal models and men showed that aging is associated with dysfunction of the Leydig cells as well as the hypothalamic-pituitary-gonadal axis. Complex interactions exist within the HPT axis. Men with HPT axis abnormalities are more obese and may recover with lifestyle changes, whereas men with primary Leydig cell dysfunction are older and tend to remain hypogonadal.

See also: Gonadotropin-Releasing Hormone (GnRH) Development and Actions. Hormone Replacement Therapy in Men. Hypogonadism and Testosterone Therapy in Elderly Men. Sexual Function in Aging Men

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Hypogonadism and Testosterone Therapy in Elderly Men

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Introduction

Male ageing is characterized by a decline in circulating testosterone concentrations and the accumulation of medical comorbidities (Harman *et al.*, 2001; Feldman *et al.*, 2002). Cause and effect are debated as to whether age per se drives the decline in circulating androgens, or whether this is a reflection or consequence of increasing ill-health. Nevertheless in epidemiological studies lower testosterone concentrations consistently predict poorer health outcomes in older men extending across multiple health domains (Yeap *et al.*, 2012a). Prominent amongst these are medical comorbidities that become more prevalent during aging, namely frailty, osteoporosis, and cardiovascular disease.

Of note, androgen deficiency due to diseases of the hypothalamus, pituitary or testes can present in elderly men and this needs to be identified and treated appropriately taking age and any coexisting medical comorbidities into account (Yeap *et al.*, 2016a, b). However the broader role of testosterone as a pharmacological intervention in elderly men without hypothalamic, pituitary or testicular disease who have low testosterone concentrations when compared to younger men continues to be debated. In this context, improvements in sexual function, anemia and bone density can be expected, but not in walking ability, vitality or cognition (Snyder *et al.*, 2016, 2017; Roy *et al.*, 2017; Resnick *et al.*, 2017). Whether or not testosterone therapy precipitates cardiovascular adverse effects in frail elderly men remains controversial (Basaria *et al.*, 2010; Budoff *et al.*, 2017). Therefore the balance of benefits and risks of testosterone therapy in elderly men in the absence of pathological hypogonadism, particularly over extended periods of treatment, remain unclear.

In this review, the longitudinal trajectories of circulating testosterone during male aging will be explored, as will the epidemiological associations of low testosterone with age-related ill-health particularly frailty, osteoporosis and cardiovascular disease in older men. The diagnosis of hypogonadism in elderly men will be discussed together with indications for treatment and treatment-related considerations. The role of testosterone in elderly men who do not have hypothalamic, pituitary or testicular disease and the potential for cardiovascular adverse events will be considered. Finally, priorities for future research will be discussed.

Testosterone in Aging Men

Several longitudinal studies in men have demonstrated a consistent decline in circulating testosterone with increasing age (Harman *et al.*, 2001; Feldman *et al.*, 2002; Camacho *et al.*, 2013; Shi *et al.*, 2013; Hsu *et al.*, 2016). In studies using mass spectrometry for measurement of testosterone concentrations, the annual rate of decline is in the order of 1%–2% per annum: slightly less in middle-aged men and slightly more in older men (Camacho *et al.*, 2013; Shi *et al.*, 2013; Hsu *et al.*, 2016). Part of this decline may relate to presence of central adiposity or to the presence of medical comorbidities resulting in a reduction in function of the hypothalamic-pituitary-testicular axis and a low circulating testosterone with a low or normal luteinising hormone (LH) concentration (Shi *et al.*, 2013; Camacho *et al.*, 2013; Rastrelli *et al.*, 2015). Nevertheless, even very healthy older men aged 70–89 years have lower circulating testosterone compared with reproductively normal men aged 21–35 years (Yeap *et al.*, 2012b; Sikaris *et al.*, 2005). Therefore there is likely to be a contribution of advancing age to the decline in circulating testosterone in men. Conversely, observational analyses have correlated higher circulating concentrations of testosterone's bioactive metabolites, dihydrotestosterone and estradiol, with longer leucocyte telomere lengths in men, thus associating sex hormones with slower biological ageing (Yeap *et al.*, 2016c). Therefore advancing age, declining circulating testosterone and the accumulation of age-associated morbidities are interrelated factors impacting on men's health.

The entity of "late onset hypogonadism" (LOH) has been proposed to describe older men with lower circulating testosterone concentrations and associated sexual symptoms such as low sexual desire and poor erectile function (Wu *et al.*, 2010). Decreased libido is a presenting symptom of androgen deficiency, but erectile dysfunction tends to have a multifactorial basis and is a less common presenting feature of androgen deficiency and then only at very low testosterone concentrations (Yeap *et al.*, 2016a). In a study of men aged 40–70 years with erectile dysfunction and baseline testosterone concentrations <11.5 nmol/L of free testosterone <173 pmol/L, sildenafil dose was optimized following which men were randomized to testosterone or placebo (Spitzer *et al.*, 2012). Sildenafil alone increased erectile function and subsequent addition of testosterone did not further improve erectile dysfunction scores.

When using a board definition of sexual activity appropriate for epidemiological studies in elderly men, about a third will report being sexually active (Hyde *et al.*, 2010a). The proportion falls from $\sim$40% at age 75–79, to $\sim$10% at age 90–95 years. Low testosterone concentrations do predict reduced sexual activity in elderly men (Hyde *et al.*, 2010a); and causality is supported by the result of the T-Trials in which testosterone therapy in men aged ≥ 65 years with baseline testosterone <9.5 nmol/L improved sexual function (Snyder *et al.*, 2016). However multiple other factors are associated with reduced sexual activity in older men including advanced age, not cohabiting with a partner, cohabiting with a disinterested partner or a partner with physical

limitations, and the presence of medical comorbidities (Hyde *et al.*, 2010a). Thus while the concept of LOH can be utilized informatively for epidemiological studies, its clinical utility remains to be determined. A logical approach to men classified as having LOH on the basis of lower testosterone concentrations and three or more sexual symptoms (reduced sexual thoughts, weaker morning erections, and erectile dysfunction) would comprise lifestyle modification, reduction of excess weight, and good treatment of comorbid diseases, with evidence currently lacking for the benefits of testosterone treatment in this setting (Huhtaniemi, 2014).

Low testosterone with an elevated LH is an indicator of primary hypogonadism (Wu *et al.*, 2010; Yeap *et al.*, 2016a). Cross-sectional studies have reported higher LH concentrations in older men (e.g., Baker *et al.*, 1976). In a longitudinal study of 1,025 men who had a median age of 75.1 years at baseline followed for 8.6 years, the longitudinal decline in dihydrotestosterone which was −7.2%/year was more marked than the decline in testosterone of −2.0%/year (Yeap *et al.*, 2018). Declines in both dihydrotestosterone and testosterone were correlated with increases in LH. Of note, annualized increases in LH of 7.5%/year and sex hormone-binding globulin (SHBG) of 5.6%.year were present in these men (Yeap *et al.*, 2018). The proportion of men with an elevated LH (>16 IU/L) increased from 2.4% at baseline to 17.4% at follow-up. Therefore, there is some evidence to support the concept of longitudinal impairment of testicular endocrine function occurring in men transitioning from the eighth to ninth decades of life (Yeap *et al.*, 2018). The clinical implications of this observation require further clarification.

Low Testosterone and Frailty

Frailty can be characterized as a decline in multiple organ systems leading to loss of function, diminished physiological reserve and hence ability to cope with stressors, with an increased risk of disability and death (Fried *et al.*, 2001; Ahmed *et al.*, 2007). Frailty is increasingly prevalent in elderly adults and contributes to the poorer health outcomes (Ahmed *et al.*, 2007). A feature of frailty is unintentional weight loss, in particular of lean or muscle mass, with accompanying reductions in physical performance (Fried *et al.*, 2001). Thus there is an overlap between frailty and sarcopenia or loss of muscle mass or strength, leading to loss of mobility and function, and to falls and mortality (Morley *et al.*, 2014). Reduced muscle mass and strength is a recognized presenting feature of androgen deficiency (Yeap *et al.*, 2016a). In epidemiological studies in middle-aged and older men, lower circulating testosterone concentrations have been associated with frailty both cross-sectionally and longitudinally (Cawthon *et al.*, 2009; Hyde *et al.*, 2010b; Travison *et al.*, 2011). This is consistent with the actions of testosterone in older men to promote gain of lean or muscle mass and strength (Bhasin *et al.*, 2005; Page *et al.*, 2005; Sattler *et al.*, 2009). Therefore in elderly men, lower circulating testosterone, sarcopenia and frailty are interrelated conditions with implications for health.

In a randomized clinical trial (RCT) in men aged ≥65 years with low–normal baseline testosterone concentrations and mobility limitations, testosterone therapy over a 6 month intervention period improved measures of physical performance (Travison *et al.*, 2011). However, that trial was discontinued prematurely due to excess adverse events in the testosterone arm (Basaria *et al.*, 2010). Of note, a similar RCT in men aged ≥65 years with baseline testosterone ≤12 nmol/L who were intermediate-frail or frail fond that testosterone therapy over a 6 month duration improved lower limb muscle strength, with an improvement in physical function in a subgroup of older and more frail men (Srinivas-Shankar *et al.*, 2010). No signal for excess cardiovascular adverse events was seen in that study, but the benefits of testosterone therapy were not sustained at 6 months posttreatment (O'Connell *et al.*, 2011). In a secondary analysis of a 3 year RCT in healthy older men, testosterone treatment in men aged ≥60 years with baseline testosterone 3.5–13.9 nmol/L or free testosterone <174 pmol/L resulted in increased muscle strength compared with placebo (Storer *et al.*, 2017). However, in that study muscle strength peaked at 6 months, and declined toward baseline levels by month 36 despite continuation of treatment. In the Physical Function Trial of the T-Trials, there was no significant increase in the proportion of men who increased their walking distance by ≥50 m in the 6 min walk test in the testosterone arm compared with placebo (Snyder *et al.*, 2016). There was an increase in walking distance at 12 months when the entire T-Trials cohort was analyzed. Therefore while testosterone therapy in elderly men can be expected to have beneficial anabolic actions on muscle, the magnitude and durability of such effects and their functional benefits remain unclear. Thus further RCTs are needed to define the role of testosterone in elderly men with or at risk of frailty with regards to optimal timing and durability of therapy.

Low Testosterone and Osteoporosis

Osteoporosis is an important cause of morbidity in adult men and as with women its prevalence increases with age (Ebeling, 2008). Osteopenia, osteoporosis and fracture, or loss of height, are presenting clinical features of androgen deficiency (Yeap *et al.*, 2016a). In an epidemiological study of men aged >60 years, low circulating testosterone was associated with increased risk of low-trauma fracture (Meier *et al.*, 2008). In a study of men with an average age of 75 years, low serum estradiol and high SHBG concentrations were associated with increased risk of fractures (Mellstrom *et al.*, 2008). However other studies in older men have implicated higher concentrations of SHBG but not circulating sex hormones with increased risk of vertebral fracture (Cawthon *et al.*, 2016; Vandenput *et al.*, 2016). While the association of low estradiol and higher SHBG with fracture risk has been confirmed in a recent study, measurement of sex hormones did not add substantially to clinical fracture risk prediction as afforded by clinical

parameters such as age, body mass index and femoral neck bone mineral density, or tools such as the Fracture Risk Assessment Tool (FRAX) with measurement of bone mineral density (Orwoll *et al.*, 2017).

RCTs of testosterone therapy in men aged >65 years with reasonable baseline testosterone concentrations in the range of 12–14 nmol/L have not shown consistent increases in bone mineral density (Snyder *et al.*, 1999; Kenny *et al.*, 2001; Christmas *et al.*, 2002). Other studies in men aged 60 years or more with lower baseline testosterone concentrations in the range of 9–10 nmol/L have reported increases in primarily lumbar spine bone mineral density (Amory *et al.*, 2004; Basurto *et al.*, 2008). A meta-analysis of 29 testosterone RCTs, most of which were not designed with bone mineral density as the primary outcome, showed an improvement in lumbar spine but not femoral neck bone mineral density (Isidori *et al.*, 2005). In the T-Trials which recruited men aged ≥65 years with baseline testosterone <9.5 nmol/L, the Bone substudy demonstrated increases in volumetric bone mineral density and estimated bone strength and both spine and hip sites following 12 months of treatment with testosterone (Snyder *et al.*, 2017). Increases at the spine were consistently greater than those seen at the hip. However, it is important to recognize that testosterone has not been proven in RCTs to reduce incidence of fracture in older men. Until such RCT evidence is available to clarify the effect of testosterone on incidence of osteoporotic fracture, men at high risk of fracture should be treated with osteoporosis therapies proven to reduce incidence of fracture (Watts *et al.*, 2012). However, an improvement in bone mineral density could be considered as an additional benefit in men receiving testosterone therapy for treatment of androgen deficiency due to underlying hypothalamic, pituitary or testicular disease.

Low Testosterone and Cardiovascular Disease

Advanced age is a powerful predictor for incidence of cardiovascular disease encompassing cardiac events such as myocardial infarction and cerebrovascular events such as stroke (Roger *et al.*, 2011). Epidemiological studies have associated lower circulating testosterone concentrations with increased incidence of cardiovascular events (for review, see Yeap, 2015). For example, in a longitudinal analysis of 2416 men aged 69–81 years, men with higher testosterone concentrations had a lower incidence of cardiovascular events during follow-up compared to men with lower testosterone concentrations (Ohlsson *et al.*, 2011). No corresponding association was found in an analysis of predominantly middle-aged men (Chan *et al.*, 2016), suggesting that the relationship is more apparent in older men. There may be possible differences according to the vascular territory of interest. In a longitudinal analysis of 3690 men aged 70–89 years, lower circulating testosterone concentrations were independently associated with increased risk of stroke (Yeap *et al.*, 2009, 2014a). There was a parallel association of lower dihydrotestosterone with higher risk of stroke (Yeap *et al.*, 2014a). By contrast, a longitudinal analysis of 1032 men aged 76 years reported a U-shaped association of dihydrotestosterone with stroke (Shores *et al.*, 2014a). Similarly, epidemiological studies have demonstrated associations of lower circulating testosterone with deaths from cardiovascular disease (Tivesten *et al.*, 2009), and lower circulating dihydrotestosterone with deaths from cardiovascular disease (Shores *et al.*, 2014b) or ischemic heart disease (Yeap *et al.*, 2014b).

While the epidemiological data are suggestive of a protective effect of endogenous androgens in older men against the incidence of cardiovascular events particularly stroke, no RCT has been undertaken with the prespecified primary outcome of incident cardiovascular events. In light of this evidence gap, analyses have been undertaken of insurance databases of men prescribed testosterone and testosterone RCTs in which cardiovascular adverse events have been reported. Retrospective analyses comparing men who were prescribed testosterone with men who were not have reported conflicting results: suggesting an increased risk of cardiovascular adverse events (Vigen *et al.*, 2013; Finkle *et al.*, 2014), neutral or reduced risk of myocardial infarction (Baillargeon *et al.*, 2014; Sharma *et al.*, 2015), or a reduced risk of major cardiovascular events (Anderson *et al.*, 2016; Wallis *et al.*, 2016; Cheetham *et al.*, 2017). Several studies have associated testosterone prescription with lower mortality (Sharma *et al.*, 2015; Shores *et al.*, 2012; Muraleedharan *et al.*, 2013; Wallis *et al.*, 2016). However, retrospective analyses of this type have recognized limitations particularly the absence of randomization and multiple potential sources of bias and confounding.

One RCT of testosterone in older men with mobility limitations was stopped due to an excess of adverse events in the testosterone arm (Basaria *et al.*, 2010), another in a similar population of older men who were frail or intermediate frail did not show any signal for excess cardiovascular adverse events (Srinivas-Shankar *et al.*, 2010). The T-Trials Cardiovascular substudy reported an increase in noncalcified coronary atheromatous plaque in men in the testosterone group (Budoff *et al.*, 2017). However that finding is difficult to interpret because the groups were unbalanced: men in the placebo group had more plaque at baseline and at the end of the trial, compared with men in the testosterone group (Budoff *et al.*, 2017). By contrast, in a 3 year RCT testosterone treatment did not alter carotid intima-media thickness (Basaria *et al.*, 2015). In the T-Trials itself with 790 men allocated to either testosterone or placebo for 12 months, there were 7 major cardiovascular adverse events in each arm of the study (Snyder *et al.*, 2016), a low overall rate of events with no signal for any excess risk in the testosterone arm of the trial. Analyses of testosterone RCTs which have reported cardiovascular adverse events have generally not shown any increase in cardiovascular events associated with testosterone therapy (Albert and Morley, 2016; Onasanya *et al.*, 2016; Alexander *et al.*, 2017). However, in one analysis there was a possible signal for adverse events in the first 12 months of treatment predominantly in men aged ≥65 years (Albert and Morley, 2016).

Therefore, at present it is not possible to draw any definitive conclusion as to whether testosterone treatment would have a beneficial, neutral or adverse effect on the incidence of cardiovascular disease in older men. Epidemiological data suggest an association of endogenous testosterone with reduced cardiovascular risk, but a RCT with cardiovascular events as the prespecified primary endpoint is needed to provide clarity. Pending such an RCT it would be prudent to counsel older men particularly those

with preexisting cardiovascular disease that there is an evidence gap and there may be a possibility of precipitating adverse events with treatment, and to ensure that cardiovascular risk factors and disease are optimally managed irrespective of whether testosterone therapy is being considered.

Diagnosis of Hypogonadism in Elderly Men

The diagnosis of hypogonadism in elderly men should be made following a careful clinical assessment to determine whether symptoms and signs of androgen deficiency are present (Yeap *et al.*, 2016a). A comprehensive medical history including details of any systemic illnesses, pituitary conditions or testicular surgery or injury, use of medications including glucocorticoids and opioids, and a full physical examination are essential elements. In the context of an aging population there will be increasing proportions of elderly men with existing medical comorbidities contributing to reduced health and well-being (Vos, 2015). Thus several nonspecific symptoms of androgen deficiency such as lethargy, fatigue, low mood, impaired short term memory and sleepiness may overlap with symptoms often found in elderly men even in the absence of disease affecting the gonadal axis (see **Table 1**). Organ-specific symptoms need to be interpreted with care: osteoporosis and sarcopenia are common in elderly men and whilst their presence should prompt evaluation for androgen deficiency, they may be due to other causes (Johnell and Kanis, 2005; Ebeling, 2008; Rolland *et al.*, 2008). The symptom of decreased libido should also be interpreted in the context of aging: sexual activity remains an important consideration for many men in their early seventies, but less so for men in their late 80s and early 90s (Hyde *et al.*, 2010a). The time course over which symptoms have evolved and additional information from partners can be informative. A thorough physical examination should be conducted which includes assessment of lean and fat mass, presence of gynaecomastia, and testicular volumes.

In men with symptoms and signs indicative of androgen deficiency, confirmatory biochemical testing is indicated. Collection of an early morning blood sample for assay of testosterone together with LH and follicle-stimulating hormone (FSH) concentrations is recommended (Yeap *et al.*, 2016a). An accurate and reliable testosterone assay is important: automated immunoassays can exhibit nonspecificity and method dependent bias and mass spectrometry is preferred where available (Taieb *et al.*, 2003). Elderly men as a group will have lower testosterone concentrations compared with middle-aged or younger men (Handelsman *et al.*, 2015, 2016). Therefore an age-appropriate reference range for circulating testosterone should be utilized. Bearing in mind that each result needs to be interpreted with reference to the individual patient and that reference ranges are not necessarily 100% sensitive and specific, lower cut-offs for serum testosterone concentrations are reported as 10.4 nmol/L in reproductively normal men aged 21–35 years and 6.4 nmol/L in very healthy men aged 70–89 years (Sikaris *et al.*, 2005; Yeap *et al.*, 2012b). For men between the ages of 35 and 70 years interpolation would provide a cut-off at 8 nmol/L, which is concordant with previous cohort studies (Travison *et al.*, 2017). Confirmatory testing should be performed also with an early morning sample and fasting if practicable. In the setting of a low circulating testosterone, elevated LH, and FSH are indicative of primary testicular disease. The role of age-appropriate cut-offs for elevated LH is unclear: conventional immunoassays typically cite an upper cut-off of 8 IU/L whereas a value of 9.4 IU/L has been reported from a reference group of 40–44 year old men (Wu *et al.*, 2010) and one of 16 IU/L from a reference group of 71–87 year old men (Yeap *et al.*, 2018).

Testosterone circulates bound to SHBG and also to albumin with a small fraction unbound or free with ongoing controversy over the clinical utility of free testosterone concentrations for the evaluation of hypogonadism (Goldman *et al.*, 2017; Handelsman, 2017). Free testosterone concentration has been proposed as a potentially informative marker of androgen status in men with total testosterone concentrations near the lower limit of normal with alterations in SHBG concentrations (Bhasin *et al.*, 2010; Goldman *et al.*, 2017). In an epidemiological study of men aged 40–79 years, calculated free testosterone was associated with sexual symptoms even in the presence of normal (total) testosterone (Antonio *et al.*, 2016). However there are two major limitations of using free testosterone concentrations for clinical diagnostic purposes. Firstly, to measure free testosterone using equilibrium dialysis is labor-intensive (and expensive) therefore most laboratories calculate free testosterone from total testosterone and SHBG with results that can be variable and not wholly accurate (Sartorius *et al.*, 2009; Ly *et al.*, 2010). Secondly, validated reference ranges for calculated free testosterone are lacking, and such reference ranges would need to specify and utilize

Table 1 Presenting clinical features of androgen deficiency (postpubertal onset)

Nonspecific symptoms	*Organ specific symptoms*
Lethargy, fatigue	Bone: osteopenia, osteoporosis, fracture/loss of height
Decreased energy and/or endurance	Muscle: reduced muscle mass and strength (sarcopenia)
Low mood, irritability, poor concentration, impaired short-term memory, sleepiness	Adipose: increased fat mass
Deteriorating work performance	Gynecomastia
Hot flushes	Sexual/reproductive:
	Decreased libido
	Erectile dysfunction (uncommon as a presenting feature of androgen deficiency, and then only at very low serum testosterone)

both accurate measurement of total testosterone and accurate calculation of free testosterone. An alternative approach is to interpret the (total) testosterone result in the light of SHBG concentrations. SHBG concentrations can be markedly elevated by hyperthyroidism, liver disease and antiepileptic medications, or suppressed in the setting of obesity, insulin resistance and exposure to exogenous androgens (Yeap *et al.*, 2016a). In the first scenario, (total) testosterone concentrations may be high without androgen excess being present, in the second scenario (total) testosterone concentrations may be low without necessarily being indicative of androgen deficiency.

Testosterone Therapy in Elderly Men—Indications

Testosterone therapy should be considered in elderly men who have the clinical syndrome of androgen deficiency due to underlying hypothalamic, pituitary or testicular disease (see **Table 2**). In such men the indication for testosterone therapy would be to improve the symptoms and signs of androgen deficiency (Yeap *et al.*, 2016a). Acquired causes of androgen deficiency are more likely to present in older age. Of note, hypogonadism may present with both reduced fertility due to impaired spermatogenesis as well as with androgen deficiency from inadequate testosterone production: in such situations management of fertility precedes consideration of testosterone replacement therapy (Yeap *et al.*, 2016a). In elderly men, fertility is unlikely to be a presenting complaint, whereas the age of children fathered implies a timeframe for when gonadal function was previously adequate.

Age per se should not be regarded as a barrier to testosterone treatment in elderly men with androgen deficiency from hypothalamic, pituitary or testicular disease. However, age and the presence of a circulating testosterone that is low when compared to younger men, is not an indication for testosterone therapy in the absence of pathological hypogonadism (**Table 2**). Similarly the presence of sexual dysfunction or nonspecific symptoms in the absence of proven androgen deficiency is not an indication for testosterone therapy. Elderly men with LOH, or those with low testosterone in the setting of obesity or medical comorbidities, should be encouraged to adopt healthy lifestyle behaviors and have existing comorbidities optimally managed with more evidence needed to determine whether testosterone therapy would be efficacious and safe in that setting (Huhtaniemi, 2014; Grossmann and Matsumoto, 2017). Treatment with glucocorticoids or opioids is associated with reduction in function of the endogenous hypothalamic-pituitary-gonadal axis (Morrison *et al.*, 1994; Fraser *et al.*, 2009; Vuong *et al.*, 2010). Where ongoing treatment with glucocorticoids or opioids at substantive doses is medically indicated and symptoms or signs suggestive of androgen deficiency are present, specialist endocrine review to provide individualized assessment and care may be recommended.

The T-Trials enrolled men aged ≥ 65 years with baseline testosterone < 9.5 nmol/L who had symptoms consistent with, but not diagnostic of, hypogonadism (Snyder *et al.*, 2016). Those men did not have apparent hypothalamic, pituitary or testicular disease. Testosterone treatment in the T-Trials resulted in a modest improvement in sexual function although the difference between testosterone and placebo groups was diminishing at 12 months (Snyder *et al.*, 2016). Improvements in anemia and bone density were seen, with negative primary outcomes for the physical function, vitality, and cognition trials (Snyder *et al.*, 2016, 2017; Roy *et al.*, 2017; Resnick *et al.*, 2017). These findings provide important information on the effects of testosterone therapy, but in isolation are insufficient to constitute a new indication for testosterone therapy in the general population of elderly men. The T-Trials were conducted in response to the recommendation from the Institute of Medicine for efficacy trials to establish whether testosterone therapy would benefit this group of men (Liverman and Blazer, 2004). The second part of the Institute of Medicine's recommendation was for a larger trial to determine possible risks only if benefits were found. It is this second, larger trial that is now awaited before the benefits and risks of treating elderly men with testosterone in the absence of hypothalamic, pituitary or testicular disease can be fully defined.

Table 2 Use and misuse of androgens

Use: physiological treatment with testosterone replacement for androgen deficiency in men with pathological hypogonadism	*Subject of ongoing research: use in these conditions remains to be fully evaluated for safety and efficacy in randomized placebo-controlled clinical trials*	*Misuse: use without a valid medical indication*
Primary testicular failure • Klinefelter syndrome • Testicular trauma, torsion, removal • Testicular infection • Testis atrophy of any cause Hypogonadotrophic hypogonadism (Secondary testicular failure) • Congenital: Kallmann's, variants without anosmia • Acquired: pituitary tumor[a], surgery, radiotherapy	• Middle aged and older men • Androgen deficiency secondary to chronic disease and ill-health • Hormonal male contraception	• Male infertility • Sexual dysfunction/impotence (in the absence of proven androgen deficiency) • "Male menopause," "andropause," "low T," "late onset hypogonadism" • Nonspecific symptoms (lethargy, tiredness, low energy)

[a]In the case of prolactinoma, dopamine agonist therapy may normalize testosterone concentrations without requirement for testosterone therapy.

Testosterone Therapy in Elderly Men—Expected Benefits

In men who are hypogonadal due to underlying hypothalamic, pituitary or testicular disease, the expected benefit of testosterone therapy is to improve the symptoms and signs of androgen deficiency (**Table 1**). Many of these beneficial effects will extend across the spectrum of middle-aged to elderly men. With regard to sexual symptoms such as loss of libido, in the T-Trials there was an improvement in sexual function spanning a range of sexual behaviors in testosterone-treated men (Cunningham *et al.*, 2016). Clearly cohabitation with a willing partner is a potentially vital modulator of this effect (Hyde *et al.*, 2010a). With regard to reduced muscle mass and strength, older men are as responsive as younger men to the anabolic action of testosterone (Bhasin *et al.*, 2005; Page *et al.*, 2005). With respect to bone mineral density, older men with low–normal testosterone concentrations exhibit increases in bone mineral density in response to testosterone (Amory *et al.*, 2004; Basurto *et al.*, 2008). Frankly hypogonadal men show a similar improvement with the effects of testosterone more prominent in trabecular bone (Benito *et al.*, 2005). Therefore elderly men with pathological hypogonadism should expect improvement in the clinical features of androgen deficiency with appropriate testosterone replacement therapy.

Of note, nonspecific symptoms and even organ-specific symptoms which are due not to androgen deficiency but to other medical conditions, should not be expected to respond comprehensively to testosterone therapy (**Table 1**). Symptoms such as lethargy, fatigue, reduced energy and decreased endurance are symptoms of many medical illnesses which are common in elderly men. Clearly such underlying medical conditions need to be identified and appropriately treated in men whether or not testosterone therapy is being considered. The T-Trials vitality substudy reported a negative primary outcome (Snyder *et al.*, 2016), which may reflect the fact that multiple comorbidities contribute to symptoms such as fatigue. Of note, despite there being an association between low circulating testosterone and poorer performance on tests of cognitive function in aging men (for review, see Yeap, 2014), the T-Trials cognition substudy did not demonstrate any benefit of testosterone therapy in men classified as having age-associated memory impairment (Resnick *et al.*, 2017). This is concordant with two other recent RCTs in older men which found no benefit of testosterone therapy on cognition (Emmelot-Vonk *et al.*, 2008; Huang *et al.*, 2016). Therefore, while it is possible that men who are overtly hypogonadal with very low circulating testosterone concentrations might show improvement in symptoms relating to concentration or short-term memory when testosterone treatment is instituted, RCTs in men without overt pathological hypogonadism do not support a beneficial role for testosterone in that setting.

As noted earlier, sarcopenia and osteoporosis are manifestations of androgen deficiency but are also common in elderly men for a range of reasons even when there overt gonadal pathology is absent (Ahmed *et al.*, 2007; Ebeling, 2008). Factors such as poor nutrition, lack of physical activity, presence of systemic illnesses or other medical conditions such as thyrotoxicosis may contribute to weight loss and osteoporosis (Rolland *et al.*, 2008; Ebeling, 2008). Therefore, the presence of sarcopenia, or osteopenia or osteoporosis in elderly men should prompt clinical evaluation for possible underlying causes, with androgen deficiency being an important cause not to be overlooked. An improvement in muscle mass and in bone mineral density would be an expected benefit of testosterone therapy in elderly men with androgen deficiency, particularly when other contributing factors are absent or appropriately managed.

Contraindications, Precautions, and Potential Adverse Effects

The presence of hormone-sensitive malignancy such as prostate or breast cancer is a recognized contraindication to testosterone therapy in men (**Table 3**). Prostate cancer is more prevalent in elderly men compared to younger or middle-aged men (Heinzer and Steuber, 2009). Therefore it is advisable to screen elderly men for the presence of prostate cancer using digital rectal examination and measurement of prostate specific antigen (PSA) prior to commencing testosterone therapy (Yeap *et al.*, 2016b). In systematic reviews and meta-analyses of testosterone RCTs in men across a range of ages with duration up to 3 years, testosterone therapy was not associated with increased incidence of prostate cancer (Fernandez-Balsells *et al.*, 2010; Cui *et al.*, 2014). A similar result was

Table 3 Contraindications and precautions to testosterone treatment

Contraindications	*Precautions*
Advanced, metastatic or incurable prostate cancer Breast cancer	Undiagnosed palpable prostate abnormalities, with or without elevated serum PSA[a] Severe lower urinary tract symptoms (International Prostate Symptom Score >19) Untreated polycythemia Untreated severe obstructive sleep apnoea[b] Unstable or inadequately treated cardiac disease (e.g., poorly controlled cardiac failure or ischemia, recent cardiovascular events) When fertility is desired When subject to occupational drug testing

[a]Urological evaluation may be required. Testosterone treatment may be acceptable in men with screen-detected organ-specific prostate cancer after definitive or clinically adequate prostate treatment.

[b]Testosterone treatment only transiently worsens severity of obstructive sleep apnoea: this is not an absolute contraindication.

reported from a registry study of newly-diagnosed hypogonadal men comparing men treated with testosterone with those who did not receive treatment over a period of 3 years (Debruyne *et al.*, 2017). Benign prostatic hyperplasia is also more common in elderly men (Berry *et al.*, 1984). Thus the presence of severe lower urinary tract symptoms which might relate to impediment of urinary outflow due to benign prostatic hyperplasia is a precaution to be considered (**Table 3**). Urological review may be required where undiagnosed palpable prostate abnormalities are present or an elevated PSA is found.

As testosterone may increase hemoglobin concentrations (Fernandez-Balsells *et al.*, 2010), untreated polycythemia needs to be evaluated and managed prior to considering testosterone therapy. Conversely anemia, whether related to nutrient deficiency, chronic inflammation or renal disease or of uncertain etiology, is more prevalent in elderly compared to younger or middle-aged men (Guralnik *et al.*, 2004). Testosterone therapy in the T-Trials increased hemoglobin concentrations and improved anemia in those older men (Roy *et al.*, 2017). Thus an increase in hemoglobin concentration may be an ancillary benefit of testosterone therapy in elderly men with low–normal hemoglobin concentrations. Obstructive sleep apnoea may itself result in symptoms of fatigue and obesity is a risk factor for both sleep apnoea and lower testosterone concentrations (Wittert, 2014). Testosterone therapy only transiently worsens the severity of obstructive sleep apnoea (Hoyos *et al.*, 2012; Killick *et al.*, 2013) therefore the presence of untreated severe obstructive sleep apnoea can be regarded as a precaution rather than an absolute contraindication to testosterone therapy (**Table 3**). Given the ongoing debate over the effects of exogenous testosterone on the cardiovascular system and the possible concerns over adverse effects in older men, cardiovascular risk factors and disease should be adequately managed and stabilized prior to consideration of testosterone therapy. Desire for fertility and occupational drug testing are less likely to be of concern to elderly men.

As with any medical intervention, potential adverse effects of treatment need to be considered. Polycythemia and elevation in PSA are recognized adverse effects of testosterone therapy (Fernandez-Balsells *et al.*, 2010; Yeap *et al.*, 2016b). Appropriate safety monitoring for hemoglobin concentration, hematocrit, and PSA should be undertaken. A small reduction in high-density lipoprotein cholesterol may be noted (Fernandez-Balsells *et al.*, 2010). Gynaecomastia, male pattern balding, weight gain, changes in mood or libido, difficulty passing urine, muscle pain, priapism and increased blood pressure are recognized potential adverse events (Yeap *et al.*, 2016b). Postinjection cough due to pulmonary oil microembolism is an uncommon but recognized side effect of depot intramuscular testosterone injections (Middleton *et al.*, 2015), and some men report pain following injection (Sartorius *et al.*, 2010). Men using transdermal formulations must be educated on appropriate steps to prevent inadvertent transfer of residual medication to others by skin contact.

In elderly men testosterone therapy can be monitored clinically by improvement in symptoms and signs of androgen deficiency, and also by measurement of testosterone concentrations achieved on treatment. Generally trough testosterone concentrations are assessed prior to the next injection of long-acting depot testosterone, or in the morning prior to the next application of daily transdermal formulations (Yeap *et al.*, 2016b). Given that higher starting doses of testosterone have been associated with adverse events in men $\geq$65 years with limited mobility (Basaria *et al.*, 2010), these should be avoided when treating elderly men. In an epidemiological study of men aged 70–89 years, there was a U-shaped association of endogenous circulating testosterone with mortality with optimal survival seen in men with testosterone concentrations in the middle of the range (9.8–15.8 nmol/L) (Yeap *et al.*, 2014b). Ideally RCTs should be conducted to test the longer term efficacy and safety of different dosing regimens of testosterone in elderly men. Pending the availability of such information, it seems prudent to maintain testosterone concentrations within the physiological range for elderly men, likely with trough concentrations toward the lower part of that range. Excessive doses that might result in elevated concentrations of testosterone should be avoided.

Conclusions and Priorities for Future Research

In men, circulating testosterone concentrations decline with age. In elderly men, nonspecific symptoms such as lethargy, fatigue, decreased energy, decreased sexual activity, and the presence of sarcopenia or osteoporosis are not uncommon and may or may not be due to androgen deficiency. Careful clinical assessment is warranted including evaluation for systemic illnesses and medication use. Biochemical confirmation of androgen deficiency should be via early morning blood sampling and measurement of testosterone concentrations using an accurate assay, preferably mass spectrometry where available. Age-appropriate reference ranges should be used to interpret testosterone results in conjunction with LH and SHBG. Men with the clinical syndrome of androgen deficiency due to underlying hypothalamic, pituitary or testicular disease merit appropriate investigation and treatment. Testosterone therapy should be considered in such men and age alone is not a barrier to treatment. Expected benefits of testosterone therapy are resolution of symptoms androgen deficiency, and an increase in lean mass and bone mineral density. Prior to commencing testosterone treatment, elderly men should be screened for underlying prostate disease and have management for cardiovascular risk factors and disease optimized. Ongoing monitoring for efficacy and safety of testosterone therapy should be undertaken.

In elderly men, low testosterone concentrations predict poorer health outcomes including frailty, osteoporosis and cardiovascular events. However, the role of testosterone therapy in elderly men with low testosterone concentrations compared to younger men in the absence of hypothalamic, pituitary or testicular disease remains uncertain. The T-Trials have shown a modest benefit of testosterone therapy for sexual function, and improvements in bone density and anemia, in men aged $\geq$65 years with baseline testosterone $<$9.5 nmol/L with symptoms consistent with but not diagnostic of hypogonadism. These results do not provide an indication for testosterone therapy, as further evidence needs to be provided regarding longer term benefits and risks. Priorities for future research are to clarify the longer term benefits and risks of testosterone treatment in elderly men in the absence of hypothalamic, pituitary or testicular disease, particularly whether in this context testosterone therapy has a beneficial, neutral or adverse effect on the cardiovascular system.

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See also: Androgens and Benign Prostatic Hyperplasia. Gonadotropins and Testicular Function in Aging. Hormone Replacement Therapy in Men. Male Sexual Dysfunction. Prostate Cancer. Sexual Function in Aging Men

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Sexual Function in Aging Men

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Abbreviations

5αr	5 alpha reductase
Cft	Calculated free testosterone
Cvd	Cardiovascular disease
Dht	Dihydrotestosterone
D-psv	Dynamic peak systolic velocity
Emas	European male aging study
Fsh	Follicle stimulating hormone
Ft	Free testosterone
Gnrh	Gonadotropin releasing hormone
Hg	Hypogonadism
Hpt	Hypothalamus–pituitary-testicular
Iief-efd	International index of erectile function-erectile function domain
Lcd	Low calorie diet
Lh	Luteinizing hormone
Loh	Late onset hypogonadism
Mace	Major adverse cardiovascular events
Nshap	National social life, health and aging project
Pge_1	Prostaglandin e_1
Rct	Randomized clinical trial
Shbg	Sex hormone binding globulin
Siedy	Structured interview on erectile dysfunction
T	Testosterone
Tt	Total testosterone
Tth	Testosterone therapy

Introduction

The Encyclopedia Britannica defines aging as "progressive physiological changes in an organism that lead to senescence or a decline of biological functions and of the organism's ability to adapt to metabolic stress" (https://www.britannica.com/science/aging-life-process). In fact, for evolutionary biologists aging is an inevitable process that results in a progressive structural and functional decline from the cellular level to the whole organism to adapt to environmental stress and changes. This leads, finally, to an increased age-specific mortality rate (i.e., a decrease in survival rate), along with a decrease in age-specific reproductive rate (Flatt, 2012). Hence, among the age-dependent or age-progressive decline in intrinsic physiological function there is the reduced ability to cope with stressors and to reproduce.

In most eukaryotic species, sexual reproduction is the leading strategy to allow reproduction and it is essentially based on sexual activity. Sexual activity consists of those physiological, anatomical, behavioral, and psychological functions that favor the union of male gamete to the female one, thus ensuring continuation of the species. Although it is not essential for individual survival, it is pivotal for species survival. The World Health Organization (WHO) recognizes that sexuality and reproduction are fundamental components of being healthy and human (World Health Organization, 2006). According to the WHO, sexual health is a state of physical, mental, and social wellbeing in relation to sexuality (World Health Organization, 2006). It requires a positive and respectful approach to sexuality and sexual relationships, as well as the possibility of having pleasurable and safe sexual experiences (World Health Organization, 2006). Considering that the aging population is growing worldwide, understanding senescence-associated modification in the sexual and reproductive attitudes represents a relevant task for the sanitary systems. In fact, sexual wellbeing is an often neglected dimension that may contribute directly and indirectly (via physical and emotional health) to successful aging.

Andropause Does Not Exist

In most mammalian species studied so far, there is a diminution of reproductive functioning during mid-through late-life (Packer *et al.*, 1998). In mammals, ovary and testis are the key actors orchestrating reproductive and sexual processes. These two endocrine glands secrete sexual hormones and produce mature germinal cells in a time- and gender-specific way. Ovary and testis are, however, very different since an early stage of fetal development and remain different lifelong. A palindromic gender translation of reproductive or sexual problems from one gender to the other is not a product of sexual revolution, that is, a change in the cultural perception of sexual morality and sexual behavior, but a mere medical mistake. A good example of this medical mistake is the exportation of the concept of the female menopause to male andropause. In the female, the number of germinal cells is determined prenatally, and during the post pubertal life, the continuous oocyte cell death is irreplaceable, finally leading to a true ovarian (germinal and endocrine) failure paralleled by the termination of menstruation (i.e., the menopause) well before it is reached the maximum lifespan of the species. This is not the case in males. In fact, in the testis, gametogenesis and steroidogenesis

do not abruptly stop and, in the large majority of cases, does not stop at all. Hence, andropause does not exist (Wu *et al.*, 2008; Perheentupa and Huhtaniemi, 2009). In fact, some men remain reproductively competent at ages approaching the maximum lifespan of the species. These biological differences might underpin some of the differences in the aging-associated modification in sexual life between genders. In this article, we will focus on sexual function in the aging men.

Testis Physiology and Aging

The male gonad, the testis, can be considered as a factory exporting two distinct groups of locally derived products: sex steroids and sperms. Sex steroids and spermatozoa are produced into two distinct areas within the testis (although in close proximity and in continuous cross talk): Leydig cells and seminiferous tubules. Active testicular production is stimulated by intra-testicular factors (including testosterone; T) and tuned by extra-testicular trophic hormones secreted by the pituitary, the gonadotrophins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). LH and FSH secretion from anterior pituitary is finely regulated by inhibitory influences coming from the testis itself (sex steroids and inhibin B) and stimulated by the secretion in the portal vessels of the pituitary stalk of a hypothalamic decapeptide, the gonadotropin-releasing hormone (GnRH). Among sex steroids produced by the testis, T is exported into the bloodstream in relatively high concentrations (10–30 nmol/L), mainly bound to a protein: sex hormone binding globulin (SHBG). Free T (FT) is the SHBG unbound fraction of total T (TT) and it represents its bioactive fraction. In target tissues, T bioactivity is increased by its reduction to dihydrotestosterone (DHT), through two distinct 5α reductase (5αR) isoforms, 5αR type 1 (SRD5A1), which is androgen-independent, and a more tightly androgen-controlled one, 5αR type 2 (SRD5A2). More recently, with the development of genome-wide profile analyses for gene expression, a third 5αR (SRD5A3) gene was identified, with a not yet characterized biological function. A small fraction of TT is converted to estrogen, through the action of P450 aromatase. T, either directly or through its effectors (DHT estrogens), exerts different actions throughout male life. In the fetus, T stimulates the differentiation of the Wolffian duct into male internal genitalia (epididymis, vas deferens, and seminal vesicles) and of the urogenital sinus in male external genitalia. At puberty, it induces the development of libido, enlargement of the vocal cords, muscle and bone mass, penile growth, and the initiation of spermatogenesis. During adult life, its main role is maintaining some of the aforementioned functions, however without resulting of vital relevance. In fact, lifespan of castrated individuals is not shorter from their eugonadal counterpart (Min *et al.*, 2012).

Male hypogonadism (HG) is the failure of the testis to produce sperm, sex steroids (T and, to a lower extent, estrogen), or both, because of a distant (pituitary/hypothalamus) or a local (testicular) failure. However, a HG-like syndrome can also derive from an impaired action of the sex steroids, as in the case of a resistance to androgens or estrogens, because of alterations in receptor sensitivity or in free hormone bioavailability. Based on these considerations, male HG can be classified in three main forms: primary HG (testis failure), secondary HG (hypothalamic/pituitary failure) or impaired androgen action (biological action failure). The most common form of impaired androgen action is due to increased level of SHBG (Rastrelli *et al.*, 2017).

Recently, HG has been also classified according to its reversibility and to the demonstration of an organic perturbation to the hypothalamus–pituitary-testis (HPT) axis (Grossmann and Matsumoto, 2017). When the latter condition is verified, HG is termed "organic"; otherwise, HG is considered "functional." In particular, functional HG is characterized by "no recognizable structural intrinsic HPT axis pathology" and by the lack of "pathologic aetiologies" (Grossmann and Matsumoto, 2017). Hence, it a diagnosis of exclusion. Because of its functional nature, it may be reversible (Grossmann and Matsumoto, 2017). Functional HG is often due to a deterioration of the HPT activity, due to morbidities impairing HPT axis function or to aging (Corona *et al.*, 2016; Grossmann and Matsumoto, 2017). In contrast to HG orchestrating its effect during prenatal life or puberty, HG with an exordium in late life (late onset HG, LOH), shows a scanty symptomatology, with a phenotype almost superimposable to that of eugonadal men. Symptoms and signs associated to LOH could be physical (weakness, decreased muscle mass, visceral obesity, osteoporosis), psychological (depression, fatigue, impaired memory) or sexual (decreased libido and impaired spontaneous or sex-related erections). In a cross-sectional survey of the European general population (the European Male Aging Study; EMAS), only a triad of sexual symptoms (low libido and reduced spontaneous and sex-related erections) syndromically associates with decreased T levels, whereas other proposed HG symptoms did not segregate with androgen deficiency (Wu *et al.*, 2010). This has been confirmed in a longitudinal extension of the same study, where it was demonstrated that worsening or development of sexual symptoms was associated with incipient secondary (Rastrelli *et al.*, 2015) or primary (Ahern *et al.*, 2016) HG. According to these and previous results (Rastrelli *et al.*, 2015; Ahern *et al.*, 2016) almost all the andrology Scientific Societies recommend the presence of sexual symptoms, along with the biochemical demonstration of low T, to define a clinically relevant LOH (Wang *et al.*, 2009; Bhasin *et al.*, 2010; Khera *et al.*, 2016).

By using ultrasonography, a recent retrospective research on 147 relatively healthy subjects indicate that testis volume decreased by only 2.8% from the fourth to seventh decade (Yang *et al.*, 2011). Accordingly, even if a T decline as a function of aging has been described in several longitudinal studies (Corona *et al.*, 2013a), this is relatively modest and clinically insignificant. In the European general population, an annual age trend of TT decrease was estimated of 0.09 nmol/L year (Wu *et al.*, 2008; Camacho *et al.*, 2013). Paternal aging is associated with a decrease in both the quality and quantity of spermatozoa, along with genetic and epigenetic changes in sperm structure (Gunes *et al.*, 2016). However, although some semen parameters decrease with age, no clinically significant alterations in the fertilizing capacity have been described in aging men (Perheentupa and Huhtaniemi, 2009). A detailed description of testicular alteration according to aging is behind of the scope of this review.

Sexual Activity in the Aging Couple

Although there is the common belief that a physiological sexual inertia characterizes the elderly state, epidemiological studies indicate that this is not always the case. The prevalence of sexual activity, behaviors, and problems in relation to aging was recently reinvestigated (Waite *et al.*, 2009; Lindau and Gavrilova, 2010) from two previously published US datasets: the national social life, health and aging project (NSHAP) and the national survey of midlife development in the United States 1995–96. The former is a national probability sample of 3005 adults (1550 women and 1455 men) 57–85 years of age (Lindau *et al.*, 2007), while the latter reports data from 3032 adults aged 25–74 (1561 women, 1471 men) (Lindau and Gavrilova, 2010). From the analysis of the two cohorts it was found that men were more likely to be sexually active than women at all ages. In particular, among the 75–85 year old group: 38.9% of men compared with 16.8% of women were sexually active, being sexual activity strongly associated with a reported very good or excellent health. In NSHAP (Lindau *et al.*, 2007), the most common sexual dysfunctions in aging men were erectile dysfunction (ED; 37%), lack of interest (28%), and inability to climax (27%). Conversely, the most prevalent sexual problems among women were low desire (43%), difficulty with vaginal lubrication (39%) and inability to climax (34%).

The Wisconsin longitudinal study is a study of the life course from late adolescence through the early to mid-1960s in a random sample of 10,317 men and women who graduated from Wisconsin high schools in 1957 (Syme *et al.*, 2013). In a case-control analysis Syme *et al.* (2013) reported that approximately two-thirds (64.2%) of subjects aged from 63 to 67 years had sex from once a month or less to once a day or more with males being more active than females. The latter data are in line with the aforementioned evidence suggesting that many older adults continue to enjoy, value, and engage in sexual activity.

The successful aging evaluation study is a population-based study on 606 community-dwelling adults in San Diego County, aged 50–99 years and who had a partner (Wang *et al.*, 2015). Over 80% of respondents had engaged in sexual activity in the past year, over 70% engaged in sexual activity weekly or more than once a week, and over 60% were somewhat or very satisfied with their sex lives. No sex differences were evident on dimensions of sexual health except for a higher rate of rejection of sexual overtures by women. Depressive symptoms were negatively associated with all assessed aspects of sexual health even after adjusting for confounders (Wang *et al.*, 2015).

In conclusion, aforementioned epidemiological evidence suggest that a large number of elderly men and women remain sexually active throughout their lives, although self-reported sexual activity is indeed more common in men, regardless of marital status and age. The validity of self-reported measures is however to be regarded with caution because quite often men over- and women under-estimate their sexual attitudes.

Sexual Problems in the Aging Man

The Krimpen study, a population-based study involving 1688 men enrolled in a Dutch municipality near Rotterdam, found the prevalence of ED increased from 3% in men aged 50–54 to 26% in men aged 70–78.25 (Blanker *et al.*, 2001). Similarly, the percentage of men who reported being sexually active declined with increasing age and was lower in men with ED and ejaculatory dysfunction and in those without a partner. In sexually active men, 17%–28% did not have normal erections, indicating that with advancing age, normal erections are not an absolute prerequisite for having sexual activity (Blanker *et al.*, 2001).

A more recent European survey (Corona *et al.*, 2010a) confirms that sexual problems are progressively increasing as a function of age, including ED (**Fig. 1**), low sexual desire and dissatisfaction with orgasm (**Table 1**). In the oldest age group (>70 years) two out of three men reports ED of moderate/severe entity. However, in the same age band, 49% report at least one sexual intercourse, 24% autoeroticism, 58% petting, and 75% thinking about sex. Hence, it is confirmed that valid erection are not a prerequisite for the continuation of sexual activity. In the same study, it was reported that only a fraction of men with ED has concerns about this sexual problem (**Fig. 1**). Interestingly, this fraction was higher at mid-life, declining thereafter and involving only a quarter of men over 70. In conclusion, whereas ED is a common problem in the aging male, only a minority is worried and, therefore, potentially asking for a medical consultation. In the EMAS study, the risk of ED was clearly associated to comorbidities, including organic (cardiovascular diseases, diabetes, obesity, lower urinary tract symptoms) and psychological (depression) disturbances (Corona *et al.*, 2010a). In the same survey, it was also found that relational factors, including satisfaction in the dyadic sexual relationship and partner's health, increase the risk of ED (Corona *et al.*, 2010a). Organic factors are less relevant for the other sexual complaints, while depression has a negative impact in all the aspect of sexuality of the aging male, including sexual desire, orgasm, and intercourse frequency.

Considering that ED is the most common form of sexual dysfunction but only for a minority of subjects, ED represents a bothering issue, it will be useful to describe the correlates of ED in subjects consulting an andrology service for ED.

Correlates of ED in the Aging Man Consulting for Sexual Dysfunction

SIEDY (Structured Interview for Erectile Dysfunction) is a structured interview we developed 15 years ago for the assessment of the components concurring with ED (Petrone *et al.*, 2003). It is a 13-item interview, with three factor analysis-derived scales, which

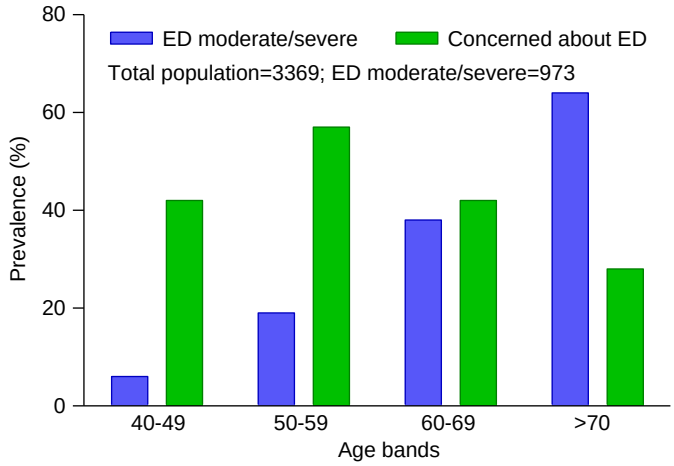

Fig. 1 Prevalence of moderate/severe erectile dysfunction (ED) and related concern as a function of age in the European male aging study population. Data adapted from Corona, G., Lee, D.M., Forti, G., et al. (2010a). Age-related changes in general and sexual health in middle-aged and older men: Results from the European male ageing study (EMAS). *The Journal of Sexual Medicine* **7**, 1362–1380.

Table 1 Prevalence of decreased sexual desire and related concern as a function of age in the European Male Ageing Study population

	Age bands			
	40–49 (n = 796)	*50–59 (n = 904)*	*60–69 (n = 839)*	*> 70 (n = 830)*
Thinking about sex less than once weekly	8.2% ($n = 64$)	17.5% ($n = 154$)	33.5% ($n = 273$)	52.8% (410)
Concerned about decreased sexual thoughts	14.1% ($n = 9$)	14.3% ($n = 22$)	18.3% ($n = 50$)	10.2% ($n = 42$)
Dissatisfied about timing of orgasm	4% ($n = 34$)	5% ($n = 45$)	10% ($n = 68$)	16% ($n = 82$)
Concerned about timing of orgasm	11.8% ($n = 4$)	42.2% ($n = 19$)	27.9% ($n = 19$)	19.5% ($n = 16$)

Data adapted from Corona, G., Lee, D.M., Forti, G., *et al.* (2010a). Age-related changes in general and sexual health in middle-aged and older men: Results From the European Male Ageing Study (EMAS). The Journal of Sexual Medicine 7, 1362–1380.

measure the organic (Scale 1), relational (Scale 2), and intrapsychic (Scale 3) components of ED. These three factors are often simultaneously present in ED patient and mutually contribute to the pathogenesis of ED. It is noteworthy that, in contrast with previously described instruments, scales of SIEDY were identified through factor analysis, and not predetermined by the authors Petrone *et al.* (2003). The ability of each scale in detecting organic, relational and intrapsychic problems in ED subjects was validated in subsequent studies (Boddi *et al.*, 2012; Corona *et al.*, 2012).

In a previous analysis on the contribution of the three scales in explaining ED as a function of aging, we found that both organic (Scale 1) and relational (Scale 2) issues were progressively increasing as a function of aging, whereas intrapsychic problems were more relevant in mid-life but declining thereafter (Corona *et al.*, 2013a). **Fig. 2** represents, as an error bar graph, the variation of each scale scoring (represented as percentage of maximal score) as a function of age bands, in a largest series of subjects than before. It is evident that organic (Scale 1) and relational (Scale 2) factors are the most relevant issues associated to ED in the elderly. However, in a previous study (Corona *et al.*, 2013a), we demonstrated that in the oldest tertile (60–88 years old) of the population referring for ED, the most relevant factor is Scale 1, that is, the organic component. Hence, we will focus on it.

Fig. 2 shows an impressive age-dependent decrease of penile blood flow, as assessed at penile color Doppler ultrasound, after PGE_1 (prostaglandin E_1) stimulation (dynamic peak systolic velocity, D-PSV) in our population. In fact, in the oldest age band, D-PSV is almost one half than in the youngest band. Considering that penile erection is a neurovascular phenomenon, this indicates that an objectively observed vascular impairment is orchestrating its effect in age-associated ED.

Age-dependent accumulation of morbidities could explain the high score of Scale 1 and the decrease of D-PSV that characterize male senescence. As shown in **Fig. 3**, in the European general population (EMAS study), the number of morbidities is progressively increasing as a function of age, being adverse cardiovascular conditions the most represented morbidities of the elderly (Corona *et al.*, 2010a). The link between organic ED and cardiovascular diseases (CVD) has been apparent since long time; however, only in the last 20 years ED has been recognized as a harbinger of underlying CVD, representing, therefore, a valid marker of risk for incident major adverse cardiovascular events (MACE) (Corona *et al.*, 2010b; Rastrelli *et al.*, 2014). It has been hypothesized that ED becomes evident earlier than MACE because the smaller penile arteries reach critical narrowing, with insufficient blood flow, earlier than larger vessels (Montorsi *et al.*, 2006).

We have collected longitudinal information regarding CVD in a subset of the previous population, attending our unit for ED for the first time between 2000 and 2007 (consecutive series of 1687 patients). The main characteristics of this study have been

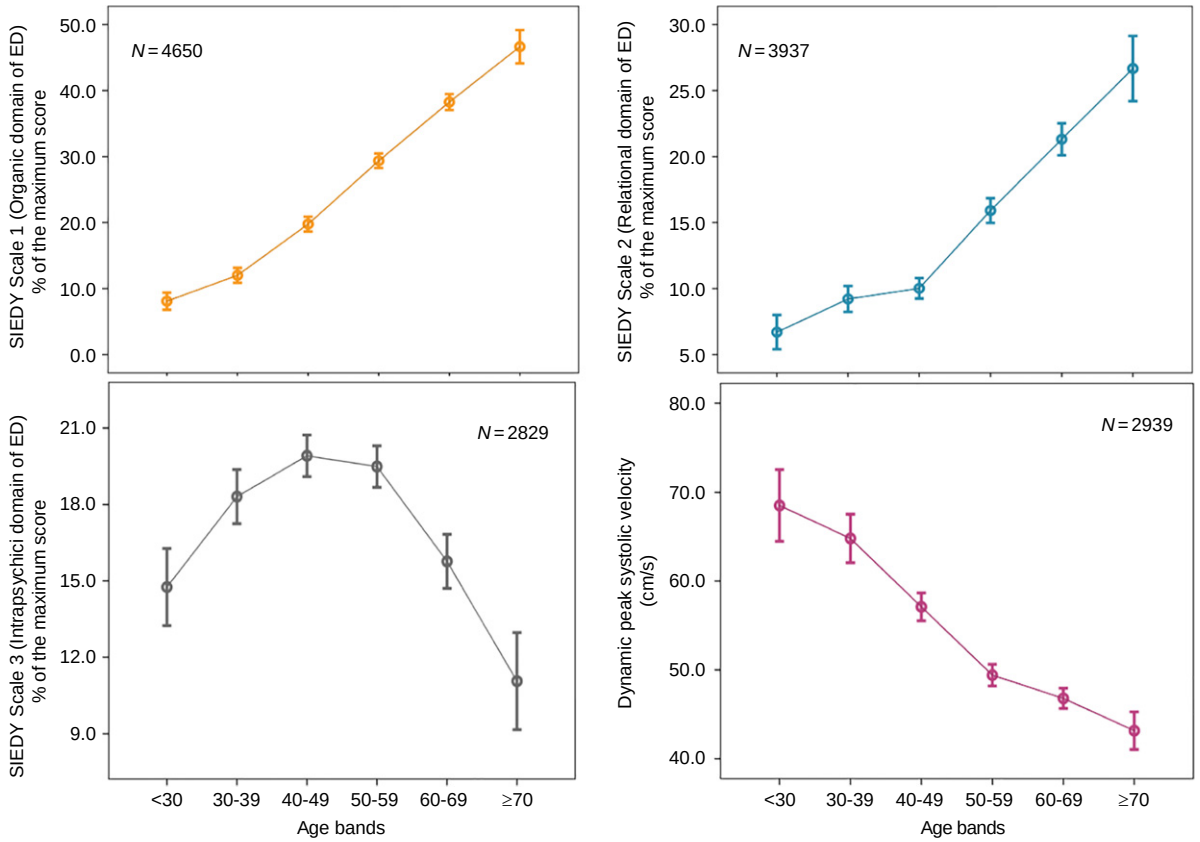

Fig. 2 Age related change in SIEDY scales and dynamic peak systolic velocity at penile color Doppler ultrasound in a population of men seeking medical care for sexual dysfunction at the Sexual Medicine and Andrology Unit of the University of Florence. The Structured Interview on Erectile DYsfunction (SIEDY) includes three scales, which quantify the organic (Scale 1), relational (Scale 2), and intrapsychic (Scale 3) pathogenic components of erectile dysfunction. The components are more important in erectile dysfunction pathogenesis as the scales increase.

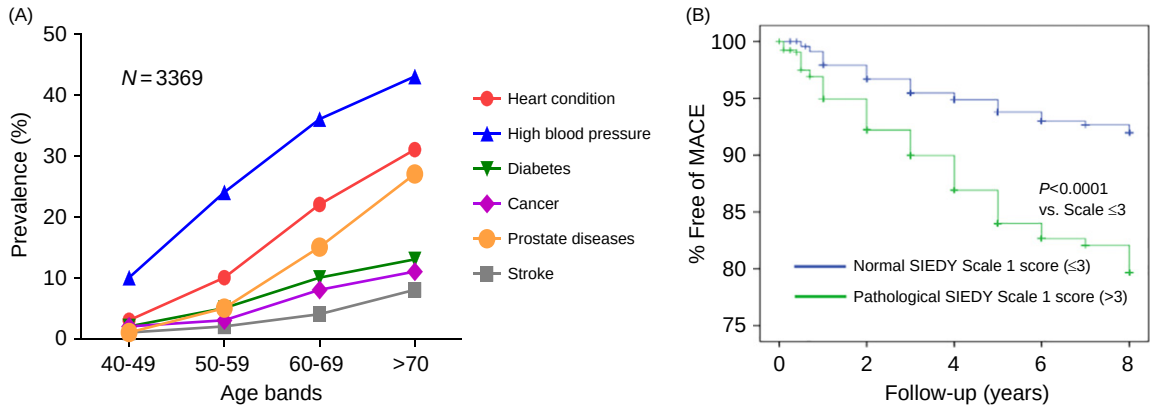

Fig. 3 (Panel A) Prevalence of several morbidities as a function of age in the European male aging study population. (Panel B) Percentage of men free from MACE (major adverse cardiovascular events) according to SIEDY Scale 1 score (organic pathogenic component of erectile dysfunction) in a population of 1687 men seeking medical care for sexual dysfunction followed-up for 4.3 ± 2.6 years. SIEDY: Structured Interview on Erectile Dysfunction. Data adapted from Corona, G., Lee, D.M., Forti, G., et al. (2010a). Age-related changes in general and sexual health in middle-aged and older men: Results from the European male ageing study (EMAS). *The Journal of Sexual Medicine* **7**, 1362–1380.

previously reported (Corona *et al.*, 2013a). Events were identified through the regional hospital discharge system and the City of Florence Register Office. Information on mortality up to 31 December 2007, including causes of death, was obtained from the City of Florence Registry Office, which contains complete and updated records of all persons living within city boundaries. During a mean follow up of 4.3 ± 2.6 years, 139 MACE, 15 of which were fatal, were observed, with a yearly rate of 0.23%. **Fig. 3** also

shows that, when the ED population was categorized according to a pathological score in Scale 1 (i.e., >3), the presence of an organic component of ED was associated with an increased risk of MACE.

Andro-Pause or Lean-Pause?

Low T represents another putative determinant of an impaired sexuality in the aging male. Two recent meta-analyses on the effect of T therapy (TTh) on sexual symptoms in randomized controlled trials (RCTs) involving HG subjects indicate that TTh significantly improved erectile function, libido, intercourse satisfaction, orgasm, and overall sexual satisfaction. This was evident when the effect of T on sexual function was explored in RCTs through different self-reported measures (Corona *et al.*, 2014) and when RCTs strictly using the International Index of Erectile Function (IIEF-EFD, i.e., the most frequently used validated tool to assess erectile function) was scrutinized (Corona *et al.*, 2017). We found that, on the average, TTh was able to improve IIEF-EFD score of 2.31 [1.41; 3.22] points, suggesting efficacy of clinical significance, at least in the milder ED form. Patients with more severe HG (TT <8 nmol/L) reported greater improvement in IIEF-EFD score when compared with those with a milder T deficiency. In addition, the effect of TTh on IIEF-EFD was less apparent in obese and diabetic subjects (Corona *et al.*, 2017), but not affected by age (Corona *et al.*, 2014). Conversely, TTh was completely without effect on ED when studies involving eugonadal subjects were selected (Corona *et al.*, 2014). These results indicate that, although TTh can be proposed at any age, it is meaningful only when an overt T deficiency is present and when other metabolic disturbances, such as diabetes and obesity, are absent, most probably because these morbidities are per se associated to ED, independently from low T.

Fig. 4 shows the age-dependent decline of TT in a large series ($n = 4883$) of subjects consulting our Center for sexual dysfunction. A stepwise decrease in TT is evident from the youngest age bands until mid-life. Thereafter, there is a more smoothed T decline. However, considering that SHBG is progressively increasing, the resultant fraction of unbound FT is linearly decreasing as a function of age also in the more aged individuals. In youngest subjects, the age-dependent T decline is not associated with a concomitant rise of LH, suggesting a central deficiency. Only in subjects older than 60 years there are evidence for a LH surge, indicating a testicular deficiency. Hence, it is possible to conclude that a mixed form of HG—due to a central and peripheral defect in T production along with an impairment of T bioactivity—is more often present in the aged male. However, several considerations should be done concerning aging and T deficiency. First, as shown in Fig. 4, the age-associated T decline never reaches, on average, a true pathological value and only approaches it in the oldest subjects, when calculated FT (cFT) is considered. Second, such a decline is not apparent when only obese individuals were considered. In fact, in nonobese individuals we observed a small, but significant, decrease in TT of 0.07 nmol/L per year, while in the obese

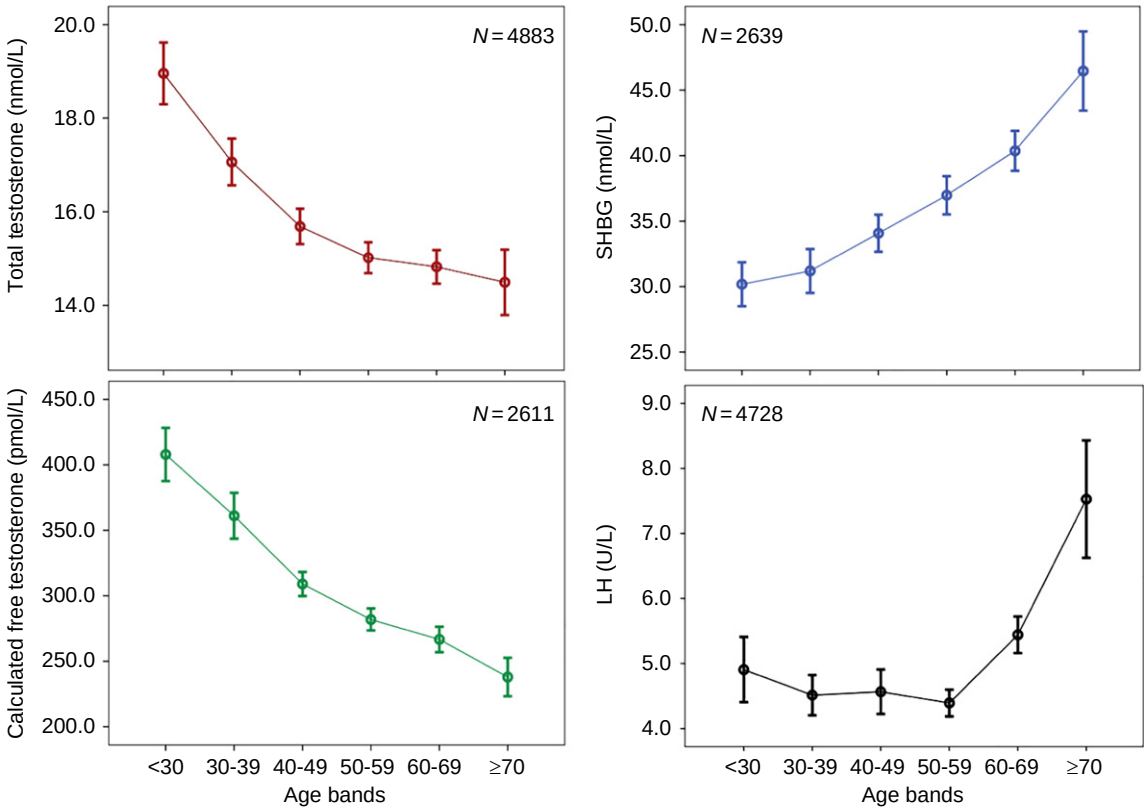

Fig. 4 Age related change in total and free testosterone, sex hormone binding globulin (SHBG) and luteinizing hormone (LH) in a population of men seeking medical care for sexual dysfunction at the Sexual Medicine and Andrology Unit of the University of Florence.

counterpart the regression line is almost flat with an annual decline of 0.02 nmol/L. This is tantamount to say that an obese young individual at the age of 20 years has a lower T than a lean subject at the age of 80 (13 vs. 14.3 nmol/L). This is even more evident when subjects were categorized according to waist circumference (102 cm). Hence, it is possible to say that more than an andropause the lean-pause is the real problem underlying T deficiency in the aging male. Considering that obesity, and in particular visceral obesity, is an age-dependent growing phenomenon (see **Fig. 5**), shifting from the regression line of obese individuals to that of lean subjects will greatly help to age more safely and with higher T levels. Intervention studies strongly support this point. A meta-analysis on the effect of low calorie diet (LCD) on TT indicates that LCD can induce an average increase of 2.87 (1.68–4.07) nmol/L (Corona *et al.*, 2013a,b). A greater T increase can be achieved through bariatric surgery, which results in an average increase of 9.19 (6.75–11.64) nmol/L and 88.85 (62.99–114.72) pmol/L in TT and cFT, respectively. The increase in T was accompanied by an increase in gonadotropin levels of more than one unit per liter (Corona *et al.*, 2013b), thus suggesting that weight loss partially restores HPT axis. This effect might be related to the decrease in circulating estradiol (on average 23 pmol/L), which could result in a decreased negative feedback on the axis (Corona *et al.*, 2015a,b). In obese men, also physical activity, in particular moderate-intense aerobic exercise, induces an increase in TT and cFT levels (Ari *et al.*, 2004; Hayes *et al.*, 2017; Kumagai *et al.*, 2017). Although reported by only one study, this was associated with an improvement in sexual functioning (Khoo *et al.*, 2013). The effect of physical activity on T seems to be greater than LCD (Kumagai *et al.*, 2016). Hence, lifestyle changes are strongly suggested as the first intervention in subjects with age-associated T decline, particularly in those with obesity.

Conclusions

A consistent fraction of aged males still consider sexuality as an important issue, although sexuality in the elderly might have behavioral, social and intrapsychic paradigms that differ from those typical of reproductive age. For instance, penetrative sexual intercourse might not necessarily reflect the hope and the need of aged individuals that can easily consider other forms of intimacy in their relationships. A fountain of youth does not exist, however for thirsty individuals to halt the senescence-associated degeneration of sexual function the only applicable formula is exercise more, eat less and adopt a healthier lifestyle.

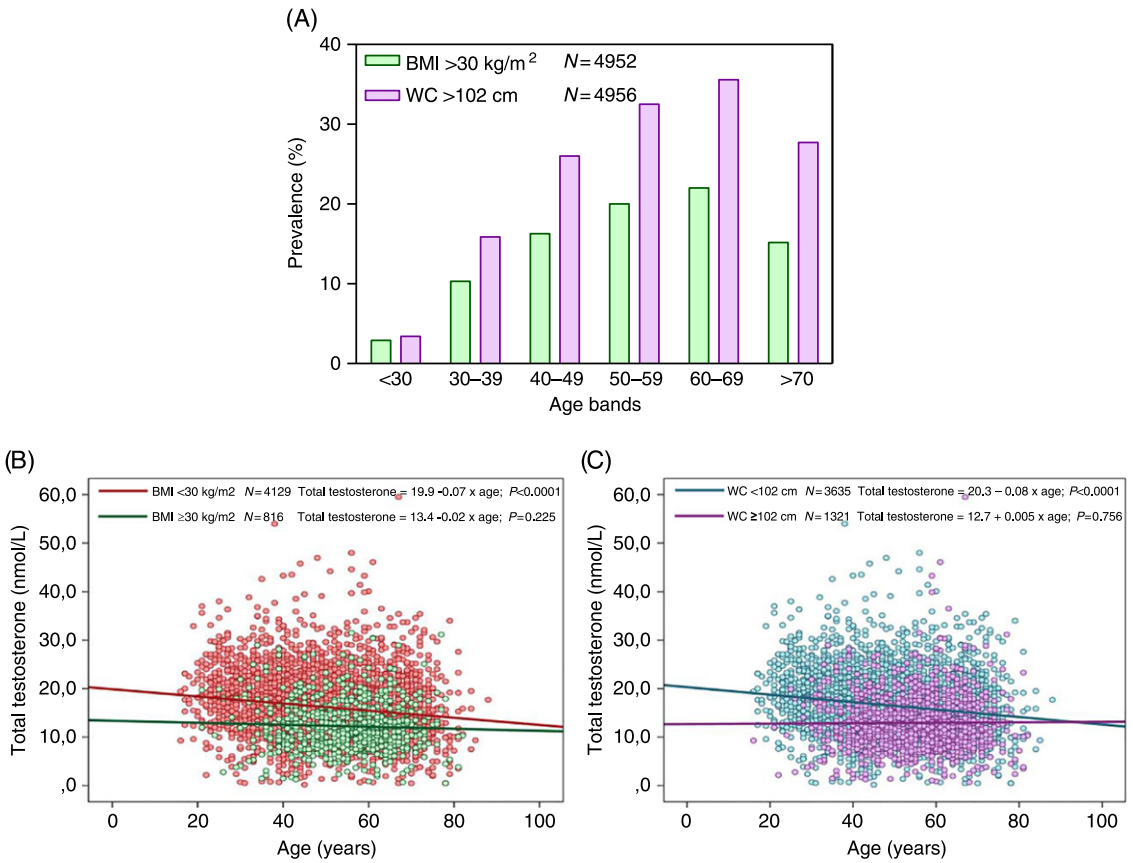

Fig. 5 (Panel A) Prevalence of obesity as defined by body mass index (BMI) or waist circumference (WC) as a function of age in a population of men seeking medical care for sexual dysfunction at the Sexual Medicine and Andrology Unit of the University of Florence. (Panel B and C) Age related change in total testosterone according to BMI or WC in a population of men seeking medical care for sexual dysfunction at the Sexual Medicine and Andrology Unit of the University of Florence.

See also: Hormone Replacement Therapy in Men. Hypogonadism and Testosterone Therapy in Elderly Men

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Osteoporosis in the Oldest Old

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Epidemiology of Osteoporosis and Osteoporotic Fractures in Old Age

Osteoporosis is a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and fracture risk (Cosman *et al.*, 2014). The incidence of osteoporotic fractures increases with age, and osteoporosis in old age is a challenge because of the extent of the problem and the major burden in terms of morbidity and mortality. Today, the cumulative incidence of hip and vertebral fractures in women at the age of 80 years is close to 30% and more than 40% respectively, and women over the age of 80 account for more than 30% of all osteoporotic fractures and for even more than 60% of all non-vertebral fractures (Cooper *et al.*, 1992; Grados *et al.*, 2004; Seeman *et al.*, 2010). In future, the burden of osteoporosis will only increase because of the aging of the population.

Thus, age is one of the main determinants of the absolute risk of fracture. It is also one of the main determinants of the type of osteoporotic fracture. Between the ages of 55 and 75, women are most at risk of vertebral fractures, but beyond the age of 75, they become increasingly at risk of nonvertebral fractures, such as hip fractures (Sambrook and Cooper, 2006).

Elderly Persons With Osteoporotic Fractures Are Frail

Osteoporosis and osteoporotic fractures tend to occur in a frail subset of the older population (Boonen *et al.*, 2004a). Indeed, elderly with osteoporotic fractures are not "average" elderly, but frail elderly with a high prevalence of underlying comorbidities and at risk of disabilities (Gielen *et al.*, 2012). This frailty is reflected in the poor postfracture outcomes of these patients. For example, 1 year after a hip fracture, 40% of the patients is still unable to walk independently, 33% is institutionalized or totally dependent and mortality is about 20% (Leibson *et al.*, 2002; Schnell *et al.*, 2010). Similar observations have been made in elderly with other types of nonvertebral fractures and even vertebral fractures (Gielen *et al.*, 2011). In hip fracture patients, the excess mortality is not only observed within the first year after the fracture but continues to be observed in the years thereafter because of the underlying comorbidities of these frail elderly (Haentjens *et al.*, 2010).

Diagnosis of Osteoporosis and Intervention Thresholds in Old Age

Despite the high prevalence and significant burden of osteoporosis in the elderly, this condition continues to be underdiagnosed and undertreated, even in those admitted to the hospital with documented fractures. This is particularly so in women over the age of 80 (Feldstein *et al.*, 2003).

Yet, the operational definition of osteoporosis also applies to the very elderly. The World Health Organization (WHO) defines osteoporosis as a bone mineral density (BMD) value at the lumbar spine or the proximal femur of at least -2.5 standard deviations (SD) below the peak bone mass of a young healthy individual (Kanis *et al.*, 1994). Dual energy X-ray absorptiometry (DXA) has, however, low sensitivity for fracture as the majority of fractures occurs in persons with a T-score above -2.5. This indicates that factors other than BMD contribute to fracture risk (Thomas-John *et al.*, 2009). In particular, an existing osteoporotic fracture is the most significant predictor of future fracture risk, doubling fracture risk compared to persons without previous fractures. Also age independently increases fracture risk, with, over a four SD interval of BMD ($+1$ to -3 SD), a 14-fold increase in hip fracture risk at the age of 50, but a 145-fold increase at the age of 80 (Melton *et al.*, 1999; Kanis *et al.*, 2001). Therefore, tools based on age and other clinical risk factors are increasingly being used to target osteoporosis therapy. Among these is the FRAX-algorithm, developed by the WHO Collaborating Centre for Metabolic Bone Disease at Sheffield, UK, which integrates the weight of various clinical risk factors for fractures, with or without femoral neck BMD, to estimate the 10-year probability of hip fracture and major osteoporotic fracture (clinical spine, forearm, hip or shoulder fracture) (FRAX ®). The objective of FRAX is to identify individuals at higher fracture risk who qualify for osteoporosis treatment. Three approaches can be distinguished, which, especially in the elderly, differ in the threshold above which treatment is recommended.

Fixed FRAX Intervention Threshold (National Osteoporosis Foundation (NOF), USA)

According to the NOF guidance, treatment is recommended in individuals with a prior hip or vertebral fracture and for those with osteoporosis (T-score ≤ -2.5) at the femoral neck, total hip or lumbar spine. In persons with osteopenia (T-score between -1.0 and -2.5), the 10-year fracture probability should be calculated and a fixed intervention threshold is recommended for all ages and both genders (Cosman *et al.*, 2014). This fixed intervention threshold is based on the 10-year hip fracture probability at which

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treatment becomes cost-effective (Kanis *et al.*, 2013; Tosteson *et al.*, 2008). Among women, this 10-year hip fracture probability ranges from 2.5% at the age of 50 to 4.7% at 75 years and among men from 2.4% at 50 years to 4.9% at 75 years. Thus, the intervention threshold slightly increases at older age, which can be explained by the increased competing mortality risks from other disorders at higher age (Tosteson *et al.*, 2008). The NOF guidance recommends treatment in a person with osteopenia and a 10-year probability of a major fracture of ≥20% or a hip fracture of ≥3% (Rizzoli *et al.*, 2014; Cosman *et al.*, 2014). Importantly, these specific intervention thresholds are valid in the United States but not in other regions of the world because of differences in the epidemiology of fracture risk, the health care budget, health economic considerations, practice guidelines and reimbursement criteria (Kanis *et al.*, 2016). Therefore, the thresholds need to be country-specific, although several national osteoporosis guidelines just recommend the thresholds for hip (≥3%) and major osteoporotic fractures (≥20%) of the NOF (Kanis *et al.*, 2013, 2016).

Age-Dependent FRAX Intervention Threshold (National Osteoporosis Guideline Group (NOGG), UK)

The position of the NOGG is to start osteoporosis treatment in an individual when his or her fracture probability exceeds the FRAX intervention threshold, defined as the age-specific fracture probability of a men or women with a previous fragility fracture, no other risk factors, an average BMI (24 kg/m^2) and without knowledge of BMD (Compston *et al.*, 2013; McCloskey *et al.*, 2015). The rationale is that, in general, a person with a previous fragility fracture is considered to qualify for treatment. Therefore, at any age, an individual with the same fracture probability, but without previous fracture, should also be eligible (Kanis *et al.*, 2016). Thus, the intervention threshold is the age-dependent "fracture threshold," and, by consequence, rises with age.

Hybrid FRAX Intervention Threshold

The age-dependent intervention threshold of the NOGG is designed to avoid under-prescription of treatment in young patients as well as over-prescription in older patients that could arise from a fixed threshold such as the intervention threshold of the NOF. The NOGG guideline, however, induces inequalities in access to therapy especially at older age, depending on the presence or absence of a prior fracture (McCloskey *et al.*, 2015; Kanis *et al.*, 2016). An age-dependent threshold indeed requires, for older persons without a previous fracture to be qualified for treatment, a higher fracture risk as compared to those qualifying for treatment because of a previous fracture (Johansson *et al.*, 2012). Therefore, an alternative threshold using a hybrid model has been proposed. According to this model, the threshold rises with age, but remains constant from the age of 70, being at 20% for a major osteoporotic fracture and 5.4% for a hip fracture (Kanis *et al.*, 2016; McCloskey *et al.*, 2015).

Treatment of Osteoporosis in the Oldest Old

In this section, we will evaluate the efficacy and safety of the treatment options for osteoporosis in the oldest old (≥80 years). Both non-pharmacological and pharmacological interventions will be discussed. Osteoporosis treatment should be proven to be effective in the elderly, not only against vertebral fractures but even more so against non-vertebral fractures, as these account for most of the morbidity and mortality. Treatment should also be safe in elderly who are frail, with underlying comorbidities and at increased risk of adverse events.

Physical Exercise Therapy in Old Age

Whether physical exercise reduces the incidence of osteoporotic fractures has, so far, not been assessed in well-designed randomized controlled trials (RCTs) with fractures as the primary end point. Limited evidence, however, suggests that physical exercise is associated with reduced fracture risk. In a meta-analysis of 10 trials (randomized or not), physical exercise significantly reduced overall but not vertebral fracture risk (RR = 0.49; 95% CI 0.31–0.76 and RR = 0.56; 95% CI 0.30–1.04 respectively) (Kemmler *et al.*, 2013). Another meta-analysis of 13 prospective cohort studies showed that moderate-to-vigorous physical activity is associated with reduced hip fracture risk in men and women (RR = 0.55; 95% CI 0.44–0.69 and RR = 0.62; 95% CI 0.56–0.69 respectively) (Moayyeri, 2008). It should be noted that both meta-analyses included young individuals (>45 and >40 years respectively) and did not focus exclusively on (very) elderly.

Physical exercise therapy may reduce the risk of fractures by increasing BMD and by improving sarcopenia, the age-related loss of muscle mass and function.

Physical exercise therapy increases BMD from childhood into old age

Reaching an optimal peak bone mass at the end of the critical period of bone accrual in childhood and young adulthood is an essential part in the prevention of fractures later in life. Physical exercise in *children* and *young adults* may be of major importance to reach this optimal peak bone mass (Gunter *et al.*, 2008; Magkos *et al.*, 2007; Martyn-St James and Carroll, 2010). After the critical period of bone accrual, physical exercise remains important in maintaining and even increasing BMD. Indeed, several meta-analyses have been published about the effect of physical exercise, such as impact exercises with mixed loading, walking or high-intensity resistance training, on BMD in *postmenopausal women* (Martyn-St James and Carroll, 2006, 2008, 2009; Ma *et al.*, 2013).

The most recent Cochrane review on this topic showed a relatively small, but statistically significant effect of physical activity on BMD in postmenopausal women (Howe *et al.*, 2011). The most effective interventions were a combined exercise program for lumbar spine BMD and progressive resistance training for femoral neck BMD. In 2012, the first systematic review with meta-analysis of physical exercises focusing on *older adults* has been published (Marques *et al.*, 2012). This meta-analysis includes 1577 subjects in 19 RCTs, with a median value for mean age of 69 years and a mean age ranging from 65 to 83 years. Exercises of mixed loading impact were associated with significant increases in BMD at the lumbar spine (weighted mean difference [WMD] = 0.011 g/cm^2, 95% CI 0.003–0.020; $P = .007$) and femoral neck (WMD = 0.016 g/cm^2, 95% CI 0.005–0.027; $P = .004$), while impact exercises combined with resistance training were effective at the lumbar spine (WMD = 0.016 g/cm^2; 95% CI 0.002–0.036; $P = .028$) (Marques *et al.*, 2012). Although these findings support the role of exercise to increase BMD at the lumbar spine and femoral neck in older adults with a mean age around 70 years, evidence in the *very elderly* is, to our knowledge, still very limited. In frail community-dwelling elderly with a mean age of 83 years, a 9-month exercise training program consisting of exercises to improve balance, flexibility and coordination as well as progressive resistance training and endurance exercises did not significantly increase BMD at the lumbar spine and proximal femur (Villareal *et al.*, 2004). There was, however, a trend toward reduction in BMD in the control group who only performed the balance, flexibility and coordination exercises. Several limitations, such as insufficient power, may, at least partly, explain the lack of significant results of this RCT (Villareal *et al.*, 2004). Thus, more research is needed to determine the effect of physical exercise on BMD in very old, frail individuals.

Effect of physical exercise therapy on sarcopenia

Physical exercise therapy may not only decrease fracture risk by increasing BMD, but also by decreasing the propensity to falls in the elderly, which is due, among other factors, to sarcopenia and impaired balance.

Numerous studies have shown that progressive resistance training is an effective intervention to improve muscle mass, muscle strength and functional limitations, even in the elderly (Liu and Latham, 2009; Peterson *et al.*, 2011). That even frail elderly may benefit from resistance training is clear from a randomized placebo-controlled trial of Fiatarone *et al.* (1990). 10 frail, institutionalized elderly aged 90 $\pm$1 years who undertook an 8-week progressive resistance training program improved their muscle strength by 174% ($\pm$31%) and muscle mass by 9.0% ($\pm$4.5%), while their mean gait speed improved by 48%. The training program consisted of three times a week three series of eight repetitions with a resistance of 80% of 1 repetition maximum (RM, the maximum weight that can be lifted). Progressive resistance training has also been reported to improve *balance performance*. In a systematic review of 29 RCTs, totalizing 2174 participants with a mean age of $\geq$60 years, 14 studies reported improvements -significantly greater than controls- in balance performance after progressive resistance training (Orr *et al.*, 2008).

In conclusion, physical exercise may increase BMD and improve sarcopenia and balance control, also in the (very) elderly. This supports the recommendation of physical training in order to, ultimately, reduce fracture risk. Although more research is needed to establish the optimal training schedule, the general recommendation is to perform a combination of aerobic exercise and resistance training, both targeted to sites predominantly affected by osteoporotic fractures. High-impact bone loading activities on 3 days a week have to greatest potential to increase BMD, but low to medium weight-bearing exercises on 5 days a week are recommended for those not used to exercising and those over 50 years. The intensity of resistance training should be moderate to high (60%–80% of 1 RM) in order to minimize the risk of injuries as well as to ensure a sufficient strength-building stimulus (Nelson *et al.*, 2007; Chodzko-Zajko *et al.*, 2009).

Calcium and Vitamin D Supplementation in Old Age

Notwithstanding some controversy about the threshold level of 25-hydroxyvitamin D (25OHD), vitamin D deficiency is commonly defined as a serum level of 25OHD below 20 ng/mL (50 nmoL/L) (Rosen *et al.*, 2012). Low levels of 25OHD occur in all age groups, but elderly are at increased risk of hypovitaminosis D because of low dietary intake and decreasing capacity of the skin to produce vitamin D_3, together with less outdoor activities and sun exposure (Lips, 2001; Holick *et al.*, 1989). Therefore, especially frail elderly and those in institutions have lower levels of 25OHD, with a gradual decline of 25OHD from healthy adults over independent elderly to hip fracture patients and institutionalized persons (Lips, 2001).

Calcium and vitamin D deficiency is one of the main determinants of bone loss and fracture risk in old age, and poor vitamin D status may also increase fracture risk by increasing the risk of falling through an effect on muscle strength (Scott *et al.*, 2010). Adequate calcium and vitamin D status is therefore essential in the prevention of bone loss and osteoporotic fractures. There is, however, much controversy about the threshold level of serum 25OHD for optimal bone health. The Task Force of the Endocrine Society recommends a serum 25OHD level above 30 ng/mL (75 nmoL/L), while the Institute of Medicine (IOM) recommends a daily intake of 800 IU of vitamin D for ages $\geq$71 years in order to achieve, in at least 97.5% of the population, a serum 25OHD level of 20 ng/mL (Holick *et al.*, 2011; Ross *et al.*, 2011; Institute of Medicine, 2011). A serum 25OHD level above 20 ng/mL is indeed sufficient to normalize surrogate endpoints of bone health such as 1.25-dihydroxyvitamin D ($1,25(OH)_2D_3$), parathyroid hormone (PTH), intestinal calcium absorption and BMD (Bouillon *et al.*, 2013).

There is, however, an ongoing intense debate whether vitamin D, in combination with calcium, does reduce the risk of osteoporotic fractures (Bolland *et al.*, 2014; Bischoff-Ferrari *et al.*, 2014). A recent meta-analysis for example found that vitamin D coadministered with calcium reduces hip fracture risk in institutionalized but not in community-dwelling elderly (Bolland *et al.*, 2014). This and other meta-analyses and individual RCTs may have failed to demonstrate a reduction in fracture risk with calcium

and vitamin D because of an insufficient dose of supplementation, therapeutic incompliance or lack of targeting of supplementation to persons at risk of vitamin D deficiency or a negative calcium balance (Jackson *et al.*, 2006; Grant *et al.*, 2005; Boonen *et al.*, 2006b; Bouillon *et al.*, 2013). Calcium and vitamin D supplementation indeed needs to be directed to persons with documented or at risk of calcium and/or vitamin D deficiency, which is, as mentioned before, highly prevalent in osteoporosis patients, home-bound frail elderly and institutionalized persons (Lips, 2001).

Thus, combined supplementation with calcium and vitamin D has become an essential component to reduce bone loss and fracture risk in elderly individuals. Osteoporosis treatment, on top of calcium and vitamin D, should be considered in elderly with osteoporosis and osteoporotic fractures.

Pharmacological Osteoporosis Treatment in Old Age

Issues concerning drug therapy in the elderly include reduced intestinal absorption, metabolism and excretion of drugs as well as polypharmacy, therapeutic incompliance and concomitant disorders (Rizzoli *et al.*, 2014). Aging is, for example, associated with a decline in renal function. Since $\pm$50% of the bisphosphonates are excreted by the kidneys, impaired renal function may result in accumulation of these drugs, which potentially affects efficacy and safety (Vondracek and Linnebur, 2009). In this section, the evidence in the oldest old about the efficacy (**Table 1**) and safety of the currently available osteoporosis treatment options will be discussed. This evidence is mostly based on subgroup analyses of the landmark osteoporosis trials that have been published in recent years.

Alendronate

Originally, the efficacy of Alendronate was established by the Fracture Intervention Trial (FIT) in postmenopausal women. The FIT Vertebral Fracture Arm (FIT I) included women with a prevalent vertebral fracture, while the FIT Clinical Fracture Arm (FIT II) included women with a T-score ≤ -1.6 at the femoral neck (mean age 70.8 years and 67.7 years, respectively) (Black *et al.*, 1996; Cummings *et al.*, 1998).

Two sub-analyses of the FIT trials have been undertaken to determine the anti-fracture efficacy and safety of Alendronate in the oldest old. A post hoc analysis of FIT I has evaluated the efficacy of Alendronate in postmenopausal women with the highest fracture risk, including a subgroup of women aged $\geq$75 years (range 75–82 years) (Ensrud *et al.*, 1997). After 3 years, Alendronate significantly reduced the risk of vertebral fracture by 38% (RR = 0.62; 95% CI 0.41–0.94) in women aged $\geq$75 years, compared to 51% in the younger population (RR = 0.49; 95% CI 0.35–0.68). To prevent one new vertebral fracture, eight women aged $\geq$75 years needed to be treated compared with nine women aged $<$75 years. In another analysis, based on pooled data from both FIT arms, age-specific fracture rates by treatment group (55 to $<$65 years, 65 to $<$70 years, 70 to $<$75 years and 75 to 85 years) were calculated (Hochberg *et al.*, 2005). Relative risk reductions for hip (RR = 0.47; 95% CI 0.27–0.81; $P < .01$) and vertebral (RR = 0.55; 95% CI 0.37–0.83; $P < .01$) fractures were constant among age groups, with an even greater absolute risk reduction as age increases, which can be explained by the age-related increase in fracture risk in the placebo group (Hochberg *et al.*, 2005).

Finally, a more recent study showed that Alendronate treatment in patients with a mean age of 82.4 $\pm$ 8.3 years and a prior fracture was associated with a reduced hip fracture risk (HR = 0.72; 95% CI 0.61–0.85; $P < .001$) (Axelsson *et al.*, 2016). The reduction in hip fracture risk was maintained across all age quartiles and the absolute risk reduction after 5 years increased substantially by quartile of age.

The post hoc analysis of FIT I and the pooled analysis from both FIT arms did not report safety data in the very elderly (Ensrud *et al.*, 1997; Hochberg *et al.*, 2005). In the more recent trial in fracture patients, adverse events such as esophagitis, dyspepsia or acid reflux were not more common in the higher than in the lower age quartiles (Axelsson *et al.*, 2016). These data indicate that Alendronate is a safe treatment option in the elderly, with proven vertebral and hip fracture reduction, and also indicates that elderly, who have a higher baseline fracture risk, benefit even more from osteoporosis treatment than younger persons.

Risedronate

The VERT (vertebral efficacy with risedronate therapy)-trials (VERT-NA and VERT-MN) have demonstrated the efficacy of risedronate to prevent vertebral and non-vertebral fractures in postmenopausal women with one or more prevalent vertebral fractures (mean age $\pm$70 years) (Harris *et al.*, 1999; Reginster *et al.*, 2000). In addition, the HIP-trial has investigated the effect of Risedronate on hip fracture risk in two different arms, of which the first arm included postmenopausal women with a mean age of 74 years (McClung *et al.*, 2001).

To determine the efficacy of risedronate in the very elderly, the second arm of the HIP-trial included 3886 women, aged $\geq$80 years (mean age 83 years). These elderly women were included based on the presence of a low BMD at the femoral neck or at least one nonskeletal risk factor for hip fracture (e.g., poor gait or a propensity to fall) (McClung *et al.*, 2001). After 3 years, no significant reduction in the risk of hip fractures was observed (RR = 0.8; 95% CI 0.6–1.2; $P = .35$). Importantly, the majority (58%) of the participants was included solely on the basis of a nonskeletal risk factor, while only 16% was included based on low BMD. A second analysis focusing on the elderly was a pooled analysis of the VERT-NA, VERT-MN and HIP-trials (Boonen *et al.*, 2004b). This analysis included 1392 women aged $\geq$80 years (mean age 83 years) with osteoporosis (T-score < -2.5 or at least one vertebral fracture). The risk of vertebral fractures was reduced by 44% (HR = 0.56; 95% CI 0.39–0.81; $P = .003$) after 3 years. For nonvertebral fractures, the incidence was not significantly different in the treatment group and the placebo group ($P = .66$).

Table 1 Relative risk (95% CI) of new vertebral, hip and nonvertebral fractures compared with placebo in very elderly women receiving currently available osteoporosis treatments

	RCT	*Included participants*	*N*	*Mean age (years)*	*Vertebral fractures*	*Hip fractures*	*Nonvertebral fractures*
Alendronate	Post hoc analysis FIT I (3 years) (Ensrud et al., 1997)	Women aged 75–82 y	539	Not specified	*RR = 0.62; 95% CI 0.41–0.94; P < .05* $p_{interact} < 75$ & ≥ 75 y NS	–	–
	Pooled analysis FIT I and FIT II (3–4 years) (Hochberg et al., 2005)	Women aged 55–80 y 55- < 65 y 65- < 70 y 70- < 75 y 75–85 y	3658		*RR = 0.55; 95% CI 0.37–0.83* (constant RR)	*RR = 0.47; 95% CI 0.27–0.81* (constant RR)	–
	Axelsson et al. (5 years) (Axelsson et al., 2016)	Women aged 71.1–92.3y with a prior fracture	110.190	82.4 y	–	*HR = 0.72; 95% CI: 0.61–0.85; P < .001*	–
Risedronate	HIP—arm 2 (3 years) (McClung et al., 2001)	Women aged $\geq$80 y with at least one nonskeletal risk factor for hip fracture or T-score at FN < -4 or $< -3 +$ hip axis length of $\geq$11.1 cm	3886	83 y	–	RR = 0.8; 95% CI 0.6–1.2; $P = 0.35$	10.8% (Risedronate) vs. 11.9% (placebo); $P = .43$
	Post hoc pooled analysis VERT-NA, VERT-MN and HIP (3 years) (Boonen et al., 2004b)	Women aged $\geq$80 y with T-score < -2.5 at FN or at least one prevalent vertebral fracture	1392	83 y	*HR = 0.56; 95% CI 0.39–0.81; P = .003* $p_{interact} < 80$ & ≥ 85 y NS	–	14.0% (Risedronate) vs 16.2% (placebo); $P = .66$
Zoledronic acid	Post hoc analysis HORIZON-PFT and RFT (3 years) (Boonen et al., 2010)	Women aged $\geq$75 y with T-score ≤ -2.5 at FN or ≥ 1 vertebral or hip fracture	3888	79.4 y	*HR = 0.34; 95% CI 0.21–0.55; P < .001* $p_{interact} < 75$ & ≥ 75 y NS	HR = 0.82; 95% CI 0.56–1.2; $P = .297$ $p_{interact} < 70$ & ≥ 75 y SS	*HR = 0.73; 95% CI 0.60–0.90; P = .002* $p_{interact} < 70$ & ≥ 75 y NS
Denosumab	Post hoc analysis FREEDOM (3 years) (Boonen et al., 2011)	Women aged $\geq$75 y	2471	78.2 y	–	*0.9% (denosumab) vs. 2.3% (placebo); P < .01; ARR 1.4%* $p_{interact} < 75$ & ≥ 75 y NS	–
	Preplanned analysis FREEDOM (3 years) (McClung et al., 2012)	Women aged $\geq$75 y	2471	78.2 y	*3.1% (denosumab) vs. 8.6% (placebo); RR = 0.36; 95% CI 0.25–0.53* $p_{interact} < 75$ & ≥ 75 y NS	–	7.9% (denosumab) vs 9.0% (placebo); RR = 0.84; 95% CI 0.63–1.12 $p_{interact} < 75$ & ≥ 75 y NS
	FREEEDOM Extension (6 years) (Papapoulos et al., 2012)	Women aged $\geq$75 y	662	78 y	3.6% (denosumab) (no placebo group)	1.0% (denosumab) (no placebo group)	5.4% (denosumab) (no placebo group)
Strontium ranelate	Preplanned pooled analysis SOTI and TROPOS (3 years) (Seeman et al., 2006)	Women aged 80–100y	1488	83.5 y	*RR = 0.68; 95% CI 0.50–0.92; P = .013*	RR 0.68; 95% CI 0.42–1.10; $P = .112$ (not powered)	*RR = 0.69 95% CI 0.52–0.92; P = .011*
	Preplanned pooled analysis SOTI and TROPOS (5 years)	Women aged 80–100 y	1489	83.5 y	*RR = 0.69; 95% CI 0.52–0.92*	RR 0.76; 95% CI 0.50–1.15	*RR = 0.73; 95% CI 0.57–0.95*

(*Continued*)

Table 1 Continued

	RCT	*Included participants*	*N*	*Mean age (years)*	*Vertebral fractures*	*Hip fractures*	*Nonvertebral fractures*
	(Seeman et al., 2010)					(not powered)	
Teriparatide	Prespecified subgroup analysis FPT (19 months) (Boonen et al., 2006a)	Women aged ≥75 y	244	78.3 y	*RR = 0.35, P < .05* $p_{interact} < 75$ & ≥ 75 y NS	–	RR = 0.75; *P* = .661 (not powered) $p_{interact < 75 \& \geq 75 y}$ NS

FN = Femoral neck; LS = lumbar spine; y = years; ITT = intention to treat; NS = not significant. Results in italics indicate significant results.
Table, with permission, adapted from Gielen, E., Bergmann, P., Bruyere, O. *et al.* (2017). Osteoporosis in frail patients: A consensus paper of the Belgian Bone Club. Calcified Tissue International.

This difference in benefit for vertebral vs. nonvertebral fractures in both analyses in the very elderly might be explained by the fact that bisphosphonates impact on BMD, proven by the significant reduction of vertebral fractures (McClung *et al.*, 2001; Boonen *et al.*, 2004b). Bisphosphonates, however, do not impact on the nonskeletal risk factors of fractures such as gait disturbances, impaired balance and fall risk. These nonskeletal factors are of particular importance in the occurrence of non-vertebral fractures, such as hip fractures, in the elderly, who are more prone to falling (Tom *et al.*, 2013). In contrast, vertebral fractures are often atraumatic, thus less influenced by these nonskeletal risk factors. An additional explanation for the discrepancy between the older and younger population in preventing nonvertebral fractures might be insufficient statistical power in the older age group (Boonen *et al.*, 2004b).

Women aged ≥80 years in the HIP trial had a slightly higher incidence of death, other serious adverse events and withdrawal due to adverse events as compared to younger women (McClung *et al.*, 2001). However, the overall frequency and types of adverse events, including those involving the upper gastrointestinal tract, were similar in the Risedronate and placebo group, regardless of age. Also in women aged ≥80 years in the pooled analysis of the VERT-NA, VERT-MN and HIP trials, adverse events were similar in the Risedronate and placebo group (Boonen *et al.*, 2004b). Even in the very elderly with active gastrointestinal tract disease at baseline or on aspirin, nonsteroidal antiinflammatory drugs (NSAIDs) or proton pump inhibitors (PPIs), the incidence of adverse events was the same in both treatment groups.

Thus, according to the safety data of both Alendronate and Risedronate, oral bisphosphonates are well tolerated in the very elderly, and, even in those at risk of gastrointestinal events, not associated with an increased risk of adverse events as compared to placebo. However, in clinical practice, treatment with oral bisphosphonates may be challenging, as the stringent intake instructions (e.g., to stay upright for at least 30 min after the intake) may be difficult for physically or cognitively impaired elderly. Inappropriate administration, however, increases the risk of adverse events (Vondracek and Linnebur, 2009; Cryer and Bauer, 2002). Furthermore, oral osteoporosis medication increases the pill burden in the elderly, which contributes to low compliance. Indeed, almost 50% of those who have been prescribed an oral bisphosphonate, have discontinued treatment after 1 year (Lo *et al.*, 2006).

Zoledronic acid

An intravenous bisphosphonate, such as Zoledronic acid, is an alternative in elderly that cannot tolerate or adhere to oral bisphosphonates (Boonen *et al.*, 2010). The Health Outcomes and Reduced Incidence with Zoledronic Acid Once Yearly Pivotal Fracture Trial (HORIZON-PFT) has shown that Zoledronic acid significantly reduces the risk of vertebral, hip and nonvertebral fractures in postmenopausal women with osteoporosis (mean age 73 years) (Black *et al.*, 2007). Also in a subsequent analysis in patients with surgical repair of a low-trauma hip fracture (mean age 74.4 years), the HORIZON-Recurrent Fracture Trial (HORIZON-RFT), Zoledronic acid significantly reduces the risk of new vertebral and nonvertebral fractures (Lyles *et al.*, 2007).

A post hoc pooled analysis of the HORIZON-PFT and HORIZON-RFT focused on the elderly, by including postmenopausal women aged ≥75 years (mean age 79.4 years) with osteoporosis (T-score ≤ −2.5 at the femoral neck or ≥1 prevalent vertebral or hip fracture) (Boonen *et al.*, 2010). After 3 years, the incidence of vertebral and nonvertebral fracture was significantly lower in the Zoledronic acid group compared to the placebo group (HR = 0.34; 95% CI 0.21–0.55; $P < .001$ and HR = 0.73; 95% CI 0.6–0.9; $P = .002$ respectively). This benefit was comparable with the relative risk reduction in subjects younger than 75 years in the HORIZON-PFT and HORIZON-RFT, presenting Zoledronic acid as an effective treatment option for the prevention of vertebral and non-vertebral fractures in the elderly. However, in patients aged ≥75 years, the incidence of hip fractures was lower with Zoledronic acid, but this did not meet statistical significance (HR = 0.82; 95% CI 0.56–1.2; $P = .297$), contrary to the statistical significance that was obtained in patients <75 years. Possibly the sample size was not statistically powered to detect hip fracture risk reduction in this older age group. An alternative explanation is the greater influence of nonskeletal risk factors for hip fractures with increasing age (Boonen *et al.*, 2004b, 2010).

In the post hoc analysis of the HORIZON-PFT and RFT trial in women aged ≥75 years, the incidence of adverse events and postdose symptoms occurring within 3 days of the infusion (pyrexia, myalgia and influenza-like illness) was higher with

Zoledronic acid than with placebo (Boonen *et al.*, 2010). However, the incidence of serious adverse events and death were similar in the two treatment arms. An increased risk of atrial fibrillation, suggested in the HORIZON-PFT, which could be a concern in the elderly, was not observed in the very elderly on Zoledronic acid (Boonen *et al.*, 2010).

Denosumab

Denosumab has been established as an effective intervention by the FREEDOM trial, with a significant reduction in the risk of vertebral, hip and non-vertebral fractures in postmenopausal women (mean age 72.3 years) (Cummings *et al.*, 2009).

A post hoc analysis of the FREEDOM trial has evaluated the effect of Denosumab in high risk populations, among which women aged ≥75 years (Boonen *et al.*, 2011). In this age group (mean age 78.2 years), Denosumab significantly reduced the risk of hip fractures by 62% (2.3% placebo vs. 0.9% denosumab; $P < .01$). This risk reduction in elderly patients is comparable with the risk reduction in the overall study population of the FREEDOM trial. Another analysis of the FREEDOM trial confirmed that Denosumab reduces the risk of vertebral fractures to the same extent in subjects aged ≥75 years (RR = 0.36; 95% CI 0.25–0.53) as in subjects <75 years (RR = 0.30; 95% CI 0.22–0.41, interaction *p*-value 0.482) (McClung *et al.*, 2012). Also the effect on non-vertebral fractures was similar in persons older (RR = 0.84; 95% CI 0.63–1.12) and younger than 75 years (RR = 0.78; 95% CI 0.63–0.96, interaction p-value 0.642). In addition, the FREEDOM Extension trial showed that, also in women aged ≥75 years at baseline, Denosumab for up to 6 years was associated with a persistently low incidence of new vertebral, hip and nonvertebral fractures, with a fracture incidence similar to that observed after 3 years (Papapoulos *et al.*, 2012).

Denosumab is thus effective to prevent vertebral *and* hip fractures in the elderly (McClung *et al.*, 2012; Boonen *et al.*, 2011). This is in contrast to the bisphosphonates where no significant reduction in hip fracture risk could be shown for Risedronate and Zoledronic acid in the elderly, although, as mentioned, this might be explained by lack of statistical power in these subgroup analyses (Boonen *et al.*, 2010; Ensrud *et al.*, 1997; Hochberg *et al.*, 2005). However, it is tempting to speculate that this observation of hip fracture reduction with Denosumab is due to the mechanism of action of Denosumab, different from the mechanism of action of bisphosphonates, with distinct effects on cortical bone (Genant *et al.*, 2013). Cortical porosity is indeed one of the main determinants of non-vertebral fracture risk, including hip fracture risk.

In general, Denosumab was well tolerated in postmenopausal women in the FREEDOM trial (Cummings *et al.*, 2009). In the post hoc analysis in women aged ≥75 years, no significant differences were noted in the safety profile between placebo- and Denosumab-treated subjects, and the incidence of the reported adverse events was similar to those in the overall FREEDOM population (Boonen *et al.*, 2011). Importantly, the efficacy of Denosumab is not affected by renal function, so elderly with renal impairment will experience the same anti-fracture efficacy as patients with normal renal function (Jamal *et al.*, 2011). However, since the use of Denosumab is associated with a high rate of severe hypocalcemia in patients with advanced chronic kidney disease, close monitoring and replacement of calcium and vitamin D is required to avoid hypocalcemia in these patients. Finally, Denosumab, but also the bisphosphonates, may be associated with very rare but severe adverse events, such as atypical femoral fractures and osteonecrosis of the jaw. To our knowledge, there is no evidence that these events are independently associated with age (Low, 2012).

Strontium ranelate

The anti-fracture efficacy of Strontium ranelate, which has a dual mode of action of increasing bone formation and reducing bone resorption, was established by the spinal osteoporosis therapeutic intervention (SOTI) trial (mean age 69.3 years) and the TReatment Of Peripheral OSteoporosis (TROPOS) (mean age 76.7 years) (Meunier *et al.*, 2004; Reginster *et al.*, 2005).

In a preplanned pooled analysis of both SOTI and TROPOS in women aged ≥80 years (mean age 83.5 years), Strontium ranelate significantly reduced the risk of both vertebral and nonvertebral fractures (Seeman *et al.*, 2006, 2010). However, the finding of an increased risk of cardiac events, including myocardial infarction, in additional clinical studies in male osteoporosis and osteoarthritis, led the European Medicines Agency (EMA) to recommend a change in the indication of Strontium ranelate (European Medicines Agency, 2013). It remained a useful option in elderly with severe osteoporosis and unable to take other osteoporosis treatments, but contraindicated in case of uncontrolled hypertension, established, current or past history of ischemic heart disease, peripheral arterial disease and/or cerebrovascular disease (Reginster, 2014). Due to this restricted indication leading to a continuous decrease of patients treated with Strontium ranelate, the worldwide and permanent cease of marketing of Strontium ranelate became effective starting from August 2017 (Servier, 2017).

Teriparatide

Daily subcutaneous injections of teriparatide, a biosynthetic parathyroid hormone analog, reduces the risk of vertebral and nonvertebral fractures, as shown in the fracture prevention trial (FPT) in postmenopausal women with a prior vertebral fracture (mean age 69.5 years) (Neer *et al.*, 2001).

A prespecified subgroup analysis of the FPT-study was undertaken to investigate the effect of Teriparatide in persons aged ≥75 years after 19 months of treatment (mean age 78.3 years) (Boonen *et al.*, 2006a). 5.2% of elderly in the Teriparatide group and 15.1% in the placebo group had a new vertebral fracture (RR = 0.35, $P < .05$). Treatment-by-age interaction was not significant ($P = .99$), indicating that the effect of Teriparatide was not statistically different in younger versus older patients. Likewise, 3.2% of the elderly women in the Teriparatide group and 4.2% in the placebo group had a new non-vertebral fracture (RR = 0.75; $P = .661$). The treatment- by-age interaction was again not significant ($P = 0.42$). The nonsignificant effect on nonvertebral fracture risk in the very elderly can be explained by the small number of nonvertebral fractures in the older subgroup.

As a result, this analysis was not sufficiently powered to show a statistically significant reduction in the risk of nonvertebral fractures in women aged ≥75 or to detect small differences in the relative treatment effect on nonvertebral fractures in the younger and older subgroups (Boonen *et al.*, 2006a). Hip fracture incidence was not a primary endpoint in this trial. So, age does not affect the efficacy of Teriparatide in preventing vertebral and non-vertebral fractures.

In the post hoc analysis of the FPT trial in women aged ≥75 years, there was no increase in adverse events in women treated with Teriparatide compared to placebo (Boonen *et al.*, 2006a). On the contrary, back pain, cataract and pruritus were significantly less common in those treated with Teriparatide. Treatment-by-age interaction (≥75 vs. <75 years) was not significant for the important adverse events. Only diarrhea was reported more frequent, while cataract, deafness, pruritus and weight loss were reported less frequent in the older compared to the younger age group. Thus, in the elderly, the safety profile of Teriparatide is, in general, comparable to placebo. In clinical practice, the major disadvantages of Teriparatide are the cost and the daily subcutaneous administration which may be a burden for older patients.

Conclusion

Osteoporosis is one of the most common age-associated conditions and a major cause of fracture risk. In old age, osteoporosis and osteoporotic fractures tend to occur in a frail subset of the population. Treatment of osteoporosis is of particular concern in the elderly because of the substantial burden of osteoporotic fractures in terms of morbidity, mortality and economic cost.

It is never too late to treat osteoporosis, not even in elderly patients with the most severe degree of osteoporosis and not even in those who have already sustained an osteoporotic fracture. Calcium and vitamin D supplementation is an essential but not sufficient component in the management of osteoporosis in old age. Adding osteoporosis treatment reduces the risk of fractures even more, at least in older individuals with documented osteoporosis and at least for vertebral fractures and possibly also for hip fractures. In frail elderly with documented osteoporosis, osteoporosis treatment may even be more effective than in younger patients, with more fractures averted and lower numbers needed to treat. Nonpharmacological interventions such as physical exercise therapy may contribute to reduce fracture risk by increasing BMD and improving muscle mass and function.

Data in the very elderly show that currently available osteoporosis therapies are relatively safe, with no significant differences in the incidence of most adverse events in the treated group compared to the placebo group. The incidence of adverse events in the very elderly is also similar to that reported in the general population. In real life, specific issues such as comorbidity and polypharmacy should be taken into account in the elderly and may influence the choice of therapy. However, all together, these efficacy and safety data in the elderly indicate that age in itself is no reason to withhold osteoporosis treatment in very elderly with osteoporosis or at high fracture risk.

See also: Osteoporosis; Prevention and Ca—Vitamin D Treatment. Bone Mass Measurement. Nutrition and Bone. Bone Turnover Markers. Epidemiology of Fractures. Bone Muscle Interactions and Exercise. Osteoporosis Treatment: Bone-Forming Agents. Role of Estrogens and Androgens in Osteoporosis. Osteoporosis Treatment: Sequential and Combination Therapy. Osteoporosis: Treatment Gaps and Health Economics. Vitamin D

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Aging and the Thyroid Gland☆

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Introduction

The aging process may affect the hypothalamic–pituitary–thyroid (HPT) axis and increase the prevalence of thyroid disorders. Moreover, the comorbidities and polypharmacy that typically accompany aging often complicate the diagnosis and treatment of thyroid dysfunction in this population. These two factors strongly point to the need for a specialized and individualized approach. The aim of the present critical review, which includes the most relevant studies of the last few years, is to highlight the effects of age on thyroid function, thus offering a concise update to clinicians on the epidemiology, pathogenesis and challenges in diagnosing and treating thyroid disease in aging persons.

Aging and the Hypothalamic–Pituitary–Thyroid Axis

Advancing aging is associated with decreasing synthesis, secretion pattern and activity of thyrotropin-releasing hormone (TRH), the core regulator of the HPT axis. Consequently, thyrotropin-stimulating hormone (TSH) secretion rate and its nocturnal peak slowly decline with age in a manner dependent upon sex, body composition, stress, drugs, nutrition, exercise and comorbidity (Veldhuis, 2013). The changes become prominent by the eighth decade of life, with a tendency, this being more evident in women than in men, towards higher basal TSH as well as to appearance of thyroid autoantibodies. In addition, taking into consideration that TRH is a trophic factor for the diencephalon and that it acts as a neuroprotectant against oxidative stress and glutamate toxicity, decreasing TRH may be involved in the development of dementia in long-standing hypothyroidism (Daimon *et al.*, 2013).

Thyroid function in aging is usually sufficient to sustain normal serum thyroid hormone levels, which is also supported by reduced thyroxine degradation rates. On the other hand, evaluation of the HPT axis in aged patients may often be challenged by non-thyroid illness (NTI), malnutrition and medication(s) used, all of them resulting in altered 5′-iodothyronine deiodination and impaired thyroid function test (low to normal T4, low T3, high reverse T3 and inappropriately normal or low TSH) (Lado-Abeal, 2015). The pathogenesis of long-term NTI is multifactorial, marked by the suppression of TRH, resulting in persistently reduced secretion of TSH despite low plasma thyroid hormone levels (Fliers *et al.*, 2015). In some cases distinguishing between NTIS and severe hypothyroidism can be challenging. In patients with NTI, infusion of hypothalamic-releasing factors can reactivate the thyroid axis (Fliers *et al.*, 2015).

With regard to medication, a number of drugs may interfere with thyroid testing by affecting the binding of thyroid hormone (TH) to binding proteins, or can displace TH from binding sites, impair T4 conversion to T3 or decrease TSH secretion (Table 1).

Thyroid Disease in the Elderly

Overt and Subclinical Hypothyroidism

The prevalence and incidence of hypothyroidism increase with aging and contribute to total morbidity in the old. However, it has been shown that among the elderly, a thyroid disorder may be underdiagnosed due to less obviously manifested symptoms and comorbidities in conjunction with the confounding effects of medications, this finding strongly supporting the concept of targeted screening for those above 65 years old. Notably, a very recent population-based study carried out in young and older hypothyroid patients investigated to what extent a high prevalence of symptoms predicts overt hypothyroidism in these two cohorts. The results demonstrated that while applying a hypothyroid symptom score was useful in diagnosing hypothyroidism in the young (<50 years), it failed to indicate hypothyroidism in older (>60 years) patients (Carlé *et al.*, 2016). As mentioned above, thyroid function test interpretation may often be difficult due to the presence of NTI which can bring about alterations in the metabolic clearance of thyroid hormone, drug interactions and potential adverse reactions (Kim, 2000–2014).

Subclinical hypothyroidism (SCH) is defined as elevated TSH with normal levels of free thyroxine (FT4) and triiodothyronine (T3). SCH is classified into a minimal disease with TSH levels between 4.0 and 6.0 mU L^{-1}, a mild form with TSH levels between 6 and 10 mU L^{-1} and a severe form with TSH >10.0 mU L^{-1}. SCH is a common condition in the elderly at a rate of up to and over 15%, the majority of individuals with SCH having a TSH between 4.5 and 6.9 mU L^{-1}. The rates of reversion to euthyroidism or progression to overt hypothyroidism depend on baseline TSH and antithyroid peroxidase antibody (TPOAb) positivity (Somwaru

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Table 1 Drugs that interfere with thyroid hormone (TH) tests, by variably acting at different levels of the pituitary thyroid axis, and with thyroid hormone metabolism

Drugs	*Interference*
Estrogens	TBG[a] ↑
Tamoxifen	TBG ↑
Fluorouracil	TBG ↑
Anabolic Steroids	TBG ↓
Salicylates and NSAD (Fenclofenac)	TH displacement
Heparin	TH displacement
Diuretics (Furosenide)	TH displacement
Amiodanone	Decrease T4:T3 conversion
Iopanoic acid	Decrease T4:T3 conversion
B-blockers (propranolol)	Decrease T4:T3 conversion
Glucocorticoids	Decrease T4:T3 conversion
Dopamine	TSH ↓
Somatostatine and its analogues	TSH ↓
Glucocorticoids	TSH ↓
Ferroursulfate	LT4 absorption ↓
Cholestiramine	LT4 absorption ↓
Soy bean	LT4 absorption ↓

Drugs	*Interference*	
Antiepileptics	*TSH*	*FT4*
Valproate	↑	↓
Phenobarbital	↑	↓
Carbamazepine	↑	↓
Retinoids	↓	
Metformin (diabetes/insulin resistance)	↓	
Tyrosine kinase inhibitors (TKIs)	Hepatic metabolism T4	↑

[a]TBG, thyroxine binding globulin.

et al., 2012). Thus, a reversion to euthyroidism at 2 year was more common, up to 46%, for those with a TSH of 4.5–6.9 mU L^{-1} as compared to about 10% in subjects with a TSH between 7 and 9.9 mU L^{-1} and only 7% of those with a TSH at 10 mU L^{-1}, which latter is also independently associated with progression to overt hypothyroidism.

TSH levels within the population reference range are not associated with risk of CHD events or CHD mortality, suggesting that subtle differences in thyroid function are not likely to influence the risk of CHD. In the elderly, age-specific population-derived normal TSH reference ranges are not commonly used to identify thyroid dysfunction, thus rendering an upper limit of normal TSH inappropriate in diagnosing SCH in persons older than 65 years (Hennessey and Espaillat, 2015). Consequently, this may lead to overestimation of SCH in the elderly and premature or unnecessary initiation of treatment. In a recent study in community-dwelling men, 3885 men aged 70–89 years were analyzed according to the 2.5th and 97.5th centiles (Yeap *et al.*, 2016). It was shown that the reference interval for TSH in older men moves upward, to 0.64–5.9 mIU L^{-1}, whereas the reference interval for FT4 is compressed, at 12.1–20.6 pmol L^{-1} (0.94–1.60 ng dL^{-1}, compared with the conventional reference ranges (Yeap *et al.*, 2016)). Thus, the application of the conventional reference interval in older patients may result in misinterpretation of results, classifying patients as having overt or subclinical thyroid disease when in fact this is not the case.

Should the disease, however, be left untreated, the consequences of SCH in the elderly are, in the absence of a consensus, controversial, while it is most likely that the effects vary depending on age, duration of disease and gender. Accordingly, some data show an increased risk for hip fracture in men and for Alzheimer's disease in women, while there are conflicting results as to cardiovascular risk (Somwaru *et al.*, 2012; Biondi, 2008). Nevertheless, in a meta-analysis of 10 studies including 14,449 participants investigating risks associated with SCH (2134 CHD events and 2822 deaths), it was estimated that SCH is associated with a modestly increased risk for CHD (Ochs *et al.*, 2008). In another study, the association of subclinical thyroid disease and mortality was evaluated in 17,440 individuals older than 65 years and followed up over a decade (Grossman *et al.*, 2016). Of the 17,440 individuals, 1956 had SCH [11.2%], 538 had subclinical hyperthyroidism (SCHyP) [3.1%], while 14,946 were normal euthyroid [85.7%]. Both SCH and SCHyP were associated with significantly increased mortality, which was persistent following multivariate analysis (Grossman *et al.*, 2016). Importantly, crude mortality was elevated at 1 (the most significant association), 2 and 5 years, but this association is likely to decrease as time from initial evaluation increases. The highest mortality rate was associated with TSH levels above 6.38 mIU L^{-1} in patients with SCH, whereas no threshold for increased mortality was identified in SCHyP.

SCH has been associated with dyslipidemia, although the pathogenesis is complicated, while high TSH levels, even within the reference levels, have been linked to modestly higher levels of blood pressure and serum lipids (Asvold *et al.*, 2013). The SCH-lipids

association is likely to be influenced by age and gender, as younger women show more detrimental effects of high TSH on lipids than those in menopause and men. Meanwhile, the reported connection with atherosclerosis is not as yet fully understood from a pathogenetic point of view. In this regard, the recent findings of increased LDL peroxidation, according to plasma concentrations of hydroxy-octadecadienoic acids (HODEs) and hydroxy-eicosatetraenoic acids (HETEs) in patients with overt (OH) and SCH warrant further investigation to elucidate the issue (Zha *et al.*, 2015). In this line of evidence, high homocysteine levels were reported in OH and SCH, resulting in decreased concentrations of apolipoprotein A-I (Apo-AI) and high density lipoprotein cholesterol (HDL-C) (Yang *et al.*, 2016). These effects may substantially contribute to development of atherosclerosis and cardiovascular disease (CVD). By inhibiting Apo-AI, SCH deranges its antioxidant capacity and mitigates oxidative stress (Yang *et al.*, 2016). Moreover, hyperhomocysteinemia propagates insulin resistance and impairs endothelial function in patients with hypothyroidism and arterial hypertension, thereby appreciably contributing to CVD. It is also noteworthy that women with SCH display higher remnant lipoproteins (RLP) levels. These however rapidly and significantly decrease following replacement therapy with levothyroxine (Brenta *et al.*, 2016), a possible mechanism behind this response being the increased hepatic lipase activity observed after levothyroxine treatment.

Subclinical thyroid dysfunction has been considered as a risk factor for cognitive impairment in aging patients. The association between subclinical thyroid dysfunction and cognition was investigated in the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) (Winkler *et al.*, 2015), a aged 70–82 years ($n = 5154$ patients) with pre-existing vascular disease. SCH and SCHyP were registered in 65 and 161 participants, respectively, but no consistent association was established of SCH and/or SCHyP with impaired cognitive performance, compared to euthyroid participants.

In contrast, an analysis was made of a population-based study, including 2563 euthyroid participants (aged 50–80 years) from the second examination of the population-based Heinz Nixdorf Recall study, to investigate the gender-specific association of low- and high-normal TSH association with mild cognitive impairment (MCI) (Wijsman *et al.*, 2013). The results did not show any association with low-normal TSH concentration in women, and no association of either low- or high-normal TSH concentration with MCI in men, thus indicating that women with high-normal TSH concentration ($TSH > 4.50$ mU L^{-1}) might be at higher risk of MCI.

Also of note, an observational cohort study based on the records of a number of Danish health registers disclosed that patients with hypothyroidism are at increased risk of being diagnosed with a psychiatric disorder and being treated with antidepressants, antipsychotics and anxiolytics both before and after the diagnosis of hypothyroidism (Thvilum *et al.*, 2014). Therefore, considering the several objective and practical difficulties, as enumerated above, in diagnosing hypothyroidism among the elderly, it seems clear that even a small clinical sign should lead to monitoring (Carlé *et al.*, 2014).

Indications for treatment of SCH remain controversial. While treatment of SCH may improve lipid profile, there is however no evidence that it is associated with decreased cardiovascular or all-cause mortality in elderly patients. In patients above 70 years with a high risk of progression from SCH to overt disease close monitoring should be considered as the best practice. Treatment of the elderly aged 70 years and over should be recommended when serum TSH is > 10 mU L^{-1} (repeatedly measured), independent of the presence of symptoms and signs of hypothyroidism: this is of particular importance in females, and especially in those with positive TPOAB, dyslipidemia, diastolic arterial hypertension and/or MCI. In cases with $TSH < 10$ mU L^{-1} an individual approach is advocated.

Overt and Subclinical Hyperthyroidism

Hyperthyroidism is usually oligosymptomatic in the elderly, this being due to alterations of thyroid hormone production, metabolism and action and, importantly, to the fact that hyperadrenergic symptoms are largely absent in older patients with hyperthyroidism (Engels *et al.*, 2015). This notion was confirmed by an experimental study in which euthyroid mice that were rendered hyperthyroid were seen to undergo age-dependent changes in thyroid hormone transporter mRNA expression (Engels *et al.*, 2015).

The most common physiological causes of hyperthyroidism in the elderly are Graves' disease and toxic multinodular goiter. Hyperthyroidism is a serious clinical condition among older subjects, associated as it is with high morbidity, and it is moreover a disorder that is frequently challenging to diagnose due to the confounding effects of drugs and NTIs, as mentioned earlier, on the interpretation of thyroid function tests (Samuels and Franklyn, 2000–2015).

There is also the problem of iatrogenic hyperthyroidism. Notably in this respect, the increased risk of atrial fibrillation (AF) in older patients prescribes the use of amiodarone or administration of iodinated contrast agents, which, however, can disrupt thyroid function and often induce hyperthyroidism (Samuels and Franklyn, 2000–2015). Meanwhile, there is the problem arising from the presence of SCHyP, defined as normal thyroxine (FT4) levels with decreased TSH, since its management is a debatable subject in the old due to the lack of symptoms. Among the elderly, however, it is linked to an increased risk of total mortality, coronary heart disease (CHD) mortality, heart failure and AF, particularly in patients with suppressed TSH levels < 0.10 mIU L^{-1} making it particularly dangerous for this age group. This fact was demonstrated by a systematic review of pooled data collected from more than 70,000 participants carried out in order to arrive at a precise estimation of the risks of CVD outcomes that were linked to subclinical thyroid dysfunction (Gencer *et al.*, 2013). Prospective cohort studies, including thyroid function data and ensuing fractures, have also been analyzed to evaluate the association between subclinical thyroid dysfunction and hip, spine, or any fractures (Blum *et al.*, 2015). SCHyP was associated with an increased risk of hip and other fractures, again among those with TSH levels of less than 0.10 mU L^{-1} and those with endogenous SCHyP (Blum *et al.*, 2015). In a prospective multicenter study, thyroid hormones and thrombophilic biomarkers were evaluated 1 year after acute venous thromboembolism (VTE) and it was found that in elderly patients, SHyP may be associated with lower rVTE risks (Segna *et al.*, 2016).

In summary, the above findings are of considerable importance to physicians and clinicians since, by establishing a firm basis of estimated risk of clinical outcomes and of potential TSH thresholds (TSH < 0.10 mU L^{-1}), they considerably facilitate decision-making for initiation of treatment.

Epicrisis

Both SCH and SCHyP are common and on the rise in the elderly. This is due to the increasing aging population, to more frequent screening as well as to possibly adverse environmental factors. Because the diagnosis can be hampered by non-specific or mild symptomatology and by confounders, it should be made with special care. Treatment should be seriously considered in patients with a TSH > 10 mU L^{-1}, while in elderly patients with TSH < 10 mU L^{-1} as well as in older patients with a TSH between 0.1 and 0.4 mU L^{-1} an individualized approach is strongly recommended.

See also: Causes of Hypothyroidism. Hypothyroidism Subclinical. Nontoxic Goiter. Radioactive Iodine. Subclinical Hyperthyroidism. Thyroid Disorders in the Elderly. Thyroid Nodule. Thyrotoxicosis; Treatment. Toxic Adenoma. Toxic Multinodular Goiter. Treatment of Hypothyroidism

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Introduction to Endocrine Toxicology and Endocrine Disruption

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The World Health Organization defines an endocrine disruptor as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations". In 2017 European Union adopted this definition for the basis of scientific criteria to identify endocrine disruptors in the field of plant protection products. This was considered an important step towards greater protection of citizens from harmful substances. However, there are also other definitions for endocrine disruptors, for example, Endocrine Society uses an operational working definition of an endocrine disrupting chemical as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Zoeller et al., 2012; Gore et al., 2015). The important difference between the definitions is, whether the effect of the chemical needs to be "adverse" to make it endocrine disruptor. According to WHO any chemical with an effect on the endocrine system is a potential endocrine disruptor. The distinction is significant from the regulatory point of view.

Several scientific societies have published their opinions, reviews, and analyses on endocrine disruptors during the recent years: the American College of Obstetricians and Gynecologists (ACOG), the American Society for Reproductive Medicine, Endocrine Society, European Society of Endocrinology, European Society for Paediatric Endocrinology, Pediatric Endocrine Society, The International Federation of Gynecology and Obstetrics (FIGO), the Royal College of Obstetricians and Gynecologists. These reviews present a comprehensive picture of the environmental problems that we face in endocrinology.

The list of endocrine disruptors is enlarging and includes now more than 1000 chemicals. However, only a small percentage of the more than 100,000 chemicals that are currently produced have been studied for their endocrine disrupting activity. Endocrine disruption can occur at several levels. Receptor interference is the simplest and easiest to analyze of the effects. Multiple receptor assays are available for all hormones. Quantitative structure–activity relationship (QSAR) analyses can be done with computer modeling, which has helped to recognize putative receptor interactions without wet laboratory work. However, endocrine disruption is not limited to receptor interactions. Hormone biosynthesis or metabolism can be affected, as well as hormone transport and elimination. The endocrine system is built with several feedback and feedforward circuits that are fine-tuned by paracrine and autocrine regulation. Disconnecting these circuits also leads to endocrine disruption, which explains the complex dose–effect relationships of endocrine disruptors. In contrast to many classical toxicological models that appear to follow the Paracelsus paradigm, according to which "dose makes the poison," the dose responses are often nonlinear in endocrinology. Therefore no adverse effects in experiments with high doses do not rule out effects at low doses. An example of nonlinear exposure–outcome relationship comes from the effects of testosterone treatment: a low testosterone dose does not interfere with spermatogenesis, but higher doses inhibit sperm production by blocking LH secretion from the pituitary, which leads to decline in intratesticular testosterone concentration. If testosterone dose is further increased, sperm production starts again, because the exogenous testosterone now fully replaces testicular hormone. The dose–response curve in this case is U-shaped. Animal experiments are still needed to cover the complex metabolism and interactions in testing of the chemicals.

Hormones guide organogenesis and growth, and therefore developmental endocrine disruption is particularly devastating. Exposure to an endocrine disruptor at a fragile stage of development can be much worse than the same exposure in later life. Critical developmental windows are known, and organogenesis during the first trimester of fetal life is the most vulnerable time. During the last decades we have learned about developmental origin of adult diseases, which is based on the programming events occurring during early development. Much of our current burden of noncommunicable diseases appears to have a connection to fetal dysregulation where endocrine disruptors may play an important role.

Endocrine toxicology started to gain more attention in the 1990s when the term endocrine disruptor was coined. The focus was first on estrogenic compounds, since the adverse effects of developmental exposure to diethylstilbestrol (DES) were shown both in rodents and humans, and many of the mouse findings were recapitulated in the analyses of old randomized controlled trials. The appearance of clear cell adenocarcinoma of vagina in teenager girls who had been exposed to DES in utero caused an alarm that led to the ban of DES in the 1970s. Although this tumorigenic effect was not occurring in mice, similar structural anomalies in the reproductive tract of both male and female were evident in humans and experimental animals. Furthermore, DES adversities were observed also in the second generation after exposure, suggesting epigenetic inheritance. At the same time, estrogenic effects in wildlife fish became evident. High levels of estrogen-induced vitellogenin in male fish and a high proportion of intersex fish in English rivers pointed to pollution of water with estrogenic compounds, such as alkylphenols and traces of human contraceptives. Alkylphenols were used for example in textile industry and their release to waste water was soon regulated when the hormonal activity was identified. Yet another concern was raised, when the unknown estrogenic activity released from polycarbonate plastic flasks in laboratories was identified as bisphenol A (BPA). The activity was weak but caused problems in experiments where blank controls showed clear hormonal activity. Ever since bisphenol A has been one of the most studied endocrine disruptors raising a lot of controversy. In many countries baby bottles are not allowed to contain any bisphenol A, and industry has started to advertise bisphenol A-free products. In the United States, a large research program CLARITY is on-going to solve the open questions concerning endocrine toxicity of BPA. In this Encyclopedia, there are plenty of examples of endocrine disrupting effects of BPA for

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example, in the pancreatic beta cells (Alonso-Magdalena et al., 2018), hypothalamus (Fudvoye et al., 2018), adipose tissue (Willner and Blumberg, 2018), and control of behavior (Arambula and Patisaul, 2018).

Increased incidences of hormone-related cancers, such as testicular, prostate, and breast cancer raised the question, whether endocrine disrupting chemicals had contributed to the increase. Developmental origin of cancers was intensively studied and for testicular cancer a lot of evidence accumulated showing its fetal origin. When the incidence of congenital birth defects, such as hypospadias and cryptorchidism had also increased, and semen quality had declined, an obvious question was, whether these had something to do with each other. Indeed, they do, since all of them can have the same cause, for example, defective androgen production or androgen receptor mutation. Male reproductive health problems turned endocrine disruptor research also to antiandrogens. The list of these compounds has grown long. One of the first was *p,p′*-DDE, the most persistent congener of DDT, an old pesticide that was banned in the developed world because of its ecotoxicity, extinction of birds at the high level of food chain. DDE was shown to be an androgen receptor antagonist (Kelce et al., 1995). Many others followed, including procymidone and vinclozolin, two fungicides. The latter did not bind androgen receptor itself, but its two metabolites were receptor blockers. Systematic research on antiandrogens demonstrated also that their effects were additive, even when the mechanism of action varied, that is, receptor antagonists and inhibitors of hormone synthesis worked together accumulating the effect. Although estrogenic compounds were in the forefront of male reproductive toxicology first, studies have switched almost totally to antiandrogens during this century.

Research on endocrine disruptors has been hampered by political agendas. Chemical industry has tried to defend its markets by denying many early warnings of adverse effects associated to its products. On the other side, some non-governmental organizations have been ready to ban chemicals at the first sign of any danger. Argumentation has often deviated from scientific rigor to naming of the opposite site with phrases like “green humbug” or “fake science.” Nevertheless, European Union, United States, and Japan have aimed at better identification and proper regulation of endocrine disruptors. Although the progress has been slow, REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) legislation in EU gave some basis for that work. Clinical endocrinologists have also become aware of the problem, and the endocrine societies are now actively involved in advocating research on endocrine disruptors. Since human experimentation with endocrine disruptors is usually not possible, except for some compounds used in pharmaceutical products, we have to base our evaluation of the chemicals on their physicochemical characteristics, in silico, in vitro and in vivo testing, ecotoxicology and epidemiology, which together form the weight of evidence basis to our decisions.

The impact of endocrine disruptors can be also measured in money. Leo Trasande and coworkers (2015) made a series of health economic analyses of the expenses caused either directly or indirectly by endocrine disruptors and inaction in their regulation in EU. The overall estimate was 157 billion euro per year. By far the highest costs derived from adverse effects on neural development by thyroid disruptors. Thomas Adams stated already 1618: “Prevention is better than healing, because it saves us from the trouble of being sick”. Thus, identification and elimination of the endocrine disruptors should be our aim. European Union chemical legislation took a step to this direction when REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) was launched, but it is only the beginning.

Encyclopedia of endocrine diseases presents now for the first time a section on environmental disruption of different endocrine systems. Endocrine disruption is not limited only to these endocrine systems but affects all our endocrine organs. In the future endocrine disruptors should always be considered when solving problems in endocrine diseases.

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Further Reading

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Toxic Effects of Common Environmental Pollutants in Pancreatic β-Cells and the Onset of Diabetes Mellitus

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The Endocrine Pancreas

The endocrine pancreas constitutes 1%–2% of the total human pancreatic weight and involves multicellular structures, called the islets of Langerhans, that are dispersed throughout the pancreas (In't Veld and Marichal, 2010). Each islet ranges in size from 100 to 300 μm and is comprised of 1000–3000 cells across different endocrine populations: glucagon-secreting α-cells, insulin-secreting β-cells, somatostatin-releasing δ-cells, pancreatic polypeptide PP cells, and ghrelin-releasing ε-cells. About 90% of the islet is composed of α- and β-cells. In mouse, pancreatic β-cells constitute 65%–80% of the islet, while α-cells represent about 15%–20%. However, the α-to-β-cell ratio is increased in human islets (Cabrera *et al.*, 2006). The major function of these two cell types is to regulate glucose homeostasis. When plasma glucose levels decline, α-cells secrete glucagon, which mainly induces hepatic glucose output by activation of gluconeogenesis and glycogenolysis. In contrast, when plasma glucose levels rise, pancreatic β-cells secrete insulin, which activates glucose uptake by skeletal muscle and adipose tissue and inhibits hepatic glucose production. This feedback loop allows for the maintenance of plasma glucose within physiological limits. The extensive microvasculature of the islet facilitates rapid sensing of ambient glucose by islet cells and their prompt hormone release into the circulation (In't Veld and Marichal, 2010). In addition to nutrients and hormones, islet cells are also regulated by sympathetic, parasympathetic, and sensory nerves (Rodriguez-Diaz and Caicedo, 2014). Among the different islet cell types and hormones, the pancreatic *β*-cell and the secretion of insulin play a key role in the pathophysiology of diabetes.

The Pancreatic β-Cell

The insulin-secreting pancreatic β-cell is an electrically excitable cell that acts as a glucose sensor (Rorsman *et al.*, 2011; Rorsman and Braun, 2013). Glucose enters the β-cell cytosol via plasma membrane glucose transporters, increasing metabolic activity. Considered the molecular sensor of the β-cell, glucokinase phosphorylates glucose molecules and is a rate-limiting step for glycolytic flux. In the pancreatic β-cell, glucose is efficiently metabolized by the mitochondrial pathway, increasing the cytosolic ATP/ADP ratio. This rise induces closure of ATP- sensitive K^+ (K_{ATP}) channels. The resting membrane potential of the pancreatic β-cell (about -70 to -75 mV) is mainly due to the opening of these channels. In the presence of stimulatory glucose concentrations, the cytosolic rise in the ATP/ADP ratio blocks the K_{ATP} channel, inducing plasma membrane depolarization until a threshold at which voltage-operated Ca^{2+} channels are activated (Rorsman *et al.*, 2011; Rorsman and Braun, 2013; Yang *et al.*, 2014). Ca^{2+} entry through these channels raises cytosolic Ca^{2+} concentration that activates the exocytosis and extracellular release of insulin secretory granules. Thus, extracellular changes in plasma glucose are sensed via β-cell metabolism and translated into electrical activity and calcium signals that induce insulin secretion. In addition to calcium, other intracellular messengers such as NADPH, glutamate, and cAMP among others potentiate and/or amplify the secretory process by increasing the transport and exocytosis of secretory vesicles (Yang *et al.*, 2014). In vivo glucose-induced insulin secretion is biphasic with a transient rapid peak of insulin release (first phase) followed by gradual secretion towards a plateau (second phase).

The regulation of insulin biosynthesis in the pancreatic β-cell is also critical to maintain an adequate insulin content and secretory response to the different stimuli and physiological situations (Fu *et al.*, 2013). Insulin transcription is regulated by numerous transcription factors that interact with the promoter region of the insulin gene, establishing numerous pathways that sensitively respond to nutrients and hormones. The insulin gene encodes the preproinsulin protein that is further processed to proinsulin via the endoplasmic reticulum and Golgi. This highly efficient process facilitates insulin synthesis in response to physiological demands, but may lead to endoplasmic reticulum stress in pathological conditions. Finally, proinsulin is sorted into the secretory vesicles and converted to insulin by prohormone convertases during granule maturation (Fu *et al.*, 2013). Thus, all these highly regulated processes are necessary of β-cells to satisfy insulin demand.

Diabetes Mellitus

Diabetes mellitus is a chronic metabolic disease with a high prevalence worldwide. Diabetes occurs either when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. According to the World Health

Organization (WHO), this disease has already reached the status of a global epidemic and has grown in almost all social strata and niches, especially in low- and middle-income countries where more than 80% of diabetes deaths occur. The 2016 WHO global report on diabetes estimated that approximately 422 million individuals have diabetes worldwide (prevalence of 8.5%) with this number projected to reach 552 million by 2030, making diabetes the 7th leading cause of death (WHO, 2016). Diabetes is therefore considered one of the most serious current public health problems, making understanding its pathophysiology essential for improving societal health.

The WHO classifies diabetes into the following types: type 1 diabetes (T1D, previously known as insulin-dependent, juvenile or infantile); type 2 diabetes (T2D, previously known as noninsulin dependent or adult), and gestational diabetes (GDM).

T1D (5%–10% of cases) is a tissue-specific, chronic autoimmune disease characterized by severe impairments in insulin synthesis resulting from immune-mediated β-cell death (Herold *et al.*, 2013). Genetic predisposition and environmental factors are the main known contributors to T1D onset. The interaction between these two factors induces the activation of several signaling pathways and transcription factors in the *β*-cells, leading to the production and secretion of cytokines and chemokines, expression of major histocompatibility complex (MHC) class II antigens, and eventually β-cell apoptosis (Morgan and Richardson, 2014; Op de Beeck and Eizirik, 2016).

T2D is the most prevalent type of diabetes accounting for approximately 90%–95% of cases. Insulin resistance resulting from the combination of obesity and low-grade, chronic inflammation in metabolic tissues, such as skeletal muscle and adipose tissue, is an important mechanism underlying the development of T2D. In addition, T2D is also considered a complex multifactorial polygenic disease, with a strong genetic component (Gregor and Hotamisligil, 2011; Bonnefond and Froguel, 2015). During its initial phase, pancreatic β-cells compensate for increased peripheral insulin resistance by progressively increasing insulin secretion (hyperinsulinemia). However, if insulin resistance persists for years, especially in genetically predisposed individuals, β-cell secretory capacity gradually deteriorates, leading to a progressive increase in fasting glucose levels and, consequently, hyperglycemia (DeFronzo *et al.*, 1992; Kahn, 2001).

Additionally, persistent metabolic stress leads to a decrease in β-cell mass (around 30%–60%) in patients with T2D. This is mainly due to an increase in apoptosis resulting from chronic exposure to free fatty acids and high glucose (glucolipotoxicity), and/or islet amyloid deposits. Furthermore, local, mild inflammation has been observed in pancreatic islets during the course of T2D. Similarly to T1D, the presence of immune cells (e.g., macrophages) surrounding β-cell contributes to β-cell dysfunction and, therefore, apoptosis (Velloso *et al.*, 2013; Marchetti, 2016).

In recent years there has been a generalized global increase in diabetes prevalence that cannot be explained by genetic factors. Regarding the development of T2D, dietary changes (i.e., consumption of high energy foods), sedentary lifestyles and, subsequently, a rise in the incidence of obesity cannot explain the rapid increase in T2D prevalence. Thus, other environmental factors likely contribute to this alarming rise in the number of diabetes cases.

Epidemiological, cellular, and animal studies indicate that chemical pollutants, including endocrine disrupting chemicals (EDCs), affect β-cell function and should be considered diabetes risk factors (Heindel *et al.*, 2016; Mimoto *et al.*, 2017; Neel and Sargis, 2011; Alonso-Magdalena *et al.*, 2011). In the current article, we will focus on the effects of widespread chemical pollutants on pancreatic β-cells in in vitro cellular models and in vivo animal models. The alterations that these chemicals induce in this critical cell type should advance understanding of their impact on glucose regulation and diabetes risk.

Nonpersistent Pollutants

This group of pollutants includes chemicals that do not strongly accumulate in fat and are generally rapidly metabolized and excreted in urine or feces within approximately 24 h. However, many of these chemicals are used in a wide variety of commonly used products resulting in extensive exposure that results in a high percentage of the human population having detectable levels in urine and/or serum.

Phthalates

Phthalates and phthalate esters are a group of EDCs commonly named plasticizers since they have been extensively used to impart flexibility and elasticity to rigid plastics such as polyvinyl chloride (PVC). They are not covalently bound to the plastic matrix so they can easily leach and be released into the environment. These chemicals are commonly used in the production of many consumer products including medical devices (intravenous tubing and containers for blood, dialysis or nutrients), toys, vinyl flooring materials, building materials, cosmetics, and food containers. Of note, European Union has banned the use of phthalates in toys for children under 3 years of age and in cosmetics. Human exposure is widespread via ingestion, inhalation, and dermal contact (Halden, 2010). In their fourth National Report on Human Exposure to Environmental Chemicals, the Centers for Disease Control and Prevention (CDC) reported detectable urinary concentrations of 13 phthalate metabolites, including mono-benzyl phthalate (MBzP), mono-isobutyl phthalate, mono-ethyl phthalate (MEP), mono-(2-ethylhexyl) phthalate (MEHP), and mono-butyl phthalate (MBP) among others (Centers for Disease Control and Prevention (CDC), 2015).

Initial concerns about phthalates focused on their developmental and reproductive effects. Human and animal studies suggested a correlation between phthalate exposure and increased risk of male reproductive abnormalities such as hypospadias, low

sperm count, or shorter anogenital distance (Meeker *et al.*, 2009). More recently, phthalates have been linked to T2D and associated disorders, including increased waist circumference, overweight, and insulin resistance (Stahlhut *et al.*, 2007; Gore *et al.*, 2015; Heindel *et al.*, 2016; Kuo *et al.*, 2013). Nevertheless, the underlying mechanisms contributing to metabolic complications are still unresolved. In an attempt to unravel this phenomenon a number of studies have explored the possibility that phthalates disrupt glucose metabolism by altering pancreatic β-cell function.

In vitro studies have evaluated the effects of the high volume phthalate di-(2ethylhexyl) phthalate (DEHP) on the rat pancreatic β-cell line INS-1. At micromolar concentrations (100–400 μM), DEHP decreased cell viability in INS-1 cells treated for 24 h. This was associated with an increase in the production of reactive oxygen species (ROS) and a decline in the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase. In addition, altered lysosomal membrane permeability and a reduction in the mitochondrial membrane potential were reported. Overall these data suggest that both oxidative stress and lysosome-mitochondrial damage could be responsible for DEHP effects (She *et al.*, 2017). This reduced cellular viability was also found in studies performed by Sun and collaborators who observed that INS-1 cells treated with DEHP (25, 125, or 625 μM) for 24 h showed significant oxidative damage and activation of ER stress response that ultimately promoted increased apoptosis and decreased glucose-stimulated insulin secretion (GSIS) (Sun *et al.*, 2014). The phthalate metabolites, monoisobutyl phthalate (MiBP), mono-*n*-butyl phthalate (MnBP), and mono-(2-ethylhexyl) phthalate (MEHP) have also been reported to impair GSIS and cell viability (Weldingh *et al.*, 2017). In contrast, other studies did not find any effect of DEHP on GSIS but did report a decline in basal insulin secretion (Hectors *et al.*, 2013).

Curiously, in the human pancreatic β-cell line 11B4, 72 h treatment with monoethyl phthalate (MEP) (1–1000 nM) augmented cellular proliferation and insulin content after glucose induction with enhanced expression of the transcription factor PDX-1 (Guven *et al.*, 2016).

Animal studies have shown that in utero exposure to DEHP may disrupt pancreatic β-cell function in the offspring. Treatment with DEHP (1.25 and 6.25 mg/kg/d) throughout gestation and lactation provoked the development of glucose intolerance in the female offspring at 27 weeks of age. This was accompanied by lower insulin release in response to glucose as well as reduced pancreatic β-cell mass and degranulation (Lin *et al.*, 2011). Similarly, perinatal administration of DEHP, at doses between 1 and 100 mg/kg/d, resulted in glucose intolerance with decreased insulin secretion and content at postnatal day 60. Interestingly the changes were associated with an increment of global DNA methylation that led to a downregulation of several genes involved in pancreatic β-cell function and differentiation such as Pdx-1, Pax-4, and Pax-6 (Rajesh and Balasubramanian, 2014).

Bisphenol-A

Bisphenol-A (BPA) is a widespread EDC that has been extensively used as the base compound in the production of polycarbonate plastics and the resin lining of food and beverage cans. It is commonly found in a variety of plastic items including plastic dinnerware, water bottles, children's toys, medical and dental devices, dental sealants, and household electronic equipment among others. It is also relatively abundant in thermal paper cash register receipts (Schecter *et al.*, 2010; Lopez-Cervantes and Paseiro-Losada, 2003; Hehn, 2016). BPA has been detected in 93% of urine samples in the United States (Calafat *et al.*, 2008). It has been found to be present in urine, amniotic fluid, neonatal blood, placenta, cord blood, and human breast milk at levels that are known to be biologically active (Vandenberg *et al.*, 2010, 2007). Levels found in urine range from 2.38 to 4.32 μg/g creatinine (Centers for Disease Control and Prevention (CDC), 2015). General population exposure to BPA occurs via ingestion but also by dermal contact and inhalation (Vandenberg *et al.*, 2010; Zalko *et al.*, 2011). Nowadays BPA is accepted as a contributing factor in the increasing incidence of diabetes and obesity worldwide (Gore *et al.*, 2015; Alonso-Magdalena *et al.*, 2011; Casals-Casas and Desvergne, 2011). The mechanisms for the diabetogenic effects of BPA are multifactorial but mounting evidence suggests that the disrupting effects of BPA on pancreatic β-cells is key. This has been demonstrated in a series of in vitro and in vivo studies.

In the rat β-cell line INS-1, BPA effects are variable depending on the dose. INS-1 cells cultured in the presence of BPA (1–100 nM) showed increased basal insulin release but no alteration in GSIS (Hectors *et al.*, 2013). On the contrary BPA (100 and 1000 nM) increased both basal secretion and GSIS in the TC-6 β-cell line leading to ER stress as indicated by the activation of Hsp70 (Makaji *et al.*, 2011). Effects into the micromolar range (0.0020–2 μM) have also been explored in the INS-1 cell line. At a dose of 0.002 μM, BPA increased GSIS (Lin *et al.*, 2013), while higher doses (0.2, 2 μM) had the opposite effect (Lin *et al.*, 2013; Weldingh *et al.*, 2017). Similar directional changes in insulin gene and protein expression were observed (Lin *et al.*, 2013). Furthermore, BPA treatment has been observed to decrease cell viability and augment apoptosis while reducing expression of some genes essential for β-cell metabolism and mitochondrial function (Lin *et al.*, 2013). In another study BPA was shown to increase ROS production and DNA damage (Xin *et al.*, 2014). Another mechanism by which BPA may promote β-cell failure in the INS-1 cell line is via human islet amyloid aggregation coupled with an enhancement of the cytotoxic properties of this peptide (Gong *et al.*, 2013).

In vitro experiments have also shown that BPA can disrupt the function of isolated pancreatic islets. In a rapid manner BPA (100 pM–10 nM) provoked closure of the pancreatic β-cell K_{ATP} channels, increasing the frequency of $[Ca^{2+}]_i$ oscillations, which in turn resulted in a rapid increase in insulin secretion. This effect has been demonstrated to be mediated by the activation of extranuclear estrogen receptor β (ERβ) (Nadal *et al.*, 2000; Soriano *et al.*, 2012). Longer exposures to BPA promoted insulin gene transcription and biosynthesis after extranuclear binding to the estrogen receptor α (ERα) (Alonso-Magdalena *et al.*, 2008). At higher doses in the micromolar range, BPA effects on insulin secretion and content exhibited an inverted U-shape with an

augmentation at lower doses (0.1–2.5 μM) followed by a significant decline at higher concentrations (25 and 250 μM) (Song *et al.*, 2012). In contrast, other reports have observed a monotonic response with similar augmentation at 10 and 100 μM (Adachi *et al.*, 2005).

Animal studies have largely demonstrated that maternal exposure to BPA during gestation, or gestation and lactation, have profound effects on glucose homeostasis and pancreatic *β*-cell function in the offspring (Alonso-Magdalena *et al.*, 2010; Wei *et al.*, 2011; Liu *et al.*, 2013; Angle *et al.*, 2013; Garcia-Arevalo *et al.*, 2014) while paternal exposure may not have any effect (Ding *et al.*, 2014).

Male offspring of mice exposed to BPA at a dose of 10 μg/kg/d from day 9 to 16 of gestation displayed glucose intolerance and insulin resistance at 6 months of age. Pancreatic β-cells from these animals were more sensitive to extracellular glucose with enhanced GSIS that could be a compensatory mechanism in response to insulin resistance (Alonso-Magdalena *et al.*, 2010). Similar results were observed at the BPA dose of 50 μg/kg/d with augmented pancreatic β-cell mass (Wei *et al.*, 2011). At later stages of life, insulin secretion has been reported to decrease in BPA-treated offspring (Liu *et al.*, 2013). Importantly, the impairment of glucose and lipid metabolism was exacerbated by combining prenatal BPA exposure with later feeding of a high fat diet (HFD). On a HFD, BPA exposure led to diminished insulin secretory response to glucose suggesting that the compensatory changes in the islets could not overcome insulin demands (Garcia-Arevalo *et al.*, 2014; Wei *et al.*, 2011).

BPA has also been shown to alter pancreas development. A recent study has demonstrated that exposure to low doses of BPA during gestation led to increased pancreatic β-cell mass and area (Garcia-Arevalo *et al.*, 2016) and to a higher number of islet-cell clusters (Whitehead *et al.*, 2016) within the early life of the offspring. These early changes were associated with a rise in cell division, decreased apoptosis, and marked hyperinsulinemia (Garcia-Arevalo *et al.*, 2016). Increased insulin secretion leading to excessive insulin signaling has been proposed to be responsible for the alterations in the adult offspring glucose homeostasis previously described (Garcia-Arevalo *et al.*, 2016).

Transgenerational effects from early developmental exposures to BPA have also been reported. F0 maternal exposure to BPA provoked an impairment of insulin secretion, epigenetic changes in islets (hypermethylation of Igf 2), and reduced β-cell mass in the F2 offspring through the male germ line (Mao *et al.*, 2015).

Of note, metabolic abnormalities were observed not only in the offspring but in the mother. BPA-exposed pregnant females develop glucose intolerance, impaired pancreatic β-cell function, and decreased β-cell mass 7 months after delivery (Alonso-Magdalena *et al.*, 2015).

In adults, BPA exposure at a dose of 100 μg/kg for 4 days increased insulin content and insulin release favoring hyperinsulinemia and insulin resistance (Alonso-Magdalena *et al.*, 2006). Conversely, longer treatments (20 days) resulted in decreased insulin secretion, enhanced apoptosis, and lower gene expression of Glut 2, Pdx1 among others (Ahangarpour *et al.*, 2016). In animal models of T1D (i.e., NOD mice) BPA treatment exacerbated insulitis and hyperglycemia (Bodin *et al.*, 2013).

Alkylphenols

Alkylphenols are synthetic surfactants found in detergents, cleaning products, pesticides, antistatic agents, lubricants, hair care products, solubilizers, and as additives in plastics such as PVC, and polystyrene. 4-*n*-Nonylphenol (NP) and 4-*n*-octylphenol (OP) are some of the most common. Human exposure to these compounds may occur through ingestion of contaminated food and drinking water as well as from contact with some personal care products and detergents (Soares *et al.*, 2008). Biomonitoring studies, based on the representative subsamples of National Health and Nutrition Examination Survey (NHANES) 2005–10, have reported urinary detectable levels of OP in the North American population (Centers for Disease Control and Prevention (CDC), 2016).

Very few studies have explored the potential alterations in the pancreas arising from alkylphenol exposure. Rat islets cultured for 24 h in the presence of OP or NP at doses of 25 and 250 μg/L showed increased basal insulin secretion as well as insulin content (2.5 μg/L), while higher doses (250 μg/L) decreased insulin content (Song *et al.*, 2012). In a similar manner, 24 h islet treatment with NP (0.1–1000 μg/L) increased insulin secretion in response to stimulatory glucose concentration (16.7 mM) (Adachi *et al.*, 2005).

Persistent Organic Pollutants (POPs)

POPs are organic chemicals of significant concern because of their physical and chemical properties: they resist chemical and biological degradation so they remain intact for extremely long periods of time, they have high lipid solubility that promotes accumulation in the fatty tissue of living organisms, they bioaccumulate in ecosystems and they are transported far from their original sources resulting in worldwide distribution. The most commonly encountered POPs are organochlorine pesticides (aldrin, dieldrin, chlordane, *o,p′*-dichlorodiphenyltrichloroethane (DDT), endrin, heptachlor, mirex, toxaphene), industrial chemicals (polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB)) and unintended chemical by-products (polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/PCDF)). More recently other compounds such as polyfluoroalkyl compounds (PFCs) and flame retardants have been added to the list of POPs under the Stockholm convention (Convention, 2008).

Due to the large number of compounds classified as POPs we will focus on those whose effects on pancreatic β-cell are best studied.

Dioxins

Dioxins mainly consist of polychlorinated dibenzodioxins (PCDDs), the polychlorinated dibenzofurans (PCDFs), certain polychlorinated biphenyls (PCBs), and other related compounds. They are mainly by-products of industrial processes including chlorine bleaching of paper pulp, incineration of chlorine-containing substances such as polyvinyl chloride (PVC) and the manufacturing of some pesticides and herbicides. They can also result from natural processes like volcanoes and forest fires (EPA, 2017). The PCDD 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most studied and toxic of all dioxins. It was a major contaminant in the industrial accident in Seveso, Italy, and during the Vietnam War. The main toxic effects, sources of exposure, and reference doses are described in reviews by the Environmental Protection Agency (EPA) (EPA, 2012) and CDC (Centers for Disease Control and Prevention (CDC), 1984).

In vitro studies have evaluated the effect of TCDD on pancreatic β-cell physiology although the conclusions are somehow discrepant. A significant increase of cytosolic calcium influx via T-type channels as well as enhanced insulin secretion at basal glucose concentrations (2.8 mM) were observed on INS-1E cells cultured in the presence of TCDD (50 and 100 nM) (Kim *et al.*, 2009). On the contrary, lower doses of TCDD (0.05–1 nM) have been shown to severely impair GSIS and to dose-dependently decrease cell survival (Piaggi *et al.*, 2007). The toxicity of TCDD on this cell line was prevented by epigallotacatechin-3-gallate, an inhibitor of the aryl hydrocarbon receptor (AhR) (Martino *et al.*, 2013). In primary islets TCDD did not alter insulin secretion; however, a significant reduction of insulin content was observed at all doses tested (0.1–100 nM) (Kurita *et al.*, 2009).

Few animal studies have evaluate TCDD effects on glucose homeostasis but they all indicate that TCDD exposure in adult mice promote an impairment of GSIS and insulin content (Kurita *et al.*, 2009; Novelli *et al.*, 2005), decreased plasma insulin levels (Kurita *et al.*, 2009; Ebner *et al.*, 1988) and transient hyperglycemia (Ebner *et al.*, 1988). Importantly, the impaired insulin secretion following TCDD administration has been proposed to be mediated by AhR (Kurita *et al.*, 2009).

Polychlorinated Biphenyls (PCBs)

PCBs are a subset of the synthetic organic chemicals known as chlorinated hydrocarbons that have been produced on an industrial scale from the late 1920s until they were banned in 1979. Despite being banned they remain widespread. They have been commonly used in a variety of materials as insulators in transformers and capacitors, as heat exchange fluids and as plasticizers in paints, plastics and rubber products as well as in carbonless copy paper, adhesives, and inks. A total of 209 possible PCB congeners exist (Fisher, 1999; Gore *et al.*, 2015). Biomonitoring information of each of them can be found in the Fourth CDC report (Centers for Disease Control and Prevention (CDC), 2015).

The effects of two PCB congeners (2,2′,4,4′-tetrachlorobiphenyl and 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB153)) and the commercial PCB mixture Aroclor-1254 have been examined in the rat β-cell line RINm5F. Treatment with each of these agents (5–10 μg/mL) increased insulin release in a calcium-dependent mechanism, which required the activation of a calcium/calmodulin-dependent kinase II (Fischer *et al.*, 1996, 1999).

In vivo intranasal PCB126 exposure (10 μg/kg) for 15 days in male rats induced insulin resistance, hyperinsulinemia, and hypertriglyceridemia together with increased oxidative stress on the islets of Langerhans. The analysis of the proteomic profile of the islets revealed that PCB126 exposure modified the expression of some proteins related to oxidative stress (Loiola *et al.*, 2016). In addition, PCB126 (2–5 nM) altered pancreas morphology during development in zebrafish. These included hypomorphic islets, altered islet migration, the appearance of ectopic β-cells, and islet fragmentation (Timme-Laragy *et al.*, 2015). Other PCBs have also been studied. For example perinatal exposure to PCB 153 in mice (0.09–1406 μg/kg/d) resulted in decreased pancreas weight in the female offspring and enhanced glucagon levels; however, no differences in glucose tolerance or insulin sensitivity were observed (van Esterik *et al.*, 2015). In contrast, chronic exposure to Aroclor 1254 (0.5, 5, 50, and 500 μg/kg) in adult mice caused a significant increment of pancreatic β-cell mass and reduced α-cell mass together with hyperinsulinemia, glucose intolerance, and hyperglycemia (Zhang *et al.*, 2015).

p,*p*′-Dichlorodiphenyltrichloroethane (DDT)

DDT is an organochlorine insecticide with a long half-life that was commonly used in the United States until 1972 when it was banned. It is still used in certain countries of Africa, South America, and Asia mainly to control malaria (EPA, 2016b).

Little is known about the direct effects of this compound on the pancreatic β-cell. In a recent study the effects of DDT and DDE, a metabolite of DDT (0.1, 1, and 10 μM), have been explored in the human pancreatic β-cell line NES2Y. Prolonged exposure to these pollutants decreased cellular viability at the highest concentration, and downregulated expression of some cytoskeletal and glycolysis-involved proteins (Pavlikova *et al.*, 2015).

Polyfluoroalkyl Compounds (PFCs)

Polyfluoroalkyl compounds (PFCs) is the collective name for a series of synthetic fluorinated organic compounds widely used in a range of industrial applications and the manufacture of products including nonstick cookware, coatings of some food packaging as well as components of fire-fighting foam. They are generally hydrophobic and lipophobic and will not tend to accumulate in fatty tissues; however, they can bind noncovalently to plasma proteins and distribute throughout the body. Among PFCs, perfluorooctane sulfonate (PFOS), and perfluorooctanoic acid (PFOA) have been the most extensively produced and studied. They are very stable to metabolic and environmental degradation (EPA, 2016a). Using data from CDC's 2003–2004 NHANES, four PFCs (PFOS, PFOA, PFHxS, or perfluorohexane sulfonic acid, and PFNA or perfluorononanoic acid) have been detected in over 98% of the serum samples collected (Calafat *et al.*, 2007). The mean levels found in serum for PFOA was 2.08 μg/L for PFOA and 6.31 μg/L for PFOS (Centers for Disease Control and Prevention (CDC), 2015).

Exposure of mice to PFOA in the range of 0.5–5 mg/kg/d for 7 days led to oxidative damage in pancreas and liver which was manifested by increased lipid peroxidation. An upregulation of several antioxidant pancreatic genes (Sod1, Sod 2, Gpx2, and Nqo1) was observed as a compensatory mechanism to alleviate oxidative stress. Interestingly accumulation of PFOA was observed not only in the serum but also in the liver and pancreas (Kamendulisa *et al.*, 2014). PFOS has been shown to disrupt pancreas organogenesis in zebrafish. Embryos exposed to micromolar concentrations of PFOS during development exhibited decreased islet area and size as well as pancreas length following a U-shaped dose response curve (Sant *et al.*, 2016, 2017).

Organotins

Organotins are a diverse class of ubiquitous environmental contaminants widely used in agriculture and industry as biocides, fungicides, molluscicides, rodent repellents, algaecides, wood preservatives, antifouling paints as well as stabilizer additives in PVC products. So far, major attention has been given to the organotin tributyltin chloride (TBT) because of its exceptional toxicity to aquatic life. It constitutes one of the first examples of endocrine disruption: TBT was shown to induce irreversible sexual abnormalities in female marine gastropods, a phenomenon known as imposex. Other organotins such as triphenyltin chloride (TPTC) have also been shown to be highly toxic biocides (Appel, 2004; Anastasiou *et al.*, 2016). The major route of human exposure to these pollutants comes from consumption of contaminated drinking, beverages, fish, and seafood. There is scarce information on levels of human exposure to organotins. A few studies have established dietary exposure levels of 2–5 μg/d, although levels can be as high as 375 μg/d in some demographic groups (Grun, 2014).

Acute exposure to TBT (0.1–0.2 μM) in the β-cell line RIN-m5F resulted in an increment of Ca^{2+} concentration, GSIS as well as elevated levels of ROS production; an effect that was abrogated in the presence of a protein kinase C (PKC) inhibitor. The same results were obtained in mouse and human islets (Chen *et al.*, 2017). The in vivo effects of TBT on the endocrine pancreas were variable depending on the dose, gender, and duration of treatment. Male mice treated with TBT for 45 days at doses of 0.5, 5, and 50 μg/kg/d showed morphological changes in the pancreas with increased apoptosis and reduced proliferation of islet cells. This caused a reduction in the relative islet area at day 60 of treatment. At this time point important metabolic alterations were observed, including glucose intolerance, decreased plasma insulin and glucagon levels as well as increased fasting glucose levels (Zuo *et al.*, 2014). Shorter exposures in female rats (0.1 μg/kg/d) resulted in a very mild glucose intolerance, hyperinsulinemia, and a greater number of islets (Bertuloso *et al.*, 2015).

A single oral dose of triphenyltin fluoride (TPTF) (100 mg/kg) in rabbits was shown to promote lower insulin release in response to glucose, glucagon or arginine. In addition, the animals showed higher fasting plasma glucose levels with lower basal insulin levels, likely due to reduced pancreatic insulin secretion. However, few abnormalities of the insulin secretory granules in pancreatic β-cells were observed by electron microscopy (Manabe and Wada, 1981). Similarly, decreased GSIS was observed after oral administration of triphenyltin chloride (TPTC) in hamsters with no change in the morphology of the pancreas (Miura and Matsui, 1987; Matsui *et al.*, 1984; Ogino *et al.*, 1996). This has been proposed to occur due to a diminution of Ca^{2+}-influx through voltage-gated Ca^{2+} channels promoted by reduced Na^{+} permeability via a protein kinase A-dependent mechanism as well as by a reduction of NAD(P)H and ATP production in pancreatic islets (Miura *et al.*, 1997, 2012; Miura and Matsui, 2006).

Heavy Metals

Heavy metals are defined as metallic chemical elements with relatively high density and atomic weight that are toxic or poisonous at low concentrations. Although they are found naturally on the earth, most environmental contamination and subsequent human exposure result from anthropogenic activities.

Multiple epidemiological reports have shown evidence suggesting that certain heavy metals exhibit diabetogenic characteristics (Gonzalez-Villalva *et al.*, 2016; Menke *et al.*, 2016; Hectors *et al.*, 2011). We summarize below the studies that reveal effects of heavy metals on glucose homeostasis with regard to metabolic function of pancreatic islets and, more specifically, insulin-secreting β-cells.

Arsenic

Because of its high degree of toxicity, arsenic is ranked first on the priority list of hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR) (2017). Natural arsenic sources include numerous mineral species and volcanos, which release it into water, air, and soil. Of these different sources of exposure, ingestion is the major one within the general population. Arsenic levels in soil range from about 1 to 40 parts of arsenic to a million parts (ppm) of soil, with an average of 3–4 ppm. EPA reduced the maximum contaminant level (MCL) standard for arsenic in drinking water from 50 to 10 μg/L (Agency for Toxic Substances and Disease Registry (ATSDR), 2007a).

Research conducted in rodent pancreatic islets and single β-cells suggests that arsenic may contribute to the development of T2D by impairing β-cell function. Numerous in vitro studies have observed inhibition of insulin secretion after either acute (90 min) (Ahangarpour *et al.*, 2017) or prolonged (48–72 h) (Douillet *et al.*, 2013; Diaz-Villasenor *et al.*, 2006) arsenic exposure at 100 and $\leq$5 μM, respectively. Apart from reporting reduced GSIS (Diaz-Villasenor *et al.*, 2006; Douillet *et al.*, 2013; Fu *et al.*, 2010), some of these studies also detected diminished insulin release at basal glucose concentrations (Ahangarpour *et al.*, 2017; Diaz-Villasenor *et al.*, 2006). The mechanisms by which insulin secretion is inhibited include, among others, reduced intracellular Ca^{2+} oscillations and decreased Ca^{2+}-dependent calpain-10 proteolysis of SNAP-25 (Diaz-Villasenor *et al.*, 2008). Insulin synthesis is also impaired in single rat β-cells exposed to 5 μM arsenic for 72 h (Diaz-Villasenor *et al.*, 2006) and in islets exposed to methylarsenite (MAsIII) and dimethylarsenite (DMAsIII) at $\leq$1 μM for 48 h (Douillet *et al.*, 2013). In contrast to the findings described in primary cultures, Fu *et al.* described elevated insulin secretion at 3 mM glucose after exposure of the β-cell line INS-1 (832/13) to arsenic, likely due to increased insulin mRNA and protein levels (Fu *et al.*, 2010). Other studies performed in β-cell lines have also associated arsenic exposure with enhanced mitochondrial mass (Fu *et al.*, 2010), autophagic β-cell death (Zhu *et al.*, 2014), increased caspase-3 activity and apoptosis (Yen *et al.*, 2007; Yao *et al.*, 2015), augmented antioxidant levels (Yen *et al.*, 2007; Fu *et al.*, 2010), and increased reactive oxygen species (ROS) content (Yen *et al.*, 2007; Zhu *et al.*, 2014) as well as decreased intracellular ATP levels (Yen *et al.*, 2007). Hence, numerous arsenic-induced effects may blunt β-cell function.

In mice, the exposure to 3 mg/L arsenic in drinking water for 16 weeks increased plasma insulin (Liu *et al.*, 2014), whereas 10 mg/L increased plasma insulin at early stages and reduced it from week 5 to week 12 (Yen *et al.*, 2007). Other symptoms observed following arsenic exposure include pancreatic inflammation, decreased Irs1 and Glut4 as well as increased Adipoq mRNA levels (Liu *et al.*, 2014), and islet damage due to mild accumulation of inflammatory cells in the pancreas (Yen *et al.*, 2007). In parallel, in diabetic leptin deficient (*db*/*db*) mice, prolonged arsenic exposure caused increased fasting blood glucose, reduced plasma insulin, impaired glucose handling and insulin sensitivity as well as pancreatic inflammation (Liu *et al.*, 2014).

Cadmium

Cadmium is a metal of considerable health concern due to its high toxicity. Natural sources involve emissions from volcanic eruptions or forest fires, among others. It is present in the earth's crust at approximately 0.1–0.5 ppm, commonly associated with other metals, and its levels in ocean water have been reported between $<$5 and 110 ng/L (Agency for Toxic Substances and Disease Registry (ATSDR), 2012). It is frequently used in industrial processes including the production of alkaline batteries, stabilizers for plastics, nonferrous alloys and pigments. Cadmium bioaccumulates in the food chain, and consumption of cadmium-contaminated food is a major source of human exposure. In addition, smoking is a significant and potentially modifiable source of human cadmium intake (Agency for Toxic Substances and Disease Registry (ATSDR), 2012).

In vitro experiments revealed that cadmium impairs GSIS in both MIN6 cells (1 μM) and primary mouse islets (0.1 μM) following 48 h exposure (El Muayed *et al.*, 2012). Although neither cell death nor oxidative stress seemed to be responsible for the decrease in insulin release (El Muayed *et al.*, 2012), a different study showed augmented generation of intracellular ROS and malondialdehyde (MDA) production after cadmium exposure of the rat β-cell line RIN-m5F, which contributed to mitochondrial dysfunction (Chang *et al.*, 2013). In addition, the authors reported JNK-MAPK activation and apoptotic-related signals that led to β-cell death (Chang *et al.*, 2013). Thus, cadmium exposure, and its subsequent accumulation within β-cells (El Muayed *et al.*, 2012; Chang *et al.*, 2013), causes cellular toxicity and β-cell dysfunction.

Numerous studies conducted in animal models confirmed the development of hyperglycemia after oral (Merali and Singhal, 1980; Trevino *et al.*, 2015) and parenteral (Ithakissios *et al.*, 1975; Merali and Singhal, 1976, 1975; Bell *et al.*, 1990; Kanter *et al.*, 2003; Edwards and Prozialeck, 2009) cadmium administration. In addition to increased blood glucose levels, cadmium also produced impaired glucose tolerance (Merali and Singhal, 1975; Chang *et al.*, 2013), reduced plasma insulin levels (Ithakissios *et al.*, 1975; Kanter *et al.*, 2003; Edwards and Prozialeck, 2009; Chang *et al.*, 2013), and decreased in vivo insulin release in response to a glucose load (Merali and Singhal, 1976), although one study observed hyperinsulinemia accompanied by hyperglycemia after cadmium administration (Trevino *et al.*, 2015). Further ex vivo research showed that both isolated islets (Merali and Singhal, 1980) and perfused pancreas (Ithakissios *et al.*, 1975) from cadmium-treated rats secreted less insulin in response to high glucose concentrations compared to controls. In addition, oral cadmium administration increased proinflammatory cytokines (TNF-α, IL1β, and IFN-γ) and cleaved caspases levels, and reduced cellular defense protein levels (Nrf2 and HO-1) as well as glucose transporters (GLUT2 and GLUT4) in rat pancreas (Bashir *et al.*, 2016). Moreover, cadmium exposure produced morphological changes in the pancreas that include degranulation, degeneration, and necrosis (Demir *et al.*, 2006; Kanter *et al.*, 2003), and possible loss of cell–cell adhesion (Edwards and Prozialeck, 2009).

Lead

Lead is a toxic metal and considered a major public health risk due to its widespread use; it is actually the second most toxic compound according to the Agency for Toxic Substances and Disease Registry (ATSDR) (2017). Despite the fact that it occurs naturally, anthropogenic sources are mostly responsible for the 1000-fold increase in environmental lead levels that have occurred over the past three centuries (Agency for Toxic Substances and Disease Registry (ATSDR), 2007b). Lead has multiple industrial and domestic applications and it is found in plumbing pipes, leaded gasoline, lead-based paints, and storage batteries among other uses. The main sources of human exposure are inhalation of lead-contaminated dust particles and ingestion of lead-contaminated water and food. EPA limited the concentration of lead in breathed air to 1.5 $\mu g/m^3$ averaged over 3 months, and to 0.015 mg/L in drinking water (Agency for Toxic Substances and Disease Registry (ATSDR), 2007b).

Mouse isolated islets exposed to lead (1–10 mM) ex vivo displayed reduced cell viability and GSIS, and exhibited potentiated glycogen synthase kinase-3 beta (GSK-3β) activity and ROS levels (Mostafalou *et al.*, 2015), thus indicating that lead exposure impairs β-cell viability and function.

The administration of lead (0.05% and 0.2%, for 32 days) in drinking water slightly increased blood glucose levels, and potentiated fasting insulin levels, and HOMA-IR in rats (Mostafalou *et al.*, 2015). While these findings suggest an effect on insulin responsive tissues, the lack of studies in the pancreas restricts knowledge of whether in vivo lead exposure also impairs islet function as part of the mechanisms by which it dysregulates glucose homeostasis.

Mercury

Mercury occurs naturally, and it is mainly present in three forms: metallic mercury, inorganic mercury salts, and organic mercury compounds. Methylmercury, mainly produced from methylation of inorganic mercury by microorganisms, is among the most common natural forms of mercury. It is of particular concern because methylmercury is linked to several health disorders. The major sources of mercury accumulation in the general population result from the ingestion of methylmercury-contaminated fish or seafood, and from dental amalgam fillings containing metallic mercury (Agency for Toxic Substances and Disease Registry (ATSDR), 1999). The limits set by EPA and Food and Drug Administration (FDA) are 2 parts of inorganic mercury per billion (ppb) parts of water in drinking water, and 1 part of methylmercury in a million parts (ppm) in seafood products (Agency for Toxic Substances and Disease Registry (ATSDR), 1999).

In vitro exposure of β-cell derived HIT-T15 cells (Chen *et al.*, 2006a,b, 2010) and mouse islets (Chen *et al.*, 2006a,b) to both organic (methylmercury) and inorganic mercury inhibited GSIS. Moreover, these mercuric compounds triggered ROS generation (Chen *et al.*, 2006a,b), activation of PI3K and Akt phosphorylation (Chen *et al.*, 2006b), disruption of the mitochondrial membrane potential and activation of caspase-3 (Chen *et al.*, 2006a). In addition, mercury decreased cell viability (Chen *et al.*, 2006a, 2010) and increased both apoptosis and necrosis in the β-cell line (Chen *et al.*, 2010).

In agreement with the outcomes reported in vitro, the oral administration of mercuric compounds from 2 to 6 consecutive weeks raised blood glucose (Chen *et al.*, 2006b, 2012; Maqbool *et al.*, 2016), reduced plasma insulin (Chen *et al.*, 2006b, 2012; Maqbool *et al.*, 2016), and C-peptide (Maqbool *et al.*, 2016) levels, produced glucose intolerance (Chen *et al.*, 2012, 2006b; Maqbool *et al.*, 2016), insulin resistance (Maqbool *et al.*, 2016), and plasma lipid peroxidation (Chen *et al.*, 2006b; Maqbool *et al.*, 2016), and increased plasma MDA levels (Chen *et al.*, 2012, 2006b) in rats. Further research conducted in pancreas from mercury-treated animals detected increased ROS levels and apoptotic cells within the islets (Chen *et al.*, 2012). In line with these findings, gene expression studies revealed increased p53, caspase-3, and caspase-7 (apoptotic genes) and decreased Bcl-2, Mcl-1, and Mdm-2 (antiapoptotic genes) and Nrf2, GPx, and NQO1 (antioxidant genes) mRNA levels within the islets (Chen *et al.*, 2012). Altogether, these results indicate that mercuric compounds induce oxidative stress and cause apoptosis and islet dysfunction.

Nickel

Nickel is a naturally occurring element, found in all soil, water, and air, primarily in combination with oxygen and sulfur. Nickel is frequently alloyed with iron, copper, chromium, and zinc, which are later used in making metal coins, jewelry, valves, or heat exchangers (Agency for Toxic Substances and Disease Registry (ATSDR), 2005). Humans are exposed to nickel from air and polluted food and drinking water. Common amounts of nickel in soil are between 4 and 80 ppm, and between 2 and 4.3 ppb in drinking water in the United States (Agency for Toxic Substances and Disease Registry (ATSDR), 2005).

Various animal studies have reported hyperglycemia following nickel administration (Cartana and Arola, 1992; Alvarez *et al.*, 1993; Gupta *et al.*, 2000; Horak *et al.*, 1978; Horak and Sunderman, 1975). In fact, just a single intraperitoneal injection of 12–85 μmol/kg body weight (Horak and Sunderman, 1975; Horak *et al.*, 1978; Gupta *et al.*, 2000; Cartana and Arola, 1992) is sufficient to significantly and transiently raise blood glucose levels within 30 min. Further research suggested that nickel-induced hyperglycemia could be associated with hyperglucagonemia (Horak *et al.*, 1978; Cartana and Arola, 1992; Horak and Sunderman, 1975; Alvarez *et al.*, 1993), hypoinsulinemia (Cartana and Arola, 1992; Alvarez *et al.*, 1993) caused by α-adrenergic activation (Alvarez *et al.*, 1993), and increased inducible nitric oxide synthase (iNOS) protein in the pancreas (Gupta *et al.*, 2000). A decrease in insulin release following nickel exposure was also observed in isolated islets stimulated at both low and high glucose concentration (Dormer *et al.*, 1974).

Conclusion

Diabetes is a common terrible problem with a multifactorial origin. Pollutants are likely contributors to its etiology and, therefore, understanding the mechanisms by which EDCs promote diabetes is important to develop therapies for exposed individuals. In addition, improved environmental regulations are essential for mitigating environmental drivers of diabetes risk.

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Endocrine Disruptors and Obesity

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Introduction

Obesity, defined as a BMI greater than 30 kg/m^2, is an epidemic disease in children and adults around the world (reviewed in Heindel *et al.*, 2017; Hales *et al.*, 2017; Flegal *et al.*, 2016; Janesick and Blumberg, 2016; Legler *et al.*, 2015). The incidence of obesity in U.S. adults has increased over the past four decades from 13% to 35% (Flegal *et al.*, 2016). The most recent statistics from the period 2015–2016 put this number at 39.8% for U.S. adults and 20.6% in youths (Hales *et al.*, 2017). Obesity is linked not only to accumulation of adipose tissue, but also to many components of the endocrine system including the gastro-intestinal tract, pancreas, muscle, liver, and brain (reviewed in Heindel *et al.*, 2017). Obesity is associated with a wide variety of other comorbidities, including diabetes, metabolic syndrome, hypertension, coronary heart disease, gall bladder disease, and cancer. There is a strong correlation between an increase in childhood obesity and an increase in diagnoses of type 2 diabetes in children and adolescents (Dabelea *et al.*, 2014). Moreover, obesity is a risk factor for many components of type 2 diabetes and other metabolic diseases particularly when visceral fat is increased (Despres, 2012). Remarkably, nearly 40% of all cancers are diagnosed in overweight or obese people in the United States (Steele *et al.*, 2017). Together, the diseases associated with obesity are not only dangerous to individual health, but also very costly to society (Legler *et al.*, 2015; Attina *et al.*, 2016).

The current clinical paradigm for obesity is one of energy intake versus energy expenditure (Hall *et al.*, 2012), with clinical management of obesity consequently focused on diet and exercise (Hill and Peters, 1998). Other well-studied risk factors implicated in obesity include genetics (Herbert, 2008; Li *et al.*, 2010), smoking during pregnancy (Behl *et al.*, 2013; Power and Jefferis, 2002), and stress (Garruti *et al.*, 2008; Torres and Nowson, 2007). A provocative recent paper based on analysis of data derived from the U.S. National Health and Nutrition Examination Study (NHANES, 1971–2008) showed that leisure time physical activity has increased 47%–120% between 1988 and 2006, rather than having decreased as is often stated (Brown *et al.*, 2016). Moreover, for an equivalent amount of caloric intake, macronutrient intake, and leisure time physical activity, average adult BMI was up to 2.3 kg/m^2 higher in 2006 than in 1988 (Brown *et al.*, 2016). This paper makes two strong points. The first is that exercise is increasing, rather than decreasing. The second is that changes in diet and exercise are not adequate to explain the increase in BMI between 1988 and 2006 (Brown *et al.*, 2016). Together, these results cast serious doubt on the sufficiency of the energy balance model of obesity and strongly implicate the importance of other risk factors in obesity (Brown *et al.*, 2016).

Obesity is also largely intractable once established. More than 83% of obese individuals who lost substantial amounts of weight gained it back within a few years (Fildes *et al.*, 2015; Fothergill *et al.*, 2016; Kraschnewski *et al.*, 2010). The obesity epidemic is not limited to humans—it also affects animals living in close proximity to humans. These include domestic dogs and cats, feral rats living in cities, and, crucially, primates and rodents living in research colonies where diets are carefully controlled (Klimentidis *et al.*, 2011). It is difficult to argue that all of these animal populations suffer from inappropriate lifestyle choices. Rather, it is clear that it is counterproductive to frame the issue of obesity in terms of a single cause, energy balance. Regulation of body weight is complex and regulated by a variety of hormones that modulate glucose homeostasis, metabolism, energy balance, appetite and satiety, as well as the development of adipose tissue itself (reviewed in Heindel *et al.*, 2017). Considering the ever-increasing rise in the incidence of obesity and its broad contribution to adverse health outcomes, it is crucial to identify and understand the variety of factors that contribute to obesity beyond the simplistic energy balance model.

The Developmental Origins of Health and Disease Hypothesis

David Barker's eponymous "Barker Hypothesis," also known as the Fetal Origins Hypothesis, proposed that prenatal nutrition is an important factor in determining whether a child will be more or less vulnerable to metabolic diseases and cardiovascular disorders later in life (Barker, 2007; Hales and Barker, 2001). He proposed that prenatal malnutrition led the fetus to become adapted to a nutritionally poor environment and that if the environment was nutrient rich, instead, the child would be more susceptible to chronic diseases later in life, such as type 2 diabetes, high blood pressure, heart disease and obesity due to this mismatch. His hypothesis suggests that there are critical periods of development during which environmental factors can cause changes in phenotype that can lead to permanent biological dysfunctions and increased susceptibility to diseases later in life (Barker, 2007; Hales and Barker, 2001). Gluckman and Hanson recognized that the critical period was not restricted to fetal development and extended the fetal origins model, renaming it as "The Developmental Origins of Health and Disease" (Gluckman and Hanson, 2004; Gluckman *et al.*, 2009; Hanson and Gluckman, 2015).

The Thrifty Phenotype

James Neel first proposed the "thrifty gene" hypothesis, which suggested that particular gene variants evolved to favor efficient use of nutrients in the calorie-limited environment in which humans evolved, but that promote obesity and type 2 diabetes in the

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modern, calorie-rich environment (Neel, 1962). However, none of the common obesity-related gene variants yet identified were judged to confer any properties or traits that could be considered to provide a survival advantage (Wang and Speakman, 2016). At the same time, it is quite clear that there is a "thrifty phenotype" (Hales and Barker, 2001). A large number of studies in animals and humans linked poor prenatal nutrition with subsequent predisposition to disease, including obesity later in life and in subsequent generations (Barker, 2007; Hanson *et al.*, 2011). Essentially, prenatal programming is thought to adapt the fetus to poor, but not plentiful nutrition later in life. Despite considerable study, there are very few concrete details on the molecular mechanisms that promote a thrifty phenotype, although, there are some indications that leptin resistance (Friedman, 2014) and epigenetic mechanisms are involved (Godfrey *et al.*, 2011; Lillycrop, 2011).

Endocrine System and EDCs

The endocrine system strongly influences obesity because many endocrine organs and hormones work together to regulate metabolism and, in turn, weight. For example, insulin and glucagon are produced in the pancreas and act to modulate affect glucose uptake and usage; ghrelin and cholecystokinin affect metabolism in the gastrointestinal tract; and glucagon, insulin, and fibroblast growth factor 21 (FGF21) act in the liver to control metabolism, hunger, and satiety. Sex hormones such as estradiol also contribute to energy metabolism and obesity by affecting food intake, body weight, fat distribution, the balance of glucose and insulin, lipogenesis, and lipolysis (reviewed in Heindel *et al.*, 2017). The brain acts as an endocrine organ that contributes to obesity through its food reward mechanisms which are affected by hormones, growth factors, and neurotransmitters (Jager and Witkamp, 2014; Schellekens *et al.*, 2012; Volkow *et al.*, 2013). Hormones and growth factors also contribute to fat accumulation by affecting appetite, satiety, and energy (reviewed in Heindel *et al.*, 2017).

Furthermore, there are many ways in which the endocrine system acts directly on adipocytes to contribute to obesity. Growth factors and hormones, including estrogens, androgens, glucocorticoids, insulin, and thyroid hormones, play a part in controlling the number and content of adipocytes. Adipose tissue development and function is affected by steroid hormones including estrogens, androgens, and glucocorticoids, and is connected to the immune system through the function of adipokines (reviewed in Heindel *et al.*, 2017). Adipocytes, themselves, secrete over 20 endocrine, paracrine, and autocrine chemicals; and metabolism in adipocytes is affected by hormones such as glucagon, insulin, and FGF21 (reviewed in Heindel *et al.*, 2017). In this way, the endocrine system may have a direct effect on development of obesity by affecting the functioning of adipocytes within the body.

Endocrine Disrupting Chemicals

According to the Endocrine Society, an endocrine disrupting chemical (EDC) is "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action" (Zoeller *et al.*, 2012). While the original definition of EDCs focused on estrogens, antiandrogens and thyroid disruptors, it is now recognized that any endocrine signaling system can be disrupted by exogenous chemicals that target various places in endocrine signaling pathways (Gore *et al.*, 2015). EDCs can alter the rate of production and metabolism of hormones, affect the way hormones are transported to their target tissues and the action of the specific receptors for individual receptors (reviewed in Diamanti-Kandarakis *et al.*, 2009; Gore *et al.*, 2015). Most recently, Jun Kanno has introduced the concept of "signal toxicity," which proposes that essentially any receptor-based cellular signaling system can be disrupted by chemicals that act on the receptor (Kanno, 2016). A key feature of this model is that such chemicals act on receptors and without the receptor, there is no "toxicity." Rather than eliciting toxicity by damaging cellular components, chemicals that cause signal toxicity do so by interfering with signaling itself (usually by acting on the receptor). Such signaling disruptors are expected to act at low doses and not to exhibit threshold effects—both of which are characteristics of EDCs (Vandenberg *et al.*, 2012).

Currently, there are approximately 1000 known chemicals that are considered to be EDCs, although no systematic analysis of chemicals for endocrine disrupting effects has yet been undertaken (reviewed in Diamanti-Kandarakis *et al.*, 2009; Gore *et al.*, 2015). These chemicals are used in a wide variety of consumer products. They have been found in food packaging, building materials, pesticides, clothing, upholstery, detergents and cleaning agents, plastics, thermal paper, and medical equipment (Zoeller *et al.*, 2012; Vandenberg *et al.*, 2012; Gore *et al.*, 2015; Diamanti-Kandarakis *et al.*, 2009; Hormann *et al.*, 2014; Bergman *et al.*, 2013). EDCs also contaminate food, air, and water, due to their use in food production and industrial processes. EDCs can make their way into the body orally, through dermal contact, through inhalation, or in the case of contamination of medical equipment, intravenously or subcutaneously (reviewed in Diamanti-Kandarakis *et al.*, 2009; Gore *et al.*, 2015). Often, only low levels of EDCs are necessary to affect the endocrine system and the effects often do not scale linearly with dose, that is, are nonmonotonic (Vandenberg *et al.*, 2012). This means that even limited exposure to these EDCs may lead to significant effects later in life.

The Obesogen Hypothesis

In 2006, we proposed the existence of endocrine disrupting chemicals that could influence adipogenesis and obesity and be important, yet unsuspected players in the obesity epidemic. These "obesogens" are functionally defined as chemicals that promote

obesity. Obesogens can act directly to increase the number of fat cells and/or the storage of fat into existing cells. Obesogens can act indirectly by changing basal metabolic rate, by shifting energy balance to favor calorie storage, and by altering hormonal control of appetite and satiety (reviewed in Janesick and Blumberg, 2011; Heindel *et al.*, 2017). Evidence has accumulated linking EDCs to obesity, and obesogens have been detected in humans (Stahlhut *et al.*, 2007; Hatch *et al.*, 2008; Trasande *et al.*, 2012; Li *et al.*, 2013; Tang-Peronard *et al.*, 2011) and animals (Newbold *et al.*, 2009; Vom Saal *et al.*, 2012; Grun *et al.*, 2006; Hines *et al.*, 2009). Because these chemicals are pervasive in the environment, it is crucial to understand how they disrupt developmental programming, predisposing individuals to obesity and related disorders

EDC exposure during development can cause lasting changes in physiology, similar to the case with early life nutrition in the DOHaD model. EDCs alter hormonal levels and function; hormones and growth factors are of utmost importance in development, as they affect gene expression, number of cells, cell location, and the balance between cell types. Moreover, EDCs that affect organ structure and endocrine signaling between cells can cause increased susceptibility to disease and organ dysfunction in the developed organism (reviewed in Heindel *et al.*, 2017; Lughetti *et al.*, 2015). Thus, obesogenic EDCs work by affecting nuclear factors or other endocrine pathways during development in ways that lead to obesity later in life. Indeed, exposure to EDCs during critical periods of development, such as when cells are differentiating into adipocytes, has been shown to contribute to diseases such as obesity in adulthood (reviewed in Heindel *et al.*, 2017).

Adipogenesis

Adipose tissue, comprised of white, brown, and beige fat, is a source of energy and an important regulator of energy balance and homeostasis in the body (reviewed in Heindel *et al.*, 2017). It aggregates in depots located subcutaneously, viscerally, and intrathoracically (reviewed in Heindel *et al.*, 2017). White adipose tissue develops in humans by the 14th week of gestation (Poissonnet *et al.*, 1984). A second period during which more adipose cells are developed occurs after birth and continues through adolescence (Spalding *et al.*, 2008). After this second period of adipose tissue development, the minimum number of white adipose cells (which store energy as triglycerides) in the body is thought to be fixed (Rosen and Spiegelman, 2014), but fat can continue to accumulate within those cells, increasing total fat mass in the body, into adulthood.

The development of adipocytes starts with mesenchymal stem cells (MSCs), which first must commit to the adipocyte cell lineage. Commitment is followed by differentiation into mature adipocytes (reviewed in Rosen and Spiegelman, 2014). Commitment of MSCs to the adipocyte cell lineage is mediated by transcription factors Zfp423, Zfp467, Schnurri2, Tcf7l1, and the mTORC1 effector S6K1 (Gupta *et al.*, 2010; Quach *et al.*, 2011; Carnevalli *et al.*, 2010; Cristancho and Lazar, 2011). One effect of these genes in contributing to adipogenesis is promoting the expression of the gene PPARγ, the master regulator of adipogenesis (reviewed in Rosen and Spiegelman, 2014; Tontonoz and Spiegelman, 2008). Other genes that contribute to adipocyte differentiation and development include Runx2 and CCAAT-enhancer-binding proteins -α, -β, and -δ (Siersbaek and Mandrup, 2011; Tontonoz and Spiegelman, 2008; James, 2013). Most recently, it was shown that activation of the nuclear receptor, RXR, commits MSCs to the adipose lineage by decreasing genome-wide expression of the repressive histone mark, histone 3, lysine 27 trimethylation (H3K27me3), in proximity to that regulate adipose commitment (Shoucri *et al.*, 2017). This "primes" the genes for subsequent activation by PPARγ and differentiation into adipocytes.

How Do Obesogens Act?

Although about 50 obesogens or potential obesogens have been identified, there is little mechanistic understanding of how most function. We know the most about Tributyltin (TBT). TBT is widely used in industry and triphenyltin (TPT) in agriculture. Human exposure occurs through dietary sources from contaminated seafood and shellfish, from organotin use as fungicides and miticides on food crops, in wood treatments, and industrial water systems. TBT contaminates plastics (e.g., polyvinyl chloride) and house dust (Antizar-Ladislao, 2008; Fromme *et al.*, 2005; Kannan *et al.*, 2010). TBT is a nanomolar affinity ligand that activates the so-called "master regulator" of adipogenesis, peroxisome proliferator activated receptor gamma (PPARγ) and its heterodimeric partner, 9-cis retinoic acid receptor (RXR) to promote adipogenesis and alter lipid homeostasis (Le Maire *et al.*, 2009; Grun *et al.*, 2006; Kanayama *et al.*, 2005). Human and mouse mesenchymal stem cells (MSCs) and preadipocytes are induced to differentiate into adipocytes via a PPARγ-dependent pathway after exposure to TBT at environmentally-relevant (nanomolar) levels or to PPARγ agonists such as rosiglitazone (ROSI) (Li *et al.*, 2011; Kirchner *et al.*, 2010). Importantly, TBT exposure during pregnancy can to lead to obesity in the F1 offspring and in F2 and F3 generations without additional exposure (Chamorro-García *et al.*, 2013). Three other groups have shown that TBT induces obesity in mice in both sexes when treated at any age (Bo *et al.*, 2011; Penza *et al.*, 2011; Zuo *et al.*, 2011; He *et al.*, 2014). Others showed similar effects in rats (He *et al.*, 2014), goldfish (Zhang *et al.*, 2016), and zebrafish (Riu *et al.*, 2014; Tingaud-Sequeira *et al.*, 2011). Therefore, the obesogenic effects of TBT exposure, both developmentally and in adulthood, are well-supported in the literature across model systems.

In vivo experiments identified several other chemicals as known obesogens. Exposure to the fungicides triflumizole (Li *et al.*, 2012), tolylfluanid (Regnier *et al.*, 2015) or the plasticizer diethylhexyl phthalate (Hao *et al.*, 2013) also led to obesity later in life. Triflumizole was shown to act through PPARγ (Li *et al.*, 2012), whereas tolylfluanid is thought to act via the glucocorticoid receptor (Regnier *et al.*, 2015). It is not known which receptor diethylhexyl phthalate acts through, but it may be PPARγ.

Other potential obesogens have been identified that are known to act on preadipocytes or MSCs to promote adipogenic differentiation (Janesick *et al.*, 2016). These include the agricultural chemicals quinoxyfen, spirodiclofen, fludioxonil, tebupirimfos, flusilazole, forchlorfenuron, acetamaprid and pymetrozine (Janesick *et al.*, 2016). It remains to be shown which of these chemicals are capable of inducing fat accumulation and obesity, in vivo, but one might reasonably expect that at least some will turn out to be bona fide obesogens.

As previously mentioned, only low levels of EDCs are necessary to alter development. This may be the case because protective mechanisms that exist in a developed organism—such as the ability to repair DNA, a competent immune system, detoxifying enzymes, liver metabolism, the blood–brain barrier, and a normal (slower than fetal) metabolic rate—may not yet be developed (reviewed in Heindel *et al.*, 2017; Newbold *et al.*, 2007). However, it is postulated that more instances of EDC exposure, occurring postdevelopment, also contribute to obesity in adulthood (Legler *et al.*, 2015; Heindel *et al.*, 2017).

Direct vs. Indirect Mechanism of EDCs

While some EDCs directly affect commitment and differentiation of MSCs and preadipocytes, others act indirectly to affect obesity by altering other parts of the endocrine system. For example, the brain hormone kisspeptin and its receptor KISS1R play a role in regulating glucose homeostasis, insulin secretion, food intake, and body composition (Song *et al.*, 2014). Studies in rodents have shown kisspeptin to be a potential target of EDCs. EDCs were shown to affect timing of pubertal onset, estrous cycles, and sociosexual behaviors (Anderson *et al.*, 2013; Mueller *et al.*, 2015; Panzica *et al.*, 2011), and it is possible that these chemicals can, in turn, affect the development of obesity. Moreover, while exposure to EDCs affects cells and development at a genomic level, effects are often not seen until later in life, "when the system is challenged by over-nutrition and/or lack of exercise" (reviewed in Heindel *et al.*, 2017).

Transgenerational Effects of Obesogen Exposure

More evidence that EDCs function by altering genes during development comes from the observation that obesogenic effects can be passed from generation to generation, from F0 through F3 and beyond, the latter having not received any direct exposure to the EDC (Chamorro-García *et al.*, 2013). For example, one study showed that exposure to low doses of TBT in mice led to a reprogramming of MSCs to differentiate into adipocytes, as well as a consequent increase in fat depot size (Kirchner *et al.*, 2010). These effects were seen through the F3 generation (Chamorro-García *et al.*, 2013). As a result, F1 through F3 generations of mice were all predisposed to obesity later in life (Chamorro-García and Blumberg, 2014; Chamorro-García *et al.*, 2013)

In order to elicit transgenerational effects, EDC exposure must make permanent changes in the genome, or epigenome (Janesick *et al.*, 2014b; Ozgyin *et al.*, 2015; Szyf, 2015; Walker and Ho, 2012; Skinner, 2015). EDCs are hypothesized to affect DNA methylation, histone methylation (reviewed in Chamorro-García and Blumberg, 2014; Janesick *et al.*, 2014b; Skinner, 2015; McCarrey *et al.*, 2016) and higher order chromatin structure (Chamorro-García *et al.*, 2017). One model for the transgenerational inheritance of obesity holds that altered DNA methylation is passed from one generation to the next. However, considering that there are at least two waves of demethylation and remethylation that occur during germ cell development, an important unanswered question is just how EDC-elicited changes in methylation are propagated to subsequent generations. The general thinking is that these EDC-dependent changes in methylation enable the altered methylation marks to evade germline reprogramming (McCarrey *et al.*, 2016; Skinner *et al.*, 2015) but the underlying mechanisms remain unclear. It is clear that DNA methylation is changed in subsequent generations after F0 exposure to EDCs (Haque *et al.*, 2016; Manikkam *et al.*, 2013; Nilsson *et al.*, 2012; Skinner *et al.*, 2013, 2015; Tracey *et al.*, 2013).

A recent study provides a potential answer to this dilemma. It showed that exposure of F0 dams to 50 nM TBT throughout pregnancy and lactation (but not in any subsequent generation) predisposed F4 male offspring to become obese and this was exacerbated by a moderate increase in dietary fat (Chamorro-García *et al.*, 2017). Moreover, the ancestrally TBT exposed group was not able to mobilize fat as well as controls during fasting, suggesting that these animals store more of the calories they consume as fat and resist mobilizing this fat when needed (Chamorro-García *et al.*, 2017). A detailed genomic analysis showed that TBT group showed persistent changes in DNA methylation that were associated with alterations in the expression of metabolic genes, including the satiety hormone, leptin (which was over-expressed). Overexpression of leptin together with obesity is clinically presumed to indicate a state of leptin resistance. Ancestral exposure to the EDC, TBT, can thus alter metabolic set points making the exposed individual more likely to gain weight and retain this weight on the same diet as unexposed controls, the classic "thrifty phenotype."

Surprisingly, instead of being associated with particular promoter sequences, the methylation changes observed in this study were located in blocks of DNA in which methylation was all in the same direction (over- or under-methylated compared with controls). These were denoted as isoDMBs and represent regions of large-scale changes in genomic structure and chromatin accessibility because they are also associated with regions of biased GC content already known to be associated with higher order chromatin structure (Chamorro-García *et al.*, 2017). In this model, EDC exposure leads to genome-wide changes in chromatin accessibility that alter the ability of DNA methylases and demethylases to modify cytosine bases (Chamorro-García *et al.*, 2017). Intriguingly, these changes in chromatin structure are associated with regions of highly biased GC sequence composition that also contain a much higher number of metabolic genes than would be expected by chance (Chamorro-García *et al.*, 2017). Thus, EDC

exposure alters chromatin structure and accessibility which secondarily alters DNA methylation to regulate gene expression and this disproportionately affects genes involved in metalism. The altered chromatin structure is what is inherited, rather than specific changes in DNA methylation.

In addition to our studies with TBT (Chamorro-García *et al.*, 2013, 2017). heritable effects of several environmental chemicals on obesity have been demonstrated, albeit at relatively high doses. Plastic components such as BPA, diethylhexyl and dibutyl phthalates (Manikkam *et al.*, 2013), the pesticide methoxychlor (Manikkam *et al.*, 2014), a mixed hydrocarbon mixture (jet fuel JP-8) (Tracey *et al.*, 2013) and the once widely used pesticide, DDT (Skinner *et al.*, 2013) all induce transgenerational obesity in rats. Thus, there is strong experimental support for transgenerational effects of environmental chemicals on obesity, which may be highly relevant for humans. There is evidence that altered DNA methylation may be transmitted across generations (Hanson and Skinner, 2016; Skinner *et al.*, 2015), but the underlying mechanisms remain unclear and the phenomenon controversial (Iqbal *et al.*, 2015; Whitelaw, 2015).

Effects of Exposure to Specific EDCs During Perinatal Period

Many studies in animals, investigating specific EDCs, have shown that exposure to these chemicals during the perinatal period (during gestation or early life) leads to obesity later in life. Below we summarize what is known about the effects of various classes of EDCs on obesity and point the reader to other recent reviews for a more detailed elaboration of this subject (Heindel *et al.*, 2015, 2017; Janesick *et al.*, 2014a). While the strength of evidence varies for different chemicals, together, they support the notion that exposure to obesogenic EDCs during development leads to obesity later in life.

Fungicides

A variety of fungicides have been shown to act as EDCs, leading to obesity. Discussed previously, TBT is a well-studied obesogenic EDC found in fungicides. It works as an agonist of RXR and PPARγ, which work together to promote adipogenesis. In multiple studies, TBT was shown to stimulate adipogenesis in preadipocytes in vitro (Heindel *et al.*, 2017; Grun *et al.*, 2006; Kanayama *et al.*, 2005; Pereira-Fernandes *et al.*, 2014; Watt and Schlezinger, 2015). In vivo, studies in mice have shown that prenatal exposure to TBT increases lipid accumulation and adipose tissue mass in adulthood (Grun *et al.*, 2006; Kirchner *et al.*, 2010), effects which are passed down to multiple generations (Chamorro-García and Blumberg, 2014; Chamorro-García *et al.*, 2013, 2017). TBT is one chemical with strong evidence of obesogenic properties in animal models, yet human biomonitoring is lacking. Furthermore, TBT not only promotes adipocyte accumulation by acting on nuclear receptors, but also has been shown to lead to hepatic steatosis, hyperglycemia with reduced circulating insulin and increased islet cell apoptosis, and reduced cellular proliferation—all aspects of metabolic dysfunction (Zuo *et al.*, 2014).

Triflumizole is another fungicide, used on food crops, known to promote obesity. Like TBT, it works through activating PPARγ which, in turn, promotes adipocyte differentiation. In 3T3-L1 cells, triflumizole was shown to induce adipogenesis, even at nanomolar concentrations. Downstream, it induced adipogenic target genes including FABP4 and ADIPOQ expression (Li *et al.*, 2012). In contrast, tolylfluanid is a commonly used fungicide, especially in Europe. Rather than acting through PPARγ activation, it works to induce preadipocyte differentiation by activating the glucocorticoid receptor (Sargis *et al.*, 2010). In mice, tolylfluanid exposure led to increased weight gain after 35 days of exposure, with increased perigonadal, perirenal, and mesenteric fat pad weight (Regnier *et al.*, 2015). These mice also experienced disruption to their adipokine profile, with increased leptin to adiponectin ratio. Moreover, genes important for fatty acid oxidation were downregulated by tolylfluanid in mice, including two isoforms of acetyl-coA dehydrogenase (Regnier *et al.*, 2015).

BPA

BPA is an obesogenic EDC to which we are commonly exposed through polycarbonate plastics, epoxy resins lining food and beverage cans, and thermal papers such as cash register receipts. BPA has been shown to promote fat accumulation and weight gain after developmental exposure in rats and mice (reviewed in Heindel *et al.*, 2017). Mice treated with BPA showed a substantial increase in weight gain and a significantly higher perigonadal fat pad weight as compared with nonexposed mice by 28 weeks (Garcia-Arevalo *et al.*, 2014). BPA treated mice also tended to have an impaired glucose tolerance as compared to controls, linking the effects of BPA exposure to not only obesity, but potential development of type 2 diabetes. Expression of genes involved in glucose and lipid metabolism in BPA-treated mice also supported the correlation between BPA exposure and adiposity. In white adipose tissue, BPA-treated mice had significantly higher levels of expression of *Strebp1c*, which is involved in regulation of lipid biosynthesis and lipogenic enzyme expression. *Pparα* expression, a gene associated with lipid detection and hypolipidemic effects, was decreased in WAT, as was expression of *Cpt1β*, which important for fatty acid oxidation. *Cd36*, a gene involved in fatty acid uptake, also showed decreased expression. In the liver of BPA-treated mice, *Pparγ* levels were increased (Garcia-Arevalo *et al.*, 2014).

In humans, prenatal exposure to BPA was shown to be associated with increased fat mass index (FMI) and body fat percentage in children at 7 years of age (Hoepner *et al.*, 2016). Prenatal BPA exposure has also been shown to correlate with BMI and waist

circumference in children at 4 years of age (Valvi *et al.*, 2013), as well as with an increase in postnatal growth without affecting BMI in children ages 2–5 (Braun *et al.*, 2014). Diet may be a confounding factor in these studies because BPA is found in food packaging; therefore, a higher food intake, especially of processed, packaged foods could be associated with both a higher caloric intake and a higher intake of BPA (Hoepner *et al.*, 2016).

Phthalates

Phthalates are obesogenic EDCs, found in many plastics such as tubing and vinyl flooring, as well as in household and personal care products, fragrances, and food packaging (reviewed in Heindel *et al.*, 2017). The EU has already taken steps to reduce the use of certain phthalates (such as DEHP). Phthalates work by activating PPARs as well as causing antiandrogenic affects, both of which can lead to obesity (Kim and Park, 2014). Indeed, multiple studies in animals, testing various phthalates, showed that phthalate exposure led to increases in body fat and. For example, diethyl-hexyl-phthalate (DEHP) was shown to induce expression of genes associated with adipogenesis, including PPARγ, C/EBPα, and Srebf1. In vivo, perinatal exposure to DEHP led to increased adipose tissue deposition, serum lipids, glucose, levels, and body weight in offspring (Hao *et al.*, 2013). Mono-(2-ethylhexyl)-phthalate (MEHP), a metabolite of DEHP, was shown to activate PPARG, the protein product of the PPARγ gene, and its downstream target genes including PPARγ. In this way, MEHP promoted preadipocyte differentiation and adipogenesis. In the liver, MEHP was shown to activate PPARG and induce its downstream targets, including aP2, LPL, and FAS. Together, these genes stimulate fatty acid uptake and TAG synthesis (Hao *et al.*, 2012a). Studies in humans have shown varied effects associated with adipogenesis and obesity, including one finding that phthalate exposure during pregnancy was associated with elevated triglyceride levels in cord blood with increased body mass at 3 months, in boys (Kim *et al.*, 2016). Levels of phthalate metabolites are associated with waist diameter, insulin resistance and BMI in humans (Hatch *et al.*, 2008; Stahlhut *et al.*, 2007). Overall, studies suggest an association of phthalate exposure with weight gain in humans (reviewed in Heindel *et al.*, 2017).

Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are another well-studied group of chemicals that act as obesogenic EDCs. They are byproducts of fossil fuel burning, found in diesel exhaust, cigarette smoke, and general air pollution (reviewed in Heindel *et al.*, 2015). PAHs are have lipophilic properties, so they accumulate in organs such as the liver, kidney, and fat cells. They alter expression of PPARγ, fatty acid synthase, and adiponectin mRNAs after prenatal exposure in mice. These mice experienced increased weight and fat mass (Yan *et al.*, 2014). A mouse-model study using benzo[*a*]pyrene, a high molecular weight PAH, showed that the PAH impaired adipose tissue lipolysis, leading to increased weight gain and fat mass (Irigaray *et al.*, 2006). Other studies in mice have also been conducted, with results indicating an association between PAH exposure and obesity, but varying with experimental design (Heindel *et al.*, 2017). Some studies in humans show an association between exposure to PAHs and childhood obesity. Scinicariello and Buser (2014) showed an association between childhood exposure to low-molecular weight PAHs and obesity, as measured by BMI and waist circumference (Scinicariello and Buser, 2014). Rundle *et al.* showed a link between maternal exposure to PAHs and childhood obesity (Rundle *et al.*, 2012). PAHs not only induce obesity, but also have been shown to promote insulin resistance and inflammation in adult mice on a high fat diet (Bolton *et al.*, 2014; Strakovsky *et al.*, 2015). They may function in the endocrine system by acting like estrogens (Scinicariello and Buser, 2014; Wenger *et al.*, 2009).

Diethylstilbestrol (DES)

DES is an obesogenic EDC that promotes weight gain in females, at puberty, after exposure during development (Newbold *et al.*, 2008, 2009). One study in mice showed that DES works to increase obesity by increasing the number of adipocytes in gonadal fat pads (Angle *et al.*, 2013). Studies in humans have shown that prenatal exposure to DES promoted childhood obesity at age 7 (Jensen and Longnecker, 2014), and that at low doses, it promoted obesity in adult women (Hatch *et al.*, 2015). As with other obesogens, DES has been shown to work through PPARγ activation and resultant preadipocyte differentiation; as well as through activation of glycerol-3-phosphate dehydrogenase (GDPH), a lipid biosynthesis enzyme (Hao *et al.*, 2012b).

Persistent Organic Pollutants (POPs)

POPs are a broad class of chemicals that act as obesogenic EDCs. POPs are persistent in the environment because they resist degradation. Many are lipophilic and, as such, accumulate in adipose tissue (Jackson *et al.*, 2017). POPs include pesticides such as HCB and DDT, as well as industrial chemicals such as PCBs (Heindel *et al.*, 2017). Prenatal exposure to POPs HCB, βHCH, and PCB-138 and -180, was shown, in each case, to lead to increased BMI in children (Agay-Shay *et al.*, 2015). These, along with many other POPs, have been studied in humans, consistently showing that prenatal exposure is associated with obesity later in life (Heindel *et al.*, 2017; Vafeiadi *et al.*, 2015; Tang-Peronard *et al.*, 2014; Valvi *et al.*, 2012; Mendez *et al.*, 2011). Interestingly, while many studies confirm an association between prenatal POP exposure and obesity, there is little understanding of the underlying mechanisms.

Future Investigations

Many chemicals have been shown to have obesogenic properties in vitro, or in vivo. Some have been heavily studied in animals, but human data are sparse. For example, animal studies have shown that TBT may be an EDC that contributes to the obesity epidemic; yet the data regarding exposure levels in the human population (Kannan *et al.*, 1999) and the potential effects of these exposures in human longitudinal studies are few (Rantakokko *et al.*, 2014, 2008). Longitudinal studies of EDCs in human populations are the gold standard for linking results in animal studies with potential effects on humans (Trasande *et al.*, 2015; Attina *et al.*, 2016; Legler *et al.*, 2015).

The study of chemical obesogens is still in its infancy and many important gaps in our knowledge remain. For example, while we and others have shown that TBT exposure leads to increased adiposity in mice, rats and zebrafish, our recent study on the effects of altered diet on male F4 descendants of TBT treated animals is the first to test the interactions between diet and obesogen exposure (Chamorro-García *et al.*, 2017). Thus, there is an important gap in our understanding of how current or ancestral obesogen exposure interacts with diet, which could be very significant for human health. We have shown that some effects of obesogen exposure on MSCs (Shoucri *et al.*, 2017; Kirchner *et al.*, 2010) and on mice (Chamorro-García *et al.*, 2013, 2017) are likely to be epigenomic, yet relatively little is known about the underlying mechanisms. How are effects of maternal TBT exposure transmitted to offspring and to subsequent generations? How does maternal TBT exposure lead to a transgenerational thrifty phenotype? How does TBT exposure elicit large-scale changes in chromatin structure apparently only in the male germline? Why are effects in females more prominent in the F1 generation? At least one paper shows that TBT at high doses can also modulate the estrogen receptor, in vivo (Penza *et al.*, 2011). Which molecular targets mediate the effects of TBT on metabolic programming, in vivo? TBT exposure appears to "whiten" brown adipose tissue (BAT), in vivo, and exposure to the EDC obesogen, DDT, impairs thermogenesis in rats (La Merrill *et al.*, 2014). Do TBT and other EDCs impair thermogenesis, in vivo and if so, through what mechanisms?

Conclusion

EDCs are pervasive in our diets and environment. Obesogenic EDCs act to promote obesity, and can function by altering levels of hormones and consequently the action of their receptors during development. Some EDCs act directly on adipocytes, creating developmental changes that lead to increased propensity for obesity later in life. EDCs can also act indirectly to promote fat accumulation and obesity, by acting on other endocrine pathways (such as those in the brain and liver) (reviewed in Heindel *et al.*, 2017). A very conservative estimate of the impact of three obesogens on the cost of obesity and type 2 diabetes in the EU put the cost at $18 billion Euro per year (Legler *et al.*, 2015). The estimated cost in the United States was lower (Attina *et al.*, 2016), but this was primarily due to the dearth of longitudinal studies in humans that could be used to link animal experiments with human outcomes. We know very little about the extent to which a confirmed obesogen such as TBT impacts the human obesity epidemic in the United States and worldwide?

There is an urgent, unmet need to understand the mechanisms underlying the predisposition to obesity and related disorders. Obesity adds more than $200 billion to US healthcare costs (Cawley and Meyerhoefer, 2012) and the number of obese individuals continues to increase (Ogden *et al.*, 2014; Hales *et al.*, 2017). Evidence implicating environmental influences continues to mount, but the study of environmental factors in obesity remains in the early stages, and the mechanisms of environmentally initiated obesity (i.e., other than by foods or lifestyles) remain largely unknown. Recent results show that ancestral TBT exposure exacerbates the effects of increased dietary fat on fat accumulation in the F4 generation and renders the animals resistant to fasting-induced fat mobilization (Chamorro-García *et al.*, 2017). This suggests that descendants of obesogen-exposed individuals may respond to calories differently—they will store proportionately more calories as fat than nonexposed individuals and will resist losing this fat, even when calories are restricted as in dieting or fasting. This is similar to the "dieter's lament"—gaining weight despite not eating as much as others and resisting losing this weight even with dieting. We predict that these observations may have important implications for understanding how to treat obesity in humans.

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Endocrine Disruptors and Thyroid Function

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Nomenclature

ADRA2	α2-Adrenergic receptor
AKT	serine/threonine kinase protein kinase B
ATC	Anaplastic thyroid carcinoma
Dehal	Dehalogenase
DIO	Deiodinase
DUOX	Dual oxidase
EDC	Endocrine disrupting compounds
FTC	Follicular thyroid carcinoma
IGF1	Insulin-like growth factor 1
K_m	Michaelis–Menten constant
LAT	L-type amino acid transporter
MCT8	Monocarboxylate transporter 8
MTC	Medullary thyroid carcinoma
NIS	Sodium-iodide symporter
OATP	Organic anion transporter
ODC	Ornithine decarboxylase
PCB	Polychlorinated biphenyls
Pi3K	PI3-kinase
PTC	Papillary thyroid carcinoma
PTEN	Phosphatase and tensin homolog, tumorsuppressor gene
PTU	6-n-Propyl-2-thiouracil
RET	Proto-oncogene tyrosine-protein kinase receptor
RXR	Retinoic acid receptor
TAAR	Trace amine-associated receptor
TBG	Thyroxine-binding globulin
Tg	Thyroglobulin
TPO	Thyreoperoxidase
Tetrac	Tetraiodothyro-acetic acid
TH	Thyroid hormone (T4,T3)
THM	Thyroid hormone metabolites
Triac	Triiodothyro-acetic acid
T4	3,3′,5,5′-Tetraiodo-L-Thyronine (Thyroxine)
T3	3,3′,5-Triiodo-L-Thyronine
rT3	3,3′, 5′-Triiodo-L-Thyronine (reverse T3)
3,5-T2	3,5-Diiodo-L-Thyronine
3-T1 AM	3-Iodo-thyronamine
T1Ac	3-Iodo-thyroacetic
TR	T3 Receptor
TRAK	TSH receptor stimulating antibodies
TRH	Thyrotropin releasing hormone
Triac	3,3′,5-Triiodothyro-acetic acid
TRPM8	Transient receptor potential cation channel subfamily M member 8
TSH	Thyrotropin, thyroid stimulating hormone
TTR	Transthyretin

Glossary

Iodothyronines Iodinated derivatives of the thyroid hormones (TH) 3,3′,5,5′-Tetraiodo-L-Thyronine (T4) and 3,3′,5-Triiodo-L-Thyronine (T3) that are formed by sequential deiodination of T4 and T3 or that are sulfated or glucuronidated metabolites. Apart from T3, the other iodothyronines, including T4, do not bind to the nuclear T3 receptor in vivo and therefore are not thyromimetically active. 3,5-T2 also exerts thyromimetic activity probably via mitochondrial mechanism of action and at high concentrations via T3 receptors.

Thyroid hormone The thyroid produces and secretes T4 and T3, which are iodinated derivatives of the amino acid tyrosine. T4 is considered as prohormone that acts primarily after 5′-deiodination to T3, the active TH. Most TH, that is, T3, actions are mediated by its binding to T3 receptors (TR) TRα and TRβ, which are encoded by different genes. TR are members of the nuclear receptor family, which modulate transcription of nuclear and mitochondrial T3-responsive genes as ligand-activated transcription factors. Recently, also "nonclassical" T3 actions have been demonstrated, which are rapidly initiated by TR interactions with intracellular kinase signaling cascades. Some rapid T4 effects may be exerted via binding and activation of $\alpha v\beta 3$ integrin receptors at the plasma membrane. TH regulate the development, differentiation, growth, energy, and structural metabolism of higher vertebrates, including humans. TH act in a permissive way in close cooperation with other hormonal, neuronal, and nutritive signals. Deficiency of iodine or TH during pregnancy leads to severe impairment of the development of the central nervous system and growth (cretinism) and impairment of many metabolic functions in the adult organism.

Thyroid hormone metabolites This term comprises the classical TH T_4 and T_3, their deiodinated metabolites and their recently re-discovered decarboxylated metabolites, the thyronamines 3-iodo-thyronamine and the iodine-free thyronamine, as well as the oxidized metabolites of the latter two compounds, that is, 3-iodo-thyroacetic acid and thyroacetic acid, all of which show various pharmacological actions distinct from those of the classical THs T_3 and T_4.

The Thyroid, the largest human endocrine gland, is located in front of the larynx and trachea below the thyroid cartilage. Shaped like a butterfly with two lobes connected by an isthmus it weighs between 18 g (female) and 25 g (male) and may grow to form a goiter under conditions of nutritional iodine deficiency, exposure to nutritional goitrogens or by autoantibody stimulation during Graves' disease (Basedow goiter). The main vital function of this highly vascularized gland is to accumulate the trace element iodine from blood and to generate, store and release the iodine-containing thyroid hormones (TH) L-thyroxine (3,3′,5,5′-Tetraiodo-L-thyronine, T4) and L-T3 (3,3′,5-Triiodo-L-thyronine). TH synthesis occurs in the angiofollicular units (**Fig. 1**), which comprise a tightly sealed epithelial monolayer of polarized thyrocytes and are organized in microspheres with diameters up to 1 mm, all of them completely surrounded by a microcapillary network. Its endothelial cells connect to each follicular cell, thus delivering iodide anions and nutrients to the follicles and disposing TH, released by follicular cells, to their target tissues and cells via the bloodstream. The follicular lumen, the site of TH synthesis and storage of the TH precursor protein thyroglobulin (Tg), contains the colloid, mainly composed of semisolid, polymerized, highly concentrated iodinated Tg, the major protein of this extracellular space. The thyroid gland is drained by lymphatic structures and receives sympathetic and parasympathetic nerval signals. The close location of the thyroid to the recurrent laryngeal nerve is a major challenge during thyroid surgery.

Regular development, migration and function of this gland and its adequate production of TH are essential for normal fetal and perinatal development as well as for healthy growth and adult life. Genetic disorders caused by mutations of several transcription factors, relevant for fetal thyroid development, migration of the thyroid anlage to its final destination, growth and proper function of the gland or mutations of genes coding for the individual components of TH biosynthesis, storage, and secretion may lead to major developmental delays and impairments known as cretinism. Developmental disorders affecting the thyroid may present with ectopic location (e.g., sublingual gland), hemiagenesis, too small size or even complete athyreosis. Cretinism may also result from inadequate maternal, fetal or postnatal iodine intake, still a problem affecting two thirds of the world population today! However, such adverse outcome can efficiently and completely be prevented by neonatal and life-long supplementation with adequate doses of the TH T4 to the newborn starting during the first week of life. Worldwide screening (by measurement of thyrotropin [TSH] or T4 concentration in neonatal heel prick blood) for this condition of congenital hypothyroidism, which is the most frequent but also preventable congenital endocrine disease occurring in ca. 1:3000 live births, has been successfully introduced around 1980. Affected adequately treated individuals have normal brain and physical development and body functions. Thyroid function may also be impaired by several nutritional and environmental factors leading to goiter formation similar to iodine deficiency. Among those goitrogens, several of them characterized by bitter taste, are various secondary metabolites of Brassicacea plants and cruciferous vegetables (goitrin, genistein, (iso-)flavones), especially if such food is inadequately prepared and cooked. In addition, environmental disrupting compounds (EDC, such as perchlorate, nitrate, thiocyanate and several other components of tobacco smoke), synthetic chemicals with properties of endocrine disruptors (flame retardants, plasticizers, PCBs) and a high number of pharmaceuticals used as routine drugs also directly interfere with the function of thyroid gland or the action of its hormones.

As the lifelong TH synthesis fully depends on adequate availability of the essential trace element iodine, proper organization of the angiofollicular units of the gland, as well as normal function and regulation of the complex biochemical and structural

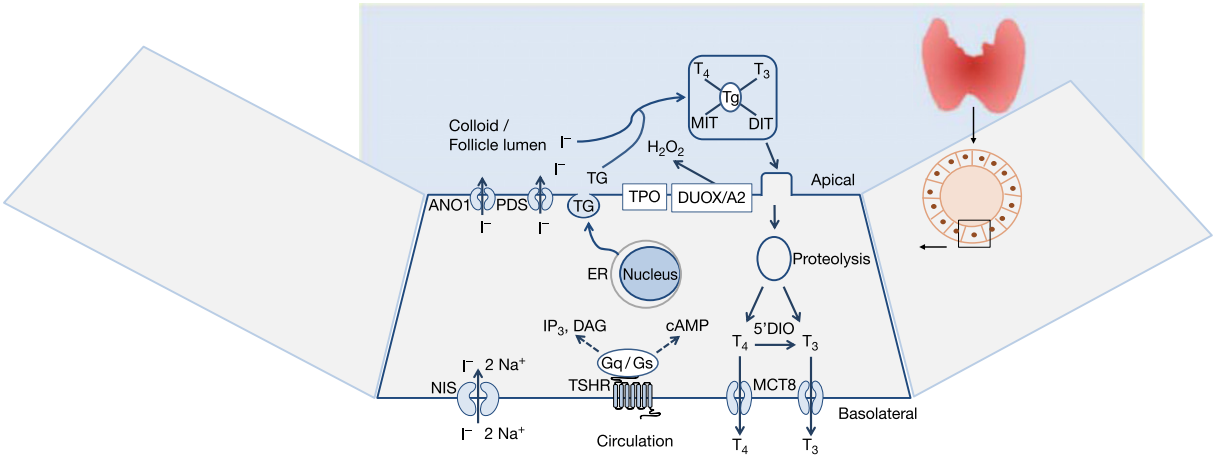

Fig. 1 The thyroid is composed of the angiofollicular units, where synthesis, storage, and secretion of thyroid hormones occurs. The complex sequence of reactions generating TH and depending on iodide uptake from blood, organification of oxidized iodine species and TH synthesis on thyroglobulin, stored in the colloid lumen, and secretion of proteolytically released TH T_4 and T_3 into the circulation is described in the text. Abbreviations: 5'-DIO, 5'-Deiodinase; cAMP, cyclic AMP; DAG, Diacylglycerol; DIT, diiodotyrosine; DUOX/A2, Dual oxidase/Activator 2; ER, endoplasmic reticulum; Gq/Gs, G-proteins; H_2O_2, hydrogen peroxide; I^-, iodide; IP_3, Inositol trisphosphate; MCT8, Monocarboxylate transporter 8; MIT, monoiodotyrosine; NIS, Sodium-iodide symporter; PDS, Pendrin; ANO1, Anoctamin1; TG, Thyroglobulin; TPO, thyreoperoxidase; T_4, 3,3',5,5'-Tetraiodo-L-Thyronine (Thyroxine); T_3, 3,3′,5-Triiodo-L-Thyronine; TSHR, thyroid stimulating hormone receptor.

machinery of the cascade of TH synthesis, storage and secretion, every defect or malfunction of this potentially "dead end street" may result in a congenital or postnatally acquired hypothyroid condition with its fatal sequelae, however treatable by T4 supplementation. The first step of TH biosynthesis (Fig. 1) is the accumulation of iodide from the blood against a concentration gradient via the sodium–iodide symporter (NIS), located in the basolateral membrane of thyrocytes and importing one iodide anion together with two Na^+ cations into the thyrocyte cytosol. Energy required for this symporter comes from ATP-driven Na^+, K^+–ATPase, which exports the excess Na^+ for K^+ import. Thus, inhibitors of Na^+,K^+–ATPase such as ouabain as well as inhibitors of NIS can interfere with adequate iodide uptake. Perchlorate, an environmental EDC released from fireworks, rockets, airbags, etc. and contaminating drinking water, rivers and lakes, is a potent competitor of NIS-dependent iodide uptake. Perchlorate is also used in medical practice for acute treatment of hyperthyroidism or blockage of the thyroid gland. Accumulated iodide immediately leaves the thyrocyte via apical ion channels (Pendrin, Anoctamin 1) into the follicular lumen. A fraction of this iodide may be "organified" and bound to unsaturated fatty acids forming iodolipids and iodolactons in thyrocyte membranes. The main protein synthetized by thyrocytes is Tg, a dimeric large glycoprotein ($2 \times 330{,}000$ Da) secreted into the thyrocyte lumen and representing the key synthesis and storage protein for TH. Tg is a cysteine-rich (ca. 120 cysteine residues) and tyrosine rich protein (60–120 tyrosine residues depending on species) with a domain structure containing four "hormonogenic" sites, three of them generating T4 at the N-terminus and one C-terminal site generating T3. Of the 66 tyrosines in the human Tg protein sequence around 15 are mono- or di-iodinated (monoiodotyrosine [MIT], diiodotyrosine [DIT]) while still being part of the polypeptide sequence of the huge protein. Iodination of the Tg tyrosine residues is an enzymatic reaction catalyzed at the luminal extracellular surface of the apical thyrocyte membranes by the multifunctional hemoprotein thyreoperoxidase (TPO). TPO is anchored in the apical membrane with its active site facing the lumen where it receives oxidized "iodine species" as cosubstrate for iodination of tyrosyl-residues of Tg. TPO also oxidizes iodide to I^0 or iodonium using H_2O_2 as oxidizing agent. This H_2O_2 is generated from H_2O by the integral apical membrane enzyme dual oxidase (DUOX), a Ca^{2+}-regulated, NADPH-dependent oxidase, which requires a maturation factor DUOXA protein as coregulatory subunit. The relay-type organization of this peculiar reaction sequence catalyzed by this multienzyme-complex has been termed "thyroxisome" based on observations, that several fine-tuned interactions are required for efficient Tg-iodination and TH biosynthesis. The third step influenced by TPO is the coupling of iodinated tyrosine residues resulting in iodothyronines, which are still part of the iodinated Tg polypeptide protein chain. Head-to-tail coupling of two DIT residues in the N-terminal hormonogenic domains generates T4, while coupling of one DIT with one MIT residue in the C-terminal hormonogenic site generates T3. One DIT or the MIT residues migrates under formation of the diphenyl-ether-ring structure of the newly formed iodothyronines producing a dehydroalanine residue at its former polypeptide sequence position. Iodinated Tg, containing MIT, DIT, T3 and T4-residues in its still intact protein sequence is deposited, oligomerized and polymerized forming compact solid globules (up to 500 μm size) in the colloid lumen at the highest protein concentration (up to 600 mg/mL) so far known for human tissues. Details of these polymerization and the depolymerisation reactions subsequently required for mobilization, degradation and cellular uptake of deposited Tg colloid content are less well studied. Probably, selenoproteins such as glutathione peroxidase 3, also secreted by thyrocytes into the apical luminal space together with several secreted cathepsins are required, which solubilize and degrade Tg in redox-regulated manner. Oligomeric Tg is then engulfed by micropinocytosis at the apical thyrocyte membrane, secondary lysosomal vesicles are formed and contained iodinated and hormone-containing Tg undergoes complete proteolysis mainly catalyzed by members of the cathepsin protease family. MIT and DIT residues liberated hereby are immediately routed towards the active site of the apical membrane associated dehalogenase enzyme, which regenerates the essential iodide to be channeled into the apical space for another round of Tg iodination and TH biosynthesis. During the lysosomal degradation of one Tg unit also up to four T4 and one T3 molecules are liberated and released into circulation via the monocarboxylate transporter 8 (MCT8), a transmembrane TH transporter located in the basolateral thyrocyte membrane. Thyrocytes not only produce a certain fraction of the active TH T3 in the C-terminal T3-hormonogenic site of Tg but also express high levels of the type 1 and type 2 5′ deiodinase (DIO1,2) selenoproteins which generate T3 from T4 inside the thyrocytes. Recent evidence suggests that the relative fraction of T3 produced during TH biosynthesis in Tg and also by DIO activities are higher under conditions of iodine deficiency and TSH stimulation. The complete process of iodide uptake, oxidation, H_20_2 generation as well as Tg secretion, iodination, storage, proteolysis, that is, TH biosynthesis, is under control of the anterior pituitary glycoproteohormone thyrotropin (TSH). TSH binds and activates the G-protein coupled TSH receptor at the basolateral thyrocyte membrane. TSH receptor signals both via Gs, activation of adenylate cyclase and protein kinase A to stimulate thyroid proliferation and TH release by the angiofollicular units and also via Gq, activation of protein kinase C and elevation of cytosolic Ca^{2+} to enhance H_20_2 production and the various steps required for Tg iodination and TH biosynthesis. Thus, TSH and its receptor are key regulators of thyroid function. Apart from TSH also the high concentrations of the pregnancy hormone human chorion gonadotropin, also a glycoproteohormone, bind and activate the TSH receptor during the first trimester of pregnancy, hereby securing maternal thyroid function and adequate fetal T4 supply during the first trimester, when fetal thyroid has not yet developed. TSH receptor stimulating autoantibodies (TRAK) represent the third type of activating ligands for the TSH receptor. TRAK, the hallmark of Graves' disease (M. Basedow), circulating in the blood of affected patients (mainly females) and entertaining continuous stimulation of the angiofollicular units and TH production, are generated by B-lymphocytes, which in contrast to the hypothalamus pituitary axis are not under negative feedback regulation of TH. Antithyroid drugs, surgical removal of the thyroid gland or its destruction by radioactive iodine isotope (radioiodine therapy) may cure this hyperthyroid disease state. Apart from the above mentioned NIS competitor perchlorate currently only two antithyroid drugs are available to treat hyperthyroidism, the thiouracil based agents 6-n-Propyl-2-thiouracil (PTU) or methimazol and its prodrug carbimazol. These agents are potent inhibitors of the hemoprotein TPO. To limited extent lithium cations are also clinically used as inhibitors of TH

release, which however may exert adverse effects as antidepressants at higher concentrations. Apart from TRAK-induced Graves' disease hyperthyroidism might originate from very rare germ line or more frequent somatic acquired activating mutations of the individual components of the TSHr signaling cascade. This might lead to the rare form of neonatal (e.g., germ line activating mutation of TSH receptor or Gs protein) or postnatal hyperthyroidism with development of autonomous ("hot") nodules in the progeny of those mutated angiofollicular units that gain proliferative and functional advantage over the rest of the thyroid gland due to the growth and functional stimulation of components of the TSH receptor signaling cascade. Autonomous nodules are preferably removed by surgery or radioiodine therapy. On the other hand several inactivating mutations of components of the TSH receptor signaling cascade are known to impair or prevent the complex process of TH biosynthesis and release, thus causing hypothyroidism, which can be efficiently treated with lifelong T4 supplementation similar to congenital hypothyroidism. A more prevalent cause of hypothyroidism is an autoimmune thyroid disease (AITD) slowly destroying thyroid structure and function. This form of hypothyroidism may present with lymphocytic infiltration of the gland (Hashimoto thyroiditis) and typically antibodies directed against TPO and Tg and measurable in the blood are formed as epiphenomenon of the slow ongoing thyroid destruction process. Again life-long treatment with T4 is required and this disease also mainly affecting women in their reproductive age is the main reason why T4 ranks among the top 5 of medical prescriptions world-wide. The underlying primary causes of both autoimmune thyroid diseases are still unknown.

Apart from these benign thyroid related diseases the thyroid is also affected by malignant alterations and thyroid cancer is the most frequent cancer of the endocrine system, again with higher prevalence in females compared to males. Four main variants of thyroid cancer originating from thyrocyte cells are known, which can be distinguished according to their molecular, morphological and clinical features: follicular carcinoma, follicular variant of papillary carcinoma, papillary carcinoma and anaplastic undifferentiated thyroid cancer. The latter variant has a very poor prognosis, while papillary thyroid cancer meanwhile is not a deadly disease anymore. Molecular basis of thyroid cancer has been partially identified during the last two decades and several mutations of oncogenes (e.g., BRAF V600E and RAS point mutations or RET-translocations) and tumor suppressor genes (P53, PTEN) involved in the carcinogenic process are known and targets for personalized treatment with selective inhibitors or anticancer drugs. The known tumor causing mutations lead to aberrant activation of mitogen-activated protein kinase and phosphoinositide 3-kinase-PTEN-AKT signaling, some aspects of which can be targeted with novel antitumor drugs. Thyroid cancer and metastases which still accumulate iodide can be effectively treated with radioiodide therapy and some current treatment attempts are directed towards pharmacological restoration of NIS function ("redifferentiation therapy"), which again allows application of efficient and long-established radioiodide therapy.

Apart from the essential trace element iodine, which plays the key role in thyroid-related biology and function, two other essential trace elements are also required for efficient synthesis, metabolism and action of TH, that is, iron and selenium. Iron is the central atom of the prosthetic heme group of TPO, which catalyzes the three steps of iodine oxidation, iodination of Tg tyrosyl-residues and the coupling of iodinated tyrosyl-residues to form iodothyronines in the Tg polypeptide chain. Iodine utilization and TH biosynthesis is more efficient with adequate iron supply. Nutritional deficiency of iron, more frequent in women during their reproductive years due to iron loss during menstruation as well as in underdeveloped regions with low intake of animal protein sources, is associated with inadequate TH production and resulting goitrogenesis. The third essential trace element required of adequate function of the TH axis and cellular action TH is selenium. Selenium deficiency alone or in combination with iodine deficiency and/or concomitant exposure to goitrogenic agents is involved pathogenesis of two forms of endemic cretinism, myxedematous or neurological cretinism, which can be prevented by adequate iodine intake, followed by selenium supplementation and avoidance of goitrogen exposure. The essential and beneficial role of adequate nutritional selenium intake has been attributed to its key function in several indispensable selenocysteine-containing proteins, which are involved in cellular redox control (e.g., thioredoxin reductases), antioxidant function (glutathione peroxidases), quality control of ribosomal protein biosynthesis, cellular signaling and enzymatic deiodination of TH (all three deiodinase isoenzymes). Remarkably, most of these selenoproteins are highly expressed in the thyroid and this gland also retains its selenium content even under nutritional selenium depletion. It has been hypothesized but not yet formally proven, that the lifelong production of H_2O_2 in the angiofollicular units, starting after week 12 in utero, requires an efficient antioxidative and redox-control system to maintain the vital function of this organ for TH production. Thus it does not come as a surprise, that selenium supplementation is also applied as adjuvant but not curative medication in the two autoimmune diseases described above.

Another major influence on thyroid follicular growth and function is exerted by the insulin/growth hormone/IGF-1 signaling cascades. Enhanced thyroid proliferation and goiter growth are well known from acromegaly patients and insulin and IGF-1 receptor signaling is markedly enhanced under conditions of low iodine supply. Probably, iodinated lipids and iodolactones, mentioned above, exert an inhibitory effect on this thyroid growth pathway, thus representing a local autoregulatory loop depending on adequate iodine intake and organification.

Finally, a further hormone producing cell type located in the thyroid gland needs to be considered, that is, the calcitonin-producing C-cells or parafollicular cells, which are found scattered between but not as integral part of the angiofollicular units. C-cells also drain their hormone into the microcapillary network of the gland and they originate from progenitor cells different from those of thyrocytes. C-cells are of clinical importance as they may evolve to medullary thyroid carcinoma (MTC), a rather aggressive, highly metastasising cancer, which already may develop during early childhood. Activating point mutations of the RET oncogene with clinically important genotype-phenotype relationships have been identified as driver mutations of this tumor. Several drugs inhibiting this RET-dependent mitogen-activated protein kinase and phosphoinositide 3-kinase-PTEN-AKT signaling or early complete thyroidectomy may cure this

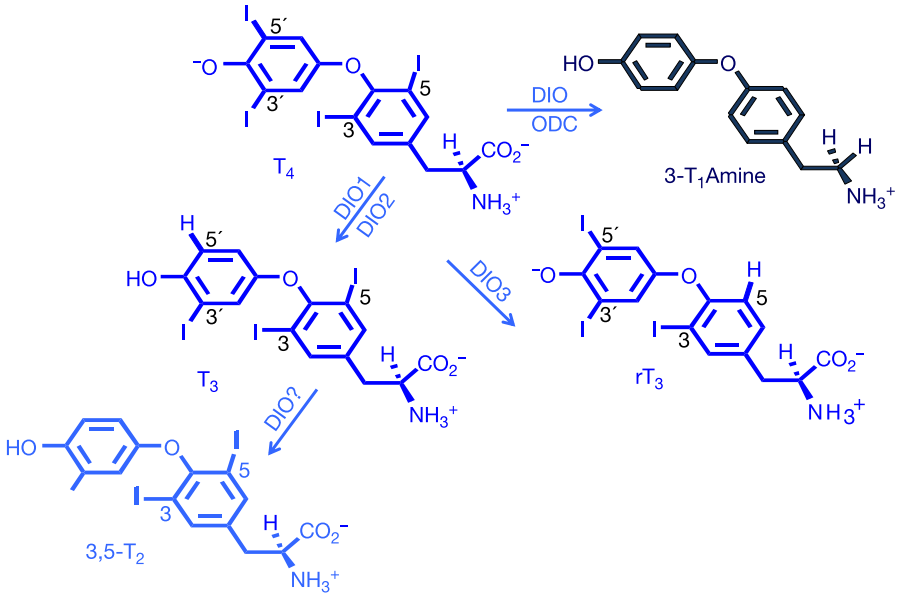

Fig. 2 Structures of the main biologically active thyroid hormones secreted by the thyroid gland (T4, T3) or generated extrathyroidally by deiodinase selenoenzymes (DIO) and ornithine decarboxylase (ODC). Tyrosine-derived iodinated thyroid hormones are charged molecules, which pass cellular membranes via various TH transmembrane transporters and act via several cellular receptors.

cancer if detected before its metastasis. Interestingly, the "hormone" calcitonin is an evolutionary dispensable relict in humans (but not in aquatic life forms). Calcitonin must not be replaced after complete thyroidectomy that does not disrupt calcium-phosphate homeostasis. However, calcitonin administration is a valuable treatment option in osteoporosis.

Thyroid Hormones

The term thyroid hormones (TH) typically comprises the two main secretory products of the angiofollicular units of the thyroid gland, that is, T_4 and T_3 (Fig. 2). Recently some more TH metabolites (THM) need to be included into this term. Two further THM are enzymatically generated outside the thyroid gland in peripheral target tissues/cells. (i) 3,5-diiodo-L-thyronine (3,5-T_2) also exerts rapid thyromimetic effects similar to the classical TH T_3, while (ii) 3-iodo-thyronamine (3-T_1AM) exhibits a spectrum of pharmacological actions quite distinct and mostly antagonistic to that of the thyromimetic T_3. A third class of THM with hormonal activity comprises the acetic acid side chain metabolites of iodothyronines and thyronamines, that is, 3,3′,5,5′-tetra-iodo-thyroacetic acid (Tetrac), 3,3′,5-tri-iodo-thyroacetic acid (Triac), and 3-iodo-thyroacetic (T_1Ac). T4 is the main secretory product (ca. 90–110 µg/day) of the normal thyroid gland under adequate iodine intake. Approximately 10–20 µg of T_3 are directly secreted by the thyroid per day, while extrathyroidal type 1 and type 2 deiodinase activities contribute another 30–40 µg of T_3 per day via enzymatic deiodination of the "prohormone" T_4 in target tissues including the brain. Molecular details of enzymatic formation of the minor THM 3,5-T_2 are not yet unraveled and 3,5-T_2 may represent a THM mainly generated and acting intracellularly on mitochondria and at higher concentrations also via the classical intracellular T_3 receptors. Remarkable are the efficient antisteatotic actions of 3,5-T_2 in obese rodent experimental animal models.

3-iodo-thyronamine (3-T_1AM) is a recently re-discovered THM circulating in nanomolar concentration in human blood. 3-T_1AM is generated from TH via deiodination in combination with decarboxylation by the enzyme ornithine-decarboxylase. One site of production during absorption of orally ingested TH might be the gastrointestinal mucosa. 3-T_1AM exhibits a remarkably broad spectrum of actions after its pharmacological administration (i.p., i.v., i.c.v.) to rodents and other experimental animals. Among those are effects, in part antagonizing those of thyromimetic T3, on the heart, body temperature, learning, memory, pain and other sensory functions. Which of these effects can also be exerted by endogenous 3-T_1AM, remains to be established by further studies. 3-T_1AM effects are not mediated by classical T3 but by cell membrane receptors such as members of the families of trace amine associated receptors (TAAR), G-protein coupled alpha-adrenergic receptors (ADRA2A), and transient receptor potential cation channel subfamily M member 8 (TRPM8).

Similarly, enzymes generating the acetic acid THM metabolites have not been characterized, albeit Tetrac and Triac are well known compounds circulating in blood and acting either via $\alpha v\beta 3$ integrin receptors at the cell membranes (Tetrac) or via the intracellular T3 receptor (Triac). Compared to the classical TH T_4, T_3 and 3,5-T_2 the acetic acid THM metabolites have very short half-lives as they exhibit low affinity to TH distribution proteins in the blood. Thus, Triac is used as thyromimetic drug to suppress elevated TSH in the conditions of TH resistance caused by inactivating mutations of TRβ and for amelioration of some hyper-metabolic aspects of tissue-specific hyperthyroidism in the X-chromosome linked Allan–Herndon–Dudley syndrome caused by inactivating mutations of the TH cell membrane transport protein MCT8.

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Neuroendocrine Disruption of Reproduction

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Neuroendocrine Disruption of Reproduction

Introduction: Peripheral Versus Central Effects of Endocrine Disrupting Chemicals on Reproduction

Reproductive functions involve the coordinated action of different central and peripheral structures including the hypothalamus, pituitary gland, gonads, uterus, vagina, breast, prostate, etc. Several studies have shown that exposure to endocrine disrupting chemicals (EDCs) is associated with reproductive disorders such as alteration of the timing of puberty, infertility, abnormal cyclicity, premature ovarian failure, testicular cancer or adverse pregnancy outcome (Gore *et al.*, 2015). Some of those disorders have been shown to involve direct effects of EDCs on the gonads and other peripheral tissues. For instance, studies have reported that early exposure to bisphenol A altered fetal ovarian steroidogenic gene and microRNA expression in sheep (Veiga-Lopez *et al.*, 2013; Rivera *et al.*, 2011) and inhibited germ cell nest breakdown (Wang *et al.*, 2014). Prenatal exposure to diesthylstilbestrol (DES) leads to an increased expression of the homeobox A10 gene in the uterus through epigenetic mechanisms (Bromer *et al.*, 2009). The testis is also directly targeted by EDCs during early human life, as indicated by intratesticular anomalies described in the testicular disgenesis syndrome in human (Skakkebaek *et al.*, 2001) which are very similar to testicular anomalies described in rodents after early exposure to phthalates (Sharpe *et al.*, 1995). Central endocrine disruption initially received relatively less attention but increasing evidence during the past decade has shown that the neuroendocrine system is also targeted by EDCs (Gore, 2001; Bourguignon *et al.*, 2010; Frye *et al.*, 2012). This article will summarize the data illustrating the effects of EDCs on sexual differentiation of the brain as well as the hypothalamic control of puberty and reproduction.

Neuroendocrine Disruption of Sexual Differentiation of the Brain

Sex steroids such as estradiol or testosterone organize the sexual differentiation of the brain during critical developmental windows (Schwarz and McCarthy, 2008; Gore, 2008). This early organization at the hypothalamic level allows the coordination of sex appropriate reproductive physiology and sexual behavior. Sexual differentiation of the brain is mostly dependent on the action of fetal and maternal hormones on steroid hormone receptors, but other factors participate to the establishment of the sex differences. The aromatization of testosterone into estradiol by the enzyme p450 aromatase (present in specific brain regions during critical periods of perinatal development) is one of the mechanisms whereby testosterone acts to organize and sexually differentiate brain structures and chemistry in males (Roselli *et al.*, 2009). Alpha fetoprotein is another factor involved in the sexual differentiation of the brain. This circulating protein is produced by the fetal liver and binds estrogens with high affinity. In addition to its known neuroprotective role, it specifically delivers estrogens to target brain cells to ensure female differentiation as described in rodents (Bakker and Baum, 2008). Although most of the sex differences in the brain are established during the prenatal and neonatal periods, it appears that pubertal hormones also contribute to the addition of new cells in the sexually dimorphic regions (Ahmed *et al.*, 2008).

Two hypothalamic regions play a crucial role in the regulation of sexually dimorphic reproductive physiology: the anteroventral periventricular hypothalamic nucleus (AVPV) and the sexually dimorphic nucleus of the preoptic area (SDN-POA). The AVPV volume is sexually dimorphic: the region has been described as larger in female than in male rats. Testicular androgens, after aromatization into estrogens, appear to be responsible for this defeminisation/masculinization of the AVPV (Wright *et al.*, 2010). AVPV neurons project onto and directly stimulate GnRH neurons in the preoptic area. In rodents, AVPV plays a well-established role in the control of the preovulatory LH surge (Wiegand *et al.*, 1982; Ronnekleiv and Kelly, 1986). The SDN-POA is approximately fivefold larger in male than in female rats (Gorski *et al.*, 1978, 1980). The volume of the SDN-POA is increased by estradiol aromatized from testicular androgens perinatally. It is thought to play a role in male reproductive behavior and mate choice (Rhees *et al.*, 1999).

Because those nuclei express nuclear hormone receptors (Estrogen receptor alpha, Estrogen receptor beta, androgen receptor, and progesterone receptor) (Chakraborty *et al.*, 2003; Orikasa *et al.*, 2002; Mann and Babb, 2005; Simerly *et al.*, 1990; McAbee and DonCarlos, 1999; Quadros *et al.*, 2002, 2007), they are potential direct targets of endocrine disrupting chemicals. Initial studies illustrated the effects of early exposure to phytoestrogens on sexually dimorphic nuclei in the hypothalamus. Phytoestrogens are naturally occurring plant compounds structurally or functionally similar to oestrogens. Isoflavones are the most hormonally active and most characterized phyto-oestrogens. They are found in soy beans but also in berries, wine, grain, nuts, and soya-fortified foods (Kurzer and Xu, 1997). Most isoflavones bind nuclear estrogen receptors (ER) and act as partial agonists. They appear to have a higher affinity for estrogen receptor beta (ERβ) (Kuiper *et al.*, 1998) which could explain varying activity depending on the tissue since ERβ expression varies across cell types and is sexually dimorphic (Cao and Patisaul, 2011, 2013). Some isoflavones such as genistein have been shown to bind to GPR30 (Thomas and Dong, 2006). Recent data suggests that isoflavones can also

alter DNA and histone methyltransferases as well as other modifiers of chromatin structure (Shanle and Xu, 2011; Li and Tollefsbol, 2010). While not always in accordance, several studies have reported an effect of isoflavones which is consistent with an estrogenic effect with an increased volume of the POA in males. For instance, genistein administered prenatally until adulthood leads to an increased volume of the POA in exposed males but not females (Scallet *et al.*, 2004). When the exposure is shorter, this increase is not observed or not maintained (Lewis *et al.*, 2003). Neonatal exposure to genistein increased tyrosine hydroxylase immunoreactivity in the male anteroventral periventricular nucleus (Patisaul *et al.*, 2006, 2007), indicating that genistein leads to a demasculinization of the AVPV through interference with endogenous estrogens. Genistein has also been shown to decrease kisspeptin fiber density in the female anteroventral periventricular nucleus and arcuate nucleus (Losa *et al.*, 2011).

Synthetic endocrine disrupting chemicals have been shown to affect sexual differentiation of the brain as well. Rubin *et al.* (2006) have shown that perinatal exposure to low doses of bisphenol A (BPA) reduced the sex differences in the population of tyrosine hydroxylase (TH) neurons in the AVPV, primarily through a decline in the number of labeled neurons in females. Another study from McCaffrey *et al.* showed a defeminization of the female AVPV (evaluated through a decrease of TH positive cell numbers) after perinatal exposure to a wide range of BPA doses (McCaffrey *et al.*, 2013). Evidence of a demasculinization of the male SDN-POA (reduced volume as labeled by calbindin) was also observed after such exposure (McCaffrey *et al.*, 2013). The volume of the sexually dimorphic nucleus of the preoptic area is larger in males exposed perinatally to low doses of BPA but not in female, suggesting a sex specific effect of BPA exposure on SDN-POA volume (He *et al.*, 2012). However, a study from Kwon *et al.* has yielded discordant results since perinatal administration of BPA did not produce detectable effects on the SDN-POA volume (Kwon *et al.*, 2000). Differences among results may be due to differences in the age of analyses, the timing of exposure, the route of exposure or the chosen doses of EDCs.

Neuroendocrine Disruption of Pubertal Timing

Secular Trend in Age at Puberty and Environment

Puberty is a life period characterized by the maturation of the hypothalamic- pituitary-gonadal axis. It involves both physiological and behavioral changes that ultimately lead to the achievement of reproductive capacity. Puberty results from the activation of a complex neuroendocrine machinery which leads to an increase in frequency and amplitude of gonadotropin-releasing hormone (GnRH) secretion in the hypothalamus (Herbison, 2016).

A reduction in menarcheal age has been reported between 1850 and 1960 in Scandinavian countries (Tanner, 1962) and further in many European countries and United States (reviewed in Parent *et al.*, 2003, 2015). These observations were thought to be the result of the improvement in health and nutritional status with the changes in socio-economical conditions. After a constant advance between 1850 and 1960, it appears that, in several countries with relatively stable and uniform condition of life, menarcheal age has shown only minor progression during the past decades while breast development seems to still be advancing (Parent *et al.*, 2015). Some studies have identified a secular trend towards early onset of puberty, irrespective of the level of BMI (Aksglaede *et al.*, 2009), suggesting the involvement of other environmental influences, among which endocrine disruptors chemicals. Detailed analysis reveals that the pattern of age distribution is affected both in boys and girls. This justifies a revision of the belief according to which current changes correspond to an advancement of pubertal timing in females. Current variations in pubertal timing actually involve a trend toward earliness for initial pubertal stages and toward lateness for final pubertal stages. This data suggests a role for environmental influences including nutrition, stress, and endocrine disruptors. This hypothesis is supported by some observations of association between early exposure to endocrine disrupting chemicals or other environmental factors and the age at onset of puberty as well as rodent data which will be described below.

Challenges in Demonstrating the Endocrine Disruption of Puberty Timing

Before discussing the available data linking exposure to EDCs and neuroendocrine disruption of pubertal timing, it is important to be aware of some challenges that we face in this area.

A growing body of evidence demonstrates that fetal and early postnatal life is a critical period of sensitivity to the effects of EDCs (Gore *et al.*, 2015; Fudvoye *et al.*, 2014). The capacity of environmental factors to shape the neuroendocrine system is thought to be maximal during fetal and early postnatal life and possibly less important when approaching the time of onset of puberty (Parent *et al.*, 2015). However, most studies on the role of environmental factors in triggering onset of puberty focused on the period immediately preceding puberty (Parent *et al.*, 2015). The current recommendations of the Organization of Economic Co-operation and Development (OECD) for testing effects of EDCs on rodent female puberty, define postnatal days 22–42 as the validated exposure period with timing of vaginal opening and increase in uterine weight as evidence of puberty. Those guidelines ignore the earlier period before 25 days that is critical for neuroendocrine maturation. The effects of environmental stressors on pubertal timing depend on the period of occurrence or exposure. For instance, prepubertal underfeeding may lead to delayed puberty (Biro *et al.*, 2005; Dunger *et al.*, 2006; Ong et al., 2004, 2006; Cheng *et al.*, 2012; Himes, 2006; Roa and Tena-Sempere, 2010)whereas intrauterine growth restriction is associated with early puberty (Ibáñez *et al.*, 2006, 2011). Likewise, psychosocial stress shortly before or during puberty may cause delayed menarche or amenorrhea (van Noord and Kaaks, 1991; Tahirović, 1998) whereas advancement of puberty has been described in girls who had experienced such stress in early postnatal life or infancy

(Moffitt *et al.*, 1992; Wierson *et al.*, 1993). Those data are consistent with the concept that environmental clues affect pubertal timing differently depending on the life period when they come into action. In the early phase of organization of adaptive mechanisms, adverse conditions can be interpreted centrally as a risk for species survival and are translated into need of early reproductive fitness. Reversely, in a period closer to puberty, similar adverse conditions can be interpreted as a risk for quality and outcome of pregnancy and are translated into a need for delayed reproductive fitness.

Another challenge comes from observations that are inconsistent with the classical toxicology perspective according to which there will be no effect of a chemical below a threshold of exposure. This principle implies that the dose–response relationship is linear. However, hormones have complex concentration-response patterns which lay the foundation for dose–response characteristics exhibited by EDCs (Gore *et al.*, 2015). We have shown that neonatal exposure to BPA for 2 weeks leads to a delayed maturation of GnRH secretion after a low environmentally relevant dose and advanced maturation after exposure to a higher dose (Franssen *et al.*, 2016). Opposing effects on male rat puberty have also been observed after exposure to lower or higher doses of phthalates (Ge *et al.*, 2007; Saillenfait *et al.*, 2008). In addition, low-dose mixtures, consistent with human exposure, can have effects not conforming to simple additive models (Kortenkamp, 2008; Christiansen *et al.*, 2012). However, most animal and human studies so far have focused on one or a single group of EDCs.

EDCs exposure, by modifying the hormonal environment, can affect pubertal timing either through central mechanisms, acting at different levels of the hypothalamic-pituitary system or through interaction with the peripheral target tissues such as the breast, uterus, testis or ovaries. Peripheral mechanisms can coexist with central mechanisms or secondarily facilitate them. Such a concept is supported by the dissociation between advancement in age at onset of breast development in Denmark, the Netherlands, and Belgium without parallel change in menarcheal age (Parent *et al.*, 2015). A single pubertal event can be influenced by different endocrine pathways. For instance breast development can be due to ovarian estrogen secretion under the stimulation by pituitary gonadotropins and/or estrogenic effects of EDCs independently of hypothalamic-pituitary maturation. Moreover, EDCs can interfere with the physiological inhibitory feedback mechanisms of sex steroids at the hypothalamic-pituitary level while they can also stimulate neuroendocrine maturation of GnRH secretion (Rasier *et al.*, 2006).

Disruption of the Hypothalamic Control of Puberty

Several animal studies have reported alterations of pubertal timing after exposure to EDCs. Some models allowed the identification of neuroendocrine mechanisms leading to such effects.

In females, the effects of EDCs on the timing of vaginal opening depend on the period of exposure (Parent *et al.*, 2015). When the animals are exposed to BPA during pregnancy only, age at vaginal opening is either not affected (Tinwell *et al.*, 2002; Murray *et al.*, 2007; Howdeshell *et al.*, 1999) or early (Honma *et al.*, 2002; Nikaido *et al.*, 2004). When exposure takes place between birth and 15 days of age, age at vaginal opening is either not affected (Adewale *et al.*, 2009; Losa-Ward *et al.*, 2012; Nagao *et al.*, 1999; Yu *et al.*, 2010) or early (Adewale *et al.*, 2009; Losa-Ward *et al.*, 2012; Fernández *et al.*, 2009; Nah *et al.*, 2011). A dose of 50 μg/kg causes advancement of puberty while 50 mg/kg has no effect, indicating a nonlinear dose–response relationship (Adewale *et al.*, 2009; Losa-Ward *et al.*, 2012). We also showed that a very low doses of 25 ng/kg/day from postnatal day 1–15 delayed maturation of GnRH secretion while a high dose of 5 mg/kg/day led to an accelerated maturation (Franssen *et al.*, 2016). Taken as a whole, those studies show that BPA effects depend markedly on the window of exposure and the dose, with possible nonlinear dose–response relationship. Several studies have provided evidence of neuroendocrine changes after early exposure to BPA in the female rat. These will be discussed further in the mechanistic article below. Most studies investigating the consequences of neonatal exposure to genistein led to equivocal conclusions since age at vaginal opening was either normal with no dose-related effect (Nagao *et al.*, 2001; Jefferson *et al.*, 2005; Kouki *et al.*, 2003) or early and dose- or duration-dependent (Lewis *et al.*, 2003; Kouki *et al.*, 2003) or even late and dose-dependent (Jefferson *et al.*, 2009). Using phthalates, the period of exposure appears crucial since prenatal exposure accounts for late vaginal opening (Salazar *et al.*, 2004) whereas postnatal exposure is associated with normal timing (Gray *et al.*, 2006; Moral *et al.*, 2007) or early vaginal opening irrespective of the dose (Hu *et al.*, 2013; Ma *et al.*, 2006). Polybrominated EDCs appear to cause either no change or delay in vaginal opening depending on the dose and irrespective of the period of exposure (Stoker *et al.*, 2004; Lilienthal *et al.*, 2006; Ceccatelli *et al.*, 2006).

In male rodents, balano-preputial separation is commonly used as a marker of puberty. With the exception of early balano-preputial separation after exposure to phthalates (Ge *et al.*, 2007; Saillenfait *et al.*, 2008), EDCs appear to cause either no effect or a delay of sexual maturation in the male rodent. BPA does not show any effect whatever the dose and the window of exposure (Tinwell *et al.*, 2002; Nagao *et al.*, 1999; Tan *et al.*, 2003; Ashby and Lefevre, 2000; Kato *et al.*, 2006). Similarly, using a very low dose of BPA neonatally for 5 days, we did not see any effect of exposure on balano-preputial seperation. However, when pulsatile GnRH secretion from hypothalamic explants is studied at 25 days in such exposed animals, GnRH pulse frequency is reduced, indicating a delayed maturation of GnRH secretion caused by BPA neonatally. This indicates that GnRH secretion could be extremely sensitive to disruption without being sufficient or lasting sufficiently to account for phenotypic changes. When similar doses of different EDCs are used at different periods of exposure in the male rodent, they are found to be effective in delaying puberty after postnatal (after weaning) exposure as opposed to no effect after prenatal exposure. Such findings are obtained using dichlorodiphenylichloroethylene (DDE), vinclozolin (Warner *et al.*, 2004) or diethylstilbestrol (DES) (Warner *et al.*, 2004; Mol *et al.*, 2002) suggesting that the fetus is less sensitive than the juvenile animal. The newborn rat can also be more sensitive than the fetus as found after exposure to DES (Warner *et al.*, 2004). Such a conclusion was also drawn in a review of several studies on BPA

effects in the female rat since gestational exposure has no effect on age at vaginal opening whereas neonatal exposure is followed by early puberty (Guo *et al.*, 2004). Importantly, both periods of exposure fall into the so-called "programming window" indicating that, even within this particular period, there may be differences in sensitivity to endocrine disruption. The dose of EDC however plays a critical role since, when investigated at a given period of life, higher doses appear to be more effective such as shown after prenatal exposure for DDE (Leijs *et al.*, 2008) and phthalates (Denham *et al.*, 2005; Den Hond *et al.*, 2002), or after lactational exposure for DDE, vinclozolin or DES (Warner *et al.*, 2004) or following exposure after weaning for DDE (Grandjean *et al.*, 2012; Warner *et al.*, 2004), vinclozolin (Warner *et al.*, 2004; Adgent *et al.*, 2012), DES (Warner *et al.*, 2004; Kim *et al.*, 2011), phthalates (Deng *et al.*, 2012; Strom *et al.*, 2001), and polybrominated diphenyl ethers (PBDE) (Giampietro *et al.*, 2004). It is noteworthy that in two studies using phthalates, opposing effects are observed since lower doses are associated with early puberty and higher doses with delay (Deng *et al.*, 2012; Denham *et al.*, 2005). Interestingly enough, we have reported similar opposing effects using DES neonatally in the female rat (Fernandez-Rhodes *et al.*, 2013).

Neuroendocrine Disruption of Ovulation

Ovulation is under the control of GnRH secretion which is tightly regulated by the positive and negative feedbacks of gonadal steroids. In both males and females, GnRH secretion is suppressed by the negative feedback taking place at the level of the arcuate nucleus. The positive feedback of estrogens is mediated by the AVPV and potentiates the surge in GnRH and subsequently luteinizing hormone (LH) that precedes ovulation. In rodents, the sexual differentiation of this positive feedback takes place during the perinatal period and is particularly sensitive to disruption by hormones and EDCs. It has been shown that the administration of testosterone or estradiol benzoate during the neonatal period can alter the positive feedback by estradiol (Padmanabhan and Veiga-Lopez, 2014). Such data suggests that early exposure to EDCs could alter the programming of the preovulatory LH surge. In addition, exposure to EDCs during adulthood could alter the central control of ovulation. This article will summarize the effects of neonatal or adult exposure to EDCs on the hypothalamic control of ovulation. As discussed above for puberty, it is sometimes difficult to differentiate the effects of EDCs on the central versus the ovarian control of ovulation. Some animal models can help to shed light upon such aspects.

The sheep offers a good model for studying the effects of EDCs on the neuroendocrine control of ovulation thanks to the availability of noninvasive approaches to monitor follicular dynamics as well as surgical approaches to obtain hypophyseal portal blood (Padmanabhan and Veiga-Lopez, 2014). Prenatal exposure of female sheep to excess androgens has been shown to lead to adult reproductive disorders with LH excess, multifollicular ovarian morphology and corpus luteus dysfunction. Such early exposure to testosterone disrupted the estradiol negative feedback (Wood and Foster, 1998; Sharma *et al.*, 2002), estradiol positive feedback (Wood and Foster, 1998; Sharma *et al.*, 2002), and progesterone negative feedback (Robinson Forsdike and Taylor, 1999). Neonatal exposure to BPA leads to reproductive disorders similar to those found in testosterone exposed sheep with LH excess and severly dampened preovulatory LH surge (Savabieasfahani *et al.*, 2006).

Neonatal exposure to BPA in rodents has been shown to disrupt pubertal timing but also leads to premature anestrus and ovarian anomalies. As described for the sheep, these anomalies could result from disrupted organization at the level of the hypothalamus/pituitary or at the level of the ovaries. Adewale *et al.* have shown that alterations of estrus cycle caused by a relatively low dose of BPA was not accompanied by a reduction of the number of GnRH neurons expressing the immediate early gene c-fos suggesting that steroid-positive feedback was not defeminized by BPA. However, this study did not rule out the possibility that other regulation of the GnRH neurons could have been altered (Adewale *et al.*, 2009). Other studies suggest that neonatal exposure to BPA decreased basal and GnRH induced LH secretion and increased GnRH pulsatility in adult rats, suggesting a permanent effect on GnRH pulsatility and pituitary signaling (Fernández *et al.*, 2009). There is relatively less information on the neuroendocrine disruption of ovulation caused by EDCs other than BPA. Exposure to TCDD (2, 3, 7, 8-tetrachlorodibenzo-para-dioxin) has been associated with abnormal ovulation and premature ovarian failure (Gregoraszczuk and Ptak, 2013). While some studies showed that TCDD disrupted estradiol secretion in human ganulosa cells (Baldridge *et al.*, 2015), others have reported alteration of the hypothalamic-pituitary axis. Takeda *et al.* (2014) have shown that exposure of pregnant rats to TCDD led to decreased expression of gonadotropins in pups and LH and FSH levels appeared to be decreased in virgin female mice (Huang *et al.*, 2011). Tributyltin chloride is used as a biocide in antifouling paints which exposure has been associated with obesity. It has recently been shown to alter estrus cyclicity and cause decreased surge LH levels and GnRH expression (Sena *et al.*, 2017). Very few data are available regarding the effects of phthalates on estrus cyclicity and ovulation. One study showed that exposure to DEHP during adult life led to increased duration of estrus in mice (Hannon *et al.*, 2014).

Mechanisms of EDCs Effects on the Overall Neuroendocrine Control of the Reproductive System

The hypothalamic-pituitary-gonadal axis is responsible for the development of puberty and acquisition of the reproductive function. A subset of neurons in the anterior hypothalamus secrete gonadotropin-releasing hormone (GnRH) which, through its pattern of release, controls all aspects of reproductive function throughout life. The secretory activity of GnRH neurons depends on trans-synaptic and glial inputs mediated by neurotransmitters and cell–cell signaling molecules. New methods have provided more information regarding the dynamic coordination of network contributing to the central control of the pubertal process and

reproduction (Ojeda and Lomniczi, 2014). When studying the central mechanisms potentially involved in the neuroendocrine disruption of puberty or ovulation, GnRH neurons appear to be a potential target of exposure to endocrine disruptors. Using a murine model of neonatal exposure to dichlorodiphenyltrichloroethane (DDT), our team has confirmed the involvement of neuroendocrine disruption in the alteration of pubertal timing. Pulsatile GnRH secretion was accelerated after early exposure to DDT and this effect was followed by early sexual maturation (Rasier *et al.*, 2007). Further studies have indicated that such effects involve the estrogen receptor, the orphan dioxin (AhR, arylhydrocarbon) receptor and a subtype of glutamate receptor (Rasier *et al.*, 2008). Another study of our group showed that exposure to DES (10 μg/kg/day) during the first 5 days of life was associated with early vaginal opening while a dose of 1 μg/kg day was associated with delayed vaginal opening and delayed developmental increase in GnRH pulse frequency (Franssen *et al.*, 2014). DES is a potent synthetic estrogen which was prescribed to prevent miscarriages during the 1950s and 1960s. Its prescription was banned because of the increased risk of vaginal cancer after in utero exposure. DES is still used as a paradigmatic EDC in animal models. Our observations counteract the classical hypothesis that EDCs cause sexual precocity. Although precocity is indeed observed in several studies and possibly reflects both peripheral and central mechanisms, delayed female puberty after neonatal exposure to a potent oestrogenic EDC is a new finding that is likely to be of neuroendocrine origin. In addition, it appears that prenatal food restriction could have additive effects to neonatal DES exposure on the neuroendocrine effects of leptin. When females were exposed in utero to prenatal food restriction and postnatally to DES, the stimulatory effect of leptin on GnRH secretion in vitro was completely blunted (Franssen *et al.*, 2014). This reinforces the demonstration of a neuroendocrine disruption of GnRH secretion and illustrates the potential additive effects of different environmental stressors.

Other studies have shown effects of EDCs on GnRH secretion. Fernandez et al. demonstrated an increase of GnRH pulsatility in infantile rats after neonatal exposure to BPA (Fernández *et al.*, 2009). Interestingly, GnRH pulsatility remained disrupted in adult rats neonatally exposed to BPA (Fernández *et al.*, 2009). Losa-Ward *et al.* (2012) have reported morphological alteration of RFamide-related peptide-3 (RFRP3) neurons known to inhibit GnRH neuron activity after neonatal exposure to BPA. They showed reduced RFRP3 perikaria, fiber density and contacts on GnRH neurons, suggesting that BPA-induced premature puberty could result from decreased inhibition of GnRH neurons. GnRH neurons response to steroid-positive feedback, however, was not affected by neonatal exposure to BPA in the study from Adewale et al. (2009). More recently, our group has reported that the developmental increase in frequency of GnRH release at the time of puberty is delayed in female rats exposed neonatally to a low dose of BPA (25 ng/kg/day). Interestingly, the effects were opposite with a high dose of BPA (5 mg/kg/day). The mechanism involved respectively increased or reduced tone of γ-aminobutiric acidergic (GABAergic) neurotransmission which is known to play an inhibitory control on GnRH secretion (Franssen *et al.*, 2016).

Neuroendocrine disruption of reproduction can act through interference with the production or the action of brain neuropeptides which regulate GnRH secretion. Kisspeptin, encoded by the KISS1 gene, is known to stimulate GnRH secretion and plays a crucial role in the onset of puberty (Seminara *et al.*, 2003; de Roux *et al.*, 2003). Navarro et al. have shown a reduction in hypothalamic expression of *KiSS1* mRNA at puberty after neonatal exposure to a high dose of BPA as it was observed after exposure to estradiol benzoate (Navarro *et al.*, 2009). Similarly, Patisaul et al. found a reduction in kisspeptin immunoreactivity (estimated in term of fibers density) in the arcuate nucleus in adult female rats (Patisaul *et al.*, 2009) after neonatal exposure. Regarding the earlier vaginal opening that occurs after exposure to a high dose of BPA (Franssen *et al.*, 2016), those observations were unexpected since Kiss1 is considered as a major gatekeeper of puberty onset. The reduction in kisspeptin expression could then be part of a reactive mechanism to BPA exposure. Similarly, a study performed in the sheep showed a decrease of Kiss1 mRNA levels at the rostral, mid and caudal regions of the hypothalamus in fetus exposed in utero to a complex mixture of EDCs (Bellingham *et al.*, 2009). Such effects were not observed after adult exposure, supporting the hypothesis that the period of exposure is critical for such regulatory processes. Xi *et al.* (2011) showed that perinatal exposure to a high dose of BPA resulted in the upregulation of the expression levels of KiSS-1 and GnRH in both male and female pups. This observation was not replicated after postnatal exposure to BPA, highlighting once again the critical perinatal period for BPA affecting reproductive neural circuits in hypothalamus (Xi *et al.*, 2011). Acute infusion of BPA in the stalk-median eminence of mid to late pubertal female rhesus monkeys led to a suppression of GnRH and kisspeptin secretion for the highest doses while lower doses did not have any apparent effects (Kurian *et al.*, 2015).

GnRH neurons themselves express ERβ but also G protein coupled receptor 30 (GPR30) and estrogen-related receptor γ, all potential receptors for endocrine disrupting chemicals. Thus GnRH neurons could be directly targeted by EDCs. A recent study has shown that BPA significantly decreased calcium activity in GnRH neurons in explants. Blockade of GABAergic and glutamatergic input did not abrogate the inhibitory effect of BPA, suggesting a direct effect of BPA on GnRH neurons (Klenke *et al.*, 2016). Other studies in fish suggest that early exposure to BPA or bisphenol S could increase the number of GnRH3 neurons during development which suggests a broader effect of BPA and BPS on neurodevelopment (Qiu *et al.*, 2016).

Conclusion and Perspectives

The hypothalamus provides a unique site for finely tuned integration of endogenous and environmental factors influencing the control of puberty and reproduction. While initial studies focused on the effects of EDCs on peripheral organs, recent data have shown that the hypothalamic-pituitary control of reproduction can be targeted by environmental pollutants. While such effects are extremely difficult to study in humans, animal models play a crucial role in deciphering the neuroendocrine mechanisms of action

of such compounds. Neuroendocrine effects of EDCs can be opposing and involve different mechanisms depending on the dose or whether they take place early, during fetal and neonatal life or late during prepubertal life.

These last few years, the knowledge of molecular and genetic mechanisms controlling the pubertal process has accelerated considerably. Puberty results from coordinated changes in a multiplicity of genes organized into functional networks. Recent evidence suggests that a dual mechanism of epigenetic regulation affecting the transcriptional activity of neurons involved in stimulating GnRH release plays a fundamental role in the timing of puberty (Ojeda and Lomniczi, 2014; Lomniczi *et al.*, 2015). In addition, a multilayered microRNA-operated switch appears to be involved in the increase in GnRH expression during the prepubertal period (Messina *et al.*, 2016). Epigenetic repression and activation of gene transcription might be a mechanism by which environmental factors affect pubertal development and reproduction. Because EDCs have been recently shown to alter DNA methylation, histone modifications and microRNA expression (Gore *et al.*, 2015), further studies will be needed in order to explore the involvement of such mechanisms in the neuroendocrine disruption of reproduction.

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Association of Endocrine Disrupting Chemicals With Male Reproductive Health

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Abbreviations

BPA Bisphenol A
CI Confidence interval
EDC Endocrine disrupting chemical
GCNIS Germ cell neoplasia in situ
GWAS Genome-wide association study
HCB Hexachlorobenzene
INSL3 Leydig cell hormone insulin-like factor 3
OR Odds ratio
PBDEs Polybrominated diphenyl ethers
PCBs Polychlorinated biphenyls
PCDD Polychlorinated dibenzo-*p*-dioxins
PCDF Polychlorinated dibenzofurans
PFCs Perfluorinated chemicals
PFOA Perfluorooctanoic acid
PFOS Perfluorooctanesulfonic acid
***p,p′*-DDE**
1,1′-Dichloro-2,2′-bis(*p*-chlorophenyl)ethylene
***o,p′*-DDT**
1,1,1-Tricholoro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl) ethane
***p,p′*-DDT**
1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane
SGA Small for gestational age
TDS Testicular dysgenesis syndrome
TCDD 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TGCTs Testicular germ cell tumors

Glossary

AGD Anogenital distance, the distance between anus and genitals.
ASD Anoscrotal distance the distance between anus and scrotum.
Cryptorchidism Undescended testis.
Hypospadias In hypospadias urethral opening is located on the ventral side of the penis due to incomplete formation of the urethra. This is often associated with an abnormal ventral penile curvature and the absence of the ventral foreskin.
TEQ WHO-recommended 2,3,7,8-TCDD equivalent.
Total-TEq WHO-recommended 2,3,7,8-TCDD equivalent for 17 dioxins and 12 dioxin-like PCBs.

Introduction

This article focuses on cryptorchidism, hypospadias, testicular germ cell cancer, and poor semen quality in humans and their associations with environmental exposure to endocrine disrupting chemicals (EDCs). These disorders have common risk factors [such as being born as small for gestational age (SGA)], they show heritability in families and some of them are also risk factors for each other (Trabert *et al.*, 2013; van der Horst and de Wall, 2017; Lee and Coughlin, 2001; Toppari *et al.*, 2010; Thorup *et al.*, 2014; Weidner *et al.*, 1999). According to the testicular dysgenesis syndrome (TDS) hypothesis, cryptorchidism, hypospadias, testicular germ cell cancer, and poor semen quality may have a common origin in fetal life and thus represent symptoms of testicular dysgenesis (Skakkebaek *et al.*, 2001). Also short anogenital distance (AGD) and impaired testosterone production are currently regarded as possible symptoms of TDS (**Fig. 1**) (Skakkebaek *et al.*, 2016). In the rat model fetal exposure to phthalates (which are antiandrogenic chemicals) during the masculinization programming window leads to cryptorchidism and hypospadias, reduced testicular and penile size, shortened anogenital distance, compensated Leydig-cell dysfunction and focal testicular dysgenesis (van den Driesche *et al.*, 2017). Exposure of the fetal testis to environmental factors such as EDCs is regarded as one of the strongest risk factors for male reproductive disorders of TDS (Skakkebaek *et al.*, 2016).

Cryptorchidism

Congenital cryptorchidism, that is, undescended testis, is one of the most common urogenital malformations of newborn boys. Thus, this congenital malformation has been investigated more frequently than other reproductive diseases in infancy. The descent of the testis from the abdomen to the scrotum has often been described to occur in two phases during fetal development (Hutson, 1985; Hutson *et al.*, 2015). The first, transabdominal phase, is according to animal studies dependent on the Leydig cell hormone insulin-like factor 3 (INSL3) (Nef and Parada, 1999; Zimmermann *et al.*, 1999; Gorlov *et al.*, 2002). The second, the inguinoscrotal

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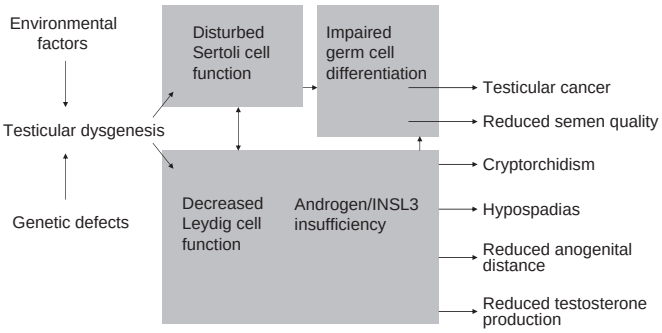

Fig. 1 Schematic presentation of testicular dysgenesis syndrome. Genetic factors and environmental endocrine disrupting chemicals can disturb fetal development of Sertoli cells and Leydig cells. This leads to impaired germ cell development and reduced Leydig cell hormone production. Testicular cancer, poor semen quality, cryptorchidism, hypospadias, reduced anogenital distance, and low testosterone levels are the possible outcomes of the developmental defect.

phase, is dependent on androgens (Hutson, 1986; Hutson *et al.*, 2015). Normally, testes have descended into the scrotum by the time of birth in a full-term baby (Hutson *et al.*, 2015). In prospective cohort studies the prevalence of congenital cryptorchidism has varied between 1% and 8% among full-term boys. The prevalence is higher among premature babies and in boys born as SGA (reviewed in Virtanen and Toppari, 2008). Cryptorchidism has been associated with shortened AGD (Thankamony *et al.*, 2014; Jain and Singal, 2013). Undescended testes often descend spontaneously during the first few months of life possibly due to temporary increase in reproductive hormone levels caused by the postnatal activation of the hypothalamus–pituitary–gonadal (HPG) axis (Raivio *et al.*, 2003; Suomi *et al.*, 2006; Virtanen and Toppari, 2008; Kuiri-Hänninen *et al.*, 2011). Referral for surgical correction (orchiopexy) is recommended if a testis in not descended by the age of 6 months and orchiopexy is to occur by the age of 12–18 months to preserve fertility (Ritzen *et al.*, 2007; Kolon *et al.*, 2014; Tekgül *et al.*, 2016). Early orchiopexy has been associated with improved testicular growth and development (Kollin *et al.*, 2007, 2012).

Some ecological and epidemiological studies have shown associations between cryptorchidism and the use of pesticides in the families' residence area, parental occupation in farming or gardening or parental occupational exposure to pesticides (Garcia-Rodriguez *et al.*, 1996; Kristensen *et al.*, 1997; Pierik *et al.*, 2004; Agopian *et al.*, 2013; García *et al.*, 2017; Weidner *et al.*, 1998; Andersen *et al.*, 2008). However, other studies have been published that did not find statistically significant positive associations (Carbone *et al.*, 2006, 2007; Restrepo *et al.*, 1990; Morales-Suárez-Varela *et al.*, 2011; Kurahashi *et al.*, 2005; Zhu *et al.*, 2006; Biggs *et al.*, 2002). Direct measurement of chemical exposure levels in biological matrices gives a much more specific information on possible exposure of an individual, but such studies are considerably more expensive and laborious to perform. Some of such case–control studies have suggested an association between levels of pesticides in maternal breast milk or in boy's adipose tissue and the risk of cryptorchidism (Damgaard *et al.*, 2006; Hosie *et al.*, 2000; Brucker-Davis *et al.*, 2008), but not all (Chevalier *et al.*, 2015). Other studies evaluating pesticide levels in maternal serum or in cord serum did not find a statistically significant positive association with cryptorchidism or remained inconclusive (Longnecker *et al.*, 2002; Trabert *et al.*, 2012; Bhatia *et al.*, 2005; Pierik *et al.*, 2007; Brucker-Davis *et al.*, 2008). Similarly, most studies evaluating association between levels of polychlorinated biphenyls (PCBs) in different matrices (breast milk, maternal serum, placenta, umbilical cord, cord serum, or boy's adipose tissue) and cryptorchidism have remained negative (Hosie *et al.*, 2000; Mol *et al.*, 2002; Brucker-Davis *et al.*, 2008; McGlynn *et al.*, 2009a; Virtanen *et al.*, 2012; Koskenniemi *et al.*, 2015; Chevalier *et al.*, 2015). Only one study evaluating colostrum levels of PCBs suggested a positive association with congenital cryptorchidism (Brucker-Davis *et al.*, 2008). The risk of cryptorchidism has been positively associated with the sum of dioxins and total-TEq levels [the WHO-recommended 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) equivalent for 17 dioxins and 12 dioxin-like PCBs] in boy's adipose tissue, but not in placenta (Koskenniemi *et al.*, 2015; Virtanen *et al.*, 2012). Maternal hair and breast milk levels of flame retardants have shown positive associations with the risk of cryptorchidism (Main *et al.*, 2007; Goodyer *et al.*, 2017), whereas studies on other matrices (maternal serum, placenta) have remained negative (Main *et al.*, 2007; Small *et al.*, 2009). In a combined analysis of breast milk levels of 106 environmental chemicals, dioxins, and polybrominated diphenyl ethers (PBDEs) were found to be risk factors of cryptorchidism, whereas PCBs were associated with reduced risk of cryptorchidism (Krysiak-Baltyn *et al.*, 2012). These statistically significant associations were observed in the Danish, but not in the Finnish cohort (Krysiak-Baltyn *et al.*, 2012). Placenta levels of organotins showed a positive association with the risk of cryptorchidism in Denmark, whereas in Finland the association was negative (Rantakokko *et al.*, 2013). Studies evaluating levels of perfluorinated chemicals (PFCs) in amniotic fluid or cord blood found no significant association with cryptorchidism (Vesterholm Jensen *et al.*, 2014; Toft *et al.*, 2016). Some studies have evaluated associations between cryptorchidism and nonpersistent chemicals, such as phthalates and bisphenol A (BPA). For phthalates, a study evaluating levels in maternal urine during pregnancy suggested a positive association with cryptorchidism (Swan, 2008), but statistically significant positive associations were not found in studies evaluating levels in amniotic fluid, cord serum, or maternal breast milk (Jensen *et al.*, 2015; Main *et al.*, 2006; Brucker-Davis *et al.*, 2008; Chevalier *et al.*, 2015). Total BPA levels in boy's serum have been positively associated with cryptorchidism (Komarowska *et al.*, 2015), whereas studies evaluating free BPA levels in cord blood or boy's serum have not reported a statistically significant positive association (Fénichel *et al.*, 2012; Chevalier *et al.*, 2015; Komarowska *et al.*, 2015).

Different matrices may also give different results within the same chemical class. Furthermore, small studies are more prone to yield negative findings than large ones. However, a combined analysis of different studies has also been performed. A meta-analysis of 10 case–control studies evaluating different chemical classes [pesticides, PBB, PCBs, PFCs, phthalates] in different matrices (maternal serum, colostrum, cord blood, amniotic fluid) and countries reported a summary estimate of an odds ratio (OR) for congenital cryptorchidism of 1.03 (95%CI 0.72–1.47) (Bonde *et al.*, 2016). Available data was too limited to allow a meta-analysis of associations with specific chemicals. More data needs to be collected before more specific conclusions can be drawn. This may, however, be challenged by the declining levels of older chemicals (Bergman *et al.*, 2013). Furthermore, legislation leads to phasing out of some chemicals whilst new ones are introduced, which makes combination of old and new data difficult. No study has the possibility to analyze all the chemicals we are exposed to. EDCs may also predispose to cryptorchidism (and other disorders of male reproductive health) via affecting fetal growth (Wohlfahrt-Veje *et al.*, 2011).

Hypospadias

In hypospadias the urethral opening is located on the ventral side of the penis due to incomplete formation of the urethra during embryonic weeks 11–16 during which the urethral folds fuse to form the penile urethra (Blaschko *et al.*, 2012). The formation of the glanular urethra, the most distal part, is however, controversial (van der Zanden *et al.*, 2012). In the majority of hypospadias the urethral opening is located on the ventral side of the glans or the coronal sulcus (this is called an anterior or mild hypospadias). In more severe cases, the urethral orifice is located on the penile shaft (middle or moderate hypospadias) or at the scrotum or perineum (called posterior or severe hypospadias) (Carmichael *et al.*, 2012; van der Zanden *et al.*, 2012). Another classification divides hypospadias into only two classes: mild (distal) hypospadias (cases with preoperative urethral opening on the glans or distally on the penile shaft) and severe (proximal) hypospadias (all other cases) (van der Horst and de Wall, 2017). Hypospadias is often associated with an abnormal ventral penile curvature (chordee) and the absence of the ventral foreskin (Blaschko *et al.*, 2012; van der Horst and de Wall, 2017). Approximately 70% of cases have a mild type hypospadias. Although hypospadias is usually an isolated finding, it may be combined with cryptorchidism or micropenis (combination may be called microphallus), and hypospadias has also been associated with a shortened AGD (Thankamony *et al.*, 2014; van der Horst and de Wall, 2017). In animal models, several gene defects have been identified with abnormal embryonic development of the urethra (Blaschko *et al.*, 2012). However, the etiology of hypospadias in humans is unknown in most cases, but both genes and the environment are thought to play a role (Thorup *et al.*, 2014; van der Zanden *et al.*, 2012). According to registry data, the prevalence of hypospadias in Europe has been approximately 19 (range 5–37) per 10,000 total births (Bergman *et al.*, 2015) during this century. Surgical correction of hypospadias between the ages of 6 and 18 (24) months is currently recommended with the objective to obtain normal function and also to improve cosmetic appearance (van der Horst and de Wall, 2017; Tekgül *et al.*, 2016).

As for cryptorchidism, also for hypospadias associations with parental residential or occupational exposure to pesticides have been suggested. A meta-analysis reported an average risk ratio of 1.36 (95%CI 1.04–1.77) for maternal occupational exposure to pesticides and 1.19 (95%CI 1.00–1.41) for paternal occupational exposure (Rocheleau *et al.*, 2009). The suggested association of hypospadias with fetal exposure to pesticides has been supported by some more recent studies (García *et al.*, 2017; Kalfa *et al.*, 2015; Agopian *et al.*, 2013), but not all (Nassar *et al.*, 2010; Morales-Suárez-Varela *et al.*, 2011; Rocheleau *et al.*, 2011; Carmichael *et al.*, 2013). It has also been suggested that parental occupational exposure to heavy metals or parental exposure to any EDCs may be risk factors of hypospadias (Nassar *et al.*, 2010; Morales-Suárez-Varela *et al.*, 2011; Giordano *et al.*, 2010; Kalfa *et al.*, 2015; Haraux *et al.*, 2016).

Most of the studies evaluating the association between hypospadias and chemical levels (pesticides, PCBs, flame retardants, phthalates, or PFCs) in maternal serum or amniotic fluid have remained negative (Longnecker *et al.*, 2002; Bhatia *et al.*, 2005; Carmichael *et al.*, 2010; Trabert *et al.*, 2012; Small *et al.*, 2009; Jensen *et al.*, 2015; Toft *et al.*, 2016). A few studies have suggested a positive association between hypospadias and some pesticides or the sum of PCBs in maternal serum or in the boy's serum (Shekharyadav *et al.*, 2011; Giordano *et al.*, 2010; McGlynn *et al.*, 2009a; Rignell-Hydbom *et al.*, 2012). One study evaluated cryptorchidism and hypospadias together and suggested a positive association with placental levels of pesticides, BPA, and benzophenones (Fernandez *et al.*, 2007; Fernández *et al.*, 2016). The often small number of subjects and therefore limited statistical power has been a serious drawback in several hypospadias studies. However, as for cryptorchidism, also for hypospadias a combined analysis has been performed. In that meta-analysis, a summary estimate of an OR for hypospadias was 1.13 (95%CI 0.86–1.50) [10 case–control studies evaluating different chemicals (pesticides, PCBs, flame retardants, phthalates, PFCs) in different matrices (maternal serum, amniotic fluid) and in different countries] (Bonde *et al.*, 2016). Available data also allowed a meta-analysis of the association with specific chemicals—for 1,1′-dichloro-2,2′-bis(*p*-chlorophenyl)ethylene (DDE) the OR was 1.38 (4 studies, 95%CI 0.93–2.04) and for PCBs the OR was 1.11 (4 studies, 95%CI 0.61–2.01). Thus, no statistically significant associations were observed.

Testicular Cancer

Testicular germ cell tumors (TGCTs) represent the majority of testicular cancers and they are the most common cause of cancer in young men in developed countries (Rajpert-De Meyts *et al.*, 2016; Litchfield *et al.*, 2015; Trabert *et al.*, 2015). TGCTs of young

adults comprise seminomas and non-seminomas, both arising from germ cell neoplasia in situ (GCNIS). GCNIS is considered to develop from gonocytes that failed to mature to spermatogonia and transformed into malignant cells (Rajpert-De Meyts *et al.*, 2016). According to genome-wide association studies (GWAS) genes related to the *KIT*/*KITLG* signaling, male germ cell development/differentiation, telomerase function, microtubule assembly, and repair of DNA damage are associated with the TGCT risk loci (Litchfield *et al.*, 2015). Currently, no animal model for TGCT exists (van den Driesche *et al.*, 2017). The incidence of testicular cancer has been reported to be highest in Northern Europe (estimated age-standardized incidence rates of 12.5 per 100,000 in Denmark, and 12.7 per 100,000 in Norway) (Ferlay *et al.*, 2013) and an increasing incidence of testicular cancer has been observed in several countries worldwide during the last decades (Znaor *et al.*, 2014). The incidence has been predicted to also increase in several Southern European countries by the year 2025, whereas a plateau or a decrease in incidence is expected in historically high-risk European countries (Le Cornet *et al.*, 2014). Risks factor of TGCTs include cryptorchidism, hypospadias, inguinal hernia, and other genital malformations (Trabert *et al.*, 2013).

A follow-up of a U.S. pregnancy cohort observed that mothers of men with testicular cancer had a higher ratio of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p′*-DDT, primary component of commercial pesticide DDT) to *p,p′*-DDE (primary metabolite of *p,p′*-DDT) and lower levels of 1,1,1-tricholoro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p′*-DDT, low level contaminant of commercial DDT) than control mothers in postpartum serum samples (Cohn *et al.*, 2010). This suggests a higher historical exposure of case mothers to DDT or differences in the elimination of DDT (Cohn *et al.*, 2010). Other studies have evaluated differences between men with testicular cancer and controls or between case mothers and control mothers in serum chemical levels around the time of the cancer diagnosis, that is, in adulthood. In a Swedish case–control study men with testicular cancer had significantly higher serum levels of *cis*-nonachlordane, but for other chemicals (PCBs, pesticides and PBDEs) no significant differences were observed (Hardell *et al.*, 2003, 2004, 2006). However, mothers of cases had significantly increased levels of PCBs, hexachlorobenzene (HCB) and PBDEs (sum of 3 congeners) and *trans*- and *cis*-nonachlordanes, but not of *p,p′*-DDE when compared to control mothers. This suggested a possibly increased prenatal exposure of men with TGCT to PCBs and HCB, as these chemicals have very long half-lifes in humans (Hardell *et al.*, 2003, 2004, 2006).

Case control studies using serum samples taken before the diagnosis of testicular cancer have suggested an association between testicular cancer and exposure to some organochlorine pesticides, but the results regarding the association with exposure to PCB congeners have been conflicting (Purdue *et al.*, 2009; McGlynn *et al.*, 2008, 2009b). In addition, two studies in which serum samples of patients with testicular cancer were taken after diagnosis have given different results on the association between the exposure to organochlorine pesticides and testicular cancer (Giannandrea *et al.*, 2011; Biggs *et al.*, 2008).

A meta-analysis of eight case–control studies evaluating the association between exposure to endocrine disrupting chemicals and testicular cancer reported a summary estimate of an OR of 1.20 (95%CI 0.78–1.89) (Bonde *et al.*, 2016) and thus, found no significant association. Available data allowed a congener-specific meta-analysis for exposure to DDT and its metabolite DDE and also here no significant association was found (the respective ORs were 1.19 (95%CI 0.60–2.36) and 1.12 (95%CI 0.82–1.55)) (Bonde *et al.*, 2016).

Semen Quality

It has been suggested that sperm concentrations have been decreasing in the 20th century and at the beginning of 21st century in Western countries (Carlsen *et al.*, 1992; Swan *et al.*, 1997, 2000; Levine *et al.*, 2017). Also several, but not all, original studies published recently have suggested that the sperm concentration or total sperm counts are decreasing (reviewed in Virtanen *et al.*, 2017). However, significant geographical differences in sperm concentration have been observed in studies using similar study protocols (Virtanen *et al.*, 2017).

Several studies have evaluated associations between fetal or adult exposure to environmental chemicals and semen quality. Pre- or perinatal accidental exposure to high levels of persistent chemicals like PCBs and their pyrolytic products and to 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD) has been associated with reduced semen quality in adulthood (motility, morphology, sperm count, or concentration) (Guo *et al.*, 2000; Mocarelli *et al.*, 2011). In addition, exposure to TCDD during infancy after the Seveso accident has been associated with reduced semen quality (motility, concentration) (Mocarelli *et al.*, 2008). In a prospective Russian study, serum levels of TCDD and toxic equivalents for polychlorinated dibenzo-*p*-dioxins (PCDD TEQ) at the age of 8–9 years were associated negatively with sperm concentration, total sperm count, and total motile sperm count at the age of 18–19 years, although the TCDD levels were much lower than in the Seveso study (Mínguez-Alarcón *et al.*, 2017). Sums of PCDDs, polychlorinated dibenzofurans (PCDFs), coplanar-PCBs, or PCBs, and also toxic equivalents for PCDFs (PCDF TEQs), coplanar PCB TEQs, and total TEQs showed no significant associations with semen variables (Mínguez-Alarcón *et al.*, 2017).

Exposure to background levels of perfluoro-octanoic acid in utero has been associated negatively with semen quality (sperm concentration, total sperm count), whereas no such association has been described for perfluorooctanesulfonic acid, PCBs or *p,p′*-DDE (Vested *et al.*, 2013, 2014b). Prenatal exposure to nonpersistent phthalates has been associated negatively with semen volume in adulthood (Axelsson *et al.*, 2015b).

Meta-analyses and more recent studies have suggested that adult exposure to nonpersistent phthalates is associated adversely with semen quality (Cai *et al.*, 2015; Axelsson *et al.*, 2015a; Thurston *et al.*, 2015; Wang *et al.*, 2016). However, their role is likely to be small in determining classical sperm variables (sperm concentration, percent motile spermatozoa, and percent spermatozoa with normal morphology, total sperm count and total motile sperm count) (Thurston *et al.*, 2015). Some studies have suggested a

negative association between adult exposure to nonpersistent bisphenol A and semen quality, but the results are limited and inconclusive (reviewed in Mínguez-Alarcón *et al.*, 2016). Adult exposure to persistent chemicals has been associated negatively with semen quality (motility (PCBs) and morphology (perfluorinated compounds)) and with an increased time to pregnancy (PCBs and *p,p′*-DDE) (Vested *et al.*, 2014a; Buck Louis *et al.*, 2013). Some studies have suggested a negative association between *p,p′*-DDE exposure and sperm motility (Aneck-Hahn *et al.*, 2007; De Jager *et al.*, 2006; Toft *et al.*, 2006), but other studies did not find a significant association (Haugen *et al.*, 2011; Hauser *et al.*, 2003).

Anogenital Distance (AGD)

AGD in boys is measured as either the distance between the anus and scrotum (also called as anoscrotal distance, ASD) or as the distance between the anus and upper (or sometimes lower) base of the penis (Swan *et al.*, 2005; Salazar-Martinez *et al.*, 2004; Dean and Sharpe, 2013; Longnecker *et al.*, 2007). AGD is believed to reflect fetal exposure to androgens (Dean and Sharpe, 2013). Maternal urine phthalate levels during pregnancy have been associated negatively with AGD and anogenital index (AGI, AGD divided with weight) in their newborn and infant sons (Swan, 2008; Swan *et al.*, 2005, 2015; Suzuki *et al.*, 2012; Bustamante-Montes *et al.*, 2013; Bornehag *et al.*, 2015). Another study in a population with low exposure remained negative (Jensen *et al.*, 2016). Also a small study evaluating amniotic fluid phthalate levels remained negative (Huang *et al.*, 2009). The first trimester has been suggested to be the critical window of susceptibility for changes in AGD (Martino-Andrade *et al.*, 2016). Maternal serum levels of DDT and DDE showed no significant association with the anogenital distance of their sons (Bornman *et al.*, 2016; Longnecker *et al.*, 2007). Maternal plasma dioxin-like activity at delivery has been negatively associated with the AGD of their newborn sons (Vafeiadi *et al.*, 2013). Boys with cryptorchidism and hypospadias show a reduced AGD (Thankamony *et al.*, 2014; Hsieh *et al.*, 2012), and AGD has been positively associated with semen quality and testosterone levels in adult men (Mendiola *et al.*, 2011; Eisenberg *et al.*, 2011, 2012).

Hormone Levels

Chemical exposures have also been associated with changes in reproductive hormone levels. Free BPA levels in cord blood have shown negative associations with INSL3 levels, but associations with testosterone levels have been mixed (Fenichel *et al.*, 2012; Chevalier *et al.*, 2015). Maternal breast milk phthalate and PBDE levels have shown a positive association with LH levels and the LH:free testosterone ratio of infant boys at the age of 3 months, when the hypothalamus–pituitary axis is active (Main *et al.*, 2006, 2007). Exposure to TCDD perinatally after the Seveso accident was associated with an increased FSH level and decreased Inhibin B level in adulthood (Mocarelli *et al.*, 2011). Also in utero exposure level of PFOA (background levels) has shown positive associations with LH and FSH levels in adult men, whereas exposure levels of PFOS, PCBs or *p,p′*-DDE showed no association with reproductive hormone levels (Vested *et al.*, 2013, 2014b). Prenatal exposure levels of phthalates have shown positive association with LH and FSH levels in young adult men (Axelsson *et al.*, 2015b).

Combined Exposures and Human Risk Assessment

All manifestations of TDS can be seen in humans with genetic disorders of androgen production or action, such as 5alpha-reductase or 17beta-hydroxysteroid-dehydrogenase type 3 mutations, and androgen receptor mutations (Toppari *et al.*, 2010; Juul *et al.*, 2014; Ahmed and Rodie, 2010). Animal experiments show also similar adverse effects of exposure to antiandrogenic chemicals (Rider *et al.*, 2008; Christiansen *et al.*, 2008). Dioxins have a different mechanism of action but have similar effects to those of antiandrogens (Rider *et al.*, 2010). Furthermore, dioxins and antiandrogens have additive effects, and therefore even doses that are below the no-adverse-effect level (NOAEL) contribute to the toxicity (Rider *et al.*, 2010, Jacobsen *et al.*, 2010). This challenges human epidemiology where we typically analyze exposure-outcome associations chemical by chemical. Both nature examples of defects in androgen action and ample evidence from animal experiments point to potential risks of all antiandrogenic and dioxin-like compounds. In the absence of robust epidemiological evidence we have to rely on biological plausibility and animal experiments in the risk analysis (Hauser *et al.*, 2015).

Conclusions

In conclusion, despite clear evidence of adverse effects of antiandrogenic and dioxin-like chemicals on the male reproductive system, only few human studies have been able to detect associations between fetal exposure to EDCs and congenital cryptorchidism, hypospadias, testicular cancer, and reduced semen quality. Small study sizes due to time and budget constraints and the use of different biological matrices to measure exposure levels, which may not always be suitable, contribute to the divergence in observations. Furthermore, humans are exposed to a mixture of chemicals from conception to adult life making the

identification of specific chemicals with adverse effects difficult. Thus, future research challenges include further investigations of associations between cocktail exposures and male reproductive health.

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Endocrine Disrupting Chemicals and Behavior

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Introduction

Inexorably connected, the endocrine system and nervous system work in tandem to regulate the development and expression of behavior. This relationship is best exemplified by the early actions of steroid hormones on the mammalian brain, which induce enduring changes in the brain that impact sexually dimorphic behavior (Cooke *et al.*, 1998; De Vries, 2004; Knudsen, 2004; McCarthy, 2008). Although brain development is a tightly regulated and orchestrated process, it is vulnerable to exogenous substances that interfere with the action of natural hormones.

Endocrine disrupting chemicals (EDCs) are a diverse group of exogenous compounds that have been found to interfere with the endocrine system and produce adverse health effects in exposed individuals or their offspring. Numerous classes of chemicals including plasticizers, flame-retardants, fungicides, pesticides, pharmaceuticals, heavy metals, and even naturally occurring compounds such as phytoestrogens are endocrine disrupting (**Table 1**). These and other EDCs dampen, block, or potentiate the action of endogenous hormones through a variety of direct and indirect mechanisms. For example, they may agonize or antagonize hormone receptors, interfere with hormone biosynthesis, or alter the number of hormone receptors (Gore *et al.*, 2015). The long-term consequences of EDCs depend on a variety of factors including the genetic susceptibility of the organism and the dose, duration, and developmental window of exposure.

Since the term was first coined nearly three decades ago, EDCs have received considerable attention from both the scientific community and the public (Hotchkiss *et al.*, 2008; McLachlan, 2016). While highly debated, endocrine disruption provides a plausible explanation for the global increase in the prevalence of sex-biased neurodevelopmental disorders, most notably attention-deficit hyperactivity disorder and autism spectrum disorder, which cannot be fully explained by genetic factors alone. Although the etiology of these disorders are not well understood, there is increasing concern that developmental exposure to EDCs may enhance risk by disrupting sexual differentiation of the brain. Indeed, extensive experimental and epidemiological evidence supports associations between early-life exposure to environmental contaminants and sex-specific neurobehavioral outcomes.

The goal of the current article is to examine the evidence for altered behavior as a consequence of EDC exposure. Although hundreds of suspected EDCs have been identified, bisphenol A (BPA) is arguably one of the most widely used and extensively studied. Thus for illustrative purposes, we will concentrate on the effects of early exposure to this notorious chemical.

BPA: A Landmark Endocrine Disrupting Chemical

Our focus on BPA is significant because this well recognized EDC is widely used as a component of polycarbonate plastics and epoxy resins and commonly incorporated into numerous household products. Human exposure occurs primarily from contaminated food and beverages (von Goetz *et al.*, 2010), and in industrialized countries, more than 90% of individuals are estimated to have detectable amounts of BPA in their bodies (Bushnik *et al.*, 2010; Calafat *et al.*, 2005, 2008; Casas *et al.*, 2013; LaKind and Naiman, 2015). Early-life exposure is of major concern because this period is characterized by rapid growth and has long been recognized as a critical window of vulnerability to the effects of neurotoxic and neuroendocrine disrupting agents. In humans, BPA has been detected in fetal plasma (Ikezuki *et al.*, 2002), amniotic fluid (Engel *et al.*, 2006), fetal liver (Nahar *et al.*, 2013), and placenta tissue (Schonfelder *et al.*, 2002), demonstrating the capacity for significant gestational exposure. Moreover, the fetal brain is particularly susceptible to environmental exposures because the blood–brain barrier is not fully formed, and thus provides limited protection (Adinolfi, 1985; Perera and Herbstman, 2011). Accordingly, levels of chemical exposures that have no obvious effects on the adult nervous system can pose a significant risk when exposure occurs developmentally.

A litany of studies provide evidence that developmental exposure to BPA results in neurobiological and mood-related behavioral consequences, even at doses below the current US Food and Drug Administration No Observed Adverse Effect Level (NOAEL) of 5 mg/kg body weight (bw)/day. It should be emphasized that despite an abundance of literature, there is a general lack of understanding concerning the specific cellular mechanisms by which BPA alters human neurodevelopment. Initially developed as a synthetic estrogen (Dodds and Lawson, 1936), BPA has long been considered weakly estrogenic. Until recently, BPA was primarily believed to exert its effects by engaging estrogen receptors (ERs). However, the binding affinity of BPA for both ERα and ERβ in cell culture assays is 10,000–100,000-fold lower than the endogenous estrogen (Blair *et al.*, 2000; Kuiper *et al.*, 1998; Waller *et al.*, 1996), making it improbable that BPA mediates its effects solely through classical ER-dependent nuclear pathways. In fact, BPA has also been demonstrated to have a low binding affinity for other steroid receptors (Rubin, 2011; Zoeller, 2005) and to have rapid, nongenomic actions via membrane-bound ERs (Belcher and Zsarnovszky, 2001; Thomas and Dong, 2006; vom Saal *et al.*, 2007). Most recently, studies have shown developmental BPA exposure can induce sex- and region-specific changes in DNA methylation patterns in the brain that are accompanied by decreased expression of ERs (Kundakovic *et al.*, 2013,

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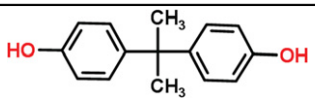
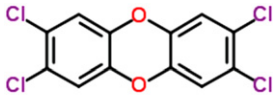
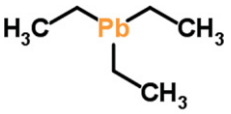
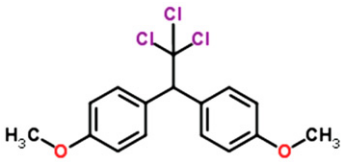
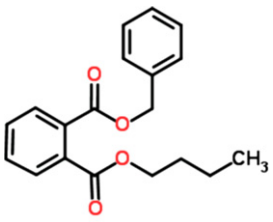
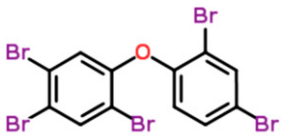
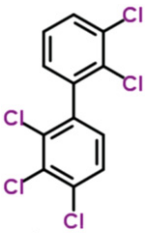
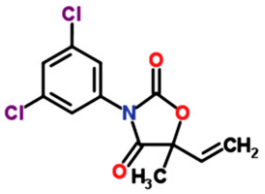
Table 1 Structures and potential sources of endocrine disrupting chemicals with published behavioral effects

EDC	*Structure*	*Potential sources of human exposure*
Bisphenol A (BPA)		• Food packaging • Medical devices • Thermal receipt paper • Epoxy resins and polycarbonate plastics
Chlorpyrifos		• Insecticide in agricultural and commercial settings • Residue on fruits and vegetables
Dioxins	 Tetrachlorodibenzo-*p*-dioxin	• High fat food (e.g., dairy products, animal fat, and eggs) • Industrial processes (e.g., municipal waste incineration)
Lead	 Triethyl lead	• Paint • Lead-based gasoline • Dust • Drinking water • Children's jewelry and toys
Methoxychlor (MXC)		• Insecticide used on pets, home gardens, crops, and livestock • Air, soil, water contaminant
Phthalates	 Benzyl butyl phthalate	• Plastics • Food packaging • Personal care products and pharmaceuticals • Vinyl flooring, wall covering, and carpet backing • High-fat foods (e.g., dairy products animal fat, and eggs)
Polybrominated diphenyl ethers (PBDEs)	 Pentabromodiphenyl ether	• Flame retardants • House dust contaminant • Fish, meat, and dairy products • Soil and sediments • Outdoor air
Polychlorinated biphenyls (PCBs)	 2,2',3,3',4-PCB	• High-fat foods (e.g., dairy products animal fat, and eggs) • Ground water contaminant • Electrical transformers, capacitors, and other industrial waste • Outdoor air
Vinclozolin		• Fungicide • Food and drinking water contaminant • Ground water • Outdoor air

2015). These alternative modes of action emphasize the complex, multimodal routes by which EDCs can impact brain and behavior across the lifespan.

Sexually Dimorphic Behaviors as Indicators of Endocrine Disruption

Sexual differentiation is the process by which the brain becomes structurally and functionally different between males and females. Sexual differentiation of the brain was once thought to hinge almost entirely upon gonadal hormones: generally, the brain develops as male in the presence of these hormones, and as female in their absence. However, recent evidence indicates this process is more nuanced and involves multiple sex-specific hormonal, genetic, and epigenetic factors that influence sexually dimorphic physiology and behavior thorough a variety of mechanisms (McCarthy and Nugent, 2013; McCarthy, 2016; Schwarz and McCarthy, 2008).

Disruption of sexually dimorphic behavior is a common outcome of developmental exposure to EDCs, particularly BPA. Indeed, sex-specific behavioral impacts of BPA have been demonstrated in numerous animal models and human epidemiological studies (Frye *et al.*, 2012; Inadera, 2015; Rochester, 2013). Considering their complex, multifactorial origin, it is not surprising that BPA has been found to decrease, eliminate, and even reverse behavioral sex differences. It is unclear, however, whether the differential effects of BPA in males and females are due to disruption of already existing behavioral dimorphisms or whether they reflect sex-specific vulnerability.

Evidence From Animal Models

The majority of experimental studies on neurobiological and behavioral consequences of developmental BPA exposure (gestational and/or neonatal) employ rodent models. Here we provide a summary of these findings and limit our discussion to studies that use BPA doses at or lower than the current FDA NOAEL (5 mg/kg bw/day).

Exploratory and Affective Behaviors

Experimental animal studies in rodents provide compelling evidence that developmental BPA exposure can increase the expression of anxiety-related and exploratory behaviors. However, effects vary across sex, animal model, and age at testing. Studies examining developmental BPA exposure in juveniles typically demonstrate sex-specific effects, but results are inconsistent. For example, two studies on juvenile C57BL/6J mice conclude that gestational and/or neonatal BPA exposure increased anxiety in males but had no effect in females (Cox *et al.*, 2010; Matsuda *et al.*, 2012), while another study with CD-1 mice reported the opposite (Gioiosa *et al.*, 2007). Additionally, a recent study in juvenile Sprague Dawley rats found no effects of perinatal BPA exposure on exploratory or anxiety activity, in either sex (Rebuli *et al.*, 2015).

Studies in adults are more consistent and in general, females display more robust anxiogenic effects following early-life BPA exposure compared to males (Gioiosa *et al.*, 2013; Hicks *et al.*, 2016; Ryan and Vandenbergh, 2006; Sullivan *et al.*, 2014). For example, neonatal exposure to 10 μg/kg of BPA was found to decrease exploratory-behavior and increase anxiety-like behavior in adult female CD-1 mice, resulting in a behavioral phenotype similar to that of adult control males (Gioiosa *et al.*, 2013). This is consistent with other studies that show developmental BPA exposure can decrease or eliminate sex differences typically observed in adult rodents, on a number of behavioral paradigms used to assess anxiety (Gioiosa *et al.*, 2007; Hicks *et al.*, 2016; Jones and Watson, 2012; Kubo *et al.*, 2003; Kundakovic *et al.*, 2013). Experiments using other animal models (including zebrafish, voles, and other alternative rodent species, and nonhuman primates) provide further evidence that developmental BPA exposure induces anxiety-related behaviors (Kinch *et al.*, 2015; Kundakovic *et al.*, 2013; Nakagami *et al.*, 2009; Patisaul *et al.*, 2012; Sullivan *et al.*, 2014). This consistency across studies and varying animal models prompted the World Health Organization to conclude that there is some concern about impacts of developmental BPA exposure on brain and behavior (FAO/WHO, 2011). While the underlying mechanisms remain unclear, this behavioral disruption is commonly associated with perturbation of ER-related gene expression in the hypothalamus and the amygdala (Arambula *et al.*, 2016, 2018; Cao *et al.*, 2012, 2013, 2014; Kundakovic *et al.*, 2013; Patisaul *et al.*, 2012; Rebuli *et al.*, 2014; Rebuli and Patisaul, 2015). Moreover, a recent study found that prenatal BPA exposure induced sex-specific effects on anxiety-like behaviors in adult BALB/c mice that corresponded to changes in DNA methylation and mRNA levels of ERα in the hypothalamus (Kundakovic *et al.*, 2013). These data provide intriguing evidence that BPA-induced disruption of anxiety behavior may be mediated through an epigenetic mechanism.

Other EDCs shown to alter anxiety-related and exploratory behaviors include phthalates (Carbone *et al.*, 2013; Quinnies *et al.*, 2017), polychlorinated biphenyls (thyroid disrupting) (Bell *et al.*, 2016; Gillette *et al.*, 2017), some flame-retardants (thyroid disrupting) (Baldwin *et al.*, 2017; Patisaul *et al.*, 2013), and vinclozolin (androgen disrupting) (Gillette *et al.*, 2014; Skinner *et al.*, 2008). Perhaps best characterized is the spectrum of adverse neurobehavioral outcomes associated with developmental lead exposure. Considerable and consistent research, in a variety of animal models including rodents, cats, and nonhuman primates, shows that developmental lead exposure can induce hyperactivity, impulsivity, and aggression (Burright *et al.*, 1989; Cervantes *et al.*, 2005; Delville, 1999; Hahn *et al.*, 1991; Holloway and Thor, 1987; Laughlin *et al.*, 1991; Li *et al.*, 2003). While not an EDC in

the classic sense, lead and other metals can be endocrine disrupting in some circumstances (Chen *et al.*, 2017; Henson and Chedrese, 2004; Hirsch *et al.*, 2010; Miao *et al.*, 2015).

Learning and Memory

Impairments in cognitive abilities have also been observed following developmental exposure to BPA. Under normal conditions, male rodents typically perform significantly better than females on spatial learning and memory tasks and, interestingly, early-life BPA exposure has repeatedly been shown to reduce this sex difference (Carr *et al.*, 2003; Jones and Watson, 2012; Xu *et al.*, 2007). Several studies in rats and mice suggest that gestational and/or neonatal exposure to BPA can negatively impact spatial memory in both juvenile and adult males (Kumar and Thakur, 2014; Kuwahara *et al.*, 2013; Liu *et al.*, 2016; Tian *et al.*, 2010; Xu *et al.*, 2007, 2010a,b). As an example, exposure to BPA (0.5 and 5 mg/kg bw/day) during the perinatal period significantly impaired spatial memory in juvenile and adult male ICR mice (Xu *et al.*, 2010b). In contrast, data on the effects of BPA on spatial learning and memory in females is sparse and mixed results have been reported (Carr *et al.*, 2003; Liu *et al.*, 2016; Xu *et al.*, 2007). Notably, the described changes in spatial memory were associated with BPA-induced alterations in dendritic spine density and morphology, as well as reduced expression of *N*-methyl-D-aspartic acid (NMDA) glutamatergic receptors and ERβ (Eilam-Stock *et al.*, 2012; Liu *et al.*, 2016; Tian *et al.*, 2010; Xu *et al.*, 2010a) in the hippocampus.

Experimental research also shows that dioxin (Kakeyama and Tohyama, 2003; Seo *et al.*, 1999), polychlorinated biphenyls (Eriksson and Fredriksson, 1996; Schantz *et al.*, 1995), polybrominated diphenyl ethers (PBDEs) (Costa and Giordano, 2007; Viberg *et al.*, 2003a), chlorpyrifos (Gomez-Gimenez *et al.*, 2017; Levin *et al.*, 2001), and other EDCs can result in altered learning and memory behaviors. As an example, perinatal exposure to PDBE slowed motor skill development in adolescent and adult CD-1 mice (Branchi *et al.*, 2002). Similarly, a series of experiments in mice found neonatal exposure to multiple PBDE congeners caused significant adult learning and memory deficits that corresponded to inhibition of the hippocampal cholinergic system (Viberg *et al.*, 2003b, 2006).

Paternal, Social, and Sexual Behaviors

In rodents, changes in parental, social, and sexual behaviors have been reported after developmental exposure to BPA, but evidence is sparse and inconsistent (Adewale *et al.*, 2009, 2011; Farabollini *et al.*, 2002; Hicks *et al.*, 2016; Jones *et al.*, 2011; Monje *et al.*, 2009; Picot *et al.*, 2014; Porrini *et al.*, 2005; Ryan *et al.*, 2010; Sullivan *et al.*, 2014; Wolstenholme *et al.*, 2011). To date only two studies have examined the relationship between developmental BPA exposure and subsequent maternal behavior. One of these found that prenatal exposure to BPA 10 μg/kg bw/day decreased the amount of time female CD-1 mice spent huddling over or nursing their offspring (Palanza *et al.*, 2002b). The second study, which was conducted in Wistar rats, reported similar effects of gestational and lifelong BPA exposure to 5 μg/kg bw (Boudalia *et al.*, 2014). The impact of developmental BPA exposure on paternal behavior is unknown. This is likely due to the fact that traditional rodent models used in toxicology do not display biparental care.

Evidence that developmental BPA exposure can alter behaviors related to sociality is limited and highly discordant, but existing literature suggests that female social behavior may be more sensitive to disruption than males. For example, exposure to 1.25 mg of BPA during the prenatal period increased male and female juvenile social play in C57Bl6J mice (Wolstenholme *et al.*, 2011). In contrast, another report perinatal exposure to 40 μg/kg BPA reduced social play in female juvenile Sprague–Dawley rats (males were not assessed) (Porrini *et al.*, 2005). A study in prairie voles, a more prosocial animal model than laboratory rats or mice, found sex- and age-specific effects on social behavior. Neonatal exposure to 5 and 50 μg/kg bw/day decreased social investigation in juvenile males and slightly inhibited partner preference formation in adult females. These behavioral outcomes were accompanied by sex-dependent changes in the number of dopaminergic-, oxytocin-, and vasopressin neurons in the paraventricular nucleus of the hypothalamus and dopaminergic neurons in the bed nucleus of stria terminals (Sullivan *et al.*, 2014).

Some published data suggests early-life BPA exposure can induce subtle changes in adult sexual behavior, but supporting evidence is mixed (Adewale *et al.*, 2009, 2011; Farabollini *et al.*, 2002; Jones *et al.*, 2011; Monje *et al.*, 2009; Picot *et al.*, 2014; Ryan *et al.*, 2010). Two studies in rodents have found a slight impairment in male sexual performance in terms of latency, intromission, and ejaculation (Farabollini *et al.*, 2002; Jones *et al.*, 2011), where others have found none (Picot *et al.*, 2014). Female data is similarly mixed but generally indicates female sexual behavior is unaffected by developmental BPA exposure. In rodents, female proceptive and receptive behaviors are often determined by hopping and darting and the lordosis response. Exposure to 0.05 mg/kg of BPA during the neonatal period decreased hopping and darting rate in adult female Wistar rats, while lordosis behavior was unaffected (Monje *et al.*, 2009). Another study, conducted in Sprague–Dawley rats, observed a modest increase in lordosis behavior in adult females following perinatal exposure to 40 μg/kg of BPA (Farabollini *et al.*, 2002). Other studies have observed no effects of developmental exposure on proceptive or receptive behaviors in females (Adewale *et al.*, 2009, 2011; Ryan *et al.*, 2010).

Animal data also indicates effects of developmental exposure to other EDCs including methoxychlor (Gray *et al.*, 1988; Palanza *et al.*, 2002a; Suzuki *et al.*, 2004), polychlorinated biphenyls (Simmons *et al.*, 2005; Steinberg *et al.*, 2007; Wang *et al.*, 2002), phthalates (Dalsenter *et al.*, 2006; Lee *et al.*, 2004), phytoestrogens (Patisaul and Adewale, 2009; Patisaul and Jefferson, 2010; Patisaul, 2017), and chlorpyrifos (De Felice *et al.*, 2015; Venerosi *et al.*, 2009, 2012) on parental, social, and sexual behaviors. For

instance, a number of studies in rodents provide evidence that PCBs can adversely impact sociosexual behavior and, in general, suggest that females may be more vulnerable to disruption than males. In rats gestational and neonatal exposure reduces receptive and proceptive sexual behaviors such as lordosis and likelihood to mate (Chung and Clemens, 1999; Steinberg *et al.*, 2007; Suzuki *et al.*, 2004).

Evidence From Epidemiological Studies

Although the health impacts of developmental BPA exposure remain controversial, during the last decade several epidemiological studies have reported adverse behavior in children developmentally exposed to BPA (Bellinger *et al.*, 2007; Braun *et al.*, 2009, 2011, 2017b; Casas *et al.*, 2015; Evans *et al.*, 2014; Harley *et al.*, 2013; Hong *et al.*, 2013; Maserejian *et al.*, 2012; Perera *et al.*, 2012; Roen *et al.*, 2015).

The first was a longitudinal cohort study (the Health Outcomes and Measures of the Environment Study; HOMES) in which maternal BPA urine concentrations were measured during pregnancy (gestational weeks 16 and 24) and around the time of birth. When the children were 2 years old, their behavior was evaluated using a questionnaire designed to assess adaptive and problem behaviors in community and home settings. In girls, but not boys, significant associations were found between higher levels of maternal BPA during gestation and increased externalizing behaviors, specifically hyperactivity and aggression (Braun *et al.*, 2009). In a follow-up study on the HOMES cohort, behavior and executive function were examined at 3 years of age. Higher levels of maternal BPA during gestation were correlated with increased anxiety, hyperactivity, and depressive behavior, particularly in girls (Braun *et al.*, 2011). Of note, two subsequent studies on the HOMES cohort found no associations between maternal levels of gestational BPA and autistic behaviors in children 4–5 years old (Braun *et al.*, 2014) or visual spatial ability in children 7 years old (Braun *et al.*, 2017a). This outcome highlights that while chemical exposures can be linked to measureable and meaningful decrements in behavior and cognition that can have life-long implications, manifestation of a clinically defined disorder, such as autism, is highly unlikely and would be notoriously difficult to prove.

Other longitudinal cohort studies have found stronger associations between prenatal urinary BPA levels and adverse childhood behavior in boys than in girls. In cohort of African-American and Dominican women and their children (Center for Children's Environmental Health Cohort; CCCEH), higher maternal urinary BPA concentration was associated with increased aggression and emotionally reactive behavior in boys at 3–5 years of age (Perera *et al.*, 2012). A follow-up study on the CCCEH cohort, found 7–9 year old boys exposed to higher concentrations of BPA during gestation had increased internalizing (symptoms of anxiety and depression) and externalizing (aggression and rule-breaking) problems (Roen *et al.*, 2015). Similarly, three additional cohort studies have reported positive correlations between higher gestational BPA concentrations and behavioral problems in boys at 6–10 years of age including increased symptoms of anxiety, depression, and inattention (Casas *et al.*, 2015; Evans *et al.*, 2014; Harley *et al.*, 2013). Lastly, the most recent epidemiological study found a relationship between maternal urinary BPA concentration during pregnancy and poorer working memory and increased internalizing behavior in boys at 3 years of age (Braun *et al.*, 2017b).

Collectively these epidemiological studies strongly suggest that developmental BPA exposure may have adverse neurobehavioral effects in children, which may differ between boys and girls. This conclusion is concordant with the abundant animal data although the mechanisms of action remain poorly understood. In general, the conclusions from the described studies are strengthened by their use of large mother–child cohorts, BPA measurements across several gestational time points, and multiple observed behavioral outcomes. However, it is important to recognize the limitations inherent to longitudinal cohort studies that may have contributed to the discrepant results, particularly demographic differences across cohorts. Additionally, differences in neuropsychological assessments and substantial within-person variation in urinary BPA concentrations may also contribute to the heterogeneity in the literature (Braun *et al.*, 2012; Thayer *et al.*, 2015).

Robust evidence from epidemiological studies support a relationship between early-life EDC exposure and neurobehavioral outcomes. Behavioral deficits, including impairments in learning, memory, and social skills, have been linked with developmental exposure to PCBs, numerous flame retardants, and other persistent organic pollutants (POPs). Of these, the evidence for PCB-related effects on neurodevelopment is particularly compelling. Several longitudinal cohort studies have observed associations between developmental PCB exposure and impairments in measures of executive functioning such as processing speed, verbal abilities, and visual recognition memory (Boucher *et al.*, 2009; Jacobson and Jacobson, 1996; Lai *et al.*, 2002; Ribas-Fito *et al.*, 2001). Similar impairments in executive functioning are also linked to developmental lead and methylmercury exposure (Grandjean and Landrigan, 2006; Winneke, 2011). Because humans are exposed to a complex cocktail of EDCs and other toxicants continuously, combined effects of multiple exposures are a significant and growing concern.

Conclusions

The experimental evidence summarized above supports the hypothesis that developmental exposure to BPA, even at doses below the current NOAEL, may interfere with some aspect of sexual differentiation of the nervous system, thereby resulting in disruption of both reproductive and nonreproductive behaviors. During fetal and child development, the brain is particularly susceptible to environmental stressors such as EDCs. A recent study found that children of parents who were concerned about EDCs had

decreased urinary concentrations of BPA, which suggests that by exercising precaution we may be able to reduce our exposure to chemicals (Pell *et al.*, 2017). While developmental exposure to BPA and other EDCs may contribute to neural disorders in children, it should be emphasized that available literature does not provide direct causal evidence. Further mechanistic and epidemiological studies are needed to clarify the relationship between EDC exposure and human health. Greater information is also needed about the effect of mixtures and repeated exposures over multiple critical periods.

Remaining Challenges and Future Directions

Accumulating evidence suggests that developmental EDC exposure is contributing to the rising rate of neurobehavioral disorders. However, there are many obstacles that make it difficult to establish direct causal relationships. First, the intervening period between neurodevelopmental insult and the manifestation of a resulting behavioral dysfunction can be very long. During this period, which may take years or decades in humans, behavior is concomitantly influenced by other factors including genetics, experience, and lifestyle. Consequently, ascertaining the contribution of single chemical exposure is extraordinarily difficult. Moreover, humans are exposed to varying amounts and mixtures of EDCs throughout their lifetime. Already an area of increased interest, modeling "real world" human exposure (i.e., chronic, low-dose mixtures) will greatly enhance the translational value of animal studies in the EDC field.

In humans, studies on the neurobehavioral changes following early-life EDC exposure are constrained by practical and ethical limitations. An obvious limitation is the relative inaccessibility of the human brain. Both in vivo and in vitro models can be used to identify peripheral biomarkers of EDC exposure and associated diseases, which can be incorporated into new and existing epidemiological studies. Reliable biomarkers could also have important implications for identifying at-risk populations.

Since the advent of the endocrine disruption concept nearly 30 years ago (Colborn *et al.*, 1993), the field has made substantial progress in identifying several unique mechanisms of EDC action on the brain but gaps remain in establishing direct relationships between changes in brain development and consequential changes in behavior. Improved understanding will require deeper investigation into the basic neural and molecular mechanisms underlying complex behaviors such as activity, sociability, and executive function. As understanding their biological origins grows, so does the capacity to more reliably predict and prevent injury from EDCs and other developmental neurotoxicants.

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Editors in Chief
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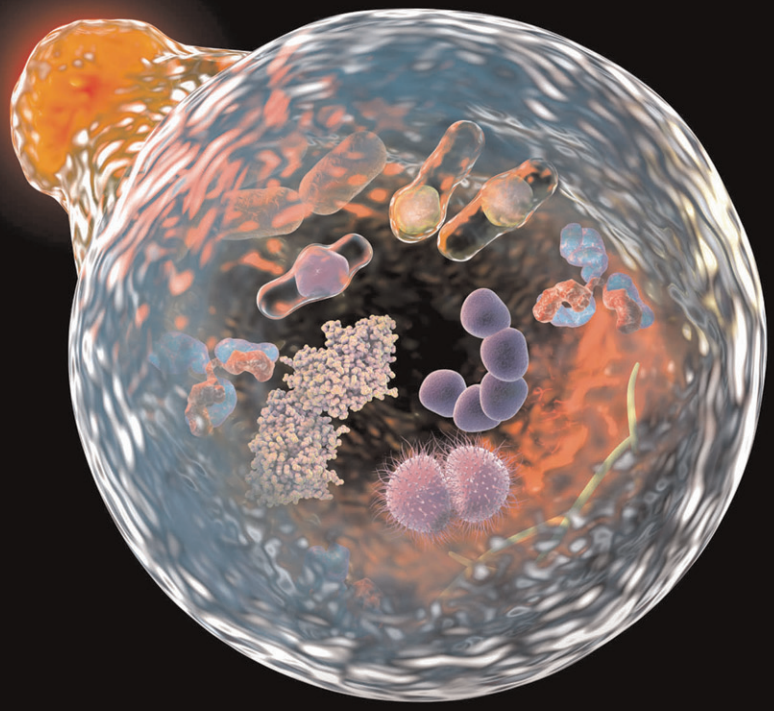

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DEDICATION

Professor Luciano Martini, 1927–2017

The other Editor in Chief of the Encyclopedia, Professor Luciano Martini, passed away on July 13th, 2017. He was an internationally acclaimed authority in the field of endocrinology, in particular neuroendocrinology, a brilliant and imaginative scientist, and an impressive and erudite scholar.

Luciano achieved the venerable age of 90, and his long career was full of outstanding scientific achievements, leadership positions in academia and in scientific societies, academies, and committees.

Luciano received his MD degree from the University of Milan in 1950. He then rapidly progressed through junior academic ranks up to the position of Professor and Chairman of the Department of Pharmacology at the University of Perugia in 1968, and subsequently, in 1972, he returned to his *alma mater*, the University of Milan, as full Professor and Chairman of the Department of Endocrinology, a post he held until 2001. He served in Milan as director of the training and research program entitled Physiology of Reproduction for nearly 20 years and attracted to his team top-class Italian and foreign scientists to address his main research interests of neuroendocrine regulation of reproductive functions.

Scientific severity, ethical integrity, fine perception, and deep farsightedness describe best Luciano's character as a scientist. He created in his institute a scientific research group devoted to experimental endocrinology, which grew over the years in size and visibility and became widely recognized internationally. Luciano published more than 400 peer-reviewed and highly cited papers mainly in the fields of neuroendocrinology, endocrine oncology, physiology of reproduction, and steroid and energy metabolisms.

Luciano was a prolific editor of scientific books and journals, which include the two volumes of *Neuroendocrinology* and the nine biennial volumes of *Frontiers in Neuroendocrinology*. He was Editor in Chief of *Comprehensive Endocrinology* published in 12 volumes and the first Edition of *Encyclopedia of Endocrine Diseases*. He served as President in many national and international scientific societies including the International Society of Neuroendocrinology, the Italian Society of Endocrinology, the International Society of Endocrinology, and the European Federation of Endocrine Societies. For his scientific achievements Luciano received honorary doctorates in the universities of Liège, Santiago de Compostela, Pécs, and Milan, and he was the recipient of numerous scientific awards and invited academy memberships.

Luciano's portrait could not be complete if one forgets to mention his life-time passion for music. He was a well-trained and accomplished pianist, a passionate music listener, and an enthusiastic connoisseur of all types of music. He also was an amateur in visual arts and deeply interested in history.

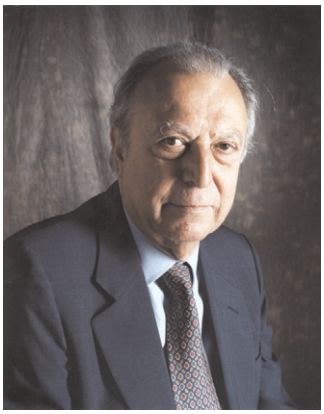
All of us who knew Professor Luciano Martini deeply mourn the loss of a great scientist and friend, the real "Il Maestro", teacher, colleague, and pioneer of modern neuroendocrinology. I trust Luciano would have been proud of this new edition of the Encyclopedia of Endocrine Diseases, and all of us having worked on its production would like to dedicate it to his memory.

Ilpo Huhtaniemi

Editor in Chief
Encyclopedia of Endocrine Diseases, 2nd edition

EDITORS IN CHIEF

Ilpo Huhtaniemi received his MD and PhD at University of Helsinki, Finland, did postdoctoral training in United States (UC San Francisco and NIH, Bethesda), and has been on sabbatical leave in Germany, United States and Scotland. In 1986–2002 he held the post of Professor and Chairman of Physiology at University of Turku, Finland. He moved in 2002 to UK to a Chair in Reproductive Endocrinology at Imperial College London, from which position he retired in 2015. He has received several national and international honors, amongst them a fellowship of The Academy of Medical Sciences, United Kingdom, and a Doctor Honoris Causa at the Medical University Łódź, Poland, and University of Szeged, Hungary. He was the Chief Managing Editor of *Molecular and Cellular Endocrinology* 1999-2017, has served in the Editorial Board of *Endocrinology and Endocrine Reviews* and is/has been the Editor or Editorial Board Member of several other scientific journals (e.g., *European Journal of Endocrinology*, *Clinical Endocrinology*, *Human Reproduction Update*, *Journal of Endocrinology*, *Molecular Human Reproduction*, *Reproduction*, *Asian Journal of Andrology*). He has extensive experience as Official of international scientific organizations (e.g., Past President of International Society of Andrology). His research interests include clinical and basic reproductive endocrinology, in particular the function of gonadotrophins and male reproductive endocrinology. He also has long-term interests in development of male contraception, hormone-dependent cancer, and the endocrinology of aging. He has authored about 700 peer-reviewed research articles and reviews, and his H-factor is 78.



Luciano Martini was born on May 14, 1927, in Milan, Italy. He obtained the degree of Medical Doctor "summa cum laude" on November 24, 1950, from the Faculty of Medicine of the University of Milan, Italy. He was Emeritus Professor of Pharmacology of the University of Perugia, Italy, and Emeritus Professor of Endocrinology of the University of Milan, Italy. He was Doctor Honoris Causa in Medicine of the Universities of Liège, Belgium, Santiago de Compostela, Spain, and Pécs, Hungary, and Doctor Honoris Causa in Biotechnological Sciences of the University of Milan, Italy. He was an author of more than 400 peer-reviewed scientific publications in the fields of endocrinology, neuroendocrinology, pharmacology, physiology of reproduction, steroid biochemistry, and basic oncology. He was elected member of the Accademia Nazionale dei Lincei (Italian National Academy) and of the American Academy of Arts and Sciences (Honorary Foreign Member).

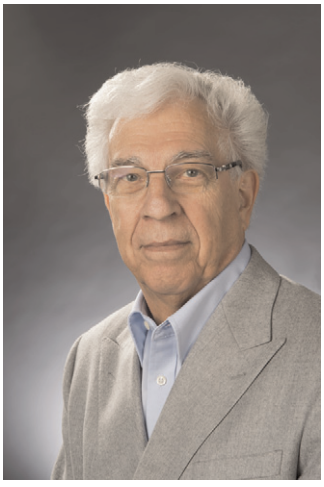
Luciano Martini acted as Editor in Chief of the journal *Frontiers in Neuroendocrinology* from 1990 to 2001, and was a Member of the Editorial Board of *Endocrinology* (Foreign Consulting Editor, 1961–65), as well as of several other speciality journals, such as *Experimental and Clinical Endocrinology*, *Biochemistry*, and *Steroids*. He has acted as Editor of several textbooks (e.g., *Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1966–67, and *Clinical Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1977–82) as well of a series of books under the name *Comprehensive Endocrinology* (13 volumes), Raven Press, New York, 1979–84. He acted as Editor in Chief for the first edition of *Encyclopedia of Endocrine Diseases* (4 volumes), Academic Press-Elsevier, San Diego, 2004.

EDITORIAL BOARD

SECTION EDITORS

Professor **Jean-Jacques Body** has been trained as an endocrinologist and a medical oncologist. He was Head of the Department of Medicine at University Hospital Brugmann in Brussels and Full Professor of Medicine (Internal Medicine) at the Free University of Brussels, (ULB), Brussels, Belgium. He was previously Head of the Internal Medicine Clinic at Institute J. Bordet (Cancer Center of ULB). He has also developed the "Supportive Care Dept" at the same Institute. His particular research interests are osteoporosis and bone metastases. He has a long-standing interest for bone metabolism and turnover in osteoporosis and tumor bone diseases. He has authored or co-authored more than 250 international peer-reviewed papers and he counts more than 200 invited lectures for international meetings.

Felipe F. Casanueva is Professor of Medicine at University of Santiago de Compostela and Head of Department of Endocrinology and Nutrition at University Hospital Santiago. He has been President of the scientific societes, such as: European Federation of Endocrine Societies (EFES), The Pituitary Society, International Society of Endocrinology (ISE) and, Sociedad Española para el Estudio de la Obesidad (SEEDO). Has written more than 50 chapters in international books and published more than 700 papers in international journals. He has received several awards for research at national and international level, such as: Rey Jaime I to the Medical Research, Geoffrey Harris Prize in Neuroendocrinology, Fundación Lilly of Bio-medical Research Clinic, Fundacion Danone – Professional Career – Dr Carlos Martí Hennberg, European Hormone Medal by the European Society for Endocrinology (ESE); he has been named Honorary Doctorate in Medicine of the University of Łódź, Erciyes, and Belgrade, and Honorary Member of the European Society of Endocrinology.



Dr. **Jean-Louis Chiasson** is currently Full Professor of Medicine at the University of Montreal. He is Head of the Research Group on Diabetes and Metabolic Regulation at the Research Center of the Centre hospitalier de l'Université de Montréal (CRCHUM).

Dr. Chiasson obtained his MD at Laval University in Quebec City in 1967. He did his specialty training in Internal Medicine at Laval Univesity and in Endocrinology at McGill University. He then did a research Fellowship in Diabetes at Vanderbilt University in Nashville, Tennessee. In 1974–76 and 1978–80, he was appointed Assistant Professor in the Department of Medicine and Physiology respectively at Vanderbilt University. In 1980, he returned to Montreal as Assistant Professor in the Department of Medicine at the University of Montreal and as Endocrinologist at Hotel-Dieu Hospital, now merged into the Centre hospitalier de l'Université de Montréal.

Dr. Chiasson's research interests include the regulation of carbohydrate metabolism in health and diabetes, as well as the development and evaluation of new strategies for the treatment and prevention of diabetes and its vascular complications. He has contributed over 250 scientific publications and lectures nationally and internationally on various topics on diabetes mellitus, its pathogenesis, its treatment, and its prevention. His scientific contribution puts him in the prestigious club of the 100 most cited publications in the world in the field of diabetes.

Sophie Christin-Maitre received her MD at University of Paris XI and her PhD at University Paris VI, Pierre and Marie Curie, France. She did a postdoctoral training in United States (Massachusetts General Hospital, Harvard University, Boston); she specialized in reproductive medicine. She holds the post of Professor of Endocrinology at University of Sorbonne, Paris, France. She has been the head of the Adult Endocrine Unit, in Hôpital Saint-Antoine, Assistance-Publique Hôpitaux de Paris, since 2011. She is a member of the INSERM research unit UMR S_933, specialized in identifying new genes in reproductive disorders. Her interests include clinical and basic reproductive endocrinology, in particular the management of patients with Turner syndrome, patients with primary ovarian insufficiency, patients with hypogonadisms, and patients with abnormalities of sex development. She has authored approximately 150 peer-reviewed research articles and reviews.

Ulla Feldt-Rasmussen is Professor at Copenhagen University and Chief of Medical Endocrinology, National University Hospital. Her research interests involve the thyroid gland and autoimmunity, as well as pituitary and adrenal dysfunction.

She has published more than 410 papers in peer-reviewed journals on e.g., thyroid hormones and body composition, thyroid autoimmunity and cancer, cytokines as regulators of endocrine cells, influence of thyroid disrupting chemicals on thyroid cells, growth hormone deficiency related to body composition, bone metabolism and other pituitary axes, and transition from adolescent to adult care, as well as several aspects of Fabry disease. In recent years her group has embarked on studies on pituitary function after traumatic brain injury in a nationwide setting, and focusing on diagnostic accuracy of pituitary testing procedures. She has further authored numerous proceedings, textbook chapters, and other publications; as well as organized numerous international meetings and postgraduate courses, and has led several European projects and other collaborations within many areas of endocrinology.

Professor Feldt-Rasmussen reviews for international journals, and is an editorial board member of several endocrine journals. She belongs to many international professional organizations, including the Endocrine Society, ETA, ATA, ENEA, and GRS; she has served as Secretary-Treasurer of ETA and as President of the ETA Cancer Research Network.

Professor Feldt-Rasmussen serves on the advisory boards of several ad hoc endocrine committees, and has received many prestigious prizes including the Mayo Clinic's Haynes Lecturer's Award and ETA's Pinchera Research Prize.

Wouter W. de Herder M.D. Ph.D. (1960) is Professor of Endocrine Oncology at the Erasmus MC in Rotterdam, the Netherlands. In this University Hospital he is chairman of a multidisciplinary group for endocrine oncology (tumorwerkgroep endocriene tumoren) and he is head of the ENETS centre of excellence for neuroendocrine tumors. His major research interests are neuroendocrine and endocrine tumors.

Professor de Herder received his M.D. in 1985 and his Ph.D. in 1990 from the Erasmus University in Rotterdam, the Netherlands.

He is a member of several international and Dutch national societies, such as the Dutch Society for Endocrinology (NVE), the Endocrine Society (USA), the European Society of Endocrinology (ESE), European Neuroendocrine Tumor Society (ENETS) and the North American Neuroendocrine Tumor Society (NANETS). He served as a board member of the Dutch Society for Endocrinology (NVE) (2009–14). He served as chairman (2006–08) and vice-chairman of ENETS (2008–10) (European Neuroendocrine Tumour Society). He is member of the advisory boards of ENETS and NANETS.

Professor de Herder has (co-)published over 400 peer-reviewed papers and book chapters and is a reviewer for many international journals.

He is a member of the editorial boards of *Neuroendocrinology; Endocrinology, Diabetes & Metabolism Case Reports; Clinical Endocrinology*, and *Endocrine-Related Cancer*.

Professor de Herder has given over 200 invited presentations at Dutch national and international meetings.

Ieuan Hughes is currently Emeritus Professor of Pediatrics at the University of Cambridge and Honorary Consultant Pediatrician at Cambridge University Hospitals NHS Foundation Trust and Cambridge Biomedical Campus. He is the author of more than 300 papers and chapters across the whole range of paediatric endocrinology. His particular expertise is in disorders of sex development for which he coordinated the International Consensus on the approach to the investigation and management of this broad topic. Research interests focus on steroid enzyme deficiencies and molecular mechanisms of androgen action.
Professor Hughes has served on the editorial boards of several journals, including *Clinical Endocrinology*, *Journal of Clinical Endocrinology*, and *Metabolism* and *Archives of Disease in Childhood* where he was also the Associate Editor. He is Past-Secretary and President of the European Society for Pediatric Endocrinology and a recipient of the highest award of the Society, the Andrea Prader Prize. Professor Hughes is a James Spence Medallist of the Royal College of Pediatrics and Child Health for outstanding contributions to paediatric knowledge. He is a Fellow of the Academy of Medical Sciences, a Council Member of the Learned Society of Wales and a Trustee of two charities. The chapter on Disorders of Sex Development in *Williams Textbook of Endocrinology* (now in its 14e) by Hughes and co-authors is considered to be a definitive and up to date regular review of this topic, specific and key to pediatric endocrinology.

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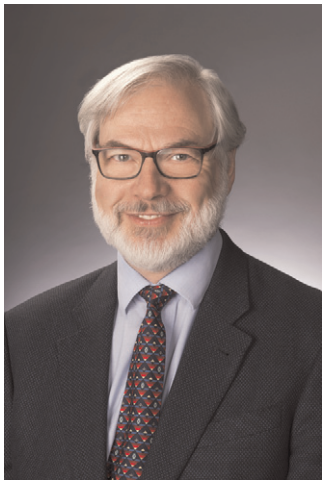

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Dr. Wang has been involved in many funded basic and clinical research studies. Her current clinical research studies include androgen replacement therapy, hormonal male contraceptive development, late onset hypogonadism, accurate assessment of serum androgens, and diet and androgen metabolism. Her basic research studies focus on the regulation of spermatogenesis and mitochondrial derived peptides in spermatogenesis.

She has authored over 300 peer-reviewed publications, 67 chapters and reviews mainly on male reproductive biology including characterization of the pharmacokinetics and efficacy of androgens in men, trials of hormonal male contraceptive, regulation of germ cell apoptosis, and reproductive aging. Dr. Wang served on the Executive Council, several committees and was the President of the American Society of Andrology (2006–07). She also served the International Society of Andrology as Secretary (2001–05) and Chair of the Program Organizing Committee (2005–09). She was President of the International Society of Andrology (2009–13). She is a member of the Research Group on Methods for the Regulation of Male Fertility of the World Health Organization since 1984 and Chairperson (1991–2002).

She has mentored many physician and scientist and is an advocate of young investigators. Dr. Wang has been invited speaker and distinguished lecturer at many national and international endocrinology, reproductive endocrinology, and andrology conferences.

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PREFACE

The first Edition of the *Encyclopedia of Endocrine Diseases* was published in 2004. Because of the enormous development in the field it was found important to produce a completely revised and updated Second Edition of the Encyclopedia. The new Edition is a must-have one-stop reference covering every aspect of the physiological background, pathogenesis, clinical diagnostics, and therapeutic aspects of the wide array of endocrine and related metabolic diseases.

The functional balance of the body (homeostasis) is maintained by two regulatory circuits, i.e., the nervous and the endocrine systems. Among the most complex constructs in the body, the endocrine system comprises a group of glands that secrete hormones directly into the bloodstream, where they reach their specific receptors in other parts of the body, evoking specific intracellular signaling pathways leading to their biological effect. Many classically non-endocrine organs (e.g., the heart) have also turned out to have endocrine functions. The endocrine system maintains and regulates the body's homeostasis by using hormones to control metabolism, temperature, biological cycles, internal fluid volume, reproduction, growth, body composition, and development. The system is a marvel when functioning optimally, i.e., maintaining the body homeostasis. Unfortunately, there is a myriad of ways these processes, actions, and functions can go awry, resulting in various endocrine and metabolic diseases, which form the over-arching theme of the Encyclopedia.

The Encyclopedia is not meant as a primer on the subject of endocrinology, but instead intended to provide a comprehensive reference work on the extensive spectrum of diseases and disorders that can occur within the endocrine and metabolic system. The updated version of this groundbreaking encyclopedia is especially timely, as it covers the dramatic discoveries in the field of endocrinology and metabolism over the past 10 years, particularly with respect to novel diagnostic techniques and treatment approaches. In particular, there have been tremendous advancements in our understanding of the molecular basis of endocrine and metabolic diseases (mutations, epigenetics, signaling), as well as pathogenesis and therapy of the common forms of these diseases (e.g., diabetes, obesity, and endocrine malignancies).

The Encyclopedia offers a unique source of up-to-date information for the physicians and basic scientists working in the field. It is an essential resource for every clinician diagnosing and treating endocrine patients. The Encyclopedia also offers the prime source of information for students of medicine and science around the world, as well as basic research workers in academia, the pharma industry, and in other areas in need of information on endocrinology and metabolism. It also offers useful information for the lay public about normal and abnormal functions of hormones.

The Encyclopedia is intended to serve as a useful and comprehensive source of information spanning the many and varied aspects of the endocrine and metabolic system. The chapters have been written to be accessible to both clinical and nonclinical readers. The articles have been formatted in similar fashion and each is intended as a stand-alone presentation. Each article begins with a glossary list defining key terms that may be unfamiliar to the reader and are important for understanding the article. The body of the article begins with a brief introduction to the subject under discussion, bold headings lead the reader through the text, and figures and tables explain and illuminate most articles. The main text is followed by referenced citations to provide the reader with access to additional information on the topic, and cross-references lead the reader to related entries in the encyclopedia. The relatively short stand-alone articles have allowed us to recruit the best experts available for each topic.

Unlike the first Edition, where the articles were arranged in alphabetical order, the 2nd Edition is arranged in organ-based thematic order, where each organ-based group of diseases is presented as cluster of articles in the first four volumes. The fifth volume is a stand-alone compilation of all articles on pediatric endocrinology. The thematic organization gives the reader a better general view of the coverage of articles on a specific endocrine organ or disease type.

The Second Edition of the Encyclopedia builds of the first edition. Nevertheless, to bring a major reference work with such a broad scope from initial conception to final publication involved a great deal of planning and organization, together with the efforts of innumerable individuals. The authors of the first edition were invited to update their earlier texts. If this was not possible, the Section Editors invited another expert in the topic either to update the previous text or to write a de novo text; the latter happened in most of these cases. Hence, the Second Edition contains to a large extent totally new information, or at least the fluency of all texts has been scrutinized. Furthermore, all manuscripts have undergone peer-review arranged by the Section Editors.

Assembling a large volume of articles with the purpose to cover all essential topics of endocrine diseases posed multiple challenges. Coverage was a significant problem: on one hand some redundancy of the topics was almost impossible to avoid in places while, on the other, there were inevitable gaps. Some of these arose from late cancellations; others from oversights on our part. We can only promise to fill these gaps in future editions. We also note that as can be expected for a large multi-author compilation the individual articles do differ in detail and approach. We considered it more important to allow our experts substantial latitude in deciding how to present their topics than to apply rigid guidelines.

Most of the editing work of the Encyclopedia has been carried out by a highly competent board of 16 Section Editors, each of them internationally renowned experts in their respective field within clinical endocrinology. First, the broadest possible list of topics was compiled, aiming at the best possible coverage. Throughout the editorial process, the Section Editors supervised their subject area of expertise, recommended and corresponded with fellow editors and article contributors, reviewed the manuscripts, and continuously helped to refine the final list of topics. This has made the task of the Editor in Chief easy, mainly entailing the supervision of smooth progress of the project.

The Section Editors and their fields deserve being listed here: *Jean-Jacques Body* (Belgium, bone endocrinology), *Felipe F. Casanueva* (Spain, metabolism and obesity), *Richard N. Clayton* (United Kingdom, pituitary gland), *Jean-Louis Chiasson* (Canada, diabetes), *Sophie Christin-Maitre* (France, female reproduction), *Wouter W. de Herder* (The Netherlands, neuroendocrinology), *Ulla Feldt-Rasmussen* (Denmark, thyroid gland), *Ieuan Hughes* (United Kingdom, pediatric endocrinology), *Gregory Kaltsas*, Greece, and *Martin O. Weickert*, United Kingdom, (gastrointestinal hormones), *Jean-Marc Kaufman* (Belgium, endocrinology of aging), *André Lacroix* (Canada, adrenal cortex), *Franco Mantero* (Italy, adrenal medulla and endocrine hypertension), *Jorma Toppari* (Finland, endocrine disruptors), *Jacquetta Trasler* (Canada, endocrine epigenetics) and *Christina Wang* (United Kingdom, male reproduction).

The Elsevier editorial staff, *Will Smaldon*, *Laura Escalante Santos*, and *Kate Miklaszewska-Gorczyca*, have been of enormous help to the editors at every step during this long project. I admire the professionalism of everyone and am deeply indebted to all for their dedication and hard work to make the Encyclopedia the leading reference book of clinical endocrinology.

The authors of the individual chapters, more than 450 in total, were specifically selected by the Section Editors to represent the best available knowledge on the topic available. They all should be thanked for their dedication and the excellent quality of their contributions.

Ilpo T. Huhtaniemi
Editor in Chief

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Corticotropin-Releasing Hormone (CRH)

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Nomenclature

ACTH	Adrenocorticotropic hormone
AVP	Arginine vasopressin
CBP	CREB-binding protein
CRH	Corticotropin-releasing hormone
CRH-R	Corticotropin-releasing hormone receptor
DHEA	Dehydroepiandrosterone
HDAC1	Histone deacetylase-1
HPA axis	Hypothalamic–pituitary–adrenal axis
IL-6	Interleukin-6
MSH	Melanocyte-stimulating hormone
NF-κB	Nuclear factor kappa b
PACAP	Pituitary adenylate cyclase activating polypeptide
PVN	Paraventricular nucleus
POMC	Proopiomelanocortin
sAC	Soluble adenylyl cyclase

Glossary

Hypothalamic–pituitary–adrenal axis A set of stimulating and inhibitory signals between the hypothalamus, the anterior lobe of pituitary gland, and the adrenal cortex.

Receptor Protein molecules on the cell surface or inside the cell that recognizes a specific ligand or hormone.

Introduction

A hypothalamic factor that increases adrenocorticotropic hormone (ACTH) release from pituitary cells was discovered as an active principle in 1955 (Guillemin and Rosenberg, 1955; Saffran and Schally, 1955). In 1981, ovine corticotropin-releasing hormone (CRH) was isolated and sequenced (Vale *et al.*, 1981). Subsequently, a small peptide was isolated from rat hypothalami and was found to have 83% sequence homology to ovine CRH. Finally, 2 years later, the human CRH was identified with identical sequence to that of rat CRH (Rivier *et al.*, 1983; Vale *et al.*, 1983a,b).

CRH is a 41-amino acid peptide that is the major physiologic ACTH secretagogue (Rivier *et al.*, 1982; Vale *et al.*, 1983a,b). There is considerable sequence homology of CRH among species, particularly in the amino-terminal region, which is required for biologic activity. CRH belongs to a family of peptides that includes the nonmammalian sauvagine and urotensin I, the mammalian urocortins 1, 2 (stresscopin-related peptide, SRP) and 3 (stresscopin, SCP), the last three of which also are found in humans (**Fig. 1**) (Spiess *et al.*, 1981; Lederis, 1987; Donaldson *et al.*, 1996). All of these peptides have activities similar to but also distinct from those of CRH (Lederis, 1987).

CRH is processed from a prepro-CRH molecule (196 amino acids in humans) from which it is cleaved at flanking basic amino acid pairs (Shibahara *et al.*, 1983). The single human CRH gene is located on chromosome 8 (Arbiser *et al.*, 1988), those of human urocortins 1, 2, and 3 on chromosomes 2, 3 and 10, respectively (Donaldson *et al.*, 1996).

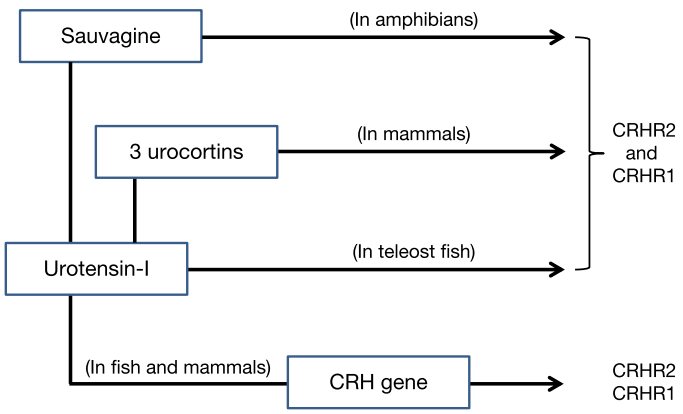

Fig. 1 CRH family of neuropeptides and receptors. *CRHR*, corticotropin-releasing hormone receptor.

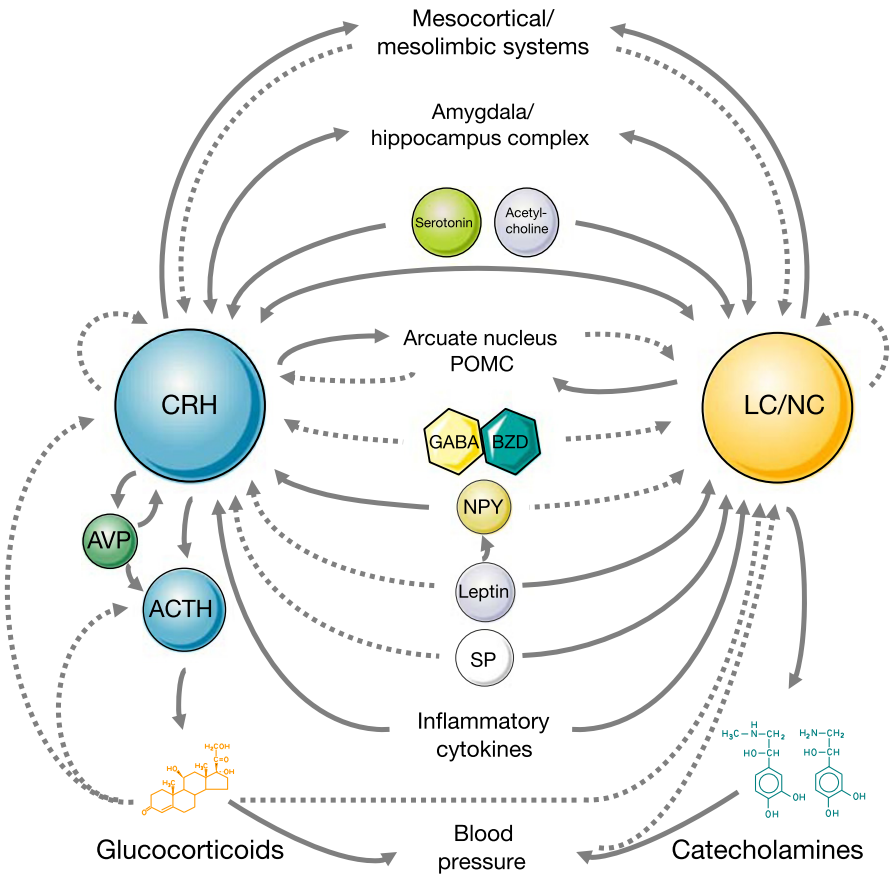

Fig. 2 Central and peripheral components of the stress system. CRH is influenced by several factors in a positive or negative fashion. Activation is represented by *solid lines* and inhibition by *dashed lines*. *ACTH*, adrenocorticotropic hormone; *AVP*, arginine vasopressin; *BZD*, benzodiazepine; *CRH*, corticotropin-releasing hormone; *GABA*, γ-aminobutyric acid; *LC/NE Symp Syst*, locus caeruleus-norepinephrine/sympathetic nervous system; *NPY*, neuropeptide Y; *POMC*, proopiomelanocortin; *SP*, substance P. Adapted from reference Chrousos, G.P. and Gold, P.W. (1992). The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis. *Journal of American Medical Association* **267**, 1244–1252 with permission.

Synthesis, Regulation of Secretion, and Distribution of CRH

CRH is synthesized by neurons in the parvocellular (small cell) division of the hypothalamic paraventricular nuclei (PVN) (Swanson *et al.*, 1983). Their axons project to the median eminence, where CRH is secreted into the hypophysial portal blood. These neurons may also contain other ACTH secretagogues (e.g., arginine vasopressin (Kiss *et al.*, 1984) and cholecystokinin (Mezey *et al.*, 1986)), opioid peptides (e.g., met-enkephalin (Hökfelt *et al.*, 1983) and dynorphin-1-8 (Roth *et al.*, 1983)), and PHI-27, a prolactin secretagogue (Hökfelt *et al.*, 1983), particularly after adrenalectomy. In addition, CRH is synthesized by anterior pituitary corticotrophs and acts in an autocrine or paracrine manner to stimulate ACTH secretion (Giraldi and Cavagnini, 1998).

Hypothalamic CRH secretion is regulated by inputs from higher centers and is mainly influenced by the circadian pacemaker, stress and glucocorticoid negative feedback (**Fig. 2**). Glucocorticoid negative feedback inhibition occurs at both the pituitary and hypothalamic levels, and perhaps at higher centers, such as the hippocampus. Interestingly, at the level of the central nucleus of the amygdala, glucocorticoids stimulate rather than inhibit CRH secretion (Makino *et al.*, 1994). Although, in the anterior pituitary, glucocorticoids inhibit both ACTH secretion and POMC gene transcription (Lundblad and Roberts, 1988), these hormones decrease—to a lesser extent—CRH mRNA and peptide levels in the hypothalamic paraventricular nuclei (Davis *et al.*, 1986; Itoi *et al.*, 1987; Beyer *et al.*, 1988; Kageyama and Suda, 2009). Glucocorticoids also block the stimulatory effect of CRH on POMC gene transcription (Eberwine *et al.*, 1987) and acute ACTH release (Oki *et al.*, 1991), and inhibit anterior pituitary CRH receptor expression (Holmes *et al.*, 1987; Luo *et al.*, 1995). *CRH* gene expression is also influenced by several other factors. Pituitary adenylate cyclase activating polypeptide (PACAP) was demonstrated to increase CRH mRNA levels in the parvocellular region of the hypothalamic PVN. Moreover, interleukin (IL)-6, produced in the PVN, influences *CRH* gene expression in a direct and indirect fashion. Indeed, IL-6 stimulates directly the expression of CRH, while it is also involved in the PACAP-induced *CRH* gene transcription in an autocrine fashion (Nezi *et al.*, 2000–2015). Moreover, estradiol was found to increase the expression of CRH in response to stress (Kageyama and Suda, 2009). Furthermore, leptin, which inhibits adrenal glucocorticoid biosynthesis, may also

inhibit CRH secretion (Cherradi *et al.*, 2001). Since glucocorticoids stimulate leptin secretion, this may represent another feedback loop regulating hypothalamic–pituitary–adrenal function.

CRH is also present in some oxytocin-containing neurons in the magnocellular (large cell) division of the paraventricular nuclei that project to the posterior pituitary (Sawchenko *et al.*, 1984). In addition, CRH is widely distributed throughout the brain and spinal cord (Swanson *et al.*, 1983; Olschowka *et al.*, 1982) and in peripheral tissues, such as the adrenal medulla (Nicholson *et al.*, 1987), testis (Yoon *et al.*, 1988), ovaries (Mastorakos *et al.*, 1993), gastrointestinal tract (Nieuwenhuyzen Kruseman *et al.*, 1984; Suda *et al.*, 1984), pancreas (Petrusz *et al.*, 1983), skin (Slominski *et al.*, 2001), myometrium (Clifton *et al.*, 1998), endometrium (Di Blasio *et al.*, 1997), placenta (Grino *et al.*, 1987) and in inflammatory sites (Chrousos, 1995; Mastorakos *et al.*, 1994, 1995).

The presence of CRH and its two receptor types CRHR1 and CRHR2 (De Souza, 1987; Chen *et al.*, 1993; Potter *et al.*, 1994; Stenzel *et al.*, 1995) in the brain suggests that CRH functions as a neuromodulator. CRH also may have local autocrine or paracrine regulatory actions in other tissues. As an example, CRH inhibits chorionic gonadotropin-stimulated androgen production by the testis (Ulisse *et al.*, 1989) and facilitates embryo implantation in the endometrium. Furthermore, CRH may act as a hormone in the hypophysial portal system and as a systemic hormone in the pregnant woman (Makrigiannakis *et al.*, 2001).

Mechanism of Action

The pleiotropic biological effects of CRH are mediated by binding to both type 1 (a, b, c, d, e, f, g, h) and type 2-alpha, -beta, and -gamma CRH receptors and CRH-binding protein (Stenzel *et al.*, 1995; Lovenberg *et al.*, 1995; Potter *et al.*, 1992; Woods *et al.*, 1996; Kostich *et al.*, 1998). CRH-R2 are splice isoforms that differ in their amino-terminal sequences, tissue distribution, pharmacology (i.e., their relative affinities for CRH, sauvagine, urotensin, and the urocortins), and, presumably, their function (Hauger *et al.*, 2006). CRH-R2-beta and gamma are most abundantly expressed in the limbic system, for example, which suggests they may mediate the behavioral effects of CRH and the urocortins (Kostich *et al.*, 1998). CRH-R1 and CRH-R2 are members of the class B family of G-protein coupled receptors (Perrin and Vale, 1999). The *CRH-R1* gene is located on human chromosome 17q21, while the *CRH-R2* gene on human chromosome 7p14 (Vamvakopoulos and Sioutopoulou, 1994; Nezi *et al.*, 2000–2015).

CRH acts on the anterior pituitary corticotrophs by binding to CRH-R1 cell-surface receptors (Chen *et al.*, 1993; Millan *et al.*, 1987) and activating adenylyl cyclase, thereby increasing intracellular cAMP concentrations. Cyclic AMP activates cAMP-dependent protein kinase A, increasing the influx of extracellular calcium via L-type calcium channels and the production of lipoxygenase metabolites of arachidonic acid (Giguère *et al.*, 1982; Won and Orth, 1990, 1994; Oki *et al.*, 1991). This results in secretion of ACTH and other proopiomelanocortin (POMC)-derived peptides (Watanabe and Orth, 1987) and later in increased POMC gene transcription and POMC biosynthesis (Lundblad and Roberts, 1988). Although CRH-R1 activation results in increased levels of cAMP, the activated downstream effector molecules and the time course of the molecular response vary depending on the cell type. Interestingly, in hippocampal neurons and fibroblast-derived cell lines, the cAMP response may be sustained, whereas this signaling response may be transient in corticotroph cells (Inda *et al.*, 2017).

Research progress in CRH signaling has revealed an important role of β-arrestins in CRH-R internalization and termination of signaling. CRH-bound CRH-R1 was demonstrated to bind to both β-arrestin-1 and β-arrestin-2 (Oakley *et al.*, 2007), whereas activated CRH-R2 was shown to preferentially recruit β-arrestin-2 (Markovic *et al.*, 2008). In addition, accumulating evidence suggests that CRH-R1 continues to signal once internalized in endosomes, possibly through the newly identified soluble adenylyl cyclase (sAC) and/or the intracellular β-arrestin (reviewed in Inda *et al.*, 2017).

Chronic CRH stimulation causes corticotroph hyperplasia (Gertz *et al.*, 1987). Corticotroph CRH receptor number may modulate the ACTH response. CRH receptor messenger RNA and number are transiently reduced in the anterior pituitary, but not in the brain, by the administration of CRH and arginine vasopressin (AVP), whose effects are additive, by immobilization stress, and by adrenalectomy (Wynn *et al.*, 1985, 1988; Hauger *et al.*, 1987, 1988; Holmes *et al.*, 1987; Hauger and Aguilera, 1993; Pozzoli *et al.*, 1996).

The effects of adrenalectomy appear to be mediated at least in part by increased hypothalamic AVP and CRH secretion and are reversed by replacement doses of glucocorticoids. However, high doses of glucocorticoids suppress pituitary CRH receptor messenger RNA and number. These complex effects on pituitary CRH receptor expression help modulate the plasma ACTH response to stress on the one hand or adrenal insufficiency on the other.

CRH also stimulates pituitary intermediate lobe melanotrophs to synthesize POMC mRNA (Loeffler *et al.*, 1985) and secrete alpha-melanocyte-stimulating hormone (MSH) (Proulx-Ferland *et al.*, 1982).

CRH in Plasma

The contribution of hypothalamic CRH to peripheral plasma CRH concentrations is small; most of the plasma CRH presumably comes from nonhypothalamic sources (Orth, 1992; Sasaki *et al.*, 1987a,b; Bruhn *et al.*, 1987; Cunnah *et al.*, 1987). However, under certain circumstances, such as insulin-induced hypoglycemia or during major surgery, small increments in plasma CRH concentrations may reflect hypothalamic CRH release (Ellis *et al.*, 1990). Plasma CRH concentrations increase markedly during the third trimester in pregnant women because the placenta secretes CRH (Sasaki *et al.*, 1987a,b; Campbell *et al.*, 1987).

Binding Protein

CRH circulates in human plasma bound to a high-affinity binding protein (Orth and Mount, 1987; Potter *et al.*, 1991), which reduces its bioactivity and increases its clearance (Linton *et al.*, 1988). In women with complicated pregnancies, plasma CRH-binding protein concentrations may be reduced (Petraglia *et al.*, 1996) at the same time as plasma CRH concentrations are increased (Jeske *et al.*, 1990; Laatikainen *et al.*, 1991). Despite binding to this protein, the plasma half-life of CRH in humans is only 4 min (Schurmeyer *et al.*, 1984). A similar circulating binding protein has not been found in other species (Orth and Mount, 1987) except primates (Xu *et al.*, 2006).

Ovine CRH is used for most clinical studies because it does not bind to the binding protein (Orth and Mount, 1987), has a plasma half-life of 55 min and, consequently, a prolonged duration of action (Nicholson *et al.*, 1983; DeBold *et al.*, 1983). Thus, the specific binding of human CRH to CRH-binding protein may facilitate the removal of CRH from plasma (Woods *et al.*, 1994).

Effects on Pituitary Hormone Secretion

CRH increases the secretion of ACTH by the anterior lobe and of POMC-derived peptides by the intermediate lobe.

Anterior Lobe ACTH Secretion

Exogenously administered CRH produces a rapid, dose-dependent increase in plasma ACTH concentrations. This effect is specific; plasma concentrations of other anterior pituitary hormones, vasopressin, or catecholamines do not change (Schurmeyer *et al.*, 1984; Orth *et al.*, 1983). Human CRH is equipotent to ovine CRH, but because of its shorter circulating half-life, the duration of the elevation in plasma ACTH concentrations is shorter and the total amount of ACTH secreted is less (Schurmeyer *et al.*, 1984; Trainer *et al.*, 1995). However, the serum cortisol responses are similar because the plasma ACTH responses to both human and ovine CRH are maximally stimulatory for the adrenal cortex (Trainer *et al.*, 1995).

The ACTH released by CRH stimulates the secretion of cortisol and other adrenal steroids, such as dehydroepiandrosterone (DHEA) and, transiently, aldosterone (Conaglen *et al.*, 1984; Pavlov *et al.*, 1986). There is a very small if any sex or age difference in the plasma ACTH or serum cortisol response to CRH (Pavlov *et al.*, 1986; Sheldon Jr *et al.*, 1985; Ross *et al.*, 1986), but the serum DHEA response is reduced in elderly men and the serum cortisol response is blunted in obese subjects (Pavlov *et al.*, 1986; Kopelman *et al.*, 1988). As compared with white subjects, black subjects have greater plasma immunoreactive but not bioactive ACTH responses after CRH; as a result, the serum cortisol responses are similar in the two groups (Yanovski *et al.*, 1996).

The hour of day has little effect on the plasma ACTH response to CRH, but the response of serum cortisol is much greater in late afternoon or evening than in the morning (DeCherney *et al.*, 1985). In healthy persons a major factor influencing the magnitude of the plasma ACTH response to CRH is the serum cortisol concentration. The response to CRH varies inversely with the basal serum cortisol concentration (DeCherney *et al.*, 1985) due to glucocorticoid inhibition of CRH action on the corticotrophs (Vale *et al.*, 1983a, b). Thus, the ACTH response is increased during metyrapone-induced hypocortisolemia and reduced by glucocorticoid administration (DeBold *et al.*, 1989).

CRH-deficient knockout mice lack a normal diurnal serum corticosterone rhythm and have severely impaired adrenocortical response to stress (Muglia *et al.*, 2000). Pituitary POMC mRNA and ACTH levels and plasma ACTH concentrations are not increased. Pituitary POMC mRNA levels increase in adrenalectomized CRH-deficient mice, but plasma ACTH concentrations do not, indicating that CRH is required to stimulate ACTH release.

Intermediate Lobe POMC Peptide Secretion

A functional intermediate lobe exists only during fetal life and in late pregnancy in humans. Regulation of secretion of alpha-MSH, beta-endorphin (beta-END), and other POMC-derived peptides from the intermediate lobe differs from that in the anterior lobe. The secretion of these peptides is mainly under tonic inhibitory control by the hypothalamic neurotransmitter dopamine, and is not inhibited by glucocorticoids (Munemura *et al.*, 1980). Intermediate lobe hormone secretion is also inhibited by gamma-aminobutyric acid (GABA), another hypothalamic neurotransmitter (Tomiko *et al.*, 1983).

CRH and beta-adrenergic agonists stimulate alpha-MSH secretion through a cAMP-mediated mechanism, which is inhibited by dopamine. Dopamine also enhances the expression of intermediate lobe CRH receptors (Shiver *et al.*, 1992).

Extrapituitary Effects

CRH has several peripheral and central actions that may be mediated by binding to CRH-R1, CRH-R2 and CRH-binding protein (Stenzel *et al.*, 1995; Lovenberg *et al.*, 1995; Potter *et al.*, 1992; Woods *et al.*, 1996; Kostich *et al.*, 1998). Below, we discuss some representative examples of extrapituitary CRH effects:

- Increased activity of the hypothalamic–pituitary–adrenal axis may play a role in insomnia. Twenty-four-hour ACTH and cortisol secretion was increased in six young men and five young women with chronic insomnia (Vgontzas *et al.*, 2001a). The

greatest increase was during the evening and first half of the night. In 12 middle-aged men, intravenous CRH given after sleep onset caused more wakefulness and allowed less slow wave sleep than in young men (Vgontzas *et al.*, 2001b).

- Systemic administration of CRH causes mesenteric vasodilatation, which at high doses can result in hypotension and tachycardia (Orth *et al.*, 1983; Hermus *et al.*, 1987), and stimulates respiration through a central effect (Oppermann *et al.*, 1987).
- Intracerebroventricular injection of CRH activates the autonomic nervous system (Brown *et al.*, 1982), resulting in increased serum catecholamine, glucagon, and glucose concentrations, elevated blood pressure and heart rate, decreased gastric acid secretion (Lenz *et al.*, 1985) and behavioral changes in animals (Sutton *et al.*, 1982).
- CRH may modulate reproductive function, since it reduces sexual receptivity in female rats (Sirinathsinghji *et al.*, 1983), lowers serum luteinizing hormone concentrations by inhibiting the secretion of gonadotropin-releasing hormone (Petraglia *et al.*, 1987) and inhibits chorionic gonadotropin-stimulated testosterone production by cultured Leydig cells (Dufau *et al.*, 1989). In addition, CRH was demonstrated to promote blastocyst implantation and maintain early pregnancy by potentiating apoptosis of activated T-cells (Makrigiannakis *et al.*, 2001). A recent study examined human placental cytotrophoblasts and showed that glucocorticoids induces the expression of both CRH and COX-2, two key regulators of human parturition, through the noncanonical NF-κB pathway. The GR/RelB/p52 signaling crosstalk results in a dynamic histone 3 Lys9 acetylation and deacetylation (H3K9ac) of the *CRH* and *COX-2* gene promoters through recruitment of the histone acetyltransferase CREB-binding protein (CBP) and histone deacetylase-1 (HDAC1) (Di Stefano *et al.*, 2015; Zannas and Chrousos, 2015).
- CRH exerts multiple actions in gestation via binding to CRH type 1 receptors. It directly stimulates CRH-1 receptor synthesis and DHEA-S secretion by human fetal adrenocortical cells (Smith *et al.*, 1998; Sirianni *et al.*, 2005). It regulates placental blood flow and prostaglandin synthesis (Challis *et al.*, 1995), increases in myometrium during labor and potentiates oxytocin- and prostaglandin F2-alpha-induced myometrial contractility (Clifton *et al.*, 1998), and facilitates parturition (Challis *et al.*, 1995; Chang *et al.*, 1998).
- CRH and/or urocortin are secreted by a number of other peripheral tissues including spleen, thymus, synovial epithelial cells, inflammatory cells, and peripheral nerve endings and modulate the immune response via CRH type 1 and 2 receptors (Baigent, 2001; Jessop *et al.*, 2001; Radulovic and Spiess, 2001; McEvoy *et al.*, 2002). Urocortin, but not CRH, is present in gastric epithelial cells (Chatzaki *et al.*, 2003). Among their actions, CRH and urocortin modulate secretion of cytokines and proliferation of lymphocytes and macrophages and degranulation of mast cells (Radulovic and Spiess, 2001; Kempuraj *et al.*, 2004). CRH and urocortin also have potent cardiovascular actions (Parkes *et al.*, 2001).
- CRH and CRH-1 receptor are synthesized by human dermal fibroblasts and hair follicles; CRH and ACTH also stimulate cortisol production in the hair follicle and corticosterone production in dermal fibroblasts (Slominski *et al.*, 2005). CRH enhances lipid secretion from human sebocytes (Zouboulis *et al.*, 2002).
- CRH plays an important role in the initiation and and/or propagation of the local inflammatory response (Karalis *et al.*, 1991). In rats that were subcutaneously injected with carrageenan causing local aseptic inflammation, the intraperitoneal administration of rabbit antiserum to CRH resulted in suppression of the inflammatory process. CRH was locally found in the inflammatory site upon treatment with carrageenan but not in systemic circulation, suggesting an autocrine or paracrine, but not endocrine role in inflammation (Karalis *et al.*, 1991).

CRH in the Epigenetic Era

Several studies in animal models and humans have convincingly demonstrated that early environmental adverse experiences might have an important role in reprogramming the hypothalamic–pituitary–adrenal (HPA) axis activity via epigenetic modifications of genes encoding molecules participating in the stress response. More than three decades ago, the pioneer studies of M. Meaney and his colleagues showed that high maternal licking/grooming (LG) resulted in increased expression of glucocorticoid receptor in the hippocampus, leading to a less potent stress response via a higher negative feedback inhibition of hypothalamic CRH and pituitary ACTH (Meaney *et al.*, 1985). On the contrary, low maternal LG caused higher hippocampal cytosine methylation of the 1_7 promoter of the *NR3C1* gene, thereby reducing hippocampal expression of the glucocorticoid receptor and altering the adaptive stress response (Weaver *et al.*, 2004).

In addition to glucocorticoid receptor, recent studies revealed epigenetic alterations in the *CRH* gene associated with stress-related conditions, including hypersexual disorder and suicide attempts (Jokinen *et al.*, 2017, 2018). A genetic region 48 bp upstream of the transcription start site of the *CRH* gene was significantly hypomethylated in hypersexual male patients compared to healthy male volunteers (Jokinen *et al.*, 2017). Moreover, two CRH-associated CpG-containing sites were hypomethylated in a high-risk group of suicide attempters compared to a low-risk group (Jokinen *et al.*, 2018). Future studies will shed light on the role of epigenetic alterations of the *CRH* gene in human pathophysiology.

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Neurotensin☆

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Glossary

Ileum The distal three-fifths of the small intestine, where bile salts and many nutrients are absorbed.

messenger RNA (mRNA) An RNA molecule that specifies the amino acid sequence of a protein.

Peptide Any member of a class of compounds of low molecular weight that yield two or more amino acids on hydrolysis and that form the constituent parts of proteins.

Chemistry, Isolation, and Distribution

During the isolation of substance P from bovine hypothalami, S. E. Leeman and colleagues in 1973 isolated a 13-amino acid peptide that was termed "neurotensin" after its vasodilatory and cyanotic effects. Neurotensin was subsequently shown to have a widespread distribution in the central nervous system, pituitary, and intestine. Furthermore, neurotensin and neurotensin-related peptides are represented across the phylogenetic tree (Table 1). The C terminus, which is the bioactive core, is conserved, whereas the N-terminal peptide, NT (1–8), may be an inactive degradation product generated following cleavage at the dibasic arg^8–arg^8 residues. The ileum contains the highest concentration of neurotensin, with lesser amounts found in the jejunum and duodenum. The neurotensin is present in specific endocrine cells, in N cells, and in nerves predominantly in the myenteric plexus. Overall, the intestine contains more than 90% of neurotensin. Most of the remaining NT is in the brain, particularly the hypothalamus and pituitary. Neurotensin is synthesized and stored in neurosecretory cells in a number of hypothalamic areas, including the arcuate nucleus and the parvocellular paraventricular nucleus. The median eminence has a dense plexus of neurotensin-containing nerves arising predominantly from these nuclei. Anterior pituitary neurotensin is present in gonadotropes and thyrotropes.

Gene and Precursor Structure

Neurotensin is synthesized within a 170-amino acid precursor that also encodes the related peptide neuromedin N. These two peptides are located near the C terminus and are separated by basic residue pairs. The rat neurotensin/neuromedin N gene has 10.2 kb and is divided into four exons, with exon 4 containing the coding region for both neurotensin and neuromedin N. Two species of mRNA have been identified (1.0 and 1.5 kb), with the difference being the lengths of the 3′ untranslated tail. A similar pattern is seen in the human. The 1.0-kb form predominates in the intestine and pituitary, with equivalent amounts of the 1.0- and 1.5-kb forms in the brain and in tumors.

Secretion

Ingestion of fat is the most potent stimulant of gastrointestinal neurotensin release. However, the majority of neurotensin is rapidly metabolized into biologically inactive N-terminal fragments, predominantly NT(1–8), with the bioactive C-terminal

Table 1 The Neurotensin Family

Bovine/Human/Canine/Porcine/Rat NT	*pGlu*	*Leu*	*Tyr*	*Glu*	*Asn*	*Lys*	*Pro*	*Arg*	*Arg*	*Pro*	*Tyr*	*Ile*	*Leu*
Guinea pig NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Ser	Arg	Arg	Pro	Tyr	Ile	Leu
Chicken NT	pGlu	Leu	His	Val	Asn	Lys	Ala	Arg	Arg	Pro	Tyr	Ile	Leu
Possum NT	pGlu	Leu	His	Val	Asn	Lys	Ala	Arg	Arg	Val	Tyr	Ile	Leu
Amphibian xenopsin						pGlu	Gly	Lys	Arg	Pro	Trp	Ile	Leu
Turkey/Canine xenopsin					Phe	His	Pro	Lys	Arg	Pro	Trp	Ile	Leu
Porcine neuromedin N								Lys	Ile	Pro	Tyr	Ile	Leu
Chicken Lys Asp NT6 (LANT6)								Lys	Asn	Pro	Tyr	Ile	Leu

☆*Change History:* July 2014. A Shulkes added Additional sentences in the following sections. Biological Effects: food intake, intestinal inflammation, analgesia. Pathology: oncogenic effects, potential biomarker role of proneurotensin. Further Reading: added newer references and deleted some older ones.

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fragment NT(9–13) being very rapidly metabolized. Thus, the increase in circulating bioactive neurotensin following a meal is quite modest and less than that required for a biological effect such as inhibition of acid secretion or gastric emptying. Nevertheless, neurotensin may function in concert with other regulatory peptides, such as cholecystokinin, to influence gastrointestinal functions. Neurotensin is also secreted from neurosecretory cells in the hypothalamus and nerve terminals in the median eminence, with elevated levels seen in hypophyseal–portal blood of the rat. In contrast, the neurotensin originating from the gonadotropes and thyrotropes of the anterior pituitary probably functions as paracrine/autocrine agents.

Receptors

Following the first demonstration of neurotensin-binding sites in rat brain synaptic terminals in 1977, there have been many biochemical and autoradiographic studies on the nature and distribution of neurotensin-binding sites. These have culminated with the cloning of three receptor subtypes, all of which recognize the C-terminal, biologically active part of neurotensin.

Neurotensin Type 1 (NTR1)

The cDNA clone was obtained from the rat brain cDNA library using an electrophysiological assay in Xenopus oocytes as the end point. The cDNA encodes a 424-amino acid protein (similar in size to that reported earlier by chemical isolation) and, as suggested by pharmacological studies, is a member of the G protein-coupled receptor family. The human receptor has been cloned from the colonic adenocarcinoma cell line HT29. The receptor has a high affinity (0.2 nm), is insensitive to levocabastine, and is expressed in the intestine and brain. Signal transduction mechanisms are primarily intracellular Ca and inositol 1,4,5-trisphosphate.

Neurotensin Type 2 (NTR2)

This is a low-affinity (5–7 nm) receptor that shares about 60% homology with NTR1 and is sensitive to levocabastine. Although it has the characteristics of a G protein-coupled receptor, the transduction mechanisms have not been completely characterized and appear to be system and species specific. Furthermore, neurotensin antagonists have paradoxical agonist functions in some species. The receptor is expressed mostly in the brain, but in a distribution pattern different from NTR1, so that there is rarely co-expression.

Neurotensin Type 3 (NTR3)

A third neurotensin-binding site was isolated by affinity cross-linking of brain extracts and following cloning shown to have 100% homology with the previously cloned human gp95/sortilin receptor. This protein has a large luminal domain, a single transmembrane domain, and a short cytoplasmic tail. The signal transduction mechanism and biological role remain to be determined.

Biological Effects

Neurotensin has a variety of biological effects, with more than 30 different *in vitro* and *in vivo* effects known. Peripheral effects include vasodilation, cyanosis, increased histamine release, stimulation of the endocrine and exocrine pancreas, effects on gastrointestinal tract smooth muscle activity and motility, stimulation of intestinal secretion, and inhibition of blood flow to adipose tissue. Centrally, neurotensin has hypothermic and antinociceptive effects, and it modulates brain dopamine systems, luteinizing hormone (LH), and prolactin release. Both central and peripheral administration of neurotensin inhibits food intake.

Gastrointestinal Effects

Neurotensin secretion is stimulated by a fat-containing meal and has been postulated as the mediator of many of the effects of fat ingestion. These include inhibition of gastric acid secretion and motility, stimulation of pancreatic and intestinal secretions, decrease in adipose tissue blood flow, and increase in small intestinal blood flow. Neurotensin potentiates the actions of secretin, cholecystokinin, and the vagus on pancreatic secretion and acts in concert with other enterogastrones, such as secretin, to inhibit gastric acid secretion. Neurotensin and its receptors have been implicated in the pathogenesis of intestinal inflammation and inflammatory bowel disease.

Central Effects

The neuroendocrine effects of neurotensin are extremely complex and dependent on the route of administration, the sex steroid status, and the species. Furthermore, there are outstanding issues of separating the physiological effects from the pharmacological effects and the mode of action (neurocrine, autocrine, paracrine, or endocrine). In this regard, the development of specific

receptors and the use of anti-neurotensin antiserum have been most useful. SR48692 blocks NTR1-mediated effects, such as potassium-evoked dopamine release and turning behavior but not analgesia or hypothermia. These latter effects appear to be mediated by the NTR2 and are blocked by R142948A, an inhibitor of both NT1 and NT2. However, recent studies using NTR1 knockout mice suggest that this receptor does contribute to the analgesic and hypothermic responses. Neurotensin has powerful analgesic effects but the requirement for injection has limited its usefulness.

There are intimate and multiple anatomical and functional relationships between neurotensin and dopamine throughout the central nervous system, often with reciprocal modulation of their activities. Neurotensin activates hypothalamic dopaminergic and noradrenergic activity in the basal state but not in the fasted state. Central administration increases plasma adrenocorticotropic hormone (ACTH) and corticosterone by enhancing the release of cortical-releasing factor (CRF) into the hypophyseal portal system. Studies with neurotensin antagonists implicate neurotensin in the stress-initiated activation of the hypothalamic pituitary adrenal axis. In ovarectomized animals (but not in male ones), injection of neurotensin in the preoptic area stimulates LH secretion, whereas injection of NT into the cerebral ventricular system inhibits LH secretion. However, no effect is seen in humans following intravenous administration.

As for gonadotropin release, the route of administration and the experimental conditions are critical in determining the effects of neurotensin on prolactin release. Intracerebroventricular administration decreases circulating prolactin, whereas intravenous administration increases prolactin concentrations. These effects appear to be specific in that they can be reversed by the administration of anti-neurotensin serum. Interestingly, estrogen increases neurotensin mRNA in specific regions of the hypothalamus, further implicating hypothalamic neurotensin in the estrogen-dependent prolactin secretion. In contrast, estrogen decreases anterior pituitary levels of neurotensin. Despite some conflicting data, studies comparing the effects of neurotensin and neurotensin antiserum administration are consistent with a stimulatory effect of neurotensin on thyroid-stimulating hormone (TSH) secretion via an autocrine or paracrine mode of action.

In summary, neurotensin regulates anterior pituitary function both by its release from the nerve terminal from the median eminence and by autocrine/paracrine effects from neurotensin synthesized within the anterior pituitary. The expression of hypothalamic and pituitary neurotensin is under the regulation of gonadal and steroid hormones.

Pathology

Pancreatic tumors producing neurotensin have been reported, but these tumors often produce excess amounts of other peptides, particularly vasoactive intestinal peptide. Because vasoactive intestinal peptide and neurotensin both stimulate intestinal secretion, it is difficult to assess the role, if any, of neurotensin in gastrointestinal symptoms of pancreatic tumors. No clinical syndromes that could be attributed to neurotensin were evident in patients with either lung or hepatic fibrolamellar tumors containing neurotensin as the only detected bioactive peptide. However, the presence of neurotensin in some types of tumors may be of some relevance given that, in experimental studies, neurotensin promotes gastric and colonic carcinogenesis and stimulates the growth of small cell lung cancer cell lines. Neurotensin antagonists inhibited basal and neurotensin stimulated small cell lung cancer growth. Neurotensin is absent from normal prostate but is synthesized and secreted from primary prostate cancers. Taken together it appears that neurotensin is oncogenic and that many of these oncogenic effects are mediated by NTR1. This receptor is over expressed in a number of tumors including lung and pancreas. The proliferative effect of neurotensin may be of theoretical benefit in patients who have atrophy of the bowel associated with long-term parenteral feeding. However, this has not been tested in the human. Neurotensin has been implicated in the pathogenesis of schizophrenia, and a subset of schizophrenic patients has low cerebrospinal fluid neurotensin concentrations that are restored by effective antipsychotic drug treatment. In a recent observational study by Melander and colleagues, fasting proneurotensin was significantly associated with the development of diabetes, cardiovascular disease and breast cancer. However a causal effect of proneurotensin or its end product, neurotensin has not been demonstrated.

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Carcinoid Syndrome

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Introduction

Neuroendocrine neoplasms (NENs), previously known as carcinoid tumors, are considered to be rare neoplasms with an estimated incidence of 3–5 cases/100,000 inhabitants but their incidence has been rising over time as a result of improved sensitivity of currently employed diagnostic tools (Modlin *et al.*, 2008; Huguet *et al.*, 2017). NENs arise mainly in the gastrointestinal tract and bronchopulmonary system but in rare cases they can also occur in the ovaries, the urinary bladder and other organs (Yao *et al.*, 2008). NENs generally follow an indolent course but can develop metastatic disease whereas a subset can behave in an aggressive malignant manner. According to the WHO classification of 2000, the following types of NENs are recognized: (1) well-differentiated endocrine tumor, (2) well-differentiated endocrine carcinoma, (3) poorly differentiated endocrine carcinoma and (4) mixed endocrine-exocrine carcinoma (Solcia *et al.*, 2000; Kloppel *et al.*, 2014). In addition, NENs display significant heterogeneity according to their proliferative behavior. Thus, NENs are classified as grade 1 or 2 (G1 or G2) if their proliferative index (determined by immunohistochemical staining for nuclear ki-67 protein expression) is below 3% or between 3% and 20% respectively. NENS with a proliferative index higher than 20% are classified as grade 3 (G3) neuroendocrine carcinomas (NECs) (Klimstra *et al.*, 2010). Recently, the G3 tumors have shown heterogeneity that relies mostly on their degree of differentiation and are subdivided as G3 NETs (well differentiated) and G3 NECs (poorly differentiated) (Sorbye *et al.*, 2013; Basturk *et al.*, 2015).

NENs may be "functioning" or "nonfunctioning" based on the presence or absence of symptoms attributed to hypersecretion of metabolically active substances. However, some nonfunctioning tumors may be immunohistochemically positive for certain compounds without causing clinical syndrome or secrete substances that are metabolically inert. In addition, NENs that were considered initially nonfunctioning may acquire the ability to secrete substances and become functioning while functioning tumors may change their secretory component leading to a different clinical phenotype (Kaltsas *et al.*, 2004; Crona *et al.*, 2016).

NENs can secrete a variety of substances, peptides, amines or prostaglandins. Carcinoid syndrome (CS) is the constellation of symptoms resulting from the action of several humoral compounds secreted by NENs, the most prominent of them being serotonin, kallikrein, tachykinins, histamine and prostaglandins. Carcinoid syndrome is associated with acute and chronic complications that can impact on the quality of life and overall survival. The most important manifestations of CS are carcinoid crisis, fibrosis and carcinoid heart disease (CHD) that can be fatal if not correctly diagnosed and treated (Kaltsas and Grossman, 2006; Crona *et al.*, 2016).

Pathophysiology

Carcinoid syndrome is the result of synergistic action of tumor products entering the systemic circulation. NENs of the small intestine are the main cause of CS that occurs in 20%–30% of patients with liver metastases from these tumors (Kaltsas *et al.*, 2017).

The most prominent secretory products of neuroendocrine tumors considered to be related with CS are 5-hydroxytryptamine (5-HT, serotonin), histamine, tachykinins, kallikrein and prostaglandins. Serotonin is synthesized from tryptophan through its precursor, 5-hydroxytryptophan (5-HTP), by the enzyme aromatic acid decarboxylase and is involved in smooth muscle contraction, blood pressure regulation and neurotransmission. In normal subjects, 2% of dietary tryptophan is converted to serotonin and is stored in enterochromaffin cells of the gastrointestinal tract, in platelets and in serotoninergic neurons of central nervous system. The urinary breakdown metabolite of serotonin is 5-hydroxyindoleacetic acid (5-HIAA) which is excreted in the urine (Caplin *et al.*, 1998; Kulke and Mayer, 1999; Oberg *et al.*, 2017). In patients with CS there is a shift towards serotonin and 5-HIAA production. When 5-HT and other tumor products are secreted into the portal circulation they are metabolized and inactivated by the liver. However, when hepatic spread from a primary gastrointestinal NEN exceeds the hepatic degradation capacity, CS becomes evident. The syndrome is also occasionally developed in patients with large lymph nodes, peritoneal and ovarian lesions or bronchial carcinoids, where secretory products by-pass the liver via the thoracic duct or retroperitoneal venous collaterals or are released directly into the systemic circulation (Kulke and Mayer, 1999; Grozinsky-Glasberg *et al.*, 2015).

Some foregut NENs (gastric and bronchial) lack the aromatic amino acid decarboxylase and produce 5-HTP and histamine instead of serotonin. Hindgut (distal colon and rectum) and pancreatic neoplasms cannot convert tryptophan to 5-HT and other products and can rarely cause CS (Kaltsas *et al.*, 2004; Tsoukalas *et al.*, 2017).

The altered tryptophan metabolism may account for the majority of symptoms of CS. Serotonin acts via seven types of G-protein-coupled receptors and is considered to be involved in cell motility, fluid secretion and blood circulation in gastrointestinal and bronchopulmonary tract. In particular, serotonin stimulates intestinal motility and secretion and inhibits intestinal absorption accounting for the diarrhea of CS. Serotonin has also mitogenic effects in fibroblasts resulting in peritoneal and cardiac valvular fibrosis. The increased conversion of tryptophan to serotonin may lead to tryptophan deficiency with subsequent decreased protein synthesis, hypoalbuminemia and nicotinic acid deficiency leading to the clinical

symptoms of pellagra (skin rash, glossitis, angular stomatitis, mental confusion). However, serotonin is not directly associated with flushing, which is considered to be mediated by bradykinins, prostaglandins, tachykinins, substance P or histamine while bronchial constriction seems to be related to tachykinins and bradykinins (Caplin *et al.*, 1998; Kulke and Mayer, 1999; Kaltsas *et al.*, 2004).

Clinical Presentation

The principal symptoms of CS are diarrhea and flushing. Patients with the classic (typical) carcinoid syndrome develop cutaneous flushing (60%–85%), secretory diarrhea (60%–80%), intermittent bronchial wheezing (< 10%), venous telangiectasia, right heart valve fibrosis (in up to 20%) and pellagra. Typical flushing occurs suddenly, is of pink or red color, involves the face, neck and upper chest and lasts a few minutes (**Fig. 1**. It can be accompanied by increased heart rate and a burning sensation. Onset of symptoms may be spontaneous and occur several times a day or precipitated by alcohol and tyramine-containing foods (chocolate, bananas, walnuts), pharmacological agents and physical or emotional stress (Caplin *et al.*, 1998; Kulke and Mayer, 1999; Niederle *et al.*, 2016).

The diarrhea of CS is typically watery and nonbloody, sometimes explosive and frequently accompanied by abdominal cramping. Initially, diarrhea may be intermittent caused by secretory and dysmotility factors, but on later stages may become continuous caused by gut lymphangectasia and bacterial overgrowth. In contrast with other diarrhea causes, carcinoid syndrome leads to diarrhea that persists with fasting. It is considered to be the result of action of 5-HT, tachykinins, kallikrein, prostaglandins and histamine which result to increased gut peristalsis and secretion (von der Ohe *et al.*, 1993; Kaltsas *et al.*, 2004).

Bronchospasm occurs in patients with CS who present with wheezing and dyspnoea. Venous telangiectasia, primarily on the face and neck, appears late in the course of the disease due to prolonged vasodilatation. Due to the fact that dietary tryptophan is used for serotonin synthesis, patients with carcinoid syndrome develop pellagra which is characterized by angular stomatitis, glossitis, skin rash and mental confusion. In addition, poor protein synthesis may lead to hypoalbuminemia and muscle wasting (Caplin *et al.*, 1998; Kulke and Mayer, 1999; Kaltsas *et al.*, 2004).

Occasionally, an atypical syndrome is developed, which consists of purplish flush that is severe and prolonged and frequently leaves telangiectasia and hypertrophy of the skin of the face and upper neck but also of the limbs which may become acrocyanotic. Hypotension, headache, lacrimation, cutaneous oedema and bronchoconstriction may also develop. This variant of CS is associated with bronchial neuroendocrine tumors and is probably attributed to histamine production. Another rare type of histamine-mediated flush is a red, patchy and pruritic flush that develops in patients with gastric NEN (Caplin *et al.*, 1998; Kulke and Mayer, 1999; Kaltsas and Grossman, 2006).

Diagnosis

The diagnosis of CS is suspected when the patient reports suggestive symptoms, such as chronic diarrhea or flushing. The biochemical confirmation of diagnosis is based on measurement of 24-h urinary 5-HIAA levels. Consumption of certain drugs and

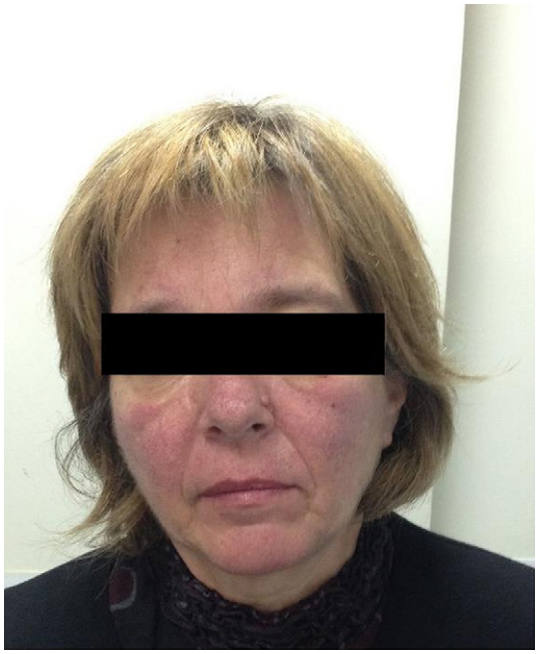

Fig. 1 Facial flushing from typical CS secondary to a small intestine NEN.

foods rich in tryptophan/serotonin can cause false elevated results and patients should collect urine with strict dietary restrictions. In addition, malabsorption syndromes (such as celiac disease, Whipple disease, cystic fibrosis) can lead to falsely increased 5-HIAA levels (Meijer *et al.*, 2000; Oberg *et al.*, 2017). 5-HIAA has a sensitivity of 100% and a specificity of 85%–90% for detecting CS and a specificity of approximately 100% for the diagnosis of a primary tumor in the small intestine (Feldman and O'Dorisio, 1986; Ardill and Erikkson, 2003). A low-level cut-off value (2,8 mmol/mol creatinine) yielded a specificity of 89%, while a high cut-off level (6,7 mmol/mol creatinine) improved specificity (98%) at the expense of a lower sensitivity (52%) (Meijer *et al.*, 2000). In certain cases, CS can be developed without increased 5-HIAA levels and this is attributed to secretion of biologically active molecules other than serotonin. Recent studies that examined the role of 5-HIAA levels as a prognostic factor show controversial results (Turner *et al.*, 2006; Formica *et al.*, 2007). Serum serotonin is less sensitive and specific while platelet serotonin is considered a sensitive test which however is not widely available (Niederle *et al.*, 2016). Recent studies have demonstrated high correlation of urine and serum 5-HIAA which could represent a useful diagnostic tool for CS in the future (Adaway *et al.*, 2016; Oberg *et al.*, 2017).

Serum chromogranin A (CgA) is a sensitive marker for neuroendocrine tumors irrespectively of primary origin and can be elevated in functional and nonfunctional NENs. In general, CgA levels increase in parallel with tumor burden. It is recommended to measure CgA in the same laboratory or with the same assay as it has been observed that CgA levels may differ significantly between different assays. However, CgA is a nonspecific marker as various clinical situations such as atrophic gastritis, chronic renal failure, liver cirrhosis, congestive heart failure or proton pump inhibitor use lead to CgA levels elevation. Specificity of CgA in the diagnosis of NENs depends on the tumor type and burden (100% specificities have been reported in patients with metastatic disease) and the cut-off values employed. Elevated CgA was found to be more sensitive than high urine 5-HIAA levels in patients with metastatic midgut lesions (87% vs. 76%, respectively) (Niederle *et al.*, 2016; Oberg *et al.*, 2017).

In general, when assessing a patient for CS, 5-HIAA is a specific marker while CgA levels are useful in case of established diagnosis to assess disease recurrence after complete surgical resection and disease response or progression during treatment.

Carcinoid syndrome is primarily associated with metastatic NENs that originate in small intestine while it can also develop in patients with ovarian, lung or gastric NENs. Multiphasic contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI) of the abdomen are useful tools not only for tumor detection but also for following-up the response to treatment. Chest CT may be necessary to detect a bronchial NEN (Niederle *et al.*, 2016). Whereas liver metastases are easily detected, localization of primary tumor may be difficult. CT detects approximately half of the lesions when the size of the mesenteric mass exceeds 1.5 cm. For gastrointestinal NENs, MRI is able to detect around two-thirds of lesions. Typically, NENs appear as T1 hypointense and T2 hyperintense lesions. Similarly, hepatic metastases are well depicted on MRI and MRI is often used to further characterize lesions that are equivocal on CT. Like the primary lesions, hepatic metastases appear as T1 hypointense and T2 hyperintense (Tan and Tan, 2011). Transabdominal ultrasound (TAUS) may be used for screening of liver metastases (Dörffel and Wermke, 2008) but for primary tumor localization and long-term follow-up CT or MRI display higher sensitivity. Newer techniques such as contrast enhanced ultrasound (CEUS) and endoscopic ultrasound (EUS) are more advantageous methods of assessment of NENs especially for pancreatic, gastric and liver lesions (Tan and Tan, 2011).

Direct visualization with colonoscopy may be possible in tumors located in terminal ileum but for more proximal parts of ileum or for the jejunum more sophisticated techniques such as CT-water enteroclysis, video-capsule endoscopy or double-balloon enteroscopy may be required. These methods are especially suggested in case of metastatic NENs of unknown primary origin (Niederle *et al.*, 2016).

Many NENs express high levels of somatostatin receptors (SSTRs) and can therefore be detected with a form of the somatostatin analog octreotide which is linked with radionuclides that can be detected by somatostatin receptor scintigraphy (SRS, ^{111}Indium) or by positron emission tomography (PET, ^{68}Gallium). This technique has the advantage of whole body scanning which also allows detection of metastases outside of the abdominal region. SRS is considered to display an overall sensitivity of approximately 80%–90% in patients with gastrointestinal NENs. Normal uptake is seen in thyroid, spleen, liver and pituitary while bowel and kidney uptake can also be observed (Strosberg *et al.*, 2009; Tan and Tan, 2011). A recent study showed that ^{68}Ga-DOTATOC PET/CT is superior compared to ^{111}In-DTPA octreotide SPECT/CT when attempting to localize the primary tumor of metastatic disease or clinically suspected disease (Schreiter *et al.*, 2014).

Fluorodeoxyglucose (FDG) PET imaging is an imaging technique that addresses the high glucose metabolism of malignant tissues. ^{18}FDG-PET/CT has a low sensitivity for the detection of well-differentiated low-grade NEN but can identify NENs characterized by aggressive growth or behavior. New traces such as ^{11}carbon-5-hydroxytryptophane and ^{18}fluoro-dihydroxyphenylalanine (DOPA) have shown promising results but are not widely available and further studies are needed before recommending their standard use (Koopmans *et al.*, 2006; Tan and Tan, 2011).

Treatment

Somatostatin is a small peptide hormone that is present in human body in two molecular forms, consisting of 14 or 28 amino acids and has various biological effects in different tissues. These effects are mediated through specific SSTRS that are present in diverse tissues including the human gastrointestinal mucosa. Five different SSTR subtypes have been recognized. Somatostatin regulates the secretion of intestinal hormones, the gastrointestinal transit time and the resorption and secretion of intestinal fluid (Lamberts *et al.*, 1996; Kaltsas *et al.*, 2004).

As the majority of carcinoid tumors express SSTRs, very early it was observed that somatostatin ameliorated the symptoms of CS (Thulin *et al.*, 1978). Native somatostatin has a short half-life and synthetic structural analogues which mimic somatostatin action but display longer half-life have been synthesized. Octreotide was the first synthetic analogue and can be administered by multiple subcutaneous injections or continuous subcutaneous or intravenous infusion. It was shown that octreotide can result to symptomatic control of functioning NENs and also prevent the development of carcinoid crisis in patients with CS submitted to invasive procedures (Wood *et al.*, 1985; Kvols *et al.*, 1985). Also, a slow-release depot intramuscular formulation of octreotide (Sandostatin LAR) has been released and has to be administered once every 4 weeks. Another octreotide analogue, lanreotide, has also become available as a slow-release deep subcutaneous depot preparation (Somatuline Autogel) that has to be administered every 4 weeks (Rubin *et al.*, 1999; Modlin *et al.*, 2010). Octreotide and lanreotide have comparable binding profiles for SSTRs; octreotide binds with a high, low and moderate affinity to $SSTR_2$, $SSTR_3$ and $SSTR_5$ respectively (Hofland and Lamberts, 2003). In addition, a new synthetic analogue, pasireotide, with high affinity for all SSTRs except $SSTR_4$, has been observed to be efficacious in cases of inadequate control with octreotide LAR (Kvols *et al.*, 2012).

In patients with CS both diarrhea and flushing improve or even disappear after treatment with long acting somatostatin analogues (SSAs), usually within the first week. When SSAs are initiated, concomitant therapy with short-acting octreotide should be prescribed for the first 2–3 weeks. Frequent side effects of SSAs are nausea, diarrhea, steatorrhea, abdominal pain, biliary sludge or gallstone formation and rarely, hyperglycaemia and rash (Kaltsas *et al.*, 2004). Although SSAs are the best available treatment for CS, it has been observed that an escape phenomenon of tachyphylaxis may occur after a few months or years of treatment. The mechanism is related to either down-regulation of somatostatin receptors or an increase in tumor load. In this case, significant increase of dosage may be required while adding top-up doses of short-acting octreotide can also be useful (Kaltsas *et al.*, 2004; Strosberg *et al.*, 2014).

It has been observed that treatment with interferon-a (IFN-a) controls the symptoms of CS in 40%–70% of patients and exerts anti-tumor action. However, IFN-a has been associated with clinically significant adverse effects including flu-like symptoms, fatigue, auto-immune diseases and immunosuppression (Oberg, 2000; Kaltsas *et al.*, 2004). A newer agent, pegylated interferon, is considered to result to fewer side effects while a number of studies address the probable synergistic use of SSAs and lower doses of IFN-a (Fazio *et al.*, 2007).

Telotristat etiprate is an oral inhibitor of the enzyme tryptophan hydroxylase, the rate-limiting step in the conversion of tryptophan to serotonin. Telotristat does not have central nervous system side effects as it does not cross the blood–brain barrier and does not affect the serotonin levels in the brain (Pavel *et al.*, 2015). Recent studies have shown that addition of telotristat in patients with inadequate symptom control despite treatment with SSAs, results to significant reduction of bowel movements frequency and 5-HIAA levels while flushing episodes were also improved (Kulke *et al.*, 2017; Pavel *et al.*, 2015). Adverse events possibly related to telotristat are mild and include abdominal pain, nausea and a low rate of depression. It is thus suggested that telotristat can be used in patients with CS refractory to SSAs while further investigation needs the assumption that the decrease of serotonin levels might reduce the development of peritoneal and cardiac valvular fibrosis (Pavel *et al.*, 2015; Kulke *et al.*, 2017).

In NEN G1 or G2, surgery with the intention to cure can be considered even if liver or lymph node metastases are present. In patients with advanced disease, tumor debulking techniques such as hepatic artery embolization and palliative hepatic cytor-eductive surgery can decrease tumor load and result to symptomatic improvement of CS (Gupta *et al.*, 2003; Strosberg *et al.*, 2006). Peptide receptor radionuclide therapy (PRRT) can be used in patients with tumors expressing SSTRs and it has been observed that CS refractory to octreotide can be ameliorated (Bushnell *et al.*, 2010). Furthermore, treatment with octreotide therapy should be instituted before any manipulation in order to prevent carcinoid crisis (Kaltsas *et al.*, 2017).

Antidiarrheal medications such as loperamide and codeine can be useful in cases of refractory diarrhea while patients should avoid known precipitants of flushing and diarrhea (exercise, alcohol intake, consumption of spicy or tyramine-containing foods). Histamine-2 receptor antagonists may be proved useful agents as sometimes symptoms are related to histamine action. Furthermore, other causes of diarrhea should be considered and treated. In patients who have been submitted to distal bowel resection, bile acid loss and bacterial overgrowth can occur and treatment with cholestyramine or antibiotics may be required (Kaltsas *et al.*, 2004). In addition, pancreatic enzymes can be used to treat steatorrhea after pancreatic resection or due to therapy with SSAs while dehydration, electrolyte abnormalities and vitamin B deficiency should also be treated (Kaltsas *et al.*, 2004).

Carcinoid Heart Disease

Carcinoid heart disease (CHD, Hedinger syndrome) occurs in about 60% of patients with NENs and CS and typically involves the right side of the heart (Fox and Khattar, 2004). CHD develops more frequently in patients with NENs of the small bowel, followed by NENs of the lung, large bowel, pancreas, appendix or ovaries. In 18% of cases the primary tumor is unknown (Pellikka *et al.*, 1993).

The precise mechanism behind CHD remains unknown but it is generally thought to be mediated by serotonin, prostaglandins, histamine, bradykinin and other substances secreted by the tumor. Patients with CS and CHD have higher levels of 5-HIAA and other vasoactive substances (Pellikka *et al.*, 1993; Kulke and Mayer, 1999). In addition, serotoninergic drugs used in the treatment of obesity, migraine and Parkinson's disease lead to development of similar heart valvular fibrosis while it has been observed in cell cultures that serotonin has mitogenic effects on fibroblasts, smooth muscle cells and endothelial cells (Grozinsky-Glasberg *et al.*, 2015). 5-HIAA levels above 300 μmol/24 h and >3 episodes of flushing per day have been demonstrated to be predictors of CHD development or progression (Bhattacharyya *et al.*, 2011).

Heart lesions are characterized by plaque-like, fibrous endocardial thickening that leads to retraction and fixation of the valve leaflets. The most common lesions are tricuspid regurgitation followed by tricuspid stenosis, pulmonary regurgitation and pulmonary stenosis. Approximately 10% of patients develop left-sided heart disease because hormonal mediators are cleared or inactivated in the lungs. However, left sided lesions can be observed in case of patent foramen ovale, lung tumors or high tumor burden (Pellikka *et al.*, 1993; Kulke and Mayer, 1999).

Early in the disease course, the patients present with subtle symptoms, mainly fatigue and dyspnoea on exertion but ultimately they develop significant dyspnoea, anasarca and cardiac cachexia. However, some patients remain asymptomatic despite cardiac involvement (Dobson *et al.*, 2014).

The principal method of CHD diagnosis and follow-up is two dimensional echocardiography and Doppler examination. Typically, in the early stages thickening of tricuspid valve leaflets is observed which ultimately leads to tricuspid regurgitation and stenosis while all the heart valves can become progressively involved (**Fig. 2**). Regular echocardiographic screening of patients with carcinoid syndrome is recommended as mandatory for patients with CS (Grozinsky-Glasberg *et al.*, 2015). Cardiac MRI may be helpful but its usefulness has not been proven. N-terminal probrain natriuretic peptide (NT-proBNP), a neurohormone released by the atria and ventricles in response to increase in volume and pressure, has been shown to be a sensitive and specific marker for CHD prediction and be correlated with patient survival (Korse *et al.*, 2009). Thus, it is highly recommended to measure NT-proBNP levels for diagnosis and follow-up of CHD in patients with intestinal NENs and in all patients with CS (Grozinsky-Glasberg *et al.*, 2015; Niederle *et al.*, 2016). Furthermore, plasma activin A levels have been observed to be an independent predictor in advanced but also in early stages of CHD (Bergestuen *et al.*, 2010).

Treatment with SSAs and tumor debulking techniques may decrease the release of vasoactive agents and the development of heart failure but there is no evidence that can prevent CHD progression. Telotristat etiprate has been observed to improve diarrhea and reduce 5-HIAA levels and could be promising for patients with CHD. In addition, therapy for heart failure with loop diuretics, fluid and salt restriction and compression stockings should be introduced and may initially improve the symptoms of right heart failure (Grozinsky-Glasberg *et al.*, 2015). However, surgical valve replacement is the definitive treatment and is now performed earlier with low perioperative mortality. Median survival after valve replacement varies between 6 and 11 years (Moller *et al.*, 2005). In patients unsuitable for cardiac valve surgery, balloon valvuloplasty is an alternate technique for tricuspid or pulmonary stenosis but the clinical benefit is of short-term. Moreover, as a patent foramen ovale has been reported to coexist with CHD and may cause left-sided heart lesions, such a lesion should be ruled out and its closure should be considered prior to cardiac surgery (Grozinsky-Glasberg *et al.*, 2015). In the perioperative period, continuous infusion of octreotide is required to reduce complications and carcinoid crisis incidence (Kaltsas *et al.*, 2017).

Carcinoid Fibrosis

Midgut carcinoid tumors are commonly associated with large mesenteric lymph node metastases that are characterized by mesenteric fibrosis (**Fig. 3**). The release of growth factors and other substances from the metastatic lesions is considered to be the

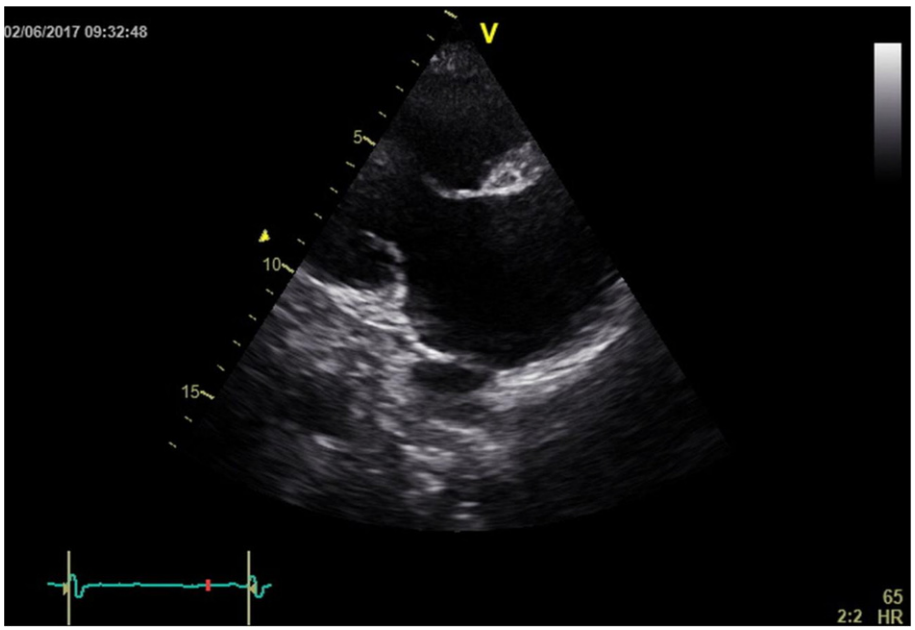

Fig. 2 Transthoracic echocardiogram showing thickened and rigid tricuspid valve leaflets in a patient with CS.

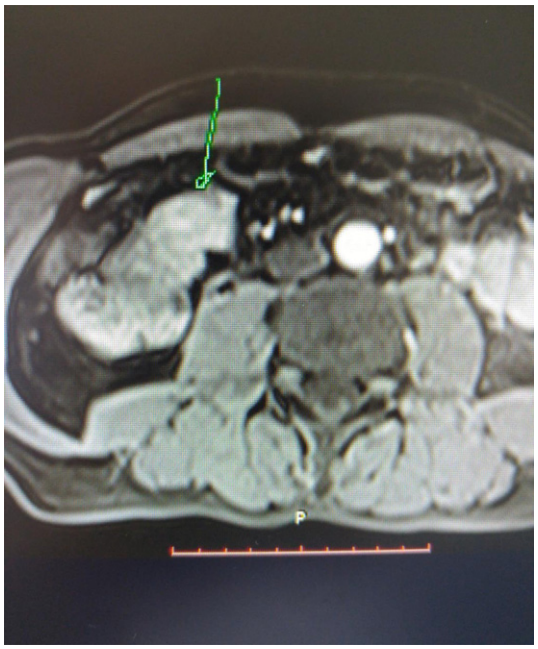

Fig. 3 Abdominal MRI showing desmoplastic reaction in a patient with CS secondary to a small intestine NEN.

cause of this fibrosis. Fibrotic tissue causes shrinkage and fixation of the mesentery and the mesenteric root to the retroperitoneum and may ultimately lead to partial or complete small bowel obstruction. Additional complications associated with carcinoid fibrosis are obstructive uropathy, mesenteric ischaemia, Peyronie's disease of the penis and carcinoid arthropathy. Surgical treatment with debulking of tumor mass from the mesenteric root preserving the vascular supply of intestine is required in order to avoid complications (Oberg, 1999; Kaltsas and Grossman, 2006).

Carcinoid Crisis

Carcinoid crisis is a life-threatening condition characterized by excessive flushing, bronchospasm, alterations in thermoregulation, hemodynamic instability mainly in the form of hypotension and tachycardia and central nervous system dysfunction. Some patients may develop hypertension provoked by excessive release of catecholamines. The carcinoid crisis can occur spontaneously or can be precipitated by severe emotional stress, anesthesia, surgery or other types of intervention and medication (chemotherapy or radiopharmaceuticals causing tumor lysis) that result to release of significant amounts of biologically active compounds in the systemic circulation. Sometimes, crisis presents with postoperative instability or fail to recover after surgery. CHD and high 5-HIAA levels have been observed to represent predictors of an emerging carcinoid crisis. Large tumor load and high CgA or 5-HIAA levels are associated with higher probability of developing a carcinoid crisis during surgery (Caplin *et al.*, 1998; Kulke and Mayer, 1999; Kaltsas *et al.*, 2017).

Pre- and perioperative treatment with intravenous octreotide is required during surgery or other minor interventions to prevent carcinoid crisis. Continuous octreotide infusion is administered 12 h before the procedure at a starting dose of 50–100 μg/h with gradual escalation until symptom control and continued for at least 48 h afterwards with dose titration as required; doses up to 500 μg/h may occasionally be required. Various schemes with subcutaneous administration of octreotide are also currently used by some centers but this approach is not considered safe (Kaltsas *et al.*, 2017). If patients are already on treatment with long-acting SSAs, these medications should be continued but a recent meta-analysis has suggested that these patients may require even higher doses to prevent carcinoid crisis (Seymour and Sawh, 2013). In case of atypical carcinoid syndrome, treatment with octreotide should be instituted but also antihistamines and corticosteroids may be required in order to decrease histamine release and its peripheral actions. In addition, it is strongly recommended to minimize the use of drugs that stimulate sympathetic nervous system or precipitate release of vasoactive products by the tumor (opioids, dopamine, d-tubocurarine) (Kaltsas *et al.*, 2017).

Conclusion

Carcinoid syndrome is a clinical entity attributed to the secretion of various humoral substances from NENs and is primarily associated with metastatic NENs of the small intestine while it can also develop in patients with ovarian, lung and gastric NENs. Flushing, diarrhea and bronchospasm are the most common manifestations of CS but an atypical CS can develop in patients with

bronchial and gastric NENs. Biochemical diagnosis is based in measurement of urine 5-HIAA levels and further investigation with imaging studies is required for tumor detection and follow-up. SSAs are the mainstay of treatment of CS while telotristat etiprate that reduces the production of serotonin is considered a promising option in cases of refractory CS. CS is associated with clinically significant and probably fatal complications the most important being carcinoid heart disease, fibrosis and carcinoid crisis that need to be timely diagnosed and treated. In addition, treatment with octreotide must be instituted before any manipulation in order to prevent carcinoid crisis.

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Catecholamines☆

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Glossary

Adrenal medulla It produces and secretes predominantly epinephrine.

Autonomic dysfunction Autonomic dysfunction comprise several disease entities including those characterized by orthostatic hypotension due to autonomic failure.

Catecholamines Catecholamines comprise norepinephrine, epinephrine and dopamine and are produced by the adrenal medulla and the central and sympathetic nervous systems. Catecholamines exert a wide array of physiological functions through adrenergic receptors, which are distributed in nearly all organs.

Catechol-*O*-methyltransferase This is an enzyme involved in the extraneuronal breakdown of catecholamines.

Monoamine oxidase It plays a pivotal role in the intraneuronal metabolism of norepinephrine.

Pheochromocytoma and paraganglioma Pheochromocytoma and paraganglioma are chromaffin cell tumors that produce excessive quantities of catecholamines leading to considerable cardiovascular morbidity and mortality if not diagnosed and treated in a timely manner.

Sympathetic nervous system Sympathetic nervous system produces exclusively norepinephrine, a neurotransmitter involved in the homeostatic modulation of numerous systems, but particularly the cardiovascular system.

Introduction

Long before first identification of the catecholamines, it were George Oliver and Edward Albert Schäfer who in 1895 were the first to demonstrate that administration of an extract of the adrenal medulla to a dog was able to increase blood pressure. In 1901 John Jacob Abel, Thomas Aldrich and Takamine isolated and identified epinephrine. It was then not until 1945 when norepinephrine was identified as the neurotransmitter of sympathetic nerves by the Swedish scientist Ulf von Euler. This was followed in 1952 by Arvid Carlsson's discovery of dopamine as a crucial neurotransmitter in the brain.

The naturally occurring catecholamines comprise norepinephrine, epinephrine and dopamine. These compounds have in common a catechol group consisting of a benzene ring with two hydroxyl groups at positions three and four and a connection to an ethylamine side chain. The distinct functional differences between these compounds reside in the ethylamine side chain (**Fig. 1**).

Whereas epinephrine is a hormone exclusively produced in the adrenal medulla, norepinephrine and dopamine act as neuromodulators or transmitters in central and peripheral nervous systems. However, most peripheral dopamine is produced in nonneuronal systems. All three are biologically active compounds, exerting their functions through adrenergic and dopaminergic receptors located in nearly all organs and tissues. Their different and varying physiological effects are not only determined by the intensity of secretory processes, but also by different affinities to receptors as well as different distributions or receptors in relation to different sources of catecholamines.

Catecholamines play a central role in the adaptation to stressful stimuli such as hypoglycemia, hypoxemia and emotions. They are also pathophysiologically involved in many diseases such as autonomic failure and pheochromocytoma. Pheochromocytoma is a neuroendocrine tumor characterized by excessive production of one or more catecholamines, exposing patients to a high risk of catastrophic complications. Reliable measurement of the *O*-methylated metabolites of catecholamines is instrumental for the diagnosis and posttreatment monitoring of these tumors.

Biosynthesis and Storage

The amino acid L-tyrosine is the precursor for the synthesis of catecholamines. Three enzymes are responsible for the final synthesis of norepinephrine: tyrosine hydroxylase (TH), aromatic-L-amino acid decarboxylase (LAAD) and dopamine-beta-hydroxylase (DBH) (Eisenhofer *et al.*, 2001). TH catalyzes the first rate-limiting step in catecholamine synthesis converting L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) (Nagatsu, 1995). By way of phosphorylation of serine residues TH can strongly and rapidly respond to various stimuli in order to speed up the production of norepinephrine when needed. After conversion of L-DOPA to dopamine by LAAD, dopamine is transported into the vesicular storage granules by vesicular monoamine transporters (VMAT). Within vesicles dopamine is

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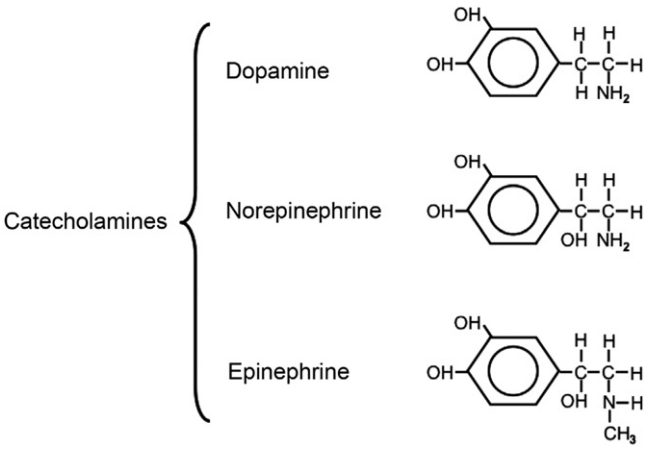

Fig. 1 The chemical structure of all three catecholamines.

hydroxylated to norepinephrine by DBH, requiring ascorbic acid and copper as cofactors. This biosynthetic pathway takes place in central and peripheral sympathetic neurons as well as chromaffin cells associated with the paraganglial chain and adrenal medulla.

Adrenomedullary cells uniquely express the enzyme phenylethanolamine *N*-methyltransferase (PNMT). This enzyme methylates norepinephrine, which leaks from storage vesicles into the cytoplasm, leading to production of epinephrine. Expression of PNMT is induced by the adrenocortical glucocorticoid, cortisol. Close proximity of the adrenocortical cells with centripetal blood flow from the cortex to the medulla guarantees continuous availability of cortisol. Following synthesis in the cytoplasm, epinephrine is actively translocated by VMAT into chromaffin storage granules.

Norepinephrine and epinephrine are stored in electron-microscopically distinct vesicles where they are available for secretion when required. These vesicles have varying size and appearance depending on the type of stored catecholamine. The ongoing dynamic exchange of stored catecholamines between granules and cytosol is based on active VMAT-dependent uptake from the cytosol and passive leakage from the vesicular granules back into the cytosol.

Regulation of Release

The secretory process of exocytosis takes place in terminal parts of sympathetic nerves (varicosities) and in the adrenal medullary cells. The storage granules containing catecholamines fuse with the plasma membrane, thus expelling catecholamines into the extracellular space. This fusing mechanism is a calcium-influx dependent regulated process, initiated by sympathetic nerve pulses or secretagogues. Since calcium channels may be affected by many other compounds such as peptides (e.g., angiotensin), many other stimuli than nerve pulses may contribute to or modulate exocytosis. Finally, under pathological (e.g., ischemia) and pharmacological conditions (e.g., tyramine), catecholamines can be secreted constitutively as a calcium-independent process. Neuronal release of catecholamines is also tightly regulated by catecholamines themselves through presynaptic adrenergic receptors by inhibition (alpha$_2$-adrenergic receptors) or stimulation (beta$_2$-adrenoreceptors) (Nagatsu and Stjarne, 1998).

Uptake and Metabolism

The human body possesses several defense mechanisms to protect against excessive catecholamine exposure (Eisenhofer *et al.*, 2004). The primary mechanism to discontinue quickly catecholamine action comes from cell membrane transporters. Nearly 90% of neuronally released norepinephrine is taken back up by the prejunctional norepinephrine transporters into the sympathetic neurons. Extraneuronal monoamine transporters play a minor role in terminating the actions of norepinephrine released by sympathetic nerves, but are more important than neuronal transporters for removal of circulating catecholamines. The rapid clearance of catecholamines from the circulation by these transport mechanisms results in a very short half-life for circulating catecholamines of about 2 min (Eisenhofer, 2001).

After neuronal reuptake, most norepinephrine is returned by VMAT in vesicular granules while a smaller proportion is metabolized to 3,4-dihydroxyphenylglycol (DHPG) in the cytosol by monoamine oxidase (MAO). Under resting conditions most DHPG is formed from norepinephrine passively leaking from storage vesicles. Similar processes occur in adrenal medullary cells where the additional presence of catechol-methyltransferase (COMT), leads also to production of *O*-methylated metabolites. Norepinephrine is converted to normetanephrine, epinephrine to metanephrine and dopamine to 3-methoxytyramine. Since COMT is not present in sympathetic neurons, metabolism of catecholamines produced from different locations results in different metabolic patterns.

COMT is also present at numerous extraneuronal sites, besides the adrenal medulla, where most *O*-methylated metabolites are deaminated to 3-methoxy-4-hydroxyphenylglycol (MHPG). Much of the DHPG produced in sympathetic neurons is also *O*-methylated at these sites leading also to production of MHPG.

The final end product of norepinephrine and epinephrine metabolism is vanillylmandelic acid (VMA), whereas homovanillylmandelic acid is the main end product of dopamine metabolism. Almost all VMA is produced in the liver through the actions of alcohol dehydrogenase on MHPG taken up into liver cells from the circulation. Additionally, all catecholamines and their metabolites except VMA are conjugated in various proportions by a specific sulfotransferase (SULT1A3) to sulfate conjugates. This enzyme is located in gastrointestinal tissues and serves as protection also against exogenous food derived amines. All metabolites, both free and conjugated are finally removed by urinary excretion. Due to their slow circulatory clearance the conjugated *O*-methylated metabolites of catecholamines are present in plasma at much higher concentrations than free metabolites, which are rapidly cleared by extraneuronal monoamine transporters.

Physiological Function

Catecholamines have profound physiological effects while almost all metabolites are biologically inactive. Even under baseline conditions without apparent stress plasma levels of catecholamines may vary considerably. Under stressful conditions, increased sympathoneural and adrenomedullary activity may induce extremely high circulating levels of plasma catecholamines. Catecholamines play an essential role in homeostasis and serve not only to adapt to such stressful conditions but also to protect vital organs. There are functional differences between catecholamines: in contrast to norepinephrine, which plays a pivotal role in cardiovascular regulation, epinephrine plays a more dominant role in many metabolic processes such as in glucose and lipid metabolism.

At the cellular level catecholamines exert their biological activity through interaction with cell membrane pre-, post-, and extrajunctional adrenergic receptors which can be classified into alpha-adrenergic receptors ($\alpha1_a$, $\alpha1_b$, $\alpha1_d$, $\alpha2_a$, $\alpha2_b$, $\alpha2_c$), beta-adrenergic receptors ($\beta1$, $\beta2$, and $\beta3$) and dopaminergic receptors (D1–D5) based on ligand-binding studies and responses to agonists and antagonists (Guimaraes and Moura, 2001). The resulting physiological effects depend on differences in affinity of receptors for different catecholamines, on the tissue distribution of the receptors and on the levels of catecholamines in plasma and in neuroeffector junctions. For instance, stimulating vascular $\beta2$-adrenergic receptors during stress in healthy subjects cause vasodilation. However, in a situation of extremely elevated plasma epinephrine levels such as in patients with an epinephrine secreting pheochromocytoma, vascular α-adrenergic receptors are stimulated, causing intense vasoconstriction, thus overriding the $\beta2$-adrenergic receptor mediated vasodilatory effects, and resulting in a severe increment in blood pressure.

Numerous classes of drugs exist that either block or stimulate adrenergic receptors. Typical examples are beta-adrenergic receptor blockers; these drugs result in reductions in heart rate, blood pressure and cardiac oxygen consumption and are commonly used in patients with cardiac ischemia. Beta2-adrenergic receptor agonists stimulate beta2-adrenergic receptors which are abundant in the bronchial tree, this resulting in bronchial dilation, which is the targeted therapeutic effect in patients with bronchial asthma. Some drugs have the potential to stimulate also central $\alpha2$-adrenergic receptors (such as clonidine), which results in a decrease in central sympathetic activity with an ensuing drop in vascular tone and blood pressure.

Understanding catecholamine physiology may also help in appreciating some side effects of drugs. Prejunctional located $\alpha2$-adrenergic receptors are supposed to have a constraining role in neuronal norepinephrine release. Blocking the $\alpha2$-adrenergic receptors by nonselective α-adrenergic blockers such as phenoxybenzamine will result in an increase in norepinephrine release, resulting in tachycardia, a well-known side effect of this drug. For a more detailed review of adrenergic receptors and their naturally and synthetic agonists and antagonists, we refer to previous reviews (Guimaraes and Moura, 2001).

Measurements Methods

The assay methodology for measurement of plasma or urinary catecholamines has evolved over the last three decades from spectrophotometric and radioenzymatic assays to liquid chromatography techniques with electrochemical detection. The latter technique, although superior to the earlier ones, requires preanalytical extraction and purification steps and suffered also from analytical interference by drugs. The latest development is liquid chromatography with tandem mass spectrometry (LC-MS/MS). The major advantages of LC-MS/MS over other methods are not only a higher and more rapid throughput of samples and a better signal-to-noise ratio but features in particular a higher analytical specificity. Immunoassays are still used in many places but suffer from inferior accuracy and analytical interference.

An essential aspect of measurements of catecholamines for obtaining reliable test results relates to how blood and urinary samples are collected and stored. As mentioned earlier plasma catecholamines are sensitive to sympathoadrenal activation, such as associated with stressful physical and mental stimuli. A typical example is the position of the patient when taking a blood sample; plasma catecholamines are nearly twice as high in the standing position as compared to the supine position due to an elevated sympathetic tone. It is therefore recommended that blood samples are drawn after supine rest for at least 20 min with an indwelling venous cannula. This minimizes stress-induced fluctuations and circumvents false-positive test results. Twenty-four hour urinary collections are less sensitive to stressful stimuli, but are inconvenient for the patient and the collections are not always

complete. Collecting night-time urine or spot urine may be provide better alternatives but should be normalized against urinary creatinine excretion.

Since catecholamines are sensitive to oxidative degradation, blood samples should be collected into tubes containing heparin or EDTA as anticoagulant and should be placed immediately on ice before centrifugation. For prolonged storage the samples should be kept frozen at $-80\ ^{\circ}C$. For urine samples it is advised to collect urine in containers that have hydrochloric acid to prevent degradation. For measurements of metabolites less stringent procedures are required.

Although analytical interference is minimal with LC-MS/MS there remains a risk of pharmacophysiological interference when drugs are used that may affect uptake transporter proteins, metabolizing enzymes or clearance of catecholamines. A typical example are the tricyclic antidepressants, which block the presynaptic norepinephrine uptake transporter mechanism, thus causing elevated plasma concentrations of norepinephrine and normetanephrine. Effects of drugs interacting with metabolizing enzymes or clearance are more relevant for metabolites of catecholamines such as metanephrines than catecholamines themselves.

Clinical Application

An increased secretion of catecholamines can be demonstrated in many clinical conditions in which the sympathoadrenal system is strongly activated. Typical examples are hypoglycemia (epinephrine), heart failure (norepinephrine), and acute cardiac ischemia. In these cases measurement of catecholamines has no significant diagnostic utility and is merely reflecting the severity of these conditions. There are other clinical conditions in which measurements of catecholamines and/or metabolites are indispensable for the diagnosis and clinical management. One main example is patients who are suspected to have a catecholamine-producing chromaffin cell tumor: pheochromocytoma, paraganglioma or neuroblastoma (Eisenhofer and Peitzsch, 2014). For many years, the diagnosis of pheochromocytoma or paraganglioma depended on measurements of catecholamines in plasma or urine. Due to a better diagnostic accuracy, measurement of metanephrines instead of catecholamines now provide the backbone for the biochemical diagnosis of these tumors (Lenders *et al.*, 2014). The underlying pathophysiological explanation for the superior diagnostic accuracy of metanephrines over catecholamines is the continuous intratumoral production of metanephrines, in contrast to the catecholamines, which may be released in paroxysms or in only relatively low amounts compared to the independent production of metanephrines.

An other clinical area in which measurements of catecholamines may be useful are specific causes of autonomic dysfunction such as is the case in patients with autonomic failure. In such cases, the disordered production of catecholamines is of pathogenic relevance. Common examples are patients with autonomic failure due to multiple system atrophy (MSA), less commonly patients with pure autonomic failure (PAF) and very rarely patients with genetically determined DBH deficiency. In all three groups a core clinical feature is orthostatic hypotension. Measurement of plasma catecholamines can be instrumental for distinguishing between these three orthostatic hypotensive groups. A correct diagnosis is pivotal for both prognosis and choice of treatment. In the first group of MSA patients plasma catecholamines may be normal but respond inadequately to sympathetic activation (e.g., supine to upright posture). The second group of PAF patients shows reduced plasma catecholamine levels at rest due to sympathetic nerve degeneration. In the last group of DBH deficient patients, plasma norepinephrine and epinephrine are not measurable while plasma dopamine is elevated. This pattern of catecholamines is pathognomonic for DBH deficiency, a genetically inherited syndrome with multiple other clinical features (Biaggioni *et al.*, 1987). There are additional genetic syndromes in which synthesis or metabolism of catecholamines is affected such as in Menke's disease (Kaler *et al.*, 1998). In this disease a gene coding for a copper-transporting ATP is mutated. As copper is a cofactor for DBH, this results in less activity of the enzyme DBH with an ensuing decreased conversion of dopamine to norepinephrine. Thus, measurements of catecholamines in addition to clinical features may be very helpful for the differential diagnosis of patients with syndromes of autonomic dysfunction.

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Pineal Tumors☆

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Pineal Physiology

The pineal gland is innervated mainly by sympathetic nerve fibers which inform the gland of the prevailing light–dark cycle and acts as a neuroendocrine transducer; it is located behind the third ventricle in the center of the brain and is a highly vascular organ formed by neuroglial cells and parenchymal cells or pinealocytes; the latter synthesize melatonin, as well as other indoleamines and peptides.

The main pineal hormone melatonin (*N*-acetyl 5-methoxy-tryptamine) exhibits an endogenous circadian rhythm, reflecting signals originating in the suprachiasmatic nucleus; environmental lighting entrains the rhythm, by altering its timing. Independently of sleep, pineal melatonin is inhibited by light and stimulated during darkness. Melatonin deficiency may produce sleeping disorders, behavioral problems, or be associated with precocious or delayed puberty in children, while chronically elevated melatonin has been observed in some cases of hypogonadotropic hypogonadism (Macchi and Bruce, 2004).

Pineal Tumors

The main clinical problem related to the pineal gland is that of pineal tumors (**Table 1**). They are rare, 10 times more common in children than in adults, and mainly derive from the following three types of cells (Brat et al., 2008; Louis et al., 2007; Osborn et al., 2012):

1. Astrocytic tumors (20%).
2. Pineal parenchymal tumors (15%–30%): The 2016 WHO classification grades them from I (pineocytomas, being mostly benign) to IV (pineoblastomas, highly malignant) (**Table 2**). However, most parenchymal tumors show intermediate differentiation (WHO grades II and III, **Table 2**) (Nazato *et al.*, 2016).
   - Pineocytomas (grade I) present more often in adults with a mean age of 43 years, with a male to female ratio of 0.6/1, evolve slowly locally, do not invade contiguous tissue or seed the cerebrospinal fluid (CSF); the major prognostic factor is the extent of surgery.
   - Pineoblastomas (grade IV) typically appear before the age of 20 years, most often in young children, with a slight male preponderance, being more rapidly progressive and of shorter duration (interval between initial symptoms and surgery may be less than a month). Median postsurgical survival varies from 24 to 30 months. They are locally invasive and prone to disseminate through the CSF, often fatal. They may occur in familial bilateral retinoblastoma known as trilateral retinoblastoma syndrome, or in patients with familial adenomatous polyposis. They may also present in patients with *DICER1* germline mutations (Nazato *et al.*, 2016). Negative prognostic predictors are disseminated disease at diagnosis, young patient's age, and partial surgical resection.
   - Intermediate grades II and III represent different degrees of differentiation and prognosis (**Table 2**).
   - Papillary tumors of the pineal region were included as a new entity in the 2007 WHO classification. They are very rare neuroepithelial tumors, macroscopically indistinguishable from pineocytomas, combining papillary and solid areas; however, microscopically, these tumors are easily distinguished. The biological and clinical behavior of these tumors is variable and may correspond to WHO grades II or III, but definite histological grading criteria remain to be defined. The median age at diagnosis is 35 years, but may affect children and adults, with no sex predilection (Fauchon *et al.*, 2000; Fevre-Montange *et al.*, 2010; Jouvet *et al.*, 2000; Mena *et al.*, 1997; Patel *et al.*, 2012; Riis *et al.*, 2013; Schild *et al.*, 1993; Nazato *et al.*, 2016). Local recurrences are common, especially if resection is incomplete, but spinal dissemination is rare. Gross total resection and younger age are associated with overall survival, while radiotherapy and chemotherapy have no significant impact. Higher Ki-67 proliferation index (>10%) or >3 mitoses per 10 high-power fields have a shorter progression-free survival (Nazato *et al.*, 2016).

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Table 1 Classification of pineal tumors

Neuroglial cells (20%)
– Low-grade astrocytomas (juvenile pylocytic)
– Intermediate diffuse and anaplastic astrocytomas
– High-grade malignant glioblastomas
Parenchymal tumors (15%–30%) (see **Table 2**)
Germ cell tumors (80% in Japan, 30%–50% in Western Europe and United States)
– Germinomas
– Nongerminomatous germ cell tumors
– Embryonal carcinoma
– Yolk sac tumor
– Choriocarcinoma
– Teratomas
– Benign teratomas
– Immature
– Mature
– Teratoma with malignant transformation
– Mixed germ cell tumors

Table 2 Parenchymal pineal tumor classification (WHO 2016 grades; Nazato et al., 2016)

Pineocytoma	I
Pineal parenchymal tumor of intermediate differentiation	II or III
Pineoblastoma	IV
Papillary tumor of the pineal region	II or III

3. Germ-cell tumors (30%–50%), with more (teratomas) or less differentiation (germinomas), as well as intermediate degrees (yolk sac tumors). An increased risk of intracranial germ cell tumors has been associated with Klinefelter syndrome, Down syndrome, and neurofibromatosis type 1.

Clinical Presentation

Clinical presentation of pineal tumors depends on age at onset and histology (Bailey *et al.*, 1996; Chang *et al.*, 1995; Gaillard and Jones, 2010). Over 90% present with raised intracranial pressure, often with obstructive hydrocephalus; initial symptoms are frequent headaches, nausea, vomiting, and decreased vision; 50%–70% of patients refer visual signs like diplopia, cranial nerve palsies, papilledema and ptosis, or Parinaud syndrome (failure of upward gaze, pupillary dilatation, and diminution of pupillary light reflex) due to pressure on the pretectal region. Compression on the brain, cerebellum, hypothalamus, and pituitary may cause paralysis of other cranial nerves, ataxia, diabetes insipidus, and hypopituitarism. Pineal tumors may interfere with puberty, due to either pressure of the tumor on the hypothalamic centers, which govern gonadotropin secretion, excessive melatonin secretion by pinealocyte tumors causing delayed puberty in adolescents, or reduction of the potential antigonadotropic effect of melatonin, which, together with beta-hCG secretion by destructive germ cell tumors, could explain precocious puberty in prepubertal children.

Diagnosis

An appropriate tissue specimen for accurate histological diagnosis and determining tumor type is critical to optimize subsequent management. Serum alpha fetoprotein (synthesized mainly by yolk sac tumors, and teratomas) and beta-hCG (in choriocarcinomas or germinomas) concentrations are of diagnostic utility if markedly elevated in serum and/or CSF. Measurement of these markers in CSF for initial staging, and if positive, for follow-up is useful. CSF cytological examination should be delayed at least 2 weeks after surgery to increase the chance of reflecting true dissemination of viable tumor rather than postoperative tumor spillage. If these markers are clearly raised, histological verification may not be required.

Biopsies may be obtained by classical surgical routes (posterior interhemispheric transcallosal, suboccipital transtentorial, and infratentorial-supracerebellar routes) or by microsurgical techniques, with significantly reduced perioperative mortality rates ($<2\%$); a neuroendoscopic or stereotactic biopsy is reasonably safe and well tolerated, in experimented hands, but the diagnosis of mixed or intermediate tumors may be difficult without extensive tissue sampling (Oi *et al.*, 2000). In any case, operative risk

Table 3 Treatment guidelines for pineal tumors

Tumor type	*Radiotherapy*	*Chemotherapy*[a]	*Surgery*
Glial origin			
Juvenile pylocytic astrocytoma	No	No	Complete resection
Intermediate/diffuse/anaplastic/ astrocytomas/glioma	Local	No	Debulking
Malignant glioblastoma	Local	No	Debulking
Parenchymal tumors			
Pineocytoma	Local	No	Biopsy
Intermediate or mixed tumor	Local ± craniospinal	Yes in more undifferentiated tumors	Biopsy
Pineoblastoma	Local Routine craniospinal not always indicated Age <5 years: Lower dose, after initial chemotherapy	Yes (role on final outcome unclear)	Biopsy
Papillary tumors	Local	No	Complete resection
Germ cell tumors			
Germinoma	Local + craniospinal (unless convinced of negative staging)	Yes (alone not curative)	Biopsy
Non-germinatous tumors	Local + craniospinal	Yes (pre- or post-surgery)	Resection as much as possible, without increased morbidity

[a]Chemotherapy includes cisplatin, etoposide, and cyclophosphamide or isofosfamide.

Surgery for histologic biopsy and subtyping, and if necessary CSF diversion (ventriculoperitoneal shunt or ventriculostomy) should always be performed, with the possible exception of germ cell tumors with diagnostically elevated tumor markers.

should be balanced with the risk of not obtaining an accurate histological diagnosis, with prognostic implications. In cases of nondiagnostic or equivocal biopsies or indicative of a benign tumor (mature teratoma, meningioma), surgery is recommended.

Imaging

An MRI will disclose the size and extension of the tumor and possible metastases, but cannot accurately identify the histological nature, which relies on biopsy or serum/CSF tumor markers. In the more malignant tumors (pineoblastomas, germinomas, teratomas) the spine as well as the brain should be imaged, since spread into the subarachnoid space and the spine are frequent.

Treatment

Surgery, chemotherapy, and radiation are used in the treatment of pineal region tumors (Cohen *et al.*, 1995; Echevarría *et al.*, 2008; Jackacki *et al.*, 1995). Surgery, either open, stereotactic, or endoscopic, is used to obtain a biopsy, mandatory in the majority of cases to obtain a definite histological diagnosis. Morbidity and cure rates have improved over the last years thanks to a greater understanding of the nature of the different tumors, more accurate neurosurgical experience, selective use of chemotherapy, and the introduction of modern irradiation techniques. However, the rarity of pineal tumors makes the obtaining of large prospective multicenter international studies to define their optimal management difficult.

Treatment depends on histology obtained after surgery, which apart from the biopsy can resolve intracranial hypertension with a ventricular shunt (atrial or peritoneal) and perform partial debulking of the tumor if possible; total resection is rarely possible. The use of radiotherapy and chemotherapy depends on the type of tumor (**Table 3**).

The optimal treatment for papillary tumors of the pineal region remains controversial, as no definitive treatment strategy exists for this lesion. It has been described that a minimally invasive strategy (radiotherapy or stereotactic radiosurgery) resulted in a favorable response to treatment, avoiding the risks of aggressive surgical removal. However, incomplete resection tended to be associated with decreased survival and with recurrence. In an updated retrospective series of 44 patients, only gross total resection and younger patient age were associated with overall survival; radiotherapy and chemotherapy had no significant impact (Nazato *et al.*, 2016).

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Further Reading

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Vasopressin

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Introduction

Vasopressin is a small, nonapeptide hormone, synthesized in the hypothalamus, and released into the circulation from the posterior pituitary gland. The main physiological stimulus to vasopressin secretion is rising plasma osmolality, though significant reductions in arterial blood pressure and blood volume can also stimulate vasopressin secretion, by unloading of arterial baroreceptors. Although historically named as a result of its potent vasopressor actions, these actions only occur when plasma vasopressin is present in the plasma in supraphysiological concentrations. The most important action of vasopressin is its antidiuretic action on the collecting ducts of the kidney. Vasopressin binds to V2 receptors on the cell surface of tubular cells, initiating an intracellular cascade which results in the generation of the water channel, aquaporin-2. Preformed aquaporin-2 also migrates and inserts into the luminal membrane of the tubule cells, where it acts a conduit for water to be reabsorbed from the urine, through the cell, and back into the circulation. This leads to a decrease in renal free water clearance, concentration of urine, and a reduction in urine volume. The net effect is the reabsorption of water into the blood, which, along with thirst-generated water intake, leads to normalization of plasma osmolality.

Regulation of vasopressin secretion and action thus represents a key homeostatic process which protects the osmotic milieu of the body, allowing normal cellular function. In this chapter we will review the synthesis, secretion and action of this crucial hormone.

Synthesis and Secretion of Vasopressin

Vasopressin is synthesized in the ribosomes of the cell bodies of the magnocellular neurones of the supraoptic and magnocellular nuclei, in the hypothalamus. Once the pre-pro-hormone complex of vasopressin, copeptin and neurophysin II (**Fig. 1**) is synthesized, it is packaged in granules, for axonal transport to the posterior pituitary. The pre-pro-hormone, which has a molecular weight of approximately 20,000 Da progresses down the axons of the magnocellular neurones. During axonal transport, the large molecule is enzymatically cleaved into the three component parts; vasopressin, neurophysin II and copeptin. Copeptin is thought to modulate the three dimensional unfolding of vasopressin into the functioning molecule (Acher et al., 2002). The secretory granules, which contain all three component molecules are then stored in secretory granules at the neuronal terminals in the posterior pituitary, in preparation for secretion into the circulation, in response to osmotic, baroregulatory, or other stimuli. Stimulatory neural inputs, from the osmoreceptors in the anterior hypothalamus, or from the baroreceptors in the arterial circulation, lead to electronic impulses along the magnocellular neurones, and depolarisation of the cell membrane, releasing vasopressin and its copeptides into the systemic circulation.

Some of the vasopressin-containing neurosecretory neurones, mainly from the paraventricular nucleus, terminate in the median eminence (Zimmerman, 1981). Depolarisation of these neurones causes secretion of vasopressin into the portal blood supply to the anterior pituitary gland. Vasopressin is known to augment the CRF-dependent release of ACTH from the anterior pituitary gland (Scott and Dinan, 1998). Other vasopressinergic neurones from the paraventricular nuclei terminate throughout the nervous system, in the lateral and third ventricles, the medulla and the amygdala.

Osmoregulation of Vasopressin Secretion

The principle physiological determinant of plasma vasopressin secretion is plasma osmolality. Rises in plasma osmolality are detected in specialized osmoreceptor cells in the anterior hypothalamus, located in the subfornical organ (SFO) and the organum vasculosum lamina terminalis (OVLT) (Thrasher et al., 1982; Ferguson and Kasting, 1986) (**Fig. 2**). It has been hypothesized that fenestrations in the blood brain barrier in the region of the anterior hypothalamus allows plasma access to these magnocellular neurones, and when plasma osmolality rises, its causes depolarisation of the neurones in the SFO and OVLT. The osmoreceptors for vasopressin release and thirst are situated in close anatomical proximity in this area, so that it is usual for lesions in this area to affect both modalities simultaneously.

Physiological studies in healthy humans has shown that there is a close linear relationship between plasma osmolality and plasma vasopressin concentrations (Robertson et al., 1976; Thompson et al., 1986). Higher experimental rates of rise in plasma osmolality may alter the relationship to a curvilinear vasopressin response, but in physiological conditions, vasopressin rises in a linear fashion (**Fig. 3**, panel A). Experimental studies also confirm that there is a linear response between plasma osmolality and thirst ratings, as measured by a well validated linear analogue scale (**Fig. 3**, panel B) (Thompson et al., 1986; Phillips et al., 1984). When linear regression analysis is applied to the results of osmotic stimulation of thirst and vasopressin release, using hypertonic

Fig. 1 Structure of vasopressin pre-pro-hormone.

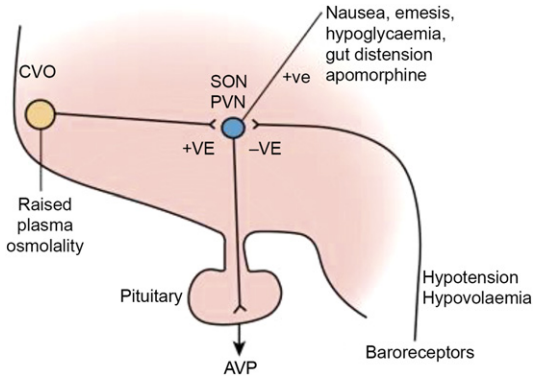

Fig. 2 Factors governing vasopressin secretion. *CVO,* Circumventricular organ; *PVN,* Paraventricular nuclei; *SON,* Supraoptic nuclei. From Hannon, M. J. and Thompson, C. J. (2016). Vasopressin, diabetes insipidus, and the syndrome of inappropriate antidiuresis. *In:* Jameson, J. L. & de Groot, L. J. (eds.) *Endocrinology: Adult and Paediatric.* 7th edn. Philadelphia: Elsevier.

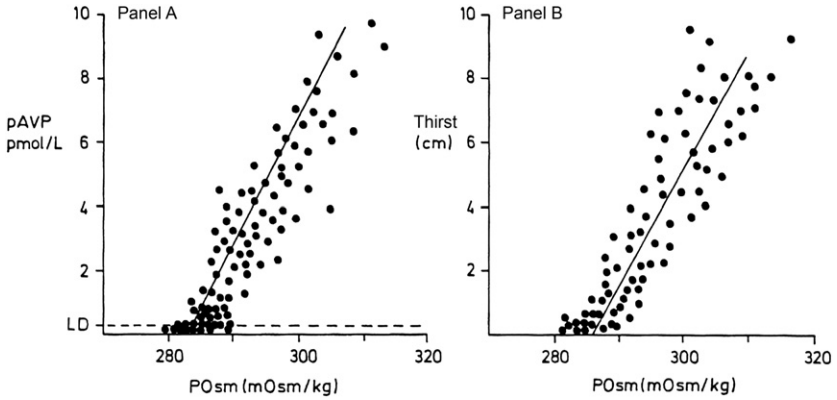

Fig. 3 The relationship between plasma osmolality and plasma vasopressin in healthy man is shown in panel A, and is described by the regression line pAVP = 0.43 (pOsm—284.3), $r = +0.92$, $P < .001$. LD represents the limit of detection of AVP, 0.3 pmol/L (Rooke and Baylis, 1982). Panel B shows the relationship between plasma osmolality and thirst and is defined by the regression line Thirst = 0.39 (pOsm—285), $r = +0.95$, $P < .001$. From Thompson, C. J. (1989). Polyuric states in man. *In:* Baylis, P. H. (ed.) *Baillieres Clinical Endocrinology and Metabolism: Water and salt homeostasis in health and disease.* 1989/08/01 ed. London: Bailliere Tindal & Cox.

saline infusion, it can be demonstrated that the osmotic threshold at which onset of thirst and release of vasopressin begin, are similar in healthy man (Phillips et al., 1984; Thompson et al., 1986). The relationship between plasma osmolality, expressed as both the slope (sensitivity) or the abscissal intercept (osmotic threshold) of the linear regression line seem highly reproducible within an individual, although there is significant interindividual variation (Thompson et al., 1991). Twin studies have shown that there is a high degree of concordance in both slope and intercept of the osmoregulatory line in monozygote, but not dizygote, twins (Zerbe, 1985), suggesting that the characteristics of the osmoregulatory relationship between plasma osmolality and vasopressin release are strongly genetically determined.

The original osmoregulatory studies showed that although the hypothalamic nuclei are osmotically sensitive, they do not respond in a similar way to all osmolytes; in other words they are solute specific. The osmolyte with the most powerful vasopressin secretagogue properties is sodium; urea is less powerful, whereas glucose seems to have little capacity to cause release of vasopressin except in the presence of insulin deficiency (Zerbe and Robertson, 1983; Thompson et al., 1988; Thompson et al., 1989). The inability of acute hyperglycaemia to provoke either thirst or vasopressin secretion is interesting, and perhaps suggests that the thirst associated with uncontrolled diabetes is due to volume depletion, rather than hyperglycaemia. The osmoregulatory relationship between plasma osmolality and vasopressin can also be altered by blood volume, in that the vigor of the vasopressin response to osmotic stimulation is enhanced by volume depletion (Dunn et al., 1973). Teleologically this would represent a

greater need for water conservation, and perhaps additional vasoconstrictor effects, when blood volume or blood pressure is compromised, and therefore it points to a positive adaptive response.

Lowering plasma osmolality has the opposite effect of hyperosmolarity, in that vasopressin and thirst are suppressed. Once plasma osmolality falls below the osmotic threshold indicated by regression analysis—usually around 284–286 mOsm kg^{-1}—plasma vasopressin concentrations become undetectable, even on the most sensitive radioimmunassays, though ultrasensitive cytochemical assays are still able to document low concentrations of vasopressin in the plasma (Baylis et al., 1986). The appearance of a hypotonic aquaresis at low plasma osmolalities does demonstrate that the theoretical concept of an osmotic threshold for vasopressin secretion has clinical relevance however. Although osmotic suppression of vasopressin secretion and thirst occurs, careful experimental studies also suggest an important role for neuroinhibitory reflexes, initiated by the act of drinking. Healthy volunteers allowed unlimited access to water show an immediate switch off of thirst appreciation and vasopressin secretion almost immediately after drinking, while plasma osmolality remains in the elevated range (Seckl et al., 1986; Thompson et al., 1987). Studies in the dog show a similar rapid fall in plasma vasopressin concentration soon after drinking; if water is instilled directly into the stomach via nasogastric tubes, this inhibitory effect does not occur, suggesting that the inhibition of vasopressin occurs more proximally, in the oropharynx (Thrasher et al., 1987). Rat studies, in which dehydration is followed by sham rehydration, suggest that preabsorptive reflexes regulate the activity of vasopressinergic neurones (Knight et al., 2010); Stricker and colleagues concluded that oropharyngeal signals inhibiting vasopressin were dependent upon the volume of the ingested fluid (Stricker and Hoffmann, 2007).

Nonosmotic Stimulation of Vasopressin Secretion

Baroregulation of vasopressin secretion in response to hypovolemia or hypotension is by far the most important of the nonosmotic stimuli. A considerable decline in blood pressure, in the order of 15%–20% is necessary to stimulate significant baroregulated vasopressin. This assumes homeostatic importance in shock, when the drop in blood pressure unloads the baroreceptors, leading to massive release of vasopressin from the posterior pituitary, at plasma concentrations sufficient to cause compensatory vasoconstriction. Baroreceptors are of two main types; low pressure receptors in the left atrium and high pressure receptors in the sino-aortic area. The high pressure receptors send inhibitory signals to the SON and PVN, but when blood pressure or blood volume drop significantly, these receptors are unloaded, and the inhibition of vasopressin secretion is removed. This is a very powerful stimulus to vasopressin secretion which produces plasma concentrations significantly higher than those produced by dehydration. Baroreceptors also modulate the vasopressin responses to osmotic stimuli, with higher plasma vasopressin concentrations per unit rise in plasma osmolality, when blood volume reduction is present (Baylis and Thompson, 1988).

Hypoglycemia (Baylis et al., 1981) causes modest elevations in plasma vasopressin concentrations. Although this could be interpreted as a homeostatic response to hypoglycemia, it is unlikely to be significant, as activation of glycogen breakdown and elevation of blood glucose concentration, has only been demonstrated at plasma vasopressin concentrations many times higher than that achieved by experimental hypoglycemia (Spruce et al., 1985). Nausea is a powerful vasopressin secretagogue.

Actions of Vasopressin

Vasopressin has various actions throughout the body which are mediated by binding to action-specific receptors on the cell surface. The subtypes and tissue-specific actions are shown in **Table 1**.

V1 Receptors

V1 receptors are found in high density on the cell surface of vascular smooth muscle cells, and vasopressin/receptor bindings generates intracellular calcium, via the phosphatidyl-inositol intracellular cascade. This leads to vasoconstriction, though this effect

Table 1 Subtypes and tissue specific actions of vasopressin receptors

Receptor	*Gene*	*Anatomical distribution*	*Intracellular signaling*	*Physiological action*
V1	AVPR1	Vascular smooth muscle Platelets, hepatocytes, myometrium	G-protein coupled phosphatidylinositol and calcium	Vasoconstriction Platelet aggregation Glycogenolysis Uterine contraction Cardiac hypertrophy
V2	AVPR2	Renal collecting duct Vascular endothelium Vascular smooth muscle	Adenyl cyclase and cAMP	AQP-2 synthesis Migration of preformed AVP into luminal cell membrane Secretion of von Willebrand factor and factor VIII
V3	AVPR3	Anterior pituitary gland	G-protein coupled phosphatidylinositol and calcium	ACTH release

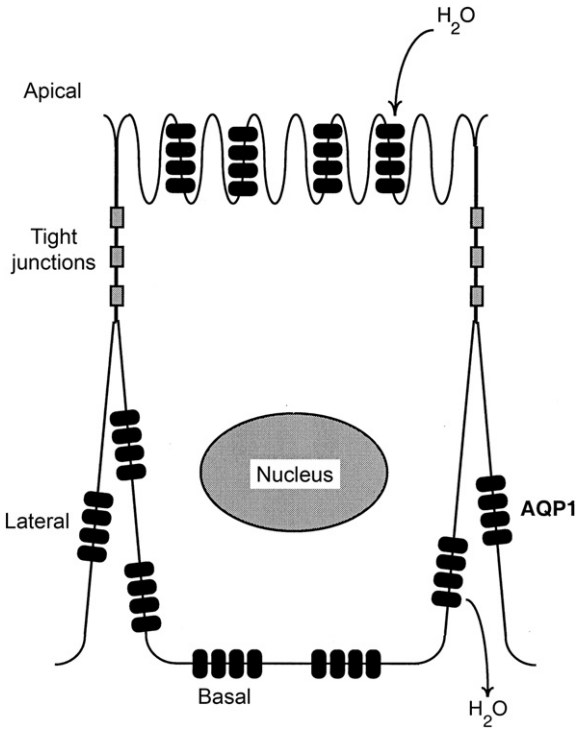

Fig. 4 Diagram representing transcellular water permeation of proximal tubule cell via AQP1. From Agre, P. (2000). Aquaporin water channels in kidney. *Journal of the American Society of Nephrology*, **11**, 764–777.

is not significant when vasopressin is present in the circulation at physiological concentrations. V1 receptors are also located on the cell surface of platelets and receptor binding induces thrombotic effects.

V2 Receptors

V2 receptors are found on the basolateral surface of the cells of the collecting tubules. Activation of these receptors initiates the key homeostatic action of vasopressin, namely water reabsorption from the urine. Binding of vasopressin to the cell surface V2 receptor initiates an intracellular cascade, which activates adenyl cyclase and generates cAMP and protein kinase A. This leads to the increased gene expression, and synthesis of, the Aquaporin water channel AQP-2 (Fushimi et al., 1993; Agre, 2000). In addition, it stimulates the movement of preformed AQP-2 vesicles to the apical cell membrane, where AQP-2 is inserted into the membrane, from which position it allows passage of water from the urine into the interior of the cells (**Fig. 4**). With reabsorption of water from the urine, urine volume falls, free water clearance is reduced, and urine osmolality increases. Failure of AVP/receptor binding, or failure to generate AQP-2, causes inability to concentrate urine, leading to nephrogenic diabetes insipidus. Genetic mutations of the V2 receptor, or, less commonly, mutations in the gene that encodes for AQP-2 production, can produce renal resistance to vasopressin, or congenital nephrogenic diabetes insipidus (Bichet, 2009). Acquired nephrogenic diabetes insipidus also occurs relatively commonly in patients on chronic lithium therapy (Moeller et al., 2013).

There is a sigmoid relationship between plasma vasopressin concentration and urine osmolality. When plasma osmolality has been lowered to below the osmotic threshold for vasopressin secretion and plasma vasopressin is undetectable on conventional radioimmunoassays, urine osmolality is low, usually at <100 mOsm/kg. As plasma vasopressin concentrations rise, there is a progressive urine concentration, with urine osmolalities rising to >800 mOsm/kg (**Fig. 5**). Urine osmolality therefore acts as a sensitive bioassay for the presence of circulating vasopressin. This is used in the diagnosis of SIAD, where urine concentration inappropriate for the low plasma concentration, represented by urine osmolality >100 mOsm/kg, is one of the key diagnostic criteria.

V3 Receptor

The V3 receptor is located in the anterior pituitary and vasopressin binding to this receptor stimulates ACTH secretion. Although in experimental conditions, vasopressin can independently stimulate ACTH secretion, in physiological conditions the role of vasopressin is to augment the secretagogue effect of CRF.

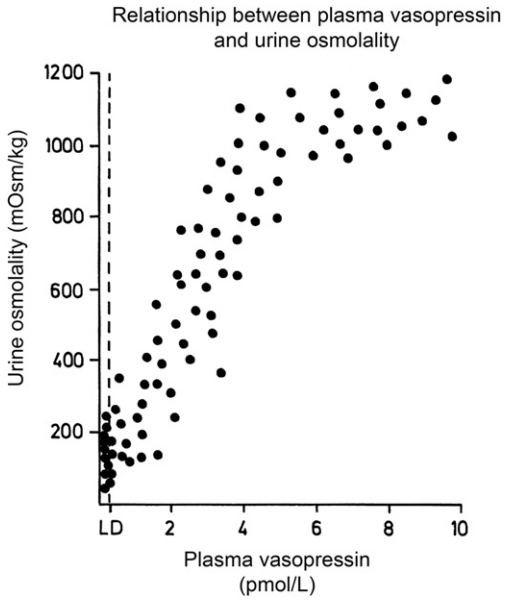

Fig. 5 The relationship between plasma vasopressin and urine osmolality in healthy volunteers during water loading and various stages of water deprivation. LD represents the limit of assay detection for AVP, 0.3 pmol/L (Rooke and Baylis, 1982). From Thompson, C. J. (1989). Polyuric states in man. *In:* Baylis, P. H. (ed.) *Baillieres Clinical Endocrinology and Metabolism: Water and salt homeostasis in health and disease.* 1989/08/01 ed. London: Bailliere Tindal & Cox.

Measurement of Vasopressin

The original experiments of VB Verney proposed the existence of an endogenous antidiuretic substance, after he demonstrated that the intracarotid injection of hypertonic saline into the conscious dog caused a prompt antidiuresis (Verney, 1947). However it was only with the subsequent development of antibodies to arginine vasopressin, which allowed the development of radioimmunoassays for vasopressin (Robertson et al., 1973; Rooke and Baylis, 1982), that the functional characteristics of vasopressin in health and in disease states, could be defined. However, despite the development of these early, highly sensitive radioimmunoassays, there are considerable logistic difficulties in the routine measurement of vasopressin, as either a research tool, or as an adjunct to clinical practice.

Vasopressin is a small molecule, which is present in minute quantities in physiological states. This is particularly difficult in humans, in whom social drinking suppresses vasopressin concentrations to close to the limit of even the most sensitive of assays. It is therefore very difficult to distinguish between physiological plasma concentrations and biologically suppressed concentrations; many of the commercial antibodies available for the development of radioimmunassays are completely unable to measure basal hormone levels with any degree of accuracy. The low plasma concentrations of vasopressin mean that the hormone must be extracted from plasma prior to assay, and the delicate nature of the hormone means that samples must be flash frozen in a $-70^{\circ}C$ freezer. Finally the assay is ponderous, time consuming and only available in a few highly specialized laboratories where it is principally used as a research tool.

This has led to interest in the measurement of copeptin as an alternate to vasopressin. Copeptin is a 39 amino acid peptide, which is a larger molecule, with a longer half life than vasopressin (Fenske et al., 2018). It is cosecreted into the plasma with vasopressin, and although it has no intrinsic biological activity, it offers a surrogate for plasma vasopressin concentrations, which is quicker, easier and cheaper to measure (Szinnai et al., 2007). Although it has been proposed that measurement of copeptin may be of value in the differential diagnosis of hyponatraemia (Fenske et al., 2009), it is unlikely that this will be of clinical value, as plasma vasopressin concentrations are elevated in almost all cases of hyponatraemia, and measurement of plasma vasopressin concentration does not constitute an important component of the diagnostic process. There has been far more interest in the use of copeptin in the differential diagnosis of polyuric states (Balanescu et al., 2011; Christ-Crain and Fenske, 2016); suppressed plasma levels in diabetes insipidus correlate with undetectable plasma vasopressin concentrations, though both hormones rise during osmotic stimulation in patients with primary thirst disorders. Given that copeptin can be readily measured in most competent laboratories, measurement of this peptide is likely to replace the occasional measurement of vasopressin in clinical practice.

Disorders of Vasopressin Secretion

Subnormal or absent vasopressin secretion gives rise to the passage of high volumes of hypotonic urine, a condition known as diabetes insipidus. A wide variety of neoplastic, inflammatory, vascular and infective conditions in the hypothalamo-pituitary area

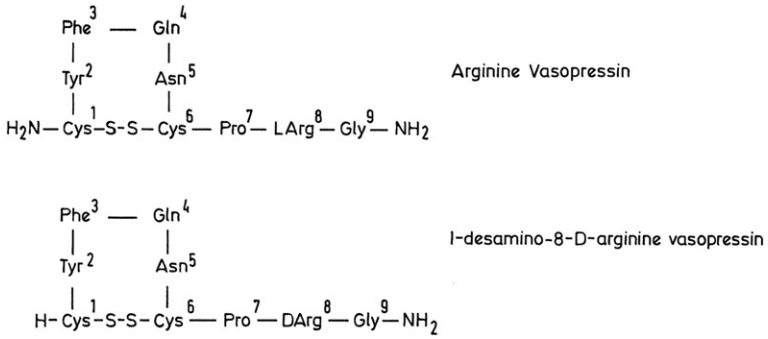

Fig. 6 Structures of arginine vasopressin and the structural analogue 1-desamino-8-D-arginine vasopressin.

can impair vasopressin secretion and produce central diabetes insipidus. In addition, genetic defects of the vasopressin V2 receptor, and a number of metabolic abnormalities, as well as lithium therapy, can cause insensitivity to the antidiuretic actions of vasopressin, leading to nephrogenic diabetes insipidus.

Conversely, abnormal or exaggerated secretion of vasopressin leads to reduction in free water clearance, accumulation of extracellular water, and the development of hyponatraemia.

These conditions are covered in depth in the relevant chapters of this textbook.

Clinical Uses of Vasopressin

The pressor properties of vasopressin are used to treat hypotensive shock, particularly due to bleeding oesophageal varices. Because the V1 receptors are widespread in the circulation, vasoconstriction is not confined to bleeding areas, and therapeutic use of intravenous vasopressin may lead to mesenteric and coronary artery vasoconstriction, leading to abdominal cramps or angina.

Because of the short half life of endogenous vasopressin (approximately 5 min), the native molecule has to be altered to make it useful for long-term treatment of diabetes insipidus. Desmopressin (ddAVP) is synthesized with two modifications to the molecular structure of native vasopressin; the amino group in the cysteine has been removed to prolong the plasma half life (Sawyer et al., 1974b) and D-arginine has been substituted for L-arginine to minimize pressor activity (**Fig. 6**) (Sawyer et al., 1974a). Desmopressin is available in intranasal and oral form. The most common side effect of therapy is dilutional hyponatraemia, occurring in 27% of ambulatory blood samples in patients with diabetes insipidus, and causing admission with hyponatraemia in 5.8% of patients with normal thirst appreciation (Behan et al., 2015). Dilutional hyponatraemia can be effectively prevented by delaying taking desmopressin once or twice weekly; this allows an aquaresis to occur, which prevents accumulation of water reabsorbed by the antidiuretic actions of desmopressin. Alternatively patients can delay every dose of desmopressin until clinically needed to prevent polyuria, though this method often leads to unacceptable disruptions to life routines.

Summary

This article explores the physiology of vasopressin synthesis, secretion and action, in order to provide the basis for the disorders of secretion and action which will be described in later articles. Vasopressin is a unique hormone; despite the small molecular weight, it has a fundamental role in maintaining cellular homeostasis.

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Effects of Steroid Hormones on Brain☆

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Glossary

Dendrite Branching neurites that emanate from the neuronal cell body.
Dendritic spine A small protrusion of the cell membrane of the dendrite that receives excitatory input.
Glucocorticoids Steroid hormones released from the cortex of the adrenal gland such as cortisol and corticosterone.
Neurogenesis The production of new neurons.
Sex steroids Steroid hormones released from the gonads such as androgens, estrogens, and progestins.
Steroid hormones A family of hormones derived from cholesterol and characterized by their three six'carbon rings and one five'carbon ring.

Introduction

The brain receives a rich supply of blood and is constantly in contact with circulating hormones in the bloodstream. Thus, it is not surprising that the brain is influenced and modulated by hormones. One major class of hormones is the steroid hormones, which gain access to the brain because of their hydrophobic chemical structure that allows them to pass across the blood–brain barrier. Steroid hormones have a wide range of effects on the brain. For instance, steroids help to shape the brain during perinatal development and continue to modulate the structure and function of the central nervous system (CNS) into adulthood and throughout the life span. Specifically, steroids can influence factors such as neuronal survival, neurogenesis, neurite outgrowth, synaptogenesis, receptor expression, RNA synthesis, and neuronal excitability. These effects at the cellular level affect many aspects of mood, cognition and behavior. The effects of the steroid hormones on the development, structure, and function of the brain are the focus of this article.

The Mechanisms of Steroid Hormone Action in the Brain

Steroid hormones can be classified into the gonadal sex steroids (androgens, estrogens, progestins) and the adrenal corticosteroids. Corticosteroids in turn are either mineralocorticoids involved in salt homeostasis (e.g., aldosterone), or the glucocorticoid stress hormones (e.g., cortisol). During pregnancy, the placenta also produces steroid hormones. The biosynthesis of all steroid hormones starts with enzymatic cleavage of cholesterol. All endocrine organs can convert cholesterol to pregnenolone inside mitochondria and further convert pregnenolone to progesterone in the endoplasmic reticulum. Further enzymatic reactions are performed by tissue specific enzymes that are expressed only in some of the endocrine organs (Hanukoglu, 1992). For example, testosterone is produced in the gonads by a series of enzymatic reactions that convert cholesterol to progesterone, and then via other intermediates to testosterone (Payne and Hales, 2004). Testosterone can be converted to estrogen by the aromatase enzyme in a reaction called aromatization. Although testosterone is considered the "male sex steroid" and estrogen is considered the "female sex steroid," it is important to note that testosterone and estrogen are found in both males and females. In fact, estrogen formed locally in the brain of males by the aromatization of testosterone is responsible for masculinization of the male brain and plays a major role in activating male sexual behavior during adulthood. Such pre-receptor metabolism also occurs for glucocorticoids, that may be inactivated or regenerated by the 11β-hydroxysteroid dehydrogenase 1 or type 2 (11β-HSD1/2). 11β-HSD1 is highly expressed in the hippocampus converting inactive cortisone into active cortisol. Conversely, 11β-HSD2 converts active cortisol into inactive cortisone to exclude cortisol from binding the mineralocorticoid receptors in, for example the kidney and a limited number of brain areas to enable aldosterone rather than cortisol to bind the mineralocorticoid receptor (Seckl and Walker, 2001).

Localisation

The steroid hormones bind to specific receptors located throughout the brain. All steroid hormones can bind to cognate nuclear receptors; intracellular receptor that can bind to DNA to regulate gene expression. This allows for both the "activational" effects

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within hours of activation and "organizational" effects during development that are very long lasting. The structure of the different steroid receptors is very similar, with the two estrogen receptors (ER) ERα and ERβ being a bit more distant from the androgen, progesterone, glucocorticoid, and mineralocorticoid receptors. Each of the six classical steroid receptors has a unique expression pattern in the brain, and this allows for a large number of steroid effects, that may show considerable region specificity even for a particular steroid (Mahfouz *et al.*, 2016).

The gonadal steroid progesterone binds to the progesterone receptor (PR), which has two isoforms that are derived from the same gene: PRA and PRB. Both PRA and PRB are expressed in the hypothalamus, hippocampus, and cortex. In addition, progesterone binds to a G-protein coupled seven transmembrane receptor (GPCR) known as 7TMPR or mPR. mPR has three isoforms α, β and γ, of which the γ isoform is specifically expressed in neural tissues (Brinton *et al.*, 2008). Also estrogen can activate a classical GPCR, GPR30, adding to the signaling repertoire of the steroids (Prossnitz *et al.*, 2008). High expression of the classical ERα and ERβ is found in amygdala, hypothalamus, prefrontal cortex, hippocampus, thalamus, and cerebral cortex (Hara *et al.*, 2015). The other gonadal steroid testosterone binds to the androgen receptor (AR), which is expressed in the brain both in hypothalamus and in higher brain regions, such as the hippocampus (Beyenburg *et al.*, 2000).

Cortisol can, in the brain, bind two types of receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Reul and de Kloet, 1985). Aldosterone also binds to the MR, but is outcompeted by corticosterone (in rats and mice) and cortisol unless these are enzymatically degraded (Wyrwoll *et al.*, 2011; Funder, 2005). In the brain most MRs are cortisol preferring. Cortisol has a higher binding affinity for MR than GR. As a consequence, at low cortisol levels MRs are activated and with high cortisol levels, for example after stress, GRs get activated. MR is abundantly expressed in limbic neurons, most notably the principal neurons of the hippocampus, while GR is expressed in neurons and glial cells throughout the brain. In the hippocampus, MR and GR are co-localized and can mediate opposite effects of cortisol. Given its high affinity and pre-stress occupancy, MR is important in the onset of the stress response and in appraisal of stimuli, while GR is involved in memory storage (de Kloet *et al.*, 2005). More effects of the individual steroids hormones will be discussed later in this article.

Signaling Mechanisms

There are three mechanisms through which steroid hormones exert their actions on neurons: genomic, indirect genomic, and nongenomic. The various mechanisms of steroid hormone action are presented in **Fig. 1**. The fastest, but least understood mode of action is non-genomic. They may be mediated by dedicated membrane type receptors in case of the sex steroids, but also by membrane associated variants of the classical nuclear receptors. For instance, steroids have been shown to bind to receptors located in the plasma membrane of neurons, and rapidly affect synaptic (re-)activity, for example, spontaneous glutamate release in the hippocampus (Karst *et al.*, 2010). Extranuclear estrogen receptors were found in axon terminals and spines of hippocampal CA1 pyramidal cells of the female rat. Various mRNAs for synaptic proteins and translation machinery have been localized in spines, suggesting that translation of such proteins can occur locally in the dendrite. Thus, estrogens may act directly on the synaptic apparatus and mRNAs to affect synaptic function and synaptogenesis. These non-genomic effects of steroids become manifest within minutes after receptor activation.

Eventually, events at the cell membrane may also via activation of second messenger pathways lead to the activation of transcription factors in the cell nucleus. The "classical" and slower genomic mechanism of steroid hormone action begins by the hormone diffusing through the plasma membrane of the neuron and binding to its intracellular receptor located in either the cytoplasm or the nucleus. This hormone–receptor complex then undergoes a conformational change allowing the hormone–receptor complex to migrate, dimerize, and bind to particular hormone response elements (HREs) on the DNA. Once the hormone–receptor complex binds to its HRE, it subsequently binds co-activators or co-repressors to activate or repress gene transcription, ultimately affecting the production of proteins that may alter the functioning of the neuron, or glia cell. This mode of signaling allows cross talk between different steroid receptors, as MR, GR, AR, and PR recognize (in part) the same HRE elements (Falkenstein *et al.*, 2000).

Steroids receptors may also interact with other, non-receptor, transcription factors. For example, GR may interact with other, non-receptor, transcription factors. For example, GR may interact with AP-1, where only the latter factor is bound to the DNA. Genome wide analysis of GR and MR binding at the chromatin for now indicates that direct HRE binding of the receptors is the dominant mode of action in the rodent hippocampus (van Weert *et al.*, 2017).

Organization Versus Activation

Steroid hormones not only allow for the activation of certain behavioral and physiological events during adulthood but also shape the nervous system during development. During perinatal development, the brains of males and females are exposed to different hormonal milieus that ultimately lead to many of the sex differences observed in the brain and behavior of males and females during adulthood. For instance, males experience increases in testosterone production and secretion during both prenatal and early neonatal development. This, on conversion in the brain to estradiol via the aromatase enzyme, organizes the brain in a masculine fashion. This early masculinization of the brain then allows the hormonal stimulation received during adulthood to act on these organized neural pathways to activate the appropriate male behaviors. In the perinatal female, the estrogen produced by the mother and the prenatal ovary does not masculinize her brain because this estrogen is bound by alpha fetoprotein and is

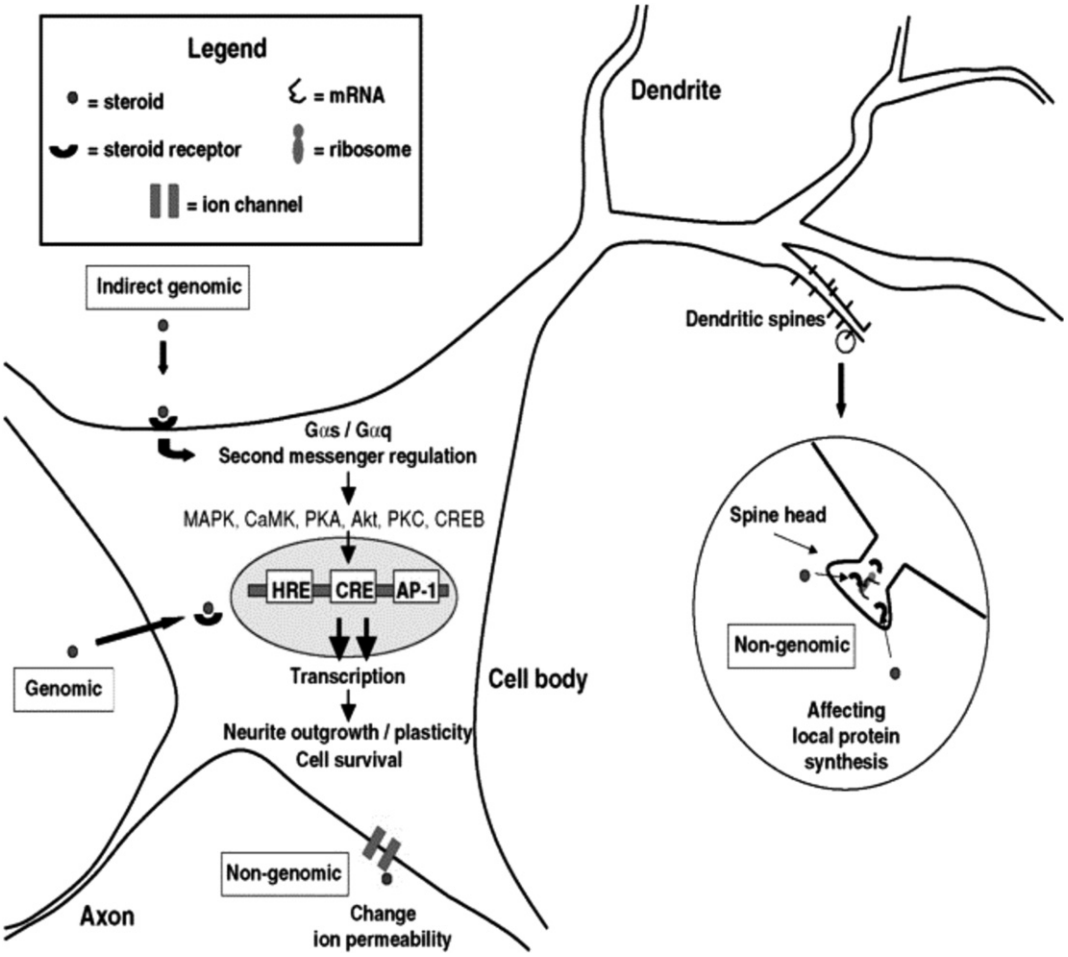

Fig. 1 The genomic, indirect genomic, and nongenomic mechanisms of steroid hormone action on neurons. Indirect genomic mechanisms include the activation of steroid receptors linked to second messenger cascades that activate gene transcription through DNA-binding domains such as cAMP response element (CRE) and activator protein-1 (AP-1), ultimately altering neuronal function. In the genomic mechanism, the steroid binds to the intracellular form of the receptor, permitting the steroid–receptor complex to translocate to the nucleus. This steroid–receptor complex then binds to the hormone response element (HRE), activating transcription of the genome and altering neuronal function. Nongenomic mechanisms of steroid action include the ability of steroids to influence ion permeability and possibly to affect local protein synthesis in the spine head. This later mechanism presumably would allow for the rapid regulation of spine-specific mRNAs and proteins. *Akt*, protein kinase B; *CaMK*, Ca^{2+}/calmodulin-dependent protein kinase; *CREB*, cAMP response element-binding protein; *Gαs and Gαq*, guanosine triphosphate (GTP)-binding proteins; *MAPK*, mitogen-activated protein kinase; *PKA*, protein kinase A; *PKC*, protein kinase C.

unable to cross the blood–brain barrier. Indeed, the feminized brain appears to be the default pattern of development in that a brain that does not receive androgenic and estrogenic stimulation during development results in a feminized brain.

The organizing influences of steroid hormones on the perinatal brain and the resulting morphological and functional differences between the sexes can be profound. Indeed, the organizing effects of steroids result in certain sexually dimorphic nuclei in the nervous system that may be as much as five times larger in one sex than in the other. Likewise, early life stress (during the first weeks of life in the rodent) can have long lasting consequences for the organization of brain circuits. However, in these cases the role of the actual steroid exposure is less well understood, as there are many other mediators activated upon stress. The effects of steroids on the adult brain are much more subtle than these organizing effects during perinatal development. However, steroids are still capable of shaping the adult brain in important ways (Becker *et al.*, 2002).

Effects of Steroids Hormones During Adulthood

Steroid hormones allow for the activation of certain behavior and psychological events during adulthood. For example in rats, female sexual lordosis behavior does not occur in absence of estrogens, but can be primed by a single estrogen treatment 2 h before the encounter with a male rat (Becker *et al.*, 2002). Sex steroids do not only affect reproduction but also other mental domains, such as testosterone effects on aggression. In male mice, aggression is positively correlated with increased circulating testosterone levels and castration of male mice show decreased aggressive behavior (Book *et al.*, 2001).

The stress hormone cortisol has substantial effects on behavior. Cortisol binding to the MR influences emotional and cognitive processes, such as memory retrieval, appraisal of potentially stressful situations and the response to stress. Binding to GR will lead to behavioral adaptation and memory storage, and can effect domains as different as reward, eating behavior, anxiety and mood, decision making and sleep (Meijer and de Kloet, 2017). These immediate effects of cortisol are beneficial, but chronic stress with long-term exposure to high cortisol levels has negative effects, such as impaired learning, disturbed anxiety and aggression regulation, increased risk for depression, cardiovascular problems, osteoporosis and obesity (de Kloet *et al.*, 2005). The negative effects of chronic exposure to high levels of cortisol are underscored by the many psychiatric and cognitive complaints in Cushing's disease patients, some of which can persist for many years (Andela *et al.*, 2013).

Steroid-Induced Neuronal Plasticity During Adulthood

Sex steroids have been shown to affect the density and morphology of dendritic spines. Spines are membranous protrusions emanating from dendrites and are sites of synaptic contact between neurons. Neurons receive excitatory input via these dendritic spines, which contain various types of glutamate receptors, scaffolding proteins, and signaling molecules. Spines come in various sizes and shapes that may be indicative of their maturity. Different steroids can affect the growth and development of spines, thereby modulating excitatory neuronal signaling.

The hypothalamus was the first brain area in which sex steroids were shown to influence synapse formation and spine maturation. Ovariectomized females have significantly fewer synaptic contacts in their ventromedial nucleus (VMN) than do ovariectomized females treated with estrogen. Furthermore, females in the proestrous stage of their estrous cycle (i.e., when estrogen levels are high) have a greater spine density than do females in diestrus (i.e., when estrogen levels are relatively low). The VMN is a fundamental component of the neural circuit that mediates the expression of female reproductive behavior. Thus, it is not surprising that estrogenic stimulation that activates female mating behavior would also promote spine density and, hence, increase excitatory input to the VMN. In males, VMN spine density increases after castration, whereas estrogen-treated males exhibit a relatively low number of spines, similar to the density observed in intact males. Thus, the effect of estrogen on VMN spine density in males is opposite that in females.

Similar effects have been observed in the arcuate nucleus (ARC) of the hypothalamus, which plays an important role in mediating the release of the gonadotropins from the anterior pituitary. The dendrites of these neurons in hormone-dependently females have twice as many spines than males, and presumably the greater excitatory input to the female ARC, may help to modulate the luteinizing hormone surge that causes ovulation in females.

Interestingly, such estrogen effects also occur in the hippocampus, and may in this way affect the organism's ability to remember. In females with higher estrogen levels significantly greater numbers of these dendritic spines were found compared with low estrogen levels. Additional studies have shown that this increased spine density is accompanied by an increased synaptic input to the apical dendrites of the hippocampal CA1 pyramidal cells. These structural alterations are paralleled by physiological and behavioral changes as well. Specifically, adult female rats experiencing high levels of estrogen show enhanced hippocampal long-term potentiation (LTP), a putative electrophysiological correlate of learning and memory, and improved memory retention on a hippocampal-dependent spatial memory task.

Whether estrogen increases hippocampal spine formation in humans is unknown, but estrogen replacement therapy has been shown to promote cognition in postmenopausal women (Bean *et al.*, 2014; Hara *et al.*, 2015). It also has been suggested that estrogen may aid in the prevention or amelioration of neurodegenerative diseases such as Alzheimer's disease. Another study has shown that estrogen can induce greater spine density in the prefrontal cortex of nonhuman primates. Given that the prefrontal cortex of primates is involved in many aspects of cognitive function, it is possible that the memory-enhancing effects of estrogen are mediated, at least in part, by its actions on both the hippocampus and the cortex.

Males do not show the same increase in spine density in response to estrogen. However, testosterone has been shown to increase the number of spines on the CA1 pyramidal cells of males. In addition, it was shown that neural AR deletion selectively impairs hippocampal LTP (Picot *et al.*, 2016).

Cortisol daily rhythmicity helps to sustain a normal rate of spine turnover. However, in contrast to the spine-promoting effects of estrogen and testosterone, high levels of corticosterone induced by repeated stressors, have been shown to decrease the branching of hippocampal CA3 pyramidal cells and to cause dendritic atrophy. Animals experiencing chronic stress also tend to perform more poorly on learning and memory tasks than do non-stressed animals. It is important to note that low to intermediate levels of "stress hormones" enhance performance on learning and memory tasks. Studies have found that high levels of corticosterone suppress LTP, whereas intermediate levels enhance plasticity. In general, MRs and GRs activation lead to behavioral adaptation, but with chronic stress MR and GR gene expression seem to adapt (de Kloet *et al.*, 2005). Both animal studies, and genetic variants in humans point to GR overactivation as a risk factor for mood disorders. Interesting, the MR gain of function genetic variant consistently protects against depression in several studies (Meijer and de Kloet, 2017). However, more research is needed to establish and explain the causal link.

Another mechanism that can alter MR and GR response is glucocorticoid medication. Use of synthetic glucocorticoids, like dexamethasone, can cause severe adverse effects, especially when high dosage is used. Severe neuropsychiatric consequences like psychosis, mania, depression, suicide (attempt), delirium, panic disorder and confusion or disorientation are reported (Judd *et al.*, 2014). In addition, cognitive and memory impairments, changes in mood and sleep disturbances (Meijer and de Kloet, 2017; Judd

et al., 2014). Normally, MR and GR activation is balanced, but glucocorticoid medication can disturb this balance. For example, dexamethasone is highly selective for GR and high dosage will lead to a suppressed cortisol production, depleting the MR of its ligand and resulting in a GR MR imbalance. It is thought that co-administration of cortisol with dexamethasone might prevent these adverse effects, since MR is re-activated and this way restoring the GR MR balance (Meijer and de Kloet, 2017).

Neuronal Protection and Neurogenesis

As discussed above, steroids can have profound influences on the structure and function of neurons early in development and into adulthood. In addition to these influences, steroids can alter the survival and production of new neurons, even in the adult brain. For instance, at high concentrations, estrogen can protect neurons by working as an antioxidant. Estrogen has also been shown to diminish the neural damage induced by stroke and reduced blood flow to the cortex. These neurotropic effects after stroke may be through the antioxidant properties and/or through the influence of estrogen on growth factor synthesis and receptor expression.

Steroid hormones have also been shown to affect neurogenesis. Estrogen has been demonstrated to increase the production of new neurons in the dentate gyrus of the female hippocampus. Conversely, animals experiencing high levels of corticosterone show less cellular proliferation in the dentate gyrus than do animals experiencing low levels of corticosterone. This corticosterone-induced decrease in neurogenesis suggests that high levels of corticosterone not only promote atrophy (and even loss) of neurons in the hippocampus but also slow the production of new neurons that may be needed to replace the lost cells.

Conclusion

Steroid hormones secreted from the gonads and adrenals act on the brain and are known to influence diverse functions such as reproduction and learning and memory. These hormones can act on neurons through a genomic, an indirect genomic, or a nongenomic mechanism of action. The sex steroids have been shown to organize the male and female perinatal brain so that the proper physiology and behavior can be activated by these hormones during adulthood. Furthermore, steroids continue to shape the adult brain by affecting factors such as synaptogenesis, spine maturation and growth, neuronal branching, neuroprotection, and neurogenesis. Therefore, steroids not only contribute to the development of the nervous system but also continue to influence neuronal structure and function throughout the life span.

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Memory and Epigenetics: Role of Estrogen

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Introduction

Evidence that hormones could regulate behavior was first demonstrated in 1849 when Arnold Berthold showed that castration and reimplantation of the testes in roosters affected normal male development, sexual desire, and aggression. However, only in the last 30 years has it been demonstrated that sex steroid hormones are poised to mediate memory formation. Some of the first evidence supporting this notion came from the discovery of estrogen receptors in the dorsal hippocampus and the entorhinal cortex (Loy *et al.*, 1988; Maggi *et al.*, 1989) and from seminal work demonstrating that spine density in the CA1 region of the hippocampus could be regulated by estrogens, including the potent 17β-estradiol (E_2), and progesterone in female rats (Gould *et al.*, 1990; Woolley *et al.*, 1990; Woolley and McEwen, 1992, 1993). In the nearly three decades since the initial demonstration that ovarian hormones regulate CA1 dendritic spine density, preclinical research has provided evidence that E_2 can enhance learning and memory in adults of a variety of species, including songbirds, rodents, nonhuman primates, and humans (for reviews see Maki, 2012; Hammond and Gibbs, 2011; Frick, 2009, 2012; Schlinger and Remage-Healey, 2012; Bimonte-Nelson *et al.*, 2010). Recent work has demonstrated that E_2 employs numerous cellular and molecular mechanisms, including epigenetic processes, to influence memory formation, and these epigenetic mechanisms will constitute the focus of this review. A general overview of the beneficial effects of E_2 on memory will first be described below, followed by a discussion of the epigenetic mechanisms through which E_2 exerts these effects.

The Role of E_2 in Cognitive Function

Estradiol and Cognitive Function in Humans

Clinical studies investigating the estrogenic regulation of memory have focused largely on the effects of estrogens in the context of aging (i.e., as it relates to menopause), or changes in performance in cognitive tasks across the menstrual cycle. With respect to aging, numerous studies have shown that ovarian hormone loss due to natural or surgical menopause impairs various aspects of cognitive function, including verbal and spatial memory (Sherwin and Henry, 2008) (**Fig. 1**). Menopausal women are also at increased risk of Alzheimer's disease relative to men, even when accounting for women's longer lifespans (Dye *et al.*, 2012; Launer *et al.*, 1999). Indeed, longer periods of lifetime estrogen exposure are significantly and negatively correlated with the likelihood of

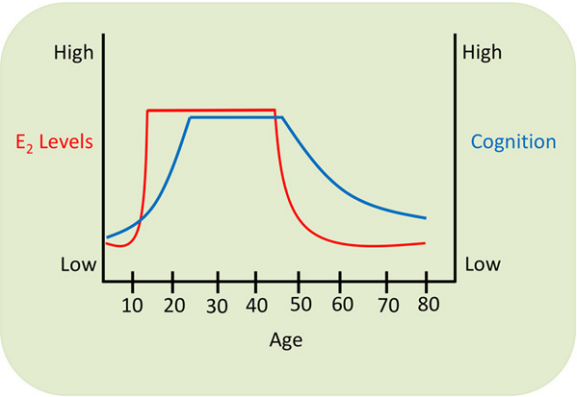

Fig. 1 Schematic illustration of changes in estradiol (E_2) levels and cognitive abilities across the life span in women. E_2 levels surge upwards at puberty and remain elevated (although fluctuating across the menstrual cycle) during the reproductive years. In the early 50's, E_2 levels drop substantially at menopause. Cognitive function reaches adult levels in the early-mid 20s and then remains relatively stable until middle-age, at which point certain aspects of cognitive function (e.g., episodic memory, divided attention, spatial navigation) decline gradually with age.

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developing Alzheimer's disease in women (Fox *et al.*, 2013). The deleterious effects of menopause on cognition and risk of Alzheimer's disease can be reduced by E_2 therapy (Rocca *et al.*, 2007, 2011), suggesting not only that E_2 loss at menopause contributes to memory dysfunction, but also that maintaining E_2 levels at menopause may help prevent Alzheimer's disease and reduce age-related cognitive decline.

Evidence also indicates that E_2 fluctuations across the month-long menstrual cycle in premenopausal women impacts certain aspects of cognitive function. For example, women experiencing high E_2 levels during the midluteal phase of the cycle perform better in tests of verbal fluency, fine motor, and perceptual speed than women experiencing low E_2 levels during the menstrual phase (Hampson, 1990; Maki *et al.*, 2002). Some evidence suggests that visual memory is also poorer during the menstrual phase (Phillips and Sherwin, 1992). High levels of E_2 do not always correlate with better performance on cognitive tasks, however, as women's performance appears to be best in tasks in which men traditionally outperform females (e.g., those related to spatial ability) when E_2 levels are low (Hampson, 1990; Maki *et al.*, 2002).

In addition to its role in regulating normal cognitive function, E_2 has also been implicated in several disorders that disproportionately affect women relative to men, including Alzheimer's disease, posttraumatic stress disorder (PTSD), and certain aspects of addiction. Although beyond the scope of this article, reviews on the role of E_2 in Alzheimer's disease (Dye *et al.*, 2012), PTSD (Albert *et al.*, 2015; Milad *et al.*, 2010), traumatic brain injury (Asl *et al.*, 2013; Day *et al.*, 2013b), and substance abuse (Becker, 2016) provide important insights into the ways in which sex-steroid hormones may impact disorders characterized by memory dysregulation, and suggest directions for future research to better understand underlying neural mechanisms.

Animal Models Demonstrate the Importance of E_2 in Cognitive Function

Basic scientific research conducted in animal models is critical for mechanistic investigations into the role of E_2 in cognitive function. E_2-mediated actions in brain regions that support cognition, such as the hippocampus, are complex and impacted by numerous factors, including dose, duration of treatment, age, length of ovarian hormone deprivation prior to treatment, type of cognitive task, timing of administration relative to testing, task difficulty, and reproductive history (Acosta *et al.*, 2009, 2010; Luine, 2014; Daniel, 2006; Frick, 2009). However, some generalizations can be drawn, particularly among studies utilizing rodent models. The majority of rodent studies support the conclusion that E_2 facilitates learning and memory in behavioral tasks that require the hippocampus (Packard and Teather, 1997; Fader *et al.*, 1998; Daniel *et al.*, 1999; Luine *et al.*, 2003; Walf *et al.*, 2008; Fernandez *et al.*, 2008; Lewis *et al.*, 2008; Zhao *et al.*, 2010; Daniel, 2006; Fan *et al.*, 2010); see (Daniel, 2006; Tuscher *et al.*, 2015) for review. For example, young adult female rodents treated with exogenous E_2 exhibit enhanced spatial memory in the object placement, Morris water maze, radial arm maze, and T-maze tasks (Sandstrom and Williams, 2004; Luine *et al.*, 1998; Fader *et al.*, 1998, 1999; Daniel *et al.*, 1997; Bowman *et al.*, 2002; Bimonte *et al.*, 2002). E_2 can also facilitate memory in a number of nonspatial tasks, as demonstrated in object recognition (Fernandez *et al.*, 2008; Fortress *et al.*, 2013; Boulware *et al.*, 2013), social recognition (Phan *et al.*, 2012), inhibitory avoidance (Singh *et al.*, 1994; Rhodes and Frye, 2004), fear conditioning (Lebron-Milad and Milad, 2012; Chang *et al.*, 2009; Barha *et al.*, 2010; Zeidan *et al.*, 2011; Milad *et al.*, 2010), and trace eyeblink conditioning (Leuner *et al.*, 2004). Collectively, these studies provide evidence that E_2 treatment can benefit hippocampal memory in numerous behavioral tasks. The molecular mechanisms through which E_2 exerts these beneficial effects will be discussed in greater detail below.

Estrogen Receptor Expression and Cell-Signaling Mechanisms

Protein synthesis is an essential component of memory formation, and E_2 regulates the synthesis of new proteins through at least two different estrogen receptor (ER)-mediated mechanisms: the classical genomic pathway and the rapid nonclassical activation of cell-signaling pathways. Many of the brain regions that support memory formation express classical intracellular ERs (ERα and ERβ), which are found within the cytoplasm and nucleus of the cell. Both ERα and ERβ have their own distinct patterns of expression in the cerebral cortex, basal forebrain, amygdala, prefrontal cortex, and hippocampus in a variety of species, including mouse, rat, nonhuman primates, and humans (Gillies and McArthur, 2010). The classical "genomic" action of E_2 is initiated once the hormone diffuses through the target cell's outer membrane and binds ERα or ERβ within the cytoplasm (Nelson, 2000). Once the E_2-ER complex is formed, it translocates to the nucleus, where it binds to estrogen response elements on the DNA. Here, the complex acts as a transcription factor, and can initiate the transcription of E_2-sensitive genes that help maintain the neural circuitry that ultimately influences behavior (Heldring *et al.*, 2007; Jensen, 1962). Changes in gene expression elicited by such nuclear ER-hormone interactions via the genomic mode of action occur slowly (on the scale of hours—days) and are thought to yield long lasting changes.

E_2 can also influence cell function in a nonclassical manner by binding to membrane-associated ERs (mERs; e.g., GPER, Gq-mER; Srivastava and Evans, 2013), classical ERs located near the membrane (Boulware *et al.*, 2005, 2013), or by interacting with neurotransmitter receptors (e.g., mGluRs, NMDARs; Lewis *et al.*, 2008; Boulware *et al.*, 2005, 2013) to rapidly activate intracellular signaling pathways on the order of seconds to minutes (Gillies and McArthur, 2010). Although these mechanisms are often referred to as "nongenomic," this designation should not be taken literally, as activation of mERs can ultimately influence gene transcription. Rather, it should be thought of as way to distinguish between the effects of mERs and classical nuclear ER activation.

Work in our own lab has focused on the cell-signaling pathways through which E_2 regulates function in the dorsal hippocampus (DH) and medial prefrontal cortex, brain regions critical for learning and memory and whose function is compromised in various neuropsychiatric disorders and during aging (Small *et al.*, 2011; Maillet and Rajah, 2013; Sampath *et al.*, 2017). Rapid activation of cell-signaling cascades in these regions allows for acute modulation of cellular function in response to an experience (i.e., learning). Several pathways previously identified in studies focused on memory modulation in male rodents, such as extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK), phosphatidylinositol 3-kinase (PI3K), protein kinase A (PKA), calcium calmodulin kinase II (CaMKII), and mammalian target of rapamycin (mTOR) (Adams and Sweatt, 2002; Horwood *et al.*, 2006; Impey *et al.*, 1998; Silva *et al.*, 1992; Hoeffer and Klann, 2010) are also regulated by E_2 in ovariectomized females. E_2-mediated activation of these pathways has been linked to changes in neuronal excitability (Woolley, 2007), long-term potentiation (LTP) (Smith and McMahon, 2005), spinogenesis (Tuscher *et al.*, 2016a), and memory enhancement (Fernandez *et al.*, 2008; Fan *et al.*, 2010; Fortress *et al.*, 2013) in ovariectomized females. Specifically, E_2 infusion directly into the DH rapidly activates cell-signaling cascades like ERK, PI3K, PKA and mTOR within 5 min of DH infusion (Fernandez *et al.*, 2008; Fan *et al.*, 2010; Fortress *et al.*, 2013). Further, activation of ERK, PI3K, PKA, and mTOR are all required for the beneficial mnemonic effects of E_2 in hippocampus-dependent tasks, as pharmacological inhibition of any of these pathways prevents the memory-enhancing effects of E_2 in ovariectomized female mice (Fernandez *et al.*, 2008; Fan *et al.*, 2010; Fortress *et al.*, 2013) (**Fig. 2**). As will be discussed below, E_2-mediated activation of certain pathways, such as ERK, can also alter gene transcription by regulating downstream epigenetic modifications such as DNA methylation and histone acetylation. Before addressing these data, the next section will first review the major epigenetic mechanisms that regulate hippocampal memory.

Epigenetic Modifications Related to Synaptic Plasticity and Memory

The term "epigenetics" refers to the idea that the expression of genes can be regulated "above the gene" by altering accessibility to genes rather than the genetic code itself (Crick, 1984; Holliday, 1999). Chromosomes are comprised of DNA tightly coiled around nucleosome subunits. Each nucleosome consists of an octamer containing eight histone (H) proteins, two each of H2A, H2B, H3, and H4 (**Fig. 3**). Each histone protein has a N-terminal tail that can be altered through posttranslational modifications such as acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation (Rothbart and Strahl, 2014) (**Fig. 3**). Chromatin is formed from the supercoiling of adjacent nucleosomes which are connected by the linker protein H1 (Mazzio and Soliman, 2012).

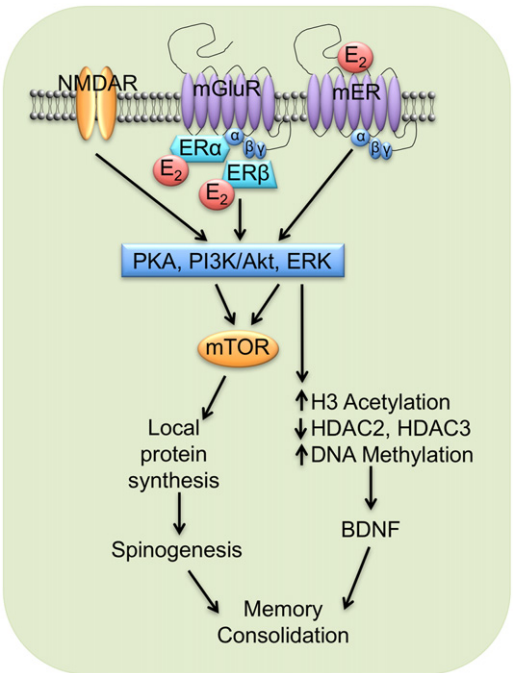

Fig. 2 Schematic representation of the cellular mechanisms through which E_2 regulates memory in ovariectomized mice. In the dorsal hippocampus, E_2 interacts with intracellular ERs (ERα and ERβ), mERs, and neurotransmitter receptors to rapidly activate PKA, PI3K/Akt, ERK, and mTOR cell signaling, which then triggers spinogenesis via local protein synthesis. Similarly, phosphorylation of ERK can enhance gene transcription via alterations in H3 acetylation, HDAC protein levels, and DNA methylation. These changes all contribute to the memory-enhancing effects of acute dorsal hippocampal E_2 infusion on memory consolidation. Abbreviations: *NMDAR*, *N*-methyl-D-aspartate receptor; *mGluR*, metabotropic glutamate receptor; *mER*, membrane estrogen receptor, *PKA*, protein kinase A; *PI3K*, phosphatidylinositol 3-kinase; *mTOR*, mammalian target of rapamycin; *H3*, histone 3; *HDAC*, histone deacetylase; *BDNF*, brain derived neurotrophic factor.

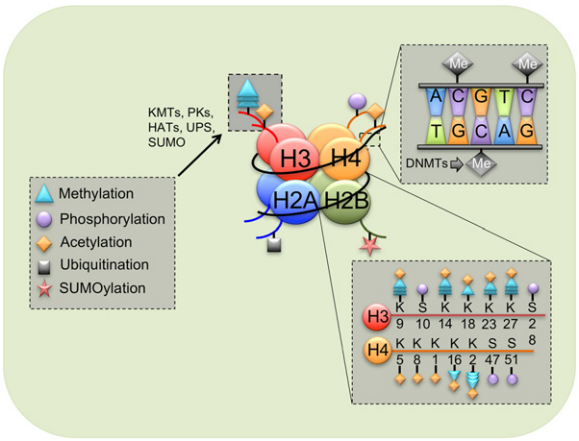

Fig. 3 Diagrammatic illustration of the nucleosome and common epigenetic alterations. The histone octamer consists of two each of histones H2A, H2B, H3, and H4. Abbreviations: *K*, lysine; *S*, serine; *KMTs*, lysine methyltransferases; *PKs*, protein kinases; *HATs*, histone acetyltransferases; *UPS*, ubiquitin proteasome system; *SUMO*, small ubiquitin-like modifier; *Me*, methyl group; *DNMTs*, DNA methyltransferases. Adapted from Fortress, A. M. and Frick, K. M. (2014). Epigenetic regulation of estrogen-dependent memory, *Frontiers in Neuroendocrinology*, **35**(4), 530–549.

The ability of a certain segment of chromatin to be modified depends on the posttranslational modification state of its histone tail, which controls how tightly wound the chromatin structure is and, therefore, whether the 3D structure is permissive to binding factors that regulate transcription. Each major modification and its individual role in memory are reviewed elsewhere (Fortress and Frick, 2014). Histone acetylation, phosphorylation, and methylation, along with DNA methylation, are summarized briefly below.

Histone modifications

Histone modifications can alter accessibility of DNA to the transcriptional machinery required for regulating the expression of genes, including those relevant to neuroplasticity and cognition. Histones are primarily modified at the N-terminal portion of their tails that extends beyond the nucleosome. The histone tail can interact with regulatory proteins, as well as neighboring histones and the DNA itself (Tsankova *et al.*, 2007; Marmorstein, 2001). A number of alterations are possible including methylation, phosphorylation, ubiquitination, SUMOylation, and acetylation. Of these, acetylation is perhaps the most well characterized in the neuroepigenetic literature. Acetylation is carried out by histone acetyltransferase (HAT) enzymes, which facilitate the transfer of an acetyl group from acetyl-CoA to lysine residues present on histone tails. In the majority of cases, acetylation of histone tails yields a transcriptionally active state, conferring open euchromatin that is accessible to transcriptional machinery (Tsankova *et al.*, 2007). Conversely, hypoacetylation generally has the opposite effect, largely because replacement of hydrogen with an acetyl group reduces the net positive charge of the histone, reducing its interaction with the negatively charged DNA backbone and increasing the likelihood of nucleosome displacement (Carrozza *et al.*, 2003). This displacement opens up the chromatin, making it readily accessible to transcriptional machinery and regulatory factors that can influence transcription and, thereby, regulate gene expression.

As mentioned earlier, activation of signal transduction pathways can facilitate epigenetic changes such as histone acetylation, which may be a mechanism contributing to the precision and the stability of a memory. One example is the ERK/MAPK signaling pathway, which stimulates CREB binding protein (CBP), a transcriptional coactivator with intrinsic HAT activity (Ait-Si-Ali *et al.*, 1999). Evidence that histone acetylation can influence learning was first demonstrated in rodents using the conditioned taste aversion paradigm. Using this task, mice that developed a conditioned taste aversion had increased ERK/MAPK activation and histone acetyltransferase activity in the insular cortex 48 h later (Swank and Sweatt, 2001). Further evidence for the role of histone acetylation in memory was determined in mice with reduced levels of CBP ($cbp^{+/-}$mice), which exhibit impaired long-lasting-LTP, as well as deficits in hippocampus-dependent object recognition memory and contextual fear conditioning (Alarcón *et al.*, 2004). Similar behavioral results were obtained in mice with an inducible dominant-negative system to turn off intrinsic CBP-mediated HAT activity. Interestingly, a histone deacetylase inhibitor rescued memory deficits in these mice, suggesting that reduced histone acetylation could underlie memory deficits in numerous disorders (Korzus *et al.*, 2004). Indeed, mice overexpressing the histone deacetylase HDAC2, which removes acetyl groups from histones, have poor hippocampus-dependent spatial memory and reduced synaptic plasticity (Guan *et al.*, 2009). The behavioral deficits induced by overexpressing HDAC2 were reversed by administering an HDAC2 inhibitor, suggesting that memory disorders accompanied by elevated HDAC2 levels could be treated by HDAC2 inhibitors (Guan *et al.*, 2009). Cellular mechanisms responsible for histone acetylation in the hippocampus were determined using contextual fear conditioning in rats. In a series of studies, it was demonstrated that ERK/MAPK signaling, acting upstream of mitogen and stress-activated protein kinase 1 (MSK1), is necessary for the acetylation of H3 (Levenson *et al.*, 2004; Chwang *et al.*, 2007). This evidence collectively suggests that rapid cell-signaling events can lead to changes in epigenetic modifications, such as histone acetylation, to regulate gene transcription and memory.

In addition to histone acetylation, histone phosphorylation is another posttranslational modification poised to regulate memory formation. Protein Serine/Threonine phosphatase 1 (PP1), which dephosphorylates proteins, has been implicated in hippocampal memory impairment and reduced plasticity in rodents (Genoux *et al.*, 2002). In general, phosphorylation is associated with the activation of proteins responsible for a sequence of events that promotes memory and, therefore, interfering with phosphorylation processes could disrupt memory formation. In support of this notion, mice with inducible deficiency of forebrain PP1 demonstrated enhanced long-term memory in the object recognition, object placement, and Morris water maze tasks. This enhanced memory performance coincided with an increase in total phosphorylation of histone H3 on Serine 10 (phospho-H3S10) in addition to increased phospho-H3S10 at the CREB promoter (Koshibu *et al.*, 2009). In addition to its role in regulating histone acetylation, ERK/MAPK signaling can also mediate histone phosphorylation. Activation of the upstream ERK/MAPK regulators PKC and PKA, as well as contextual fear conditioning training, all increased phospho-H3S10 in the hippocampus of rats (Chwang *et al.*, 2006). Further, inhibition of ERK/MAPK prevented phosphorylation of H3S10, suggesting that ERK/MAPK signaling is necessary for H3S10 phosphorylation and contextual fear memory consolidation (Chwang *et al.*, 2006). Notably, H3S10 phosphorylation is also increased in the hippocampus following both retrieval of a fear memory and the phosphorylation of ERK/MAPK (Besnard *et al.*, 2014). Although site-specific dephosphorylation on histone tails has not been demonstrated as a cause of memory impairment, these studies collectively suggest that histone phosphorylation contributes to memory formation.

Whereas histone acetylation and histone phosphorylation are associated with a permissive transcriptional state, histone methylation can be transcriptionally permissive or repressive depending on the residue and number of sites affected. The most commonly examined residue is lysine, which can be mono-(me), di-(me2), or tri-methylated (me3) (Ng *et al.*, 2009). Methyl groups are added to residues by histone (lysine) methyltransferases, which are specific to the residue they modify (Black *et al.*, 2012) and are removed by lysine demethylases. For example, unmethylated lysine9 on H3 (H3K9) is converted to H3K9me1 and H3K9me2 by the G9a methyltransferase, but only the MLL methyltransferase can convert H3K4 to H3K4me3 (Jarome and Lubin, 2013). H3K4me3 and H3K9me2, associated with transcriptional activation and repression, respectively, are both increased in the hippocampus following contextual fear conditioning (Gupta *et al.*, 2010). In support of the role of H3K9me2 in memory formation, inhibition of the G9a methyltransferase with BIX01294 impairs contextual fear memory formation (Gupta-Agarwal *et al.*, 2012). The importance of the H3K4 MLL methyltransferase in memory has been demonstrated in two independent studies in which mice deficient in *Mll1* exhibited selective impairments in hippocampus-dependent contextual fear memory (Gupta *et al.*, 2010), and mice deficient in *Mll2* displayed impaired hippocampus-dependent object recognition and object placement memory (Kerimoglu *et al.*, 2013). Although these studies suggest that methylation can affect histones in a site-specific manner, future studies will need to investigate the extent to which demethylation is site-specific.

DNA methylation

DNA methylation occurs when a methyl group is added to cytosine residues predominantly within cytosine-guanine (CpG, where 'p' refers to the phosphodiester bond) dinucleotides by a methyl donor (e.g., *S*-adenosyl methionine; SAM). CpG dense areas are referred to as CpG islands, (Mastroeni *et al.*, 2011). DNA methylation typically represses transcriptional activity when it occurs within the promoter region of a gene, although this is not always the case (Chahrour *et al.*, 2008). The addition of methyl groups results in the recruitment of corepressor complexes to the DNA, inducing a repressive state by conferring a state of tightly packed heterochromatin that prevents binding of transcription factors and molecules necessary for the initiation of transcription (Tsankova *et al.*, 2007). This often includes the recruitment of histone deacetylases (HDACs), which can act concurrently to affect the acetylation state of neighboring histones, further compacting the chromatin and reducing transcriptional activity. The addition of methyl groups occurs with the aid of DNA methyltransferases or DNMTs (**Fig. 3**), of which at least two types have been identified. Maintenance methylation, which preserves DNA methylation patterns throughout cellular replication, is catalyzed by DNMT1 (Chouliaras *et al.*, 2010). De novo methyltransferases DNMT3a and DNMT3b are responsible for methylation throughout development regardless of previous methylation status (Okano *et al.*, 1999). DNMT2 is not involved in DNA methylation, but instead plays a role in the methylation of aspartic acid transfer RNA (Chouliaras *et al.*, 2010). Together, these enzymes influence transcription and the expression of a number of genes that are critical for neuroplasticity and memory formation (Miller and Sweatt, 2007; Day *et al.*, 2013a). The dysregulation of these enzymes can lead to detrimental changes in gene expression, which may impair neuronal function and contribute to any number of neuropsychiatric disorders.

Early demonstration for the role of DNA methylation in memory came from evidence showing that contextual fear conditioning increased *Dnmt3a* and *Dnmt3b* mRNA in the hippocampus of rats 30 min after training, and that infusion of a DNMT inhibitor into the hippocampus impaired consolidation of contextual fear memory (Miller and Sweatt, 2007). Interestingly, the increase in methylation was at least partially due to hypermethylation of the memory suppressing *PP1* gene (resulting in a net decrease in expression of *PP1*). The persistence of contextual fear memories were later determined to be maintained through DNA methylation in the dorsomedial prefrontal cortex, a brain region important for long-term memory storage (Miller *et al.*, 2010). Additional evidence for the role of DNA methylation in regulating memory consolidation came from work linking contextual fear memory with expression of brain derived neurotrophic factor (BDNF), a neurotrophin well known to promote synaptic plasticity and memory (Cowansage *et al.*, 2010). The *Bdnf* gene is unique in that it has multiple exons that can be independently and differentially regulated. Contextual fear conditioning increased the mRNA expression of exon IV, which was associated with hypomethylation at CpG islands corresponding to exon IV (Lubin *et al.*, 2008). Similarly, treatment with a DNMT inhibitor was sufficient to impair memory, increase methylation of the *Bdnf* gene, and decrease *Bdnf* gene transcription (Lubin *et al.*, 2008). These findings support the conclusion that manipulation of DNA methylation is yet another method of regulating memory.

Estrogenic regulation of histone modifications and effects on memory

As mentioned earlier in this chapter, the ability of a dorsal hippocampal E_2 infusion to enhance hippocampal memory consolidation depends on the rapid activation of numerous cell-signaling cascades, including ERK/MAPK, in the hippocampus (Fernandez *et al.*, 2008; Fortress *et al.*, 2013). Activation of ERK in the hippocampus leads to an increase in the acetylation and phosphorylation of the histone protein H3, and these increases are associated with memory formation (Levenson *et al.*, 2004; Chwang *et al.*, 2006). Thus, our laboratory reasoned that E_2 might enhance memory by triggering ERK-mediated histone alterations. Indeed, direct infusion of E_2 into the DH increases HAT activity in the DH within 15 min and increases DH H3K9,14 acetylation within 30 min (Zhao *et al.*, 2010, 2012). However, E_2 infusion into the DH does not affect acetylation of H2BK12 or H4K12 (Zhao *et al.*, 2010, 2012), suggesting an effect specific to H3K9,14. Additionally, DH infusion of an ERK inhibitor impaired memory and blocked the E_2-induced increase in H3K9,14 acetylation (Zhao *et al.*, 2010), suggesting that H3 acetylation is critical for estrogenic regulation of hippocampal memory consolidation. To determine the extent to which histone acetylation is necessary for E_2 to enhance memory, the HAT inhibitor garcinol was coinfused with E_2. Garcinol prevented DH-infused E_2 from elevating H3K9,14 acetylation and enhancing object memory consolidation (Zhao *et al.*, 2012), demonstrating that H3 acetylation is necessary for the estrogenic enhancement of object memory formation.

E_2 may regulate histone acetylation in the long term by altering the expression of HDAC proteins. Four hours after DH infusion, E_2 significantly decreases levels of HDAC2 and HDAC3 protein (Zhao *et al.*, 2012; Fortress *et al.*, 2014), both of which have been implicated as negative regulators of hippocampus-dependent memory (Guan *et al.*, 2009; McQuown *et al.*, 2011; Graff *et al.*, 2012). E_2-induced downregulation of HDAC2 protein expression was blocked by garcinol (Zhao *et al.*, 2012), suggesting that HAT activity regulates expression of this memory repressing HDAC. Collectively, these findings suggest that E_2 may exert its beneficial effects on memory by increasing H3 acetylation and reducing the expression of negative regulators of memory such as HDAC2 and HDAC3 (**Fig. 2**).

Many of the specific gene targets affected by E_2-mediated H3 acetylation remain to be defined, although genes involved in neuroplasticity are likely candidates. One such example is the neurotrophin BDNF, which has an established role in regulating spine density, synaptic plasticity, and memory (Scharfman *et al.*, 2003; Heldt *et al.*, 2007; Spencer *et al.*, 2008; Luine and Frankfurt, 2013). Recent evidence demonstrates that E_2 infusion directly into the DH specifically increases H3 acetylation at *Bdnf* promoters pII and pIV 30 min after infusion in both young and middle-aged ovariectomized mice (Fortress *et al.*, 2014). These alterations in H3 acetylation at pII and pIV precede a significant increase in BDNF and pro-BDNF protein levels that occurs in the DH 4 and 6 h after infusion. Thus, one potential mechanism through which E_2 exerts its beneficial mnemonic effects is via epigenetic regulation of BDNF in the hippocampus. However, many genes are involved in memory formation, so additional research is needed to elucidate how histone alterations may impact the expression of other genes that contribute to the beneficial effects of E_2 on memory.

Estrogenic regulation of DNA methylation and effects on memory

DNA methylation can also be modified by E_2 treatment. For example, infusion of E_2 directly into the DH significantly increases *Dnmt3a* and *Dnmt3b* mRNA levels 45 min later, however, only DNMT3b protein levels were significantly elevated 4 h later (Zhao *et al.*, 2010). Protein and mRNA levels of the maintenance enzyme DNMT1 remained unaffected after direct DH infusion of E_2. Interestingly, E_2-mediated increases in DNMT3b protein were blocked by coadministration of the HAT inhibitor garcinol (Zhao *et al.*, 2012), suggesting that histone acetylation is required for E_2-mediated changes in DNMT3b protein. This finding is consistent with other research demonstrating that interactions between DNA methylation and histone acetylation are critical for modulating cognition and neuroplasticity (Miller *et al.*, 2008). The E_2-mediated enhancement of object memory consolidation is also blocked by a DH infusion of the DNMT inhibitor 5-aza-2′-deoxycytidine (Zhao *et al.*, 2010), suggesting that DNA methylation is also critical for the memory-enhancing effects of E_2 (**Fig. 2**). However, the specific genes and CpG sites methylated by E_2 in the DH remain unknown and this is an area ripe for future research.

Gaps in Knowledge and Implications for Future Studies

Understanding the mechanisms through which E_2 can orchestrate epigenetic processes to regulate memory is important for understanding cognitive function in both health and disease. Thus far, these mechanisms have only begun to be elucidated as outlined in this chapter. The extent to which E_2 regulates other posttranslational modifications such as histone phosphorylation, ubiquitination, methylation, or SUMOylation in the hippocampus through various cell-signaling mechanisms remains to be identified. Further, only very few gene targets important for memory whose expression is epigenetically regulated by E_2 have been identified to date. Thus, understanding which epigenetic modifications are regulated by E_2 and the downstream transcriptional and translational consequences of these modifications will be critical next steps. Finally, understanding the functional implications of estrogenic regulation of neuroepigenetics will be imperative to better understanding the etiology of disease and improving the design of drugs for reducing memory dysfunction in the future.

It's also important to consider that it is very unlikely that the epigenetic effects of E_2 on memory are restricted to the hippocampus. Parallel evidence for estrogenic regulation of epigenetic processes exists in other brain regions outside of the hippocampus, including the amygdala and the prefrontal cortex. For example, both the prefrontal cortex and the amygdala are actively being investigated for sensitivity to epigenetic modifications in neuropsychiatric disorders, including PTSD (Pizzimenti

and Lattal, 2015). E_2 facilitates extinction learning following fear conditioning, which corresponds to decreased activation in the amygdala and increased activation in the ventromedial prefrontal cortex in rats (Zeidan *et al.*, 2011). Although a necessary role for E_2 in regulating epigenetic mechanisms in the fear memory circuitry has not been tested, a necessary role of histone acetylation in fear memory consolidation was demonstrated using direct infusion of the HAT inhibitor garcinol into the lateral amygdala, which impaired fear memory consolidation in rats (Maddox *et al.*, 2013). Mice that were in the metestrus phase of the estrous cycle (low estrogen state) or were ovariectomized exhibited increased *Hdac4* mRNA in the amygdala following cued fear conditioning, suggesting a potential mechanism through which low levels of E_2 could impair processing of cues related to PTSD (Maddox *et al.*, 2017). These findings suggest that E_2 may promote fear memory consolidation by increasing histone acetylation in the other brain areas, however direct evidence has not been provided.

Finally, the role of de novo neurosteroidogenesis in regulating the epigenetic processes that govern memory formation remains unknown and is an area of increasing clinical relevance. All sex steroid hormones, including E_2, are synthesized in the brain, including the hippocampus, and suppressing endogenous E_2 synthesis with aromatase inhibitors prevents memory formation in female mice and male song birds (Bailey *et al.*, 2013; Tuscher *et al.*, 2016b). Similarly, clinical studies suggest that aromatase inhibition may negatively impact cognitive function in human females. Aromatase inhibitors such as letrozole are used to treat hormone-receptor positive forms of breast cancer (Geisler *et al.*, 2002; Puddefoot *et al.*, 2002), and some findings suggest that such treatments compromise working memory, concentration, and performance in verbal and visual memory tasks (Collins *et al.*, 2009; Bender *et al.*, 2007, 2015). Epigenetic regulation of the aromatase enzyme and downstream epigenetic changes in E_2-mediated memory in response to aromatase inhibitors have yet to be identified, but may prove to be important given increasing evidence that aromatase inhibition leads to memory impairment in females.

Summary

The past 30 years has provided exciting new information about the molecular mechanisms underlying memory formation and dysfunction. In particular, neuroepigenetic studies have illuminated the complexities of gene regulation and revealed a multitude of ways in which epigenetic processes can alter behavior without changing the genetic code. Combined with behavioral neuroendocrinology, neuroepigenetics is advancing our understanding of how hormones regulate the epigenetic processes that influence behavior. Such regulation could explain how environmental, chromosomal, and psychological factors determine individual responses to specific situations. Although information on E_2-mediated epigenetic alterations in the brain remains limited, there is ample potential to explore how sex-steroid hormones modulate behavior and disease in numerous brain regions with implications across a spectrum of disorders. It is our hope that this is where the future will take us and other investigators.

See also: Diagnosis of Menopause. Effects of Steroid Hormones on Brain

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VIPoma, Glucagonoma, and Somatostatinoma

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Abbreviations

PTHrp Parathyroid hormone related peptide

VIP Vasoactive intestinal polypeptide

Vipoma

Vasoactive intestinal polypeptide (VIP) derives its name from the splanchnic vasodilatory effect of VIP after systemic administration (Said and Mutt, 1970). It was first hypothesized that VIP is secreted by endocrine cells, but VIP is now known to be found in the (central) nervous system and thus VIP is a neurotransmitter (Fahrenkrug, 1989). Peripherally, VIP has been localized in neurons in the intestine, lung, adrenals, pancreas, and liver (Larsson *et al.*, 1976). In the gastrointestinal tract, VIP stimulates contraction of enteric smooth muscle cells, pancreatic exocrine secretion, gastrointestinal blood flow and inhibition of gastric acid secretion (Holst *et al.*, 1984; Robberecht *et al.*, 1976; Barbezat and Grossman, 1971). In the circulation VIP has a half-life of less than 1 min and concentrations are normally very low (0–190 pg/mL). This concentration reflects the overflow of VIP from the nerves into the circulation. A VIPoma is a neuroendocrine tumor secreting VIP, significantly increasing the overflow of VIP in to the circulation. The combination of elevated plasma VIP concentrations and symptoms reflecting the actions of VIP (see clinical presentation) is named the VIPoma syndrome.

History

The first two patients with a VIPoma were described by the US physician John V. Vermer Jr. and the Irish-US pathologist Ashton B. Morrison (1922–2008) in 1958. They presented a 67-year-old male and a 19-year-old male with the typical symptoms of VIPoma: watery diarrhea and hypokalemia. Both patients died of arrhythmias secondary to refractory hypokalemia and autopsy revealed pancreatic tumors (Verner and Morrison, 1958). Through this case report the two acronyms for the syndrome of VIP hypersecretion arose: Verner–Morrison syndrome, after the first to discover the syndrome or watery diarrhea hypokalemia achlorhydria (WDHA)-syndrome. It took until 1970 to isolate VIP from pigs' small intestines. This enabled Steve Bloom and colleagues to develop a specific radioimmunoassay to demonstrated elevated plasma VIP levels in patients with the VIPoma syndrome and for positive immunohistochemistry for VIP in the pancreatic tumor (Bloom *et al.*, 1973). Finally a proof of principle was provided by Kane in 1983. Injecting five healthy subjects with porcine VIP, resulting in levels resembling VIPoma patients, resulted in profuse diarrhea in all patients within 4 h (Kane *et al.*, 1983).

Clinical Presentation

The incidence of VIPomas is estimated to be 1–2 per 10 million per year (Halfdanarson *et al.*, 2008). Patients with a VIPoma experience symptoms which are in line with the action of VIP, which are mainly gastro-intestinal. When the tumor is small, and thus secretion is still limited, symptoms are often temporary. Patients suffer only from intermittent diarrhea. However, with tumor growth and increasing VIP secretion, the complaints become more frequent and severe. In severe cases, diarrhea becomes profuse and patients lose over 6–8 L of watery, tea-colored stools per 24 h. The diarrhea is of the secretory type, indicating it will persist even when the patient is fasting. Eventually the diarrhea will lead to electrolyte disturbances. Patients lose large amounts of bicarbonate and potassium in the stools and then often require intravenous supplementation of these electrolytes. Other symptoms include facial flushing and, inhibition of gastric acid secretion. Around 50% of patients have hypercalcemia but the mechanism of action is unknown.

VIPomas are mostly diagnosed in a work-up for diarrhea and the differential diagnosis includes all kinds of secretory diarrhea. This is mainly infectious colitis or laxative abuse, but also other neuroendocrine syndromes like gastrinoma, medullary thyroid carcinoma and the carcinoid syndrome.

Diagnosis and Clinical Work-Up

The diagnosis of a VIPoma is made on the combination of the clinical presentation and elevated plasma VIP levels. One measurement showing a non-elevated plasma VIP concentration does not exclude a patient from having a VIPoma since especially small tumors can secrete VIP intermittently. On the other hand, a marginally elevated plasma VIP level should be confirmed by a second measurement.

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A VIPoma is either localized in the pancreas (75%), or in the sympathetic ganglia (Soga and Yakuwa, 1999c). As 80% of the pancreatic VIPomas are larger than 2 cm they can easily be detected with a magnetic resonance imaging (MRI)-scan or a CT-scan. Smaller lesions can be detected with an endoscopic ultrasound. Functional imaging includes a somatostatin-receptor scintigraphy or PET–CT with gallium-labeled somatostatin analogues (Nikou *et al.*, 2005; Virgolini *et al.*, 2005).

Treatment and Prognosis

The first goal in patients with a VIPoma is to correct the fluid and electrolyte disturbances, for which often intravenous supplementation is required. Diarrhea can be effectively reduced by intermediate, or long-acting somatostatin analogues and should be started as soon as possible in all patients (Song *et al.*, 2009). In the acute setting octreotide can be administered subcutaneous three times daily, or octreotide can be given as a continuous iv infusion. All patients presenting with a VIPoma should be considered for curative surgery, if disease is localized or all metastases are accessible for resection. If resection is not feasible several therapeutic options are available in the palliative setting. As VIPomas are rare all therapies are either based on case series or extrapolated from other, more common pancreatic neuroendocrine tumors. When diarrhea is well controlled a patient can be switched to long-acting depot somatostatin analogues, like lanreotide autosolution, or octreotide LAR. In patients who do not respond to somatostatin analogues debulking can be considered with surgery, embolization (bland- or radioembolization), or peptide receptor radiotherapy (PRRT) with radiolabeled somatostatin analogues (Kwekkeboom *et al.*, 2008; Moug *et al.*, 2006; Peng *et al.*, 2004). The tyrosine kinase inhibitor Sunitinib has also been described in some patients to effectively decrease diarrhea (de Mestier *et al.*, 2014).

The prognosis of patients with a VIPoma is highly dependent on the stage. Patients with a localized VIPoma have an average 5-year survival of 94% but this declines to 60% in metastatic VIPoma (Soga and Yakuwa, 1999c).

Glucagonoma

Glucagon is a peptide hormone, secreted from alpha cell of the pancreas and it has a major role in glucose regulation. It is one of the many hormones which can increase plasma glucose levels. The glucagon receptor can be found in the liver, brown and white adipose tissue, brain, heart, kidney and gastrointestinal tract. Increase of glucagon levels results in increased hepatic glucose output, lipolysis of adipose tissue and satiety.

In the non-fasting state, alpha cells only secrete low levels of glucagon. In response to hypoglycemia glucagon secretion increases and this counteracts the metabolic effects of insulin, restoring euglycemia. Through this effect it can also be used as a drug in patients with hypoglycemia due to a (relative) overdose of insulin (Campbell and Drucker, 2015). Glucagonomas present a rare kind of pancreatic NETs characterized by the hypersecretion of glucagon.

History

The first studies on the endocrine function of the pancreas were performed in the early 1920 when dogs were pancreatectomized and were found to develop diabetes mellitus. However, in 1922 *John R. Murlin* discovered that certain peptides from the pancreas were also able to increase glucose levels in dogs and rabbits (Kimball and Murlin, 1923). It took until the 1950s to isolate glucagon and to report the complete amino acid sequence (Staub *et al.*, 1953). The typical skin lesions associated with glucagonomas were then already described by S. William Becker in 1942 (Becker *et al.*, 1942). The skin eruption was thereafter linked to a metastatic pancreatic tumor by Mcgavran *et al.* (1966). This female patient presented with diabetes mellitus, anemia, and skin eruptions. She was diagnosed with a metastatic pancreatic tumor and later in the course of the disease strongly elevated glucagon levels were measured. The UK dermatologist Darrell Wilkinson was the first to name the typical skin lesions "necrolytic migratory erythema" in 1971(Wilkinson, 1971).

Clinical Presentation

The hypersecretion of glucagon associated with glucagonomas has several distinct clinical features. In a majority of patients there is either a new onset, or worsening of diabetes mellitus. This is accompanied by significant weight loss trough the catabolic effect of glucagon in 70%–80% of patients. Also, cheilosis, glossitis and stomatitis can occur in 30%–40% of patients and glucagomas are highly associated with thromboembolic events (Kaltsas *et al.*, 2004; Soga and Yakuwa, 1999a). But the most distinct feature remains the typical skin manifestations named necrolytic migratory erythema. The rash can start as an erythema or dermatitis-like lesion, but in later stages bullae can form with secondary infections. (**Fig. 1**A–C) Necrolytic migratory erythema usually starts in the groin and perineum but can spread over the entire body in severe cases (Wermers *et al.*, 1996). The cause of the skin lesions is unknown, but it is thought to be associated with zinc, fatty acid, and amino acid deficiency. In patients with a glucagonoma, plasma glucagon is often increased over 1000 pmol per liter (Soga and Yakuwa, 1999a). Hyperglucagonemia can also be found in

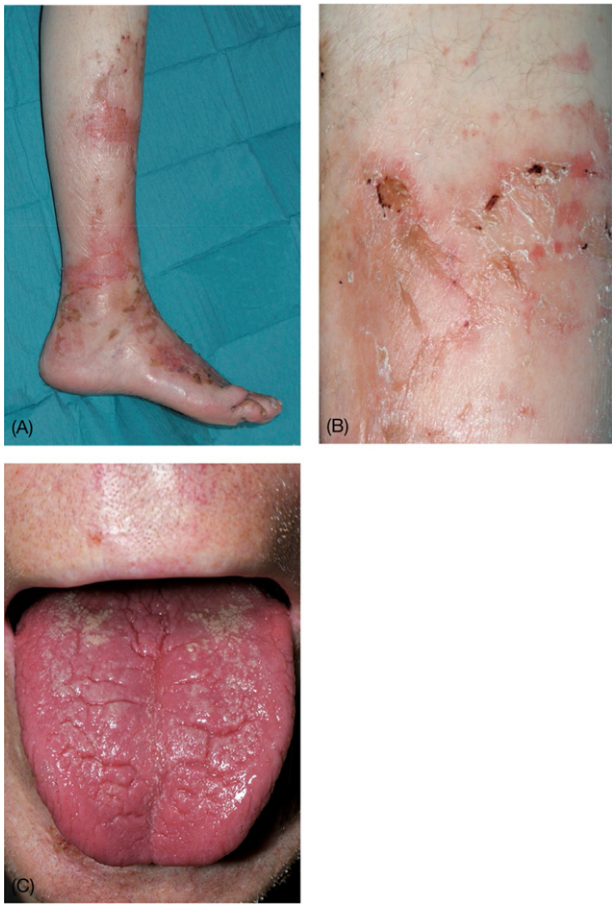

Fig. 1 (A–C) Necrolytic erythema and glossitis caused by glucagonoma.

patients with diabetes mellitus, insufficiency of glucagon catabolism (renal or liver failure) or serious disease with catabolism, however in these conditions plasma glucagon is rarely higher than 500 pmol per liter (Bloom and Polak, 1987). The incidence of glucagonomas is low, and only around 2% of all pancreatic NETs.

Diagnosis and Clinical Work-Up

A glucagonoma is diagnosed when a patient is found to have (part of) the previously described clinical syndrome in combination with an elevated plasma glucagon level and a histological diagnosis of a neuroendocrine tumor (preferably with a pancreatic lesion on imaging). Fasting plasma glucagon levels have been described to be 4–150 times elevated in patients with a glucagonoma, but are usually more than 10–20 times elevated (Stacpoole, 1981). A biopsy should always be taken for the histopathological diagnosis and grading.

Imaging of the primary tumor in the pancreas is best performed with an MRI, CT or an endoscopic ultrasound (**Fig. 2**). In metastatic disease, somatostatin-receptor scintigraphy or PET-CT with gallium-labeled somatostatin analogues has a high probability of detecting glucagonomas and their metastatic spread (Eldor *et al.*, 2011; Kindmark *et al.*, 2007).

Treatment and Prognosis

First step in treatment of patients with a glucagoma is correction of diabetes mellitus, mineral deficiencies and the catabolic state of the patient often requiring insulin treatment and intravenous supplementation of minerals and amino acids (Doherty, 2005). Administration of somatostatin analogues can result in weight increase and large reductions of skin lesions and is therefore advised in all patients. Also treatment with low-molecular-weight heparins should be started to prevent thrombosis. After improving the clinical condition of the patient, surgical resection should be considered if a radical resection seems feasible. However as around 50%–80% of patients have metastatic disease at diagnosis this is often not feasible (Soga and Yakuwa, 1999a; Stacpoole, 1981).

For unresectable glucagonomas, palliative treatment with lanreotide autosolution or octreotide LAR should be commenced, because of the reduction in glucagon secretion and thus symptoms. It is likely that somatostatin analogues also have an

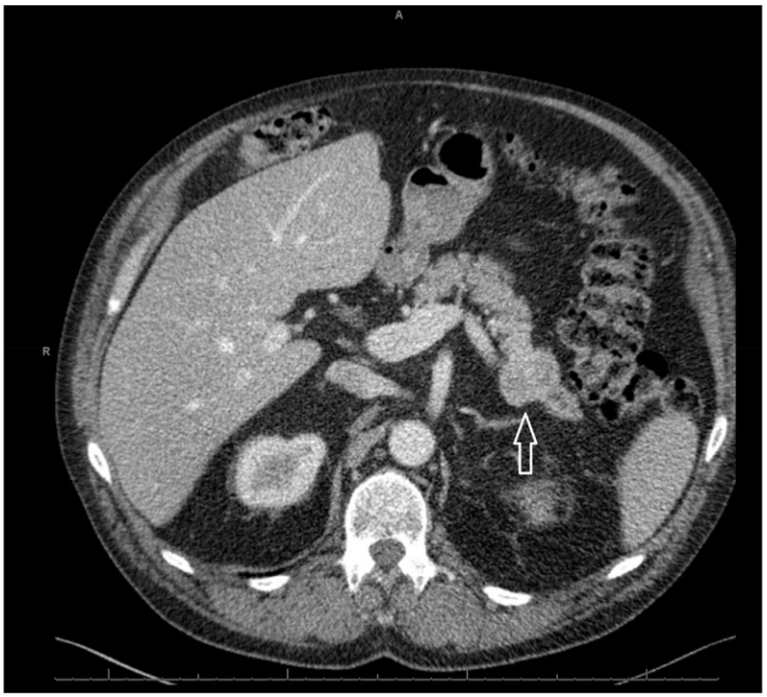

Fig. 2 CT scan demonstrating a pancreatic neuroendocrine tumor. From Müller, R., Gravert, M., Kern, T., Madl, T., Peschek, J., Sattler, M., Groll, M. and Buchner, J. (2013). High-resolution structures of the IgM Fc domains reveal principles of its hexamer formation. Proceedings of the National Academy of Sciences of the United States of America **110**, 10183–10188.

anti-proliferative effect in glucagomas, but because of the low incidence of glucagonomas there is no evidence for this. In high-grade glucagonomas, treatment with chemotherapy can be considered. PRRT with radiolabeled somatostatin analogues has also been successfully used in the treatment of glucagonomas (Eldor *et al.*, 2011). Futhermore, Everolimus and Sunitinib have been successfully tried. Prognosis is excellent in patients after surgical resection, however in patients with metastatic glucagonomas 5-year survival is around 50%–70% (Soga and Yakuwa, 1999a; Chu *et al.*, 2003, Kindmark *et al.*, 2007).

Somatostatinoma

Somatostatin is a regulatory peptide found throughout the central nervous system and in most large organs. There are two major sub-forms of somatostatin, namely somatostatin-14 and somatostatin-28. Somatostatin-14 is found in hypothalamus and has an inhibitory effect on neurotransmitter release as well as growth hormone and thyroid stimulating hormone secretion from the pituitary. Luteinizing hormone, follicle-stimulating hormone, and adrenocorticotropic hormone (ACTH) secretion are not inhibited in healthy subjects, but interestingly ACTH secretion can be inhibited by somatostatin in patients with Cushing's disease (ACTH-secreting pituitary adenoma). In the gastro-intestinal tract somatostatin can be secreted form the delta cells in the bowel mucosa or pancreas, but also in specific bowel neurons. Somatostatin inhibits the secretion of insulin and glucagon in the pancreas, inhibits gall bladder and bowel movement, but also inhibits the exocrine secretion of bile and gastric acid.

There are five subtypes of somatostatin receptors (SSTRs). Pituitary tumors mainly express the subtypes 2 and 5 while neuroendocrine tumors mainly express the SSTR subtype 2. The inhibitory effect of somatostatin on varies physiological processes led to development of drugs targeting this effect. Somatostatin itself only as a half-life of 3 min and therefore varies analogues where developed. Octreotide was the first analogue to be developed (half-life: 1.5 h) and later the slow release analogues lanreotide autogel and octreotide LAR were developed. These drugs are mainly used for the treatment of acromegaly and neuroendocrine tumors (Oberg and Lamberts, 2016; Patel, 1999).

A somatostatinoma is a neuroendocrine tumor arising from the delta-cells in the pancreas or bowel.

History

The somatostatinoma syndrome has a relative short history. Krulich and McCann, in 1968, were the first to describe the effect of a hypothalamic extract inhibiting the secretion of growth hormone (Krulich *et al.*, 1968). The exact nature and structure of the extract was discovered later. The group of Roger Guillemin was the first to isolate somatostatin from sheep hypothalami and for this work the Frenchman received the Nobel Prize in 1977 (together with Andrew Scally "for their discoveries concerning the

peptide hormone production of the brain") (Brazeau *et al.*, 1973). In that same year the first cases of somatostatinoma were described by Om Ganda as well as Lars-Inge Larsson, followed by the publication of the full description of the syndrome by Günter Krejs in 1979 (Larsson *et al.*, 1977; Ganda *et al.*, 1977; Krejs *et al.*, 1979).

Clinical Presentation

The syndrome of somatostatin hypersecretion is well defined in the WHO guidelines. It consist of markedly elevated somatostatin levels in the plasma and/or tumor, diabetes mellitus of recent onset, decreased gastric acid secretion, cholelithiasis, steatorrhoea, and anemia/weight loss (Bosman and World Health Organization, 2010). All these symptoms are the result of the inhibitory effects of somatostatin. However, this distinct presentation is very rare, with only 10% of patients presenting with the somatostatinoma syndrome (Garbrecht *et al.*, 2008; Soga and Yakuwa, 1999b). Abdominal pain as presenting symptom is more common in 50% of patients. Many of these patients are diagnosed as somatostatinoma based on positive immunohistochemical staining for somatostatin, without the corresponding syndrome and this makes the diagnosis somewhat controversial. This contributes to the rare nature of somatostatinomas with an estimated incidence of only 1 per 40 million persons per year.

Diagnosis and Clinical Work-Up

Somatostatinomas are most often localized in the pancreas (60%) or in the duodenum (40%). Pancreatic somatostatinomas tend to be larger with an median size of 43 mm compared to duodenal tumors of 15–20 mm (Soga and Yakuwa, 1999b). The diagnosis is (as in VIPoma and glucagonoma) based on the clinical picture of the somatostatinoma syndrome, elevated plasma somatostatin and the histopathological diagnosis of a neuroendocrine tumor. The pancreatic and duodenal lesions can best be visualized with CT, MRI or endoscopic ultrasound. Despite of elevated plasma somatostatin levels, somatostatinomas can still be visualized with functional imaging with somatostatin receptor scintigraphy or gallium-labeled somatostatin PET–CT scans.

Treatment and Prognosis

Surgery is the only curative treatment for somatostatinomas and should always be considered if a radical resection can be performed. In metastatic disease treatment with somatostatin analogues can result in decrease of plasma somatostatin levels (Angeletti *et al.*, 1998). Due to the rare character of somatostatinomas no evidence is available for treatment with PRRT or immunotherapy. Prognosis is excellent after radical resection and 5 year survival was 59.9% in metastatic disease (Soga and Yakuwa, 1999b).

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Insulinoma

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Glossary

MEN-1 Multiple endocrine neoplasia-1.

PRRT Peptide receptor radionuclide therapy.

Introduction

Plasma glucose levels need to be maintained within a narrow range for the proper functioning of several body functions. There are several hormones that increase plasma glucose (growth hormone, cortisol, epinephrine, and glucagon), but only insulin directly decreases plasma glucose. The pancreas has an important role in glucose regulation, mainly through the islets of Langerhans. Insulin is secreted from β-cells located in these islets together with the glucagon secreting α-cells and the somatostatin-secreting δ-cells. After the ingestion of glucose or carbohydrates there is a subsequent rise in plasma glucose, but the swift secretion of insulin from the β-cells keeps plasma glucose within narrow margins in healthy subjects. In the fasting state plasma glucose levels are mainly regulated by the insulin/glucagon ratio in the portal vein of the liver: insulin secretion decreases at plasma glucose levels lower than 4.5 mmol/L and plasma insulin should be immeasurable at plasma glucose levels under 2.5 mmol/L. Glucagon and epinephrine are secreted when plasma glucose levels drop below 3.8 mmol/L and with this defense mechanism hypoglycemia's only rarely occur. One specific cause of hypoglycemia is an insulinoma: a neuroendocrine tumor arising from the pancreatic β-cells causing hypoglycemia through hypersecretion of insulin (Cryer and Gerich, 1985; Kittah and Vella, 2017).

History

Paul Langerhans was the first to describe the pancreatic islets in 1869 and with that discovery the islets where named after this German pathologist (Langerhans, 1869). Several years later, Lane from Chicago first described the different α- and β-cell within the islets (Lane, 1907). The peptide insulin was then first isolated by the Canadian group of, Frederick Banting and Charles Best, James Collip and John MacLeod (Banting and Best, 1922). During one of their experiments they removed the pancreas in dogs and they observed all symptoms of diabetes and increase of plasma glucose. Administering to these dogs a pancreatic extract (then named "isletin") reversed hyperglycemia, thereby identifying the important role of the pancreas in glucose regulation. John MacLeod and Frederick Banting were awarded the Nobel Prize for Physiology in 1923 for this discovery. However, Nicolae Paulescu from Rumania actually discovered insulin a year before the Canadian group but was never credited for this fact (Paulescu, 1921).

The first case of a patient with hypoglycemia caused by an insulinoma was described by the surgeon Harris and William Mayo operated this patient but found a unresectable pancreatic tumor with multiple metastases. In this case, extracts of the liver nodules were said to cause hypoglycemia in rabbits and thus must have been producing insulin (Wilder *et al.*, 1927). The first to cure a patient with an insulinoma was the Roscoe Graham, a surgeon from Canada (Howland *et al.*, 1929). An excellent case series on insulinomas was later published by the surgeon Allen Whipple in 1935 describing the symptoms on which we currently still base our diagnosis of hypoglycemie (Whipple's triad) (Whipple, 1938).

Clinical Presentation

Since an insulinomas is a neuroendocrine tumor arising from the pancreatic β-cells, hypoglycemia caused by hypersecretion of insulin by the β-cells, is its main symptom. The diagnosis can be very challenging because the symptoms can be aspecific and very prevalent while insulinomas only occur rarely. In some cases patients suffer from seizures or abnormal behavior and only after long neurological or psychiatric treatment the insulinoma is diagnosed.

The symptoms of hypoglycemia start with the so-called autonomic symptoms secondary to catecholamine release and activation of the cholinergic system when plasma glucose drops below 3.3 mmol/L (60 mg/dL). The adrenergic symptoms due to catecholamine release include tremors, palpitations and anxiety. Among the cholinergic symptoms are sweating, hunger and nausea. When plasma glucose drops below 2.8 mmol/L (50 mg/dL) cognitive dysfunction can occur with symptoms as weakness, behavioral changes, confusion, amnesia, lethargy and in severe cases coma or seizures. A patient with an insulinoma typically experiences weight gain because of the increased appetite to prevent hypoglycemic episodes and the anabolic effect of insulin. Hypoglycemia occurs during fasting, but patients can also experience hypoglycemia after a meal because of the overshoot of insulin secretion. Some patients also present with a pancreatic incidentaloma found to be a neuroendocrine tumor and hypoglycemia occurs only later in the course of the disease.

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The estimated incidence of insulinomas is around 4 per million person years. Approximately 10% of insulinomas is associated with MEN-1 and about 5% is metastatic (Service *et al.*, 1991).

Diagnosis and Clinical Work-Up

The first step in diagnosis of an insulinoma is the diagnosis of endogenous hyperinsulinemia, meaning an inappropriately elevated insulin during hypoglycemia. The hypoglycemia should fulfill Whipple's triad: symptoms or signs of hypoglycemia at which time a low plasma glucose is measured and symptoms should resolve when euglycemia is restored (Whipple, 1938; Cryer *et al.*, 2009). It is important to first rule out any drugs that can cause hypoglycemia. These drugs are mainly antihyperglycemic drugs like insulin or sulfonylurea derivatives used to treat diabetes. But is also important to look for other comorbidities like severe sepsis, adrenal insufficiency or liver failure that can cause hypoglycemia. If these factors seem unlikely testing for endogenous hyperinsulinemia should be performed (Cryer *et al.*, 2009).

If a spontaneous hypoglycemia occurs one can always draw blood for the required measurements but often a 72-h fast is required to provoke a hypoglycemia. In this test a hypoglycemia is defined as plasma glucose below 2.2 mmol/L (40 mg/dL) in the presence of Whipple's triad. Some guidelines define hypoglycemia as plasma glucose below 3.0 mmol/L (54 mg/dL), but this will decrease specificity with more false positive results (Service, 1995; de Herder *et al.*, 2006). Almost all patients with an insulinoma develop hypoglycemia during this test. A majority even develops the hypoglycemia within the first 12–24 h and at 48 h the sensitivity is already near 100% (Hirshberg *et al.*, 2000; Service and Natt, 2000; van Bon *et al.*, 2009; Dizon *et al.*, 1999). When hypoglycemia occurs blood should be drawn to test for plasma insulin, C-peptide, pro-insulin, beta hydroxybutyrate and a screening for hypoglycemic drugs should be performed. Thereafter hypoglycemia should be corrected with intravenous glucagon or glucose. Patients with an insulinoma typically have elevated plasma insulin (>18 pmol/L) and C-peptide (>0.2 nmol/L). Occasionally an insulinoma secretes pro-insulin instead of insulin. The combination of laboratory assessments helps to differentiate between the causes of hypoglycemia (**Table 1**).

If no hypoglycemia occurs after 72 h the fast is ended. Some advocate that at that time glucagon should be administered to test the hepatic glycogen stores, as in hyperinsulinemia the hepatic glycogen stores are preserved during fasting and a higher increase in glucose will be seen.

After the demonstration of endogenous hyperinsulinemia the next step is to find a neuroendocrine tumor in the pancreas. The first step is often conventional imaging with a MRI- or CT-scan. A transabdominal ultrasound is not sensitive, only detecting 34.4% of insulinomas, while an abdominal CT-scan is able to detect the insulinoma in around 30%–85% of patients and the MRI detects 85%–95% of insulinomas (**Fig. 1**) (Mehrabi *et al.*, 2014; de Herder *et al.*, 2006). In case these scans are negative or if additional imaging is needed for localization an endoscopic ultrasound can be used: it allows for a transducer to be placed very close to the pancreas in the duodenum and can thereby detect lesions as small as 5 mm. The endoscopic ultrasound alone has a sensitivity of around 95%, but sensitivity is lower in detecting lesions in the tail of the pancreas. In combination with CT, the endoscopic ultrasound is able to detect hardly all insulinomas (de Herder *et al.*, 2006).

Nuclear imaging can also be very useful in detecting insulinomas. The somatostatin scintigraphy or PET-CT has only limited detection rates in localized insulinoma, most probably because the low expression of somatostatin receptors in localized insulinoma (Mehrabi *et al.*, 2014). There is however high expression of the glucagon-like peptide 1 (GLP-1) receptor making it an excellent target for radiolabeled exendin4. This radiolabeled GLP-1 receptor agonist was shown to be superior than CT, MRI or somatostatin receptor imaging detecting 95% of insulinomas (Christ *et al.*, 2013). In metastatic insulinoma, the expression of somatostatin receptors is usually more prevalent and therewith the sensitivity of the somatostatin scintigraphy or PET-CT increases (**Fig. 2**) (Wild *et al.*, 2011). It seems that GLP-1 receptor imaging is superior in localized insulinoma and the somatostatin receptor imaging in metastatic insulinoma.

Table 1 Patterns of results during 72 h fast

Cause	*Insulin (pmol/L)*	*C-peptide (nmol/L)*	*Proinsulin (pmol/L)*	*Beta-hydroxybutyrate (mmol/L)*	*Circulating oral hypoglycemic drugs*	*Antibody to insulin*
Exogenous insulin	$\gg 18$	<0.2	<5	≤ 2.7	No	No
Endogenous hyperinsulinemi[a]	≥ 18	≥ 0.2	≥ 5	≤ 2.7	No	No
Oral hypoglycemic drugs	≥ 18	≥ 0.2	≥ 5	≤ 2.7	Yes	No
Insulin auto-immune	$\gg 18$	$\gg 0.2$	$\gg 5$	≤ 2.7	No	Yes
IGF-mediated	<18	<0.2	<5	≤ 2.7	No	No

[a]Endogenous hyperinsulinemia: nesidioblastosis, post-gastric bypass hypoglycemia.

IGF: Insulin-like growth factor.

Based on Cryer, P.E., Axelrod, L., Grossman, A.B., Heller, S.R., Montori, V.M., Seaquist, E.R., Service, F.J., Endocrine Society. (2009). Evaluation and Management of Adult Hypoglycemic Disorders: an Endocrine Society Clinical Practice Guideline. The Journal of Clinical Endocrinology and Metabolism 94, 709–728.

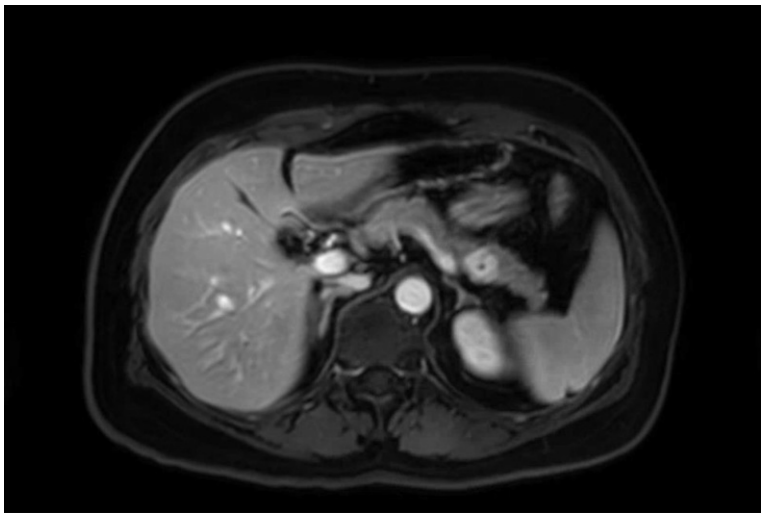

Fig. 1 MRI-scan showing a hypervascular lesion in the tail of the pancreas found to be an insulinoma.

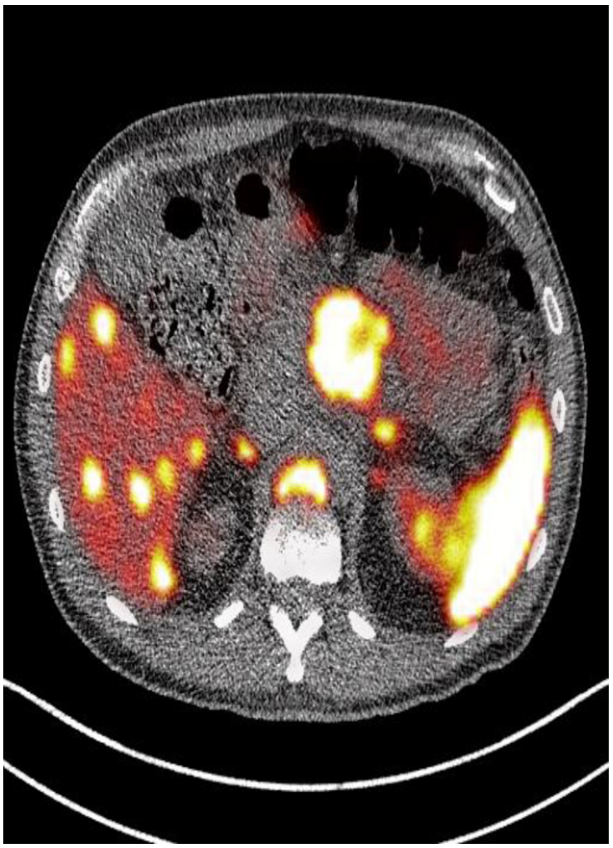

Fig. 2 68-Gallium-DOTATATE PET/CT showing intense uptake in pancreatic tumor and multiple liver metatstases. Physiologic uptake in the spleen.

In patients with negative imaging noninsulinoma pancreatogenous hypoglycemia syndrome (NIPHS) can be considered. The disease caused by hypertrophy of pancreatic islet can be distinguished from an insulinoma with angiography combined with calcium stimulation and transhepatic portal venous sampling. Hypersecreting β-cells release insulin upon stimulation with calcium where normal functioning β-cells do not. This results in an increase of insulin measured in the portal vein. Through selective catheterization of an artery a specific anatomical part of the pancreas can be stimulated. An insulinoma will typically only

secrete insulin upon stimulation of one region while NIPHS reacts to stimulation of the entire pancreas (Jackson, 2005). In children a F-DOPA-PET can be used to diagnose nesiodioblastosis (Stanley, 2016).

Treatment and Prognosis

Surgery is the first line treatment of patients with a localized insulinoma. In 90% of patients the insulinoma is smaller than 5 cm and restricted to the pancreas. After enucleation or (partial) pancreatectomy overall survival and curation rates are often near 100% (Mehrabi *et al.*, 2014; Tsutsumi *et al.*, 2013).

In patients with a metastasized insulinoma surgery should be considered if the primary tumor and all metastasis can be resected. In other cases treatment is aimed at preventing hypoglycemia and stabilizing tumor growth.

Hypoglycemia can be prevented with diazoxide which activates the ATP-sensitive potassium channels of the β-cells and reduces insulin secretion (Altszuler *et al.*, 1977). The most common side effect are fluid retention (well treatable with diuretics) and hirsutism (Gill *et al.*, 1997). Octreotide or the longer acting analogs lanreotide and octreotide LAR can also be used in metastatic insulinoma. They require adequate expression of somatostatin receptor subtypes (preferably the SSTR2 subtype and SSTR3 and SSTR5 subtypes) on the tumor to reduce insulin secretion but, if somatostatin receptor subtype-expression is low these drugs can also paradoxically increase hypoglycemias by decreasing glucagon secretion, relatively more than they decrease insulin secretion. A short clinical trial with short-acting octreotide is, therefore, recommended when starting with somatostatin analogues (de Herder *et al.*, 2011). Lanreotide and octreotide LAR can also potentially increase progression-free survival as has been demonstrated in non-functioning neuroendocrine tumors (Caplin *et al.*, 2014).

As a second-line treatment, when tumor progresses or hypoglycemias persist, Peptide Receptor Radionuclide Therapy (PRRT) with radiolabeled somatostatin has been shown to be highly effective. Patients are usually treated with four doses of PRRT with radiolabeled somatostatin and this has been reported to result in long lasting euglycemia and progression-free survival (van Schaik *et al.*, 2011). Also, everolimus was shown to be effective in preventing hypoglycemia in metastatic insulinoma (Bernard *et al.*, 2013; Kulke *et al.*, 2009). In high-grade insulinoma the chemotherapy combination with streptozotocin and 5-fluorouracil can be considered (Moertel *et al.*, 1980).

The prognosis of metastatic insulinoma has not been studied in the current era with contemporary treatment options. Older series report a median survival of approximately 2–3 years, but survival of over 30 years has been described in single patients (Danforth Jr. *et al.*, 1984; Hirshberg *et al.*, 2005; Starke *et al.*, 2005). It is however likely that survival is currently in the same range as non-functioning pancreatic neuroendocrine tumors: approximately 6–8 years (Martin-Perez *et al.*, 2013).

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Melatonin☆

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Glossary

Circadian Occurring or recurring about (latin-circa) once per day (dian). Biological circadian rhythms are internally generated and, in humans, have a period which is usually slightly longer than 24 h.

Period The duration of one complete cycle of a rhythmic variation.

Phase A distinct stage in a process of change, in this case a circadian rhythm.

Photoperiod Strictly the length of the light phase of a particular light–dark cycle, but may be used to describe the whole light–dark cycle.

Photoperiodism The response of an organism to changes in the lengths of the daily periods of light.

Introduction

The discovery of melatonin (N-acetyl-5-methoxytryptamine) in 1958 by Lerner and colleagues was the major stimulus for an enormous amount of work. Research on this small molecule has led to much current knowledge, not only concerning the function of the molecule itself as an internal hormonal time cue (the 'hormone of darkness'), but also to our fundamental understanding of rhythmic functions in species as diverse as microorganisms and humans. Substantial therapeutic benefits of melatonin treatment, particularly in the field of rhythm-related sleep disorder, are emerging.

Lerner and colleagues were searching for an amphibian skin lightening factor known to be present in the cow pineal gland. He and colleagues collected hundreds of thousands of cow pineal glands from abattoir material and processed the tissue through a series of extractions and other separation procedures. The skin lightening activity was monitored by an amphibian skin lightening bioassay. Two years of effort were necessary before a few micrograms of pure material were isolated. Chemical synthesis led to N-acetyl-5-methoxytryptamine (Fig. 1) which proved to be the most potent skin lightening substance ever tested.

It is now evident that melatonin is a hormone, synthesised from tryptophan (Fig. 1) and secreted, in mammals, largely by the pineal gland. In a normal environment secretion takes place essentially during the dark phase of the light dark cycle in virtually all species (including unicellular organisms). The evening increase and morning decline of melatonin serve to define 'biological night.' Its primary function in mammals is to convey information concerning the length of the night for the organisation of daylength dependent functions, and in consequence it has been called the 'darkness' hormone. Other sites of synthesis exist, for example the retina and the gut, however pinealectomy leads to undetectable or very low circulating levels at all times of day and night. Thus other sites of synthesis contribute very little to circulating melatonin (in mammals).

Synthesis and Metabolism of Melatonin

Melatonin synthesis in rodents and humans is initiated through noradrenergic stimulation of adrenergic beta1 receptors within the pineal, potentiated by alpha1 receptor stimulation. Administration of beta receptor antagonists such as propanolol and atenolol will suppress melatonin production in humans. In most circumstances the rate limiting step in synthesis (Fig. 1) is the activity of serotonin-N-acetyltransferase (arylalkylamine N-acetyl transferase, AA-NAT) with a major increase (7–150 fold) in the activity during the dark phase. Serotonin availability may also play a role. The rhythm of melatonin production is endogenous, being generated by clock genes in the suprachiasmatic nuclei (SCN), the major central rhythm-generating system or 'clock' in mammals (the pineal gland itself, and also the retina are self sustaining 'clocks' in some if not all lower vertebrates). The rhythm is synchronized to 24 h primarily by the light–dark cycle acting via the retina and the retinohypothalamic projection to the SCN (Fig. 1 and 2).

The cDNAs encoding both AA-NAT and the O-methylating enzyme hydroxyindole-O-methyl transferase (HIOMT) (Fig. 1) have been cloned. It is likely that the human enzyme is regulated primarily at a post transcriptional level, whereas in rodents the key event appears to be cyclic AMP-dependent phosphorylation of a transcription factor that binds to the AA-NAT promoter. Rapid decline in activity with light treatment at night appears to depend on proteasomal proteolysis.

In humans and rodents melatonin is metabolized to 6-sulphatoxymelatonin (aMT6s), primarily within the liver, by 6-hydroxylation, followed by sulphate conjugation. A number of minor metabolites are also formed, including the glucuronide

☆*Change History:* August 2014. J Arendt added an abstract, text on melanospin, some text to receptors section, a new paragraph to agonists section, a new paragraph to melatonin actions in the pars tuberalis, a new paragraph on melatonin and diabetes, text on cardiovascular disorder and autism, a new paragraph on light at night, cancer and melatonin, otherwise minor corrections and eight new references. Added Fig. 1, 5, 7(a) and 7(b), correct Figure 7(a), added 9(a) and 9(b) and 11. Note new numbering of most figures and updating of old figures.

This article is an update of Josephine Arendt, Melatonin, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 228-237.

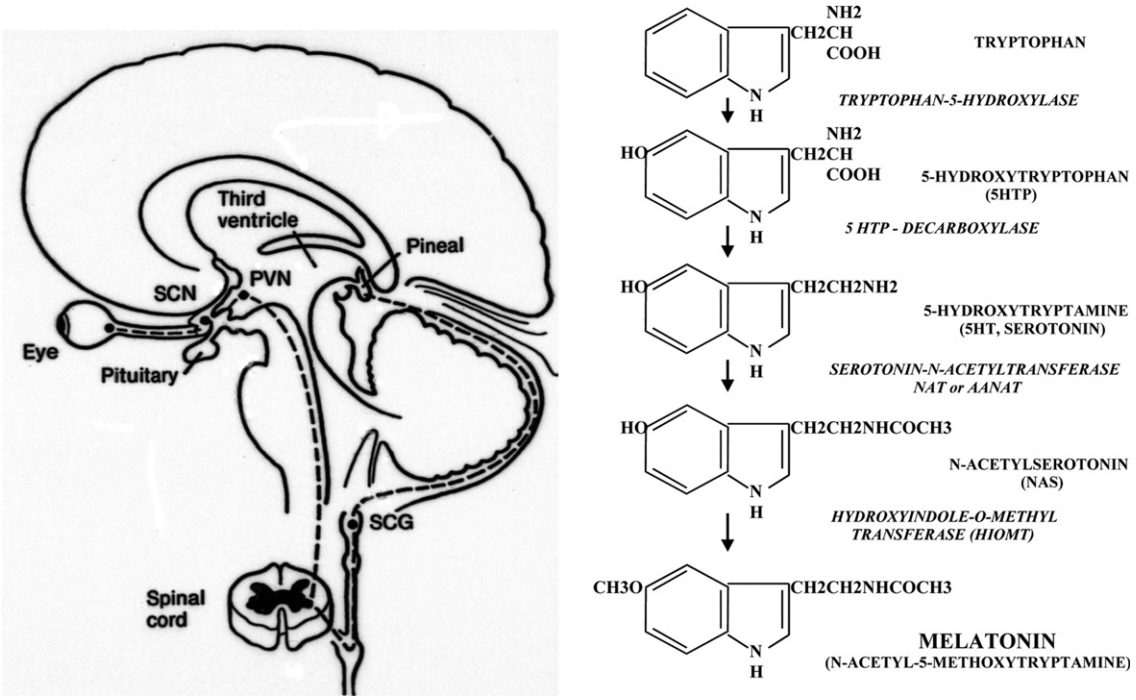

Fig. 1 Melatonin synthesis and the principle neural pathways innervating the pineal gland. SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; SCG, superior cervical ganglion. Left panel from Tamarkin, K., Baird, C.J., Almeida, O.F.X. (1985) Melatonin: a coordinating signal for mammalian reproduction. Science, 227, 714–720.

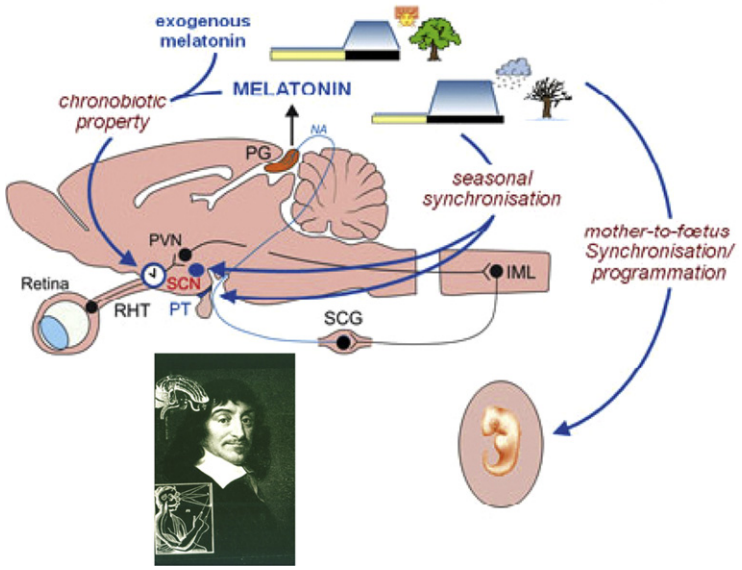

Fig. 2 A summary of the major effects of melatonin and the control of its secretion. The French philosopher Descartes considered, during the 17th century, that the pineal (PG) was controlled by light input from the eyes, and today we know that light does indeed regulate the timing of the pineal melatonin rhythm by its synchronizing effects on the activity of the central circadian clock in the suprachiasmatic nucleus (SCN), as well as its ability to suppress melatonin secretion depending on intensity and spectral composition. Light (daylength) also regulates the duration of melatonin secretion at night: long duration in short photoperiod and vice-versa. This daylength signal is used to time seasonal events in photoperiodic species. Prenatal maternal melatonin can influence postnatal circadian rhythmicity (rodents) and the timing of pubertal development (sheep). Endogenous and exogenous melatonin influence the timing of sleep and other circadian rhythms as well as having a direct influence on sleep propensity in humans. In many lower vertebrates, the pineal is effectively a 'third eye,' registering overall illuminance and acting itself as a central circadian clock. RHT, retinohypothalamic tract; PT, pars tuberalis; PVN, paraventricular nucleus; SCG, superior cervical ganglion; IML, intermediolateral column of the spinal cord. Adapted from an original diagram by Valerie Simonneaux, CNRS, Strasbourg, France, by permission.

conjugate. Exogenous oral (fast release) or intravenous melatonin has a short metabolic half-life (20–60 min, depending on author and species), with a large hepatic first-pass effect and a biphasic elimination pattern.

Very large interindividual variations in melatonin and aMT6s production are seen with intra-individual stability of both amplitude and timing. This stability leads to the extensive use of melatonin in plasma or saliva, and aMT6s in urine, as marker rhythms for circadian phase, for example in the investigation of sleep disorders, and evaluating adaptation to abrupt phase shifts as in shift work and jet lag. The large individual variations have been ascribed to the size of the pineal gland rather than to variations in enzymic activity.

Physiological Role of the Pineal Gland

The pineal is essentially part of the visual system. Probably the most famous of ancient texts on the pineal is that of the influential French philosopher Descartes. In 1662 Descartes considered that by movements, the pineal controlled the flow of 'animal spirits' into motor nerves and thus influenced movements of the body. He thought that the stimulus for pineal function came from visual input to the retina. The latter is a most remarkable insight, as effectively this is true today. It is usually Descartes to whom is attributed the concept of the pineal as the seat of the soul, although this idea probably derives from Herophilos. In lower vertebrates it is directly photoreceptive and often functions as a 'third eye' measuring overall illuminance in the environment for the organisation of circadian and seasonal functions. Pineal glands of lower vertebrates are innervated by both afferent and efferent fibres. In reptiles and birds the gland has a mixed photoreceptor and secretory function. Direct neural connections to the brain exist in mammals, but the sympathetic nervous system provides the main input (**Fig. 1** and **2**). Mammalian pineals consist largely of pinealocytes and glia.

The mammalian pineal is secretory and has lost its capacity for direct photoreception. Remnants of its ancient visual role remain in the presence of, for example, opsins in pinealocytes. However light indirectly controls mammalian melatonin production via the retino-hypothalamic projection (RHT) and the SCN.

Melatonin Secretion Characteristics

In the absence of time cues, for example light–dark alternation, the melatonin rhythm, like all circadian rhythms, 'free-runs' i.e. it assumes a period which is individually variable and genetically determined, usually somewhat longer than 24 h (on average about 24.2–24.3 h). Time cues synchronise or 'entrain' the rhythm to 24 h. The most potent time cue is the light dark cycle, and many blind people with no light perception at all show 'free-running' melatonin and other rhythms (e.g. sleep, cortisol, core temperature) in a normal environment (**Fig. 3**).

In addition to entraining the rhythm, daylength (photoperiod) determines the duration of night time secretion both by direct suppression of melatonin and by determining the length of the signal emitted by the SCN. Light of suitable intensity, duration and spectral quality, suppresses melatonin production at night, short wavelengths (ca 480 nm) are most effective, acting via a novel opsin–melanopsin-in directly photosensitive retinal ganglion cells. Short wavelengths are also most effective for entrainment of the rhythm, and indeed other circadian rhythms, to 24 h.

In mammals melatonin is secreted into the bloodstream and also probably into the CSF. CSF concentrations are reported to be higher than those of plasma in some species. In humans the evening melatonin onset is usually about 2100–2200 h, it peaks around 0300–0500 h, and declines to daytime values by about 0800–0900 h (**Fig. 4**). The effective cessation of secretion is earlier according to various models of the secretion characteristics. The melatonin onset and offset correspond to a number of important events related to biological dawn and dusk, including the evening increase in sleep propensity and decline in core body temperature. In winter, particularly in high latitudes and polar regions, the timing of the rhythm may be delayed compared to summer and this has been attributed to the weaker light dark cycle in winter. The melatonin rhythm is extensively used as a marker for the timing of the circadian system (**Fig. 5**).

Melatonin Receptors

Melatonin receptors have now been cloned and three subtypes were initially named Mel 1a, Mel 1b and Mel 1c. The Mel 1a receptor gene has been mapped to human chromosome 4q35.1. Its primary expression is in the pars tuberalis of the pituitary and the SCN. Mel 1b has been mapped to chromosome 11q21-22 and its expression is in the retina and the brain. Mel 1c is not found in mammals. Two cloned mammalian receptors (Mel 1a, Mel 1b) have been renamed MT1 and MT2. They are a new family of G protein coupled receptors, have high affinity (Kd 20–160 picomolar) and inhibit forskolin-stimulated cyclic AMP formation. Using gene knockout technology and pharmacological manipulations, the results to date suggest that the phase shifting receptor is MT2, whilst MT1 is associated with acute suppression of SCN electrical activity in addition to its actions within the pars tuberalis. However there is apparent overlap of function in some studies. Many other

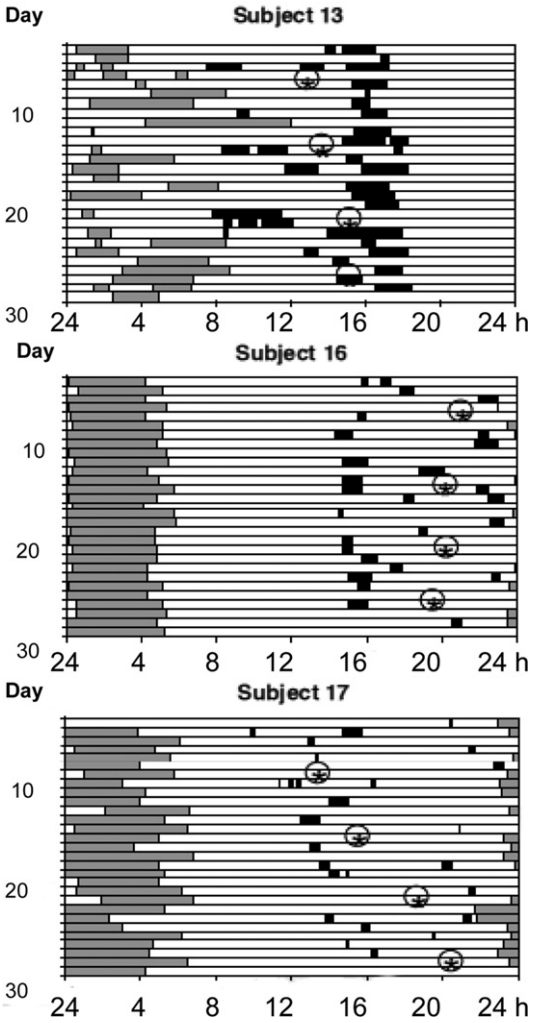

Fig. 3 Three blind subjects (no perception of light) showing abnormal sleep and melatonin rhythms. Subjectively recorded sleep is shown by the grey bars and naps (classified as such by the subjects) by the black bars. Melatonin was assessed as 6-sulphatoxymelatonin (aMT6s) in sequential urine collections for 48 h weekly and the asterisks indicate the calculated peak time (acrophase) of the aMT6s rhythm. Note the abnormal timing of aMT6s which normally peaks between 2400 and 0600 h, and the association of naps with daytime melatonin production. Subject 17 is clearly 'free-running' i.e. showing endogenous periodicity, with a steady daily delay of the aMT6s rhythm. Blind subjects with no eyes have all shown this phenomenon to date. Subject 16 is synchronized to 24 h but with an abnormal phase position. Cortisol and core body temperature rhythms show similar abnormalities to melatonin. These and other observations in the blind underline the importance of light for synchronizing rhythms and the association of daytime melatonin production with poor night time sleep and daytime naps. From Lockley, S.A., Tabandeh, H., Skene, D.J., Buttery, R., Bird, A.C., Defrance, R. and Arendt, J. (1995) Daytime naps and melatonin rhythms in blind people. Lancet, 346, 1491, by permission.

physiological responses have been ascribed to MT1 and MT2 receptors, including (MT1) melatonin-mediated potentiation of adreneregic vasoconstriction and (MT2) modulation of dopamine release in the retina. Mel1c, renamed MT3, belongs to the family of quinone reductases.

Melatonin Agonists and Antagonists

Large numbers of putative and actual melatonin agonists together with some antagonists have now been described. The most interesting have similar effects to melatonin on rhythm physiology in both rodents and humans. The primary indication is sleep disorders. A therapeutic role for the agonist agomelatine (valdoxan) in depression has emerged. Importantly this particular agonist acts as an antagonist at $5HT_{(2c)}$ receptors as well as via MT1 and MT2 receptors.. It appears to have both anti-depressant and chronobiotic effects. At the moment of writing, the marketing application for another agonist ramelteon (rozerem) has been withdrawn in Europe 'in patients best interests.' The latest agonist tasimelteon has just been approved by the FDA for use in non-24 h sleep-wake disorder, common in totally blind individuals. A recent review summarises melatonin receptor pharmacology.

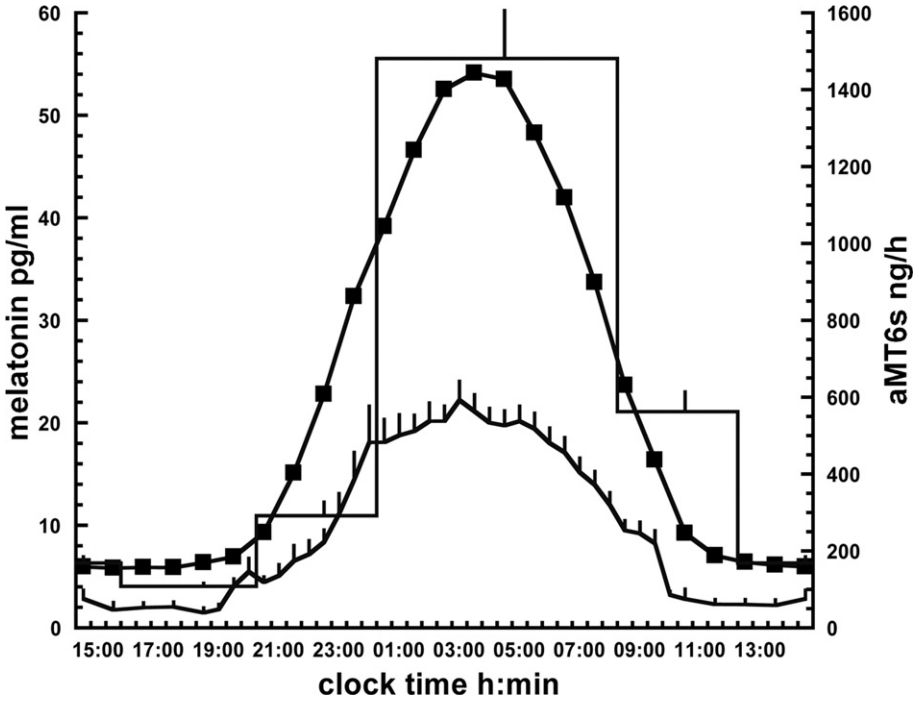

Fig. 4 Average concentrations + SEM of melatonin in plasma (squares, average N = 133, error bars within the symbol), saliva (no symbol, average N = 28) and 6-sulphatoxymelatonin (aMT6s) in urine (histogram, average N = 88), all measurements by radioimmunoassay values (healthy men and women over 18 years of age) from the author's laboratory.

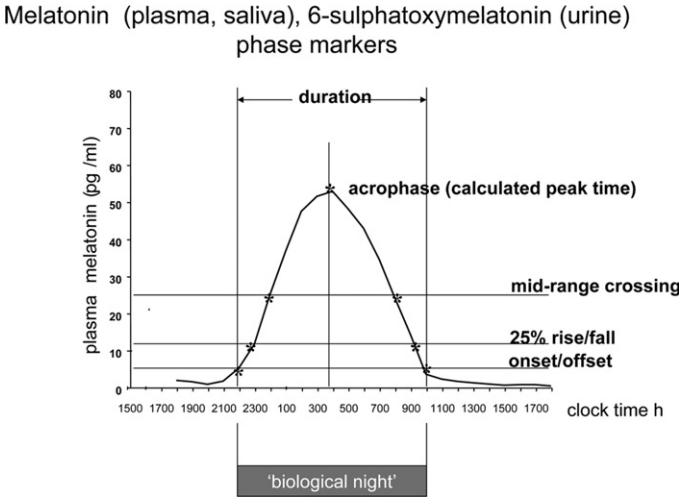

Fig. 5 The markers used to characterize melatonin and aMT6s (6-sulphatoxymelatonin) rhythms are illustrated diagrammatically. Area under the curve or total 24-h excretion (aMT6s) is used to assess total secretion. At present, there is no standard definition of onset-offset (and hence duration). The evening rise is often referred to as the dim light melatonin onset (DLMO) and the morning decline as the DLMOff, but these have variable definitions.

Physiological Role of Melatonin in Mammals

Photoperiodism

In all species studied melatonin appears to act as a timekeeping hormone. It has receptors both within and without the central nervous system and can modify gene expression in certain target organs. Pinealectomy abolishes the ability to respond to

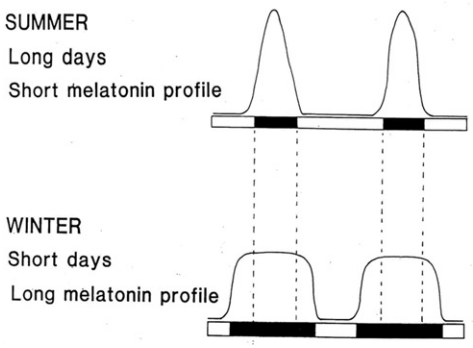

Fig. 6 The duration of melatonin secretion changes with daylength providing an internal time cue for the organisation of daylength dependent seasonal functions in photoperiodic species. This changing duration can be seen in humans if they are maintained in different durations of total darkness, however domestic intensity light at night is sufficient to greatly diminish or eliminate this photoperiodic response.

changing daylength in photoperiodic seasonal breeders. In mammals the primary role of melatonin is to provide information about photoperiod for the timing of seasonal functions in daylength dependent (photoperiodic) seasonal species. These functions include timing of breeding, of pubertal development, seasonal changes in coat growth, behaviour and body weight. The critical signal for the timing of photoperiodic events is the changing duration of melatonin secretion according to daylength, together with previous photoperiodic history (Fig. 6). Artificial generation of winter (long night) melatonin profiles in summer is equipotent with artificial long nights in changing the timing of seasonal functions. Melatonin implants (which are 'read' as long nights) are used in agriculture to time the season of birth of, for example, lambs and the growth of winter coat of cashmere goats and mink.

Melatonin influences production of gonadotrophins and gonadal hormones via actions within the hypothalamus. There is recent evidence for a molecular mechanism within the pars tuberalis of the pituitary, which links the short duration, long day melatonin signal to a hypothalamic increase of triiodothyronine (T3) through a thyroid-stimulating hormone/deiodinase2 paracrine mechanism.The local synthesis of type 2 deiodinase (Dio2) promotes triiodothyronine (T3) production and summer biology, whereas type 3 deiodinase (Dio3) promotes T3 degradation and winter biology. However melatonin controls seasonal variations in prolactin by a direct action on the pars tuberalis of the pituitary. This structure is the major site of melatonin receptors (MT1) in most species. Photoperiod-dependent gene expression in the pars tuberalis (PT) is directly modified by melatonin (Fig. 7(a) and 7(b)). The PT directly controls seasonal variations in prolactin secretion via the effects of melatonin on clock genes. In the PT, melatonin onset at dusk activates cryptochrone (Cry1) gene expression and melatonin offset at dawn activates period (Per1) gene expression, reflecting the photoperiod. It is proposed that the melatonin signal is decoded through circadian clock genes. Multiple effects of melatonin on clock genes have been reported in the PT and reports continue to accumulate of effects on clock genes in other tissues.

Role in the Mammalian Circadian System

Pinealectomy in mammals leads to subtle effects on circadian rhythms. The speed of adaptation to forced phase shift is enhanced and the activity-rest cycle becomes fragmented in continuous light in rodents. There is good evidence for the importance of the maternal melatonin rhythm in setting circadian phase in neonatal rodents. This may be the most important role for melatonin in the mammalian circadian system since an essential physiological role in adult mammals remains to be defined. The effects of pinealectomy in humans remain to be clarified: confounding factors are the possible concomitant damage of surrounding structures during surgery. However suppression of melatonin by the beta-adrenergic antagonist atenolol leads to an enhanced rate of phase shift to applied light.

The peak night time levels of melatonin are temporally associated with the nadir in core body temperature (cBT), maximum sleepiness/fatigue, lowest alertness, increased blood lipid (triacylglycerol), glucose, and possibly insulin (Fig. 8). These are correlative associations, however in the case of cBT and sleep propensity there is good evidence that melatonin contributes to the night time decline and increase respectively. Light suppression of melatonin at night leads to an increase in cBT and alertness. The increase in alertness correlates to the degree of melatonin suppression, but evidence for the quantitative contribution of endogenous melatonin to these effects is lacking. Exogenous melatonin clearly influences sleep. Possibly the best evidence for a causal role of melatonin in human sleep comes from studies in free-running blind subjects. When they are out of phase, i.e. when melatonin is secreted during the daytime, the peak levels are strongly associated with an increase in daytime napping (Fig. 3). In general melatonin reinforces functions associated with darkness. In nocturnal animals its production is associated with activity not sleep.

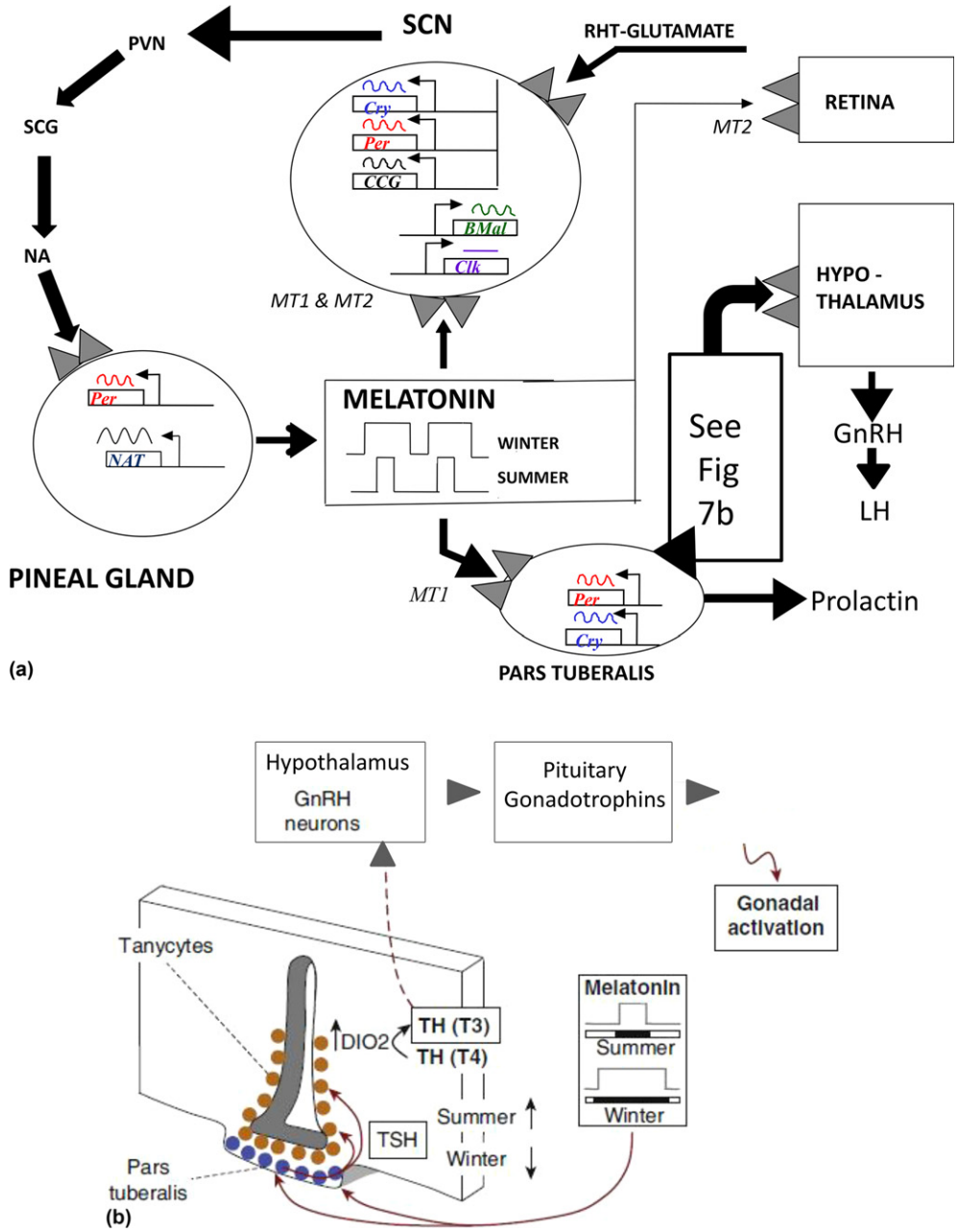

Fig. 7 (a) Diagrammatic representation of the control of production and the functions of melatonin with regard to seasonal and circadian timing mechanisms. RHT, retino-hypothalamic tract; NA, noradrenalin; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; SCG, superior cervical ganglion; MT1 and MT2, melatonin receptor subtypes. The melatonin rhythm is generated by a closed-loop negative feedback of clock gene expression in the SCN. Clk and BMal, positive stimulatory elements; Per and Cry, negative elements; CCG, clock controlled genes. Per and NAT mRNA oscillate in the pineal, although posttranscription control is evident in some species. Melatonin influences SCN activity via two or more receptors. MT2 appears to be primarily the phase-shifting receptor in rodents, whereas MT1 is associated with suppression of SCN electrical activity. The MT2 receptor was first characterized in the retina and influences dopamine release. Melatonin conveys photoperiodic information influencing the patterns of clock gene expression in the pars tuberalis for the control of seasonal prolactin variations via an MT1 receptor. Mechanisms concerning the control of seasonal gonadotrophin changes are shown in Figure 11b. Based (with permission) on an original diagram by Elisabeth Maywood, MRC Laboratory of Molecular Biology, Neurobiology Division, Cambridge, United Kingdom. Modified from Encyclopedia of Endocrine diseases. (b) The mechanisms whereby melatonin influences gonadotrophin production according to season. Circulating melatonin binds to the MT1 receptors on the pars tuberalis. Here it induces TSH production under long photoperiods. The high levels of TSH cause the hypothalamic tanycytes in the third ventricle wall to produce thyroid hormone (T3) from the inactive form (T4) through increased expression of the enzyme DIO2 (type II thyroid hormone deiodinase). T3 then by a direct or indirect pathway drives the production of GnRH within the hypothalamus, to stimulate gonadotrophin production from the anterior pituitary once a critical length of photoperiod is present. Modified in part from Hut RA, Photoperiodism: Shall *EYA* Compare Thee to a Summer's Day? Current Biology 2011, 21:R22–R25, by permission.

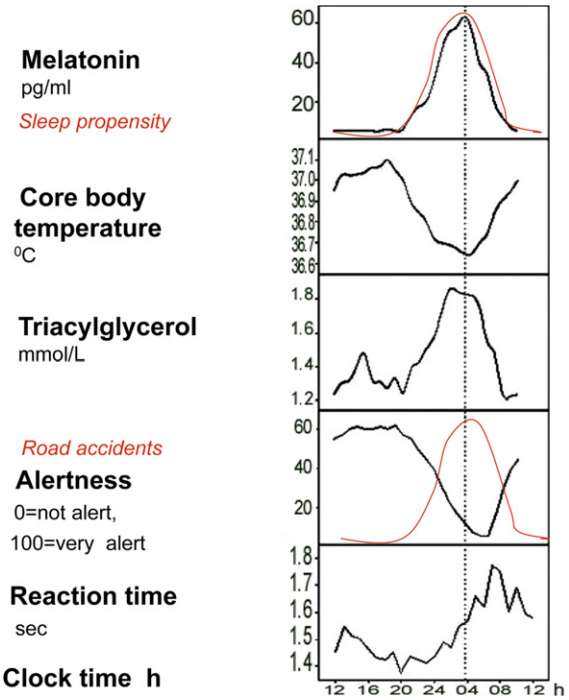

Fig. 8 Relationship of plasma melatonin to other major circadian rhythms. Note the close correspondence between the core temperature nadir and the melatonin peak. Reproduced from Rajaratnam SMW and Arendt J. Lancet 358:999–1005, 2001 by permission.

Melatonin and Human Reproduction

Melatonin declines during development, peaking at 3–4 years, with a plateau circa 18–35 years, followed by a decline into old age (although it should be noted that very healthy elderly subjects have not shown this decline). Early work suggested that it might have a role in the timing of human puberty, acting in an inhibitory capacity. Certainly exogenous melatonin is able to delay pubertal development in rats.

Pineal tumours are frequently associated with precocious/delayed puberty, but melatonin production has not been firmly implicated in normal or abnormal pubertal development in humans. Pineal tumors are heterogeneous and may arise from germ cells (teratomas, germinomas, choriocarcinomas, endodermal sinus tumors, mixed germ cell tumors), pineal parenchymal cells (pineoblastoma and pineocytoma), and the supporting stroma (gliomas). All are rare (less than 1% of intracranial space-occupying lesions) and tend to occur below 20 years of age with the exception of parenchymal cell tumors, which occur equally in adults and children. Most of the evidence now suggests that precocious puberty is due to the production of human chorionic gonadotrophin (hCG) by germ cell tumors of the pineal. Precocious puberty is associated with abnormally low melatonin levels whereas in delayed puberty, and in hypothalamic amenorrhoea melatonin levels are high in comparison to age matched normal subjects.

There is no doubt that suitable administration of melatonin can modify human reproductive function, generally speaking in an inhibitory capacity. It is able to suppress the ovulatory LH peak and to potentiate testosterone-induced LH suppression in very large doses. It can modulate the ultradian properties of LH and FSH secretion and acutely increases PRL (although chronic administration lowers prolactin in animals, corresponding to the effects of long nights). A series of studies in males with and without hypogonadism has reinforced the perception that melatonin is essentially inhibitory to human reproductive function. An (unsuccessful) attempt has been made to develop it as an oral contraceptive in combination with a progestin minipill, with very large daily doses (80 mg). These observations fuel worries concerning the possible reproductive side effects of over the counter availability of melatonin as a sleep aid in some countries.

General Human Pathology

Numerous reports exist of variations in melatonin secretion as a function of endocrine and other diseases. A problem with many publications is the possible influence of ambient light conditions and posture, together with sleep-wake characteristics of the subjects, especially if a hospitalised group is compared with a non-hospitalised control population. Liver disease such as cirrhosis, which impairs metabolic function, leads to higher than normal plasma concentrations and abnormal profiles of melatonin. Drugs that stimulate or suppress hydroxylation and conjugation mechanisms or that compete for metabolic pathways can be expected to

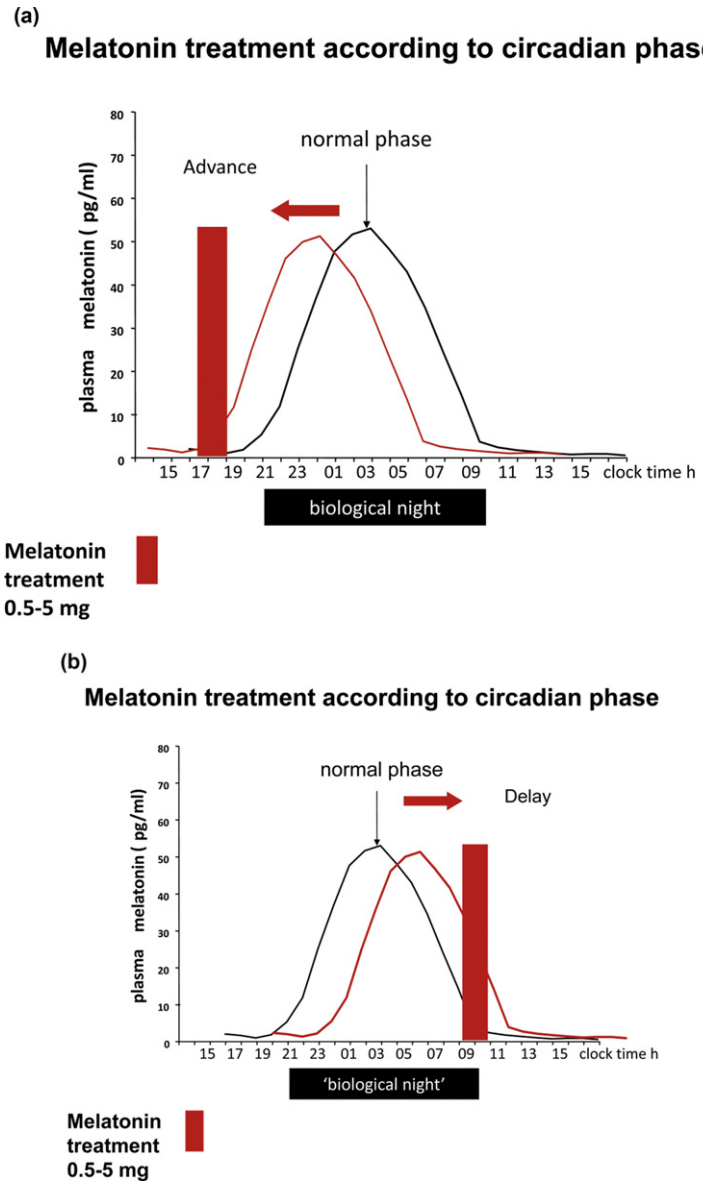

Fig. 9 (a and b) Diagrammatic representation of phase shifts induced by melatonin. Melatonin treatment, specifically timed according to internal circadian phase (shown here by the timing of the endogenous melatonin rhythm) can advance or delay the circadian clock as evidenced by shifts in its own endogenous rhythm, or that of core body temperature. The shift can be predicted by a 'Phase Response Curve' which describes the magnitude and direction of shift that can be obtained with specific circadian timing of treatment. Other rhythms that can be phase-shifted by melatonin include sleep, alertness, cortisol and TSH. The oral doses used are usually in the range of 0.5–5 mg fast release. The phase shift obtained is dose dependent, but individually variable.

affect circulating melatonin. There is little evidence for a disturbance of melatonin secretion in major sleep disorders such as narcolepsy and Klein–Levine syndrome (intermittent sleeping for days at a time). One very interesting genetic disorder, Smith–Magenis syndrome is caused by a deletion in chromosome 17p11.2. The syndrome is associated with various physical, developmental and behavioural disabilities, but it is not fully understood. It is associated with daytime melatonin production and poor nighttime sleep, but with normal cortisol rhythms. Pharmacological suppression of the daytime melatonin leads to improved daytime alertness and night time sleep. For some time sporadic publications have associated melatonin with aspects of diabetes and metabolism. For example light treatment to suppress melatonin and/or change its timing of secretion may reduce the need for insulin in diabetes mellitus. Recently a rare variant of the *MTNR1B* gene that encodes the receptor MT2 and impairs its function, has been reported and is associated with impaired glucose homeostasis, reduced insulin secretion, and an increased risk of developing type 2 diabetes. MT1 and MT2 knock-outs have provided some evidence that melatonin may synchronise the functions of the major organs involved in blood glucose regulation. Melatonin is also implicated in cardiovascular function with therapeutic

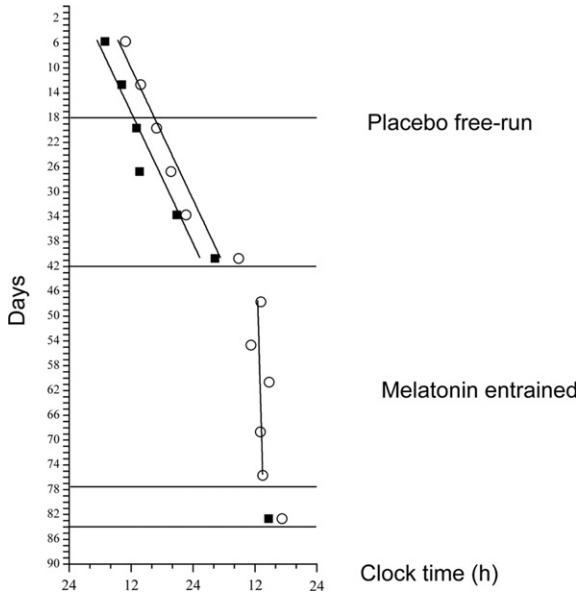

Fig. 10 Melatonin can phase shift and, in some cases, synchronise circadian rhythms in some sighted and blind subjects with suitable timing of treatment and dose. Shown are the times of the calculated peaks (acrophases) of urinary 6-sulphatoxymelatonin (squares) and cortisol (circles) of a free-running blind subject with a period of 24.57 h treated with placebo or 5 mg melatonin daily, timed to phase advance the internal clock. Note that with melatonin a 24 h period is maintained. Significant beneficial effects on sleep are found using melatonin treatment in non-24 h sleep disorder of the blind. Redrawn from Lockley SW, Skene DJ, James K, Thapan, K, Wright J and Arendt J. Melatonin administration can entrain the free running circadian system of blind subjects. J Endocrinol 164:R1–R6, 2000, reproduced by permission of the Society for Endocrinology.

possibilities in hypertension and other cardiovascular problems. Numerous attempts have been made to relate melatonin suppression to exposure to electromagnetic fields, with no consistent data in humans.

Melatonin has been extensively investigated in psychiatric disorder, particularly winter depression or seasonal affective disorder (SAD) where a causal role has been proposed. No firm conclusions can be drawn at present. Many antidepressant drugs acting on serotonergic or catecholaminergic systems will acutely increase or otherwise modify melatonin production. It is also invoked as a possible factor in autism and in Asperger syndrome.

There is some evidence for an involvement of melatonin and the pineal gland in vasopressin production.

Possible Role of Melatonin in Cancer

Melatonin suppression has been linked to infertility and increased incidence of certain cancers. There is evidence for low levels of melatonin in hormone dependent cancer (oestrogen receptor positive breast cancer, prostate cancer). Some cancers are photoperiod dependent in rodents and melatonin may have a role here. Pinealectomy in rats leads to a shortened survival time in dimethylbenzanthracene-induced mammary cancer: melatonin administration reverses this effect. There is good evidence for melatonin inhibition of growth of some hormone dependent cancer cell lines and also evidence for stimulation of immune function. Combined melatonin therapy with tamoxifen for breast cancer is being investigated in humans. It has been proposed that light at night during night shift work suppresses melatonin and that this loss of melatonin 'activity' is responsible for the increased cancer risk. However, to attribute any detrimental effects of shift work directly to loss of melatonin is overspeculative. The average drop in melatonin in rotating night shift workers at present appears to be around 20% and the meaning of such a (small) change is unknown). Moreover light at night has numerous other effects. The mere fact of frequent disruption of all circadian rhythms, not just melatonin, is effectively a physiological insult. In animals frequent forced change of phase (by manipulating the light–dark cycle) leads to increased vulnerability to development of cancer.

Melatonin as Anti-oxidant-Free Radical Scavenger

The melatonin molecule is easily oxidised, and therefore it is not particularly surprising that it demonstrates anti-oxidant/free radical scavenging effects. The concentrations required are, in general, orders of magnitude higher than physiological levels. However there is evidence that endogenous circulating melatonin may be responsible for some of the antioxidant status of blood. Many reports exist of its neuroprotective and cardioprotective ability which is attributed to free-radical scavenging activity. Some recent reports include its ability to improve in vitro sperm viability, oocyte competence and blastocyst development.

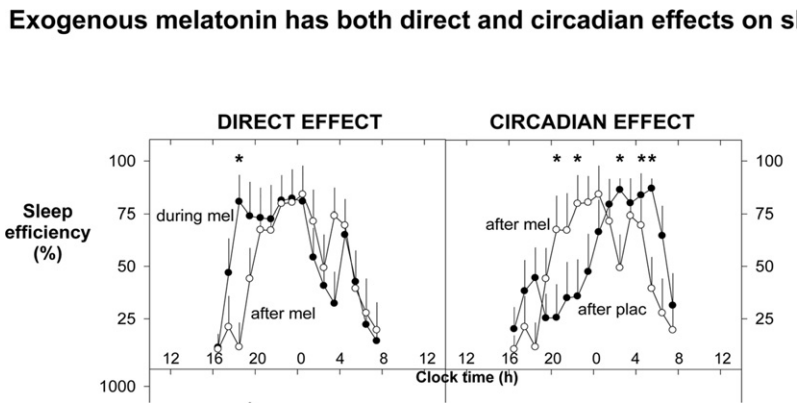

Fig. 11 Exogenous melatonin has both direct and circadian effects on sleep. Healthy young men (n = 8) received 1.5 mg surge sustained release melatonin or placebo in a double blind cross-over design at 1600 h daily for 8 days, recumbent, <5 lux, 1600–0800 h, sleep was evaluated by polysomnography on the night after the last dose of melatonin or placebo and on the subsequent night after melatonin washout. These data confirm the utility of melatonin for the treatment of delayed sleep. In a treatment situation the two effects can be maximised by suitable timing of the dose. Hormones (melatonin, cortisol, TSH) and heart rate variability showed similar substantial phase advances as seen for sleep. From Rajaratnam SMW, Middleton B, Stone BM, Arendt J, Dijk D-J. J Physiol 2004; 561:339–351, by permission.

Effects of Exogenous Melatonin in Humans

The sleepiness-inducing properties of melatonin have been known since its discoverer, the dermatologist Aaron Lerner, reported this effect after self administration. A substantial body of evidence indicates that taken during biological daytime (i.e. when endogenous melatonin is very low or absent), melatonin induces sleepiness/sleep and lowers alertness, cognitive performance and cBT. These effects are posture dependent: subjects must be recumbent and preferably in very dim light for maximum responses to be seen. Accompanying these direct effects is the important ability of melatonin to change the timing of the internal circadian clock as evidenced by shifts in the timing of endogenous melatonin itself, cortisol, cBT, TSH and sleep rhythms (Fig. 9–**11**). Administration during the (biological) afternoon/early evening (where biological night is the period of melatonin secretion) leads to phase advances, whereas taken in the early morning phase delays can be elicited. The existing data have been summarised as a 'phase response curve' from which specific timing of administration to induce a specific shift can be predicted.

Therapeutic Uses

In view of its ability to induce sleepiness, change the timing of sleep and shift the timing of the internal clock melatonin has been extensively investigated as a treatment for circadian rhythm related sleep disorders. It has proved successful, when correctly timed, for the alleviation of jet lag, improving daytime sleep in shift workers and normalising sleep time in delayed sleep phase syndrome. It is able to synchronise free-running sleep and other rhythms of some blind subjects with suitable dose and timing of treatment (**Fig. 10**). It is the latter indication which underlines the importance of such timed treatment. Circadian sleep disorders can normally be treated with appropriate exposure to bright light to shift the clock, inducing a resetting to a normal or near normal pattern of melatonin secretion. In the blind this is not possible and melatonin is the treatment of choice.

It is likely that SCN receptors mediate the circadian effects of melatonin, those in the pars tuberalis influence photoperiodic seasonal reproduction with regard to gonadotrophin secretion and prolactin, and those in the retina mediate the retinal processes influenced by melatonin (**Fig. 7(a)**). The physiological functions of the multiplicity of melatonin binding sites in other areas remain to be clarified.

Conclusions

Melatonin acts as both an internal clock and an internal calendar to influence the timing of circadian and seasonal rhythms. It is likely that any aspect of physiology which depends on perception of daylength change, and/or which is driven by the master circadian clock in the SCN, is susceptible to the effects of melatonin. These effects may be modulatory and complementary to the effects of light, in the case of the adult circadian system. In the perinatal period maternal melatonin may serve to set the timing of circadian rhythms in the offspring.

Suitable timing of melatonin administration is critical for therapeutic benefit in rhythm disorders. The acute effects of melatonin on sleep, in particular, reinforce its therapeutic effects. The possible clinical applications of anti-oxidant activity remain to be explored.

The effects of melatonin on photoperiod dependent functions are critically important and include, for example, the timing of pubertal development in photoperiodic species. It is used commercially in agriculture to modify the timing of breeding and seasonal changes in pelage.

Current research focuses on the mechanisms of action with particular emphasis on receptor mechanisms and modification of gene expression in target tissues. Melatonin is the marker rhythm of choice for the investigation of disturbed circadian rhythms in, for example, sleep disorder, shift work and jet lag. It is recommended by the American Academy of Sleep Medicine for the treatment of circadian rhythm sleep disorders, including jet lag, delayed sleep phse, free-running sleep-wake cycle and shift work.

Acknowledgments

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Overview of Neurotransmitters☆

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Glossary

Acetylcholine The neurotransmitter for all autonomic ganglia and the terminal links of the parasympathetic nervous system, the neuromuscular junction, and large components of the central nervous system.

Anandamides A group of naturally occurring lipid amides whose receptors provide the molecular sites at which cannabinoid drugs (e.g., marijuana) produce their regulation of emotional responsiveness.

Catecholamines The chemical family of monoamine neurotransmitters, which are all produced from the amino acid tyrosine by the enzyme tyrosine, thus adding the second hydroxy to the phenyl ring characteristic of catechols.

Dopamine The main catecholamine neurotransmitter innervating the basal ganglia and some parts of the hypothalamic nuclei controlling pituitary secretion.

Endorphins One of the subfamilies of neuropeptide transmitters constituting the class whose receptors provide the molecular sites at which synthetic opiates provide pain relief and other effects.

GABA The major inhibitory neurotransmitter in the mammalian brain; the reaction product of glutamate decarboxylase.

Glutamate The major excitatory neurotransmitter in the mammalian brain; a dicarboxylic amino acid also used to synthesize proteins.

Histamine A significant neurotransmitter for neurons of the basal hypothalamus, with lengthy projections to the forebrain. Histamine is a diamine and chemically one of the aminergic neurotransmitters.

Neuropeptides A chemical class of neurotransmitters composed of three or more amino acids in peptide linkages, generally working in concert with amino acid or amine neurotransmitters. All hypothalamic hypophysiotrophic hormones are neuropeptides.

Norepinephrine The second major catecholamine neurotransmitter innervating most regions of the mammalian brain, including the magnocellular hypothalamic nuclei, hippocampus, cerebral cortex, olfactory bulb, and cerebellum.

Introduction

Neurotransmitters can be grouped within three main chemical categories: amino acids, amines, and neuropeptides. Other substances that may participate in central synaptic transmission include purines (e.g., adenosine and ATP), nitric oxide, and arachidonic acid derivatives.

Amino Acids

The central nervous system (CNS) contains uniquely high concentrations of two highly potent amino acids capable of affecting neuronal activity: glutamate and GABA. Neuroscientists were once quite reluctant to accept these or other amino acids as central neurotransmitters because of their broad distribution and their ability to produce powerful effects on virtually every neuron tested. Thus, glutamate and other dicarboxylic amino acids produce consistent excitation, whereas GABA, glycine, and other monocarboxylic amino acids produce consistent inhibition. There is general acceptance that the amino acids GABA, glycine, and glutamate are central transmitters.

GABA

GABA was recognized as a unique chemical component of brain in 1950, long before its functions were understood. Its neurotransmitter properties were first validated for the inhibitory nerves of crustacean (lobster) limb muscles, in which GABA content was shown to account for the inhibitory effects of extracts of these nerve fibers, and release of GABA with increased firing of the nerve fibers correlated with the inhibitory effects on the muscle.

In the mammalian CNS, GABA is considered the major mediator for local interneurons and for presynaptic inhibition within the spinal cord. Presumptive GABAergic inhibitory synapses have been demonstrated most clearly between cerebellar Purkinje neurons and their targets in Deiter's nucleus; between small interneurons and the major output cells of the cerebellar cortex, olfactory bulb, cuneate nucleus,

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hippocampus, and lateral septal nucleus; and between the vestibular nucleus and the trochlear motoneurons. GABA also mediates inhibition within the cerebral cortex and between the caudate nucleus and the substantia nigra. GABAergic neurons and nerve terminals have been localized with immunocytochemical methods that visualize glutamic acid decarboxylase, the enzyme that catalyzes the synthesis of GABA from glutamic acid. GABA-containing neurons have frequently been found to coexpress one or more neuropeptides. The most useful selective antagonists for demonstration of likely GABAergic synapses are bicuculline and picrotoxin.

GABA receptors exist in two main molecular forms, each with a functional representation. The more prominent GABA receptor subtype, the $GABA_A$ receptor, is a ligand-gated Cl^- ion channel that opens in the presence of GABA. As a typical ion channel receptor, akin to the classical cholinergic nicotinic receptor, the $GABA_A$ receptor is composed of four or five subunits, each of which is composed of proteins that exhibit four transmembrane domains and cluster to form the ion channel for Cl^-. Although there are four main classes of subunits, there are multiple variations, providing for an enormous number of possibly similar but distinct classes of GABA receptors. Many neuroactive drugs, such as benzodiazepines, ethanol, and barbiturates, act on $GABA_A$ receptors. The $GABA_B$ receptor is a G protein-coupled receptor able to activate second-messenger signal transduction pathways, and like other receptors of this class, it exhibits a structure composed of seven transmembrane domains. Many of the features described for the $GABA_A$ receptor family also apply to the glycine receptor, which appears to be the major inhibitory neurotransmitter in the brainstem and spinal cord.

Glutamate and Aspartate

Glutamate and aspartate are both abundant in brain, and both are powerful excitants for neurons in every brain region of the CNS, where they are considered the principal fast (classical) excitatory transmitters. Glutamate receptors, like GABA receptors, exist in two forms – as ligand-gated ion channel receptors with multiple possible combinations of a few isoforma of subunits or as G protein-coupled receptors. The ligand-gated glutamate ion channel receptors are further classified according to the agonists that selectively activate them, including α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and *N*-methyl-D aspartate (NMDA) receptors, each of which has selective agonists and antagonists. The diversity of gene expression and, consequently, of the protein structure for glutamate receptors also arises by alternative splicing and RNA editing.

AMPA and kainate receptors mediate fast depolarization at most glutamatergic synapses in the brain and spinal cord. NMDA receptors are also involved in normal synaptic transmission, but activation of NMDA receptors is more closely associated with the induction of various forms of synaptic plasticity. AMPA or kainate receptors and NMDA receptors coexist at many glutamatergic synapses. Activation of NMDA receptors is obligatory for the induction of the form of plasticity known as long-term potentiation. Activation of NMDA receptors requires both the binding of glutamate and the simultaneous depolarization of the postsynaptic membrane to displace a Mg^{2+} normally bound within the channel. High concentrations of glutamate are lethal to neurons because of elevation of intracellular Ca^{2+} levels and induction of apoptosis. Studies suggest that local depletion of Na^+ and K^+, as well as small increases in extracellular Zn^{2+}, will activate both necrotic and proapoptotic cascades of neuronal death. Disordered glutamatergic transmission has been postulated as a mechanism of chronic neurodegenerative diseases and schizophrenia.

Amine Transmitters

In the following sections, all the neurotransmitters that are chemically amines are discussed, including the monoamines dopamine, norepinephrine, and epinephrine (which together comprise the catecholamines); 5-hydroxytryptamine (or serotonin); the diamine histamine; and the quaternary amine acetylcholine. However, all of these have been studied for far longer than their chemical similarities have been apparent.

Acetylcholine

Acetylcholine (ACh) became the first established chemical neurotransmitter when it was demonstrated to be the substance released by the vagus nerve to slow the heart rate as the terminal step in the parasympathetic control of heart rate. ACh was later identified as the transmitter at both neuromuscular and parasympathetic neuroeffector junctions, as well as at the major synapse within autonomic ganglia. Eventually, ACh received recognition as a central neurotransmitter, based on its irregular distribution within the CNS and the behavioral effects exerted by cholinergic drugs after central administration. The first established cholinergic central synapse was the recurrent excitatory synapse from Renshaw interneurons to spinal motoneurons.

As for the amino acid receptors, the classical pharmacological forms of the cholinergic receptors were also shown by molecular cloning and sequence determinations to be represented by ligand-gated ion channels (nicotinic receptors) and by G protein-coupled forms (muscarinic receptors). As is the case for the amino acids, nicotinic receptors have multiple combinations of four transmembrane domain subunits, whereas the muscarinic receptors, like all known G protein-coupled receptor forms, exist as a single seven-transmembrane domain. In most regions of the CNS, the effects of ACh, assessed either by iontophoresis or by radioligand receptor-displacement assays, appear to be generated by interaction with a mixture of nicotinic and muscarinic receptors. Several sets of presumptive cholinergic pathways have been described in addition to that of the nicotinic spinal Renshaw cell to motoneuron. Eight major clusters of ACh neurons and their pathways have been characterized, with four separate groups of

cell bodies located in the basal forebrain, two groups in the upper pons provide the major cholinergic innervation of thalamus and striatum, and there are two groups for the midbrain and brainstem. The intense cholinergic projections to neocortex and hippocampal formation are dependent on the trophic growth factors provided to them by retrograde axonal transport from their target neurons, and their loss has been the sole target of treatments for Alzheimer's disease through inhibition of the central catabolic enzyme, acetylcholinesterase.

Catecholamines

The brain contains separate neuronal systems that use three different catecholamines – dopamine, norepinephrine, and epinephrine. These monoamines are derived from the common precursors tyrosine and L-dihydroxyphenylalanine (L-DOPA), and they form a three-step synthetic metabolic pathway. Tyrosine is converted to L-DOPA by tyrosine hydroxylase, L-DOPA is converted to dopamine by DOPA decarboxylase, dopamine is converted to norepinephrine by dopamine β-hydroxylase, and norepinephrine is converted to epinephrine by the synthetic enzyme phenylethanolamine *N*-methyl transferase. When these enzymatic steps were determined, antibodies raised against these proteins were found to be useful cellular markers and defined three catecholamine-containing neuronal systems: Tyrosine hydroxylase defined dopamine- and norepinephrine-synthesizing neurons, antibodies to dopamine β-hydroxylase revealed exclusively the norepinephrine-synthesizing neurons, and antibodies to phenylethanolamine *N*-methyl transferase defined the epinephrine-synthesizing neurons. Each system is anatomically distinct and serves separate but similar functional roles within their fields of innervation.

Dopamine

Originally, dopamine was considered to be only a precursor for norepinephrine; however, assays of distinct regions of the CNS eventually revealed that the distributions of dopamine and norepinephrine are markedly different. In fact, more than half the CNS content of catecholamine is dopamine, and extremely large amounts are found in the basal ganglia (especially the caudate nucleus), the nucleus accumbens, the olfactory tubercle, the central nucleus of the amygdala, the median eminence, and restricted fields of the frontal cortex. The anatomical connections of the dopamine-containing neurons are categorized into three major morphological classes: (i) ultrashort neurons within the amacrine cells of the retina and periglomerular cells of the olfactory bulb; (ii) intermediate-length neurons within the tuberobasal ventral hypothalamus, critical for many endocrine regulatory processes within the median eminence and intermediate lobe of the pituitary, that connect the dorsal and posterior hypothalamus with the lateral septal nuclei and other hypothalamic nuclei; and (iii) long projections between the major dopamine-containing nuclei in the substantia nigra (named for the black pigment that these neurons exhibit in the postmortem human and monkey brain and that is lost when these neurons die in Parkinson's disease) and the ventral tegmentum. These dopamine neurons project to precise targets in the striatum, in the cerebral cortex, and in other major limbic structures except the hippocampus. All dopamine receptors are represented by two functional classes of G protein-coupled receptors – those that activate adenylate cyclase (D1 and D5) and those that inhibit the activation of adenylate cyclase (D2–D4). The D2 receptors couple to multiple effector systems, including the inhibition of adenylyl cyclase activity, suppression of Ca^{2+} currents, and activation of K^{+} currents. D2 dopamine receptors have been implicated in the pathophysiology of schizophrenia and Parkinson's disease.

Norepinephrine

The noradrenergic innervation of the forebrain, including the hypothalamus, cortex, hippocampus, and olfactory bulb, arises from the major cluster of noradrenergic neurons in the pontine nucleus called the locus ceruleus because of the blue pigment they contain in the brains of primates. There are relatively large amounts of norepinephrine within the hypothalamus and in certain zones of the limbic system, such as the central nucleus of the amygdala and the dentate gyrus of the hippocampus. However, norepinephrine is also present in significant but lesser amounts in most brain regions. Detailed mapping studies indicate that most noradrenergic neurons arise either in the locus ceruleus of the pons or in neurons of the lateral tegmental portion of the reticular formation. From these neurons, multiple branched axons innervate specific target cells in a large number of cortical, subcortical, and spinal fields. The pharmacological properties of such synapses are complex, with evidence of mediation by both α- and β-adrenergic receptors (both G protein-coupled receptors; the β-adrenergic receptors activate adenylate cyclase, whereas α-adrenergic receptors inhibit cyclic AMP synthesis, and each form has three or more subtypes). For example, stimulation of the locus ceruleus depresses the spontaneous activity of target neurons in the cerebellum mediated by a slowly developing hyperpolarization and a decrease in membrane conductance. However, activation of the locus ceruleus also enhances the higher firing rates produced by stimulation of excitatory inputs to these convergent target neurons to a lesser degree. The afferent circuits innervating the locus ceruleus neurons include medullary cholinergic neurons, opioid peptide neurons, raphe (5-HT) neurons, and corticotropin-releasing hormone neurons from the hypothalamus. Tricyclic antidepressant drugs act on noradrenergic neurons, augmenting levels in the extracellular space by inhibiting the norepinephrine transporter.

Epinephrine

Epinephrine-containing neurons are found in the medullary reticular formation and make restricted connections to a few pontine and diencephalic nuclei, such as the paraventricular nucleus of the dorsal midline thalamus. Their physiological properties have not been established.

5-Hydroxytryptamine

The early history of this neurotransmitter brought together two sets of previously unconnected work on factors extracted from serum (serotonin) and gut (enteramine) identified chemically as 5-hydroxy-tryptamine (5-HT), which is functionally detected in large amounts in brain. Through various cytochemical approaches, 5-HT-containing neurons have been characterized in nine pontine and medullary nuclei lying in or adjacent to the midline (raphe). The more rostral raphe nuclei innervate forebrain regions, whereas the caudal raphe nuclei project within the brainstem and spinal cord with some overlap. The median raphe nucleus makes a major contribution to the innervation of limbic cortical structures, and the dorsal raphe nucleus makes a similar contribution to the cerebral cortex and the neostriatum.

Molecular biological approaches have led to the identification of more than 12 distinct mammalian 5-HT receptor subtypes, all of which are G protein-coupled receptors linked to a variety of signal transduction cascades except for the 5-HT_3 receptor, which is a ligand-gated ion channel receptor. These subtypes exhibit characteristic ligand-binding profiles, couple to different intracellular signaling systems, exhibit subtype-specific distributions within the CNS, and mediate distinct behavioral effects of 5-HT. The 5-HT_{1A} receptors are abundantly expressed on 5-HT neurons of the dorsal raphe nucleus, where they are thought to be involved in temperature regulation. They are also found in regions of the CNS associated with mood and anxiety, such as the hippocampus and amygdala. 5-HT_{1D} receptors are potently activated by the drug sumatriptan, which is prescribed for acute management of migraine headaches.

The 5-HT_2 receptor class has three recognized subtypes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. 5-HT_{2A} receptors are enriched in forebrain regions such as neocortex and olfactory tubercle as well as in several brainstem nuclei, including most cranial motoneurons. The 5-HT_{2C} receptor, which is very similar in sequence and pharmacology to the 5-HT_{2A} receptor, is expressed abundantly in the choroid plexus, where it regulates cerebrospinal fluid production. The hallucinogen LSD interacts through the 5-HT_2 receptors. Serotonin-containing pathways have been implicated in sleep disturbances, appetitive problems, pain, and depression. The class of drugs termed serotonin selective reuptake inhibitors is very effective in some patients with major depression.

Histamine

Although not recognized as a neurotransmitter for much of the recent history of such signaling molecules, histamine and antihistaminic drugs were long known to possess potent effects on animal behavior. The subsequent biochemical detection of histamine synthesis by neurons, as well as direct cytochemical localization of these neurons, established the existence of a histaminergic system in the CNS. Most histaminergic neurons are located in the ventral posterior hypothalamus and give rise to long ascending and descending tracts to the entire CNS. Histaminergic neurons function in the regulation of arousal, body temperature, and vascular dynamics.

Peptides

During the 1980s, numerous novel peptides were discovered in the CNS, each capable of regulating one or another aspect of neural function. Furthermore, certain peptides previously thought to exist only in gut or endocrine glands were also found in the CNS. Most CNS peptides are thought to act mainly in concert with coexisting transmitters, both amines and amino acids. Each neuropeptide system has significant implications for endocrinology and endocrine diseases. Three general approaches to the continuously growing families of neurons containing one or more neuropeptides are discussed here.

Organization by Peptide Families

Peptides can be grouped into families based on the possession of significant homology in amino acid sequences, with each having its own genetic representation through evolutionary gene duplication and subsequent divergence of functions. Such relationships are well illustrated by the tachykinin or the vasotocin (vasopressin/oxytocin) families, in which species differences can be correlated with modest variations in peptide structure. The multiple members of the tachykinin/substance P family coexist within mammalian neurons in the brain and in the intestines and may account for the apparent existence of subsets of receptors for each of these peptides. The mammalian representatives of the vasotocin family show two concurrent products, vasopressin and oxytocin, each having evolved to perform separate functions once executed in amphibia by a single vasotocin-related peptides and receptor. A different divergent evolutionary pathway is illustrated by the endorphin and by the glucagon–secretin peptide families. In the endorphin superfamily, three major genetically distinct systems of endorphin peptides (proopiomelanocortin, proenkephalin, and prodynorphin) exist in independent neuronal circuits. These natural opioid peptide families have emerged from independent but homologous genes. The peptides all share some actions at receptors once classed generally as ‘opioid’ but that are undergoing progressive refinement with molecular resolution of their structures, functions, and cellular expression patterns. In the glucagon–secretin family, multiple and somewhat homologous peptides are found simultaneously in different cells of the same organism but in separate organ systems: glucagon and vasoactive intestinal polypeptide (VIP) in pancreatic islets; secretin in duodenal mucosa; VIP and related peptides in enteric, autonomic, and central neurons; and growth hormone-releasing factor in central neurons only. The general metabolic effects produced by this family lead to increased blood glucose.

Organization by Anatomic Pattern

Some peptide systems follow consistent anatomical organizations. Thus, the hypothalamic peptides oxytocin, vasopressin, proopiomelanocortin, gonadotropin-releasing hormone, and growth hormone-releasing hormone all tend to be synthesized by single large clusters of neurons that give off multibranched axons to several distant targets. Others, such as systems that contain somatostatin, cholecystokinin, and enkephalin, can have many forms, with patterns varying from moderately long, hierarchical connections to short-axon, local-circuit neurons that are widely distributed throughout the brain.

Organization by Function

Since almost all peptides were initially identified on the basis of bioassays, their names reflect these biologically assayed functions (e.g., thyrotropin-releasing hormone and vasoactive intestinal polypeptide). These names will become trivial if more ubiquitous distributions and additional functions are discovered. Some general integrative role might be hypothesized for widely separated neurons (and other cells) that make the same peptide. However, a more parsimonious view is that each peptide has unique messenger roles at the cellular level and that these are used repeatedly in functionally similar pathways within large systems that differ in their overall functions. Cloning of the major members of the opioid–peptide receptors revealed unexpected and unexplained conservation of sequences with receptors for somatostatin, angiotensin, and other peptides.

Comparison with Other Transmitters

Peptides differ in several important respects from the monoamine and amino acid transmitters. Synthesis of a peptide is performed in the rough endoplasmic reticulum of the cell body, while monoamines and amino acids are synthesized in the nerve terminals, only with the rough endoplasmic reticulum of the neuronal cell body can the mRNA for the propeptide be translated into an amino acid sequence. The propeptide is cleaved (proteolytically processed) to the form that is secreted as the secretory vesicles are transported from the perinuclear cytoplasm to the nerve terminals. In addition, no active reuptake mechanisms for peptides have been described. Thus, peptidergic nerve terminals are dependent for their signaling on distant sites of synthesis. Much progress has been made in to the development of drugs that act as antagonists for neuropeptides and that are orally active and not peptides, thereby advancing roles for neuropeptides in human diseases such as stress and depression.

Modern Modes of Neuropeptide Discovery

While the vast majority of neuropeptides were discovered by exploiting brain abstracts for bioassay actions on presumptive end organs, that is, the hypophysiotrophic factors action on specific pituitary cell groups, new methods of discovery emerged in the 1990s. The initial drafts of the human genome revealed putative G-protein coupled receptors with no known ligands. Searching for factors in brain extracts that could bind to or activate these orphan receptors led to the discovery of at least 2 endocrinological important neuropeptides. Orphanin F/Q (also known as Nociceptin) is a 17 amino acid peptide, structurally related to Dynorphin A,but does not activate the classical opioid receptors, or do any of the endorphin or enkephalin peptides or their antagonists interact with the orphanin F/Q receptor termed the: NOP (Nociceptin Opioid Peptide) Receptor. Yet the Orphanin peptide is itself an anti-opioid peptide in terms of preventing opioid induced analgesia at supra-spinal levels. This peptide and synthetic antagonist analogs seem mainly to interact with actions of glutamate within reward circuits. Hypocretin, by amino acid sequence homology is a member of the secretin-VIP peptide family. It was discovered by searching for genes that were uniquely expressed in rat hypothalamus. Arising from neurons in the perifornical nucleus of the dorsal hippocampus, it projects heavily to midbrain structures regulating, sleep, blood pressure and appetite. Hypocretin receptors are mutated in narcolepsy, and hypocretin antagonists are under developments as sleep aids. The same peptide was discovered at the same time by another group panning for ligands for orphan receptors and named orexin for its ability to stimulate appetite in food satiated animals.

Other Regulatory Signals

In addition to the previously discussed major families of neurotransmitters, other endogenous substances have also gained attention as interneuronal signaling molecules that, if valid, will expand the definition of this process. For example, the purines adenosine monophosphate, adenosine triphosphate, and free adenosine have helped define a purinergic signaling system with two large families of purinergic receptors. ATP-regulated responses have been linked pharmacologically to a variety of supracellular functions, including anxiety, stroke, and epilepsy.

Although all the classical neurotransmitters described previously were detected on the basis of the ability to extract them as factors from their storage sites in brain, gut, or other organ systems, studies have revealed potent classes of signaling molecules that are apparently synthesized on demand and act at significant distances from their sites of synthesis and release through diffusion. Arachidonic acid, normally stored within the cell membrane as a glycerol ester, can be liberated when phospholipases are activated by a variety of neurotransmitter receptors and then converted to highly reactive signals by one of three enzymatic pathways:

cycloxygenases (leading to prostaglandins and thromboxanes), lipoxygenases (leading to the leukotrienes and other transient catabolites of eicosatetraenoic acid), and cytochrome P450 (which is inducible but expressed at low levels in brain). These arachidonic acid metabolites have been implicated as diffusible modulators in the CNS, particularly for long-term potentiation and other forms of plasticity, and include the anadamides, which are endogenous lipid signals identified on the basis of their interactions with the cannabinoid receptors.

Last, nitric oxide was initially recognized as an important regulator of vascular and inflammatory mechanisms and was later recognized to play analogous signaling roles in the brain after determination of the various forms of the enzyme nitric oxide synthase (NOS), through which NO is made and released. At least four isoforms of this biosynthetic enzyme have been identified in the brain: a constitutive form present in some neurons, capillary endothelial cells, and macrophages, as well as inducible forms of the enzyme. The availability of potent activators of NOS has led to reports of the presumptive involvement of NO in a host of phenomena in the brain, including long-term potentiation, guanylyl cyclase activation, neurotransmitter release and reuptake, and enhancement of glutamate (NMDA)-mediated neurotoxicity.

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Anatomy of Hypothalamus

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Glossary

Anterior lobe of the pituitary gland (anterior pituitary) The major part of this organ; synthesizes hormones that control body growth, thyroid, adrenocortical, and gonadal functions, among others.

Brainstem The medulla oblongata, pons, and mesencephalon parts of the brain.

Hypophysiotropic neurohormones Well-characterized substances synthesized by neurons projecting to the median eminence and carried by the portal vascular system to the anterior pituitary.

Hypothalamic nuclei Areas of neurons of higher density than those in the neighborhood.

Neurohormones Bioactive substances synthesized by neurons and released into the blood.

Neurons The nerve cells.

Pathways (fiber tracts) Large number of nerve fibers running together, at least for some distance, and arising from and terminating in the same region, but with their origins and final destinations not necessarily always being identical.

Pituitary gland (hypophysis) An endocrine organ connected to the hypothalamus and being composed of an anterior lobe, an intermediate lobe, and a posterior lobe.

Posterior lobe (neural lobe or posterior pituitary) of the pituitary gland A small part of this organ that stores and releases hormones synthesized by neurons in the paraventricular and supraoptic nuclei.

The hypothalamus, a fairly small part of the brain being connected with the pituitary gland, is an extremely complex, highly integrated structure of the diencephalon that plays a key role in the control of homeostasis and reproduction.

Introduction

The hypothalamus, part of the diencephalon, is a rather small region of the brain. The human hypothalamus weighs about 4 g and, therefore, represents only 0.3% of the whole brain. In spite of its small size, the hypothalamus plays a fundamental role in the control of homeostasis as well as in reproduction. The structural organization of this diencephalic region is extremely complex, manifold interrelated, and integrated. The hypothalamus is closely and reciprocally linked to other parts of the brain (e.g., the autonomic nervous system), is connected to the pituitary gland, and produces neurohormones.

This article describes the basic structural organization of the hypothalamus, summarizing hypothalamic regions, cell groups within regions, and connections to the hypothalamus. Subsequently, it deals with the hypothalamo–anterior pituitary and hypothalamo– posterior pituitary systems. Finally, it describes the blood supply of the hypothalamus.

Basic Organization

Topography of the Hypothalamus: Hypothalamic Regions

From a morphological point of view, the hypothalamus can be subdivided into three longitudinal parts in the sagittal plane: periventricular (around the third ventricle), medial, and lateral. Both the periventricular hypothalamus and medial hypothalamus are cell rich, whereas the lateral hypothalamus is dominated by the longitudinal fiber system of the medial forebrain bundle. In the rostrocaudaldirection, the hypothalamus can be subdivided into five regions (**Fig. 1**): preoptic region, anterior hypothalamus, middle hypothalamus, premammillary region (also called the posterior hypothalamus), and mammillary region (topographically a part of the hypothalamus but functionally belonging to the limbic system that is not dealt with here). In the human hypothalamus, only three major parts can be distinguished: supraoptic region (corresponding to the preoptic and anterior hypothalamic regions of the rat), tuberal region (middle hypothalamic region), and mammillary region.

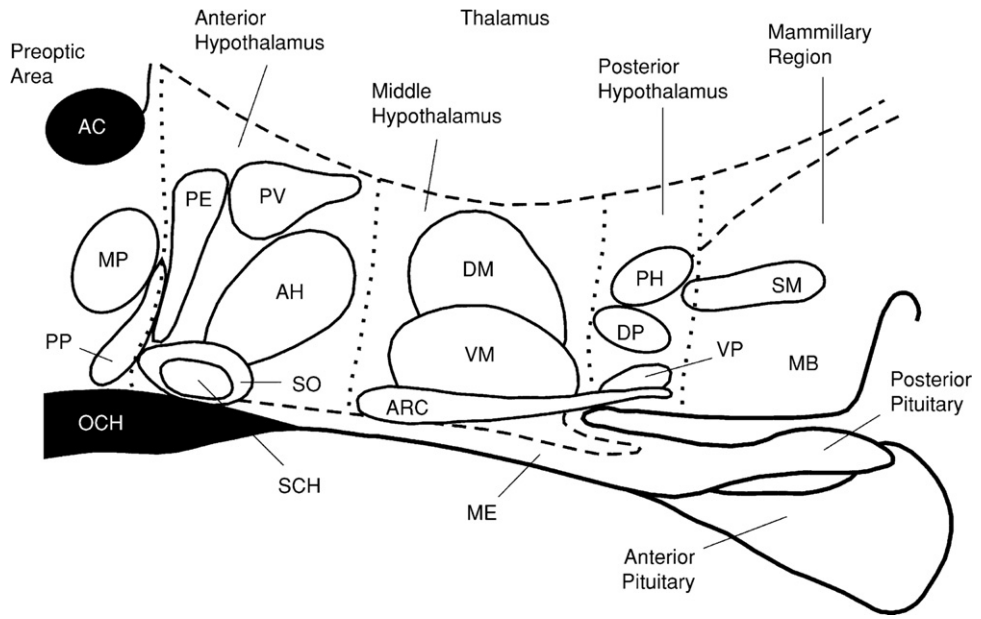

Fig. 1 Hypothalamic regions and cell groups within regions projected into the midsagittal section of the rat hypothalamus. AC, anterior commissure; AH, anterior hypothalamic nucleus; ARC, arcuate nucleus; DM, dorsomedial nucleus; DP, dorsal premammillary nucleus; MB, mammillary body; ME, median eminence; MP, medial preoptic nucleus; OCH, optic chiasm; PE, periventricular nucleus; PP, preoptic periventricular nucleus; PV, paraventricular nucleus; SCH, suprachiasmatic nucleus; SM, supramammillary nucleus; SO, supraoptic nucleus; PH, posterior hypothalamic nucleus; VM, ventromedial nucleus; VP, ventral premammillary nucleus.

Cell groups and areas

Preoptic region

The preoptic region extends from the basal forebrain and lamina terminalis to the level of the rostral half of the optic chiasm and continues into the anterior hypothalamus. Dorsally, it is bordered by the anterior commissure and the bed nucleus of the stria terminalis.

The cell-rich medial part of the preoptic region contains several cell groups, including the medial preoptic and preoptic periventricular nuclei, and contains gonadotropin-releasing hormone (GnRH) neurons. The lateral part is occupied by the medial forebrain bundle. In the midline, a circumventricular organ, the organum vasculosum laminae terminalis (also called the supraoptic crest) can be found around the tip of the third ventricle. The organ is outside of the blood–brain barrier.

Anterior hypothalamus

The anterior hypothalamus is a continuation of the preoptic region and extends caudally to the level of the arising of the median eminence. Its medial part comprises the anterior periventricular, suprachiasmatic, anterior hypothalamic, and paraventricular nuclei. The anterior periventricular nucleus contains somatostatin immunoreactive neurons projecting to the median eminence and dopaminergic neurons terminating in the posterior and intermediate lobe of the pituitary gland (periventriculo–hypophyseal dopaminergic system). The suprachiasmatic nucleus (a key structure in the control of biological rhythms) is situated just above the optic chiasm on the two sides of the third ventricle. It receives direct input from the retina and indirect neuronal input from the retina via the lateral geniculate body and from brainstem serotonergic neurons. The supraoptic nucleus is located along and around the lateral edge of the optic chiasm and is composed of magnocellular neurons. In humans, the supraoptic nucleus is situated above the rostral end of the optic tract. Neurons of this cell group synthesize oxytocin and vasopressin and project to the posterior pituitary. The paraventricular nucleus lies close to the two sides of the third ventricle, above the anterior hypothalamic nucleus. Magnocellular and parvocellular divisions can be distinguished. Magnocellular neurons synthesize oxytocin and vasopressin and project to the posterior pituitary. The cell group contains corticotropin-releasing hormone (CRH)- and thyrotropin-releasing hormone (TRH)producing neurons that terminate in the median eminence as well as medium-sized neurons that give rise to long descending projections to the brainstem and spinal cord. The lateral part of this hypothalamic region contains the supraoptic nucleus and fibers and cells of the medial forebrain bundle. Caudally, the so-called retrochiasmatic area containing loosely packed cells and fibers occupies the ventral part of the anterior hypothalamus. In both the medial and lateral parts of the anterior hypothalamus, magnocellular neurons form small groups (called accessory nuclei).

Middle hypothalamus (tuberal region)

The middle hypothalamus, starting behind the retrochiasmatic area, ends at the level of the separation of the pituitary stalk. The medial basal part of the region contains the arcuate and ventromedial nuclei and the median eminence, whereas the medial dorsal part contains the dorsomedial nucleus. The lateral part is called the lateral hypothalamic area and is largely occupied by the medial forebrain bundle.

The arcuate (also called the infundibular) nucleus, made up of five subdivisions, is an elongated cell group in the most ventromedial part of the middle hypothalamus and posterior hypothalamus, mostly on the two sides of the third ventricle (infundibular recess). The cell group contains, in addition to several other neuropeptides, pro-opiomelanocortinsynthesizing neurons and growth hormone-releasing hormone (GHRH) as well as dopaminergic neurons. These latter two project to the median eminence, forming the tubero–infundibular pathway. The ventromedial nucleus is a major cell group composed of five subdivisions that contain morphologically and functionally distinct neurons with neuronal connections to various components of the limbic system. The dorsomedial nucleus divided into three subdivisions is caudal to the paraventricular nucleus.

Posterior hypothalamus (premammillary region)

The main cell groups of the posterior hypothalamus, a relatively small part of the hypothalamus, are the ventral and dorsal premammillary, tuberomammillary, supramammillary, and posterior hypothalamic nuclei. The ventral premammillary nucleus is a small cell group in continuation with the ventromedial and partly the arcuate nuclei. The dorsal premammillary nucleus is located caudal to the ventromedial nucleus at the sides of the inframammillary recess of the third ventricle. The tuberomammillary nucleus is located in the most ventral and medial part of the posterior hypothalamus. Some neurons of the cell group synthesize histamine, providing the only source of neuronal histamine in the brain. The posterior hypothalamic nucleus is a fairly large group of cells occupying the dorsal part of the periaqueductal central gray. The unpaired supramammillary nucleus is dorsal to the mammillary body and projects to the hippocampus, dentate gyrus, and medial septum diagonal band nuclei.

Characteristics of hypothalamic cell groups

The hypothalamic cell groups are not at all homogenous, and in most cases subgroups can be distinguished. For example, in the arcuate nucleus, there are dopamine, GHRH, enkephalin, galanin, substance P, g-aminobutyric acid (GABA), atrial natriuretic peptide, neurotensin, gastrin-releasing peptide (GRP), and glutamate-containing neurons. The suprachiasmatic nucleus, representing a key structure of the biological clock, is composed of vasopressin, vasoactive intestinal peptide, somatotropin release-inhibiting hormone (SRIH), substance P, GABA, and GRP containing nerve cells. The situation is further complicated by the colocalization of various neuropeptides in the same neurons. The functional significance of colocalization of substances in the nerve cells needs to be clarified.

The paraventricular nucleus is unique among hypothalamic cell groups in housing substantial populations of cells that participate in the control of anterior and posterior pituitary secretions and also is associated with the autonomic nervous system. This nucleus is the predominant source of CRH and TRH. Neurons of the cell group synthesize oxytocin and vasopressin for release into the general circulation from terminals in the posterior pituitary. A third major cell type contains neurons that give rise to long descending projections to the brainstem and spinal cord that include sensory and motor structures associated with the autonomic nervous system. These three visceromotor populations are essentially separate and exhibit a high degree of topographic organization. On this high degree of anatomical organization is imposed a somewhat imprecise manner of chemical coding.

The neurons of a cell group display rich intrinsic connections. A local network of fibers seems to be a common feature of hypothalamic nuclei. Local circuit neurons may synchronize the activities of peptidergic neurons in a hypothalamic nucleus to integrate or coordinate them as a functional unit.

Hypothalamic Connections

Intrahypothalamic connections

A large number of intrahypothalamic connections exist between hypothalamic cell groups, and in most cases these connections are reciprocal. For example, arcuate neurons, in addition to projecting to the outer layer of the median eminence, project to several other regions such as the ventromedial nucleus, lateral hypothalamic area, anterior hypothalamic area, and preoptic area and also receive afferents from the preoptic area, the ventromedial nucleus, and several other hypothalamic regions.

The abundant intranuclear connections, as well as the rich connections among the various hypothalamic cell groups, support the general impression that the hypothalamus should be considered a neuronal network of quasirandom internal connections. In this network, where the impulses leave the hypothalamus through the main axons, excitation can spread from a given focus in any direction and can establish an infinite number of closed, self-reexciting chains.

Extrahypothalamic connections

Connections with telencephalic and diencephalic structures

A large number of regions project to the hypothalamus, including various parts of the limbic system (e.g., amygdaloid complex, hippocampus, septum), thalamus, basal ganglia, and cortex. Although the hypothalamus receives a large amount of sensory input through the regions mentioned, there are also some more or less direct pathways. There is a direct projection from the retina to the

hypothalamus, primarily to the suprachiasmatic nucleus. The effect of light on the hypothalamus, particularly on its control of the anterior pituitary, may be mediated by this pathway. Inputs from the olfactory bulb have relatively free access to the hypothalamus, although direct connections from the olfactory bulb to the hypothalamus are not known. The piriform cortex, which receives fibers from the olfactory bulbs, sends projections directly to the hypothalamus. Other olfactory pathways reach the hypothalamus mainly via the amygdala.

The efferent pathways of the hypothalamus appear to reciprocate several of the major afferent hypothalamic connections. Many such reciprocating connections are contained in the medial forebrain bundle, the dorsal longitudinal fasciculus, and the stria terminalis. These pathways appear to close neuronal circuits between the hypothalamus and several of the limbic forebrain structures. The major brain structures receiving hypothalamic efferents are the amygdala, hippocampus, and septum.

Hypothalamic connections with the brainstem

The hypothalamus has extremely rich connections with the brainstem. A significant part of the ascending fibers are aminergic, and they terminate in various hypothalamic cell groups. Noradrenalin-containing fibers arise from the medulla oblongata (from the so-called A1 and A2 catecholaminergic cell groups) and pons (from the locus coeruleus). Adrenalinsynthesizing neurons are in the medulla oblongata (C1 and C2 adrenergic cell groups). Ascending serotonergic fibers arise from the pons and midbrain raphe nuclei.

Descending fibers from the hypothalamus, primarily from the paraventricular, arcuate, and medial preoptic nuclei, terminate in the brainstem and spinal cord.

Recent findings indicate that there are polysynaptic neuronal connections between hypothalamic structures and endocrine glands such as the gonads, adrenal, and pancreas. The neural connections of the hypothalamus are summarized in **Fig. 2**.

The reciprocal connections of the hypothalamus with limbic forebrain structures and the brainstem are of such magnitude that it appears possible to interpret the hypothalamus, at least partly, as a way station in both the ascending and descending limbs of a polysynaptic neural circuit that extends between the limbic forebrain, on the one hand, and the primarily paramedian mesencephalic region, on the other. It may be assumed that the functional state of the hypothalamus is determined, to a significant

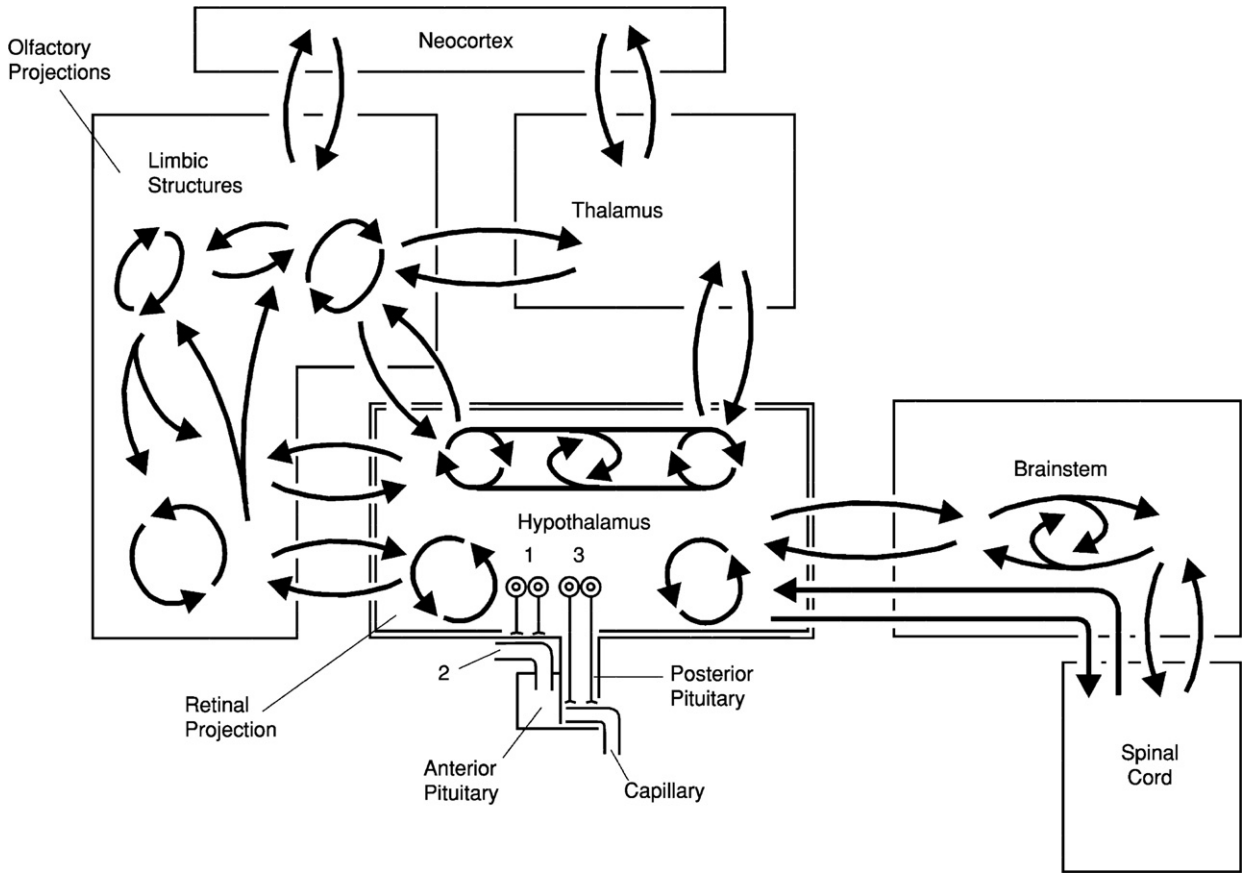

Fig. 2 A simplified drawing of the bidirectional neural connections within the hypothalamus (intranuclear and internuclear) and between hypothalamic and extrahypothalamic structures. The relations of the hypothalamus to the anterior and posterior pituitary are also indicated. (1) Neurons synthesizing hypophysiotropic neurohormones. (2) Portal capillary. (3) Neurons of the supraoptic and paraventricular nuclei terminating in the posterior pituitary.

extent, by the neural events that take place in the limbic structures and the lower brainstem, with both having a very integrated structural organization, including several reciprocal interconnections and neural circuits. In addition, they receive a vast amount of information from both the external and internal environments flowing in along neural and humoral pathways (there are hormone receptors in the hippocampus and amygdala). It should be mentioned that the hypothalamus itself also contains hormone receptors as well as several other types of receptors.

The extremely complex neuronal network of the hypothalamus and its reciprocal connections suggests that, apart from a few exceptions such as the supraoptico- and paraventriculohypophyseal system, there are not well-defined regions and pathways that are specifically and exclusively concerned with a discrete hypothalamic function. Of course, this does not exclude the predominance of one or the other hypothalamic area in the involvement of a particular hypothalamic function. Instead of a mosaic-type pattern, the hypothalamus can rather be envisaged as some kind of computer. This computer has a number of built-in programs, and its elements are involved in several processes. It elaborates the solution for each actual situation on the basis of a wealth of information that is partly stored and partly streaming in continuously. The results are then distributed over a number of neural and humoral output channels.

Major neuroanatomical pathways connecting the hypothalamus with extrahypothalamic structures

Medial forebrain bundle

The medial forebrain bundle running longitudinally andoccupyingthemajorityofthelateralhypothalamus contains a high number of fibers with different destinations from hypothalamic nuclei and from limbic, cortical, and brainstem structures directed to hypothalamic cell groups. It also contains many fibers that just pass through the hypothalamus.

Stria terminalis

The stria terminalis is a major pathway between the amygdala and the hypothalamus that provides reciprocal connections between the two structures.

Fornix

The fornix is a main link of the limbic system connecting the hippocampus, the septum, and the mammillary body. Some of its fibers deviate from the main bundle and terminate in the preoptic area as well as around the ventromedial nucleus of the hypothalamus.

Medial corticohypothalamic tract

The medial corticohypothalamic tract connects the hippocampus with the arcuate, ventromedial, and ventral premammillary nuclei.

Dorsal longitudinal fasciculus

The dorsal longitudinal fasciculus contains ascending and descending fibers connecting the dorsal hypothalamus and posterior hypothalamus with the periaqueductal central gray of the mesencephalon.

Hypothalamus–Anterior– Pituitary System

Neurovascular Contact Between the Hypothalamus and Anterior Pituitary

Theconnectionsbetweenthehypothalamusandanterior pituitary are neurovascular (Fig. 3). Hypophysiotropic substances, called trop hormone-releasing hormones (factors) or release-inhibiting hormones (factors), are produced by the hypothalamus. The

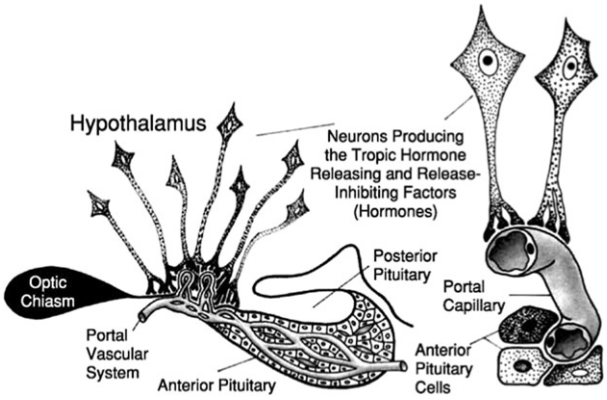

Fig. 3 Schematic illustration of the neurovascular contact between the hypothalamus and the anterior pituitary.

chemically identified hypophysiotropic substances are CRH or corticotropin-releasing factor (CRF); GnRH, also called luteinizing hormone-releasing hormone (LHRH) or gonadoliberin; GHRH; SRIH, somatostatin, or growth hormone-inhibiting factor (GIF); TRH; and prolactin-inhibiting factor (PIF), at least one of which is dopamine (DA). These substances are transported by the hypophyseal portal vascular system to the anterior pituitary cells. The median eminence and the proximal part of the pituitary stalk are the site where the axons of the neurons synthesizing the hypophysiotropic neurohormones are very close to the portal vessels.

Location of Neurons Synthesizing the Hypophysiotropic Neurohormones and Projecting to the Median Eminence

Corticotropin-releasing hormone

The most prominent CRH-containing cell group of the hypothalamus is the paraventricular nucleus, mainly its medial parvocellular part. The vast majority of the CRH terminals in the median eminence and pituitary stalk arise from here.

Gonadotropin-releasing hormone

In general, there are two areas that contain a significant amount of immunoreactive GnRH nerve cell bodies projecting to the median eminence: (1) The septal– preoptic–suprachiasmatic region and (2) the mediobasal area of the middle hypothalamus and posterior hypothalamus, especially the arcuate and premammillary nuclei. But therearegreatvariations inthe number of such cells in these two regions of various species. In humans and primates, they are concentrated mainly in the second region. In the rat, many GnRH cells are in the medial preoptic area, the diagonal band of Broca, and the septal nuclei, whereas some cells are in the anterior hypothalamic area.

GnRH neurons originate in the medial olfactory placodal epithelium of the developing nose, migrate across the nasal septum, and enter the forebrain with thenervusterminalis,acranialnervethatisapartofthe accessory olfactory system and projects directly from the nose to the septal–preoptic area and hypothalamus. This migratory route for GnRH-expressing neurons could explain the deficiency of gonadotropins seen in hypogonadotropic hypogonadism with anosmia.

Growth hormone-releasing hormone

The majority of the GHRH immunoreactive neurons projecting to the median eminence are in the arcuate nucleus.

Somatotropin release-inhibiting hormone

SRIH immunoreactive neurons terminating in the median eminence are located mainly in the medial preoptic and anterior periventricular areas.

Thyrotropin-releasing hormone

Although TRH immunoreactive neurons are widely distributed in the central and peripheral nervous systems, those projecting to the median eminence are gathered mostly in the medial parvocellular division of the paraventricular nucleus.

Prolactin-inhibiting factor

At least one PIF is dopamine and is produced by the tuberoinfundibular dopaminergic neurons. These neurons are situated in the arcuate nucleus and in the ventral part of the anterior periventricular nucleus.

The location of neurons synthesizing the hypophysiotropic neurohormones and projecting to the median eminence is summarized in **Fig. 4**.

The hypophysiotropic neurons receive very significant neural input mediated by several chemical messengers. Various peptidergic, monoaminergic, and GABAergic axons terminate on perikarya and dendrites of these nerve cells, providing the structural basis for the assumption that the actions of the neurotransmitters or neuromodulators are at least partly exerted directly on the structures producing the hypophysiotropic neurohormones. In addition, axons containing one or the other hypophysiotropic neurohormone form synaptic connections with neurons synthesizing the same peptide. This may be the morphological basis for an ultrashort feedback mechanism or may indicate an intrinsic circuit.

However, it should be kept in mind that neurons containing the troph hormone-releasing or releaseinhibiting neurohormones are widely distributed in the central nervous system; some of them are even present in other tissues. Not all of these neurons in the brain terminate in the hypothalamic median eminence and pituitary stalk; instead, some project to other brain structures.

Hypophysial Portal Vascular System

Besides the trop hormone-releasing and releaseinhibiting hormones, the portal vascular system represents the key structure required for the operation of the neurohumoral (neurovascular) mechanism controlling pituitary tropic functions. It transports the substances released from the nerve terminals in the median eminence to the pituitary.

The main features of the hypophyseal portal vascular system are the following (**Fig. 5**). The so-called superior hypophyseal arteries form a dense plexus, largely of precapillary character, within the so-called pars tuberalis, a small part of the pituitary gland. This plexus is especially dense on the contact surface between the median eminence and the pars tuberalis (mantleplexus). From this plexus arise the capillary loops that penetrate into the tissue of the median eminence and infundibular stem. The mantleplexus

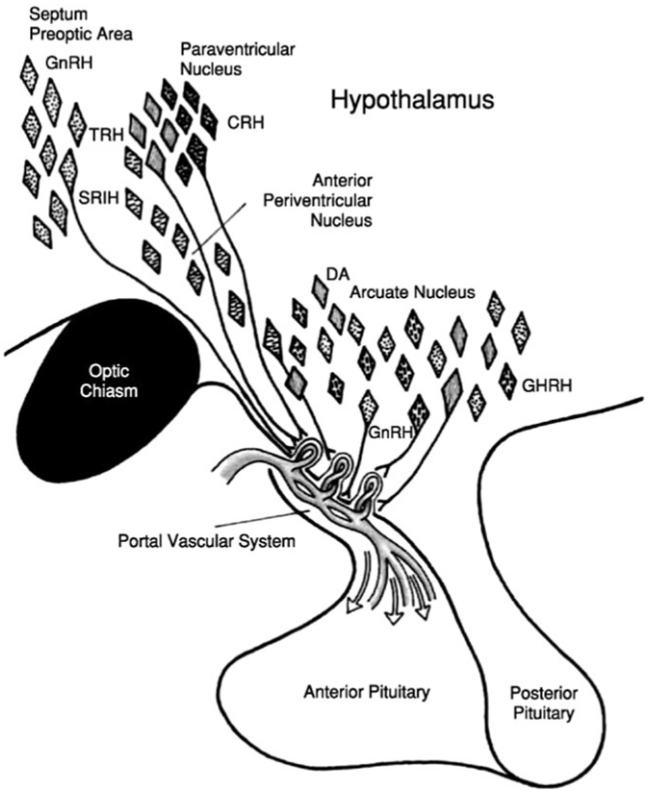

Fig. 4 Schematic drawing of midsagittal section of the hypothalamo–pituitary unit showing the location of neurons producing hypophysiotropic neurohormones and projecting to the outer layer of the median eminence. CRH, corticotropin-releasing hormone; DA, dopamine; GHRH, growth hormone-releasing hormone; GnRH, gonadotropin-releasing hormone; SRIH, somatostatin release-inhibiting hormone; TRH, thyrotropin-releasing hormone.

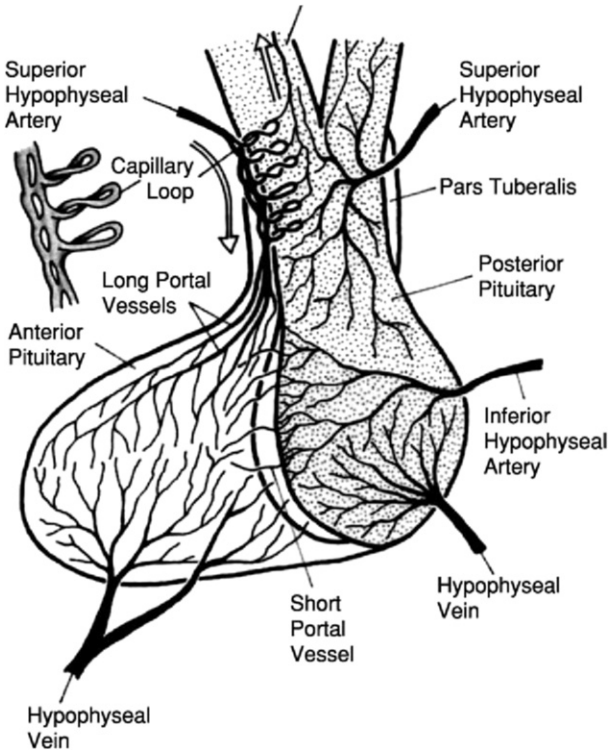

Fig. 5 Schematic illustration of the hypophyseal portal vascular system.

and capillary loops drain toward the portal vessels (some of the capillary loops drain toward the subependymal plexus of the third ventricle) that lie on the ventral surface of the pituitary stalk. These vessels are called the long portal vessels. Part of the blood from the posterior pituitary reaches the anterior pituitary by way of vessels known as short portal vessels. Each portal vessel supplies a certain part of the pituitary. Anastomoses between these vessels are rare. The majority of the portal blood is directed from the median eminence toward the pituitary, but some blood may flow in the reverse direction, toward the hypothalamus.

The presence of trop hormone-releasing and release-inhibiting substances in the portal blood is well documented, as is the fact that the concentration of these substances is much higher in the portal blood than in the peripheral plasma and that changes occur under certain experimental conditions.

Structure of the Median Eminence

The median eminence arising from the ventral surface of the tuberal region of the hypothalamus is a slight midline prominence. It continues into the pituitary stalk and represents the contact area between the nerve terminals of the neurons synthesizing the hypophysiotropic neurohormones and the precapillaries and capillaries of the portal vascular system. The inner surface of the median eminence is covered by ependymal cells. Two layers can be distinguished in the median eminence. The inner layer contains the fibers of the supraoptico – and paraventriculo–hypophysial system terminating in the posterior pituitary. The outer layer contains the trop hormone-releasing and release-inhibiting hormones and the vessels of the portal vascular system. It must be mentioned that in addition to the terminals of the neurons synthesizing the hypophysiotropic neurohormones, there are many other neurons containing chemical messengers different from these compounds (most of them are also peptides, but "classic" neurotransmitters are also present), which also terminate in the median eminence. The functional significance of these other chemical messengers is not known. The possibility of interactions of the various substances at the median eminence level exists.

Hypothalamus–Posterior Pituitary System

Magnocelluler neurons of the supraoptic and paraventricular nuclei and parvocellular neurons of the paraventricular nucleus project to the posterior pituitary, forming the so-called supraoptico- and paraventriculohypophyseal tract (Fig. 6). The neurons synthesize oxytocin and vasopressin. The hormones are synthesized in the cell bodies of the nerve cells and are transported down the axons of these neurons to their endings in the posterior pituitary. Some of the neurons make oxytocin and others synthesize vasopressin. Oxytocin- and vasopressin-containing cells are evident in both cell groups. Vasopressin- and oxytocin-producing neurons of the paraventricular nucleus project not only to the posterior pituitary but also to the brainstem and spinal cord and may be involved in cardiovascular control.

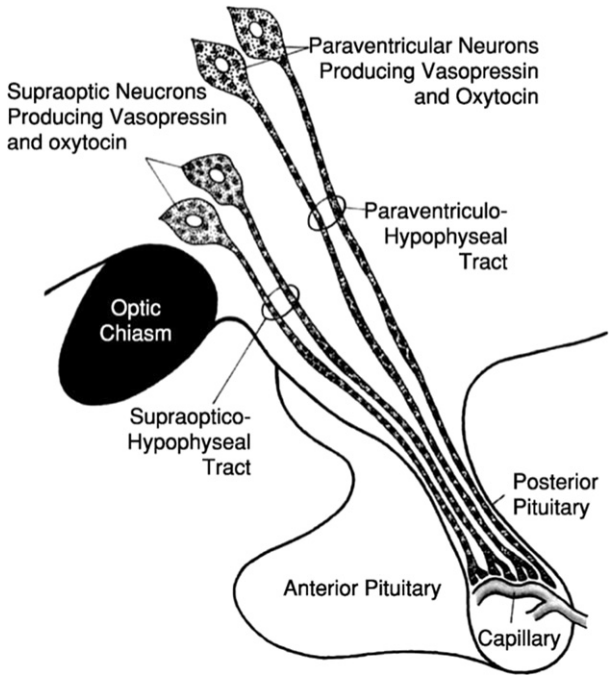

Fig. 6 The hypothalamus–posterior–pituitary system.

Neurosecretion

The term "neurosecretion" was originally coined to describe the secretion of hormones by neurons, but the term is now somewhat misleading because it became evident that nearly all neurons secrete chemical messengers.

Blood Supply

The hypothalamic arteries arise directly from the circle of Willis (circulus arteriosus Willisi). Six major groups of arteries supply the hypothalamus. The arteries cover each other like shells in a mediolateral direction. The vessels entering at the midline supply the medial and basal parts of the hypothalamus, whereas those entering laterally supply the lateral and dorsal parts of the hypothalamus. None of the hypothalamic nuclei is supplied by a single artery.

Most of the hypothalamic veins enter into the anterior cerebral, basilar, and interpeduncular veins. The anterior cerebral vein drains into the basilar vein. The venous blood is drained by the basal vein, which enters the great cerebral vein of Gallen.

See Also the Following Articles

Hypothalamic Disease • Hypothalamic Hypogonadism • Hypothalamic Hypothyroidism • Hypothalamic Regulation of Appetite and Obesity • Hypothalamic-Pituitary Unit • Hypothalamus–Pituitary–Thyroid Axis • Pituitary Gland Anatomy and Embryology • Pituitary Tumors, Molecular Pathogenesis

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Pituitary Gland Anatomy and Embryology

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Glossary

Adenohypophysis The glandular part of the hypophysis (pituitary) containing the hormone-producing cells and composed of the anterior lobe (pars distalis), the intermediate lobe, and the pars tuberalis.

Anterior lobe (pars distalis) of the pituitary A subdivision of the adenohypophysis; it constitutes approximately 80% of the gland and contains somatotroph, mammotroph, gonadotroph, corticotroph, thyrotroph, and folliculostellate cells.

Neurohypophysis The neural part of the pituitary composed of the posterior lobe (infundibular process), the neural stalk, and the median eminence.

Posterior lobe of the pituitary A subdivision of the neurohypophysis that develops as an evagination of the third ventricle of the brain: It stores and releases oxytocin and vasopressin.

Rathke's pouch The evagination of the roof of the primitive oral cavity (stomodeum), giving rise to the adenohypophysis.

The pituitary or hypophysis is an unpaired endocrine gland located in a midline depression (sella turcica) of the base of the skull under the hypothalamus and linked to it.

Introduction

The pituitary is an oval, symmetrical, unpaired organ. Its weight in adults ranges between 400 and 900 mg. On average, it measures 13 mm transversally, 6 mm vertically, and 9 mm anteroposteriorly. The gland is somewhat larger in women than in men and enlarges during pregnancy. It is usually heavier in multiparous women than in nulliparous women and decreases in size during advanced aging.

Anatomy

Divisions and Subdivisions

The pituitary has two main divisions: the adenohypophysis and the neurohypophysis (Fig. 1). Both the adenohypophysis and the neurohypophysis are composed of three subdivisions. The subdivisions of the adenohypophysis are the pars distalis or anterior lobe, the pars intermedia or intermediate lobe, and the pars tuberalis, which encircles the neural stalk. The anterior lobe constitutes approximately 80% of the pituitary. The subdivisions of the neurohypophysis are the posterior lobe, also called the neural lobe or infundibular process, the neural stalk, which connects the hypothalamus with the posterior lobe, and the median eminence, a midline eminence on the ventral surface of the hypothalamus, which is the portion of the neural stalk that ascends from the hypothalamus. The neural stalk and the pars tuberalis of the adenohypophysis form the pituitary stalk. The anterior lobe and posterior lobe are clearly separated and can easily be distinguished with the naked eye. The two main divisions of the pituitary have different origins.

Topography

The hypophysis is located under the brain in the sella turcica at the base of the skull (Fig. 2(A)). The sella turcica is the saddlelike part of the sphenoid bone. It is composed of three parts: an olive-shaped swelling, called the tuberculum sellae, which is located anteriorly; a seatlike depression, called the hypophysial fossa, for the pituitary gland; and the posterior part of the saddle, known as the dorsum sellae. The gland is surrounded laterally and inferiorly by the sphenoid bone and is covered from above by the diaphragma sellae, which is a dural sheath forming the roof of the sella (Fig. 2(B)). There is a central opening on the sellar diaphragm through which the hypophysial stalk passes through in connecting the hypothalamus to the pituitary. The optic chiasma lies directly above the sellar diaphragm ahead of the hypophysial stalk. Growth of a pituitary tumor may compress the chiasma and impair vision. The tuber cinereum of the hypothalamus also lies above the roof of the sella. Space-occupying lesions in the pituitary may compress and compromise the tuber cinereum and cause hypothalamo-hypophysial dysfunctions. The lateral walls of the sella are close to the cavernous sinuses containing the internal carotid arteries and several nerves including the oculomotor, trochlear, and abducens nerves and the first two branches of the trigeminal nerve. The sphenoid sinus, separated from the sella by a thin layer of bone, is inferior to the pituitary. In the case of a pituitary tumor, this bone may be resorbed and eroded, leading to tumor penetration into the sinus.

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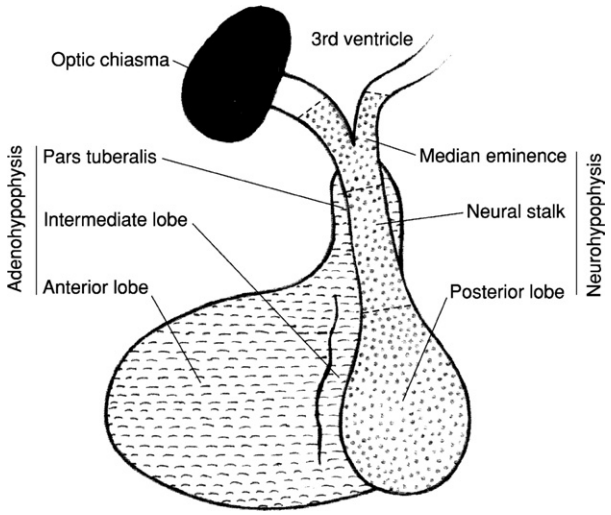

Fig. 1 Schematic of the divisions and subdivisions of the pituitary as seen in a midsagittal section of the gland.

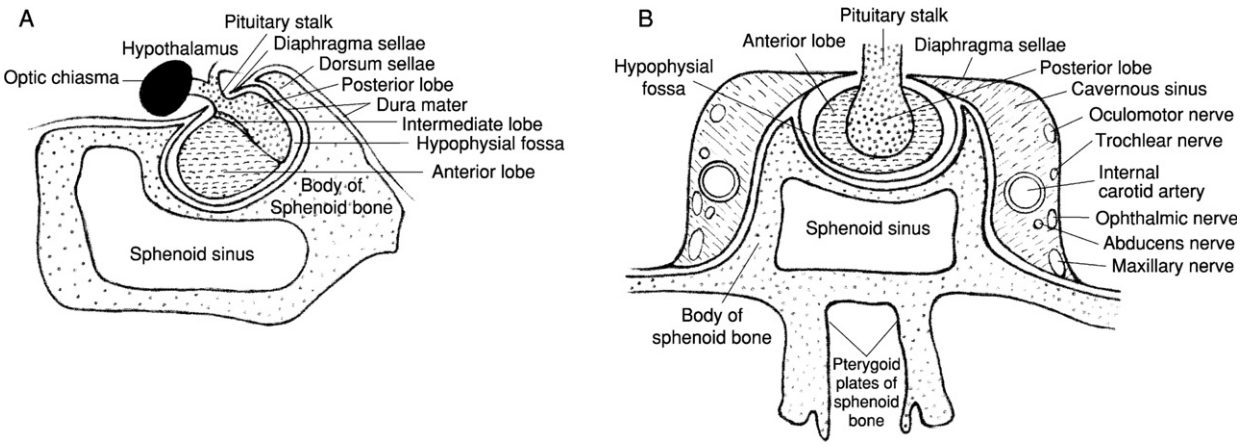

Fig. 2 Schematic of the location and topography of the pituitary in a midsagittal section (A) and a coronal section (B) of the sphenoid bone.

Blood Supply

The blood supply of the pituitary derives from two groups of arteries: from above, the right and left superior hypophysial arteries; from below, the right and left inferior hypophysial arteries. Both groups of arteries arise from the internal carotid arteries. The superior hypophysial arteries supply blood to the median eminence and proximal portion of the pituitary stalk. Here these arteries break up into a primary capillary plexus. The capillaries of this plexus rejoin to form the long portal vessels that traverse the pituitary stalk and break up into a secondary capillary plexus in the anterior lobe of the pituitary in close relationship to the cells of anterior pituitary. This hypophysial portal vascular system is of the utmost importance in regulating hormone secretion of the anterior pituitary. The posterior pituitary receives its blood supply from the inferior hypophysial arteries. Some vessels from the posterior lobe penetrate into the anterior pituitary (short portal vessels).

Venous blood leaves the pituitary through dural channels and enters the cavernous sinuses, which drain to the inferior petrosal sinus and the internal jugular vein. There may be anastomoses between the petrosal sinuses, which can lead to confusing observations with petrosal sinus sampling.

Although most blood flow is from the hypothalamus to the pituitary, there is evidence that some blood may flow in the opposite direction, from the anterior pituitary to the hypothalamus.

Innervation

The anterior lobe is poorly innervated. A few nerve fibers reach the anterior pituitary along the blood vessels. These nerve fibers are not believed to have major importance in the control of hormone secretion. The posterior pituitary contains the axon terminals of

the supraoptic and paraventricular neurons, synthesizing and releasing oxytocin and vasopressin. In addition, the intermediate pituitary and the posterior pituitary are innervated by hypothalamic dopaminergic neurons of the periventriculo-hypophysial and the tuberohypophysial dopaminergic system.

Embryology

The adenohypophysis originates from an evagination of the epithelium covering the vault of the stomodeum (primary oral cavity). The neurohypophysis develops as a process growing downward from the floor of the diencephalon, i.e., as an evagination of the third ventricle of the brain (Fig. 3). During embryonic development, the adenohypophysial primordium becomes located anterior to the neural primordium.

Development of the Adenohypophysis

The epithelium of the stomodeum (primitive oral cavity) becomes thicker just ahead of the pharyngeal membrane (Fig. 3(A)). This flat primordium invaginates and penetrates the connective tissue in the direction of the diencephalon, forming a diverticulum, Rathke's pouch (Fig. 3(B)). Initially, Rathke's pouch is composed of a small, thin-walled vesicle in the roof of the primitive oral cavity. It subsequently expands in the direction of the evagination of the third ventricle and will be situated just ahead of the evagination (Fig. 3(C)) and then it adheres to it. Rathke's pouch is attached to the stomodeal vault by a stalk (craniopharyngeal canal), which regresses, is obliterated, and usually disappears (Fig. 3(D)). Parts of it, however, may persist in a more or less differentiated and sometimes functional condition. Nests of adenohypophysial tissue may be deposited along the route of the craniopharyngeal canal. Remnants of the pharyngeal hypophysis may be capable of hormone synthesis and may give rise to ectopic adenomas. Occasionally, craniopharyngiomas (tumors) develop in the pharynx or in the sphenoid bone of the skull, but most often they form in or above the sella turcica of the sphenoid bone at the base of the skull.

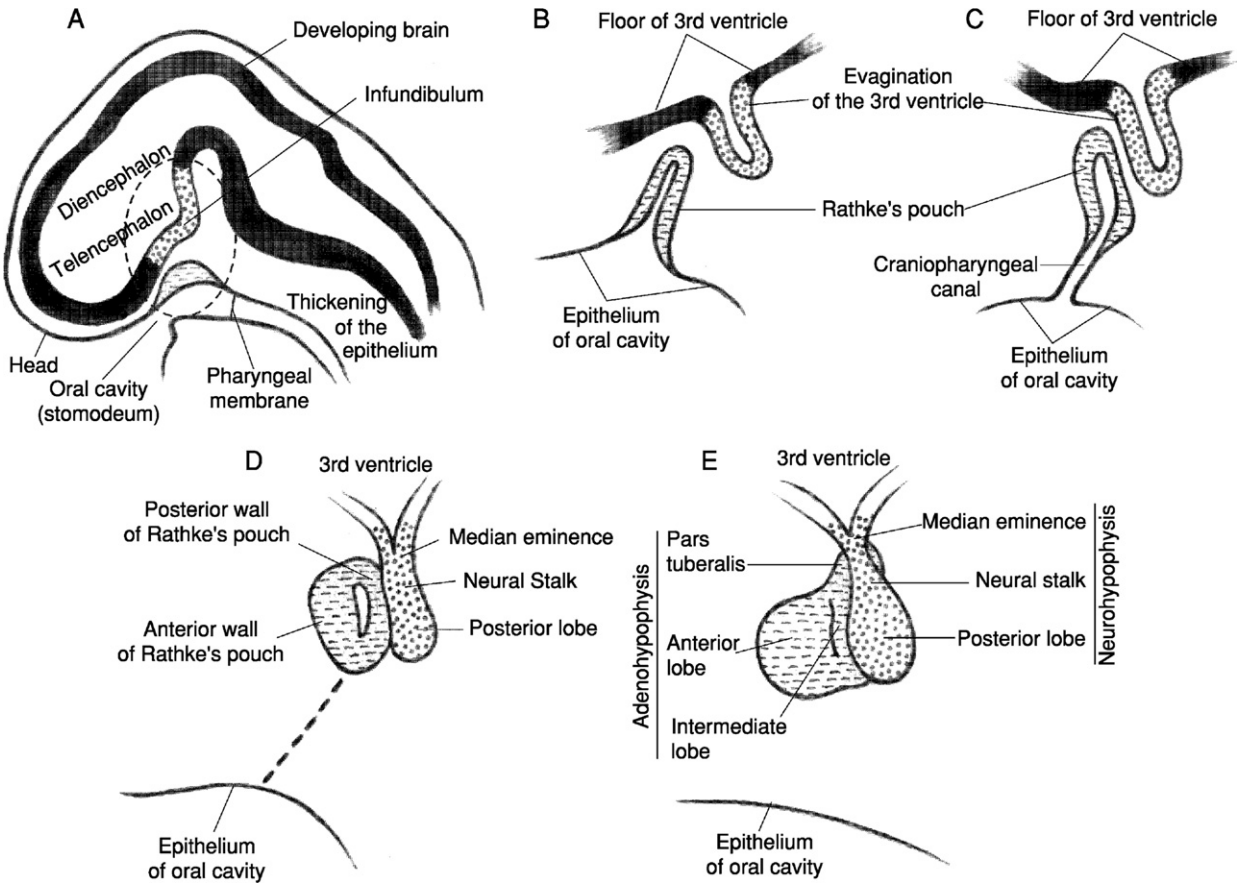

Fig. 3 Schematics of the development of the pituitary as seen in midsagittal sections. The drawings show the various stages of development: (A) very early stage; (B, C) intermediate stage; (D) nearly developed stage; and (E) completely developed stage.

Just behind the pharyngeal membrane, the entodermal epithelium also forms a pouch, the pouch of Sessel. This structure is involved in the formation of the adenohypophysis in lower vertebrates. However, this involvement decreases as the evolutionary scale increases and disappears completely in primates and human. In human, it sometimes persists and may cause certain tumors.

Development of the anterior lobe

The anterior lobe develops from the anterior wall of Rathke's pouch. Cells of this wall proliferate actively and give rise to the anterior lobe of the pituitary gland (Fig. 3(D)). Proliferation occurs in such a way that a small basin is formed, separated into two compartments by a median cellular septum. The compartments disappear progressively, due to the growth of the wall. The median septum forms the pars medialis and the lateral portions form the lateral part of the anterior lobe. The extensive proliferation of the anterior wall of Rathke's pouch reduces the lumen to a narrow residual cleft (Fig. 3(E)). It is usually not recognizable in the adult gland and is represented by a zone of cysts.

Acidophiliccellsaredetectableintheanteriorlobeof the human embryo at approximately the third month of gestation; basophilic cells can be detected somewhat later. Adrenocorticotropic hormone (ACTH)- and growth hormone (GH)-synthesizing cells are identifiable by the end of the second month of gestation. This is followed by the production of glycoprotein hormones. Blood vessels grow into the anterior lobe and establish a direct neurovascular link between the anterior lobe and the hypothalamus at approximately the eighth week of gestation. The mammotroph cell type (prolactin-producing) appears late, at approximately the fifth month of gestation. Hormone-producing cells differentiate in the absence of a hypothalamic influence. In anencephaly, all cell types of the anterior lobe except corticotrophs develop and are capable, to some extent, of hormone synthesis and release.

Development of the intermediate lobe

The posterior wall of Rathke's pouch gives rise to the intermediate lobe of the adenohypophysis (Fig. 3(D and E)). In humans, cells of the posterior wall of Rathke's pouch do not proliferate; instead, they form the poorly defined intermediate lobe, which becomes an inconspicuous, discontinuous layer.

Development of the pars tuberalis

The pars tuberalis of the adenohypophysis develops from the anterior wall of Rathke's pouch. The cells of the median septum of the anterior wall of Rathke's pouch proliferate upward along the pituitary stalk, which becomes gradually encircled by the cellular expansion of the developing pars tuberalis (Fig. 3(E)).

Development of the Neurohypophysis

The neurohypophysis develops from the evagination of the wall of the third ventricle (Fig. 3). The floor of the third ventricle becomes depressed and produces the infundibulum (Fig. 3(A)). This depression penetrates progressively toward the adenohypophysial primordium. Its ventral end forms a diverticulum (Fig. 3(B and C)). The wall of the diverticulum thickens and its lumen gradually fills, forming the neural or posterior lobe, which is attached to the posterior wall of Rathke's pouch (Fig. 3(D)). It remains to be connected to the diencephalon part of the brain by the thin neural stalk. The ascending part of the infundibulum is the median eminence. The median eminence, the neural stalk, and the posterior lobe (or neural lobe or infundibular process) form the neurohypophysis (Fig. 3(E)). The neural lobe differentiates and specific neuroglial cells, called pituicytes, appear in it. The neurohypophysis is then colonized by axons coming from the hypothalamic paraventricular and supraoptic nuclei. In the human, neurosecretory material, characteristic of the posterior pituitary, is demonstrable at approximately the fifth month of gestation.

Histology

Adenohypophysis

Anterior lobe (or anterior pituitary or pars distalis)

The anterior lobe accounts for approximately 75%–80% of the whole gland. It is highly vascular and contains various cell types. The lobe is largely enclosed by a dense collagenous capsule and is composed of glandular cells arranged in irregular cords or clumps, which are intimately related to an extensive system of dilated capillaries (sinusoids) of the blood vascular system. Reticular fibers surround the cords of parenchymal cells and are also present close to the wall of the sinusoids.

The glandular cells were originally classified as chromophilic and chromophobic on the basis of their avidity or lack of affinity for the dyes used in routine staining of histological sections. The chromophilic cells were subdivided into acidophilic and basophilic cells on the basis of staining with combinations of an acidic dye and a basic dye. In the human pituitary, acidophilic cells are most numerous in the posterolateral portions of the anterior lobe. They are rounded, small cells with a well-developed Golgi complex and rod-shaped mitochondria. The basophilic cells are larger and less numerous.

The most meaningful method applied at both the light and electron microscopic levels for identification of the cells producing various hormones involves the use of immunocytochemical procedures. Antibodies to a specific hormone induced in another species are conjugated with horseradish peroxidase or with fluorescence dyes. These labeled antibodies are reacted with sections of

the anterior lobe and the sites of the antigen in the tissue are localized by the histochemical method for peroxidase or by fluorescence microscopy.

The anterior pituitary produces six hormones: GH (or somatotropin), prolactin, thyrotropin (TSH), ACTH, and two gonadotropins – follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The acidophilic cells secrete simple proteins (somatotropin and prolactin) and the basophils secrete glycoprotein hormones (TSH, FSH, LH, and the precursor of ACTH). It has become a common practice to use the terms of the hormone secreted (ACTH cell, TSH cell, FSH/LH cell) or the name of the target organ stimulated (corticotroph, thyrotroph, gonadotroph) to denote the cell type. In addition to these cells, some other cell types, such as folliculostellate cells and null cells, are also found in the pituitary.

Somatotrophs

Somatotrophs represent approximately 50% of the anterior lobe cells. They are found in groups along the sinusoids and are located mainly in the two lateral wings of the anterior lobe. The cells are usually medium-sized and contain numerous spherical, evenly electron-dense granules, the majority being 350–500 nm in diameter. They have a well-developed endoplasmic reticulum and secrete GH. Immunocytochemical observations strongly suggest that the somatotrophs are not a uniform mass of cells. It appears that they consist of several subpopulations, all of which express somatotropin, but are also capable of producing other hormones, such as prolactin and TSH, in special circumstances. There is evidence that transdifferentiation of GH-/prolactin-synthesizing cells (somatomammotrophs) may contribute to the mass of prolactin-producing cells during development of prolactin cell hyperplasia in human pregnancy and further, that somatotrophs may transform into large thyrotroph cells in rats during experimental hypothyroidism.

Mammotrophs

Mammotrophs (or lactotrophs or prolactin cells) produce prolactin and constitute 10%– 25% of the anterior lobe cells. They are relatively small cells, are distributed individually in the interior of the cell cords, and are located throughout the entire anterior lobe. The cells are especially numerous at the posterolateral and posteromedial edges of the adenohypophysis. Two types of lactotrophs can be distinguished. The majority of the cells are small or medium in size and sparsely granulated. The secretory granules are spherical and evenly electron-dense, measuring 150–350 nm in diameter. The other, less frequently occurring type is densely granulated and the granules are larger (300–600 nm). The number of prolactin cells varies considerably. In pregnancy and lactation, lactotrophs are increased in number. During pregnancy, the cells undergo considerable hypertrophy: their Golgi complex enlarges and the endoplasmic reticulum becomes more extensive; multiple layers parallel to the cell membrane develop and the granules become larger (550–600 nm in diameter) and often irregular in outline. The mammotrophs are most active during lactation. After weaning has occurred, lysosomes play an important role in the elimination of excess secretory granules and the hypertrophied cellular organelles involved in the lactation period of active protein synthesis. Lysosomes fuse with the secretory granules to form autophagic vacuoles. The secretory granules are degraded by hydrolytic enzymes in these vacuoles. This procedure of disposal of secretory product that is no longer needed is called crinophagy. Excess cytomembranes and ribosomes are also enclosed in vacuoles and degraded by autophagy.

Thyrotrophs

Thyrotrophs, secreting TSH, are located mainly in the anteromedial part of the anterior lobe and constitute less than 10% of the lobe. The cells are medium or large in size and polygonal with long cytoplasmic processes. In electron micrographs, they are characterized by spherical secretory granules, are variably electron-dense, measure 100–200 nm in diameter, and often line up along the cell membrane, with short stacks of rough endoplasmic reticulum membranes and a Golgi complex with flattened sacculi and several vesicles. In hypothyroidism, thyrotrophs increase in size and number and transform into so-called thyroidectomy cells or thyroid-deficiency cells. These cells are large and contain widely dilated endoplasmic reticulum membranes, conspicuous Golgi complexes, and a varying number of secretory granules.

Gonadotrophs

Gonadotrophs synthesize both gonadotropic hormones, FSH and LH. These cells are larger than other cells of the anterior lobe, constitute approximately 15%–20% of the lobe, and are located throughout the anterior pituitary. They are near capillaries and often in close proximity to mammotrophs, suggesting the possibility of paracrine action between the two cell types. FSH and LH are located mostly in the cytoplasm of the same cells, but there are also gonadotrophs that show only FSH or LH positivity. In electron micrographs, the rough endoplasmic reticulum is prominent and forms slightly dilated stacks; the Golgi complexes are conspicuous. The secretory granules represent two populations: the diameter of one granule type is 150–250 nm and that of the other type is 350–450 nm. Following castration, the size and number of gonadotrophs increase and the cells show enlargement of the cytoplasm, proliferation, and dilation of the endoplasmic reticulum membranes.

Corticotrophs

Corticotrophs (or corticotropin- or ACTH-producing cells) are located mainly in the central part of the pituitary. Some cells are scattered in the lateral wings. They represent 10%–15% of the anterior lobe cells. Corticotrophs are medium-sized or large oval cells and show ACTH, β-lipotropin, and β-endorphin immunoreactivity (proopiomelanocortin-derived peptides). In electron micrographs, they have welldeveloped rough endoplasmic reticulum membranes and are usually numerous, spherical, irregular

secretory granules measuring 250–400 nm, showing varying electron density. The granules tend to be located adjacent to the cell membrane. In addition, the presence of type 1 filaments, which are bundles of filaments located mainly in the perinuclear area, are a characteristic feature of corticotrophs.

Folliculostellate cells

Folliculostellate cells, called also follicular cells, are mostly agranular cells with branching processes among the secretory cells. There are data indicating that they are derived from granulated cells after having been joined by junctional complexes around damaged and ruptured adenohypophysial cells and are capable of forming follicles. Folliculostellate cells produce several substances (interleukin G, follistatin, etc.). It is assumed that these cells play an important role in the paracrine mechanisms controlling pituitary functions.

Null cells

Null cells are relatively small, chromophobic cells that do not contain known anterior pituitary hormones in their cytoplasm. The cells have all the cytoplasmic organelles necessary for secretion. They contain secretory granules that may contain hormone fragments, precursors, or biologically inactive substances. They may represent resting cells, precursors of various cell types, or an unknown cell type.

It should be mentioned that the majority of what were originally called chromophobic cells contain specific granules with hormone content. The cells classified as chromophobes by light microscopy are not a homogenous population. Some are evidently chromophils agranulated to the point at which their specific nature is not detectable. There seems to be a considerable degree of cytological specialization among the cells normally classified as chromophobes. It is probable that many of the apparent chromophobes have already been determined and are capable of differentiating into only one of the chromophil types.

Intermediate lobe

There is considerable variation among species in the degree of development of the intermediate lobe. It is poorly developed in the human. It contains a few dilated cavities lined by a single layer of cuboidal or columnar epithelial cells and is filled with an amorphous proteinaceous material. Many cells lining the cystic cavities and cells between the cysts give a positive immunostaining for proopiomelanocortinderived peptides. These cells are smaller than the corticotrophs in the anterior lobe.

Pars tuberalis

The pars tuberalis is an upward extension of the anterior lobe and is attached to the neural stalk. It contains small groups of cells that produce mainly glycoprotein hormones, gonadotropins and thyrotropin. The functional significance of the pars tuberalis is not known.

Neurohypophysis

The human neurohypophysis consists of approximately 10,000 axons and terminals of neurosecretory cells located in the hypothalamic supraoptic and paraventricular nuclei. The unmyelinated fibers of the neurosecretory cells descend, converge, and form the supraoptico- and paraventriculohypophysial tract or pathway. These fibers descend through the median eminence and neural (or infundibular) stalk into the neural lobe of the hypophysis and make up the bulk of the substance of the lobe.

Median eminence and infundibular stalk

The median eminence is made up of two layers: the external layer and the internal layer. Not the capillary loops but the hypophysiotrophic neurons whose axon terminals are in the external layer are producing the neurohormones. This layer is a key structure of the neurovascular contact between the hypothalamus and the anterior pituitary.

The internal layer of the median eminence and the infundibular stalk contain the descending unmyelinated axons of the supraoptico- and paraventriculohypophysial tract, terminating in the posterior pituitary, transporting the neurosecretory material from the hypothalamic neurons to the posterior lobe at a rate of 1 to 4 mm/h. In histological preparations stained with chromalum hematoxylin, deeply stained neurosecretory material is seen in aggregations of varying sizes throughout the infundibular stalk and neural lobe. These aggregates are called Herring bodies. In electron micrographs, they are found to be large aggregations of the small secretory granules. The axons of these neurosecretory neurons vary greatly in caliber and have numerous dilations along their length. Approximately 60% of all neurosecretory material resides in these dilations and approximately 30% resides in axon endings in the posterior pituitary.

Posterior lobe (or neural lobe, or infundibular process)

The posterior pituitary is made up of axons and axon endings of the neurosecretory neurons of the hypothalamic supraoptic and paraventricular nuclei synthesizing oxytocin and vasopressin (called also antidiuretic hormone) and also contains an intrinsic population of cells called pituicytes, which resemble astrocytes and do not appear to be secretory. Axons of the neurosecretory neurons terminate blindly in close relation to the basal lamina of a rich capillary plexus.

The neurosecretory material is stored in granules at the dilated blind endings of the axons and are released as needed. The neurosecretory granules have a diameter of 100–200 nm, are surrounded by a membrane, and are more numerous apposed to fenestrated blood capillaries. The neurosecretory material consists of oxytocin or vasopressin and a binding protein (neurophysin) specific for each hormone. The hormone– neurophysin complex is synthesized as a single, long peptide on ribosomes attached to the membranes of the rough endoplasmic reticulum. The endoplasmic reticulum is a cell organelle from where the synthesized material is passed onto the Golgi complex. As the granules pass down axons of the supraoptico- and paraventriculo-hypophysial tract, proteolysis of the precursor occurs, yielding the hormone and its specific neurophysin. Vasopressin and oxytocin are stored in the posterior pituitary and are released into the blood by impulses in the nerve fibers from the hypothalamus.

In addition to the axons from hypothalamic nerve cells, approximately 25%–30% of the volume of the posterior pituitary consists of a distinctive type of glial cell, called a pituicyte. Pituicytes are highly branched cells with processes that form a three-dimensional network ensheathing the neurosecretory axons. Their cytoplasmic processes meander among groups of preterminal secretory axons and often intimately envelop their granule-filled terminal expansions. In the human, they are highly variable in size and shape. The cytoplasm of the pituicytes may contain lipid droplets and pigment. The processes of pituicytes are connected by gap junctions. Pituicytes are believed to have a trophic and supportive function and to maintain the appropriate ionic composition of the extracellular fluid compartment.

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Hypothalamic Disease☆

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Glossary

Carcinoma A malignant growth of epithelial cells that exhibits the tendency to infiltrate surrounding tissues and gives rise to metastases.

Chordoma A malignant tumor arising from the embryonic remnants of the notochord.

Craniopharyngioma A tumor arising from the cell rests derived from embryonic Rathke's pouch that gives rise to the hypophysis (pituitary).

Cyst A closed cavity or sac that contains liquid or semisolid material (e.g., Rathke's cleft cyst, arachnoid cyst, and dermoid/epidermoid cysts in the hypothalamic region).

Diabetes insipidus A metabolic disorder due to deficient production or release of antidiuretic hormone, resulting in failure of tubular resorption of water by the kidney and thus causing excretion of abundant urine of low specific gravity and dehydration.

Gangliocytoma (ganglioneuroma) A benign neoplasm composed of nerve fibers and mature ganglion cells.

Germinoma (germ cell tumor) A tumor of germ tissue of the testis or ovary, arising in these sites or in ectopic sites along the midline.

Glioma A tumor composed of neurological tissue of any type, including astrocytes and ependymal cells.

Meningioma A hard, slowly growing tumor arising from the meninges.

Neurocytoma A tumor of small, poorly differentiated hypothalamic neurons.

Pituicytoma A tumor of the modified glia of the posterior lobe of the pituitary.

Pituitary tumor A tumor composed of adenohypophysial epithelial cells. Pituitary tumors may secrete hormones and may be infiltrative and locally aggressive but do not metastasize.

Sarcoidosis A chronic progressive systemic granulomatous inflammation of unknown etiology.

Sarcoma A malignant tumor composed of connective tissue elements (mesenchyme).

Schwannoma A neoplasm originating from Schwann cells of the myelin sheath of nerves.

Syndrome of inappropriate antidiuretic hormone (SIADH) Persistent hyponatremia, inappropriately elevated urine osmolality, and no discernible stimulus for ADH release.

Teratoma A neoplasm composed of a number of different types of tissue, none of which is native to the area in which it occurs. It is thought to derive from germ cells.

Introduction

The hypothalamus is a phylogenetically primitive structure that plays an important role in the regulation of autonomic or vegetative functions, behavior, and emotion. Its importance in physiology is highlighted by its key role in the regulation of most endocrine functions. The pituitary has been called "the conductor of the endocrine orchestra"; using the same analogy, the hypothalamus would have to be considered "the composer that writes the music."

Normal Hypothalamic Anatomy and Physiology

The hypothalamus is a poorly defined anatomical structure with arbitrary delineations: the anterior commissure, lamina terminalis, and optic chiasm define the anterior border, and the midbrain tegmentum and mamillary bodies identify the posterior boundary. Laterally, it is demarcated by the substantia innominata, internal capsule, subthalamic nucleus, and cerebral peduncle. The superior border is the hypothalamic sulcus and inferiorly it is defined by the optic chiasm and median eminence that gives rise to the pituitary stalk (Fig. 1A).

The hypothalamus is composed of nuclei, which are clusters of functionally distinct neuronal cell populations. These neurons are large and polyhedral cells known as ganglion cells (Fig. 1B). The nuclei of the hypothalamus are topographically distinct in lower-order animals and they are relatively well demarcated in the developing human fetus, but in the adult human they are ill defined. From animal studies, it is known that individual nuclei have important physiological functions; for example, there are specific nuclei implicated in hunger or satiety control, temperature regulation, olfaction, circadian rhythms, sexual drive, ovarian regulation, and parenting behaviors. However, functionally, a given hormone is often produced in more than one nucleus, and in

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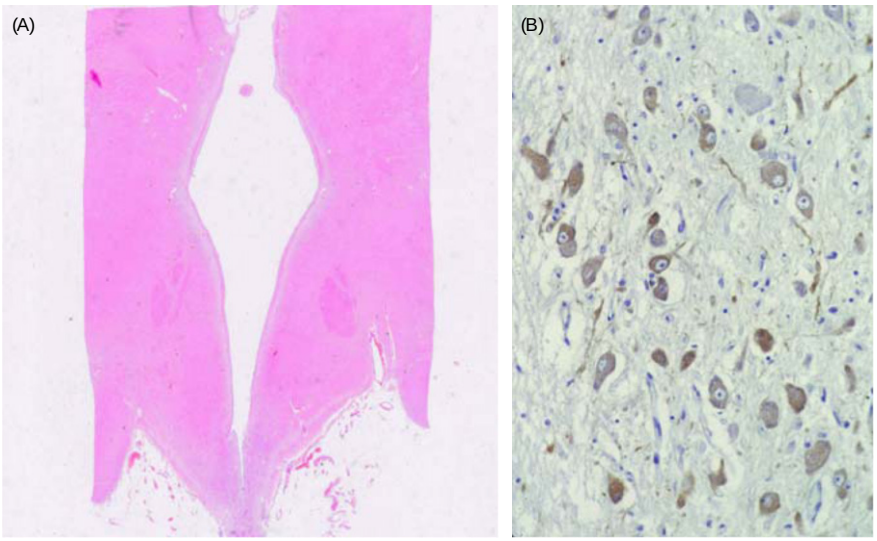

Fig. 1 Normal hypothalamus. (A) The third ventricle is lined by the lateral hypothalamus that harbors the paraventricular nuclei. Inferiorly, the infundibulum carries nerve fibers to the pituitary stalk. (B) The nuclei of the hypothalamus are collections of ganglion cells; in this supraoptic nucleus, the neurons contain vasopressin immunoreactivity.

many instances, a single nucleus produces more than one hormone. These data raise doubts about the concept of individual nuclei as designated with exclusive functional entities.

Clinical Manifestations of Hypothalamic Disease

Lesions of the hypothalamus cause headache, nausea, vomiting, somnolence, behavioral alterations, psychosis, and dementia. Hypothalamic destruction can result in bulimia or anorexia. Visual disturbance can result from oculomotor alterations or optic nerve damage. Hypopituitarism and diabetes insipidus are common manifestations. In severe cases, patients can develop hydrocephalus. Inflammation can result in meningitis.

Diabetes insipidus is the most common and often the initial manifestation. The presentation of hypopituitarism varies with age. In children, hypothalamic dysfunction may present with dwarfism. In adults, sexual dysfunction is the most common endocrine complaint, with impotence in males and primary or secondary amenorrhea in females. If the disease is predominantly hypothalamic or causes interruption of the pituitary stalk, pituitary hypofunction is associated with hyperprolactinemia due to interruption of the dopaminergic neurons that maintain tonic inhibition of that pituitary hormone, and stimulation confirms an intact pituitary response. If the lesion causes destruction of hypophysial tissue as well as hypothalamic disease, there is a reduction of all basal pituitary hormones or more subtle changes with reduced response to stimulation.

Some tumors are associated with pituitary hormone excess. Occasionally, this is due to the production of hormones stimulating pituitary function, such as in hypothalamic gangliocytomas that secrete adenohypophysiotrophic hormones. Alternatively, the clinical manifestations may be due to the production of substances that simulate or mimic pituitary hormones. For example, germ cell tumors associated with precocious puberty produce β-chorionic gonadotropin. Occasionally, patients manifest excessive secretion of vasopressin, resulting in the syndrome of inappropriate antidiuretic hormone.

Developmental Disorders

The failure of development of areas of the hypothalamus can lead to variable clinical manifestations. Major defects are incompatible with life.

Septo-optic dysplasia or de Morsier's syndrome is a complex developmental disorder with variable manifestations of aplasia of the septum pellucidum, hypoplasia of the optic nerves, and endocrine dysfunction as well as vegetative alterations. The disorder has been associated with mutations of a homeobox transcription factor, *Hesx1*, which is required for normal development of the affected regions of the central brain, and alterations in OTX2, SOX2, and PAX6 have also been implicated (Lopes, 2016).

Kallman syndrome is an X-linked developmental defect causing gonadal insufficiency in males due to hypothalamic GnRH deficiency associated with anosmia. It was initially attributed to mutation of the *KAL-1* or ANOS1 gene that encodes an extracellular glycoprotein, anosmin-1, that is expressed during the period of human organogenesis in the early olfactory system and is required for normal migration of gonadotropin-releasing hormone (GnRH)-containing neurons. Other genetic alterations associated with this syndrome involve CHD7, FGF8/FGFR1, PROK2/PROKR2, IL17RD, NELF, HS6ST1, FLRT3, SPRY4, DUSP6, SEMA3A, and WDR11 genes that play a role in neuronal migration (Valdes-Socin *et al.*, 2014).

Hypothalamic hypogonadotropic hypogonadism also occurs with normosmia and is classified separately. It is attributed to mutations in some of the same genes as Kallman syndrome (PROKR2, FGF8, FGFR1, CHD7, DUSP6, WDR11) as well as in mutations in KISS1/KISSR, GNRH1/GNRHR, TAC3/TACR3, LEP.LEPR, HESX1, and the gonadotropins (Valdes-Socin *et al.*, 2014).

Laurence–Moon–Biedl syndrome is a highly polymorphic disorder that includes pigmentary retinopathy, mental retardation, spinal paraplegia and hypogonadism associated with obesity, and digital anomalies. The genetic basis had remained unknown and the variable clinical manifestations suggest incomplete penetrance with differential expression of the anomaly. More recently, several mutations in families with Laurence–Moon syndrome were identified in the PNPLA6 gene, which encodes neuropathy target esterase (Hufnagel *et al.*, 2015).

Prader–Willi syndrome is characterized by obesity, short stature, delayed puberty, infertility, mental retardation, muscle hypotonia, hypopigmentation, and seizure disorder. It has been linked with deletions of chromosome 15q11–q13. This region contains an imprinting center that regulates paternally expressed genes whose expression is lost; some cases are attributed to maternal uniparental disomy. However, the precise causative gene(s) are unknown.

Wolfram syndrome (*WS*) is characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (hence the acronym DIDMOAD). This rare neurodegenerative disorder exhibits autosomal recessive inheritance usually due to compound heterozygous mutations of the *WFS1* gene, a member of a novel gene family that encodes wolframin, an endoglycosidase H-sensitive membrane glycoprotein that localizes in the endoplasmic reticulum. *WFS1* is expressed predominantly in selected neurons in the hippocampus, amygdala, olfactory tubercle, and superficial layer of the allocortex—components of the limbic system or structures closely associated with this system that account for the psychiatric, behavioral, and emotional abnormalities of this syndrome, as well as in the pancreas where it causes diabetes, and in the heart, bones, muscle, lung, liver, and kidneys. Heterozygous mutations are associated with nonsyndromic, low-frequency sensorineural hearing loss affecting only the 2000-Hz and lower range. This is an unusual disorder that worsens over time without progressing to profound deafness. Occasional cases have been associated with mutations of CISD2 that encodes a mitochondrial protein of unknown function.

Idiopathic growth hormone (*GH*) *insufficiency* is most often due to a defect in the synthesis or secretion of growth hormone-releasing hormone (GHRH) since patients usually have normal pituitary GH somatotroph structure and hormone content, and they respond to GHRH administration (Attie, 2000).

Hypothalamic Inflammation

Infectious Lesions

Acute and chronic infections of the hypothalamus are rare but they do occur, usually in association with sphenoid sinus infection, cavernous sinus thrombosis, otitis media mastoiditis, or peritonsillar abscess. Pituitary tumors have been associated with the development of pituitary abscess that can spread to the hypothalamus. It has been suggested that bony erosion by the tumor predisposes such patients to the spread of sinonasal infection. Rarely, infection results from vascular seeding of distant or systemic infection.

Noninfectious Inflammatory Lesions

Sarcoidosis is a multisystem granulomatous disease of unknown etiology. It has long been attributed to an infectious agent; however, none have been identified. Disease onset usually occurs in adults, and there is a predilection for blacks and females. There are usually systemic manifestations, and neural involvement is rare; however, it can occur in the hypothalamus, usually involving the meninges at the infundibulum and floor of the third ventricle. The granulomatous inflammation has a subacute or protracted course of tissue destruction that may respond to steroid suppression, and there rare reports of spontaneous resolution.

Neuroinfundibulohypophysitis is a rare inflammatory condition that affects the infundibulum, the pituitary stalk, and the neurohypophysis and may be part of a range of autoimmune disorders, including lymphocytic hypophysitis. Lymphocytic hypophysitis occurs mainly in women and most often presents in the later stages of pregnancy (Asa, 2011; Asa and Mete, 2016). Infundibulohypophysitis shows no sexual predilection and usually presents with diabetes insipidus. The cause is unclear but is thought to be autoimmune in nature (Asa, 2011; Asa and Mete, 2016).

Inflammatory pseudotumor, a lesion similar to orbital pseudotumor characterized by chronic inflammation and fibrosis, can involve the parasellar tissues and may be associated with other sclerosing lesions, such as Riedel's thyroiditis, retroperitoneal fibrosis, and sclerosing cholangitis that form the disorder known as IgG4 disease (Asa, 2011; Asa and Mete, 2016). The etiology and appropriate management of these disorders are uncertain.

Metabolic Lesions

Neurodegenerative Processes

Alzheimer's disease, Parkinson's disease, Huntington's chorea, and other neurodegenerative diseases can involve the hypothalamus, resulting in variable endocrine, behavioral, and vegetative abnormalities.

Systemic Processes

Wernicke's encephalopathy can alter hypothalamic function, but usually the manifestations are due to mamillary body degeneration. Hemochromatosis results in hypogonadotropic hypogonadism, but this is thought to be due to iron deposition in the pituitary gonadotrophs rather than a principally hypothalamic lesion.

Cystic Lesions

Rathke's Cleft Cysts

These cysts originate in the remnants of Rathke's pouch of the pituitary. Rathke's cleft arises from the oropharynx and migrates upward with an anterior and posterior limb that ultimately gives rise to the anterior and intermediate lobes of the adenohypophysis, respectively. In the human, the intermediate lobe is vestigial, and its remnants line small cystic cavities that are remnants of the cleft and usually <5 mm in diameter. When they enlarge and become detectable, they can cause symptoms. Initially the symptoms are hypopituitarism and diabetes insipidus, due to sellar compression (Shin *et al.*, 1999). When they develop suprasellar extension, symptoms of hypothalamic involvement ensue. There are rare cases of purely suprasellar Rathke's cleft cysts, and occasionally abscess formation develops within these lesions.

They usually present in adults but do occur in infants and young children. Computed tomography (CT) scans reveal low-density cystic areas with capsular enhancement in most cases; the magnetic resonance imaging (MRI) appearance is variable. Morphologic examination reveals a cyst lining characterized by ciliated cuboidal or columnar epithelium resembling respiratory epithelium, with occasional goblet cells and squamous elements. The degree of ciliation and the propensity for squamous metaplasia distinguish these cysts from the neuroepithelial-derived colloid cysts of the third ventricle.

The management of these lesions involves surgical drainage with or without partial excision. The recurrence rate is low. Most symptoms and signs are relieved postoperatively, but permanent hypopituitarism and diabetes insipidus require hormone replacement therapy.

In some patients, the cyst can rupture and the release of cyst contents induces a reaction that can vary from granulation tissue to xanthomatous inflammation, an accumulation of clear, lipid-laden macrophages with a characteristic appearance. Xanthomatous hypophysitis was initially described as an idiopathic phenomenon but has increasingly been recognized to be attributable to a ruptured Rathke's cleft cyst (Duan *et al.*, 2017).

Arachnoid Cysts

These lesions may be congenital anomalies or acquired cysts in the arachnoid of the sellar and suprasellar region. The cystic nature of these lesions evident on CT or MRI scans may make it difficult to distinguish them from other cysts that occur in this area (Shin *et al.*, 1999). These cysts are filled with clear, colorless fluid and are lined by arachnoid laminar connective tissue with a single layer of flattened epithelium. These lesions are also managed by drainage, with partial cyst wall excision.

Dermoid and Epidermoid Cysts

Dermoid and epidermoid cysts arise from epithelial cells that are misplaced during embryologic development or, rarely, traumatically. Epidermoid cysts are also known as cholesteatoma (Asa, 2011). These epithelial cysts can occur intracranially, most commonly at the cerebellopontine angle but often in the suprasellar area.

The cystic nature of these lesions evident on CT or MRI scans may make it difficult to distinguish them from other cystic lesions in this area. Epidermoid cysts are lined by keratinizing squamous epithelium; dermoid cysts are distinguished by the additional presence of skin appendages, including hair follicles and sweat glands.

Management usually involves surgical resection. Complications include rupture with chemical meningitis due to keratin debris or the development of squamous carcinoma.

Aneurysms

Aneurysms of the carotid arteries can expand to give rise to masses in the suprasellar region that compress hypothalamic structures and result in endocrine and other hypothalamic manifestations. It is important to distinguish these from other cystic lesions prior to surgery.

Meningoencephalocele

Encephaloceles involving the hypothalamus have been reported to cause symptoms of a mass lesion with variable endocrine manifestations.

Primary Hypothalamic Neoplasms

Craniopharyngioma

Craniopharyngiomas are thought to derive from the remnants of Rathke's pouch (Asa, 2011). They represent up to 13% of intracranial neoplasms and are the most common sellar tumor of childhood. They can occur at any age from infancy to old age, but the peak incidence is from 5 to 20 years. A second small peak occurs in the sixth decade. In some series, males are more often affected than females.

The majority of tumors occur in the suprasellar region; 15% have an intrasellar component. Craniopharyngiomas are usually cystic or may be cystic and solid, and approximately half have radiologically detectable calcification. Although they may be as small as 1 cm, the majority are much larger at the time of diagnosis. Grossly, they are well-circumscribed tumors, but there may be little or no capsule at the interface with brain parenchyma. The lesions usually contain a thick oil-like fluid that is described as "black sludge." Cholesterol crystals and calcification may be seen grossly. Rarely, these tumors may contain bone and/or teeth. Microscopically, they are composed of cords or islands of epithelial cells in a loose fibrous stroma and with intervening cysts (**Fig. 2**A). The epithelium usually has an outer palisaded layer, a midzone of stellate epithelial cells, and a superficial keratinizing layer. Masses of keratin often form the nidus for calcification. Occasionally, there is an inflammatory component. Microscopically, the borders are frequently irregular and may be associated with gliosis in the adjacent brain tissue.

Two subtypes of craniopharyngioma have been recognized. The adamantinomatous type has a predominance of stellate components, which yields a pattern resembling the dental ameloblastic organ and is similar to that seen in adamantinomas. The papillary variant is less common, rare in children, and believed to have a better prognosis. It is characterized by solid or cystic growth of pseudopapillary squamous epithelium and lacks the palisading, fibrosis, and cholesterol accumulation that characterizes the typical craniopharyngioma. These two variants have differing genetic alterations that have implications for management when complete surgical resection is not possible. Adamantinomatous craniopharyngiomas have mutations of beta-catenin that result in nuclear translocation of that protein that activates Wnt-signaling and can be detected by immunohistochemistry (**Fig. 2**B). In contrast, papillary craniopharyngiomas harbor BRAFV600E mutations (Brastianos *et al.*, 2014) that can be detected by mutant-specific immunohistochemistry using the VE1 antibody and that can be targeted by medical therapy using debrafinib and trametinib (Brastianos *et al.*, 2016).

Craniopharyngiomas can be extremely infiltrative lesions. They may cause extensive tissue damage, extending into the hypothalamus and as high as the third ventricle; obstruction of the ventricle may result in hydrocephalus. Rarely, craniopharyngiomas may spontaneously rupture or form abscesses; a rare form of hypophysitis has been described in this setting and is called sellar xanthogranuloma (Paulus *et al.*, 1999). Because of their highly infiltrative nature, they are often incompletely excised surgically. There is a 10%–40% recurrence rate, particularly in younger patients. Many surgeons advocate postoperative radiation. There is a single report of malignant transformation of a craniopharyngioma. This occurred on the fifth recurrence after a 35-year history of disease and 8 years following radiotherapy.

Neuronal Tumors

Hypothalamic neuronal tumors are very rare neoplasms that have been reported in the literature as "*gangliocytomas*" or "*ganglioneuromas*." These lesions are composed of mature neurons resembling hypothalamic ganglion cells and are capable of producing hypothalamic peptides (**Fig. 3**). Some researchers prefer to consider these hamartomas because they occur in children and it is thought that they represent developmental anomalies. They are commonly called ganglioneuromas or gangliocytomas because there is evidence that they represent neoplasms that have their onset in adulthood and are capable of continued growth. Such

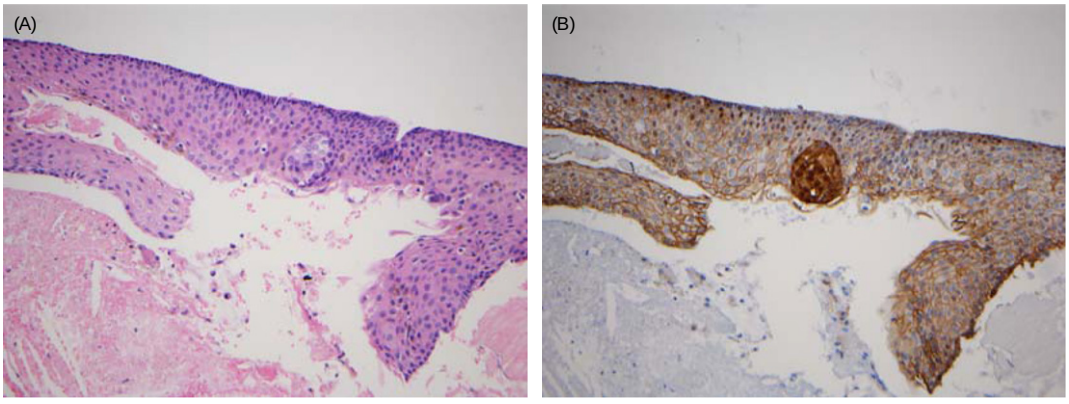

Fig. 2 Craniopharyngiomas. (A) These tumors are composed of squamoid epithelium that derives from remnants of Rathke's pouch. The infiltrative lesions destroy hypothalamic parenchyma. (B) This adamantinomatous craniopharyngioma has characteristic nuclear translocation of beta-catenin in morules, compared with the membranous pattern in the squamous component; this feature correlates with mutation.

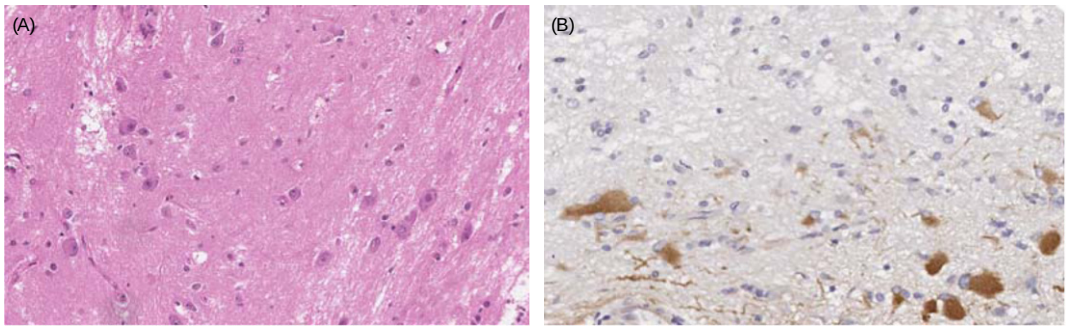

Fig. 3 Hypothalamic gangliocytoma. (A) The tumor is composed of ganglion cells resembling those of the normal nuclei of the hypothalamus but lacking organization and containing abnormal nuclei. (B) The tumor cells of this gangliocytoma associated with acromegaly contain GHRH immunoreactivity.

tumors have also been described in other parts of the brain as extrahypothalamic gangliocytomas. The term choristoma has been applied to describe collections of hypothalamic neurons within the adenohypophysis.

In addition to the usual symptoms of a hypothalamic mass lesion, some of these tumors are hormonally active and cause endocrinopathies that are usually mediated by the pituitary (Asa, 2011; Asa and Mete, 2016). They have been associated with acromegaly, precocious puberty, Cushing's disease, and amenorrhea–galactorrhea. The most common localization of these peculiar neoplasms is in the hypothalamus or tuber cinereum, with variable involvement of the third ventricle. They may be nodular, pedunculated, sessile, solid, or cystic. They may be within, attached to, or completely detached from the hypothalamus. These tumors may also be found within the parenchyma of the adenohypophysis. They vary from microscopic lesions composed of only a few cells and measuring a few millimeters to masses that measure >5 cm. In particular, hypothalamic hamartomas associated with acromegaly often present as a solitary intrasellar mass that mimics a simple pituitary GH-producing pituitary neuroendocrine tumor; microscopic examination reveals a gangliocytoma associated with a pituitary adenohypophysial tumor in such cases. They are composed of randomly oriented large mature ganglion cells with large nuclei and very prominent nucleoli; binucleated and even multinucleated cells are seen. Most of the ganglion cells contain abundant cytoplasm, and Nissl granules are conspicuous at the periphery of the cell body. Some tumors have a glial stroma.

In patients with acromegaly, the ganglion cells may contain GHRH (**Fig. 3**B), glucagon, somatostatin, vasoactive intestinal peptide (VIP), corticotropin-releasing hormone (CRH), GnRH, or gastrin; associated pituitary adenohypophysial neuroendocrine tumors generally contain GH, but some patients have somatotroph hyperplasia and, rarely, no pituitary abnormality is identified. In patients with amenorrhea–galactorrhea, prolactin and VIP have been localized in the cytoplasm of ganglion cells. One patient had an associated lactotroph tumor; another had a mixed GH- and prolactin-producing pituitary tumor. Tumors associated with Cushing's disease contain CRH and may also express ACTH, ß-lipotropin, and somatostatin; they have been associated with corticotroph hyperplasia or corticotroph neoplasia. Children with precocious puberty have neuronal tumors that contain GnRH but may also contain immunoreactivity for CRH, β-endorphin, and oxytocin. Some gangliocytomas not associated with clinical evidence of hormone excess show multiple immunoreactivities, including positivity for VIP, galanin, α-subunit of glycoprotein hormones, somatostatin, and serotonin. In a few cases, there is ultrastructural evidence of an intimate association between neurons and adenohypophysial cells, both neoplastic and nontumorous, supporting the theory of an endocrine relationship.

The pathogenesis of these lesions is not known. Their association with pituitary adenohypophysial tumors in some patients suggests that there may be a common causative mechanism. It has been suggested that sellar gangliocytomas may arise by abnormal differentiation during neoplastic proliferation of adenohypophysial cells. However, it is more likely that these tumors have a true hypothalamic origin. It may be that the same tumorigenic stimulus results in tumors of both hypothalamus and pituitary.

These tumors are diagnosed at the time of surgery, and cure has resulted from complete surgical resection in several published cases. However, despite the apparent cure of the endocrine disturbance, there may be radiographic evidence of residual tumor. When surgery cannot accomplish total resection of the lesion, the prognosis is varied. Most of these neuronal tumors have an extremely low proliferative potential, and the overall prognosis is good. A few have caused severe hypothalamic destruction and have resulted in the demise of the patient. Radiotherapy appears to ameliorate hormone excess in these patients, but it does not appear to be effective in preventing regrowth of the neuronal tumor.

Much more rare immature neuronal tumors known as "*neurocytomas*" occur in this region (Asa and Mete, 2016). They are composed of small round cells with variable but usually scant cytoplasm, and poorly developed neuropil. Morphologically they resemble small immature neuronal tumors in the regions of the cerebral ventricles that were called "central neurocytoma"; subsequent reports identified "extraventricular neurocytomas." The small primitive neurons express neuronal markers NeuN and scant neurofilaments, as well as synaptophysin and chromogranin. The hypothalamic ones also express TTF-1, a feature of hypothalamic cells that derived from the basal hypothalamus. Occasional tumors present with features of hormone excess due to dysregulated production of vasopressin.

Pituicytomas

Tumors of the posterior pituitary are composed of modified glial cells that form the sustentacular network surrounding the axons of hypothalamic neurons (Asa, 2011; Asa and Mete, 2016). These tumors clinically resemble pituitary adenohypohysial tumors but are distinguished from them morphologically and by their immunohistochemical profile. Pituicytes have five morphologic variants: dark, light, oncocytic, granular, and ependymal (Mete *et al.*, 2013). The tumors that derive from these cells also have a spectrum of morphology, including the usual spindle cell variant that is composed of dark and light cells, the oncocytic variant that was initially called "spindle cell oncocytoma" because of its large round cells with numerous mitochondria creating a granular basophilic cytoplasm, the granular cell variant that is also known as "granular cell tumor" with large granular complex lysosomes, and the rare "ependymal variant" that has elongated cells forming ependymal and perivascular pseudorosettes (Kamil *et al.*, 2013). These tumors are characterized by expression of TTF-1, GFAP, and occasionally epithelial membrane antigen (EMA), but they are completely negative for keratins, pituitary transcription factors, and hormones.

Gliomas

Gliomas are neoplasms derived from and composed of neuroglial cells. They include astrocytomas and glioblastoma multiforme, oligodendrogliomas, and ependymomas. These tumors show a wide range of biologic behaviors.

Aggressive, rapidly lethal malignant gliomas or glioblastoma multiforme in the hypothalamus occur following radiotherapy for pituitary neuroendocrine tumor, craniopharyngioma, or suprasellar germinoma. These tumors can occur 5–25 years after conventional radiation doses of 42.5–66 Gy. Glioma of the optic nerve is rare and usually occurs in children or adolescents who are predisposed due to neurofibromatosis or Beckwith–Wiedemann syndrome.

The radiologic features vary with the tumor type. On CT scan, low-grade astrocytomas are low-density lesions that do not enhance with contrast; enhancement suggests malignant transformation, particularly if it is peripheral to a central hypodense area of necrosis, as seen in glioblastoma multiforme. On MRI, astrocytomas usually have high T2-weighted signal intensity.

The most common glioma in the sellar region is the pilocytic astrocytoma (Asa, 2011; Asa and Mete, 2016). Most common in children, hence the term juvenile type, this is a relatively discrete, often cystic mass with prominent enhancement on CT scan. These tumors usually arise in the wall of the third ventricle, where they displace and compress the optic chiasm; they may also arise in the optic nerve, usually at the chiasm. The low-grade lesions in children have a relatively good prognosis because they grow slowly, but chiasmal lesions can be more aggressive. In contrast, optic gliomas that occur sporadically in adults are usually rapidly fatal tumors.

Meningiomas

Meningiomas are tumors derived from the meninges and their derivatives in the meningeal spaces. They may arise from dural fibroblasts or pial cells, but the most common are of arachnoid origin. Approximately 20% of tumors of arachnoid and meningothelial cells occur in the sellar and parasellar area (Asa, 2011; Asa and Mete, 2016); they are most common in the sphenoid ridge and tuberculum sellae but also occur in the clivus. Occasionally, meningiomas arise in the sellar region following irradiation of the area for pituitary neuroendocrine tumor or craniopharyngioma. These tumors are more common in women than in men, possibly due to their expression of progesterone receptors and estrogen receptors.

Meningiomas are histologically diverse tumors. The more familiar types and the most common in the suprasellar hypothalamic area are the meningothelial, fibrous or fibroblastic, and transitional variants. Papillary patterns and anaplastic changes portend a more aggressive behavior and the possibility of metastases.

The management of these lesions is generally surgical resection. These highly vascular lesions can create problems with intraoperative bleeding.

Schwannomas

Tumors composed of spindle-shaped Schwann cells derive from cranial nerves; they are also known as neurilemmoma or neurinoma. Hypothalamic and parasellar schwannomas occur rarely (Asa, 2011; Asa and Mete, 2016). On CT scan, these lesions are visualized as sellar or parasellar masses that enhance with contrast and can mimic pituitary neuroendocrine tumor. They are hypovascular on angiography.

The morphology of these lesions in the pituitary region is identical to that of schwannomas elsewhere. These tumors are usually encapsulated and sometimes cystic lesions composed of spindle-shaped cells arranged in compact Antoni type A and loose Antoni type B areas. The former may exhibit palisading that leads to the formation of Verocay bodies. Immunohistochemical staining for S100 protein is strong in these lesions. Using electron microscopy, these tumors are recognized by the prominent basal lamina surrounding individual cells and by the characteristic "long-spacing" collagen of the stroma.

Similar to Schwannomas elsewhere, these lesions are usually benign and are amenable to surgical resection unless they involve critical structures in the parasellar region, in which case conservative surgical resection is indicated.

Chordomas

Chordomas are rare lesions thought to derive from remnants of the notochord. They occur in the midline, most often in the sacral region, but also in the region of the clivus and occasionally in vertebrae and within the sella turcica (Asa, 2011; Asa and Mete, 2016). These slowly growing but locally aggressive neoplasms usually occur in patients older than 30 years of age, but patients with the unusual sphenoid lesions tend to be younger.

Chordomas are usually lobulated, calcified, and expansile osteolytic lesions that may cause characteristic elevation of the periosteum, in which case they may be suspected on the basis of the radiologic findings. They have a characteristic gelatinous gross appearance and may have areas of calcification. Histologically, they are composed of large polyhedral cells with a characteristic bubbly or "physaliphorous" vacuolation. The tumor cells have immunohistochemical markers of epithelial differentiation, keratins, and EMA, as well as S100 protein and vimentin. Some exhibit positivity for carcinoembryonic antigen. High-grade sarcomatous areas may herald dedifferentiation and are sometimes seen in metastatic deposits. These aggressive variants are rare in intracranial chordomas. In contrast, the spheno-occipital region is the preferred site of the "chondroid" variant, which exhibits areas of cartilaginous differentiation that may dominate the histologic picture. These chondroid chordomas lack keratin and EMA positivity and are considered by some to be chondrosarcomas. This variant has a better prognosis than the usual clival chordomas.

Surgery is the preferred initial therapeutic approach; however, the location and extent of tumor may make complete extirpation impossible. Radiotherapy is indicated for incompletely resected lesions. Mean survival is approximately 4 or 5 years, and metastases to the lung, liver, bone, and lymph nodes occur rarely.

Vascular and Mesenchymal Tumors

These tumors arise from vessel walls, fibrous connective tissue and fat, bone, and cartilage. They are rare in this area and can be benign or malignant.

The development of sarcoma in this region may be sporadic but more commonly is the result of previous ionizing irradiation. There are several reports in the literature of osteosarcoma and fibrosarcoma of the sella developing 4–21 years after irradiation for pituitary adenohypophysial tumor or craniopharyngioma (Asa, 2011; Asa and Mete, 2016). These aggressive neoplasms result in the rapid demise of the patient.

Germ Cell Tumors

These tumors are derived from germ cells that are residual along the midline; they are identical to germ cell tumors of the gonads and mediastinum. Intracranial germ cell tumors represent $<1\%$ of intracranial neoplasms, but in children they constitute up to 6.5% of such lesions. After the pineal, the suprasellar region is the second most common site of involvement. These tumors occur most often before the age of 20 years and occur more often in males than in females.

The most common type, the germinoma, is usually a well-demarcated tumor that has high density on CT scan and enhances with contrast. Teratomas may exhibit fat densities and calcifications that are recognized radiologically. Whereas the CT appearance of some of the other germ cell tumors may not be distinctive, MRI is more sensitive and can show features that are obscured on CT scan.

The classification of parasellar and intrasellar germ cell tumors (Asa, 2011; Asa and Mete, 2016) includes teratomas, germinomas, embryonal carcinomas, endodermal sinus tumors, and choriocarcinomas. Germinomas and teratomas predominate, and mixed germ cell tumors that show features of more than one tumor subtype are frequent, usually germinoma combined with one of the other tumor types or a combination of embryonal carcinoma and immature teratoma, known as teratocarcinoma. Recognition of even minor components that are characterized by more aggressive elements can predict a worse prognosis and may alter management.

If the diagnosis is suspected preoperatively, biopsy can be performed to type the lesion and determine whether surgery or other modalities, such as radiation or chemotherapy, are indicated. Germinomas are uniquely radiosensitive and long-term remission is achieved in approximately 70% of patients. The recognition of more aggressive elements in mixed tumors can predict failure of radiotherapy. Other tumors are more aggressive, and despite surgery, radiation, and chemotherapy, they may recur or metastasize both within and beyond the central nervous system. Seeding via ventriculoperitoneal shunt may also result in dissemination.

Secondary Neoplasms

Pituitary Neuroendocrine Tumors

Pituitary adenohypophysial cell tumors, recently classified as "pituitary neuroendocrine tumors (PitNETs) (Asa *et al.*, 2017), are common lesions, occurring in 20%–27% of the population, and at least one-third of these give rise to clinical manifestations. Many of these tumors exhibit suprasellar or parasellar invasion involving the hypothalamus.

Metastatic Carcinoma

Cranial metastasis is a manifestation of a systemic and usually terminal malignancy (Fig. 4A). The most common primary sites of these lesions are the lung, breast, and gastrointestinal tract. Most hypothalamic metastases are not associated with specific clinical symptomatology and are therefore found incidentally at autopsy. Patients may present with diabetes insipidus, and large tumors may cause headache, visual field defects, ophthalmoplegia, and ptosis. Rarely is there an endocrine manifestation, usually resulting in hypopituitarism. A few reported cases have identified hormone excess, and usually the culprit is ectopic ACTH production in a location that mimics primary pituitary Cushing's disease. A fascinating case of CRH production by a hypothalamic metastasis of prostate small cell carcinoma resulted in pituitary corticotroph hyperplasia.

Since these patients have disseminated malignancy, therapy is aimed at palliation. Surgical decompression with or without radiotherapy can relieve symptoms.

Hematologic Neoplasms

Tumors of leukocytic, lymphocytic, or plasmacytic differentiation are usually systemic disorders. Very rarely, lesions in the region of the hypothalamus appear to be solitary and primary at that site (Asa, 2011; Asa and Mete, 2016). As in the central nervous system in general, lymphoma or leukemia is most often meningeal and extradural.

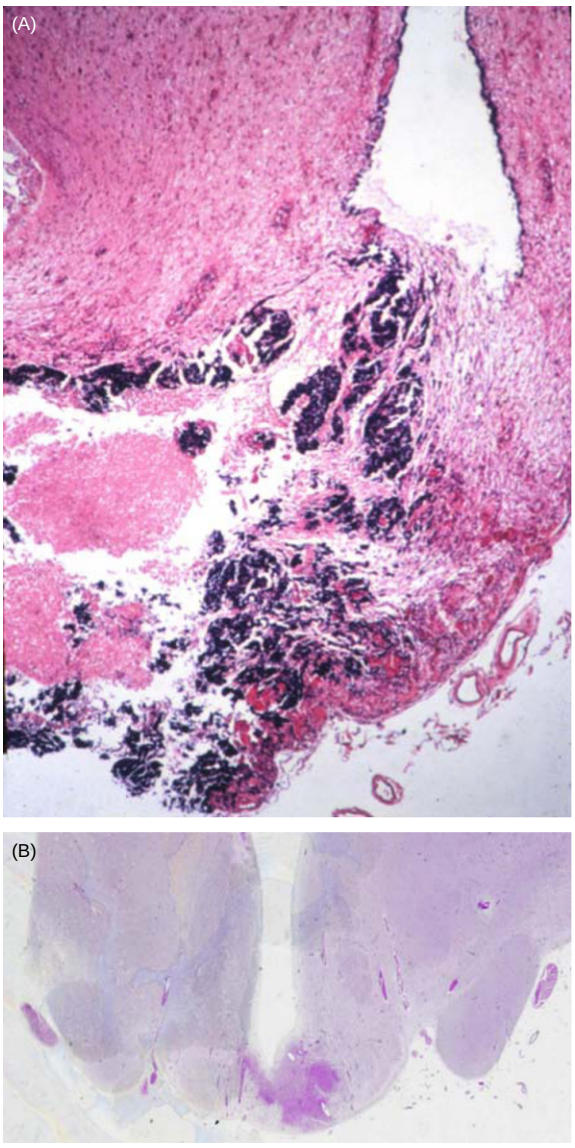

Fig. 4 Metastatic tumor. (A) This metastatic small cell lung carcinoma has replaced the arcuate nucleus and invades the infundibulum and superior pituitary stalk. (B) In this case of Langerhans cell histiocytosis, the lesion infiltrates and destroys normal hypothalamus.

Lymphomas are seen on MRI as masses with signals of similar intensity to those of their surroundings on T1-weighted images, but they are hyperintense on T2-weighted views. They enhance with contrast media and usually in a uniform pattern.

Histologically, the lymphomas of the central nervous system resemble systemic lymphomas. They are almost always non-Hodgkin lymphomas with a diffuse rather than follicular pattern; the majority are B cell tumors. Plasmacytomas are composed of well-differentiated plasma cells.

Langerhans cell histiocytosis includes the disorder formerly known as histiocytosis X, as well as eosinophilic granuloma, Hand–Schüller–Christian disease, Letterer–Siwe disease, Langerhans cell granuloma, and several other variants. This clonal proliferation of epithelioid histiocyte-like Langerhans cells may be localized, multifocal, or disseminated i. The classical presentation of Hand–Schüller–Christian disease involves the hypothalamus (**Fig. 4**B) and the disorder may involve the pituitary gland.

CT scans of Langerhans cell histiocytosis are characterized by ill-defined contrast-enhancing hypodense masses with areas of edema. MRI can detect the small multifocal lesions of this disorder more readily than CT scan; these lesions may be hypointense but can have slightly increased T1 contrast and intense T2-weighted images. Involvement of the pituitary stalk is characteristic of this disorder and may precede clinical manifestations of the disease.

Langerhans cells are characterized by an epithelioid, histiocyte-like appearance. They have kidney-shaped nuclei and abundant cytoplasm and stain for CD1 and S100 proteins. They are admixed with chronic inflammatory cells, foamy macrophages, and eosinophils. By electron microscopy, they contain pathognomonic Birbeck granules that allow a definitive diagnosis.

The prognosis of this disorder is variable. When systemic involvement is manifest, it is frequently lethal and apparently isolated lesions can progress rapidly to widely disseminated disease that is unresponsive to any form of therapeutic intervention. Surgery has been reported to be curative of localized lesions, and radiotherapy has been used postoperatively with success in cases of isolated involvement of this region.

Other rare hematologic neoplasms in this region include Rosai–Dorfman disease, Erdheim–Chester disease, hemophagocytic lymphohistiocytoses, and juvenile xanthogranuloma.

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Diabetes Insipidus☆

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Glossary

Gene A segment of DNA within the chromosome that contains all of the information required for the synthesis of a product. It is the individual functional unit of heredity responsible for inherited characteristics that distinguish one individual from another. Genes may be passed from parent to progeny in one of several different methods, including autosomal and X-linked modes of transmission, in dominant or recessive fashion.

Hypertonicity An increase in the "effective" osmotic pressure of body fluids above the normal range, general considered to be a function of the plasma osmolarity or plasma concentration of sodium and its anions.

Magnetic resonance imaging An imaging technique used to image internal structures of the body. It employs the influence of a large magnet to polarize hydrogen atoms within tissues and then monitors the summation of these dipoles within cells. It is a particularly useful tool for imaging soft tissues, such as the brain, spinal cord, joints, and abdominal structures.

Osmolarity The concentration of osmotically active particles expressed in terms of osmoles of solute per liter of solution. One osmole represents the molecular weight of the solute, in grams, divided by the number of particles into which it dissociates in solution.

Pituitary gland A small, oval-shaped endocrine gland situated at the base of the brain, in the fossa of the sphenoid bone. Its role is to coordinate and regulate growth and metabolism. It is divided into anterior and posterior portions, each responsible for the production of its own set of unique hormones.

Polyuria The passage of an abnormally high volume of urine within a given period of time. Pathologic conditions are typically in excess of 45 mL of urine per kilogram of body weight over a 24-h period.

Water intoxication A state of dilutional hyponatremia induced by an inability to effectively excrete free water. The resultant reduction in sodium concentration may be marked by lethargy, nausea, and mild mental status alterations, with severe cases progressing to convulsions, coma, and even death.

Introduction

Diabetes insipidus (DI) is a clinical syndrome characterized by the production of abnormally large volumes of dilute urine (polyuria). It is usually associated with symptoms of urinary frequency, nocturia (awakening to urinate), and/or enuresis (bedwetting) and is almost always accompanied by a commensurate increase in fluid intake (polydipsia) which may or may not be attributed to thirst. DI is customarily divided into four types based on fundamental differences in the cause. The most common type is due to a primary deficiency in pituitary secretion of the antidiuretic hormone, arginine vasopressin (AVP). It is referred to here as pituitary DI but may also be called neurohypophyseal, neurogenic, hypothalamic, central, or cranial DI. Another type of DI is due to a deficiency of AVP caused by increased degradation of the hormone by an enzyme made in the placenta. It differs from pituitary DI not only in its cause but also in its clinical setting, history, and prognosis. It is referred to here as gestational DI. The third type of DI results from insensitivity of the kidneys to the antidiuretic effect of AVP and is called nephrogenic DI. The fourth type of DI is due to suppression of AVP secretion by excessive fluid intake. It is generally referred to as primary polydipsia and can be subdivided into three subtypes depending on whether the polydipsia is due to abnormal thirst (dipsogenic), a cognitive defect (psychogenic), or treatment of other real or imagined health problems (iatrogenic). All four types of DI and the methods used to differentiate and manage them will be described.

Normal Regulation of Water Balance

Arginine vasopressin (AVP) is the chemical name of the antidiuretic hormone (AVP). It is a nonapeptide containing a six member disulfide ring and a three membered tail in which the terminal glycine is amidated (**Fig. 1**). It is produced and secreted by the neurohypophysis, a collection of large neurons that originate in the supraoptic and paraventricular nucleus of the hypothalamus and project downward to form the posterior pituitary (Swaab, 1993). AVP is produced in these neurons through a series of steps directed by a gene located on chromosome 20 (Schmale *et al.*, 1987). Initially, it is synthesized as part of a large protein that is

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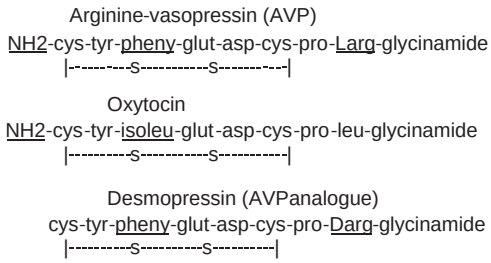

Fig. 1 Structure of vasopressin, oxytocin, and desmopressin, a synthetic analogue of vasopressin with enhanced antidiuretic and diminished pressor effects.

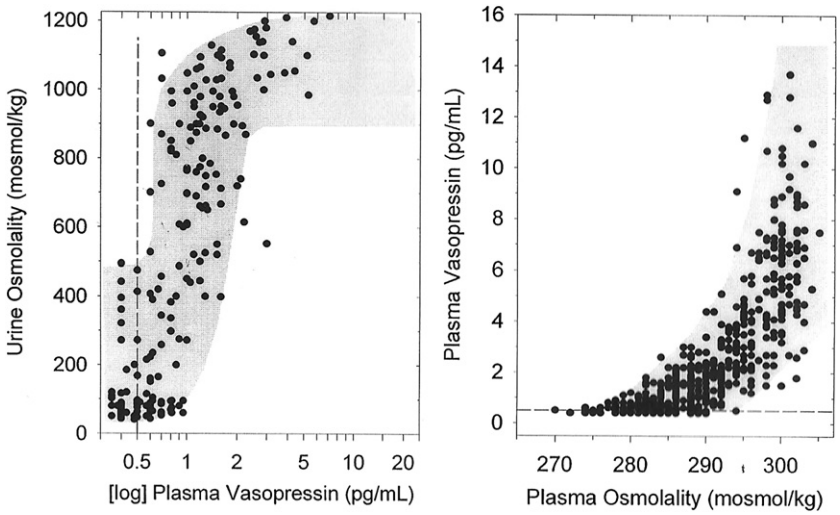

Fig. 2 The relation of plasma AVP to plasma osmolarity (*right panel*) and to urine osmnolarity (*left panel*) in healthy adults. Modified from Robertson, G.L. (2013). *Thirst and Vasopressin in Seldin and Giebisch's The Kidney*, 5th edn., vol. 1, Alpern, R. W., Caplan, M. J., Moe, O. W. eds. Elsevier Inc, p. 1445.

folded and packaged in vesicles for transport down the axon. Within these vesicles, the precursor protein is cleaved to yield AVP and two other components, neurophysin-2 and co-peptin, all of which are stored together in bulbous nerve terminals until they are co-secreted into the circulatory system. Oxytocin is produced in other magnocellular neurons of the neurohypophysis, is secreted in response to different stimuli than AVP, and has little if any antidiuretic effect.

The measurement of AVP in plasma requires a radioimmunoassay of extraordinary sensitivity and specificity. Under physiologic conditions, it circulates at a concentration of only 1–3 pg/mL, is structurally similar to oxytocin and accompanied by large amounts of other, unidentified immuno-reactive material that does not come from the posterior pituitary and must be removed before assay. Until these problems were solved, physiologic and pathophysiologic data were limited and measurements for the purpose of differential diagnosis were impractical if not impossible. Therefore, indirect estimates using measurements of urine osmolarity in response to various procedures and treatments were widely employed. About 30 years ago, a relatively simple, sensitive, and specific radio-immunoassay was developed and used to better define the physiology and pathophysiology of AVP secretion and action (Robertson *et al.*, 1973). These findings have both confirmed and altered several widely held concepts and provided new insights and more accurate methods of differential diagnosis in several disorders of AVP function including DI.

In healthy adults, AVP secretion is regulated primarily by the effective osmotic pressure of extracellular fluid (**Fig. 2**, right panel) (Robertson, 1977; Robertson and Christensen, 2012). This variable, referred to as osmolarity, is determined by the concentration of solutes that do not readily cross cell membranes. In healthy humans, these solutes are principally sodium and its anions. The plasma and urine osmolarities usually measured by depression of the freezing point or vapor pressure. The measurements in plasma reflect the concentration not only of sodium and its anions but also those of other solutes such as glucose and urea. The latter normally do not contribute to effective osmotic pressure across cell membranes but their effect on the measurements of plasma osmolarity is customarily ignored because it is small and relatively constant from sample to sample. The effective osmotic pressure of plasma can also be estimated by doubling the molar concentration of sodium and the values so obtained correlate closely with measurements of plasma osmolarity unless other solutes such as glucose or urea are abnormally elevated.

The influence of plasma osmolarity/sodium on AVP secretion is mediated by specialized sensory cells known as osmoreceptors that are thought to be located in the hypothalamus near the supraoptic nucleus. They are capable of detecting minute changes in the plasma concentration of sodium, chloride and certain other exogenous solutes, such as mannitol, which do not readily cross cell membranes. The level of plasma osmolarity/sodium at which AVP secretion begins is known as the osmotic threshold or set point. It differs slightly among healthy adults, ranging from a plasma osmolarity of 270–290 mOsm/L and of sodium from 135 to 145 mEq/L (**Fig. 2**, right panel).

These small individual differences in set-point are reproducible and appear to be due to genetic influences. However, the set-points are also lowered by pregnancy, menstruation, and large decreases in blood volume or pressure. When plasma osmolarity rises above the threshold or set-point, AVP secretion also rises in direct proportion (**Fig. 2**, right panel). The magnitude of the rise in plasma AVP per unit increment in plasma osmolarity/sodium varies as much as fivefold between healthy individuals and these variations also appear to be genetically determined. However, like the set-point, the sensitivity can also be altered by large changes in blood volume or blood pressure.

The effect of blood volume and pressure on AVP secretion is mediated via specialized sensors known as baroreceptors located in the heart, aorta, and carotid arteries. These hemodynamic variables have little or no detectable effect on AVP secretion until blood volume and/or pressure fall by more than 10%–20% at which point plasma AVP begins to rise exponentially. However, hemodynamic stimuli do not abolish the osmoregulation of AVP. Rather, they appear to lower the threshold and/or increase the magnitude of the response to a given osmotic stimulus. Other nonosmotic stimuli, such as nausea, cortisol deficiency, and a rapid fall in blood sugar, may also increase AVP release. Nausea is one of the most powerful stimuli, resulting in 50- to 100-fold increases in plasma AVP. The potent effect of nausea requires special attention to symptoms in all studies of AVP function since even a mild, transient vasovagal reaction induced by the simple act of drawing blood from an anxious subject can result in massive release of the hormone that elevates plasma levels for hours.

The principal if not the only important action of AVP in humans is to reduce the rate of urinary water excretion by increasing its concentration (**Fig. 2**, left panel). The increase in urine osmolarity is achieved by selectively promoting the reabsorption of water from dilute filtrate as it passes through the collecting ducts of the kidney (**Fig. 3**) (Bichet, 2012).

When plasma AVP is very low, typically less than 0.5 pg /mL, the luminal membranes of the cells lining the collecting ducts are impermeable to water. Hence, the large volume of dilute filtrate that is not reabsorbed in the proximal nephron flows unmodified through the collecting ducts to be excreted as urine. In this condition, known as a water diuresis, the volume of urine output is very high (500–1000 mL/h depending on the solute load) and its concentration or osmolarity is very low (30–60 mOsm/L). At these rates of water excretion, it is nearly impossible to drink and retain enough water to reduce the osmolarity of body fluids below the normal range. Hence, the capacity to suppress AVP secretion and maximally dilute the urine provides a powerful barrier to over-hydration. When plasma AVP rises, it binds to receptors, designated AVPR-2, located on the serosal surface of cells lining the collecting ducts (**Fig. 3**). This binding increases the production of a substance known as cyclic AMP which then stimulates the

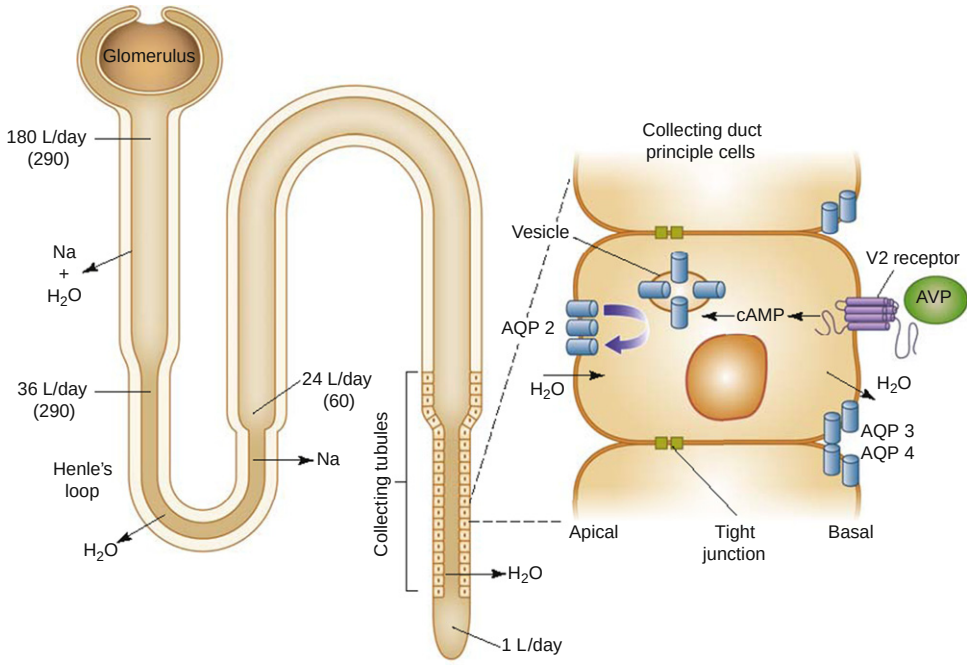

Fig. 3 Schematic diagram of a renal tubule showing the formation of urine by filtration of blood and reabsorption of water and solutes at different parts including the principle cells in the collecting tubules where AVP acts to regulate the rate of water reabsorption (Hay, 1996; Knepper, 1997; Bichet, 2012).

translocation and production of a protein, aquaporin-2 (AQP2), the production of which is directed by a gene on chromosome 12q13. The molecules of AQP2 associate into groups of four to form water channels that fuse with membranes on the side of the cell facing the lumen of the collecting duct. The insertion of these channels permits water to passively diffuse back from the lumen through the cell into the hypertonic environment of the renal medulla and thence back into the body. The fluid that remains in the collecting duct is excreted as urine that is more concentrated and smaller in volume than in the absence of AVP. AVP also acts via AVPR2 to stimulate readsorption of urea in the terminal inner medullary collecting ducts, thereby increasing the magnitude of urine concentration by enhancing the hypertonicity of the renal medulla. The magnitude of this antidiuretic effect varies as a function of plasma AVP (**Fig. 2**, left panel). In healthy adults, plasma AVP concentrations in the range of 2–4 pg/mL raise urine osmolarity to levels ranging from 900 to 1400 mOsm/L and reduce urine flow to less than 50 mL/h, the maximum levels of antidiuresis permitted by the hypertonicity of the renal medulla. This capability serves to minimize the amount of body water that must be excreted to eliminate solutes such as sodium, chloride, and urea. However, it does not completely prevent water loss from the kidneys and has no effect on evaporation from skin or lungs. Hence, some other mechanism to ensure replacement of these obligatory water losses is essential.

Thirst

In health humans, thirst is the principal mechanism for ensuring that water intake is always sufficient to replace obligatory losses from kidneys, skin, and lungs. It is regulated by hypothalamic osmoreceptors in much the same way as AVP. The only difference is that the osmotic threshold for thirst seems to be "set" slightly higher (5–15 mOsm/L) than that for AVP release. Thus, in a healthy adult, thirst is usually not stimulated until plasma osmolarity rises to a level at which AVP secretion is sufficient to produce a maximum antidiuresis. This arrangement ensures that the antidiuretic mechanism serves as the first line of defense against hypertonic dehydration and thirst provides essential backup protection that comes into play whenever the water ingested with food and provided by fat metabolism is insufficient to replace minimum obligatory losses. The thirst mechanism is normally so effective in this regard that, if an adequate supply of potable water is available, it increases intake sufficiently to keep plasma osmolarity and sodium within the normal range even when urinary output is abnormally high due to a defect in AVP secretion or action.

Pathophysiology

Pituitary Diabetes Insipidus

When the capacity of the posterior pituitary to secrete AVP is reduced by 80% or more, urinary osmolarity falls below that of plasma and the rate of excretion rises. The polyuria, in turn, reduces body water and raises plasma osmolarity and sodium thereby stimulating thirst and an increase in fluid intake that compensates for the loss of body water and keeps plasma osmolarity/sodium within the normal range (Christensen and Robertson, 2012). However, if anything intervenes to prevent the increase in fluid intake for even a few hours, plasma osmolarity and sodium rise, delivering a stronger osmotic stimulus for both thirst and AVP release. If the loss of secretory capacity is severe (greater than 90%), the increase in osmotic stimulation has little or no effect on plasma AVP or urine osmolarity. Hence, the polyuria persists, the deficit in body water increases and plasma osmolarity and sodium rise to abnormally high levels. However, if the reduction in secretory capacity is less severe, the increase in osmotic stimulation caused by fluid deprivation produces a rise in plasma AVP that is subnormal but still sufficient to concentrate the urine, sometimes to near maximum levels. This condition, known as partial pituitary DI, is difficult to distinguish from primary polydipsia and partial nephrogenic DI by traditional fluid deprivation tests. Hence, some other approach to differential diagnosis is needed (see below).

The causes of pituitary DI are numerous, varied and often undetermined (**Table 1**). About half are attributable to acquired, congenital or genetic disorders. The acquired forms are usually due to trauma or diseases involving the brain and occur at any age. The congenital forms are due to various brain malformations that result in hypogenesis of the posterior pituitary and DI from birth. The genetic forms can be transmitted in an autosomal dominant, autosomal recessive or X-linked recessive manner. The autosomal dominant form is the most common. It is due to mutations of the AVP–neurophysin-2 gene that interfere with processing of the precursor causing it to accumulate and eventually destroy the neuron. The DI develops gradually, usually a few months or years after birth but sometimes later. The autosomal recessive forms appear to be rare. One is due to a mutation in AVP moiety of the precursor that results in production of a biologically inactive form of the hormone. In the homozygous state, it causes pituitary DI that manifests early in life. Two other forms are due to mutations in the AVP–neurophysin-2 precursor that interfere with its production and cause early onset DI in the homozygous state. Pituitary DI also develops at some stage in many patients with Wolfram syndrome, an inherited disorder commonly known as DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, deafness, and other degenerative neurological disorders). It is caused by recessive mutations in the WFS1 gene on chromosome 4. One family in which pituitary DI is inherited in an X-linked recessive manner has also been reported in preliminary form. It causes DI in males but not in obligate female carriers. The mutation responsible co-segregates with markers for Xq28 but has not yet been identified. In the remaining patients, almost half, the cause of the pituitary cannot be determined and they are classified as idiopathic.

Table 1 Causes of pituitary DI

Acquired
Head trauma (closed and penetrating)
Neoplasm
Primary
Craniopharyngioma, adenoma, dysgerminoma, meningioma)
Metastatic
Lung, breast
Hematologic
Lymphoma, leukemia
Granuloma
Sarcoidosis, histiocytosis
Infectious
Chronic meningitis, viral encephalitis, toxoplasmosis
Inflammatory
Lymphocytic infundibuloneurohypophysitis, Wegener's granulomatosis, lupus erythematosis, scleroderma
Vascular
Sheehan syndrome, aneurysm (internal carotid), infarction
Congenital malformations
Septo-optic dysplasia, midline craniofacial defects
Holoprosencephaly
Hypogenesis, ectopic pituitary
Idiopathic
Genetic
Autosomal dominant
Neurotoxic mutations of AVP–neurophysin gene
Autosomal recessive
Inactivating mutation of AVP
Nonsynthesis of AVP-NP
WFS1 (DIDMOAD)
X-linked recessive
Mechanism unknown

Gestational Diabetes Insipidus

A decline in plasma AVP due to increased degradation of the hormone by an enzyme produced in the placenta results in a series of changes similar to those in pituitary DI (Durr, 1987; Barron, 1987). The laboratory findings differ only in that plasma osmolarity/sodium tends to be 1%–2% lower than in pituitary DI because the osmotic thresholds for thirst and AVP secretion are also reduced during pregnancy. The natural history differs from that in a pregnant patient with preexisting pituitary DI in that gestational DI begins during pregnancy, usually in the second trimester, and remits spontaneously 4–6 weeks after delivery of the placenta. There may also be a form of gestational DI that is caused by renal resistance to the antidiuretic effect of AVP during pregnancy. Differentiating between the two forms may be difficult. Assay of plasma AVP is problematic because pregnant plasma contains the enzyme that rapidly degrades the hormone in vitro, necessitating special precautions in sample collection. Evaluating the antidiuretic effect of a therapeutic trial of AVP is unreliable for the same reason. However, a therapeutic trial of standard doses of desmopressin, a synthetic analogue of vasopressin, may differentiate because it is more resistant than AVP itself to degradation by the enzyme. Thus, it is fully effective in controlling the DI if is due solely to increased degradation of the natural hormone but may be less effective if decreased renal sensitivity is the cause.

Although the primary cause of gestational DI is increased destruction of AVP by an enzyme made in the placenta, an underlying subclinical defect in secretory capacity appears to contribute in some patients. In at least one of these patients, the subclinical deficiency in AVP secretion was attributed to damage caused by prior treatment for a prolactinoma of the anterior pituitary.

Nephrogenic Diabetes Insipidus

Decreased renal sensitivity to the antidiuretic effect of AVP results in a series of changes similar to those in pituitary DI (Bockenhauer and Bichet, 2015). Urine osmolarity falls and the rate of excretion increases resulting in a decrease in body water and a slight rise in plasma osmolarity/sodium. The latter, in turn stimulates thirst which, if an unlimited supply of potable water is available, produces an increase in water intake that prevents overt dehydration. The main difference is that, in nephrogenic DI, plasma AVP is normal or elevated instead of low or undetectable relative to the concurrent level of plasma osmolarity/sodium. If fluid intake is restricted, body water decreases, and plasma osmolarity/sodium rise providing an even stronger stimulus to thirst and AVP secretion. The effect of the further rise in plasma AVP varies widely, depending on the

Table 2 Causes of nephrogenic DI

Acquired
Drugs
Lithium
Demeclocycline
Methoxyflurane
Amphotericin B
Aminoglycosides
Cisplatin
Rifampin
Foscarnet
Metabolic disorders
Hypercalcemia, hypercalciuria
Hypokalemia
Urologic
Postobstruction of ureter or urethra
Hematologic
Sickle cell disease or trait
Granulomas
Sarcoidosis
Neoplastic
Sarcoma
Infiltrative
Amyloidosis
Genetic
X-linked recessive
Mutations of AVPR2 receptor gene in chromosome Xq28
Autosomal recessive
Mutations of AQP2 gene on chromosome 12q13
Autosomal dominant
Mutations of AQP2 gene on chromosome 12q13

severity of the defect in renal sensitivity. If it is severe, the large increases in plasma AVP produced by fluid restriction have little or no effect on urine concentration. Hence, the deficit in body water increases and plasma osmolarity/sodium rise to abnormally high levels that vary depending on the duration of the restriction on intake. However, if the reduction in renal sensitivity to AVP is less severe, the rise in plasma AVP produced by fluid deprivation results in concentration of the urine, sometimes to near maximum levels. This condition, known as partial nephrogenic DI, is difficult if not impossible to distinguish from partial pituitary DI and primary polydipsia DI by traditional fluid deprivation tests. Hence, some other approach to differential diagnosis including assay of plasma AVP and magnetic resonance imaging (MRI) of brain is usually necessary (see below).

The causes of nephrogenic DI are also numerous but distinctly different from those of pituitary DI (**Table 2**).

The acquired forms of nephrogenic DI can occur at any age, are often caused by drugs or a metabolic disorder, vary in severity and may remit if the offending drug or condition is eliminated. The genetic forms manifest at birth, are caused by many different mutations of the genes that encode the AVP2 receptor or the AQP2 water channels and rarely if ever remit. However, the severity of the DI sometimes declines with aging. Over 200 different AVPR2 gene mutations have been identified. They usually occur in the trans-membranous portion of the receptor protein, impair receptor function by a number of different mechanisms and produce either a severe or partial defect in the antidiuretic response to AVP. The recessive AQP2 mutations also tend to occur in the trans-membranous portion of the protein. They impair the ability to join with other molecules to form water channels and move to the surface of the cell. They almost invariably result in a severe defect in antidiuresis. The dominant AQP2 mutations, on the other hand, alter the C-terminal portion of the protein causing most of the water channels to be misrouted to the wrong surface of the principal cell resulting in a less severe and partial defect in antidiuretic function.

Primary Polydipsia

Abnormal thirst or any other motivation for increased fluid intake produces a sequence of changes that are the reverse of those in pituitary or nephrogenic DI. The increase in fluid intake expands body water, slightly reduces plasma osmolarity/sodium, and suppresses AVP secretion to low levels (<0.1 pg/mL) indistinguishable from those in pituitary DI. The suppression of AVP, in turn, reduces urine concentration and increases urinary water excretion, thereby compensating for the increased water intake. The clinical picture differs from pituitary and nephrogenic DI only in that basal plasma osmolarity/sodium tends to be slightly lower in primary polydipsia but the overlap between the three disorders is considerable owing to individual differences in the set and

sensitivity of the osmoregulatory system. The only exception occurs in severe psychogenic polydipsia in which the rate of water intake is often so enormous that the kidneys cannot keep pace and frank hyponatremia develops. Administering desmopressin or AVP to a patient with primary polydipsia also produces hyponatremia making it very important to differentiate between the three disorders before embarking on treatment.

There are three causes or types of primary polydipsia. One is psychogenic (Barlow and De Wardener, 1959). It does not appear to be due to abnormal thirst but to a psychological or cognitive defect such as schizophrenia or obsessive compulsion. A second type, possibly related, may be termed iatrogenic since it seems to be motivated by a belief, wide spread on the internet, that a high fluid intake improves health. The third type of primary polydipsia appears to be motivated by abnormal thirst (Robertson, 1987). It is termed dipsogenic DI and has been observed in association with head trauma, lithium treatment, or certain acquired diseases of the brain including sarcoidosis, tuberculous meningitis, and multiple sclerosis. Like pituitary DI, dipsogenic DI is often idiopathic. Genetic forms have not been reported.

Diagnosis

When symptoms or signs such as urinary frequency, enuresis, nocturia, or thirst suggest the possibility of DI, the diagnosis should be confirmed before embarking on studies to determine the type. This cannot be done reliably from a single voided urine sample because false positive or negative results are common owing to large differences in the severity of the antidiuretic defects and other variables that impact urine production in the course of a normal day. Therefore, it is best to collect, pool, and test all the urine excreted over a 24 h period when the food and fluid intake are unrestricted. If the collection has a volume greater than 40 mL/kg body weight, an osmolarity of less than 300 mOsm/L and does not contain glucose, the diagnosis of DI is established and the evaluation should continue to determine the type. For this purpose, inquiries about the duration of symptoms as well as any injuries, disorders, or treatments prior to the onset of DI and a family history is often helpful. Plasma osmolarity and/or sodium should be measured to detect the rare patient with severe dehydration or over-hydration. In most cases, however, they are of little help in differential diagnosis because the values found in pituitary DI, nephrogenic DI, and primary polydipsia overlap extensively within the normal range.

Except in the rare patient who presents with hypernatremia under basal conditions, the traditional method of differential diagnosis is a fluid deprivation test. There are several versions of this procedure but the basic principle in all of them is to determine if with-holding fluids results in concentration of the urine *before* plasma osmolarity/sodium rise above the normal range. If it does not, primary polydipsia is excluded and AVP or its analogue desmopressin can be given to determine if it results in concentration of the urine. If it does, the DI is due to a severe deficiency in production of the hormone (pituitary DI). If it does not, the DI is due to severe defect in the antidiuretic effect of the hormone (nephrogenic DI). This test is simple in concept but has certain difficulties in practice that limit if not discourage its use. One is the need for constant close monitoring by well-trained medical, nursing, and laboratory staff for periods of 3–6 h or even longer. Another is the often intense discomfort of the patient due to thirst. But the most significant problem with the procedure is that the findings cannot be interpreted reliably if fluid deprivation alone results in concentration of the urine. That result, which is relatively common, is consistent with either primary polydipsia, partial pituitary DI, or partial nephrogenic DI. Some differentiation between them is possible if low doses of AVP or desmopressin are given and the percent increase in urine osmolarity is considered. Often, however, the results still not correlate well with other evidence or the response to therapy.

The development of a sensitive and specific assay for plasma vasopressin made it possible to differentiate more reliably between primary polydipsia, partial pituitary DI, and partial nephrogenic DI. When used in conjunction with the fluid deprivation/hypertonic saline infusion test, plotting the plasma AVP assay values in relation to the concurrent plasma and urine osmolarity permits a clear distinction between the three types of DI (**Fig. 4**).

For best results, the measurements of plasma AVP should be made when plasma osmolarity/sodium is raised above the normal range. Since this level of hypertonicity is often difficult to achieve by fluid deprivation alone when urinary concentration occurs, it is usually necessary to follow it with an infusion of 3% saline (0.1 mL/kg/min for 60–90 min) and repeat the AVP assay when plasma osmolarity/sodium reach the requisite levels at or above 295 mOsm/L and 145 mEq/L. While this method permits reliable differentiation between primary polydipsia, partial pituitary DII, and partial nephrogenic DI, it is even more involved and demanding of the time of trained staff, especially when the infusion of 3% saline is required. It also requires an AVP assay of extraordinary sensitivity and specificity. These requirements have severely limited the availability of an immunoassay suitable for diagnostic purposes. Therefore, a simpler much less demanding method of differential diagnosis is required if it is to be employed more widely in clinical medicine.

A new, much easier but equally reliable method of differential diagnosis has evolved in recent years (Robertson, 2016). It also entails the assay of plasma AVP but the requirements for sensitivity and specificity are slightly less stringent, the sample can be collected almost anytime under basal conditions of ad libitum fluid intake and the results can be interpreted with reference only to the concurrent urine osmolarity. If plasma AVP is clearly normal or elevated (≥ 1 pg/mL) when urine osmolarity is low (<300 mOsm/L), pituitary DI, and primary polydipsia are excluded and the cause of the DI is renal insensitivity to the antidiuretic action of the hormone (nephrogenic DI). No other diagnostic procedure is required other than a search for the cause. However, if plasma AVP is undetectable (<0.5–1 pg/mL), nephrogenic DI is very unlikely and an MRI of the brain without infusion can determine if the DI is due to a primary defect in the production of AVP (pituitary DI) or suppression of AVP secretion by excessive

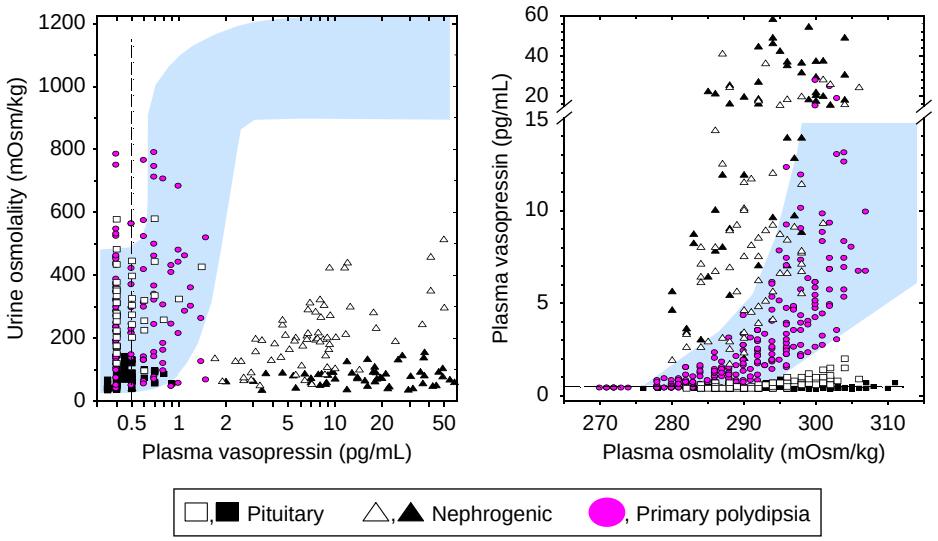

Fig. 4 Relationship of plasma vasopressin to the concurrent plasma osmolarity (right) and urine osmolarity (left) in healthy adults (*blue shaded* areas) and three types of DI. Plasma osmolarity was raised above 295 mOsm/L by fluid deprivation followed, when necessary, by infusion of hypertonic saline for 60–90 min. *Open* and *closed squares* indicate values in patients with severe and partial pituitary DI, respectively. *Open* and *closed triangles* indicate severe and partial nephrogenic DI. *Purple symbols* represent patients with primary polydipsia.

intake of fluids (primary polydipsia). Presence of the hyperintense signal (bright spot) normally emitted by the posterior pituitary on T1 weighted mid-saggital images indicates the gland is intact and the low plasma AVP levels are due to suppression by excessive fluid intake (primary polydipsia). On the other hand, absence or diminution of the bright spot indicates the low plasma AVP levels are due to impaired production of the hormone (pituitary DI). The posterior pituitary bright spot is also absent or feint in nephrogenic DI, probably as a result of hypersecretion and reduced stores of AVP. However, it is easily distinguished by elevated levels of plasma AVP under basal conditions. There are two potential sources of error in this method of differential diagnosis. One is confusion of the bright spot with the signal emitted by fat in the marrow of the dorsum sella. This problem can be reduced by use of fat suppression techniques with the MRI. The other potential problem is empty sella. It is usually associated with small or absent bright spot even if AVP secretion is normal. Therefore, if a patient with DI has empty sella, the functional state of the posterior pituitary cannot be reliably assessed by MRI and definitive diagnosis may require another approach such as repeat measurement of plasma AVP after a suitable stimulus such as hypertonic saline infusion.

As noted above, ordinary measurements of plasma AVP cannot be used to determine the mechanism of gestational DI owing to the presence of enzymes that rapidly destroy the hormone during sample collection. In this setting, assessing the antidiuretic effect of standard therapeutic doses of the AVP analogue, desmopressin, may suffice to differentiate renal insensitivity from a deficiency of the hormone.

Management

The first objective in the management of DI is to eliminate or at least reduce the symptoms of polyuria and polydipsia. In pituitary and gestational DI, this can be achieved by treatment with desmopressin acetate (dDAVP, Ferring), a synthetic analogue of AVP that is more resistant to degradation and has less vascular and other smooth muscle effects than the native hormone (Oisoi *et al.*, 2013). It is formulated for administration orally (po), intranasally (in), subcutaneously (sq), intramuscularly (im), or intravenously (iv). The optimum doses of desmopressin vary considerably due to individual differences in bioavailability and elimination. In an adult, they range from 50 to 200 mg po, 5–20 mg in, or 1–2 mg iv or sq. two to three times per day as needed to maintain the 24 h urine osmolarity and volume within the normal range–that is, from 400 to 800 mOsm/L and 15–30 mL/kg body weight. The resultant decrease in water excretion produces a small, 1%–2% increase in body water and a commensurate decrease in plasma osmolarity/sodium that reduces thirst and water intake, thereby maintaining water balance. Plasma osmolarity/sodium should be checked within a day or two of starting treatment and periodically thereafter to verify that they remain within the normal range. Desmopressin should not be given in a dose large enough to produce maximum antidiuresis "around the clock" because it may fix urine output below the level required to excrete all the water consumed in a normal diet even when thirst is completely suppressed. For the same reason, during desmopressin therapy, fluid intake should be limited to the amounts needed to satisfy thirst. Drinking for social or other reasons should be avoided because an increase in body water does not produce a compensatory increase in urine output when the level of antidiuresis is determined solely by the dose and frequency of desmopressin treatment. If following these guidelines does not prevent recurrent episodes of hypoosmolar hyponatremia,

consideration should be given to discontinuing therapy and undertaking more detailed diagnostic studies to rule out some form of primary polydipsia.

The treatment of nephrogenic DI differs from that of pituitary and gestational DI. In 5- to 10-fold higher doses, desmopressin may be effective if the insensitivity to AVP is partial as it is in many patients. However, the expense and inconvenience of this approach to therapy makes it impractical for long term use. When nephrogenic DI is caused by exposure to a drug such as lithium or demeclocycline or to a metabolic disorder such as hypercalcemia or hypokalemia, eliminating the drug or correcting the imbalance may also eliminate the defect at least if it has been present for no more than a year or two. If nephrogenic DI is of longer duration or is due to genetic mutation, other treatments may be used to reduce if not eliminate the polyuria, thirst, and polydipsia. In standard doses, thiazide diuretics have a paradoxical effect to increase urine osmolarity and reduce urine output and fluid intake in nephrogenic DI. This effect is the result of two related mechanisms; interference with the readsorption of sodium in the ascending limb of Henle's loop, which reduces urinary dilution followed by a compensatory increase in sodium reabsorption in the proximal tubule, which reduces urine volume by about 50%. The thiazides also produce potassium loss and hypokalemia which itself produces nephrogenic DI but this effect can be avoided by giving potassium supplements with the diuretics. Prostaglandin synthetase inhibitors such as indomethacin also reduce polyuria and polydipsia in nephrogenic DI. When given with a thiazide diuretic, the combination usually reduces urine output by about 75% and in some cases normalizes it completely. As in pituitary DI, reducing polyuria in nephrogenic DI also reduces the slight deficiency in body water, thereby decreasing the osmotic stimulus to thirst and polydipsia and keeping water balance within the normal range. Hyponatremia is rare.

The treatment of primary polydipsia differs completely from that of pituitary, gestational and nephrogenic DI. Desmopressin or any other medication that reduces urine output is contraindicated because these drugs have little or no effect on the excessive water intake that is the cause of the disorder. Consequently, reducing water excretion produces water intoxication, a syndrome that manifests as hyponatremia accompanied by symptoms and signs of confusion, headache, nausea, vomiting, coma, convulsions, and even death. If the polydipsia is motivated by a belief in the health giving value of drinking more water (iatrogenic), it can sometimes be cured by patient education. However, there is no documented way to reduce the polydipsia if it is motivated by abnormal thirst (dipsogenic) or a psychiatric disorder (psychogenic). In these conditions, the only help that can be provided is to try to minimize the risk of severe water intoxication by warning the patient and/or caregivers of the many drugs and diseases that can induce it and of the early signs and symptoms that should prompt them to seek immediate medical care.

The other objective in the management of DI is to try to determine the cause. In many cases it is obvious from the clinical setting but in others it can be ambiguous or misleading. For example, head trauma or certain infiltrative diseases of the brain can be associated with either pituitary DI or dipsogenic DI and lithium therapy can cause either nephrogenic DI or dipsogenic DI. Therefore, a careful search including a family history, physical examination, routine laboratory tests and, depending on the type of DI, an MRI of the brain should be undertaken. If these investigations are unrevealing, appropria1te genetic analyses should be undertaken, particularly if the DI dates from birth or the first few years of life, because absence of a family history does not rule out a recessive or de novo mutation.

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Growth Hormone (GH)☆

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Glossary

ADAM Acronym for "a disintegrin and metalloproteinase"; designates a family of zinc-dependent metalloproteinases involved in proteolytic modulation at the cell surface and in the extracellular matrix.
Growth hormone (GH) A pituitary hormone important for somatic growth, development, metabolism, and body composition.
Insulin-like growth factor 1 (IGF-1) Mediator of many actions of growth hormone.
Pituitary gland A master hormone gland located at the base of the brain and serving as the interface between the brain and peripheral hormone glands.
Somatotropin Synonym for growth hormone.
TACE A membrane-resident protease, also known as ADAM 17, cleaves ectodomains from several transmembrane proteins.

Introduction

Growth hormone (GH) is a pituitary hormone that serves as the master anabolic hormone during postnatal life. It is also known as somatotropin, perhaps a better name since GH has many actions beyond the promotion of body growth. GH is evolutionarily conserved from shark to humans. It is a polypeptide hormone whose primary structure varies among species while maintaining an overall physical similarity. A special feature is the so-called one-way species specificity, a term indicating that primate GH is biologically active in most if not all vertebrates, whereas nonprimate GH is biologically inert in primates, including humans. Minor differences in biopotency exist among the GHs of other species. Because description and discussion of all the chordate GHs are beyond the scope of this article, the principal focus is on human GH (hGH), one of the best studied GHs. A treatise on hGH is applicable to many other GHs because GH biology and action is similar among species despite structural differences in the hormone. Where applicable, important species differences are discussed.

The hGH Gene Cluster

In humans, there are two GH genes, *GH1* and *GH2*, located on the long arm of chromosome 17 (17q22–q24) (Baumann, 2009, 1991). They form part of a cluster of GH-like genes thought to arise by gene duplication that also includes two chorionic somatomammotropin (*CS* or *PL* [placental lactogen]) genes and a *CS* pseudogene. This 67-kb gene cluster consists of *GH1*, *CSHL1*, *CSH1*, *GH2*, and *CSH2* in a 5′ to 3′ orientation (*GH1* is also known as *GH-N*, and *GH2* is also known as *GH-V*). Each of these genes is composed of five exons and four intervening sequences. *GH1* is expressed in the pituitary (somatotrope cells), and the other members of the gene cluster are expressed in the placenta (syncytiotrophoblast). Thus, GH2 is also called placental GH. The intergenic DNA regions between the members of the cluster are highly homologous, rendering them vulnerable to gene deletions due to recombination.

GH Structure

Mature hGH, in its principal molecular form, is a 22-kDa, 191-amino acid, single-chain polypeptide with two intramolecular disulfide bridges. Its tertiary structure is that of a twisted bundle of four α-helices akin to the overall structure of many cytokines. GH contains two distinct receptor-binding epitopes (site 1 and site 2) allowing it to interact with the extracellular region of two GH receptors (GHR) in a dimerized complex (Gent *et al.*, 2003, 2002).

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GH Isoforms

Several molecular forms of hGH exist (Baumann, 2009). The most abundant form (except during pregnancy) is pituitary GH1, also known as GH-N or 22-kDa GH. GH2 (also known as GH-V or placental GH) differs from pituitary GH1 at 13 of the 191 amino acid residues. In addition, GH2 contains an N-linked glycosylation site, and indeed, GH2 exists as both a glycosylated and a nonglycosylated form. During pregnancy, placental GH progressively supplants pituitary GH in the maternal circulation. The *GH-1* gene also generates an internally deleted GH (lacking 15 internal amino acids and named 20-kDa GH) through alternative mRNA splicing. A secondary or cryptic splice acceptor site within exon 3 is used for that purpose. This 20-kDa variant accounts for 5%–10% of GH production. A second postulated splice variant arises from complete exon 3 skipping, owing to the relatively weak splice donor site in intron 3. Although the mRNA for this so-called 17.5-kDa GH has been demonstrated, it is not entirely clear whether the corresponding protein is produced in significant amounts in normal individuals. Additional GH molecular variants arise from posttranslational processing. These include glycosylated (especially placental GH), acetylated, deamidated, and (in some animals) phosphorylated GHs. Further molecular heterogeneity results from oligomeric GH forms (up to at least pentameric GH), with both noncovalently associated oligomers and disulfide-linked oligomers. The biological roles of the GH variants are largely unknown. They vary in biological activity, with monomeric 22-kDa GH1 and GH2 being the most biologically active.

Regulation of GH Production and Secretion

Expression of the *GH1* gene in pituitary somatotropes is positively regulated by GH-releasing hormone (GHRH), glucocorticoids, and (in rodents) thyroid hormones (Gunawardane *et al.*, 2000). It is negatively regulated by insulin-like growth factor-1 (IGF-1) and somatostatin. GHRH is also very important for normal somatotrope cell proliferation during pituitary development. GH is stored in secretory granules and released in response to GHRH; its release is inhibited by somatostatin. Ghrelin or related GH secretagogues, in pharmacological amounts, are potent releasers of GH, but the physiological role of ghrelin in GH regulation appears to be minor at best. The various GH isoforms appear to be cosecreted, and no isoform-specific stimulus has been identified.

GH secretion is under complex hypothalamic control by at least two hypophysiotropic hormones: GHRH (stimulating) and somatostatin (inhibitory) (Gunawardane *et al.*, 2000; Giustina and Veldhuis, 1998). GH secretion is pulsatile, with a marked ultradian rhythm of pulses of widely varying amplitude occurring every 1–2 h. The highest secretory pulses are linked to slow wave sleep and typically occur during the first 2 h after sleep onset. However, major pulses can also occur at other times due to stimuli such as stress, exercise, pain, and other acute events. Feedback inhibition of GH secretion is provided by IGF-1 as well as by GH itself. Daily GH production is high and largely unregulated during fetal and neonatal life, falls after birth under the regulatory influence of IGF-1, is again high during puberty, and falls progressively thereafter by about 15% per decade. Physiological and metabolic regulators of GH secretion are nutrition, estrogen (stimulatory), glucocorticoids (the inhibitory effect at the hypothalamic level predominates over the stimulatory effect at the pituitary level), and thyroid hormone (stimulatory, especially in rodents) (Giustina and Veldhuis, 1998). In humans, undernutrition stimulates GH production, whereas overnutrition dampens GH production (the opposite is generally true in rodents). There is marked sexual dimorphism in the GH secretory rhythm, with women showing generally higher values, higher basal secretion, and more "noisy" circadian profiles. This difference can be largely attributed to an estrogen effect. In rats, the male secretion pattern is characterized by very high pulses interrupted by nearly complete quiescence, whereas the female secretion pattern is characterized by lower pulses but higher tonic secretion between pulses.

In contrast to this complex regulation of GH production in the pituitary gland, the expression and secretion of placental GH during pregnancy appear to be constitutive and largely unregulated.

The GHR- and GH-Induced Signal Transduction

The GHR is a 620-amino acid single-chain glycoprotein that belongs to the family of cytokine receptors. It has a large, 246-residue extracellular domain, a single-transmembrane domain, and a 350-amino acid cytoplasmic domain. The extracellular ectodomain is folded into two subdomains; the amino-terminal subdomain 1 contains the GH binding site, and the carboxy-terminal subdomain 2 is involved in receptor dimerization. A short ($\sim$10 residue) linear stem region between subdomain 2 and the transmembrane domain serves as the substrate for enzymatic cleavage that, in humans, yields the circulating GH binding protein (GHBP) (Baumann, 2001; Cunningham *et al.*, 1991; Kelly *et al.*, 1991; Wang *et al.*, 2002). The cytoplasmic domain contains several features involved in GH-induced signaling. Among those, a membrane-proximal proline-rich region (Box 1) is most important; it serves as a docking site for Janus kinase 2 (JAK2). Another important region in the cytoplasmic portion is the internalization domain.

The single *GHR* gene resides on the short arm of human chromosome 5 (5p13–p12), spans 298 kb, and consists of 10 exons, of which exons 2–10 encode the protein (Baumann, 2001). The 22-residue portion encoded by exon 3 is not necessary for function, and inclusion or exclusion of exon 3 occurs as a form of *GHR* polymorphism. Several alternative exons 1 can be used to express the GHR in a tissue- or metabolic state-specific manner. Two truncated splice variants of the GHR are known; they lack most of the cytoplasmic domain, represent a small part ($\sim$1%–5%) of the total GHR complement, and have an unknown function. The GHR is expressed ubiquitously, although the level of expression varies among tissues. The liver is the tissue with the highest GHR content.

Signal transfer along single-transmembrane cell-surface receptors is not well understood. It has long been thought that these receptors, such as the GHR, are activated by ligand binding through ligand-induced dimerization of the receptors (the "dimerization model") (Uchida *et al.*, 1999). Thus, GH would bind to a GHR monomer and prompt dimerization by recruiting a second GHR monomer. However, evidence now suggests that prior to ligand binding, the GHR exists predominantly as a constitutively dimerized, yet inactive, homodimer (Gent *et al.*, 2003, 2002). It is believed that GH interaction with the preformed GHR dimer induces a conformational repositioning, which allows for one receptor subunit to rotate relative to the other, the so-called rotation model (Brown *et al.*, 2005; Brooks and Waters, 2010; Waters *et al.*, 2006). The rearrangement of the intracellular domains unmasks the catalytic parts of adjacent JAK2 molecules, which are constitutively associated with the cytoplasmic GHR Box 1 motif. The JAK2 molecules are activated by transphosphorylation and subsequently phosphorylate distal cytoplasmic tyrosine residues on the GHR, which enables Src homology 2 (SH2) domain molecules, such as signal transducer and activator of transcription (STAT), to dock to these sites (Wang *et al.*, 1996). The STAT pathway has been regarded the most prominent, with STAT-5b in particular being an important mediator of the biological effects of GH. Following tyrosine phosphorylation by JAK-2, STAT-5b molecules dimerize and translocate to the nucleus where they bind DNA and regulate gene transcription. However, GHR activation also results in activation of Src family kinases (SFK) independent of JAK2. SFK activates the mitogen-activated protein kinase (MAPK) pathway through phospholipase Cγ/protein kinase C (PKC) and probably other signaling pathways (Herrington and Carter-Su, 2001; Lanning and Carter-Su, 2006).

The various pathways may subserve different components of the GH bioactivity spectrum. The growth-promoting/IGF-1-generating action is primarily mediated by signaling through the JAK2 pathway, whereas some metabolic activities are mediated by signaling through PI3K. A whole host of genes are activated or deactivated in response to GH-induced signaling. Among those best recognized are serine protease inhibitor 2.1 (Spi 2.1), c-Fos, and IGF-1. GH-induced signaling also has nongenomic effects in some cultured cells, such as enhanced glucose transport.

GH-induced signaling is negatively regulated by several mechanisms to prevent runaway cellular stimulation, with some acting in a classic feedback loop. Suppressors of cytokine signaling (SOCS) proteins and cytokine-inducible SH2 (CIS) proteins facilitate downregulation and termination of GHR activation by various unique mechanisms. Additionally, activation of phosphatases, GHR downregulation through GHR internalization, and perhaps GHR inactivation by proteolytic GHR decapitation are involved in termination of the GHR signal cascade.

Blood Transport of GH

GH circulates in blood partly bound to two different GHBPs. The principal carrier is the high-affinity GHBP that corresponds to the soluble ectodomain of the membrane-anchored GHR, and because of its origin, GHBP binds GH with similar affinity as the GHR (Baumann and Frank, 2002). In humans and many other species, the GHBP is produced by proteolytic cleavage of the GHR homodimer in its juxtamembranous stem region (between 8 and 9 residues outside the membrane), with shedding of the ectodomain from the cell as the GHBP (Baumann, 1995; Baumann and Frank, 2002). The cleavage is mediated by tumor necrosis factor-alpha converting enzyme (TACE), a transmembrane zinc metalloproteinase also known as ADAM-17. TACE is activated by MAPK- and PKC-dependent pathways, and is responsible for cleavage of various transmembrane proteins. The regulation of TACE activity and ectodomain shedding is poorly understood. Recent studies have suggested that intracellular domain modifications of the predimerized GHR may lead to positional rearrangements in the extracellular domains that enable ectodomain cleavage. Thus, GHR predimerization may be a requirement for ectodomain shedding. The GHR remnant protein, consisting of the transmembrane and intracellular domain, is subject to additional processing and may have biological functions of its own.

In rodents, the GHBP is generated from the *ghr* gene as an alternative mRNA spliced product. Exon 8 of the *ghr* gene encodes a 30-amino acid transmembrane domain sequence specific to the receptor, as well as exon 9–10, which encode the intracellular GHR domain. The soluble GHBP contains the extracellular domain of the GHR, but has a unique hydrophilic tail, which is encoded by a GHBP-specific exon in the *ghr* gene, designated exon 8A. Differential splicing of exon 7 to either exon 8 or the interposed exon 8A yields the GHR or the GHBP, respectively.

Under basal conditions, approximately half of circulating GH is bound to GHBP. A minority of GH ($\sim$5%) is bound to another low-affinity GHBP that appears to correspond to a modified form of α_2-macroglobulin. The GHBPs prolong the plasma half-life of GH and act to provide a circulating GH reservoir. The plasma half-life of free 22 kDa GH in humans is about 10 min; elimination occurs primarily through glomerular filtration and GHR-mediated clearance. However, due to the large size of the GH/GHBP complex, overall clearance is lower than that of free GH, with a half-life of about 18 min. In addition to its role in the circulation, the GHBP acts as a local modulator of GH action by competing with GHRs for GH binding. Thus, at the local tissue level, GHBP acts primarily as an inhibitor of GH action.

Biological Actions of GH

Many, but not all, of the activities of GH are mediated by its second messenger, IGF-1 (Leroith *et al.*, 2001). IGF-1 is generated in response to GH in liver and many other target tissues. It is primarily responsible for the growth-promoting action, serving as both a mitogen and a metabolically active hormone. For some GH actions, it is still not clear whether they are mediated by IGF-1, by GH

directly, or by both. In fact, mouse growth has been attributed to distinct and overlapping actions of GH, IGF-1, and IGF-2 (Lupu *et al.*, 2001). **Table 1** lists the principal bioactivities of GH. The diverse manifestations of GH action are evident from the table. All tissues and organs are targets for GH action; hence, the term "somatotropin" describes this hormone more aptly than does the term "growth hormone." The most prominent net effects of GH actions in vivo are somatic growth, loss or maintenance of fat mass, muscle anabolism, increase or maintenance of bone mineral density, fluid retention, and insulin antagonism.

IGF and IGF-Binding Proteins

GH is the most well characterized regulator of the IGF system. The primary components of the IGF system include IGF-1 and IGF-2 that are structurally related polypeptides of about 7.5 kDa, and the IGF-1 receptor (IGF-1R), which shares homology with the insulin receptor and is ubiquitously expressed on most cell types (Hjortebjerg *et al.*, 2014; Hjortebjerg and Frystyk, 2014). IGF-1 is more GH dependent than IGF-2, and in humans IGF-1 is the main IGF involved in mediating GH action. IGF activity is strictly regulated by six high-affinity IGFBPs (1 to 6), which bind most of the circulating IGFs, thereby prolonging their half-life and modulating their ability to activate the IGF-1R. Foremost among those is IGFBP-3, which complexes the great majority of IGFs in a 150-kDa ternary complex composed of IGF, IGFBP-3, and the acid-labile subunit (ALS). Besides the IGFs, GH is a potent regulator of circulating IGFBP-3 and ALS. Insulin may be the second most important regulator of the IGF system and although GH and insulin are able to modulate the hepatic IGF-1 production independently, GH significantly regulates and influences the biological actions of insulin and vice versa. Several other IGFBPs are likely to be GH dependent. IGFBP-1 is the most metabolically regulated IGFBP and is the primary acute regulator of IGF-1. IGFBP-2 is also metabolically regulated, albeit not as rapidly as IGFBP-1. Both IGFBP-1 and -2 are inhibited by insulin, and evidence suggests that GH regulates them both either directly or indirectly (Hjortebjerg *et al.*, 2014).

Abnormalities in the GH/IGF Axis

Disease States and Treatments in Humans

GH Deficiency

GH deficiency can result from a variety of causes such as genetic defects, birth trauma, and organic lesions affecting the pituitary or the hypothalamus (Baumann, 2002). The most common cause is a pituitary or hypothalamic tumor resulting in destruction of somatotropes or GHRH-producing neurons as well as interruption of hypothalamo–pituitary communication. In patients with pituitary tumors, GH deficiency usually develops before the other pituitary hormones are compromised. Genetic causes include inactivating mutations in the *GH-1* gene, the *GHRH receptor* gene, or genes involved in pituitary development such as *PROP1*,

Table 1 Biological activities of GH

A. *Direct GH effects*
Lipolysis
Inhibition of lipogenesis
Prechondrocyte differentiation
Preadipocyte differentiation
Amino acid transport in muscle
Glucose transport (acute hypoglycemic effect, so-called insulin-like effect)
IGF-1, IGFBP-3 and ALS generation
Hypothalamic somatostatin secretion (short-loop feedback)
Milk production (human GH only)
B. *IGF-1 mediated effects*
Sulfate and thymidine incorporation into growth cartilage
Clonal expansion of chondrocytes
Linear bone growth
DNA and RNA synthesis
Renal Na and phosphate retention
C. *Unknown or combined GH/IGF-1 effects*
Nitrogen retention
Somatic growth[a]
Insulin antagonism
Beta cell hyperplasia
Erythropoiesis
Immune system stimulation

[a]Somatic growth involves not only bone elongation but also concomitant muscle and organ growth. Somatic growth is possible with IGF-1 alone, but differs both quantitatively and qualitatively from that induced by GH.

POU1F1 (PIT1), *HESX1*, *PITX2*, *LHX3*, and *LHX4* (Baumann, 2002). Deficiency of the placental GH appears to have no adverse effects on either mother or fetus, as has been learned from naturally occurring cases with a deletion of the *GH2* gene.

One relatively common type of GH deficiency in childhood is called "idiopathic," meaning that no cause is evident (Bang, 2011). In general, this is a diagnosis of exclusion in a child with poor growth and subnormal serum GH levels. The delineation between this entity and normal variation of growth patterns is difficult.

The clinical manifestations of GH deficiency depend, in part, on whether it develops during childhood or during adult life. Childhood GH deficiency is characterized by growth retardation, a feature that does not apply to adults. Other clinical manifestations of GH deficiency include moderately delayed puberty, childhood hypoglycemia, increased adiposity, osteopenia, decreased lean body mass, low exercise tolerance and stamina, extracellular volume depletion, impaired psychosocial functioning, and decreased quality of life. In humans, immune function is not sufficiently affected to be clinically relevant. Except for growth retardation and hypoglycemia, the manifestations of GH deficiency are relatively subtle and not readily recognized unless sought out specifically. Diagnosis is suspected in the proper clinical setting and is confirmed by the failure of serum GH to rise in response to standard pharmacological stimuli. Typical biochemical features include low levels of serum IGF-1, IGFBP-3, and ALS as well as an elevated level of IGFBP-2.

Treatment of children and adults with GH deficiency consists of replacement therapy with recombinant hGH (Baumann, 2002; Carroll *et al.*, 1998). Starting in 1960 in the US, hGH was extracted from cadaveric human pituitaries because there was no other source of GH that was biologically active in humans. Only children were recipients of this GH because supplies were limited by necessity. In 1985, after the occurrence of cases of Creutzfeldt–Jacob disease attributed to GH contaminated with prions, the use of cadaveric hGH was discontinued. The simultaneous arrival of recombinant human GH (rhGH) in unlimited quantities resulted in a universal switch to rhGH, and today, numerous pharmaceutical companies manufacture 22-kDa rhGH, which is highly effective despite lacking all the other GH isoforms that are normally produced by the pituitary. Availability of GH has enabled large-scale use of GH therapy in all patients with GH deficiency as well as conditions not associated with GH deficiency (e.g., chronic renal failure, Turner syndrome, intrauterine growth retardation, Prader–Willi syndrome, and idiopathic short stature). Indications for GH continue to expand beyond classical GH deficiency, and much has been learned about GH biology from the availability of large quantities of chemically defined, highly pure GH preparations as well as from clinical trials and more intense scrutiny of the manifestations of GH deficiency and the effects of GH replacement therapy.

GH treatment improves height, body composition, bone density, and overall life quality. GH is administrated daily and injected subcutaneously. Especially in children, the daily administration may be inconvenient and discomforting and lead to low compliance and adherence. This problem has fueled the search for GH agents with longer absorption profiles, longer half-lives, and alternative routes of administration. Numerous long-acting GH preparations have been invented and are under development, all displaying different pharmacodynamics (Borras Perez *et al.*, 2017). The early published data are promising, but an improvement compared to currently available options remains to be demonstrated. Furthermore, in view of the nature of the pulsatile GH secretion, the long-acting GH preparations may result in altered levels of circulating GH and IGF-1 that may uniquely affect GH's biological actions. More research in this area of long-acting GHs is required.

With an increase in the use of GH over the past decades, a concomitant increase in the potential safety concerns has occurred. The mitogenic and antiapoptotic activity of GH and IGF has primarily raised concerns regarding the risk of malignancy. Notably, the GH-induced signaling pathways are among the most important in most types of cancers, and numerous studies have provided evidence that the GH/IGF-1 axis may be involved in cancer development (Boguszewski *et al.*, 2016; Brooks and Waters, 2010). The concerns are further supported by findings in various GH mouse strains with increased or decreased cancer occurrence. However, thus far, rhGH treatment of GHD patients has shown to be safe, and despite theoretical worries, various studies have failed to consistently demonstrate a causal relationship between GH therapy and cancer development in patients. However, further work will continue in this area as the age of rhGH-treated patients increase.

GH Insensitivity

GH insensitivity or resistance shows a clinical picture that is similar, although not identical, to GH deficiency. In a GH insensitive state, there is no shortage of GH; rather, there is an inability of GH to promote its various actions. In humans, GH insensitivity results in Laron syndrome (LS) (Laron *et al.*, 1968, 1966), a genetic disorder caused by inactivating mutations in the *GHR* gene (Rosenbloom, 2016; Brooks and Waters, 2010; Rosenfeld *et al.*, 1994). LS patients show all of the physical manifestations of GH deficiency, but serum GH levels are high, while IGF-1, IGFBP-3, and ALS levels are low. In most, but not all, cases, the serum GHBP level is low or undetectable (the type of mutation in the GHR determines the presence or absence of GHBP). The phenotype is one of complete absence of GH activity and resembles that of patients with the most severe degrees of GH deficiency. Diagnosis is suspected in the proper familial setting by measuring serum GH, IGF-1, and GHBP; it is usually confirmed by genetic analysis. Other, postreceptor forms of GH insensitivity are due to inactivating mutations in genes encoding downstream elements in the GH signaling cascade, such as Stat5b, IGF-1, ALS, and the IGF1R. Treatment in most cases consists of IGF-1 replacement therapy.

A mild to moderate form of acquired GH resistance is frequently seen in catabolic states. This appears to be an adaptive response, with low serum IGF-1 and elevated GH levels. The nutritional deprivation that frequently accompanies such conditions explains part, but not all, of this phenomenon. This is a reversible derangement in the GH–IGF axis that returns to normal when the underlying disease process is corrected. Treatment with GH is not recommended because GH therapy, in severe cases of illness, has been associated with increased mortality. However, one partially GH-resistant condition where GH treatment is beneficial is chronic renal failure.

GH Excess

Persistent GH hypersecretion in humans leads to a condition called acromegaly, where patients exhibit overgrowth of hands, feet, and facial structures, as well as internal organs (Dal *et al.*, 2016; Melmed *et al.*, 2009; Katznelson *et al.*, 2014). If the condition starts during childhood and is left untreated, it leads to gigantism, in which overall somatic growth is accelerated. In the vast majority of cases, the condition is caused by a GH-secreting pituitary adenoma. At least half of these adenomas have somatic mutations in the $G_s\alpha$-subunit of the signal-transducing G-protein, through which GHRH normally signals. The mutation renders the G-protein constitutively active and leads to tumor formation and unchecked GH production (Gadelha *et al.*, 2017a). A germ line variant of this type of G-protein activation is seen in McCune–Albright syndrome, which has as one of its manifestations the occurrence of acromegaly. In rare cases, acromegaly can be caused by overproduction of GHRH, either by a eutopic source (e.g., a hypothalamic lesion) or (more frequently) in an ectopic site by tumors of neuroendocrine lineage (e.g., carcinoids, islet cell tumors). The high levels of systemic unregulated GHRH lead to somatotrope hyperplasia and GH overproduction. Only one convincing case of ectopic production of GH itself—by an islet cell tumor—has been reported.

The diagnosis is typically delayed because of the insidious onset of clinical signs. Biochemical findings include increased serum GH and IGF-1 levels. The diagnosis is supported by demonstrating a pituitary tumor and confirmed by showing that serum GH is not normally suppressible by administration of glucose (an oral or intravenous glucose tolerance test). In cases of ectopic GHRH production, a high GHRH level in peripheral blood is diagnostic.

Besides the overgrowth of the extremities and organs, the clinical aspects of acromegaly include hypertension, nerve entrapment manifestations, insulin resistance or diabetes mellitus, and increased cardiovascular morbidity and mortality. Although GH/IGF-1 exhibits mitogenic and antiapoptotic effects in many tissues, it still remains to be clarified whether cancer incidence and prevalence are increased in acromegalic patients. Patients with acromegaly do have an increase in colon polyps, which is a risk factor for colon cancer.

First-line treatment of acromegaly consists of surgical removal of the pituitary adenoma (or, rarely, the ectopic tumor) (Buchfelder and Schlaffer, 2017). Surgical resection is frequently not curative, especially when the adenoma exceeds the confines of the sella turcica. In such cases, postsurgical radiation or medical therapy is used to acquire disease control. Radiotherapy has numerous adverse side effects and since more efficacious and safe medical treatments have emerged, it is only used in patients with persistent disease or where the medication is not tolerated.

Medical Therapy in Acromegaly

After noncurative surgery, medical therapy is often necessary to reduce GH and/or IGF-1into the normal range. Currently, medical treatment of acromegaly comprises three drug classes: somatostatin analogues, GHR antagonists, and dopamine agonists (**Fig. 1**).

Long-acting somatostatin analogues are recommended for first-line adjuvant use. These analogues act on the somatostatin receptors (SSTRs) in the pituitary gland and exhibit inhibition on the secretion of GH by the somatotrophs (Gadelha *et al.*, 2017b). The drugs also have antiproliferative effects on the pituitary adenomas. The first compounds approved for use in acromegaly were octreotide and lanreotide, both considered preferential ligands of the pituitary SSTR2 and, to a lesser extent, SSTR5. However, the different SSTR subtypes are heterogeneously expressed in pituitary adenomas, and SSTR distribution, tumor size, and tumor location all influence treatment outcome. At present, oral preparations of octreotide are also being tested and clinical trials are under way to evaluate the efficacy of oral octreotide in acromegaly (Tuvia *et al.*, 2012). With the aim to generate compounds with a broader binding profile, the next-generation multireceptor-targeting somatostatin compound, pasireotide, was developed and approved in 2014 (Mckeage, 2015). The formulation appears as a promising and more effective alternative to the first-generation therapies, and in a study of 358 medically naïve acromegaly patients, pasireotide demonstrated superior efficacy over octreotide (Colao *et al.*, 2014).

Somatostatin analogue treatment, however, exerts additional effects because SSTRs are ubiquitously expressed in many tissues. In particular, it has the disadvantage of inhibiting insulin secretion and inducing hyperglycemia, with resulting increased propensity to promote diabetes. Pasireotide is particularly prone to inducing hyperglycemia (Neggers *et al.*, 2011). These side effects of somatostatin analogues limit their clinical usefulness in certain patients, and if an inadequate response is achieved with one drug, combination therapy may improve efficacy and minimize the potential adverse effects associated with increased doses of somatostatin analogues.

The GHR antagonist, pegvisomant, which was licensed in the USA in 2003, represents another effective agent with which it is possible to achieve medical control of acromegaly (Kopchick *et al.*, 2002; Freda *et al.*, 2015; Berryman *et al.*, 2007), and it is the medical treatment that has the highest reported efficacy (Neggers *et al.*, 2014). Pegvisomant is a recombinant GH analogue that differs from wild-type GH by a single Gly120Lys substitution in GH binding site 2 and eight amino acid substitutions in GH binding site 1. The Gly120Lys change results in GHR site 2 being altered and likely prevents proper dimerization by sterically inhibiting the conformational rearrangement of the GHR dimers. Thus, whereas somatostatin analogues function in the pituitary to directly target pituitary tumor GH secretion and tumor growth, pegvisomant acts on the ubiquitous GHR to block GH-induced signaling. Furthermore, the eight amino acid substitutions in GH binding site 1 result in an enhanced affinity of it for the GHR. Additionally, pegvisomant is conjugated with 4–5 polyethylene glycol moieties, which reduces its immunogenicity and extends its

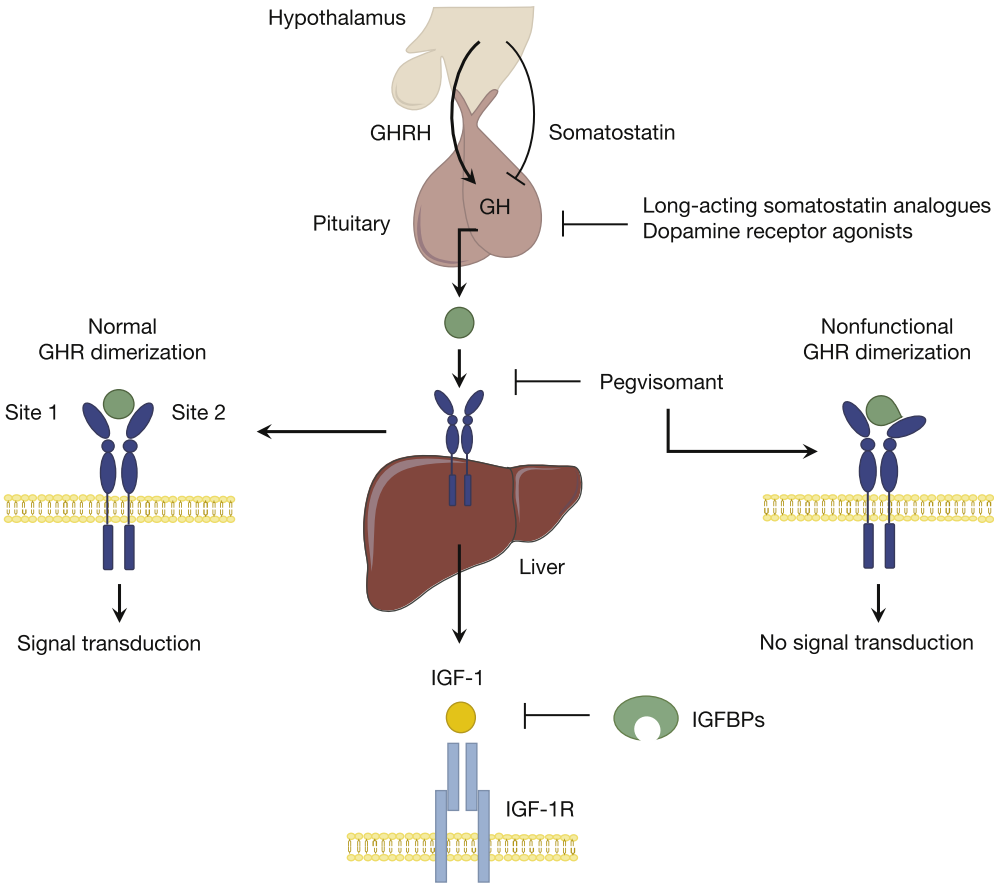

Fig. 1 The growth hormone (GH)-insulin-like growth factor (IGF) axis with therapeutic entry points for the treatment of acromegaly. Release of GH from the pituitary is regulated primarily by growth hormone releasing hormone (GHRH) and somatostatin. A single GH molecule binds to a constitutively dimerized, yet inactive, GH receptor (GHR) homodimer through sites 1 and 2. GH interaction with the GHR induces a conformational change and initiates downstream signal-transduction pathways, which promotes peripheral IGF-I secretion from the liver and other tissues. IGF-I binding to the IGF-I receptor (IGF-IR) mediates growth and metabolic functions. The IGF-IR binding is modulated by IGF-binding proteins (IGFBPs). Medical treatment of acromegaly comprises three drug classes: somatostatin analogues, dopamine agonists, and GHR antagonists. Long-acting somatostatin analogues act on the somatostatin receptors in the pituitary gland to inhibit GH secretion by the somatotrophs. Dopamine receptor agonists suppress GH hypersecretion through binding to D_2 receptors on adenomas. The GH analogue Pegvisomant contains amino-acid substitutions in the GHR binding sites, which blocks GH-induced signaling. Site 1 contains eight amino acid substitutions that improve receptor binding and site 2 contains a single amino acid substitution that blocks the conformational rearrangement of the GHR.

half-life from approximately 20 min to 2.5 days (Clark *et al.*, 1996). Overall, it prevents endogenous GH from binding to the receptor (Ross *et al.*, 2001).

The aim of pegvisomant therapy is to lower serum IGF-1 levels to the normal range. However, pegvisomant does not lower circulating GH levels; thus serum IGF-1 levels are used as a biomarker of drug efficacy. In most cases, it is possible to achieve IGF-1 normalization, and thus, pegvisomant represents a unique advance in treating the peripheral clinical sequelae of increased IGF-1 levels (Van Der Lely *et al.*, 2001). In contrast to somatostatin analogues, pegvisomant improves glucose tolerance and decreases fasting glucose levels, and thus, it is the safer choice in patients with diabetes mellitus. Effective treatment of acromegaly with pegvisomant is successful in reversing the soft tissue changes, improving cardiovascular risk parameters and insulin sensitivity, reducing the mortality rate, and improving quality of life. However, it is only partially effective in reversing the bony changes, and thus, early diagnosis and curative intervention are of paramount importance.

Pegvisomant can be used in the treatment of acromegaly both as a single agent and in combination with somatostatin analogues (Neggers and Van Der Lely, 2009; Muhammad *et al.*, 2016; Neggers *et al.*, 2014). Most studies on pegvisomant monotherapy efficacy have been based on clinical trials that evaluated the daily administration (Van Der Lely *et al.*, 2001; Trainer *et al.*, 2000), whereas few studies have assessed the weekly injection regimen (Higham *et al.*, 2009; Muhammad *et al.*, 2016). It has been suggested that conversion to weekly pegvisomant administration can be achieved with no loss of efficacy, and this may increase compliance and reduce the costs of medical treatment. Alternatively, pegvisomant and somatostatin analogue combination therapy shows promising results with regard to long-term efficacy and safety. The treatment appears highly effective at normalizing IGF-1 levels in most patients, and while pegvisomant monotherapy does not lead to a decrease in pituitary tumor

size, combination therapy with somatostatin analogues results in a decrease in tumor size in about 20% of patients (Neggers *et al.*, 2009). In 2005, the first study on the combined treatment of acromegaly with monthly high-dose long-acting somatostatin analogue therapy and weekly subcutaneous pegvisomant administration proved as effective as daily pegvisomant monotherapy (Feenstra *et al.*, 2005).

In patients with tumors cosecreting prolactin or only modestly increased IGF-1 levels and mild symptoms of GH excess, long-acting dopamine D_2 receptor agonists, usually cabergoline, may provide sufficient biochemical control (Sandret *et al.*, 2011). Physiologically, dopamine stimulates GH secretion, but dopamine receptor agonists paradoxically suppress GH hypersecretion in acromegalic patients through binding to D_2 receptors on adenomas (Chiodini *et al.*, 1974; Bernabeu *et al.*, 2013). However, clinical trials suggest that cabergoline monotherapy only normalizes IGF-1 levels in one-third of patients with acromegaly, and despite initial efficacy of cabergoline, the drug response appears to decrease with time.

Diagnostics

GH deficiency is diagnosed by pituitary stimulation test because a random serum GH level is largely uninformative due to the normally pulsatile nature of GH secretion. Various pharmacological tests are used for stimulation of GH secretion. The insulin tolerance test is often the first choice, as it measures the entire hypothalamo–pituitary axis. However, as it is attended by the risks of hypoglycemia, it must be administered under close supervision, and is contra-indicated in the elderly. The GHRH–arginine and pyridostigmine-GHRH tests are reliable and appear to have few drawbacks. Ghrelin is a strong GH secretagogue, and the GHRH–ghrelin and GHRH–hexarelin (a synthetic ghrelin analogue) tests are safe and convenient, but need further evaluation as to their diagnostic usefulness. Other less reliable tests include arginine alone, GHRH alone, L-dopa, glucagon, and clonidine. Clonidine appears to be a more potent secretagogue in children than in adults, where it is considered a weak stimulus. The absolute GH response to these provocative stimuli (in terms of serum GH levels achieved) varies depending on the GH assay used. Typically, monoclonal immunoassays yield lower GH values than do polyclonal assays. Diagnostic guidelines have been published by the GH Research Society (Ho and Participants, 2007) and by the American Association of Clinical Endocrinologists.

Acromegaly is readily diagnosed in the right clinical setting by an elevated serum IGF-1 level and a high GH level (the latter is not diagnostic by itself due to the normally pulsatile GH secretion) (Katznelson *et al.*, 2014). The confirmatory test, both in overt acromegaly and especially after surgical treatment, is the glucose tolerance test. Serum GH should fall below 1 ng/mL after glucose administration. In addition, IGF-1 should be within the normal age-adjusted range. Frequently, evidence for low-grade acromegaly persists after treatment even in cases where these criteria are met, as GH secretory dynamics remain disordered when examined carefully. For practical clinical purposes, an IGF-1 level in the mid-normal range is a reasonable criterion for a cure or biochemical control.

Studies in Mouse Strains With Altered GH-Induced Signaling

Most of our current understanding about the functions of GH has come from decades of studies in humans and animals. However, relatively recent reports dealing with mice with genetic mutations that alter GH action have increased our knowledge and appreciation of its multifunctional properties (Berryman *et al.*, 2008, 2016; Hjortebjerg *et al.*, 2017). Some of these mouse strains represent the murine counterparts of human diseases. A thorough description of all mouse lines that illustrate different aspects of GH actions is beyond the scope of this article, and thus, only three strains will be mentioned: the transgenic GH mouse with excess GH biological action; the GHR gene disrupted mouse (GHR $-/-$) with complete inability to mediate GH actions; and the transgenic GHR antagonist mouse (GHA) with reduced GH/GHR-induced action.

Genetically engineered mice with a GH transgene (human, bovine etc.) overexpress the GH gene and exhibit high GH and IGF-1 levels (Bartke *et al.*, 1994; Chen *et al.*, 1997). Depending on the origin of the transgene, there are modest variations in the phenotypes. However, all have organomegaly and display a giant phenotype that resembles the excessive somatic growth observed in acromegaly and gigantism. The mice are lean, but since GH is a diabetogenic hormone, they suffer from a number of pathological changes associated with glucose and lipid metabolism. Major metabolic characteristics of young GH transgenic mice are hyperinsulinemia, insulin resistance, hypercholesterolemia, and hypertension. These changes are the probable cause of the significantly reduced life span observed in these mice.

The GHR $-/-$ mouse displays a phenotype that is opposite to that of the GH transgenic mouse (Zhou *et al.*, 1997; List *et al.*, 2011). These mice lack both the GHR and the GHBP, and thus, they are GH insensitive, have low circulating IGF-1 levels, and suffer from severe growth retardation. Due to the absence of GH- and IGF- mediated feedback inhibition on GH secretion, circulating levels of GH are elevated, yet the mouse is GH insensitive. In many ways, this mouse strain mimics human LS. In this regard, most patients with LS harbor inactivating mutations in the GHR gene. The GHR $-/-$ mice are very insulin sensitive, and contrary to the transgenic GH mice, they are obese, mostly due to an increased subcutaneous adipose tissue accumulation. Interestingly, the subcutaneous adiposity has no negative influence on health and longevity, suggesting an importance of depot-specific lipid accumulation. Longevity is significantly increased in GHR $-/-$ mice when compared to wild-type littermates, and the increased life span is partly due to a reduction in malignant diseases, as well as a decrease in the occurrence of diabetes (Bartke *et al.*, 2016). A similar phenotype is found in LS patients, which are generally protected against cancer and diabetes.

A single codon change in the bovine *GH* transgene led to the generation of a transgenic mouse that expressed a GH analogue, namely, a molecule in which glycine in the 3rd alpha helix was replaced by arginine (Chen *et al.*, 1997; Kopchick *et al.*, 2014). Mice that expressed this mutated gene were dwarf with low levels of IGF-1. Subsequently, this GH analogue was found to act as a GHR antagonist. The dwarf GHA mouse phenotype is an intermediate between that of wild type and GHR −/− mice and illustrates the physiological impact of partial GH deficiency as opposed to the complete lack of GH actions (Chen *et al.*, 1991; Coschigano *et al.*, 2003). Again, the GHA mouse is small with low levels of IGF-1 compared to wild-type controls, but does not exhibit an extended life span like the GHR −/− mouse. Besides the usefulness of this novel mouse strain in assessing the impact of reduced GH action, the generation of the GHA mouse also resulted in the discovery of the drug pegvisomant used for the treatment of patients with acromegaly (Kopchick *et al.*, 2014).

Major results from studies of GH activity in mice over the last two decades are: the discovery of GHR antagonist (pegvisomant); the discovery of the influence of the GH/IGF-1 in aging and longevity, that is, low GH action can result in improved health and life span (at least in mice) (Junnila *et al.*, 2013); and the discovery of the effect of GH on "partitioning" of white adipose depots.

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Natural and Synthetic Growth Hormone Secretagogues☆

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From the First Growth Hormone Secretagogues to the Endogenous One, Ghrelin

Growth hormone secretagogues (GHSs) are a family of synthetic peptidyl and nonpeptidyl molecules that bind specific receptors (GHS-Rs) in the hypothalamus and pituitary with potent stimulatory effect on somatotrope secretion. Interestingly, the activity of GHSs is not fully specific for GH release; in fact, after acute administration they also possess a weak prolactin (PRL-), adrenocorticotropin hormone (ACTH-) and cortisol-releasing effect, but they have also been reported to be able to influence food intake and sleep patterns (Ghigo *et al.*, 1998). However, over the last 30 years, relevant scientific research has been focused on identifying synthetic GHSs in order to treat GH deficiency. The first GHSs were nonnatural synthetic peptides (also known as GH-releasing peptides, GHRP) endowed with a potent GH-releasing action. Invented in 1977 as met-enkephalin derivatives, they were devoid of any opioid activity and they did not affect the release of pituitary hormones (Bowers *et al.*, 1977; Ghigo *et al.*, 1998). The GHS family includes peptidyl analogues, such as GHRP-1, GHRP-2, GHRP-6, hexarelin (Bowers *et al.*, 1993), and nonpeptidyl molecules. A series of hexarelin analogues that contain one or several unnatural amino acids have been synthesized (Vodnik *et al.*, 2016). The peptidyl analogues possess strong GH-releasing activity, but they have the disadvantage of having an oral bioavailability of less than 1% with a short-lasting effect (Ghigo *et al.*, 1998). In the nineties, research at Merck & Co. led to the synthesis of an orally active nonpeptidyl molecule, MK-0677 also known as spiroindoline, one of the most potent peptidomimetic GHSs (Patchett *et al.*, 1995). MK-0677 shows an oral bioavailability of more than 60% and seems endowed with a long-lasting effect (Copinschi *et al.*, 1996). GHSs bind to specific sites in the rat forebrain and other brain regions, although the greatest density of binding sites is the hypothalamus and pituitary gland. GHS-R was in fact initially cloned in 1996 from pig pituitaries (Howard *et al.*, 1996). It is expressed in humans as two isoforms from the same gene on chromosome 3q26.2: GHS-R type 1a and GHS-R type 1b. GHS-R type 1a is a G protein-coupled receptor, composed of 366 amino acids with seven transmembrane regions and a molecular mass of approximately 41 kD, while GHS-R type 1b is a splice variant of type 1a made up of a truncated polypeptide of 289 amino acids with five transmembrane domains and a molecular mass of approximately 32 kDa (Laviano *et al.*, 2012). Whereas GHS-R type 1a binds both peptidyl and nonpeptidyl GHSs, GHS-R type 1b does not bind GHSs and its functional role is unknown. It has been hypothesized that the latter receptor would play a critical role in modulating the activity of GHS-R type 1a by forming hetero-dimeric complexes which attenuates trafficking of the active variant to cell surface (Laviano *et al.*, 2012; Chan and Cheng, 2004). The GHS-R1a is expressed predominantly in the anterior pituitary gland and in the hypothalamus, but also in extrahypothalamic areas of the central nervous system (ventral tegmental area, nucleus accumbens, hippocampus, and amygdala), in multiple peripheral endocrine (pancreatic islets, adrenal gland, thyroid) and nonendocrine organs and in different neoplastic tissues (Gnanapavan *et al.*, 2002).

Although GHS-R had been designated as an orphan receptor specific for the synthetic GHS molecules, in 1999 Kojima *et al.* isolated and characterized a natural bioactive ligand for this receptor, named ghrelin. The name ghrelin derives from the word "ghre," the Proto-Indo-European root of the word "grow" (Kojima *et al.*, 1999). It derives from the 117 amino acid precursor "preproghrelin" encoded by the gene GHRL located on chromosome 3p25–26, with five exons and four introns. Mature ghrelin consists of 28 amino acid residues, mainly produced from the enteroendocrine (X/A like) cells in the stomach wall (oxyntic mucosa), but lower levels of expression have been found in several other peripheral tissue, such as the gastrointestinal tract, adrenal gland, thyroid, breast, ovary, placenta, fallopian tube, testis, prostate, liver, gallbladder, fat tissue, human lymphocytes, spleen, kidney, lung, skeletal muscle, myocardium, vein, and skin (Date *et al.*, 2000; Kojima *et al.*, 2001; Gnanapavan *et al.*, 2002; Ghelardoni *et al.*, 2006). Ghrelin is also produced by neurons in several areas of the central nervous system, particularly in the nucleus arcuatus of the hypothalamus and in a group of neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei (Kojima *et al.*, 1999; Korbonits *et al.*, 2001; Cowley *et al.*, 2003; Kojima and Kangawa, 2005). Ghrelin is the first peptide isolated from natural sources in which the hydroxyl group of its third serine residue (namely, serine 3) is acylated by *n*-octanoic acid, catalyzed by ghrelin-*O*-acyltransferase (GOAT). The acylation of the N-terminal tetra-peptide is critical for the peptide to be able to cross the blood–brain barrier and is essential for binding GHS-R type 1a and for endocrine actions (Banks *et al.*, 2002; Lorenzi *et al.*, 2009). This special feature is conserved across species (Muccioli *et al.*, 2007). Ghrelin circulates in two major forms, acylated ghrelin (AG) and nonacylated ghrelin (UAG), which is the most abundant and stable form. UAG does not bind GHS-R type 1a and is devoid of typical AG endocrine activities. Nevertheless, UAG is an active peptide sharing some of the activities of AG, either enhancing or opposing AG. In fact, UAG has been demonstrated to have opposite effects on glucose and insulin metabolism in healthy humans (Benso *et al.*, 2012). Moreover, several studies have shown that UAG exhibits biological activities on cell proliferation and binds to cell membranes of cardiomyocytes, adipocytes, prostatic,

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and skeletal muscle cells (Cassoni *et al.*, 2001; Baldanzi *et al.*, 2002; Muccioli *et al.*, 2004; Filigheddu *et al.*, 2007). These effects are likely mediated through different GHS-R subtypes or completely different receptor families (Cassoni *et al.*, 2001; Baldanzi *et al.*, 2002; Ghigo *et al.*, 2005; Giovambattista *et al.*, 2008).

It is possible that ghrelin is not the sole natural GHS-R ligand and that GHS-R type 1a is one of a group of receptors for such ligands. Indeed, GHS-R is also bound by other molecules, such as Des-Gln14-ghrelin, which is a gastric homologue of ghrelin, with the exception that one glutamine is missing, and which has the same biological activities as ghrelin; adenosine, which binds but does not activate the receptor; and cortistatin, which is a neuropeptide that is homologous to somatostatin and is unable to recognize GHS-R type 1a. Moreover, there is strong evidence suggesting the existence of additional receptor subtypes. Specific binding sites for peptidyl GHS only, with very low binding affinity for ghrelin and nonpeptidyl GHS, have been shown in a wide range of nonendocrine peripheral human tissues.

The regulation of ghrelin secretion is still largely unknown, but it has already been shown that circulating ghrelin levels are increased in fasting states and declined 60–120 min after meals (Tschöp *et al.*, 2001; Cummings *et al.*, 2001), suggesting that ghrelin may act as an initiation signal for food intake (Ariyasu *et al.*, 2001; Tschöp *et al.*, 2001; Cummings *et al.*, 2001; Klok *et al.*, 2007; Lorenzi *et al.*, 2009). A diurnal and nocturnal rhythmicity of ghrelin levels in humans has also been observed by some (Cummings *et al.*, 2001; Koutkia *et al.*, 2004), but not by other authors (Barkan *et al.*, 2003). In addition, ghrelin levels seem to be influenced by age, gender, BMI, GH, glucose, and insulin (Ariyasu *et al.*, 2001; Tschöp *et al.*, 2001; Cummings *et al.*, 2001; Barkan *et al.*, 2003; Klok *et al.*, 2007). Notably, leptin has also been suggested to have influence on circulating ghrelin levels. In fact, it has been hypothesized that the satiety-inducing effects of leptin include the suppression of ghrelin secretion. In agreement with the critical influence of nutrition on ghrelin secretion, circulating ghrelin levels are increased in anorexia and cachexia but reduced in obesity (Yildiz *et al.*, 2004).

A comprehensive summary of ghrelin actions in vivo is provided in **Table 1**.

In the following paragraphs we will discuss the main endocrine and nonendocrine ghrelin activities.

Ghrelin and GHS Endocrine Activities

GH Releasing Action

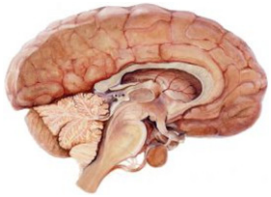
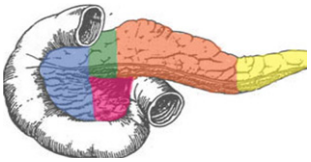
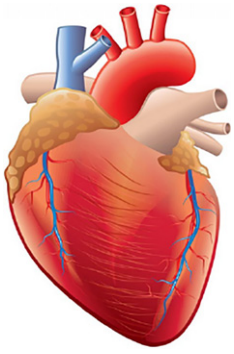
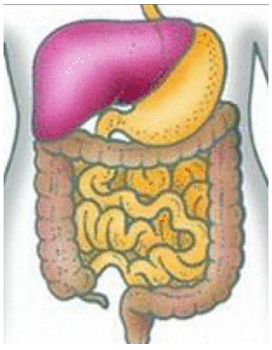
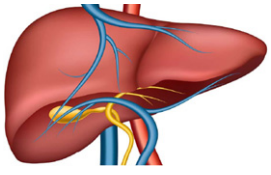
Ghrelin GH-releasing property was its first recognized effect (Kojima *et al.*, 1999). Ghrelin, as well as synthetic GHS, possesses a strong and dose-related GH-releasing activity, both in vitro and in vivo, which is more marked in humans than in animals (Van der Lely *et al.*, 2004; Broglio *et al.*, 2006). Intravenous ghrelin administration stimulates GH release which peaks at 5–15 min and returns to basal levels after 1 h (Ghigo *et al.*, 2005).

AG GH-releasing effect is mediated by actions on two different sites: on the somatotrophs of the anterior pituitary gland and, within the hypothalamus, on GHRH secreting neurons (Benso *et al.*, 2013). The GHS-R is expressed in about one fourth of GHRH mRNA containing neurons in arcuate nucleus and ventromedial nucleus of the hypothalamus suggesting that ghrelin may influence GH secretion through interaction with the GHS-R and that the release of GHRH into the hypophyseal portal blood may also be influenced by ghrelin neurons (Khatib *et al.*, 2014). At the hypothalamic level, ghrelin and GHS act via mediation of GHRH-secreting neurons as indicated by evidence that passive immunization against GHRH, as well as pretreatment with GHRH antagonists, reduces their stimulatory effect on GH secretion (Osterstock *et al.*, 2010). Moreover, the GH-releasing effect of GHS is markedly reduced in animals with lesions of the pituitary stalk (Van der Lely *et al.*, 2004). The GH response to ghrelin bolus has been shown to be more robust than the response after GHRH bolus (Arvat *et al.*, 2000) or hexarelin (Arvat *et al.*, 2001) and is synergistic with the GHRH response (Arvat *et al.*, 2001; Veldhuis *et al.*, 2012), suggesting a potential therapeutic use of ghrelin as a GH secretagogue. Moreover, it was recently discovered that the somatotroph releasing effect of AG is refractory to the direct inhibitory effect of a short-term elevation of GH levels, while it is markedly inhibited in the presence of increased IGF-I levels induced by 4-day rhGH administration. This finding might suggest the possibility of a selective IGF-I-mediated feedback (Benso *et al.*, 2013).

The AG and GHS GH-releasing effect undergoes marked age-related variations, increasing at puberty, persisting similar in adulthood and decreasing with aging (Broglio *et al.*, 2003a); variations in estrogenic levels, the reduced expression of the hypothalamic GHS receptors in the aged human brain, GHRH hypoactivity and somatostatinergic hyperactivity would explain these age-related changes (Ghigo *et al.*, 2005). AG GH releasing effect is independent of gender, does not vary with the menstrual cycle (Messini *et al.*, 2013), and occurs in a dose-dependent manner (Takaya *et al.*, 2000).

Differently from GHRH, AG stimulatory effect on GH secretion is reduced both in obese and in anorectic patients (Tassone *et al.*, 2003; Broglio *et al.*, 2004), in polycystic ovary syndrome (Fusco *et al.*, 2007), hyperthyroidism (Molica *et al.*, 2010), Cushing's disease (Correa-Silva *et al.*, 2006), and primary hyperparathyroidism (Cecconi *et al.*, 2008). Moreover, the GH response to a ghrelin bolus is reduced by centrally acting cholinergic antagonism (Maier *et al.*, 2004), but is not affected by peripherally acting cholinergic blockade (Broglio *et al.*, 2003b), cholinergic agonist (Broglio *et al.*, 2003b; Maier *et al.*, 2004), oxytocin (Coiro *et al.*, 2011), dopamine receptor blockade or by the most important inhibitory inputs on GH secretion such as glucose, free fatty acids and b-adrenergic agonist, all acting to increase the hypothalamic somatostatin release. In healthy postmenopausal women, estradiol or combination estradiol–progestin replacement increases GH secretion in response to a ghrelin bolus (Veldhuis *et al.*,

Table 1 In vivo endocrine and nonendocrine ghrelin actions

Site of action	*Acylated ghrelin actions*	
	Hypothalamus ↑ GHRH secretion ↑ CRH secretion ↓ GnRH pulse generator ↑ Food intake (via NPY) and appetite	Pituitary ↑ GH secretion ↑ ACTH secretion ↑ PRL secretion ↓ LH in men/↓FSH and LH in women ↓↔ TSH
	Pancreas ↓ Insulin secretion (spontaneous and glucose stimulated) ↑ Glucose levels ↑ Glycogenolysis ↑ Glucagone secretion	
	Adipose tissue ↑ Lipogenesis	
	Cardiovascular system ↑ Cardiac output ↑ Cardiac contractility ↓ Systemic vascular resistances ↑ Vasodilation	
	Gastrointestinal system ↑ Gastric emptying ↑ Gastric acid secretion ↑ Gastric motility ↑ Intestinal motility	
	Liver ↑ IGF-I	

2006; Villa *et al.*, 2008), and estradiol replacement therapy increases basal, but not pulsatile, GH secretion in response to a ghrelin infusion (Kok *et al.*, 2008).

AG, as well as synthetic GHS, could have diagnostic and therapeutic implications based on GH-releasing effects. Since a damage to the pituitary stalk or to the pituitary reduces the GH response to a ghrelin bolus (Maghnie *et al.*, 2007; Popovic *et al.*, 2003), ghrelin and GHS, particularly when combined with GHRH, could be used as a potent and reliable provocative test to evaluate the capacity of the pituitary to release GH for the diagnosis of GH deficiency (Gasco *et al.*, 2012). Long-acting

and orally active ghrelin analogs might represent an anabolic treatment in frail elderly subjects or in catabolic patients. At present, however, there is no definite evidence showing the therapeutic efficacy of ghrelin analogs as GH/IGF-I axis-mediated anabolic agents in humans.

Potential use in cancer cachexia

Ghrelin and its agonists are the perfect candidate to treat cachexic and frail states because of the anabolic biology of the hormone (Vodnik *et al.*, 2016). Ghrelin has a very short half-life but the peptide can be engineered for a more sustained delivery and better pharmacokinetic properties (Müller *et al.*, 2015).

Few studies demonstrate that ghrelin or ghrelin mimetic administration in advanced incurable cancer and anorexia increases energy intake and appetite (Strasser *et al.*, 2008; Lundholm *et al.*, 2010). Anamorelin-ONO-7643 (ANAM) is a novel, orally active, ghrelin receptor agonist under clinical development for the treatment of cancer cachexia and has advanced to phase III studies (Garcia *et al.*, 2015; Vodnik *et al.*, 2016). It is found to be associated with significant appetite-enhancing activity and resultant improvements in body weight, lean body mass. However, further studies are needed to confirm the significant potential of ANAM in cancer anorexia-cachexia syndrome (Pietra *et al.*, 2014).

The composite preclinical literature indicates beneficial effects of ghrelin-based intervention in cancer-cachexia models and with an increase in lean body mass. Preliminary clinical data show that ghrelin maintains its GH releasing and orexigenic effect in the setting of cancer. However, further investigations should evaluate the effects of ghrelin administration on tumor growth (Guillory *et al.*, 2013).

Potential use in AIDS associated cachexia

Cachexia has also been found in AIDS and alterations in GHRH-GH-IGF-I axis are common in the complex of HIV and AIDS, particularly in case of lipodystrophy. The processes involved in lipodystrophy are related to the suppression of GH production. The mechanism of low GH levels is due to increased somatostatin tone and decreased ghrelin secretion. The GHRH analogue Tesamorelin is the only therapeutic option, which is FDA approved, to reduce abdominal fat excess in patients with HIV-associated lipodystrophy (Stanley *et al.*, 2014). On the other hand, elevated GH and low IGF-I levels are present in AIDS wasting syndrome, suggesting GH resistance (Jain *et al.*, 2013).

Lower ghrelin levels have been reported in HIV-infected lipodystrophic patients, but to date, no reports of ghrelin or GHS use in this clinical setting are available (Guillory *et al.*, 2013), even if ghrelin GH releasing effect could be used to improve lipodystrophy.

ACTH Releasing Action

The stimulatory effect of ghrelin and synthetic GHS on the hypothalamus–pituitary–adrenal (HPA) axis in humans is remarkable and similar to that of the administration of naloxone, vasopressin, and even corticotropin-releasing hormone (CRH) (Arvat *et al.*, 2001; Van der Lely *et al.*, 2004; Ghigo *et al.*, 2005). Interestingly, the effect of ghrelin on ACTH and cortisol secretion is even more pronounced than that elicited by synthetic GHS, such as hexarelin, as demonstrated by Arvat *et al.* (2001).

The ACTH-releasing effect of GHS is acute, being attenuated during prolonged treatment, is independent of gender, but shows peculiar age-related variations, increasing at puberty, then showing a reduction in adulthood and, again, a trend toward an increase in aging, when the GH-releasing activity of GHS is clearly reduced (Arvat *et al.*, 1997; Ghigo *et al.*, 1998, 2001; Broglio *et al.*, 2003a).

Under physiological conditions, ghrelin ACTH-releasing activity is entirely mediated via the central nervous system (CNS) (Ghigo *et al.*, 1998, 2001). In fact, transection of pituitary stalk decreased the stimulatory effect of ghrelin on HPA axis in animal models (Van der Lely *et al.*, 2004). These mechanisms via the CNS not only include CRH and/or vasopressin-mediated actions (Ghigo *et al.*, 1998, 2001; Ishizaki *et al.*, 2002) but also neuropeptide Y and/or γ-aminobutyric acid (GABA) (Arvat *et al.*, 1998; Korbonits *et al.*, 1999). More recently, Kageyama *et al.* found that ghrelin stimulates both CRH and AVP mRNA levels in incubated 4B rat hypothalamic cells, suggesting that ghrelin does not directly stimulate ACTH release from the pituitary cells (Kageyama *et al.*, 2011). Moreover, ghrelin does not have direct stimulatory effects on adrenal hormone secretion. While ghrelin stimulates the HPA axis, hypercortisolemia is associated with reduced ghrelin levels, as a consequence of a feedback mechanism (Otto *et al.*, 2004; Sherif *et al.*, 2011).

Since GHS and ghrelin ACTH releasing activity is sensitive to the negative glucocorticoid feedback in healthy individuals and in patients with ACTH independent Cushing syndrome (Ghigo *et al.*, 1997, 1998, 2001; Arvat *et al.*, 1998), it is remarkable and intriguing that in patients with pituitary ACTH-dependent Cushing's disease (Arvat *et al.*, 1998; Leal-Cerro *et al.*, 2002; Martinez-Fuentes *et al.*, 2006) and in patients with ectopic ACTH-dependent Cushing syndrome (Korbonits *et al.*, 1998; Ghigo *et al.*, 1998), despite chronic hypercortisolism, the responsiveness to ghrelin and GHSs is remarkably enhanced. An over-expression of ghrelin receptors on corticotroph adenoma cells may at least in part account for this finding (Rotondo *et al.*, 2011). Only two studies have investigated the ACTH and cortisol responses after the combined administration of GHS hexarelin + CRH in healthy subjects (Korbonits *et al.*, 1999) and in patients with Cushing disease (Arvat *et al.*, 1999). Korbonits *et al.* have shown that hexarelin stimulates HPA axis independently of CRH in healthy subjects, while Arvat *et al.* reported additive mean ACTH responses after combined CRH + hexarelin administration in eight female patients with Cushing's disease. In the following in vitro study by

Pecori-Giraldi *et al.* the ghrelin-stimulated ACTH response in cell cultures of human pituitary corticotroph cells collected from ACTH secreting adenomas was significantly lower than the one induced by CRH, suggesting that ghrelin acts on both hypothalamic and pituitary sites to stimulate ACTH secretion in Cushing's disease (Pecori-Giraldi *et al.*, 2007). In line with this, evidences from in vitro animal studies suggest potent AVP, CRH, and NPY releasing effects of ghrelin from rat hypothalamic explants (Mozid *et al.*, 2003). These complex interactions may account for CRH and ghrelin synergistic effects on ACTH release at the hypothalamic and pituitary level. Miljic and colleagues have recently demonstrated that ghrelin in combination with CRH additionally enhances ACTH secretion without further additive effects on cortisol output (Miljic *et al.*, 2017). These data suggest uncoupling of ACTH and cortisol after achieving maximal cortisol values similar to that observed in states of psychological or physical stress (Veldhuis *et al.*, 2005) or during pharmacological stimulation (Keenan *et al.*, 2003). The expression of ghrelin receptors in some neuroendocrine tumors with ectopic ACTH secretion may reduce its potential for use in the differential diagnosis of ACTH dependent Cushing syndrome (Miljic *et al.*, 2017).

PRL Releasing Action

According to the previous in vitro and in vivo studies on synthetic GHS (Ghigo *et al.*, 1999), ghrelin significantly stimulates PRL secretion in humans in a gender- and age-dependent manner (Takaya *et al.*, 2000). The ghrelin effect on PRL secretion is higher than that of hexarelin (Arvat *et al.*, 2001).

On the contrary, an inhibitory effect on PRL secretion acting primarily at the hypothalamus has been shown in prepubertal male and female rats (Tena-Sempere *et al.*, 2004), even if the reason for this discrepancy is unclear. The species specificity could be explained by higher levels of mammosomatotroph cells in humans than in animals. Data in mice have shown that different parts of the brain, including the hypothalamus, contain neurons that coexpress the dopamine receptor subtype 1 and GHS-R (Jiang *et al.*, 2006). In such neurons, ghrelin had the capacity to amplify dopamine-induced cyclic adenosine monophosphate accumulation, thus providing temporal control over the magnitude of dopamine signaling (Jiang *et al.*, 2006). Additional data have shown that ghrelin can activate the mesoaccumbal dopamine system in the ventral tegmental area of mice and rats (Abizaid *et al.*, 2006; Jerlhag *et al.*, 2006) and protect nigral dopaminergic neurons by reducing apoptosis (Jiang *et al.*, 2008).

Concerning the effects in humans, Messini *et al.* (2010) have showed that bromocriptine, which is a dopamine agonist, blocked the ghrelin stimulating effect on PRL release and attenuated the GH response to the same stimulus in women. The same authors also evaluated the effect of exogenous thyrotropin-releasing hormone (TRH) on ghrelin-induced PRL release in women. They showed that ghrelin induced a smaller PRL increase than TRH did and that the ghrelin stimulating effect on PRL secretion was not additive with that of TRH (Messini *et al.*, 2009). More recently, the same research group demonstrated that physiological secretion of ovarian steroids does not affect the GH and PRL response to ghrelin in a group of 10 women during menstrual cycle (Angelidis *et al.*, 2012). Fasting serum ghrelin levels had no relationship with PRL in patients diagnosed with prolactinoma, suggesting that ghrelin levels have no significant effect on weight gain and could not explain increased obesity prevalence in prolactinoma (Delibaşı *et al.*, 2015).

Influence on Gonadal Function

Ghrelin regulates the hypothalamus–pituitary–gonadal (HPG) axis acting both at the central and at the peripheral level (Tena-Sempere *et al.*, 2002; Caminos *et al.*, 2003) and several in vitro or in vivo studies indicate that ghrelin negatively influences the gonadal axis (Rak-Mardyla, 2013), as summarized in **Table 2**.

AG suppresses LH pulsatility in rodent, ovine and primate models (Furuta *et al.*, 2001; Kawamura *et al.*, 2003; Barreiro and Tena-Sempere, 2004; Vulliémoz *et al.*, 2004; Fernández-Fernández *et al.*, 2005; Iqbal *et al.*, 2006). However, ghrelin infusion decreased LH pulse frequency but not pulse amplitude in adult ovariectomized rhesus monkeys, suggesting that ghrelin could inhibit the GnRH pulse activity (Vulliémoz *et al.*, 2004). Ghrelin can suppress not only LH, but also FSH secretion in male and female rats and this effect may depend on the manner of ghrelin administration (Fernández-Fernández *et al.*, 2006; Martini *et al.*, 2006).

Table 2 Ghrelin effects on HPG axis

	Effect	*References*
Animal studies		
Ovariectomized rat	↓ GnRH ↓ LH	Furuta *et al.* (2001)
Female and male rat	↓ LH ↓ FSH	Fernández-Fernández *et al.* (2006) and Martini *et al.* (2006)
Sheep	↓ LH	Iqbal *et al.* (2006)
Rhesus monkey	↓ LH	Vulliemoz *et al.* (2004)
Human studies		
Men and women	↓ GnRH ↓ LH ↔ GnRH-stimulated LH	Kluge *et al.* (2007), Messini *et al.* (2014), and Lanfranco *et al.* (2008a,b)

Ghrelin regulation of gonadotropin secretion in humans has been investigated mainly in male subjects. While in the first published study (Takaya *et al.*, 2000) different dosages of ghrelin increased GH but did not affect LH concentrations in normal males, two studies in men showed a delay and a suppression in LH pulse amplitude following acute i.v. ghrelin administration (Kluge *et al.*, 2007) and an inhibitory effect of ghrelin infusion on LH pulsatility (Lanfranco *et al.*, 2008a). More in details, Lanfranco *et al.* showed that a prolonged AG infusion quantitatively and qualitatively inhibits LH but not FSH secretion in healthy young males (Lanfranco *et al.*, 2008a). Morerover, in contrast with in vitro data showing that ghrelin reduces the LH response to GnRH in rodents (Fernández-Fernández *et al.*, 2005), the LH response to GnRH in humans is not modified by the exposure to AG (Lanfranco *et al.*, 2008a). These findings are therefore against the hypothesis that ghrelin plays a direct inhibitory role on pituitary gonadotropic cells. As AG inhibits the LH response to naloxone in humans, this clearly points toward a CNS-mediated inhibitory action on the HPG axis (Lanfranco *et al.*, 2008a). In addition, ghrelin decreases GnRH release by hypothalamic explants/fragments ex vivo (Fernández-Fernández *et al.*, 2005), reinforcing the contention of a complex mode of action of ghrelin with inhibitory effects at the central level and a direct stimulatory action on basal gonadotropin secretion. Whether ghrelin action on the GnRH pulse generator takes place directly on GnRH neurons or through indirect regulatory pathways is yet to be determined (Muccioli *et al.*, 2011). Some evidences suggest that ghrelin indirectly decreases gonadotropin secretion acting on central NPY, AgRP, or orexin expression (Tena-Sempere *et al.*, 2002; Rak-Mardyla, 2013), which exhibit inhibitory effect on LH secretion (Muccioli *et al.*, 2011). On the other side, Forbes *et al.* (2009) demonstrated that ghrelin administration significantly reduces LH pulsatility and suppresses kisspeptin mRNA expression in ovariectomized rats and suggested that downregulation of kisspeptin expression may play a critical role in the transduction of ghrelin-induced suppression of the reproductive function often observed during caloric restriction.

In women experimental studies, Messini *et al.* (2009) demonstrated for the first time the inability of a ghrelin bolus to affect basal and GnRH-induced LH and FSH, suggesting that ghrelin does not play a major physiological role in gonadotropin secretion in female subjects (Messini *et al.*, 2009). However, more recent studies have shown opposite results: a pharmacological dose of ghrelin, divided in four boluses, suppressed LH and FSH secretion in young women (Kluge *et al.*, 2012). Ghrelin decreased the frequency of LH pulses in all women and of FSH pulses in the one woman who exhibited clear FSH pulses resulting in a diminished secretion. This pattern further corroborates the widely accepted concept that ghrelin inhibits the hypothalamic GnRH pulse generator (Kluge *et al.*, 2012). The inhibitory effect of ghrelin has been recently demonstrated to be more evident in the late follicular phase of the cycle (Messini *et al.*, 2014).

It is well known that ghrelin is an important signal of energy insufficiency. In fact anorexia, malnutrition and cachexia are generally associated to hypogonadism that reflects a functional impairment of neuroendocrine mechanisms (Vanhorebeek *et al.*, 2006). The pathophysiological conditions mentioned above are associated with ghrelin hypersecretion (Shimizu *et al.*, 2003; Broglio *et al.*, 2004) and this could have a role in the functional hypogonadism connoting anorexia, malnutrition, and cachexia.

Influence on Thyroid Function

In humans plasma ghrelin levels have been reported to be reduced or unaffected in hyperthyroidism and unaffected or increased in hypothyroidism (Kosowicz *et al.*, 2011). A few contrasting published data on the relationship between thyroid status and circulating ghrelin are available in patients affected by Hashimoto's thyroiditis (HT). Recently it was demonstrated that euthyroid HT is associated with a decrease in plasma ghrelin levels and altered body fat distribution and increased anti-TPO levels do not seem to be directly involved in lower ghrelin levels in euthyroid HT patients (Biyikli *et al.*, 2014).

Concerning ghrelin administration, Molica *et al.* demonstrated that GH values after ghrelin boluses results lower in hyperthyroidism, suggesting that thyroid hormones are likely to affect ghrelin neuroendocrine action (Molica *et al.*, 2010). Ghrelin decreases energy expenditure and suppresses TSH secretion, but published literature shows contrasting results. Although Takaya *et al.* had shown no significant variation of TSH levels in humans after intravenous ghrelin administration (Takaya *et al.*, 2000), a more recent study by Kluge *et al.* has demonstrated that consecutive boluses of AG decrease the activity of hypothalamus–pituitary–thyroid axis in 20 healthy adults (Kluge *et al.*, 2010). Ghrelin may exert a stimulating effect at the thyroid level, as suggested by an increase of thyroxine (Kluge *et al.*, 2010; Morillo-Bernal *et al.*, 2011), but a suppressing effect at the hypothalamus level, as indicated by a TSH and TRH decrease (Kluge *et al.*, 2010).

Ghrelin and GHS Activities on the Endocrine Pancreas

Extensive research established a role for ghrelin and GHS in the regulation of insulin release and glycaemia (Yada *et al.*, 2014). GHS-R mRNA and protein are expressed in pancreatic islets of rats and humans (Volante *et al.*, 2002) and in beta cell lines (Wierup *et al.*, 2004).

Ghrelin modulates glucose metabolism in humans and in animal models and glucose homeostasis in a GH-independent manner, suggesting a link between ghrelin and the endocrine pancreas. In particular, both a ghrelin bolus and a ghrelin infusion have been shown to increase glucose levels in healthy young men and women (Broglio *et al.*, 2001, 2003c, 2008; Gauna *et al.*, 2005), and in obese subjects (Tassone *et al.*, 2003). This effect is blunted in patients affected by anorexia nervosa (Broglio *et al.*, 2004; Miljic *et al.*, 2007) and malnourished dialysis patients (Ashby *et al.*, 2009; Garin *et al.*, 2013). A clear relationship between

ghrelin and insulin secretion has been found in humans: AG pharmacological doses inhibit spontaneous, glucose- and arginine-stimulated insulin secretion (Broglio *et al.*, 2001, 2003c), despite increasing or unchanged glucose levels (Mori *et al.*, 2006). Insulin resistance was found in healthy subjects (Vestergaard *et al.*, 2008) and gastrectomized patients (Damjanovic *et al.*, 2006) in hyperinsulinemic euglycemic clamp studies after ghrelin infusion (Garin *et al.*, 2013).The hyperglycemic response to ghrelin is independent of GH (Vestergaard *et al.*, 2008) and the vagus nerve (Huda *et al.*, 2010): ghrelin may likely block insulin inhibitory effects on gluconeogenesis and have a direct, non-GHS-R type 1a-mediated, stimulatory effect on glycogenolysis, since this activity is not shared by synthetic GHS; instead, a direct effect on both insulin resistance and insulin secretion occurs. AG diminishes insulin secretion from beta cells lines or the isolated pancreas in vitro (Granata *et al.*, 2010), whereas it exerts a positive effect on glucagon release, acting on alpha cells (Qader *et al.*, 2008).

In contrast to AG, UAG infusion decreased fasting glucose and increased postprandial insulin in healthy humans (Benso *et al.*, 2012). Administration of AG and UAG in GH deficient men increased fasting and postprandial glucose levels, whereas coadministration of AG and UAG increased insulin sensitivity (Gauna *et al.*, 2004).

Concerning fat metabolism, acute ghrelin administration increased lipolysis in healthy subjects (Vestergaard *et al.*, 2008, 2011) and potentiated a beta-agonist-induced increase in free fatty acids (FFAs) (St-Pierre *et al.*, 2010). AG infusion was associated with a rise in FFA before each meal in healthy, obese and gastrectomized patients (Huda *et al.*, 2011). On the contrary, long term exogenous administration of AG has adipogenic properties (Tschöp *et al.*, 2000; Wells, 2009), inhibiting the sympathetic nervous system through stimulation of orexigenic NPY neurons in order to promote fat deposition (Wells, 2009).

Lipolysis is ghrelin dose-dependent and independent of circulating insulin and GH levels (Vestergaard *et al.*, 2011). On the contrary, UAG infusion decreased circulating FFA levels in healthy volunteers (Benso *et al.*, 2012) and the coadministration with AG decreased postprandial FFA in GH deficient men (Gauna *et al.*, 2004).

Potential Use of Ghrelin Blockade in Glucose Intolerance/Diabetes

The ghrelin blockade counteracts the obesity-associated glucose intolerance in two different models: in ob/ob mice, a genetic model of obesity due to leptin deficiency, ablation of ghrelin augmented insulin release and reduced hyperglycaemia (Sun *et al.*, 2006); in ghrelin Knock Out mice, fed high fat diet for 4 weeks, plasma insulin increased and blood glucose levels did not increase, in comparison with wild-type mice (Dezaki *et al.*, 2006). Thus, under conditions of insulin resistance, the lack of ghrelin may increase the maximal capacity of glucose-induced insulin release in beta cells, enabling them to secrete more insulin in order to prevent glucose intolerance (Yada *et al.*, 2014). Suppression of ghrelin actions could have a potential to counteract diabetes, hyperphagia, and obesity simultaneously, with a specific therapeutic role against the metabolic syndrome (Yada *et al.*, 2014). However, a direct antagonism of the receptor does not always have an antidiabetogenic effect, since it can cause body weight gain (Delhanty and van der Lely, 2011). Pharmacological suppression of the AG/UAG ratio by treatment with UAG may also be a viable alternative approach which appears to improve insulin sensitivity (Van der Lely, 2009; Benso *et al.*, 2012). A promising approach appears to be the GOAT activity blockade using a specific inhibitor, although the longer term effects of this treatment remain to be investigated (Barnett *et al.*, 2010).

Ghrelin and GHS Nonendocrine Actions

Effects on Energy Balance

Ghrelin is the first known peripheral hormone to display orexigenic effects through its action on the hypothalamic appetite-regulating pathways (Nakazato *et al.*, 2001).

The clear preprandial rise and post prandial fall in plasma ghrelin levels support the hypothesis that ghrelin acts as a hunger signal triggering meal initiation in humans (Cummings *et al.*, 2001). This action would be mediated by GHS-R nontype 1a subtypes, as suggested by evidence that GHS analogues devoid of any GH-releasing effect stimulate food intake. Because the rate at which peripheral ghrelin passes the blood–brain barrier has shown to be very low, peripheral ghrelin must activate the appropriate hypothalamic regions via an indirect pathway. The GHS-R localizes on vagal afferent neurons in the rat nodose ganglion. Then, ghrelin signals from the stomach are transmitted to the brain via vagus nerve (Date *et al.*, 2002). This peripheral signaling through vagus nerve, reaches arcuate nucleus of the hypothalamus (Date *et al.*, 2006). In the arcuate nucleus the ghrelin—containing neurons send efferent fibers onto NPY- and AgRP expressing neurons in order to stimulate the release of orexigenic peptides and onto proopiomelanocortin (POMC) neurons in order to suppress the release of anorexigenic peptides (Cowley *et al.*, 2003; Sato *et al.*, 2014). Thus, ghrelin is produced primarily in the stomach in response to hunger and starvation, circulates in the blood and serves as a peripheral signal, informing the arcuate nucleus of CNS to stimulate feeding.

Published literature has demonstrated that exogenous ghrelin and GHS central or peripheral administration has an orexigenic effect in humans as well as in animal models. AG infusion and/or bolus increases appetite and/or food intake in healthy volunteers, obese patients (Druce *et al.*, 2005), cancer patients (Lundholm *et al.*, 2010), and patients with some form of cachexia (Nagaya *et al.*, 2005). The only patient populations in which a single infusion of AG does not exert appetite stimulation are postvagotomy or gastrectomy patients (Huda *et al.*, 2009; le Roux *et al.*, 2005).

Potential ghrelin use in obesity

Plasma ghrelin levels inversely correlate with body mass index (BMI). Thus, ghrelin is reduced in obesity compared to normal weight as an adaptation to a long-term positive energy balance (Williams *et al.*, 2006; Sherif *et al.*, 2011). Recent evidence suggests that one of the mechanisms of diet-induced obesity is that ghrelin resistance arises by reducing NPY/AgRP responsiveness to plasma ghrelin and suppressing the neuroendocrine ghrelin axis in order to limit further food intake (Sato *et al.*, 2014). It has been shown that selective ghrelin blockade in diet-induced obese mice results in reduction of food intake, body weight, and body fat mass (Shearman *et al.*, 2006; Sherif *et al.*, 2011).

Both ghrelin and its related substances have been investigated as targets against obesity (Sato *et al.*, 2014). Neutralization of ghrelin by the specific antibody reduced body weight gain with the reduction of fat mass in rats (Zorrilla *et al.*, 2006).

Spiegelmers, antisense polyethylene glycolmodified L-oligonucleotides, have the ability of specifically binding a target molecule. Inhibitory effects of ghrelin-induced GH release in rats were exerted by NOX-B11-3 (Helmling *et al.*, 2004; Becskei *et al.*, 2008). Thus, vaccination against ghrelin and the use of ghrelin spiegelmers may be useful in the treatment of obesity.

GHS-R is also an useful target for obesity drugs. For many years, several classes of GHS-R1a antagonists have been developed (Sato *et al.*, 2014). A representative GHS-R1a antagonist is [D-Lys3-]GHRP-6. By treatment of [D-Lys3-]GHRP-6, food intake is decreased and weight gain is reduced in lean and obese mice (Asakawa *et al.*, 2003; Beck *et al.*, 2004). Piperidine-substituted quinazolinone derivatives were identified as a novel class of small GHS-R1a antagonist molecules (Rudolph *et al.*, 2007). A piperidine substituted quinazolinone derivative, YIL-781, improved glucose-stimulated insulin secretion and reduced food intake in diet-induced obese mice (Esler *et al.*, 2007). Thus, a certain result on obesity is expected from GHS-R1a antagonists.

Inverse GHS-R1a agonist may also be one of the candidates. In fact, an inverse agonist, such as [D-Arg1, D-Phe5, D-Trp7,9, Leu11] substance P, may be a candidate to regulate ghrelin action (Holst and Schwartz, 2004). Reduction of the GHS-R1a constitutive activity by an inverse agonist could increase the sensitivity to anorexigenic hormones like leptin, and then, inhibit food intake.

GOAT has been implicated as another attractive and potential target for the development of antiobesity treatment. Barnett *et al.* described the design, synthesis, and characterization of GO-CoA-Tat, a peptide-based bisubstrate analog that antagonizes GOAT (Barnett *et al.*, 2010). Intraperitoneal administration of GO-CoA-Tat improves glucose tolerance and reduces weight gain in wild-type but not ghrelin-deficient mice (Barnett *et al.*, 2010); thus, its beneficial metabolic effects are due specifically to GOAT inhibition. Moreover, quantitative magnetic resonance measurements showed that, relative to controls, GO-CoA-Tat treated animals displayed significantly lower fat mass, but not lean mass (Barnett *et al.*, 2010). GOAT is therefore a potentially useful target for future development of therapeutic compounds.

Bariatric surgery, weight loss and improvement in glucose tolerance and role of ghrelin

Currently used bariatric procedures are classified as restrictive, malabsorptive, or combined, according to the mechanism of weight loss. Restrictive procedures, such as laparoscopic adjustable gastric banding (LAGB), vertical banded gastroplasty (VBG), and sleeve gastrectomy (SG) greatly reduce the volume of the stomach in order to decrease food intake and induce early satiety. In particular, SG totally removes the gastric fundus. Malabsorptive techniques, such as biliopancreatic diversion with duodenal switch (BPD/DS), shorten the small intestine to decrease nutrient absorption while combined procedures, such as the Roux-en-Y gastric bypass (RYGB, considered as gold standard procedure for bariatric surgery), incorporate both restrictive and malabsorptive elements. RYGB additionally creates malabsorption by the rapid shunt of undigested food to the distal small intestine (Vetter *et al.*, 2009).

Both short and long-term effects of bariatric surgery upon ghrelin concentration are still unclear. Different bariatric procedures seem to have different effects on ghrelin secretion, possibly due to anatomical variations influencing the stomach volume and the degree of contact between ingested nutrients and gastric mucosa where ghrelin producing cells are located (Meek *et al.*, 2016).

Although an empty stomach is associated with an increased ghrelin level in the short term, it is possible that the permanent absence of food in the stomach and duodenum causes a continuous stimulatory signal that ultimately suppresses ghrelin production through a process of "override inhibition" (Cummings *et al.*, 2002). Moreover, ghrelin levels are affected by the vagus nerve and tend to be higher after procedures that preserve the vagal fibers, such as LAGB, in comparison with RYGB (Vetter *et al.*, 2009).

The effects of RYGB on ghrelin levels are contradictory (Frühbeck *et al.*, 2004): although many studies report decreased ghrelin levels after surgery (Cummings *et al.*, 2002), others report no changes (Faraj *et al.*, 2003; Yousseif *et al.*, 2014) or increased concentrations (Holdstock *et al.*, 2003; Kalinowski *et al.*, 2017). Moreover, Nergård and colleagues demonstrated that the density of ghrelin cells and ghrelin mRNA levels did not differ 12 months after RYGB (Nergård *et al.*, 2015). These disparate effects could be due to variations in surgical technique resulting in different dimensions and consequent different amount of intact ghrelin-producing tissue (Vetter *et al.*, 2009). In fact, when present, the reduction in fasting ghrelin after RYGB occurs as early as after surgery (2–6 weeks), suggesting that its reduction is mostly a consequence of altered anatomy (Jeon *et al.*, 2004).

Sleeve gastrectomy may decrease circulating AG concentrations, possibly due to the removal of ghrelin-producing cells in the stomach (Anderson *et al.*, 2013; Yousseif *et al.*, 2014; Kalinowski *et al.*, 2017). Hypothetically, the additional mechanism of reduced appetite post SG, due to the significant lowering of ghrelin levels, could compensate the lack of the malabsorption effect which does not occur with a restrictive surgery as SG (Makris *et al.*, 2017).

However, weight loss itself may play a central role in regulating plasma ghrelin levels. The increase in fasting ghrelin after LAGB seems to occur over time, suggesting a predominant role of weight loss instead of restriction of nutrient flow (Korner *et al.*, 2009). Ghrelin levels after RYGB remain unchanged in weight-stable patients but increase by approximately 60% in

weight-reducing subjects, suggesting that weight loss and negative energy balance may be an important determinant of postsurgical ghrelin levels (Faraj *et al.*, 2003). No correlations between decrease in ghrelin concentrations and change in appetite were found (Bužga *et al.*, 2014).

These data suggest that the role of ghrelin in the induction and maintenance of weight loss after bariatric surgery might be marginal although procedures in which ghrelin is decreased could benefit from the additional inhibition of feeding resulting from a reduced ghrelin action (Ionut *et al.*, 2013).

A summary of the described effects of bariatric surgery on ghrelin levels is provided in **Table 3**.

Changes in ghrelin concentrations induced by bariatric surgery may contribute to improvement of glycaemic metabolism and remission of diabetes (Ramón *et al.*, 2012).

Beneficial effects on glucose metabolism, including remission of type 2 diabetes mellitus (T2DM), occurs in 48% and 84% of patients who underwent LAGB and RYGB respectively (Korner *et al.*, 2009), but those percentages can vary depending on the different thresholds used to define the improvement of glycaemic control.

The rate of remission also depends on the phenotype of the patients: short diabetes duration, reliable β-cell function (assessed by C-peptide measurements), and absence of insulin requirement are predictors of type 2 diabetes remission after bariatric surgery (Madsbad *et al.*, 2014). In fact, in earlier studies of RYGB, longer duration of diabetes (>10 years), poor preoperative glycaemic control and preoperative insulin use reduced the probability of diabetes resolution (Schauer *et al.*, 2003). Similarly, Dixon and colleagues reported that a short duration of disease (<3 years) could predict T2DM remission after LAGB (Dixon *et al.*, 2003).

Another important factor involved in improvement of glycaemic control is the percentage in excess weight loss (%EWL) after surgery (Hady *et al.*, 2012); a remission of symptoms of T2DM after LAGB was observed in patients with higher %EWL (Polat *et al.*, 2010). In comparative studies for LAGB, RYGB, and BPD/DS the percentage of patients with T2DM symptoms remission in a 5 year follow-up was similar for all procedures, excluding differences in %EWL (Hady *et al.*, 2012).

Changes in plasmatic concentration of gut hormones (GLP-1 and GIP) might also play a central role in T2DM remission. Korner *et al*. reported that postprandial GLP-1 levels after RYGB were significantly greater than presurgical values and higher when compared with LAGB (Korner *et al.*, 2009). Furthermore, GLP-1 cell density was 4.9-fold higher 12 months after RYGB; a larger increase in GLP-1 cell density was evident in patients with longer biliopancreatic limb (200 cm) (Nergård *et al.*, 2015). Finally, low deterioration in beta cell function may maximize the effect of surgery in increasing plasmatic levels of gut peptides (mainly GLP-1) that enhance insulin secretion (Vetter *et al.*, 2009).

In conclusion, improvements in glycaemic control in diabetic patients undergoing bariatric surgery are probably mediated by several mechanisms, including caloric restriction, increased satiety and change in plasmatic concentration of hormones such as ghrelin, GLP-1, GIP, and PYY. Collectively, caloric restriction and alterations in the enteroinsular axis probably affect both insulin secretion and sensitivity, resulting in improvement or remission of T2DM (Vetter *et al.*, 2009).

Effects on the Cardiovascular System

Ghrelin has been proposed to act on the cardiovascular system in a direct and an indirect way. Ghrelin could have beneficial effects on the cardiovascular system in a direct way, through an increase in GH activity, similar to what is seen during GH replacement therapy in GH-deficient patients (Amato *et al.*, 1993). Ghrelin could act directly on the cardiovascular system, binding GHS receptors in the myocardium and blood vessels (Katugampola *et al.*, 2001). In fact, ghrelin is demonstrated to dilate human arteries (Okumura *et al.*, 2002) in an endothelium-independent mode. In addition, ghrelin inhibits apoptosis of cultured cardiomyocytes and endothelial cells possibly through activation of extracellular signal-regulated kinase-1/2 and Akt serine kinases (Baldanzi *et al.*, 2002).

In humans, Nagaya *et al*. studied the cardiovascular effects of 10 mg/kg ghrelin in healthy volunteers (Nagaya *et al.*, 2001). In this study, plasma GH levels increased 15-fold 20 min after i.v. ghrelin administration and there was also an increase in epinephrine concentrations, while norepinephrine levels did not change. It was noted that ghrelin treatment produced a decrease of

Table 3 Effects of bariatric surgery on ghrelin levels compared with the condition of obesity

	Ghrelin	*References*
Obesity (without surgery)	↓ Fasting and postprandial	Sherif *et al.* (2011)
Laparoscopic adjustable gastric banding (LAGB)	↑	Korner *et al.* (2009)
Sleeve gastrectomy (SG)	↓ Fasting and postprandial	Anderson *et al.* (2013), Yousseif *et al.* (2014), and Kalinowski *et al.* (2017)
Roux-en-Y gastric bypass (RYGB)	↓	Frühbeck *et al.* (2004)
	↓	Cummings *et al.* (2002)
	↔	Faraj *et al.* (2003)
	↔	Yousseif *et al.* (2014)
	↑	Kalinowski *et al.* (2017)
	↑	Holdstock *et al.* (2003)

systemic vascular resistance and mean arterial pressure and increased cardiac index and stroke volume index in normal subjects and in patients with chronic heart failure (Nagaya *et al.*, 2001). Thus, since the administration of ghrelin has been demonstrated to decrease blood pressure, reduce cardiac afterload and increase cardiac output without affecting heart rate in humans and in animals (Matsumura *et al.*, 2002; Nagaya *et al.*, 2001), the therapeutic potentials of ghrelin in different cardiac diseases have been speculated. In patients with congestive heart failure, for instance, ghrelin intravenous administration for 3 weeks significantly improved left ventricular ejection fraction (from 27% to 31%; $P < .05$) and increased peak workload and peak oxygen consumption during exercise, which was accompanied by a dramatic decrease in plasma norepinephrine (from 1132 to 655 pg/mL; $P < .001$) (Nagaya *et al.*, 2004; Kishimoto *et al.*, 2012).

Antiproliferative Effects

Ghrelin and its receptor are now known to be expressed not only in normal, but also in malignant tissues. GHS-R have been also found in neoplastic endocrine and nonendocrine tissues even from organs that do not express these receptors under physiological conditions, such as the breast. The first suggestion that the ghrelin/GHSR1a axis could be involved in cancer progression, before the discovery of ghrelin itself, was the finding that the GHSR1a is expressed not only in normal pituitary, but also in pituitary and neuroendocrine tumors (Korbonits *et al.*, 1998). Then, soon after its discovery, it was speculated that ghrelin and GHSR1a could play a role in pituitary tumor pathogenesis through an autocrine or paracrine pathway (Kim *et al.*, 2001) by modulating pituitary hormone release (Korbonits *et al.*, 2001). Ghrelin was later demonstrated to increase cell proliferation in the rat pituitary somatotroph GH3 cell line through the ERK1/2 pathway (Nanzer *et al.*, 2004).

Ghrelin and its receptor may be an autocrine/paracrine growth factor in a number of cancer tissues (Chopin *et al.*, 2012). The first functional study indicating that ghrelin treatment stimulated cell proliferation was about the HepG2 hepatoma cell line (Murata *et al.*, 2002). Then other studies followed: human leukaemic cell lines, in adrenocortical carcinoma, in pancreatic adenocarcinoma, in colorectal cancer, in prostate, breast and endometrial cell lines (Chopin *et al.*, 2011). However, some reports indicate that ghrelin may inhibit cell proliferation: in thyroid, prostate, breast, and small cell lung carcinoma cell lines (Chopin *et al.*, 2011). These discrepancies could be explained by the different ghrelin concentration used: for example the application of supraphysiological doses of ghrelin in prostate cancer cell lines could have an inhibitory effect, while physiological levels could stimulate cell proliferation (Lanfranco *et al.*, 2008b). As there have been a number of conflicting results, it is now unclear whether ghrelin promotes cancer or inhibits its development and further studies are required.

Conclusions

Ghrelin, a 28-residue acylated peptide predominantly produced by the stomach, displays a strong GH-releasing action but also shows other central and peripheral endocrine and nonendocrine actions via the activation of the GHS-R. Indeed, ghrelin isolation helped elucidate GH–IGF-I physiology and the hypothalamic appetite-regulating pathways but also opened new promising perspectives in other medical fields, such as internal medicine, oncology, and cardiology. Nowadays ghrelin and/or GHS analogues have certainly a diagnostic application and researchers are working on the possibility that ghrelin has a potential therapeutic role, mostly in glico-metabolic fields.

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Luteinizing Hormone (LH)☆

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Introduction

Luteinizing hormone (LH) is a glycoprotein secreted by the pituitary gland in a pulsatile fashion and, in concert with follicle-stimulating hormone (FSH), plays a central role in reproduction. LH acts directly on the gonads, binding to its receptor on the surface of the target cells and initiating a cascade of molecular events beginning with the activation of specific signaling pathways and ending with the expression of gene products. In the ovary, LH stimulates (1) the outer layer of the primary follicle, the theca cells, to produce androgens to be converted to estrogens during folliculogenesis; (2) ovulation; and (3) the formation of the corpus luteum, which secretes progesterone. These structures and their products prepare the female reproductive tract for fertilization, implantation, and pregnancy. In the testis, this gonadotropin stimulates the synthesis of androgens by the Leydig cells. Androgens are required for fetal development of the male phenotype, for sexual maturation of male mammals, and for spermatogenesis in the adult testis. In both sexes, the gonadal products of LH stimulation that enter the circulation regulate the secretion of this gonadotropin by modifying the release of the hypothalamic peptides, kisspeptin and gonadotropin-releasing hormone (GnRH), or acting directly on the pituitary gland.

Molecular Structure

LH is a protein composed of two glycosylated polypeptide subunits, designated α and β, and has a molecular weight of approximately 28 kDa, varying with the species and the length of the added oligosaccharides. The α-subunit (14 kDa) is common to two other glycoprotein hormones, namely, FSH, chorionic gonadotropin (CG) and thyroid-stimulating hormone (TSH) (Ascoli *et al.*, 2002). Especially, in primates, the placenta secretes CG during pregnancy, that has more oligosaccharide moieties and a longer carboxyl-terminal peptide in its β-subunit. These modifications confer a longer half-life on CG in the circulation, leading to a more sustained action (Fournier *et al.*, 2015). Acting on the LH receptor (LHCGR), human CG (hCG) may replace LH during treatments of infertility. The β-subunit is unique to each glycosylated hormone and is specific for receptor binding, thus conferring biologic activity to the hormone. Although the α-subunit alone may be secreted and may achieve relatively high concentrations in the blood, no function for the free α-subunit per se has been demonstrated. However, α-subunit interacts with LHCGR during binding (Kreuchwig *et al.*, 2013).

Carbohydrate groups are present at specific locations on each subunit and are important determinants of the biological activity and metabolic clearance of LH. The oligosaccharide moieties are coupled through *N*-acetylglucosamine at a specific amino acid, asparagine. The α-subunit of human LH has two sites, while one site is in the β-subunit. The oligosaccharides, containing the monosaccharides mannose, galactose, glucosamine, *N*-acetylgalactosamine, and sialic acid, are branched, heterogeneously, and terminate mainly in sulphate and fucose. These differences are largely responsible for the variation in isoelectric point (6–10) of this gonadotropin. The alkaline forms are biologically more active in bioassays, but are also more rapidly cleared from the circulation. Removal of terminal sialic acid residues from the oligosaccharides reduces the half-life of LH in circulation, but has little effect on the binding of LH to its receptor (Arey and López, 2011).

Synthesis and Secretion

Human LH β-subunit is encoded by the *LHB* gene located on chromosome 19q13.3. It falls within a genetic cluster spanning about 40 Kbase-pairs and comprising eight genes, encoding hCG β-subunits (*CGBs*), likely originated by repeated duplications of an ancestral *LHB* gene. It was theorized that, during ancient evolution of primates, *CGB* genes were subjected to frame-shift mutations and nucleotide insertions producing 24 additional codons and a variety of CG molecules (Hallast *et al.*, 2005).

The pituitary cells that synthesize LH are called gonadotropes and they can be placed in one of three categories based on the results of immunocytochemical staining. The majority of the gonadotropes synthesize both LH and FSH, whereas the two other categories are composed of cells that exclusively synthesize either FSH or LH. The gonadotropes are stimulated by the

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hypothalamic polypeptide, GnRH, which secretion is pulsatile. GnRH is released by neurons that terminate on the blood vessels running along the pituitary stalk. GnRH release occurs under the stimulation of a peptide product named kisspeptin, which was described in several mammalian species as a stimulator of the gonadotrophic axis and an activator of GnRH neurons at puberty (Tena-Sempere, 2006). Kisspeptin is produced mainly in the hypothalamus and regulated upon a feedback mechanism played by steroid hormones and acts through a G protein-coupled receptor (GPCR) (**Fig. 1**). It supports the pulses of GnRH to the gonadotropes in the pituitary gland and stimulate the synthesis and release of LH. Accordingly, LH is also released from the pituitary gland in pulses. The frequency of the GnRH pulses results in concurrent, selective pulsatile secretion of LH or FSH (Thompson and Kaiser, 2014). GnRH binds to a GPCR on the surface of the gonadotrope cells, activating the Gq/11 proteins, which, in turn, stimulate phospholipase C. This enzyme generates inositol 1,4,5-triphosphate and diacylglycerol. The increased levels of these intracellular messengers activate protein kinase C (PKC) and increase intracellular calcium also. PKC and calcium regulate the expression of the *LHB* gene, whereas the secretion of LH is regulated by the increase in intracellular calcium (Millar, 2005; Caunt *et al.*, 2006).

Differently to LH, hCG is released by trophoblast cells during pregnancy in a constant, increasing manner, achieving the peak at about 3 months of gestation. Several hCG isoforms and glycosylation variants are produced, resulting in a number of molecules featuring different half-life and functions not completely characterized (Evans *et al.*, 2015). This hormone mediates trophoblast invasion of maternal tissues, angiogenesis and progesterone production acting through LHCGR. Moreover, it mediates thyroid hormones production, at least in part, cross-reacting with the TSH receptor. A relatively low amount of pituitary hCG was also described, but its function is still to be elucidated.

Receptor and Receptor Defects

LHCGR is predominantly in the gonads of each sex, although extragonadal receptors with not fully clear functions have been found in tumors as well as other tissues. LHCGR (85–95 kDa) is a member of the family of GPCRs; therefore, it is composed of three domains: a transmembrane domain, an extracellular domain, and an intracellular domain (Troppmann *et al.*, 2013). The transmembrane domain is composed of seven hydrophobic stretches of 20–25 amino acids that form α-helices traversing the cellular membrane alternating between intracellular and extracellular loops. The extracellular domain of the receptor binds LH, which, in turn, causes the conformational change transmitted through a hinge region to the intracellular domain, activating the Gαs protein. This is the first step in an intracellular cascade that continues with the activation of the enzyme adenylyl cyclase and the conversion of adenosine tri-phosphate (ATP) to 3′,5′-cyclic adenosine mono-phosphate (cAMP). In turn, protein kinase A (PKA) is activated and phosphorylation of the transcription factor CREB (cAMP regulatory element-binding protein) leads to the expression of genes coding for several factors regulating steroid synthesis, cell proliferation and differentiation. The activation of this intracellular signaling cascade, known as cAMP/PKA-pathway, may be linked to pro-apoptotic signals via a complex intracellular cross-talk, resulting in TP53 gene expression, p53 activation, procaspase 3 cleavage and apoptosis (Amsterdam *et al.*, 1999;

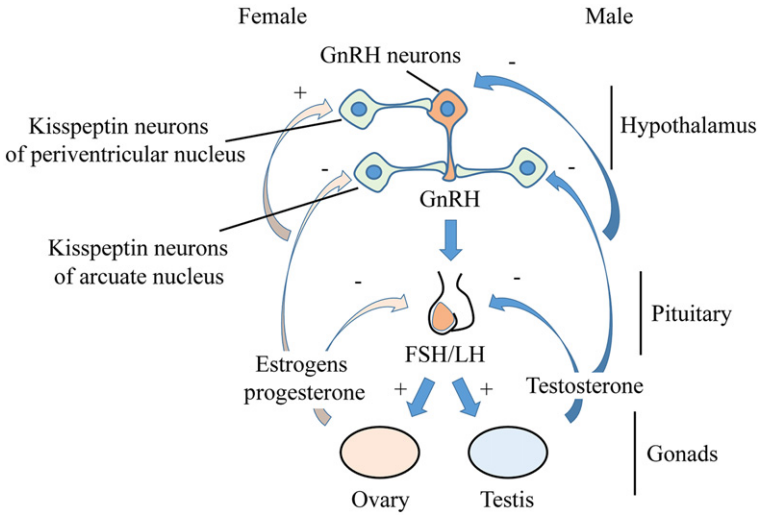

Fig. 1 Feedback controls of gonadotropins' production. In the hypothalamus, kisspeptin neurons stimulate GnRH secretion under the control of steroid feedback. GnRH release is pulsatile and induces the release of LH and FSH by the anterior pituitary. In the gonads, gonadotropins promote gamete formation and the production of steroid hormones, which, in turn, regulate GnRH release by a feedback mechanism. In females, steroid hormones estrogens and progesterone exert a positive feedback on kisspeptin neurons of the periventricular nucleus, inducing the preovulatory surge of GnRH and LH. On the contrary, estrogens and progesterone inhibit kisspeptin production in the arcuate nucleus. In the male, testosterone exerts a negative feedback on kisspeptin neurons of the arcuate nucleus, as well as on GnRH neurons and pituitary, inhibiting GnRH and gonadotropins release.

Casarini *et al.*, 2016a,b). This pathway has been expanded to include various isoforms of adenylyl cyclase, phosphodiesterase, and anchoring proteins of PKA. Other transcription factors, for example, stimulatory protein 1, upstream stimulatory factor, and estrogen receptor α and β, have been shown to be activated by high intracellular concentrations of cAMP. In addition, CREB can be phosphorylated by kinases other than PKA, such as extracellular-regulated kinase 1/2 (ERK1/2) (Menon and Menon, 2012). ERK1/2 is rapidly recruited by PKA (1–5 min after ligand-receptor binding) and has the dual role to amplify the steroidogenic signal mediated through CREB, and, in parallel, trigger the recruitment of GPCR kinases, which catalyze the phosphorylation of LHCGR at the intracellular level. The phosphorylated receptor is a target of other intracellular interactors, such as β-arrestins, which bind LHCGR before internalization as a system for cell desensitization (Musnier *et al.*, 2010). Moreover, β-arrestins mediate a late ERK1/2 phosphorylation (5–15 min), important for the maintenance of steroidogenic and proliferative signals. Since LHCGR may be coupled to different types of G proteins, several signaling pathways may be simultaneously activated upon hormone binding, such as the antiapoptotic phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, thus resulting in a complex balance between life and death signals and determining the cell fate (Amsterdam *et al.*, 2003).

The gene encoding LHCGR contains 11 exons, and several splice variants have been identified. Exons 1–10 encode the N-terminal and extracellular domain, as well as part of the hinge region, while exon 11 encodes the remaining part of the hinge region, the transmembrane and intracellular G protein-activating domains. Several LHCGR mutations have been identified, resulting either in constitutive activation or in inactivation (Latronico and Arnhold, 2012; Ulloa-Aguirre *et al.*, 2014). Activating mutations of LHCGR in boy results in precocious puberty observed at 1–4 years of age. Activating mutations in girls do not lead to precocious puberty because the gonadotropin FSH is necessary to stimulate folliculogenesis. However, polymorphic LHCGR or LHβ variants may impact on LH signaling resulting in hyperandrogenic females affected by pathological conditions linked to androgen excess, such as polycystic ovary syndrome (Rosenfield and Ehrmann, 2016). Mutations that completely inactivate LHCGR in boys result in genetically male infants displaying female external genitalia but having abdominal testes and lacking Müllerian structures. Interestingly, inactivation of LH-mediated signaling may occur due to mutations within untranslated *LHCGR* short variants, causing male pseudohermaphroditism (Kossack *et al.*, 2008). These variants are produced at the mRNA level as truncated forms at the level of exon 6 and display an additional, primate-specific exon (exon 6A) featuring stop codons. They are highly expressed in Leydig and granulosa cells where trigger nonsense-mediated mRNA decay (NMD). Mutations leading to aberrant amount of exon 6A transcripts result in *LHCGR* destruction by NMD and are linked to the pathological phenotype. Mutations that do not completely inactivate the LH receptor in boys, however, result in male infants with micropenis sometimes associated with hypospadia, but the intra-abdominal testes have few, if any, androgen-secreting Leydig cells. Deletions of *LHCGR* exon 10 are associated to hypogonadism due to impaired LH signaling (Müller *et al.*, 2003). In patients carrying homozygous mutant LHCGR, testosterone synthesis may be restored upon treatment by hCG, revealing that exon 10 encodes part of the receptor fundamental for differentiating LH- and hCG-specific signaling (Grzesik *et al.*, 2015). Interestingly, in the New World monkey *Callithrix jacchus*, pituitary messenger RNAs corresponding to choriogonadotropin transcripts were found. This may be an endocrine adaptation of the pituitary-gonadal axis, which molecules able to bind Lhcgr naturally lacking the amino acid sequence from exon 10 were encoded (Gromoll *et al.*, 2003). In girls, inactivating mutations of the LH receptor, either complete or incomplete, result in normal primary and secondary female characteristics, but also result in amenorrhea because LH is required for folliculogenesis.

Action in the Ovary

During fetal development of the female mammal, the primitive germ cells in the putative ovary enter meiosis and arrest in the prophase of meiosis I. Each oocyte is surrounded by a single layer of granulosa cells, and soon after formation, these primordial follicles enter a resting phase. The mechanisms that stimulate the primordial follicles to initiate folliculogenesis are unknown, but the process begins at once and continues until all follicles are depleted from the ovary. During fetal life, at least in primates and ruminants and within the first 2 weeks after birth in rodents, some of the primordial follicles are activated and become primary follicles. Two layers of cells develop around the oocyte: the outermost are the theca cells that are separated from the innermost layers of granulosa cells by a basement membrane.

The rupture of the follicle, along with the release of the oocyte and some of the granulosa cells surrounding it, is called ovulation. During the periovulatory period, antiinflammatory factors are produced and appear to aid in rapid healing and vascularization of the ruptured follicle. The theca and granulosa cells remaining in the ruptured follicle differentiate into luteal cells, forming the corpus luteum, which characterizes the luteal phase of the ovarian cycle. If no pregnancy occurs, after a finite time specific to each species, the corpus luteum dies, marking the end of the luteal phase. The rapid involution of the corpus luteum is called luteolysis and is regulated by specific factors. Luteolysis is necessary before a subsequent ovarian cycle can begin. The follicular phase, ovulation, the luteal phase, and luteolysis constitute the ovarian cycle and LH plays some role in all phases of the cycle (Atwood and Vadakkadath Meethal, 2016).

LH has three functions in the ovary: (1) stimulation of androgen synthesis during the follicular phase; (2) stimulation of ovulation; and (3) following ovulation, differentiation of the thecal and granulosa cell remnants of the ruptured follicle into luteinized cells. The luteal cells constitute the steroidogenic cells of the corpus luteum, shifting from predominantly estrogen-producing cells to progesterone-producing cells.

LH binds to its receptor on the theca cells of the follicle and stimulates the synthesis of androgens. Steroidogenesis begins with the movement of cholesterol from its storage site on the outer mitochondrial membrane to the inner mitochondrial membrane, where the cytochrome P450-associated enzyme removes the side chain of cholesterol, producing pregnenolone. This mobilization of cholesterol is the rate-limiting step in steroidogenesis and LH, as well as FSH, regulates the production of a protein called steroidogenic acute regulatory (StAR) protein (Miller and Auchus, 2011). The tertiary structure of this 30 kDa protein has a domain containing a cholesterol-binding hydrophobic tunnel that may permit StAR to shuttle cholesterol across the mitochondrial membranes. The steroids are lipid-soluble, unlike cholesterol, and the steroidogenic enzymes are readily accessible for production of the androgen, androstenedione. This androgen diffuses out of the thecal cells and into the granulosa cells, where the enzyme aromatase, stimulated by FSH action, converts androstenedione into estradiol. As the theca and granulosa cells of the maturing follicles proliferate, a greater amount of estradiol is produced, which triggers proliferative signaling in the follicles. Moreover, the number of LH receptors on each granulosa cell is increased under FSH stimulation, leading to increased sensitivity to the hormone mediating both proliferative and antiapoptotic signals via ERK1/2, AKT-, and other pathways (Kayampilly and Menon, 2009). With the increased number of LH receptors on the theca and granulosa cells, only those follicles with sufficient LH stimulation will continue folliculogenesis. Once the circulating estradiol produced by the antral follicle is elevated, this steroid causes an abrupt release, or surge, of LH from the pituitary. The surge of LH causes ovulation by increasing the expression of PKA and increasing the inositol lipid hydrolysis that activates PKC, shifting the pattern of gene expression in the granulosa cells. The shift results in the suppression of cell division and change of the intracellular enzymatic milieu of the granulosa cells, signaling of the oocyte to reenter meiosis, and rupture of the follicle wall.

After ovulation, the luteinized granulosa and theca cells remaining in the ruptured follicle begin to synthesize progesterone. Although the luteal cells of some species, for example, human, are able to produce small amounts of estrogen, the cellular differentiation leads to primarily, or, in some species, only progesterone production by luteal cells. This hormone prepares the endometrium of the uterus to be receptive for the blastocyst, if fertilization of the oocyte occurs, and exerts a negative feedback on GnRH and gonadotropin production by pituitary. The corpus luteum under LH regulation continues to survive for a specific period of time, depending on the species. If, on one hand, no blastocyst is formed, then the corpus luteum involutes through programmed cell death, as a consequence of declined LH levels. If, on the other hand, implantation of a blastocyst occurs, hCG secreted by the blastocyst and later by the placenta binds to LHCGR on the corpus luteum, at least in human, rescuing it from luteolysis and maintaining the production of progesterone. hCG has a greater potency in activating cAMP-mediated signals (**Fig. 2**), such as steroidogenesis, and a longer half-life in the circulation than LH itself, which, instead, retains higher proliferative and antiapoptotic potential, at least in granulosa cells (Casarini *et al.*, 2012; Riccetti *et al.*, 2017; Casarini *et al.*, 2017). These gonadotropin-specific features reflect the different physiological role of the two hormones, providing LH as a mediator of proliferative stimuli occurring in folliculogenesis, while hCG is suitable for massive progesterone synthesis supporting pregnancy (Casarini *et al.*, 2016a,b).

Action in the Testis

In the testis, LH acts through binding to its receptor expressed in the Leydig cells, stimulating the production of the androgenic steroid, testosterone. The adult mammalian testis is an ovoid structure covered by a capsule called the tunica albuginea. The parenchyma of the organ is composed of two compartments, the seminiferous tubules and the interstitium between the tubules. The seminiferous tubules contain the epithelium where spermatogenesis takes place. The interstitium contains the Leydig cells, fibroblasts, macrophages, nerves, blood, and lymphatic vessels.

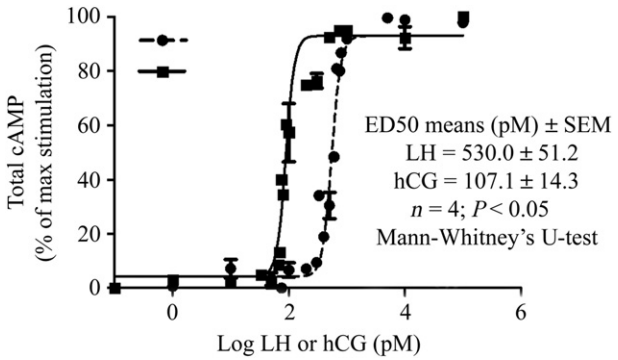

Fig. 2 Different potency of LH and hCG in activating cAMP. In LHCGR-transfected COS-7 cells, total cAMP production was evaluated upon 3-h treatment by recombinant LH and hCG. Statistical analysis revealed different effective dose 50% (ED50) between LH and hCG, demonstrating that hCG is about fivefold more potent than LH in inducing cAMP production (Mean ± SEM; Mann-Whitney's U-test; $P < .05$; $n = 4$). Image extracted and modified from the original article under Creative Commons Attribution license (Casarini, L., Lispi, M., Longobardi, S., *et al.* (2012). LH and hCG action on the same receptor results in quantitatively and qualitatively different intracellular signalling. *PloS One*, **7**, e46682).

Steroidogenesis begins in the Leydig cell, as it does in the theca cell of the ovary as a counterpart of female, with the movement of cholesterol from its storage site on the outer mitochondrial membrane to the inner mitochondrial membrane, where the cytochrome P450-associated enzyme removes the side chain of cholesterol, producing pregnenolone. This mobilization of cholesterol is the rate-limiting step in steroidogenesis and LH regulates the production of StAR protein. The steroids are lipid-soluble, unlike cholesterol, and the steroidogenic enzymes are readily accessible for production of the androgen, testosterone (**Fig. 3**). This androgen is a major stimulator of spermatogenesis in the adult male mammal. Since hCG binds LHCGR and stimulates StAR production, exogenous administration of the pregnancy hormone results in testosterone synthesis by male Leydig cells, providing a strategy to treats the hypogonadotropic hypogonadism in males (Pitteloud and Dwyer, 2014).

Testosterone is an important regulator of the spermatogenesis. This process starts from diploid, spermatogenetic stem cells called spermatogonia which, in human, exist as type Ad and the more differentiated Ap spermatogonia. The latter undergo mitosis forming type B spermatogonia. This final generation of differentiated spermatogonia undergoes mitosis and produce the primary spermatocytes. Spermatocytes are the germ cells that undergo meiosis and the products of this cell division are the haploid spermatids. The spermatids enter spermiogenesis, during which the nuclear contents condense, the specialized lysosome, the acrosome, is formed, a tail is produced, and the amount of cytoplasm is reduced. The duration of the whole spermatogenesis is of about 74 days in human and is structurally and metabolically supported by Sertoli cells.

Sertoli cells mediate hormonal effects on the seminiferous epithelium and on spermatogenesis, and their activities are regulated mainly by FSH. These cells are the only somatic component of the epithelium and provide the cytoarchitecture of the seminiferous epithelium, nourish the germ cells, and secrete the hormone inhibin B for pituitary control of the spermatogenesis. Moreover, Sertoli cells produce the androgen-binding protein (ABP), which binds testosterone resulting in the accumulation of the hormone in proximity of gametes. Testosterone plays a central role in the regulation of spermatogenesis, especially for the progress of meiotic phases. The hormone acts through the nuclear, androgen receptor (AR) expressed in Sertoli cells, sperms and other peripheral tissues. The functional significance of testosterone on spermatogenesis was provided by studies in complete AR knockout (ARKO) and Sertoli cell-selective AR knockout (SCARKO) mice (Tan *et al.*, 2005). All these knockout models displayed impairment of normal spermatogenesis, but ARKO revealed a more severe phenotype than SCARKO mice. Especially, Sertoli and Leydig cell number was extremely decreased in ARKO mice compared to controls, while it was slightly reduced in SCARKO mice, which even maintained the capacity to support spermatogonia by Sertoli cells. These data support the concepts that AR is not expressed exclusively in Sertoli cells and androgens may acts on other gonadal cells.

Most importantly, endocrine disrupting chemicals have recently attracted the attention of several researches, since they may impact on fertility affecting steroid hormones signaling and, therefore, resulting in low sperm quality and decreased sexual functions. For instance, the organic synthetic compound bisphenol A mimics the effects of estrogens and acts as an androgen antagonist injuring Sertoli cells (Rochester, 2013).

Pituitary plays a central role in stimulating spermatogenesis (Steinberger, 1971). Surgical removal of the pituitary gland of adult rats led to a decrease in testicular weight and the seminiferous epithelium comprised Sertoli cells and germ cells as mature as spermatids. Hypophysectomy of adult rhesus monkeys resulted in a precipitous decline in testicular size associated with the complete regression of the seminiferous epithelium to the extent that the tissue comprised only Sertoli cells and type A spermatogonia, that is, only stem cells. Further experiments on adult rodents and monkeys have confirmed these earlier observations. GnRH receptor antagonist, which suppresses all gonadotropin secretion, chemical hypophysectomy achieved the same results as observed using a surgical hypophysectomy (Matthiesson and McLachlan, 2006). Namely, the rodent seminiferous epithelium

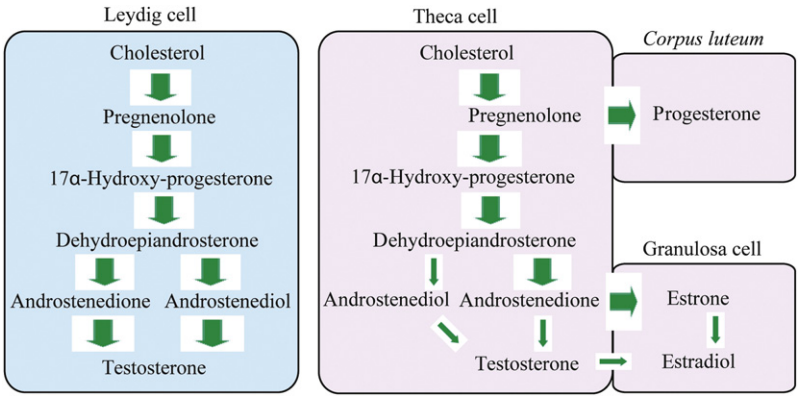

Fig. 3 Comparison between Leydig and theca cells steroidogenesis. In Leydig cells, cholesterol is efficiently converted to testosterone, as a major product obtained *via* both androstenedione and androstenediol under stimulation by LH. In the ovary, granulosa cells convert cholesterol to progesterone and its precursor pregnenolone. The latter is the substrate for progesterone production in the *corpus luteum*, during the luteal phase, as well as to androgens in theca cells stimulated by LH. Androstenedione is the primary product of theca cells, serving as a substrate for the aromatase enzyme and estradiol production by FSH-stimulated granulosa cells. *Large arrows* indicate major steroidogenic pathways, minor pathways are shown by *thin arrows*.

comprised Sertoli cells and germ cells as mature as spermatids, whereas the primate seminiferous epithelium comprised only Sertoli cells and stem spermatogonia.

Physiological replacement of testosterone in rats that had been previously rendered hypogonadotropic results in full restoration of the seminiferous epithelium containing the normal number of spermatogenic cells. Testosterone alone is sufficient to stimulate spermatogenesis in this and related species (Huhtaniemi, 2015). Replacement of testosterone in hypogonadotropic, hypogonadal rhesus monkeys, however, results in stimulation of testicular growth but to less than normal size. The gonadal growth is due primarily to the stimulation of spermatogenesis by androgen, but morphometric analysis of the seminiferous epithelium of the monkeys revealed that the smaller testicular size was accounted for by a deficit in the number of all differentiated, or type B, spermatogonia. Replacement of FSH in testosterone-treated hypophysectomized adults results in a greater number of all four generations of type B spermatogonia. These observations led to the conclusion that testosterone alone stimulates spermatogenesis, but FSH is necessary to restore spermatogenesis completely in primates (Ramaswamy and Weinbauer, 2014). This action of FSH in primates is posited to be the rescue of the differentiated spermatogonia from programmed cell death.

Testosterone is also secreted into the circulatory system and transported to the male reproductive tract, stimulating growth and differentiation. The androgen also causes the increased body size, muscle mass, and behavior characteristic of male mammals. In the case of humans, beard growth and male pattern baldness are also manifestations of testosterone action. Testosterone acts to reduce the secretion of LH exerting a negative feedback at different levels. It induces the inhibition of LH secretion by direct action on the anterior pituitary, as well as on the GnRH- and kisspeptin-secreting neurons in the hypothalamus.

Fetal Development of the Male Reproductive System

Fetal development of the male reproductive system requires the presence of a testis capable of secreting two hormones, testosterone and Müllerian inhibiting hormone. The formation of a testis requires the action of the testis-determining factor (SDF) protein, encoded by the sex-determining region Y gene (*SRY*) (Windley and Wilhelm, 2016). This factor causes the undifferentiated gonad to develop into a testis. If this factor is absent or defective, a testis will not form and a female phenotype will be produced. The fetal testis produces testosterone, which stimulates the Wolffian ducts to differentiate into the male reproductive tract consisting of the epididymis, vas deferens, seminal vesicle, and prostate. Testosterone also causes the formation of the penis and scrotum into which the testes descend. Testosterone is produced in the fetal testis by Leydig cells and Müllerian inhibiting hormone is produced by the fetal Sertoli cells.

The stimulus for the fetal Leydig cells to produce testosterone cannot be the fetal pituitary since the secretion of testosterone precedes the effective secretion of LH by the fetal pituitary. In the case of higher primates, hCG may cause the fetal testis to form Leydig cells as well as stimulate those cells to secrete testosterone (O'Shaughnessy *et al.*, 2006). Interestingly, placental LH is produced in equids during pregnancy and is also called equine CG (eCG) (Hoppen, 2009). This molecule is differently glycosylated from pituitary LH and has longer half-life, although the β-subunit is encoded by the same *LHB* gene of pituitary LH. The situation in the other mammalian genera, however, must be different because these species do not produce a placental gonadotropin. In fact, human qualitative LH- and hCG-specific signaling and steroid synthesis is lost in primary Leydig cells expressing the rodent LH receptor, although it retains binding capability for both the hormones (Peltoketo *et al.*, 2011). Anyway, in rodents, the fetal Leydig cells seem to be autonomous; that is, they synthesize testosterone without the stimulus of a gonadotropin. In all mammalian species, the fetal Leydig cells cease producing androgens soon after birth and seem to disappear in the interstitium of the testes of newborns.

During sexual maturation at the time of puberty, the adult Leydig cells form from undifferentiated cells, called mesenchymal-like cells, in the interstitial spaces between the seminiferous tubules. In rats, these cells actively proliferate from 2 to 4 weeks after birth, but their numbers diminish, suggesting differentiation into a new type of cell. The interstitial cells that give rise to the adult Leydig cell population have been posited to be part of the population of mesenchymal-like cells that during uterine life produced the fetal Leydig cells. Nonetheless, these mesenchymal cells have a very high mitotic index of rapid and multiple cell divisions. The next generation of differentiated cells express the proteins of the adult Leydig cell, 3β-hydroxyl dehydrogenase and LH receptors. These latter cells have been called immature Leydig cells and divide once and produce the adult Leydig cell (Benton *et al.*, 1995).

Feedback controls of gonadotropins' production. In the hypothalamus, kisspeptin neurons stimulate GnRH secretion under the control of steroid feedback. GnRH release is pulsatile and induces the release of LH and FSH by the anterior pituitary. In the gonads, gonadotropins promote gamete formation and the production of steroid hormones, which, in turn, regulate GnRH release by a feedback mechanism. In females, steroid hormones estrogens and progesterone exert a positive feedback on kisspeptin neurons of the periventricular nucleus, inducing the preovulatory surge of GnRH and LH. On the contrary, estrogens and progesterone inhibit kisspeptin production in the arcuate nucleus. In the male, testosterone exerts a negative feedback on kisspeptin neurons of the arcuate nucleus, as well as on GnRH neurons and pituitary, inhibiting GnRH and gonadotropins release.

Different potency of LH and hCG in activating cAMP. In LHCGR-transfected COS-7 cells, total cAMP production was evaluated upon 3-h treatment by recombinant LH and hCG. Statistical analysis revealed different effective dose 50% (ED50) between LH and hCG, demonstrating that hCG is about fivefold more potent than LH in inducing cAMP production (Mean $\pm$ SEM; Mann-Whitney's U-test; $P < .05$; $n = 4$). Image extracted and modified from the original article under Creative Commons Attribution license (Casarini *et al.*, 2012).

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Follicle-Stimulating Hormone (FSH)☆

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Glossary

Azoospermia The complete lack of spermatozoa in an ejaculate.

Gonadotropin receptor A G-protein coupled receptor, belonging to a wide family characterized by seven transmembrane domains.

Oligospermia Less than 39 millions of sperms in an ejaculate, or a less than 15 millions/mL of sperm concentration.

Receptor A protein that resides either on the surface or in the cytoplasm of a cell. The protein configuration allows interaction with a particular hormone. The binding of the hormone elicits a cascade of reactions in the cell that result in the expression of genes that affect the cell's function.

Single nucleotide polymorphism (SNP) A nucleotide point change with a frequency more than 1% within a population.

Introduction

Follicle-stimulating hormone (FSH) is a glycoprotein secreted by the pituitary gland and, in concert with luteinizing hormone (LH), plays a central role in mammalian reproduction (Simoni and Casarini, 2014). FSH acts directly on the gonads in both sexes, binding to its receptor on the surface of target cells, initiating a cascade of molecular events beginning with the activation of specific signaling pathways and ending with the expression of gene products (Hansson *et al.*, 2000). The gene products, in turn, set in motion processes specific to each gonad. In the ovary, FSH stimulates folliculogenesis and steroidogenesis, which, in turn, prepare the female reproductive tract for fertilization, implantation, and pregnancy (Messinis *et al.*, 2014). In the testis, this gonadotropin plays a central role in testicular development and spermatogenesis (Huhtaniemi, 2015). In both sexes, the products of FSH stimulation regulate the secretion of FSH either by modifying the release of the hypothalamic secretagogue, gonadotropin-releasing hormone (GnRH), or by acting directly at the pituitary gland.

Molecular Structure

FSH is a protein composed of two glycosylated polypeptide subunits, designated α and β, and has a molecular weight of approximately 33 kDa, varying with the species and the length of the added oligosaccharides. The α-subunit (14 kDa) is common to other glycoprotein hormones produced by the pituitary gland, LH and thyroid-stimulating hormone (TSH), as well as chorionic gonadotropin (CG), the latter produced mainly by trophoblast cells during pregnancy. The α-subunit may be secreted and in certain circumstances may achieve high concentrations in the blood, but no function for the free subunit has been demonstrated. The β-subunit is unique to each glycosylated hormone and, when associated with the α-subunit, confers biologic activity on the hormone (Berger and Lapthorn, 2016).

Carbohydrate groups are present at specific locations on each subunit with two oligosaccharide moieties on each subunit (Bousfield and Dias, 2011). These carbohydrate groups are important determinants of the biologic activity and metabolic clearance of FSH. The oligosaccharide moieties are coupled through *N*-acetylglucosamine to specific asparagine groups in the α- and β-subunits. The oligosaccharides contain the monosaccharides mannose, galactose, glucosamine, *N*-acetylgalactosamine, and sialic acid. In addition, the oligosaccharides are branched and heterogeneously terminate mainly in sialic acid and fucose. These differences are largely responsible for the variation in the isoelectric point (4.5–5) of this gonadotropin. The alkaline forms are biologically more active in bioassays, but are also more rapidly cleared from the circulation. Moreover, acidic FSH isoforms consisting in hyperglycosylated hormones display higher activity in the human granulosa KGN cell line in vitro than those fully glycosylated. These results were confirmed using *Fshb* knock-out male mice, where hyperglycosylated human recombinant FSH induced marked transcription of target genes and proliferation of Sertoli cell compared to the less acidic isoforms. Removal of terminal sialic acid residues from the oligosaccharides reduces the half-life of FSH in circulation and decreases its ability to activate target cells in both the ovary and testis (Bousfield *et al.*, 2014).

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Synthesis and Secretion

Gonadotropes are the pituitary cells synthesizing FSH, and they could be classified in three categories, based on the results of immunocytochemical staining (Simoni *et al.*, 1997). The majority of the gonadotropes belong to the category of cells synthesizing both FSH and LH, while other categories are composed of cells that exclusively synthesize FSH or LH. The secretion of gonadotropins is constitutive and it is mainly stimulated by the hypothalamic polypeptide GnRH. GnRH is released in a pulsatile fashion by neurons that terminate on the blood vessels running along the pituitary stalk. These vessels constitute a specialized form of the circulatory system, called "portal system." Portal systems are characterized by special veins, arising in one organ system and terminate in another before returning to the heart. They carry the pulses of GnRH to the gonadotropes in the pituitary gland and stimulate the synthesis and pulsatile release of both FSH and LH, according to the GnRH pulses. Changing the pattern of the GnRH pulses results in coincidental changes in the pattern of pulsatile secretion of FSH. GnRH binds to a G protein-coupled membrane receptor on the surface of the gonadotrope, activating the $G_{q/11}$ proteins that, in turn, stimulate phospholipase C. This enzyme generates inositol-1,4,5-triphosphate and diacylglycerol. The increased levels of these intracellular messengers activate protein kinase C (PKC) and increase intracellular calcium. PKC and calcium regulate the expression of the β-subunit gene, whereas secretion of FSH is regulated by the increase in intracellular calcium.

Recently, together with the known GnRH function, the role of kisspeptin in regulation of gonadotropin release was demonstrated. Kisspeptin is a 54-amino acid peptide, which is encoded by the *KISS-1* gene (Ratnasabapathy and Dhillo, 2013). Kisspeptin activates the G protein-coupled receptor (GPCR)54, regulating the mammalian and human reproduction. GPCR54-deficient mice showed abnormal sexual development, whereas inactivating GPR54 mutations in humans are responsible for normosmic hypogonadotrophic hypogonadism (Ratnasabapathy and Dhillo, 2013; Lanfranco *et al.*, 2005). On the other hand, the GPR54 signaling activation is associated with premature puberty (Ratnasabapathy and Dhillo, 2013). Moreover, several studies demonstrated that the acute intravenous administration of kisspeptin in healthy humans potently increases both LH and FSH serum levels (Ratnasabapathy and Dhillo, 2013). These results suggest the important role of the kisspeptin/GPR54 system in the regulation of gonadotropin secretion.

FSH Receptor Structure and Signaling

The first FSH receptor (*FSHR*) gene cloned and functionally expressed in mammalian cells was of rat Sertoli origin and obtained from a cDNA library (Sprengel *et al.*, 1990). Its predicted structure displayed high similarity with other GPCRs and was characterized by a relatively large extracellular domain and seven transmembrane segments. The human *FSHR* gene is located on chromosome 2p21 (Rousseau-Merck *et al.*, 1993). It spans 10 exons encoding for a mature protein, which consists of 678 amino acids (75–80 KDa). Mature FSHR is located in the gonads and belongs to the superfamily of rhodopsin-like GPCRs. Like all members of this family it is characterized by seven transmembrane domains, totally of 264 amino acids. These hydrophobic stretches are of 21–24 amino acids connected by intracellular and extracellular loops (Simoni *et al.*, 1997). The extracellular domain consists of 349 amino acids and serves for FSH binding, while the intracellular domain spans 65 amino acids and interacts with G proteins and other cytoplasmic molecules (Simoni *et al.*, 1997). Upon hormone binding, the receptor undergoes a conformational change leading to dissociation of G protein heterotrimer at the intracellular level, thus inducing signal transduction. Especially, the activation of Gαs protein subunit induces the conversion of ATP to cAMP by adenylyl cyclase. This is the first step in the intracellular signaling cascade leading to protein kinase A (PKA) activation which, in turn, induces the phosphorylation of the mediator of proliferative signals extracellular-regulated kinase (ERK1/2) (Gloaguen *et al.*, 2011). Downstream to PKA and ERK1/2 activation, the phosphorylation of the transcription factor cAMP-responsive element binding protein (CREB) (Hunzicker-Dunn *et al.*, 2012) induces the expression of steroidogenesis-related target genes, such a *STARD1* and *CYP19A1* (Freimann *et al.*, 2004). *STARD1* encodes for steroidogenic acute regulatory protein (StAR) (Amsterdam *et al.*, 2003), a mediator of cholesterol transport within the mitochondria, as a rate-limiting step of the steroidogenesis (Miller and Auchus, 2011). FSHR binding to the ligand is linked to simultaneous activation of different G protein subunits (Gloaguen *et al.*, 2011), such as the Gαi protein, which induces the phosphorylation of ERK1/2 and inhibits cAMP synthesis (Saltarelli, 1999). Moreover, Gαi protein mediates receptor desensitization via activation of GPCR-specific kinases (Pitcher *et al.*, 1999). Other FSHR cytoplasmic interactors were found. β-arrestins are involved in phosphorylated GPCR internalization (Benovic *et al.*, 1987; Ferguson *et al.*, 1996), and mediates both proliferative and antiapoptotic signals via ERK1/2 signaling (Casarini *et al.*, 2016; Kara *et al.*, 2006; Cassier *et al.*, 2017). The result of these intracellular pathways, simultaneously activated by FSH, range from steroidogenesis, to cell differentiation, proliferation, and apoptosis (Dias *et al.*, 2010).

In 2005, the structure of FSH combined with its receptor was studied with a 2.9-A-resolution structure (**Fig. 1**) (Fan and Hendrickson, 2005). FSH binds the FSHR into the concave face of the curved extracellular receptor domain, similar to a hand clasp (**Fig. 1**) (Fan and Hendrickson, 2005).

FSHR and FSHβ Polymorphisms and Mutations

More than 2000 *FSHR* and *FSHB* single nucleotide polymorphisms (SNP) were described. SNP is characterized by a nucleotide point change with frequency $>1\%$ within a population. Most of these SNPs fall within introns or are synonymous, while a

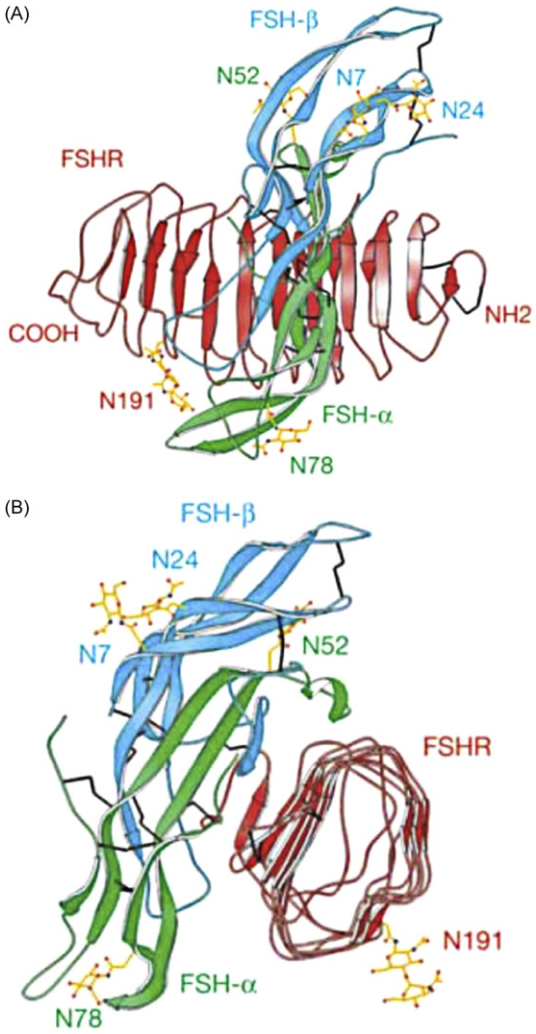

Fig. 1 Crystal structure of human FSH bound to FSHR. Ribbon diagram of the complex structure shown in two views related by a 90 degree rotation about the vertical axis (panel A and B). FSH a-chains and b-chains are in *green* and *cyan*, respectively. FSHR is in *red*. Modified from Fan, Q. R. and Hendrickson, W. A. (2005). Structure of human follicle-stimulating hormone in complex with its receptor. *Nature* **433**, 269–277.

number of them lead to amino acid change. Among the SNPs located within the exons of the *FSHR* gene, only two were associated with the gonadal response to FSH. These SNPs are the threonine to alanine change at position 307 of the amino acid sequence (p.T307A; rs6165, in the National Center for Biotechnology Information SNP database) and the asparagine to serine at position 680 (p.N680S; rs6166). They are associated with different kinetics of cAMP/PKA- and ERK1/2-pathway activation, as well as progesterone synthesis in vitro (Casarini *et al.*, 2014), suggesting that the FSHR p.N680S "S" is a variant less sensible to FSH stimulation. Indeed, p.N680S S homozygous women display higher FSH serum levels and require higher hormone doses than N homozygous for ovarian stimulation (Grigorova *et al.*, 2013; Perez Mayorga *et al.*, 2000). In fact, the FSHR gene is located in a genomic region which is a "hot spot" of the gonadal physiopathology (Chen *et al.*, 2011). Interestingly, the SNP p.N680S seems to be linked to sperm DNA fragmentation index (Simoni *et al.*, 2016) and testes volume (Grigorova *et al.*, 2013) in males, confirming the relevance of the genetic *locus* for the gonadal physiology.

Another common SNP falling within the *FSHR* gene is the amino acid change G to A, located 29 base pairs upstream the transcription start codon ($-29G>A$; rs1394205). It is located in the promoter region of the receptor gene modulating the transcription levels of the gene (Desai *et al.*, 2011), resulting in relatively low FSHR protein and, therefore, affecting the gonadal response to FSH (Desai *et al.*, 2013). Since the distribution of the FSHR genotypes is variable worldwide, the cumulative effects of FSHR $-29G>A$ in association with p.T307A and p.N680S was discussed, as well as their role in the evolution of different endocrine phenotypes among humans was suggested (Simoni and Casarini, 2014).

Among the more than 20 *FSHB* gene SNPs described, the c.$-211G>T$ (rs10835638), located within the promoter region and influencing FSH production, is one of the most commonly studied (Benson *et al.*, 2013). Due to the low gene transcription of the promoter featuring the "T" genotype, the SNP modulates the FSH circulating levels and is linked to age of menopause and follicle growth in women (Busch *et al.*, 2016; Ruth *et al.*, 2016). In men, c.$-211G>T$ SNP is linked to FSH serum levels as well

(Grigorova *et al.*, 2010) and age at testicular growth (Busch *et al.*, 2017). The potential, combined effect of the *FSHB* c. − 211G > T and the *FSHR* SNPs on human fertility were investigated, suggesting the existence of haplotypes associated with "less sensitive" FSH trait (Grigorova *et al.*, 2014; Tuttelmann *et al.*, 2012).

Several inactivating and activating *FSHR* gene mutations have been identified, providing a wide spectrum of effects depending on the nature of amino acid change and location. Mutations differ from SNPs due to their frequency (< 1%) in the population. An example of inactivating mutation is given by the C to T nucleotide point mutation found in Finnish families (Aittomaki *et al.*, 1995). This mutation results in the alanine to valine substitution, falling within the FSHR extracellular domain, and in reduced binding capacity and signal transduction. The women of these families have hereditary hypergonadotropic ovarian failure, with amenorrhea and atrophic ovaries with follicles that do not develop beyond primary follicles. Men homozygous for this mutation are normally virilized and have sufficient testosterone to initiate and maintain spermatogenesis, although displaying variable degrees of spermatogenic failure (Tapanainen *et al.*, 1997).

Only few *FSHR* gene activating mutations were described so far, most of them located at the transmembrane domain of the receptor. For example, the first case was reported in 1996 in a hypophysectomyzed hypogonadotropic man with normal fertility in spite of undetectable gonadotropin serum levels, due to an activating mutation on the *FSHR* gene (Gromoll *et al.*, 1996). It was an aspartic acid to glycine substitution at position 566 displaying constitutive activity in vitro. This mutation falls within the third intracellular loop of FSHR, which is a crucial region for glycoprotein hormone receptor functioning. In a woman, the same mutation induced ovarian hyperstimulation syndrome during pregnancy, moreover revealing that the mutant receptor mediated hCG- and TSH-induced signaling (Smits *et al.*, 2003). *FSHB* gene mutations were reported as well. Especially, five inactivating mutations leading to the insertion of a stop codon or to a loss of residual cysteine were described so far (La Marca *et al.*, 2013). All these mutations compromise the subunit folding and lead to abnormal puberty or primary amenorrhea in women, and altered pubertal development and azoospermia in men.

Action in the Ovary

FSH acts on the ovary by stimulating folliculogenesis (Rimon-Dahari *et al.*, 2016) (**Fig. 2**). During fetal development of the female mammal, the primitive germ cells in the ovary enter meiosis and arrest in the prophase of meiosis I. Each oocyte is surrounded by

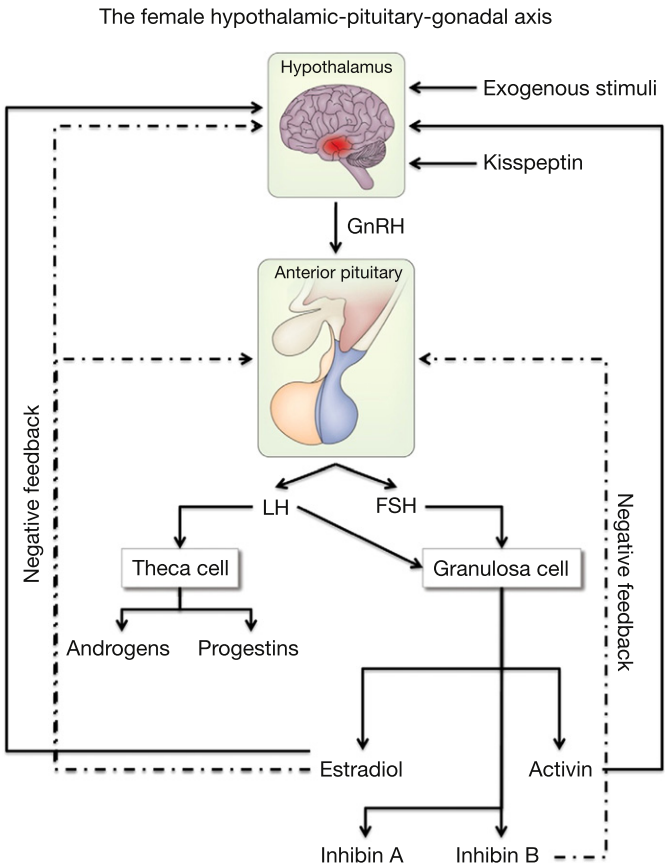

Fig. 2 The female hypothalamic-pituitary-gonadal axis. Abbreviations: *GnRH*, gonadotropin-releasing hormone; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone.

a single layer of cells, and enters in the resting phase. During fetal life in primates and ruminants and within the first 2 weeks after birth in rodents, some of the primordial follicles are activated and become primary follicles. The mechanisms stimulating the primordial follicles to initiate folliculogenesis are unknown, but are clearly independent of gonadotropin action. Two layers of somatic cells develop around the oocyte. The outermost layer is composed of theca cells that are separated from the innermost layer, comprising granulosa cells, by a basement membrane. Both types of cells proliferate, increasing the size of the follicle until a fluid-filled cavity, or antrum, forms in the layers of the granulosa cells. The mature, or antral, follicle ruptures, marking the end of folliculogenesis and representing the ovulation phase, results in releases of the oocyte and some of the granulosa cells surrounding it. During the periovulatory period, antiinflammatory factors are produced and appear to aid in rapid healing and vascularization of the ruptured follicle. The theca and granulosa cells remaining in the ruptured follicle differentiate into luteal cells, forming the corpus luteum that characterizes the luteal phase of the ovarian cycle. If no pregnancy occurs, after a finite time specific to each species, the corpus luteum dies, marking the end of the luteal phase. The rapid involution of the corpus luteum is called luteolysis and is regulated by specific factors. Luteolysis is necessary before a subsequent ovarian cycle can begin. The follicular phase, ovulation, luteal phase, and luteolysis constitute the ovarian cycle and FSH plays an important role only during the first phase of the ovarian cycle.

The theca cells of the developing ovary produce androgens in response to LH. FSH stimulates the granulosa cells to produce the enzyme aromatase, which converts the androgens produced by the theca cells into estradiol, thus exerting a negative feedback on gonadotropins production by pituitary. The number of granulosa cells increases by mitosis occurring under FSH-induced proliferative stimuli, which are preferentially mediated by FSHR at low density on the cell surface (Tranchant *et al.*, 2011; Casarini *et al.*, 2016). In growing follicles, FSH also increases estradiol production and the number of LH receptors (LHCGR) on theca and granulosa cells. With the increase in LH receptors on the granulosa cells of one or more follicles in the presence of decreased FSH, only those follicles with sufficient gonadotropin and estrogen stimulation will continue folliculogenesis. As the follicle enlarges, a cavity forms around the oocyte so that layers of granulosa cells and fluid surround it, but a peduncle of granulosa cells remains attached to the rest of the follicle. Once the level of circulating estradiol produced by the Graafian follicles is elevated, estradiol cause an abrupt release of LH and FSH, which results in ovulation, that is, the extrusion of the oocyte and the granulosa cells immediately surrounding it, which is subsequently referred to as the oophorus cumulus. Once ovulation has occurred, the role of FSH is greatly diminished and the formation of the corpus luteum and maintenance of the endometrium of the uterus are under the influence of LH.

Action in the Testis

The role of FSH in regulating spermatogenesis in adult mammals has been controversial for several decades (Huhtaniemi, 2015) (**Fig. 3**). Although the direct role of FSH in adult male rodents on spermatogenesis is limited, it remains the primary determinant of the spermatogenic capacity. This capacity is defined as the number of sperm potentially produced by the male gonad and intuitively this must directly relate to the number of spermatogenetic stem cells and the capacity of the structure of the gonad to nourish and support the progeny of these stem cells.

The Sertoli cells of the mammalian testis are the only somatic cells in the seminiferous epithelium and are known to proliferate only during fetal and peripubertal development in rodents. A third period of Sertoli cell proliferation occurs during infancy in higher primates. After these, somatic cells have attained a population size associated with adulthood, proliferation ceases, and the cells terminally differentiate. The mature Sertoli cells serve to organize and nourish the germ cells that make up the remainder of the cell types in this epithelium. Moreover, they are the mediators of FSH action because they are the only cells in the male that have FSHR. FSH stimulates Sertoli cell proliferation in the rodent testis, but, in primates, both FSH and LH are equally effective in stimulating the proliferation of these cells. Because the Sertoli cells provide the nutrients and structural support to spermatogenesis, it was immediately recognized that FSH regulates the spermatogenic capacity of the testis. In contrast to the situation in rodents, FSH plays a more direct role in regulating spermatogenesis in primates.

Spermatogenesis is a complex process occurring in the seminiferous tubules, with the final outcome of the mature male gamete production. This network comprises several phases, such as spermatogonia proliferation, spermatogonial differentiation into spermatocytes, spermatocytes meiotic division producing spermatids, maturation of round spermatids; and the release of highly specialized mature spermatozoa into the testicular tubule lumen (Neto *et al.*, 2016). This process is allowed by the stem cell continuous renewal, which ensures a large and undiminishing number of undifferentiated germ cells available for the subsequent waves of spermatogenesis (Heller and Clermont, 1963; Misell *et al.*, 2006). The spermatogenetic stem cells are called type A spermatogonia, which are continuously renewed and differentiate forming type B spermatogonia. The entire spermatogenic process requires approximately 74 days, varying between 42 and 76 days, with an overall daily production from 150 to 275 millions of sperms (Misell *et al.*, 2006). In this process, several testicular structures are involved, from which Sertoli cells are indispensable for spermatogenesis (Neto *et al.*, 2016). Their function is regulated by hormonal, nonhormonal, and paracrine factors. In this context, FSH represents the most important regulator of Sertoli cells action.

The importance of pituitary in stimulating spermatogenesis was demonstrated by hypophysectomy of adult rats, which led to a decrease in testicular weight and impairment of germ cells maturation to spermatids. Hypophysectomy of adult rhesus monkeys resulted in a precipitous decline in testicular size associated with the complete regression of the seminiferous epithelium to the extent that the tissue comprised only Sertoli cells and type A spermatogonia. In hypogonadotropic hypogonadal rhesus monkeys,

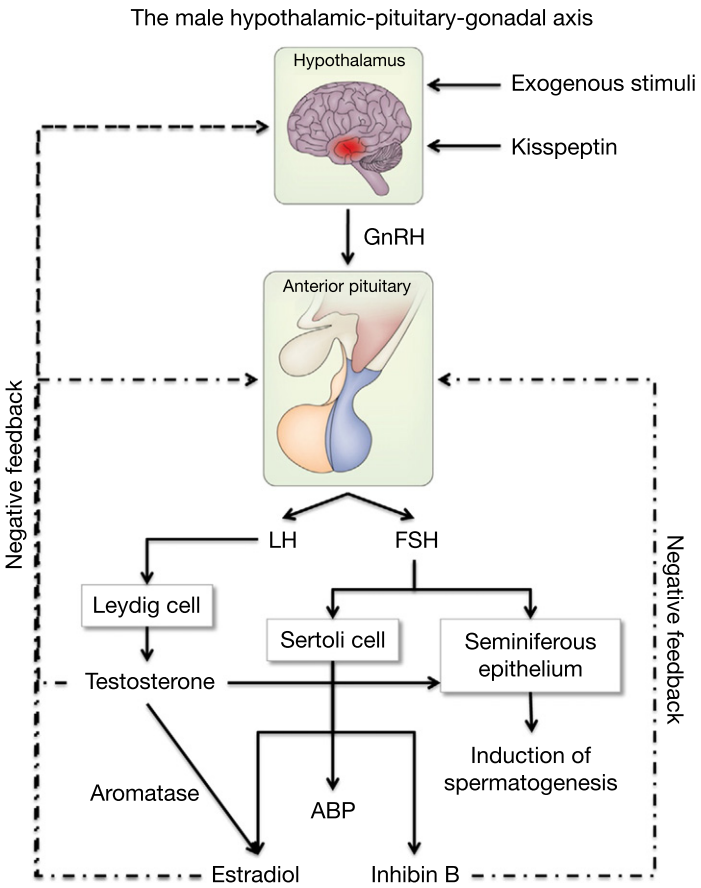

Fig. 3 The male hypothalamic-pituitary-gonadal axis. Abbreviations: *ABP*, androgen bindingprotein; *GnRH*, gonadotropin-releasing hormone; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone.

testicular growth is partially recovered by testosterone replacement. Therefore, gonadal growth was due primarily to the stimulation of spermatogenesis by androgen, but morphometric analysis of the seminiferous epithelium of the monkeys revealed that the smaller testicular size was accounted for by a deficit in the numbers of all type B spermatogonia. Replacement of FSH in testosterone-treated hypophysectomized adults resulted in a greater number of all four generations of type B spermatogonia. These results led to the conclusion that testosterone alone stimulates spermatogenesis, but FSH is necessary to restore spermatogenesis completely. This action of FSH is posited to be the rescue of type B spermatogonia from programmed cell death.

Unilateral orchidectomy in adult macaques results in a compensatory growth of the remaining testis. The number of Sertoli cells per testis was identical in the gonad removed at the time of unilateral orchidectomy and the gonad that remained in the animal for 45 days. The number of all germ cells more mature than type A (subtype "p"; Ap) spermatogonia was greater in the remaining testis than in the removed testis. Moreover, the removal of one testis was occasioned by a transient decline in testosterone that in turn led to a transient increase in LH. By 4 days after surgery, the testosterone and LH concentrations were restored to those measured prior to surgery. The removal of one gonad in these primates was marked also by a decline in inhibin B, which is secreted by Sertoli cells into the circulatory system. The concentrations of FSH in the circulation increased, confirming the role of inhibin B in the negative feedback on FSH secretion by the pituitary gland of the primate (**Fig. 3**). The role of inhibin B is less clear in rodents and the steroid testosterone may play a greater role in the regulation of FSH secretion in those species. On the contrary, other investigators have presented results indicating that FSH increases the number of germ cells in the primate testis indirectly, and FSH administration to adult macaques resulted in an increase in spermatogonia in the seminiferous epithelium.

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Prolactin: Regulation of Secretion and Action☆

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Glossary

Cathepsin Cathepsins are proteases (enzymes that degrade proteins) found in all animals as well as other organisms.

ERK Protein kinase whose action can mediate numerous cellular responses.

GABA gamma-Aminobutyric acid (γ-aminobutyric acid). It is a neurotransmitter in the central nervous system of mammals.

JAK proteins Janus kinase (JAK) is a family of intracellular, nonreceptor tyrosine kinases that transduce cytokine-mediated signals via the JAK-STAT pathway.

Kallikrein Kallikreins are a subgroup of serine proteases, enzymes capable of cleaving peptide bonds in proteins.

MAPK A mitogen-activated protein kinase (MAPK or MAP kinase) is a type of protein kinase that is specific to the amino acids serine, threonine, and tyrosine (i.e., a serine/threonine-specific protein kinase). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines.

PACAP Pituitary adenylate cyclase-activating peptide. 38-amino acid C-terminally alpha-amidated peptide known to act throughout the body and brain, modulating the activity of the central nervous system, metabolism, blood pressure, pain sensitivity and immune function.

Pit-1 PIT1 is a pituitary-specific transcription factor responsible for pituitary development and hormone expression.

RAS Ras family of proteins belong to a class of proteins involved in signal transmission within cells.

STAT proteins Members of the signal transducer and activator of transcription (STAT) protein family are intracellular transcription factors that mediate many aspects of cellular immunity, proliferation, apoptosis and differentiation.

Tubero-infundibular dopaminergic system (TIDA) The tuberoinfundibular pathway refers to a population of dopamine neurons that project from the arcuate nucleus in the tuberal region of the hypothalamus to the median eminence.

Definition

Prolactin (PRL) is a hormone primarily secreted by the anterior pituitary gland involved in regulation of reproductive and nonreproductive processes.

Structure

The PRL gene, consisting of five exons and four introns, was cloned in 1981 and it is located on chromosome 6 in humans. Human PRL cyclic DNA is 914 nucleotides long and contains a 681-nucleotide frame encoding for PRL pro-hormone (227 amino acids) (Freeman *et al.*, 2000). PRL is a single chain polypeptide of 199 amino acids (molecular weight 23 kD, half-life 50 min) with three intramolecular disulfide bonds between six cysteine residues (Cys4-Cys11, Cys58-Cys174, and Cys191-Cys199 in humans), primarily synthesized and secreted by lactotrope cells in the anterior pituitary gland (Freeman *et al.*, 2000). Lactotropes represent 40%–50% of the total pituitary cell population and are mostly aggregated in the postero-lateral areas of the gland. The PRL pituitary content is relatively low (approximately 135 micrograms per gland). The disparity between the content of PRL and the amount of lactotrope cells is probably due to the fact that the PRL has a turnover considerably high with consequent limited storage in pituitary gland.

PRL structure has a remarkable degree of homology in amino acids sequence with human growth hormone (hGH) and placental lactogen (hPL). Specifically, human mRNAs of PRL and GH have 42% of homology, consisting with the hypothesis that they arose by consecutive duplications of a common ancestral gene (Ben-Jonathan *et al.*, 2008).

PRL is secreted in different forms depending on post-translational glycosylation, proteolysis and phosphorylation processes (Sinha, 1995). Phosphorylated PRL form represents about 5%–30% of the PRL released by the anterior pituitary gland with

☆*Change History*: March 2018. F Maffezzoni, G Mazziotti, and Andrea Giustina was updated in the text and references concerning the neuroregulation of PRL secretion (pages 7–11) and extra-gonadal effects of PRL (14–16). Moreover, new 2 Figures (#2,4) and one Table (#1) were included.

This article is an update of Nira Ben-Jonathan, Robert Hnasko, Prolactin (PRL), In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 99–103.

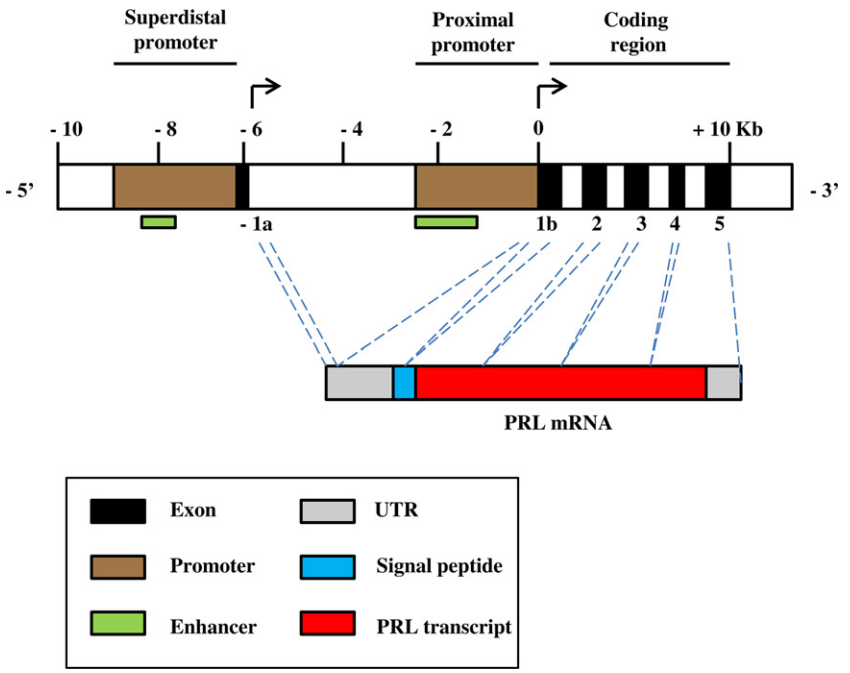

Fig. 1 Diagram of the human PRL gene, mRNA transcript, and mature PRL. The *left* and *right arrows* in the upper panel designate extrapituitary and pituitary transcription start sites, respectively, and the numbers designate the exons. The locations of the enhancers in both promoters is also shown. The extrapituitary PRL mRNA is longer in the 50 untranslated region (UTR). The structure of the PRL protein is composed of four up-up-down-down alpha helices.

both agonistic and antagonistic properties. Moreover, PRL fragments may derive from post-secretion proteolytic cleavage of full hormone. For instance, kallikrein and cathepsin D were shown to produce the 22 and 16 kD fragments, respectively, which may be important in regulating non reproductive functions (see, section on physiological actions). Finally, large forms of PRL hormone may result from dimerization and multimerization processes. In blood stream several forms of circulating PRL may be found:

- Small PRL (molecular weight 23 kD), which corresponds to the monomeric nonglycosylated hormone with high receptor affinity, biological and immunological complete activities.
- Two glycosylated forms (G^1 and G^2) of PRL (~ 25 kD), different in carbohydrate units chain and both with reduced immunoreactivity (G^1-PRL has only a quarter of the bioactivity of the G^2-PRL).
- Big PRL (molecular weight ~50 kD), which consists of a mixture of forms dimeric and trimeric glycosylated PRL (G-PRL).
- Big–Big PRL (~100 kD), probably represented by G-PRL, combined with immunoglobulins by covalent bond.

Regulation of Secretion

Two independent promoters have been identified in PRL gene with tissue- and cell-specific response to regulatory mediators (Marano and Ben-Jonathan, 2014). Specifically, the proximal promoter is the 5000-bp region involved in regulation of PRL secretion by pituitary gland whereas the downstream promoter region is responsible for regulation of extrapituitary expression of the hormone (**Fig. 1**). Interestingly, the two promoters regulate expression of the same PRL isoforms. The pituitary-specific expression of PRL is regulated by the Pit-1 (pituitary transcriptional factor-1), which is a member of the POU domain family (a family of proteins that have well-conserved homeodomains). Pit-1 exerts similar effects on PRL, GH, and thyroid stimulating hormone (TSH) expression. Indeed, point mutations in the human gene encoding for Pit-1 is responsible for a congenital disease characterized by combined deficiencies of GH, PRL, and TSH. In extra-pituitary sites, such as decidua and lymphocytes, expression of PRL occurs independently of Pit-1.

PRL is secreted by pituitary gland in a pulsatile manner, but it also has a circadian fluctuation (Mancini *et al.*, 2008). PRL secretion is high during the nighttime sleep (non rapid eye movement—REM phase). Specifically, PRL release begins to increase rapidly (10–60 min) after the onset of sleep with several secretory peaks (i.e., from three to eight) determining high PRL levels persistently during sleep. In the first hour after waking up, plasma PRL concentration falls rapidly reaching the lowest levels in late morning (i.e., between 9 and 11 a.m.). Interestingly, the biologically active form of PRL (23 kD) is produced during the day, whereas the glycosylated form of the hormone is released predominantly during sleep.

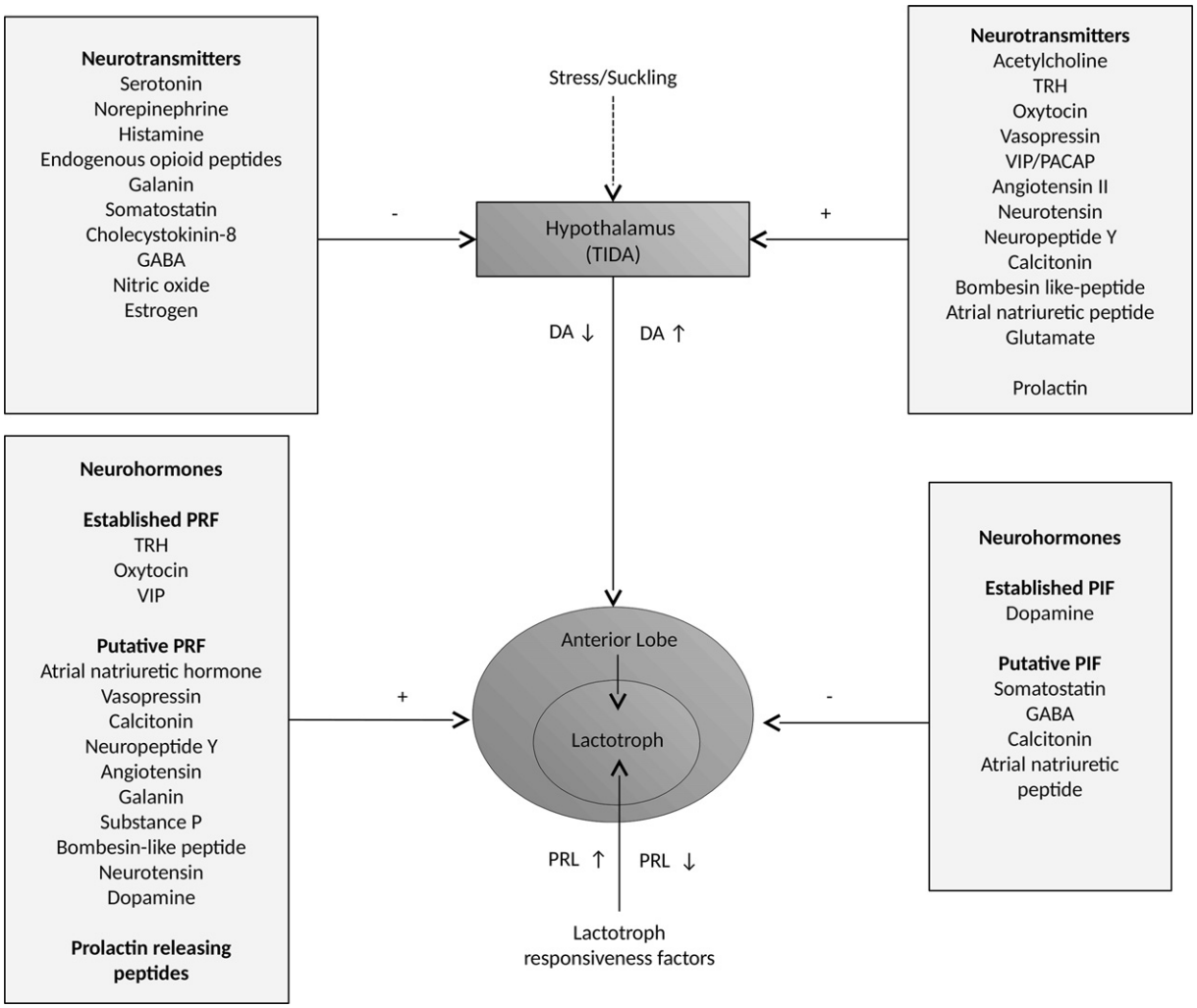

Fig. 2 Regulation of PRL secretion. Permission is required from Mancini, T., Casanueva, F.F., Giustina, A. (2008). Hyperprolactinemia and prolactinomas. *Endocrinology & Metabolism Clinics of North America*. **37**, 67–99.

Secretion of PRL from pituitary gland is regulated by endocrine, paracrine and autocrine factors. Endocrine regulators originate from the hypothalamus and the peripheral glands and reach the lactotrophs via the blood. Paracrine factors reach the lactotropes by diffusion from neighboring pituitary cells. Autocrine agents are synthesized by the lactotropes themselves. The regulation is complex involving neurotransmitters, neurohormones, neuropeptides, metabolic substrates and systemic hormonal signals divided in PRL-inhibiting factors (PIFs) and PRL-releasing factors (PRFs) (**Fig. 2**) (Mancini *et al.*, 2008). Consequently, the overall secretory activity of the lactotropes reflects a balance between local and distant PIFs and PRFs which can be influenced by physiological and pathological conditions (**Table 1**). Pregnancy and lactation are two physiological conditions associated with increase in PRL secretion. In fertile women, a progressive and significant increase in serum PRL is observed during the late follicular phase, with a maximal value concomitant to the LH peak. Serum PRL levels is also higher during the luteal phase than during early follicular phase. The overall pattern of serum PRL during the menstrual cycle resembles that reported for circulating 17beta-estradiol. This is relevant to the timing of blood sample collection which should be done in the early follicular phase when PRL is at nadir. Moreover, PRL levels increase after exercise, meals, psychological stress and stimulation of the chest wall. As predicted from the physiologic dopaminergic inhibition of PRL secretion (see below), treatment with dopamine receptor antagonist drugs commonly induce hyperprolactinemia (**Table 1**). Twelve to thirty percentage of women taking higher estrogen containing oral contraceptives may have a small increase in serum PRL. This effect was shown to be often transient resolving spontaneously in about 50% of women (Luciano *et al.*, 1985). Similarly, occupational exposure to heavy metals and some over-the-counter herbal or alternative remedies may cause an increase in PRL (Mancini *et al.*, 2008).

Unlike GH, lactotropes have spontaneously high secretory activity and the hypothalamic control of PRL secretion is predominantly inhibitory mediated by dopamine (DA). DA is secreted by neurons in the arcuate nucleus and released in the portal vessels from the tubero-infundibular dopaminergic system (TIDA). DA exert its action by binding to dopamine receptors (DRs) on lactotrope cells (Ben-Jonathan and Hnasko, 2001). Five receptor subtypes of DRs (DR1, DR2, DR3, DR4, DR5) have been characterized. DRs receptors are also

Table 1 Physiological and pathological conditions influencing prolactin (PRL) secretion

Factors increasing PRL secretion	*Factors inhibiting PRL secretion*
Neurohormones	
• TRH	• Dopamine (DA)
• VIP	• Somatostatin
• Oxytocin	• GABA
• ADH	• Atrial natriuretic peptide (ANP)
Hormones	
• Estradiol	• Glucocorticoids (endogenous—exogenous)
	• Calcitonin
	• Prolactin
Neurotransmitters	
• Serotonin, norepinephrine, histamine, galanin, GABA, nitric oxide, endogenous opioid peptides	• Acetylcholine
Drugs	
• Antipsychotics (haloperidol, phenothiazines, risperidone, amisulpride, chlorpromazine)	• Dopamine agonists (cabergoline, bromocriptine, quinagolide, pergolide)
• Antihypertensive (α metil dopa, reserpine, verapamil)	• Indirect D2 receptor activators (amphetamines)
• Prokinetics (metoclopramide, domperidone)	• Antipsychotics (aripiprazole)
• H2-receptor antagonists (cimetidine, ranitidine)	
• Antidepressants (amitriptyline, sertraline, fluoxetine, paroxetine)	
• Opiates (morphine)	
Psychological conditions	
• Acute psychological and physical stress	
• Pregnancy/Breastfeeding	
• Sleep	
• Physical/sexual activity	
• Hypoglycemia	
Pathological conditions	
• Hypothalamic/pituitary diseases (primitive tumors, metastases, lymphocytic hypophysitis, infiltrative diseases, cranial irradiation, aneurisms)	• Hypothalamic/pituitary diseases (primitive tumors, metastases, lymphocytic hypophysitis, infiltrative diseases, postpartum pituitary necrosis)
• Genetic syndromes (multiple endocrine neoplasia, Carney complex, McCune–Albright syndrome)	• Congenital (familial puerperal alactogenesis, traumatic brain injury)
• Primary hypothyroidism	• Autoantibodies against PRL (lymphocytic hypophysitis, Hashimoto's thyroiditis)
• Kidney failure	
• Hepatic cirrhosis	
• Polycystic ovary syndrome	

classified into two subclasses (DR1-like) and (DR2-like), based on the phylogenetic classification and the type of pathway used for the post-receptor transduction signals. DR1 class (DR1 and DR5 subtypes) has a stimulatory effect on intracellular signaling pathways, while DR2 class (DR2, DR3, and DR4 subtypes) has mainly inhibitory activity on cAMP production. Type DR2 is the predominant dopamine receptor in pituitary lactotrope cells with essential role in maintaining physiological PRL levels. Indeed, pituitary hyperplasia with persistent hyperprolactinemia was reported in receptor knock-out (KO) mice for DR2. Two alternatively spliced isoforms of DR2 exist, termed DR2-short (DR2-S) and DR2-long (DR2-L) receptors, differing for the insertion of 29 amino acids of the third cytoplasmic loop. The two isoforms of DR2 dopamine receptor are both present on lactotrope cells. After interaction of DA to DR2, inhibition of cAMP production occurs along with activation of voltage-gated calcium channels and inhibition of potassium channels, finally resulting in membrane either depolarization or hyperpolarization changes.

A short positive feed-back between PRL and DA exists, consisting in increase of DA release in TIDA in response to increase in PRL secretion by pituitary gland. Other neuromediators and neuropeptides may influence the cross-talking between DA and PRL (Mancini *et al.*, 2008). For instance, melanocyte-stimulating hormone (MSH), a product of pro-opiomelanocortin (POMC) gene was shown to activate TIDA neurons and then favoring the release of DA and inhibition of PRL secretion by lactotropic cells. Similarly, acetylcholine causes a decrease in serum PRL levels through the TIDA stimulation. By contrast, β-receptors activation by norepinephrine and epinephrine inhibits DA release in TIDA with consequent increase in PRL secretion (**Fig. 2**).

Glucocorticoids were shown to exert inhibitory effects on PRL secretion (Giustina and Wehrenberg, 1992). In experimental models, glucocorticoids suppress the differentiation of lactotropes whereas hypocortisolism post-adrenalectomy significantly increased plasma levels of PRL (Brann *et al.*, 1990). Interestingly, the effect of adrenalectomy were reversed by the administration of exogenous corticosteroids.

Although the hypothalamic control of PRL secretion is mediated by a tonic inhibitory mechanism, a PRF is necessary for the acute secretory activity (Mancini *et al.*, 2008) (**Fig. 2**). TRH is a powerful stimulator of pituitary PRL release both in vivo and in vitro conditions. The concentration of TRH request to cause a PRL release in vitro is within the range found in the portal blood

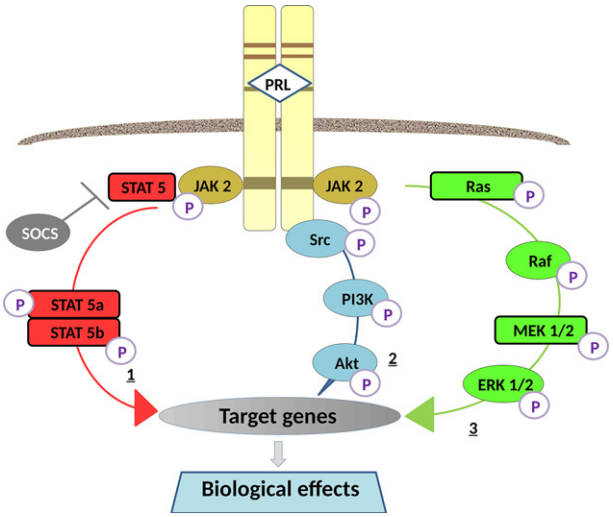

Fig. 3 The PRL receptor (PRLR) and its signaling cascade. PRL binds to the extracellular domain of the receptor and rapidly phosphorylates both Jak2 and the intracellular domain of the PRLR. PRL activates three major signaling pathways: Jak2/Stat (1), PI3K/Akt (2), and Ras/MAPK (3). P designates activation by phosphorylation.

(from 200 to 500 pg/mL). Specific receptors for TRH are present on lactotrope cells and stimulate the transcription of PRL gene in a few minutes. Consisting with these effects, hyperprolactinemia is commonly observed in patients with primary hypothyroidism due to the lack of negative feed-back of thyroid hormones on TRH. Similarly, estradiol increases PRL secretion, modulating PRL gene expression, modifying its sensitivity to physiologic stimuli but also inhibiting the activity of hypothalamic neuroendocrine dopaminergic neurons (Mancini *et al.*, 2008) (**Fig. 2**). The presence of VIP and oxytocin in high concentrations in pituitary portal blood and the presence of specific anterior pituitary receptors suggests that these neuropeptides may be involved in the regulation of anterior pituitary function. In fact, VIP stimulates PRL secretion in the rat, acting at both the hypothalamus and pituitary gland. The effects of VIP seem to be mediated by oxytocin. At pituitary level, VIP was shown to interfere with the inhibitory action of DA on the adenylate cyclase-cAMP system in an autocrine and paracrine manner. Angiotensin II (AII) is a potent secretagogue of PRL both in vivo and in vitro. It acts on specific receptors of lactotrope cells and PRL release may be blocked by an antagonist of the IIA. The stimulation of PRL release by the IIA is more intense, faster and transient as compared to the effects of TRH.

PRL secretion can also be affected by many different molecules that act indirectly on response of lactotropes to PIFs and PRFs (**Fig. 2**). For example, galanin increases PRL secretion (De Marinis *et al.*, 2000; Giustina *et al.*, 1994), and somatostatin inhibits baseline and induced PRL secretion in vitro. Somatostatin neurons receive abundant efferent connections from galanin, neurotensin, neuropeptide Y, g-aminobutyric acid (GABA), serotonin, enkephalin, substance P, TRH, and the catecholaminergic system (Giustina and Veldhuis, 1998). Moreover, γ-aminobutyric acid (GABA) is in part responsible for the nondopaminergic PIF activity. Finally, hypoglycemia and arginine stimulate PRL secretion.

PRL acts also as a leukocyte-derived cytokine in the immune system and is regulated by cytokines in an autocrine and paracrine way. In T-lymphocytes, interleukin (IL)-2, IL-1β, and IL-4 reduces PRL mRNA (Gerlo *et al.*, 2006). On the other hand, in myeloid leukemic cells, the PRL alternative promoter is activated by the proinflammatory cytokine tumor necrosis factor alpha (TNFα). A TNFα responsive region was located between -1842 and -1662 of the extrapituitary PRL promoter. Interestingly, the stimulatory effect of TNFα upon PRL is blocked by a protein kinase C (PKC) inhibitor. Since TNFα is a proinflammatory cytokine, its involvement in PRL stimulation might have clinical relevance in view of several studies indicating that leukocyte-derived PRL may be involved in autoimmune and hematological disorders.

Prolactin Receptors

PRL receptor (PRL-R) is a trans-membrane protein belonging to the superfamily of cytokine receptors of type 1, which also includes the receptors of GH and IL-1 (Bole-Feysot *et al.*, 1998). PRL-R is encoded by a gene located on chromosome 5 near the gene associated with the GH receptor. The size of human PRL-R gene exceeds 200 kb and contains 11 exons, the 3–10 exons being those encoding the full length of receptor (599 aminoacids). Several isoforms of human PRL-R have been identified including the full length receptor and at least eight other variants depending on the different splicing in the gene transcription. The long PRL-R is the major form that transmits PRL signaling, while the short isoforms, when co-expressed with the long isoform, can act as dominant negatives.

As a member of the cytokine receptor family, PRL-R consists of an extracellular binding domain, a single transmembrane domain and an intracellular domain required for signal transduction that lacks intrinsic kinase activity (**Fig. 3**). The extracellular

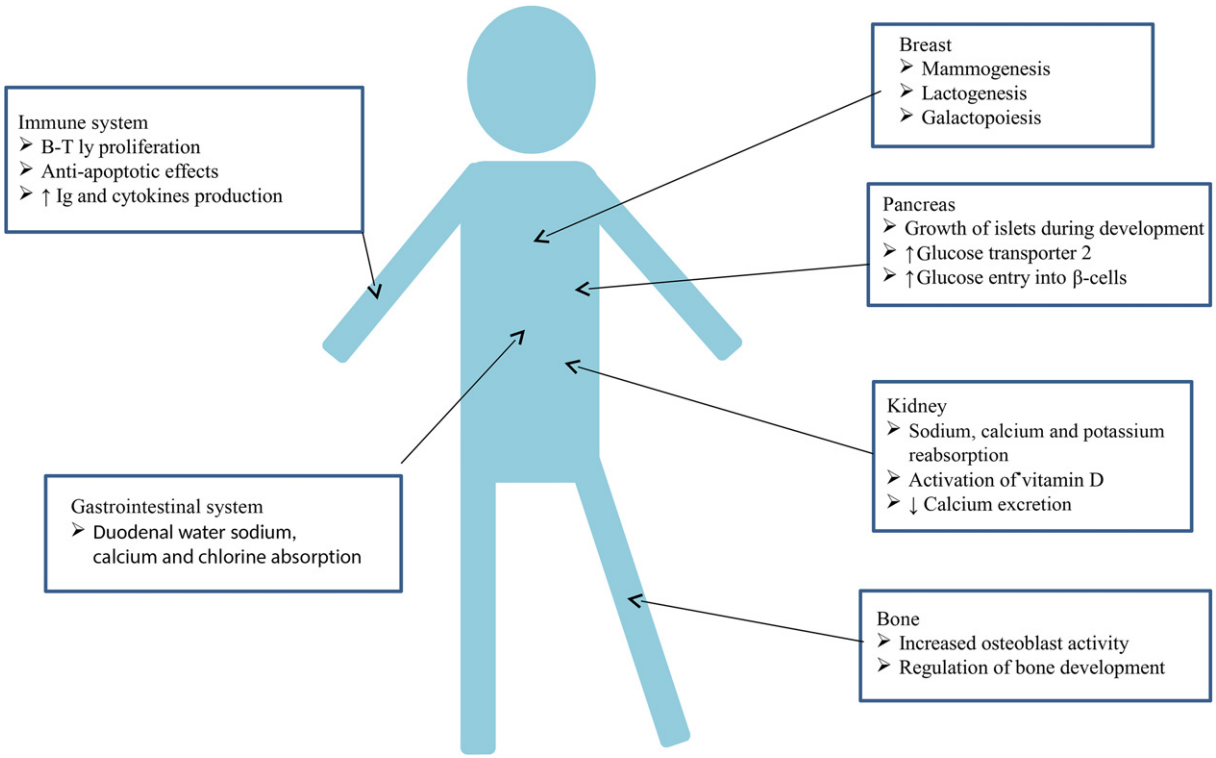

Fig. 4 Extragonadal effects of PRL. Ly, lymphocytes, Ig, Immunoglobulin.

segment of PRL-R is homologous to that of GH, whereas the intracytoplasmic segments of PRL-R are different and shorter than those of GH.

After binding, PRL induces receptor dimerization which is needed for intracellular signal transmission, being the Jak2 (Janus kinase 2)-STAT (Signal transducers and activators of transcription) pathway the main signaling cascade. Jak2 is rapidly phosphorylated upon PRL binding and induces the phosphorylation of the receptor itself, other associated kinases and STAT proteins. The phosphorylation of STAT proteins promotes their dimerization that is required for the final signal transduction in the nucleus, where they regulate gene expression. Other signals used by PRL-R include the pathways of the MAP kinases (MAPK), second messengers phosphatidylinositol and calcium channels.

Physiological and Pathophysiological Actions of Prolactin

PRL-Rs are present in the mammary gland, ovaries, pituitary gland, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, skin and areas of the central nervous system supporting the concept that PRL may exert physiological effects on several tissues and organs (Bole-Feysot *et al.*, 1998) (**Fig. 4**).

Effects on Mammary Gland

PRL exerts different effects on the mammary gland stimulating the growth and development (mammogenesis), the synthesis of human milk (lactogenesis) and maintenance of milk secretion (galactopoiesis). During pregnancy, circulating levels of PRL increase in a progressive manner but lactation occurs only after the completion of delivery. This phenomenon is due to high levels of estrogens during pregnancy, which exert a dual effects on PRL secretion (i.e., stimulation) and activity (i.e., blocking). After delivery, the postpartum drop of estrogen secretion removes the blocking effects on PRL activity.

Gonadal Effects

While hyperprolactinemia effects on gonadal function are well known in males and females (Mancini *et al.*, 2008), less is known about PRL physiological effects. The physiological effect of PRL on the female gonad is represented by an increase in progesterone secretion favoring the LH stimulation of granulosa cells and inhibiting the 20-α-hydroxysteroid dehydrogenase activity (i.e., the enzyme which inactivates progesterone) (Zhong *et al.*, 1997). Interestingly, effects of PRL on progesterone production depend on hormone concentration since hyperprolactinemia was shown to be associated with inhibitory effects on progesterone secretion. In

males, PRL could act modulating the binding of gonadotropins to Leydig cell receptors and interfering with the enzymatic cascade of steroidogenesis. Moreover, in experimental conditions, normal levels of PRL were needed for spermatogenesis (Hair *et al.*, 2002). These physiological effects of PRL on progesterone synthesis in females and steroidogenesis and spermatogenesis in males may be clinically relevant in the specific setting of patients with prolactinomas (over)-treated with DA agonists.

Effects on Immune System

The relationship between PRL and the immune system has been studied and characterized over the last 20 years, opening important horizons in the field of immunoendocrinology. PRL-R is expressed in splenocytes, thymocytes, bone marrow cells, peripheral blood mononuclear cells, lymphocytes and monocytes. Interestingly, PRL was shown to stimulate B and mainly T lymphocyte proliferation. Moreover, PRL induces an anti-apoptotic effect, enhances the proliferative response to antigens and mitogens, and increases the production of immunoglobulin, cytokines and autoantibodies (**Fig. 4**). These effects may at least in part explain the association already reported in literature between PRL excess and several autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Sjogren syndrome, Hashimoto's thyroiditis, and multiple sclerosis (Savino, 2017; Wang *et al.*, 2017).

Metabolic Actions

PRL exerts a well-defined role on osmotic adjustment in numerous animal species. This is particularly evident in fish and in many invertebrates in which there is a direct relationship between PRL, sodium levels and volume of body fluids. Its action as osmotic-controller would involve renal and extra-renal targets through the stimulation of the transport of solutes across the cell membranes such as duodenal absorption of water, sodium, calcium and chlorine and resorption of sodium, potassium and water in the kidney (**Fig. 4**). This mechanism is more easily detectable during the late stage of pregnancy, facilitating the subsequent lactation.

PRL also appears to play a role in pancreatic and adipose development. In adipose tissue, PRL is essential in adipogenesis, adipocyte differentiation and lipid metabolism through the stimulation of leptin and inhibition of adiponectin production (Ben-Jonathan *et al.*, 2006; Carre and Binart, 2014). At pancreatic level, PRL promotes growth of islets during development, and glucose-stimulated insulin secretion. It also increases expression of glucose transporter 2 and promotes glucose entry into β-cells (**Fig. 4**). These actions are important during pregnancy and lactation with adaptive changes in lipid metabolism and glucose homeostasis (Ladyman *et al.*, 2012). Failure of this adaptive response may result in gestational diabetes.

PRL-Rs have been found in the arcuate, ventromedial and paraventricular hypothalamic nuclei involved in regulation of food intake. An excess in PRL secretion, as physiologically occurs during pregnancy, may contribute to the rapid increase in food intake commonly observed during this period and during lactation.

PRL favors renal activation of vitamin D, stimulates intestinal calcium absorption and inhibits the fractional excretion of calcium by the kidney (Rojas-Vega and Gamba, 2016) (**Fig. 4**). All these effects are important for guaranteeing adequate calcium levels for fetal development during pregnancy and breastfeeding during lactation. Over the recent years, there has been convincing evidence that PRL may have also direct effects on osteoblasts which results seem to be variable in relationship to the duration of exposure and likely to the hormone concentrations. Physiological PRL levels are likely required for normal bone development, as suggested by the experimental evidence that knock-out mice for PRL-R have decreased bone mass and deformed skeletal development. However, a prolonged exposure of osteoblasts to PRL excess can have an inhibitory effect on osteoblast number, mineralization and differentiation (Seriwatanachai *et al.*, 2009). This direct effect of PRL on osteoblasts may explain the clinical observation that skeletal fragility may occur in males and females with prolactinomas regardless of coexistent hypogonadism (Mazziotti *et al.*, 2011a,b, 2015).

PRL and Cancer

PRL promotes the proliferation of breast epithelial cells and the differentiation of alveoli in physiological conditions. Moreover, PRL may modulate angiogenesis with variable effects depending on the PRL isoform. Whereas native PRL has angiogenic properties, the 16 K-PRL fragment is a potent antiangiogenic factor inhibiting the effects of vascular endothelial growth factor and fibroblast growth factor (Clapp and De La Escalera, 1997).

An association between circulating PRL levels and risk in breast cancer has been reported in both pre- and post-menopausal women. Interestingly, expression of PRL and PRL-R has been found in breast cancer cells suggesting that a paracrine–autocrine action of PRL may occur in this setting (Shemanko, 2016). Moreover, PRL-PRL-R pathway was involved in determining resistance of breast cancer cells to anti-neoplastic drugs (Idelman *et al.*, 2011). PRL has been studied in other type of cancers with rather discordant results. For instance, in hepatocellular carcinoma, PRL was shown to either impeding liver carcinogenesis by promoting the innate immune response or to promote tumor progression leading to a worse survival of patients (Kong *et al.*, 2016).

However, the emerging evidence that PRL-PRL-R pathway may be involved in some forms of carcinogenesis has highlighted its potential role as target for anti-neoplastic drugs (Wen *et al.*, 2014; Kelly *et al.*, 2017).

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Molecular Pathogenesis of Pituitary Tumors☆

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Glossary

Adenohypophysial Pertaining to the adenohypophysis or anterior pituitary.
Epigenetic Functionally relevant changes to the genome that do not involve changes to the nucleotide sequence.
Heterozygous Pertaining to heterozygosity.
microRNA Small non-coding RNAs.
Monoclonal Derived from a single cell.
Nonsense Pertaining to altered recognition or identification.
Somatic mutations Genetic changes pertaining to a specific cell lineage.
Transgenic Pertaining to the experimental splicing of a segment of DNA from one genome onto DNA of a different genome.

Introduction

This article is an update of Shereen Ezzat, Pituitary Tumors, Molecular Pathogenesis, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 681-686.Pituitary tumors are common neoplasms that exhibit a wide range of biological courses as evidenced by hormonal and proliferative activities (Asa *et al.*, 2017a). Traditionally, there was an on-going controversy regarding the basis of pituitary tumorigenesis. Two prevailing theories confronted hormonal stimulation as an extrinsic stimulus against a somatic mutation as an intrinsic pituitary defect. While a minority of these tumors harbors genetic mutations, several animal models and unique clinical cases have provided support for hormonal stimulation in the development of these tumors. These findings along with evolving experimental evidence favor a theory that accounts for epigenetic dysregulation of the balance between stimulatory and inhibitory influences, irrespective of their origin (Asa, 2011; Asa and Ezzat, 2009). In this review, we highlight recent insights into mechanisms implicated in the dysregulated balance of signals that control pituitary adenohypophysial cell growth and function.

Pituitary Tumor Classification by Cytogenesis

Pituitary tumors have been recognized to produce hormones and immunohistochemistry has complemented the clinical measurement of circulating hormone excess to classify pituitary tumors that cause hormone excess. The clinical features of these functioning tumors are discussed elsewhere. Morphologically, classification of such tumors entails the identification of subcellular features that identify the cell lineage, transcription factors, hormones, and other structural parameters (Asa, 2011) that indicate altered signaling pathways. Acromegaly due to pituitary growth hormone (GH) excess is indeed not one disease, but rather comprises several distinct disorders that have different clinical manifestations, radiologic characteristics, and responses to therapeutic agents (Asa *et al.*, 2017b); the importance of recognizing sparsely granulated somatotroph tumors and distinguishing them from densely granulated somatotroph or mammosomatotroph neoplasms has implications for the postoperative management of residual or recurrent disease. Similarly, although most tumors that are called "prolactinoma" represent sparsely granulated lactotroph tumors, those with unusual clinical manifestations and that fail to respond to dopaminergic agents are frequently discovered to be rare variants such as acidophil stem cell or poorly differentiated Pit-1 lineage tumors (Gomez-Hernandez *et al.*, 2015). In patients with Cushing disease, there are several morphologic variants; the classical microadenoma that causes florid Cushingoid features is usually a densely granulated tumor, whereas the sparsely granulated tumors are usually large when discovered and may not be associated with obvious hypercortisolemia, a phenomenon that has been called a "whispering" rather than "silent" tumor. Rarely, corticotroph tumors may present when large and invasive and with an unusual history that sometimes is considered to be "cyclical" Cushing syndrome, and these tumors are found to be composed of corticotrophs with Crooke's hyaline change.

Clinically nonfunctioning tumors are equally if not more complex. The vast majority of these is now known to be of gonadotroph differentiation; although functioning gonadotroph tumors are rare, the silent tumors usually have focal gonadotropin reactivity and with the use of immunohistochemistry for steroidogenic factor-1, are proven to be of gonadotroph lineage (Asa, 2011). However, those that are not, whether they are silent corticotroph tumors, silent tumors of Pit-1 lineage, or true "null cell" adenomas that have no evidence of any lineage differentiation, are more aggressive (Nishioka *et al.*, 2015).

Armed with the knowledge that pituitary tumors are a complex family of different neoplastic cell types (**Fig. 1**), it is not surprising to find that their pathogenesis is equally complex, often with distinct alterations in different tumor types.

☆*Change History:* September 2017. S Ezzat and SL Asa updated references in all sections. A new list of Further Reading was also updated.

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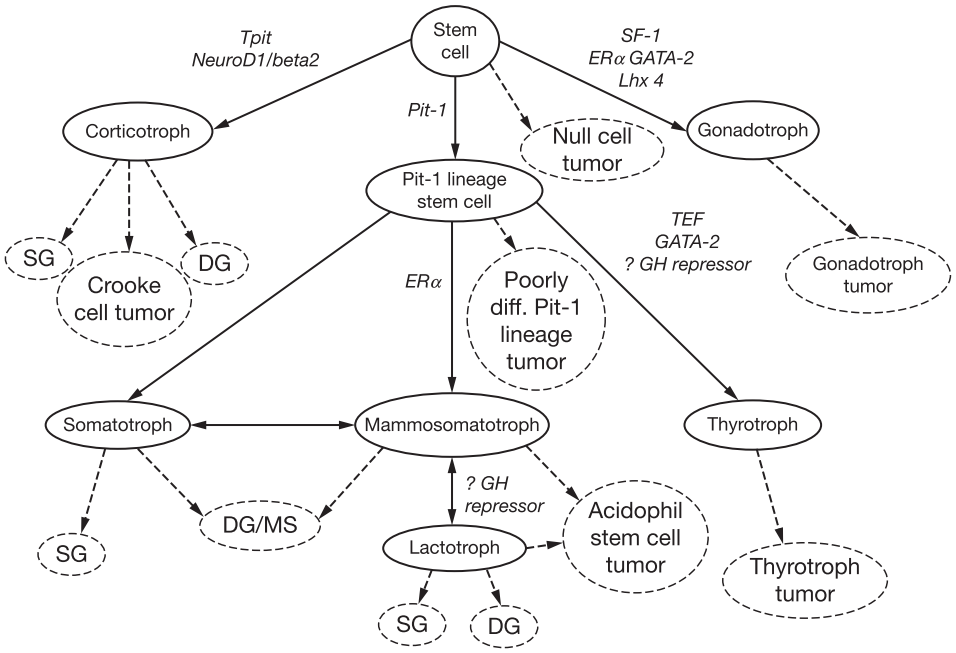

Fig. 1 Pituitary cytodifferentiation. Pituitary stem cells differentiate along three major pathways of pituitary differentiation that are determined by expression of transcription factors (italics); the Pit-1 lineage is more complex, giving rise to four cell types again determined by expression of additional transcription factors. Each cell type can give rise to specific tumor types. *SG*, sparsely granulated; *DG*, densely granulated; *MS*, mammosomatotroph.

Molecular Events

Evidence in favor of an intrinsic pituitary defect accounting for the development of pituitary tumors has been based primarily on the monoclonal composition of these tumors. Although pituitary tumors are monoclonal, somatic mutations in genes that have been identified in other malignancies are usually absent. For example, mutations in codon 12 of *H-RAS* have been reported in an aggressive prolactinoma (Cai *et al.*, 1994; Karga *et al.*, 1991) and in rare metastasizing pituitary carcinomas, but are exceptionally uncommon in the usual pituitary tumors, even when locally invasive. The mutations leading to pituitary tumorigenesis primarily involve components of the hormone regulatory signaling pathways.

Molecular alterations have been identified in the setting of familial pituitary neoplasia (Lyssikatos *et al.*, 2016). Several of the important clinical entities, including multiple endocrine neoplasia type 1 (MEN1) and type 4 as well as most patients with Carney complex, are due to mutations in known genes, specifically MEN1 (Chandrasekharappa *et al.*, 1997; Zhuang *et al.*, 1997), p27 (Georgitsi *et al.*, 2007; Pellegata *et al.*, 2006) or p18 (Georgitsi, 2010), and PRKAR1A (Sandrini *et al.*, 2002), respectively. Familial somatotropinoma and familial isolated pituitary adenoma (FIPA) syndromes are associated with aryl hydrocarbon receptor-interacting protein (AIP) mutations (Beckers *et al.*, 2013; Beckers and Daly, 2007). More recently, the 3PA syndrome (pituitary adenoma with paraganglioma/pheochromocytoma) has been attributed to SDH complex mutations (Xekouki and Stratakis, 2012). Mosaic germline GNAS mutations have been known to be associated with McCune–Albright syndrome (Weinstein *et al.*, 1991) and recent identification of Xq26 microduplications and GPR101 mutation associated with early childhood gigantism is now classified as X-linked acrogigantism (X-LAG) (Trivellin *et al.*, 2014). Dicer mutations have been implicated in the extremely unusual pituitary blastoma (de Kock *et al.*, 2014).

These mutations and their impact will be discussed later. Many of them are implicated in altered signaling pathways that are important in adenohypophysial cells. However, with a few exceptions, these mutations are not found as somatic events that can explain the much more common sporadic pituitary tumors.

Hormone Regulatory Pathways

Activation of Stimulatory Hormone Receptor Pathways

Growth hormone-releasing hormone and its receptor

GH-releasing hormone (GHRH) can cause somatotroph proliferation as evidenced by somatotroph hyperplasia in patients with endocrine carcinomas ectopically secreting GHRH (Asa, 2011). Intrapituitary GHRH expression is well documented and GHRH may be overexpressed in some aggressive tumors. Human somatotroph tumors respond to GHRH stimulation in vitro, consistent

with functional GHRH receptors (GHRHR) on these tumors. In some instances, however, they may lose the downregulation characteristic of normal somatotrophs. Thus, GHRH stimulation can contribute to the development of some pituitary neoplasms. Moreover, older transgenic mice overexpressing GHRH develop pituitary neoplasia. However, in situations of GHRH excess, GH-producing tumors are usually accompanied by somatotroph hyperplasia, which is distinctly unusual in human pituitary GH tumors. Moreover, prolonged exposure to GHRH does not always lead to true neoplastic transformation in humans.

Cloning of the GHRHR has permitted examination of the role of GHRH in somatotroph function. Indeed, loss of GHRHR signaling is recognized as the genetic basis for the little (lit/lit) dwarf mouse. A truncated alternatively spliced form of the GHRHR with limited signaling properties has been identified in GH-producing pituitary tumors. Unlike the case with other examples of endocrine hyperfunction where constitutive activation of the relevant receptor has been described, no intrinsic constitutively active forms of the GHRHR have thus far been identified in pituitary tumors. A truncated GHRHR identified in GH-producing pituitary tumors reduces signaling; there are no reports of activating mutations of *GHRHR* in pituitary tumors (Asa, 2011; Asa and Ezzat, 2009, 2014).

Estrogen

Estrogen excess is implicated in the physiological lactotroph proliferation of pregnancy. In rat models, estrogen-induced lactotroph hyperplasia is associated with enhanced expression of other growth factors such as vascular endothelial growth factor (VEGF) and galanin (Lloyd, 1983). In contrast, a dominant-negative ER induces apoptosis. Although estrogen may be implicated in the development of lactotroph neoplasms in rare situations, such as in a male-to-female transsexual, the administration of estrogen in contraceptive agents or in postmenopausal hormone replacement is not associated with any increased risk of the development of pituitary lactotroph tumors.

Thyrotropin-releasing hormone and the thyroid axis

Primary hypothyroidism is a well-recognized cause of pituitary thyrotroph and lactotroph hyperplasia. Patients with untreated primary hypothyroidism exhibit the spectrum of hyperplasia to neoplasia, suggesting that continuous stimulation by thyrotropin-releasing hormone (TRH) may lead to thyrotroph tumor (Asa, 2011; Asa and Ezzat, 2009). TRH has also been shown to be expressed in the pituitary and by the different types of pituitary tumors.

TRH signaling appears to be intact in pituitary tumors as evidenced by intact binding and normal release of thyroid stimulating hormone (TSH) and prolactin (PRL) in response to TRH exposure. TRH receptor expression and structure are grossly unaltered even in thyrotroph tumors. TRH mRNA is alternatively spliced in some pituitary tumors with deletion of exon 3, resulting in a truncated product that does not bind TRH. The relatively higher levels of the truncated forms compared to the full-length form of the TRH receptor in lactotroph tumors may explain some of the paradoxical in vivo responses to TRH administration.

Thyroid hormones mediate feedback suppression through dedicated nuclear DNA-binding thyroid hormone receptors (TRs), α and β, each of which undergoes alternative splicing. TRβ2 is predominantly expressed in hypothalamus and pituitary; the other isoforms are ubiquitously expressed. Mice with disrupted *TRβ* exhibit elevated thyroid hormone levels but do not develop pituitary tumors. Rare *TRα* missense mutations have been reported, one that was specific to the TRα2 and two that would affect both TRαs isoforms. Thus far, no somatic TRβ alterations have been reported in pituitary tumors (Asa, 2011; Asa and Ezzat, 2009).

Corticotropin-releasing hormone and the glucocorticoid axis

Tumor-associated overproduction of corticotropin-releasing hormone (CRH) causes pituitary corticotroph hyperplasia and Cushing syndrome; however, most CRH-producing tumors also express ACTH, resulting in pituitary suppression. A mouse model of systemic CRH excess similarly caused a Cushing syndrome phenotype but there was no evidence of pituitary corticotroph hyperplasia, because multiple other sources of ACTH respond to CRH. Activating mutations of the CRH receptor have not been reported in corticotroph tumors (Asa, 2011; Asa and Ezzat, 2009).

Conversely, loss of glucocorticoid inhibition in primary adrenal cortical deficiency can result in corticotroph hyperplasia. Corticotroph adenomas have been associated with germline *GCR* mutations and a somatic inactivating *GCR* mutation (Karl *et al.*, 1996a,b).

G proteins

The family of seven transmembrane domain G-protein coupled receptors signal through a family of G proteins to transduce signaling to downstream effectors. Stimulatory G proteins (Gs) have three subunits; on ligand binding, the α-subunit dissociates from the β- and γ-subunits, thereby stimulating adenylyl cyclase to convert ATP to cyclic AMP (cAMP). Activating mutations in the *GNAS* gene alter two domains of Gsα to interfere with GTP hydrolysis, constitutively increasing cAMP. These G-protein oncogenes (*gsp*s) were the first somatic mutations described in endocrine tumors, including some pituitary densely granulated somatotroph neoplasms, as well as hormone-secreting tumors of other endocrine glands including thyroid and adrenal cortex (Landis *et al.*, 1989; Lyons *et al.*, 1990). The resulting elevation of cAMP is thought to be the reason for the clinical responsiveness of densely granulated somatotroph tumors to somatostatin (SS) analog therapy (Bhayana *et al.*, 2005).

Germline mosaicism of these *GNAS* mutations causes McCune–Albright syndrome, which features somatotroph hyperplasia or neoplasia (as well as thyroid and adrenal cortical autonomously functioning adenomas) (Salpea and Stratakis, 2014; Weinstein *et al.*, 1991). This gene exhibits monoallelic imprinting and the mutations usually involve the maternal allele.

More recently, germline and somatic changes in the orphan G-protein receptor GPR101 have been implicated in childhood gigantism and adult acromegaly respectively. Indeed, germline duplication of Xq26.3 with overexpression of GPR101 has been implicated in a rare form of pediatric GH excess termed X-LAG (Trivellin *et al.*, 2014). Somatic mutations of GPR101, however, do not appear to play a role in sporadic somatotroph tumors that develop in adolescence and adulthood.

Protein kinase-A

The role of cAMP signaling in somatotroph proliferation and transformation is evident from Carney's complex, an autosomal dominant disorder in which affected patients develop spotty pigmentation of mucosal surfaces, cardiac myxomas, and hypersecreting endocrine tumors of the pituitary (somatotroph and mammosomatotroph), adrenal cortex, and gonads. Most Carney complex patients have germline inactivating mutations in the PRKAR1Aα gene that encodes the protein kinase-A (PKA) regulatory subunit 1Aα, one of four components of the tetrameric holoenzyme that is critical for intracellular protection from unrestrained catalytic activity and elevation of cAMP (Kirschner *et al.*, 2000). However, this gene is not implicated in the pathogenesis of sporadic pituitary tumors (Salpea and Stratakis, 2014). A second locus at 2p16 has been implicated but the responsible gene remains unknown. Patients with this disorder develop pituitary somatotroph or mammosomatotroph tumors (Asa, 2011). Animal models proved that loss of PRKAR1A activity can lead to pituitary tumor development (Yin *et al.*, 2008).

The aryl hydrocarbon receptor-interacting protein

AIP is a chaperone protein with multifunction properties, including modulation of the transcriptional activity of the aryl hydrocarbon receptor, which mediates toxicological and carcinogenic dioxin effects. Recent studies have shown that AIP regulates cAMP concentrations through Gαi-2 and Gαi-3 proteins that inhibit cAMP synthesis (Beckers *et al.*, 2013). Germline mutations in AIP have been identified in some families with the isolated familial somatotropinoma syndrome where acromegaly and gigantism predominate, and in those with FIPA syndrome, with various types of pituitary tumors but usually including some GH- and PRL-secreting tumors (Beckers *et al.*, 2013). Loss of heterozygosity (LOH) of the *AIP* gene in the tumors suggests that it acts as a tumor suppressor, consistent with its role in cAMP modulation. Nevertheless, numerous studies have shown that AIP mutations are not found in sporadic pituitary tumors (Asa and Ezzat, 2014).

Inactivation of Inhibitory Hormone Receptor Pathways

Dopamine

The role of diminished hypothalamic inhibition was first suggested based on the observation of neovascularization in lactotroph tumors (Bergland and Page, 1979). It was speculated that neovascularization would allow lactotrophs to escape from tonic dopaminergic inhibition. Dopamine signal transduction is mediated through D1 receptors that stimulate adenylyl cyclase activity and D2 receptors (D2R) that inhibit this enzyme. The family of dopamine receptors is complex in biochemical, physiological, and pharmacological diversity. Nevertheless, it appears that the predominant anterior pituitary dopamine receptor is the D2R. Targeted disruption of D2R activity in D2R knockout mice results in lactotroph hyperplasia and, subsequently, lactotroph neoplasia in female D2R-deficient mice (Schuff *et al.*, 2002). Interestingly, these lesions are monohormonal PRL-immunoreactive neoplasms that display a characteristic juxtanuclear Golgi pattern of PRL staining and loss of the reticulin fiber network. Several of these tumors have been noted to be much larger than normal glands, with marked suprasellar extension and invasion of brain but no gross evidence of distant metastases.

Although some pituitary tumors have been shown to be responsive to dopamine suppression, the dopaminergic resistance that is found in some tumors implicates diminished D2R activity as a putative factor in pituitary tumorigenesis. Thus far, however, investigation of the D2R gene has revealed it to be structurally intact in human lactotroph tumors (Asa and Ezzat, 2009).

Prolactin

The autoregulation of PRL secretion in the rat has been demonstrated at the levels of both the hypothalamus and the pituitary. In transfected somatolactotrophs, PRL exerts a strong and specific inhibition of PRL gene transcription. These effects appear to be mediated by intermediate and long forms of the PRL receptor (PRL-R). Deletions of the PRL promoter indicated that the autoregulatory effect of PRL requires the same regulatory domains that have been described for the other PRL gene regulators. These studies support an extra-short loop of PRL transcriptional autoregulation. In the normal human pituitary, PRL and lutei-nizing hormone-expressing cells exhibit the highest levels of PRL-R mRNA expression, whereas PRL-producing pituitary tumors have significantly higher levels of PRL-R mRNA than other types of pituitary neuroendocrine tumors. Dopamine agonist treatment decreases the levels of PRL-R mRNA in PRL tumors. Although *PRL-R* mutations are not found in human prolactinomas (Asa, 2011; Asa and Ezzat, 2009), an inactivating germline mutation has been reported in familial hyperprolactinemia (Newey *et al.*, 2013).

Somatostatin

GH secretion is under contrasting influence from hypothalamic stimuli including GHRH, which stimulates GH secretion, and SS, which inhibits GH secretion. Specific receptors for SS (SSTRs) are expressed by somatotroph tumors. Earlier studies suggested a relationship between the density of SS receptors on GH tumors and the secretory response to this analog both in vitro and in vivo. Binding sites for SS, however, have been identified by autoradiography in tumors resistant to the GH-lowering effects of octreotide.

These findings are consistent with differential adenylyl cyclase coupling by the five known subtypes of SSTRs and their heterogeneous expression in pituitary neoplasia. A germline SSTR5 inactivating mutation has been associated with acromegaly in one patient but no other mutations have been described to account for loss of SS inhibition. Expression of SS itself in large invasive GH tumors appears to be diminished compared to that in the normal pituitary (Lania *et al.*, 2008). These findings suggest multiple paracrine, autocrine, and endocrine mechanisms for SS-mediated control of somatotroph function and proliferation.

Growth hormone

GH feedback on somatotrophs has been shown in mice, where interruption causes mild somatotroph enlargement and hyperplasia. Pharmacologic inhibition of GH receptor signaling with the GH antagonist pegvisomant induces the development of keratin aggresomes known as "fibrous bodies" that are the hallmark of sparsely granulated somatotroph adenomas, and a rare somatic histidine-to-leucine mutation that destabilizes the GH receptor have been reported in tumors of that morphology, suggesting that altered STAT signaling is implicated in their development (Asa *et al.*, 2007; Asa and Ezzat, 2009).

Thyroid hormones

The development of pituitary thyrotroph adenomas in patients with prolonged primary hypothyroidism has provided further evidence supporting the hypothalamic role in pituitary tumorigenesis. Thyroid hormones mediate their actions via nuclear TRs that bind to specific regulatory hormone-response elements. There are two major classes of TRs, designated α and β, each of which undergoes alternative splicing to generate $\alpha 1$ and $\alpha 2$ and $\beta 1$ and $\beta 2$ isoforms. With the exception of the $\beta 2$ form, which is predominantly expressed in the hypothalamic–pituitary system, these receptor isoforms are ubiquitously expressed. Of interest, in the pituitary, the $\beta 1$ and $\beta 2$ isoforms appear to be expressed to a lesser extent in tumors than in the normal gland. Screening TR-α mRNA identified three novel missense mutations, two in the common TR-α region and another that was $\alpha 2$ specific. TR-β-response elements failed to show any differences from published sequences.

Mice with targeted disruption of the entire TR-β locus exhibit elevated thyroid hormone levels as a result of abnormal central regulation of thyrotropin but do not develop pituitary tumors (Abel *et al.*, 1999). Thus, the putative differential hormone regulatory and mitogenic effects of the different THR isoforms in the pituitary remain unclear.

The TGF-β family, activins, inhibins, and follistatins

TGF-β has been implicated in the regulation of normal and neoplastic cell function. TGF-β regulates the expression of various proteins, including the cell-cycle inhibitory protein p27Kip1 (p27). TGF-β 1/2/3 isoforms and the TGF-β receptor are expressed in normal and neoplastic pituitaries. Dispersed pituitary tumor cells show a biphasic response to TGF-β with changes in follicle-stimulating hormone (FSH) secretion. The TGF-β family, however, is represented in at least three different forms in the pituitary. Inhibins and activins consist of two homo- or heterodimeric polypeptide subunits derived from a common precursor; inhibin A (α-βA) and inhibin B (α-βB) selectively inhibit the release of FSH from pituitary gonadotroph cells, whereas activin (βA-βB), activin A (βA-βA), and activin B (βB-βB) stimulate its release. Inhibin subunits are expressed by pituitary gonadotroph tumors and activin is known to stimulate hormone secretion by these tumors. Activin, however, inhibits cell proliferation in a variety of cell types including pituitary tumors. These actions are mediated through type I and type II serine/threonine kinase receptors. The predominant form is type I (ActRIB or Alk4). Truncated forms of the type I receptors expressed by gonadotroph tumors interfere with wild-type receptor function and block the antiproliferative effects of activing (Zhou *et al.*, 2000). In addition, follistatin expression is diminished in these tumors (Penabad *et al.*, 1996), which represent the vast majority of clinically nonfunctioning pituitary neoplasms and almost 40% of pituitary tumors (Asa, 2011). These findings suggest an important role for the activin/follistatin/activin receptor balance in modulating pituitary tumor cell replication.

Gonadal steroids

The development of pituitary gonadotroph neoplasia in patients with prolonged primary hypogonadism (Asa, 2011) suggests that the lack of hormone negative feedback may facilitate pituitary tumor development. The role of GnRH stimulation cannot be easily distinguished from that of gonadal hormone inhibition in the development of these tumors.

Glucocorticoid hormones

Loss of glucocorticoid inhibition has been reported to cause corticotroph hyperplasia and neoplasia in rare patients with prolonged primary adrenal insufficiency. Germline inactivating mutations in the human glucocorticoid receptor (GCR) cause familial glucocorticoid resistance. A small number of pituitary corticotroph tumors causing Cushing's disease may be attributed to novel germline GCR mutations or rare somatic GCR mutation that can interrupt glucocorticoid inhibition (Karl *et al.*, 1996b).

Growth Factor Regulatory Pathways

Growth Factors and Their Receptors

Transforming growth factor-α

Transforming growth factor-α (TGF-α) is expressed as a membrane-anchored protein by human adenohypophysial cells and tumors (Ezzat *et al.*, 1995). TGF-α may alter pituitary production of GH, PRL, and TSH as well as cell proliferation. Estrogen stimulation has been implicated in pituitary tumorigenesis and TGF-α appears to mediate some estrogenic effects (Liu *et al.*, 1987). Targeted overexpression of TGF-α to the pituitary results in lactotroph tumor (McAndrew *et al.*, 1995), providing compelling evidence for the significance of this growth factor in pituitary tumorigenesis.

Epidermal growth factor and its receptor

Epidermal growth factor (EGF) is detectable by immunohistochemistry in most adenohypophysial cells and its mRNA is expressed with marked variation in all types of functional and nonfunctional adenohypophysial tumors. EGF potently stimulates PRL and adrenocorticotropic hormone secretion with variable effects on rat pituitary cell proliferation. The selective expression and specific effects of EGF suggest that the pituitary is an important target site for this growth factor's action.

The common receptor of EGF and TGF-α, EGF receptor (EGF-R), is a 170 kDa plasma membrane tyrosine kinase product of the proto-oncogene v-*erb*B. EGF-R is overexpressed in several types of human cancers and in most instances this overexpression is accompanied by TGF-α expression; expression of this receptor appears to correlate with tumor aggressiveness. EGF-R is expressed by human pituitary tumors with the highest levels in recurrent somatotroph tumors and suggesting a selective mechanism for the EGF/EGF-R family in the growth of aggressive somatotroph tumors (LeRiche *et al.*, 1996). The importance of the EGF-R in the somatotroph is further supported by the selective loss of somatotrophs in transgenic mice overexpressing a dominant-negative EGF-R mutant lacking the intracellular protein kinase domain (EGFR-tr) (Roh *et al.*, 2001). These findings point to EGF-R as an integral component in the differentiation and proliferation of somatotrophs. However, unlike other cancers, no mutations of the EGF-R have been identified in pituitary neuroendocrine neoplasms.

The human EGF-R family includes four members, the prototypic EGF-R (ErbB1, HER1), as well as p185Her2/neu (ErbB2 and HER2), ErbB3 (HER3), and ErbB4 (HER4). Recently, mutations in the deubiquitinase USP8, a component of the proteasomal degradation pathway, were shown to impair EGF-R degradation thereby augmenting signaling in some corticotroph tumors (Hayashi *et al.*, 2016; Ma *et al.*, 2015; Reincke *et al.*, 2015). Like EGF-R, the ErbB2 receptor is expressed by all tumor subtypes and is upregulated in invasive tumors but there is no evidence of mutation or gene amplification as in other cancers. ErbB3 is expressed in lactotroph tumors and upregulated in aggressive forms, and Heregulin (HRG), the ligand for ErbB3 and ErbB4, is involved in PRL regulation. ErbB4 is expressed in the normal adenohypophysis but its expression and possible function in pituitary tumors is not known (Cooper *et al.*, 2011). Recent studies involving pharmacologic approaches that interrupt Erbb2 signaling have highlighted the potential role of exploiting this pathway in pituitary tumors (Liu *et al.*, 2015).

Fibroblast growth factors and their receptors

Basic fibroblast growth factor (also known as FGF-2) is one of an expanding family of at least 23 members of ligands with variable mitogenic, angiogenic, and hormone regulatory effects. FGF-2 immunoreactivity was described originally in nonhormone-producing bovine pituitary folliculo-stellate cells and has since been shown to regulate GH, PRL, and TSH secretion by the rodent pituitary. FGF-2 is produced by adenohypophysial cells in human pituitary tumors. Pituitary-derived FGF-2 has been shown not only to stimulate replication of PRL-secreting cells but also to inhibit DNA synthesis in pituitary tumor cells, suggesting that some forms of the growth factor or its receptor may act as growth inhibitors. Elevated concentrations of FGF-2-like immunoreactivity have been documented in patients with MEN1 and in patients with sporadic pituitary tumors. The FGF-related *hst* has been found in transforming DNA of human PRL-secreting tumors and facilitates lactotroph proliferation. Transgenic mice expressing FGF-2 under the control of the GH and the α-subunit promoters develop hyperplasia of several adenohypophysial cell types but not frank neoplastic transformation. FGFs or homologous family members may, therefore, play an important role in pituitary tumorigenesis.

FGF signaling is mediated through FGF receptors (FGFRs). Four FGFR genes encode a complex family of transmembrane receptor tyrosine kinases. Multiple forms of cell-bound or secreted receptors are produced by the same gene. Tissue-specific alternative splicing, variable polyadenylation sites, and alternative initiation of translation result in truncated receptor forms. Structural alterations of FGFRs may play a role in human tumorigenesis; for example, antisense targeted interruption of FGFR1 reduces malignant melanoma cell proliferation and differentiation. The normal pituitary expresses mRNAs for FGFR1, 2, and 3 (Abbass *et al.*, 1997). FGFR2-IIIb is expressed in normal adenohypophysial cells; it acts as a tumor suppressor and is downregulated in pituitary tumors, associated with increased proliferation and release of expression of the cancer/testis-melanoma associated antigen MAGE-A3 (Zhu *et al.*, 2008).

Altered FGFR4 expression in pituitary tumors (Qian *et al.*, 2004) results in distinctive cytoplasmic localization that is implicated in dysadhesion of pituitary tumor cells (Ezzat *et al.*, 2006). Similarly PSA-NCAM, which is associated with tumor growth and invasiveness, likely alters the FGFR4 complex that maintains adhesion and normal acinar architecture of adenohypophysial cells (Ezzat *et al.*, 2006). A common germline polymorphism in the transmembrane domain of the FGFR4 gene has also been implicated in modulating growth and function of pituitary tumor cells; the FGFR4-R388 variant yields an isoform whose signaling

relies heavily on Src and STAT3 activation to enhance pituitary cellular growth and impair normal hormone regulation (Nakano-Tateno *et al.*, 2014; Tateno *et al.*, 2011) and may play a role in determining response to SS analog action (Ezzat *et al.*, 2017b).

Angiogenesis and hypoxia

VEGF plays a role in angiogenesis and is thought to be important in tumorigenesis. It is expressed in some pituitary tumors (Takano *et al.*, 2014); however, it does not correlate with microvascular density that varies between tumor types but is generally much lower in tumors than in the normal adenohypophysis (Jugenburg *et al.*, 1995). This interesting observation is consistent with the reduced gadolinium enhancement seen in pituitary tumors during magnetic resonance imaging and indicates a hypoxic environment.

Hypoxia-driven gene and protein changes are implicated in epithelial-to-mesenchymal transition, drug resistance, cancer stem cell formation, and evasion from immune surveillance. These were not traditionally considered to be features of pituitary tumors; however, the evidence for reduced blood flow led to studies of hypoxic responses in pituitary tumors. C-terminal binding protein, a transcriptional corepressor that mediates development and tumorigenesis, interacts with Ikaros isoforms in pituitary cells and both are induced by hypoxia (Dorman *et al.*, 2012) and target the metalloproteinase ADAMTS10 (Shen *et al.*, 2017). Mammalian sterile-20-like kinase (MST4) is also overexpressed in tumors compared with normal pituitaries and is thought to mediate proliferation and survival in a hypoxic environment (Xiong *et al.*, 2015).

Cell-Cycle Regulatory Pathways

Loss of Tumor Suppressors

Multiple endocrine neoplasia type 1

According to the two-hit model of tumorigenesis, both copies of a gene situated on opposite alleles must be inactivated, such as by deletion, rearrangement, or silencing through methylation, to confer a selective growth advantage to a precursor cell, which may subsequently proliferate in a clonal neoplastic fashion. Such genes that require homozygous inactivation of both copies are termed tumor suppressor genes (TSGs), anti-oncogenes, or recessive oncogenes. Examples of TSGs include retinoblastoma (Rb) and p53. In the affected tumors, allelic loss of the gene in question is invariably noted.

After a decade of search, the MEN1 gene was identified by positional cloning with germ-line mutations characterized in kindreds with familial MEN1. The gene product was coined menin to describe the 610-amino-acid protein product. Targeted gene inactivation of menin results in prolactinomas characterized by somatic loss of the wild-type allele with remarkable similarity to humans with MEN1. As predicted, mutations in sporadic pituitary tumors have been described. One copy of the MEN1 gene was found to be deleted in 10% of sporadic tumors examined. Missense mutations have been described throughout the gene of the remaining allele. Thus, as predicted by Knudson's two-hit TSG hypothesis, mutational inactivation of one copy of MEN1 coupled with deletion of the second allele strongly indicates that this gene is involved in the pathogenesis of hereditary (familial) pituitary tumors and in a subset of sporadic pituitary tumors. Up to 20% of sporadic pituitary tumors exhibit LOH at the 11q13 locus even in the absence of menin mutations, suggesting additional TSG at this locus. The characterization of normal menin function will likely prove to be of evolving significance in pituitary tumorigenesis. Thus far, menin has been shown to modulate a number of growth-regulatory pathways including that of the inhibitory transforming growth factor-β (TGF-β) as a result of interaction with the Smad3 transcription factor.

The Rb gene

The Rb gene is another member of the family of TSGs that has been implicated in several neoplasms including Rb and osteosarcoma. Mice heterozygous for an Rb mutation develop pituitary tumors of intermediate lobe corticotroph differentiation. In contrast, proteoglycan NG2-mediated Rb inactivation leads to anterior pituitary tumors highlighting the cell-type selective actions of this TSG (Tateno *et al.*, 2016). Nevertheless, no such mutations have been identified in human pituitary adenohypophysial tumors. Instead, there is LOH at sites telomeric and centromeric to the Rb locus in some aggressive pituitary neoplasms. These data argue for an independent TSG on 13q, which is closely linked with but distinct from Rb. In addition, methylation of the Rb promoter region has been described in association with diminished detectable Rb protein expression.

Cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors

A series of cyclins and cyclin-dependent kinases (cdks) plays a central role in the regulation of cell cycle progression. The cyclin D (cdk4) and E (cdk2) complexes are catalytically active during the late G_1 phase and are implicated in the regulation of G_1/S progression. The Rb protein is one of the putative substrates of the cdks. Rb phosphorylation abrogates the ability of these proteins to inhibit transactivation of transcription factors important in cell cycle control. In turn, cdk activity is modulated by cdk inhibitors. These include $p27^{kip1}$, $p57^{kip2}$, $p16^{ink4A}$, $p15^{ink4B}$, $p18^{ink4c}$, and $p19^{ink4D}$.

Prompted by the potentially pivotal role of the Rb protein in regulating pituitary cells (Tateno *et al.*, 2016) and the negative findings involving the *Rb* gene itself, factors governing Rb phosphorylation became obvious candidates. Specifically, cdk inhibitors have received extensive attention. As with Rb itself, p16 has not been found to be mutated in pituitary tumors, but is frequently

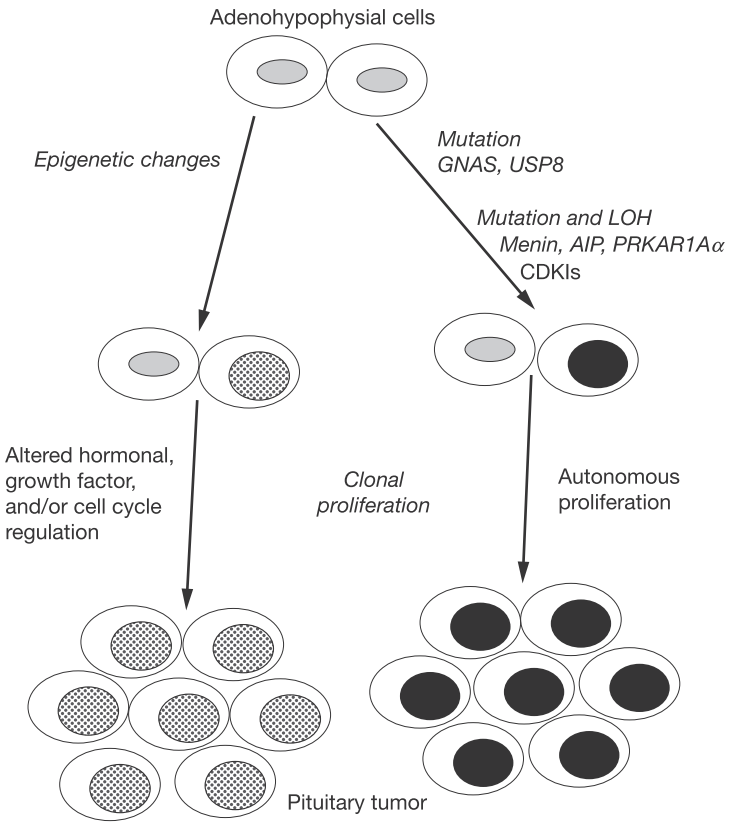

Fig. 2 Proposed model of pathogenesis of pituitary tumorigenesis. While some tumors undergo the classical mutation of oncogenes or mutation and loss of heterozygosity leading to clonal proliferation (right), the majority of pituitary tumors have no known mutations. Instead, epigenetic changes are implicated in cell transformation and subsequent clonal proliferation (left).

silenced by means of extensive methylation in human pituitary neoplasms. These findings suggest an alternative mechanism in modulating Rb-related protein control of the pituitary tumor cell cycle.

Mice lacking $p27^{kip1}$ have an increased propensity for the development of multiorgan neoplasia including pituitary tumors. Mutations in the CDKNIB/p27Kip1 gene were found in a kindred with Cushing disease and hyperparathyroidism and mutations in the CDKN2C/p18INK4c gene have been associated with an MEN-1-like syndrome classified as MEN-X or MEN-4. As with the *Rb* gene, however, intragenic mutations of these genes do not appear to play a role in sporadic human pituitary tumorigenesis. There is a single report of a pituitary tumor with reduced p27 translation due to a mutation of *DKC1* that encodes dyskerin, a pseudouridine synthase implicated in translation of p27 (Bellodi *et al.*, 2010).

p53

This nuclear protein is critical in the control of G_1/S phase progression by regulating the gene expression of p21, the first of the group of cdk inhibitors to be identified. Following DNA damage, p53 can induce growth arrest during the G_1 or G_2 phase of the cell cycle or by stimulating apoptosis, thereby protecting the normal cell from replicating damaged DNA. Conversely, p53 mutations render the cell genome more vulnerable to mutations that represent the rate-limiting steps in tumor progression. Indeed, inactivating mutations of p53 have proven to be among the most commonly encountered gene alterations in human malignancies. However, studies have failed to identify p53 mutations in human pituitary tumors.

Epigenetic Events

The vast majority of sporadic pituitary adenohypophysial tumors lack mutations of candidate genes; however there is common pattern of altered expression levels. The various changes in signaling pathways of key hormonal and growth factors and the silencing of cell cycle regulators discussed earlier are likely attributable to epigenetic factors (Ezzat *et al.*, 2017a).

A number of mechanisms have been implicated in the epigenetic dysregulation that underlies pituitary tumors. DNA methylation has been identified as a mechanism of silencing several TSGs including Rb, p27, p16, and p18 (Yacqub-Usman *et al.*, 2012), and the mediators of this, the DNMT enzymes, are also altered in these tumors. Histone modifications are mediated by

changes in multiple chromatin remodelers, including Ikaros (Ezzat and Asa, 2008) and HMGA proteins (De Martino *et al.*, 2009). Alterations in miRNA expression are also becoming discovered as putative mechanism of epigenetic dysregulation (Li *et al.*, 2014).

In contrast to mutational events, epigenetic alterations are potentially reversible, offering novel therapeutic approaches for the management of pituitary tumors.

Conclusions

Accumulating evidence suggests that the control of pituitary cell multiplication rests on a precarious balance between growth factor stimulation and inhibition with dominant contributions from the FGF/FGFR and TGF-β systems. Mutations in oncogenes and TSGs have been identified as causative of neoplastic transformation in several familial syndromes and in a small subset of sporadic tumors, but the events leading to pituitary tumorigenesis in the vast majority of tumors remain to be validated. Epigenetic changes that alter cell cycle regulators and the signaling pathways induced by hormones and growth factors are frequent but their cause is unknown (**Fig. 2**). Differences between pituitary and other endocrine and nonendocrine neoplasms will likely emerge to explain the remarkably varied spectrum of biologic behavior in terms of invasiveness and metastatic potential, which is unique to human pituitary adenohypophysial neoplasms.

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Further Reading

Melmed, S., 2016. Pituitary medicine from discovery to patient-focused outcomes. Journal of Clinical Endocrinology and Metabolism 101 (3), 769–777.

Classification of Pituitary Neuroendocrine Tumors (PitNets)

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Introduction

Since the first description of acromegaly by Pierre Marie (1902) and Cushing's disease by Cushing (1932), these endocrine pituitary diseases are now considered oncologic pathologies. Recently, use of the term pituitary neuroendocrine tumors (PitNETs) rather than pituitary adenomas has been proposed (Asa *et al.*, 2017). These pituitary tumors, arising from the adenohypophyseal cells, are common intracranial neoplasms, affecting 1/1000 of the population (Daly, *et al.*, 2006; Fernandez *et al.*, 2010).

Over the years, the increase in technology and knowledge has meant pathological classification has evolved from a tinctorial classification (three types: acidophilic, basophilic, and chromophobic) to a morphofunctional classification (five main types: somatotroph, lactotroph, corticotroph, gonadotroph, and thyrotroph). The great majority of these tumors are benign. However, 30–45% of them invade the cavernous or the sphenoid sinus, and a substantial proportion (10%) are considered aggressive, based on recurrence during follow-up. The absence of histological signs of malignancy means that only the tumors that develop metastasis during follow-up (0.2%) are considered to be malignant and termed carcinomas. Recent evidence suggests that the benign status of the pituitary tumors must be reconsidered. A prognostic clinicopathological five-tiered classification has been proposed, as well as the term "atypical adenoma" to describe tumors suspected of malignancy. These classifications, and several pathological, molecular, and genetic markers associated with tumor behavior, are presented in this chapter.

Nomenclature and Definitions

From Adenoma to PitNET

The current classifications do not accurately reflect the clinical spectrum of pituitary tumor behavior, because the simplistic distinction between adenoma and carcinoma is based solely on metastatic spread. Recently, members of the International Pituitary Pathology Club present at the 14th meeting in Annecy (France) in November 2016 proposed new terminology: the pituitary neuroendocrine tumor (Asa *et al.*, 2017). This revision of nomenclature is not intended to negate the well-established morphofunctional classification that encompasses five main subtypes (somatotroph, lactotroph, corticotroph, gonadotroph, and thyrotroph). Instead, it is intended to modify the term from "adenoma" to "tumor". The new nomenclature is consistent with that used for other neuroendocrine neoplasms and should favor the establishment of tumor registries to capture data on these tumors. Indeed, PitNETs are not simply endocrine diseases, but should be considered tumors with endocrine manifestations within the context of oncology. This is not only a semantic interest. In most jurisdictions, these tumors are not currently reported in cancer registries. Patients and healthcare providers have long expressed frustration that these lesions are considered rare, benign, and inconsequential, and they are often denied access to health insurance.

We will use this new terminology in this chapter.

Pituitary Tumors

According to the WHO 2017 classification (Osamura *et al.* 2017), "a pituitary tumor is a neoplastic proliferation of the anterior pituitary hormone-producing cells. The tumors are typically benign, but can be aggressive and invasive into adjacent structures" (ICD-O-Code 8272/0).

Ectopic Tumors and Invasive Tumors

Most pituitary tumors are found in the sella turcica, but may sometimes occur in the sphenoid sinus, separated from the normal pituitary. These tumors are named "ectopic" pituitary tumors (Thompson *et al.*, 2012) and are sometimes difficult to differentiate from the invasive tumors that originate from the pituitary.

A total of 30–45% of tumors invade adjacent structures, particularly the sphenoid and the cavernous sinuses (Meij *et al.*, 2002; Zada *et al.*, 2011). From anatomical studies (Francois *et al.*, 2010), suprasellar extension is differentiated from "true" invasion into the cavernous or the sphenoid sinus. Based on endoscopic verification, only grade 3b and 4 tumors as defined by the Knosp's classification are now considered to be invasive into the cavernous sinus (Micko *et al.*, 2015).

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Aggressive Tumors and Carcinoma

The definition of aggressiveness is clinically defined, based on the clinical characteristics of the tumor during the follow-up period (review by Raverot *et al.* 2012). Aggressive Pit-NETs are typically large with radiological signs of invasion and, despite optimal standard therapies (surgery, radiation therapy, and conventional medical treatments), exhibit an unusually rapid growth rate, and/or clinically relevant tumor growth or recurrence. The frequency of aggressive tumors is not well established; recent studies suggest it is around 10% (Raverot *et al.*, 2017). Although aggressive PitNETs are usually large and invasive, invasion alone does not automatically indicate aggressiveness, and in fact recurrence does not occur in all invasive PitNETs (Chatzellis *et al.*, 2015). Moreover, some giant lactotroph tumors, which regress under dopamine agonist treatment, are not considered to be aggressive tumors.

According to the WHO 2017 classification (Osamura *et al.*, 2017), "Pituitary carcinoma is strictly defined as a tumor of adenohypophyseal cells that metastasizes craniospinally or is associated with systemic metastasis" (ICD-O code 8272/33). This subtype is detailed in a specific chapter. We will discuss this definition in a following section on the prognostic classifications.

Functioning, Nonfunctioning, and "Silent" Tumors

Pituitary endocrine tumors are clinically classified as functioning tumors, when the tumor induces symptoms caused by hormonal hypersecretion (acromegaly with elevated plasma growth hormone (GH) levels, amenorrhea-galactorrhea or hypogonadism with hyperprolactinemia, or Cushing's disease with hypercortisolism), or nonfunctioning tumors, where there are no signs of hypersecretion; these are mainly gonadotroph tumors. These nonfunctioning tumors should be differentiated from silent tumors, where the patient shows no clinical sign or hormonal elevation in the plasma, but immunohistochemistry (IHC) detects tumor hormone production. Somatotroph and corticotroph silent tumors are the most common and may be aggressive.

Classifications of PitNETs

Morphofunctional Classifications

Previous classifications

Until the 1980s, based on their tinctorial properties with hematoxylin eosin/phloxine saffron and their correlation with clinical disease, pituitary tumors were classified as eosinophilic or acidophilic tumors with acromegaly, basophilic tumors with Cushing's disease, or chromophobic tumors without signs of hypersecretion. Later, the development of electron microscopy (EM) and IHC resulted in tumor classification based on the appearance of their organelles (such as granulations, mitochondria) and their hormonal secretion (Asa and Kovacs, 1983; Trouillas *et al.*, 1974; Trouillas and Girod, 1996). Taking into account the clinical signs, and the ultrastructural and IHC data, subtypes were described: lactotroph (prolactin (PRL) adenoma or prolactinoma (review by Nosé *et al.*, 2017), somatotroph (GH) adenoma with both the sparsely and densely ultrastructural subtypes (review by Mete *et al.*, 2017), gonadotroph (FSH/LH) adenoma, with hypersecretion (review by Snyder, 1985) or with normal plasma values (Trouillas *et al.*, 1981; Trouillas *et al.*, 1986), null cell adenoma (Kovacs *et al.*, 1980; Yamada *et al.*, 2017), also termed immunonegative adenoma (Trouillas and Girod, 1996), and thyrotroph (TSH) adenoma (review by Beck-Peccoz *et al.*, 1996; Bertholon-Grégoire *et al.*, 1999).

The pathological characteristics of these types are not described here. Illustrated descriptions can be found in the chapters of the WHO 2017 classification (Lloyd *et al.*, 2017), and a detailed history of these different classifications are available in a recent review (Trouillas 2015).

The WHO 2004 and 2017 classifications

The WHO 2004 (Lloyd *et al.*, 2004) and 2017 (Osamura *et al.*, 2017) classifications remain morphofunctional, and keep the term "adenoma". The pituitary "adenomas" are classified into five main types using immunocytochemistry: GH-, PRL-, FSH/LH-, ACTH-, and TSH-producing adenomas in the WHO 2004 classification, and somatotroph, lactotroph, corticotroph, gonadotroph, and thyrotroph adenomas in the WHO 2017 classification. Many of the 12 ultrastructural subtypes in the WHO 2004 classification are abandoned in the WHO 2017 classification, because the EM technique is expensive, time-consuming, and not in routine use. Recognizing the role of transcription factors in the differentiation of the anterior pituitary cells and regulation of specific pituitary hormones (Pit-1, SF-1, Tpit), the 2017 classification concentrates on pituitary-cell lineage for designation of adenomas. For example, the term somatotroph adenoma defines this as a group of tumors that secrete GH and are derived from a Pit-1 lineage, rather than being a GH-producing adenoma.

The differentiation of normal pituitary cells during embryonic development depends on transcription factors. Pit-1 is the transcription factor responsible for somatotroph differentiation, Pit-1 and the estrogen receptor alpha are associated with lactotroph differentiation, and Pit-1 and GATA-2 control thyrotroph differentiation. Corticotroph differentiation depends on Tpit and NeuroD1, and gonadotroph differentiation depends on SF-1 and GATA-2 (Asa *et al.*, 1996; Sanno *et al.*, 1998; Nishioka *et al.*, 2015; Umeoka *et al.*, 2002). IHC detection of these transcription factors remains difficult because of technical problems related to antibody specificity. In our opinion, IHC detection increases the costs of pathological analysis without giving any more useful

Table 1 Morphofunctional classification of PitNETs (adapted from the WHO 2017 classification)

Tumor types	*Cytological aspects*	*Immunophenotypes*	*Transcription factors and receptors*
Somatotroph	Densely granulated[a]	GH ± PRL ± α-subunit LMW CK: perinuclear or diffuse pattern	Pit-1, SSTR
	Sparsely granulated	GH ± PRL LMW CK: fibrous bodies	Pit-1, ±SSTR
	Mammosomatotroph	GH + PRL (in same cell) ± α-subunit	Pit-1, ERα, ±SSTR
	Mixed somatotroph-lactotroph	GH + PRL (in different cells) ± α-subunit	Pit-1, ERα, ±SSTR
Lactotroph	Sparsely granulated[a]	PRL	Pit-1, ERα
	Densely granulated	PRL	Pit-1, ERα
Thyrotroph	Sparsely granulated	βTSH, α-subunit	Pit-1, GATA2, SSTR
Corticotroph	Densely granulated[a]	ACTH, βend LMW CK: diffuse pattern	Tpit, ±SSTR
	Sparsely granulated	ACTH, βend LMW CK: diffuse pattern	Tpit
	Crooke's cell	ACTH, βend LMW CK: ring-like pattern	Tpit
Gonadotroph	Sparsely granulated	βFSH, βLH, α-subunit (various combinations)	SF-1, GATA2, ERα, SSTR (variable)
Immunonegative[b]	Sparsely granulated	None	None
Plurihormonal[c]		GH, PRL, βTSH ± α-subunit	Pit-1
Double tumors	Distinct tumors	Usually PRL and ACTH	Pit-1 and Tpit,

[a]Most common subtype.
[b]Also termed null cell adenoma.
[c]Previously termed silent subtype 3 adenoma.
LMW CK, low-molecular weight cytokeratin; *PitNETs*, pituitary neuroendocrine tumors.

information on the functional type. However, it is useful in the case of immunonegative or plurihormonal tumors, such as plurihormonal Pit-1-positive tumors.

We adapted the WHO 2017 classification in **Table 1**, using the term tumor instead of adenoma, and immunonegative tumor instead of null cell adenoma. This tumor type is now found very rarely (down from 10% in 1992 to 1% in 2012 in Lyon's pathological series). Many studies find a high percentage of these tumors, at around 30%. However, these tumors correspond to gonadotroph tumors with a low intracellular hormonal content that is undetected by some antibodies, but that can be identified in culture or with more sensitive IHC techniques. We have not mentioned oncocytoma (Kovacs and Horvath, 1973; Trouillas *et al.*, 1975); it is not an entity, but ultrastructural aspect of gonadotroph tumor with numerous mitochondria. This is also the case for acidophilic stem cell adenoma (Horvath *et al.*, 1981), which are likely to be lactotroph tumor with giant and dilated mitochondria.

Prognostic Classifications

Typical and atypical adenomas

In the decade after the millennium, it appeared that some tumors without metastasis behaved differently from benign tumors. MRI indicated that some of these tumors invaded the skull base or both cavernous sinuses; however, pathologists could not histologically detect the invasion and metastasis to ascertain the malignancy.

The WHO 2004 classification (Lloyd *et al.*, 2004) classified all benign tumors as typical adenomas (ICD-0 8272/0) and identified "atypical" adenomas showing "borderline or uncertain behavior" (ICD-0 8272/1). Such tumors were described to have "atypical morphologic features suggestive of aggressive behavior such as invasive growth. Other features include an elevated mitotic index and a Ki-67 labeling index greater than 3%, as well as extensive nuclear staining for p53 immunoreactivity". Likely due to these vaguely defined criteria, the reported incidence of atypical adenoma varies widely ranging from 2.9% (Miermeister *et al.*, 2015) to 18.7% (Chiloiro *et al.*, 2015). In addition, the few small studies that examined the prognostic usefulness of this classification delivered controversial results (Zada *et al.*, 2011; Saeger *et al.*, 2007; Yildirim *et al.*, 2013; Del Basso De Caro *et al.*, 2017).

As stated in the WHO 2017 edition (Osamura *et al.*, 2017), "the use of the term atypical adenoma is therefore no longer recommended". Moreover, some special subtypes that commonly show aggressive behavior must also be taken into account. These include sparsely granulated somatotroph tumor, lactotroph tumor in men (Delgrange *et al.*, 1997; Delgrange *et al.*, 2015), Crooke's cell tumor (Kovács *et al.*, 2013), silent corticotroph tumor (review in Raverot *et al.*, 2010a), and silent plurihormonal Pit-1-positive tumor (Chinezu *et al.*, 2017; Mete *et al.*, 2016).

A clinicopathological five-tiered classification

In 2013, we proposed a new clinicopathological, five-tiered classification of PitNETs (Trouillas *et al.*, 2013), that considers invasion assessed by pituitary imaging, the immunohistological subtype, and proliferative markers (Ki-67, mitosis, p53), as shown in **Fig. 1** (review in Raverot *et al.* 2014; Raverot *et al.* 2015; Vasiljevic *et al.* 2016). The tumors were classified according to size (micro, macro, and giant tumors), type (somatotroph, lactotroph, corticotroph, gonadotroph, and thyrotroph), and grade (grade 1a: non-

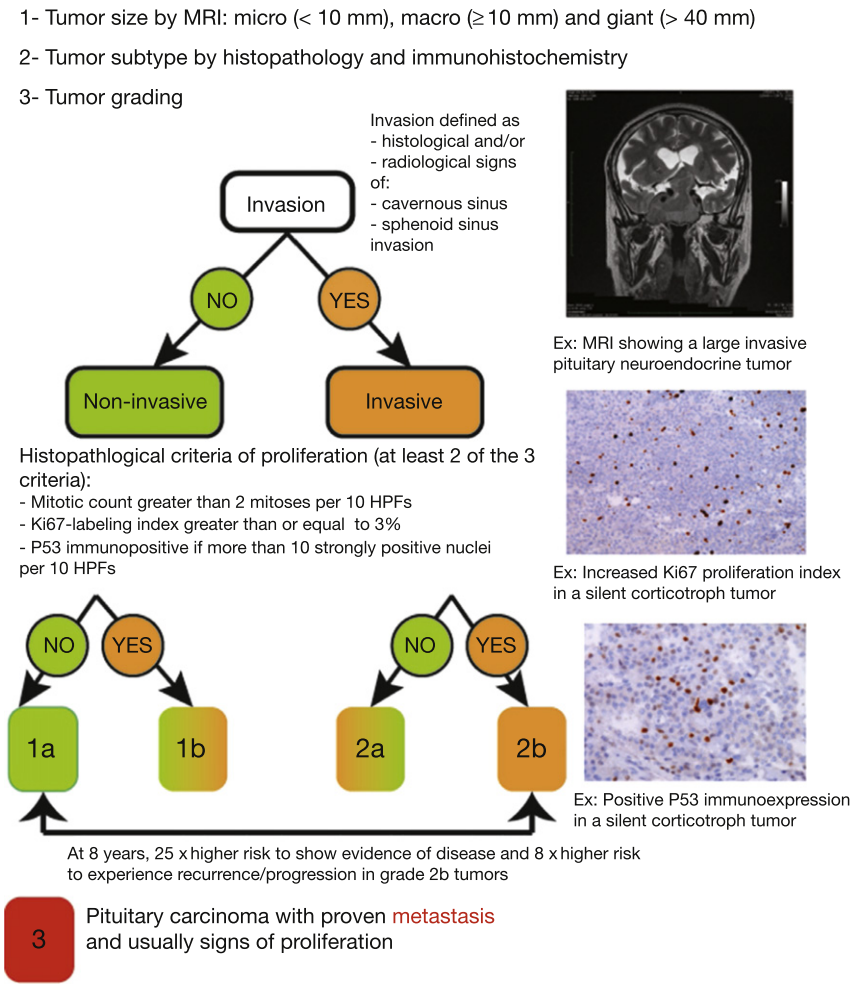

Fig 1 Clinocopathological five-tiered classification of Pit Nets. Adapted from Vasiljevic A., Jouanneau E., Trouillas J., Raverot G. (2016). Clinicopathological prognostic and theranostic markers in pituitary tumors. *Minerva Endocrinologica* **41**, 377–389.

invasive tumor; 1b: non-invasive and proliferative; 2a: invasive; 2b: invasive and proliferative; 3: metastatic tumor). A retrospective case-control study of 410 patients at 8 years after surgery (Trouillas *et al.*, 2013) and a prospective study of 365 patients at 3.5 years after surgery (Raverot *et al.*, 2017) validated the prognostic value of this classification. These two studies showed that grade 1a tumors were the most common (51.2%), followed by grade 2a (32.3%), 2b (8.8%), and 1b (7.7%). The frequency of invasive tumors (grades 2a and 2b: 41%) is similar to other large surgical series. At 3.5 or 8 years after surgery, 30–40% of tumors had recurred or progressed. Multivariate analyses of disease-free status and recurrence/progression status revealed the significant prognostic value ($p < 0.001$) of age, tumor type, and grade across all tumors and for each tumor type. The risk of recurrence was respectively 12- and 3.5-fold higher for a grade 2b tumor (invasive and proliferative) compared to grade 1a (non-invasive and non-proliferative) tumor. The lower risk found in the prospective study is related to the shorter duration of follow-up and the exclusion of patients treated by initial postoperative radiotherapy and second surgery for recurrence. Indeed, many patients had grade 2b tumors. In these two studies, thyrotroph tumors and silent GH and ACTH tumors were not classified separately because the number of cases was too small. There were no cases of grade 3 tumors or carcinoma in these two cohorts.

Discussion of the criteria

Invasion

Some pathologists did not agree with including invasion in a pathological classification of pituitary tumors because, as previously stated, histological proof of invasion is very rare (9%), requiring the pathologist to consider MRI data. While it is true that the pathologist cannot directly interpret the MRI, the diagnosis of the neuroradiologist and the preoperative data should be considered (Vasiljevic *et al.*, 2016). This requires the data to be available before the pathologist's diagnosis; this is in fact the case in the majority of the expert centers where patients typically receive surgery. As for all malignant tumors, invasion is an important criterion. In our most recent study (Raverot *et al.* 2017), differences in the Kaplan-Meier progression-free survival curves obtained

for different grades for invasion and for the immunocytochemical profile for the grade were mainly due to the effect of invasion; the effect of proliferation was lower than the effect of invasion.

Proliferative markers

The Ki-67 index, a proliferative marker, has been used in pituitary tumor analysis since 1996 (Thapar *et al.*, 1996). It is now routinely evaluated, but its value in determining PitNET prognosis remains controversial. This could be because various cut-offs for the Ki-67 index, ranging from 1.3% (Gejman *et al.*, 2008) to 10% (Kovacs, 2006) have been applied to identify tumors with a high risk of recurrence and have sometimes been adapted to tumor subtype (Righi *et al.*, 2012). Furthermore, most studies were based on a limited number of cases, an expert opinion, or a retrospective analysis. The Ki-67 index alone is regarded as insufficient to predict tumor behavior. The prognostic value of immunopositivity for p53, a criterion of the WHO classification, has also been debated, as a reliable method of quantification had not been established (Di Ieva *et al.*, 2014). Recent studies, however, defined p53 staining as positive if >10 nuclei per 10 high-power fields were strongly labeled (Miermeister *et al.*, 2015, Trouillas *et al.*, 2013, Gejman *et al.*, 2008). A prospective study (Raverot *et al.*, 2017) validated the choice of criteria. Indeed, 80% of proliferative tumors (grade 1b and 2b) displayed two proliferative markers and 20% had three proliferative markers. The Ki-67 index above 3% has major prognostic value. First, a Ki-67 value $\geq$3% was found in the majority of proliferative grade 1b and 2b tumors and only rarely (10%) in non-proliferative grade 1a and 2a tumors. Second, p53 positivity was much more common in tumors with Ki-67 $\geq$ 3% (83.3%) than in those with a Ki-67 index below 3% (11.7%). For grade 1a and 2a tumors, the great majority did not present any proliferative markers. In 21.6% of tumors, only one marker was present (Ki-67 $\geq$ 3%: 9.5%; mitotic count >2: 3.0%; p53: 9.2%).

This classification is easy to use, but some parameters, such as cellular differentiation, silent subtype, and sex of the patient for lactotroph tumor, are not considered. A scoring system such as that used for other neuroendocrine tumors, especially pancreatic tumors (Rindi *et al.*, 2006), could be proposed. However, early identification of those pituitary tumors with a high risk of recurrence associated with a malignant potential is now possible. In such cases (grade 2b), an optimized therapeutic strategy should be proposed, taking into account new therapeutic options in addition to conventional therapies associating including surgery and radiotherapy (Zemmoura *et al.* 2013).

Grade 3 tumors or pituitary carcinomas

Pituitary tumors with metastases, otherwise known as pituitary carcinomas, are rare and account for about 0.2% of pituitary tumors (reviews by Kaltsas *et al.*, 2005 and Heaney, 2011). In 40 years, we have only observed eight carcinomas with metastasis out of around 4000 pituitary tumors studied (0.2%), and 132 cases were reported in the literature between 1961 and 2009 (Dudziak *et al.* 2011). Most pituitary carcinomas are functioning and in the majority of cases secrete PRL (36% of cases) or ACTH (30% of cases). Nonfunctioning carcinomas are less common (23% of cases). Histological signs of malignancy were once thought to be absent, but the frequency of certain signs reported in previous pathological studies of pituitary carcinomas (Pernicone *et al.*, 1997; Scheithauer *et al.*, 2005) means we can make reasonable assumptions with regards to potential malignancy. These assumptions would be based on the following pathological signs which, when combined, might be considered criteria of malignancy: invasion, neoangiogenesis, vascular invasion, abnormal mitoses, very high indices for Ki-67 (i.e. >10%) and p53 (i.e. >5%), and genomic alteration (chromosome 11 deletion in lactotroph tumors). However, these cut-off values have not been validated, and this, together with the absence of some of the above signs in particular metastatic pituitary carcinomas, is a concern (Lubke and Saeger, 1995; Scheithauer *et al.*, 2005). Based on analyses comparing human lactotroph tumors to our animal model (Trouillas *et al.*, 1994), and on the observations that in our cohort (Trouillas *et al.*, 2013) none of the eight carcinomas were grade 1a at the time of the first surgery, and six of them were grade 2b, we suggested that grade 1a tumors are benign and do not transform into carcinoma, and grade 2b tumors are malignant tumors without metastases (Wierinckx *et al.*, 2007; Wierinckx *et al.*, 2010; Wierinckx *et al.*, 2011).

Molecular and Genetic Biomarkers

Biological Markers Detected by IHC

Expression of several biological markers, analyzed using IHC, correlates with invasiveness and/or aggressive behavior, but no single marker has been found that can predict tumor behavior, and until now their detection remains in the research setting. As three recent and exhaustive reviews expand this subject (Gurlek *et al.*, 2007; Mete *et al.*, 2012, Raverot *et al.*, 2014), we focus here on biomarkers that seem to correlate with invasive and aggressive behavior.

Fibroblast growth factors (FGFs) and their receptors (FGFRs) regulate growth, differentiation, migration, and angiogenesis. The expression of a truncated isoform of FGFR4, known as pituitary tumor-derived FGFR4 (ptd-FGFR4) induces invasive growth of pituitary tumor cells in vivo with loss of membranous N-cadherin expression (Ezzat *et al.*, 2004). Moreover, ptd-FGFR4 cross-talks with a PolySiAlilated form of Neuro Cell Adhesion Molecule (PSA-NCAM), which correlates with invasiveness (Trouillas *et al.*, 2003). Loss of E-cadherin correlates with larger size, invasiveness, and progression of pituitary tumors (Fougner *et al.*, 2010; Evang *et al.*, 2011). Expression of endocan, a proteoglycan secreted by endothelial cells, is associated with size and progression of pituitary tumors (Cornelius *et al.*, 2012). Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that cleave extracellular matrix molecules and play a role in the invasiveness of many neoplasms. MMP9 expression is significantly higher in invasive pituitary

tumors (Kawamoto *et al.*, 1996) and there may be a correlation with protein kinase C activation, which is involved in pituitary tumor invasion. The pituitary tumor transforming gene (PTTG) is part of the securin family that regulates sister chromatid separation during mitosis, and its expression is higher in invasive pituitary tumors than their non-invasive counterparts (Zhang *et al.*, 1999). In the lactotroph subtype, low ERα expression characterized dopamine agonist-resistant tumors. The expression of ERα is lower in men than in women and is inversely correlated with tumor size and grade (Delgrange *et al.*, 2015).

Gene Expression by Transcriptomic Analyses

Initial transcriptomic studies compared normal pituitary with pituitary tumors as a whole or according to tumor subtype. A meta-analysis on these published results (Wierinckx *et al.*, 2010) identified only six genes (GADD45B, SAT1, ID1, VIM, IGFBP5, and ZFP36L1) with proven roles in cell proliferation or tumorigenesis. The functional involvement in the pituitary tumor has been determined for only two genes: GADD45γ, and a novel noncoding RNA gene, termed the maternally expressed gene 3 (MEG3) identified by Zhang *et al.* (Zhang *et al.*, 2002; Zhang *et al.*, 2003). Indeed, downregulation of these two genes occurs more frequently in non-functioning adenomas than in functioning tumors, and MEG3, as a tumor suppressor gene, interacts with both p53 and Rb to control cell proliferation (Zhou *et al.*, 2007). However, none gene has been associated with prognosis (Farrell, 2006).

Only a small number of studies analyzed the differential gene expression according to tumor characteristics. Galland *et al.* (Galland *et al.*, 2010) identified a stronger overexpression of MYO5A in invasive than in non-invasive nonfunctioning tumors. In lactotroph tumors, a set of nine genes (PTTG, ASK, CCNB1, AURKB, CENPE, PITX1, ADAMTS6, CRMP1, and DCAMKL3) was associated with pathological tumor classification (Wierinckx *et al.*, 2007) and seven (ADAMTS6, CRMP1, PTTG, ASK, CCNB1, AURKB, and CENPE) correlated with a higher risk of recurrence or progression during a long-term follow-up of over 10 years (Raverot *et al.*, 2010b).

MicroRNAs (miRNAs) are a class of small noncoding RNAs deregulated in many types of cancer. They act as tumor suppressors or oncogenes depending on the function of the targeted genes, and appear to be involved in the regulation of many steps of pituitary tumor progression (review by Wierinckx *et al.*, 2017). At this time, transcriptomic analysis is a very difficult and expensive technique that remains in the research setting, and cannot be carried out extensively in pituitary tumors. However, the technique could help to find new markers.

Chromosomal Alteration and Genetic Mutations

In contrast, genetic analysis seems very promising. Use of whole genome sequencing or comparative genomic hybridization methods has identified a high frequency of chromosomal alterations in somatotroph tumors (Välimäki *et al.*, 2015). In tumors exhibiting aggressive behavior, an allelic deletion is clustered at four loci: 11q13 (multiple endocrine neoplasm type 1 (MEN1) and aryl-hydrocarbon receptor-interacting protein (AIP) gene), 13q12–14, 10q26, and 1p (Bates *et al.*, 1997). T53 mutations have occasionally been reported in pituitary tumors (Levy *et al.*, 1994). Trisomy of chromosome 12 has been also found in lactotroph tumors (Finelli *et al.*, 2000). Wierinckx *et al.* showed that a deletion of the 11p region occurred more commonly in lactotroph aggressive tumors than in non-aggressive ones, and the three aggressive tumors that developed metastasis during follow-up showed a loss of the whole chromosome 11 and a gain of the 1q arm (Wierinckx *et al.*, 2011). It therefore appears that the accumulation of genomic alterations may transform an aggressive pituitary tumor into a pituitary carcinoma.

Pituitary tumors are mostly sporadic. However, somatic activating mutations of G-protein alpha sub-unit (GNAS) have been identified in 40% of sporadic somatotroph tumors, resulting in upregulation of the cAMP pathway (Landis *et al.*, 1989), and in the *USP8* gene in 36–62% of corticotroph tumors, leading to an upregulated EGFR pathway (Perez-Rivas *et al.*, 2015; Reincke *et al.*, 2015). Moreover, some familial cases have been identified, leading to the characterization of a genetic form of pituitary tumors. Germline AIP gene mutations are observed in 20% of familial isolated pituitary adenoma (FIPA), and various other mutations in genes encoding MEN1, MEN4, and Carney complex and in those linked to syndromic diseases such as succinyl dehydrogenase (SDH) in paraganglioma, and phaeochromocytoma. Genetic testing for MEN1 or AIP mutations is recommended where there is a familial history of pituitary tumors (Beckers *et al.*, 2013; Verges *et al.*, 2002; Vierimaa *et al.*, 2006), or in cases of large macro-adenomas in young patients (Cazabat *et al.*, 2007) or of unusual plurihormonality and double adenomas (Trouillas *et al.*, 2008).

Theranostic Factors

Theranostics is defined as a diagnostic process to stratify patients who will respond to a specific treatment. Predicting sensitivity to a specific treatment is important for the choice of postoperative therapeutic strategy. However, this analysis is highly dependent on the definition of the therapeutic response and on the techniques and methods used to analyze them. Somatostatin receptors, especially subtypes 2 and 5, are expressed in almost all the somatotroph tumors, all thyrotroph tumors, and around 20% of gonadotroph tumors (Chinezu *et al.*, 2014). In somatotroph tumors, the effects of somatostatin analogs seem positively correlated with somatostatin receptor expression (Casar-Borota *et al.*, 2013; Gatto *et al.*, 2013).

O^6-methylguanine DNA methyltransferase (MGMT), a DNA repair enzyme that counteracts the effects of temozolomide by removing alkylating adducts from DNA, is the most frequently studied to attempt to predict the tumor response to temozolomide treatment. Results of the studies are still debated (Bush *et al.*, 2010; Bengtsson *et al.*, 2015, McCormack *et al.*, 2011), but a high MGMT expression level is generally associated with a poor tumor response.

Perspective and Conclusions

Taken separately, clinical symptoms, biochemical hormone levels, radiological features, and histopathology have failed to effectively predict the behavior of pituitary tumors. It is a combination of all these data that allow the optimal management of patients affected by pituitary tumors. The development of a "mixed" classification (invasion assessed via MRI/endoscopy and histopathological characteristics) is a way to improve the prognostic assessment of aggressive pituitary tumors. It requires multidisciplinary collaboration between pathologist, endocrinologist, and neurosurgeon. The role of the pathologist may be more active in the clinicopathological assessment of patients with pituitary tumors than previously appreciated. By identifying potentially aggressive pituitary tumors, and by analyzing theranostic factors, the pathologist can directly guide the clinical choice of the optimal postoperative management of these patients.

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Genetic Causes of Familial Pituitary Tumors

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Introduction

Pituitary adenomas (PA) are frequent benign monoclonal tumors representing approximately 15% of all primary intracranial tumors, being the third most frequent tumor type after meningioma and glioma (Scheithauer *et al.*, 2006). The majority of the lesions identified incidentally remain clinically silent; however, clinically relevant adenomas are found in approximately 1:1100 patients in the general population (Daly *et al.*, 2006; Fontana and Gaillard, 2009; Fernandez *et al.*, 2010; Raappana *et al.*, 2010; Gruppetta *et al.*, 2013; Agustsson *et al.*, 2015).

PA can be divided by size into microadenoma (<1 cm) and macroadenoma (>1 cm), the latter being responsible for 40% of the cases (Aflorei and Korbonits, 2014). They can also be classified as hormone secreting or clinically nonfunctioning adenomas (NFPA) according to whether or not they secrete hormones. Approximately 66.2% of PA are prolactinomas, 14.7% are NFPA, 13.2% are somatotropinomas, and 5.9% are corticotropinomas, with the other subtypes being rarer (Daly *et al.*, 2006).

Although only 0.2% of pituitary tumors are classified as malignant, defined by distant metastasis, they cause significant morbidity. PA are associated with hormonal disturbances and compression symptoms like headaches, visual disturbances, and hypopituitarism due to mass effect. Due to pituitary gland localization, they can put pressure on the optic chiasm and the pituitary stalk, as well as invading areas like the cavernous sinus and suprasellar area.

Most of PA appear to occur sporadically, but about 5% occur due to an inherited condition, either isolated or as a part of an endocrine tumor syndrome.

Several conditions are associated with a known genetic defect causing a predisposition to familial PA. Familial PA can be considered when at least two family members have a PA, but in cases of low penetrance seemingly simplex cases may have an inherited germline mutation, or "de novo" mutations can establish the potential for familial disease in further generations. The two most frequent forms are familial isolated pituitary adenomas (FIPA) and multiple endocrine neoplasia type 1 syndrome (MEN1), which are responsible for 2%–3% and 3%, respectively, of PA (Daly *et al.*, 2006). X-linked acrogigantism (XLAG) syndrome, Carney Complex (CNC), multiple endocrine neoplasia type 4 (MEN4), pheochromocytomas/paragangliomas and pituitary adenomas, and pituitary blastoma (PitB) are other rare causes of familial PA (**Fig.1**). McCune–Albright syndrome is

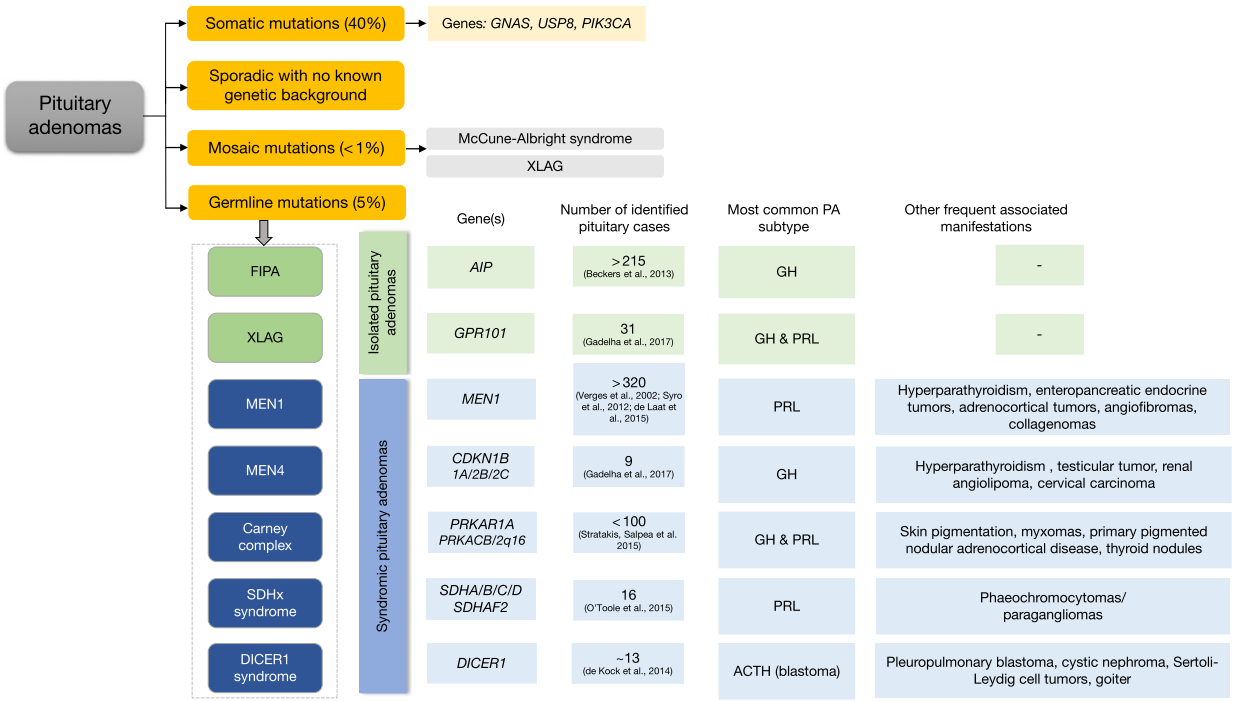

Fig. 1 Pituitary adenomas due to somatic, mosaic, and germline mutations. In the group of germline mutations, the estimated number of described cases of pituitary adenomas for each condition is presented.

caused by postzygotic activating mutations in the guanine nucleotide-activating alpha-subunit gene (*GNAS*), which could lead to PA, but no familial has been reported to date.

Familial Isolated Pituitary Adenomas

Overview

FIPA syndrome is defined as the occurrence of at least two cases of PA in a family that does not exhibit any other abnormality (Chahal *et al.*, 2010; Beckers *et al.*, 2013). However, there are many simplex cases with germline mutations, either due to low penetrance or due to "de novo" mutations, where the possibility of familial disease is present.

Based on data of over 400 families, FIPA appears to be more common than initially thought, but the exact prevalence is unknown (Beckers and Daly, 2007; Stiles and Korbonits, 2011). It is the most common familial cause of acromegaly/gigantism (Gadelha *et al.*, 2017).

FIPA can be divided into three subgroups of patients with significant phenotypic differences among them: patients with mutations in the *AIP* gene, patients with XLAG due to duplication of *GPR101*, and patients with a family history of PA with no known genetic cause, which represent the largest subgroup of FIPA (Caimari and Korbonits, 2016). Not all cases have a known family history, either due to low penetrance (*AIP* mutation-positive subgroup) or due to "de novo" mutation (XLAG subgroup) (Caimari and Korbonits, 2016).

The same type of PA can be present in all affected family members (homogeneous family, 60%) or affected members can have different types of tumors (heterogeneous family, 40%). These families show an autosomal dominant inheritance with incomplete penetrance (Daly *et al.*, 2006; Vierimaa *et al.*, 2006; Georgitsi *et al.*, 2008; Leontiou *et al.*, 2008; Daly and Beckers, 2015).

Germline *AIP* mutations are identified in 20% of patients with FIPA or 40% when only somatotropinoma families are considered (Chahal *et al.*, 2010; Daly *et al.*, 2010; Beckers *et al.*, 2013; Hernandez-Ramirez *et al.*, 2015).

The *AIP* gene is located at the 11q13 chromosome region and consists of 6 exons, encoding a 330 amino acid cytoplasmic cochaperone protein (Dull *et al.*, 2002). *AIP* is composed of an N-terminal domain similar to the immunophilin proteins, three tetratricopeptide repeats (TPR) of 34 amino acids comprising two antiparallel helices, and an α-helix in the C-terminal. These helices are essential for interaction with AIP partners (Trivellin and Korbonits, 2011).

AIP was identified in 1996, but heterozygous germline mutations were found in PA only in 2006, in a large Finnish family with acromegaly and prolactinoma.

AIP is widely expressed in the human body (Petrulis and Perdew, 2002). In the normal pituitary gland, *AIP* is expressed only in somatrotroph and lactotroph cells, associated with secretory vesicles (Leontiou *et al.*, 2008). In sporadic PA, *AIP* is expressed in all cell types. Higher *AIP* expression levels are observed in NFPA, and low expression was described in invasive somatotropinomas (Leontiou *et al.*, 2008; Jaffrain-Rea *et al.*, 2009).

More than 90 *AIP* mutations have been reported in the literature, with some of the variants with uncertain clinical significance (Korbonits *et al.*, 2012; Beckers *et al.*, 2013) (**Fig. 2**; **Tables 1–3**). Missense, nonsense, splice-site, promoter mutations, segmental duplication, small deletions and insertions, and large genomic deletions have been described (**Tables 1–2**). 10% of *AIP* mutations are large genomic deletions and can only be detected with specific techniques such as multiplex ligation-dependent probe amplification (MLPA) (Georgitsi *et al.*, 2008; Gadelha *et al.*, 2013). About two-thirds of *AIP* mutations occur at the C-terminal end, resulting in a truncated protein, while missense variants and in-frame segmental changes mostly affect the TPR domain or the C-terminal α-helix (Chahal *et al.*, 2010, Alband and Korbonits, 2014). There are hotspot mutations mostly affecting CpG sites such as codons 81, 271, 304 (Daly *et al.*, 2006; Tichomirowa *et al.*, 2009; Chahal *et al.*, 2010).

All known mutations are predicted to lead to loss of function of the protein (Gadelha *et al.*, 2013). The loss of function together with the loss of heterozygosity in pituitary tumors of FIPA patients supports its role as a tumor suppressor gene in the pituitary gland (Vierimaa *et al.*, 2006; Leontiou *et al.*, 2008; Heliovaara *et al.*, 2009; Igreja *et al.*, 2010). According to the Knudson two-hit hypothesis, the first hit is due to a germline mutation of an allele and the second hit is a somatic deletion of the other allele (Knudson, 1971).

Numerous proteins have been identified to interact with *AIP* directly, which suggests that *AIP* is involved in various cellular pathways (Gadelha *et al.*, 2013). *AIP* has numerous binding proteins, such as viral proteins (HBVX and EBNA-3) AhR, Hsp90, Hsc70, phosphodiesterases (PDE4A5 and PDE2A3), nuclear (AhR, PPARa, TRb1), and transmembrane (RET) receptors, G proteins, mitochondrial import receptor (TOMM20), survivin, cardiac-specific kinase (TNNI3K), ubiquitin ligase FBXO3, cytoskeletal proteins (actin), and growth factor receptor (EGFR) (Trivellin and Korbonits, 2011). However, the way in which *AIP* exerts its tumor-suppressive action in the pituitary is not fully clear. The most studied association is with AhR, which is a transcription factor whose best-known function is as a mediator in the toxic effects of the environmental toxin 2,3,7,8-tetrachloro-*p*-dioxin although it is unclear whether this is involved in pituitary tumorigenesis. AhR binds environmental toxins and translocates to the nucleus to induce the transcription of xenobiotic enzymes. AhR has many roles, including regulation of the activity of nuclear receptors, transcription factors, and protein kinases and modulation of the cell cycle, cell adhesion, and migration, as well as alteration of multiple intracellular signaling pathways (Denison *et al.*, 2011). *AIP* forms a complex with AhR/Hsp90/p23 and appears to act in part by stabilizing AhR in cytoplasm. Low levels or loss of AIP correlate with low expression of AhR (Lees *et al.*, 2003; Jaffrain-Rea *et al.*, 2009). Cyclic adenosine 3′, 5′-monophosphate (cAMP) functions as an intracellular second messenger in several signaling pathways, including stimulation of cell proliferation in somatotroph cells via GHRH receptors (Formosa and Vassallo, 2017). The association between cAMP levels and PA has been made in CNC and McCune–Albright syndrome. PDEs degrade the phosphodiesters bond, inactivating cAMP. *AIP* interacts with different PDEs, but the role of this interaction is still uncertain (Stork and Schmitt, 2002; Horvath and Stratakis, 2008). Another study showed that the lack of *AIP*

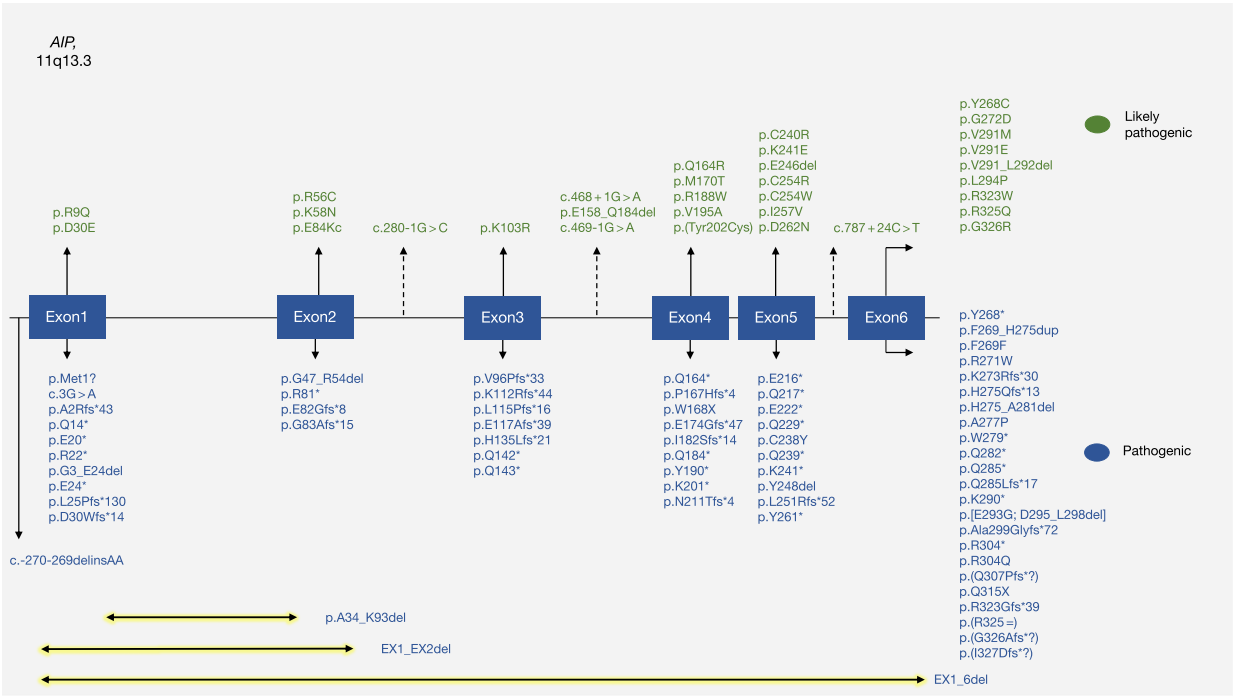

Fig. 2 AIP gene and likely pathogenic and pathogenic variants. *Blue boxes* represent exon (6) and introns are represented by *black line*. Mutations are distributed according to the localization.

leads to reduced inhibitory G protein Gα1–2 expression with the result of increased levels of cAMP, suggesting that this could be a major contributor to the development of somatotropinomas in *AIP* mutation carriers (Tuominen *et al.*, 2015). *AIP* also interacts with survivin, an inhibitor of apoptosis, and forms a complex with it in addition to Hsp90. The result is the prevention of ubiquitinations, thus stabilizing it. This interaction can be altered by another partner, RET, which will prevent the formation of the AIP/survivin complex (Kang and Altieri, 2006).

No other tumor types have been consistently associated with *AIP* mutation. Somatic mutations have not been found in PA either (Barlier *et al.*, 2007; Leontiou *et al.*, 2008).

Pituitary Adenomas

In families with multiple affected generations, the children/grandchildren present earlier than their ancestors, maybe due to patient education regarding the symptoms and surveillance among later generations (Daly *et al.*, 2006; Tichomirowa *et al.*, 2009).

Macroadenomas are seen in 63% of cases, which is a higher percentage compared with sporadic PA. PRL in FIPA are mainly microadenomas in women and macroadenomas in males (Tichomirowa *et al.*, 2009). Most *AIP* mutation-positive PA are somatotropinomas and prolactinomas (Daly *et al.*, 2010; Hernandez-Ramirez *et al.*, 2015). In a cohort of FIPA with *AIP* mutation, 80% were somatotropinomas and somatolactotropinomas, 14% were prolactinomas, and 6% were NFPA and gonadotropinomas (Korbonits *et al.*, 2012). Overall management of PA in FIPA families does not differ from the management of sporadic cases.

There are phenotypic differences in *AIP* mutation-positive families when compared with *AIP* mutation-negative families (Tichomirowa *et al.*, 2009; Gadelha *et al.*, 2013):

- The penetrance in *AIP* mutation-positive families is calculated 22.7% (range 15%–45%), being higher than in *AIP* mutation-negative families (Igreja *et al.*, 2010; Occhi *et al.*, 2010; Chahal *et al.*, 2011; Hernandez-Ramirez *et al.*, 2015);
- The mean age of diagnosis in *AIP* mutation-positive families is 20–24 years, 12–13 years lower than in *AIP* mutation-negative families and sporadic adenomas. The majority of PA from *AIP* mutation-positive families are diagnosed before the age of 30 (Chahal *et al.*, 2010; Daly *et al.*, 2010; Igreja *et al.*, 2010; Williams *et al.*, 2014; Hernandez-Ramirez *et al.*, 2015) (**Fig. 3**). Childhood-onset disease is present in 11% of *AIP* mutation-negative families, while it is demonstrated in 80% of *AIP* mutation-positive families (Cain *et al.*, 2010; Daly *et al.*, 2010; Hernandez-Ramirez *et al.*, 2015);
- The majority of studies reported a predominance of male patients in *AIP* mutation-positive families, but this was not reaching significance when extended fully studied families were considered (Hernandez-Ramirez *et al.*, 2015);
- Patients in *AIP* mutation-positive families are harbor either pure somatotropinoma or mixed somatotroph and lactotroph adenomas, while in *AIP* negative families there is more variability, although prolactinoma and somatotropinoma are also the most common tumor type seen (Chahal *et al.*, 2010; Daly *et al.*, 2010; Igreja *et al.*, 2010; Chahal *et al.*, 2011; Korbonits *et al.*, 2012);

Table 1 Pathogenic variants of the AIP gene

Region	*Chr. position*	*dbSNP rs ID*	*ExAc (Allele frequency)*	*DNA level*	*Protein level*	*Variant type*	*References*
5′ near gene	67250360–67250361			c.-270-269delinsAA/ g.4856_4857CG>AA	–	Promoter	Soares *et al.* (2005), Leontiou *et al.* (2008), and Igreja *et al.* (2010)
Exon1	67250632	67250632		c.2T>C	p.Met1?	Start codon	Personnier *et al.* (2011)
Exon1	67250633	67250633		c.3G>A	p.?	Start codon	Radian *et al.* (2017)
Exon1	67250633–67250634	rs267606547		c.3_4insC	p.A2Rfs*43	Frameshift	Xekouki *et al.* (2013)
Exon1	67250669	rs104894194/EF066510		c.40C>T	p.Q14*	Nonsense	Vierimaa *et al.* (2006), Georgitsi *et al.* (2007a), Raitila *et al.* (2007), and Hernandez-Ramirez *et al.* (2015)
Exon1	67250687			c.58G>T	p.E20*	Nonsense	Cuny *et al.* (2013)
Exon1	67250693	rs121908357		c.64C>T	p.R22*	Nonsense	Barlier *et al.* (2007), Tichomirowa *et al.* (2011), and Cuny *et al.* (2013)
Exon1	67250695–67250700	rs267606567		c.66_71delAGGAGA	p.G23_E24del	In-frame deletion	Georgitsi *et al.* (2007a)
Exon1	67250699	rs267606568/EF643644		c.70G>T	p.E24*	Nonsense	Gadelha *et al.* (1999), and Leontiou *et al.* (2008)
Exon1	67250703–67250710	rs104895074		c.74_81delins7	p.L25Pfs*130	Frameshift	Pestell *et al.* (1989), and Igreja *et al.* (2010)
Exon1	67250717–67250718	GQ847775.1		c.88_89delGA	p.D30Wfs*14	Frameshift	Tichomirowa *et al.* (2011) and Cuny *et al.* (2013)
Exon2	67254517–67254541	rs267606537/EF066504		c.140_163delGCACCGTGCTG-GACGACAGCCGGG	p.G47_R54del	In-frame deletion	Daly *et al.* (2007)
Exon2				c.240_241delinsTG	p.M80_R81delinsIG		Iacovazzo *et al.* (2016)
Exon2	67254618	rs267606541/EF643647		c.241C>T	p.R81*	Nonsense	Luccio-Camelo *et al.* (2004), Soares *et al.* (2005), Toledo *et al.* (2007), Leontiou *et al.* (2008), and Guaraldi *et al.* (2012)
Exon2	67254622–67254626	rs267606542/FJ514477.1		c.245_249delAAGGG	p.E82Gfs*8	Frameshift	Tichomirowa *et al.* (2011)
Exon2	67254626	rs104895072		c.249G>T	p.G83Afs*15	Splice site	Igreja *et al.* (2010)
Exon3	67256744–67256745	rs267606545		c.286_287delGT	p.V96Pfs*33	Frameshift	Yamada *et al.* (1997) and Iwata *et al.* (2007)
Exon3	67256791			c.333delC	p.K112Rfs*44	Frameshift	Hernandez-Ramirez *et al.* (2015)
Exon3	67256796–67256799			c.338_341dup	p.L115Pfs*16	Frameshift	Stratakis *et al.* (2010) and Hernandez-Ramirez *et al.* (2015)
Exon3	67256808	rs267606549/GQ847774.1		c.350delG	p.E117Afs*39	Frameshift	Tichomirowa *et al.* (2011), Cazabat *et al.* (2012), and Cuny *et al.* (2013)
Exon3				c.376_377del	p.Q126fs	Frameshift	Coxson *et al.* (2015) and Iacovazzo *et al.* (2016)
Exon3	67256862	rs267606551/EF553637		c.404delA	p.H135Lfs*21	Frameshift	Cazabat *et al.* (2007)
Exon3	67256882	rs267606552/EF066506		c.424C>T	p.Q142*	Nonsense	Daly *et al.* (2007)
Exon3	67256885			c.427C>T	p.Q143*	Nonsense	Hernandez-Ramirez *et al.* (2015)
Exon4	67257530	rs104895073		c.490C>T	p.Q164*	Nonsense	Igreja *et al.* (2010)

(*Continued*)

Exon4	67257540	rs267606557		c.500delC	p.P167Hfs*4	Frameshift	Khoo *et al.* (2009)
Exon4	67257546			c.504G>T	p.W168*	Missense	Bautista *et al.* (2017), Unpublished our group
Exon4	67257561–67257565	rs267606558/EF066503		c.521_525delAGAAG	p.E174Gfs*47	Frameshift	Daly *et al.* (2007) and Naves *et al.* (2007)
Exon4	67257583	rs267606559		c.543delT	p.I182Sfs*14	Frameshift	Georgitsi *et al.* (2007a)
Exon4	67257590	rs267606560/GQ403802.1		c.550C>T	p.Q184*	Nonsense	Tichomirowa *et al.* (2011)
Exon4	67257610			c.570C>G	p.Y190*	Nonsense	Hernandez-Ramirez *et al.* (2015)
Exon4	67257641	rs267606563/EF553639		c.601A>T	p.K201*	Nonsense	Cazabat *et al.* (2007)
Exon4	67257670			c.630delG	p.N211Tfs*4	Frameshift	Cuny *et al.* (2013)
Exon5	67257787	rs267606565		c.646G>T	p.E216*	Nonsense	Villa *et al.* (2011)
Exon5	67257790	rs267606566/EF066507		c.649C>T	p.Q217*	Nonsense	Daly *et al.* (2007) and Cai *et al.* (2013)
Exon5	67257803–67257804	rs104895075		c.662dupC	p.E222*	Nonsense	Igreja *et al.* (2010)
Exon5	67257826			c.685C>T	p.Q229*	Nonsense	Urbani *et al.* (2014)
Exon5	67257854	rs267606569/EF643648	0.00000842	c.713G>A	p.C238Y	Missense	Gadelha *et al.* (1999) and Leontiou *et al.* (2008)
Exon5	67257856	rs267606571/EF066508		c.715C>T	p.Q239*	Nonsense	Daly *et al.* (2007)
Exon5	67257862	rs267606573		c.721A>T	p.K241*	Nonsense	Stratakis *et al.* (2010)
Exon5	67257883–67257885	rs267606574		c.742_744delTAC	p.Y248del	In-frame deletion	Georgitsi *et al.* (2008,b)
Exon5	67257893			c.752delT	p.L251Rfs*52	Frameshift	Cazabat *et al.* (2012)
Exon5	67257924	GQ403803.1		c.783C>G	p.Y261*	Nonsense	Cazabat *et al.* (2012), Cuny *et al.* (2013), and Hernandez-Ramirez *et al.* (2015)
Exon6	67258275	rs121908356		c.804C>A	p.Y268*	Nonsense	Toledo *et al.* (2007), Jorge *et al.* (2001), and Hernandez-Ramirez *et al.* (2015)
Exon6	67258276–67258296	rs267606578/EF643650		c.805_825dup	p.F269_H275dup	In-frame insertion	Prescott *et al.* (1982), Soares *et al.* (2005), Leontiou *et al.* (2008), and Cazabat *et al.* (2012)
Exon6	67258278	rs139407567/EF643649		c.807C>T	p.F269F	Splice site	McCarthy *et al.* (1990), Cazabat *et al.* (2007), Georgitsi *et al.* (2008), Leontiou *et al.* (2008), Igreja *et al.* (2010), Tichomirowa *et al.* (2011), and Oriola *et al.* (2013)
Exon6	67258282	rs267606579/EF066502		c.811C>T	p.R271W	Missense	Daly *et al.* (2007), Jennings *et al.* (2009), Igreja *et al.* (2010), and Tichomirowa *et al.* (2011)
Exon6	67258287			c.816delC	p.K273Rfs*30	Frameshift	Hernandez-Ramirez *et al.* (2015)
Exon6	67258295–67258296	rs267606580		c.824dupA	p.H275Qfs*13	Frameshift	Georgitsi *et al.* (2007b)
Exon6	67258296–67258316			c.825_845delCGCGGCCGTGT-GGAATGCCCA	p.H275_A281del	In-frame deletion	Belar *et al.* (2012)
Exon6	67258300	rs267606581		c.829G>C	p.A277P	Missense	Jaffrain-Rea *et al.* (2009) and Tichomirowa *et al.* (2011)
Exon6	67258332			c. 836G>A	p.W279*	Nonsense	Cansu *et al.* (2016)
Exon6	67258315			c.844C>T	p.Q282*	Nonsense	Schofl *et al.* (2014)

Table 1 Continued

Region	*Chr. position*	*dbSNP rs ID*	*ExAc (Allele frequency)*	*DNA level*	*Protein level*	*Variant type*	*References*
Exon6	67258324			c.853C>T	p.Q285*	Nonsense	Beckers *et al.* (2013)
Exon6	67258325–67258328	rs267606582/EF066509		c.854_857delAGGC	p.Q285Lfs*17	Frameshift	Daly *et al.* (2007)
Exon6	67258339			c.868A>T	p.K290*	Nonsense	Hernandez-Ramirez *et al.* (2015)
Exon6	67258349–67258362	rs267606583, rs267606585		c.[878_87c.878_879delinsGT; 884_895delCTGGACC-CAGCC]	p.[E293G; D295_L298del]	In-frame deletion	Georgitsi *et al.* (2007a)
Exon6	67258381	rs104894195/AM236344		c.910C>T	p.R304*	Nonsense	Vierimaa *et al.* (2006), Cazabat *et al.* (2007), Daly *et al.* (2007), Leontiou *et al.* (2008), Occhi *et al.* (2010), Igreja *et al.* (2010), Tichomirowa *et al.* (2011), Chahal *et al.* (2011), Cazabat *et al.* (2012), and Cuny *et al.* (2013)
Exon6	67258382	rs104894190/EF203236	0.001458	c.911G>A	p.R304Q	Missense	Cazabat *et al.* (2007), Georgitsi *et al.* (2007a), Leontiou *et al.* (2008), Igreja *et al.* (2010), Occhi *et al.* (2010), Tichomirowa *et al.* (2011), Cazabat *et al.* (2012), and Cuny *et al.* (2013)
Exon6	67258390	rs267606589		c.919dupC	p.(Q307Pfs*?)	Frameshift	Stratakis *et al.* (2010)
Exon6	67258414			c.943C>T	p.Q315X	Nonsense	Iwata *et al.* (2014)
Exon6	67258438			c.967delC	p.R323Gfs*39	Frameshift	Hernandez-Ramirez *et al.* (2015)
Exon6	67258444			c.973C>A	p.(R325=)	Synonymous	Lecoq *et al.* (2016)
Exon6	67258447			c.976_977insC	p.(G326Afs*?)	Frameshift	Hernandez-Ramirez *et al.* (2015)
Exon6	67258449			c.978dupG	p.(I327Dfs*?)	Frameshift	Hernandez-Ramirez *et al.* (2015)
				c.-1213_279+578del (ex1_ex2del)	–	Large genomic deletions	Georgitsi *et al.* (2008) and Igreja *et al.* (2010)
				c.100-1025_279+357del (ex2del)	p.A34_K93del	Large genomic deletions	Georgitsi *et al.* (2008)
				c.1-?_993+?del	–	Large genomic deletions	Igreja *et al.* (2010)

Table 2 Likely pathogenic variants of the *AIP* gene

Region	*Chr. position*	*dbSNP rs ID*	*ExAc (Allele frequency)*	*DNA level*	*Protein level*	*Variant type*	*References*
Exon1	67250655	rs139459091	0.0002146	c.26G>A	p.R9Q	Missense	Cazabat *et al.* (2012) and Oriola *et al.* (2013)
Exon1	67250719			c.90T>G	p.D30E	Missense	Cai *et al.* (2013)
Exon2	67254543	rs267606538/GU969040	0.00001651	c.166C>T	p.R56C	Missense	Tichomirowa *et al.* (2011)
Exon2	67254549	rs267606539/GQ847773.1		c.174G>C	p.K58N	Missense	Tichomirowa *et al.* (2011) and Cazabat *et al.* (2012)
Exon2	67254627	rs267606543/GQ403801.1		c.250G>A	p.E84K	Missense	Tichomirowa *et al.* (2011)
Intron2	67256737	rs267606544		c.280-1G>C	–	Splice site	Georgitsi *et al.* (2007a)
Exon3	67256766	rs267606548		c.308A>G	p.K103R	Missense	Stratakis *et al.* (2010)
Intron3	67256927	rs267606554		c.468+1G>A	–	Splice site	Occhi *et al.* (2010)
Intron3	67257507	rs267606556/EF553638		c.469-2A>G	p.E158_Q184del	Splice site	Cazabat *et al.* (2007), Cazabat *et al.* (2012), and Martucci *et al.* (2012)
Intron3	67257508	rs267606555/AM236343		c.469-1G>A	–	Splice site	Vierimaa *et al.* (2006)
Exon4	67257531			c.491A>G	p.Q164R	Missense	Aflorei Ed *et al.* (2015)
Exon4	67257549			c.509T>C	p.M170T	Missense	Cazabat *et al.* (2012)
Exon4	67257602		0.0000343	c.562C>T	p.R188W	Missense	Hernandez-Ramirez *et al.* (2016)
Exon4	67257624	rs267606561	0.00002522	c.584T>C	p.V195A	Missense	Jaffrain-Rea *et al.* (2009), Naves *et al.* (2010), and Tichomirowa *et al.* (2011)
Exon4	67257645			c.605A>G	p.(Tyr202Cys)	Missense	Iacovazzo *et al.* (2016)
Intron4	67257686			c.645+1G>C	p.?	Splicing	Caimari *et al.* (2016)
Exon5	67257859			c.718T>C	p.C240R	Missense	Nozieres *et al.* (2011)
Exon5	67257862	rs267606573/EF066505	0.00004212	c.721A>G	p.K241E	Missense	Daly *et al.* (2007)
Exon5	67257877–67257880			c.736_738delGAG	p.E246del	In-frame deletion	Beckers *et al.* (2013) and Cuny *et al.* (2013)
Exon5	67257901			c.760T>C	p.C254R	Missense	Hernandez-Ramirez *et al.* (2016)
Exon5	67257903			c.762C>G	p.C254W	Missense	Hernandez-Ramirez *et al.* (2016)
Exon5	67257910	rs267606575		c.769A>G	p.I257V	Missense	Daly *et al.* (2010)
Exon5	67257925			c.784G>A	p.D262N	Missense	Cai *et al.* (2013)
Intron5	67257952	rs373727233		c.787+24C>T	–	Intronic	Cazabat *et al.* (2007) and Oriola *et al.* (2013)
Exon6	67258274	rs267606577/GQ412196.1		c.803A>G	p.Y268C	Missense	Tichomirowa *et al.* (2011)
Exon6	67258286		0.000008463	c.815G>A	p.G272D	Missense	Karaca *et al.* (2015) and Radian *et al.* (2017)
Exon6	67258342			c.871G>A	p.V291M	Missense	Occhi *et al.* (2010,b)
Exon6	67258343			c.872T>A	p.V291E	Missense	Cazabat *et al.* (2012)
Exon6	67258343–67258348			c.872_877delTGCTGG	p.V291_L292del	In-frame deletion	Ramirez-Renteria *et al.* (2016)
Exon6	67258352			c.881T>C	p.L294P	Missense	Cuny *et al.* (2013)
Exon6	67258438	rs188965257	0.00007992	c.967C>T	p.R323W	Missense	Cai *et al.* (2013)
Exon6	67258445		0.0000585	c.974G>A	p.R325Q	Missense	Cazabat *et al.* (2012) and Garcia-Arnes *et al.* (2013)
Exon6	67258447			c.976G>A	p.G326R	Missense	Cai *et al.* (2013)
Exon6	67258461			c.991T>C	p.X331R	Missense	Imran *et al.*, unpublished data, manuscript in preparation

– *AIP* mutation-positive patients with acromegaly often exhibit sparsely granulated somatotropinomas, a subtype that has been previously suggested to respond less to somatostatin analogues and to be more aggressive (Leontiou *et al.*, 2008; Daly *et al.*, 2010; Chahal *et al.*, 2012). They also have an aggressive clinical behavior, being large (88% macroadenomas), 80% of them being extrasellar, with frequent invasion (56% of cases) and submitted to more surgery and radiotherapy (Daly *et al.*, 2010; Martucci *et al.*, 2012); *AIP* mutation-positive tumors responded more poorly to somatostatin analogues (Leontiou *et al.*, 2008; Daly *et al.*, 2010; Kasuki Jomori de Pinho *et al.*, 2011; Chahal *et al.*, 2012; Gadelha *et al.*, 2013). The first generation of somatostatin analogues increases *AIP* expression, and this upregulates the expression of *ZAC1*, a tumor suppressor gene known to be upregulated by somatostatin-analogues and linked to their antiproliferative effects. This fact can explain the poor response to SRLs in *AIP* mutation-positive patients (Gadelha *et al.*, 2017). They also possibly have a lower IGF-1 control on pegvisomant therapy (Daly *et al.*, 2010). *AIP* mutation-negative patient response to treatment is poor although not as bad as in *AIP* positive (Beckers and Daly, 2007).

Regarding *AIP* mutation-positive PA, there is only one genotype–phenotype correlation for age: patients with truncating mutations are significantly younger at disease onset and diagnosis in comparison with patients with nontruncating *AIP* mutation (Cazabat *et al.*, 2009; Gadelha *et al.*, 2013; Xekouki *et al.*, 2013; Hernandez-Ramirez *et al.*, 2015).

Low *AIP* expression has been shown to be a better predictor of tumor invasion in somatotropinomas than Ki-67 or p53 (Korbonits and Kumar, 1993; Kasuki Jomori de Pinho *et al.*, 2011). They also have an increased risk for pituitary apoplexy (Daly *et al.*, 2010; Xekouki *et al.*, 2013).

In sporadic pituitary tumor, *AIP* mutations have been found in 2%–3.6% of patients (Tichomirowa *et al.*, 2009; Chahal *et al.*, 2010; Cazabat *et al.*, 2012; Preda *et al.*, 2014). PA in these patients present at a younger age and mainly with somatotropinoma (Georgitsi *et al.*, 2007a). In apparently sporadic PA patients, 20% of childhood-onset and 11.7% of macroadenoma in patients with <30 years harbor an *AIP* mutation (Tichomirowa *et al.*, 2011; Cazabat *et al.*, 2012). The lack of family history is probably due to incomplete penetrance, absence of information about family, or lack of diagnosis in family members in previous generations (Alband and Korbonits, 2014).

Who Should Be Tested?

Most reviews agree that there are three groups of patients that should do molecular genetic testing for an *AIP* mutation: (1) patients fulfilling the criteria for FIPA, (2) patients with PA diagnosed before the age of 18, and (3) patients with macroadenoma before the age of 30 (Korbonits *et al.*, 2012; Beckers *et al.*, 2013).

Mutation testing includes sequencing and testing for large deletions/duplications using MLPA if the first study is negative (Korbonits *et al.*, 2012).

Patients should be advised about the low penetrance of the disease, and the fact that only 20% of FIPA families harbor an *AIP* mutation.

Management

Treatment of pituitary adenomas is not significantly different between AIP positive patients, AIP-negative FIPA patients, or sporadic patients although we need to keep in mind that AIP-positive cases are more aggressive and respond poorly to SSA therapy, so other treatment modalities need to be considered early (e.g., pegvisomant, radiotherapy).

(A) *AIP* mutation-positive carriers:

Family member screening of *AIP* mutation carriers was shown to identify 24% of previously "unaffected" carriers with biochemical or radiological abnormalities (Korbonits *et al.*, 2012).

– Children: Based on the youngest known affected AIP positive case (3 years symptoms and 4 years diagnosis with a large invasive adenoma), the screening should start at the age of 4 years (Korbonits *et al.*, 2016). Yearly measurements of height and weight with calculation of height velocity and documentation of development of puberty together with pituitary function are recommended. MRI scanning is suggested around the age of 10 and then every 5 years if pituitary tests remain normal (Korbonits *et al.*, 2012; Williams *et al.*, 2014).
– Adult: Baseline clinical assessment with pituitary function tests and MRI followed by yearly pituitary function test, and consider repeat MRI every 5 years until the age of 30 if clinical and biochemical tests are normal. Due to the fact that the vast majority of patients are diagnosed before the age of 30 and due to the likelihood of the development of PA after the age of 50 years being low, the screening can be reduced by the age of 30 years and potentially stopped above the age of 50 (Chahal *et al.*, 2010; Korbonits *et al.*, 2012; Williams *et al.*, 2014).

(B) *AIP* mutation-negative:

The screening and follow-up of *AIP* mutation-negative families is more difficult due to a low penetrance and the fact that asymptomatic carriers cannot be identified by genetic screening. Some authors suggest education regarding symptoms, and clinical, laboratory, and MRI screening could be performed upon patients' request and/or under a research protocol (Korbonits *et al.*, 2012; Gadelha *et al.*, 2013).

Table 3 Variants of Uncertain Significance of the *AIP* gene

Region	*Chr. position*	*dbSNP rs ID*	*ExAc (Allele frequency)*	*DNA level*	*Protein level*	*Variant type*	*References*	*Comments*
5′ near gene	67248532	rs1638579		g.3028T>G	–	Promoter		
5′ near gene	67248638	rs11828274		g.3134C>A	–	Promoter		
5′ near gene	67248677	rs1638580		g.3173delA	–	Promoter		
5′ near gene	67248677	rs370928135		g.3173A>T	–	Promoter		
5′ near gene	67248970	rs181461909		g.3466C>T	–	Promoter		
5′ near gene	67249058–67249059	rs67365326		g.3554_3555insG	–	Promoter		
5′ near gene	67249155	rs373500637		g.3651T>G	–	Promoter		
5′ near gene	67249220	rs369248237		g.3716C>T	–	Promoter		
5′ near gene	67249234	rs1849113675		g.3730C>A	–	Promoter		
5′ near gene	67249296	rs118100488		g.3792T>A	–	Promoter		
5′ near gene	67249612	rs200878413		g.4108G>A	–	Promoter		
5′ near gene	67249694	rs71457777		g.4190T>C	–	Promoter		
5′ near gene	67249740	rs148149334		g.4236C>T	–	Promoter		
5′ near gene	67249760	rs1638582		g.4256A>C	–	Promoter		
5′ near gene	67249778	rs1638583		g.4274A>C	–	Promoter		
5′ near gene	67249801–67249805	rs201681859		g.4297_4301delTTTAA	–	Promoter		
5′ near gene	67249814	rs189761044		g.4310T>A	–	Promoter		
5′ near gene	67249818	rs376631316		g.4314T>G	–	Promoter		
5′ near gene	67249827	rs35580926		g.4323delT	–	Promoter		
5′ near gene	67249827	rs35580926		g.4323T>G	–	Promoter		
	67249911	rs181072302		g.4407C>G	–	Promoter		

Table 3 Continued

Region	Chr. position	dbSNP rs ID	ExAc (Allele frequency)	DNA level	Protein level	Variant type	References	Comments
5′ near gene								
5′ near gene	67250127	rs185280967		g.4623C>T	–	Promoter		
5′ near gene	67250231	rs201817175		g.4727delT	–	Promoter		
5′ near gene	67250289	rs369010349		g.4785delC	–	Promoter		
5′ near gene	67250329	rs369752687		g.4825T>C	–	Promoter		
5′ near gene	67250368			g.4864G>A	–	Promoter	Leontiou *et al.* (2008)	
5′ near gene	67250443	rs374248735		g.4939C>G	–	Promoter		
5′ near gene	67250464	rs12271280		g.4960G>A	–	Promoter		
5′UTR	67250544	rs1049506		c.-86G>A	–			
5′UTR	67250553	rs377565228		c.-77C>G	–			
5′UTR	67250573	rs369451483		c.-57T>C	–			
5′UTR	67250628	rs377710724		c.-2G>A	–			
Exon1	67250667	rs376913545	0.000008254	c.38T>A	p.I13N	Missense	Salvatori *et al.* (2014)	
Exon1	67250681	COSM194557		c.52A>G	p.I18V	Missense	http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=mut_summary&id=194557	Intestine carcinoma
Exon1	67250692	rs199913396		c.63C>T (p.(=))		Synonymous		
Exon1	67250697	rs116940576	0.001015	c.68G>A	p.G23E	Missense	Georgitsi *et al.* (2007a), Cazabat *et al.* (2007), and Raitila *et al.* (2007)	Follicular adenoma of thyroid, colorectal tumor
Exon1	67250701	rs201958318	0.0001651	c.72G>C	p.E24D	Missense		
Exon1	67250713	rs371423932	0.00002478	c.84T>C (p.(=))		Synonymous		
Exon1	67250719	rs374324200		c.90T>C (p.(=))		Synonymous		
Exon1	67250722	rs371636632	0.000008266	c.93G>T (p.(=))		Synonymous		
Intron1	67250737	rs374003812	0.00001658	c.99+9C>T	–	Intronic		
Intron1	67250738	rs187051018		c.99+10T>C	–	Intronic		
Intron1	67250753	rs201382386	0.000008323	c.99+25C>T	–	Intronic		
Intron1	67250755	rs200264737	0.0001249	c.99+27C>G	–	Intronic		
Intron1	67250766	rs373339489	0.00005845	c.99+38T>G	–	Intronic		
Intron1	67250906	rs143968953		c.99+178T>G	–	Intronic		
Intron1	67250913	rs371177056		c.99+185C>T	–	Intronic		
Intron1	67251033	rs191443582		c.99+305G>T	–	Intronic		
Intron1	67251034	rs376184207		c.99+306C>T	–	Intronic		
Intron1	67251278	rs183589142		c.99+550T>C	–	Intronic		

(*Continued*)

Intron1	67251320	rs374706460		c.99 + 692G > A	–	Intronic
Intron1	67251422	rs188081771		c.99 + 694C > T	–	Intronic
Intron1	67251454	rs146909894		c.99 + 726A > G	–	Intronic
Intron1	67251478	rs139310569		c.99 + 750G > A	–	Intronic
Intron1	67251678	rs190085354		c.99 + 950G > A	–	Intronic
Intron1	67251819	rs182330632		c.99 + 1091C > G	–	Intronic
Intron1	67251855	rs79806126		c.99 + 1127A > G	–	Intronic
Intron1	67251999	rs376096915		c.99 + 1271C > T	–	Intronic
Intron1	67252058	rs186475786		c.99 + 1330C > T	–	Intronic
Intron1	67252069	rs150811507		c.99 + 1341A > G	–	Intronic
Intron1	67252174	rs372698845		c.99 + 1446A > G	–	Intronic
Intron1	67252314	rs192602031		c.99 + 1586C > T	–	Intronic
Intron1	67252442	rs374195233		c.99 + 1714G > A	–	Intronic
Intron1	67252450	rs185429777		c.99 + 1722T > G	–	Intronic
Intron1	67252471	rs188992142		c.99 + 1743T > G	–	Intronic
Intron1	67252502	rs368371842		c.99 + 1774G > T	–	Intronic
Intron1	67252631–67252632	rs35670062		c.100-1845_1846insG	–	Intronic
Intron1	67252653	rs192960256		c.100-1824C > T	–	Intronic
Intron1	67252785	rs138143051		c.100-1692C > T	–	Intronic
Intron1	67252904	rs149598350		c.100-1573T > C	–	Intronic
Intron1	67253088	rs201456911		c.100-1389C > A	–	Intronic
Intron1	67253136–67253137	rs57126355		c.100-1340_1341insA	–	Intronic
Intron1	67253195–67253196	rs149993602		c.100-1282_100-1281delTT	–	Intronic
Intron1	67253195–67253197	rs139303676		c.100-1282_100-1280delTTT	–	Intronic
Intron1	67253215	rs57783261		c.100-1262delT	–	Intronic
Intron1	67253215	rs57783261		c.100-1262delTinsTT	–	Intronic
Intron1	67253215	rs57783261		c.100-1262delTinsTTT	–	Intronic
Intron1	67253232	rs185275707		c.100-1245C > T	–	Intronic
Intron1	67253244	rs144276045		c.100-1233C > T	–	Intronic
Intron1	67253307	rs374334224		c.100-1170C > T	–	Intronic
Intron1	67253351	rs188842535		c.100-1126T > C	–	Intronic
Intron1	67253382	rs59524104		c.100-1095G > A	–	Intronic
Intron1	67253401	rs139329931		c.100-1076T > C	–	Intronic
Intron1	67253637	rs144079780		c.100-840G > T	–	Intronic
Intron1	67253919	rs76605055		c.100-558C > G	–	Intronic
Intron1	67253921	rs75081450		c.100-556C > G	–	Intronic
Intron1	67253921	rs75081450		c.100-556C > T	–	Intronic
Intron1	67253927	rs146033798		c.100-550G > A	–	Intronic
Intron1	67254028	rs183782643		c.100-449G > T	–	Intronic
Intron1	67254350	rs188172321		c.100-127G > A	–	Intronic
Intron1	67254405	rs200750110		c.100-72G > A	–	Intronic
Intron1	67254434	rs377538917	0.00001661	c.100-43G > T	–	Intronic
Intron1	67254461	rs200754493	0.00000828	c.100-16C > T	–	Intronic
Intron1	67254470	rs371268619	0.000008273	c.100-7C > T	–	Intronic

Table 3 Continued

Region	Chr. position	dbSNP rs ID	ExAc (Allele frequency)	DNA level	Protein level	Variant type	References	Comments
Exon2	67254493	rs139947406	0.00002479	c.116G>A	p.R39Q	Missense		
Exon2	67254496	rs142044984		c.119C>T	p.T40M	Missense		
Exon2	67254522	rs1063385	0.0002395	c.145G>T	p.V49L	Missense		
Exon2	67254582	rs141223463	0.00001649	c.205A>G	p.K69E	Missense		
Exon2	67254585			c.208C>A	p.L70M	Missense	Beckers *et al.* (2013)	
Exon2	67254594			c.217T>C	p.W73R	Missense	Baciu *et al.* (2012)	Male, 29 years, GH secreting PA
Intron2	67254679	rs375562070	0.00008243	c.279+23C>T	–	Intronic		
Intron2	67254701			c.279+45T>C	–	Intronic		
Intron2	67254861	rs371535524		c.279+205G>A	–	Intronic		
Intron2	67254862	rs375719530		c.279+206C>T	–	Intronic		
Intron2	67254863	rs187332576		c.279+207G>A	–	Intronic		
Intron2	67255272	rs142218826		c.279+616C>T	–	Intronic		
Intron2	67255343	rs75105505		c.279+687T>C	–	Intronic		
Intron2	67255388	rs151153923		c.279+732C>T	–	Intronic		
Intron2	67255448	rs140077772		c.279+792T>A	–	Intronic		
Intron2	67255513	rs149912027		c.279+857C>T	–	Intronic		
Intron2	67255616	rs192187404		c.279+960A>G	–	Intronic		
Intron2	67255753	rs114749639		c.280-985G>A	–	Intronic		
Intron2	67255802	rs147581050		c.280-936C>T	–	Intronic		
Intron2	67256049	rs181893050		c.280-689T>G	–	Intronic		
Intron2	67256306	rs370857260		c.280-432G>A	–	Intronic		
Intron2	67256351	rs35286380		c.280-387_280-386insC	–	Intronic		
Intron2	67256355	rs71461650		c.280-386dupC	–	Intronic		
Intron2	67256605	rs146679495		c.280-133G>A	–	Intronic		
Intron2	67256638	rs139351231		c.280-100T>G	–	Intronic		
Intron2	67256653	rs368021930		c.280-85G>T	–	Intronic		
Intron2	67256657			c.280-81G>A	–	Intronic	Cazabat *et al.* (2007)	
Intron2	67256701	rs370499402	0.00002442	c.280-37C>T	–	Intronic		
Exon3	67256741	rs374539726		c.283G>A	p.V95M	Missense		
Exon3	67256749	rs146865791		c.291G>A (p.(=))		Synonymous		
Exon3	67256759	rs147931650	0.0002221	c.301G>A	p.V101M	Missense		
Exon3	67256774	rs369414668	0.00003519	c.316C>T	p.R106C	Missense		
Exon3	67256775	rs373950586	0.0000263	c.317G>A	p.R106H	Missense		
Exon3	67256783			c.325G>A	p.A109T	Missense	Barlier, France	Male, 15 years, macroprolactinoma
Exon3	67256813	rs368933035	0.00003387	c.355C>T	p.R119W	Missense	Cai *et al.* (2013)	Female, 32 years, sporadic GH secreting PA
Exon3	67256824		0.00003375	c.366C>T (p.(=))		Synonymous	Unpublished data from our group	
Exon3	67256840	rs140530307	0.0001347	c.382C>T	p.R128C	Missense	Caimari *et al.* (2015)	
Exon3	67256861	rs150487522	0.0000842	c.403C>T	p.H135Y	Missense		
Exon3	67256873	rs138312605	0.000008433	c.415G>T	p.D139Y	Missense		
Exon3	67256875	rs149570102	0.000008437	c.417C>T (p.(=))		Synonymous		

(*Continued*)

Exon3	67256915	rs376193405	0.000008547	c.457G>A	p.E153K	Missense	http://www.cbioportal.org/index.do?tab_index=tab_visualize&cancer_study_id=all_stjude_2013&genetic_profile_ids_PROFILE_MUTATION_EXTENDED=all_stjude_2013_muta	
Intron3	67256930			c.468+4A>G	–	Intronic		
Intron3	67256934	rs368923226	0.000008852	c.468+8G>A	–	Intronic		
Intron3	67256935	rs373159347	0.00005295	c.468+9C>T	–	Intronic		
Intron3	67256939	rs375823655	0.00007144	c.468+13C>T	–	Intronic		
Intron3	67256940	rs369766409		c.468+14G>A	–	Intronic		
Intron3	67257062	rs79270719	0.0008043	c.468+136A>G	–	Intronic		
Intron3	67257144	rs199777404		c.468+218C>T	–	Intronic		
Intron3	67257169	rs149018552		c.468+243C>T	–	Intronic		
Intron3	67257201	rs186922971		c.468+275G>A	–	Intronic		
Intron3	67257251	rs190123945		c.469-258C>T	–	Intronic		
Intron3	67257292	rs182438052		c.469-217C>A	–	Intronic		
Intron3	67257294	rs75842186		c.469-215G>T	–	Intronic		
Intron3	67257357	rs142119195		c.469-152C>G	–	Intronic		
Intron3	67257358	rs140960814		c.469-151G>T	–	Intronic		
Intron3	67257359	rs144412971		c.469-150A>T	–	Intronic		
Intron3	67257359–67257360	rs5792416		c.469-150_469-149insTGTT	–	Intronic		
Intron3	67257360	rs33941803		c.469-149_469-148insTGTT	–	Intronic		
Intron3	67257365	rs10625132		c.469-144_469-143insTTTG	–	Intronic		
Intron3	67257362–67257363	rs33961879		c.469-143_469-142insTTTG	–	Intronic		
Intron3	67257366	rs76392311		c.469-143A>T	–	Intronic		
Intron3	67257398	rs143812313		c.469-111G>A	–	Intronic		
Intron3	67257413	rs372075021		c.469-96G>A	–	Intronic		
Intron3	67257454	rs374519806		c.469-55C>T	–	Intronic		
Intron3	67257458	rs368797185	0.00006314	c.469-51C>T	–	Intronic		
Intron3	67257474	rs377621889	0.00009149	c.469-35C>T	–	Intronic		
Intron3	67257475	rs371043120	0.0000305	c.469-34C>T	–	Intronic		
Intron3	67257492			c.469-17T>C	–	Intronic	Unpublished data from our group	
Exon4	67257532	rs148095197	0.00001429	c.492G>A (p.(=))		Synonymous		
Exon4	67257557	rs138902236	0.0001377	c.517G>A	p.E173K	Missense		
Exon4	67257601	rs142037029	0.00002576	c.561C>T (p.(=))		Synonymous		
Exon4	67257603			c.563G>A	p.R188Q	Missense	Cazabat *et al.* (2012)	Female, 24 years old, microprolactinoma
Exon4	67257611	rs189861025	0.00004243	c.571C>T	p.R191C	Missense		
Exon4	67257613		0.00001693	c.573C>T (p.(=))		Synonymous	Unpublished data from our group	
Exon4	67257631		0.0001091	c.591G>A (p.(=))	p.E197=	Synonymous	Tichomirowa *et al.* (2011)	

Table 3 Continued

Region	*Chr. position*	*dbSNP rs ID*	*ExAc (Allele frequency)*	*DNA level*	*Protein level*	*Variant type*	*References*	*Comments*
		rs202006716/ GQ403806.1						
Exon4	67257633	COSM215485		c.593C>A	p.A198D	Missense	http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?	
action-=mut_-summary&id=215485		Neuroectodermal tumour medulloblastoma						
Exon4	67257649	rs146317385	0.0001004	c.609C>T (p.(=))		Synonymous		
Exon4	67257652	rs182746617		c.612T>C (p.(=))		Synonymous		
Exon4	67257680	rs3210041		c.640A>C	p.M214L	Missense		
Intron4	67257697	rs373778965	0.000008375	c.645+12A>G	–	Intronic		
Intron4	67257722			c.645+37G>A	–	Intronic	Cazabat *et al.* (2007)	
Intron4	67257723	rs376846946	0.00003361	c.645+38G>C	–	Intronic		
Intron4	67257726	rs370717327	0.00001681	c.645+41G>C	–	Intronic		
Intron4	67257726			c.645+41dup	–	Intronic	Cai *et al.* (2013)	Female, 47 years, NFPA
Intron4	67257744	rs199980884	0.000101	c.646-43C>G	–	Intronic		
Intron4	67257761	rs367957036	0.000008408	c.646-26C>G	–	Intronic		
Intron4	67257764	rs370364419	0.00003365	c.646-23G>A	–	Intronic		
Exon5	67257834	rs141715817	0.0002271	c.693G>A (p.(=))		Synonymous		
Exon5	67257836	rs150594019	0.000008411	c.695C>T	p.P232L	Missense		
Exon5	67257837			c.696G>C (p.(=))		Synonymous	Georgitsi *et al.* (2007a)	ACTH secreting PA
Exon5	67257861			c.720C>T (p.(=))		Synonymous	Cazabat *et al.* (2007)	
Exon5	67257879	rs149677884	0.000008436	c.738G>A (p.(=))		Synonymous		
Exon5	67257894	rs147351993	0.0001943	c.753G>A (p.(=))	p.L251=	Synonymous	Caimari *et al.* (2015)	
Intron5	67257937		0.00004289	c.787+9C>T	–	Intronic	Unpublished data from our group	
Intron5	67257954	rs368265892	0.000008764	c.787+26G>C	–	Intronic		
Intron5	67257974–67257975			c.787+46_787+47del	–	Intronic		
Intron5	67258216	rs376695792		c.788-43del	–	Intronic		
Intron5	67258238	rs138846272	0.00005971	c.788-21G>A	–	Intronic		
Intron5	67258244			c.788-20_788-14dup	–	Intronic		
Intron5	67258247	rs376713535	0.00003402	c.788-12C>A	–	Intronic		
Exon6	67258293	rs145142121	0.00002539	c.822C>A (p.(=))		Synonymous		
Exon6	67258302		0.00003384	c.831C>T (p.(=))		Synonymous	Unpublished data from our group	
Exon6	67258360	rs373922286	0.00001707	c.889G>A	p.A297T	Missense		
Exon6	67258391	rs4930199		c.920A>C	p.Q307P	Missense		
Exon6	67258411	rs375740557		c.940C>T	p.R314W	Missense	Baciu *et al.* (2012)	Acromegaly
Exon6	67258420	rs145025838		c.949G>T	p.D317Y	Missense		
Exon6	67258422	rs138814192	0.00001078	c.951C>T (p.(=))		Synonymous		
Exon6	67258426			c.955G>A	p.E319K	Missense	Cai *et al.* (2013)	Male, 11 years, GH secreting PA
3′UTR	67258478	rs142567224	0.0004784	c.*14C>A	–			

(*Continued*)

3′UTR	67258498		c.*34C>G	–	Cazabat *et al.* (2007)
3′UTR	67258520		c.*56C>G	–	Cazabat *et al.* (2007)
3′UTR	67258528	rs115346238	c.*64G>A	–	
3′UTR	67258534		c.*70C>T	–	Occhi *et al.* (2010)
3′UTR	67258541		c.*77C>A	–	Leontiou *et al.* (2008)
3′ near gene	67258633	rs369548473	c.*169A>G	–	
3′ near gene	67258720	rs12361982	c.*256G>C	–	
3′ near gene	67258857	rs193245280	g.*393C>G	–	
3′ near gene	67258884	rs149754566	c.*420C>T	–	
3′ near gene	67258960	rs144589607	c.*496C>T	–	
3′ near gene	67258963	rs375110677	c.*499G>A	–	
3′ near gene	67259006	rs148408490	c.*542G>A	–	

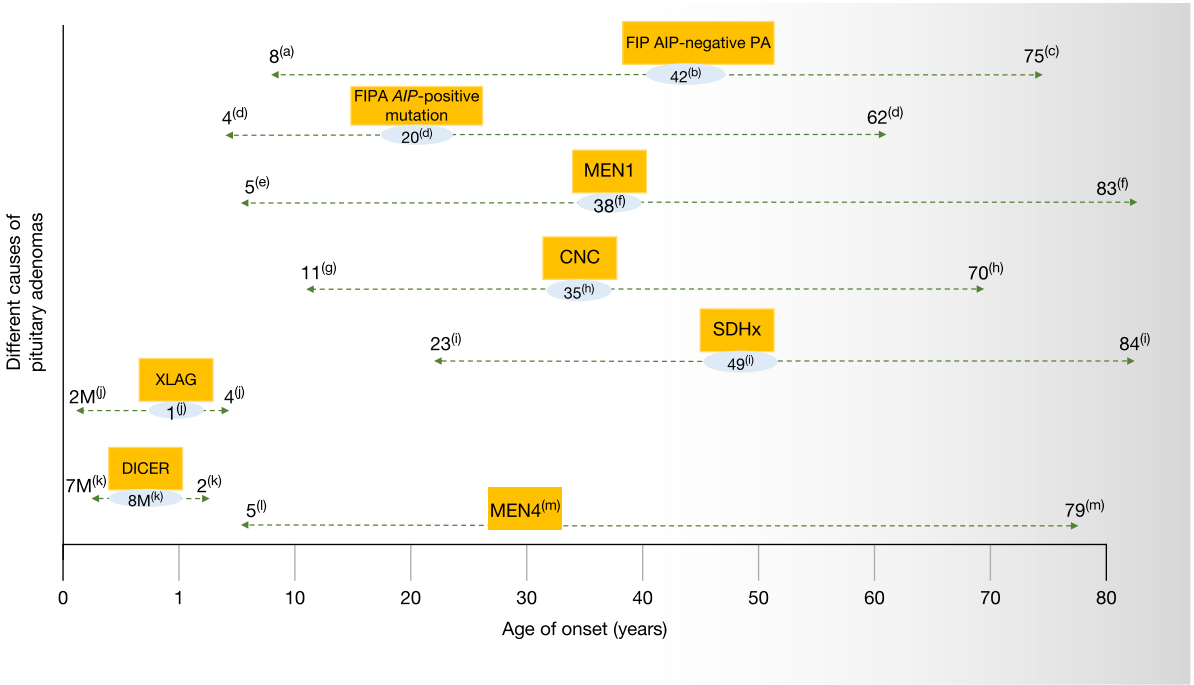

Fig. 3 Schematic representation of age of onset for familial pituitary adenoma. Mean age of onset is shown (in *light blue oval*), except for DICER1, where median is shown. Very few patients were reported with MEN4 with age of onset ranging between 5 and 79 years (median of 9 cases). The arrows represent the range of age of onset. References: (a) Hernandez-Ramirez *et al.* (2015), (b) Cazabat *et al.* (2012), (c) Martucci *et al.* (2012), (d) Hernandez-Ramirez *et al.* (2015), (e) Stratakis *et al.* (2010), (f) Verges *et al.* (2002), (g) (Watson *et al.* (2000), (h) Boikos and Stratakis (2006), (i) O'Toole *et al.* (2015), (j) Beckers *et al.* (2015), (k) de Kock *et al.* (2014), (l) Sambugaro *et al.* (2015), (m) (Lee and Pellegata (2013b).

X-Linked Acrogigantism (XLAG) Syndrome

Overview

XLAG syndrome presents an early-onset of pituitary gigantism, caused by duplications of the *GPR101* gene located at chromosome Xq26.3 (Trivellin *et al.*, 2014). Current data suggest that there are no other conditions associated up till the age of 50 years (Iacovazzo *et al.*, 2016).

This group represents the smallest group of FIPA and is responsible for 8%–10% of the cases of the patients with no syndromic pituitary gigantism, representing the second largest group of patients with childhood-onset acromegaly, after *AIP* positive-mutation patients (Rostomyan *et al.*, 2015; Iacovazzo *et al.*, 2016; Iacovazzo and Korbonits, 2016). Altogether 31 patients have been reported to date (Gadelha *et al.*, 2017).

The usually duplicated Xq26.3 region codes four genes, but only one of them, *GPR101*, was overexpressed in the patients with a microduplication of this region. There is also a patient described with duplication affecting only the *GPR101* (Iacovazzo *et al.*, 2016). The *GPR101* gene encodes an orphan G protein-coupled receptor that is significantly expressed in the hypothalamus and in pituitary samples of XLAG patients whose physiological function and endogenous ligand are unknown. This receptor, when activated, causes increase in cAMP levels, which represents a key factor involved in the regulation of GH secretion and cell proliferation in response to GHRH (Bates *et al.*, 2006; Trivellin *et al.*, 2014; Iacovazzo *et al.*, 2016). However, the exact mechanism how this duplication leads to tumorigenesis is unclear.

XLAG occurs as a simplex condition due to a "de novo" mutation in the majority of cases, but it can also be associated with FIPA: there are two familial cases described with transmission from affected mother to son (Beckers *et al.*, 2015). In addition, mosaic somatic mutation cases have also been described in males where the mutation was identified in the pituitary tissue and/or at low-level germline. The phenotype of somatic and germline *GPR101* duplication patients is the same (Daly *et al.*, 2016; Iacovazzo *et al.*, 2016; Rodd *et al.*, 2016).

Genetic testing could include CGH array or GPR101-specific droplet PCR. Negative genetic tests on peripheral blood DNA do not exclude the diagnosis of XLAG as the disease can result from mosaic somatic mutation. If the phenotype is characteristic of XLAG, testing of DNA samples from pituitary or other tissues should be considered (Iacovazzo *et al.*, 2016).

Pituitary Abnormalities Related to XLAG

Patients usually present with increased growth velocity before the end of the first year (range 0.5–2 years) and with a median age at diagnosis of 3 years old (range 1–22). Their growth curves significantly exceed the 97th percentile and acral enlargement and

coarsened facial features can be noticed (Trivellin *et al.*, 2014; Beckers *et al.*, 2015; Iacovazzo *et al.*, 2016; Rodd *et al.*, 2016). The majority of the cases are females (Trivellin *et al.*, 2014; Beckers *et al.*, 2015; Iacovazzo *et al.*, 2016).

Pituitary macroadenoma has been identified in most cases, but a significant minority of the patients have pituitary hyperplasia. All patients have elevated growth hormone levels with accompanying hyperprolactinaemia in most cases. The pituitary tissue shows a characteristic sinusoidal and lobular architecture and contains densely and sparsely granulated somatotrophs and microcalcifications, and follicle-like structures are also commonly observed with usually a low Ki-67 staining, but higher Ki-67 has also been described (Iacovazzo *et al.*, 2016; Naves *et al.*, 2016).

Hyperprolactinemia accompanies the GH excess in about 80% of the cases, and about 50% present with clinic of hyperprolactinemia. GHRH levels can be normal or slightly elevated, and in some patients a paradoxical response is seen in the TRH test (Iacovazzo and Korbonits, 2016).

XLAG typically occurs earlier than *AIP* mutation-positive PA, presenting with larger height *Z*-scores at diagnosis and lower rate of pituitary adenoma invasion (Iacovazzo and Korbonits, 2016).

Treatment of XLAG is challenging (Naves *et al.*, 2016). Treatment includes surgery, medical therapy, and, in some cases, radiotherapy as responses to somatostatin analogues are poor. Dopamine agonists effectively control prolactin excess although they seem to have no significant effect on GH/IGF-1 levels (Beckers *et al.*, 2015, Iacovazzo *et al.*, 2016).

GH receptor antagonists have been used successfully even in patients who were not controlled by surgery or somatostatin analogues. Patients with pituitary hyperplasia, where extensive pituitary surgery was not considered due to the risk of hypopituitarism, can be treated with a combination of SSA, dopamine agonist, and pegvisomant (Beckers *et al.*, 2015; Rodd *et al.*, 2016). Permanent hypopituitarism is common due to a need for aggressive treatments (Iacovazzo and Korbonits, 2016).

Who Should Be Tested?

The frequency of XLAG in nonsyndromic pituitary gigantism supports the need for testing for *GPR101* duplication in patients with early-onset (<5 years) pituitary gigantism (Beckers *et al.*, 2015; Iacovazzo *et al.*, 2016).

Management

XLAG patients represent a significant management problem. In cases of hyperplasia, although the rest of the pituitary function is normal, only hypophysectomy was shown to be effective surgical option for the GH excess (Moran *et al.*, 1990). More recently combined SSA, carbergoline, and pegvisomant achieved normal biochemistry and growth velocity (Rodd *et al.*, 2016).

For patients with pituitary tumors, surgery and, if necessary, combined medical therapy (as above) or radiotherapy need to be employed (Beckers *et al.*, 2015; Iacovazzo and Korbonits, 2016).

Multiple Endocrine Neoplasia Type 1 Syndrome

Overview

MEN1 is characterized by development of tumors mainly in endocrine but also in nonendocrine organs in a single patient. It is an autosomal dominant condition with high penetrance but may also occur sporadically (Wermer, 1954). The penetrance is different by the various components of the disease, overall it is >50% by age of 20 years (Brandi *et al.*, 2001; Machens *et al.*, 2007; Thakker, 2010).

The incidence of MEN1 is around 0.25% from postmortem studies and affects about 1/30.000 individuals, usually in a familial context (Alband and Korbonits, 2014).

The *MEN1* gene is located on chromosome 11q13 and has 10 exons, of which exons 2–10 encode a 610 amino acid nuclear protein called menin (Chandrasekharappa *et al.*, 1997; Marx, 2005). MENIN directly regulates the expression of the cyclin-dependent kinase-inhibiting (CDKI) genes, *CDKN1b* (encoding p27) and *CDKN2C* (encoding p18), and thereby negatively regulates the proliferation of the cells (Giusti *et al.*, 1993). The correlation of menin with p27 is particularly interesting because mutations in the *CDKN1B* are associated with a MEN4 syndrome (Caimari and Korbonits, 2016). Decreased p27 expression, whose main function is to control the progression from G1 to S phase, is enough for tumor development in p27Kip1 $\pm$ mice (Fero *et al.*, 1998; Lee and Pellegata, 2013a). A reduction in *CDKN1B* expression has been described in some endocrine malignant and benign tumors (Pellegata *et al.*, 2007; Marinoni and Pellegata, 2011). Inheritance of germline *MEN1* mutation predisposes carriers to the development of a tumor that arises after somatic mutation, leading to loss of heterozygosity in 11q13 in the tumor DNA, consistent with the Knudson two-hit hypothesis and a tumor suppressor role for menin (Lemos and Thakker, 2008; Thakker, 2010). LOH in 11q13 occurs in around 90% of MEN1 tumors, with deletions and point mutations representing other mechanisms by which the second hit may occur (Thakker *et al.*, 2012).

MENIN is located in the nucleus and interacts with the promoter regions of thousands of genes, which indicate that it may be implicated in the regulation of gene transcription, DNA repair, and cell division and proliferation (Lemos and Thakker, 2008; Tichomirowa *et al.*, 2009; Syro *et al.*, 2016).

Mutations in the *MEN1* gene were first described in 1997, and more than 1500 mutations identified (Chandrasekharappa *et al.*, 1997; Lemos and Thakker, 2008). These mutations include frameshift mutations, nonsense mutations, missense mutations, splice site mutations, and large deletions. There are mutational hotspots in exons 2, 3, 9, 10, and intron 4 (Lemos and Thakker, 2008). Seventy percent of *MEN1* mutations cause truncated forms of menin (Lemos and Thakker, 2008).

MEN1 mutations can be identified in 75%–95% of all cases, and about 10% of the mutations are "de novo" mutations (Lips *et al.*, 1984; Thakker, 2010). The absence of an identified mutation may be due either to other currently unknown disease-causing genes, phenocopies or to lack of full assessment of the known disease causing-gene. Phenocopies have been reported in around 5% of MEN1 patients (Turner *et al.*, 2010).

In addition, there are 24 *MEN1* polymorphisms identified that may not be easily distinguished from pathological variants (Brandi *et al.*, 2001; Lemos and Thakker, 2008).

MENIN has many known partners that include JunD, NF-kB, Pem, SIN3A, HDAC, elements of the Smad family, Runx2, MLL, estrogen receptor-α hostel cell factor 2, and RBBP5 (Thakker, 2014). Menin activates the transcription of *CDKN1B* and *CDKN2C* by recruiting the histone methyltransferase MLL protein to the promoters and coding regions of these genes. The *CDKN1B* and *CDKN2C* are predominantly expressed in endocrine organs, which may explain the preference for endocrine tumors in patients with MEN1 (Taguchi *et al.*, 2011). Menin also regulates transcription of homeobox (HOX) genes (Wu and Hua, 2011). In the pituitary menin negatively regulates the cell proliferation and secretion of PRL, GH, and ACTH. The expression of IGFBP-2, IGF-2, and PTHrp is negatively modulated by menin (La *et al.*, 2004; Fontaniere *et al.*, 2006).

The three main components of the syndrome are hyperparathyroidism (in almost all patients by the age of 50), enteropancreatic endocrine tumors (in about 60% of cases), and PA (in about 30%–40% of cases) (Wermer, 1954). Other endocrine manifestations include foregut carcinoids, adrenal cortex tumors, and rarely pheochromocytoma. Lipomas, facial angiofibromas, collagenomas, and ependymomas are nonendocrine tumors also associated with MEN1 (Brandi *et al.*, 2001; Caimari and Korbonits, 2016).

A diagnosis of MEN1 may be established in an individual by one of the three criteria: (1) occurrence of two or more primary MEN1-associated endocrine tumors; (2) the occurrence of one of the MEN1 associated tumors in a first degree relative of a patient with a clinical diagnosis of MEN1; (3) an identification of a germline *MEN1* mutation even in the absence of a clinical or biochemical manifestation of MEN1 (Turner *et al.*, 2010).

Pituitary Adenomas

Overall about 2.7%–3% of all pituitary adenomas are due to MEN1 (Chandrasekharappa *et al.*, 1997; Thakker, 2010; Alband and Korbonits, 2014). Approximately 30%–40% of *MEN1* gene mutation carriers develop PA, with a slight female preponderance. This is the presenting problem in 15% (ranging from 10% to 25%) of MEN1 cases although underdiagnosed hyperparathyroidism is often already present (Skogseid *et al.*, 1991; Burgess *et al.*, 1996; Marx *et al.*, 1998; Verges *et al.*, 2002; Lecoq *et al.*, 2015).

The most common clinically manifest form are prolactinomas (Verges *et al.*, 2002; Benito *et al.*, 2005), but small NFPA have also been seen in prospective screening studies (de Laat *et al.*, 2015). On the other hand, the proportion of plurihormonal adenomas and multiple adenomas is significantly higher in MEN1 patients (Trouillas *et al.*, 2008). In patients with *MEN1* mutation and clinical signs of acromegaly, the possibility of GHRH-secreting NET should be considered and GHRH level should be assessed (Garby *et al.*, 2012; Saleem *et al.*, 2012).

PA in MEN1 patients appear to occur at younger ages when compared to sporadic PA: the earliest age reported is 5 years and the mean age of onset has been reported to be 38 ± 15.3 years (Farrell *et al.*, 2011). They are also larger and more aggressive than sporadic tumors, with 85% of macroadenoma cases, lower rate of hormonal normalization and higher recurrence rates (Verges *et al.*, 2002; Trouillas *et al.*, 2008; Tichomirowa *et al.*, 2009; Thakker, 2010; Syro *et al.*, 2012; Toledo *et al.*, 2013). However, one study concluded that PA in MEN1 seems to be much less aggressive than previously suggested by their demonstrations of a good response to the prolactinoma treatment and a limited growth of NFPA (de Laat *et al.*, 2015).

The clinical management of PA is not different from sporadic cases although it is likely that surgery will be required more frequently (Thakker *et al.*, 2012). Untreated patients with MEN1 have a decreased life expectancy, but even with treatment 70% of MEN1 patients die due to MEN1 complications (Dean *et al.*, 2000; Geerdink *et al.*, 2003; Goudet *et al.*, 2010).

Who Should Be Tested?

The *MEN1* germline mutation screening is recommended for index cases and their relatives, either asymptomatic or with clinical manifestations of MEN1. *MEN1* germline mutation testing of asymptomatic relatives should be offered at the earliest opportunity because manifestation may occur by the age of 5 (Brandi *et al.*, 2001; Thakker *et al.*, 2012).

MEN1 mutation testing can be considered in patients with PA and no other MEN1 manifestation in childhood or young-onset cases (de Laat *et al.*, 2015), and may be recommended in individuals with atypical MEN1 phenotypes like multigland hyperparathyroidism or multiple pancreatic NET at any age (Thakker *et al.*, 2012; Alband and Korbonits, 2014).

Management

MEN1-related pituitary adneomas could be challenging, where multimodal therapy is needed especially in aggressive pediatric cases (Gan *et al.*, 2015; Syro *et al.*, 2016). Regarding pituitary screening in *MEN1* mutation-positive carriers, yearly clinical assessment and plasma prolactin and IGF-1 measurements starting at the age of 5 years and pituitary MRI every 3 years are recommended (Brandi *et al.*, 2001, Thakker *et al.*, 2012).

Multiple Endocrine Neoplasia Type 4 Syndrome

Overview

There are patients with MEN1-like phenotype and no identifiable *MEN1* mutations. A small minority of these patients have been identified with loss-of-function mutations in the *CDKN1B* gene, establishing a distinct entity called MEN4. The *CDKN1B* gene is a tumor suppressor gene localized on chromosome 12q13 (Pellegata *et al.*, 2006; Agarwal *et al.*, 2009; Igreja *et al.*, 2009). There are only a handful of different discovered heterozygous frameshift, nonsense, or missense mutations (Thakker, 2014) and two cases of alterations in the 5′ open reading frame (Occhi *et al.*, 2013; Sambugaro *et al.*, 2015). Loss of p27 is frequently observed in PA especially in corticotropinomas and pituitary carcinomas (Lidhar *et al.*, 1999) although somatic *CDKN1B* mutations have not been found in PA (Dahia *et al.*, 1998; Pellegata *et al.*, 2006; Marinoni and Pellegata, 2011). Mutations in other cyclin-dependent kinases inhibitors, encoding p15 (*CDKN2B*), p18 (*CDKN2C*), and p21 (*CDKN1A*), were also implicated in rare cases of MEN1-like phenotype (Agarwal *et al.*, 2009).

Human cyclin-dependent kinases inhibitor-related MEN1-like cases are very rare. They are inherited in a dominant manner and due to their rarity, and it is not possible to determine the penetrance of the syndrome, frequency of familial and sporadic cases, and phenotype–genotype correlation.

Hyperparathyroidism is present in almost all the cases, but PA are only reported in about a third of these cases (Gadelha *et al.*, 2017). Other tumors described include renal angiomyolipoma, adrenal nonfunctional tumor, uterine fibroma, gastrinoma, neuroendocrine cervical carcinoma, bronchial carcinoid, papillary thyroid carcinoma, and gastric carcinoma (Georgitsi *et al.*, 2007b; Molatore *et al.*, 2010; Malanga *et al.*, 2012).

Pituitary Adenomas

The most common tumor type is somatotropinoma, but corticotropinoma, NFPA, and a possible prolactinoma have also been described (Lee and Pellegata, 2013b; Gadelha *et al.*, 2017). A childhood-onset somatotropinoma case has been described (Sambugaro *et al.*, 2015).

Who Should Be Tested?

In cases that are negative for *MEN1* mutation but display a MEN1 phenotype, screening for *CDKN1B* gene mutation could be considered (Tichomirowa *et al.*, 2009; Georgitsi, 2010). *CDKN1B* mutation is rare but can be found in 1.5%–2.8% of patients with MEN1 features and negative *MEN1* testing and represents 0.1%–0.2% of all MEN1 cases (Ozawa *et al.*, 2007; Agarwal *et al.*, 2009; Owens *et al.*, 2009; Georgitsi, 2010).

Carney Complex

Overview

Carney Complex (CNC) is a rare condition characterized by endocrine, including pituitary hyperplasia or adenoma, and other tumor types (Stratakis *et al.*, 2001). Nowadays there are approximately 700 people described (Stratakis *et al.*, 2015). It is an autosomal dominant disease with unknown prevalence and high penetrance for some of the manifestations (>95% by age 50 years) (Horvath *et al.*, 2010). It is familial in 70% of cases and the diagnosis is made at a median age of 20 years (Stratakis *et al.*, 2001; Boikos and Stratakis, 2007).

Originally two distinct loci for CNC have been identified: one on chromosome 17q22-24 and the other on chromosome 2p16 (Stratakis *et al.*, 1996; Kirschner *et al.*, 2000). The gene located on 2p16 has not been identified, while the chromosome 17 locus is associated with the gene encoding the protein kinase A regulatory subunit Iα (*PRKAR1A*) and is the cause of the vast majority (>70%) of CNC cases (Stratakis *et al.*, 1996; Veugelers *et al.*, 2004; Horvath *et al.*, 2010). There is now a third locus also identified on chromosome 1p31.1, coding for the enzymatic subunit of PKA (Forlino *et al.*, 2014).

PKA is implicated in a wide range of cellular processes including hormone release, transcriptional regulation, cell cycle progression, and apoptosis. Loss of regulatory activity of PKA results in enhanced activity of PKA and therefore enhances the response to cAMP signaling in affected tissues. PKA is a tetramer composed of 2 homodimers of regulatory and 2 catalytic subunits (Horvath and Stratakis, 2008; Stratakis *et al.*, 2010).

PRKAR1A comprises 11 exons and over 125 pathogenic variants have been identified (Kirschner *et al.*, 2000; Correa *et al.*, 2015). Most *PRKAR1A* mutations lead to mRNA instability, decreased or absent protein expression, and *PRKAR1A* haploinsufficiency in CNC tumors (Kirschner *et al.*, 2000). Large *PKRAR1A* deletions, 21.6% of patients with CNC, seem to have a more severe phenotype (Salpea *et al.*, 2014) and specific testing with MLPA or long-PCRs need to be performed to identify these mutations. LOH at 17q22-24 and allelic loss have been shown in CNC tumors, supporting a tumor suppression function, and the loss of PRKAR1A function enhances intracellular response to cAMP in CNC tumors (Bossis and Stratakis, 2004).

Recent studies have shown gene duplication affecting the catalytic B subunit of PKA in association with elements of the CNC phenotypes (Forlino *et al.*, 2014; Syro *et al.*, 2016).

Variants of the phosphodiesterase type 11A (*PDE11A*) showed association with a higher incidence of hyperplasia and large-cell calcifying Sertoli tumors, suggesting that PDE11A can modify the phenotype (Libe *et al.*, 2011).

The diagnosis of CNC is established in a proband with two or more major clinical manifestations and/or when a pathogenic variant is identified in *PKRAR1A*. Major criteria include: spotty skin pigmentation with typical distribution (lips, conjunctiva and inner or outer canthi, vaginal and penile mucosal); cutaneous and mucosal myxoma or cardiac myxoma; sporadic isolated primary pigmented nodular adrenocortical disease or paradoxical positive response of urinary glucocorticoid excretion to dexamethasone administration during Liddle's test; acromegaly as a result of GH-producing adenoma; large-cell calcifying Sertoli cell tumor or characteristic calcification on testicular ultrasound, thyroid carcinomas (at any age) or multiple hypoechoic nodules on thyroid ultrasound in prepubertal child; psammomatous melanotic schwannomas; blue nevus; breast ductal adenoma and osteochondromyxoma. The supplemental criteria include affected first-degree relative and inactivating mutation of the *PRKAR1A* gene (Correa *et al.*, 2015; Stratakis *et al.*, 2015).

Pituitary Adenomas

PA in CNC are somatotropinoma and prolactinoma and rarely stain positively for TSH, LH, and alpha-subunit (Pack *et al.*, 2000). Acromegaly is rare and occurs in 10%–12% of cases, but 75% of patients have biochemical abnormalities like elevation in GH, IGF-1, or PRL levels or abnormal responses to dynamic pituitary testing due to pituitary hyperplasia (Pack *et al.*, 2000; Raff *et al.*, 2000; Correa *et al.*, 2015). Histology more commonly shows multifocal hyperplasia of somatomammotrophic cells which may lead to formation of GH/PRL adenoma. Most of the patients do not have an aggressive PA and acromegaly usually develops insidiously. The mean age of onset of acromegaly in CNC patients is 35.8 years; however, a few cases of gigantism have been described. Microadenomas are more common than macroadenomas, and they are frequently multiple and surrounded by hyperplastic tissue (Stratakis *et al.*, 2001; Pack *et al.*, 2005; Boikos and Stratakis, 2007; Caimari and Korbonits, 2016).

Who Should Be Tested?

Genetic testing should be offered to patients with two or more CNC major manifestations or relatives of CNC patients (Stratakis *et al.*, 2015).

Management of CNC-Related Pituitary Adenoma

The treatment of PA in CNC does not differ from sporadic cases. Selective adenomectomy is the preferred treatment for PA, but in CNC cases where multiple adenomas can occur, partial or complete hypophysectomy may be necessary to achieve biochemical remission (Lonser *et al.*, 2016).

Regular screening for the manifestations of the disease is recommended for patients with CNC and known carriers of *PRKAR1A* mutations. Regarding pituitary abnormalities, for postpubertal patients, annual serum IGF-1, GH, and PRL should be performed (Stratakis *et al.*, 2001; Correa *et al.*, 2015) with pituitary MRI and 3-h oral glucose tolerance test for GH alterations in suspected GH excess (Stratakis *et al.*, 2015).

Pheochromocytoma/Paraganglioma and Pituitary Adenoma

Overview

The coexistence of pheochromocytoma/paraganglioma is rare. In the recent years a common genetic basis has been described in a number of cases now termed 3PA syndrome or the "three P association" of pituitary adenoma with pheochromocytoma/paraganglioma (Denes *et al.*, 2015; Xekouki *et al.*, 2015).

Of the 74 cases reviewed (O'Toole *et al.*, 2015), 30% have mutations in predisposing pheochromocytoma/paraganglioma or PA genes, *SDHA*, *SDHB*, *SHDC*, *SDHD*, *SDHAF2*, and *MEN1*, with the most common mutation (15 patients) being in the *SDHx* gene (especially the *SDHB* mutation), and 31% are patients with a personal or family history that is suggestive of a hereditary case, but without confirmed pathogenic genetic mutation (O'Toole *et al.*, 2015).

The presence of *SDHx* mutations in PA is rare but is more likely if pheochromocytoma/paraganglioma are also present or if there is a positive family history of pheochromocytoma/paraganglioma (Papathomas *et al.*, 2014; Xekouki *et al.*, 2015).

Succinate dehydrogenase (SDH) is a multimeric enzyme bound to the inner mitochondrial membrane. SDH consists of two hydrophilic subunits, a flavoprotein (SDHA) and an iron–sulfur protein (SDHB), which form the catalytic core of the enzyme with its associated assembly factor (SDHAF2), and two hydrophobic subunits, SDHC and SDHD, which anchor the homotetramer to the membrane and serve as the ubiquinone-binding site (Oyedotun and Lemire, 2004). It plays a critical role by transferring electrons directly to the ubiquinone pool in the Kreb's cycle and the respiratory chain. Mutations in the genes encoding subunits A, B, C, and D are associated with the development of pheochromocytoma, paraganglioma, gastrointestinal stromal tumors, renal cancer, papillary thyroid cancer, neuroblastoma, adrenal medullary hyperplasia, testicular seminoma, and PA (Papathomas *et al.*, 2014; Xekouki *et al.*, 2015).

The loss of heterozygosity demonstrated at the *SDH* locus in PA and a reduction of the respective SDH protein expression in PA tissue support the causality between these genes and PA tumorigeneses (Xekouki and Stratakis, 2012; Papathomas *et al.*, 2014; Denes *et al.*, 2015; Xekouki *et al.*, 2015). Inactivation of SDH can lead to activation of the hypoxia-inducible factor (HIF-1α) pathway and a pseudohypoxic state. It was shown that an increased HIF-1α level in a *SDHD* mutation case was linked to PA (Xekouki and Stratakis, 2012).

In the differential there are also cases described of this association in *MEN1* positive-mutation patients, with the demonstration of LOH at the MEN1 locus together with absent menin staining in the pheochromocytoma samples (Denes *et al.*, 2015). There are a few cases of MEN2 and VHL syndrome and concomitant PA; however, there is insufficient evidence available at present to conclude whether they play a role in pituitary tumorigenesis (O'Toole *et al.*, 2015).

Pituitary Adenomas

Prolactinoma are the most common followed by somatotropinoma and NFPA. They usually present as macroadenomas with aggressive behavior and resistant to medical therapy and most often require surgery (O'Toole *et al.*, 2015; Syro *et al.*, 2016). A pituitary carcinoma has also been described in a patient with paraganglioma and *SDHB* mutation (Tufton *et al.*, 2017).

PA of these patients have an unusual appearance of extensive vacuolization of cytoplasm (Denes *et al.*, 2015). The age of onset of PA is comparable with that of sporadic tumors. The penetrance of PA in patients with *SDHx* mutation is low.

Who Should Be Tested?

SDHx mutations are very rare in unselected sporadic PA, and therefore genetic screening is not justified routinely in patients with PA, unless there is coexistence of and/or family history of pheochromocytoma/paraganglioma (Xekouki *et al.*, 2015).

Management of Pituitary Adenoma in *SDHx* Positive Mutation Carriers

In patients with *SDHx* positive mutation under MRI surveillance for pheochromocytoma/paraganglioma, pituitary MRI should be added to at least the first MRI screening of the neck and skull base (Caimari and Korbonits, 2016).

DICER1

Overview

DICER1 syndrome is a rare condition of multiple tumors arising from infancy with unknown prevalence caused by a heterozygous germline mutation in the *DICER1* gene, which is located on chromosome 14q32.13 and is comprised of 1922 amino acids and 27 exons (Foulkes *et al.*, 2011). DICER1 is an RNA processing endoribonuclease that cleaves precursor microRNAs (miRNA) into active miRNA, which in turn posttranscriptionally regulate cellular protein production (Foulkes *et al.*, 2011).

DICER1 germline mutations usually lead to truncated proteins, whereas the "second hit" somatic mutations are typically in the metal-binding sites of the catalytic RNAase IIIa and b domains, which are hotspot mutations (Rio Frio *et al.*, 2011). It is currently unclear how *DICER1* mutation leads to tumorigenesis in general or specifically to pituitary blastomas (Sahakitrungruang *et al.*, 2014; Caimari and Korbonits, 2016).

DICER1 syndrome is characterized by autosomal-dominant inheritance with variable clinical presentation and with a low penetrance (Foulkes *et al.*, 2011; de Kock *et al.*, 2014).

The main manifestations of the DICER1 syndrome include pleuropulmonary blastoma, rhabdomyosarcoma, nasal hamartoma, cystic nephroma, multinodular goiter, and Sertoli–Leydig cell tumors. Pituitary blastoma is a rare manifestation but a pathognomonic one (Foulkes *et al.*, 2011; Slade *et al.*, 2011; Sabbaghian *et al.*, 2012; de Kock *et al.*, 2014).

Pituitary Blastoma

The first description of pituitary blastomas was in 2008 in a 13-month-old child with Cushing's disease and diabetes insipidus (Scheithauer *et al.*, 2008; Scheithauer *et al.*, 2012).

In the histological composition it is possible to find Rathke epithelium cells, folliculostellate cells, and differentiated pituitary hormone secretory cells (Scheithauer *et al.*, 2008). Pituitary blastomas always express ACTH in at least a subset of cells and sometimes also express GH (de Kock *et al.*, 2014). Its ultrastructure resembles that of 10- to 12-week embryonic pituitary (Scheithauer *et al.*, 2008).

There are only a few cases described in the literature, but all confirmed cases are due to the DICER1 syndrome (Scheithauer *et al.*, 2012; de Kock *et al.*, 2014; Sahakitrungruang *et al.*, 2014). The penetrance appears to be below 1% (de Kock *et al.*, 2014).

Pituitary blastoma development occurs early in life, usually before the age of 2 years, and is associated with ACTH secretion and severe Cushing's diseases in the majority of the cases. They frequently extend to the suprasellar and parasellar regions, and ophthalmoplegia and strabismus are present in about 50% of cases (de Kock *et al.*, 2014). In an overview of all known cases, 61.5% were female, the age of pathological diagnosis ranged from 7 $\pm$ 24 months, and in 61.5% patients pituitary blastoma was the only manifestation at the time of the study (de Kock *et al.*, 2014). Mortality rate of pituitary blastomas is around 40%, and it could be due to mass effect or due to a severe Cushing syndrome (de Kock *et al.*, 2014).

Who Should Be Tested?

ACTH-dependent Cushing syndrome during infancy (under the age of 2 years) is very rare, so in its presence the clinician should consider pituitary blastomas and look for other *DICER1* mutation-related disease in the patient or relatives.

Management

Due to its rarity and its recent discovery, clinical experience is very limited. The prognosis is unfavorable, but recent studies showed better outcomes with the association of surgery, polychemotherapy, and adjuvant radiotherapy (de Kock *et al.*, 2014; Sahakitrungruang *et al.*, 2014).

Conclusion

Several advances have been made in the genetic aspects of PA in the last few years. The knowledge of particular aspects of each disease may facilitate the diagnosis, allowing for genetic screening. Genetic screening and clinical follow-up of patients with PA of genetic origin allow early identification, diagnosis, and treatment of its manifestations and consequently decrease its significant complications both in patients and in family members.

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Clinical Features of Acromegaly☆

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Glossary

Arthropathy Abnormality in the condition of a joint of the body; enlargement, swelling, degeneration, or other such disturbance in a joint.

Growth hormone (GH) A polypeptide hormone that is secreted mainly by the pituitary gland, although it is also produced by other cell types, such as lymphoid cells. Its actions are related mainly to growth (soft tissues, long bones, etc.) and to metabolism. It belongs to a family of hormones that includes prolactin and placental lactogens as well as other placental factors. Acromegaly results from GH hypersecretion.

Insulin-like growth factor type 1 (IGF-1) A hormone structurally related to insulin, exhibiting strong proliferative effects and facilitating differentiation of tissues., IGF-1 overproduction is associated with acromegaly.

Multiple endocrine neoplasia syndrome type 1 (MEN-1) A group of genetically distinct familial diseases in which two or more endocrine glands develop excess normal tissue (hyperplasia) and/or adenoma (tumor).

Sleep apnea A sleep disorder in which the subject has intermittent periods of a failure to automatically control respiration; these involuntary pauses in breathing may occur repeatedly during a given period of sleep.

Introduction

Clinical Characteristics of Acromegaly

Acromegaly is due to the hypersecretion of growth hormone (GH) and the resultant secondary overproduction of insulin-like growth factor-1 (IGF-1). In the vast majority of the cases (>99%), the source of excessive GH is a benign pituitary tumor of purely somatotroph or mixed somatotroph or lactotroph origin. Rarely, somatotroph tumors arise in an ectopic pituitary, a remnant of the primitive Rathke's pouch, and are found in the posterior pharynx, sphenoid bone, or sphenoid sinus or even within the sella, separate from the normal pituitary gland. Ectopic production of GH-releasing hormone (GHRH) leads to pituitary somatotroph hyperplasia. Carcinoids and islet cell tumors are the most frequent sources of ectopic GHRH. Hypothalamic/pituitary gangliocytomas or choristomas, or in one case the pituitary adenoma itself, were shown to be a source of excessive GHRH production. Ectopic GH secretion was documented in only very few cases: one by a malignant islet cell tumor and another by a non-Hodgkin's lymphoma. Acromegaly may be part of endocrine tumor syndromes, such as MEN-1 and MEN-4 (parathyroid, pituitary, pancreas), McCune–Albright syndrome and Carney complex. In some instances, the clinical syndrome of acromegaly may be overshadowed by other manifestations of a malignant or polyglandular diseases.

Clinical manifestations of acromegaly correlate better with the prevailing levels of IGF-1 than with GH, and the duration of GH/IGF-1 excess may play a major role. A clinical and biochemical syndrome of acromegaly may be transiently expressed during normal puberty or pregnancy. This is due to physiological overproduction of GH by the normal pituitary gland during sexual maturation or by the placental synthesis of placental GH, a variant of pituitary GH.

In most cases, acromegaly is an insidious disease, and its early clinical manifestations usually go unnoticed by the patient, the patient's family, and/or the patient's physician. Retrospective questionnaires and the inspection of old photographs usually set the clinical onset of disease at 5-10 years prior to the diagnosis.

Clinical presentation of acromegaly consists of the mass effects of the tumor itself, the manifestations of the abnormal growth affecting virtually all organs and tissues, and the metabolic derangements effected by GH itself.

Mass Effects of the Tumor

The majority of GH-secreting adenomas (60–80%) are ≥10 mm at the time of diagnosis (macroadenoma) and are often invasive towards the sphenoid sinus, cavernous sinus or 3rd ventricle (Potorac *et al.*, 2015). Headache is present in over half of the patients and can be severe enogh to significantly interfere with everyday activities. Headache may be present even in patients with relatively small tumors, where mass effect is unlikely to provide an explanation. It has been suggested that headache is typical in patients with previous predisposition or family history of headaches. The headache can be the result of a combination of factors, including the hormonal activity of the tumour, relationship to the cavernous sinus and patient predisposition to headache (Levy *et al.*, 2005). Hypopituitarism

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(ACTH, TSH and LH/FSH deficiency leading to hypogonadism) can also occur due to the mechanical effects of the pituitary adenoma. Symptomatic pituitary apoplexy occurs in less than 5% of patients, often presenting as sudden onset of severe headache with or without ophthalmoplegia (III, IV, VI, and V_1, V_2 nerves), but it is significantly more common in *AIP*-mutation positive cases, especially in children (Hernández-Ramírez *et al.*, 2015). Asymptomatic pituitary hemorrhage may occur in 30-40% of the cases. Photophobia occurs in about half of cases and is most troublesome in bright sunlight and during night driving.

Abnormal Growth

If abnormal GH hypersecretion occurs before puberty, rapid linear growth may lead to gigantism. Combination of high GH levels with hypogonadism due to mass effect of the tumor damaging LH/FSH levels or due to prolactin secretion inhibiting LH/FSH via inhibition of kisspeptin and GnRH, delays epiphyseal closure and continued growth. Gigantism is a defined as pituitary adenoma with (i) abnormally high growth velocity in children or teenagers with abnormal IGF-1 and OGTT, (ii) height >3SD above the mean height for age, (iii) >2SD over the calculated midparental height, using country-specific growth charts. Height increase is often seen if disease starts later in puberty, and height increase might not reach the threshold of gigantism. Pre-pubertal onset of disease with delayed puberty leads to disproportionate growth (arm span/body height is $>1+5$ cm, sometimes referred as eunuchoidal-infantile gigantism or daddy-longlegs syndrome. The typical dysmorphic features of acromegaly usually occurs in postpubertal disease, or can develop later in patients with prepubertal disease, although the very high GH levels recorded in XLAG syndrome can produce these features in young children as well (Beckers *et al.*, 2015).

Face

The combination of bone and soft tissue overgrowth leads to a typical acromegalic face: widened and thickened nose, thick lips, macroglossia, coarsening of the features and facial and infraorbital puffiness, thickened oily skin sometimes with acne, exaggerated nasolabial and forehead skin furrows, maxillary widening, mandibular overgrowth and prognathism. The face and skull changes also include thickening of the calvarium, increased size of the frontal sinuses, which leads to prominence of the supraorbital ridges and frontal bossing. Patients may seek care for dental problems (teeth separation, malocclusion), and the diagnosis is often raised by a doctor, such as a locum doctor, who has never seen the patient before.

Patients may have asymmetrical faces, especially in cases due to McCune–Albright syndrome. Exophthalmos may be noted; however, it might be masked by frontal bossing. Hypertrophic tissue surrounding the canal of Schlemm may impede aqueous filtration, leading to open-angle glaucoma (Melmed *et al.*, 2011). Photographs show a slow, insidious transformation of the facial features over several years. These features are seen in 98-100% of patients (see Fig. 2).

Although the clinical diagnosis of acromegaly can be easy, in case the physician is considering this disease, the typical features are often under-recognized leading to long delay in diagnosis (4–10 years). An attempt has been made to recognize acromegalic face by a computer program using a model that establishes a three-dimensional model from a regular 2D photograph (Fig. 1). Despite having several limitations, the study of Schneider *et al.* suggest that acromegaly can be detected by computer software using photographs of the face. The accuracy of acromegaly diagnosis by software was higher than by medical experts or general internists, particularly in patients with mild features of acromegaly, making this software a promising tool to help detecting acromegaly (Schneider *et al.*, 2011).

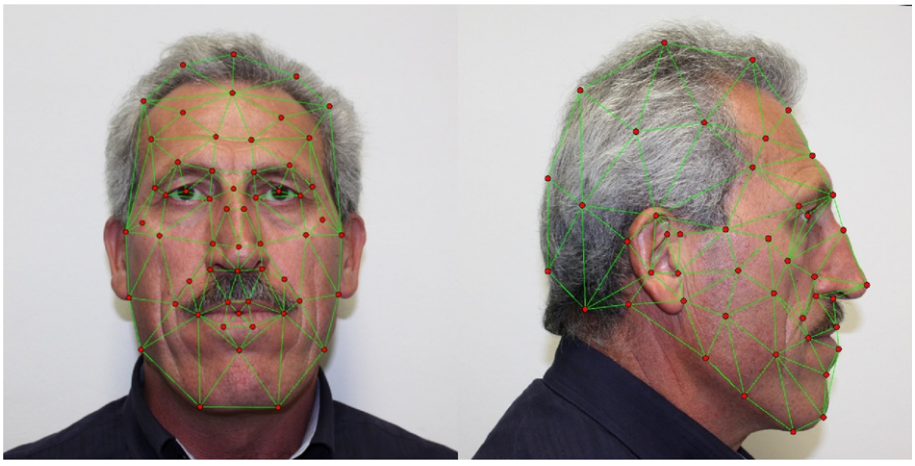


Fig. 1 Computer software recognizing acromegaly based on detecting of texture of the skin under the marked nodes and on the geometry of the face by analyzing edge length and distances between the nodes depicted on this example (courtesy of Dr Schneider following from Schneider *et al.* JCEM 2011)

Extremities

Hands and feet become very "fleshy" (Fig. 2). An increase in finger circumference and a widening of the hands and feet develop in 98-100% of patients (Fig. 3). Patients routinely recall repeated resizing of rings and changes in shoe size (mostly widening). Patients with acromegaly often have a characteristic handshake: a large, soft, slight sweaty doughy hand often makes the diagnosis relatively easy. Diagnosis of acromegaly is often made by orthopedic, rheumatologic or neurologist colleagues (Table 1) due to joint abnormalities and pain.

Skin and appendages

Skin is characteristically thickened because of excessive deposition of the glycosoaminglycans, hyaluronic acid, chondroitin sulfate, and dermatan sulfate in the papillary and upper reticular dermis. These compounds are very hydrophilic, causing the development of a non-pitting edema and consequently leading to the appearance of facial wrinkles and prominent nasolabial folds. The heel pads are thickened, this sign was used in the past form diagnosing the condition. Body hair may become coarsened (Ben-Shlomo and Melmed, 2006). Skin thickening at the vertex causes a peculiar appearance of cutis verticis gyrata (skin folds at the top of the head). Similar skinfolds can be seen in patients pachydermaperiostosis or elevated IGF-II levels. Hair growth is increased, and women complain of hirsutism or hypertrichosis. As opposed to the androgen-related hirsutism, this is pronounced even on the forearms and forelegs. Many women with acromegaly have exceedingly thick scalp hair growth. Hair loss after successful therapy is often a cause of concern but is essentially a physiological return to normal hair growth (Barkan, 2004).

The functional capacity of sweat and sebaceous glands is increased, resulting in excessive perspiration, often with offensive odor, in acne and sebaceous cysts, hyperhidrosis and malodorous oily skin occurring in up to 70% of patients. Skin tags are frequently present, particularly on the neck and may be markers for the adenomatous colonic polyps (Leavitt *et al.*, 1983). Acanthosis nigricans of the axillae and neck is common as well. Raynaud's phenomenon is reported in up to one third of patients.

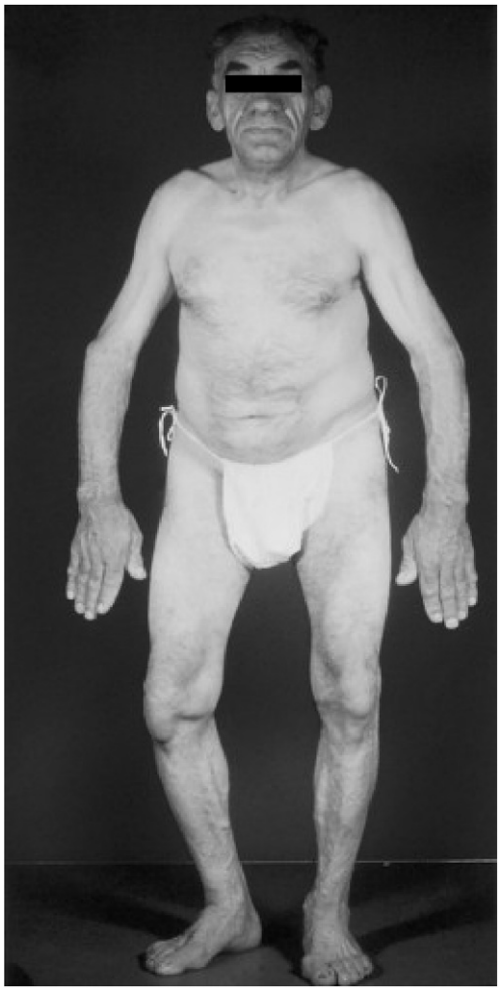


Fig. 2 Appearance of a patient with florid acromegaly. Courtesy of Professor Stefan S. Fajans.

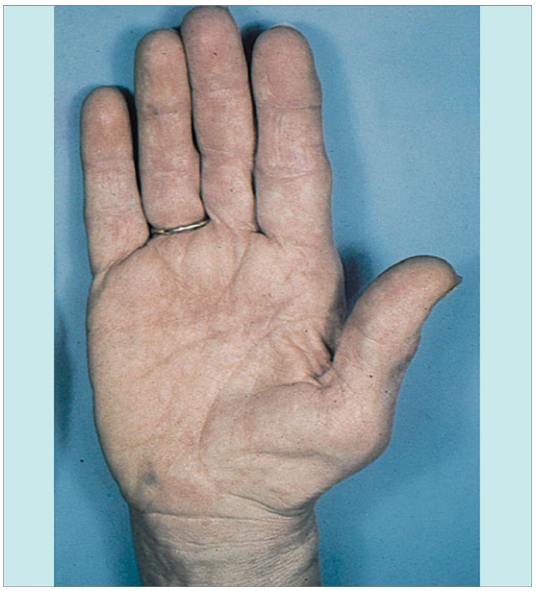


Fig. 3 Hand of a patent with acromegaly showing thickened sausage like fingers (representing a pathognomonic feature of acromegaly with a diagnostic odds ratio of 131, according to a recent study (Prencipe *et al.*, 2016)) and atrophy of the thenar muscles due to carpal tunnel syndrome (Photo from Comprehensive Clinical Endocrinology, Besser and Thorner, Elsevier).

Neuromuscular

The muscle mass is increased, but this is primarily due to increased intracellular water, so that muscle strength is either normal or low. In fact, many patients have clinically obvious proximal myopathy. Muscle biopsy often shows hypertrophy of type I and/or atrophy of type II fibers. Proximal myopathy may also be accompanied by myalgias, cramps, and nonspecific myopathic changes on electromyography.

Neural enlargement and wrist tissue swelling may lead to carpal tunnel syndrome in up to half of all patients, causing acroparesthesias. Patients may have thenar atrophy (Fig. 3) due the median nerve paresis. Compression neuropathies are common (30–50%), the median nerve being most often affected. Median and ulnar nerve cross-sectional areas increase, and nerve conduction is abnormal (Tagliafico *et al.*, 2008). Occasionally, distal symmetric polyneuropathy may be present. Peripheral acroparesthesias and symmetric peripheral neuropathy should be distinguished from diabetic neuropathy, which may occur secondarily to acromegaly (Jenkins *et al.*, 2000).

Oral cavity and larynx

Prognathism and widening of the interdental spaces are typical signs of acromegaly. The size of the tongue is usually enlarged and contributes to the obstruction of the pharynx, with the resultant sleep apnea and impaired mastication. The abnormal oral anatomy often results in speech disturbances. The diameter of the trachea is increased, and the vocal cords are thickened. Together with grossly enlarged sinus cavities, this results in a low and hollow voice. Salivary glands are typically enlarged.

Joints and spine

Acromegalic arthropathy can affect up to 84% of the cases (depending on the affected joint), especially older patients or females. Both axial and appendicular skeleton may be affected. Carpal tunnel syndrome occurs often bilaterally. Musculoskeletal pain is extremely common in patients with acromegaly and adversely impacts the quality of life (Miller *et al.*, 2008).

GH receptors are present on all major cell types comprising the skeletal system: fibroblasts, chondrocytes, and osteoblasts. These cells readily produce IGF-1 and are targets for both endocrine and autocrine IGF-1 effects (Barkan, 2004). Knees, hips, shoulders, lumbosacral joints, elbows, and ankles are affected as monoarticular or polyarticular arthritis, but joint effusions rarely develop. The degree and severity of arthropathy best correlate with the duration of disease. However, it is unclear whether higher GH levels correlate with increased articular disease activity. Joint pain and low back pain may be experienced soon after the clinical onset of acromegaly but are often reversible with successful therapy. However, clinical duration of acromegaly in excess of 10 years is often associated with clinical and radiological joint deformities that are only minimally affected by GH-lowering therapy. There is a high prevalence of self-reported joint complaints which persist despite successful long-term treatment of acromegaly (Biermasz *et al.*, 2005). These joint problems significantly contribute to the impaired quality of life of patients with acromegaly (Biermasz *et al.*, 2004). Therapeutic responses usually depend on the degree of irreversible bony changes already in place.

The most frequently affected appendicular joint is the knee, followed by the shoulder, hip, ankle, elbow, and small hand joints. Radiological changes are usually seen even in clinically unaffected joints. Initially, GH and IGF-1 excesses are responsible for replication of articular chondrocytes and increased matrix synthesis, growth of periarticular structures and synovial hypertrophy.

Table 1 Reasons for visits to physicians before diagnosis of acromegaly (Ilonka Kreitschmann-Andermahr *et al.*, 2014)

General practitioner (67.5%)
Headache (10.9%)
Changes of hands and feet (7.3%)
Lack of energy (6.7%)
Hypertension (5.5%)
General unwellness (5.5%)
Orthopedist (35.6%)
Troubles with hands and feet (8.5%)
Back pain (7.3%)
Joint pain (7.3%)
Carpal tunnel syndrome (6.7%)
Headache (3%)
Neurologist (27.0%)
Headache (7.9%)
Carpal tunnel syndrome (7.9%)
Depression (1.8%)
Numbness/Tingling (1.8%)
Depression (1.2%)
Endocrinologist (26.4%)
Suspected acromegaly (5.5%)
Hormone investigation (2.4%)
Diabetes (2.4%)
Thyroid disease (2.4%)
Other diagnostics (1.8%)
Internist (23.3%)
Heart disease (4.8%)
Hypertension (3%)
Sleep apnea (2.4%)
Thyroid disease (1.8%)
Lack of energy (1.8%)
Ophtalmologist (22.7%)
Disturbance of vision (10.9%)
Defective vision (10.9%)
Check-up (1.8%)
Headache (1.8%)
Photosensitivity (1.8%)
Dentist (20.9%)
Jaw pain (3%)
Change of bite occlusion (3%)
Enlargement of interdental spaces (3%)
Damaged teeth (3%)
Malposition of teeth (1.8%)
Gynecologist (20.2%)
Menstrual disturbances (10.3%)
Abnormal hair growth (3%)
Menopause (2.4%)
Lactation (1.8%)
Weight gain (1.2%)
Cardiologist (14.7%)
Heart disease (8.5%)
Hypertension (4.8%)
Dermatologist (8.0%)
Abnormal hair growth (2.4%)
Skin changes (2.4%)
Acne (1.2%)
Warts (1.2%)
Neurosurgeon (6.1%)
Carpal tunnel syndrome (2.4%)
Brain tumor (1.8%)
Psychiatrist (4.3%)
Depression (3%)
Anxiety (1.2%)

As a result, there is thickening of the cartilage, widening of the joint space, alteration of normal joint geometry and hypermobility, leading to abnormal mechanical loading of the joint. At this earlier phase of arthropathy the process could be reversible by controlling GH and IGF-1 hypersecretion. With disease progression, cartilage surface fissures develop and progressively enlarge with regenerative fibrous cartilage proliferating disproportionately, more than in osteoarthritis not associated with GH excess, presumably as a result of GH stimulation. The regenerative fibrocartilage frequently becomes calcified, resulting in osteophyte formation. In advanced cases, fissures extend to the subchondral bone, widen, and produce ulceration of the joint cartilage. The underlying bone shows an accelerated turnover, eburnation, and subchondral cyst formation. Finally, the articular cartilage becomes thinned with narrowing of the joint space, a process that shares many features with osteoarthritis. At this phase, arthropathy becomes irreversible and biochemical control of acromegaly, as documented by a normal IGF-1, will have a little effect improving the clinical status (Barkan, 2004; Killinger *et al.*, 2012).

The deformations can also affect the spine resulting in dorsal kyphosis with deformation of rib cage, leading to the classical "punchinello" aspect, especially when GH hypersecretion begins prior to closure of the epiphyses. Lumbar involvement is the most common spine abnormality, followed by thoracic and cervical arthropathy. Overall, approximately 50% of patients complain of back pain and limitation of movements. In early phase of the disease widened intervertebral spaces and vertebral enlargement may be present on spine X-ray (Killinger *et al.*, 2012). Thickened intervertebral discs and lax paraspinal ligaments contribute to abnormal joint mobility. End-stage arthropathy is characterized by the narrowing of the intervertebral space. Ossification of the anterior aspect of the vertebral bodies with exuberant osteophyte formation often bridges the disc space, mimicking diffuse idiopathic skeletal hyperostosis (DISH) (Barkan, 2004).

Bone metabolism

GH has anabolic bone effects so bone mass is usually increased compared to normal subjects (Kaji *et al.*, 2001), unless untreated hypogonadism is also present (Capatina and Wass, 2015). Biochemical markers of bone remodeling are increased in patients with acromegaly. However, histomorphometric data are conflicting; cortical bone shows predominance of bone formation over resorption, whereas trabecular bone has the opposite pattern. As many patients with acromegaly have concomitant hypogonadism, a combination of acromegaly and hypogonadism induced bone changes may be identified.

Bone density studies are equally controversial, but overall it appears that cortical bone mineral density may be normal or even increased, whereas trabecular bone mineral density (BMD) is normal or decreased. Bone density measurements could be influenced by interference of osteophytes. However, a recent meta-analysis of the literature revealed a high risk of vertebral fractures in active acromegaly even in patients with normal BMD regardless of gender suggesting a possible low quality of the bone despite a high bone mass (Mazziotti *et al.*, 2015).

Cardiovascular

Symptomatic cardiac disease is present in about 20% of patients with acromegaly and is a major cause of morbidity and mortality (Colao *et al.*, 1999a). Moreover, increased mortality of untreated or poorly treated acromegaly is almost completely attributable to cardiovascular disease. Early stages of acromegaly, typical of young patients with short disease duration, are characterized by tachycardia and increased systolic output (hyperkinetic syndrome). Arterial hypertension is among the most frequent complications in subjects with acromegaly, present in 36–40% of patients (Mestron *et al.*, 2004; Reid *et al.*, 2010). Left ventricular hypertrophy is observed in half of patients with hypertension and also in about half of normotensive patients with acromegaly. Therefore, the suspicion of acromegaly is sometimes raised by cardiologists.

Valvulopathies and arrhythmias are also more prevalent in these populations (Colao *et al.*, 2004; Pereira *et al.*, 2004) and in approximately 20% of patients the development of hemodynamic abnormalities is augmented by valvular involvement. Rhythm abnormalities, occurring in 40% of patients, are more ominous. Ectopic beats, paroxysmal atrial fibrillation, paroxysmal supraventricular tachycardia, sick sinus syndrome, ventricular tachycardia, and bundle branch blocks all are seen with increased frequencies in patients with acromegaly and are exacerbated by physical exercise. In one study, almost a half of a small cohort of acromegaly patients presented complex ventricular arrhythmias (Lown III–IV), especially those with a long disease duration (Kahaly *et al.*, 1992). A multicentric study performed in Italy using 24-h ECG-Holter monitoring reported a lower but still very significant rate of ventricular extrasystoles (in 33% of the cases with acromegaly evaluated) (Lombardi *et al.*, 2002). 24-h ECG-Holter monitoring might be useful in the preoperative evaluation of the patients, especially those with clinically detected abnormalities (Fedrizzi and Czepielewski, 2008).

If untreated, the presence of these significant morphological and functional changes leads to a complex cardiomyopathy characterized by concentric hypertrophy (predominantly affecting the left ventricle, found hypertrophic in 60% of cases) (Colao *et al.*, 2004), diastolic dysfunction and progressive systolic impairment eventually leading to heart failure (Mosca *et al.*, 2013). Cardiac enlargement may be secondary to hypertension, atherosclerotic disease, or, rarely, to acromegalic cardiomyopathy. Other cardiovascular risk factors such as elevated triglycerides, lipoprotein(a), fibrinogen, plasminogen activator inhibitor, and small dense LDL particles are also often present in acromegaly (Colao *et al.*, 2004). Although the prevalence of coronary artery disease is not clearly increased in these patients and most subjects belong to the low-risk category if assessed with the Framingham score (Bogazzi *et al.*, 2007), aggressive management of all cardiovascular risk factors is indicated. The aim is to reduce the cardiovascular morbidity and mortality (Katznelson *et al.*, 2014). Normalization of GH and IGF-1 can reduce the degree of left ventricular hypertrophy within 2-4 weeks. Disturbingly, biochemical control of acromegaly might not improve conduction abnormalities.

Late ventricular potentials (low-amplitude, high-frequency waves in the terminal phase of QRS complexes) are strong predictors of future arrhythmic events and are seen in 50% of patients with active acromegaly.

Plasma renin levels are suppressed and endogenous plasma digitalis-like activity with chronic volume expansion has been identified in acromegaly (Deray *et al.*, 1987). Renal sodium channel activity is induced by GH at the aldosterone-sensitive distal nephron (Kamenicky *et al.*, 2008).

The presence of cardiovascular disease at the time of diagnosis portends a high risk of mortality despite improved cardiac function after effective GH and IGF-1 control.

Sleep apnea

Sleep apnea has been reported at varying frequencies from 13% to over 50-75% of cases (Capatina and Wass, 2015; van Haute *et al.*, 2008), more frequently in diabetic or hypertensive patients. Most patients have obstructive sleep apnea (mediated by the GH-related soft tissue growth around the upper airways), and a minority of cases have either pure central apnea or mixed apnea (Roemmler *et al.*, 2012). More specifically, in approximately one-third of patients, there is a central component of sleep apnea associated with higher GH and IGF-1 levels.

Prognathism, thick lips, macroglossia, and hypertrophied nasal structures may obstruct airways. Irregular laryngeal mucosa, cartilage hypertrophy, tracheal calcification, and cricoarytenoid joint arthropathy lead to unilateral or bilateral vocal cord fixation or laryngeal stenosis with voice changes. Tracheal intubation may be particularly difficult in patients undergoing anesthesia, and tracheostomy may be required. A suggested predictor of difficult direct laryngoscopy and endotracheal intubation is Mallampati score (Table 2), a high Mallampati score (class 3 or 4) being associated with more difficult intubation as well as a higher incidence of obstructive sleep apnea. Obstructive sleep apnea, characterized by excessive daytime sleepiness, fatigue, habitual excessive snoring and headache with at least five episodes of apnea per hour of sleep, causes daytime somnolence, especially in men with acromegaly who also have a ventilation-perfusion defect with hypoxemia (Melmed *et al.*, 2011). There are several methods for measuring sleepiness, among which the Epworth sleepiness scale is used widely to assess the risk of sleep apnea. The higher the score, the higher the person's level of daytime sleepiness. Sleep apnea is a strong predictor of future cardiovascular events, hypertension, or stroke. Many patients are unaware of their snoring; family members provide markedly more reliable information. However, both subtypes of apnea may be ameliorated by GH-lowering therapy.

Renal

Kidney size and glomerular filtration rate are characteristically increased in acromegaly. GH enhances glomerular filtration through an IGF-1-mediated decrease in renal vascular resistance, leading in turn to increased glomerular perfusion. GH, in concert with IGF-1, stimulates epithelial sodium channel (ENaC)-mediated sodium transport in the late distal nephron, accounting for the pathogenesis of sodium retention when GH/IGF-1 is secreted in excess. Patients with acromegaly frequently have phosphate and calcium abnormalities. Mild hyperphosphatemia due to both increased calcitriol-stimulated dietary phosphate absorption and to a direct anti-phosphaturic action of IGF-1 in the proximal tubule is often seen. There is also a tendency toward increased plasma calcium levels, and hypercalciuria. In these cases PTH should be measured and the diagnosis of MEN1 should be ruled out.

Hypercalciuria may lead to kidney stone formation in approximately 10% of patients.

The hypercalciuria observed in patients with acromegaly is classically related to increased calcitriol-driven intestinal calcium absorption via the intestinal epithelial calcium channel TRPV6. In addition to this absorptive mechanism, increased bone turnover may also participate in hypercalcemia. IGF-1 induced activation of renal epithelial calcium channel TRPV5 results in increased distal calcium reabsorption. Disturbances in calcium and phosphate handling in acromegaly may well contribute to the increased spinal skeletal fragility in acromegaly (Kamenicky *et al.*, 2014). In patients with kidney stones and hyperparathyroidism Multiple Endocrine Neoplasia syndrome type 1 should be considered.

Gastrointestinal

Liver and spleen sizes are normal. Hepatomegaly in patients with acromegaly always should be assumed to result from another disease process and should be investigated. Similarly, the incidence of cholelithiasis is not increased relative to that in the general population. Patients with acromegaly often suffer from constipation due to long and tortuous colon and many years of strain could lead to rectal prolapse. The incidence of colonic polyps appears to be increased (see below).

Metabolic

Acromegaly is frequently associated with impaired glucose tolerance (IGT) and type 2 diabetes mellitus because the GH excess is associated with insulin resistance (in the liver and in periphery), hyperinsulinemia, increased gluconeogenesis, and decreased

Table 2 Mallampati score

Class 0: Ability to see any part of the epiglottis upon mouth opening and tongue protrusion
Class I: full visibility of tonsils, uvula and soft palate
Class II: Soft palate, upper portion of tonsils and uvula visible
Class III: Soft palate and hard palate, base of uvula visible
Class IV: Soft palate not visible at all

peripheral glucose uptake. Diabetes prevalence has been variably reported (reflecting the significant heterogeneity with respect to ethnicity, disease status, age, etc.), but the highest prevalence rates were 40–52% (Biering *et al.*, 2000; Kasayama *et al.*, 2000; Fieffe *et al.*, 2011); similarly, IGT can be found in up to 28–46% of cases The GH-lowering effect of some of the treatment methods used in acromegaly (surgery, pegvisomant) improves the glucose tolerance, while somatostatin analogs may have milder (SSTR2 agonists) or stronger (pasireotide) deleterious effect (Mazziotti *et al.*, 2009). Hyperglycemia was noted in 33 or 31% of patients treated with 40 or 60 mg pasireotide and 14% subjects with SSTR2 agonist, while diabetes was found in 21, 26 and 8%, respectively (Gadelha *et al.*, 2014).

Moderate weight gain may be noted in patients with acromegaly. Other metabolic abnormalities in acromegaly include hypertriglyceridemia, hypercalciuria, and hyperphosphatemia. Hypercalcemia is not a feature of acromegaly per se, and its presence should suggest another pathological process, such as MEN1 syndrome.

Neuropsychiatric

The patients often complain of lethargy and fatigue. Additionally, similar to any chronic disease associated with physical discomfort and lifelong therapy, acromegaly is associated with decreased quality of life. Progressive facial and bodily disfigurement often leads to lowered self-esteem. Depression, apathy and mood swings may occur secondarily to physical deformity (Furman and Ezzat, 1998) or chronic pain from headache or joints.

Neoplasia

Acromegaly has been associated with an increased risk of certain tumors, presumably due to the stimulatory effect of the IGF-1 on tumorigenesis. However, it is challenging to study the incidence of cancer in a population of people with a rare disease.

The best-documented data are those regarding colorectal neoplasia. However, the precise mechanism for the increased risk of colon tumors is not known. Potential mechanisms include direct GH or IGF1 actions on the colonic epithelial cells, altered bile acid secretion pattern, impaired immune response mechanisms in the colon mucosa, and increased colonic length (Renehan *et al.*, 2003). The pooled odds ratio (OR) is increased for both benign tumors (2.48 for adenomas and 3.557 for hyperplastic polyps) and colon cancer (2.04–4.351) (Renehan *et al.*, 2003; Rokkas *et al.*, 2008). The mortality rate for colon cancer is also higher in active acromegaly compared to the general population. The timing of the baseline colonoscopy is still debated. Some only consider it at 50–55 years (Renehan *et al.*, 2001), but even younger patients harbor colonic tumors, so initial colonoscopy at acromegaly diagnosis has been suggested (Terzolo *et al.*, 2005). Repeated colonoscopy every 10 years in controlled acromegaly with a normal initial result and every 5 years in those with benign tumors or uncontrolled disease appears reasonable (Dworakowska *et al.*, 2010).

The risk of thyroid nodular disease and thyroid cancer is also raised in acromegaly, with an OR of 6.9 and 7.5, respectively, and a prevalence of 59 and 4.3% respectively (Wolinski *et al.*, 2014). Whether this reflects a specific effect of the GH/IGF-1 excess or the increased worldwide availability and use of precise diagnostic techniques is controversial. Only a circumstantial relationship between acromegaly and prostate or breast cancer has been described to date. Until large epidemiological studies to clarify this relationship become available, it seems prudent to offer prostate cancer surveillance to older uncontrolled male patients and routine breast cancer prevention in females (Webb *et al.*, 2002).

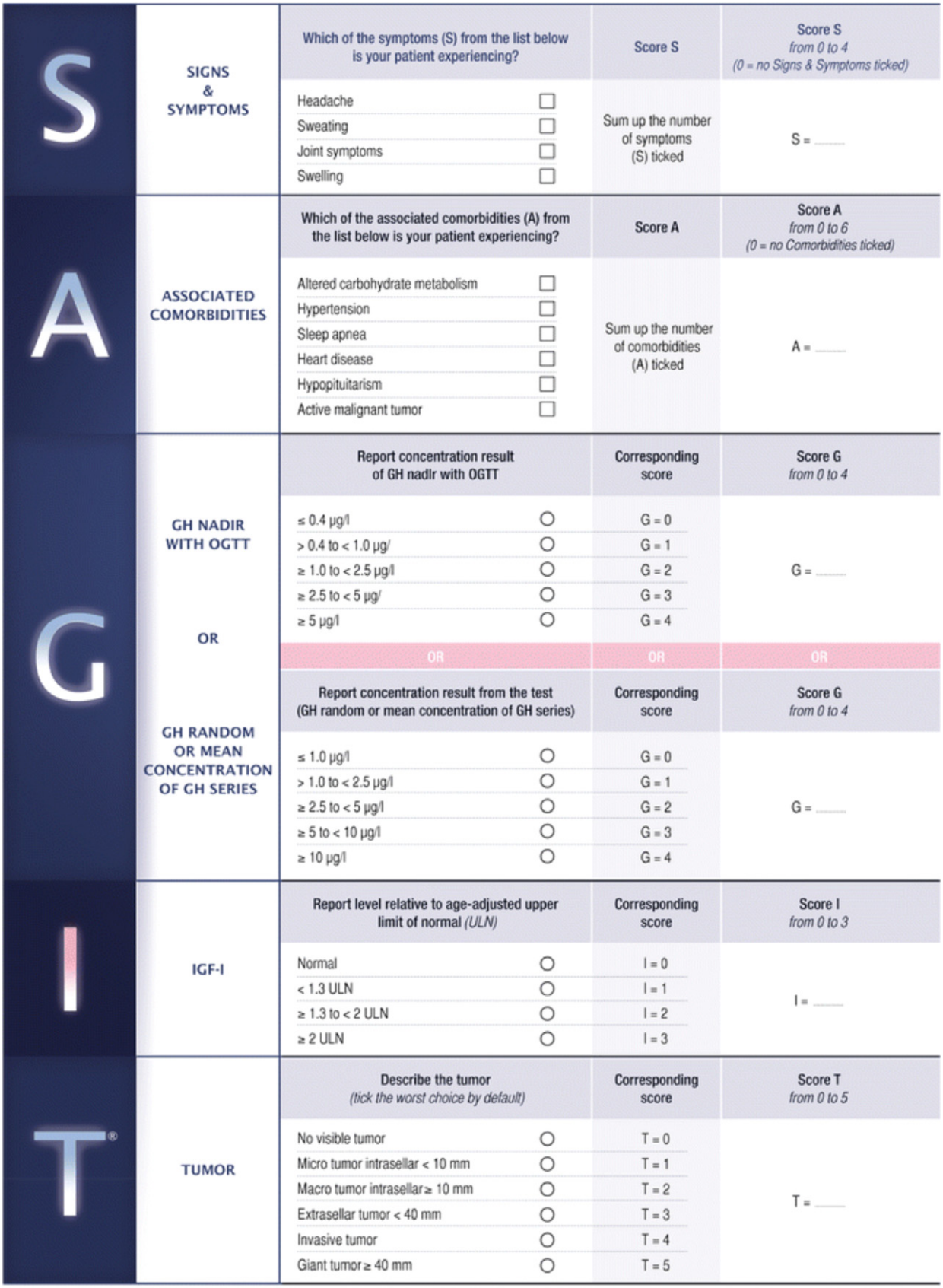
The analysis of the German Acromegaly Registry did not find higher cancer incidence compared to the general population overall, nor did it suggest higher incidence specifically for colorectal, breast, thyroid, or prostate cancer. There was no correlation between GH levels and cancer (Petroff *et al.*, 2015).

Endocrine complications

About 30% of patients with acromegaly present elevated serum PRL levels (up to 100 $\mu g\,L^{-1}$ or more), with or without galactorrhea (Barkan *et al.*, 1989). Functional pituitary stalk compression by a pituitary mass prevents access of hypothalamic dopamine to the lactotroph, releasing the cell from tonic hypothalamic inhibition. Moreover, a mixed GH-cell and PRL-cell adenomas secrete both GH and PRL. Additionally, the tumor may cause galactorrhea in the face of normal PRL levels because of the agonist effect of

Table 3 ACROSCORE parameters

Sex
Headache
Tumor size (macro/micro)
Type 2/secondary diabetes
Hyperhidrosis
Thyroid hyperplasia
Colorectal polyps
Spaced teeth
Carpal tunnel syndrome
Fingers widening



S	SIGNS & SYMPTOMS	Which of the symptoms (S) from the list below is your patient experiencing?	Score S	Score S *from 0 to 4* *(0 = no Signs & Symptoms ticked)*
		Headache ☐ Sweating ☐ Joint symptoms ☐ Swelling ☐	Sum up the number of symptoms (S) ticked	S = ____
A	ASSOCIATED COMORBIDITIES	Which of the associated comorbidities (A) from the list below is your patient experiencing?	Score A	Score A *from 0 to 6* *(0 = no Comorbidities ticked)*
		Altered carbohydrate metabolism ☐ Hypertension ☐ Sleep apnea ☐ Heart disease ☐ Hypopituitarism ☐ Active malignant tumor ☐	Sum up the number of comorbidities (A) ticked	A = ____
G	GH NADIR WITH OGTT	Report concentration result of GH nadir with OGTT	Corresponding score	Score G *from 0 to 4*
		≤ 0.4 µg/l ○ > 0.4 to < 1.0 µg/ ○ ≥ 1.0 to < 2.5 µg/l ○ ≥ 2.5 to < 5 µg/ ○ ≥ 5 µg/l ○	G = 0 G = 1 G = 2 G = 3 G = 4	G = ____
	OR	OR	OR	OR
	GH RANDOM OR MEAN CONCENTRATION OF GH SERIES	Report concentration result from the test (GH random or mean concentration of GH series)	Corresponding score	Score G *from 0 to 4*
		≤ 1.0 µg/l ○ > 1.0 to < 2.5 µg/l ○ ≥ 2.5 to < 5 µg/l ○ ≥ 5 to < 10 µg/l ○ ≥ 10 µg/l ○	G = 0 G = 1 G = 2 G = 3 G = 4	G = ____
I	IGF-I	Report level relative to age-adjusted upper limit of normal *(ULN)*	Corresponding score	Score I *from 0 to 3*
		Normal ○ < 1.3 ULN ○ ≥ 1.3 to < 2 ULN ○ ≥ 2 ULN ○	I = 0 I = 1 I = 2 I = 3	I = ____
T®	TUMOR	Describe the tumor *(tick the worst choice by default)*	Corresponding score	Score T *from 0 to 5*
		No visible tumor ○ Micro tumor intrasellar < 10 mm ○ Macro tumor intrasellar ≥ 10 mm ○ Extrasellar tumor < 40 mm ○ Invasive tumor ○ Giant tumor ≥ 40 mm ○	T = 0 T = 1 T = 2 T = 3 T = 4 T = 5	T = ____

Fig. 4 SAGIT scoring system to assess disease activity in patients with acromegaly (Giustina *et al.*, 2016).

GH for breast PRL-binding sites. Tumor mass compressing surrounding normal pituitary tissue may also cause hypopituitarism. More than half of all patients have menstrual abnormality or amenorrhea, decreased libido or impotence and secondary thyroid or adrenal failure is present in about 20% of patients (Melmed *et al.*, 2011). Hypogonadism occurs in 60% of female and 46% of male patients and is of multifactorial origin; tumor growth and compression may impair pituitary gonadotropin secretion, and associated hyperprolactinemia or the PRL-like effect of excessive GH secretion may impair gonadotropin and gonadal function. Gynecomastia occurs in about 10% of men. Thyroid dysfunction in acromegaly may be caused by diffuse or nodular, toxic or nontoxic goiter or by Graves' disease, especially because IGF-1 is a major determinant of thyroid cell growth (Kasagi *et al.*, 1999). Associated MEN1 features may be present in affected individuals, including hypercalcemia with hyperparathyroidism or pancreatic tumors. Benign prostatic hypertrophy has been documented in acromegaly with no apparent increase in prostate cancer rates (Melmed *et al.*, 2011; Colao *et al.*, 1999b).

Visceromegaly

Enlarged tongue, bones, salivary glands, thyroid, bowels, heart are the effects of generalized visceromegaly.

Facilitating optimal acromegaly diagnosis and management

The delay in acromegaly diagnosis is a fundamental issue: the longer the delay, the higher the risk of progression, tumor invading the cavernous sinus and therefore being inoperable, the risk of comorbidities and the mortality rate. Great efforts have been made to reduce the time between first symptoms and diagnosis. Currently, it is a commonly accepted opinion that the best strategy to shorten the time to acromegaly diagnosis is to build awareness amongst general practitioners and the specialists who generally take care of the comorbidities associated with the disease (cardiologists, orthopedic physicians, dental practitioners and ophthalmologists, etc., Table 1) so they consider acromegaly as a diagnosis. As part of this strategy, a scoring system (ACROSCORE), focused on the cardinal symptoms and signs of acromegaly (Table 3), has been developed for physicians (Prencipe *et al.*, 2016). Each symptom/sign, if present, is given a rating, and the sum of these ratings determines the final score. ACROSCORE classifies the patient as having a low, medium or high risk of disease suspicion and might become a new tool for GPs and non-endocrinology specialists in the clinical screening for acromegaly, if further validation in a general population suggests its usefulness.

Furthermore, aiming to provide a reference instrument for acromegaly staging in clinical practice, a score system has been developed, SAGIT (Giustina *et al.*, 2016) (Fig. 4). This acronym represents signs and symptoms (S), associated comorbidities (A), GH levels (G), IGF-1 levels (I), and the Tumor profile (T). The higher the number, the more severe the disease. The SAGIT instrument is intended to be used as an instrument offering a standardized classification, with potential for distinguishing clinical stages of acromegaly, assessing treatment response and aid therapeutic decision-making. Future studies are needed to assess its usefulness.

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Diagnosis of Acromegaly

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Clinical Diagnosis

The major challenge in acromegaly is early diagnosis of a disease that develops insidiously. Once the suspicion of acromegaly has been arisen, further assessments can rapidly decide on the diagnosis. What takes a long time is to have the suspicion in the back of the mind of clinicians seeing the patient for various complains related to acromegaly. Clinical assessment and diagnosis of patients with suspected acromegaly should focus particularly on the characteristic symptoms and signs as well as on common complications of acromegaly, as mentioned in the "Clinical features of acromegaly" chapter. A thorough physical examination including detailed observation of the skin, cardiovascular and neurological testing with confrontal visual field assessment is important in the diagnosis of the main symptoms and complications. Description of the past medical history and assessment of previous photographs of the patient can rapidly turn the clinical suspicion to firm clinical diagnosis. Biochemical tests and imaging studies are needed to establish the diagnosis of acromegaly. In addition, syndromic presentation, positive family history and young-onset disease should prompt appropriate genetic testing to identify a possible genetic background of the disease.

Biochemical Diagnosis

GH secretion is pulsatile, with six to ten GH pulses over 24 h separated by long periods of low or virtually undetectable GH levels. While this secretory pattern would suggest that measurement of a random serum GH concentration has little value, in clinical practice morning GH correlates well with overall GH status in the majority of the cases, although this is not used in the diagnosis of acromegaly. Insulin-like growth factor 1 (IGF-1), mediates many of the actions of GH and is considered a marker of integrated GH secretion. Age-related normal range needs to be established for the particular GH assay used, and various factors influencing circulating IGF-1 levels need to be taken into account (Table 1). The biochemical diagnosis of acromegaly requires the confirmation of persistently elevated serum levels of IGF-1. Lack of suppression of GH in response to oral glucose tolerance test (OGTT) (GH $< 1\ \mu g\ L^{-1}$) is diagnostic. Therefore, the biochemical diagnosis is usually straightforward. However, the assessment can be challenging in some cases, such as with mild disease, in teenagers and in pregnancy.

Measurement of IGF-1 Levels

Measuring an IGF-1 level is recommended as the initial screen for patients with clinical suspicion of acromegaly, in cases with pituitary masses and in selected patients where suspicion of acromegaly is instigated by combination of several comorbidities commonly present in acromegaly (Katznelson *et al.*, 2014a).

Serum IGF-1 levels are invariably elevated in active acromegaly, are stable during the day (its half-life is approximately 15 hours), independent of food intake, and correlate well with the GH levels.

IGF-1 levels peak at adolescence and are lower before and after puberty. Therefore, all levels must be assessed in relationship to age-matched normal values (adults) or age- and sex-matched levels (children and adolescents) for the specific assay being used. There is significant inter-assay variability for measurement of IGF-1 that needs to be taken into account if values are assessed by different laboratories (Bidlingmaier *et al.*, 2014; Frystyk *et al.*, 2010). In fact, the diagnosis of acromegaly was inaccurately excluded in 30% of single samples assayed for IGF-1 in 23 different laboratories (Pokrajac *et al.*, 2007). Active acromegaly is associated with elevated IGF-1 levels compared to normal subjects, so an age-matched normal IGF-1 excludes the diagnosis in most patients (Katznelson *et al.*, 2014a). However, certain pathological conditions (Table 1), apart from assay variability or inaccurate reference ranges (Pokrajac *et al.*, 2007), can lead to false negative results, especially in mild cases. Systemic illnesses, severe infection, hepatic or renal failure, malnutrition, diabetes mellitus and oral estrogens (which may render the liver less responsive to GH) may decrease IGF-1 levels which might result in false-negative interpretations (Katznelson *et al.*, 2011; Isotton *et al.*, 2012). Elevated IGF-1 levels during pregnancy and the wide normal range in adolescence may render the diagnosis of acromegaly difficult.

Moreover, in patients with acromegaly and poorly controlled diabetes mellitus, the OGTT is also not reliable and serum IGF-I levels should be re-assessed when glycemic control has been established (Muhammad *et al.*, 2015).

This chapter is based on the chapter by Prof Klaus von Weder (von Werder K. diagnosis of Acromegaly. In Encyclopedia of Endocrine Diseases. Elsevier Inc 2004;1).

Table 1 Factors affecting IGF-1 and GH

Factors interfering with IGF-1 concentration
Low IGF-1
● Poorly controlled diabetes mellitus
● Liver or renal failure
● Exogenous estrogen
● Malnourishment
● Systemic illness
High IGF-1
● Adolescence/puberty
● Pregnancy
● Hyperthyroidism (note: acromegaly can be associated with hyperthyroidism due to goiter or due to TSH co-secretion)
High GH and low IGF-1
● Kidney failure: GH ↑ or ↔, IGF-1 ↓
● Liver failure - GH↑, IGF-1↓
● Anorexia nervosa GH ↑, IGF-1↓
● GH insensitivity - GH↑, IGF-1↓
False-positive oral glucose challenge
● Diabetes mellitus
● Liver disease
● Renal insufficiency
● Malnutrition
● Adolescence
● Women receiving estrogen or pregnant

Measurement of Growth Hormone Levels

Although an elevated random GH level in a patient with suspicious clinical features is suggestive of acromegaly, random serum GH is more used for follow-up of patients after treatment rather than for diagnosis. Random serum GH level $<0.4\ \mu g\ L^{-1}$, on the other hand, is very suggestive of lack of acromegaly, especially with corresponding normal IGF-1 (Giustina *et al.*, 2000; Giustina *et al.*, 2010).

The serum GH concentration measured during an OGTT (with 75 g of oral glucose) is the gold-standard diagnostic test and should be used to confirm the diagnosis. In acromegaly, GH levels may decrease, increase, or show no change in response to glucose load; however, they do not decrease to less than $1\ \mu g\ L^{-1}$ and this lack of suppression establishes the diagnosis (Dimaraki *et al.*, 2002).

The cutoff for nadir GH during OGTT is highly dependent on the assay used.

A nadir serum GH $<0.4\ \mu g\ L^{-1}$ after an oral glucose load has been considered for establishing the diagnosis (Freda *et al.*, 2003). However, although current GH assays have improved sensitivity, many assays do not have sufficient accuracy at GH levels $<1\ \mu g\ L^{-1}$, and it has been suggested that a cutoff GH $<1\ \mu g\ L^{-1}$ after the glucose load is sufficient for excluding the diagnosis (Katznelson *et al.*, 2014b). On the other hand, it should always be remembered that using this cutoff could lead to the inaccurate exclusion of the diagnosis in a significant percentage of cases (25%) (Giustina *et al.*, 2010) with mild active disease, since significant GH suppression during OGTT, as low as $0.33\ \mu g\ L^{-1}$ was noted in some cases of active mild acromegaly (Dimaraki *et al.*, 2002; Freda *et al.*, 2003; Freda *et al.*, 1998), these cases sometimes mentioned as "micromegaly".

Increasing age, female gender, obesity, and elevated body mass index may be associated with abnormal post-glucose GH suppression, and there is a need to define normal ranges for these variables (Arafat *et al.*, 2008; Carmichael *et al.*, 2009).

Accurate measurement of GH and IGF-I is important for the diagnosis and monitoring of acromegaly. Despite the use of international reference preparations of GH, the commercially available immunoassays produce heterogeneous values. We face a lack of uniform assay standardization, poor reproducibility between laboratories and assays, imprecise standards, and there is also a lack of robust normal control values using sensitive immunometric assays (Clemmons, 2011). In an illustrative paper, the same GH sample was measured in 104 centers across the UK using different assays (Pokrajac *et al.*, 2007). The results varied more than threefold for GH. Even when using the same automated immunoassay, significant intra-individual variability still existed.

Another test to assess GH burden is a GH day curve (GHDC), which significantly correlates with the GH nadir during OGTT and with the IGF-1 level (Dobrashian *et al.*, 1993; Minuto *et al.*, 2012). In normal subjects, at least one GH value is usually undetectable over repeated sampling during the day. In active acromegaly, nadir GH levels during a GHDC do not suppress below $1\ \mu g\ L^{-1}$(Ho and Weissberger, 1994). As a research tool mean integrated 24-h levels can also be used being significantly elevated in active acromegaly. Barkan *et al.* evaluated the correlation between plasma IGF-1 and the degree of GH hypersecretion assessed by multiple GH determinations throughout a 24-h period before and during therapy with a long acting somatostatin analog. They noted that when mean plasma GH was higher than $12.0 \pm 0.6\ \mu g\ L^{-1}$, plasma IGF-1 concentrations plateaued, whereas below this

GH value it was accompanied by a linear decrease in IGF-1 levels (Barkan *et al.*, 1988). In addition, there was also a lack of correlation between GH and IGF-1 in patients with high IGF-1 and clinical acromegaly but low (<1 μg L^{-1}) nadir GH on OGTT. These data suggest that while GH and IGF-1 change in a linear fashion in the mid-range of GH values, at high GH (>12 on integrated GH values (Barkan *et al.*, 1988)) and low GH (<1 μg L^{-1} on OGTT) GH and IGF-1 do not change in a linear fashion (Dimaraki *et al.*, 2002; Ribeiro-Oliveira *et al.*, 2011).

Further complicating the diagnosis in cases with mildly active disease (who could derive a great benefit from an early diagnosis and treatment) are the frequent (around 30% of cases) discrepancies between the GH and IGF-1 hypersecretion (Capatina & Wass, 2015). A finding of an elevated IGF-1 with normal GH values needs to be interpreted based on the clinical findings because this may reflect earlier disease (Dimaraki *et al.*, 2002) and usually should prompt a thorough investigation (including imaging) to clarify the diagnosis. (Fig. 1)

IGFBP3

The major binding protein of IGF-1, IGFBP3 is not useful for diagnosis or treatment follow-up of patients with acromegaly as significant overlap occurs between normal controls and patients (Wass, 1997).

Measurement of other anterior pituitary hormones in patients with acromegaly

Patients with suspected acromegaly should have a full pituitary workup. Hyperprolactinemia is common either due to prolactin co-secretion (up to 1/3 of patients) or due to the stalk effect. PRL levels higher than 200 μg L^{-1} are usually indicative of a co-secreting adenoma. GH can also be co-secreted with TSH, with symptomatic secondary hyperthyroidism. Large tumor mass or prolactin hypersecretion can lead to the hypofunction of the adrenal, thyroid or gonadal axes.

Assessment of complications and related issues

Electrocardiogram and echocardiography could inform of the degree of the cardiac involvement (Katznelson *et al.*, 2011). X-ray of the spine and bone density measurement, especially with history of hypogonadism, informs of spine abnormalities (Wassenaar

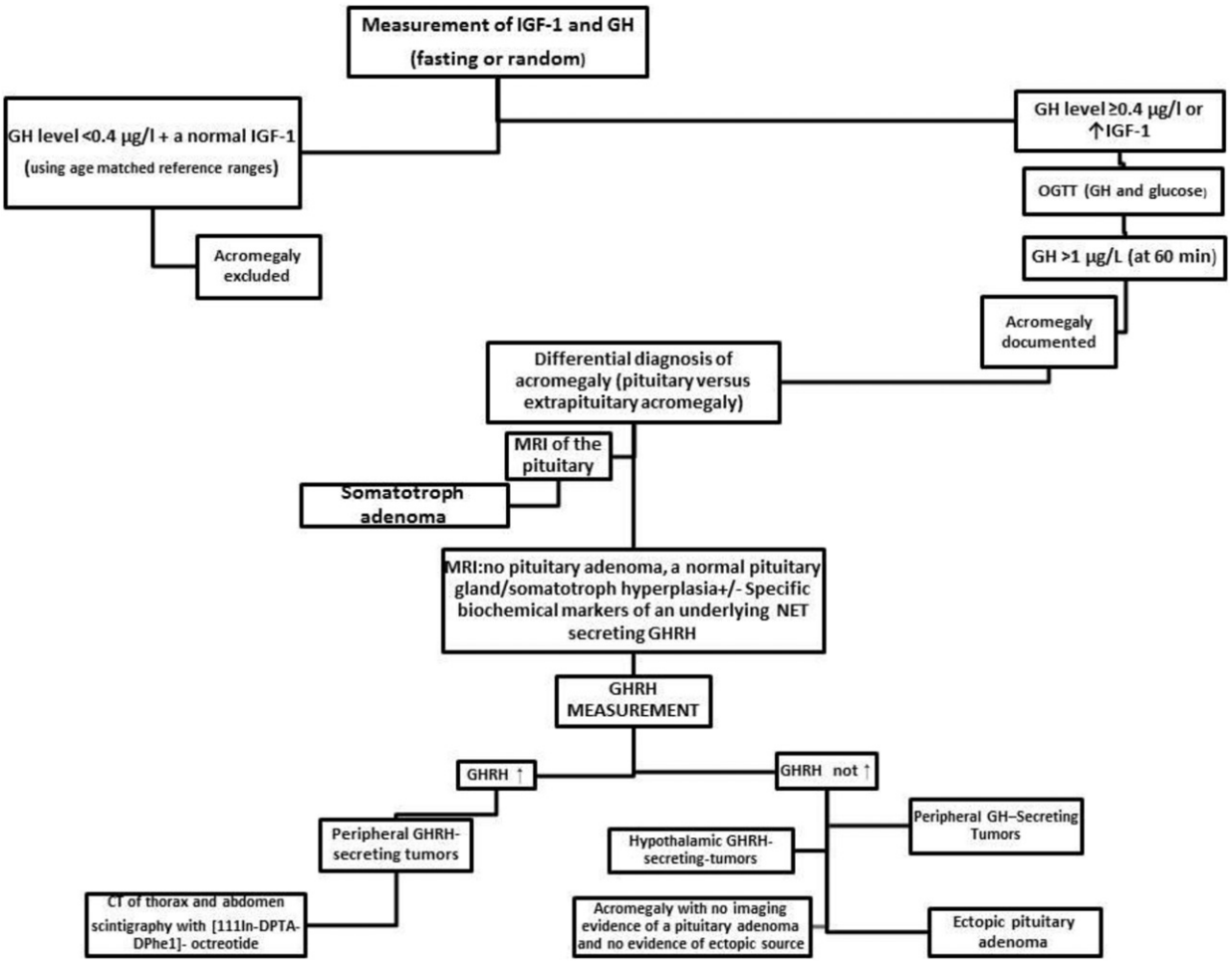

Fig. 1 Algorithm for the diagnostic workup in acromegaly

et al., 2011; Claessen *et al.*, 2013) and abnormal bone density. In young patients bone age assessment and follow-up of height velocity is necessary, and some of these patients need rapid intervention. Assessment of painful joints could be necessary. Presence of sleep apnea should be investigated and finding of hypercalcemia should prompt for search for MEN1 syndrome. Syndromic presentation, childhood-onset disease and positive family history for pituitary adenomas should prompt genetic assessment.

Neuroradiological and Neuro-Ophtalmological Investigations

Following biochemical diagnosis of acromegaly, a contrast-enhanced T1 and T2 pituitary MRI is recommended with thin slices as the imaging modality of choice to identify a pituitary adenoma. This will assist to establish tumor size and appearance, as well as parasellar extension. High-resolution T1-weighted sections in the coronal and sagittal planes, obtained both before and after gadolinium pentetic acid contrast administration, are the mainstay of pituitary imaging and will identify most pituitary masses causing acromegaly. After administration of gadolinium, microadenomas usually appear hypodense compared with the normal gland, especially when multiple thin-section echo sequences are examined during the first few minutes after contrast injection. In contrast, macroadenomas, which are significantly more vascular than microadenomas, have a higher affinity for gadolinium. They often enlarge the sella turcica by remodeling the bony fossa; they can grow upward toward the optic apparatus and cause draping of the nerves over the tumor, but especially somatotroph adenomas often go downwards and can extend into the sphenoid sinus. Invasion of the cavernous sinuses are significant as can hinder total tumor removal (Nishioka *et al.*, 2014).

Coronal T2 weighted sequences can also provide added information. It has been suggested that the signal characteristics of GH-secreting adenomas on T2 weighted sequences, whether they are of lower or higher signal than the adjacent grey matter (Heck *et al.*, 2012) or the adjacent normal pituitary (Potorac *et al.*, 2015), can be an indicator of their histological composition: densely granulated GH-secreting adenomas are typically hypointense, whilst sparsely granulated GH-secreting adenomas are hyperintense. Furthermore, it has been described that the low T2 signal (densely granulated) adenomas are seemingly responding better to somatostatin analogues (Fougner *et al.*, 2012).CT does not provide such excellent soft tissue resolution as MR but can be a very useful investigation if MR is contraindicated.

More than 99% of patients with acromegaly harbor a GH secreting pituitary adenoma. However, in rare circumstances, patients with acromegaly may not have imaging evidence of a pituitary adenoma and an ectopic source should be ruled out. Management of these patients poses special challenge, and once ectopic source of GH/GHRH is ruled out, surgical exploration of pituitary might be useful (Khandelwal *et al.*, 2011; Daud *et al.*, 2011; Lonser *et al.*, 2010).

While confrontal visual field testing is part of the physical examination, formal visual field testing should be performed when the tumor is found to abut the optic chiasm on an imaging study.

Special considerations

Diagnosis of Gigantism

Pituitary gigantism is usually a spectacular disorder, although delayed diagnosis is still well-described in this patient group. Pituitary gigantism should be considered in children who are more than 3 standard deviations above normal mean height for age or more than 2 standard deviations over their adjusted mean parental height. The biochemical diagnosis is similar to that for acromegaly: GH levels are in excess of 1 $\mu g\ L^{-1}$ after a glucose load, and age-adjusted IGF-1 concentrations are elevated. However, in healthy children undergoing pubertal growth spurts, GH suppression to glucose could be lacking in upto 1/3 of tall pubertal children (Holl *et al.*, 1999) and GH can even paradoxically rise after glucose (Hindmarsh *et al.*, 1986). Serum IGF-1 concentrations are often physiologically elevated and they must be interpreted with an age- and sex-specific reference range (Holl *et al.*, 1999; Davies & Cheetham, 2014). Therefore, the diagnosis requires clear-cut MRI evidence for a pituitary lesion.

Acromegaly in pregnancy

An initial diagnosis of acromegaly during pregnancy could be problematic due to placental secretion of GH that binds to GH receptors increasing IGF-1 secretion by $>30\%$ over pre-pregnancy levels as well as to marked alteration of GH response to suppressive testing. Pregnancy is associated with a pseudo-acromegaloid state, because of production of placental GH (GH-2 or GH-V) from syncytiotrophoblast. Placental GH binds to hepatic GH receptor stimulating IGF-1 production, which on its turn decreases pituitary GH production. If GH-like activity is measured during pregnancy, only 3% of the activity is explained by maternal GH and 12% by placental lactogen. There is a decrease in the number of somatotroph cells during pregnancy, resulting in decreased circulating pituitary GH levels. Maternal levels of IGF-1 are slightly elevated. A lack of GH suppression to oral glucose challenge would be expected during pregnancy, since estrogen facilitates GH secretion (Cheng *et al.*, 2012). However, high levels of estrogen block the normal stimulation of IGF-1 production by GH (Laway & Mir, 2013).

GHRH-induced or ectopic GH excess

In more than 99% of cases, acromegaly is due to a GH-secreting pituitary adenoma (Fig. 2).

GHRH Hypersecretion

Neuroendocrine tumors, usually located in the lungs, endocrine pancreas (often associated with MEN1 syndrome) or thymus can secrete GH-releasing hormone (GHRH), but rarely phaeochromocytoma (Vieira Neto *et al.*, 2007) or paraganglioma (Ghazi *et al.*, 2013) could also be the source (Fig. 2).

GHRH secretion is responsible for 0.5% of all cases of acromegaly (Losa & von Werder, 1997). In the rare situation where a non-pituitary etiology is suspected, the serum GHRH level should be measured. Plasma GHRH levels are usually elevated in patients with peripheral GHRH-secreting tumors, and are normal or low in patients with pituitary acromegaly (Gola *et al.*, 2006). Measuring GHRH plasma levels therefore provides a precise and cost effective test for the diagnosis of ectopic acromegaly. In case of GHRH-related acromegaly, the pituitary gland may appear normal or slightly bulky on MRI. Elevated levels of other hormones usually secreted from neuroendocrine tumors of the pancreas, such as insulin, calcitonin, gastrin, chromogranin could draw attention to ectopic GHRH-secreting tumors. Somatostatin receptor scintigraphy and PET scanning could be helpful to identify the source of the ectopic hormone secretion. The diagnosis of MEN1 should be considered. CT scan of the thorax and abdomen could be used to identify a neuroendocrine tumor (Gola *et al.*, 2006).

Rarely, hypothalamic tumors, including hamartomas, choristomas, gliomas, and gangliocytomas, may produce GHRH with subsequent somatotroph hyperplasia or even a pituitary GH-cell adenoma and resultant acromegaly (Sano *et al.*, 1988) (Fig. 2). Peripheral GHRH levels are not elevated in patients with hypothalamic GHRH-secreting-tumors, supporting the notion that excess eutopic hypothalamic GHRH secretion into the hypophyseal portal system does not appreciably enter the systemic circulation.

GH excess originating from outside the pituitary fossa

Ectopic acromegaly due to a GH-secreting adenoma arising from abnormally located pituitary tissue is a rare entity. The most frequent localization for these ectopic tumors has been the sphenoid sinus, followed by the clivus and isolated cases of tumors in the cavernous sinus or the suprasellar region (Ramirez *et al.*, 2013; Appel *et al.*, 2012; Gondim *et al.*, 2004; Warner *et al.*, 1982; Guerrero *et al.*, 2007).

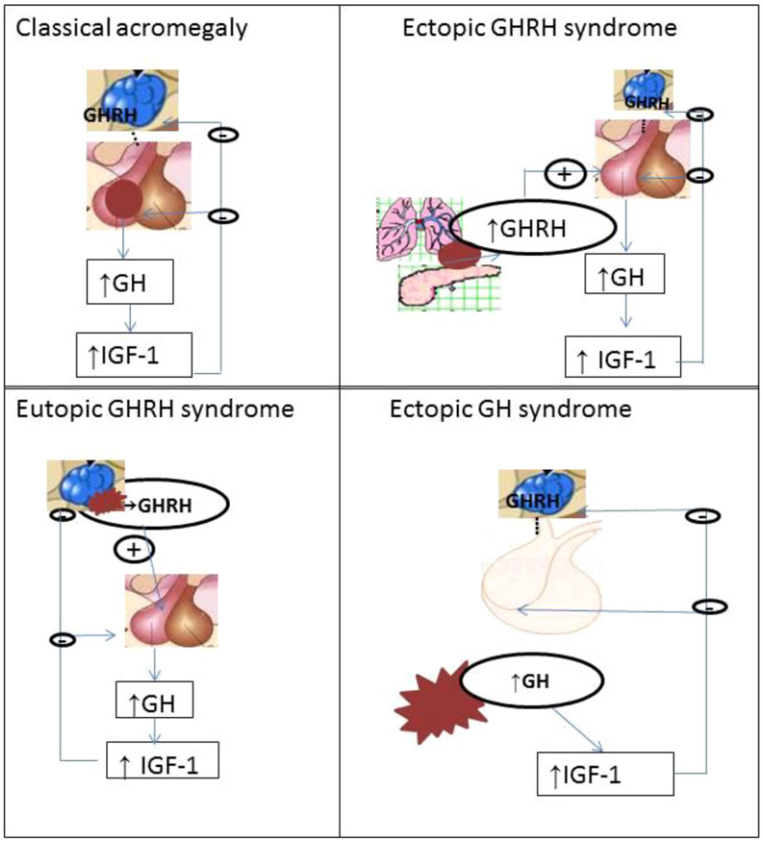

Fig. 2 Pituitary acromegaly (over 99% of cases) versus extrapituitary acromegaly: peripheral GHRH-secreting tumors (ectopic GHRH syndrome), hypothalamic GHRH-secreting-tumors (eutopic GHRH syndrome) and ectopic GH syndrome, respectively.

Table 2 Genetic alterations leading to GH excess

Syndromic disease	*Isolated GH-secreting pituitary adenoma*
MEN1 syndrome (*MEN1*)	FIPA (*AIP*)
MEN4 syndrome (*CDKN1B*)	FIPA – X-linked acrogigantism (XLAG) (*GPR101*)
SDHx (*SDHA*, *SDHB*, *SDHC*, *SDHD*)	Unknown gene(s)
Carney complex (*PRKAR1A*, *PRKACB*)	
McCune–Albright syndrome (mosaic *GNAS*)	
Neurofibromatosis (very rare (Crawford & Buckler, 1983; Drimmie *et al.*, 2000)) (*NF1*)	
Von Hippel–Lindau syndrome (single case report (Tudorancea *et al.*, 2012)) (*VHL*)	

Cases of GH secretion from non-pituitary tissue are exceptionally rare (Fig. 2). Two cases have been described: one with GH secretion from a pancreatic cancer and the other in a patient with non-Hodgkin lymphoma (Werder., 2004). Elevated GH and IGF-1 levels with no evidence of a pituitary lesion (either adenoma or somatotroph hyperplasia), and normal GHRH levels could suggest the diagnosis, but XLAG needs to be considered in very young patients.

Acromegaly due to genetic predisposition

Acromegaly is the most common type of pituitary adenoma associated with a genetic abnormality. Predisposing factors include mutations causing syndromic diseases (Table 2) or isolated pituitary adenomas. Family history may not be positive due to low penetrance, such as in AIP mutation kindreds, or due to *de novo* mutations (often in XLAG).

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Therapy for Acromegaly☆

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Abbreviations

ACTH	Adrenocorticotropin
CSF	Cerebrospinal fluid
DA	Dopamine agonist
FSH	Follicle-stimulating hormone
GH	Growth hormone
IGF-I	Insulin-like growth factor-I
LH	Luteinizing hormone
MRI	Magnetic resonance imaging
PRL	Prolactin
SA	Somatostatin analogs
SMR	Standardized mortality ratio
Sst	Receptors subtypes
TSH	Thyroid-stimulating hormone

Acromegaly is a rare disease characterized by progressive somatic modifications, mainly involving the face and extremities, together with systemic manifestations related to organ overgrowth and fluid retention. Acromegaly is associated with severe comorbidity and premature death if not adequately treated. It is due to excessive secretion of growth hormone (GH), originating from a pituitary adenoma in the vast majority of cases and stimulating production of insulin-like growth factor-I (IGF-I). Management of acromegaly is now rather consensual (Ribeiro-Oliveira and Barkan, 2012; Chanson and Kamenicky, 2012; Sherlock *et al.*, 2011; Giustina *et al.*, 2010, 2014; Melmed, 2009; Katznelson *et al.*, 2014). If surgical removal of the pituitary GH-secreting adenoma is usually the first line therapy, it is able to cure the disease only in microadenomas or small non invasive macroadenomas. In the other cases persistent postoperative GH/IGF-I hypersecretion needs to be treated with medical therapy, which is now generally preferred to radiation therapy for second line treatment. The various therapeutic options are presented on **Fig. 1**.

Objectives of Treatment

Clinical Aims

The clinical aims are to relieve symptoms, to reduce the volume of the pituitary tumor, to avoid tumor relapse, and to improve long-term morbidity and mortality (Melmed *et al.*, 2002).

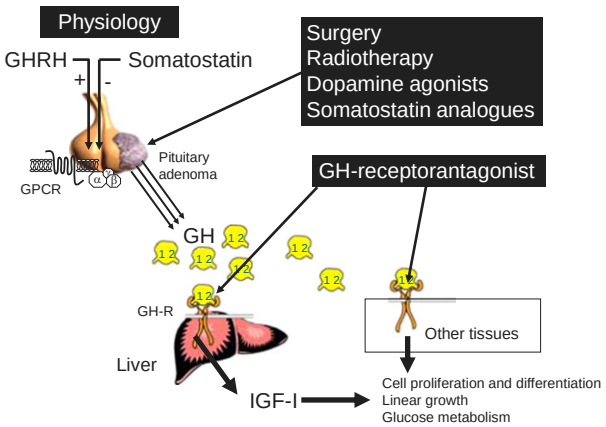

Fig. 1 Therapeutic options in acromegaly. Site of action of the different therapeutic tools in acromegaly. Surgery, radiotherapy, somatostatin analogs and dopamine agonists act at the level of the pituitary adenoma, while GH-receptor antagonists act in the periphery by blocking the GH receptor and thus impairing the effects of GH on the different tissues. Reproduced with permission from Chanson, P., Salenave, S., Kamenicky, P., Cazabat, L. and Young, J. (2009b). Pituitary tumours: Acromegaly. *Best Practice & Research. Clinical Endocrinology & Metabolism*, **23**, 555–574.

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Biochemical Criteria of Successful Treatment

The 2000 consensus on the definition of acromegaly cure

More than 15 years ago, an expert consensus group considered that control of acromegaly was achieved when, biochemically, circulating IGF-I levels are reduced to the age-adjusted normal range and when the nadir GH level after an oral glucose load is less than 1 $\mu g\ L^{-1}$ (Giustina *et al.*, 2000). However, many endocrinologists preferred to assess "random" GH levels, particularly in therapeutic trials using somatostatin analogs (SA), as criterion of disease control. Level of 2 or 2.5 $\mu g\ L^{-1}$ to define disease control were chosen with regard to earlier epidemiological studies linking mortality to GH serum levels, and because the majority of patients reaching these levels also have normal IGF-I levels (Freda *et al.*, 2005; Chanson *et al.*, 2009b; Sherlock *et al.*, 2011). A meta-analysis of all published studies providing estimates of mortality, published in 2008 (Holdaway *et al.*, 2008), confirmed the prognostic relevance of this cutoff (GH $<2.5\ \mu g\ L^{-1}$), as mortality among patients with a final GH level below 2.5 $\mu g\ L^{-1}$ was very close to that expected in the general population, while a final GH level above 2.5 $\mu g\ L^{-1}$ was associated with a 1.7-fold excess risk of death. The same was true for IGF-I, final normal IGF-I being associated with absence of excess mortality while a 2.5-fold mortality risk was found for patients with final IGF-1 levels above the age-adjusted normal range (**Fig. 2**).

However, one of the main drawbacks of former epidemiological studies is that they were based on old radio-immunoassays for serum GH measurement. Modern assays (two-site antibody "sandwich" methods), have better sensitivity (Cazabat *et al.*, 2008). European experts, followed by a larger international consensus group, recommended the use of the World Health Organization (WHO) international standard (WHO IS 98/574 for GH and WHO IS 02/254 for IGF-I) (Trainer *et al.*, 2006; Clemmons, 2011).

The 2010 and 2014 consensus on the definition of acromegaly cure

A subsequent expert consensus meeting (Giustina *et al.*, 2010) defined optimal disease control (i.e., posttreatment remission of acromegaly) as follows: "an IGF-I level (determined by a reliable standardized assay) in the age-adjusted normal range and a GH level less than 1.0 $\mu g\ L^{-1}$ from a random GH measurement (using an ultrasensitive assay). Normalization of IGF-I is the only reliable marker of disease control under pegvisomant. In patients with acromegaly undergoing surgical management of GH-secreting tumors, OGTT can be used to assess the outcome. There is substantial evidence to suggest that nadir GH levels less than 0.4 $\mu g\ L^{-1}$ (with ultrasensitive assays) may define control in these circumstances. In the case of discrepant biochemical results, multiple GH sampling may be useful."

The Endocrine Society Clinical Practice Guideline proposed by a Task Force sponsored by the Endocrine Society and the European Society of Endocrinology (Katznelson *et al.*, 2014) also suggests "a biochemical target goal of an age-normalized serum IGF-1 value, which signifies control of acromegaly" and "using a random GH $<1.0\ \mu g\ L^{-1}$ as a therapeutic goal, as this correlates with control of acromegaly."

Surgical Treatment

Surgery Aims to Reduce Mass Effects

Surgery is generally the first-line treatment, as tumor excision, usually by the trans-sphenoidal route, is the most rapid way of reducing tumor symptoms, particularly visual defects. However, it must be acknowledged that it may be possible to reduce the

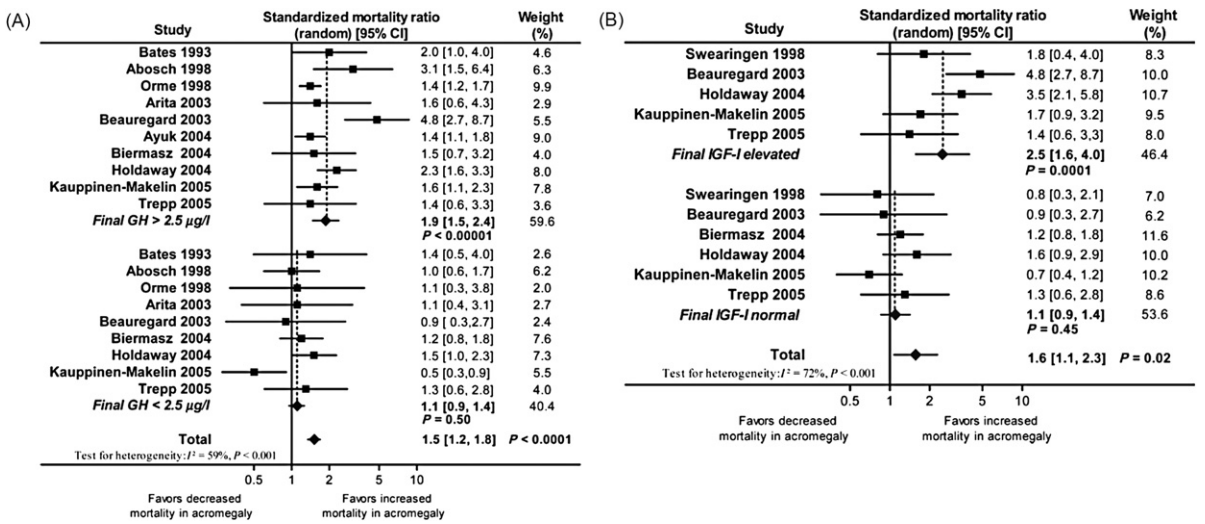

Fig. 2 Pooled standardized mortality ratios (SMRs) in studies of acromegaly grouped according to GH (A) or to IGF-I (B) level at final follow-up. Data are SMR (95% confidence interval). Reproduced with permission from Holdaway, I. M., Bolland, M. J. and Gamble, G. D. (2008). A meta-analysis of the effect of lowering serum levels of GH and IGF-I on mortality in acromegaly. *European Journal of Endocrinology* **159**, 89–95.

tumor volume by medical treatment with SA, particularly when the tumor is distant from the optic chiasm (see the paragraph "SA as first-line treatment").

Before initiating treatment, patients with acromegaly must undergo a workup focusing on tumor mass effects (headaches, visual field and acuity, magnetic resonance imaging, MRI). This will guide therapeutic decisions, particularly the use of surgery. The mass effect of the tumor can also affect other pituitary functions, and other pituitary hormones should thus be measured to determine if they are deficient or, on the contrary, secreted in excess. Another aim of surgical treatment, besides the control of GH/IGF-I levels, is to improve hormone deficiencies or PRL hypersecretion related to mass effects on the normal pituitary or the pituitary stalk. Indeed, hyperprolactinemia is present in 10%–30% of patients, and may be either functional (secondary to impaired hypothalamic dopamine production or tumor compression of the pituitary stalk, hindering dopamine delivery to the pituitary) or due to a mixed adenoma (Grynberg *et al.*, 2010; Petrossians *et al.*, 2017).

Surgery Aims to Reduce/Normalize Serum GH/IGF-I Levels

Tumor resection, usually by the trans-sphenoidal route, is also the most rapid way of reducing GH and IGF-I concentrations in patients with acromegaly. Percent reduction of GH levels (but not of IGF-I levels) correlates closely with percent resected tumor volume (Schwyzer *et al.*, 2015). Nevertheless, GH and IGF-I levels normalize in only 40%–70% of cases (Fahlbusch *et al.*, 1992; Swearingen *et al.*, 1998; Biermasz *et al.*, 2000; Nomikos *et al.*, 2005, Jane *et al.*, 2011; Roelfsema *et al.*, 2012; Abu Dabrh *et al.*, 2014), depending on the size of the tumor (microadenomas are more amenable to cure), the preoperative GH concentration (the success rate is higher when GH concentrations are low, i.e., $<10\ \mu g\ L^{-1}$), the length of follow up and the surgeon's experience. After true surgical cure, true recurrence of acromegaly is very rare, $<5\%$ (Roelfsema *et al.*, 2012). When surgery fails to achieve good disease control, or when surgery is impossible or contraindicated, patients are now generally offered medical treatments (Katznelson *et al.*, 2014; Giustina *et al.*, 2014).

Adverse Effects of Surgery

Surgical removal of pituitary adenomas can occasionally have severe adverse effects such as cerebrospinal fluid (CSF) leak with a risk of meningitis or epistaxis in fewer than 5% of patients. In a meta-analysis of 92 studies enrolling 6988 patients, Carvalho *et al.* found weighted incidence rates of 13% for hypopituitarism, 2.5% for panhypopituitarism, 6.5% for ACTH deficiency, 4.4% for TSH deficiency, 6.7% for FSH/LH deficiency, 10% for transient and 2.4% for permanent diabetes insipidus (Carvalho *et al.*, 2015). Endoscopic surgery does not markedly improve these rates compared with microscopic series (Jane *et al.*, 2011). If pituitary functions are deficient, the aim of replacement therapy, is to achieve euthyroidism, eucortisolism, and eugonadism (Fleseriu *et al.*, 2016; Higham *et al.*, 2016). A major determinant of the rate of surgical complications is the neurosurgeon's experience (Ahmed *et al.*, 1999; Bates *et al.*, 2008). Thus, when surgery is indicated, one precondition is to find a good surgeon!

Medical Treatment

Dopamine Agonists (DAs)

Dopamine receptors

Physiologically, dopamine stimulates GH secretion (Giustina and Veldhuis, 1998), but DAs paradoxically suppress GH hypersecretion in patients with acromegaly (Chiodini *et al.*, 1974). Dopamine binding sites were found on the surface of GH-secreting adenomas (Bression *et al.*, 1982), and expression of dopamine type 2 (D2) receptor (D2R), the predominant DR subtype in somatotropinomas, has later been confirmed (Stefaneanu *et al.*, 2001; Missale *et al.*, 1998; Neto *et al.*, 2009). D2 receptors and type 5 somatostatin receptors (SSTR5) can heterodimerize, thus enhancing the functional activity of both agonists (Rocheville *et al.*, 2000).

Cabergoline is now the most used DA

DAs were the first drugs to be used in acromegaly. Bromocriptine moderately attenuates the symptoms of acromegaly and reduces the GH concentration, but it normalizes the IGF-I level in only some 10% of patients (Jaffe and Barkan, 1992). Cabergoline is an ergot derivative which is more selective for D2R and with a long duration of action allowing once- or twice-weekly administration. Cabergoline has been used for more than three decades in the treatment of hyperprolactinemia (Gillam *et al.*, 2006). It was also tested in acromegaly (Kuhn and Chanson, 2017) but its potential value was overshadowed by the advent of SAs such as octreotide and lanreotide. Marketing authorization has never been sought for cabergoline in this indication, meaning that the drug is used off-label in acromegaly.

A systematic review, in 2011, of clinical trials using cabergoline in acromegaly showed that available studies were generally small, non randomized or placebo-controlled and their results variable (Abs *et al.*, 1998; Colao *et al.*, 1997b; Cozzi *et al.*, 1998; Ferrari *et al.*, 1988; Freda *et al.*, 2004; Jackson *et al.*, 1997; Moyes *et al.*, 2008; Muratori *et al.*, 1997; Sherlock *et al.*, 2009; Vilar *et al.*, 2002). The meta-analysis (Sandret *et al.*, 2011) of cabergoline monotherapy in a total of 149 patients showed that 48% and 34% of patients achieved a GH level below $2.5\ \mu g\ L^{-1}$ and a normal serum IGF-I level, respectively (**Fig. 3**). In multivariate analysis, the

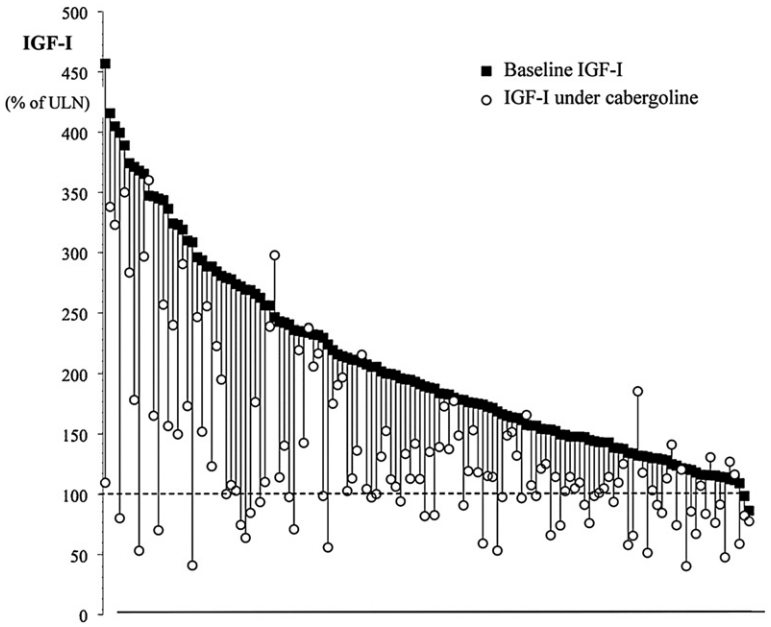

Fig. 3 Individual IGF-I levels, expressed as a percentage of the upper limit of the age-adjusted normal range (ULN) before (*black squares*) and after treatment with cabergoline (*open circles*) in patients with acromegaly. Data from a meta-analysis. Reproduced with permission from Sandret, L., Maison, P. and Chanson, P. (2011). Place of cabergoline in acromegaly: A meta-analysis. *Journal of Clinical Endocrinology and Metabolism*, **96**, 1327–1335.

decline in IGF-I was related to the baseline IGF-I concentration and to the baseline PRL concentration and a trend towards a relationship with the cabergoline dose was also found (Sandret *et al.*, 2011). A retrospective study of patients enrolled in the UK Acromegaly Register published after the meta-analysis confirmed these results (Howlett *et al.*, 2013).

The effect of cabergoline on tumor volume was examined prospectively in only five studies (Ferrari *et al.*, 1988; Jackson *et al.*, 1997; Colao *et al.*, 1997b; Muratori *et al.*, 1997; Abs *et al.*, 1998. On monotherapy, tumor shrinkage was observed in about one third of patients (Sandret, 2011). Tumor shrinkage was linked to a higher baseline PRL concentration, a higher baseline IGF-I concentration, and prior treatment status. The study population was too small for multivariate analysis.

Thus, cabergoline is mainly recommended for patients with borderline or moderately increased plasma IGF-I levels (< 150% X the upper limit of normal) (Giustina *et al.*, 2014; Katznelson *et al.*, 2014), even though it is sometimes effective (leading to normal IGF-I levels) in patients with high baseline IGF-1 levels (**Fig. 3**) and also in patients with normal PRL levels (Sandret *et al.*, 2011).

Adverse effects

The adverse effects of carbergoline are rare, usually mild, and include nausea, headache, constipation, postural hypotension, dizziness, nasal stuffiness, and Raynaud's phenomenon. They tend to occur after the initial dose and at each dose increment, but can be minimized by initiating the drug at a low dose at bedtime, and then escalating dosage very progressively. Symptoms of psychosis (or exacerbation of pre-existing psychosis) (Boyd, 1995) and impulse control disorders (such as pathological gambling, hypersexuality, compulsive shopping or eating) have been observed under bromocriptine and cabergoline in patients with prolactinomas (Bancos *et al.*, 2014; Noronha *et al.*, 2016; Barake *et al.*, 2014; Moore *et al.*, 2014). These reactions usually resolve within 72 h of drug interruption.

Mixed GH-PRL secreting adenomas or mammosomatotroph adenomas treated with cabergoline may shrink and, rarely be complicated by CSF leak (Lam *et al.*, 2012; Thakur *et al.*, 2011).

The use of very high doses of DAs, and particularly cabergoline, in patients with Parkinson's disease has been linked to an increased risk of cardiac valve disease (Schade *et al.*, 2007; Zanettini *et al.*, 2007). Since these publications, several retrospective cross-sectional studies have focused on cardiac complications in patients treated with cabergoline for prolactinoma: in fact, this occurrence seems extremely rare as a maximal prevalence of 0.17% patients with typical cabergoline-associated valvulopathy was found on more than 1800 patients reviewed (Caputo *et al.*, 2015). The same concern has been raised regarding the use of cabergoline in acromegaly, particularly as acromegaly itself is associated with an increased prevalence of cardiac valve disease (Colao *et al.*, 2003, 2008; Pereira *et al.*, 2004; van der Klaauw *et al.*, 2006). Data on cardiac valve status in acromegalic patients treated with cabergoline are scarce (Lafeber *et al.*, 2010; Izgi *et al.*, 2010). Cardiac valve status was studied in a large population of cabergoline-treated patients with acromegaly (Maione *et al.*, 2012). The cross-sectional study (42 patients who had received cabergoline at a median cumulative dose of 203 mg for a median of 35 months, compared to 46 acromegalic patients who had never received cabergoline and who were matched for age, sex and disease duration) and the longitudinal study (26 patients studied before and during cabergoline treatment, compared with a group of acromegalic patients who were not receiving

cabergoline and who were managed during the same period) did not find a higher prevalence or incidence of valve abnormalities (Maione *et al.*, 2012). Thus, at the doses used, cabergoline was not associated with an increased risk of cardiac valve regurgitation or remodeling.

Medical Treatment With Somatostatin Analogs (SAs)

Somatostatin receptors subtypes

SAs suppress GH secretion by binding to somatostatin receptors (Patel and Srikant, 1998; Hofland and Lamberts, 2003; Jaquet *et al.*, 2000; Kubota *et al.*, 1994; Reisine and Bell, 1995). There are five somatostatin receptors subtypes (sst), on somatotroph adenoma cells. First generation somatostatin analogs such as octreotide or lanreotide, exert their antisecretory and antitumoral effects by acting on sst2 and 5 on which they bind with high affinity (**Table 1**).

Antisecretory effects of first generation SAs

SAs analogs have been widely used in the treatment of acromegaly (Chanson, 2016). Octreotide (Sandostatin®) can be injected subcutaneously (SC) at a dose of 100–500 μg two or three times a day. This was the first such analog to be marketed, in the 1980s (Lamberts *et al.*, 1985), and represented a major therapeutic advance (Lamberts *et al.*, 1996). Sustained-release lanreotide (Somatuline® LP 30 mg) was the first slow-release preparation to be marketed. It was injected intramuscularly every 10–14 days (the frequency of injections being adjusted to GH/IGF-I concentration). Lanreotide is now available for deep SC injection every 28 days, at doses of 60, 90, and 120 mg (Somatuline® Autogel®). Octreotide LAR (Sandostatin® LAR 10–20 or 30 mg), the sustained-release version of octreotide, is administered intramuscularly, once a month. SAs are usually initiated at the median dose and are then titrated (decreased or increased) according to the GH/IGF-I concentration.

The two SAs have largely similar efficacy (Murray and Melmed, 2008; Carmichael *et al.*, 2014). They allow to achieve GH concentrations below 2 $\mu g\,L^{-1}$ in 60%–70% of patients and normalize IGF-I levels in 50%–80% of patients (Freda *et al.*, 2005; Bevan, 2005; Chanson, 2008). The wide range of SAs efficacy from one study to the other was recently analyzed (Carmichael *et al.*, 2014). This meta-analysis showed that overall achieved control rates were 56% for mean GH and 55% for IGF-1 normalization (**Fig. 4**). If treatment duration, prior SA therapy and year of study publication were related to biochemical control, no significant differences in GH or IGF-I response rates were observed for multicenter versus single center, retrospective versus prospective, study drug, preselection for SA responsiveness, dosing scheme, GH response criterion, or switch study design (Carmichael *et al.*, 2014). However, when studies reported a composite end point (GH + IGF-I), this lowered the remission rate: the mean difference between the composite and individual GH and IGF-1 efficacy rates were 13 ± 14.5% and 8 ± 9.3%, respectively (thus, around 43%–47%) (Carmichael *et al.*, 2014). This lower efficacy rate (compared to what was claimed previously) was confirmed by another recent meta-analysis of the effects of SAs in an unbiased group, that of treatment-naïve patients: using strict combined cutoff criteria (normal serum IGF-I levels *and* serum GH less than 1 $\mu g\,L^{-1}$), remission was observed in only 45% of these patients (Abu Dabrh *et al.*, 2014). It must be noted that 25%–40% of treated patients exhibit discordant GH and IGF-I concentrations (Alexopoulou *et al.*, 2008; Ayuk *et al.*, 2004; Carmichael and Bonert, 2008; Kanakis *et al.*, 2016), with a higher proportion of patients considered as in remission who have normal IGF-I and who keep increased GH levels (Kanakis *et al.*, 2016). This may be related to the GH assays which are now more sensitive or to the effect of sex (female patients have increased GH levels compared with males) or younger age (Alexopoulou *et al.*, 2008).

Several long-term studies have shown that the cure rate tends to improve over time (Ayuk *et al.*, 2002; Cozzi *et al.*, 2003; Maiza *et al.*, 2007). SA therapy must be continued indefinitely because, theoretically, it only suspends GH hypersecretion. In fact, it seems possible, in a handful of very good responders, to lengthen the interval between injections, or even to stop the treatment permanently with no subsequent increase in GH/IGF-I concentrations (Ronchi *et al.*, 2008; Ramirez *et al.*, 2012).

An oral formulation of octreotide is currently in development. Absorption to the circulation is apparent within 1 h after dose administration and escalating doses resulted in dose-dependent increased plasma octreotide concentrations, with an observed rate of plasma decay similar to parenteral administration; both 20 mg oral octreotide and injection of 0.1 mg sc octreotide resulted in equivalent pharmacokinetic parameters supporting an oral octreotide alternative to parenteral octreotide treatment for patients with acromegaly (Tuvia *et al.*, 2012).

Table 1 Binding affinities of SRIF-14, first generation somatostatin analogues (lanreotide and octreotide), and second-generation somatostatin analogue (pasireotide) for the receptor subtypes, hsst1–5

Compound	*hsst1*	*hsst2*	*hsst3*	*hsst4*	*hsst5*
SRIF-14	0.93 ± 0.12	0.15 ± 0.02	0.56 ± 0.17	1.5 ± 0.4	0.29 ± 004
Lanreotide	180 ± 20	0.54 ± 0.08	14 ± 9	230 ± 40	17.5 ± 5
Octreotide	280 ± 80	0.38 ± 0.08	7.1 ± 1.4	>1000	6.3 ± 1.0
Pasireotide	9.3 ± 0.1	1.0 ± 0.1	1.5 ± 0.3	>100	0.16 ± 0.01

Results are the mean ± S.E.M. IC50 values expressed as nmol L^{-1}.

Reproduced in part with permission from Bruns, C., Lewis, I., Briner, U., Meno-Tetang, G. and Weckbecker, G. (2002). SOM230: A novel somatostatin peptidomimetic with broad somatotropin release inhibiting factor (SRIF) receptor binding and a unique antisecretory profile. *European Journal of Endocrinology* **146**, 707–716.

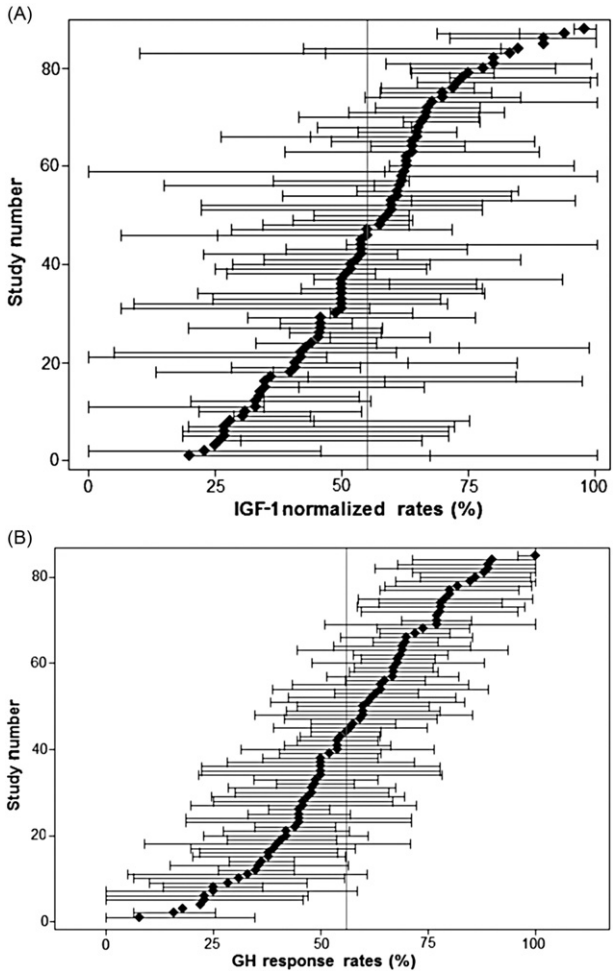

Fig. 4 Meta-analysis of clinical trials assessing biochemical efficacy parameters of somatostain analogues in acromegaly. (A) IGF-I response rates and 95% confidence intervals for the 90 analyzed cohorts and (B) GH response rates and 95% confidence intervals for the 90 analyzed cohorts. Median response rates for GH and IGF-I noted by the vertical lines. Figures are sorted from least to greatest percent response rate. Reproduced with permission from Carmichael, J. D., Bonert, V. S., Nuno, M., Ly, D. and Melmed, S. (2014). Acromegaly clinical trial methodology impact on reported biochemical efficacy rates of somatostatin receptor ligand treatments: A meta-analysis. *Journal of Clinical Endocrinology and Metabolism*, **99**, 1825–1833.

Anti tumoral effects of first generation SAs

SAs also have effects on tumor volume (**Fig. 5**), which decreases in 20%–70% of patients (Melmed *et al.*, 2005; Bevan, 2005). A meta-analysis showed that, on average, this was achieved in 53% of patients treated with octreotide (Giustina *et al.*, 2012), and in 66% of patients treated with octreotide LAR. The overall weighted mean percentage reduction in tumor size was 37.4%, rising to 50.6% with octreotide LAR (Giustina *et al.*, 2012). The reduction in tumor volume is larger when a SA is the first-line treatment (Colao *et al.*, 2001). Even when SA therapy does not lead to a reduction in tumor volume, it does at least control tumor volume in the vast majority of cases (Bevan, 2005). The antitumoral effects of SAs vary from one study to the other, probably for methodological reasons. In a recent multicenter study, where the primary endpoint was the proportion of patients with clinically significant ($\geq$20%) tumor volume reduction under lanreotide administered at a dose of 120 mg every 4 weeks, in treatment naive patients, it was shown that 62% of patients reached this criterion after one year of treatment (Caron *et al.*, 2014). The main anti-tumoral effect is generally achieved within the first three months and slowly progresses thereafter (Bevan *et al.*, 2002; Caron *et al.*, 2014).

First generation SAs as first-line therapy

SA therapy is indicated after surgical failure, but it can sometimes be used for first-line treatment, especially when severe comorbidities (heart failure or respiratory problems) provide a risk of perioperative complications (Colao *et al.*, 2004; Attal and Chanson, 2010). This has been introduced in the new recommendations for the treatment of acromegaly (Giustina *et al.*, 2014; Katznelson *et al.*, 2014). In some cases, when the tumor is very large and invasive, and is not completely resectable by surgery (which would justify postoperative SA therapy), a SA can be administered first in the hope of controlling GH hypersecretion and

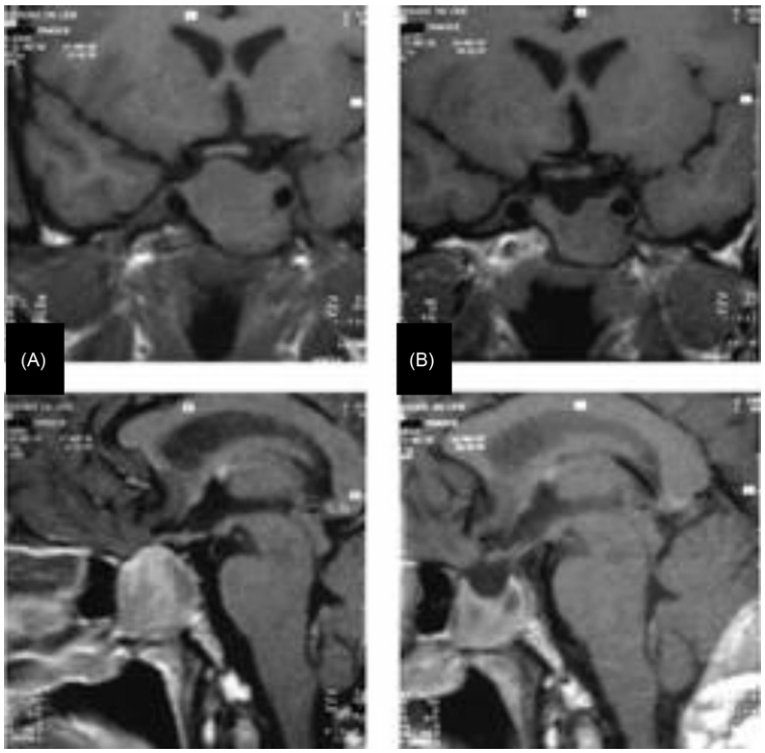

Fig. 5 Effect of treatment with first generation somatostatin analogue on tumor volume as assessed by MRI. (A) Before treatment; (B) after 3 months of treatment with somatostatin analogues. Upper panels, coronal view; lower panels, sagittal views.

tumor growth, thus avoiding the need for surgery (Giustina *et al.*, 2014; Katznelson *et al.*, 2014). Indeed, it has long been considered that the effectiveness of first-line medical therapy (in treatment-naive patients) is equivalent to that of second-line treatment after surgery or radiotherapy (Newman *et al.*, 1998; Sheppard, 2003; Colao *et al.*, 2001; Bevan *et al.*, 2002; Attanasio *et al.*, 2003). In fact, according to the meta-analysis published by Freda *et al.*, IGF-I is more likely to normalize after second-line treatment than after first-line drug therapy (Freda *et al.*, 2005). In this setting, it is interesting to know how primary medical or surgical therapy compare in terms of efficacy, both in secretory (GH/IGF-I) and tumoral terms. Retrospective data from the German Registry indicate that SA treatment is less effective than surgical treatment (Petersenn *et al.*, 2008). This has not been confirmed by the only prospective study which did not find significant differences in terms of efficacy and adverse events (Colao *et al.*, 2009). In a systematic review and meta-analysis to synthesize the existing evidence comparing these two approaches in treatment-naive patients, surgery was associated with a higher remission rate (67% vs. 45%), particularly when follow-up periods were long ($\geq$24 months) and when performed by a single operator (Abu Dabrh *et al.*, 2014).

Strategy in case of first generation SAs partial resistance

When first generation SA therapy fails to achieve remission, some authors report that an increase in the SA dose or a shorter interval between injections, whether by octreotide or by lanreotide, can rescue some patients (Giustina *et al.*, 2009, 2017).

When first generation SA therapy is inadequate, another possibility is debulking surgery for lowering hormone levels, in which case retreatment with an SA often provides disease control (Petrossians *et al.*, 2005; Karavitaki *et al.*, 2008).

It is also possible to combine first generation SAs with other drugs (see below "Combination therapy") or to shift to a GH receptor antagonist such as pegvisomant (see below).

The availability of second generation SAs such as pasireotide may also be helpful for controlling some patients resistant to first generation SAs (see below).

Effects of preoperative first generation SA treatment on surgical outcome

There is some controversy about the ability of preoperative SA therapy to improve surgical outcome (Jacob and Bevan, 2014): some studies (Stevenaert *et al.*, 1992; Colao *et al.*, 1997a; Barkan *et al.*, 1988; Abe and Ludecke, 2001; Lucas *et al.*, 2003; Carlsen *et al.*, 2008; Mao *et al.*, 2010; Shen *et al.*, 2010) indicate that, in some patients with somatotroph macroadenomas, surgery provides better control of acromegaly when patients have been pretreated with an SA, compared with those operated immediately, without SA preparation, while other studies showed no difference (Biermasz *et al.*, 1999; Kristof *et al.*, 1999; Losa *et al.*, 2006). A recent meta-analysis of all controlled trials showed a borderline effect, on short term with a pooled odds ratio (OR) of 1.62 (95% CI, 0.93–2.82) for biochemical cure with SA treatment; analysis of the three prospective controlled trials showed a statistically

significant effect (OR 3.62, 95% CI, 1.88–6.96) (Pita-Gutierrez *et al.*, 2013). However, after a longer follow up, even the studies which were initially in favor of preoperative SA treatment did not confirm any differences in terms of acromegaly control after surgery between patients treated preoperatively or not with SAs (Shen *et al.*, 2010; Fougner *et al.*, 2014). In any case, patients with minimally invasive macroadenomas (e.g., minor cavernous sinus invasion) represent the subgroup most likely to benefit from improved remission rates after preoperative SA therapy, particularly in centers with low surgical results (Jacob and Bevan, 2014).

Second-generation SAs: pasireotide

First generation SAs bind with high affinity to somatostatin receptor subtypes sst2 and 5. A significant proportion of somatotroph adenomas appear partially or totally resistant to octreotide or lanreotide, possibly owing to variable expression or a reduced density of receptor subtypes (Hofland and Lamberts, 2003). Pasireotide (Signifor®) is a new SA that binds with high affinity to sst 1, 2, 3, and 5 (**Table 1**) (Bruns *et al.*, 2002) and seems more effective than octreotide in terms of the GH/IGF-I levels achieved in patients with acromegaly (Petersenn *et al.*, 2010). Pasireotide LAR is administered every 28 days at a dose of 40–60 mg. In a head-to head prospective, randomized, double-blind trial led in treatment naive patients, biochemical control was achieved by significantly more pasireotide LAR patients than octreotide LAR patients (31.3% vs. 19.2% and 35.8% vs. 20.9% when including patients with IGF-I below the lower normal limit) (Colao *et al.*, 2014). This drug may be particularly interesting in patients with partial resistance to first-generation SAs such as octreotide or lanreotide. This has been recently studied in a large multicenter randomized trial performed in patients inadequately controlled with maximal doses of octreotide LAR or lanreotide LAR: 15% of patients in the pasireotide 40 mg group and 20% of patients in the pasireotide 60 mg group achieved biochemical control, compared with no patients in the active control group (who pursued their previous SA treatment) (**Fig. 6**) (Gadelha *et al.*, 2014).

Side-effects of SAs

First generation SAs have mild adverse effects, consisting mainly of transient gastrointestinal disorders (abdominal bloating, nausea, diarrhea), and also gallstones in 10%–20% of cases (ursodeoxycholic acid has no proven effectiveness in this setting) (Attanasio *et al.*, 2008). Gallstone complications are rare, and guidelines for their monitoring have been much relaxed in recent years (Chanson *et al.*, 2009a). Changes in glucose metabolism are sometimes observed, including impaired glucose tolerance or even diabetes in patients who are overweight. In other cases, however, glucose tolerance improves following the reduction in insulin resistance due to lowering of GH concentrations. Overall, according to a recent meta-analysis, the consequences are very minor in terms of fasting glucose and HbA1c levels (Mazziotti *et al.*, 2009). Regular metabolic monitoring is nevertheless justified.

On pasireotide, gastrointestinal adverse events occurred at a similar frequency compared to first generation SAs but glucose metabolism was more strongly altered (57.3% vs. 21.7% in the head-to-head comparison study) (Colao *et al.*, 2014). This was confirmed in the study conducted in patients resistant to first generation SAs (Gadelha *et al.*, 2014) where the most common adverse events were hyperglycemia (33% under 40 mg pasireotide and 31% under 60 mg pasireotide, vs. 14% with active control), and diabetes (21% under 60 mg and 26% under 40 mg pasireotide vs. 8% in active controls). Diarrhea was also more frequent during pasireotide in this trial (Gadelha *et al.*, 2014).

Medical Treatment With GH-Receptor Antagonists

Pegvisomant (Somavert®) has a different mechanism of action. It acts in the periphery, blocking the effects of GH on its target organs by binding to GH receptors and preventing their dimerization (**Fig. 7**); this blocks GH signal transduction and inhibits GH activity, including IGF-I production (Kopchick *et al.*, 2002). As pegvisomant inhibits the action of GH but not its secretion, GH concentrations cannot be used to evaluate treatment efficacy. IGF-I is used as a surrogate marker, together with clinical parameters. Pegvisomant is administered subcutaneously at a daily dose of 10, 15 or 20 mg (or even more), the dose being titrated according to hormone response (IGF-I normalization). Pegvisomant is highly effective, as IGF-I levels normalize in more than 90% of patients (Trainer *et al.*, 2000; van der Lely *et al.*, 2001a). In fact, efficacy may be slightly lower in routine practice, as shown by observational studies (Moore *et al.*, 2009), where normalization of IGF-I was achieved in around 70% of cases (Schreiber *et al.*, 2007; Colao *et al.*, 2006; Marazuela *et al.*, 2009; van der Lely *et al.*, 2012; Chanson *et al.*, 2015). For the moment this treatment is reserved for patients in whom SAs fail. In a series of 304 patients in whom tumor volume was monitored for at least 3 years, an increase in tumor volume occurred in 9 cases, within 8 months after commencing pegvisomant. This is likely related to rebound expansion after discontinuation of SAs and/or to the natural history of aggressively growing pituitary tumors (Jimenez *et al.*, 2008); this latter situation may justify combination with a SA to reduce tumor volume (van der Lely *et al.*, 2001b). Tumor volume must therefore be monitored (by MRI) during this treatment. Available clinical data on pegvisomant concern a relatively small number of patients and relatively short treatment periods. Adverse effects are limited to rare liver enzymes elevations: in 2.5% of patients according a large surveillance study (van der Lely *et al.*, 2012), that generally normalize either spontaneously or after treatment interruption. Exceptional cases of true hepatitis have been reported (Schreiber *et al.*, 2007; van der Lely *et al.*, 2012). Gilbert disease has been suggested as a risk factor for severe hepatitis (Bernabeu *et al.*, 2009, 2010) but this was not confirmed by an Italian study (Filopanti *et al.*, 2014). Lipohypertrophy has also been observed, likely related to repeat injections in the same site without rotating the sites of injections (Maffei *et al.*, 2006).

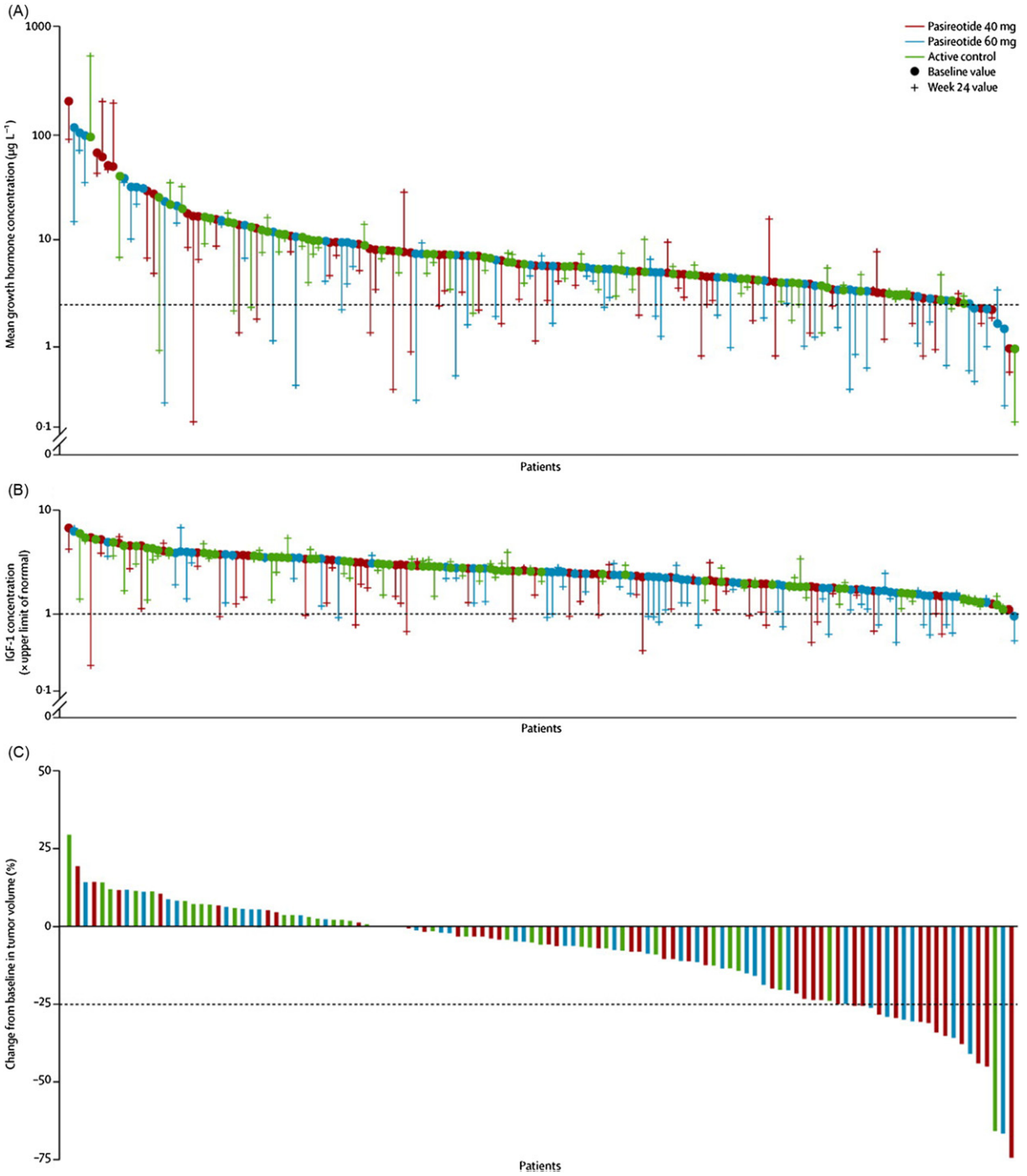

Fig. 6 Effect of treatment with pasireotide. Change in GH (A), IGF-1 concentration (B), and tumor volume (C) from baseline to week 24 of treatment with pasireotide 40 mg (in *red*), 60 mg (in *blue*) or active controls (in *green*) administered monthly in patients not controlled by their first generation somatostatin analog (octreotide or lanreotide). Data are individually shown for patients with available data at baseline and week 24. The dashed line represents $2 \times 5\ \mu g\ L^{-1}$ for GH concentration, the upper limit of normal for IGF-I concentration, and 25% reduction for tumor volume. Reproduced with permission from Gadelha, M. R., Bronstein, M. D., Brue,T., Coculescu, M., Fleseriu, M., Guitelman, M., Pronin, V., Raverot, G., Shimon, I., Lievre, K. K., Fleck, J., Aout, M., Pedroncelli, A.M., and Colao, A. (2014). Pasireotide versus continued treatment with octreotide or lanreotide in patients with inadequately controlled acromegaly (PAOLA): A randomised, phase 3 trial. *Lancet Diabetes Endocrinol* **2**, 875–884.

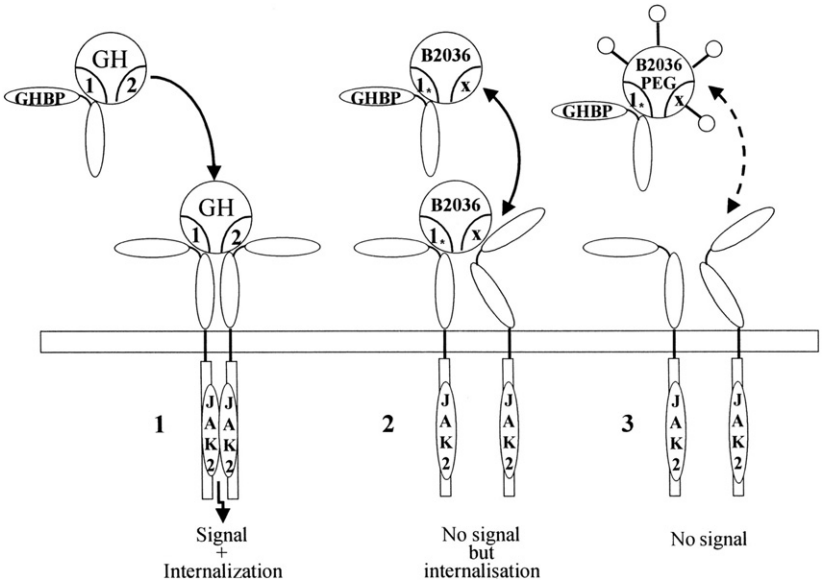

Fig. 7 Mode of action of pegvisomant. Hypothesis for interaction of GH, B2036, and B2036-PEG (pegvisomant) with the GH receptor (GHR). (1) GH binds, through site 1, to GH binding protein (GHBP); and binding to the GHR, through sites 1 and 2, triggers a conformational change, signaling, and internalization. (2) B2036 binds preferentially to GHBP, because of the site 1 mutations (1*), but this advantage is lost in the GHR dimer, where other interactions are revealed within the trimeric complex. The site 2 mutation (x) blocks the conformational change required for signaling, but internalization still occurs. (3) B2036-PEG (pegvisomant) binds to GHBP through site 1, which is protected from pegylation by the mutation of the site 1 lysines, but the PEG moieties do conjugate in site 2 to the G120K mutation. Pegylation sterically reduces binding to the GHR dimer at the cell surface, although binding is sufficient, at high concentration of B2036-PEG, to block GHR signaling. Reproduced with permission from Ross, R.J., Leung, K.C., Maamra, M., Bennett, W., Doyle, N., Waters, M.J., Ho, K.K. (2001). Binding and functional studies with the growth hormone receptor antagonist, B2036-PEG (pegvisomant), reveal effects of pegylation and evidence that it binds to a receptor dimer. *Journal of Clinical Endocrinology and Metabolism* **86**, 1716–1723.

Combined Medical Therapy

Cabergoline and SAs combinations

In a systematic review published in 2011, out of 77 patients who failed to normalize IGF-I under SA treatment alone (in three prospective studies and two retrospective studies), 40 (52%) achieved normal IGF-I levels on combined treatment with cabergoline (**Fig. 8**); mean IGF-I and GH serum concentrations fell by respectively 30% and 19%. Multivariate analysis confirmed the predictive value of the baseline IGF-I level but not that of the baseline GH level. Four more recent studies also reported the effects of adding cabergoline to SAs, with very similar results (Mattar *et al.*, 2010; Vilar *et al.*, 2011; Suda *et al.*, 2013; Puig-Domingo *et al.*, 2016). Cabergoline adjunction to ongoing SA therapy seems particularly beneficial for patients with borderline or moderately elevated serum IGF-I levels (<150% the upper limit of normal). A Japanese retrospective study reported a significant decrease in tumor volume after combination therapy (Suda *et al.*, 2013). Finally, a Spanish retrospective study showed that IGF-I levels normalized after cabergoline adjunction to lanreotide in 48% of patients (Puig-Domingo *et al.*, 2016).

The synergistic effect of DA and SA has led to develop chimeric agonists binding both DA and SRIF receptors (particularly sst2 and sst5) (Saveanu *et al.*, 2002). One of this compound BIM 23A760 (Jaquet *et al.*, 2005), with an in vitro, synergistic effect on GH secretion, showed promising results in studies led in the monkey or in man after unique dose. Unfortunately, in patients with acromegaly, the multicenter trial which was initiated in 2008 was prematurely interrupted due to insufficient efficacy.

Somatostatin analogues and pegvisomant combination therapy

SA-pegvisomant combination therapy has also been developed (Feenstra *et al.*, 2005), a long acting SA being given once a month at the highest marketed dose (30 mg octreotide LAR or 120 mg lanreotide Autogel) and pegvisomant being injected once a week at escalating doses until the IGF-I level normalizes. IGF-I normalization was obtained in all the patients with a median weekly pegvisomant dose of 60 mg (Neggers *et al.*, 2007). This decrease in dose requirement during combined therapy might be partially explained by an increase of about 20% in serum levels of pegvisomant (Jorgensen *et al.*, 2005). Biochemical hepatic anomalies were frequent (although always transient) (Neggers *et al.*, 2009). By comparison with octreotide monotherapy, this combination appears to have a greater positive impact on quality of life for a given degree of IGF-I normalisation (Neggers *et al.*, 2008), an effect that might be due to an extrahepatic action of pegvisomant (Neggers and van der Lely, 2009).

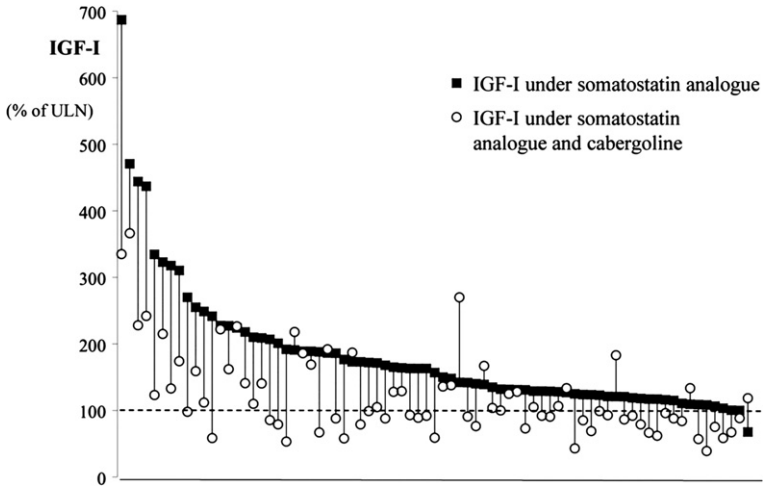

Fig. 8 Individual IGF-I levels, expressed as a percentage of the age-adjusted ULN range during treatment with somatostatin analogs alone (*black squares*) and after cabergoline adjunction (*open circles*) in patients with acromegaly. Reproduced with permission from Sandret, L., Maison, P. and Chanson, P. (2011). Place of cabergoline in acromegaly: A meta-analysis. *Journal of Clinical Endocrinology and Metabolism* **96**, 1327–1335.

Cabergoline and pegvisomant combination therapy

Cabergoline–pegvisomant combination therapy has been used to treat patients in whom cabergoline monotherapy failed to achieve adequate serum GH/IGF-I levels, but few data are available. In an observational retrospective cross-sectional study of 14 patients with acromegaly who were on pegvisomant monotherapy for partial resistance to SA, the IGF-I level was normal in four patients (28%) 18 months after cabergoline adjunction (Bernabeu *et al.*, 2013), but all the patients whose serum IGF-I level normalized by the end of the study had received radiotherapy. A better response to the combined therapy was associated with a lower baseline IGF-I level, a higher PRL level, female gender, and lower body weight (Bernabeu *et al.*, 2013). In a prospective study of 24 acromegalic patients, Higham *et al.* found that the cabergoline–pegvisomant combination was more effective than either drug used alone (Higham *et al.*, 2012). Thus, cabergoline adjunction may be of interest when pegvisomant alone does not achieve a normal IGF-I level in a patient with moderately elevated serum IGF-I.

Radiotherapy

Techniques

Radiotherapy techniques have evolved. Radiation can be delivered in a single session by stereotactic radiosurgery or in fractionated form. Radiosurgery is a term used to define radiation delivered at a high dose to a typically small target in a single or few sittings. Fractionated radiotherapy refers to radiation therapy delivered at smaller doses delivered over typically 5–6 weeks in 25–30 sessions. In order to minimize the dose to surrounding tissue, techniques for stereotactic localization are now used. Different forms of stereotactic radiosurgery (SRS) are available, with radiation delivered as photons (Gamma Knife, Linac, CyberKnife) or charged particles (protons). Stereotactic fractionated radiotherapy (SFR) is a hybrid form that has been developed which employs stereotactic localization techniques with fractionated therapy, administered by 3D-conformal radiation therapy, intensity modulated radiation therapy or proton radiation therapy (Colin *et al.*, 2005; Shih and Loeffler, 2008; Kuhn and Chanson, 2015).

For acromegaly, SFR delivers an average total dose of 50 Gy in about 25 daily sessions. SRS delivers 20–25 Gy in one (or few) session(s). The choice between the two methods, SFR and SRS, depends on the size of the tumor and its vicinity with the optic apparatus (Loeffler and Shih, 2011). If the lesion measures less than 3 cm and is situated more than 3–5 mm from the optic chiasm, SFR is indicated, while SRS can be proposed in other cases.

Results of Radiotherapy

Fractionated irradiation

Fractioned irradiation resulted in GH concentrations below 2 $\mu g\ L^{-1}$ and normal IGF-I levels in 22% of patients at 2 years, 60% at 10 years, and 77% at 20 years in a British study (Jenkins *et al.*, 2006), and respectively 23% of patients at 5 years, 42% at 10 years and 61% at 15 years in an Italian study (Minniti *et al.*, 2005). Results of a French study using SFR showed similar results: remission (defined as normal IGF-I without medical treatment) was 25% at 5 years, 43% at 10 years, and 50% at 15 years (Diallo *et al.*, 2015). Here again, the baseline GH concentration seemed to be predictive of treatment outcome (Barrande *et al.*, 2000; Jenkins *et al.*, 2006; Diallo *et al.*, 2015). Control of tumor growth is excellent (>90% of cases) with SFR (Minniti *et al.*, 2016).

Stereotactic radiosurgery

SRS provides more focused irradiation. In a French series of over 80 patients, the efficacy of gamma-knife irradiation was close to that of fractionated radiotherapy (Castinetti *et al.*, 2005). In a recent meta-analysis, good control of acromegaly (without complementary treatment) was achieved in 48%–53% of cases after a mean follow-up of 4 years. This apparently better efficacy of SRS is probably explained by the fact that tumors treated with this approach are relatively small ($2.11 \pm 1.16\ cm^3$)(Yang *et al.*, 2010). However, in another meta-analysis (Abu Dabrh *et al.*, 2015), the difference in the percentage of patients in remission at last follow up between fractionated radiotherapy and SRS appeared to be not significant (52% vs. 36%; $P = .14$).

Whether or not SRS achieved a faster remission rate than SFR, remains a matter of discussion (Minniti *et al.*, 2016). The efficacy of the different SRS techniques seems to be similar in terms of tumor control which is excellent in >95% of cases (Yang *et al.*, 2010; Loeffler and Shih, 2011; Castinetti *et al.*, 2005, 2009; Minniti *et al.*, 2016).

Side-Effects of Radiotherapy

Whatever the technique, radiotherapy leads to variable degrees of anterior pituitary insufficiency in 50%–80% to 100% of patients after 10–15 years. SRS may have a lower incidence of hypopituitarism than fractionated radiotherapy; however, the difference was not statistically significant (32% vs. 51%, respectively; $P = .05$) in a recent meta-analysis (Abu Dabrh *et al.*, 2015).

Complications such as radionecrosis and optic neuropathy are now very rare. In contrast, the risk of stroke may be increased, sometimes many years after irradiation (Brada *et al.*, 1999): stroke may be 1.7–2.8 times more frequent (Loeffler and Shih, 2011; Minniti *et al.*, 2011) and is considered to be an important factor of excess mortality in these patients (Sherlock *et al.*, 2010), together with cortisol deficiency and inadequate hormonal substitution. If this increased cerebrovascular risk is related to older techniques of radiotherapy and will not be associated with modern procedures used nowadays is presently unknown.

Trends in the Therapeutic Strategy in the Previous Years

As exemplified by national registries, the treatment strategy is multimodal in the majority of cases: most patients undergo surgery and receive adjuvant medical treatment. As in other registries, almost 80% of patients in the French Acromegaly Registry (Maione *et al.*, 2017) underwent at least one neurosurgery. This proportion did not change over time, indicating that surgery remains the preferred strategy for cure, or at least for removing as much tumor as possible in order to enhance the efficacy of adjuvant therapy. In contrast, the proportion of patients who received radiation therapy fell gradually with time (**Fig. 9**), as in other national registries and series (Abosch *et al.*, 1998; Bex *et al.*, 2007; Kauppinen-Makelin *et al.*, 2005). The majority of patients received medical treatment. A variety of drugs were being used at the last visit, as in other registries, most patients still being treated with SAs. The percentage of patients on SA declined, while the percentage of patients on pegvisomant increased; this latter drug being more recently available.

Current Therapeutic Strategy

The advantages, disadvantages and costs of the different treatment options must be taken into account.

A therapeutic strategy is proposed in **Fig. 10**. Currently, if surgical treatment fails to cure acromegaly, medical treatment with SAs is preferred to radiotherapy. In some selected patients (those with moderately increased IGF-I), cabergoline may be tried first. If SA therapy fails, it may be interesting to propose repeat surgery in order to remove a large tumor remnant, before trying a SA

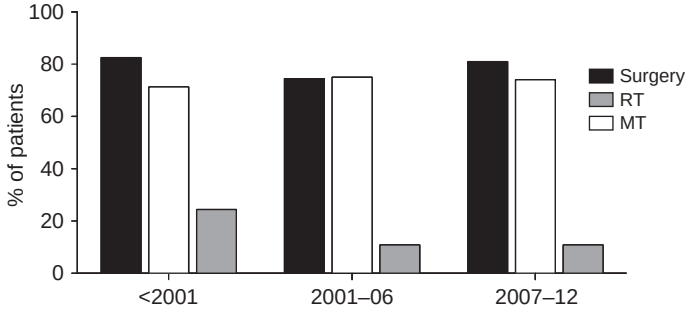

Fig. 9 Evolution of treatment strategies adopted for acromegaly according to time in the French Acromegaly Register. Data are the percentages of patients. Distribution of treatment approaches in different follow-up periods. *MT*, medical treatment; *RT*, radiotherapy. Reproduced with permission from Maione, L., Brue, T., Beckers, A., Delemer, B., Petrossians, P., Borson-Chazot, F., Chabre, O., Francois, P., Bertherat, J., Cortet-Rudelli, C., Chanson, P., and French Acromegaly Registry Group (2017). Changes in the management and comorbidities of acromegaly over three decades: the French Acromegaly Registry. *European Journal of Endocrinology* **176**, 645–655.

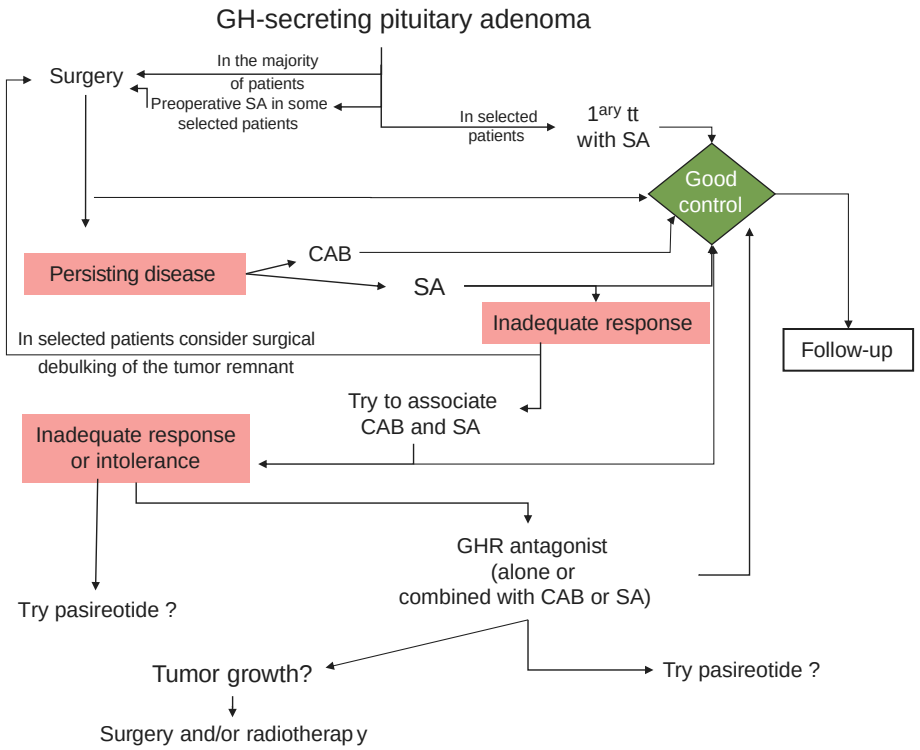

Fig. 10 Strategy proposed by the author for the current management of acromegaly. *SA*, somatostatin analogs; *CAB*, cabergoline; *GHRA*, GH-receptor antagonist (pegvisomant). Adapted from Chanson, P., Salenave, S., and Kamenicky, P. (2014). Acromegaly. *Handbook of Clinical Neurology* **124**, 197–219.

again. Combination with cabergoline may also be interesting if a SA is only partially effective. If this does not allow good control of GH/IGF-I levels, pegvisomant is generally proposed, either in combination with SAs if they are partially effective, in particular in tumoral terms or in replacement of SAs. The recent introduction of second-generation SAs such as pasireotide might change these strategies in the future if their superiority to first-generation SAs is confirmed. However, side-effects, particularly on glucose metabolism of these new SAs will need to be taken into account for the choice of the SAs to be used. Finally, the cost of these medical treatments, which may be required indefinitely (except if a radiotherapy susceptible to progressively decrease the GH production is proposed and presumably lead to decrease the requirement for medical treatment) needs also to be considered in the therapeutic decision.

If surgery is contraindicated, first-line SA therapy may be proposed.

All these treatments must be re-assessed on a yearly basis if the treatment is effective but as requested if the treatment is ineffective or in case of titration. After radiotherapy, if a medical treatment is necessary in waiting for the effects of irradiation, its regular withdrawal is necessary for assessing the persistence of active disease. Moreover regular evaluation of other pituitary functions (when normal at time of radiotherapy) is also mandatory during the years following radiotherapy for diagnosis of hypopituitarism to be replaced.

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ACTH-Secreting Pituitary Tumors☆

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Abbreviations

11β-HSD2	11β hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotropic hormone
AMP	Adenosine monophosphate
AVP	Arginine vasopressin
AVPR1B	AVP receptor
brg1	ATPase subunit of the Swi/Snf complex
BIPSS	Bilateral inferior petrosal sinus sampling
BmAH	Bilateral micronodular adrenal hyperplasia
BMAH	Bilateral macronodular adrenal hyperplasia
CBG	Cortisol binding globulins
CD	Cushing disease
CRH	Corticotrophin releasing hormone
CRH-R1	CRH receptor
CRT	Conventional radiotherapy
CS	Cushing syndrome
DDAVP	Desmopressin
DMX	Dexamethasone
DST	Dexamethasone suppression test
ECS	Ectopic Cushing syndrome
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FIPA	Familial isolated pituitary adenomas
GC	Glucocorticoid
GR	Glucocorticoid receptor
HPA	Hypothalamus–pituitary–adrenal
HPLC	High performance liquid chromatography
IA	Immunoassay
LCMS/MS	Liquid chromatography tandem mass spectrometry
LINAC	Linear accelerator system
LNSC	Late-night salivary cortisol
m	Mean
MC2-R	ACTH receptor
MEN1	Multiple endocrine neoplasia type 1
MEN4	Multiple endocrine neoplasia type 4
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
POMC	Pro-opiomelanocortin
SRT	Stereotactic radiotherapy
UFC	Urinary free cortisol

Introduction

The chronic exposure to excessive concentrations of circulating glucocorticoids (GCs), mainly cortisol, also named hypercortisolism, determines a chronic and severe disease named Cushing syndrome (CS). Adrenocorticotropic hormone (ACTH)-secreting pituitary tumors (or corticotroph pituitary tumors) induce adrenal cortisol hypersecretion and represent the most frequent form (around 70%) of endogenous CS (**Table 1**). This serious endocrine disorder is known as Cushing's disease (CD) (Pivonello *et al.*, 2008; Lacroix *et al.*, 2015; Pivonello *et al.*, 2016b).

Since 1932, when Harvey Cushing firstly described the clinical picture of glucocorticoid excess in a young woman, CS has continued to attract great interest among endocrinologists from both a clinical and an experimental point of view. Although, the low prevalence of the syndrome and its variable clinical and laboratory features have slowed down the progress in understanding pathophysiology and in developing treatments for this rare endocrine disorder, in recent times progresses have been made in this field (Pivonello *et al.*, 2008; Lacroix *et al.*, 2015; Pivonello *et al.*, 2016b, 2017).

This article reviews the clinical features of CD and the diagnostic procedures required to establish the diagnosis of CS, and, subsequently, to differentiate between pituitary (CD) and non-pituitary (ectopic and adrenal) causes of CS. Finally, currently available therapeutic approaches, including surgery, radiotherapy and medical therapy, are illustrated.

Clinical Picture

The typical clinical picture of CD usually presents with the classical stigmata of CS which includes central obesity, with round face ("moon face"); cervical ("buffalo hump") and supraclavicular fat pad; weakening of the upper and lower limbs associated with proximal muscle atrophy and asthenia; thinning of the skin with purplish striae and easy bruising. CD is associated with several complications including hypertension, glucose intolerance or diabetes mellitus and dyslipidemia which can constitute a specific form of metabolic syndrome; osteoporosis; myopathy; coagulopathy (hypercoagulopathy/hemostatic abnormalities);

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Table 1 Causes of Cushing syndrome (CS)

Type	*Form*	*Etiology*	*Estimated percentage (%)*
ACTH-dependent CS			
	Cushing disease	● ACTH-secreting pituitary adenomas (in most cases) ● Corticotroph hyperplasia (not associated to a CRH producing tumor) ● Corticotroph pituitary carcinomas	70
	Ectopic CS	● ACTH-secreting extra-pituitary tumors (in most cases) ● CRH-secreting extra-pituitary tumors	10
ACTH-independent CS			
	Adrenal CS	● Cortisol secreting adrenocortical tumors (adrenocortical adenomas and less frequently adrenocortical carcinomas) ● BMAH ● BmAH	20

ACTH, adrenocorticotropic hormone; *BMAH*, macronodular adrenal hyperplasia; *BmAH*, bilateral micronodular adrenal hyperplasia; *CRH*, corticotrophin releasing hormone; *CS*, Cushing syndrome.

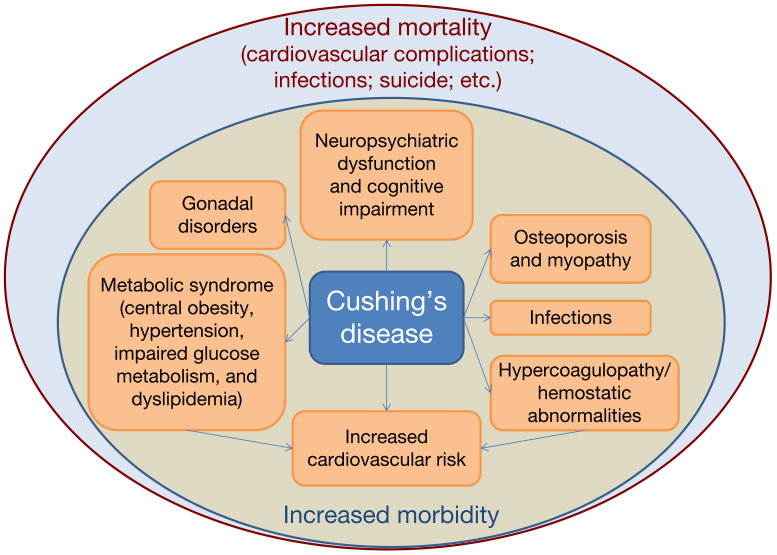

Fig. 1 Consequences of untreated or uncontrolled Cushing disease.

neuropsychiatric dysfunction; cognitive impairment and susceptibility to infections which are responsible for an impairment of quality of life and an increased morbidity and mortality (**Fig. 1**; Pivonello *et al.*, 2008; Lacroix *et al.*, 2015; Pivonello *et al.*, 2016a, 2016b, 2017; Newell-Price *et al.*, 2006; Isidori *et al.*, 2015a; Faggiano *et al.*, 2003; Pivonello *et al.*, 2005; Colao *et al.*, 1999; Scillitani *et al.*, 2014). In addition, gonadal disorders, mainly characterized by hypogonadism and increased androgen levels in women, manifesting with menstrual disturbances associated with hirsutism, acne, and alopecia, and hypogonadism with decrease of testosterone levels in men, manifesting with loss of libido and erectile dysfunction, can be also observed (**Fig. 1**; Pivonello *et al.*, 2016b, 2017).

CD has to be differentiated from diseases such as metabolic syndrome, visceral obesity, polycystic ovary syndrome, major depression, and chronic alcoholism which can present clinical and laboratory findings resembling those of Cushing syndrome and represent the so-called "pseudoCushing's states"(Newell-Price *et al.*, 1998; Findling *et al.*, 2017).

Epidemiology, Etiology and Physiopathology of Cushing Disease

Overall CS has an estimated prevalence of around 40 cases per million and an estimated incidence of 0.7–2.4 cases per million per year. The incidence of CD has been estimated to range from 1.2 to 2.4 per million. However, the worldwide epidemiology of both, CS and CD, has not been fully determined. CD is at least three times more prevalent in women than in men, and mainly occurs during the fourth to sixth decades of life (Pivonello *et al.*, 2015, 2016b).

In approximately 80% of cases, endogenous CS is a consequence of an ACTH hypersecretion (ACTH-dependent CS), generally due to a corticotroph pituitary tumor (pituitary-dependent CS, or CD, 70%), and, rarely, to an ACTH-secreting or corticotrophin releasing hormone (CRH)-secreting extra-pituitary tumor (ectopic CS, ECS, 10%) (Pivonello *et al.*, 2008; Newell-Price *et al.*, 2006; Pivonello *et al.*, 2016b). In the remaining 20% of cases, CS is independent from ACTH hypersecretion (ACTH-independent CS), and cortisol excess is a direct consequence of autonomous overproduction by the adrenal glands, because of unilateral benign or malignant adrenocortical tumors or bilateral macronodular adrenal hyperplasia (BMAH), or bilateral micronodular adrenal hyperplasia (BmAH) (**Table 1**; Pivonello *et al.*, 2008; Newell-Price *et al.*, 2006; Pivonello *et al.*, 2016b).

Cortisol, the main GC in humans, is produced from the adrenal glands under the control of the hypothalamus–pituitary–adrenal (HPA) axis. In physiological conditions, in response to several changes in the external and internal environment, including the stimulation from light or stress exposure, biological clocks regulation and nutrients balance, the hypothalamus produces CRH and arginine vasopressin (AVP), which, after the binding to their specific receptors (CRH-R1 and AVPR1B receptor, respectively), stimulate the corticotroph cells of the anterior pituitary to produce ACTH through the cleavage of the pro-opiomelanocortin (POMC). In turn, the ACTH, binding its specific receptor (MC2-R) on cells of the adrenal cortex and increasing the intracellular levels of cyclic AMP, stimulates the production of cortisol. The 24-hour profiles of ACTH and cortisol show a circadian rhythm with an early morning maximum, declining levels throughout daytime, a quiescent period of minimal secretory activity usually centered around midnight, and a sharp elevation during late sleep culminating in an early morning maximum (Oster *et al.*, 2017). The main regulation mechanisms of HPA axis includes: the circadian rhythm of ACTH and cortisol secretion; the negative feedback of cortisol on CRH and ACTH production and the response to stress (Papadimitriou and Priftis, 2009; Jacobson, 2005). These regulation mechanisms are generally compromised in CS (Raff *et al.*, 2014; Newell-Price *et al.*, 2006).

In *ACTH-dependent CS*, the hypercortisolism is consequence of an increased ACTH stimulation of adrenal gland. This ACTH stimulation involving both the fasciculate and reticularis adrenal layers can also promote the production of adrenal androgens and can determine bilateral adrenal hypertrophy and hyperplasia with possible micro/macronodular modifications (Raff *et al.*, 2014; Newell-Price *et al.*, 2006). As above mentioned, in the majority of cases the reason of the abnormal ACTH secretion is CD, generally caused by corticotroph pituitary adenomas, although corticotroph hyperplasia (not associated to a CRH producing tumor) or rarely corticotroph pituitary carcinomas are also possible (Newell-Price *et al.*, 2006; Biller *et al.*, 1992; Mampalam *et al.*, 1988; Young *et al.*, 1988; Holthouse *et al.*, 2001; Colao *et al.*, 2010). The majority of corticotroph pituitary tumors are microadenomas (maximal diameter <10 mm), whereas macroadenomas (maximal diameter >10 mm) are less frequent (about 10%) (Pivonello *et al.*, 2015; Newell-Price *et al.*, 2006; Raff *et al.*, 2014; Hofmann *et al.*, 2008). These tumors secrete an increased amount of ACTH, although macroadenomas can also secrete unprocessed POMC. Rarely, corticotroph pituitary tumors can be silent corticotroph tumors being unable to secrete large amounts of ACTH, but capable to secrete unprocessed POMC, and potentially causing tumor mass effects (Newell-Price *et al.*, 2006). CRH-R1 and AVPR1B receptors are expressed on the majority of corticotroph pituitary tumors that can therefore secrete ACTH in response to CRH and vasopressin, whereas these tumors have an impaired sensitivity to the negative feedback of cortisol on CRH and ACTH production (Newell-Price *et al.*, 2006; Raff *et al.*, 2014). These features give the rationale to the tests currently used in the diagnostic algorithm of CD.

Recently, progress has been made in understanding the genetic background of corticotroph pituitary tumors although the pathogenesis of these tumors still need to be better addressed (Lacroix *et al.*, 2015; Newell-Price *et al.*, 2006; Raff *et al.*, 2014; Perez-Rivas and Reincke, 2016). Pituitary corticotroph tumors derive from the clonal expansion of a single cell manifesting adaptive advantages acquired through one or few mutations and are generally sporadic; rarely pituitary corticotroph tumors are caused by germline gene alterations and arise in the context of familial syndromes (Lacroix *et al.*, 2015; Perez-Rivas and Reincke, 2016). Indeed, CD has been described in the context of multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant disease caused by germline mutations in the *MEN1* gene (encoding menin); multiple endocrine neoplasia type 4 (MEN4), a very rare condition caused by germline mutations in *CDKN1B* gene (encoding p27/KIP1), and familial isolated pituitary adenomas (FIPA), a syndrome, characterized by the familial occurrence of isolated pituitary tumors and caused, in about 20% of cases, by germline mutation in *AIP* gene (encoding aryl hydrocarbon receptor interacting protein) (Lacroix *et al.*, 2015; Perez-Rivas and Reincke, 2016). Recently, in pituitary corticotroph tumors, frequent (35%–62%) somatic mutations in the *USP8* gene have been found (Reincke *et al.*, 2015). The product of *USP8* gene is a deubiquitinase, which enhances the epidermal growth factor receptor (EGFR) signaling, by protecting activated EGFR from lysosomal degradation. This discovery has suggested a role of EGFR signaling in the pathogenesis of corticotroph pituitary tumors (Lacroix *et al.*, 2015; Perez-Rivas and Reincke, 2016; Reincke *et al.*, 2015). A potential role in the pathogenesis of corticotroph pituitary tumors has also been attributed to factors causing a reduced sensitivity to the cortisol feedback, such as an increased inactivation of cortisol by 11β hydroxysteroid dehydrogenase type 2 (11β-HSD2); an altered expression of brg1 (ATPase subunit of the Swi/Snf complex) or histone deacetylase-2 proteins, which are involved in glucocorticoid receptor (GR) signaling or rarely to mutations in *GR* gene (encoding glucocorticoid receptor) (Lacroix *et al.*, 2015; Perez-Rivas and Reincke, 2016; Dworakowska and Grossman, 2012). Other rare mutations have been described in *TP53* gene, which encodes for the tumor suppressor protein p53 (Lacroix *et al.*, 2015; Perez-Rivas and Reincke, 2016). Considering the increased incidence of CD in women, a role of estrogens in the pathogenesis of corticotroph pituitary tumors might also be hypothesized (Newell-Price *et al.*, 2006).

ACTH-dependent CS can also be caused by a large spectrum of non-pituitary ACTH-secreting or CRH-secreting tumors, mostly represented by small cell lung carcinomas and neuroendocrine tumors of the lung, thymus and pancreas, and less frequently medullary thyroid carcinomas, pheochromocytoma or other tumors. This type of CS is known as ECS (Lacroix *et al.*, 2015; Newell-Price *et al.*, 2006; Raff *et al.*, 2014; Alexandraki and Grossman, 2010).

In *ACTH-independent CS,* the hypercortisolism is consequence of an autonomous adrenal cortisol production, independent from the ACTH stimulation and caused by a heterogeneous group of diseases. In these conditions the GC negative feedback on hypothalamus and pituitary is preserved determining a reduction in circulating ACTH levels. Particularly, in adrenal tumors, hypercortisolism can be associated with hyperandrogenism because of a possible autonomous androgen production by the tumor (Lacroix *et al.*, 2015; Newell-Price *et al.*, 2006; Raff *et al.*, 2014). The most common causes of endogenous ACTH-independent CS are the cortisol secreting adrenocortical tumors (adrenocortical adenomas and less frequently adrenocortical carcinomas). Additional rare causes include BMAH and BmAH (Lacroix *et al.*, 2015; Newell-Price *et al.*, 2006).

In physiological conditions most of the effects of cortisol on peripheral tissues are mediated by its specific receptor GR, whereas the binding of cortisol to the mineralocorticoid receptor (MR) is prevented by 11β-HSD2 enzyme that converts cortisol to cortisone, which is unable to activate the MR. In CS the increased cortisol levels can overcome the capacity of 11β-HSD2 to inactivate cortisol, leading to potential MR activation with possible induction of systemic blood hypertension and hypokalemia (Newell-Price *et al.*, 2006). Cortisol regulates several physiological functions including glucose, lipid and protein metabolism, blood pressure, calcium and electrolyte balance, coagulation, immune, endocrine and reproductive functions, mood and cognition. These pleiotropic actions of GCs explain the metabolic, cardiovascular, musculoskeletal and neuropsychiatric diseases and the immune, endocrine, reproductive and sexual disorders observed in CS, and are responsible for the increased morbidity and mortality observed in CS syndrome (Pivonello *et al.*, 2016b; Newell-Price *et al.*, 2006).

Diagnosis of Cushing Syndrome

Although patients with CS usually present with the classical stigmata of hypercortisolism, the diagnosis of these patients can be challenging, because the clinical features of CS can largely overlap with the above mentioned "pseudoCushing's states" (Newell-Price *et al.*, 1998; Findling *et al.*, 2017). Despite several tests have been extensively used, none of them has been proven to be fully able to distinguish the affected patients from the non-affected subjects (Nieman *et al.*, 2008; Arnaldi *et al.*, 2003).

Therefore, the first step in the diagnostic algorithm of CD is the confirmation of CS (endogenous chronic hypercortisolism), trying to exclude "pseudoCushing's states." This step is based on a set of tests defined *screening tests*, characterized by an high sensitivity and including (1) urinary free cortisol (UFC); (2) late-night salivary cortisol (LNSC); (3) 1 mg-overnight dexamethasone (DMX) suppression test (DST); (4) low-dose DST (**Table 2**; Nieman *et al.*, 2008; Arnaldi *et al.*, 2003).

Urinary free cortisol (UFC) consists in the measurement of cortisol concentration in the urine of patients collected over a 24-h period. Due to the high intra-individual and inter-individual variability, at least two UFC measurements are required and their mean value is generally considered (Nieman *et al.*, 2008; Arnaldi *et al.*, 2003). Depending on the assay methods used, UFC sensitivity and specificity range from 38% to 100% and from 44% to 100%, respectively, with higher sensitivity and specificity reported with the use of new methodologies, such as the high performance liquid chromatography (HPLC) or liquid chromatography tandem mass spectrometry (LCMS/MS), and with lower sensitivity and specificity reported with the use of classical immunoassay (IA) methodologies (El-Farhan *et al.*, 2017; Elamin *et al.*, 2008; Nieman *et al.*, 2008).

An high fluid intake (more than 5 L/day), the use of substances as liquorice or carbenoxolone that inhibit the 11β-HSD2, hampering the conversion of cortisol in cortisone, the use of specific drugs such as carbamazepine and fenofibrate (if HPLC is

Table 2 Diagnosis of Cushing disease

Aim	*Phase of diagnosis*	*Type of test*
Confirmation of hypercortisolism/diagnosis of CS	Screening tests	• Urinary free cortisol • Late-night salivary cortisol • 1 mg-overnight DST • Low-dose DST
Confirmation of hypercortisolism/diagnosis of CS	Additional screening tests	• Midnight serum cortisol • DMX-CRH test • DDAVP test
Differential diagnosis	Noninvasive tests and procedures for differential diagnosis	• Plasma ACTH levels • High dose DST • CRH stimulation test • DDAVP stimulation test • Pituitary MRI • Other imaging techniques such as TC and/or MRI of the chest and/or abdomen (in case of CS different from CD)
	Invasive procedure for differential diagnosis	• BIPSS

ACTH, adrenocorticotropic hormone; *BIPSS*, bilateral inferior petrosal sinus sampling; *CD*, Cushing disease; *CRH*, corticotrophin releasing hormone; *CS*, Cushing syndrome; *DDAVP*, desmopressin; *DMX-CRH*, dexamethasone-CRH; *DST*, dexamethasone suppression test; *MRI*, magnetic resonance imaging; *TC*, computerized tomography.

used), or the use of some synthetic GCs such as prednisolone and methylprednisolone (if IA is used) might cause false positive results in UFC measurement, whereas moderate to severe renal failure (creatinine clearance <60 mL/min) might cause false negative results (Nieman *et al.*, 2008).

Late-night salivary cortisol (LNSC) is a simple test to measure cortisol through saliva collections. Because the physiological reduction of cortisol secretion at evening is generally compromised in CS patients, salivary cortisol is evaluated at late-night and at least two separate samples are collected between 23.00 and 24.00 h. The sensitivity and specificity of this test range from 92% to 100% and from 93% to 100%, respectively, with a variability attributed to the different assays used (Nieman *et al.*, 2008). Normal validated ranges are assay-dependent, requiring local laboratory validations (Nieman *et al.*, 2008; Arnaldi *et al.*, 2003; El-Farhan *et al.*, 2017). Although further validation are required, according to Endocrine Society Clinical Practice Guidelines, the best validated assays are enzyme-linked immunosorbent assay (ELISA) and LCMS/MS; considering these two techniques, salivary cortisol levels at bedtime, or between 23.00 and 24.00 h, higher than 145 ng/dL (4 nmol/L) suggest CS (Nieman *et al.*, 2008).

The chew of liquorice or tobacco, which contain the 11β-HSD2 inhibitor glycyrrhizic acid, smoke of cigarettes, variability in bedtime, night work, depression, critical illness, eating, stress, salivette contamination with steroid contained in lotions or oral gels, sample contamination with blood (including blood leakage, periodontal diseases and vigorous tooth brushing) might cause false positive results in LNSC (Nieman *et al.*, 2008). Lastly, although the influence of gender, age, and coexisting medical conditions has not been fully characterized, men aged 60 years or older, diabetic and hypertensive subjects seem to have a high percentage of false positive results (Nieman *et al.*, 2008).

Overnight dexamethasone (DMX) suppression test (DST) test, also known as 1 mg DST or *Nugent test* is performed administrating to the patient 1 mg of DMX between 23.00 and 24.00 h, and measuring serum cortisol between 8.00 and 9.00 h on the following morning. Patients with CS are less sensitive to the negative feedback exerted by GC, therefore in these patients the administration of 1 mg of DMX is not followed by an inhibition of endogenous cortisol production. The test is considered positive and suggestive of CS when serum cortisol levels are equal or above the cut-off of 1.8 μg/dL (50 nmol/L) with a sensitivity of 98%–100%, and a specificity of 58%–80% (Nieman *et al.*, 2008; Arnaldi *et al.*, 2003; Wood *et al.*, 1997; Pecori Giraldi *et al.*, 2007).

Variable absorption and metabolism of DMX might influence the DST results. Particularly, drugs such as phenobarbital, phenytoin, carbamazepine, rifampicin and alcohol, accelerate DMX metabolism, reducing the plasma DMX concentrations, and causing false positive results. All conditions that can increase the cortisol binding globulins (CBG) such as the use of oral contraceptive pill or mitotane might also cause false positive results (Nieman *et al.*, 2008). Conversely, drugs such as itraconazole, ritonavir, fluoxetine, diltiazem and cimetidine, decrease DMX metabolism causing false negative results. False negative results might also occur in case of liver and/or renal failure, due to decreased DMX metabolism (Nieman *et al.*, 2008).

Low-dose dexamethasone suppression test, also known as 48 h 2 mg DST or *low-dose Liddle test* consists in the administration of 0.5 mg of DMX every 6 h, for 48 h, starting at 9.00 h or at 12.00 h of the first day. Serum cortisol should be assessed at 9.00 h or at 8.00 h, 6 or 2 h after the last DMX dose, accordingly to different protocols (Nieman *et al.*, 2008). As the above reported Nugent test, also the low-dose Liddle test is considered positive and suggestive of CS when serum cortisol levels are equal or above the cut-off of 1.8 μg/dL (50 nmol/L) with a sensitivity of 98%–100% and specificity of 70%–88% (Nieman *et al.*, 2008; Wood *et al.*, 1997; Martin *et al.*, 2006). False positive and negative results might occur in the conditions reported above for Nugent test.

In case the performed screening test results abnormal, or in case of normal test results associated with the persistence of a high clinical suspicion of CS, the diagnostic algorithm should continue performing additional screening tests among the above-mentioned tests, or one of the following tests: (1) midnight serum cortisol; (2) dexamethasone-CRH test; (3) desmopressin (DDAVP) test (**Table 2**; Nieman *et al.*, 2008; Arnaldi *et al.*, 2003). These procedures require an appropriate expertise in the execution and interpretation, therefore they should be performed in specialized endocrine centers.

Midnight serum cortisol can be performed in a sleeping or awake state. The physiological reduction of cortisol secretion at evening is generally compromised in CS patients. In case of sleeping procedure, blood must be drown through an in-welling line or within 5–10 min of waking patients. Values higher than 1.8 μg/dL (50 nmol/L) are suggestive of CS, with a high sensitivity (98%–100%) but poor specificity (18%–26%) (Nieman *et al.*, 2008; Pecori Giraldi *et al.*, 2007; Vilar *et al.*, 2007).

In case of awake procedure, values higher than 7.5 μg/dL (207 nmol/L) are suggestive of CS, with a high sensitivity (56%–100%) (Nieman *et al.*, 2008; Papanicolaou *et al.*, 1998; Friedman *et al.*, 2007) and specificity (83%–100%) (Nieman *et al.*, 2008; Vilar *et al.*, 2007; Friedman *et al.*, 2007). Hospitalization stress and all conditions that can increase CBG levels might cause false positive results in the measurement of midnight serum cortisol (Nieman *et al.*, 2008).

Dexamethasone-CRH test (DMX-CRH test) is performed administering 48 h 2 mg-DMX followed by 1 μg/kg/i.v. CRH 2 h after the last DMX dose, with measurement of serum cortisol 15 min after CRH injection. This combined test has been developed with the scope to improve the diagnostic accuracy of DST particularly in the discrimination between patients with CS as compared with pseudo-Cushing's states. Patients with CS as compared with pseudo-Cushing's states are expected to have an altered sensitivity to both DMX and CRH, therefore a response to DMX-CRH test is expected in individuals with CS but not in those with pseudo-Cushing's states and a mild degree of hypercortisolism (Yanovski *et al.*, 1993; Newell-Price *et al.*, 1998). CRH after DMX elevates plasma ACTH and cortisol levels in the majority of patients with CD but not in normal individuals or patients with pseudo-Cushing's states. Serum cortisol levels higher than 1.4 μg/dL (38 nmol/L) have been proposed to be suggestive of CS, with sensitivity rates of 94%–100% and specificity rates of 60%–100% (Nieman *et al.*, 2008; Martin *et al.*, 2006; Pecori Giraldi *et al.*, 2007; Yanovski *et al.*, 1993; Reimondo *et al.*, 2008). However, the diagnostic accuracy and the advantages of DMX-CRH test over the other screening tests remain to be validated. False positive or false negative results might occur in the same conditions above mentioned for other DMX tests (Newell-Price *et al.*, 1998; Nieman *et al.*, 2008).

Desmopressin (DDAVP) test is performed administrating 10 μg i.v. DDAVP, a vasopressin analogue with measurement of plasma ACTH levels 30 or 15 min before, and at time 0, 15, 30, 45, 60, 90 and 120 min after the injection of DDAVP. DDAVP elevates plasma ACTH and cortisol levels in the majority of patients with Cushing's disease but not in normal individuals or patients with pseudoCushing's states. Using as criteria to suggest CD diagnosis a Δ-ACTH increase ≥6 pmol/L (>27 pg/mL) versus the baseline, this test has shown a sensitivity of 75%–87%, and a specificity of 90%–91% (Findling and Raff, 2017), however it remains still poorly validated and therefore is not recommended as a routinely screening diagnostic test (Nieman *et al.*, 2008).

Diagnosis of Cushing Disease

Once the diagnosis of CS has been established the following step in the diagnostic algorithm is to distinguish CD from different possible causes of endogenous CS (differential diagnosis) and possibly to visualize the anatomical cause by imaging techniques. To this purpose several methodologies are required, including: (1) the measurement of plasma ACTH levels; (2) the high dose DST; (3) the CRH stimulation test; (4) the DDAVP stimulation test; (5) pituitary magnetic resonance imaging (MRI) and (6) the bilateral inferior petrosal sinus sampling (BIPSS) (**Table 2**; (Arnaldi *et al.*, 2003; Newell-Price *et al.*, 1998).

Plasma ACTH is measured at morning using non-haemolysed EDTA-coated plastic tubes mostly by fully automated IA. Samples have to be preserved at cool temperature prior to be rapidly processed (Arnaldi *et al.*, 2003; Newell-Price *et al.*, 1998). ACTH measurement represents the first step in differential diagnosis of CS. Suppressed ACTH levels (<2 pmol/L or 10 pg/mL) suggests an adrenal ACTH-independent CS form, whereas non-suppressed ACTH levels (>4 pmol/L or 20 pg/mL) suggests an ACTH-dependent CS form (Arnaldi *et al.*, 2003).

In case of intermediate ACTH levels (2–4 pmol/L or 10–20 pg/mL), the differential diagnosis between ACTH-dependent versus ACTH-independent CS forms requires a CRH stimulation test, which shows an emphatic ACTH response in ACTH-dependent forms and a blunted ACTH response in ACTH-independent forms (Arnaldi *et al.*, 2003).

Baseline ACTH levels may also suggest the origin of ACTH secretion given that ACTH levels tend to be higher in ECS than in CD, but this distinction requires further investigation with high dose DST or CRH test as reported below. These tests rely on the fact that the tumoral corticotroph cells of pituitary tumors, although partly autonomous, retain some features of normal corticotroph cells (i.e., sensitivity to glucocorticoid feedback and CRH stimulation). In contrast, tumors causing ECS are generally different from corticotroph cells and are not likely to respond to glucocorticoid or CRH administration.

High-dose DST or 8 mg DST or high-dose Liddle test is performed administrating DMX according with one of the following protocols: (1) oral administration of 2 mg of DMX every 6 h, for 48 h, starting at 9.00 h or at 12.00 h of the first day and assessment of serum cortisol at 9.00 h or at 8.00 h or alternatively, 6 or 2 h after the last dose of DMX; (2) oral administration of 8 mg DMX in one single dose at 23.00 h and assessment of serum cortisol between 8.00 and 9.00 h on the following morning; (3) intravenous DMX infusion at a rate of 1 mg/h for 5–7 h (5–7 mg) and assessment of serum cortisol before and at the end of infusion (Newell-Price *et al.*, 1998). A suppression of serum cortisol (>50% vs. basal values after oral 8 mg DST and after 5 mg intravenous test; or >190 nmol/L vs. basal values after 7 mg intravenous test) suggests CD with a sensitivity and specificity within the ranges of 65%–100% and 60%–100%, respectively (Newell-Price *et al.*, 1998). False positive or false negative results might occur in the same conditions above mentioned for other DMX tests. Additionally, a false-positive response might be observed in some cases of ECS, especially some benign differentiated carcinoid tumors, which can be sensitive to GC feedback inhibition.

CRH stimulation test is performed administrating 1 μg/kg or 100 μg of CRH i.v. with measurement of ACTH and serum cortisol 15 min before at time 0, 15, 30, 45, 60, 90 and 120 min after the CRH injection (Arnaldi *et al.*, 2003; Newell-Price *et al.*, 1998). In the majority of patients with CD a marked rise in plasma ACTH and cortisol is observed, whereas the hormonal response is modest or absent in patients with ECS. A rise over the baseline in plasma ACTH> of 50% or > of 35% at time 15′–30′ or a rise in cortisol>20% over the baseline can identify patients with CD with a sensitivity and a specificity of 86%–93% and 95%–100% respectively (Arnaldi *et al.*, 2003; Newell-Price *et al.*, 1998). False positive response to CRH test might be rarely observed in ECS particularly in some bronchial carcinoid tumor (Newell-Price *et al.*, 1998).

DDAVP stimulation test is performed as above mentioned. As for the CRH test, in the majority of patients with CD a rise in plasma ACTH and cortisol is expected. A rise from baseline in plasma cortisol of 20% or more than four times the intra-assay coefficient of variation suggests CD with a reported sensitivity of 84% and specificity of 83%. A rise in plasma ACTH of 35% suggests CD with a reported sensitivity of 77% and specificity of 73% (Newell-Price *et al.*, 1998). A false positive DDAVP response might be observed also in 20%–50% of ECS. Because the limited experience with this test, CRH test should be preferred (Arnaldi *et al.*, 2003; Newell-Price *et al.*, 1998).

Pituitary MRI should be performed in case of biochemical suspect of CD. Pituitary corticotroph tumors are visualized by MRI with gadolinium enhancement in up to 70% of cases due to their extremely small size (mostly 3–5 mm). In patients with classic clinical presentation and dynamic biochemical studies compatible with CD, the presence of a focal lesion (>6 mm) on pituitary MRI may provide a definitive diagnosis (Arnaldi *et al.*, 2003; Vitale *et al.*, 2017). However, in about 10%–20% of the general population pituitary imaging might also visualize small lesions (e.g., incidentalomas, which are incidentally discovered pituitary tumors), that might be erroneously interpreted as ACTH-secreting pituitary tumors (Arnaldi *et al.*, 2003; Scangas and Laws, 2014). For this reason, in patients showing discordant biochemical tests and/or a pituitary MRI negative or showing a pituitary tumor <6 mm, an additional evaluation, by using BIPSS, should be performed (Arnaldi *et al.*, 2003). In case of CS different from CD,

other imaging techniques such as computerized tomography and/or MRI of the chest and/or abdomen are required (Arnaldi *et al.*, 2003; Isidori *et al.*, 2015b).

BIPSS. When the diagnosis of CD cannot be firmly established by previously reported noninvasive procedures, BIPSS needs to be performed, preferably in a highly specialized center. This technique requires the catheterization of both the right and left inferior petrosal sinuses, which drain the respective hemipituitaries, and the collection of ACTH samples simultaneously from both petrosal sinuses and a peripheral vein. The ratio between petrosal and peripheral plasma ACTH concentrations, obtained before and after stimulation with CRH, permits to distinguish CD from ECS. If the gland harbors a corticotroph tumor, higher ACTH levels in the veins draining the pituitary are observed, whereas if the source of ACTH is outside from pituitary, petrosal ACTH levels does not differ from peripheral concentrations. A baseline center/periphery ratio greater than 2 and/or greater than 3 after CRH stimulation supports a pituitary source of ACTH hypersecretion with high sensitivity and specificity (95%–99%), representing the "gold standard criteria" in the diagnosis of CD (Arnaldi *et al.*, 2003). However, false negative results might occur in CD patients with anomalous venous drainage. IPSS might also provide useful information for identification of the site of the tumor within the pituitary, but its predictive value remains controversial. Despite the fact that BIPSS is an invasive procedure, the occurrence of adverse events is extremely rare (severe adverse events have a prevalence lower than 1%), particularly if it is performed by experienced operators in referral centres. They mainly include: deep vein thrombosis, pulmonary emboli and brain stem vascular damages (Arnaldi *et al.*, 2003; Pecori Giraldi *et al.*, 2015; Zampetti *et al.*, 2016).

Therapy

The aims of treatment OF CD are: (1) the normalization of cortisol secretion; (2) the reversal of clinical picture in terms of clinical signs and symptoms; (3) the prevention or improvement of concomitant comorbidities; and (4) the long-term disease control without recurrence (Nieman *et al.*, 2015; Pivonello *et al.*, 2015). The main treatment approaches include pituitary surgery, which usually represents the first-line treatment, and repeat pituitary surgery, pituitary radiotherapy, adrenal surgery and medical treatment, which usually represent second-line approaches (Nieman *et al.*, 2015; Pivonello *et al.*, 2015).

Pituitary surgery is the first line treatment for CD, consisting in selective removal of the pituitary tumor, mainly performed by a transsphenoidal approach with microscopic or endoscopic techniques (Nieman *et al.*, 2015; Pivonello *et al.*, 2015). Currently, the historical transcranial approach is rarely used for selected patients with tumors of large size (Pivonello *et al.*, 2015). The overall mean (m) initial remission rate of pituitary surgery is 77.8%, ranging from 25% to 100%. Initial remission rate is lower for macrodenomas ($m = 62.3\%$; 30.8%–100%) than microdenomas ($m = 82.1\%$; 48.7%–100%). Persistence or recurrence of disease are common accounting for the high rate of long-term failure of pituitary surgery ($m = 31.7\%$; 0%–75%). Particularly, macroadenomas show a higher recurrence rate ($m = 18.8\%$; 0%–59%) than microadenomas ($m = 11.7\%$; 0%–36.4%). The recurrence risk is higher within the first 10 years, but it is still present even 30 years later (Mampalam *et al.*, 1988; Hofmann *et al.*, 2008; Lambert *et al.*, 2013; Jagannathan *et al.*, 2009; Invitti *et al.*, 1999; Patil *et al.*, 2008a; Cavagnini and Pecori Giraldi, 2001). Factors that might negatively influence pituitary surgery outcome include: male gender, tumor size (>2 cm), tumor with suprasellar extension or cavernous sinus invasion, undetected pituitary tumor at pre-surgical MRI or during surgical procedure, the absence of histological confirmation, the presence of peritumoral Crooke's cells and the inexpertise of surgeon (Pivonello *et al.*, 2015; Hammer *et al.*, 2004; Cannavo *et al.*, 2003; Wagenmakers *et al.*, 2013; Ciric *et al.*, 1997; Bigos *et al.*, 1980; Mampalam *et al.*, 1988; Invitti *et al.*, 1999; Jagannathan *et al.*, 2009). Particularly, when performed by skilled pituitary surgeons pituitary surgery has a low risk of complications, mainly including hypopituitarism and diabetes insipidus, occurring in 29.6% (0.9%–93.3%) and in 12.3% (0.9%–32.5%) of cases, respectively (Mampalam *et al.*, 1988; Pivonello *et al.*, 2015; Hofmann *et al.*, 2008; Cavagnini and Pecori Giraldi, 2001; Invitti *et al.*, 1999; Jagannathan *et al.*, 2009; Lambert *et al.*, 2013; Patil *et al.*, 2008a). Myocardial infarction, pneumonia infection and meningitis, are the main mortality causes, with a mean mortality rate of 0.6% (0%–7.1%) (Pivonello *et al.*, 2015).

Repeat pituitary surgery is one of the second line treatment options currently available for patients with persistent or recurrent CD after a first pituitary surgery. This approach is mainly suggested in patients with a clear evidence of a residual tumor at pituitary imaging (Pivonello *et al.*, 2015; Nieman *et al.*, 2015), although the remission rates (58%, 30%–87.5%) are lower and the recurrence rates ($m = 16.1\%$; 0%–60%) are higher than those of the first surgery (Pivonello *et al.*, 2015; Benveniste *et al.*, 2005; Hofmann *et al.*, 2008; Patil *et al.*, 2008b). Main negative predictive factors are residual tumor localization and size (Pivonello *et al.*, 2015; Benveniste *et al.*, 2005; Hofmann *et al.*, 2008; Patil *et al.*, 2008b). Hypopituitarism is the most common complication ($m = 38\%$; 9.1%–78.6%) (Pivonello *et al.*, 2015; Benveniste *et al.*, 2005; Hofmann *et al.*, 2008; Patil *et al.*, 2008b).

Pituitary radiotherapy is generally suggested as a second line option in case of pituitary surgery failure, especially with aggressive and/or invasive tumors, although it might rarely represent a first line approach, in patients without indication for surgery, with contraindications to surgery, and in patients who refuse surgery (Pivonello *et al.*, 2015; Nieman *et al.*, 2015). Currently, the pituitary radiotherapy is mainly used as third line option, in patients who are unresponsive or intolerant to medical treatment, or after a period of hypercortisolism control by medical therapy (Pivonello *et al.*, 2015). The historically used technique was conventional radiotherapy (CRT), which delivers ionizing radiation to the target tumor in small daily doses, over a period of 25–30 days. In terms of hypercortisolism control, the mean remission rate of CRT is 63.8% (19.6%–100%) (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994). In terms of tumor growth control (growth arrest or tumor size reduction) the mean remission rate is 98.5% (53%–100%) (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and

Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994). Currently, the most commonly used technique is stereotactic radiotherapy (SRT), which provides the precise identification of tumor localization, with three spatial coordinates. SRT can be delivered as a fractionated treatment (stereotactic conformal radiotherapy) or as a single treatment (stereotactic radiosurgery) using a multi-headed cobalt unit (Gamma Knife), a linear accelerator system (LINAC), or a proton-beam system (Proton-beam) (Pivonello *et al.*, 2015). In terms of hypercortisolism control, the mean remission rate of SRT is 60.8% (10%–100%), with a mean recurrence rate of 12.3% (0%–100%) (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994); whereas, in terms of tumor growth control, the mean remission rate is 90.9% (50%–100%) (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994). Treatment response rates appear similar using the different SRT methods (Pivonello *et al.*, 2015). Potential predictors of SRT outcome include radiation dose and the use of medical treatments with a radioprotective effect. However, these predictors need further validation (Pivonello *et al.*, 2015).

The most frequent complication of radiotherapy is hypopituitarism, which ranges from 0% to 100% ($m = 39.3\%$) using CRT, from 0% to 66% ($m = 22.9\%$) using Gamma Knife, from 0% to 40% ($m = 17.\%$) using LINAC, and from 0% to 52% ($m = 26\%$) using proton-beam method (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994). The incidence and severity of hormonal deficiencies increases over the time from the procedure (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994). Other complications include cerebrovascular accidents, mainly reported after CRT (incidence of 4%, 11% and 21% after 5, 10 and 20 years, respectively), an higher incidence of secondary brain tumors, especially in patients who receive high radiation doses and CRT (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994), and optic neuropathy, a specific complication of SRT (incidence of up to 11.1% of cases) (Pivonello *et al.*, 2015; Estrada *et al.*, 1997; Orth and Liddle, 1971; Minniti *et al.*, 2007; Tsang *et al.*, 1994).

Bilateral adrenalectomy is currently considered as a second or third line option, after failure of previous treatments and is reserved to a limited number of patients with a very severe CD. Very rarely bilateral adrenalectomy can be used as first line option to obtain an immediate hypercortisolism resolution (Nieman *et al.*, 2015). In CD patients, the most commonly used technique is the laparoscopic approach, which represents the gold standard, whereas the laparotomic approach is currently used only in case of contraindications for laparoscopic surgery (Pivonello *et al.*, 2015). The mean remission rate is 96.8% (78.9%–100%), with a mean persistence or recurrence rate of 1.7% (0%–12%), mainly due to adrenal rests (Pivonello *et al.*, 2015; Ernest and Ekman, 1972; Kelly *et al.*, 1983; Welbourn, 1985; Nagesser *et al.*, 2000; Chow *et al.*, 2008; Smith *et al.*, 2009; Ding *et al.*, 2010). The most important complication of bilateral adrenalectomy is the permanent adrenal insufficiency in all patients, which requires lifelong replacement treatment with GC and sometime also mineralocorticoids (Pivonello *et al.*, 2015). Another important complication ($m = 18.6\%$; 0%–34.6%) is the possible ACTH-secreting tumor progression (Nelson syndrome) due to the loss of the negative feedback exerted by GCs on ACTH-secreting cells (Pivonello *et al.*, 2015; Ernest and Ekman, 1972; Kelly *et al.*, 1983; Welbourn, 1985; Nagesser *et al.*, 2000; Chow *et al.*, 2008; Smith *et al.*, 2009; Ding *et al.*, 2010). Cardiovascular events, mainly myocardial infarction and secondary postoperative hemorrhages, are the main mortality causes, with a mean mortality rate of 1.8% (0%-–11.1%) (Pivonello *et al.*, 2015).

Medical treatment is gaining a more important role in different steps of the treatment algorithm particularly in patients with CD (Ferone *et al.*, 2014; Pivonello *et al.*, 2015). Currently, medical treatment plays a role in several situations: as preoperative treatment, in order to improve the clinical picture; as second line treatment after pituitary surgery, in patients with persistent or relapsed CD; before or after pituitary radiotherapy, to control hypercortisolism while awaiting for radiation effects and more recently as first line alternative treatment in case of contraindication to surgery or in case of patients refusal of surgery (Pivonello *et al.*, 2015). Available drugs can be divided in three main categories: (1) the pituitary-directed drugs; (2) the adrenal-directed drugs (steroidogenesis inhibitors); (3) the glucocorticoid receptor antagonist (Pivonello *et al.*, 2015).

Pituitary-directed drugs include two main compounds: the dopamine agonist, cabergoline, and the somatostatin analogue, pasireotide. Although they are effective only in a group of patients with CD, these compounds represent the ideal medical approach for CD management because they act directly on the cause of CD (Ferone *et al.*, 2014; Pivonello *et al.*, 2015).

Cabergoline is currently an off-label treatment for CD. This drug is an agonist of dopamine receptors type 2, which are often expressed in ACTH-secreting pituitary tumors (Pivonello *et al.*, 2015). In terms of hypercortisolism control, cabergoline (orally administered; dosages of 0.5–7 mg/week) has shown a mean remission rate of 32.7% (25%–40%) associated with a clinical improvement, particularly in hypertension and glucose metabolism, and with potential tumor shrinkage. However, a mean escape rate of 25% (18.2%–33.3%) has also been reported (Pivonello *et al.*, 2009, 2015; Vilar *et al.*, 2010; Barbot *et al.*, 2014; Lila *et al.*, 2010; Godbout *et al.*, 2010; Ferriere *et al.*, 2017). Cabergoline is very well tolerated with rare adverse events reported, including hypotension and severe asthenia ($m = 14\%$; 10%–20%), dizziness and nausea ($m = 16.5\%$; 5%–25%) (Godbout *et al.*, 2010; Pivonello *et al.*, 2009; Vilar *et al.*, 2010; Ferriere *et al.*, 2017). The increased risk of cardiac valve diseases, reported using cabergoline at the higher doses for neurological disorders, seems to be less important at the dosage used for CD (Pivonello *et al.*, 2009; Vilar *et al.*, 2010; Barbot *et al.*, 2014; Lila *et al.*, 2010; Godbout *et al.*, 2010). Because the limited experience with cabergoline in CD, further investigation are still required to define the role of this compound in CD management.

Pasireotide is the first medical therapy officially approved for the treatment of adult CD patients, which experienced a failure of pituitary surgery, or are not candidates for pituitary surgery and require medical therapeutic intervention. This compound is a recently developed multi-ligand somatostatin analogue with high affinity for somatostatin receptor type 5, which is highly expressed in ACTH-secreting pituitary tumors (Pivonello *et al.*, 2015). Pasireotide (s.c. administered twice daily; dosage of 300–900 μg), has shown remission rates, in terms of hypercortisolism control, ranging from 17% to 22.2% in the short-term

follow-up (shorter or equal 12 months), and reaching 68.8% of cases in the long-term follow-up (up to 5 years) (Boscaro *et al.*, 2009; Colao *et al.*, 2012; Schopohl *et al.*, 2015; Petersenn *et al.*, 2017; Trementino *et al.*, 2016). Pasireotide is generally well tolerated and it has also been associated with a clinical improvement (facial rubor, bruising, supraclavicular and dorsal fat pads, waist circumference, weight, body mass index, blood pressure, total and LDL-cholesterol levels, depression and QoL) and a reduction of pituitary tumor volume (in patients at 12-months follow-up; $m = 73.2\%$; 46.3%–100%) (Pivonello *et al.*, 2014, 2015; Simeoli *et al.*, 2015; Colao *et al.*, 2012). The most frequently reported significant adverse events include hyperglycemia-related events (72.8%), diarrhea (58%), nausea (52%), cholelithiasis-related events (30%), mild transient elevations in liver enzyme levels (29%), headache (28%), abdominal pain (24%), fatigue (19%) and asthenia (11%), hypocortisolism-related events (8%) and prolongation of the corrected QTc interval (2%) (Colao *et al.*, 2012). During treatment, glycemia need to be carefully monitored, and when appropriate an antidiabetic therapy should be initiated to maintain glycemic control (Colao *et al.*, 2014).

Pasireotide long acting release (LAR), a new formulation with monthly administration at a dosage of 10–30 mg, has been recently investigated and it seems to be an efficacious treatment option for some patients with CD (Lacroix *et al.*, 2018).

Adrenal-directed drugs include several compounds that reduce cortisol production by inhibiting different enzymes involved in steroidogenesis (steroidogenesis inhibitors). Among these compounds ketoconazole, metyrapone and mitotane are detailed in this chapter, but few other compounds (aminoglutethimide and etomidate) have been historically used with evidence of effects in subgroups of patients (i.e., etomidate in patients with conditions of emergency) and two new compounds (osilodrostat and levoketoconazole) are currently under investigation. These drugs can be used not only for CD, but also for other forms of endogenous CS (Pivonello *et al.*, 2015).

Ketoconazole, officially approved for the treatment of patients older than 12 years, suffering from CS, is an imidazole derivative, initially used as antifungal drug (Loose *et al.*, 1983; Feldman, 1986; Sonino, 1987). Apart from adrenal blocking effects, some preclinical studies suggest that ketoconazole might also have direct effects on corticotroph tumor cells in patients with CD (Feelders *et al.*, 2010b; Jimenez Reina *et al.*, 1989). Ketoconazole (orally administered; twice/thrice daily; dosage of 200–1200 mg), has shown a mean remission rate of 68.4% (44.7%–92.9%), although a mean escape rate of 14.4 (7.1%–22.7%) has been reported (Pivonello *et al.*, 2015; Sonino *et al.*, 1991; Moncet *et al.*, 2007; Castinetti *et al.*, 2008; van den Bosch *et al.*, 2014; Castinetti *et al.*, 2014; Espinosa-de-Los-Monteros *et al.*, 2017). This treatment has also been associated with clinical improvement particularly in body weight, hirsutism, myopathy and muscle weakness, bone status, psychiatric symptoms, glucose metabolism and blood pressure (Sonino *et al.*, 1991; Moncet *et al.*, 2007; Castinetti *et al.*, 2008; van den Bosch *et al.*, 2014; Castinetti *et al.*, 2014). The most severe and frequent adverse event related to ketoconazole is hepatotoxicity (2.6%–18.7%) which requires a careful monitoring of liver enzymes, particularly within the first month of treatment, although fatal hepatitis have been only rarely reported (Sonino *et al.*, 1991; Moncet *et al.*, 2007; Castinetti *et al.*, 2008; van den Bosch *et al.*, 2014; Castinetti *et al.*, 2014; Duarte *et al.*, 1984; Pivonello *et al.*, 2015). Additional adverse events include gastrointestinal disturbances, skin rash, adrenal insufficiency, pruritus, fatigue, headache, and gynecomastia (Sonino *et al.*, 1991; Moncet *et al.*, 2007; Castinetti *et al.*, 2008; van den Bosch *et al.*, 2014; Castinetti *et al.*, 2014). Ketoconazole can inhibit gonadal testosterone synthesis, resulting in hypogonadism, for this reason it should not be preferred in men (Pivonello *et al.*, 2015).

Metyrapone, officially approved for treatment of patients suffering from CS, is a pyridine derivative (Chart *et al.*, 1958; Liddle *et al.*, 1958; Carballeira *et al.*, 1976; Gower, 1974). Metyrapone (orally administered four to six times daily; dosage of 500-–6000 mg) has shown a mean remission rate of 71% (45.4%–100%), in terms of hypercortisolism control, although a mean escape rate of 7.8% (0%–18.7%) has been reported (Jeffcoate *et al.*, 1977; Thoren *et al.*, 1985; Verhelst *et al.*, 1991; Valassi *et al.*, 2012; van den Bosch *et al.*, 2014; Pivonello *et al.*, 2015). This treatment has also been associated with clinical improvement particularly in facial plethora, round face, muscle weakness, psychiatric symptoms, glucose metabolism and blood pressure (Jeffcoate *et al.*, 1977; Thoren *et al.*, 1985; Verhelst *et al.*, 1991; Valassi *et al.*, 2012; Igaz *et al.*, 2008). The most frequent adverse events are hyperandrogenism in women (4.5%–71.4%), together with hypertension (48.4%) and hypokalemia (6.7%–13.6%) (Jeffcoate *et al.*, 1977; van den Bosch *et al.*, 2014; Thoren *et al.*, 1985; Verhelst *et al.*, 1991; Valassi *et al.*, 2012; Igaz *et al.*, 2008; Chart *et al.*, 1958; Liddle *et al.*, 1958; Carballeira *et al.*, 1976; Gower, 1974; Pivonello *et al.*, 2015). These adverse events are related to the accumulation of androgens and cortisol or aldosterone precursors stimulated by the ACTH increase, which is consequent to the reduction of cortisol feedback on pituitary. Because of the potential increase of androgens, this drug should not be preferred in women (Feelders *et al.*, 2010b; Igaz *et al.*, 2008). Less frequently reported adverse events include dizziness, headache, arthralgia, myalgia, fatigue, gastrointestinal disturbances, adrenal insufficiency and skin rash (Jeffcoate *et al.*, 1977; Thoren *et al.*, 1985; Verhelst *et al.*, 1991; Valassi *et al.*, 2012; van den Bosch *et al.*, 2014; Feelders *et al.*, 2010b).

Mitotane officially approved for treatment of patients suffering from adrenocortical carcinoma, has been occasionally used in CD treatment. Mitotane, a dyphenilmethane derivative, inhibits different enzymes involved in adrenal steroidogenesis and shows a direct adrenolytic effect (Vilar and Tullner, 1959; Young *et al.*, 1973). Mitotane (orally administered, three daily; dosage of 1–12 g/day), has shown a mean remission rate of 86.9% (71.6%–100%), in terms of hypercortisolism control, without escape phenomenon (Orth and Liddle, 1971; Luton *et al.*, 1979; Schteingart *et al.*, 1980; Baudry *et al.*, 2012; Pivonello *et al.*, 2015). However, this compound has many interactions with other drugs (mainly through the activation of CYP3A4) and is associated with important adverse events, mainly including gastrointestinal disturbances, lipid disorders, neurological manifestations, gynecomastia, liver enzymes increase, leukopenia, skin rash, hypersialorrhea and chloasma (Orth and Liddle, 1971; Luton *et al.*, 1979; Schteingart *et al.*, 1980; Baudry *et al.*, 2012; Pivonello *et al.*, 2015). Therefore, mitotane plasma concentrations should be monitored, and maintained between 8.5 and 18 mg/L. Additionally, to avoid adrenal insufficiency, hydrocortisone should be co-

administered at dosage higher than those used for other causes of hypocortisolism (Baudry *et al.*, 2012; Luton *et al.*, 1979; Pivonello *et al.*, 2015).

The only *glucocorticoid receptor antagonist* currently developed is mifepristone, officially approved in USA for the treatment of patients suffering from CS associated with diabetes mellitus or impairment of glucose tolerance. This compound acts by blocking central and peripheral GR, and consequently antagonizing cortisol receptor binding. This effect determines a rise in plasma ACTH and serum cortisol levels, making the measurement of these hormones inadequate to monitor the treatment efficacy (Jung-Testas and Baulieu, 1983; Bertagna *et al.*, 1984). For this reason, treatment efficacy can only be evaluated by monitoring clinical parameters (Morgan and Laufgraben, 2013). Mifepristone (orally administered, once daily; dosage of 300–1200 mg), has shown an efficacy rate up to 87%, in terms of clinical picture improvement, including decrease in body weight, waist circumference and body fat, and increase in insulin sensitivity (Sitruk-Ware and Spitz, 2003; Castinetti *et al.*, 2009; Fleseriu *et al.*, 2012). The most important adverse events include endometrial thickening (38.5%), due to the antagonism of mifepristone on progesterone and androgen receptors, hypokalemia (25%–34%) and hypertension (24%–25%), both due to the effect of hypercortisolism on MR. Additional common adverse events include gastrointestinal disturbances, fatigue, headache, arthralgia, peripheral edema, adrenal insufficiency and dizziness (Castinetti *et al.*, 2009; Fleseriu *et al.*, 2012; Castinetti *et al.*, 2012; Castinetti *et al.*, 2010; Fleseriu *et al.*, 2013; Pivonello *et al.*, 2015).

Combined treatment aims at improving the hypercortisolism control and at reducing the risk of adverse events by administrating lower doses of compounds than those typically used in monotherapy (Pivonello *et al.*, 2015). Among treatment schedules reported, the combination of ketoconazole, metyrapone and mitotane, showed a rapid hypercortisolism control in all severe ACTH-dependent CS patients studied, associated with a rapid clinical improvement (Kamenicky *et al.*, 2011). The reported adverse effects were hypokalemia (100%), nausea and vomiting (63.7%), acute adrenal insufficiency (36.4%), dizziness and confusion (9.1%), and increases in liver enzymes (18.2%–81.8%) (Kamenicky *et al.*, 2011). Association of drugs acting at adrenal and pituitary levels, have also shown interesting results (Vilar *et al.*, 2010; Feelders *et al.*, 2010a; Barbot *et al.*, 2014). Combination of cabergoline and ketoconazole has shown remission rates, in terms of hypercortisolism control, ranging from 75% to 79%, associated with good tolerability (Vilar *et al.*, 2010; Barbot *et al.*, 2014). Stepwise combination approach using pasireotide, cabergoline and ketoconazole has reported remission rates up to 88%, in terms of hypercortisolism control, with potential positive effects on tumor mass, and it has been associated with a good safety profile (Feelders *et al.*, 2010a). These results are encouraging but further studies are needed to define the role of combined treatment in the management of CD patients (Pivonello *et al.*, 2015).

Conclusions

ACTH pituitary tumors cause the most frequent form of endogenous hypercortisolism named CD, a chronic and severe endocrine disease associated with an increased morbidity and mortality. The diagnosis of CD is a multistep process, still challenging, and requiring several tests and procedures adequately performed only in highly specialized centers.

The main treatment approaches for CD include pituitary surgery, which usually represents the first-line treatment, and repeat pituitary surgery, pituitary radiotherapy, adrenal surgery and medical treatment, which usually represent second-line approaches.

Medical treatment is gaining a more important role in different steps of the treatment algorithm and is represented by three cathegories of drugs: the pituitary-directed drugs; the adrenal-directed drugs (steroidogenesis inhibitors) and the glucocorticoid receptor antagonist. These drugs alone, or sometimes in combination, can help to control hypercortisolism or antagonize the hypercortisolism effects and seem to positively affect the morbidity of CD patients, but further experience are still required to optimize their use in the management of CD.

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TSH-Secreting Pituitary Adenomas☆

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Glossary

Hyperthyroidism Clinical disorder resulting from increased production and/or secretion of thyroid hormones from the thyroid gland.

Neoplastic inappropriate secretion of thyrotropin (TSH) Secondary or central hyperthyroidism due to a TSH-secreting pituitary tumor.

Nonneoplastic inappropriate secretion of thyrotropin Secondary hyperthyroidism due to a disorder of thyroid hormone transport, metabolism or action (e.g., syndrome of resistance to thyroid hormone (RTH)).

Primary hyperthyroidism Hyperthyroidism due to a primary disorder originating within the thyroid gland.

Secondary hyperthyroidism Hyperthyroidism due to stimulation of the thyroid gland by thyrotropin secreted from the anterior pituitary gland.

Introduction

Thyrotropin (TSH)-secreting pituitary adenomas have traditionally been considered rare tumors, accounting for approximately 1%–2% of all pituitary adenomas based on historical surgical and pathological series (Beck-Peccoz *et al.*, 2009). Central hyperthyroidism secondary to a TSH-secreting pituitary adenoma is sometimes referred to as neoplastic inappropriate secretion of TSH (thereby differentiating it from nonneoplastic inappropriate TSH secretion, as is observed in the syndrome of resistance to thyroid hormone (RTH, caused by mutations in *THRB*, encoding the β isoform of the thyroid hormone receptor)). Following the first case report in 1960, fewer than 300 cases had been described by the mid-1990s; however, in the last two decades TSH-secreting pituitary adenomas have been recognized with increasing frequency. Several factors likely account for this, including the development of ultrasensitive and specific TSH assays, the routine measurement of free thyroid hormone and TSH levels when screening for suspected thyroid dysfunction, and improvements in (coupled with more widespread use of) pituitary imaging. Analysis of data from a national Swedish registry covering the interval 1990–2010 revealed an approximate fivefold rise in the incidence of the condition (Ónnestam *et al.*, 2013).

Earlier recognition of the disorder offers the prospect of reducing the delay between the onset of hyperthyroidism and treatment (with the potential to reduce complications such as atrial fibrillation), and may increase the percentage of patients with surgically-remediable adenomas, while helping to avoid an inappropriate thyroid ablative procedure. On the other hand, the identification of an increasing number of TSH-secreting microadenomas raises new diagnostic challenges (e.g., when there are equivocal biochemical abnormalities, and magnetic resonance imaging (MRI) is unremarkable). The utility of long-acting somatostatin analogues as an alternative to the surgical treatment of these pituitary tumors is also increasingly recognized.

Pathogenesis and Pathology

The etiology of TSH-secreting pituitary tumors (which are monoclonal in nature) is still largely unknown. Previous studies have examined expression of the Gsp oncogene, and screened for mutations in the ras oncogene, and mutations or deletions in the Rb or p53 genes, but have proved largely uninformative in terms of causation. The pituitary-specific transcription factor (Pit-1) is overexpressed in TSH-secreting pituitary adenomas, and Pit-1 might play a role in cell proliferation as well as heterogeneous hormone expression in such adenomas. TSH-secreting pituitary adenomas have also rarely been observed in the context of multiple endocrine neoplasia type I and familial isolated pituitary adenoma (due to an aryl hydrocarbon receptor-interacting protein (AIP) mutation) (Burgess *et al.*, 1994; Hernández-Ramírez *et al.*, 2015).

TSH-secreting pituitary adenomas are heterogeneous with possible hormonal cosecretion (e.g., growth hormone, prolactin, gonadotrophins), suggesting that these tumors might arise from primordial stem cells with multidirectional differentiation potential. On the other hand, TSH-secreting macroadenomas are often accompanied by unbalanced hypersecretion of α-subunit,

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and double-immunostaining studies have suggested the existence of two different types of cells: one secreting α-subunit alone and another cosecreting α-subunit and β-TSH.

TSH-secreting pituitary adenomas are benign, and only two cases of a malignant tumor, one with brain metastases and another with widespread metastases to lung, liver, and bone, have been reported (Brown *et al.*, 2006; Mixson *et al.*, 1993). These tumors are more fibrotic than other pituitary tumors, which has been linked to the secretion of basic fibroblast growth factor by the adenoma cells (Ezzat *et al.*, 1995). Such fibrosis may make surgical resection more challenging and explain relatively poorer surgical results in patients with macro-/invasive adenomas.

TSH-secreting pituitary adenomas constitutively secrete TSH and exhibit a defect in negative feedback regulation of TSH by thyroid hormones. High thyroid hormone concentrations in the face of a "normal" TSH level may be explained, at least in part, by an increased bioactivity of neoplasm-derived TSH. A somatic mutation of *THRB* (encoding TRβ), as well as aberrant expression of a TRβ4 isoform, have been proposed as potential mechanisms for the defective regulation of TSH by triiodothyronine (T3) (Tagami *et al.*, 2011; Ando *et al.*, 2001).

Thyrotropin-releasing hormone (TRH) receptors are present on the majority of tumor cells. However, the attenuated/absent response of TSH and α-subunit to TRH in the majority of patients with TSH-secreting pituitary adenomas suggests that the TRH receptor signaling pathway is impaired/nonfunctional in these tumors. Several studies have revealed high concentrations of different subtypes of somatostatin receptors on TSH-secreting adenomas, explaining the in vivo response to somatostatin analogue administration. Dopamine D2 receptors have been detected in TSH-secreting tumors, but the effect of long-term bromocriptine therapy is limited due to incomplete suppression of inappropriate TSH secretion. Finally, estrogen receptors have been found on TSH-secreting tumors and, at least in theory, could predispose to growth of these tumors during a hyperestrogenic state such as pregnancy.

Clinical Presentation

Clinical manifestations of central hyperthyroidism secondary to a TSH-secreting pituitary adenoma are similar to those found in the other forms of hyperthyroidism such as Graves' disease, multinodular goiter, and toxic nodular goiter, but without disease-specific (e.g., autoimmune Graves') manifestations. However, in many instances the clinical features are milder, with some patients appearing relatively asymptomatic (although following successful treatment these patients may report an improvement in their wellbeing, coinciding with resolution of previously unrecognized clinical symptoms). TSH-secreting pituitary adenomas can occur at any age and affect both genders (without a female predominance in the majority of series). Clinical features of hyperthyroidism may be progressive (although some patients report little change over time intervals as long as a decade), and the mean latency between onset of hyperthyroidism and correct diagnosis is four years. A wide spectrum of clinical presentations, ranging from mild to moderate, and even severe thyrotoxicosis with atrial fibrillation and cardiac failure, are observed in such patients. Goiter and/or thyroid nodules may be present and are likely due to sustained TSH stimulation of thyroid follicular cells over many years. The cooccurrence of differentiated thyroid carcinoma has been reported in a small number of cases. Historically, symptoms due to compression of surrounding nervous structures, such as visual field defects (80%) and headaches (20%), prevailed over those due to hyperthyroidism. However, this is no longer the case, as many patients present at an earlier stage and the incidence of microadenomas is also on the rise. Unilateral exophthalmos due to orbital invasion by the pituitary tumor is very rare, but must be distinguished from asymmetric exophthalmos of Graves' disease. In mixed secreting tumors, clinical findings are dependent on the nature of the hormone cosecreted (e.g., acromegaly features, amenorrhea and/or galactorrhea). In macroadenomas, concomitant hypopituitarism (e.g., hypogonadism, hypoadrenalism) may be present. TSH-secreting pituitary adenomas have been reported in pregnant women, in patients with multiple endocrine neoplasia type I, and in atypical McCune–Albright syndrome (Burgess *et al.*, 1994; Caron and Gerbaud, 1996; Gessl *et al.*, 1994).

Hormonal Evaluation

TSH-secreting pituitary adenomas are now more readily diagnosed following the introduction of ultrasensitive assays for TSH. The classical biochemical signature is one of a detectable or elevated TSH concentration in the face of biochemical hyperthyroidism (with increased free thyroxine (FT4) and triiodothyronine (FT3)). Once potential confounding intercurrent illness and medications (e.g., heparin, amiodarone) have been ruled out, and assay interference excluded, a diagnosis of hyperthyroidism with inappropriate secretion of TSH (IST) is made (Gurnell *et al.*, 2011). The next task is to differentiate neoplastic (TSH-secreting pituitary adenoma) and nonneoplastic (TRβ RTH) causes (see differential diagnosis below).

Classically, an increased plasma α-subunit level and increased α-subunit/TSH molar ratio were considered important diagnostic markers, but recent findings indicate that the α-subunit level is frequently normal in patients with TSH-secreting pituitary microadenomas. Some studies have reported a relationship between hypersecretion of other pituitary hormones and tumor volume; for example, hyperprolactinemia and/or elevated growth hormone and insulin-like growth factor-1 (IGF-1) concentrations are observed more commonly in the context of macroadenomas. In many patients with TSH-secreting pituitary adenomas, dynamic testing reveals a reduced or absent response of TSH during the TRH test, impaired negative feedback of thyroid hormone

Table 1 Distinguishing between TSH-secreting pituitary adenoma and TRβ resistance to thyroid hormone (TRβ RTH)

	TSH-secreting adenomas	*Resistance to thyroid hormone*
Family history	Usually absent	Possible
Increased free thyroid hormone	Yes	Yes
Normal or high TSH	Yes	Yes
Circadian TSH secretion	Absent	Preserved
SHBG	Increased	Normal
α-subunit level	Increased/normal	Normal
α-subunit/TSH molar ratio	Increased/normal	Normal
Response to TRH test	Absent/attenuated	Preserved
Response to T3 test	Absent	Present*
Hormonal cosecretion	+	−
MRI	Adenoma	Normal
Genetic analysis	−	+

Key: MRI, magnetic resonance imaging; SHBG, sex hormone-binding globulin; T3, triiodothyronine; TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; * signifies that TSH suppression following exogenous T3 may not be complete.

on TSH secretion (which forms the basis of the T3 suppression test), and a marked decrease in TSH levels after treatment with somatostatin analogues (see below).

Differential Diagnosis

Elevated total thyroid hormone (TT4, TT3) levels, with inappropriately normal or elevated TSH, may be observed when thyroid hormone binding capacity is increased (e.g., during pregnancy when thyroxine binding globulin (TBG) levels are raised, or in individuals with familial dysalbuminemic hyperthyroxinemia (FDH), where point mutations in the *ALB* gene increase albumin's affinity for thyroid hormone). FDH may also perturb commonly used FT4 ($\pm$FT3) assays to yield falsely elevated levels. Close liaison with the laboratory is therefore required to exclude these and other conditions (e.g., heterophilic antibodies or anti-iodothyronine antibodies) which are capable of producing false positive TSH or FT4 ($\pm$ FT3) assay readouts. In addition, careful consideration must be given to other conditions and medications (e.g., heparin, amiodarone) that may be associated with a blood profile suggestive of central hyperthyroidism.

Once genuine hyperthyroxinemia and nonsuppressed TSH have been confirmed, the main challenge is to distinguish a TSH-secreting pituitary adenoma and resistance to thyroid hormone (*THRB*) syndrome. Age, sex distribution, TSH and free thyroid hormone levels do not differ significantly between the two conditions. However, the diagnosis of a TSH-secreting pituitary adenoma is more likely when: serum α-subunit is raised (with a high α-subunit/TSH molar ratio); serum sex hormone-binding globulin (SHBG) is increased; the TSH response to TRH is attenuated/absent; TSH fails to suppress after oral administration of liothyronine (L-T3); treatment with depot somatostatin analogue (minimum of 2–3 injections) leads to a sustained reduction in free thyroid hormone levels; and in the absence of a family history of similar abnormal thyroid function tests (**Table 1**).

In contrast, patients with TRβ RTH exhibit resistance to thyroid hormone action in tissues predominantly expressing the TRβ isoform (principally the hypothalamus, pituitary, liver and kidney). Accordingly, the set point of the hypothalamic-pituitary-thyroid axis is altered (due to central resistance), but retains the ability to respond to stimuli such as TRH and T3. SHBG is also typically normal (reflecting hepatic resistance), while only a transient reduction in thyroid hormone levels is observed during depot somatostatin analogue therapy (**Table 1**).

The diagnosis of central hyperthyroidism secondary to a TSH-secreting pituitary adenoma is likely when there is an obvious pituitary lesion on magnetic resonance imaging (MRI) and genetic analysis has excluded a mutation in the *THRB* gene (**Table 1**). However, clinicians should be alert to the fact that around 10%–15% of patients with apparent TRβ RTH do not harbor a readily identifiable mutation in *THRB*, and it is also possible for a patient with TRβ RTH to have a pituitary incidentaloma. In addition, in a significant proportion of TSH-secreting pituitary microadenomas, a lesion cannot be readily visualized on pituitary MRI (see below). Finally, several cases of coexisting autoimmune thyroid disease (both Graves' and Hashimoto's thyroiditis) have been reported with coexistent RTH or a TSH-secreting pituitary adenoma (Arai *et al.*, 2017; Kamoun *et al.*, 2014; Losa *et al.*, 2006).

Pituitary Imaging

The presence of a pituitary microadenoma in a patient with inappropriate secretion of TSH, although strongly suggestive, is not diagnostic of a TSH-secreting pituitary tumor given that pituitary incidentalomas are found on MRI in $\sim$10% of normal individuals. In contrast, incidental macroadenomas are much less commonly encountered. In patients with TSH-secreting pituitary adenomas there is no correlation between serum TSH levels and tumor size. In older case series, patients commonly presented

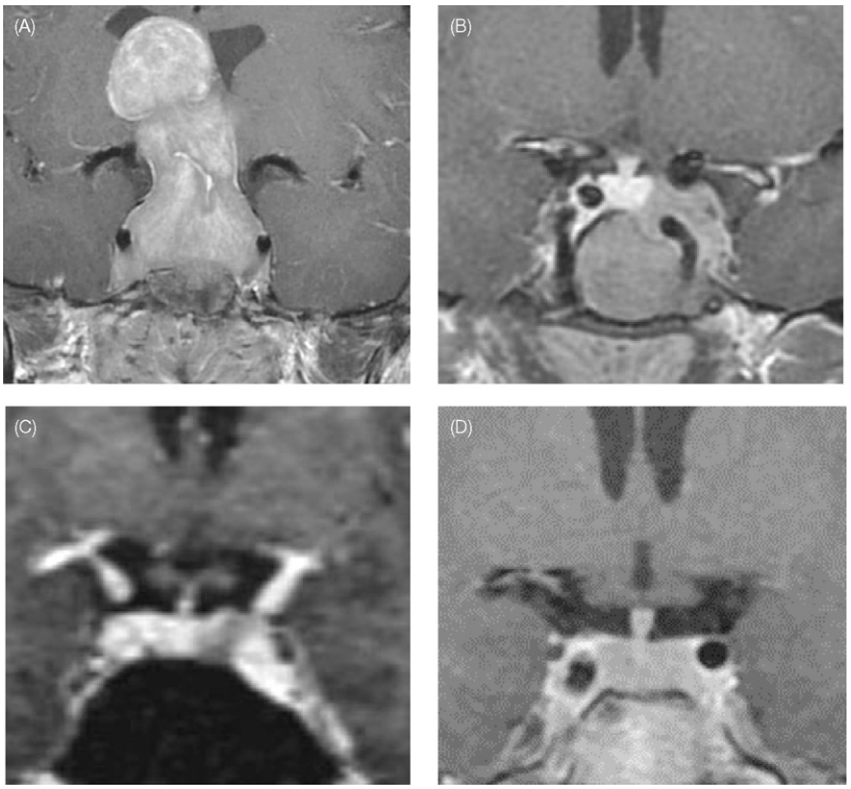

Fig. 1 *Spectrum of TSH-secreting pituitary adenomas.* (A) Giant adenoma. (B) Macroadenoma with cavernous sinus and sphenoid extension. (C) Left-sided microadenoma. (D) No visualized adenoma.

with macroadenomas with suprasellar and sphenoidal extension, whereas in more recent series microadenomas feature much more frequently. Indeed, it is now clear that thyrotropinomas exhibit a spectrum ranging from large invasive (even giant) tumors through to microadenomas that cannot be readily visualized using conventional MR sequences (analogous to some Cushing's microadenomas) (**Fig. 1**). For the latter, various approaches have been tried to aid localization of the tumor (including inferior petrosal sinus sampling with TRH stimulation) and octreotide scintigraphy. However, the most promising approach appears to be functional imaging with ^{11}C-methionine PET-CT coregistered with volumetric MRI, which may aid precise localization of the tumor, especially when performed pre-and postsomatostatin analogue therapy (**Fig. 2**) (Koulouri *et al.*, 2016).

Treatment

In patients with TSH-secreting pituitary adenomas, the goal of therapy is to restore euthyroidism and to eliminate the symptoms of mass effect in patients with large tumors. Early diagnosis and correct treatment of these rare tumors helps prevent complications, such as visual abnormalities due to compression of the optic chiasm and hypopituitarism, and may also improve surgical cure rates.

The success of treatment depends on the criteria used. It has been suggested that an early test of cure might be an undetectable TSH concentration seven days after surgery (when the normal thyrotrophs remain suppressed as a consequence of prolonged exposure to raised circulating thyroid hormone levels). However, many patients are now treated with somatostatin analogues prior to surgery, often with rapid normalization of hyperthyroidism, which may therefore allow recovery of normal thyrotroph function prior to theater. Normalization of dynamic tests (e.g., TRH test, T3 suppression test) might therefore represent a better (later) test of cure. In any case, long-term follow-up is necessary to detect relapse and recurrence.

Transsphenoidal surgery remains the treatment of choice in many patients with TSH-secreting pituitary adenomas. Historically, pituitary surgery alone has been reported to result in normalization of thyroid hormone secretion and resolution of the pituitary mass in approximately 50% of patients, achieve normalization of thyroid parameters despite incomplete tissue removal in approximately 25% of patients, and is unsuccessful in nearly 30% of cases. However, an increase in the surgical cure rate has been reported in more recent series, probably reflecting improved surgical techniques and earlier diagnosis.

Medical treatment is an alternative option to pituitary surgery in patients with TSH-secreting pituitary tumors. Dopamine agonists are an effective treatment in true mixed TSH/prolactin-secreting pituitary adenomas, whereas success is limited when bromocriptine is used in patients with pure TSH-secreting pituitary adenomas. In contrast, depot somatostatin analogues

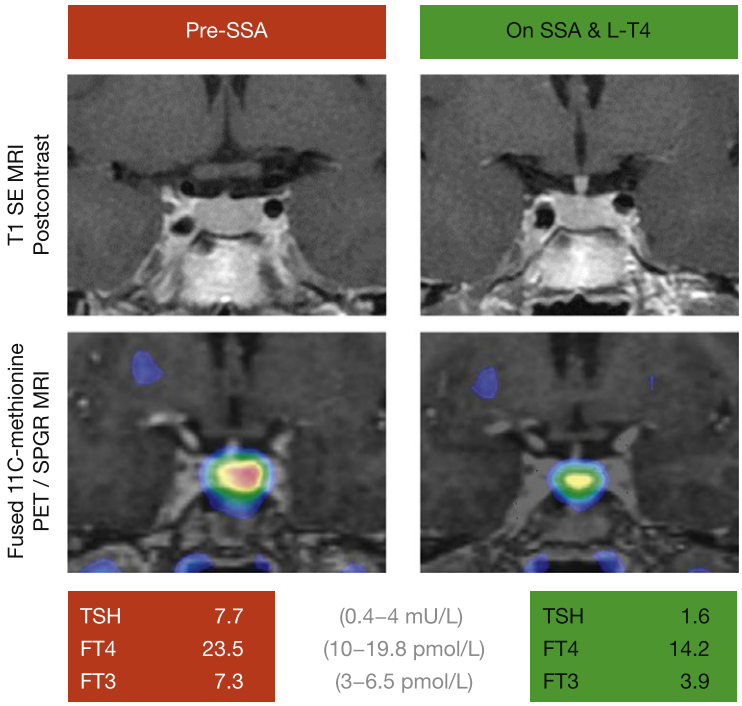

Fig. 2 *Confirmation of the site of a microadenoma using functional PET imaging.* Spin echo (SE) MRI and ^{11}C-methionine PET coregistered with spoiled gradient recalled acquisition (SPGR) MRI before (left panels) and after (right panels) treatment with somatostatin analogue (SSA) in a patient with a suspected microTSHoma. Coregistered ^{11}C-methionine PET/SPGR MRI reveals asymmetric tracer uptake with maximum intensity on the left side of the gland. Treatment with SSA leads to extinction of tracer uptake from this area, leaving only physiological (central) tracer uptake by the normal pituitary gland. Transsphenoidal surgery and histological analysis confirmed a left-sided TSH secreting microadenoma.

(octreotide LAR or lanreotide ATG) suppress TSH secretion in more than 90% of TSH-secreting pituitary adenomas, and normalize thyroid hormone levels in approximately 75%–90% of patients. However, significant shrinkage of the adenoma is only observed in approximately 50% of cases, and total resolution of a macroadenoma with maintenance of long term cure in response to somatostatin analogue therapy has been reported in only a single case (Fliers *et al.*, 2012). As a general rule, the effects of somatostatin analogues are reversible, with the need for long-term administration, with possible tachyphylaxis requiring increasing doses of the drug to maintain good control of the disease in up to 10% of cases, and with true resistance in a small number of patients. The second generation somatostatin receptor ligand pasireotide may offer an alternative for treatment resistant cases, although experience to date is limited to a single patient in whom it was not clear whether there was true resistance to first line SSA therapy (van Eersel *et al.*, 2017). Somatostatin analogues remain expensive and may be associated with side effects such as cholelithiasis and carbohydrate intolerance. Accordingly, although some patients and their clinicians might elect to pursue long-term primary SSA therapy, for many patients these remain an important bridge to surgery (allowing anesthesia to proceed safely) and as adjunctive therapy when surgery is unable to achieve full remission, or while awaiting the beneficial effects of radiotherapy.

It is appropriate to briefly mention conventional antithyroid drug (ATD) therapy, which may be used for a short duration in preparation for surgery. However, concerns remain that longer term use may predispose to tumor expansion (Nelson's-like phenomenon), and for that reason permanent thyroid ablation is also generally best-avoided, although may occasionally be indicated in those with recalcitrant arrhythmias or cardiac failure.

Conventional radiotherapy and stereotactic radiosurgery have also been employed in the management of TSH-secreting pituitary tumors, especially when surgery and/or medical therapy has proved unsuccessful. However, the number of reported cases, especially using modern radiotherapy techniques, remains relatively small. As with other pituitary tumor subtypes, and taking into consideration the long time period necessary for the full effect to be realized and the well-known late side effects (e.g., hypopituitarism, impaired cognitive function, second tumor), radiotherapy is now generally reserved for the small number of cases in whom combination surgery and medical therapy is unable to achieve satisfactory biochemical and/or tumor control.

Conclusion

TSH-secreting pituitary adenomas are a rare cause of hyperthyroidism. The main prognostic factors of these adenomas are size and invasiveness of the tumors, duration of symptoms, and intensity of hyperthyroidism. Early recognition of central hyperthyroidism, and localization of the causative lesion should now be readily achieved in most cases, reflecting the availability of ultrasensitive

TSH assays and improvements in pituitary imaging. Reflecting this, the spectrum of disease and approach to the treatment of these rare pituitary tumors has changed significantly during the past decade. Patients typically present with mild or moderate symptoms and signs of hyperthyroidism, hormonal evaluation shows increased free thyroid hormone concentrations with detectable serum TSH levels, and MRI increasingly identifies patients with micro- rather than macro-adenomas. Transsphenoidal surgery remains the treatment of choice, although depot somatostatin analogue therapy is now established as a key component in the management pathway: either pre- and, in selected cases, postsurgery or sometimes as long-term primary medical therapy.

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Surgery for Pituitary Tumors☆

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Glossary

Acromegaly A chronic disease marked by the progressive enlargement of the hands, feet, face, head, and thorax due to abnormally high secretion of growth hormone secreted by the anterior lobe of the pituitary gland.

Adenomas Various benign epithelial tumors in which the cells form glandlike structures or develop from the glandular epithelium.

Amenorrhea The absence or abnormal stoppage of menstruation.

Cushing's disease ACTH dependent hypercortisolemia caused by excess ACTH secretion from a pituitary adenoma.

Galactorrhea An excessive flow of milk or a persistent secretion of milk, irrespective of breastfeeding.

Sella turcica A shallow depression, located in the superior surface of the body of the sphenoid bone, that contains the pituitary gland.

Introduction

Surgery for pituitary adenomas has evolved over 120 years. Largely performed via the transsphenoidal route, this procedure carries an acceptably low mortality and morbidity rate. Surgical excision is considered first-line treatment for most secretory pituitary tumors (causing acromegaly and Cushing's disease), except for prolactinomas, where surgery is usually reserved for cases of medication-resistant tumors. Surgery for non-functioning tumors is strongly indicated for symptoms of mass effect, especially visual loss due to pressure on the optic apparatus, and may be recommended when tumors are >1 cm (macroadenomas), are growing, or are causing pituitary insufficiency. Long-term outcomes for these tumors are favorable, although medical management following surgery may be required for biochemical control of acromegaly and Cushing's disease (CD) and radiation treatment of tumors may sometimes be necessary for growth control and/or biochemical remission as well.

History

Since the late 1800s, surgery has been a mainstay of treatment for pituitary disease, when Sir Victor Horsley performed the first craniotomy for treatment of a patient with acromegaly. Surgeons quickly realized that the proximity of the sella turcica (translated as "Turkish saddle") to the sphenoid sinus provided a relatively noninvasive approach to the pituitary and began to devise alternative routes. In 1907, Hermann Schloffer developed an approach through the sphenoid sinus via a lateral rhinotomy incision, while A. E. Halstead and Oskar Hirsch approached sublabially and transnasally, respectively. Harvey Cushing modified Halstead's approach and performed more than 1000 transsphenoidal procedures in the early 1900s (Schmidt *et al.*, 2012; Liu *et al.*, 2001). His careful clinical observations led to significant advances in the field, especially in his description of the syndrome of cortisol excess that bears his name. Difficulties with visualization and orientation were overcome by the introduction of the operating microscope by Cushing's pupil Dott and the intraoperative use of the lateral fluoroscope by G. Guiot in the 1950s. With J. Hardy's microscopic excision of the first microadenoma in 1962, transsphenoidal surgery entered the modern era.

Surgical Anatomy and Approach

The pituitary is enclosed in a bony cavity, the sella turcica, at the base of the skull. It is bounded inferiorly and anteriorly by the sphenoid sinus, laterally by the cavernous sinuses, posteriorly by the dorsum sella, and superiorly by the diaphragm sella, which is in close proximity to the suprasellar cistern, optic nerves, and chiasm above. There are multiple variants on the transsphenoidal approach to this region. These include sublabial or transnasal approaches, either submucosally along the nasal septum or directly endonasally through the face of the sphenoid sinus at the level of the sphenoid ostia. The endonasal approach can be performed with a microscope or an endoscope. It is critical to maintain awareness of the midline, since lateral deviations can lead to unintentional encroachment on the carotid or optic canals, with potentially severe complications. Intraoperative navigation or intraoperative imaging, as traditionally performed with lateral fluoroscopy, will ensure the correct trajectory in the sagittal plane

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and avoid inadvertent penetrations through the skull base. The sella can be markedly expanded, as is seen with large macroadenomas, or can be completely normal, as is seen with the tiny microadenomas of Cushing's disease. The sella is opened with a drill and the tumor is removed using an array of ring curettes. Suprasellar tumors can often be delivered into the operative field and removed, whereas cavernous sinus invasion usually results in an incomplete resection. The tumor bed is then packed with fat, muscle, or fascia lata, to avoid a cerebrospinal fluid (CSF) leak. Nasal packing may be required for a short period of time, but the procedure is usually well-tolerated with minimal morbidity.

Modern Advances

Technological advancements have improved preoperative diagnosis, both radiographically and endocrinologically, have aided intraoperative orientation, and have improved intraoperative visualization.

Imaging

The major advance in surgical planning over the past 50 years has been the introduction of sophisticated radiographic imaging techniques. Computerized tomography was introduced in the early 1980s, followed by magnetic resonance imaging (MRI) shortly thereafter. Current MRI scans will delineate all but the smallest microadenomas and demonstrate their proximity to surrounding structures. MRI scanners have been incorporated into the operating room environment and can provide near-real-time MRI for both intraoperative orientation and determination of the adequacy of resection. Whether this expensive technology leads to improvements in patient care and outcome remains to be shown.

Surgical Techniques

Advances in surgical techniques have aided both intraoperative orientation and visualization. Navigational devices which correlate intraoperative localization with preoperative imaging may offer some benefits when the traditional anatomical landmarks have been destroyed, as in cases of recurrent tumors. Improved lateral visualization with a wider field of view is offered by the use of the endoscope, though traditional operative microscopy offers better optics, depth of field, and binocular vision.

Surgical Results

Secretory Tumors

Cushing's disease

Transsphenoidal surgery remains the primary mode of treatment for Cushing's disease, with cure rates approaching 90% in experienced hands (Valassi *et al.*, 2010; Aranda *et al.*, 2015). After surgical cure, patients will demonstrate profound hypoadrenalism for some months postoperatively and require cortisol replacement during this time. Tumors may recur at a rate of at least 10% at 5 years; reoperation for tumor recurrence is less successful (Shimon *et al.*, 2002; Nakane *et al.*, 1987; Prevedello *et al.*, 2008). Here, adjuvant therapy, including radiosurgery, adrenalectomy, or medical therapy, may play a role. Since MRI is able to detect microadenomas in this disease in at best 60% of cases, accurate endocrine diagnosis, including inferior petrosal sinus catheterization, is necessary (Jagannathan *et al.*, 2009; Ciric *et al.*, 2012; Hall *et al.*, 1994).

Acromegaly

Patients with acromegaly tend to present with larger, more invasive tumors, and transsphenoidal surgery is less successful in achieving cure. The remission rate is 50%–60% in the typical macroadenomas, whereas the remission rate may approach 90% in the less common microadenomas (Sun *et al.*, 2014). Newer medical treatments are available, including the use of somatostatin analogues and growth hormone receptor antagonists, are useful in controlling growth hormone levels in those patients not surgically cured. Aggressive multimodality therapy (e.g., surgery combined with medical treatment and possibly radiotherapy) is sometimes necessary to control the increased mortality risk associated with the disease. The role of medical pretreatment, or primary medical therapy for acromegaly, remains unclear, but has been suggested for those patients who are not optimal surgical candidates or who have a low chance of surgical cure (Fougner *et al.*, 2014).

Prolactinomas

Women who present with amenorrhea–galactorrhea, mild elevations in prolactin level, and microadenomas on MRI scanning are usually best treated with dopamine agonist therapy. Surgery, though effective in approximately 90% of patients with microadenomas (Babey *et al.*, 2011), is usually reserved for those patients who are unable to tolerate or who fail to respond to medical treatment. With newer, better-tolerated dopamine agonists (cabergoline), patients only rarely require surgical treatment. Men with

prolactinomas tend to present with either impotence or visual abnormalities and their tumors are more likely to be large and invasive. Dopamine agonist therapy remains effective in these cases, with surgery reserved for those cases that fail to respond. Even patients with bitemporal field defects will often respond dramatically after a few weeks of treatment with a dopamine agonist. It must be remembered that mild elevations in prolactin (less than $\approx$200 ng/mL) may have causes other than prolactin-secreting tumors (e.g., pregnancy, medication effects). A large tumor with a mild increase in prolactin level may represent the so-called "stalk effect" from compression of the pituitary stalk and normal gland, resulting in blockade of intrinsic dopamine inhibition and an elevated prolactin level. This tumor is unlikely to be a prolactinoma and may well require surgical removal. Dopamine agonist therapy in these cases may well normalize the prolactin level, but will do nothing to shrink the tumor.

Nonfunctioning tumors

The term "nonsecreting" may well be a misnomer in these cases, as many of these tumors secrete inactive hormone fragments, especially the α-subunit. Because of their endocrine inactivity, they can become large and invasive before being detected. They usually present with signs of mass effect, either with visual abnormalities, especially the classic bitemporal field defect of chiasm compression, or with endocrine abnormalities from destruction of the remaining gland. Surgery is required to decompress the optic apparatus and approximately 70% of patients will regain some degree of visual loss. Decompression of the remaining gland results in improvements in hormone function in approximately 30%–50% of these patients. A small amount of residual tumor may be followed with serial MRIs, whereas an extensive amount of residual should be treated with radiotherapy. An incidentally found nonfunctioning tumor (the "incidentaloma") has been reported in up to 10% of MRI studies (Sivakumar *et al.*, 2011). Microadenomas (<1 cm) can usually be followed with serial MRI; macroadenomas (>1 cm) often merit resection, though some can be followed for a time with MRI to determine the rate of growth before recommending surgery.

Complications

Transsphenoidal surgery in experienced hands carries a low risk of serious complications. New hormone insufficiency may occur in 5%–10% of cases, although this may be considerably greater in cases of Cushing's disease, in which extensive exploration of the gland is sometimes required to find a tiny microadenoma. The incidence of permanent diabetes insipidus is approximately 1%–2%. Serious complications are unlikely. Carotid artery injury occurs at a rate of approximately 0.1%, whereas permanent postoperative visual decline occurs at a rate of approximately 0.5%. Postoperative CSF rhinorrhea is seen in approximately 1%–2% of cases and may lead to meningitis if untreated. Approximately 5% of patients report persistent sinus disease postoperatively (Murad *et al.*, 2010; Ciric *et al.*, 1997).

Conclusion

Transsphenoidal surgery remains the therapy of choice for nonfunctioning adenomas, especially in cases of optic chiasm compression or hormonal insufficiency. Secretory tumors, except for prolactinomas, require surgical therapy in most cases, although new medical treatments and radiosurgery may provide useful adjuncts.

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Clinical Features, Diagnosis, and Management of Nonfunctioning Tumors of Pituitary

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Introduction

Pituitary tumors constitute 10%–15% of all intracranial neoplasms with an increasing prevalence over the last 20 years due to various factors (advances in imaging techniques and hormonal measurements, increased awareness of the medical community, more frequent use, and easier access to imaging facilities). Numerous types of neoplasms can be found in the sellar/parasellar region, including both benign and malignant tumors.

Patients with sellar/parasellar tumors can present with manifestations of mass effects to surrounding structures or can be discovered on imaging performed for an unrelated reason. Treatment depends on the diagnosis and includes surgery, radiotherapy (RT), chemotherapy, or combination of these.

Nonfunctioning Pituitary Adenomas

Nonfunctioning pituitary adenomas (NFPAs) are histologically benign neoplasms that constitute the majority of nonfunctioning pituitary tumors, and on the basis of maximum diameter on imaging, are classified into microadenomas (<1 cm) and macroadenomas (≥ 1 cm).

Epidemiology

The prevalence of NFPAs is difficult to be reliably assessed given that many patients do not experience any symptoms and remain undiagnosed. According to recent epidemiological studies, their prevalence is 22–41 per 100,000 inhabitants, with an annual incidence of 1–1.8 new cases per 100,000 inhabitants per year (Agustsson *et al.*, 2015; Tjörnstrand *et al.*, 2014; Gruppetta *et al.*, 2013; Fernandez *et al.*, 2010; Raappana *et al.*, 2010). NFPAs comprise 28%–43% of all pituitary adenomas and they are the second most frequent type after prolactinomas, although one study reported that NFPAs were the most common pituitary adenomas with a percentage of 54% (Tjörnstrand *et al.*, 2014). They are mainly diagnosed between the fifth and sixth decade of life, 70%–80% of them are macroadenomas on presentation, and their incidence increases with age.

Pathogenesis

NFPAs are monoclonal in origin and their pathogenesis remains unknown: genetic and epigenetic changes that result in cell cycle dysregulation, signaling defects, or loss of tumor suppressor factors have been proposed as possible underlying mechanisms (Melmed, 2011). Although the majority of NFPAs are sporadic, rare cases related to hereditary syndromes have been reported. Familial isolated pituitary adenomas and multiple endocrine neoplasia type 1 and type 4 are the most recognized syndromes associated with NFPA (Thakker, 2014; Beckers *et al.*, 2013).

Clinical Features and Diagnosis

The absence of clinical manifestations due to hormonal hypersecretion translates into delays in the diagnosis of NFPAs and patients usually present with symptoms due to local mass effects. These depend on the size of the tumor and its anatomical position and extensions. Headaches, caused by dura stretching and increased intracranial pressure, are the most common reported presenting manifestation of NFPAs (Rogers *et al.*, 2014). In case of suprasellar extension with compression of the anterior visual pathways (optic nerves, chiasm, and tracts), neuro-ophthalmological symptoms can present, mainly bitemporal hemianopia, although unilateral or even central visual loss can be present (Newman *et al.*, 2016). If the pressure on the optic chiasm is prolonged, optic atrophy and decreased visual acuity can be evident. Lateral expansion of the NFPA and invasion of the cavernous sinus can lead to ocular nerve palsies (particularly III, IV, and VI cranial nerves), resulting in ptosis, ophthalmoplegia, and diplopia. Erosion of the sellar floor and extension of the adenoma to the sphenoid sinus may cause cerebrospinal fluid (CSF) rhinorrhea and meningitis. Finally, in rare cases, pituitary apoplexy due to hemorrhage or infarction of an adenoma can be the first presentation of a NFPA, characterized by severe headache, sudden onset of visual disturbance, ophthalmoplegia, meningism, and impaired consciousness (Capatina *et al.*, 2015).

Patients with NFPA can also complain about manifestations attributed to hypopituitarism such as fatigue, decreased libido, impotence, and menstrual disturbances (Greenman and Stern, 2009). Anterior pituitary dysfunction is generally present in most patients with macroNFPA, with growth hormone (GH) deficiency being the most common deficit, followed by gonadotropins, and lastly thyrotropin-stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH) deficiencies. Prolactin levels can be increased due to interruption of the inhibitory effect of dopamine on prolactin release or can be decreased indicating severe pituitary damage.

Magnetic resonance imaging (MRI) is the preferred imaging modality, used not only for the differential diagnosis of a pituitary adenoma from other sellar/parasellar masses but also for identifying expansion of the adenoma to the optic chiasm, anterior cerebral vessels, and cavernous sinuses (Buchfelder and Schlaffer, 2014). The diagnosis of NFPA is established by pathological examination demonstrating the presence of adenomatous cells; on immunohistochemistry, the majority of them stain for pituitary hormones. About 70% of NFPAs stain for gonadotropins or their subunits, followed by null-cell adenomas, that are negative on immunohistochemistry for any pituitary transcription factor and adenohypophyseal hormone; the remaining are either silent adenomas (corticotropinomas, prolactinomas, thyrotropinomas, and somatotropinomas) or stain for multiple hormones (Mete and Lopes, 2017; Neto *et al.*, 2016).

Treatment

Treatment of patients with NFPA includes monitoring, surgery, and RT. A "watch and wait" policy can be adopted in cases of NFPAs without neuro-ophthalmological symptoms that do not compress the optic chiasm. Macroadenomas are at higher risk of enlargement compared with microadenomas and regular imaging surveillance is recommended, initially yearly and less frequent thereafter. Monitoring for new pituitary dysfunction or for visual deterioration is also advised (Fernández-Balsells *et al.*, 2011; Freda *et al.*, 2011).

Pituitary surgery, especially via the transsphenoidal route, is the treatment of choice in NFPAs with neuro-ophthalmological manifestations and/or compression of the optic pathways, as well as in cases of pituitary apoplexy with severe visual acuity and visual field impairment or deterioration of visual or neurological signs. The aim of surgical treatment is to improve or resolve the mass effects on adjacent structures, especially the optic pathways, and the preservation of the normal pituitary tissue (Capatina *et al.*, 2015; Dekkers *et al.*, 2008).

RT, conventional or stereotactic, is mainly used as adjuvant treatment postoperatively in selective cases, aiming to reduce the risk of tumor enlargement, or in case of tumor recurrence (Molitch, 2017). Consideration of irradiation adverse sequelae, particularly hypopituitarism, needs to be taken into account (Loeffler and Shih, 2011).

Finally, no medical therapy is currently approved for the treatment of NFPAs; dopamine agonists and somatostatin analogs are potential options but their place in the management algorithm remains to be established (Greenman, 2017). Temozolomide is also used in the treatment of aggressive NFPAs that show continuous growth despite surgery and irradiation (Halevy and Whitelaw, 2017).

Rathke's Cleft Cysts

Rathke's cleft cysts (RCCs) are benign, sellar, and/or suprasellar cystic lesions originating from the remnants of Rathke's pouch. They are the most common type of sellar mass after pituitary adenomas (Famini *et al.*, 2011).

Epidemiology

The prevalence of RCCs is uncertain, considering that most of them are asymptomatic and escape diagnosis. However, they are detected more frequent nowadays as the quality of brain imaging has improved and its use is more extensive in clinical practice. RCCs are mainly discovered during adulthood and a female preponderance has been described, although this is probably attributed to their earlier detection in women due to menstrual irregularities (Trifanescu *et al.*, 2012).

Clinical Features and Diagnosis

RCCs are found incidentally when they have a small size but become clinical apparent when they are large enough to cause mass effects to adjacent structures or when they rupture. Headaches are the most prevalent manifestation of RCCs and are not correlated with the size or the location of the cyst (Nishioka *et al.*, 2006). Visual field defects and impairment of visual acuity are also evident in most symptomatic cases. Anterior pituitary hormone deficiencies affecting all axes and hyperprolactinemia are commonly found in patients with RCCs and in contrast to NFPAs, diabetes insipidus can be also present. In rare cases, apoplexy can be the first presentation of a RCC, characterized by manifestations similar of pituitary apoplexy. Rupture of the cyst leading to aseptic meningitis due to leakage of cystic contents on the subarachnoid space is also rare (Larkin *et al.*, 2014).

On MRI, RCCs often appear as well-circumscribed, spherical or ovoid lesions, located between the anterior and posterior pituitary gland in the region of the pars intermedia (Trifanescu *et al.*, 2012). The intensity of the signal of the cyst depends on its

contents. A cyst filled with clear, CSF-like fluid appears hypointense on T1- and hyperintense on T2-weighted images, whereas a cyst containing thick mucoid material made up of cholesterol and protein is hyperintense on T1- and isointense on T2-weighted imaging (Han *et al.*, 2014). In addition, an intracystic nodule with high signal intensity on T1-weighted images and low signal intensity on T2-weighted images can also be demonstrated.

Treatment

The natural history of RCCs is not known and the percentage of nonoperated cysts that grow during follow-up varies between studies. Cases of spontaneous involution or decrease of the size of cysts under monitoring has also been described (Amhaz *et al.*, 2010; Sanno *et al.*, 2003). However, given that RCCs are slowly growing, surveillance seems to be the most appropriate management for patients with small and asymptomatic cysts.

Surgical therapy, aiming to complete drainage of the cyst and removal of a proportion of the capsule as safely as possible, avoiding damage to the posterior pituitary and pituitary stalk, is recommended in symptomatic patients and is performed mainly with a transsphenoidal approach (Zada, 2011). Craniotomy may be required in complex cases of giant or purely suprasellar RCCs or when transsphenoidal surgery is contraindicated. Cyst decompression results in improvement or resolution of headaches and visual disturbances in the majority of the patients, while the recovery of pituitary function varies.

Relapse of the RCCs is possible after surgery and in a recent meta-analysis a recurrence rate of 12.5% was reported (Mendelson *et al.*, 2014). Although there is no consensus on the optimal therapeutic approach in recurrent RCCs, asymptomatic cases are usually followed up with imaging surveillance, while repeat surgery is performed in the symptomatic ones. RT has also been used in a small number of recurrent cases (Mukherjee *et al.*, 1997).

Craniopharyngiomas

Craniopharyngiomas are benign (World Health Organization grade I) epithelial tumors arising along the path of the craniopharyngeal duct. These tumors can be aggressive, infiltrating the parasellar structures and resulting in significant morbidity and mortality. The majority of them have a suprasellar component whereas the purely intrasellar ones represent the least common type (Müller, 2014).

Epidemiology

Craniopharyngiomas are rare tumors with an overall incidence of 1.34 cases per million persons per year in all ages and 1.44 cases among children; approximately 70% of them are diagnosed during adulthood (Zacharia *et al.*, 2012; Nielsen *et al.*, 2011). They can be detected at any age with peak incidence rates demonstrated in children between 5 and 14 years old and adults between 50 and 74 years old (Bunin *et al.*, 1998). No sex predisposition has been described.

Pathology

Histologically, there are two primary subtypes, the adamantinomatous and the papillary, but mixed or transitional forms have also been described (Prieto and Pascual, 2013). The adamantinomatous is the most common and is mainly detected in children and young patients, in contrast to the papillary one which is almost predominantly found in adults. Macroscopically, adamantinomatous craniopharyngiomas have cystic and/or solid components and calcifications (especially in children), while papillary tumors are mainly solid or mixed with cystic and solid parts with rare calcifications; they are also differences in the cystic contents between the two types (Karavitaki *et al.*, 2006). Although craniopharyngiomas are considered benign tumors, there are reports of malignant transformation in the literature (Sofela *et al.*, 2014).

Clinical Features and Diagnosis

The clinical manifestations of craniopharyngiomas are mainly due to pressure effects of the tumor to the adjacent structures including the optic pathways, the parenchyma of the brain, major blood vessels, and the hypothalamic-pituitary axis. Symptoms of increased intracranial pressure (headaches, nausea, vomiting, and papilledema; mainly in children), visual impairment (mostly bitemporal hemianopia), endocrine defects (growth failure, sexual immaturity in children; hypogonadism in adults), and cranial nerve palsies (also mainly found in children) are the most frequent features (Karavitaki *et al.*, 2005). In the majority of cases, anterior pituitary function is compromised at the time of diagnosis, while diabetes insipidus is present in 17%–27% of the patients (Müller, 2014).

MRI with contrast enhancement is valuable for the detection of craniopharyngiomas and for the differential diagnosis from other sellar/parasellar masses, mainly including RCC and other cysts (epidermoid, dermoid, arachnoid), pituitary adenomas, hamartomas, gliomas, intracranial germinomas, and other inflammatory/infiltrative diseases. The signal intensity of

craniopharyngioma on MRI is highly variable and depends on the proportion of the solid and cystic components, the content of the cysts, and the presence of calcifications, which are ideally detected with the use of computed tomography (CT).

Treatment

Surgery combined or not with adjuvant RT is the mainstay of treatment. The extent of the resection depends on many factors, including the location, the consistency, the shape and size of the tumor, its infiltration to adjacent neurovascular structures, as well as on the surgeon's preference and experience (Karavitaki, 2014). Complete resection may be attempted in craniopharyngiomas that do not expand to surrounding brain areas and can be safely removed without damage of the hypothalamus and the optic pathways (Müller, 2014). On the other hand, a hypothalamus-sparing subtotal resection of the tumor may be performed in cases of craniopharyngiomas that invade the hypothalamus (Elowe-Gruau *et al.*, 2013).

The transsphenoidal surgical route is mainly preferred for smaller intrasellar-infradiaphragmatic tumors, while craniotomy through a number of different approaches is used for all other cases (Buchfelder *et al.*, 2013). When large cystic components are present in the neoplasm, fluid aspiration facilitates the removal of the solid portion of the tumor and provides relief of the obstructive symptoms.

RT plays an important role in the management of patients with craniopharyngiomas for controlling tumor growth. It is usually offered postoperatively in cases of subtotal resection of the neoplasm or in tumor recurrence, which is common in patients with craniopharyngioma particularly after partial resection (Kortmann, 2011). However, in cystic tumors treated with RT, there is a risk of enlargement during the radiotherapy which may necessitate further intervention.

Given that the majority of the craniopharyngiomas is comprised of a cystic component, intracavitary instillation of beta-emitting radioisotopes or antineoplastic agents (bleomycin, interferon-a) has been used as alternative treatment (Julow, 2013; Steinbok and Hukin, 2010). A potential benefit of systemic chemotherapy in craniopharyngiomas has also been investigated in a very small number of patients.

Long-Term Morbidity and Mortality

Craniopharyngiomas are related with significant morbidity due to the damage of critical structures by the primary and/or recurrent tumor and/or to the adverse effects of the therapeutic interventions. Pituitary hormone deficiencies are common at the time of diagnosis of craniopharyngioma and their recovery after the surgical removal of the neoplasm is rare; in addition, aggressive surgery leads to more frequent pituitary dysfunction. Compromised vision is evident in a significant number of patients treated by surgery (combined or not with RT) and is adversely affected by the presence of visual symptoms at diagnosis and by daily irradiation doses >2 Gy (Karavitaki *et al.*, 2006).

Hypothalamic damage may lead to devastating comorbidities including hyperphagia and uncontrollable obesity, imbalances in regulation of body temperature, of thirst, of heart rate, and blood pressure, behavioral changes, cognitive impairment, and disorders in the sleep pattern. Obesity is the most common morbidity and is caused by the disruption of the mechanisms that control satiety, hunger, and energy balance (Karavitaki, 2014). Finally, other long-term irradiation-attributed morbidities, such as vasculopathy or secondary brain tumors, have been proposed as rare consequences of RT in patients with craniopharyngioma, but the risk of developing these has not been as yet defined (Liu *et al.*, 2009; Rajan *et al.*, 1993).

The mortality rates of patients with craniopharyngioma have been reported to be 3–5 times higher than that of the general population (Müller, 2014). Deaths are mainly attributed to the tumor and/or its recurrences, the treatment interventions, the hypothalamic and pituitary dysfunction, and to cardio/cerebrovascular and respiratory diseases.

Meningiomas

Meningiomas are the most common intracranial tumors and originate from the meningothelial cells of the arachnoid. They are slowly growing neoplasms which can arise from the dura at any site, including sellar/parasellar region. In imaging series, meningiomas account for 15% of nonadenomatous sellar masses (Famini *et al.*, 2011). According to World Health Organization, they are classified as benign (grade I), which constitute the majority of menigiomas, atypical (grade II), and malignant (grade III), which are the least common type.

Their clinical manifestations are attributed to compression of adjacent structures. Visual deterioration is the most common symptom, with varying types of field defects depending on the location of the tumor, followed by headaches. Pituitary dysfunction with mild hyperprolactinemia can also be evident. The diagnosis is facilitated by the characteristics of meningiomas on imaging. The typical MRI signal intensity consists of isointensity to slight hypointensity relative to gray matter on the T1-weighted sequences and a homogeneous, intense contrast enhancement (Watts *et al.*, 2014). However, there can be areas of necrosis or calcification (best demonstrated with CT) that do not enhance. The presence of a linear, enhancing dural tail, extending away from the neoplasm, is useful to distinguish meningiomas from other parasellar masses (Raoa *et al.*, 2008).

Surgery is the treatment of choice for symptomatic or growing meningiomas, while asymptomatic tumors can be managed conservatively with active surveillance (Oya *et al.*, 2011). The aim of surgical treatment is total excision of the tumor, although

many of them cannot be totally removed due to encasement of vital vascular or neural structures. In addition, given that meningiomas are high vascular neoplasms, major bleeding can occur during surgery (Kwancharoen *et al.*, 2013). RT can be used as primary treatment in patients with inoperable meningiomas, as adjuvant therapy in order to slow tumor growth after incomplete resection of grade I tumors or after complete removal of grade II and III neoplasms and as salvage therapy for recurrent and progressive disease (Rogers *et al.*, 2015).

Germcell Tumors

Intracranial germcell tumors (GCTs) represent a rare and heterogeneous group of neoplasms, primarily affecting children, adolescents, and young adults (<20 years old) and mostly males. They are mainly midline tumors, usually arising in the pineal gland, followed by the neurohypophyseal/suprasellar region but they can also be found in other intracranial sites (McCarthy *et al.*, 2012). Although the majority of patients have an isolated mass in either the pineal or the neurohypophyseal region, in some cases synchronous lesions in both locations may be found (Aizer *et al.*, 2013).

Primary central nervous system GCTs share histologic and genetic similarities to extracranial GCTs, and based on histology, they are classified as germinomas, which account for 2/3 of the cases, and nongerminomatous GCTs, which include embryonal carcinoma, yolk sac tumors, choriocarcinoma, teratoma, and mixed GCT with more than one type of histology (Echevarría *et al.*, 2008). GCTs may secrete the tumor markers alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (β-hCG) in the serum and/or CSF; high levels of AFP are found in yolk sac tumors, while β-hCG is very much increased in choriocarcinomas and mildly raised in germinomas which contain syncytiotrophoblastic elements (Bromberg *et al.*, 2013). Increased levels of one or both tumor markers can also be seen in mixed GCTs and immature teratomas.

The clinical presentation varies based on the location and size of the tumor. The majority of patients with suprasellar tumors presents with hypothalamic-pituitary dysfunction and visual disturbances, caused by compression or invasion of the optic chiasm. Endocrine manifestations include diabetes insipidus (the commonest sign which may precede all other abnormalities), followed by anterior pituitary hormone deficits and precocious puberty, especially in males due to increased β-hCG levels (Jorsal and Rørth, 2012). It should be also noted that the presence of the endocrinopathies often appear prior to radiological findings rendering the diagnosis of the tumor delayed (Sethi *et al.*, 2013).

MRI is the best imaging modality for intracranial GCTs. Suprasellar germinomas usually appear as ill-defined contrast enhancing masses, with similar or slightly low signal intensity on T1-weighted images and being isointense to slightly hyperintense on T2-weighted sequence (Wang *et al.*, 2010). On the other hand, teratomas appear as heterogeneous masses, due to their contents (fatty tissue, cystic components, and calcifications); they are also enhanced after gadolinium administration, except for the cystic areas.

The diagnosis of intracranial GCTs depends on clinical manifestations, tumor markers, neuroimaging characteristics, and/or histology. In patients with consistent radiological findings and increased serum and/or CSF AFP and β-hCG levels (above nationally defined thresholds), surgical biopsy is not required and the diagnosis can be based on the tumor markers (Murray *et al.*, 2015). Biopsy for an intracranial GCT diagnosis should be performed in patients who are marker-negative, regardless of imaging findings.

Treatment depends on the type of GCT. Pure germinomas are radiosensitive and can be treated with RT alone or a combination of lower dose RT and chemotherapy (Fu *et al.*, 2017). Patients with malignant nongerminomatous GCTs have a worse prognosis compared to those with pure germinomas and should receive a combination of chemotherapy and RT, to maximize their chance of cure; if metastatic disease is present, craniospinal RT should be performed (Murray *et al.*, 2015). Finally, radical surgical resection, where feasible, is the treatment of choice for intracranial mature and immature teratomas without malignant transformation.

Chordomas

Chordomas are rare tumors that are derived from embryonic remnants of the notochord. Their cranial sites include mostly the clivus region. They are slow-growing neoplasms that are locally destructive and may metastasize. Their symptoms depend on the direction of tumor growth and they more commonly manifest with headaches and asymmetric visual disturbances (mainly diplopia) (Kaltsas *et al.*, 2008). Pituitary dysfunction, although uncommon, may be present, as well as neck pain and nasopharyngeal obstruction in large tumors.

MRI reveals an invasive lesion in the clivus which enhances after contrast administration and is heterogeneously hyperintense on T2-weighted images (Rennert and Doerfler, 2007). A honeycomb appearance of the tumor after gadolinium injection can also be revealed due to low T1 signal areas within the neoplasm (Doucet *et al.*, 1997). Osteolytic bony erosion of the scull base and calcifications inside the tumor, which are common in chordomas, are better demonstrated with CT. In some cases, the normal pituitary gland can be distinguished from the tumor, which is helpful for the differential diagnosis of chordomas from other pituitary masses.

Treatment includes a maximal safe surgical resection of the tumor, leading to symptomatic improvement of the patient, although a complete removal of the neoplasm is usually not possible due to locally aggressive growth pattern of chordomas.

Considering the difficulty in achieving gross total resection of the tumor and its high recurrence rate, postoperative RT is important for local tumor control (Fernandez-Miranda *et al.*, 2014). However, issues regarding the type of adjuvant RT and the timing of the treatment (in all patients after surgery or only in those with residual or recurrent disease) remain unresolved.

Pituitary Malignancies

Primary pituitary malignancies are extremely rare and comprise pituitary carcinomas, primary lymphoma, and melanoma. On the other hand, metastatic involvement of the pituitary gland, although uncommon, has been increasingly reported during the past decades.

Pituitary Carcinoma

Pituitary carcinomas are tumors of adenohypophyseal origin demonstrating metastatic spread by either craniospinal dissemination or systemic metastases (Mete and Lopes, 2017). They are extremely rare, accounting for only 0.1% of all pituitary tumors, and most of them are hormonally active; prolactin-secreting tumors are the most frequent, followed by ACTH-secreting ones (Heaney, 2011). They tend to metastasize mainly hematogenous and through the lymphatic system rather than via craniospinal spread and are associated with a poor prognosis that depends on the extent of the metastatic disease (Heaney, 2014).

Pituitary tumors that will ultimately become carcinomas can follow different courses; they can be aggressive and unresponsive to treatment from their initial diagnosis, progressing rapidly to carcinoma, or they can remain stable, responsive to standard therapy and progress to carcinomas after a long period. No single morphological feature is able to predict pituitary tumor behavior but Ki-67 $\geq$3%, p53 positivity and a mitotic count $>$2 mitoses/10HPFs are frequently observed in aggressive pituitary tumors and carcinomas (Raverot *et al.*, 2017).

A multimodal approach is required in pituitary carcinomas including surgery, RT, chemotherapy, and medical therapy for the control of the biochemical secretion (in cases of functioning tumors) and tumor growth. Temozolomide can be used as the first-line chemotherapeutic treatment in pituitary carcinomas with documented progressive disease due to its reported effectiveness (Raverot *et al.*, 2017). Also, new potential targeted therapies are being studied such as mammalian target of rapamycin (mTOR), vascular endothelial growth factor (VEGF), and epidermal growth factor receptor 2 (EGFR2) inhibitors (Di Leva *et al.*, 2014).

Pituitary Lymphoma

Primary pituitary lymphoma defined as isolated involvement of the pituitary gland without evidence of systemic disease is extremely uncommon and can be found either in immunocompetent or in immunocompromised patients. They mostly affect patients in their 5th–6th decade of life, although adult cases from all age groups have been reported, and the majority of them are of B-cell origin (Tarabay *et al.*, 2016).

The clinical manifestations are those of expanding intracranial masses with headache, diplopia, visual field defects, and cranial nerve deficits; hypopituitarism is also a common finding (Giustina *et al.*, 2001). In addition, fever has been described in a small proportion of patients (Liu *et al.*, 2007). On imaging, primary pituitary lymphomas are usually contrast enhancing lesions, isointense on T1-MR images, and hypo- to isointense on T2-weighted sequences, and can demonstrate suprasellar extension, invasion of the sphenoid and cavernous sinuses, and pituitary stalk thickening (Tarabay *et al.*, 2016).

After the final diagnosis, which is established histologically, a differential diagnosis between primary and secondary intracranial pituitary lymphoma should be performed. Treatment is comprised of surgical resection of the tumor, RT, and chemotherapy.

Metastases

Although many kinds of malignancies can metastasize to the pituitary, breast, and lung cancers are the most common, followed by prostate and renal cancer (He *et al.*, 2015). Pituitary metastases occur mostly in patients with systemic dissemination but they can also be the first manifestation of an occult primary tumor or the only evident metastatic lesion (Komninos *et al.*, 2004).

The majority of pituitary metastases is asymptomatic and found incidentally. In symptomatic cases, the most common presenting manifestations are similar to those of pituitary macroadenomas, including headaches, visual field deficits, cranial nerve palsies, and anterior pituitary dysfunction but with higher prevalence (Al-Aridi *et al.*, 2014). Diabetes insipidus, however, which is absent in patients with pituitary adenomas, is a frequent finding in pituitary metastatic disease, given that the posterior pituitary is a preferred site for bloodborne metastatic spread due to its direct arterial supply from the hypophyseal arteries.

Imaging is not very helpful in the differential diagnosis of metastases from other pituitary masses, although some findings can be useful, including a dumbbell-shaped tumor (due to indentation by the diaphragma sella), invasion of the infundibular and bony erosion (He *et al.*, 2015; Komninos *et al.*, 2004). Although the diagnosis is ultimately confirmed by pathology, the possibility of pituitary metastasis is high in patients with a rapidly increasing sellar mass, sudden onset of diabetes insipidus and quick development of mass effect symptoms, especially in patients with a history of malignancy.

Treatment depends on the symptoms and the extent of systemic disease, as well as on the prognosis of the patient. The available management modalities include surgery, RT, and chemotherapy or a combination of them. In patients with metastatic mass effects causing visual impairment and diplopia or ophthalmoplegia, transsphenoidal surgery combined with postoperative radiotherapy may provide symptomatic relief and local disease control (Burkhardt *et al.*, 2016). However, resection of the pituitary metastases can be difficult due to their invasive, infiltrative, and vascular nature.

Gliomas and Pituicytomas

Gliomas in the sellar, parasellar, and suprasellar region may arise from the hypothalamus, the optic chiasm, nerve or tract, the brainstem, and the neurohypophysis or the pituitary stalk (pituicytoma). Optic pathway/hypothalamic gliomas are mainly diagnosed during childhood, with a significant proportion found in patients with neurofibromatosis type 1, and the majority is low-grade malignant tumors (Goodden *et al.*, 2014). On the other hand, pituicytomas are very rare intrasellar or suprasellar low-grade gliomas (about 100 cases in the literature) that originate from the neurohypophysis or infundibulum in adults (Wang *et al.*, 2016). Finally, brainstem gliomas, which generally affect young adults, can extend to the parasellar region (Hu *et al.*, 2016).

The clinical manifestations of gliomas depend on their location and they are the results of local tumor effects. The presenting features of pituicytomas include headaches, visual impairment, anterior pituitary hormone deficits, diabetes insipidus, and mild hyperprolactinemia due to pituitary stalk effect (El Hussein and Vincentelli, 2017). Patients with hypothalamic-optochiasmatic tumors can present with all the aforementioned manifestations, as well as hypothalamic dysfunction, obstructive hydrocephalus, and other signs of increased intracranial pressure (headaches, papilledema). Precocious puberty and diencephalic syndrome can also be found in children (Binning *et al.*, 2007).

Hypothalamic–optochiasmatic gliomas are suprasellar masses which may infiltrate the adjacent structures and on MRI they demonstrate high signal intensity on T2-weighted images. Larger tumors can be heterogeneous with both cystic and solid components, with the solid component showing a significant contrast enhancement. On the other hand, pituicytomas are generally well-defined, solid, round, or oval masses in the intrasellar and/or suprasellar region with the same MRI characteristics as hypothalamic-optochiasmatic gliomas (Yang *et al.*, 2016). The presence of a cystic component in pituicytomas is uncommon.

Complete tumor resection is the mainstay of treatment for symptomatic pituicytomas, although not possible most of the times due to their potential for infiltration and their high vascularization which can lead to extensive bleeding during the surgery limiting the extent of removal (Pirayesh Islamian *et al.*, 2012). RT has been used as adjuvant treatment in a minority of cases with residual disease but its efficiency has not been as yet established. Treatment of hypothalamic-optochiasmatic gliomas depends on the size, location, and presenting symptoms of the patients and can include observation, chemotherapy, RT, and surgery (Bornhorst *et al.*, 2016).

Gangliocytomas

Gangliocytomas are well-differentiated slow-growing neuroepithelial tumors composed by mature neurons that are very rarely discovered in the sellar region. The majority consists of mixed adenomatous and neuronal tissue and are usually found in association with a hormone secreting pituitary adenoma (in particular GH-secreting adenoma, followed by prolactin- and ACTH-secreting adenomas), although isolated sellar gangliocytomas have also been reported (Qiao *et al.*, 2014). Interestingly, the latter tumors can also demonstrate endocrine manifestations, especially hyperprolactinemia, acromegaly, and Cushing's.

The clinical presentation of gangliocytomas depends on their secreting activity and includes symptoms and signs due to pituitary hormone hypersecretion and/or local mass effects. Imaging reveals a sellar tumor that can extend to the suprasellar region or infiltrate the cavernous and sphenoid sinuses. Treatment is surgical and consists of the safe resection of the gangliocytoma, mainly via the transsphenoidal route. However, if hormonal resolution is not achieved with surgery (in cases of hormone secreting tumors), adjuvant medical treatment or RT may be needed (Cossu *et al.*, 2016).

Granular Cell Tumors

Granular cell tumors arising in the posterior lobe of pituitary or the stalk are rare entities and mostly affect adults in their 4th–5th decade of life and most frequently women (Covington *et al.*, 2011). They are purely suprasellar or intrasuprasellar lesions that can be asymptomatic and be discovered incidentally or present with visual impairment and headaches. Endocrine disturbances, mainly hyperprolactinemia, can also be evident, although hypopituitarism is uncommon; despite their location in the neurohypophysis and the infundibulum, diabetes insipidus is also rare (Ahmed *et al.*, 2017).

On MRI, granular cell tumors usually appear isointense to the brain on T1-weighted sequence and isointense to the white matter on T2-weighted images with a heterogeneous or homogeneous enhancement after gadolinium administration (Gagliardi *et al.*, 2016). In symptomatic cases, surgical resection of the tumor is the treatment of choice and the approach (transsphenoidal or transcranial) depends on the location of the lesion. The use of RT must be individualized, considering that this treatment modality has not shown clear benefits in cases which it has been used till now (Ahmed *et al.*, 2017).

Hypothalamic Hamartomas

Hypothalamic hamartomas are rare, congenital, benign mass lesions, composed of abnormally distributed but cytologically normal neurons and glia and are mostly found in children and adolescents (Coons *et al.*, 2007). They arise from the floor of the third ventricle, the tuber cinereum, or the mammillary bodies and they mainly present with epileptic syndromes, behavioral and cognitive impairment, psychiatric disorders and, from an endocrine point of view, isosexual central precocious puberty. A strong association between the clinical manifestations of the hypothalamic hamartomas and their connection to normal hypothalamic and surrounding tissues has been recognized (Mittal *et al.*, 2013).

High-resolution MRI scans reveal a hypo- to isointense mass on T1-weighted images, that is hyperintense on T2-weighted sequence and do not enhance following administration of contrast. Surgical treatment is usually required for the treatment of epilepsy associated with hypothalamic hamartomas, for halting the progressive decline in neurocognitive function and improving the coexisting behavioral and psychiatric syndrome. On the other hand, central precocious puberty is treated medically with GnRH-agonist therapy (Harrison *et al.*, 2017). Other methods of treatment, including stereotactic radiosurgery, brachytherapy, and radiofrequency ablation, can also be used in specific cases.

Cysts

Apart from RCCs, which constitute the majority of the cystic lesions found in the sellar/suprasellar region, other congenital cysts can also be rarely discovered in the same anatomical area, including arachnoid, epidermoid, and dermoid lesions. Arachnoid cysts arise from a splitting of the arachnoid membrane which fills with CSF, while epidermoid and dermoid cysts are of ectodermal origin, containing keratin and cholesterol, derived from desquamated epithelial cells; dermoid cysts contain epidermal appendages as well, such as hair follicles, sweat glands, and sebaceous glands (Lynch *et al.*, 2014).

Symptoms usually develop when the cysts are large enough to cause compression of the adjacent brain parenchyma or the cranial neurovascular elements. Headaches and visual disturbances are the most commonly presenting manifestations, while pituitary dysfunction is less frequently observed. Spontaneous rupture of a dermoid (mainly) or a epidermoid cyst (in rare cases) can cause aseptic chemical meningitis; there are also reports for these two types of cystic lesions describing a clinical presentation mimicking pituitary apoplexy (Tuna *et al.*, 2008; El-Bahy *et al.*, 2006).

On imaging, arachnoid cysts are pure cystic lesions with clear defined borders, no contrast enhancement or calcifications, and with a typical CSF-like signal behavior. Epidermoid cysts are usually isointense or slightly hyperintense to CSF on both T1- and T2-MR images, although lesions with high protein content can show reversed signal intensity. They generally do not enhance after contrast administration, although some minimal rim enhancement can occur, and calcification may be present (Osborn and Preece, 2006). High signal intensity on diffusion-weighted images is helpful for the differential diagnosis of epidermoid cysts from arachnoid ones (Hakyemez *et al.*, 2005). Finally, dermoid cysts are hyperintense on T1-weighted images due to the lipid content and they have heterogeneous signal intensity on T2-weighted sequence due to their mixed composition; they do not show contrast enhancement and foci of calcification can also be evident (Warakaulle and Anslow, 2003).

Sellar/parasellar cystic lesions not causing manifestations do not require treatment and can be followed up, while surgical treatment is preferred in symptomatic cases. In arachnoid cysts, the operative aims include the evacuation of the cystic fluid, the safe excision of all or part of the cyst membranes, and the enlargement of the communication of the lesion with the suprasellar CSF spaces (Dubuisson *et al.*, 2007). On the other hand, in epidermoid and dermoid cysts, a gross-total resection is desirable but not feasible many times, given that these tumors can often be adhesive to vital vascular and nervous structures lesions and the cyst capsule and contents cannot be dissected without significant morbidity (Zada *et al.*, 2010). Intraoperatively, the removal of the contents of the cysts should be performed with caution in order to avoid a spillage of fluid that can cause chemical meningitis.

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Surgical Treatment of Nonfunctioning Pituitary Tumors

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Abbreviations

3D	3-Dimensional
BMI	Body mass index
CT	Computerized tomography
CSF	Cerebrospinal fluid
ERS	Extensive resection surgery
HSS	Hypothalamus-sparing surgery
MRI	Magnetic resonance imaging
NFPA	Nonfunctioning pituitary adenoma
RCC	Rathke's cleft cyst

Glossary

Adenomas A type of benign epithelial tumors of glandular origin, glandular characteristics, or both, which can grow from glandular organs, such as the pituitary gland and others.

Craniopharyngioma Squamous epithelium tumors develope from residual cells of Rathke's pouch, which often arises from the anterior superior margin of the pituitary. It does not undergo malignant degeneration but has a tendency to recur.

Endoscope In contrast to the microscope, endoscopy usually has a long and thin body that carries the camera and light source on the tip, which can be inserted into a cavity or organ and inspect the inside, providing an image via a digital screen.

Hypopituitarism The deficient (hypo) secretion of one or more of the hormones normally produced by the pituitary gland.

Hypothalamic syndromes Malfunctions of the hypothalamus, causing cachexia, disturbed sleep–wake rhythm, altered temperature regulation, the gain of weight, etc.

Non-functioning tumor or hormonally inactive tumor Neoplasm that does not produce excess hormones or just biologically inactive hormone fragments.

Operating microscope An optical microscope specifically designed to be used in a surgical setting is typically used to perform microsurgery.

Pituitary tumor Tumors which are located in the intra-, para-, or supra-sellar region, such as pituitary adenomas, craniopharyngiomas, and meningiomas.

Suprasellar region The region above the pituitary (the level of the chiasmatic cistern), below the third ventricle. It includes the suprasellar cisterns, the visual pathways, and the inferior hypothalamus.

Visual compromise Tumors in sellar region may compress the optic chiasm, thus causing deterioration of vision, such as bitemporal visual field defects and decreased visual acuity.

History

Pituitary surgery began with subtemporal craniotomy approaches to the sellar region performed by Horsley in 1887. Almost 20 years later, Schloffer and Von Eiselberg described a new nasal approach for pituitary surgery via the sphenoid sinus, which was thus called the transsphenoidal approach. Oscar Hirsch improved the technique further. He and Harvey Cushing, who had tremendous interest in pituitary physiology and pathophysiology, designed instruments for this operation. Cushing performed the subtemporal approach, first described the sublabial transsphenoidal approach, and later the "transfrontal" (i.e., subfrontal) approach for pituitary adenoma resection. However, he abandoned the transsphenoidal approach since with the transcranial approach, he had a lower recurrence rate and a better recovery of vision. For a while, only Dott and Guiot continued to practice the transsphenoidal approach. With the introduction of the image intensifier and the operating microscope by Jules Hardy, from 1965 on, the transsphenoidal approach experienced a renaissance and became the operative approach of choice for most of the sellar tumors. Large patient series documented its efficiency and safety. More recently, with the development of magnetic resonance imaging (MRI), the endoscope, and navigation technology, transsphenoidal surgery is considered to be the first choice operation for pituitary surgery. It is efficient and safe for most patients (Buchfelder and Schlaffer, 2009; Couldwell, 2004).

Indications of Surgery

Tumors that compress the visual pathways or neurovascular structures on MRI, and cause corresponding symptoms like deterioration of vision and diplopia, respectively, are considered absolute indications for surgery (Chanson *et al.*, 2015; Freda *et al.*, 2011; Marosi *et al.*, 2008). Tumors, which abut or compress the visual pathways, or neurovascular structures on MRI, without causing any corresponding symptoms, are also recommended to undergo surgical resection as primary treatment (Freda *et al.*,

2011; Marosi *et al.*, 2008). Patients with hypopituitarism in the endocrinological assessment should be considered for surgery (Chanson *et al.*, 2015; Freda *et al.*, 2011). When the tumors are close to the chiasm (<5 mm) but as yet without any contact, decisions can be made on an individual basis, particularly taking into account the age of the patients and their health status (Chanson *et al.*, 2015). However, the risk of apoplexy and a patient's desire for pregnancy in nonfunctioning pituitary adenomas (NFPAs) should be considered (Freda *et al.*, 2011). Patients without ophthalmological or endocrinological symptoms, but with other symptoms that are caused by the mass effect of the tumor, such as a persistent headache, may also be good candidates for elective surgery (Chanson *et al.*, 2015; Freda *et al.*, 2011; Marosi *et al.*, 2008). Tumors with documented size increase during follow-up imaging might be considered for surgery (Freda *et al.*, 2011). Incidental, asymptomatic tumors which are remote to the chiasm (≥5 mm) should rather undergo serial follow-up with MRI and endocrinological assessments, with or without ophthalmological testing (Chanson *et al.*, 2015; Freda *et al.*, 2011; Igarashi *et al.*, 1999; Marosi *et al.*, 2008). Other conditions including pituitary apoplexy, progression, or recurrence of residual tumors during the follow-up period should be managed according to the criteria mentioned above. In some situations, surgery may be needed to establish the diagnosis of an unknown lesion (Glezer *et al.*, 2008; Hadziahmetovic *et al.*, 2008; Huang and Castillo, 2005).

Preoperative Evaluation

Pituitary MRI with T1-weighted coronal and sagittal (with and without contrast enhancement) and T2-weighted axial sequences are the most valuable radiological studies, especially with high-field MRI. Computerized tomography (CT) may also provide some information. However, the MRI depicts any intracranial lesion with the highest soft tissue contrast and precisely shows its relationship to the adjacent anatomical structures. Sometimes, in consideration of preoperative evaluation, CT angiography or MR angiography is recommended to rule out aneurysms or other abnormal vascular conditions. An ophthalmological evaluation, in particular, perimetry and visual acuity testing, is required, at least, once there is impingement of the visual pathways on MRI. Of course, clinical and laboratory endocrinological evaluation is needed before surgery to exclude hormonally active adenomas and to assess pituitary function. Pre- and perioperative substitution therapy should be initiated in case of partial or total hypopituitarism. Clinical symptoms, such as diplopia, headache, and other cranial nerve functions, should be documented as well. These radiological, ophthalmological, and endocrinological evaluations are crucial for the optimal management of an individual lesion and the assessment of surgical outcome (Buchfelder and Schlaffer, 2009). Additional testing might be required for the differential diagnosis.

Differential Diagnosis

Pituitary adenomas, craniopharyngiomas, supra- and parasellar meningiomas, and various cystic lesions are frequent space-occupying lesions in the sellar region. Secreting pituitary adenomas can be easily identified by measuring hormones. Thus, the entire differential diagnosis of space-occupying lesions within the sellar region is related to nonfunctioning tumors.

Non-Functioning Pituitary Adenomas

In adults, an adenoma is by far the most common pituitary lesion. Nonfunctioning adenomas are rare in childhood. The tumors usually present as macroadenomas (>10 mm diameter). Only a minority are incidentally found as microadenomas (<10 mm diameter). The classical manifestations are visual compromise and hypopituitarism. Loss of vision may be gradual (with gradual tumor growth) or sudden (apoplexy). Characteristically, the mass lesion sits in the sella turcica and expands from there (Buchfelder and Schlaffer, 2009).

Craniopharyngiomas

They are common pituitary tumors in children, but also occur even in aged adults. Craniopharyngiomas tend to arise from the anterior superior margin of the pituitary, the infundibulum, and from the third ventricle. Many craniopharyngiomas have cystic and solid components, as well as calcifications in imaging. Some craniopharyngiomas might leak cystic component and thus present with aseptic meningitis. Clinically, patients may present with visual compromise (optic chiasm compression), hypothalamic syndromes (compression on hypothalamus), hypopituitarism (compression on the pituitary gland), and symptoms due to hydrocephalus (when the tumor occludes the foramina of Monro). Intraoperatively, the normal pituitary gland is usually found to be compressed at the sellar floor level, and the "machine-oil" cyst fluid contains cholesterol crystals. Once the diagnosis of craniopharyngioma is suspected, most surgeons would recommend an operation (Karavitaki *et al.*, 2006).

Supra- and Parasellar Meningiomas

Patients with meningiomas in the sellar region usually present with progressive visual loss due to optic nerve or chiasm compression. Characteristic features in imaging are a wide dural attachment at the planum sphenoidale in suprasellar meningiomas

and a narrowing of the carotid artery in parasellar meningiomas. The mass of the lesion homogenously enhances in postcontrast T1-weighted sequences on MRI and the major portion of the lesion is usually not located within the sella turcica. They can also cause hormonal deficiencies as the tumor impinges on normal pituitary gland and stalk, however, much less frequent than pituitary adenomas or craniopharyngiomas. Symptomatic meningiomas and those with signs of compression in imaging should undergo surgery. Asymptomatic patients without neurovascular compression in imaging are recommended to undergo serial follow-up. Patients who are not suitable candidates for surgery can be considered for radiotherapy (Marosi *et al.*, 2008).

Cystic Lesions

Rathke's cleft cyst (RCC) is thought to derive from remnants of Rathke's pouch. It appears as low-density cystic lesions in the CT and low signal intensity in the MRI. RCC is radiologically differentiated from craniopharyngioma by the presence of only a cyst without solid components or calcification. It can cause visual compromise and/or hypopituitarism just as with craniopharyngioma. Intraoperatively, the cyst content resembles motor oil. The differentiation from craniopharyngioma is essentially based on the histology of the cyst membrane (Huang and Castillo, 2005).

Colloid cysts are the macromanifestations of "pars intermedia's" cysts, which are frequently found in minute size. They do not at all enhance in either CT or MRI, and are located between the anterior and posterior lobe of the pituitary. As intrasellar space-occupying lesions, they might cause disturbances of pituitary function. They are ideal targets for selective resection because they lack membranes (Nomikos *et al.*, 1999).

Rare Parasellar Tumors

Hypophysitis refers to inflammation of the pituitary gland, with a sellar mass lesion in the MRI. It presents mostly with headaches, followed by visual disturbances. Nontumorous hyperprolactinemia is a common feature in hypophysitis. Patients who are considered to have hypophysitis should have a histologic confirmation via a biopsy. When the patients suffer from cranial nerve deficits and severe headache, pituitary surgery can be an option as active treatment, and partial resection is preferred over gross total resection. It is a matter of debate whether corticosteroids should be used for primary treatment (Glezer *et al.*, 2008; Gutenberg *et al.*, 2006).

Germinomas are the most common subtype of central nervous system germ cell tumors that can mimic craniopharyngiomas and other nonfunctioning tumors when located within the sellar region, frequently causing diabetes insipidus and severe hypopituitarism. The MRI shows contrast-enhanced lesion with unclear boundaries, mostly in young patients. Patients with germinomas may develop spinal metastasis. Therefore, a spinal MRI could assist in the diagnosis. There are tumor markers like alpha-fetoprotein, β-human chorionic gonadotrophin and placental alkaline phosphatase, which can be assessed in the serum and cerebrospinal fluid (CSF). The diagnosis is confirmed by biopsy. Radiotherapy is usually curative because of the high radiosensitivity (Hadziahmetovic *et al.*, 2008).

Chordoma is a semimalignant tumor that arises from the clivus. It presents with symptoms of mass effect, like other nonfunctioning tumors, such as headaches, visual deterioration, diplopia, and hormonal changes. Characteristically, the clivus is distended in postcontrast MRI, and shows heterogeneous enhancement and frequently also brain stem compression. Surgical treatment usually results in incomplete resection and requires postoperative irradiation (Glezer *et al.*, 2008).

Moreover, there are other neoplastic, inflammatory, and vascular rare space-occupying sellar and parasellar lesions that are even less frequent (Glezer *et al.*, 2008; Huang and Castillo, 2005; Petrakakis *et al.*, 2016).

Surgical Anatomy

The pituitary gland sits in the sella turcica, which lies almost in the center of the head. The sella turcica is a bony cavity in the skull base. Bone thus forms the anterior and posterior walls and the floor of the pituitary fossa. The cavernous sinuses are the lateral walls. The roof of the sella is called diaphragm sella. It has a small opening for pituitary stalk just enough wide for it to pass through. The dura from the frontal basis splits into two layers that hug the pituitary gland. Anteriorly and inferiorly to the pituitary fossa is the sphenoid sinus, and superiorly of the pituitary gland is the suprasellar region with the optic pathways, blood vessels (anterior cerebral artery and anterior communicating artery). As the nonfunctioning tumor grows, it will increasingly compress and distort the normal pituitary gland and thus gradually cause hypopituitarism. Subsequently, the tumor is likely to cause enlargement of the sellar fossa and expand laterally and inferiorly, to finally reach and compress surrounding neurovascular structures resulting in cranial nerve palsy (compression on cranial nerves III, IV, V, and VI which run through the cavernous sinuses), and deterioration of vision (compression on optic chiasm or optic nerves).

In order to reach the sellar region and remove the tumors, a passage to this region has to be dissected either from inferiorly, that is, through all the bony structures of the sphenoid sinus and sellar floor (transsphenoidal approaches), or from superiorly via a craniotomy (transcranial approaches) along the skull base or through the ventricles to reach the sellar region (Buchfelder and Schlaffer, 2009; Couldwell, 2004).

Operative Approaches

Transsphenoidal Approach

Transsphenoidal surgery is to date the main procedure for pituitary tumor resection since it suits most of the cases. There are many variants of the transnasal approach, such as a submucosal dissection along the nasal septum or a direct endonasal access by cutting the mucosa only at the surface of the sphenoid sinus. A sublabial submucosal approach is another popular approach to reach the sphenoid sinus, which provides a perfect visualization of the entire sella when compared with the direct transnasal approach. 95% of pituitary adenomas can be operated via this approach. The patient is positioned supine, with the head slightly extended. A septal mucosal incision in the right nostril (based on the surgeon's dominant hand) is made after submucosal injection of diluted adrenaline to expose the nasal cartilage. The septal mucosa is detached from the cartilaginous and osseous septum by creating a submucosal pouch, which finally exposes the crest of the sphenoid bone. The perpendicular plate of the vomer is pushed aside and partially removed. A speculum is introduced to maintain the corridor open and to allow microscopic visualization of the sphenoid sinus. The septations are resected to expose the sellar floor, and the mucosa of the sphenoid sinus is removed (Buchfelder and Schlaffer, 2009). The C-arm or neuronavigation can be used to confirm the location. The sellar floor is removed exposing the dura. Then a "cross" incision or square window is made in the dura and tumor is removed with different microsurgical instruments. Tumors with soft consistency or fluid components can be easily removed with suction. Tumors with firm texture or attachment to surrounding structures need to be dissected gently piece by piece or "en bloc." The goal is to achieve total resection or maximal safe resection. The normal pituitary should be identified and preserved during surgery, which gives the name "selective adenomectomy" to the procedure. During the surgery, the descent of the arachnoid is evidence of decompression, but occasionally tumor may hide between the arachnoidal folds. An elevation of intracranial pressure (ICP) by using ventilation with positive end-expiratory pressure (PEEP) or compression of the jugular veins may help to make the residual tumor descend, but also increases the risk of CSF leak.

Many different modifications of the transsphenoidal approach are described and both microscope and endoscope are potential visualization aids in this approach (Couldwell, 2004). The classical endonasal transsphenoidal approach can be extended by additionally drilling parts of clivus or anterior fossa for the resection of large pituitary tumors (Di Maio *et al.*, 2011). Extreme variants, mostly supported by the endoscopic technique, are opening of the frontal base with posterior ethmoidectomy, wide sphenoidotomy, removal of large parts of the sphenoid, and drilling of the clivus, laterally only limited by the optic canal. With the extended approaches, the risk of postoperative CSF leaks is high.

When the tumor has been extracted as much as possible, the sellar floor must be reconstructed. This can be accomplished by gelfoam, fat, fascia lata, or bone fragments. The nasal septum and mucosa are repositioned and the mucosal incision is sutured. Many authors recommend the introduction of nasal tamponades into both nostrils. Modifications might not require tamponades. In case of CSF leak, an autologous graft with fascia lata or fat and harvested bone has been used to close the defects of the skull base. Fibrin glue is often used as an adhesive (Buchfelder and Schlaffer, 2009).

In craniopharyngioma or meningioma and in many of the rare tumors in the sellar region, the adenohypophysis might be the first structure to be seen after dura opening. A dissection of the adenohypophysis in the anterior–posterior dimension or even a partial resection of the gland might be needed to expose the tumor. The tumors are usually quite firm and need to be gradually dissected. The optic chiasm, the optic nerve and tract, the pituitary stalk, the anterior communicating artery, and the hypothalamus are important structures that should be detached cautiously because any mechanical damage could cause postoperative complications (Buchfelder and Schlaffer, 2009; Couldwell, 2004).

Transcranial Approach

Although the transsphenoidal approach is sufficient for many cases, transcranial approaches offer better access when there is only minimal enlargement of the sella but extensive suprasellar extension, or if the tumor has a larger extrasellar than intrasellar portion. For fibrous tumors like meningiomas in this region or recurrent tumor following transsphenoidal resections, transcranial approaches are occasionally more suitable. However, the disadvantage of transcranial microsurgery is the direct brain exposure, retraction, and the inconvenience that surgeons have to sharply dissect the tumor from the optic nerve and cerebral arteries, which exposes these to the threat of damage.

Usually, a frontolateral or frontotemporal (pterional) craniotomy is performed with a curvilinear skin incision behind the hairline. The skin flap is retracted and a small craniotomy is performed, placed maximally near the skull base. A dura flap is retracted to expose and dissect the Sylvian fissure and to release CSF to slacken the brain. It is suitable for tumors that also have a significant lateral extension. A subfrontal approach provides access to both optic nerves and does not require the dissection of the Sylvian fissure. The view, however, is pretty much restricted to the midline. Self-retaining retractors are used to maintain a corridor of access to the sella and visual pathways. The tumor is resected piece by piece through this corridor. For tumors that extend significantly into the third ventricle or have a large retrosellar component, a trans-lamina terminalis approach could be considered with bifrontal skin incision and a midline craniotomy. This approach is most frequently used for craniopharyngioma, which may extend into the third ventricle. The olfactory nerves need to be gently dissected. For complex tumors, one approach may not be sufficient. Thus, a transsphenoidal approach followed by a transcranial approach is sometimes preferred in two stages to resect such tumors (Buchfelder and Schlaffer, 2009; Couldwell, 2004).

Surgical Results

The goals of surgery for nonfunctioning tumors in the sellar region include the elimination of mass effect, normalization and preservation of endocrinological functions, and ideally total resection. The extent of resection is normally evaluated with pre- and postoperative MRI and used as a measure of surgical results. Pre- and postoperative ophthalmological and endocrinological functions should also be appreciated as surgical results.

Nonfunctioning Pituitary Adenomas

For all tumors, utmost resection should be attempted. However, many NFPAs are usually already large at the time of diagnosis. Over the years of progression, para- and suprasellar regions might be invaded. Invasion into the cavernous sinus and encasement of major arteries are factors that force surgeons to leave some tumor on purpose, which might be treated by irradiation. The evaluation of the extent of resection, the existence of a residual tumor, and the occurrence of recurrence are entirely based on MRI since there are no hormonal tumor markers. There is some controversy in the literature in consideration of the total resection and recurrence rates at 5 or 10 years postoperatively. One study reported that 23 out of 35 patients (66%) who underwent transsphenoidal surgery had residual tumor according to postoperative imaging (Marazuela *et al.*, 1994). Likewise, in another study, 34 out of 51 patients (67%) who underwent transsphenoidal surgery had tumor residuals (Soto-Ares *et al.*, 2002). In contrast, a large individual series reporting the results of 1140 patients with several types of pituitary adenomas who underwent primary transsphenoidal surgery demonstrated a total resection rate of 64.8% in patients with NFPA (Mortini *et al.*, 2005). In respect to long-term results, 98 out of 132 (74.2%) patients who were treated with transsphenoidal surgery had a total resection, and 73 of the 98 total resection patients were followed by serial pituitary imaging without irradiation therapy, showing a 90% recurrence-free survival at 5 years (Bradley *et al.*, 1994). A follow-up study of 65 of the 73 patients from the same cohort showed freedom from recurrence in 82% at 5 years and 56% at 10 years based on life table analysis of nonirradiated patients (Turner *et al.*, 1999). Another study described a 5-year recurrence-free rate of 84.8% and 10-year recurrence-free rate of 49.5% in nonirradiated patients (Park *et al.*, 2004), which was lower than that in the irradiated patients (in these, 5- and 10-year recurrence-free rates were both 97.7%).

Recovery of vision is probably the first sign of a successful decompression achieved through surgery. In a study with 2000 eyes, it was documented that a visual recovery occurred gradually after 3 months postoperatively, and peaked at 6 months to 1 year in patients who had giant adenomas with ophthalmological symptoms for >2 years (Dutta *et al.*, 2016). They reported a 93% improvement of vision at 1 year after pituitary tumor resection in both visual acuity and visual fields.

After removing the tumor and the decompression of the normal pituitary, endocrinological improvements were found in a variable degree of the individual axes. A study from Spain reported that all but one patient who had normal pituitary functions preoperatively retained their functions, while for the patients with preoperative abnormal pituitary function, 46% had improvements of variable degree and 54% had persistent deficits (Marazuela *et al.*, 1994). We previously reported improvement in endocrinological function in 49.7% of the patients who underwent transsphenoidal surgery, whereas 11.3% of the patients who underwent transcranial surgery had at least some recovery of pituitary function (Nomikos *et al.*, 2004).

The outcome is much more associated with tumor characteristics and surgical experience than with the visualization tool utilized for the resection. Moreover, asymptomatic patients with NFPA have better surgical results and the 5-year tumor recurrence rate was found much lower than that of symptomatic patients (Losa *et al.*, 2013). Moreover, patients who were younger at the time of surgery had a higher 5-year recurrence risk than older patients (Losa *et al.*, 2013; Reddy *et al.*, 2011).

Craniopharyngiomas

Craniopharyngiomas are benign tumors with a high tendency to recur, and local recurrences occur mostly within the first 3 years after complete resection. The main controversy in the management for craniopharyngioma is whether radical resection or subtotal resection followed by radiation therapy would be the better choice (Karavitaki *et al.*, 2006; Shi *et al.*, 2008). A realistic chance for tumor excision exists only during primary surgery. An attempt of radical resection is preferred by some neurosurgeons even if the sacrifice of the pituitary stalk and the need of postoperative substitution therapy are unavoidable sequelae. There are three major types of surgeries for craniopharyngioma: transcranial microsurgery, transsphenoidal microsurgery, and transsphenoidal endoscopic surgery. Unlike NFPA, craniopharyngiomas frequently have extra-sellar extensions and intense involvement with the surrounding neurovascular structures and the hypothalamus, and thus, an operation via a transcranial procedure is more frequent than via the transsphenoidal route. As a result, overall severe complications in craniopharyngiomas, such as postoperative visual defects, partial or complete hypopituitarism, hypothalamic dysfunctions, are higher than that in NFPA. Surgical results have been reported with variable outcomes in the literature, but with similar surgical indications and postoperative complications. Transsphenoidal approaches are documented with more efficiency in improving ophthalmological symptoms than transcranial approaches, but with a significantly higher risk of postoperative CSF leak. However, postoperative seizures and permanent diabetes insipidus rate are significantly more frequent than after transsphenoidal approaches. Transsphenoidal endoscopic and microscopic surgeries reveal a similar amount of resection and postoperative complications. The extended endoscopic endonasal approach is popular in some centers, but frequently there are difficulties in the reconstruction of the skull base. A study from China (Shi *et al.*,

2008) reported on an impressively high resection rate of craniopharyngioma using microscopic techniques. They extracted 97% of the tumors with a diameter of up to 6 cm. In recent years, another management strategy is proposed by an initiative from France (Elowe-Gruau *et al.*, 2013), who advocates hypothalamus-sparing surgery (HSS), a maximal resection sparing the optical and hypothalamic structures, for craniopharyngioma especially in children to decrease surgery-related postoperative hypothalamic dysfunctions and to provide a better postoperative quality of life. They reported an 18.5% complete resection rate by the first operation, and 37% of the patients required at least one additional intervention for tumor residuals or recurrences. In contrast, a 60% complete resection rate after the first extensive resection surgery (ERS) was observed (Elowe-Gruau *et al.*, 2013). Interestingly, the body mass index (BMI, indicates the risk of obesity) of patients with HSS was significantly lower after 6 and more months postoperatively than after ERS with no significant difference in the local recurrence rate, and patients who underwent HSS tended to have less postoperative endocrinological deficiencies (Elowe-Gruau *et al.*, 2013; Karavitaki *et al.*, 2006; Mortini *et al.*, 2013).

Complications of Surgery

Transsphenoidal surgery has a lower general risk of complications than transcranial surgery. Permanent diabetes insipidus is more frequent in transcranial approaches, for the direct dissection of the infundibulum. Transient diabetes insipidus usually occurs within 24 h after surgery and abates within several days. Damage to the normal gland might result in long-term hypopituitarism, which requires hormonal substitution therapy. Hypothalamic injury is feared after craniopharyngioma radical resection and results in an uncontrollable gain of weight and behavioral abnormalities. Other complications, such as loss of vision and carotid artery injury, are relatively rare (Mortini *et al.*, 2005). Modern technologies such as neuronavigation or application of the laser Doppler probe allow the surgeon to localize the carotid artery and avoid such injuries. Rhinological complications may cause persistent nasal discomfort after transnasal surgeries. Infection, meningitis, and subarachnoid hemorrhage are other rare but possible complications for both transsphenoidal and transcranial surgeries. Individual reports of selected patient series from experienced surgeons suggest extremely low cumulative complication rates in primary interventions (Mortini *et al.*, 2005). However multicenter studies reveal much higher, countrywide figures. In a recent multicenter analysis of 5886 patients who underwent microsurgical or endoscopic transsphenoidal surgery, the overall cumulative complication rate of transsphenoidal surgery was 40.0%. It was 35.3% with microscopic surgery and 45.8% with endoscopic surgery (Asemota *et al.*, 2017). The most common postoperative complications after transsphenoidal surgery were hypopituitarism (19.1%), particularly diabetes insipidus (16.9%), followed by fluid and electrolyte disorders (11.2%) and postoperative CSF leaks (10.4%). Multivariate regression analysis and propensity score matching showed that endoscopic surgery was more likely to cause diabetes insipidus and postoperative CSF leaks than microscopic surgery (Asemota *et al.*, 2017). Other medical complications, such as acute respiratory failure, deep vein thrombosis, pulmonary embolism, and mortality, were not significantly different in microscopic and endoscopic surgery. The overall complication rate of transsphenoidal surgery increased by 18.5% from 2010 to 2014, while neither the endoscopic-related complication rate nor the microscopic-related complication rate changed over this period. This increase in overall complication might be due to a better recording of complications (Asemota *et al.*, 2017). A convincing relationship between the surgeon's experience, the centers' caseload, and the cumulative complication rate was documented in an analysis based on insurance data in the United States (Barker *et al.*, 2003).

Follow-Up

Regular repeat radiological, ophthalmological, and endocrinological examinations are recommended for nonfunctioning pituitary tumors.

The first endocrinological and ophthalmological testing is usually performed 3–7 days after surgery, and the initial postoperative MRI 3 months after surgery, together with endocrinological and ophthalmological testing. Ophthalmological follow-up is repeated every 6 months until maximal improvement is achieved. The frequency of hormonal follow-up is adapted to the requirements (Chanson *et al.*, 2015). Further radiological follow-up intervals should be 1 year (Chanson *et al.*, 2015). For patients without postoperative residuals, they may be repeated at intervals of 5 years, then at 7, 10, and 15 years after surgery (Chanson *et al.*, 2015). One study reported alarmingly high relapse rates of 23.1%, 46.7%, and 67.9% at 5, 10, and 15 years respectively, and a significant increase in the regrowth rates in patients either with tumor remnants in the first postoperative imaging or a younger age at initial surgery (Reddy *et al.*, 2011). Thus, patients with postoperatively documented or suspected residual tumor should undergo a yearly follow-up MRI for at least 5 years, and then every 2 or 3 years (Chanson *et al.*, 2015). Ideally, life-long follow-up should be performed (Reddy *et al.*, 2011). Irradiation is recommended for those patients who harbor residual tumor to decrease the risk of recurrence (Park *et al.*, 2004). After irradiation, ophthalmological follow-up in yearly and hormonal follow-up in 6 monthly intervals should be continued (Chanson *et al.*, 2015). Symptomatic recurrences should be submitted to further surgical management or irradiation.

For asymptomatic adenomas that do not reach criteria for surgical resection, follow-up with pituitary MRI and endocrinological screening is recommended over many years (Chanson *et al.*, 2015; Freda *et al.*, 2011).

Modern Advances

New Technology

Endoscope

The development of the endoscope and its introduction into pituitary surgery provided surgeons with a closer view and better illumination in the deep-seated pituitary region. With an endoscope, the "eye" of the surgeon is virtually introduced close to the sellar floor or tumor cavity, whereas the lens of the microscope remains far outside and the surgeon's view is restricted by the straight beam of light. It is feasible for both transsphenoidal and transcranial approaches. Various angled lenses are available for endoscopes that allow the surgeons to have a more panoramic and lateral visualization. It can be combined with the microscope to compensate its visual limits, or the surgery can be conducted purely by using the endoscope. The advantages of endoscopic transsphenoidal surgery are avoidance of a septal dissection, a nasal speculum, and of postoperative tamponades, as well as better visualization. However, the insertion of the endoscope may take valuable space when micro instruments must be manipulated through the corridor (Buchfelder and Schlaffer, 2016b). Thus, some surgeons use both nasal cavities for this procedure and the endoscope will be held by an assistant. Most of the endoscopes do not offer three-dimensional (3D) views, and endoscopic surgery requires special training. Experience with endoscopic surgery is quite crucial because the surgical orientation is different from conventional microsurgery. Many series reported a longer duration of endoscopic surgery than microsurgery, the results of endoscopic surgery are comparable to conventional microsurgery (Mortini, 2014).

Intraoperative MRI

A surgical room with access to MRI allows the acquisition of MRI during a surgical procedure. Mostly, the surgery is interrupted and an MRI scan is performed when the surgeon estimates that he has achieved satisfactory tumor resection. This intraoperative MRI reveals the extent of tumor excision, and in case of a residual lesion, guides re-resection. Numerous data from different countries have shown that intraoperative MRI has the advantage of improving the extent of resection, and increase the proportion of complete excisions. This intraoperative feedback can certainly speed up the learning curve of young surgeons with little experience in this region. High-field MR systems (≥ 1.5 T) have a better resolution and allow a better depiction of the parasellar space in comparison to the low-field system (≤ 0.5 T). However, the measurement during a surgical procedure is time-consuming. Moreover, the tumor cavity can be filled with blood, fluid, and hemostasis material, and it is sometimes difficult to interpret the intraoperative situation (Buchfelder and Schlaffer, 2012, 2016a). Although 1.5 T and 3 T intraoperative systems are commonly available, the cost of the system restricts the general use of this technology. In addition, the interpretation of intraoperative imaging should always refer to surgical conditions, rather than simple radiological manifestation.

Neuronavigation

Neuronavigation utilizes preoperative MRI and/or CT to guide the surgery in a 3D anatomical orientation. This is extremely helpful in patients with incomplete or lacking pneumatization of the sphenoid sinus and during reoperations. The recognition of the midline is crucial in transsphenoidal surgery. A proper opening on the sellar floor avoids complications. Crucial structures are generated with computer software based on preoperative imaging, and the navigation system superimposes these contours into the real surgical field. Although it cannot directly depict tumor residuals like intraoperative MRI, it increases the comfort of the surgeon and the safety of the patient. No brain shift is expected at the level of the skull base. Thus, the location provided by the navigation system can be trusted, particularly during transsphenoidal surgery (Buchfelder and Schlaffer, 2016b).

Ultrasound and Doppler system

Pituitary adenomas are mostly hyperechoic masses in ultrasound images. Ram and coworkers described that ultrasound allowed visualizing the interface between tumor and normal pituitary gland and enabled depiction of small tumors (Ram *et al.*, 1999). However, the inconsistency of findings, the low resolution, and the prerequisite for specialized ultrasound skills made ultrasound less popular in the recent years. Doppler systems are commonly used to localize the carotid arteries at the level of the sellar floor (Buchfelder and Schlaffer, 2016b). They are extremely helpful in reducing the risk of damaging the carotid arteries, especially when the tumor infiltrates the cavernous sinus (Solheim *et al.*, 2010).

Conclusions

Transsphenoidal surgery to date is the established procedure of the first choice for pituitary tumor resection. Transcranial surgery has advantages in tumor with extensive extrasellar components. Endoscopic surgery is becoming more popular, but as yet no improved resection rate or reduction of complications has been documented when compared with microscopic surgery. Intraoperative MRI and navigation increase the safety of the procedure and the rate of maximal resections, especially in complicated cases. Serial imaging, endocrinological testing, and assessment of vision can be used to evaluate the surgical results, as well as to detect tumor recurrence. Residual and recurrent tumor can be either observed or treated with secondary surgery or irradiation, depending on the patient's condition.

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Pituitary Radiotherapy

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Introduction

Various modalities can be employed in the treatment of patients with secreting or nonsecreting pituitary adenomas, including medical treatment, surgical intervention, or either radiotherapy or radiosurgery. Concerning these latter two modalities, it is however, sometimes difficult to distinguish between different patient profiles that may benefit from such treatments. The principles of radiotherapy and radiosurgery being different, it is valid to consider each of them individually rather than in opposition: their patient profile as a general rule is different and each modality has a place in the treatment of pituitary adenomas. The aim of this article is to clarify the respective indications for each modality, their advantages and disadvantages in terms of their antisecretory and antitumoral effects, as well as their patient tolerance, and to better define whether radiotherapy and radiosurgery maintain a place in therapeutic plans for pituitary adenomas in the 21st century.

The Different Modalities of Radiotherapy

The general principle of radiotherapy is the delivery of radiation in a concentrated manner to a defined zone, termed the target zone. There are several radiotherapy modalities available, which vary in the way that they deliver the radiation to the tissue or in the precision with which they strike the target zone. So-called conventional radiotherapy is in general delivered in fractionated doses from 160 to 200 centiGray (cGy), four to five times per week for a total duration of 5–6 weeks (giving a total dose of 45–50 Gy). This modality of radiotherapy has seen major improvements over the last 10 years including better techniques for visualization of the target or for immobilization of the head (Minniti *et al.*, 2009). The risk of side effects is correlated with the total radiation dose and the dose delivered by fraction. Fractionated radiotherapy can be delivered via a noninvasive, repositionable stereotactic frame to improve the precision of target definition: this is termed fractionated stereotactic radiotherapy, for which the total dose of radiation delivered is the same (in the order of 45–50 Gy). The aim of destroying the target zone is achieved due to the phenomenon of cellular DNA damage and repair. DNA damage that is induced by the procedure is repaired in healthy cells more so than in tumoral cells, this being possible due to the fractionation of the total dose. Classically, fractionated radiotherapy is contrasted with stereotactic radiosurgery, in which the total radiation dose is delivered in a single session using a stereotactic frame. One such modality is Gamma Knife (GK) radiosurgery in which gamma radiation is delivered by 201 sources of Cobalt-60 with an isodose 50 in the order of 20–30 Gy. Other modalities are possible using a linear accelerator (LINAC), which delivers photons. These intervention methods theoretically allow the target zone to be irradiated while sparing surrounding or peripheral healthy tissue. In contrast to radiotherapy, the goal here is focused on destruction of the target rather than the greater capacity for recovery in healthy cells compared to tumor cells. Lastly, the Cyberknife is a LINAC which allows stereotactic radiotherapy without a frame, thanks to a robotic arm and real-time analysis of information that means the target can be followed during movement. In each endocrine pathology, we will outline the principal published data in relation to the modality (radiotherapy, radiosurgery) by which radiation is delivered. As the majority of published studies on radiosurgery are based on GK surgery, we will limit our analysis to these data. It should be noted that studies on other modalities, though fewer, have reported similar values for efficacy and side effects to those for GK.

Three criteria need to be specified when evaluating a radiotherapy or radiosurgery treatment:

- Antisecretory efficacy, which needs to be defined in the absence of any other antisecretory treatment. This parameter needs to take into account the delay required for obtaining maximal efficacy: after therapy, hormone levels will progressively fall until they reach a plateau phase. This delay varies depending on the modality used and thus means that an efficient antisecretory treatment must be used in the interim. In studies published to date, this delay is often overestimated due to the lack of regular withdrawal of antisecretory treatment.
- Antitumoral efficacy, which consists of stabilization or reduction of tumor volume.
- Pituitary and extra-pituitary side effects, which are generally observed and in some cases appear several years after the procedure.

Antisecretory Efficacy

Conventional Radiotherapy

Overall, the results with conventional radiotherapy are similar regardless of the type of hypersecretion. However, the values for remission vary over time and depending on the study, either because of stricter criteria for remission or because of shorter or longer

periods of patient monitoring. Thus for acromegaly, the majority of studies published prior to 1997–2000 report totals for remission of 80%–100%, based solely on mean growth hormone (GH) levels, without taking into account IGF-1 levels. The first study to evaluate both parameters was published in 1997, and reported normalization in only 5% of cases. However, this study has been the subject of much criticism due to the small number of patients who had prolonged monitoring (mean of 5 years), the major variability between patients in terms of the radiation techniques used and variability in dose, and multicenter modalities of treatment (Barkan *et al.*, 1997). A single study that was published since found similar results with 10% of patients normalized during 10 years of follow-up (Cozzi *et al.*, 2001). The largest published study to date, on 884 patients, found 60% of patients in remission after a mean duration of monitoring of 10 years, in agreement with the majority of studies published since 1997 (remission showing a spread of values between 35% and 76% of cases with a mean follow-up of 10 years) (Jenkins *et al.*, 2006). No cases of later relapse were reported after conventional radiotherapy. Results are comparable in Cushing's disease (53%–82% rates of remission, depending on the study) (Losa *et al.*, 2010). It is much more difficult to show such data in the case of prolactinoma, since in this setting, radiotherapy is rarely considered a therapeutic option. The few published studies on the subject report comparable values for remission.

The principle limiting factor with conventional radiotherapy is the delay before obtaining maximal efficacy, in the order of 5–10 years, though some studies have reported a maximal antisecretory effect up to 20 years after radiotherapy. There is a progressive decrease in hormone levels until a plateau is reached. This delay in obtaining eventual remission means that effective antisecretory treatment is necessary during the intervening period. Equally, it explains that the principal predictive factor for remission is the initial hormone level: even very high initial levels can be controlled by radiotherapy, but after a consequently longer period of time.

Radiosurgery

In the case of radiosurgery, as for radiotherapy, antisecretory efficacy is generally comparable regardless of the type of hypersecretion, being approximately 50%. In acromegaly, published studies report antisecretory efficacy for GK surgery in 17%–100% of cases. As with radiotherapy, this high degree of variability can be explained in the main by the use of different remission criteria: for example in the case of acromegaly, more recent studies that have used strict remission criteria have reported figures for remission in the order of 40%–50% (Castinetti *et al.*, 2010; Lee *et al.*, 2014; Pollock *et al.*, 2008a; Ronchi *et al.*, 2009). In addition to the 50% with remission, around 20% of patients should be added who were uncontrolled prior to the procedure and are perfectly controlled by administration of antisecretory drugs after the procedure (the reduced hormone levels allowing control of the hypersecretion). The number of studies on Cushing's disease is smaller, probably due to the large number of relapses in patients in whom a residual mass is not seen by MRI, and thus lack a target for further radiosurgery: the number of remissions reported varies between 10% and 83% of cases (Castinetti *et al.*, 2009; Jagannathan *et al.*, 2007; Sheehan *et al.*, 2013b). However, the higher numbers for remission correspond to older studies, where evaluation of the target was by CT scan rather than MRI: the efficacy is increased since the target is defined more approximately, at the cost of panhypopituitarism in the majority of cases (radiosurgery being similar in this case to conventional radiotherapy). Recently, the first large international multicenter retrospective study on the outcome of patients treated by radiosurgery for Cushing's disease was reported: out of 278 patients, cumulative control of hypercortisolism was obtained in 80% at 10-year follow-up. Such results should however be toned down by the fact that 11% of these patients were controlled by medical treatments at last follow-up (while they were not before GKS), and that 18% recurrences were observed on a long-term basis (Mehta *et al.*, 2017). This means that GKS allowed cure in 50% of the 278 patients treated after a mean follow-up of 5 years. The time to obtain remission was shorter than usually expected, roughly a year (14.5 months). The total for remission after GK surgery for prolactinoma ranges from 15% to 100% of cases (Landolt and Lomax, 2000; Pouratian *et al.*, 2006). As for radiotherapy, the precise place for radiosurgery in this type of adenoma is probably fairly limited: the majority of studies reporting on small numbers of subjects. A single group has reported on their experience with a large cohort of 128 patients; however, the low efficacy reported in this study (15%) suggests that some indications for GK were poorly evaluated (Pan *et al.*, 2000).

The principal predictive factor is the preradiosurgery hormone level (Castinetti *et al.*, 2010). The main limitation of radiosurgery is, as for radiotherapy, the existence of a delay between the procedure and the plateau of antisecretory efficacy. This delay period is of the order of 18–36 months, and thus requires the use of drug treatment during this time to control hypersecretion. This also limits the use of GK surgery which cannot be used in the case of massive hypersecretion, which requires rapid, efficient treatment. This more rapid efficacy (compared to radiotherapy) is obviously counterbalanced by a reduced overall antisecretory efficacy (50 vs. 60%–80%). Therefore, the ideal profile of patients who could benefit from radiosurgery needs to be defined, the classical criteria being a target of small volume that is well defined and not highly secretory. However, somewhat surprisingly, the target volume and the dose delivered to the target have been inconsistently reported to be predictive factors. Contradictory data have been reported on administration of somatostatinergic and dopaminergic drugs having a radio-protective effect, when taken during radiosurgery. It was hypothesized that in reducing the proliferation rate of the adenoma these treatments may reduce the efficacy of the radiosurgery (Landolt *et al.*, 1998; Landolt and Lomax, 2000). Only two of seven major long-term follow-up studies reported a deleterious effect in subjects with acromegaly, and this effect has not been reported in any studies of prolactinoma (Castinetti *et al.*, 2010). Even if it seems improbable that such an effect exists, we feel that it may be useful to stop antisecretory treatments 1–3 months before radiosurgery with the aim of knowing initial hormone levels after withdrawal of treatment since

this constitutes a predictive factor for remission. Lastly, a crucial difference compared to radiotherapy is the possibility of late relapse, three studies have reported on cases of relapse in 10%–20% patients suffering from Cushing's disease, 12–60 months after radiosurgery efficacy (Castinetti *et al.*, 2009; Mehta *et al.*, 2017; Sheehan *et al.*, 2013b). Taking into consideration the plateau of efficacy that is reached very late, prolonged surveillance, including after remission, is justified by the possibility of late relapse. The explanation for this phenomenon is unclear, but needs to be compared with the possibility of relapse after surgery for Cushing's disease which can occur even where the neurosurgeon believes that they have completely excised the tumor.

Antitumor Efficacy

Antitumor efficacy is a crucial point in the case of residual nonsecretory adenomas, where the aim is to stabilize or indeed reduce the volume of residual tissue. Antitumoral efficacy is similar for all techniques. For radiosurgery, the theoretical dose for antitumoral control is lower than the dose necessary for antisecretory efficacy, thus exposing less healthy pituitary tissue to radiation toxicity. Stabilization or a reduction in tumor volume was observed in 70%–100% of cases. However, there are no detailed studies of tumor volume in patients monitored long term who were treated by radiotherapy or radiosurgery in the setting of nonsecreting pituitary adenomas. Given the very slow evolution of these adenomas, it is possible that some may increase in size despite treatment by radiotherapy or radiosurgery. One theory that is still under discussion is the possible eventual transformation of an adenoma that was treated several years before by radiotherapy, into a carcinoma. To date, there are no data that support this hypothesis and additionally the exact mechanisms by which an adenoma may transform into a carcinoma are not completely understood. The antitumoral effect is rapid, appearing in the first year after treatment by GK surgery (Chen *et al.*, 2013; Sheehan *et al.*, 2013a). A large follow-up study reported on the outcomes of 62 patients treated by GK after incomplete surgery on a nonsecretory adenoma, who were then monitored for more than 5 years. The tumoral volume decreased in 60% of cases and was stabilized in 37% of cases, while only two patients presented an increase in tumor volume despite the radiosurgery procedure (Pollock *et al.*, 2008b). In the short term, there is likely edema with inflammation postradiosurgery. MRI data from a short time after the procedure is lacking, but one study has reported a transient increase in volume immediately after the procedure, probably due to a transient edema.

Side Effects

All of the studies reporting side effects of radiotherapy are based on very long surveillance of patients who were treated using modalities of radiotherapy that have since been greatly modernized (Brummelman *et al.*, 2011). Therefore it is difficult to determine if these "classic" side effects are still present in patients that are currently treated with conformal radiotherapy (rather than conventional radiotherapy). There are numerous side effects of conventional radiotherapy and their risk increases proportionally with the time elapsed since the radiotherapy. These side effects include an increased risk of hypopituitarism (estimated at 25%–80% of treated patients), vascular effects, radiation-induced tumors, and cognitive problems. The cerebrovascular effects of conventional radiotherapy are well known, with an increased risk of stroke, $3\times$ greater compared to the general population, in patients who have undergone conventional radiotherapy of more than 50 Gy, and $1.7\times$ greater for those receiving doses of 45–50 Gy. This risk of stroke is also increased with the duration of hypopituitarism in these patients (Ayuk, 2012). The risk of radiation induced optic neuropathy is estimated to be around 5% in subjects treated with conventional radiotherapy. Lastly, in studies on conventional radiotherapy, the risk of radiation induced tumors has been estimated to be low (Burman *et al.*, 2017). However, these tumors can occur after a long delay, estimated at 7–24 years after conventional radiotherapy.

In the case of radiosurgery, side effects, in particular late side effects are difficult to evaluate since the majority of studies have had a mean follow-up of <5 years. A single study reported results from 20-year follow-up, but was based on CT scan and not on MRI imaging of the target (this poor definition of the target resulting in panhypopituitarism in the majority of patients) (Hoybye *et al.*, 2001). Current studies underline that the principal risk of radiosurgery is the occurrence of panhypopituitarism which affects around 20% of patients (Castinetti *et al.*, 2010; Marek *et al.*, 2011). This risk increases with (i) time elapsed since radiosurgery; (ii) radiosurgery subsequent to surgery or radiotherapy (possibly due to increased fragility of the tissue), and (iii) with dose. In general it is also correlated with efficacy (the studies showing the highest rates of remission also show the highest levels of hypopituitarism). As outlined earlier, the initial definition of the target is fundamentally important in diminishing the risk of side effects. Older studies have reported a high risk of optic neuropathy, with this risk being low if the distance between the target and the optic chiasm (>5 mm) and the dose at the optic chiasm (<8–10 Gy) are adhered to. A review described the occurrence of optic neuritis and cranial (oculomotor, trochlear, trigeminal) neuritis in around 1% of patients treated with GK surgery for pituitary adenoma, with half of these cases being regressive (Leavitt *et al.*, 2013). Lastly, intense headaches are classically reported in the 48 h after radiosurgery, with these generally being well controlled by administration of level 1 or 2 analgesics.

Other potential side effects (radiation-induced tumors, cognitive deficits, vascular abnormalities), are unlikely due to the improved definition of the procedure (more targeted), and have not been described to date in the treatment of pituitary adenoma (Muracciole and Regis, 2008). A review has described the occurrence of cerebral necrosis or inflammation (hypothalamic or temporal) in 13 cases out of 1567 patients, on average 1 year after radiosurgery (though six of these patients had prior treatment with conventional radiotherapy) (Brada *et al.*, 2004). However, as with radiotherapy, it is likely that these effects, if they occur, will

occur late. Therefore, studies with very long surveillance of patients need to be performed to definitively assess the safety of radiosurgery. Similarly, studies with very long surveillance of patients treated with conformal radiotherapy will be required to show if technical progress in radiotherapy results in a reduction in the number of complications, particularly extra-pituitary complications.

The Place of Radiotherapy and Radiosurgery in Treatment of Pituitary Adenoma

Are the principle reasons for which patients were treated by radiotherapy 20 years ago or by radiosurgery since the start of 2000, still the same? The last 20 years have seen the development of multiple antisecretory treatments for acromegaly, Cushing's disease, and prolactinoma. These treatments have shown variable degrees of efficacy (in the order of 30%–50% control for somatostatinergics and 60%–80% for GH receptor antagonists in acromegaly; in the order of 50% for the main anticortisol drugs in Cushing's disease; and around 80% for dopaminergics in the treatment of hyperprolactinemias) and have shown good patient tolerance. If the role of radiotherapy in treatment of prolactinoma has always seemed to be an accessory one (and more so since dopaminergic drugs are very efficient), this question remains current in acromegaly and Cushing's disease for at least two reasons: the first being that the number of patients controlled by antisecretory treatment is at best equivalent to that for radiosurgery, and at least 50% of patients treated with drugs are not controlled; the second is that radiotherapy or radiosurgery may result in cure of the pathology, whereas antisecretory treatments may control hypersecretion via a lifetime of treatment. In the current conditions of reducing the load on our health systems, it is valid to suggest radiotherapy or radiosurgery for those patients with a profile suitable to achieve the best outcome: small tumor volume (for radiosurgery), low level of hypersecretion and absence of risk of major side effects (for example sufficient distance from optic tracts, in the case of radiosurgery). If the model of surgery as first-line treatment remains logical for acromegaly and Cushing's disease, and antisecretory treatments remain crucial for treatment of patients where surgery is not possible or contraindicated, then radiotherapy and radiosurgery maintain a role in the treatment of secretory pituitary adenomas, particularly as new modalities of treatment will drastically reduce the risk of side effects, particularly extra-pituitary side effects. In this setting, even though indications are rare, we have reported efficacy of radiosurgery as first-line treatment (in cases of refusal or contraindication for surgery, intolerance of drug treatments). We have described treatment of 13 patients, who underwent first-line treatment with radiosurgery and were monitored for >60 months, who had values for remission that were comparable, being approximately 50%.

The discussion is different for treatment of nonsecretory adenomas, where no drug treatment is available. Here surgery remains the first-line treatment in the case of increased tumor volume. Radiotherapy or radiosurgery can be considered when there is persistence of and an increase in volume of residual tumor. Because of the rapid antitumoral efficacy of the procedure, and assuming a sufficient distance from the optic chiasm, these treatments can be considered after surgery without great urgency: studies with long follow-up report an increase in volume of residual tumor, postsurgery, in around 20% of cases. Systematic radiotherapy after incomplete surgery for a nonsecretory adenoma is no longer indicated except in cases where there is a large amount of residual tumor that is inaccessible for further surgery. One last point is the problem of occurrence of Nelson syndrome after bilateral adrenalectomy for Cushing's disease. The objective here is to limit possible expansion of the tumor rather than secretory level. Systematic radiotherapy was, for a long time, recommended in this case. More precise studies now allow better characterization of this phenomenon and the patients at risk so that radiotherapy no longer needs to be performed systematically in these patients. In all of these cases (nonsecretory adenomas, Nelson syndrome) surveillance by MRI is indispensable when deciding on the potential usefulness of radiotherapy or radiosurgery.

Perspectives and Conclusions

It is difficult to determine whether treatment for pituitary adenoma in the future will use radiotherapy and radiosurgery. Pharmacological advances may lead to the percentage of patients being controlled by a combination of surgery/drug treatments being close to 100% (compared to a value currently in the order of 80%). The lack of perfect treatments at the moment, however, means it is almost mandatory that some patients are treated with all available treatment modalities. Improvements in drug treatments will in any case go together with improvements in radiotherapy and radiosurgery techniques. Assuming equal efficacy, and conditional on a reduction in late side effects, economic criteria mean that the use of radiotherapy or radiosurgery will likely continue with a view of curing young patients and avoiding a lifetime of drug treatment. However, for young patients, more than any other age group, the safety of modern techniques of radiotherapy and radiosurgery needs to be formally demonstrated. Research to better characterize the theoretical risk of late side effects, particularly for radiosurgery, is currently ongoing.

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Diagnosis and Clinical Management of Aggressive Pituitary Tumors

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Introduction

Pituitary neuroendocrine tumors (PitNETs) are the most common intracranial neoplasms, affecting 1/1000 of the worldwide population (Daly *et al.*, 2007; Fernandez *et al.*, 2010). PitNETs are largely benign tumors. However, tumor growth occurred in about half of the nonoperated nonfunctioning PitNET macroadenomas followed up for a period of 5–10 years (Dekkers *et al.*, 2007; Karavitaki *et al.*, 2007). Recent evidence suggests that while metastasis is rare (Heaney, 2014), the benign status of PitNETs requires revision. Indeed, a small subset of pituitary tumors are defined as aggressive pituitary tumors, based on their resistance to medical treatment and multiple recurrences despite standard therapies combining surgical, medical, and radiation therapy treatment approaches. The identification of this group of aggressive pituitary tumors is of major clinical importance, as they are associated with an elevated morbidity and mortality despite the absence of metastasis (Lasolle *et al.*, 2017; Losa *et al.*, 2016). Pituitary carcinoma, defined by the presence of systemic metastasis, occurs in the setting of an initially aggressive pituitary tumor. Management of these tumors is based on optimizing conventional medical treatment, in combination with radiation therapy in some instances. Use of temozolomide resulted in a good response rate when conventional treatment (including chemotherapy in the case of pituitary carcinomas) had failed to control tumor growth (Lasolle *et al.*, 2017).

Clinical Markers of Aggressiveness

The WHO 2004 classification proposed the term "atypical" pituitary tumors following the identification of potential pathological prognostic markers. The term "atypical" tumor is not synonymous with "aggressive" tumor. It is a pathological term whose prognostic value has not been evaluated in a prospective study. As it is presented in a specific chapter, we will not discuss this pathological classification here. However, we would emphasize that a prognostic classification of PitNETs allowing early identification of aggressive pituitary tumors would be of great importance in personalizing therapeutic strategies (Raverot *et al.*, 2017; Trouillas *et al.*, 2013). In a recent prospective study, we demonstrated that around 10% of PitNETs were classified as having a high risk of recurrence or progression within three years based on the pathological classification (Raverot *et al.*, 2017).

The definition of an aggressive PitNET is based on clinical characteristics and behavior during the follow-up period. Aggressive PitNETs are usually large and radiologically confirmed invasive tumors with an unusually rapid growth rate, and/or clinically relevant tumor growth or recurrence, despite optimal standard therapies.

Tumor Size: Giant Tumors

Recent surgical studies (Dallapiazza *et al.*, 2015; Raverot *et al.*, 2017) demonstrate that tumor size does not significantly impact surgical outcomes thanks to the development of new surgical procedures. Transsphenoidal endoscopy allows large tumor resection and, in some instances, for example when combined with a transcranial approach for giant PitNETs with large suprasellar extensions, is able to remove almost all, if not all, of the tumor (**Fig. 1**). Moreover, giant prolactinomas, which are usually invasive, respond reasonably well to medical treatment (**Fig. 2**). As for all prolactinomas, dopamine agonists are the first-line treatment and result in rapid alleviation of neurologic symptoms in the majority of cases, and a significant reduction in tumor size and prolactin normalization in three-quarters of patients (Maiter and Delgrange, 2014).

Invasion

Parasellar invasion into the cavernous sinus structures is the most common reason for incomplete tumor removal. There is a good correlation between radiological and surgical (via endoscopic judgment) classification of the parasellar growth of tumors (Micko *et al.*, 2015). Cavernous sinus invasion is graded from grade 1 to 4, with grade 4 being encasement of the internal carotid artery (**Fig. 3**A). Grading of tumor growth into the cavernous sinus could potentially identify different outcomes. Invasion of the cavernous sinus is associated with a high risk of residual tumor and is predicative of future tumor progression or recurrence (Cortet-Rudelli *et al.*, 2015; Monsalves *et al.*, 2014). It is significantly associated with recurrence (HR: 2.98, 95%CI: 1.89–4.70) (Raverot *et al.*, 2017). Progression of postoperative residual tumors occurred in 30%–60% of patients compared to recurrence in 10% of patients in the absence of tumor remnants after a long follow-up period (Dallapiazza *et al.*, 2015; Tampourlou *et al.*, 2017). However, invasion alone is not a synonym for PitNET aggressiveness, and not all invasive PitNETs are associated with recurrence (Chatzellis *et al.*, 2015; Raverot *et al.*, 2017).

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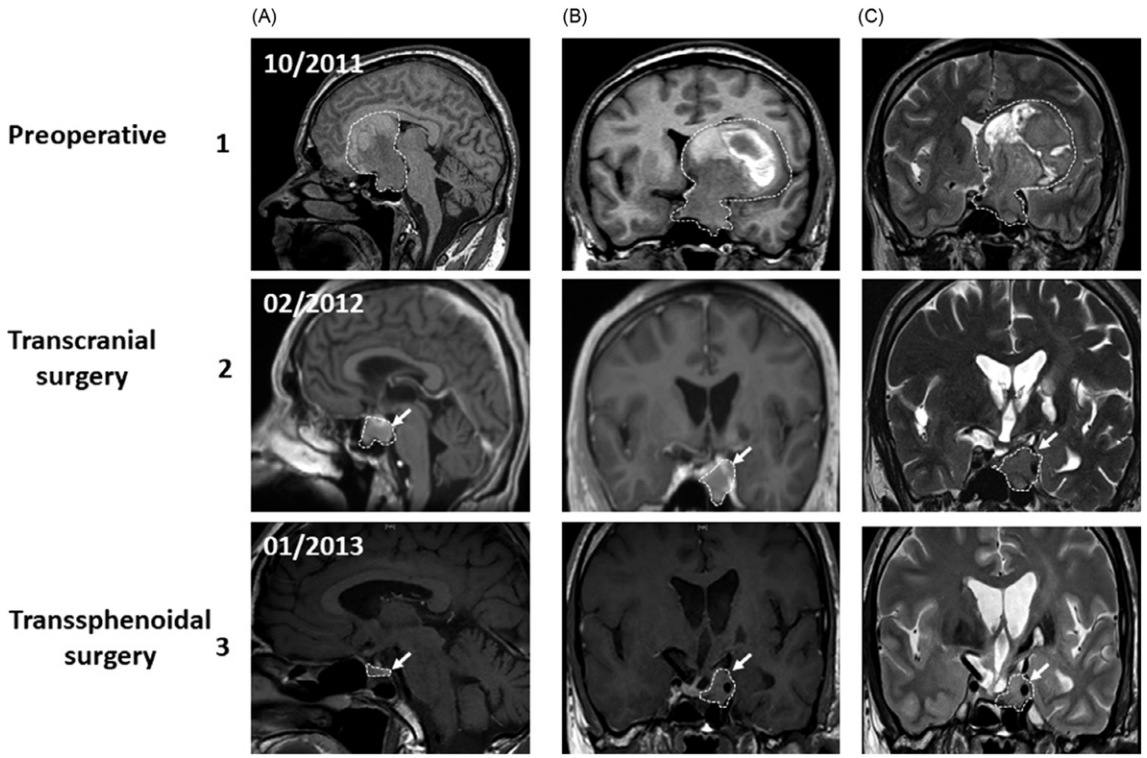

Fig. 1 Effect of surgical treatment of a giant gonadotroph pituitary tumor after a combined transcranial and transsphenoidal approach. Sagittal T1 (A); coronal T1 views after gadolinium (B), and coronal T2 (C) MRIs; before surgery (1) and after a transcranial (2) and transsphenoidal procedure (3).

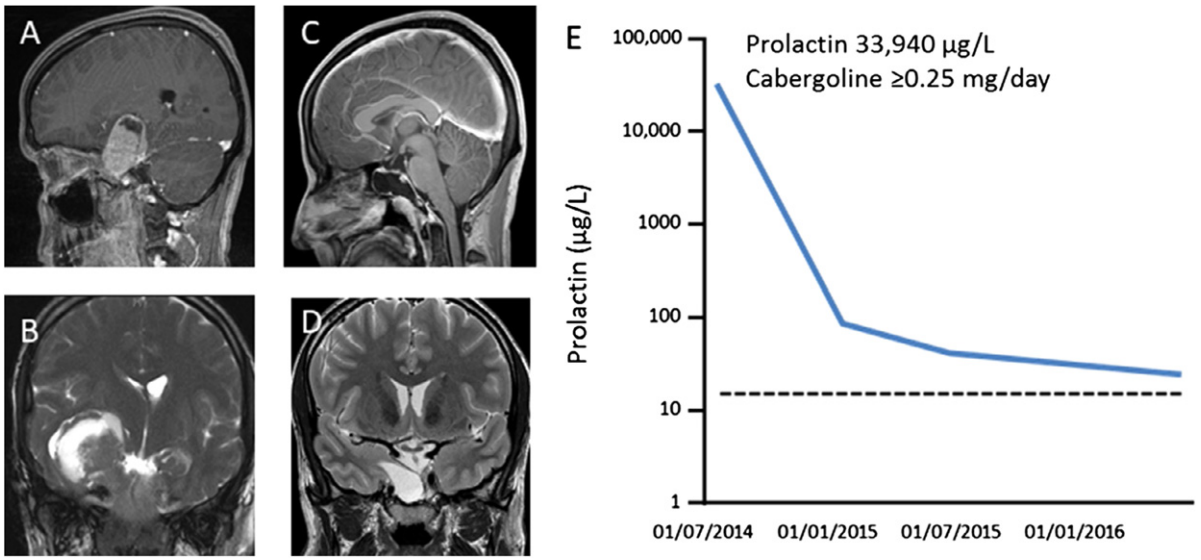

Fig. 2 Effect of dopamine agonist treatment on a giant prolactinoma revealed by a temporal seizure. Sagittal T1 (A) and T2 coronal (B) MRI view before cabergoline treatment, sagittal T1 (C) and coronal T2 (D) demonstrating massive tumor shrinkage associated with rapid prolactin decrease after six months of cabergoline (0.25 µg/day) (E).

Growth Rate

Aggressive tumors recur more frequently and earlier. One study suggests that the preoperative growth rate is associated with age, suprasellar growth, and the presence of a cyst/hemorrhage (Monsalves *et al.*, 2014). This study calculated a preoperative tumor volume doubling time (VDT) of 1147 ± 870 days (range 60–3478 days). For patients with a residual tumor, there was no

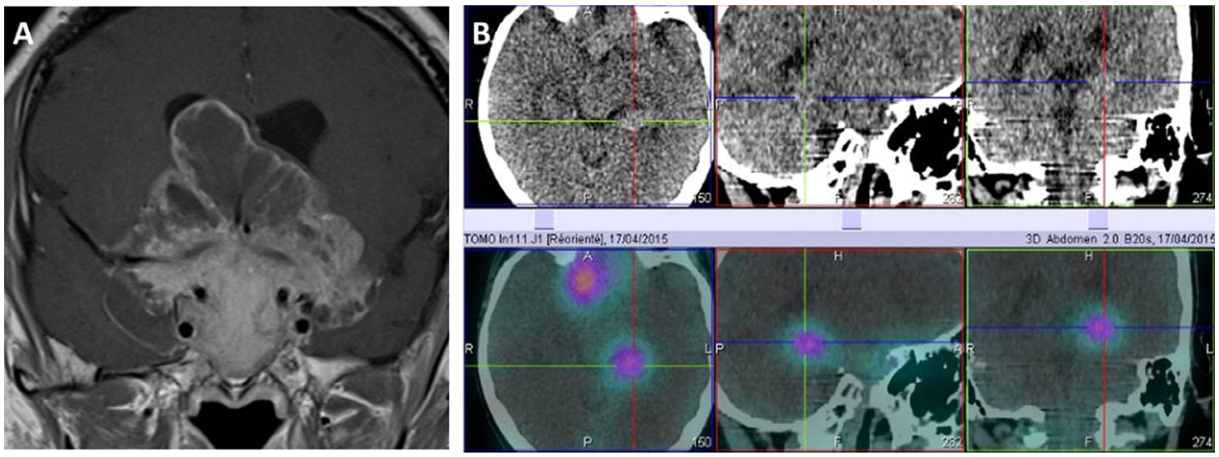

Fig. 3 A giant silent corticotroph tumor encompassing both cavernous sinuses, coronal T1 view after gadolinium (A). 111In-DTPA-octreotide scintigraphy imaging demonstrating high uptake in the sella and in brain metastatic deposits in the presence of a growth hormone-secreting pituitary carcinoma (B).

difference in the postoperative VDT compared to the preoperative (Monsalves *et al.*, 2014). However, the preoperative growth rate of PitNET is rarely known and is difficult to estimate in the absence of multiple MRI scans. In the absence of preoperative information on tumor growth, close postoperative follow-up is mandatory to identify PitNETs associated with rapid growth. Ratnasingam *et al.* suggest that the postoperative growth rate of nonfunctioning PitNETs in the first three years of imaging is the best marker to identify potentially aggressive tumors, and can be used to tailor long-term follow-up to optimize the use of health resources (Ratnasingam *et al.*, 2017). The progression rate of PitNETs is difficult to estimate, and growth kinetics can be described either by an exponential growth model or by a logistic model with initial exponential growth followed by deceleration of growth (Honegger *et al.*, 2008). Some PitNETs exhibit slow growth and necessitate a long observation time; others are more aggressive and invade the neighboring structures, thereby requiring rapid neurosurgical intervention to prevent long-term impairment on the visual field or pituitary deficiency. Moreover, recurrent tumors are more prone to regrowth during follow-up. A study of 90 patients with recurrent tumors determined a 5-year second regrowth rate of 35.3%; the probability of regrowth was influenced by the regrowth management approach (Tampourlou *et al.*, 2017).

Pituitary Carcinomas

Until recently, there has been limited data on pituitary carcinomas. A review from Dudziak *et al.* published in 2011 identified 132 cases without gender predilection. The mean age at diagnosis was 40 years, with a latency of 89 months between initial diagnosis and occurrence of distant metastasis. In this review, all pituitary carcinomas evolved from invasive macroadenoma, and, in most cases, had proliferative features. To our knowledge, there are no reports of de novo pituitary carcinomas revealed by the presence of metastasis at diagnosis.

Since 2006, >50 pituitary carcinomas have been reported in published case reports or in small and large studies of pituitary tumors treated with temozolomide, suggesting that pituitary carcinomas may be more common than initially thought. In patients with aggressive pituitary tumors, where site-specific symptoms occur (neck/back pain, neurological complaints), or where there are discordant biochemical and radiological findings, screening for metastatic disease is recommended. Appropriate structural (MRI or CT) and/or functional (FDG- and/or SSTR-PET) (**Fig. 3**B) imaging may be considered. Pituitary carcinomas disseminate via lymphatic, hematologic, and craniospinal spread, and range from a single lesion to widespread dissemination.

Lactotroph and corticotroph carcinomas are the most commonly encountered subtypes, representing 36% and 30%, respectively, of published cases (Dudziak *et al.*, 2011) and together representing around 80% of patients treated with temozolomide reported in the literature. These two subtypes seem more likely to behave aggressively. Lactotroph tumors are expected to be more frequently observed, as they represent the most common subtype in the general population. However, this is not the case for corticotroph tumors, which represent <15% of all pituitary tumors, and most of these are microadenomas. Special attention should be given to "silent" ACTH tumors, which are very common in corticotroph tumor population. "Silent" ACTH tumors are ACTH tumors without Cushing's disease. These tumors express ACTH or other peptide products of the pro-opiomelanocortin gene (*POMC*) (Trouillas *et al.*, 1984). Despite the absence of clinical and biochemical signs of hypercorticism, elevated plasma ACTH levels can resemble those found in cases of macroadenoma with Cushing's disease (Raverot *et al.*, 2010b), and a number of patients with "silent" ACTH tumors may present with clinical symptoms of Cushing's disease at some stage during disease evolution (Cooper and Melmed, 2012; Raverot *et al.*, 2010b). A phenotype change from a "silent" to a "secreting" tumor should be considered a marker of aggressiveness and a risk factor for malignancy (Bradley *et al.*, 2003; Jouanneau *et al.*, 2012). Indeed, most reported ACTH carcinomas were classified as "silent" ACTH tumors at diagnosis (Dudziak *et al.*, 2011; Heaney, 2011).

Resistance to Medical Treatment

A distinction should be made between resistance to treatment and an absence of secretion normalization. Moreover, a discrepancy between hormonal and tumor response has been noted in some instances. Numerous therapeutic options are available to control tumor secretion or the hormonal effect; however, few treatments are able to induce tumor shrinkage or control tumor growth.

In somatotroph PitNETs, treatment with somatostatin analogs is considered the cornerstone of currently available treatment options. First-generation somatostatin analogs (lanreotide, octreotide) act on the somatostatin receptor (sst), predominantly on sst2, and can normalize levels of insulin-like growth factor 1 (IGF1) and reduce growth hormone levels to <2.5 μg/L in about 20%–40% of cases (Caron *et al.*, 2014; Colao *et al.*, 2014), while second-generation analogs (pasireotide), acting on sst1–3 and particularly sst5, achieve biochemical control in 15%–20% of patients who had inadequate control with first-generation somatostatin analogs (Gadelha *et al.*, 2014). More importantly, treatment with somatostatin analogs achieves $\geq$20% tumor volume reduction in 70%–85% of patients, with the maximal decrease occurring within the first 6 months of treatment (Caron *et al.*, 2014; Colao *et al.*, 2016). Despite the absence of tumor shrinkage in a high proportion of patients during treatment with somatostatin analogs, an increase in tumor volume is rarely observed (1%–2% patients) (Freda *et al.*, 2005). Such tumor growth during somatostatin analog treatment is considered a sign of aggressiveness. In the presence of biochemical resistance without evident tumor reduction (volume increase or $<$20% volume reduction compared with baseline) or when tumor regrowth is observed, somatostatin analogs should not be continued and alternative therapies should be considered. Although dopamine is reported to normalize IGF1, its effect on tumor growth still requires evaluation (Kuhn and Chanson, 2017).

Cabergoline, a dopamine agonist, is the most effective and the best-tolerated drug for the treatment of lactotroph PitNETs (Colao and Savastano, 2011). For doses of cabergoline up to 3.5 mg/week, complete resistance, defined as a failure to normalize prolactin and a $<$50% decrease in size, occurs in $<$10% of these tumors (Delgrange *et al.*, 2009). In addition, higher doses of cabergoline (up to 11 mg/week) successfully treated patients in one study (Ono *et al.*, 2008).

Data on the antitumoral effect of pasireotide monotherapy on corticotroph PitNETs are very limited (Colao *et al.*, 2012). The rare thyrotroph PitNETs are well controlled by somatostatin analogs (Amlashi and Tritos, 2016). There is currently no treatment available for gonadotroph or immunonegative PitNETs (Raverot *et al.*, 2014).

Radiation Therapy

Fractionated radiation therapy, γ-knife radiosurgery, proton-beam radiosurgery, and linear accelerator radiosurgery should be discussed when surgery fails to cure a patient in cases of recurrent tumors or tumors with pathological markers of aggressiveness (Cortet-Rudelli *et al.*, 2015; Ding *et al.*, 2014). Most studies showed a significant improvement in the nonrecurrence rates at five years for functioning (Ding *et al.*, 2014) and nonfunctioning tumors following the use of radiation therapy; postoperative radiation therapy is the only factor that limits the risk of recurrence in recurrent tumors (Cortet-Rudelli *et al.*, 2015; Tampourlou *et al.*, 2017).

Radiation therapy, irrespective of the technique, is associated with an increased risk of hypopituitarism, radiation-induced cerebral tumors, and cognitive disorders, and may also increase vascular risk (Cortet-Rudelli *et al.*, 2015). Indications for radiation therapy should take account of risk factors for regrowth, patient age and history, and any hypopituitarism. In most cases, regular surveillance can be the first-line approach, with treatment postponed until the residual tumor progresses and/or becomes threatening. Postoperative adjuvant radiation therapy should be considered in the setting of a clinically relevant invasive tumor remnant with pathological markers (high Ki67/high mitosis count/p53-positive tumor). Radiation therapy is also recommended for patients with clinically relevant tumor growth despite surgery in nonfunctioning tumors, or despite surgery and standard medical treatment in functioning tumors. In these cases, the risk of hypopituitarism is not a major problem.

Chemotherapies

Systemic chemotherapy is indicated when conventional therapeutic modalities fail, or in the presence of metastasis confirming the diagnosis of pituitary carcinoma. Several therapeutic regimens have been used in the past (procarbazine-etoposide-lomustine; cyclo-hexyl-chloroethyl-nitrosourea; and lomustine-doxorubicin) (Kaltsas *et al.*, 1998), all resulting in occasional, sporadic, and short-lasting responses. None of them was able to control tumor progression or improve survival (Kaltsas *et al.*, 2005).

This paradigm fortunately changed after the first publication reporting the successful use of temozolomide (Fadul *et al.*, 2006; Lim *et al.*, 2006; Syro *et al.*, 2006), a second-generation alkylating agent routinely used for the treatment of glioblastomas (Stupp *et al.*, 2005). Temozolomide is an imidazotetrazine derivative that is rapidly converted at physiological pH to methyl-triazeno-imidazole-carboxamide, which is the active drug. It exerts its action by attaching a methyl group to the O6 position of guanine bases, causing a mispair with thymine bases, DNA damage, proliferation arrest, and cell death (apoptosis). Temozolomide can cross the blood–brain barrier, and its action is not cell cycle-specific. It therefore inhibits all stages of tumor cell growth, even in slow-growing tumors such as pituitary tumors.

Temozolomide is relatively well tolerated. The most common side effects are fatigue, nausea, vomiting, and hematologic effects (thrombocytopenia, rarely neutropenia). A dose reduction or delay in treatment cycles can allow the patient to continue treatment. The

preferred dosing regimen is 200 mg/m^2 daily for 5 days every 28 days. However, recent publications reported cases of temozolomide treatment given according to the Stupp protocol (Stupp *et al.*, 2005) that is used routinely in the treatment of glioblastomas—a daily dose of 75 mg/m^2 for 6 weeks in combination with radiation therapy, followed by 6–12 months of "standard regimens."

Following the initial publications on temozolomide, numerous case reports and studies on relatively large series (Bengtsson *et al.*, 2015; Lasolle *et al.*, 2017; Losa *et al.*, 2016; Raverot *et al.*, 2012) have been published. Limiting the literature review to publications, including at least three patients, a total of 149 patients have been reported on, all showing a similar response rate despite heterogeneity in the populations. The largest series reported partial or complete tumor regression in about 50% of patients at the end of treatment. Most tumors were corticotroph or lactotroph tumors.

To date there is no clearly identified predictive marker of responsiveness to temozolomide. The response rate is similar in aggressive pituitary tumors and carcinomas, but silent pituitary tumors appear to be associated with a worse prognosis. Concomitant radiation therapy, given according to the Stupp protocol, seems to be associated with a high rate of tumor response. However, the number of patients is too limited to be conclusive (Lasolle *et al.*, 2017).

Evidence of tumor shrinkage after three cycles, as evaluated by MRI, is the only undebatable prognostic marker of later response. Indeed, all responders showed tumor shrinkage after three cycles, while a delayed tumor response was never observed in the absence of an initial response. However, an initial response is not a guarantee of long-term tumor control. In some patients, tumor growth occurred during subsequent cycles, and, more frequently, tumor progression occurred after temozolomide cessation. After treatment ended, responders exhibited improved overall survival compared to nonresponders (44 months (42–infinity) (95% CI) vs. 16 months (9–25), with an estimated survival probability of 51.3% (32.4–70.3) at 3 years (Lasolle *et al.*, 2017)). Due to the limited data on long-term follow-up, the optimal duration of temozolomide treatment remains unclear. Remissions are rare, secondary resistance common (Bengtsson *et al.*, 2015; Campderá *et al.*, 2016; Lasolle *et al.*, 2017; Losa *et al.*, 2016; Raverot *et al.*, 2010a), and all new treatment attempts with temozolomide in cases of relapse failed (Campderá *et al.*, 2016; Lasolle *et al.*, 2017).

The expression of proteins implicated in DNA repair mechanisms has been studied to identify tumors more likely to be sensitive to temozolomide treatment. O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme that counteracts the effects of temozolomide by removing alkylating adducts from DNA, is the most frequently studied. There is debate regarding the results of these studies (Bengtsson *et al.*, 2015; Bush *et al.*, 2010; Lasolle *et al.*, 2017; Losa *et al.*, 2010; McCormack *et al.*, 2009; Raverot *et al.*, 2010a,b); however, it is now agreed that MGMT status should not be used to select patients for treatment with temozolomide, but that patients with high MGMT expression have a lower probability of tumor response. Studies on other DNA mismatch repair proteins (MLH1, MSH2, MSH6, and PMS2) are too limited to draw conclusions.

Alternative Therapies

Temozolomide treatment failed to control at least 50% of these tumors, and progression or recurrence after temozolomide cessation is common. Identification of therapeutic alternatives is therefore needed. Unfortunately, clinical experiences are uncommon. Only single case reports have demonstrated the efficacy of antivascular endothelial growth factor (anti-VEGF) therapy (bevacizumab 10 mg/kg every 2 weeks for 10 months) (Ortiz *et al.*, 2012), or tyrosine kinase inhibitor treatment (lapatinib 1250 mg daily for 6 months) (Cooper *et al.*, 2014). Despite encouraging preclinical data (Chanal *et al.*, 2016; Lee *et al.*, 2015), treatment with a mTOR inhibitor (everolimus 5 mg daily) does not seem effective in humans (Jouanneau *et al.*, 2012).

Conclusion

Aggressive PitNETs are not especially rare (10%), and cases of carcinoma are perhaps not as exceptional as once thought. These tumors, associated with poor prognosis and high morbidity, should be managed by an expert multidisciplinary team. Recent studies focusing on clinical, radiological, and pathological presentation allowed early identification of these tumors, which are associated with a high risk of recurrence and resistance to medical treatment. Better classification of these patients will improve stratification of the different treatment options, including radiation therapy. Moreover, more clinical evidence of the efficacy of temozolomide treatment in controlling tumor growth may change the prognosis for these patients. The upcoming publication of the European Society of Endocrinology clinical practice guidelines for the management of aggressive pituitary tumors and carcinomas will provide the tools for the treatment of these tumors. However, despite this progress, more studies, in particular international collaborative studies, are needed to decipher PitNET tumor behavior and to identify new treatment options for patients with tumors that are not controlled by temozolomide.

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Hypopituitarism, Causes, Diagnosis, Management and Mortality☆

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Introduction

The pituitary gland together with the hypothalamus control many endocrine and metabolic functions essential for survival, growth, and reproduction. Hypopituitarism is caused by conditions that reduce or completely damage the pituitary function or interfere with the hypothalamic secretion of pituitary-releasing hormones, leading to complete or partial pituitary dysfunction (Schneider *et al.*, 2007).

Hypopituitarism is associated with significant morbidities, compromised quality of life and increased mortality, especially if it is not timely diagnosed and treated (Jasim *et al.*, 2017; Ehrnborg *et al.*, 2000). Given that many of the presenting manifestations of pituitary dysfunction are nonspecific, early diagnosis can be challenging.

Epidemiology

Accurate data on the prevalence of hypopituitarism are very limited. In a study including 146,000 adult inhabitants in northwestern Spain (Regal *et al.*, 2001) the prevalence of hypopituitarism was increased from 29.0 to 45.5 cases per 100,000 people between 1992 and 1999 with a mean annual incidence of 4.21 cases per 100,000 persons. However, these figures probably underestimate the actual incidence of this disorder. It should be also noted that with the advances in pediatric oncology, the number of patients diagnosed with hypopituitarism in childhood or later during monitoring in adult life is increasing.

Etiology

Hypopituitarism can be caused by a plethora of etiologies shown in **Table 1**.

Neoplastic Causes

Pituitary insufficiency can be caused by sellar and parasellar lesions. Pituitary tumors account for 10%–15% of all intracranial tumors (Terada *et al.*, 1995); and 90% of them are pituitary adenomas with a prevalence of 77.6 cases per 100,000 inhabitants in the United Kingdom (Fernandez *et al.*, 2010). Pituitary adenomas are defined as clinically functioning or nonfunctioning (depending on the presence of secretory activity that can cause clinical manifestations), and into microadenomas (<1 cm) and macroadenomas (≥ 1 cm) on the basis of maximum diameter on imaging. Prolactinomas are the most common functioning pituitary adenomas, followed by those causing acromegaly and Cushing's disease, with functioning gonadotropinomas and thyrotropinomas occurring only rarely.

Both functioning and nonfunctioning adenomas may result in hypopituitarism due to pressure effects to the normal gland (possible mechanisms include mass effect of the tumor on the vascular portal system, raised intrasellar pressure affecting portal circulation, and focal pituitary necrosis (Arafah *et al.*, 2000; Arafah, 1986)) or the effects of hormonal hypersecretion on other pituitary hormones. The typical sequence of hyposecretion starts with growth hormone (GH) deficiency, followed by loss of gonadotropins before progressing to loss of thyrotropin-stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH) reserves. Mild hyperprolactinemia may be detected in nonprolactin secreting adenomas due to interruption of the inhibitory effect of dopamine from the hypothalamus on prolactin release. Pituitary adenomas do not present with vasopressin deficiency.

Parasellar masses related with hypopituitarism are craniopharyngiomas, gliomas, hamartomas, meningiomas, germinomas, chordomas and astrocytomas. Craniopharyngiomas account for up to 4% of all intracranial tumors and although are often thought to be childhood tumors, 50% of all of them present in patients over 16 years of age (Karavitaki *et al.*, 2006). They have the potential of aggressive behavior with increased morbidity and mortality compared with patients with other sellar/parasellar masses.

☆*Change History:* November 2017. A. Fountas and N. Karavitaki updated the whole text of the manuscript, added the mortality section, citations in the manuscript, as well as a reference section. In addition, they deleted the old Table 1; they updated the old Tables 2 and 3 (with the new Tables 1 and 2) and added a new table (Table 3). Finally, they deleted Figs. 1, 2 and 3, as well as the further reading section.

This chapter is an update of K.J. Bradley and J.A.H. Wass, Hypopituitarism, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 652–661.

Table 1 Causes of hypopituitarism

Neoplastic	Sellar and parasellar masses (e.g., pituitary adenomas, craniopharyngiomas, cysts, gliomas, meningiomas, chordomas, metastases)
Iatrogenic	Pituitary surgery
	Pituitary radiotherapy
Vascular	Pituitary apoplexy
	Sheehan syndrome
	Carotid artery aneurysm
	Subarachnoid hemorrhage
	Snake bite
Inflammatory/infiltrative	Hypophysitis
	Hemochromatosis
Infections	Bacterial
	Fungal
	Parasital
	Viral
	Tuberculosis
Traumatic	Head injury
Anatomic	Empty sella syndrome
Congenital	Genetic (*POU1F1, PROP1, HESX1, LHX3, LHX4, TPIT, SIX6, SOX2, SOX3, FGF8, PROKR2, ARNT2, GLI2, NFKB2, PAX6*)
	Developmental central nervous system defects
	Perinatal insults (traumatic delivery, birth asphyxia)
	Interrupted pituitary stalk
Medications	Opiates
	Somatostatin analogs
	CTLA4 and PD1 inhibitors
	Interferon-alpha
	Glucocorticoids
Idiopathic	

(*CTLA4: cytotoxic T-lymphocyte antigen 4/PD1: programmed cell death protein 1*).

Rathke's cleft cysts are benign sellar and/or suprasellar lesions constituting the majority of the cystic lesions in this area. They arise from remnants of Rathke's pouch with a varying size and content (Fountas and Karavitaki, 2017). Other less common cystic lesions include arachnoid, epidermoid and dermoid cysts. Finally, primary malignancies of the sella are extremely rare and they comprise of pituitary carcinomas, primary lymphoma and melanoma. On the other hand, metastatic deposits at the pituitary do occur, usually arising from malignancies of the lung, breast, kidney and prostate (He *et al.*, 2015).

Iatrogenic Causes

Hypopituitarism is often the result of pituitary surgery (transsphenoidal or transcranial) and its frequency depends on the tumor size, invasion on the surrounding structures, as well as the skills and experience of the surgeon. In addition, cranial irradiation for various reasons (head and neck tumors, pituitary masses, brain tumors, prophylactic radiotherapy in children with leukemia, total body irradiation for various malignancies) is a causative factor for pituitary insufficiency. The risk of hypopituitarism is time- and dose-dependent with the deficits presenting with a typical manner (Pekic and Popovic, 2017). Deficiency of GH axis is the most common first manifestation, followed by the gonadotropin axis; ACTH and TSH deficiencies are the least frequent whilst diabetes insipidus (DI) does not occur as a consequence of radiation treatment (Fernandez *et al.*, 2009). The effects of radiotherapy may take many years to manifest fully; consequently, it is vital that irradiated patients at risk require regular assessment (usually at yearly intervals). The impact of new radiation techniques (e.g., protom beam) on the hypothalamo-pituitary function remains to be elucidated.

Vascular Causes

Pituitary apoplexy is the most frequent vascular cause of hypopituitarism and is attributed to damage of the pituitary gland due to infarction or hemorrhage, usually in the background of a pituitary adenoma. Its clinical features include acute, severe headache, accompanied by nausea and vomiting, reduced visual acuity and visual field defects, and ocular palsies (Capatina *et al.*, 2015a). However, hemorrhage in a pituitary adenoma may lack the typical acute clinical manifestations and be evident only on imaging.

Postpartum ischaemic pituitary necrosis (Sheehan syndrome), remains an important cause of hypopituitarism in developing countries, but is relatively rare in the western countries due to the advances in obstetric care (Karaca *et al.*, 2016). Another known vascular etiology of pituitary insufficiency is subarachnoid hemorrhage with variable frequencies reported among published studies (Robba *et al.*, 2016; Kronvall *et al.*, 2015; Gardner *et al.*, 2013). Carotid artery aneurysm is also a rare cause mainly due to

mass effect on the hypothalamus and the pituitary gland (Hanak *et al.*, 2012). Finally, cases of pituitary insufficiency after snake bite have been described (Golay *et al.*, 2014).

Inflammatory/Infiltrative Conditions

Primary hypophysitis is an inflammatory disease that can affect both lobes of the pituitary, as well as the stalk and can be divided in four different types: lymphocytic hypophysitis, mainly reported in women during or after parturition, granulomatous, xanthomatous and IgG-4-related (in patients with IgG-4-related disease). The clinical features are similar to those of pituitary adenomas, comprising local mass effects (headache, visual deterioration) and anterior hypopituitarism. However, in contrast to the patients harboring pituitary adenomas, DI can be present when the posterior lobe or the stalk are involved (Caturegli *et al.*, 2005).

Granulomatous conditions, such as sarcoidosis, Langerhans cell histiocytosis and granulomatosis with polyangiitis (previously known as Wegener's granulomatosis) can also cause anterior hypopituitarism and DI. Moreover, hereditary and secondary hemochromatosis can affect pituitary function, especially hypogonadotropic hypogonadism by iron deposits in the gland (Lewis *et al.*, 2009).

Infections

Pituitary infections, although rare, need to be considered in the differential diagnosis of hypopituitarism. They can be primary in origin, secondary due to hematogenous spread (especially in patients with immunoinsufficiency) or by direct dissemination of infection in adjacent sites, and iatrogenic after pituitary surgery. Pathogens responsible for these infections are bacteria (including mycobacterius tuberculosis), viruses, parasites and fungi. Pituitary abscess can also develop with magnetic resonance imaging (MRI) usually revealing a sellar cystic or partially cystic mass, with an enhanced rim following gadolinium administration (Wang *et al.*, 2014).

Traumatic Causes

Traumatic brain injury (TBI) is another recognized cause of pituitary insufficiency. The prevalence widely varies between different studies and depends on the methods and criteria used for the diagnosis of hypopituitarism (Kokshoorn *et al.*, 2010). A recent review reported prevalence of pituitary deficiency in TBI patients at the chronic phase (at least 3 months after the injury) of 15% when confirmatory tests were used after the initial abnormal testing (Tanriverdi *et al.*, 2015). GH deficiency is the most common deficit, followed by ACTH, gonadotropins and TSH deficiency. DI can also occur, especially in the acute phase of the injury (Capatina *et al.*, 2015b). Whether the severity of TBI is a predictor of hypopituitarism in these patients remains a matter of debate (Tan *et al.*, 2017).

Anatomic Malformations

Empty sella syndrome may be associated with variable clinical conditions ranging from normal pituitary function to hypopituitarism. It may be congenital secondary to arachnoid herniation through a diaphragmatic defect or acquired postsurgery, postradiotherapy, or postpituitary infarction (De Marinis *et al.*, 2005). Imaging reveals an enlarged empty pituitary fossa.

Congenital Causes

Congenital hypopituitarism can be attributed to many underlying causes, particularly genetic ones. Pituitary development requires interplay between pituitary transcription factors and signaling pathways; mutations of the genes coding for these transcription factors result in pituitary developmental anomalies and hypopituitarism (Castinetti *et al.*, 2016). These mutations are often associated with extrapituitary defects, mainly affecting craniofacial/midline development, including septo-optic dysplasia and holoprosencephaly (McCabe and Dattani, 2014).

Pituitary insufficiency may present in the neonatal period, in childhood or young adulthood and can be associated with single or multiple pituitary hormone deficiencies (Kelberman *et al.*, 2009). Many genes have been identified as responsible for hypopituitarism including *POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *TPIT*, *SIX6*, *SOX2*, *SOX3*, *FGF8*, *PROKR2*, *ARNT2*, *GLI2*, *NFKB2*, *PAX6*, *KAL* and *KISS1R*. Each of these gene mutations is related with a different clinical phenotype, and appropriate genetic testing should be performed accordingly.

Pituitary stalk interruption syndrome also results to congenital hypopituitarism, ranging from isolated GH deficiency to combined pituitary hormone deficiencies, and belongs to the spectrum of midline abnormalities. It is characterized by the presence of a thin or absent pituitary stalk, commonly associated with an absent or hypoplastic anterior pituitary lobe and/or an ectopic posterior lobe (Reynaud *et al.*, 2011). The underlying mechanism is not known. However, mutations of genes involved in pituitary development have been found in some patients indicating a possible genetic background (Bar *et al.*, 2015). Finally, congenital hypopituitarism can be also caused by perinatal insults, such as birth asphyxia or traumatic delivery (Webb and Dattani, 2011).

Table 2 Main clinical manifestations and laboratory findings of hypopituitarism

Hormone deficiency	*Clinical features*
Growth hormone	Fatigue and weakness Reduced muscle strength and exercise capacity Impaired psychological well-being Reduced lean body mass and increased visceral adiposity Decreased bone mass density Increased cardiovascular risk Impaired sleep quality Dyslipidemia
Luteinizing hormone and follicle-stimulating hormone	Women ● Oligomenorrhea/amenorrhea ● Vaginal dryness, dyspareunia ● Breast atrophy Men ● Erectile dysfunction ● Testicular atrophy ● Diminished facial and body hair ● Fine facial wrinkles ● Gynecomastia ● Anemia (normocytic, normochromic) Both sexes ● Loss of libido ● Hot flushes ● Infertility ● Fatigue and weakness ● Reduced muscle strength and exercise capacity ● Impaired sleep quality ● Impaired psychological well-being ● Decreased bone mass density
Adrenocorticotropic hormone	Fatigue and weakness Reduced exercise capacity Impaired psychological well-being Anorexia Nausea and vomiting Weight loss Orthostatic hypotension Diarrhea Cognitive impairment Pallor Impaired cardiac function Oligomenorrhea/amenorrhea Decreased hair in armpits and pubic area (women) Decreased libido (women) Hypoglycemia Hyponatremia Anemia (normocytic, normochromic) Eosinophilia and lymphocytosis
Thyroid-stimulating hormone	Fatigue and weakness Reduced muscle strength and exercise capacity Impaired psychological well-being Weight gain Cold intolerance Constipation Dry skin and hair Cognitive impairment Hyporeflexia Oligomenorrhea/amenorrhea Bradycardia, impaired cardiac function and increased cardiovascular risk

(*Continued*)

Table 2 Continued

Hormone deficiency	*Clinical features*
	Hypertension
	Dyspnea
	Impaired sleep quality
	Insulin resistance
	Dyslipidemia
	Anemia (mainly normocytic, rarely macrocytic)
Prolactin	Failure of lactation
Vasopressin	Polydipsia
	Polyuria
	Nocturia
	Hypernatremia

Medications

Hypopituitarism can be caused by a variety of drugs. Opioid-induced changes in pituitary function are common, leading to hypogonadotropic hypogonadism, by decreasing the secretion of gonadotropin-releasing hormone (GnRH) and limiting the production of the luteinizing hormone, and to central hypoadrenalism, by reducing the secretion of corticotropin releasing hormone (CRH) and the pituitary responsiveness to it (Brennan, 2013). It is also well known that somatostatin and its analogs inhibit GH secretion acting directly on the somatotrophs and indirectly by antagonizing the production of growth hormone-releasing hormone (GHRH), as well as by inhibiting the secretion of ghrelin (Tannenbaum *et al.*, 2003). In addition, these agents inhibit the production of TSH and the stimulation of its secretion by the thyrotropin-releasing hormone (TRH) (Siler *et al.*, 1974). Immune checkpoint inhibitors are recently developed drugs that have proved effective for the treatment of a variety of malignancies, especially melanoma and lung cancer. Ipilimumab is the most known agent of this category followed by pembrolizumab and nivolumab. Use of these inhibitors has been related with multiple endocrinopathies, including hypophysitis. Although, the precise mechanism for this is not known, there are many proposed pathways that may lead to the development of hypophysitis including an autoimmune process targeting unidentified pituitary antigens and a direct binding of ipilimumab to the pituitary cells (Faje, 2016). In addition, there are reports in the literature of interferon-induced hypopituitarism (Concha *et al.*, 2003). Finally, suppression of the hypothalamus-pituitary-adrenal (HPA) axis is a common side effect of prolonged exogenous glucocorticoid administration (Crowley *et al.*, 2014).

Symptoms and Signs Related to Hypopituitarism

Clinical features of hypopituitarism (**Table 2**) depend on many factors, including the severity and the number of hormonal deficiencies and the underlying pathology that leads to pituitary failure. If the cause of hypopituitarism is a pituitary mass, apart from the manifestations of the pituitary hormone deficits, tumor effects (headache, visual impairment) will probably be also evident. Also, the rapidity of onset of hypopituitarism affects its clinical presentation. In cases of acute pituitary deficiency, such as pituitary apoplexy, central hypoadrenalism can be life threatening if not diagnosed and treated timely. However, most patients exhibit a slow and progressive loss of pituitary function with relatively mild and often nonspecific clinical symptoms.

Diagnosis

The diagnosis of hypopituitarism is established on appropriate hormonal investigations (**Table 3**). For the interpretation of the results, the limitations of the currently used assays, their sensitivity and specificity, as well as situations where interference is expected (drugs, disorders affecting binding globulins) need to be taken into account.

In any patient with suspected pituitary or hypothalamic pathology, imaging of the area, ideally with MRI scanning is mandatory. Additional investigations will be dictated by the imaging findings and the suspected etiology of hypopituitarism. Diagnosis is based on the measurement of the basal levels of the anterior pituitary and the target organ hormones, as well as on dynamic testing. The confirmation of hypotonic polyuria combined with exclusion of hypokalaemia or hypercalcaemia and a water deprivation test are valuable tools for the diagnosis of diabetes insipidus related to vasopressin deficiency (**Table 3**).

Table 3 Diagnosis of pituitary hormone deficits

GH deficiency

- GH stimulation testing is mandatory (exceptions: patients with clinical features of GHD and deficiencies in at least three other pituitary hormones, young adults with GHD and multiple hormone deficiency due to structural lesions or genetic disease with low IGF-1 levels after discontinuation of GH therapy for at least 1 month)
- One stimulation test is sufficient and prior to testing all other pituitary hormones must have been replaced adequately
- Insulin tolerance test: gold standard, GHD if peak GH levels $\leq$3–5 μg/L
- GHRH and arginine stimulation test: well tolerated, does not cause hypoglycemia; cut-off GH levels are BMI-dependent
- Glucagon stimulation test: GHD if peak GH levels $<$3 μg/L
- Normal IGF-1 levels do not exclude the diagnosis

FSH/LH deficiency

Men

- Low morning serum testosterone levels (measured before 10:00 h) in conjunction with low or normal gonadotropins
- Second measurement of testosterone with the same assay when initial levels in the mildly hypogonadal range
- Measurement of free or bioavailable testosterone in cases with alterations of SHBG or albumin concentration or when testosterone concentrations close to the lower limit of normal

Women

- Postmenopausal: Absence of high levels of gonadotropins
- Premenopausal: Low serum estradiol levels along with low or normal gonadotropins in the presence of oligomenorrhea or amenorrhea; other causes of oligomenorrhea or amenorrhea should be excluded particularly when dysfunction of other pituitary axes is not evident

ACTH deficiency

- Serum cortisol levels at 08:00–09:00 h $<$ 3 μg/dL (in the absence of glucocorticoid therapy) are indicative of adrenal insufficiency
- If morning cortisol values are 3–15 μg/dL, dynamic test is required
- Interpretation of cortisol values should be done with caution when alterations of cortisol-binding globulin levels are expected
- Inappropriately low or normal ACTH levels in the presence of low morning cortisol concentrations ($<$3 μg/dL) suggest ACTH deficiency
- Insulin tolerance test: adequate cortisol reserve if cortisol levels $>$18.1 μg/dL
- Standard dose short Synacthen test: easier to perform, less invasive, serum cortisol $>$18.1 μg/dL at 30 or 60 min excludes hypoadrenalism, false negative results in patients with recent onset ACTH deficiency
- Glucagon stimulation test: alternative to insulin tolerance test, associated with false-positive results

Central hypothyroidism

- Low free thyroxine concentrations in conjunction with low, normal, or mildly elevated TSH in the setting of pituitary disease
- Absence of nonthyroidal illness syndrome
- Milder cases, characterized by free thyroxine levels still within the normal range, can remain undiagnosed

Central diabetes insipidus

- Hypotonic polyuria ($>$50 mL/kg of body weight/24 h) must be evident and other causes of polyuria should be excluded (e.g., diabetes mellitus)
- Initiation of glucocorticoid replacement therapy unmasks diabetes insipidus
- Urine osmolality of $<$600 mOsm/kg together with serum osmolality of $>$295 mOsm/kg (in the absence of glycosuria) confirms the diagnosis
- In less clear-cut cases or when the differential diagnosis from nephrogenic diabetes insipidus or primary polydipsia is required, a formal water deprivation test is helpful

(*GH*: growth hormone/*GHD*: growth hormone deficiency/*IGF-1*: insulin-like growth factor 1/*GHRH*: growth hormone-releasing hormone, *BMI*: body mass index/*FSH*: follicle-stimulating hormone/*LH*: luteinizing hormone, *SHBG*: sex hormone binding globulin/*ACTH*: adrenocorticotropic hormone/*TSH*: thyroid-stimulating hormone).

Assessing Growth Hormone Reserve

GH is secreted in a pulsatile fashion with peaks at night during sleep and in response to stress, exercise and other factors (Muller *et al.*, 1999). Therefore, random blood GH measurements are of no diagnostic value. In addition, measurements of insulin-like growth factor 1 (IGF-1) concentrations, which is released in response to GH secretion and mediates many of the GH actions, are useful but not sufficient enough to establish the diagnosis of GH deficiency (GHD). IGF-1 levels in the blood are also affected by a number of other parameters (age, gender, oral estrogen therapy, prolactin levels, severity of GHD) (Mukherjee and Shalet, 2009). For the aforementioned reasons provocative tests that stimulate GH release are needed for the diagnosis of GHD.

Insulin tolerance test (ITT) is still regarded as the gold standard for assessing GHD with GH levels $>$3–5 μg/L indicating normal GH reserve (Fleseriu *et al.*, 2016). However, ITT is associated with risks in patients with a history of epilepsy, cardiovascular disease, as well as in the elderly. In these cases, the GHRH + arginine stimulation test can be used which is generally well tolerated without provoking hypoglycemia and with similar with the ITT sensitivity and specificity (Aimaretti *et al.*, 1998). The cut-off levels for the diagnosis of GHD using this test are body mass index (BMI)-dependent and they are divided in three main categories: for

BMI <25 kg/m^2, peak GH <11 μg/L, for BMI 25–30 kg/m^2, peak GH <8 μg/L and for BMI >30 kg/m^2, peak GH <4 μg/L (Ho, 2007). However, GHRH is no longer available in many countries including the United States. Furthermore, it should not be used in patients with suspected GHD of hypothalamic origin (e.g., after irradiation) due to high rate of false negative results (Darzy *et al.*, 2003). When an ITT is contraindicated and GHRH is not available, the glucagon stimulation test is the alternative method, with GH levels <3 μg/L confirming GHD (Gómez *et al.*, 2002). Other tests such as clonidine, L-DOPA, and arginine alone are not useful in adults.

One stimulation test is sufficient for the diagnosis of adult GHD and all other pituitary hormone deficits should have been replaced adequately before it is performed. Furthermore, a number of factors (including age, gender, BMI, exercise and uncontrolled diabetes mellitus) may affect the results of these tests and need to be kept into account when interpreting their results (Molitch *et al.*, 2011). It has been also proposed that in patients with clinical features of GHD and deficiencies in at least three other pituitary axes, provocative testing is not required (Fleseriu *et al.*, 2016). In addition, in children with GHD and multiple hormone deficits due to structural lesions or a genetic condition, a stimulation test is not necessary in adulthood: low IGF-1 levels after discontinuation of GH therapy for at least 1 month is sufficient proof of persistent GHD (Molitch *et al.*, 2011). On the other hand, young adults that received GH therapy during childhood due to short stature (not attributed to structural lesions or genetic conditions) should be retested for GHD, as many of them show normal GH reserve in adulthood.

Assessing the Pituitary–Gonadal Axis

The diagnosis of patients with central (secondary) hypogonadism is based on the measurement of basal levels of gonadotropins and their sex steroids (testosterone and estradiol), combined with menstrual history in females. GnRH stimulation test has no use in the diagnosis of hypogonadism in adult patients (Cheer and Trainer, 2014).

Men

Low morning serum testosterone levels in conjunction with low or normal gonadotropins confirm the diagnosis of central hypogonadism in males. Testosterone demonstrates a circadian secretion rhythm with higher levels in the morning necessitating blood sampling between 8 and 10 am (Brambilla *et al.*, 2009). A second measurement of testosterone should be performed using the same assay when the initial value is in the mildly hypogonadal range (Bhasin *et al.*, 2010). Normal ranges for testosterone concentration vary among laboratories and assays and clinicians need to rely on reference ranges established in their laboratory.

In addition, most of the circulating testosterone is bound to sex hormone binding globulin (SHBG) and to albumin, with only the free hormone being biologically active. In cases where alterations of SHBG or albumin concentration are expected or when total testosterone concentrations are close to the lower limit of the normal range, measurement of free or bioavailable testosterone should be considered, especially if an accurate and reliable method is available (Silveira and Latronico, 2013). Finally, many conditions can cause hypogonadotropic hypogonadism including illness, eating disorders, extensive exercise, drugs (glucocorticoids, opiates, marihuana, alcohol or anabolic steroids abuse) and hyperprolactinemia (Rahnema *et al.*, 2014; Kalyani *et al.*, 2007).

Women

In postmenopausal women, central hypogonadism is defined by the absence of the high levels of gonadotropins. In premenopausal women though, the diagnosis requires a combination of menstrual irregularities (oligomenorrhea or amenorrhea) attributed to anovulation and relevant hormonal findings. The latter include low serum estradiol and low or normal gonadotropins (which in case of oligomenorrhea, need to be measured during the follicular phase of the menstrual cycle (2nd-5th day)). Also, other causes of oligomenorrhea or amenorrhea should be excluded (pregnancy, hyperandrogenism, thyroid disease, drugs) (Fleseriu *et al.*, 2016).

Assessing the Hypothalamic–Pituitary–Adrenal Axis

Cortisol secretion follows a circadian rhythm reaching its peak levels in the morning after awakening. Due to this reason, measurement of serum cortisol concentration should be performed between 08:00–09:00 am. Cortisol levels <3 μg/dL are sufficient to diagnose adrenal insufficiency, whereas levels >15 μg/dL usually exclude the diagnosis (Fleseriu *et al.*, 2016). Patients with morning values between these limits warrant further investigation. Inappropriately low or normal ACTH levels in the presence of low morning cortisol concentrations (<3 μg/dL) suggest ACTH deficiency.

The majority of circulating cortisol is bound to cortisol-binding globulin (CBG) and serum cortisol measurements are, therefore, affected by changes in CBG concentrations. Conditions that increase (oral estrogen therapy, pregnancy, mitotane) or decrease CBG (genetic diseases, nephrotic syndrome, cirrhosis, inflammation) need to be kept in mind when interpreting total cortisol levels in patients with suspected hypopituitarism (Bae and Kratzsch, 2015).

The main tests for assessing ACTH reserve are the ITT and the short Synacthen test (SST). In patients already on glucocorticoid treatment, discontinuation of hydrocortisone for 24-h (or longer for synthetic glucocorticoids) is required. As for GHD, ITT is the gold standard for the assessment of ACTH reserve with peak serum cortisol >18.1 μg/dL considered to be adequate response for most assays. Adequate hypoglycemia (serum blood glucose <40 mg/dL) should be accomplished in order for valid results to be obtained (Crowley *et al.*, 2014).

The SST it the most common test in diagnosing adrenal insufficiency. Patients with primary or secondary hypoadrenalism do not reach cortisol levels >18.1 μg/dL 30 or 60 min following parenteral administration of 250 μg of synthetic ACTH. The SST is easier to perform and less invasive than ITT but is not of value in patients with recent onset of ACTH deficiency (immediately post-surgery or pituitary apoplexy). In these cases, it can lead to false negative results as it takes approximately 6 weeks for the adrenals to lose responsiveness to the stimulatory effect of ACTH. Given the widely accepted safety profile of the SST, most endocrinologists reserve the ITT for patients with borderline results on SST or for cases where simultaneous assessment of GH reserve is required. The low-dose SST, using 1 μg of synthetic ACTH instead of the supraphysiological 250 μg dose, has become increasingly popular during recent years. However, a recent meta-analysis has not confirmed superiority in its diagnostic performance compared with the 250 μg test for the diagnosis of central hypoadrenalism (Ospina *et al.*, 2016).

Other tests include the glucagon stimulation test and the overnight metyrapone stimulation test. The glucagon test has the advantage, as does the ITT, of being able to assess ACTH and GH reserves simultaneously. Glucagon, however, is a weaker stimulus for ACTH secretion and, consequently, is more often associated with false-positive results (Garrahy and Agha, 2016). On the other hand, metyrapone stimulation test is based on the principle that decrease of serum cortisol concentrations (by blocking 11b–hydroxylase and thereby inhibiting conversion of 11-deoxycortisol to cortisol) will lead to increase of ACTH secretion. In subjects with normal HPA axis, the increased ACTH production leads to rising levels of 11-deoxycortisol, whilst in patients with central ACTH deficiency no increase in both hormones is evident. However, this test is rarely used in clinical practice nowadays.

Assessing the Pituitary-Thyroid Axis

Baseline assessment of TSH and free thyroxine is usually sufficient for the diagnosis of secondary (central) hypothyroidism. Measuring free thyroxine instead of total is essential, especially in cases where abnormalities of thyroid-hormone binding proteins are possible (pregnancy, drugs that affect thyroxin-binding globulin, inherited diseases) (Pappa *et al.*, 2015). Free thyroxine levels below the reference range together with a low, normal, or mildly elevated TSH in the presence of pituitary disease usually confirm the diagnosis (Garber *et al.*, 2012). However, with this definition, the milder defects of central hypothyroidism, characterized by free thyroxine levels still within the normal range, can remain undiagnosed (Persani, 2012).

The differential diagnosis of secondary hypothyroidism due to pituitary disease is mainly from nonthyroidal illness syndrome, associated with critical systemic illness; the latter can also induce central hypothyroidism by suppression of TRH production and inhibition of TSH release (Warner and Beckett, 2010). The TRH test (assessment of TSH response to administration of intravenous TRH) has not any clinical use in the diagnosis of central hypothyroidism and has been practically abandoned.

Posterior Pituitary Assessment

The establishment of the diagnosis of DI relies on the confirmation of hypotonic polyuria (>50 mL/kg of body weight/24 h). Other causes of polyuria, even those related with osmotic diuresis (e.g., diabetes mellitus), need to be excluded. It should also be kept in mind that in concomitant ACTH deficiency, glucocorticoid replacement therapy may unmask DI, as lack of cortisol compromises free water clearance.

Paired plasma and urine osmolalities and accurate estimation of fluid balance may be sufficient for the diagnosis; urine osmolality of <600 mOsm/kg together with serum osmolality of >295 mOsm/kg (in the absence of glycosuria) confirms the diagnosis (Cheer and Trainer, 2014). However, in less clear-cut cases, or when the differential diagnosis from nephrogenic diabetes insipidus or primary polydipsia is required, a formal water deprivation test is helpful.

Treatment

Management of hypopituitarism includes appropriate hormone replacement therapy combined with addressing the underlying cause of pituitary dysfunction. Hormonal replacement should be optimized and monitored regularly aiming to avoid suboptimal treatment or the risks associated with over-replacement.

GH Deficiency

GH replacement is administered as a daily subcutaneous injection at a starting dose of 0.2–0.4 mg daily for patients aged below 60 years and 0.1–0.2 mg daily for older ones (Fleseriu *et al.*, 2016). Females usually need higher doses, especially if they are on oral estogens (Johannsson and Bengtsson, 1998). The dose is titrated up gradually guided by clinical and biochemical parameters. IGF-1 is the currently used marker and the aim is achievement of levels within age-adjusted reference range (Molitch *et al.*, 2011). It has been suggested that GH replacement in GHD adults is associated with improvement in mood, lipid metabolism, bone mineral density, exercise tolerance, quality of life, increase in lean body mass and reduction in fat mass (Reed *et al.*, 2013).

Side effects of treatment are arthralgias, myalgias, fluid retention, peripheral edema, carpal tunnel syndrome and sleep disturbances. Also, given that GH reduces insulin sensitivity, GH therapy has been implicated with the development of diabetes, although the relevant published data are conflicting (Weber *et al.*, 2017; Luger *et al.*, 2012). The safety profile in

terms of developing new cancers of GH treatment on appropriate replacement doses appears reassuring in surveillance studies (Fleseriu *et al.*, 2016). Finally, active malignancy is a contraindication for offering GH.

Gonadotropin Deficiency

In patients with central hypogonadism, fertility, sex steroid replacement therapy and careful induction of puberty (if the onset of the deficiency is in childhood or in cases of arrested pubertal progress) are the areas requiring focus.

Men

Androgen replacement in men is available as intramuscular testosterone injections (at intervals depending on the type of preparation used), oral tables, transdermal gels or patches, and subcutaneous implants, with different advantages and disadvantages (Aversa and Morgentaler, 2015). Contraindications for offering testosterone replacement are breast and metastatic prostate cancer, unevaluated prostate pathology, high levels of prostatic specific antigen (>4 ng/mL or >3 ng/mL in high risk individuals), hematocrit $>50\%$, severe lower urinary tract symptoms associated with benign prostatic hypertrophy and poorly controlled congestive heart failure (Bhasin *et al.*, 2010).

The monitoring of patients on testosterone therapy includes clinical response, measurement of serum testosterone levels in various times depending on the preparation used, as well as screening for possible side effects. When fertility is desired, testosterone therapy should be discontinued (it reduces spermatogenesis), and induction of spermatogenesis therapy with human chorionic gonadotropin and/or recombinant FSH is initiated (Anawalt, 2013).

Women

Any form of estrogen (mainly oral or transdermal) in combination with a cyclical progestagen (to avoid endometrial hyperplasia and risk of malignancy in women with intact uterus) or combined estrogen–progestogen preparations are suitable for sex steroid treatment in female patients with central hypogonadism. Unopposed estrogens are used for women who have undergone hysterectomy. The choice of the preparation offered relies on the risk of adverse effects, cost, patient convenience and preference. Estrogens should not be used in women with undiagnosed abnormal genital bleeding, known breast cancer or other estrogen-dependent malignancy or a history of these conditions, previous or active thromboembolic disease, liver disease and thrombophilic disorders (Stuenkel *et al.*, 2015).

Follow-up includes evaluating symptoms and potential side effects. Replacement is advised until the mean age of natural menopause and following this, decisions on continuing treatment should be individualized. Finally, induction of ovulation is achieved with established protocols using gonadotropins or pulsatile GnRH (Yasmin *et al.*, 2013).

ACTH Deficiency

A number of glucocorticoids are available for the treatment of hypoadrenalism. Hydrocortisone is the most commonly used; it is converted to cortisol in the body and can be easily measured in the serum. Alternatives include cortisone acetate or the synthetic glucocorticoids prednisolone, methylprednisolone and dexamethasone. Cortisone acetate is metabolized to cortisol by 11-hydroxysteroid dehydrogenase type 1 in the body. Due to the short half-life of hydrocortisone, two-three doses are required daily, whilst the synthetic glucocorticoids have a longer duration of action and can be administered once daily being a choice of treatment in patients with compliance issues. Dexamethasone is the least favorable option due to difficulties in dose titration and higher risk of side effects (Bornstein *et al.*, 2016). Patients with ACTH deficiency do not require mineralocorticoid supplementation as the renin–angiotensin–aldosterone pathway is intact.

Hydrocortisone in a total dosage of 15–20 mg/24 h divided into two or three doses is the most recommended approach, although patients with partial cortisol insufficiency may need lower doses. The highest dose should be given in the morning on waking and the second in the afternoon (two-dose regimen), or the second and third at lunchtime and in the late afternoon, respectively (three-dose regimen). Splitting of the dose aims to mimic the natural circadian rhythm of cortisol secretion. A newly marketed dual-release hydrocortisone preparation, comprising an immediate-release coating and an extended-release core, administered once daily, resembles the daily normal cortisol profile more closely than conventional hydrocortisone and has been associated with improvements of cardiometabolic factors (blood pressure, serum glucose and lipid levels, BMI) compared with the three-dose hydrocortisone regimen (Giordano *et al.*, 2016; Johannsson *et al.*, 2012). However, it cannot replace the physiological rise in early morning cortisol. Also, a new modified-release, multiparticulate, oral formulation of hydrocortisone is in clinical development; a two dose regimen (07:00 and 23:00 h) seems to provide a more physiological cortisol exposure, as well as an overnight rise in cortisol levels (Whitaker *et al.*, 2014).

Monitoring patients with hypoadrenalism is mainly comprised of clinical assessment for the presence of signs and symptoms of hypocortisolism or glucocorticoid excess. Cortisol day curves are useful when malabsorption or increased steroid metabolic clearance is suspected (Husebye *et al.*, 2014). Furthermore, compliance to treatment should be regularly checked, as well as a concomitant use of medications that may impair glucocorticoid metabolism, such as antiepileptics (Paragliola *et al.*, 2015). Close follow-up of patients with recent initiation of GH replacement is also important as GH inhibits 11β-hydroxysteroid

dehydrogenase type 1 that converts cortisone to cortisol (Stewart *et al.*, 2001). In these cases, adrenal insufficiency may be unmasked or in patients that are already ACTH deficient an increase of the hydrocortisone dose may be needed.

Patient education is vital in optimizing management and outcomes. Written information on sick day rules (adjustment of hydrocortisone dose in case of stress e.g., fever, infection requiring antibiotics), offering and educating on the emergency kit with the injectable hydrocortisone (aiming to avoid adrenal crisis, particularly in cases of vomiting or diarrhea), as well as some form of identification, such as a medic alert bracelet, to inform medical staff if they are found unconscious are vital points. In case of a surgical procedure or severe illness necessitating hospital admission, the cortisol requirements are increased and steroid cover is offered according to specific protocols (Allolio, 2015).

Finally, ACTH deficiency results in compromised secretion of adrenal androgen precursors (including dehydroepiandrosterone (DHEA) and androstenedione), which are a major source of androgens in women. Studies addressing the DHEA replacement in women with adrenal insufficiency have given conflicting results. A systematic review and meta-analysis suggested that the evidence regarding the use of DHEA in this group of women was not sufficient (Alkatib *et al.*, 2009). In addition, in the recent Endocrine Society guidelines on androgen therapy in women, the Taskforce recommended against the routine DHEA replacement in women with low androgen levels due to hypopituitarism (Wierman *et al.*, 2014).

TSH Deficiency

Levothyroxine is the treatment of choice for secondary hypothyroidism on an average adult replacement dose of 1.6 μg/kg/day (Slawik *et al.*, 2007). There is no evidence of beneficial use of triiodothyronine in these patients. Starting dose of levothyroxine and dose titration depends on age and comorbidities, with lower initiation doses and slower increments used in elderly and those with ischemic heart disease. Primary biochemical treatment goal is usually serum free thyroxine values in the upper half of the reference range (before the daily intake of levothyroxine) (Jonklaas *et al.*, 2014). In contrast to primary hypothyroidism, serum TSH is an unreliable indicator in patients with central hypothyroidism and has no value in the monitoring of replacement.

In cases of multiple pituitary hormone deficits, adequate steroid replacement should be initiated prior to commencing levothyroxine; otherwise, a potentially dangerous adrenal crisis may be precipitated. In addition, GH treatment increases the conversion of thyroxine to triiodothyronine (Jørgensen *et al.*, 1994). In these cases, monitoring of thyroid function is needed for the risk of developing central hypothyroidism or a decline in free thyroxine levels. Furthermore, women with secondary hypothyroidism and hypogonadism may require adjustment of their levothyroxine dose when alterations of estrogen levels are expected (e.g., change of estrogen formulation) due to the estrogen-induced increase of thyroid-binding globulin.

Vasopressin Deficiency

Desmopressin, a synthetic analogue of arginine vasopressin, is the replacement drug of choice. It is available in oral, intranasal, sublingual and parenteral forms and is generally administered between one and three times per day. Desmopressin should be started at bedtime to prevent nocturia and gradually titrated up during the day until polyuria is controlled. Monitoring serum urea and sodium, as well as monitoring of fluid balance are essential, particularly during the early stages and with dose adjustments.

In patients with central DI and intact thirst response, severe dehydration and hypernatremia are usually avoided because adequate fluid intake is maintained. In contrast, hyponatremia as a consequence of lack of free water excretion due to overtreatment with desmopressin is a potentially more serious complication. In order to avoid this side effect, a weekly phase of free diuresis is suggested in all patients by omitting a desmopressin dose (Fleseriu *et al.*, 2016). On the other hand, patients with adipsic DI are at risk of developing severe hypertonic hypernatremia. The management of these patients is challenging and includes a stable dose of desmopressin, a fixed amount of fluid intake (under normal circumstances), daily weighing and frequent monitoring of serum sodium levels (Cuesta *et al.*, 2017).

Mortality

Studies assessing the long-term mortality of patients with hypopituitarism have not given consistent results, mainly due to confounding factors, as selection bias, heterogeneity of population studied and variable treatment modalities. Furthermore, the etiology of the pituitary insufficiency is of major importance, as in a number of conditions like acromegaly, craniopharyngioma and Cushing's disease, mortality is affected by the original diagnosis and its management (Sherlock *et al.*, 2010). Despite these drawbacks, two recent meta-analyses suggest an increase premature mortality in adult patients with hypopituitarism (Jasim *et al.*, 2017; Pappachan *et al.*, 2015) with deaths mainly attributed to cardio/cerebrovascular causes, infections and malignancies.

Multiple predictive factors of increased mortality have been recognized: younger age at diagnosis, female gender, an underlying diagnosis of a craniopharyngioma, type of surgery, and prior radiotherapy treatment (Ntali *et al.*, 2016; Olsson *et al.*, 2015; Chang *et al.*, 2008; Tomlinson *et al.*, 2001; Nilsson *et al.*, 2000). In addition, the type of pituitary hormone deficit affects mortality. ACTH deficiency has been correlated with reduced survival, as well as hydrocortisone replacement dose of >20 mg/day, whilst no difference has been found between patients without secondary adrenal insufficiency and those receiving hydrocortisone dose

≤20 mg/day (Hammarstrand *et al.*, 2017; Zueger *et al.*, 2012). Finally, untreated gonadotropin deficiency has also been described as a risk factor of increased mortality (Tomlinson *et al.*, 2001).

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Diagnosis of Prolactinoma and Causes of Hyperprolactinemia

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Introduction

Prolactin is a hormone which is secreted by the pituitary gland in mammals. It is well recognized as having a major role in mammalian reproduction.

Common in Clinical Practice

Hyperprolactinemia is a common problem in clinical endocrine practice. Hyperprolactinemia, a raised serum prolactin level, is typically associated with disorders of the reproductive system. It is important to understand the causes of hyperprolactinemia before making a diagnosis. With careful clinical and laboratory assessment the cause of hyperprolactinemia may be identified and appropriate treatment may be given.

Clinical Scenario

A 25 year old woman presented with secondary prolactinoma. She had had no menstruation for 4 months. A blood test showed serum prolactin was raised at 1112 mU/L. She had researched her condition and the blood test result, and wondered if she had a prolactin-secreting pituitary adenoma (prolactinoma).

Prolactin is secreted by the lactotroph cells of the anterior pituitary gland. The lactotrophs comprise up to 25% of the normal anterior pituitary gland. The key actions of prolactin in women are to promote lactation and to inhibit menstruation: this is an important physiological process after childbirth.

Human Prolactin: Key Facts

- Protein hormone
- Secreted by pituitary lactotroph cells
- Length: 199 amino acids
- Size: 23 kDa
- 82% homology with growth hormone
- Plasma half-life 20 min
- Normal serum prolactin levels:

<530 mU/L (<25 ng/mL) in women.
<420 mU/L (<20 ng/mL) in men.

Secretion of prolactin is regulated by a variety of endocrine factors, including estrogen, dopamine and thyrotropin releasing hormone (TRH). The high levels of estrogen found in pregnancy promote lactotroph hyperplasia and an increase in prolactin secretion; this is important in preparing the breast for lactation. Dopamine is secreted by the hypothalamus into the pituitary portal circulation, and inhibits prolactin secretion by the anterior pituitary gland; disruption of this circulation may interrupt the inhibition and lead to an increase in prolactin secretion and hyperprolactinemia. TRH is secreted by the hypothalamus and regulates the hypothalamic–pituitary–thyroid axis; primary hypothyroidism leads to an increase in TRH secretion, and this may result in hyperprolactinemia. Each of these three endocrine regulatory mechanisms is relevant in understanding the causes of hyperprolactinemia.

In clinical practice hyperprolactinemia most commonly presents in women of reproductive age. In women hyperprolactinemia typically causes amenorrhea (loss of menstruation) with estrogen deficiency. In some cases there is also galactorrhea (inappropriate lactation). In men hyperprolactinemia typically causes erectile dysfunction and loss of libido. In both women and men fertility is reduced.

Prolactinoma or Another Cause?

In common with other endocrine glands, the anterior pituitary gland may form a functioning adenoma, leading to autonomous secretion of a specific hormone. Prolactinoma, a benign adenoma secreting an excess of prolactin, is a common and well recognized cause of hyperprolactinemia. In clinical practice it is tempting to assume that a patient with hyperprolactinemia has a

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prolactinoma. However, several other important causes of hyperprolactinemia must be considered before prolactinoma is diagnosed.

Physiological Causes

A raised serum prolactin is found in pregnancy and lactation. The degree of hyperprolactinemia is variable; there is no clear consensus on a normal reference range for serum prolactin in pregnancy and lactation. Physiological stress also leads to a rise in serum prolactin.

Clinical Case 1
A 17 year old woman was referred with secondary amenorrhea. She had had a normal menarche, and the menstrual cycle had been regular until 4 months earlier, when menstruation ceased. Initial investigations had shown serum prolactin 2560 mU/L, LH 1.8 U/L, FSH 1.6 U/L. She had mild weight gain and breast enlargement. She denied the possibility of pregnancy. On examination the uterus was enlarged. A urinary hCG pregnancy test was positive, and pregnancy was confirmed by ultrasound scan.

Pharmacological Causes

Many prescribed medications have been reported to cause hyperprolactinemia. The major causes of drug-induced hyperprolactinemia are the medications which act as dopamine antagonists: these include antiemetics (such as metoclopramide and domperidone) and antipsychotic agents (such as chlorpromazine and risperidone).

Clinical Case 2
A 34 year old woman with psychosis was treated with risperidone. She developed menstrual irregularity and galactorrhea. Serum prolactin was 1112 mU/L. There was no disturbance of vision. It was not clinically appropriate to stop treatment with risperidone. A pituitary MRI scan excluded the presence of a mass lesion. The hyperprolactinemia was attributed to the effects of risperidone acting as a dopamine antagonist.

Other Nonpituitary Medical Conditions

Primary hypothyroidism may cause hyperprolactinemia as a result of increased hypothalamic TRH production, which stimulates lactotrophs. In marked hypothyroidism there may be significant pituitary enlargement due to lactotroph and thyrotroph hyperplasia.

Chronic kidney disease is associated with hyperprolactinemia; this is partly due to reduced metabolic clearance of prolactin.

Clinical Case 3
A 42 year old woman was referred with menstrual irregularity. Investigations showed serum prolactin 1516 mU/L, creatinine 246 μmol/L. She was not aware of kidney disease. She had suffered recurrent urinary tract infections for many years. After further investigation she was found to have chronic kidney disease due to chronic pyelonephritis.

Polycystic ovary syndrome is a common reproductive disorder in women. It is characterized by menstrual irregularity and hyperandrogenism. Estrogen is generally abundant rather than deficient. Mild hyperprolactinemia is commonly seen in polycystic ovary syndrome: this may be due to the stimulatory effect of oestrogen on pituitary lactotrophs.

Clinical Case 4
A 19 year old woman presented with irregular menstruation. She had had oligomenorrhoea since menarche, with menstruation around every 3–4 months. She had mild hirsutism and acne. A blood test showed serum oestradiol 345 pmol/L, LH 10.8 mU/L, FSH 5.9 mU/L, prolactin 928 mU/L. Pelvic ultrasound revealed ovaries of polycystic appearance. Administration of an oral progestogen for 1 week led to a withdrawal bleed. The hyperprolactinemia was attributed to polycystic ovary syndrome.

Pitfalls in Measurement of Serum Prolactin

Laboratory assays of serum prolactin by immunoassay methods are generally reliable. However there are two important circumstances in which a serum prolactin level measured by immunoassay may be misleading.

The hook effect

In patients with a large prolactinoma the serum prolactin level may be markedly elevated: serum prolactin may be $>10{,}000$ mU/L, and in some cases $>100{,}000$ mU/L. In some serum prolactin assays the extremely high level of serum prolactin exceeds the

linearity of the prolactin assay, causing failure of the assay performance: in this situation the prolactin assay result may be much lower than expected, such as around 1000–3000 mU/L. This assay result, in the context of radiological evidence of a large pituitary tumor, may lead to the erroneous diagnosis of a nonfunctioning pituitary adenoma with disconnection hyperprolactinemia (see below), and the diagnosis of large prolactinoma may be missed. This error can be prevented by reassay of the serum sample after dilution: the assay performance returns to linearity, revealing the very high serum prolactin level.

Macroprolactin

In some people serum prolactin exists in two distinct molecular forms, simple prolactin (23 kDa) and a high molecular mass form of prolactin, typically around 150 kDa. This larger form of prolactin is known as macroprolactin. Macroprolactin is usually formed by the association of simple prolactin with an IgG immunoglobulin; this form of prolactin has reduced metabolic clearance, leading to accumulation of prolactin and hyperprolactinemia. Macroprolactin is generally thought to have reduced or absent bioactivity: in people with hyperprolactinemia solely due to the presence of macroprolactin the raised serum prolactin level does not lead to amenorrhea, galactorrhea, reduced libido or hypogonadism. Laboratories can detect hyperprolactinemia by polyethylene glycol (PEG) precipitation, which removes higher molecular mass forms of prolactin, or by gel filtration chromatography. Clinicians need to know when macroprolactin is present, to avoid misinterpretation of a raised serum prolactin level; for this reason laboratories should routinely include a test for macroprolactin on all samples where a raised serum prolactin result indicates a new finding of hyperprolactinemia.

Clinical Case 5

An 18 year old woman presented with secondary amenorrhea. Her menstrual cycle was usually regular; she had missed one menstrual period, and had no galactorrhea. A urinary pregnancy test was negative, and a blood test showed serum prolactin 1812 mU/L, LH 3.8 U/L, FSH 4.1 U/L. On reassay of the serum after PEG precipitation the serum prolactin was 272 mU/L, indicating that the raised serum prolactin was due to the presence of macroprolactin. The menstrual cycle had returned to normal, and no further investigation was indicated.

Pituitary and Para-pituitary Causes

As noted above, prolactin secretion is inhibited by dopamine secreted by the hypothalamus into the hypothalamic–pituitary portal circulation. Interruption of this inhibition by disruption of the portal circulation may lead to hyperprolactinemia. A mass lesion in the pituitary gland or in the suprasellar space is an important cause of hyperprolactinemia. This phenomenon is sometimes referred to as stalk-compression hyperprolactinemia or disconnection hyperprolactinemia. The degree of hyperprolactinemia is usually modest, typically <2000 mU/L and rarely up to 5000 mU/L. The commonest cause is a nonfunctioning pituitary adenoma. Para-pituitary mass lesions causing hyperprolactinemia include craniopharyngioma and meningioma of the sphenoid wing. Disruption of the portal circulation, along with direct pressure on the anterior pituitary gland, may also lead to varying degrees of loss of pituitary function.

Clinical Case 6

A 53 year old man presented with erectile dysfunction and loss of libido. Blood tests showed serum testosterone 5.2 nmol/L, LH 1.2 U/L, FSH 2.5 U/L, prolactin 1712 mU/L. Anterior pituitary function was otherwise intact. A pituitary MRI scan revealed a large mass arising from an expanded pituitary fossa, extending into the suprasellar space and elevating the optic chiasm (see Fig. 1). He underwent pituitary surgery to protect his vision: on histological analysis the mass was a nonfunctioning pituitary adenoma.

Acromegaly is also a recognized cause of hyperprolactinemia. Acromegaly arises from autonomous secretion of growth hormone from an adenoma of the somatotroph cells of the pituitary gland. Somatotroph and lactotroph cells have a common origin, and in some cases of somatotroph adenoma there is cosecretion of growth hormone and prolactin. In acromegaly hyperprolactinemia may be due to cosecretion of growth hormone and prolactin by the adenoma, or alternatively the mass effect of the somatotroph adenoma inhibiting pituitary stalk function and leading to disconnection hyperprolactinemia.

Clinical Case 7

A 42 year old man was referred with loss of libido and erectile dysfunction. A blood test had shown serum testosterone 5.2 nmol/L, LH 1.8 U/L, FSH 2.1 U/L, prolactin 1119 mU/L. On examination he had coarse facial features, large hands and feet and prognathism. On further investigation he was found to have growth hormone excess due to acromegaly. He was treated for acromegaly, and the serum growth hormone and prolactin levels fell in response to treatment.

Prolactinoma

Prolactinoma is an important and clinically significant cause of hyperprolactinemia. Prolactinoma develops as a result of monoclonal proliferation of the lactotroph cell population. It causes autonomous secretion of prolactin, which leads to the

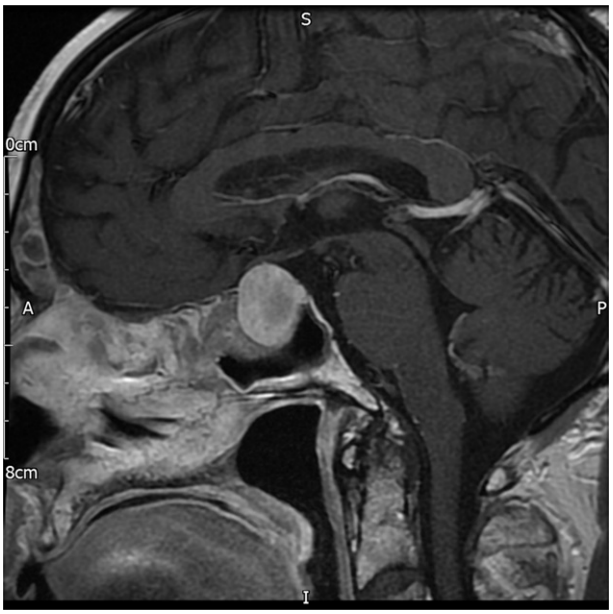

Fig. 1 MRI pituitary, sagittal view with contrast. Serum prolactin 1712 mU/L. The large smooth, round, high signal mass lesion arising from the pituitary fossa is larger than would be expected for a prolactinoma. The diagnosis is nonfunctioning pituitary adenoma with stalk-compression hyperprolactinemia.

hyperprolactinemia syndrome. In women of reproductive age the clinical manifestations are amenorrhea, sometimes galactorrhea, estrogen deficiency and loss of libido. In men the clinical manifestations are loss of libido and erectile dysfunction. As outlined above, it is important for other causes of hyperprolactinemia to be excluded before a diagnosis of prolactinoma is made.

In prolactinoma the degree of hyperprolactinemia is usually commensurate with the size of the adenoma. A large pituitary mass with mild hyperprolactinemia is usually due to a nonfunctioning adenoma rather than a prolactinoma. The diagnosis of prolactin-secreting pituitary adenoma is supported by finding a pituitary adenoma on MRI scan, of a size which is proportionate to the degree of hyperprolactinemia, and by a fall in serum prolactin and shrinkage of the adenoma after treatment with a dopamine agonist. In rare cases which are not responsive to dopamine agonist therapy the diagnosis of prolactinoma may be confirmed by surgical removal of the adenoma and immunohistological analysis showing adenoma with a predominance of cells which stain positively for prolactin.

Clinical Case 8

A 23 year old woman presented with secondary amenorrhea for 6 months. She had noticed mild galactorrhea. A urinary pregnancy test was negative. She was not taking any medication. There were no clinical features of androgen excess, hypothyroidism or acromegaly. The visual fields were full. Investigations revealed serum prolactin 1614 mU/L (with a similar result after PEG precipitation), oestradiol $<$70 pmol/L, LH 1.2 mU/L, FSH 1.5 mU/L, TSH 1.23 mU/L, fT4 12.1 pmol/L, creatinine 79 μmol/L. A pituitary microprolactinoma was seen on MRI pituitary scan (see Fig. 2). She was treated with a dopamine agonist, the serum prolactin level returned to normal, and menstruation was restored, supporting the diagnosis of prolactin-secreting pituitary microadenoma (microprolactinoma).

Summary

The clinical approach to assessing a patient with a raised serum prolactin level requires consideration of the several different causes of hyperprolactinemia. In each situation a disturbance in reproductive function may indicate the effect of the hyperprolactinemia. Prolactinoma is a common and well recognized cause of reproductive disturbance and hyperprolactinemia; however, several other causes of hyperprolactinemia should be considered before a diagnosis of prolactinoma is made.

Summary

Causes of hyperprolactinemia to be considered in diagnosis:

- Physiological
- Pharmacological

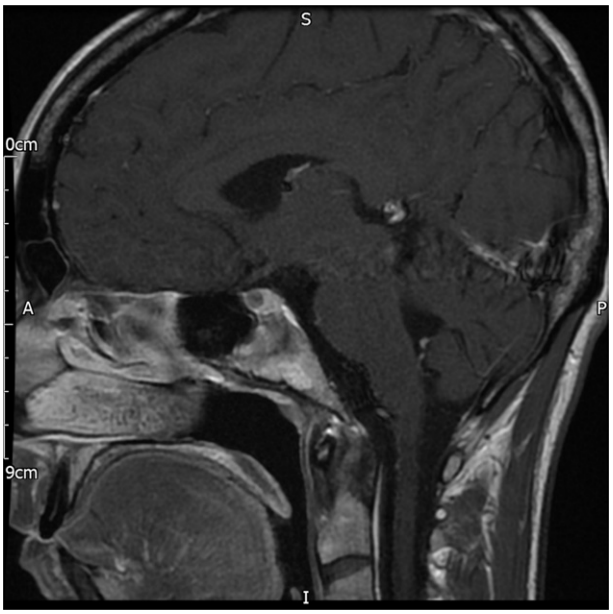

Fig. 2 MRI pituitary, sagittal view with contrast. Serum prolactin 1614 mU/L. The small low signal lesion within the pituitary gland is characteristic of a microprolactinoma.

- Nonpituitary disease
- Pitfalls in measurement
- Other pituitary and para-pituitary disease
- Prolactinoma

Further Considerations

It is generally recognized that the main physiological role of prolactin in women is in the regulation of reproduction. Its role in men is less clearly understood. It should be noted that prolactin is also secreted by tissues other than the anterior pituitary gland, and that there are prolactin receptors in a wide variety of tissues outside the reproductive system. Several functions for prolactin other than in reproduction have been recognized, including actions in the immune system and in relation to angiogenesis. Furthermore, prolactin is found in almost all vertebrates, including fish and birds, where its physiological role is clearly quite different. It should be remembered that our understanding of prolactin is incomplete, and that further research may reveal additional effects of hyperprolactinemia not generally recognized in current clinical practice.

Further Reading

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Prolactinomas: Clinical Manifestations and Therapy

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Glossary

Amenorrhea Lack of menstruation. Primary (never had menses) or secondary (loss of previously established menses).
Dopamine agonists Drugs that activate dopamine receptors.
Galactorrhea Discharge of milk from the breasts.
Hyperprolactinemia Raised levels of prolactin.
Oligomenorrhea Infrequent menses.
Prolactinoma A prolactin-producing pituitary adenoma.
Prolactin (PRL) Pituitary hormone whose physiological role is to cause lactation in women in the postpartum period.
Prolactinoma Prolactin-secreting pituitary adenoma.

Introduction

Prolactinomas are the commonest type of pituitary tumor, accounting for 40% of all pituitary adenomas. Symptoms and signs may arise from hyperprolactinemia per se and/or from local mass effects. Microadenomas (<10 mm in diameter), which predominate in women, are much commoner than macroadenomas (>10 mm in diameter), which have a more equal gender split. Local mass effects can cause symptoms in the latter, dependent on adenoma size and extension outside the sella.

Clinical Manifestations

Table 1 summarizes the commonest clinical findings on presentation.

Galactorrhea

Nonpuerperal galactorrhea describes any discharge of milk from the breasts, and may be spontaneous or expressible on stimulation. Lactation is considered inappropriate when it occurs outside pregnancy or if it persists for more than a year after completion of breast feeding. Galactorrhea is reported in 5%–10% of women with regular menses, nearly all of whom have normal prolactin

Table 1 Presenting symptoms at diagnosis according to tumor size and gender

	Women	*Men*
Macroprolactinoma		
Hypopituitarism	51%	59%
Visual field defects	37%	48%
Headache	69%	38%
Galactorrhea	75%	22%
Infertility	24%	19%
Menstrual disturbance	100%	–
Reduced libido	–	83%
Weight gain	94%	53%
Microprolactinoma		
Headache	31%	6%
Galactorrhea	43%	0%
Infertility	54%	19%
Menstrual disturbance	76%	–
Reduced libido	–	88%
Weight gain	59%	19%

Adapted from Colao, A., Sarno, A. D., Cappabianca, P., *et al.* (2003). Gender differences in the prevalence, clinical features and response to cabergoline in hyperprolactinemia. European Journal of Endocrinology 148(3), 325–331.

levels. In contrast, 75% of women will have hyperprolactinemia when galactorrhea coexists with oligo/amenorrhea. Galactorrhea is much less common in men (10%–20% of cases) but when present is almost pathognomonic of a prolactinoma.

Female Reproductive Function

Hyperprolactinemia affects reproductive function by a number of mechanisms. Hypogonadotropic hypogonadism is the best understood of these, whereby gonadotropin secretion is reduced as a consequence of suppressed gonadotropin-releasing hormone (GnRH) activity from upstream inhibition of hypothalamic kisspeptin-producing neurons. Other mechanisms include a direct inhibitory action of hyperprolactinemia on estrogen and progesterone production in the ovaries, and decreased estrogen production secondary to reduced ovarian aromatase activity from blockade of the stimulatory actions of FSH. As a consequence of these effects, hyperprolactinemia shortens the luteal phase, such that most women become anovulatory, with consequent oligo/amenorrhea and infertility. Menstrual disturbance was reported in 94% of >1400 patients undergoing transsphenoidal surgery for prolactinoma. This usually manifests as secondary oligo/amenorrhea although can present as primary amenorrhea if the adenoma develops prepubertally, at which age macro- lesions predominate.

Many patients with hyperprolactinemia present with infertility, which is inevitable when gonadotropins are suppressed. Most patients in this context have amenorrhea and galactorrhea but a small proportion do not. Dopamine agonist therapy may still be helpful in restoring fertility in such circumstances. Reduced libido may also be apparent, and usually improves when prolactin levels are normalized.

Male Reproductive Function

The mechanisms by which hyperprolactinemia results in hypogonadism in men are similar to those in women, and include reduced LH and FSH pulsatility. Testosterone concentrations, which are typically low or in the low-normal range, improve with correction of hyperprolactinemia provided there is no structural compromise of the pituitary gland. Reduced libido and erectile dysfunction are present in some 90% of patients with chronic hyperprolactinemia, although other hypogonadal symptoms such as reduced beard growth and loss of muscle strength are rare. Sperm count and function may be affected but this usually improves with restoration of normoprolactinemia.

Bone Mineral Density

Long-standing hypogonadism secondary to hyperprolactinemia can lead to osteoporosis in both men and women. Women with hyperprolactinemia, but normal menstruation, do not display a reduced bone mineral density (BMD). Bone mass improves in both sexes with correction of hyperprolactinemia and restoration of eugonadism.

Mass Effects

As with any macroadenoma, macroprolactinomas may induce symptoms through local mass effects. Macroprolactinomas are generally larger in men. Whilst delayed presentation may account for this in part, macroprolactinomas in men tend to be more invasive and resistant to dopamine agonist therapy, and display greater proliferative potential.

The risk of visual compromise increases with the extent of suprasellar extension. Visual field defects may range from classical bitemporal hemianopia (which is not always symmetrical) to smaller quadrantic defects or scotomas. Visual field loss is usually gradual and may go unnoticed by the patient. Lateral extension into the cavernous sinuses may lead to varying degrees of ophthalmoplegia and cavernous sinus syndrome as a result of entrapment of cranial nerves III, IV, V_1, V_2 and/or VI. Invasive tumors causing bony destruction in the skull floor can also cause cranial nerve entrapment, in addition to compression of other base-of-skull structures. Giant prolactinomas are rare, comprising $\sim$2%–3% of all prolactinomas and presenting most commonly in young to middle-aged men. Exact definitions vary, but common criteria are a diameter of >4 cm with significant extrasellar extension and markedly raised prolactin levels. Massive extrasellar extension in such tumors can cause hydrocephalus and temporal lobe epilepsy.

Moderate to severe headaches are most commonly reported by women with macroprolactinomas. Sudden onset of a severe headache, with or without visual compromise and reduced consciousness, should raise suspicion of pituitary apoplexy. This can rarely occur as the first presentation of a macroprolactinoma, but can also develop following initiation of dopamine agonist therapy. Dopamine agonists can also rarely induce a CSF leak as a result of significant tumor shrinkage.

Local mass effects can cause hypopituitarism, either due to direct compression of anterior pituitary cells or as a result of hypothalamic/pituitary stalk compression. The risk of hypopituitarism increases with tumor size, and the pattern of anterior pituitary loss follows that of other tumor types (GH > TSH > ACTH). In children, hypopituitarism may result in pubertal and growth delay. Rarely, clinical manifestations of prolactinomas can result from co-secretion of other hormones. Most commonly, this occurs in the context of Acromegaly, but has on occasion been reported with TSH or ACTH co-secretion.

Additional Features

Up to 5% of clinically diagnosed pituitary adenomas occur due to hereditary tumor syndromes. Prolactinomas are the most common, being found in approximately 40% of MEN-1 and FIPA (familial isolated pituitary adenoma) patients. Carney Complex patients can develop prolactinomas, but these usually also cosecrete GH.

Therapy

The goals of treatment are to restore gonadal and sexual function, to improve symptomatic well-being and, in macroprolactinomas, to reduce tumor size. All macroprolactinomas and most microprolactinomas require treatment. Specific indications for therapy include troublesome galactorrhea, infertility, neurological effects (especially visual field loss), hypogonadism, reduced BMD and delayed puberty. Women seeking fertility with normal menses and mild hyperprolactinemia may also benefit from treatment. Treatment may not be necessary in women with regular cycles and nontroublesome galactorrhea nor in postmenopausal women with microprolactinomas and nontroublesome galactorrhea. Regular surveillance with monitoring of prolactin levels is nevertheless important as an indicator of potential tumor expansion.

Medical Therapy

Dopamine agonists are the primary therapy for patients with prolactinomas. Whilst all dopamine agonists are effective, pergolide and quinagolide are much less commonly used than cabergoline and bromocriptine. A systematic review conducted by the Endocrine Society (Wang *et al.*, 2012) showed that dopamine agonists improve many important patient outcomes including normalization of prolactin levels (median 68%, range 40%–100%), resolution of galactorrhea (86%; 33%–100%), resolution of amenorrhea (78%; 40%–100%) and improvement of sexual function (67%; 6%–100%). In macroprolactinomas, a benefit on reduction in tumor size (62%; 20%–100%) and resolution of visual field defects (67%; 33%–100%) was also confirmed.

A number of studies have shown that cabergoline is more effective than bromocriptine in normalizing prolactin levels, restoring gonadal function and reducing tumor size, potentially due to a higher binding affinity for receptor binding sites. Cabergoline is also better tolerated and more convenient to administer; treatment adherence may therefore be better with cabergoline. For these reasons, cabergoline is generally preferred as first line therapy.

Cabergoline therapy is usually begun at a dose of 0.25–0.5 mg given once or twice weekly, with the dose increased monthly until normoprolactinemia is achieved. Doses in excess of 3 mg weekly are rarely needed. Postmenopausal women should be reassessed for the need for ongoing treatment as prolactin levels may normalize.

Bromocriptine is commenced at a dose of 0.625–1.25 mg daily which may be increased weekly to a typical maintenance dose of 2.5 mg twice or three times daily. Side-effects of nausea and postural hypotension are not uncommon but may be minimized by a gradual dose increment and taking tablets with a snack before bedtime.

Ergot-derived dopamine agonists (cabergoline, bromocriptine and pergolide) have been associated with an increased risk of cardiac valve fibrosis and regurgitation when used in high doses in patients with Parkinson's disease. In contrast, nearly all studies conducted in patients with prolactinomas have found no evidence of an increased risk of clinically significant valve disease, and no relationship of valvular abnormalities with cumulative dose exposure. Nevertheless it is prudent to keep the dose of dopamine agonist therapy at the lowest needed to control prolactin levels, and periodic echocardiography may still be needed in patients receiving high doses for prolonged periods of time.

Microprolactinomas

Dopamine agonist therapy is highly effective in restoring gonadal function and fertility in microprolactinomas. Tumor shrinkage is not a treatment goal because microprolactinomas by definition are confined to the sella and only 5%–10% will increase in size. Annual prolactin measurements and clinical review is often all that is required once normoprolactinemia is achieved. In some patients, gonadal function is restored even if prolactin levels are not normalized. Complete normalization of prolactin levels is therefore not always necessary and decisions on dose adjustment should be made based on clinical response as much as biochemical review. Estrogen replacement, most commonly given as the combined oral contraceptive pill, may be an alternative approach to dopamine agonist therapy in oligo/amenorrheic women with microprolactinomas not seeking pregnancy. Whilst studies have shown that estrogen therapy does not appear to be associated with an increase in tumor size, prolactin levels should be measured annually and an MRI repeated if these rise significantly. Many microprolactinomas shrink or even disappear in response to long-term therapy such that a trial of dopamine agonist withdrawal is recommended after 2 years of treatment in patients who no longer have a raised prolactin. Sustained normoprolactinemia is achieved in 20%–35% of patients under such circumstances.

Macroprolactinomas

Dopamine agonists will normalize prolactin levels and reduce tumor size in the majority of patients with macroprolactinomas. Tumor shrinkage usually occurs rapidly within a few weeks of starting therapy but may be delayed for a few months in some patients (**Fig. 1**). An MRI undertaken at 3 months following therapy commencement is helpful in confirming this response, with

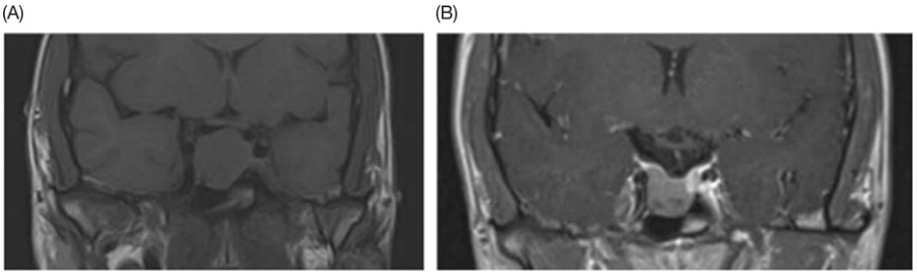

Fig. 1 MRI pituitary showing a macroprolactinoma (A) at diagnosis and (B) following 3-months of cabergoline therapy.

scanning intervals extended thereafter and guided by prolactin response. Similarly rapid improvements in visual function are usually evident, such that surgery is not required for patients with visual field defects in the absence of resistance or intolerance to dopamine agonist therapy. Hypopituitarism, especially growth hormone deficiency, may also recover hence it is important to consider retesting to confirm ongoing need for anterior pituitary hormone replacement. Treatment should aim to restore normoprolactinemia; once tumor shrinkage is achieved doses can be tapered gradually until the lowest dose which maintains a normal prolactin is reached. In contrast to microprolactinomas, cessation of dopamine agonist therapy is rarely followed by sustained remission. If a trial of drug withdrawal is to be considered in patients with significant tumor shrinkage and a normal prolactin, close follow-up is needed to look for recurrence of hyperprolactinemia and tumor reexpansion.

Resistant Prolactinomas

Whilst outcomes in response to dopamine agonists are usually excellent, some patients do not respond adequately. Intolerance to a dopamine agonist due to side-effects may be managed by switching to an alternative preparation or by undertaking transsphenoidal surgery. Dopamine agonist resistance should be distinguished from intolerance and may include a failure to restore normal prolactin levels in response to a maximally tolerated dose of dopamine agonist and a failure to achieve a significant ($>50\%$) reduction in tumor size. The molecular basis of dopamine agonist resistance varies and may include dopamine D2 receptor and postreceptor mechanisms. Cabergoline fails to restore normoprolactinemia in approximately 10% of microprolactinomas and 18% of macroprolactinomas. Men are also more likely to display resistance than women. Options for treatment include maximization of the dopamine agonist dose, switching to a different dopamine agonist and consideration of transsphenoidal surgery. The evidence for a benefit in switching dopamine agonist is greatest for changing bromocriptine to cabergoline. Up to 25% of patients are resistant to bromocriptine, 80% of whom will restore normal prolactin levels in response to cabergoline. Tumor shrinkage is also more profound with cabergoline.

Surgery

The success rates of transsphenoidal surgery, in common with all types of pituitary adenoma, are dependent on tumor size and skill of the surgeon. In specialized centers, normalization of prolactin levels occurs in about 75% of microprolactinomas but is significantly worse for macroprolactinomas, especially if there is cavernous sinus extension. A 20% recurrence rate of hyperprolactinemia is also reported. Transsphenoidal surgery is therefore usually reserved for patients showing dopamine agonist intolerance or resistance (including a failure of visual function to improve). Less commonly, surgery may be considered in cystic macroprolactinomas with neurological compromise (as these often fail to shrink in response to dopamine agonists), apoplexy with neurological signs and where patient preference is for surgery.

Radiotherapy

Radiotherapy is rarely required to treat prolactinomas, because medical therapy is so effective and the side-effects of radiotherapy are not inconsiderable (including hypopituitarism, increased risk of stroke and secondary brain tumors, and optic nerve damage). In practice, radiotherapy is therefore reserved for resistant tumors not cured by surgery, or for malignant prolactinomas. When used, radiotherapy restores normal prolactin levels in approximately one-third of patients and may stabilize tumor growth, albeit that it may take many years for maximum benefit.

Malignant Prolactinoma

Malignant prolactinomas are very rare and cannot be distinguished from adenomas on histological grounds. They are defined as malignant when metastatic disease develops elsewhere in the central nervous system or further afield. Only very few are malignant at presentation, and most arise on a background of a previously invasive macroprolactinoma that has already been treated with dopamine agonists, surgery and radiotherapy. Resistance to dopamine agonists is common and there is often a discordance

between tumor mass and prolactin levels. Treatment is particularly difficult. Whilst debulking surgery may be considered to reduce symptomatic local compression, outcomes from 'conventional' chemotherapy (e.g., vincristine, procarbazine, cisplatin, etoposide) are poor. However, although experience is limited to case series, the oral alkylating agent temozolomide appears to be effective in reducing prolactin levels and controlling tumor growth. Nevertheless, responses are not usually sustained and prognosis is usually poor.

Prolactinoma and Pregnancy

The management of hyperprolactinemia prior to and during pregnancy presents several challenges, including the effects of hyperprolactinemia on fertility, the safety of dopamine agonists in pregnancy, effects of pregnancy on tumor expansion, and lactation.

Dopamine agonists are highly effective in restoring a regular menstrual cycle and ovulation. Patients must thus be warned that restoration of fertility may occur rapidly, even before menses resume. Women not wishing to become pregnant should thus use appropriate methods of contraception. When pregnancy is desired in a woman with a macroprolactinoma, it is preferable to ensure that conception is planned once prolactin levels have normalized and tumor shrinkage has ensued, in order to reduce the risks of optic chiasmal compression with tumor expansion during pregnancy.

Both cabergoline and bromocriptine cross the placenta but to date no safety concerns have been raised with respect to fetal outcomes with either drug. In excess of 6000 pregnancies have been reported in women treated with bromocriptine, with no differences in congenital abnormalities, ectopic pregnancies or spontaneous abortions compared to the background population. Long-term follow-up studies have also been reassuring with respect to child neurodevelopmental outcomes. Although the experience with cabergoline use in pregnancy is much less, the data to date are also reassuring with respect to fetal outcomes. In contrast, quinagolide does appear to be associated with an increased risk of congenital malformations in the relatively small number of pregnancies reported, and its use in this setting is not recommended.

Although the safety data of both bromocriptine and cabergoline in pregnancy appear reassuring, the first trimester is the time when any drug-induced teratogenic effects are likely to be at their highest. It is thus appropriate for most women to be advised to stop these drugs as soon as they miss a menstrual period and pregnancy is confirmed.

Pregnancy is accompanied by an estrogen-stimulated expansion of the lactotroph population, such that prolactin levels increase 10-fold and pituitary volume twofold compared to prepregnancy values. This expected rise in prolactin during pregnancy, coupled with the observation that some prolactinomas can enlarge without a significant rise in prolactin, limits the diagnostic

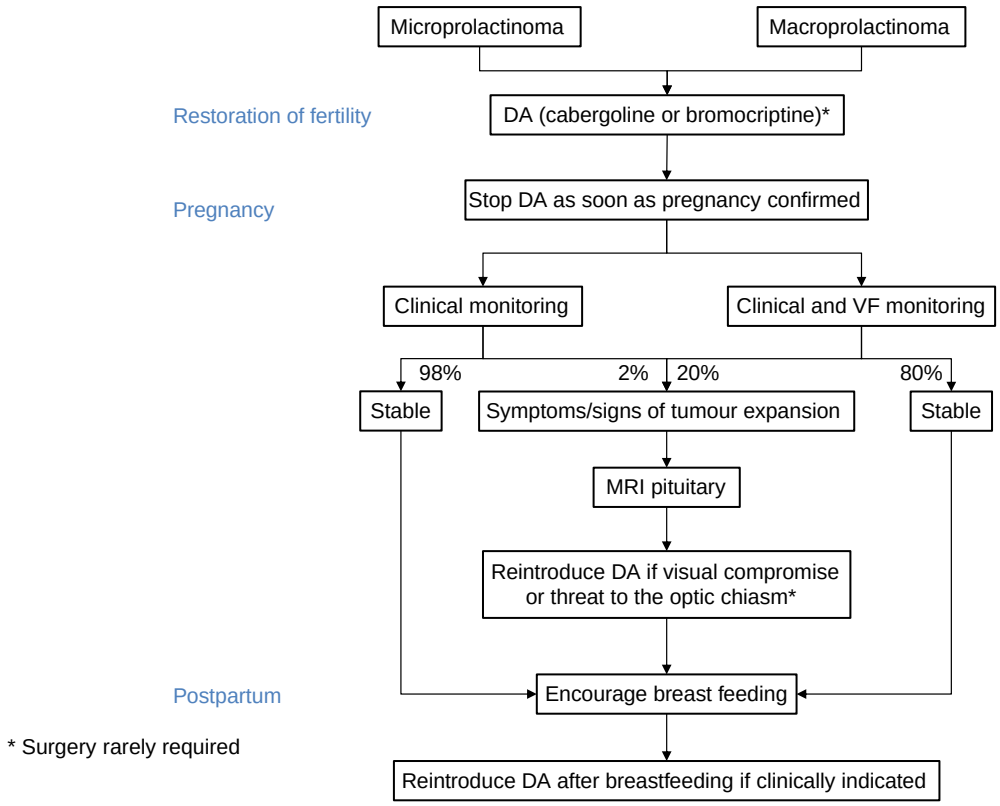

Fig. 2 Management algorithm for prolactinoma in pregnancy.

value of prolactin measurement as a marker of tumor enlargement in pregnancy. Serum prolactin should not therefore be measured during pregnancy.

The risk of clinically significant tumor expansion in pregnancy is dependent on tumor size and prior treatment. Only about 2.5% of microprolactinomas expand to an extent that symptoms ensue, such that clinical examination to include a bedside assessment of visual fields is all that is usually required in each trimester. Formal visual field assessment and MRI can then be reserved for those rare patients in whom there is clinical evidence of tumor growth. In contrast, the risk of symptomatic expansion in macroprolactinomas is 20%–30%, although this is reduced to 5% in patients who have previously been treated with surgery or radiotherapy. An individualized approach is needed in this setting dependent on the position of the macroprolactinoma with respect to the optic chiasm and its previous responsiveness to dopamine agonist therapy. Some clinicians might recommend debulking surgery before pregnancy in women with macroprolactinomas to reduce the risk of tumor expansion, but this runs the risk of inducing hypopituitarism and infertility, and is best reserved for patients who are intolerant or resistant to dopamine agonists. More common approaches include discontinuing the dopamine agonist once pregnancy is confirmed, with regular clinical surveillance and formal visual field testing, or continuing the dopamine agonist throughout pregnancy (recognizing that the safety data of dopamine agonists continued in this manner are still comparatively limited). MRI without gadolinium should be undertaken in women reporting worsened headaches and/or changes in their visual fields. In cases of confirmed tumor expansion, reintroduction of dopamine agonists is usually successful in reversing tumor enlargement, but transsphenoidal surgery or early delivery may rarely be required.

Women expressing a wish to breast feed their children should not be recommenced on dopamine agonists because the fall in prolactin levels will impair lactation. Breastfeeding need not be restricted since it does not cause an increase in prolactin levels nor are there data to suggest an increase in tumor size.

A flow chart outlining a potential approach to the management of prolactinoma during pregnancy is shown in **Fig. 2**.

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Hypophysitis

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Abbreviations

ACE	Angiotensin conversion enzyme
ACTH	Adrenocorticotropic hormone
APA	Antipituitary antibodies
AI	Autoimmune
βHCG	Beta human chorionic gonadotropin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computerized tomography
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DI	Diabetes insipidus
EMA	European Medical Agency
FDA	Food and Drug Administration
FDG	Fluoro-deoxy-glucose
FSH	Follicle-stimulating hormone
GCT	Germ cell tumor
GH	Growth hormone
GrH	Granulomatous hypophysitis
HIV	Human immunodeficiency virus
IgG4-R	IgG4-related
IgG4-RD	IgG4-related disease
IgG4-RH	IgG4-related hypophysitis
IIF	Indirect immunofluorescence
irAEs	Immune-related adverse events
LCH	Langheran's cell histiocytosis
LH	Luteinizing hormone
LYH	Lymphocytic hypophysitis
mAb	Monoclonal antibody
MRI	Magnetic resonance imaging
PA	Pituitary adenoma
PD1	Programmed cell death protein 1
PET	Positron emission tomography
PRL	Prolactin
RCC	Rathke's cleft cyst
TS	Transsphenoidal
XH	Xanthomatous hypophysitis
XGrH	Xanthogranumomatous hypophysitis

Introduction

Hypophysitis is defined as the presence of any inflammatory process of the pituitary gland, that may involve the anterior lobe, the posterior lobe and/or the infundibulum. A rare localized or predominant involvement of the hypothalamus has been reported as "hypothalamitis." The etiopathogenesis of hypophysitis is complex (Caturegli *et al.*, 2005, 2008). Pituitary inflammatory infiltration was first recognized in Brissaud *et al.* (1908), while associated aspects (i.e., pituitary atrophy, concomitant extra-pituitary autoimmune (AI) disorders and the temporal association with pregnancy) were described in 1962 in an autopsy report of a young female who died of postsurgical adrenal crisis 14 months after delivery in a context of hypopituitarism (Goudie and Pinkerton, 1962). Soon after, the first experimental animal model of AI hypophysitis was reported (Levine, 1967), followed by the identification of autoantibodies against PRL-secreting pituitary cells (Bottazzo *et al.*, 1975). Then, the clinical, histological, and radiological features of different forms of hypophysitis have been progressively characterized (Carpinteri *et al.*, 2009; Guaraldi *et al.*, 2017).

Nowadays, the term "hypophysitis" encompasses a broad variety of primary and secondary inflammatory diseases, some of which are being increasingly recognized. In particular, in the last decade, hypophysitis has emerged as a frequent complication of anticancer immunotherapy, raising interest far outside the limited community of pituitary specialists. Hypophysitis in the setting of the systemic IgG4-related (IgG4-R) inflammatory syndrome or infective disorders in immunocompromised patients have also gained interest among physicians in different fields of internal medicine. As most of these disorders can be suspected according to the clinical context, the indications for diagnostic neurosurgery have decreased over time, but histopathological diagnosis remains a cornerstone in many cases. The clinical implications of hypopituitarism, in particular the risk of adrenal insufficiency, which is more common than in pituitary masses of noninflammatory origin, are relevant and should be promptly recognized and treated in order to prevent morbidity and mortality. Diabetes insipidus and mass effects are additional issues in affected patients.

This article presents a synthetic update on the physiopathology, clinical presentation, diagnosis, and treatment of the different forms of hypophysitis, in order to provide a useful tool for any practitioner who may need to recognize and manage this condition in clinical practice.

Pathogenesis and Natural History of Hypophysitis

Hypophysitis can be schematically classified according to three essential criteria: (1) the anatomical site of the inflammatory process: adenohypophysitis, infundibulum/neurohypophysitis, panhypophysitis (both adeno- and neuro-hypophysitis), and

infundibulum/hypothalamitis; (2) its etiology: primary hypophysitis, an intrinsic pituitary process, mostly of autoimmune origin, and secondary hypophysitis, which may be reactive to adjacent lesions (i.e., Rathke's cleft cysts), a part of systemic inflammatory or infectious diseases, or side effect of immunomodulatory therapies; (3) histopathology: lymphocytic (LYH), granulomatous (GrH), xanthomatous (XH), mixed forms (lympho- or xantho-granulomatous—LGrH or XGrH), and IgG4-R lymphoplasmacytic form. In clinical practice, overlap between the different anatomical localizations and/or underlying pathological conditions may occur and acute, subacute or chronic forms may be observed. We will use a transitional approach to link the pathogenic mechanisms to the clinical presentation and histological characterization of this heterogeneous condition.

Primary Hypophysitis

Primary hypophysitis is considered as a rare clinical entity. The estimated annual incidence is 1/9 million inhabitants (Caturegli *et al.*, 2005), accounting for 0.24%–0.88% of pituitary diseases and $<1\%$ of operated pituitary masses (Lee *et al.*, 2017). However, the real epidemiology remains uncertain due to the heterogeneity of criteria used for its definition and data collection.

Lymphocytic hypophysitis (LYH) is by far the most common form and is considered as an autoimmune (AI) disorder based on a series of findings: pituitary infiltration by inflammatory cells (typically T lymphocytes, occasionally organized in follicles, in combination with B lymphocytes and macrophages) (**Fig. 1**), the progressive replacement of glandular tissue by fibrotic tissue, the reproducibility of the disease in animal models, the common association with other autoimmune diseases or immunomodulatory treatments, the presence of antipituitary autoantibodies (APA) in most cases, and the frequent resolution of symptoms by immunosuppressive treatments (Caturegli *et al.*, 2005). LYH may be isolated or associated with additional AI disorders, in particular thyroiditis, including polyendocrine AI syndromes (Caturegli *et al.*, 2005; De Bellis *et al.*, 2008). It typically affects young patients (mean age 35 ± 13 years in females, 45 ± 13 years in males), women more than males (F:M ratio up to 6:1), especially in adenohypophysitis, which often occurs in the late pregnancy or within 6 months postpartum (Caturegli *et al.*, 2005). The association of hypophysitis with pregnancy could be due to the expression of common autoantigens expressed by pituitary and placenta tissues (i.e., α-enolase, CSH1–2), increased release of pituitary antigens and/or increased accessibility of pituitary to immune cells due to estrogen-driven pituitary enlargement and changes in blood flow, or unmasking of preexisting silent condition by pregnancy. Noteworthy, contrasting with other AI diseases, LYH is not transmitted from the mother to the fetus and does not appear to impair pregnancy outcome, fetus health or subsequent fertility, nor predispose to disease recurrence in subsequent pregnancies (Landek-Salgado *et al.*, 2010). LYH has been more recently reported in children (Kalra *et al.*, 2011). Pediatric LYH has no gender predominance, DI and GH deficiency are the leading symptoms and the diagnosis is usually delayed. The pituitary mass typically shrinks in some years, whereas hypopituitarism commonly persists, requiring long-term replacement therapy (Kalra *et al.*, 2011).

Idiopathic granulomatous hypophysitis (GrH) is the second most common form of primary hypophysitis but remains very rare, and it is still debated whether isolated GrH is a distinct physiopathological entity or may represent a late stage of LYH. In a systematic review of 82 cases of GrH reported in the literature (Hunn *et al.*, 2014), a female predominance was observed (72%) and the mean age at diagnosis was around 45 years. Mass effects were frequent and might include cranial nerve palsies. Multiple anterior pituitary insufficiency was common, ACTH deficiency being the most frequent ($>70\%$), whereas DI was present in about 25% of the cases. Importantly, attention should be paid to recognize secondary forms of GrH, which are more frequent and may

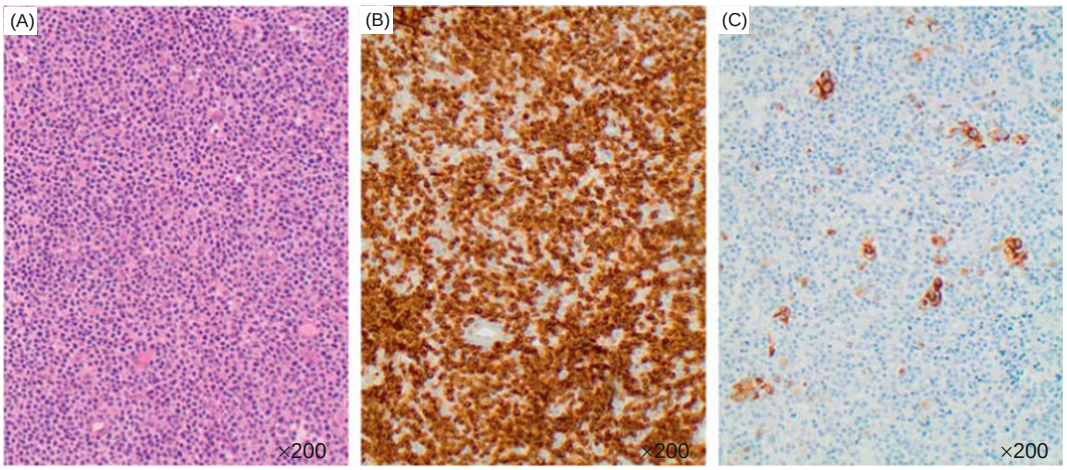

Fig. 1 Lymphocytic hypophysitis. The pituitary gland is intensively infiltrated by lymphocytes obscuring the normal architecture. (A) Hematoxylin-eosin; (B) Inflammatory cells are strongly expressing CD3 (T-cells); (C) Normal endocrine pituitary cells are visualized by anticytokeratin staining (Cam 5.2) By courtesy of Prof Jacqueline Trouillas, University of Lyon, France.

be associated to ruptured Rathke's cleft cyst (RCC), craniopharyngioma or microbial agents. Moreover, granulomatous involvement of pituitary gland may be part of a systemic, neoplastic or reactive granulomatous disorder (Carpinteri *et al.*, 2009).

Xanthomatous hypophysitis (XH) is the rarest histological type with less than 20 cases reported in the literature (Hanna *et al.*, 2015). Both XH and mixed XGrH are controversial as primary entities and probably represent spectrum of reactive changes related to other primary pituitary lesions, mainly RCC (Paulus *et al.*, 1999; Kleinschmidt-DeMasters *et al.*, 2017).

Finally, a pure necrotizing hypophysitis, characterized by an acute onset, has been reported, though it appears as an exceptional entity of unclear etiology (Gutenberg *et al.*, 2012). As such, its existence as a definite entity has been subsequently questioned.

IgG4-related hypophysitis (IgG4-RH) will be described with systemic diseases potentially involving pituitary gland.

Hypophysitis and Pituitary Gland Involvement In Systemic Diseases

The pituitary gland may be involved in a variety of rare inflammatory and/or neoplastic systemic diseases. As it may represent the first clinical manifestation, physicians should be aware of this eventuality in order to appropriately recognize and treat the systemic condition and endocrine deficiencies as appropriate.

Systemic granulomatous diseases such as sarcoidosis, granulomatosis with polyangiitis (Wegener's granulomatosis), Takayasu's disease and even Crohn's disease may rarely infiltrate the pituitary gland (Carpinteri *et al.*, 2009). Neurosarcoidosis with hypothalamic-pituitary involvement is the best characterized, with DI, hyperprolactinemia and hypogonadotropic hypogonadism being the most frequent symptoms, while hypothalamic manifestations are less common (Martin-Grace and Murialdo, 2015).

Langherhan's cell histiocytosis (LCH) and other histiocytic diseases. The classification of histiocytic disorders has been recently reviewed by the World Health Organization (WHO) (Pileri *et al.*, 2017). LCH, previously designated as histiocytosis X, is a clonal neoplastic proliferation of Langerhans-type cells that express CD1a, langerin and S100 protein, and shows Birbeck granules by ultrastructural examination (Weiss *et al.*, 2017). LCH may develop at any age but is more frequent in children. Most pediatric cases are diagnosed at the age of 1–5 years and adult cases in their 3rd–4th decade (Donadieu *et al.*, 2004a; Girschikofsky *et al.*, 2013). The severity of the disease depends on its localization and extension (bone, skin, central nervous system, lungs, liver, spleen, lymph nodes). Aggressive and potentially fatal disseminated forms are rare in adults. Hypothalamic-pituitary involvement is frequent (Donadieu *et al.*, 2004a; Girschikofsky *et al.*, 2013). DI is the most common endocrine symptom and may be the first manifestation of the disease, whereas hypopituitarism, which rarely occurs in the absence of DI, leads to progressive and permanent endocrine dysfunction. GH and gonadotroph deficiencies are the most frequent (40%–60%), and translate into impaired growth and delayed puberty in children (Donadieu *et al.*, 2004a). Hyperprolactinemia occurs mostly in adults. Hypothalamic symptoms may be present, including morbid obesity (Kaltsas *et al.*, 2000). Neurodegenerative radiological lesions are being increasingly recognized and may lead to neuropsychological disabilities (Fahrner *et al.*, 2012). Radiologically, an isolated thickening of the stalk or sellar/suprasellar masses can be found (Fahrner *et al.*, 2012; Girschikofsky *et al.*, 2013). Diagnostic criteria should possibly include at least a pathological proof of LCH (Girschikofsky *et al.*, 2013). Both Erdheim–Chester disease, a systemic, clonal proliferation of histiocytes and disseminated juvenile xanthogranuloma, a rare proliferation of xanthomatous histiocytes can affect the pituitary gland as isolated localization of the diseases or as a result of their dissemination to the central nervous system (CNS) (Brousse *et al.*, 2017a,b). Rosai–Dorfman disease, another histiocytic disorder, usually localizes to the skin, but may present as an intra/suprasellar lesion composed of a mixed inflammatory infiltrates composed of histiocytes, lymphocytes, and plasma cells (Paulus *et al.*, 2016). The differential diagnosis between primary (xantho)granulomatous hypophysitis and pituitary localization of systemic, frequently neoplastic, histiocytic disorders may be challenging and requires a multidisciplinary approach to avoid serious and potentially fatal consequences if these latter remain untreated, especially in the context of a CNS dissemination (Ferguson *et al.*, 2015).

IgG4-R hypophysitis was more recently characterized and appears as a rare disease characterized by fibrosis and lymphoplasmacytic proliferation including IgG4-reactive cells. Following its first description in van der Vliet and Perenboom (2004) and the first histological proof of this entity (Wong *et al.*, 2007), less than a hundred of cases were reported until 2015, many observations coming from Japan (Shikuma *et al.*, 2017). The typical localizations of the disease are retroperitoneum, pancreas, salivary glands, lymph node, lung, while isolated hypophysitis may not represent more than 10% of the cases. Men are more frequently affected, with a mean age at diagnosis around 67 and 56 years-old in men and in women, respectively (Shikuma *et al.*, 2017). Diagnostic criteria avoiding the need for a histological proof of the pituitary disease if sufficient additional elements are present have been proposed (Leporati *et al.*, 2011).

Cancer Immunotherapy and Hypophysitis

Immune checkpoints inhibitors aim to release constraints on immune cells and promote their antitumor activity. Not unexpectedly, immune-related adverse events (irAEs) often occur as side-effects and frequently involve endocrine glands (Bertrand *et al.*, 2015). Pituitary dysfunction is being increasingly recognized in cancer patients treated with a variety of monoclonal antibodies (mAbs) used as targeted therapies (Torino *et al.*, 2013). Hypophysitis is the most significant endocrine irAE induced by mAbs raised against CTLA-4 and may also be induced by anti-PD1 mAb (Faje, 2016; Boutros *et al.*, 2016; Joshi *et al.*, 2016). Ipilimumab (Ipi) and tremelimumab target CTLA4 whereas pembrolizumab and nivolumab target PD1. As Ipi and pembrolizumab/nivolumab were approved by FDA and EMA for the treatment of metastatic melanoma in 2011 and 2014/2015,

respectively, and their indications are extending to the treatment of additional malignancies, the number of patients treated with these molecules has dramatically increased in the last years. The pathogenesis and peculiarities of hypophysitis arising in this setting are being increasingly understood.

CTLA-4 is expressed on activated T-cells and negatively regulates their proliferation and activation. CTLA-4 is also expressed on endocrine pituitary cells and anti-CTLA-4 induced hypophysitis was reproduced in an experimental model whereas anti-TSH antibodies, followed in order by anti-FSH and anti-ACTH antibodies were also observed in humans and strongly associated with the subsequent development of hypophysitis (Iwama *et al.*, 2014). Yet, a single human pathological report of Ipi-induced hypophysitis is available as a postmortem observation showing evidence of type II and IV hypersensibility (Caturegli *et al.*, 2016). Faje (2016) recently summarized the results of three large longitudinal series of melanoma patients receiving Ipi (Faje *et al.*, 2014; Albarel *et al.*, 2015; Min *et al.*, 2015). Hypophysitis was recorded in 11.0%–17.2% of the cases, which is much higher than reported in oncological trials (0%–6.5%) (Barroso-Sousa *et al.*, 2018). Ipi-induced hypophysitis generally develops after 2–3 months of treatment, though delayed presentation may occur, with headache and hypopituitarism as leading symptoms, uncommon visual impairment, and exceptional DI (Faje, 2016). The dose effect (10 mg/kg vs. 3 mg/kg) remains uncertain. Overall, TSH, ACTH, FSH/LH, and GH deficits were reported in 96.3%, 85.7, 74.1%, and 28.6% of the patients, respectively (data on GH deficiency were partial but poorly relevant in this context), and hypoprolactinemia was more frequent than hyperprolactinemia (Faje, 2016). Of note, moderate pituitary enlargement was found to precede pituitary dysfunction and improve over time, explaining the absence of surgical indication and pathological reports. In addition, contrasting with frequent recovery of thyrotroph and gonadotroph functions, ACTH deficiency commonly persisted ($>80\%$) (Faje, 2016).

PD1 is expressed by activated T and B cells and its ligands PDL1 and PDL2 by antigen presenting cells (APC) and some tumor cells. Clinical experience with anti-PD1 is more recent but irAEs appear to be less frequent than reported with Ipi (Boutros *et al.*, 2016). This may reflect the differential role of CTLA4 and PD1, with a more restricted population of T cells affected by PD1 blockade, and is in agreement with the milder autoimmune phenotype of PD1–deficient mice as compared to CTLA-4-deficient mice (Boutros *et al.*, 2016). In a meta-analysis of nine randomized studies, the pooled risk of hypophysitis was estimated to 0.47% (Costa *et al.*, 2017). However, as delayed and even isolated ACTH deficiency is being reported on long-term treatment with nivolumab (Okano *et al.*, 2016; Kuru *et al.*, 2017; Takaya *et al.*, 2017), longitudinal follow-up studies are needed to better evaluate the prevalence of pituitary dysfunction and prevent acute adrenal failure.

Hypophysitis and Tumors of the Sellar Region

Hypophysitis may occur secondarily to a pathological process of nearby structures. Inflammation is frequently associated with RCC, which leakage, rupture or bleeding may promote xanthomatous or xanthogranulomatous reaction (Paulus *et al.*, 1999; Kleinschmidt-DeMasters *et al.*, 2017; Lee *et al.*, 2017). LH, XH or XGrH may also be observed in the proximity of a craniopharyngioma (Paulus *et al.*, 1999; Kleinschmidt-DeMasters *et al.*, 2017; Lee *et al.*, 2017). Germ cell tumors (GCT) are rare intracranial tumors mainly arising in the pineal and sellar regions (Duron *et al.*, 2018). Because of their rich lymphocytic infiltration, GCTs may be misdiagnosed as primary hypophysitis (Bettendorf *et al.*, 1999; Endo *et al.*, 2007; Ozbey *et al.*, 2006). Rarely, pituitary localization of lymphoma may present with a concomitant hypophysitis (Huang *et al.*, 2005). Slight lymphocytic infiltration or cholesterol clefts may occasionally be seen in pituitary adenomas (PA) (Lupi *et al.*, 2010; Yokoyama *et al.*, 1998), which should be distinguished from the true coexistence of primary hypophysitis and PA (Ballian *et al.*, 2007; Saeger *et al.*, 2007). Exceptionally, hypophysitis may coexist with meningioma, as in a case of a young pregnant woman with previously unrecognized sellar meningioma who developed lymphocytic hypophysitis. The meningioma, in combination with physiological lactotroph hyperplasia and additional pituitary enlargement due to the inflammatory changes by the end of pregnancy led to the critical increase of a sellar mass, requiring transsphenoidal debulking and surgical termination of pregnancy. (A consultation case, kindly provided by Dr. Pia Burman, University of Lund, Sweden) (**Fig. 2**).

Hypophysitis In Infective Conditions

Hypophysitis related to infectious agents still represents an important cause of transient or permanent hypopituitarism in tropical and developing countries. An increasing incidence can be expected worldwide due to migrations and a rising prevalence of predisposing conditions such as diabetes mellitus, immunosuppressive therapies and other immunocompromised conditions, including HIV infections (Harbeck *et al.*, 2011; Spinner *et al.*, 2013). Secondary abscesses in pituitary lesions such as RCC or PA, or following pituitary surgery, have also been reported (Awad *et al.*, 2014; Wang *et al.*, 2014; Yang *et al.*, 2017). Infectious agents can reach the sellar region via blood or by direct spreading from neighboring structures (brain, paranasal sinuses, cavernous sinus, and meninges). The most important forms of chronic bacterial infections of pituitary gland are granulomas associated with tuberculosis (Sharma *et al.*, 2000; Dhanwal *et al.*, 2010), less commonly syphilis (Bricaire *et al.*, 2015), which may also cause acute hypophysitis (Spinner *et al.*, 2013). Pituitary abscesses due to Gram-positive or Gram-negative bacteria are potentially life-threatening conditions which may affect patients at any age (Vates *et al.*, 2001; Altas *et al.*, 2013; Karagiannis *et al.*, 2016; Gao *et al.*, 2017). Infections by Hantaviruses are zoonoses spread by rodents causing hemorrhagic fever with renal syndrome, especially in Asia and America. The Puumala hantavirus (PUUV)—endemic in Nordic countries and other parts of Europe—causes a less severe systemic disease but may directly target the pituitary determining secondary hypophysitis and/or ischemia/necrosis

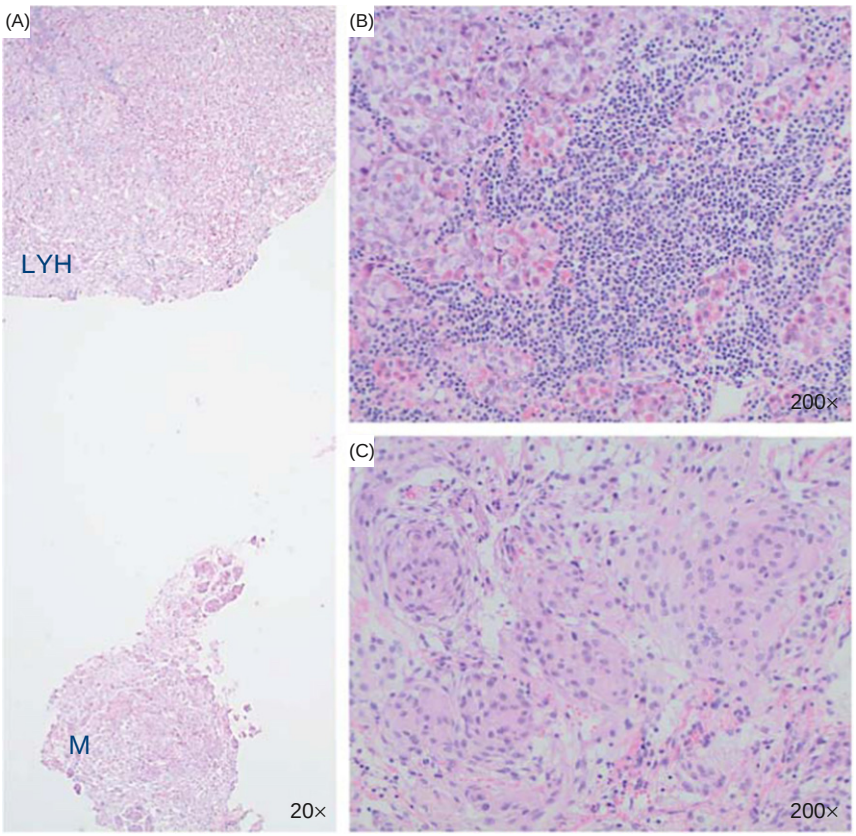

Fig. 2 Coexistence of a pregnancy-related lymphocytic hypophysitis (LYH) and a meningioma (M) (hematoxylin-eosin), (A) Both LYH and M are present in the same surgical specimen (provided by courtesy of Dr Pia Burman, see text), (B) LYH: the pituitary gland is infiltrated by lymphocytes; (C) The adjacent meningioma has a typical appearance and is devoided of lymphocytic infiltration.

leading to hypopituitarism (Hautala *et al.*, 2010; Partanen *et al.*, 2016; Pekic and Popovic, 2017). Aspergillus, Nocardia, Candida may occasionally cause fungal abscesses (Beatrice *et al.*, 2013; Hao *et al.*, 2008, Pekic and Popovic, 2017). Hypophysitis related to *Toxoplasma gondii* infection was recently reported in immunocompromised patients (Hamdeh *et al.*, 2015).

Headache is the most common symptom of pituitary infection (70%–90% in most series), whereas the prevalence of a suggestive subacute onset with fever and meningism may not exceed 25% (Karagiannis *et al.*, 2016). Rather, long-standing pseudo-tumoral symptoms may be present, implying that pituitary abscesses should be considered in the differential diagnosis of a pituitary mass, in particular if cystic. Endocrine dysfunction is frequent, due to variable degrees of destruction of the anterior pituitary (30%–85%), with a classical progressive hypopituitarism starting from GH and FSH/LH insufficiency, and DI in about half of the cases (Vates *et al.*, 2001; Karagiannis *et al.*, 2016; Gao *et al.*, 2017). Cavernous sinus thrombophlebitis may complicate the clinical outcome (Karagiannis *et al.*, 2016).

Diagnosis

Clinical Presentation of Hypophysitis

The clinical manifestations of hypophysitis are heterogeneous and nonpathognomonic, ranging from asymptomatic to severe clinical pictures, depending on the stage and evolution of the disease, its anatomic localization and extension. In its acute phase, adenohypophysitis may be revealed by mass effects due to rapid pituitary enlargement by the inflammatory process and associated edema, such as headache, visual disturbances (chiasmatic syndrome), and more rarely, nausea, vomiting, or diplopia secondary to III, IV, or VI cranial nerve palsies (cavernous sinus involvement), as well as hypopituitarism. Isolated pituitary deficiency may be present and limited to ACTH insufficiency, potentially leading to adrenal crisis. Variable degrees of menstrual abnormalities and hypogonadism are also frequent and may reflect primary impairment of gonadotroph cells or functional inhibition by hyperprolactinemia of unequivocal origin—impaired dopamine delivery to the anterior pituitary (pituitary stalk compression or infiltration), abnormal PRL release into the circulation due to massive cellular destruction, or PRL secretion by infiltrating immune cells (Caturegli *et al.*, 2005; Guaraldi *et al.*, 2017). Inability to lactate can also occur due to hypoprolactinemia in the context of hypopituitarism, mimicking Sheehan syndrome. DI typically occurs in the presence of neuro-infundibular localization of the

inflammatory process, though it may occur in adenohypophysitis, likely through an inhibition of the axonal transport of ADH due to swelling of the *pars tuberalis* (Caturegli *et al.*, 2005). Interestingly, the severity and rapidity of onset of hypopituitarism are poorly correlated with the degree of pituitary enlargement, suggesting the predominant role of immunomediated cell destruction over pituitary compression (Caturegli *et al.*, 2005). In the chronic phase of LYH, replacement of the functional gland by fibrosis and progressive atrophy lead to irreversible hypopituitarism, typically starting from ACTH, followed by FSH/LH, GH, TSH, and PRL secretion. Central DI may also persist as a permanent sequela. Of note, LYH may have a polyphasic course with remissions and relapses (Caturegli *et al.*, 2005). Rarely, hypophysitis manifest with mood disorders, alterations of hypothalamic functions (i.e., thermo-, weight and sleep regulation), epilepsy, or is asymptomatic and incidentally discovered (Guaraldi *et al.*, 2017).

Clinical Evaluation of a Patient With Suspect Hypophysitis

In clinical practice, hypophysitis often presents as a nonsecreting pituitary lesion revealed by mass effects, in particular headache, DI and/or various degrees of hypopituitarism. In the eventuality of surgery, the diagnosis relies on pathological examination, which remains the gold standard. In the absence of a pathological proof of the disease, the diagnosis may be challenging due to its heterogeneous, nonpathognomonic presentation and variations from acute to chronic forms (Caturegli *et al.*, 2005; Carpinteri *et al.*, 2009; Guaraldi *et al.*, 2017). The presence of predisposing factors has to be considered first. Then, a combination of clinical, radiological, and laboratory findings may be put together in order to reasonably approach the diagnosis. In the absence of a suggestive setting or in front of an atypical clinical presentation, the differential diagnosis with other nonsecreting pituitary masses requires specific multidisciplinary expertise and a surgical biopsy is generally indicated. Age and sex are not uniformly distributed among patients with hypophysitis and the clinical context may be highly suggestive, provided that differential diagnosis is appropriately thought. For example, primary LYH should be first considered in young women, especially in relation with pregnancy and/or in a context of autoimmunity, and in recent series the indications for diagnostic surgery have decreased (Honegger *et al.*, 2015; Kyriacou *et al.*, 2017; Wang *et al.*, 2017). Systemic LCH has a typically young onset whereas IgG4-R diseases generally occurs in elderly patients, and in both conditions additional localizations may be present and accessible for a diagnostic biopsy. The presence of fever, recent migration from tropical countries, immunosuppression may be suggestive of an infectious origin, which should be thought before any glucocorticoid trial is planned, since pituitary infections are potentially life-threatening conditions. Immunotherapy in cancer patients may induce transient hypophysitis as an irAE, which should not be confounded with pituitary metastasis, which may also induce DI, hypopituitarism and mass effects including cranial nerve palsies (Gilard *et al.*, 2016). Hypophysitis secondary to neighboring lesions should be considered, with special attention to GCTs masquerading as hypophysitis in children and young adults (Bettendorf *et al.*, 1999; Endo *et al.*, 2007; Ozbey *et al.*, 2006). Importantly, the spectrum of endocrine deficiencies varies according to the etiology and the typical order of progressive hypopituitarism in noninflammatory sellar masses such as PA (GH, followed by FSH/LH, and ACTH/TSH deficiencies) may be inverted and impaired ACTH secretion may be the earliest to occur, as in LYH or immunotherapy-related hypophysitis. Despite a variable prevalence according to the etiology of hypophysitis, DI is an important clinical argument against a PA.

Radiological Evaluation

Magnetic resonance imaging (*MRI*) is the gold standard method for the evaluation of sellar masses. Although no single radiological feature is specific of hypophysitis, a combination of criteria can be highly suggestive (Gutenberg *et al.*, 2009). These include symmetric and homogeneous enlargement of the pituitary gland; intense and homogeneous gadolinium enhancement, thickening of the pituitary stalk, disappearance of the posterior pituitary bright spot in T1 (usually accompanying DI), integrity of the sellar floor and, occasionally, hypothalamic infiltration. A radiological score, including patient gender, age and relation to pregnancy has been proposed to facilitate differential diagnosis between primary LYH and a PA, which is by far the most common pituitary mass (Gutenberg *et al.*, 2009). Similar aspects, including isolated pituitary stalk thickening, can also be found in GrH (Hunn *et al.*, 2014) and in secondary hypophysitis. Of note, intrasellar germinomas may be indistinguishable from a primary hypophysitis and attention should be paid to a potential pineal localization, since bifocal GCTs are not infrequent (Duron *et al.*, 2018). Peculiar MRI findings may be present in hypophysitis secondary to a RCC or a craniopharyngioma, and a thick peripheral enhancement surrounding a cystic lesion may be suggestive of a pituitary abscess (Gutenberg *et al.*, 2009; Guaraldi *et al.*, 2017). Subsequently, regression of the inflammatory process may be followed-up by MRI and secondary empty sella may be the late stage in hypophysitis of different etiologies (Gutenberg *et al.*, 2009; Guaraldi *et al.*, 2017). Whole brain MRI may reveal additional abnormalities in systemic diseases, especially in histiocytic disorders (Fahrner *et al.*, 2012), whereas hypothalamic-pituitary involvement may not be radiologically evident in neurosarcoidosis (Martin-Grace and Murialdo, 2015).

Additional imaging may include PET-^{18}FDG, since inflammatory lesions are typically associated with increased FDG uptake due to their high metabolic activity (Sher *et al.*, 2017; Wachsmann *et al.*, 2017). Combined PET-TC may be very useful in systemic diseases as it may help identifying additional lesions, some of them being more easily accessible to obtain a pathological diagnosis (Martin-Grace and Murialdo, 2015). Whole body PET-TC is also useful during follow-up and PET-MRI was recently proposed to minimize radiation in pediatric cases (Sher *et al.*, 2017). Of note, it is essential in cancer patients to distinguish between systemic localizations of irAE and metastatic lesions, as both may induce abnormal ^{18}FDG uptake (Wachsmann *et al.*, 2017).

Laboratory Evaluation

Diagnostic of primary hypophysitis

The identification of antipituitary autoantibodies (APA) contributes, but is not sufficient per se to diagnose AI hypophysitis, because of some important methodological and theoretical issues (Caturegli *et al.*, 2005, 2008; Ricciuti *et al.*, 2014). The role of APA in the pathogenesis of AI LYH and their clinical significance remains uncertain, since they have been identified in several conditions and no correlations have been detected between APA and disease severity or prognosis (Caturegli *et al.*, 2008; Guaraldi *et al.*, 2017). However, they may represent a useful tool in the presence of a suggestive clinical context. Indirect immunofluorescence (IIF) is currently considered the gold standard technique for APA evaluation (**Fig. 3**). Despite the low sensitivity and the subjective interpretation of results with respect to other immunological methods, IIF is characterized by a relatively low cost, rapidity and simplicity of application, and is useful when the target antigen is unknown, as in LYH. Indeed, although several antigenic pituitary targets have been postulated, including hormones (i.e., GH, chorionic somatomammotropin hormone 1 and 2, CSH 1–2; proopiomelanocortin, POMC), enzymes (i.e., α-enolase), enzymes (i.e., prohormone convertase PC 1/3 and 2; carboxypeptidase PC2 regulatory protein, 7B2), pituitary factors (i.e., PGSF 1a and 2), transcription factors (i.e., Tpit), cytosolic and nuclear proteins (i.e., rabphilin-3A, secretogranin II, chromosome 14 open reading frame 166, C14orf166; Tudor Domain Containing Protein 6, TDRD6), none of these appeared pathognomonic for the identification of primary hypophysitis, and, when injected in animal models, they were not able to induce the disease (Guaraldi *et al.*, 2017). An anti-Pit1 syndrome with combined GH, TSH, and PRL insufficiency (Yamamoto *et al.*, 2011) and antihypothalamic antibodies directed against AVP-secreting cells (De Bellis *et al.*, 2012) have also been identified. The reliability of pituitary IIF is limited by a high tissue background due to natural pituitary autofluorescence and nonspecific antigen-antibody binding, and there is no consensus yet regarding appropriate pituitary controls, tissue processing, and the interpretation of APA (Caturegli *et al.*, 2008; Ricciuti *et al.*, 2014). Such limits should be considered, and the determination of cut-off titers and attention to peculiar type of immunostaining may help improving the specificity of the results (Bellastella *et al.*, 2016; Guaraldi *et al.*, 2017).

An additional attractive tool is HLA haplotyping. Although the most frequently reported alleles were initially HLA-DR4 and DR5, HLA-DQ8 has been recently identified as the most reliable marker of AI LYH, being present in 87% versus 20% of the patients with AI versus non AI pituitary diseases; HLA-DR53 is also frequent but less specific than DQ8 (Heaney *et al.*, 2015). If these data are confirmed, HLA haplotyping could help differentiating AI LYH from other pituitary lesions.

Diagnosis of secondary hypophysitis

Systemic diseases can sometimes be suspected on the basis of circulating markers, though these may have an insufficient sensibility and specificity. Histologically proven IgG4-R lesions have been reported in the presence of normal circulating IgG4 and in a recent meta-analysis, the sensitivity and specificity of serum IgG4 measurement for the diagnosis of IgG4-R

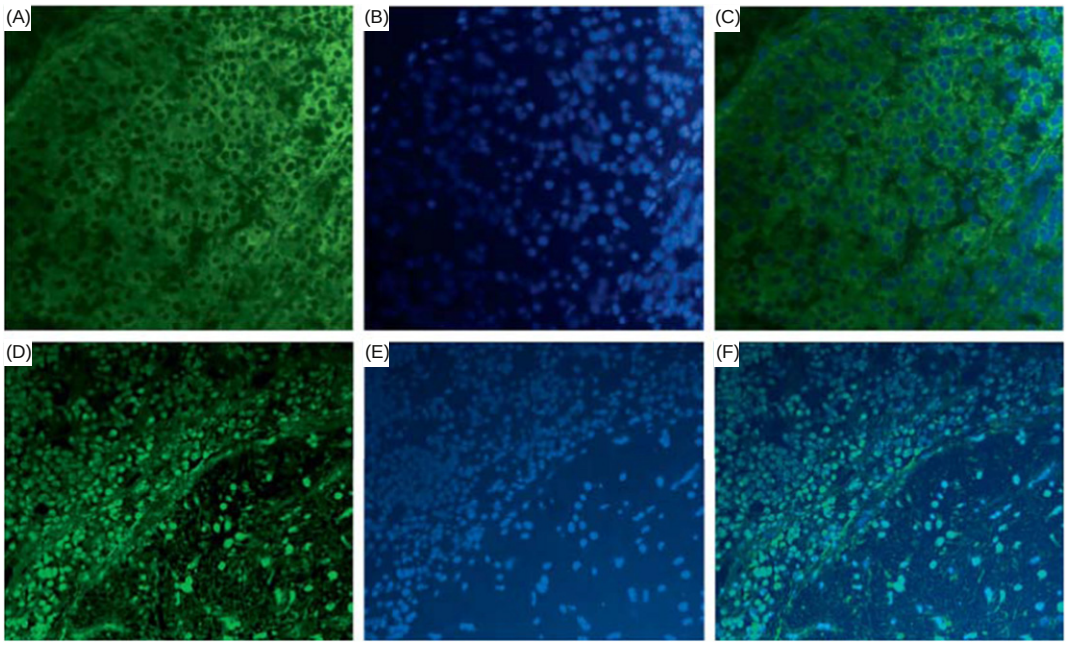

Fig. 3 Detection of antipituitary antibodies (APA) by indirect immunofluorescence (IIF), 20 × magnification. Double staining is based on the use of FITC-conjugated secondary antibodies (*green*, A and D), and DAPI counterstaining of cell nuclei (*blue*, B and E), with merged imaging (C and F). APA may have a cytosolic (A and C) or nuclear (D and F) pattern. On the lower panel (D—F), the anterior (top left) and posterior (bottom right) pituitary portions can be distinguished morphologically by cell distribution.

conditions were 87.2% and 82.6%, respectively (Hao *et al.*, 2016). Since they are rapidly normalized by glucocorticoid therapy, an early determination of IgG4 should be obtained. Elevated circulating ACE levels are found in a minority of patients with neurosarcoidosis and CSF analysis may be more sensitive, but not specific enough to make the diagnosis (Martin-Grace and Murialdo, 2015). Beta-human chorionic gonadotrophin (βHCG) and alpha-fetoprotein (AFP) are classical markers of germ cell tumors (GCTs). However, the majority of GCTs localized in the central nervous system are not accompanied by elevated serum values and CSF analysis should be performed in suspect cases (Endo *et al.*, 2007). A diagnostic biopsy may reveal the tumor even in the presence of normal CSF values (Ozbey *et al.*, 2006). Elevated white blood cell count may be present in patients with a pituitary abscess and a septic presentation, haemocultures and/or CSF biochemical and bacteriological analysis can be helpful, however the causal infective agent is difficult to identify and cultures of the pus removed from the cavity are often negative (Altas *et al.*, 2013; Vates *et al.*, 2001; Zhang *et al.*, 2012). Specialized infective work-up is advisable, especially in the case of atypical infections or granulomas of specific origin.

Pathological Diagnosis

Histopathological examination of surgical pituitary samples remains the gold standard in the diagnosis of hypophysitis, though it is not always available. Traditionally, the three main histological patterns of hypophysitis are lymphocytic, granulomatous, and xanthomatous (Sautner *et al.*, 1995; Caturegli *et al.*, 2005; Carpinteri *et al.*, 2009; Hanna *et al.*, 2015). Both primary and secondary hypophysitis can manifest as one of these histological patterns. Combinations, such as mixed Gr-LYH (Madsen and Karluk, 2000) and X-LYH (Gopal-Kothandapani *et al.*, 2015) have been reported. IgG4 hypophysitis has also been histologically characterized (Leporati *et al.*, 2011). In daily practice, the small size and fragmentation of pituitary surgical specimens may hamper the identification of the whole spectrum of inflammatory changes and/or a potential causative disease. Immunohistochemistry (IHC) may provide important additional clues. Finally, as similar aspects may be encountered in primary and secondary hypophysitis, multidisciplinary work-up may be essential to achieve a final etiological diagnosis.

Microscopic examination

In LYH the pituitary gland is richly infiltrated by polyclonal reactive lymphocytes and plasma cells surrounding the adenohypophysial cells (Beressi *et al.*, 1999) (**Fig. 1**). Occasionally, lymphoid follicles with germinal cents may form (Caturegli *et al.*, 2005). Fibrosis becomes a dominant feature in the later phase of LYH, making the histological diagnosis difficult. GrH is characterized by the presence of granulomas composed of epitheloid histiocytes and macrophages, including multinuclear giant cells, usually surrounded by a mixture of lymphocytes and plasma cells. Primary idiopathic GrH is very rare (Hunn *et al.*, 2014). Thus, secondary granulomatous process due to microbial agents or as a part of systemic granulomatous disorders should be considered (Carpinteri *et al.*, 2009), and specific staining for tuberculosis or fungal infections may be performed (Al-Haddad *et al.*, 2011). In XH, the rarest histological type, pituitary gland is infiltrated by foamy macrophages, also known as xanthomatous cells, with an admixture of T- and B-lymphocytes and plasma cells. Occasionally, XGrH has been reported, with a characteristic combination of epitheloid giant cell granulomas, xanthomatous cells surrounding cholesterol clefts and lymphoplasmacytic infiltration (Tashiro *et al.*, 2002; Burt *et al.*, 2003; Yokoyama *et al.*, 2004; Gopal-Kothandapani *et al.*, 2015). Causative primary pituitary lesions, such as ruptured RCC, may be recognized in XH and XGrH (Paulus *et al.*, 1999) (**Fig. 4**). Differential diagnosis between (X)GrH and a pituitary manifestation of systemic histiocytic disorders may require multidisciplinary expertise.

The histopathological characteristics of IgG4-RH were described in Wong *et al.* (2007) and an example is shown in (**Fig. 5**). Lymphoplasmacytic infiltration with a high proportion of IgG4-reactive plasma cells, storiform fibrosis and occasional obliterative phlebitis are common to all IgG4-R lesions, although organ-specific variations may occur (Deshpande *et al.*, 2012; Kamisawa *et al.*, 2015). Among the criteria reported by Leporati *et al.* for the diagnosis of IgG4-RH, criterion 1 consists of a histological proof of the disease and is therefore sufficient for the diagnosis. It is defined by fibrosis and lymphoplasmacytic infiltration with at least 10

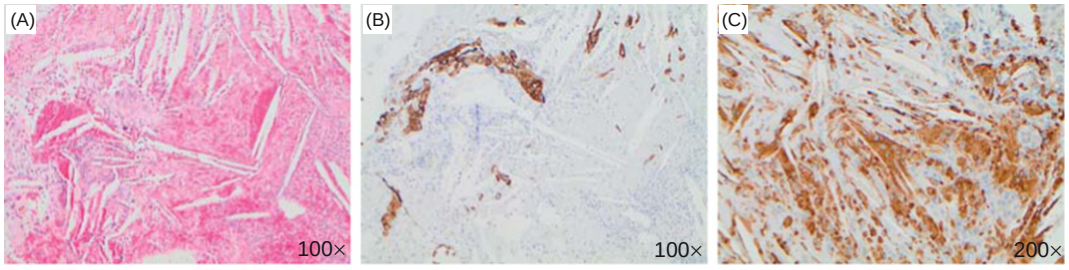

Fig. 4 Secondary xanthogranulomatous hypophysitis. Foamy histiocytes and multinuclear giant cells surround cholesterol crystals and bleeding resulting from a ruptured cyst. (A) Hematoxylin-eosin; (B) fragments of the epithelial cyst wall are identified by cytokeratin immunostaining (Cam5.2); (C) CD68 immunostaining is present in the foamy histiocytes and multinuclear giant cells.

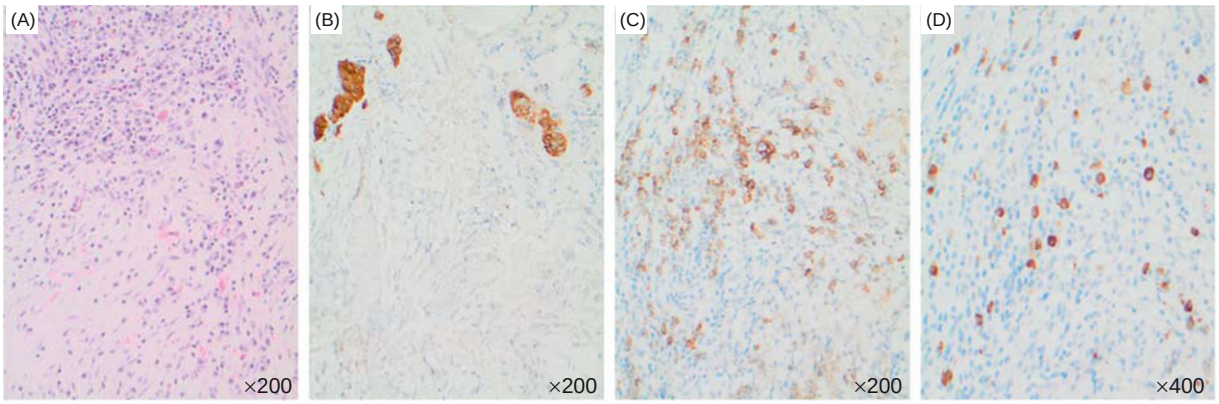

Fig. 5 IgG4-related hypophysitis. An abundant fibrosis is present with a mixed infiltration of lymphocytes, plasma cells, and eosinophils. (A) Hematoxylin-eosin; (B) Residual endocrine pituitary cells are identified by chromogranin A immunostaining; (C) A predominance of plasma cells among inflammatory cells is shown by CD138 immunostaining; (D) IgG4 immunostaining reveals IgG4 reactive cells.

IgG4 cells/HPF (high power field) (Leporati *et al.*, 2011). An IgG4 + /IgG + cells ratio above 40% is an additional criterion in other organs (Deshpande *et al.*, 2012). Of note, IgG4-positive cells have been observed in some localizations of granulomatosis with polyangiitis (GPA) (Wegener's), although its relationship to IgG4-RD is uncertain (Chang *et al.*, 2013; Bando *et al.*, 2015).

What can we expect from immunohistochemistry?

IHC is useful in the differential diagnosis of inflammatory pituitary lesions, especially in the presence of atypical pathological features and/or limited biopsy material. It may also help identifying normal pituitary endocrine cells in specimens with prominent inflammatory changes and/or fibrosis (**Figs.1**C and **5**B). In AI-LYH, inflammatory cell infiltrates are dominated by CD3 + T-cells (**Fig. 1**B), including CD8 + cytotoxic and CD4 + helper T-cells, whereas CD20 + B-cells, CD138 + plasma cells and CD68 + macrophages are less common. In GrH and XGrH, reactive epitheloid histiocytes and multinuclear giant cells are CD68 + (**Fig. 4**C), but do not stain for CD1a and Langerin, which are markers of LCH-type neoplastic histiocytes. More than 50% of LCH (Badalian-Very *et al.*, 2010; Sahm *et al.*, 2012) and Erdheim–Chester cases (Haroche *et al.* 2012) are related to a BRAF V600E mutation and immunopositive with a BRAF V600E monoclonal antibody. Since for unclear reasons normal corticotrophs and corticotroph adenomas may also show strong BRAF V600E immunolabeling in the absence of mutation (Mordes *et al.*, 2014), a BRAF V600E mutation gene analysis is advisable where a systemic histiocytic disorder is suspected. Another challenging issue may be the identification of GCTs, since the lymphocytic reaction may be largely predominant with a limited number of tumor germ cells. These latter can be identified by IHC panel of markers including PLAP, OCT3/4, CD117 (c-kit) for pure germinomas, and AFP, βHCG, cytokeratin AE1/AE3, and CD30 for other types of GCTs (Gao *et al.*, 2014). In IgG4-RH, IHC is used to identify and confirm the polyclonality of CD138 plasma cells and to quantify the population of IgG4 + cells (**Fig. 5**C and D).

Treatment

The wide clinical spectrum of hypophysitis in terms of etiology and symptoms accounts for the diversity of their management, which may range from follow-up to active medical or neurosurgical treatment—typically by a transsphenoidal (TS) route-, exceptionally radiotherapy. Due to its relative rarity, some guidelines are available as panel expert consensus rather than evidence-based. Indeed, no randomized trials are available to compare the outcome of surgical versus medical management where active treatment is thought necessary and the optimal treatment of primary hypophysitis is still a matter of debate. Improved knowledge of some secondary forms of hypophysitis has contributed to refine their diagnosis and management. Importantly, hypopituitarism should be actively treated in all cases, hormone replacement being mandatory in the presence of DI and/or secondary adrenal and thyroid deficiencies. Surgery may be considered for diagnostic or debulking purposes, upon multidisciplinary evaluation by a pituitary team. Microscopic or endoscopic TS is typically used where an intrasellar component of the lesion is present (Honegger *et al.*, 2015). More recently, extended TS and intraventricular endoscopic approaches (Jinguji *et al.*, 2013) and TS biopsy of the posterior lobe (Kinoshita *et al.*, 2017) have been proposed in alternative to transcranial surgery in the presence of lesions involving the stalk or the infundibular region.

Primary Hypophysitis

The decision to treat patients with a primary hypophysitis depends on the clinical symptoms and/or diagnostic challenges. In a nationwide retrospective survey conducted in Germany including 76 patients with primary hypophysitis (Honegger *et al.*, 2015), a wait-and-see option was elected in 40% of the cases, leading to spontaneous mass reduction in 46% and improvement of pituitary

dysfunction in 27% of the cases, whereas radiological progression and worsening of endocrine dysfunction were reported in 27% and 18%, respectively. The median time to disease progression was 1.2 years with wide variations. Active treatment included surgery and/or glucocorticoids (GC) and was generally elected in the most symptomatic cases, diagnostic surgery being performed in a minority of patients. Postoperative mass reduction was present in 68% of the cases, with early regrowth or later relapse leading to an overall recurrence rate of 25%. In contrast, GC induced a 97% rate of response, but recurrences occurred in 38% of the cases and side-effects in 63%. Despite a wide variability in the follow-up duration, this study provides relevant information on the natural course of primary hypophysitis and the potential limits of active treatment, supporting conservative management unless severe or progressive symptoms develop (Honegger *et al.*, 2015). The wait-and-see option was less frequently adopted in other recent studies, which tended to favor a medical versus surgical approach, since GC are effective on headache and may improve pituitary function (Kyriacou *et al.*, 2017; Wang *et al.*, 2017). However, there is no consensus on the optimal GC schedule in primary hypophysitis and a variety of protocols have been reported, ranging from high dose pulse therapy (up to 500 mg prednisone equivalent daily) (Kristof *et al.*, 1999; Honegger *et al.*, 2015) to continuous oral therapy (starting from 20 mg prednisone equivalent daily) (Honegger *et al.*, 2015; Wang *et al.*, 2017), with wide variations in the duration of treatment (from a few days to several weeks or months). In a former review of the literature, oral and i.v. GC were associated with pituitary mass reduction in 87% and 75% of the cases, respectively (Lupi *et al.*, 2011). Progressive tapering of the dose is always recommended. A slow tapering rate and a longer duration of treatment (>6 months) were recently associated with a lower rate of recurrences (Wang *et al.*, 2017). This should clearly be balanced with the long-term side effects of GC. Resistance to GC may occur in a minority of patients and surgery can be proposed as a second choice (Honegger *et al.*, 2015). Radiotherapy or other immunosuppressive therapies (mainly azathioprine) have been used in a minority of cases (Curtò *et al.*, 2010; Lupi *et al.*, 2011; Honegger *et al.*, 2015). An important long-term issue is potential endocrine sequelae. In the German survey, GC were associated with an equal rate of improvement or worsening of pituitary insufficiency (15% each), although worsening could accompany relapse (Honegger *et al.*, 2015). These results compare unfavorably with a previous review reporting an overall improvement of hypopituitarism and DI in 45% and 41% of medically treated cases, respectively (Lupi *et al.*, 2011), so this may reflect the treatment of the most symptomatic cases. Worsening of pituitary function appears to be more frequent after surgery (Honegger *et al.*, 2015).

Secondary Forms of Hypophysitis

The therapeutic options depend on the etiology of the disease. Systemic therapy, including chemotherapy, may be considered in LCH patients (Donadieu *et al.*, 2004a; Girschikofsky *et al.*, 2013), and if regression of the hypothalamic-pituitary lesions is seen in a significant proportion of patients, endocrine sequelea are the rule (Fahrner *et al.*, 2012). The first line treatment for IgG4-R conditions is GC, typically oral GC with a starting dose depending on disease severity; alternatively GC-sparing immunosuppressive drugs or rituximab, aiming at B-cell depletion, can be used (Kamisawa *et al.*, 2015). As extensive fibrosis is associated with poorer responses, early recognition of the disease is desirable (Kamisawa *et al.*, 2015). Rituximab was successfully used in recurring IgG4-RH (Gu *et al.*, 2017). Where a pituitary abscess is suspected, TS evacuation is highly recommended with antiseptic washing of the cavity and broad-spectrum antibiotherapy, keeping in mind that a transcranial approach, if strictly necessary, is endowed with a significant risk of cerebral spread of the infection (Zhang *et al.*, 2012; Gao *et al.*, 2017).

Hormone Replacement Therapy

Hormone replacement should be managed by an endocrinologist and is mandatory for DI and secondary adrenal and thyroid deficiencies, whereas gonadal replacement may be indicated according to the context. Recent clinical guidelines from the Endocrine Society are available for the diagnosis and treatment of adult hypopituitarism (Fleseriu *et al.*, 2016). As ACTH deficiency may occur first in hypophysitis, appropriate treatment with hydrocortisone should be started promptly and instructions for the prevention of adrenal crisis should be provided. Of note, treatment of adrenal insufficiency may unmask partial DI, whereas treatment of thyroid insufficiency may unmask adrenal insufficiency (Fleseriu *et al.*, 2016). Secondary adrenal insufficiency should also be thought when antiinflammatory corticoid therapy is withdrawn. An emergency card/bracelet/necklace is useful in patients with ACTH deficiency and DI for any situation in which they are unable to refer about their medical conditions. There is little experience with GH replacement therapy in patients with hypophysitis, though it may be considered where documented GH deficiency persists as an endocrine sequela. The diagnosis relies on diagnostic testing with age-related parameters and adjustment for BMI in adults (Corneli *et al.*, 2005, 2007; Fleseriu *et al.*, 2016; Grimberg *et al.*, 2016) and GH replacement may require adjustment of other replacement therapies (Fleseriu *et al.*, 2016). No adverse effect of GH replacement on the course of the disease was reported in pediatric LCH (Donadieu *et al.*, 2004b).

Conclusion

With the identification of new entities such as IgGR-4R diseases and the increasing use of immune checkpoint inhibitors, the epidemiology of hypophysitis has significantly changed over the past 15 years and this heterogeneous disease is gaining interest among the medical community, extending well beyond the specialized pituitary teams. The frequent clinical complexity of patients

affected by hypophysitis should encourage multidisciplinary collaboration to ensure appropriate diagnosis and treatment of this condition. The optimal treatment of endocrine deficiencies, including appropriate prevention of adrenal crisis and long-term management of endocrine sequelea, should be best managed by an endocrinologist, and endocrinologists should collaborate with internists/oncologists for the appropriate management of systemic/oncological diseases. Despite increasing knowledge of the different forms of hypophysitis has reduced the indications for diagnostic surgery, pathology remains the gold standard for the definitive diagnosis. Thus, evaluation by a multidisciplinary pituitary team should best define the need for a pathological specimen and evaluate the best surgical approach. In the future, new markers may improve the diagnostic of some entities, for example, the development of reliable panels of APA. Therapeutic neurosurgery may be indicated in the most symptomatic cases, such as visual fields defects, or if a peculiar etiological setting (e.g., a pituitary abscess or associated tumor) is suspected. Alternatively, GC may be proposed. In all cases, the diagnostic challenge and the choice of active treatment should be discussed with the patient.

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Radiology of the Pituitary

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Anatomy and Embryology

The pituitary gland sits within the sella turcica which is a cup shaped depression in the sphenoid bone. The sphenoid air sinus lies below and anterior to the sella turcica (**Fig. 1**). Lying above the pituitary gland is a CSF space, the suprasellar system, which contains the optic chiasm (**Fig. 1**A and B). The lateral walls of the pituitary fossa are formed by the cavernous sinuses (**Fig. 1**B) which contain the internal carotid arteries as well as the 3rd, 4th, and 6th cranial nerves as well as the 1st and 2nd divisions of the 5th cranial nerve. The pituitary gland is connected via the pituitary stalk to the hypothalamus which is a thin plate of tissue making up the floor of the anterior part of the 3rd ventricle (**Fig. 1**A and C).

The appearance and size of the pituitary gland changes during life. At birth it is typically globular in shape and shows high signal on T1 weighted images (Dietrich *et al.*, 1995). By approximately 6 weeks of age this high signal has diminished and the anterior pituitary tissue has a similar signal to brain tissue. The posterior pituitary tissue, however, retains a bright signal on T1 weighted sequences. This so-called "posterior pituitary bright spot" is a normal appearance thought to be due to the high neurophysin content (**Fig. 1**).

The size of the pituitary gland varies with age and sex. On average it is between 3 and 8 mm in height but is generally larger in females than males. The height increases during adolescence due to normal physiological hypertrophy (Tsunoda *et al.*, 1997). There is also a slight increase in size seen during the sixth decade in females. The most striking physiological changes are seen during pregnancy when the gland progressively enlarges reaching a maximal height immediately after birth when it may reach 10 mm in height (Dinc *et al.*, 1998).

Embryologically, the anterior and posterior pituitary lobes are distinct. The anterior lobe forms from an invagination of the oral ectoderm known as Rathke's Pouch. The posterior pituitary forms from a protrusion of the neural ectoderm of the diencephalon. Between the anterior and posterior lobes lies an intermediate lobe which is vestigial and known as the pars intermedia. This is a potential site for small nonfunctional Rathke's cysts (**Fig. 2**).

MR Imaging

MR is the imaging of choice for the pituitary gland. Thin sections (2 mm or 3 mm) targeted to the ptituitary fossa and performed in both the sagittal and coronal planes are needed. T1 weighted sequences before and after intravenous contrast are the main-stay of pituitary imaging (**Fig. 1**A–C) (Steiner *et al.*, 1989). Coronal T2 weighted sequences can also give added information (cystic change or tumor consistency) but are less sensitive overall in the detection of adenomas (Heck *et al.*, 2012). CT does not provide such excellent soft tissue resolution as MR but can be a very useful investigation if MR is contraindicated or if it is important to identify the presence of calcification (e.g., if a craniopharyngioma is suspected). Dedicated CT study should be performed utilizing no more than 1 mm slice thickness in the axial plane and then reconstructed in the sagittal and coronal planes.

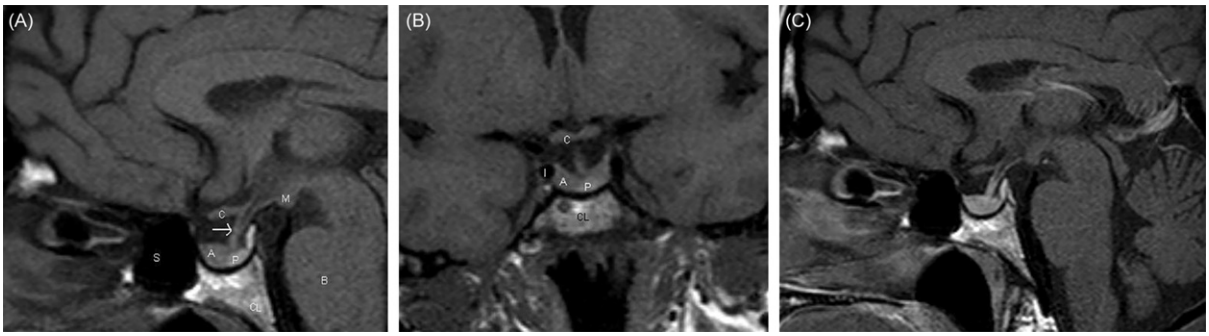

Fig. 1 (A) Sagittal T1W unenhanced images of the pituitary fossa demonstrate normal anatomy. The anterior pituitary tissue, A, is visible within the sella and the posterior pituitary bright spot, P, is evident behind it. The stalk (*arrow*) is well seen with a small cleft of CSF visible within it superiorly—the infundibular recess of the third ventricle. The optic chiasm, C, and mamillary bodies, M, are seen in the suprasellar region. *B*, Brainstem; *S*, sphenoid air sinus; *CL*, clivus. (B) Coronal T1W unenhanced images of the pituitary fossa. The anterior pituitary gland, A, is within the fossa. The posterior pituitary bright spot is visible centrally, P. The stalk is seen extending up into the suprasellar region. The optic chiasm, C, is visible. The cavernous segments of the carotids arteries, I, are seen within the cavernous sinuses, which form the lateral boundaries of the pituitary fossa. (C) Sagittal T1W enhanced image of the pituitary. The pituitary tissue has enhanced, as has the pituitary stalk.

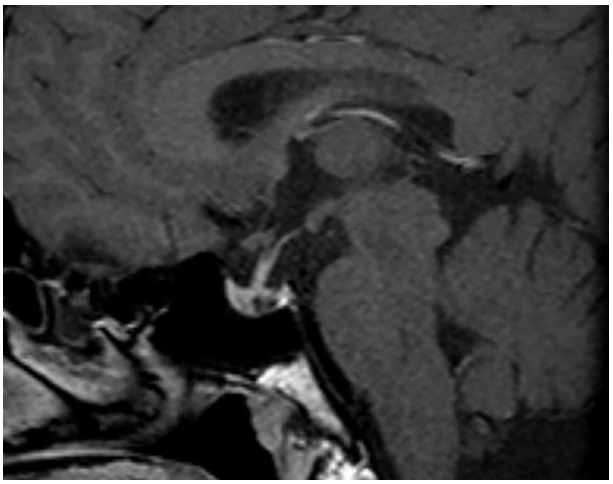

Fig. 2 Sagittal T1W enhanced image demonstrating a small Rathke's cyst. This is seen to lie just below the insertion of the pituitary stalk and centrally within the gland. Although it is possible that a small cystic adenoma could have these appearances, this is a very typical location for a Rathke's cyst arising in the pars intermedia.

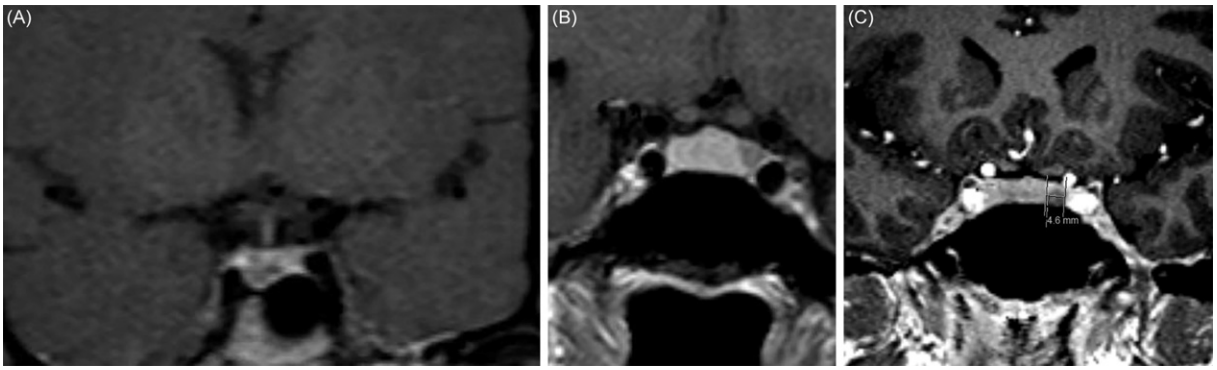

Fig. 3 (A–C) Coronal T1W after contrast images of microadenoma in three different cases. (A) Dynamic acquisition with a central lesion, (B) standard postcontrast with a small left far lateral lesion, and (C) a volumetric acquisition with a left lateral lesion.

There can be some benefit in performing the postcontrast MR sequences in a dynamic fashion (within the first 60 s) after contrast injection. This can maximize the conspicuity of adenomas which typically enhance less than the normal highly vascular pituitary tissue, and this differential enhancement is sometimes best appreciated within the first arterial phase of the contrast injection (**Fig. 3**A) (Kucharczyk *et al.*, 1994). However in the majority of cases lesions are adequately demonstrated on a standard acquisition after contrast (**Fig. 3**B) (Kucharczyk *et al.*, 1994).

Use of a postcontrast volumetric T1 weighted acquisition allows for higher resolution images with slice thickness around 1 mm and may improve the sensitivity for small microadenomas (Patronas *et al.*, 2003) and is therefore particularly useful in the investigation of Cushing's disease, when it should be used routinely (**Fig. 3**C).

Pituitary Adenomas

Pituitary adenomas are by far the most common mass lesion seen in the sella and parasellar region. They are slow growing benign neoplasms arising from the anterior pituitary tissue; radiologically they are simply classified by size: lesions smaller than 10 mm transversely are termed microadenomas and those greater than 10 mm are macroadenomas.

Pituitary Macroadenomas

Pituitary macroadenomas can extend superiorly into the suprasellar cistern (**Fig. 4**A and B) and impinge on the optic apparatus to produce visual field abnormalities (typically a bitemporal hemianopia). Pituitary macroadenomas with a large suprasellar com-

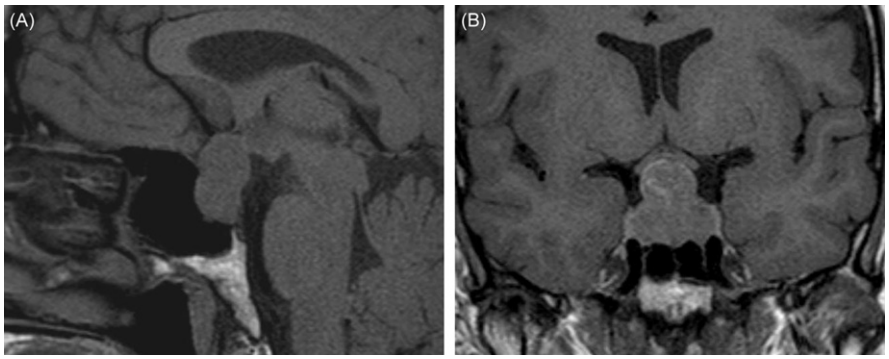

Fig. 4 (A) and (B) Sagittal and coronal T1W enhanced images of a macroadenoma. The sella is enlarged and the macroadenoma is seen to extend upwards into the suprasellar cistern with the optic chiasm stretched and deformed over the surface of the macroadenoma. (C) Coronal T1W enhanced image shows diffuse enhancement of the macroadenoma, no normal pituitary issue can now be identified. The optic chiasm is easily identified; it does not enhance and is seen to be stretched over the superior aspect of the adenoma.

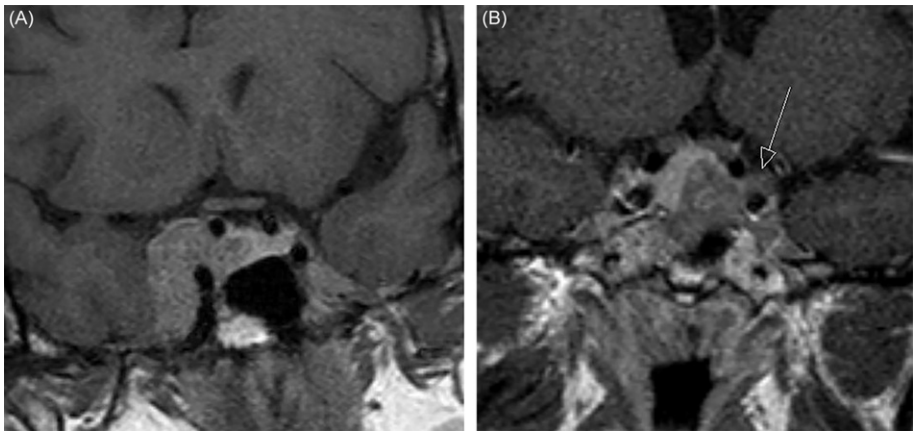

Fig. 5 (A) Coronal enhanced T1W images shows a large Rt sided pituitary adenoma that has invaded laterally into the Rt cavernous sinus. A significant component of the tumor lies lateral to the cavernous carotid artery and must therefore be within the cavernous sinus. (B) Coronal enhanced T1W images show a left sided lesion that has extended into the superior compartment of the left cavernous sinus and is seen to extend beyond the lateral border of the left cavernous carotid artery.

ponent characteristically show the appearance of "waisting" (**Fig. 4**B) as they pass through the opening in a sheet of dura—the diaphragma sellae.

Lateral growth of a macroadenoma is initially seen to cause deformity of the cavernous sinus; however, adenomas can invade into the cavernous sinus (**Fig. 5**A and B). If adenoma tissue extends beyond the lateral border of the cavernous internal carotid artery (**Fig. 5**A and B) then it is more likely that there is tumor directly within the cavernous sinus (Knosp *et al.*, 1993) although this remains a contentious issue. Recent studies suggest that the rate of direct invasion as identified at endoscopic surgery may be lower than previously thought using MR criteria (Micko *et al.*, 2015).

Occasionally macroadenomas will show very extensive involvement of the central skull base (**Fig. 6**) and, exceptionally, can extend out into the infratemporal fossa. Significant skull base involvement is associated with an increased risk of CSF leak after treatment (surgery or medical).

Macroadenomas may be heterogeneous in their MR signal characteristics and areas of cystic change and focal areas of hemorrhage are not infrequently identified. A significant hemorrhage with necrosis in an adenoma can produce the syndrome of *pituitary apoplexy*; sudden onset of headache often associated with visual disturbance and 3rd, 4th, or 6th cranial nerve palsies. The MRI shows an enlarged sella containing a macroadenoma with areas of high T1 signal representing the hemorrhage (**Fig. 7**A and B). Often there is suprasellar extension and lateral involvement of the cavernous sinus.

Pituitary microadenomas are confined within the sella and can be identified within the normal pituitary gland as an area of lower T1 signal than the normal pituitary tissue and reduced enhancement (**Fig. 3**C) (Kucharczyk *et al.*, 1986). Local remodeling of the floor of the sella and remodeling of the dorsum are also useful features. Although there may be displacement of the pituitary stalk by a lesion this is not a very reliable indicator (Ahmadi *et al.*, 1990).

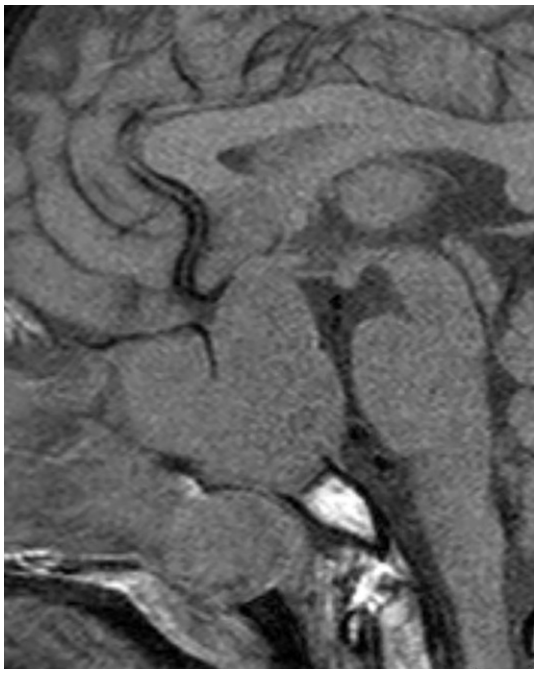

Fig. 6 Sagittal T1W unenhanced image shows an invasive macroadenoma which has involved much of the central skull base. It has invaded the sphenoid air sinus which can no longer be identified and has also extended down the clivus and into the nasopharynx. There is suprasellar extension with elevation of the optic chiasm.

After pituitary surgery it takes around 3–4 months for the postoperative changes within the sella to regress to allow for assessment of the true volume of residual pituitary tissue (Kremer *et al.*, 2002).

Rathke's Cleft Cysts

These benign, nonfunctioning cysts arise from remnants of squamous epithelium from Rathke's cleft. They typically arise close to the insertion of the stalk (**Fig. 2**). They are a common incidental finding reported in up to 11% of pituitary glands at autopsy (Teramoto *et al.*, 1994) and are found at all ages. On imaging they may appear proteinaceous, reflected as a hyperintense signal on T1 weighted sequence. It is not uncommon for these cysts to lie in the suprasellar region on the surface of the gland where they are usually found immediately anterior to the pituitary stalk (**Fig. 8**A and B). If they are of significant size then it may be hard to distinguish a Rathke's cyst from a cystic craniopharyngioma. However the wall of a Rathke's cyst shows no more than minimal enhancement, there are no solid enhancing areas and no calcification, which are all typical features of a craniopharyngioma.

Granular Cell Tumors

Granular cell tumors are rare lesions most typically seen in the suprasellar region. They arise from the neurohypophysis and/or the pituitary stalk. They have also been known as pituicytomas, infundibulomas, or choristomas (Cohen-Gadol *et al.*, 2003). Histologically they appear to arise from the pituicyte which is the main posterior pituitary cell. These are seen as well-defined suprasellar masses related to the pituitary stalk with homogenous enhancement (**Fig. 9**) (Cohen-Gadol *et al.*, 2003). Despite their intimate relationship to the pituitary stalk diabetes insipidus is not usually present although other endocrine disturbance may be present and these lesions may even be asymptomatic. If they have reached significant size they may be associated with visual disturbance or headache. They are benign and slow growing with a low recurrence rate after surgery.

Hypophysitis

Hypophysitis is an inflammatory lesion of the pituitary gland which is usually idiopathic rather than secondary to a known infection or systemic disease. Although the underlying cause of hypophysitis is not known, three distinct histopathological conditions are recognized; lymphocytic hypophysitis, granulomatous hypophysitis, and the very rare xanthomatous hypophysitis

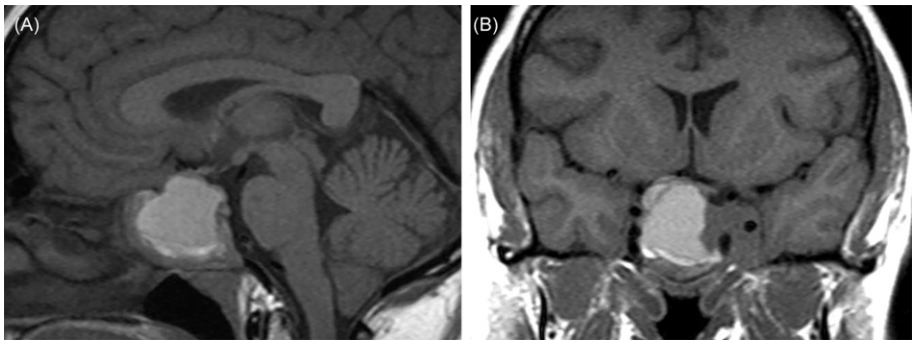

Fig. 7 Sagittal (A) and coronal (B) T1W noncontrast images showing hemorrhage into an existing pituitary macroadenoma. The area of high T1 signal represents the recent hemorrhage. There is a component of the tumor extending into the left cavernous sinus which does not show hemorrhage. The suprasellar extension is compressing the chiasm particularly on the right side.

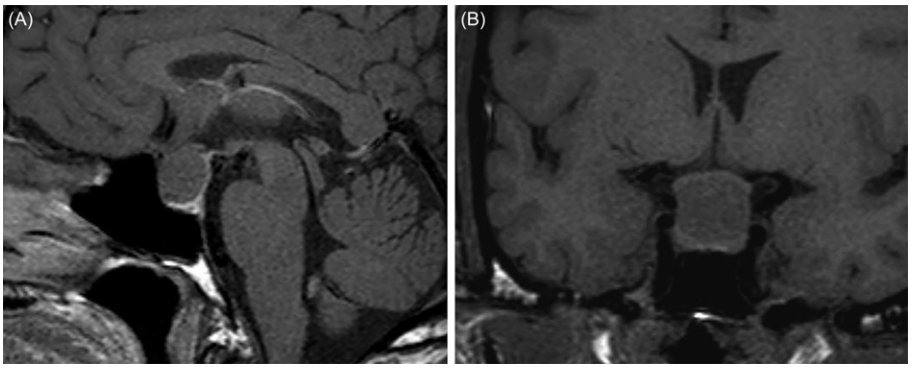

Fig. 8 Sagittal enhanced (A) T1W and coronal unenhanced (B) T1W images of a large Rathke's cyst. The cyst is seen to be sitting on the superior aspect of the pituitary tissue which appears flattened within the sella. The optic chiasm is stretched over the surface of the cyst. After contrast the cyst does not show enhancement. (A) The cyst shows higher signal than the CSF indicating that it has a higher protein content.

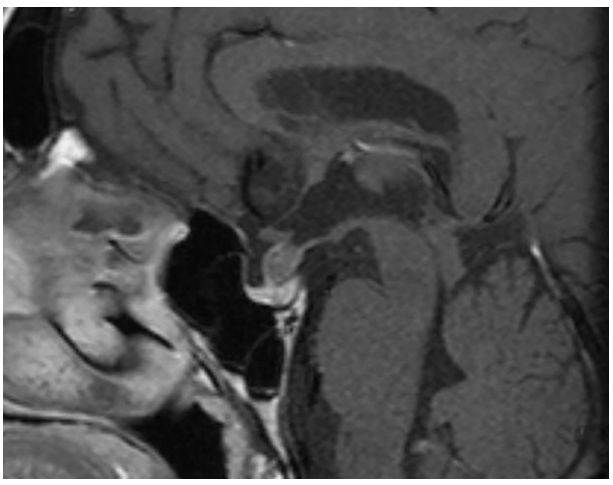

Fig. 9 Sagittal enhanced T1W image shows a well-defined enhancing mass within the pituitary stalk. The pituitary gland is normal, as is the hypothalamus. This was a granular cell tumor.

(Gutenberg *et al.*, 2006). Lymphocytic and granulomatous hypophysitis have similar indistinguishable MR imaging features; enlargement of the gland producing the appearance of a sellar mass lesion with involvement of the stalk (**Fig. 10**A and B). The gland may appear heterogeneous after contrast and there may be distortion of the chiasm if the suprasellar component is sizeable, although that is uncommon. Lymphocytic hypophysitis is the most common form occurring more often in females and classically presenting at the end of pregnancy or in the postpartum period with endocrine dysfunction (Gutenberg *et al.*, 2006). There may

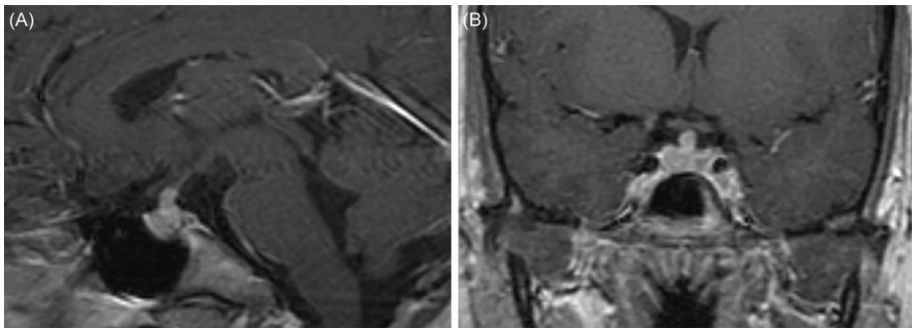

Fig. 10 Sagittal (A) and coronal (B) T1W enhanced images of lymphocytic hypophysitis. There is slight enlargement and heterogeneity of the gland with thickening of the lower part of the stalk. The fossa is not enlarged.

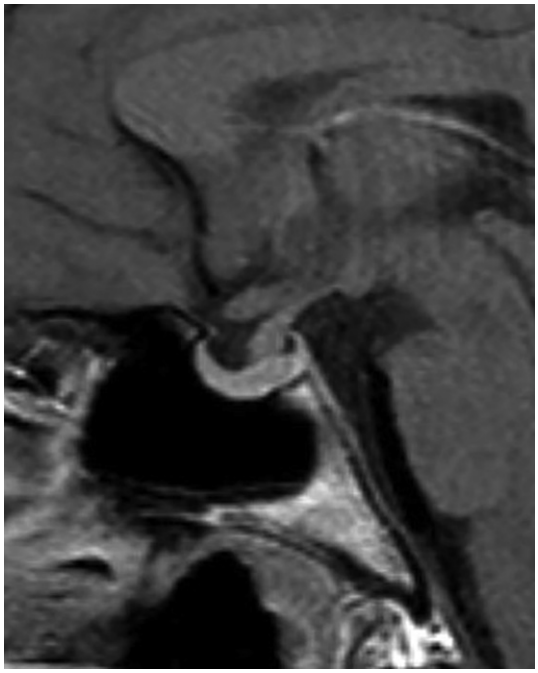

Fig. 11 Sagittal enhanced T1W image of neurohypophysitis. The stalk is thickened in its lower and mid portions, the pituitary gland is normal.

also be diabetes insipidus which may correlate with loss of the posterior pituitary bright spot, this is a helpful distinguishing feature from a pituitary adenoma.

Granulomatous hypophysitis is less common and is found equally in males and females. MR appearances are the same as those described for lymphocytic hypophysitis (Gutenberg *et al.*, 2006).

A variant of this condition is the so-called lymphocytic infundibulo-neurohypophysitis where the inflammatory process selectively involves the pituitary stalk and the posterior pituitary tissue and imaging reflects that—showing enlargement of only the posterior gland and stalk (Abe, 2008). Diabetes insipidus is the dominant clinical feature (**Fig. 11**).

All forms of hypophysitis can ultimately result in hypopituitarism with a small volume pituitary gland on imaging.

Newly described forms of hypophysitis are the rare IgG4 plasma cell hypophysitis (Hsing *et al.*, 2013) and hypophysitis caused by anticytotoxic T-lymphocyte antigen 4 antibody therapy for cancer.

Ipilimumab-related hypophysitis is now well recognized in up to 11% of patients (Faje *et al.*, 2014). MR often shows a diffuse enlargement of the gland (more rarely the stalk) which variably regresses after discontinuation of treatment (**Fig. 12**). Pituitary function may not recover despite imaging normalization (Faje *et al.*, 2014).

The Empty Sella

An empty sella contains only CSF without any visible pituitary tissue (anterior or posterior). The pituitary stalk will be visible and typically extends down to the floor of the sella (**Fig. 13**). An empty sella may be the result of previously documented pituitary/

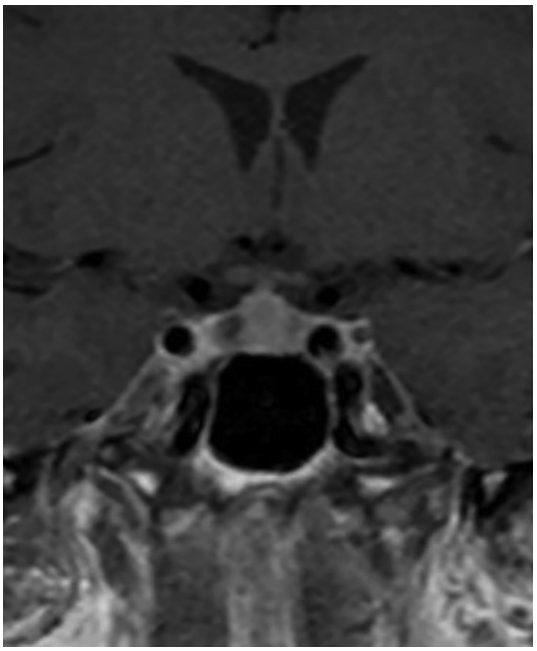

Fig. 12 Coronal T1W enhanced image of drug-induced hypophysitis. The gland is bulky and the enhancement is heterogenous.

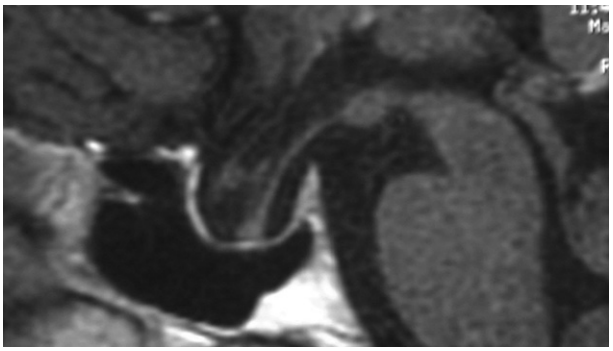

Fig. 13 Sagittal enhanced T1W images show an empty sella. No pituitary tissue is visible and the stalk extends down to the floor of the sella. The optic chiasm has prolapsed inferiorly; a not uncommon appearance after a large mass has been removed.

hypothalamic pathology or treatment (e.g., surgery, radiotherapy, hypophysitis, Sheehan syndrome, or pituitary apoplexy). However, it may also be discovered incidentally during MR scanning or in the course of investigating a new endocrine problem, then referred to as a "primary empty sella" (Del Monte *et al.*, 2006). A partially empty sella implies that some residual pituitary tissue can still be seen along the floor of the fossa. In the cases of a primary empty sella it is thought that a defect in the diaphragma sella (the sheet of dura over the surface of the sella through which the stalk passes) has allowed increased communication with the pulsatile CSF in the subarachnoid space. The primary empty sella is commoner in women and has been associated with idiopathic intracranial hypertension (IIH) obesity, visual disturbance, and spontaneous CSF leaks. Endocrine problems may be seen in up to 25% of cases with a primary empty sella (Del Monte *et al.*, 2006), the majority within this group being specifically investigated for a suspected endocrine abnormality. However, a small proportion of patients found to have an empty sella unexpectedly may have endocrine abnormalities on detailed testing.

Congenital Pituitary Abnormalities

Congenital hypopituitarism can manifest as isolated growth hormone deficiency (IGHD) or combined pituitary hormone deficiency (CPHD), which can be related to anatomical abnormalities of the hypothalamic/pituitary structures on MR imaging. Mutations associated with pituitary transcription factors (e.g., PROP1) can be associated with congenital hypopituitarism although most are sporadic (Mehta *et al.*, 2009). Imaging features include an ectopic location to the posterior pituitary which is seen to be

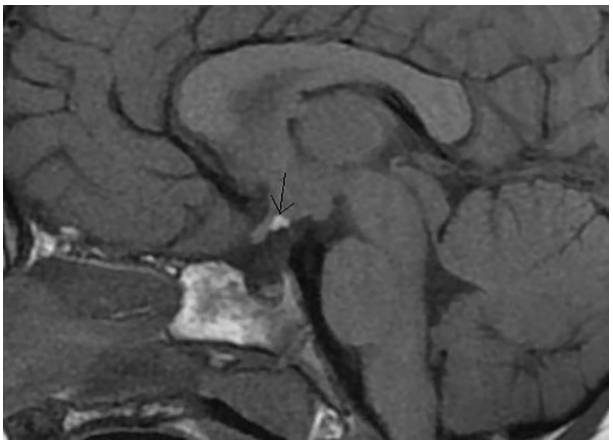

Fig. 14 Sagittal T1W unenhanced image. This demonstrates a small pituitary fossa containing a reduced volume of anterior pituitary tissue. The stalk is absent and the posterior pituitary bright spot (*arrow*) is lying in an ectopic location within the hypothalamus. The corpus callosum appears normal.

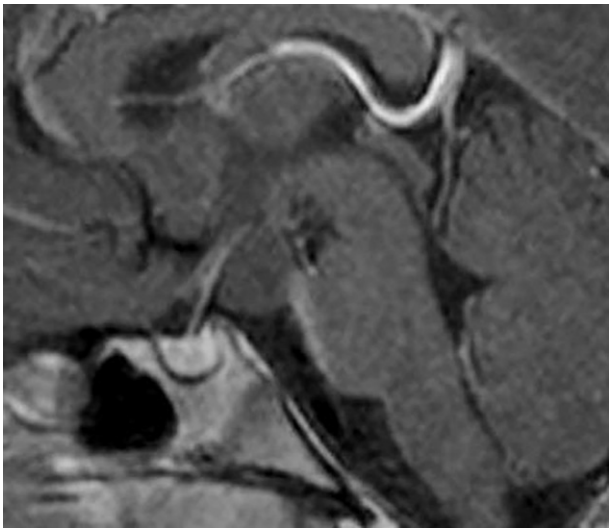

Fig. 15 Sagittal T1W enhanced image of a hypothalamic hamartoma. There is a nonenhancing mass visible arising from the undersurface of the hypothalamus and lying behind the pituitary stalk.

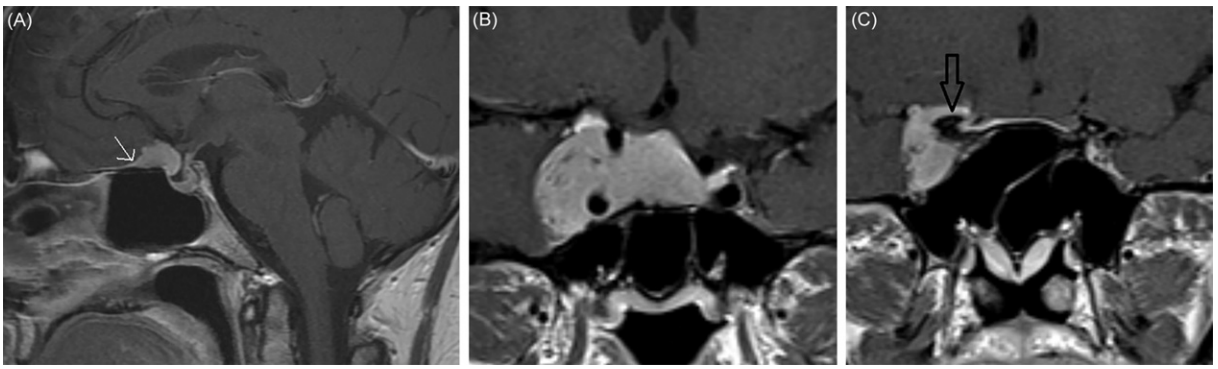

Fig. 16 (A) Sagittal enhanced T1W images show a meningioma arising from the floor of the anterior cranial fossa and extending into the pituitary fossa, lying anterior to the stalk, but separate from the pituitary tissue. There is a dural tail of enhancement extending forward (*arrowed*). (B) and (C) Coronal enhanced T1W image shows a large Rt sided meningioma involving the Rt cavernous sinus and filling most of the sella. This could be a large macroadenoma but (C) shows a more anterior image demonstrating the associated bony hyperostosis of the Rt anterior clinoid process (*arrowed*) confirming that this is a meningioma.

undescended and is visible as a high T1 signal area in the region of the median eminence (Fig. 14). There may be absence of the pituitary stalk and/or hypoplasia of the anterior pituitary tissue and a small pituitary fossa. Other congenital abnormalities of midline structures may be associated with these hypothalamic/pituitary features; optic nerve hypoplasia, absence of the septum pellucidum, and corpus callosum abnormalities (Mehta *et al.*, 2009). CPHD is more often associated with callosal and stalk abnormalities than the milder forms of hypopituitarism such as IGHD (Mehta *et al.*, 2009).

Hypothalamic Hamartomas

These are benign developmental mass lesions that arise in the tuber cinereum of the hypothalamus and can be associated with central precocious puberty, gelastic (laughing) seizures, and sometimes developmental delay. The lesions are easily identified on MR imaging as they have a distinctive appearance as an almost pedunculated rounded mass hanging from the hypothalamus. They are of similar signal to the gray matter of the cerebral cortex and do not show enhancement after contrast injection (Fig. 15). Histologically, they are composed of well differentiated neurones and glial cells. They are sometimes approached surgically if the epilepsy is proving refractory to treatment.

Stalk and Suprasellar Abnormalities

Pathological processes that primarily involve the pituitary stalk and suprasellar structures, rather than the pituitary gland itself, have a wide differential diagnosis. The commoner neoplastic processes include meningiomas, craniopharyngiomas, and germinomas. Meningiomas are benign tumors that can arise from any dural surface around the sella and features such as a dural tail, bony hyperostosis, calcification, and carotid artery constriction are useful distinguishing radiological features (Kaltsas *et al.*, 2008) (Fig. 16A–C). Craniopharyngiomas characteristically have both proteinaceous cystic and enhancing solid components with calcification and CT may be helpful to identify the calcification. Germinomas manifest as solid, noncalcified enhancing masses involving the upper part of the stalk/hypothalamus (Fig. 17A and B) and an associated pineal mass or ependymal enhancement indicates more extensive disease (Packer *et al.*, 2000).

Metastatic disease from systemic malignancy can also involve the stalk and sellar region, the typical clinical manifestation of diabetes insipidus may prompt pituitary imaging.

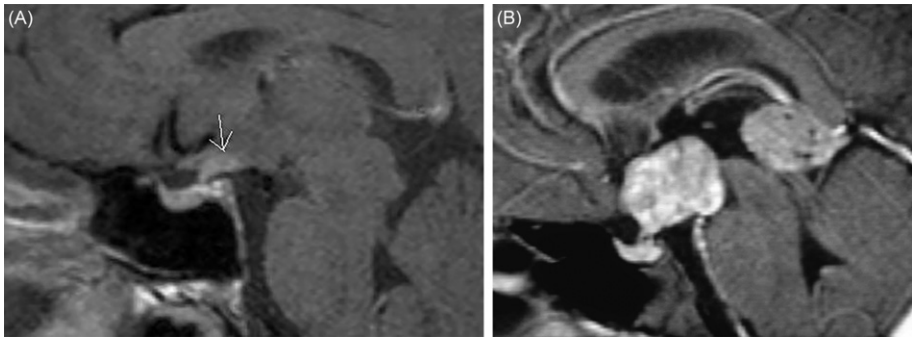

Fig. 17 Sagittal T1W enhanced images show a small germinoma (A) with thickening of the hypothalamus (*arrowed*) and a large suprasellar germinoma (B) with a synchronous pineal lesion.

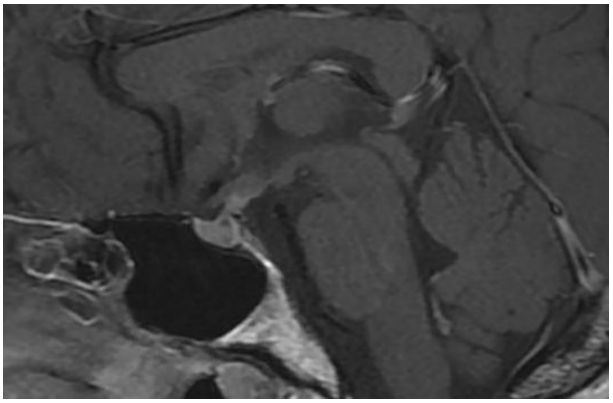

Fig. 18 Sagittal T1W image of Langerhans cell histiocytosis—visible only as thickening of the upper stalk and hypothalamus.

Processes such as sarcoidosis (Lexa and Grossman, 1994) and Langerhans cell histiocytosis can involve the pituitary stalk. Widespread basal leptomeningeal enhancement is one typical manifestation of neurosarcoidosis. Langerhans cell histiocytosis may show just isolated stalk thickening but the presence of extra cranial involvement should aid diagnosis (D'Ambrosio *et al.*, 2008) (**Fig. 18**).

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Hyponatraemia

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Introduction

Hyponatraemia is the commonest electrolyte disturbance encountered in clinical practice. Estimates vary according to study populations (Sturdik *et al.*, 2014; Frenkel *et al.*, 2010; Funk *et al.*, 2010), but an overall view would suggest a figure of 15%–30% of hospitalized patients (Upadhyay *et al.*, 2006), with community rates of 2%–10% (Miller *et al.*, 1996; Liamis *et al.*, 2013; Gisby *et al.*, 2016; Mohan *et al.*, 2013). Hyponatraemia is habitually underinvestigated and undertreated in routine clinical practice (Greenberg *et al.*, 2015). However, the explosion of interest in hyponatraemia over the last 10 years has led to the publication of data which indicates that there is substantial morbidity, mortality and prolonged hospital stay in patients whose hospital admission is complicated by hyponatraemia (Corona *et al.*, 2013; Zilberberg *et al.*, 2008). Although mild hyponatraemia may be asymptomatic, subtle cognitive effects, which result in poor attention, predispose to unsteady gait (Renneboog *et al.*, 2006), leading to increased falls (Fehlberg *et al.*, 2017), and increased fracture rate (Kinsella *et al.*, 2010) are well recognized. The increase in fracture rate may reflect the association with osteoporosis, which animal studies suggest is directly related to hyponatraemia (Verbalis *et al.*, 2010).

In addition to excess morbidity, almost every published study has shown that mortality is increased in hyponatraemia (Corona *et al.*, 2013; Sturdik *et al.*, 2014; Waikar *et al.*, 2009), though there is still a debate regarding the causality of hyponatraemia, with some authors suggesting that hyponatraemia is only a marker of severity of the underlying disease (Chawla *et al.*, 2011). Severe hyponatraemia (plasma sodium [pNa] <125 mmol/L) is a medical emergency and can lead to seizures, coma, and death.

Hyponatraemia can complicate a diverse variety of pathologies with different pathophysiological mechanisms. Volume status of the patient and duration of hyponatraemia determine the treatment approach. Elucidating the underlying cause of hyponatraemia requires meticulous clinical examination to establish extracellular volume status as well as ancillary biochemistry including spot urine analysis for sodium and osmolality. Failure to do so can lead to inappropriate treatment which may lower plasma sodium concentration [pNa] further. On the other hand, over-zealous correction of chronic hyponatraemia can result in permanent neurological damage.

In this article, we will discuss the prevalence of hyponatraemia in hospital and in the community, explore the literature on the morbidity and mortality associated with hyponatraemia, offer a practical approach to the differential diagnosis and summarize treatment options for acute and chronic hyponatraemia.

Prevalence of Hyponatraemia

Hyponatraemia is the commonest electrolyte disorder encountered in clinical practice—rates vary according to the patient population, health care setting and diagnostic criteria. Large population-based studies from the United States (Mohan *et al.*, 2013), Sweden (Gisby *et al.*, 2016), and Netherlands (Liamis *et al.*, 2013) have reported rates of 2%–8%, with the highest prevalence reported in females, older patients and those with co-morbidities (Mohan *et al.*, 2013). Rates of 8%–10% percent are reported in ambulatory patients over 60 years attending a geriatric outpatient clinic, and of 18% in a similarly aged cohort of nursing home patients (Miller *et al.*, 1995, 1996), of whom over half had at least one episode of hyponatraemia in the preceding 12 months.

The incidence of hyponatraemia in hospital inpatients is 13%–37% (Frenkel *et al.*, 2010; Sturdik *et al.*, 2014); up to 5% have significant hyponatraemia, defined as [pNa] <130 mmol/L (Upadhyay *et al.*, 2006). Low plasma sodium concentrations are common in intensive care units (ICU, 15%–34%) (Padhi *et al.*, 2014; Nicolini *et al.*, 2017; Funk *et al.*, 2010), neurosurgical (Hannon *et al.*, 2013, 2014; Qureshi *et al.*, 2002; Sherlock *et al.*, 2006, 2009; Agha *et al.*, 2004b; Olson *et al.*, 1997), and oncology units (34%–47%) (Castillo *et al.*, 2012; Doshi *et al.*, 2012; Abu Zeinah *et al.*, 2015). Hyponatraemia is a common complication of chronic disease—more than one third of patients receiving specialist palliative care have low plasma sodium concentrations, which are associated with greater symptom burden compared with eunatremic patients (Kremeike *et al.*, 2018). Hyponatraemia occurs in 8%–31% of adult patients hospitalized with pneumonia (Kruger *et al.*, 2014; Zilberberg *et al.*, 2008; Nair *et al.*, 2007). Data from our own center have shown that 40% of cases are due to SIAD, 40% to hypovolemic hyponatremia, and the remainder hypovolemic (Cuesta, personal communication). Hypervolemic hyponatraemia occurs in up to 24% of patients with heart failure (Gheorghiade *et al.*, 2007a,b; Bettari *et al.*, 2012), and more than 50% of patients with cirrhosis and ascites (Angeli *et al.*, 2006).

Morbidity and Mortality of Hyponatraemia

The severity of symptoms of hyponatraemia depends on how low the plasma sodium concentration is and, more importantly, the rate at which [pNa] falls. Mild hyponatraemia ([pNa] 130–135) can be asymptomatic, but moderate hyponatraemia ([pNa]

125–130) can present with nausea, headache and vomiting. Severe hyponatraemia ([pNa] <125 mmol/L) is a often a medical emergency, particularly if the rate of fall has been rapid, over 1–3 days, and can present with symptoms of cerebral irritation—agitation, confusion, and impaired mental status (Smith *et al.*, 2000). Animal studies, including the classic experiments of Arieff *et al.* (1976), have demonstrated that when hyponatraemia develops over several days, intracerebral fluid, sodium and potassium, followed by organic solutes, are lost from the brain, decreasing intracerebral osmolality and preventing the development of cerebral oedema (Verbalis and Gullans, 1991; Arieff *et al.*, 1976). It is because of this adaptive mechanism that chronic severe hyponatraemia may present with only modest symptoms. Symptoms are far more likely if the drop in [pNa] is rapid. In this setting the osmotic gradient across the blood–brain barrier is increased, the ability of the brain to compensate is exceeded; water is osmotically shifted into the brain and cerebral oedema ensues. This can lead to raised intracranial pressure, cerebral herniation and death, if hyponatraemia is not corrected promptly and effectively (Hannon and Thompson, 2014). The nature of the underlying illness may also affect presentation, for example, neurosurgical patients are more vulnerable to developing symptomatic hyponatraemia due to co-existence of other factors which cause cerebral irritation; hyponatraemic seizures may occur at a higher [pNa] in these patients than might be expected in other settings (Hannon and Thompson, 2014).

Even mild chronic hyponatraemia is associated with gait instability, poor attention span (Renneboog *et al.*, 2006), and increased risk of falls (Harianto and Anpalahan, 2017; Fehlberg *et al.*, 2017; Rittenhouse *et al.*, 2015; Gunathilake *et al.*, 2013). Hyponatraemia is a risk factor for fractures, independent of the presence of osteoporosis (Kinsella *et al.*, 2010). Up to one in five patients admitted with hip fracture are hyponatraemic, and low plasma sodium concentrations are associated with a delay in time to definitive hip surgery (Aqil *et al.*, 2016; Tinning *et al.*, 2015), increased length of stay (Mc Causland *et al.*, 2014), and increased postoperative mortality (Tinning *et al.*, 2015; Madsen *et al.*, 2016; Hagino *et al.*, 2013). Hyponatraemia also has deleterious effects on bone density, as chronic hyponatraemia can increase the odds of having osteoporosis fourfold (Usala *et al.*, 2015). Rat models of SIAD have demonstrated a 30% reduction in bone mineral density (BMD) over 3 months, when compared with eunatremic control rats, while analysis of subjects >50 years of age in the Third National Health and Nutrition Examination Survey (NHANES III) database showed that mild hyponatraemia is associated with increased odds of osteoporosis at the hip when adjusted for other confounders (Verbalis *et al.*, 2010).

Hyponatraemia has been shown to be associated with increased mortality in almost every published series in the literature, spanning a diverse range of medical conditions (Corona *et al.*, 2013; Sturdik *et al.*, 2014; Waikar *et al.*, 2009; Gheorghiade *et al.*, 2007a). An inverse relationship between [pNa] and mortality risk has been proven in population studies such as the NHANES database (Mohan *et al.*, 2013), in ambulatory (Gankam-Kengne *et al.*, 2013), and hospitalized patients (Corona *et al.*, 2013), and in a 2013 metaanalysis (Corona *et al.*, 2013). A large cohort study of 53,236 patients admitted to an academic medical center reported significantly increased odds ratios for inpatient mortality, remarkably at [pNa] cut-off of 138 mmol/L; risk of death was increased by 2.3% for each 1 mmol/L decline in [pNa] (Wald *et al.*, 2010). Not all analyses have supported this inverse relationship however, particularly when [pNa] falls below 120 mmol/L (Chawla *et al.*, 2011; Waikar *et al.*, 2009); the authors of these papers have contended that the underlying illness rather than the [pNa] value accounts for much of the mortality and that patients die "with hyponatraemia" rather than "from hyponatraemia."

Almost all studies published in the literature have produced mortality data associated with hyponatraemia due to all causes. However, a large prospective study which divided patients presenting to hospital with hyponatraemia according to blood volume status has challenged this traditional view. Using the careful application of well-described diagnostic criteria, they showed that although mortality was increased in all three categories of hyponatraemia, there were considerable differences in mortality according to the blood volume categorization (Cuesta *et al.*, 2017a). The data revealed that patients with SIAD had lower mortality rate (RR 1.8) than those with hypervolemic or hypovolemic hyponatraemia (RR 4.9 and 2.8 respectively), when death rate was compared with eunatremic controls (**Fig. 1**). To a certain extent this reflected the seriousness of the disease processes causing hypervolemic hyponatraemia, particularly end stage cardiac and hepatic failure, and the Charlson comorbidity index was significantly higher in this group compared with the other groups. However, this was not the entire explanation for the excess mortality in the hyponatraemic groups. The Charlson comorbidity index was lower in the SIAD group than the eunatraemic group, and despite this, the mortality in the SIAD group was significantly higher. This provides strong circumstantial evidence to support the view that the relationship between hyponatraemia and mortality is partly causal.

Dynamic plasma sodium changes during hospital admission may also increase the mortality risk. Higher mortality rates have been reported in patients whose [pNa] fall during hospital admission, a group Gill and colleagues described as a "sicker" cohort, and whom they suggested would benefit from more intensive monitoring (Gill *et al.*, 2006). On the other hand, resolution of hyponatraemia during hospitalization has been associated with lower mortality risk compared to hyponatraemia that persists or is acquired during admission (Waikar *et al.*, 2009).

Differential Diagnosis of Hyponatraemia

The differential diagnosis of hyponatraemia is wide and there are a number of recommended diagnostic approaches and algorithms. Our clinical approach is to classify patients by extracellular fluid status into hypo-, eu- and hypervolemic hyponatraemia (**Table 1**). While a definitive diagnosis is not always possible at the time of presentation—the distinction between euvolaemia and mild hypovolemia is not always easy—categorizing patients according to extracellular fluid (ECF) volume status and urine electrolyte excretion does allow the initiation of etiology-specific treatment.

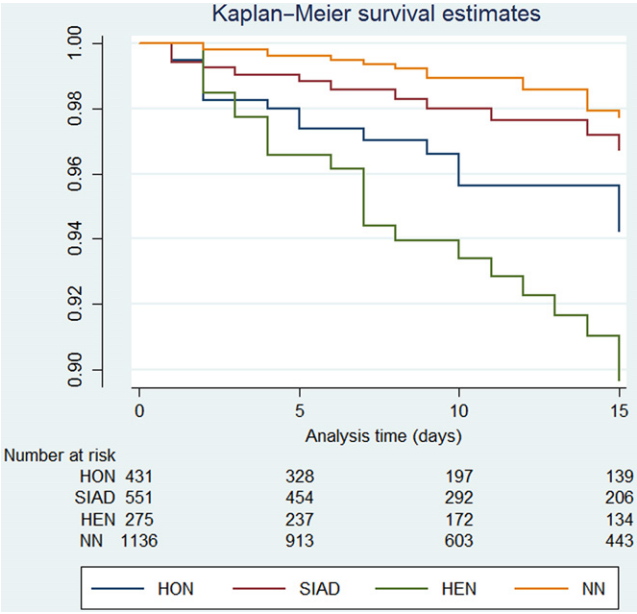

Fig. 1 Kaplan–Meier survival curve in patients with hypovolemic hyponatraemia (HON), SIAD, hypervolemic hyponatraemia (HEN), and normonatraemia (NN). With permission Cuesta, M., Garrahy, A., Slattery, D., Gupta, S., Hannon, A. M., Mcgurren, K., Sherlock, M., Tormey, W. and Thompson, C. J. (2017a). Mortality rates are lower in SIAD, than in hypervolaemic or hypovolaemic hyponatraemia: Results of a prospective observational study. *Clinical Endocrinology* 87, 400–406.

Table 1 Differential diagnosis of hyponatraemia

	Clinical features	*Urine sodium* $<$ *20 mmol/L*	*Urine sodium* $>$ *40 mmol/L*
Hypovolemic	Dry mucus membranes, decreased skin turgor, tachycardia, hypotension (in particular orthostatic), low CVP, raised urea	Dehydration	CSW Diuretics Addison's disease Salt losing nephropathy
Euvolemic	Normal pulse and blood pressure	SIAD with FR	SIAD ACTH insufficiency Hypothyroidism
Hypervolemic	Peripheral oedema, ascites, pulmonary oedema	Inappropriate IV fluids Cardiac failure Cirrhosis	Renal failure

Hypovolemic Hyponatraemia

Hypovolemic hyponatraemia is characterized by ECF depletion secondary to a deficit in total body sodium and water. This loss can occur through the skin, gastro-intestinal tract or kidney; the cause is often suggested by the history. Off-loading of baroreceptors stimulates vasopressin (AVP) release, and together with inappropriate administration of hypotonic fluids (either oral or parenteral), hyponatraemia may develop. Measurement of central venous pressure (CVP) offers the most reliable confirmation of hypovolemia but is invasive. More often a clinical diagnosis is arrived at on the basis of orthostatic hypotension, tachycardia, dry mucous membranes, decreased skin turgor, and laboratory clues such as raised urea to creatinine ratio and uric acid concentration. Urinary sodium concentration is nearly always <20 mmol/L, and often <10 mmol/L in hypovolemia due to activation of the renin–aldosterone system, and mineralocorticoid action at the kidney. Hypovolemia with elevated urine sodium concentrations indicates that site of volume/sodium loss is the kidneys, or that the patient is taking diuretics.

Diuretics frequently cause hyponatraemia and the diagnosis is often obvious from the history. Thiazides are particularly likely culprits, due to their effects on urine diluting capacity; loop diuretics are rarely implicated (Sonnenblick *et al.*, 1993). The reported incidence of thiazide-induced hyponatraemia is 14%–30% (Leung *et al.*, 2011; Clayton *et al.*, 2006). Older age and female gender are associated with increased risk. Three mechanistic explanations are proposed—baroreceptor stimulated AVP secretion due to volume depletion, impairment of diluting capacity at the distal tubule, and depletion of potassium leading to cellular uptake of

sodium (Verbalis *et al.*, 2013). Some patients with thiazide-induced hyponatraemia may appear euvolemic and present with a SIAD-like picture, however expert guidelines would caution against making a diagnosis of SIAD in a patient treated with diuretics and suggest deferring evaluation for SIAD until at least 2 weeks following cessation of the drug (Verbalis *et al.*, 2013).

Addison's disease is another important cause of hypovolemic hyponatraemia. Combined glucocorticoid and mineralocorticoid deficiency leads to renal salt and water loss, and baroreceptor stimulated AVP release. This clinical picture differs from that of central adrenal insufficiency in which mineralocorticoid secretion is retained preventing such profound hypovolemia. Clinical clues to the diagnosis of Addison's disease include hyperpigmentation, history of other autoimmune conditions, metabolic alkalosis, and hyperkalemia (Oelkers, 1996).

Hypervolemic Hyponatraemia

Hypervolemic hyponatraemia is characterized by clinical evidence of fluid overload. Clinical signs include the presence of peripheral or sacral oedema, ascites and elevated central and jugular venous pressures. The underlying pathophysiological process is arterial underfilling, either due to reduced cardiac output in heart failure or splanchnic vasodilation in liver failure (Schrier, 2006). This leads to a baroreceptor mediated increase in AVP secretion and antidiuresis, activation of the renin–angiotensin system and secondary hyperaldosteronism, and increased sympathetic tone. Secondary hyperaldosteronism leads to sodium retention at the distal tubule, made worse by failure of the normal physiological "escape" from the natriuretic effect of high aldosterone levels (Smith *et al.*, 2000). However, it is the relative excess of total body water to sodium, resulting from increased plasma AVP concentrations and up-regulation of aquaporin-2 water channels, that is responsible for the development of hyponatraemia. This notion is supported by the observation that administration of AVP antagonists results in aquaresis and prompt rise in [pNa] in patients with heart and liver failure (Schrier *et al.*, 2006).

Euvolemic Hyponatraemia

Patients without clinical evidence of volume expansion or depletion should be considered euvolemic. Spot urinary sodium concentration is >20–30 mmol/L in most euvolemic patients, with the important caveat that because regulatory mechanisms of sodium excretion are intact, dietary sodium restriction can lead to misleadingly low urinary sodium levels. Other supporting laboratory parameters in euvolemia include low blood urea and low serum uric acid (Decaux and Musch, 2008). The majority of cases of euvolemic hyponatraemia are due to the syndrome of inappropriate antidiuresis (SIAD). Hyponatraemia due to central adrenal insufficiency presents with a similar biochemical picture and should always be considered.

Syndrome of Inappropriate Antidiuresis

SIAD is a clinical and biochemical syndrome characterized by inappropriate urinary concentration and antidiuresis, in the setting of euvolemic hyponatraemia.

Pathogenesis of SIAD

In SIAD, unregulated AVP secretion leads to water retention, with concentrated urine, and manifests biochemically as hypotonic euvolemic hyponatraemia. Although SIAD is classified as euvolemic hyponatraemia, physiologically it is characterized by slight blood volume expansion, characterized by slight suppression of plasma renin and elevation of plasma natriuretic peptide concentrations (Cuesta and Thompson, 2016). Plasma concentrations of AVP have been shown to be elevated in the great majority of cases of SIAD (Robertson *et al.*, 1982). Rarely, gain-of-function mutations in the V2 receptor can lead to the nephrogenic syndrome of inappropriate antidiuresis, in which AVP levels are undetectable (Feldman *et al.*, 2005); therefore syndrome of inappropriate antidiuresis (SIAD) may be more a more precise term than syndrome of inappropriate antidiuretic hormone secretion (SIADH). SIAD can be subclassified into four types defined by the pattern of AVP secretion (**Fig. 2**) (Zerbe *et al.*, 1980).

1. Type A is the commonest cause of SIAD (40%) and is characteristic of lung and nasopharyngeal tumors. Ectopic AVP secretion is unregulated with loss of the close linear relationship between plasma osmolality and plasma AVP. Furthermore, plasma AVP concentrations are not suppressed by drinking and these patients demonstrate a lower osmotic threshold for thirst appreciation —therefore they are at risk of severe hyponatraemia.
2. Type B ("Reset osmostat") is the second most common cause of SIAD in which the osmotic threshold is reset at a lower plasma osmolality. Thus while secretion of AVP occurs at a lower plasma osmolality that normal, it is suppressed below the lower threshold, protecting the subject from severe hyponatraemia.
3. Type C is a rare form of SIAD, thought to be due to dysfunction of hypothalamic inhibitory neurons, in which the linear relationship between plasma osmolality and AVP levels is maintained at normal plasma osmolality, but there is failure to suppress AVP secretion at plasma osmolality below the threshold leading to persistent low grade AVP secretion.
4. Type D is biochemically identical to SIAD but with undetectable circulating AVP and is thought to be due to gain-of-function mutations in the V2 receptor.

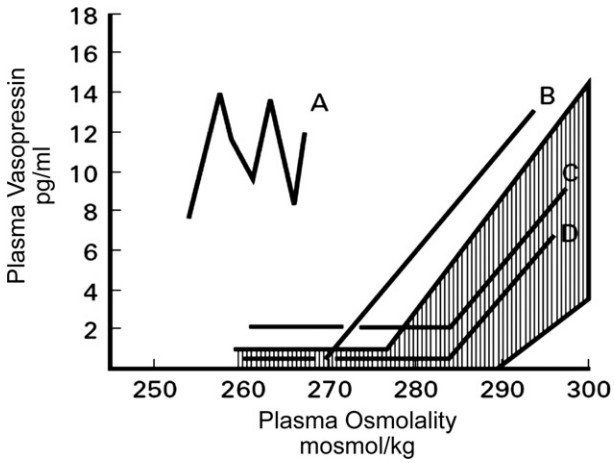

Fig. 2 Patterns of abnormal vasopressin (AVP) secretion in SIAD (A–D). With permission Robertson, G. L., Aycinena, P. and Zerbe, R. L. (1982). Neurogenic disorders of osmoregulation. *The American Journal of Medicine* 72, 339–353.

Table 2 Diagnostic criteria for SIAD (Schwartz *et al.*, 1957)

1. Hypo-osmolality: plasma osmolality <275 mOsm/kg
2. Inappropriate urine concentration: urine osmolality >100 mOsm/kg
3. Elevated urine sodium >40 mmol/L with normal salt and water intake
4. Euvolemia
5. Exclusion of glucocorticoid and thyroid hormone deficiency

Diagnostic criteria

The diagnostic criteria for SIAD are outlined in **Table 2**. In healthy physiology, plasma AVP should be suppressed to undetectable concentrations at plasma osmolalities <280 mOsm/kg; therefore any urine osmolality greater than 100 mOsm/kg in this setting reflects inappropriate urine concentration, and therefore, inappropriate plasma AVP concentrations. Measurement of plasma AVP levels is not performed in day-to-day practice due to limited availability of reliable radioimmunoassays and impractical sample turn-around times, but urine osmolality can be interpreted as a surrogate biomarker of AVP action—the higher the plasma AVP the more concentrated the urine (Robertson *et al.*, 1976).

Ascertainment of the key diagnostic parameters for SIAD in routine clinical practice has invariably been poor (Tzoulis and Bouloux, 2015). The report of the large hyponatraemia registry revealed that in the 1524 patients specifically entered into the registry with a diagnosis of SIAD the triumvirate of key diagnostic parameters—plasma osmolality, urine osmolality and urine sodium concentration—were measured in less than half of patients. In 11% of patients, none of these parameters were measured at all, and plasma cortisol concentrations were measured in only one third of patients (Greenberg *et al.*, 2015). This indicates that the diagnostic accuracy in the largest published registry is open to reasonable question. In our own carefully planned, single-site, prospective study, including over 500 patients with SIAD, only 88% had adequate testing to exclude glucocorticoid deficiency (Cuesta *et al.*, 2016). It is clear that full ascertainment of the diagnostic parameters for SIAD presents significant logistic challenges in both clinical practice and research studies. The clinical importance of measuring these parameters in the workup of SIAD extends beyond making the correct diagnosis—measurement of urinary electrolytes (and subsequent calculation of Furst formula) and urine osmolality have been proposed as predictors of poor sodium response to FR (Cuesta *et al.*, 2017b).

Etiology of SIAD

SIAD has been reported in a wide variety of medical conditions (**Table 3**) and can present to many different specialties; in an single center study performed in an Israeli tertiary center, the diagnosis of SIAD was made in 555 patients across 14 different departments (Shepshelovich *et al.*, 2015).

Of the neoplastic causes, SIAD occurs most commonly in small cell lung cancer (SCLC) with reported rates of up to 15% (Castillo *et al.*, 2012); incidence in nonsmall cell lung cancer is much lower. SIAD occurs due to ectopic secretion of AVP from SCLC tumor tissue—George *et al.* using immunostaining of surgically excised SCLC samples, demonstrated evidence of in-vitro synthesis of AVP (George *et al.*, 1972). In addition, mRNA for AVP has been isolated from tumor cells (Gross *et al.*, 1993). SIAD has been reported in 3% of cases of head and neck cancer (Talmi *et al.*, 1992), and less commonly in a wide variety of other solid tumors and hematological malignancies. SIAD is present in 3% of patients with intracranial tumors; the incidence increases to 15% in the immediate period after neurosurgical intervention (Cuesta and Thompson, 2016). Chemotherapeutic agents may also cause SIAD; vincristine, and to a lesser extent, vinblastine exert neurotoxic effects on the hypothalamus, disrupting osmoreceptor control of AVP release, while cyclophosphamide potentiates the action of AVP at the kidney (Liamis *et al.*, 2008). Opioid

Table 3 Causes of SIAD (Cuesta *et al.*, 2016)

	Cause of SIAD	*n*
Intracranial ($n = 108$, 30%)	Subarachnoid haemorrhage	20
	Ischaemic stroke	12
	Intracranial tumor	18
	Traumatic brain injury	9
	Haemorrhagic	9
	Hydrocephalus	9
	Miscellaneous	31
Pulmonary ($n = 96$, 27%)	Lobar pneumonia	45
	Other respiratory tract infections	31
	Bronchiectasis	9
	Miscellaneous: pneumothorax, pulmonary fibrosis, pulmonary embolism etc.	11
Oncology ($n = 90$, 25%)	Lung cancer	30
	• SCLCA: $n = 14$ • NSCLCA: $n = 21$	25 9
	Gastrointestinal malignancy	9
	Urological malignancy	15
	Haematological malignancies	
	Other malignancies	
Iatrogenic ($n = 31$, 9%)	Carbamazepine	14
	Oxcarbazepine	4
	SSRI	8
	Other	4
Complications of surgery ($n = 28$, 8%)	Orthopedic surgery	20
	Other surgeries	8

Description of the aetiology in patients with SIAD in a prospective cohort of 353 patients in Beaumont Hospital, Dublin, Ireland. *CNS*, central nervous system, *SSRI*, selective serotonin reuptake inhibitor, *CA*, carcinoma.

analgesics, antidepressants, antipyschotics, and antiseizure medications used for symptoms control may also cause SIAD (Castillo *et al.*, 2012), while pain and nausea are nonosmotic triggers for AVP release (Rowe *et al.*, 1979).

SIAD is frequently reported in respiratory illness. In our own prospective study of 1723 patients admitted to our hospital with community acquired pneumonia, 8% had [pNa] <130 mmol/L; 43% of these had SIAD (Cuesta, personal communication). SIAD usually resolves with successful treatment of infection, without specific treatment. Failure of hyponatraemia to resolve with antimicrobial therapy should alert the clinical to the possibility of alternative underlying causes of SIAD, such as bronchiectasis, pulmonary fibrosis or malignancy.

Medications account for 8%–27% of cases of SIAD in hospital inpatients (Shepshelovich *et al.*, 2015; Cuesta *et al.*, 2016), and an even greater proportion in elderly cohorts. Underlying physiological mechanisms include an increase in AVP production in the hypothalamus, potentiation of AVP effect at the renal medulla or a lowering of the osmotic threshold for AVP secretion (reset osmostat). Commonly implicated drugs include selective serotonin reuptake inhibitors, phenothiazine antipsychotics, carbamazepine, sodium valproate, chemotherapeutic agents particularly vincristine and cyclophosphamide, opioid analgesics, and non-steroidal antiinflammatory agents (NSAIDS). More recently, proton pump inhibitors and ACE inhibitors have been recognized as rare causes of drug-induced SIAD (Izzedine *et al.*, 2002; Brewster and Perazella, 2007).

Establishing the underlying cause of SIAD

Once the diagnosis of SIAD is confirmed, identification of the underlying cause is required. The depth of investigation for the etiology of SIAD is made on an individual basis. A careful medication history can identify offending medications that should be discontinued and replaced by alternative agents which are osmotically neutral. Clinical examination, baseline blood-work and radiography may reveal the likely cause for example, lung mass in a smoker. However, in the absence of an obvious candidate pathology, we perform computed tomography of the brain, chest, abdomen, and pelvis as a routine workup for the causation of SIAD. A chest radiograph alone is not sufficient to out-rule a bronchogenic malignancy.

ACTH/Cortisol Deficiency

Secondary adrenal insufficiency can produce a biochemical picture identical to SIAD, with euvolemic hyponatraemia and inappropriate urinary concentration. Mineralocorticoid secretion is preserved as it is under the control of the renin–angiotensin–aldosterone system. This biochemical and clinical picture contrasts with patients with primary adrenal insufficiency, or classical

Addison's disease, in whom combined glucocorticoid and mineralocorticoid deficiency lead to renal salt loss, hypovolemia, and baroreceptor mediated "appropriate" AVP release, manifest as hypovolemic hyponatraemia and hyperkalemia (Oelkers, 1996).

Circulating cortisol is required to excrete free water in the renal medulla. This interplay is exemplified in hypopituitary patients, when diabetes insipidus is often only unmasked when coexisting ACTH deficiency is treated. In animal studies too, there is impairment of water diuresis in adrenalectomised rats with inherited diabetes insipidus; the administration of glucocorticoids and mineralocorticoids restored normal free water clearance (Green *et al.*, 1970).

AVP secretion also plays a role in the hyponatraemia of cortisol deficiency. Experimental animal models of glucocorticoid deficiency have demonstrated an increase in hypothalamic AVP mRNA expression and upregulation of aquaporin-2 mRNA expression in basal conditions, and failure to suppress plasma AVP and downregulate aquaporin-2 mRNA expression after a water load (Boykin *et al.*, 1978; Pyo *et al.*, 1993; Saito *et al.*, 2000, 2009). Administration of AVP antagonist results in restoration of urinary dilution, further supporting the central role of AVP in the development of antidiuresis in ACTH/cortisol deficiency (Ishikawa and Schrier, 1982; Saito *et al.*, 2000).

One to three percent of unselected hyponatraemic patients have underlying ACTH/cortisol deficiency (Winchester Behr *et al.*, 2012; Katoch *et al.*, 2013; Nigro *et al.*, 2015). In a recent prospective series, with full ascertainment of the minimum diagnostic criteria for SIAD in 83% of cases, we have shown that 3.8% of patients presenting with euvolemic hyponatraemia to our institution had adrenal insufficiency. Undiagnosed pituitary disease was responsible for one third of cases while the remaining two-thirds developed hyponatraemia due to adrenal suppression after therapeutic chronic glucocorticoid excess, including the use of inhaled steroids (Cuesta *et al.*, 2016). ACTH/cortisol deficiency should be particularly suspected in patients who develop hyponatraemia after neurosurgical conditions such as posttraumatic brain injury (Hannon *et al.*, 2013) and subarachnoid hemorrhage (Hannon *et al.*, 2014).

Hypothyroidism

Hypothyroidism is an exceptionally rare cause of euvolemic hyponatraemia. The mechanism by which hypothyroidism induces hyponatraemia is incompletely understood but may be due in part to nonosmotic stimulation of AVP release, reduced renal capacity for free water excretion due to impaired renal perfusion, and water retention due to accumulation of mucopolysaccharides (Hierholzer and Finke, 1997; Liamis *et al.*, 2017). The evidence for an association between chronic hypothyroidism and hyponatraemia is, however, unconvincing (Croal *et al.*, 1997). So, while confirmation of normal thyroid function remains a diagnostic criterion in SIAD, and is recommended as part of routine workup in all clinical guidelines (Verbalis *et al.*, 2013; Runkle *et al.*, 2013; Grant *et al.*, 2015), it is our opinion that hyponatraemia due to hypothyroidism is seen only in rare cases of profound myxedema.

Neurosurgical Hyponatraemia

Hyponatraemia is commonly encountered in neurosurgical units. In the national neurosciences center at our hospital, hyponatraemia accounts for approximately 70% of endocrine consults (Hannon *et al.*, 2012). Management can be challenging—multiple potential reasons for impaired mental status in an obtunded patient may make it difficult to say for certain that symptoms are due to hyponatraemia, particularly if [pNa] is only mildly decreased. However, neurosurgical patients are prone to symptoms of cerebral irritation at [pNa]s higher than one would expect for this very reason. Therefore, close monitoring and early treatment of hyponatraemia is important.

Hyponatraemia Following Pituitary Surgery

Hyponatraemia occurs after pituitary surgery in 2%–35% of cases (Jahangiri *et al.*, 2013; Olson *et al.*, 1997; Kristof *et al.*, 2009; Taylor *et al.*, 1995; Sane *et al.*, 1994; Barber *et al.*, 2014). The majority of cases are due to SIAD, secondary to uncontrolled AVP release consequent upon mechanical irritation of the pituitary stalk or neurohypophysis during pituitary surgery. Hyponatraemia can also occur as part of the triple phase response in which initial diabetes insipidus (DI) occurs, usually within the first 48 h after surgery, and is followed by a transient period of hyponatraemia and antidiuresis due to release of pre-formed AVP, before a return to permanent DI due to gliosis of secretory neurons, usually 2 weeks postoperatively. ACTH/cortisol deficiency is an important differential. However, in many pituitary units (including our own) patients are routinely treated with stress dose glucocorticoids thus eliminating this as a cause of hyponatraemia in in the postoperative period.

Delayed hyponatraemia is a significant contributor to hospital re-admission rates following TSS (Bohl *et al.*, 2016; Jahangiri *et al.*, 2013), and it is recommended that patients should be made aware of symptoms suggestive of hyponatraemia. Peak onset is at day 7 (Zada *et al.*, 2007), and typically resolves within 3–5 days; hyponatremia may be severe and symptomatic (Guerrero *et al.*, 2007). Outpatient strategies to reduce readmission rates have given contrasting results (Burke *et al.*, 2017), with a recent study finding a standardized program of outpatient [pNa] monitoring no more effective than close symptom monitoring alone (Bohl *et al.*, 2018).

Hyponatraemia Following SAH

Hyponatraemia occurs following SAH in 30%–56% cases (Sherlock *et al.*, 2006; Qureshi *et al.*, 2002; Hannon *et al.*, 2014; Anas and Vasikaran, 2010). It accounts for one in six readmissions after hospital discharge (Greenberg *et al.*, 2016) and predicts increased morbidity and longer hospital stay (Sherlock *et al.*, 2006). Ninety percent of cases are euvolemic. SIAD accounts for approximately 70% of cases (Hannon *et al.*, 2014; Marupudi and Mittal, 2015); 8% of euvolaemic hyponatraemia patients have acute ACTH/cortisol deficiency, and plasma sodium is restored to normal with steroid therapy (Hannon *et al.*, 2014).

Most cases of acute ACTH/cortisol deficiency occur within the first 3 days. Short synacthen testing is not useful in this setting as it relies on adrenal atrophy, which can take 4–6 weeks to occur and the insulin tolerance test is not appropriate in the acute phase postintracranial hemorrhage due to risk of seizure. Measurement of serial cortisol values is the most practical approach, with empiric replacement of glucocorticoids recommended if cortisol <300 nmol/L (11 μg/dL) followed by more robust assessment once the patient has recovered (Hannon *et al.*, 2012).

Other potential causes of hyponatraemia following SAH include excessive intravenous fluid therapy (often administered to prevent vasospasm) and/or diuretic therapy. Cerebral salt wasting (CSW) is extremely rare. In a series of 187 consecutive cases of neurosurgical hyponatraemia, only 3.7% had CSW and 2.7% had CSW with SIAD (Sherlock *et al.*, 2009). A more recent prospective study of 100 cases of SAH failed to reveal a single case, even after careful clinical and biochemical assessment of volume status (Hannon *et al.*, 2014). It should be noted that SIAD and CSW share many biochemical features—both conditions present with low plasma osmolality, high urine osmolality, and high urinary sodium values. Distinguishing features are outlined in **Table 4**—key is the demonstration of an initial period of naturesis and hypovolemia preceeding the onset of hyponatraemia in CSW (Nelson *et al.*, 1981).

Hyponatraemia Following TBI

Hyponatraemia occurs in approximately 15% of patients during the recovery phase following head injury (Hannon *et al.*, 2013; Agha *et al.*, 2004a). SIAD has traditionally been thought of as the most common cause of hyponatraemia following TBI. However, transient ACTH/cortisol deficiency following TBI may account for a higher proportion of cases of hyponatraemia in this setting than previously appreciated. In a prospective study of acute pituitary dysfunction in which TBI patients underwent sequential measurements of [pNa], cortisol, urine osmolality and fluid balance, 15% of 100 patients developed hyponatraemia. Eighty five percent of hyponatraemic patients had inappropriately low plasma cortisol concentration and [pNa] corrected with glucocorticoid replacement; the remainder had SIAD (Hannon *et al.*, 2013). This raises the possibility that hyponatraemia following TBI is due to acute ACTH/cortisol deficiency rather than SIAD. Chronic hyponatraemia post TBI is rare, and often secondary to antiseizure medications for example, carbamazepine.

Treatment of Hyponatraemia

The key issues which determine optimum management of hyponatraemia are accurate assessment of the underlying cause (**Table 1**) and awareness of the chronicity of low plasma sodium concentrations. Acute hyponatraemia, which occurs over several hours, does not give the brain time to compensate; an osmotic gradient develops between brain and plasma, and cerebral oedema ensues. This is a medical emergency which untreated can culminate in transtentorial herniation and death. In contrast, when the onset of hyponatraemia is slower, the brain adapts by extruding electrolytes and osmolytes which serves to maintain an osmotic equilibrium and minimize cerebral odema. Thus, patients with chronic hyponatraemia may have minimal symptoms, and cerebral herniation is uncommon. In this scenario, it is the risk of osmotic demyelination syndrome (ODS) from overly rapid correction of [pNa] which guides treatment regimens (Verbalis *et al.*, 2013). ODS occurs when correction of chronic hyponatraemia exceeds the ability of the brain to reverse compensatory mechanisms. Failure to recapture organic solutes as [pNa] rises results in an inverse osmotic gradient leading to dehydration of the cells and possible demyelination of white matter (Singh *et al.*, 2014).

Table 4 Distinguishing features between SIAD and CSW

	SIAD	*Cerebral salt wasting*
Blood volume status	Euvolemic	Hypovolemic
Urinary Na	>40 mmol/L	>40 mmol/L
Plasma osmolality	Low	Low
Urine osmolality	High	High
Serum uric acid	Decreased	Decreased/no change
Haematocrit	No change	Raised
Treatment	FR	Fluid replacement

Acute Hyponatraemia

Acute symptomatic hyponatraemia is a medical emergency which is associated with high rates of morbidity and death (Ayus *et al.*, 1985). The immediate goals of treatment are to reduce cerebral oedema and to prevent transtentorial herniation (Ayus *et al.*, 2015; Koenig *et al.*, 2008). Published guidelines have recommended revision of traditional treatment goals, and have emphasized the importance of rapid initial correction of plasma sodium concentration of 3–5 mmol/L over 2–4 h. For severe symptoms, US/Irish guidelines recommend administering 100 mL of 3% saline as a bolus over 10 min, which can be repeated three times if no clinical improvement occurs (Verbalis *et al.*, 2013). European guidelines advocate a similar approach, recommending an initial 150 mL bolus of 3% saline, which can be repeated twice (Spasovski *et al.*, 2014). In patients with mild-moderate symptoms 3% saline infusion at a rate of 0.5–2 mL/kg/h can be administered, with the aim of a gradual increase in plasma sodium concentrations of up to 8 mmol/L over 24 h. Reliance on complex calculations to determine the volume of saline which should be administered in order to correct to target can frequently lead to over-correction (Mohmand *et al.*, 2007) and may delay the start of emergency treatment. The newer guidelines simplify and speed up life-saving treatment. Hourly monitoring of urine output to detect rapid aquaresis is useful. In true acute hyponatraemia (<48 h) the rate of correction may need not be restricted, but the duration of hyponatraemia is often indeterminate, so correction must be limited to safe parameters for the correction of chronic hyponatraemia in most cases.

In suspected ACTH/cortisol deficiency, parenteral glucocorticoids should be given immediately. Blood should be drawn for measurement of plasma ACTH and cortisol concentrations prior to administration but should not delay treatment. A prompt aquaresis after administration of glucocorticoids strongly supports the diagnosis of cortisol deficiency, and [pNa] corrects without any other specific treatment. Close monitoring of [pNa] and urine output is required due to the risk of overcorrection (Verbalis *et al.*, 2013). In Addison's disease intravenous isotonic saline is required to restore intravascular volume and replace sodium losses, while intravenous dextrose should be added if the patient is hypoglycemic.

Chronic Hyponatraemia

Rationale for treatment

Chronic hyponatraemia was traditionally considered a benign condition. However, it is now well established that chronic hyponatraemia negatively affects attention, gait (Renneboog *et al.*, 2006, 2017), bone health (Hannon and Verbalis, 2014) as well as increasing falls (Fehlberg *et al.*, 2017), and fracture risk (Kinsella *et al.*, 2010). There is now a growing body of evidence that suggests that active management of hyponatraemia can reverse these detrimental effects. Treatment of hyponatraemia leads to improvement in balance and attention; Renneboog demonstrated an improvement in tandem gait parameters and reaction time in 23 consecutive patients following treatment of "asymptomatic" chronic hyponatraemia (Renneboog *et al.*, 2017). In the SALT studies, a rise in [pNa] of 6 mmol/L, in patients randomized to treatment with tolvaptan, was associated with significant improvements in the mental component summary of the SF12 Health survey compared with placebo. The INSIGHT trial, designed specifically to look at the effect of tolvaptan on cognition and postural stability in patients with asymptomatic hyponatraemia demonstrated a trend for improvement in neurocognitive tests in the tolvaptan group associated with an increase in [pNa] from 129 to 136 mmol/L. Study numbers were small, baseline hyponatraemia was mild and their findings did not reach statistical significance; however, the observed effect tended to reverse when [pNa] fell again following withdrawal of treatment, strongly supporting a causal relationship (Verbalis *et al.*, 2016a). Based on these data, it is reasonable assume that treatment of chronic hyponatraemia could reduce falls and injury; good quality prospective trial data is, however, needed.

A recent prospective intervention study in a London hospital has suggested that active management of hyponatraemia reduces hospital length of stay (Tzoulis *et al.*, 2017), while a 2015 metaanalysis has reported, for the first time, that improvement in [pNa] in hyponatraemic patients is associated with a reduction in overall mortality (OR 0.57, $P = .002$), which persisted at 12 months. Metaregression analysis showed the reduction to be greatest in older patients and those with lowest [pNa] at baseline (Corona *et al.*, 2015). While a cause-effect relationship cannot be extrapolated from the data included in this analysis, this report provides the first evidence for a potential mortality benefit from treating chronic hyponatraemia, and emphasizes the need for studies of clinical outcomes with effective therapies in such patients.

Rate of correction

Patients with chronic hyponatraemia are much less likely to develop neurological symptoms but are at higher risk of osmotic demyelination. Therefore, risk-benefit analysis favors a slower rise in [pNa] in this patient group. Target rise in [pNa] should be set at <8 mmol/L/24 h and should not exceed 12 mmoL/L/24 h (Verbalis *et al.*, 2013). Added caution is advised in those more vulnerable to the risk of osmotic demyelination including patients with history of alcohol excess, cirrhosis, malnutrition, hypokalemia, and those with [pNa] < 105 mmol/L; target and maximum rise is <6 mmol/L/24 h and <8 mmol/L/24 h respectively in these patients (**Table 5**). Simultaneous treatment of the underlying cause of hyponatraemia, for example, antibiotic treatment of pneumonia, glucocorticoid replacement in steroid deficiency or removal of a thiazide diuretic may lead to greater rise in [pNa] than anticipated as the stimulus for AVP release is removed resulting in a rapid aquaresis (Verbalis *et al.*, 2016b); correction rate may be even higher if active therapy is used.

Table 5 Targets for elevation in plasma sodium concentration [pNa] recommended to avoid osmotic demyelination in chronic hyponatraemia (Verbalis *et al.*, 2013)

	Target rise [pNa]/24 h (mmol/L)	*Maximum rise [pNa]/24 h (mmol/L)*
Standard patient	8	12
High risk patient ([pNa] <105 mmol/L, hypokalemia, alcoholism, advanced liver disease, malnutrition)	6	8

Osmotic demyelination syndrome

Osmotic demyelination syndrome, formerly called central pontine myelinolysis, was first described in 1959 (Adams *et al.*, 1959). It is characterized clinically by spastic quadriparesis and pseudobulbar palsy, reflecting damage to the corticospinal and corticobulbar tracts. Severely affected patients may become "locked in." A rapid rise in [pNa] is the biggest risk factor for ODS; studies in experimental rats have illustrated increased likelihood and severity of brain lesions with increasing rate of correction (Verbalis and Martinez, 1991). Onset of symptoms is typically delayed until 2–6 days after excessive elevation of [pNa]. Diagnosis is based on clinical suspicion, and is supported by characteristic MRI findings of hyperintense lesions in the central pons on T2 weighted imaging (Lopez-Sendon Moreno *et al.*, 2009); up to 40% cases can involve extrapontine areas such as the cerebellum, thalamus, and midbrain. Traditionally the prognosis was reported as poor as diagnosis was made postmortem. However, with increasing use of MRI leading to detection of subclinical radiological abnormalities, it is now accepted that the majority of patients with ODS survive, though 25% are left with significant permanent disability (Singh *et al.*, 2014).

Management of excessive correction

If overcorrection is anticipated based on trend in [pNa] and urine output, further therapy should be held. Once target [pNa] concentration has been reached, [pNa] can be kept stable with the administration of hypotonic fluids (intravenous dextrose) and/or 2–4 μg parenteral desmopressin. An alternative strategy is to administer desmopressin at the onset of treatment, with titration of 3% saline according to [pNa] response. This "proactive approach" may be preferable in those at a particularly high risk of rapid water diuresis with overshooting of [pNa]. Evidence for this strategy is limited to case series (Sood *et al.*, 2013; MacMillan *et al.*, 2015).

If over-correction does occur, therapeutic re-lowering of [pNa] can be considered by administering 2–4 μg desmopressin combined with repeated infusions of 3 mL/kg of 5% dextrose over 1 h with need for further 5% dextrose infusions determined by [pNa] response. Evidence for rescue therapy comes from experimental animal studies in which early re-lowering of [pNa] prevents ODM and reduces mortality (Gankam Kengne *et al.*, 2009). High dose glucocorticoid therapy (Sugimura *et al.*, 2005; Murase *et al.*, 2006), and administration of myoinositol, a major osmolyte lost in cerebral adaptation to hyponatraemia, have been associated with improved survival and reduced myelinolysis in rats (Silver *et al.*, 2006). None of these therapies have been validated in clinical practice.

Hypovolemic Hyponatraemia

Isotonic saline is the treatment of choice for hypovolemic hyponatraemia as specific therapy for the underlying cause is put in place. Correction of hypovolemia eliminates the stimulus for AVP secretion resulting in a rapid aquaresis which can potentially lead to overcorrection. Plasma sodium concentration and urine output should be monitored closely during the first 24–48 h of replacement. Treatment of thiazide diuretic induced hyponatraemia requires particular caution as withdrawal of the drug combined with correction of hypovolemia may result in a rapid aquaresis (Liamis *et al.*, 2016).

Euvolemic Hyponatraemia

SIAD

Treatment of the underlying cause of SIAD is always the first therapeutic step. Drug-induced hyponatraemia usually responds to withdrawal of therapy, and SIAD due to pneumonia resolves quickly with antimicrobial therapy. Symptomatic hyponatraemia needs treatment irrespective of causation and a number of therapeutic options are available in clinical practice. A summary of the key aspects of available therapies for SIAD is presented below and in **Table 6**.

Fluid restriction

Fluid restriction (FR) is the most widely used therapeutic strategy employed in the management of SIAD (Verbalis *et al.*, 2016b). Remarkably, the evidence base to support its efficacy is lacking. In the Hyponatraemia Registry, the largest observational study of routine management of hyponatraemia to date, FR was used first line in half of the 1524 patients. Disappointingly however, 52% of patients with baseline [pNa] <130 mmol/L failed to correct their [pNa] by >5 mmol/L with FR; the majority required the addition of a second-line treatment (Verbalis *et al.*, 2016b). Data from our own unit has found that 60% of patients treated with FR have a criteria which clinical guidelines suggest would predict nonresponse to FR (Cuesta *et al.*, 2017b). Moreover, FR is

Table 6 Comparison of treatment options for chronic SIAD

	Evidence	*Dose*	*Advantages*	*Disadvantages*
Fluid Restriction	Nil	Aim 500 mL/day below 24 h urine volume	Inexpensive	Often ineffective Takes several days to achieve effect Difficult to achieve Can lead to caloric restriction Contraindicated in SAH
Urea	Nonrandomized observational studies	15–60 g daily	Effective	No licence or available preparation Poor palatability Reversible renal impairment
Demeclocycline	Nil	600–1200 mg daily in divided doses	Inexpensive	Takes 3–4 days to achieve effect Reversible renal impairment Rash Not licensed
V2 receptor antagonist—tolvaptan	Robust RCT data	15 mg daily (often 7.5 mg suffices), maximum 60 mg daily	Effective	Relatively expensive Not universally reimbursable Requires supervision for first dose Thirst, polyuria Raised LFTs

difficult to enforce. A target of at least 500 mL/day less than 24 h urine output is recommended—this may not be feasible in the hospital setting if intravenous medications, such as antibiotics, are essential for treatment. In patients with SAH, aggressive hydration to avoid cerebral vasospasm, is accepted as routine therapy by neurosurgeons, who are reluctant to contemplate fluid restriction.

Although fluid restriction is recommended as first line therapy for SIAD by all reputable guidelines (Grant *et al.*, 2015; Runkle *et al.*, 2013; Verbalis *et al.*, 2013), doubts about the efficacy and practicality remain, particularly as the evidence base for fluid restriction is practically nonexistent.

AVP receptor antagonists

The development of AVP receptor antagonists (vaptans) represents a major advance in the management of SIAD. Vaptans competitively bind to the V2 receptor, displacing AVP from the binding site resulting in free water clearance and in a rise in [pNa] (Plosker, 2010). Tolvaptan is an oral selective antagonist of V2 receptor available in Europe and USA while conivaptan is an intravenous antagonist of V1 and V2 receptors available in the United States. In the large multicenter randomized placebo controlled SALT trials tolvaptan has been shown to induce elevation of plasma sodium concentration (Schrier *et al.*, 2006) which is faster and more consistent than FR (Verbalis *et al.*, 2016b) in a mixed population of patients with euvolaemic and hypervolaemic hyponatremia. Plasma sodium concentration fell following cessation of the drug at 30 days, confirming that the positive effect was related to tolvaptan and not spontaneous recovery of hyponatraemia. Subsequent subgroup analysis has confirmed the efficacy of tolvaptan in the SIAD cohort in the study (Verbalis *et al.*, 2011). Thirst and dry mouth are the most commonly reported side-effects, which is unsurprising considering the mechanism of the drug is net water excretion. Administration of tolvaptan at higher doses (average dose 95 mg/day) in autosomal dominant polycystic kidney disease has been associated with increased risk of raised liver enzymes (Torres *et al.*, 2012), however the safe and effective use of long-term tolvaptan in hyponatraemia patients has been confirmed in the SALTWATER open-label 4 years follow-up studies (Berl *et al.*, 2010). Liver function should be monitored regularly (e.g., 3 monthly) and the drug discontinued if liver enzymes increase >2 ULN (Verbalis *et al.*, 2013). We have reported acquired resistance to tolvaptan in two patients with rapidly advancing small cell lung cancer, with escalating plasma vasopressin concentrations (Garrahy *et al.*, 2018); in both cases, loss of effect of the drug heralded deterioration in the underlying malignancy.

Concurrent FR is not recommended and treatment must be initiated in hospital to allow careful monitoring of [pNa] and urine output to prevent over-correction. Target rates of [pNa] correction (>0.5 mmol/L/h) were exceeded in 2% of patients randomized to Tolvaptan in the SALT studies, there were no reported cases of ODM. Patients with lowest starting [pNa] are at greatest risk, and tolvaptan should be used with caution in those with [pNa] <120 mmol/L. Using a lower starting dose of 7.5 mg may reduce the risk of overcorrection (Kenz *et al.*, 2011; Hirai *et al.*, 2016), but does not eliminate it (Shoaf *et al.*, 2017).

Urea

Urea increases solute-free water excretion and decreases naturesis, thereby increasing [pNa]. Retrospective studies have shown it to be effective in SIAD post SAH and in critical care patients (Decaux *et al.*, 2010), and in 12 patients who transitioned to urea therapy following 12 months of vaptan therapy in a clinical trial (Soupart *et al.*, 2012); it is recommended by European Guidelines as a second line agent for the treatment of SIAD. However, urea is unlicensed, difficult to obtain, unpalatable and its reconstitution requires cooperation from pharmacy.

Demeclocycline

This tetracycline derivative induces a nephrogenic form of diabetes insipidus and was historically used in the treatment of SIAD. Response to treatment is variable and it can take several days to reach effect. Adverse effects include reversible renal impairment, liver dysfunction, and photosensitive skin rash (Miller *et al.*, 1980). It is not licensed for use in SIAD.

ACTH/Steroid Deficiency and Hypothyroidism

Chronic hyponatraemia attributed to ACTH/cortisol deficiency or hypothyroidism should be treated with replacement of the deficient hormones.

Hypervolemia Hyponatraemia

In patients with heart failure, combination of FR, and loop diuretics plus neurohormonal antagonism is the recommended first line approach. Use of loop diuretics can be limited by intravascular volume depletion, renal impairment and hypokalemia; overdiuresis may stimulate AVP secretion and worsen hyponatraemia. Vaptans offer an attractive alternative or adjunct to loop diuretics; they are potassium-neutral (Xiong *et al.*, 2015), and cause less neurohormonal activation from intravascular volume depletion compared with diuretics. Several large randomized controlled trials (RCTs) have confirmed tolvaptans beneficial effect on [pNa], weight and dyspnea in heart failure patients (Konstam *et al.*, 2007; Xiong *et al.*, 2015). Posthoc analysis of the large EVEREST trial (4133 patients) demonstrated a reduction in mortality in those patients with [pNa] <130 mmol/L (Konstam *et al.*, 2007), while improvements in hyponatraemia in the smaller ACTIV in CHF trial (319 patients) were associated with improved survival at 60 days (Rossi *et al.*, 2007).

A combination of fluid and salt restriction, loop diuretics and spironolactone is recommended in the treatment of hyponatraemia due to liver failure. Demeclocycline is contraindicated in this setting due to heptatotoxicity. In patients with hyponatraemia and ascites, short term tolvaptan use has been shown to normalize [pNa] in 27%–50% of patients (Gerbes *et al.*, 2003). However, the routine use of tolvaptan is not recommended in patients with cirrhosis, based on the liver injury seen in higher doses used in the TEMPO trial (Torres *et al.*, 2012). One exception is patients awaiting liver transplant, in whom correction of [pNa] preoperatively may reduce the risk of ODS following surgery (Verbalis *et al.*, 2013).

Conclusion

Hyponatraemia is the commonest electrolyte disorder in clinical practice and has a negative impact on falls and fracture risk, bone health and mentation. It is associated with increased length of hospital stay and increased mortality. There is growing evidence that active management negates these effects, with a recent metanalysis confirming for the first time that improvement of hyponatraemia reduces mortality. Clinical evaluation of volume status, interpretation of urinary biochemistry, and confirmation of chronicity are key first steps in deciding appropriate initial fluid management, directing further investigation and ensuring [pNa] rises at a safe rate. Acute symptomatic hyponatraemia is a medical emergency and is treated with hypertonic saline. Traditional treatment options for chronic SIAD are limited by poor efficacy, side-effects and lack of supportive randomized control trial data. In the absence of prospective randomized controlled data investigating the efficacy of FR, its status as first line treatment of SIAD remains questionable. Vaptans offer a novel treatment approach for SIAD that specifically targets the pathophysiological cause of the disorder and their efficacy has been confirmed in large multicenter placebo RCTs. If future studies confirm the observations of the Hyponatraemia Registry that FR is ineffective in a significant proportion of patients with SIAD, bearing in mind that correction of hyponatraemia improves morbidity and mortality, we predict for the future that vaptan therapy will become more mainstay in the management of SIAD.

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Pituitary Disorders Following Traumatic Brain Injury

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Abbreviation

ACTH	Adrenocorticotrophic hormone
FSH	Follicle stimulating hormone
GH	Growth hormone
LH	Luteinizing hormone
TBI	Traumatic brain injury
TSH	Thyroid stimulating hormone

Introduction

A considerable body of research has emerged over the last 10 years, which points to a significant incidence of pituitary dysfunction in survivors of moderate or severe traumatic brain injury (TBI). Although the key research groups have differed in terms of patient populations studied and diagnostic tests that they employed, most papers with robust methodology estimate that chronic hypopituitarism occurs in 20%–30% of survivors of moderate/severe TBI. The prevalence in patients with mild TBI is much lower. As TBI is incredibly common, the potential number of patients with undiagnosed hypopituitarism is therefore substantial.

It is clear from autopsy studies that vascular damage to the pituitary occurs early after TBI, so that patients may develop hypopituitarism, which is clinically significant, in the acute phase of after brain injury. Some patients recover normal endocrine function in the ensuing months, whereas new hormone deficits may appear in the same time period; pituitary status is therefore unstable following brain injury. The challenge to the endocrine community is how to manage screening and treatment of such a large population of patients, in whom secretory function may vary over several months. Glucocorticoid and growth hormone deficiency are optimally defined by dynamic tests of hormone secretion. As TBI is common, there is a logistic difficulty in identifying which patients should be selected for screening for hypopituitarism. Screening all survivors of moderate/severe TBI with dynamic testing using insulin tolerance testing (ITT) or glucagon stimulation testing (GST) would overwhelm the diagnostic capacity of most endocrine units, so the outcome of studies to identify who does—or, more importantly, who does not—require dynamic testing are important in order to rationalize our approach to this common, and treatable, complication of TBI. In this article, we will document the evidence for posttraumatic hypopituitarism and discuss strategies to investigate and manage this endocrine challenge.

Prevalence of Pituitary Disorders Following TBI

Anterior Pituitary Disorders

Acute hypopituitarism

Post mortem studies conducted in cases of fatal brain injury show that vascular pituitary damage occurs very soon after TBI. Although victims of TBI who die immediately on brain injury display no evidence of pituitary vascular damage, histological features of infarction or hemorrhage occur as early as 3 h after brain injury (Salehi *et al.*, 2007). It is reasonable to expect, therefore, that pituitary dysfunction may occur early after TBI, and this hypothesis is borne out by experimental data; Agha and colleagues showed that blunted cortisol and GH responses to glucagon testing occurred as early as 7–10 days after TBI (Agha *et al.*, 2004a). Hyperprolactinemia and hypogonadism also occurred, though both of these hormonal abnormalities are seen in patients in intensive care patients, as part of the response to acute stress, and so it is difficult to know how much these hormone abnormalities reflect structural pituitary damage.

In a prospective study of 100 patients with moderate to severe TBI, Hannon and colleagues reported plasma cortisol concentrations were measured at day 1,3,5,7, and 10, and compared the results to those derived from a control group of patients, who were admitted to the intensive care unit following major vascular surgery (Hannon *et al.*, 2013). The patients in the control group all had the expected stress cortisol response to surgery, with plasma cortisol concentrations >500 nmol/L (18.2 μg/dL) at day 1, and consistently >300 nmol/L (10.87 μg/dL) for the subsequent days of hospitalization. In contrast, 78 of the TBI cohort had at least one plasma concentration <300 nmol/L, and some displayed evidence of severe cortisol deficiency, for patients recovering from life-threatening stress. In some patients, the dip in plasma cortisol concentrations were very transient, compatible with an acute, reversible, contusion injury, whereas in others, the ACTH/cortisol deficiency persisted; 6/32 patients had chronic glucocorticoid deficiency, as measured by impaired cortisol responses to ITT or GST and SST. The results of this study suggest that acute glucocorticoid deficiency is more common than previously believed and it is frequently transient in nature; it raises the interesting theory that identification and treatment of early glucocorticoid deficiency may lead to therapy which could improve the clinical outcome from TBI. However, this remains to be tested by intervention studies.

Natural history of post TBI hypopituitarism

The results of carefully conducted prospective studies indicate that pituitary function is inconsistent in the months after TBI, with acute deficiencies recovering in some patients, whereas other patients only develop hypopituitarism well after the acute event. Aimaretti and colleagues evaluated 70 patients with TBI (including mild TBI) 3 months following the injury and then repeated the testing at 12 months. At 3 months 32.8% of the patients had at least one pituitary hormone deficiency. When testing was repeated at 12 months this figure had fallen to 22.2%. Interestingly, in 5.5% of TBI patients with no deficit at 3 months, an isolated pituitary deficit was recorded at retesting. Early panhypopituitarism was diagnosed in 5.7% which persisted in all when they underwent repeat evaluation at 12 months. In contrast, isolated pituitary deficiencies, lasted until repeat testing in only 25% of patients (Aimaretti *et al.*, 2005). This study would suggest that more severe early hypopituitarism is more likely to persist and develop into chronic hypopituitarism.

In our own unit, 50 patients with severe or moderate head injury who were evaluated during the acute phase of TBI, underwent repeat dynamic pituitary stimulation at 6 months, and at 12 months after injury. Forty patients had gonadotrophin deficiency in the acute phase, 85% of these patients had recovered by 12 months, as would be expected with an acute adaptive response to critical injury. Evaluation of the growth hormone response to glucagon showed a lower incidence of acute deficiency however (18%), with a more erratic picture of recovery. At 6 months, five patients recovered function and two new deficiencies were subsequently detected at 12 months. The cortisol response was also inconsistent, 16% ($n=8$) of patients had suboptimal responses to glucagon testing in the acute phase, when these patients were retested however, four patients recovered and five developed new deficiencies. All nine patients that were had an insufficient cortisol response at 6 months persisted at 9 months. These results are depicted in **Fig. 1**. This highlights that pituitary dysfunction post TBI may be transient and that periodic evaluation is required even if the initial endocrine evaluation is normal (Agha *et al.*, 2005a).

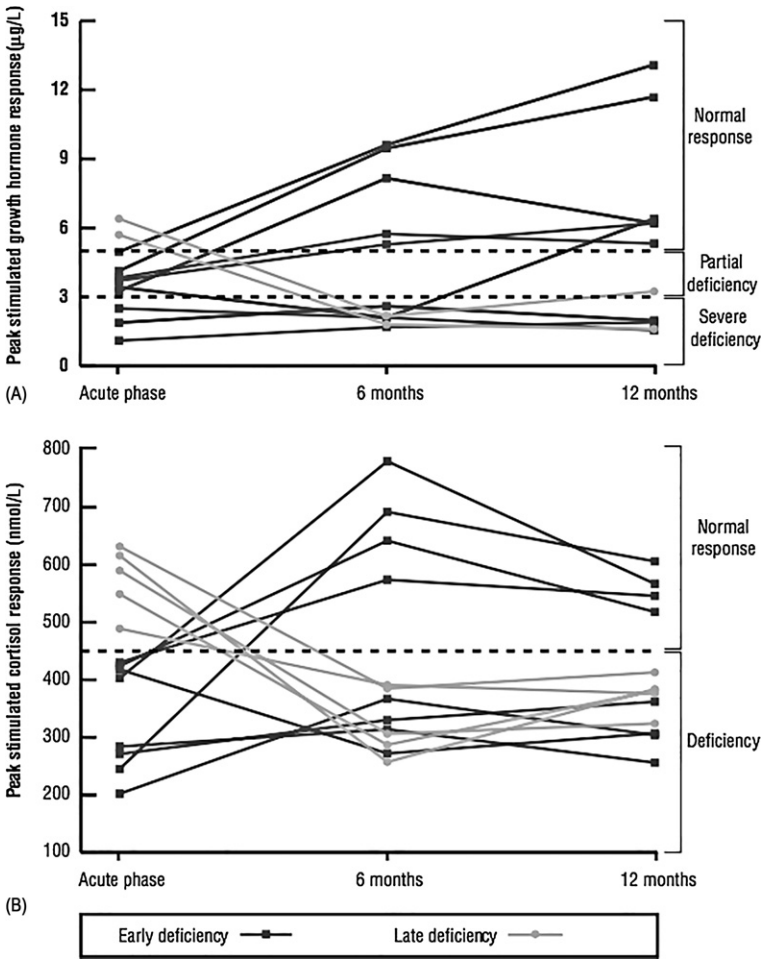

Fig. 1 Peak growth hormone (A) and cortisol (B) responses to glucagon stimulation in patients with early and late growth hormone and adrenocorticotropin hormone (ACTH) deficiencies. Reprinted with permission Agha, A., Phillips, J., O'Kelly, P., Tormey, W. and Thompson, C. J. (2005a). The natural history of post-traumatic hypopituitarism: Implications for assessment and treatment. *The American Journal of Medicine* **118**, 1416.

A similar picture of fluctuating pituitary function was demonstrated were found by Tanriverdi, who assessed pituitary function in the acute phase and after 12 months in 52 patients with mild/moderate or severe TBI. Five patients demonstrated subnormal cortisol plasma concentrations in the acute phase (<7 μg/dL), four of these recovered however nine patients developed new ACTH deficiency when they were retested at 12 months by low dose synacthen test. The GH axis showed a similar response; 10% were deficient in the acute phase (IGF-I concentration <84 ng/mL), 5% recovered and 13% had a new deficiency at 12 months (combined test, GHRH and GH-releasing peptide (GHRP-6)). Overall, new deficiencies were present in 51.9% of patients on repeat testing (Tanriverdi *et al.*, 2006).

The different recovery rates observed in these studies likely relates to the timing of testing (acute phase vs. 3 months post injury), the diagnostic criteria used and the severity of TBI. It is therefore difficult to predict if hypopituitarism will be transient or chronic. The Aimaretti data suggests however, that if a patient has panhypopituitarism on initial testing, these deficiencies are unlikely to recover.

Chronic hypopituitarism

The prevalence rate of chronic pituitary dysfunction in long term survivors of TBI is the subject of vigorous debate in the literature. **Table 1** shows the results of published studies. Some of the studies report wildly discrepant estimations of the frequency of hormonal deficits, which mainly reflect differences in patient populations studied and methods of screening for hypopituitarism.

One of the earliest attempts to estimate the prevalence of anterior pituitary dysfunction following moderate/severe TBI examined the response of 22 patients, a median of 26 months post injury, to gold standard insulin tolerance testing. The results revealed GH deficiency in four patients, ACTH deficiency in one patient and one patient had gonadotrophin deficiency, suggesting a significant prevalence of chronic hypopituitarism. This landmark study prompted a more widespread investigation of pituitary function after TBI. A subsequent larger study measured the GH response to glucagon stimulation in 70 survivors of TBI, and confirmed a significant minority (15%) had severe GH deficiency (peak GH <3 μg/L). This suggested that almost one patient in six survivors of TBI might benefit from GH treatment. The effect of choice of diagnostic test was underlined by the discrepant cortisol data in this study; although 45% of subjects had a low morning cortisol at <7 μg/L (193 nmol/L), only five patients failed to mount a normal cortisol response of >500 nmol/L to stimulatory testing. This underlines therefore the need for dynamic testing for GH and ACTH/cortisol deficiencies.

A comprehensive study of a large number of chronic survivors of moderate/severe TBI (102 patients), who were studied at a median of 19 months post injury confirmed the significant incidence of anterior pituitary hormone deficits. The glucagon stimulation test (GST) was used as an initial screening test for both GH and ACTH deficiency, but any identified deficits were confirmed by a second test; the ITT or synacthen (where there was a history of seizures) for ACTH, and a GHRH/arginine test for GH deficiency. Patients had to fail both dynamic tests before confirmation of hormone deficits. Using these rigorous criteria, and robust testing, 11% of patients were shown to have severe GH deficiency, 13% had ACTH deficiency, 12% gonadotrophin deficiency, and 1% had TSH deficiency. Several other studies have since confirmed these findings (Aimaretti *et al.*, 2004; Alavi *et al.*, 2016; Bondanelli *et al.*, 2004; Popovic *et al.*, 2004; Leal-Cerro *et al.*, 2005; Schneider *et al.*, 2008) (see **Table 1**).

Other studies however have found a lower prevalence of hypopituitarism post TBI. Kokshoorn *et al.* evaluated 112 patients using ITT and fasting hormone profile, at least 1 year after TBI. Hypopituitarism was diagnosed in only 5.4% of patients (Kokshoorn *et al.*, 2011), prompting the authors to speculate that the prevalence of hypopituitarism after TBI was much lower than previously published. However, they excluded over 90% of eligible patients, and included a large number of patients with mild (Glasgow coma scale 14–15) TBI, who would be expected to have minimal hypopituitarism. Thus, although gold standard testing with ITT was used, the selection of mild TBI patients meant that they were predetermined to identify lower rates of pituitary dysfunction than previous studies.

Table 1 Reported prevalence of chronic hypopituitarism

Author	*Median duration (months)*	*N*	*Total HPit (%)*	*GH (<3 ng/mL)*	*ACTH*	*GN*	*TSH*
Kelly *et al.* (2000)	26	22	36	18	5	23	5
Lieberman *et al.* (2001)	13	70	69	15	46	1	22
Agha *et al.* (2004b)	17	102	28	8	13	12	1
Aimaretti *et al.* (2004)	3	100	35	21	8	17	5
Bondanelli *et al.* (2004)	12–64	50	54	8	0	14	10
Leal-Cerro *et al.* (2005)	>12	99	24	6	6	17	6
Klose *et al.* (2007a)	13	104	15	15	5	2	2
Schneider *et al.* (2008)	12	70	36	10	9	21	3
Tanriverdi *et al.* (2008)	36	30	30	23	7	0	0
Park *et al.* (2010)	16	45	31	20	13	18	7
Kokshoorn *et al.*, 2011	50[a]	112	5	3.6	2	1	0
Hannon *et al.* (2013)	14	100	34	19	18	3	0
Silva *et al.* (2015)	40	166	31	21	10	12	8

[a]Mean duration.

Kokshoorn proceeded to review 14 studies with reported pituitary function testing post TBI, and concluded that the chosen dynamic test influenced the likelihood of identifying hormone deficits. The prevalence of GH deficiency varied from 2% to 39%, with higher rates in studies using the GST compared with studies using GHRH-arginine; only two studies used a combination of two tests (Kokshoorn *et al.*, 2010). The tests used to diagnose GH deficiency therefore can significantly impact the prevalence of GH deficiency; this applies to all disease processes which can potentially altered pituitary function.

Klose *et al.* evaluated the GH axis in 439 patients with a history of TBI and 124 health controls, and found a prevalence of severe GHD of 19%, when the standard guidelines were applied and testing was confirmed with the pyridostigmine (PD)—GHRH test (Klose *et al.*, 2014). However when local cut-off values were used derived from the healthy population the prevalence was 12%. When a combined PD-GHRH test and ITT were performed the prevalence was estimated at only 1%. This is significantly lower than all previous studies. Sixty-nine percentage of the patients in this study suffered mild traumatic brain injury, whereas previous studies, which published high rates of pituitary damage, had limited their analysis to patients with moderate to severe TBI. However, this study does highlight that the test used to diagnose the deficiency may have a significant impact on the prevalence of observed hypopituitarism and that when reporting data on GH and ACTH deficiency it is important to report the results from age matched controls to establish normative data.

A meta-analysis of 19 studies published in 2007 found that the pooled prevalence of anterior hypopituitarism after TBI was 27.5% (95% CI 22.8%–28.9%) with a range between studies of 37.5% to 55%. Associated aneurysmal subarachnoid hemorrhage (SAH) is associated with a significantly higher prevalence of pituitary dysfunction, with a prevalence of 47% (95% CI 37.5%-–56.8%) (Schneider *et al.*, 2007a). GH deficiency and gonadotrophin deficiencies were the most commonly observed pituitary deficiencies following TBI in this meta-analysis (Schneider *et al.*, 2007a). A further meta-analysis of 27 studies reported that the prevalence of anterior pituitary dysfunction, when all pituitary axes were interrogated, was 31.6% (95% CI 23.6%–40.1%) (Lauzier *et al.*, 2014). Significant heterogeneity in time of testing, and method of assessment for each anterior pituitary axis was considered to account for the large part of interstudy discrepancy.

It is clear therefore that several factors affect the prevalence of TBI, including the time interval following the injury, the cut-off values used for diagnosis of hypopituitarism and the severity of brain injury studied (pituitary dysfunction is more common after severe TBI) (Klose *et al.*, 2007a). Whilst there are significant challenges with diagnosing pituitary dysfunction post TBI, these prevalence studies would indicate that hypopituitarism is a reasonably frequent occurrence post moderate to severe TBI.

Posterior Pituitary Disorders

As the posterior pituitary gland secretes the antidiuretic hormone, vasopressin (AVP), posterior pituitary damage may result in disordered water balance, manifesting either as diabetes insipidus or hyponatremia. Although both disorders have been clinically recognized complications of TBI for many years, there is relatively little hard data available regarding the prevalence of posterior pituitary dysfunction following head injury.

Diabetes insipidus

Diabetes insipidus manifests as the passage of large volumes of hypotonic urine. Although most patients with new onset diabetes insipidus can respond to rising plasma sodium concentrations with appropriate thirst (Thompson and Baylis, 1987), patients in intensive care, who are either unable to recognize thirst due to cognitive impairment, or who cannot access sufficient fluid replacement, may develop marked hypernatremia (Hannon *et al.*, 2011). As a result, poorly treated diabetes insipidus can contribute to delayed recovery, and even increased mortality in TBI.

In an initial study of 50 patients with acute moderate to severe TBI, Agha and colleagues found that 13 patients (26%) had acute cranial diabetes insipidus (CDI) in the early post TBI phase. This resolved in nine of these patients by 6 months (Agha *et al.*, 2005a). Subsequent studies would suggest a prevalence of 3%–26% of patients of acute CDI post TBI (Agha *et al.*, 2004a; Hadjizacharia *et al.*, 2008; Boughey *et al.*, 2004).

In a larger prospective study of 100 TBI patients who were assessed daily for development of abnormal water balance, diabetes insipidus was diagnosed using the well described Seckl and Dunger criteria (Seckl and Dunger, 1989). A surprising large number of patients (51%) developed hypernatremia, due mainly to acute diabetes insipidus, though some cases had simple dehydration; 21.6% had persistent DI after day 10 of admission (Hannon *et al.*, 2013). Persistent diabetes insipidus in this cohort was strongly predictive of mortality. Other studies have confirmed both transient and permanent DI are poor prognostic indictors post TBI and are associated with an increased mortality, particularly when hypernatremia develops (Maggiore *et al.*, 2009; Hadjizacharia *et al.*, 2008).

Most cases of posttraumatic diabetes insipidus (DI) are transient. However, a significant proportion have persistent DI. Agha *et al.* evaluated 102 patients who suffered moderate or severe TBI, using standard water deprivation test at a median of 17 months post injury (Agha *et al.*, 2004c). Although 21.6% of patients had evidence from the case notes of diabetes insipidus in the acute period following TBI, only 6.9% of patients had abnormal water deprivation tests. This data suggested a higher incidence of post TBI DI than other studies, which reported a prevalence of 0%–2.9% (Aimaretti *et al.*, 2005, Kreitschmann-Andermahr *et al.*, 2004); the earlier studies did not use formal water deprivation tests and so may have underreported the true incidence. However, it should be recognized also that only 2% of patients in Agha's study had DI of sufficient severity to warrant therapy with desmopressin; it may be that the true figure for symptomatic DI is closer to 2%. Patients with both transient and permanent diabetes insipidus in

this cohort were more likely to have a more severe traumatic brain injury, as assessed by Glasgow coma scale, and evidence of cerebral edema on imaging studies (Agha *et al.*, 2004c).

Whilst the majority of patients with diabetes insipidus will have an intact thirst response and will increase their fluid intake to replace renal water losses, patients with traumatic brain injury are particularly prone to the development of hypernatremia. This is largely due to decreased level of consciousness secondary to brain injury, cerebral edema, sedation for airway management or a combination of factors. Adipsic DI (absent thirst response) has also been reported following head injury (Cuesta *et al.*, 2017). In studies where thirst response was formally measured however, the thirst response is typically intact and adipsic DI is a rare complication of TBI (Agha *et al.*, 2004c).

Rarely, diabetes insipidus may progress to transient SIADH followed by recurrent diabetes insipidus. This is known as the triple phase response. It is hypothesized that the initial phase of DI is an acute contusion injury, with a period of SIAD occurring due to the release of preformed AVP from damaged neurohypophyseal cells, resulting in antidiuresis and water retention. Permanent DI follows as neuronal gliosis attenuates vasopressin secretion. This is thought to occur in less than 5% of neurosurgical diabetes insipidus (Hannon *et al.*, 2012). However, it is important to monitor for this potential complication as continued desmopressin administration during the development of SIADH may lead to the development of profound hyponatremia.

Hyponatremia

Hyponatremia has been reported to occur in 2.3%–36.6% of cases of TBI (Lieberman *et al.*, 2001; Born *et al.*, 1985; Sherlock *et al.*, 2009). The differential diagnosis for hyponatremia is substantial (**Table 2**), and includes Syndrome of Inappropriate ADH secretion (SIADH), glucocorticoid deficiency, and hypotonic intravenous fluids. SIADH is the commonest cause of hyponatremia following TBI, and occurs in approximately 12.7% of patients (Hannon *et al.*, 2014). SIADH typically develops 1–2 days after the trauma, and is usually transient, with complete return to normality. It is important to differentiate SIADH from other causes of hyponatremia as incorrect management may have significant detrimental effects; severe symptomatic hyponatremia, masquerading as SIADH, has been reported to be the principle manifestation of acute glucocorticoid deficiency following TBI (Agha *et al.*, 2005c). **Table 2** demonstrates the different treatment strategies for post TBI hyponatremia, according to etiology.

In a prospective study of 100 patients with moderate to severe TBI, 15% developed hyponatremia. Thirteen of these patients had evidence of glucocorticoid deficiency, as defined by a 09.00 am plasma cortisol <300 nmol. All 13 patients normalized their serum sodium concentrations with the administration of glucocorticoid therapy. The remaining two patients had SIADH, which responded to fluid restriction and resolved. All 15 patients had a normal serum sodium concentration when re-evaluated at 6 months; there was no case of persistent hyponatremia (Hannon *et al.*, 2013). Although hyponatremia following TBI is self-limiting, it may be severe and present with features of cerebral irritation. It is therefore important to monitor plasma sodium concentration following TBI and to implement appropriate evaluation to determine the cause of the hyponatremia.

Cerebral salt wasting is a rare condition that is characterized by marked natriuresis, resulting in hypovolemic hyponatremia. Some authorities have questioned whether cerebral salt wasting really exists, or whether it represents escape from antidiuresis. Although we think that cerebral salt wasting is rare, and our prospective studies of hyponatremia post TBI and SAH, have revealed only very rare cases (Hannon *et al.*, 2014), the occasional presentation of patients with profound diuresis and natriuresis, associated with hypotension and decreased central venous pressure, is so characteristic, and so different from the classical presentation of a case of SIADH, that we feel it merits a special, though rare, diagnostic status.

Pathophysiology of Pituitary Disease Following TBI

There are several potential pathological mechanisms of hypopituitarism post TBI; pituitary gland compression due to raised intracranial pressure and cerebral edema, skull fracture with disruption of the vascular supply to the pituitary gland, hypoxic insult, direct mechanical injury and autoimmunity with antipituitary and antihypothalamus antibodies have all been studied.

The pituitary gland is situated in the median section of the middle cranial fossa in the sphenoid bone. The majority of the vascular supply to the anterior lobe of the pituitary is from the long hypophyseal vessels. The hypophyseal vessels become the hypophyseal portal circulation that transports hypothalamic neuropeptides from the hypothalamus to the pituitary gland.

Table 2 Differential diagnosis of hyponatremia in TBI

Causes of hyponatremia following TBI	*Treatment*
SIADH	Fluid restriction, vaptans
Acute glucocorticoid deficiency	IV hydrocortisone
Inappropriate intravenous fluids	Frusemide, cessation of diuretics
Cerebral salt wasting	High volume IV saline
Drugs, for example, desmopressin, carbamazepine, loop diuretics	Cessation of drug
Triple phase response of acute diabetes insipidus	dDAVP/fluid restriction/dDAVP

The corticotroph and thyrotroph cells are located anteriorly and medially in the anterior pituitary, and derive their vascular supply from the short portal veins. The somatotroph cells are located more laterally and are typically supplied by the long portal veins (Gorczyca and Hardy, 1987). The blood supply to the posterior lobe of the pituitary gland differs and is supplied by an anastomotic arterial circle derived from the inferior hypophyseal vessels, which arise from the internal carotid artery, as they enter the cavernous sinus.

The vascular insult theory for the pathophysiology of pituitary injury post TBI is currently the foremost hypothesis. The long hypophyseal vessels and the portal circulation in the stalk are particularly vulnerable to injury from mechanical and shearing forces at the time of brain injury, as they arise in the subarachnoid space and travel through the diaphragm sella. Interruption of this vascular supply will result in anterior lobe infarction with resultant hypopituitarism. This theory is supported by autopsy studies from the 1960s to 1970s which demonstrated tissue necrosis in the anterior pituitary of patients with traumatic brain injury (Daniel *et al.*, 1959; Ceballos, 1966). Subsequently more recent studies have shown 43% of patients who survive severe traumatic brain injury for longer than 3 h will have pituitary infarction (Salehi *et al.*, 2007) (**Fig. 2**).

The vascular hypothesis is further supported by the pattern of hormonal loss observed in pituitary dysfunction post TBI. Somatotroph and gonadotroph cells lie laterally and are supplied by the long hypophyseal portal vessels, interruption of this blood supply will result in infarction of these cells (Dusick *et al.*, 2012), the GH and Gonadotrophic axes therefore, are the most commonly affected pituitary axes (Schneider *et al.*, 2007a). By contrast, the inferior hypophyseal arteries are much less vulnerable to injury and therefore infarction of the posterior pituitary is therefore rare. Furthermore, magnetic resonance imaging studies of the pituitary gland in the acute phase post TBI have demonstrated an enlarged pituitary when compared to healthy controls with or without associated hemorrhage, infarction or signal loss (Maiya *et al.*, 2008). Schneider *et al.* demonstrated that patients with TBI in the chronic phase often demonstrate loss of pituitary volume, empty sella, loss of pituitary gland heterogeneity or absent posterior pituitary signal. When compared to TBI patients without hypopituitarism these abnormalities occurred in 80% of the patients with hypopituitarism in comparison to 29% of those without hypopituitarism ($P = .032$) (Schneider *et al.*, 2007b).

Secondary insults from hypoxia, hypotension, and cerebral edema frequently complicate major trauma and may contribute to the development of hypopituitarism. Evidence for this is derived from autopsy studies that failed to demonstrate vascular injury in patients with TBI who died less than 3 h. In contrast to this, 43% of patients who survived for between 3 h and 1 week after injury had evidence of pituitary infarction (Salehi *et al.*, 2007). Harper and colleagues examined pituitary infarcts in 38 of 100 consecutive patients with fatal closed head injuries. All patients with large- or medium-sized pituitary infarctions had evidence of raised ICP at some point. The authors concluded that disruption of the portal blood supply, due to raised ICP or direct vascular insult, was the possible mechanism of anterior lobe infarction (Tanriverdi *et al.*, 2015; Harper *et al.*, 1986). It is important to consider that these studies focused on fatal TBI. These patients were likely to have severe injuries with associated hypotension, hypoxia, ischemia and cerebral edema, which limits the ability to generalize these findings to patients with nonfatal TBI.

Autoimmunity has also been hypothesized as a possible contributing mechanism. Antipituitary (APA) and antihypothalamic antibodies (AHA) have been demonstrated in idiopathic and autoimmune hypopituitarism (De Bellis *et al.*, 2014). Observational studies in TBI have demonstrated that pituitary dysfunction post TBI was higher in APA positive patients (46.2%) versus negative patients (12.5%) (Tanriverdi *et al.*, 2008). This association was also observed in 61 boxers when they were compared to normal controls; pituitary dysfunction was significantly higher in AHA positive boxers (46.2%) than in AHA negative boxers (10.4%) with an odds ratio of 7.37, 95% CI (1.8–30.8) (Tanriverdi *et al.*, 2010). Whilst it is possible that autoimmune processes play a role in the development of hypopituitarism post TBI, the exact mechanism behind this is unknown. Furthermore, as this is observational data, causality has not been shown, therefore this observed high incidence of APA may be a response to tissue damage rather than a causative factor.

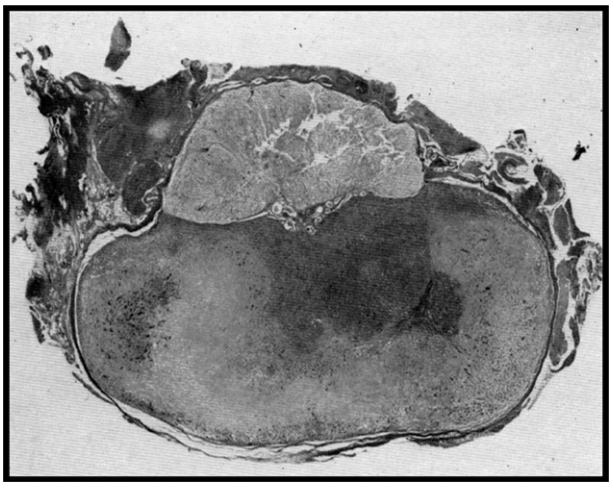

Fig. 2 Pituitary pathological specimen—demonstrating pituitary infarction in the long hypophyseal vein distribution (Daniel *et al.*, 1959).

Whilst the exact mechanism remains unclear, it is likely that the pathophysiology is multifaceted and is likely an interplay between mechanical injury to the pituitary gland or hypothalamus, complicated by secondary insults such as hypotension and raised intracranial pressure and the transient stress of critical illness with resultant inflammatory response.

Predictors of Hypopituitarism Following TBI

Identifying predictors of hypopituitarism in TBI is of paramount importance in order to design appropriate screening strategies for patients with TBI. The suggested predictors of hypopituitarism post TBI include early pituitary dysfunction, raised ICP, radiological changes, ITU admission, and severity of injury.

Early pituitary dysfunction as a predictor

Several studies have evaluated early pituitary dysfunction as a predictor of the development of chronic hypopituitarism. In a prospective study of pituitary dysfunction in the acute setting following TBI, pituitary deficiencies were common in the acute phase (80% of patients had gonadotrophin deficiency in the acute phase), but 85% recovered on repeat testing at 12 months. In fact when patients were retested, some patients who had intact pituitary dysfunction earlier had developed new deficiencies (Agha *et al.*, 2005a). The authors were unable to identify factors predictive of persistent hypopituitarism in this study. Similar data was presented by Tanriverdi and colleagues, who reported that in the acute phase following TBI, 56.5% of patients had at least one pituitary hormone deficiency, with gonadotrophin deficiency the most common (41.6%). They described a recovery rate of 57.7% of patients (Tanriverdi *et al.*, 2006). In this article, there was no discernible pattern to identify those at higher risk of permanent pituitary deficiencies.

Neither of these studies had a control group of critically unwell patients without known pituitary dysfunction. Hannon *et al.* compared sequential plasma cortisol levels in acute TBI patients and compared them to matched controls who had undergone vascular surgery and were cared for in the ICU. All 15 of the control patients had a plasma cortisol concentration >300 nmol/L on repeated testing, however 78% of the TBI patients had at least one plasma cortisol concentration <300 nmol/L. However, when repeat testing was performed 12 months later 6 of 32 patients tested were cortisol deficient, all of whom had acute cortisol deficiency and/or cranial diabetes insipidus in the acute phase. Lower mean plasma cortisol (over the 10 days of the study) and lower plasma cortisol level at day 10 of the study (final day) were strongly associated with the development of chronic hypopituitarism of any pituitary axis ($P=.049$, $P=.015$, respectively) (Hannon *et al.*, 2013).

Severity of trauma as a predictor

The pre-resuscitation Glasgow coma score (GCS) is the most commonly used method to assess the severity of traumatic brain injury. It is score out of a maximum score of 15, mild TBI is graded 13–15, moderate 9–12 and severe is 3–8. Increased trauma severity (as measured by low initial GCS) and number of days of intubation and hospitalization (Herrmann *et al.*, 2006) have been shown to be independent predictors of posttraumatic hypopituitarism in some studies, however, the results in individual studies were not conclusive (Klose *et al.*, 2007a). However, two meta-analysis have been undertaken, both of which identified GCS as a predictor of the development of hypopituitarism following TBI (Schneider *et al.*, 2007a; Lauzier *et al.*, 2014).

Radiological changes as a predictor

Agha *et al.* did not find any association between the initial cranial CT imaging results and hypopituitarism in patients with moderate and severe TBI (Agha *et al.*, 2004b), but some other studies did find an association between chronic hypopituitarism and abnormal cranial CT findings during the acute phase. A meta-analysis of six studies, including 357 patients, found that skull fractures on CT imaging were associated with an increased risk of developing anterior pituitary dysfunction (Lauzier *et al.*, 2014). Brain edema on CT imaging has not been shown to be predictive of pituitary dysfunction in larger studies (Agha *et al.*, 2004b), however one smaller study demonstrated an association between diffuse brain swelling on CT and hypopituitarism (Kelly *et al.*, 2000). Two separate studies have quantitatively reported the effect of secondary brain injuries (hypoxemia and hypotension) on pituitary function, but neither study demonstrated a predictive association (Krahulik *et al.*, 2010; Bavisetty *et al.*, 2008).

Testing/Screening for Pituitary Disease Post TBI

Screening for pituitary disease in TBI presents significant logistical and resource difficulties. This reflects the large number of patients who, as roads become more congested, develop brain injuries, and the requirement for dynamic testing for GH and cortisol deficiency. Whilst the optimal timing for screening for pituitary insufficiency is relatively straight forward, establishing which groups to screen—or not to screen—remains challenging. There continues to be much debate as to the ideal approach to screening in traumatic brain injury.

Acute hypopituitarism

The priority in acute hypopituitarism is to identify the urgent and potentially life-threatening pituitary deficiencies of ACTH and AVP. Failure to correctly identify and treat affected patients may result in increased morbidity and mortality.

Dynamic testing is not appropriate in the acute setting of TBI. The short synacthen test requires time for the adrenal glands to atrophy and is therefore not suitable in the acute setting. The insulin tolerance test may precipitate seizures and as patients with TBI have a reduced seizure threshold this should be avoided. In practice, a 09:00 am plasma cortisol measurement is a reasonable alternative. In critical illness, the normal diurnal variation of cortisol is typically lost, however a 09.00 am plasma cortisol concentration >300 nmol/L has been determined as normal response in our institution (Hannon *et al.*, 2013). Establishing a safe cut-off value for basal cortisol levels requires a high sensitivity due to the potential repercussions of a missed diagnosis. It is also important to consider that serum cortisol concentrations of between 300 and 500 nmol/L do not necessarily exclude acute ACTH deficiency and empiric glucocorticoid cover should be considered if there is a clinical suspicion of hypoadrenalism, particularly in the presence of hyponatremia, hypoglycemia or profound hypotension.

Serum sodium concentrations and urine output should be monitored closely for the development of CDI. The diagnosis of CDI should be made if the Seckl and Dunger criteria are met; plasma sodium >145 nmol/L in the presence of hypotonic (urine osmolality <300 mosmol/kg) polyuria (>300 mL/h for two consecutive hours or >3 L/day) (Hannon *et al.*, 2012). Other potential causes of polyuria, such as diuretic use, steroid induced hyperglycemia or renal impairment should also be excluded. TSH, GH, and Gonadotrophin axes should not be tested in the acute setting as, deficiency in these axes is typically transient, in response to stress (Agha *et al.*, 2005a) (**Table 3**).

Chronic hypopituitarism

Patient selection

Identifying patients that require screening is imperative and is the subject of ongoing debate. A number of guidelines exist which have suggested criteria for screening, which include moderate and severe TBI, long duration of hospital admission and symptom based screening (Aimaretti and Ghigo, 2007; Ghigo *et al.*, 2005; Tan *et al.*, 2017; Garrahy *et al.*, 2017).

In order to determine whether symptom-based screening would improve the diagnostic yield of dynamic testing, we compared the rate of hypopituitarism identified from a referral strategy based only on symptoms suggestive of pituitary dysfunction. When compared with universal screening, whether or not symptoms were present, nonspecific symptoms such as weight loss and anergia produced no higher rate of pituitary dysfunction. In contrast, patients who were referred on the basis of symptoms of gonadal dysfunction, such as erectile dysfunction, menstrual disturbance and loss of libido, were highly likely to be diagnosed to have hypopituitarism (Cuesta *et al.*, 2016). This evidence supports pituitary screening in any patient with gonadal dysfunction following traumatic brain injury but questions the utility of non-specific symptom based screening suggested by some guidelines (Gasco *et al.*, 2012; Aimaretti and Ghigo, 2007; Ghigo *et al.*, 2005; Tan *et al.*, 2017). As the rate of hypopituitarism was no different in patients with nonspecific symptoms and asymptomatic patients, it also suggests that a symptom-based screening process may miss some patients who have asymptomatic hypopituitarism.

Screening based on the severity of injury is also advocated by expert guidelines (Ghigo *et al.*, 2005; Tan *et al.*, 2017). Schneider's meta-analysis reported showed pooled prevalence rates of hypopituitarism in mild, moderate, and severe TBI of 17%, 11%, and 35%, respectively (Schneider *et al.*, 2007a). Therefore, if screening is performed only on those with moderate and severe injury, a significant subset of patients in the mild TBI group will be misdiagnosed.

Timing of screening

Pituitary function after TBI is dynamic; some acute deficiencies will resolve on retesting and other patients may develop new deficiencies over time (Agha *et al.*, 2005a; Tanriverdi *et al.*, 2006). It is reasonable therefore to retest all patients who present with acute deficiencies to assess for resolution. New deficiencies rarely appear after 6 months; therefore, it is sensible to retest at this stage. Although rare, late recovery has been reported 5 years after TBI therefore clinicians should be aware of this and consider retesting if symptoms change (Agha *et al.*, 2005b).

Screening test selection

The expert guidelines recommend basal hormone testing as the initial screening test (see **Fig. 3**) (Tan *et al.*, 2017; Ghigo *et al.*, 2005). If there is any abnormality noted on these tests or if there is a high index of suspicion from symptoms, then provocative testing should be performed. The insulin tolerance test remains the gold standard for the diagnosis of ACTH and GH deficiency. If the insulin tolerance test is contraindicated (due to seizures or heart disease), then the glucagon stimulation, GHRH-arginine test or GHRH + GHRp-6 should be considered as an alternative.

Table 3 Diagnostic tests to consider for anterior pituitary function assessment in each time frame

Timing of assessment	*Adrenal axis*	*GH axis*	*Thyroid axis*	*Gonadotrophin axis*
Acute phase	Early morning cortisol	n/a	n/a	n/a
Midterm (3–6 months)	Insulin tolerance test GST (if ITT C/I) ACTH stimulation test	Insulin Tolerance test GST (if ITT C/I) GHRH-Arg stimulation	TSH and T4	Testo/E2 + FSH/LH
Long term (>12 months)	Insulin tolerance test GST (if ITT C/I) ACTH stimulation test	Insulin tolerance test GST (if ITT C/I) GHRH-Arg stimulation	TSH and T4	Testo/E2 + FSH/LH

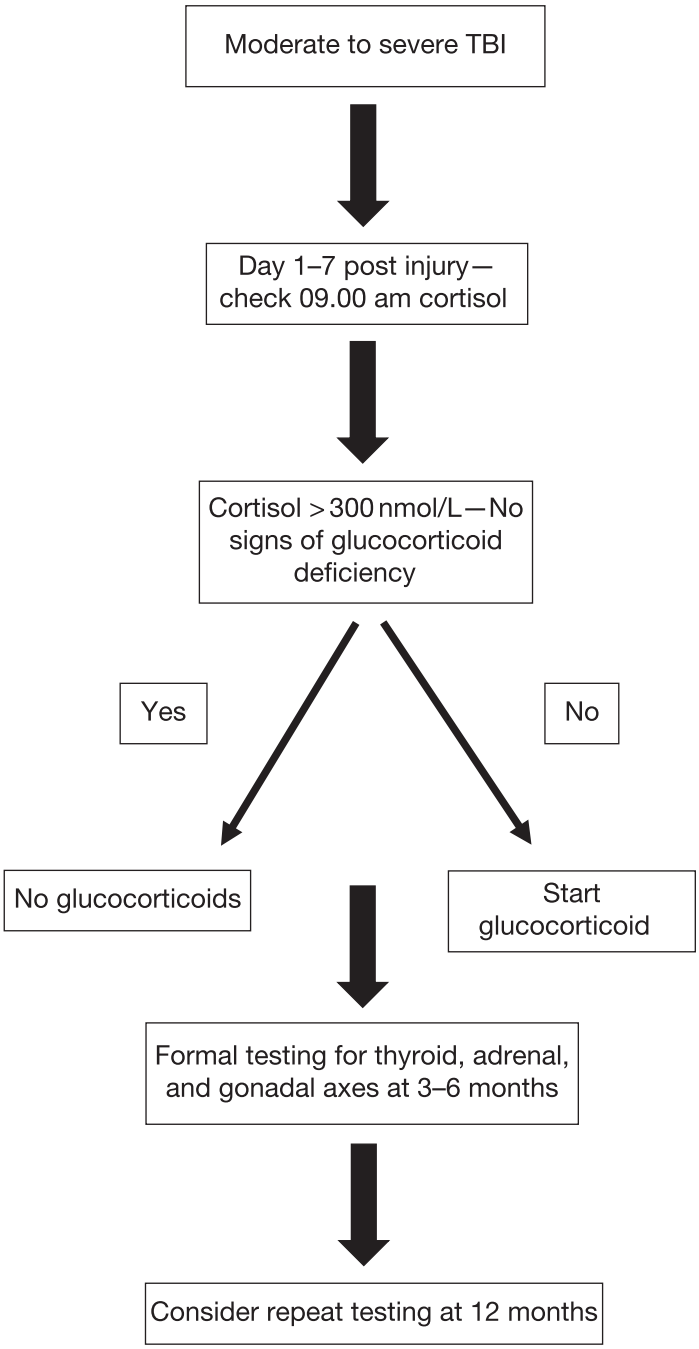

Fig. 3 Proposed algorithm for assessment of patients post TBI.

The screening tests used to make the diagnosis will affect the prevalence rate of hypopituitarism post TBI as discussed earlier. Therefore, robust universal definitions of normal cut-off values need to be developed before systematic screening can be considered in TBI.

Clinical Outcome of Pituitary Disease Post TBI

Outcome of Quality of Life/Cognitive Function

GH deficiency

Data from the KIMS database—an international database metabolic database of patients with GHD—was reported in an observational study in 2015. The authors compared patients with GHD following TBI to patients with GHD and a nonfunctioning pituitary adenoma. TBI patients showed a greater improvement in quality of life following GH therapy (change in QoL-AGHDA

score 5.0 vs. 3.5, respectively, $P = .04$) than patients with pituitary adenomas, an improvement which was sustained over 8 years of follow up (Gardner *et al.*, 2015).

A randomized, placebo-controlled assessment of the impact of GH replacement on cognitive function, in patients with GHD after TBI showed improvements in various functional and in the GH treated group. This suggests that some of the cognitive and functional disturbances seen post TBI may in fact be due to GH deficiency, and offers evidence that these disturbances may a be improved with growth hormone replacement (High *et al.*, 2010).

Other outcomes

There is increasing interest in the impact of TBI on quality of life (QOL) and other social and behavioral sequelae. A number of observational studies have sought to demonstrate an association of hypopituitarism post brain injury and QOL and cognitive outcomes. However, the results of these studies have been conflicting.

A large study of 70 survivors of TBI, conducted 6–9 months after injury, revealed that patients with hormonal deficits had a higher BMI ($P < .01$) and a worse Disability Rating Scale score (multivariate $P = .04$) compared to patients with intact pituitary function post TBI (Bavisetty *et al.*, 2008). Another study reported that patients with hypopituitarism were more likely to be overweight at follow up testing; this was attributed to GH and/or sex steroid deficiency. Otherwise there was no association found between hypopituitarism and quality of life and functional outcome in this cohort (Ulfarsson *et al.*, 2013).

Post TBI hypopituitarism is associated with an adverse lipid profile, unfavorable body composition and poorer quality of life, as measured by the Nottingham Health profile, compared to patients with intact pituitary function post TBI (Klose *et al.*, 2007b). This adverse metabolic profile was found to be independent of other confounding factors such as age, gender and BMI and was unrelated to 12 months IGF-1 and IGF-1 SD scores.

Other studies have described the metabolic effects of growth hormone deficiency and at least some of the adverse metabolic profile observed in patients post TBI likely occurs due to the decreased lipolytic activity associated with growth hormone deficiency (Klose *et al.*, 2007b; Popovic *et al.*, 2004). Furthermore, Mossberg *et al.* evaluated aerobic capacity in patients post traumatic brain injury and found reduced capacity in all patients with a greater reduction observed in GH deficient patients (Mossberg *et al.*, 2008). Similarly, Silva *et al.* found that patients with pituitary insufficiency post TBI were less likely to be working after TBI ($P = .002$), and had lower Global Assessment of Functioning (GAF) scores ($P = .03$) (Silva *et al.*, 2015).

Klose *et al.* published patient-reported outcomes in hypopituitarism post brain injury, using QOL questionnaires in 84 patients. This study failed to demonstrate an independent relationship between pituitary deficiency and QOL/fatigue (Klose *et al.*, 2015). The mixed there are undoubtedly multiple other factors that will influence QOL and fatigue after head trauma, therefore it is currently difficult to definitively conclude the precise impact of pituitary dysfunction on recovery post TBI. Further study is required but data from other causes of pituitary dysfunction may be extrapolated and it is therefore logical that pituitary dysfunction confers an adverse clinical outcome.

Conclusions

Traumatic brain injury is an important cause of morbidity and mortality, particularly in the younger population. The reported prevalence of pituitary dysfunction following brain injury varies depending on research protocol, but seems to consistently occur in 20%–30% of patients. Screening for hypopituitarism post TBI is complicated by the large volume of patients presenting with brain injury and other than severity of brain injury, there are no reliable predictors of the development of pituitary dysfunction post TBI. Therefore, all clinicians must be vigilant for signs and symptoms of pituitary dysfunction and all patients with moderate-severe TBI should be considered for pituitary evaluation.

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Folliculogenesis

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Abbreviations

ADAMTS1 ADAM metallopeptidase with thrombospondin type 1 motif 1
AIRE Autoimmune regulator
AKT AKT serine/threonine kinase
AMH Anti-Mullerian hormone
AMPK Protein kinase AMP-activated catalytic subunit alpha 1
BMP Bone morphogenetic protein
AR Androgen receptor
BMPR Bone morphogenetic receptor
CDKN1B Cyclin-dependent kinase inhibitor 1b
CEBPB CCAAT/enhancer-binding protein beta
CTGF Connective tissue growth factor
CYP11A1 Cytochrome P450 family 11 subfamily A member 1
CYP17A1 Cytochrome P450 family 17 subfamily A member 1
CYP19A1 Cytochrome P450 family 19 subfamily A member 1
EGF Epidermal growth factor
EGFR Epidermal growth factor receptor
ESR Estrogen receptor
FGF Fibroblast growth factor
FOXL2 Forkhead box l2
FSHR Follicle-stimulating hormone receptor
FOXO3A Forkhead box O3
GDF9 Growth differentiation factor 9
GJA Gap junction protein
GNRH1 Gonadotropin releasing hormone 1
HSD3B2 Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
IGF1 Insulin-like growth factor 1
IGFBP IGF binding proteins
IL1 Interleukin 1
INHA Inhibin alpha subunit
INHB Inhibin beta subunit
KIT KIT proto-oncogene receptor tyrosine kinase
KITLG Kit ligand
LHCGR Luteinizing hormone/choriogonadotropin receptor
Lhh/Dhh Hedgehog ligands
LHX8 LIM homeobox 8
MAPK Mitogen-activated protein kinase
MMP19 Matrix metallopeptidase 19
mTORC Mammalian target of rapamycin complex
NGF Nerve growth factor
NOBOX NOBOX oogenesis homeobox
NPR2 Natriuretic peptide receptor 2
PAPPA Pappalysin 1
PDE4D Phosphodiesterase 4D
PDPK1 3-Phosphoinositide-dependent protein kinase 1
PGRMC1 Progesterone receptor membrane component 1
PI3K Phosphatidylinositol 3 kinase
PLCL Phospholipase C like
PPAR Peroxisome proliferator activated receptor
PR Progesterone receptor
PTEN Phosphatase and tensin homolog
PTGER2 Prostaglandin E receptor 2
PTGS2 Prostaglandin-endoperoxide synthase 2
PTX3 Pentraxin 3
RPS6 Ribosomal protein S6
RPTOR Regulatory associated protein of MTOR complex 1
SMAD SMAD family member
SOHLH Spermatogenesis- and oogenesis-specific basic helix-loop-helix
SOX3 SRY-box 3
STAR Steroidogenic acute regulatory protein
TGF Transforming growth factor
TNF Tumor necrosis factor
TNFAIP6 TNF alpha-induced protein 6
TSC Tuberous sclerosis
VCAN Versican
VEGF Vascular endothelial growth factors
ZP Zona pellucida glycoproteins

The Ovarian Reserve of Primordial Follicles

The reserve of primordial follicles is set up before birth in humans. The formation of oocytes, a process also designed as oogenesis, precedes the formation of follicles (reviewed by Hartshorne *et al.*, 2009; Kerr *et al.*, 2013; Ackert *et al.*, 2001; Hutt *et al.*, 2006). In the human ovary, oogenesis begins at 2 weeks postconception (wpc), also referred as 4 weeks of gestation, when the first primordial germ cells are formed within the epiblast. Then, these cells migrate to the hind gut, colonize the gonadal ridges before 7 wpc and proliferate as oogonia into ovarian cysts (also called nests). The cysts also contain the

precursor granulosa cells, derived from the gonadal ridges (Hummitzsch *et al.*, 2013). After 8.5 wpc, the oogonia can cease mitosis, enlarge and develop into primary oocytes, which initiate meiotic prophase. The breakdown of the cysts, occurring between 13 and 30 wpc, leads to the formation of 30-µm-diameter primordial follicles, each consisting of a primary oocyte arrested at the diplotene stage of prophase I of meiosis and surrounded by one layer of flattened granulosa cells. It is worth noting that there is a marked asynchronicity of germ cell development in the human ovary. Even after primordial follicles have started to form, mitosis continues in more peripherally located germ cells for many weeks thereafter (Fulton *et al.*, 2005).

Abnormal follicle formation is associated with a massive loss of oocytes in the fetal ovaries from 22 wpc. At this age, a maximal number of 6.8 million germ cells (healthy and atretic) per pair of ovaries was reported (Baker, 1963). The ovarian reserve of primordial follicles has been estimated at 100,000 primordial follicles/ovary at 15 wpc, and it increases to 350,000 to 1.1 million at birth, the average being about 700,000 (Baker, 1963; Block, 1952; Forabosco and Sforza, 2007) (**Fig. 1**).

Once formed, the primordial follicles may begin to grow immediately or become quiescent. In the latter case, they will either degenerate or resume their growth several months or years later. The activation of primordial into growing follicles occurs throughout life until complete exhaustion of the ovarian reserve. All the growing follicles degenerate by atresia before puberty, whereas a tiny proportion of them (about 0.01%) ovulate in adults.

The isolation of oogonial stem cells from adult mouse and human ovaries has been reported; these cells exhibit both germ and stem cell markers in culture (White *et al.*, 2012). When reintroduced into an ovarian somatic environment, the mouse oogonial stem cells have generated follicles capable of producing healthy offspring (Zou *et al.*, 2009). However, these stem cells are unable to sustain by themselves the ovarian function into an advanced age, which may be partly due to age-related changes in the ovarian microenvironment (reviewed by Truman *et al.*, 2017). Moreover, there are no data on their potential physiological role within the

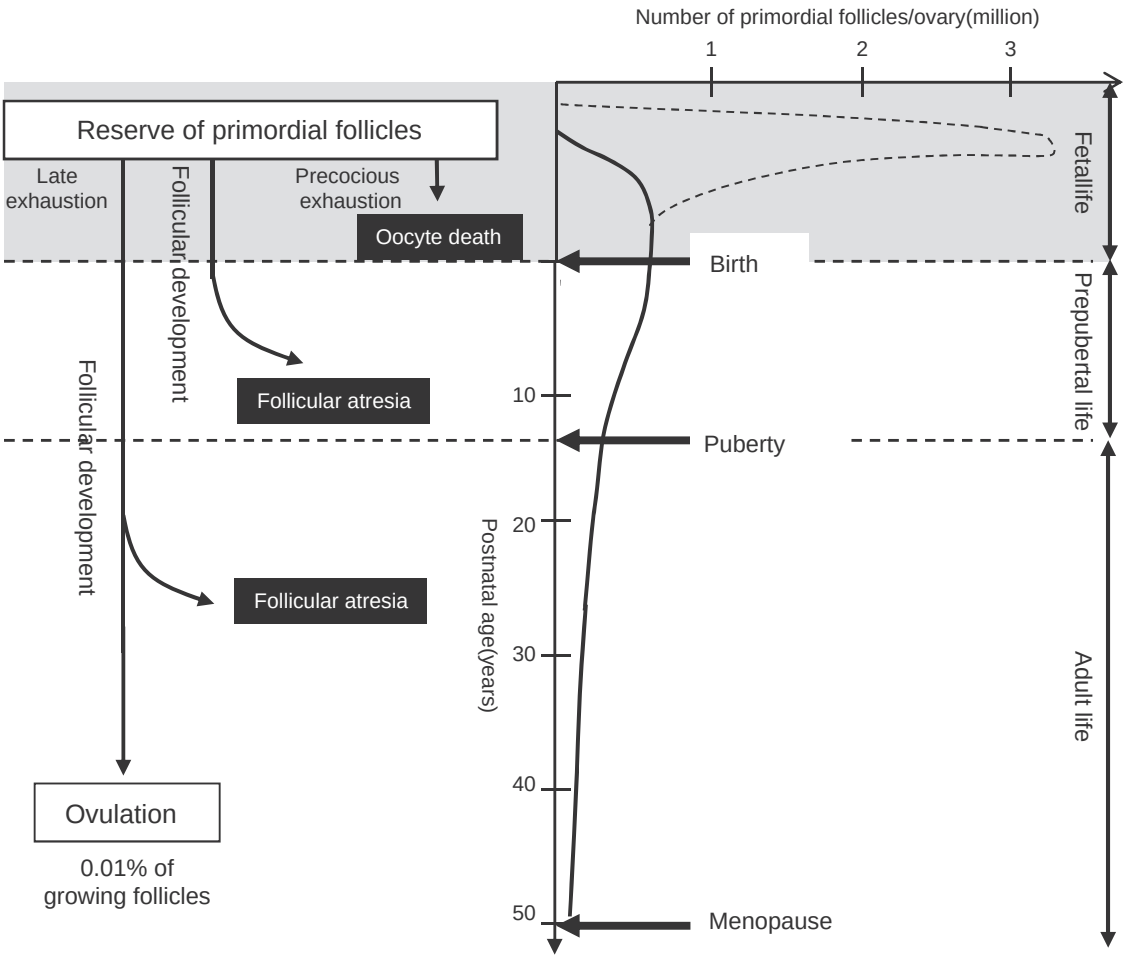

Fig. 1 Changes in the ovarian reserve of primordial follicles throughout life in humans. In the right part of the figure, the *solid* and *dotted lines* represent the number of primordial follicles and the total number of germ cells per ovary, respectively (from the compiled data of Block (1952), Baker (1963), and Forabosco and Sforza (2007)). The main mechanisms leading to the exhaustion of the reserve are indicated in the *left* part of the figure.

ovary, and specifically no evidence that they can contribute to the primordial follicle pool, hence to later stages of follicle development (reviewed by Grieve *et al.*, 2015).

From the Primordial to the Ovulatory Follicle: Morphological and Functional Features

Follicular Morphogenesis and Growth Rates

When a quiescent primordial follicle is activated to grow, its granulosa cells increase in number and change shape; they become cuboidal, while the oocyte starts to enlarge (primary follicle stage). Thereafter, multiple layers of proliferating granulosa cells develop around the oocyte, while the oocyte grows further. At this stage of secondary (or preantral) follicle, a vascularized theca differentiates by recruitment of progenitor cells present in the ovarian cortex (Liu *et al.*, 2015) and surrounds the granulosa tissue. In follicles of about 250 μm in diameter, small cavities filled with follicular fluid appear due to the movement of fluid originating from the thecal vasculature and powered by an osmotic gradient generated by hyaluronan and proteoglycans (reviewed by Rodgers and Irving-Rodgers, 2010). Then, these cavities merge into a central cavity (called the antrum) lined by the granulosa wall. In antral follicles, those granulosa cells closely connected with the oocyte form the cumulus–oocyte complex. The morphology of the follicles at different stages of follicles is illustrated in **Fig. 2** (reviewed by Monniaux *et al.*, 2009).

The duration of folliculogenesis from the initiation of follicular growth to ovulation has been estimated to about 200 days by histological observations in humans (Gougeon, 1996). This timing agrees roughly with the average duration of 4.5 months needed for the restoration of ovarian activity after orthotopic transplantation of frozen–thawed ovarian tissue (Donnez *et al.*, 2013). More than 80% of this duration corresponds to the first part of folliculogenesis, named basal folliculogenesis, during which the number of granulosa cells doubles about 16-fold to form a 5-mm-diameter antral follicle. Terminal folliculogenesis is defined as the developmental phase strictly dependent on gonadotropin (FSH and LH) supply and corresponds to the selection, growth and final maturation of the preovulatory follicle. It covers five additional doublings of the number of granulosa cells to produce in about 14 days a 16- to 20-mm-diameter follicle able to ovulate (reviewed by Gougeon, 1996) (**Fig. 3**).

Functional Changes in Follicular Cells and Oocyte During Folliculogenesis

During basal folliculogenesis, granulosa cells have an increased mitotic activity and they synthesize gap-junction components (GJA1), cytokines (KITLG) and growth factors (EGF (epidermal growth factor), FGF (fibroblast growth factor), AMH (anti-Mullerian hormone)). FSH receptors are present on the granulosa cells from the primary follicle stage onwards, but small follicles can develop in vivo and in vitro in the absence of FSH supply (Xu *et al.*, 2011; Cadoret *et al.*, 2017; Hardy *et al.*, 2017). As they differentiate, theca cells express growth factors, LH receptors and key factors for progestagen (CYP11A1, STAR, HSD3B2) and androgen (CYP17A1) synthesis. The oocyte initiates the synthesis of growth factors, particularly GDF9 and BMP15 (both belonging to the bone morphogenetic protein family) as well as components of its zona pellucida (ZP1, 2, 3, and 4) and of gap junctions (GJA4) involved in the connections with its surrounding granulosa/cumulus cells. Concomitantly, the oocyte accumulates untranslated mRNA in ribonucleic particles.

During terminal folliculogenesis, the granulosa cells loose progressively their ability to proliferate and to produce AMH. At this stage, they become increasingly responsive to FSH, synthesize increasing amounts of inhibin and differentiate into fully steroidogenic cells expressing CYP11A1, STAR, HSD3B2 and notably CYP19A1, the aromatase enzyme catalyzing the synthesis of estradiol (E2) from the androgens produced by theca cells. The volume of the antrum increases rapidly, with the accumulation of follicular fluid formed by serum transudation and the accumulation of metabolism and secretion products from follicular cells. During terminal folliculogenesis, the concentrations of androgens, AMH and IGF binding proteins (IGFBP) decrease in the follicular fluid, while those of estradiol and inhibin increase dramatically. In the 10- to 12-mm-diameter human follicle, the granulosa cells become endowed with LH receptors, and therefore, the follicle is able to ovulate in response to an LH surge (**Fig. 4**).

When the follicle enters terminal folliculogenesis, its oocyte has reached its maximal size. During terminal folliculogenesis, the transcriptional activity of the oocyte decreases steadily and becomes undetectable in the preovulatory follicle. Meiosis resumption in the oocyte is triggered by the LH ovulatory blood surge and will terminate at fertilization. For more details, see the reviews by Monniaux et al. (1997), Hillier (2001), McGee and Hsueh (2000), Hennet and Combelles (2012), Fragouli *et al.* (2014), and Dewailly *et al.* (2016).

Hormonal and Molecular Control of Folliculogenesis

Ovarian Factors and Signaling Pathways Controlling the Different Phases of Folliculogenesis

It is now well established that basal folliculogenesis is controlled by paracrine factors involved in the dialogue existing between the oocyte and its surrounding granulosa cells, whereas terminal folliculogenesis is highly dependent on the pituitary gonadotropins

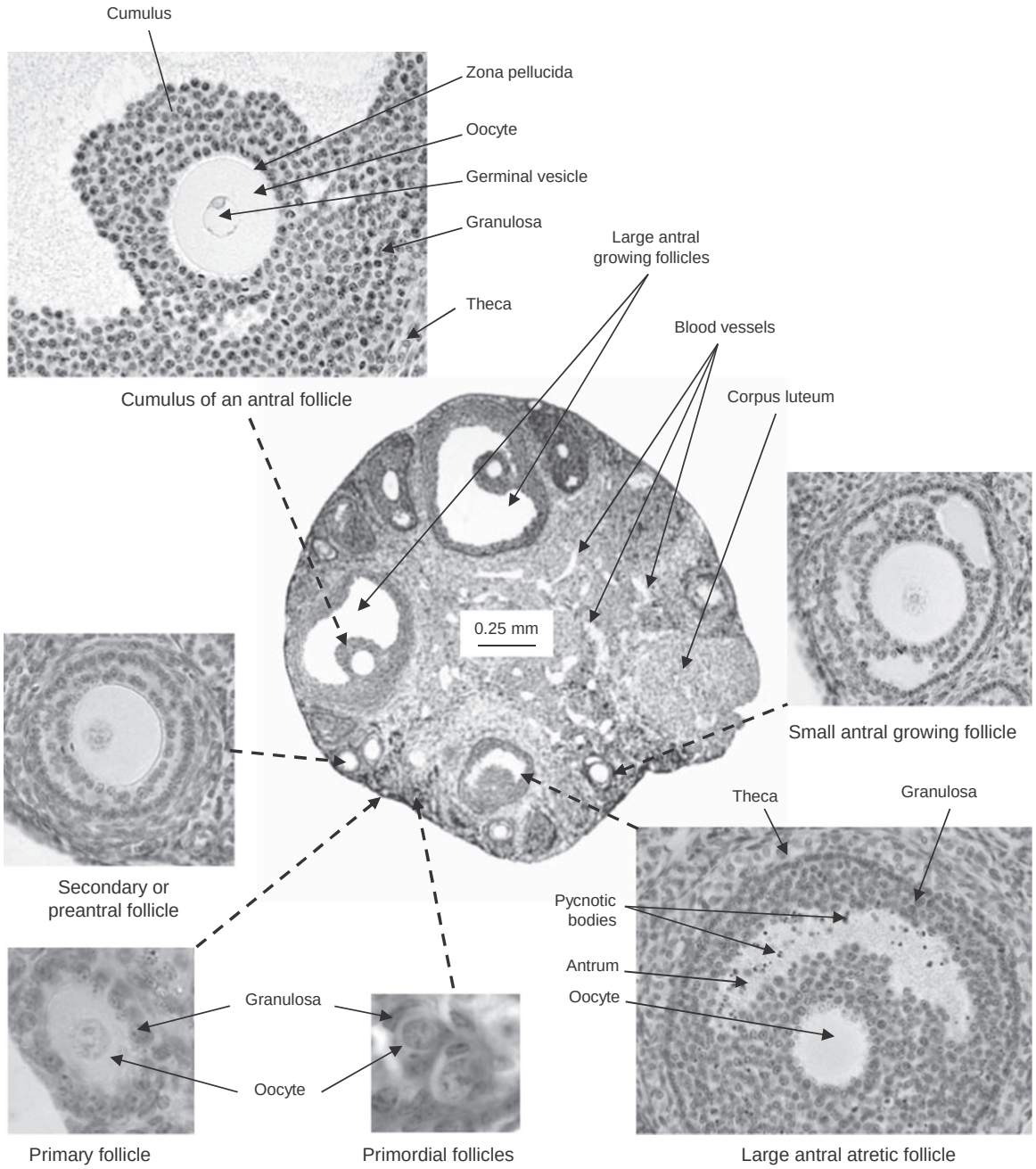

Fig. 2 Histological section of a mouse ovary and morphology of the main stages of ovarian follicles. Adapted from Monniaux, D., Dalbies-Tran, R., Fabre, S., Gérard, N., Monget, P. and Clément, F. (2014). Le développement folliculaire ovarien et l'ovulation. In Saint-Dizier, M. and Chastant-Maillard, S. (Eds.) *La reproduction animale et humaine*, pp. 41–56. Versailles, France: Quae, with permission.

FSH and LH (reviewed by Scaramuzzi *et al.*, 2011). However, the mechanisms triggering the activation of primordial follicles and the relative importance of the numerous ovarian factors able to enhance or modulate follicular development have remained misunderstood until recently. During the last decades, the role of factors specific to the ovarian function has been deciphered, thanks to the generation of transgenic mice using gene knockout, knock-in, targeted deletion or overexpression strategies (reviewed by Edson *et al.*, 2009; Matzuk and Burns, 2012). These genetic models have allowed one to identify the main factors controlling the transitions between follicular stages and involved in follicular activation, growth and maturation (**Fig. 5**A). All these factors are potential candidates for identifying mutations associated with premature ovarian failure or polycystic ovarian syndrome in women. Interestingly, most of them belong to three families of signaling pathways, the phosphatidylinositol 3 kinase (PI3K)/AKT, the SMAD and the gonadotropin signaling networks, depicted in **Fig. 5**B. As it will be detailed below, these pathways are,

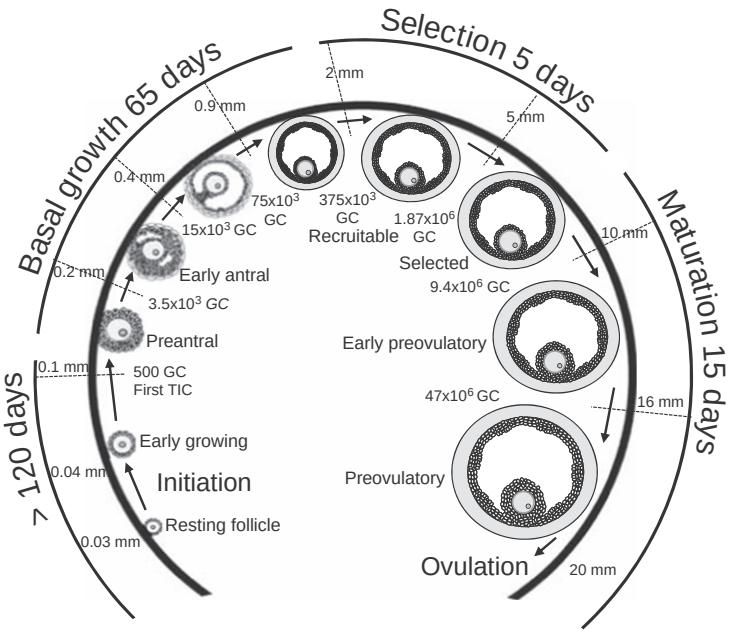

Fig. 3 Morphological and cellular trajectory of an ovulatory follicle in humans. *TIC*, theca/interstitial cells; *GC*, granulosa cells. From Gougeon, A. (1996). Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocrine Reviews* **17**, 121–155, with permission. From our current knowledge, the durations of the selection and maturation phases are more likely about 8 and 12 days, respectively.

respectively, involved in: (1) the activation of primordial follicles and survival of growing follicles, (2) the transition from primary to secondary follicles, basal follicular growth and cumulus metabolism and (3) terminal folliculogenesis up to ovulation.

Activation of Primordial Follicles

Different transcription factors have been identified in the oocyte to maintain the quiescent state (FOXO3A, LHX8) or, on the contrary, to participate in follicular growth activation (SOHLH1, SOHLH2, NOBOX) (reviewed by Jagarlamudi and Rajkovic, 2012). Interestingly, FOXO3A and LHX8 are the effectors of the PI3K/AKT signaling pathway. The oocyte-specific invalidation of genes involved in this pathway has shown that mutations in its stimulating factors (KIT, PDPK1, RPTOR, RPS6) block follicular activation and induce the atresia of primordial follicles, whereas mutations in its inhibiting factors (PTEN, CDKN1B, TSC1, TSC2) lead to the premature and massive activation of primordial follicles in the ovaries of mutant mice (**Table 1**, in Supplemental Data).

The role of the granulosa cells in follicular activation has also been demonstrated. In primordial follicles, the invalidation of the granulosa-specific transcription factor FOXL2 is associated with the failure in the differentiation of the flattened granulosa cells, followed by oocyte death (Schmidt *et al.*, 2004; Uda *et al.*, 2004). The cuboidal granulosa cells are known to synthesize KITLG, the ligand of the KIT receptor which can trigger the activation of the PI3K/AKT pathway in the oocyte. It has been shown recently that primordial follicle activation is initiated in the flattened granulosa cells by the activation of mTORC1 signaling, which induces both the flattened-to-cuboidal-shape transition of the granulosa cells and KITLG expression, triggering in turn the oocyte awakening (Zhang *et al.*, 2014; **Fig. 6**). The protein complex mTORC1 is known to be activated in tissues by various molecules such as growth factors, nutrients, oxygen and stress- or energy-induced metabolites (reviewed by Laplante and Sabatini, 2012); these signals could reach the primordial follicles via the microvascularization of the ovarian cortex. Alternatively or synergistically, the remodeling of the ovarian cortex in the vicinity of the primordial follicles could also promote follicular activation, as suggested by the impressive effects observed on the induction of follicular growth after disruption of HIPPO signaling by ovarian fragmentation (Kawamura *et al.*, 2013).

Basal Folliculogenesis

At the primary stage, the oocyte synthesizes GDF9, which supports follicular growth to the secondary follicle stage (Dong *et al.*, 1996) through the activation of the SMAD2/3 signaling pathway in granulosa cells (**Fig. 5**B). BMP15, which acts as a cofactor of GDF9 (Peng *et al.*, 2013), drives this transition in sheep (reviewed by Otsuka *et al.*, 2011) and also likely in humans (reviewed by Persani *et al.*, 2014). GDF9 is also critical to follicular theca formation since it induces the production of Hedgehog ligands by the granulosa cells of preantral follicles. These ligands act as morphogens involved in the recruitment of progenitor cells in the ovarian cortex and their commitment to the theca cell lineage (Liu *et al.*, 2015; **Fig. 7**).

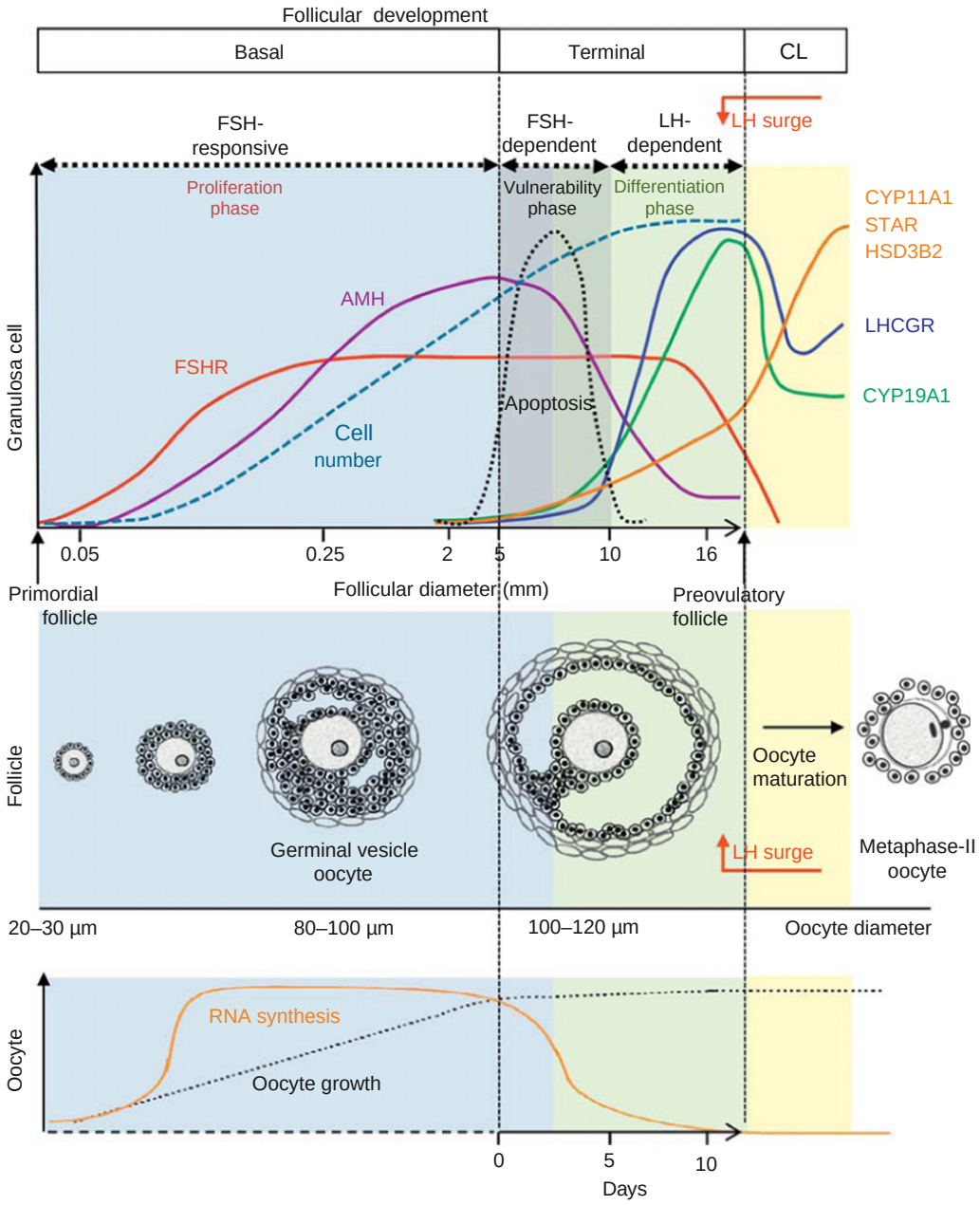

Fig. 4 Main functional changes in granulosa cells and oocyte during follicular development. FSH supply is not essential during the first phase of follicular development, named basal folliculogenesis. During this phase, the growth of FSH-responsive follicles is underlain by granulosa cell proliferation, oocyte growth, and the formation and development of theca and antrum. Terminal folliculogenesis is defined as the developmental phase strictly dependent on gonadotropin (first FSH, then LH) supply. During this phase, the antrum enlarges rapidly, the granulosa cells loose progressively their ability to proliferate and they differentiate into steroidogenic cells, while the oocyte has reached its full size and ceases its transcriptional activity gradually. The first part of this phase is a "vulnerability phase," during which the FSH-dependent follicles enter atresia (characterized by apoptosis of the granulosa cells) when FSH levels fall below a threshold. The last part of this phase corresponds to the final maturation of the LH-dependent, dominant follicle. Meiotic resumption in the oocyte and ovulation are triggered by the LH ovulatory surge. *CL*, corpus luteum. *AMH*, anti-Mullerian hormone; *CYP11A1*, cytochrome P450 family 11 subfamily A member 1; *CYP19A1*, cytochrome P450 family 19 subfamily A member 1; *FSHR*, follicle-stimulating hormone receptor; *HSD3B2*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2; *LHCGR*, luteinizing hormone/choriogonadotropin receptor; *STAR*, steroidogenic acute regulatory protein. Adapted from Monniaux, D., Dalbies-Tran, R., Fabre, S., Gérard, N., Monget, P. and Clément, F. (2014). Le développement folliculaire ovarien et l'ovulation. In Saint-Dizier, M. and Chastant-Maillard, S. (eds.) *La reproduction animale et humaine*, pp. 41–56. Versailles, France: Quae, with permission.

The balanced growth of the preantral follicle is driven by a finely tuned oocyte–granulosa cell molecular dialog. GDF9 and BMP15 produced by the oocyte activate the SMAD2/3 signaling pathway in granulosa cells and support their proliferation and survival, whereas KITLG produced by the granulosa cells activates the PI3K/AKT pathway essential for the growth and survival of

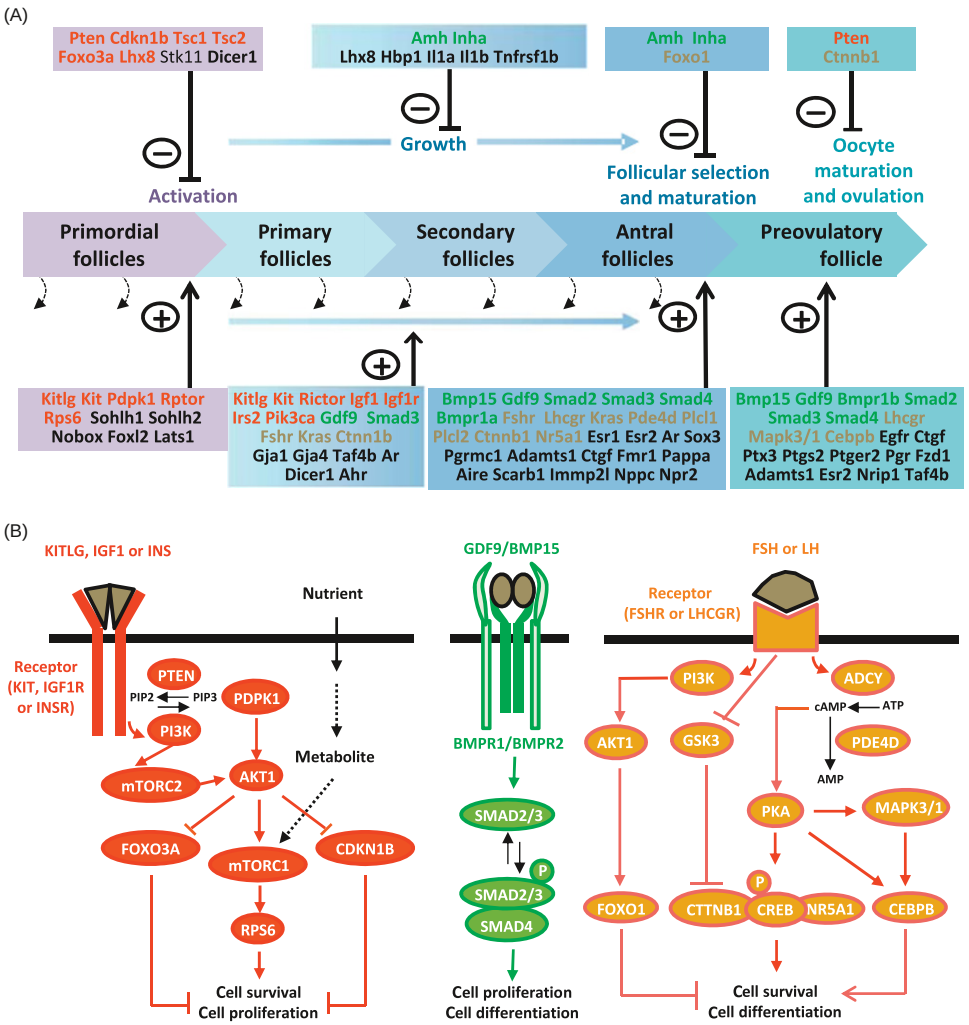

Fig. 5 Current knowledge on factors controlling folliculogenesis, according to the data available from mouse genetic models. (A) Main factors identified by in vivo transgenic approaches in mouse and their importance in controlling the transitions between the different follicular stages. (B) Schematic representation of the three main signaling pathways (PI3K/AKT, SMAD and gonadotropin signaling network) involved in the control of folliculogenesis. In both panels, factors belonging to the PI3K/AKT, SMAD and gonadotropin network are depicted in *red*, *green* and *brown* colors, respectively. In Panel A, the names of factors belonging to other signaling pathways are written in *black*. See **Tables 2–4** for gene nomenclature.

the oocyte (reviewed by Monniaux, 2016). The protein complex mTORC1 is not required for basal folliculogenesis, but a critical role of the mTORC2/AKT1/FOXO3A prosurvival signaling cascade has been recently demonstrated (Chen *et al.*, 2015; **Fig. 5**B). Moreover, the presence of functional gap junctions between the oocyte and granulosa cells (made up of GJA4, also known as connexin 37) and between granulosa cells themselves (made up of GJA1, also known as connexin 43) has been shown to maintain the follicle in a functionally integral state that is essential for its development (Carabatsos *et al.*, 2000; Ackert *et al.*, 2001).

The newly formed theca cells contribute to the regulation of basal folliculogenesis through the synthesis of androgens and growth factors (BMP4, BMP7, NGF (nerve growth factor), FGF7, EGF, TGF (transforming growth factor), etc.) which enhance granulosa cell proliferation. Conversely, granulosa cells control the gene expression and secretory activity of theca through the secretion of growth factors and cytokines, particularly KITLG (reviewed by Hutt *et al.*, 2006) as well as the development of its vascularization through the secretion of angiogenic factors (vascular endothelial growth factors, reviewed by Fraser, 2006) (**Fig. 7**).

FSH supply is not essential for the growth of small follicles up to the large preantral stage in mouse or the small antral follicle (about 5 mm in diameter) stage in humans. However, FSH enhances granulosa cell survival and proliferation and participates in antrum formation by stimulating the synthesis of osmotically active molecules (versican, hyaluronic acid) (Russell *et al.*, 2003). During basal folliculogenesis, the sensitivity of the growing follicle to FSH increases progressively, as various factors (IGF1 (insulin-like growth factor 1), insulin, androgens, aryl hydrocarbon, etc.) induce FSH receptors and/or amplify FSH action on granulosa cells (**Table 2**, in Supplemental Data). Among the factors inhibiting basal folliculogenesis, some inflammatory cytokines (IL1A, IL1B, TNF (tumor necrosis factor)) are known to modulate FSH sensitivity and induce follicular atresia (Greenfeld *et al.*, 2007; Uri-Belapolsky *et al.*, 2014). AMH inhibits both the growth of the small follicles from their activation onwards (Durlinger

Table 1 Genes involved in follicle activation, as evidenced from mouse genetic models

Gene name	*Signaling pathway*	*Genetic model*	*Ovarian phenotype and fertility*	*References*
Cdkn1b (cyclin-dependent kinase inhibitor 1b)	PI3/AKT	KO	Massive follicle activation, POF, infertility by 12 weeks of age	Rajareddy Et al. (2007)
Dicer1 (dicer 1, ribonuclease III)	miRNA regulatory pathway	cKO in granulosa	Accelerated follicle activation and increased degenerate oocytes and follicles, infertility due to oocyte impaired quality	Lei *et al.* (2010)
Foxl2 (forkhead box l2)	TF	KO	Impaired follicle activation, POF, infertility by 16 weeks of age	Schmidt *et al.* (2004), Uda *et al.* (2004)
Foxo3 (forkhead box O3)	PI3/AKT	KO	Massive follicle activation, POF, infertility by 18 weeks of age	Castrillon *et al.* (2003)
Kit (KIT proto-oncogene receptor tyrosine kinase)	PI3/AKT	Loss- and gain-of-function mutations in oocyte	Kit inactivation: failure of follicle activation, POF Kit activation: massive follicle activation, POF infertility by 6 months of age	Saatcioglu *et al.* (2016)
Kitlg (kit ligand)	PI3/AKT	Steel mutation	Impaired follicle activation, POF, infertility by 6 months of age	Kuroda *et al.* (1988)
Lats1 (large tumor suppressor kinase 1)	HIPPO	KO	Increased oocyte and primordial follicle loss, subfertility	Sun *et al.* (2015)
Lhx8 (LIM homeobox 8)	TF PI3/AKT	KO	Impaired follicle activation, POF, primary infertility	Choi *et al.* (2008a), Ren *et al.* (2015)
Nobox (NOBOX oogenesis homeobox)	TF	KO	Increased oocyte loss and impaired follicle activation, POF, primary infertility	Rajkovic *et al.* (2004)
Pdpk1 (3-phosphoinositide dependent protein kinase 1)	PI3/AKT	cKO in oocyte	Primordial follicle loss, POF, infertility by 5 months of age	Reddy *et al.* (2009)
Pten (phosphatase and tensin homolog)	PI3/AKT	cKO in oocyte	Massive follicle activation, POF, infertility by 12 weeks of age	Reddy *et al.* (2008), John *et al.* (2008)
Rps6 (ribosomal protein S6)	PI3/AKT	cKO in oocyte	Primordial follicle loss, POF, infertility by 5 months of age	Reddy *et al.* (2009)
Rptor (regulatory associated protein of MTOR, complex 1)	PI3/AKT	cKO in primordial follicle granulosa cells	Impaired follicle activation, POF, infertility by 4 months of age	Zhang *et al.* (2014)
Sohlh1 (spermatogenesis- and oogenesis-specific basic helix-loop-helix 1)	TF	KO	Impaired follicle activation, POF, infertility by 10 weeks of age	Pangas *et al.* (2006a)
Sohlh2 (spermatogenesis- and oogenesis-specific basic helix-loop-helix 2)	TF	KO	Impaired follicle activation, POF, primary infertility	Choi *et al.* (2008b)
Stk11 (serine/threonine kinase 11)	mTORC1 inhibitor	cKO in oocyte	Massive follicle activation, POF, subfertility by 18 weeks of age	Jiang *et al.* (2016)
Tsc1/2 (tuberous sclerosis 1/2)	PI3/AKT, mTORC1 inhibitors	cKO in oocyte	Massive follicle activation, POF, infertility by 12 weeks of age	Adhikari *et al.* (2009), Adhikari *et al.* (2010)

TF, transcription factor; *KO*, knock out; *cKO*, conditional KO; *POF*, premature ovarian failure.

et al., 1999) and the sensitivity to FSH of the large preantral and antral follicles (reviewed by Visser and Themmen, 2014). However, some of the AMH effects do not counteract the advance of development, since AMH has been recently shown to prevent follicular atresia (Hayes *et al.*, 2016).

Terminal Folliculogenesis and Ovulation

Terminal folliculogenesis is strictly dependent on the gonadotropins FSH and LH, which act, respectively, upon the granulosa and theca cells, in synergy with various ovarian and endocrine factors (reviewed by McGee and Hsueh, 2000; Hillier, 2001).

LH acts through specific receptors, constitutively present in thecal cells, to stimulate androgen production. These receptors mediate the short-term effects of LH by activating the STAR protein, which translocates cholesterol from the outer to the inner mitochondrial membrane where the first committed step in steroid synthesis is performed by the CYP11A1 enzyme, resulting in the production of pregnenolone (Kallen *et al.*, 1998). Through this mechanism, the pulse frequency of LH tunes the amounts of steroid hormones produced, as each pulse of LH is followed by an increase in androgens and

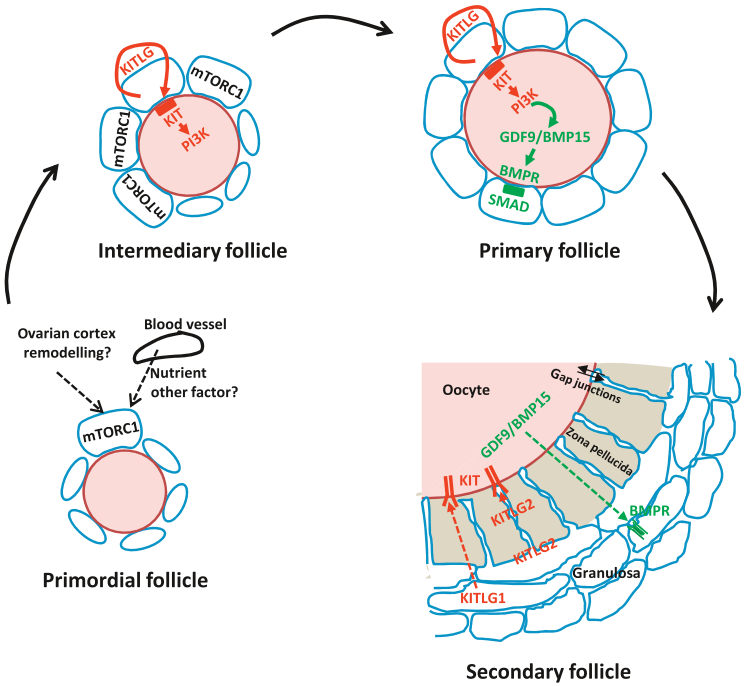

Fig. 6 The oocyte–granulosa cell molecular dialog during follicle activation and growth. The activation of primordial follicle starts with the shape change from flattened cells into cuboidal granulosa cells (through the activation of their mTORC1 protein complex) and is followed by the induction of KITLG expression in the cuboidal granulosa cells. Then, KITLG triggers the awakening of the oocyte from its quiescent state by activating the KIT receptors and PI3K signaling pathway (intermediary follicle stage). From the primary follicle stage onwards, GDF9 and BMP15 synthesized by the oocyte cooperate through SMAD signaling to sustain the proliferation, survival and differentiation of granulosa cells. Conversely, KITLG produced by the granulosa cells and acting as diffusible (KITLG1) and membrane-bound (KITLG2) isoforms can sustain the growth and survival of the oocyte. In addition, intercellular channels consisting of gap junctions located at the tips of granulosa/cumulus cell transzonal cytoplasmic projections permit molecular exchanges (ions, cAMP, cGMP, amino acids, and metabolites) with the oocyte. *BMP15*, bone morphogenetic protein 15; *BMPR*, bone morphogenetic receptor; *GDF9*, growth differentiation factor 9; *KIT*, KIT proto-oncogene receptor tyrosine kinase; *KITLG*, kit ligand; *mTORC1*, mammalian target of rapamycin complex one; *PI3K*, phosphatidylinositol 3 kinase; *SMAD*, SMAD family member.

estradiol secreted from the ovaries. Moreover, LH exerts long-term trophic effects on theca cells by enhancing the expression of factors and enzymes involved in progesterone (STAR, CYP11A1, HSD3B2) and androgen (CYP17A1) production. Insulin and locally produced intrafollicular factors such as IGF1, inhibin, or follistatin enhance the trophic effects of LH, whereas activin, TGFB and BMP exert modulating actions (reviewed by Young and McNeilly, 2010). Importantly, testosterone diffusing into the mural granulosa cell layers is the substrate for the synthesis of follicular estradiol catalyzed by FSH-induced CYP19A1 (**Fig. 8**).

FSH enhances the survival of granulosa cells and their differentiation into steroidogenic cells expressing STAR, CYP11A1, HSD3B2, and CYP19A1 and producing increasing estradiol amounts. FSH also increases the expression of many genes, in particular those encoding the inhibin/activin subunits (INHA, INHBA, INHBB), as well as the gonadotropin receptors FSHR and LHCGR. Moreover, FSH induces the production of PAPPA, a protease able to cleave specifically the IGFBP, increasing IGF intrafollicular bioavailability and thus contributing to increase the sensitivity of follicular cells to gonadotropins in the large antral follicles (reviewed by Giudice, 2001; Mazerbourg *et al.*, 2003). The final maturation of the preovulatory follicle up to ovulation is supported by LH.

Gonadotropins can activate various signaling pathways simultaneously, mainly the cAMP/PKA, PI3K/AKT, MAPK3/1 pathways, and at the same time inhibit the GSK3 pathway (**Fig. 5**B) (reviewed by Gloaguen *et al.*, 2011), which can explain their pleiotropic effects on follicular cells. Numerous factors modulate their actions at different steps of their signaling pathways, in particular through transcription factors such as ESR1 and ESR2 (for estradiol), AR (for androgens), SMAD (for BMP, AMH, activin, and other members of the TGFB family), TCF/LEF (for progesterone action mediated by its membrane receptor PGRMC1), AIRE, SOX3, etc. (**Table 3**, in Supplemental Data). Follicular survival and maturation up to ovulation are supported by a proper rate of stimulation of the gonadotropin pathways. Indeed, excessive stimulation obtained from the invalidation of genes encoding intracellular inhibitors of these pathways, such as the phosphodiesterase PDE4D or the phospholipase C-related but catalytically inactive proteins 1 and 2 (PLCL1 and 2), has led to impaired follicle development with the formation of cystic or prematurely luteinized follicles (Park *et al.*, 2003; Matsuda and Hirata, 2017).

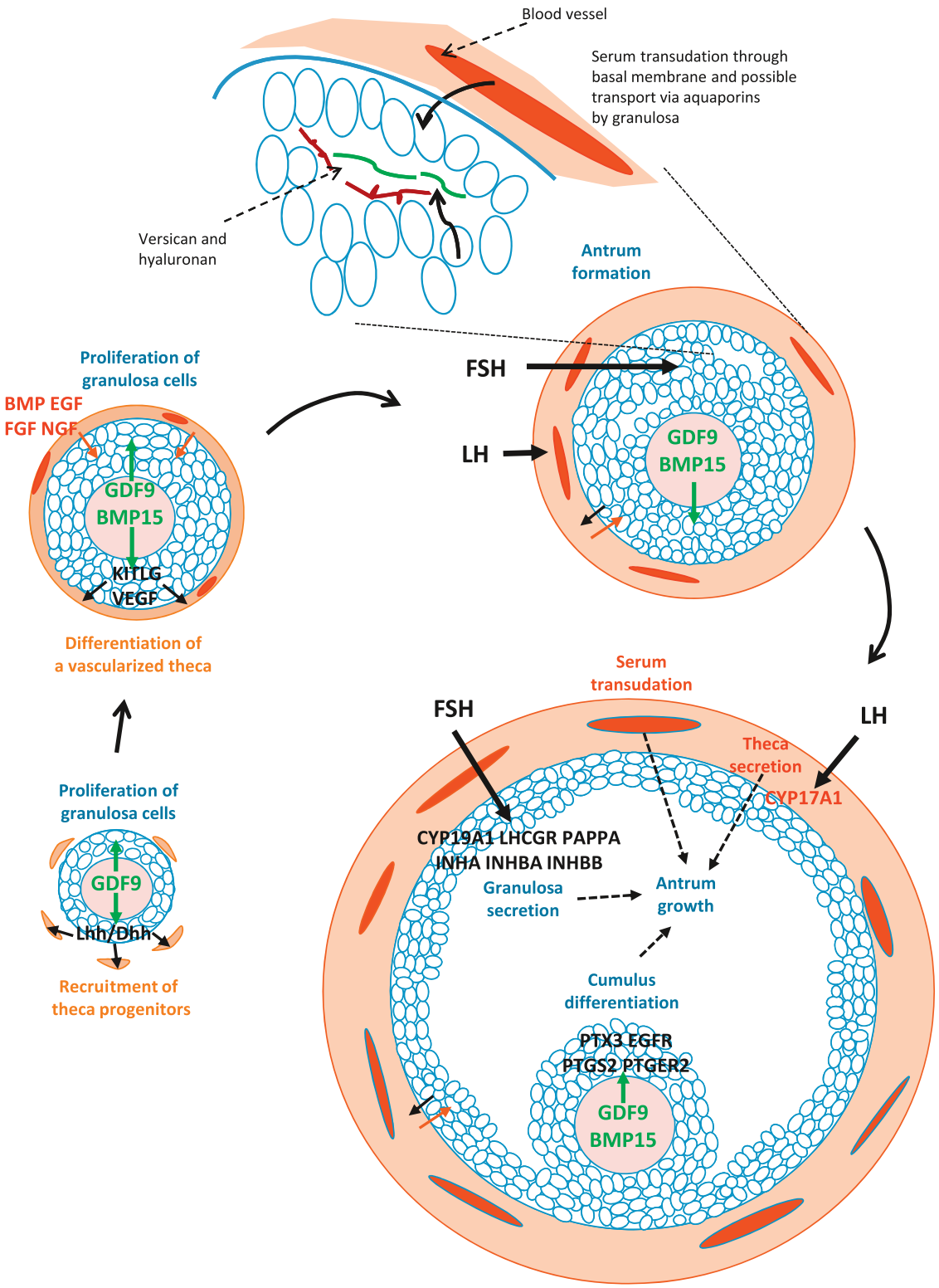

Fig. 7 Main mechanisms governing follicular morphogenesis, antrum formation and the differentiation of theca and granulosa/cumulus cells during follicular growth. *BMP*, bone morphogenetic proteins; *BMP15*, bone morphogenetic protein 15; *CYP17A1*, cytochrome P450 family 17 subfamily A member 1; *CYP19A1*, cytochrome P450 family 19 subfamily A member 1; *EGF*, epidermal growth factor; *EGFR*, epidermal growth factor receptor; *FGF*, fibroblast growth factors; *GDF9*, growth differentiation factor 9; *INHA*, inhibin alpha subunit; *INHBA*, inhibin beta A subunit; *INHBB*, inhibin beta B subunit; *KITLG*, kit ligand; *LHCGR*, luteinizing hormone/choriogonadotropin receptor; *Lhh/Dhh*, Hedgehog ligands; *NGF*, nerve growth factor; *PAPPA*, pappalysin 1; *PTGER2*, prostaglandin E receptor 2; *PTGS2*, prostaglandin-endoperoxide synthase 2; *PTX3*, pentraxin 3; *VEGF*, vascular endothelial growth factors.

Table 2 Genes involved in basal follicular growth, as evidenced from mouse genetic models

Gene name	*Signalling pathway*	*Genetic model*	*Ovarian phenotype and fertility*	*References*
Ahr (aryl-hydrocarbon receptor)	TF	KO	Slower follicular growth, decreased number of growing follicles and gonadotropin responsiveness, subfertility	Benedict *et al.* (2003), Barnett *et al.* (2007)
Amh (anti-Mullerian hormone)	SMAD	KO	Accelerated follicle activation, increased oocyte degeneration and follicular atresia, accelerated follicular depletion, fertility	Durlinger *et al.* (1999), Visser *et al.* (2007)
Ar (androgen receptor)	TF Androgen	KO and cKO in granulosa	Defective folliculogenesis, enhanced atresia, POF, subfertility	Shiina *et al.* (2006)
Ctnnb1 (catenin beta 1)	WNT Gonadotropin	cKI (dominant activating mutation) in granulosa	Defective folliculogenesis, abnormal follicles, severe subfertility	Boerboom *et al.* (2005)
Dicer1 (dicer 1, ribonuclease III)	miRNA regulatory pathway	cKO in granulosa	Accelerated follicle activation, increased oocyte degeneration and follicular atresia, infertility due to oocyte impaired quality	Lei *et al.* (2010)
Fshr (follicle stimulating hormone receptor)	Gonadotropin	KO	Absence of antral follicles, sterility	Dierich *et al.* (1998)
Gdf9 (growth differentiation factor 9)	SMAD	KO	Arrest of folliculogenesis at the primary follicle stage, sterility	Dong *et al.* (1996)
Gja1 (gap junction protein alpha 1)	Intercellular connection	KO	Arrest of folliculogenesis at the primary follicle stage, impairment of oocyte growth	Juneja *et al.* (1999), Ackert *et al.* (2001)
Gja4 (gap junction protein alpha 4)	Intercellular connection	KO	Arrest of folliculogenesis at the small preantral stage, impairment of oocyte growth and meiotic competence, sterility	Carabatsos *et al.* (2000)
Hbp1 (HMG-box transcription factor 1)	TF Mitochondria metabolism	KO and cKO in granulosa	Enhanced granulosa cell survival, decreased follicular atresia, protection of ovarian reserve, but infertility by 7 months of age	Dong *et al.* (2016)
Igf1 (insulin like growth factor 1)	PI3K/AKT	KO	Arrest of folliculogenesis at the early antral follicle stage, decreased sensitivity to FSH, sterility	Zhou *et al.* (1997)
Igf1r (insulin like growth factor 1 receptor)	PI3K/AKT	cKO in granulosa	Enhanced follicular atresia, absence of antral follicles, sterility	Baumgarten *et al.* (2017)
Il1a/b (interleukin 1 alpha/beta)	Inflammation	KO	Increased number of growing follicles, prolongation of ovarian lifespan, fertility	Uri-Belapolsky *et al.* (2014)
Inha (inhibin alpha subunit)	SMAD	KO	Increased granulosa cell proliferation, advanced granulosa differentiation and tumor formation	Myers *et al.* (2009), Nagaraja *et al.* (2010)
Irs2 (insulin receptor substrate 2)	PI3K/AKT	KO	Increased follicular atresia, impaired oocyte growth and antral cavity development, severe subfertility	Burks *et al.* (2000), Neganova *et al.* (2007)
Kit (KIT proto-oncogene receptor tyrosine kinase)	PI3K/AKT	KI	Arrest of folliculogenesis at the primary follicle stage, sterility	Kissel *et al.* (2000)
Kitlg (kit ligand)	PI3K/AKT	Natural mutations	Arrest of folliculogenesis at the primary follicle stage, sterility	Kuroda *et al.* (1988)

Table 2 Continued

Gene name	*Signalling pathway*	*Genetic model*	*Ovarian phenotype and fertility*	*References*
Kras (KRAS proto-oncogene, GTPase)	Gonadotropin	cKI in granulosa (activating mutation)	Impaired proliferation of granulosa cells, subfertility	Fan *et al.* (2008b)
Lhx8 (LIM homeobox 8)	TF	cKO in oocyte	Arrest of folliculogenesis at the primary follicle stage, oocyte death, sterility	Ren *et al.* (2015)
Pik3ca (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha)	PI3K/AKT	cKI in oocyte (activating mutation)	Increased number of growing follicles with developmentally competent oocytes, decreased follicular atresia	Kim *et al.* (2015)
Rictor (RPTOR independent companion of MTOR complex 2)	PI3K/AKT	cKO in oocyte	Extensive apoptosis in follicular cells, massive follicular loss, secondary subfertility	Chen *et al.* (2015)
Smad3 (SMAD family member 3)	SMAD	KO	Slower follicular growth, increased follicular atresia, impaired granulosa differentiation, subfertility	Tomic *et al.* (2004)
Taf4b (TATA-box binding protein associated factor 4b)	TF	KO	Decreased number of growing follicles, decreased granulosa cell proliferation, increased follicular atresia, POF, sterility	Freiman *et al.* (2001), Voronina *et al.* (2007), Lovasco *et al.* (2010)
Tnfrs1b (TNF receptor superfamily, member 1b)	Inflammation	KO	Accelerated follicular growth, increased number of growing and primordial follicles, fertility	Greenfeld *et al.* (2007)

TF, transcription factor; *KO*, knockout; *cKO*, conditional KO; *KI*, knock-in; *cKI*, conditional knock in; *POF*, premature ovarian failure.

In the large antral follicles, the BMP15/GDF9 heterodimer produced by the oocyte modulates the respective effects of FSH and LH on the differentiation of granulosa and theca cells, through the activation of the SMAD2/3 pathway (Mottershead *et al.*, 2015). In addition, BMP15 and GDF9 play a pivotal role in supporting cumulus function up to the preovulatory stage by promoting the survival and metabolism of cumulus cells (Hussein *et al.*, 2005; Sugiura *et al.*, 2007) and the expression of factors involved in cumulus integrity (PTX3), differentiation (EGFR), expansion ability (PTGS2, PTGER2), as well as in oocyte meiosis arrest (NPR2) (**Tables 3** and **4**, in Supplemental Data, and **Fig. 7**).

Before ovulation, a massive LH surge occurs, which triggers the rupture and luteinization of the wall of the preovulatory follicle, expansion of its cumulus and meiosis resumption of its cumulus-enclosed oocyte. LH activates first its receptor (LHCGR) at the surface of granulosa and theca cells. The spatial propagation of the signal to the cumulus is mediated by transactivation of the EGF network (Hsieh *et al.*, 2011) and involves the activation of the MAPK3/1 signaling pathway and CEBPB transcription factor in granulosa cells (Fan *et al.*, 2009). Ovulation is an inflammatory process induced by cytokines (interleukins, TNF) and prostaglandins (Espey, 1980) which are produced in response to LH and impact the synthesis and/or proteolytic degradation of hyaluronan binding proteins (TNFAIP6, VCAN) underlying cumulus expansion (reviewed by Richards, 2005). Concomitant with cumulus expansion, the cumulus-enclosed oocyte resumes meiosis, as a result of the partial loss of its molecular communication with the somatic cells of the follicle (Wigglesworth *et al.*, 2013). The follicular rupture and folliculo-luteal transition are dependent on the specific remodeling of the extracellular matrix, which involves various matrix metalloproteinases, plasminogen activators/plasmin and ADAMTS (reviewed by Curry and Smith, 2006). The LH-induced progesterone receptor and connective tissue growth factor participate in tissue remodeling by mediating the effects of LH on the expression of the protease ADAMTS1 (Richards *et al.*, 2005; Nagashima *et al.*, 2011). The estradiol receptor ESR2 has also been demonstrated recently to control the expression of the matrix metalloprotease MMP19 (Nalvarte *et al.*, 2016).

The Different Follicular Populations and Their Dynamics

The Dynamic Ovarian Reserve

The ovaries of the young adult contain several thousands of primordial follicles (forming the static reserve of quiescent follicles) and hundreds of growing follicles. Among the latter, a few tens of small antral follicles (with diameters between 2 and 5 mm in humans) constitute a pool of gonadotropin responsive follicles which have not yet entered terminal folliculogenesis and are the

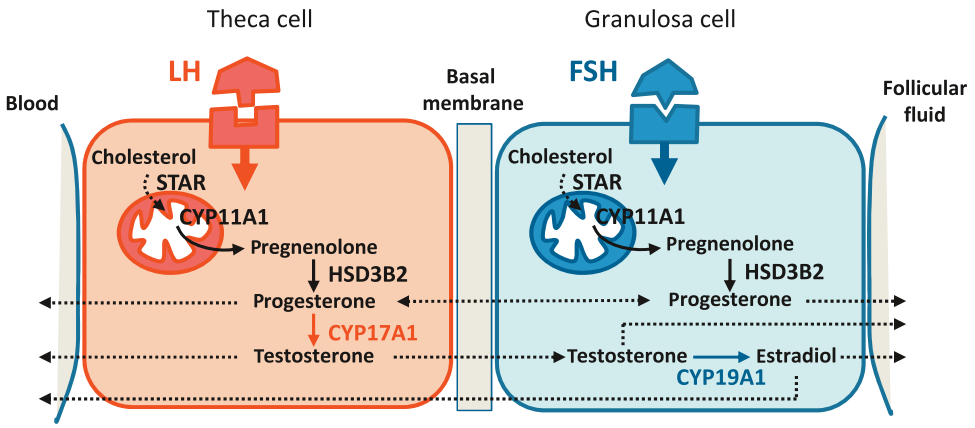

Fig. 8 The "two cell–two gonadotropin" model for steroidogenesis within the follicle during terminal folliculogenesis. *CYP11A1*, cytochrome P450 family 11 subfamily A member 1; *CYP17A1*, cytochrome P450 family 17 subfamily A member 1; *CYP19A1*, cytochrome P450 family 19 subfamily A member 1; *HSD3B2*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2; *STAR*, steroidogenic acute regulatory protein.

reserve for ovulation and ovarian biotechnologies (reviewed by Monniaux *et al.*, 2014a). The small antral follicles within this permanently renewed, dynamic reserve are the main contributors to AMH circulating levels (Gnoth *et al.*, 2015; Hagen *et al.*, 2015). Assessing the size of this reserve by ovarian ultrasonography (with the antral follicle count index) or by the measurement of AMH levels in blood (reviewed by La Marca *et al.*, 2010) is now a routine practice of primary importance to establish a diagnosis for infertility.

The size of the reserve of small antral follicles decreases with age, in parallel with the decline in primordial follicle numbers (Scheffer *et al.*, 1999; Depmann *et al.*, 2015), but it also appears to be highly variable between individuals of similar age (Broekmans *et al.*, 2004). In the young adult, the dynamic reserve has reached a stationary size (with exits broadly compensated for by entries), which is specific to each individual and correlated with the size of the static reserve.

The dynamic reserve is emptied by the cyclic, FSH-orchestrated entry of follicles in the follicular waves of terminal development and renewed by the continuous growth of smaller follicles (**Fig. 9**). The transition between the static and the dynamic reserves is favored by cytokines and growth factors (KITLG, GDF9, BMP15, and activin). AMH produced by the dynamic reserve exerts a negative endocrine feedback onto the pool of primordial and primary follicles. The survival and growth of the follicles belonging to the dynamic reserve are supported by stimulating factors acting in an endocrine (essentially FSH, insulin, IGF1) or a paracrine way (activin, BMP, EGF, FGF, NGF, testosterone) and modulated by paracrine factors (AMH, IGFBP) lowering the sensitivity of granulosa cells to FSH (reviewed by Monget *et al.*, 2002; Knight *et al.*, 2012; Visser and Themmen, 2014).

In the premenopause period, few aged primordial follicles persist in the static reserve; they can give rise to growing follicles, yet they contain low-quality oocytes. The dynamic reserve has a reduced size and produces low to undetectable AMH endocrine levels, which enhances follicular growth activation. The high FSH endocrine levels present during this period could be responsible for the increased occurrence of multiple ovulations.

Mechanisms of Selection of the Ovulatory Follicle

At each menstrual cycle, the 2- to 5-mm follicles present in the dynamic reserve can enter terminal development, and generally, a single follicle (named the dominant follicle) is selected for ovulation from this cohort (or wave) of growing follicles. From histologic, endocrinological and/or ultrasonographic data, different theories explaining the cohort recruitment have been proposed in humans: a continuous recruitment throughout the menstrual cycle, a single recruitment episode in the late-luteal or early-follicular phase of each cycle, or the recruitment of two or three cohorts during each cycle (reviewed by Baerwald *et al.*, 2012). Generally, the dominant follicle is selected in the mid-follicular phase of each cycle, but it may also be selected within the anovulatory cohorts preceding the ovulatory one in some women.

The emergence and growth of the cohort are triggered by relatively high blood levels of FSH. Each follicle of the cohort secretes increasing amounts of inhibin and estradiol, relative to its cell number and cell maturity level, as it gets more mature. The cumulated contributions of all growing follicles to the release of estradiol and inhibin by the ovaries lead to a drop in FSH secretion by the pituitary gland, and the follicle producing the highest hormonal outputs takes progressively the control of FSH secretion and becomes dominant. The drop in plasma FSH levels is at the source of the selection process for ovulation (reviewed by Clément and Monniaux, 2013; Monniaux *et al.*, 2016). All follicles except the dominant one(s) become atretic when FSH levels fall below the threshold needed to sustain their development. In fact, FSH levels are above this threshold only for a few days (reviewed by Smacklon and Fauser, 2000). Next, only the dominant, most mature follicle will continue its development even in the unfavorable FSH environment, thanks to an increased sensitivity to FSH and a highly developed vascularization. In addition, its granulosa cells become LH responsive before those of all other follicles in the cohort, so that the dominant follicle can benefit

Table 3 Genes involved in follicle selection and maturation, as evidenced from mouse genetic models

Gene name	*Signaling pathway*	*Genetic model*	*Ovarian phenotype and fertility*	*References*
Adamts1 (ADAM metallopeptidase with thrombospondin type 1 motif 1)	Extracellular matrix proteolysis	KO	Decreased number of antral follicles, follicle dysgenesis, subfertility	Brown *et al.* (2006)
Aire (autoimmune regulator)	TF Inflammation	KO	Follicular depletion, subfertility	Jasti *et al.* (2012)
Amh (anti-Mullerian hormone)	SMAD	KO	Enhanced FSH-stimulated follicular growth and increased atresia, accelerated follicular depletion	Durlinger *et al.* (2001), Visser *et al.* (2007)
Ar (androgen receptor)	TF Androgen	KO and cKO in granulosa	Defective folliculogenesis, enhanced atresia, POF, subfertility	Reviewed by Walters (2015)
Bmp15 (bone morphogenetic protein 15)	SMAD	KO double mutant with Gdf9 +/−	Defective cumulus cell development, abnormal folliculogenesis, subfertility	Yan *et al.* (2001), Su *et al.* (2004)
Bmpr1A (bone morphogenetic protein receptor type 1A)	SMAD	cKO in granulosa	Decreased number of large antral follicles, decreased ovulation rate, subfertility	Edson *et al.* (2010)
Ctgf (connective tissue growth factor)	Extracellular matrix protein	cKO in granulosa	Decreased number of antral follicles, increased atresia, disrupted steroidogenesis, subfertility	Nagashima *et al.* (2011)
Ctnnb1 (catenin beta 1)	WNT Gonadotropin	cKO and cKI (activating mutation) in granulosa	cKO: loss of sensitivity to FSH, defective FSH-induced steroidogenesis, increased sensitivity to LH cKI: increased numbers of antral follicles, decreased atresia, impaired sensibility to LH, subfertility	Hernandez Gifford *et al.* (2009), Fan *et al.*, (2010)
Esr1 (estrogen receptor 1)	TF Estrogen	cKO in theca	Increased number of antral follicles, decreased ovulation rate, POF, infertility by 6 months	Lee *et al.* (2009)
Esr2 (estrogen receptor 2)	TF Estrogen	KO	Impaired FSH-induced granulosa cell differentiation, decreased ovulation rate, subfertility	Couse *et al.* (2005)
Fmr1 (fragile X mental retardation 1)	RNA-binding protein	KI	Increased atresia, POF, subfertility	Lu *et al.* (2012), Conca Dioguardi *et al.* (2016)
Foxo1 (forkhead box O1)	Gonadotropin	cKO in granulosa	Increased granulosa cell survival, decreased granulosa cell proliferation, normal fertility	Liu *et al.* (2013)
Fshr (follicle-stimulating hormone receptor)	Gonadotropin	FSHR +/−	Impaired antral follicle development, estrogen deficiency, subfertility, early reproductive senescence	Danilovich *et al.* (2000)
Furin (furin, paired basic amino acid cleaving enzyme)	Proprotein convertase	cKO in oocyte	Decreased number of antral follicles, enhanced atresia, primary infertility	Meng *et al.* (2017)
Gdf9 (growth differentiation factor 9)	SMAD	Gdf9 +/−	Normal ovarian function and fertility. Abnormal folliculogenesis and subfertility in double mutants Bmp15 −/−; Gdf9 +/−	Yan *et al.* (2001)
Immp2l (inner mitochondrial membrane peptidase subunit 2)	Mitochondrial function	KI	Increased oxidative stress, ovarian senescence, POF	George *et al.* (2011)
Inha (inhibin alpha subunit)	SMAD	KO	Increased granulosa cell proliferation, improper granulosa differentiation and tumor formation	Myers *et al.* (2009), Nagaraja *et al.* (2010)
Kras (KRAS proto-oncogene, GTPase)	Gonadotropin	cKI in granulosa (activating mutation)	Impaired proliferation and differentiation of granulosa cells, ovulation failure, subfertility	Fan *et al.* (2008b)

(*Continued*)

Table 3 Continued

Gene name	*Signaling pathway*	*Genetic model*	*Ovarian phenotype and fertility*	*References*
Lhcgr (luteinizing hormone/chorionogonadotropin receptor)	Gonadotropin	KO	Absence of preovulatory follicles and ovulation, sterility	Pakarainen *et al.* (2005)
Nppc (natriuretic peptide C)	NPR2 ligand	Spontaneous mutations	Premature resumption of meiosis in follicle-enclosed oocytes	Zhang *et al.* (2010)
Npr2 (natriuretic peptide receptor 2)	Guanylyl cyclase receptor	Spontaneous mutations	Premature resumption of meiosis in follicle-enclosed oocytes	Zhang *et al.* (2010)
Nr5a1 (nuclear receptor subfamily 5 group A member 1)	TF Gonadotropin	cKO in steroidogenic cells	Impaired differentiation of theca cells, but normal fertility	Buaas *et al.* (2012)
Pappa (pappalysin 1)	Proteolysis of IGFBP	KO	Impaired steroidogenesis and IGFBP intrafollicular levels, decreased ovulation rate, subfertility	Nyegaard *et al.* (2010)
Pgrmc1 (progesterone receptor membrane component 1)	TCF/LEF Progesterone	cKO in granulosa	Increased granulosa cell apoptosis and follicular atresia	Peluso and Pru (2014)
Pde4d (phosphodiesterase 4D)	Gonadotropin	KO	Impaired differentiation of granulosa cells, premature luteinization of follicles, subfertility	Park *et al.* (2003)
Plcl1/2 (phospholipase C like 1/2)	Gonadotropin	Double KO	Decreased follicular atresia, multiple cystic follicles, subfertility	Matsuda and Hirata (2017)
Scarb1 (scavenger receptor class B member 1)	Receptor of high-density lipoproteins	KO	Follicular cysts with hypertrophied theca cells, sterility	Jimenez *et al.* (2010)
Smad2/3 (SMAD family member 2/3)	SMAD	Double cKO in granulosa	Decreased number of antral follicles, premature luteinization of follicles, subfertility	Li *et al.* (2008)
Smad4 (SMAD family member 4)	SMAD	cKO in granulosa	Premature luteinization of follicles, cumulus defects, subfertility	Pangas *et al.* (2006b)
Sox3 (SRY-box 3)	TF	KO	Increased atresia, ovulation of defective oocytes, subfertility	Weiss *et al.* (2003)

TF, transcription factor; *KO*, knockout; *cKO*, conditional KO; *KI*, knock-in; *cKI*, conditional knock-in; *POF*, premature ovarian failure.

from LH supply until the preovulatory stage. The large estradiol amounts secreted by the preovulatory follicle impact the secretion of GNRH1 from the hypothalamus and ultimately trigger the GNRH1 ovulatory surge. As a result, the pituitary LH surge occurs and brings about ovulation.

Nutrition and Folliculogenesis

Nutritional changes affecting the energy balance or specific nutrients can affect folliculogenesis and ovulation by acting on the hypothalamo-pituitary system and/or directly on the ovary. Their effects are mediated by metabolic sensors (adipokines, AMPK, PPAR, not detailed in this section, reviewed by Dupont *et al.*, 2012, Reverchon *et al.*, 2014, Vitti *et al.*, 2016).

Energy Balance and Fertility

Gametogenesis and reproduction are energetically expensive, especially in females, and are therefore sensitive to energy imbalance (reviewed by Chavarro *et al.*, 2015). Chronic malnutrition induced by starvation (during wars, food insecurity or due to anorexia) reduces the secretion of hypothalamic GNRH1 and leads to anovulation and an endocrine status similar to that of the prepubertal stage (reviewed by Fontana and Della Torre, 2016; Chavarro *et al.*, 2015). Although the primary effect is hypothalamic, direct ovarian effects of hypoglycemia are not excluded (Chen *et al.*, 1992). Young girls or women exposed to high energy exertion (ballet, competitive sport programs, strenuous exercise) also show delays in puberty or amenorrhea and cycle irregularities, through similar mechanisms as in malnutrition conditions (Fontana and Della Torre, 2016).

On the contrary, high body mass index (BMI) is related to an earlier puberty. Overweight women also exhibit a higher frequency of anovulation, infertility, poorer oocyte quality and maturity, and negative outcomes for obese patients undergoing in vitro fertilization (IVF) (reviewed by Fontana and Della Torre, 2016; Broughton and Moley, 2017). Increased BMI is also

Table 4 Genes involved in oocyte maturation and ovulation, as evidenced from mouse genetic models

Gene name	*Signaling pathway*	*Genetic model*	*Ovarian phenotype and fertility*	*References*
Adamts1 (ADAM metallopeptidase with thrombospondin type 1 motif 1)	Extracellular matrix proteolysis	KO	Abnormal morphogenesis of the ovulating follicle, abnormal cumulus matrix remodeling, decreased ovulation rate, subfertility	Brown *et al.* (2010)
Bmp15 (bone morphogenetic protein 15)	SMAD	Bmp15 −/− Gdf9 +/−	Defective cumulus cell development, impaired cumulus integrity and expansion, subfertility or sterility	Yan *et al.* (2001), Su *et al.* (2004)
Bmpr1b (bone morphogenetic protein receptor type 1B)	SMAD	KO	Failure of cumulus expansion, sterility	Yi *et al.* (2001)
Cebpb (CCAAT/enhancer binding protein beta)	TF Gonadotropin	cKO in granulosa (preovulatory follicles)	Impaired ovulation and luteinization, subfertility	Fan *et al.* (2009), Fan *et al.* (2011)
Ctgf (connective tissue growth factor)	Extracellular matrix protein	cKO in granulosa (preovulatory follicles)	Impaired ovulatory process, subfertility	Nagashima *et al.* (2011)
Ctnnb1 (catenin beta 1)	WNT Gonadotropin	cKI in granulosa (activating mutation)	Impaired oocyte maturation, cumulus expansion and ovulation, subfertility	Fan *et al.* (2010)
Egfr (epidermal growth factor receptor)	MAPK1/3	cKO in granulosa (preovulatory follicles)	Impaired oocyte maturation, cumulus expansion and ovulation, subfertility	Hsieh *et al.* (2011)
Esr2 (estrogen receptor 2)	TF Estrogen	KO	Impaired ovulatory process, subfertility	Nalvarte *et al.* (2016)
Fzd1 (frizzled class receptor 1)	WNT	KO	Impaired oocyte maturation and cumulus cell differentiation, subfertility	Lapointe *et al.* (2012)
Lhcgr (luteinizing hormone/choriogonadotropin receptor)	Gonadotropin	KO	Absence of preovulatory follicles and ovulation, sterility	Pakarainen *et al.* (2005)
Mapk3/1 (mitogen-activated protein kinase 3/1)	Gonadotropin	Double cKO in granulosa (pre-ovulatory follicles)	Impaired ovulation and luteinization, subfertility	Fan *et al.* (2009)
Nrip1 (nuclear receptor interacting protein 1)	TF	KO	Failure of cumulus expansion and ovulation, luteinized unruptured follicle syndrome, sterility	White *et al.* (2000), Nautiyal *et al.* (2010)
Pgr (progesterone receptor)	TF Progesterone	KO	Failure of ovulation, sterility	Robker *et al.* (2000), reviewed by Richards (2005)
Pten (phosphatase and tensin homolog)	PI3K/AKT	cKO in granulosa (preovulatory follicles)	Enhanced granulosa cell survival, increased ovulation rate, increased life span of luteal cells, fertility	Fan *et al.* (2008a)
Ptger2 (prostaglandin E receptor 2)	Prostaglandin	KO	Abortive expansion of the cumulus, reduced ovulation, subfertility	Hizaki *et al.* (1999)
Ptgs2 (prostaglandin-endoperoxide synthase 2)	Prostaglandin	KO	Failure of cumulus expansion and ovulation, sterility	Davis *et al.* (1999), reviewed by Richards (2005)
Ptx3 (pentraxin 3)	Inflammation	KO	Defects in the integrity of the cumulus cell–oocyte complex, subfertility	Varani *et al.* (2002)
Smad2/3 (SMAD family member 2/3)	SMAD	Double cKO in granulosa	Impaired cumulus expansion, subfertility	Li *et al.* (2008)
Smad4(SMAD family member 4)	SMAD	cKO in granulosa (preovulatory follicles)	Increased atresia of preovulatory follicles, ovulation defects, subfertility	Yu *et al.* (2013)
Taf4b (TATA-box binding protein associated factor 4b)	TF	KO	Abnormal cumulus, defects in oocyte maturation, sterility	Falender *et al.* (2005)

TF, transcription factor; *KO*, knockout; *cKO*, conditional KO; *cKI*, conditional knock-in.

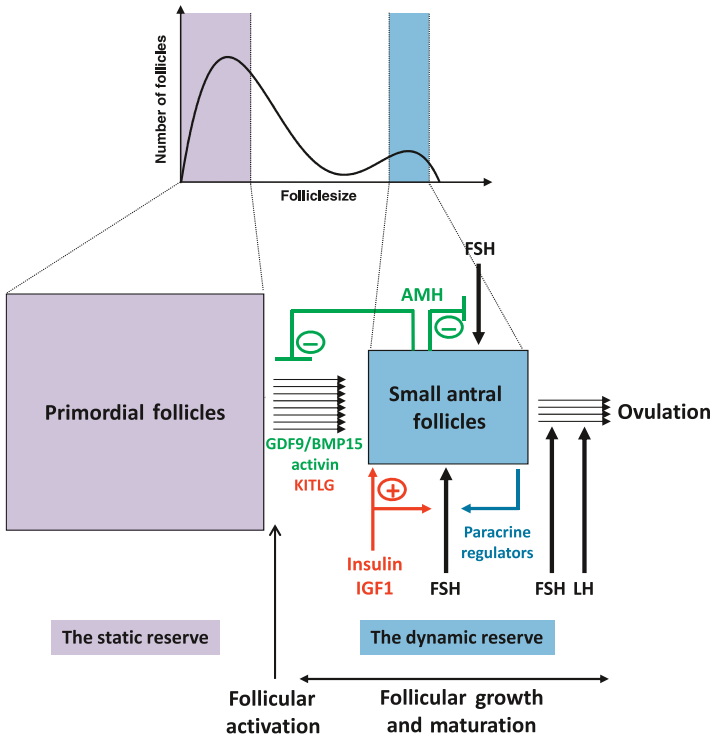

Fig. 9 The ovarian reserves of follicles and their main endocrine and paracrine regulations. The reserves are represented as boxes containing the follicle populations. The reserve of small antral follicles (dynamic reserve) is filled by growing follicles originating from the static reserve of primordial follicles and emptied by the cyclic, FSH-orchestrated entry of follicles into the follicular waves of terminal development. The quantitative distribution of follicles between the static and dynamic reserves is schematically depicted in the *upper part* of the figure. *AMH*, anti-Mullerian hormone; *BMP15*, bone morphogenetic protein 15; *GDF9*, growth differentiation factor 9; *IGF1*, insulin like growth factor 1; *KITLG*, kit ligand.

associated with quantitative alteration of endocrine factors (i.e., insulin, plasma adipokines such as leptin and adiponectin) or reproductive hormones (androgens) that negatively influence folliculogenesis and oocyte development (El-Toukhy and Osman, 2015). For example, increased insulin resistance, hyperinsulinemia or high leptin, consecutive to obesity, can adversely affect folliculogenesis and oocyte maturation by altering the expression of genes involved in meiosis and steroidogenic pathways, or the steroidogenic activity, proliferation and apoptosis rates of granulosa cells (Brannian and Hansen, 2002; Nteeba *et al.*, 2014; Lin *et al.*, 2017).

Nutrients and Folliculogenesis

Carbohydrates

Dietary glycemic load (quality/amount of carbohydrate) was shown to be related to anovulation (Chavarro *et al.*, 2015). In the gonads, glucose is essential for maintaining the quality of oocytes and regulating meiotic maturation (Dupont *et al.*, 2014). Glucose availability in follicular cells is regulated by the glucose transporter system and by glucose transfer between cumulus cells and oocyte through gap junctions (Dupont *et al.*, 2014). In sheep, a higher level of glucose increases the number of small antral (1–2 mm) follicles by a direct action (without altering insulin signaling pathways), while the effect of glucose in estrogenic follicles (reduction in aromatase level in follicular cells and reduction in estradiol level in follicular fluid) is mediated by insulin (Scaramuzzi *et al.*, 2015). Nevertheless, pyruvate is the main energy substrate oxidized in follicles, where both glycolysis and mitochondrial pyruvate oxidation occur, and pyruvate is needed to meet oocyte energetic requirement for the resumption of meiosis (reviewed by Collado-Fernandez *et al.*, 2012).

Amino acids and proteins

Despite their roles as substrates (for proteins, signaling molecules, nucleotides, energy, etc.), little is known on the effect of amino acids on folliculogenesis, contrary to their effects on ovulation or embryo development (reviewed by Collado-Fernandez *et al.*, 2012). A specific system of leucine transport has been reported in the mouse ovarian follicles during folliculogenesis (Chand and Legge, 2011). The oocyte amino acid transport and composition are related to its maturation stage and its ability to cleave once fertilized (reviewed by Collado-Fernandez *et al.*, 2012). In cow, glycine and alanine from follicular fluid were even suggested as predictors of cumulus–oocyte complex quality (Sinclair *et al.*, 2008). Finally, amino acid depletion impairs mTORC signaling and consequently disturbs folliculogenesis (reviewed by Dupont *et al.*, 2014).

Lipids

More and more data point out the role of lipids in female fertility. Indeed, lipid metabolism and in particular fatty acid β-oxidation in cumulus cells are essential for oocyte maturation depending on the species (mouse oocytes would be less sensitive to fatty acid β-oxidation impairment than swine oocyte, with an intermediate position for human) (Paczkowski *et al.*, 2013; Dunning *et al.*, 2014). A high level of fatty acids (as observed in obese patients) will lead to lipotoxicity and prevent the oocyte from maturing. Cumulus cells indeed incorporate lipids from follicular fluid and store them in lipid droplets to protect the oocyte from lipotoxicity (reviewed by Fontana and Della Torre, 2016). Dietary fat supply in bovine, especially with polyunsaturated fatty acids, has been used to improve female fertility, folliculogenesis and oocyte quality (evidenced on blastocyst transition rates after IVF) (reviewed by Leroy *et al.*, 2014).

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Further Reading

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Human Menstrual Cycle☆

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Glossary

Corpus luteum The structure formed from the ovarian follicle after ovulation that is primarily responsible for progesterone production.

Estradiol The principal estrogenic hormone secreted by the developing ovarian follicle in response to follicle-stimulating hormone; necessary for proliferation of the endometrium.

follicle The functional unit of the ovary; consists of an oocyte and surrounding granulosa and theca cells.

Follicle-stimulating hormone (FSH) A glycoprotein hormone secreted in a pulsatile fashion by the anterior pituitary that is responsible for selecting a dominant ovarian follicle and stimulating ovarian steroidogenesis, particularly estrogen production from granulosa cells.

Follicular phase Beginning with the first day of menstruation, the phase of the ovarian cycle that correlates with the process of dominant follicle selection from the pool of immature oocytes and culminates in the process of ovulation.

Gonadotropin-releasing hormone (GnRH) A peptide hormone released in a pulsatile fashion from the medial–basal hypothalamus that stimulates the release of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary.

Luteal phase The phase of the ovarian cycle that begins at ovulation and corresponds in timing to its functional production of the hormone progesterone from the corpus luteum.

Luteinizing hormone (LH) A glycoprotein hormone secreted in a pulsatile fashion by the anterior pituitary that is responsible for stimulating ovarian steroidogenesis and promoting ovulation.

Progesterone The predominant hormone produced by the corpus luteum in response to pituitary luteinizing hormone; responsible for secretory transformation of the endometrium in anticipation of embryo implantation.

Proliferative phase The phase in the endometrial cycle during which the glands of the endometrium undergo active growth and maturation in response to estradiol; corresponds temporally to the follicular phase of the ovarian cycle.

Secretory phase The phase in the endometrial cycle during which large secretory glands important to embryo implantation develop within the endometrium; corresponds temporally to the luteal phase of the ovarian cycle.

The presence of a menstrual cycle implies many interactions among the brain, the ovaries and the uterus. A menstrual cycle is defined as the interval between two episodes of menses. Its normal duration in humans is between 26 and 32 days. The first phase of the cycle is called the follicular phase or proliferative phase, the second phase, occurring after ovulation, is called the luteal phase or secretory phase (Chabbert Buffet *et al.*, 1998). The duration of the luteal phase is rather constant, around 14 days. The occurrence of the first menstrual cycle is called menarche. Its mean age of arrival is 12.5 years. The mean delay between breast development and the first menses is 2 years.

Hypothalamus and the Gonadotropin Releasing Hormone (GnRH)

The menstrual cycle is under the control of GnRH. This hormone is a decapeptide, secreted by neurons located in the arcuate nucleus of the hypothalamus. Those neurons need to migrate during fetal life, from the olfactory bulb to the hypothalamus. Failure of such migration to the arcuate nucleus results in Kallmann syndrome (Dodé and Hardelin, 2010). This congenital syndrome, commonly associates hypogonadotropic hypogonadism and anosmia, a failure in the sense of smell. Clinically, these patients present with absent puberty and primary amenorrhea. In the past 20 years, many genes involved in GnRH migration and/or GnRH secretion have been identified in patients with congenital hypogonadotropic hypogonadism. Some of the most important factors are Kiss1 receptor (Kiss1R) or GPR54, as well as its ligand, the protein Kiss. Studies have demonstrated that hypothalamic neurons secreting Kiss peptide are located upstream of GnRH neurons. Kiss neurons secrete the protein Kiss which binds to its receptors located on the membrane of the GnRH neurons (Gahete *et al.*, 2016). Therefore, they are able to regulate GnRH secretion. A subpopulation of Kiss neurons are called KNDY neurons as they release not only Kiss peptide (K) but also neurokinin (N) and dynorphin (DY). Kiss neurons are major players of the gonadotroph axis as they are able to integrate estradiol and progesterone feedbacks, as well as messages issued from fat tissue. This point is illustrated by the fact that a minimal weight is necessary in a child in order to induce puberty and therefore GnRH pulsatility. Factors released by fat tissue, such as leptin, are involved in Kiss peptide secretion and therefore GnRH secretion.

☆*Change History*: May 2018. Sophie Christin-Maitre has updated the article. This article has been totally transformed.

This article is an update of Lee Caperton and Robert Brzyski, Menstrual Cycle: An Integrative View, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 238–241.

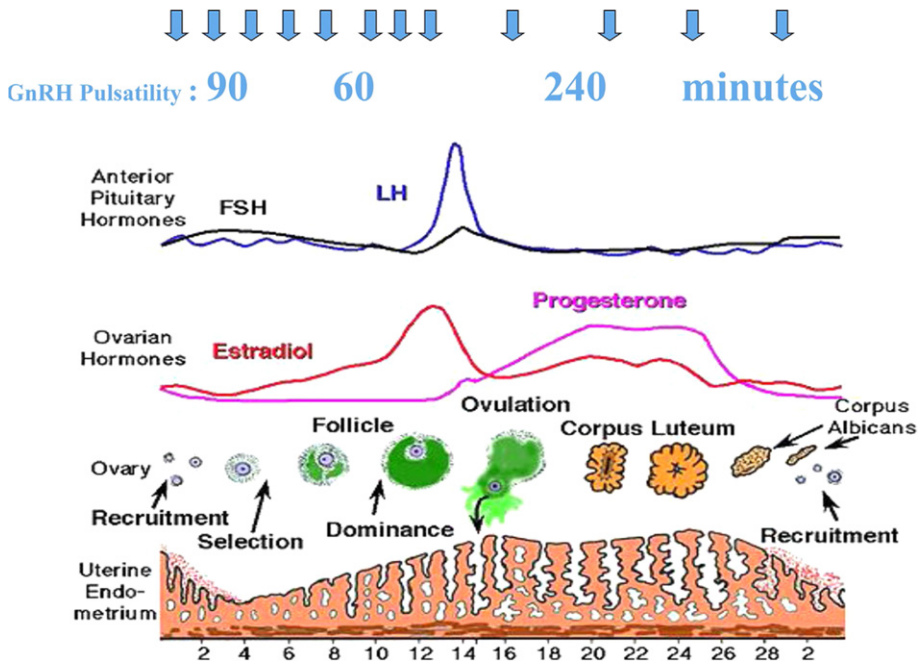

Fig. 1 GnRH pulsatility from the hypothalamus ranges from 90 min during the first phase of the follicular phase, to 60 min at the end of the follicular phase and 240 min during the luteal phase. FSH and LH are secreted by the pituitary and stimulate follicular growth and ovulation. At the beginning of the menstrual cycle, among the cohort of follicles, several are going to be recruited, selected and only one is going to become the dominant follicle. The endometrium proliferates under the effect of estradiol during the follicular phase. During the luteal phase, progesterone induces endometrium differenciation and therefore the window of implantation. In the absence of pregnancy, a decrease of estradiol and progesterone occur and menses are initiated. Reprinted from Reed, B. G., Carr, B. R. The Normal Menstrual Cycle and the Control of Ovulation. In: De Groot, L. J., Chrousos, G., Dungan, K., Feingold, K. R., Grossman, A., Hershman, J. M., Koch, C., Korbonits, M., McLachlan, R., New, M., Purnell, J., Rebar, R., Singer, F., Vinik, A., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000–2015 May 22.

Pulsatile release of GnRH appears to be an innate characteristic of neurons in the arcuate nucleus. In vitro experiments have shown that these neurons have pulsatile activity even when isolated from their normal surrounding structures and microenvironment. In vivo experiments using portal system catheterization have shown in ewes, monkeys and mice, that GnRH secretion is pulsatile. This pulsatility is directly correlated with luteinizing hormone (LH) pulse frequency. Therefore, in humans, the only way to evaluate GnRH secretion is to measure LH in serum every 10 min. It has been shown that in females, GnRH pulses occur every 90 min during the beginning of the follicular phase, every 60 min at the end of the follicular phase and every 240 min during the luteal phase (**Fig. 1**).

Environmental changes, such as stress, intense exercise, extremes of weight may disrupt normal hypothalamic GnRH secretion. Even a selective diet avoiding lipids may induce a hypothalamic hypogonadism. Patients with amenorrhea resulting from hypothalamic–pituitary dysfunction are usually hypoestrogenic.

The Gonadotropin (Follicle-Stimulating Hormone and Luteinizing Hormone) Release From the Pituitary

In response to the pulsatile release of GnRH from the hypothalamus, the anterior pituitary secretes and releases two hormones, called luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Abel *et al.*, 2014). GnRH binds to its receptor, located on the membrane of gonadotroph cells from the pituitary. This receptor belongs to the G protein related family. After its binding, GnRH induces a pulsatile secretion of FSH and LH. Few patients have been described in the literature with GnRH or GnRH receptor loss of function mutations. They have a lack of breast development and no menstrual cycle.

FSH and LH are each made of two subunits, a common alpha subunit and a specific beta subunit (Abel *et al.*, 2014). Those subunits are FSHβ and LHβ, respectively. Both proteins are glycosylated and are therefore called glycoproteins. Several types of gonadotropins are secreted, depending on the degree of their glycosylation. The different forms of gonadotropins are called isoforms. The mean half-lives of gonadotropins are respectively 1 and 5 h, for LH and FSH.

The predominant function of FSH is to stimulate follicular recruitment and development during the follicular phase of the cycle. By definition, the follicular phase starts on the first day of menstruation. Slight but significant increases in FSH secretion can be identified 1 or 2 days before the onset of menstruation, during the period called the luteo-follicular transition, occurring between two menstrual cycles. This FSH increment is a consequence of the decline of ovarian hormone production, especially inhibin A and estradiol, during the late luteal phase. The initial increase in FSH stimulates the development of a cohort of ovarian

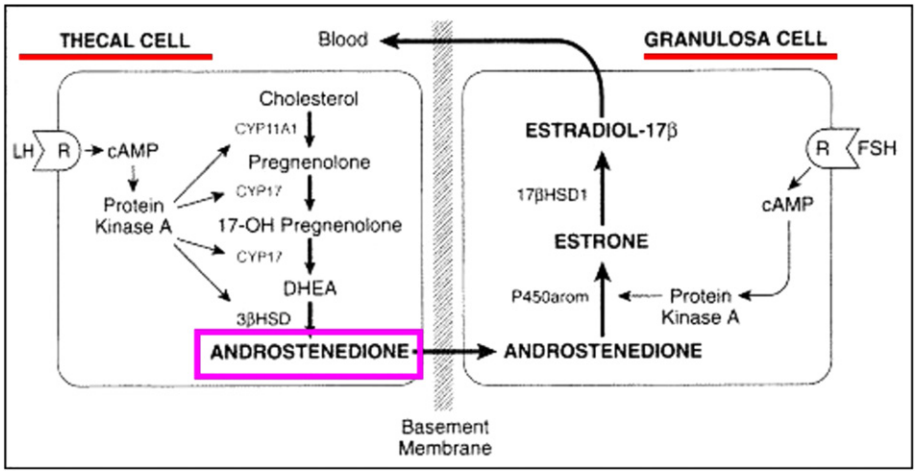

Fig. 2 The two cells- two gonadotropin theory. The two types of follicular cells are the thecal and the granulosa cells. LH binds to its receptor on the membrane of thecal cells and induce the formation of androstenedione. This androgen diffuses through the basement membrane to the granulosa cells. Within this type of cells, androstenedione is transformed to estradiol. This aromatization is under the control of FSH, after its binding on the membrane of granulosa cells. Adappted from http://www.ansci.wisc.edu/jjp1/ansci_repro/lab/lab7/lab7_2003/menstrual.html.http://www.ansci.wisc.edu/jjp1/ansci_repro/lab/lab7/lab7_2003/menstrual.html.

follicles containing immature oocytes. At the beginning of the menstrual cycle, this cohort contains an average of 5 to 10 follicles. This FSH stimulation eventually results in the selection of a single dominant follicle destined for ovulation.

Progressively increasing estradiol secretion during the second part of the follicular phase acts to inhibit FSH release prior to the mid-cycle gonadotropin surge. This negative feed-back of estradiol involves Kiss neurons. This decrease in FSH allows the dominant follicle a unique advantage over other developing follicles that will ultimately degenerate through the process of atresia. This advantage is predominantly a result of a higher density of FSH receptors, greater steroidogenic capacity, and a more profound angiogenic proliferation within the dominant follicle. This process results in mono ovulation. The rise of FSH occurring at the beginning of the follicular phase followed by its decrease during the end of the follicular phase has been called the window of FSH. It has initially described by Baird (1987). The largest is the window, the more follicles are going to be recruited. Only the follicles most sensitive to FSH are going to be able to pursue their maturation up to the dominant stage. In order to obtain a mono follicular development during ovarian stimulation induction, the biggest challenge is to mimic this FSH window and therefore to avoid ovarian hyperstimulation.

At the end of the follicular phase of the cycle, estradiol (E2) increases and exerts a positive feedback on the hypothalamus. This is a reversal of the estradiol negative feed-back to a positive feed-back. At mid-cycle, E2 serum level generally reaches around 200 pg/mL or 680 pmol/L per dominant follicle. The mean size of the dominant follicle is 20 mm in diameter. Estradiol increase promotes the stimulation of the midcycle gonadotropin surge that initiates the events resulting in ovulation of the dominant follicle. The mid-cycle LH surge is under the dependence of a GnRH surge from the hypothalamus. Although a surge in both LH and FSH occurs, LH plays the most significant role in the ensuing ovulation process.

The LH surge plays three major roles during the menstrual cycle. First, it stops granulosa cell proliferation, in the follicle. Secondly it induces granulosa cells differentiation and those cells become able to secrete progesterone. They form the corpus luteum. The third role of the LH surge is to re-initiate oocyte meiosis which has been arrested during fetal life. In summary, the LH surge is critical in regulating the production of progesterone, prostaglandins, and other local factors that contribute to follicle rupture (Richards and Pangas, 2010). The mean delay between the onset of the LH surge and ovulation is 36 h. Ovulation of the follicle occurs 10–12 h after the peak of the LH surge. In response to these conditions, the oocyte is extruded from the ovary into the fimbria of the fallopian tube. Few cases of women carrying loss of function mutation of LH receptors have illustrated the fact that LH is necessary in order to induce follicular ovulation.

Menstrual cycles are ovulatory very early after menarche. Indeed, a study performed in a large cohort of American teenagers has shown that 80% of cycles are ovulatory, as soon as after the first year after menarche (Legro *et al.*, 2000). In physiology, menstrual cycles may be irregular during the first 2 years after menarche but they should be regular after such a delay (van Hooff *et al.*, 2004).

Just after ovulation, a decrease of estradiol is observed. During the luteal phase, pulsatile LH secretion continues to promote the production of estradiol and progesterone. In case of pregnancy, hCG secreted by the placenta stimulates progesterone secretion from the corpus luteum. Progesterone secretion is essential for the induction of secretory changes within the uterine cavity. These changes are a prerequisite for proper embryo implantation.

The Follicles in the Ovary

The number of ovarian follicle is maximal during fetal life. At 20th week of gestation, the number of primordial follicles reaches around 6 million. At birth, the number of follicles is around 1 million, 500,000 at puberty and less than 1000 at menopause. The growing follicles develop from a reserve of primordial follicles constituted early in life. From this preestablished reserve, a second ovarian reserve is formed, which consists of gonadotropin-responsive small antral growing follicles and is a dynamic reserve for ovulation (Monniaux *et al.*, 2014). From this reserve of follicles, many are being recruited constantly up to the preantral stage or early antral stage. This recruitment is permanent. Cohorts of primordial follicles start growing. Primordial follicles during their maturation have to pass several stages, such as secondary, preantral, antral and finally preovulatory stages. However, more than 99.9% of follicles are not able to mature to the last stage and are submitted to apoptosis.

After puberty, FSH rises and induces a second type of recruitment occurs. It is called the cyclic recruitment of follicles. One should remember that only the last stages of folliculogenesis are FSH dependent. This has been illustrated by the rare cases of female patients carrying FSHβ or FSH receptor mutations. Those patients present a blockade in their folliculogenesis and are not able to secrete estradiol. This phenomenon is also observed in women taking combined contraceptive pills. In such cases, progestins inhibit FSH secretion and follicular growth is blocked at the early antral stage.

In the ovarian follicles, FSH receptors are expressed on the membrane of granulosa cells and LH receptors are typically expressed on the membrane of thecal cells. Furthermore, when the follicle is mature, it expresses LH receptors not only on thecal cells but also on the membrane of granulosa cells. The basis of steroid synthesis within the follicle relies on the two cells, two gonadotropins theory. The two cells are respectively granulosa cells and thecal cells. In order to synthetize steroids, the first step is the binding of LH to its receptor, localized on thecal cells (**Fig. 2**). This binding induces within thecal cells the production of androgens, originating from cholesterol metabolism. Androstenedione, the main androgen, then crosses the basal membrane separating thecal cells from granulosa cells. In the granulosa cells, androgens are aromatized to estradiol. This enzymatic transformation depends on FSH, after its binding to its receptor on the granulosa cells. The respective roles of FSH and LH in females have been illustrated in the early 90s, in studies using recombinant gonadotropins. In women with a hypogonadotropic hypogonadism, a treatment using recombinant FSH, in the absence of LH, was able to induce follicular growth. However, estradiol secretion was very low, due to the absence of endogenous LH in those patients (The European Recombinant Human LH Study Group, 1998). This study illustrated the respective roles of FSH and LH during the human cycle.

After ovulation, granulosa cells form the corpus luteum. Their main characteristic is the acquisition of 3-β-hydroxysteroid enzyme. This enzyme enables the production of progesterone. The corpus luteum appears to have a programmed life span, rapidly regressing 9–11 days following ovulation in the absence of pregnancy. Progesterone secretion by the corpus luteum exerts a negative feedback on GnRH pulsatility. This feedback involves progesterone receptors located on the membrane of hypothalamic Kiss neurons. GnRH pulses during the luteal phase occur every 240 min. This low frequency induces low levels of FSH and LH.

During early pregnancy, the conceptus produces human chorionic gonadotropin (hCG), a glycoprotein nearly identical to LH in structure and function. hCG binds to the same receptor as does LH. This sustains the steroid-producing capacity of the corpus luteum until the placenta develops sufficient steroidogenic activity.

The cessation of ovarian function, which results in menopause, is related to the depletion of ovarian follicles. This process usually occurs at approximately 51 years of age. Premature ovarian insufficiency (POI) is defined as cessation of ovarian function prior to 40 years of age. The diagnosis is established in front of a primary or a secondary amenorrhea lasting more than 4 months, with elevated FSH serum levels. FSH is higher than 25 IU/L and estradiol level is low (ESHRE POI, 2016). POI usually results from an early follicular depletion.

The Uterus and Menstruation

Distinct changes in the uterine lining are noted throughout the menstrual cycle. The proliferative phase of the menstrual cycle corresponds with the follicular phase of the ovarian cycle and is marked by estrogen-dependent proliferation of glandular structures and vasculature within the endometrium. Mitotic activity is prominent, and the endometrial lining increases in thickness as much as 10-fold.

After ovulation, the thickness of the endometrium is rather constant. Tortuosity of the vessels and secretory activity within the glands are the hallmarks of the secretory phase of the cycle. At the time of implantation of the blastocyst, marked edema can be seen within the stroma of the endometrium. Concomitantly, decidualization occurs. The decidua is derived from the stromal cells of the endometrium and is rich in glycogen and lipid content. This layer of cells displays the ability to produce a large number of autocrine and paracrine regulatory peptides. The decidua is integrally involved in the processes of implantation and placentation. The window of implantation occurs around day 21–23 of the cycle or 8 days after ovulation.

In the absence of pregnancy, the demise of the corpus luteum results in the withdrawal of estrogen and progesterone. This results in apoptosis of the endometrial cells and contraction of the spiral arteries nourishing the surface of the endometrium. Hemorrhage into the stroma and infiltration of the endometrium with inflammatory mediators follow. The decreases of estradiol and progesterone result in menstruation. The normal length of menstruation is from 3 to 6 days.

In conclusion, regular menstrual cycles involve a functional hypothalamus containing Kiss neurons and GnRH neurons, which secrete pulsatile GnRH. GnRH binds to its receptor located on the membrane of gonadotroph cells in the pituitary. Those cells

secrete both gonadotropins, FSH and LH. Those hormones bind respectively to granulosa and thecal cells, from the ovarian follicle. The follicle is able to secrete steroids such as estradiol and progesterone which regulate respectively endometrium growth and differentiation. Their feedback on the hypothalamus involves Kiss neurons. Disorders of menstrual cycle may involve defects at each level of the gonadotroph axis: the hypothalamus and/or the pituitary, the ovaries and/or the uterus. In order to evaluate the endogenous estradiol secretion, in front of an amenorrhea, a progestin test may be performed. It consists of giving to the patient, 10 days of progesterone or progestins. If menses occur within two weeks after stopping the progesterone or the progestin, the test is positive. This indicates that there is an endogenous secretion of estradiol. If the test is negative, and if the patient has a uterus, it means that the patient's endogenous estradiol level is too low. The second hypothesis in front of a negative progestin test is that the patient is pregnant.

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Implantation☆

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Glossary

Embryo Animal in the stage of development starting after the first division of fertilized oocyte; the embryo stage in humans lasts 8 weeks.

Endometrium Inner lining of mammalian uterus composed of two compartments: epithelial and stromal cells.

Human chorionic gonadotropin (hCG) A gonadotropin produced during pregnancy by the placenta; prolongs ovarian corpus luteum life span and stimulates progesterone production.

Introduction

Implantation is a process that occurs in the mammalian uterus in which the conceptus apposes, attaches itself to the maternal endometrium, and finally invades it (Fig. 1).

Apposition and Attachment

The entire implantation process can be divided into three stages—apposition, attachment, and invasion— and ends when the placenta starts to form.

Embryo during Apposition and Attachment

The fertilized oocyte, during its way through the oviduct and after several cell divisions, reaches the stage of blastocyst. On the fifth day after fertilization, the human blastocyst possesses two clearly defined groups of cells: an external single-layer group of epithelial cells attached called trophoectoderm and internally located cells gathering on one side of the blastocoelic cavity. At this stage of conceptus development, the embryo is surrounded by the zona pellucida. This envelope is degraded by lytic factors present in the uterine cavity before starting the process of implantation. Plasmin produced from plasminogen present *in utero* is probably responsible for degradation of the zona pellucida. However, the blastocyst itself seems to be also involved in the process of zona lysis. Approximately 7 days after fertilization, the human blastocyst hatches and the embryo is prepared for the next stage of implantation, that is, attaching to the endometrial epithelial cells. Cellular adhesion molecules expressed on the endometrial epithelium are responsible for contact of the hatched blastocysts. At this point, the human trophoblast starts to differentiate into two layers: inner cytotrophoblast and outer syncytiotrophoblast. The syncytiotrophoblast is responsible for human chorionic gonadotropin (hCG) production that stimulates progesterone production by the ovarian corpus luteum. In clinical practice, hCG

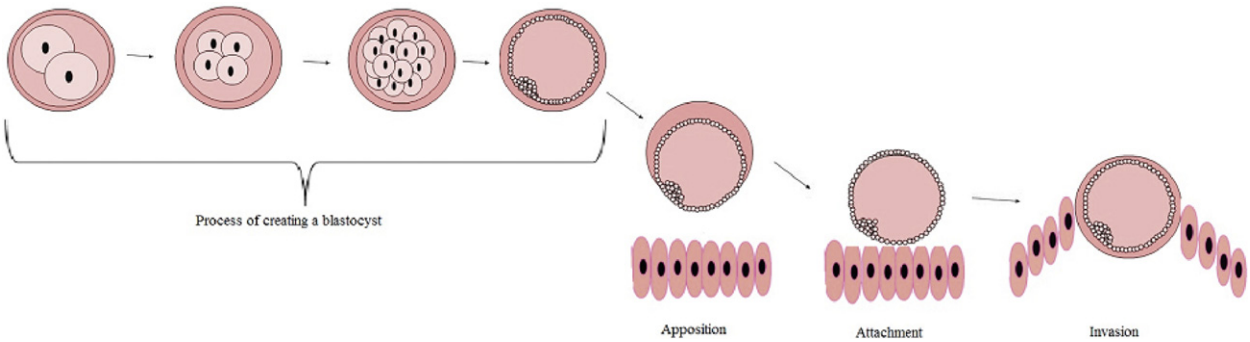

Fig. 1 Stages of blastocyst implantation.

☆*Change History:* July 2014. LT Putowski and M Nowak introduced small edits in the text of the article and Figure 1 was slightly changed.

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is used as a pregnancy marker. The cross-talk between blastocyst and endometrium results in precisely tuned regulation of cytokines production and other regulatory molecular factors expression.

Endometrium During Apposition and Attachment

Blastocysts interact with the uterine epithelium that has been properly sensitized with ovarian hormones. Cyclic histological changes in the endometrium were initially described in 1950 by Noyes and colleagues. The endometrium starts to prepare for the implantation during the follicular phase of the menstrual cycle under the influence of estradiol (E_2) secreted by the ovaries. When the ovulation occurs, rising progesterone transform the endometrium into the secretory histological type. Progesterone, in cooperation with E_2, initiates decidual transformation of the endometrium, induces prostaglandin production by endometrial macrophages, and causes stromal edema and angiogenesis in the endometrium. In addition, progesterone decreases uterine irritability and contractivity and, together with hCG, suppresses immunological response that prevents embryo rejection by the maternal immunological system.

During embryo implantation, the uterine endometrium undergoes morphological and physiological changes to host conceptus. The restricted period of time when the endometrium is receptive for the conceptus is called the 'implantation window.' The embryo being transferred to the uterus at an inappropriate time is not able to implant. In the normal human menstrual cycle lasting 28 days, the implantation window lasts between 19 and 24 days of the cycle. One of the earliest indexes for transmission of embryonic signals to the endometrium that surrounds the blastocysts is capillary permeability and local stromal edema. This leads to the changes in the endometrium termed 'decidualization.' During this process, endometrial stromal cells transform into large decidual cells that are rich in glycogen.

Attachment of the embryo to the endometrium is facilitated by the specific cell adhesion structures and molecules located on the apical epithelial plasma membrane.

Pinopodes

A characteristic feature for the status of endometrial receptivity is formation of the pinopodes. Pinopodes, a large cytoplasmatic projection located on the apical surface of endometrial epithelium, are associated with changes in cell membrane morphology during the implantation window opening. Progesterone is responsible for the formation of these structures. Pinopodes are involved in facilitating adhesion of the blastocyst to the endometrial epithelium.

Trophinin/Tastin

Trophinin, together with its adhesion molecule tastin (trophinin-assisting protein), is additional protein responsible for trophoblast cell adhesion to the endometrial epithelium. Tastin is located in the endometrial cell membrane. The cytoplasmatic domain of trophinin is bound to the cytoskeleton. The presence of highly concentrated areas of trophinin 'patches' is believed to be adhesion sites for the embryo. The trophinin is expressed exclusively when the implantation window is opened.

Mucin: MUC1

MUC1 is an integral membrane glycoprotein. MUC1 has high molecular weight, a highly glycosylated extracellular domain, and a relatively short domain associated with the cytoskeleton. The endometrial expression of MUC1 is menstrual cycle dependent. It is believed that MUC1 prevents adhesion of the embryo to endometrial epithelium adhesion and that only healthy blastocysts are able to decrease MUC1 expression leading to the contact of the cells. In addition to the endometrium, MUC1 is expressed in blastocyst during the preimplantation period.

Integrins

Cell-to-cell adhesion seems to be a crucial process in the course of implantation. Integrins are transmembrane glycoproteins responsible for this process. They are members of an immunoglobulin superfamily. Integrins are composed of two units: α and β. Between several types of integrins, heterodimers $\alpha_4\beta_3$ and $\alpha_v\beta_1$ seem to play an important role in the implantation. Integrin α_4 and integrin β_3 coexpress in the apical secretive endometrium when the implantation window is opened. For this reason, integrin β_3 is proposed to be a marker of uterine receptivity. Integrins are believed to be receptors for extracellular matrix components: fibronectin, collagen, and laminin. A sequence most often recognized by integrins is the peptide sequence arginine-glycine-aspartic acid (RGD) and this is also the sequence that fibronectin, gluten and laminin most often have. Because trophoblasts express oncofetal fibronectin, the presence of integrins on apical endometrial epithelial cells may have a major role in the initial process of embryo adhesion to decidual cells. Contact of the embryo with endometrium trigger the production of proteases by trophoblasts, and this is crucial for the next step of implantation.

Cadherins

They belong to the superfamily of adhesive glycoproteins, which are involved in inter-cell interactions. Their correct process is dependent on the presence of calcium ions. E-cadherin is the most extensively studied cadherin with the function of cell to cell adhesion. It is present on the endometrial cells throughout the entire menstrual cycle, whereas during the implantation window expression significantly increase and it participates in the adhesion of the trophoblast to the maternal cells.

Invasion

Proteases degrade the endometrial extracellular matrix, helping the embryo to invade the uterine wall. Matrix metalloproteinases and urokinase-type plasminogen activator are involved in extracellular matrix proteolysis. In addition, first-trimester cytotrophoblasts express specific enzymes called gelatinases, which are responsible for matrix degradation. Enzymes are controlled indirectly by cytokines and by interaction with the extracellular matrix. Proteolytic activity of invading trophoblasts is a result of balance between matrix metalloproteinases secreted by the embryo and specific inhibitors, including tissue inhibitors of metalloproteinases originating from decidua.

Markers of Implantation

Trophoblasts and decidua produce lots of factors and their receptors affecting events associated with the process of implantation. Changes in the expression of platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor (TGF), IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, colony-stimulating factor (CSF), leukemia inhibitory factor (LIF), and hemoglobin-binding epidermal growth factor (HB-EGF) were observed at the time of implantation.

However, in a successful implantation the most important role is played by the cells from the mother's immune system. The local (in endometrium) immune system of the mother must be reordered so as to protect the semi-allogeneic embryo from destruction by the maternal immune response. It is necessary to mute the inflammatory (effector) cells of the Th2 type. The most important role in this mechanism is played by the following factors:

Regulatory T-Cell (Treg)

Tregs are a subpopulation of lymphocytes having immunosuppressive properties with respect to effector cells. After fertilization, their amount in the decidual mucosa and the peripheral blood doubles. Tregs are involved in creating an immunologically privileged environment in the decidual mucosa which is the point of contact with the embryo, and they maintain the immune tolerance of the mother in relation to alloantigens derived from the father. Their presence is essential in a successful implantation process. It is believed that there are two possible mechanisms by which Tregs activate suppression processes in relation to the trophoblast. One of them is the recognition of maternal antigens on the surface of the semi-allogeneic trophoblast. The second one – the pre-exposure to paternal alloantigens (the so-called sensitization) in the seminal fluid. To maintain the immunosuppressive action, Tregs increase the secretion of several factors, the most important of these being IL-10 and TGF-b.

Helper Cells (Th1/Th2)

Th1 and Th2 are subpopulations of lymphocytes that secrete cytokine groups with different behaviours. They correspond to two types of immune response, cellular response (Th1) and humoral response (Th2). Both the implantation process and the entire pregnancy favor the Th2-type immune response. As a result, in the peri-implantation period during the luteal phase the secretion of Th-2 cytokines (IL-3, IL-4, IL-6, IL-10, and TGF-beta) is increased and the secretion of Th-1 cytokines (IL-2, IL-12, IFN-gamma, TNF-alpha) is reduced.

Uterine NK Cells (uNK)

The uNK cells occur in the endometrium, and in the luteal phase of the menstrual cycle, their amount in decidua increases significantly. The uNK in the decidual secrete cytokines and other factors (vascular endothelial growth factor-VEGF) that are responsible for the normal growth of the trophoblast and the successful pregnancy while playing a minor role in the implantation compared to other immune system cells.

Leukemia Inhibitory Factor

LIF is a cytokine belonging to the IL-6 family which is considered crucial for the correct implantation process. The role of this cytokine in the process of implantation is confirmed by results showing that mouse lacking LIF become infertile. An increased expression of LIF and its receptor in the uterine epithelium occurs at exactly the same time when pinopodes appear on the surface of the endometrium, which confirms the necessity of the presence of LIF for the proper implantation of the blastocyst, and points to the particular role of LIF in blastocyst apposition and attachment. LIF enhances STAT3 activation (phosphorylation pathway). Studies have confirmed the importance of the proper functioning of the STAT3 pathway for blastocyst attachment and successful implantation.

Transforming Growth Factor(TGF-Beta)

In the preimplantation period, increased expression of TGF-beta in the glandular epithelium of the uterus can be observed. At the same time, TGF-beta promotes the secretion of LIF and directly takes part in the nesting of the blastocyst.

IL-1

This cytokine stimulates the production of metalloproteinases (MMP-9) by the conceptus in the preimplantation period, which increases its invasive ability. IL-1 also stimulates the production of LIF, indirectly affecting the implantation.

The process of implantation lead to the formation of placenta and is crucial for the reproduction of mammals including humans.

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Pregnancy Endocrinology☆

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Steroid Hormones in Pregnancy

Total steroidogenic output during pregnancy results from interactions of the fetal, placental, and maternal compartments. The fetus and placenta lack certain steroidogenic enzymes and thus utilize maternal precursors and metabolic clearance of steroids.

Progesterone

The placenta, located between the mother and fetus, utilizes precursors from either of them to circumvent its own deficiencies in enzyme activities. Cholesterol and pregnenolone are obtained from the maternal bloodstream for progesterone (P) synthesis. P acting through a cytosolic receptor that belongs at steroid and thyroid superfamily (Lee *et al.*, 2016). There are two isoforms of P receptor (PR) A and B, both transcribed from the same gene located on chromosome 11q22-23. The two isoforms differ structurally in N-terminal domain. PR-B expression is more relevant than PR-A expression. Functionally, PR-A is necessary for optimal uterine physiology and reproduction, PR-B is important during gestation to maintain the quiescence of uterus (Mesiano *et al.*, 2011; Bahia *et al.*, 2017).

Until week 10 of gestation, maternal P largely originates from the corpus luteum. After a transition period of shared function between the 7th and 10th weeks, the placenta becomes the major source of P and the maternal serum levels increase progressively up to 100–200 ng mL^{-1} at term (Chasen, 2014). Most of the P produced enters the maternal circulation. Estrogen (E) stimulates placental low-density lipoprotein (LDL) uptake and cholesterol production in the fetal liver and increases its placental conversion to pregnenolone. A small amount of human chorionic gonadotropin (hCG) must be present for P production. The decidua and fetal membranes also synthesize and metabolize P, utilizing pregnenolone sulfate as the precursor.

Amniotic fluid P is maximal between weeks 10 and 20 and then decreases gradually. Myometrial levels are three times higher than maternal plasma levels in early pregnancy and gradually equalize with serum levels toward term. Two active metabolites of P, 5α-reduced P and 5α-pregnane-3,20-dione, increase significantly during pregnancy and contribute to maternal resistance to angiotensin II.

P, acting through PR-A and PR-B, regulates the development and function of the endometrium and induces changes in cells (decidualization) essential for implantation and the establishment and maintenance of pregnancy (Gellersen and Brosens, 2014). Because implantation occurs 5–6 days after ovulation and hCG must appear by the 10th day to rescue the corpus luteum, the blastocyst must successfully implant and secrete hCG within a narrow window of time. Although E levels begin to increase at 4–5 weeks due to placental secretion, P production by the placenta does not significantly increase until approximately 10–11 weeks after ovulation. During pregnancy, progesterone via the PRs promotes myometrial relaxation and cervical closure (Pabona *et al.*, 2015). Withdrawal of PR-mediated progesterone signaling triggers menstruation and parturition. At term of pregnancy, the ratio of PR-A to PR-B in myometrium increases. Specifically, the withdrawal of PR-B follows an activation of E receptor (ER) leading to an inflammatory response by expression of cytokine/chemokine, whereas the mechanism that leads to increase of PR-A is totally unclear (Bahia *et al.*, 2017).

In the fetus, P serves as the substrate for adrenal production of glucocorticoids and mineralocorticoids. Cortisol synthesis also relies in part on LDL-cholesterol obtained in the fetal circulation or synthesized in the fetal liver. The fetus lacks significant activity of the 3β-hydroxysteroid dehydrogenase, Δ4–5 isomerase system. Thus, the fetus takes up P from the placenta to synthesize corticosteroids. At the same time, the fetus supplies what the placenta lacks, that is, the 19'carbon compounds that serve as precursors for E.

There is an emerging interest in the P metabolite and neuro-steroid allopregnanolone (Brunton *et al.*, 2014). Allopregnanolone is produced in increasing amounts during pregnancy both in the periphery and in the maternal and fetal brain. Allopregnanolone protects the fetus from exposure to harmful levels of maternal glucocorticoids, released during pregnancy as a result of stress events. Furthermore, allopregnanolone prevents premature secretion of oxytocin and minimize the risk of premature delivery. Allopregnanolone also plays a key role in the fetal brain, where it promotes development and is neuroprotective.

Finally, even if still matter of research, another point of interest is the role of P in the regulation of immune response of the mother. In fact, the P plays a role in induction and maintenance of tolerance in T-cells toward the semi-allogenic fetal antigens (Areia *et al.*, 2015; Li *et al.*, 2016).

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Estrogens

E production in pregnancy is under the control of the fetus and is a fundamental signaling tool. E influences P production, uteroplacental blood flow, mammary gland development, fetal adrenal gland function, and has antiinflammatory effects. The precursors of E are 19-carbon androgens. Due to the virtual absence of 17α-hydroxylation and 17,20-desmolase activity in the placenta, 21-carbon steroids (P and pregnenolone) cannot be converted to 19-carbon steroids [androstenedione and dehydroepiandrosterone (DHEA)]. Thus, in early pregnancy, androgens are derived from the mother and later the fetus becomes the major source.

The fetus rapidly conjugates steroids with sulfate, protecting itself from the effects of high steroid levels. The placenta is extremely efficient in cleaving these sulfate conjugates. The fetal adrenal provides dehydroepiandrosterone sulfate (DHEAS) as precursor for placental production of estrone (E1) and estradiol (E2). The placenta, which lacks 16α-hydroxylation ability, also uses 16α-DHEAS produced in the fetal liver as precursor for estriol (E3), but 90% of E3 production results from fetal adrenal DHEAS production. E3 is the E produced in greatest quantity during pregnancy, whereas E1 and E2 are derived equally from fetal and maternal precursors.

E1 in the maternal plasma rises from 6 to 10 weeks and reaches 2–30 ng mL^{-1} at term. E2 rises from 6 to 8 weeks and reaches 6–40 ng mL^{-1} at term. During pregnancy, E1 and E2 excretion is increased approximately 100-fold compared to nonpregnant levels, whereas E3 excretion increases 1000-fold. The maternal level of E2 is higher than in the fetus, whereas the level of E3 is lower.

The maternal cardiovascular adaptations to pregnancy are also regulated by Es. Blood volume is increased by E stimulation of the maternal and trophoblastic renin–angiotensin systems and uteroplacental blood flow is influenced by the vasodilator effects of E (Heidari *et al.*, 2017). Placental aromatization is so efficient that no androgens from the maternal side reach the fetus, protecting it from masculinization. The Es presented to the maternal bloodstream are rapidly metabolized by the maternal liver prior to excretion into the maternal urine. Only approximately 8%–10% of the maternal blood E3 is unconjugated.

E, as well as P, are essential for the implantation process and for the regulation of immune system of the mother. In murine models the ablation of corpus luteum, such as the administration of only P, does not permit the implantation of blastocyst, whereas the implantation process is possible only with the synchronous administration of P and E in the same animal models. In addition, E induce and maintain the immune-tolerance toward semi-allogenic fetal antigens both in the innate immune response and in the adaptative immune response playing a role in the balance of Th2/Th1 response in favor of Th2 response and a prevalent Th2 immune response is useful in the maintenance of physiological pregnancy (Muzzio *et al.*, 2014; Areia *et al.*, 2015).

Vitamin D

Vitamin D are a group of steroid hormones, which play a pivotal role in enhancing intestinal absorption of calcium, phosphate, iron, magnesium, and zinc. In humans, vitamin D is mainly synthesized in the skin from 7-dehydrocholesterol (provitamin D3), which on exposure to sunlight [ultraviolet B (290–315 nm)], is isomerized into cholecalciferol (vitamin D3) (Zhou *et al.*, 2017). A small proportion of vitamin D in humans originates from dietary sources that is, fish, meat, milk, eggs, and from vegetables [ergocalciferol (vitamin D2)]. The liver enzyme, 25-hydroxylase, converts both vitamin D3 and D2 into 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2, respectively. These then enter the circulation, and almost all binds to vitamin D binding protein (VDBP), which in turn is taken up by the epithelial cells of the proximal tubules of the kidney and converted to the active hormonal form of vitamin D—di-hydroxy-vitamin D [1,25(OH)2D or calcitriol]—by the action of the mitochondrial enzyme 1-α-hydroxylase. Interestingly, by the end of the first trimester of pregnancy, 1,25(OH)2D levels are more than double what they are during the nonpregnant state without parallel alterations in circulating calcium concentrations (Aghajafari *et al.*, 2013). Furthermore, during pregnancy, the placenta is the most important site for extra-renal activation of vitamin D. However, the clinical role played by placenta as site of activation of vitamin D is little. In fact, the changes in calcitriol concentration are not significant in anephric women during pregnancy (De-Regil *et al.*, 2016).

Vitamin D acts through the interaction with a nuclear receptor called vitamin D receptor (VDR). VDR is presents in many tissues and belongs to the super-family of steroid receptors. It is transcribed from a gene located on chromosome 12q12-14 and has a large promoter region that can generate several tissue-specific isoforms of VDR.

Vitamin D is a crucial hormone in the maintenance of metabolic, cardiovascular, musculoskeletal, nervous, and immune health. In pregnancy, vitamin D impacts upon both maternal and fetal health, including fetal skeletal development, tooth enamel formation, and general fetal growth and development (Wagner *et al.*, 2012). Deficiency in vitamin D is associated with an increased risk of fetal miscarriage, preeclampsia, gestational diabetes and, preterm birth (Zhang *et al.*, 2018; von Websky *et al.*, 2018). In fact, vitamin D improves the trophoblast invasion enhancing the epithelial mesenchymal transition of extravillous trophoblast via specific signaling pathways (such as ERK pathways) (Kim *et al.*, 2017; Ganguly *et al.*, 2018). Vitamin D seems also modulates the proliferation and differentiation of preadipocytes during pregnancy influencing the methylation of several genes, and its deficiency could influence not only the birth weight, and the risk of intrauterine growth restriction (IUGR) fetus and small for gestational age (SGA) babies, but also the offspring obesity (Nguyen *et al.*, 2018; Santamaria *et al.*, 2018). Finally, recent experimental and clinical data seem to demonstrate that vitamin D deficiency can be the link between complicated pregnancy and

infertility and/or polycystic ovary syndrome (PCOS) (Wen *et al.*, 2018). However, the effects of vitamin D during pregnancy are extremely variable due to the influence of polymorphisms with particular regard for placental VDR (Colonese *et al.*, 2015).

The Fetal Adrenal Cortex

By gestational weeks 8–9, the fetal adrenal cortex is differentiated into a thick inner fetal zone and a thin definitive outer zone, the forerunner of the adult cortex (Mesiano and Jaffe, 1997). By the end of the first trimester, the gland reaches a size equal to or larger than that of the adjacent kidney. After the 20th to the 24th week, the adrenal glands slowly decrease in size until a second growth spurt at approximately 34–35 weeks. After delivery, the fetal zone (~80% of the bulk of the gland) rapidly involutes to be replaced by simultaneous expansion of the adult adrenal cortex. Experimental data suggest that the decline in fetal reticular zone DHEAS production and cortisol to DHEAS ratio is modulated by E2 (Pepe *et al.*, 2016).

The expression and the isomerase activity of 3β-hydroxysteroid dehydrogenase are low in the fetal adrenal and therefore DHEA and DHEAS are its major products. Fetal DHEA and DHEAS production rises steadily, concomitant with the increase in the size of the fetal zone and increase in adrenal weight. Maternal E levels follow the increased availability of the fetal DHEAS as a precursor. Early in pregnancy, the gland grows and functions without adrenocorticotropic hormone (ACTH), perhaps in response to hCG. After 15–20 weeks, fetal ACTH is required (Pepe and Albrecht, 1995). Paradoxically, during the last 12–14 weeks of pregnancy when fetal ACTH levels are declining, the adrenal gland quadruples in size, via processes including cell proliferation and angiogenesis at the gland periphery, cellular migration, hypertrophy, and apoptosis.

Because prolactin (PRL) is the only fetal pituitary hormone to increase throughout pregnancy, paralleling changes in the size of the fetal adrenal, it was proposed as the critical tropic substance. The development and/or function of the fetal adrenal cortex may be regulated by a repertoire of molecules, including transcription factors, extracellular matrix components, locally produced growth factors (GFs), and placenta-derived corticotropin-releasing hormone (CRH), in addition to the primary regulator, fetal pituitary ACTH. However, only ACTH exerts adrenal steroidogenesis. It activates adenylate cyclase, increases LDL receptors, and increases the expression of its own receptors. These cause increased uptake of circulating LDL-cholesterol, largely derived from the fetal liver, and de novo synthesis of cholesterol in the adrenals to sustain the high rates of DHEAS and E formation. The tropic support of the fetal adrenal gland by ACTH from the fetal pituitary is protected by the placental conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase. In late gestation, when E levels are high, less cortisol is transferred to the fetus, fetal ACTH secretion increases, the fetal adrenal gland undergoes greater maturation, and fetal cortisol synthesis from endogenous cholesterol increases. Recent data demonstrated that IUGR/SGA fetus have a smaller adrenal gland at ultrasound suggesting a relationship between adrenal steroidogenesis and fetal growth and vice versa (Ishimoto and Jaffe, 2011).

CRH production and the size of the fetal adrenal gland are closely correlated. CRH augments fetal ACTH secretion in a positive feedback mechanism, producing adrenal growth and cortisol and DHEAS secretion. CRH may also directly stimulate DHEAS production.

Adrenal steroidogenesis is subject to autocrine and paracrine regulation by various GFss, such as inhibin A, activin A, transforming GF-α, basic fibroblast GF, insulin-like GF-I (IGF-I) and IGF-II, and kisspeptin 1. As below detailed, kisspeptin1 and its receptor are expressed in the fetal adrenal cortex from 8 week of gestation and play a role in the regulation of the fetal adrenocortical development (Mesiano *et al.*, 1997; Katugampola *et al.*, 2017). In fact, kisspeptin 1 increases DHEAS production and fetal adrenal volumes in the second trimester of pregnancy.

Polypeptide Hormones

The placental villus exterior has cytotrophoblasts (CT), separate mononuclear cells that are prominent early in pregnancy and sparse late in pregnancy, and syncytiotrophoblasts (ST), cells that form a continuous multinuclear layer on the surface. CT is the basic placental stem cell from which the ST arises by differentiation. ST is the functional cell type of the placenta. Trophoblast differentiation is influenced by hCG and several other GFs. Since the surface of the ST is in direct contact with the maternal blood, placental proteins are secreted directly and preferentially to the mother. Several hypothalamic-like peptides originate in the CT and influence the ST to secrete pituitary-like hormones. In addition, locally produced hormones, GFss, and peptides work together to regulate placental function (Petraglia *et al.*, 1996).

Hypothalamic-Like Releasing Hormones

Several isoforms of GnRH and GnRH receptor (GnRHR) are described. The hypothalamic decapeptide, GnRH-I, binds to the anterior pituitary and induces the synthesis and secretion of luteinizing hormone and follicle-stimulating hormone. It is also found in extra-hypothalamic sites. A second isoform, GnRH-II, acts both in the hypothalamus and other organ systems, including placenta, breast, endometrium, and ovary. Both GnRH-I and GnRH-II signal through a single receptor, GnRHR-I, in humans. In the early pregnancy, GnRH regulates trophoblast invasion and placentation increasing the expression of matrix metalloproteinase (Peng *et al.*, 2016). Placental gonadotropin-releasing hormone (GnRH-II) regulates placental steroidogenesis and release of

prostaglandins (PGs) as well as hCG (Sasaki and Norwitz, 2011). The placental receptors for GnRH have lower affinity than that of GnRH receptors in the pituitary, ovary, and testis. GnRH receptors are present in both CT and ST in a pattern that parallels hCG secretion, suggesting that GnRH regulates hCG secretion. GnRH release is increased by E, activin A, insulin, and PGs and inhibited by P, endogenous opiates, inhibin, and follistatin.

Recently, kisspeptin and its receptor (KISS1R, also called GPR54), have been reported to be strong stimulators of GnRH neurons and are involved in various mechanisms regulating the gonadotrope axis such as puberty induction or positive and negative feedback regulation on the gonadotrope axis by gonadal steroids. They also mediate metabolic or environmental cues on the reproductive axis. Kisspeptins are secreted by hypothalamic nuclei located in the arcuate nucleus and anteroventral periventricular nucleus. This system is complex because neurons located in the arcuate nucleus coexpress many neuromediators such as neurokinin B (NKB) and dynorphin, involved in the control of the gonadotrope axis. During pregnancy, kisspeptins seems implicated in trophoblast invasion. Recent data have demonstrated an elevation of kisspeptin 1 level, as well as an enhancement of the kisspeptin 1/KISS1R signaling, in the endometrium the day of the implantation and in the decidual tissue (Fayazi *et al.*, 2015). The decidual increase of the kisspeptin 1/KISS1R signaling is related to a reduced extravillous trophoblast migration and invasiveness, and to an increased adhesion of trophoblast cell to type I collagen (Taylor *et al.*, 2014). High placental kisspeptin concentrations are related with higher risk of preeclampsia (Matjila *et al.*, 2016). Kisspeptin 1 administration in cell model reduces matrix metalloproteinases (MMP) and vascular endothelial GF (VEGF), and increases the specific tissue inhibitors. Kisspeptins are significantly secreted by the placenta (Babwah, 2015). The stimulatory effect of E on GnRH and kisspeptin expression in the placenta and the uterus is of similar magnitude. In placenta cells, GnRH is able to stimulates GnRH itself, whereas kisspeptin did not stimulate/control GnRH neither kisspeptin itself. NKB is also directly stimulated by E2, and it stimulates the GnRH and kisspeptin expression (Oride *et al.*, 2015). Both GnRH and kisspeptin seem the effectors of E/NKB system to increase hCG mRNA expression in placenta cells. Of interest, kisspeptin expression seems to have a circadian rhythm in the placenta (de Pedro *et al.*, 2015).

CRH is produced in the trophoblast, fetal membranes, and decidua. Its production is decreased by P and increased by glucocorticoids, which are responsible for the rise in ACTH and cortisol during the last weeks of pregnancy and during labor. The progressive increase in maternal CRH levels during pregnancy is due to the secretion of intrauterine CRH into the maternal circulation. Furthermore, CRH is involved in the timing of birth by modulating signaling systems that control the contractile properties of the myometrium via prostaglandins expression and release. This effect seems to be enhanced by urocortin decrease. To this regard, it seems that CRH is one of the crucial factors determining the duration of the gestation, and the onset of labor (Thomson, 2013). CRH (and hCG, see below) are controlled by cortisol, since cortisol increases aromatase expression, and E production, in the placenta tissue via their stimulatory effect. This mechanism is particularly active toward the end of gestation. In the placenta, cortisol stimulates CRH synthesis via activation of nuclear factor kappa B (NF-κB), a component in a cellular messenger system that may also be triggered by stressors such as hypoxia and infection, indicating that intrauterine stress could bring forward childbirth and cause low birth weight infants. Recently, CRH has been found to modulate glucose transporter (GLUT) proteins in placental tissue, and therefore there may be a link between CRH levels and fetal growth.

Human Chorionic Gonadotropin

hCG is a glycoprotein $\alpha\beta$-dimer. A high content of sialic acid prolongs its half-life compared to its analogue, luteinizing hormone (LH). The α-subunit is identical in hCG, LH, follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). The biological activity is specific to the β-subunit. hCG production and secretion are stimulated by GnRH, interleukin-1β, activin, and E and inhibited by endorphins, inhibin, P, and follistatin.

Blastocyst secretion of hCG has been reported to contribute to endometrial cross-talk to enable implantation (Choi and Smitz, 2014). Endometrial expression of LH/choriogonadotropin receptor (LHCGR) increases mid-luteal phase, during a short period in which the endometrium is receptive to implantation (i.e., the implantation window); it is plausible that a blastocyst producing locally high concentrations of hCG could extend the implantation window, thereby increasing the chances for a successful pregnancy.

During early pregnancy, hCG is also believed to support implantation and placentation by modulating endometrial tissue remodeling, fostering maternal immunotolerance of fetal tissue, promoting neoangiogenesis and increasing the uterine natural killer cell population (Nwabuobi *et al.*, 2017). These functions all contribute to endometrial receptivity. hCG expressed by the blastocyst stimulates receptors on the endometrium, eliciting a variety of effects on downstream molecules, including VEGF, transforming GF-β, cytokines (i.e., leukemia inhibitory factor) and proteinases (i.e., MMP), which contribute to different aspects of endometrial receptivity. For example, hCG-mediated upregulation of VEGF is believed to promote angiogenesis, whereas stimulation of MMP-9 expression by hCG is involved in regulation of tissue remodeling.

Expression of hCG continues throughout pregnancy, with peak levels occurring at 10 weeks of gestation. hCG concentrations decrease to a nadir at 18–20 weeks and remain at that level until term. Clearance of hCG is carried out mainly by renal metabolism. As many as 20–30 isoforms of hCG can be detected during pregnancy. Hyperglycosylated hCG (hCG-H) secreted by root or extravillous CT is the predominant species early in gestation, but it is gradually surpassed in proportion by regular hCG, which is produced by fused and differentiated STs. hCG is crucial to the maintenance of pregnancy by supporting progesterone production by the corpus luteum (Braunstein, 1996). Placental production of P takes over in the later part of the first trimester, at a

time when hCG levels are in decline. Beyond its role in luteal support, hCG is believed to promote angiogenesis, thereby supporting blood flow to the placenta and nutrition for the fetus. Emerging evidence also suggests that hCG is involved in umbilical cord growth and development, synchronizing uterine growth with that of the developing fetus and relaxation of myometrial contractions. In addition, the presence of LHCGR in the brain has led to speculation that gonadotropin signaling is involved in the nausea and vomiting experienced during pregnancy. hCG stimulates steroidogenesis in the early fetal testes, so that androgen production will ensue and masculine differentiation can be accomplished. It is also possible that the function of the inner fetal zone of the adrenal cortex depends on hCG in early pregnancy. The β-hCG gene is expressed in fetal kidney and adrenal gland, suggesting that hCG may affect the development and function of these organs. Detection of LHCGR in fetal organs, including the kidney, liver, lung, pancreas and gastrointestinal tract, has led to speculation that hCG may have a further role in fetal organ growth and differentiation.

Human Placental Lactogen

Human placental lactogen (hPL) is a single-chain polypeptide of 191 amino acids. hPL is very similar to human growth hormone (GH) but has only 3% of its activity. Its lactogenic contribution in human pregnancy is uncertain. Its half-life is approximately 15 min. The hPL level in the maternal circulation is correlated with fetal and placental weight, steadily increasing until it plateaus in the last month of pregnancy (5–7 mg mL^{-1}). There is no circadian variation and only minute amounts enter the fetal circulation.

Its metabolic role is to mobilize free fatty acids from lipids (Handwerger and Freemark, 2000). Relative hypoglycemia in fasting pregnant women ("accelerated starvation") is due to the transfer of glucose to the fetus by facilitated diffusion and due to the diabetogenic effect of placental hormones (E, P, and especially hPL) causing peripheral insulin resistance and hyperinsulinism. hPL and PRL increase maternal food intake by induction of central leptin resistance and promote maternal beta-cell expansion and insulin production to defend against the development of gestational diabetes mellitus. As the fasting glucose level decreases, hPL levels rise to stimulate lipolysis and increase free fatty acids, which are an alternative fuel for the mother, sparing glucose and amino acids for the fetus. With sustained fasting, maternal serum ketone levels rise. Due to limited transport of free fatty acids across the placenta, fetal tissues then utilize these ketones, which do cross the placenta. hPL also enhances the fetal uptake of ketones and amino acids. Insulin antagonism by hPL is mediated by the increase in free fatty acid levels, which, in turn, directly interfere with insulin-directed entry of glucose into cells. With a sustained state of inadequate glucose intake, maternal ketosis may impair fetal brain development and function (Wadhwani *et al.*, 2017).

hPL, despite its lower levels in the fetus, directly affects fetal tissue metabolism, including synergistic actions with insulin, especially actions on glycogen synthesis in the liver (Newbern and Freemark, 2011). The failure of fetal growth hormone to affect fetal growth suggests that hPL may be the fetal growth hormone.

Human Chorionic Adrenocorticotropic Hormone

The rise in maternal free cortisol, cholesterol, and pregnenolone is due to secretion of placental ACTH and CRH, which are not suppressible by glucocorticoids. ACTH production in the ST is stimulated by CRH from the CT. CRH levels in maternal plasma rise in the second trimester, increasing to peak values at term. Oxytocin is a potent stimulator of CRH and ACTH placental production. A decrease in CRH-binding protein near term further increases the cortisol availability during labor and delivery.

Growth Hormone

Growth hormone (GH), growth hormone-releasing hormone (GHRH), and somatostatin GHRH and somatostatin itself are found in the placenta and somatostatin alone is present in the decidua too. Somatostatin decreases with advancing gestation. Placental GHRH and somatostatin do not contribute to maternal circulating levels. During the second half of gestation, placental GH gradually replaces pituitary GH in the maternal circulation. Placental GH is not present in fetal blood. Maternal IGF-I levels increase during pregnancy in parallel with GH. Placental GH is not regulated by placental GHRH, but responds inversely to maternal glucose levels, securing glucose availability for the fetus. It also stimulates gluconeogenesis and lipolysis in the mother.

α-Fetoprotein

α-Fetoprotein (AFP) is a glycoprotein derived mainly from fetal liver and partially from the yolk sac. It is comparable in size to albumin and may serve as a protein carrier of steroid hormones in fetal blood. AFP may also be a modulator of cell proliferation, synergizing with various GFs, and play a role in fetal organogenesis.

Peak values of AFP in the fetal blood are reached at the end of the first trimester. Thereafter, levels decrease gradually until a rapid decrease begins at 32 weeks. Maternal blood levels are much lower than fetal levels, rising until week 32 and then declining

(Mizejewski, 2004). Elevated maternal levels of AFP are associated with a variety of fetal anomalies, multiple pregnancies, and increased risk of spontaneous miscarriage, stillbirth, preterm birth, preeclampsia, neonatal death, and low birth weight. Low maternal levels of AFP are associated with trisomies 21 and 18.

Relaxin

Relaxin is a peptide hormone produced by the corpus luteum of pregnancy, and by the placenta, decidua, and chorion. It rises during the first trimester and declines in the second. It may have a role in implantation, placentation and vascularization, ripening the cervix, inhibiting uterine contractions, and relaxing the pubic symphysis (Anand-Ivell and Ivell, 2014). More recently, there is emerging evidence suggesting that the vasodilator effects of relaxin is in part responsible for the increase in glomerular filtration and renal blood flow in normal pregnancy.

Prolactin

Following ovulation, the endometrium becomes a secretory organ and remains so throughout pregnancy. It secretes renin, relaxin, and PRL. PRL production requires the combined effect of P and E plus the presence of other placental and decidual factors, including relaxin, IGF-I, and specific stimulatory and inhibitory proteins (Marano and Ben-Jonathan, 2014). PRL derived from the decidua is the source of that found in the amniotic fluid. The PRL in the fetal circulation is derived from the fetal pituitary. Beginning at 8 weeks, PRL levels rise from the normal level of 10–25 ng mL^{-1} to a peak of 200–400 ng mL^{-1} at term. There is marked maternal variability and a diurnal variation as in the nonpregnant state.

Amniotic fluid concentrations parallel maternal serum levels until week 10, rise markedly until week 20, and then decrease until delivery. PRL reduces the amnion permeability from fetus to mother and contributes to the regulation of fetal water and electrolyte balance by acting as an antidiuretic hormone (Tenorio *et al.*, 1992). The increase in maternal levels represents pituitary secretion in response to E.

PRL plays a critical role in mammary gland development during pregnancy, and milk production postdelivery (Macias and Hinck, 2012). PRL also promotes neurogenesis in both maternal and fetal brains, and more recently has been implicated in pulmonary surfactant synthesis in fetal lungs (Mendelson, 2000; Pathipati *et al.*, 2011; Yarlagadda *et al.*, 2015).

Cytokines and Growth Factors

Local placental cytokine production is believed to be important for embryonic growth and in the maternal immune response essential for the survival of the pregnancy (Rutanen, 1993). A system of communication is present between maternal decidual and fetal tissue to provide GF support for the placenta, which would include fetal hematopoiesis, a known response to colony-stimulating factor-1. Decidual interleukin (IL)-1β and placental tumor necrosis factor-α synergistically release placental interleukin-6 and secrete hCG.

Cytokines are also considered as pivotal modulators of immune tolerance at the maternal-fetal interface (Robinson and Klein, 2012). Specifically, IL-10 acts in either autocrine or paracrine manner inhibiting inflammatory signals and blocking major histocompatibility complex class II expression and costimulatory molecules (such as CD80/CD86) (Cheng and Sharma, 2015). In addition, IL-10 regulates differentiation and proliferation of several immune cells (Cheng and Sharma, 2015). IL-10 is produced by several cell of immune system but also by nonimmune cells, and its levels increase during the first and the second trimester and decrease prior to labor. Alterations in cytokines production and release are associated with adverse pregnancy complications, including preterm birth, miscarriage, IUGR, and preeclampsia.

IGF-I and IGF-II are involved in placental, fetal and postnatal growth (Gluckman 1995). IGFs do not cross the placenta. The fetus can influence maternal IGF-I levels by placental secretion of hPL. During pregnancy, IGF-binding proteins (IGFBP)-2 and-3 decrease thereby promoting the bioavailability of IGF-I in maternal tissues and enhancing nutrient transfer to the fetus. Placental IGF-I production further enhances transfer of nutrients across the placenta. On the other hand, IGFBP-1 produced in the decidua rises during pregnancy, interferes with IGF-I action, and inhibits fetal growth. Other GFs, such as epidermal-GF platelet-derived-GF, nerve-GF, fibroblast-GF, and transforming-GFs, are involved in differentiation, proliferation, and growth associated with pregnancy.

With the epidemic of obesity, there has been an increasing interest in adipose tissue secreted cytokines (adipokines) and its impact on reproductive health. Leptin, the first adipokine discovered in 1994, has been shown to be important for placentation and maternal–fetal exchange processes regulating growth and development. In later stages of a healthy pregnancy, central leptin resistance occurs to allow increased nutrient availability for the fetus (Tessier *et al.*, 2013). Fetal adiponectin promotes the expansion of adipose tissue and stimulate fetal growth. Adiponectin is the most abundantly expressed adipokine in adipose tissue. In addition, regulation of placental function by adiponectin constitutes a novel physiological mechanism by which the endocrine functions of maternal adipose tissue influence fetal growth (Aye *et al.*, 2013).

Inhibin, Activin, and Follistatin

Placental inhibin A rises in the maternal circulation and peaks at 8 weeks, and at term. Activin A produced by the placenta also increases. Activin stimulates and inhibin inhibits placental production of hCG, GnRH, and steroids (Qu and Thomas, 1995). Follistatin is an activin-binding protein expressed in the placenta, membranes, and decidua. It antagonizes the stimulatory effects of activin. Both inhibin A and B are located in the ST and the intermediate trophoblast of the placenta, during early pregnancy (inhibin A) and present throughout pregnancy (inhibin B) (Kondi-Pafiti *et al.*, 2013). The inhibins, activins, and follistatins have been implicated in the pathophysiology of pregnancy disorders such as preeclampsia and premature delivery (Shelling, 2012).

Endogenous Opiates

Fetal and maternal endogenous opiates originating from the pituitary glands are secreted in parallel with ACTH, in response to CRH, which is, in part, derived from the placenta. Endorphins, enkephalins, and dynorphins are produced also by the ST in response to CRH. They inhibit oxytocin, vasopressin, and gonadotropins and increase PRL secretion.

Beta-endorphins are endogenous-opioid substances produced by the pituitary gland and placenta. Plasma levels of beta-endorphin display a significant decrease in gestational weeks 28 and 33 compared to week 10, followed by a subsequent increase between gestational weeks 28 and 37 (Dabo *et al.*, 2010). However, no difference in levels of beta-endorphin between gestational weeks 10 and 37 can be detected (Dabo *et al.*, 2010). Low levels of beta-endorphin at the end of pregnancy are associated with need for additional pain medication beyond nitrous oxide during labor, although the causal relationship is unclear (Dabo *et al.*, 2010).

The Renin–Angiotensin System

In human pregnancy, the maternal and fetal circulating renin–angiotensin–aldosterone systems (RAAS) and various tissue renin–angiotensin systems (RAS) interact to ensure a satisfactory pregnancy outcome (Lumbers and Pringle, 2014). Tissue RASs crucially involved in normal pregnancy are the ovarian, intrauterine (placental and decidual), and the intra-renal RASs. The nonrenal RASs not only play key roles in ovulation, implantation, placentation, and development of the utero-placental and umbilico-placental circulations, but they also contribute to the activity of the circulating maternal RAAS, so influencing maternal cardiovascular and renal function. The fetus also has a circulating functional renin–angiotensin system, and this system together with its intra-renal RAS is essential for normal renal development and function.

Increased maternal renin activity is the result of an E induced increase in angiotensinogen and a compensatory response to maintain blood pressure in the presence of vasodilation. Renin cleaves angiotensinogen (AGT), to form angiotensin (ANG) I. ANG I is converted to ANG II by angiotensin-converting enzyme (ACE). Furthermore, angiotensin-converting enzyme 2 (ACE2), which has 40% homology with ACE can cleave ANG II to form ANG (1–7) or ANG I to form ANG (1–9), which can subsequently be cleaved to ANG (1–7) by ACE. ACE2 is primarily localized to endothelial cells, highly expressed in early pregnancy on the ST, and may be shed into the circulation. ANG II is the main ANG peptide; its biological actions are mediated predominantly by the angiotensin 1 receptor (AT1R). Other ANG peptides in the circulation include ANG (1–7), which is formed at the fastest rate from ANG II, ANG III, and ANG IV. ANG II also binds to the type 2 receptor, AT2R. Many actions of the ANG II/AT2R interaction oppose the actions of ANG II/AT1R. ANG II/AT1R interactions cause vasoconstriction, aldosterone synthesis, secretion, angiogenesis, and cell proliferation. Those mediated by ANG II/AT2R include vasodilatation and apoptosis. ANG (1–7) interacts with MasR, a G protein-coupled receptor, and many of its actions oppose the ANG II/AT1R effects, as do the actions of ANG IV mediated via the AT4R, also known as IRAP (insulin regulated aminopeptidase). ANG IV/IRAP-induced effects include hypertrophy, vascularization, inflammation, and vasodilation, as well as actions mediated via AT1Rs. IRAP is also known as placental oxytocinase.

Prorenin, the precursor of active renin was initially regarded as being biologically inert. However, the discovery of a prorenin receptor, (P)RR, altered this notion (Mikami *et al.*, 2017). (P)RR binds prorenin, exposing its catalytic site so that ANG I can be cleaved from AGT. The fact that (P)RR knockouts are embryo lethal suggests that (P)RR plays a pivotal role in embryonic development. A soluble form of (P)RR, s(P)RR, has also been described, and may influence the rate of formation of ANG I from AGT.

Recent data suggest a close relationship between RAS and hypertensive disorders in pregnancy and preterm birth (Bertagnolli, 2017). Specifically, the RAS seems one of the major candidates for preterm birth. Decidual RAS may play a role in inhibiting inflammation and maintaining the integrity of the fetal membranes during pregnancy. Interesting, sex-specific alterations in the intrauterine RAS could contribute to the increased risk of PTB in male babies (Pringle *et al.*, 2017). RAS alterations may occur as consequence of kidney and heart immaturity to promote adaptive responses, suggesting a dual role of this system on fetal and neonatal organogenesis. Fetal exposure to deleterious stress conditions can significantly impact on this dual RAS role, contributing to the establishment of hemodynamic and structural alterations (Tkachenko *et al.*, 2014).

Atrial Natriuretic Peptide and Corin

Atrial natriuretic peptide (ANP) is produced in the heart atrium and in the placenta. It is a potent natriuretic, diuretic, and smooth muscle-relaxant peptide that contributes to the regulation of volume and electrolyte changes associated with pregnancy and delivery (Burlingame *et al.*, 2017). Corin is a transmembrane serine protease discovered in the heart, where it converts pro-ANP to active ANP. Corin is upregulated in the decidua of the pregnant uterus, suggesting a potential role of corin in pregnancy. In mice lacking corin or ANP, high blood pressure and proteinuria were found at late gestational stages (Zhou and Wu, 2013). Histological analysis indicated delayed trophoblast invasion and impaired spiral artery remodeling in the uterus. In humans, CORIN gene mutations were identified in patients with preeclampsia. An enhanced corin signal has been detected not only in case of pre-eclampsia but also in patients with unexplained IUGR without hypertension (Miyazaki *et al.*, 2016).

Prostaglandins

Thromboxane (TXA2) is the most powerful vasoconstrictor known, whereas prostacyclin (PGI2) is a potent vasodilator. Platelets predominantly synthesize TXA2 whereas PGI2 is derived mainly from the endothelium. The endothelial production of PGI2 plays an important role in the marked vasodilation/contractility, that occurs during pregnancy, with particular regard for the decidualization process (Majed and Khalil, 2012; Broegger *et al.*, 2016).

The placenta is a major source of TXA2. The predominant effect of PGs in the fetal and maternal cardiovascular system is to maintain the ductus arteriosus, the renal, mesenteric, uterine, and placental arteries, and probably the cerebral and coronary arteries in a dilated state (Hansen *et al.*, 1999). Control of ductus arteriosus patency and closure is mediated through PGs. The arterial concentration of oxygen is the key regulator of the ductus caliber. With increasing gestational age, the ductus becomes increasingly responsive to increased oxygen. Prostaglandin E2 (PGE2) is the most important functional PG in the ductus, whereas PGI2, the major product in the main pulmonary artery, is the major factor in maintaining vasodilation in the pulmonary bed. With increasing maturation, the lung shifts to TXA2 formation. With the onset of pulmonary ventilation at birth, leading to vascular changes that deliver blood to the duct directly from the lungs, TXA2 serves as vasoconstrictor to the ductus.

Prior to parturition, fetal breathing is very shallow. Placental PGE2 may suppress breathing by acting in the fetal brain. Occlusion of the umbilical cord is rapidly followed by a loss of this PGE2 influence and the onset of air breathing.

Finally, initial experimental data seems to suggest that COX-2 expression in the decidua and the elevation of PGF2α in maternal serum is negatively regulated by vitamin D.

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Placental Epigenetics and Outcomes in Children

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Abbreviations

ART	Assisted reproductive technologies
DOHaD	Developmental origins of health and disease
IGs	Imprinted genes
ICRs	Imprinting control regions
IVF	In vitro fertilization
ICSI	Intracytoplasmic sperm injection
IUGR	Intrauterine growth restriction
LBW	Low birth weight

Introduction

In mammals, the placenta is vital for gestation and fetal well-being by ensuring fetal-maternal exchange (gases, nutrients, and waste products) (Zhang *et al.*, 2008). The placenta also plays a critical role in mediating programming of the fetus for future disease (Barker and Thornburg, 2013). Modifications in placental morphology/function can predict a wide range of conditions in later life (Barker *et al.*, 2013). An increased incidence of placental anomalies has been observed in assisted reproductive technology (ART) conceptions. Notably, several human studies reviewed by Thomopoulos *et al.* (2013) found an increased risk of gestational hypertension, preeclampsia, placenta praevia, and placental abruption. These placental defects may contribute to abnormal fetal growth such as low birth weight (Jackson *et al.*, 2004) and prematurity (Jackson *et al.*, 2004; Pinborg *et al.*, 2013), which are also more frequent following ART than following natural conception (Choux *et al.*, 2015). The placenta originates from the peripheral cells of the blastocyst, the trophectoderm cells that have been directly exposed to the environmental *milieu* up to implantation. In humans, placental syncytiotrophoblasts formed by the fusion of cytotrophoblasts make up the site of exchange between the maternal and fetal circulation; this interface has specific endocrine functions, but also functions as a barrier against various stresses (oxidative, xenobiotic, and chemical) (Huang *et al.*, 2013). The finely-tuned regulation of trophoblastic invasion, essential for proper future function of the placenta and fetal development, involves molecular crosstalk between the endometrium and trophoblast tissue driven notably by epigenetic factors (Chelbi and Vaiman, 2008). Epigenetics plays an important role not only in placental development, but also in adaptation to environmental stress (Novakovic and Saffery, 2012). Because ART procedures typically coincide with epigenetic reprogramming events, they represent models of extreme exposure to periconception epigenetic stress, which could impair trophoblastic invasion and contribute to the pathophysiology of disease seen in the postnatal period.

Epigenetics is defined as a set of cell-based molecular mechanisms able to modify gene expression without DNA sequence variation. Epigenetic regulation controls DNA transcription (through DNA methylation/hydroxymethylation mechanisms) and involves the proteins around which the DNA is wrapped to constitute nucleosomes (histone posttranslational modifications) (Nelissen *et al.*, 2011). Epigenetic regulation also controls translation or mRNA stability by the expression of noncoding RNAs (such as microRNA, Piwi, Miwi, …).

Imprinted genes (IGs), representing a small percentage of all genes, are known to be involved in important biological processes to enable in utero development. IGs are preferentially expressed by one parental chromosome as a consequence of epigenetic events (DNA methylation being the most often described) in the germline. These epigenetic modifications (reprogramming) are established in a sex-specific manner during gametogenesis on regulatory sequences referred to as imprinting control regions (ICRs). After fertilization, these ICRs act in *cis* to achieve complete or partial monoallelic expression of most imprinted genes (IGs). Up to now, approximately 150 imprinted genes have been identified in mice and humans. In mice, these are under the control of 23 identified ICRs (Barbaux *et al.*, 2012; Proudhon *et al.*, 2012; Court *et al.*, 2014) (http://www.geneimprint.com/site/genes-by-species). IGs are generally not imprinted in all tissues, and the imprinted pattern can be limited to a precise developmental stage. In addition, the conservation of imprinted status or even the sex involved (i.e., whether the maternal or paternal allele is expressed for a given IG) may vary between mammalian species (Barbaux *et al.*, 2012). Interestingly, the majority of IGs identified in mice are both expressed and imprinted in the placenta (Tunster *et al.*, 2013). Current findings indicate that IGs converge on two key placental functions in placental development and the regulation of fetal growth: nutrient transport and placental signaling (Tunster *et al.*, 2013).

As discussed in this review, compelling evidence indicates the particular role of epigenetics in placental regulation and the potential link between epigenetic dysregulation and adverse pregnancy outcomes. In addition, the purpose of this review is to provide a summary of current knowledge regarding placental epigenetic regulation and consequences in children conceived after ART.

Placental Epigenetic Modifications and Adverse Pregnancy Outcomes

Disturbed placental epigenetic regulation may cause abnormal trophoblastic invasion, which may contribute to the pathophysiology of some spontaneous miscarriages, intrauterine growth restriction (IUGR), and preeclampsia (**Fig. 1**).

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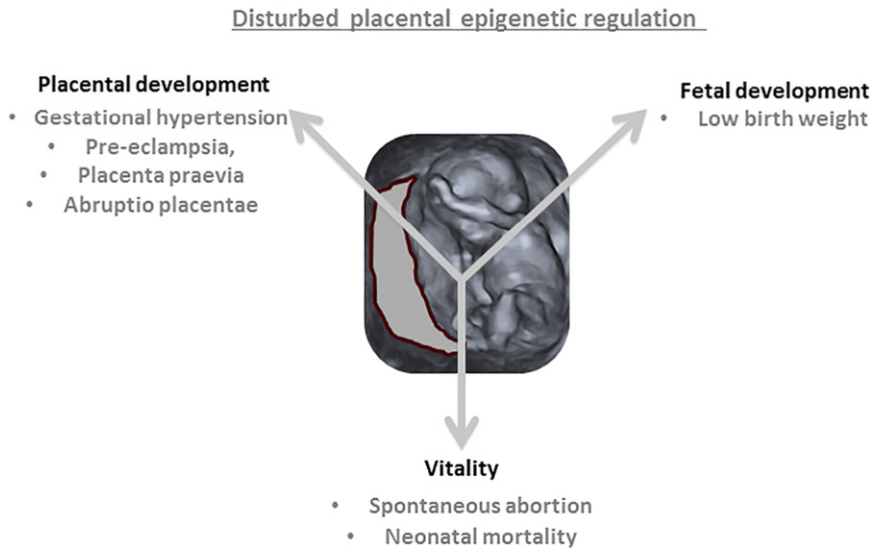

Fig. 1 Potential impact of placental epigenetic disturbance.

Miscarriages

In humans, the expression of DNA methyltransferase 1 (*DNMT1*), which is involved in DNA methylation maintenance throughout cellular divisions, and global DNA methylation were significantly lower in chorionic villi from early fetus losses than in those harvested following selective pregnancy termination (Yin *et al.*, 2012). In addition, in a large study on 165 human spontaneous abortions, aberrant DNA methylation levels were observed for some imprinted loci, suggesting that inappropriate DNA methylation status may lead to spontaneous abortions. However, the conception modes (natural vs. ART) or the fertilization methods (in vitro fertilization [IVF] vs. intracytoplasmic sperm injection [ICSI]) did not seem to affect the DNA methylation patterns of IGs (Zheng *et al.*, 2013). Earlier, in a smaller number of abortions and stillbirths, even though a significant difference was found between ART and non-ART samples for the methylation levels of *LIT1* (=*KCNQ1OT1*), no major differences were reported between ART and natural conceptions (Zechner *et al.*, 2010).

Intrauterine Growth Retardation

In humans, a large number of associations have been found between IUGR and epigenetic variations in placentas. Banister and colleagues found that the DNA methylation pattern of several loci was highly predictive of IUGR (Banister *et al.*, 2011). Some authors demonstrated that the *PHLDA2* imprinted gene was upregulated in the placenta in cases of IUGR (McMinn *et al.*, 2006; Diplas *et al.*, 2009; Kumar *et al.*, 2012) and that its expression level correlated negatively with birth weight (Apostolidou *et al.*, 2007). As *PHLDA2* is considered a negative growth regulator, the authors suggested that this IG potentially plays a direct role in the pathophysiology of IUGR.

Other imprinted genes were also upregulated (*CDKN1C*), or downregulated (*MEG3*, *GATM*, *ZAC1*, *GNAS*, *MEST*, *IGF2*) in IUGR placentas (McMinn *et al.*, 2006; Guo *et al.*, 2008; Diplas *et al.*, 2009; Koukoura *et al.*, 2011). Some of these differential expressions were associated with decreased placental methylation, as was the case for *H19/IGF2* ICR1 (Bourque *et al.*, 2010), or loss of imprinting, as was the case for *ZAC1* (=*PLAGL1*) and *H19* (Diplas *et al.*, 2009).

In addition, other examples of nonimprinted genes highlight the possibility that fetal growth potential could be negatively impacted by epigenetic dysregulation in the placenta. Ruebner and colleagues pointed out that the expression of Syncytin-1, a protein that promotes cellular fusion in the syncytiotrophoblast, was lower in human IUGR placentas than in controls (Ruebner *et al.*, 2010). The same team linked decreased expression of this protein to epigenetic hypermethylation of its promoter (Ruebner *et al.*, 2013). Additionally, another example concerns the *WNT2* gene. This gene, which is implicated in placental vascularization, exhibited decreased expression and increased DNA methylation in placentas of growth-restricted neonates as compared to controls (Ferreira *et al.*, 2011).

Interestingly, epigenetic changes were also found on repeated sequences. For example, Michels and colleagues found an increased LINE-1 methylation level in placental tissues from low birth weight infants (Michels *et al.*, 2011).

Preeclampsia

Evidence about preeclampsia reinforces the idea that epigenetic disorders may be involved in abnormal trophoblastic invasion. Besides, global DNA methylation changes were found in placentas from pregnancies with preeclampsia as compared with gestational age-matched controls (Blair *et al.*, 2013). Some of these methylation modifications correlated negatively with expression changes, especially for genes implicated in angiogenesis (*EPAS 1* and *FLT I genes*). In addition, the expression of maspin (*SERPINB5*), a serine protease

inhibitor and an inhibitor of cell migration (Khalkhali-Ellis, 2006), which may modify trophoblast cell invasion in the first trimester (Dokras *et al.*, 2002), could also be modified in preeclampsia. Additionally, SERPIN A3, a specific inhibitor of elastase, which is crucial during the implantation process, displayed decreased methylation and increased gene expression in placentas from pregnancies complicated by preeclampsia compared with controls (Chelbi *et al.*, 2007). As for IUGR, several studies highlighted the increased methylation (Ruebner *et al.*, 2013) and reduced expression of the *syncytin-1* gene, as well as the downregulation of *WNT2* in pre-eclamptic placentas. These modifications were possibly responsible for impaired placental function (Huang *et al.*, 2014). Interestingly, epigenetic modifications could also correlate with the severity of the disease (Yu *et al.*, 2009; Anton *et al.*, 2014).

Placental Epigenetic Modifications and ART Pregnancy Outcomes

Evidence from human epidemiological studies supports the hypothesis that ART could affect placentation and placental function. Indeed, an increased incidence of anomalous placentation and placental size has been observed following ART even after adjustments for length of gestation, gender, parity, and maternal age (Walker, 2000; Rinaudo and Lamb, 2008; Haavaldsen *et al.*, 2012). Indeed, two prospective studies on very large cohorts of singletons reported an almost twofold increased risk of preeclampsia following the use of in vitro fertilization (IVF) as well as a significantly higher risk of placenta praevia and abruptio placentae (Katalinic *et al.*, 2004; Kallen *et al.*, 2005). Moreover, placentas from ART pregnancies were overrepresented in the highest quartile of placenta weight (Haavaldsen *et al.*, 2012). Consequently, these placental defects may contribute to abnormal fetal growth and to low birth weight (LBW), which is also a well-described complication of ART pregnancy (Pandey *et al.*, 2012; Qin *et al.*, 2016).

These placental dysfunctions and the subsequent LBW may result from changes in epigenetic reprogramming, notably with modifications affecting IGs (Choux *et al.*, 2015).

In support of this hypothesis, it has recently been demonstrated in mice that suboptimal ART conditions could induce genomic imprinting alterations in the placenta and affect its development and function (Chen *et al.*, 2015; de Waal *et al.*, 2015).

Epigenetic Regulation Studied at Birth

In humans, several studies analyzed DNA methylation and/or gene expression in ART-conceived newborns compared with those naturally conceived. However, of these, only three studied DNA methylation (Gomes *et al.*, 2009; Rancourt *et al.*, 2012; Camprubi *et al.*, 2013) and, two DNA methylation and expression (Katari *et al.*, 2009; Turan *et al.*, 2010) of IGs in both cord blood and placenta (**Tables 1** and **2**).

Concerning the DNA methylation analysis, conflicting data were generated when the analyses were carried out on a global scale and when they focused on IGs. Indeed, for large DNA methylation analyses using arrays, one reported no variability in DNA methylation (Camprubi *et al.*, 2013) whereas another one (Katari *et al.*, 2009) described quantitative differences in global DNA methylation (briefly with a higher and a lower degree of DNA methylation in post-IVF cord blood and placenta samples, respectively) (**Tables 1** and **2**). However, the two studies are not comparable regarding the sample size (73 individuals vs. 10) and the mode of reproductive treatment (unspecified ART vs. IVF).

Moreover, as discussed above, studies focusing on the DNA methylation of specific IGs also reported conflicting results. Indeed, although some authors reported no epigenetic changes after ART (Gomes *et al.*, 2009; Shi *et al.*, 2011; Wong *et al.*, 2011; Zheng *et al.*, 2011), several authors reported variations in methylation levels in both cord blood and/or placentas for a number of IGs such as *MEST* (Tierling *et al.*, 2010; Rancourt *et al.*, 2012), *H19* (Turan *et al.*, 2010; Rancourt *et al.*, 2012; Nelissen *et al.*, 2013), *SNRPN* (Rancourt *et al.*, 2012), *GRB10* (Song *et al.*, 2015), *NDN* or for retrotransposable elements such as LINE1 (Ghosh *et al.*, 2017). However, none of them agreed on the changes in DNA methylation. Once again, comparisons between these studies are difficult given the limitations similar to those mentioned above and also given that the most of analyses were performed on different IGs or IG-sequences and that different methods were used (bisulfite pyrosequencing, methylation array, etc. …).

Concerning the analysis of IG expression, conflicting data were also found (**Table 2**). In the only two studies that analyzed both cord blood and placenta, dysregulations were reported, mainly in the placenta, but only for three IGs (*H19*, *IGF2*, *MEST*) (Katari *et al.*, 2009; Turan *et al.*, 2010; Nelissen *et al.*, 2013) (**Table 2**). Even though minimal expression changes on a few specific IGs were also found in cord blood after ART, no significant modifications in expression were observed in global analysis using microarray methodology (Feng *et al.*, 2011).

Therefore, all of these discrepancies in expression and DNA methylation results at term do not allow us to draw strong conclusions. Certainly, we still have to search for epigenetic modifications using robust methodologies that take into account potential confounding factors in a homogeneous cohort of singletons, and to determine the contribution of each reproductive technique and the role of infertility per se.

In addition, even though they are few in number, these epigenetic "defects" in ART-placentas should not be underestimated and should not simply be regarded as allelic polymorphisms. Indeed, we have to carry out more investigations to determine what they mean. They could originate from epigenetic errors at imprinted genes (de Waal *et al.*, 2014), or could correspond to epigenetic adaptation mechanisms (Choux *et al.*, 2015). Moreover, what also remains to be determined is whether epigenetic defects in the placenta are simply compensatory in nature or whether these early epigenetic modifications could be a risk factor for certain diseases later in life.

Table 1 Effects of ART on the DNA *methylation* of imprinted genes and retrotransposable elements in cord blood and/or placenta

Control group	*Manipulation group*	*Gene*	*Sample* *CB* *P*	*Results of methylation analysis* *Trends (differential methylation level)*[a]	*References*
30 NC	18 IVF or ICSI	*KCNQ1OT1*	CB	=	Gomes *et al.* (2009)
			P	=	
77 NC	35 IVF	*MEST*	CB	↑ (3.0%)	Tierling *et al.* (2010)
	77 ICSI	*KCNQ1OT1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Ex1A*	CB	=	
		MEST, KCNQ1OT1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Ex1A	CB	=	
12 NC	32 IVF, 45 ICSI	*H19*	P	=	Wong *et al.* (2011)
30 NC	61 ART	*H19*	CB	=	Shi *et al.* (2011)
60 NC	61 IVF, 40 ICSI	*KvDMR1, SNRPN, MEST, MEG3, TNDM, XIST*	CB	=	Zheng *et al.* (2011)
59 NC	59 IVF	*KCNQ1*	CB	↑ (0.6%)	Rancourt *et al.* (2012)
		MEST, GRB10, H19, IGF2 DMR0, SNRPN	CB	=	
		SNRPN	P	↑ (1.7%)	
		MEST	P	↓ (3.4%)	
		H19	P	↓ (1.3%)	
		GRB10, IGF2 DMR0, KCNQ1	P	=	
121 NC	73 ART	ALU-Yb8, LINE-1	CB	=	Camprubi *et al.* (2013)
			P	=	
		DIRAS3, NAP1L5, ZAC1, IGF2R, FAM50B, MEST, GRB10, PEG10, PEG13, INPP5Fv2, H19, KCNQ1OT1, RB1, MEG3, SNRPN, ZNF597, ZNF331, C19MC, PEG3, MCTS2, NNAT, L3MTBL, NESP, GNAS XL, GNAS Ex1A	CB	=[b]	
			P	=[b]	
8 NC	10 IVF	*GNAS, PLAGL1, ZIM2, DIRAS3*	CB	↑[b]	Melamed *et al.* (2015)
49 NC	66 ART	*GRB10, H19, MEST, SNRPN, NDN*	P	=	Song *et al.* (2015)
	22 donor oocyte ART	*GRB10*	P	↓	
		NDN	P	↑	
		H19, MEST, SNRPN	P	=	
65 NC	126 ART	LINE1	P	↓	Ghosh *et al.* (2017)
	39 ICSI		P	↓	
	87 IVF		P	↓	
	73 20%O_2		P	↓	
	53 5%O_2		P	=	
	90 fresh		P	↓	
	36 frozen		P	=	
	67 Day-3		P	↓	
	59 Day-5		P	↓	

[a]When available.

[b]By using an array method.

ART, assisted reproductive technologies; *CB*, cord blood; *ICSI*, intracytoplasmic sperm injection; *IVF*, *in vitro* fertilization; *NC*, naturally conceived; *P*, placenta; ↑, increased; ↓, decreased; =, no significant difference compared with control.

Epigenetic Regulation Studied During Childhood

Low birth weight and impaired fetal growth are assessable signs of embryonic/fetal stress, and the developmental origins of health and disease (DOHaD) hypothesis emphasizes that these features, even though within the spectrum of normal birth weight, are markers of adult disease, notably cardiometabolic defects (Bubb *et al.*, 2007; Meas *et al.*, 2010; Vrooman and Bartolomei, 2016). Currently, it is difficult to determine whether the risk of these chronic diseases in adulthood originates in the embryo as the direct

Table 2 Effects of ART on expression and DNA methylation of imprinted genes in cord blood and/or placenta

Control group	*Manipulation group*	*Gene*	*Sample CB P*	*Results of methylation analysis*	*Results of methylation analysis*	*References*
				Trends (fold change)[a]	*Trends (differential methylation level)*[a]	
13 NC	10 IVF	MEST	CB	=	↑ (21.8%)[b]	Katari *et al.* (2009)
		SLC22A2	CB	=	↓ (3.0%)[b]	
		PEG10	CB	=	↓ (4.2%)[b]	
		PEG3	CB	=	↓ (5.2%)[b]	
		GNAS	CB	=	↓ (3.0%)[b]	
		NNAT	CB	=	↓ (1.6%)[b]	
		PEG3	P	=	↑ (6.7%)[b]	
		MEST	P	↑ (2.09)	↓ (1.9%)[b]	
		SLC22A2	P	=	↓ (7.3%)[b]	
12 NC	45 ART	H19	CB	=	=	Turan *et al.* (2010)
		IGF2R	CB	↓ (0.61)	=	
		H19	P	↓ (0.72)	↑ LOI	
		IGF2	P	↓ (0.52)	NA	
		IGF2R	P	=	=	
8 NC (pooled by 2)	8 (4 IVF, 4 ICSI)		CB	=[b]		Feng *et al.* (2011)
40 NC	40 ART	SNRPN, UBE3A, TP73, GNAS, PWCR1,MEG3	CB	=		
		PEG10	CB	↑	=	
		L3MBTL	CB	↑	1/18 ART LOI versus 0/15	
		PHLDA2	CB	↓	=	
35 NC	5 IVF, 30 ICSI	MEST	P	=	↓	Nelissen *et al.* (2013)
		MEG3	P	NA	↓	
		H19	P	↑ (1.3)	↓	
		IGF2	P	=	NA	
		PEG3, SNRPN, KCNQ1OT1, IG-DMR	P	NA	=	

[a]When available.

[b]By using an array method.

ART, assisted reproductive technologies; *CB*, cord blood; *ICSI*, intracytoplasmic sperm injection; *IVF*, *in vitro* fertilization; *LOI*, loss of imprinting; *NA*, not analyzed; *NC*, naturally conceived; *P*, placenta; ↑, increased; ↓, decreased; =, no significant difference compared with control.

result of a suboptimal ART-environment, or instead in subsequent growth modifications, or both. Therefore, the possible existence of epigenetic defects in ART-offspring should not be underestimated. Specifically, data reported by our team and other teams show that the type of culture medium used in human reproduction may influence the birth weight of the children thus conceived (Dumoulin *et al.*, 2010; Nelissen *et al.*, 2012; Hassani *et al.*, 2013; Zandstra *et al.*, 2015) and may have an impact on their postnatal development (Bouillon *et al.*, 2016). Nevertheless, in humans, there is no evidence of epigenetic changes persisting into childhood. Indeed, in children conceived after IVF, reassuring data have been reported for DNA methylation for IGs and on a global scale (Kanber *et al.*, 2009; Oliver *et al.*, 2012; Puumala *et al.*, 2012; Whitelaw *et al.*, 2014). Only one study observed some epigenetic errors for the imprinted gene *SNRPN* during childhood (Whitelaw *et al.*, 2014). However, DNA methylation levels of this IG were not found to be modified at birth in ART-cord blood. Only one study found some DNA methylation modifications of *SNRPN* in the placenta after ART (Rancourt *et al.*, 2012).

However, the heterogeneity of biological samples, age range, type of reproductive technique and the analysis of methylation could hide potential underlying differences and confirm that more research is still needed.

Conclusion

This review highlights the relationship between epigenetic dysregulation and subsequent placental response. The dialogue between the endometrium and the embryo is a crucial step to achieve successful trophoblastic invasion and thus ensure a noncomplicated pregnancy and healthy offspring. The initially disturbed placentation may trigger compensatory mechanisms, in line with adaptive responses, through epigenetic mechanisms. The hypothesis is that if these mechanisms are overwhelmed, inappropriate maternal-fetal exchange could occur, potentially leading to abortion or adverse pregnancy outcomes (Choux *et al.*, 2015).

ART procedures or parental factors, for which a greater risk of placenta-related diseases is reported, could induce perturbations in genomic imprinting by impairing endometrial receptivity or by modifying the early steps in the epigenetic development of the embryo. However, studies that carried out DNA methylation analyses at birth found conflicting data. Nevertheless, these studies are not comparable regarding the mode of reproductive treatment (IVF vs. unspecified ART), notably. Concerning the analysis of IG expression, conflicting results at birth were also reported. Thus, we cannot be totally reassured and further evidence is required to determine whether alterations in the regulation of IGs induced by ART could exist in newborns. In addition, to better decipher the epigenetic mechanisms and to determine which factors/procedures are involved, analyses have to be performed jointly in cord blood and placenta. Moreover, it also remains to be determined whether some epigenetic feto-placental changes could have further consequences.

See also: Abnormal Growth: Small for Gestational Age. Control and Monitoring of Fetal Growth. In Vitro Fertilization (IVF). Pregnancy Endocrinology

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Endocrinology of Delivery

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Introduction

Parturition is a coordinated process of transition from a quiescent myometrium to an active rhythmically contractile state, requiring complex interplay between placental, fetal, and maternal compartments (Vannuccini *et al.*, 2016). It involves the synchronization between coordinated myometrial activity, cervical changes, including effacement and dilatation, and rupture of membranes (Mohan *et al.*, 2004).

Labor at term may be regarded best physiologically as a release from the inhibitory effects of pregnancy on the myometrium rather than as an active process mediated by uterine stimulants. The myometrium is normally maintained in a relaxed and quiescent state until the balance between the inhibitory effect of progesterone and pro-labor stimuli, including oxytocin and inflammatory mediators, are preserved. In fact, the mechanisms for initiation of parturition involve progesterone withdrawal, increased estrogen bioavailability, corticotrophin releasing hormone (CRH) and neuroendocrine mediators and finally, increased responsiveness of the myometrium to prostaglandins (PGs) and oxytocin (OT) (Smith, 2007). While parturition in most animals results from changes in circulating hormone levels in the maternal and fetal circulations at the end of pregnancy, in human it is both an endocrine and an inflammatory event with a complex interplay between the mother and the placenta–fetal unit.

Understanding the initiation signals and sequential set of events culminating in normal labor is still challenging. The role of fetal endocrine signals functioning as the biologic clock of organ maturation and as triggers for labor at term has been well documented. However, knowledge gaps still exist in our understanding of the initiator and effecter signals of normal term labor. In fact, recent evidences are supporting the role of other mechanisms playing a major role in triggering labor and delivery, as chorio-amniotic senescence (Menon *et al.*, 2016b).

Parturition as an Endocrine Event

Estrogens

Human pregnancy is characterized by a hyperestrogenic state and the placenta is the primary source of estrogens. Concentrations of estrogens increase in the maternal circulation with increasing gestational age. Placental estrone and 17β-estradiol are derived primarily from maternal C19 androgens (testosterone and androstenedione), whereas estriol is derived almost exclusively from the fetal C19 estrogen precursor (dehydroepiandrosterone sulfate, DHEAS) (Weiss, 2000). Studies on the effect of administered androgen to pregnant rhesus monkeys have provided evidence that estrogen synthesized from androgens plays a central role in labor and delivery in monkeys (Nathanielsz *et al.*, 1998). Estrogens do not themselves cause uterine contractions but do promote a series of myometrial changes, including increasing the number of prostaglandin receptors, oxytocin receptors (OTR), and gap junctions, and upregulating the enzymes responsible for muscle contractions (myosin light chain kinase, calmodulin) that enhance the capacity of the myometrium to generate contractions. In fact, estrogen has a key role in priming myometrium for labor by inducing expression of contraction associated proteins including OTR and PG receptors in myometrium (Challis *et al.*, 2000). However, the pathway to estrogen production in the human placenta is unique as P450c17 is not induced and the direct conversion of progesterone to estrogen does not occur. Instead, the human placenta relies on DHEAS from the fetal and maternal adrenal glands for the supply of precursor for estrogen synthesis, and bypasses the step catalyzed by P450c17 (Mesiano and Jaffe, 1997). Hence there is no reciprocal fall in plasma progesterone and rise in plasma estrogen in humans and other primates. Rather both estrogen and progesterone increase progressively toward term but the ratio of estrogen/progesterone begins to favor estrogen. Upon reaching the placenta and sequential actions by sulfatase, 3β-HSD and aromatase, DHEA, and its hydroxylated product 16-hydroxy-DHEA are converted to estrogen in placental syncytiotrophoblasts. Glucocorticoids increase the conversion of DHEA to estrogen via induction of aromatase expression in human placenta (Wang *et al.*, 2012).

CRH has been shown to stimulate placental production of estrogens and to inhibit placental synthesis of progesterone (Yang *et al.*, 2006; You *et al.*, 2006). Placental CRH is also released into the fetal circulation, and in vitro CRH directly stimulates DHEAS production from the fetal zone of the fetal adrenal, that is the precursor for placental estriol (E3) synthesis. E3 is an inhibitor of the action of the potent estrogen estradiol (E2) at low concentrations but becomes an effective agonist when the ratio of E3 to E2 exceeds 10:1. Placental CRH may therefore lead to increased E3 production and reduced progesterone synthesis. Smith *et al.* showed in a cohort of 500 women at 26 weeks' gestation and during the last weeks of pregnancy that the very rapid rise of CRH in late pregnancy is associated with an E3 surge and critically altered P/E3 and E3/E2 ratios that create an estrogenic environment at the onset of labor. Thus, a critical feature of parturition appears to be a change in the ratio of the two estrogens E2 and E3 as labor approaches, leading to a more than 10-fold excess of E3 (Smith *et al.*, 2009).

Progesterone

Progesterone is a "pro-gestational" agent; it sustains the pregnant state and promotes myometrial relaxation. The balance between the inhibitory effects of progesterone and the stimulatory actions of estrogens is pivotal in determining the contractile state of the pregnancy myometrium and the timing and process of parturition (Mesiano and Welsh, 2007). In contrast to most animal species, the circulating levels of progesterone during human labor are similar to prelabor levels (Walsh *et al.*, 1984), suggesting that the systemic withdrawal of progesterone is not a prerequisite for labor in humans. However, as RU486 treatment induces labor at all stages of human pregnancy, it is generally considered that human parturition involves a form of progesterone withdrawal that does not depend on a decrease in circulating progesterone levels. The mechanisms involved in progesterone functional withdrawal are mainly related to a decreased myometrial progesterone responsiveness mediated by changes in the levels of specific nuclear progesterone receptors (nPRs) or nPR co-activator/co-repressors (Mesiano, 2007; Mesiano *et al.*, 2002, 2011). PR signaling can be regulated through two general mechanisms: changes in ligand concentration or changes in PR responsiveness. Levels of the progesterone ligand can be regulated at the systemic level through an endocrine regulation. They can also be regulated locally through the presence of metabolic enzymes in the target tissues that increase or decrease the levels of ligand available (paracrine or autocrine regulation). Progesterone signaling can also be regulated at the receptor level through changes in the relative expression of the nuclear PR isoforms, reduced expression of membrane receptors, and changes in the expression levels of coactivators and/or corepressors, including nuclear factor kB (Byrns, 2014). Functional progesterone withdrawal in human parturition is mediated by an increase in the myometrial PR-A (or PR-C)/PR-B ratio; in addition, the progesterone responsiveness is differentially regulated in fundal (by PR-C) and lower segment (by PR-A) myometrium. Most of human pregnancy, progesterone via PR-B promotes uterine quiescence, in part by inhibiting the responsiveness of myometrial cells to pro-inflammatory stimuli and preventing tissue level inflammation, and that functional progesterone withdrawal at parturition is caused by increased PR-A-mediated trans-repression of PR-B (Merlino *et al.*, 2007). As pregnancy advances, the capacity for PR-B to mediate inhibitory and antiinflammatory actions of progesterone on the pregnancy myometrium decreases due to increased repression by PR-A (Tan *et al.*, 2012). Interestingly, the amount and trans-repressive activity of PR-A in myometrial cells is increased by pro-inflammatory stimuli suggesting a causal link between inflammation and PR-A-mediated functional progesterone withdrawal (Madsen *et al.*, 2004).

The biochemical and physical changes induced by the combined effects of progesterone withdrawal and estrogen activation are (Mesiano and Welsh, 2007): (a) increased Cx43 expression leading to increased coupling between myocytes so that contractions are synchronized across the whole uterus; (b) increased sensitivity and contractile responsiveness to stimulatory uterotonins such as OT an PGF2 due respectively to increased OTR expression; (c) increased production of PGs by the gestational tissues and decreased inactivation of PGs in the myometrium; (d) lowered threshold for myocyte excitability; (e) decreased capacity for the cAMP/PK-A signaling pathway to maintain relaxation.

CRH and Neuropeptides

The complex mechanisms controlling human parturition involves mother, fetus, and placenta, and stress is a key element activating a series of physiological adaptive responses (Petraglia, 2010; Voltolini and Petraglia, 2014). The hypothalamus-–pituitary–adrenal (HPA) axis plays a key role in the neuroendocrine response to stress and CRH, a 41-amino-acid peptide hormone discovered in the 1980s, emerged as a crucial hypothalamic hormone regulator of human labor (Petraglia *et al.*, 1987). CRH is classically synthesized in the hypothalamus, but during human pregnancy, the placenta and fetal membranes produce large amounts of CRH and its related urocortin peptides, the circulating concentration of which rise exponentially in the third trimester of pregnancy (Smith, 2007; Challis *et al.*, 2000; McLean and Smith, 2001). CRH, in turn, further drives maternal and fetal HPA activation, thereby establishing a potent positive feed-forward loop. In normal pregnancy, the increased production of CRH from decidual, trophoblastic, and fetal membranes leads to an increase in circulating cortisol beginning in mid-gestation. It has been noted that the rise of maternal CRH level occurs earlier and more rapidly in women delivered preterm and more slowly in women delivered postterm, than in women delivered at term (Challis *et al.*, 2000). Because of this, CRH has been proposed to regulate a placental clock that controls a cascade of physiological events leading to parturition (McLean *et al.*, 1995). In addition, CRH stimulates fetal ACTH release, activating fetal adrenal steroidogenesis. The increased production of glucocorticoids is responsible for fetal lung maturation and on the other hand the increased DHEA and DHEAS levels creates an estrogen-dominant environment favorable to parturition.

Plasma CRH levels rise during the second half of pregnancy, peak during labor, and rapidly decline postpartum. Near term, CRH concentrations begin to exceed concentrations of the CRH-binding protein, leading to a rapid increase in circulating concentrations of bio-active CRH at term (Perkins *et al.*, 1993). CRH also enhances prostaglandin production by amniotic, chorionic, and decidual cells. Prostaglandins, in turn, stimulate CRH release from the decidual and fetal membranes.

Thus, human parturition is achieved by a series of positive-feedback hormonal loops that operate at multiple levels to drive labor forward. On one side, placental CRH promotes fetal cortisol and DHEA-S production, and these steroids return via the umbilical circulation to the placenta where cortisol promotes further CRH secretion, and DHEA-S is converted to estrogen (Yang *et al.*, 2006). CRH has also been shown to directly stimulate placental estrogen synthesis (You *et al.*, 2006) and similarly estrogen indirectly promotes further placental CRH secretion through its action on PG synthesis. On the other side, CRH increases the synthesis and release of PGE_2 and $PGF2_\alpha$ from the cells of the amnion, chorion, and decidua. These PGs, in addition to glucocorticoids derived from fetal urine, promote further CRH secretion by the placenta and fetal membranes. Besides, recent

evidences suggest that pro-inflammatory mediators may be direct and/or indirect promoters of placental CRH release. In fact, IL-6 and CRH are both secreted in a pulsatile fashion during the active phase of human labor and their time-integrated concentrations are positively correlated (Papatheodorou *et al.*, 2013).

Urocortins belong to the CRH superfamily and have recently been implicated as novel neuroendocrine mediators in physiologic and pathologic pregnancy and parturition. Human myometrium expresses urocortin 1 (Ucn) and a twofold increase of contractility is observed when Ucn is added after $PGF2_{\alpha}$ administration. Ucn activates diverse intracellular signaling pathways that contribute to the activation of myometrial contractility (Petraglia, 2010). Moreover, Ucn stimulates MMP-9 protein level in the culture medium of chorionic trophoblast, syncytiotrophoblast, and amniotic epithelial cells (Li *et al.*, 2006), suggesting a local role in tissue remodeling and cervical ripening at the time of labor.

A role for Ucn2 in the control of myometrial contractility during human pregnancy has been demonstrated, involving binding to CRH receptor 2 (CRH-R2) and enabling actin–myosin interaction and cell contraction. Ucn2 affects placental conversion of fetal adrenal C19 steroid precursors into E2 and appears to do so by increasing P450 aromatase expression via CRH-R2, thus suggesting a putative novel role for CRH family peptides in the sequence of steps involved in estrogen biosynthesis. CRH and Ucn2 participates also in the inflammatory mechanisms of parturition. In Jeg-3 cell line has been demonstrated that Ucn2 is a potent pro-inflammatory neuropeptide via NF-kB and MAPK pathways and CRH via MAPK (Novembri *et al.*, 2015). Recent experiments in laboring/nonlaboring human gestational tissues and in mouse utero-placental tissues showed that Ucn2 is a neuroendocrine factor that is upregulated at time of parturition and acts as a pro-inflammatory agent in placenta and in myometrium. The data suggest that Ucn2 has a role in mediating inflammatory processes of parturition but also has implications for Ucn2 being involved in the pathogenesis of obstetric complications (Voltolini *et al.*, 2015).

Glucocorticoids

Glucocorticoids (GC) play a pivotal role in initiation of labor and in the maturation of fetal organs in several mammalian species (Li *et al.*, 2014). GC activity depends on the 11β-hydroxysteroid dehydrogenase family (11β-HSDs), catalyzing the interconversion between "active" cortisol into inactive cortisone. The type 1 enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD1) is bi-directional but operates mostly as an oxo-reductase, facilitating the production of active cortisol (or corticosterone, murine equivalent), in a NADPH-dependent manner. The type 2 (11β-HSD2) is a NAD^+-dependent enzyme, uni-directional and inactivating cortisol to cortisone (or corticosterone to dehydrocorticosterone) (Michael *et al.*, 2003). The biochemically distinct activities of the two 11β-HSDs are present in the placenta, where the balance between the reductase activity of 11β-HSD1 and the oxidative activity of 11β-HSD2 changes at different stages of gestation. Specifically, 11β-HSD2 appears to be the major form expressed in the human placenta, suggesting a potential importance of local glucocorticoids increase in the placenta in preparation for labor (Murphy and Clifton, 2003). During pregnancy, cortisol is synthesized in the adrenal glands and placenta and plays a primary role in the maturation of fetal organs at the end of pregnancy, thus contributing to the onset of labor (Alfaidy *et al.*, 2003). Recent evidences demonstrated in both murine and human samples that 11β-HSD1 expression in the human myometrium significantly decreases at the onset of labor (Damiani *et al.*, 2017). In addition local cortisol regeneration by 11β-HSD1 in amnion causes the downregulation of lysyl oxidase (LOX) expression, a collagen cross-linking enzyme, thereby contributing to the rupture of fetal membranes (ROM) (Liu *et al.*, 2016).

Although the maternal adrenal glands undergo chronic hyperplasia and secrete increasing cortisol with advancing gestation in pregnancy, the placental syncytiotrophoblasts express increasing amounts of 11β-HSD2 toward term (Schoof *et al.*, 2001), thus limiting transfer to the fetus and minimizing the negative feedback of maternal cortisol on the fetal HPA axis. This protects the developing fetus from the growth restrictive and programming effect of maternal GCs that is much higher than the fetal concentration. It also allows the fetal adrenal glands to secrete copious amounts of DHEAS in the presence of high maternal GC concentrations during pregnancy.

Limitation of the negative feedback of maternal cortisol on the fetal HPA axis by the placental enzyme 11β-HSD2 also allows the secretion of cortisol from the androgenic fetal adrenal glands to increase toward the end of gestation. In addition, the fetal membranes and decidua express increasing amounts of 11β-HSD1 with gestational age, and are regarded as an important extraadrenal source of GCs locally within the intrauterine tissues during gestation (Alfaidy *et al.*, 2003; Li *et al.*, 2006; Sun and Myatt, 2003).

Thus, cortisol level rises in parallel with estrogen in maternal and fetal circulations as well as in the amniotic fluid toward the end of human gestation. Considering that glucocorticoids are potent stimulants of prostaglandin synthesis, cortisol regenerated locally in the fetal membranes play a central role in the initiation of parturition in humans (Li *et al.*, 2014; Challis *et al.*, 2000; Lye *et al.*, 1998). In addition, GC also upregulate expression of CRH from the placenta and fetal membranes (Petraglia *et al.*, 1996).

The final common pathway toward parturition in the human appears to be maturation and activation of the fetal HPA. Fetal HPA axis activity starts at mid-gestation, after the maturation of the fetal pituitary. Interestingly, fetal stress responses are independent from those of the mother: the fetal hypothalamus releases CRH, together with placental CRH, inducing fetal pituitary ACTH secretion. ACTH in turn controls adrenocortical functional development, including angiogenesis and expression of steroidogenetic enzymes (Mesiano and Jaffe, 1997). Parturition is a very stressful condition for the fetus and an adequate adrenocortical secretion of steroids (mainly cortisol) enables its adaptation to extrauterine life. Stimulation of the fetal pituitary by CRH increases ACTH production and, consequently, the synthesis of cortisol by the fetal adrenal gland and maturation of fetal lungs. In turn, the rising fetal cortisol concentrations further stimulate placental CRH production. The maturation of the fetal lungs as a result of increasing cortisol concentrations represents a fundamental aspect of fetal adaptive mechanisms to extrauterine life

activated by the stress of delivery. Moreover, fetal lung maturation is associated with increased production of surfactant protein A and phospholipids, both pro-inflammatory agents, that may stimulate myometrial contractility through increased production of prostaglandins by fetal membranes and the myometrium itself (Smith, 2007).

Oxytocin

OT is synthesized in the hypothalamus and secreted by the posterior pituitary; its major target organs are the pregnant uterus and mammary glands, as it regulates myometrial contractility and milk ejection. Although placental expression and secretion of OT have been shown, their contribution to the mechanisms of parturition remains unknown at this stage. Human decidua expresses greater levels of OT mRNA than amnion, chorion, and trophoblast. Placental OT secretion is increased by several paracrine factors such as CRH, activin A, and PGs operating within human intrauterine tissues (Florio *et al.*, 1996). At the onset of labor, the uterine sensitivity to OT markedly increases in association with both an upregulation of OT receptor mRNA levels and a strong increase in the density of myometrial OT receptors, reaching a peak during early labor (Havelock *et al.*, 2005). Since OT infusion can initiate labor and an OT antagonist is effective in delaying threatened preterm labor in several species, an important role for OT in driving parturition is suggested.

Parturition as an Inflammatory Event

Parturition is associated with increased tissue-level inflammation within the myometrium, decidua, and cervix (Norman *et al.*, 2007). A pivotal role for PGs in the inflammation process leading to labor initiation has been shown. The presence of cytokines, interleukins and estrogen seems to promote the production of PGs from myometrial cells, fetoplacental unit and predominantly from the maternal decidua in pregnancies near term. In labor, PGs increase in maternal urine and blood, and in fetal membranes. PGF2α is produced primarily by the maternal decidua and acts on the myometrium to upregulate oxytocin receptors and gap junctions, thereby promoting uterine contractions. PGE_2 is primarily of fetoplacental origin and is likely more important in promoting cervical ripening (maturation) associated with collagen degradation and dilation of cervical small blood vessels and spontaneous rupture of the fetal membranes. In fact, PGE_2 stimulates uterine contractions by upregulation of oxytocin receptors (OTRs) and synchronization of contractions, and acts in collaboration with IL-8 to remodel the cervix. PGE_2 also attenuates the expression of lysyl oxidase (LOX), the enzyme that cross-links collagen fibrils in human amnion fibroblasts, thereby reducing the tensile strength of the membranes. Uncross-linked collagen fibrils also predispose the fibrils to degradation by MMPs, inducing fetal membranes rupture (Liu *et al.*, 2016).

The increase of PGs exert a positive feedback action, further stimulating placental CRH release and sustaining a positive loop that results in increased intraamniotic concentration of cortisol (influence 11b-HSD1 activity), prostaglandins, and membrane matrix metalloproteases (MMP-9) that finally lead in fetal membrane activation and initiation of parturition (Sun *et al.*, 2006).

Increasing evidence suggests that, near term, also mechanical uterine distension and fetal neuroendocrine hormonal signaling enhance the production of pro-inflammatory chemokines (cytokines IL-1b, Il-6, IL-8) in the amniotic fluid that act as chemoattractants. As a result, larger amounts of cytokines are released inducing the activation of pro-inflammatory transcription factors that promote myometrial contractility, including gap junction protein connexin 43, the oxytocin and the $PGF2_\alpha$ receptor, cycloxygenase-2 and ion channels (Mendelson, 2009). These proteins act within the uterus to facilitate coordinated, rhythmic contraction as they promote intercellular connection, excitability of myometrial cells, and propagation of the electrical activity. In addition, chemokines influx induces both calcium entry in myometrial cells and degradation of cAMP via stimulation of phosphodiesterase activity (Oger *et al.*, 2002).

Labor Mechanisms

Myometrial Contractility

During pregnancy, the myometrium undergoes changes in cellular phenotype, characterized by an early proliferative phase, an intermediate phase of cellular hypertrophy and matrix elaboration, a third phase in which the cells become highly active assuming a contractile phenotype. The phenotypic modulation of the uterine myocytes is the result of integration of endocrine signals and mechanical stimulation of the uterus by the growing fetus (Shynlova *et al.*, 2009b). The proliferative phenotype is associated with dramatic changes in the expression of IGF family proteins and coincided with an upregulation of the antiapoptotic pathway (Shynlova *et al.*, 2007). The growth and remodeling of the myometrium during pregnancy is associated with increased synthesis of extra cellular matrix (ECM) proteins and their corresponding integrin receptors (Shynlova *et al.*, 2009a). A decrease in expression of fibrillar collagens and a coordinated temporal increase in expression of components of the basement membrane near term is observed, together with decreased progesterone levels and increased mechanical tension. In the labor phase, myometrial cells are characterized by increased excitability, spontaneous activity, responsiveness to agonists, and effective coupling of the myocytes. In fact, in the contractile phase an upregulation of contraction-associated proteins and downregulation of myometrial inhibitory pathways is observed, and, finally the expression of myometrial labor genes, synthesis of uterotonic agonists, and development of

the intense contribute to a synchronous contractions of labor (Shynlova *et al.*, 2009b). Myometrial cells play also an important role in the generation and regulation of uterine inflammation, actively participating through the release of the pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1).

Rupture of Membranes

Rupture of the fetal membranes is one of the labor phenomenon where stretch forces act upon preweakened tissue (Kumar *et al.*, 2016) and although part and necessary for delivery, it is likely controlled separately from uterine contractions. In fact, the rupture of membranes is the result of a remodeling-maturation process analogous to that seen in the cervix. The physiological mechanisms which cause membranes to fail, term or preterm, are not completely understood. Changes in collagen type and matrix cause initial structural weakening (McLaren *et al.*, 2000), which is then followed by cellular apoptosis, especially in a focal area overlying the cervix (McLaren *et al.*, 1999). A biochemically remodeled para-cervical fetal membranes region seems to be physically weaker than other and have been names "physiological weak zone" (El Khwad *et al.*, 2005), where the spontaneous rupture of membranes seems to initiates, even though mechanisms are not fully understood.

Cervix

The final process of parturition is ultimately effected by cervical effacement and dilatation, allowing a progressive and controlled delivery of the fetus. In the cervix, cytokines, prostaglandins, nitric oxide, and steroids, especially androgens (Makieva *et al.*, 2014), seem to induce ripening by mediating the remodeling of the extracellular matrix and collagen (Sennström *et al.*, 2000). It is postulated that the underlying mechanism involves an increased expression of metalloproteases by cervical fibroblasts and the breakdown of collagen in the cervical tissue (Iwahashi *et al.*, 2003).

Other Mechanisms Involved in Parturition

Senescence of Chorio-Amniotic Membranes

The role played by hormones in labor and onset progression has been widely investigated, however the mechanisms underlying the initiation of parturition are still not fully understood. Recently, cellular senescence of fetal membranes has been hypothesized as the initiator of a coordinated, redundant signal cascade leading to parturition (Menon, 2016). Multiple inputs into an inflammatory load converge on common labor effectors such as progesterone withdrawal and increased sensitivity to oxytocin and PGs to transform the uterus into a laboring phenotype (Menon *et al.*, 2016b).

Cellular senescence refers to the physiologic and biomolecular mechanisms normally associated with aging (Munoz-Espin and Serrano, 2014). However, premature senescence may also lead to pathologic states. Morphologic evidence of senescence is characterized by enlargement of cells and biochemically by the presence of senescence associated (SA) b-galactosidase (gal) and formation of DNA-damaged foci with chromatin alterations. Unlike apoptosis (programmed cell death), senescent cells persist and change the tissue environment with a unique inflammatory milieu. Senescence of the chorio-amniotic membranes is a natural and physiological process and term labor can be considered as an end stage of life for chorioamnion. Morphologic evidence of cellular senescence (enlarged cells and organelles) and a higher number of SA b-galactosidase stained amnion and chorion cells were observed in chorio-amniotic membranes obtained from women in labor at term, when compared to term not in labor. Additionally, a phenomenon known as the senescence-associated secretory phenotype (SASP), characterized by the promulgation of pro-inflammatory cytokines, chemokines, growth factors, and MMPs, manifest a form of intrauterine sterile inflammation, functioning as a signal to promote labor. The concentration of pro-inflammatory SASP markers has been shown to be significantly higher in the amniotic fluid of women in labor at term than women not in labor (Behnia *et al.*, 2015). Fetal membrane senescence is also associated with oxidative stress and telomere length loss with release of telomere fragments (Polettini *et al.*, 2015), that elicit pro-inflammatory responses, acting as signals for parturition (Menon *et al.*, 2016a; Phillippe, 2014). Chorio-amniotic membrane senescence constitutes a counting device, that is, a clock, that measures maturation of the fetal organ systems and the production of hormones and other soluble mediators and that promotes inflammation and orchestrates an immune cascade to propagate signals across different uterine compartments. This mechanism in turn sensitizes decidual responsiveness and eventually promotes functional progesterone withdrawal in the myometrium, leading to increased myometrial cell contraction and the triggering of parturition (Menon *et al.*, 2016b).

Conclusion

Human labor is a multifactorial physiological event involving an integrated set of changes within the maternal tissues of the uterus (myometrium, decidua, and uterine cervix) and fetal membranes, which occur gradually over a period of days to weeks. A parturition cascade exists in humans that is responsible for the removal of mechanisms maintaining uterine quiescence and for the recruitment of factors acting to promote uterine activity (Snegovskikh *et al.*, 2006). Whereas there may be a final common pathway

for the initiation of labor, there are multiple, sometimes complementary, endocrine, paracrine, and autocrine initiating factors involved. The "final common pathway" to delivery is likely to be multiple, parallel, interactive paths that tip the balance in favor of coordinated uterine contractility and cervical dilation.

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Premenstrual Syndrome (PMS)☆

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Glossary

Premenstrual dysphoric disorder (PMDD) A subtype of premenstrual syndrome that is characterized by emotional symptoms such as depression, anxiety, and irritability that are severe enough to interfere with social or role functioning and are verified by prospective daily symptom ratings.

Premenstrual syndrome (PMS) A constellation of physical and emotional symptoms that recur repetitively during the 1 or 2 weeks before the onset of a menstrual period and cannot be accounted for by other underlying disorders.

Selective serotonin reuptake inhibitors (SSRIs) A category of antidepressant medications that increase the levels of serotonin at the junction between nerve fibers.

For centuries, many women have recognized that they experience physical and emotional symptoms during the week before the onset of their monthly menstrual period. However, it was not until 1931 that Frank and Horney validated their experience by describing a medical disorder termed premenstrual tension syndrome (Knaapen and Weisz, 2008). Twenty years later, Green and Dalton described the same syndrome but used the term premenstrual syndrome (PMS). In the 1990s, psychiatrists described a subtype of PMS that consisted of severe emotional symptoms and behavioral changes and referred to it as premenstrual dysphoric disorder (PMDD).

Epidemiology

Although mild physical and emotional symptoms may occur during the week before the onset of menstruation in up to 80%–90% of women, population studies have demonstrated that premenstrual symptoms that are severe enough to warrant medical evaluation and therapy occur in only 3%–5% of menstruating women (Segebladh *et al.*, 2009; Halbreich *et al.*, 2012; Freeman *et al.*, 2012; Yonkers *et al.*, 2008). Although women commonly present for treatment during the fourth decade of life, many of them can trace the onset of their symptoms to their adolescence. They also often describe other family members with PMS/PMDD as well as a personal history of postpartum depression or the exacerbation of their symptoms when combined oral contraceptives or progesterone alone is administered (Backstrom *et al.*, 2011).

Pathophysiology

There are numerous theories regarding the etiology of PMS/PMDD, but its pathogenesis remains elusive. Ovarian steroidal hormones play a central role, as demonstrated by the fact that symptoms resolve with anovulation and oophorectomy (Yonkers *et al.*, 2008). Also, because symptoms occur in the late luteal phase, progesterone has been implicated as a causative agent, although the mechanism by which it acts to instigate symptoms is unclear (Segebladh *et al.*, 2009). It has been suggested that progesterone and its metabolites act on γ-aminobutyric acid (GABA) receptors; stimulation of GABA-A receptors has been shown to have paradoxical effects with negative mood symptoms like those observed in women with PMS/PMDD (Backstrom *et al.*, 2011). In contrast to this theory, however, is that fact that many women with PMS/PMDD begin experiencing symptoms at the start of ovulation, before the decline in progesterone has occurred, and administration of progesterone during the late luteal phase does not ameliorate symptoms (Yonkers *et al.*, 2008). Estrogen may also play a role, although its contribution to the pathogenesis of PMS/PMDD is less well studied. Lastly, the neurotransmitter serotonin has been implicated in PMS/PMDD. Serotonin helps regulate mood and behavior, and studies in serotonin-deficient rodent led to increased sex-steroid dependent behaviors (Ho *et al.*, 2001). In addition, PMS/PMDD symptoms are improved with administration of selective serotonin reuptake inhibitors (see below) and other serotonin-enhancing agents (Yonkers *et al.*, 2008; Marjoribanks *et al.*, 2013). Individual biological, genetic, and psychological factors as well as sociocultural conditions may also play a role in the etiology of the symptoms as well as their severity.

Diagnosis

Because there is no biochemical marker for PMS/PMDD, the diagnosis is made upon the patient's clinical history. PMS and PMDD are distinguished from each other based upon the presence and severity of symptoms (see **Table 1**) (Freeman *et al.*, 2011).

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Table 1 Diagnostic criteria for PMS and PMDD

Premenstrual syndrome (PMS)	*Premenstrual dysphoric disorder (PMDD)*
At least one affective or somatic symptom[a] in the 5 days before menses	At least 5 of 11 specified symptoms[b] at a severe level in the week before menses for the past year
Remission of the symptom(s) on cycle days 4–13	Remission for 1-week post-menses
Symptoms impair functioning	Symptoms impair functioning or relationships
Symptoms confirmed in 2 cycles of prospective symptom ratings	Symptoms confirmed in at least half of the woman's menstrual cycles within the past year
Symptoms are not due to another disorder	Symptoms are not due to another disorder

[a]Affective symptoms include depression, angry outbursts, irritability, anxiety, confusion, and/or social withdrawal. Physical symptoms include breast tenderness, abdominal bloating, headache, and/or peripheral edema.

[b]Specified symptoms: depression, anxiety, mood swings, irritability, decreased interest, difficulty concentrating, fatigue, appetite changes, sleep disturbance, feeling overwhelmed, physical symptoms. Symptoms must include at least one of the first four symptoms and only one physical symptom.

Adapted from Box 1 in Freeman *et al.*

Recently, PMDD was added to the Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (Epperson *et al.*, 2012). To meet diagnostic criteria, patients must experience a premenstrual pattern of symptoms in at least half of their menstrual cycles during the previous year. Both psychological and physical symptoms typify PMS/PMDD; those most commonly reported by patients include mood lability, irritability, bloating, and breast tenderness (Epperson *et al.*, 2012).

Because some women may have selective recall of their symptoms, prospective charting of the symptoms is necessary to establish the relationship of these symptoms to the onset of the menstrual period and to establish a diagnosis of PMS or PMDD. While the DSM-5 diagnosis of PMDD also requires prospective daily self-ratings of symptoms for at least six menstrual cycles within a year, many find this tedious and unnecessary in clinical practice. Though prospective charting is ideal for establishing the diagnosis of PMS/PMDD, some women presenting for evaluation and treatment will be unwilling or unable to prospectively record their daily symptoms and it may be necessary to initiate therapy without adequate prospective charting. Finally, the fact that the symptoms never last for more than 2 weeks distinguishes PMS and PMDD from other mood or physical disorders (Epperson *et al.*, 2012).

It is important during the course of the evaluation to rule out other conditions such as anxiety disorders, depressive disorders, anemia, hypothyroidism, chronic fatigue syndrome, central nervous system tumors, or autoimmune disorders. It is also important to evaluate the impact of the symptoms on her self-esteem and relationships with family, friends, and co-workers. It is prudent to discuss stresses in the lives of these women for they may exacerbate the symptoms of PMS/PMDD.

Treatment

Overview

Because no single treatment is universally effective for PMS/PMDD, many have doubted the existence of this disorder and criticized attempts to help women with their premenstrual symptoms. However, in the past decade there have been over 200 prospective randomized clinical trials of various therapies, which have led to an evidence-based approach to the treatment of PMS/PMDD.

Initial Nonspecific Therapy

The initial treatment of PMS/PMDD can begin during the phase of evaluation using nonspecific therapies. These nonspecific therapies include non-pharmacologic approaches such as patient education, validation of symptoms through daily prospective charting, relaxation training, and self-help measures such as exercise and nutrition. Alternative and complementary therapies like acupuncture and herbal medicine may also be employed, though with limited evidentiary support (Jang *et al.*, 2014). Because social support is often essential for relief of symptoms, it may be helpful to the women with PMS/PMDD if the clinician involves family members or friends in the process of evaluation so that they may support her and help validate her symptoms. Although data supporting the effectiveness of these non-pharmacologic therapies are limited, these therapies are often used because they lack significant side effects, are very practical, and may benefit the woman's physical and emotional health (Jarvis *et al.*, 2008).

First-Line Pharmacologic Therapies

Once the diagnosis of premenstrual syndrome has been made, more specific therapy can be initiated for those women who desire pharmacologic intervention. Because of their demonstrated effectiveness in prospective randomized clinical trials, selective serotonin reuptake inhibitors (SSRIs) are considered first-line treatment (Marjoribanks *et al.*, 2013; Freeman *et al.*, 2011; ACOG, 2010; Dimmock *et al.*, 2000). A recent Cochrane review included thirty-one randomized controlled trials

(RCTs) comparing fluoxetine, paroxetine, sertraline, escitalopram, and citalopram versus placebo. SSRIs were found to be more effective in reducing self-reported symptoms than placebo, though medication withdrawal and side effects were more common in the SSRI group (Marjoribanks *et al.*, 2013). Notably, SSRIs may be taken throughout the menstrual cycle or during the luteal phase only, the latter of which clearly distinguishes PMS/PMDD from other mood disorders (Marjoribanks *et al.*, 2013; Epperson *et al.*, 2012). In addition, in contrast to the gradual and delayed response of individuals with chronic depressive disorders to SSRIs, women with premenstrual syndrome may note improvement of their symptoms within days of starting their medication (Epperson *et al.*, 2012).

Despite these positive findings, up to 40% of women with PMS/PMDD fail to respond to SSRI treatment (Freeman *et al.*, 2011). This may be related to the heterogeneity of PMS/PMDD, with a wide range of both physical and psychological symptoms. A study of 447 women found that sertraline significantly improved symptoms in women diagnosed with predominately psychological and mixed psychological/physical PMS/PMDD subtypes but was not effective in women in the predominately physical subtype (Freeman *et al.*, 2011).

Second-Line Therapies

In addition to SSRIs, combined oral contraceptives (COC) may be used to treat symptoms of PMS/PMDD. The first published RCTs comparing COC to placebo utilized a 24/4 regimen (24 days of hormonally active pills followed by 4 days of inactive pills) containing drospirenone 3 mg and ethinyl estradiol 20 mcg (Lopez *et al.*, 2012; Yonkers *et al.*, 2005; Pearlstein *et al.*, 2005). These studies found improvements in both mood and psychological symptoms as well as an improvement in overall quality of life, although sample sizes were low and there was a large placebo effect seen. Small studies have shown equivalent effect with levogestrol and levonorgestrol as the progestin component (Sangthawan and Taneepanichskul, 2005; Freeman *et al.*, 2012; Halbreich *et al.*, 2012). While effective, many women note increased symptoms while taking the hormonally inactive pills, leading to the administration of COC in an extended cycle or continuous pattern to suppress menstruation and ovarian hormonal fluctuations that are thought to precipitate PMDD.

When the above therapies are either ineffective or unacceptable to the patient, there are other treatment options that may be helpful even though scientific support for their use is not as strong as for SSRIs and COC. Second-line non-pharmacologic treatment may include cognitive behavioral therapy, which has been shown to be as effective as fluoxetine in the treatment of PMDD (Hunter *et al.*, 2002). Second-line pharmacologic agents include other agents to suppress ovulation and anxiolytics. Although obliteration of the menstrual cycle and ovulation by gonadotropin-releasing hormone (GnRH) agonists has been shown to be effective, their side effects limit their widespread use (Segebladh *et al.*, 2009). GnRH agonists are associated with menopausal symptoms including hot flashes, night sweats, and insomnia. A randomized, double-blind, crossover clinical trial evaluated three different hormonal add-back regimens to women with PMDD treated with leuprolide acetate found increased PMDD symptoms with all regiments, though symptoms were worse with add-back progesterone regimens (Segebladh *et al.*, 2009). Lastly, newer treatment options like acetazolamide may be helpful by potentiating GABAergic transmission, but evidence is limited regarding their utility and effectiveness (Sani *et al.*, 2014).

This wide array of potentially effective therapies offers new hope to women with PMS or PMDD. However, the therapy of PMS/PMDD should be more than just pharmacologic and should include self-help measures so that the patient becomes an active partner in her therapy. In addition, attempts should be made to recognize and help the patient deal with stresses in her life and to help her establish an effective social support system. The importance of validation, social support, and the elimination of stress cannot be emphasized enough. It is hoped that further research will uncover new and even more effective and safe therapies for premenstrual syndrome.

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Amenorrhea

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Definition

Amenorrhea is an absence of menses. In physiology, menses are bleeding occurring at the beginning of the menstrual cycle, due to the desquamation of the endometrium, the inner layer of the uterus. They usually last from 3 to 6 days. The occurrence of the first menses is called menarche. Its mean age is 12.5 years. The average time between the first pubertal sign, that is, the increase in breast development, and the first menses is of 2 years. Amenorrhea is called primary, when the patient has never had any episode of menses. It is called secondary, after 3 months of amenorrhea, in a patient who has already had menses.

In all cases of amenorrhea, pregnancy must be ruled out. The physiological conditions of amenorrhea are pregnancy, lactation and the time after menopause (i.e., the last menstrual bleeding). Apart from these obvious causes, an etiological assessment, including clinical and hormonal evaluations must be carried out. The different causes are of hypothalamic- pituitary, ovarian, adrenal or uterine origin.

Primary Amenorrhea

In the case of primary amenorrhea, etiological assessment must be carried out in the absence of menses, at the age of 15 years.

If there is no breast development, the assessment must be done before the age of 14. The items to look for when questioning the patient are (1) the age of the menarche of the mother and/or the sisters; (2) a past history of hypogonadism in the family; (3) the presence of eating disorders; (4) the degree of physical activity per week; (5) the presence of headaches and visual field abnormalities; (6) the absence of smell, called anosmia or a diminished smell called hyposmia; (7) a previous history of chronic disease such as renal failure, pulmonary disease or coeliac disease; and (8) a previous history of chemotherapy and/or radiotherapy. Clinically, the stage of breast development should be characterized as well as the presence of pubic and/or axillary hair, hirsutism, acne, the patient's height, her weight and her body mass index. A pelvic mass on abdominal examination favors hematocolpos which is a blood accumulation in the uterus, due to a lack of hymenal perforation.

In the initial assessment, laboratory tests include hCG, FSH, LH, estradiol (E2), prolactin and testosterone. A progestin test can be used in order to evaluate the patient's endogenous estrogen secretion. The test is positive if bleeding occurs at the end of 10 days of treatment with progestin. It is in favor of a normal endogenous E2 secretion. On the opposite, the test is negative in the absence of bleeding at the end of 10 days of treatment with progestin. The causes are endogenous estrogen deficiency, a lack of uterus and pregnancy.

The pelvic ultrasound is useful in order to measure the ovarian sizes, evaluate the presence of ovarian follicles and a uterus. A uterine length greater than 25 mm is in favor of a beginning of puberty. Transvaginal ultrasound is usually much more informative than transabdominal ultrasound. However, in some patients, vaginal ultrasound cannot be performed if the patient has not had sexual intercourse.

The diagnostic procedure is presented in **Fig. 1**. Serum FSH and estradiol levels will discriminate between hypogonadotropic and hypergonadotropic hypogonadism. Normal or low levels of FSH in the face of low E2 levels suggest a hypothalamic-pituitary disease. Congenital hypogonadotropic hypogonadism (CHH) is a rare disease. In Kallmann syndrome, CHH is associated with anosmia (see chapter on delayed puberty). High FSH levels are in favor of primary ovarian insufficiency (POI), such as Turner syndrome (see chapter on Turner syndrome).

In case of breast development, the diagnostic procedure is similar to that of secondary amenorrhea. However, some specific diagnostic procedures should be mentioned in case of primary amenorrhea. Uterine abnormalities include an absence of uterus, called Rokitansky syndrome. A second abnormality is hymenal imperforation leading to hematocolpos with a history of cyclic pelvic pains. A rare disease called the complete androgen insensitivity syndrome (CAI) is due to loss-of-function mutations of the androgen receptor, in 46 XY individuals. The diagnosis is made in a patient with primary amenorrhea, breast development with a lack of hair and high testosterone serum levels, higher than those of a normal male range.

Secondary Amenorrhea

In case of secondary amenorrhea, pregnancy must first be ruled out. The diagnosis procedure is presented in **Fig. 2**.

During the questionnaire, one should record: the age of menarche, the regularity of menses, history of sexual intercourses and previous history of chemotherapy, radiotherapy or chronic diseases. Headaches and visual field abnormalities are in favor of a hypothalamic-pituitary tumor. Medication inducing a hyperprolactinemia (see chapter on hyperprolactinemia) or an atrophy of

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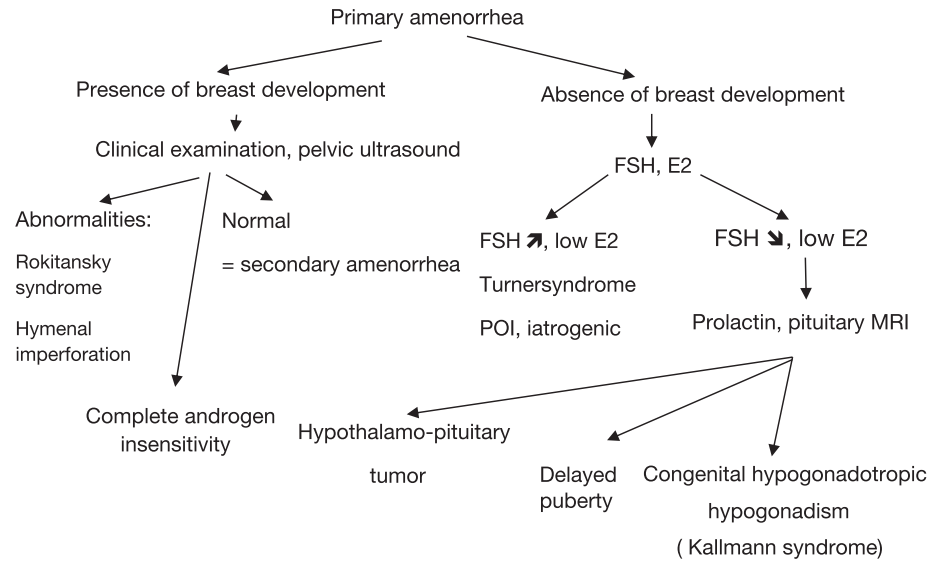

Fig. 1 Diagnostic procedure for primary amenorrhea. *FSH* = Follicle stimulating hormone, *E2* = estradiol, *POI* = primary ovarian insufficiency, *MRI* = Magnetic resonance imaging.

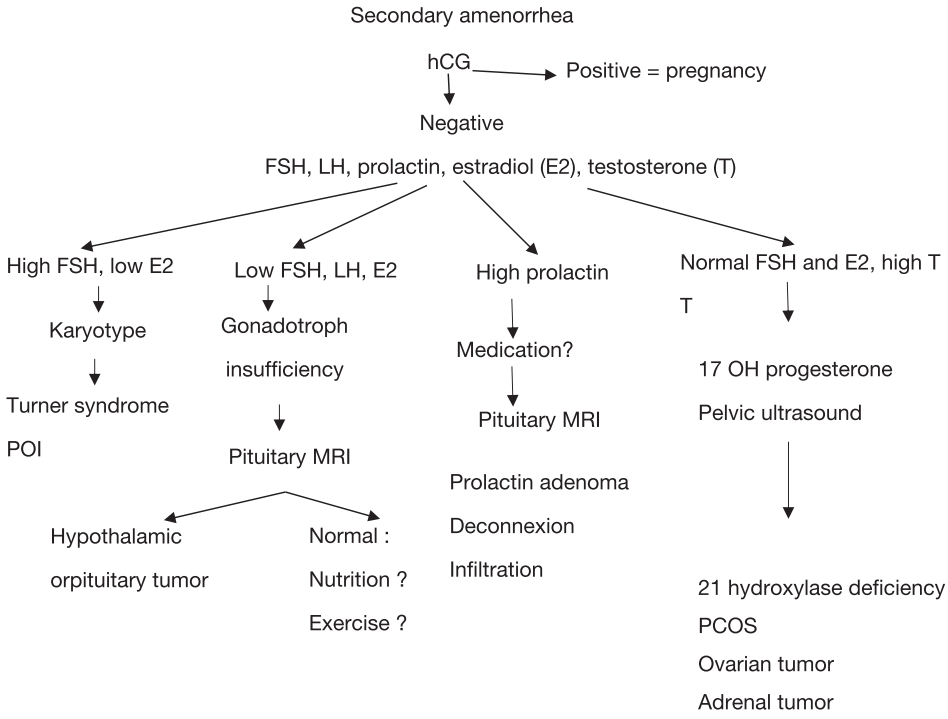

Fig. 2 Diagnostic procedure for secondary amenorrhea. *hCG* = human choriogonadotropin hormone, *FSH* = follicle stimulating hormone, *LH* = luteinizing hormone, *E2* = estradiol, *T* = testosterone, *MRI* = magnetic resonance imaging.

the endometrium should be searched for. Nowadays, acquired uterine adhesions, called synechiae are becoming quite a rare cause of secondary amenorrhea.

The laboratory evaluation includes FSH, LH, E2, testosterone (T) as well as prolactin levels. The most frequent cause of secondary amenorrhea is polycystic ovarian syndrome. It affects around 10% of the female population. Women may have biological and/or clinical hyperandrogenism with hirsutism, acne, as well as severe oligomenorrhea or amenorrhea. The ultrasound usually find a large amount of arrested follicles, higher than 20 per ovary. If testosterone serum level is very high (>1.5 ng/mL), an ovarian or an adrenal tumor should be ruled out. In young women, acquired hypogonadotropic hypogonadism is usually related to hyperprolactinemia or to an occult food selection associated with excessive physical activity.

In summary, physicians should identify the cause of primary or secondary amenorrhea before introducing any treatment. After ruling out a pregnancy, the initial laboratory assessment includes FSH, LH, E2, prolactin and T measurements. The ultrasound is useful to confirm the presence of a uterus, to measure ovarian volume and count ovarian follicles. An MRI must be requested in front of a hypogonadotropic hypogonadism.

See also: Turner Syndrome

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Congenital Hypogonadotropic Hypogonadism in Females☆

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Introduction

Congenital isolated gonadotropin-releasing hormone deficiency (GnRH), also called congenital hypogonadotropic hypogonadism (CHH) or isolated hypogonadotropic hypogonadism (IHH) is a rare condition in women and is characterized by GnRH and/or gonadotropin deficiency associated with low serum ovarian sex steroids levels and low circulating gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Boehm *et al.*, 2015). In IHH/CHH, the underlying hypothalamo-pituitary neuroendocrine abnormalities are classically divided into two main groups: 1) Molecular defects of actors involved in the gonadotrope cascade leading hypothalamic GnRH deficiency or pituitary resistance to GnRH and causing isolated normosmic CHH (nCHH), and 2) Developmental abnormalities affecting development and/or migration and the normal hypothalamic location of GnRH neurons in post natal life, but also olfactory bulbs/tracts morphogenesis and responsible for an anosmic/hyposmic IHH/CHH form called Kallmann syndrome (KS) (Boehm *et al.*, 2015; Stamou *et al.*, 2016). The main clinical sign that reveals the condition is primary amenorrhea (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017; Tang *et al.*, 2017). Knowledge of the broad clinical reproductive spectrum of IHH/CHH in women has improved during the last two decades. As a result, the detection of partial forms with significant pubertal development seems to be more effective in adolescents who consult for primary amenorrhea. Discovery of tens of genes associated with IHH/CHH/KS has provided major insights into the molecular pathways critical for the development, maturation of GnRH neurons and for function of the gonadotrope axis.

Prevalence

There is no specific epidemiologic studies on the prevalence of IHH/CHH/KS particularly in women. Two old studies suggested a prevalence comprised between 1/10000 and 1/86000 in males (Boehm *et al.*, 2015). The prevalence of IHH/CHH/KS has classically been considered to be lower in females with a male/female ratio of 5:1(Boehm *et al.*, 2015). However, a bias in the diagnosis of female IHH/CHH/KS is likely as they often are treated with hormonal therapy without etiological investigation and are not referred to teaching hospitals where prevalence is usually evaluated (Boehm *et al.*, 2015).

Clinical Presentation

In women, IHH/CHH/KS is revealed after the age of puberty by delayed pubertal development associated with primary amenorrhea in more than 95% of cases (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017; Tang *et al.*, 2017). At diagnosis, before any hormonal therapy, breast development is highly variable, absent in only a minority of cases, but often present (breast Tanner stages B2-B4) and sometimes even normally developed (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017; Tang *et al.*, 2017) (**Fig. 1**). On the same way, pubic hair may be absent, sparse or even normal. These partial forms, in majority not referred to hospital also contribute to explain the underestimated prevalence of this condition in women (Boehm *et al.*, 2015). In rare very mild forms, IHH/CHH/KS can be restricted to isolated chronic anovulation. In these IHH women estradiol secretion seems adequate for uterine and endometrial development explaining the existence of a single or few menses (primosecondary amenorrhea) (nejm) or even chronic oligomenorrhea (Sarfati *et al.*, 2015). Growth and final height in IHH/CHH/KS women have been evaluated in very few studies (Dickerman *et al.*, 1992). The scant reported data indicate that the final height in these women is similar to that of the reference population. Contrary to hypogonadal women with Turner syndrome IHH women are not at risk of small height, (Dickerman *et al.*, 1992).

IHH/CHH/KS are not always congenital lifelong disorder as initially thought, because in nearly 10% of patients the disease seems not permanent, as evidenced by partial or complete recovery of the pulsatile activity of the hypothalamic-pituitary-gonadal axis after discontinuation of treatment in adulthood (the so-called reversible CHH/IHH) (Dwyer *et al.*, 2016).

Hormonal Evaluation

Most women with IHH/CHH/KS have very low serum pituitary gonadotropin levels (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017) when sensitive LH and FSH immunoassays are used. However, circulating gonadotropins in a subset of patients can be low-normal, which is inappropriate in the setting of low estradiol and low inhibin B levels. Circulating estradiol levels in untreated

☆*Change History:* April 2018. Jacques Young updated the text and references to this article.

This article is an update of Lawrence C. Layman, Gonadotropin-Releasing Hormone Deficiency, Congenital Isolated, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 302–305.

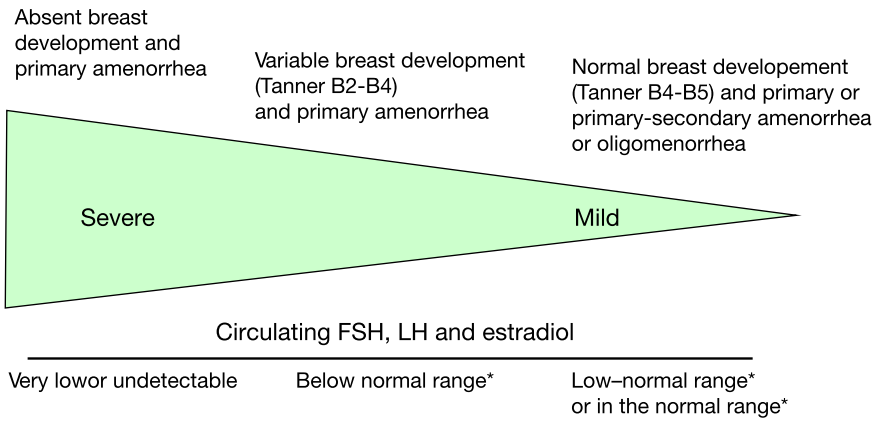

Fig. 1 Reproductive phenotype in IHH/CHH women, wide clinical spectrum at diagnosis. * In early follicular phase in normal women.

IHH/CHH/KS women are low or in the lower end of the normal range during the follicular phase when sensitive assays are used (radioimmunoassays or GCMS and LCMS methods) that allow detection of very low estradiol levels (Bry-Gauillard *et al.*, 2017). Fiew studies have investigated circulating inhibin B levels in IHH/CHH/KS female patients (81). Low Inhibin B concentrations are reported to be very low, as in prepubertal girls (Bry-Gauillard *et al.*, 2017). Circulating AMH levels are significantly lower in women with complete untreated IHH than in healthy women (Bry-Gauillard *et al.*, 2017). The severity of AMH deficiency seems related to the severity of FSH deficiency as IHH/CHH/KS women with lower circulating FSH levels have lower serum AMH levels (Bry-Gauillard *et al.*, 2017). The low AMH levels in these latter patients are associated with low antral follicular count (AFC)(see below). However and importantly, these patients' low ovarian volume, low AMH, and/or AFC should not be considered to indicate a poor fertility prognosis, as fertility can be effectively restored by both pulsatile GnRH and gonadotropin administration (Bry-Gauillard *et al.*, 2017).

In the endocrine evaluation of IHH/CHH/KS, it is important to evaluate all pituitary functions in order to rule-out hyperprolactinemia and to exclude additional pituitary deficiencies (Boehm *et al.*, 2015).

Pituitary and Pelvic Imaging

Pituitary MRI is useful to rule out expansive, infiltrative, or malformative disorders of the hypothalamo–pituitary region that can be responsible for pubertal delay associated with hypogonadotropic hypogonadism (HH) in the context of hypopituitarism (Higham *et al.*, 2016). Brain MRI can also be used to analyze olfactory bulbs (OB) and furrows in a search for signs suggesting Kallmann syndrome (unilateral or bilateral OB aplasia or hypoplasia, and effacement of the furrows), particularly when semi-quantitative olfactometry is not locally available (Boehm *et al.*, 2015; Maione *et al.*, 2013).

Studies on uterine morphology in IHH/CHH/KS women are scarse (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017; Tang *et al.*, 2017). Pelvic or transvaginal ultrasound demonstrated prevalent but variable uterine hypoplasia (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017; Tang *et al.*, 2017) which correlated with the importance of estradiol deficiency and endometrial atrophy (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017; Tang *et al.*, 2017). Ovarian volume (OV) in IHH females, evaluated in two studies, showed a significant decrease in mean OV compared to healthy adult women of similar age (Shaw *et al.*, 2011; Bry-Gauillard *et al.*, 2017). Only one recent study quantified the number of ovarian antral follicles (AF) and showed a significant decrease in the average number of AF compared to normal, age-matched women, in agreement with low levels of AMH (see above).

Differential Diagnosis

One challenge in women with IHH/CHH, particularly when the condition is sporadic and with normal sense of smell and normal hypothalamo-pituitary MRI and no identified causative mutation (see below and **Table 1**) is the differential diagnosis of functional hypothalamic amenorrhea (Caronia *et al.*, 2011; Gordon *et al.*, 2017). Therefore, in women referred for primary amenorrhea and with an hormonal profile suggesting HH but without anosmia or hyposmia, the diagnosis of IHH/CHH must only be considered with caution, after ruling out underweight, eating disorders, excessive physical activity, and chronic underlying conditions (**Fig. 2**) (Caronia *et al.*, 2011; Gordon *et al.*, 2017). When body weight or BMI are at the lower limit of normal (22 Kg/m^2), body fat measurement can also be useful to screen for functional hypothalamic amenorrhea (Caronia *et al.*, 2011; Gordon *et al.*, 2017). Another challenge in adolescent girls presenting with delayed puberty, primary amenorrhea and low pituitary gonadotropins is to differentiate an IHH/CHH from a constitutional delay of puberty (CDGP). The presence of associated clinical signs such as anosmia or other signs usually associated with Kallmann syndrome or complex syndromes including IHH/CHH help to exclude CDGP (Boehm *et al.*, 2015; Varimo *et al.*, 2017; Cassatella *et al.*, 2018). No hormonal marker can reliably differentiate these two

Table 1 Genes and loci associated with in isolated congenital hypogonadotropic hypogonadism (CHH), Kallmann syndrome and syndromic forms of CHH

Gene	*Locus*	*Mode of inheritance*	*Reproductive phenotype*	*Nonreproductive associated signs*
Isolated CHH				
GNRH1	8p21.2	AR (oligo)	nCHH	No
GNRHR	4q13.2	AR (oligo)	nCHH	No
KISS1	1q32.1	AR	nCHH	No
KISS1R	19p13.3	AR	nCHH	No
TAC3	12q13.3	AR	nCHH	No
TACR3	4q24	AR	nCHH	No
FSHB	11p14.1	AR	Isolated FSH deficiency	No
LHB	19q13.33	AR	Isolated LH deficiency	No
Kallmann syndrome				
ANOS1 (KAL)[a]	Xp22.31	X-linked (oligo?)	KS	MM-S,RA (in males)
FGFR1	8p11.23	AD/AR/Oligo/de novo	KS or nCHH	CLP,DA,BA, SHF
FGF8	10q24.32	Oligo	KS or nCHH	CLP,DA,BA
FGF17	8p21.3	Oligo	KS or nCHH	?
PROK2	3p13	AD/AR/Oligo	KS or nCHH	No
PROKR2	20p12.3	AD/AR/Oligo	KS or nCHH	No
CHD7	8q12.2	AD/AR/Oligo/de novo	CHARGE or KS (nCHH?)	CLP,HI,EEA,SCC,C
NSMF (NELF)	9q34.3	Oligo	KS or nCHH	?
HS6ST1	2q14.3	Oligo	KS or nCHH	?
WDR11	10q26.12	?	KS or nCHH	?
SEMA3A	7q21.11	AD?/Oligo?	KS	No
SEMA7A	15q24.1	Oligo	KS/CHH	?
SEMA3E	7q21.11	Oligo	KS/CHH	?
PLXNA1	3q21.3	Oligo?	KS/CHH	?
SOX10	22q13.1	AD	Waardenbourg/KS	HI,SCC, ISHH
IL17RD	3p14.3	Oligo	KS/CHH	?
FEZF1	7q31.32	AR	KS	No
WDR11	10q26.12	?	KS/CHH	?
Syndromic CHH				
DAX1 (NROB1)	Xp21.2	X-linked	CHH/AHC	AI
HESX1	3p14.3	AR	Hypopit	SOD
LHX4	1q25.2	AR	Hypopit	Phyp,ST,CM
PROP1	5q35.3	AR	Hypopit	PMass,Phyp,ST,
LEP	7q32.1	AR	Morbid obesity and HH	
LEPR	1p31.3	AR	Morbid obesity and HH	
PCSK1	5q15	AR	Morbid obesity and HH/AI	ACTH-D, HGI-D
OTUD4	4q31.21	AR	HH + Ataxia	PD
RNF216	7p22.1	AR	HH + Ataxia	PD
PNPLA6	19p13.2	AR	Gordon Holmes/Boucher-Neuhauser	CD
SOX2	3q26.33	AR	Hypopit	An

[a]Few heterozygous mutations reported in females.

Reproductive and nonreproductive main associated signs.

nCHH, Normosmic Congenital hypogonadotropic hypogonadism; KS, Kallmann syndrome; HH: hypogonadotropic hypogonadism; AR: autosomal recessive; AD: autosomal dominant; Oligo: oligogenic; Hypopit: Congenital Hypopituitarism; MM-S: mirror movements-Synkinesia; RA: renal agenesis; CLP: Cleft lip/palate; DA: dental egenesis; BA: bone abnormalities; SHF: split hand foot malformations; HI: Hearing impairment; EEA: External ear anomalies; SCD: Semicircular canal dysplasia; C: coloboma; ISHH: iris, skin, hair hypopigmentation; AHC: adrenal hypoplasia congenita; AI: andenal insufficiency; SOD, septo-optic dysplasia; Phyp: pituitary hypoplasia; ST: sella turcica abnormalities; CM: Chiari malformation; PMass: pituitary masses; ACTH-D: ACTH deficiency; HG-ID: hyperglycemia and insulin deficiency; PD: progressive dementia; CD: chorioretinal dystrophy; An: anophthalmia.

conditions. CDGP is therefore a diagnosis of exclusion, and will only be retained if spontaneous pubertal development and regular menses occur after the age of 18 (Varimo *et al.*, 2017).

Genetic Basis

Since the discovery of the first causative gene KAL1/ANOS1 in 1991 (Boehm *et al.*, 2015; Stamou *et al.*, 2016; Maione *et al.*, 2018), mutations affecting more than 30 genes have been identified to be involved in IHH/CHH and/or KS (**Table 1**). These genes account for approximately 50% of cases but some loci are exceedingly rare and/or occur in the context of complex syndromes. These genes have been identified different approaches: either via cytogenetic or candidate gene studies, linkage analysis (Boehm *et al.*, 2015;

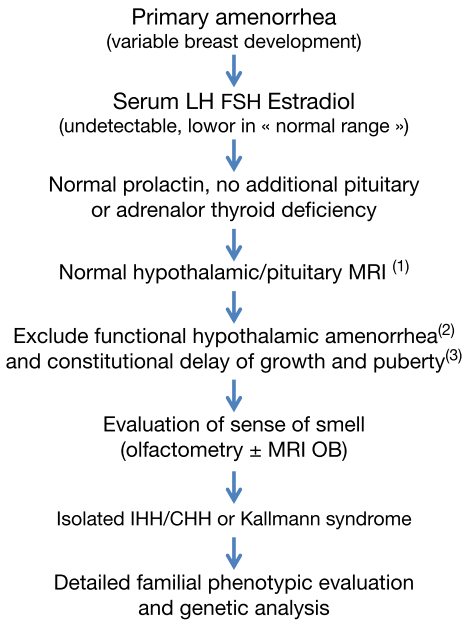

Fig. 2 Successive steps to overcome before confirming the diagnosis of IHH/CHH in a woman referred for primary amenorrhea. (1) Sometimes partial pituitary hypoplasia is observed in Kallmann syndrome. (2) Especially if Body Mass Index is below 22 kg/m^2 (Shaw et al., 2011) which requires follow-up at least until the age of 18 years. (3) MRI: magnetic resonance imaging. OB olfactory bulbs.

Stamou *et al.*, 2016; Maione *et al.*, 2018), and more recently by pathway analysis, or by next-generation sequencing (i.e., targeted or whole exome sequencing) (Boehm *et al.*, 2015; Cassatella *et al.*, 2018).

Genes involved in CHH or KS are classified according to their function in the development of the gonadotrope axis or in the neuroendocrine control of GnRH homeostasis (Stamou *et al.*, 2016): (1) GnRH differentiation; (2) GnRH neuron migration and olfactory axon guidance; (3) GnRH neuron secretion; and (Bry-Gauillard *et al.*, 2017) pituitary gonadotrope cell defects. The modes of transmission of the known genetic causes are shown in **Table 1**. In IHH/CHH/KS inheritance was initially proposed to be exclusively Mendelian with autosomal recessive, autosomal dominant, de Novo or X-linked (males) modes of transmission (**Table 1**) (Maione *et al.*, 2018). However, in many familial cases, the IHH/CHH/KS phenotype and/or associated reproductive signs are not consistent with these classical modes of transmission. Since almost a decade it gradually emerges that either the transmission and the penetrance and the phenotypic variability could be oligogenic in nature (**Table 1**). This new, complex genetic architecture is increasingly recognized by simultaneous analysis of tens or hundreds of genes with next-generation sequencing more efficient techniques (Maione *et al.*, 2018).

Identification of a genetic cause in patients with CHH/IHH is important for clarify the pathophysiological mechanism of the disease and to guide the phenotypic evaluation in propositus and relatives but is above all crucial for determine the mode of transmission within a family which is essential for genetic counseling of both the referred patient and his relatives (Maione *et al.*, 2018). Indeed, IHH/CHH/KS is responsible for a treatable form of infertility in both males and females and is then a genetic condition that can be transmitted to patients' offspring (Maione *et al.*, 2018).

Therapy

The aim of therapy in female IHH teenagers is to induce feminization and normal sexual function, to stimulate statural growth, to promote bone health and to address concerns about future fertility (Boehm *et al.*, 2015). In CHH/IHH/KS girls puberty can be induced by oral or transdermal estradiol administration associated with progestin. In adulthood, estradiol is typically given orally or transdermally (by patch or pumps gel daily) as a maintenance dose associated with a cyclic progestin regimen to avoid endometrial hyperplasia. In the majority of women with CHH, estroprogestin therapy is effective in inducing development of the breasts and genitals, which contributes to an increased sense of femininity and a satisfactory emotional and sexual life. Estrogen therapy also increases uterine size and combined estrogen/progestin therapy induces monthly menses.

Infertility in CHH/IHH women is related to insufficient follicular maturation, which leads to chronic anovulation. Ovulation induction can be achieved either with pulsatile GnRH therapy alone or, alternatively, with FSH and LH administration followed by hCG or LH to trigger ovulation and maintain the functioning of corpus luteum before the relay is taken by the embryonic/placental hCG (Kaufmann *et al.*, 2007). Infertility therapy must be preceded by genetic counseling (Maione *et al.*, 2018). The latter requires the search for abnormalities in many genes involved in this group of diseases (**Table 1**) and will be significantly facilitated by the Next Generation Sequencing (Maione *et al.*, 2018; Cassatella *et al.*, 2018).

Conclusion

Diagnosis of IHH should be considered in any teenager or young woman referred for primary amenorrhea, whatever the degree of breast and pubic hair development. The diagnosis is strengthened in the presence of concomitant low gonadotropin and estradiol levels. After excluding pituitary organic disease, multiple pituitary deficits and functional hypothalamic amenorrhea the main diagnostic challenge is to differentiate in teenagers chronic IHH from transient hypogonadism related to CDGP. The search for a genetic IHH/CHH/KS causes is currently facilitated by the simultaneous analysis of many responsible genes by next generation sequencing allowing a more reliable genetic counseling. Hormone replacement therapy is effective to both enable feminization and to allow normal sexual life as well as to successfully treat these women infertility.

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Functional Hypothalamic Amenorrhea

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Glossary

Allostasis Adaptive processes that produce a new stable physiological state through the production of mediators such cortisol and other chemical messengers. These mediators of the stress response promote adaptation in the face of acute stress, but when chronic contribute to allostatic overload and wear and tear on the body and brain.

Cognitive behavior therapy (CBT) A form of psychotherapy based on the notion that the way that individuals perceive a situation is more closely connected to their reaction than the situation itself. The aim of CBT is to help the individual modify dysfunctional emotions, behaviors, and thoughts. Unlike traditional psychoanalysis, which probes childhood wounds to get at the root causes of conflict, CBT focuses on solutions, encouraging patients to challenge distorted cognitions and change unhelpful thinking and behavior to catalyze enduring improvement in mood and functioning.

Introduction

Functional hypothalamic amenorrhea (FHA) is a form of hypothalamic hypogonadism that presents clinically as amenorrhea due to chronic anovulation. It is a diagnosis of exclusion: FHA must be distinguished from all other causes of amenorrhea and anovulation. Functional hypothalamic anovulation manifests clinically as amenorrhea. The proximate cause of chronic anovulation is insufficient hypothalamic GnRH drive to support folliculogenesis. Specifically, GnRH pulse frequency is too slow to drive sufficient release of pituitary LH and FSH secretion that is required to initiate and then sustain folliculogenesis. Chronically low levels of estradiol and progesterone lead to lack of endometrial development and thus absence of menses. The term functional implies that the condition is reversible and that the cause of the reduced GnRH drive is related to potentially modifiable factors that are viewed as stressors because they activate the hypothalamic–pituitary–adrenal axis and lead in increased cortisol secretion. We demonstrated that FHA represents an adaptation to chronic stress that results not only in reduced GnRH drive but also a constellation of concomitant neuroendocrine adjustments including hypothalamic hypercortisolism and hypothalamic hypothyroidism (Berga *et al.*, 1989). The constellation of endocrine adaptations associated with FHA represents an allostatic state that diverts and directs metabolic and psychological energy to acute and chronic challenges. Given the energetic expense of reproduction, it is no surprise that metabolic factors play a fundamental role in gating reproductive function.

Evidence that stress is the cause of FHA includes the demonstration that cortisol levels are higher in women with FHA than in those who are eumenorrheic and ovulatory (Berga *et al.*, 1989) and also higher than in women with other forms of anovulation (Berga *et al.*, 1997). Additionally, recovery from FHA is associated with a reduction in circulating cortisol levels (Berga *et al.*, 1997; Michopoulos *et al.*, 2013b). In practice, it may be difficult to detect hypercortisolemia because the elevation of cortisol is most evident at night (Berga *et al.*, 1989; Michopoulos *et al.*, 2013b) and the cortisol level from a single day time blood sample in unlikely to be outside the normal range. FHA may result from chronic or severe illness such as cancer or pneumonia. Indeed, the term functional means that the hypothalamic GnRH-pituitary gonadotropin apparatus is anatomically and physiologically intact and therefore capable of generating LH pulses and causing pituitary secretion of FSH. In clinical practice, it is often difficult to exclude insufficient GnRH drive due to mutations in genes critical to GnRH ontogeny and function and it has been hypothesized that women with genetic mutations may be more sensitive to stressors (Caronia *et al.*, 2011).

Differential Diagnosis

The causes differ for primary and secondary presentations of amenorrhea. The differential diagnosis includes: (1) Mullerian anomalies, congenital and acquired, that block the outflow tract, including vaginal agenesis, imperforate hymen, and Asherman syndrome; (2) ovarian causes of anovulation such as gonadal agenesis, Turner syndrome (45, XO), polycystic ovary syndrome, and premature ovarian insufficiency (premature menopause); (3) other endocrine conditions such as primary hyper- and hypothyroidism, Cushing syndrome and disease; (4) pituitary causes such as pituitary adenomas and hyperprolactinemia; and (5) organic central causes such as meningioma and Kallmann syndrome and variants. The causes to be investigated can be categorized as vaginal, cervical, uterine, ovarian, adrenal, thyroidal, pituitary, hypothalamic, and central. **Table 1** presents the endocrine concomitants of common causes of amenorrhea due to anovulation (Gordon *et al.*, 2017).

Defining reproductive tract anatomy is the first step in excluding anatomic causes of amenorrhea and doing so is especially important in primary amenorrhea. Outflow tract anomalies often present as primary amenorrhea and require a physical exam and

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Table 1 Differential diagnosis of anovulation

	LH (IU/L)	*FSH (IU/L)*	*LH: FSH*	*E2 (pg/mL)*	*P4 (ng/mL)*	*AMH (ng/mL)*	*Prolactin*	*TSH*	*T4*	*A'dione*	*DHEAS*	*17OHP*	*Testosterone*
Functional hypothalamic anovulation	<10	<10	~1	<50	<1	>1	Low nl	Low nl	Low nl	Low nl	nl	nl	Low nl
Ovarian insufficiency; menopause	>15	>15	FSH > LH	<50	<1	<0.5	nl	nl or ↑	nl or ↓	Low nl	nl	nl	Low nl
Polycystic ovary syndrome	<15	<10	LH > FSH	<50	<1	nl or ↑	High nl	nl	nl	High nl or ↑	High nl	nl	High nl or slight↑
Attenuated CAH	<15	<10	LH > FSH	<50	≤1	nl	nl	nl	nl	Low nl or nl	High nl	↑	↑
Hyperprolactinemia	<10	<10	LH < FSH	<50	<1	nl	↑	nl or ↑	nl	nl	nl	nl	nl
Hypothyroidism	<10	<10	LH < FSH	<50	<1	nl or ↓	High nl or ↑	↑	↓	Low nl or nl	Low nl or nl	nl	nl

often imaging with ultrasound or MRI to exclude and/or define anatomic anomalies. Asherman syndrome may present as secondary amenorrhea and is due to injury of the endometrial basal layer that is necessary for the generation of the endometrial functional layer. Findings may include intrauterine scarring, synechiae, and/or adhesions. A history of D&C for miscarriage, retained products of conception, or postpartum bleeding and/or pelvic infection should raise the index of suspicion for endometrial injury and hysteroscopy is typically needed to establish the diagnosis of Asherman syndrome. Polymenorrhea may be due to intrauterine polyps or intramural fibroids rather than functional hypothalamic hypogonadism. A karyotype is needed to evaluate the possibility of gonadal dysgenesis and Turner syndrome, although typically there are physical stigmata. Amenorrhea in the setting of a 46,XY karyotype may reflect Müllerian agenesis and either (1) 5-alpha reductase deficiency causing insufficient production of dihydrotestosterone (DHT) from testosterone or (2) androgen insensitivity syndromes (AIS) due to variable androgen receptor sensitivity. In both of these conditions, the uterus regressed during development and the gonad is a testis, but its location may be inguinal or pelvic. If the karyotype is 46,XX, a testosterone level will be in the male range in 5-alpha reductase deficiency and AIS and very low in gonadal dysgenesis (Swyer syndrome).

In the setting of secondary amenorrhea, history is important because physical examination often shows normal reproductive anatomy and external genitalia. Delineating the timing of pubertal milestones such as adrenarche and thelarche provides clues as does a history of weight gain and loss, dietary habits, and athletic endeavors. History must be supplemented with testing hormone levels as outlined in **Table 1** to establish the diagnosis. A panel that includes LH, FSH, estradiol (E2), progesterone, TSH, thyroxine, prolactin, and androstenedione detects most important causes if properly interpreted. The pattern of hormone levels is more critical than absolute values. In FHA, FSH will be in the normal range and typically it will be slightly higher than LH, which will also be in the low normal range, with E2 <50 pg/mL and progesterone <1 ng/mL. Very low LH and FSH levels suggest organic HA due to genetic mutations affecting GnRH ontogeny and function or central causes such as brain or pituitary tumors. Anosmia indicates Kallmann syndrome, which is failure of GnRH neurons to migrate from the olfactory placode to the hypothalamus. Androstenedione (or testosterone) is typically in the lower range of normal except when FHA is superimposed on polycystic ovary syndrome, both TSH and thyroxine will be in the lower range of normal indicating hypothalamic hypothyroidism or sick euthyroid syndrome, and prolactin will be in the low normal range. It is critical to exclude chronic health conditions that may be a cause of undernutrition such as food allergies including gluten enteropathy (celiac disease).

In contrast, elevated LH and FSH with low E2 and progesterone indicate low or absent ovarian reserve or premature menopause. High LH and FSH with E2 $>$ 150 pg/mL and progesterone <2 ng/mL indicates a midcycle gonadotropin surge. If TSH is low and thyroxine is high, then one must consider the possibility of autoimmune hyperthyroidism (Grave's disease). Similarly, if TSH is in upper limit of normal with thyroxine is in the lower range of normal, then autoimmune thyroiditis and hypothyroidism must be considered and the next step would be to measure antithyroid antibodies such as thyroid peroxidase and thyroid stimulating immunoglobulin. If frank hyperprolactinemia is found, additional evaluation is needed. Prolactin levels are elevated by food, sleep, exercise, coitus, nipple stimulation, physical examination, lactation, and many medications. Acromegaly may present with oligo- or amenorrhea and an elevated somatomedin-C (IGF-1). Diabetes may present as oligo- or amenorrhea due to reduced GnRH drive. Amenorrhea and anovulation associated with Cushing syndrome and disease is also due to reduced GnRH drive.

While the cause of FHA is stress, the increase in cortisol secretion in FHA is less than that seen with Cushing's syndrome and disease and the circadian pattern is preserved, so the cortisol increase is highest overnight and in the early morning hours (Berga *et al.*, 1989; Michopoulos *et al.*, 2013b). If Cushing's is suspected, a 24-h urinary free cortisol (UFC) is a reasonable screening test. Rarely secondary adrenal insufficiency presents as fatigue and anovulation. Serum dehydroandrosterone sulfate (DHEAS) will be in the lower range for age. The differential diagnosis of low normal DHEAS includes Sheehan's syndrome with partial or complete pituitary apoplexy. Provocative stimulation testing helps to establish pituitary hypofunction due to injury, tumor, autoimmune hypophysitis, or other CNS conditions. History is essential for elaborating the differential diagnosis and guiding the evaluation.

MRI of the pituitary and the brain should be considered to exclude or confirm serious CNS conditions (Lowry *et al.*, 1996). A history of trauma should raise the index of suspicion to include pituitary stalk damage. The differential diagnosis of central lesions and conditions is extensive. A high index of suspicion and a low threshold for obtaining a MRI or other relevant neuroimaging is recommended.

Certain medications and drugs of abuse suppress GnRH drive or cause other endocrine perturbations. Chronic drug use is often a marker of stress and undernutrition. An evaluation for syndromal psychiatric conditions such as eating disorders, depression, and personality disorders is critical. Formal psychiatric evaluation may be indicated. Psychiatric conditions are associated with activation of the hypothalamic–pituitary–adrenal axis and appropriate treatment may reverse the functional suppression of GnRH drive. Paradoxically, stress often drives individuals to take drugs and medications that alone may suppress GnRH drive. Opioid use is both mechanistically and clinically linked to suppression of GnRH and the development of hypothalamic hypogonadism in both men and women (Daniell, 2008). Psychotropics induce hyperprolactinemia which secondarily suppresses GnRH drive (Ajmal *et al.*, 2014). Marijuana and alcohol have been linked to inhibition of GnRH drive (Rettori *et al.*, 2010). Exercise and dietary restrictions and excesses also gate GnRH drive. In short, medications, drugs, lifestyle adjustments initiated in response to psychological stress may interact synergistically to suppress ovarian function. If the root cause for substance use and abuse is stress, then clearly the management must involve stress as well as addiction management. The suppression of GnRH by a variety of stresses can be blocked by CRH antagonists, suggesting a pivotal role for endogenous CRH (Li *et al.*, 2010; Michopoulos *et al.*, 2013a).

Pathophysiology

Functional hypothalamic amenorrhea is more than an isolated disruption of GnRH drive. Functional suppression of GnRH is universally accompanied by increased cortisol secretion due to increased hypothalamic–pituitary CRH-ACTH drive and reduced thyroxine (T4) and thyroxine (T3) due to decreased hypothalamic–pituitary TRH-TSH input. The constellation of neuroendocrine alterations characteristic of FHA reflects altered feedback sensitivity to estradiol, cortisol, and thyroxine. Other feedback sensitivities may be altered, including leptin and ghrelin signaling. Increased circulating and CSF cortisol levels are specific to FHA (Berga *et al.*, 1997; Brundu *et al.*, 2006). Many of the clinical consequences of FHA such as osteoporosis reflect the clinical impact of the full constellation of neuroendocrine aberrations that accompany FHA (Berga *et al.*, 1989; Brundu *et al.*, 2006).

Reduced GnRH input decreases circulating LH and FSH to levels too low to fully support folliculogenesis to the point of ovulatory adequacy. Amenorrhea is the most clinically recognizable manifestation of reduced GnRH and occurs when there is sustained suppression of GnRH pulsatility to $<50\%$ of expected (Berga *et al.*, 1989). Because the suppression of GnRH exists on a spectrum, so too does the clinical presentation. More clinically occult forms result from intermittent suppression of GnRH that causes partial folliculogenesis that manifests as anovulatory cycling or luteal insufficiency. In luteal insufficiency, there is embryo-endometrial asynchrony that impairs or prevents implantation. The patient may present with infertility with regular menstrual cycle intervals rather than with more clinically recognizable oligo- or amenorrhea. To complicate recognition, FHA and PCOS may coexist. The following clinical presentations may be due to functional insufficiency of GnRH drive: complete anovulation and amenorrhea, hypermenorrhea with short menstrual intervals, oligomenorrhea with long cycle intervals, menometrorrhagia, and eumenorrheic infertility. Men can also develop functional hypothalamic hypogonadism due to insufficient GnRH drive that results in oligoasthenospermia with or without reduced testosterone levels (Berga, 1997).

Risk factors for the development and persistence of FHA include any factors that chronically activate the HPA axis and commonly include greater energy expenditure than intake, excessive exercise, nutritional restriction of protein and fats, unrealistic expectations of self and others, attitudes that increase reactivity to common and uncommon stressors including perfectionism, high need for social approval, and conditional love (Berga and Girton, 1989; Giles and Berga, 1993; Marcus *et al.*, 2001). We found that the endocrine impact of stressors is synergistic rather than additive (Williams *et al.*, 2007; Berga, 2008; Sanders *et al.*, 2017). We also demonstrated that addressing problematic attitudes and behaviors using cognitive behavior therapy (CBT) not only restored ovarian function (Berga *et al.*, 2003) but also reduced cortisol levels (Berga *et al.*, 1997; Berga and Loucks, 2006; Michopoulos *et al.*, 2013b). Recovery from FHA included amelioration of hypothalamic hypothyroidism and an increase in leptin levels independent of weight gain (Michopoulos *et al.*, 2013b). These data underscore the tight link between metabolic and reproductive function (**Fig. 1**).

Acute and Chronic Health Consequences

Many of the health consequences linked to FHA are likely due to the combined alterations in metabolism, neuroendocrine function, and anovulation (Michopoulos *et al.*, 2013b). Health conditions that accrue from chronic FHA putatively include osteoporosis, syndromal psychiatric conditions, and infertility. Longer-term health consequences may include an increased risk of cardiovascular diseases (Bairey Merz *et al.*, 2003) and neurodegenerative diseases due to persistent stress and hypercortisolism (Brundu *et al.*, 2006). Treatment of osteoporosis with bisphosphonates in women intending to become pregnant is discouraged because the bisphosphonates incorporate into maternal bone and can then be mobilized during pregnancy and incorporated into

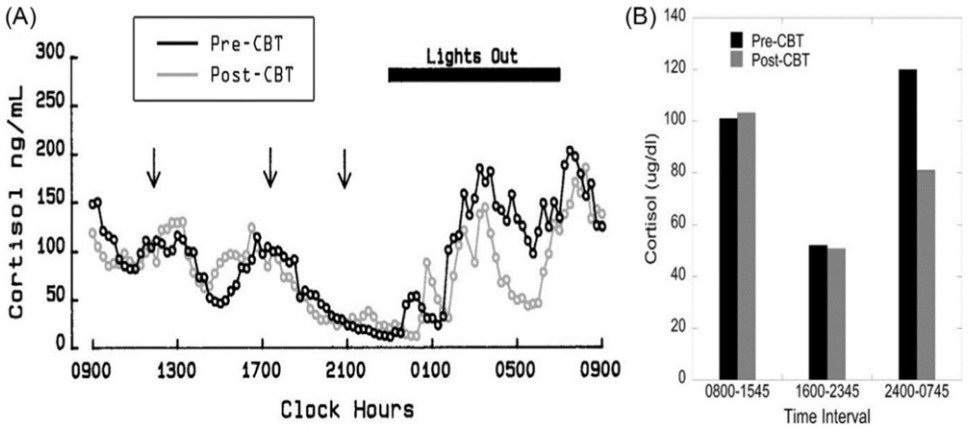

Fig. 1 (A) Circulatory cortisol concentrations (ng/mL) in 15 min intervals over 24 h in a woman with FHA before (pre) and after (post) CBT (cognitive behavior therapy). Meals are indicated by arrows. (B) Circulatory concentrations of cortisol displayed as 8-h mean in the same woman before (pre) and after (post) CBT (Michopoulos *et al.*, 2013b).

fetal bone. Hormone replacement regimens also have limitations and may not permit full bone accretion because glucocorticoids abrogate the impact of estrogens by competing for transcriptional coactivators and interfering with estrogen action (Whirledge *et al.*, 2013; Gordon *et al.*, 2017). There is no reason to assume that chronic stress is benign or that hormone replacement regimens can fully counteract the clinical impact of chronic stress or fully reverse the constellation of neuroendocrine aberrations that accompany FHA.

Management

As the term functional implies, functional hypothalamic amenorrhea improves or even remits altogether when the allostatic load is reduced. Thus, when all other causes of FHA have been excluded, stress management is indicated. The goal of treatment is to identify and reduce the neuroendocrine impact of stressors. Amelioration of FHA requires that stressors are identifiable and manageable or that they spontaneously cease. Appropriate management of stress has been shown to ameliorate hypothalamic hypercortisolism and reverse concomitant neuroendocrine adaptations (Berga *et al.*, 2003; Berga and Loucks, 2006; Berga, 2008; Michopoulos *et al.*, 2013b). Since stress is an inevitable consequence of living, psychoeducation about how to manage typical and specific stressors is more practical than trying to avoid stressors. Our research indicates that stressors are multiple and seemingly personal or minor yet interact synergistically to elicit FHA. For practical purposes, stressors are often categorized as metabolic in which energy expenditure exceeds energy intake and psychogenic or behavioral which alter energy intake or expenditure because of cognitions and attitudes but the neuroendocrine cascade that follows is similar regardless of the behavioral antecedents (Berga, 2008). Psychogenic stressors reflect cognitive expectations and often present as unrealistic attitudes that compromise coping with daily demands that are to some extent externally imposed. Typically, individuals with FHA report a combination of both metabolic and psychogenic variables (Berga and Girton, 1989; Giles and Berga, 1993; Marcus *et al.*, 2001) that we have shown interact synergistically (Williams *et al.*, 2007; Berga, 2008) to induce chronic hypothalamic hypogonadism.

Women with FHA often display greater neurobiological reactivity to stressors than individuals who are ovulatory and presumably are more stress-resilient. Metabolic stressors such as exercise and nutritional restriction are often undertaken to reduce psychological stress and yet have to potential to serve independently as metabolic or even psychogenic stressors. We found that exercise elicited a greater rise in cortisol in FHA than ovulatory women that was attributed to a drop in circulating glucose during exercise in FHA that was not observed in eumenorrheic, ovulatory women who instead showed a rise in circulating glucose during exercise (Sanders *et al.*, 2017). We acknowledge that what is stressful to one individual may be less so to another due to personal valences and underlying neurobiological reactivity. Identifying stressful attitudes and behaviors and teaching better coping styles has been shown to reduce the neuroendocrine impact of stressors (Berga *et al.*, 2003; Berga and Loucks, 2006) and results not only in a decrease in cortisol, particularly during sleep, but also an increase in TSH and leptin independent of weight gain (Michopoulos *et al.*, 2013b).

Stress management can be undertaken before or concurrently with other interventions, including infertility therapy. In our pilot study, 75% of women with FHA randomized to cognitive behavior therapy (CBT) designed to address problematic attitudes and behaviors regained ovulatory ovarian function whereas only 25% of those randomized to observation did (Berga *et al.*, 2003; Michopoulos *et al.*, 2013b). Clomiphene citrate is less effective in eliciting an increase in FSH release if the cause of anovulation is hypothalamic hypogonadism because of reduced feedback sensitivity to hypoestrogenism. The efficacy of aromatase inhibitors has not been studied in women with FHA. While antidepressants reduce hypercortisolemia in those with depression and PTSD, their use for treatment of FHA has not been studied. It is important to screen for syndromal psychiatric disorders, particularly eating disorders, and refer individuals so identified to appropriate psychiatric care.

The Endocrine Society recently published a clinical practice guideline on FHA that comprehensively addressed therapeutic options (Gordon *et al.*, 2017). The authors concluded that oral contraceptive pills should not be used for the sole purpose of improving bone mineral density and patients who do use oral contraceptive pills should be cautioned that their use may not prevent ongoing bone loss. A meta-analysis included in the clinical practice guideline reported that there were no reliable data examining the relationship between hormone use and bone fractures and very limited data on their impact on bone density in women with FHA. The authors concluded it is unlikely that hormone use will improve bone density or foster bone accretion in women with FHA because ongoing hypercortisolism, hypothyroidism, and undernutrition places women with FHA in a catabolic rather than anabolic state. As noted earlier, bisphosphonates should not be used in women intending to become pregnant as they may become incorporated into the fetal skeleton. Further, there are good data demonstrating that cortisol directly interferes with estrogen action (Whirledge *et al.*, 2013; Whirledge and Cidlowski, 2017). Since there are no data to suggest that instituting hormone therapy for other indications is harmful and women with FHA may spontaneously recover, those wishing to avoid pregnancy should use some form of birth control. However, hormonal contraception may not confer the same benefits such as cardioprotection as touted for eumetabolic women and hormone use should not be expected to reverse the metabolic impact of stress. The situation is similar for vitamin and nutrient supplementation. Regardless of menstrual status, women need sufficient dietary calcium intake and vitamin D levels. However, neither exogenous vitamin D nor increased calcium intake will overcome the hypoestrogenism and catabolism induced by chronic stress.

Women with FHA often seek treatment for infertility. However, clomiphene and letrozole may prove ineffective as the hypothalamic GnRH drive is suppressed by mechanisms other than estradiol feedback inhibition and antagonizing estradiol action does not lead to increased GnRH drive. In our recent Endocrine Society clinical practice guideline on FHA (Gordon *et al.*,

2017), we recommended exogenous pulsatile GnRH be offered for ovulation induction because the approach is more physiological and more likely to result in monofollicular development and less likely to result in multiple gestation than is the use of injectable gonadotropins (Martin *et al.*, 1993). However, many patients with FHA undergo gonadotropin therapy and assisted reproductive therapies (ART) to treat the infertility associated with FHA. The pros and cons of these approaches are open to debate. The risks are many, including multiple gestation and high expense. At the very least, women with low body mass index should not be treated for infertility. In our recent Endocrine Society clinical practice guideline on FHA (Gordon *et al.*, 2017), we recommended a minimum BMI of 18.5 kg/m^2, however, we also noted that women with a BMI < 20 kg/m^2 have a fourfold higher risk of preterm labor (ESHRE Capri Workshop Group, 2006) and the infants have lower birth weights (Koubaa *et al.*, 2005). Therefore, we recommended that clinicians limit ovulation induction to women of satisfactory body weight. Independent of weight, potential maternal and fetal consequences of ongoing maternal stress and undernutrition include preterm labor (Moutquin, 2003), intrauterine growth restriction (Koubaa *et al.*, 2005), and fetal neurodevelopmental disorders such as learning disabilities and autism spectrum disorders. ART is likely to increase, rather than decrease, metabolic and psychogenic stress. Indeed, controlled ovarian hyperstimulation with gonadotropins has been demonstrated to increase TSH levels and presumably metabolic demand due to associated with multiple folliculogenesis associated with ovarian hyperstimulation (Muller *et al.*, 2000). Women conceiving by ART had a greater increase in TSH earlier in gestation, indicating a greater need for thyroxine early in gestation (Alexander *et al.*, 2004). Thus, ART may amplify the neuroendocrine consequences of preexisting stress, exposing oocytes, the mother, and the fetus to persistent neuroendocrine aberrations including lower thyroxine and higher cortisol levels. Since the mother is the sole source of thyroxine in the first trimester and since thyroxine is critical for fetal neuronal migration and differentiation (Lavado-Autric *et al.*, 2003; Ausó *et al.*, 2004), fetal neurodevelopment may be compromised (Glinoer, 1997). Further, even intermittent hypercortisolemia may accelerate placental aging as indicated by telomere shortening (Menon *et al.*, 2012) and induce epigenetic changes in fetal DNA. Ultimately, the consequences of these exposures may be to potentiate the fetal origins of adult disease.

Conclusions

Functional hypothalamic amenorrhea (FHA) is a reversible form of anovulation that is not due to organic causes. While the proximate cause is insufficient GnRH drive, FHA is more than an isolated reduction in GnRH. Behavioral and psychological factors such as undernutrition, excessive exercise, and unrealistic cognitions serve as stressors that elicit hypothalamic hypercortisolism and a cascade of neuroendocrine adaptations including hypothalamic hypothyroidism. The neuroendocrine constellation conserves and diverts energy to perceived challenges and thereby promotes short-term survival at the expense of longer-term health. Behavioral interventions such as cognitive behavior therapy that address problematic attitudes and behaviors reduce hypothalamic hypercortisolism and restore ovulatory eumenorrhea. Hormonal therapies offer less promise but contraception should be offered for those wishing not to conceive as spontaneous recovery of ovulation precedes menses. Infertility treatment should be undertaken with caution. The role of psychotropic medications has not been appropriately studied. Because of the many health consequences of chronic stress and hypoestrogenism, women with FHA should be closely followed by an appropriate team of physicians and other health care providers. FHA is a prototypic example of psychoneuroendocrinology and illustrates how cognitions and emotions drive behaviors that eventuate in neuroendocrine allostasis.

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Polycystic Ovary Syndrome☆

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Glossary

Polycystic ovaries The characteristic morphological features of polycystic ovaries are multiple antral follicles and increased stroma. Typically, polycystic ovaries hypersecrete androgens.

Polycystic ovary syndrome Clinical and endocrine abnormalities—typically manifestations of anovulation and hyperandrogenism—associated with polycystic ovaries.

Introduction

Polycystic ovary syndrome (PCOS) is the commonest endocrine disorder in women, accounting for the majority cases of anovulatory infertility and of hirsutism (Jayasena and Franks, 2014). Polycystic ovary syndrome is heterogeneous in its presentation and this has resulted in some discussion about how to define PCOS. The classic definition includes the association of anovulatory menses (or estrogen-replete amenorrhoea) with clinical and/or biochemical evidence of excess androgen secretion. Using this definition, the estimated prevalence of PCOS is in excess of 5% of the female population of reproductive age. The range of clinical presentation of women with polycystic ovaries—as defined by pelvic ultrasonography—is, however, wide. It includes patients with anovulation who are non-hirsute and those with hirsutism who have regular menstrual cycles. Indeed, polycystic ovaries are found in over 80% of women who would otherwise have been labeled as having "idiopathic hirsutism". Acknowledgement of the broad spectrum of presenting features in women with PCOS led to reappraisal of the accepted diagnostic criteria at a consensus conference in Rotterdam (2003) featuring experts representing the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) (Rotterdam, 2004). The resulting "Rotterdam Criteria" for diagnosis of PCOS require the presence two of three features: (1) oligo- or anovulation, (2) clinical and/or biochemical hyperandrogenism, and (3) polycystic ovarian morphology. The results of ultrasound studies of "normal" populations suggest that polycystic ovaries are present in about 20% of women of reproductive age. The cause(s) of polycystic ovaries (PCO) and PCOS are not known for certain but there is strong evidence for a major genetic contribution as will be discussed in section "Genetic Studies of PCOS."

In addition to the reproductive consequences of the syndrome, PCOS is characterized by a metabolic disorder (amplified by obesity) in which hyperinsulinemia and peripheral insulin resistance are the central features (Jayasena and Franks, 2014). This metabolic dysfunction may play a part in the etiology of anovulation but also has important implications for long-term health. Women with PCOS are two to four times more likely to develop type 2 diabetes mellitus (T2D) in later life and carry risk factors for development of cardiovascular disease (although there is currently no firm evidence of an increase in cardiovascular events).

Endocrine Abnormalities in PCOS

The major endocrine abnormalities in women with PCOS are elevated serum concentrations of androgens and luteinizing hormone (LH) and, particularly in those with the classic definition of PCOS (i.e., including menstrual disturbances). Women with PCOS are relatively more insulin resistant (with compensatory hyperinsulinaemia) than normal subjects, a distinction that is amplified by obesity.

Hypersecretion of Androgens

The most common biochemical abnormality in women with polycystic ovaries is hypersecretion of androgens. The ovary appears to be the predominant source of excess androgen production although many studies have pointed to evidence for an additional adrenal abnormality. Nevertheless, the ovary is clearly the more important contributor to hyperandrogenemia because suppression of LH in women with PCOS leads to a fall of androgen concentrations to levels which are indistinguishable from those in menopausal or oophorectomized women. Cultured thecal cells from women with polycystic ovaries, regardless of presenting symptoms, produce some 20 times more androstenedione in primary culture than do cells from women with normal ovaries. Increased steroidogenic activity is, however, not confined to androgen production. All stages of the steroidogenic pathway—including progesterone production—appear to be amplified in PCO theca. These results have been confirmed in subsequent studies, using theca cells from PCO and normal theca which had undergone several passages in culture and retained the phenotype.

☆*Change History:* February 2018. Author Franks updated all sections and new sections were added.

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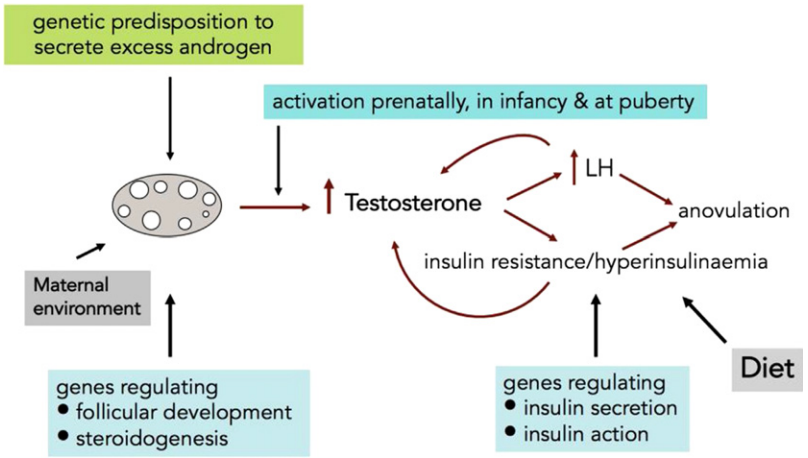

Fig. 1 Proposed developmental etiology of PCOS. It is suggested that the ovary is genetically predisposed to hypersecrete androgens, perhaps as early as intrauterine life but certainly during the activation of the hypothalamic-pituitary-ovarian axis that occurs transiently in infancy and in a sustained manner at puberty. Higher than normal circulating levels of testosterone "program" the hypothalamic-pituitary unit to produce high tonic levels of LH, and also amplify the physiological insulin resistance of puberty. Higher than normal concentrations of LH and insulin further enhance ovarian androgen production and may contribute to the mechanism of anovulation. The maternal environment, particularly metabolic dysfunction associated with obesity, may also play a part. Adapted from Franks, S. (2008). Polycystic ovary syndrome in adolescents. *International Journal of Obesity(Lond)* **32**, 1035–1041.

Androgen Programming and the Etiology of PCOS

We have proposed that PCOS has its origins in early life and that the polycystic ovary is genetically predisposed to hypersecrete androgens, certainly at puberty but possibly also in the early postnatal period or even during fetal development (Fig. 1). It is unlikely that maternal androgen excess affects fetal exposure to androgen because high circulating concentrations of sex hormone-binding globulin (SHBG) and placental aromatase activity provide an effective barrier between mother and fetus. Using evidence from studies in the prenatally androgenized (PA) rhesus monkey or sheep, we put forward the hypothesis that increased exposure to androgens during development leads to the abnormalities of endocrine and metabolic function that are characteristic of PCOS. The PA rhesus monkey, during adulthood displays abnormal ovarian morphology, ovarian hyperandrogenism (and adrenal androgen excess), hypersecretion of LH, insulin resistance and anovulation in relation to increased body weight. Studies to date in the PA sheep have yielded similar findings with respect to reproductive and metabolic sequelae. We suggest that, in human PCOS, the source of excess androgen is the ovary which may be activated during fetal life, during the "mini-puberty" of infancy or during puberty itself. The genes that may be involved in excess ovarian androgen production remain to be determined. Genes that affect insulin secretion and action have been well described but their role in glucose/insulin homeostasis in PCOS remains speculative (Fig. 1).

Metabolic Abnormalities in PCOS

In recent years, there has been a great deal of interest in the metabolic associations of PCOS (Jayasena and Franks, 2014). The classic syndrome is characterized by a distinctive form of insulin resistance. Women with PCOS have higher fasting and glucose-stimulated insulin concentrations and significantly reduced insulin sensitivity compared with weight-matched control subjects. The cause of this abnormality is unclear but clinical and laboratory-based studies in PCOS have variously pointed to abnormalities of insulin receptor binding, or more plausibly, post-receptor signaling as well as to evidence for a primary abnormality of insulin secretion. It has been demonstrated that weight reduction in obese women with PCOS results in normalization of insulin sensitivity but "first-phase" insulin secretion in response to an intravenous glucose challenge remains abnormal. These data also illustrate an important principle in understanding the etiology of PCOS which is that whatever the genetic basis for the syndrome, the phenotype can be influenced by environmental (in this case nutritional) factors. Women with PCOS are at increased risk of long health problems related to insulin resistance, including gestational diabetes (GDM), impaired glucose tolerance and type 2 diabetes (T2D) (see "Management" section below). Obesity greatly amplifies the metabolic abnormalities in PCOS and further increases the risk not only of diabetes but also of non-alcoholic fatty liver disease (NAFLD) and of sleep apnea.

Ovarian Follicular Abnormalities in PCOS

A further phenotypic feature of PCOS is the polycystic ovarian morphology itself. The polycystic ovary is characterized by the presence of an increased number not only of antral follicles but also of early growing and larger preantral follicles. Since these earlier stages of follicular development are thought to be largely independent of gonadotrophins, the implication is that local ovarian factors may have a role in genesis of the polycystic ovary (Franks *et al.*, 2008). Many growth factors have been shown to have an influence on early follicular development, including those of the transforming growth factor beta (TGFβ) superfamily and growth factors signaling through tyrosine kinase coupled receptors such as the type 1insulin-like growth receptor (IGFR-1). Indeed, results of recent studies of mouse preantral follicles in culture indicate that androgens have a direct stimulatory effect on growth and that this effect is due, at least in part, to interaction with TGFβ growth factors (Laird *et al.*, 2017).

Anovulation in PCOS is characterized by arrested development of medium sized antral follicles. Granulosa cells from these follicles display evidence of premature responsiveness to LH and increased steroidogenesis (indicative of advanced differentiation) compared with similar-sized follicles from normal ovaries. The reasons for these differences are not yet entirely clear but granulosa cells of small antral follicles from polycystic ovaries exhibit premature responsiveness to LH and this may be related to the characteristic hyperinsulinemia of PCOS, since insulin can greatly enhance the response of the granulosa cell to LH (Franks *et al.*, 2008).

Genetic Studies of PCOS

The important contribution of genetic factors in the genesis of androgen excess and etiology of PCOS is clear, with evidence of hereditability of both endocrine (including serum testosterone levels) and metabolic indices. Despite obvious candidate pathways, case-control association studies have yielded few, if any, robust candidates for susceptibility loci, and that has led to the recent interest in genome-wide association studies (GWAS). The first large scale GWAS was performed in Han Chinese women and intriguingly, significant signals have emerged at loci close to the genes on chromosome 2 coding for the gonadotropin receptors LHCGR and FSHR (Chen *et al.*, 2011). Subsequent GWAS studies in women from East Asia and in those of European origin have shown loci that are in common with the Chinese study, as well as novel loci, notably that close to the gene coding for the β subunit of FSH (Hayes *et al.*, 2015; Day *et al.*, 2015).

Clinical Management Issues

Diagnosis of PCOS

The diagnosis of PCOS is reached primarily on a clinical basis. A patient presenting with irregular menses, oligomenorrhoea or amenorrhoea and who has signs of hyperandrogenism is very likely to have PCOS. Even in the absence of hirsutism, PCOS is the most likely cause of these menstrual symptoms (it accounts for about 30% of cases of amenorrhoea overall and about 90% of amenorrhoeic women with normal estrogen levels).

The majority of patients presenting with hirsutism have polycystic ovaries, irrespective of menstrual history. Much rarer but more serious causes of hirsutism and menstrual disturbances include Cushing's disease, acromegaly, hyperprolactinemia, and tumors of the adrenal or ovary. In such cases, however, there are usually other clues, both clinical and biochemical, to the diagnosis for example, short history of increasing hirsutism and significantly raised serum testosterone (>5 nmol/L). For this reason, serum testosterone should be measured in all hirsute patients as a screening test to exclude more serious causes of hyperandrogenism. Late-onset ("non-classical") congenital adrenal hyperplasia due to 21-hydroxylase deficiency may be difficult to distinguish clinically from PCOS but it is debatable whether this makes much practical difference to management of symptoms. In our clinic, where the prevalence of non-classical 21-hydroxylase deficiency is $<5\%$, measurement of 17-alpha-hydroxyprogesterone (the biochemical marker of 21-hydroxlyase deficiency) is not performed routinely. However, its measurement is recommended in most currently available clinical guidelines.

No single test is diagnostic of the syndrome and choice of investigations should be tailored to the clinical presentation. Serum LH levels are typically elevated in PCOS (FSH is normal) but up to 50% of women with all other clinical and biochemical features of the syndrome may have normal serum LH. Measurement of LH is therefore of limited diagnostic value; it is quite specific—raised LH and normal FSH essentially occur only in PCOS—but it is not very sensitive. Pelvic ultrasonography will define the polycystic ovarian morphology but accurate assessment of the ovaries by ultrasound is a particular skill so that false negative results are not uncommon. Conversely, the presence of polycystic ovaries does not necessarily mean that the patient has polycystic ovary syndrome. Polycystic ovaries may be found coincidentally in women who have, for example, hypothalamic, estrogen-deficient amenorrhea. Anti-Müllerian hormone (AMH) is produced by the granulosa cells of the ovary, predominantly from small antral follicles and serum levels of AMH show a close correlation with antral follicle count (AFC) on ultrasound. Measurement of AMH has therefore been suggested as a surrogate of AFC in the diagnosis of PCOS but the range of serum AMH levels in women with or without PCOS is wide and its usefulness as a diagnostic test for PCOS remains in doubt.

In summary, pelvic ultrasonography and measurements of LH, FSH and testosterone may be of some diagnostic value when set in the appropriate clinical context. Routine measurements of adrenal androgens are not indicated. Measurement of sex hormone-binding globulin (which is affected by body weight) is recommended (to allow calculation of the free androgen index) in the diagnosis of hyperandrogenism when total testosterone is normal. Because of the increased risk of T2DM it is recommended that overweight and obese women with PCOS should have an oral glucose tolerance test (to identify impaired glucose tolerance as well as frank diabetes) on presentation and, if negative, at least once every 2 years thereafter. Also, in view of the associated dyslipidemia, it is advisable to check the serum lipid and lipoprotein profile at the same time.

Management

Since the physiological basis of PCOS is unknown, treatment is largely symptomatic. Patients with anovulation may require induction of ovulation (ESHRE Capri Workshop, 2012). The antiestrogen, clomiphene is usually effective but even this "simple" treatment should be monitored at a specialist center because of the risk of ovarian hyperstimulation and multiple pregnancy. Recent studies show that the use of the aromatase inhibitor, letrozole, is an effective alternative to clomiphene for induction of ovulation in PCOS. The effect of both clomiphene and letrozole is to raise endogenous levels of FSH. That may be enough to trigger an ovulatory cycle but in women who do respond to either of these oral agents, the use of low-dose, exogenous FSH is safe and effective, if carefully monitored. For those not concerned about fertility, menstrual regulation by means of the oral contraceptive or cyclical progestagens should be considered. Non-androgenic progestagens (e.g., desogestrel, gestodene) are obviously preferable to norgestrel and norethisterone in women who may anyway have symptoms of androgen excess.

Symptoms of hyperandrogenism can be managed by antiandrogens such as cyproterone acetate or spironolactone. In women with acne, mild or moderate hirsutism, this can usually be given be given in the form of co-cyprindiol (Dianette: cyproterone acetate 2 mg + ethinylestradiol 35 mg). Cosmetic advice about removal of hair should not be forgotten, whether or not antiandrogens are given.

Obese subjects with PCOS require particular attention. It has been clearly demonstrated that calorie restriction in obese women with PCOS improves insulin sensitivity and glucose tolerance. It also leads to resumption of spontaneous ovulatory cycles and normal fertility in many subjects. Significantly, such improvements in glucose-insulin homeostasis and reproductive function can be achieved with weight reduction of as little as 5% of the initial body weight. Insulin-sensitizing drugs may also have a role in reducing the risk of diabetes and improving ovarian function. The thiazolidinediones (TZDs) are insulin sensitizing drugs which have been introduced primarily for the control of type 2 diabetes. They may be effective in improving insulin sensitivity but there are serious concerns about their safety in pregnancy and are best avoided in women of reproductive age. Metformin is a well-established medication in management of type 2 diabetes. Its mechanism of action is complex but its effects include reduction of IR and insulin levels. Studies in women with PCOS have suggested that this may be a safe and effective means of improving the metabolic profile and reproductive function in both lean and obese women with PCOS. Results so far have been encouraging but by no means conclusive. What is clear is that metformin alone is associated with a low chance of pregnancy (live birth rate 7%) and adds no advantage to clomiphene therapy for induction of ovulation.

Exercise is complementary to calorie restriction in management of overweight or obese women with PCOS (Domecq *et al.*, 2013). Indeed, exercise has been shown to improve both reproductive and metabolic function although we lack large, prospective studies of long-term efficacy.

Summary

PCOS is clinically and biochemically heterogeneous the major endocrine hallmark is hyperandrogenemia and whilst it is clear that hypersecretion of adrenal androgens may contribute to the hyperandrogenemia of women with polycystic ovary syndrome, the weight of evidence favors the ovary as the major source of excess androgen secretion.

Recent studies of the genetic susceptibility to PCOS using GWAS have highlighted new, and sometimes unexpected, loci which are already providing novel insight into the etiology of PCOS. An international consortium of key centers across the globe has been established with the aim of coordinating and consolidating data from several large cohorts of women with PCOS and providing further targets for improved diagnostic and therapeutic measures in PCOS.

From the viewpoint of clinical management, the major issues relate to correction of infertility, menstrual disturbance and hirsutism. The metabolic abnormalities in PCOS have implications both for management of anovulatory infertility and of the increased risk of T2DM in later life. Diet and lifestyle measures are very important in overweight subjects but insulin-sensitizing agents may also have an important part to play in management both of anovulation and of metabolic consequences of PCOS.

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Further Reading

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Polycystic Ovary Syndrome: Implications for Cardiovascular, Endometrial, and Breast Disease

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The polycystic ovary syndrome (PCOS) is among the most common endocrine and metabolic disorders in premenopausal women, with frequencies in the 5–15% range (Yildiz *et al.*, 2012). Even though PCOS is considered mainly an androgen excess disorder nowadays (Azziz *et al.*, 2009), mounting evidence suggests that its consequences exceed largely the classic cutaneous and reproductive manifestations of hyperandrogenism. To this regards, PCOS is associated with almost all known cardiovascular risk factors and may influence the risk of gynecological cancer.

Cardiovascular Disease

Sexual dimorphism in cardiovascular disease, derived from the marked differences in body fat distribution in men and women, has been known for decades (Nedungadi and Clegg, 2009). Adult men show a predominantly central and visceral accumulation of body fat, as opposed to the more subcutaneous and peripheral fat deposition characteristic of premenopausal women (Borruel *et al.*, 2013).

Women with PCOS, on the contrary, show increased global adiposity and a masculinized pattern of body fat distribution characterized by visceral and abdominal deposition of adipose tissue (Borruel *et al.*, 2013). Recent evidence suggests that the effects of androgen excess on body fat distribution are accompanied by effects on the function of adipose tissue. The patterns of gene expression and protein abundance of subcutaneous and visceral adipose tissue of obese women with PCOS resembled that of obese men instead of being similar to that of control obese women in recent transcriptomics and protemics studies (Martinez-Garcia *et al.*, 2013; Montes-Nieto *et al.*, 2013). Hence, the androgen excess characteristic of PCOS may result in masculinization of body fat distribution and function.

We have recently proposed that a vicious circle underlies the pathogenesis of PCOS, especially when associated with obesity. This vicious circle would consist of androgen excess favoring masculinization of body fat distribution and adipose tissue dysfunction. The latter may stimulate androgen secretion by direct endocrine effects of inflammatory mediators and adipokines secreted by dysfunctional adipose tissue on the ovaries and the adrenals, or indirectly by the facilitation of insulin resistance and compensatory hyperinsulinemia (Escobar-Morreale and San Millan, 2007).

Importantly, all the steps in this vicious circle may contribute to the well-known clustering of cardiovascular risk factors in women with PCOS (Wild *et al.*, 2010). Among others, PCOS may be associated with visceral adiposity, low grade chronic inflammation, obesity, metabolic syndrome, type 2 diabetes, dyslipidemia, and abnormal blood pressure profiles (Wild *et al.*, 2010). Moreover, subclinical markers of atherosclerosis such as increased carotid intima-media thickness (Luque-Ramirez *et al.*, 2007) or coronary artery calcification (Talbott *et al.*, 2004) are also associated with PCOS. However, definite proof that these associations result into actual cardiovascular disease is still lacking (Wild *et al.*, 2010; Schmidt *et al.*, 2011) since large longitudinal prospective studies addressing the future occurrence of cardiovascular events in well characterized women with PCOS are definitely needed. Nevertheless, it must be highlighted that many patients with PCOS, especially when weight excess is absent, show no cardiovascular risk factors or metabolic dysfunction.

Considering the possible association of PCOS with cardiovascular risk factors and subclinical atherosclerotic disease current guidelines recommend assessing cardiovascular risk in certain patients with PCOS and treating modifiable risk factors as early as possible (Wild *et al.*, 2010). The Androgen Excess and PCOS Society guidelines (Wild *et al.*, 2010) consider *at risk* for cardiovascular disease patients with PCOS who are obese or show abdominal adiposity, smoke, have hypertension, dyslipidemia, subclinical vascular disease, impaired glucose tolerance or family history of premature cardiovascular disease, and at high risk those who meet criteria for the metabolic syndrome, type 2 diabetes or overt vascular or renal disease.

Accordingly, women with PCOS must be assessed for assessed for abdominal adiposity and hypertension by measuring waist circumference and blood pressure at every visit and, at least in obese patients, screening for lipid and glucose tolerance abnormalities should be considered every 2 years by obtaining a complete lipid profile and a standard oral glucose tolerance test (Wild *et al.*, 2010).

Considering that women with PCOS may be exposed to cardiovascular risk factors for many years, primary cardiovascular prevention should include aggressive treatment of cardiovascular risk factors by means of life-style modification programs, lipid

and blood pressure lowering drugs and insulin sensitizers such as metformin and, in selected cases presenting with severe obesity, bariatric surgery (Wild *et al.*, 2010; Escobar-Morreale *et al.*, 2005).

Endometrial Cancer

The theoretical basis for the association of polycystic ovary syndrome with endometrial cancer derives from the prolonged exposure of the endometrium to estrogens, in the absence of the protective effects of cyclic exposure to progesterone resulting from the oligoovulation characteristic of this disorder. This association was suggested by small observational studies and has been confirmed nowadays not only by epidemiological studies but also by meta-analyses. A recent Danish cohort study found an almost four-fold increase in the risk of endometrial cancer in patients with PCOS (Gottschau *et al.*, 2015). Such a risk was confirmed in a recent meta-analysis of 5 studies, including 138 patients with PCOS and 5593 controls, that showed that PCOS associated a 2.8 odds ratio (95% confidence interval 1.3–6.0) for endometrial cancer, a figure that increased to 4.1 (95% confidence interval 2.4–6.8) in women under 54 years old (Barry *et al.*, 2014). However, this meta-analysis showed evidence of publication bias favoring studies reporting the aforementioned association, casting doubt about its strength (Barry *et al.*, 2014). Nevertheless, considering the possible negative consequences for the health of patients with PCOS, current guidelines strongly recommend avoidance of prolonged severe menstrual dysfunction in these women by means of treatment with hormonal contraceptives or cyclic progestin administration (Conway *et al.*, 2014).

Breast Cancer

The possibility that women with PCOS could be at increased risk for breast cancer arises from their subfertility since younger age at first pregnancy, larger number of pregnancies and breast feeding are among protective mechanisms against this gynecological cancer in the general population (Layde *et al.*, 1989). However, a recent meta-analysis of 3 studies (Barry *et al.*, 2014), including 529 patients with PCOS and 39795 controls showed no evidence of increased risk for breast cancer in the former (0.95 odds ratio, 95% confidence interval 0.6–1.4), even when restricting the analysis to women under 54 years old (0.8 odds ratio, 95% confidence interval 0.5–1.3). Hence, women with PCOS should follow the same recommendations made for the general population regarding the prevention of breast cancer.

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Ovarian Androgen-Producing Tumors☆

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Glossary

Androgen A steroid hormone which produces masculinizing features. Androgens include testosterone, androstenedione, and dehydroepiandrosterone.
Crystals of Reinke Rod-shaped crystalline structures sometimes seen in the cytoplasm of Leydig cells, their presence helps confirm the diagnosis of a Leydig cell tumor or hyperplasia.
Cushingoid features Clinical features that resemble Cushing syndrome related to excess cortisol production, these include round "moon" facies, adipose deposits on the back of the neck "buffalo hump," central obesity, and abdominal striae.
Hilus of the ovary The anatomic center of the ovary.
Hysterectomy The surgical removal of the uterus.
Oophorectomy The surgical removal of the ovary.
Ovarian stroma The tissue that surrounds the ovarian follicles and comprises the bulk of the ovary. Ovarian androgens are normally produced by the stroma.
Selective venous catheterization A radiologic procedure that involves placement of catheters into an organ's venous system for blood sampling.
Virilization The appearance of secondary male sexual characteristics; in a woman this will include enlargement of the clitoris, increased acne and hair growth on the face and chest, male-type balding, as well as an increased propensity for acne.

Introduction

Androgen-producing tumors of the ovary represent $<1\%$ of all ovarian neoplasms. Their dramatic clinical presentation reflects the effect of acute increases in ovarian androgen production.

Clinical Presentation

The key feature in the clinical presentation of patients with androgen-producing ovarian tumors is the rapid onset of androgenic signs. Features include dermatologic changes with acne, oily skin, and menstrual irregularities leading to amenorrhea. Frank masculinization and virilization follow soon thereafter, with clitoromegaly, increased muscle bulk, temporal balding, decreased breast size, and deepening of the voice. This clinical presentation can occur within weeks or months. This is in marked contrast to conditions such as polycystic ovarian syndrome or congenital adrenal hyperplasia that is associated with chronic elevations of androgen production, resulting in menstrual irregularities and androgenic symptoms that develop gradually over years. However, patients with androgen-producing tumors may also present without these characteristic signs, instead presenting with pelvic pain, a pelvic mass, or abnormal uterine bleeding.

Classification of Androgen-Producing Ovarian Neoplasms

Androgen-producing ovarian neoplasms may be classified as either functional (the tumor itself secretes androgen) or nonfunctional (the mechanical pressure from the tumor triggers increased androgen production from the surrounding stroma) (Table 1). Functional tumors include Sertoli–Leydig cell tumors (formerly called arrhenoblastomas); Leydig cell, hilus cell, and lipoid cell tumors; granulosa–theca cell tumors; gynandroblastomas (in which both granulosa and Leydig cell elements exist); and luteomas of pregnancy. In addition to androgen secretion, functional tumors may also produce other steroids, including estrogen, progesterone, and cortisol. Many of these tumors respond to hormonal suppression with oral contraceptives and dexamethasone, and they may respond to gonadotropin-releasing hormone agonists and antagonists. Nonfunctional ovarian androgen-producing tumors include benign epithelial serous or mucinous cystadenomas, cystadenocarcinomas, and Brenner and Krukenberg tumors.

Sertoli–Leydig Cell Tumors

Sertoli–Leydig cell tumors (SLCTs) are the most common of the functional ovarian androgen-producing tumors. The reported incidence of androgenic symptoms in patients with SLCTs is 38%–62%. Therefore the gross tumor morphology does not always

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Table 1 Ovarian androgen-producing tumors

Functional	*Nonfunctional*
Sertoli–Leydig cell	Serous cystadenoma
Leydig cell	Mucinous cystadenoma
Hilus cell	Cystadenocarcinoma
Lipoid cell	Brenner
Granulosa–Theca cell	Krukenberg
Gynandroblastomas	
Luteoma of pregnancy	

correlate with functional or clinical pathology. In Young and Scully's review, patients' ages ranged from 4 to 67 years, with 72% in their twenties or thirties. Almost all tumors were unilateral, with sizes ranging from 9 to 25 cm. Cohen reported a patient with a very small SLCT of only 0.8 cm, but this is unusual. In Young and Scully's report, hyperandrogenic manifestations lasted from 6 months to 4 years before surgical removal. Serum testosterone levels ranged from 4.9 to 13.4 nmol/L.

Prognosis and treatment modalities are controversial because these tumors are rare and only a few studies have been published. SLCTs are classified as malignant sex cord-stromal tumors. In a series of 207 cases of SLCTs, Young and Scully reported a good correlation between pathologic differentiation and clinical outcome at FIGO stage I disease. Well-differentiated tumors behaved clinically benign, whereas 59% of poorly differentiated tumors were malignant. In tumor stage II and higher, mortality increased to 100%. Ovarian rupture had a negative impact at stage I; 7% of unruptured vs. 30% of ruptured tumors displayed malignant behavior.

Therapies for SLCTs depend on the stage, the presence or absence of rupture, and the differentiation of the tumor, as well as the patient's age and desire for preservation of fertility. In women who have completed their childbearing, hysterectomy with bilateral salpingo-oophorectomy is generally considered appropriate treatment. In young women with stage I disease, unilateral salpingo-oophorectomy is usually advised. Beyond stage I, more aggressive surgical and medical therapy is required. Hillemanns reported successful conservative management of a 17-year-old nulligravid patient using surgical excision of the SLCT without oophorectomy. Such management is reserved only for young women of low parity with stage I, well-differentiated disease, with no ovarian rupture and negative peritoneal washings.

The benefit of adjuvant chemotherapy is uncertain, but may be considered in patients with poorly differentiated or advanced stage tumors. Common regimens include BEP (bleomycin, etoposide, and cisplatin) or, more recently, paclitaxel and carboplatin. Radiotherapy has also been rarely reported as an adjunct in patients with poor prognosis or disease recurrence.

Other Functional Tumors

Leydig and hilus cell tumors are mostly composed of Leydig cells. They are usually small tumors, unilateral, occur postmenopausally, and secrete testosterone. The pathognomonic feature is crystals of Reinke. The term hilus cell tumor describes tumors localized to the hilus of the ovary. These tumors are not generally malignant, and in some cases they will be histologically classified as Leydig cell hyperplasia.

Lipoid cell tumors are larger than Leydig cell tumors and usually occur in premenopausal women. They do not contain crystals of Reinke. In addition to androgen, other steroids (e.g., estrogen, progesterone, and cortisol) can be secreted, producing Cushingoid features.

Granulosa cell tumors and thecomas, which usually secrete estrogen, may occasionally produce androgens. A pure thecoma that only produced testosterone and no estrogen, and that was also gonadotropin sensitive, has been reported.

Luteomas of pregnancy are nonneoplastic ovarian androgen-producing tumors that only occur in pregnant women. They can be unilateral or bilateral solid ovarian tumors. They are usually not palpable, asymptomatic, and found incidentally at time of cesarean section or postpartum tubal ligation. These benign tumors regress spontaneously after pregnancy, with no adverse effect on the mother's health or her future fertility. Given the challenges of accurate ultrasound diagnosis during pregnancy, MRI may be considered. Not all luteomas cause maternal virilization, but if the mother is virilized, female offspring can also be affected.

Diagnosing Ovarian Androgen-Producing Tumors

The first step is to determine tumor localization to either the ovary or the adrenal glands. No androgen is exclusively produced by the ovary. In general, testosterone serves as the best marker for ovarian androgen activity. Dehydroepiandrosterone sulfate (DHEAS), produced almost exclusively by the adrenal gland, is the best serum marker for adrenal androgen activity. Total serum testosterone levels >6.9 nmol/L denote abnormal ovarian androgen activity, and ovarian androgen-producing tumors are often found to have testosterone levels at least three times the upper normal limit. In postmenopausal women, testosterone levels >3.5 nmol/L may be abnormal. Frequent sampling of serum testosterone may be necessary due to episodic variation in

testosterone secretion and assay and tumor characteristics. DHEAS levels >27 μmol/L suggest adrenal disease. Dynamic endocrine testing has little clinical usefulness. It is important to emphasize that all virilized patients require evaluation, regardless of their serum testosterone levels.

Vaginal ultrasound is the preferred radiologic method for detecting ovarian tumors. Tumors as small as 0.5 cm can be localized using ultrasound. Color Doppler techniques may further determine tumor blood flow characteristics. Computerized tomography and magnetic resonance scans are better utilized for adrenal imaging, though may also be useful to better characterize ovarian masses. Even with the availability of high-grade imaging technology, many of the ovarian tumors cannot be localized due to their very small size. There may still be a role for selective venous catheterization in diagnosing and localizing very small tumors in difficult cases, however, even this is not universally effective in localization. Definitive diagnosis sometimes requires oophorectomy in appropriately selected patients. Due to their rarity misclassification is possible, with approximately one-third of diagnoses being reclassified after consensus review.

Increasing experience with genetic and genomic tools may allow for more accurate diagnosis, classification, prognosis, and management of ovarian androgen-producing tumors. Sertoli–Leydig cell tumors are recognized as part of the *DICER1* syndrome, an inherited disorder also classically found to involve pleuropulmonary blastoma and thyroid carcinoma. Up to 60% of Sertoli–Leydig cell tumors have been found to involve mutations in the *DICER1* gene. Further investigation into specific molecular markers is warranted.

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Sport and Menses

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Glossary

Anovulation Lack of ovulation.
Delayed menarche No menstruation by age 16.
Delayed puberty in a girl No pubertal sign by age 13.
Hirsutism Increased body hair of male type.
Hyperandrogenism Increased androgen production with associated symptoms.
Oligomenorrhea Irregular menstruation with intervals of 6–12 weeks.
Primary amenorrhea Spontaneous menstruation has never occurred.
Secondary amenorrhea Absence of menstruation for at least 3 consecutive months.
Virilization Masculinization of the body including increased muscle mass and body hair, deepening of the voice, breast atrophy and clitoromegaly.

There is clear evidence that regular physical activity has positive physical and mental health effects in both women and men. It is therefore worrying that the average physical activity level in the Western population has gradually declined. More and more people become physically inactive, that is, have sedentary employment and do not exercise in their spare time. At the same time, there has been a dramatic increase in the proportion of women who engage in sports at all levels in recent decades. The increase of female athletes is also reflected in Olympic sports with an increasing proportion of women as participants. At the summer Olympics in Munich in 1972, 15% of the participants were women. In London 2012, the proportion was 44% and this was the first Olympics in which women competed in all sports in the program. The increase of women at the elite level shows a positive trend that can hopefully lead to increased physical activity for the entire population. However, we have also gained knowledge that sports women may be at greater risk of medical complications, including menstrual disorders, loss of bone mass, and musculoskeletal injury.

Athletic Amenorrhea—Functional Hypothalamic Amenorrhea

In the late 1970s, an increased prevalence of menstrual disorders in female athletes was first reported (Loucks and Horvath, 1985). This finding and subsequent investigations gave rise to the term "athletic amenorrhea," that is, loss of menstruation due to intense physical exercise. Athletic amenorrhea can be either primary (spontaneous menstruation has never occurred) or secondary (absence of menstruation for at least three consecutive months). The estimated prevalence of secondary amenorrhea in the general population is 2%–5% (Bachmann and Kemmann, 1982). In comparison, the prevalence of athletic amenorrhea has been reported to range from 6% to 69% depending on the type of sport, age, and definition (Nattiv *et al.*, 2007). The sports with highest occurrence of athletic amenorrhea are those in which a lean body composition is regarded an advantage for physical performance, such as esthetic or endurance sports, for example, gymnastics and long-distance running. Leanness is also of critical importance for effective performance in events involving weight classes, for example, wrestling, boxing, and martial arts and gravity opposing events such as the high jump and pole vaulting. However, the frequency of menstrual disorders may be underestimated, since mild disorders with luteal phase defects and ovulatory disturbances also have been demonstrated in athletes and these disturbances often are asymptomatic and thereby not clinically detected (De Souza *et al.*, 1998).

High intensity training at an early age could affect pubertal development adversely, including a delay of growth and menarche (Warren, 1980; Abraham *et al.*, 1982; Baxter-Jones *et al.*, 1994). Delayed puberty in girls is defined as no pubertal signs by age 13 and delayed menarche is defined as no menstruation by age 16. The incidence of delayed puberty differs between sport disciplines, but is particularly common in gymnasts and ballet dancers. Some studies suggest that a genetically dependent delay in pubertal development leads to a selection bias to some disciplines such as gymnastics due to desirable physique characteristics related to delayed puberty. This would overestimate the rate of pubertal delay seen in these disciplines. However, a break from exercise due to for instance injury often results in a rapid catch-up in pubertal development, indicating that environmental factors also play a role.

Endocrine Disturbances

Athletic amenorrhea is a functional disturbance attributed to inhibition of the hypothalamic–pituitary–gonadal (HPG) axis, that is, functional hypothalamic amenorrhea, which leads to a disruption of the pulsatile release of gonadotropin-releasing hormone (GnRH). This in turn causes a reduced secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) resulting in

a low production of sex steroids, including estradiol, progesterone, and testosterone from the ovaries with ensuing anovulation and amenorrhea (Loucks *et al.*, 1989; **Fig. 1**).

Several mechanisms are involved in the inhibition of the HPG axis in female athletes. There is evidence of exercise-induced activation of the hypothalamic–pituitary–adrenal axis with increased release of stress hormones, including cortisol from the adrenal glands (Loucks *et al.*, 1989). In the normal case, cortisol increases acutely in response to physical activity to mobilize energy such as glucose and then the levels normalize at rest. In female athletes, however, chronic elevation of cortisol has been associated with increased blood glucose levels, low body fat, and amenorrhea (Lindholm *et al.*, 1995). The increased cortisol levels indicate catabolic metabolism and adaptation to negative energy balance and are probably a manifestation of a general activation of the stress response, which also includes increased secretion of corticotropin-releasing hormone (CRH) from the hypothalamus

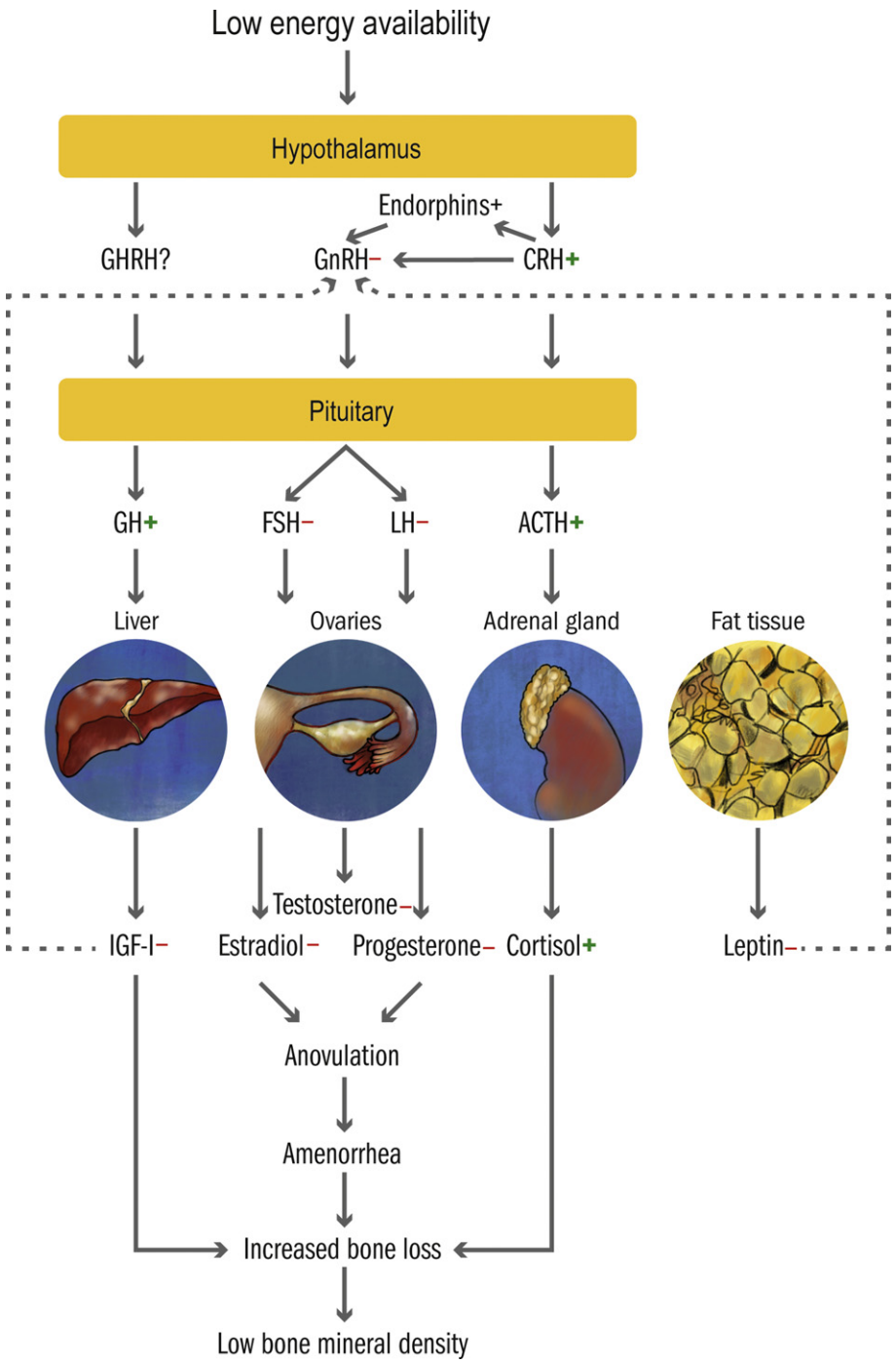

Fig. 1 A summary of endocrine disturbances associated with athletic amenorrhea. For explanations and abbreviations, see the accompanying text.

(Fig. 1). CRH has an inhibitory effect on GnRH secretion in hypothalamus. Endorphins, released in response to physical activity, might also inhibit GnRH secretion in the hypothalamus in concert with CRH (Barbarino *et al.*, 1989).

A hypometabolic state in athletes is furthermore reflected by low levels of insulin and insulin-like growth factor I (IGF-I) and high levels of growth hormone and IGF binding protein (Laughlin and Yen, 1996; Rickenlund *et al.*, 2010). IGF-I, which is secreted from the liver, is an anabolic hormone of importance for muscle and skeletal growth. Because IGF-I also stimulates GnRH and LH release, decreased IGF-I activity may reduce LH secretion in amenorrheic athletes (Fig. 1).

Leptin is another marker of energy availability. This hormone, produced in adipocytes, is an independent regulator of metabolism and levels are positively associated with body fat mass. Leptin is furthermore an important link between nutritional status and reproductive capacity. The exact mechanism is not known, however leptin receptors in hypothalamic neurons indicate that the hormone is engaged in the pulsatile secretion of GnRH. There are also leptin receptors in the ovary suggesting a direct regulatory effect on estrogen production. Leptin levels are markedly reduced in amenorrheic athletes (Laughlin and Yen, 1997; Fig. 1). Thyroid hormones are also considered as markers of energy balance and low levels of thyroxin (T4) and triiodothyronine (T3) are observed in female athletes with functional amenorrhea (Loucks *et al.*, 1992).

Taken together, athletic amenorrhea can be explained by central inhibition of the reproductive system by stress hormones and endorphins, and by reduced stimulation of GnRH due to low levels of IGF-I and leptin.

Low Energy Availability and Disordered Eating

It is today understood that the most important cause for athletic amenorrhea is low energy availability due to failure to ingest adequate energy in relation to energy expenditure (Loucks *et al.*, 1998; Loucks and Thuma, 2003). The intense physical training necessary for top athletic achievement requires a high-energy output that many athletes do not match with a corresponding caloric intake. Recent advances have therefore led to the understanding that many sportswomen are chronically energy deficient.

The reasons for this common discrepancy between energy intake and output include the difficulties involved in eating and digesting large portions of food alongside a demanding training program, and poor knowledge about energy and nutritional requirements. Another problem for athletes is that biological feedback mechanisms for energy balance are unreliable. For example, appetite does not necessarily reflect the caloric deficit induced by an intense training session (Truswell, 2001). However, a more common cause of energy deficiency is a conscious pursuit of leanness (Hagmar *et al.*, 2008), that is, a relatively low amount of body fat in relation to muscle mass, which is an important factor in sports performance in many disciplines. These include endurance sports such as long-distance running and cycling as well as sports that overcome gravity, such as high jump. A low body weight or body fat content is also often an advantage in sports that are divided into weight classes, such as wrestling, boxing, and martial arts, or in sports with esthetic considerations, such as gymnastics and figure skating. These sports are the same as those with the highest incidence of athletic amenorrhea.

Eating Disorders

Female athletes have an increased risk of developing eating disorders compared with nonathletes and male athletes (Sundgot-Borgen, 1993; Beals and Hill, 2006; Sundgot-Borgen and Torstveit, 2010). Besides optimizing body composition for performance, some female athletes are also under the pressure to attain an appealing appearance. This is particularly true for athletes in esthetic disciplines, such as gymnastics and figure skating, but is becoming increasingly important in many other disciplines, due to scrutinizing media coverage and the importance of sponsorship. Disordered eating in athletes may advance along a continuous scale ranging from a normal but often strictly controlled food intake to a disturbed eating behavior that meets the diagnostic criteria for any of the eating disorders, such as anorexia nervosa or bulimia nervosa. The prevalence of eating disorders in the general population in young women is about 1% for anorexia nervosa and 2%–5% for bulimia nervosa. In comparison, the prevalence of clinical eating disorders in female athletes has been reported to be about 20% to 30% in adult female elite athletes, and as high as 70% in weight-class sports (Gibbs *et al.*, 2013). However, it should be mentioned that most of these results are based on surveys and not on clinical interviews, and survey data usually yields higher numbers than the actual occurrence. All types of eating disorders are related to menstrual disorders.

Bone Mass

Today it is well-known that long-term amenorrhea and estrogen deficiency are associated with loss of bone mass, particularly of trabecular bone such as the lumbar spine and pelvis (Lambrinoudaki and Papadimitriou, 2010). If the condition remains untreated, it is estimated that the loss of bone mass is approximately 2%–3% per year and there is a risk of irreversible changes of bone mass (Miller *et al.*, 2006). The definition of low bone mineral density (BMD) in a premenopausal woman is a *Z*-score (i.e., standard deviation score of the mean of an age- and sex-matched reference population) lower than 2, whereas osteoporosis in this age group is defined as *Z*-score less than 2 combined with secondary clinical risk factors such as eating disorders, hypogonadism, or previous fracture (Lewiecki *et al.*, 2008). Since physical activity usually stimulates bone formation, low BMD in female athletes

was defined as a *Z*-score between −1.0 and −2.0 SD together with additional risk factors, and osteoporosis as defined earlier (**Fig. 2**; Nattiv *et al.*, 2007). It was initially considered paradoxical that elite athletes could develop reduced bone mass (Warren, 1980; Drinkwater *et al.*, 1984). The mechanism is not entirely clear but has been explained by nutritional deficiency together with its endocrine consequences such as low levels of estradiol and IGF-I and increased levels of cortisol (De Souza *et al.*, 2008).

Mechanisms of Bone Loss

Estrogen is important for bone turnover and acts through specific receptors in bone tissue to prevent bone resorption. Consequently, estrogen deficiency is associated with bone loss due to increased bone resorption (Almeida *et al.*, 2017). Chronic elevation of cortisol can also contribute to increased bone resorption (Tauchmanovà *et al.*, 2007), whereas low levels of IGF-I result in impaired bone formation (Snow *et al.*, 2000). Furthermore, energy deficiency adversely affects bone formation. Thus, even though physical activity usually increases bone mass, the overall result from energy deficiency and a catabolic hormone balance is suppressed bone formation and increased bone resorption resulting in a net loss of bone mass. Women seem to be more vulnerable than men to the consequences of energy deficiency on bone mass (De Souza *et al.*, 2014a,b). One explanation for this could be sex differences in androgen production. Testosterone exerts potent anabolic effects and has direct stimulatory effects on bone tissue (Almeida *et al.*, 2017). Since men have on average more than 10 times higher circulating levels of testosterone than women (Turpeinen *et al.*, 2008), the higher levels of testosterone might be protective against bone loss.

The prevalence of low BMD in female athletes has not been clearly established mainly because of different criteria used to define low BMD and osteoporosis. Furthermore, bone mass could vary depending on athletic population, elite level, and ethnicity. The reported prevalence to date on low BMD ranges from 0% to 40% and 0% to 13% in terms of osteoporosis (Gibbs *et al.*, 2013). In contrast, studies in Swedish female Olympic athletes have consistently shown high BMD and none athlete in these studies has presented low BMD (Hagmar *et al.*, 2009; Eklund *et al.*, 2017).

Athletic amenorrhea is also associated with an increased risk of musculoskeletal injuries (Feingold and Hame, 2006; Barrack *et al.*, 2014). The relative risk of stress fracture, for example, in the lower limb, is two to four times higher in female athletes with amenorrhea compared with regularly menstruating athletes (Bennell *et al.*, 1999). Skeletal damage also includes more severe fractures in the pelvis, hip, and spine with serious long-term consequences. It is well-known that a prior fracture increases the risk of another fracture.

The Female Athlete Triad

The concept of "the female athlete triad" was first described at a consensus conference 1992 organized by the American College of Sports Medicine (ACSM) and defined as the three associated conditions: disordered eating, functional hypothalamic amenorrhea, and osteoporosis (**Fig. 3**; Yeager *et al.*, 1993). A revised position stand was published in 2007 when the triad was redefined as a syndrome linking low energy availability (with or without disordered eating), menstrual

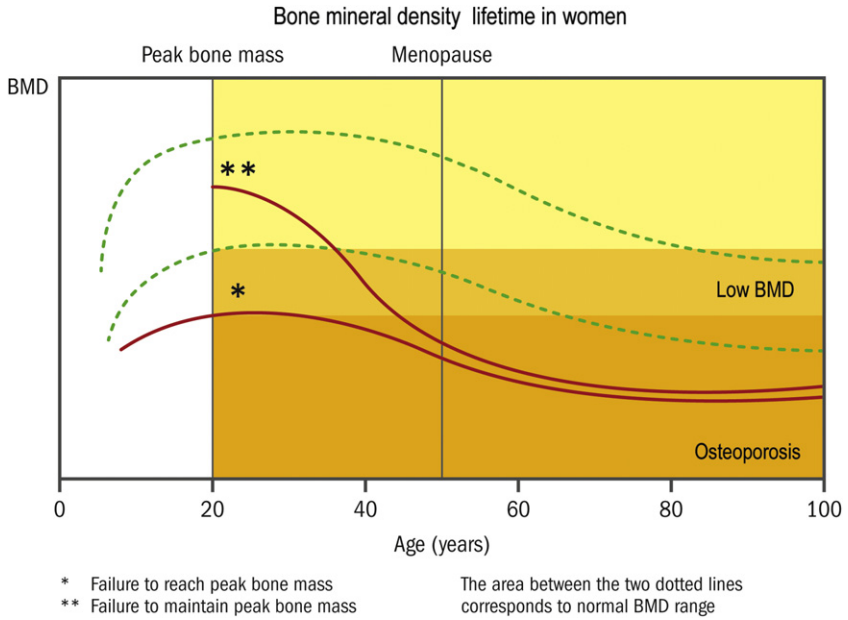

Fig. 2 Changes in bone mass in women during lifetime and the hypothetical consequences of low energy availability and estrogen deficiency.

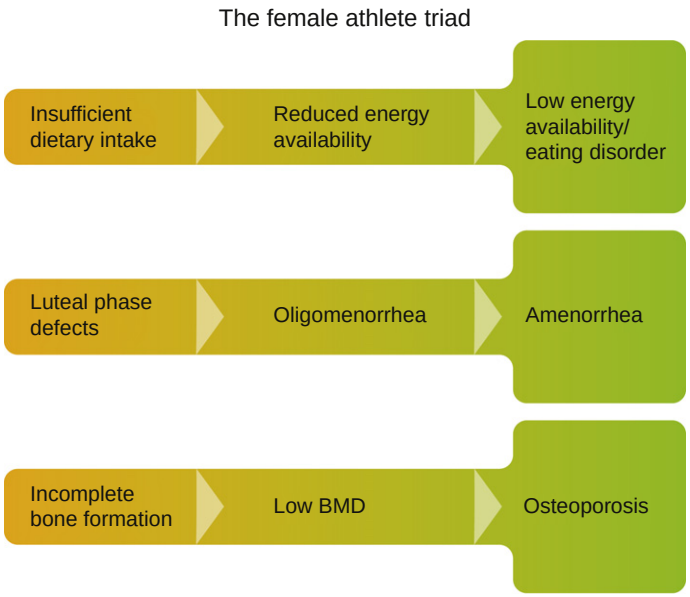

Fig. 3 The three components of the female athlete triad.

dysfunction, and low BMD (Nattiv *et al.*, 2007). It is today well established that energy deficiency plays a causal role in the development of menstrual dysfunction, and that amenorrhea and estrogen deficiency can cause increased loss of bone mass leading to low BMD (De Souza *et al.*, 2014a,b).

The triad has been highlighted as the most serious medical problem in female athletes and is considered most frequent in sports that emphasize leanness. As illustrated in **Fig. 3**, the different entities may present as symptoms along a continuous scale between normality and apparent pathology. Caloric intake and the degree of menstrual disorder may vary according to sport season, and to the amount and quality of training, age, and a variety of physiological and psychological factors. Whereas menstrual disturbances and low energy availability are more variable from time to time, the decline in BMD is slow and may partly be irreversible. The prevalence of the triad is not well documented due to methodological problems and lack of clear definitions of the criteria. However, in a review of in total 65 studies, the prevalence of all three components of the female athlete triad was reported to range from 0% to 16% (Gibbs *et al.*, 2013). In general, the prevalence of low BMD was lower than estimates of menstrual disturbances and disordered eating.

The triad is not limited to reproductive and skeletal outcomes but also to other medical consequences. For instance, amenorrhea and estrogen deficiency in athletes is associated with impaired endothelial function and lipid dysfunction (Rickenlund *et al.*, 2005a). Furthermore, problems with the central nervous system, gastrointestinal system, and renal system have been described (De Souza *et al.*, 2014a,b).

Management of Athletic Amenorrhea

Menstrual dysfunction is a sign of hormonal imbalance that should always be investigated by means of gynecological examination and endocrine evaluation. Athletic amenorrhea is an acquired condition, that is, functional hypothalamic amenorrhea, most often caused by low energy availability with or without disordered eating. This disorder can be normalized including ovulation and thereby fertility once the balance between energy intake and energy expenditure has been restored. An early intervention is essential to prevent from serious clinical consequences such as irreversible bone loss.

The typical clinical appearance is a lean woman with low body weight/body fat. A careful review of eating habits in relation to training is important, and counseling by a dietitian is recommended. In case of suspicion of eating disorders, the individual must be referred to a specialist clinic. Laboratory tests should include measurement of hormones and nutritional factors. The most characteristic endocrine pattern of functional hypothalamic amenorrhea includes suppressed levels of gonadotropins, particularly LH, estradiol, testosterone, IGF-I, and thyroid hormones, whereas cortisol levels could be elevated (Rickenlund *et al.*, 2004a,b). Delayed puberty and/or menarche in an athlete girl should be evaluated by a specialist in pediatrics or gynecological endocrinology.

Longstanding hypothalamic amenorrhea and low energy availability are associated with a loss of bone mass. Dual-energy X-ray absorptiometry (DXA) is the golden standard method for assessment of bone mass and body fat. Indications for a DXA scan include amenorrhea more than 6 months, low body weight/body fat, suspected eating disorder, and prior stress fracture. *Z*-scores for BMD spine and hip are most relevant for evaluation of bone mass. Whole body scan is used for the assessment of body fat.

Nutrition Counseling and Pharmacological Treatment

Adequate nutrition in relation to energy expenditure should always be the first line strategy of intervention (De Souza *et al.*, 2014a, b). Improved energy intake and increased body weight/fat mass have documented effect on restoration of menstrual function in functional hypothalamic amenorrhea (Misra *et al.*, 2008). A gradual increase of 200–600 kcal/day have been recommended (De Souza *et al.*, 2014a,b). In case of low bone mass, supplementation of calcium and vitamin D may also be beneficial. However, weight gain and subsequent resumption of menses are the most important changes to prevent further loss of bone mass.

If there is a lack of response to nutrition counseling and adjustment of training for at least 1 year without resumption of menses, pharmacological treatment should be considered particularly in athletes with osteoporosis and fracture history. However, bisphosphonates are not recommended since this treatment is not approved for use in premenopausal women and there are risks of atypical fractures and osteonecrosis by long-term use. There are conflicting data about to what extent estrogen substitution will restore low BMD in amenorrheic women. Reductions in BMD of amenorrheic athletes have not been fully restored by oral estrogen. This has been explained by a suppressive effect of oral estrogen on hepatic IGF-I production since IGF-I is a bone trophic factor. To overcome the first-passage effect of exogenous estrogen, the transdermal route is recommended (De Souza *et al.*, 2014a, b). Indeed, transdermal estrogen does not suppress IGF-1 and has been demonstrated to improve bone mass in combination with cyclic progesterone/progestogen in adolescent girls with anorexia nervosa (Misra *et al.*, 2011). Addition of progesterone/progestogen to estrogen substitution is necessary to avoid deleterious effects of unopposed estrogen on the endometrium.

Finally, it should be noted that low BMD is not the only indication for estrogen substitution in athletes with longstanding amenorrhea but also because of other symptoms of estrogen deficiency such as endothelial dysfunction, adverse lipid profile, urogenital symptoms, dyspareunia, and sexual dysfunction (Rickenlund *et al.*, 2005b; De Souza *et al.*, 2014a,b).

Hyperandrogenism in Female Athletes

The most common cause of amenorrhea among athletes is probably low energy availability resulting in functional hypothalamic amenorrhea. This condition is acquired and should be reversible by optimal nutrition in relation to energy expenditure. However, not all athletes with menstrual disorders have chronic energy deficiency. Research in the past decade has demonstrated essential hyperandrogenism, such as the polycystic ovary syndrome (PCOS), as an alternative etiology of menstrual disorders in female athletes.

Polycystic Ovary Syndrome

PCOS is probably the most frequent endocrine disorder in women of fertile age, affecting about 10% of the female population (Rosenfield and Ehrmann, 2016). The syndrome is characterized by increased ovarian production of androgens, anovulation, and ultrasound findings of polycystic ovaries (**Fig. 4**). The typical symptoms are oligomenorrhea (menstruations at an interval exceeding 6 weeks) or amenorrhea, hirsutism, and acne. PCOS is also associated with accumulation of abdominal fat and obesity although this is not common in athletes. The etiology of PCOS is largely unknown, but there is strong evidence for a genetic predisposition although environmental factors also play a role.

Hyperandrogenism and insulin resistance are the endocrine cornerstones in the pathogenesis of PCOS explaining the various symptoms of the disorder (Rosenfield and Ehrmann, 2016). There is evidence of a primary abnormality of increased ovarian production of androgens. This production is augmented by disordered feedback control of pulsatile GnRH secretion, resulting in elevated LH secretion and a relative FSH deficiency, which will favor androgen synthesis. The clinical consequences of increased production of androgens are the typical polycystic ovarian morphology, anovulation causing menstrual disorders and reduced fertility, hirsutism, and acne. Women with PCOS also have an increased occurrence of insulin resistance, independent of obesity, leading to secondary hyperinsulinemia. Hypersecretion of insulin directly stimulates androgen production from the ovarian theca cells. Furthermore, insulin inhibits hepatic synthesis of sex hormone-binding globulin (SHBG), and thereby increases free and bioavailable testosterone levels. Thus, hyperinsulinemia contributes to hyperandrogenism and ovarian dysfunction in PCOS women. Insulin resistance may cause metabolic symptoms including abdominal obesity. However, regular physical activity counteracts insulin resistance and is beneficial in PCOS.

It has been demonstrated that PCOS is a common disorder in female elite athletes (Rickenlund *et al.*, 2003; Rickenlund *et al.*, 2004a; Hagmar *et al.*, 2009; Coste *et al.*, 2011; Javed *et al.*, 2015). Endocrine studies have shown enhanced diurnal secretion of LH and testosterone in athletes with PCOS compared to those without (Rickenlund *et al.*, 2004a; **Fig. 5**). Thus, the hormonal profile of PCOS is completely different from that of functional hypothalamic amenorrhea. However, the levels of testosterone in PCOS typically remain within the normal upper range for women and are seldom pathologically increased. Athletes with PCOS also demonstrate a more anabolic body composition with more muscle mass and higher BMD than other athletes (Rickenlund *et al.*, 2003). Hyperandrogenism appears to provide good protection from bone loss despite oligomenorrhea/amenorrhea and relative estrogen deficiency in PCOS.

There are data to support that PCOS is advantageous for physical performance. For instance, endurance athletes with PCOS have shown higher maximal oxygen uptake and performance levels than athletes without PCOS (**Fig. 6**; Rickenlund *et al.*, 2003). It has also been reported that PCOS is overrepresented and is the most frequent cause of menstrual disorders among Olympic sportswomen (Hagmar *et al.*, 2009). These studies suggest that mild forms of hyperandrogenism like PCOS may be beneficial for

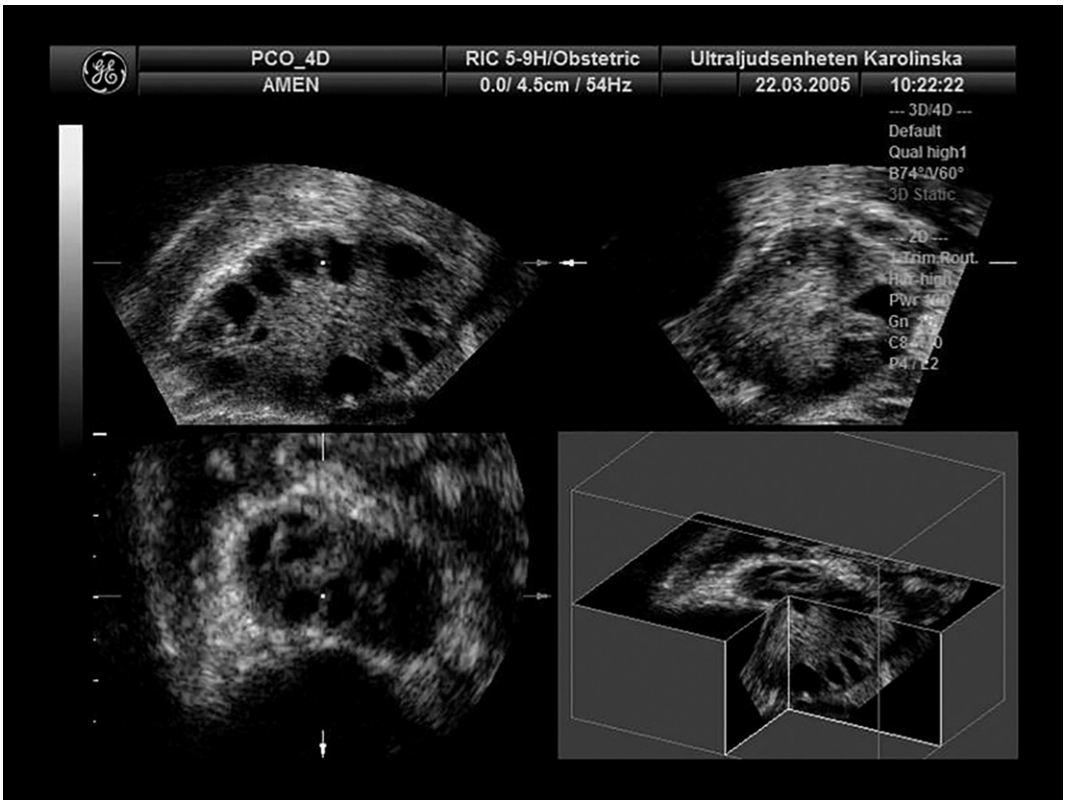

Fig. 4 Ultrasound picture of a typical enlarged polycystic ovary with an increased number of small follicles.

physical performance and could play a role in the recruitment of women to competitive sport activities. There is no support for the opposite, that is, that sport could induce PCOS. Androgen levels even within the normal range seem to play a role for physical performance. Thus, recent studies have shown associations between androgen levels, muscle mass, and physical performance in female top athletes (Eklund *et al.*, 2017).

The performance-enhancing effects of PCOS are likely explained by the anabolic effect of androgens. Testosterone exerts potent anabolic actions by direct stimulation of muscle mass and bone tissue (Mooradian *et al.*, 1987; Almeida *et al.*, 2017). Furthermore, testosterone stimulates the formation of new blood cells and the immune system, and promotes competitive behavior. All these effects of testosterone could be beneficial for physical performance.

Severe Hyperandrogenism

Men have on average more than 10 times higher concentrations of testosterone in the blood as compared to women (Turpeinen *et al.*, 2008), which is likely one reason to sex differences in physical performance. However, a few women are born with rare conditions, named disorders of sex development (DSD) in which the development of chromosomal, gonadal, and anatomic sex is atypical. These conditions may cause a greatly increased production of testosterone in the male range. If the individual has normal sensitivity to androgenic hormones, her muscle mass will develop as in males, along with increasing signs of virilization such as increased body hair, deepening of the voice, breast atrophy, and clitoromegaly.

The prevalence of such rare conditions is estimated to be about 140 times increased among elite female athletes (Bermon *et al.*, 2014). Since sports are divided into male and female classifications, many female athletes consider it unfair if they must compete against a woman who has the advantage of a male physiology. Recently, the International Association of Athletics Federations (IAAF) and the International Olympic Committee (IOC) finalized guidelines for management of hyperandrogenism in female athletes. However, the regulations are controversial and the Court of Arbitration of Sport (CAS) has requested further scientific evidence of the degree of athletic performance advantage sustained by hyperandrogenism in female athletes.

Management of Hyperandrogenism in Athletes

PCOS is a highly heterogeneous disorder, presenting a spectrum of symptoms and manifestations that vary over time. The condition is managed according to symptoms, such as menstrual disorders, infertility, hirsutism, and overweight/obesity but

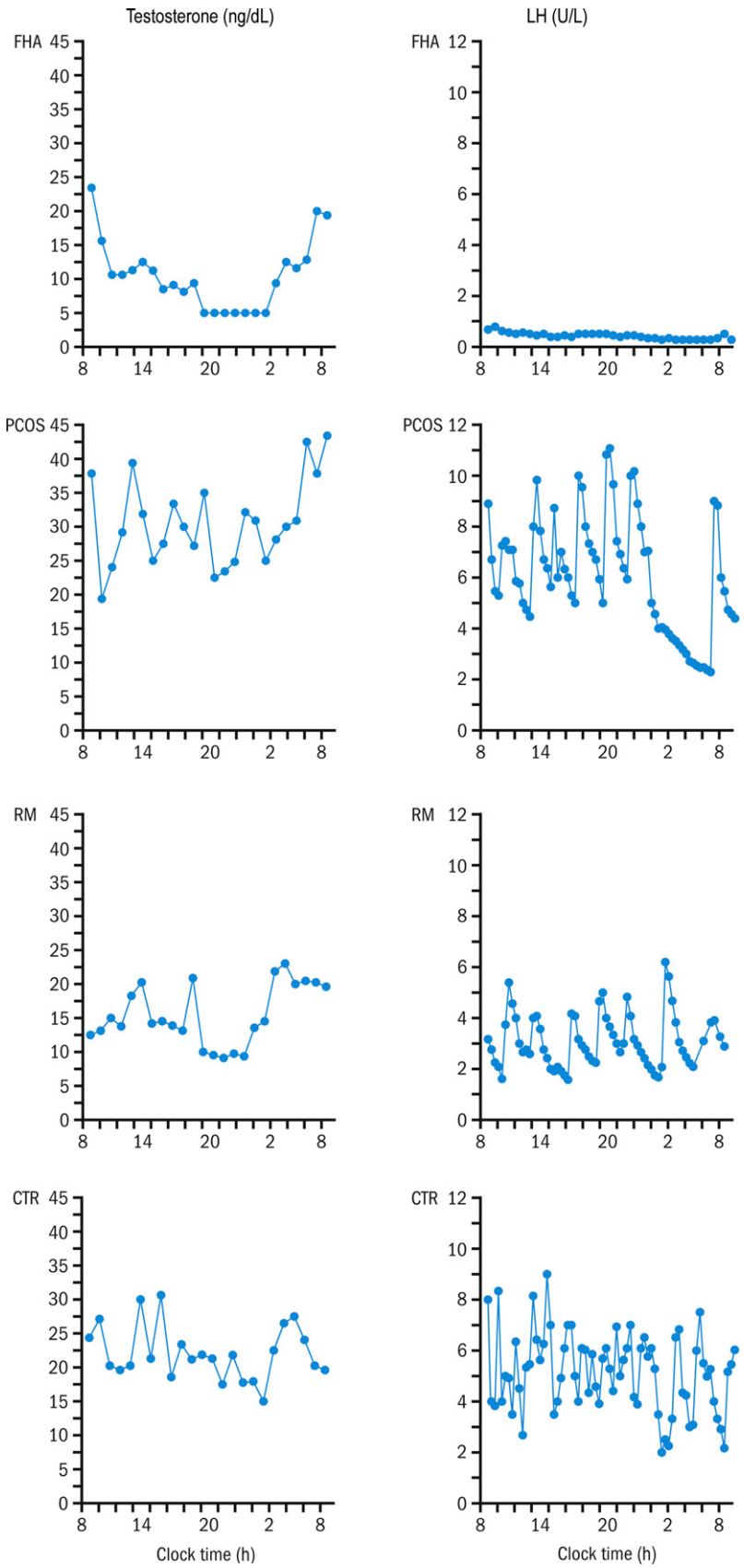

Fig. 5 Typical diurnal hormonal profiles in individual female athletes and a sedentary control woman. *FHA*, athlete with functional hypothalamic amenorrhea; *PCOS*, athlete with polycystic ovary syndrome; *RM*, athlete with regular menstruation; *CTR*, sedentary control.

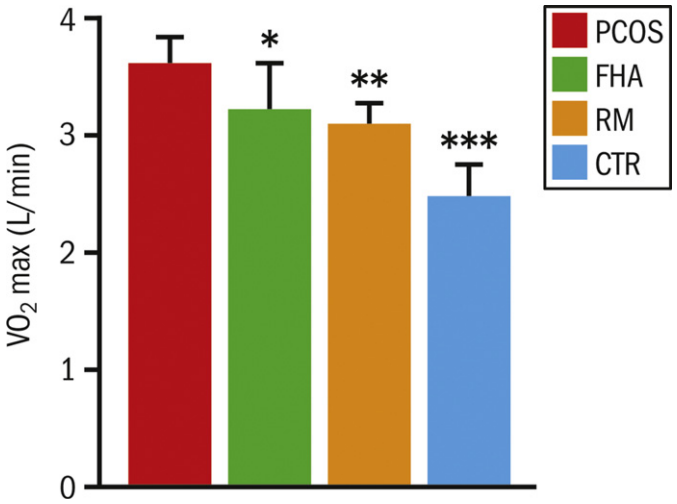

Fig. 6 Maximal oxygen uptake in groups of athletes with polycystic ovary syndrome (PCOS), functional hypothalamic amenorrhea (FHA) and regular menstruation (RM), and in a sedentary control group (CTR).

specific treatment is not always needed. The first choice of strategy to improve symptoms of PCOS in overweight and obese women is change of lifestyle including healthy diets and increased physical activity aiming at weight loss (Moran *et al.*, 2011). There is no support that weight loss improves clinical symptoms in normal-weight women with PCOS, however regular physical activity can be recommended to all women with PCOS. There is evidence that physical activity alone or in combination with dietary changes causes decreased insulin resistance, lower androgen levels, decreased hirsutism, and increased ovulation rate (Moran *et al.*, 2011). The main mechanism for improvement is probably increased insulin sensitivity, resulting in reduced compensatory hypersecretion of insulin, which in turn leads to increased SHBG and thus lower levels of free testosterone.

Longstanding anovulation should be treated due to an increased risk of endometrial hyperplasia and endometrial cancer in PCOS. The mechanism is attributed to lack of progesterone and unopposed estrogen stimulation of the endometrium. Treatment with oral contraceptives or cyclic progestogens abolishes this risk and is used to regulate menstruation. Combined oral contraceptives also inhibit androgenic effects and counteract hirsutism and acne. However, there is no evidence that oral contraceptives impair physical performance, as long as body weight/body composition is stable (Rickenlund *et al.*, 2004b).

Physical activity usually improves fertility in women with PCOS, and in contrast to women with functional hypothalamic amenorrhea, there is no support that reduction or discontinuation of sport training improves reproductive function in athletes with PCOS. For women with PCOS who do not conceive spontaneously, fertility treatment using ovulation stimulation or in vitro fertilization can be offered.

The clinical evaluation and care of women with severe hyperandrogenism like DSD should be managed by a multidisciplinary team at tertiary clinics (Lee *et al.*, 2016). Many of these individuals have already been diagnosed and treated in early childhood due to ambiguous genital development, but in some cases, they might enter puberty undiagnosed. During adolescence, individuals with DSD can present with primary amenorrhea or progressive virilization in a phenotypic girl. The evaluation includes a thorough medical history focusing on hereditary factors, a comprehensive physical examination, ultrasound or magnetic resonance tomography of internal genital organs, karyotyping and specific genetic testing, as well as endocrine evaluation.

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Primary Ovarian Insufficiency☆

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Nomenclature

MIM: Mendelian Inheritance in Man

Glossary

Primary amenorrhea Absence of menarche.

Secondary amenorrhea Absence of menstruation for at least four consecutive months.

Introduction

Premature ovarian Insufficiency (POI) is the condition of hypergonadotropic hypogonadism due to an anticipated loss of ovarian follicle reserve. It can be associated with primary amenorrhea (PA) or secondary amenorrhea (SA) lasting for >4 months in women before 40 years of age. A serum follicle-stimulating hormone (FSH) level >25 IU/L on two occasions drawn >1 month apart associated with low estradiol defines the hypergonadotropic hypogonadal state (Webber *et al.*, 2016). POI occurs in 1/100 women before the age of 40, in 1/1000 women before the age of 30 and is very rare before menarche in women with a normal karyotype. Turner syndrome (estimated frequency 1:2000 phenotypically females) is the prototype of POI generally associated with PA but also with SA in 20%–25% of the cases. Intermittent or resumptive forms of POI are not so rare (up to 20% of cases) and unexpected pregnancies have also been reported in women with the diagnosis of POI. For this reason, the previous usual terms of premature ovarian failure or premature menopause have been discarded (Gago and Ginsburg, 2004; Beck-Peccoz and Persani, 2006; Goswami and Conway, 2005; Welt, 2008; Nelson, 2009).

The clinical relevance of POI raised exponentially in the last decades because of two main reasons: (a) the frequent postponing of the fertility desire and programmed conception by women beyond their 30–35 years of age; (b) the prolongation of life expectancy.

Clinical Features and Diagnosis

The clinical features include the menstrual irregularities up to amenorrhea, anovulation and infertility, and the manifestations of hypoestrogenism (Goswami and Conway, 2005; Beck-Peccoz and Persani, 2006). The ovarian phenotype can then be associated with several different extra-ovarian clinical manifestations that depend upon the underlying cause or genetic defects.

The follicular depletion before puberty is associated with a pubertal delay, and POI should be suspected in girls that do not go through puberty by the age of 13 years. If untreated, eunuchoidism may develop. In cases with short stature a Turner syndrome should be suspected. Other particular extra-ovarian phenotypes may suggest alternative syndromic forms of POI (**Table 1**) (Persani *et al.*, 2013; Rossetti *et al.*, 2017; Martin *et al.*, 2017). In postpubertal onset, POI is not associated with a characteristic preceding menstrual history. The development of amenorrhea may be acute or it may be insidious. SA is generally accompanied by typical climacteric manifestations (flushes, sweating, vaginal dryness, urogenital manifestations, irritability/anxiety, sleep disorders). Hypoestrogenism is associated with sexual dysfunction and impaired physical performance and may then lead to osteoporosis, and predispose to neurodegenerative and cardiovascular diseases (Nelson, 2009). About one out of four POI cases are probably of autoimmune origin and POI can be part of classic polyglandular autoimmune diseases (PGA). POI can be present in about half of the patients with PGA type 1 caused by *AIRE* mutations and in about 1 out of 10 patients with PGA type 2, in both of these diseases POI is associated with Addison disease. In these cases, the patients have antisteroid cell antibodies (SC-Abs) (La Marca *et al.*, 2010). An autoimmune origin may alternatively be supported by the association of POI with other autoimmune diseases, such as Hashimoto thyroiditis (Welt, 2008; Martin *et al.*, 2017).

Because of the involvement of particular genes, POI women can be predisposed to malignancies (Rossetti *et al.*, 2017). Women with POI have a diminished life expectancy likely due to the increased risk of malignancies, cardiovascular and autoimmune diseases (Goldberg *et al.*, 2015; Tao *et al.*, 2016).

The ESHRE guidelines indicate that diagnosis of POI should be confirmed by AMH determinations and ovarian US (Webber *et al.*, 2016). The low/undetectable levels of AMH and/or absence of preantral follicles at US are indicative of a complete

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Table 1 Particular phenotypes of POI patients associated with specific gene variations

Genes	*Associated phenotypes*
HSD17B4, *HARS2*, *CLPP*, *LARS2*, *C10ORF2*, *KIAA091*, *ERAL1*	Hearing defects (Perrault syndrome)
POLG	Progressive external ophthalmoplegia and tremor and parkinsonism
FOXL2	Blepharophimosis, ptosis, epicanthus inversus (BPES)
GNAS	Resistance to multiple hormones (PTH, GHRH, LH/FSH, TSH), short stature, short IV metacarpus, overweight
AIRE	Candidiasis, Addison disease
AIRE (mutations in the PHD1 domain), *MCM8*	Hypothyroidism
FMR1 premutation	X-linked mental retardation or tremor-ataxia in relatives
LHCGR	LH elevation higher than FSH, large ovarian follicles present, anovulation
FSHR	Variable presence of small preantral follicles
NR5A1, *SOX8*	DSD (Swyer Syndrome) in male relatives
GALT	Galactosemia
EIF2B	Vanishing white matter (VWM) disease with progressive neurological deterioration
ATM	Ataxia-Teleangiectasia
BMPR1	Dehmiran syndrome
Turner mosaicism	Short stature (cardiac malformations, lymphedema)
GDF9	Recurrent spontaneous dizygotic twinning
BMP15, *MCM9*, *FSHR*, *NUP107*, *PSMC3IP*, *ATM*	46,XX ovarian dysgenesis
BLM, *WRN*, *ANTXR1*	Premature aging syndromes (Bloom syndrome, Werner syndrome, GAPO disease)

exhaustion of the follicular reserve and predict the inefficacy of attempts aimed to retrieve fertilizable oocytes from the affected women. In these cases, oocyte donation is the only solution for the desired fertility. Conversely, detectable AMH levels and preantral follicles indicate the possibility to retrieve fertilizable oocytes.

Causes of POI

The origin of POI is highly heterogeneous. The possible mechanisms at the origin of POI can be: (a) a deficient primordial follicle pool; (b) an accelerated follicular atresia; or (c) a hampered maturation/recruitment of primordial follicles (Gago and Ginsburg, 2004; Persani *et al.*, 2013). However, in most of the cases POI occurs because of a dramatic reduction in the size of the preantral follicular pool. The etiological causes that may activate such mechanisms are highly heterogeneous and include chromosomal, genetic, autoimmune, metabolic, infectious and iatrogenic factors (**Table 2**). At present, about 25% of all forms of POI can be classified as iatrogenic and related to cancer treatment, but >50% of the cases remain idiopathic. The most frequent known cause is therefore iatrogenic and mainly due to chemo- or radio-therapeutic or surgical interventions for cancer in young age. Since the number of cancer survivors is constantly increasing, so is the relevance of fertility preservation in young cancer patients. This phenomenon led to the diffusion of fertility units in cancer centers (Woodruff, 2015). Excessive and long-standing but also passive smoking, pollutants or alcohol addiction can also diminish the fertility potential of women (Ertunc *et al.*, 2015; Grindler *et al.*, 2015). Viral or bacterial infections with a potential gonadal toxicity are generally prevented by vaccination. All the other forms of POI have an autoimmune or genetic origin.

Autoimmune POI

Autoimmune POI can be mainly classified into two groups, the one associated with autoimmune adrenal disease and the one associated with other autoimmune diseases. However, a clear autoimmune ovarian disease can be demonstrated only when POI is found in patients with adrenal autoimmunity or failure, probably due to common antigens between the two glands both constituted by steroidogenic cells. This association was reported in up to 10% of the POI cases (Nelson, 2009; Martin *et al.*, 2017) and can be part of both PGA type 1 and type 2 (Welt, 2008; La Marca *et al.*, 2010). PGA1 is typically manifest since childhood and it is characterized by mucocutaneous candidiasis, hypoparathyroidism and Addison disease and is accompanied by POI in 60% of cases. PGA1 has a known genetic cause, resulting from mutations in the *AIRE* gene that is involved in regulatory T cell development and maturation (Gago and Ginsburg, 2004; Nelson, 2009). PGA2 is defined by adrenal insufficiency and hypothyroidism, and is associated with POI in approximately 10% of cases. SC-Abs and/or enzyme antibodies are present in 60%–90% of POI women with SA and Addison disease. Antibodies can be directed against enzymes common to the ovary and adrenal glands, including cytochrome P450 side-chain cleavage enzyme (P450SCC), 17-α hydroxylase/17,20-lyase (CYP17A1) and 21-hydroxylase (CYP21) enzymes. The most sensitive indicators of adrenal autoimmunity are SC-Abs or CYP21 antibodies. These antibodies represent serological indexes of the autoimmune disease and would lack a pathogenic role. A combination of antibody and cell-

Table 2 Acquired extrinsic and genetic factors accounting for an early age at menopause or POI

Acquired extrinsic factors	*Genetic susceptibility*
Chemo-, radio-therapy (e.g., Hodgkin disease, alkylating drugs, pelvic irradiation), ovariectomy	X chromosome defects (e.g., Turner syndrome and related variants)
Exposition to toxics/disruptors (e.g., heavy and/or long-lasting smoking, passive smoking, alcohol addiction, pollutants, radiations)	Autoimmunity (e.g., Polyglandular Autoimmune Syndromes type 1, 2, 3a)
Ovarian infections	Genetic defects (>50 candidate genes for isolated or complex diseases)

mediated immunity is likely to play a role in ovarian autoimmunity (Martin *et al.*, 2017). In several POI cases, the theca cells of preantral and antral follicles appear to be the main target of the autoimmune process. In these cases, the inflammatory infiltration does not affect the primordial follicles and granulosa cells are generally spared until they luteinize after ovulation. Since primordial follicles are frequently unaffected, fertility may be partially conserved in autoimmune oophoritis and intermittent ovarian failure may be more common. However, the immune reaction in the ovary may finally lead to a more generalized process and POI as the clinical end-point (Nelson, 2009; Martin *et al.*, 2017).

POI associated with other autoimmune disease is rarely characterized by SC-Abs thus it appears more difficult to document a direct connection. Nevertheless, POI is associated most commonly with thyroid autoimmunity (about 20%) when adrenal autoimmunity is absent. Other most commonly associated autoimmune alterations are parietal cell antibodies (4%), acetylcholine receptor antibodies in myasthenia gravis (2%) and insulin dependent diabetes (2%). The risk for these diseases in women with POI is higher than in the general population (Nelson, 2009; Persani *et al.*, 2013), suggesting that there may be a still unknown autoimmune component.

Because of the possible coincident adrenal failure it is important to exclude adrenal insufficiency in all women with unexplained POI in order to prevent the possible clinical consequences of Addison's disease. Basal and dynamic testing of the hypothalamus–pituitary–adrenal axis should be considered in any women with positive SC-Abs and if adrenal autoimmunity cannot be excluded.

Familial and Genetic POI

Several observations support a prevalent role of genetic mechanisms in the pathogenesis of idiopathic POI. Epidemiological evidences support the inheritance of menopausal age between mothers and daughters and genome wide studies found loci that were significantly associated with age at natural menopause. Interestingly, POI has also a frequent familial incidence. In large series of POI women, the incidence of familial forms reaches more than one third of cases if a familial history of early menopause between 40 and 45 years of age is considered (Nelson, 2009; Persani *et al.*, 2013; Martin *et al.*, 2017). Pedigree analysis demonstrates different modes of inheritance, but the more frequent maternal transmission would be consistent with an X-linked inheritance with incomplete penetrance. The presence in one pedigree of women with PA or SA, or with EM indicates that POI may be a genetic disease with a highly variable expressivity, thus supporting the view of POI as a complex multifactorial disease probably involving the contribution of several alleles (Rossetti *et al.*, 2017).

More than 50 gene loci have nowadays been associated with POI. A recent work brought evidence that POI may have an oligogenic origin (Bouilly *et al.*, 2016). Oligogenic involvement was also shown to lead to younger age at menopause. Genetic POI can occur within complex syndromes or constitute an isolated phenotype. The most relevant genetic causes of POI are briefly illustrated.

Turner Syndrome and X Chromosome Defects

Turner syndrome (TS) is the consequence of complete or partial loss of one X chromosome (Gravholt *et al.*, 2017). The resulting phenotype is that of a female with infertility due to POI and short stature, variably associated with other extra-gonadal abnormalities. The 45,X is the characteristic karyotype but 45,X/46,XX mosaicisms or structural abnormalities of the X chromosome account for about half of the patients. TS has an estimated prevalence of about 1:2000 females. In women with TS, oocyte-loss occurs in the early stages of meiotic prophase and ovarian development, resulting in OD and PA with elevated FSH levels since infancy. Nevertheless, spontaneous menarche and pregnancies have been reported in about 20% of cases (Gravholt *et al.*, 2017; Webber *et al.*, 2016). The TS phenotype may be explained by several mechanisms, but the most substantiated one is the lack of required dosage of particular X-linked gene products (like *SHOX*) that physiologically escape X inactivation. The requirement for a double dosage of X-linked genes is supported by the complete spontaneous puberty reached in about one third of patients with high mosaicism level. We documented the spontaneous puberty in one TS patient with short stature and a full duplication of the *BMP15* gene on the short arm of X chromosome in presence of low level mosaicism (<10%), suggesting a relevant role for a double dose of this gene in ovarian development (Castronovo *et al.*, 2014).

X chromosome abnormalities have long been recognized as a frequent cause of many forms of familial as well as sporadic POI. They include Triple X syndrome, Turner mosaics, deletions, isochromosomes and translocations between X chromosome and autosomes (Persani *et al.*, 2013; Gravholt *et al.*, 2017).

PGA1 or Autoimmune Polyendocrinopathy Syndrome Type 1 (APS1)

APS1 (MIM #240300) is caused by mutations in the autoimmune regulator gene (*AIRE* MIM #607358). The syndrome is characterized by having two out of three major clinical findings: Addison disease (AD), and/or hypoparathyroidism, and/or chronic mucocutaneous candidiasis. Generally in this syndrome, the Addison disease has its onset in childhood or early adulthood. APS1 is frequently associated with chronic active hepatitis, malabsorption, juvenile-onset pernicious anemia, alopecia (Persani *et al.*, 2013; Rossetti *et al.*, 2017).

Two laboratories concurred in the isolation of the causal gene for APS1 in 1997 and designated it *AIRE* (autoimmune regulator). The gene is located at 21q22.3 The AIRE protein contains two zinc finger motifs consistent with a role as transcription factor. AIRE is involved in the induction of tolerance to self-antigens by inducing the expression of peripheral tissue self-antigens in thymic stromal cells. Normally, this promotes the clonal deletion of differentiating T cells that recognize these self-antigens. In the absence of AIRE protein, many tissue-specific self-antigens fail to be expressed in the thymus, thus leading to multi-organ autoimmunity due to the failure in the negative selection of auto-reactive T cells. Negative selection normally causes death of T cells having receptors that are highly specific for self-peptides. If these auto-reactive T cells are left unchecked autoimmunity may result. The *AIRE* mutations ($n > 50$) are generally inherited in a recessive manner (Nelson, 2009; Persani *et al.*, 2013).

However, several mono-allelic mutations in the homeodomain of AIRE have been identified to exert a dominant negative effect. They were found in cases with delayed onset and milder manifestations compared to classical APS1, and follow incomplete inheritance. In this context, POI might represent the first or even the only manifestation of a nonclassical form of organ-specific autoimmunity (Persani *et al.*, 2013).

Blepharophimosis, Ptosis, Epicanthus Inversus Syndrome (BPES)

BPES (MIM #110100) is an autosomal dominant eyelid malformation that is classified as BPES type 1 when it is associated with POI. In the absence of POI, it is classified as type 2 BPES. *FOXL2* (forkhead transcription factor L2) (MIM *605597) mutations represent the cause of BPES. Animal models of human BPES, including the goat with polled/intersex syndrome (PIS) and the Foxl2 knock-out mice, were shown to replicate the human findings. FOXL2 role in granulosa cells (GC) physiology includes the promotion of granulosa cells differentiation and ovarian function (Caburet *et al.*, 2012). FOXL2 regulates: (a) AMH expression through the interaction with SF-1; (b) follistatin gene transcription by cooperating with SMAD3; (c) estradiol signaling by triggering the Activin-dependent expression of ESR2, and (d) maintenance of GC identity through the indirect repression of SOX9 (Caburet *et al.*, 2012; Rossetti *et al.*, 2017).

Numerous FOXL2 variants have been reported in individuals with BPES types 1 and 2 (*FOXL2* Mutation Database at http://medgen.ugent.be/LOVD2/home.php). *FOXL2* intragenic mutations resulting in truncated proteins before the poly-Ala tract are typically associated with BPES type 1, whereas poly-Ala expansions would rather lead to BPES type 2. However, the missense mutations mainly located within the forkhead domain are associated with discrepant ovarian phenotypes, and recently reported cases emphasize the importance of long-term clinical follow-up of ovarian function also in patients with a poly-Ala expansion. In contrast, *FOXL2* variants in women with POI but without palpebral abnormalities appear to be a rare event (Rossetti *et al.*, 2017).

Galactosemia

Galactosemia (MIM #230400) is a hereditary disorder of galactose metabolism caused by deficiency of *GALT* (MIM *606999) enzyme (galactose-1-phosphatase uridyltransferase), with an incidence in Europe and North America of about 1:30,000–50,000) (Rossetti *et al.*, 2017; Martin *et al.*, 2017). Galactosemia presents with the worst complications in organs with high GALT expression (liver, kidney, ovary and heart). Up to 80%–90% female patients with *GALT* homozygous mutations that partially or completely abolish GALT activity show a severe phenotype including early onset of POI. Several causative mutations have been described in *GALT* gene, but >70% of cases associated with impaired GALT function are caused by two common mutations (p.Q188R and p.K285N). The patients with GALT mutations accumulate galactose in the ovary leading to accelerated follicle atresia. FSH levels can be increased since birth to puberty and the disease is frequently associated with PA. Spontaneous pregnancies have seldom been reported in women with galactosemia (Coelho *et al.*, 2014).

Pseudo-Hypoparathyroidism Type 1a (PHP1A)

PHP1a (MIM #103580) is a generalized form of hormone resistance characterized by renal resistance to parathyroid hormone (PTH), resulting in hypocalcemia and hyperphosphatemia (Persani *et al.*, 2013; Rossetti *et al.*, 2017). Moreover, it is characterized by partial resistance to other hormones, including gonadotropins, and a variety of clinical features known as Albright hereditary osteodystrophy. Gonadal dysfunction with delayed or incomplete sexual maturation and infertility is frequent. In about

70%–80% of cases, PHP1a is caused by maternally inherited heterozygous loss-of-function variants in the *GNAS* gene (MIM *139320), which lays in a locus that is under a differential parental imprinting. The *GNAS* gene encodes for the protein Gsα, which is the first intracellular element downstream of PTH and gonadotropin receptors. The presence of gonadotropin resistance and POI in these patients is the consequence of the preferential expression of a mutant maternal allele in gonads as in other target tissues of hormones acting through the same Gsα-cAMP pathway.

Progressive External Ophthalmoplegia

The *POLG* gene (MIM *174763) encodes the enzyme synthesizing the mitochondrial DNA and correcting mitochondrial DNA errors. Patients with POLG mutations present with autosomal dominant (MIM #157640) or recessive (MIM #258450) progressive external ophthalmoplegia (PEO), a disease characterized by weakness of the ocular muscles and myopathy secondary to the depletion of mitochondria. The POLG mutations clustering in the polymerase domain undergo a typical dominant inheritance pattern, while those affecting the proofreading domain follow recessive inheritance. The polymerase-domain mutations have been reported in several families in cosegregation with POI and parkinsonism (Rossetti *et al.*, 2017).

Ovarioleucodystrophy

Ovarioleukodystrophies are neurological disorders characterized by the involvement of the white matter of the central nervous system associated with POI. Some of the patients have unusual association of POI with "vanishing white matter disease" (*VWM*, MIM #603896) observed on cerebral MRI, with variations in any of the five subunits of the *EIF2B* gene. This factor acts in response to cellular stress preventing the accumulation of denaturated proteins. The age at onset of neurologic degeneration correlates with the severity of ovarian dysfunction. In some cases, SA occurs before the neurological symptoms or occurs when neurological abnormalities are subclinical (Rossetti *et al.*, 2017).

Ataxia Telangiectasia

ATM (Ataxia Telangiectasia Mutated) gene (MIM *607585) encodes a cell-cycle checkpoint kinase which is involved in the cellular response to DNA damage, in the processing of the DNA strand breaks that occur during meiosis, during immune system maturation and for telomere maintenance. *ATM* mutations are the underlying causes of ataxia telangiectasia (MIM #208900), an autosomal-recessive disorder which includes cerebellar degeneration, oculomotor dysfunction, immunodeficiency, predisposition for cancer, radiosensitivity and chromosome instability as well as gonadal abnormalities and reduced germ cell pool. Loss-of-function mutations of the ATM gene have been associated with ovarian dysgenesis and defects in primordial germ cells development (Persani *et al.*, 2013; Rossetti *et al.*, 2017).

Premature Aging Syndromes

Several syndromes characterized by symptoms of "premature aging" are associated with POI (or azoospermia in males). Bloom syndrome (MIM# 21090) is a rare autosomic recessive disorder caused by mutations in the gene coding for the DNA helicase BLM (MIM# 604610), which result in genomic instability. The main symptoms of Bloom syndrome include short stature, distinctive skin rashes on sun-exposed areas, moderate immuno-deficiency, increased cancer risk and hypogonadism in both sexes. Recessive mutations in the *WRN* gene (MIM#604611), which encodes another DNA helicase, are the causatives of Werner syndrome (MIM#604611), a form of adult progeria characterized by sklerodermic-like skin, cataract, premature arteriosclerosis, increased cancer risk and atrophic gonads. Another form of syndromic premature aging associated with POI is represented by GAPO syndrome (MIM# 230740), which is caused by recessive mutations in a gene involved in cell adhesion and migration, *ANTXR1* (MIM# 606410). GAPO syndrome is characterized by severe growth retardation, alopecia, optic atrophy and distinctive facial features. Ovaries of women affected by GAPO syndrome display extensive deposition of hyaline extracellular material and premature follicular depletion.

The epidemiological observations that POI is often associated with syndromes characterized by premature aging further support the growing idea that even nonsyndromic POI could be considered as a form of ovary-specific accelerated aging (Rossetti *et al.*, 2017).

Perrault Syndrome

Perrault syndrome (PS) is a highly heterogeneous autosomal recessive syndrome mainly characterized by ovarian dysfunction and sensorineural deafness. Several genes implicated in mitochondrial functions or metabolism have nowadays been associated with PS (Chatzispyrou *et al.*, 2017; Lerat *et al.*, 2016; Demain *et al.*, 2017).

HARS2 and *LARS2* encode mitochondrial histidyl or leucyl-t-RNA synthetases involved in translation of mitochondrially encoded genes. *CLPP* encodes a highly conserved endopeptidase component of a mitochondrial ATP-dependent proteolytic complex, involved in degradation of unfolded or misfolded polypeptides. *C10orf2* encodes Twinkle, a mitochondrial primase-

helicase essential for replication of mitochondrial DNA yielding a mitochondrial DNA depletion syndrome and leads also to PEO. Very recently, mutations of *ERAL1* and *KIAA0391* were involved in PS (Chatzispyrou *et al.*, 2017). *KIAA091* encodes the metallonuclease subunit of the mitochondrial RNase P complex responsible for the 5′-end processing of mitochondrial precursor tRNAs. ERAL1 protein binds to the mitochondrial 12S rRNA and is involved in the assembly of the small mitochondrial ribosomal subunit affecting mitochondrial respiration and function. Mutations in a peroxisomal enzyme involved in fatty acid ß-oxidation and steroid metabolism, 17ß-hydroxysteroid dehydrogenase type 4 (HSD17B4), can also lead to PS.

Given the role of apoptosis in ovarian follicle functions, inappropriately timed or diffuse apoptosis is the most likely cause of POI.

Nonsyndromic POI

Fragile X Mental Retardation 1 (FMR1)

The FMR1 gene is located on the X chromosome and includes a trinucleotide repeat sequence, (CGG)n, in its 5′ untranslated region. Common alleles include 6–44 CGG repeats, typically with AGG interspersions every 9 or 10 repeats. When expanded to 55–200 repeats, this "premutation" becomes unstable when transmitted and has the potential to expand beyond 200 repeats in the next generation. The resulting "full" mutation then leads to the full silencing of the *FMR1* gene due to hypermethylation of the repeat and regulatory regions and causes fragile X syndrome, the most common inherited form of intellectual and developmental disabilities in males. The premutation is carried by about 1/250 women, with a higher frequency in the Mediterranean population. Approximately one out five women who carry the premutation will develop a POI. Differently, about 12% of women with familial POI and 3% of those with sporadic POI carry the premutation. Thus, the *FMR1* premutation has emerged as one of the main inheritable factors predisposing to POI (Wittenberger *et al.*, 2007; Martin *et al.*, 2017).

Studies conducted on women carrying the premutation allele that are still having regular menstrual cycles revealed that their hormonal hypothalamic–pituitary–gonadal axis profile is strikingly similar to that of aging ovaries with increased FSH and decreased inhibin B in the follicular phase. Low AMH levels among young premutation carriers are also consistent with an anticipated ovarian aging.

Two risk factors act as predictors of risk and severity of POI, the repeat size and mean age at menopause of first-degree relatives. The premutation carriers with the highest risk for POI turn out to be those with about 80–100 repeats. Consistently, the onset of FXPOI is earliest among those with 80–100 repeats, but rarely before 20 years. The second established risk factor is mean age at menopause of first-degree relatives. It is thus possible that modifier genes could play a substantial role in the variability of age at menopause among premutation carriers.

The mechanism leading to FXPOI is still unexplained. As the premutation repeat size increases, the level of FMR1 transcripts abnormally increases and the level of FMRP, the resulting protein, decreases. The toxic effect of the premutation could have its influence at several levels. *FMR1* mRNA studies in the mouse and FMRP expression studies in fetal ovaries indicate that FMRP is highly expressed in the germ cells of the fetal ovary. Thus, FMRP may play a role in oogonia proliferation and the determination of the initial size of the ovarian reserve. Interestingly, several studies have indicated that FMRP is also expressed in GC of maturing follicles, but not in primordial/primary stages. It was suggested that this cellular shift of FMRP expression to GC during follicle maturation after birth indicates a role for FMRP in the maturation of an oocyte.

Since FMRP appears to be involved in the control of transcripts' translation, it is possible that increased levels of FMRP in specific moments during development could lead to the insufficiency of proteins necessary in follicle development and survival (Persani *et al.*, 2013; Rossetti *et al.*, 2017; Martin *et al.*, 2017).

Gonadotropin Receptors

Rare loss-of-function mutations can cause gonadotropin resistance with hypergonadotrophic hypogonadism. The homozygous missense mutation (p.A189V) in the extracellular domain of the *FSHR* gene (MIM *136435) was the first reported to cause PA with hypoplastic ovaries in women with 46,XX karyotype. This mutation causes an intracellular retention of FSHR but appears to be particularly frequent only in the Finnish population, as result of a founder effect (Aittomaki *et al.*, 1995). Other mutations in different regions of the *FSHR* gene have been reported in women with the classic biochemical phenotype of premature ovarian insufficiency (FSH higher than LH levels). Complete FSH resistance is associated with absent pubertal development and PA, whilst postpubertal POI and SA are characteristics of partial resistance. Both the complete and partial forms undergo a typical recessive inheritance (Beck-Peccoz and Persani, 2006; Persani *et al.*, 2013; Rossetti *et al.*, 2017).

Homozygous inactivating variants of the *LHCGR* gene (MIM + 152,790) are a rare cause of POI in women with 46,XX karyotype. They represent a particular form of hypergonadotropic hypogonadism characterized by LH levels higher than those of FSH. Studies in families with Leydig cell hypoplasia showed in female carriers of complete LH resistance a POI phenotype characterized by anovulatory oligoamenorrhea or SA with evidence of multiple follicles at the antral stage at ultrasound (Persani *et al.*, 2013; Rossetti *et al.*, 2017).

Transforming Growth Factor-Beta (TGF-β) Family

Both the oocyte and GC within the ovarian follicle express several TGF-β-like factors, which promote proliferation and differentiation in the tissues where they are expressed. These factors include: growth and differentiation factors (GDFs), bone

morphogenetic proteins (BMPs), as well as inhibins, activins or anti-mullerian hormone (AMH). TGF-β-like factors are commonly expressed as pre-pro-proteins, which undergo proteolytic cleavage during the secretory pathway. The precursors are specifically cleaved to generate the "mature" ligand, which alone or in combination with other secreted factors promote the cell signaling cascade. The pro-region is important for the processing of the pro-protein by driving the dimerization and secretion of the mature peptides. Several of these factors acting within the ovarian follicles are required for maintaining the follicle homeostasis and for proper folliculogenesis. Therefore the related encoding genes are considered as candidates to be investigated in women with POI (Persani *et al.*, 2014; Rossetti *et al.*, 2017).

Inhibin A (INHA)

Inhibin is a candidate gene given its important role in regulating ovarian function either as negative modulator of pituitary FSH synthesis or as a paracrine factor within the ovarian follicles. The association between inhibin and POI was first suggested by the identification in a woman with POI of a 46,XX,t(2;15)(q32.3;q13.3) translocation causing a breakpoint in the α subunit of inhibin (INHA; MIM *147380, locus 2q33–36) therefore prompting the mutational screening of this gene. One recurrent variation of *INHA* (p.A257T) has been consistently found in women affected by POI of different ethnicities, with a prevalence of 0%–11% depending on the population studied. A meta-analysis of the random effects on the risk of POI in carriers of the INHA variant from the most relevant studies revealed a slight increase in the risk to develop POI in the female carriers of the polymorphism. Based on these results, INHA gene might be considered as a susceptibility locus for POI (Rossetti *et al.*, 2017).

Bone Morphogenetic Protein 15 (BMP15)

BMP15 (MIM *300247) is an oocyte-specific growth/differentiation factor involved in follicular development and regulation of many GC processes (Persani *et al.*, 2014). The main BMP15 actions include: (a) the promotion of follicle growth and maturation; (b) regulation of follicular GC sensitivity to FSH; (c) prevention of GC apoptosis; (d) promotion of oocyte developmental competence and (e) determination of ovulation quota. Consistent with a relevant role of *BMP15* gene in folliculogenesis and ovulation, ewes with heterozygous natural mutations have an increased ovulation rate, while homozygous carriers show infertility with complete block of folliculogenesis. Several data indicate that the role of BMP15 appears more critical in mono-ovulating species (such as sheep and human) than in the poly-ovulating ones (mice). However the overexpression of a biologically active BMP15 in mice leads to accelerate folliculogenesis and causes an early onset of ovarian failure. *BMP15* maps to a locus on Xp critical to ovarian reserve determination where several of the TS traits are located including ovarian failure. In women, mutations in BMP15 gene have been associated with both PA and SA in several POI cohorts with a prevalence ranging from 1.5% to 15%. BMP15 p.Y235C was the first mutation reported in association with hypergonadotropic ovarian insufficiency in two Italian sisters with PA and OD. The Y235 residue is highly conserved among species and corresponds to a site of positive selection in the hominidae clade during evolution (Auclair *et al.*, 2013). When functionally tested, this alteration enhances the BMP15-induced transcriptional activity and causes increased GC steroidogenesis. In contrast, BMP15 p.Y235C was unable to increase GC proliferation. Several other variants have been further identified with variable frequency in worldwide POI cohorts. Although some of these variants have also been found in low percentage in the control populations, a finding that may question their pathogenic role, a recent revision of the frequency of BMP15 variants in POI and control populations revealed a 10-fold higher frequency of heterozygous BMP15 variations in cases. In a recent study on X chromosome mosaicism in TS, a tandem duplication of the single BMP15 gene was identified in a patient with 45,X karyotype but spontaneous menarche followed by regular menses for 4 years. This BMP15 duplication would have enabled a small amount of functional follicles to survive atresia and reach pubertal age by means of a partial compensation to the haploinsufficiency for the other X-linked genes. Consistent with this, FISH and array-CGH experiments demonstrated that the presence of a mosaicism with the euploid cell line $>10\%$ would be sufficient to induce spontaneous menarche. Taken together, these data highlight how BMP15 gene dosage contributes to the ovarian phenotype of patients with TS and further support the hypothesis that BMP15 is an ovary-determining X-linked gene. This finally brings additional support to the idea that inactivating mutations in this gene can represent a predisposing event for POI (Persani *et al.*, 2014).

Growth Differentiation Factor 9 (GDF9)

GDF9 (MIM *601918) is homologous to the gene encoding BMP15. GDF9 expression is high in the oocytes, where its products can form noncovalent heterodimers with BMP15, which are active on surrounding follicular GC. Evidences from experimental animals have strongly suggested that GDF9 activity is crucial in poly-ovulating species: in mice, for instance, GDF9 is fundamental for folliculogenesis (Persani *et al.*, 2014). Concerning mono-ovulating species, natural GDF9 mutations have been described both in Cambridge and Belclare sheeps, where the ovarian phenotype was analogous to that seen in BMP15 mutants. In humans, several heterozygous missense GDF9 variations affecting the pro-region of the translated protein were described in POI women (prevalence of 1.4%). Some insertion/deletion or missense variants in GDF9 have been reported in mothers of dizygotic twins,

with an incidence around 4%, thus supporting GDF9 as a determinant of the ovulation quota also in humans (Persani *et al.*, 2014).

Genes Affecting DNA Replication, Meiosis and DNA Repair

Variations in genes involved in replication and repair of DNA double-strand breaks for recombination, DNA damage checkpoint control, cell cycle progression or formation of the synaptonemal complex may be associated with POI, as strongly suggested by mouse models showing a POI-like phenotype (Rossetti *et al.*, 2017).

Genetic variations have been described to affect DMC1 or LIM15 (MIM *602721), MSH5 (MIM *603382), *STAG3* (encoding a meiosis-specific subunit of the cohesin ring), SMC1β, REC8 (genes encoding protein of the cohesin complex and thus controlling sister chromatid cohesion and recombination between homologous chromosomes), POF1B (an X-linked gene located within the critical region for normal ovarian function, known to escape X inactivation, which encodes a protein that interacts with actin filaments, and the authors speculate that POF1B could have a role in the pairing of meiotic chromosomes), HFM1 (encoding a protein necessary for homologous recombination of chromosomes), PSMC3IP (MIM *608665) (another gene required for homologous recombination), MCM8 and MCM9 (encoding members of the highly conserved mini-chromosome maintenance proteins complex involved in homologous recombination and repair of double-stranded DNA breaks), *SYCE1* (gene encoding an essential component of the synaptonemal complex, where paired chromosome homologs closely associate in meiosis before crossover), NUP107 (MIM *607617) (encoding a component of the nuclear pore complex, which mediates nucleocytoplasmic transport of macromolecules such as transcription factors, thus promoting cell-specific gene-expression) (Caburet *et al.*, 2014; de Vries *et al.*, 2014; Wang *et al.*, 2014; Goldberg *et al.*, 2015; Al Asiri *et al.*, 2015). More recently, microdeletions involving the *CPEB1* gene (MIM *607342) (a gene encoding the cytoplasmic polyadenylation element-binding protein 1 involved in the regulation of translation and senescence) have been reported to play a relevant role in an oligogenic model of POI (Hyon *et al.*, 2016; Tšuiko *et al.*, 2016). Finally, another chromosomal instability disorder associated with POI is Fanconi Anemia, which can be caused by alterations in *FANCA* (MIM *607139), *FANCC* (MIM *613899) and *FANCG* (MIM *602956) genes (Rossetti *et al.*, 2017).

Transcription Factors

NR5A1

The *NR5A1* (MIM + 184,757) gene encodes for a nuclear receptor whose expression can be detected early in embryo development in bipotential gonads, where it plays a key role as a transcriptional regulator of genes involved in the adrenal and gonadal axis (Persani *et al.*, 2013; Rossetti *et al.*, 2017). Mutations of NR5A1 have been reported in cases of 46,XY disorders of sex development (DSD), with or without adrenal failure. The first evidence of an association between NR5A1 function and POI came from the detection of mutations in this transcription factor in members of four families with histories of both 46,XY DSD and 46,XX POI, as well as in 2/25 women with isolated ovarian insufficiency. The association between NR5A1 mutations and POI pathogenesis was further confirmed with a mutation frequency of 1.6%. Patients carrying NR5A1 mutations show a wide spectrum of ovarian anomalies, ranging from SA to PA, or even gonadal dysgenesis.

SOX8

Variations in *SOX8* gene have very recently been reported in women POI (Portnoi *et al.*, 2018). SOX8 is an HMG-box transcription factor closely related to SRY and SOX9. Deletion of the gene encoding Sox8 in mice causes reproductive dysfunction. SOX8 is expressed in the somatic cells of the early developing gonad in the human and influences human sex-determination. In fact, several individuals with 46,XY DSD were found to carry variable genetic variations leading to SOX8 defects.

Oocyte specific transcription factors

NOBOX (newborn ovary homeobox) (MIM *610934) and *FIGLA* (factor in germline alpha) (MIM *608697) both encode for oocyte-specific transcription factors. NOBOX is a homeodomain-containing, oocyte- and granulosa cell-specific protein able to directly regulate the expression of key oocyte-specific factors such as Gdf9, Oct4 and KIT-L. Heterozygous NOBOX mutations have been reported in women with sporadic POI of African and Caucasian origin at a prevalence of ~6% (POF5, OMIM #611548), suggesting to consider NOBOX as the first autosomal candidate gene involved in POI. Vice versa, mutations in the homeobox domain of NOBOX seem not to be common explanations for POI in Asian women. We recently documented that several NOBOX mutants form intracellular aggregates and are unable to enter the nucleus and activate transcription. Interestingly such mutants conserve the ability to interact with FOXL2, thus presumably reducing also its access to the nucleus (Ferrari *et al.*, 2016).

FIGLA is a basic helix-loop-helix transcription factor involved in the regulation of the expression of zona pellucida genes. A mutational study conducted on 100 Chinese women affected by POI revealed two heterozygous deletions in two unrelated cases. Functional in vitro studies confirmed that both variants might have a pathogenic role. Two additional variants, p.R83C (positioned within the functional domain bHLH) and p.S141T (located outside the functional domain, but possibly impairing the protein–protein interaction between FIGLA and TCF3), were further identified in a cohort of Indian women with POI. Both variants were predicted as potentially pathogenic and disease-causing by in silico analysis, but were not experimentally tested.

Recently, thanks to the NGS approach a rare loss-of-function variant (p.A41V) has been identified in 1 out of 100 women with idiopathic POI (Rossetti *et al.*, 2017).

Hormone Replacement Therapy (HRT)

In females with pubertal delay and PA due to POI estradiol supplementation should be started as soon as possible after diagnosis (Christin-Maitre, 2017). ESHRE guidelines and TS management guidelines recommend to start with low dose of transdermal estradiol followed by a progressive increase every 3–4 months depending upon the age and clinical evaluation of the girls (Webber *et al.*, 2016; Gravholt *et al.*, 2017). Oral progesterone should be given when an adequate uterine growth and thickening have been detected at US. Cyclic HRT is generally preferable in young POI patients, who should also be involved in the choice of the route of administration. Oral hormonal contraceptives may have positive psychological and social implications in young POI women.

The transdermal route should in principle be advised in POI women beyond 40 years of age because HRT should then be recommended to all POI women at least until the age of physiological menopause, since these women are at higher risk for osteopenia, osteoporosis, and accelerated atherogenesis (Atsma *et al.*, 2006; Gallagher, 2007). Cyclic or continuous estrogen and progestogen relieve symptoms of hypoestrogenism and maintain bone density. Additional benefits of estrogen therapy include potential cardioprotective effects, delayed onset of Alzheimer's disease, decreased incidence of colorectal cancers, and improved vulvovaginal and urethral tissue structure and function.

Absolute contraindication to HRT should be reserved only to POI women with breast cancer. The carriers of BRCA1–2 variants should receive HRT after prophylactic breast surgery. Other conditions like migraine, hypertension or increased thromboembolic risk do not represent absolute contraindications to HRT in POI women but particular caution should be applied in order to prevent the associated risks. Breast examinations with sensitive radiological techniques should always be recommended.

Low androgen supplementation can be useful to alleviate the sexual dysfunction. Calcium supplementation and weight-bearing exercise are also important for skeletal health.

Importantly, psychological support should be provided to patients afflicted with this difficult diagnosis. Frequently, these women feel that fertility potential is taken away from them before they have even had the option to consider their reproductive desires. This can be devastating and threatening to their self-esteem. Thorough education regarding their general health and fertility options is important. In certain circumstances, individual counseling may also be appropriate (Webber *et al.*, 2016; Gravholt *et al.*, 2017; Christin-Maitre, 2017).

Fertility Defect

The natural history of premature ovarian failure includes spontaneous pregnancies in 10% of women after diagnosis. Hormone replacement therapy does not provide birth control and those not desiring pregnancy should be placed on oral contraceptives. Undetectable AMH instead indicates the loss of ovarian reserve.

The infertility represents a major issue in the POI women. Nowadays, oocyte donation is the only solution if AMH is undetectable. Implantation rates using young oocyte donors are very favorable and pregnancy outcome in these women who have donated oocyte-derived embryos is excellent.

Early diagnosis or prediction of POI may instead open other possible strategies (Gago and Ginsburg, 2004; Martin *et al.*, 2017). These include harvesting of immature oocytes with their in vitro maturation and co-culture prior to in vitro fertilization. The prospective identification of women at risk for developing POI can instead allow the oocyte harvest and cryopreservation for subsequent use before the onset of clinical ovarian failure. Because of the frequent familial recurrence of POI, this strategy should be recommended in young daughters or close female relatives of a POI woman and can be further improved if a genetic diagnosis is reached (Rossetti *et al.*, 2017; Webber *et al.*, 2016).

Conclusion

As mentioned above, prospective cryopreservation of primordial follicles may become a more practical option in the future for women at risk of developing POI. This availability stresses the importance of early diagnosis and specific testing of women with POI and their younger female relatives. With increasing capabilities in molecular medicine and diagnosis, genetic therapies to correct the dysfunction or prevent the follicular loss can be envisioned. Improved measures of protecting primordial follicles during cytotoxic therapies and the ability to preserve primordial follicles in vitro and mature them successfully are also therapeutic strategies under intense investigation. Specific therapy for autoimmune POF—which could include highly specific blocking antibodies that would potentially prevent the follicular dysfunction and loss and avoid the adverse systemic effects associated with corticosteroids and other immunomodulatory therapies—may become possible in the future.

Other possible interventions include the in vitro activation (IVA) by AKT stimulators of residual dormant follicles potentially present in ovarian biopsies from POI patients (Zhai *et al.*, 2016). Ultimately, the reconstitution of complete oogenesis was obtained from induced pluripotent stem (IPS) cells in the mouse (Hikabe *et al.*, 2016). These novel technique would represent a

potential alternative for poor responders to gonadotropin stimulation but may probably prove unsuccessful in POI women affected with oogenesis defects unless coupled to gene editing techniques.

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Adult Care of Turner Syndrome

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Introduction

Knowledge of Turner syndrome (TS) is expanding rapidly, especially aspects concerning adult care are recognized, and new international guidelines have recently been published (Gravholt *et al.*, 2017). The syndrome only affects females and clinical care emphasize the need for multidisciplinary care, involving several specialties including genetics, embryology, pediatrics, gynecology and obstetrics, fertility, endocrinology, cardiology, radiology, otorhinolaryngology and ophthalmology.

Here, we review the diverse and complex clinical aspects encountered throughout adulthood in TS.

Epidimiology

Turner syndrome (TS) is a congenital syndrome, present in approximately 1 in 2000 live born girls (Nielsen and Wohlert, 1990; Stochholm *et al.*, 2006). The 45,X karyotype is still the most common karyotype identified, however during the last decades the number of females diagnosed with mosaic TS (45,X/46,XX) has increased (Stochholm *et al.*, 2006). Other karyotypes exist, including ring X chromosomes, iso chromosomes, deletions of a part of the X chromosome, or karyotypes including part of a Y chromosome. Only 30%–66% of all with TS are ever diagnosed, with a wide range of age at diagnosis, from prenatally to after postmenopausal age (Stochholm *et al.*, 2006). There is no obvious explanation for this lack of diagnosis, but new avenues for better diagnostics, such as neonatal screening programs using genome sequencing should be put in place (Gravholt *et al.*, 2017; Murdock *et al.*, 2017). The median age at diagnosis in general is 15 years, lower in those with a 45,X karyotype or a mosaic karyotype, and higher in those with "another" karyotype. Generally, the phenotype of the mosaic TS person is milder, however the overall health status varies greatly within the karyotypes and one cannot predict the phenotype from the karyotype.

Of those diagnosed, the majority is diagnosed at birth, probably due to typical TS clinical findings such as a combination of webbing of the neck, oedema of hands and feet, low set ears, and a low birth weight. During adolescence, diagnosis is due to the late or lacking pubertal development and/or absent menarche, or due to low height. Similarly, in young adulthood, diagnosis is most likely due to infertility. However, diagnosis is possible at all ages. With the increasing use of prenatal diagnosis, an identification of an increasing number of diagnosed TS fetuses is expected (Viuff *et al.*, 2015). A postnatal karyotype is always recommended for confirmation. Convincing data on the prenatally diagnosed population is lacking; however, it is most likely that their phenotype *is* affected but possibly milder than in the postnatal diagnosed population (Gunther *et al.*, 2004). Among the TS fetuses, there is a high degree of spontaneous abortion, also late abortions. Danish data show that in prenatally diagnosed TS fetuses with a median gestational age of 14 weeks, $>80\%$ with 45,X karyotype and $>40\%$ with other TS karyotypes are legally aborted (Viuff *et al.*, 2015).

Most women with TS are of average intelligence, however about 10% have some degree of intellectual disability. Most function better verbally than perceptually, and language functioning is average to above average. The most frequent cognitive problem is visuospatial, such as finding way on a map. Other problems are deficits when switching attention, reduced processing speed, social cognition (recognizing faces etc.) and attention deficits. Further, social isolation, fewer friendships, and limited sexual experience is more common in TS (Boman *et al.*, 2004; Fjermestad *et al.*, 2016; Amundson *et al.*, 2010), and sexual functioning seem slightly reduced compared to controls although data differ. They leave their parents at an older age compared to their peers, and a reduced number of TS women is in a relationship. The degree of education is similar to or higher in TS women compared to their peers. Income is lower than expected in the younger years (Stochholm *et al.*, 2012; Gould *et al.*, 2013); however, among those working there is a catch-up in the late twenties; hereafter income is similar to age-matched controls. Due to the hypogonadism and social factors, fertility is reduced, however more and more become mothers, typically using oocyte donation (Hagman *et al.*, 2011). Women with TS retire at a younger age and more frequently compared to age-matched controls. Data show that those diagnosed >30 years ago differ from those diagnosed more recently. Those diagnosed recently have more often been in a relationship, have a higher education, they become mother more often, and they retire later. It is noteworthy that the more recently diagnosed group includes relatively more with a mosaic karyotype.

Compared to the background population, morbidity and mortality is significantly increased (Schoemaker *et al.*, 2008a,b; Stochholm *et al.*, 2012; Gravholt *et al.*, 1998a). The increased morbidity in TS includes a wide range of diseases, such as recurrent otitis media in the child, congenital malformations, renal abnormalities, cardiovascular abnormalities, abnormal or absent pubertal development due to hypogonadism, hearing loss, osteoporosis, skin disorders including oedema, infertility, hypothyroidism, adiposity, type 2 diabetes, hypertension, and autoimmune diseases (Gravholt *et al.*, 1998a). There is more often need for correction of refractory errors due to hyperopia and myopia. Cancer is not increased in TS; especially the risk of breast cancer is low (Schoemaker *et al.*, 2008a). However, rare

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tumors such as gonadoblastomas are more frequent. It is found in TS women whose karyotype includes part of a Y chromosome and prophylactic gonadectomy is recommended. A Dutch study in TS women with a mean age of 31 years identified >30% lacking medical follow-up at first screening at a multidisciplinary clinic (Freriks *et al.*, 2011).

Mortality is significantly increased in TS, with a mortality three times higher than age-matched female controls at all ages, and highest in the 45,X karyotype group. In line with the almost universally increased morbidity, mortality is increased due a wide range of diseases, especially cardiovascular disorders including aortic dissection, see later. Mortality is increased in almost all diagnosis chapters in the International Classification of Diseases 10th edition, when compared with the female population in general. It is expected that mortality will decrease and hopefully normalize with careful follow-up, however sound data are still lacking.

Although morbidity and mortality is increased, quality of life (QoL) or life satisfaction seem rather unaffected in TS. Various studies show different results including a QoL worse, similar to or better than compared to controls. Thus, findings include: higher satisfaction with leisure and economy; lower self-esteem; more life strain, and better health related quality of life on social functioning. QoL seem better in those reporting normal puberty.

Cardiovascular Disorders

Congenital Heart Disease

TS is associated with a significant higher prevalence of congenital heart disease and aortic dissection than the normal population. There is an unexplained relation between the 45,X karyotype and development of the left-sided heart congenital malformations. The major common denominator is the presence of a bicuspid aortic valve, which is seen in >15%–30% of an adult Turner population (Mortensen *et al.*, 2012). Along with aortic valve disease, Turner syndrome is associated with coarctation of the aorta, aortic arch anomalies and abnormal head and neck vessels, mitral valve disease, ventricular septal defects, subaortic obstruction, abnormal pulmonary vein drainage, presence of a left caval vein, and coronary anomalies (Mortensen *et al.*, 2012a,b, 2016; Viuff *et al.*, 2016; Ho *et al.*, 2004).

Most congenital heart defects of an adult population will already have been diagnosed during fetal and pediatric care, however, the rarer defects with low morbidity may have been missed and can cause symptoms in adult life, like abnormal pulmonary vein drainage or abnormal coronary anatomy (Mortensen *et al.*, 2016; Viuff *et al.*, 2016; Gutmark-Little *et al.*, 2012).

For this reason, it is important to do a detailed cardiovascular examination when approaching the adult Turner woman who is either diagnosed for the first time, or has transitioned from pediatric care (Gravholt *et al.*, 2017). A detailed echocardiogram may in most cases be enough to determine aortic valve morphology, coronary and aortic arch morphology and the pulmonary vein anatomy. If not so, it is important to do either a magnetic resonance scan (MRI) (Mortensen *et al.*, 2011, 2013) or at computed tomography (CT-scan) to determine the cardiac anatomy anomalies (Mortensen *et al.*, 2016).

Aortic Disease

In early adult life, aortic dilatation and eventually aortic dissection is seen in significant numbers (Ho *et al.*, 2004; Gravholt *et al.*, 2006c). In clear contrast aortic dissection is a disease mostly seen among the elderly in the normal population, (Craiem *et al.*, 2017). This is probably why aortic disease is misdiagnosed and mistreated in young TS women, where the significance of aortic dilatation is underestimated and prophylactic surgery is not offered in time (Ho *et al.*, 2004; Gravholt *et al.*, 2006c).

The presence of a bicuspid aortic valve is a major determinant for aortic dilatation, as well as for the presence of elevated diastolic blood pressure, and aortic arch anomalies (Mortensen *et al.*, 2013).

Medical management of aortic dilatation has never been thoroughly examined neither in the general population or a TS population. For this reason, the common recommendation is blood pressure reduction, preferably with a betablocker, and close follow-up. If the aorta becomes significantly enlarged, prophylactic surgery should be offered with aortic root replacement, to avoid a type A–dissection (Gravholt *et al.*, 2017). Type A dissection is defined as a dissection of the proximal ascending part of the aorta, while type B dissection occurs in the distal or descending part of the aorta.

A smaller part of TS women with aortic disease (10%) develop a type B dissection. Type B dissection is also associated with hypertension, but may also originate from an aortic coarctation without hypertension (Di *et al.*, 2012). This emphasizes the importance of a full assessment of the aortic size in women with TS. Since repeated scans are important, this assessment is best done with MRI and assessed by cardiologist with special interest in TS.

In TS women with normal aortic size, full MRI-scans should be done every 3–5 years, depending on aortic valve morphology and the presence of either a repaired aortic coarctation or arch anomalies. Some TS patients may need a closer follow-up (Gravholt *et al.*, 2017).

Hypertension

Hypertension is a common phenomenon in TS. The true prevalence is uncertain but up to 50% of an adult population may have arterial hypertension (De Groote *et al.*, 2015). The cause of hypertension in TS is multifactorial. The high prevalence of overweight

and obesity in TS contributes significantly to the hypertension disease burden as well as the association to both type 1 and type 2 diabetes (Mortensen *et al.*, 2012). At present it is not known which anti-hypertensive treatment is most effective.

Congenital defects like repaired aortic coarctation or aortic arch hypoplasia or congenital kidney disease also contribute to the number of hypertensive TS patients (De Groote *et al.*, 2015).

The more unresolved issues in TS, like estrogen deficiency, increased intima media thickness (Mortensen *et al.*, 2012), aortic stiffness, and high sympathetic activity may also increase the blood pressure in women with TS. Direct causal links between these factors and the presence of hypertension is yet to be found but numerous studies implicate that these complex factors play a role in blood pressure regulation.

Ischemic Heart Disease and Stroke

Adult TS women also have a high prevalence of ischemic heart disease (Schoemaker *et al.*, 2008b). The cardiovascular morbidity and mortality in adult life is significantly higher in adult TS women, compared to the background population (Stochholm *et al.*, 2006), and comorbidity plays a significant role. The lean and well-controlled TS woman may never develop ischemic heart disease. However, the TS women with type 2 diabetes, overweight, poor estrogen substitution through life and with hypertension is very likely to develop coronary heart disease in very young age. It is therefore important to assess the TS patients risk factors and motivate the patient to exercise, weight loss, and to sustain from smoking. Additional risk factors as dysregulated diabetes, elevated blood pressure and hypercholesterolemia are also important to treat.

There is not a particular dyslipidemia associated with TS, but many patients develop an unhealthy lipid profile due to obesity, diabetes, or poor estrogen substitution (Landin-Wilhelmsen *et al.*, 2001; Gravholt *et al.*, 2000). Therefore, it is important to have a multifactorial approach to the women with TS, who presents with an unhealthy risk profile and the task will often include a cardiologist, an endocrinologist and a dietitian (Gravholt *et al.*, 2017).

Cerebrovascular disease (stroke) is also more common in TS. This is probably due to the excessive presence of hypertension, but disturbances in thrombosis and fibrinolysis in TS can also be the cause of a thromboembolic strokes. The risk of thrombotic disease in TS has never been addressed in large studies, but there seems to be an increased risk of thrombus formation (Gravholt *et al.*, 2012). On the individual level, many TS patients may have procoagulant levels of clotting and fibrinolytic factors. Especially, fibrinogen has been found elevated in approximately two thirds of females with TS, and proteins C and S have also been found in significantly lower concentrations (Gravholt *et al.*, 2012). Nevertheless, this must not deter the treating physician from treating TS women with hormone replacement therapy, consisting of 17β-estradiol and a progestin normally given in a sequential manner, since no data support that there is a specific risk of thromboembolism in TS patients in estrogen treatment.

Arrhythmia

Women with TS often have an excessive sympathetic drive. Therefore, it is not uncommon that the women with TS has an increased resting heart rate (Gravholt *et al.*, 2006a). The patients are mostly asymptomatic, but some may sense that their pulse is high.

Presence of a prolonged QT-interval is also a common electrophysiological phenomenon in TS. This can easily be missed in patients with a high resting heart rate, therefore it is advised to calculate the corrected QT-interval according to Hodge's formula over Bazett's formula because it takes higher heart rates into account (Gravholt *et al.*, 2017). The mechanism behind QT-prolongation is unresolved but an increased number of patients with polymorphisms in the QT-genes have been found among TS women with prolonged QT-intervals (Trolle *et al.*, 2013). The clinical significance of prolonged QT-intervals is unresolved, but there may be a risk in treating women with TS with QT-prolonging drugs (Nielsen *et al.*, 2017).

Ovarian Failure

TS is usually accompanied by hypergonadotropic hypogonadism leading to low levels of estrogens and compared to healthy girl's lower levels of androgens (Gravholt *et al.*, 1999; Apter *et al.*, 1982). The ovarian failure leads to pubertal delay, primary or secondary amenorrhea, poor development of secondary sex characteristics, impaired sexual functioning and in most cases infertility.

The phenotype differentiates depending on karyotype, as 45,X show more pronounced symptoms. A higher percentage of mosaicism results in milder symptoms and lesser ovarian failure.

The etiology and genetics of the ovarian insufficiency is still being investigated, with different theories. One theory is lack of a particular X-linked gene that require double dosages. Another suggesting an epigenetic component (Trolle *et al.*, 2016).

Puberty can be divided into two independent events: gonadarche and adrenarche. During gonadarche the ovaries start producing sex hormones, and thelarche (development of breasts) is usually the first sign. During adrenarche, the maturation and secretion of adrenal androgens, initiates the growth of pubic and axillary hair (pubarche). This occurs independently of the hypothalamic–pituitary–gonadal axis.

The gonadal functioning in TS is extremely variable and dependent of karyotype. Approximately 20% of monosomy X enters puberty spontaneously, compared to 70% of mosaics. About one-third of these develops secondary failure. Pubarche is in most cases the first sign of puberty, compared to 46,XX girls where the first feature is thelarche (Negreiros *et al.*, 2014).

About one third of girls with TS has spontaneous breast development (Tanaka *et al.*, 2015; Negreiros *et al.*, 2014; Pasquino *et al.*, 1997). Regular menstrual cycles occur in 6% of these subjects (Pasquino *et al.*, 1997). Mosaic karyotypes were associated with similar preservation of ovarian function, as determined by the frequency of spontaneous menarche (Tanaka *et al.*, 2015).

TS women are most often infertile, this being the greatest factor influencing their quality of life (Sutton *et al.*, 2005). Very few can become pregnant with own oocytes. Most females with TS are likely to experience premature ovarian failure and hence less likely to become pregnant.

Spontaneous pregnancies occur in 4.8%–7.6% of women with TS (Bryman *et al.*, 2011; Bernard *et al.*, 2016), however one study showed that of the fertile women registered with 45,X/46,XX, several had 45,X in $<10\%$ of analyzed cells, indicating that some of these women might not have many, if any, signs of TS (Birkebaek *et al.*, 2002).

According to the new international TS guidelines (Gravholt *et al.*, 2017), it is recommended to initiate estrogen replacement when the patient is between 11 and 12 years of age, increasing to adult dosage over 2–3 years. Low-dose estradiol is recommended and transdermal administration is preferred due to lesser side effects compared with oral use. Once bleeding occurs, progesterone should be added.

With adequate hormone replacement, it is possible to stimulate uterine growth, which will become susceptible to oocyte donation from a foreign donor. In mosaic TS women pregnancy after FSH-stimulated oocyte retrieval, freezing of own oocytes until the female becomes older, followed by IVF treatment is possible, if it is initiated in a young age before ovarian failure (Borgstrom *et al.*, 2009; Hovatta, 2012). Counseling not to postpone pregnancy is advisable.

If pregnancy is accomplished, TS women experience a higher number of complications and miscarriages. A study reported a complication rate of up to 45% (Bryman *et al.*, 2011; Bernard *et al.*, 2016). It is also clear that pregnancy after oocyte donation carries a higher risk than the rare spontaneous pregnancy among TS women (Chevalier *et al.*, 2011).

Endocrine Disorders

The risk of both type 1 and type 2 diabetes mellitus is increased (Gravholt *et al.*, 1998a). In a general cohort of patients with TS, fasting glucose levels tend not to be different from healthy peers but fasting hyperinsulinaemia is present and impaired glucose tolerance has been found in 25%–78% of adult patients with TS (Gravholt *et al.*, 1998b; Bakalov *et al.*, 2004). In addition to higher glucose levels during oral glucose loading, the insulin response is altered and a delayed insulin peak is seen. The impaired glucose homeostasis seems to be partly explained by an attenuated "first phase insulin response" which could be viewed as an inappropriately low β-cell response (Gravholt *et al.*, 1998b; Bakalov *et al.*, 2004). Body composition is altered in TS with increased BMI, decreased muscle mass and increased total fat mass and visceral fat mass (Gravholt *et al.*, 2006b; Elsheikh and Conway, 1998). A relatively sedentary lifestyle and decreased physical fitness has also been demonstrated (Landin-Wilhelmsen *et al.*, 1999). These factors may be causally linked with reduced insulin sensitivity, although this trait has only been shown in some (Caprio *et al.*, 1991; Salgin *et al.*, 2006; Stoppoloni *et al.*, 1990), but not all studies (Hjerrild *et al.*, 2011; Bakalov *et al.*, 2004), and linked with manifest diabetes (Gravholt *et al.*, 1998a; Bakalov *et al.*, 2009).

Appropriate estrogen replacement also seems to be important for glucose homeostasis even though the findings in TS diverge. Exogenous estrogen reduced fasting glucose and fasting insulin in TS (Elsheikh *et al.*, 2000), and increased fat free mass and physical fitness, although insulin sensitivity was not improved (Gravholt *et al.*, 1998b). In the latter study, a decreased glucose tolerance was seen during HRT (Gravholt *et al.*, 1998b). On balance, HRT may slightly improve glycemic control though more studies are necessary to elucidate the relation between glucose metabolism and states of deficiency of and replacement with estrogens in TS.

Insulin levels increase during GH treatment, given for growth-promoting reasons during childhood and adolescence, decrease after termination of treatment but remain higher than pre-treatment levels (Sas *et al.*, 2000). GH generally reduces insulin sensitivity in the first 6–12 months of treatment, where after it stabilizes. This stabilization could be due to changes in body composition with increasing lean body mass and decreasing fat mass. The proportion of patients with TS patients with overtly impaired glucose tolerance does not seem to increase and HbA_{1c} remains unchanged or even decreases during GH therapy (Sas *et al.*, 2000). While most of the effects on the glucose metabolism seem to reverse after cessation of GH treatment, the long-term effects of hyperinsulinism and insulin resistance induced during GH delivery are not known.

In the face of widespread abnormalities of glucose homeostasis and increased risk of type 1 and type 2 diabetes mellitus there is a need for persistent attention to these factors in clinical follow-up. Recommendations for diagnosis and treatment of diabetes adhere to general population guidelines and annual screening of fasting glucose and HbA1c should be performed (Gravholt *et al.*, 2017).

Autoimmune thyroid disease is very frequent in TS, with hypothyroidism occurring in up to 50% of women at the age of 50 years (El Mansoury *et al.*, 2005). Hyperthyroidism also occurs more frequently than among controls. Treatment follows clinical guidelines on thyroid diseases. The occurrence of all other autoimmune diseases is also increased in comparison with the background population and the reason for this increase remains obscure (Jorgensen *et al.*, 2010). Therefore, the clinician should be vigilant and examine TS women at the slightest suspicion. Currently, there are recommendations for how a clinical care program

should be tailored for adults with TS (Gravholt *et al.*, 2017). Diseases like coeliac disease, inflammatory bowel disease, and rheumatoid disease occur sufficiently frequent to warrant special attention.

Peak bone mass depends on several factors, such as genetic background, nutrition, physical activity, local growth factors and a spectrum of hormones. Estradiol secretion in TS is deficient already in childhood and adolescence, and children and younger and middle-aged adult patients with TS have low bone mineral density, and studies show that the risk of fracture and frank osteoporosis is increased (Gravholt *et al.*, 1998a, 2003; Ross *et al.*, 1991). Estrogen substitution therapy is crucial in order to induce maximal peak bone mass in adolescents and young adults and avoid a rapid decline in density (Bertelloni *et al.*, 2000). TS with spontaneous menstruation have normal BMD, while absent menarche associates with a reduced BMD (Carrascosa *et al.*, 2000). A 3-year longitudinal study of 21 women with TS (age 20–40) showed marked improvements of estrogen on bone structure (Khastgir *et al.*, 2003) resulting in estradiol levels comparable to levels in premenopausal women. The bone biopsies pointed

Table 1 Guidelines for adult health surveillance

	Action	*Suggested frequency*	*Comments*
Obesity	Weight	Annually	Many co-morbidities are weight-related, for example, diabetes, elevated cholesterol, and liver dysfunction. Weight management is the most important health intervention at annual visits. Obesity may be due to low physical fitness, sedentary lifestyle, and poor food choices
Cardiovascular	Echocardiogram	3–5 years—yearly if aortic root >3 cm	Management shared with GUCH (grown-ups with congenital heart defects) clinic preferable. Congenital malformations include bicuspid aortic valve, coarctation of the aorta, and aortic dilatation
	MRI aorta	As appropriate	Some units use echocardiography for routine monitoring and reserve MRI for ambiguous findings or as part of pre-pregnancy assessment. MR is always recommended if available.
	Blood pressure	Annually	Hypertension affects up to 50% of young adults and contributes to the risk of aortic dissection. Refer to age-specific reference data. Hypertension can be treated to normal guidelines including use of beta blockers or angiotensin-receptor blockers. For aortic root >3.0 cm and BAV, aim for systolic blood pressure <140 mmHg if tricuspid aortic valve or <120 mmHg if bicuspid valve
Bone metabolism	DEXA scan	Every 5 years	Estrogen replacement required until ~ 50 years (or older if there have been many years of estrogen deficiency) to prevent osteoporosis. Bone density of spine reads low in short stature with DEXA. Osteoporosis can be treated as in other situations
	Vitamin D and calcium profile	3–5 years	Monitor bone profile in those with low calcium and low vitamin D levels; exclude celiac disease (see below)
Liver	Liver function tests	Annually	Liver enzymes, especially gammaglutamyl transaminase (GGT), are commonly elevated. Slowly progressive, but improves with estrogen and weight loss. Consider viral screen for acute changes (rarely positive)
	Liver ultrasound	As appropriate	Liver US required for markedly raised GGT, alkaline phosphatase or transaminases. Consider special scans measuring fibrosis and steatosis, and biopsy if structural defects identified on UL
Diabetes	HbA1c $\pm$ fasting plasma glucose	Annually	Consider OGTT if HbA1c is elevated. High risk of developing impaired glucose tolerance (50%) due a combination of insulin deficiency and insulin resistance. Fasting plasma glucose underestimates defect of insulin secretion
Fertility	Adoption and oocyte donation education	As appropriate	Spontaneous pregnancy occurs in 2%–5%. Ovarian failure occurs in 90%. Medical review on advisability of pregnancy with regard to risk of aortic dissection is required
	Uterine ultrasound	As appropriate	US of the uterus should take place on arrival in the adult clinic and again during the work-up for pregnancy
Psychological	Review psychological issues	As appropriate	Increased risk of social isolation, anxiety, and obsessive behavior. Higher levels of shyness and social anxiety, and reduced self-esteem. Review problems in work place or in relationships. Problems are responsive to clinical psychology support.
Audiology	Audiogram ENT History	3–5 years	Deafness is common and under reported. Self-reporting unreliable. Otitis media is common in childhood (60%–80%) which can lead to conductive hearing loss. Sensori-neural hearing loss common and progressive in adults
Dermatology	Skin inspection	Annually	Assess for keloid and changes and in pigmented nevi
Orthodontics	Teeth inspection	Annually	Referral recommended if required
Blood tests	Thyroid function	Annually	Increased risk of autoimmune thyroiditis. In hypothyroidism (24%) or hyperthyroidism (2.5%) include TPO antibodies if previously negative
	Celiac screen	With suggestive symptoms	Increased risk (4%–6%) of celiac disease. Check transglutaminase IgA antibodies (and total IgA) and vitamin B12

From Gravholt, C.H., Andersen, N.H., Conway, G.S., Dekkers, O.M., Geffner, M.E., Klein, K.O., Lin, A.E., Mauras, N., Quigley, C.A., Rubin, K., *et al.* (2017). Clinical practice guidelines for the care of girls and women with Turner syndrome. Proceedings from the 2016 Cincinnati international Turner syndrome meeting. European Journal of Endocrinology 177, G1–G70, with permission.

towards an anabolic effect on the skeleton of high-dose estradiol substitution therapy in young adult patients with TS (Khastgir *et al.*, 2003), and growth hormone treatment may also improve bone mineral density in TS (Sas *et al.*, 2001). No very long-term studies of the effect of estradiol have been published but 5 years of appropriate HRT maintain BMD unchanged (Cleemann *et al.*, 2009). Furthermore, the optimal dosage of estrogen during adult life has yet to be determined, but recent recommendations should be followed (Gravholt *et al.*, 2017). Vitamin D-deficiency is frequent and appropriate substitution should be in place, and general recommendations on a bone healthy life style should be advised.

Co Morbidity

A wide range of comorbidities can be seen in TS and it is therefore helpful to assemble a multidisciplinary team to enable pervasive care (**Table 1**).

Hearing impairment is frequent and most TS will experience early hearing loss compared with controls and many times necessitate the need for hearing aids. The hearing loss is multifactorial. During childhood otitis media is frequent and during adulthood, many experience sensorineural hearing loss and some also conductive hearing loss.

Conclusions

Patients with TS are in need comprehensive monitoring and care preferably delivered by a multidisciplinary team. This has recently been very clearly documented in a study where the authors followed clinical guidelines in their clinical setup and found a substantial and hitherto undiagnosed morbidity in a large cohort (Freriks *et al.*, 2011), and this can be most optimally handled with centralized care. Glucose metabolism, weight, thyroid function, bone metabolism, blood pressure, liver function and cardiovascular status should be regularly assessed. Estrogen deficiency should be treated, preferably with natural estrogens and a progestin, and growth hormone treatment should be commenced early in life. Accompanying medical problems should be detected and aggressively treated.

Unfortunately, an array of important clinical questions is left to be answered regarding optimal care in patients with TS. There is a substantial deficit in our understanding of the syndrome but a hope to improve patient outcome through not only a specialized multidisciplinary clinical approach but also via a continuous effort to span disciplines in future research.

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Initial Evaluation of the Infertile Couple

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Introduction

Infertility affects around 13%–15% of couples worldwide (Mascarenhas *et al.*, 2012), seriously impacting their life and representing a common clinical issue as well as a public health problem. Infertility is clinically defined as "the inability of a couple to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse." For healthy young couples, the probability to conceive per reproductive cycle is about 20%–25% and the cumulative probabilities of conception approximate 90% within the first year (Kamel, 2010).

Infertility may be primary, occurring in couples with no previous history of conception, or secondary. The most common causes in developed countries are the result of female factors in 30% of cases, male factors in 20%, combined male and female in 40%, and in approximately 10% of couples no obvious etiologic factor can be detected (unexplained infertility).

Before applying any therapy, an evidence-based diagnostic will be performed in both members of the couple, relying on medical history, physical examination, and appropriate investigations (semen analysis, ovarian function, and female genital tract). An efficient and complete initial evaluation is essential to identify and prove the cause(s) of infertility, provide accurate information, and choose the most adequate treatment. Emotional aspects of reproductive disorders should never be overlooked and a psychosocial support should be available besides medical care.

Evaluation of Female Infertility

For women, the initial assessment should include at least hysterosalpingography, hormonal assays, and pelvic ultrasound with antral follicle count.

When to Start the Infertility Work-Up?

Time is essential: too many couples complain about a long delay in effective management. Infertility is classically defined by the absence of pregnancy after 2 years of unprotected regular intercourse. However, it is currently recommended to start infertility evaluation:

- After 1 year of regular intercourse before the women's age of 35, the majority of pregnancies occurring in the first 6 months,
- After 6 months between 35 and 37,
- Immediately from the age of 38 or in case of a major cause of infertility already known: endometriosis, genital malformation, bilateral salpingectomy, azoospermia.

Where to Start the Infertility Work-Up?

The first tests may be prescribed by a general practitioner or a gynecologist. Specialized structures such as Fertility Assessment and Counseling (FAC) Clinics are also available in some countries to inform women about their fertility and give them preventive advice (Birch Petersen *et al.*, 2017).

History

Women's age is of paramount importance: desire to succeed in professional careers, lifespan prolongation, and second unions drive more and more women to delay their first pregnancy. Older age is associated with a reduction in the pregnancy rate and an increase in miscarriages, mainly due to deterioration of the ovarian reserve and oocyte quality. Uterine aging comes later and has more limited effects, as shown by oocyte donation model. Age of the male partner and reduction in the intercourse frequency are also involved. Many infertility cases can only be explained by advanced female age, raising the question of the limits between physiological fertility decline and so-called unexplained infertility (Somigliana *et al.*, 2016).

It is also necessary to search for information about:

- Duration of infertility,
- Family and personal genetic, oncologic, or thromboembolic events,
- Weight, height, smoking, previous surgery, chemotherapy or radiotherapy,
- Gynecological history: in utero exposure to diethylstilbestrol, age at first menstruation, contraception (modalities, complications), past pregnancies, infections, pelvic pain,

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- Cycle features: duration (short/long in favor of ovarian insufficiency/polycystic ovaries), regularity, periods of amenorrhea, signs of lack of estrogens (hot flushes),
- Sexual intercourse frequency, programming, dyspareunia, or other sexual troubles,
- Lifestyle: smoking, alcohol, or other drugs use.

Physical examination

- Height, weight, and BMI,
- General morphology, secondary sexual characteristics, hirsutism,
- Malformation of the vulva or vagina,
- Congenital malformation of the womb, myomas, adenomyosis, fixed uterine retroversion suggestive of pelvic endometriosis,
- Adnexal pain at uterine mobilization (chronic or acute inflammation), increase in volume (ovarian cyst, hydrosalpinx),
- Galactorrhea in favor of functional or organic hyperprolactinemia.

Ovarian function assessment

Basal body temperature chart reflects the plasma progesterone pattern during the cycle:

- Normoovulatory cycle: middle of the cycle fast shift in 1–2 days, followed by a plateau of about 10 days,
- Dysovulatory cycle: delayed and slow shift, short and irregular plateau,
- Anovulatory cycle: temperature constantly below 37°C.

Vaginal ultrasound on the third day of the cycle evaluates:

- Volume and appearance of ovaries: polycystic ovaries, ovarian atrophy,
- Antral follicles count for ovarian reserve evaluation, taking into account previous oral contraception (Letourneau *et al.*, 2017).

Midluteal phase serum progesterone: low in dysovulation or anovulation.
Plasma hormone assays on the third day of the cycle:

- Anti-Müllerian hormone: >2 ng/mL (low in early ovarian failure), a good predictor of ovarian response in controlled ovarian stimulation, not of pregnancy (Meczekalski *et al.*, 2016),
- FSH (follicle-stimulating hormone): between 1 and 6 mIU/mL (high in early ovarian failure),
- LH (luteinizing hormone): between 1 and 6 mIU/mL (high in polycystic ovaries),
- Estradiol: <80 pg/mL (high in early ovarian failure or hormones producing ovarian cyst),
- Prolactin: <20 ng/mL (high in some drugs intake or pituitary prolactin secreting adenomas),
- Testosterone: <0.6 ng/mL and delta-4 androstenedione <2.5 ng/mL (high in polycystic ovaries or ovarian hyperandrogenism),
- Progesterone: <0.5 ng/mL (high in unknown pregnancy, corpus luteum cyst or 21 hydroxylase blockage),
- TSH (thyroid stimulating hormone) for hyper- or hypothyroidism detection, to be treated before starting a pregnancy (Cho, 2015).

More tests are needed in some conditions:

- Hypothalamic or pituitary failure, hyperprolactinemia: pituitary MRI,
- Hyperandrogenism: DHA, SDHA, 17 hydroxyprogesterone, Synacthen test, ovarian and adrenal imaging,
- Ovarian failure: genetic screening (karyotype, fragile X premutation) (Rossetti *et al.*, 2017), bone densitometry, search for associated autoimmune diseases.

Infectious assessment

Before invasive uterine examinations, bacteriological samples in the cervix, vagina, and urine look for banal germs, chlamydiae, or mycoplasma infection, leading to treat both partners.

Chlamydia antibody testing helps in identifying possible tubal factor infertility (Singh *et al.*, 2016).
Rubella, toxoplasmosis, HIV, syphilis, hepatitis B and C serology detect:

- Lack of immunization: vaccination against rubella with temporary contraception,
- Infections requiring specific management before pregnancy. Specialized tests dealing with hepatitis B and C (viral DNA or RNA, liver evaluation) or HIV (CD4, viral load) are required before ART in the context of a viral risk.

Cervical factors assessment

The Hühner postcoital test is controversial in the international literature (Hessel *et al.*, 2014), but still used in France. It consists of examining cervical mucus during the preovulatory period, 6–12 h after sexual intercourse. Its anomalies may be related to:

- insufficient mucus secretion, infection, or excessive acidity,
- sperm deficiency, to be confirmed by semen analysis,
- or disorder in their interaction, despite apparently normal mucus and sperm. It leads to cross penetration tests, using control sperm and mucus, and to search for antisperm antibodies (ASA) in mucus and sperm. All these results are taken into account in the choice between intrauterine insemination and intracytoplasmic sperm injection (ICSI).

Female genital tract assessment

Transvaginal sonography provides information about:

- Myometrial pathologies: malformation, fibroids, adenomyosis (Phillips *et al.*, 2015).
- Adnexal diseases: ovarian cysts, hydrosalpinx, endometriosis which is associated with an age-adjusted 2-fold increased risk of infertility (Prescott *et al.*, 2016).

Endo-uterine invasive examinations must be done:

- after ruling out recent hemorrhage or infection,
- in the first part of the cycle, between the end of menstruation and periovulatory period to avoid unknown pregnancy and false tubal blockage by uterotubal junction spasm.

Hystero-sonosalpingography has quite good sensitivity and specificity for evaluation of tubal patency. It is inexpensive, minimally invasive and quick, without radiation risk (Malik *et al.*, 2014).

However, conventional hysterosalpingography remains the first-line diagnostic method for most reproductive medicine experts (Capobianco *et al.*, 2015). Regarding the technique, it has recently been shown that spontaneous ongoing pregnancy and live births rates are higher among women who underwent hysterosalpingography with oil than with water contrast (Dreyer, 2017). Besides uterine cavity imaging, tubal patency can be evaluated:

- at the proximal level: postinfectious or endometriotic occlusion, endometrial polyp, or round horn aspect leading to rule out a tubal spasm by selective catheterization under radiological or laparoscopic control,
- at the middle-isthmus level: genital tuberculosis, sequelae of tubal sterilization,
- at the distal part:
  - Hydrosalpinx: complete closure with or without mucosal folds preservation,
  - Phimosis: partial patency with distension and ampullary retention on late control,
  - Peri-tubo-ovarian adhesions: patent tube but limited peritoneal scattering of the contrast.

Hysteroscopy allows direct vision of the uterine cavity and tubal ostia. It ensures:

- the correction of false positive at hysterosalpingography due to air bubbles or clots,
- a better evaluation of organic lesions, mainly their resectability by operative hysteroscopy.

The risk of perforation is low, but interpretation is operator dependent. Hysteroscopy is mostly performed as a second-line examination, according to clinical signs and imaging. Opinions are divided about the interest of systematic hysteroscopy in any infertility assessment or after a certain age (35 or 40 years): it does not belong so far to the first-line guidelines (Di Spiezio Sardo *et al.*, 2016).

Endometrial biopsy evaluates the endometrial growth, maturation, and implanting capacity, depending on the date of the cycle. A subacute endometritis can sometimes be diagnosed and treated. More specialized tests deal with numerous genomic, biochemical and immunologic markers of implantation (Katzorke *et al.*, 2016). The possible beneficial effect on pregnancy rate of endometrial microtrauma remains controversial (Shokeir *et al.*, 2016; Maged *et al.*, 2016).

Laparoscopy is no longer indicated systematically during any infertility work-up, but in cases of suspected genital endometriosis, pelvic mass, tubal abnormality, or pelvic adhesions (Hassa and Aydin, 2014; Berker *et al.*, 2015). Both diagnostic and operative, it allows a direct assessment of:

- Tubes condition: proximal node, hydrosalpinx, phimosis with the degree of conservation of the ampullary wall, and blue test for evaluating tubal permeability.
- Ovaries: size and aspect, endometrioma or other types of cysts.
- Peritoneum: subacute or chronic inflammation due to chlamydia infection, fine granulations suggestive of genital tuberculosis, pelvic adhesions, or endometriosis localization and staging.

More Specialized Examinations

- *Genitourinary malformations or deep and/or extended pelvic endometriosis*: pelvic MRI, endorectal ultrasound, rectoscopy, intravenous urography, cystoscopy.
- *Recurrent spontaneous miscarriages*: karyotype, immunological factors, and coagulation assessments.

Risk Factors for the Female Infertility Treatment

According to personal or family history, additional examinations may be necessary before starting an infertility treatment:

- Genetics: karyotype, molecular genetics,
- Thromboembolic: coagulation assessment,
- Cancer risk: mammography, BRCA1 and BRCA2 mutations,
- Risk of ovarian hyperstimulation: FSH receptors polymorphisms should make possible to better predict ovarian response to stimulation.

Evaluation of Male Infertility

In almost 50% of involuntarily childless couples, a male factor is found, either isolated or associated with female factors. Therefore, a clinical assessment is essential in all men, as well as semen analysis.

History Taking

The male partner should be questioned about:

- Age and other information relevant to male fertility (occupation …)
- Fertility history with his partner or another, duration of infertility, and any previous evaluation ± treatments
- Family history of infertility or other reproductive disorders
- General history of diseases that may influence fertility: fever exceeding 38°C and affecting spermatogenesis for ≥3 months, diabetes (retrograde ejaculation), respiratory problems as bronchiectasis (may be part of the immotile cilia syndrome), neurological disorders that may affect sexual function, any irradiation of the genital area, or any medical treatment that may impair spermatogenesis (cytotoxic, antidepressants, antihypertensive drugs, …)
- Genitourinary and andrological background: age at puberty, history testicular problem (cryptorchidism, testicular torsion, or injury), testicular and scrotal surgery, or any locoregional operation which may impact fertility (inguinal hernia repair, …), infectious diseases (sexually transmitted, mumps orchitis, tuberculosis)
- Is sexual activity normal? Libido, frequency of intercourse (a too long abstinence may decrease motility), matching with the time of the partner's ovulation, is there any erectile or ejaculatory dysfunction?
- Search for environmental or occupational toxic factors to male fertility (heat, solvents, pesticides, …). Personal habits may impair sperm count and/or motility: wearing tight pants, taking hot baths, excessive alcohol drinking, cigarette smoking, or regular cannabis use.

Clinical Examination

Aims to assess secondary sexual characteristics such as distribution of facial and pubic hair, pubertal stage, body configuration and possible gynecomastia, looking for signs of hypogonadism.

A general physical examination is also performed and body weight, height, and blood pressure are recorded.

The rest of the medical screening will be focused on the urogenital system:

- inspection of the penis and location of the urethral meatus (hypospadias will prevent the normal delivery of semen into the vagina),
- verification of the presence of both testis in the scrotum and evaluation of their volume (approximately 25 mL or 5 × 3 cm in adults), consistency as 80% of the testis mass is represented by the germinal tissue (seminiferous tubules) and possible nodes to be explored by ultrasound. Small and soft testis indicate a hypogonadism,
- palpation of the epididymis along its entire course could reveal abnormalities (partial agenesis, distension suggesting a downstream obstruction)
- presence of the vas deferens should be assessed within the spermatic cord
- varicocele is better detected while the patient is standing
- rectal examination to evaluate the prostate and seminal vesicles is only performed on warning signs (history of infection, dysuria, cancer screening in men over 50)

Semen analysis should be performed in all couples presenting with infertility and represents the primary and basic biological testing of the male partner. Abnormal values suggest a possible male factor cause, direct the etiological diagnosis requiring further clinical assessment and biological tests, and may orientate therapeutic proposals.

Standard semen analysis

A semen sample should be obtained by masturbation in the laboratory setting, after an abstinence interval of 2–5 days. The entire ejaculate should be collected in a clean, sterile, nontoxic plastic container and analyzed, according to WHO criteria (2010), within

1 h of collection, after liquefaction (occurring usually within 15–30 min at 37°C). Any loss should be recorded, keeping in mind that losing the first semen fraction (containing the spermatozoa expelled together with prostatic fluid) has more consequences on the results than spilling the rest of the ejaculate (seminal vesicular fluid).

The main parameters assessed include the volume of ejaculate, sperm concentration, progressive motility (percentage of spermatozoa displaying a progressive movement, essential for fertilizing ability), vitality, and sperm morphology. They have to be interpreted in accordance with the WHO references values (2010) (**Table 1**).

- The lower reference limits were generated from men whose partners had a time-to-pregnancy $\leq$12 months. They provide, together with clinical data, a useful guide to a patient's fertility status. Nevertheless, it would be inappropriate to consider that a man with a semen analysis below the reference limits is necessarily infertile as well as to think that semen parameters within the 95% intervals guarantee fertility. Indeed, there is a considerable overlap between semen characteristics in fertile and subfertile men.
- The two major quantifiable attributes of a semen sample in normal conditions are:
  - the total fluid volume reflecting the secretory activity of accessory glands (mainly seminal vesicles and prostate).
  - the total number of spermatozoa or total sperm count (TSC) (sperm concentration $\times$ volume of the whole ejaculate) indicative of testis sperm production. Both also reflect the patency of the male reproductive tract.
  - Vitality (assessed with the eosin test or the hypo-osmotic swelling test and quantifying living cells independently of their motility), progressive motility, and normal morphology are also essential for sperm function.

Normozoospermia refers to a semen sample with parameters of number, progressive motility, and normal morphology equal to or above the lower reference limits (**Table 1**).

- Abnormalities may be isolated or associated (oligoasthenozoospermia, asthenoteratozoospermia, etc.). Often, all three parameters are simultaneously impaired, defining the oligo-astheno-teratozoospermia syndrome, remaining frequently unexplained, contrary to azoospermia (Punab *et al.*, 2017).
- TSC, sperm concentration, and progressive motility are the semen parameters correlating the best with fertility potential (time to pregnancy and pregnancy rates) and when comparing proven fertile and infertile male cohorts (Slama *et al.*, 2002; Nallella *et al.*, 2006).
- Complete absence of motility of live spermatozoa can be seen in men with ciliary dyskinesia (Kartagener syndrome).
- Necrozoospermia may originate from testis causes (hyperthyroidism, local hyperthermia, varicocele), posttesticular causes (epididymal dysfunction, dysregulation of seminal plasma, cytotoxic ASA), or both (infection, toxic, …).

If the first semen analysis is normal, no additional investigation is required. If it is abnormal, a new testing is needed for confirmation, owing to the wide intra-individual variation in semen quality (Castilla *et al.*, 2006) and the potential problems occurring before (fever) or during semen collection. It will be performed 3 months later, according to the length of spermatogenesis cycle in humans (about 74 days).

Table 1 Main semen parameters: lower reference limits (5th centiles and their 95% confidence intervals)

Sperm parameter	*Lower reference limit (range)*	*Abnormalities*
Semen volume (mL)	1.5 (1.4–1.7)	<1.5 mL: hypospermia in case of congenital bilateral absence of the vas deferens (CBAVD) or obstruction of ejaculatory duct or loss of part of the ejaculate during collection or androgen deficiency 0 mL: aspermia (search for retrograde ejaculation)
Total sperm count (10^6 per ejaculate)	39 (33–46)	<39: oligozoospermia No spermatozoa in the ejaculate: azoospermia Sperm only observed in a centrifuged pellet: cryptozoospermia
Sperm concentration (10^6 per mL)	15 (12–16)	<15: oligozoospermia
Progressive motility (%)	32 (31–34)	<32: asthenozoospermia
Vitality (% live spermatozoa)	58 (55–63)	High percentage of immotile dead spermatozoa: necrozoospermia
Morphology (% normal forms)[a]	4	<4: teratozoospermia
Morphology (% normal forms)[b]	23	<23: teratozoospermia Usually multiple anomalies are recorded. Occasionally almost all spermatozoa have the same structural defect which may be linked to infertility. It may be the case for the absence of the acrosome: "small round-head defect or globozoospermia," microcephaly, short-tail sperm

[a]According to Kruger *et al.* (1986).
[b]According to David in: Auger *et al.* (2016).

Other semen parameters might be of interest:

- Agglutination of motile spermatozoa sticking to each other by heads, midpieces, or tails should be recorded and graded (1–4) as it may be indicative of the presence of ASA requiring further testing.
- Evaluation of sperm morphology on stained semen smears allows identification of other cell types than spermatozoa. The presence of immature germ cells may result from spermatogenesis dysfunction.

  Leucocytes can be seen in most human ejaculates and granulocytes can be quantified by their peroxidase activity. Excessive leucocyte numbers ($>1 \times 10^6$ per mL), called leukocytospermia, reflect an inflammatory condition but the association with infection remains controversial. Leucocytospermia is >2-fold higher in a group of patients with idiopathic reduced sperm counts compared to controls (Punab *et al.*, 2017).
- The semen pH (lower threshold value: 7.2; WHO, 2010) represents an equilibrium between alkaline seminal vesicular and acidic prostatic secretions. A low pH (<7) is indicative of an absent or reduced seminal vesicular secretion as seen in congenital bilateral absence of the vas deferens (CBAVD) or in ejaculatory duct obstruction.

Additional semen investigations

Biochemical analysis: markers of the secretion of epididymis and accessory glands can be measured in the seminal fluid and give information about their functional state. The amount of zinc (≥2.4 μmol/ejaculate) reflects the secretory capacity of the prostate, neutral α-glucosidase (≥20 mU/ejaculate) is the more reliable marker for epididymal condition, and fructose (≥13 μmol/ejaculate) correlates with the secretion of seminal vesicles (WHO, 2010). A decrease in the concentration of these markers may suggest an infection, an androgen deficiency, or an obstruction on the male tract. A low fructose concentration is typical of CBAVD but may also indicate an ejaculatory duct obstruction.

ASA are present in about 4% of men consulting for infertility.

- ASA may be checked in case of high sperm agglutination and/or deficient postcoital test, despite normal mucus and semen. Sperm cells display various surface antigens that may induce the synthesis of ASA when the blood-testis barrier is ruptured. Antibodies adsorbed on the surface of motile spermatozoa can be detected by immunological assays using IgG and IgA antibodies coupled to beads. The mixed antiglobulin reaction (MAR test) is performed on a fresh semen sample while the immunobead test uses washed spermatozoa.
- When 50% or more of the motile spermatozoa have adherent beads (Barratt *et al.*, 1992), except on the tail tip, a feature also seen in fertile men (Chui and Chamley, 2004), immunological infertility may be suspected and responsible for impaired in vivo and conventional in vitro fertilization.

Evaluation of complete or partial retrograde ejaculation should be performed by looking for sperm in the pellet of postejaculatory urine, in case of aspermia or low sperm volume.

Other Basic Investigations

Infectious

Semen culture will be prescribed in case of history or clinical signs of a genitourinary tract infection or semen anomalies (abnormal appearance, viscosity or pH, leukocytospermia) and is mandatory before ART. A concentration $>10^3$ CFU (colony forming unit) of known male tract pathogens is indicative of a significant bacteriospermia but it is difficult to differentiate active infection from commensal microflora.

Hepatitis B, C, and HIV serological tests are required before ART to avoid any viral risk.

Hormonal testing is recommended in case of abnormally low sperm concentration, impaired sexual function, or clinical signs of hypoandrism or endocrine disease and has to be interpreted according to standard values of reference laboratories (Sikka and Hellstrom, 2016).

- FSH levels (1–15 mIU/mL) are inversely correlated with the number of spermatogonia. LH values (1.5–13 mIU/mL) and total testosterone (2.3–9.5 ng/mL) with Leydig cell function.
- Hormonal profiles may distinguish isolated abnormal spermatogenesis (high FSH, normal LH, and testosterone), hypogonadotropic hypogonadism with low levels of LH, FSH, and testosterone (pituitary or hypothalamic origin, treatable by gonadotropins or gonadotropin-releasing hormone), and hypergonadotropic hypogonadism (complete testicular failure) with high levels of FSH and LH and decreased testosterone.
- In azoospermic men, normal FSH suggest an obstructive origin (OA, obstructive azoospermia) with normal spermatogenesis, except for men with maturation arrest at the spermatocyte or spermatid level whose FSH values are within the normal range.
- Inhibin B, product of Sertoli cells, combined with FSH assay may be of value, in nonobstructive azoospermia (NOA), to indicate the presence or absence of testicular spermatozoa.
- Prolactin has to be checked in case of impotence, gynecomastia, galactorrhea, low testosterone. High levels may indicate some drug intake or a pituitary tumor.
- TSH assay for hyper or hypothyroidism is only valuable in the presence of suggestive clinical features (Lotti *et al.*, 2016).

Ultrasonography (US)

Scrotal US can be helpful in men with anatomy particularities hindering physical examination or presenting a risk factor for testicular cancer (cryptorchidism) but not as a routine procedure. Screening for nonpalpable varicocele has no demonstrated clinical significance (ASRM, 2014).

Transrectal US may help in a minority of patients to evaluate abnormalities of the male genital tract that may adversely affect fertility.

Genetic Screening

When indicated, identifying genetic causes for infertility may greatly influence the choice and outcome of treatment. Genetic counseling must be offered to patients.

Blood karyotype: the prevalence of numerical or structural (translocation, inversion) chromosomes anomalies is increased in infertile men and inversely correlated to the sperm count (Clementini *et al.*, 2005). A karyotype is therefore indicated in men with azoospermia (NOA) and still on debate in case of severe oligozoospermia ($<5 \times 10^6$ spermatozoa/mL) (Dul *et al.*, 2012). Sex chromosome aneuploidy (essentially Klinefelter syndrome: 47, XXY and mosaic variants) represents about two-third of all aneuploidies observed in infertile patients.

Y-chromosome microdeletions: 16% of men with severely damaged spermatogenesis (spermatozoa $<1 \times 10^6$ per mL) display Yq microdeletions in the azoospermia factor (AZF) regions (AZFa, AZFb, and AZFc). Identification by polymerase chain reaction techniques of these microdeletions, too small to be detected on standard karyotype, has a diagnostic and prognostic value for testicular sperm retrieval (Krausz *et al.*, 2000). Men with AZFc deletion have severe oligozoospermia or azoospermia with presence of testicular sperm. However, in case of complete AZFa or AZFb loss, it is extremely unlikely to find any testicular sperm and testicular biopsy should not be proposed.

Cystic fibrosis mutations: almost all men with unilateral or bilateral (CBAVD) absence of the vas deferens have mutations in the cystic fibrosis transmembrane regulator gene, except men with an associated unilateral renal agenesis (McCallum *et al.*, 2001). The patient and his partner have to be tested for CF gene mutations.

Sperm DNA aneuploidy can be assessed by multicolor fluorescent in situ hybridization in men referred to ICSI and presenting with karyotype anomalies, as they have an increased risk of sperm aneuploidy. However, results can only be indicative as the sperm to be injected cannot be screened.

Advanced Investigations for Etiological or Therapeutic Approach

Sperm function tests

Selection survival test: it is required before any ART procedure and consists in selecting good-quality spermatozoa and assessing their ability to maintain progressive motility during a 24 h culture period at 37°C. Selection is performed usually using semen centrifugation over discontinuous density gradients. Seminal plasma and epithelial cells are discarded while spermatozoa with forward motility are recovered and the total number of progressively motile spermatozoa in the ejaculate is calculated and will help in choosing the appropriate ART technique. Survival is evaluated as the percentage of still motile sperm cells at the end of the culture and is indicative of the fertilizing ability of the selected spermatozoa.

Sperm chromatin integrity tests: several direct (Comet, TUNEL: Terminal deoxyUridine Nick End Labeling) or indirect (sperm chromatin structure assay) methods have been developed to measure DNA fragmentation rates. However, despite extensive studies, the clinical values of these tests remain to be proven.

Computer-aided semen analysis is still only performed in a small number of clinical laboratories, mainly for assessment of sperm movement characteristics.

Other tests of sperm function are used in research procedures but have limited clinical relevance: assessment of hyperactivation, acrosome reaction, reactive oxygen species, gamete interaction, …

New predictive seminal biomarkers for male infertility may emerge from the genomic, transcriptomic, proteomic, and metabolomics fields (Bieniek *et al.*, 2016).

Surgical sperm retrieval

First applied to OA, sperm retrieval by microsurgical or percutaneous epididymal sperm aspiration has 100% probability of finding sperm suitable for ICSI in the aspirates.

In NOA, testicular sperm extraction (TESE) allows harvesting spermatozoa from the tissue samples in about 50% of the cases, even in Klinefelter syndrome (Corona *et al.*, 2017).

Multiple biopsies and micro TESE maximize the results (Verheyen *et al.*, 2017). Retrieved sperm are microinjected immediately or cryopreserved for later use.

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Endometriosis

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Glossary

Adenomyosis The presence of endometrial glands and stroma in the uterine myometrium.
Dysmenorrhea Painful menses.
Dyspareunia Pain with sexual intercourse.
Endometrioma A cystic lesion of endometriosis, which typically involves the ovary and is filled with brown fluid; also known as a chocolate cyst.
Endometriosis A gynecologic disorder that involves the presence of endometrial glands and stroma outside of the uterine cavity.
Infertility The inability to conceive spontaneously after 1 year of unprotected intercourse.

Endometriosis is defined as the presence of endometrial glands and stroma outside of their normal anatomic location, forming a lining of the uterine cavity. Endometriosis was first described by Rokitansky in Vienna in 1860 and was later given the name endometriosis by Sampson in 1925.

Adenomyosis is characterized by the presence of endometrial glands in the uterine myometrium. Adenomyosis was previously referred to as endometriosis interna, whereas endometriosis outside the uterus was termed endometriosis externa.

Endometriosis is differentiated from endosalpingiosis, which involves only the presence of ectopic endometrial glands without stroma.

Endometriosis is a multifactorial estrogeno dependent inflammatory and chronic disease.

Epidemiology

Endometriosis is estimated to affect 5%–10% of women in general (Eskenazi and Warner, 1997), but the true prevalence of this disease may never be known unless a valuable plasmatic test becomes routinely available.

Endometriosis is a disorder which mainly afflicts women of reproductive age. Age distribution of women with a histologically confirmed endometriosis indicates that the peak incidence is between 35 and 45 years old (Haas *et al.*, 2012). Endometriosis in adolescents appears more common than formerly claimed. Endometriosis is rare in postmenopausal women. Whether or not the occurrence of endometriosis is increasing remains yet unclear.

In selected populations, such as patients with pelvic pain or infertile women, the prevalence may attain 70%.

Endometriosis is more common in first-degree relatives of affected women, but no clear pattern of genetic transmission has been established.

This disorder was long considered to be more common among Caucasian women; however, recent data suggest that the incidence is similar across ethnic origin.

Types of Lesions and Locations

Four basic types of endometriotic lesions have been described. They are frequently associated in the same patient:

- *Superficial implants*: Superficial implants (SI) are the most frequent lesions encountered. The classical appearance of SI is bluish or blue-black or "powder-burn" lesions. Many other appearance have been reported. Nonpigmented, also called atypical or subtle, lesions have been described (**Fig. 1**) (Jannsen and Russel, 1986). These latter lesions are early stages of the disease and have been proved to be very active. They are more frequently encountered in adolescents or young adult women. A biopsy with histologic analysis is mandatory to confirm. Endometriosis, because many other diseases may causes similar lesions.
- *Endometriotic cysts*: Endometriomas (OMA), frequently with associated adhesions, contain a chocolate-like fluid (**Fig. 2**). The etiology of these lesion is still debated.
- *Deep infiltrating endometriosis*: Deep infiltrating endometriosis (DIE) is defined as lesions infiltrating the peritoneum, or the serosa of vital organs, greater than 5 mm (Cornillie *et al.*, 1990). Typically DIE is a nodular blend of fibromuscular tissue and adenomyosis, located in the uterosacral ligaments or the rectovaginal septum (**Figs. 3** and **4**). DIE is apparently more and more frequent' and associated with severe pain symptoms.
- *Adhesions*: Endometriosis is considered as a very "adhesiogenic" disease, demonstrated by the frequent occurrence of associated adhesions.

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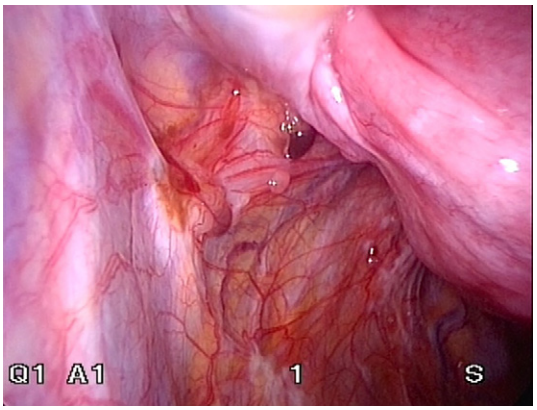


Fig. 1 Early implants in the left fossa ovarica, note the hypervascularization of the surrounding peritoneum.

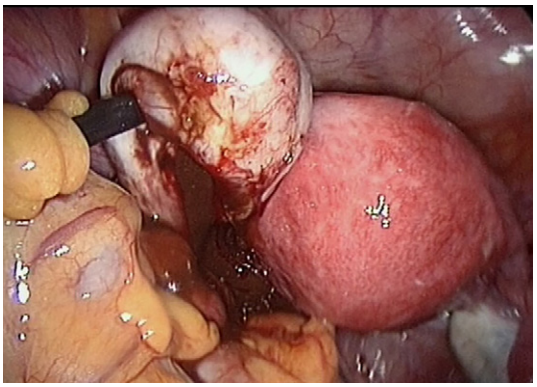


Fig. 2 Ovarian endometrioma with leakage of "chocolate" liquid content.

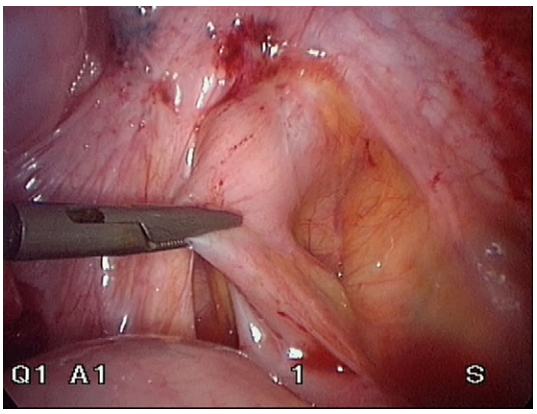


Fig. 3 Adhesion of the sigmoi to torus uterinum suggestive of associated DIE.

Many sites including the ovaries, ovarian fossae, uterosacral ligaments, and pouch of Douglas, which are the most common sites for endometriotic lesions, with a left predominance (Jenkins *et al.*, 1986). Extra-pelvic locations of endometriosis are rare (Matalliotakis *et al.*, 2017; Maccagnano *et al.*, 2013; Nezhat *et al.*, 2014). Some of the sites where endometriosis may be found are shown in **Table 1**. The myriad of involved sites explain the huge variety of symptoms associated with endometriosis.

The extent of the disease may be staged and it is recommended to use a staging system at the time of the diagnosis. The most commonly used staging system is the American Society for Reproductive Medicine (ASRM) revised classification

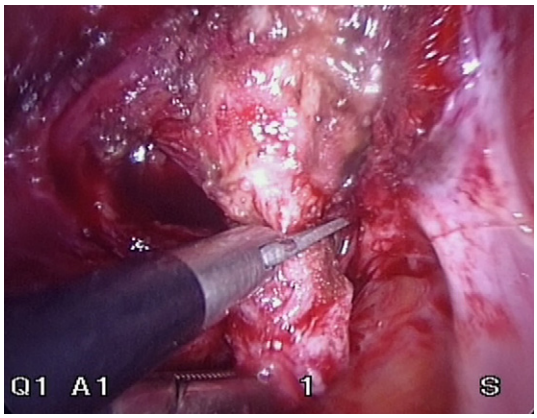


Fig. 4 Nodule of the rectovaginal septum attached to the vagina.

Table 1 Locations of endometriosis

Common sites	*Less common sites*
Ovary	Brain
Pelvic peritoneum	Lung
Uterine serosa	Bowel
Fallopian tube	Ureter
Posterior cul-de-sac	Kidney
Uterosacral ligaments	Appendix
Rectovaginal septum	Vulva
Anterior cul-de-sac	Incision

system AFS (1985). The system involves a point system based on the size, depth, and location of both implants and adhesions. Points are tallied on a form and a stage is assigned based on the number of points (**Fig. 5**). These include stage I (minimal, 1–5 points), stage II (mild, 6–15 points), stage III (moderate, 16–40 points), and stage IV (severe, 40+ points).

But the correlation between lesions and pain symptom or infertility is unclear.

The evolution of lesions is unpredictable. It is assumed that 70%–80% of lesions are stable or progress, thus endometriosis is considered as a recurrent chronic disease requiring a long term management. Degenerescence of endometriotic lesions is rare (0.5%) and related to aging.

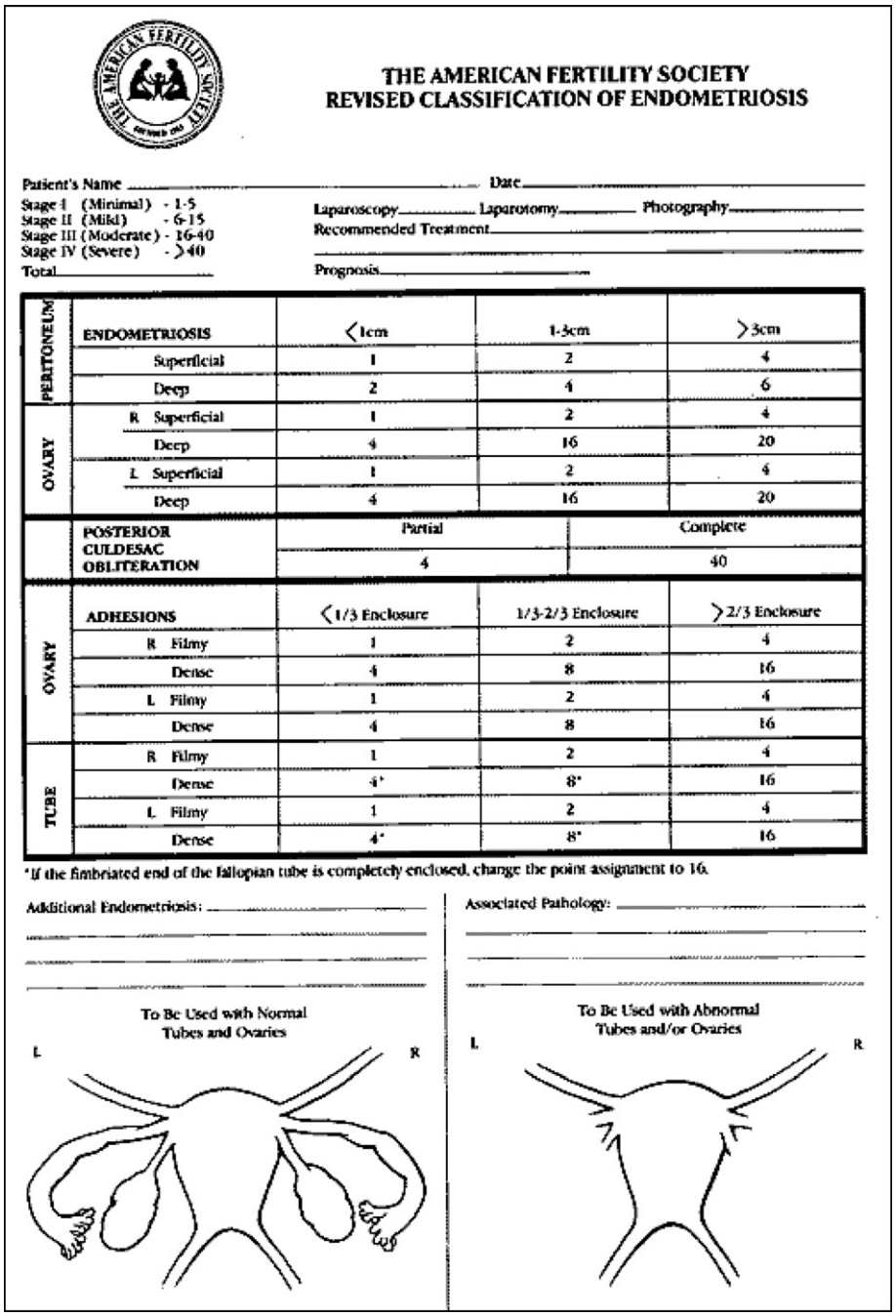
Etiology

The etiology of endometriosis remains unclear. Theories that have been put forth to explain the disorder include retrograde menstrual flow, hematogenous spread, lymphatogenous spread, coelomic metaplasia, embryonic Mullerian rests, and immunologic abnormalities (**Table 2**) (Gleicher and Pratt, 1993).

Women with uterine congenital reproductive anomalies, in particular those that involve obstruction of menstrual outflow, are at particularly high risk for endometriosis. Especially during adolescence. However, the vast majority of women with endometriosis have normal outflow tracts. Since none of the theories put forth can explain all cases, it is likely that endometriosis may be multifactorial in origin.

Several immunologic changes are noted in women with endometriosis. Women with the disease are more likely to be deficient in T cell- and natural killer cell-mediated toxicity. The peritoneal fluid of women with endometriosis contains greater numbers of macrophages and greater macrophage activity than that of normal controls. The fluid also contains higher levels of interleukin-8, a leukocyte chemotactic cytokine. The immunologic differences in these women may explain why most women experience retrograde menstruation yet not all women will develop endometriosis.

In addition many other predisposing factors have been proposed (Burney and Giudice, 2012): genetic predisposition, fetal exposure to diethylstilbestrol, environmental toxins exposition, epigenetic modifications, stem/progenitor cells….



THE AMERICAN FERTILITY SOCIETY
REVISED CLASSIFICATION OF ENDOMETRIOSIS

Patient's Name _____ Date _____

Stage I (Minimal) - 1-5
Stage II (Mild) - 6-15
Stage III (Moderate) - 16-40
Stage IV (Severe) - >40
Total _____

Laparoscopy _____ Laparotomy _____ Photography _____
Recommended Treatment _____
Prognosis _____

PERITONEUM	ENDOMETRIOSIS	<1cm	1-3cm	>3cm
	Superficial	1	2	4
	Deep	2	4	6
OVARY	R Superficial	1	2	4
	Deep	4	16	20
	L Superficial	1	2	4
	Deep	4	16	20
	POSTERIOR CULDESAC OBLITERATION	Partial	Complete	
		4	40	
OVARY	ADHESIONS	<1/3 Enclosure	1/3-2/3 Enclosure	>2/3 Enclosure
	R Filmy	1	2	4
	Dense	4	8	16
	L Filmy	1	2	4
	Dense	4	8	16
TUBE	R Filmy	1	2	4
	Dense	4*	8*	16
	L Filmy	1	2	4
	Dense	4*	8*	16

*If the fimbriated end of the fallopian tube is completely enclosed, change the point assignment to 16.

Additional Endometriosis: _____

Associated Pathology: _____

To Be Used with Normal Tubes and Ovaries

L R

To Be Used with Abnormal Tubes and/or Ovaries

L R

Fig. 5 The American Fertility Society revised classification of endometriosis.

Symptoms

Endometriosis may be asymptomatic or may present with symptoms mainly related to location of lesions. Two symptoms are predominant: pain and infertility, but these symptoms are very similar to other common conditions.

Pain Associated With Endometriosis

The cardinal symptom of endometriosis is pelvic pain, typically temporally related to menses. The severity of the pain is variable, altering markedly the quality of life when pain is intense. Pain tends to increase over time. In addition 1/5 of women with endometriosis do have concurrent pain chronic conditions. Different types of pain are common: dysmenorrhea (painful period),

Table 2 Theories regarding endometriosis pathogenesis

- Endometrial spread (tubal and vascular)
- Coelomic metaplasia
- Mullerian rests
- Immunologic changes
- Fetal exposure to DES
- Environmental toxins
- Obstruction of menstrual flow
- Genetic defects
- Stem/progenitor cells
- Epigenetic modifications

dyspareunia (pain with intercourse), chronic pelvic pain. Some pains are less common but often associated with DIE: dysuria (pain with urination), dyschezia (pain with defecation) or bowel pain.

Although all of the above symptoms are common, there is no symptom that is pathognomonic for endometriosis, but when dysmenorrhea is refractory to routine medical treatment the prevalence of endometriosis attains 70%–80%.

Other Symptoms

Many other symptoms may be associated with endometriosis: excessive bleeding, diarrhea or bloating during menstrual periods, fatigue ….

Another clinical presentation of endometriosis is pelvic mass with or without pain.

In rare cases, endometriotic foci may be located in other parts of the body, such as a surgical scar (causing cyclical incision bleeding), in the lungs (causing cyclic hemoptysis or pneumothorax), or in the brain (causing catamenial seizures). When unusual, seemingly nongynecological symptoms occur only with menses, endometriosis often becomes part of the differential diagnosis.

Infertility

Many women will present with no physical symptoms, yet endometriosis will be discovered during the course of an infertility evaluation.

The incidence of endometriosis in infertility patients undergoing diagnostic laparoscopy is relatively high (38%), yet the association between endometriosis and infertility remains unclear. Advanced cases of endometriosis (stage III and IV disease) are associated with decreased fertility, presumably on the basis of pelvic adhesions and mechanical factors. Less advanced cases (stages I and II) are considered by some authors to be causal of infertility and others as merely coincidental. The suggested mechanisms include interference of peritoneal fluid with sperm function and fertilization, decreased oocyte quality, tubal alterations associated with endometriosis (Brichant *et al.*, 2016).

Diagnosis

The only conclusive diagnosis of endometriosis is direct visual inspection preferably by laparoscopy, with histologic confirmation (Hsu *et al.*, 2010). The challenge is to select properly cases for whom laparoscopy is indicated, particularly patients with a high suspicion of endometriosis. The diagnosis of endometriosis is thus difficult and often delayed.

The first step, through a detailed analysis of past history and symptoms, is to identify risk factors, such as familial history, early menarche, pelvic pain, cyclic or not refractory to routine medical treatment, indicating patients more likely to have endometriosis.

The next step is the pelvic examination. Often normal, the pelvic examination may reveal evocative abnormalities of endometriosis such as focal tenderness, nodularity of the uterosacral ligaments, mass in the rectovaginal septum of fixed ovarian mass causing pain during palpation. Experienced clinicians can predict the presence of endometriosis in 80% of cases.

Noninvasive diagnostic tools are serum biomarkers and imaging technics.

Many serum markers have been evaluated. CA-125, a tumor marker, has been studied and was initially thought to be appropriated. Unfortunately CA-125 did no prove to have a good specificity and sensibility according to many published studies. None of the biomarkers evaluated in a review for Cochrane Database could be evaluated in a meaningful way and there was insufficient or poor-quality evidence all other marker do have a limited value (Mol *et al.*, 1998). Some hopes may result from proteomic research.

Table 3 Medical treatments for endometriosis

Oral contraceptives (continuous or cyclic)
Progestins (oral, parental or intra uterine)
Danazol
GnRH agonists (leuprolide, nafarelin, goserelin, buserelin, histrelin)
Antiprogestins (mifepristone, ulipristal acetate)
Aromatase inhibitors (anastrozole, letrozole)
Immunomodulators

Imaging technics did improve their performances for the diagnostic of endometriosis but performances are operator dependent and limited to identify adhesions or SI (Spaczynski and Duleba, 2003). Ultrasound is useful for evaluating pelvic masses for identifying genital malformation, bladder lesions, and nodular DIE.

MRI offers similar performances to identify endometriotic lesions but less operator dependent. MRI is also useful to detect associated adenomyosis present in 25% of patients with endometriosis. MRI is helpful for DIE providing an accurate staging guiding further surgical approach.

Treatment

The goal of treatment of endometriosis is either to ablate or excise lesions by surgery or to improve symptoms with a hormonal agent. Both modalities have a temporally effects. The choice is guided by individual lesions and presenting symptoms. For many years the only treatment was surgery. Then a myriad of hormonal agents were available and practically all of them have been clinically tested and proved to be more or less successful.

Treatment Modalities

- Abstention: A woman who is asymptomatic but is diagnosed with endometriosis at the time of an unrelated surgical procedure (e.g., a laparoscopic tubal sterilization) does not necessarily require treatment. In the absence of pain or infertility, the disease may be managed expectantly.
- Surgery, preferably by laparoscopy, can now treat all types of lesions including the more severe ones. Frequently, surgical treatment is performed at the time of the initial laparoscopy when the diagnosis of endometriosis is made. After the diagnosis is established, the lesions discovered may be fulgurated or resected. Various modalities have been used to ablate the implants including various lasers (CO_2, KTP, Nd-YAG), bipolar or monopolar cautery, endocoagulation, the harmonic scalpel, and sharp resection. Although many physicians have advocated the use of laser for ablation, there are no data that show better outcomes associated with a specific energy source. At the time of surgery, any pelvic adhesions noted may be lysed in order to reduce the pain. Additionally, the uterosacral ligament, which carries sensory nerve fibers from the uterus, may be ablated at this time to assist in pain relief. If an endometrioma or chocolate cyst is encountered, when possible the cyst is opened, the chocolate fluid is aspirated, and the cyst wall is excised. DIE involving digestive or urinary tract can also be excised with the laparoscopic approach. Surgery for these lesions may be difficult to perform and is associated with severe complications in less than 5% of the cases.
- Other surgical procedures are available for the treatment of endometriosis. The procedures described above for laparoscopy may also be performed at the time of laparotomy. A presacral neurectomy may be performed to interrupt the sympathetic nerves at the level of the superior hypogastric plexus. In some instances, one or both ovaries may be removed and sometimes a hysterectomy may be required. Many patients with intractable endometriosis pain and multiple prior surgeries benefit from a "pelvic clean-out," which involves total abdominal hysterectomy with bilateral salpingoophorectomy. It should be noted that most definitive of therapies are approximately 90% effective in the resolution of pain.
- Postoperative adhesions occur frequently after surgery for endometriosis (adhesions may cause pain or infertility). The operative procedure must include routine use of adjuvants measures (gel, barrier …) in order to reduce the development of postoperative adhesions.
- Medical therapies are indicated in patients presenting endometriosis-related pain.

Medical treatment for endometriosis-related pain involves the use of drugs against pain and hormonal therapies to suppress ovulation.

Anti-inflammatory drugs appears to be more efficient among drugs used for pain treatment, and none is superior to others (Allen *et al.*, 2009).

The hormonal therapies are listed in **Table 3**. The rationale for this type of treatment is that the implants of endometriosis are similar to normal eutopic endometrium and will respond to estrogen and progesterone in the same way. Thus, the same hormonal therapies that can induce amenorrhea in women with dysfunctional bleeding would be expected to decrease pain in women with pelvic endometriosis.

Oral contraceptive is the most commonly regimen used for initial treatment. Due to the benign nature of this therapy, treatment is often initiated prior to obtaining a definitive diagnosis. A standard monophasic low dose birth control pill is taken continuously, with no days off and no placebo pills. This will mimic the hormonal milieu found during pregnancy in which high levels of estrogen and progesterone are observed. In general, pregnant women with endometriosis experience a significant abatement of symptoms during pregnancy. Alternatively, progestational agents may be used (Brown *et al.*, 2012). Depot formulations of progestational agents, such as medroxyprogesterone acetate (Depo-Provera), or daily oral progestins, such as medroxyprogesterone acetate (Provera, Cycrin) and norethindrone (Aygestin), or dienogest may be used. A levonorgestrel-releasing intrauterine device LNG-IUD has demonstrated a good efficiency according to randomized control studies (Bahamondes *et al.*, 2013).

An even more effective way to treat endometriosis pain involves what is called a "pseudo-menopause" regimen. Various medications can be used to suppress the hypothalamic–pituitary axis, resulting in a hypogonadotropic, hypoestrogenic state.

One of the first pseudo-menopausal agents to be widely used was Danazol (Danocrine). Danazol is an orally active androgenic steroid believed to both suppress ovulation and act directly to cause atrophy of the endometriotic implants. Given in doses of 200—400 mg twice daily, it is accompanied by side effects that may include hot flashes, vaginal dryness, muscle aches, deepening of the voice, and hirsutism.

Gonadotropin-releasing hormone (GnRH) agonists, such as leuprolide (Lupron), goserelin (Zoladex), nafarelin (Synarel), and buserelin, have become the agents of choice to induce a pseudo-menopause. These medications are all administered parenterally, oral formulations are under investigation. Some are designed to be used monthly or even at 3-month intervals for prolonged suppression of endogenous gonadotropins. This suppression will create a hypoestrogenic milieu and will cause the involution of the endometriotic implants, which are estrogen-dependent. The side effects of the GnRH agonists include headaches, hot flashes, vaginal dryness, and bone loss. In order to prevent the sequelae of osteoporosis later in life, Danazol and GnRH analogues are limited to 6–9 months of use.

The GnRH agonists may also be used on a longer term basis when accompanied by low doses of estrogen plus progestin replacement. This type of regimen is referred to as add-back therapy and its rationale is based on the estrogen threshold hypothesis (Barbieri, 1992). This hypothesis states that endometriosis and bone loss respond to different circulating estrogen levels. The goal thus is to attain an estrogen level that is high enough to conserve bone density yet low enough to prevent regrowth of the endometriosis lesions. The patient is started on a long-acting GnRH agonist, and concurrently or at a later date, low-dose estrogen/progestin replacement therapy is added in a dose that is similar to that given to menopausal women. Alternatively, a norethindrone-only regimen may be used. Add-back therapy has been demonstrated to be safe and effective when used for up to 1 year. Although there exists no general consensus on how long a patient may be kept on such a regimen, many clinicians will continue such therapy for a number of years, with intermittent monitoring of bone density by DEXA scan to ensure that there is no significant bone loss (Surrey and Hornstein, 2002).

Treatment with antiprogestins, aromatase inhibitors or immunomodulators is considered experimental and will not be reviewed in this article (Platteeuw and D'Hooghe, 2014).

Indications and Results of Treatments

Pain treatment

When a patient presents with endometriosis-related pain, she may be treated using a myriad of various medical or surgical therapies.

Initial treatment is surgical or medical. Medical therapy is often used as a first-line therapy and can also be used in conjunction with those patients who undergo surgical therapy for pain.

Comparative studies indicate that combined oral contraceptive pills, GnRHa and progestogens are all effective and well tolerated by patients in treating endometriosis associated pain and improvement of quality of life (Apers *et al.*, 2018), though side effects have to be considered. Rates of improvement of symptoms and quality of life vary from 40% to 90%. The rates of recurrence of pain after cessation of treatment varies from 17% to 32%, depending on the initial severity of pain and lesions, the agent used, the length of follow-up and the presence of a genetic susceptibility.

Combined oral contraceptive pills and progestogens are relatively cheap and more suitable for long-term use as compared to GnRHa.

Surgical treatment of pain associated with endometriosis is improved within the same ranges compared to medical treatment. Recurrence rates of pain occur in 24%–54% of patients. Usually to improve results and reduce the risk of recurrences a medical treatment is prescribed after surgery.

Infertility treatment

A patient who presents with endometriosis and infertility may be treated using surgical means or other types of infertility treatments that are not directly related to endometriosis.

No medical therapy has proven effective for infertility. A meta-analysis by Hughes on the treatment of infertility in early stage endometriosis found no benefit of medical treatment on pregnancy rates (Hughes *et al.*, 1993). The sole exception is the use of GnRHa as a pretreatment for 3 months before proceeding to IVF, for moderate to severe endometriosis associated infertility. In fact medical treatment may be detrimental by causing a delay in pregnancy due to suppression of ovulation.

Superovulation with gonadotropins accompanied by intrauterine insemination resulted in a significant increase in pregnancy rates in women with infertility associated minimal and mild endometriosis (Tummon *et al.*, 1997; Hughes, 1997).

The use of laparoscopic surgery to treat early stage endometriosis was evaluated in two randomized controlled trials. A large Canadian multicenter study showed an increase in monthly fecundity in patients in whom endometriosis was ablated compared with those in whom it was left in situ (Marcoux *et al.*, 1997). A much smaller Italian trial failed to show any benefit (Prazzini, 1999). Thus, it appears likely though not certain that early stage endometriosis may increase the chances of future pregnancy and live birth. For more severe lesions (OMA, DIE, and adhesions) the reported rates of pregnancy is 50%.

Infertility in endometriosis patients can be treated by the same modalities used to treat those with unexplained infertility. IVF is often used in case of failure of surgical treatments and according to recent meta-analysis provides similar results in comparison with a reference group of tubal alterations (Gonzalez-Comadran *et al.*, 2017).

Summary

Endometriosis is an enigmatic disease that is defined by the presence of endometrial stissue in an ectopic location. The etiology remains unknown, though there are certain changes in the immune system as well as an association with abnormal outflow tracts. The symptoms of endometriosis include various manifestations of pelvic pain and infertility and are often temporally related to menses. Diagnosis is made by surgical visualization and treatment may involve hormonal manipulation and/or surgical ablation or resection.

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Epigenetics of Endometriosis

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Endometriosis: A Short Definition

Endometriosis is a frequent gynecological disease (affecting 10% of women) resulting in pain and infertility. It is characterized by the implantation of uterine tissue from the endometrium in various places in the abdominal cavity (de Ziegler *et al.*, 2010; Bulun, 2009; Giudice, 2010), such as the peritoneum, the ovaries, the uterosacral ligaments, etc. One way of classifying endometriosis is in three sub categories (OMA = ovarian endometriosis, SUP = superficial endometriosis where the lesions do not reach more than half a centimeter in depth, and DIE = deep infiltrating endometriosis (Borghese *et al.*, 2008)). Another way of classifying endometriosis is similar to the way cancer is classified, in four stages I–IV from moderate to severe (Johnson *et al.*, 2017). An important aspect of endometriosis pathophysiology is the cell proliferation of the ectopic implant(s). This proliferation is hormone-dependent, and women with the disease have an abnormal steroid estrogenic response at the level of the endometrium together with an abnormal expression profile for genes involved in inflammation, cell proliferation, and angiogenesis (Sherwin *et al.*, 2008; Burney *et al.*, 2007). Reciprocally, a quite general finding in endometriosis is progesterone resistance, with a very strong decrease of the PR-B receptor in the lesions (Borghese *et al.*, 2008; Attia *et al.*, 2000; Patel *et al.*, 2017). This down-regulation of the PR-B gene expression could be achieved via epigenetic deregulation; in the lesion, the inflammatory state increases the level of proinflammatory cytokines such as IL1β or TNFα. This latter molecule has been shown to increase methylation at the level of the PR-B promoter (Wu *et al.*, 2008).

Endometriosis: A Benign Proliferative Lesion

Despite its negative effect on the quality of life of women, endometriosis is considered a benign disease since it is not lethal. In fact, although recent reports associate the occurrence of certain ovarian cancers with endometriosis (Ruderman and Pavone, 2017), it seems that cancer-associated somatic mutations are found in specific genes in endometriosis lesions, without cancer development. A recent study using exome sequencing (Anglesio *et al.*, 2017) revealed the existence of somatic mutations in 19 patients out of 24 in genes previously associated with tumorigenesis (*ARID1A*, *PIK3CA*, KRAS, and *PPP2R1A*). These somatic mutations were found in the glandular epithelium of some DIE lesions; interestingly, these non-ovarian deep infiltrating lesions almost never transform into cancer. Therefore, the cellular environment of the endometriosis lesion appears generally unconducive to cancer development, probably owing to a specific epigenetic state of the cells in hormone-dependent lesions.

The Epigenetic Context in Endometriosis

Epigenetics concerns the intricate regulation and modifications able to reversibly modify gene expression, without altering the DNA sequence. In an adult organism, these modifications are transmitted stably from one cell generation to the next. Epigenetic transmission takes place through mitosis but an increasing number of reports indicate that epigenetic inheritance can in some cases also occur through meiosis, and thus can be transmitted from one generation of individuals to the next (Anway *et al.*, 2005; Carone *et al.*, 2010). Three major epigenetic pathways co-exist and interact to modulate gene expression, two pathways change the structure and accessibility of the chromatin either by chemical modifications of the DNA, or by chemical modifications of the histones, the third involves the complex cellular machinery generating and regulating the biogenesis of non-coding RNAs, mainly micro RNAs (miRNA) but also long non coding RNAs (lncRNA). Concerning epigenetic chemical modifications in mammalian tissues, DNA is mainly modified at CG sites, by methylation or hydroxymethylation, and this has been extensively studied in the context of brain development, metabolic diseases, or cancer progression, but also recently in the decidualizing uterus (Gao and Das, 2014). The epigenetic machinery also chemically modifies histones. Histones are proteins that constitute basically charged cores (nucleosomes) built from eight polypeptides (2 × HISTH2A, 2 × HISTH2B, 2 × HISTH3, and 2 × HIST4) around which 146 bp of DNA are wrapped. The N-terminal of these proteins protrudes outside the nucleosome and is the target of numerous post-translational modifications (PTM) generally associated with specific chromatin states, for instance, H3K27ac is associated with active promoters, H3K4me3 with transcriptionally active regions and H3K27me3 with transcriptionally inactive regions (Natsume-Kitatani and Mamitsuka, 2016). Finally, the epigenetic machinery can also modulate the capacity of mRNAs to be translated by affecting their stability. This is performed through the action of small RNA molecules, such as miRNAs, while longer RNAs (e.g., lncRNAs) also play a role in gene regulation. The DNA and histone chemical modification involve short

aliphatic residues with generally one (methyl-) or two carbons (acetyl-) but also phosphorylations, sumoylation and more exotic modifications generally at the level of histone lysine residues (Tan *et al.*, 2011). There is an enzymatic machinery of increasing complexity that is described to perform all these modifications. The necessary chemical residues are provided by food, making it clear that epigenetic mechanisms constitute an interface between the environment and the genome, leading to the concept of the existence of environmental metabolites. The availability of environmental metabolites may alter the offspring epigenome including that of germ cells (Sharma and Rando, 2017). Epigenetic changes in endometriosis have classically been analyzed by two types of comparison: either the comparative analysis of the eutopic endometrium between control and endometriotic women (Houshdaran *et al.*, 2016), or the comparative analysis of the ectopic versus eutopic endometrium in a given patient (Dyson *et al.*, 2014; Borghese *et al.*, 2010).

We published a recent review (Borghese *et al.*, 2017) on the genomics and epigenetics of endometriosis. Therefore, in the present article, we will concentrate on five recent topics linking endometriosis and epigenetic regulation that were not addressed in detail in the previous review:

(i) In recent papers there has been an increasing interest in histone modifications in endometriosis, modifications which were less studied previously and certainly represent a field where research will intensify due to technical improvements in ChIP-seq or ATAC-seq from tissues;
(ii) Using gene-targeted mouse models, there have been several papers focusing on the zinc finger-containing transcription factors KLF10 and KLF11, which appear to be important epigenetic regulators;
(iii) In terms of DNA methylation, recent studies have been increasingly concerned about women's cycles, and focused rather on the comparison between the eutopic endometrium, either in endometriotic or control women, as well as attempting to connect these alterations with gene expression modifications. The comparison between the lesion (ectopic endometrium) and the endometrium of patients have been the subject of numerous articles, both at the genomic level (Dyson *et al.*, 2014; Borghese *et al.*, 2010) and at the level of specific genes, such as *HOXA10*, *SF1*, *CYP19*; thus the reader can consult recent reviews on the subject (Borghese *et al.*, 2017; Koukoura *et al.*, 2016), and we will not deal extensively with this topic here;
(iv) Some recurrent investigations concern the miRNA profile in endometriosis; however, results appear highly variable from one study to another;
(v) Also, the carcinogenic properties of endometriotic lesions have recently been addressed in the aforementioned New England Journal of Medicine study (Anglesio *et al.*, 2017), and this can be considered in terms of epigenetic regulation.

These five points will be addressed in the next part of this review, and are tentatively summarized in **Fig. 1**.

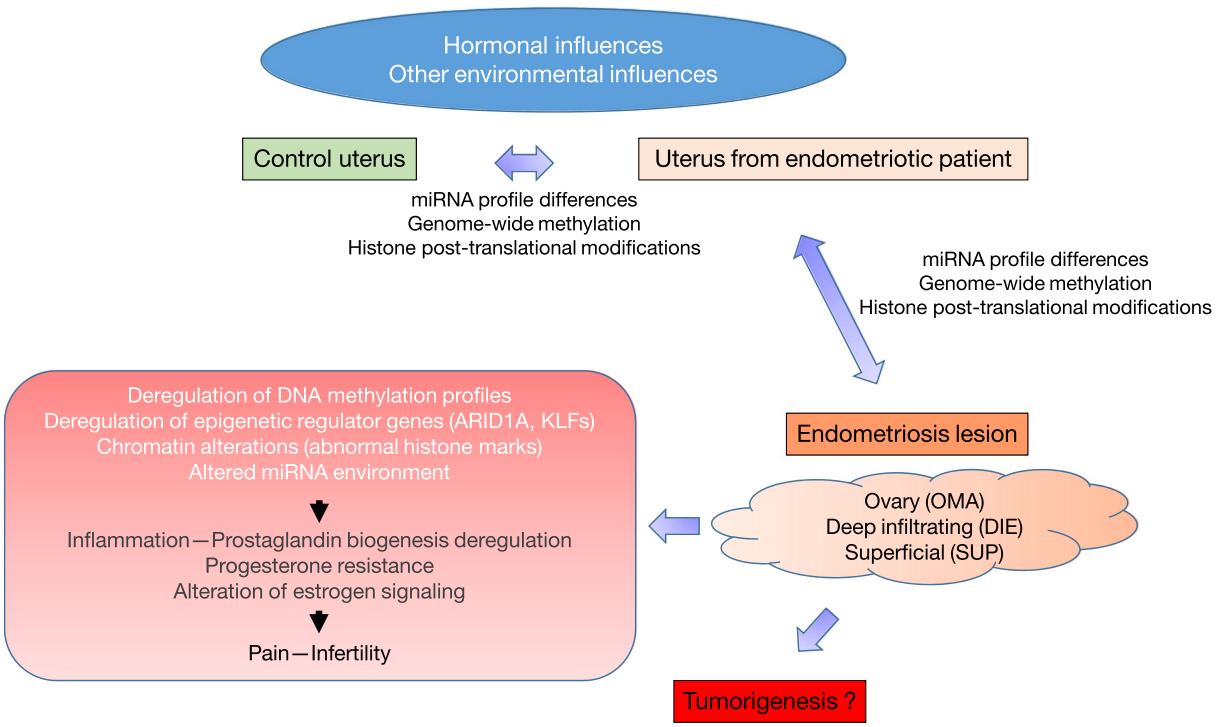

Fig. 1 Endometriosis is a complex disease where numerous genes are implicated. Their deregulation is accompanied (or sometimes caused) by epigenetic alterations and concerns both differences between the lesion and the eutopic endometrium in patients and eutopic endometrium from patients and controls. The various epigenetic alterations affect genes involved in inflammation and hormonal regulation, which may contribute to the symptoms of the disease (pain and infertility). Environmental influences, including endocrine disrupters, may contribute to the disease.

Recent Issues on Endometriosis Epigenetic Research

Histone Mark Alterations in Endometriosis

As mentioned above, endometriosis is an estrogen-dependent disease. Actually, the major therapy is estrogen inhibition using oral contraceptives, aromatase inhibitors, androgenic reagents or GnRH homologues. The symptoms reappear very often within a year after the treatment is interrupted (Kyama *et al.*, 2008). Estrogen signaling depends primarily on ~100 major genes, as illustrated by the KEGG pathway hsa04915 (http://www.genome.jp/dbget-bin/www_bget?hsa04915). To understand how the chromatin is regulated in these genes could result in novel therapeutic targets, and drugs better able to treat the symptoms, and this could be done by manipulating histone PTM marks. Several drugs are able to alter these marks (Borghese *et al.*, 2017). Indeed, specific histone marks, such as H3K27Me3, seem specifically enriched in endometriotic lesions (Colon-Caraballo *et al.*, 2015; Monteiro *et al.*, 2014). Drugs such as valproic acid (VPA), trichostatin A, or sodium butyrate, which are histone deacetylase inhibitors, but also lysine-specific demethylase inhibitors (such as tranylcypromine, which targets KMD1A), are able to decrease cell proliferation and invasiveness (Ding *et al.*, 2014), and have been shown to improve the symptoms of endometriosis in a mouse model (Sun *et al.*, 2016). Such epigenetic regulation has been studied for the *CYP19* aromatase gene in cell models with VPA treatment inhibiting histone acetylation in the promoter of *CYP19*, and suppressing its expression (Chen *et al.*, 2015). In endometriotic stromal cells, acetylation of H3 and H4 is considerably reduced compared to normal endometrial cells, while VPA was also able to increase considerably the acetylated histone marks. On the other hand, the specific analysis of the *CYP19* promoter revealed a decreased presence of acetylated H3 and H4 following VPA treatment, associated with a decreased expression of this gene, an increase in apoptosis and reduced proliferation. Therefore, this study shows that global modifications of histone PTMs may have paradoxical consequences at the level of specific genes, sometimes crucial for endometriosis progression. Recently, Hosseini and coworkers showed that in cumulus cells from endometriotic women, CYP19A1 expression was reduced compared to infertile women without endometriosis (Hosseini *et al.*, 2016), consistent with previous studies (Barcelos *et al.*, 2015). The authors also showed by ChIP-qPCR an increased presence of MeCP2 (a methylated CpG binding protein) at the more distal promoter (PII), and at the proximal promoter PI.4. They also showed a decreased H3K9ac (transcription activation mark) presence at PII, and increased H3K9 me2 (transcription repression mark) at PI.4. This was accompanied by differences in ERβ binding at the promoter element that was decreased at PII, but increased at PI.3 and PI.4. As suggested by this example, it is an intermingling of epigenetic factors that modulate promoter accessability to transcription factors in a complex way. The results suggest specificities of endometriosis-related infertility compared to other types, and show the complexity of the epigenetic regulation of gene expression, suggesting that until a general deciphering of epigenome dynamics is available, the consequences of epigenetic regulation on gene expression should be addressed on a case by case basis.

Along the same line of ideas, it has recently been shown that the downregulation of ARID1A (AT-rich Interactive Domain 1A) in endometriotic cells increases cell colony formation capacity, adhesiveness and invasive properties. ARID1A is a member of the SWI/SNF family of chromatin remodelers, known to be mutated in endometriosis precursor lesions. In these lesions, together with the phenotypic alterations of the cells where ARID1A was down-regulated, there was a strong deregulation of the distribution of H3K27ac, that increased considerably at the promoter levels for 61% of them while only 39% were unchanged and <1% decreased; at the enhancer level, on the contrary, H3K27Ac was reduced for 23.54% of the enhancers (Lakshminarasimhan *et al.*, 2017). The latter findings were compared with gene expression information, revealing that only a subset of genes was regulated by this mark (a total of 72 genes up- and 27 genes down-regulated). In summary, a discrepancy was seen between the extensive reorganization of epigenetic marks and a relatively mild effect on transcription alterations, that may occur more significantly at later stages. In analyzing more directly endometiotic lesions, Liu and coworkers revealed by histology that EZH2, H3K9me3, and H3K27me3 are more intense in OMA and DIE lesions compared to eutopic endometrium (Sutton *et al.*, 2017). In sum, the technical difficulties of analyzing the histone code in endometriosis are being overcome at an increasing pace. This suggests that in the future, both for issues of basic science and medicine, this mode of epigenetic regulation will play a pivotal role.

Epigenetic Regulation by KLF10 and KLF11 Transcription Factors

KLF11 (Kruppel Like Factor 11) is a zinc finger transcription factor involved in TGFB signaling and B cell receptor signaling, known for its involvement in MODY (Maturity-Onset Diabetes of the Young). KLF11 is known to intervene in the mechanisms achieving gene expression repression through contributing to the binding of HP1 to the chromatin. In turn, HP1 provokes the recruitment of H3K9 and its methylation by SUV39H1. This finally leads to chromatin compaction and silencing of specific genes (Lomberk *et al.*, 2012). In 2014, the team of GS Daftary focused on the function of KLF11 in the context of endometrial biology, first analyzing its effect at specific promoters of nearby genes important for endometrial biology, such as glycodelin (Tabbaa *et al.*, 2014), through the recruitment of the SIN3/histone deacetylase corepressor complex to the glycodelin promoter, glycodelin being a protein important for immnomodulation during pregnancy (Bersinger *et al.*, 2008; Seppala *et al.*, 2009). The same team went on to study the endometrial impact in *Klf11* $-/-$ KO mice on surgically provoked lesions (Daftary *et al.*, 2013). They showed in cell models that cytochrome p450 metabolic enzymes (especially *CYP3A4*, an enzyme involved in the metabolism of many drugs, steroids and carcinogens) are targets of KLF11 that alter their expression through epigenetic mechanisms (Zheng *et al.*, 2014). The same team also showed involvement of KLF11 in the regulation of TGFB-mediated cascades (Correa *et al.*, 2016), modulation of COL1A1 and fibrosis (Zheng *et al.*, 2016; Shenoy *et al.*, 2017) or dopaminergic signaling (Richards *et al.*, 2017). Paradoxically, the expression of

KLF11 in endometriosis by transcriptome analysis gives highly contrasting results and did not emerge as deregulated in many studies, at least at the mRNA level (Sha *et al.*, 2007; Hawkins *et al.*, 2011; Crispi *et al.*, 2013). According to the data presented in 2013 (Daftary *et al.*, 2013), this could be compatible with a difference occurring at the protein level and not at the mRNA level, suggesting that in endometriosis, the stability of KLF11 could be decreased, without changes in the level of the mRNA. In sum, while the effects of KLF11 on endometriosis lesions in mice appear well-documented, the position of this factor in the cascade of events leading to the lesion is still an open question. Recently, the same team showed that KLF10 also plays a role in the promotion of endometriosis (Delaney *et al.*, 2016). Contrary to KLF11, KLF10 appears modified at the transcriptomic level (Hawkins *et al.*, 2011), where the transcript NM_001032282 is induced 2.2-fold ($P = 0.003$), calculated from the GDS3975 dataset in GEO Profiles. The *Klf10* $-/-$ animals presented with an abnormal regulation of the CD40/CD154 immune pathways leading to progression without massive fibrosis as observed in the *Klf11* $-/-$ animals. The authors suggested that both transcription factors contribute to specific aspects of endometriosis progression, through epigenetic regulation of specific promoters.

DNA Methylation in the Normal and Endometriotic Uterus

A very recent paper exhaustively studied endometrium DNA methylation with the Illumina HumanMethylation450 array, in parallel with gene expression analyzed by RNAseq at two periods of the cycle (on days 2—prereceptive endometrium—and 8—receptive endometrium—after the LH surge of the same cycle for 17 women—34 biopsies). Statistically, 5% of the CpGs were modified, and 30% of those were associated with modifications of gene expression. 5′ UTR methylation was often inversely correlated with gene expression, while gene body methylation was rather positively correlated to gene expression (Kukushkina *et al.*, 2017). Another recent study analyzed the same question in the context of endometriosis (Houshdaran *et al.*, 2016), using the older Illumina platform analyzing ~27,000 CpG dinucleotides (Illumina 27 K). In terms of methylation, the authors observed no difference for most sites in terms of percentage of hypomethylation (<20%) or hypermethylation (>80%) according to the phases of the cycle. Also no obvious differences were found between control and endometriotic patients. Nevertheless, the largest differences were mainly found in the P4-dominant mid secretory phase (MSE), with 137 differential CpG sites corresponding to 125 loci, while only 58 and 39 CpG sites were found different in proliferative phase (PE) and early secretory phase (ESE), respectively. Analyzing the correlation between methylation and gene expression, the authors found that outside CpG islands (CGI), the correlation between endometriosis-increased methylation with gene expression level was mainly negative in the proliferative phase, mainly positive in CGI in the early secretory phase, rather negative outside CGI in the mid secretory phase. Therefore, the correlation of gene expression and gene methylation throughout the genome in the various menstrual phases of the cycle was shown by this recent paper to be extremely complex. Overall, these results tend to show that the uterus of endometriotic women is molecularly different than that of control women. The differences are visible at the DNA methylation level. Furthermore, various reports show that mRNA levels differ for numerous genes between patients and controls; for instance, Santulli and coworkers showed that *PTGS2*, *PTGER2*, *PTGER3*, *PTGER4* differ in terms of mRNA levels (while this can be untrue at the protein level) (Santulli *et al.*, 2014). In the same study, the authors showed that oral contraceptives may also have a strong influence on the mRNA level for PTGS1 and mRNA/protein levels for PTGS2, making the situation more complex if this parameter of oral contraception is not taken into consideration. Interestingly, it has recently been shown that *PTGS2* promoter methylation differences are present in the endometrium of endometriotic women, at the binding site of NF-IL6 (CEBPB). It may be hypothesized in this case that methylation interferes with binding and that its decrease fosters activation of PTGS2 expression.

Recent Data, Questions and Paradoxes About miRNA Expression in Endometriosis

As in most physiological or pathological processes, it is established that miRNAs can be used as biomarkers of a specific state. In addition, and interestingly compared to other epigenetic marks, the consequences of an abnormal miRNA concentration in terms of gene function may be considered as quite straightforward functionally. miRNAs are encoded by nuclear genes, generally transcribed by RNA polymerase II as a pri-miRNA (Nothnick, 2017), processed in the nucleus to form a pre-miRNA through the action of the specific ribonuclease III, Drosha, to generate a stem-loop structure of ~70 base pairs. This structure is exported from the nucleus by the XPO5 exportin, linearized as a miRNA duplex by DICER and associated to the RISC complex, where it will induce the targeted degradation of the cognate mRNA. One major issue of miRNA research in the context of endometriosis is that the panel of deregulated miRNAs either between the ectopic lesion and the endometrium, or between the eutopic endometrium in patients versus controls, varies considerably from one study to another (Saare *et al.*, 2017). This last review points to a series of 14 miRNAs discovered deregulated in endometriotic lesions compared to eutopic endometrium and in common in more than three independent studies (Hawkins *et al.*, 2011; Ohlsson Teague *et al.*, 2009; Filigheddu *et al.*, 2010; Shi *et al.*, 2014; Yang *et al.*, 2016; Braza-Boils *et al.*, 2014).

To use miRNAs as non-invasive biomarkers of endometriosis, numerous studies are still being published, with improvements in the detection techniques. For instance, next generation sequencing (NGS) has recently been carried out on the plasma of 30 patients (19 in the proliferative phase, and 11 in the secretory phase), compared to 20 controls (17 in the proliferative and 3 in the secretory phase) (Wang *et al.*, 2016). This study discovered 108 differentially expressed miRNAs, 98 down-regulated in endometriosis and 10 up-regulated, of which only 21 (all down-regulated) were shared by other studies. The most spectacular study in terms of the capability of miRNAs to be used systematically as serum biomarkers was published in 2013 (Wang *et al.*, 2013). The

authors found that miR-199a and miR-122 were up-regulated, while miR-145*, miR-141*, miR-542-3p, and miR-9* were down-regulated in the patients. Four of these miRNAs led to a sensitivity of 93.2% and specificity of 96% for revealing endometriosis, with an area under the curve (AUC) reaching an impressive value of 0.994. In common with the previous study, miR-199a-5p and miR-122-3p were found in the study of Wang *et al.* (2016), but both were down-regulated (~2.5 and ~16-fold, respectively). More consistently between the two studies, miR-145-3p, miR-141–3p were found reduced (~2.7 and ~3, respectively). MiR-542-3p was found upregulated >fivefold in the Solexa NGS study of Wang and coworkers. In summary and taking these two studies as exemplary, highly inconsistent results were surprisingly obtained between two apparently rigorously carried out studies. The authors of such studies indicate these discrepancies without providing convincing explanations, despite the question of reproducibility being at the center of every scientific process. Possibly, the technical maturity of the different experimental approaches used is still disputable in studies that were performed before 2015. If this is the case, consensus panels of miRNAs should eventually come to light in the years to come. In the latest Cochrane database census, including 15,141 participants (Nisenblat *et al.*, 2016), 9 miRNAs were selected (miR-122, miR-141*, miR-145*, miR-17-5, miR-199a, miR-20a, miR-22, miR-532-3p, and miR-9) as blood markers among 122 markers including cytokines, immune cells, etc. As discussed before, some miRNAs yielded discrepant observations, suggesting that evolution of these lists of miRNAs should be published in the future. As the authors conclude "A subset of blood biomarkers could prove useful either for detecting pelvic endometriosis or for differentiating ovarian endometrioma from other benign ovarian masses, but there was insufficient evidence to draw meaningful conclusions. Overall, none of the biomarkers displayed enough accuracy to be used clinically outside a research setting."

Endometriosis and Cancer

One important question in endometriosis research is the study of a putative link with tumorigenesis, since endometriosis shares common features with cancer. One major form of endometriosis is ovarian endometriosis (mentioned above as OMA), therefore, specific attention was paid to endometriosis-associated ovarian carcinoma. Two studies published in 2007 and 2008 by Kobayashi *et al.* (2007, 2008), evaluated the risk of developing ovarian cancer among OMA women. Forty-six women out of 6398 (0.72%) developed an ovarian cancer (RR = 8.95 [4.12–15.3]). A 2010 study indicated that two types of ovarian tumors could be distinguished, low-grade endometrioid, clear cell, mucinous and transitional carcinomas, confined to the ovary with a relatively slow progression (Type I), and another (Type II) more classical highly aggressive including high-grade serous carcinoma, undifferentiated carcinoma, and malignant mixed mesodermal tumors (carcinosarcoma) with TP53 mutations in 80% of the cases (Kurman and Shih, 2010), emphasizing the necessity to discriminate ovarian tumors. Endometriosis was then considered as a source of endometrioid and clear cell tumors. The link between endometriosis, ovarian cancer and epigenetics has recently been associated with dysfunction of the *ARID1A* gene. This chromatin remodeler is expressed ubiquitously, and particularly in the glandular cells of the uterus (https://www.proteinatlas.org/ENSG00000117713-ARID1A/tissue/endometrium#img). Its link with endometriosis has been evoked above in ovarian carcinomas since 2010 (Wiegand *et al.*, 2010). Mutations of *ARID1A*, initially found in clear cell and endometrioid carcinomas of the ovary, appear frequent in endometrial carcinomas, but infrequent in other tumors, leading the authors to conclude that loss of ARID1A is a common feature of carcinomas arising from the endometrial glandular epithelium (Wiegand *et al.*, 2011). Interestingly, a recent study of 70 samples from endometriotic patients revealed a partial loss of ARID1A (as monitored by immunohistochemistry) in some of the patients sampled, suggesting that, since only a small proportion of endometriosis leads to carcinogenesis, no direct correlation can be made between ARID1A partial loss and tumor development (Borrelli *et al.*, 2016). As mentioned before, exome sequencing revealed that mutations in genes known to be specific cancer targets in endometriosis lesions (*ARID1A*, *PIK3CA*, *KRAS*, *PPP2R1A*) do occur, but presumably the niche environment in the uterus does not lead to malignancy (Anglesio *et al.*, 2017). In a recent study, Shibuya and coworkers studied by NGS 48 samples of ovarian clear cell carcinoma tissue in comparison with adjacent non-cancerous tissue, revealing frequent mutations in *ARID1A* (66.7%), *PIK3CA* (50%), *PPP2R1A* (18.8%), and *KRAS* (16.7%). Interestingly, the authors also found clusters of mutations related to APOBEC activation (Shibuya *et al.*, 2018), APOBEC being a family of cytidine deaminases involved in mRNA editing mechanisms, recently shown to be regulated by miRNA (Cao and Wu, 2018). In summary, there is an abundant literature linking the chromatin remodeler ARID1A, endometriosis and ovarian cancer; the next step will be understanding how its loss-of function mutations change the histone profile in endometriotic lesions. Once the epigenetic chromatin status modification is deciphered, it will pave the way to understand how gene deregulation is triggered, and which genes are affected by these transitions.

Temporary Conclusions

The history of the links between epigenetics and endometriosis followed the development of technical advances; until recently, genome-wide analysis of epigenetic deregulation focused on DNA methylation and miRNA profiling. While the DNA methylation profiles obtained by different teams appear relatively consistent when lesions are compared to eutopic endometrium, the miRNA profiles either in the comparison of ectopic versus eutopic, or between patient versus control endometria appear much less consensual. This may be due to a certain immaturity in the techniques used, an issue that could be solved shortly. Histone PTM profiling is becoming more and more accessible to technical analysis and will certainly become a major issue in the years to come, with a predictable profusion of published papers on this topic.

See also: Endometriosis

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Uterine Fibroids and Infertility

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Introduction

Uterine fibroids (also known as leiomyomas or myomas) are the most common form of benign uterine tumors (Donnez and Jadoul, 2002; Islam *et al.*, 2013; Bulun, 2013; Donnez and Dolmans, 2016). They are monoclonal tumors of uterine smooth muscle, thus originating from the myometrium (Kim and Sefton, 2012). They are composed of large amounts of extracellular matrix (ECM) containing collagen, fibronectin, and proteoglycans (Kim and Sefton, 2012). Leiomyomas occur in 50%–60% of women, rising to 70% by the age of 50 (Baird *et al.*, 2003), and, in 30% of cases, cause morbidity due to abnormal uterine bleeding (heavy menstrual bleeding inducing anemia), pelvic pressure (urinary symptoms, constipation, tenesmus) (Donnez and Jadoul, 2002; Donnez *et al.*, 2014a,b), and/or infertility.

Risk Factors

The risk factors for uterine fibroids are illustrated in **Fig. 1**.

Race

Race constitutes an important risk factor for leiomyoma development (Wise *et al.*, 2004; Stewart *et al.*, 2013). A US study found that the incidence of uterine fibroids was 60% by age 35 among African-American women (Baird *et al.*, 2003). It is clear that African-American women have a greater chance of being affected by uterine fibroids, particularly at an earlier age (Wise *et al.*, 2004, 2005; Wise and Laughlin-Tommaso, 2016).

Age

The average growth rate was 9% over 6 months in a study by Peddada *et al.* (2008) who followed the size of 262 uterine fibroids for up 12 months, but growth rates differed between races when age was taken into account.

Delaying the first pregnancy until the third decade of life also places women at higher risk of uterine fibroids (Petraglia *et al.*, 2013).

Early menarche and parity

Menarche at an early age increases the risk of developing fibroids and is also considered a risk factor for other hormonally mediated diseases. Pregnancy has been found to have a protective effect but the mechanism remains unclear. During postpartum uterine remodeling, small lesions may be subject to selective apoptosis (Laughlin *et al.*, 2010).

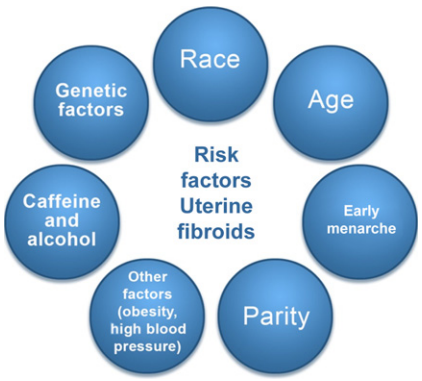

Fig. 1 Risk factors for uterine fibroid. These include race, age, delayed pregnancy, early menarche, parity (protective effect), caffeine, genetic alterations, and others, such as obesity and a diet rich in red meat.

Genetic factors

Mehine *et al.* (2013) performed whole genome sequencing and gene expression profiling of 38 uterine leiomyomas and corresponding myometrium. The common occurrence of chromothripsis in uterine fibroids suggests that it also plays a role in their genesis and progression (Mehine *et al.*, 2013, 2014).

Other factors

An association has been reported between alcohol and caffeine intake and an increased risk of developing uterine fibroids (Wise *et al.*, 2004; Wise and Laughlin-Tommaso, 2016).

General health status may also be predictive of leiomyoma growth, with factors such as obesity and high blood pressure playing a role (Kim and Sefton, 2012).

Evidence of the Crucial Role of Progesterone Pathways in the Pathophysiology of Uterine Fibroids

Genetic and epigenetic factors, sex steroids, growth factors, cytokines, chemokines, and ECM components have been identified as being implicated in the pathogenesis of leiomyomas (Islam *et al.*, 2013; Bulun, 2013; Marsh *et al.*, 2015, Protic *et al.*, 2015; Yin *et al.*, 2015; Donnez and Dolmans, 2016).

In the past, estrogen was considered to be the major growth factor in myoma development. However, already in the late 90s, Nisolle *et al.* reported increased expression of both progesterone receptor A (PR-A) and progesterone receptor B (PR-B) in leiomyoma tissue (Nisolle *et al.*, 1999). Higher proliferative activity, demonstrated by proliferating cell nuclear antigen and the mitotic index, was observed in leiomyomas during the luteal (secretory) phase (Nisolle *et al.*, 1999). There is evidence from preclinical and clinical trials, as well as from histological and pharmacological studies, that progesterone and its receptors play a key role in uterine fibroid growth (Bouchard *et al.*, 2011; Bouchard, 2014; Chabbert-Buffet *et al.*, 2005, 2012, 2014; Kim and Sefton, 2012; Bestel and Donnez, 2014; Moravek *et al.*, 2015).

Very recently, Tsigkou *et al.* (2015) showed that PR-B mRNA and PR-A and PR-B proteins were more concentrated in leiomyomas than in matched myometrium.

The PI3K/AKT pathway is mediated by progesterone which, through its receptors, can quickly activate this pathway, which is increasingly considered to be a potential promoter of leiomyoma growth. PTEN, on the other hand, should be considered as a negative regulator of AKT (Kim and Sefton, 2012). Progesterone and growth factor signaling pathways are interconnected and govern numerous physiological processes such as proliferation, apoptosis, and differentiation; nevertheless the initial event that triggers the first stages of tumorigenesis involves somatic mutations (Kim and Sefton, 2012).

Symptoms

Many fibroids are asymptomatic, but in 30%–40% of cases, they show a variety of symptoms, depending on the location and size. The FIGO classification, published (Munro *et al.*, 2011), describes eight types of fibroids, as well as a hybrid class (association of two types of myomas) (**Fig. 2**). As different types of fibroids are often present at the same time (depending on site), this classification offers a more representative 'map' of fibroid distribution and will be used further for the establishment of new algorithms (Donnez and Dolmans, 2016).

Fibroids can cause heavy menstrual bleeding with subsequent anemia, which could be life-threatening (Parker, 2007; Nelson and Ritchie, 2015). African-American women have more severe symptoms in terms of heavy bleeding and anemia compared with white women (Stewart *et al.*, 2013). Large fibroids can also result in pressure symptoms (bulk symptoms). Abdominal distention

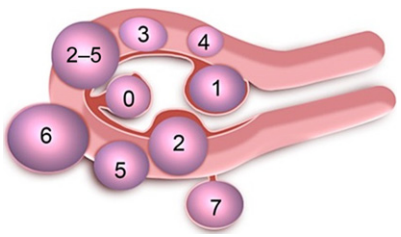

Fig. 2 FIGO classification of uterine fibroids according to Munro *et al.* (2011). Fibroid types range from 0 to 8. 0 = Pedunculated, intracavitary; 1 = submucosal, <50% intramural; 2 = submucosal, ≥50% intramural; 3 = contact with endometrium, 100% intramural; 4 = intramural; 5 = subserosal, ≥50% intramural; 6 = subserosal, <50% intramural; 7 = subserosal, pedunculated; 8 = other (e.g., cervical, parasitic). Where two numbers are given (e.g., 2–5), the first number refers to the relationship with the endometrium, while the second number refers to the relationship with the serosa; for example, 2–5 = submucosal and subserosal, each with less than half the diameter in the endometrial and peritoneal cavities, respectively. Fibroid classification cartoon republished with permission from Munro, M. G., Critchley, H. O., Broder, M. S. and Fraser, I. S. (2011). FIGO classification system (PALM-COEIN) for causes of abnormal uterine bleeding in nongravid women of reproductive age. FIGO Working Group on Menstrual Disorders. *International Journal of Gynecology & Obstetrics* **113**, 1–2.

or distortion and pelvic pressure on the ureters (causing hydronephrosis) and pelvic blood vessels (particularly pelvic veins) could also interfere with quality of life (Spies *et al.*, 2002; Donnez *et al.*, 2014a,b, 2016).

Infertility and recurrent miscarriage may also be symptoms of fibroids, depending on their location and size, especially for submucous and intramural myomas distorting the uterine cavity (Pritts *et al.*, 2009; Zepiridis *et al.*, 2016; Donnez and Dolmans, 2016).

Fibroids can impair fertility through several possible mechanisms, including (**Fig. 3**): (1) alteration of the local anatomy (anatomic distortion of the uterine cavity), subsequent alterations to endometrial function (Somigliana *et al.*, 2007); (2) impairment of the endometrial and myometrial blood supply (Donnez and Jadoul, 2002; (3) functional changes, such as increased uterine contractility; (4) changes to the local hormone milieu and paracrine molecular changes induced by fibroids, which could impair gamete transport and/or reduce blastocyst implantation (Sinclair *et al.*, 2011; Galliano *et al.*, 2015); (5) thicker fibroid capsule; and (6) changes in gene expression (↓HOXA 10).

Fibroids can affect obstetric outcomes and are significantly associated with preterm delivery, primary cesarean section, breech presentation, and lower birthweight infants (Blitz *et al.*, 2016).

Diagnosis

Examination of the pelvis may reveal an enlarged uterus or mass. An ultrasound is the gold standard test for uterine fibroids. Its widespread availability enables easy and inexpensive confirmation in almost all instances. Moreover, ultrasonography after infusion of saline into the uterine cavity can delineate submucous myomas and indicate the proximity of intramural myomas to the endometrial cavity (Seshadri *et al.*, 2015).

Hysteroscopy is usually performed in an outpatient setting and does not require any anesthesia (Bettocchi *et al.*, 2003). In case of irregular bleeding or if the patient has risk factors for endometrial hyperplasia (obesity, chronic anovulation), hysteroscopy may be combined with an endometrial biopsy.

MRI (magnetic resonance imaging) can provide information on the number of fibroids, their size, vascularization, relationship with the endometrial cavity and serosal surface, and boundaries with normal myometrium. It should nevertheless be stressed that like ultrasonography, MRI cannot diagnose malignancy with any certainty (Lumsden *et al.*, 2015; Donnez and Dolmans, 2016).

Current Surgical Management Strategies

Current management strategies involve mainly surgical interventions, but the choice of treatment is guided by the patient's age and desire to preserve fertility or avoid 'radical' surgery such as hysterectomy (Donnez and Jadoul, 2002; Donnez *et al.*, 2014a,b, 2016).

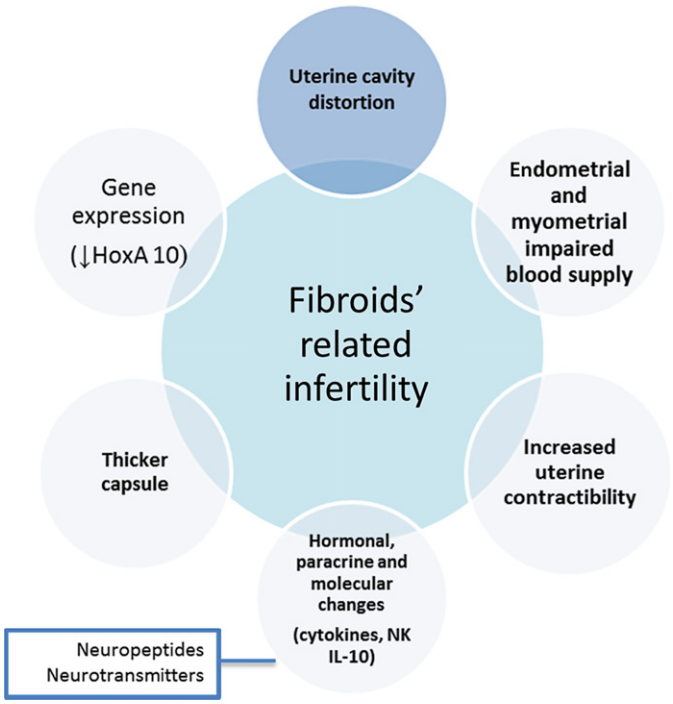

Fig. 3 Fibroids could impair fertility through several possible mechanisms.

Surgical and nonsurgical approaches include hysterectomy (vaginal, abdominal, or laparoscopic), myomectomy by hysteroscopy, myomectomy by laparotomy or laparoscopy, uterine artery embolization (UAE), and other interventions performed under radiologic or ultrasound guidance (**Fig. 4**; Donnez and Jadoul, 2002).

Contraindications to laparoscopic myomectomy usually include: the presence of an intramural myoma >10–12 cm in size or multiple myomas (≥ 4) in different sites of the uterus, requiring numerous incisions.

The dimensions and localization of the main myoma are the principal criteria for choosing the laparoscopic approach (Dubuisson *et al.*, 2000; Alessandri *et al.*, 2006; Palomba *et al.*, 2007; Nezhat *et al.*, 2009; Thomas *et al.*, 2010; Malzoni *et al.*, 2010; Segars *et al.*, 2014; Donnez *et al.*, 2014a,b; Parazzini *et al.*, 2015). Thus, depending on the skill of the surgeon and his/her ability to suture the myometrial defect without delay, either laparoscopy or minilaparotomy may be selected.

In terms of infertility, several noncontrolled studies have suggested that myomectomy yields a decrease in the miscarriage rate in women with myomas distorting the uterine cavity (Saravelos *et al.*, 2011; Bernardi *et al.*, 2014; Parazzini *et al.*, 2015). In a review of prospective and retrospective studies, Donnez and Jadoul reported a pooled pregnancy rate of 49% (95% CI 46–52) in patients who underwent laparoscopic myomectomy (Donnez and Jadoul, 2002). These postmyomectomy pregnancy rates have been confirmed by other studies, but the lack of randomized trials represents a serious drawback.

In cases of infertility related to pediculated or myomas distorting the uterine cavity, complete resection by hysteroscopy improves the pregnancy rate (Di Spiezio Sardo *et al.*, 2015; Donnez *et al.*, 2014a,b).

Why We Need New Options

Fibroids are highly prevalent and represent a high health burden. Indeed, about 30% of women with leiomyomas will request treatment due to morbidities such as heavy menstrual bleeding, abdominal pain, pressure symptoms, and/or infertility.

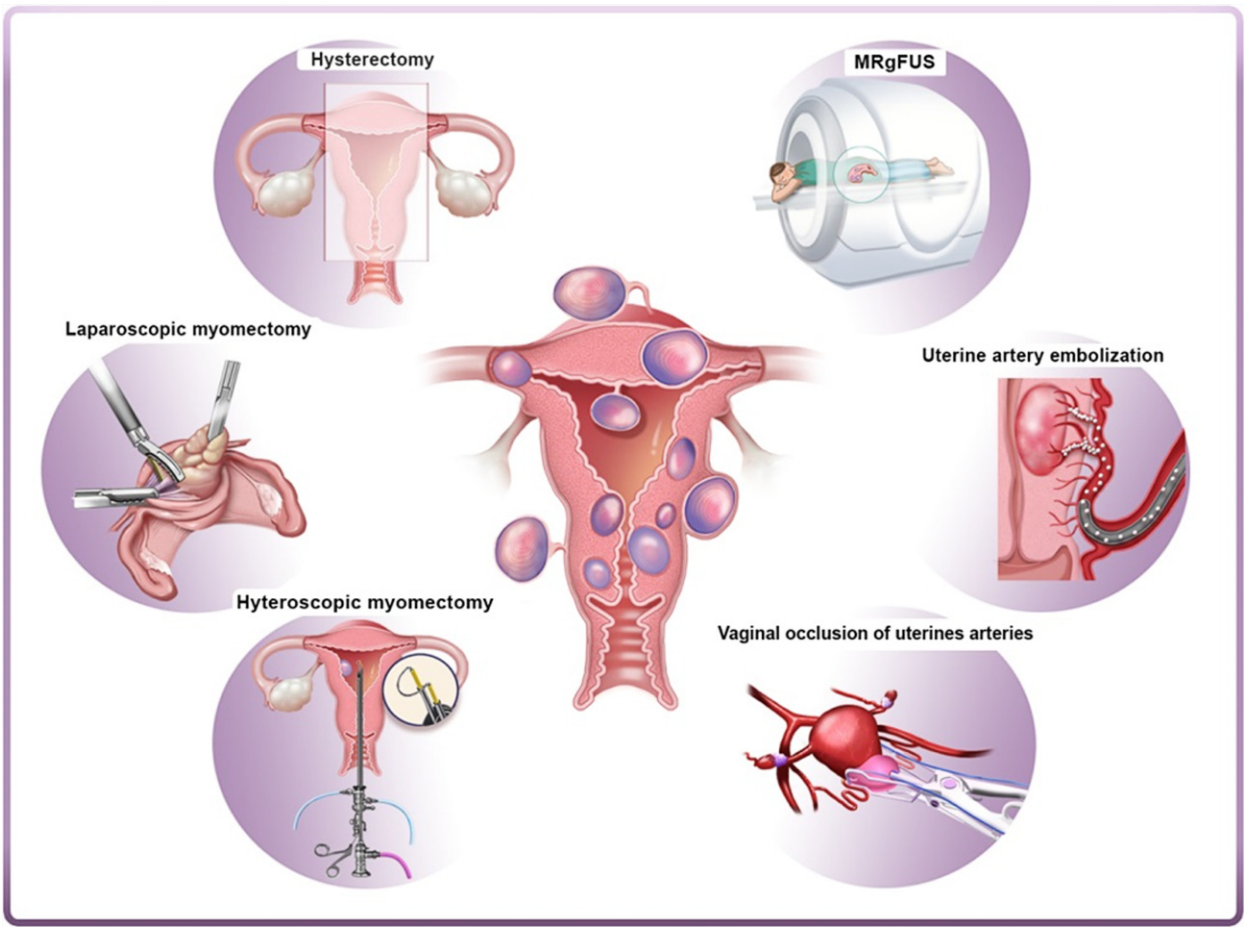

Fig. 4 Current surgical and nonsurgical management strategies of myomas. Left panel: hysterectomy, laparoscopic myomectomy, and hysteroscopic myomectomy are the most widely used surgical interventions for myomas. Right panel: alternatives to surgical intervention include uterine artery embolization (UAE), high-frequency magnetic resonance-guided focused ultrasound surgery (MRgFUS), and vaginal occlusion of uterine arteries.

Despite the lack of relevant medico-economic evaluations of the different therapeutics, it is likely that reducing the number of hysterectomies and other surgical procedures will reduce costs and morbidity. It is therefore necessary to develop and evaluate alternatives to surgical procedures especially when fertility preservation is the goal (Donnez *et al.*, 2014a,b).

Selective Progesterone Receptor Modulators and Fibroids

The crucial role of progesterone in the growth and development of myomas having been established, we can modulate the progesterone pathway by use of selective progesterone receptor modulators (SPRMs) (Donnez *et al.*, 2012a,b; Kim and Sefton, 2012; Bouchard *et al.*, 2011, Bouchard, 2014; Bestel and Donnez, 2014). SPRMs are synthetic compounds that exert either an agonistic or antagonistic effect on PRs (**Fig. 5**). Their binding allows these receptors to interact with coactivators and/or corepressors, and this is further impacted by the presence of coregulators in a particular cell type, which will dictate whether an SPRM acts more as an agonist or antagonist.

Courtoy *et al.* suggested an important role of ulipristal acetate (UPA) in collagen degradation induced by matrix metalloproteinase 2 (MMP-2), offering an explanation for the sustained beneficial effect. Indeed, this study strongly points to multifactorial mechanisms of action involving: (1) a persistently low cell death rate; (2) a limited period of cell death; and (3) ECM remodeling concomitant with stimulation of MMP-2 expression (Courtoy *et al.*, 2015).

Four members of the family of compound SPRMs have been investigated clinical trials: mifepristone, asoprisnil, UPA, and telapristone acetate.

The latest antiprogestin to be widely studied in large clinical trials, UPA, showed promising results in terms of efficiency and safety. UPA was compared to a placebo and to leuprolide acetate (a GnRH agonist) in two randomized trials (Donnez *et al.*, 2012a, b). In these first clinical studies, uterine bleeding was controlled in more than 90% of patients receiving a 3-month course of UPA, and the median times to control bleeding were shorter in the UPA group (5–7 days) than in the GnRH agonist group (21 days).

UPA was also found to have a sustained effect (up to 6 months) in women who did not undergo surgery after the 3-month study period. By contrast, those treated with GnRH agonist experienced rapid regrowth of their fibroids, whose size reached pretherapy dimensions by 6 months posttreatment (Donnez *et al.*, 2012a,b).

Importantly, the induced effects on the endometrium, now described as progesterone receptor modulator-associated endometrial changes (PAECs), present in almost 70% of patients at the end of treatment, have proved to be benign and reversible, as they disappear 2 months after the end of therapy (Donnez *et al.*, 2012a,b; Williams *et al.*, 2012).

Because of the sustained effect observed in the first two trials, additional intermittent (12-week) courses of SPRMs with off-treatment intervals may be an alternative for long-term medical management of fibroids. The results of the first long-term intermittent administration study suggested that more than one course of SPRMs can maximize its potential benefits in terms of bleeding control and fibroid volume reduction (Donnez *et al.*, 2014b, 2016).

The latest clinical trial investigated the efficacy and safety of four repeated 12-week courses of either 5 or 10 mg UPA daily for intermittent treatment of symptomatic uterine fibroids (Donnez *et al.*, 2015a,b). As a similar degree of response was observed in both treatment groups, we focus on the results in terms of efficacy and safety of this trial using the approved dose of 5 mg UPA in a

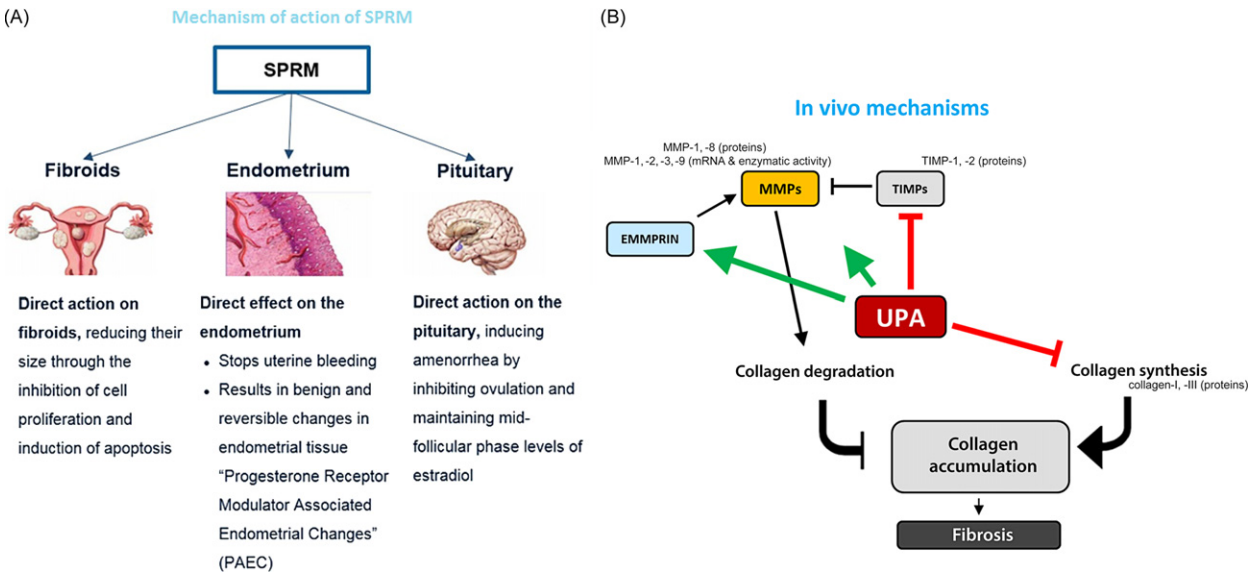

Fig. 5 (A) Mode of action of SPRMs (selective progesterone receptor modulators). SPRMs have a direct impact on fibroids, endometrium, and the pituitary. (B) In vivo mechanism of action, explaining the sustained effect of UPA treatment.

repeated intermittent therapy setting (four courses) (Donnez *et al.*, 2015b). The percentages of subjects identified as being in amenorrhea after each individual treatment courses were 75.8%, 84.1%, 86.4%, and 87.5% in the 5 mg group (Donnez *et al.*, 2015a) (**Fig. 6**). The pictorial blood assessment chart (PBAC) (Higham *et al.*, 1990) score was measured at initial screening and after 1, 2, and 4 courses to assess the level of menstrual bleeding during the off-treatment period. In the 5 mg group, (median) levels at screening were 224.0, dropping significantly with each subsequent course, and finally reaching 77.5 after course 4 (Donnez *et al.*, 2015a). The percentage of subjects with a clinically significant volume reduction of $\geq$25% increased from course 1 to course 4 (from 62.3% to 78.1%), and those with a volume reduction of $\geq$50% also increased from course 1 to course 4, proving that repeated courses considerably maximize the impact of treatment. The volume reduction of the three largest fibroids was increased from course 1 to course 4 (**Fig. 6**). The findings of this study therefore demonstrate the efficacy of 5 mg UPA treatment and further confirm the efficacy and safety of repeated intermittent administration of UPA for symptomatic myomas (Donnez *et al.*, 2015a,b,c).

Concerning endometrial safety, no increased occurrence of more serious conditions of the endometrium, such as hyperplasia with atypia or endometrial carcinoma, was noted after up to 4 treatment courses. Data further confirm the rapid reversibility of PAEC following completion of treatment and subsequent menstruation.

Novel Algorithms, With a Special Emphasis on Infertility

There is a clear need for alternatives to surgery, even the less invasive endoscopic techniques, especially when fertility preservation is the goal (Donnez *et al.*, 2014a,b, 2015b). There is no doubt that surgery remains indicated in some instances, but we must now establish whether SPRMs (UPA) allow less invasive surgery or even complete avoidance of surgery (Donnez and Dolmans, 2016).

It is crucial to consider key factors determining the management of uterine fibroids: patient age, severity of symptoms, wish to preserve the uterus and/or fertility, localization of fibroids according to FIGO classification, and myoma volume.

Women of Reproductive Age With Fibroids Related Infertility

Type 0 and Type I Myomas (Fig. 7A and B)

If type 0 myomas are present, cutting the pedicle by hysteroscopy is indicated. In the majority of cases, hysteroscopic myomectomy for type 1 myomas is relatively easily performed for experienced surgeons, especially in case of type 1 myomas $<$3 cm in size (**Fig. 8**). If a fibroid is of type 1 but larger than 3 cm, or if the patient presents with anemia, prehysteroscopic medical therapy (SPRMs or GnRH agonist) is indicated.

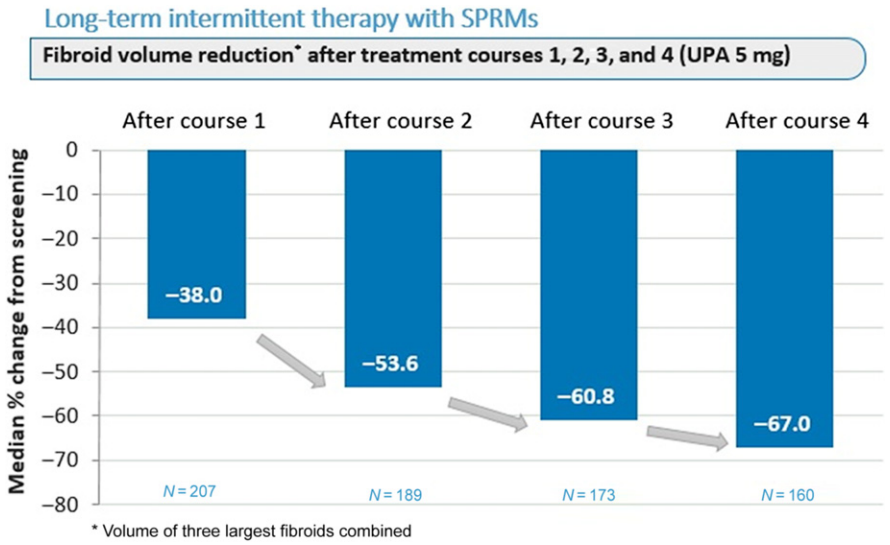

Fig. 6 Effect on fibroid volume reduction after four courses of 3 months of ulipristal acetate (UPA) 5 mg daily. The off-period between two courses was two natural cycles. Adapted from Donnez, J., Hudecek, R., Donnez, O., Matule, D., Arhendt, H. J., Zatik, J., Kasilovskiene, Z., Dumitrascu, M. C., Fernandez, H., Barlow, D. H., *et al.* (2015). Efficacy and safety of repeated use of ulipristal acetate in uterine fibroids. *Fertility and Sterility* **103**, 519–527; Donnez, J., Donnez, O., Matule, D., Ahrendt, H. J., Hudecek, R., Zatik, J., Kasilovskiene, Z., Dumitrascu, M. C., Fernandez, H., Barlow, D. H., *et al.* (2016). Long-term medical management of uterine fibroids with ulipristal acetate. *Fertility and Sterility* **105**, 165–173.

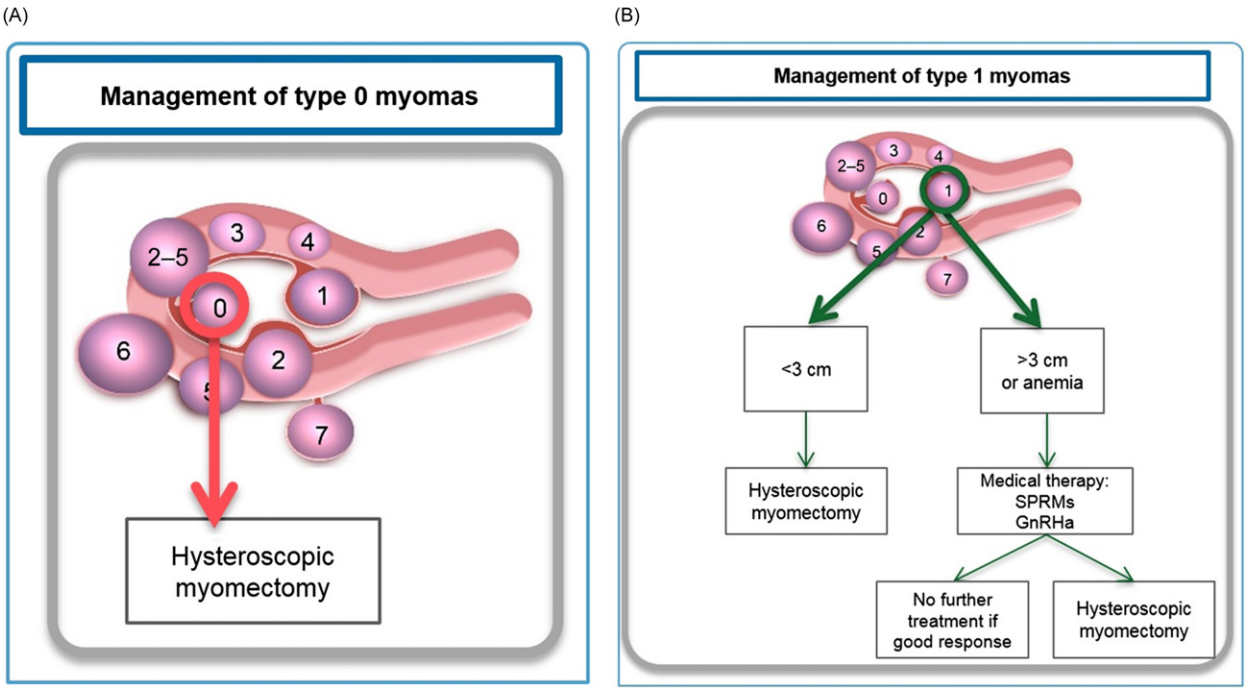

Fig. 7 (A) Management of type 0 myomas. Hysteroscopic myomectomy is the most appropriate approach. (B) Management of type 1 myomas. Depending on the myoma size, presence of anemia and the surgeon's skill, hysteroscopic myomectomy combined or not with ulipristal acetate (UPA) should be proposed.

In terms of reproductive outcomes, most studies are retrospective (Bosteels *et al.*, 2010a,b). They report postsurgery pregnancy rates ranging from 16.7% to 76.9%, with a mean of 45% (Donnez *et al.*, 2014a). Their robustness could be criticized, but the authors of a recent review (Bosteels *et al.*, 2015) acknowledge that the benefits of hysteroscopic removal of submucous myomas for improving the chances of pregnancy "cannot be excluded." In addition, one prospective randomized study (Casini *et al.*, 2006) has provided good-quality evidence that surgical therapy (hysteroscopic myomectomy) yields higher pregnancy rates than alternative treatments in women with submucous myomas.

Types 2–5, Myomas (Single or Multiple) Distorting the Uterine Cavity

Young infertile women of reproductive age and wishing to conceive

In case of type 2 myomas, medical therapy (SPRMs) can be proposed (**Fig. 9**).

If myomas are multiple (≥ 2) or of different types (types 2–5), medical therapy (SPRMs) can be given in two courses of 3 months, as described in clinical trials with UPA (Donnez *et al.*, 2014a,b, 2015a,b). After these two courses of 3 months, there are three possible outcomes. Myoma regression is very significant ($>50\%$ decrease in volume). The uterine cavity is no longer distorted, and the patient can try to conceive naturally or undergo assisted reproductive techniques, if indicated. In some cases, surgical treatment is not required and patients can conceive and deliver healthy offspring (Luyckx *et al.*, 2014; **Fig. 9**).

Myoma regression is significant ($\geq 25\%$ but $<50\%$) but the uterine cavity remains distorted. In this case, indication for surgery stand and medical treatment may allow surgery to be performed by a laparoscopic approach once the hemoglobin level is normalized, avoiding laparotomy.

The response to medical therapy is inadequate. In this case, surgery remains indicated.

Young women of reproductive age with symptomatic myomas and wishing to preserve their fertility but having no immediate desire for pregnancy

When there is no immediate wish to conceive, there is no pressing need for surgery (even if the uterine cavity remains distorted and/or large myomas are still present).

It is known that the rate of recurrence of myomas after myomectomy can reach almost 60% after an interval of 4–5 years, and that the risk of pelvic adhesions is significantly increased after a repeat myomectomy (Malone, 1969; Donnez *et al.*, 2014a,b).

Medical therapy can be proposed (**Fig. 8**): four courses of 3 months to induce a significant improvement, course upon course (decrease in myoma size and PBAC score) (Donnez *et al.*, 2015a,b). In the vast majority of cases, regression of myoma size and control of bleeding will allow avoidance of surgery and restoration of hemoglobin levels (**Fig. 10**).

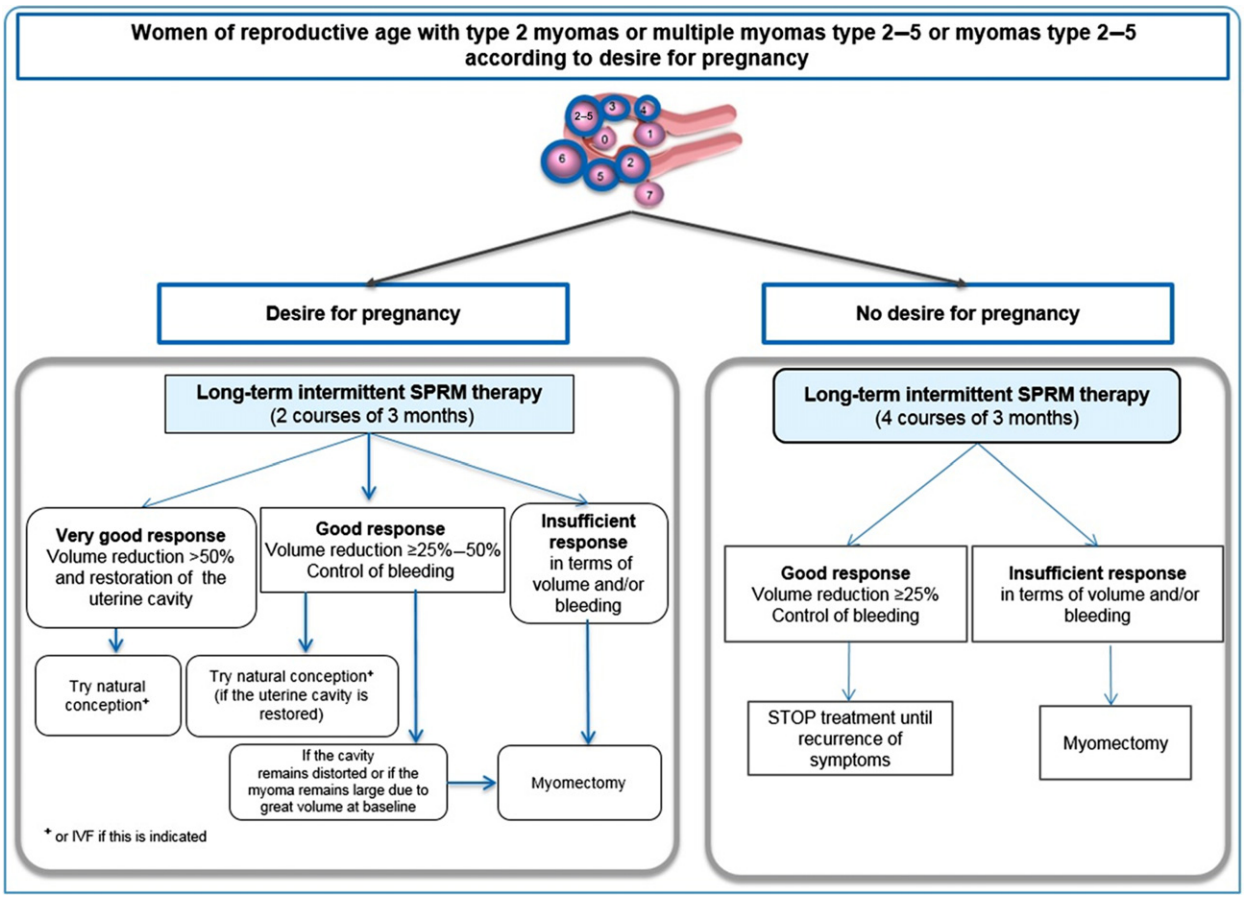

Fig. 8 Management in case of myomas or multiple myomas (types 2–5) in women of reproductive age, according to desire for pregnancy. In cases of infertility, two courses of 3 months are recommended (left panel). Subsequent therapy is determined depending on the response to treatment and restoration of the uterine cavity. If there is no desire to conceive (right panel), long-term (four courses) intermittent therapy may be proposed. In case of a good response in terms of fibroid volume reduction and bleeding, treatment is stopped and only restarted if symptoms recur (Algorithm republished with permission of HRU).

Myomectomy should only be considered when the patient wishes to become pregnant, and if really necessary according to the localization and volume of the fibroids still present. Surgery remains indicated only when the patient wishes to conceive, and if large myomas (>3–4 cm) distorting the uterine cavity are present, as these could be the cause of her infertility. This is important to take into account, especially for women of African descent who have a greater chance of developing symptomatic myomas at an earlier age than Caucasian women (Baird *et al.*, 2003). Medical treatment with SPRMs can thus be beneficial, since long-term intermittent therapy (repeated in case of symptom recurrence during the interval) may help avoid or at least postpone the need for surgery until the patient wishes to conceive (**Fig. 10**).

Asymptomatic women with myomas and undergoing IVF or oocyte donation

It could be proposed that patients with myomas be treated with one or two courses of SPRMs before IVF or oocyte donation, in order to reduce the size of myomas and restore the uterine cavity and subsequently improve implantation rates (**Fig. 8**). A meta-analysis by Pritts *et al.* (2009) showed a significant drop in pregnancy and implantation rates in the presence of myomas, especially submucous and/or intramural myomas distorting the uterine cavity. In another meta-analysis, Sunkara *et al.* (2010) demonstrated their impact on fertility, even in case of intramural myomas not distorting the uterine cavity. A recent study by Yan *et al.* (2014) confirmed that intramural fibroids >2.85 cm in size significantly decreased the delivery rate of patients undergoing IVF/intra-cytoplasmic sperm injection.

Future Prospectives for Medical Therapy

Future clinical trials should focus on prevention strategies, such as preventing occurrence in women genetically predisposed to this condition, and avoiding recurrence after surgery in women at high risk (i.e., those of a young age or with a family history).

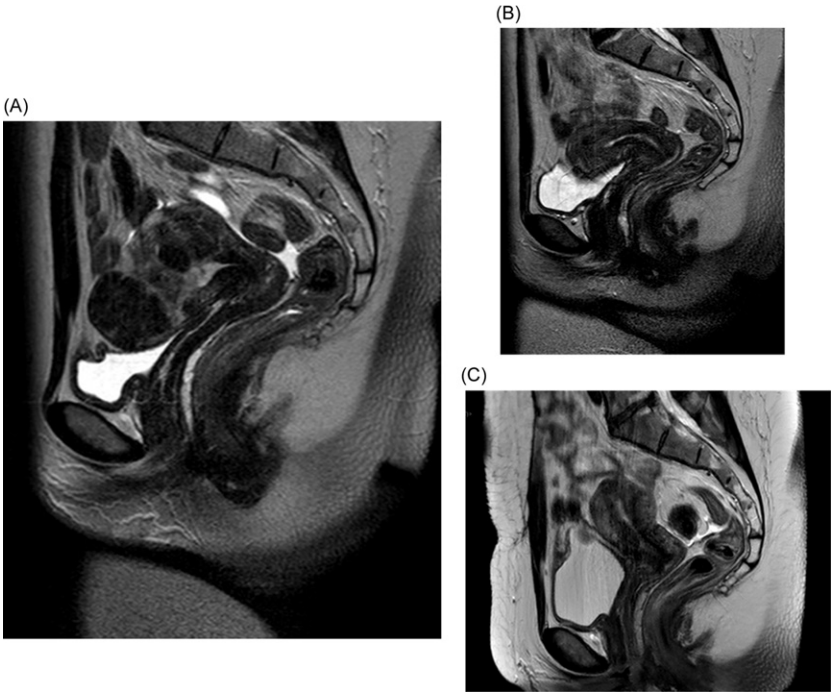

Fig. 9 Considerable shrinkage of all myomas after four courses of intermittent ulipristal acetate (UPA) therapy. A patient aged 30 years presented with heavy menstrual bleeding and an unclear desire for pregnancy. (A) Before treatment, a midline sagittal T2-weighted magnetic resonance image (MRI) demonstrated the presence of multiple myomas: types 2, 3, 4, and 6. (B) Upon completion of treatment (intermittent UPA therapy (four courses of 3 months), the uterine cavity was no longer distorted. (C) One year after delivery of a healthy baby, no fibroid regrowth was observed after delivery. Adapted from Donnez, J. and Dolmans, M. M. (2016). Uterine fibroid management: From the present to the future. *Human Reproduction Update* **226**, 665–686; Donnez, J., Donnez, O., Matule, D., Ahrendt, H. J., Hudecek, R., Zatik, J., Kasilovskiene, Z., Dumitrascu, M. C., Fernandez, H., Barlow, D. H., *et al.* (2016). Long-term medical management of uterine fibroids with ulipristal acetate. *Fertility and Sterility* **105**, 165–173.

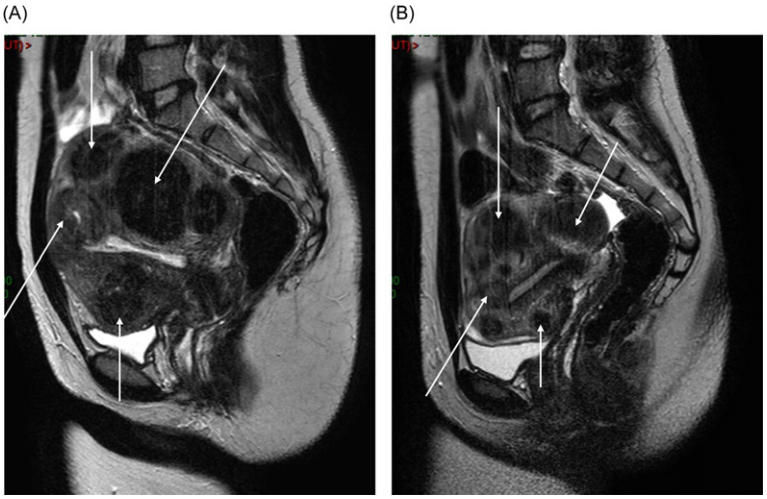

Fig. 10 Important shrinkage of the submucosal myoma was obtained after two courses of 3 months of intermittent ulipristal acetate (UPA) therapy. (A) Coronal T2-weighted MRI image illustrated the presence of multiple myomas (type 2, types 2–5) distorting the uterine cavity in a 19-year-old nulligravid patient, who presented to the emergency department, with heavy menstrual bleeding and anemia (hemoglobin level of 7.4 g/L). The patient received two courses of UPA (5 mg) and iron. (B) At the end of therapy, MRI demonstrated a significant reduction in myoma volume ($<50\%$) and restoration of the uterine cavity. Amenorrhea was achieved, with a hemoglobin level of 11.9 g/L. The patient was free of symptoms and did not wish to conceive; therefore, surgery was avoided.

Conclusion

The choice between less invasive techniques (uterine-sparing options such as myomectomy) is guided by the size, number, and location of fibroids, as well as the personal experience of the gynecologist and available equipment. Other surgical techniques, such as laparoscopic cryomyolysis, thermocoagulation, or uterine artery occlusion, are rarely used. Nonsurgical interventions, such as UAE and MRgFUS, are also available but the desire for future pregnancy is a relative contraindication.

The need for medical therapy remains a reality and it is indeed essential that new treatments be developed to be able to offer as there is a pressing need for alternatives to surgical intervention, particularly when fertility preservation is the goal.

We demonstrated that more than one 3-month course of UPA maximizes its potential benefits in terms of bleeding control and fibroid volume reduction. SPRMs should be considered an alternative to surgical therapy, or at least an adjunct to surgery.

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Environmental Factors and Female Reproduction

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Abbreviations

AhR	Aryl hydrocarbon receptor
AMH	Anti-Müllerian hormone
ANM	Age at natural menopause
ART	Assisted reproductive technology
BMI	Body mass index
BPA	Bisphenol A
CYP17A1	Cytochrome P450 17 hydroxylase gene
DDE	*p,p′*-Dichlorodiphenyldichloroethylene
DES	Diethylstilbestrol
DOHaD	Developmental Origin of Health and Diseases
EDCs	Endocrine-disrupting chemicals
ERα, ERβ	Estrogen receptor α, β
IVF	In vitro fertilization
LH	Luteinizing hormone
miRNA	MicroRNA
NCOR1	Nuclear corepressor factor 1
PCB	Polychlorinated biphenyls
PCOS	Polycystic ovaries syndrome
PCO	Polycystic ovaries
PFOA	Perfluoro octanoic acid
PFOS	Perfluoro octane sulfonate
PPARγ	Peroxysome proliferator-activated receptor γ
P450 scc	P450 side chain
SHBG	Sex hormone-binding globulin
StAR	Steroidogenic acute regulatory protein

Introduction

The total fertility rate has declined dramatically over the past decades in developed countries reaching such a low rate for Europe and Japan (around 1.5 children per couple) that it might contribute for the first time in Japan, while life expectancy is still increasing, to a decrease of the population while life expectancy is still increasing (Skakkebaek *et al.*, 2016). Cultural changes, including the widespread use of contraception by women, a deliberate choice to raise fewer children, and delayed childbearing (Richardson *et al.*, 2014; Hart, 2016), may partly explain this continuous decline. However, there are many indices suggesting that impaired female reproduction linked to environmental factors may also be contributing to this trend. In most developed countries, the use of assisted reproductive technology (ART) is growing, as reported in Denmark, where the percentage of women between 20 and 40 years old has decreased, yet the percentage of couples resorting to ART has dramatically increased (Skakkebaek *et al.*, 2016). Association between environmental factors and decreasing trend of sperm characteristics and increasing male reproductive abnormalities have been widely explored and documented over the last two decades (Skakkebaek *et al.*, 2016).

The next challenge is to verify if female reproduction is also influenced by the changing environment, including exposures to deleterious nutritional factors and/or endocrine-disrupting chemicals (EDCs). There are limited data concerning the prevalence of diseases that affect women's reproductive health. It is less easy to evaluate the trend of female fertility than that of male fertility since gametes are less accessible, and objective markers of ovarian reserve are all imperfect. Identifying the time taken to become pregnant could be a relatively good approach, even though it also depends on the male partner. Most female reproductive disorders such as polycystic ovaries syndrome (PCOS), premature ovarian decline and/or failure, endometriosis, and uterine fibroids share several interesting characteristics, which are as follows: prevalence is relatively high and possibly increasing although not yet demonstrated; the disorders are all associated with impaired fertility with an unclear pathophysiology, which cannot be explained by genetic factors alone; and for all of the disorders, simple and efficient therapeutic solutions are lacking (**Fig. 1**). The most recent hypotheses suggest that interactions between genetic, epigenetic, and environmental factors could also play an important role. These hypotheses are supported by epidemiological and experimental evidence that we analyze in the following sections. Annual worldwide chemical production is dramatically increasing (Neel and Sargis, 2011) and these chemicals are present in large quantities in our domestic environments especially in food. We focus on nutrition and exposure to EDCs, two main environmental factors, depending on lifestyle changes which may have important implications in development of fetal and adult reproductive disorders. We exclude premature puberty and hormone-dependent cancers which may also be influenced by environmental factors, infectious factors through sexually transmitted diseases leading to tubal obstruction, and the consequences of surgery or radiochemotherapy.

Nutrition

Adipose Mass, Energetic Balance, and Female Reproduction

The role of nutrition in female reproduction has long been known; Rosa Frisch linked the onset of puberty with the increasing fat mass in girls more than 40 years ago (Frisch and McArthur, 1974). The mechanisms linking energetic balance and the

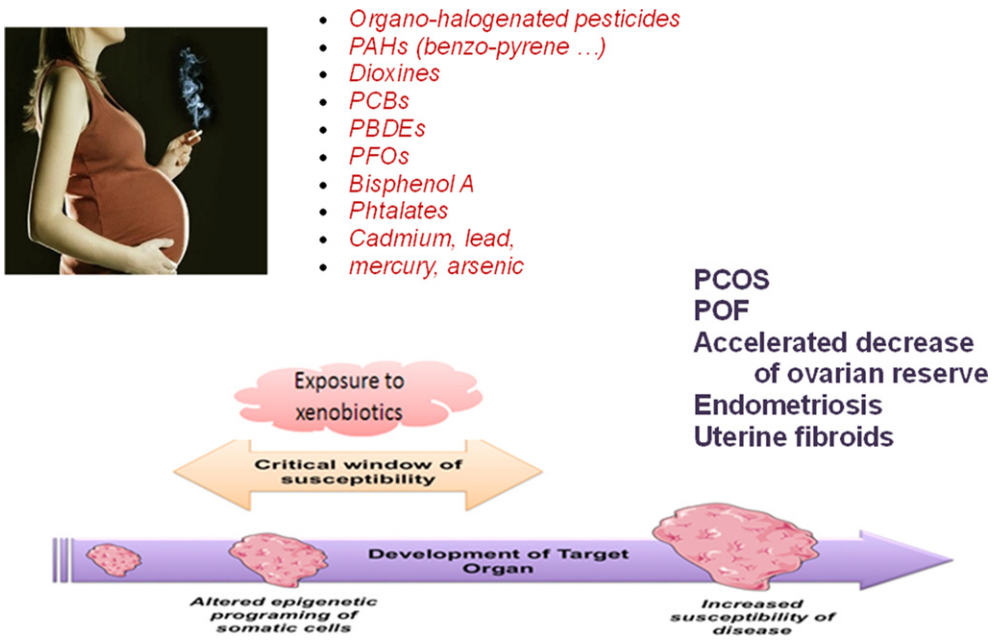

Fig. 1 Exposure to EEDs and impaired ovarian function.

hypothalamic control of the pituitary–ovarian axis were elucidated first with the discovery of leptin (Welt *et al.*, 2004), an adipokine, then with the integrative role of kisspeptin neurons (Tena-Sempere, 2010). Low levels of leptin and insulin indeed played an important inhibitory role in ancient times when the risk of starvation was frequent and women therefore needed to avoid conceiving, as they would not be able to produce enough free fatty acids to ensure healthy fetal growth and subsequently to breastfeed. This security system likely led to a self-selecting bias toward women with a low basal metabolic rate, a greater ability to store adipose tissue, and insulin-resistance susceptibility. These women would survive and conceive, but their offspring would be susceptible to obesity or the development of metabolic syndrome and diabetes when exposed to a more favorable environment, where food was no longer scarce. This selective bias may have contributed in developed or developing countries with improving economic conditions to the dramatic increase in cases of obesity, metabolic diseases, and polycystic ovaries (PCO) all around the world (Corbett and Morin-Papunen, 2013).

Impaired reproductive function may still occur in pathological conditions associated with a negative energetic balance such as that observed in addictive behaviors, like excessive restrictive diet, intensive sport practice, or drug use, associated with severe general diseases or with anorexia nervosa. Less severe restricted food-intake behavior is relatively common among young women and may be associated with disrupted menstrual cycles and decreased fertility rate (Miller *et al.*, 1998), even in cases of women with normal weight.

Fetal Nutritional Environment and Female Reproductive Disorders

One susceptible period during which nutrition seems particularly important for the future reproductive axis is the fetal stage. It is well established that physiological development during prenatal life is influenced by the intrauterine environment and that deleterious nutritional, metabolic, toxicological, or stressful conditions linked to the mother's exposure could increase the risk of adult onset of diseases as first proposed by David Barker in the 1980s (Barker and Osmond, 1986). Barker *et al.* showed that intrauterine growth retardation could cause a predisposition later to the development of adult metabolic and cardiovascular diseases, which is a hypothesis known as the Developmental Origin of Health and Diseases (DOHaD) (Barker, 2007). The question of whether poor maternal nutrition could have an adverse effect on ovarian reserve has been reviewed by Richardson *et al.*(2014). Reduced ovarian volume, low ovulation rates and increased FSH have been described in young women born small for gestational age (SGA) (Ibanez *et al.*, 2002), and the age at natural menopause (ANM) has been associated with both extremes (<2.5 kg or >4 kg) of birth weight standardized for gestational age (Tom *et al.*, 2010). However, these results were not confirmed by two other studies examining ovarian reserve related to birth weight (Hart *et al.*, 2009 Shayeb *et al.*, 2014). One of those studies found a correlation between weight at 2 years and ANM, but not between weight at the time of birth and ANM (Hardy and Kuh, 2002). However, the potential link between fetal or child nutrition and development of ovarian reserve is supported by experimental data in sheep, pigs, and cows (Richardson *et al.*, 2014), associating restricted maternal food intake with reduced ovary size, ovarian reserve, and adult ovulation rate. Fetal epigenetic ovarian modifications are likely to be responsible for this.

Obesity and Reproduction

Obesity and female fertility

Although a minimum of adipose mass may indeed be necessary for a woman to activate and maintain her hypothalamic–pituitary–ovarian axis (Frisch and McArthur, 1974), it seems that obesity, especially visceral obesity with insulin resistance and low-grade inflammation, is associated with: (1) a lower rate of ovulation, meaning less chance to conceive spontaneously or after ART (Broughton and Moley, 2017); (2) an increased risk of miscarriages (Meldrum, 2017); and (3) more adverse pregnancy outcomes including premature labor, stillbirth, and perinatal risks such as gestational diabetes and hypertension, need for operative delivery, wound infection, and thromboembolism (Meldrum, 2017). This is becoming a real public health concern with the occurrence of worldwide pandemics of obesity; in 2009, 23% of American women of reproductive age were classified as obese (Vahratian, 2009).

Obese women take longer to become pregnant, as shown in large prospective studies such as the Danish cohort (Wise *et al.*, 2010), even in the case of normal ovulating obese women included in the large American cohort (Gesink Law *et al.*, 2007), or the large Dutch cohort (van der Steeg *et al.*, 2008), which reported a linear declined probability of spontaneous conception for each body mass index (BMI) point over 29. Obese women undergoing in vitro fertilization (IVF) also have oocytes and embryos of poorer quality (Shah *et al.*, 2011) and have decreased pregnancies and/or a decreased live birth rate (Luke *et al.*, 2011) when compared to nonobese women.

Obesity, insulin resistance and PCOS

Prevalence of PCOS among obese women has been estimated at around 30% (Alvarez-Blasco *et al.*, 2006), and between 38% and 88% of women with PCOS were overweight or obese (Barber *et al.*, 2006). This obesity is most often "visceral" or "metabolically active." It is associated with other metabolic markers including insulin resistance, which is in fact the common starting point, regardless of whether or not the woman is already overweight. Obesity contributes to insulin resistance and exacerbates the symptoms of PCOS. Hyperinsulinism potentiates luteinizing hormone (LH) stimulation of the internal theca LH receptor, activating CYP17A1, increasing hyperandrogenism, and disrupting follicular maturation. Hyperinsulinism also increases circulating hyperandrogenism via the decrease of sex hormone-binding globulin (SHBG) and potentiates the action of insulin growth factor-1 via the decrease of insulin growth factor-binding proteins. Whatever its origin, hyperinsulinism is a gateway through which environmental factors such as nutrition, energy balance, intestinal microbiota (Guo *et al.*, 2016), and chemical pollutants (Fenichel *et al.*, 2017) are able to influence the chances of infertility. As for its origin, it may be genetic; thus insulin receptor gene variants were associated with PCOS (Lee *et al.*, 2008). It can also integrate into a developmental origin and preprogramming by impairment of the fetal environment (Fenichel *et al.*, 2017). In fact, both metabolic syndrome and PCOS may have an intrauterine developmental origin (DOHaD). An intrauterine growth retardation, known (Barker, 2007) to be associated with an increased risk of developing metabolic and cardiovascular diseases in adulthood, has been reported in adolescents with premature pubic hair and ovarian hyperandrogenism (Ibanez *et al.*, 2007; Melo *et al.*, 2010), as well as in a prospective cohort of 165 women with PCOS born at term (Cresswell *et al.*, 1997).

Obesity and female infertility: which mechanisms?

As discussed earlier, obesity is an aggravating factor of PCOS. Hyperinsulinemia, hyperandrogenism, and increased adipokines like leptin contribute to a central impairment of the pituitary–ovarian axis and the follicular maturation. However, infertility in obese women is not only due to lack of ovulation. Both human IVF results and animal experimental studies have shown that obesity has a direct and complex effect on the microenvironment of oocytes, preimplantation embryos, and endometrium mediated by insulin resistance, low-grade inflammatory state, oxidative stress, or lipotoxicity by higher levels of circulating free fatty acids and deleterious adipokines (Broughton and Moley, 2017). In obese woman, the use of IVF has resulted in altered follicular fluid biomarkers with a pro-inflammatory environment. Observation of oocytes that failed to fertilize in severely obese women has revealed high rates of meiotic aneuploidy with fragmented disorganized meiotic spindles with misaligned metaphase chromosomes (Machtinger *et al.*, 2012). Similar observations have been made in diet-induced obesity (DIO) mouse models with more apoptotic follicles, smaller and less mature oocytes with high rates of meiotic aneuploidy (Luzzo *et al.*, 2012), and alteration of mitochondrial function in the oocyte (Igosheva *et al.*, 2010). A similar pro-inflammatory and lipotoxic state can affect the microenvironment of the preimplantation embryo, as observed in the same DIO model with lower expression of insulin-like growth factor 1 receptor, influencing insulin sensitivity and glucose transport (Jungheim *et al.*, 2010), and contributing later to higher frequency of preeclampsia, due to abnormal trophoblast invasion.

In the oocyte donation model in relation to obesity, out of three reports on the pregnancy rate, one (Dessolle *et al.*, 2009) showed the receiver's BMI to be an independent negative predictor, illustrating the direct effect of obesity on the endometrium receptivity, as confirmed in the DIO mouse model (Rhee *et al.*, 2016).

Fetal outcomes and transgenerational effects

A very recent and large Swedish cohort study of 1.2 million singletons has shown that major congenital malformations and several subgroups of organ specific malformations increased with maternal overweight and increasing severity of obesity (Persson *et al.*, 2017).

There is also an increasing body of evidence suggesting that maternal obesity may contribute to pandemics of metabolic diseases by conferring a transgenerational risk (Van Dijk *et al.*, 2016) through in utero epigenetic modifications (Jungheim *et al.*, 2010). Moreover, in the DIO mouse model, it has been shown that metabolic dysfunction mediated through impaired mitochondrial function can be transmitted through the maternal germline from aberrant oocytes to several generations of offspring (Saben *et al.*, 2016). These recent data and the impaired fertility associated with obesity highlight the importance of medical programs to be proposed to obese women who want to conceive.

Medical interventions before pregnancy

It is obvious that weight loss should be proposed to every obese woman before they conceive and that efforts should be made to reduce adiposity through efficient educational programs. In fact, such programs still have room for improvement. Many ART centers from different countries set a weight cutoff before accepting obese women for fertility treatment. However, this rule could be ineffective and even harmful in some cases, as discussed recently by Legro (2016). There were no evidence-based theories to support the idea that weight loss before IVF had a beneficial effect on outcomes when comparing a 6-months evidence-based lifestyle intervention to immediate ART (Mutsaerts *et al.*, 2016). It is possible that BMI is a poor marker of fat distribution, insulin resistance, and low-grade inflammatory state (Legro, 2016). Instead of significant weight loss, which is difficult to obtain rapidly, efficient lifestyle changes from 3 months before IVF, and then during and after that IVF, should improve the rate of success, as was shown by regular exercise (Palomba *et al.*, 2014) and healthy diet (Vujkovic *et al.*, 2010) reducing oxidative stress inflammation and insulin resistance. Legro (2016) concluded that "weight limits used to deny women access to fertility care are not only arbitrary but discriminatory and not evidence-based." The best program and strategy to be proposed remain to be determined. Bariatric surgery in the case of morbid obesity is the right solution. However, excessive rapid weight loss may be associated with SGA offspring, which can itself increase the risk of adult metabolic diseases (Johansson *et al.*, 2015). It may also rapidly release persistent pollutants with EDC effects (Kim *et al.*, 2011) in the circulation. For all these reasons, it is recommended that a woman waits for 1 year after bariatric surgery before attempting start spontaneous or medically induced conception.

EDCs and Female Reproductive Health

Endocrine-Disrupting Chemicals

The human population is exposed to a large number of chemical pollutants that may interfere with physiological functions. Since worldwide annual production of synthetic chemical is dramatically increasing (Neel and Sargis, 2011), questions have arisen in the last few decades about the role of chronic exposure to low doses of chemicals pollutants in terms of animal and human health and diseases; these questions focus particularly on reproductive and neurodevelopmental outcomes, but also on chronic diseases including metabolic disorders or cancers. The first observations were made on animals and concerned the reproductive system, after exposure to chemicals showing estrogenic or anti-androgenic effects. Together with the diethylstilbestrol (DES) story (discussed below), this led to the concept of *endocrine disruptors*. EDCs are natural or synthetic chemical compounds, present in the everyday domestic environment, that interfere with hormonal regulation systems critical for development or homeostasis (Diamanti-Kandarakis *et al.*, 2009). Many EDCs are present in the food chain, and will after absorption be sequestered in adipose tissue. They may represent, after low-dose exposure during critical windows or via chronic exposure to cumulative doses, one aspect of the genetic/environment interface involved in many chronic diseases. They will act through different nuclear and membrane receptors specific to the female reproductive function, estrogen receptors including ERα and ERβ (estrogen receptor α, β) (Crain *et al.*, 2008), G protein-related receptor 30 (Bouskine *et al.*, 2009, Chevalier *et al.*, 2012), or the aryl hydrocarbon receptor (AhR) (Cavallini *et al.*, 2016), an orphan nuclear receptor able to bind dioxins and dioxin-like endocrine disruptors.

Many EDCs are lipophilic chemicals called persistent organic pollutants (POPs), for example (**Table 1**), many organohalogenated pesticides such as estrogenic DTT and its persistent metabolite *p,p'*-dichlorodiphenyldichloroethylene (DDE), which is

Table 1 Endocrine-disrupting chemicals and female reproduction which evidence?

(1) The "diethylstilbestrol story"
(2) Epidemiological human studies
● Occupational exposures
● Acute exposures
● Cross-section studies
● Prospective longitudinal studies
(3) Experimental animal studies
● In vivo
● In vitro
(4) Observations during IVF

antiandrogenic, phenols such as polychlorinated biphenyls (PCBs) used as electric isolants, polycyclic aromatic hydrocarbons (PAHs) present in tobacco or industrial dust such as dioxins, polybromated compounds used as flame retardants, and polyfluoroalkyls compound used as impermeants or flame retardants. Others are more rapidly eliminated after oxidation and conjugation in the liver, and are called nonpersistent pollutants such as Bisphenol A (BPA) and phthalates; both are used in plastics but found in blood and urine since we are continuously exposed to them. BPA is used in many plastics, resins, and PVC. It is found in the everyday domestic environment (receipts, infusion bags, CDs) and in the food chain (packaging, plastic bottles, plastic bottles, cans, etc.). BPA was discovered as an estrogen in the wake of DES but was soon used as a monomer to polymerize plastic polycarbonate chains with interesting properties. Some heavy metals such as cadmium, arsenic, lead, or mercury are nonessential and toxic. They are resistant to degradation, may persist or bioaccumulate in the environment, and may be toxic as EDCs for female reproduction and offspring (Hart, 2016; Choy *et al.*, 2002).

Cigarette smoke contains several thousand toxic components with diverse effects; these components include EDCs such as polycystic aromatic hydrocarbons acting through the AhR receptor or cadmium with estrogenic effects. Cigarette smoking in females is associated with lower fecundity rates, deleterious reproductive outcomes, and a higher risk of IVF failures depending on the time of exposure (in utero development or adult) and with multisites of action (ovaries, endometrium, uterus, tubes) (Dechanet *et al.*, 2011). The prevalence of cigarette smoking among women of reproductive age is still important and was estimated around 25% in the United States and 23% in the United Kingdom (WHO, 2009 in: World Health Organization (Ed), Prevalence of tobacco use among adults and adolescents 2014) and represents a real concern for female reproduction (Camlin *et al.*, 2014).

EDCs are found in the blood (Diamanti-Kandarakis *et al.*, 2009), including cord blood (Fenichel *et al.*, 2012; Woodruff *et al.*, 2011; Brucker-Davis *et al.*, 2008), milk (Brucker-Davis *et al.*, 2008), adipose tissue, amniotic fluid (Fini *et al.*, 2017), and placenta (Fernandez *et al.*, 2016). The ability of EDCs to alter female reproductive function and health is illustrated by the DES story, and is also supported by epidemiological and experimental studies and direct observations during IVF.

The DES Story

DES is a synthetic estrogenic compound; discovered in 1938, it was given to pregnant women until the 1970s to prevent miscarriages. Occurrence of very rare and severe cervical-vaginal cancers in young girls who had been exposed to DES in utero led to its banishment. However, this dramatic experience has also provided unexpected progress by suggesting new pathophysiological concepts such as fetal programming of adult diseases and/or transgenerational transmission of environmental effects through epigenetic modifications induced in selective windows by fetal exposure to xenoestrogens (Fenichel *et al.*, 2015). In the second generation, enhanced risk of early cervical-vaginal cancer, impaired reproducctive function and adult breast cancer (after 40 years) have been reported in large prospective follow-up. This follow-up includes significant hazard ratios of uterine malformations, infertility, spontaneous abortion, ectopic pregnancy, stillbirth, preeclampsia, and early menopause after exposure in utero to DES. Experimental studies in rodents have confirmed such an impact and allowed the identification of molecular mechanisms such as epigenetic changes (Fenichel *et al.*, 2015).

Fecundity and EDCs: Time to Become Pregnant

Time to become pregnant after stopping contraception is a useful way to explore the link between exposure to EDCs and female fertility. Epidemiological studies have concerned either occupational exposures or the general population. Danish female workers in flower greenhouses with potential exposure to pesticides had a reduced fecundity in terms of increased time to pregnancy, and this was related to their more or less direct exposure to pesticides (Abell *et al.*, 2000). In order to assess the real role of environmental factors in human fecundity, Buck Louis *et al.* (2013) designed a large prospective Longitudinal Investigation of Fertility and the Environment (LIFE) study on the effects of POPs on both partners in a couple. Women were recruited prior to conception and were followed through 12 observed cycles. Adjusted reduced fecundity was associated with the increase of several POPs in serum, PCBs 118, 167, and 209 and perfluoro octane sulfonate (PFOS) in females, and *p*,*p*′-DDE and PCBs 138, 156, 157, 167, 170, 172, and 209 in males. The same stringent methodology adapted to non-POPs like BPA and phthalates measured in urine did not show any correlations for females, but did indicate a positive correlation in males for selective phthalate metabolites (Buck Louis *et al.*, 2014). A similar difference was found for heavy metals related to time to pregnancy only in males (Buck Louis *et al.*, 2012). In the French PELAGIE cohort, which included more than 3000 pregnant women in Britain, time to pregnancy was significantly increased when seafood was consumed more than twice weekly when compared to less than eight times monthly or when total serum PCBs or *p*,*p*′-DDE were at the highest tercile, compared to the lowest one (Chevrier *et al.*, 2013). POPs and heavy metals bioaccumulate easily in fish and seafood. However, the most interesting epidemiological evidence supporting a developmental role for exposure to POPs in the decrease of female fertility was reported by Cohn *et al.* (2003). These authors measuredmaternal serum levels of DTT, an estrogenic pesticide, in a historical biobank of serum collected 30 years before in an American maternity. The levels were then correlated to the time to become pregnant taken by the daughters 30 years later. The concept that fetal or perinatal exposure to toxicants is able to influence the reproductive capacity of a young woman later in her life is supported by the DES story and by several animal models that we describe in the next section.

EDCs and Ovarian Reserve: Premature Ovarian Failure: Effects on Oocytes

Premature ovarian failure

Ovarian reserve depends on two kinds of factors: (1) the follicular pool established definitively before birth and (2) the velocity at which the ovarian reserve decrease. Premature ovarian failure (POF), a hypergonadotropic and hypoestrogenic amenorrhea, is present in 2% of women under the age of 40. There are no objective data to verify if the prevalence of premature decline of ovarian reserve or real POF is increasing. Spontaneous POF is idiopathic in more than 75% of cases. Environmental, nutritional, and/or toxicological factors could play a role in some of these cases, as suggested by epidemiological and experimental evidence (Richardson *et al.*, 2014). There are no reliable ovarian reserve biomarkers in adulthood, even though basal serum FSH level at the beginning of the menstrual cycle, serum anti-Müllerian hormone (AMH) level, antral follicle count, and ANM are usually assessed.

Human data

Hairdressers who are exposed to chemicals have been shown to be at increased risk of POF when compared with women employed in other occupations (Gallicchio *et al.*, 2009). POF has been associated with exposure to a solvent containing 2-bromopropane (Koh *et al.*, 1998), but the causal link was discussed by Beranger *et al.* (2012). Exposure to perfluorocarbons (perfluoro octanoic acid (PFOA), PFOS) was associated with an earlier ANM in a very large population of women aged over 42 years (Knox *et al.*, 2011). Using the US population National Health and Nutrition Examination Survey (NHANES) database in a cross-section study including 31,575 females, and analyzing 111 EDCs focusing on persistent chemicals, women with high levels of 15 identified EDCs had mean ages of menopause 1.9–3.8 years earlier than women with lower levels. Women exposed to EDCs were up to six times more likely to have menopause than nonexposed women (Grindler *et al.*, 2015). Classical measures of ovarian reserve among patients undergoing fertility treatments showed an association with urinary phthalate monoester concentrations (Souter *et al.*, 2011). Loss of ovarian reserve linked to cigarette smoking is supported by several studies reviewed by Richardson *et al.* (2014), reporting correlations with elevated FSH levels, earlier onset of menopause, and reduced retrieval of oocytes in IVF cycles. A causal relation is enhanced by the significant FSH elevation and inhibin B decrease, which are both correlated with increased serum cadmium, a heavy estrogenic metal present in cigarette smoke (Gallagher *et al.*, 2010). Maternal exposure to cigarette smoke was associated with earlier ANM in daughters (Strohsnitter *et al.*, 2008). While conflicting data have been published on the developmental effect of maternal smoking (Richardson *et al.*, 2014), several experimental studies have confirmed such a possible link (see later). In the Avon Longitudinal study of parents and children, paternal but not maternal smoking prior to and during pregnancy was inversely associated with AMH levels in female adolescents (Fraser *et al.*, 2013). Antenatal exposure to monoethyl phthalate, widely found in cosmetics, measured in maternal serum, correlated negatively with AMH levels in adolescent girls (Hart *et al.*, 2014).

Animal studies

There are many experimental studies reporting the effects of EDCs exposure on an ovarian reserve, especially during the developmental period. Animal studies enable one to study a single chemical alone or in a cocktail, identifying the dose and the time of exposure and the molecular mechanisms involved. Fetal and perinatal exposure to low environmentally relevant doses of methoxychlor, an organochlorine pesticide, impaired ovarian folliculogenesis in adult rats (Armenti *et al.*, 2008) with morphological and molecular changes leading to an accelerated ovarian failure. Hypermethylation of the CPG islets of the ERβ gene promoter was involved in this developmental induced POF (Zama and Uzumcu, 2009). Perinatal exposure to environmentally relevant levels of BPA decreased fertility and fecundity in CD61 mice (Cabaton *et al.*, 2011) and reduced the pool of primordial follicles in rat ovaries (Rodriguez *et al.*, 2010) by acting as an activator of the neonatal recruitment. This effect in granulosa cells got through an estrogenic pathway involving ERβ and ERα. Prenatal exposure of female mice to a phthalate mixture which mimics human exposure to phthalates present in consumer products, including personal care products and plastics, disrupted estrus cycles and reduced fertility related indices in F1 offspring (Zhou *et al.*, 2017b), and impaired female reproduction in F2 and F3 through a transgenerational effect (Zhou *et al.*, 2017a). AhR could be involved since Chen *et al.* (2012) had shown that benzyl butyl phthalate induced necrosis by AhR mediation of CYP1B1 expression in human granulosa cells. Exposure of adult mice to concentrations of cigarette smoke proportional to those involved in human exposure resulted in a significant primordial follicle loss. This provides further support for the idea that cigarette smoke is a reproductive toxicant resulting in POF, but curiously without enhancing apoptosis even with isolated high doses of benzopyrene (BaP), a major PAH present in tobacco (Tuttle *et al.*, 2009). In fact, the molecular mechanisms involved depend on the time of exposure. Maternal exposure during pregnancy or lactation, to PAHs in mice, induced POF in female offspring by activation of the aromatic hydrocarbon receptor driving bax-dependent apoptosis (Matikainen *et al.*, 2002). This signaling pathway including Harakiri activation is also involved in human ovaries as xenografted human ovarian cortex exposed to PAHs, caused by activation of the identical cell death cascade (Jurisicova *et al.*, 2007). This raises concerns about the transgenerational impact of maternal smoking on ovarian function in human.

Fecundity and EDCs: observations during IVF

Many persistent and nonpersistent organic pollutants have been found in follicular fluids collected during IVF and correlated with IVF outcomes. PCB congeners and DDE were identified in follicular fluids and inversely related to number of oocytes retrieved and live births obtained (Bloom *et al.*, 2017). BPA was detectable in follicular fluid in the majority of women undergoing IVF and inversely associated with the number of oocytes retrieved (Mok–Lin *et al.*, 2010). Higher urinary BPA concentrations were found to

be associated with decreased ovarian response, number of fertilized oocytes, and decreased blastocyst formation (Ehrlich *et al.*, 2012). Cigarette smoking is associated with lower fertility rates, adverse reproductive outcomes, and a higher risk of IVF failures (Dechanet *et al.*, 2011). Many pollutants with endocrine-disrupting effects and/or toxicological effects have been identified in follicular fluids such as cadmium, PAH like BaP, or cotidine (Dechanet *et al.*, 2011), which have been shown in vivo or in vitro in rodents to induce deleterious effects on ovarian function. A recent French study reported a significant correlation between abnormal oocytes collected by IVF, showing centrally located cytoplasm granulation and domestic exposure to pesticides in an intensive agriculture area, assessed by geolocation (Merviel *et al.*, 2017). All these observations and epidemiological correlations are supported by experimental studies. Several studies in mice reviewed by Richardson *et al.* (2014) have reported meiotic abnormalities including synaptic defects and increased levels of recombination in oocytes linked to exposure to BPA either in utero or at the adult stage (Susiarjo *et al.*, 2007). BPA similarly altered oogenesis in the fetal ovary of the rhesus monkey. BPA has also been shown to impair steroidogenesis in vitro in rat theca-interstitial and granulosa cells (Zhou *et al.*, 2008).

A new and important question concerns the potential reprotoxicity of nanoparticles. Preliminary studies have shown, for example, that cerium dioxide nanoparticles widely used as a diesel additive or as promising therapy in cancerology are able to reduce fertilization rates in mice during IVF when added in low doses in a culture medium (Preaubert *et al.*, 2016). Decreased fertilization rates could result from genotoxicity observed in gametes, a mechanical effect disrupting gamete interaction and oxidative stress (Preaubert *et al.*, 2016). Regarding the increasing use of nanoparticles in everyday life, there is an urgent need for evaluation of their toxicity, including reprotoxicity by in vivo studies after low-dose exposure.

Polycystic Ovaries Syndrome and Endocrine-Disrupting Chemicals

PCOS: an environmental disease?

PCOS, the most common female endocrine disorder, affects 7%–10% of women of childbearing age. It includes ovarian hyperandrogenism, impaired follicular maturation, anovulation, and subfertility. Insulin resistance is present in most cases and increases with obesity. The origin of hyperandrogenism and hyperinsulinemia has a genetic component, as demonstrated by familial aggregation studies and recent identification of associated genomic variants, conferring a particular susceptibility to the syndrome (Fenichel *et al.*, 2017). However, experimental and epidemiological evidences also support a developmental origin via a deleterious fetal environment, concerning the nutritional level (intrauterine growth retardation), the endocrine status (fetal hyperandrogenism), or the toxicological exposure (endocrine disruptors). Epigenetic changes recently reported in the literature as being associated with PCOS enhance this hypothesis of fetal reprogramming by environmental factors of the future adult ovarian function (Fenichel *et al.*, 2017).

Hyperandrogenism

It has been well demonstrated in the rhesus monkey that fetal exposure to an excess of androgens induced an irreversible and predictable sequence of physiological changes. These changes included delayed puberty, ovarian dysfunction (Dumesic *et al.*, 1997), ovarian hyperandrogenism with anovulation (Abbott *et al.*, 1998), increased BMI within adulthood, and insulin resistance (Eisner *et al.*, 2000), all of which closely belong to the PCOS phenotype (Eisner *et al.*, 2002). Works carried out in ewes treated with testosterone at different times of pregnancy have shown similar results (Padmanabhan and Veiga-Lopez, 2014). In this model, induction of prepubertal obesity by overfeeding could amplify ovarian disorders (Steckler *et al.*, 2009). Women with adrenal hyperplasia due to a congenital enzymatic block in 21-hydroxylase often present a PCOS phenotype (Dunaif, 1992) with hypertonic LH, which may be consecutive to prenatal androgen exposure. Excluding this case, the question arises of the origin of such fetal hyperandrogenism, since maternal SHBG is usually elevated during pregnancy (Morel *et al.*, 2016) and placental aromatase activity theoretically protects the fetus from maternal androgens. However, one cannot exclude a decrease of SHBG due to hyperinsulinemia (Sir-Petermann *et al.*, 2002 Nestler, 1987) in the case of maternal PCOS or due to the presence of a specific genetic variant (Xita *et al.*, 2003 Hogeveen *et al.*, 2001 Cousin *et al.*, 2004), which could also explain a decreased aromatase activity (Balakrishnan *et al.*, 2010). Several potential mechanisms have been proposed to explain the fetal programming linked to hyperandrogenism whatever its origin (Petry *et al.*, 2005): modifications of the neuroendocrine control of the gonadotropic axis leading to permanent excessive LH secretion or some epigenetic changes concerning ovarian genes involved in follicular maturation.

Endocrine disruptors and PCOS

EDCs generally have less affinity to the binding proteins like maternal SHBG, are not transformed in estrogens by aromatization, and easily cross the placenta (Corbel *et al.*, 2014). In addition, the fetal liver keeps an enzymatic immaturity, with a weak detoxifying power by oxidation and conjugation. Both experimental and epidemiological evidence support a role for EDCs in PCOS through an in utero exposure via the mother or through chronic adulthood exposure interfering with follicular maturation, ovarian steroidogenesis, and/or occurrence of insulin resistance. Fetal exposure to BPA may determine the changes of the hypothalamic-pituitary-ovarian axis (Tena-Sempere, 2010) in adults as demonstrated in ewes (Collet *et al.*, 2010) or rhesus monkeys (Kurian *et al.*, 2015) and also act on the oocyte maturation with increased follicular atresia, as reported in the mouse in vivo (Susiarjo *et al.*, 2007; Eichenlaub-Ritter *et al.*, 2008) or in vitro (Peretz *et al.*, 2012). Neonatal exposure to BPA in rats results in a similar picture of PCOS, with hyperandrogenism, anovulation, infertility, and PCO (Fernandez *et al.*, 2010). All these

experiments with perinatal exposure of the female gonads are accompanied in adult by impaired steroidogenesis with modifications in the expression of key enzymes such as P450c17, P450scc, and StAR (Abbott *et al.*, 2008): activity which is known to be impaired in human PCO (Nelson *et al.*, 1999).

Moreover, many chemical pollutants with endocrine-disrupting activity are considered to be able to play a role in obesity and type 2 diabetes through an in utero fetal effect, leading in adulthood to insulin resistance. BPA is one of them and is particularly relevant since it can, at low concentrations, be found in human cord blood (Fenichel *et al.*, 2012), and can promote both ovarian hyperandrogenism and hyperinsulinemia in animals, two major features of PCOS (Chevalier and Fenichel, 2015). Measures of BPA performed at the time of diagnosis have been reported in humans. They are higher in men than in women and higher in women with PCOS than in those with normal cycles. The measures correlate positively with total or free testosterone (Takeuchi and Tsutsumi, 2002), suggesting an effect of hyperandrogenism on BPA metabolism. Free serum BPA has been found to be higher in women with PCOS, regardless of whether or not obesity was present and correlated with both testosterone levels and insulin resistance (Kandaraki *et al.*, 2011). These findings were confirmed in a group of adolescents aged 13–19 years with PCOS (Akin *et al.*, 2015). Potential bi-directional interactions between BPA and hyperandrogenism were modeled by Palioura and Diamanti-Kandarakis (2015). Hyperandrogenism can slow down BPA metabolism in the liver. BPA can interfere with hyperandrogenism at various levels such as circulating SHBG, androgen receptor, synthesis via steroidogenic enzymes, and androgen metabolism (Peretz *et al.*, 2012). However, formal evidence supporting a role of in utero exposure to BPA in the occurrence of PCOS has yet to be confirmed in humans. In vitro experiments have shown that methoxychlor (an organochlorine pesticide) and dioxin (derived from the combustion of industrial products or wastes) are able to inhibit follicular maturation at the antral stage and to disrupt steroidogenesis (Patel *et al.*, 2015) by stimulating the production of AMH (Uzumcu *et al.*, 2006). In a recent US case/control study assessing blood and urine levels of different classes of EDCs a correlation was found in PCOS with two perfluorinated components, PFOA and PFOS in blood after correction for age, BMI, and ethnic origin (Vagi *et al.*, 2014).

Epigenetic modifications in PCOS

It seems likely that PCOS occurs as a result of the conjunction of both genetic and environmental factors (**Fig. 1**). The protective or susceptible genomic variants involved (Fenichel *et al.*, 2017) may also be influenced by environmental factors through epigenetic modifications. In the PCOS model induced in the rat by fetal hyperandrogenism, hypermethylation of the *PPARγ* gene and hypomethylation of the nuclear corepressor factor NCOR1 have been reported (Qu *et al.*, 2012). The LH/CG receptor, which regulates the thecal steroidogenesis, has several variants that represent a susceptibility factor, as found in several studies of the genome-wide association study type (Hayes *et al.*, 2015 Wang *et al.*, 2014). It is therefore quite consistent that methylation changes have also been found on the promoter of the *LH/CG* receptor gene in the PCOS mouse model induced by fetal exposure to androgens (Zhu *et al.*, 2010), as well as in a series of 85 women with PCOS who had an excess of hypomethylated sites compared to 88 control subjects (Wang *et al.*, 2014), with overexpression of the *LH/CG* gene on the surface of the thecal cells. Several authors have attempted to analyze methyloma more systematically in the case of PCOS. Shen *et al.* (2013) reported 79 genes with differential methylation in a PCOS group with insulin resistance compared to a group of women with PCOS without insulin resistance and 43 genes with a different methylation profile in PCOS compared to controls. Using the DNA chip method, Kokosar *et al.* (2016) in Sweden found 1720 genes differentially expressed in adipose tissue in 64 women with PCOS compared to 30 controls. Thirty-three of these genes exhibited corresponding modifications at some of their methylation sites. In a similar Chinese study (Wang *et al.*, 2014), comparing DNA methylation profile and transcriptional analysis in the ovarian tissue of women with or without PCOS, a match could be established in the case of PCOS for 54 genes between the perturbation of the ovary gene expression and the methylation modifications at the CpG sites of their promoter. Some of these genes were involved in molecular signaling, inflammation, metabolic control, or proliferation processes consistent with their involvement in the development of PCOS. Several recent studies have analyzed the profile of microRNAs (miRNAs) in PCOS (Sorensen *et al.*, 2014 Ding *et al.*, 2015) in serum or in follicular fluid (Roth *et al.*, 2014). Differences have been found for some miRNAs involved in the control of genes necessary for androgen synthesis, inflammation, adipogenesis, and signaling, thus being able to integrate into the pathophysiology of PCOS (Ilie and Georgescu, 2015 Murri *et al.*, 2013). Two miRNAs appear to be of particular interest as biomarkers in PCOS; one (miR-222) is positively correlated with insulin and the other (miR-146a) is negatively correlated with testosterone, after multivariate adjustment for age and BMI (Long *et al.*, 2014). These results, which need to be confirmed and consolidated, therefore strongly suggest that epigenetic changes induced, for example, by environmental factors (Anway *et al.*, 2005) could influence the molecular mechanisms leading to PCOS (**Fig. 2**).

EDCs and Endometriosis

Endometriosis, defined by the presence of functional ectopic endometrial glands and stroma outside the uterine cavity, affects 6%–10% of women of reproductive age, causing chronic pain and infertility; it remains a pathophysiological and therapeutic enigma. Recent experimental studies suggest a developmental in utero origin with epigenetic changes (Borghese *et al.*, 2017), which could be induced by exposure to EDCs (Smarr *et al.*, 2016) acting through the AhR and target genes controlling estrogen metabolism (Bulun *et al.*, 2000; Huang *et al.* 2016). In a case–control study of women enrolled in a large health care system in the US Pacific Northwest, serum concentrations of *β*-HCH (lindane) and mirex were positively associated with endometriosis (Upson

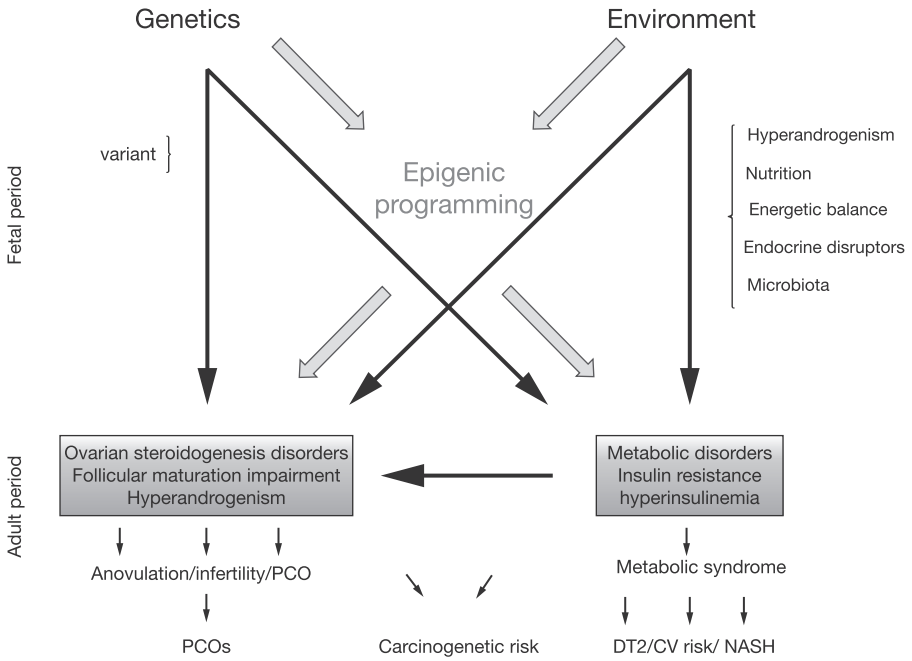

Fig. 2 PCOS physiopathology: interactions between genetic, epigenetic, and environmental factors (Fénichel *et al.* 2017).

et al., 2013; Sofo *et al.*, 2015). The role of dioxins, dioxine-like PCBs (Martinez-Zamora *et al.*, 2015), phthalates (Reddy *et al.*, 2006), DES, benzophenones, and some metals/trace elements are supported by clinical and experimental studies (Smarr *et al.*, 2016; Crain *et al.*, 2008), with some methodologic concerns needing confirmation.

Uterine Fibroids

Uterine fibroids (leiomyomas), the most common tumor of the female reproductive tract occur in 25%–50% of all women (Cramer and Patel, 1990), and in some cases can impair fertility. Several risk factors such as genetic susceptibility or hormonal climate have been reported. Both in vitro and in vivo studies suggest a role for estrogenic EDCs in promoting leiomyomas in women (Crain *et al.*, 2008). Only one human study with ultrasound detection clearly showed a significant relationship with prenatal exposure to DES (Baird and Newbold, 2005). Further investigations are needed.

Conclusion

Environmental factors seem to contribute significantly to physiological and pathophysiological aspects of female reproductive function. While a minimal fat mass is necessary to maintain the menstrual cycle, obesity is associated with impaired fertility and obstetrical, fetal, and postnatal complications. EDCs present in the domestic sphere may influence fetal development by epigenetic imprinting and later by the occurrence of current gynecological diseases associated with impaired reproductive function. It is important to evaluate the effect of the environment on female reproduction in order to identify simple preventive strategies, including advices to be given to young women before they become pregnant, as proposed recently by the Endocrine Society and FIGO, in order to set in motion a virtuous cycle to limit toxicological exposure for future generations.

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Miscarriages

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Introduction

Miscarriages are the most frequent complication of pregnancy. They happened in at least 12% of pregnancy (one woman out of four before 39 years old) (Blohm *et al.*, 2008). They cause maternal death in a small number of cases (0.05–0.22/100000) (Cantwell *et al.*, 2011).

They are defined by a pregnancy loss before 14 weeks of gestation. The risk factors for miscarriage are maternal age (with a higher risk after 35 years old) (Nybo Andersen *et al.*, 2000; Maconochie *et al.*, 2007), paternal age (after 45 years old) (Maconochie *et al.*, 2007), maternal body mass index (with a higher risk for body mass index more than 25) (Metwally *et al.*, 2008), toxic consumption (coffee, tobacco and alcohol) (Henriksen *et al.*, 2004; Maconochie *et al.*, 2007; Weng *et al.*, 2008; Waylen *et al.*, 2009) and exposure to magnetic fields and ionizing radiation (Fucic *et al.*, 2008; Wang *et al.*, 2013; Delabaere *et al.*, 2014). A previous miscarriage or a previous abortion are also risk factors such as infertility and alteration of ovarian reserve (Brigham *et al.*, 1999; Maconochie *et al.*, 2007; Sahu *et al.*, 2010).

Aim of this article is to recall how to manage first trimester pregnancy loss (i.e., miscarriage) from diagnosis to therapeutic but also how to manage repeat miscarriages.

Diagnostic Criteria (Lavoue *et al.*, 2014)

Gold standard for diagnosis of miscarriage is pelvic sonography (Wieringa-de Waard *et al.*, 2002; Doubilet *et al.*, 2013). Sensibility and specificity of evaluation of symptoms (pain and bleeding), history and clinical examination are poor compared to sonography. Then a sonography is recommended in case of bleeding or pain at the first trimester of pregnancy. Transvaginal sonography might be useful for very early pregnancies (Jain *et al.*, 1988; Cacciatore *et al.*, 1989; Pennell *et al.*, 1991; Ferrazzi *et al.*, 1993). First of all, diagnostic of an intrauterine pregnancy has to be done. The diagnosis of a gestational sac can be made only in case of a yolk sac or an embryo inside the sac. Others signs such as double decidual sac or intradecidual sign cannot allow a diagnosis of an intrauterine pregnancy (Mueller, 1979; Bradley *et al.*, 1982; Doubilet and Benson, 2013).

A gestational sac larger than 25 mm without embryo and with or without a yolk sac allows a diagnosis of miscarriage such as the presence of a gestational sac with an embryo of more than 7 mm without cardiac activity (Abdallah *et al.*, 2011; Jeve *et al.*, 2011).

In case of pregnancy of uncertain viability, defined by a gestational sac less than 25 mm, a sonographic control has to be performed after 14 days when there is no yolk sac in the gestational sac and 11 days if there is a yolk sac. After this delay, the diagnosis of miscarriage can be done if there is still no embryo with cardiac activity. When an embryo is present, less than 7 mm, a sonographic control after 7 days is recommended to search for cardiac activity and to allow the diagnosis of miscarriage if there is still no cardiac activity (Bree *et al.*, 1989; Doubilet and Benson, 2013).

For pregnancy of unknown location (no gestational sac inside uterus and no visible ectopic pregnancy), the threshold of hCG above which an ongoing intra uterine pregnancy can be excluded if there is no gestational sac inside uterus is 3510 UI/mL. At this threshold, a gestational sac is visible in 99% of cases of ongoing pregnancy. However, above this threshold, diagnosis between a miscarriage and an ectopic pregnancy can be done only with sonography. An ongoing pregnancy can also be excluded under the threshold of 3.2 ng/mL for progesterone rate (Verhaegen *et al.*, 2012; Connolly *et al.*, 2013). But no conclusion between intrauterine pregnancy and ectopic pregnancy can be done on hCG or progesterone rates.

When initial hCG rate is less than 20,00 UI/L, a control of sonography and hCG rate after 48 h allow elimination of an ongoing intrauterine pregnancy if increase of hCG rate is less than 15%. The two possible diagnoses are then ectopic pregnancy or miscarriage. For initial hCG rate between 2000 and 3510 UI/L, published data didn't allow any conclusion on the evolution of hCG rate to conclude between an ongoing pregnancy or not.

In case of women with abnormal uterine bleeding or pain with an intrauterine ongoing pregnancy, there is no published data about the period before a sonographic control. There is no evidence of the benefit of any therapeutic (progesterone, vitamins, hCG, bed rest) for threatened miscarriage (Aleman *et al.*, 2005; Rumbold *et al.*, 2011; Haas and Ramsey, 2013; Morley *et al.*, 2013).

Management of a First Trimester Miscarriage (Beucher *et al.*, 2014)

There are three options for the management of a first trimester miscarriage: expectant management, medical treatment or surgery.

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For asymptomatic women with a first trimester miscarriage, expectant management led to higher risk of spontaneous expulsion but the delay for expulsion is often prolonged (2–6 weeks) with a risk of emergency consultation or surgery in 28%–80% of cases and a higher risk of blood transfusion (1.4% vs. 0%) (Graziosi *et al.*, 2004; Sotiriadis *et al.*, 2005; Neilson *et al.*, 2006; Trinder *et al.*, 2006; Nanda *et al.*, 2012).

When a medical treatment is decided, vaginal misoprostol (800 μg) should be preferred because of shorter delay for expulsion (Neilson *et al.*, 2006). A second administration 24 to 48 h later is required in up to 30% of cases (Lister *et al.*, 2005; Zhang *et al.*, 2005). Administration of mifepristone doesn't improve results (Gronlund *et al.*, 2002; Stockheim *et al.*, 2006; Kollitz *et al.*, 2011; Torre *et al.*, 2012). It leads to a success rate of more than 80%. When gestational bag is still present at the end of the medical treatment, a delay before surgical management should be respected (few days to 2 weeks). Medical management reduces the costs with less hospitalization and less surgery and anesthesic appointment (Graziosi et al., 2005b; Rausch *et al.*, 2012). Women satisfaction is equivalent (more than 80%) between medical management and surgery (Neilson *et al.*, 2006; Nice, 2012). Surgery consists of an aspiration followed by curettage; success rate are between 95% and 98% significantly higher than those after medical management (Graziosi et al., 2005a; Sotiriadis *et al.*, 2005).

Women Have to be Informed About the Two Methods and Their Results

For women with an incomplete first trimester miscarriage, an expectant management can be performed leading to success rates between 71% and 100% without a higher risk of complications (Luise *et al.*, 2002; Graziosi et al., 2005a; Sotiriadis *et al.*, 2005; Trinder *et al.*, 2006; Casikar *et al.*, 2010). However, the delay of expectant management cannot be specified. Medical treatment (misoprostol) doesn't improve these rates and should not be performed for incomplete miscarriage (Neilson *et al.*, 2013). Surgical management led to high success rate (around 98%) (Graziosi et al., 2005a; Neilson *et al.*, 2006; Trinder *et al.*, 2006). Curettage should be avoided and aspiration has to be preferred as it is significantly less painful, led to less bleeding and is shorter (Tuncalp *et al.*, 2010).

When retention is diagnosed later, hysteroscopic management is probably the method of choice leading to less synechiae, less incomplete treatment and a higher pregnancy rate but published data are scarce (Cohen *et al.*, 2001; Faivre *et al.*, 2009; Rein *et al.*, 2011).

After a previous miscarriage, synechiae are frequent in case of surgical management (between 16% and 23% of cases) (Hooker *et al.*, 2014). If there are any reasons at all for concern, an office hysteroscopy has to be performed.

Subsequent fertility is equivalent whatever the treatment received (expectant management, medical treatment or surgery) (Tam *et al.*, 2005; Smith *et al.*, 2009). There is however less miscarriage when next pregnancy occurs within the next 6 months after the miscarriage (El Behery *et al.*, 2013).

Finally, psychological aspect has to be taken into account, as risk of subsequent miscarriage is higher in women with stress and depression after previous miscarriage. This risk is lower with repeated medical consultation and sonographies at the beginning of subsequent pregnancy (Legendre *et al.*, 2014). A feeling of grief after a miscarriage as after the loss of a loved one is frequent. Depression is frequent (10%–50% of women in the first months but less than 1 year), such as anxiety (20%–40% of women until 6 months). Depression or anxiety doesn't seem different depending on the treatment received and systematic psychological support doesn't revel any benefit at 1 year from miscarriage. Some time ago, without any support, depression and anxiety scores are similar to general population (Legendre *et al.*, 2014).

Repeat Miscarriages (Gallot *et al.*, 2014)

Repeat miscarriage is defined as the repetition of no less than three consecutive miscarriages before 14 weeks of gestation. It concerns 1%–5% of couples looking for pregnancy (Berry *et al.*, 1995; Regan and Rai, 2000; Hogge *et al.*, 2003; Rai and Regan, 2006). Numerous risk factors have been identified such as maternal age over 36 years old (Stefanidou *et al.*, 2011), alteration of ovarian reserve (Trout and Seifer, 2000), uterine malformation (Homer *et al.*, 2000; Kowalik *et al.*, 2011; Jaslow and Kutteh, 2013), obesity with BMI higher than 30 (Lashen *et al.*, 2004), folic acid or B12 vitamin deficiency (Reznikoff-Etievant *et al.*, 2002; Sikora *et al.*, 2007; Puri *et al.*, 2013), hyperhomocysteinemia (Robertson and Greer, 2005; Jaslow and Kutteh, 2013; Puri *et al.*, 2013), maternal tobacco consumption (Kumar, 2011; Stefanidou *et al.*, 2011), coffee consumption more than one cup per day (Stefanidou *et al.*, 2011). In women with repeat miscarriage, an increased prevalence of chromosal parental abnormalities is noticed (Gardner and Sutherland, 1996).

Out of these risk factors, chronic endometritis is more frequent in women with repeat miscarriage without it being possible to conclude for such a cause effect relationship (Zolghadri *et al.*, 2011).

In case of repeat miscarriage, it is recommended to realize the following examinations: a morphological diagnosis of the uterus (for septate uterus particularly but also T-shaped uterus), plasma dosage of B9 and B12 vitamins and homocysteinemia, evaluation of ovarian reserve (follicular count and anti-Mullerian hormone), karyotype of both man and woman, search for an antiphospholipid syndrome (lupus anticoagulant screening, beta1GP1 and anticardiolipid antibody), dosage of TSH, search for TPO and TG antibody, blood glucose and blood prolactin.

Management of repeat miscarriage should begin with eviction of toxic (tobacco and coffee particularly). In case of any abnormality, an appropriate management will have to be performed: vitamin B9 or B12 supplementation in case of deficiency or of hyperhomocysteinemia (Quere *et al.*, 2001), aspirin and low molecular weight heparin for antiphospholipid syndrome (Rai *et al.*, 1997; Mak *et al.*, 2010), levothyroxine supplementation for hypothyroidism or thyroid antibody (Negro *et al.*, 2010), diabetes management in case of high sugar blood concentration (Wahabi *et al.*, 2010), bromocriptine in case of hyperprolactinemia (Hirahara *et al.*, 1998). Preimplantation diagnostic in case of anomalies of parental karyotype and oocyte donation in case of alteration of ovarian reserve will be discussed in multidisciplinary meeting (Budak *et al.*, 2007; Molina Gomes *et al.*, 2009; Hammoud *et al.*, 2010; Jungheim *et al.*, 2013).

The correction of a uterine abnormality has to be performed in case of repeat miscarriage (Homer *et al.*, 2000; Giacomucci *et al.*, 2011). A metroplasty has to be performed in septate uterus as it seems to enhance ongoing intrauterine pregnancies (Bendifallah *et al.*, 2013). For T-shaped uterus, published data are scarce but also suggest a positive effect of metroplasty (Fernandez *et al.*, 2011).

For women with repeat miscarriage with normal results, a folic supplementation 0.4 mg/day until 14 WG combined with progesterone until 14 WG (no recommendation can be done about dose and way of administration) and psychological support with repeated sonography (Haas and Ramsey, 2013).

Evaluation is not recommended after one miscarriage. However, published studies are controversial whether to perform examinations after two or three repeated miscarriages. It seems that risk of subsequent miscarriage is almost the same after two or three previous miscarriage (24%–29% vs. 31%–33%) (Jaslow *et al.*, 2010). More data need to be published to clarify whether we have to wait for three miscarriages or not.

Conclusion

Miscarriage is a very frequent pregnancy complication and should be managed following recommendations for diagnosis. It will avoid numerous sonographic controls before diagnosis. For management, surgery and medical treatment are equivalent for asymptomatic women but expectant management should be avoid whereas, all of these treatments can be performed in women with bleeding.

When there are three or more miscarriages, the diagnosis of repeat miscarriages can be done and required a restricted medical check to diagnose abnormalities that can be corrected to avoid a subsequent miscarriage. When the medical check is normal, folic acid and progesterone supplementation should be given until 14 weeks of gestation.

In all cases, women with a previous miscarriage need to be reassure and repeated early sonographies for the next pregnancy might be necessary all the more in case of repeat miscarriage.

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Obesity and Reproduction

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Glossary

Adipokines Term that refers to inflammatory and immune mediators, including several hormones, that are secreted by adipose tissue. Adipsin was the first adipokine described back in 1987.

Bariatric surgery Surgical intervention which main purpose is obtaining a quick and marked weight loss. Roux-en-Y by-pass gastric and vertical gastrectomy ("gastric sleeve") are the most widely used techniques nowadays.

Decidualization Process characterized by significant changes that endometrium cells suffer in preparation for future pregnancy. Morphological and functional changes of the endometrial stromal cells, presence of decidual white blood cells, and also, vascular changes to maternal arteries will conduct the endometrium to become into a structure called the decidua.

Idiopathic hyperandrogenism Functional androgen excess disorder in which there is no evidence of ovarian dysfunction: ovulation is present and ovarian morphology is normal.

Male obesity-associated secondary hypogonadism Functional form of hypogonadism typical of obese men that results from decreased secretion of gonadotropins and testosterone and may resolve with weight loss.

Miscarriage Death of an embryo or fetus before the 20th week of gestation.

Obesity-associated gonadal dysfunction Subset of patients presenting with polycystic ovary syndrome in women or secondary hypogonadism in men, in whom obesity and visceral adipose tissue excess and dysfunction appear to play a major causative role, considering the frequent resolution of these gonadal disorders following weight loss.

Polycystic ovary syndrome Functional androgen excess disorder characterized by the association of clinical and/or biochemical hyperandrogenism with ovarian dysfunction in the form of chronic oligo-ovulation and/or polycystic ovarian morphology.

Stillbirth Death of the fetus from weeks 20th to 28th of pregnancy.

Introduction

Obesity, defined as a body mass index (BMI) over 30 kg m^{-2}, is a health concern with an increasing worldwide incidence and prevalence in children, adolescents and adults. The American Association of Clinical Endocrinologist and the American College of Endocrinology (AACE/ACE), estimate that approximately 500 million adults are obese all over the world, with an overall prevalence around 33% (Garvey *et al.*, 2016). Moreover, prevalence between children and adolescents is approximately 17%, with a trend to continue rising in future years (Ogden *et al.*, 2012). Obesity impacts negatively on healthcare because of the risk of developing metabolic complications such as insulin resistance, type 2 diabetes, hypertension, sleep apnea and cardiovascular disease.

Infertility is common in the general population and is usually associated with reduced quantity and quality of ejaculated semen in men, occurrence of pregnancy at older ages in women, and also with environmental conditions that include overweight and/or obesity (Calderón *et al.*, 2016). The links between obesity and reproductive impairment gained attention in the last decades. Increased waist circumference and/or increased waist-hip ratio in women have been reported to impact negatively fertility through combined effects on ovulation, oocyte development, implantation, pregnancy loss and endometrial development (Brewer and Balen, 2010). In addition, obesity will also impair male fertility though a deterioration of in sperm quantity and quality. **Table 1** summarizes the impact of obesity on male and female reproduction.

During puberty, the correct balance of sex between androgens and estrogens leads to a dimorphic sexual distribution of adipose tissue. As a result, premenopausal women at have greater adipose tissue than lean mass and a typical deposition of body fat in the subcutaneous and gluteal femoral areas; conversely, in men adipose tissue is stored in the visceral and abdominal compartments (Wells, 2007).

This article will focus on the impact of obesity on reproduction, the reproductive disorders associated with obesity in women and men and the main mechanisms and hormonal imbalances underlying them.

Effects of Obesity on Female Reproductive Function

For ages, undernutrition was the main responsible of subfertility in women, explaining why obese women represented fertility goddesses in many ancient civilizations. However, with the sudden change in environmental conditions occurred in the past century in most parts of the world, it has become apparent that, compared with normal weight women, obesity actually impairs

Table 1 Impact of obesity in male and female reproduction

Women	
Androgen-circulating concentrations	Increased free testosterone and adrenal androgens
	Low—normal gonadotropin concentrations
	Decreased SHBG plasmatic concentrations
Menstrual cycle	Oligomenorrhea and/or amenorrhea
	Anovulatory cycles
Effects on conception and implantation	Quality and maturation of the oocyte impaired
	Abnormal folliculogenesis
	Impaired endometrium decidualization and oocyte implantation. Placentation process is also disturbed
Effects on pregnancy	Increased rates of early pregnancy loss and miscarriages
	Metabolic complications: hypertension, pre-eclampsia, venous thromboembolism, gestational diabetes
	Macrosomic fetus
Metabolism	Increased prevalence of PCOS, hyperandrogenism, hyperinsulinemia, and metabolic syndrome
Men	
Androgen-circulating concentrations	Decreased circulating testosterone concentrations
	Increased estradiol concentrations
	Low—normal gonadotropin concentrations
	Decreased circulating SHBG concentrations
Sperm parameters	Subnormal quantity and quality of sperm
	Increased sperm DNA damage
	Increased sperm oxidative stress
Impact on testicular function	Increased testicular temperature
	Reduced intra-testicular testosterone
	Abnormal spermatogenesis

Abbreviations: PCOS: Polycystic ovary syndrome; *SHBG*: sex-hormone-binding globulin.

fertility in affected women. Women with weight excess and obesity associate decreased fecundity, increased incidence of anovulatory cycles, and prolongation of the time to pregnancy when fertility is desired (Brewer and Balen, 2010).

The mechanisms involved in the impairment of fertility in obese women include gonadal dysfunction characterized by androgen excess leading to ovulatory and menstrual disturbances (Escobar-Morreale and San Millán, 2007). However, subfertility is also characteristic regularly menstruating women presenting with overweight or obesity suggesting that other mechanism may collaborate with androgen excess in the impairment or reproductive function in these women (Gesink Law *et al.*, 2007). Accordingly, pregnancy loss and elevated miscarriage rates are also observed in obese women after spontaneous or assisted conception (Fedorcsák *et al.*, 2004). These findings may be associated with gestational insulin resistance and diabetes, pre-eclampsia, hypertension, venous thromboembolism, gestational diabetes and fetal macrosomia (Dutton *et al.*, 2018).

Influence of Obesity on Regulatory Hormones in Women

Sex Hormones

The influence of weight and fat mass on the balance of sex steroids has been studied extensively. Sex hormone-binding globulin (SHBG) binds both testosterone and estradiol with high affinity thereby determining the concentrations of free hormones available to target tissues (Pugeat *et al.*, 1996). The affinity of SHBG is higher for testosterone and dyhidrotestosterone (DHT) than for estradiol (Pasquali, 2006). Weight excess and obesity decrease the circulating concentrations of SHBG (Pasquali, 2006) because of the combined effects of portal hyperinsulinism and inflammatory mediators secreted by abdominal visceral adipose tissue and hepatic ectopic fat (Simo *et al.*, 2015). Hence, circulating SHBG concentrations are correlate inversely with body mass index and waist hip ratio in women (Pasquali, 2006). Decreasing levels of SHBG contribute to the increase in unbound testosterone concentrations in women, especially in those with abdominal visceral adipose deposition.

Adiposity also contributes to androgen excess in predisposed women by stimulating the secretion of androgens by the ovaries and the adrenals by the direct effect of proinflammatory cytokines secreted into the circulation and also through the indirect effects of insulin resistance (and the compensatory hyperinsulinism) on these glands (Escobar-Morreale and San Millán, 2007). Moreover, androgen excess may favor further deposition of adipose tissue in the visceral abdominal fat depot, which in turns facilitates further androgen excess closing a vicious circle that is particularly severe in obese hyperandrogenic women (**Fig. 1**).

Sex Steroid Metabolism and Fat Tissue

Obese women, especially those with visceral abdominal adiposity, have increased secretion of androgens such as androstenedione and dehydroepiandrostenedione—that are not bound to SHBG—and also an increased metabolic clearance of these substances (Kirschner *et al.*, 1990). In addition, since most androgens concentrate within adipose tissue, obese women have larger amounts of

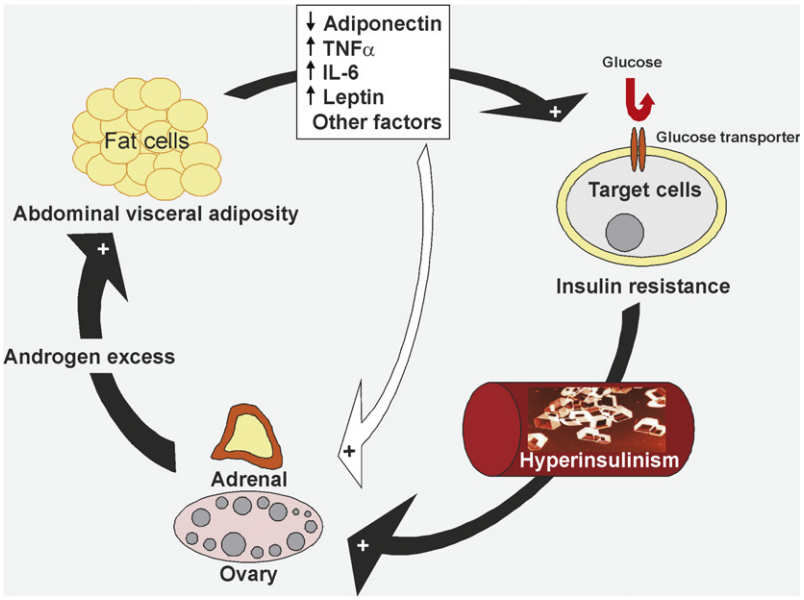

Fig. 1 The interplay between the polycystic ovary syndrome and abdominal adiposity may be the result of a vicious circle represented by the *black arrows*: androgen excess favors the abdominal deposition of body fat, and visceral fat facilitates androgen excess of ovarian and/or adrenal origin by the direct effects (*white arrow*) of several autocrine, paracrine and endocrine mediators, or indirectly by the induction of insulin resistance and hyperinsulinism. Reproduced with permission from Escobar-Morreale, H. F. and San Millán, J. L. (2007). Abdominal adiposity and the polycystic ovary syndrome. *Trends in Endocrinology and Metabolism* 18, 266–272, Copyright Elsevier, 2007.

sex-steroids stored in their fat compared with their lean counterparts. Moreover, adipose tissue has a direct influence on the metabolism of sex steroids, especially with regards to the conversion of androgens into estrogens mediated by aromatase. Hence, the larger the fat mass, the higher the production of sex-steroids will be (Pasquali, 2006).

Finally, proinflammatory cytokines synthesized by adipocytes favor androgen excess by a direct effect on the ovaries and the adrenal glands (Escobar-Morreale and San Millán, 2007). Adipose tissue is considered as an active endocrine and metabolic organ, playing its role by producing several proinflammatory and immune mediators, known as "adipokines." The dysfunctional secretion of this molecules correlates with the amount of visceral adipose tissue (Kershaw and Flier, 2004) and its excess may impact negatively upon reproductive function and the balance among sex steroids.

Taken together, all these mechanisms contribute to explain why androgen excess is very frequent in obese women. The polycystic ovary syndrome (PCOS) is the most frequent disorder of female androgen excess, showing a prevalence in the general population in the 5%–20% range depending on the population studied and the criteria used for its diagnosis (Azziz *et al.*, 2016). The prevalence of PCOS increases to 28% in overweight and obese women seeking medical advice for weight loss (Alvarez-Blasco *et al.*, 2006) and reaches 36% in women with severe obesity submitted to bariatric surgery (Escobar-Morreale *et al.*, 2017). Women with PCOS are at risk of developing metabolic abnormalities such as insulin resistance, hyperinsulinemia, gestational and type 2 diabetes, especially when obese (Wild *et al.*, 2010). Importantly, patients with PCOS have increased global and visceral adiposity regardless of obesity, yet obesity worsens both the reproductive and metabolic consequences of androgen excess (Escobar-Morreale *et al.*, 2017).

Insulin

Insulin is synthesized by pancreatic β-cells and their main role in adults is to regulate glucose homeostasis. Ovary, adrenal glands and pituitary express receptors for insulin, playing an important role by stimulating ovarian steroidogenesis in a direct way by acting on ovarian theca and granulosa cells, increasing the biosynthesis and androgen secretion, and in an indirect way by enhancing the sensitivity of pituitary cells to the actions of gonadotropin-releasing hormone that will lead to impair the quality of ovarian secretion (Poretsky *et al.*, 1999). In turn, androgen excess will promote visceral fat deposition, situation that will impact negatively upon insulin sensitivity and adipocyte dysfunction (Luque-Ramírez *et al.*, 2009). In fact, it has been well studied the association between overweight and obesity and resistance to insulin, relation that will derive into a compensatory insulin secretion and hyperinsulinemia. Moreover, hyperinsulinemia will also inhibit the synthesis of SHBG, and this will contribute to the dysregulation of sex steroids bioavailability, hyperandrogenemia, and, finally, impaired gonadal function and infertility.

Leptin and Kisspeptin

Leptin is a signaling proteic hormone that is secreted by adipocytes after food intake stimulus. As obese people have increased fat amounts, higher levels of leptin are expected in this population (Isidori *et al.*, 1999). Leptin is involved in several roles in body

homeostasis, including metabolic and reproductive functions. In relation to the first, leptin induces a reduction of appetite and an increase of energy expenditure (Brewer and Balen, 2010). Since increased leptin concentrations in obese humans are not associated with any of these effects, leptin resistance may occur in these subjects (Myers *et al.*, 2010).

Regarding reproductive function, leptin deficiency results in delayed puberty and hypogonadotropic hypogonadism in animal models and humans, conditions that may be reversed by leptin administration (Clarke *et al.*, 2015). Albeit the central effect of leptin on the HPG axis appears to be stimulatory, its peripheral effects on gonadal steroidogenesis are mostly inhibitory (Greisen *et al.*, 2000; Tena-Sempere *et al.*, 2001).

Moreover, even though leptin has been proposed to link nutrition with fertility, GnRH neurons lack receptors for leptin and kisspeptin has been recently proposed to participate in the link between adipose tissue and the reproductive axis (Clarke *et al.*, 2015). Kisspeptin is the neuropeptide product of the *KISS1* gene and plays a central role in the modulation of the hypothalamic—pituitary—gonadal axis by a direct stimulating effect on GnRH secretion, and therefore, on LH and FSH secretion (Meczekalski *et al.*, 2011; Avendano *et al.*, 2017; Roseweir and Millar, 2009). In fact, since kisspeptin neurons express both leptin and insulin receptors, kisspeptin might mediate some of the effects of these hormones on the HPG axis (Avendano *et al.*, 2017; George *et al.*, 2010; Smith *et al.*, 2006).

Adipokines and Inflammatory Mediators

Adipose tissue is considered nowadays as an endocrine organ because of the secretion of cytokines and adipokines from adipocytes and stromovascular cells (Kershaw and Flier, 2004). Adipose tissue dysfunction in obesity may include decreased adiponectin secretion and excessive production of tumor necrosis factor α, interleukin-6 and plasminogen activator inhibitor type-1 among other mediators which may influence are described in the literature as HPG function in the obese population (Kershaw and Flier, 2004). These mediators will favor insulin resistance, impaired folliculogenesis, gonadotropins dysregulation and disturbances in the metabolism of sex steroids. Other pro-inflammatory and immune mediators may also contribute to HPG dysfunction and cardiometabolic disturbances by indirect effects upon renin–angiotensin-system, cortisol hypersecretion, hypercoagulability, low-grade chronic inflammation, and by and increased sympathetic tone (Escobar-Morreale *et al.*, 2017). **Fig. 2** summarizes the effect of the principal adipokines upon metabolism and reproductive parameters.

Effects on the Oocyte

Quality and maturation of the oocyte in obese women after natural or assisted conception is compromised. Several studies performed in overweight and obese women who underwent in vitro fertilization (IVF) reported a diminished number, quality and maturation of oocytes retrieved from obese women (Shah *et al.*, 2011; Wittemer *et al.*, 2000; Dokras *et al.*, 2006). Furthermore, folliculogenesis is also disturbed in women with weight excess, a fact that might be related to an abnormal follicular environment characterized by excessive insulin, triglycerides, inflammatory mediators and oxidative stress (Broughton and Moley, 2017).

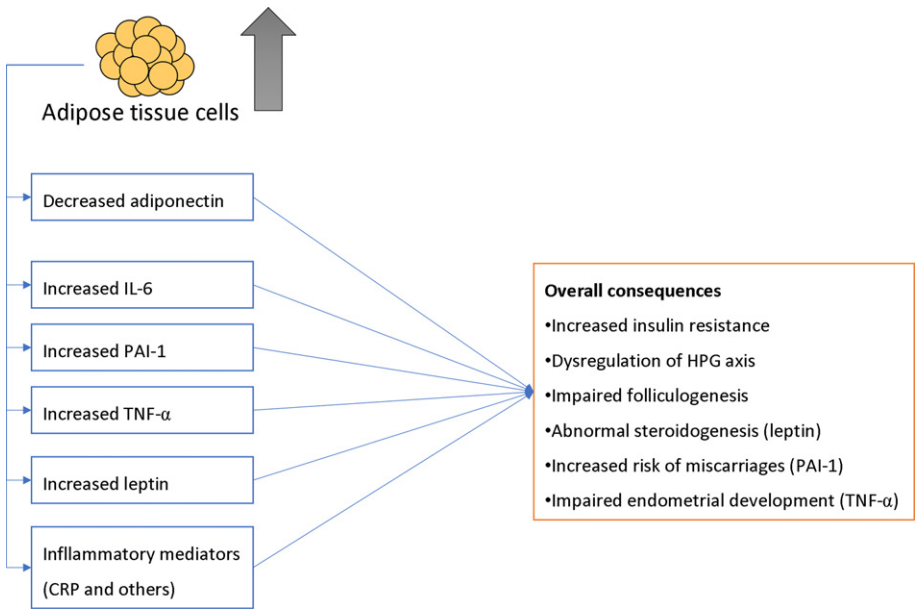

Fig. 2 Adipose tissue dysfunction, adipokine and inflammatory mediators, and reproductive consequences of obesity. Abbreviations: *CRP*, C-reactive protein; *HPG*, hypothalamic pituitary axis; *IL-6*, interleukin 6; *PAI-1*, plasminogen activator inhibitor type 1; *TNF-α*, tumor necrosis factor alpha.

Effects on Endometrium

Impaired endometrium decidualization—an essential step during the implantation process—may contribute to the increased risk of miscarriages before the 20th week of gestation observed in women with weight excess and obesity (Broughton and Moley, 2017). This may contribute to abnormalities in placentation, that will consequently lead to gynecological comorbidities along pregnancy, such as, placental dysfunction, pregnancy hypertension or pre-eclampsia (Brewer and Balen, 2010). In addition, insulin resistance and hyperinsulinemia may also impact on the endometrium, because of the association between of hyperinsulinemia and reduced levels of glycodelin and insulin growth factor binding protein 1. The decrease in the levels of these molecules is linked to higher rates of pregnancy loss in relation to impaired implantation (Carrington *et al.*, 2005).

Obesity and the Embryonic and Fetal Development

The same mechanisms impairing folliculogenesis and oocyte maturation may interfere with the normal embryonic development. Among them, lipotoxicity has been proposed as one of the most important mechanisms because higher levels of free fatty-acids, particularly α-linoleic acid, are associated with decreased pregnancy rates in women undergoing IVF (Broughton and Moley, 2017).

Obesity also interferes with fetal development. Fetal abnormalities are more frequent in obese women, including spina bifida and omphalocele in spite of folic acid supplementation and heart anomalies (Ramsay *et al.*, 2006). The impact of obesity upon endometrium, oocyte and embryo and fetus are summarized in **Fig. 3**.

Reversibility of Reproductive Dysfunction in Obese Women

For the reasons described above, possibility exists that, by targeting weight excess, the gonadal and reproductive dysfunction associated with female obesity improve and even resolve after weight loss (Escobar-Morreale *et al.*, 2017).

Increasing exercise and reducing caloric intake are the mainstay of life-style recommendations focused on weight reduction, and should be offered to all obese women. Modest weight loss may result into improvement in the metabolic and reproductive comorbidities of obesity (Moran *et al.*, 2011) yet the evidence supporting the long-term efficacy of life-style modification for obesity is scarce (Tam and Yeung, 2017). Hence, for severely obese women in whom life-style modification strategies have failed, bariatric surgery is a valid and effective long-term approach, not only to warrant substantial weight loss but, more importantly, because of the potential for resolution of any comorbidity associated with obesity, including gonadal and reproductive dysfunction (Escobar-Morreale *et al.*, 2017). Of note, a recent meta-analysis reported resolution of hirsutism and of menstrual

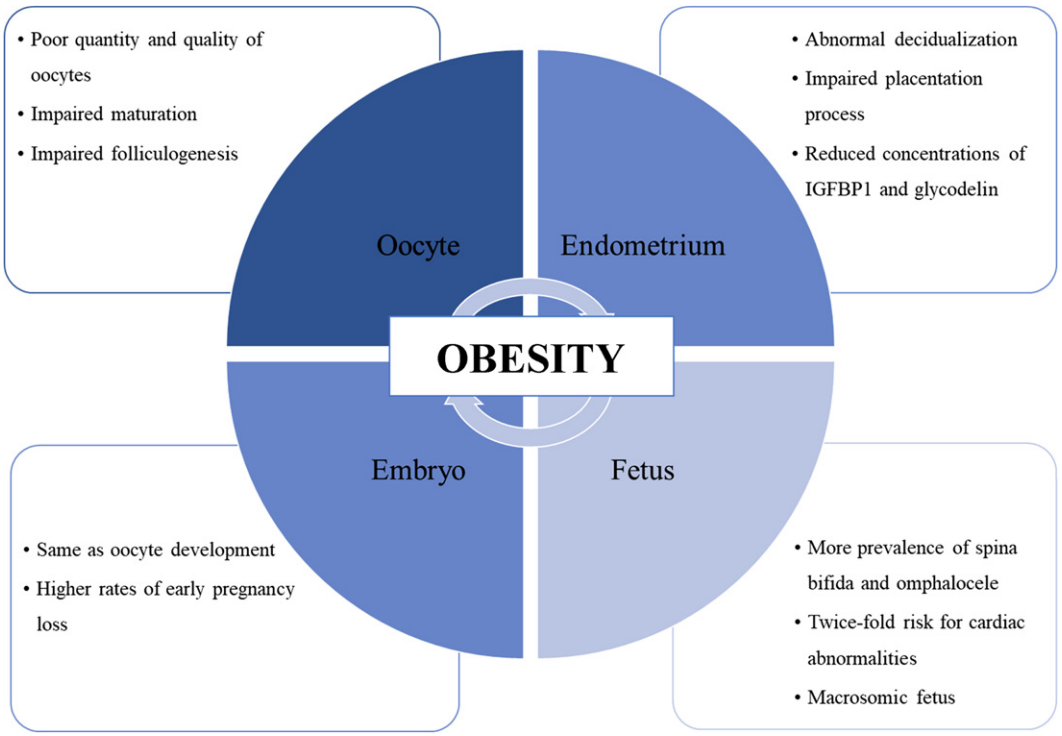

Fig. 3 Negative effects of obesity on fecundity and pregnancy. Abbreviations: *IGFBP1*, Insulin growth factor binding protein 1.

dysfunction after bariatric surgery in 53% and 96% of women showing those symptoms, leading to an overall 96% resolution rate of PCOS in these morbidly obese women. A shortened follicular phase and the improvement of sexual function after bariatric surgery have been also reported in response to obesity surgery in obese women (Legro *et al.*, 2012). Overall, the prevention of obesity-associated infertility and obstetrical complications such as gestational diabetes, macrosomia and pre-eclampsia in women submitted to bariatric surgery before pregnancy clearly compensate for the increased incidence of intrauterine growth restriction and the risk of mechanical complications of the previous abdominal surgery, provided that pregnancy is delayed for at least 1-year after surgery to avoid the initial phase or rapid weight loss, and appropriate nutritional support and obstetrical follow-up is offered throughout pregnancy (Guelinckx *et al.*, 2009).

Impact of Obesity on Male Reproduction

The sexual dimorphism in the consequences of obesity for reproduction also translate into the ancient imagery of fertility deities, which for the male sex was usually represented by lean men showing large or even monstrous penises. As opposed to women, adiposity does not associate any apparent survival advantage for fertility and, in fact, the negative consequences of obesity for gonadal function are even more frequent in men than in women (Escobar-Morreale *et al.*, 2017).

Strikingly, androgen deficiency is the main gonadal abnormality in obese men, as opposed to androgen excess in women. The main mechanism underlying androgen deficiency is the inhibition of gonadotropin secretion leading to male obesity-associated secondary hypogonadism (MOSH), a disorder that reaches a 64% prevalence in severely obese men submitted to bariatric surgery (Escobar-Morreale *et al.*, 2017). The hormonal profile of MOSH consists of low concentrations of total testosterone, free testosterone, FSH and LH resulting from dysfunctional GnRH secretion (Saboor Aftab *et al.*, 2013; Corona *et al.*, 2013). Moreover, in men reduced androgen concentrations facilitate body fat deposition in the abdominal and visceral depots and a decrease in lean mass. This might result into a vicious circle whereby abdominal adiposity leads to androgen deficiency by the mechanisms described above, and reduced androgen concentrations facilitate visceral fat accumulation and decreased lean body mass (**Fig. 4**) (Escobar-Morreale *et al.*, 2014).

One of the major causative mechanisms that underlie MOSH derives from the increase in aromatase activity in adipose tissue that occurs with obesity (Saboor Aftab *et al.*, 2013). An increased activity of this enzyme may convert androstenedione to estrone and testosterone to estradiol that will result into higher estrogen concentrations in obese men, with reduced circulating. SHBG concentrations amplifying the increase in circulating free estradiol concentrations. The latter may impact negatively on the HPG axis leading to alterations of LH pulsatility (Corona *et al.*, 2013; Palmer *et al.*, 2012). The causative role of increased aromatase activity on MOSH is supported by the

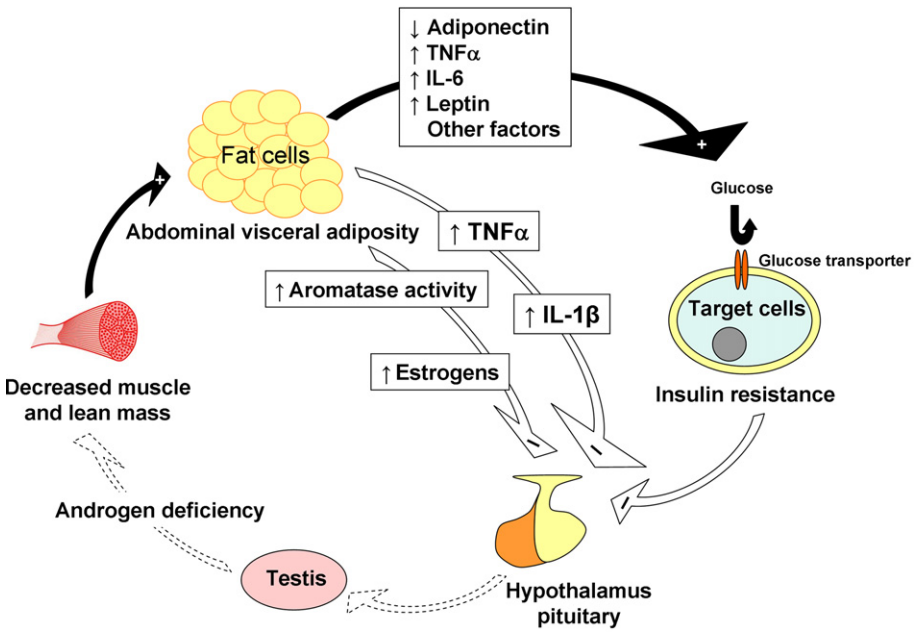

Fig. 4 The interplay between male obesity-associated hypogonadism and abdominal adiposity may be the result of a vicious circle represented by the arrows: androgen deficiency decreases lean and muscle mass thereby increasing the relative amount of visceral adipose tissue, and visceral fat facilitates androgen deficiency by inhibiting gonadotropin secretion at the hypothalamic–pituitary level through the combined action of increased aromatase activity and estrogen generation and increased secretion of inflammatory cytokines in visceral fat, and through the induction of insulin resistance. Reproduced with permission from Escobar-Morreale, H. F., Alvarez-Blasco, F., Botella-Carretero, J. I. and Luque-Ramirez, M. (2014). The striking similarities in the metabolic associations of female androgen excess and male androgen deficiency. *Human Reproduction* 29, 2083–2091; Copyright Oxford University Press, 2014.

favorable effects of aromatase inhibitors such as letrozole on the gonadal dysfunction of obese men (de Boer *et al.*, 2005). The reduction in total testosterone concentrations in parallel to the increase in estrogen concentrations will contribute to subfertility and gonadal dysfunction in severe obese male patients (Saboor Aftab *et al.*, 2013). Moreover, MOSH and the concomitant androgen deficiency will decrease lean mass and increase visceral fat mass as exemplified in **Fig. 4** (Escobar-Morreale *et al.*, 2014).

Hyperinsulinism derived from insulin resistance might also play a role in MOSH because circulating testosterone concentrations correlate negatively with BMI, insulin resistance and inflammatory markers in diabetic patients (Saboor Aftab *et al.*, 2013). On the one hand, hyperinsulinemia may also reduce the hepatic secretion of SHBG thereby contribution to the increase in free estradiol derived from aromatase activity. Moreover, hyperinsulinemia in parallel with hypogonadism will increase the activity of lipoprotein lipase, and as consequence, enhance the uptake and storage of triglycerides into the visceral fat cells (Saboor Aftab *et al.*, 2013). On the other hand, inflammatory mediators secreted by the excess in dysfunctional adipose tissue may not only contribute to insulin resistance and hyperinsulinemia (Fernandez-Real *et al.*, 2003) but also to decreased SHBG concentrations (Simo *et al.*, 2015) and to impairment of hormone signaling within hypothalamic neurons (Saboor Aftab *et al.*, 2013).

Other mechanisms that may contribute to the effects of obesity upon the male hypothalamic–pituitary–gonadal axis are mediated through the effect of leptin, kisspeptin and other adipokines. **Fig. 5** represents the complex mechanisms in relation to the effect of obesity upon male fertility and hormonal regulation.

Role of Obesity in Male Infertility

Despite sperm abnormalities not being necessary for the diagnosis of MOSH, semen alterations are common in severely obese men, who may present with decreased sperm volumes, concentrations and total counts possibly contributing to subfertility (Calderón *et al.*, 2016; Liu and Ding, 2017; Ramaraju *et al.*, 2017). These findings appear to be more frequent in the subset of patients with very high BMI. To these regards, low ejaculate volume is associated with weight excess and ejaculate volume correlates inversely with BMI and waist circumference (Calderón *et al.*, 2014).

However, the pathophysiology of sperm abnormalities in obese men is poorly understood. In line with this, MOSH may contribute directly because high intra-testicular testosterone concentrations are necessary for optimal spermatogenesis (Michalakis *et al.*, 2013; Eisenberg *et al.*, 2014). Oxidative stress and chronic inflammation characteristic of obese men may also contribute to impair sperm quality (Liu and Ding, 2017). Other possible mechanism involves the excess of scrotal adiposity and the consequent increase of testicular temperature (Palmer *et al.*, 2012). Because of the marked sensitivity of spermatogenesis to temperature changes, this mechanism may contribute to decrease sperm motility and to increase sperm oxidative stress and sperm DNA damage (Palmer *et al.*, 2012). Finally, fertility may also be impaired by mechanical factors that difficult effective coitus in severely obese patients, and by obesity-associated negative psychosocial factors that may impair sexual function (Kolotkin *et al.*, 2012; Han *et al.*, 2011). **Table 2** describes the alterations of sperm parameters due to obesity.

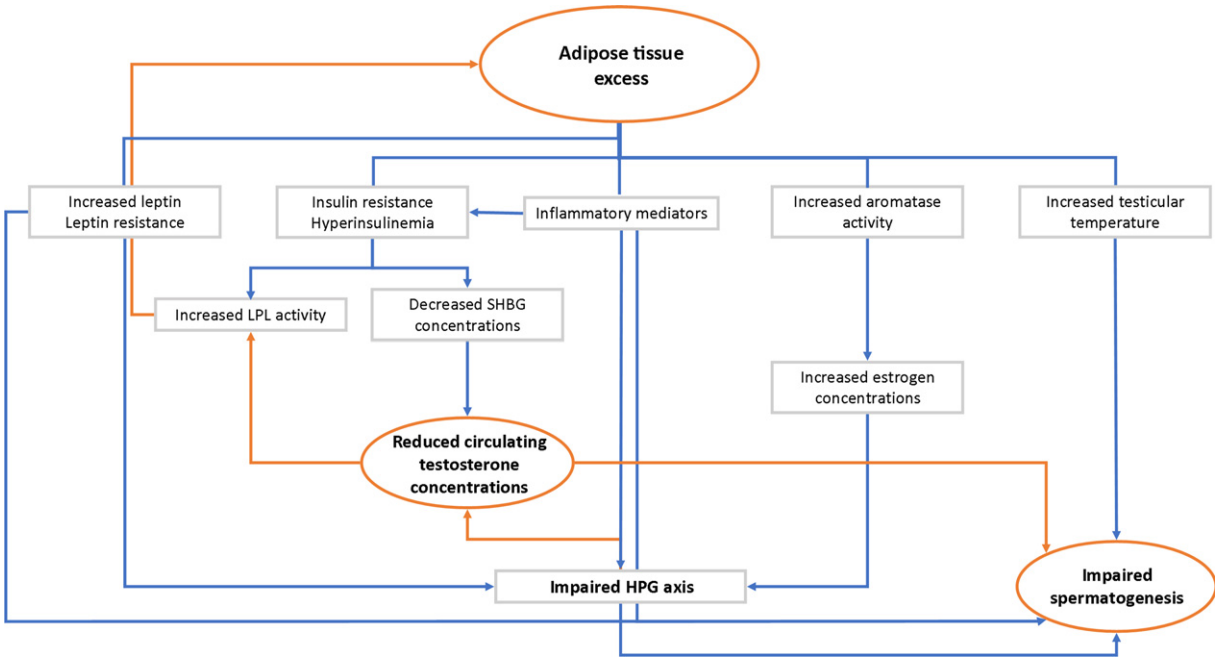

Fig. 5 The complex link between obesity and reproductive function in obese men. A direct negative effect upon HPG axis leads to androgen deficiency, which collaborates with other mechanisms in the impairment of spermatogenesis. Abbreviations: *HPG*, hypothalamic–pituitary–gonadal axis; *LPL*: lipoprotein lipase.

Table 2 Sperm abnormalities in severely obese men

Semen parameters	*Observed effect*
Volume	Reduced ejaculate quantity
Sperm count	Reduced
Concentration	Reduced
Progressive motility	Reduced
Total motility	Reduced
Average curve and path velocity	Reduced
Morphological alterations	Thin heads, pyriform heads and mid-piece defects

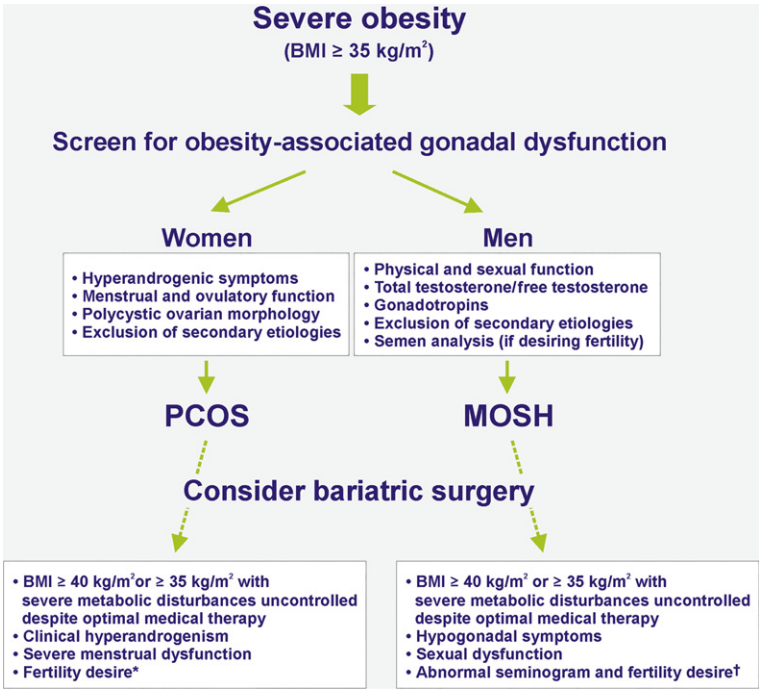

Fig. 6 Relevance of obesity-associated gonadal dysfunctions for the clinical management of severe obesity in men and women. *BMI*, Body mass index; *MOSH*, male obesity-associated secondary hypogonadism; *PCOS*, polycystic ovary syndrome. *Women desiring fertility must be advised to postpone pregnancy for at least 1 year after bariatric surgery and to maintain close nutritional and obstetric support throughout pregnancy. †The impact of surgically-induced weight loss on male fertility and sterility is unclear at present and men presenting with fertility desire and an abnormal semen analysis may need hormonal treatment or even assisted reproductive techniques.

Reversibility of Reproductive Dysfunction in Obese Men

As happens with obese women, MOSH may improve or even resolve with weight loss (Calderón *et al.*, 2016). Total and free testosterone concentrations rise after weight loss interventions (Corona *et al.*, 2013) and weight loss is one of the factors associated with spontaneous recovery of MOSH (Rastrelli *et al.*, 2015). Bariatric surgery is quite effective in improving all the factors associated with the development of MOSH and, accordingly, a recent meta-analysis reported that obesity surgery resolved MOSH in 87% of severely obese men presenting with this disorder (Escobar-Morreale *et al.*, 2017).

On the contrary, the impact of weight loss and particularly bariatric surgery on semen parameters is unclear. Large prospective studies reported benefits upon fertility and the quality of semen (Bardisi *et al.*, 2016), whereas other small case-control series have found the opposite effect (di Frega *et al.*, 2005; Lazaros *et al.*, 2012; Sermondade *et al.*, 2012). Screening for obesity-induced gonadal dysfunction and bariatric surgery indications are schematized in **Fig. 6**.

Conclusions

Obesity is associated with impairment of the reproductive dysfunction in women and men, showing a marked sexual dimorphism in the gonadal consequences of weight excess. In women, obesity associates androgen excess disorders—mostly PCOS—whereas in

men, androgen deficiency—namely, MOSH—is the dominant gonadal dysfunction associated with weight excess. The very large prevalence of obesity-associated gonadal dysfunction in obese women and men warrant screening strategies in these populations, especially in the subset of patients submitted to bariatric surgery. Weight loss therapies, such as life-style intervention or bariatric surgery, may improve and even resolve these disorders in obese men and women, normalizing circulating androgen concentrations and improving gonadal and reproductive function.

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Developmental Epigenetics and the Contribution of Parental Diet to Offspring Outcomes

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Metabolic diseases contribute a significant burden to human health throughout the world. Although a large number of Mendelian disorders of metabolism have been identified, the vast majority of metabolic disease burden stems from complex diseases such as diabetes, which have both heritable genetic components as well as environmental contributions. However, the genetic variants identified by GWAS studies typically contribute a small fraction of the heritability of a given complex trait such as diabetes. A number of potential factors could explain this so-called "missing heritability," including many rare variants contributing to a given phenotype, and epistasis. In addition to these, another emerging theme is the potential contribution of epigenetics—the inheritance of information beyond DNA sequence—to the heritability of such diseases. Indeed, increasing evidence links parental nutritional status to metabolic traits in offspring, potentially providing an explanation for a subset of missing heritability in metabolic diseases.

The development of disease later in life has been linked to exposure to an adverse intrauterine environment, as observed in offspring of pregnancies complicated by intrauterine growth restriction (IUGR), obesity, or diabetes (Hales and Barker, 1992, 2001). The period from conception to birth is a time of rapid growth, cellular replication and differentiation, and functional maturation of organ systems. These processes are very sensitive to alterations in nutrient availability and an abnormal intrauterine metabolic milieu can have long-lasting effects on the offspring.

The metabolic or nutritional state of the organism directly influences epigenetic modifications, as essentially all known epigenetic modifications rely upon substrates derived from intermediary metabolism such as S-adenosyl methionine (SAM), acetyl CoA, α-ketoglutarate, and nicotinamide adenine dinucleotide (NAD^+) (Kaelin and McKnight, 2013). A role for nutritional regulation of DNA methylation in offspring is best exemplified by experiments performed in *agouti viable yellow* (A^{vy}) mice or *axin fused* ($Axin^{Fu}$) mice (reviewed in Martin *et al.*, 2008). A^{vy} mice carry an intracisternal A particle (IAP) retrotransposon upstream of the *Agouti* gene, and animals exhibit a range of coat colors which is linked to repression of *Agouti* transcription in some animals by encroaching cytosine methylation from the IAP element. When pregnant agouti-colored female A^{vy} mice are fed a diet supplemented with methyl donors, a larger percentage of offspring have a wild-type coat color compared to offspring of mothers fed standard chow. These phenotypic changes are associated with changes in DNA methylation at the IAP element (Cooney *et al.*, 2002; Dolinoy *et al.*, 2006; Waterland *et al.*, 2006), suggesting that early-life access to specific metabolites can stably change gene expression via epigenetic modifications, thus affecting the phenotype of the adult.

A number of animal studies in non-agouti models have shown that maternal nutritional status and fetal nutrient availability induce epigenetic modifications at numerous key loci across the genome. In uteroplacental insufficiency models, fetal access to glucose, amino acids, and fatty acids is reduced, and diabetes develops in adult animals at approximately 15–26 weeks of age with underlying β-cell secretory defects and insulin resistance (Simmons *et al.*, 2001; Stoffers *et al.*, 2003). Genome-wide DNA hypomethylation is observed in postnatal IUGR liver, along with an increase in total H3 acetylation (MacLennan *et al.*, 2004). Acetylation of multiple lysines on the histone H3 N-terminal tails are increased at the promoters of *Ppargc1* and *Cpt1* in neonatal IUGR liver (Fu *et al.*, 2004), and this hyperacetylation persists in male offspring at least until postnatal day 21. Whether hyperacetylation at these sites influences transcription of *Ppargc1* or *Cpt1*, and how these findings relate to a phenotype in the offspring, remains to be determined.

Studies in the IUGR rat also demonstrate that fetal growth restriction induces epigenetic modifications of key genes regulating β-cell development such as *Pdx1* (Park *et al.*, 2008). Repression of *Pdx1* occurs in two waves, as early repression of this gene involves recruitment of the mSin3A histone deacetylase complex, followed later by H3K9 dimethylation and eventual recruitment of DNMT3A and cytosine methylation. Prior to cytosine methylation—at the neonatal stage—this epigenetic process is reversible and may define an important developmental window for therapeutic approaches. Indeed, early reversal of *Pdx1* deacetylation can prevent the onset of diabetes (Pinney *et al.*, 2011), demonstrating that IUGR-induced epigenetic modifications are responsible for the development of diabetes in this animal model.

The pancreatic transcription factor Hnf4α is also epigenetically regulated by maternal diet and aging in rat islets from offspring of protein restricted dams (Sandovici *et al.*, 2011). Increased DNA methylation and repressive histone modifications at the P2 promoter of Hnf4α were associated with a significant reduction in expression, while reversal of DNA methylation and histone modifications could re-activate transcription of *Hnf4α* via the P2 promoter. Another relatively well-characterized epigenetic target of caloric restriction during pregnancy and lactation is the *Glut4* promoter in skeletal muscle, where altered levels of activating and repressing TFs in response to caloric restriction lead to repression via diminished histone acetylation and increased H3K9 methylation, although in this case there is no apparent increase in cytosine methylation (Raychaudhuri *et al.*, 2008). These events effectively create a metabolic knockdown of *glut4*, an important regulator of peripheral glucose transport and insulin resistance and contributing to the adult T2D phenotype.

There are numerous studies in humans examining the relationship between fetal nutrient availability and epigenetic modifications in the offspring (Rakyan *et al.*, 2011). Many of these are confounded by small sample size, cellular heterogeneity of

tissues examined, and lack of validation. Moreover, most DNA methylation assays are performed in total peripheral blood monocytes, where the unique methylation profiles of the various cellular lineages complicate interpretation of the data. Despite these issues, multiple studies in diverse populations repeatedly show changes in DNA methylation associated with low birth weight and or altered nutrient availability. Thus, it is likely that an adverse in-utero milieu does indeed induce epigenetic modifications in the offspring, but whether these modifications have biological relevance remains to be determined.

Obesity is a growing threat worldwide, and the prevalence has risen dramatically over the last two decades with many studies indicating that early life exposures are important in promoting adult obesity. It is becoming increasingly evident that the prenatal stage represents a window of susceptibility for early life exposures (Bayol *et al.*, 2005; Chang *et al.*, 2008; Guo and Jen, 1995; Jungheim and Moley, 2010; Simmons, 2005; Sullivan *et al.*, 2011), as offspring of obese humans and animals exhibit increased fat mass very early in life. In fact, several studies suggest that obesity has adverse effects on the oocyte and embryo (Jungheim and Moley, 2010; Jungheim *et al.*, 2010; Marquard *et al.*, 2011; Sen and Simmons, 2010), suggesting the possibility that exposure to an adverse metabolic milieu even prior to pregnancy could account for some of the metabolic outcomes observed in offspring. Using reciprocal embryo transfer studies, we showed that a pre-gestational exposure to maternal obesity impaired fetal and placental growth despite the conceptus being exposed to a normal gestational milieu after transfer, with changes in placental gene expression being observed for offspring generated from high fat oocyte donors (Sasson *et al.*, 2015), as well as the brain reward system (Grissom *et al.*, 2014), suggesting that obesity prior to pregnancy may program food preferences and or food intake. These results have profound implications as it is possible that the effects of maternal obesity may be transmitted to subsequent generations.

While the impact of maternal environment on their children has long been clear, the father's contribution to offspring is less understood. Several studies have linked paternal environmental conditions—largely stress and dietary paradigms—to offspring phenotypes. Interestingly, in humans paternal body mass appears to be a better predictor of childhood metabolic traits than is maternal BMI (Figueroa-Colon *et al.*, 2000).

The timing of exposure is a key factor to take into account when considering paternal effect paradigms, as primordial germ cell (PGC) development occurs during the last week of gestation in male mice. A number of major epigenomic transactions, such as erasure of previous imprints and establishment of male-specific cytosine methylation patterns, occur during this period (Feng *et al.*, 2010), meaning that dietary paradigms starting after birth (generally after weaning) are less likely to influence the epigenome. That said, much remains to be learned about the plasticity of the epigenome and the ability of spermatogonial stem cells to respond to environmental alterations.

Many different metabolic phenotypes have been measured in offspring and reported to change in response to paternal diet. The most common metabolic phenotypes measured are related to glucose control, and include fasting glucose, glucose clearance, insulin release in response to glucose, and glucose clearance in response to insulin (Jimenez-Chillaron *et al.*, 2009; Ng *et al.*, 2010; Watkins and Sinclair, 2014; Carone *et al.*, 2010). Beyond glucose control phenotypes, cholesterol and lipid metabolism, and other cardiovascular phenotypes such as blood pressure, are reportedly altered in response to paternal dietary conditions.

Sperm cytosine methylation patterns have been reported to change at a number of loci in both an in utero undernutrition paradigm (Radford *et al.*, 2014), and in response to paternal prediabetes (Wei *et al.*, 2014). Methylation differences were generally modest changes of ~20% between sperm samples. However, even if the methylation status of a cytosine in question were *completely* responsible for some phenotype in offspring, at best this 20% methylation change would alter the fraction of a rodent's litter expressing the phenotype from 1 out of 5 to 2 out of 5. Thus, a 20% change in methylation at a cytosine is unlikely to result in penetrant changes in offspring.

Several paternal effect studies in mammals have documented changes in small RNA profiles in the sperm of treated versus control males (Rodgers *et al.*, 2013; Gapp *et al.*, 2014). However, mammalian sperm carry extremely low levels of RNA, and considering the volume of a sperm relative to the oocyte suggests that sperm are unlikely to carry enough RNAs to significantly alter the concentrations of a given RNA species in the oocyte, unless the RNA in question is absent or nearly so from the oocyte. Thus, although small RNAs represent a very strong candidate for the molecular basis of dietary information in sperm based on their roles in other model organisms, many mechanistic questions remain to be resolved to consider sperm delivery of small RNAs to be a credible influence over offspring metabolism.

How then might sperm alter maternal resource provisioning? Intriguingly, numerous studies have found effects of brief embryo culture on metabolic phenotypes in offspring (Rinaudo and Wang, 2012). One of the earliest cell fate decisions in mammalian embryos is cell fate allocation between the inner cell mass (ICM) and the trophectoderm (TE) of the blastocyst, which give rise to the embryo and to the extraembryonic tissues, respectively. It is thus theoretically possible that molecular changes in sperm induced by paternal diet somehow influence cell fate allocation between ICM and TE—by altering cell cycle dynamics for the first few cleavage divisions, for instance—with resulting effects on placental development then resulting in metabolic changes in offspring as detailed above.

In summary, the combined epidemiological, clinical, and animal studies clearly demonstrate that the intrauterine environment influences both growth and development of the fetus and the subsequent development of adult diseases. There are specific critical windows during development, often coincident with periods of rapid cell division, during which a stimulus or insult may have long-lasting consequences on tissue or organ function postnatally.

See also: Environmental Factors and Female Reproduction. Management of Obesity in Children and Adolescents: Lifestyle and Exercise Options. Obesity and Reproduction. Obesity, Childhood, and Adolescence. Pregnancy Endocrinology

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Anti-Müllerian Hormone (AMH) in Adults

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Introduction

Anti-Müllerian hormone (AMH) is the latest marker for the assessment of the ovarian reserve (OR) in women. Although the physiology and clinical utility of AMH are not completely established, this hormone appears to best reflect the OR. Despite the lack of an international standard for serum AMH assay and some technical issues in the past, serum AMH assay now tends to become an essential tool for the evaluation of the OR and for the treatment of infertility.

Physiology of AMH

AMH is a dimeric glycoprotein and a member of the transforming growth factor β family of growth and differentiation factors (Cate *et al.*, 1986). AMH has been predominantly known for its role in male sexual differentiation (Jost, 1947). From castration experiments in the fetal rabbit, Jost demonstrated that a testicular factor distinct from testosterone was responsible for the regression of the Müllerian ducts (Jost, 1947). In later years, it was demonstrated that this factor was produced by Sertoli cells in the testis (Josso *et al.*, 1993).

AMH was only isolated and purified in 1984. Genes for AMH and its receptor were sequenced and cloned in 1986 and 1994, respectively (Rajpert-De Meyts *et al.*, 1999). In women, AMH expression is restricted to one cell type: the granulosa cells of the ovary. It starts around the 25th week of gestation continuing until menopause (Rajpert-De Meyts *et al.*, 1999; Kuiri-Hanninen *et al.*, 2011). AMH is expressed at all steps of folliculogenesis. It is initiated as soon as primordial follicles are recruited to grow into small preantral follicles and its highest expression is observed in preantral and small antral follicles. AMH expression then decreases with the selection of follicles for dominance and is no longer expressed during the FSH-dependent stages of follicular growth (except in the cumulus cells of pre-ovulatory follicles) or in atretic follicles (Salmon *et al.*, 2004; Durlinger *et al.*, 2002a; **Fig. 1**; Dewailly *et al.*, 2014a; van Houten *et al.*, 2010).

The functional role of AMH in early follicular growth has been characterized by the study of "knocked out" models for the AMH gene (AMHKO) (Durlinger *et al.*, 1999, 2001, 2002a,b). When there is no AMH, primordial follicles are recruited faster, resulting in more growing follicles and thus in exhaustion of primary follicle pool at younger age than wild-type animals. AMH therefore has an inhibitory effect on early follicular recruitment by preventing the entry of primordial follicles into the growing pool and thus premature exhaustion of follicles/oocytes (Iliodromiti *et al.*, 2013). AMH also has an inhibitory effect on cyclic follicular recruitment in vivo by reducing the follicle sensitivity to FSH (Durlinger *et al.*, 2001). In vitro AMH inhibits FSH induced preantral follicle growth (Salmon *et al.*, 2004; Durlinger *et al.*, 1999, 2001, 2002a).

AMH also reduces the number of LH receptors in granulosa cells, also an FSH induced process (Teixeira *et al.*, 2001). Thus, it is clearly established that AMH is involved in the regulation of follicle growth initiation and in the threshold for follicle sensitivity to FSH.

AMH Assay

In clinical application, AMH presents many opportunities but unfortunately there are difficulties due to several biological features of this molecule (Pigny, 2014). First, there is a molecular heterogeneity of the circulating AMH level with a noncleaved biologically inactive form and a cleaved biologically active form (Pankhurst and McLennan, 2013; Nachtigal and Ingraham, 1996). Also, there is variable sensitivity of the immunoassays to interference by complement C1q and C3 (Han *et al.*, 2014). Then, the stability of AMH samples during the storage is not well known (Rustamov *et al.*, 2012). Moreover, AMH concentration varies according to the situation: in adults they are low (from 10.7 to 98 pmol/L) whereas in male newborns they are much higher (749–1930 pmol/L) (Pigny, 2014). Measurement thus involves different assays with different sensitivities. The last but not the least technical problem is the interlaboratory variability, mainly for low values of serum AMH. The difficulty lies in the fact that there are currently different ELISA immunoassays used worldwide; mainly the Gen II (Beckman Coulter) and the AL-105-i (Anshlabs), which use different monoclonal antibody and different standards (Pellatt *et al.*, 2011). The lack of agreement between these assays explains the absence of consensual reference values and decision thresholds between teams in the literature (Iliodromiti *et al.*, 2013). But progresses were made and the main two assays would now be superimposable (Pigny *et al.*, 2016). Likewise, automation on immuno-analyzers (Access Dxi of Beckman Coulter and Elecsys of Roche Diagnostics) yield nearly identical values (van Helden and Weiskirchen, 2015; Nelson *et al.*, 2015; Anderson *et al.*, 2015). The development of an ultrasensitive assay ("pico AMH" kit,

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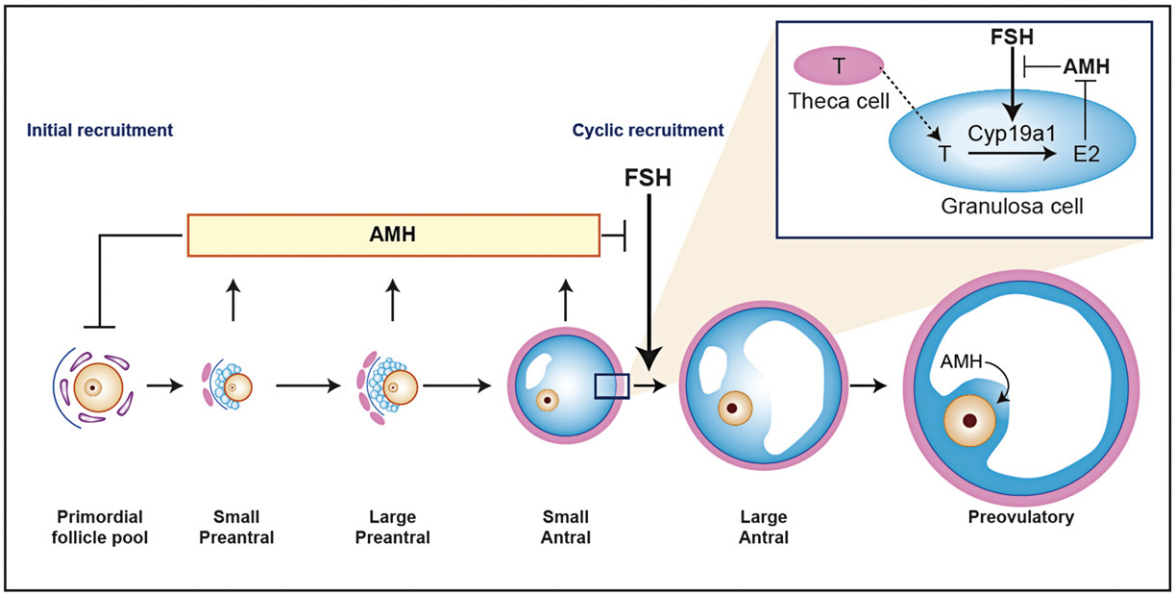

Fig. 1 Schematic model of AMH actions in the ovary. AMH, produced by the granulosa cells of small growing follicles, inhibits initial follicle recruitment and FSH-dependent growth and selection of preantral and small antral follicles. In addition, AMH remains highly expressed in cumulus cells of mature follicles. The inset shows in more detail the inhibitory effect of AMH on FSH-induced CYP19a1 expression leading to reduced estradiol (E2) levels, and the inhibitory effect of E2 itself on AMH expression. Reproduced from Dewailly, D., Andersen, C. Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R., Griesinger, G., Kelsey, T. W., La Marca, A., Lambalk, C., Mason, H., Nelson, S. M., Visser, J. A., Wallace, W. H. and Anderson, R. A. (2014). The physiology and clinical utility of anti-Mullerian hormone in women. *Human Reproduction Update* 20, 370–385.

Anshlabs) is also a progress. Hopefully, an international standard for serum AMH assay will soon be established in order to maximize its clinical utility.

Variability of Serum AMH

Serum AMH assay has many benefits over the other markers of OR (Iliodromiti *et al.*, 2013). First, its plasmatic level is quite stable from one cycle to another and throughout the same cycle since the dominant follicle and corpus luteum do not secrete AMH (Fanchin *et al.*, 2005; van Disseldorp *et al.*, 2010). Conversely, the antral follicle count (AFC) and the FSH E2 pair have to be measured on the first 5 days of the cycle (Hehenkamp *et al.*, 2006; La Marca *et al.*, 2006; Tsepelidis *et al.*, 2007). Also, a recent study has shown that ethnicity does not influence serum AMH level, contrary to previous studies (Bhide *et al.*, 2015). Besides its minor variability, serum AMH is also useful when the AFC cannot be done such as in obese, virgin, or poorly echogenic patients (Dewailly *et al.*, 2014a). Moreover, serum AMH level is rather independent from the hypothalamic pituitary axis and as such is not modified in pathologies such as hyperprolactinemia, functional hypothalamic amenorrhea, or in incomplete and recent hypogonadotropic hypogonadism, providing serum FSH level remains normal or subnormal (Tran *et al.*, 2011). The question thus actually arises about the replacement of the conventional markers of OR by the AMH assay (La Marca *et al.*, 2009).

However, serum AMH can be influenced by many factors and some controversies persist. Obesity is often associated with a significantly lower level of serum AMH, but not in all studies (Freeman *et al.*, 2007; Steiner *et al.*, 2010; Lefebvre *et al.*, 2017). There is also a controversy regarding the influence of hormonal contraception: to some authors, combined estrogen progestin does not change AMH serum levels whereas others have recently reported a decrease of 29%–50% that could be explained by the suppression of gonadotropin secretion (Bentzen *et al.*, 2012; Somunkiran *et al.*, 2007; La Marca *et al.*, 2004; Dolleman *et al.*, 2013; Plouvier *et al.*, 2016). Similarly a decrease of serum AMH has been shown 7 days after injection of depot leuprolide 3.75 mg, a GnRH agonist (HI *et al.*, 2013).

AMH as Indicator of Ovarian Follicular Status

Ovarian Reserve

The OR refers to the number of primordial follicles defined at birth (around 1 million). This follicular capital decreases gradually throughout reproductive life, with the continuous initiation of growth of some follicles, and then mostly their apoptosis. There are

about 400,000 follicles in adolescents' ovaries (leading roughly to 400 ovulations), whereas only a thousand remains at the time of menopause.

Serum AMH concentration is strongly correlated with the number of growing follicles since it represents AMH secretion from all developing follicles (Laven *et al.*, 2004; Pigny *et al.*, 2003). Considering that the rate of initiation of follicle growth is deeply related to the initial follicular pool, we can assume that serum AMH is an indirect reflection of OR. There is actually a very good correlation between serum AMH levels and ultrasonographic measure of the AFC (van Rooij *et al.*, 2002; Fanchin *et al.*, 2003). This is explained by the fact that circulating AMH is mostly produced by granulosa cells of follicles from 2 to 9 mm in diameter (60%), and those small follicles are precisely the ones counted on the ultrasound when the AFC is done (Jeppesen *et al.*, 2013). Measurement of serum AMH is even more sensitive and specific than the AFC as it also reflects pre-antral and small antral follicles (<2 mm), which are hardly seen in ultrasound. Serum AMH is therefore a deeper "probe" for the growing follicular pool than the AFC (Dewailly *et al.*, 2014a).

Most published studies that report AMH in normal girls and women include only a relatively small age range; thus a "data-driven" approach has been used (Kelsey *et al.*, 2012). This involved extracting data using a semi-automated procedure, and combined it with other unpublished data. The resulting combined dataset ($n = 3260$; age range -0.3 years to 54 years) (Kelsey *et al.*, 2011) forms a representative sample of AMH levels in the population of healthy female humans, and can therefore be used as a basis for a predictive model of serum AMH level with changing age and was used to generate and validate the model. Analysis of the model shows that the dynamics of circulating AMH levels throughout life can be split into several distinct phases (**Fig. 2**; Dewailly *et al.*, 2014a; Kelsey *et al.*, 2012; Wallace and Kelsey, 2010). A peak shortly after birth confirms that girls also undergo a "mini puberty" of the neonate, following which there is a sustained rise to about 9 years of age. There is an inflection with even a slight decline during the pubertal ages (9–15 years), followed by a second growth phase to a peak at an age of about 25 years. After this, there is a steady decline to undetectable levels at an average age of 50–51 years, corresponding to the menopause.

When nongrowing follicle (NGF) recruitment dynamics are considered and compared to AMH levels (**Fig. 2**) there is a strong and positive correlation ($r = 0.96$) between declining AMH and declining numbers of recruited NGFs after age 25 (the average age of peak AMH). This observation underpins the use of serum AMH level as an indirect indicator of human OR for ages after the

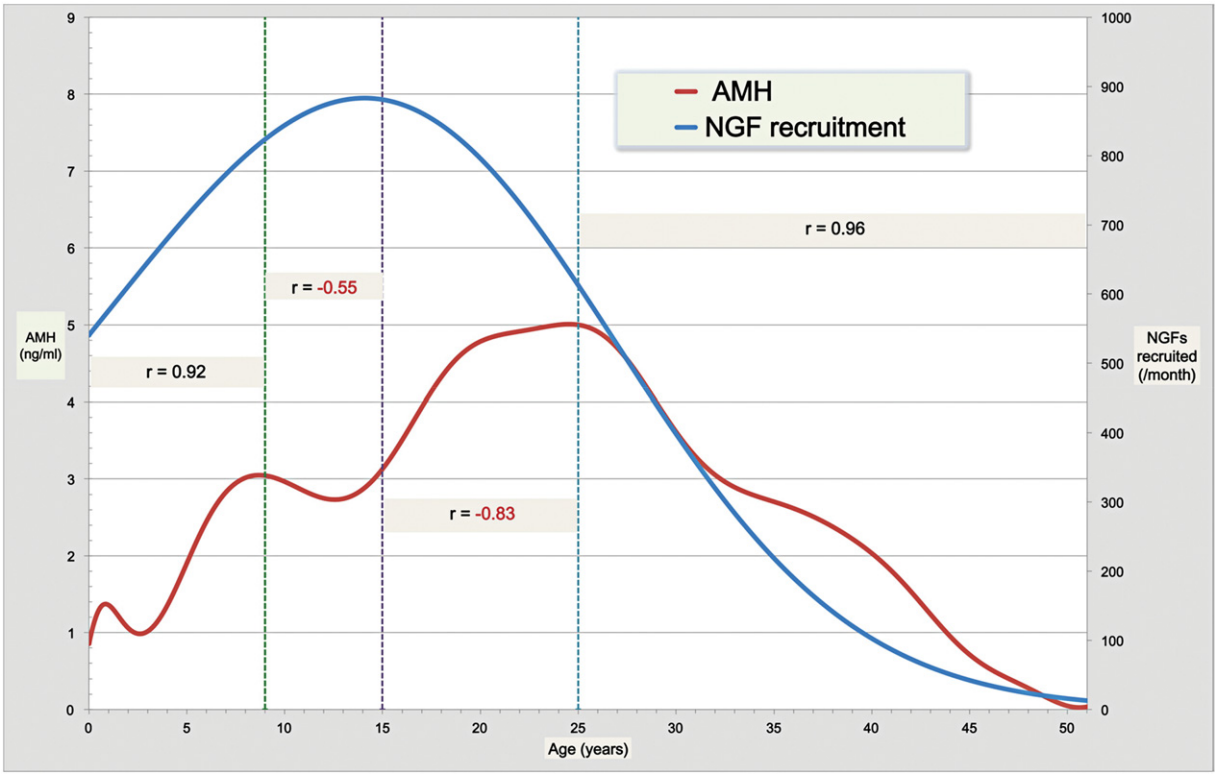

Fig. 2 AMH and follicular recruitment profile across the human reproductive lifespan. The *red line* is the log-unadjusted validated AMH model, peaking at 24.5 years. The *blue line* denotes the numbers of nongrowing follicles (NGF) recruited per month for the maturation population (Wallace and Kelsey, 2010), with peak numbers lost at age 14.2 years on average. Correlation coefficients (r) are given for AMH concentrations against follicular recruitment for each developmental phase; from birth to puberty (age 9 years), during puberty (9–15 years), postpuberty (15–25 years), and mature adults (0.25 years). Reproduced from Dewailly, D., Andersen, C. Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R., Griesinger, G., Kelsey, T. W., La Marca, A., Lambalk, C., Mason, H., Nelson, S. M., Visser, J. A., Wallace, W. H. and Anderson, R. A. (2014). The physiology and clinical utility of anti-Mullerian hormone in women. *Human Reproduction Update* 20, 370–385.

mid-20s. Before the age of 25, the relationships between AMH and OR are more complex with overall a positive relationship between rising AMH and increasing follicle growth activation, and thus we would recommend caution in the interpretation of AMH concentrations in girls and young women as an indirect indicator of OR.

Predicting the Time of Menopause

The value of predicting age at menopause serves multiple targets. First of all, the ability to assess the future OR status, and thereby the reproductive lifespan of an individual women, will have implications for female infertility. Because of the fixed time interval that is believed to be present, prediction of age at menopause will predict the age of natural end of fertility. If such predictions could be made early in life, with sufficient accuracy, this could have a great influence on individual women making decisions regarding career and a wish to have children. Genetic factors have proven to play a major role in determining the variation in menopausal age, as demonstrated in several mother–daughter, twin and sib-pair studies. Next to genetic factors, several environmental and life-style factors like smoking, body mass index, use of alcohol, and parity have claimed to influence menopausal timing as well. Thus, menopausal age is considered a complex genetic trait.

To study the value of AMH in the assessment of the future OR status, long-term follow-up studies are required, where several factors assessed at initiation of the follow-up are linked to the final outcome age at menopause. As menopause has a fixed time relation to earlier events such as onset of cycle irregularity (average age 46 years) and the loss of natural fertility (average age 41 years), a woman's reproductive lifespan can be predicted from forecasting age at menopause. To date, a total of four datasets are available addressing this issue and show that women with low age-specific AMH will have menopause earlier and vice versa (Tehrani *et al.*, 2009; Broer *et al.*, 2011a; Tehrani *et al.*, 2013; Freeman *et al.*, 2007). All these datasets however have very wide confidence intervals in the predictive value of a single AMH measurement. The role for AMH as a predictor of the age of menopause is therefore not yet assessed.

Assessment of Ovarian Damage

The relationship between serum AMH and the number of small growing and indeed primordial follicles has made it a prime potential tool for the investigation of gonadotoxicity of cancer therapy and of loss of the OR from ovarian surgery. AMH offers the possibility of a more accurate assessment, revealing partial loss of the OR, as well as ovarian failure. AMH was decreased in a study of ovarian function in young adults following treatment for childhood Hodgkin lymphoma with a clear dose–response relationship demonstrated between the number of chemotherapy cycles and the serum AMH level (van Beek *et al.*, 2007). FSH also rose with increasing treatment, but AMH appeared to have greater sensitivity to detect ovarian damage at lower doses of chemotherapy. Radiotherapy is also widely recognized to cause ovarian damage even at low doses and women treated with radiotherapy that includes the pelvis (including abdominal pelvic therapy in children or total body irradiation) generally have very low or undetectable AMH concentrations (Gracia *et al.*, 2012; Lie Fong *et al.*, 2009). The predictive value of AMH for postchemotherapy ovarian function has subsequently been confirmed (Anderson *et al.*, 2013) allowing the development of prediction tools combining age and AMH. It therefore appears that in addition to reflecting postchemotherapy (or radiotherapy) damage, AMH is also able to predict on-going ovarian activity after such treatment, and the existing data suggest it is likely to be more robust than either FSH or inhibin B in this regard. Consistent with this, a study in younger women has demonstrated that pre-treatment AMH predicts postchemotherapy recovery, with a more rapid recovery in women with higher pretreatment AMH (Dillon *et al.*, 2013).

The impact of ovarian surgery on the OR as measured by AMH has also been investigated, and two systematic reviews of the impact of ovarian surgery for endometriosis have been published (Raffi *et al.*, 2012; Somigliana *et al.*, 2012). Both analyses highlight the heterogeneity of study design and the difficulty in pooling data. However both conclude that ovarian endometrioma surgery is associated with a decline in serum AMH, indicating the removal of a significant part of the OR. A subsequent large retrospective analysis has confirmed the impact of endometrioma surgery on the OR as detected by serum AMH (Streuli *et al.*, 2012), and these findings should be taken in to account in the planning and decision-making process relating to ovarian surgery in women desirous of future pregnancy.

Substantial prospective studies are required to develop a clearer analysis of the predictive value of AMH in all those different circumstances and it may be of value in information provision for example regarding the need for fertility preservation strategies. However, low serum AMH level does not predict the chance of spontaneous fertility. A recent analysis showed a high prevalence of successful pregnancy in childhood lymphoma survivors despite low AMH concentrations (Hamre *et al.*, 2012). Another study on oocyte donor also showed that low serum AMH does not predict the chance of spontaneous pregnancy (Catteau-Jonard *et al.*, 2017).

Polycystic Ovary Syndrome and AMH

Polycystic ovary syndrome (PCOS) is the most common cause of chronic anovulation and hyperandrogenism in young women and affects 5%–10% of the female population (Franks, 1995; Norman *et al.*, 2007). PCOS is a diagnosis of exclusion and is defined by the Rotterdam classification (Rotterdam ESHRE/ASRM sponsored PCOS Consensus Workshop Group, 2003) requiring at least two out of the three following characteristics: (i) cycle disorder, (ii) clinical or biological hyperandrogenism, (iii) antral follicular excess on ultrasound with $\geq$12 follicles from 2 to 9 mm per ovary and/or ovarian volume $\geq$10 mL. PCOS is almost

certainly a genetic condition but the precise causes of hyperandrogenism and anovulation, which are not always associated, are still under investigations (Cui *et al.*, 2015; Kosova and Urbanek, 2013).

PCOS is characterized by an increased number of follicles at all growing stages (Hughesdon, 1982; Webber *et al.*, 2003; Maciel *et al.*, 2004). This increase is particularly seen in the pre-antral and small antral follicles, those which primarily produce AMH (Weenen *et al.*, 2004; Bhide *et al.*, 2014). Thus elevated serum AMH level, as a reflection of the stock of pre-antral and small antral follicles, is two- to fourfold higher in women with PCOS than in healthy women (Pellatt *et al.*, 2007; Azziz *et al.*, 2009). This increase in serum AMH was first thought to be only due to the higher number of pre-antral and small antral follicles. However, production of AMH by granulosa cells was found in vitro to be 75-fold higher in anovulatory PCOS and 20-fold higher in normo-ovulatory PCOS than in normal ovaries (Pellatt *et al.*, 2007). This suggests that increased serum AMH levels in PCOS would also reflect an intrinsic dysregulation of the granulosa cells, in which AMH, itself, could be involved since an over expression of the AMH receptor type II (AMHRII) has also been described (Catteau-Jonard *et al.*, 2008; Alebic *et al.*, 2015).

The cause of such high production of AMH in antral follicles from PCO is currently unknown, but there is evidence to support a role played by androgens. Indeed, a positive correlation between serum androgen and AMH levels has been reported and the overproduction of androgens could be an intrinsic defect of thecal cells in PCOS (Laven *et al.*, 2004; Pigny *et al.*, 2003; Gilling-Smith *et al.*, 1994; Carlsen *et al.*, 2009; Eldar-Geva *et al.*, 2005).

Studies demonstrated contradictory results concerning AMH regulation by gonadotropins. For some authors, gonadotropins (especially FSH) inhibit serum AMH production in vivo in normal ovaries (Panidis *et al.*, 2011). Pellatt *et al.* (2007) demonstrated a reduced AMH production in granulosa cells from women with PCOS stimulated by FSH, but no such effect was found in "normal" women. On the contrary, others demonstrated a stimulating effect of FSH on AMH expression in normal ovaries as well as in PCOS (Pierre *et al.*, 2013). The recent finding that E2 inhibits AMH expression could reconcile those different results (Grynberg *et al.*, 2012). FSH may directly stimulate AMH in small antral follicles, as long as they do not express aromatase. But in larger follicles, by increasing E2 production with the recruitment of a dominant follicle, FSH would have an indirect inhibitory effect on AMH expression through the negative feedback of E2 via its receptor ERβ (**Fig. 3**; Grynberg *et al.*, 2012; Dumont *et al.*, 2015).

It has also been demonstrated that AMH significantly decreases not only the FSH receptor expression but also ovarian aromatase expression (Pellatt *et al.*, 2011). This allows protection of the small follicles from premature aromatase expression. However, when this protective effect exceeds its physiological role, because of AMH excess and/or because it lasts longer than it should in larger follicles, this could result in a defect in the selection of the dominant follicle, thus causing the so called the "follicular arrest." The fact that AMH is inhibitory to FSH-dependent factors required for follicle dominance adds considerable

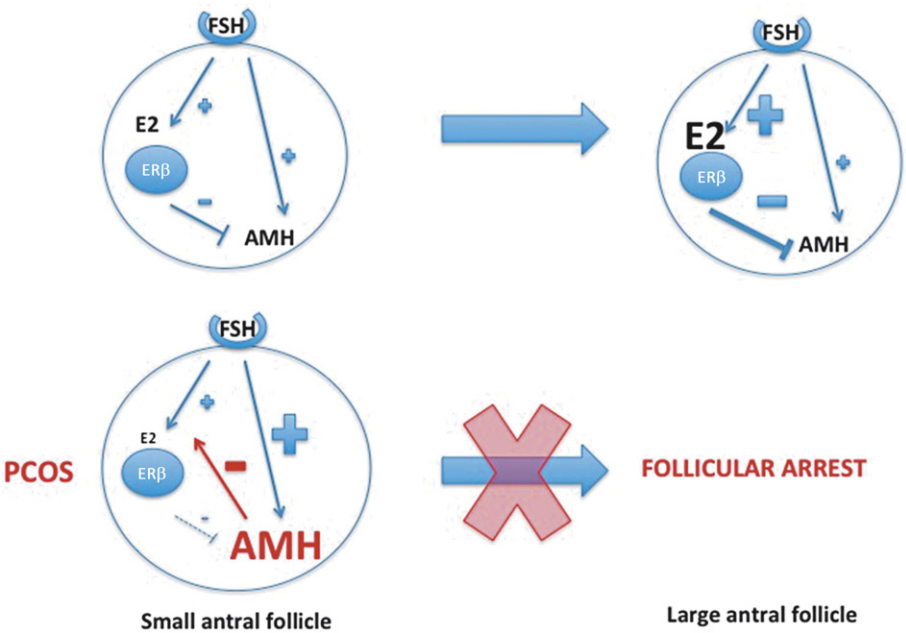

Fig. 3 Schematic diagram of AMH regulation by FSH and E2 in GC of small and large antral follicles. Until the small antral stage, AMH secretion is stimulated by different factors like FSH. Estradiol (E2) production under the influence of FSH is impaired by the inhibiting effect of AMH on aromatase. When estradiol concentration reaches a certain threshold in large antral follicles, it is capable of completely inhibiting AMH expression through ERβ, which predominates in growing follicles, thus overcoming the stimulation by FSH. In large follicles from PCOS, the lack of FSH-induced E2 production and the high level of AMH impair the shift from the AMH to the E2 tone, thus leading to the follicular arrest. Reproduced from Dumont, A., Robin, G., Catteau-Jonard, S. and Dewailly, D. (2015). Role of anti-Mullerian hormone in pathophysiology, diagnosis and treatment of polycystic ovary syndrome: a review. *Reproductive Biology and Endocrinology* 13, 137.

significance to the high serum AMH expression found in PCOS and makes AMH a putative central actor of the "follicular arrest." In good agreement, clinical studies have shown a relationship between high AMH and ovulatory disorder (Pellatt *et al.*, 2007). Besides FSH, acquisition of LH receptors by the granulosa cells happens sooner in PCOS (Willis *et al.*, 1998). Some authors demonstrated that LH reduces AMHRII expression in granulosa luteal cells in normal ovaries and in women with normo-ovulatory PCOS, whereas it cannot do so in women with anovulatory PCOS (Pierre *et al.*, 2013; Pellatt *et al.*, 2010). This lack of LH-induced downregulation of AMHRII expression in women with anovulatory PCOS could contribute to anovulation. Therefore, the premature action of LH might also contribute to the "follicular arrest" through a mechanism involving the AMH system (Willis *et al.*, 1998; Jakimiuk *et al.*, 2001).

To sum up, in PCOS, there are many abnormalities of folliculogenesis: (i) an increased number of small growing follicles (Webber *et al.*, 2003), (ii) an inhibition of the terminal follicular growth (Maciel *et al.*, 2004), resulting in a lack of selection of the dominant follicle, so-called the follicle arrest (Jonard and Dewailly, 2004), and (iii) a follicular apoptosis defect aggravating the excess of growing follicles (Das *et al.*, 2008; Webber *et al.*, 2007).

The robust association between AMH and AFC has led some authors to compare their performance in the diagnosis of PCOS (Eilertsen *et al.*, 2012). Even though serum AMH would be theoretically more accurate than AFC, as it reflects also the excess of small follicles nonvisible on ultrasound (Dewailly *et al.*, 2011, 2014a; **Fig. 4**), it is still considered premature to make this diagnostic transition.

The results from the current literature on the subject are not homogeneous due to the lack of well-defined populations. In particular, some authors have used the PCOS definition established in 2003 at the Rotterdam conference, using 12 follicles of 2–9 mm diameter per ovary for the polycystic ovaries morphology (PCOM) (Balen *et al.*, 2003). This cut-off is highly dependent on ultrasound equipment and operator skill, as demonstrated by Dewailly *et al.* (2014b). Therefore, with the latest ultrasound generation, the threshold has evolved and is now up to 19 or 25 (Dewailly *et al.*, 2011; Lujan *et al.*, 2013; Robin *et al.*, 2012). This threshold will probably continue to increase as newer ultrasound technologies and equipment are developed. Additionally, there are critical issues regarding what populations are included or excluded in the normative population. Lastly, technical issues remain regarding serum AMH assays. It is therefore impossible, so far, to propose a consensual and universal diagnostic threshold for serum AHM in the prediction of PCOS. However, in our experience, a cut-off at 35 pmol/L (4.9 ng/mL) with the enzyme immunoassay AMH-EIA (EIA AMH/MIS kit) ("Immunotech," ref. A16507) provided by Beckman Coulter (France) had a good specificity (97%) and a better sensitivity than the AFC (92%) to distinguish women with PCOS from normal women (Dewailly *et al.*, 2011). Pigny *et al.* (2016) have also compared the five serum AMH assays (as described earlier) for the diagnosis of PCOS. They proposed, with manual ELISAs assays, a higher cut-off at 5.6 ng/mL (40 pmol/L), as biological criteria indicative of PCOM, corresponding to the 95th percentile of "pure" controls. They also proposed a threshold at 4.2 ng/mL (30 pmol/L) for the automatic assays. If confirmed with the new automatized serum AMH assays or the ultrasensitive assay, a high serum AMH level could then become a reliable and accurate marker for PCOM, and eventually replace the AFC.

Serum AMH level is also correlated to the severity of PCOS symptoms (Pellatt *et al.*, 2010) and is higher when hyperandrogenism or oligo-anovulation is present (Pellatt *et al.*, 2007; Eldar-Geva *et al.*, 2005; Piouka *et al.*, 2009). By a principal component analysis, it has been shown that a high serum AMH level can be considered as a marker of hyperandrogenism and could also be used as a substitute for this item in the Rotterdam classification (Dewailly *et al.*, 2010). This would reconcile the different classifications currently available for PCOS because some require hyperandrogenism as a necessary criterion. In 2011, the

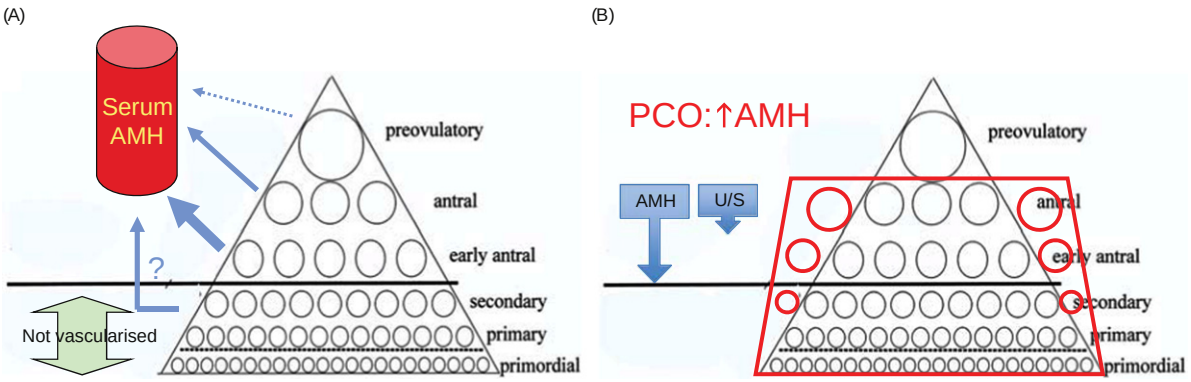

Fig. 4 Rationale for the use of serum AMH assay as a probe for PCOM. (A) All growing follicles secrete AMH but serum AMH reflects only the secretion from bigger follicles that are in contact with the vascular bed. As the numbers of follicles in all growth stages are strongly related to each other, serum AMH is considered to reflect the sum of growing follicles but not the number of primordial follicles that do not secrete AMH. (B) In PCO, the numbers of all growing follicles are increased, resulting in a marked increase in serum AMH level. AMH may be considered as a deeper and more sensitive probe to define follicle excess than the follicle count by ultrasound (U/S) since it appraises more follicle classes (*blue arrows*). Reproduced from Dewailly, D., Andersen, C. Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R., Griesinger, G., Kelsey, T. W., La Marca, A., Lambalk, C., Mason, H., Nelson, S. M., Visser, J. A., Wallace, W. H. and Anderson, R. A. (2014). The physiology and clinical utility of anti-Mullerian hormone in women. *Human Reproduction Update* 20, 370–385.

following strategy was proposed (Dewailly *et al.*, 2011): for the diagnosis of PCOS, hyperandrogenism and oligo-anovulation should be first sought. If one is missing, PCOM (i.e., high FNPO or/and high serum AMH level) can be used instead (Dewailly *et al.*, 2011). Thus, there are four PCOS phenotypes and it is important to differentiate them, as they do not involve the same reproductive concerns and/or metabolic consequences. Many studies have tried to identify a predictive level of serum AMH for the different phenotypes but results remain mixed (Hwang *et al.*, 2013; Sahmay *et al.*, 2013). Although it is clear that complete phenotypes of PCOS (phenotype A) have the highest serum AMH levels, it was observed that nonhyperandrogenic oligo-anovulatory phenotypes (phenotype D) had higher median serum AMH levels than hyperandrogenic normo-ovulatory phenotypes (phenotype C) (Dewailly *et al.*, 2014b). Alebic *et al.* (2015), described a steadily increase in median serum AMH levels across the phenotypes (PCOM < phenotype C < phenotype D < phenotype A). They also used the ratio AMH/AFC as a marker of per-follicle AMH production and found it to be significantly increased in a stepwise manner: low in controls, intermediate in eumenorrheic women (PCOM/phenotype C), and high in oligo-amenorrheic women, regardless of androgen status (phenotype A/phenotype D) (Alebic *et al.*, 2015). Recently, Carmina *et al.* (2016) reported that FNPO was significantly more sensitive than serum AMH in the overall diagnosis of PCOS (93% vs. 79%) and especially in nonhyperandrogenic phenotypes (93% vs. 53%) or ovulatory phenotypes (95% vs. 50%). AMH appeared to be mostly helpful in anovulatory phenotypes (sensitivity 91% vs. 92%) (Carmina *et al.*, 2016).

AMH and Assisted Reproductive Technologies

In addition to its diagnostic role, serum AMH level is useful to establish treatment protocols and to define the best strategy for ovulation induction and stimulation in ART.

Clomiphene citrate (CC) is the first-line treatment for ovulation induction in PCOS. So far, there are very few studies that have examined the predictive power of serum AMH level in the response to CC. Mahran *et al.* (2013) have proposed a threshold at 3.4 ng/mL above which a resistance to CC is highly expected, suggesting a higher starting dose should be used.

Laparoscopic ovarian drilling (LOD) is currently recommended as a successful second-line treatment for ovulation induction in women with PCOS. It is considered to be an alternative to gonadotropin stimulation in the case of CC resistance (Thessaloniki Eshre Asrm-Sponsored PCOS Consensus Workshop Group, 2008). The aim is to trigger spontaneous ovulation by destroying small amounts of ovarian stoma. The physiological mechanism remains unknown, but drilling significantly alters the hormonal environment within the ovary. The utility of the AMH assay as a predictor for LOD outcome has been recently questioned (Abu Hashim, 2015). Elmashad *et al.* (2011) showed LOD was followed by lower serum AMH levels and less elevated Doppler flow indices. Amer *et al.* (2009) also showed that women who ovulated after LOD had lower preoperative AMH levels. They identified a pretreatment AMH level cut-off of 7.7 ng/mL (sensitivity 78%, specificity 76%), which predicted failure of LOD.

Concerning the use of gonadotropins in in vitro fertilization, there is a linear relationship between AMH and the number of oocyte yield (Nelson *et al.*, 2007; La Marca *et al.*, 2010). The fact that AMH can predict ovarian response accurately (Broer *et al.*, 2009, 2011b) enables clinicians to avoid iatrogenic complications and to choose the optimal stimulation strategy (treatment protocol and doses). For example, we can identify women at risk of ovarian hyperstimulation syndrome (PCOS), and maximize the protocols to prevent this risk (Broer *et al.*, 2011b; Al-Inany *et al.*, 2011). Conversely, maximizing follicular recruitment in women with low OR seems appropriate because a poor response is anticipated, although the optimal strategy for the poor responder remains debated (Ferraretti *et al.*, 2011). This also ensures that patients are counseled appropriately with realistic expectations of the outcome of their ovarian stimulation. Given that many women do not fully appreciate the detrimental effect of age on oocyte number, the ability to guide them on overall success using a combination of their age as a surrogate for oocyte quality, and AMH for oocyte yield would be a powerful tool (La Marca *et al.*, 2011). It is likely in the future that with standardization of AMH measurement and stimulation strategies, multivariate prediction models with tight confidence intervals will be able to be created and individualized reports generated. Steps on this path have already been made with optimal prediction of excessive response achieved by combining age, AMH and AFC (Broer *et al.*, 2011b) and refinement of gonadotropin dosing by combining AMH with FSH and age (La Marca *et al.*, 2012).

Conclusion

It is now undeniable that serum AMH is one of the most important ovarian hormones and the best marker of the ovarian function. Although knowledge of its precise roles in ovarian physiology still requires extensive fundamental and clinical studies, it is already clear that AMH is crucial in maintaining the right tempo of folliculogenesis in the ovary, making it one of the most crucial factors underpinning female fertility. As for its benefit in the treatment of infertility, there may be an advantage in therapeutic decision support, but this needs to be confirmed by further studies.

However, the current technical difficulties to set up consensual serum AMH thresholds (stability and heterogeneity of circulating AMH, wide range of values, interlaboratory variability, different immunoassays used worldwide) may have curbed the enthusiasm of some clinicians to make it "THE" marker of PCOM. But we must remain optimistic regarding the latest progress made.

Competing Interest

The authors declare that they have no competing interests.

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Ovarian Stimulation with Clomiphene Citrate☆

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Glossary

Follicle-stimulating hormone (FSH) The pituitary hormone that causes the development and maturation of the oocyte and stimulation of the granulosa cells to produce estrogen.

Gonadotropin-releasing hormone (GnRH) A hypothalamic decapeptide released in a pulsatile manner that causes the release of FSH and LH by the pituitary.

Luteinizing hormone (LH) The pituitary hormone that completes oocyte maturation, stimulates oocyte release and initiates corpus luteum progesterone production.

World Health Organization group 1 anovulation Women with hypogonadotropic hypogonadism (low levels of estrogen and low FSH and LH). They tend to be thin and have a limited response to either clomiphene citrate or progestin withdrawal.

World Health Organization group 2 anovulation Women with normal gonadotropins or elevated LH/FSH ratios. They may have polycystic ovaries and have a tendency toward obesity, hyperandrogenism or insulin resistance.

Pathophysiology

Clomiphene citrate is a triphenylethylene derivative chemically related to DES and tamoxifen. The novelty of this compound is that it produces a chemical rather than a hormonal stimulation for ovulation induction. Although there are receptors in the ovaries and pituitary, it is generally believed that its therapeutic action is derived through hypothalamic stimulation increasing gonadotropin-releasing hormone (GnRH) pulse frequency and/or amplitude in order to stimulate pituitary release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). A simplistic view is that it binds to the estradiol receptors in the medial basal hypothalamus, antagonizing the negative feedback mechanism of estrogen. The increase in FSH then stimulates follicular development, eventually allowing dominant follicle predominance.

Clomiphene citrate is a racemic mixture distributed only in 50-mg tablet form. A typical prescription is 1–3 tablets per day for 5 days. In more resistant cases, 4–5 tablets per day are suggested. Other dosing schedules may require longer intervals at lower doses, such as 1–2 pills a day for 8–12 days. Slowly increasing the dose every 5 days in a given cycle has also been suggested in resistant cases. The objective is to achieve the desired response (ovulation) with the least amount of medication and minimal number of side effects. Starting on Day 5 may interfere with the use of LH kits (ovulation predictor tests). If an elevation in LH occurs immediately following the last pill, a false-positive result will occur. Patients will think they "ovulated" when they have not. This could lead to inappropriate timing for coitus, post coital tests, or inseminations. An alternative approach consists of beginning clomiphene on Day 2 or 3 of the patient's cycle. This allows for earlier recruitment of the dominant follicle and typically increases the time from the last pill to the time of the LH surge. Decreasing the number of days and increasing the dosage, such as 3 pills for 3 days starting on Day 3, can accomplish the desired result while decreasing the amount of medication and unpleasant side effects.

Indications

Ideally, patients who have a recognizable hormonal imbalance should use clomiphene citrate. Clinically, these patients are World Health Organization (WHO) group 2 anovulatory individuals who are estrogenized and have normal gonadotropins. Typically, these patients may also have hyperandrogenism and/or insulin resistance. Appropriate evaluation for these problems, along with examinations for thyroid abnormalities, pregnancy, hyperprolactinemia and adrenal disorders, should be carried out initially. Weight loss and the addition of an insulin sensitizer in these individuals help to limit the amount and duration of medication.

The age-related decrease in fecundity, the "biological clock," is a common problem for couples seeking fertility assistance. The clomiphene challenge test is used to evaluate decreased ovarian reserve. Serum FSH and estradiol levels are obtained on Days 3 and 10 of the patient's cycle. Clomiphene citrate (100 mg, two tablets) is given for 5 days starting on Day 5. If the FSH level on Day 3 or Day 10 is elevated, a problem with follicular recruitment can be expected. Occasionally, the use of clomiphene citrate in the perimenopausal patient may stimulate a better response than injectable medications by using the patient's own stimulated endogenous gonadotropins.

☆*Change History:* December 2015. J Gianfortoni updated the text and further readings to this entire article.
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The natural continuum of follicular development results in the formation of a single mature dominant follicle. In the in vitro fertilization setting, it is hoped that additional follicles will develop so that a reasonable number can be retrieved for fertilization and transfer. Clomiphene citrate has been used with variable success compared to injectable menotropins. It is a less expensive means of stimulation and has a decreased risk of ovarian hyperstimulation. Unfortunately, fewer follicles develop. Another difficulty is the development of a spontaneous LH surge causing premature oocyte release and luteinization. The use of newer GnRH antagonists may help resolve this problem.

Clomiphene citrate is most commonly used, and abused, by patients who have an undetermined etiology of their infertility. This includes the group of patients who report difficulty conceiving, leading the physician to treat them prior to completing a thorough evaluation. This occasionally works if subtle endocrinopathies are corrected by the development of multiple follicles.

Clomiphene citrate has also been suggested for treatment of a luteal-phase defect. Theoretically, the increase in follicle number produces increased levels of estrogen and progesterone, thereby stimulating better endometrial development. Paradoxically, the estrogen antagonistic effect on the endometrium can diminish endometrial thickening and receptivity. Other indications for its use include better timing of inseminations and the development of follicles on the side of a single functioning tube.

Side Effects

The side effects of clomiphene citrate are generally associated with their estrogenic antagonist properties. Alerting the patient to potential problems allows better adjustment to this therapy in an already stressful situation. Side effects can be seen within any organ that is estrogen sensitive. Thus, thickened hostile cervical mucus interfering with sperm mobility, dyssynchronous endometrial development leading to problems with implantation, vaginal dryness and hypothalamic changes (eg, hot flashes, headaches and visual changes) may be seen. Visual changes are the most frightening. The most frequently described are blurred vision and flashing lights. It is usually recommended that clomiphene be discontinued if these symptoms occur, although at lower doses they typically will not return.

Mood changes can occur with varying degrees of intensity. Because of its association with irritability and an altered sense of well-being, clomiphene has sometimes been called the patient's "mean pills."

Mature follicular cysts at the time of ovulation are larger than average when the patient is taking clomiphene. The change in diameter from a 2-cm follicle to a 3-cm follicle increases the volume from 4 to 13 cm^3. If several large follicles develop at the same time, it is understandable that the patient may have increasing pelvic pain. Once formed, these corpus lutea may persist into the next cycle. Evaluation prior to restarting clomiphene citrate is generally suggested. Although rare, hyperstimulation syndrome has been reported with the use of clomiphene citrate.

In the general population, the multiple birthrate is 1–2%, whereas it is 5–12% with clomiphene use. Although the vast majority are twins, higher order multiples have been reported.

Ovarian Cancer Risk

Clomiphene as associated with an increase in the rate of ovarian cancer in two early studies. Theoretically, increased ovarian activity would lead to additional epithelial changes, thereby elevating malignant neoplastic potential. Subsequent analysis and other studies failed to find similar results. During the past 40 years, the use of ovulation-stimulating agents has not resulted in an increase in the U.S. mortality rate for ovarian cancers. In contrast, the marketing of tobacco products to women during this same period has resulted in a dramatic increase in the rate of lung cancer. This killer has surpassed breast cancer as the leading cause of cancer deaths in women.

Alternatives

After correction for other endocrinopathies, such as hyperprolactinemia, hypo- or hyperthyroidism and hyperandrogenism, the addition of clomiphene citrate usually aids ovarian stimulation in anovulatory patients. For some WHO group 2 individuals, metformin has been shown to be of assistance with or without overt insulin resistance. The addition of injectable FSH after 5–10 days of clomiphene citrate treatment can assist in the continued maturation of an appropriate number of follicles while lowering the overall cost to the patient.

Tamoxifen and letrozole have proven to be successful alternatives for stimulating otherwise clomiphene-resistant patients. Injectable gonadotropins or the use of GnRH offer the best solution for WHO group 1 anovulatory women.

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Ovulation Induction With Gonadotropins

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Introduction

Ovarian stimulation represents a major management tool used very commonly nowadays for many conditions in the field of reproductive medicine. Gonadotropins have been available for clinical use for >60 years and are essential for ovarian stimulation including for ovulation induction in anovulatory women as well as for multi-follicular recruitment necessary for assisted reproductive technology (ART) procedures. Multiple preparations of gonadotropins have been developed over the years and are currently widely available on the market for ovulation stimulation.

Historical Background

In this section, we will briefly review the major landmarks that lead to the understanding of the physiology and pharmacology of gonadotropins, as well as the pioneering works that created the wide use, range and availability of pharmaceutical gonadotropin preparations we have today.

Year	*Advancement*
1927–30	Aschheim and Zondek, two German gynecologists, were the first to identify the endocrine function of the pituitary gland and to discover the hormonal action on the human ovary of two forms of gonadotropins they labeled "prolans" A and B. The substances they identified were extracted from pregnant, postmenopausal women and castrated individuals' samples. Notably, they injected pregnant women's urine subcutaneously into immature female mice, causing the development of follicular cysts and hemorrhage into the ovarian stroma and this became known as the Aschheim Zondek pregnancy test. But they believed that the substance responsible for this originated from the anterior pituitary (Aschheim and Zondek, 1927)
1931	Fevold et al. confirmed the roles of the two hormones, and "prolan A" was labeled "follicle-stimulating factor" whereas "prolan B" became "luteinizing factor." The roles of gonadotropins FSH (follicle stimulating hormone) and LH (luteinizing hormone) in being responsible for the rhythmic function of the ovary, and in turn controlling the proliferation and function of the endometrium in preparation for an eventual implantation of a fertilized oocyte, were identified at this point (Fevold *et al.*, 1931)
1937	Cartland and Nelson were the first to extract a purified version of gonadotropins from urine. Their material was stable and sterile enough to initiate use in laboratory and clinical research (Cartland and Nelson, 1937)
1941	Mazer and Ravetz introduced the concept of a "two-step protocol," where anterior pituitary gland extracts are used during the follicular phase for ovarian stimulation followed by urinary pregnancy extracts for ovulation induction. They were the first to report on the resumption of menstrual flow in previously amenorrheic patients, the concept that ovarian response to exogenous gonadotropins is based on individual ovarian receptivity, as well as the first to describe three cases of iatrogenic ovarian hyperstimulation syndrome (OHSS) in regularly menstruating women (Mazer and Ravetz, 1941)
1943	Seegar-Jones et al. were the first to confirm that one of the "prolans" described by Aschheim and Zondek had a placental origin and became known as human chorionic gonadotropin (hCG) (Lunenfeld, 2004; Seegar-Jones *et al.*, 1943)
1945	Hamblen et al. were the first to publish on the use of gonadotropins in a physiologic temporal sequence form of the "two-step protocol" followed by planned intercourse that was compatible with fertility and that resulted in multiple pregnancies (Hamblen *et al.*, 1945)
1949	Until the early 1940s, most of the available gonadotropin extracts were of animal origin. At the end of the 1940's, gonadotropins started to be extracted from urine of post-menopausal women. Multiple methods have been reported and improved over the years, including the early works of Bradbury et al. in 1949 and later Albert in 1955, which were used by many laboratories for years to extract material from large pools of human urine to produce gonadotropins (Bradbury *et al.*, 1949; Albert, 1955)
1950	The method of urinary extraction was further perfected and introduced for clinical use which led to the extraction of purified forms of FSH and LH at the final stages of production (Donini et al., 1964, 1966)
Late 1950s–early 1960s	The first successful inductions of ovulation leading to pregnancies were reported in hypogonadotropic anovulatory women (Lunenfeld, 1963)

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	Extraction of gonadotropins for clinical use from human pituitary glands (hPG) was developed and compared to urinary extraction with the increasing demands for pharmaceutical gonadotropins. However, hPGs were later removed from the market in the 1980s with the appearance of cases of dementia and death secondary to Creutzfeldt-Jacob disease (Gemzell *et al.*, 1958; Buxton and Hermann, 1961; Lazarus, 1985)
Early 1970s	Technological advances have permitted the development of highly purified "third generation gonadotropin" FSH preparations with <0.1 IU of LH activity and <5% of unidentified urinary proteins (Lunenfeld, 2004). The enhanced purity equated to only a very small amount of protein becoming required for injection, therefore permitting highly purified FSH preparations to be injected subcutaneously. Also, with the batch-batch variability having been almost excluded at this point, new ovulation induction regimens have started to emerge with lower doses, lower dose increments, as well as more consistent and predictable responses (Lunenfeld, 2004)
1975–76	Rathnam and Saxena were the first to describe the amino-acid sequence of the alpha and beta subunits of FSH (Rathnam and Saxena, 1975; Saxena and Rathnam, 1976)
1977	Steptoe and Edwards' pioneering work from the UK led to the birth of the world's first baby conceived by IVF, Louise Brown on 25 July 1978. This work was awarded a Nobel Prize in 2010 and contributed heavily to the wide use of gonadotropins
1980s	Howard W. Jones and Georgeanna Seegar Jones from the Eastern Virginia Medical School in Norfolk incorporated the use of urinary gonadotropins for ovarian stimulation for IVF purposes. This became known and widely utilized worldwide as "controlled ovarian hyperstimulation" (COH), with the addition of pituitary down-regulation with GnRH agonists in the 1980s that can prevent a premature LH surge and ovulation during gonadotropin stimulation
Late 1980s–90s	With the advent of recombinant DNA technology, human FSH was successfully expressed in a Chinese hamster ovary (CHO) cell line in 1988 (Keene *et al.*, 1989). This first recombinant FSH (follitropin alpha) was first used in the clinic in 1991 (Mannaerts *et al.*, 1991). Another similar recombinant form of FSH (follitropin beta) was later introduced and marketed in 1996 (Olijve *et al.*, 1996)
2000–01	Recombinant human LH (lutropin alpha) was released, and with the launch of recombinant hCG (choriogonadotropin alpha) in 2001, the full recombinant gonadotropin range became available
2010	An analogue of FSH with long-acting activity, corifollitropin alpha was introduced on the market. The molecule product labeled is a fusion molecule of recombinant human FSH and the C-terminal peptide of the beta subunit of hCG, which allows for a prolonged absorption and a longer half-life for up to a week with a single subcutaneous injection in women undergoing controlled ovarian stimulation (Croxtall and McKeage, 2011)
2016	A new form of human recombinant FSH, follitropin delta was derived from a human fetal retinal cell line (Arce *et al.*, 2014). Due to differences in glycosylation profile, it has a lower clearance and induces a higher ovarian response than follitropin alpha when administered at equal doses of biological activity (Arce *et al.*, 2014). An individualized dosing algorithm was developed for follitropin delta incorporating body weight, which influences drug exposure and pretreatment anti-müllerian hormone (AMH) levels, which predict ovarian response (Nyboe Andersen *et al.*, 2017)

Choice Considerations for Pharmaceutical Gonadotropins

Multiple gonadotropin preparations have been developed over the years and are currently on the market.

The three main types of urinary gonadotropin extracts used for ovarian stimulation are human Menopausal Gonadotropins (menotropins or HMGs), purified FSH preparations (urofollitropins or FSH-P), and highly purified FSH preparations (FSH-HP). The older preparations used to contain >95% protein impurities and varying amounts of FSH, LH, and hCG, but improvements in purification techniques resulted into a standardization of the FSH and LH activities to 75 IU for each type of gonadotropin, although <5% extraneous urinary proteins still exist even in today's hMG products. It is important to underline that the LH-activity in hMG derives primarily from the hCG component (Practice Committee of American Society for Reproductive Medicine, Birmingham, Alabama, 2008).

Different forms of pure recombinant FSH gonadotropin preparations have been developed for ovarian stimulation: follitropin alpha, beta, and, more recently, follitropin delta (Arce *et al.*, 2014). Lutropin alpha is the only pure recombinant LH preparation available on the market. A combined preparation of recombinant FSH and LH (follitropin alpha and lutropin alpha) is available in a single injection.

Corifollitropin alpha is the only long-acting recombinant FSH analogue available, which allows for a half-life of up to 1 week with a single subcutaneous injection. Corifollitropin alpha is only used for IVF protocols because a single dose can initiate and sustain multifollicular growth, whereas other urinary and recombinant FSH can be used for both ovulation induction and IVF.

Ovulation triggering and induction of final oocyte maturation is performed with hCG, which binds the same receptor as LH.

Efficacy

Treatment of hypogonadic hypogonadal patients, characterized by low gonadotropin and low estrogen levels, requires exogenous LH activity in addition to FSH. In these patients, because of low LH levels, treatment with FSH alone leads to follicular development but not pregnancy (Schoot *et al.*, 1992). Until recently, the only option was hMG (which contains equivalent amounts of FSH and LH IU), but the recent development of recLH and rechCG offered new possibilities of treatment.

For all other indications, several systematic reviews compared recombinant gonadotropins with urinary gonadotropins (HMG, purified FSH, and highly purified FSH) for ovulation induction as well as for controlled ovarian hyper-stimulation for ART. Concerning ovulation induction, a recent Cochrane review found no evidence of a difference in live birth rates among women with polycystic ovary syndrome (PCOS) and CC resistance, between urinary-derived preparations and recombinant FSH. Evidence for all outcomes was, however, still considered to be of "low or very low quality" by the authors (Weiss *et al.*, 2015). For IVF/ICSI, the latest Cochrane review included 28 trials and the meta-analysis showed no evidence of a statistically significant difference in terms of live birth rate when comparing recombinant FSH to any of the other gonadotropins, irrespective of the down-regulation protocol used (van Wely *et al.*, 2012). The authors also mention in their conclusion that "further research on these comparisons is unlikely to identify substantive differences in effectiveness" among the different preparations.

Regarding the effectiveness of co-administrating recombinant LH to recombinant FSH during ovarian stimulation for IVF and ICSI cycles, no evidence of statistical difference in pregnancy outcomes exists with the addition of recombinant LH (Broekmans *et al.*, 2006).

Principles of Ovarian Stimulation With Gonadotropins

Ovarian stimulation encompasses ovulation induction in anovulatory patients and ovarian hyperstimulation, often used to treat ovulatory women in a context of subfertility or infertility. While ovulation induction is intended to induce a monofollicular ovulation in order to restore fertility in anovulatory women, ovarian hyperstimulation aims at inducing a multifollicular ovulation to increase a couple's chance of conception. Before timed intercourse, ovarian hyperstimulation aims at inducing the development of two or three follicles in order to enhance the overall chance of conception. The same principle of ovarian hyperstimulation can be combined with intra-uterine insemination (IUI), whereby a sperm preparation is brought into proximity of two to three oocytes. In the case of in vitro fertilization (IVF), conventional protocols of ovarian stimulation aim at multi-follicular development in order to maximize oocyte yield, thereby increasing the number of embryos and the subsequent cumulative pregnancy rate.

Whatever the indication, it must be emphasized that no stimulation should be administered without a careful infertility work-up including ovarian function assessment, verification of the fallopian tubes permeability, and a sperm exam.

Ovulation Induction in Anovulatory Patients

Anovulatory disorders account for around 25% of causes of infertility (Hull *et al.*, 1985). According to serum levels of gonadotropins and estrogens, they can be classified in one of the three categories of the World Health Organization (WHO) classification. Low gonadotropins and low estradiol refer to WHO class 1 anovulation encompassing disorders at the hypothalamic or pituitary level. The WHO class 2 anovulation is characterized by normal estradiol and FSH levels. The vast majority of patients in this last category have PCOS. The last category, WHO class 3, is characterized by high gonadotropins and low estradiol levels associated with ovarian insufficiency.

Ovulation induction with gonadotropins applies to WHO classes 1 and 2, while it is not effective in patients with ovarian failure (WHO class 3).

Indications

Women with class 1 anovulation who have an intact pituitary function can be treated with pulsatile GnRH therapy or exogenous gonadotropins. However, it should be emphasized that for many of these patients, hypothalamic anovulation is functional and results from stress-inducing conditions such as excessive dieting or intense exercise training. Increased caloric intake or reducing exercise in order to match caloric intake to energy expenditure should be considered before using pharmacological agents. Pulsatile GnRH therapy is highly efficient in restoring the periodic release of FSH and LH. Compared to gonadotropins, it has a reduced risk of multiple folliculogenesis, multiple pregnancies, and ovarian hyperstimulation syndrome (OHSS) (Martin *et al.*, 1993). However, it has the disadvantage of being administered through a portable mini-pump that has to be worn during the whole period of stimulation, which can extend up to several weeks.

The vast majority of women with WHO class 2 anovulation have PCOS, which is the most common endocrine disorder among reproductive-aged women and the main cause of infertility due to anovulation. In 2003, a consensus workshop between the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine in Rotterdam indicated PCOS in the presence of two of the following three features: oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries at ultrasound and exclusion of other endocrinopathies (such as hyperprolactinemia, Cushing syndrome, 21α hydroxylase deficiency) (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004).

Obesity is common in women with PCOS and can lead to or exacerbate insulin resistance, which plays a key role in the pathogenesis of ovarian dysfunction in many PCOS patients. Obesity is also associated with an absence of response to all

pharmacological agents used to induce ovulation. Weight loss is therefore recommended in all obese women with PCOS because it leads to higher spontaneous ovulation rates (Legro *et al.*, 2015) and natural conception rates (Mutsaerts *et al.*, 2016).

Anti-estrogen therapy with clomiphene citrate (CC) is generally considered as the first-line treatment in women with anovulation due to PCOS.

However, a recent systematic review and meta-analysis aiming at comparing the effectiveness of alternative first line treatments of ovulation induction for women with WHO class 2 anovulation concluded that letrozole (aromatase inhibitor) was more efficient than CC in terms of ovulation, pregnancy, and live birth rates (Wang *et al.*, 2017). Insulin-sensitizing agents as metformin can be used alone or as adjuvant therapies for ovulation induction. The meta-analysis concluded that the combination of CC and metformin was superior to CC alone in terms of ovulation and pregnancy, but that there was no evidence of an improved live birth rate with the combined treatment, which could be related to the reduced sample size of studies reporting live births (Wang *et al.*, 2017). Although letrozole and the combination of CC and metformin appear superior to CC alone as first-line treatment of ovulation induction in terms of ovulation and pregnancy, it must be emphasized that neither letrozole nor metformin are approved for the treatment of anovulation in many countries, and continue to be used off-label (Vitek *et al.*, 2015; Usadi and Merriam, 2015). As a result, CC remains the front-line agent to induce ovulation and achieve pregnancy in patients with PCOS. Around 25% of PCOS women will not respond at all to CC and are considered to be CC resistant (White *et al.*, 1996).

Women with PCOS who fail to ovulate or conceive following ovulation induction with anti-estrogens or aromatase inhibitors can be offered ovarian stimulation with gonadotropins or laparoscopic ovarian diathermy as a second-line treatment (Balen *et al.*, 2016). In the absence of pregnancy after 6–9 ovulation cycles with CC or letrozole, however, it is appropriate to offer the couple IVF, taking into consideration the age and resources of the patient (Balen *et al.*, 2016).

Treatment Regimens

The aim of the treatment is to restore normal ovulation and promote monofollicular development, while avoiding major side-effects such as high-order multiple pregnancies and OHSS. Whatever the indication, it is therefore essential to perform a strict monitoring of the cycle by measuring serum estradiol levels and assessing the number and size of developing follicles by repeated transvaginal ultrasounds.

Initial protocols used to start with a dose of 75 IU FSH incremented every 5–7 days, but they were associated with multiple follicular development resulting in an unacceptable incidence of multiple pregnancies (Homburg and Howles, 1999). Several investigators have attempted to reduce the rate of complications of multiple follicular development by proposing a stepwise administration of gonadotropins to achieve the FSH threshold required for initiation and maintenance of follicle growth (Brown et al., 1978), without exceeding the individual threshold requirement of the ovary (Seibel *et al.*, 1984; Polson *et al.*, 1987; Buvat *et al.*, 1989). Different studies have shown that chronic low-dose regimens could induce ovulation while considerably reducing multifollicular development compared to conventional regimen (Homburg and Howles, 1999). The current standard protocol is the low-dose step-up protocol (Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2008). For the first cycle, it is recommended to use low starting doses of FSH of 37.5–50 IU/day (Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2008) and to increase the dose very progressively in order to reach the adequate threshold. In overweight patients (BMI $\geq$ 25 kg/m^2) a starting dose of 75 IU/day can be used (Yildizhan *et al.*, 2008). The dose is increased by 50% if, after 14 days, no response is observed on echography and estradiol monitoring (no follicle $\geq$10 mm, no increase in plasma estradiol level). As a follicle reaches the size of 10 mm, the ongoing dose is maintained until the dominant follicle reaches the size of at least 17 mm, when ovulation can be triggered by the administration of hCG (5000 IU of hCG or 250 μg of recombinant hCG). The threshold dose or a dose slightly below can be used as the starting dose in subsequent cycles (Homburg and Howles, 1999) (**Fig. 1**).

Overall, low-dose step-up regimens result in a monofollicular ovulation rate in approximately 70% of the cycles, a pregnancy rate of 20%, and a multiple live birth rate of about 6% (Homburg and Howles, 1999).

In order to mimic more closely the events of the normal ovulatory cycle, with a decrease in FSH during the late follicular phase allowing follicular dominance, decremental dose regimen have been proposed starting with a FSH dose of 150 IU continued until a follicle $\geq$10 mm is observed. The dose is then lowered to 112.5 IU/day followed by a later decrease to 75 IU/day 3 days later, which is continued until ovulation is triggered with hCG (Schoot *et al.*, 1995; van Santbrink *et al.*, 1995). Preliminary studies reported that both step-up and step-down regimens achieved similar high rates of monofollicular development (van Santbrink

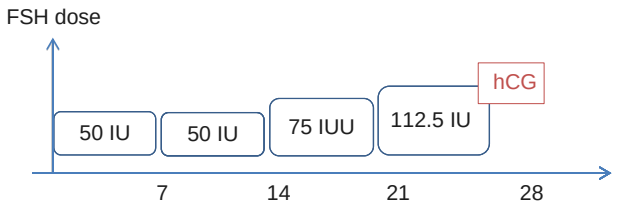


Fig. 1 Chronic low-dose step-up regimen.

and Fauser, 1997). However, it has been subsequently shown in larger series that the step-down protocol was associated with a higher rate of multifollicular development, multiple pregnancies, and a higher incidence of OHSS (Christin-Maitre *et al.*, 2003). A combined approach of sequential step-up and step-down regimens has been shown to help reduce the risk of over-response observed in step-down regimen (Hugues et al., 1996, 2006). It is generally accepted that the step-down regimen should be used only after the response threshold has been established in one or more previous stimulation cycles with a low-dose step-up regimen.

Adjunction of insulin-sensitizing agents to gonadotropins as potential adjuvant therapy has been addressed in several studies for PCOS patients. In a recent Cochrane review, metformin co-treatment during ovulation induction with gonadotrophins was associated with a higher cumulative live birth rate when compared with FSH alone (odds ratio (OR) 2.31, 95% confidence interval (CI) 1.23–4.34), but the conclusion was considered to be based on low-quality evidence (Bordewijk *et al.*, 2017). There was insufficient evidence to show an effect of the adjunction of metformin to FSH on multiple pregnancy rates and adverse events (Bordewijk *et al.*, 2017).

In hypogonadic hypogonadal patients, a dose of 75 IU of recLH in addition to daily FSH was observed to be the right dose to induce follicular development and pregnancy (Burgués and Spanish Collaborative Group on Female Hypogonadotrophic Hypogonadism, 2001).With an LH-like activity driven by hCG, HMG appears to be as effective as the combination of recFSH and recLH in ovulation induction in WHO class 1 anovulation infertility (Carone *et al.*, 2012).

Monitoring

Intense ovarian response monitoring is required in all cases of ovulation induction with gonadotropins. Strict adherence to the chronic low-dose regimen of FSH should markedly reduce the likelihood of multiple follicular development and hence high-order multiple pregnancies and OHSS. The multiple birth rate has been shown to increase from 5% in the presence of one follicle $\geq$16 mm to 20% in the presence of three follicles $\geq$16 mm and to 50% if more than three follicles $\geq$16 mm were observed (Homburg and Howles, 1999). It is advised to cancel the cycle and to ask the patient to refrain from sexual activity or to use a barrier contraception in the presence of more than two follicles $\geq$16 mm and two additional follicles $\geq$14 mm, in order to minimize the risk of multiple pregnancies in women with a history of anovulation, under the age of 38, and without any other infertility factor (Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2008). It is in any case mandatory to inform the patient about the risks associated with multiple pregnancies following ovulation induction with gonadotropins.

Ovarian Stimulation in Intra-Uterine Insemination

IUI is a simple assisted reproductive technology procedure in which semen is prepared in the laboratory and inserted in the uterine cavity using a small catheter at the time of ovulation. IUI can be performed with or without ovarian stimulation. It is the first-line therapy for moderate male factor (asthenospermia), cervical factor (malformations, cervical conization), mild endometriosis, and unexplained infertility. It can also be considered after failed ovulation induction cycles with gonadotropins in women with dysovulation, although if pregnancy has not occurred after six ovulatory cycles, the patient may be offered IVF (Balen *et al.*, 2016).

Before starting ovarian stimulation in the presence of unexplained infertility (attempt of conception for at least 1 year with a fertility work-up showing patent fallopian tubes, ovulatory menstrual cycles, and a normal semen analysis), it is important to consider the likelihood of spontaneous pregnancy and expectant management (Hunault *et al.*, 2004; van Eekelen *et al.*, 2017). Ovarian stimulation in this context aims at inducing the development of two or possibly three follicles in order to enhance the overall chance of conception. In the case of unexplained infertility, the efficacy of ovarian stimulation with gonadotropins followed by programmed intercourse has not been demonstrated and IUI is usually proposed from the outset (Veltman-Verhulst *et al.*, 2016). The place of IUI in combination with ovarian hyperstimulation for unexplained infertility is, however, still a matter of debate, the main concern being the increase in multiple pregnancy rate. A recent Cochrane review confirmed the increase in live birth rate after IUI in stimulated cycles versus natural cycles (Veltman-Verhulst *et al.*, 2016). However, in cycles with three or four dominant follicles, the multiple pregnancy rate increases without a substantial gain in overall pregnancy rate (van Rumste *et al.*, 2008). Therefore, IUI with ovarian stimulation should ideally aim for no more than two follicles (van Rumste *et al.*, 2008). One mature follicle should be the goal if safety is the primary concern, whereas two follicles may be accepted after careful patient counseling. However, the age of the patient, the duration of infertility, and the number of previous failed IUI cycles should also be taken into account.

Exogenous gonadotrophin administration is usually started around day 2–4 of a menstrual cycle at daily dose of 50–75 IU FSH per day. Careful cycle monitoring should be performed by ultrasound and estradiol measurement, and in the presence of an excessive number of follicles, the cycle should be canceled or supernumerary follicles should be punctured transvaginally before insemination (Albano *et al.*, 2001). Monitoring is also necessary for correct timing of the insemination by assessing preovulatory luteinizing hormone rise in blood or urine. In stimulated cycles, ovulation is often induced by an injection of hCG, which improves timing possibilities.

The live birth rate is around 10%–15% per IUI. It is recommended to limit IUI treatment to 6 cycles (Collège National des Gynécologues et Obstétriciens Français (CNGOF), 2010; National Institute for Health and Care Excellence (NICE) guideline,

2013), in line with reduced pregnancy rates with increasing IUI rank (Custers *et al.*, 2008). Nevertheless, the woman's age should be taken into account to optimize the duration of the IUI treatment and ensure timely transfer to IVF if indicated.

Ovarian Stimulation in In Vitro Fertilization

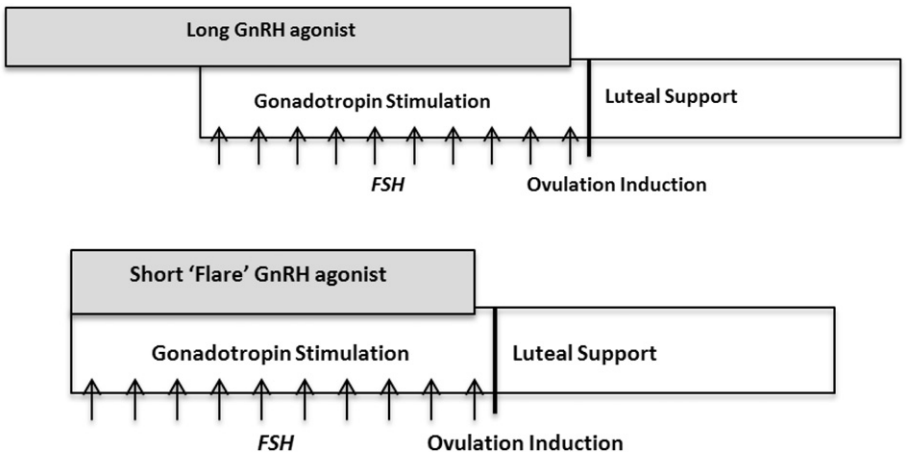
Ovarian stimulation for IVF aims at multi-follicular development in order to maximize oocyte yield, thereby increasing the number of embryos and the subsequent cumulative pregnancy rate. The advent and addition of pituitary down-regulators (GnRH agonists since the 1980s and GnRH antagonists since 2001) that can prevent a premature LH surge and ovulation during gonadotropin stimulation cycles contributed heavily to a wider use of gonadotropins for IVF purposes. Indeed, before the addition of GnRH agonists, the risk of premature LH rise occurred in about 20% of the cycles. The premature LH surge used to occur secondary to the positive feedback triggered by a high serum estrogen level in the mid-follicular phase. Pituitary inhibition is therefore part of the large majority of ovarian stimulation protocols for IVF.

GnRH Agonist Protocols

When the GnRH agonist is used, a phenomenon of initial positive "flare" in pituitary response is observed, followed by down-regulation through clustering and internalization of pituitary GnRH receptors. Making use of the latter part of this phenomenon, the so-called "long down-regulation protocol" of gonadotropin stimulation with a preliminary and continuous GnRH agonist administration has been established in IVF practice since the late 1980s, and has contributed significantly in reducing the rate of cycle cancellation and improving outcomes because of its practically nullified risk of premature LH surge. The "long protocol" requires the GnRH agonist to be started in the luteal phase of the run-in cycle or at the first day of the menstrual cycle and to be continued until the ovulatory hCG trigger. Gonadotropin stimulation is typically started about 10–14 days later, when pituitary down-regulation is established. An important advantage of this protocol relies on the flexibility it provides in the scheduling of egg retrieval (Hayden, 2008). Some women will experience hypo-estrogenic side effects of varying severity secondary to pituitary down-regulation; however, the major disadvantage of this protocol is its relatively prolonged duration with additional costs secondary to increased consumption and injections of gonadotropins, as well as hormonal and ultrasound follow-up measurements.

The "short" or "flare" protocol is another frequently used protocol of ovarian gonadotropin stimulation for IVF purposes that employs a GnRH agonist. Indeed, it makes use of the GnRH agonist-induced positive flare of endogenous FSH pituitary secretion, in addition to the exogenous gonadotropin administration. The agonist is typically started on day 2 of the menstrual cycle while gonadotropin stimulation is initiated on day 3. Follicular growth and maturation take about 10–12 days to occur, which gives enough time for adequate pituitary down-regulation to be established in order to prevent a premature LH surge. Therefore, a shorter duration of treatment and decreased amounts of gonadotropins and injections are required compared to the "long protocol." However, one disadvantage is the reliance on the lowered scheduling flexibility encountered with this approach, given that the start of stimulation is dependent on the start of menses.

A 2015 Cochrane review compared short and long protocols IVF outcomes, and found "no conclusive evidence of a difference in live birth and ongoing pregnancy rates, but there was moderate quality evidence of higher clinical pregnancy rates in the long protocol group" (Siristatidis *et al.*, 2015).

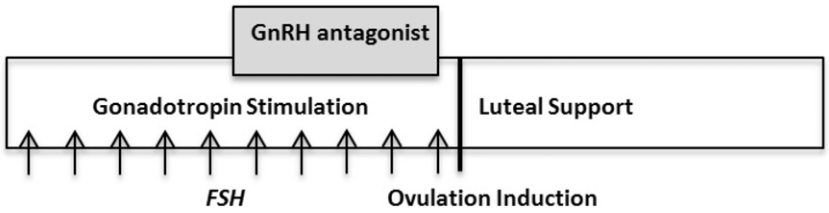


GnRH Antagonist Protocol

It took a considerable amount of time and research to generate clinically useful GnRH antagonists. The current third-generation compounds, Ganirelix and Cetrorelix, can competitively and reversibly bind to GnRH receptors in the pituitary gland, therefore inducing immediate pituitary suppression and subsequent rapid recovery of function, which makes them particularly useful. Indeed, this property allows for a more patient-friendly GnRH antagonist protocol compared to the GnRH agonist protocols, as it requires pituitary down-regulation injections that can be restricted only to the days when a premature LH surge is likely to occur (Duijkers *et al.*, 1998; Felberbaum *et al.*, 1995; Huirne *et al.*, 2007). Typically, multiple small daily doses are used from cycle day 6 as a fixed order, or when the leading follicle reaches 14 mm of diameter, and continued until the day of ovulation induction.

The antagonist protocol offers clear advantages that increase its favor with IVF patients, including a more compact total treatment length that is also physiologically confined to a single month as well as a more favorable side-effect profile. More importantly, the risk of hospital admission due to OHSS is reduced with the GnRH antagonist protocol. In fact, in 2016 a Cochrane review compared the use of the GnRH antagonist with long GnRH agonist protocols and found moderate-quality evidence of a substantial reduction in OHSS with the use of the antagonist without a reduction in the likelihood of achieving live birth.

The main disadvantage of the GnRH antagonist is the lowered flexibility for scheduling that is encountered given that the start of stimulation is dependent on the start of menses. One way to bypass this problem is to use pretreatment with estrogen, which does not compromise the IVF outcome (Cédrin-Durnerin *et al.*, 2012). Another way of programming antagonist cycles is to use the combined oral contraceptive pill to manipulate the length of the follicular phase of the menstrual cycle preceding IVF.



Correct individualization of the gonadotropin's starting dose is a critical decision that clinicians usually make according to multiple clinical criteria including the patient's characteristics like age and BMI, objective markers of ovarian reserve like follicular phase FSH level, antral follicular count (AFC), and anti-Mullerian hormone (AMH), as well as previous history of outcomes of recent ovarian stimulation cycles (La Marca and Sunkara, 2014; Fauser *et al.*, 2008; Alviggi *et al.*, 2012). AMH levels and AFC have been found to be the most reliable examination tools for the measurement of the potential ovarian reserve (Broekmans *et al.*, 2006; La Marca *et al.*, 2010; Nelson et al. 2007). AMH is a glycoprotein secreted into the circulation by primary, pre-antral, and small antral ovarian follicles. Its serum levels correlate strongly with antral follicles as counted by ultrasound, and with the number of retrieved oocytes in IVF cycles where it has been commonly used (La Marca *et al.*, 2010).

Risks and Counseling Issues

Multiple Pregnancies

With the advent of ART, the incidence of multiple gestation has increased drastically. Indeed, while only 1%–2% of all births result from naturally conceived twin pregnancies (ESHRE Capri Workshop Group, 2000), up to 26% of ART-related live births in the USA (Kissin *et al.*, 2015) and 18% in Europe are multiple gestations (Calhaz-Jorge *et al.*, 2017).

Perinatal outcome is known to be severely affected in multiple gestations as the risks for prematurity and low birth weight are elevated in such instances. Many reports have shown fetal mortality to be increased four- to seven-fold in twin gestations and as much as 20 times in triplet gestations (Fauser *et al.*, 2008; Nugent *et al.*, 2000; Verberg *et al.*, 2007).

In IVF, multiple gestation can be easily controlled for and practically eliminated by limiting the number of embryos transferred. Over the past decade, multiple studies have shown that repeated single embryo transfer (SET) was as efficient as the replacement of 2–3 embryos in terms of cumulative pregnancy rates, but was associated with a significant decrease in the incidence of multiple pregnancies (Gerris *et al.*, 1999; Martikainen *et al.*, 2001; Thurin *et al.*, 2004; Pandian *et al.*, 2013). In some European countries like Belgium and Sweden, SET has even become mandatory in good prognosis couples. These guidelines are also increasingly being adopted worldwide as new technologies for embryo selection and successful embryo preservation through freezing were developed and are becoming widely available.

Since this option is not available after timed-intercourse and IUI, monofollicular recruitment through rigorous ultrasound monitoring should remain the goal for those cases.

OHSS

The development of OHSS is mainly an iatrogenic side effect secondary to the use of exogenous gonadotropin for ovarian stimulation. It is unusual with other stimulation agents like CC (Delvigne and Rozenberg, 2002).

Patients at the highest risk of OHSS are typically the young and lean PCOS patients (Navot *et al.*, 1992) who display elevated results on ovarian reserve testing with AMH levels and AFC, and present a hyper-response to ovarian stimulation (Tarlatzi *et al.*, 2017).

OHSS can be classified into mild, moderate, or severe states. Truly clinically relevant instances of OHSS, that is, moderate and/or severe cases, occur at an estimated rate of 3%–8% of ART cycles (3%–6% moderate and 0.5%–5% severe forms) (Delvigne and Rozenberg, 2002; Golan *et al.*, 1989; Schenker and Ezra, 1994). Milder forms of OHSS are more common, of less clinical importance, and occur in an estimated 20%–33% of ART cycles (Mourad *et al.*, 2016).

In the milder presentation of OHSS, women experience mild abdominal pain and bloating in addition to mild nausea and vomiting, and ascites present mainly around the ovaries. However, when the condition progresses to severe OHSS, massive ascites are observed in addition to increased fluid in the pleural and pericardial spaces, concomitant with intravascular volume depletion, hemoconcentration, hypoalbuminemia, and electrolyte imbalances. Severe OHSS can be associated with severe morbidity including thromboembolic events (Kligman *et al.*, 1995), respiratory distress, and liver and/or renal failure. Very rarely, when untreated or inefficiently managed, OHSS can rapidly progress to potentially lethal complications (Braat *et al.*, 2010).

Human chorionic gonadotropin plays a crucial role in the development of the syndrome as severe forms are restricted to cycles with exogenous hCG (to induce ovulation or as luteal phase support) or with endogenous pregnancy-derived hCG (Delbaere *et al.*, 2004).

Avoiding endogenous and exogenous exposure to hCG in the stimulated cycle at risk can be performed by using a GnRH antagonist protocol with a GnRH agonist to trigger ovulation and freezing all embryos for later use. The development of this stimulation strategy in patients at risk has drastically decreased the incidence of OHSS with only anecdotal reported cases (Devroey *et al.*, 2011; Banker *et al.*, 2015).

Cancer

The issue of a potential risk of cancer has been addressed in several studies. However, these concerns have not been validated objectively, since all published reviews and meta-analysis that focused on such issues have consistently found a lack of convincing causal relationship between ART fertility drugs and cancers whether in the breasts, ovaries, or uterus (Siristatidis *et al.*, 2012; Li *et al.*, 2013; Saso *et al.*, 2015; Sergentanis *et al.*, 2013; Salhab *et al.*, 2005; Rizzuto *et al.*, 2013).

A possible increased risk of borderline ovarian tumors in subfertile women treated with IVF has been reported (Rizzuto *et al.*, 2013). However, this is still questioned since infertility itself was found to be an independent risk factor for ovarian malignancy, therefore possibly explaining the above mentioned observation (Venn *et al.*, 1995; Ness *et al.*, 2002; Brinton *et al.*, 2004).

Conclusions and Future Perspectives

Ovarian stimulation with gonadotropins encompasses different clinical situations aiming at increasing the chance of conception whether by restoring ovulation in anovulatory women or by increasing the follicular recruitment in subfertile patients in association with timed intercourse, IUI, or IVF. Whatever the indication, it is associated with complications, particularly multiple pregnancies, which can be drastically reduced by a strict monitoring and cancellation policy in ovulation induction cycles and controlled by a SET policy in IVF. OHSS is another complication that can be largely minimized by proper monitoring in ovulation induction or in the case of empirical treatment of unexplained infertility. The incidence of OHSS is much more frequent in IVF cycles, but recent therapeutic approaches and individualized algorithms have nearly eliminated this potentially life-threatening complication. Controlled ovarian hyperstimulation and assisted reproduction should not become a substitute for ovulation induction for anovulatory patients, as this first-line treatment offers good live birth rates with fewer complications. In the future, gonadotropins could be taken orally and replace the injectable forms currently available, which would contribute to increased comfort for the patient.

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Ovarian Hyperstimulation Syndrome

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Abbreviations

ARDS	Adult respiratory distress syndrome
CC	Clomiphene citrate
E2	Estradiol
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
HES	Hexaethyl starch
hCG	Human chorionic gonadotropin
IVF	In vitro fertilization
LH	Luteinizing hormone
OHSS	Ovarian hyperstimulation syndrome
PCOS	Polycystic ovarian syndrome
RCTs	Randomized control trials
VEGF	Vascular endothelial growth factor

Introduction

Ovarian hyperstimulation syndrome (OHSS) is the most serious iatrogenic complication associated with ovulation induction or multifollicular development for in vitro fertilization (IVF) (Abramov *et al.*, 1999b). OHSS is triggered by the exogenous administration of human chorionic gonadotropin (hCG), used for induction of final oocyte maturation and/or by its endogenous production in cases of pregnancy. Depending on the timing of onset, OHSS is characterized as either early, occurring within 10 days from oocyte retrieval (Mathur and Jenkins, 2000), or late, occurring after that initial 10 day period, usually in patients who become pregnant (Fiedler and Ezcurra, 2012). Most OHSS cases occur during ovarian stimulation for IVF. However, OHSS is also possible in patients undergoing ovulation induction or enhancement of ovulation.

Incidence of OHSS

Europe Versus United States

Based on data provided by the European IVF-Monitoring Consortium, the incidence of OHSS (for which, however, no definition is given) in Europe is estimated to be 0.78% (95% CI: 0.74–0.82) of all stimulated IVF cycles (Kupka *et al.*, 2016). On the other hand, the incidence of severe OHSS in the United States is reported to be 0.28% (95% CI: 0.25–0.31) (Kawwass *et al.*, 2015).

Ovulation Induction and OHSS

In anovulatory women, such as those with polycystic ovarian syndrome (PCOS), several approaches exist in order to induce monofollicular growth (Birch Petersen *et al.*, 2016). The most common ones are clomiphene citrate (CC) and aromatase inhibitors, while less frequently low-dose gonadotropins have also been used (Kar, 2012; Melo *et al.*, 2015). The incidence of OHSS with the use of both CC and aromatase inhibitors is very rare (Franik *et al.*, 2014). However, the incidence of OHSS in PCOS patients with the use of low-dose recombinant follicle stimulating hormone (FSH) is reported to be 2% (95% CI: 1–3) (Weiss *et al.*, 2015).

Enhancement of Ovulation and OHSS

CC, aromatase inhibitors, as well as low-dose gonadotropins have also been used for enhancement of ovulation in women with unexplained infertility. However, since in these patients the target of ovarian stimulation is to allow the development of two to three follicles in order to increase the probability of conception, CC and aromatase inhibitors are less frequently used. No cases of OHSS after CC administration for unexplained fertility were reported in meta-analyses of relevant randomized control trials (RCTs) comparing CC with aromatase inhibitors (Liu *et al.*, 2014). On the other hand, based on data reported in a meta-analysis of RCTs where low dose gonadotropins were used for the treatment of women with unexplained infertility, male infertility or mild endometriosis, the incidence of OHSS (for which, however, no definition was given) was 2% (95% CI: 0–3) (Cantineau *et al.*, 2011).

Ovarian Stimulation for IVF and OHSS

Based on published meta-analyses of RCTs comparing gonadotropin-releasing hormone (GnRH) agonists with antagonists (Al-Inany *et al.*, 2011b) and recombinant daily FSH versus corifollitropin alfa (Tarlatzis *et al.*, 2012), the incidence of OHSS varies from 1.3% to 20.3% according to the characteristics of the patients treated and the type of IVF protocol used (**Table 1**).

Table 1 The incidence of OHSS according to the characteristics of the patients treated and the type of IVF protocol used

Protocol	*Population*	*Source*	*Incidence %*	*95% CI*
GnRH agonists	All women	Al-Inany *et al.* (2011b)	9	7.7–10.5
	PCOS		20.3	16–25.5
GnRH antagonists	All women	Al-Inany *et al.* (2011b)	3.2	2.6–4
	PCOS		4.8	2.9–7.9
Corifollitropin alfa	Normal responders	Tarlatzis *et al.* (2012)	1.8	1.1–2.8
rFSH			1.3	0.7–2.2

Pathophysiology of OHSS

The pathophysiology of OHSS has been related to increased vascular permeability, caused by the extensive production of several vasoactive substances secreted by the ovaries, such as vascular endothelial growth factor (VEGF), angiotensin II, insulin-like growth factor 1, interleukins, and tumor necrosis factor-alpha, following the luteinization of granulosa cells (Goldsman *et al.*, 1995; Tollan *et al.*, 1990; Ata and Tulandi, 2009).

The key factor for OHSS occurrence is hCG, which is administered to simulate endogenous luteinizing hormone (LH) surge for triggering final oocyte maturation. HCG is thought to increase serum VEGF levels, leading to fluid shift in the third space (Ata and Tulandi, 2009). Unlikely to LH, hCG is characterized by a longer half-life, stimulating the existing corpora lutea, enhancing ovarian angiogenesis, and triggering vascular permeability in ovarian vessels (Neulen *et al.*, 1995; Pellicer *et al.*, 1999).

Clinical Presentation of OHSS

The symptoms of OHSS are not specific and the clinical diagnosis has been classified into different grades based on its severity as mild, moderate, severe, or critical (Navot *et al.*, 1992). Clinical presentation of OHSS includes abdominal distension, due to accumulation of intraperitoneal fluid, nausea, vomiting, diarrhea, and abnormal laboratory values (Mathur and Jenkins, 2000; Golan and Weissman, 2009).

As OHSS severity increases, a further increase in vascular permeability and aggravation of ascites lead to hypovolemia, hemoconcentration, oliguria, thromboembolism, and electrolyte disturbance. At that point, enlarged ovaries with multiple large follicles can be detected by ultrasound. In critical cases, patients may present with tense ascites, pericardial effusion, hydrothorax, severe dyspnea, and even adult respiratory distress syndrome (ARDS) (Abramov *et al.*, 1999b; **Fig. 1**).

Management of OHSS

The management of OHSS depends on its severity and the presence or absence of pregnancy. Mild and moderate OHSS do not require specific treatment and patients can be treated symptomatically on an outpatient basis (Practice Committee of the American Society for Reproductive Medicine, 2016). On the other hand, severe OHSS usually requires patient hospitalization since it might lead to serious complications.

Outpatient Management of OHSS

Women undergoing outpatient management of OHSS should be appropriately counseled and provided with information regarding fluid intake and output monitoring. Nonsteroidal antiinflammatory agents should be avoided, as they may compromise renal function (Royal College of Obstetricians and Gynaecologists, 2016). In women with severe OHSS ultrasound-guided paracentesis has been suggested as a safe alternative to hospitalization for the management of OHSS in an outpatient setting (Practice Committee of the American Society for Reproductive Medicine, 2016; Royal College of Obstetricians and Gynaecologists, 2016). In this case thromboprophylaxis is required.

Hospitalization for OHSS

In patients hospitalized with severe OHSS, assessment of severity usually involves physical examination, recording of vital signs, body weight, abdominal circumference, ultrasound examination, and laboratory tests such as hemoglobin, hematocrit, serum creatinine, electrolytes, liver enzymes, and coagulation (Mathur and Jenkins, 2009). Since underlying symptomatic moderate or severe OHSS is a hypovolemic state, treatment involves fluid administration, supportive care, paracentesis, and prophylactic anticoagulation (Royal College of Obstetricians and Gynaecologists, 2016).

There are no trials on the optimum method for maintaining fluid balance in women with severe OHSS (Mathur and Jenkins, 2009). Women with hemoconcentration and dehydration may need more intensive initial rehydration and may benefit from

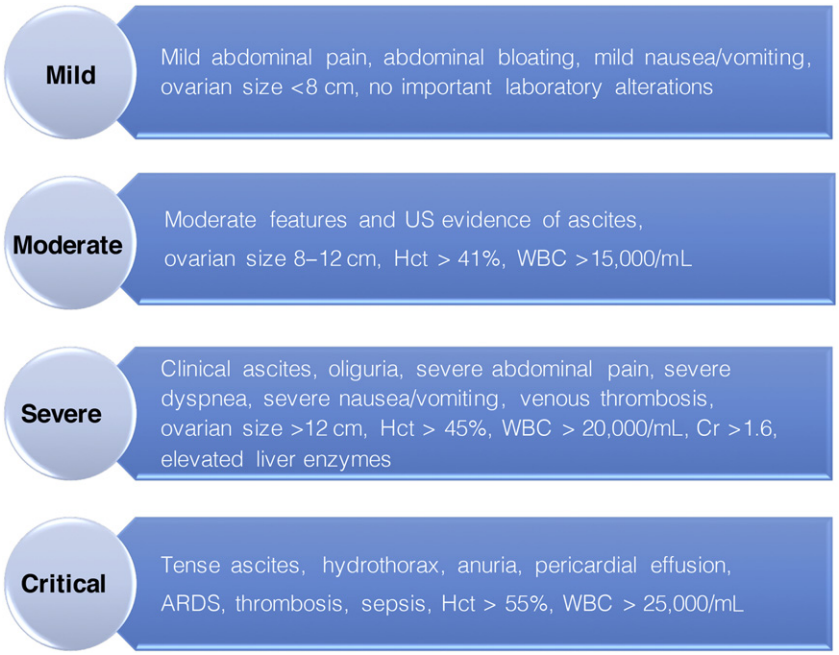

Fig. 1 Classification of OHSS into different grades based on its severity (Abramov et al., 1999b; Mathur and Jenkins, 2000; Golan and Weissman, 2009).

colloids such as human albumin and hexaethyl starch (HES) (Practice Committee of the American Society for Reproductive Medicine, 2016). Paracentesis under ultrasound guidance should be carried out for patients with severe discomfort, abdominal pain, respiratory distress, or oliguria due to the large amount of ascites (Royal College of Obstetricians and Gynaecologists, 2016). Symptomatic hydrothorax persisting despite abdominal paracentesis may be drained directly (Mathur and Jenkins, 2009).

All patients hospitalized with OHSS should be provided with prophylactic anticoagulation to avoid potentially serious thromboembolism. The duration of thromboprophylaxis should be individualized according to patient risk factors and outcome of treatment (Royal College of Obstetricians and Gynaecologists, 2016; Practice Committee of the American Society for Reproductive Medicine, 2016). Since the risk of adnexal torsion or ovarian rupture appears to be increased in women with OHSS, particularly in the presence of pregnancy, surgery is indicated as a treatment option in these patients (Royal College of Obstetricians and Gynaecologists, 2016; Practice Committee of the American Society for Reproductive Medicine, 2016).

Complications of OHSS

OHSS may result in significant morbidity and mortality due to life-threatening complications including renal insufficiency, ARDS, thromboembolism, and hemorrhage from ovarian rupture (Braat *et al.*, 2010; Zosmer *et al.*, 1987; Abramov *et al.*, 1999a; Al Omari *et al.*, 2011).

Mortality rates due to OHSS are difficult to be estimated due to inadequate reporting. Thus, currently reported mortality rates are likely to represent an underestimation of the real problem. Three deaths from OHSS have been reported out of 100,000 IVF cycles performed in the Netherlands during the period 1984–2008 (Braat *et al.*, 2010). Moreover, four deaths, three of which maternal, were reported in 100,000 women following IVF, according to the Confidential Enquiry into Maternal and Child Health in the period 2003–2005 in the United Kingdom (Lewis, 2007), while no maternal deaths due to OHSS in more than 200,000 IVF/ICSI cycles were reported later in the 2011 triennial report of the Confidential Enquiries into Maternal Deaths in the period 2006–2008 (Cantwell *et al.*, 2011).

Prevention of OHSS

Identification of Risk Factors

In recent years, the introduction of GnRH antagonists led to a substantial decrease in the incidence of severe OHSS as compared to GnRH agonists. Although the incidence of severe OHSS is significantly decreased in GnRH antagonist as compared to GnRH agonist cycles based on the latest meta-analysis by the Cochrane group (Al-Inany *et al.*, 2011a), severe OHSS is still possible even with the use of GnRH antagonists, especially in patients at high risk, such as those with PCOS.

Prevention of OHSS can be attempted at different time points during patients' evaluation and treatment but preferably it should be performed before initiation of ovarian stimulation. This is feasible by identifying known risk factors for its occurrence and by classifying patients accordingly. For this purpose, important variables to consider include history of OHSS, assessment of antral follicles count and of

anti-Mullerian hormone. Following assessment of OHSS risk, selection of appropriate stimulation protocols, dose individualization, and tight monitoring of ovarian stimulation are essential. Alarming signals for the occurrence of OHSS during ovarian stimulation are a rapid rise of estradiol (E2) levels, very high E2 levels, or an excessive number of developing follicles. In an attempt to predict high-risk patients for developing severe OHSS, a cut-off value for ≥ 19 follicles of ≥ 11 mm on the day of triggering final oocyte maturation has been proposed, yielding a sensitivity of 74.3% and a specificity of 75.3% (Griesinger *et al.*, 2016). In a more recent retrospective cohort study performed in consecutive IVF/ICSI cycles, a cut-off value for ≥ 15 follicles of ≥ 10 mm on the day of triggering final oocyte maturation appears to offer high sensitivity (89.5%) and specificity (82.9%) for the prediction of severe OHSS (Tarlatzi *et al.*, 2017).

Post hCG Strategies

Several strategies have been suggested to minimize the risk of severe OHSS after hCG administration, including co-administration of dopamine agonists, intravenous administration of albumin, HES, low-dose aspirin, ketoconazole, corticosteroids, metformin, "coasting," continuation of GnRH agonist administration after HCG combined with embryo cryopreservation, or reduction of the hCG dose used to trigger final oocyte maturation. The use of these strategies can only decrease the incidence of severe OHSS but not result in its elimination (Aboulghar and Mansour, 2003).

Replacement of hCG With GnRH Agonist

The use of GnRH antagonists for suppressing premature LH surge has made feasible the substitution of hCG for final oocyte maturation with GnRH agonists. This has changed dramatically clinical practice in assisted reproductive technologies, resulting in the virtual elimination of severe OHSS and in an increased safety of ovarian stimulation (Devroey and Adriaensen, 2011). The replacement of hCG with a GnRH agonist for triggering final oocyte maturation avoids the sustained hCG stimulation for 10 days of corpora lutea. With the use of GnRH agonist triggering, the endogenously elicited LH lasts only 24–36 h, decreasing in this way the occurrence of severe OHSS as compared to hCG. However, the absence of corpora lutea stimulation results in low steroids levels and is associated with a decreased probability of pregnancy after fresh embryo transfer (Tarlatzis *et al.*, 2017).

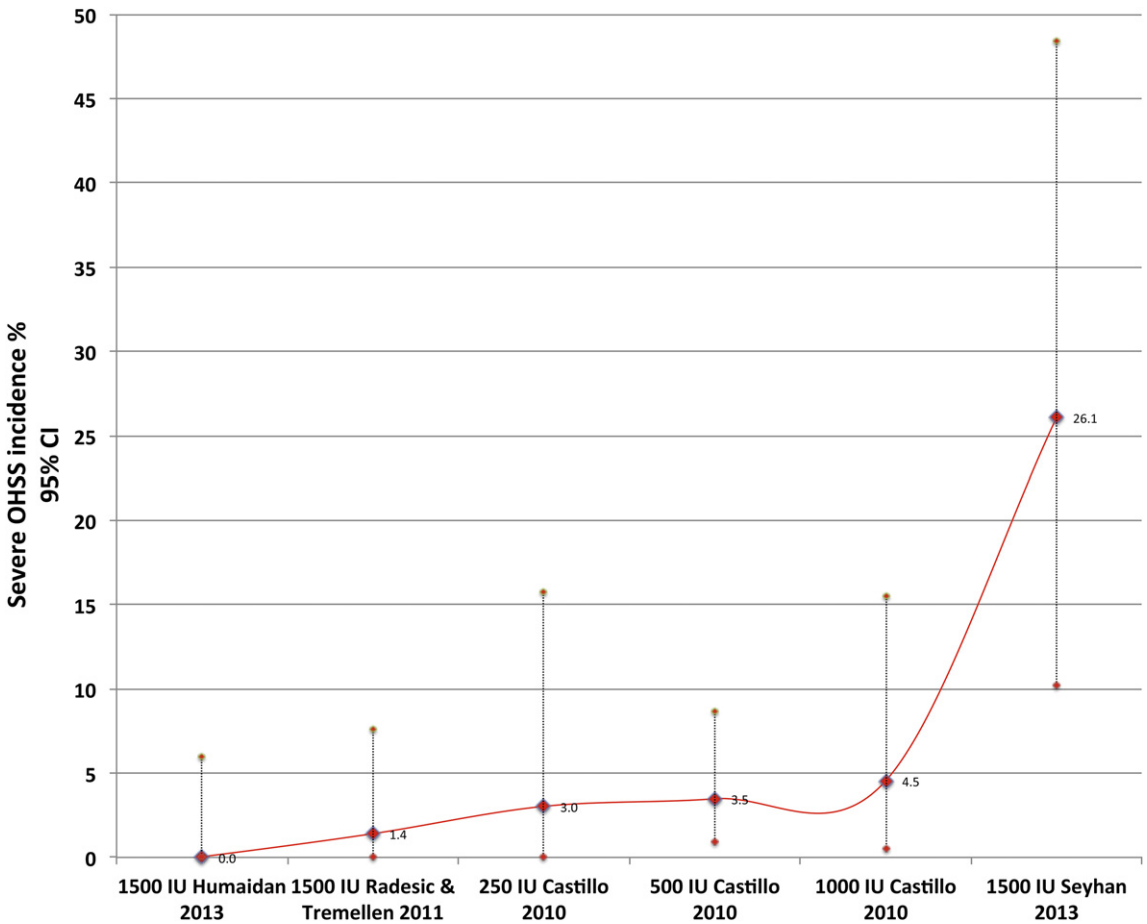

Fig. 2 Incidence of severe OHSS in studies evaluating hCG supplementation in the luteal phase after triggering with GnRH agonist.

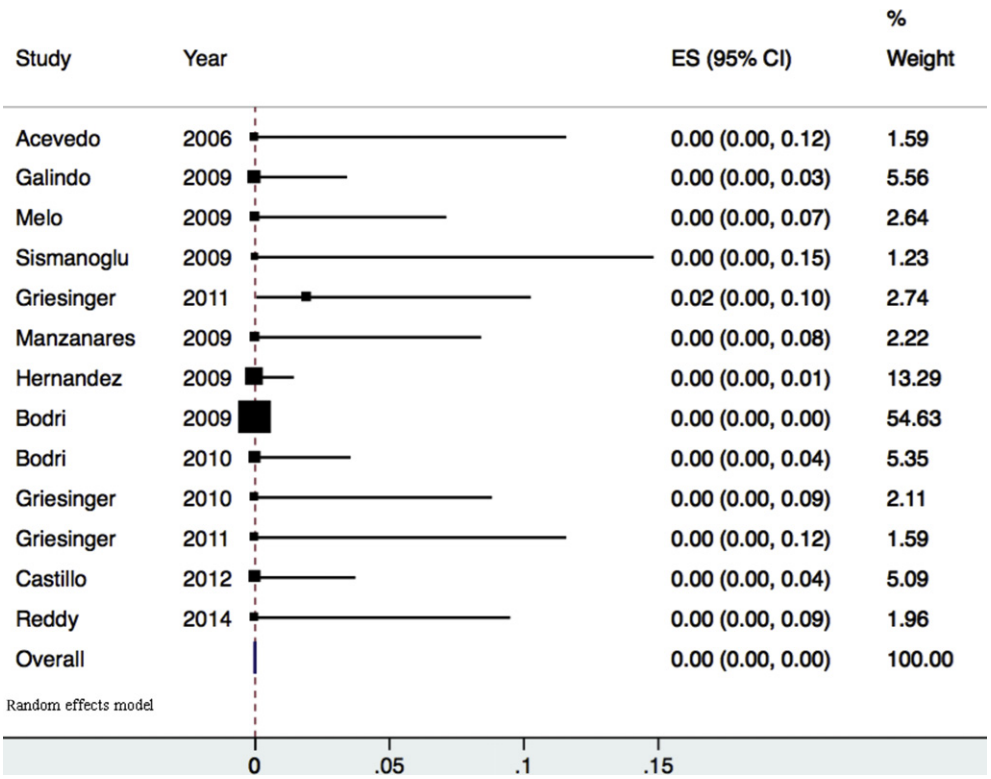

Fig. 3 Estimated probability of severe OHSS in patients triggered with GnRH agonist, by synthesis of the results from all available trials in which GnRH agonist was used for triggering final oocyte maturation and no luteal phase support was administered.

To manage the problem of the decreased probability of pregnancy after GnRH agonist triggering in ovarian stimulation for IVF, different approaches were proposed. These include luteal supplementation with recombinant LH after triggering with GnRH agonist (Papanikolaou *et al.*, 2011), the dual triggering of final oocyte maturation with the combination of GnRH agonist and hCG (Decleer *et al.*, 2014), the stimulation of endogenous LH levels with repeated GnRH agonist administration (Pirard *et al.*, 2006), the intensive luteal phase steroid supplementation after GnRH agonist triggering (Engmann *et al.*, 2008), or the administration of GnRH antagonist in the luteal phase and cryopreservation of all embryos (Lainas *et al.*, 2012; Lainas *et al.*, 2013). Although no severe OHSS cases were observed following these approaches, no firm conclusions regarding the incidence of severe OHSS can be drawn, due to the small number of both studies performed and of participants included (Tarlatzis *et al.*, 2017).

Currently several studies have been performed evaluating hCG supplementation in the luteal phase after triggering with GnRH agonist, in order to both decrease the incidence of severe OHSS and maintain the probability of pregnancy. However, this approach based on the revival of corpora lutea will inevitably lead to severe OHSS in a proportion of high-risk patients (Humaidan *et al.*, 2013; Seyhan *et al.*, 2013; Castillo *et al.*, 2010; Radesic and Tremellen, 2011; **Fig. 2**).

In order to eliminate OHSS, freezing of all embryos is the preferred approach. By synthesizing the results from all available studies until November 2015, prospective and retrospective (Bodri *et al.*, 2009, 2010; Castillo *et al.*, 2012; Hernandez *et al.*, 2009; Acevedo *et al.*, 2006; Galindo *et al.*, 2009; Melo *et al.*, 2009; Sismanoglu *et al.*, 2009; Griesinger *et al.*, 2011a; Griesinger *et al.*, 2011b; Reddy *et al.*, 2014; Manzanares *et al.*, 2010), in which GnRH agonist was used for triggering final oocyte maturation and no luteal phase support was administered (Tarlatzis *et al.*, 2017), the incidence of severe OHSS was 0% (95% CI: 0–0) (**Fig. 3**). In such a scenario, deferring embryo transfer to a subsequent cycle is mandatory and probably beneficial since subsequent embryo transfer will take place during a normal luteal phase. This is the basis of the so called "freeze all" approach after GnRH agonist triggering (Griesinger *et al.*, 2007).

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Fertility and Pregnancy in Patients With 21-Hydroxylase Deficiency

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Abbreviations

21OHD	21-Hydroxylase deficiency
CAH	Congenital adrenal hyperplasia
CVS	Chorionic villi sampling
DEX	Dexamethasone
FSH	Follicle stimulating hormone
GC	Glucocorticoid
HC	Hydrocortisone
LH	Luteinizing hormone
MC	Mineralocorticoid
NC-CAH	Nonclassic form of CAH
SV	Simple-virilizing form
SW	Salt-wasting form

Congenital adrenal hyperplasia (CAH MIM 201910) corresponds to a group of inherited autosomal recessive disorders that arise from defective steroidogenesis and results from a deficiency in one or several of the enzymes of cortisol biosynthesis. CAH due to 21-hydroxylase deficiency (21OHD) is the most common form of CAH, accounting for more than 95% of the cases and is one of the most common known autosomal recessive disorders (Auchus, 2015). The impaired glucocorticoid feedback inhibition via the classic negative feedback loop leads to increased secretion of adrenocorticotropic hormone (ACTH) and to subsequent adrenal hyperplasia and increased production of adrenal androgens and steroid precursors prior to the enzymatic defect (**Fig. 1**). 21OHD is the result of deletions or deleterious mutations in the active gene *CYP21A2* (Auchus, 2015). There are many mutations of the *CYP21A2* gene identified so far, which cause of varying degrees of impairment of 21-hydroxylase activity, and most patients are compound heterozygotes. The clinical phenotype, related to the less severe mutated allele, is classified as classic for the severe form, or nonclassic for the mild or late-onset form (Auchus, 2015). Classic CAH encompasses salt-wasting (SW) or simple-virilizing (SV) forms, depending on the degree of aldosterone deficiency. Increased production of adrenal-derived androgens and progesterone in CAH women interfere with their reproductive function and their fertility in many different ways, depending of the severity of the disease (**Fig. 2**).

Fertility and Pregnancy in Women With Classic Form of CAH

Classic 21OHD CAH is characterized by severe mutations in the *CYP21A2* gene, leading to cortisol deficiency, androgen and progesterone overproduction and virilization of the female external genitalia due to prenatal androgen excess (**Fig. 2**). Sexuality and fertility in women with classic CAH has been described form many years as impaired, especially for the patients with the SW form (Bachelot *et al.*, 2017). This results of several issues such as biological (poor hormonal balance), mechanical (related to surgeries), psychological factors.

Fertility in Women With Classic Form of CAH

One of the main factor implicated in the reduce fertility of the CAH women is the consequence of the childhood genital surgery (Wang and Poppas, 2017). In CAH, 46XX female fetuses are virilized in utero due to their increase exposure to androgens. Genital phenotype of 46,XX CAH patients at birth includes an increased development of the genital tubercle along with an increased length of the urethra, the opening of which is usually located on the ventrum of the genital tubercle. The vaginal cavity opens into the posterior wall of the urethra at a variable distance from the bladder neck. Aims of surgery are to restore functional genital anatomy to allow future penetrative intercourse, to facilitate future reproduction. Surgery can include clitoroplasty, vaginoplasty and labioplasty (Wang and Poppas, 2017). There are few long-term follow-up studies evaluating the outcome of surgery in CAH women, and most of those studies have small sample sizes. Urinary incontinence, vaginal stenosis and inadequate introitus, poor cosmetics, anorgasmia and painful intercourse have been reported in adults and currently remain relevant issues (Wang and Poppas, 2017). It is well established that there is a relationship between sexual activity and vaginal function, thus genital surgery may result in sexual dissatisfaction. Surgical techniques for genital feminization in female CAH patients have nonetheless evolved significantly over time. There are nowadays new surgical procedures which, for instance, preserve innervation and clitoral sensation in order to conserve erotic sensitivity and orgasmic capacity secondary to the clitoroplasty and improved vaginoplasty techniques. Moreover, to date, the choice of the timing of the surgery (early or late surgery) remains therefore a matter of debate. Unfortunately, there are few data in the literature about the outcomes of this surgery in terms of sexual function and the outcomes of the current techniques will take time to emerge. In a cohort study of 138 CAH patients, Arlt et al. have shown that 92 women had undergone genital reconstruction, 43% of who had more than one surgery, and 23% during adulthood (Arlt *et al.*, 2010). Among these patients, 46% have stated being unhappy about their sexual life. A French cross sectional study showed that, despite expert

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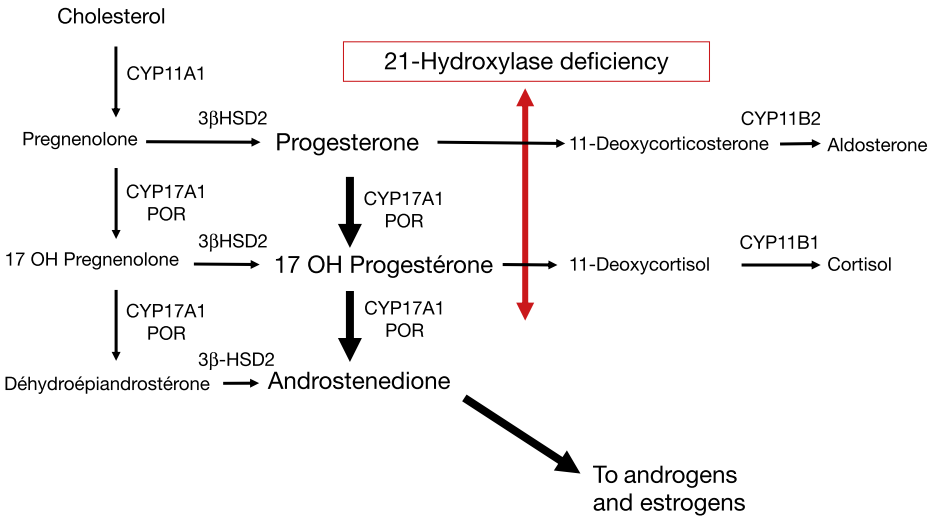

Fig. 1 Steroidogenesis in patients with CAH due to 21-hydroxylase deficiency.

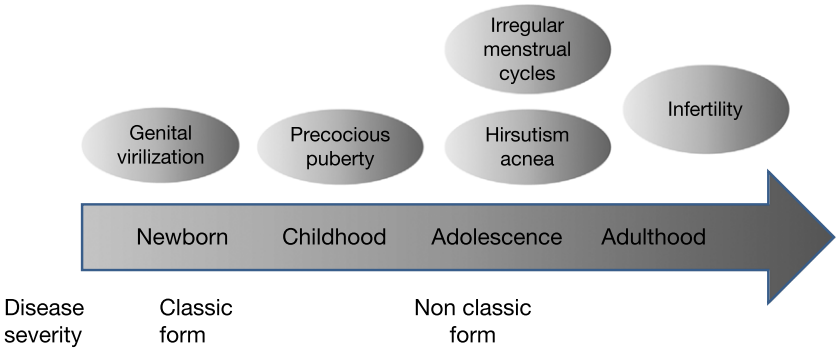

Fig. 2 Clinical aspects of classic and non-classic forms of 21-hydroxylase deficiency according to the age of the patients.

medical and surgical care by physicians dedicated to this rare disease, women with CAH still suffer major limitations in their sexual function and reproductive life. In this study, 37% of CAH women said they never had heterosexual intercourse with vaginal penetration, and for those who had, 81% experienced pain during vaginal penetration. Sexual functioning was much lower in CAH women than controls and lowest in CAH women with high Prader stages (Bachelot *et al.*, 2017). In a more recent review including 151 patients with genitoplasty, assessments of cosmetic results have shown that the majority of patients (between 60% and 94%) reported good or excellent outcomes (Wang and Poppas, 2017). When the physician was the person who assessed the cosmetic outcomes, 59%–94% reported satisfactory results (Wang and Poppas, 2017). Data on the youngest adult generation who may have benefited from improved insights in surgical methods are needed, especially with respect to adult sexual function and patient's satisfaction with the treatment. All these data also underscore the importance of psychological support in the treatment of children with CAH. Recent studies have shown that these women have normal sexual identification, and do not have gender identity confusion. Sexual preference has been studied, but the results are conflicting, with a large variability of heterosexuality and homosexuality rates probably due to the different methods of these studies (Bachelot *et al.*, 2017).

Another factor related to CAH female subfertility is related to the menstrual irregularities and anovulation, which are frequent in CAH women, affecting from 30% to 70% of women (Bachelot *et al.*, 2017). Menstrual cycle control represents therefore an important therapeutic target in these patients (**Fig. 3**). Several factors (androgen and progesterone overproduction, prenatal exposure to sexual steroid) are suspected to disturb the reproductive axis in CAH females (Bachelot *et al.*, 2017). Some authors have therefore suggested that high levels of fetal or perinatal androgens favor LH hypersecretion at puberty, leading to anovulation and polycystic ovaries (Bachelot *et al.*, 2012). However, the conclusions of these studies are almost all speculative, as there are not based on LH pulsatility study. More recently, LH pulsatility pattern has been described in women with classic CAH (Bachelot *et al.*, 2012), therefore highlighting the mechanism of anovulation in these women. It showed two different profiles in CAH women: one group of patients had LH pulsatility patterns similar to the controls; the other one had reduced LH pulses amplitude and frequency, and presented more frequently with menstrual cycle disturbances, higher 17OHP, testosterone, progesterone and androstenedione levels and lower FSH levels. LH pulsatility profiles, with reduced pulse frequency and amplitude, suggest progesterone action on the gonadotropic axis rather than androgen action. This was corroborated by the plot analysis of

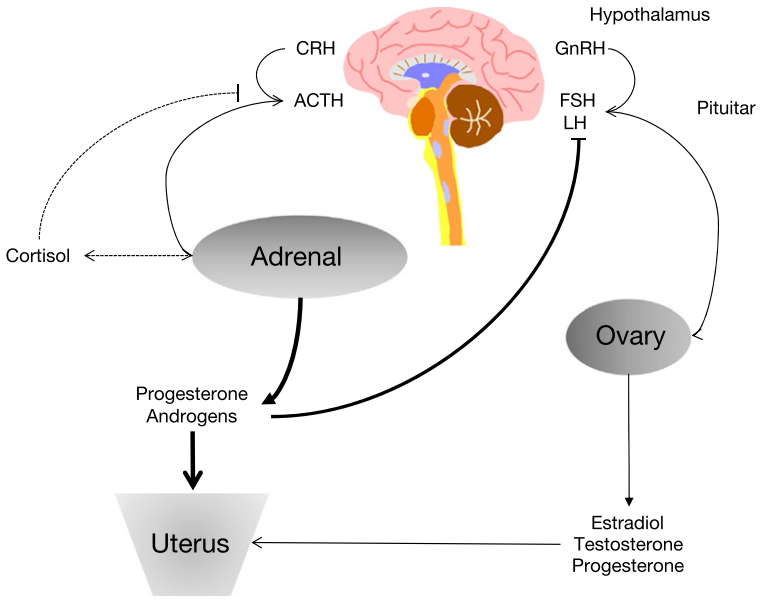

Fig. 3 Hormonal mechanism of female infertility in 21OHD. The impaired glucocorticoid feedback inhibition via the classic negative feedback loop leads to increased secretion of ACTH and to subsequent adrenal hyperplasia and increased production of adrenal androgens and steroid precursors prior to the enzymatic defect. Adrenal-derived androgens and progesterone interfere with gonadotropin pulse secretion. Adrenal progesterone prevent thickening of the endometrium, failure of endometrial breakdown and impermeability of the cervical mucus leading to infertility. *Dashed lines* indicate the absence of steroid production or feedback inhibition.

progesterone concentrations versus testosterone concentrations. None of CAH women exhibit polycystic ovarian syndrome like-LH pulse pattern, with elevated LH pulse levels and amplitude. This study thus demonstrated that hormonal control, especially progesterone overproduction control, is a key factor of gonadotrop function and therefore fertility in adult CAH women (Bachelot *et al.*, 2012). Optimized glucocorticoid (GC) and mineralocorticoid (MC) regimens during fertility monitoring should thus be thus an important concern in CAH women and in particular, suppression of serum progesterone concentrations during the follicular phase of the menstrual cycle should be a major objective in these patients, because it is probably responsible for reduced LH pulsatility and anovulation. Moreover, this elevated adrenal progesterone can prevent thickening of the endometrium in the follicular phase, failure of endometrial breakdown and impermeability of the cervical mucus leading to infertility (Bachelot *et al.*, 2017) (**Fig. 3**).

Analogous to testicular adrenal rest tumors, ovarian adrenal rest tumors have been described, but only in case reports (Bachelot *et al.*, 2017). It is very likely that ovarian adrenal rest tumors, if present, could impair ovarian function in CAH females by displacing normal ovarian tissue and by locally producing steroids, which interfere with normal ovarian function. Systematic study of ovarian adrenal rest tumors by pelvic ultrasonography and MRI was not able to detect them in any of the 13 CAH women studied, according to the diagnostic criteria derived from the imaging features of testicular adrenal rest tumors. This suggests that ovarian adrenal rest tumors in CAH females are rare, in contrast to the high prevalence of testicular adrenal rest tumors in CAH males.

Better fertility and fecundity in CAH women will be largely dependent on surgical advances in genital reconstruction, earlier treatment, optimized compliance to therapy, availability of psychological support, organization of transition from pediatric to adult specialist care, procuration of menstrual cycle control and sexual well-being. At last, considering that these women carrier two severe mutations and the high rate of heterozygotes for *CYP21A2* mutations in the general population, it is essential to genotype the partner of these women, to predict the risk of classical CAH in the offspring and offer genetic counseling.

Pregnancy in Women With Classic CAH

Fertility rate, that is, live births per woman, is significantly lower in CAH women than in the general female population (Bachelot *et al.*, 2017). The first large study has shown that among 25 women with SV form and adequate vaginal opening, fertility rate was 60%, meanwhile among the 15 women with SW form with adequate vaginal opening, fertility rate was only 7%. However, more recent data suggest that fertility rates have significantly improved, largely owing to earlier treatment of CAH, improvement of compliance with therapy and surgical advances in genital reconstruction leading to increased percentage of patients with sexual activity (Bachelot *et al.*, 2017). A recent study has presented for the first time the pregnancy rate as the proportion of women who were actively trying to conceive, and reported a near normal conception rate for women with classical congenital adrenal hyperplasia (Casteràs *et al.*, 2009). In this cohort of 103 CAH women among whom 25% wanted to conceive, the pregnancy estimate was 54% (Casteràs *et al.*, 2009). Pregnancies were most often spontaneous, obtained after a good hormonal control with

optimized GC and MC regimens. Indeed, to achieve pregnancy, they focused on combined suppression of serum progesterone concentrations in the follicular phase of the menstrual cycle and plasma renin activity. This was confirmed in a prospective cross-sectional study of adults with CAH attending specialized endocrine centers across the United Kingdom. In this cohort, 25% of classic CAH women had sought pregnancy. 28 of 47 CAH women reported 46 live births, including six conceived after fertility treatment. The success rate of CAH women seeking pregnancy was 54% (Arlt *et al.*, 2010). Recently, a large population-based epidemiological study on psychosocial outcomes in CAH patients was conducted in Sweden that showed that women with the SW form were less often married [OR 0.5 (0.2–1.1)] and had fewer partnerships compared with controls. CAH patients were less likely to have biological children than controls [OR 0.3 (0.2–0.3)] and when assessing women with the SW and SV forms, it was still significantly decreased [SW OR 0.05 (0.0–0.1); SV OR 0.4 (0.2–0.7)] (Bachelot *et al.*, 2017).

There are few empiric data concerning the management of GC doses in the pregnant CAH patient. There are recent guidelines from the Endocrine Society (Speiser *et al.*, 2010). Consideration may be given to increasing the dose of GC and/or fludrocortisone in advance of the development of clinical evidence of adrenal insufficiency in difficult to manage pregnant SW CAH patients. Symptoms of adrenal insufficiency, including postural hypotension, may rarely develop in pregnant women with classic CAH. GC and/or fludrocortisone doses should be increased if such signs and symptoms occur. During labor and delivery, stress doses of GCs should be given, but there are no controlled studies regarding optimal dosing. CAH women are at increased risk for gestational diabetes; thus, glucose tolerance should be monitored throughout pregnancy. Overall, the treatment of the pregnant CAH patient should be individualized.

Female Fertility in Women With Nonclassic Form of CAH

Contrasting with numerous data on classical CAH, fertility in NC-CAH has been rarely studied. Subfertility seems nevertheless to be relative in this population, based on the three main published cohort (Moran *et al.*, 2006; Bidet *et al.*, 2010; Eyal *et al.*, 2017).

Reproductive Axis of NC-CAH Women

In girls and women with nonclassic CAH, the excess of androgens is responsible for varying manifestations (**Fig. 2**). Hirsutism is the most common sign, ranging from 60% to 80% of the women (Bidet *et al.*, 2009; Livadas *et al.*, 2015). It usually appears in the pubertal period, while the need for cortisol increases. Other signs of hyperandrogenism such as acne and alopecia may occur. Clitoromegaly has been reported in 6%–20% of adult women with NC-CAH. Menstrual cycles irregularities has been reported in about 50% of the patients at diagnosis, are a reflection of these disturbances of the gonadotropic axis (Bidet *et al.*, 2009; Livadas *et al.*, 2015). These disorders of the cycle are variable, ranging from primary (8%) or secondary (4%) amenorrhea to oligomenorrhea (46%) in a large cohort of 190 patients (Bidet *et al.*, 2009). Women with NC-CAH may present with signs of hyperandrogenism suggestive of a diagnosis of polycystic ovary syndrome. It is therefore important for any polycystic ovary syndrome chart to eliminate non classic 21-hydroxylase deficiency, according to the Rotterdam criteria.

In the majority of the affected subjects with NC-CAH, ACTH production is normal (Bidet *et al.*, 2009). Cortisol response to ACTH may be normal or slightly impaired (Bidet *et al.*, 2009). However, adrenal androgens secretion is increased. These patients exhibit elevated adrenal androgens and testosterone levels, but within a wide range and similar to the levels found in PCOS patients (Bidet *et al.*, 2009; Livadas *et al.*, 2015). Other mechanisms may contribute to hyperandrogenism of NC-CAH patients including ovarian dysfunction, which may contribute to androgen excess in these women, as demonstrated by the improvement of clinical and biochemical hyperandrogenism after ovarian suppression. The overproduction of adrenal androgens appears to be the main disruptive factor of the gonadotropic axis. This may have a direct or indirect (via aromatization on estrogens) effect on ovary and gonadotrop axis function. This results in a loss of pulsatility of gonadotropin secretion, leading to anovulation or dysovulation. It has been also suggested that androgens may play a role in ovarian folliculogenesis via androgen receptors on granulosa cells. In addition, 21-hydroxylase deficiency can cause increased levels of progesterone. As it has been showed in women with classic 21-hydroxylase deficiency, increased adrenal progesterone production could disturb gonadotrop axis. The negative impact of elevated progesterone levels at the end of the follicular phase on embryo implantation rates has been shown in in vitro fertilization cycles. In addition, a "mini-pill" effect with inhibition of endometrial proliferation, a negative effect on cervical mucus and feedback on the gonadotropic axis may also occur. The inhibitory effect of adrenal androgens and/or progesterone overproduction on the pituitary-ovary axis is confirmed by the normalization of menstrual cycles subsequent to the suppression of adrenal androgen and progesterone oversecretion by glucocorticoid treatment in women with NC 21-hydroxylase deficiency. Indeed, Bidet et al. showed that menstrual cycle or ovulation disorders observed in NC-CAH women who consulted for infertility were in most cases corrected by hydrocortisone treatment, which led to simultaneous lowering of plasma testosterone and androstenedione levels and rapid occurrence of pregnancy (Bidet *et al.*, 2010).

Fertility in Women With NC-CAH Form

Subfertility is relative in NC CAH women. Indeed, only 10% and 30% of NC CAH women of reproductive age complain of infertility (Moran *et al.*, 2006; Bidet *et al.*, 2010). Anovulation was the main cause of subfertility in NC CAH women. In an

international retrospective study of 203 identified pregnancies, 138 (68%) occurred before CAH diagnosis and treatment (Moran *et al.*, 2006). Similarly, in a recent French study on a cohort of 190 women, only 11% of women consulted for infertility, including 2.6% for secondary infertility (Bidet *et al.*, 2010). This rate was comparable to rates reported in the general population. They described 187 pregnancies, 110 without and 77 with glucocorticoid treatment, occurred in 85 of the 95 women (90.5%) who wished pregnancy, leading to 141 births. These results are in agreement with previous reports (Moran *et al.*, 2006). The study of the cumulative incidence of pregnancies showed that 67% of women wishing to have conceived in the first 6 months and that 75.9% had conceived at 1 year. That is slightly less than in the general population (80 and 92%, respectively). However when considering the 85 patients who conceived, the cumulative incidences of all pregnancies (83.5%) and pregnancies resulting in birth (82%) at 1 year or less are similar to those in the general population (Bidet *et al.*, 2010). These reassuring data have been confirmed in a retrospective study included 75 women diagnosed with NC CAH who sought fertility, 72 women succeeded in conceiving (187 pregnancies) (Eyal *et al.*, 2017). The 96% pregnancy rate among this cohort of NC CAH females was similar to the 95% rate reported for the normal population. 17 pregnancies were achieved by glucocorticoid therapy after failure to conceive spontaneously.

Pregnancy in Women With NC-CAH Form

Apart to this relative subfertility, increase in the frequency of the occurrence of miscarriage has been described in NC CAH women, in the absence of glucocorticoid treatment (Moran *et al.*, 2006; Bidet *et al.*, 2010; Eyal *et al.*, 2017). In the French study, the rate of miscarriage in NC CAH women in the absence of glucocorticoid treatment was 19.4% when abortions are excluded. This rate is higher than the 10%–15% rate reported in the general population. Contrariwise, this rate was significantly lower in NC CAH women under glucocorticoid treatment (Bidet *et al.*, 2010). It seems therefore possible that this treatment is likely to improve the fertility of these patients and the prognosis of their pregnancies. Moran et al., (2006) observed also a markedly lower rate of miscarriages (25.4% vs. 6.2%) after NC CAH diagnosis among 107 patients and 206 pregnancies. However, in the study, some patients received glucocorticoid before NC CAH diagnosis and some others ovulation inducers after NC CAH diagnosis, which may dilute the specific glucocorticoid effect. The high percentage of miscarriages may therefore reflect the poor quality of ovulation and/or the existence of an inadequate corpus luteum with its hormonal consequences on the uterine environment. In a recent retrospective study, of 187 pregnancies, 38 (20.3%) ended in spontaneous miscarriages during the first trimester, but there was no significant difference in the rate of miscarriages between glucocorticoid-treated and untreated pregnancies, but the majority of pregnancies (68%) involved women who had been treated before conceiving (Eyal *et al.*, 2017). That difference might be due to differences in the sample sizes of this study and the others. Therefore, glucocorticoid drug therapy should be considered in hyperandrogenic women with NC CAH who have failed to conceived spontaneously and demonstrate overt or subclinical ovulatory dysfunction, as it has been demonstrated that it prevents infertility and recurrent miscarriages, by lowering adrenal androgen and progesterone secretion and normalizing menstrual cycles (Speiser *et al.*, 2010). Dexamethasone should be avoided during pregnancy, and glucocorticoids such as hydrocortisone and prednisone, which are easily metabolized by placental 11 beta-hydroxysteroid dehydrogenase type 2, must be use (Speiser *et al.*, 2010). Cortisol-binding globulin concentrations increase in pregnancy, and as hydrocortisone administered exogenously is bound by this globulin, hypothetically the dose of hydrocortisone should be increased in pregnant patients with NC-CAH. However, in actual practice there are no biochemical markers and few clinical markers that should be followed in pregnancy, and doses are rarely adjusted. Prospective controlled trials will be needed to determine the true benefit and the mechanism of action of glucocorticoid therapy on pregnancy outcome in NC CAH.

All studies suggest that using a physiological dose of glucocorticoids does not affect birth weight. There were also no differences in the rate of ectopic pregnancy, preterm birth, stillbirths, twins or multiple pregnancies (Moran *et al.*, 2006; Bidet *et al.*, 2010). The prevalence of NC CAH among children of NC CAH mothers (19.2%) was much higher than the predicted rate of 3%–5%. This finding is similar to that of Moran et al. (2006).

At last, considering that NC CAH women might carrier a severe mutation, with a frequency ranging from 9% to 60%, depending of the population, and the high rate of heterozygotes for *CYP21A2* mutations in the general population, it is essential to genotype the partner of nonclassic CAH patients carrier a severe mutation, to predict the risk of classical CAH in the offspring and offer genetic counseling (Moran *et al.*, 2006; Bidet *et al.*, 2010).

Prenatal Management of CAH

The aim of prenatal treatment is to avoid the need for surgery in the little girl and to relieve the emotional distress and anxiety of the parents that may be caused by an external genitalia anomaly in their child. Prenatal treatment was first introduced in the early 80's, using dexamethasone (DEX) which is a synthetic GC with a long half-life, not deactivated by the placental 11β-hydroxysteroiddehydrogenase type 2 and that crosses the placenta and becomes bioavailable to the fetus (review in Bachelot *et al.*, 2017). In the CAH fetus, DEX leads to ACTH suppression and reduction of androgen excess, which blocks the virilization of the external genitalia in female fetuses. The dose of DEX used is 20 μg/kg maternal body weight (preconception)/day, divided in two or three daily doses, without exceeding 1.5 mg/day. This dose corresponds to about six times the physiologic GC needs of the mother (Bachelot *et al.*, 2017). Studies with lower doses have not been performed, but some data show that the DEX dose could

probably be reduced when poorly tolerated in the mother (Tardy-Guidollet *et al.*, 2014). DEX must be initiated before the presumed date of genital sensitivity to androgens, at the latest at the 7th week of gestation (WG) or 9th week of amenorrhea and continued until birth in CAH females to ensure its efficacy (New *et al.*, 2001; Tardy-Guidollet *et al.*, 2014). The timing of DEX initiation seems to play an essential role in the genital morphology of CAH girls (New *et al.*, 2001; Tardy-Guidollet *et al.*, 2014).

Early DEX initiation before 7 WG, ideally at the latest at 6 WG, and its maintenance during the whole gestation have resulted in normal feminine genitalia in CAH girls in 80%–85% cases, failure being usually observed when treatment was started after 8 WG (New *et al.*, 2001; Tardy-Guidollet *et al.*, 2014). A recent small study from French surgeons has suggested that prenatal DEX therapy could potentially be limited to the period of the partitioning window, during the time of urogenital cleavage, which would both reduce total fetal exposure to DEX, yet still facilitate easier surgical correction. Enlargement of the genital tubercle continues to occur in late pregnancy without ongoing antenatal treatment, but it is generally responsive to postnatal treatment (Bachelot *et al.*, 2017.

Prenatal DEX exposure has decreased over the years. Circulating cell-free DNA in maternal serum allows fetal sex determination by detecting the Y chromosome (SRY test). It has recently been shown that the sensitivity of the SRY test was guaranteed just after 4 WG in 96% cases (Tardy-Guidollet, 2015). Moreover, trophoblast retrieval and isolation from the cervix was recently proven to be an approach that noninvasively and correctly identifies male fetal DNA in fetuses at risk for CAH as early as 5 WG. In addition, the development of chorionic villi sampling (CVS) performed earlier than amniocentesis and with a shorter response delay, has allowed minimizing DEX exposure of non-CAH females. Very interestingly, the first demonstration of non-invasive prenatal diagnosis of CAH using cell-free fetal DNA in maternal plasma, as early as 6 WG, has recently been published (Bachelot *et al.*, 2017). Even though large-scale prospective studies are needed, this technique offers the possibility to only treat the affected female fetus.

Prenatal DEX however continues to be a subject of debate. Rare adverse events have been reported in treated children, but no harmful effects have been documented that can be clearly attributed to this treatment (New *et al.*, 2001; Bachelot *et al.*, 2017). A large study on 600 CAH-affected pregnancies where infants were treated prenatally with DEX, reported no significant difference in head circumference, birth weight or length, compared to untreated affected siblings (Bachelot *et al.*, 2017). A recent large French retrospective study confirmed the absence of malformations and of growth restriction at birth (Tardy-Guidollet, 2015). Maternal side effects of prenatal DEX include weight gain, edema, mood change, sleep disturbance, acne and striae. But there has not been a confirmed association with major pregnancy complications such as hypertension, gestational diabetes, stillbirth or spontaneous abortions.

Concerns have been raised in regards to the GC effects on the fetal brain, which arise from studies of other conditions rather than direct studies on prenatal treatment of CAH. A small study from M. New's group described children prenatally exposed to DEX compared to untreated children from CAH at-risk pregnancies, and showed no significant differences in cognitive abilities but demonstrated an increase in internalizing behaviors, such as being more shy, more emotional and less sociable (Mercè Fernández-Balsells *et al.*, 2010). Another large study from the same group was unable to find any adverse effects of prenatal DEX on motor and cognitive outcome. Recently, the same American group published a large study evaluating the long-term effects of prenatal DEX in affected and unaffected CAH patients, and found no adverse effects such as increased risk for cognitive defects, disorders of gender identity and behavior or sexual function in adulthood (Mercè Fernández-Balsells *et al.*, 2010). Conversely, in a small-sample Swedish study of 26 children prenatally treated with DEX compared to 35 matched controls, the authors from S. Lajic's group found no effects on intelligence, lateralization, memory encoding, or long-term memory, but short-term treated CAH-unaffected children had significantly poorer performance than controls on a test of verbal working memory (Mercè Fernández-Balsells *et al.*, 2010). These patients also had lower questionnaire scores in self-perceived scholastic competence and social anxiety. However, parents described these children as more sociable than controls, without significant difference in psychopathology, school performance, adaptive functioning or behavioral problems. A large neuropsychological American study also found that subjects treated with short-term prenatal DEX actually performed better than controls in most areas of mental processing and memory performance; however, girls treated with DEX in the long-term had slower mental processing (Bachelot *et al.*, 2017).

Despite these inconsistencies, a meta-analysis has found no significant difference in neuropsychological outcomes of children treated prenatally with DEX, although only four eligible observational studies were identified, which in addition had low methodological quality (Mercè Fernández-Balsells *et al.*, 2010). Since the early 2000, several medical societies have issued opinions concerning prenatal treatment of CAH based on data presented above and agreed that it is experimental and should only be done in Institutional Review Boards-approved prospective research protocols, with written informed consent, and that this treatment is inappropriate for use in community practice (Speiser *et al.*, 2010). The Swedish group has stopped recruiting patients due to concerns regarding abnormal behavioral development in children exposed to prenatal DEX and notified the Regional Ethics Committee in Stockholm.

It is certain that DEX safety in children treated in utero remains controversial and needs to be better assessed. However, the review of the literature shows an overall efficacy of prenatal DEX. The actual perspective of only treating affected girls will be a major improvement in the care of these at risk pregnancies.

Conclusion

Better fertility and fecundity in classic CAH women will be largely dependent on surgical advances in genital reconstruction, earlier treatment, optimized compliance to therapy, availability of psychological support, organization of transition from pediatric to

adult specialist care, procuration of menstrual cycle control and sexual well-being. Contrasting with numerous data on classical CAH, fertility in NC-CAH has been rarely studied but subfertility seems nevertheless to be relative in this population. Glucocorticoid drug therapy should be considered in hyperandrogenic women with NC CAH who have failed to conceived spontaneously and demonstrate overt or subclinical ovulatory dysfunction, as it has been demonstrated that it prevents infertility and recurrent miscarriages. At last, considering that these women carrier one or two severe mutations and the high rate of heterozygotes for *CYP21A2* mutations in the general population, it is essential to genotype the partner of these women, to predict the risk of classical CAH in the offspring and offer genetic counseling. Recent years have brought new insights in the description of CAH reproductive function and fertility. The use of DEX during pregnancy remains another matter of debate. Its use to prevent or diminish the risk of virilization of the young girl has to be discussed taking account the potential long term use of such molecules on brain function or metabolism.

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In Vitro Fertilization (IVF)☆

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Glossary

Agonist Drug that has affinity for and stimulates physiological activity at cell receptors normally stimulated by naturally occurring substances.

Antagonist Substance that tends to nullify the action of another as a drug that binds to a cell receptor without eliciting a biological response.

Cryopreservation Maintenance of the viability of embryos or spermatozoa at extremely low temperatures.

Diagnostic ultrasound Use of ultrasound to obtain images for medical diagnostic purposes, employing frequencies ranging from 1.6 to approximately 10.0 MHz.

Donor gamete/embryo Eggs, spermatozoa, or embryos contributed from one person (or couple) to assist in establishing pregnancy in another couple.

Endometriosis Condition in which cells similar to those that form the inside of the uterus are found outside of the uterus; may cause internal bleeding, tissue inflammation, and subsequent scarring, pain, and infertility.

Hysterosalpingography Insertion of a small tube into the cervix followed by injection of dye; the resulting X-ray is inspected for evidence of blockage or irregularity of the uterus or fallopian tubes.

Laparoscopy Inserted through a small incision made just under the navel; looking through the scope, one can examine the ovaries, fallopian tubes, uterus, and other internal structures.

Meiosis Consists of two cell divisions that result in four cells, each of which contains half of the number of chromosomes found in somatic cells.

Ovarian hyperstimulation syndrome (OHSS) Side effect that can occur during infertility treatment with ovulation-inducing drugs; symptoms include ovarian enlargement, accumulation of fluid in the abdomen, and gastrointestinal disorders (e.g., nausea, vomiting, diarrhea).

Surrogate pregnancy Purposely established pregnancy in which the woman who bears the pregnancy does not intend to raise the child.

Unexplained infertility Diagnosis of exclusion when the standard investigation of both the female and male partners has ruled out other infertility diagnoses.

Introduction

In vitro fertilization (IVF) is an assisted reproductive technology (ART) used to treat infertility, to prevent the transmission of some genetic diseases, or to preserve and re-establish fertility potential after fertility-damaging treatments of diseases. IVF involves a number of complementary steps. In brief, oocyte retrieval (usually after controlled ovarian stimulation) and sperm collection, both required for the fertilization of oocytes outside the human body, are followed by the in vitro culture of embryos obtained under proper conditions. The whole process culminates into the transfer of selected embryos in the uterus to start a pregnancy.

History of IVF

During the late 19th century scientists began study of the physiology of mammalian fertilization (**Table 1**). Because there was limited technology for observing events at a cellular level, study of mammalian fertilization did not progress further until after 1930 when Pincus began to study rabbit eggs and sperm under the binocular microscope. In 1944, Rock and Menkin reported in vitro fertilization of a human egg but did not intend to use the resulting embryo in an attempt to start a pregnancy. In 1967, Edwards and Steptoe began work on a technique for harvesting and fertilizing human eggs in vitro with the intention of treating infertile couples. Edwards reasoned that he could "simply pluck the egg from the ovary and fertilize it in the laboratory." In 1969, Edwards, Bavister, and Steptoe reported that they had achieved human fertilization in vitro. The first IVF human pregnancies were achieved in 1975, although they were not successful in reaching term. Then, on July 25, 1978, Louise Joy Brown was born by caesarian section at Oldham General Hospital in England. Her birth was the first to demonstrate that conception in the laboratory could result in the birth of a normal baby. The first IVF baby in the United States was born in 1980. Shortly thereafter, in response to patient demand, numerous clinics offering ART procedures were established all over the world, ushering in a new epoch in helping infertile couples to achieve pregnancy.

☆*Change History*: April 2018. Renato Fanchin updated the text.

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Table 1 History of in vitro fertilization

Year	*Investigator(s)*	*Event*
1870–80	S. Schenk	Observed rabbit sperm stripping away cumulus cells from egg under the microscope
1890	Walter Heap	Successful transfer of fertilized rabbit oocytes flushed from donor rabbit to foster mother rabbit
1930	Gregory Pincus	Observed rabbit sperm penetrating the egg with binocular microscope
1934	Gregory Pincus and E. V. Enzmann	Mixed rabbit sperm and eggs and returned them to the fallopian tube with birth of young; fertilization not observed
1944	John Rock and Miriam Menkin	Harvested human eggs just before ovulation, combined them with human sperm, and observed the results
1954	Charles Thibault	Reported in vitro fertilization of rabbit eggs; first report of mammalian fertilization in vitro
1959	M. C. Chang	Fertilized rabbit eggs in vitro and transferred four-cell embryos with birth of healthy young
1969	Robert Edwards, Barry Bavister, and Patrick Steptoe	Human in vitro fertilization with embryo division
1971	Robert Edwards and Patrick Steptoe	First transfer of in vitro fertilization embryo with intent of establishing a pregnancy
1978	Robert Edwards and Patrick Steptoe	Birth of Louise Brown
1992	Gianpiero Palermo	Development of intracytoplasmic sperm injection

IVF Procedures

At first, most women treated with IVF procedures had damaged fallopian tubes. As confidence with the new technique increased, clinicians began to use IVF to treat other causes of infertility. Today, ART procedures are used to help women with damaged fallopian tubes, endometriosis, couples who are infertile because of low sperm count or poor sperm function, unexplained infertility, and some genetic disorders.

Ovulation Induction

Edwards and Steptoe obtained a single egg, which would become Louise Brown, with a retrieval timed just before her mother's natural ovulation. Today, most women are treated with follicle-stimulating hormone (FSH) to promote the maturation of multiple eggs before their retrieval. This treatment is known as "ovulation induction" or "controlled ovarian hyperstimulation." FSH treatment for ovulation induction is administered by daily injection for several days, beginning on the third day of menstrual bleeding. Many women are treated with gonadotropin-releasing hormone (GnRH) agonists, GnRH antagonists, or oral contraceptives to suppress their hypothalamic–pituitary–ovarian axis and, consequently, their reproductive cycle. Women undergo monitoring of their serum estradiol concentration and sonogram imaging to determine the growth of their follicles. When the largest follicle has a diameter of greater than 18 mm, the egg is approaching maturity. Final egg maturation is triggered by injection of human chorionic gonadotropin (hCG) or GnRH agonist administration to induce a luteinizing hormone (LH) peak. The clinician retrieves the eggs 34–37 h after this final injection.

Egg Retrieval

Until the late 1980s, laparoscopy was used as the primary method of egg retrieval. To achieve pregnancy, some women would undergo multiple laparoscopies in a single year. Laparoscopic egg retrieval required about 45 min of general anesthesia and was more difficult when pelvic scars covered the ovaries. Today, physicians use ultrasound imaging to guide a needle through the vaginal wall to the ovary. Introduction of ultrasound-guided egg retrievals during the mid-1980s eliminated the need for laparoscopy and a full operating room to complete egg retrievals, allowing ART services to be provided in outpatient settings. Using ultrasound, each follicle is imaged on the display. The retrieval needle is passed through the needle guide, which is parallel to the vaginal ultrasound probe. The needle passes through the vagina and into the peritoneal cavity. The follicles are punctured, and their fluid is suctioned through the needle to a sterile container. The follicular fluid is brought to the embryology laboratory, where the eggs are identified under low-power magnification. The eggs are removed from the fluid, carefully examined by the embryologist, and evaluated for maturation. An egg must have completed its first meiotic division to be successfully fertilized. After the eggs are inspected, the embryologist places each egg in a small bubble of media along with a suspension of washed sperm.

Embryology Laboratories

Embryology laboratories have evolved greatly over the years. Most successful embryology laboratories have protocols to ensure quality control and to maintain a sterile, toxin-free environment. Techniques have been developed in the embryology laboratories to assist in fertilization, embryo growth, and embryo selection and safe transfer.

Fertilization

Expansion of assisted reproductive indications to help couples with poor sperm production or function led to methods of gamete micromanipulation to improve the chance of fertilization in the laboratory. A clear shell called the zona pellucida surrounds human eggs. Zona drilling and subzonal insertion were techniques used to help spermatozoa pass the zona pellucida barrier and bind to the oocyte cell membrane. Working in Belgium in 1992, Palermo achieved fertilization of a human egg by injecting a sperm cell through the egg cell membrane into the cytoplasm, a procedure that became known as intracytoplasmic sperm injection (ICSI). Until then, many embryologists believed that intracytoplasmic injection of sperm would disrupt the basic mechanisms of fertilization. Palermo showed that an egg could be successfully fertilized by ICSI and that a healthy embryo and successful pregnancy could result.

Incorporation of ICSI techniques into the IVF procedures has allowed successful treatment of couples who in the past could only have been offered adoption or artificial insemination. A few living sperm cells retrieved from testicular biopsy or epididymal aspiration can be used for intracytoplasmic injection of oocytes. Use of laboratory techniques to overcome such natural barriers to fertility provoked concern that children resulting from these procedures might be adversely affected. Studies have shown that men with deletions or mutations of genes needed to produce spermatozoa are likely to pass on those traits when they have children by ART procedures. Some genetic traits associated with male infertility are closely linked to other inherited diseases such as cystic fibrosis. For these reasons, couples with severe male factor infertility are encouraged to seek genetic counseling as part of their preparation for infertility treatment.

Embryo Culture

Over the first 5 days after fertilization, the embryo grows rapidly from a single-cell zygote to a hollow ball of many cells, called a blastocyst. During this crucial interval, the embryo makes its transition from a metabolism based on maternal genes to its own genetic program. The media used for embryo culture is a simple solution of salts and nutrients. High-quality embryos can grow in this standard media with good implantation and pregnancy rates.

The metabolic needs of the embryo change as the embryo grows. Sequential culture uses media of different types depending on the stage of embryo development. These more complex media are used to respond to the changing metabolic requirements of the embryo.

Embryo Transfer

Over time, embryologists have learned to produce healthier embryos. In 1985, it was not unusual to transfer six to eight embryos in a single procedure. Today, to decrease the chance of multiple gestations, clinics commonly transfer no more than three high-quality embryos. Embryo transfer is typically done after 3 days of culture. In some cases, embryo culture may be extended to allow transfer of a single blastocyst. Some clinics will transfer more embryos if the overall embryo quality is poor. Embryos are transferred in a thin flexible straw that is threaded carefully through the cervix. The optimal transfer places the embryos in the mid-uterine cavity, together with a minimal amount of transfer media. The transfer is performed carefully to avoid uterine bleeding, which can decrease the chance of implantation. In most cases, the transfer procedure is painless. Embryo transfer may fail if the embryos become trapped in the cervical mucus or do not leave the transfer catheter. Each of these problems can be overcome by careful technique. Some clinicians use sonographic imaging to confirm intrauterine placement of the embryos.

A normal uterine cavity and endometrium will encourage implantation. A physician will often evaluate the patient's endometrial cavity with hysterosalpingogram or ultrasound imaging before the ART cycle begins. Endometrial polyps or fibroids inside the uterine cavity are frequently removed in preparation for treatment. Progesterone supplementation is used after embryo transfer to promote normal secretory endometrium. Embryo hatching is another way in which to assist implantation. Hatching is performed by making a slit in the zona pellucida just before the embryo is transferred. Hatching is thought to make it easier for the embryo to emerge from the zona and may improve signaling between the embryo and the endometrium.

Cryopreservation

As mentioned previously, to reduce the risk of a multiple pregnancy, most clinics transfer only two or three embryos. Because an average ART cycle produces more than three embryos, there are often healthy embryos left over after the transfer. It is possible to freeze the leftover embryos to preserve them. This process is called cryopreservation. If a couple does not achieve pregnancy after IVF, the partners can attempt pregnancy again by transfer of thawed embryos remaining from their first treatment cycle. Pregnancy rates after cryopreservation and thaw of human embryos are less than those following a fresh embryo transfer. The chance of pregnancy will depend on the quality of the embryos and on the maternal age at the time that the embryos were frozen. Live births have occurred after as long as 7 years of cryopreservation, although in most cases couples will use their frozen embryos within a short time.

More recently, progresses in cryobiology have led to vitrification techniques that have been applied to both oocytes and embryos with results surpassing conventional methods. Today, vitrified-thawed oocytes or embryos offer comparable results to fresh oocytes or embryos. This has open the possibility of vitrifying all embryos and delaying embryo transfer to improve uterine readiness for implantation.

Treatment Success and Risks

Even though ART is expensive and invasive when compared with other therapies, ample empirical evidence supports the use of ART procedures for treatment of infertility. Today, live birth rates per initiated cycle often surpass 35%. Chance of success is greatest with younger maternal age and evidence of normal ovarian function. Women over 40 years of age or with high concentrations of FSH early in the menstrual cycle have little chance of achieving a live born child. Multiple pregnancy rates after ART treatment average 25%–40% and can be as high as 60% in some series. The high rate of multiple pregnancy after ART treatment contributes to an increase in spontaneous pregnancy loss (25%), ectopic pregnancy (5%), and preterm delivery. Ovarian hyperstimulation syndrome (OHSS) occurs in 5% of cycles and is another significant consequence of ART treatment, although the use of new methods for ovulation triggering (in particular, GnRH agonists) have practically reduced this prevalence to zero.

Future Implications of Art Techniques

It is likely that we have only just begun to see the direction that these technologies can take us. ART techniques, combined with a growing knowledge of molecular and cell biology, will allow further development of preimplantation diagnosis and possible preimplantation gene therapy. The goal of helping to reverse the effects of aging on reproductive potential may one day be realized through the application of techniques borrowed from cloning and stem cell research. Further improvement in embryo culture technique and embryo selection should increase the number of established pregnancies, reduce the number of multiple pregnancies and pregnancy losses, and further increase the live birth rate per initiated ART cycle.

Ethical Concerns

Some have expressed concern that the expense and risks of ART may outweigh the benefit to the few couples who achieve successful live births. Further concern about expense, risk, and commercialization arises if an egg donor or gestational surrogate, who bears procreative risk in return for payment, is involved. There is consensus that surrogates and donors should be paid for their services but not paid so much that the participants are blinded to their potential risks. In many countries, ART procedures are available only to those who can afford them. Does a society have responsibility to provide infertility care to all of its members as it would to any individual suffering with disease? Some governments have dictated the maximum number of eggs to be retrieved and fertilized and the maximum number of embryos to be transferred per case in an effort to decrease the chance of a multiple gestation. Is this intrusion on the right of individuals to have control over their medical care justified by a concern for patients' safety and the safety of their potential fetuses? Do governments have a right to limit the types of reproductive services available to their citizens?

In July 1978, after Louise Brown was born, religious leaders complained that doctors did not have the right to "play god." Steptoe replied, "I don't know what the fuss is all about. I'm just glad we were able to help Mrs. Brown to have such a happy healthy baby." Today, because of ART, millions of couples and their physicians have had the joyful opportunity of echoing Steptoe's sentiment.

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Ovarian Failure Treatment Strategies: Egg Donation☆

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Glossary

Embryo transfer The process by which an embryo is placed into the uterine cavity. The embryo is loaded into a fine cannula that is placed into the uterine cavity. The location of the cannula may be identified by transabdominal ultrasonography. The embryo is expelled into the uterine cavity and the cannula is removed.

Endometrial receptivity The uterine endometrium undergoes continual remodeling. In the early luteal phase of the menstrual cycle, under the influence of progesterone, the endometrium is in a permissive state for the embryo to implant.

Gonadotropin A peptide hormone, follicle-stimulating hormone or luteinizing hormone, that is synthesized and secreted in the pituitary.

Ovarian reserve The extent of residual follicles in the ovary that determines the ovarian response to gonadotropin stimulation.

Superovulation The use of gonadotropin stimulation to increase ovarian follicle recruitment and development.

Window of implantation The limited period of time during which the endometrium is receptive for an embryo to implant.

Introduction

Improvements in life expectancy, obstetrical practice, and neonatal care and advances in assisted reproductive technology have enabled conception and successful childbirth in women with ovarian failure, the majority of whom are peri- and postmenopausal. Assisted reproductive techniques and ovum donation are essential for the establishment of pregnancy in the setting of ovarian failure.

The egg donor is identified and undergoes the process of superovulation and oocyte retrieval to obtain the egg. The egg is fertilized in vitro. Concomitantly, the endometrium of the woman with ovarian failure (recipient) is synchronized to a secretory endometrium receptive for implantation by the use of exogenous sequential estrogen and progesterone administration. When the embryo has cleaved and is ready for transfer, it is placed into the recipient's uterus, and exogenous hormone treatment with estradiol and progesterone is continued.

Pregnancy rates are in the range of 40%–50% per cycle; the highest rates are observed with in vitro fertilization (IVF) and are comparable to those seen with IVF in women of similar age to the egg donor. Even with improvements in antenatal care, obstetrical consequences of childbirth in women older than 40 years of age using egg donation include hypertensive complications and gestational diabetes, an increase in intrauterine fetal deaths, and at least a twofold greater likelihood of cesarean delivery. However, as demonstrated by the fertility and obstetrical outcomes of older women using egg donation, the aging uterus is not a limiting factor in reproduction, pregnancy, or successful delivery of a live-born infant.

Pregnancy rates in OD programs are 50%–60% and live birth rates are around 40%. However, these results are accompanied with high twin pregnancy rates (30%–35%). Multiple pregnancies, commonly associated with poor perinatal outcomes, are considered the most frequent complication of assisted reproductive technique (ART). According to the Spanish Fertility Society (SEF) registry, a remarkable decrease in triplet delivery in OD has been observed in recent years, reaching 0.5% in 2012, yet twin pregnancy rates remain high, being 27.8% in the same year.

Most oocyte donation programs consider a high clinical pregnancy rate as the most important outcome to be taken into account. Pressure to obtain good results, as well as the difficulty of the patients both emotionally and in economic terms in repeating cycles (e.g., advanced age, lack of donors), might explain that some centers do not take into consideration the costs and risks associated with complications caused by multiple gestations.

Natural Limits of Reproductive Capacity

Birth records in the United States and Great Britain prior to the current era of assisted reproductive technology indicate that the maximum recorded age for a successful pregnancy in a woman was 52 years, and only 1/20,000–60,000 births occur naturally past the age of 50. Peak fertility in women occurs between the ages of 20 and 25. After age 30, an insidious decline in fertility ensues

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and becomes clinically evident after age 35. In reality, although the ovaries continue to produce steroids during the 10 years preceding menopause, effective gamete production decreases significantly during this time. The loss of ovarian sensitivity to gonadotropins heralds the cessation of reproductive capacity. A woman is born with all the ovarian follicles she will ever have. As a woman ages, follicle depletion occurs as a result of ovulation and follicular atresia within the ovary. Concurrently, the secretion of estradiol and inhibin from the ovarian granulosa cells declines. Subsequently, follicular growth becomes more infrequent despite increasing pituitary gonadotropin secretion, and ovulation occurs more erratically. Ultimately, menopause, the absence of ovulation and the cessation of menses, occurs.

When the numbers of follicles contained within the ovaries of similarly aged women but with differing menstrual status (regular cycles, perimenopausal, and postmenopausal) are compared, a marked depletion in the remaining follicles is evident in the perimenopausal ovary compared to the ovary of a woman who has been menstruating regularly. Loss of follicles accelerates prior to menopause, and the ovarian follicular reserve is practically expended in menopause. Oocyte chromosomal abnormalities are more common in aged women.

In addition to chronologic age, genetic and other factors such as endometriosis, previous ovarian surgery, systemic chemotherapy, or radiotherapy may affect the ovarian reserve and consequently the biologic response of the ovary. A biological marker of ovarian reserve is the basal follicle-stimulating hormone (FSH) level, obtained on menstrual cycle day 3. Other markers of ovarian reserve include. AMH levels and ultrasonographic evaluation of antral follicle count (AFC)

Egg Donation

Egg (ovum) donation is a process in which eggs (ova) provided by one woman (egg donor) are given to a recipient with the intent to establish a pregnancy in the recipient. The egg donor can be a friend, family member, or an anonymous donor. The donated eggs may be used solely by one recipient or shared by two recipients without reducing the chance of pregnancy. The process of obtaining eggs is similar to that for IVF.

Although the recommendations are based on fresh embryo transfer cycles advances in the methods of cryopreservation (vitrification) and increased survival of embryos vitrified at blastocyst stage will allow better results in the transfer cycles and thus, assumes that the accumulated rates are increasingly high. It will be necessary then that the recommendations designed to consider cumulative pregnancy rates and especially live birth rates.

Candidates for Egg Donation

Women who may consider egg donation include those who have reduced ovarian reserve and impaired folliculogenesis, those who have undergone menopause, those who have ovarian dysgenesis (Turner syndrome), or those who have ovarian failure related to radiation therapy or chemotherapy to treat a malignancy. Any woman who has a uterus but absent or nonfunctioning ovaries is a potential egg recipient. There are approximately 100,000 women in the United States who are unable to bear children due to their inability to produce eggs or whose eggs may not fertilize normally. The majority of women using egg donation are those of advanced reproductive age (>39 years) who are peri- or postmenopausal. Screening of the general health status of the recipient prior to the establishment of pregnancy is advised and should include a full physical examination, screening mammogram, and, in women older than age 40, a cardiac stress test. In addition, before initiating the process of egg donation, the recipient's uterus must be evaluated to exclude intrauterine pathology. Finally, a trial cycle of sequential hormone treatment to prepare the endometrium may be undertaken to determine whether adequate endometrial development occurs in response to estradiol and progesterone administration.

Appropriate preparation of the recipient's endometrium is achieved with the administration of exogenous estrogen in a stepwise fashion, followed by estrogen and progesterone administration synchronized with the timing of hCG administration to the ovum donor. If pregnancy occurs, exogenous administration of estrogen and progesterone is continued for 10 weeks until the placenta has grown sufficiently to produce an ample amount of progesterone (the luteal–placental shift of estrogen and progesterone production generally occurs at 9 or 10 weeks of pregnancy). In 2000, in the United States donor eggs were used in approximately 10% (10,389) of all assisted reproductive technology cycles. More than 70% of all IVF cycles used donor eggs among women older than age 46.

Requirements for an Egg Donor

An egg donor is generally 21–34 years old and has a satisfactory physical examination, including a normal Pap smear, a negative medical history, and genetic screening history, including karyotype negative screening tests for infectious agents, and satisfactory psychological screening and counseling. Appropriate screening of potential egg donors consists of a complete history and physical examination; detailed review of genetic and infectious diseases, including testing for syphilis, gonorrhea, chlamydia, HIV-1 and HIV-2, and hepatitis B surface antigen and hepatitis C antibody; psychological evaluation; and extensive counseling regarding the risks of the procedure prior to signing an informed consent form. If the egg donor is married, both she and her husband should

sign an informed consent. Compensation is provided for time and effort expended and varies depending on geographic area. Screening of the candidates was carried out as described in, incorporating the modifications established in RD-Law 9/2014. The genetic test of carrier of recessive and X-linked autosomal diseases (qCarrier test) was incorporated, based on the Next Generation Sequencing technique, which allows the detection of 240 genetic diseases (Abulí et al., 2016).

Pregnancy and Live Birth Rates

The pregnancy success rate for IVF when donor eggs are used is related to the age of the woman who donated the egg. Since egg donors are typically younger than age 30, the pregnancy rate of an egg donor cycle is approximately 40%–50% per cycle, comparable to that of a young woman undergoing IVF. The cumulative live birth rate is approximately 90% after four cycles. Although the rate of pregnancies conceived using embryos that developed from an egg provided by a donor is excellent, the live birth rate may differ slightly from that of a young woman because maternal factors, such as an increased rate of gestational diabetes and hypertension, contribute to the viability of pregnancy.

Whereas in naturally conceived pregnancies, an age-related increase in spontaneous abortion is observed with advancing age, the rate of spontaneous abortion in pregnancies conceived with egg donation is related to the age of the egg donor, who is generally younger than age 30. Thus, an increase in the spontaneous abortion rate is not an added risk for these older women. In addition, the age of the egg donor determines the risk of fetal chromosome abnormalities such as trisomies. Consequently, since the egg donor is typically a young woman, the risk of chromosomal abnormalities tends to be lower than that observed in women older than age 40.

Uterine Factors

The structure and function of the uterus are dependent on the ovarian production and secretion of estrogen and progesterone and the delivery of these hormones to the endometrium and myometrium via the uterine vasculature. After age 50, uterine weight decreases by $>50\%$, accompanied by changes in collagen and elastin. Because women in their 50s can conceive and carry pregnancies to viability with egg donation, structural changes in the uterus related to hormonal changes of ovarian failure or aging are likely reversible with the administration of exogenous steroid hormones in high doses or they are not of sufficient magnitude to prevent pregnancy.

The effects of aging on the human uterus and consequently on conception, pregnancy course, and spontaneous abortion have not been fully explored. The best predictor of endometrial receptivity and successful implantation is full endometrial thickness as determined in the longitudinal plane by transvaginal ultrasound. As more data are obtained through clinical experience with ovum donation programs, transvaginal ultrasonographic measurements of endometrial thickness and uterine blood flow, and basic research on uterine function, identification, and modulation of critical uterine factors may improve pregnancy outcomes in all women.

Obstetrical Consequences of Delayed Childbearing

Obstetrical consequences of egg donation in women older than age 40 include antenatal medical complications, an increase in intrauterine fetal deaths, and at least a twofold greater likelihood of operative delivery. Medical complications, including hypertensive complications and gestational diabetes, occur with higher frequency in older women. Hypertension occurs 2–10 times more often in older women. In addition, cardiovascular, respiratory, renal, and autoimmune disorders and neoplasia result in more frequent hospitalizations in the older gravida, and when pregnancy is complicated by a medical problem, the complication tends to be more serious than in a younger parturient. According to the report of the ESHRE consensus meeting on "Risks and complications in assisted reproduction techniques" held in Maastricht in 2002, the success of assisted reproduction technology should be measured by the birth of one single healthy child, and multiple pregnancy should be considered as a major complication (Clua et al., 2010, 2016). For oocyte donation, most scientific societies recommend that the age of the donor should be used to determine the number of embryos for transfer. Since in Spain, Law 14/2006 establishes that all donors must be under 35 years of age, it would be logical to recommend single-embryo transfer (SET) in oocyte donation programs as long as good-quality embryos were available, similarly to the recommendation made for young IVF patients. In Spain, according to the SEF guidelines, it is advisable to transfer one or two embryos in oocyte donation recipients (Clua et al., 2012, 2015).

Malpresentation occurs more frequently during labor and delivery, as do placenta previa, uterine myoma, and multiple gestation. The presence of these conditions increases the likelihood of operative delivery. In addition, an increased incidence of labor abnormalities is observed, including prolonged latent phase, prolonged labor (slow active phase, active phase arrest, and prolonged second stage), and failure of decent. Labor abnormalities also contribute to the increased risk of operative delivery.

Perinatal Consequences in Women of Advanced Maternal Age

Age of the mother does not appear to affect birth weight or the incidence of preterm births in women in the United States. These outcomes are determined by social factors such as nutrition, socioeconomic status, smoking, and prior obstetrical history. There is

a three or fourfold increase in perinatal mortality with older maternal age. This is due primarily to an excess of fetal deaths (stillbirths) both before and during labor and not to neonatal deaths. Congenital anomalies, maternal hypertension, uteroplacental insufficiency including large placental infarcts, abruptio placenta, and placenta previa are contributing conditions. The contribution of age independent from having undergone egg donation is unknown. When obstetrical outcome in women who conceived with the assistance of IVF was compared to that of a control group matched by maternal age and multiplicity, an increased incidence of intrauterine growth retardation, placenta previa, and preterm delivery was found among IVF singleton pregnancies. As more women of various ages undergo egg donation, age-related factors influencing the course of pregnancy and delivery will be distinguished from IVF-related risk factors and will contribute to our understanding of the obstetrical consequences of both IVF and aging.

In the past century, medical advances have contributed to the reality of pregnancy in peri- and postmenopausal women. These include the development of IVF and related assisted reproductive technologies, the identification and synthesis of estrogen and progesterone, and their availability as pharmaceutical preparations that can be used to artificially prepare an endometrium for implantation, and developments in obstetrical and neonatal practice that have resulted in significant improvements in maternal health and neonatal outcome. In addition, improvements in overall general health status as reflected by increased life expectancy and decreased infant mortality in developed countries have made parenthood at an older age a feasible option as people live longer and healthier lives.

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Fertility Preservation in Women

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Introduction

Recent advances in fertility preservation (FP) techniques now allow to consider cryopreservation of oocytes, embryos, and ovarian tissue. Initially developed for patients suffering from cancer, FP has rapidly invaded others medical fields, and should be included in the management of all women of reproductive age diagnosed with diseases that could threaten fertility or having to receive gonadotoxic treatments. Thus, FP should now be discussed for non-oncological at risk for premature ovarian insufficiency (POI) due to gonadotoxic iatrogenic treatments, repeated ovarian surgery, auto-immune, endocrine or genetic disorders.

After a description of the existing techniques of FP, the main FP indications will be discussed.

Techniques for Fertility Preservation in Women

Among FP techniques, some are considered as well established such as oocytes or embryos cryopreservation after ovarian stimulation while others still remain experimental.

Oocyte/Embryo Cryopreservation After Controlled Ovarian Stimulation

Ovarian stimulation

Prior to embryos/oocytes cryopreservation, a controlled ovarian stimulation (COS) is commonly requires. It consists in the administration of exogenous FSH for 10–15 days to produce a multifollicular growth. The use of high doses of gonadotropins overrides the mechanism of follicle dominance and thereby achieves the growth of several large follicles up to the preovulatory stage (Verberg *et al.*, 2009). COS is usually performed using GnRH antagonist protocols until three to four follicles have reached 16–22 mm in diameter. Final follicular maturation is therefore triggered using hCG or GnRH agonist administration. Thirty-six hours later, oocytes are retrieved by transvaginal ultrasound-guided aspiration of follicular fluid from follicles. Mature oocytes obtained are frozen by vitrification. For PF patients, the objective is to yield a maximum number of oocytes while avoiding risk of ovarian hyperstimulation syndrome. Their use will require a thawed procedure and further in vitro fertilization (IVF) and embryo transfer.

GnRH antagonists have modified the way patients are stimulated, enabling a significant reduction in the duration of treatment. Indeed, in contrast to agonists, GnRH antagonists immediately suppress pituitary FSH and LH releases and do not require the 10–14 days of GnRH agonist administration before gonadotropins administration. Exogenous FSH is usually started on cycle day 2–3 and GnRH antagonists will be initiated when the lead follicle reaches 12–14 mm in diameter, at approximately day 6 of ovarian stimulation. As a result, the timing between the beginning of gonadotropins administration and oocytes pick-up will often not exceed 14 days on average. Numerous studies and meta-analyses have shown similar live birth rates with antagonist and agonist protocols (Toftager *et al.*, 2017). Antagonist protocol offers the advantage of reducing the risk of ovarian hyperstimulation syndrome (OHSS) especially with GnRHa ovulation triggering (Bodri *et al.*, 2009; Humaidan *et al.*, 2011).

Recently, new protocols have been developed offering the possibility to perform ovarian stimulation irrespective of the phase of the menstrual cycle ("random start" protocols) or to realize a double stimulation. Additionally, it is possible to use alternative stimulation protocols without exogenous FSH-related increase in serum estradiol levels or to carry out natural cycles. At least new procedures of ovulation triggering have almost canceled risks of severe ovarian hyperstimulation syndrome.

The "random start" protocol, starting gonadotropins administration regardless of the day in the menstrual cycle have been proposed and is especially interesting for patients having to initiate gonadotoxic treatment very soon (Sönmezer *et al.*, 2011; von Wolff *et al.*, 2009). The total number of oocytes recovered did not differ whatever the phase of the cycle at which ovarian stimulation was started. However, no data is currently available in this population regarding oocytes quality, pregnancy or live birth rates.

The concept of the double stimulation has recently emerged. Kuang *et al.* (2014), first showed, in infertile poor responders, the efficiency of a double stimulation during the same menstrual cycle for increasing the number of oocytes available for IVF. Further, this concept has been proposed for FP candidates.

The supraphysiologic estrogen serum levels (10–20-fold) induced by conventional protocols might have a detrimental effect on hormonal-sensitive diseases such as breast or endometrial cancers. The risk of cancer recurrence directly related to COS remains theoretical even though current available data remain reassuring (Azim *et al.*, 2008; Goldrat *et al.*, 2015). To reduce this risk as much as possible, innovative protocols of ovarian stimulation have been described, with a major objective: reducing estrogens production or action using tamoxifen or aromatase inhibitors (letrozole) (Oktay *et al.*, 2005).

The concomitant administration of letrozole and gonadotropins allows maintaining serum estradiol levels into the physiologic ranges, by preventing conversion of androstenedione and testosterone respectively to estrone and estradiol. In the absence of estrogenic negative feedback on the gonadal axis, FSH secretion is increased stimulating follicles growth (Oktay *et al.*, 2005, 2006). Despite lower serum estradiol levels on the day of ovulation triggering, the number of oocytes and embryos obtained with letrozole-FSH protocols did not differ when compared with conventional COS (Oktay *et al.*, 2005). Moreover, follow-up of breast cancer patients having undergone ovarian stimulation for FP with letrozole has not showed increased risk of recurrence or detrimental effect on short-term survival rates (Azim *et al.*, 2008; Oktay *et al.*, 2006, 2015a). Kim *et al.* (2016) confirmed in a recent prospective study the safety of controlled ovarian stimulation with letrozole prior to chemotherapy with 5-year follow-up after breast cancer diagnosis. Moreover, a recent investigation showed comparable pregnancy rates between patients having undergone ovarian stimulation with aromatase inhibitors and non-cancer patients included in conventional protocols (Oktay *et al.*, 2015). Finally, no increase in congenital malformations and chromosomal abnormalities was observed in newborns with letrozole protocols (Oktay *et al.*, 2015).

Tamoxifen is a competitive antagonist within the breast by competitive binding to estrogens receptors. Yet, tamoxifen, by inhibiting the negative feedback of estrogens over the hypothalamic-pituitary system, causes increase in GnRH secretion and further FSH release (Meirow *et al.*, 2014). In FP candidates, the detrimental effect of tamoxifen on endometrium is no longer relevant, since no fresh embryo transfer is planned. Oktay et al. first proposed a tamoxifen-FSH protocols for breast cancer patients. When compared with letrozole protocols, the use of tamoxifen yield significantly higher peak serum estradiol levels and is less effective in term of number of oocytes and embryos obtained (Oktay *et al.*, 2005).

At least, given the short half-life of GnRH agonist-induced endogenous LH surge, it is now recommended to use these drugs as an alternative to hCG in FP candidates stimulated with GnRH antagonist protocols. In cancer patients, GnRH agonists trigger have shown to be as effective as hCG, while reducing the risks of moderate/severe OHSS (Reddy *et al.*, 2014). Avoiding hyperstimulation complications is critical in order not to delay initiation of cancer treatment.

Natural-cycle IVF have been proposed as an alternative to conventional COS for poor responders (Lainas *et al.*, 2015; Pelinck *et al.*, 2002). However, it is now well established that the overall pregnancy rates achieved with modified natural-cycle IVF in patients >35 years are low, probably as a result of the poor oocyte quality. However, iterative natural cycles could be used for young FP candidates (excluding cancer indications) with already impaired ovarian reserve. The main limit for this strategy may be the high number of cycles required for obtaining a "sufficient" number of frozen oocytes, which may slow down the FP process.

Embryo cryopreservation

Cryopreservation of embryos using IVF is a well-established technique largely used worldwide for the last decades. Live birth rates are comparable with fresh and frozen embryos (Roque *et al.*, 2013). Most IVF outcomes appear comparable for cancer patients and age-matched controls (Cardozo *et al.*, 2015). However, cryopreservation of embryos requires a male partner or a sperm donor.

Oocyte cryopreservation

The first live birth obtained after thawing of a frozen embryo was published 30 years ago (Kuleshova *et al.*, 1999). Since then, improvement in cryopreservation techniques especially in vitrification has allowed the development of oocytes cryopreservation. With slow-freezing, survival rates of oocytes were about 65%. Vitrification consists in a rapid cooling, requiring a higher concentration of cryoprotectants and preventing formation of freezing crystals which can damage cell structures. This is especially important for egg freezing, since oocytes are large size cells with a high content of water. Vitrification has superior post-thaw survival and fertilization outcomes compared with oocytes that were slow-frozen (Rienzi *et al.*, 2017; Glujovsky *et al.*, 2014). Oocyte vitrification produces similar IVF outcomes compared with fresh oocytes and is not associated with further obstetrical or perinatal morbidity (Crawford *et al.*, 2017; Rienzi *et al.*, 2010). Moreover, concerning neonatals outcomes, no increase in chromosomal abnormalities or birth defects has been noted in children born from cryopreserved oocytes (Noyes *et al.*, 2009; Cobo *et al.*, 2014).

This safe and efficient technique is no longer considered as experimental according to the criteria by the ESHRE Special Interest Group "Ethics and Law" (Provoost *et al.*, 2014; ISFP Practice Committee *et al.*, 2012; Practice Committees of American Society for Reproductive Medicine, Society for Assisted Reproductive Technology, 2013; Loren *et al.*, 2013; Schmidt *et al.*, 2012).

Number of oocytes required

The minimal number of vitrified oocytes required to achieve a live birth remains unknown. Vaughan *et al.* (2017) observed that pregnancy rate was significantly higher when ≥15 oocytes were retrieved (41.3%) than <15 oocytes (36.5%). For Cobo *et al.* (2016), in adult women having frozen their gametes for nonmedical reasons, 10–15 eggs were associated with a 85% chances of live birth. Age at time of FP is a major factor influencing success rates: with 15 mature oocytes, the pregnancy rates were 40%, 36%, 27% and 16% in women <35, 35–37, 38–39 and >40 year-old, respectively (Sunkara *et al.*, 2011). More recently, in women having undergone autologous IVF-ET treatment using vitrified and warmed oocytes, the overall vitrified-

warmed oocyte to live birth efficiency was 6.4% (Doyle *et al.*, 2016). Based on these data, the authors recommend the cryopreservation of 15–20 or 25–30 mature oocytes in women aged <38 years or 38–40 years, respectively (Doyle *et al.*, 2016; Stoop *et al.*, 2014a). Thus, the higher the number of oocytes yields, the higher the probability to achieve a live birth after utilization (Drakopoulos *et al.*, 2016). Until now, after cancer treatment, <5% of patients returned to use their oocytes (Martinez *et al.*, 2014).

Ovarian Tissue Cryopreservation OTC

This technique consists in the removal of a part or an entire ovary during a laparoscopy. Then, small fragments of ovarian cortex containing primordial follicles are cryopreserved. The ovarian tissue can later be orthotopically transplanted into a peritoneal window or into one of the ovaries, with the purpose of restoring both endocrine and exocrine ovarian function with possibility of pregnancies natural or medically induced. Several transplantations may be performed successively in a single patient. Some heterotopically transplantations have been experienced (subcutaneous tissue of the abdominal wall, forearm or chest wall) with no result in terms of fertility.

The major issue with OTC is the great follicular loss due to ischemic events during the freezing and thawing procedures of cortical strips. As a consequence, the function of frozen-thawed ovarian tissue after grafting remains uncertain, even though the chances of restoring optimal ovarian function and fertility depend on the follicular density at the time of cryopreservation. Amount of ovarian tissue harvested (biopsies/cortical strips or whole ovary) is still debated. It may depend on the pathology, the expected gonadotoxicity of treatment, patient's age and ovarian reserve. Actually, partial or total oophorectomy automatically implies the irreversible loss of a part of the primordial follicular pool, possibly reducing the chance of natural pregnancies.

The first birth achieved with this technique was published more than 10 years ago by Donnez *et al.* (2004). To date, around 90 births have been reported worldwide (Jensen *et al.*, 2017) and OTC is performed in many countries.

Ovarian function, i.e., regular menstruation, was observed in two-third of transplantation cases (Van der Ven *et al.*, 2016). The mean duration of ovarian endocrine function after transplantation is about 5 years, offering a treatment of the negative effects of POI on quality of life such as osteoporosis, cardiovascular diseases and depression (ISFP Practice Committee *et al.*, 2012). Few complications are reported with those procedures. Live birth rates per transplantation are around 20% (Jensen *et al.*, 2017; Van der Ven *et al.*, 2016; Dittrich *et al.*, 2015; Donnez *et al.*, 2013). In a recent Belgian publication with more than 500 patients who underwent OTC between 1997 and 2013, <5% of patients underwent autotransplantation with 33% live birth rate per transplantation (Jadoul *et al.*, 2017). In the meta-analyze of Pacheco and Oktay (2017), with 309 ovarian tissue transplantations performed, cumulative live birth rate and endocrine restoration rate was 37.7% and 63.9% respectively.

The age limit to perform OTC still remains controversial. For many authors, OTC should not be proposed from women older than 35. However, pregnancies have been observed for women aged 36–39 (Meirow *et al.*, 2016). As a consequence, ovarian reserve and thus follicle density at the time of tissue cryopreservation may be a better criterion than age. OTC should be performed theoretically before chemotherapy. In some cases, tissue can be removed after gonadotoxic treatment and live births have been obtained among patient with IVF following transplantation of ovarian tissue removed after chemotherapy, nevertheless with apparently lower live birth rate than chemotherapy-naïve patients (Meirow *et al.*, 2016).

Moreover, some cancers might preclude transplantation owing to a high risk of reintroducing malignant cells (leukemia …) (Dolmans *et al.*, 2010). Some cases reported of leukemia survivors and transplantation after control of the absence of leukemic cells within the graft with no cancer recurrence observed (Meirow *et al.*, 2016). Authors highlight the fact that in hematologic malignancies, chemotherapy before OTC reduces the risk of malignant cells in the graft by clearing blood. At least, no methods can guarantee the absence of cancer cells in tissue.

Risks of surgery should be considered each time, especially in context of leukemia with potential platelet dysfunction and hemorrhagic complications.

After a large increase, the number of OTC procedures decreases with the improvement and spreading of oocyte vitrification. It remains hard to assess efficacy of OTC with only few publications concerning his effectiveness. This method still remains considered as experimental but with regard to recent results, OTC seems a viable option.

In Vitro Maturation of Oocytes

In vitro maturation consists in the retrieval of immature oocytes by transvaginal puncture under ultrasound guidance. Then, immature oocytes are put in a culture media for 24–48 h. Around 60% of maturation rate are observed (Shalom-Paz *et al.*, 2010) and allowed the obtaining of mature oocytes. This FP technique requires no ovarian stimulation and could be used without delay whatever the cycle phase, in case of emergency or when contraindications to ovarian stimulation exist.

The number of immature oocytes recovered is strongly correlated with the number of antral follicles visible into the ovaries and anti-Müllerian hormone (AMH) level (Sonigo *et al.*, 2016). As a result, IVM alone may not be suitable in patients with a low ovarian reserve.

The competence of an oocyte obtained from IVM is certainly reduced compared with one retrieved after in vivo maturation occurring during COS. And there are very few data concerning IVM for FP with only two pregnancies obtained after IVM practicing

during ovarian tissue removal (Prasath *et al.*, 2014; Uzelac *et al.*, 2015). However, data from thousands of children born from this technique realized for infertile women are promising especially with no increased congenital abnormalities (Buckett *et al.*, 2007).

GnRH Analogs

Some authors proposed the use of GnRH agonists (GnRHa) in order to prevent premature ovarian insufficiency induced by cytotoxic treatment. A monthly therapy leads to a reversible resumption of menses. The role of ovarian function suppression with use of GnRHa in terms of fertility preservation has remained controversial for years and is not recommended by numerous guidelines. GnRHa are used with the hypothesis that the drop induced in FSH levels will limit the growing initiation of follicles and thus avoid the destruction of growing follicles observed with exposition to cytotoxic drugs. Moreover, they could lead to an hypoperfusion of the ovary and thereby a lowered exposition to chemotherapy agents. At least, the sphingosine 1 phosphate up-regulation induced by GnRHa could have anti-apoptotic effects.

In most studies the primary endpoint analyzed was the resume of menses. The OPTION study provides evidence that GnRHa reduce the risk of POI (amenorrhea and elevated FSH) to 18.5% versus 34.8% in the control group ($P = 0.048$) (Leonard *et al.*, 2017). The meta-analyze of Lambertini *et al.* (2015), similarly observed a reduced risk of POI with agonist with a trend to an increase in pregnancy rate. In the POEMS study, administration of GnRHa during chemotherapy appeared to protect against ovarian failure and pregnancies occurred in more women in the GnRHa group than in the chemotherapy alone group (21% vs. 11%; OR: 2.45; 95% CI 1.09–5.51 $P = 0.03$) (Moore *et al.*, 2015).

However GnRHa do not provide long term protection on ovarian reserve after chemotherapy (Demeestere *et al.*, 2016). In the OPTION study, a marked fall in AMH was observed with no difference between groups (Leonard *et al.*, 2017). At least, concerning pregnancy rate, Yang *et al.* (2013) showed in a meta-analyze including 5 RCT and 528 breast cancers that GnRHa reduce the risk of POI chemotherapy-induced without improving live birth rate.

In conclusion, temporary ovarian suppression with GnRHa might reduce chemotherapy-induced gonadotoxicity and can be considered for women interested in preserving ovarian function and fertility, but not as an exclusive option.

FP Techniques in the Future

OTC and in vitro activation

Kawamura *et al.* (2013) demonstrated that the disruption of hippo signaling pathway in the ovary and acting on the AKT pathway leads to promotion of primordial follicle growth. Suzuki *et al.* (2015) proposed an in vitro activation (IVA) of residual follicles from the ovarian tissue before grafting, via stimulation of the AKT pathway, in order to improve the ovarian response to stimulation for women with POI. IVA allowed a successful production of mature oocytes among women with POI who as previously received ovarian stimulation with no follicular growth. Two successful live births have already been reported (Suzuki *et al.*, 2015).

In vitro folliculogenesis

In vitro culture of pre-antral follicles still remains a key challenge but could allow the utilization of primordial resting follicles (Telfer and Zelinski, 2013).

Germ line cells

Isolation of oogonial stem cells have been reported without data on their true role in the ovary. The utilization of germ line cells could represent an option in the future offering an unlimited source of primordial follicles.

Artificial ovary

The development of transplantable artificial ovary is on study. A team has recently demonstrated that isolated human pre-antral follicles embedded in fibrin formulations and xenografted into peritoneal pockets in nude mice are all viable after 1 week grafting. Fibrin seems to be a promising matrix for obtain an artificial ovary, supporting follicle survival and development (Paulini *et al.*, 2016). This could represent an option in pathologies with risk of re-introduction of malignant cells in disease free survivors with ovarian tissue transplantation.

Ovarian protective agents

Given that chemotherapy also triggers activation of dormant follicle growth, immune modulator that acts on the PI3K/PTEN/AKT follicle activation pathway and AMH, a negative regulator of follicle activation, are candidates to prevent depletion of the primordial follicles pool chemotherapy-induced (Kalich-Philosoph *et al.*, 2013; Roness *et al.*, 2016).

Oncological Indications

It is now well-established that all women in reproductive age facing with cancer should be referred early to reproduction specialist in order to discuss PF options before initiating gonadotoxic treatment. Fertility impairment could deeply affect cancer patients' long term quality of life. As a consequence, FP is considered as a fundamental part of cancer treatment. Numerous cancers are diagnosed in women in reproductive age, but breast cancers, lymphomas, leukemia or gynecologic malignancies represent the most encountered cancers.

Chemotherapy has detrimental effects on gonads and is associated with risk of POI for cancer survivors. Various mechanisms are implied (1) follicle stockpile destruction; (2) a "burn out" effect with increasing of growing follicle then undergoing atresia (3) vascular injuries and (4) ovarian fibrosis (Meirow *et al.*, 2001). Damages on the ovarian function depend on the drug employed, the dose, the association with other drugs or radiotherapy and above all, patient's age and ovarian reserve when initiating gonadotoxic treatment (Meirow *et al.*, 2010). Alkylating agents, such as cyclophosphamide, are highly gonadotoxic. Conditioning by busulfan or total body irradiation, used before hematopoietic stem cells transplantation (HSCT), is also highly gonadotoxic and causes impairment of fertility in most of cases.

The gonads are very sensitive to radiotherapy. The dose required to destroy 50% of the primordial follicles (LD50) is <2 Gy (Wallace *et al.*, 2003). Moreover, the dose of fractionated radiotherapy at which ovarian failure occurs was found to decrease with increasing age at treatment: at birth was 20.3 Gy; at 10 years, 18.4 Gy; at 20 years, 16.5 Gy; and at 30 years, 14.3 Gy (Wallace *et al.*, 2003). At least, radiotherapy causes reduction in uterine volume, myometrial fibrosis, vascular damages, and endometrial injury, which are behind the risk for pregnancy-related complications, including spontaneous miscarriages, preterm labor and delivery, low birth weight, and placental abnormalities (Meirow and Nugent, 2001).

When it is possible (i.e., sufficient delay before initiating treatment and no contra-indication to hormonal ovarian stimulation), oocytes cryopreservation after COS represents the technique of choice for female fertility preservation. In the literature, ovarian response to stimulation among cancer patients is matter to debate. In a recent study, it has been demonstrated that cancer patients have similar response to ovarian stimulation compared with controls of the same age and ovarian reserve parameters (Quinn *et al.*, 2017). Patients could also be candidate to a co-treatment by GnRHa. In case of emergency to initiate gonadotoxic treatment, OTC and/or IVM should be discussed.

Some cancers imply specific managements.

Breast Cancer

Breast cancer is the most encountered cancer among young women. Chemotherapy is often indicated. If surgery is first planned, ovarian stimulation with anti-aromatase in order to preserve oocytes could be proposed before chemotherapy. If neoadjuvant chemotherapy is proposed, OTC should be discussed considering the fact that data are reassuring concerning the risk of reintroducing malignant cells with grafting in this context. The possibility of BRCA 1 or BRCA 2 mutation (around 10% of young women with breast cancer) (Wang *et al.*, 2014) should be mentioned and that the graft of ovarian tissue is not suitable in these women at high risk. IVM represent another option in case of neoadjuvant therapy, alone or associated with OTC. At least, GnRHa is now recommended for breast cancer irrespective of hormone receptors status (Paluch-Shimon *et al.*, 2016; Lambertini *et al.*, 2017) but not as an alternative option to embryo/oocyte cryopreservation.

Ovarian stimulation is theoretically contra-indicated in breast cancer patients receiving neoadjuvant therapy. However, a single and recent study reported data for patients who underwent COS for FP before neoadjuvant chemotherapy (Chien *et al.*, 2017). Thirty-four women, with BC stage II–III, aged 35 on average, almost half with positive hormonal receptors, were stimulated and compared with controls. No significant difference was observed in terms of mean time from diagnosis to initiate chemotherapy, rates of pathologic complete response, recurrence and death between the two groups with a median follow-up of 79 months (Chien *et al.*, 2017).

For some authors, fertility preservation should be systematically proposed to women with BRCA mutations without cancer history, due to the high risk of breast cancer at a young age with gonadotoxicity of chemotherapy and duration of hormonotherapy with necessity to postpone pregnancy. Authors suggest an accelerated loss of primordial follicles and increased apoptosis in response to chemotherapy among those patients. Especially when considering the absence of risk due to stimulation in BRCA ½ carriers. Furthermore, recent studies showed that BRCA mutation carriers may have a lower ovarian reserve with higher risk of premature ovarian insufficiency and poorer reproductive outcomes. Moreover, some authors found poorer ovarian response to hormonal stimulation among BRCA mutations carriers with less oocytes recovered (Oktay *et al.*, 2010). Several studies show earlier menopause (Lin *et al.*, 2013; Finch *et al.*, 2013; Rzepka-Górska *et al.*, 2006) and lower AMH concentrations in BRCA mutations carriers (Wang *et al.*, 2014; Titus *et al.*, 2013).

Haematologic Malignancies

Chemotherapy is commonly indicated from low to high gonadotoxicitiy as before hematopoietic stem cells transplantation (HSCT) with risk of intensification.

For Hodgkin and non-Hodgkin lymphomas, oocytes and/or embryos cryopreservation is recommended when 10–12 days are available before initiating chemotherapy (ISFP Practice Committee *et al.*, 2012). OTC could be realized in case of emergency to start the chemotherapy or in case of highly gonadotoxic treatment, even when patients have already received chemotherapy. The risk of reintroducing cancer cell is considered as moderate (Dolmans *et al.*, 2010).

For women with leukemia, OTC is not recommended owing to the high risk of malignant cell reintroduction (Dolmans *et al.*, 2010). However, for very young women, OTC should be considered given the future advances in in vitro folliculogenesis, when a highly gonadotoxic chemotherapy is planned.

Other Gynecologic Cancers

Ovarian cancer is a rare cause of cancer among women of reproductive age. In stage 1, conservative surgery could be performed. Likewise, in Borderline tumors, conservative surgery should be offered if possible and ovarian stimulation is often possible after surgery in order to cryopreserve oocytes and/or embryos (Daraï *et al.*, 2013). OTC is not recommended owing to the risk of reintroducing malignant cells.

For endometrial cancer in early stage, fertility-sparing treatment using progestin could be introduced for women in reproductive age instead of radical surgery. Patients should follow-up with hysteroscopy and endometrial sampling. Then, patients could obtain pregnancy (spontaneous or medically assisted). When the parental plan is achieved, hysterectomy should be performed.

Pelvic Radiotherapy

Pelvic irradiation can be indicated in colo-rectal or cervix cancers. The option is oocytes and/or embryos freezing associated with ovarian transposition. This last procedure is hard to realize with stimulated ovaries. Ovarian transposition could only preserve endocrine function.

OTC should be discussed.

Patients must be informed that pelvic irradiation is associated with a high morbidity when pregnancy occurs (spontaneous miscarriages, preterm labor and delivery, low birth weight, and placental abnormalities).

For cervical cancer, in case of nonconservative surgery, patients should turn toward adoption or surrogacy. Uterus transplantation is an experimental technique with few live births reported worldwide and with high complications (Brännström *et al.*, 2015).

Medical Indications

Women at Risk of Premature Ovarian Insufficiency

Premature ovarian insufficiency is defined by resumption of cycle before the age of 40: primary or secondary amenorrhea >4 months with low level of estradiol (<50 pg/mL) and high level of gonadotrophins (FSH $>$ 25UI/L on two analysis spaced of 1 month). It concerns 1% of women (Nelson, 2009). Early exhaustion of ovarian follicle stockpile can be due to chromosomic, genetic, or auto-immune diseases as well as consequence of surgery, chemotherapy or radiotherapy. However, in <70%, POI appear idiopathic with no cause identified. Among chromosomal abnormality associated with POI, the most common is Turner syndrome.

Turner syndrome

During Turner syndrome, primary or secondary POI is due to premature and accelerated exhaustion of the follicular stockpile. Various phenotypes are encountered owing to the possibility of mosaïcism. Natural pregnancies are observed among 5.6% of patients with 30% risk of miscarriages (Bernard *et al.*, 2016). Pregnancies with TS is associated with obstetrical complications (caesarian section, hypertension, pre-eclampsia, …) and fetal abnormalities (Hovatta, 2012). Pre-conceptional evaluation is needed in order to diagnose cardiovascular contra-indications to pregnancy. If pregnancy occurs, specialized follow-up is required.

Until now, egg donation was the most common option for TS women to achieve pregnancy. Given the noteworthy advances in FP, ovarian stimulation could be considered in order to cryopreserve oocytes for TS young women with spontaneous puberty. When ovarian reserve is already reduced with risk of ovarian unresponsiveness to hormonal stimulation, natural cycle could be planned. During childhood, when follicles are still present, ovarian tissue cryopreservation should be discussed (Oktay *et al.*, 2016).

Whatever the technique use, the patient and/or their parents should be informed of the lack of data concerning gametes or ovarian tissue competency in this context. Moreover, the utilization of gametes freezing will be function of the patient status.

FMR1 premutation

Females carrying a premutation do not present any sign of the classical Fragile X symptoms, but 20% of them will experience a primary ovarian insufficiency (POI) leading to infertility before the age of 40 (Sherman, 2000). FMR1 premutation is more often encountered in case of familial history of POI and the highest risk of POI is observed for premutation alleles in the range of about 80–100 repeats (Allen *et al.*, 2007). Oocyte vitrification after ovarian stimulation is possible for premutated women given the risk of POI. Pre-implantation diagnosis could be proposed to patients.

Others genes are associated with POI such as BMP15, SF1, NOBOX, FOXL2. Identifying mutations should lead to fertility counseling and discussion about FP options.

Congenital galactosemia

Congenital galactosemia is a rare metabolic disease causes by abnormalities of galactose metabolism. Despite diet without galactose, most girls will develop POI (Rubio-Gozalbo *et al.*, 2010), due to a reduced number of primordial follicle at birth and an accelerated loss of follicles (Rubio-Gozalbo *et al.*, 2010). Patients are encouraged not to postpone pregnancy. Moreover, oocyte vitrification should be discussed after puberty. In the same way as for TS girls, ovarian tissue preservation could be discussed for prepubertal girl before exhaustion of follicles stockpile.

Auto-Immune Diseases

The natural history of systemic disease such as systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), scleroderma, Sjögren syndrome, vascularitis often encounter fertility issues. Indication of FP is clearly established when a gonadotoxic treatment, most of the time alkylating agents, such as cyclophosphamide (CTX), is planned. The gonadotoxicity of CTX depend on dose and age at time of treatment (Carré-Pigeon and Schubert, 2007). Moreover, a delay of 6 months is necessary after the end of treatment and conception owing to the risk of congenital abnormalities. Other treatments like methotrexate or corticosteroids are not gonadotoxic and there is no data concerning monoclonal antibodies or anti-TNF.

Before initiating a treatment with CTX, patients should be counseled about the risk of ovarian insufficiency. The minimal dose sufficient should be employed and FP be discussed. Oocytes vitrification after ovarian stimulation is possible but could be contraindicated in case of antiphospholipid syndrome for example. It should be emphasized that FP in such a context often requires a multidisciplinary management.

If the indication to FP in case of alkylating treatment is well established, the use of FP technique for auto-immune disease is still debated. It is not clear if all systemic diseases per se lead to diminished ovarian reserve. Ovarian reserve exhaustion is mostly the consequence of alkylating agents. However, some disease such as lupus polyarthritis rheumatoid, Behçet disease and spondyloarthritis are associated with diminution of the ovarian reserve regardless of the treatment received. At least, sarcoidosis, granulomatosis vascularitis and histiocytosis could lead to hypogonadotropic hypogonadism due to hypophysis infiltration. Moreover, patients should be advised that the use of cryopreserved oocytes/embryos will be conditioned by evaluation of the risk of pregnancy obstetrical adverse outcomes.

Systemic lupus erythematosus

It is well known that infertility is associated with APS and SLE (Carp *et al.*, 2012) via ovarian failure, implantation failure and pregnancy losses. But, it is not proven that SLE per se is responsible for an exhaustion of primordial follicle stockpile (Andreoli *et al.*, 2017). Data on AMH levels in lupus women are conflicting, but many studies suggesting a lower ovarian reserve in those patients. AMH appeared to be reduced in pre-menopausal lupus patients during reproductive age compared to controls, even in women never treated with CTX and despite mild disease activity, suggesting a decreased ovarian function in lupus women regardless of a regular menstrual cycle (Lawrenz *et al.*, 2011; Ma *et al.*, 2013). Phenomenon of auto-immune ovaritis could be hypothetized. On the other hand, other authors found no difference in AMH between female patients and controls (Morel *et al.*, 2013). Recently, Gasparin *et al.* (2016) reported similar levels of AMH in 40 pre-menopausal women and 40 healthy controls, even after adjusting for age. It appears not clear whether SLE per se has a negative influence on ovarian reserve, regardless of the exposure to cytotoxic treatment. In a cross-sectional study on 216 lupus women, AMH levels were found to correlate with age and cumulative dose of CTX (Mok *et al.*, 2013).

To conclude, women with a diagnosis of APS and/or SLE should be properly counseled on fertility, especially on the negative impact of disease-unrelated factors as tobacco use, alcohol consumption and increasing age. According to EULAR recommendations, when multiple risk factors for impaired fertility coexist, ovarian reserve should be assessed in female lupus patients at a younger age than recommended for the general population (Andreoli *et al.*, 2017). If an alkylating agent is indicated, cryopreservation of oocytes/embryos is an option (Henes *et al.*, 2012). GnRH agonists could be introduced prior to initiation of CTX (Brunner *et al.*, 2015). IVF techniques with ovarian stimulation are safe and efficacy for women with quiescent SLE and/or APS (Orquevaux *et al.*, 2017; Bellver and Pellicer, 2009) with few flare of disease and thrombo-embolic events. In case of positive aPL, a prophylaxis should be initiated with low molecular weight heparin and/or low dose of aspirin and will be stop at least 12 h prior to oocytes pick-up (Andreoli *et al.*, 2017). Ovarian hyperstimulation should be avoided using antagonist protocol with GnRHa triggering. In case of active disease with contraindication to ovarian stimulation, IVM should be indicated avoiding any ovarian stimulation and its detrimental consequences.

Diseases Unresponsive to Immunosuppressive Therapy

It could concern hematological diseases such as sickle cell anemia, thalassaemia major or plastic anemia. When HSCT is indicated, oocytes vitrification must be proposed and/or OTC depending on the gonadotoxicity expected of the conditioning treatment. In case of ovarian stimulation, thrombosis risk must be evaluated due to the hyperestrogenia induced and a prophylaxis could be recommended.

Endometriosis and Benign Ovarian Disease

Endometriosis is a frequent hormone dependent disease encountered in around 5%–10% of women in reproductive age (Coccia *et al.*, 2010). Endometriosis can cause infertility, pain and premature ovarian insufficiency.

Various mechanisms can lead to infertility: (1) reduced number of antral follicle consequence of direct toxicity of endometriosis on healthy adjacent ovarian tissue occasioning burn out effect and mechanical distension, and iterative ovarian surgeries with the removal of healthy adjacent ovarian tissue because endometriomas are coherent to the ovary and it turns out to be hard to separate capsule and healthy ovarian tissue, (2) chronic pelvic inflammation, (3) adhesions and anatomic changes, (4) tubal alteration, (5) and adenomyosis often encountered during this disease (Vercellini *et al.*, 2014). As a consequence, several women with endometriosis will require ART to obtain pregnancy. This pathology tends to recur after surgical treatment (Donnez *et al.*, 2012a). The alteration of ovarian reserve manifests as a decrease in AMH level observed immediately after surgery when bilateral (Raffi *et al.*, 2012).

PF for women with endometriosis remains controversial. More and more teams include FP in endometriosis management but without existing consensus. According to Somigliana *et al.* (2015), women with bilateral endometriomas, recurrent cysts or iterative surgeries might benefit of PF especially for women more than 35 years or presenting risk factors for POI.

Elizur *et al.* (2009) reported the first case of oocytes cryopreservation in a young woman with endometriosis in 2009. Some authors have suggested to use ovarian tissue cryopreservation for endometriosis (Oktay and Oktem, 2010). In 2012, Donnez et al., have reported the first case of live birth after grafting of ovarian tissue retrieved during bilateral ovariectomy for endometriosis (Donnez *et al.*, 2012b). There is no data on the risk of disease recurrence when transplantation of ovarian tissue.

The first step of PF in the field of endometriosis consists in minimizing ovarian surgery with reasonable indication of surgical treatment in order to preserve ovarian reserve. When surgery is required, the intervention should be performed by experienced surgeons with minimal surgery energy preventing thermal injury during hemostasis procedure and when it is possible cystectomy should be performed rather than ovariectomy (Roman *et al.*, 2011).

Otherwise, oocyte vitrification should be discuss when it is possible. Realization of ovarian stimulation in order to cryopreserve oocyte should be undertaken when ovarian reserve is not too much compromise ovarian reserve. Indeed, ovarian response is diminished after preexisting ovarian surgery (Somigliana *et al.*, 2009). The presence of large endometriomas (≥ 5 cm) at time of IVF significantly decreases the number of oocyte retrieved compared with the contralateral healthy ovaries (Ferrero *et al.*, 2017). But, most of the time, women are referred too late with and ovarian reserve is yet reduced compromising de facto the efficacy of oocytes vitrification procedure. Several ovarian stimulations could be done in order to obtain a sufficient oocytes number but with increasing in both cost and impact of procedure.

Moreover, ovarian stimulation appear to be safe in endometriosis context with few complications, particularly no increased risk of ovarian abscess after oocytes harvesting in this context (Benaglia *et al.*, 2008). The risk of pain during hormonal treatment should be explained to women. Literature data are scarce but reassuring concerning complication occurring during ovarian stimulation in endometriosis context as well as the risk of recurrence (Coccia *et al.*, 2010).

To conclude, women with endometriosis diagnosis should be counseled about PF possibilities. Further studies should be done in order to evaluate the benefice of FP for endometriosis regarding cost-effectiveness aspect, impact on the use of egg donation, on the number of IVF performed for elderly women. It is important to define which group of endometriosis patient would benefit from PF.

Benign Gynecologic Diseases

Every pelvic surgery could lead to diminution of the follicular stockpile without alteration of oocytes quality. When a risk of recurrences exists (teratoma, endometriosis …), FP should be discussed. In case of single ovary, the risk of stimulation with ovarian torsion should be underlined. Oocyte vitrification could be indicated for women at risk to develop POI: bilateral cysts, unilateral with single ovary or recidive on remaining ovary. It could be performed before or after surgery depending on predictive response to ovarian stimulation and ovarian accessibility.

Gender Reassignment Procedures

A counseling about FP is recommended before starting hormone therapy or undergoing surgery to remove reproductive organs' even at a younger age (Martinez and International Society for Fertility Preservation–ESHRE–ASRM Expert Working Group, 2017). FP techniques should be undergone prior to puberty blockage in adolescents and before treatment with cross-hormones in both adolescents and adults (Committee on Adolescent Health Care, 2017). Currently, there is no data in prepubertal or pubertal adolescents who will never develop reproductive function in their natal sex.

Oocytes Banking for Anticipated Gamete Exhaustion

In many countries, oocytes vitrification is available for women wanting to postpone maternity due to the decline of follicle stockpile with aging. The goals are to bank oocytes and to anticipate gamete exhaustion (Stoop *et al.*, 2014b). The spreading of this "societal" FP could lead to a diminished use of egg donation.

Conclusion

Given recent improvements in terms of techniques and results, fertility preservation is now offered in numerous situations threatening ovarian reserve. Appropriate counseling by trained team should be provided to candidates for fertility preservation in order to support them in making decisions. Ethical issues should always been considered and further researches are warranted.

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Female Hormonal Contraception

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Abbreviations

CHC	Combined hormonal contraceptives	**VTE**	Venous thromboembolism event
EE	Ethinyl-estradiol	**POC**	Progestin only contraceptives
IUD-LNG	Intra-uterin device with levonorgestrel	**OR**	Odd ratio
		IC	Confidence interval

Introduction

Female hormonal contraceptives are one of the most commonly proposed birth control methods, used by several million women worldwide (United Nations, 2013). The use of contraceptive methods aims to prevent the occurrence of an unwanted pregnancy during a given period and this, reversibly. The ideal contraception should be totally effective, acceptable, perfectly well tolerated, while preserving the subsequent fertility (United Nations, 2013). Despite a very wide choice of contraceptive methods currently available, such contraception does not exist.

Initially, combined hormonal contraceptives (CHC) delivered a daily dose of 150–100 μg of ethinyl-estradiol (EE) or mestranol and a progestin such as norethisterone acetate or norethindrone (Christin-Maitre, 2013). Due to results having early showed that these drugs increased the risk of cardiovascular disease, formulations of CHC have dramatically changed over the past 50 years (Bitzer and Simon, 2011). Subsequent developed newer-generation progestins have resulted in a stronger progestogenic activity and decreased androgenic effects such as acne, hirsutism and deleterious lipid changes (Sitruk-Ware and Nath, 2010). Finally, nonoral delivery methods represent a recent advancement in CHC, including the contraceptive vaginal ring and the transdermal contraceptive patch.

These different types of hormonal contraceptives have advantages and disadvantages. The main deleterious effect of combined estrogen/progestin contraception remains vascular effects. The impact on the risk of cancer is either protective or deleterious. Finally, noncontraceptive benefits are important in some clinical circumstances. After a review of classification of hormonal contraceptive, this article details these effects.

Classification of Hormonal Contraceptives

It is important to distinguish combined hormonal contraceptives (CHC) and progestin only contraceptive (POC), each with their indications, contraindications and potential side effects.

Combined Hormonal Contraceptives

Composition

These contraceptions combine two types of molecules: an estrogen (EE or estradiol) and a progestin. The latter is classically classified into generation (first, second, third) or "other progestins," depending on their date of commercialization and their pharmacological properties (**Table 1**). The estrogen-progestin balance depends on the dose and the type of the two hormones. This balance induces very different hormonal climates. The different indications and side effects derive directly from it. The early CHC consisted of high doses of synthetic estrogen (150 μg of EE or mestranol) and androgenic progestin such as norethisterone acetate or norethindrone. The contemporary CHC now deliver 50–15 μg per day of EE or 1–3 mg of estradiol or estradiol valerate (Bitzer and Simon, 2011). Progestogens used in combined hormonal contraception are mainly progestins derived from 19 nortestosterone (norsteroids). Derivatives of norethisterone, norethisterone acetate, lynestrenol, ethynodiol acetate or norethynodrel are the first generation and are no longer used. The currently available oral contraceptives are both 2nd and 3rd generation pills. Second generation pills contain norgestrel or levonorgestrel. Since the beginning of the 1980s, newer progestins (desogestrel and gestodene) consist of the 3rd generation pills (Sitruk-Ware and Nath, 2010). Norgestimate is a progestin, initially classified as 3rd generation but it is probably between 2nd and 3rd generation. These compounds have significantly reduced the EE dose due to their higher antigonadotropic activity. Moreover, their clinical use is theoretically associated with less androgenic adverse effects. Drospirenone, an aldosterone antagonist, and cyproterone acetate are molecules providing high-antiandrogenic effects and are classified as other generation pills with chlormadinone acetate and dienogest (Sitruk-Ware and Nath, 2010). These products have theoretically no androgenic properties and are antiandrogens for drospirenone and cyproterone acetate (Sitruk-Ware, 2004).

Classification according to generations is irrespective of EE doses and is thus debated. In January 2014, The EMA published a new classification depending on specific molecule of progestin and not on generation (EMA, 2014).

Table 1 Classification of different types of CHC

Types	*Molecules of estrogen*	*Molecules of progestin*	*Route of administration*
First generation	EE	Norethisterone	Oral
Second generation	EE	Norgestrel or levonorgestrel	Oral
Between 2nd and 3rd generation	EE	Norgestimate	Oral
Third generations	EE	Desogestrel	Oral
		Gestodene	
Others generations	EE	Drospirenone	Oral
		Cyproterone acetate	
		Chlormadinone acetate	
	Estradiol	Dienogest	Oral
	Estradiol valerate	Nomegestrol acetate	
	EE	Norelgestromin	Transdermal patch
	EE	Etonogestrel	Vaginal ring

Finally, hormonal contraceptives can be also classified according to the route of administration and include nonoral administered drugs such as the combined EE/norelgestromin transdermal patch and EE/etonogestrel vaginal ring. These two new methods provide continuous hormone dosing and simplify compliance.

Mechanism of action

The mechanism of action of these combined pills is mainly through the inhibition of ovulation, the antigonadotropic effect resulting in the absence of LH and FSH peaks. Moreover, the pill induces an inhibition of the follicular growth, a thickening of the cervical mucus by the progestin and an atrophy of the endometrium which becomes unfit for a possible implantation (Black *et al.*, 2015).

Progestin Only Contraception

Composition

Although the original development of oral contraceptives focused on progestin-only products, current POCs are less used than CHC because of its poorer uterine tolerance. Indeed, the menstrual cycle is less well controlled and bleeding such as spotting are common (Grimes *et al.*, 2013). However, POCs are contraceptives which may be an attractive option for women with contraindication for CHC. Several routes of administration are available. Four types of progestin-only contraceptives are currently available (**Table 2**):

- Progestin only pill that deliver low daily doses of progestin (Norethindrone, Levonorgestrel or desogestrel).
- Injectable 3-month contraceptives (depot medroxyprogesterone acetate [DMPA]) administered intramuscularly.
- Levonorgestrel implant or more recently etonogestrel single-rod implant which provide effective contraception for 3 years.
- Intrauterine system containing levonorgestrel (SIU-LNG), effective for 5 years or 3 years for the shorter device.

Mechanism of action

The contraceptive mechanisms are essentially peripheral action exerting an antimucus effect, antinidatory and by modification of tubal mobility (Black *et al.*, 2015). An atrophy of endometrium is associated with the use of SIU-LNG. An antigonadotropic effect is observed in some women using desogestrel only pill, but not all. The contraceptive implants (levonorgestrel or desogestrel) and the intra-uterine device delivering small doses of levonorgestrel, are called long acting reversible contraception. Its major advantage is perfect compliance and is therefore indicated in women who frequently forget their pill.

Efficacy of Hormonal Contraceptives

The effectiveness of a contraceptive method is measured by the Pearl index which corresponds to the ratio of the number of pregnancies per 100 women after 12 months of use. It is expressed in percentage of years. **Table 3** summarizes the Pearl indices for each method (Trussell, 2011).

Hormonal Contraceptives and Vascular Risk

Venous Thrombosis Risk

Venous thrombosis event (VTE) is a common disease, with an annual incidence of 1–4 per 1000 persons. Before menopause its incidence is low but strongly increases with age (Oger, 2000; Naess *et al.*, 2007). Among pill users, annual VTE incidence is 10 times more common than arterial events (Lidegaard *et al.*, 2009). Since early 1960s, it has been clearly shown that CHC increased

Table 2 Type of progestin-only contraceptives

Molecules	*Route of administration*	*Efficacy duration*	*Progestin doses (mg)*
Norethindrone	Oral	24 h	0.35
Levonorgestrel			0.03
Desogestrel			0.075
Etonogestrel	Subcutaneous implant	3 years	68
Levonorgestrel	Intrauterine	5 years	52
		3 years	13.5
Medroxyprogesterone	Intra muscular	3 months	150

Table 3 Efficacity of hormonal contraceptives

Methods	*Percentage of women experiencing an unintented pregnancy during the first year of use*	
	Typical use	*Perfect use*
No methods	85	85
Oral CHC, patch, vaginal ring or oral POC	9	0.3
Progestin implant	0.05	0.05
MPA injectable	3	0.3
SIU-LNG	0.2	0.2

the risk of VTE including deep vein thrombosis, pulmonary embolism or venous cerebral thromboembolism. Hormonal contraceptive use explains a substantial part of the venous thrombotic events among childbearing-aged women and VTE is the most important determinant of the benefit/risk profile of hormonal contraceptive. Several metaanalysis have evaluated the VTE risk according to the types of CHC and POC.

Oral combined hormonal contraception

The increase in thrombotic risk is the highest the first year of CHC use and 3rd generation pill use is associated with an increased VTE risk as compared to 2nd generation pill use (OR: 1.7; 95% confidence interval 1.4–2.0) (Plu-Bureau *et al.*, 2013; Stegeman *et al.*, 2013). Specific molecules combined with EE (drospirenone or cyproterone acetate) have been now investigated. Current users of drospirenone or cyproterone acetate containing CHC are at increased risk of VTE by around 1.5–2.2 compared with levonorgestrel users (Plu-Bureau *et al.*, 2013; Stegeman *et al.*, 2013). Many biological studies have consistently shown blood coagulation activation among pill users and thus providing a biological support to the increased thrombotic risk associated with the contraceptives use (Rosing *et al.*, 2001). The third generation progestins, drospirenone and cyproterone acetate in combination with EE appear to induce a resistance to activated protein C (APC) more pronounced than biological changes observed with 2nd generation pill use (Rosing *et al.*, 1999; Kemmeren *et al.*, 2004). Sex hormone-binding globulin (SHBG), which has been recently positively correlated to APC resistance among pill users (Odlin *et al.*, 2002; Raps *et al.*, 2012), would also be another useful marker to estimate the thrombotic safety of a CHC. Overall, estrogenic climate of a contraceptive pill could be estimated by plasma SHBG and could reflect the level of thrombotic risk associated with this pill use (Raps *et al.*, 2012; Gerstman *et al.*, 1991).

The risk associated with contraceptives containing norgestimate is debated (Plu-Bureau, 2015). Indeed, the level of risk seems similar to that of levonorgestrel. This is in accordance with metaanalysis of nine published studies regarding VTE risk associated with CHC containing norgestimate which shows no difference in risk as compared with levonorgestrel (Dragoman *et al.*, 2018). However, according to data on sex hormone binding globulin and activated protein C-resistance, similar VTE risks between norgestimate and levonorgestrel CHC are not biological plausible (Hugon-Rodin *et al.*, 2014), since studies on hemostasis markers show more deleterious changes in hemostasis among users of norgestimate than levonorgestrel. These discrepancies between epidemiological results and biological data need further research.

Nonoral combined hormonal contraception

Two methods of nonoral combined contraceptives are available such as the combined ethinyl-estradiol/norelgestromin transdermal patch and ethinylestradiol/etonogestrel vaginal ring. These contraceptives have recently been evaluated on thrombotic risk. In the context of contraceptive use, nonoral route of EE administration seems to be more thrombogenic than oral route. A recent metaanalysis shows an overall assessment of VTE risk with a significant OR of 1.5 (95% CI: 1.2–1.8), suggesting that contraceptive patch is associated with a higher thrombotic risk than CHC containing the same progestin (Plu-Bureau *et al.*, 2013; Lidegaard *et al.*, 2012; Tepper *et al.*, 2017). The vaginal ring delivering levonorgestrel seems to be also associated with an higher VTE risk as compared to 2nd generation pills that contain the same progestogen (pooled OR = 1.7; 95% CI: 1.3–2.3) (Plu-Bureau *et al.*, 2013). These results are in agreement with biological data. In a randomized cross-over study aimed to compared the impact of oral

and vaginal EE administration on haemostatic variables and estrogen-sensitive liver proteins, we showed that vaginal EE was associated with similar decreased activity of the coagulation inhibitory system and similar increased SHBG levels then oral route (Sitruk-Ware *et al.*, 2007). In addition, few years later, other studies found that APC resistance was higher among users of the vaginal ring than among users of contraceptives containing levonorgestrel (Fleischer *et al.*, 2009; van Vliet *et al.*, 2010; Kluft *et al.*, 2008).

Progestin only contraceptives

Progestin only pill is a good alternative of contraception for women with contraindication of estrogen use. By contrast, low doses of both oral progestin contraceptives and intra-uterine levonorgestrel could be safe with respect to VTE risk (Blanco-Molina *et al.*, 2012; Tepper *et al.*, 2016). A recent metaanalysis has shown no increase in VTE risk among users of oral POC as compared to nonusers (OR = 1.45; 95% CI: 0.92–2.26) (Blanco-Molina *et al.*, 2012). On contrary, the use of medroxyprogesterone acetate intramuscular contraception is associated with an increased VTE risk (OR 3.2; 95% CI: 1.8–2.7) (Plu-Bureau *et al.*, 2013). This increase in VTE risk could be consistent with deleterious glucocorticoid effect of MPA on vascular risk factors. The impact of levonorgestrel IUD has been investigated and pooled OR show a significant decrease in VTE risk as compared with nonuse (OR = 0.6; 95% CI: 0.4–0.8). Finally, the Danish study provided the first assessment of the VTE risk in relation to the etonogestrel implant use (Lidegaard *et al.*, 2012) but the low sample size (only 5 cases of confirmed VTE events occurred among 29,497 person-year) did not allow concluding that etonogestrel was not associated with an increased thrombotic risk (OR = 1.4; 95% CI: 0.58–3.38).

Women with biological thrombophilia

Congenital thrombophilia increases the risk of VTE. Two metaanalysis have evaluated the VTE risk associated with CHC use and biological thrombophilia. Compared to nonusers without thrombophilia, the risk of VTE among CHC users was 15.6 (95% CI: 8.7–28.1) for carriers of factor V Leiden, 12.6 (95% CI: 1.4–115.8) for antithrombin deficient, 6.3 (95% CI: 1.7–23.9) for protein C deficient and 4.9 (95% CI: 1.4–17.1) for protein S deficient. This comparison did not reach the significance for carriers of G20210A prothrombin mutation because of a too low sample size (OR = 6.1; 95% CI: 0.8–45.6) (Wu *et al.*, 2005). In the most recent metaanalysis, the VTE risk among carriers-CHC users was sixfold as compared with noncarriers-CHC users (Van Vlijmen *et al.*, 2016). Such observations have led international guidelines to advise against the use of CHC in women with known biological thrombophilia.

Arterial Risk

Combined hormonal contraceptives

Arterial disease is an uncommon disease among childbearing-aged women. Annual incidence of myocardial infarction (MI) is 2 per million among healthy women aged 30–34-year-old and rises to 20 per million between 40 and 44 year (Farley *et al.*, 1999). With respect to stroke, the annual incidence also increases with age (6 per million at age 20–24, 10 per million at age 30–34, and 16 per million at age 40–44) (Farley *et al.*, 1999). But given the potential consequences of these arterial diseases, these are an important determinant of the benefit/risk profile of hormonal contraception.

Most epidemiological studies have shown an increased risk of myocardial infarction and stroke in users of CHC. Overall, current use of oral combined contraceptives increased the risk of MI and ischemic stroke (pooled OR: 1.7; 95% confidence interval [95% CI]: 1.2–2.3 and OR: 1.8; 95% CI: 1.2–2.8, respectively), but was not associated with the risk of hemorrhagic stroke (Plu-Bureau *et al.*, 2013). The increase in ischemic arterial disease was higher among 1st generation pill users as compared to 2nd or 3nd generation pill users. By contrast, the risk of ischemic arterial disease among current users of 2nd or 3nd generation pill was similar for MI risk and for ischemic stroke. In conclusion, newer generation formulations of CHC as well as the nonoral hormonal contraceptive do not seem to be safer than 2nd generation hormonal contraceptives. Surprisingly, a more recent network metaanalysis (Roach *et al.*, 2015) with selected articles and including 24 studies show that CHC users were not at increased risk of myocardial infarction or ischemic stroke compared with nonusers (OR 1.0; 95% CI 0.9–1.0). The risks did not vary according to the generation of progestogen or according to progestogen type. However, when the authors stratified preparations according to estrogen dose, the risk of myocardial infarction or ischemic stroke seemed to increase with higher doses of estrogen. These two metaanalyses are not comparable because included studies are not the same. In our review, the included studies were set up after 1990 and only assessed preparations containing low doses of estrogen.

Progestin only contraceptives

Very few studies have evaluated arterial risk in relation to the use of progestin-only contraceptives. Two metaanalyses were performed on the risk of IDM and stroke. These do not show an increase in arterial risk associated with the use of this type of contraception (Chakhtoura *et al.*, 2011; Chaktoura *et al.*, 2009).

Arterial risk factor

Metabolic adverse effects include changes in the lipid profile, carbohydrate and blood pressure. The effect of combined contraception on lipids depends on the dose of estrogen and the type of progestogen used in its composition (Sitruk-Ware and Nath,

2013). If it is an estrogen-dominated pill, there is a larger increase in triglycerides compared to progestin-only pills. This increase still exists with the pills dosed at 20 μg EE, as well as an increase in HDL-cholesterol.

Some oral contraceptives may cause some degree of insulin resistance and hyperinsulinism. These are primarily oral contraceptives with a high dosage of estrogen and progestin derived from 19-nortestosterone. The contemporary contraceptives have limited effects on carbohydrate metabolism (Sitruk-Ware and Nath, 2013).

The frequency of arterial hypertension under estrogen/progestogens is poorly evaluated. However, there is a moderate increase in blood pressure by stimulation of renin in about 5% of cases (Tepper *et al.*, 2013). But there are large individual variations in this response. For some authors, oral contraceptives most often only reveal or aggravate a predisposed terrain. The risk of high blood pressure due to oral contraceptives increases with age, becoming of real concern only after age 35.

In practice, the evaluation of arterial risk factors is important. **Table 4** summarizes the risk factors to be assessed before prescribing any CHC.

Hormonal Contraceptives and Others Risks and Benefits

Risk of Cancers

Breast cancer is the most common cancer in terms of incidence, although events are very rare at the ages of use of CHC. Other cancers are also important to evaluate the benefit balance of hormonal contraception.

Breast cancer

The metaanalysis of the Collaborative Group on Hormonal Factors in Breast Cancer published in 1996 showed a slight increase in breast cancer risk associated with the use of CHC (OR 1.24; 1.15–1.33; 95% IC) (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Studies published after this metaanalysis showed overall similar results (Cibula *et al.*, 2010). The recently published Danish study evaluates contemporary contraceptives and provides valuable information on this level of risk (Morch *et al.*, 2017). The relative risk of breast cancer associated with all current and recent users of hormonal contraceptives was 1.20 (1.14–1.26; 95% IC). This risk varies between 1.0 and 1.6 according to the type of CHC. This risk is similar with the use of progestin only pills. This slight increase of risk must be put into perspective with the absolute risk. Thus for a woman younger than 35 years of age, the absolute increase in breast cancer would be 2 per 100,000 persons–year.

Others cancers

The use of combination pills would reduce the risk of endometrial cancer by approximately 50% (Collaborative Group on Epidemiological Studies on endometrial Cancer, 2015). In some studies, the greatest reduction in risk has been observed for women using highly progestin-only pills. Moreover, it should be noted that the use of oral contraception reduces the risk of ovarian cancer by about 40% (Collaborative Group on Epidemiological Studies of Ovarian Cancer *et al.*, 2008). This effect increases with duration of use and persists for several years after stopping oral contraception. Hormonal contraceptives are also associated with a decreased risk of colorectal cancers (Bosetti *et al.*, 2009).

Finally, a recent study have suggested that the global effect of the use of CHC for 5 years or longer is a slight reduction in the total risk of cancer (Bassuk and Manson, 2015).

Hormonal Contraceptives and Noncontraceptive Benefits

Hormonal contraceptives offer noncontraceptives benefits. Two recent reviews have evaluated the different clinical entities associated with a benefit impact of CHC or POC (Schindler, 2013; Bahamondes *et al.*, 2015). Clinical effectiveness of CHC has been shown for the treatment or the prevention of menstrual bleeding disorders. The use of SIU-LNG controls heavy menstrual bleeding

Table 4 Arterial and venous risk factors

Arterial risk factors	*Venous risk factors*
Age >35 years	Age >35 years
Dyslipidemia	Overweight-obesity
Smoking	Biological thrombophilia
Diabetes	Familial history of VTE
Overweight-obesity	
Hypertension	
Migraine with aura	
Familial history of myocardial infarction or ischemic stroke	

and the subsequent associated anemia. CHC is currently used for alleviating menstrual related pain symptoms (dysmenorrhea) and is the first choice treatment of endometriosis. Moreover, CHC are useful to improve symptoms of hyperandrogenia like that acne, hyperseborrhea, hirsutism, and some alopecia.

In Practice

Before the prescription of hormonal contraceptives, the research of vascular risk factor is the main key of the first medical consultation of contraception.

The clinical examination must include at least blood pressure measurement and body mass index calculation. The gynecological examination is not always necessary, in particular for the young woman.

The choice of contraceptive method suitable for each woman is possible given the wide choice of hormonal contraceptives available. The WHO publication of the medical eligibility criteria for contraception use allows help for the prescription of contraception in various clinical situations (WHO, 2015).

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Progesterone Receptor Modulators: Current Applications and Perspectives

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Introduction

The development of progesterone receptor modulators (PRMs) (**Table 1**) started in the early 80's, when mifepristone, the lead compound, allowed medical management of unwanted pregnancies (Christin-Maitre *et al.*, 2000). Further development in search for additional potential fields of application has been hampered for some time by political interferences (Bouchard, 2014; Bouchard *et al.*, 2011; Chabbert Buffet *et al.*, 2012; Ulmann, 2000; Ulmann *et al.*, 1990). PRMs are now used also for emergency contraception (since 2009 in Europe and United States) and treatment of myoma-related symptoms (since 2014). Further applications are currently under evaluation, and these compounds are likely to play a considerable role in Gynecology.

SPRMs Development and Mechanisms of Action

Initially synthesized in 1980 at Roussel Uclaf company, (RU 486 or mifepristone) for the treatment of depression, based on its antiglucocorticoid capacity, the antiprogesterone activity of mifepristone and its potential applications in obstetrics and gynecology was subsequently developed under the direction of Etienne Baulieu. Mifepristone was approved in France in 1988 in medical termination of pregnancy, in combination with a prostaglandin.

Progesterone (P) regulates uterine maturation for implantation and sustains pregnancy, but also exerts multiple roles in physiology through its nuclear receptor (PR) isoforms A and B (Bouchard, 2014; Bouchard *et al.*, 2011; Chabbert Buffet *et al.*, 2012). Progesterone can stimulate breast cell proliferation while it can exert antiproliferative effects in the uterus, preventing estradiol-induced endometrial growth.

It also regulates the GnRH pulse regulator by acting on KNDY neurones and the release of Kiss 1, the crucial regulator of GnRH pulse frequency (Bouchard, 2014; Ulmann *et al.*, 1990; Chabbert-Buffet *et al.*, 2000). While acute administration of P can trigger the release of GnRH, its continuous administration results is anovulation due to the slowing down of the GnRH pulse generator.

It became therefore tempting to study the effects of PRM into antagonizing progesterone known and unknown effects.

Table 1 Main PRMs and gynecology: in clinical use, currently or previously under development

Compound	*Therapeutic applications*	*Current status*
Mifepristone (RU-486)	Termination of pregnancy	Launched
	Emergency contraception	Phase III
	Long-term contraception	Phase II
	Uterine fibroids	Phase II
	Endometriosis	Phase II
	Endometrial cancer	Phase I
Ulipristal acetate (CDB-2914; VA2914)	Emergency contraception	Launched
	Uterine fibroids	Phase III
	Long-term contraception (vaginal ring)	Phase II
Asoprisnil (J-867)	Uterine fibroids	Withdrawn
	Endometriosis	Withdrawn
	Long-term contraception	Withdrawn
Telapristone acetate (CDB-4124)	Uterine fibroids	Phase III trials terminated
	Anemia	Phase I/II trial initiated
	Endometriosis	Phase III trials terminated
		Phase I/II trial initiated
		Phase II trials terminated
		Phase I/II trial initiated
Lonaprisan (ZK230211)	Breast cancer	Phase II
Onapristone (ZK98299)	Endometriosis	No development since the 1990s
	Cancer	
ORG-31710 & ORG-31806	Contraception	Preclinical; no development since the 1990s
	Cancer	

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Current applications of PRMs include termination of pregnancy and management of preterm and term delivery, emergency contraception and medical treatment of uterine myomas.

PRMs Use in Pregnancy

The introduction of mifepristone in obstetrics and pregnancy termination has provided a safe medical method of first trimester pregnancy termination, which is a potential major progress especially in health-care resource-deprived areas, where abortion is a source of maternal mortality. In other countries it has allowed the development of the procedure in outpatients for very early pregnancy termination, reducing costs and improving patients comfort. Mifepristone is currently available in over 30 countries (Christin-Maitre *et al.*, 2000) for pregnancy termination.

Prostaglandins, used in association to mifepristone for pregnancy termination, induce uterine contraction, resulting in embryo expulsion, while mifepristone impairs two crucial functions of progesterone during early pregnancy, synchronization of the endometrium and the conceptus, and myometrial contractions inhibition. The antiprogestin activity may also act on endometrial blood vessels, and compromise embryo development.

A review of the Cochrane database (Kapp *et al.*, 2010) has analyzed the efficacy of the different available protocols showing that combined regimens associating mifepristone and PgE1 analogs are more effective even with the mifepristone dose reduced to 200 mg. Finally misoprostol, a prostaglandin analog, is efficient using the vaginal route, although this has been associated with very rare cases of fatal septic shocks related to Clostridium Sordellii in the United States and in Canada (Niinimaki *et al.*, 2017).

Mifepristone also causes cervical dilation and can be used in the management of fetal miscarriage (Baev *et al.*, 2017) and full term labor management (Singh Susheela *et al.*, 2009).

SPRMs for Emergency Contraception

Globally, 55‰ of pregnancies are estimated to be unintended in women aged 15–44 (Singh Susheela *et al.*, 2009), resulting in termination of pregnancy or unwanted birth, with their associated personal and social burden, especially in countries where abortion is not available. Emergency contraception (EC) offers women an important strategy to prevent unintended pregnancy after intercourse in cases of contraceptive misuse, nonuse or failure, as well as in situations of sexual violence (Jones *et al.*, 2002). Although use of EC is constantly increasing, it remains limited (KFF Foudation, 2003). Available data on the reduction of abortion rates since EC was developed, and made available over-the-counter, have not demonstrated a marked reduction in abortion rates (ESHRE CapriWorkshop group, 2015). However, this may be due to insufficient knowledge about pregnancy risk, inappropriate use of EC or a gap between the theoretical and practical efficiency of EC in current conditions.

In a strategy to increase access to and safety of EC, hormonal EC initially based on the administration of high doses of combined oral contraceptives (Jatlaoui *et al.*, 2016), was then replaced by levonorgestrel only administration, which does not increase thrombotic risk and could therefore subsequently be released over the counter.

PRMs share with levonorgestrel the advantage of low to null thrombotic risk. Mifepristone (Baird *et al.*, 1995), Ulipristal Acetate (UPA) (Glasier *et al.*, 2010) interrupt the LH surge and eventually follicular rupture. Further research has shown that PRMs remain highly active to prevent ovulation up to 120 h after unprotected intercourse, while levonorgestrel is only 50% active after 72 h (Brache *et al.*, 2013b). UPA is at least as safe as LNG when taken up to 72 h after sexual intercourse (Glasier *et al.*, 2010; Cheng *et al.*, 2012; Creinin *et al.*, 2006; Richardson and Maltz, 2012).

Today mifepristone, despite its proven efficacy as an EC (Cheng *et al.*, 2012), is available for this indication only in China, Vietnam, Russia and Armenia, while ulipristal acetate (EllaOne 30 mg) is available worldwide, over the counter. Tolerance of LNG and UPA is good (Thomin *et al.*, 2014), with side effects in 9% of cases and comparable (Creinin *et al.*, 2006), mainly headache, dysmenorrhea, intermenstrual bleeding and nausea. There are no contraindications to LNG or UPA use for EC, except for hypersensitivity to the compound or its excipient and ongoing pregnancy (Creinin *et al.*, 2006). No metabolic side effects have been demonstrated with UPA (Cameron *et al.*, 2015) or levonorgestrel.

Different aspects of hormonal EC still need improvement or evaluation however.

PRMs activity on ovulation is restricted to the preovulatory period, and disappears if the LH surge is already ongoing (Brache *et al.*, 2013b). Due to their action on progesterone receptors, interaction of PRMs with subsequent use of estroprogestins or progestins may require caution in patients taking ongoing contraceptive medication (Salcedo *et al.*, 2013). While UPA has been shown to have no impact on the efficacy of subsequent estroprogestin use in one study (Cameron *et al.*, 2015), recent data show that subsequent use of continuous desogestrel reduces the efficacy of UPA as an EC (Brache *et al.*, 2015). On the other hand, UPA did not appear to have an impact on desogestrel efficacy. Currently, the use of barrier contraceptives is advisable for 7 days after EC use. Some enzyme inducers may reduce the effectiveness of UPA or LNG, as well as certain drugs that increase gastric pH (Pohl *et al.*, 2013a,b). Body mass index (BMI) was shown to have a significant impact on EC efficiency as well (Glasier *et al.*, 2010, 2011). The impact of obesity appears more pronounced with LNG compared to UPA. In obese women, UPA may thus be recommended instead of LNG if possible; alternatively, the use of a copper intrauterine device (Cu-IUD) is recommended.

SPRMs for Myoma-Related Symptoms

Uterine myomas, present in 70%–80% of women by age 50, are the leading cause of hysterectomy (400,000 every year in the USA), and their main complication bleeding, is responsible for a severe alteration of quality of life (Stewart, 2015; Chabbert Buffert *et al.*, 2014a). They are benign clonal smooth muscle cell tumors (Bulun, 2013), and estradiol is considered as the leading growth signal.

The effects of PRMs in women with uterine myomas associate two independent characteristics: rapid control of uterine bleeding and sustained reduction in myoma volume as initially shown by Anna Murphy and SSC Yen with mifepristone in a short term study (Murphy *et al.*, 1993). K Chwalisz, a pioneer in the field of PRM, obtained similar results with the PRM asoprisnil (Chwalisz *et al.*, 2005). However, as mentioned initially by Murphy et al., the long term administration of these PRMs created some nonphysiological changes in endometrium morphology with a pattern initially described as glandular hyperplasia.

The development of UPA for uterine myoma treatment was subsequently achieved in several steps showing the rapid cessation of uterine bleeding and the decrease in uterine fibroids (Chabbert-Buffet *et al.*, 2007; Donnez *et al.*, 2012a,b; Williams *et al.*, 2012; Donnez *et al.*, 2014, 2015, 2016). Daily administration of 5 or 10 mg UPA in normal women volunteers studied in a randomized controlled study of 3 months, confirmed the cessation of menstrual bleeding in 90% of women, the absence of ovulation in 80% of volunteers. Interestingly, estradiol levels were not blunted (Chabbert-Buffet *et al.*, 2007).

Treatment of women with symptomatic uterine myomas using 5 mg UPA for 3 months was evaluated in a randomized trial versus placebo (Pearl I) showing the rapid cessation of bleeding, within 7 days in 90% of treated women. In addition, fibroid volume decrease reached 50% within the 3 months of the study, and was sustained for 6 months following cessation of treatment, while normal menstruation resumed within a month following cessation of treatment (Donnez *et al.*, 2012a).

The study Pearl II was a randomized controlled trial, comparing the effect of UPA and Leuprolide, a GnRH agonist (Donnez *et al.*, 2012b). This study confirmed the results of Pearl I and showed that the rapidity of the cessation of bleeding was shorter under UPA. The decrease in uterine volume was similar in both groups, but was not sustained in the GnRH-agonist group compared to the UPA group. Finally, the GnRH-agonist treatment induced estradiol deprivation symptoms as compared to UPA. As with Asoprisnil, the key issue was the interpretation of the endometrial changes observed following the treatment. They are now widely recognized as PAECs (Williams *et al.*, 2012): PRM associated endometrial changes. The endometrium histology shows altered glandular architecture including extensive glandular epithelium dilatation. The epithelium appeared essentially inactive with rare mitoses. Mild reversible thickening rarely occurred (Williams *et al.*, 2012) (**Fig. 1**).

These changes proved to be reversible following cessation of therapy and associated with no sign of proliferation. Curiously enough, PAECs are also observed in a significant number of untreated patients. Their significance is unknown (Williams *et al.*, 2012).

UPA was initially approved as a preoperative treatment (3 months). In Pearl III study and extension (Donnez *et al.*, 2014), each course of UPA was followed by a sequence of Progestin: Norethisterone Enanthate (10 mg daily for 10 days), in order to limit the development of PAEC, believed to be a consequence of unopposed estradiol effects. This study showed that the progestin course does not change the appearance of PAEC, which were confirmed to be benign aspects more frequent after PRM treatment and disappearing after cessation of treatment.

In Pearl IV study (Donnez *et al.*, 2015) intermittent courses of 3 months treatment with UPA were simply separated by sequences of 2 normal cycles in 451 women. Eight courses of 3 months 10 mg UPA daily were finally administered in 64 women. This study showed 9.1% of PAEC after cessation of the study, similar to what is observed in untreated women. All histological analysis showed benign lesions. Endometrial thickness usually did not exceed 7 mm after course 5–8. This lead to the authorisation for intermittent long term therapy using 3 month courses separated by 2 cycles interval. This strategy is particularly useful in women with fibroids near the menopausal transition (Donnez *et al.*, 2016). Seven hundred thousands women have been treated so far. Recently the European Medicine Agency is reviewing few cases of severe liver injury. Such cases were never observed, neither in animal nor in preclinical studies. This toxicity seems linked to probable prescriptions in women with liver disease. Recommendations will be provided by the EMA by June 2018.

Perspectives

Perspectives for PRMs use include new molecules, new routes of administration and new indications (**Table 1**).

Vilaprisan is a new steroid PRM currently in development for myoma treatment (Seitz *et al.*, 2017; Schultze-Mosgau *et al.*, 2017; Schütt *et al.*, 2016) with a very similar profile as compared to ulipristal acetate.

PRMs are currently evaluated in intrauterine systems or vaginal rings for contraception. Data on intrauterine systems have been published in primates (Heikinheimo *et al.*, 2007; Nayak *et al.*, 2007) showing a suppression of progesterone withdrawal induced bleeding and estradiol induced endometrial proliferation. However the latter remains to be confirmed in women, since the endometrial effects of systemic administration of PRM in primates also results in endometrial atrophy, while the aspect observed in women is specific as previously discussed. Prototypes of ulipristal acetate releasing IUS are being designed and developed for a preclinical toxicology study. The initial proof of concept study in users of the vaginal ring (Schütt *et al.*, 2016) has shown that the doses of ulipristal acetate had to be increased to reach pharmacologically effective circulating levels. These studies are currently undergoing. This form of vaginal ring could offer a convenient estradiol free contraceptive. The endometrial impact of such a system is unknown.

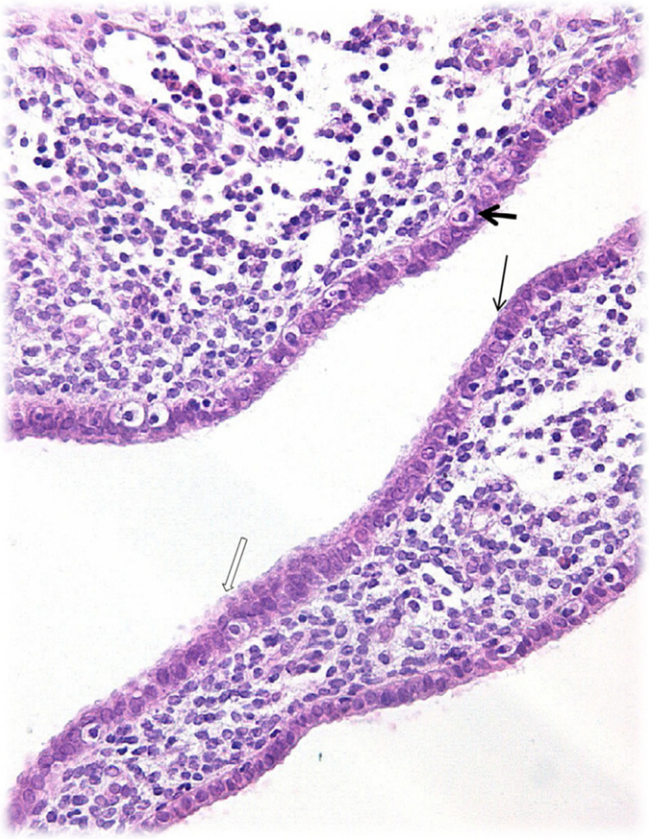

Fig. 1 Histological aspect of the PRM associated endometrial changes (PAECs). Cystic dilation of uterine glands is observes. The glandular epithelium shows a coexistence of apoptotic cells (*bold arrow*) and mitotic cells (*black arrow*), together with features of secretory differentiation (*white arrow*). Courtesy of Pr Pintiaux.

Finally, in addition to long term contraception, future indications may include dysfunctional uterine bleeding in the absence of myoma, adenomyosis and endometriosis. In addition the use of PRMs priori to IVF, to reduce myoma volumes and possibly improve IVF outcomes, is currently under evaluation, since successful pregnancies occurred in women with fibroids not requiring IVF after treatment with ulipristal acetate (Luyckx *et al.*, 2014).

Conclusion

Despite political interferences resulting in the slowing of their development, PRMs have allowed major improvements in women's health and comfort, such as medical termination of pregnancy, which is now doable at home, extended efficacy for emergency contraception, and finally medical treatment of myoma-related bleeding with the additional benefit of a very rapid cessation of bleeding and myoma shrinking, without estradiol deprivation. Development towards new molecules and application is actively ongoing.

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Diagnosis of Menopause

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Glossary

Amenorrhea Absence of menstruation—one or more missed menstrual periods.

Menopause The permanent cessation of menses due to the loss of ovarian follicular activity. The final menstrual period is retrospectively assigned after 12 months of amenorrhea, in the absence of other pathological or physiological causes.

Menopause may be spontaneous (natural menopause) or iatrogenic (secondary menopause). The latter can be induced earlier by medical intervention such as bilateral oophorectomy (surgical menopause) or iatrogenic ablation of ovarian function by chemotherapy, radiotherapy or treatment with gonadotrophin-releasing hormone analogues. In the absence of surgery, induced premature ovarian failure may be permanent or temporary.

The recently published NICE menopause guidelines recommends that diagnosis is made without laboratory tests: it's possible to diagnose in healthy women aged over 45 years with menopausal symptoms:

- perimenopause based on vasomotor symptoms and irregular periods;
- menopause in women who have not had a period for at least 12 months (who are not using hormonal contraception);
- menopause based on symptoms in women who have undergone hysterectomy (Sarri *et al.*, 2015).

For women aged over 45 years, laboratory tests, particularly follicle-stimulating hormone (FSH), do not help with the diagnosis because hormone levels fluctuate during the perimenopause. Knowing these levels will not change management (Roberts and Hickey, 2016).

Clinical Diagnosis

The menopausal transition marks a period of physiologic changes as women approach reproductive senescence. Evidence supports the clinical importance of the transition for many women as a period of temporal changes in health and quality of life (vasomotor symptoms, sleep disturbance, depression) and longer-term changes in several health outcomes (urogenital symptoms, bone, lipids) that may influence women's quality of life and the likelihood of healthy aging (Davis *et al.*, 2015). The 2001 Stages of Reproductive Aging Workshop (STRAW) proposed nomenclature and a staging system for ovarian aging including menstrual and qualitative hormonal criteria to define each stage. The STRAW staging system is widely considered the gold standard for characterizing reproductive aging through menopause. As a standardized staging system for reproductive aging, STRAW made a substantial contribution to women's health research by providing consistent classification of menopause status for studies of midlife women. Importantly, STRAW facilitated research that aimed to distinguish the health effects of ovarian versus somatic aging. The STRAW staging system also serves as a clinical tool for women and their healthcare providers to guide the assessment of fertility, contraceptive needs, and healthcare decision making.

As women progress to the late menopausal transition (STRAW Stage -2, characterized by variability in menstrual cycle length and increased levels of FSH; to -1, characterized by onset of skipped cycles or amenorrhea of at least 60 days and continued elevation of FSH), their menstrual cycle length increases to >60 days, anovulation becomes more likely and periods of time with little or no estrogen secretion occur. At this time, all of the commonly observed menopausal symptoms worsen acutely. Many symptoms peak in their intensity at this time, and women may require treatment (Harlow *et al.*, 2012) (**Table 1**).

A woman is considered postmenopausal when she is over the age of 45 and has gone at least 12 months without a spontaneous menstrual period (World Health Organization, 1996). No specific diagnostic testing is required unless the clinical presentation is atypical. Atypical presentations would include substantial mood changes, new-onset anxiety or fatigue, and arthralgia without flushes or sweats. Earlier ages of onset of prolonged amenorrhoea require consideration of other diagnoses such as polycystic ovary syndrome, secondary hypogonadotropic hypogonadism, hyperprolactinaemia, pituitary tumors or uterine problems such as Asherman syndrome. A menopausal woman who has undergone at least 12 months of amenorrhoea is unlikely to ever have another menstrual period, but it can occur in about 10% of women.

Menopausal Symptoms

Most women entering menopause experience vasomotor symptoms. The term vasomotor symptoms is often used to cover hot flashes and accompanying sweating.

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Table 1 The stages of reproductive aging workshop +10 staging system for reproductive aging in women

MENARCHE						*FMP (0)*				
Stage	*−5*	*−4*	*-3b*	*-3a*	*−2*	*−1*	*+1a*	*+1b*	*+1c*	*+2*
Terminology	*Reproductive*				*Menopausal transition*		*Postmenopause*			
	Early	Peak	Late		Early *Perimenopause*	Late	Early		Late	
Duration	*Variable*				*Variable*	1–3 years	2 years		3–6 years	Remaining lifespan
Principal criteria										
Menstrual cycle	Variable to			regular	Regular	Regular	Sub-	tle ch-	anges in flow/ length	Variable length, Persistent >7 days difference in length of consecutive cycle
Interval of amenorrhea of >60 days										
Supportive criteria										
Endocrine										
FSH			Low	Variable[a]	>Variable[a]	>25 IU/L[b]	>Variable[a]		Stabilizes	
AMH										
Inhibin B			Low	Low	Low	Low	Low		Very low	
				Low	Low	Low	Low		Very low	
Antral follicle count			Low	Low	Low	Low	Very low		Very low	
Symptoms						Vasomotor symptoms *Likely*	Vasomotor symptoms *Most Likely*			Increasing symptoms of urogenital atrophy

[a]Blood draw on cycle day 2–5.

[b]Approximate expected level based on assays using current international pituitary standard.

Modified from Harlow, S. D., Gass, M., Hall, J. E., *et al.* (2012). Executive summary of the stages of reproductive aging workshop þ 10: Addressing the unfinished agenda of staging reproductive aging. Menopause (New York, NY) 19(4), 387–395.

A hot flush is a sudden episode of vasodilatation in the face and neck, which lasts 1–5 min and is accompanied by profuse sweating. Women experiencing hot flushes are reported to have a narrower thermo neutral zone, such that subtle changes of core temperature elicit thermoregulatory mechanisms such as vasodilatation, sweating or shivering. Declining levels of oestrogens and inhibin B, as well as increasing FSH levels, explain only part of the disturbed thermoregulation, which is associated with changes in brain neurotransmitters and peripheral vascular reactivity. Although initial studies reported that LH pulses occur during hot flashes in postmenopausal women, a causative link was never found. Learning more about the mechanisms of hot flushes may allow to identify novel therapeutic targets to better treat patients affected by these symptoms. It has recently emerged this hypothesis: kisspeptin, neurokinin B and dynorphin (KNDy) neurons, which project to the preoptic thermoregulatory area, regulate the hypothalamic GnRH pulse generator, most likely by mediating estrogen-dependent negative feedback of LH secretion. At postmenopause, KNDy neurons undergo hypertrophy, and the expression of the genes encoding neurokinin B and kisspeptin increases as a result of estrogen withdrawal, leading to increased signaling to heat dissipation effectors in the CNS and to GnRH neurons (Rance *et al.*, 2013).

Hot flushes usually occur in late perimenopause and the first postmenopausal years. Some women, however, may continue to experience vasomotor symptoms for many years after menopause. Occasionally, hot flushes occur in the late reproductive years, or several years after menopause. The occurrence and intensity of menopausal symptoms vary widely between women and depend on genetic, environmental, racial, lifestyle and anthropometric factors. Black race, smoking and overweight—in particular central obesity—increase the prevalence and severity of vasomotor symptoms (Sturdee *et al.*, 2017).

Sleep disturbances are also very common during the menopausal transition and they are mainly attributed to frequent arousals due to night sweats and occurring secondarily to psychological factors. Mood disorders such as depression and anxiety are not caused by menopause; vulnerable women, however, may have their first episode or a relapse during the transition. Muscle and joint pain is also part of the menopausal symptomatology and it is highly correlated with hot flushes and depressed mood (Davis *et al.*, 2015).

Genitourinary Syndrome of Menopause

The anatomy and function of the female lower genital tract is estrogen-dependent. Vulvovaginal atrophy and urinary tract atrophy, due to estrogen deficiency, are collectively called the genitourinary syndrome of menopause (GSM). With declining estrogen, the mucosa of the cervix, the epithelium of the vagina and vulva thin and become susceptible to injury. The vaginal rugae diminish, leading to a smoother appearing vaginal wall accompanied by diminished blood flow and a rise in pH (6–8). Together, these changes result in a pale appearance which may contain small petechiae and/or other signs of inflammation. The proportion of superficial to parabasal cells decreases with loss of glycogen and loss of lactobacilli (Panay, 2015). The loss of secretions leads to vaginal dryness and irritation. Women experiencing sexual and urinary symptoms due to vaginal atrophy should be diagnosed and treated without delay in order to avoid a cascade of events which do not resolve spontaneously. The diagnosis of GSM is often made on symptoms alone—many health-care professionals avoid examination of the patient, which is a mistake. There are other vulval and vaginal conditions which can produce similar symptoms such as vulval dermatoses, for example, lichen sclerosis, and vulval/vaginal malignancy. The diagnosis has been largely subjective with few objective measures used to confirm the diagnosis and monitor progression and response to treatment. The measurement of vaginal pH and the vaginal maturation index from vaginal smears provides some objective evidence. Although these measurements are commonly used in GSM studies, they are rarely used in day-to-day clinical practice. Unlike hot flushes and night sweats, which improve over time, symptoms of urogenital atrophy persist throughout postmenopausal life and may have a serious impact on sexual health and quality of life (QoL). Pain during intercourse, secondary to vulvovaginal atrophy, leads to diminished sexual desire, arousal difficulties, relationship problems, and diminished physical and emotional sexual satisfaction. Although the majority of women have signs of urogenital atrophy upon physical examination, less than half of the postmenopausal population report bothersome complaints. Age, sexual activity, ethnicity and attitudes towards menopause influence the occurrence and the severity of urogenital symptoms (Portman and Gass, 2014).

Assessment tools have been developed to facilitate the formal diagnosis and classification of the severity of GSM. One of the most commonly used tools for assessing vaginal health has been the Vaginal Health Index (Bachmann *et al.*, 1992). The user is asked to rate both the appearance of the vaginal mucosa and production of secretions on a scale from 1 to 5 (**Table 2**).

There has also been an attempt to introduce a quality-of-life impact modality (pain on intercourse) in this tool although the value of assessing only one modality is limited (The vulval health index, 2015) (**Table 3**).

A more comprehensive tool has recently been developed following the Consensus Group development of the term GSM. The GSM assessment tool consists of three general categories of elasticity, lubrication and tissue integrity, an anatomical section which includes vulval, vaginal and urethal anatomy, and two objective measures, vaginal pH and vaginal maturation. There is a score from 0 to 3 for each one of these seven components describing severity of symptoms (**Table 4**) to provide a semiobjective measure of vulval and vaginal atrophy. A numeric score is calculated by adding the individual scores to give a total out of 21 (0–7 mild atrophy, 7–14 moderate atrophy, and 14 severe atrophy) (Portman and Gass, 2014).

But, there is some inaccuracy in the tool assesses GSM. Both vulva health index and GSM tool still require validation and publication of clinical trial usage.

Biochemical Assessment

Reproductive aging coincides with endocrine changes which primarily originate from a diminished pool of follicles being recruited during each cycle. This leads to a reduction in produced estradiol, lower levels of inhibin B resulting in elevated FSH concentrations. Elevated FSH during the early follicular phase of a regular menstrual cycle, was traditionally considered a first sign of reduced ovarian reserve (Davis *et al.*, 2015). A more advanced stage of decrease in the amount of FSH-sensitive follicles results in

Table 2 Vaginal health index

	Score				
	1	*2*	*3*	*4*	*5*
Elasticity	None	Poor	Fair	Good	Excellent
Fluid volume (pooling of secretion)	None	Scant amount, vault not entirely covered	Superficial amount, vault entirely covered	Moderate amount of dryness (small areas of dryness on cotton tip applicator	Normal amount (fully saturates on cotton tip applicator)
pH	>6.1	5.6–6.0	5.1–5.5	4.7–5.0	4.6
Epithelial integrity	Petechiae noted before contact	Bleeds with light contact	Bleeds with scraping	Not friable–thin epithelium	Normal
Moisture (coating)	None, surface inflamed	None, surface not inflamed	Minimal	Moderate	Normal

Modified from Bachmann, G. A., Notelovitz, M., Kelly, S. J., *et al.* (1992). Long-term nonhormonal treatment of vaginal dryness. Clinical Practice in Sexuality 8, 12.

Table 3 Vulva health index

	Normal: 0	Mild: 1	Moderate: 2	Severe: 3
Labia majora	Normal	Mild loss	Moderate loss	Severe loss or disappeared
Labia minora	Normal	Mild loss	Moderate loss	Severe loss or disappeared
Clitoris	Normal size	Mild decrease in size	Moderate decrease in size	Severe loss or undetected
Introitus and elasticity	Normal	Mild decrease or stenosis	Moderate decrease or stenosis	Severe decrease or stenosis
Color	Normal	Mild pallor	Moderate pallor	Severe pallor
Discomfort and pain	None	Mild during intercourse	Moderate during intercourse	Severe during intercourse and any discomfort intensity beyond intercourse
Other findings (petechiae, excoriation, ulceration, etc.)	None	Mild	Moderate	Severe

Modified from Palacios, S. (2015). The vulval health index. Personal communication.

Table 4 Genitourinary syndrome of the menopause assessment tool

	Normal: 0	Mild: 1	Moderate: 2	Severe: 3
Elasticity	Stretchable, elastic tissue	Slightly diminished elasticity	Moderately diminished	Absent, fibrotic
Lubrication	Normal secretions	Moisture slightly decreased moisture	Mostly dry, some moisture	Very dry
Tissue integrity	Intact epithelium, no friability or petechiae	Some friability with vigorous contact, no petechiae	Moderate friability or petechiae with some contact	Significant friability, bleeding, petechiae with minimal contact
Anatomy				
Introitus	3-Dimensional	Mostly 3-dimensional	Some contraction, stenosis, rather flat	Mostly contracted, stenotic, flat
Labia majora, minora	Normal for parity, coital activity, and anatomic variation	Most definition present	Some resorption, especially inferior aspect of labia minora	Significantly decreased size; minora mostly resorbed
Urethra	Normal size and position	Normal to slightly prominent	Moderately prominent urethral meatus	Eversion present; inner aspect protruding
Rugae	Normal	Present to slightly diminished	Moderately diminished but visible	Significantly diminished to absent
Color	Normal	Some faint pallor	Moderate pallor	Complete pallor
Supportive				
pH	<5		5–6.5	
Maturation index	No parabasal	Decrease in number of superficial cells, increase in number of parabasal cells	Cells fewer superficial cells, more parabasal cells	Few to no superficial cells, many parabasal cells

Modified from Portman, D. J., Gass, M. L. Vulvovaginal Atrophy Terminology Consensus Conference Panel. (2014). Genitourinary syndrome of menopause: New terminology for vulvovaginal atrophy from the International Society for the Study of Women's Sexual Health and the North American Menopause Society. Climacteric 17, 557–563].

increased menstrual cycle irregularity, and characterizes the menopausal transition. A combination of the menstrual cycle pattern, FSH serum levels and the antral follicle count (number of ovarian follicle-like structures assessed by transvaginal ultrasound) have been widely adopted in routine clinical practice to assess the current status of reproductive aging in women (Broekmans *et al.*, 2009). However, the capacity of these conventional measures to predict the course of reproductive aging over time—including timing of age at natural menopause (ANM)–is limited.

Although specific diagnostic testing for menopause is not recommended, clinical situations may arise in which it is beneficial to document primary gonadal failure. Usually, an increased FSH level will suffice to confirm this. Changes in FSH, oestradiol and inhibin B have been well documented in population-based samples of women undergoing observational research. Measurement of oestradiol during the perimenopause is not clinically useful. Inhibin B is the first hormone to decline, and its drop precedes a rise in FSH. The compensatory rise in FSH causes follicles to continue to grow and leads to a shortening of the follicular phase of the menstrual cycle. Eventually, the follicle pool becomes depleted and folliculo-genesis no longer occurs reliably. This juncture is when the late menopausal transition begins. Follicle failure is intermittent in the late transition and is eventually superseded by permanent amenorrhoea. After menopause has happened, oestradiol levels are expected to be consistently low (<20 pg mL^{-1}); progesterone is no longer produced. Anovulation leads to the loss of progesterone production. Of note, there is no acute change in testosterone levels in relation to the menopause transition. During the reproductive years, both ovaries and the adrenal glands contribute to circulating androgens. The postmenopausal ovary continues to contribute substantially to the circulating levels of testosterone for years, even if after menopause, the adrenals become the major source of androgen production. Serum

concentrations of adrenal androgens in midlife women might variably and transiently increase in the late menopausal transition. In particular, a rise in mean serum concentrations of dehydroepiandrosterone sulfate (DHEAS) occurs in most women between the early menopausal transition and early postmenopause stages. This changing hormonal environment produces a cascade of CNS-related and peripheral symptoms of variable severity for an unpredictable amount of time (McConnell *et al.*, 2012).

Over the past years extensive research has been performed regarding anti-Mullerian Hormone (AMH) as a predictor of ANM. AMH—formerly known as Mullerian-inhibiting substance (MIS)—has previously been studied extensively in the context of male gonadal differentiation. In the female fetus, the production of AMH commences around 36 weeks of gestation. AMH is a member of the transforming growth factor-β (TGFβ) superfamily of proteins, and is produced by granulosa cells within the follicles during the early stages of preantral and antral follicular development. Hence, AMH is a direct product of the growing cohort of follicles. Detectable serum AMH levels rise during early puberty up until they reach a plateau around the age of 20–25 years (Sowers *et al.*, 2008). Thereafter serum AMH levels gradually decline with advancing age, resulting in undetectable concentrations around 5 years prior to menopause. AMH might be useful for predicting the time of menopause. The development of a sufficiently sensitive AMH assay should be useful to help forecast the final menstrual period with better accuracy. Furthermore, several confounders which may influence AMH levels such as smoking, ethnicity, BMI, vitamin D and oral contraceptive pill usage, have been identified. AMH is currently also under investigation as a marker of ovarian function in a whole variety of clinical conditions, such as chemotherapy induced ovarian damage, premature menopause, ovarian endometriosis, ovarian surgery, polycystic ovary syndrome and many others (Daan and Fauser, 2015). Finally, AMH has recently been shown to predict the amount of exogenous FSH required for adequate ovarian stimulation for in vitro fertilization, allowing for individualized treatment regimens. Routine measurement of anti-Mullerian hormone (AMH) is not recommended to diagnose menopause in women over the age of 45, but may be helpful in women with suspected premature ovarian failure to assess ovarian reserve. However, while AMH has a relationship to age at menopause, the marked variability in levels needs further exploration and improved assay validity is required.

Diagnostic Imaging

Other measurements have also been proposed for forecasting the timing of permanent ovarian failure, including both antral follicle counts (assessed by transvaginal ultrasonography), recording of all follicles measuring $\leq$7 mm in diameter (usually done in the early follicular phase of the cycle), and functional ovarian reserve, which is ascertained by dynamic testing of the ovary with a stimulating agent (either clomiphene citrate or FSH). However, these methods are better predictors of the loss of fertility than they are of menopause per se. Transvaginal ultrasonography is a very useful method for assessing fertility status and can provide information about ovarian aging when appropriate. Measurement of the number of antral follicles can provide useful information about the likelihood of pregnancy in women who are of advanced reproductive age and wish to conceive. However, antral follicle counts have not been as useful in predicting menopause. Other ovarian measurements such as total ovarian volume and stromal thickness have also been proposed, but they currently lack the sensitivity and specificity to be clinically useful (Hansen *et al.*, 2012).

Differential Diagnoses of Menopausal Symptoms

If a woman is $>$50 years of age, has stopped menstruating and has classic estrogen deficiency symptoms, a diagnosis other than menopause is very unlikely. Other causes of amenorrhoea should be considered in younger women and in women aged $>$50 years who have atypical symptoms. Secondary amenorrhoea has to be investigated: progestin challenge, or progesterone withdrawal test is rarely used to evaluate amenorrhea. The test is performed by administering progesterone either as an intramuscular injection or oral medroxyprogesterone acetate. If serum estradiol is $>$40 pg/mL, withdrawal bleeding should occur 2–7 days after the progestin is withdrawn, indicating that amenorrhea is due to anovulation. However, if no bleeding occurs after progesterone withdrawal, then the patient's amenorrhea is likely to be due to either low serum estradiol, hypothalamic-pituitary axis dysfunction, a nonreactive endometrium or a problem with the uterine outflow tract, such as cervical stenosis or uterine synechiae (Asherman syndrome). In order to distinguish between hypoestrogenism or a uterine outflow tract problem/nonreactive endometrium, estrogen may be administered followed by a course of progestin in order to induce withdrawal bleeding (Ryosuke *et al.*, 1979). Pregnancy should always be considered in the setting of amenorrhoea: pregnancy needs to be excluded.

Other causes include thyroid disease, prolactinoma, severe depression or stress, and substantial weight loss. Each of these may be associated with vasomotor symptoms and mood changes. Other causes of vasomotor symptoms should be excluded if the presentation is atypical. Hormonal hot flushes do cause a significant rise in core temperature. If a woman records an increase in her oral temperature with flushing or night sweats, infective causes must be investigated. Thyrotoxicosis can mimic menopause, with women presenting with agitation and anxiety, sleep disturbance, overheating, sweating and palpitations. These symptoms may precede the classic thyrotoxic symptoms of weight loss and tremor. A careful medical and medication history should identify other possible causes. Serotonin-producing carcinoid tumors can present with nocturnal diarrhea and episodic flushing without sweating. Phaeochromocytomas release the catecholamines adrenaline and noradrenaline, and are characterized by persistent hypertension, flushing and profuse sweating (Lenders *et al.*, 2014). Measurement of thyroid stimulating hormone (TSH) and prolactin are also helpful in investigating menstrual irregularity. Estimates of the levels of luteinizing hormone, estradiol,

progesterone and testosterone are of no value in the diagnosis of ovarian failure, but may provide information about other menstrual cycle disorders. Some women may present with oligomenorrhoea and mood changes or depression (American Psychiatric Association, 2013). Perimenopausal depression needs to be differentiated from major depression and simple dysphoria. The diagnosis of major depression requires at least 2 weeks of depressed mood, or loss of interest or pleasure in nearly all activities for most of the day, nearly every day, accompanied by at least four of the following symptoms: change in appetite or sleep, fatigue, psychomotor agitation or retardation, feelings of worthlessness and/or guilt, diminished concentration or indecisiveness, and suicidal ideation. By contrast, perimenopausal depression is usually accompanied by irritability, hostility or agitation, and anxiety. Clinically, it resembles the mood changes of premenstrual syndrome, with negative mood, negative self-concept and less effective coping abilities. The lability of perimenopausal depression is a distinguishing feature, in contrast to the pervasive low mood that is seen in major depression.

In younger women with suspected premature ovarian failure or early menopause serial follicle-stimulating hormone (FSH) measurements should be undertaken. In menstruating women, measurement of FSH should be performed at the beginning of the follicular phase (days 2–5 of the cycle) to avoid ovulation-induced elevations of FSH.

Factors Hindering Diagnosis

Although one of the defining features of menopause is the cessation of the menstrual period, it more broadly encompasses the permanent cessation of ovarian reproductive function. Thus, the traditional definition is not useful in the setting of iatrogenic causes of amenorrhoea, such as hysterectomy, endometrial ablation or progestin intrauterine devices. Biochemical investigations may be warranted for women with iatrogenic amenorrhoea (notably, measurement of FSH and oestradiol) and for younger women (measurement of AMH). The latter is less useful in women >50 years of age, as most women will have low AMH levels by this time. Some women may report vasomotor symptoms when using combined oral contraception. In this instance, menopause can only be effectively diagnosed if the oral contraception is stopped for several weeks, after which menopausal status can be assessed both clinically and biochemically.

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Menopausal Treatment

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Introduction

Definition: menopause is defined by the final menstrual period (FMP) and corresponds to the extinction of ovarian steroids secretion and loss of follicles.

Menopause is thus associated with a drastic decrease in estradiol secretion and its consequences at short and long terms. Mean age of Menopause is 51 years in the United States and France.

Menopause usually occurs after a period of menstrual irregularity and hormonal changes. This phase is called perimenopause or menopausal transition (MT).

Perimenopause and MT

Before the extinction of estradiol secretion by the ovary, occurs a period with cycle irregularity potentially associated with clinical symptoms. The first description of the pattern of menstrual cycles during the perimenopausal period was made by Treolar *et al.* (1967). The classical view is that the first stigma of perimenopause is a shortening of the follicular phase due to the decrease in follicular content and subsequently an increase in follicular stimulating hormone (FSH) levels which are associated with a more rapid growth of the dominant follicle. The cycles are still ovulatory but of a mean duration of 21–25 days. Then ovulation occurs less frequently and with alternate normal cycles and long anovulatory cycles. Then, approaching the menopause, number and length of anovulatory cycles increase. Estradiol secretion fluctuates and can be high or low according to time. FSH is also fluctuating and progressively increases as a signature of ovarian insufficiency.

Several recent studies have updated the descriptive analysis of cycle's abnormalities during this phase. Wide use of modern tools (antimullerian hormone (AMH) and follicular antral count) developed in infertile patients has shown ovarian insufficiency defined from fertility patterns to begin much earlier than cycles irregularity. A group of experts evaluated the data available for hormonal and cycle modifications occurring during the late reproductive stages and up to the postmenopause and published it as "Stages of Reproductive Aging Workshop (STRAW)" (Soules *et al.*, 2001). They proposed to classify the gonadal female activity in three periods, reproductive, MT, and postmenopause. The reproductive phase (RP) was described into three stages corresponding to early, peak, and late phase. The MT phase was divided into two stages, early and late and the postmenopause phase (PM) into two stages, early and late. The late phase of RP was characterized by regular menstrual cycles and increasing levels of FSH. Going through the subsequent stages, increased variability in menstrual cycle length and increased levels of FSH were observed (Soules *et al.*, 2001). Ten years later, the same group reanalyzed the data and included more specific description of hormonal events, including ovarian peptides as AMH and inhibin B (Harlow *et al.*, 2012). They proposed that in the late RP, initial hormonal features of perimenopause consist in low AMH and low follicular antral counts with no modification in length of cycles and FSH levels. Then a second later subphase is characterized by increasing values of FSH and shortened cycles (Harlow *et al.* 2012). In early MT the variability in menstrual cycle length was defined as a persistent difference of 7 days or more in the length of consecutive cycles with recurrence within 10 cycles of the first variable length cycle (Harlow *et al.*, 2012). The late MT is characterized by increased variability in cycle length, extreme fluctuations in hormonal levels, and increased prevalence of anovulation. FSH fluctuates but is higher during this phase than previously and can reach menopausal levels. The duration is estimated to be 1–3 years and climacteric symptoms are frequently present. A recent study gave few more insights from data collected from the Study of Women's Health Across the Nation (SWAN) (Santoro *et al.*, 2017). SWAN is a multiracial/multiethnic observational study of the MT among 1455 women enrolled at seven US sites. Among them, a population of middle-aged women with normal cycles collected their urines every day, 1 month year^{-1} until the FMP or 10 years after inclusion. The results mostly confirmed what was already known from smaller studies. Ovulation persisted up to 5/4 years before the FMP in most of the patients and then its frequency declined rapidly up to 1 year before the FMP where only 23% of the cycles were still ovulatory. Obese women displayed much more anovulation and menstrual cycle irregularity. The menstrual cycles remained regular up to 5 years before FMP (Santoro *et al.*, 2017). Curiously in this study the shortened cycles were not observed. The methodology of this study and/or the selected population can explain this discrepancy with the rest of the literature and our clinical experience. One of the consequences is to reaffirm the necessity of contraception up to the LMP.

Clinical consequences of MT can be important. It is a period when progesterone secretion decreases, estradiol levels can be rather high, and thus endometrial and uterine diseases can occur. Menorrhagia and metrorrhagia are frequently observed. Benign uterine diseases such as myoma, adenomyosis, polyps, and endometrial hyperplasia are more frequent than endometrial cancer but abnormal breakthrough bleeding always needs a rigorous evaluation by echography ± hysteroscopy (Goldstein and Lumsden, 2017). Mastalgia, premenstrual syndrome, and dysmenorrhea can also reappear or worsen. Climacteric symptoms can preexist to

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menopause which can start far beyond the menopause as demonstrated by several longitudinal studies. A systematic study analyzing 66 papers on climacteric symptoms all over the world reported hot flushes in 3%–86% of premenopausal women, with a median of 21.5% (Freeman and Sherif, 2007). In the perimenopausal women, the prevalence was between 40% and 60% and remained stable in early menopause (Freeman and Sherif, 2007). The prevalence of symptoms was different according to the regions of the world. More recently, three big longitudinal studies also reported on the prevalence of climacteric symptoms during MT. The US Penn Ovarian Aging Study from a 9-year longitudinal, population-based cohort study of 403 women was studied from pre- to postmenopause (Freeman *et al.*, 2007). Hot flushes, vaginal dryness, sleep disorders, aches, joint pain, and stiffness, and depressed mood were observed before and across MT. The most prevalent symptoms were aches, joint pain, and stiffness and hot flushes. They increased during the late transition phase and the early postmenopause from about 30% of the patients up to 75% in MT and PM; whereas depression was more frequent in MT than after and poor sleep and vaginal dryness were not significantly associated with menopausal stages (Freeman *et al.*, 2007). This study demonstrated that even more prevalent after menopause, several symptoms can be present and severe far beyond FMP. SWAN study among the 1455 women enrolled reported on early onset of climacteric symptoms in 247 of them (Tepper *et al.*, 2016). The Australian Longitudinal Study on Women's Health (Mishra and Dobson, 2012a) also described symptoms through early, late MT and menopause in a sample of 5295 Australian women. Eleven percent of women had an early severe profile (according to a score for vasomotor symptoms (VSM)) while still premenopausal and in addition to them, some other reported moderate or mild symptoms before FMP (Mishra and Dobson, 2012a).

MT can be associated with metabolic pejorative features. Changes in fat distribution, with predominant abdominal fat, are associated with reduction in lean mass, all of which can contribute to insulin resistance. Waist circumference (WC) or WC/hip circumference ratio correlates with cardiovascular (CV) and breast cancer (BC) risks. Women often complain of gaining weight without modifying their nutrition. Actually, reduction of energy expenditure, insulin resistance, and modifications in orexigens are features of E2 loss, at least well demonstrated in mice. But these alterations usually occur few years before LMP, and are less clearly explained in the human. Overweight/obesity, dyslipidemia, hypertension, metabolic syndrome, and possibly diabetes are more prevalent in the perimenopausal period and after menopause. These metabolic dysfunctions contribute to the risk of CV events and have to be taken into account. Thus lifestyle recommendations on nutrition and regular exercise are the first line of management of women at this period of life (Khan *et al.*, 2014; Baber *et al.*, 2016). If menorrhagia/metrorrhagia are present, levonorgestrel-releasing intrauterine system (norg-IUS) is an interesting option after eliminating a cause which should necessitate a specific treatment (submucous myoma, polyp) (Lethaby *et al.*, 2015).

Management of Perimenopause

The management of symptoms in this period is different from what it is after menopause. Imbalance between estrogens and progesterone is responsible for the various symptoms and consequences of target organs. In addition, contraception is mandatory in women without tubal ligation or equivalent. Combined estro–progestin contraception is associated with increased risk of CV events and their use is not recommended in women with any risk factor for arterial or venous disease. Age is a factor increasing the risk of both events. Given the frequency of metabolic, endometrial, and uterine disorders, there is a place for treatment by IUS or progestins. If mastalgia or fibrocystic diseases or premenstrual syndrome occur, then antigonadotropic progestins could help. These agents can help to prevent endometrial cancer and breakthrough bleeding. However they can also be associated with VMS. When approaching the menopause, in periods of long cycles if contraception can be taken over by barrier contraceptives, then 10 days months^{-1} of progesterone can help to alleviate VMS, improve sleep disorders and irritability, and provide regular menstrual cycles up to the FMP.

Menopause

Menopause is associated with a drastic decline in ovarian steroids secretion and its consequences at short and long terms. Since menopause is associated with aging, it is somehow difficult to discriminate between the specific consequences of estradiol deficiency and natural senescence since they can also synergize on certain endpoints.

Diagnosis of Menopause

As previously mentioned, the diagnosis of menopause relies on clinical evaluation as the last menstruation. The classical definition for menopause is 1 year without menstruation with a FSH level above 40 UI mL^{-1}. This definition is somehow awkward or at least theoretical. Indeed in the clinical practice it is not worthy to recommend women consulting with various complains to wait 1 year before getting a medication to relieve their VSM. As stated by several textbooks including menopause management (Bricaire and Sitruk-Ware, 1998; Speroff, 2010), the best clinical practice to diagnose menopause is to administrate 10 days of progestin. If bleeding occurs, then this treatment has to be continued as long as bleeding follows the end of the 10 days treatment. Indeed, this treatment can prevent endometrial dystrophies (Gambrell *et al.*, 1980) which occur frequently in the MT as reported earlier. The

benefit of the treatment by progesterone/progestin treatment is also as mentioned earlier to alleviate VSM during this period and even after (Prior and Hitchcock, 2012; Loprinzi *et al.*, 2006).

The short-term consequences are onset or aggravation of climacteric symptoms. In addition to VSM symptoms, and joint pains, urogenital symptoms increase also in prevalence. The longitudinal studies cited earlier have shown that these symptoms can last several years after the menopause; severity of symptoms and their duration vary according to patients with some having early and severe symptoms which can last for long, and some others moderate or mild symptoms with long- or short-term duration (Mishra and Dobson, 2012b; Freeman *et al.*, 2011; Avis *et al.*, 2015). In the SWAN study, median total VMS duration was 7.4 years, but women who experienced early frequent VMS had more than 11.8 years of symptoms (Avis *et al.*, 2015).

Long-term consequences of menopause consist in osteoporosis, CV diseases, urogenital symptoms, sexual difficulties, and decrease in cognitive functions.

Osteoporosis affects about 30% of women in Europe and the United States (International Osteoporosis Foundation, 2017) with increasing risk of vertebral, wrist, and cervical fractures as aging is increasing. The role of estrogens is clearly demonstrated on osteoporosis, whereas its efficacy in CV prevention is less clear.

CV diseases are the first cause of mortality according to (WHO 2017). Men are at higher risk than women before menopause, but after menopause women have an increasing prevalence of CV events. There are myriad of preclinical and animal data showing that estradiol is protective against atherosclerosis (Morselli *et al.*, 2017). If most of observational studies including women treated by menopause hormonal treatment (MHT) have shown a significant decrease in CV events, randomized data are less favorable. One of the explanations is that estrogen administration close to menopause acting on vessels containing estradiol receptors can be protective but with aging, ERs are progressively lost, and estrogens cannot longer exert their effects or be deleterious (Clegg *et al.*, 2017). In the observational studies, women were mostly young postmenopausal whereas in the women health initiative (WHI) they were far from menopause in their great majority. This leads to the concept of "window of opportunity or intervention." A further explanation could be that the women treated had less risk factors than the women in the WHI study, thus this selection suggests "a healthy bias." During MT, metabolic disorders become prevalent and another beneficial effects of estrogens are to maintain insulin sensitivity and secretion and thus decrease prevalence of metabolic syndrome and diabetes (Hevener *et al.*, 2015).

Urogenital symptoms directly associated with a lack of E2 can associate vaginal itching, vaginal discharges, urethritis, and urinary incontinence. Incontinence can be urgent incontinence, stress incontinence, or mixed incontinence. Their prevalence increases with aging. Women are not always reporting them spontaneously and they have to be searched for systematically. Similarly, sexual dysfunctions increase with aging and have to be asked for. Loss of libido is frequent as well as dyspareunia because of vaginal dryness; if systemic treatment is not fully efficient on libido, topical treatment can help to improve sexual intercourses (see following). Androgens have positive effects on libido in some studies, but with controversial results according to the type of treatment. The patch no more available was the most effective treatment (Shifren and Davis, 2017). Australia is the only country to have approved for use a 1% testosterone cream with the recommended dose being 0.5 g day^{-1} gel of testosterone (Shifren and Davis, 2017). Androgens are not recommended in most of countries because of a lack of long-term safety data.

Cognitive disorders are the consequences of aging. They are a frequent complain of women during MT and after menopause, especially memory loss (Maki and Henderson, 2016). In preclinical studies in the animal, estradiol or progesterone can exert neuroprotection. Observational studies have suggested that an early MHT could protect against Alzheimer but randomized control trials (RCT) did not demonstrate any beneficial effect; furthermore, more dementia were observed in the WHI (Shumaker *et al.*, 2003, 2004). The negative impact can be interpreted as deleterious vascular effects of the treatment on the brain in relatively old patients. Similarly to a beneficial effect on CV events, MHT can have some benefits on cognitive disorders if administered during a window of opportunity that is to say early after menopause. There is, however, no proof of any protective effect of MHT on Alzheimer (Maki and Henderson, 2016).

Principles of MHT

In women with a uterus, the most efficient treatment on climacteric symptoms is an estrogen combined with a progestin. In women without a uterus estrogen only is recommended (Stuenkel *et al.*, 2015a).

Type of estrogen, progestin, and regimen of administration may vary, but all have proven their high efficacy to alleviate climacteric symptoms and increase quality of life in symptomatic women. In addition, RCTs and observational studies have consistently shown a benefit of MHT on fractures. The side effects however can be modulated by the choice of specific regimens (see further).

- It is now recommended by most of drug agencies (FDA, EMEA, HAS) (Office of the Commissioner, 2017; Haute Autorité de Santé—Traitements Hormonaux de La Ménopause, 2017). MHT should be administered at the "lower dose for the shortest period of time, according to symptoms, in symptomatic women" and that reevaluation of the need of MHT should be made regularly. Scientific societies of Endocrinology involved in menopause have regularly reevaluated the benefit/risk balance of the treatment and have somehow a different view. In addition to alleviate climacteric symptoms and prevent osteoporosis, they all agree on benefit over risk in women 50–60 years old or at <10 years since menopause (Stuenkel *et al.*, 2015b; Baber *et al.*, 2016; Lumsden *et al.*, 2016; The NAMS 2017 Hormone Therapy Position Statement Advisory Panel, 2017). The benefits can decrease in women over 60 years but still if women have no specific CV and BC risks if they are symptomatic continuation of

MHT can be discussed. If a woman is willing to take MHT after full information on benefits and risks, then MHT can be prescribed (Stuenkel *et al.*, 2015a).

Results From the Main RCTs, the WHI Trials

Until 2002, when the first publication from the randomized trial WHI was released (Rossouw *et al.*, 2002), MHT was considered as beneficial on climacteric symptoms but also on endpoints such as osteoporosis and CV protection. The view supported by numerous observational studies as well as preclinical data was that administration of estrogens should prevent the deleterious effects of the decline in the ovarian secretion on CV events. The successive publications from the WHI combined E + P trial (combined Hormone therapy, CHT) and the corresponding one using estrogens alone (ET) in postmenopausal hysterectomized women (Anderson *et al.*, 2004) have fully transformed the scope of MHT and as a consequence the management of climacteric symptoms and long-term consequences of E2 loss. These RCT were designed to follow women during 10 years after the inclusion by randomization between the active treatment and placebo. In order to get a low rate of dropout were included women far from menopause as they were less often symptomatic. The treatment consisted in conjugated equine estrogens (CEE) at the dose of 0.625 mg day^{-1} irrespective of the age of the patients and with or without medroxyprogesterone acetate (MPA) at 2.5 mg day^{-1} orally and continuously. The results were evaluated as primary outcome coronary heart disease (CHD) (fatal and nonfatal myocardial infarction) and BC as the primary adverse effect; a global index of morbidity, including, BC, pulmonary embolism, colorectal cancer, endometrial cancer, hip fractures, and death from other causes, was also followed (Rossouw *et al.*, 2002). In the CHT, 16,608 women of a mean age of 63.3 years were included; after a mean follow-up of 5.2 years, the study was stopped because of risks exceeding benefits (Rossouw *et al.*, 2002). Only 33% of the population had less than 60 years and more than 20% were over 70 years old. More than 30% were obese and 36% had hypertension. The ET trial included 10,739 hysterectomized women with a mean age of 63.3 years and an average follow-up of 6.8 years (Anderson *et al.*, 2004). Similarly to the CHT trial, 30% of the women were below 60 years and more than 25% were above 70 years. Even a bigger proportion of women was obese (40%) and had hypertension (53%). The main results of the CHT study were an increase in CHD, stroke, venous thromboembolism (VTE), and BC incidence and a decrease in colorectal cancers and osteoporotic fractures (**Table 1**). These outcomes were significantly using nominal confidence intervals (CI) but only the risk of VTE remained significantly using adjusted CI (Rossouw *et al.*, 2002). In the ET trial, there was an increase in stroke and VTE but not in BC. Several subsequent publications from the WHI reanalyzed these data according to the categories of age of the patients and time since menopause. Indeed, these studies have some important limitations: the ages of the included women do not correspond to the real life where MHT is indicated primarily to alleviate climacteric symptoms in recent postmenopausal women.

More recent analysis from the WHI, as shown in **Table 1**, reported a decrease in global mortality in women aged 50–59 years old (Manson *et al.*, 2013, 2017). Furthermore, it showed that expressed—in terms of excess risk or benefit—1.45 (−6.6 to +4.2) fewer CV events occurred per 1000 women per 5 years of treatment in the ET trial and 3.9 (0.15–8.0) more events per 1000 patients per 5 years in the CHT trial (Rossouw *et al.*, 2007). Furthermore, there were less BC in the ET group. In the CHT trial, the hazard ratio (HR) for BC was 1.24 (1.01–1.53) and increased with duration whereas in the ET trial, the HR was 0.79 (0.61–1.02) with no effect of time since inclusion (Manson *et al.*, 2013). CV events were significantly increased only in women over 70 years. The venous and arterial events were observed only in current users. The increase in CV events and VTE was more important the first year of treatment as it is in all procoagulant hormone-dependent events. Women with CV risk factors had an increased chance of events (Bassuk and Manson, 2014). BC risk as detected after at least 5 years of treatment increased with duration and decreased progressively after last use. This is interpreted as a promoter effect on preexisting lesions (Gompel and Santen, 2012). It is mostly ER-positive BC including lobular which are promoted by MHT. About 30% of the women in the CHT had previously used an HT. Only these women had a significant increase in the risk of BC after at least 2 years of previous use of CHT and an additional use of at least 3/5 years (Anderson *et al.*, 2006). The relative risk (RR) of BC was increased in women with mastalgia and high breast

Table 1 Health outcomes in the WHI RCT during treatment (from Manson *et al.*, 2013) expressed as difference in number of events per 10,000 person-years

	CEE + MPA		*CEE*	
	Total	*50–59 years*	*Total*	*50–59 years*
CHD	6	5	−3	−11
BC	9	6	−7	−5
Stroke	9	5	11	−1
PE	9	6	4	3
DVT	12		7	
Colorectal cancer	−6	−1	2	3
Endometrial cancer	−1	0	NA	NA
Hip fracture	−6	−3	−6	3
All cause mortality	−1	−10	3	−11

CHD, coronary heart disease; *BC*, breast cancer; *DVT*, deep vein thrombosis.

density (Crandall *et al.*, 2012; Byrne *et al.*, 2017).

Other endpoints were analyzed in the WHI trials; as an important benefit of MHT, significant less diabetes type 2 were observed in the CHT and ET trials (Manson *et al.*, 2013). Gallbladder disease and urinary incontinence were worsened by the systemic treatment whether ET or CHT (Manson *et al.* 2013). As stated earlier, more dementia were observed in a substudy of the WHI, the women's health initiative memory study (WIMS) which addressed specifically cognitive disorders (Shumaker *et al.*, 2003, 2004).

Differences Between CEE ± MPA and Other Available Treatments in Terms of Side Effects

The route of estrogen administration matters for the CV risk

Venous thrombotic and CV risks can be decreased by using E2 administered by a nonoral route compared with oral estradiol. Estrogens used in the WHI, CEE, are known as thrombogenic for years (Stangel *et al.*, 1977; Elkik *et al.*, 1982a). In Europe, estradiol (E2) is the first-line prescription. It can be administered by oral or transdermal/percutaneous (TTS) route. A major difference exists between these two routes: TTS administration at standard doses used for MHT (**Table 2**) has no effect on VTE risk and risk of stroke. This evidence is coming from observational and case–control studies conducted in France, United Kingdom, and the United States (Scarabin *et al.*, 1997; Canonico *et al.*, 2007, 2008, 2010, 2016; Renoux *et al.*, 2010a,b; Simon *et al.*, 2016). Unfortunately, there are no RCT on the venous events with TTS, but the biological plausibility of a lower effect on coagulation of TTS is very likely since studies show less activation of blood clotting (Scarabin *et al.*, 1997; Canonico, 2015). Similarly other hepatic proteins such as lipids, angiotensinogen, and C reactive protein (CRP) are less modified with this route (Elkik *et al.*, 1982b; Lacut *et al.*, 2003). The interpretation is that oral route is associated with a greater hepatic impact than TTS E2 administration; this is known as the hepatic first-pass effect. The intrahepatic concentrations are far greater when an oral administration and thus trigger activation of coagulation. Using TTS route, the concentration of estrogens which reaches the liver is equivalent to the rest of the circulation, as in physiologic state of E2 secretion by the ovaries. E2 is the main circulating estrogen whereas using oral administration E2 is metabolized to estrone at the level of digestive mucosa and increasing concentrations of estrogens then reaches the liver (Dupont *et al.*, 1991). Induction of blood clotting is also dose dependent. Increasing administration of estrogens even by an extradigestive route can be associated with activation of coagulation. This can explain that in the study on stroke from United Kingdom (Renoux *et al.*, 2010a, b) use of patch >50 μg was associated with an increased risk. Stroke seems to be more directly dependent on hypercoagulability than MI. In addition in the large E3N cohort, transdermal and oral E2 had favorable effects on DT2 (de Lauzon-Guillain *et al.*, 2009). Concerning BC risk, both routes of administration carry the same risk (Lyytinen *et al.*, 2006; Stuenkel *et al.*, 2015b).

The type of progestin and the regimen may be important to decrease some events; VTE, metabolic risk, endometrial cancer, and BC risk

Progestins are nonselective steroids which have different properties according to their binding to and agonist or antagonist effects on the androgen, glucocorticoid, and mineralocorticoid receptors. Progesterone, retroprogesterone (dydrogesterone), or synthetic progestin can be used in MHT. The aim is to protect the endometrium against the proliferative effects of estrogens in women with uterus. It was validated long time ago that in sequential treatment, progestins have to be administered at least 12 days months^{-1} in association with estrogens. More recently, several studies reported that a sequential administration was at higher risk for the endometrial cancer than continuous combined administration. Interestingly, in obese/overweight women, CHT decreases the RR

Table 2 Risk factors for breast cancer

High risk ≥fourfold

- BRCA mutation, high family history without mutation (premenopausal)
- Biopsy with atypical hyperplasia, LCIS, DCIS
- Thoracic radiotherapy especially at a young age
- Increased breast density (Birad 4) (four categories breast imaging-reporting and data system (BIRADS) according to American College of Radiology)

Intermediate risk ≤twofold

- Family history at older age
- Obesity (postmenopause), × 1.2–2.5
- Diabetes × 1.2–2
- Birad 3 × 2
- Reproductive factors:
  - young age at menarche (premenopausal breast cancer > postmenopausal) × 1.20–1.50
  - age at FFTP ≥35 years (premenop > postmenop) × 1.36
  - Nulliparity × 1.3
- Sedentarity
- Alcohol × 1.05–1.4

of endometrial cancer in a large observational study (Beral *et al.*, 2005). Synthetic progestins appear to equally protect the endometrium (Allen *et al.*, 2010). However, progesterone has been reported to be associated with an increased risk of endometrial cancer (Allen *et al.*, 2010; Fournier *et al.*, 2014). One of the possible explanations is a problem of compliance since progesterone does not exist in association with E2, women can forget to take the pill. Indeed in two randomized trials, progesterone was not associated with an excess of endometrial hyperplasia compared with a synthetic progestin (The writing group for the PEPI trial, 1996; Jondet *et al.*, 2002). Consequently, it is important to explain the importance of compliance to the progestin in combination with estradiol to the treated women (Gompel, 2012). The risk of endometrial cancer is remnant during long term, contrary to BC. At the opposite, progesterone (and dydrogesterone) has different CV impact and can also carry different level of BC risk. From observational and case–control studies, it is observed that micronized progesterone and dydrogesterone are neutral in terms of metabolic risks and risk of VTE (Canonico, 2015; Fineberg, 2000). In addition, they are less associated with BC than combined treatment with synthetic progestins at least up to >5 years of treatment (Fournier *et al.*, 2005, 2008; Lyytinen *et al.*, 2009; Cordina-Duverger *et al.*, 2013; Asi *et al.*, 2016). Conversely, norpregnane derivatives were associated with a higher risk of VTE (Canonico *et al.*, 2010).

The population in which is prescribed MHT and lifestyle can modify the risk of BC

The importance of breast density in the risk of BC is reported by several studies which suggest that women with high breast density have an increased risk of BC without MHT but even greater with MHT (Kerlikowske *et al.*, 2010; Hou *et al.*, 2013). Women with atypical hyperplasia can have a greater risk from MHT but with conflicting results (Dupont *et al.*, 1989; Arthur *et al.*, 2017). In the WHI ET, women with family history of BC and a personal history of benign breast disease (BBD) did not experience a decrease in BC risk (Anderson *et al.*, 2012). Exercise and losing weight can decrease the risk of BC, whereas sedentarity and putting on weight increase the risk (Pizot *et al.*, 2016; Rosner *et al.*, 2017). Women with high BMI do not seem to have a greater risk of BC than their baseline risk if taking MHT in observational studies (Ritte *et al.*, 2012).

Other Endpoints

- Ovarian cancer (serous and endometrioid differentiation) is reported to be mildly increase with MHT in a metaanalysis but with possible bias (Collaborative Group on Epidemiological Studies of Ovarian Cancer *et al.*, 2015). In most observational studies, estrogens alone are at higher risk than CHT on the ovarian cancer but not in this metaanalysis and a minority of studies (Reid *et al.*, 2017). Some studies show an effect with duration whereas others do not (Collaborative Group on Epidemiological Studies of Ovarian Cancer *et al.*, 2015; Reid *et al.*, 2017). The effect decreases after cessation of the treatment (Reid *et al.*, 2017). Interestingly in survivors of invasive high grade ovarian cancer, MHT (mostly estrogens) in three RCTs improved survival of the patients (Guidozzi and Daponte, 1999; Li *et al.*, 2012; Eeles *et al.*, 2015).
- Gastrointestinal cancers: In addition to colorectal cancers which are decreased in women using MHT, upper Gastro-intestinal (GI) and liver cancers were recently reported to be also of a lower prevalence in women using MT.
- Tibolone: it is a progestin belonging to the normethyltestosterone derivative with metabolites with androgenic and estrogenic properties. It was shown to be effective on climacteric symptoms to a less extent than CHT, and in a RCT was associated with increased of stroke in older women (Formoso *et al.*, 2016). It probably carries the same level of BC risk than CHT (Stuenkel *et al.*, 2015a, b). It was shown to prevent fractures in a RCT of osteoporotic women (Cummings *et al.*, 2008) but increased the risk of recurrence in BC patients (Kenemans *et al.*, 2009). It does not increase mastalgia and has beneficial effects on libido due to its androgenic potency. It is a second-line prescription in young postmenopausal symptomatic women without contra-indication to MHT, displaying mastalgia using estrogens, loss of libido not restored by MHT, or breakthrough bleeding.
- An association between CEE (0.45 mg) and a selective estrogen receptor modulator (SERM), bazedoxifene (20 mg), is labeled in the United States and Europe (DUAVIVE). The concept is to combine CEE at low dose with a SERM which will antagonize the proliferative effects on the endometrium and breast. Mastalgia are less prevalent than the standard treatment and breast density is less increased; data are lacking however on BC risk (Harvey *et al.*, 2013; Mirkin *et al.*, 2016). The efficacy on VSM symptoms is also lower than with CEE alone. The incidence of endometrial hyperplasia is low and it increases BMD at the spine and hip (Pinkerton *et al.*, 2014). The RR of VTE and arterial diseases is not evaluated but both agents can activate coagulation.
- Estetrol is an estrogen physiologically produced during pregnancy, under development. It has a lower affinity for ERα than E2. Alone it has estrogenic activity at higher concentrations than E2 (100-fold); it is an antagonist in combination with E2 in preclinical models of breast (Gérard *et al.*, 2015). It is currently evaluated in the treatment of menopause and was shown to decrease VSM (Coelingh Bennink *et al.*, 2016). Its endometrial and breast safeties remain to be studied.

In Practice

- The first consultation is the time to evaluate the importance and type of climacteric symptoms, including urogenital disorders and sexual dysfunction. Individual and familial risk factors of the woman have to be gathered. CV, metabolic, cancer, and osteoporosis risks have to be evaluated. Increased CV risk factors and BC risk need to be managed and could contraindicate MHT. Information on nutrition, including calcium intake and alcohol, benefits of exercise, stop smoking, gynecological follow-

up, and cervical and breast screening, is also delivered. Measure of BMI and blood pressure with breast and gynecological examination are performed as well as pap smears (according to the local guidelines) and a prescription of mammogram in the context of national screening if it exists or as opportunistic screening if not usually every 2 years after 50 years. Lipid fractions and glycaemia levels are measured. Measure of vitamin D levels and of bone mineral density (BMD) is not systematically recommended. It is however mandatory in case of risk factors for osteoporosis such as family history of osteoporotic fracture, personal history of amenorrhea, low BMI, corticosteroid treatment, excessive alcohol intake and smoking, or a previous spontaneous fracture. Following menopause, the decrease in BMD is maximal during the first 2 years so that a BMD can be performed after this term in the absence of MHT. However, some recommendations state to perform it once in older women (>65 years) (Shifren *et al.*, 2014). The FRAX score developed by the World Health Organization and validated in many countries can help to calculate a 10-year risk of fracture and the level of risk which could beneficiate from a treatment (FRAX Tool, 2017). There are also some scores which can help to predict CV risk at 5 or 10 years, based mostly on gender, age, BP, HDL/total cholesterol, and in some smoking, usCRP (HeartScore France, 2017; Framingham Coronary Heart Disease Risk Score, 2017; Reynolds Risk Score, 2017). They are not all validated in all populations. In the United States, the Reynolds score or the Framingham score are most used, whereas the "SCORE" (from European Society of cardiology) has been validated in most European countries. If the lipid and BP values are normal and if there is neither diabetes nor smoking, then the risk is low. In case of diabetes, metabolic syndrome, smoking, and high risk maybe present and evaluation of the presence of plaques is recommended even if the tools for doing so are not consensual. In intermediate situations, according to the age of the patients and duration of risk factors, such evaluation can also be useful. Echography of the arteries, exercise tolerance test, stress echography, coronary calcium score, etc. can be used (Stuenkel *et al.*, 2015a). The actual consensus from WHI results and preclinical data is that MHT prescribes before the apparition of plaques can be safe but in the presence of significant atherosclerosis, estrogens are proapoptotic on the instable plaques which can then migrate and induce a CV event. Concerning BC risk, similarly, the risk can be stratified as low, moderate, or high risk. This quantification is, however, more complex for BC than for CV risk. The risk factors are many (**Table 2**) and scores do not predict with a very good accuracy of the risk. High risk exists in the presence of a strong family history of BC, atypical hyperplasia at a biopsy, breast density grade D at mammogram, and history of thoracic irradiation (**Table 2**). In those patients, MHT is not recommended and nonhormonal treatment should be privileged. Moderate/intermediate risk (**Table 2**) is associated with postmenopausal BC at first degree in the family, reproductive factors increasing the risk, breast density grade C, and biopsy with proliferative disease. In those patients, thorough evaluation of the balance between benefits and risk is necessary as well as a deep discussion with the patient (**Table 4**).

- If the woman is willing to take hormone treatment and has no contraindication, we thus recommend to start at medium doses by a combination of transdermal (37.5 μg day^{-1}) or percutaneous E2 (0.75 mg–1 mg day^{-1}) and micronized progesterone or dydrogesterone. A continuous administration is associated with less bleeding than sequential administration. In France, we use a combined administration during 25 days of the month which minimizes the breakthrough bleeding compared with a nonstop administration. However, some patients cannot stop during 5 days since they are symptomatic of having an increased prevalence of migraine. There are no safety data to oppose to a continuous administration in those patients rather a stop of 5 days. Continuous administration using synthetic progestins has been reported in observational studies to be associated with less endometrial cancer and more BC (Lyytinen *et al.*, 2009; Bakken *et al.*, 2011; Beral *et al.*, 2005; Allen *et al.*, 2010). There is no data for progesterone and dydrogesterone comparing sequential and continuous administration side effects. Then adaptation of the dose of treatment has to be made in a next consultation which also will monitor efficacy, clinical tolerance, and occurrence of side effects including metrorrhagia; those have to be systematically explored by echography ± hysteroscopy. Regular follow-up at least once a year will help to diagnose any new event and adapt the need of the treatment to the symptoms.
- In case of estrogen intolerance (mastalgia, benign breast disease, adenomyosis/bleeding), progesterone, progestin, or tibolone can be of some efficacy on VSM (see above for tibolone). Progesterone has some demonstrated efficacy on sleep disorders and probably anxiety.
- If the woman is not willing to take an MHT or have contraindications alternatives can be prescribed. This concerns in particular women who are BC survivors. The most studied agents are selective serotonin receptor inhibitors (SSRI) and selective noradrenaline receptor inhibitors (**Table 3**). They are less effective than estradiol on climacteric symptoms but RCTs have shown a greater efficacy than placebo. Venlafaxine, fluoxetine, paroxetine, citalopram, or gabapentin (a gamma-aminobutyric acid (GABA) receptor ligand) have been evaluated in RCTs (Stuenkel *et al.*, 2015a, b). Paroxetine cannot be associated with tamoxifen because of their interaction with the CYP2D6 cytochrome and the metabolism. Their efficacy is slow, it takes at least 2/3 months to be optimal, and side effects can occur. Doses have to be progressively increased in case of failure (**Table 3**). Transdermal clonidine appears to have some efficacy (Stuenkel *et al.*, 2015a) but is not available outside of United States (**Table 5**).
- Most of complementary therapies have not been validated in sufficient trials. Their efficacy remains controversial. Phytoestrogens cannot be prescribed to women with BC. Yoga, acupuncture, relaxation, and meditation can help in some extent and have been particularly studied in BC survivors (Koch *et al.*, 2017; Chien *et al.*, 2017). Black cohosh and St John's Wort have been associated with adverse effects and interactions with medications and should therefore be used with caution. Stellate ganglion blockade has been shown to reduce VSM, but the follow-up is still short (Walega *et al.*, 2014).
- Treatment of vulvovaginal atrophy (VVA).

Table 3 Products used in MHT (not available in all countries)

(1) Estrogens
Oral estrogens
Micronized estradiol(E2): 0.5, 1.0, 2.0 mg day^{-1}
E2 valerate: 1.5 mg day^{-1}
Conjugated estrogens (CEE): 0.3, 0.45, 0.625 mg day^{-1}
Transdermal estrogens
E2 patch 0.025–0.1 mg once or twice weekly
E2 percutaneous gel 0.25–1.5 mg
E2 transdermal spray 1.5 mg
Vaginal ring
E2 acetate 0.05–0.10 mg day^{-1} systemic levels of estradiol provide relief of VMS
90-day duration/ring

(2) Progestin
Oral progestin
Micronized progesterone: 100, 200 mg day^{-1}
Dydrogesterone: 10 mg day^{-1}
Medroxyprogesterone acetate (MPA): 2.5, 5, 10 mg day^{-1}
Norethindrone: 0.35 mg day^{-1}
Norethisterone acetate: 5.0 mg day^{-1}
Megestrol acetate: 20, 40 mg day^{-1}
Chlormadinone acetate: 5, 10 mg day^{-1}
Medrogestone: 5 mg day^{-1}
Nomegestrol acetate: 3.75, 5 mg day^{-1}
Promegestone: 0.125, 0.25, 0.5 mg day^{-1}
Intrauterine system progestin
LNorg 20 μg released per day IUS for 5-year use
6 μg day^{-1} IUS for 3-year use
Vaginal gel progesterone 4%, 8% 45- or 90-mg applicator

(3) Combination hormone therapies
Oral
CEE + MPA 0.3–0.625 mg per 1.5–5 mg day^{-1} cyclic or continuous
E2 + Neta 0.5–1 mg per 0.1–0.5 mg day^{-1} continuous
E2 + drospirenone 0.5–1 mg per 0.25–1 mg day^{-1} continuous
E2 + norgestimate 1 mg per 0.09 mg day^{-1}
E2 + dydrogesterone 1–2 mg per 5–10 mg day^{-1} cyclic and continuous
E2 + cyproterone acetate 2 mg per 1 mg day^{-1} continuous
E2 + MPA 1–2 mgper 2–10 mg day^{-1} continuous
CEE + BZA 0.45 mg per 20 mg day^{-1} continuous
Transdermal
E2 + Neta 50 g per 0.14–0.25 mg per patch twice weekly
E2 + LNorg 45 g per 0.015 mg per patch once weekly

Table 4 Absolute or relative contraindications to MHT

(1) CV risk factors
If >10% high risk (SCORE), absolute contraindication
Diabetes
Metabolic syndrome
5%–10% moderate risk (to be discussed)
(2) VTE history or risk: E2 oral route
(3) BC survivors
High risk
Moderate BC risk (to be discussed)
(4) Endometrial cancer survivors (according to their stage)
(5) Meningioma

Table 5 Nonhormonal alternatives to MHT and their doses

Paroxetine: 7.5–25 mg
Venlafaxine : 65–150 mg
Desvenlafaxine:100–150 mg
Citalopram: 10–20 mg
Gabapentin: 900–2400 mg
Pregabalin: 150–300 mg

It is a prevalent symptom, often not told, which can be associated with pain, discharges, and dyspareunia (Gandhi *et al.* 2016). VVA may also worsen incontinence. Topical estrogens, moisturizers, and lubricants can help to treat the symptoms and improve dyspareunia. Administration of topical estrogens as estradiol, estriol, and promestriene twice a week is usually sufficient and carries no proliferative effect on the endometrium. A vaginal ring which delivers 5 μg of E2 and last 3 months is also available. They are indicated in the various type of incontinence in combination with physiotherapy. Adjunction of a progestin is not necessary. Recent data suggests no increase in the risk of BC, CV events, nor endometrial cancer in women who have used topical estrogens (Crandall *et al.*, 2017). In women with BC using aromatase inhibitors, however, use of topical estrogens is not recommended but moisturizers and lubricants can be used. In some countries, ospemifene (60 mg day^{-1}) is available for the treatment of VVA moderate to severe. It is a SERM with significant agonist activity and thus activates the coagulation. In addition it can worsen VSM symptoms. The endometrial safety is still to be established at long term but they were no significant hyperplasia at 1 year of treatment (Constantine *et al.*, 2015).

Two new therapies for VVA need more long-term safety data. Vaginal LASER therapy is developed to treat vaginal dryness, sexual dysfunction, but the long-term efficacy and safety remains to be demonstrated (Pitsouni *et al.*, 2017). Daily intravaginal 0.50% DHEA (6.5 mg) (PRASTERONE) is under study. It seems of a certain efficacy but safety data still remain to be shown.

Conclusions

MHT is still the most effective treatment for climacteric symptoms and help protecting against osteoporosis. The choice of route of estrogen and type of progestin can decrease side effects of the treatment. It is mandatory to evaluate the individual risks of the patient before administrating MHT. Alternatives exist but of lower efficacy. The consultation is a great opportunity for giving information about lifestyle and prevention of cancer and CV risk to women consulting at the time of menopause.

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Hormonal Treatment of Breast Cancer

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Abbreviations

AIs	Aromatase inhibitors
AKT	Protein kinase AKT
CDK4/6	Cyclin-dependent kinases 4 and 6
DFS	Disease-free survival
DMFi	Distant metastasis free interval
DNA	Deoxyribonucleic acid
ER	Estrogen receptors
ERK	Extracellular signal-regulated
ESR1–ESR2	Estrogen receptor alpha and beta
FDA	Food and Drug Administration
GnRH (LH-RH)	Gonadotropin releasing hormone
HR	Hormone receptor
MEK	MAPK-kinase
mTOR	Mammalian target of rapamycin
NASBA	Nucleic acid sequence-based amplification
OFS	Ovarian function suppression
OS	Overall survival
PFS	Progression-free survival
PI3K	phosphatidylinositol 3-kinase
PR	Progesterone receptor
RAF	Rapidly accelerated fibrosarcoma
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator

Introduction

Breast cancer is the most common cancer worldwide, and the second leading cause of cancer death (Aguas *et al.*, 2005).

Many factors, such as age, genetics, family history, diet, alcohol, and obesity, have been implicated in its etiopathogenesis.

Endogenous and exogenous steroid hormones, such as estrogens and progesterone, have also been implicated in its pathogenesis, due to their significant effects on cell growth, differentiation, and function in the breast and other tissues.

The first medical evidence was the suppression of estrogen levels through oophorectomy to cause regression of metastatic breast cancer (Beatson, 1983). Similar effects were observed after adrenalectomy and hypophysectomy in postmenopausal women with breast cancer (Jordan, 2009).

This led to the development of endocrine therapies, with the principal goal of depriving tumor cells of estrogen to induce tumor regression. Endocrine therapies can be given preoperatively (neoadjuvant), postoperatively (adjuvant), and in the metastatic/advanced disease setting (palliative treatment).

Common classes of drugs used for this purpose include the selective estrogen receptor modulators (SERMs), selective estrogen receptor downregulators (SERDs), aromatase inhibitors (AIs), and luteinizing hormone releasing hormone (LH-RH) agonists like buserelin and goserelin (**Fig. 1**).

Steroid Hormones

Steroid Hormones and Their Receptors

Steroid hormones and their receptors include estrogens, progesterone, and androgens. They are manufactured from one common parent molecule, cholesterol, via a reaction catalyzed by several enzymes (Weinberg *et al.*, 2005).

Estrogen is produced by two different organ systems.

In premenopausal women, the vast amount of estrogen (estradiol-17 β and estrone) is produced by the ovaries, in response to the pituitary-derived luteinizing and follicles-stimulating hormones.

In postmenopausal women, precursors of estrogen (testosterone and androstenedione) are produced by the adrenal gland and converted to estradiol and estrone via aromatization.

Steroid hormone receptors bind to steroid hormones such as estrogens and progesterone and relay their signals. Cancers dependent on steroid hormones include breast, prostate, ovarian, and endometrial cancer.

Estrogen Receptors

Estrogen signaling occurs via the standard classical steroid receptor mechanism.

Estrogen diffuses through the cellular membrane and binds monomeric ER protein. This binding leads to a conformational change in ER and induces receptor dimerization. Then, the ligand/receptor complex binds to estrogen response elements in the promoter regions of estrogen-responsive genes enhancing transcription.

The cellular response depends on tissue-specific nuclear ER coregulatory proteins: coactivators and corepressors.

 doi:10.1016/B978-0-12-801238-3.64351-9

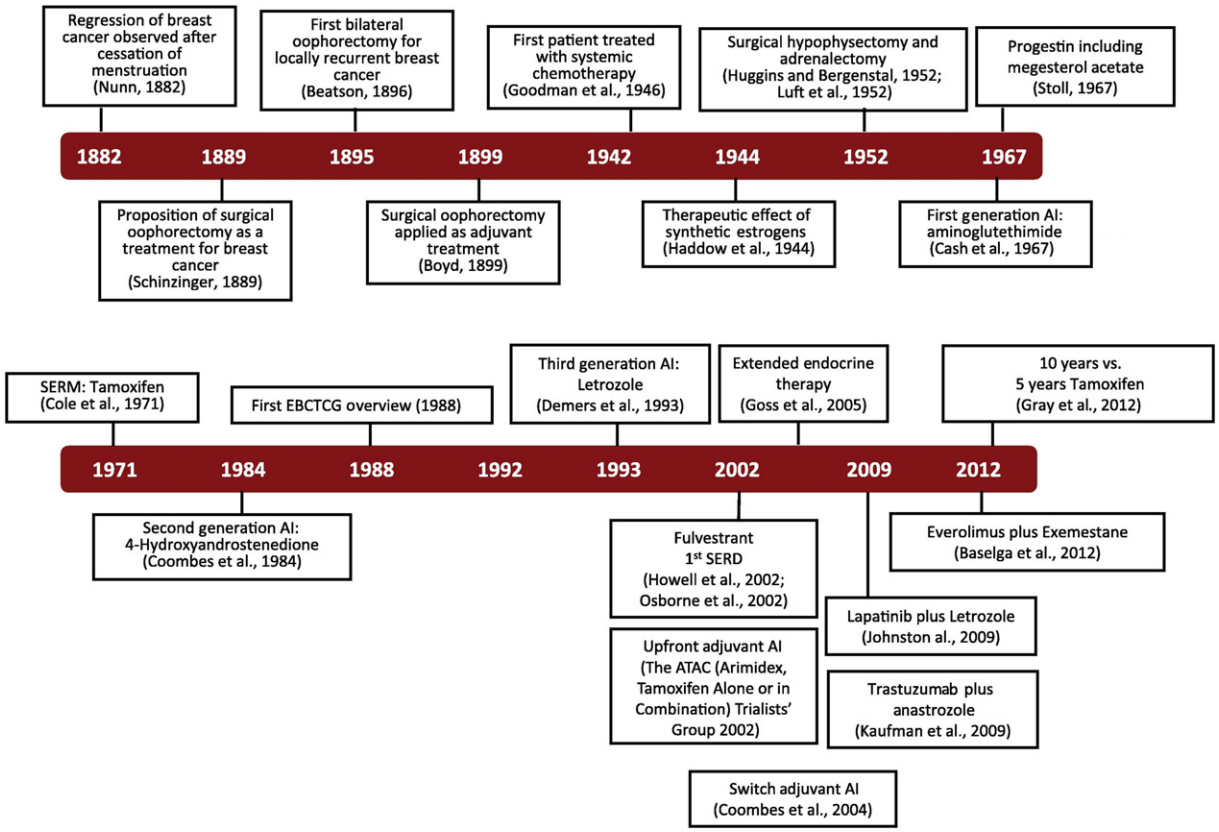

Fig. 1 Historical overview of the development of endocrine therapy and targeted therapy for breast cancer.

There are two separate homologous ER isoforms, ER-α and ER-β, encoded by two separate genes, ESR1 and ESR2, respectively (Kato *et al.*, 2005).

ER has been identified as a nuclear protein, associated with the breast cancer cell membrane, and its effects is mediated through a cascade of tyrosine kinases and phosphatases, resulting in a gene activation through nuclear transcription factor modulation (Massarweh *et al.*, 2008).

The receptor–ligand interaction causes significant effects on growth, differentiation, and functioning of many tissues (such as mammary gland, uterus, bone, cardiovascular system, and brain). Prolonged or excessive receptor–ligand interaction may lead to hyperproliferation and malignant transformation, especially in the breast and uterus.

Measurement of Hormone Receptors

During the more than 30 years of analysis of ER, several methods with differing biochemical principles, analytic sensitivity, and analytic precision have been used (Wittliff *et al.*, 1980).

Until about 1990, ER protein was quantified using a variety of ligand-binding assay (Lamy *et al.*, 2006), with homogenization of fresh-frozen tumor followed by centrifugation.

In the 1980s, the availability of monoclonal antibodies to ER had a profound impact on the methodologies for ER assay. Quantification was initially by enzyme immunoassay, with cutoff values approved by the US Food and Drug Administration (FDA) of ER positivity >10 fmol/mg cytosol protein.

In the 1990s, immunohistochemical assays were developed for the assessment of ER/progesterone receptor (PR) status (Malara *et al.*, 2006). The cutoff to distinguish "positive" from "negative" cases, and to consider endocrine therapy in patients, is $\geq 1\%$ ER-positive tumor cells in the United States, whereas it is $\geq 10\%$ ER-positive tumor cells in France.

The intensity of the marking is quoted as follows: 0 (no staining), 1+ (weak and incomplete membrane staining), 2+ (strong, complete membrane staining in $<30\%$ of the invasive tumor cells or weak/moderate heterogeneous complete staining in more than 10% of invasive tumor cells), or 3+ (strong complete homogenous membrane staining in more than 30% of the invasive tumor cells).

Some new methods, like NASBA (nucleic acid sequence-based amplification) or analysis by RT-PCR can also be performed, with high sensitivity and specificity (Lamy *et al.*, 2006).

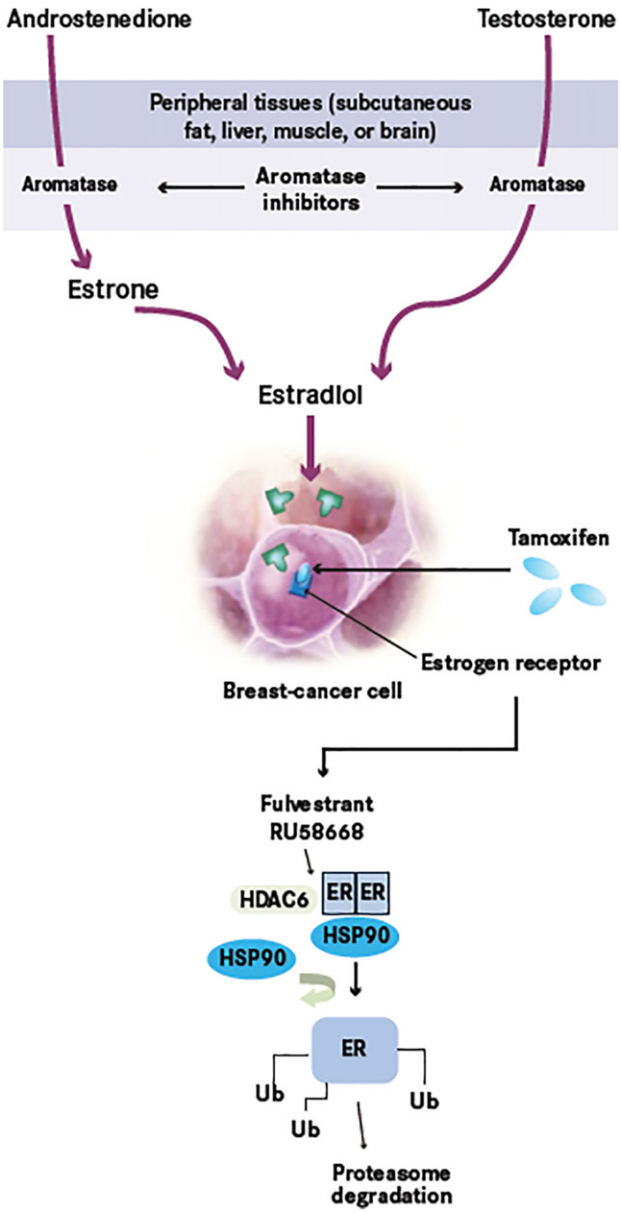

Fig. 2 Principles of hormonal therapy. From Smith, I. E. and Dowsett, M. (2003). Case vignettes in metastatic breast cancer, partially adapted -Aromatase inhibitors in breast cancer. *N Engl J Med*. **348**, 2431–2442.

Hormonal Therapy: Principles (Fig. 2)

➢ ER targeting
- By competitive inhibition of ER: SERM (selective estrogen receptor modulator)
- By ER degradation: SERD (selective estrogen receptor downregulator)

➢ Decreasing circulating estrogen levels
- LH-RH agonists before menopause
- AIs in postmenopausal women

ER Targeting

- By competitive inhibition of ER: SERM

These drugs act as receptor binding competitors of estrogen and block their effects. They bind to the ligand-binding domain of the ER and cause a conformational change in this domain.

The most common is tamoxifen, a nonsteroidal antiestrogen used in treatment of breast cancer, and in prevention in some countries (like the United States) (Cole *et al.*, 1971). It exerts a strong antagonist action at the mammary level and partial agonist on other tissues (endometrium, bones, vessels) (Weinberg *et al.*, 2005).

- By ER degradation: SERD

They are antiestrogens with no agonist activity, and more potent than SERMs.

The most common is fulvestrant, a steroidal antiestrogen. Fulvestrant has a 100-fold higher affinity than tamoxifen to the ER, with no agonist activity in the uterus (Howell *et al.*, 2000).

It is routinely used at the metastatic stage in postmenopausal patients.

Decreasing Circulating Estrogen Levels

- *AIs* in postmenopausal women

AIs block the enzyme involved in estrogen biosynthesis (aromatase cytochrome P450), to stop the production of estrogens from androgens, which is the main pathway of estrogen production in postmenopausal women.

Third-generation AIs have been developed; nonsteroidal (anastrozole, letrozole) and steroidal (exemestane).

These drugs are active in postmenopausal women, or undergoing suppression of ovarian function.

- LH-RH agonists

LH stimulates the ovaries to produce estrogen. GnRH, such as LH-RH, downregulates its own production in the hypothalamus through a reversible reaction. Common examples include buserelin, goserelin, leuprorelin, and triptorelin.

Strategies in the Use of Endocrine Therapy in the Management and Prevention of Breast Cancer

Adjuvant Monotherapy

Tamoxifen

At the end of the 1970s, the antiestrogen tamoxifen was introduced and became the mainstay of adjuvant treatment for patients with estrogen receptor (ER)-positive breast cancer (Jordan, 2008).

It has been first used in advanced breast cancer, then in early breast cancer and ductal carcinoma in situ, and more recently in breast cancer chemoprevention in some countries (Clemons *et al.*, 2002).

In ER-positive early breast cancer, 5 years of adjuvant tamoxifen significantly reduce breast cancer recurrence and mortality throughout the first 10 years and 15 years, respectively (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) *et al.*, 2011).

The most described adverse effects of tamoxifen are menopausal symptoms including hot flashes, fatigue, weight gain, and more rarely vaginal dryness, low libido, mood swings, and nausea. It leads to a significant increase in the risk of uterine cancers and thromboembolic events.

Aromatase inhibitors

Despite the clinical success of tamoxifen, development of drug resistance and endometrial cancer led to the requirement of alternative hormonal therapy, such as nonsteroidal AIs (anastrozole and letrozole) and steroidal AIs (exemestane).

Several randomized trials have compared AIs to 5 years of tamoxifen as primary adjuvant treatment of postmenopausal women with early breast cancer.

The ATAC trial compared 5 years of tamoxifen with 5 years of anastrozole, and showed at the 100 months analysis a significantly improved DFS (HR 0.85, $P = 0.003$) in the anastrozole group, but no improvement in overall survival (Arimidex, Tamoxifen, Alone or in Combination (ATAC) Trialists' Group *et al.*, 2008).

The Breast International Group 1–98 study (BIG 1–98) compared 5 years of tamoxifen with 5 years of letrozole, and showed at the 51 months analysis a significant improvement of DFS (HR 0.82, P = 0.007), and a nonsignificant trend to improved overall survival (Coates *et al.*, 2007).

The TEAM (Tamoxifen, Exemestane Adjuvant Multicenter) trial, comparing 5 years of tamoxifen to 5 years of exemestane, showed no significant difference in DFS, but the trial recorded a high rate of treatment discontinuation and unplanned crossover (Derks *et al.*, 2017).

A meta-analysis of the ATAC and BIG trials revealed that the AIs, compared with tamoxifen, achieved a 2.9% absolute decrease in recurrence (9.6% for AIs vs. 12.6% for tamoxifen; $P < 0.00001$) and a nonsignificant reduction in breast cancer mortality (Dowsett *et al.*, 2010).

The most described adverse effects of AIs are arthralgia, myalgia, tendinitis, carpal tunnel syndrome, osteoporosis and fractures, cardiovascular and cardiac toxicity, hypercholesterolemia, hypertension, vaginal dryness, and libido disorders.

Adding an ovarian function suppression (OFS) to treatment

OFS, with triptorelin or goserelin, and ovarian ablation, with bilateral oophorectomy or bilateral ovarian irradiation, have been investigated in premenopausal women in the TEXT trial (Tamoxifen and EXemestane Trial) and SOFT trial (Suppression of Ovarian Function Trial) (Regan *et al.*, 2013).

The TEXT trial evaluated the outcome between patients receiving 5 years of exemestane plus triptorelin versus tamoxifen plus triptorelin.

The SOFT trial evaluated 5 years of OS in patients receiving tamoxifen plus triptorelin, exemestane plus triptorelin, or tamoxifen alone.

A combined analysis of TEXT and SOFT trials, including 4690 patients, showed an improved DFS among patients receiving exemestane plus OFS (91.1%) versus tamoxifen plus OFS (87.3%) (HR 0.72, $P < 0.001$). There was no difference in OS. In the SOFT trial, there was no difference in DFS between patients receiving tamoxifen plus OFS compared with tamoxifen alone ($P = 0.10$).

Sequential Therapy

The BIG 1–98 trial (BIG 1–98 Collaborative Group *et al.*, 2009) compared letrozole monotherapy to tamoxifen monotherapy and sequential therapy of 2 years of letrozole followed by 3 years of tamoxifen or 2 years of tamoxifen followed by 3 years of letrozole. After a follow up of 71 months, no significant difference was observed in terms of DFS or OS.

The EBCTCG meta-analysis (Dowsett *et al.*, 2010), involving 9856 women, pooled these two trials (ATAC and BIG 01–98 trials) and showed lower recurrence rates with AIs, alone or after 2 or 3 years of tamoxifen, compared with tamoxifen.

Several trials investigated the effect of switching (2–3 years of tamoxifen followed by 2–3 years of AI for a total of 5 years) versus continuing with tamoxifen. The EBCTCG meta-analysis pooled four trials (German Adjuvant Breast Cancer Group, Arimidex-Nolvadex, Intergroup Exemestane Study/BIG 02–07, Italian Tamoxifen Anastrozole Trial), and demonstrated overall superiority of the switch scheme with a decrease of recurrence and in breast cancer mortality (Dowsett *et al.*, 2010).

Extended Adjuvant Endocrine Therapy

Clinical studies have shown that women with early-stage hormone-positive breast cancer have a prolonged risk for recurrence, and half of them happen after 5 years of adjuvant endocrine therapy (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) *et al.*, 2011).

A recent study (Pan *et al.*, 2017) showed that breast-cancer recurrences continue to occur steadily throughout the study period from 5 to 20 years, after 5 years of adjuvant endocrine therapy.

Several trials have investigated the potential therapeutic benefits of extended endocrine therapy beyond the traditional 5 year period either with tamoxifen, AIs, or a combination.

Tamoxifen

Five trials have evaluated the role of extended tamoxifen therapy beyond 5 years.

The three first trials did not show any additional benefits beyond the 5-years period: the NSABP B-14 trial (2001) (Benson, 2001), the Eastern Cooperative Oncology Group (ECOG, 1996) (Tormey *et al.*, 1996), and the Scottish Adjuvant Tamoxifen Trial (2001) (Stewart *et al.*, 2001).

Two larger trials, Adjuvant Tamoxifen: Longer against Shorter (ATLAS) (Davies *et al.*, 2013) trial and Adjuvant Tamoxifen—To Offer More (aTTom) (Azim and Saadeldeen, 2014) trial, showed a statistically significant benefit for disease recurrence ($P < 0.0001$), breast cancer mortality ($P = 0.002$), and overall survival ($P = 0.005$) in their combined results.

A meta-analysis published in PloS One in 2014 (Al-Mubarak *et al.*, 2014), including five trials, has compared the benefits of extended adjuvant tamoxifen (>5 years) with adjuvant tamoxifen (5 years). There was no difference in the risk of recurrence or in all-cause death. Subgroup analysis suggested a greater effect size among lymph-node positive patients.

AIs and tamoxifen

Several phase III clinical trials evaluated the effect of AI use in the extended adjuvant therapy setting.

In the MA.17 trial (Goss *et al.*, 2005), 5187 postmenopausal patients were randomized, receiving 5 years of letrozole or placebo after 5 years of adjuvant tamoxifen. It showed, in patients with the extended letrozole treatment, a significantly improved DFS, a reduction in recurrence risk of 42%, a reduced risk of distant metastasis, and an improved overall survival in node-positive patients ($P = 0.004$).

The NSABP B-33 trial (Mamounas *et al.*, 2008) studied extended AI therapy using exemestane, and showed a nonsignificant improvement of DFS ($P = 0.07$).

A meta-analysis of some trials (Chia *et al.*, 2008) found that AI therapy after 5 years of adjuvant tamoxifen was associated with a 2.9% decrease in recurrence rate and a 0.5% reduction in breast cancer mortality rate.

The IDEAL trial (Blok *et al.*, 2018) showed no superiority in terms of DFS, OS, or DMFi of 5 years over 2.5 years of extended adjuvant letrozole, after an initial 5 years of adjuvant endocrine therapy.

The phase 3 DATA trial (Tjan-Heijnen *et al.*, 2017) assigned postmenopausal women with hormone receptor-positive early breast cancer after 2–3 years of adjuvant tamoxifen to either 3 or 6 years of anastrozole treatment. The 5-year DFS was not significantly different between the two groups ($P = 0.06$), and patients in the 6-year treatment group had more adverse events (arthralgia, myalgia, osteopenia, osteoporosis).

Indications of extended endocrine therapy

The optimal combination and length of adjuvant endocrine therapy is controversial. There is no formal criteria to define the patients that would benefit most from the treatment.

Extending hormonal therapy should be decided based on patient's clinicopathological characteristics and available molecular signatures, such as Prosigna (PAM50) (Filipits *et al.*, 2014) and EndoPredict (Dubsky *et al.*, 2013), which can predict risk for late distant recurrence after endocrine therapy in postmenopausal women.

Side effects and adherence of the patient to treatment are essential to take into account before deciding.

Mechanisms of Resistance to Hormonal Therapy

Introduction

Approximately 70% of advanced breast cancers are considered "hormone responsive," defined by expression of the ER, PR, or both.

In reality, not all of these tumors are truly sensitive to manipulation of the ER pathway, with approximately 20% of HR+ metastatic breast cancers proving refractory to first-line endocrine therapy (McGuire *et al.*, 1977).

The emergence of resistance to endocrine manipulation is inevitable with advanced breast cancer. Although clinicians are encouraged to consider second- and third-line endocrine therapies for patients who initially benefited from first-line treatment (NCCN 2016), the clinical benefit rate declines from approximately 70% for first-line to around 30% for second or more lines of therapy (Chia *et al.*, 2008).

The mechanisms of endocrine resistance are becoming better understood, and treatment strategies are developed.

Mechanisms of Resistance and Emerging Therapies for HR+ Breast Cancer

ESR1 mutations

Mechanism

Loss of ER expression occurs in only 10% of metastatic tumors (Sighoko *et al.*, 2014).

Mechanisms of resistance can be driven by the emergence of additional mutations in the target oncogene.

Attention has recently focused on mutations in the gene *ESR1*, which encodes ERα, resulting in ligand-independent activation of ERα.

ESR1 mutations are rare in a treatment-naïve setting, and become more frequent in metastatic and pretreated ER+ breast cancers (Toy *et al.*, 2013). Robinson *et al.* (2013) indicate an *ESR1* mutation rate in advanced HR+ breast cancer of approximately 22%. PALOMA 3 study found 25% of mutation rates among patients with progression on endocrine therapy, and the SoFEA study 39% of mutation rates.

Highly recurrent mutations were noted at two residues in the ligand-binding domain: p.Tyr537Ser/Asn and p.Asp538Gly (Li *et al.*, 2013).

Emerging therapies

In the case of identification of mutations of ESR1, a group of agents resulting in degradation of ER: the selective estrogen receptor downregulators (SERDs), represented by fulvestrant, seem to have efficacy. They act as ER antagonists without any tissue-specific agonist properties. Fulvestrant binds to the ligand-binding domain of ERα, resulting in a conformation incompatible with transcriptional activation (Wardell *et al.*, 2011).

Prospective and retrospective analysis of ESR1 mutation status in plasma circulating tumor DNA indicated improved outcomes with fulvestrant compared with exemestane among patients with ESR1 mutations, whereas no difference was seen in patients who were ESR1wildtype (Fribbens *et al.*, 2016).

The dose of fulvestrant revealed to be superior in the phase III CONFIRM study is 500 mg on days 1, 15, 29, and every 28 days thereafter (Di Leo *et al.*, 2014). It requires intramuscular administration because of its poor oral bioavailability.

Growth factor receptors, PI3K/AKT/mTOR and RAF/MEK/ERK pathway activation

Mechanisms

Overexpression and/or amplification of growth factor receptors are associated with the emergence of endocrine resistance (Frogne *et al.*, 2009; Turner *et al.*, 2010). They converge on the PI3K/AKT/mTOR and RAF/MEK/ERK pathways.

PI3K pathway hyperactivation promotes estrogen-independent ER transcriptional activation, whereas PI3K inhibition results in increased estrogen dependence, providing a rationale for combined endocrine therapy and PI3K inhibition in endocrine-resistant breast cancer (Ghayad *et al.*, 2008; Bosch *et al.*, 2015).

The PIK3CA gene is mutated in up to 40% of human breast cancers. The frequency of PIK3CA mutations is not increased in metastatic compared with primary breast cancers (Meric-Bernstam *et al.*, 2014; **Fig. 3**).

Emerging therapies

- mTOR inhibitors, represented by everolimus

mTOR position in the PI3K/AKT pathway makes it an attractive target to reverse emerging endocrine resistance.

The phase II TAMRAD study (Bachelot *et al.*, 2012) evaluated the oral mTOR inhibitor everolimus in combination with tamoxifen versus tamoxifen alone in patients with metastatic ER+ HER2-negative breast cancer progressing after prior AI therapy. It showed a significant improvement in time to progression and overall survival in patients with secondary endocrine resistance.

The phase III Bolero 2 trial evaluated the mTOR inhibitor everolimus in combination with the steroidal AI exemestane versus exemestane alone in patients with advanced ER+ HER2-negative breast cancer in progression after prior nonsteroidal AI therapy. It showed better PFS with everolimus-exemestane versus exemestane alone (6.9 vs. 2.8 months), but increase in grade 3–4 toxicities including stomatitis, anemia, dyspnea, fatigue, and pneumonitis.

- PI3K inhibitors

Inhibition of PI3K may obviate some of the feedback activation, improving pathway inhibition (Chandarlapaty, 2012). Pictilisib and buparlisib are pan-class 1 PI3K inhibitors.

The LOTUS phase 2 study, published in the LANCET Oncology in 2017, compared ipatasertib (an oral AKT inhibitor) plus paclitaxel versus placebo plus paclitaxel as first-line therapy for metastatic triple-negative breast cancer. It showed a significantly longer progression-free survival in patients who received ipatasertib.

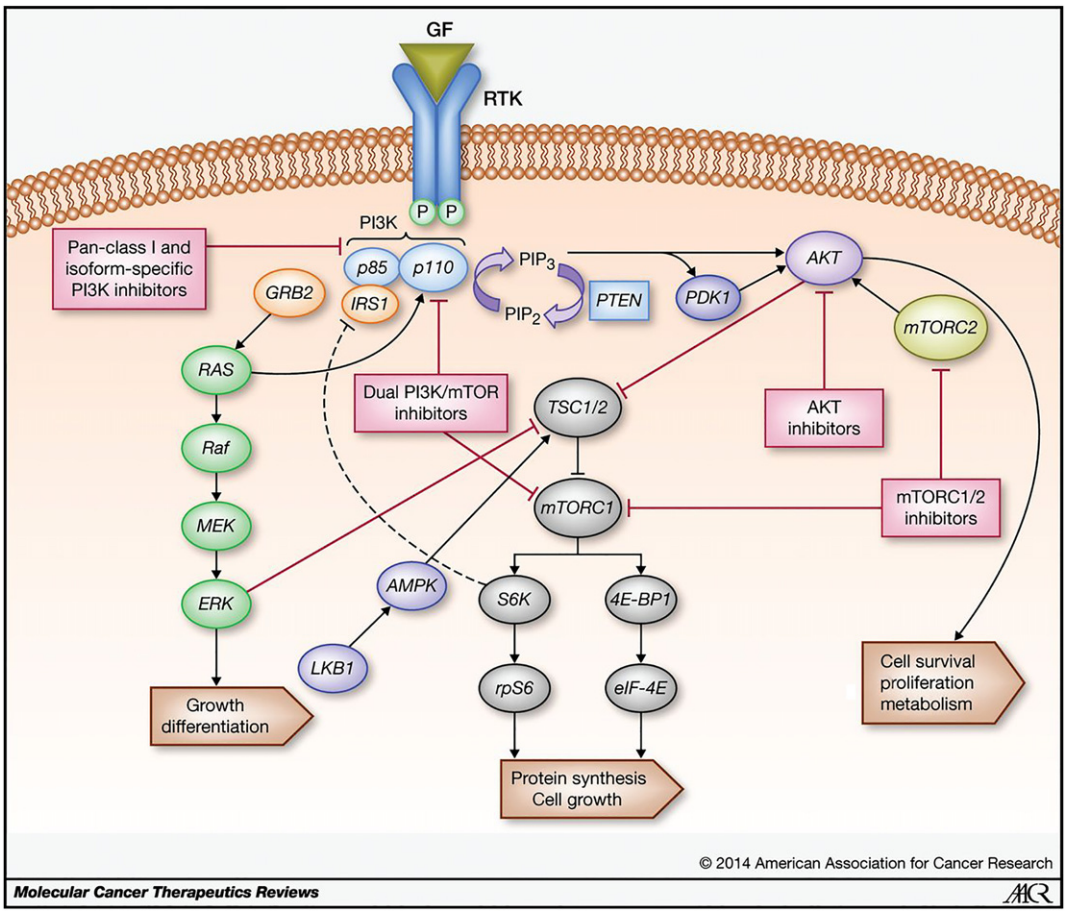

Fig. 3 The PI3K/AKT/mTOR pathway and drug targets.

Cell cycle checkpoint alterations

Mechanisms

Progression through the G1-S phase requires phosphorylation of the retinoblastoma protein (Rb) by the cyclin-dependent kinase CDK4 or CDK6, a family of serine-threonine protein kinases, in complex with cyclin D1, D2, or D3 (Morgan, 1997).

Many tumors increase cyclin D-dependent activity to escape senescence via multiple mechanisms (Cancer Genome Atlas Network, 2012).

Several studies have shown the particular role for CDK4/6 inhibition in ER+ breast cancer cells, including estrogen-sensitive and estrogen-resistant models (Murphy and Dickler, 2015; **Fig. 4**).

Emerging therapies

- CDK4/6 inhibitors

CDK4/6 inhibitors are a new class of selective drugs, offering an effective and tolerable treatment. They induce cell cycle arrest in the G1 phase by preventing phosphorylation of Rb, and may thereby prevent tumor progression.

Today, there are three highly selective CDK4/6 inhibitors in clinical development—palbociclib (Ibrance), ribociclib (Kisqali), and abemaciclib (LY2834219).

Palbociclib and ribociclib were recently approved by the US FDA in combination with letrozole for the treatment of metastatic breast cancer in a first-line setting, as well as palbociclib in combination with fulvestrant for hormone-receptor positive metastatic breast cancer that had progressed on previous endocrine therapy according to the PALOMA-1 (Finn *et al.*, 2015), MONALEESA-2 (Sonke *et al.*, 2017) and PALOMA-3 trials (Turner *et al.*, 2015), respectively.

In the phase III MONARCH 2 trial (Sledge *et al.*, 2017), abemaciclib in combination with fulvestrant significantly improves PFS and ORR, with a tolerable safety profile, in women with hormone receptor-positive and human epidermal growth factor receptor 2-negative advanced breast cancer who progressed while receiving endocrine therapy.

In the MONARCH 3 trial (Goetz *et al.*, 2017), abemaciclib plus a nonsteroidal AI was effective as initial therapy, significantly improving progression-free survival and objective response rates in advanced HR-positive breast cancer.

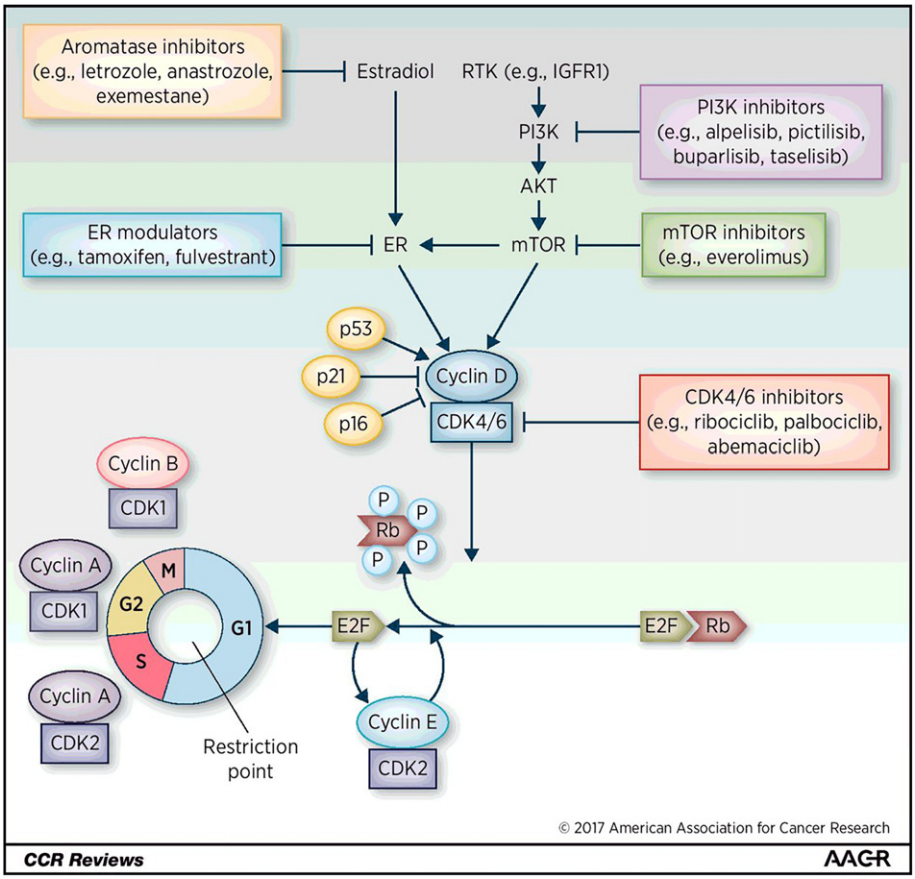

Fig. 4 The role of cyclin-dependent kinase 4/6 inhibition in the cell cycle.

Enhanced autophagy

Autophagy represents a key mechanism by which cancer cells and normal cells deal with various stresses in their environment. Cancer cells demonstrate persistent high levels of basal autophagy. Inhibition of autophagy has been linked to restoration of endocrine sensitivity in preclinical models of endocrine-resistant breast cancer.

Conclusion

Endocrine therapy forms a central modality in the treatment of ER-positive breast cancer. The widespread usage of adjuvant therapy has had a positive impact on overall survival.

Given that women with ER-positive breast cancer remain at long-term risk of relapse, some data have revealed that longer durations of adjuvant therapy result in further survival gains. The optimal duration of adjuvant endocrine therapy remains controversial, with recent data supporting 10 years rather than 5 years of adjuvant tamoxifen in case of bad prognosis.

Endocrine therapy is limited by the development of resistance, whose mechanisms can be modulated by emerging therapies, with the aim of preventing or delaying them.

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Hormonal Treatment of Male to Female Transgender

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Background

According to the Diagnostic and Statistical Manual of Mental Disorders, *transgenderism* refers to the broad spectrum of people who transiently or persistently identify with a gender different from their natal sex (DSM 5, APA, 2013). Indeed, male-to-female transgender (or transwomen or transgender women) describes individuals assigned male at birth but who later in life identify and live as women (Hembree *et al.*, 2017). Many, but not all transgender people, seek a social or a somatic transition in order to alleviate the significant distress (called Gender Dysphoria) resulting from the incongruence between the body and the gender identity.

Somatic interventions aim at aligning the body with the perceived gender, and this medical approach should include gender-affirming hormonal treatment. In line with the recent nonbinary multidimensional conceptualization, gender identities and expressions are diverse (Coleman *et al.*, 2011). Thus, gender dysphoric individuals (GDs) could benefit from flexibility in treatment, according to their final goals (Fisher *et al.*, 2014). Indeed, treatment of GDs should be individualized based on a person's desires (Coleman *et al.*, 2011; Fisher *et al.* 2014). This is particularly the case when transwomen's hormonal treatment is considered. Personalizing hormonal treatment is best done in the context of a multidisciplinary team in collaboration with the referring mental health professional and following accepted safety recommendations. Evidence-based protocols for people located somewhere along a dimensional continuum have not yet been developed, however (Hembree *et al.*, 2017).

Gender-affirming hormone therapy has two major aims: (i) to reduce the secondary sex characteristics of the natal sex and (ii) to induce those of the desired gender (Hembree *et al.*, 2017), in line with client's wishes.

In order to reach these goals, in transwomen, it is necessary to decrease endogenous sex hormone levels (androgens) and to replace them with those of the reassigned sex (estrogens, Hembree *et al.*, 2017). Administration of estrogens alone will suppress gonadotropin secretion incompletely (and, consequently, androgen production). Combined therapy, with both estrogens and antiandrogens, is more effective in reducing endogenous androgens into the female range, thus increasing the feminizing effects of estrogens (Gooren *et al.*, 2008).

Antiandrogens

Several compounds are available to inhibit androgen secretion or action (see **Table 1**). *Cyproterone acetate* (CPA, 50–100 mg/d) is the most common antiandrogen used in Europe for male-to-female gender-affirming hormonal treatment. It has also strong progestogenic proprieties, which result in a negative hypothalamic feedback, leading to a reduction in gonadotropin release and, thus, testicular androgen secretion. In addition, CPA competes with androgens for binding to the androgen receptor and inhibits adrenal steroidogenesis (Hembree *et al.*, 2017; Gooren, 2005; Levy *et al.*, 2003). The recommended dose for transwomen is 50–100 mg/d (Hembree *et al.*, 2017); however, because CPA is highly lipophilic and stored in the subcutaneous tissue, lower doses may also be effective in suppressing androgen levels (Tangpricha and den Heijer, 2017). A variety of hepatotoxic reactions have been reported in men receiving a higher dose of CPA (200–300 mg/day) for prostate cancer (Savidou *et al.*, 2006). However, in transgender populations, only a transient elevation of liver enzymes during hormonal treatment has been reported (Wierckx *et al.*, 2014a,b). In line with this data, no significant differences were found in transaminase levels between transwomen under hormonal treatment compared to those not receiving gender-affirming hormones (Fisher *et al.*, 2016). In addition, for its slight glucocorticoid activity, CPA may contribute to the weight gain observed in transwomen during hormonal treatment. An increased risk of meningiomas, which are hormone-sensitive tumors expressing progesterone receptors, has also been reported in CPA users compared to nonusers (Gil *et al.*, 2011; Ter Wengel *et al.*, 2016). Finally, according to some authors, CPA may contribute to the elevation in prolactin levels induced by estrogens (Defreyne *et al.*, 2017).

Spironolactone (100–300 mg/d), an aldosterone antagonist with antiandrogenic proprieties, is mostly used in the United States, where CPA is not available. It has antiandrogenic effects by blocking the androgen receptor, directly inhibiting testosterone secretion and increasing metabolic clearance of testosterone (Moore *et al.*, 2003; Tangpricha *et al.*, 2003). Its use increases the risk of hyperkalemia and has been associated with gastrointestinal bleeding (Gulmez *et al.*, 2008).

GnRH agonists, taken as monthly injections (3.75 mg) or every 3 months (11.25 mg), are very effective in reducing testosterone levels with a low risk of adverse effects (Dittrik *et al.*, 2005); however, they are extremely costly and, therefore, their use is limited.

Nonsteroidal antiandrogens, such as *flutamide* (50–75 mg/day)—which blocks the binding of androgens to the androgen receptor—can theoretically be used; they induce gonadotropin secretion, with a consequent rise of testosterone and estradiol levels (the latter may be desirable in this circumstance). However, due to their liver toxicity and the unavailability of efficacy and safety data in transgender populations, their use is not recommended (Levy *et al.*, 2003).

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Table 1 Gender-affirming hormonal treatment protocols in male-to-female transgenders

Antiandrogen regimens	
Cyproterone acetate	25–100 mg/d
Spironolactone	100–300 mg/d
GnRH analogs	3.75 mg sc/monthly or 11.25 mg sc/3 months
Estrogen regimens	
Oral estradiol	2–6 mg/d
17-β-Estradiol patch	100–400 mcg/24 h (changing the patch as directed once or twice weekly)
17-β-Estradiol hemihydrate gel	2 mg twice daily
17-β-Estradiol gel	3–4.5 mg daily
Parental estradiol valerate or cypionate	5–30 mg IM every 2 week 2–10 mg IM every week

Finally, the 5-alpha reductase inhibitor *finasteride* (5 mg daily)—which inhibits the conversion of testosterone to 5α-dihydrotestosterone—may be considered as an additional antiandrogen, particularly to slow male pattern balding.

Progestins have also been proposed to suppress gonadotropins and, thus, testosterone secretion. In addition, transgender women often believe that progestins will augment breast development, yet well-designed studies demonstrating additional feminization effects are not available in transgender populations (Wierckx *et al.*, 2014b; Hembree *et al.*, 2017). In addition, breast cancer and cardiovascular diseases have been reported when used in postmenopausal women together with estrogens (Chlebowski *et al.*, 2013; Mueller and Gooren, 2008; Gooren *et al.*, 2004; Rossouw *et al.*, 2002). Finally, other side effects have been reported with progestins, such as water retention and the consequent elevation of blood pressure and weight gain, detrimental lipid changes and depression (Gooren, 2011). Therefore, in consideration of the lack of efficacy and the associated adverse effects, their use is not recommended in gender transition (Hembree *et al.*, 2017).

Estrogens

In order to induce female secondary sexual characteristics, estrogens are required. Typical estrogen dosage in transgender women needs to be two to three times as high as the recommended doses for hormone replacement therapy in postmenopausal women (Moore *et al.*, 2003).

A wide range of estrogenic compounds is available (see **Table 1**), of which 17beta-estradiol represents the treatment of choice. Indeed, the most widely accepted estrogen treatment in Europe consists of oral estradiol 2–6 mg daily (Dekker *et al.*, 2016). Transdermal and injectable routes of administration, avoiding the first-pass effect, have a more favorable lipidic, inflammatory and coagulative profile (Asschemann *et al.*, 2011, 2014). Therefore, transdermal 17beta-estradiol (100 mcg/d) is particularly recommended for transwomen at higher thromboembolic risk (i.e., those older than 40 years, smokers and/or with diabetes or liver disease, Hembree *et al.*, 2017; Van Kesteren *et al.*, 1997; Asscheman *et al.*, 1989). Injectable estrogens are not routinely recommended because they can generate rapid peaks of circulating estrogens, a prolonged time to reach a steady state and a potential risk of patient abuse (Moore *et al.*, 2003).

Oral ethinyl estradiol should be avoided in consideration of the 20-fold increased risk of venous thrombosis and the threefold increase in cardiovascular mortality (Toorians *et al.*, 2003; Asscheman *et al.*, 2011). Moreover, the fact that it is impossible to monitor its blood levels represents an additional important and practical limitation for its use.

Recommendations Before Starting Treatment

Before prescribing gender-affirming hormonal therapy, the treating endocrinologist needs to confirm that the person fulfills diagnostic criteria for GD/gender incongruence and criteria for hormonal treatment (**Table 2**, Hembree *et al.*, 2017).

Moreover, before starting treatment, the treating clinician should evaluate and address conditions that can be exacerbated by this treatment, such as thromboembolic diseases (*high risk*), breast cancer, macroprolactinoma, coronary artery disease, cerebrovascular disease, cholelithiasis and hypertriglyceridemia (*moderate risk*, Hembree *et al.*, 2017). Pretreatment screening is presented in **Table 3**.

Cessation of tobacco use should be strongly recommended to avoid increased risk of thromboembolism and cardiovascular complications (Hembree *et al.*, 2017).

Fertility preservation options should also be discussed before starting treatment (Hembree *et al.*, 2017).

Table 2 Criteria for cross-sex hormone treatment as reported in guidelines on gender-dysphoric/gender-incongruent persons (Hembree *et al.*, 2017)

1. Persistent, well-documented Gender Dysphoria/Gender Incongruence;
2. The capacity to make a fully informed decision and to consent for treatment;
3. Legal age in a given country;
4. If significant medical or mental health concerns are present, they must be reasonably well controlled.

Table 3 Monitoring schedule for transgender females

Pretreatment
Physical examination: weight, blood pressure, waist, body mass index, hair distribution, balding pattern.
Fasting lipid, renal and liver function, glucose, glycosylated hemoglobin, complete blood count, serum estradiol, testosterone, prolactin.
For those taking spironolactone: serum electrolytes (particularly potassium).
Consider BMD testing (according to the natal sex) at baseline, particularly in individuals with existing osteoporosis risk (previous fracture, family history, glucocorticoid use, prolonged hypogonadism, not compliance with hormonal therapy, older than 60 years).
During the first year of hormonal treatment, every 3 months
Physical examination: weight, blood pressure, waist, body mass index, hair distribution, balding pattern, breast development.
Fasting lipid and liver function, glucose, serum estradiol, testosterone, prolactin
Electrolytes (for those taking spironolactone)
During second year of treatment, every 6–12 months
Physical examination: weight, blood pressure, waist, body mass index, hair distribution, balding pattern, breast development.
Fasting lipid and liver function, glucose, glycosylated hemoglobin, complete blood count, hemoglobin, serum estradiol (ideal between 100 and 200 pg/mL) and testosterone (ideal $<$55 ng/dL), prolactin
Electrolytes (for those taking spironolactone)
PSA and digital rectal prostate exam when older than 50 years.
After genital-affirming surgery (when requested), every 12 months
Physical examination: weight, blood pressure, waist, body mass index, hair distribution, balding pattern, breast development,
Fasting lipid and liver function, glucose, glycosylated hemoglobin, complete blood count, hemoglobin, serum estradiol (ideal 100–200 pg/mL) and testosterone (ideal $<$55 ng/dL), prolactin
Additional screening
BMD according to natal sex (if osteoporosis risk exists)
PSA, digital rectal prostate exam according to establish guidelines for biological sex
Mammogram/breast ultrasound according to establish guidelines for assigned sex

Approach in Specific Conditions

Before an Elective Surgical Intervention

Estrogens should be stopped 3–4 weeks before any elective surgical intervention (including gender-affirming surgery) and can be resumed once the person is fully mobilized (Gooren and Delemarre-van de Waal, 2007). After gonadectomy, estrogen treatment should be continued, in order to avoid the signs and symptoms correlated with hypogonadism and to avoid osteoporosis. In those still complaining of male typical sexual hair growth, antiandrogens may remain effective, although their dose may be reduced compared to presurgery.

Postmenopausal Age

Up to now, there is no consensus whether hormonal treatment has to be stopped when client gets older, mirroring the postmenopausal milieu. No data are available in this regard.

Treatment Monitoring

Clinical and laboratory monitoring has to be performed every 3 months during the first year of hormonal treatment and then every 6–12 months (**Table 3**). Routine breast and prostate cancer screening is recommended as in nontransgender individuals (Hembree *et al.*, 2017).

Both serum estradiol and testosterone levels have to be monitored regularly in order to avoid supraphysiologic levels and to minimize the risk of adverse effects (Knezevich *et al.*, 2012). Adequacy of estrogens levels can be monitored by measurement of

serum estradiol levels when oral, transdermal and intramuscular estradiol or its esters are used, but not with conjugated or synthetic estrogens. Theoretically, serum estradiol should be maintained at the mean daily for premenopausal women (100–200 pg/mL) and testosterone levels should be in the female range (<50 ng/dL, Hembree *et al.*, 2017). Treatment doses should be adjusted accordingly. Moreover, body feminization changes should be monitored in order to guide treatment.

Efficacy of Hormonal Treatment

Breast Growth

An increase in breast size usually starts within the first 3 months after the initiation of hormonal therapy and shows a significant progression over 2 years of treatment (Meyer *et al.*, 1986; Fisher *et al.*, 2016). According to these observations, the Clinical Practice guidelines of the Endocrine Society for the endocrine treatment of transgender persons recommend delaying surgical breast augmentation until the person has completed at least 2 years of estrogen treatment (Hembree *et al.*, 2017). Fifty to sixty percent of transgender women have reported their breast formation as quantitatively unsatisfactory and seek augmentation mammoplasty (Gooren and Asscheman, 2013). This may also be the consequence of the fact that the attained size could be disproportionate to the typical male body frame. In addition, in transgender women, breasts are located more laterally on the chest wall and thus the breasts may appear smaller than in natal women with the same size (Wierckx *et al.*, 2014a).

Skin

The reduction of sebaceous gland activity due to androgen deprivation often results in softening and decreased oiliness of skin (Giltay *et al.*, 2000).

Body Hair

Hormonal treatment induces a reduction of sexual hair growth and hair shaft diameter, with hair usually becoming thinner and less pigmented (Giltay *et al.*, 2000). However, facial hair is more resistant to hormonal therapy, particularly in the Caucasian population where a medium Ferriman-Gallwey score of 8 (indicative for hirsutism in natal females, Martin *et al.*, 2008) has been observed after 24 months of treatment (Fisher *et al.*, 2016). Indeed, additional measures, such as electrolysis or laser, are usually necessary to eliminate beard growth.

Voice

Hormonal treatment has no effects on transgender women's voice. Voice therapy can be considered an option to develop a voice within the frequency ranges for a biological female (de Bruin *et al.*, 2000). Alternatively, laryngeal surgery may be suggested in order to change the pitch of the voice, even if it reduces its range.

Testes

Hormonal treatment results in a significant reduction of testis volume (by 40% and 50% after 1 and 2 years of therapy, respectively, Fisher *et al.*, 2016), making it easier to hide male genitals, and, thus, may help alleviating the psychological distress perceived by transwomen.

Sexual Effects

Decrease of sexual desire, spontaneous erections and male sexual dysfunction, often desired by patients, are usually observed within 1–3 months after starting hormonal treatment (Hembree *et al.*, 2017).

Body Composition

Hormonal treatment causes a more feminine body fat distribution, with a decrease of lean body mass and an increase of subcutaneous body fat (Elbers *et al.*, 1999; Van Caenegem *et al.*, 2015; Wierckx *et al.*, 2014a) and a lower waist-to-hip ratio mainly due to an increase in hip circumference. Cardiovascular risk factors may deteriorate, and it is therefore essential to encourage a healthy lifestyle.

Adverse Effects of Hormonal Treatment

Hormonal therapy for transgender females may be associated with adverse effects particularly when supraphysiologic or inadequate doses of sex hormones are used.

Bone Mineral Density

At baseline, transgender females may show significantly lower baseline bone mineral density (BMD) compared to age-matched control men (Van Caenegem *et al.*, 2013). The lower bone mass, together with the often lower vitamin D status, may be explained by the more sedentary lifestyle and less outdoor activities observed in transwomen (Van Caenegem *et al.*, 2013). However, different studies have reported an increase in lumbar spine BMD in transwomen treated with antiandrogens and estrogens (Van Caenegem *et al.*, 2015; van Kesteren *et al.*, 1996, 1998; Wiepjes *et al.*, 2017; Haraldsen *et al.*, 2007) or treated with estrogens and gonadotropin-releasing hormone agonists (Mueller *et al.*, 2005). One prospective study did not find a change in BMD in a small sample transwomen (Haraldsen *et al.*, 2007).

Venous Thromboembolism

A 20-fold increased risk of venous thromboembolism (VTE) has been reported in a large cohort of transgender women receiving hormonal therapy (Van Kesteren *et al.*, 1997). According to a later study from the same group, this increase was found mostly to be associated with the use of ethinyl estradiol than with the use of 17beta-estradiol (Toorians *et al.*, 2003). The more deleterious effect of ethinyl estradiol compared to 17beta-estradiol seems probably linked to effects on activated protein C resistance, due to its molecular structure rather than first-pass liver effect (Toorians *et al.*, 2003). The risk of VTE was also found to be associated with age, being higher in persons older than 40 years, similar to the figure observed in natal females treated with estrogens (Asscheman *et al.*, 1989; Scarabin *et al.*, 2003).

Thus, the use of ethinyl estradiol should to be avoided, and 17beta-estradiol should be considered the treatment of choice. Transdermal preparations are preferred, particularly in subjects older than 40 years. Finally, thrombophilia screening in transgender individuals starting CHT can be restricted to those with a personal or family history of VTE (Hembree *et al.*, 2017; Otto *et al.*, 2010).

Hyperprolactinemia

Estrogen treatments can induce hyperplasia of pituitary lactotropic cells. In the literature, elevations in serum prolactin levels (>1000 mU/L), associated with an enlargement of the pituitary gland, are reported in up to 20% of transwomen during hormonal treatment (Asscheman *et al.*, 1988). Usually, serum prolactin levels return to the normal range after reducing or discontinuing hormonal therapy (Gooren *et al.*, 1985; Bunck *et al.*, 2009). In addition, a recent study has shown a possible contribution of CPA in the prolactin elevation observed in transwomen receiving gender-affirming hormones (Defreyne *et al.*, 2017).

Despite several reports of prolactinomas occurring after high-dose long-term hormonal affirming therapy in transgender women having been described (Gooren *et al.*, 1988; Kovacs *et al.*, 1994; Serri *et al.*, 1996; Cunha *et al.*, 2015; Garcia-Malpartida *et al.*, 2010), the overall risk of prolactinoma can be considered very low.

However, it is recommended to measure prolactin levels before starting hormonal treatment and then at least annually during the first year and, after that, every 2 years (see also **Table 3**). If levels of prolactin are extremely high or persistently increase despite stable or reduced levels of estrogens, a pituitary magnetic resonance imaging (MRI) is recommended (Hembree *et al.*, 2017). In fact, it is of note that the majority of hyperprolactinemia signs and symptoms (such as hypogonadism hypoactive sexual desire, sexual dysfunction and gynecomastia) may not be apparent in transgender women. Another common cause of drug-induced elevated prolactin is the use of psychotropic drugs, and concomitant treatment should be considered during hyperprolactinemia evaluation.

Cancer Risk

Breast cancer has been reported in relatively few cases of hormonally treated transgender women (Ganly and Taylor, 1995; Pritchard *et al.*, 1988; Symmers, 1968; Brown, 2015), though numbers may be rising (Gooren and Asscheman, 2013; Gooren *et al.*, 2015). The data suggest that estrogen therapy does not increase the risk of breast cancer substantially in the short term, but a definitive conclusion cannot be reached particularly in terms of long-term risk. As in natal females, routine breast self-examination, as well as mammogram/breast ultrasounds, according to the established guidelines for assigned sex should be suggested.

It has been assumed that castration in early life protects against prostate cancer. However, a few cases of prostate carcinoma have been reported in transgender females (Dorff *et al.*, 2007; Thurston 1994; van Haarst *et al.*, 1998; Turo *et al.*, 2013; Miksad *et al.*, 2006; Gooren and Morgenthaler, 2014). In addition, some cases have reported of benign prostatic hyperplasia in transgender women treated with estrogens for 20–25 years (Brown and Wilson, 1997; Casella *et al.*, 2005). According to Endocrine Society

guidelines, prostate-specific antigen (PSA) levels and digital rectal prostate examination should be checked according to established guidelines for natal sex (Hembree *et al.*, 2017).

Cardiovascular Health

In transwomen, hormonal treatment showed apparent favorable changes in metabolic profile, with an increase of high-density lipoprotein cholesterol (HDL) and decrease of low-density lipoprotein cholesterol (LDL, Elbers *et al.*, 2004). However, the latter was also associated with transition to smaller, denser and more deleterious LDL, high in triglyceride content (Elbers *et al.*, 2004). In addition, estrogen and antiandrogen treatment induces an increase in weight, body mass index, total body fat, blood pressure, triglycerides and markers of insulin resistance (van Kesteren *et al.*, 1997; Elbers *et al.*, 2004; Elamin *et al.*, 2010; Fisher *et al.*, 2016). Finally, oral, but not transdermal, administration of estrogens was associated with an increase in inflammatory markers involved in the pathogenesis of vascular disease (Giltay and Gooren, 2000; Wilson *et al.*, 2009).

Regarding the cardiovascular risk of estrogens in transgender women, results are still conflicting. The largest cohort of transgender females, followed for a mean of 10 years, showed no increase in cardiovascular risk (Van Kesteren *et al.*, 1997); however, two more recent cohort studies showed an increased risk of mortality due to cardiovascular disease among transwomen following gender-affirming procedures (Dhejne *et al.*, 2011; Asscheman *et al.*, 2011). Indeed, the Dutch cohort study, with a median follow-up of 18.5 years, showed that current, but not past, ethinyl estradiol use was associated with an independent threefold increased risk of cardiovascular death (Asscheman *et al.*, 2011). No increased risk was found in former users who had changed to other formulations and lower doses of 17beta-estradiol (Asscheman *et al.*, 2011); however, it should be noted that only a small percentage of clients studied by Asscheman and colleagues were treated with 17beta-estradiol.

Thus, further research is needed to better clarify the cardiovascular effects of hormonal treatment in transwomen. In any case, the available evidence stresses the importance of avoiding ethinyl estradiol prescription and directing clients toward a healthy lifestyle.

Reproductive Health

A prolonged estrogen treatment induces a reduction of testis volume and has a suppressive effect on sperm motility and density in a cumulative dose-dependent manner (Payer *et al.*, 1979). Similarly, hormonal treatment in transwomen results in a reduction of testis volume (Meyer *et al.*, 1986; Fisher *et al.*, 2016) and in a high incidence of oligozoospermia, asthenozoospermia, and teratozoospermia (Hamada *et al.*, 2014; Lubbert *et al.*, 1992).

Thus, clients requesting hormonal treatment need adequate information about the options available to preserve (e.g., banking of spermatozoa) their fertility potential, before actual treatment takes place (De Roo *et al.*, 2016; Hembree *et al.*, 2017; Wierckx *et al.*, 2012).

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Testes Embryology: Cellular Molecular Changes☆

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Glossary

Cryptorchidism A developmental defect characterized by failure of the testes to descend into the scrotum.
Embryo The term embryonic refers here to the entire period from fertilization to birth, as gonadal development typically spans the transition between embryo and fetus, and the staging is often poorly defined in many mammals.
Germ cells A general term for the reproductive cells of multicellular organisms. Precursor cells of spermatogonia and oogonia.
Interstitial tissue The tissue between the testis cords, which comprise endocrine Leydig cells, vasculature, mesenchymal cells and peritubular (myoid) cells.
Leydig cell tumor The most common non-germinal tumor of the testis; derived from Leydig cells. It is rarely malignant. It appears among 1-3% of testicular tumors and, although they may occur in children, the median age of appearance is 60 years.
Leydig cells Steroid-producing endocrine cells in the interstitial tissue of the testis. They are regulated by gonadotropic luteinizing hormone from the pituitary and produce testosterone, the major androgenic hormone.
Meiosis A special method of cell division whereby haploid gametes are produced from diploid gonocytes; that is, each daughter nucleus receives half the number of chromosomes characteristic of the somatic cells of the species.
Primordial germ cells The precursors to gonadal germ cells (gonocytes) not yet having colonized the gonadal ridges.
Seminoma A radiosensitive, malignant neoplasm of the testis, thought to be derived from primordial germ cells. They account for roughly half of all testicular germ cell tumors.
Sertoli cells Organizers of initial testis differentiation, later the supporting cells of the testis cord with cytoplasms projecting inward from the basement membrane. They surround and nourish the developing male germ cells (gonocytes) and secrete androgen-binding protein and anti-Müllerian hormone (AMH). Sertoli-Sertoli, Sertoli-spermatogonia and -spermatocyte tight junctions provide a blood-testis barrier
Spermatogonia Euploid male germ cells of an early stage of spermatogenesis, derived from pre-spermatogonia. With the onset of puberty, spermatogonia at the basement membrane (BM) of the seminiferous tubule proliferate by mitotic, then meiotic divisions and give rise to the haploid secondary spermatocytes.
Testicular neoplasms Tumors or cancer of the testis. Approximately 95% of all testicular neoplasms are germ cell tumors.
Testis cords In the embryonic testis; the convoluted solid cylindrical precursors of seminiferous tubules where, after puberty, sperm are produced (spermatogenesis). Testis cords (seminiferous tubules) are composed of developing germ cells and supporting Sertoli cells.

Introduction

The testis has two key functions, spermatogenesis and androgen production. The reproductive function – sperm production – is in a dormant phase until puberty, whereas the androgenic function – male sex hormone production – is central to the regulation of sexual differentiation and development of an individual from a very early stage, as illustrated in **Fig. 1** and **2**. This chapter focuses on embryonic development of the testis and the involvement of testis-derived hormones on masculinization of the embryo.

The testes influence the embryonic development of genital organs and brain by specific hormones (Bocklandt and Vilain, 2007; Svechnikov et al., 2010), as illustrated in **Fig. 3**. These testis-derived hormones are largely produced by Leydig cells, one exception being AMH that is produced and secreted by Sertoli cells. However, before they can be produced and fulfill their androgenic roles, the embryonic testis must first differentiate into a compartmentalized organ through a series of biochemical, cellular, structural, and functional events (Svingen and Koopman, 2013).

The Gonadal Ridge

In the developing embryo the testicular anlagen initially consists of epithelium-covered ridges of mesenchymal cells on the surface of the two mesonephroi running parallel on either side of the body midline (**Fig. 2**). At this stage they are typically referred to as gonadal (or genital) ridges. They are morphologically indistinguishable between the sexes until the seventh week of gestation in

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Table 1 Gene names and their corresponding symbols (abbreviations) as used in the text

Symbol	*Name*
AMH (MIS)	Anti-Müllerian hormone (Müllerian inhibing substance)
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1
DAX1(NR0B1)	Dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on chromosome X, gene 1 (Nuclear receptor subfamily 0, group B, member 1)
DHH	Desert hedgehog
FGF9	Fibroblast growth factor 9
FOXL2	Forkhead box L2
HSD3B1(3β-HSD)	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
INSL3	Insulin-like 3 (Leydig cell)
RXFP2(LGR8)	Relaxin/insulin-like family peptide receptor 2 (Leucine-rich repeat-containing G protein-coupled receptor 8)
PGDS	Prostaglandin D2 synthase
PTCH1	Patched 1
RSPO1	R-spondin 1
SF1(NR5A1)	Steroidogenic factor 1 (Nuclear receptor subfamily 5, group A, member 1)
SOX9	SRY (sex determining region Y)-box 9
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2
SRY	Sex determining region Y
WNT4	Wingless-type MMTV integration site family, member 4

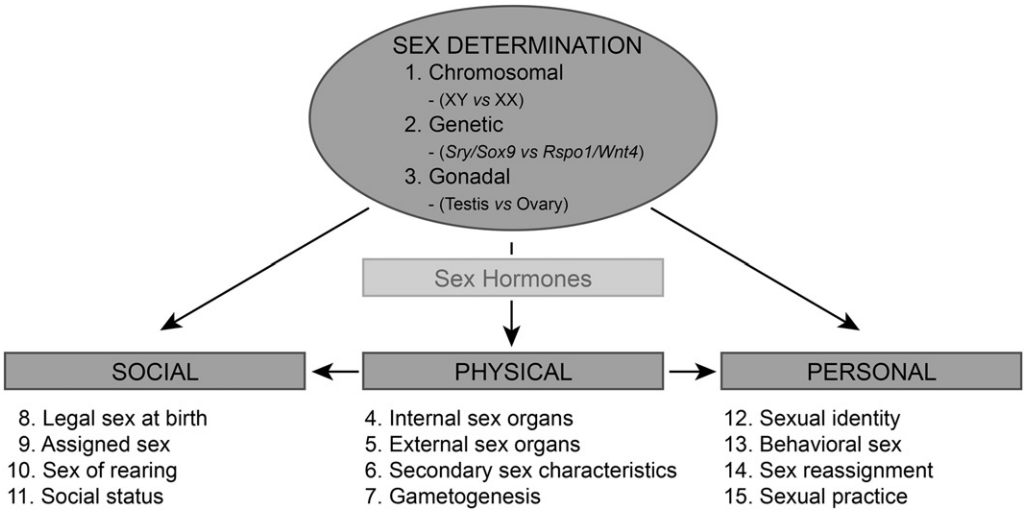

Fig. 1 The central role of testis (or ovary) differentiation in the regulation of sex development, reproductive function, and sexual manifestation in humans and other mammals. The different modalities of sex at various developmental and organizational levels are numbered from one to 15 roughly according to the developmental sequences. Mammalian sex determination is a three-tiered process. First, chromosomal sex (1) is determined at the time of fertilization by the fusion of haploid sperm and egg cells to form a diploid zygote. Second, genetic sex (2) refers to gonadal sex determination, triggered by the Y-linked gene *Sry* (which triggers *Sox9* expression) in XY embryos, or the absence thereof in XX embryos (which allows for expression of pro-ovary genes such as *Rspo1* and *Wnt4*). Third, gonadal sex (3) refers to the differentiation of the indifferent gonads into either testes or ovaries in response to genetic cues, ultimately allowing sex-specific hormones to be produced. Following gonad development, the internal sex organs (4) such as epididymis, ductus deferens and urethra, and the external sex organs (5), the penis and scrotum in males, differentiate in response to hormonal signals. The secondary sex characteristics (6) include shaping of the body and breasts as well as the growth pattern of body hair, which takes place at puberty under the control of testes (or ovaries) together with the pituitary hormones. Gametogenesis (7) refers to the ability of the testes (or ovaries) to produce sperm (or egg) cells. Partially parallel with the development of physical sex, social and personal aspects of sexual development are formed, both by direct and indirect control of sex hormones. The legal sex (8) is determined at birth in normal cases according to the anatomy of the external genitalia. In the case of ambiguity, assigned sex (9) is usually given following more detailed analysis of the chromosomal and genetic status of the individual. The sex of rearing (10) comprises the behavior of parents and/or other significant adults in relation to the growing child. The sex, or perceived sex, of an individual, can also impact on social status (11), including employment opportunities, sporting endeavors, and religious activities to mention a few. Individuals also develop a sexual identity (12) according to internal physical and functional development and the behavior of relatives. This is soon reflected in the manifestation of behavioral sex (13) of a child, or later in adults, according to established roles in each society. In some cases, sex reassignment (14) is performed owing to misinterpretation of the sex at birth or change of subjective sex-identity later in the life. After maturation, sex can also be identified according to anatomical and functional roles in sexual practice (15). From biological, medical, and practical experiences and knowledge, it is evident that any of the modalities constitute a spectrum from normal female through different intersex states into normal male, and that successive developmental phases can be independent and different from the previous phases.

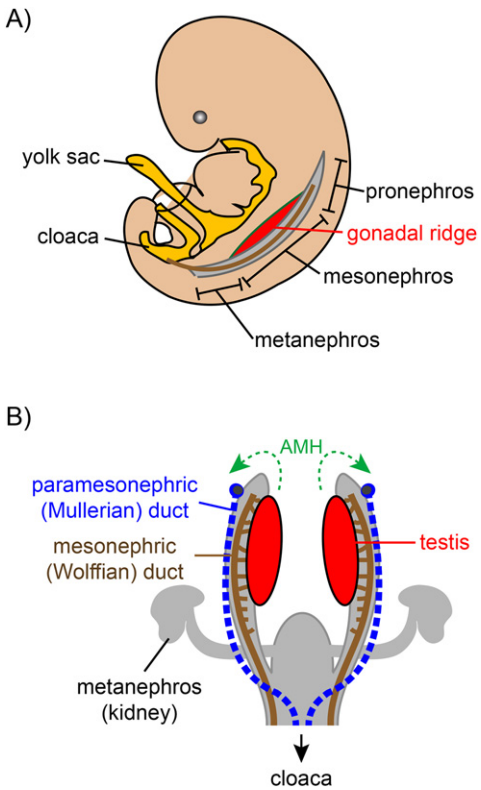

Fig. 2 Gonadal ridge outgrowth, testis differentiation, and establishment of the male reproductive tracts. (a) The gonadal ridges (red) first appear as regional thickenings on the ventral side of the mesonephros, the middle section of the nephrogenic cord that runs parallel on each side of the body midline. The anterior section of the cord is the pronephros (rudimentary kidney) that regresses in mammals, and the posterior section is the metanephros that differentiates into the adult functional kidney. (b) During early embryo development, the rudimentary ductal systems for both male (mesonephric, or Wolffian ducts; brown) and female (paramesonephric, or Müllerian ducts; blue) are present. As the testis differentiates, Sertoli cells produce and secrete anti-Müllerian hormone (AMH; green), which will cause the paramesonephric (Müllerian) ducts to regress (**Fig. 3**), leaving only the mesonephric (Wolffian) ducts to differentiate into the male urogenital structures such as epididymis, vas deferens, and seminal vesicles.

man and by embryonic day 12 in the mouse, after which testicular cords become apparent (Pelliniemi and Fröjdman, 2001; Svingen and Koopman, 2013).

The Testis

Testis development is a complex process involving numerous molecular and cellular events that bring about a sequential differentiation of many cell types that all have specific functions (Svingen and Koopman, 2013). In simple terms, mammalian testis differentiation is triggered by the expression of the sex determining gene *SRY* (located on the Y chromosome) in a few cells within the gonadal ridges of XY embryos. This activates a regulatory cascade that drives differentiation of Sertoli cell, which in turn orchestrate the initial phase of testis development. The Sertoli cells start to aggregate around clusters of germ cells to form irregular cord-like structures, effectively dividing the gonad into two distinct compartments; the testis cords (future seminiferous tubules) and the interstitium. The testis cords grow into loop-shaped tubes connected in each end to the mesonephric base of the testis, which later becomes the rete testis. Peritubular myoid cells then differentiate from interstitial precursors and form a continuous simple epithelial sheath around the testis cords. Together with the developing Sertoli cells, they secrete components that make up a basement membrane that serves both as physical barrier and as structural support for the testis cords. As the cords continue to grow, they buckle in on themselves to form a network of tubules resembling a bowl of spaghetti. The germ cells, now shielded from the interstitial space, enter mitotic arrest and lie more or less dormant until the initiation of meiosis during puberty.

Concomitant with testis cord development, early vascularization takes place with the formation of a distinct artery, the coelomic vessel, along the length of the gonad at the coelomic side (opposite the mesonephric side). Smaller arteries develop between the testis cords, with development of venous and lymphatic networks occurring later. The steroidogenic Leydig cells differentiate within the interstitial space and start producing androgens under the stimulation of human chorionic gonadotropin

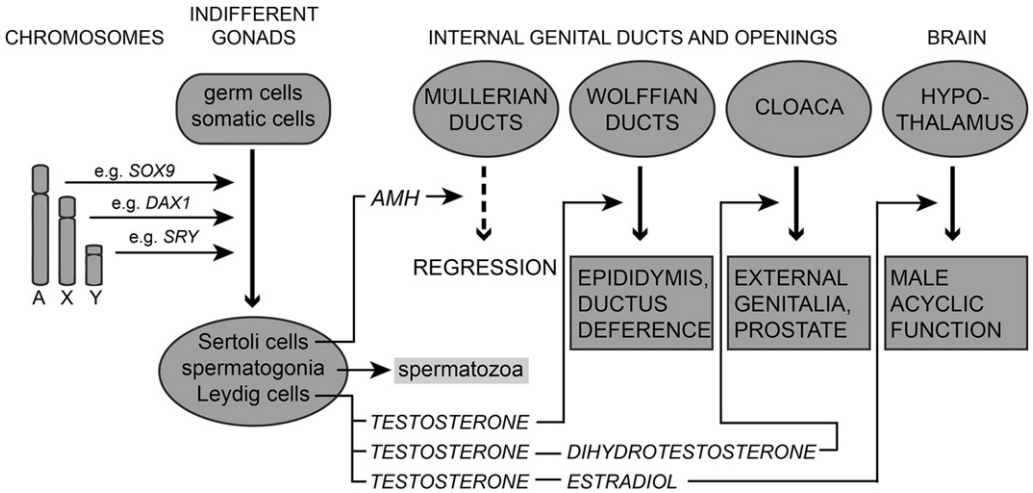

Fig. 3 The differentiation of testis and its subsequent activities in regulating the development of genital organs and brain in the male direction. The regulatory genes controlling testis differentiation are located on the autosomes (A), X chromosomes (X), and the Y chromosome (Y), exemplified by *SOX9*, *DAX1*, and *SRY* respectively. Genes involved in sex determination and differentiation typically have male (M) or female (F) promoting (or directing) functions. In most mammals, including humans, the gonadal sex determining factor is the Y-linked gene *SRY*. Its upregulation in XY gonadal ridges leads to expression of the crucial testis differentiation factor *SOX9*, which again regulates the expression of several downstream testis differentiation factors, for instance *AMH, DHH* and *PGDS*. The expression of SOX9 also suppresses ovary-promoting factors such as *WNT4*, *RSPO1*, and *FOXL2*. Following early differentiation, the fetal testis produces factors (primarily testosterone and AMH) that act on peripheral tissues and organs ultimately directing the fetus to develop into a male phenotype. Under the influence of these gene products, the indifferent gonads consisting of germ cells and undifferentiated somatic cells develop into a microscopically identifiable testis with cylindrical cords (consisting of Sertoli cells and spermatogonia) and the interstitial tissue (containing the Leydig cells). The Sertoli cells secrete AMH, which causes the female paramesonephric duct to regress and thus prevents the development of the oviduct, uterus, and vagina. The Leydig cells secrete testosterone, which via the circulation reaches the embryonic mesonephric duct and stimulates its differentiation into epididymis and ductus deferens. In a similar fashion, after conversion of testosterone into dihydrotestosterone, the prostate and external genitalia develop from the cloacal region of the embryo. Another conversion of testosterone into estradiol takes place in the brain, where the regulatory neurons in the hypothalamus will develop male acyclic function, thus preventing the later start of a menstrual cycle that develops in the female brain without perinatal exposure to testosterone.

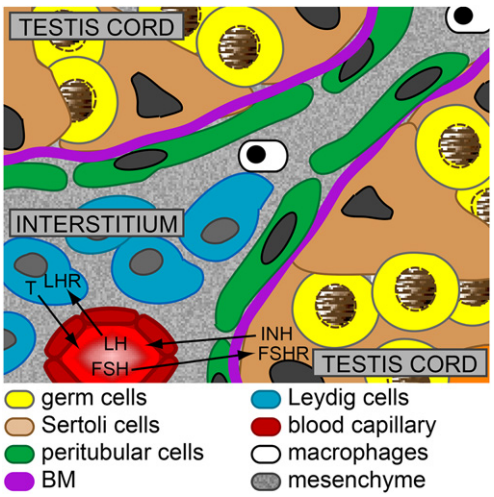

Fig. 4 Histological organization in the embryonic testis. The testicular cords consist of Sertoli cells (light brown) and spermatogonia (yellow). Together with peritubular myoid cells (green) encasing the testis cords, they deposit a basement membrane (BM; purple). The testis cords, future seminiferous tubules, are elongated and curved cylinders, much like spaghetti in a bowl. The tissue between the testis cords is referred to as the interstitium and consists of several cell types, the most prominent being the Leydig cells (blue). The interstitium also contains embryonic connective tissue (mesenchyme; grey background), vascular structures (red), macrophages (white), and peritubular myoid cells (green). The embryonic Sertoli cells produce anti-Müllerian hormone (AMH), which diffuses into the adjacent paramesonephric (Müllerian) ducts and causes their regression (see **Figures 2(b)** and **3**). During the late embryonic period the Sertoli cells have a receptor (FSHR) for the follicle-stimulating hormone (FSH) produced by the pituitary gland. The Sertoli cells also secrete inhibin (INH), which via the circulation is carried into the pituitary gland, where it inhibits the secretion of FSH. The interstitial Leydig cells secrete testosterone, which is locally transported into the peritubular cells and Sertoli cells, whereas testosterone reaches the other more distant target organs by testosterone secretion into the blood circulation through the testicular capillaries. In humans, the fetal Leydig cells are first regulated by hCG via the luteinizing hormone receptor (LHR) in their plasma membrane and later by luteinizing hormone (LH) via the same receptor.

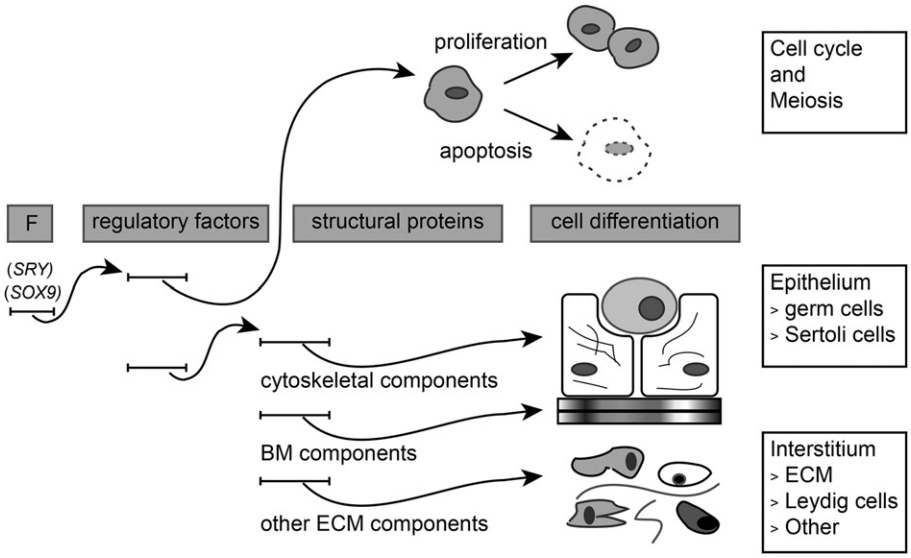

Fig. 5 Regulatory pathways driving testis organogenesis. Initially, sex determining and differentiation factors (F) drive the bipotential gonadal ridges toward a testicular fate. In most mammals, the Y-linked gene *SRY* acts as the male sex determining factor, leading to upregulation of *SOX9* and subsequent testis differentiation. In turn, SOX9 is a direct regulator of several genes in the Sertoli cells such as *AMH* and *DHH*; factors that directly act on other cell types and tissues. In simple terms, upstream regulators such as SOX9 act on other regulators that guide and regulate successive differentiation and proliferation events in other testicular cells, culminating in a compartmentalized and fully functional endocrine organ. During these events, some cells are also cleared by apoptosis, itself a crucial mechanism aiding morphogenesis. Although some primary targets of the testis differentiation factors (e.g., SOX9) are known, most remain to be identified. The segment lines depict genes and the arrows show the route and fate of the gene products, encompassing transcription factors, intermediate signaling molecules, as well as structural, secretory, and other proteins required by cells and the extracellular matrix (ECM). For instance, cytoskeletal components regulating cell shape and motility, basement membrane (BM) components separating and supporting epithelial tissues such as testicular cords in the testis, and ECM components such as collagens and fibronectin in the interstitium of the testis. The interstitial cells have protein components different from those in epithelial cells. The rate of cell cycle, apoptosis, and later meiotic divisions determine testicular growth-rate. The internal organization of the originally undifferentiated epithelial tissue into the testicular cords, which comprise the germ cells and Sertoli cells, is a diagnostic feature of testicular differentiation at the light microscopic level.

(hCG) from the placenta. Notably, hCG has emerged in primates and is typically not present in other mammals where the pituitary luteinizing hormone (LH) likely exerts similar actions.

Testosterone is necessary for proper somatic masculinization of the embryo. The most important regulatory hormones and their functions are described in **Fig. 4**. The interstitium also contains connective mesenchymal cells and macrophages.

Regulatory Mechanisms

The genetic and hormonal pathways that are involved in gonadal sex determination, testis differentiation and ultimately regulation of testicular function are extensive. Here we will highlight some of the most central and best studied, starting with testis determination by *SRY* expression. Once the transcription factor SRY has been activated in a subpopulation of somatic cells, it transcriptionally activates *SOX9*, which has emerged as the key regulator of Sertoli cell differentiation and thus testis development more generally. In turn, SOX9 (an SRY-related transcription factor) activates downstream target genes that themselves regulate various aspects of Sertoli cell differentiation, as well as juxtacrine and paracrine signal transduction pathways acting on other cells and tissues. For instance, regulatory loops involving the para-/autocrine factors FGF9 and PGD2 help ensure the differentiation and recruitment of a critical number of Sertoli cells necessary for testis development, whereas SOX9/SF1 synergistic transactivation of *AMH* ensures the secretion of AMH required for regression of the (female) paramesonephric (Müllerian) ducts (Svingen and Koopman, 2013; Wilhelm and Koopman, 2006). Another secreted factor produced by Sertoli cells is DHH, which influences the differentiation of both peritubular myoid cells and Leydig cells by acting on PTCH1 receptors that are present on most interstitial cells in the early embryonic testis (Franco and Yao, 2012).

The Leydig cells are the source of androgens (the most important being testosterone), which are synthesized *de novo* from cholesterol by the action of several enzymes including HSD3B1 and CYP17A1. Testosterone is in turn secreted into the endocrine circulation where it acts to stimulate the development of accessory sex organs and secondary sex characteristics, including those of the brain (Bocklandt and Vilain, 2007; Virtanen and Toppari, 2014). Leydig cells also produce the peptide hormone INSL3, a key factor inducing testicular descent by acting on the RXFP2 receptor located on the gubernaculum ligaments (**Fig. 5**).

Diseases

Diseases or disorders affecting testis development and/or function can include everything from failure to develop testes altogether during embryonic life, to infertility issues or testicular cancer in adulthood. Here, we will only briefly discuss disorders linked to embryonic development of the testis and not diseases caused by external factors later in life.

In contrast to the embryonic ovaries, the testes produce hormones that play a cardinal role in the regulation of sexual differentiation of the individual, before as well as after birth. Therefore, abnormal development or function of the embryonic testis can often manifest as partial or complete feminization of reproductive organs at birth. The most severe form of testicular disorder is the total lack of testes, as in XY pure gonadal dysgenesis, or Swyer syndrome (Michala et al., 2008). The underlying cause is mutations in genes necessary for early growth and differentiation of the genital ridges, such as *SRY* and *SF1*. As the testes fail to develop, there is no production of either AMH or testosterone, resulting in the formation of female rather than male internal and external reproductive organs and genitalia. This is one of many so-called disorders of sex development (DSD), congenital conditions in which 'development of chromosomal, gonadal, or anatomical sex is atypical' (Hughes et al., 2006). Other examples could include Leydig cell hypoplasia caused by loss-of-function mutations in the luteinizing hormone receptor (LHR), resulting in obstructed androgen production (and mild-to-severe feminized phenotypes such as hypospadia, micropenis or ambiguous genitalia); Klinefelter syndrome (Aksglaede and Juul, 2013), the presence of at least one extra X chromosome in the male karyotype, with typically mild (often undetected) phenotypic traits, although infertility is common; 5-alpha-reductase 2 deficiency (Costa et al., 2012), which is caused by mutations in the *SRD5A2* gene in genetic males and where affected individuals often are born with ambiguous or female external genitalia.

The most common disorder associated with the testis is cryptorchidism (undescended testis) (Virtanen and Toppari, 2014), where one or both of the testes are still located in the abdominal cavity or in the inguinal canal of the newborn. If the testis remains undescended after 4–6 months, medical intervention is important, as the undescended testis is prone to reduced (or lack of) spermatogenesis causing fertility issues, and potentially cancer development later in life.

Testicular neoplasms are fortunately rare and account for around 1% of cancers in men (Ferlay et al., 2010). Most common are germ cell tumors (95%) that, despite comprising a heterogeneous group of tumors, all are believed to originate from the primordial germ cells (Oosterhuis and Looijenga, 2005; Rajpert-De Meyts, 2006). The remaining 5% are somatic tumors and include cancers such as testicular lymphoma, Sertoli- or Leydig cell tumors. Consisting of hormone-producing cells, the Leydig cell tumors can cause strange symptoms by stimulating peripheral organs such as the genitals, hair follicles, and pituitary gland.

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Spermatogonial Physiology and Regulation of the Niche

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Abbreviation

ABP Androgen binding protein
AR Androgen receptor
Arp Actin-related proteins
AKT Protein kinase B
α1 Alpha1
ß6 Beta6
BMP4 Bone morphogenetic protein 4
BTB Blood-testis-barrier
C-Kit Receptor tyrosine kinase
CSF Colony stimulating factor
CXCR4 Chemokine receptor-4
EGF Epidermal growth factor
FGF Fibroblast growth factor
FSH Follicle stimulating hormone
GDNF Glial cell line-derived neurotrophic factor
GFRA1 Glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-1
GnRH Gonadotropin hormone
IGF1 Insulin like growth factor 1
IGF1R Insulin like growth factor 1 receptor
LH Luteinizing hormone
LIF Leukemia inhibitory factor
MAP Mitogen-activated protein
NGF Nerve growth factor
RA Retinoic acid
RAC1 RAS-related C3 botulinum substrate 1
Rho-GTPase Rho family of GTPases
SCF Stem cell factor
SSC Spermatogonial stem cells
TGF Transforming growth factor
VEGF Vascular endothelial growth factor
Taf4b TATA box-binding protein-associated factor 4b
3D Three dimensional

Glossary

Androgens Steroid hormones regulating development of male sexual characteristics.
Blood-testis-barrier *Cellular* barrier (tight band gap junction between Sertoli cells) separating the seminiferous tubules into a basal and adluminal compartment.
Cytokines Cell signaling molecules involved in autocrine, paracrine and immune regulation.
Follical Stimulating Hormone, FSH Gonadotropic hormone produced in the pituitary which stimulates Sertoli cells.
Leydig cell Interstitial cells producing testosterone on LH stimulation.
Luteinizing hormone, LH *Gonadotropic* hormone produced in the pituitary which stimulates testosterone production in Leydig cells.
Peritubular cell Smooth muscle cells surrounding seminiferous tubules, involved in testicular cord formation and transport (by peristaltic movement) of mature sperm into the epididymis.
Sertoli cell Testicular somatic cells and epithelial constituents forming the structural backbone of the seminiferous tubules. Sertoli cells form a syncytial-like epithelium with embedded layers of differentiating germ cells.
Spermatogenesis The process of male gametogenesis from undifferentiated spermatogonia to mature spermatozoa including sequential mitotic and meiotic divisions.
Testosterone Male sex hormone stimulating androgen dependent cellular functions and the development of male-specific sexual characteristics.

Stem Cell Niche

Term "Stem cell niche" was first introduced by Schofield in 1978 to describe the role of microenvironment in tissue-specific stem cell function (Schofield, 1978). Niches are exclusively specific to the tissue-specific stem cell populations (e.g., intestinal/hair-follicle/neural/testicular/bone marrow stem cells). Niche function is regulated by somatic cells depending on the developmental stage-specific requirements of the tissue (as in neuronal stem cells, symmetric or asymmetric divisions occur depending on the developmental stage-specific functional necessity) (Mione *et al.*, 1997)

Testicular Stem Cell Niche

The in vivo microenvironment of testicular stem cells also referred to as the "testicular stem cell niche," plays a significant role in maintaining the "stemness" and regenerative potential of these cells. The niche provides adequate nourishment, nutritional and

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structural support to spermatogonial stem cells (SSCs) to maintain their self-renewal and differentiation potential (Fuchs *et al.*, 2004; Kanatsu-Shinohara and Shinohara, 2013). The testicular niche is presumably composed of components and growth factors secreted by Sertoli cells, peritubular cells, interstitial cells, germ cells, immune cells like macrophages, vasculature, blood-testis barrier (BTB) and basement membrane. Niches are constituted before the ultimate expansion and differentiation of SSC initiates (Oatley and Brinster, 2008; De Rooij, 2009). Recolonization of germ cells after transplantation experiments substantiates the presence of germ cell niches in testis. Depending on the distinct testicular organizational patterns (like U shaped, cystic and epithelial testis in Nematodes, *Drosophila* and Mammals, respectively), niche structures may vary in various organisms (Ramm *et al.*, 2014). Testicular niches are well studied in model organisms like *Drosophila* (Sheng and Matunis, 2011). However, the structural organization and functional role of niches in regulating spermatogenesis in other species, has yet to be completely understood. Some studies in mouse investigating spermatogonial populations provide evidence that SSC niche is most likely avascular. In contrast, lineage tracing studies in mouse, equine species (stallions and mules) and 3D-remodeling of rodent niches provide evidence that testicular niches are localized in close proximity to vasculature and interstitial region (Chiarini-Garcia and Russell, 2001; Chiarini-Garcia *et al.*, 2003; Yoshida *et al.*, 2007; Chan *et al.*, 2014; De Falco *et al.*, 2015). It has been shown that relocation of spermatogonia results in modified vascular dynamics, thus indicating a role of vascular components in defining the niche region. Although some additional molecular factors (like vascular endothelial growth factor (VEGF)) might be involved in linking vasculature in the interstitial region to the germinal epithelium (Nowak *et al.*, 2008; Yoshida *et al.*, 2007; Potter and DeFalco, 2017) (**Fig. 1**).

Different studies investigated the role of the somatic microenvironment and growth factors secreted by testicular somatic cells in development of the germline stem cell niche, and in SSC colonization, self-renewal, differentiation, apoptosis, de-differentiation and trans-differentiation of stem cells (**Table 1**). Amongst the somatic cells, Sertoli cells are considered to be the most significant constituent of the niche. Thus the number of Sertoli cells is supposedly key determinant of the number of germ cell niches and SSC numbers present in the testis. This has been substantiated by several clinical studies which show correlation of reduced Sertoli cell numbers with impaired spermatogenesis in humans (Hai *et al.*, 2014). Transplantation studies show a significant role of Sertoli cells in efficient SSC colonization of the testis. Recolonization was observed to be more efficient in neonatal recipients compared to adults (Carlson and Conboy, 2007). Studies show that SSCs associate with Sertoli cells by using laminin receptor (using transmembrane proteins α6 and ß1-integrin), chemokine receptor (CXCR4), RAS-related C3 botulinum substrate 1 (RAC1), a Rho-GTPase, translocate and recolonize the niche (Kanatsu-Shinohara *et al.*, 2008, 2012; Takashima *et al.*, 2011). Transplantation studies indicate the role of "adhesion molecules: α6 and ß1-integrin" in SSC homing in the basement membrane (De Rooij *et al.*, 2008; Kanatsu-Shinohara *et al.*, 2008). Kit ligand produced by Sertoli cells and its receptor C-kit is present in type A spermatogonia. Expression of C-kit is observed in differentiating germ cells (Rossi *et al.*, 1993; Sorrentino *et al.*, 1991).

In vitro studies show that maintenance of adequate levels of "Glial cell-line derived neurotrophic factor (GDNF)," a ligand of Gfra1 produced by Sertoli cells is necessary for maintenance of SSC numbers in the testes (Yomogida *et al.*, 2003). A specific feedback mechanism is supposedly involved in regulating adequate GDNF levels in the testis. An increase in GDNF levels leads to a reduced germ cell load and results in an increase of clusters of undifferentiated SSCs. A decrease in GDNF levels evokes a depletion of SSCs (Tadokoro *et al.*, 2002). It is speculated that other growth factors including fibroblast growth factor (FGF2), epithelial growth factor (EGF), leukemia inhibitory factor (LIF), insulin-like growth factor 1 (IGF1) may be complimenting GDNF in regulating SSC numbers (Oatley and Brinster, 2012). GDNF shows a fluctuating expression profile during specific epithelial stages (low during stage 7–8 and 9–11, and high during stage 2–6) (Johnston *et al.*, 2011; Sato *et al.*, 2011; Grasso *et al.*, 2012). In rodents and hamsters, Sertoli cells during different developmental stages show specific FSH expression patterns. FSH is considered to stimulate GDNF expression which is involved in SSC self-renewal; high GDNF expression corresponds with high FSH production. Both FSH and GDNF are thus reportedly involved in SSC localization (De Rooij, 2009; Oatley *et al.*, 2011). In primates, expression of GDNF in peritubular cells has been reported in response to androgen action, but not in Sertoli cells (Potter and DeFalco, 2017). GFRA1 expression has been observed in primate spermatogonia (Ad and Ap); although a heterogeneous expression profile was detected in humans. The functional role of GDNF/GFRA1/Ret pathway in primates is not completely understood.

"Retinoic acid (RA)" also acts on Sertoli cells via RA receptors (RARs), thus fine-tuning Sertoli cell-germ cell interactions. Local RA concentration fluctuates with the epithelial cycle and appears to initiate differentiation of SSCs (Sugimoto *et al.*, 2012). RA induction reportedly stimulates undifferentiated SSCs towards meiotic differentiation; meiotic arrest in germ cells was observed when RA synthesis was disrupted (Potter and DeFalco, 2017). Direct RA action in inducing differentiation is reported by rise in Stra8 and Kit expression and drop in POU5F1 and ZBT16 expression (Dann *et al.*, 2008; Zhou *et al.*, 2008; Busada *et al.*, 2015; Yang *et al.*, 2016). Vitamin A (source for RA) deficiency in mouse testis leads to impaired spermatogenesis (Griswold, 2016). RA is also known to be involved in BTB restructuring and spermatogenesis (Chung and Wolgemuth, 2004; Chung *et al.*, 2005, 2010; Hasegawa and Saga, 2012).

"Fibroblast-like growth factor (FGF2)" is secreted by Sertoli cells. Some studies indicate a significant role of FGFR receptors in spermatogonial selection and clonal expansion. In adult humans, FGFR is found to be expressed by small clones and undifferentiated spermatogonia which seldom divide (Von Kopylow *et al.*, 2012). It significantly enhances SSC self-renewal as patients with higher FGF2 levels show disturbed testicular function beginning at the spermatogonial level especially at older ages (Apert Syndrome, Goriely *et al.*, 2005).

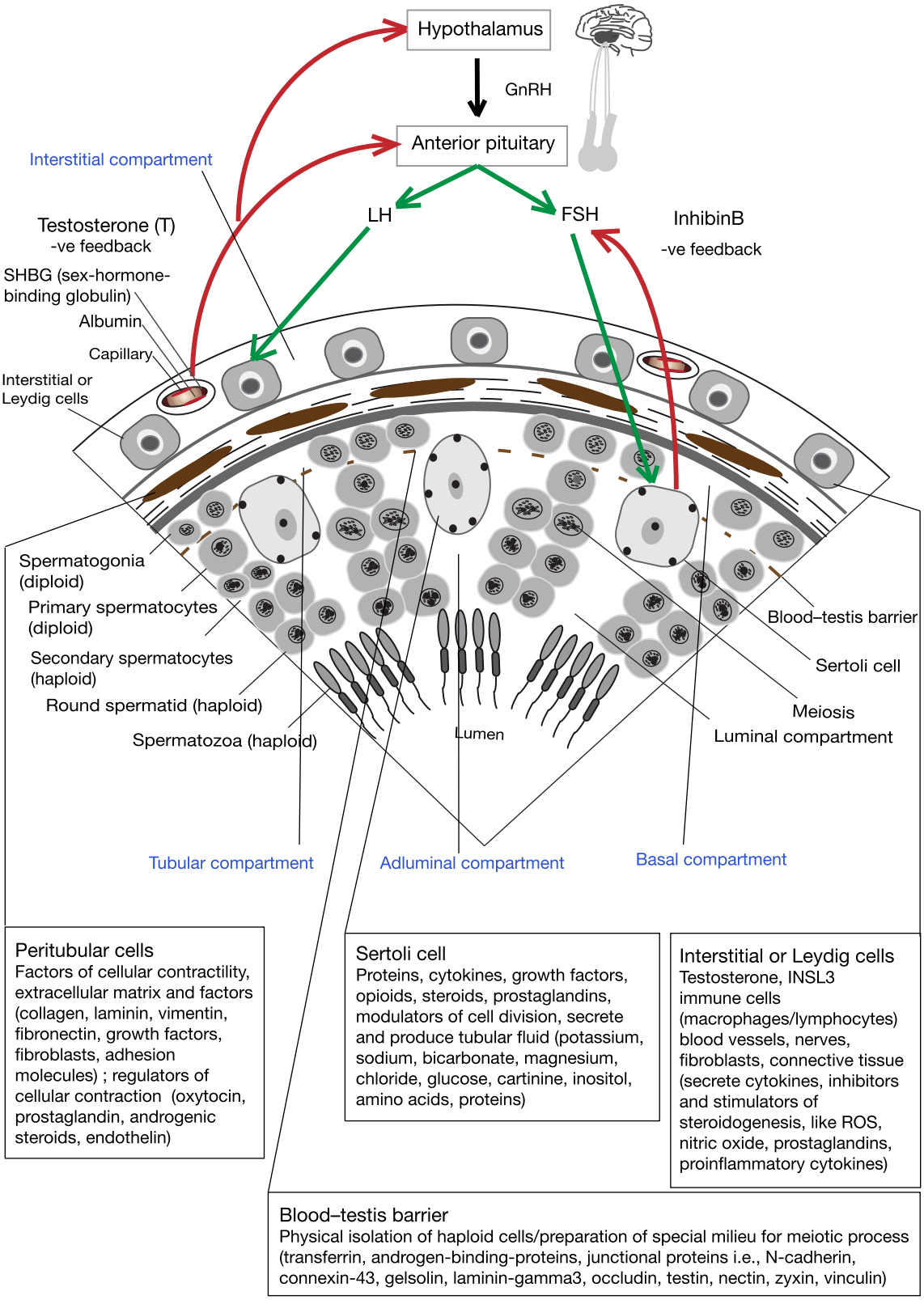

Fig. 1 Testicular niche with structural and functional organization of a testis. Testis comprises of dual compartments: Tubular and Interstitial. Tubular compartment is further subdivided into basal and adluminal compartment, which comprise of undifferentiated and differentiating cell types, respectively. Sertoli cell and peritubular cells are the two somatic cell types present in the tubular compartment. Interstitial compartment comprises of interstitial and immune cells. Endocrine regulation is controlled by the HPG axis. Growth factors secreted by testicular somatic cells either act independently or work synergistically with other niche factors to regulate Spermatogenic function, SSC renewal, proliferation and differentiation.

Table 1 Role and Function of testicular niche factors

Secreted niche component	*Name*	*Secreted/ expressed by*	*Role/function*	*Potentially affected by*	*References*
Growth factors	*GDNF*	Sertoli cells	SSC self-renewal, maintains SSC numbers in testis	FSH	Meng *et al.* (2000), Yomogida *et al.* (2003), and Kubota *et al.* (2004)
	FGF	Multiple testicular cells	Clonal expansion and spermatogonial selection, self-renewal, mitotic divisions, Sertoli-germ cell interactions	FSH	Von Kopylow *et al.* (2012), Goriely *et al.* (2005), Takashima *et al.* (2015), and Kubota *et al.* (2004)
	CSF1	Leydig cells, perivascular compartment	SSC self-renewal, proliferation, niche regulation, adult spermatogonial behavior	Unknown	Kokkinaki *et al.* (2009), Oatley *et al.* (2009), and Potter and DeFalco (2017)
	SCF	Multiple testicular cells	Apoptosis, cell proliferation, differentiation and migration	Unknown	De Rooij (2009)
	LIF	Peritubular cells	Maintenance of pluripotency, Cell proliferation, differentiation of gonocytes to SSCs	Unknown	Pellegrini *et al.* (2003) and Jung *et al.* (2010)
	IGF1	Multiple testicular cells	SSC self-renewal and survival, stimulates steroidogenesis	LH	Kubota *et al.* (2004), Oatley *et al.* (2009), Wang *et al.* (2015), and Cannarella *et al.* (2018)
	NGF	Multiple testicular cells	Involved in meiotic division, tubular integrity	LH	Nieschlag *et al.* (2010)
	TGFα & ß	Multiple testicular cells	Stimulates steroidogenesis	LH	Nieschlag *et al.* (2010)
	EGF	Sertoli cells	SSC proliferation, Meiotic initiation	LH	Oatley *et al.* (2009) and Chen *et al.* (2016)
	VEGF	Multiple testicular cells	SSC renewal, endothelial cell proliferation, survival, migration and permeability	Unknown	Oatley *et al.* (2009) and Potter and DeFalco (2017)
	SDF1 (or CXCL12)	Somatic cells	PGC migration	Unknown	Oatley *et al.* (2009)
	Activin A/B	Multiple testicular cells	Differentiation, steroidogenesis	FSH, LH	Nagano *et al.* (2003), Li *et al.* (2017), and Stanton (2016)
	BMP4	SSCs	Differentiation	Unknown	Nagano *et al.* (2003), and Pellegrini *et al.* (2003)
	Retinol	Multiple testicular cells	Initiation of meiosis, Differentiation of gonocytes, spermatogonia	Unknown	Hogarth and Griswold (2010, 2013) and Agrimson *et al.* (2016)
Transcription factors	e.g., *Ckit, Neuregulin1*	SSCs	Differentiation	Unknown	Stassen *et al.* (1999) and Oatley *et al.* (2009)
	Plzf	SSCs	SSC renewal and differentiation	Unknown	De Rooij (2009)
	Taf4b	SSCs	SSC maintenance	Unknown	De Rooij (2009)
Adhesion molecules	*Cadherin and Integrins (like α6 and ß1 Integrin, CDH1)*	SSCs	SSC homing (?), maintenance of undifferentiated SSC pool	Unknown	Oatley *et al.* (2009) and Chen *et al.* (2013)
Cytokines	Like ROS, nitric oxide, prostaglandin, pro-inflammatory cytokines	Immune cells (like macrophages, lymphocytes)	Inhibitors and stimulators of steroidogenesis	LH	Nieschlag *et al.* (2010)
Junctional Proteins	N-cadherin, connexin-43, gelsolin, laminin gamma-3, occludin, testin, nectin, vinculin, zyxin, claudin	BTB, Sertoli cells	Maintenance of BTB integrity and function	Androgens, LH, FSH	Holstein *et al.* (1996) and Stanton (2016)
Extracellular matrix and factors	Collagen, laminin, vimentin, fibronectin, growth factors, fibroblasts	Basement membrane, Peritubular cells	Maintenance of tubular and BTB integrity, BTB function	Unknown	Nieschlag *et al.* (2010)
Regulators of cellular contraction	Oxytocin, prostaglandin, androgenic steroids, endothelin, panactin, desmin, smooth muscle, actin	Multiple testicular cells (primarily peritubular cells)	Transport of mature sperm	Unknown	Holstein *et al.* (1996), Romano *et al.* (2005), and Nieschlag *et al.* (2010)

Table 1 Continued

Secreted niche component	*Name*	*Secreted/ expressed by*	*Role/function*	*Potentially affected by*	*References*
Non receptor protein kinases	c-Src, c-Yes	BTB	BTB integrity	Unknown	Cheng and Mruk (2002, 2012)
Steroids	Testosterone	Leydig cells	Regulates spermatogenesis, maintenance of BTB integrity and function	LH	Cheng and Mruk (2012) and Walker (2009, 2010)
	Estradiol-17ß	Multiple testicular cells	Regulates BTB function, influences spermatogenesis	LH, FSH	Cheng *et al.* (2011) and Cheng and Mruk (2012)

Interstitial cells stimulate 17-β HSD (hydroxysteroid dehydrogenase) secretion which influences rise in testosterone production. In testis, testosterone binds to "Androgen Receptor (AR)" or "Androgen-Binding Protein (ABP)" and influences spermatogenesis (Potter and DeFalco, 2017). By binding to cytoplasmic AR, testosterone allows secretion and shift of cytoplasmic AR to nuclear location and facilitates its DNA-binding transcription factor function (like modulation of transcription factors *Igf1r*) (Blok *et al.*, 1992; Zhu *et al.*, 2000). In adults, AR is involved in effecting synergistic function of Sertoli cells and adhesion molecules involved in BTB (Blood-testis barrier) formation (Chang *et al.*, 2004; Meng *et al.*, 2005; Wang *et al.*, 2006). Interstitial cells also secrete INSL3 (Insulin-like Factor 3), which presumably influences spermatogenesis via LGR8, found to be expressed in interstitial and mature germ cells (Foresta *et al.*, 2004; Anand-Ivell *et al.*, 2006).

"Insulin-like growth factor 1 (IGF1)" is involved in proliferation of testicular cells showing IGF1R expression (Sertoli cells, Interstitial cells, SSCs and differentiating germ cells). In vitro studies show that interstitial cells regulate their expansion, differentiation and testosterone production through IGF1 secretion in culture. IGF1 influences SSC propagation in conjunction with GDNF, it cannot influence SSC proliferation independently. IGF1 influences synthesis of DNA and acts through IGF1R and AKT signaling pathway, instead of MAP kinases employed by GDNF (Kubota *et al.*, 2004; Wang *et al.*, 2015).

"Colony stimulating factor 1 (CSF1)" expressed in interstitial and peritubular cells is supposedly involved in increasing colony forming potential of spermatogonia, SSC self-renewal, colonization and niche regulation (Oatley *et al.*, 2009). Together with GDNF/FGF, it influences SSC proliferation without impacting rate of proliferation (Oatley *et al.*, 2009). Increased expression of Colony stimulating factor 1 (CSF1) receptor was observed in undifferentiated SSCs (Kokkinaki *et al.*, 2009; Oatley *et al.*, 2009; Potter and DeFalco, 2017). Different studies also report the role of macrophages in influencing spermatogenesis through secretion of CSF1, RA, other cytokines and chemotactic factors (Kokkinaki *et al.*, 2009; Oatley *et al.*, 2009; De Falco *et al.*, 2015).

Growth factors in the niche like "Activin A, BMP4 and SCF" are reportedly involved in germ cell differentiation. In vitro studies show role of these factors in inhibiting self-renewal, and stimulating differentiation (De Rooij, 2009). "Regulatory proteins" including Cdc42, Rho, Rac GTPases, Arp2/3 involved in cellular motility, are secreted by both Sertoli and germ cells. Although Sertoli cells are nonmotile in vivo, they supposedly form pores and extensions in vitro when cultured (Mruk *et al.*, 1997). Actin proteins and signaling molecules secreted by germ cells are reportedly involved in germ cell locomotion (Xiao *et al.*, 2014).

In addition, "intercellular adhesion molecules" ICAMs (1, 2, 3, 4, 5) c-Yes and c-Src (members of nonreceptor protein tyrosine kinase family) expressed by both Sertoli and germ cells and Focal adhesion kinase (FAK) expressed in basal membrane also influence germ cell transport (Xiao *et al.*, 2014). Translocation of preleptotene spermatocytes at epithelial stage 8, through the BTB to the adluminal compartment results in disintegration of old BTB and formation of a new BTB (Xiao *et al.*, 2014). "Cytokines" like Transforming Growth factors TGFß2 and ß3, Tumor necrosis factor TNFα, Interleukin-IL1α play a significant role in regulating BTB restructuring and dynamics (Cheng and Mruk, 2002).

Soma-germline interactions play a significant role in regulating testicular homeostasis through signaling pathways. These factors might also influence aging processes affecting testicular stem cells in older males. Studies show that during aging, somatic components do not retain their innate functional and nourishment potential, thus resulting in decreased functional efficiency of stem cells. However, when stem cells from older males were transplanted in younger hosts, transplanted cells demonstrate normal regenerative potential and function (Carlson and Conboy, 2007). This substantiates a significant role of niche factors in functional regulation of stem cells. The role of stem cell niches and growth factor enriched microenvironment appears to be central in determining the fate of germ cells (Oatley and Brinster, 2012). Decline in niche function causes impaired spermatogenesis, thus giving rise to subfertility or infertility in men (Oatley and Brinster, 2012).

In the past few years, several in vivo and in vitro studies investigated the role of different transcriptional regulators and testicular factors as illustrated in **Table 1** in maintaining homeostasis and modulating stem cell fate decisions (like self-renewal or differentiation) (Kanatsu-Shinohara and Shinohara, 2013; Kanatsu-Shinohara *et al.*, 2014, 2016b; Takashima *et al.*, 2013; Chan *et al.*, 2014; Kimura *et al.*, 2014; Takashima *et al.*, 2015; Morimoto *et al.*, 2015). However, there is very limited understanding on biological function and components of the niche, how the niche regulates the stem cell pool and which specific factors are functionally involved in maintenance of homeostasis, self-renewal and differentiation (Kanatsu-Shinohara and Shinohara, 2013).

Endocrine Regulation

GnRH synchronously regulates spermatogenesis and steroidogenesis, two autonomous feedback mechanisms which control LH-testosterone and FSH-inhibin axis (Plant and Marshall, 2001). LH-testosterone axis controls androgen production, whereas FSH-inhibin axis controls sperm production (Schlatt and Ehmcke, 2014). LH and FSH are produced in the anterior pituitary. These heterodimers are composed of noncovalently linked α and β subunits, α subunits are common in both hormones; however distinct β subunits regulate their specific biological functions (Combarnous, 1992). LH action on Leydig cells stimulates androgen production which in turn controls androgenization, by stimulating male-specific phenotypic features. FSH receptors are exclusively expressed in Sertoli cells (Dankbar *et al.*, 1995). FSH action on Sertoli cells regulates the expansion of Sertoli cells and thereby determines the number of niches and the size of the SSC pool, which limits maximal sperm production. In primates, FSH works in concert with testosterone for maintenance of spermatogenic function (Schlatt and Ehmcke, 2014). Inhibin B levels are considered an important indicator for the pituitary and serve as a feedback signal to the hypothalamus, to determine sperm production capacity (Schlatt and Ehmcke, 2014; Ramaswamy *et al.* 2000a,b, 2003).

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Germ Cell Differentiation, Male

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Introduction

Spermatogenesis is the process by which males produce spermatozoa. This process takes place within the seminiferous tubules that contain both germline cells and somatic cells. Sertoli cells are large columnar somatic cells that span from the basement membrane to the lumen of the seminiferous tubules and serve as support cells that surround the developing germ cells. Sertoli cells provide structural support, essential nutrients and regulatory factors for germinal cell development (Griswold, 1998; de Rooij and Russell, 2000). Between the seminiferous tubules in the interstitial space are macrophages, Leydig cells, blood and lymphatic vessels. The steroidogenic Leydig cells are of central importance as these cells generate and secrete the male hormone testosterone that is required to initiate and maintain spermatogenesis.

Most of what is known about the spermatogenetic process has been obtained using rodent models because numerous gene ablation/overexpression models exist, in which spermatogenesis is arrested at different developmental stages. Despite the immense amount of interest that has been devoted to understanding the molecular controls committing spermatogonia to their differentiation pathway, much still remains to be learned. The focus of this article is to provide a brief review of the key signaling pathways and molecules implicated in regulating spermatogonial differentiation.

Male Germ Cell Development: A Primer

In the male mouse, germ cell competence is induced at embryonic day 6.5 (E6.5) in the proximal epiblast as directed by signals arising from the extraembryonic ectoderm (Lawson and Hage, 1994). These precursor cells will transition into the primordial germ cells (PGCs) and will then migrate through the dorsal mesentery to reach the genital ridges between E10.5 and E12.5 (Ginsburg *et al.*, 1990; Lawson and Hage, 1994; Richardson and Lehmann, 2010; Saitou, 2009; Molyneaux *et al.*, 2001). Once at the genital ridges, PGCs become surrounded by the developing Sertoli cells, forming the seminiferous cords. The PGCs, now called gonocytes or preferably, prospermatogonia that will proliferate for several days until around 15–16 days post coitum (dpc) when they enter quiescence (Donovan *et al.*, 1986; Tam and Snow, 1981; Kluin and de Rooij, 1981; Vergouwen *et al.*, 1991; Moreno *et al.*, 2010). Two to three days after birth, the arrested gonocytes will: (1) resume mitotic proliferation, (2) migrate from the center of the tubules to the basement membrane, and (3) transition into spermatogonia (Ginsburg *et al.*, 1990).

Shortly after birth the germ cells within the developing testis consist of stem cells and progenitor cells or undifferentiated spermatogonia. The spermatogonial stem cells (SSCs) can self-renew or divide to form two progenitor spermatogonia (A_{paired} or A_{pr}) that are connected by an intercellular bridge (de Rooij and Russell, 2000). These A_{pr} spermatogonia can undergo additional divisions to form syncytial chains of 4, 8, 16, and 32 bridge-connected cells, collectively called the $A_{aligned}$ (A_{al}) spermatogonia (de Rooij and Grootegoed, 1998). The proliferation of the syncytial chains is random with regard to the cycle of the seminiferous epithelium. The progression of A_{al} undifferentiated spermatogonia to differentiating (A1) spermatogonia (A to A1 transition) is a critical regulatory point, as it represents the irreversible commitment of spermatogonia to proceed through meiosis (de Rooij, 2001; Griswold 2016).

In response to an extrinsic signal (retinoic acid), the undifferentiated population will differentiate without a mitotic division to form the first differentiating A_1 spermatogonia. This differentiation step and the subsequent mitotic divisions primes the germ cells to become competent to enter meiosis and eventually results in the production of spermatozoa. Thus, the A to A_1 transition is tightly controlled both temporally and spatially and it is this transition that leads to the cycle of the seminiferous epithelium. In the mouse the A_1 spermatogonia will undergo five additional mitotic divisions to form chains of A_2, A_3, A_4, intermediate (In), and the B spermatogonia (Aponte *et al.*, 2005; Phillips *et al.*, 2010; de Rooij and Russell, 2000). The B spermatogonia will transition into the preleptotene spermatocytes, which will proceed through the first meiotic division to form the secondary spermatocytes. The second meiotic division follows rapidly and gives rise to haploid round spermatids that will then undergo drastic morphological changes during spermiogenesis to become immature spermatozoa. These spermatozoa will the complete their maturation process as they transit through the epididymis.

FSH, Testosterone and Retinoic Acid

Both FSH and testosterone are known to be necessary for normal spermatogenesis in mammals and both have their primary action on Sertoli cells. Gene deletion experiments for the FSH receptor have shown that FSH action is not normally required for fertility but that it ultimately governs the number of Sertoli cells and the size of the germ cell pool (Krishnamurthy *et al.*, 2000). In

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androgen receptor gene deletions, progression of round spermatids to elongating spermatids was inhibited when the androgen receptor function was deleted in Sertoli cells, but progression through meiosis was surprisingly not completely blocked. In one of these studies the numbers of spermatocytes, round spermatids, and elongated spermatids were reduced to 64%, 3%, and 0% respectively, when compared to WT (de Gendt *et al.*, 2004).

In contrast to androgens and gonadotropins, retinoic acid (vitamin A) can act directly on germ cells and promote the development of A spermatogonia to A1 differentiating spermatogonia (for review see (Griswold, 2016)). The essential role of vitamin A for spermatogenesis was established in 1925, when researchers found that rodents fed a vitamin A deficient (VAD) diet were infertile (Wolbach and Howe, 1925). Morphological examination of VAD testes demonstrated the loss of the advanced germ cells and the presence of only Sertoli cells and undifferentiated spermatogonia within the seminiferous tubules indicated the presence of a block in spermatogonial differentiation (van Pelt and de Rooij, 1990, 1991; Gaemers *et al.*, 1996, 1998; Huang and Hembree, 1979; Ismail *et al.*, 1990; Morales and Griswold, 1987; Mitranond *et al.*, 1979). An exogenous injection or dietary replenishment of retinoids reinitiated spermatogenesis by stimulating the arrested A_{al} spermatogonia to differentiate into A_1 spermatogonia, resulting in the production of spermatozoa within retinoid rescued testes (van Pelt and de Rooij, 1990; Clagett-Dame and Knutson, 2011; Griswold *et al.*, 1989; Huang and Hembree, 1979; Morales and Griswold, 1987; Wilson *et al.*, 1953; Wolbach and Howe, 1925). These foundational experiments demonstrated that retinoic acid plays a critical role in regulating spermatogonial differentiation.

The action of RA on the expression of target genes is mediated through two families of nuclear hormones receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Each of these two receptor families consists of three subtypes, α, β, and γ (Chambon, 1996). In cells, RA binds and activates these nuclear receptors which are bound as RAR–RXR heterodimers to retinoic acid response elements (RAREs) in the regulatory regions of target genes (Bastien and Rochette-Egly, 2004). Mouse models with a depletion of either RARα or RXRβ are sterile (Lufkin *et al.*, 1993; Kastner *et al.*, 1996). Although loss of the RARγ receptor located in developing germ cells does not result in sterility, morphological analysis demonstrated that depletion of the RARγ receptor does result in an impairment of the A to A1 transition (Gely-Pernot *et al.*, 2012). Accordingly, ectopic expression of *Rar*γ induced the differentiation of RARγ-negative undifferentiated spermatogonia (Ikami *et al.*, 2015). Studies of mutant mice lacking multiple RARs or RXRs have also provided evidence that retinoid signaling is essential for spermatogonial differentiation (Gely-Pernot *et al.*, 2012, 2015). In addition, when a dominant-negative mutant of RAR was used to conditionally block retinoid signaling specifically in germ cells, the corresponding mutant was not only sterile but also had a complete block in spermatogonial differentiation (Chen *et al.*, 2016).

Testicular Gene Products Directly Affecting Germ Cell Differentiation

Recent technical advances have allowed for a more detailed description of some of the gene products made by testicular germ and/or somatic cells that directly affect germ cell differentiation. Some of the more central factors and well-studied gene products are discussed below.

Sertoli Cell Ligands/Germ Cell Receptors That Regulate Germ Cell Differentiation

Kit/*Kitl*: A commonly used marker of spermatogonial differentiation is KIT (c-KIT), a tyrosine kinase receptor that is located on the surface of germ cells (Besmer *et al.*, 1993). KIT is expressed in a variety of cell types in the postnatal testis ranging from A_{al} spermatogonia to preleptotene spermatocytes and Leydig cells (Manova *et al.*, 1990; Yoshinaga *et al.*, 1991; Schrans-Stassen *et al.*, 1999; de Rooij *et al.*, 1999). The KIT receptor is activated by the binding of its ligand, KITL (also called stem cell factor, SCF), secreted by neighboring Sertoli cells. Upon activation via KITL binding, the KIT receptor stimulates a signaling cascade across the plasma membrane to regulate cellular events (Motro *et al.*, 1991; Marziali *et al.*, 1993). This signaling cascade initiated by KIT has been shown to be critical for the survival, proliferation, and differentiation of spermatogonia. Mutations in either *Kit* (such as *Dominant White Spotting, W*) or *Kitl* (such as *Steel, Sl*) disrupt the KIT/KITL signaling pathway and lead to sterility. Accordingly, analysis of mouse models with heterozygous mutations in the *Dominant White Spotting* locus (*Kit*) or *Steel* locus (*Kitl*) resulted in a block at the A to A1 transition (de Rooij *et al.*, 1999; Koshimizu *et al.*, 1991; Sawada *et al.*, 1991). These observations were further confirmed when another mutant line, *Sl17h/Sl17h* ($Kitl^{Sl}$), containing a splicing defect in the cytoplasmic tail of the KITL protein was shown to contain only Sertoli cells and actively proliferating undifferentiated spermatogonia within the seminiferous epithelium (de Rooij and Grootegoed, 1998; Brannan *et al.*, 1992; de Rooij *et al.*, 1999). Transplantation experments in which wild-type germ cells were transplanted into $Kitl^{Sl}$ mutant recipents revealed that the transplanted spermatogonia expanded and proliferated, however no differentiaion of donor spermatogonia into A_1 spermatogonia was observed (Ohta *et al.*, 2001). Collectively, these studies with mutated KIT or KITL proteins demonstrate that the signaling cascade regulated by KIT/KITL receptor ligand complex is necessary for the irreversible transition of undifferentiated spermatogonia into A_1 differentiating spermatogonia.

Glial cell line-derived neurotropic factor (GDNF): GDNF, an extrinsic factor secreted by the Sertoli cells, is a distant member of the transforming growth factor-β (TGF-β) superfamily of proteins (Saarma, 2000). GDNF is a key factor involved in the self-renewal of undifferentiated spermatogonia but also modulates the ability of spermatogonia to respond to differentiation signals (Meng *et al.*, 2000). Since mice with a null mutation in *Gdnf* die during the first postnatal days, mice with a heterozygous null mutation in *Gdnf*

were examined for the effects of GDNF on spermatogenesis. Testes from those animals displayed a progressive loss of spermatogonia, often resulting in Sertoli cell-only seminiferous tubules (Meng *et al.*, 2000). However, mice containing a transgene overexpressing *Gdnf* displayed an accumulation of undifferentiated spermatogonia, correlating to a block in the ability of spermatogonia to differentiate (Meng *et al.*, 2000). Intriguingly, attempts to rescue this block in differentiation by RA administration resulted in apoptosis of the undifferentiated spermatogonia (Meng *et al.*, 2000). This impaired ability of spermatogonia in the GDNF overexpressed testes to respond to differentiation signals and proceed along their differentiation pathway resulted in infertility (Meng *et al.*, 2000). GDNF mediates its signaling through a receptor complex comprising of the GDNF family receptor alpha 1 (GFRα1) and a ret. receptor tyrosine kinase (RET) (Golden *et al.*, 1999; Yomogida *et al.*, 2003). These receptor complexes are localized to the surface of the undifferentiated spermatogonia (Buageaw *et al.*, 2005; He *et al.*, 2008; Jijiwa *et al.*, 2008; Johnston *et al.*, 2011). Mutations in any of these signaling components results in spermatogenic phenotypes that are analogous to that of GDNF null mice (Buageaw *et al.*, 2005; Jijiwa *et al.*, 2008; Naughton *et al.*, 2006).

Regulatory Factors Endogenous to Germ Cells

PLZF (promyelocytic leukemia zinc finger) or Zbtb16: PLZF is a transcriptional repressor that is expressed in undifferentiated progenitor spermatogonia and was the first gene shown to be essential for stem cell self-renewal in the mouse testis. Homozygous mutant mice produced low numbers of spermatozoa and the germ line was increasingly depleted with age (Buaas *et al.*, 2004).

Inhibitor of differentiation 4 (ID4): ID4 is the first clear marker for spermatogenic stem cells. In adult mouse testes ID4 is expressed exclusively in single cells presumably representing the stem cell population (Chan *et al.*, 2014). These observations suggest that the spermatogenic stem cell population consists of a subset of the A_s or A_{single} cells. When germ cell populations containing ID4-GFP genes were isolated, sorted by ID4-GFP expression and transplanted, all of the transplantation success that indicated the presence of stem cells was in the ID4-GFP positive population (Chan *et al.*, 2014) (**Fig. 1**).

Deleted in azoospermia-like (DAZL): DAZL is an RNA-binding protein belonging to the DAZ (deleted in azoospermia) family of proteins (Reijo *et al.*, 1995). Unlike the *Daz* gene cluster which is found only on the Y chromosome in humans and old-world primates, the single-copy *Dazl* gene is an autosomal homolog that is highly conserved throughout evolution in humans, monkeys, rodents, flies, frogs, fish, and worms (Yen *et al.*, 1996; Reijo *et al.*, 1995; Ruggiu *et al.*, 1997; Cooke *et al.*, 1996; Saxena *et al.*, 1996; Houston *et al.*, 1998; Maegawa *et al.*, 1999; Eberhart *et al.*, 1996). As the name infers, deletions in the *Dazl* gene are strongly associated with infertility in both sexes (Tung *et al.*, 2006; Reijo *et al.*, 1995; Vogt *et al.*, 1996). Due to the clinical significance of mutations in the *Dazl* gene, a lot of interest has been devoted to understand the underlying cause of spermatogenic arrest. Studies have shown that expression of DAZL is germ cell specific and it can be detected postnatally in B spermatogonia, preleptotene spermatocytes, zygotene spermatocytes, and pachytene spermatocytes (Ruggiu *et al.*, 1997, 2000). It has been suggested that DAZL enables germ cells to initiate meiosis in response to retinoic acid (Lin *et al.*, 2008).

Spermatogenesis and oogenesis specific helix-loop-helix (SOHLH): The SOHLH proteins are basic helix-loop-helix transcription factors that play critical roles in cell differentiation. SOHLH1 is expressed in A_{al} through B spermatogonia while SOHLH2 is expressed in A_s through A_4 differentiating spermatogonia (Ballow *et al.*, 2006a,b; Toyoda *et al.*, 2009). As with mutants that display a disruption in spermatogonial differentiation, *Sohlh1* null testes lack differentiating populations; however, proliferating

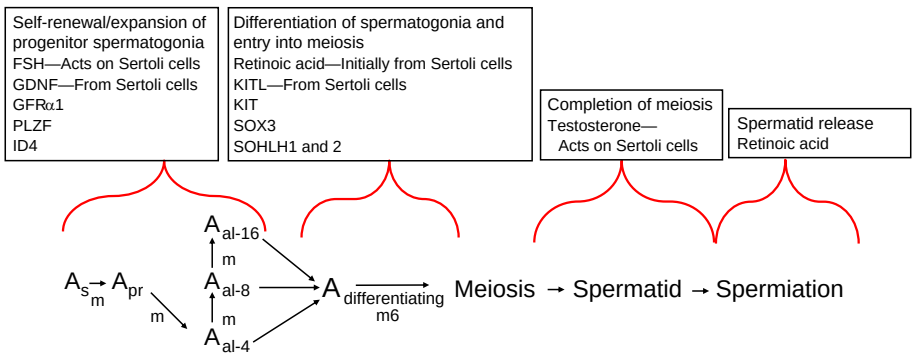

Fig. 1 Summary of male germ cell development from stem cells to sperm formation in the mouse. The major steps in this development and the factors influencing the process are shown. The first steps involve the expansion (m) of cell numbers from A_s (spermatogenic stem cells) into syncytial chains of A_{al} (A aligned) spermatogonia up to as many as 16 cells. This pool of progenitor spermatogonia is influenced by the actions of FSH and GDNF through the Sertoli cells and directly by GFRα1, PLZF and ID4 expressed in the germ cells (see text). In the presence of retinoic acid most of the pool of progenitor cells become differentiating spermatogonia that undergo 6 mitoses (m6), express KIT, Sox3 and the SOHLH proteins and ultimately form preleptotene spermatocytes. In the first wave of cells becoming differentiating spermatogonia the source of the retinoic acid is the Sertoli cells. In subsequent waves of cells the source of retinoic acid may be the more advanced *gem* cells (Griswold 2016). The advancement of preleptotene spermatocytes into meiosis also appears to require retinoic acid. Testosterone acting through Sertoli cells is required for the completion of meiosis and retinoic acid is required once again for the release of spermatozoa into the lumen of the seminiferous tubules.

undifferentiated spermatogonia can still be observed in 8-month old testes (Ballow *et al.*, 2006a). *Sohlh2* null mice show degenerating colonies of A_2–A_4 differentiating spermatogonia and have a disrupted appearance of KIT+ spermatogonia, which is also suggestive of a block in spermatogonial differentiation (Hao *et al.*, 2008; Toyoda *et al.*, 2009). Since these transcription factors can form homo- or heterodimers and mRNA expression of one factor increases when the other is disrupted, it was proposed that they may: (1) function together and (2) functionally compensate for each other to regulate the expression of genes involved in spermatogonial differentiation (Massari and Murre, 2000; Ballow *et al.*, 2006a; Hao *et al.*, 2008; Toyoda *et al.*, 2009; Suzuki *et al.*, 2012). These SOHLH proteins are additional transcriptional regulators that play essential roles in regulating the A to A_1 transition.

SRY-box containing gene 3 (SOX3): Sox3 is a member of the high mobility group (HMG) family of transcription factors (Collignon *et al.*, 1996). The single-exon gene encoding *Sox3* is located on the X-chromosome and is closely homologous with the SoxB1 family of genes, which includes *Sox1* and *Sox2* (Collignon *et al.*, 1996; Schepers *et al.*, 2002). Immunohistochemistry analysis showed that SOX3 is specifically expressed in the undifferentiated spermatogonia (A_s, A_{pr}, and A_{al}) (Rizzoti *et al.*, 2004; Raverot *et al.*, 2005). Laronda and colleagues conditionally eliminated *Sox3* from germ cells using the vasa-cre (Laronda and Jameson, 2011). These mice displayed an accumulation of spermatogonia and a drastic reduction in the number of differentiating spermatogonia. It is worth noting that this block in spermatogonial differentiation induced by elimination of *Sox3* is not permanent, as adult mice testis tubules displayed relatively normal spermatogenesis. Clearly, more research will be needed to determine the mechanism by which *Sox3* influences spermatogonial differentiation and the mechanism by which *Sox3* null males are able to recover.

Conclusions

Spermatogonial differentiation marks the irreversible commitment of spermatogonia to undergo meiosis and results in the production of spermatozoa. Despite the central importance of spermatogonial differentiation to male fertility, we still remain largely in the dark about the molecular controls which regulate this critical transition. It is becoming increasingly clear that this regulatory point is highly sensitive to perturbations, as disruptions to key signaling pathways and molecules (some of which were discussed above) result in a halt in spermatogenesis at this transition. The identification of these signaling molecules and pathways has provided us with considerable insights; however, the leaky phenotypes that are observed in some null models (e.g., SOHLH and SOX3 proteins) have made interpretations of the exact point at which spermatogonial differentiation is arrested difficult to ascertain. Our understanding of these arrest phenotypes is further hampered by our limited understanding and ability to distinguish between the different subpopulations of differentiating spermatogonia. Future studies aimed at dissecting the molecular details underlying each of the differentiating spermatogonia subpopulations may further elucidate the molecular mechanisms regulating the commitment of spermatogonia into meiosis and through spermiogenesis.

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Endocrine Control of Spermatogenesis☆

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Glossary

Apoptosis Programmed cell death; benign process of cell suicide.

Azoospermia, oligozoospermia, and teratozoospermia Absence of spermatozoa in the ejaculate, low concentration of spermatozoa in the ejaculate, and sperm with abnormal morphology, respectively.

Paracrine factor Growth factor or signaling molecule released by cells into the extracellular medium and acting on neighboring cells.

Spermatids Haploid germ cells, differentiating progressively from round spermatids to elongated spermatids and sperm in a process known as spermiogenesis.

Spermatocytes Tetraploid germ cells undergoing two successive meiotic divisions to produce haploid round spermatids.

Introduction

The adult testis has two main functions: the production of sperm and the production of androgens (primarily testosterone). These functions are intimately coupled and are controlled by two gonadotropins secreted by the anterior pituitary: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both of the gonadotropins are composed of two peptide subunits, a common α-subunit and a unique β-subunit that confers biological activity on each heterodimer. LH acts on the Leydig cells located in the interstitial compartment of the testis to stimulate the synthesis of testosterone, which is required to initiate spermatogenesis at puberty and maintain the process in the adult. An important action of testosterone is on the Sertoli cells to promote germ cell proliferation and development. Sertoli cells are the somatic component of the seminiferous tubules where spermatogenesis occurs. FSH acts in concert with testosterone, also by an action on the Sertoli cells. The production of the gonadotropins, in turn, depends on intermittent stimulation by the neuropeptide, gonadotropin-releasing hormone (GnRH), which is synthesized in the hypothalamus and is secreted in a pulsatile manner into the hypophyseal-portal circulation under the control of the GnRH pulse generator in the arcuate nucleus. Kisspeptin is considered to relay the output of the pulse generator to the GnRH neuronal network. The gametogenic and steroidogenic output of this neuroendocrine axis is regulated by testicular feedback signals, primarily testosterone and inhibin B (Fig. 1). This article first describes the fundamental aspects of spermatogenesis and then discusses the endocrine control of the process with a particular focus on testosterone and FSH, the major hormones that are directly involved.

Organization of Spermatogenesis

Spermatogenesis is the process through which diploid stem cells (spermatogonia) multiply and divide into haploid cells that then differentiate into mature spermatozoa. In humans, and in many other species, this is a continuous process resulting, from puberty onward, in the production of vast numbers of spermatozoa (some 10^8 spermatozoa/day or 1000 per heartbeat!). The spermatogenic process is complex but can be divided into five major phases or steps: (1) balance of spermatogonial stem cell (SSC) differentiation and self-renewal; (2) mitotic amplification and differentiation of the spermatogonial progeny of SSC differentiation; (3) entry of differentiating spermatogonia into meiosis with subsequent formation of primary spermatocytes, secondary spermatocytes, and (haploid) round spermatids; (4) differentiation of round spermatids leading to the formation of elongated spermatids and spermatozoa – a complex process known as spermiogenesis and characterized by major morphological changes to the cell, such as the appearance of an acrosome, the formation of a flagellum, and nuclear condensation; and (5) spermiation, the release of spermatozoa into the tubular lumen.

The spermatogenic process is remarkably organized, both spatially and temporally (Figs. 2 and 3). Spatially, developing germ cells move from the periphery of the seminiferous tubules toward the central lumen. In doing so, they are supported by Sertoli cells with which they develop intimate morphological and functional interactions. During their migration and before completion of meiosis, germ cells cross a very tight barrier (blood–testis barrier) formed by specialized junctions situated at the base of

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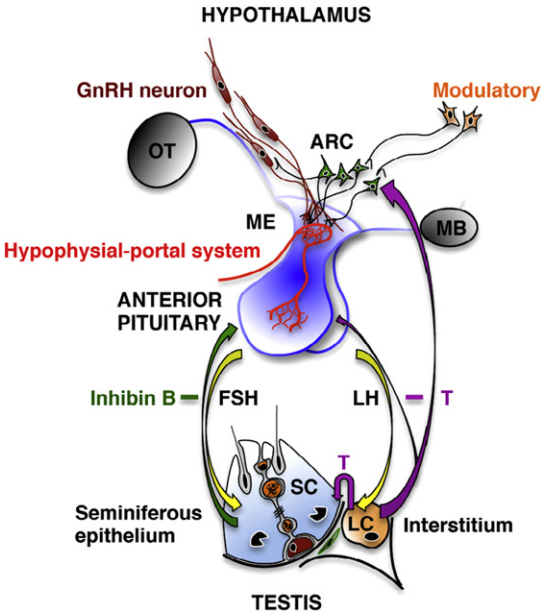

Fig. 1 The neuroendocrine axis governing testicular function. The GnRH pulse generator, resident in the arcuate nucleus (ARC) of the hypothalamus, drives pulsatile release of GnRH into the hypophyseal-portal system. GnRH, in turn, stimulates the synthesis and secretion of the gonadotropic hormones (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) from the anterior pituitary. LH stimulates the secretion of testosterone (T) by the Leydig cell (LC) in the interstitium of the testis. High intratesticular levels of T exert a paracrine action on the Sertoli cell (SC), which is also targeted by circulating FSH, and together, T and FSH act in concert to control spermatogenesis. LH secretion is regulated by a negative feedback action of T, which is exerted primarily on the GnRH pulse generator to decelerate the frequency of pulsatile GnRH release. FSH, on the other hand, is regulated by a direct negative feedback action of inhibin B at the level of the anterior pituitary. The axis is modulated by many factors such as nutrition, stress, and metabolism. While modulation occurs at all three levels of the hypothalamic–pituitary–testicular axis, the role of the hypothalamus in this regard is particularly important. OT, optic tract; ME, median eminence; MB, mammillary body.

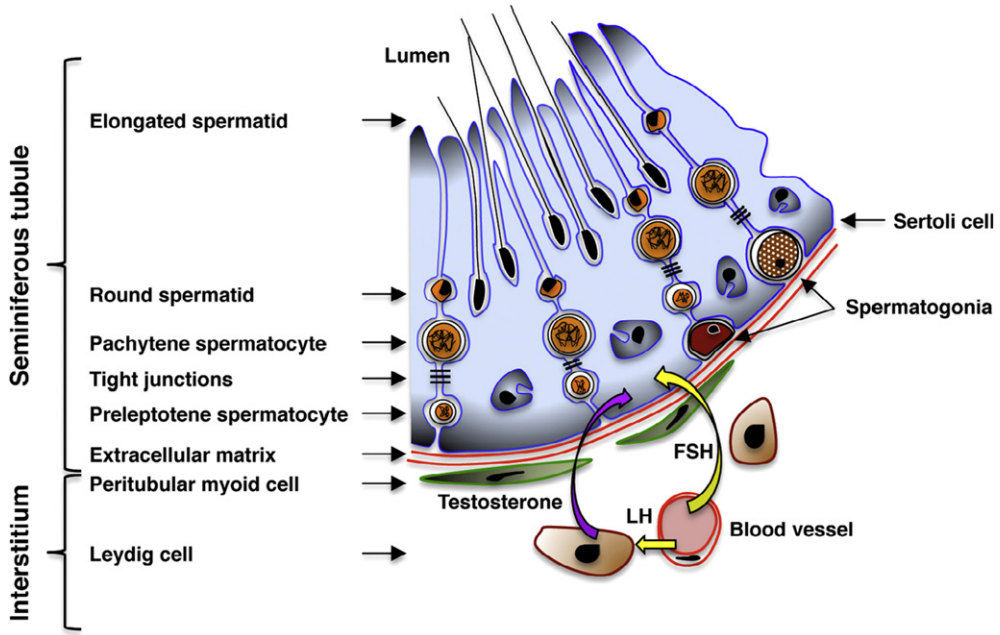

Fig. 2 Schematic of the organization of the seminiferous tubule and interstitium and the endocrine inputs to the germinal epithelium (blue): note that the action of LH on the Sertoli cell is indirect and mediated by the paracrine action of testosterone secreted from the Leydig cell.

neighboring Sertoli cells. This structure is very important since it enables a unique immune, ionic, protein, and nutrient milieu to be maintained in the adluminal compartment, which is stringently controlled by the Sertoli cell and is essential for completion of spermatogenesis.

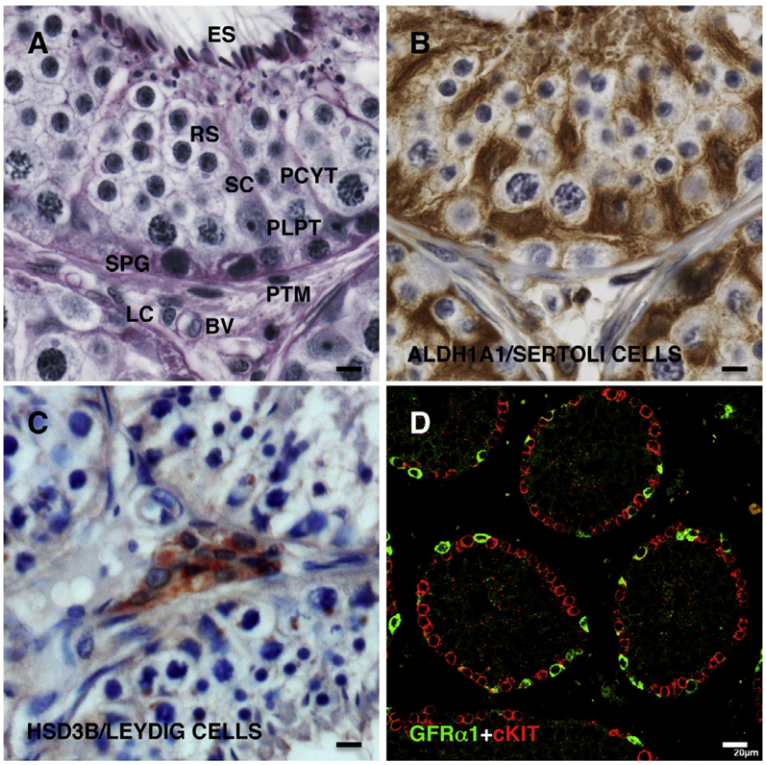

Fig. 3 Cellular architecture of the testis of the adult rhesus monkey. (a) Cross section of a seminiferous tubule stained with periodic acid–Schiff–hematoxylin to show the germ cell and somatic cell types in the adult testis. (b) Cross section of a seminiferous tubule highlighting Sertoli cell architecture by immunohistochemical staining for ALDH1A1 (brown), a Sertoli cell protein. (c) Immunohistochemical staining for HSD3B (brown), a steroidogenic enzyme showing the distribution of Leydig cells in the interstitium. (d) Confocal projection of dual immunofluorescence image of seminiferous tubules stained for GFRα1 (green) marking undifferentiated spermatogonia and cKIT (red) marking differentiating spermatogonia. LC, Leydig cell; BV, blood vessel; PTM cells, peritubular myoid cells; SC, Sertoli cell; SPG, spermatogonia; PLPT, preleptotene spermatocyte; PCYT, pachytene spermatocyte; RS, round spermatid; ES, elongated spermatid; ALDH1A1, aldehyde dehydrogenase 1 family member A1; HSD3B, hydroxysteroid dehydrogenase 3β. Scale bars, 10 μm (a–c) and 20 μm (d). All sections, 5 μm.

Spermatogonial differentiation is initiated in small distinct areas of the tubules and spreads as a synchronized wave to adjacent tubular segments. This temporal organization, coupled with invariant cell cycle times of each of the cell types in the spermatogenic lineage, results in a wave of a species-specific set of germ cell associations moving through the tubules. Each of the cellular associations is known as a stage of spermatogenesis. In human, six stages are typically described. The duration of a spermatogenic cycle (16 days in human) is the time required for a given segment of the tubule to pass through all of the successive stages. The complete development from spermatogonium to spermatozoon in human requires approximately 74 days (more than four cycles), and the spermatogenic stages are arranged in an intertwining helical pattern within the tubules. As a result, a transverse tubular section may display several different stages. In rats and mice (14 and 12 stages, respectively), the situation is simpler as transverse tubular sections reveal only one particular spermatogenic stage. Not surprisingly, Sertoli cell gene expression and function are tightly correlated with the stages of the spermatogenic cycle, with the germ cells dictating the kinetics of the Sertoli cell cycle. Such a temporal organization of spermatogenesis guarantees that, in nonseasonal breeders, sperm are continuously produced and the male is always fertile.

SSC Biology

SSCs, which reside on the basement membrane of the seminiferous tubule, are responsible for the ability of the adult testis to maintain the production of sperm over protracted periods of time, a characteristic that is exemplified in our own species. SSCs are defined by their longevity and ability to produce differentiating progeny and, concomitantly, to self-renew to maintain their own population. Under physiological conditions, mitoses leading to differentiation on the one hand and to self-renewal on the other are balanced by control mechanisms that are poorly understood. SSC fate (i.e., the decision to self-renew or to differentiate) is viewed to be governed by an interplay between the SSC and a specialized microenvironment known as a SSC 'niche' that is created by somatic elements of the seminiferous tubule primarily by neighboring Sertoli cells. The progeny of SSCs that is committed to the path of differentiation undergoes a species-dependent number of amplifying mitotic divisions that lead to spermatogonial clones of increasing size as the lineage progresses prior to meiosis. Early generations of spermatogonia are viewed as undifferentiated cells, while later generations are classified as differentiating spermatogonia. This classification is based on nuclear

morphology revealed by chromatin staining patterns and by expression of molecular markers of cell differentiation revealed by immunohistochemistry. The massive rate of sperm production by the mammalian testis may be achieved by utilizing differing strategies. For example, in rodents, a large number of amplifying spermatogonial divisions are the dominant determinant of sperm output, while in primates, the size of the SSC population appears to take on greater importance. Our understanding of the biology of SSC has been obtained largely from studies of rodents and, particularly, the mouse, a genetically tractable species. In these species, isolated undifferentiated spermatogonia, known as A single (A_s) spermatogonia, are considered to be the SSC, although an element of 'stemness' is apparently retained by the early generations of undifferentiated spermatogonial clones. Self-renewal results from the division of an A_s that is immediately followed by completion of cytokinesis, while commitment to the pathway of differentiation is indicated by an incomplete cytokinesis, which leads to a pair of undifferentiated spermatogonia know as 'A_{pr}.' The molecular determinants of such decision-making by SCCs are poorly understood, but adhesion molecules and members of the transforming growth factor-β family have been implicated. As clonal amplification progresses A_{pr} become A aligned (A_{al}) spermatogonia with clones of 4, 8, and 16 cells represented as A_{al4}, A_{al8}, and A_{al16}, respectively. Several signaling molecules, such as GDNF family receptor alpha-1 (GFRα1), promyelocytic leukemia zinc finger, basic fibroblast growth factor (FGF2), and nanos homolog 2 (NANOS2), have been identified that support the proliferation of undifferentiated spermatogonia, while other signaling molecules such as spermatogenesis- and oogenesis-specific basic helix–loop–helix 1 and 2 (SOHLH1 and 2) and Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) are associated with spermatogonial differentiation.

In highly evolved primates, such as the monkey and human, a somewhat different strategy for the sustained production of premeiotic cells appears to have evolved that enables male fertility in these species to be maintained for decades. From classical studies by Clermont, two major types of undifferentiated spermatogonia are recognized: A dark (Ad), characterized by dense, homogeneous staining chromatin, and A pale (Ap), characterized by granular heterogeneous staining chromatin. Under physiological conditions, Ad divide rarely in the postpubertal testis, but Ap, on the other hand, are mitotically active with a cell cycle of approximately 10 days in the monkey. The majority of the Ap population divides each cycle in synchrony: half of the dividing cohort producing the first generation of differentiating spermatogonia while the other half of the cohort completing a self-renewing division. It is likely that Ap are not immortal, as is effectively the case for A_s in rodents, but rather have a finite life span and when lost are replaced from the pool of Ad, which are considered 'reserve' stem cells. The molecular signals that underlie (1) the decision of Ap to commit to differentiation or to self-renew and (2) maintenance of Ad in a quiescent state, presumably G0, have not been investigated.

Blood–Testis Barrier

The blood–testis barrier is formed between adjacent Sertoli cells at the time of puberty and divides the seminiferous epithelium into a basal compartment, containing spermatogonia and early spermatocytes, and an adluminal compartment containing all later germ cell types. This physicobiochemical barrier provides an immune-privileged site within the seminiferous epithelium for the completion of meiosis and production of round spermatids and completion of spermiogenesis and spermiation. The molecular composition of the blood–testis barrier includes actin-filament bundles, tight junction proteins, ectoplasmic specializations, desmosomes, and gap junctions. Complex mechanisms allow the germ cells to enter the adluminal compartment through the blood–testis barrier. This breach of the barrier occurs at a specific stage of the cycle when preleptotene spermatocytes (basal compartment) differentiate to pachytene spermatocytes (adluminal compartment). Breakdown of the barrier on the adluminal side of the spermatocyte and the restructuring of a new barrier on the basal side unfold in a simultaneous manner. Disintegration of the barrier requires endocytosis by the Sertoli cells of tight junction proteins (e.g., occludin), gap junction proteins, actin filaments, and basal ectoplasmic specializations. Recreation of the barrier may involve recycling of the junctional complex proteins or the synthesis of newly formed components. The simultaneous dissociation of the old barrier and the recreation of a new barrier allow for the maintenance of the unique environment of the adluminal compartment of the seminiferous epithelium.

Factors Controlling Quantity and Quality of Sperm Output

The efficiency and quality of sperm production depend on at least three determinants: a genetic program inherent to the germ cells themselves, a number of variables defining maximal spermatogenic capacity, and a series of regulatory factors deciding whether the system is used at maximal capacity or not. This article focuses only on the second and third of these determinants in which hormones play a definite role. The role of factors inherent to the germ cells themselves is probably illustrated most clearly by germ cell transplantation experiments showing that essential characteristics, such as the duration of the spermatogenic cycle, are maintained when, for example, rat germ cells are transplanted into a recipient mouse testis.

The capacity of the testes to produce sperm differs considerably from species to species. In humans, for instance, total daily sperm production expressed per gram of testis is five times lower than that in rat, rabbit, or rhesus monkey. Maximal spermatogenic capacity depends on the number of germ cells that can be supported by an individual Sertoli cell, the total number of Sertoli cells, the number of undifferentiated germ cells entering the spermatogenic cycle, and the type and number of germ cells that are lost during the entire process. The number of germ cells that can be nurtured by an individual Sertoli cell varies considerably from species to species. It is three times lower in human than in rabbit or rat. The total number of Sertoli cells

determines the ceiling of sperm output and the maximal size of the testis. Sertoli cell proliferation is maximal in the perinatal period and ceases at the time of puberty concordant with Sertoli cell maturation and the formation of tight inter-Sertoli cell junctions. In primates, the onset of puberty is associated with a second peak in Sertoli cell proliferation. An important regulator of Sertoli cell replication is FSH, and, accordingly, absent or reduced levels of FSH will limit Sertoli cell numbers, testis size, and sperm output. There is evidence for the monkey that testosterone also regulates Sertoli cell number. Thyroid hormone may also affect testicular size. Hypothyroidism restricted to the prepubertal period delays Sertoli cell maturation, thereby prolonging the period of Sertoli cell proliferation, and increases testicular size as well as sperm output in the adult if a euthyroid condition is restored postpubertally.

The number of undifferentiated spermatogonia entering the spermatogenic cycle varies with species. In rodents, but not in primates, this number is such that the adult testes operate at their spermatogenic ceiling and explains why unilateral orchidectomy postpubertally in rodents is not associated with compensatory hypertrophy of the remaining testis, as it is in primates (see later text for mechanism).

Finally, large numbers of germ cells undergo apoptosis during germ cell development. In rat, cell losses are observed mainly during spermatogonial stages and may exceed 75%. In human, losses seem to be most pronounced during meiosis and may reach as high as 40%. This reduction in germ cells probably reflects the limited germ cell supportive capacity of Sertoli cells and may also prevent further progression of chromosomally aberrant germ cells through spermatogenesis. Disturbances in the endocrine and local control mechanisms affecting spermatogenesis may markedly increase germ cell apoptosis.

Endocrine Control of Spermatogenesis

An overwhelming amount of data indicates that gonadotropins are the major hormones controlling spermatogenesis. Partial or complete inhibition of germ cell production is observed after procedures that suppress gonadotropin secretion (e.g., hypophysectomy, administration of GnRH receptor antagonists or long-acting agonists, and immunization against GnRH) or selectively reduce the secretion or action of either LH (e.g., supraphysiological doses of testosterone) or FSH (FSH antibodies). None of these procedures are completely effective and selective, however. Moreover, the effects of a particular intervention may differ depending on the species studied and whether the selected end point is initiation, maintenance, or restoration of spermatogenesis. It is safe to say that quantitatively and qualitatively, normal spermatogenesis requires both LH (testosterone) and FSH, but that the individual actions and interactions of these hormones remain only partially understood. Reports of rare mutations of the gonadotropin β-subunits and receptors in men and the development of analogous knockout animal models have offered, and are continuing to provide, additional insight into the relative role of FSH and testosterone in regulating spermatogenesis.

Testosterone and Spermatogenesis

Testosterone plays a critical role in driving spermatogenesis in mice, rats, and highly evolved primates, and this is best illustrated by the findings that this androgen alone can initiate and restore spermatogenesis and fertility in a number of experimental models where FSH is extremely low or absent, including severely hypogonadotropic mice deficient in GnRH (*hpg*) and hypophysectomized monkeys. Interestingly, in the Djungarian hamster, a seasonally breeding rodent, FSH treatment alone has been reported to stimulate spermatogenesis in regressed testis in the absence of an increase in intratesticular testosterone levels. Stereological approaches to the study of spermatogenesis and particularly the so-called optical disector model have contributed considerably to a more quantitative evaluation of the effects of androgens on germ cell development. In rats, testosterone seems to be essential for the adhesion of round spermatids to Sertoli cells. In the absence of the steroid, round spermatids are sloughed from the epithelium and spermatid elongation fails. Spermiation is also under the control of testosterone as is the cyclical disintegration and reorganization of the blood–testis barrier. Rodent and primate models provide little evidence that androgens promote spermatogonial proliferation.

In many target organs, androgen action is mediated by so-called active metabolites. Testosterone can be 5α-reduced to form dihydrotestosterone (DHT), a more potent androgen with a higher affinity for the androgen receptor (AR). Given the low level of 5α-reductase in testicular target cells, however, testosterone rather than DHT is considered to be the active androgen in the testis. Intratesticular levels of testosterone are 20–50 times higher than those observed in the circulation. While these high levels of the androgen have been considered to be required for initiation and maintenance of spermatogenesis, recent studies with mice have challenged this view.

The effects of testosterone are primarily mediated by AR, a member of the nuclear receptor superfamily. Most studies, however, have failed to demonstrate AR in germ cells indicating that androgens drive spermatogenesis indirectly by an action on somatic cells in the testis. This view is supported by transplantation experiments where AR-deficient germ cells (^{Tfm}X/Y mice) introduced into the seminiferous tubules of azoospermic mice expressing a functional AR resulted in qualitatively normal donor-derived spermatogenesis. Elegant studies over the last decade of the consequences of transgenetically ablating AR in specific somatic cells within the testis of mice have provided much needed information regarding the cell biology of androgen action underlying spermatogenesis. The Sertoli cells, which express AR, are considered the major target of testosterone action. Sertoli cell AR knockout mice are infertile with spermatogenesis arrested during meiosis, although, interestingly, the testis of these mice contain normal numbers of Sertoli cells. Peritubular myoid cell AR knockout mice are also infertile, but how these somatic cells affect the germ cells is poorly understood. It is important to recognize that in most transgenic mouse models, ablation of AR occurs

perinatally, and therefore, under these conditions, it is not possible to separate the impact of deficits in AR signaling during development from those required for maintenance of spermatogenesis in the adult. Nevertheless, it is probably reasonable to conclude that androgen-sensitive somatic cells in the testis act in concert to produce an environment that permits spermatogenesis to occur at 'basal' levels (see succeeding text) sufficient to maintain fertility.

The identity of specific genes and associated molecular events in the somatic cells of the adult testis that are regulated by AR to drive spermatogenesis remain incompletely understood. However, it is clear that testosterone's action in this regard is complex and that multiple families of genes are involved. Moreover, it has recently emerged that AR, in addition to its classical action within the nucleus to regulate gene transcription, may also mediate testosterone action on the Sertoli cell as a membrane receptor coupled to established signal transduction pathways.

Testosterone can also be aromatized to 17β-estradiol, which can act via one of the estrogen receptors (ER-α or ER-β). Aromatase activity and estrogen receptors are widely distributed in the somatic and germinal elements of the testis and in the efferent ducts. Moreover, fertility defects have been described in mice with a knockout of the ER-α or aromatase. Some of these defects may be related to defective fluid resorption at the level of the efferent tubules and retrograde accumulation of fluid, causing Sertoli cell dysfunction. Only two men with mutations resulting in aromatase deficiency and one man with a mutated ER-α have been described. Data available are too limited to allow definitive conclusions, but there are indications that here too germ cell development may be impaired.

FSH and Spermatogenesis

As may be inferred from the discussion of testosterone and spermatogenesis earlier in the text, FSH action in mice, rats, monkey, and man is unlikely to be essential for the initiation of spermatogenesis at puberty and for maintenance of adult fertility. This view is reinforced by studies of FSHβ or FSH receptor knockout mice, which are fertile, although they have small testes with reduced Sertoli cell number, and by a report of five Finnish men who are homozygous for a mutation in the FSH receptor that severely impairs incorporation of the protein into the membrane and therefore signal transduction. As expected, these men have reduced testicular volume but two of them had fathered two children each. Semen examination revealed a variable degree of oligozoospermia and teratozoospermia, but none of the five men displayed azoospermia. Taken together, these observations suggest that FSH is required for quantitatively and qualitatively normal spermatogenesis, but that, in the presence of normal testicular testosterone production, fertility is possible in the absence of FSH action. It should be noted, however, that the foregoing view appears to be at odds with reports on two men with loss of function mutations in FSHβ that were associated with azoospermia in the face of adequate androgen action as indicated by normal virilization. Moreover, under some conditions, FSH surprisingly may be able to maintain spermatogenesis in the presence of low concentrations of androgens. Relevant to this issue is the anecdotal description of a hypophysectomized patient undergoing testosterone treatment to maintain normal circulating levels of this steroid. The subject was fertile despite undetectable levels of gonadotropins; spermatogenesis appeared to be maintained by an activating mutation of the FSH receptor.

As is the case with testosterone, FSH does not act on the germ cells directly but activates specific receptors on the Sertoli cells. The FSH receptor belongs to a large family of membrane receptors that are coupled to G proteins and that are characterized by seven hydrophobic helices forming the transmembrane domain. This family also comprises the receptors for LH and TSH. Ligand binding results in the activation of the adenylate cyclase signaling pathway and other signaling pathways and induces a cascade of protein phosphorylations.

Stereological studies in rodents and in monkeys suggest a major and specific role for FSH in the proliferation of spermatogonia. In addition, FSH seems to favor spermatocyte and round spermatid development. In both cases, germ cell survival is most likely supported. Finally, and in cooperation with testosterone, FSH may promote the release of mature spermatozoa from Sertoli cells. The last two effects might be related to the actions of FSH on cytoskeletal elements and on the specialized Sertoli cell junctional apparatus.

Interactions of Testosterone and FSH

The data discussed in the preceding text indicate that both testosterone and FSH affect multiple steps in the spermatogenic process, though to a variable degree. Although under some conditions one of these hormones may maintain spermatogenesis in the absence of the other, qualitatively and quantitatively normal spermatogenesis requires both hormones. A major determinant of the relative signaling by AR and FSH receptor throughout the spermatogenic process appears to involve feedback controls between germ cells and the Sertoli cells. This is reflected by the findings that phasic but nonparallel changes in AR and FSH receptor concentrations (and presumable signaling) in the Sertoli cell are observed during the spermatogenic cycle. In the rat, for example, stages VII and VIII of the spermatogenic cycle, when elongated spermatids are being released, exhibit maximal AR expression and low FSH receptor content. Additional interactions between signaling pathways activated by the two hormones are observed. For example, AR concentration in Sertoli cells is upregulated by FSH, whereas maturation of Leydig cells and production of androgen are promoted by Sertoli cell-derived paracrine regulators produced under the influence of FSH. Again, the importance of these interactions may vary with species. The exact molecular and cellular mechanisms by which testosterone- and FSH-induced changes in Sertoli cell function ultimately govern germ cell development remain far from understood.

Feedback Control of Sperm Output

Studies of the monkey employing an experimental model known as a testicular clamp that allows for a selective, physiological increase in either FSH or LH stimulation to be imposed on the testis have provided results that have led to the hypothesis that the rate of sperm production by the primate testis under normal conditions is set by the circulating concentration of FSH. In this model, the role of LH is posited to produce the high intratesticular concentrations of testosterone that guarantee a basal level of sperm production that is amplified in direct relationship to the intensity of the circulating FSH signal. The primary action of FSH to stimulate spermatogenesis is to amplify the first generation of differentiating B spermatogonia. Whether this is achieved by increasing the proliferation of the undifferentiated precursors or by facilitating the survival of B spermatogonia or by both mechanisms remains to be determined.

The blood concentration of FSH is, in turn, set by a negative feedback action of inhibin B that is exerted directly at the level of the anterior pituitary to selectively suppress secretion of this gonadotropin. Accordingly, a reduction in inhibin B, as may be achieved experimentally by hemicastration, leads to an increase in FSH levels. Interestingly, the error signal in the feedback loop, that is, the reduction of circulating inhibin B, is maintained, and as a consequence, the hypersecretion of FSH is sustained resulting in hypertrophy of the remaining testis as spermatogenesis is driven to its ceiling by the exaggerated and sustained FSH signal. The dynamics of the LH–testosterone negative feedback are more plastic, and compensatory cellular mechanisms are rapidly recruited resulting in the remaining testis doubling its testosterone output and LH secretion returning to control levels within 48 h.

Osteocalcin

Since the publication of the previous edition of this book, the bone has been shown to function as an endocrine organ. In the context of the testis, recent studies of transgenic mice have provided evidence that osteocalcin, an osteoblast-specific secreted protein, plays a role in maintaining testosterone secretion by the Leydig cell. Osteocalcin signals via a G-protein receptor (GPRC6A) that is expressed by the Leydig cell. Moreover, two men with primary testicular failure exhibiting low testosterone levels and sperm counts have recently been reported with loss of function mutation of GPRC6A. The precise contribution of this bone hormone to the classical negative feedback loop regulating testosterone secretion remains to be established.

Male Contraceptives

The most widely investigated approach to hormone-based male contraception rests on the idea that the administration of physiological or slightly supraphysiological amounts of testosterone suppresses LH secretion, reduces the high intratesticular concentrations of testosterone, and inhibits spermatogenesis. At the same time, libido and potency are maintained by the 'normal' circulating levels of the androgen. The specific type of testosterone or testosterone analog used has been varied and is on occasion combined with a progestin to amplify the feedback suppression of gonadotropin. Impairment of spermiation may be a key to achieving azoospermia with androgen-based contraceptives. As explained earlier in the text, testosterone is considered to be directly responsible for the maintenance of spermatogenesis under normal conditions. However, under conditions in which intratesticular testosterone levels are reduced, an upregulation of 5α-reductase activity has been observed. These findings suggest that differences in the ability to activate this reaction could contribute to the observed interracial and interindividual differences in the efficiency of testosterone-based contraceptives. To date, testosterone-based contraception has generally been associated with poor acceptance due to complications and concerns of toxicity and poor efficacy, and this has led in recent years to a search for novel, nonhormonal, and testis-specific contraceptives.

Conclusion

Quantitatively and qualitatively normal spermatogenesis requires both FSH and high local concentrations of testosterone, but it appears that sperm production and fertility may be maintained by high concentrations of androgens in the absence of FSH. During prepubertal and early pubertal development, FSH plays a key role in determining the ultimate number of Sertoli cells and the spermatogenic capacity of the adult testis. Testosterone and FSH influence many steps in the spermatogenic linage including the maintenance of the blood–testis barrier, which is obligatory for the completion of meiosis and maturation and release of the haploid progeny. The extent to which hormones regulate SSC behavior, however, is less clear. Testosterone and FSH affect germ cell development indirectly by modulating the function of somatic testicular cells and particularly of Sertoli cells, which express both AR and FSH receptors. The genes, gene families, adhesion molecules, and paracrine signals that underlie the harmony between Sertoli cells and germ cells as they mature and move toward the tubular lumen are only partially understood, but contemporary technologies for (1) high-throughput sequencing of DNA and RNA, (2) examining cell-specific RNA profiles, and (3) transgenic manipulation are beginning to clarify these issues. Androgen-based contraceptives, which act both by suppressing intratesticular androgen levels and by lowering circulating concentrations of FSH, have been explored for many years, but consistent efficacy, in the absence of side effects and with wide acceptance, has not been achieved.

Acknowledgments

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Gonadotropin-Releasing Hormone (GnRH) Development and Actions

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Introduction

Over the last 25 years, research has demonstrated that the GnRH neuronal population originates in the olfactory placode and the neurons migrate with olfactory neurons during embryogenesis to the cribriform plate and then diverge to their final home in the forebrain (Wierman *et al.*, 2011). They extend their processes to the anterior pituitary to 5%–10% of the pituitary cells, termed gonadotropes. GnRH receptors in the gonadotropes receive the intermittent secretory signal from GnRH neurons and tranduce the secretory pattern to differentially regulate the production of FSH and LH. Fast, high amplitude GnRH pulses favor LH secretion, whereas low frequency and amplitude GnRH pulses favor FSH secretion (Spratt *et al.*, 1986). This differential pattern is particularly critical in the female reproductive axis to ensure a normal ovulatory cycle.

Developmental Ontogeny of the GnRH Neuronal System

In contrast to other hypothalamic releasing factors that control pituitary hormone secretion, GnRH neurons are not born in the brain but have a unique developmental migratory program to reach their final destination in the hypothalamus in a dispersed neuronal network. In rodents, GnRH neuronal migration begins on embryonic day 10.5–11 and is completed by day 15–16 resulting in about 800 neurons (Schwanzel-Fukuda and Pfaff, 2002). During migration, the neurons express low levels of GnRH mRNA and protein. Identification of the many factors that modulate this early migration and later control of GnRH secretion at the time of sexual maturation are areas of active research. Cell adhesion molecules, intracellular kinases and other factors control neuronal survival and movement along the developmental pathway. Soluble factors such as γ-amino butyric acid (GABA), semaphorins and netrins are examples of components of the early migratory environment (Wierman *et al.*, 2011).

In the human, a few thousand GnRH neurons are arranged in a neuronal network. Secretion of GnRH requires a pulsatile pattern of hormone delivery to appropriately activate the GnRH receptor. Immortalized GnRH neuronal cells and primary neurons demonstrate that the GnRH "pulse generator" is intrinsic to the neurons (Mellon *et al.*, 1990). However, extensive new information has outlined the role of other hypothalamic factors including Kisspeptin, NPY, dynophin and tachychinins from KNDy neurons and other neurotransmitters as well as microRNAs to modulate GnRH neuronal activity centrally (Seminara, 2006; Navarro *et al.*, 2007; Messina *et al.*, 2016; Terasawa *et al.*, 2018). These factors play important roles during sexual development and in the adult. Neuronal decreases in GABAergic activity and increased Kisspeptin, glutaminergic, norepinephrine, and neuropeptide Y activities are important in the activation of the GnRH neuronal pulsatile release at the time of sexual maturation. Transforming growth factor-α/erbgB-1 and neuregulin/erbB-4 secretion from associated glial cells and nitric oxide from adjacent endothelial cells are additional factors and modulate GnRH release (Prevot *et al.*, 2010).

GnRH Receptors

The pituitary GnRH receptor is a G protein-coupled receptor that couples with Gqα to activate the inositol phosphate signaling pathway (Bliss *et al.*, 2010). Unlike most GPCRs, the GnRH receptor lacks a large cytoplasmic C-terminal tail. Receptor phosphorylation in the C-terminus responds to intermittent compared to continuous activation. Constant activation leads to receptor desensitization and internalization. Phospholipase C transmits the signal to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates the intracellular protein kinase C pathway, and IP3 stimulates release of intracellular calcium. Protein kinase C activation in response to GnRH also leads to increases in the mitogen-activated protein kinase (MAPK) pathways in pituitary gonadotropes. These pathways control gonadotropin subunit synthesis and gonadotropin (FSH and LH) release into the circulation to act at the gonads to ensure reproductive development and function.

Disorders of the GnRH/GnRH Receptor System

Failure of the GnRH neurons to migrate, survive and/or secrete appropriately results in a central disorder of the reproductive axis. There has been an explosion of new factors identified that impact the GnRH neuronal migratory path or secretory program to cause pubertal disorders in the human (Balasubramanian *et al.*, 2010; Sykiotis *et al.*, 2010; Shaw *et al.*, 2011). Hypogonadotropic hypogonadism (HH) refers to patients who present with significantly delayed or absent pubertal development, hypogonadism and

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infertility. HH reflects a defect in GnRH secretion or GnRH signaling to the pituitary. HH occurs in 1/5000 men and 1/8000 women and reflect genetic mutations which can be autosomal dominant, recessive, X-linked or sporadic. Kallmann syndrome refers to HH with anosmia and is due to mutations in genes that impact early GnRH neuron migration along the olfactory neurons. Defects in the KAL-1 gene and protein anosmin-1 results in an aborted migration of the dual system early in development. Mutations in fibroblast growth factor 8 (FGF8) or its receptor FGFR1, the sempahorin SEMA-3A, the prokineticins PROK2 or its receptor PROKR2 among others can result in HH or Kallmann syndrome (Balasubramanian *et al.*, 2010; Sykiotis *et al.*, 2010). Surprisingly, only recently have mutations in the GnRH gene itself been discovered to cause absent pubertal development. Despite intensive investigation only about 30% of the underlying mutations are currently known suggesting additional research is needed to both understand the defects and give clues to the complexity of the control of the reproductive axis.

Other causes of HH involve defective signaling pathways downstream of the GnRH neuron. Mutations in GnRH receptor expression alter the ability of GnRH to activate pituitary gonadotropin synthesis and secretion to a variable extent (Balasubramanian *et al.*, 2010). Rare patients have been found to have mutations of the LHβ or FSHβ gonadotropin subunit genes resulting in defective sexual maturation.

In contrast to a lack of pubertal development, precocious puberty results from premature reactivation of the HPG axis. In girls it is usually idiopathic and in boys often results from a central nervous system tumor. The exact mechanism of premature pubertal development has been of great interest to investigators. Some have suggested that premature activation of the glial production of TGFα to activate GnRH neuronal secretion. Recently, investigators have discovered that a constitutive activating mutation in the Kisspeptin receptor which transduces Kisspeptin secretion to activate GnRH neurons can induce precocity (Abreu *et al.*, 2013; Bessa *et al.*, 2017). Others have shown that mutations in pathways of protein degradation may result in familial precocity (Shi *et al.*, 2014). Additional research is needed.

Clinical Use of GnRH and GnRH Agonists and Antagonists

Once scientists understood the importance of intermittent signal transduction to active the reproductive axis and that continuous exposure to GnRH desensitized the GnRH receptor and function and blocked hormonal secretion, manipulation of the GnRH decapeptide was performed to produce activators and inhibitors of the reproductive axis. In research centers, GnRH which has a short half-life can be administered via a small infusion pump worn at the waist to activate reproductive function in women with HH to induce ovulation and allow pregnancy (Spratt *et al.*, 1986; Santoro *et al.*, 1988). In men, the pulsatile GnRH can be given subcutaneously by an infusion pump to activate spermatogenesis over a 12–24 month period (Spratt *et al.*, 1986). Because of cost and availability, most patients are instead treated with exogenous gonadotropins (recombinant FSH and human chorionic gonadotropin (hCG)) to mimic LH to treat these patients. GnRH agonists when given chronically turn off gonadotropin secretion and are a mainstay of inducing a medical castration in various situations (Yen, 1983; Lahlou *et al.*, 2000; Newton *et al.*, 2016). They are used to turn off early puberty in children with precocity as well as modulating hormone dependent disorders such as endometriosis or leiomyomata in the uterus. In addition, GnRH agonists have been used extensively in hormone dependent malignancies predominantly in prostate cancer patients instead of orchiectomy or sometimes in breast cancer patients. More recently, short acting pure GnRH antagonists have been used to directly block GnRH receptor function to inhibit LH and FSH secretion. They are used occasionally as an adjunct to prevent ovarian hyperstimulation during in vitro fertilization.

Conclusions

GnRH is the hypothalamic hormone that stimulates the pituitary gland to produce FSH and LH. The reactivation of GnRH neuronal secretion triggered by Kisspeptin and other transmitters secreted by KNDy neurons are responsible for the initiation of puberty and completion of sexual maturation. The KnDy neurons may modulate hot flashes in the menopause or other states of gonadal failure and be a target for new treatments (Dacks *et al.*, 2011; Rance *et al.*, 2013). The GnRH–GnRH receptor system has been a target for drug therapies to treat early or delayed pubertal development. These agents also are used to turn on or off sex steroid production in hormone dependent tumors of the prostate and breast. Understanding the complexities of the development and function of GnRH neurons and action allows us to further identify causes of reproductive disorders and devise novel therapies for our patients.

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Genes and Gene Defects Affecting Gonadal Development and Sex Determination

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Disorders of Sex Development (DSDs)

DSDs are a heterogeneous group of birth defects that are defined as conditions in which the development of the chromosomal, gonadal, or anatomical sex is atypical (Hughes *et al.*, 2006). They often present with abnormal genitalia at birth or failure/delay of puberty, and are estimated to occur in approximately 1:4500 live births. If defects in external genitalia are included, DSDs exist in up to 1:300 newborns, as is observed for hypospadias (misplacement of the urethral opening in boys) (Ahmed *et al.*, 2004). Based on a consensus statement in 2005 (Hughes *et al.*, 2006), DSDs are classified into three broad groups; sex chromosome DSD, 46,XY DSD, and 46,XX DSD. The latter two are further divided into (i) disorders of gonadal development, (ii) disorders in androgen synthesis/action (46,XY DSD) and androgen access (46,XX DSD), and (iii) others, which include, for example, severe hypospadias and vaginal atresia.

In recent years, even though considerable progress has been made in identifying genes that drive sex determination and sex differentiation (for review see Eggers and Sinclair, 2012; Ostrer, 2014), many DSDs are still unexplained at the molecular level. While most 46,XX DSDs are due to 21-hydroxylase deficiency, which causes congenital adrenal hyperplasia, the genetic etiology is unknown for more than 50% of 46,XY DSDs (Ahmed *et al.*, 2013; Baxter *et al.*, 2015; Eggers *et al.*, 2016). The genes that have been described to be associated with DSD were identified through genetic studies of DSD patients and/or animal models, especially genetically modified mice. In this article, we will review genes that are implicated in the development of testis and ovary in mice and humans, and that, when mutated, can lead to DSDs (see **Table 1**).

Bipotential Genital Ridge

The development of testes and ovaries starts with a common precursor, the bipotential genital ridges. They arise through proliferation of the coelomic epithelium at the ventro-medial surface of the mesonephros within the intermediate mesoderm. The genital ridges are first visible at around 10 days post coitum (dpc) in mouse, and during the fourth week postfertilization in human (Byskov, 1986; Satoh, 1991). The somatic cells are joined by primordial germ cells (PGCs) that were specified at the base of the allantois and migrated via the hindgut and the dorsal mesentery to the developing genital ridges (Molyneaux *et al.*, 2001; Saitou *et al.*, 2005).

A number of genes are known to play important roles in the formation of the genital ridges. These include genes that encode the transcription factors GATA4 (GATA binding protein 4), LHX9 (LIM homeobox protein 9), and WT1 (Wilms' tumor suppressor 1) as well as the orphan nuclear receptor NR5A1 (also known as SF1, steroidogenic factor 1). Null mutations in any of these in mice result in gonadal dysgenesis or agenesis (Birk *et al.*, 2000; Hu *et al.*, 2013; Kreidberg *et al.*, 1993; Luo *et al.*, 1994). Of these four genes, *GATA4* functions the earliest, leading to a complete failure of genital ridge formation in mouse, and 46,XY gonadal dysgenesis in human (Lourenco *et al.*, 2011). In contrast, the coelomic epithelium in mice lacking WT1, NR5A1 or LHX9 thickens initially, but then regresses soon after. The hierarchy of these genes has been elucidated in mice with WT1, together with LHX9, upregulating *Nr5a1* through binding in its proximal promoter (Wilhelm and Englert, 2002).

WT1 was initially identified to cause Wilms' tumor, a pediatric kidney cancer, but subsequently shown that mutations can cause 46,XY DSD, including Denys–Drash, Frasier, and WAGR (Wilms' tumor, aniridia, genital anomalies, and mental retardation) syndrome (Barbaux *et al.*, 1997; Jadresic *et al.*, 1990; Pelletier *et al.*, 1991; van Heyningen *et al.*, 1990). Similarly, mutations in *NR5A1* lead to 46,XY DSD with embryonic testicular regression syndrome and 46,XX premature ovarian failure (Achermann *et al.*, 1999; Domenice *et al.*, 2016; Lourenco *et al.*, 2009). *NR5A1* mutations are one of the major causes of 46,XY DSD with a frequency of 10%–15% (Baxter *et al.*, 2015; Lin and Achermann, 2008). In contrast, no mutations in *LHX9* have been associated with human DSD to date.

Sex Determination

The fate of the bipotential genital ridges is decided with the expression of the male-determining gene *Sry*/*SRY* (sex determining region of chromosome Y), which encodes an HMG domain transcription factor, the founding member of the SOX (SRY-box) protein family (Schepers *et al.*, 2002). In mice, *Sry* is expressed during a narrow time window between 10.5 and 12.5 dpc with a peak at 11.5 dpc (Hacker *et al.*, 1995). In humans, *SRY* starts to be expressed between 41 and 44 days post-ovulation (dpo), reaching a peak at 44 dpo, and remains expressed at low levels beyond 52 dpo (Hanley *et al.*, 2000). It has been shown in mice

Table 1 Genes involved in mammalian gonadal development and sex determination

Gene	*Gonadal phenotype in mice*	*Human DSD phenotype*	*References*
Genes important for the development of the bipotential genital ridge			
Lhx9	Arrest of gonad development, resulting in absence of gonads and XY sex reversal (LOF)	–	Birk *et al.* (2000)
Nr5a1/Sf1	Arrest of gonad development, resulting in absence of gonads and XY sex reversal (LOF)	46,XY DSD with embryonic testicular regression syndrome (LOF); 46,XX premature ovarian failure (LOF)	Achermann *et al.* (1999), Domenice *et al.* (2016), Lourenco *et al.* (2009), and Luo *et al.* (1994)
Gata4	Failure of genital ridge formation (LOF); XY sex reversal in *Gataki/ki* embryos (LOF)	46,XY DSD with ambiguous genitalia (LOF)	Hu *et al.* (2013), Lourenco *et al.* (2011), and Tevosian *et al.* (2002)
Wt1	Arrest of gonad development, resulting in absence of gonads (LOF)	46,XY DSD; Denys–Drash, Frasier, and WAGR syndromes (LOF)	Barbaux *et al.* (1997), Jadresic *et al.* (1990), Kreidberg *et al.* (1993), Pelletier *et al.* (1991), and van Heyningen *et al.* (1990)
Genes important for testis development			
Gadd45γ	XY sex reversal with reduced *Sry* expression (LOF)	–	Gierl *et al.* (2012) and Warr *et al.* (2012)
Map3k4	XY sex reversal with reduced *Sry* expression (LOF)	–	Bogani *et al.* (2009)
Map3k1	Minor abnormalities of testis development (LOF)	46,XY DSD with partial or complete gonadal dysgenesis (GOF)	Pearlman *et al.* (2010) and Warr *et al.* (2011)
p38 Mapk family	XY sex reversal with reduced *Sry* expression in *p38α*;*p38β* double KO mice (LOF)	–	Warr *et al.* (2012)
Zfpm2/Fog2	XY sex reversal with reduced *Sry* expression (LOF)	46,XY DSD with ambiguous genitalia or complete sex reversal (LOF)	Bashamboo *et al.* (2014) and Tevosian *et al.* (2002)
Cbx2/M33	Delayed genital ridge formation and XY sex reversal with virtually absent *Sry* expression (LOF)	46,XY DSD with complete sex reversal (LOF)	Biason-Lauber *et al.* (2009) and Katoh-Fukui *et al.* (1998, 2012)
Kdm3a/Jmjd1a	XY sex reversal with reduced *Sry* expression (LOF)	–	Kuroki *et al.* (2013)
Sry	XY sex reversal (LOF) XX sex reversal (GOF)	46,XY ovarian DSD (LOF) 46,XX testicular DSD (GOF)	Koopman *et al.* (1991) and Sinclair *et al.* (1990)
Sox9	XY sex reversal (LOF) XX sex reversal (GOF)	46,XY DSD with ambiguous genitalia or complete sex reversal associated with Campomelic Dysplasia (LOF); 46,XX DSD with ambiguous genitalia (GOF)	Barrionuevo *et al.* (2006), Chaboissier *et al.* (2004), Cox *et al.* (2011), Foster *et al.* (1994), Shankara Narayana *et al.* (2017), Vidal *et al.* (2001), and Wagner *et al.* (1994)
Sox10	XX sex reversal (GOF)	46,XX DSD with ambiguous genitalia or complete sex reversal (GOF)	Aleck *et al.* (1999), Nicholl *et al.* (1994), Polanco *et al.* (2010), and Seeherunvong *et al.* (2004)
Sox3	XX sex reversal (GOF)	46,XX DSD with complete sex reversal (GOF)	Sutton *et al.* (2011)
Fgf9	XY sex reversal due to a failure to maintain SOX9 expression (LOF)	–	Colvin *et al.* (2001), and Schmahl *et al.* (2004)
Fgfr2	XY sex reversal due to a failure to maintain SOX9 expression (LOF)	46,XY DSD with complete sex reversal (LOF)	Bagheri-Fam *et al.* (2008, 2015) and Kim *et al.* (2007)

(*Continued*)

Table 1 Continued

Gene	*Gonadal phenotype in mice*	*Human DSD phenotype*	*References*
Ptgds	Delayed testis cord organization due to transient reduced *Sox9* expression and aberrant SOX9 nuclear localization (LOF)	–	Moniot *et al.* (2009)
Dhh	Defective fetal and adult Leydig cell differentiation (LOF)	46,XY DSD with partial or complete gonadal dysgenesis (LOF)	Canto *et al.* (2004), Clark *et al.* (2000), Umehara *et al.* (2000), and Yao *et al.* (2002)
Hhat	Reduced number of testis cords and Leydig cells (LOF)	46,XY DSD with complete sex reversal (LOF)	Callier *et al.* (2014)
Genes important for ovary development			
Rspo1	XX partial sex reversal with ectopic testis-specific coelomic blood vessel, androgen-producing cells, and loss of germ cells; development of testis cords around birth (LOF)	46,XX DSD with complete sex reversal and palmoplantar hyperkeratosis (LOF)	Chassot *et al.* (2008), Parma *et al.* (2006), Tomizuka *et al.* (2008)
Ctnnb1/β-catenin	XX partial sex reversal with ectopic testis-specific coelomic blood vessel, androgen-producing cells, and loss of germ cells (LOF); XX sex reversal (GOF)	–	Liu *et al.* (2009) and Maatouk *et al.* (2008)
Wnt4	XX partial sex reversal with ectopic testis-specific coelomic blood vessel, androgen-producing cells, and loss of germ cells (LOF); XX sex reversal in *Foxl2; Wnt4* double KO mice	46,XX DSD with ambiguous genitalia or complete sex reversal (LOF)	Biason-Lauber *et al.* (2004, 2007) and Jeays-Ward *et al.* (2003), Mandel *et al.* (2008), Philibert *et al.* (2008), and Vainio *et al.* (1999)
Foxl2	Premature ovarian failure with loss of germ cells (LOF); XX sex reversal in *Foxl2;Wnt4* double KO mice	Premature ovarian failure in blepharophimosis/ptosis/ epicanthus inversus syndrome (BPES) (LOF)	Crisponi *et al.* (2001), Ottolenghi *et al.* (2007), Schmidt *et al.* (2004), and Uda *et al.* (2004)

GOF, gain-of-function; *LOF*, loss-of-function.

that if SRY is not expressed, like in an XX individual, or its expression is too late or too low, the bipotential genital ridge develops into an ovary (Bullejos and Koopman, 2005; Warr *et al.*, 2012). Similarly, mutations in human *SRY* result in 46,XY ovarian DSD. In addition, *SRY* is the major cause of 46,XX testicular DSD in cases in which abnormal X–Y exchange during recombination caused the transfer of Y chromosomal DNA, including the *SRY* gene, to the X chromosome. It is estimated that the *SRY* gene accounts for 10%–15% of 46,XY ovarian DSD and 80%–90% of 46,XX testicular DSD (Cameron and Sinclair, 1997; Hawkins, 1993; McElreavey *et al.*, 1993; Sinclair *et al.*, 1990).

Even though *Sry* was identified as the testis-determining gene in 1990/1991 the regulation of its expression is still not completely understood. Factors regulating its expression are expected to be present in both the XY and XX genital ridge, as a 14 kb genomic fragment, including only the *Sry* gene and its regulatory region, is sufficient to drive male development in XX transgenic mice (Koopman *et al.*, 1991). It has been shown that a critical transcriptional activator of *Sry* expression is the GATA4/ZFPM2 (zinc finger protein, FOG family member 2) complex (Tevosian *et al.*, 2002) and recent data in mice have shown that GATA4 needs to be activated by phosphorylation through p38 MAPK (mitogen activated protein kinase), which is part of an intracellular signaling cascade (Gierl *et al.*, 2012; Warr *et al.*, 2012). This cascade includes the small, acidic protein GADD45γ, which binds to and activates MAP3K4, which in turn results in the phosphorylation and activation of p38 MAPK (**Fig. 1**) (Gierl *et al.*, 2012; Warr *et al.*, 2012). While mouse models with mutations in the genes encoding any of these factors result in greatly reduced or abolished *Sry* expression and subsequent male-to-female sex reversal, none of these genes, with the exception of *GATA4* and *ZFPM2* (Bashamboo *et al.*, 2014; Eggers *et al.*, 2016; Lourenco *et al.*, 2011), has been associated with human 46,XY DSD to date. However, mutations in *MAP3K1*, encoding a kinase closely related to MAP3K4, can lead to 46,XY DSD. Recent studies have shown that human *MAP3K1* mutations are common and cause 10%–18% of cases of 46,XY gonadal dysgenesis (Baxter *et al.*, 2015; Eggers *et al.*, 2016; Ostrer, 2014; Pearlman *et al.*, 2010). Interestingly, in contrast to *Map3k4* in mouse, which results in sex reversal due to loss-of-function of the kinase, the mutations identified in human *MAP3K1* appear to be gain-of-function mutations (Bogani *et al.*,

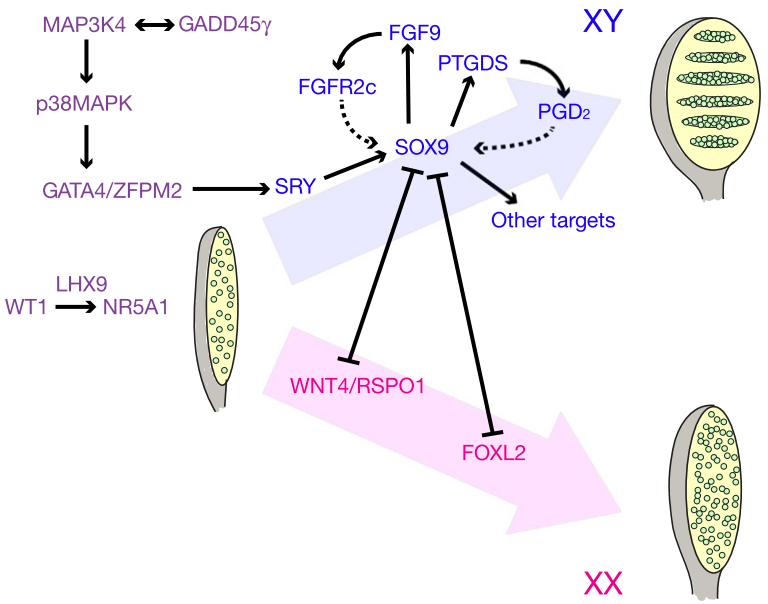

Fig. 1 Sex determination pathways in mouse. Schematic representation of the sex determination pathways in the developing mouse gonads. WT1 together with LHX9 is responsible for the upregulation of NR5A1 in the indifferent genital ridges. All three genes together with GATA4 and ZFPM2 are necessary for the formation of the genital ridges in both sexes. Activation of GATA4/ZFPM2 by MAP kinase signaling results in the up-regulation of SRY expression in XY gonads which in turn up-regulates the expression SOX9. Expression of SOX9 is then maintained by two positive feedback loops involving FGF9 and PGD2, which drives the differentiation of the genital ridges into testes marked by the formation of testis cords that enclose germ cells (indicated as *small circles*). In the absence of SRY, female pathways are activated including WNT4/RSPO1 signaling and the forkhead transcription factor FOXL2, leading to the formation of an ovary. See text for more details.

2009; Loke *et al.*, 2014; Pearlman *et al.*, 2010). Accordingly, deletion of *Map3k1* in mice does not result in sex reversal (Warr *et al.*, 2011), and it would be interesting to test if ectopic activation of MAP3K1 in mice mimics the human phenotype.

Other factors that have been implicated in the regulation of *Sry* expression include WT1 and NR5A1, as well as the epigenetic regulators CBX2 (chromobox 2) and KDM3A (lysine (K)-specific demethylase 3A, also known as JMJD1A, jumonji domain containing 1A). CBX2 is a component of the polycomb multiprotein complex, which is required to maintain the transcriptionally repressive state of many genes throughout development via chromatin remodeling and modification of histones. Disruption of *Cbx2* in mice results in male-to-female gonadal sex reversal due to low *Sry* expression, while in humans a compound heterozygous *CBX2* mutation was found in a female patient with 46,XY ovarian DSD (Biason-Lauber *et al.*, 2009; Katoh-Fukui *et al.*, 1998, 2012). The histone demethylase KDM3A has only been shown in mice, but not in humans to date, to be important for removing the repressive H3 histone methylation mark at lysine 9 within the *Sry* regulatory region (Kuroki *et al.*, 2013). In addition, a recent study has demonstrated that reduced activity of the H3K9 methyltransferase GLP/G9a complex restores *Sry* expression and rescues XY gonadal sex reversal in *Kdm3a* knockout mice. These data indicate that *Sry* expression is controlled by a fine balance between H3K9 demethylase and methyltransferase activities (Kuroki *et al.*, 2017). In summary, genetic studies have identified critical factors involved in *Sry* expression in mouse, as well as bioinformatics analysis described binding sites within the *Sry* promoter (Larney *et al.*, 2014). However, these sites have not been confirmed in vivo to be functional.

Testis Differentiation

The main, or possibly the only, function of SRY is the upregulation of *Sox9* (SRY-box 9; **Fig. 1**) encoding another member of the SOX family of transcription factors (Schepers *et al.*, 2002; Sekido and Lovell-Badge, 2008). Like *Sry*, *Sox9* is both necessary and sufficient for testis development in mouse (Barrionuevo *et al.*, 2006; Chaboissier *et al.*, 2004; Lavery *et al.*, 2011; Vidal *et al.*, 2001). Recent studies provided evidence that duplication of the human *SOX9* upstream regulatory region results in 46,XX ovotesticular DSD with ambiguous genitalia (Cox *et al.*, 2011; Shankara Narayana *et al.*, 2017). In addition, heterozygous mutations in the human *SOX9* gene lead to campomelic dysplasia, a disorder that is characterized by the bowing of long bones and is associated with ambiguous genitalia or male-to-female sex reversal in approximately 75% of XY cases (Foster *et al.*, 1994; Mansour *et al.*, 1995; Wagner *et al.*, 1994). Two closely related SOX factors, SOX8 and SOX10, have been suggested to function redundantly in the remaining patients. Accordingly, duplication of at least the region including *SOX10* in humans leads to ambiguous genitalia or complete sex reversal in 46,XX individuals (Aleck *et al.*, 1999; Nicholl *et al.*, 1994; Seeherunvong *et al.*, 2004). Similarly, in mice ectopic expression of *Sox10* in XX gonads caused female-to-male sex reversal (Polanco *et al.*, 2010). In addition, another member

of the SOX family, SOX3, has also been shown to result in 46,XX testicular DSD when duplicated in humans, and female-to-male sex reversal in mice when ectopically expressed in XX gonads (Sutton *et al.*, 2011).

The expression of *Sox9*, after the initial up-regulation by SRY, is maintained through different positive feedback loop mechanisms, at least in mouse. Firstly, SOX9 binds to its promoter to maintain its own expression (Sekido and Lovell-Badge, 2008). Secondly, *Sox9* expression results in the upregulation of *Fgf9* (fibroblast growth factor 9), and *Ptgds* (prostaglandin D synthase) (Kim *et al.*, 2006; Moniot *et al.*, 2009; Wilhelm *et al.*, 2007). FGF9 binds to its receptor FGFR2c to indirectly activate *Sox9* expression through repression of the ovarian program (Jameson *et al.*, 2012); whereas PTGDS produces prostaglandin D_2, which through binding to its DP receptor leads to the nuclear translocation of SOX9 protein and subsequent up-regulation of *Sox9* transcription (**Fig. 1**) (Malki *et al.*, 2005; Wilhelm *et al.*, 2005). Accordingly, *Fgf9* $^{-/-}$ and conditional *Fgfr2* knockout mice show partial to complete XY sex reversal, while *Ptgds*$^{-/-}$ XY gonads display abnormalities in testis cord organization (Bagheri-Fam *et al.*, 2008, 2017; Colvin *et al.*, 2001; Kim *et al.*, 2007; Schmahl *et al.*, 2004). However, only *FGFR2* has been implicated in 46,XY DSDs in human to date (Bagheri-Fam *et al.*, 2015).

Both, *Sry* and *Sox9*, are expressed in the supporting cell precursors in the developing testis. Expression of *Sox9* drives their differentiation into the first testis-specific cell type, the Sertoli cells. Sertoli cells only proliferate during fetal and neonatal development, and their final number will determine the number of germ cells that can be supported during spermatogenesis to form sperm (Rebourcet *et al.*, 2017; Svingen and Koopman, 2013). Sertoli cells are considered the coordination centers of the developing testis. They will organize themselves around clusters of germ cells to form testis cords, the precursors of the seminiferous tubules of the adult testis. They also produce factors to direct the differentiation of other cell types in the developing testis, including steroidogenic Leydig cells and peritubular myoid (PM) cells (Svingen and Koopman, 2013).

Fetal Leydig cells differentiate in the interstitium between testis cords at around 12.5 dpc in mouse and at 8–9 weeks of human development. They are responsible for the production of androgens, which drive the development of secondary sexual characteristics including the male reproductive tract and external genitalia. Two signaling pathways are important for Leydig cell specification and differentiation, DHH (desert hedgehog) secreted by Sertoli cells, which binds to its receptor PTCH1 (patched 1) on Leydig cell precursors, and Notch signaling. Accordingly, XY *Dhh*-null mice have major defects in fetal Leydig cell differentiation, and the vast majority of adult mice are feminized lacking adult-type Leydig cells (Clark *et al.*, 2000; Yao *et al.*, 2002). Similarly, mutation of *DHH* in humans results in 46,XY DSD (Canto *et al.*, 2004; Umehara *et al.*, 2000). In addition, mutations in an enzyme that is important for hedgehog protein modification and function, HHAT (hedgehog acyltransferase), in mouse and human lead to testicular dysgenesis (46,XY DSD) in addition to other skeletal, neuronal, and growth defects (Callier *et al.*, 2014). Fetal XY *Hhat* knockout mice display smaller testes with a reduced number of testis cords, and fetal Leydig cells are almost completely absent (Callier *et al.*, 2014). The second pathway, Notch signaling has only been shown in mouse to be important for Leydig cell differentiation. Inhibition and constitutive activation of Notch signaling resulted in an increase and decrease of Leydig cell numbers respectively (Tang *et al.*, 2008).

DHH is not only important for the differentiation of Leydig cells, but also for proper development of PM cells. PM cells are long, flattened cells that surround Sertoli cells and together they produce a basal lamina between them which is important for testis cord integrity. PM cells at later stage become contractile and help pumping the sperm from the seminiferous tubules into the epididymis.

Ovary Differentiation

In the absence of the Y chromosome, and therefore the *Sry* gene, the bipotential genital ridges develop into ovaries (**Fig. 1**). In contrast to the developing testis, the ovary does not display major morphological changes during fetal development. However, there is an active gene expression program that drives the development of ovaries. The main drivers are the WNT4 (wingless-type MMTV integration site family member 4)/RSPO1 (R-spondin 1)/β-catenin pathway and the transcription factor FOXL2 (forkhead box L2). Both WNT4 and RSPO1 have been shown to function through the canonical WNT signaling pathway in the developing ovary, inducing the stabilization and accumulation of β-catenin. Loss-of-function of any of the three molecules in mice leads to partial female-to-male sex reversal (Chassot *et al.*, 2008; Jeays-Ward *et al.*, 2003; Liu *et al.*, 2009; Tomizuka *et al.*, 2008; Vainio *et al.*, 1999). In addition, ectopic expression of a constitutive active β-catenin in the developing XY mouse gonads results in male-to-female sex reversal (Maatouk *et al.*, 2008). Similarly, in human, homozygous mutation in *WNT4* or *RSPO1* can lead to 46,XX DSD with female-to-male sex reversal, while heterozygous mutations in *WNT4* are associated with 46,XX DSD and ambiguous genitalia (Biason-Lauber *et al.*, 2004, 2007; Mandel *et al.*, 2008; Parma *et al.*, 2006; Philibert *et al.*, 2008). These findings demonstrate the importance of this signaling pathway for ovarian development.

The second pathway, marked by the transcription factor FOXL2 (**Fig. 1**), appears to work largely independent of the WNT signaling pathway in driving ovarian development. The importance of FOXL2 to the development of ovaries is dependent on the species. Loss of FOXL2 results in complete female-to-male sex reversal in goats (Boulanger *et al.*, 2014; Pailhoux *et al.*, 2001), while it leads to premature ovarian failure, that is, a premature loss of oocytes, in postnatal mice and humans (Crisponi *et al.*, 2001; Schmidt *et al.*, 2004; Uda *et al.*, 2004). Nevertheless, XX double knockout mice that have *Foxl2* deleted together with either *Wnt4* or *Rspo1* show a more severe sex reversal phenotype than the *Wnt4* or *Rspo1* single-knockouts (Auguste *et al.*, 2011; Ottolenghi *et al.*, 2007), demonstrating that *Foxl2* also plays a role in the fetal mouse ovary.

Germ Cell Differentiation

PGCs, once they have reached the developing genital ridges, stop migrating, but keep proliferating. They start their sexual dimorphic differentiation at around 13.5–14.5 dpc in mouse and at week 11–12 in human development, when PGCs in an ovary enter meiosis, whereas PGCs in a testis arrest in mitosis. This bifurcation in the differentiation pathway between males and females is solely dependent on the environment, that is, ovary or testes, PGCs are in. It has been shown that retinoic acid (RA) produced by the mesonephros is able to induce entry into meiosis. In a testis, Sertoli cells, which surround PGCs within testis cords, produce the RA-degrading enzyme CYP26B1 (cytochrome P450, family 26, subfamily b, polypeptide 1) (Bowles *et al.*, 2006; Koubova *et al.*, 2006; Li *et al.*, 2009; MacLean *et al.*, 2007). Therefore, even though RA is also produced by the mesonephros in males, PGCs do not enter meiosis until after birth.

Antagonism Between the Male and Female Pathway

For a long time it was believed that once the decision to make a testis or an ovary is made, it is irreversible. However, in recent years, it became clear that the sex-specific program needs to be actively maintained through repression of the opposite sex not only during fetal development, but also postnatally into adulthood (**Fig. 1**). In the fetal XY gonad, SOX9 is required for the initiation of the male pathway, but it also has been suggested to suppress, directly or indirectly FOXL2 expression (Barrionuevo *et al.*, 2006; Georg *et al.*, 2012). Conversely, FOXL2 has been shown to bind and repress the regulatory region of *Sox9* in the postnatal mouse ovary (Uhlenhaut *et al.*, 2009). In addition, FGF9 and WNT4 appear to act antagonistically to promote the fetal testicular and ovarian developmental pathways respectively (Jameson *et al.*, 2012; Kim *et al.*, 2006). Postnatally, the testis-specific transcription factor DMRT1 (doublesex and mab-3 related transcription factor 1) together with SOX9 repress FOXL2, whereas FOXL2 together with the estrogen receptors directly repress *Sox9* expression (Barrionuevo *et al.*, 2016; Matson *et al.*, 2011; Minkina *et al.*, 2014; Uhlenhaut *et al.*, 2009). If either of these factors is deleted in mice after birth Sertoli cells transdifferentiate into granulosa-like cells and granulosa cells transdifferentiate into Sertoli-like cells respectively.

Conclusions

Since the discovery of the male-determining gene *Sry*, extensive progress has been made in identifying genes and gene regulatory mechanism that drive the differentiation of testes and ovaries, so that in humans mutations in the three major genes *SRY*, *NR5A1* and *MAP3K1*, together with mutations in additional genes, such as *DHH*, *GATA4*, *ZFPM2* and *WT1*, account for up to 43% of all 46,XY DSD (Baxter *et al.*, 2015; Eggers *et al.*, 2016). However, we are still unable to explain the other half of these cases at the molecular level, suggesting that additional, important factors are involved. One of the reasons to explain why we have not identified more crucial genes is that emphasis was placed on sexually dimorphically expressed genes, with the rational that a gene important for testis development should not be expressed in an ovary. However, this disregards the possibility that a gene might be expressed in both testis and ovary, but its target is sexually dimorphic, as has been shown for the DHH-modifying enzyme HHAT (Callier *et al.*, 2014), or that any gene that is expressed in both testis and ovary might play important roles in both organs.

With the development of high-throughput technologies such as whole exome and whole genome sequencing to identify potentially pathogenic gene variants in familiar and sporadic DSD cases, in combination with the establishment of new mouse models using the latest genome editing techniques such as the TALEN and CRISPR/CAS9 systems, a more rapid discovery of important players is expected.

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Mutations of the Gonadotropin-Releasing Hormone Receptor Gene☆

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Glossary

Digenicity Protein-altering variants in two or more alleles of different genes.
Domain An independently folded portion of a protein that has a recognizable or characterizable structure of its own; in large proteins, each domain is connected to other domains by flexible regions of polypeptide.
Effector A molecule that couples a ligand-occupied receptor to a second messenger.
Ligand A molecule that binds specifically to a receptor.
Mutation A heritable or de novo change in the nucleotide sequence of a gene.
Receptor A molecule, usually a protein, that binds a specific extracellular signaling molecule and initiates or blocks a response in the cell.
Second messenger Usually a small molecule that is formed in or mobilized into the cytoplasm in response to an extracellular signal; this molecule relays the signal to the interior of the cell.
Signal transduction The process whereby a cell converts an extracellular signal into a biochemical response.
Pharmacoperone (from "pharmacological chaperone") A small molecule that enters cells and serves as molecular framework in order to cause otherwise-misfolded mutant or wild type proteins to fold and route correctly within the cell.

Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide produced by specialized neurons predominantly located in the arcuate nucleus of the mediobasal hypothalamus and in the preoptic area of the anterior hypothalamus. The axons of these neurons are projected to various regions of the brain, where the decapeptide acts as neurotransmitter or neuromodulator of reproductive behavior, and to the median eminence, where the GnRH peptide enters the portal circulation, eventually reaching and interacting with its membrane receptor in the gonadotropes. Activation of the GnRH receptor (GnRHR; OMIM 138850; Genomic coordinates (GRCh38):4:67,737,374-67,756,085) (https://www.omim.org/entry/138850?search=gnrhr&highlight=gnrhr) leads to both synthesis and release of the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Chronic exposure to GnRH promotes synthesis of the gonadotropins, which will be stored in secretory vesicles until exocytosis stimulated by GnRH exposure. The coordinated release of LH and FSH allows for an extremely precise control of gonadal function, including gametogenesis and steroidogenesis. The secretion and subsequent interaction of GnRH with its cognate receptor occurs in a pulsatile and intermittent manner. Pulsatile release of GnRH is regulated indirectly by gonadal steroids and directly by a complex network of interactions between the GnRH neurons and other neurons located in the mediobasal hypothalamus, including mainly those belonging to the Kiss1/neurokinin B/dynorphin system (Beltramo *et al.*, 2014; Goodman *et al.*, 2014; Narayanaswamy *et al.*, 2016). Intermittent exposure of the GnRHR to the releasing hormone is extremely important from the functional point of view. First, it prevents the desensitization that leads to refractoriness of the gonadotrope to a subsequent stimulus that may result as a consequence of the continuous exposure of the cell to the decapeptide. Second, it allows for the occurrence of distinct rates and patterns of synthesis and secretion of LH and FSH that follow GnRH exposure.

Decreased synthesis of pituitary gonadotropins and/or alterations in their episodic release from the pituitary may lead to impaired gonadal function and reproductive failure, a condition known in humans as hypogonadotropic hypogonadism (HH) (Chevrier *et al.*, 2011; Noel and Kaiser, 2011; Silveira *et al.*, 2010; Ulloa-Aguirre *et al.*, 2004; Boehm *et al.*, 2015).

The presence of GnRH and a normally plasma membrane (PM) expressed and functioning GnRHR is crucial for pubertal development, sexual maturation, and reproductive competence (Silveira *et al.*, 2010). Mutations in the *GNRHR* may lead to abnormal synthesis of the GnRHR and/or to structural alterations that may potentially alter its functional properties and/or PM expression, the latter making GnRH entirely inaccessible to the receptor (Chevrier *et al.*, 2011; Conn and Ulloa-Aguirre, 2010). Inactivating mutations in the *GNRHR* represent, in fact, a well-known cause of normosmic HH (Chevrier *et al.*, 2011; Ulloa-Aguirre *et al.*, 2004). This article describes the inactivating mutations of the *GNRHR* detected to date and the molecular physiopathogenesis of GnRHR mutants for which data is available. Also included is information on pharmacological approaches that in experimental studies have proved to overcome the functional defect provoked by the mutations, and briefly discuss the impact of

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the mutations on the phenotypic expression of HH in individuals bearing such mutant GnRHs as well as the currently available therapeutic options.

The GnRH Receptor

The type 1 GnRHR belongs to the superfamily of G protein-coupled receptors (GPCRs), specifically the β-group of the Rhodopsin family, according to the phylogenetic classification proposed by Fredriksson *et al.* (2003), which is the best-known family in terms of its structural and functional characteristics. Seven transmembrane hydrophobic domains (TMDs) oriented roughly perpendicular to the plasma membrane plane, with an extracellular NH_2-terminus, an intracellular COOH-terminus, and three alternating intra- and extracellular hydrophilic loops connecting the TMDs, characterize the structure of these receptors (**Fig. 1**B and C). The

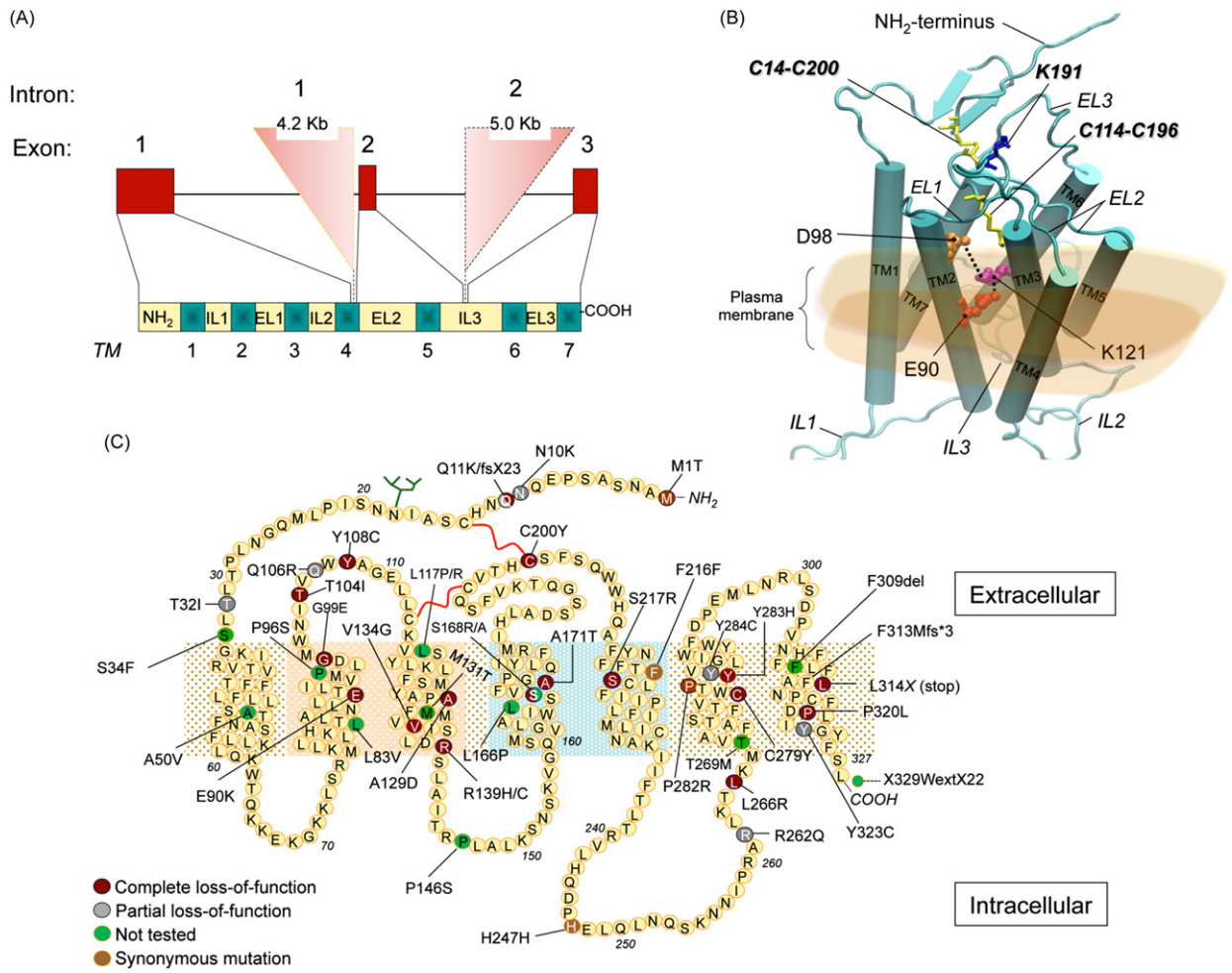

Fig. 1 The human GnRH receptor. (A) Schematic of the human *GnRHR* gene. The open reading frame is distributed among 3 exons (*maroon rectangles*) spanning 18.9 Kb, that encode amino acids 1–174, 175–248, and 249–328, respectively. Intron 1 is located between amino acids 174 and175 in the putative transmembrane domain (TM) 4, and intron 2 is located between amino acids 248 and249 in the intracellular loop (IL) 3 (*shaded inversed triangles*). *EL*, extracellular loop. (B) Predicted model of the human GnRHR showing the seven transmembrane helices (displayed as *rods*) connected by the extracellular (EL) and intracellular (IL) loops (Jardón-Valadez *et al.*, 2008). C14–C200 and C114–C196 disulfide bridges are shown as *yellow sticks*; K191 is represented by *blue sticks*. E90 (at the TM2; *red spheres*) forms a salt bridge with K121 (at the TM3; *purple spheres*) which is eliminated by the E90K mutation which leads to complete HH. Pharmacoperones act to stabilize the E90K mutant by bridging residues D98 (at the extracellular face of TM1; *orange spheres*) and K121 (*discontinuous line*). (C) Sequence of the human GnRHR and location of the loss-of-function mutations identified to date. *Circles* represent amino acid residues. *Red curved lines* represent the position of the C14–C200 and C114–C196 disulfide bridges. The *light orange rectangle* corresponds to the portion of the receptor where the TMs 2 and 3 are located and that are stabilized by the conserved E90–K121 salt bridge or the surrogate D98–K121 bridge (see B) resulting from pharmacoperone action; the *light green rectangle* corresponds to TMs 4 and 5, where mutations are completely recalcitrant (S168R and S217R) or marginally responsive (A171T) to pharmacoperones (Ulloa-Aguirre et al., 2004). Reproduced from Conn, P. M., and Ulloa-Aguirre, A. (2011). Pharmacological chaperones for misfolded gonadotropin-releasing hormone receptors. *Advances in Pharmacology* ***62***, 109–141, with permission from Elsevier.

mammalian GnRHR exhibits more than 85% amino acid identity among the several species that have been cloned. Unlike other members of the Rhodopsin family of GPCRs, the GnRHR exhibits several unique features, including the reciprocal exchange of the conserved D and N residues in TMDs 2 and 7, the replacement of Y with S in the D–R–Y motif located in the junction of the TMD 3 and the intracellular loop (IL) 2, and the lack of the COOH terminal domain (Millar *et al.*, 2004). This latter feature, which is not exhibited by GnRH receptors from nonmammalian vertebrate species such as fish and avian GnRH receptors, is unique among the thousands of members of mammalian GPCRs and is apparently associated with differential physiological regulation (internalization, desensitization, and cell surface PM expression) of the receptor in mammals and nonmammals. Another important feature of the human GnRHR is the presence of a lysine residue at position 191 (K191) in the extracellular loop (EL) 2 (**Fig. 1**B and C). In nonprimate mammals, glutamic acid or glycine (occasionally) are found instead of lysine; in fact, in rodent (rat and mice) GnRHRs the orthologous amino acid is absent, thereby yielding a structure that is one residue smaller (327 amino acid residues). The absence of lysine in this position confers the rodent GnRHRs with an increased PM expression, whereas its presence in the human GnRHR (328 amino acids) limits the amount of GnRHR protein that reaches the PM after synthesis. The mechanism subserving the effect of K191 on PM expression of the primate receptor includes interfering with formation of the C14–C200 disulfide bridge, which is necessary to stabilize the GnRHR in a conformation compatible with endoplasmic reticulum (ER) export. In the rat GnRHR, this disulfide bridge is not essential for receptor expression, as replacement of either of the cysteine residues of the bridge does not affect PM expression (Ulloa-Aguirre *et al.*, 2006). In humans, the GnRHR is located on 4q13.2-3 and consists of three exons and two introns that encode for a 328-amino acid protein (**Fig. 1**A).

The GnRHR is preferentially coupled to the trimeric $G_{q/11}$ protein, localized in the cytoplasm, and associated with the intracellular domains of the receptor. There is evidence that GnRHR also may regulate the activity of the adenylyl cyclase/cyclic AMP/protein kinase A pathway through G_s or G_i proteins, depending on the cell context and experimental paradigm employed. Activation of the GnRHR by its ligand is associated with conformational changes in the receptor molecule that lead to activation of the $G\alpha_{q/11}$ protein subunit followed by its dissociation from the $G\beta\gamma$ complex. The activation of the GnRHR–$G\alpha_{q/11}$ protein complex stimulates the effector enzyme phospholipase-Cβ, which in turn provokes phosphatidylinositol 4,5-biphosphate hydrolysis, leading to formation of the second messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol. The former diffuses through the cytoplasm, promoting the release of intracellular Ca^{2+} from intracellular stores followed by Ca^{2+} influx via L-type voltage-gated Ca^{2+} channels and acute, calmodulin-mediated secretion of gonadotropins, mainly LH. Diacylglycerol, along with Ca^{2+}, activates the enzyme protein kinase C, triggering a cascade of protein–protein phosphorylation and interactions, including activation of other kinase cascades (e.g., mitogen-activated protein kinase—MAPK- cascade), which eventually lead to the expression of several genes, such as those encoding the gonadotropin subunits and the GnRHR (Naor and Huhtaniemi, 2013). Other signaling molecules involved in GnRH-mediated signaling include phospholipases D and A2, which promote phosphatidic acid and arachidonic acid synthesis, respectively, as well as nitric oxide-stimulated cGMP, and molecules involved in the Wnt/β-catenin signaling cascade and AMP-activated protein kinase (AMPK), the latter apparently involved in LHβ gene transcription (Andrade *et al.*, 2013).

Mutations in the *GNRHR*

Structural alterations in key residues of the receptor molecule or the G proteins may potentially lead to altered function of the receptor–G protein system. Mutations in sites involved in ligand binding usually result in altered receptors unable to recognize the ligand and to become activated (loss-of-function mutations), whereas mutations in sites involved in receptor activation or G protein coupling may lead either to loss-of-function or to constitutive activation (activation in the absence of ligand or gain-of-function) of the receptor molecule. Only one naturally occurring mutation of this latter type has been detected in the human GnRHR (E90K mutation) (**Fig. 1**B and C). This particular mutation leads to a complete form of HH due to the drastic inability of the mutant receptor to traffic from the ER to the PM. The constitutive activity of this mutant could be identified only after promoting its PM expression by exposure to pharmacoperones (see below) (Janovick and Conn, 2010).

Resistance to GnRH by inactivating (loss-of-function) mutations in the *GNRHR* is the most common known genetic cause of normosmic congenital HH and leads to distinct forms of autosomal recessive HH (Chevrier *et al.*, 2011; Ulloa-Aguirre *et al.*, 2004). *GNRHR* mutations may also occur in individuals with sporadic HH, but with a lower frequency (Beranova *et al.*, 2001). Interestingly, patients harboring some *GNRHR* mutations, also present alterations in other genes that lead to HH, including the *PROKR2* or the *FGFR1* (Pitteloud *et al.*, 2007; Silveira *et al.*, 2010; Sykiotis *et al.*, 2010; Gonçalves *et al.*, 2017). Nearly 43 inactivating mutations (including deletions of large sequences and synonymous mutations) in the *GNRHR* have been described as a cause of partial or complete forms of HH (**Fig. 1**C and **Tables 1** and **2**). The detected mutations in the human *GNRHR* are distributed along the entire coding sequence of the receptor, except the IL1 and the extracellular loop (EL) 3. However, two "hot spots" have been identified at residues Q106 and R262 (Q106R and R262Q mutations) (Chevrier *et al.*, 2011).

Although expression of mutated GnRH receptors (*maroon* and *gray circles* in **Fig. 1**C) in in vitro heterologous cell systems has shown that these mutations may influence ligand binding and/or intracellular signal transduction (**Table 2**) (which initially suggested that such mutations disrupted motifs involved in agonist binding, receptor activation and/or interaction with G proteins), studies on several mutants (**Table 2**) have shown that the loss-of-function is rather due to folding defects that lead to impaired intracellular trafficking and reduced PM expression (Conn and Ulloa-Aguirre, 2010). In fact, in a number of these mutant receptors the function may be partially or completely restored in vitro and in vivo by genetic and/or pharmacologic (employing

Table 1 Genotype/phenotype correlates in patients with normosmic HH due to GnRHR mutations or in subjects with simple heterozygous mutations exhibiting reproductive abnormalities or somatic defects associated to HH

Compound heterozygous	*Phenotype (HH)*	*Simple heterozygous*	*Phenotype (HH)*	*Homozygous*	*Phenotype*
M1T + R139H/Q106R (Gianetti *et al.*, 2012)	Complete (♀)	S34F (Riaz *et al.*, 2017)	CDP (♂)	Q11fsX23 (Gianetti *et al.*, 2012)	Complete (♀)
N10K + Q11K/Y283H (Beneduzzi *et al.*, 2014)	Complete (♂)	A50V (Gianetti *et al.*, 2012)	CDP (♀)	E90K (Soderlund *et al.*, 2001)	Complete (♀ and ♂)
N10K + Q11K/P320L (Meysing *et al.*, 2004)	Partial (♀)	Q106R (Gianetti *et al.*, 2012; Beneduzzi *et al.*, 2014)	Partial or complete (♀ and ♂), only CLP (♀) or CDP (♂)	G99E (Krausz *et al.* 2017)	Complete (♂)
T32I/C200Y (Beranova *et al.*, 2001)	Complete (♂)	Q106R + S217R (Gianetti *et al.*, 2012)	CDP (♂)	Q106R (Gianetti *et al.*, 2012; Beranova *et al.*, 2001; Tommiska *et al.*, 2013)	Partial (♂ and ♀); LOH (♂)
T104I/Y108C (Antelli *et al.*, 2006)	Partial (♂) or complete (♀)	L117P (Gianetti *et al.*, 2012)/R (Zhu *et al.*, 2015)	CDP (♂)	L117R (Gürbüz *et al.*, 2012)	Complete (♂)
Q106R/N10K (Costa *et al.*, 2001)	Partial or complete (♀ and ♂)	R139H (Gianetti *et al.*, 2012; Tommiska *et al.*, 2016)	Anosmia (♀); LOH	M131T (Gürbüz *et al.*, 2012)	Complete (♂)
Q106R/L83V (Gianetti *et al.*, 2012)	Complete (♀)	P146S (Gianetti *et al.*, 2012)	Complete (♂) or partial (♀)	R139C (Topaloglu *et al.*, 2006)	Complete (♀)
Q106R/P96S (Gianetti *et al.*, 2012)	Partial (♂; fertile eunuch)	L166P (Gianetti *et al.*, 2012)	CDP (♂)	R139H (Beneduzzi *et al.*, 2014; Costa *et al.*, 2001)	Complete (♀)
Q106R/S168A (Gianetti *et al.*, 2012)	Partial (♀)	A171T (Gianetti *et al.*, 2012)	Complete (♂)	M1T + R139H/R139H (Wolczynski *et al.*, 2003)	Complete (♂)
Q106R/A171T (Karges *et al.*, 2003)	Complete (♂)	F216F (Gianetti *et al.*, 2012)	HH (♂), not specified	S168R (Pralong *et al.*, 1999)	Complete (♂)
Q106R/R262Q (Beranova *et al.*, 2001; de Roux *et al.*, 1997; Seminara *et al.*, 2000)	Partial (♀ and ♂) or complete (♀)	H247H (Cerrato *et al.*, 2006)	HH (♂), not specified.	R262Q (Gianetti *et al.*, 2012; Lin *et al.*, 2006; Tommiska *et al.*, 2013)	Partial (♂) or complete (♂ and ♀); reversal followed by LOH
Q106R + S217R/Q106R (Gianetti *et al.*, 2012)	Partial (♂; fertile eunuch) (♀)	R262Q (Gianetti *et al.*, 2012)	Complete (♂) and partial or complete (♀)	C279Y (Gianetti *et al.*, 2012; Beranova *et al.*, 2001)	Complete (♀ and ♂)
Q106R + S217R/R262Q (de Roux *et al.*, 1999)	Complete (♂) or partial (♀)			Y283H (Gonçalves *et al.*, 2017)	Complete (♀)
Q106R/L266R (Beranova *et al.*, 2001)	Partial (♀)			Exon 2 deletion[a] (Silveira *et al.*, 2002)	Complete (♀ and ♂)
Q106R/F313Mfs[a]3 (Gonçalves *et al.*, 2017)	Complete (♂ and ♀)				
Q106R/L314*X*(stop) (Kottler *et al.*, 2000)	Complete (♀)				
V134G/R262Q (Beneduzzi *et al.*, 2014)	Partial (♂)				
V134G/Q106R (Beneduzzi *et al.*, 2014)	Complete (♂)				
V134G/R139C (Gonçalves *et al.*, 2017)	Complete (♂)				
R139H + T269M (Zernov *et al.* 2016)	Partial (♀)				
R139H/T32I (Gianetti *et al.*, 2012)	Complete (♂)				
R262Q/Y284C (Layman *et al.*, 1998)	Complete (♀ and ♂)				
R262Q/A129D (Caron *et al.*, 1999)	Complete (♀ and ♂)				

Table 1 Continued

Compound heterozygous	*Phenotype (HH)*	*Simple heterozygous*	*Phenotype (HH)*	*Homozygous*	*Phenotype*
R262Q/L166P (Gianetti *et al.*, 2012)	Partial (♂; fertile eunuch)				
R262Q/F313Mfs*3 (Gonçalves *et al.*, 2017)	Complete (♂)				
R262Q/F309del (Vaaralahti *et al.*, 2011)	CDP (♂)				
R262Q/X329WextX22 (Gürbüz *et al.*, 2012)	Complete (♂)				
P282R/Y323C (Tello *et al.*, 2012)	Complete (♂)				

[a]G to A transversion in the acceptor splice site at the intron 1–exon 2 boundary.
CDP, Constitutional delay of puberty; *CLP*, cleft lip/palate; *LOH*, late-onset hypogonadism.

pharmacological chaperones or pharmacoperones, Conn *et al.*, 2013) means (Ulloa-Aguirre and Conn, 2016), whenever the mutation does not replace critical residues involved in agonist binding or effector activation (Conn and Ulloa-Aguirre, 2011). For example, the E90K mutation profoundly affects membrane receptor density as a consequence of disruption of trafficking to the PM (Maya-Nunez *et al.*, 2002). Nevertheless, either deletion of K191 (which increases membrane expression and reduces the rate of internalization of the human GnRHR) or exposure to pharmacoperones, efficiently restored membrane expression and agonist-induced, receptor-mediated intracellular signaling of this mutant in vitro (for the mechanism of action of pharmacoperones, see legend for **Fig. 1**B) (Brothers *et al.*, 2004; Conn and Ulloa-Aguirre, 2010; Maya-Nunez *et al.*, 2002). Further, recent studies in an animal model of HH due to the E90K mutation demonstrated that administration of the pharmacoperone IN3 reversed the phenotypic and biochemical abnormalities provoked by the mutation (Janovick *et al.*, 2013; Ulloa-Aguirre and Conn, 2016). The E→K substitution at position 90 prevents the formation of an E90–K121 salt bridge destabilizing the interaction between TMD2 and TMD3 (**Fig. 1**B) required to pass the quality control system of the ER. Consequently, the E90K mutant is retained in the ER. Gaining or losing a disulfide bridge also may result in misfolded GnRHRs. This is the case of the C200Y and the Y108C GnRHR mutants. The C200Y mutation prevents formation of the disulfide C14–C200 bridge required in the human GnRHR to pass the quality control system of the ER, while the Y108C mutation leads to formation of an aberrant disulfide bridge between C108 and C200, provoking gross receptor distortion, which severely impairs its PM expression (Ulloa-Aguirre *et al.*, 2006; Maya-Nunez *et al.*, 2011). The function of the Y108C mutant can be partially rescued by deleting K191 or by pharmacoperone treatment (**Table 2**), and complete rescue is possible only when both rescuing strategies are simultaneously applied (Maya-Nunez *et al.*, 2011). In the case of the A171T mutation (in the TMD4 of the GnRHR) the replacement presumptively disrupts receptor function by impeding conformational mobility of the TMD3 and 4, resulting in stabilization of the receptor in its inactive conformation (Karges *et al.*, 2003); in this particular mutant, exposure to pharmacoperones minimally rescued function (Ulloa-Aguirre *et al.*, 2004). In the S168R and S217R mutations, the thermodynamically unfavored substitutions provoke twisting of the corresponding α-helices moving the EL2 away from the NH_2-terminal domain, impeding the formation of the C14–C200 bridge essential for correct folding and trafficking of the receptor from the ER to the PM (Janovick *et al.*, 2006). Thus, both mutants are completely refractory to genetic or pharmacologic rescue approaches (Conn and Ulloa-Aguirre, 2010). In the L314*X*(Stop) mutation, GnRH binding is practically abolished. In this latter mutation, which leads to partial deletion of the TMD7, the mRNA levels of the receptor are reduced considerably, suggesting that the truncated protein might not be adequately translated and expressed in vivo (Kottler *et al.*, 2000). A truncated nonfunctional GnRHR also has been reported for the homozygous splice junction mutation (G to A replacement) at the intron 1–exon 2 boundary, resulting in a transcript showing splicing of exon 1 to exon 3 (i.e., complete deletion of exon 2) (Silveira *et al.*, 2002).

Individuals with normosmic HH due to *GNRHR* mutations exhibit a strikingly wide spectrum of clinical and biochemical phenotypes, including delayed puberty, variable alterations in pubertal development, late-onset hypogonadism, low or low-normal plasma gonadotropin and sex steroid levels, and impaired response to exogenous GnRH administration and pulsatile pattern of gonadotropin release (de Roux *et al.*, 1999; Abel *et al.*, 2013; Beranova *et al.*, 2001; Gianetti *et al.*, 2012; Tommiska *et al.*, 2013). These alterations usually occur in the absence of anatomical or functional abnormalities of the hypothalamic–gonadotrope axis. Thus, the hypogonadism due to GnRHR mutations can be complete or partial (**Table 1**). The differences between phenotypes in HH due to inactivating *GNRHR* mutations could be related to the particular allelic combination of the coexisting mutations, with the functional activity of a given mutant being more or less severely affected than that exhibited by the other (Leaños-Miranda *et al.*, 2005). In fact, studies in large populations of patients with HH due to GnRHR mutations, found that the reproductive phenotype in patients with biallelic mutations correlated better with the functional severity of the GnRHR mutant than in those harboring monoallelic mutations (Gianetti *et al.*, 2012) (**Table 1**). Nevertheless, the fact that different phenotypes may be present within patients bearing the same molecular alteration or in subjects with simple heterozygous *GNRHR* mutations suggests that other genetic (e.g., coexistence of other mutations also leading to HH (*PROKR2* or *FGFR1*), Pitteloud *et al.*, 2007; Silveira *et al.*, 2010; Sykiotis *et al.*, 2010) or nongenetic (e.g., dominant-negative effects of the mutant GnRHR on its wild-type counterpart,

Table 2 Function of GnRHR mutants as disclosed by in vitro or in silico studies

Mutant	*PME*	*Binding*	*Signalling*	*RPh (Leaños-Miranda et al., 2005; Topaloglu et al., 2006; Tello et al., 2012; Ulloa-Aguirre et al., 2004)*	*Mutant*	*PME*	*Binding*	*Signalling*	*RPh (Leaños-Miranda et al., 2005; Topaloglu et al., 2006; Tello et al., 2012; Ulloa-Aguirre et al., 2004)*
M1T (Gianetti *et al.*, 2012)	Predicted complete LOF[a]			ns	L166P (Gianetti *et al.*, 2012)	Predicted LOF[a]			ns
N10K (Costa *et al.*, 2001)	+	+ Bmax/ ↑Kd	↓	C	S168R (Pralong *et al.*, 1999)	+	–	–	–
Q11K (Gianetti *et al.*, 2012)	Predicted partial LOF[a]			ns	S168A (Gianetti *et al.*, 2012)	Predicted LOF[a]			ns
Q11fsX23 (Gianetti *et al.*, 2012)	Predicted complete LOF[a]			ns					
N10K + Q11K (Meysing *et al.*, 2004)	+	↓ Bmax	+	ns	A171T (Karges *et al.*, 2003)	+	–	–	P
T32I (Bedecarrats *et al.*, 2003a)	+	–	↓	C	C200Y (Bedecarrats *et al.*, 2003a)	+	–	↓↓	P
S34F (Riaz *et al.*, 2017)	ns	ns	ns	ns	F216F (Gianetti *et al.*, 2012)	Predicted benign[a]			
A50V (Gianetti *et al.*, 2012)	Predicted benign[a]			ns	S217R (de Roux *et al.*, 1999)	ns	–	–	–
					H247H (Cerrato *et al.*, 2006)	ns	ns	ns	ns
L83V (Gianetti *et al.*, 2012)	Predicted benign[a]			ns	R262Q (Bedecarrats *et al.*, 2003b; Layman *et al.*, 1998)	+	+	↓	C
E90K (Maya-Nunez *et al.*, 2002; Brothers *et al.*, 2004)	–	–	–	C	L266R (Bedecarrats *et al.*, 2003a)	+	–	–	P
					T269M (Zernov *et al.*, 2016)	Predicted pathogenic[b]			ns
P96S (Gianetti *et al.*, 2012)	Predicted LOF[a]			ns	C279Y (Bedecarrats *et al.*, 2003a)	+	–	–	P
G99E (Krausz *et al.*, 2017)	–	↓	↓	ns	P282R (Tello *et al.*, 2012)	↓	–	–	–
T104I (Maya-Nunez *et al.*, 2011)	ns	↓	↓↓	P	Y283H (Beneduzzi *et al.*, 2014)	ns	ns	–	ns
Q106R (Bedecarrats *et al.*, 2003b; de Roux *et al.*, 1997; Leaños-Miranda *et al.*, 2005)	+	↓	↓	P	Y284C (Layman *et al.*, 1998; Layman *et al.*, 2001)	ns	↓	↓↓	C
					F309del (Vaaralahti *et al.*, 2011)	ns	ns	ns	ns
Y108C (Maya-Núñez *et al.*, 2011)	ns	–	–	P	F313Mfs*3 (Gonçalves *et al.*, 2017)	Predicted LOF[a]			ns
L117P (Gianetti *et al.*, 2012)/R (Gürbüz *et al.*, 2012)	Predicted LOF[a]			ns	L314X (Kottler *et al.*, 2000)	ns	–	–	–
A129D (Caron *et al.*, 1999)	ns	–	–	M	P320L (Meysing *et al.*, 2004)	+	–	–	ns
M131T (Gürbüz *et al.*, 2012)	Predicted LOF[a]	ns			Y323C (Tello *et al.*, 2012)	↓	↓	–	M
V134G (Beneduzzi *et al.*, 2014)	ns	ns	–	ns	Exon 2 deletion (Silveira *et al.*, 2002)	ns	ns	ns	ns
R139H (Costa *et al.*, 2001)	+	–	–	M	X329WextX22 (Gürbüz *et al.*, 2012)	Predicted LOF[a]			ns
R139C (Topaloglu *et al.*, 2006)	ns	–	↓↓	P					
P146S (Gianetti *et al.*, 2012; Vagenakis *et al.*, 2005)	Predicted LOF[a]			ns					

[a]Studied in silico.

[b]By ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/intro).

PME, Plasma membrane expression studied by immunocytochemistry of tagged-GnRHR or by confocal microscopy of GnRHR-green fluorescent protein (GFP) chimera (E90K). Caution should be exercised in the interpretation of these studies (see Brothers *et al.*, 2003); *RPh*, rescue with pharmacoperones [*C*, complete rescue (response at or above that shown by the Wt GnRHR in the absence of pharmacoperone); *P*, partial rescue (response below that shown by the Wt GnRHR in the absence of pharmacoperone); *M*, marginal rescue]. *LOF*, loss-of-function; *ns*, not studied; +, comparable with Wt GnRHR; –, no measurable effect; ↓ decrease compared to Wt GnRHR.

Leaños-Miranda *et al.*, 2003, 2005) factors may be implicated on the phenotypic expression of the GnRH-resistant HH. Thus, both mechanisms (digenic mutations and dominant negative effects of the mutant GnRHR protein) may account for the severity and variable phenotypic expression of GnRHR mutations. Finally, it is worth mentioning that the HH provoked by certain GnRHR mutations (Q106R, R262Q) can revert spontaneously (Laitinen *et al.*, 2012; Raivio *et al.*, 2007; Sidhoum *et al.*, 2014; Tommiska *et al.*, 2013); the mechanisms subserving this spontaneous reversion, in the absence of coexisting mutations in other genes, are uncertain.

Treatment

Although treatment of HH due to mutations in the *GNRHR* will depend on the particular clinical manifestations and biochemical phenotype of the patient, it may, in general, follow those recommended for other forms of HH due to GnRH deficiency (Ulloa-Aguirre and Lira-Albarrán, 2016; Boehm *et al.*, 2015), keeping in mind that the response to pulsatile GnRH in partial HH might be variable and require a more careful patient selection as well as administration of higher GnRH doses than those usually employed for non GnRH-resistant forms of HH, particularly for ovulation induction (de Roux *et al.*, 1999; Caron *et al.*, 1999; Seminara *et al.*, 2000). In male patients with complete HH, treatment options include administration of testosterone or, preferably, exogenous gonadotropins [FSH and human chorionic gonadotropin (hCG), or human menopausal gonadotropin (hMG)], the latter to promote testicular growth and maturation, stimulate spermatogenesis, normalize serum testosterone levels, and induce development of secondary sexual characteristics. Once puberty has initiated, the patient can be switched to treatment with testosterone indefinitely or until fertility is desired, when gonadotropin treatment should be reinstated. In incomplete forms of HH, treatment with testosterone is usually sufficient to complete sexual maturation and maintain virilization, sexual function, and bone and muscle mass.

Here is important to mention that in patients with GnRHR mutations, HH may revert spontaneously, so a brief withdrawal of treatment with either testosterone or gonadotropins and reassessment of the reproductive axis function with close monitoring of serum testosterone levels are advisable to seek for spontaneous reversion of the hypogonadal state. This will ensure that, in fact, the HH condition reverted and that normalization of reproductive function has occurred. Since reversion may be temporal, long-term monitoring of reproductive function is recommended.

In females, unopposed oral or transdermal estrogen administration may allow optimal breast development. Thereafter, cyclic estrogen-progestin treatment is advisable to provide endometrial protection. When fertility is desired, exogenous gonadotropins may be administered to induce follicular maturation and ovulation, and if spontaneous conception fails, then the patient may undergo IVF treatment (Zernov *et al.*, 2016).

Although pharmacoperones represent the most promising agents to treat HH due to conformationally-, trafficking-defective GnRHR mutants, the development of this particular class of drugs still is under investigation.

Conclusion

GnRH resistance due to inactivating mutations in the *GNRHR* is a common cause of congenital normosmic HH. The reproductive phenotype of patients harboring mutations in the *GNRHR* is highly variable and depends on the allelic distribution of the mutations (mono- vs. biallelic) as well as the coexistence of mutations in other genes also associated to HH (digenic inheritance). Pharmacoperones represent a unique potential therapeutic strategy to overcome the functional defect of the abnormal receptor, whenever the mutation leads to misfolding, impaired intracellular trafficking and altered PM expression. Naturally occurring mutations in the *GNRHR* represent unique models for the analysis of the structure–activity relationships of this particular receptor.

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Androgen Biosynthesis and Gene Defects☆

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Glossary

Cryptorchidism The condition in which the testes fail to descend into the scrotum and are retained within the abdomen or inguinal canal.

Follicle-stimulating hormone (FSH) A gonadotropin secreted and released by the anterior pituitary. FSH stimulates the ripening of the follicles in the ovary and formation of sperm in the testes by acting on granulosa and Sertoli cells, respectively.

Haploinsufficiency A mutation in only one of the two alleles for an autosomal gene, resulting in a reduced gene dosage for the protein encoded on the normal allele.

Human chorionic gonadotropin (hCG) A hormone similar to the pituitary gonadotropin luteinizing hormone; it is produced by the placenta during pregnancy.

Hypospadias A congenital abnormality in which the labioscrotal folds have not completely fused, so that the opening of the urethra is on the underside of the penis.

Leydig cells The cells interspersed between the seminiferous tubules of the testis. They secrete androgens in response to luteinizing hormone.

Luteinizing hormone (LH) A gonadotropin secreted by the anterior pituitary. LH stimulates androgen synthesis by the Leydig cells of the testis and the theca cells of the ovary; it also stimulates the "luteinization" of ovarian cells after ovulation, forming the corpus luteum, which makes progesterone.

Male pseudo-hermaphroditism A congenital abnormality in which the genitalia of a 46,XY infant are not completely masculinized, despite the presence of testes.

Müllerian structures Structures that develop from the paramesonephric duct in normal females. These include the fallopian tubes, uterus, and upper part of the vagina.

Sertoli cells Cells found in the walls of the seminiferous tubules of the testis. They anchor and nourish the developing germ cells.

Wolffian structures Structures that develop from the mesonephric duct in normal males. These include the epididymis and vas deferens.

Normal Androgen Biosynthesis

All steroid hormone production begins with the conversion of cholesterol to pregnenolone. A "mobile pool" of free cholesterol in the outer mitochondrial membrane (OMM) is physically inaccessible to the side chain cleavage enzyme (CYP11A1, P450scc), which resides in the inner mitochondrial membrane (IMM). Stimulation of Leydig cells with chorionic gonadotropin (hCG) or luteinizing hormone (LH), both of which bind to the same LH/CG receptor, increases intracellular cyclic AMP (cAMP). The rise in cAMP induces the expression and activation of the labile steroidogenic acute regulatory (StAR) protein, which allows the cholesterol to flow from the OMM to the IMM, where CYP11A1 converts the cholesterol to pregnenolone. The microsomal enzyme CYP17A1 (P450c17) sequentially oxygenates pregnenolone (the 17α-hydroxylase reaction) and cleaves the C17–C20 bond (the 17,20-lyase reaction), yielding dehydroepiandrosterone (DHEA). Although human CYP17A1 hydroxylates pregnenolone and progesterone with comparable efficiencies, its 17,20-lyase activity is much more efficient for the Δ^5-steroid 17α-hydroxypregnenolone (17Preg) than for its Δ^4-congenor 17α-hydroxyprogesterone (17OHP). Consequently, DHEA is the substrate for 17β-hydroxysteroid dehydrogenase type 3 (17βHSD3), which reduces DHEA to Δ^5-androstenediol, and 3β-hydroxysteroid dehydrogenase/isomerase type 2 (3βHSD2) converts this Δ^5 steroid to testosterone (T). The sequence of these latter two reactions (17βHSD3 and 3βHSD2) may also proceed in reverse, with androstenedione (AD) as the intermediate. T produced by the testis diffuses into peripheral tissues and those tissues that contain the enzyme 5α-reductase type 2 (SRD5A2) (i.e., prostate, genital skin) metabolize T to the potent androgen dihydrotestosterone (DHT). The importance of all of these steps and the lack of adequate redundancy are demonstrated by the clinical disorders caused by mutations in the genes encoding the key proteins described herein (Fig. 1).

Disorders of Leydig Cell Stimulation

Hypogonadotropic Hypogonadism

Because fetal T synthesis during weeks 8–12 of gestation is driven primarily by placental hCG, 46,XY children with defects in gonadotropin-releasing hormone (GnRH) or gonadotropin production are born with relatively normal male external genitalia. In

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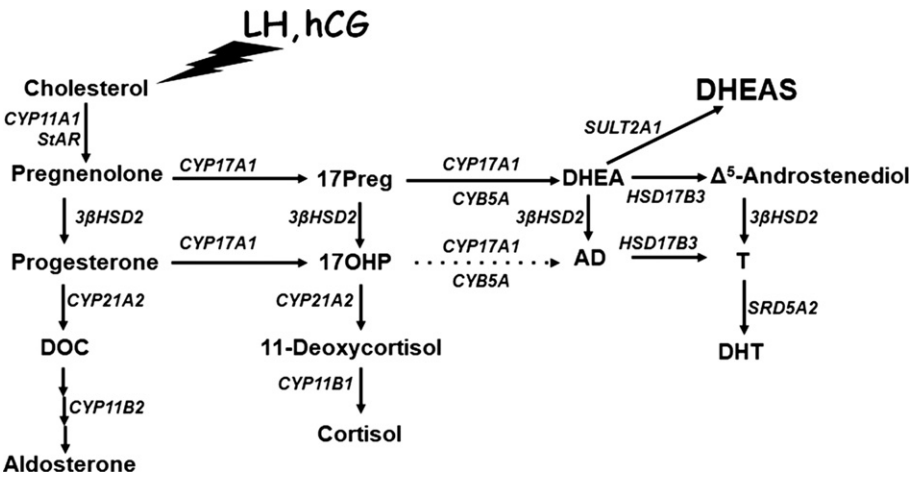

Fig. 1 Human steroid biosynthesis. Abbreviations include *LH*, luteinizing hormone; *hCG*, human chorionic gonadotropin; *StAR*, steroidogenic acute regulatory protein; *17Preg*, 17-hydroxypregnenolone; *17OHP*, 17-hydroxyprogesterone; *DHEA*, dehydroepiandrosterone; *DHEAS*, dehydroepiandrosterone sulfate; *AD*, androstenedione; *T*, testosterone; *DHT*, 5α-dihydrotestosterone; *DOC*, 11-deoxycorticosterone. Enzymes and cofactors are given in script adjacent to *arrows* representing reactions catalyzed.

contrast, LH drives T production in late gestation, so micropenis is often present, and these individuals fail to experience puberty. These conditions are introduced below but not discussed in detail, as the complex genetics and physiology are beyond the scope of this section.

Kallmann Syndrome and Congenital Normosmic Hypogonadotropic Hypogonadism

Kallmann syndrome refers to the combination of hypogonadotropic hypogonadism and anosmia. Consequently, 46,XY patients are born with normal male genitalia except for micropenis, and they fail to initiate puberty. The diagnosis is made clinically with endocrine and olfactory evaluation and confirmed with low LH and FSH, FSH > LH, and lack of gonadotropin rise in response to a bolus of GnRH analog. Magnetic resonance imaging (MRI) with attention to the olfactory bulb hypoplasia and midline structure defects is helpful but not essential. At the time of expected puberty, testosterone replacement is commenced. Treatment with pulsatile GnRH using a programmable pump will not only induce sexual maturation, but may restore fertility in individuals of both sexes.

Kallmann syndrome can be sporadic, autosomal dominant, autosomal recessive, or X-linked. The best understood form of Kallmann syndrome results from mutations in the *KAL1* gene, which accounts for 5%–10% of cases and up to half of familial cases with X-linked inheritance. This gene encodes an extracellular matrix protein called anosmin-1 that guides the migration of both the olfactory and GnRH-producing neurons from the olfactory placode to their definitive location in the head and brain. Forms of hypothalamic hypogonadism with or without anosmia may result from mutations in at least 35 other genes, which is beyond the scope of this discussion. In approximately half the cases of Kallmann syndrome and its variants, the molecular basis remains unknown.

Combined Pituitary Hormone Deficiencies and Septo-Optic Dysplasia

Developmental defects in midline structures often ablate hypothalamic–pituitary axes, and the growth hormone and GnRH–LH axes are particularly vulnerable. Severe defects, such as holoprosencephaly, characteristically involve large portions of the brain, but milder developmental defects can involve few structures. Combined pituitary hormone deficiency refers to a group of conditions in which growth hormone deficiency occurs with one or more additional pituitary hormone deficiencies. As the spectrum of these disorders expands, 20–30 genes have been identified, and the phenotype has been found to vary from isolated growth hormone deficiency to panhypopituitarism to hypogonadotropic hypogonadism and many intermediate combinations. Septo-optic dysplasia refers to the combination of optic nerve hypoplasia and hypothalamic–pituitary maldevelopment. Evaluation and management are similar to those for Kallmann syndrome, although vision testing and MRI evaluation are important for guiding follow-up and for prognosis. Mutations in several genes, including *HESX1*, *LHX3*, *FGF8*, *SOX2*, and *PROKR2* have been identified in patients with various forms of congenital hypopituitarism, from isolated hypogonadotropic hypogonadism or growth hormone deficiency to septo-optic dysplasia with multiple pituitary hormone deficits. The clinical severity roughly correlates with the impairment in activity of the mutant proteins when tested in vitro.

Leydig Cell Agenesis or Hypoplasia (Testicular Unresponsiveness to hCG/LH)

In a 46,XY fetus with a mutation in the LH/CG receptor, the Leydig cells fail to develop appropriately, and T production is impaired from conception. This defect leads to varying degrees of genital anomalies at birth, depending on the amount of hCG-

independent T production prior to the tenth week of gestation and the severity of the LH/CG receptor dysfunction. Because secretion of anti-Müllerian hormone by the Sertoli cells is intact, 46,XY children with Leydig cell hypoplasia do not retain Müllerian structures. The testes lack distinct Leydig cells on biopsy, and Sertoli cells may appear at puberty. However, the seminiferous tubules, if present, often show spermatogenic arrest, and the testes degenerate progressively.

The 46,XY infants born with completely female genitalia may not be identified until puberty, when they present with failure to develop breasts and to undergo menarche. Milder forms in 46,XY infants cause undervirilization, including hypospadias, micropenis, and cryptorchidism. 46,XX females homozygous for LH/CG receptor defects will have normal female genitalia and may experience some breast development at puberty, but with amenorrhea and infertility. The diagnosis is confirmed by low or absent T, androstenedione (AD), and 17OHP production in response to hCG stimulation testing. Basal and GnRH-stimulated gonadotropin values are elevated in pubertal subjects.

Management depends on the age of diagnosis and the degree of virilization. When the defects are severe enough to produce phenotypically female genitalia, assignment of the female gender, with gonadectomy and estrogen replacement therapy at the time of expected puberty, is usually recommended. For less severely affected individuals with undervirilized male genitalia, surgery may be necessary to correct hypospadias, and testosterone therapy is used to stimulate phallic development and to virilize the patient at puberty.

Leydig cell hypoplasia is an autosomal recessive condition due to mutations in the gene encoding the LH/CG receptor. Several genetic defects have been reported, including missense, nonsense, and null mutations. The null mutations, such as Arg554Stop, are associated with the most severe clinical phenotypes.

Variants of Congenital Adrenal Hyperplasia

The most common form of congenital adrenal hyperplasia (CAH) is 21-hydroxylase (CYP21A2, P450c21) deficiency, but CYP21A2 is not expressed in the gonads and does not participate in T biosynthesis. Other, less common forms of CAH that involve enzymes or proteins expressed both in the adrenals and the gonads are discussed below.

Lipoid CAH

StAR (Steroidogenic Acute Regulatory protein) Deficiency

Because StAR facilitates the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, inactivating mutations in StAR block the production of pregnenolone and thus impair all steroidogenesis, both in the adrenals and in the gonads. Under the stimulation of adrenocorticotropic hormone (ACTH) and LH, cholesterol esters massively accumulate in the adrenal glands and testes, respectively, affording the characteristic enlarged, lipid-laden adrenals from which the name lipoid CAH derives. Secondarily, sterol auto-oxidation products accumulate in the adrenals and Leydig cells, altering cell structure and ultimately leading to cell destruction.

Both 46,XY and 46,XX individuals will have female external genitalia at birth. Affected 46,XY individuals have abdominal, inguinal, or intralabial testes, a blind vaginal pouch, and no uterus or fallopian tubes. Wolffian duct remnants may be preserved in 46,XY individuals secondary to low levels of StAR-independent steroidogenesis. All reported patients are diffusely hyperpigmented from pro-opiomelanocortin and ACTH excess. Because the theca cells of the ovary do not normally make steroids during fetal and neonatal life, the ovaries of 46,XX subjects do not suffer lipid accumulation and cell death during childhood. Consequently, 46,XX subjects may produce enough estrogens in early puberty to undergo some breast development and may even menstruate until lipid accumulation and auto-oxidation obliterate ovarian function as well.

The diagnosis of lipoid CAH is confirmed by low or absent glucocorticoids, mineralocorticoids, gonadal steroids, their precursors, and their metabolites in plasma and/or urine, even after stimulation. In particular, 3βHSD2 deficiency is excluded by documenting low concentrations of not only the active Δ^4 steroids but of the Δ^5 precursors pregnenolone and 17Preg. On abdominal computed tomography scan or MRI, the lipid-laden adrenals are strikingly enlarged, displacing the kidneys in the caudal direction.

Treatment requires replacement doses of gluco- and mineralocorticoids in the newborn period, which must be continued throughout life. All affected 46,XY males have been reared as females, and orchidectomy is advised. Estrogen replacement therapy for individuals of both genotypes is required at puberty to initiate female secondary sexual characteristics, and low-dose testosterone may be used to elicit a female pattern of sexual hair growth. Lipoid CAH is an autosomal recessive disease with a male/female ratio of approximately 3/1; however, this ratio may be skewed by ascertainment bias. This condition is rare in the United States and Europe, but is the second most common form of CAH in Japan and Korea. In one series, mutation Q258X accounted for 80% of the affected alleles from Japanese and Korean subjects, suggesting a founder effect that causes the relatively high incidence of lipoid CAH in these countries. Mutation R182L was found in 78% of affected alleles from Palestinian subjects in the same series. Less severe mutations cause a partial or "nonclassic" form of lipoid CAH, in which sex steroid production is normal or mildly impaired, yet cortisol production is deficient, leading to clinically significant adrenal insufficiency. This discrepancy is likely due to the much larger amounts of cortisol synthesis necessary to sustain normal physiology, compared to testosterone or estradiol.

Side Chain Cleavage Enzyme (CYP11A1 or P450scc) Deficiency

For many years, it was hypothesized that homozygous CYP11A1 deficiency caused lipoid CAH, but an absence of CYP11A1 would preclude placental progesterone synthesis and promote spontaneous abortion after the eight to tenth week. However, haploinsufficiency of CYP11A1 has been shown to produce a milder clinical picture of lipoid CAH than in StAR deficiency. Tajima and associates described a 46,XY patient with clitoromegaly, a blind vaginal pouch, hyperpigmentation, and absent Müllerian structures. This patient was raised as a female, and testes were removed from the inguinal region. Adrenal insufficiency with hyperplasia did not occur until the child was 4 years old, and no mutation was found in the gene for StAR. Instead, one allele of the gene for CYP11A1 had a 6 bp in-frame insertion, adding G-D between D271 and V272. This mutant enzyme had no activity and appeared to impair the function of wild-type CYP11A1 when expressed in the same cells, suggesting a partial, dominant-negative mode of action, leading to less severe disease in early childhood rather than infancy.

Treatment is similar to that for StAR protein deficiency, with gluco- and mineralocorticoid replacement to prevent life-threatening adrenal insufficiency at the time of diagnosis, plus estrogen replacement therapy at the time of puberty.

3βHSD2 Deficiency

The 3βHSD enzymes catalyze the conversion of the Δ^5 steroids pregnenolone, 17Preg, DHEA, and Δ^5-androstenediol to their corresponding Δ^4 steroids progesterone, 17OHP, AD, and T, respectively. One of these conversions is required in the biosynthesis of all active steroid hormones, so severe 3βHSD2 deficiency will also result in a form of CAH with impaired androgen production.

46,XY individuals with 3βHSD2 deficiency most frequently exhibit male pseudo-hermaphroditism with a small phallus, hypospadias, partial labioscrotal fusion, and possibly a urogenital sinus with a blind vaginal pouch. Testes usually lie in the lower inguinal region, and Müllerian structures are absent. Paradoxically, 46,XX individuals often have trace clitoral enlargement and progressive masculinization if undertreated. Severe 3βHSD2 deficiency can present with salt-wasting crisis from glucocorticoid and mineralocorticoid insufficiency within the first week of life. Less severe forms of 3βHSD2 deficiency are usually diagnosed in genetic males because of genital abnormalities, but may be difficult to diagnose in females. Androgen production increases at puberty in both sexes but at a rate that is intermediate for males and females; consequently, girls show signs of androgen excess, but boys often develop gynecomastia. Fertility has been reported in affected individuals of both sexes (**Table 1**).

The diagnosis of 3βHSD2 deficiency hinges on elevated ratios of Δ^5 steroids to their Δ^4 congeners. These ratios, which are already increased at baseline, are accentuated by cosyntropin stimulation and should reach >12 SD above normal (17Preg > 3000 ng dL^{-1} $= 90$ nmol L^{-1}). Adult females with idiopathic hirsutism or polycystic ovary syndrome often have high circulating DHEA-S concentrations with high ratios of Δ^5 to Δ^4 steroids, so extremely elevated ratios are required to confidently diagnose 3βHSD2 deficiency.

Therapy includes early glucocorticoid and mineralocorticoid replacement in severely affected individuals to prevent life-threatening adrenal insufficiency, and this treatment also limits sexual precocity caused by increased synthesis of adrenal androgen precursors. Females require estrogen replacement, while males require testosterone supplementation to achieve full development of secondary sexual characteristics.

Two functional 3βHSD genes are encoded on chromosome 1p13. The type 2 enzyme is the dominant isoform expressed in the adrenals and gonads, and its gene is mutated in 3βHSD2 deficiency. Adult females who present with hirsutism, infertility, and relatively high DHEA-S concentrations do not have mutations in the cognate *HSD3B2* gene. Mutations in the *HSD3B2* gene have been found from residues A10 to G294, with no mutations dominating large series. Salt-wasting subjects have completely inactive alleles, and non-salt-wasting individuals can harbor mutations that retain partial activity when expressed in heterologous systems. The type 1 enzyme is expressed in the placenta but also in liver and skin, and this enzyme accounts for the peripheral conversion of Δ^5 precursors to Δ^4 steroids in 3βHSD2 deficiency, leading to the paradoxical androgen excess in females. Mutations in the gene for the type 1 enzyme have not been reported, although the common N367T gain-of-function allele resists degradation and confers a poor prognosis in patients with castration-resistant prostate cancer.

CYP17A1 (P450 17A1) Deficiencies

CYP17A1 catalyzes both the 17α-hydroxylase and 17,20-lyase reactions. Severe mutations will ablate both activities, but milder mutations may either partially impair both activities or preferentially impair 17,20-lyase activity with 17α-hydroxylase activity remaining relatively normal. Furthermore, the 17,20-lyase activity is particularly dependent on the interaction of CYP17A1 with its redox partners, cytochrome P450 oxidoreductase (POR) and cytochrome b_5. In particular, the presence of cytochrome b_5 increases the 17,20-lyase activity 10-fold but minimally influences 17α-hydroxylase activity. Thus, alterations in redox partners and/or their interactions with CYP17A1 may preferentially impair 17,20-lyase activity.

Combined 17α-Hydroxylase/17,20-Lyase Deficiency

The classical description of severe, combined, 17α-hydroxylase/17,20-lyase deficiency is sexual infantilism and hypokalemic hypertension in both genetic sexes. The presentation in 46,XY individuals varies from completely female external genitalia with a blind vaginal pouch to an undervirilized male genital phenotype with hypospadias and a small phallus. Testes in these subjects

Table 1 Compares clinical manifestations in 46,XY children born with these disorders

	Leydig cell hypoplasia	*Lipoid CAH*	*3βHSD2 deficiency*	*17-OHase deficiency*	*Isolated 17,20-lyase deficiency*	*POR deficiency*	*17βHSD3 deficiency*	*5α-Reductase 2 deficiency*
Possible appearance of genitalia	Female/ambiguous/hypoplastic male	Female	Ambiguous/hypospadic male	Female/ambiguous/hypospadic male	Female/hypoplastic male ± hypospadias	Female/ambiguous/hypospadic male	Female/	ambiguous
Hypospadias, small phallus								
Wolffian duct derivatives	Absent/hypoplastic	Absent/hypoplastic	Normal	Absent/hypoplastic	Hypoplastic/normal	Hypoplastic/normal	Hypoplastic	Normal
Müllerian duct derivatives	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Gonads in 46, XY	Testes, no Leydig cells[a]	Testes lipid laden	Testes	Testes	Testes	Testes	Testes	Normal testes
Adrenal			insufficiency?	No	Severe	Severe–mild	No	No
Partial	No	No						
Virilization at puberty?	None to poor	None	Poor to mild	None to poor	Poor	No	Yes	Yes
Increased hormone concentrations	LH, FSH	LH, FSH, renin	LH, FSH, pregnenolone, 17Preg, DHEA	LH, FSH, DOC, B, Progesterone	LH, FSH	LH, FSH, Prog, 17OHP, B	LH, FSH, Estrone, AD	T
Decreased hormone				concentrations	T, DHT	All adrenal and gonadal steroids	T, DHT	Renin, cortisol, 17OHP, DHEA, AD, T, DHT
DHEA, AD, T, DHT	Cortisol, DHEA, AD, T, DHT	T, DHT	DHT					
Chromosomal location	2p21	StAR: 8p11.2 CYP11A: 15q23–q24	1p13	10q24.3	10q24.3	7q11.2	9q22	2p23

[a]Small undescended testes with absent or decreased numbers of Leydig cells to descended testes of normal size with decreased numbers of Leydig cells. Hypogonadotropic hypogonadism is not included, because boys with this disorder generally have normal genitalia, except for micropenis.

may be intra-abdominal, in the inguinal canal, or in labioscrotal folds. Müllerian structures are absent, and Wolffian derivatives are usually hypoplastic. Severely affected patients will fail to develop secondary sexual characteristics, including pubic and axillary hair. With milder disease, males may develop gynecomastia at puberty and females may progress to Tanner stage 5 with ovulatory menses but infertility. Based on studies with recombinant enzyme, it has been estimated that at least 25% of normal fetal CYP17A1 activities are required for masculinization of the external genitalia. Hypertension with hypokalemia occurs in childhood and is often severe, with myopathy and hypertensive complications at a young age.

The diagnosis should be entertained not only in undervirilized males but also in any phenotypic female with hyporeninemic hypertension, hypokalemic alkalosis, and a suppressed aldosterone. This diagnosis may be confirmed by finding elevated circulating levels of ACTH and the precursors that accumulate proximal to the block in 17-hydroxylation: progesterone, 11-deoxycorticosterone (DOC), corticosterone (B), 18-hydroxy-DOC, and 18-hydroxy-B. Both DOC and B have mineralocorticoid activity, leading to hypertension and hypokalemia. However, signs of adrenal insufficiency rarely develop, because the weak glucocorticoid B is present in abundance. Gonadotropins are elevated at puberty, and serum concentrations of aldosterone, 17OHP, cortisol, and sex steroids are low or absent.

Treatment includes replacement of glucocorticoids to suppress DOC and B secretion and thereby to normalize potassium homeostasis and blood pressure. Mineralocorticoid receptor antagonists, such as spironolactone, can be used to reduce the doses of glucocorticoids and to prevent iatrogenic Cushing syndrome. At puberty, sex steroid replacement is indicated, and gonadectomy should be performed in 46,XY patients assigned a female sex of rearing due to increased risk of malignancy. One live birth has been reported in a 46,XX patient with incomplete deficiency, which required progesterone suppression with dexamethasone, ovulation induction, and in vitro fertilization.

Mutations have been identified throughout the *CYP17A1* gene, and some of the encoded proteins with missense mutations retain partial activity when expressed in heterologous systems. Most mutations that yield a completely inactive enzyme also destabilize the enzyme structure and ablate heme binding. Recurrent mutations include deletion of F53; R96W or Q; R239Q or X; Y329D, X, or ins/del; H373D, N, or L; P406R; a CATC duplication following I479; and deletions or duplications starting at D487. In Brazil, 17-hydroxylase deficiency is the second most common cause of CAH, due to the founder mutations W406R and R362C.

Isolated 17,20-Lyase Deficiency

When only the 17,20-lyase activity is deficient, adrenal glucocorticoid and mineralocorticoid synthesis is normal, but testosterone synthesis is impaired. Therefore, serum potassium and blood pressure are normal, but sexual development may be hampered. In 46,XY patients, the external genitalia are that of an undervirilized male, due to some residual 17,20-lyase activity. Müllerian structures are absent, Wolffian derivatives are either hypoplastic or normal, and testes may be intra-abdominal, inguinal, or in the scrotum. In 46,XX females, failure to progress through adrenarche and puberty suggest the diagnosis, but the few cases so far identified have been ascertained as siblings of affected 46,XY affected individuals.

Cosyntropin stimulation tests yields normal or elevated 17-hydroxysteroid values, including cortisol and 17OHP, but DHEA and AD do not rise proportionately. Similarly, hCG stimulation testing will produce an increase in 17OHP concentrations, but AD and T do not rise normally. The ratio of the rise in the C_{19} steroids to their C_{21}, 17-hydroxy precursors is the most discriminatory parameter for diagnosing isolated 17,20-lyase deficiency, such as 17OHP/AD >50. Detailed hormonal testing can demonstrate a partial loss of 17-hydroxylase activity, but usually not enough to cause hypertension or hypokalemia, and the nearly complete absence of androgens and estrogens dominates the clinical presentation.

A few cases of isolated 17,20-lyase activity have been confirmed with molecular genetic and biochemical studies. Patients homozygous for mutations R358Q and R347H have been identified in Brazil, and others heterozygous for a null allele and one copy of R347C were described in the Netherlands. These residues, R347 and R358, are both located on the redox-partner-binding surface of the CYP17A1 enzyme. Consistent with the known dependence of the 17,20-lyase activity on the interaction of CYP17A1 with redox partners POR and cytochrome b_5, these mutations at this binding surface appear to preferentially impair 17,20-lyase activity. In contrast, a large kindred from Israel with isolated 17,20-lyase activity carries the homozygous mutation E305G, which is found in the active site and impairs 17Preg binding.

Mutations in the *CYB5A* gene encoding cytochrome b_5 also cause isolated 17,20-lyase deficiency, with or without congenital methemoglobinemia. A deletion in the *CYB5A* gene was described in an undervirilize male, but hormonal analyses were not performed. Subsequently, individuals homozygous for mutations W27X or H44L were identified and characterized endocrinologically, which confirmed isolated 17,20-lyase deficiency. H44 is one of the axial histidine ligands for the heme prosthetic group of cytochrome b_5, and the stop codon at W27 yields a small peptide missing the critical residues for CYP17A1 stimulation. A third genetic form is described in the section on POR deficiency below.

Gonadectomy is recommended in 46,XY subjects raised as females and gender-appropriate sex steroid replacement will be necessary at the time of puberty. Optimal management in 46,XX subjects has not been established.

P450 Oxidoreductase (POR) Deficiencies

POR is a membrane-bound flavoprotein widely expressed in human tissues, which serves as a reductase for all microsomal P450 enzymes, including the steroid hydroxylases CYP17A1 (P450 17A1), CYP21A2 (P450 21A2), and aromatase (CYP19A1, P450 19A1). POR transfers two electrons from NADPH to the receiving enzymes.

Patients with POR deficiency may exhibit features similar to deficiencies of CYP17A1, CYP21A2, and aromatase, in various combinations and severities. Patients of both sexes may present with ambiguous genitalia, similar to 3βHSD deficiency. The function of 17,20-lyase is predominantly disrupted by POR defects, resulting in low C_{19} steroids and consequently in underdeveloped genitalia in 46,XY fetuses. Homozygous mutation G539R yields a steroid profile very similar to isolated 17,20-lyase deficiency due to mutations in *CYP17A1* or *CYB5A*. POR mutations that simultaneously disrupt aromatase function prevent the placental conversion of fetal C_{19} steroids to estrogens, thus contributing to maternal virilization. Furthermore, excessive 17OHP accumulation upstream from the enzymatic dysfunction can be diverted via the so-called "backdoor pathway" to the most potent androgen, DHT, which is generated bypassing AD and T. This DHT production causes the paradoxical virilization of 46,XX cases despite low C_{19} steroid production postnatally.

POR deficiency should be suspected when clinical and hormonal evidence support dysfunction of multiple P450 enzymes, most commonly of CYP17A1 and CYP21A2. Additionally, these patients may present skeletal malformations, such as craniosynostosis, radiohumeral or radio-ulnar synostosis, and femoral bowing, as part of the Antley–Bixler syndrome (ABS) or other eponymic conditions. ABS cases with disordered steroidogenesis all have POR mutations and autosomal recessive inheritance. Approximately 50% of ABS cases lack steroidogenesis defects and derive from gain-of-function mutations in fibroblast growth factor receptor type 2 (*FGFR2*), with autosomal dominant inheritance.

Two missense mutations account for most POR deficiencies: A287P, encountered in patients of European ancestry, and R457H, the most common mutation in Japan and Korea. Deficiency of aromatse has been associated with mutation R457H but not mutation A287P.

Defects Affecting Only Testosterone and Dihydrotestosterone Production

The final two genetic disorders discussed involve the terminal steps of T and DHT biosynthesis. These two conditions are unique in that only males (46,XY) experience clinical manifestations that are solely due to androgen deficiency in utero. Furthermore, other enzymes partially compensate for the genetic deficiencies, but only at puberty.

17β-Hydroxysteroid Dehydrogenase Type 3 (17βHSD3) Deficiency

The human genome contains several 17βHSD isoforms, but 17βHSD3 is the enzyme that is defective in the clinical entity "17βHSD deficiency," also known as "17-ketosteroid reductase deficiency." The 17βHSD3 enzyme catalyzes the conversion of C_{19}, 17-ketosteroids to 17β-hydroxysteroids using NADPH as cofactor: AD to T, DHEA to Δ^5-androstenediol, 5α-androstanedione to DHT, and 5α-androsterone to 5α-androstanediol. Because 17βHSD3 is expressed exclusively in the testes, the loss of this enzyme impairs androgen biosynthesis only in males.

Most affected 46,XY individuals with 17βHSD3 deficiency have predominantly female external genitalia with a blind vaginal pouch. Surprisingly, Wolffian derivatives, such as the epididymis, vas deferens, seminal vesicles, and ejaculatory duct, are present, suggesting that an alternate pathway in these tissues enables some testosterone production, perhaps mediated by the 17βHSD type 5 isoform. Testes are usually located in the inguinal canal, and Müllerian structures are absent.

Most of these children are raised as females. At puberty, testicular AD production increases, and significant extraglandular conversion of this AD to T elicits marked physical changes. The phallus enlarges and can reach lengths of 4–8 cm; the voice deepens, male pattern body hair develops, and muscle mass increases. Several affected individuals have changed gender role from female to male in adolescence because of the prominent physical and psychological masculinization they experience. GnRH agonist therapy will stop these changes. In contrast, 17βHSD3 is not expressed in the human ovary, so 46,XX patients with this disorder are asymptomatic.

The diagnosis is based on markedly elevated AD concentrations in the face of low T in the neonatal period or in adolescence. The discrepancy in the AD/T ratio is accentuated with HCG stimulation. In the past, affected 46,XY males were frequently raised as females and underwent castration followed by estrogen substitution therapy at puberty, but infants with adequate phallic structures and mild hypospadias may be reared as males and undergo genitoplasty if necessary. This approach anticipates the tendency for gender reversal associated with virilization at puberty. Even within members of a kindred with identical genotypes, however, affected individuals vary in their decisions about gender reversal at puberty when reared initially as females. If the patient is reared as a male, T replacement at puberty might be necessary to achieve full masculinization and to prevent the development of gynecomastia. Spermatogenesis is absent if the deficiency is complete or nearly complete, due to lack of intratesticular T synthesis.

Most mutations in the gene for 17βHSD3 are located on exon 9 and impair all enzyme functions. One common mutation, identified in both Brazilian and Palestinian subjects, is R80Q, which lies in the nucleotide-binding domain and primarily disrupts the binding of cofactor but not of steroid. Genotyping is often necessary to establish the diagnosis when steroid testing is ambiguous or if gonadectomy has been performed.

5α-Reductase Type 2 (SRD5A2) Deficiency

The disorder 5α-reductase deficiency (also known as pseudo-vaginal perineoscrotal hypospadias) provides strong genetic evidence that DHT is required for complete formation of the male external genitalia in human beings. The 5α-reductases catalyze the conversion of T to its 5α-reduced metabolite DHT, and the type 2 isoform executes this transformation in the prostate and genital skin. Consequently, 46,XY male infants with a deficiency in the type 2 enzyme are born with hypospadias and a phallic structure that resembles an enlarged clitoris, often bound in chordee. The urogenital sinus with a blind vaginal pouch opens on the perineum, and the scrotum is bifid, with testes located in the inguinal canal or labioscrotal folds. With the abundance of T, Wolffian structures are well differentiated, and Müllerian structures are absent. The ejaculatory ducts terminate in the blind vaginal pouch or onto the perineum next to the urethra, and the prostate is hypoplastic.

As is the case in 17βHSD3 deficiency, masculinizing changes occur at puberty as circulating T concentrations rise into the normal adult male range. The voice deepens, muscle mass increases, the phallus grows to 4–8 cm, and the subject may experience erections. The testes enlarge, the scrotal structure becomes rugated and pigmented, and spermatogenesis may occur, but is often impaired from cryptorchidism. Facial hair and body hair are sparse and acne and temporal hair recession do not occur, presumably because DHT production is low. As in 46,XY infants with 17βHSD3 deficiency, most 46,XY individuals with 5α-reductase deficiency are reared as females but reverse gender role with the masculinizing changes of puberty, and in some cultures where the disorder is endemic, this process has achieved a socially acceptable status. However, unlike 17βHSD3 deficiency, gynecomastia does not develop. The 46,XX females with 5α-reductase deficiency are phenotypically normal at birth, but at puberty they have decreased body and pubic hair and delayed menarche yet normal fertility.

Diagnostic testing includes measurement of serum T and DHT, and a T/DHT ratio >30 unambiguously confirms the diagnosis. One pitfall of testing is that after puberty, the activity of the type 1 isozyme may provide measurable levels of DHT, emphasizing the importance of the T/DHT ratio. In contrast, a T/DHT of 8.5 should be used to increase the sensitivity in early infancy, when androgen levels are low compared with puberty. Although not always necessary, molecular diagnostics of the *SRD5A2* gene aids in making the diagnosis definite in selected cases, particular when androgens are low and testing is equivocal. Analysis of urinary steroids using gas chromatography/mass spectrometry is also used for diagnosis, even following gonadectomy, using the ratio of 5α-/5β-reduced cortisol metabolites.

Treatment of 5α-reductase deficiency is DHT therapy, often applied as a cream to the genitalia, to increase phallic length and to facilitate hypospadias repair. Supraphysiologic dosing of T in adults may generate adequate DHT via a partially functional type 2 enzyme and/or via the type 1 isoform.

Approximately 40% of children with 5α-reductase deficiency are born to consanguineous parents; uniparental disomy has also been described. The founder mutation R246W is prevalent in the Dominican Republic, where the disease is common, but other mutations are found in other kindreds there. A 20 kb deletion that includes the *SRD5A2* gene is prevalent in Papua New Guinea, and an A insertion into amino acid 251 causes 5α-reductase deficiency in a Turkish cluster. Several mutations are found in Brazil, and Q126R is the most common. The type 1 isozyme is expressed in the liver up to 2–3 years of age and in nongenital skin. Mutations in the type 1 isozyme have not been described.

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Reproductive and Nonreproductive Actions of Testosterone

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Introduction

Testosterone is an important regulator of male sexual differentiation, reproductive and nonreproductive behaviors, development of secondary sex characteristics, spermatogenesis, muscle and bone mass, erythropoiesis, and metabolism (Bhasin and Jameson, 2018; Bhasin *et al.*, 2018). Testosterone may exert its biologic effects directly by itself or indirectly through its conversion to its two major metabolites: 5α dihydrotestosterone (DHT) and estradiol 17β (Bhasin and Jameson, 2018; Bhasin *et al.*, 2018). The beneficial effects of testosterone on bone resorption, high-density lipoprotein cholesterol, atherosclerosis progression, sexual desire, and fat mass require its conversion by CYP19A1 (aromatase) to estradiol 17β (Bhasin and Jameson, 2018; Bhasin *et al.*, 2018; Finkelstein *et al.*, 2013, 2016; Vanderschueren *et al.*, 2014). Conversion of testosterone to DHT by a family of steroid 5α-reductase enzymes is obligatory for mediating its effects on the differentiation of the urogenital sinus and the genital tubercle (Bhasin and Jameson, 2018; Bhasin *et al.*, 2018). Testosterone's conversion to DHT also is believed to mediate its effects on hair growth, scalp hair loss, prostate growth, and acne; however, it is not clear whether this conversion is obligatory for mediating its effects on the prostate, skin, and the hair follicle (Bhasin and Jameson, 2018; Bhasin *et al.*, 2012, 2018). Testosterone's conversion to DHT is not required for testosterone's effects on muscle and bone, plasma lipids, and erythropoiesis (Bhasin *et al.*, 2012; Page *et al.*, 2005; Amory *et al.*, 2004).

Testosterone and the Androgen Receptor

Many actions of testosterone and DHT, including its effects on sexual differentiation of urogenital structures, spermatogenesis, muscle and bone, and erythropoiesis, are mediated through the classical androgen receptor (AR) (Bhasin and Jameson, 2018; Bhasin *et al.*, 2018). As a member of the nuclear receptor superfamily, AR is a 919 amino-acid (aa), 110 kDa, ligand-inducible intracellular transcription factor that mediates most of the biological effects of testosterone and its androgenic derivatives, including DHT (Roy *et al.*, 2001; Tan *et al.*, 1988; Jenster *et al.*, 1991; Trapman *et al.*, 1988). AR possesses three characteristic structural domains: ligand binding domain (LBD, CTD), DNA binding domain (DBD), and transactivation domain or N-terminus domain (NTD) (Roy *et al.*, 2001; Tan *et al.*, 1988; Jenster *et al.*, 1991; Trapman *et al.*, 1988), The LBD in the C-terminus (690–919 aa) is highly conserved and confers ligand specificity. The DBD (545–675 aa) also is well conserved and binds to the androgen response elements or other transcription factors bound to the androgen response elements in the AR promoter. The NTD (1–514 aa) is not well conserved, has been difficult of crystallize, and contains several unstructured domains. Unliganded AR is primarily located in the cytoplasm; in response to ligand binding, AR undergoes distinct conformational changes, leading to recruitment of co-regulators and chaperone proteins, translocation to the nucleus, dimerization, chromatin remodeling, DNA binding and eventual regulation of transcriptional activity of AR-responsive genes (Kuil and Mulder, 1994, 1995; Tyagi *et al.*, 2000; Kemppainen *et al.*, 1992; Trapman *et al.*, 1990). The three domains of AR act in concert to conformationally gate the response to agonist or antagonist binding.

Crystal structure of C-terminus LBD of the nuclear hormone superfamily displays highly conserved arrangement of 12 α helices (Sack *et al.*, 2001; Pereira de Jésus-Tran *et al.*, 2006; Bohl *et al.*, 2007). While AR contains 11 helices, they are still numbered from 1 to 12 (helix 2 is skipped) to reflect the shared functional organization with helix 12 acting as a mobile, conformational gate. Ligand binding is associated with significant rearrangement in the immediate vicinity of the involved residues as well as in distant residues in other domains. The LBD is attached to the 80 aa DBD organized in two zinc-coordinated, tetra-cys sequence repeats through a hinge region (Verrijdt *et al.*, 2003, 2006). The DBD binds with high affinity to the androgen response elements (AREs) to trigger transcriptional activity. While AR shares affinity for consensus reads 5′-TGTTCT-3′ (clARE) with other nuclear receptors, such as GR and PR, it also has selective ARE (selARE), including PB-ARE2 and Slp-HRE2. These interactions of the DBD and ARE regulate the recruitment of enhancers/promoters to the macromolecular transcriptional assembly (Cato *et al.*, 1987; Schoenmakers *et al.*, 2000). The crystal structures of liganded LBD and DBD have been described, but no crystallographic or solution NMR structures have been solved for the NTD or its fragments. It is conceivable that the conformational flexibility of NTD enables intra- and interdomain steric interactions.

AR utilizes two activation function regions located in the N-terminal domain (AF-1) and the C-terminal LBD (AF-2). These surfaces regulate NTD/CTD interaction as well as the recruitment of co-activator and co-repressor proteins in a ligand-specific manner (Heinlein and Chang, 2002; Bevan and Parker, 1999; Heemers and Tindall, 2007; Estébanez-Perpiñá *et al.*, 2007). The AR

AF-2 plays two functional roles: (a) in the unliganded state, it associates with FxxLF (residues 23–27), WxxLF (residues 433–437) motifs observed in the NTD to keep the AR in an inactive conformation; and (b) in the ligand-bound state, it recruits coregulators that possess LxxLL and FxxLF motifs, including p160 family, ARA55, ARA55, ARA70, NCoRs, and SRCs. Selective recruitment of the coregulators elicits ligand-specific, AR mediated transcriptional activity. Within the AR-AF1, two transcription activation units, Tau-1 and Tau-5 have been described (Jenster *et al.*, 1995). The presence of both Tau-1 and Tau-5 is obligatory and sufficient for activity of the isolated AR-NTD and the full-length AR; mutations in Tau-1/Tau-5 render the AR protein inactive.

Collectively, the transcriptional activity of AR is regulated by a spatiotemporal interplay between domains and motifs. Ligand binding and coregulator protein-induced conformational dynamics enable the domains to exhibit multiple functional roles.

Modulation of Testosterone' Bioavailability and Action by Binding Proteins

Circulating testosterone is bound with high affinity to sex hormone–binding globulin (SHBG) (30%–45% in men and 70% in women) and with substantially lower affinity to albumin (50%–68% in men and ~25% in women), cortisol binding globulin, and orosomucoid; only 1%–4% of circulating testosterone is in the unbound or free form (**Fig. 1**) (Bhasin and Jameson, 2018; Bhasin *et al.*, 2018; Goldman *et al.*, 2017; Zakharov *et al.*, 2015). Free testosterone refers to the circulating unbound fraction of testosterone; according to the free hormone hypothesis, this is the fraction of circulating testosterone that is able to cross the cell membrane and exert biologic effect. The term bioavailable testosterone refers to the fraction of circulating testosterone that is not bound to SHBG, and reflects the view that albumin-bound testosterone may dissociate at the capillary level, especially in tissues and organs with long transit time such as the liver and brain, and become "bioavailable" (Bhasin and Jameson, 2018; Bhasin *et al.*, 2018; Goldman *et al.*, 2017). Under certain situations, SHBG may transport testosterone intracellularly through a megalin-mediated endocytic process (Goldman *et al.*, 2017). The binding proteins may regulate the transport, bioavailability, and clearance of testosterone and modulate its biologic effects.

The Effects of Testosterone on Sexual Function

Testosterone regulates many domains of sexual function, including sexual desire; spontaneous sexual thoughts and fantasies; attentiveness to erotic cues; the duration, magnitude, and frequency of nocturnal penile erections; volume of ejaculate; and, overall sexual activity (**Table 1**) (Bhasin *et al.*, 2007; Bhasin and Basson, 2016). Davidson and colleagues established that the primary role of testosterone is to increase sexual motivation and thoughts; their studies demonstrated that hypogonadal men experienced fewer sexual thoughts and fantasies, fewer spontaneous erections, and lower overall sexual activity than eugonadal men (Kwan *et al.*, 1983). However, hypogonadal men and eugonadal men exhibited similar erectile responses to visual erotic stimuli. When androgen-deficient men were treated with testosterone, the frequency of sexual fantasies and thoughts, number of spontaneous erections, and overall sexual activity increased (Kwan *et al.*, 1983). However, their erectile response to visual erotic stimuli did not change. These pioneering experiments by Davidson led to the prevalent view that spontaneous but not stimulus-induced erections are androgen-dependent. The primary role of testosterone is to increase sexual motivation and thoughts (*libido*).

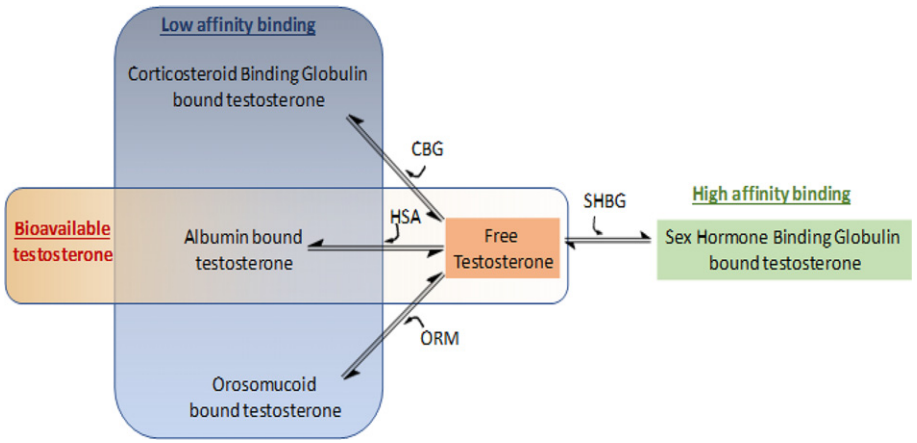

Fig. 1 The regulation of the bioavailability of circulating testosterone by its cognate binding proteins. Most of the circulating testosterone is bound to at least one of four binding proteins: sex-hormone binding globulin (SHBG), albumin, cortisol binding globulin, and albumin, and only 2%–4% of circulating testosterone is free. Testosterone binds with high affinity to SHBG and with substantially lower affinity to albumin, CBG, and orosomucoid. The term bioavailable refers to that fraction of circulating testosterone which is not bound to SHBG and reflects the belief that non-SHBG bound testosterone, being bound with low affinity to albumin can dissociate at the capillary level and become "bioavailable."

Table 1 Effects of testosterone on sexual function in men

- Increases the frequency of spontaneous sexual thoughts and sexual fantasies
- Increases attentiveness to erotic cues
- Increases the frequency, duration, and magnitude of nighttime erections
- Increases the frequency of spontaneous erections
- Increases overall sexual activity
- Improves libido, erectile function, satisfaction with intercourse, and overall sexual activity in hypogonadal men

Randomized large trials of testosterone have shown that testosterone treatment of hypogonadal men with low libido improves libido, erectile function, and overall sexual activity more than placebo (Steidle *et al.*, 2003; Brock *et al.*, 2016; Cunningham *et al.*, 2016; Snyder *et al.*, 2016); systematic reviews and meta-analyses have confirmed these findings (Corona *et al.*, 2014; Boloña *et al.*, 2007; Jain *et al.*, 2000). Testosterone does not improve sexual function and activity in men who do not have low testosterone levels (Basaria *et al.*, 2015).

Testosterone replacement therapy does not improve ejaculatory function in men with low testosterone levels and ejaculatory dysfunction (Paduch *et al.*, 2015). There have been no randomized trials of testosterone in men with orgasmic disorders.

Testosterone regulates a number of processes that culminate in penile erection (Lugg *et al.*, 1995; Prigo-Rocha *et al.*, 1993; Chamness *et al.*, 1995; Giuliano *et al.*, 1993; Reilly *et al.*, 1997a,b; Heaton and Varrin, 1994; Shabsigh *et al.*, 1998; Mills and Lewis, 1999; Mills *et al.*, 1998). Testosterone regulates nitric oxide synthase expression and activity in the cavernosal smooth muscle of rats (Lugg *et al.*, 1995; Prigo-Rocha *et al.*, 1993; Chamness *et al.*, 1995). There is additional evidence that non-NO-dependent pathways may also be androgen sensitive in the erectile response (Giuliano *et al.*, 1993; Reilly *et al.*, 1997a,b; Heaton and Varrin, 1994). Androgens exert a trophic effect on cavernosal smooth muscle (Shabsigh *et al.*, 1998). Castration results in impaired erectile response to central and peripheral stimulation and a decrease in penile tissue concentration of nitric oxide synthase-containing nerves; testosterone replacement reverses these abnormalities (Heaton and Varrin, 1994). Testosterone has trophic effects in maintaining the mass of the bulbospongiosus and ischiocavernosus muscles; the contraction of these muscles contributes to the achievement of peak rigidity just prior to ejaculation (Mills and Lewis, 1999). Testosterone enhances blood flow into the penis (Mills and Lewis, 1999; Mills *et al.*, 1998; Aversa *et al.*, 2003). Furthermore, during ganglionic stimulation, veno-occlusion fails to occur in castrated rats (Mills *et al.*, 1998; Fournier *et al.*, 1987). Collectively, these data indicate that testosterone plays an important role in regulating erectile mechanisms at multiple levels.

Androgen deficiency and erectile dysfunction are two independently distributed conditions, each with a distinct pathophysiology. However, the two conditions often overlap, particularly in older individuals and in those with comorbid conditions (Korenman *et al.*, 1990). Phosphodiesterase 5 inhibitors can improve erectile function in eugonadal and hypogonadal men (Tsertsvadze, 2009). Randomized controlled trials have failed to demonstrate further improvements in erectile function with the addition of testosterone to an optimized regimen of phosphodiesterase 5 inhibitors (PDE5I) (Spitzer *et al.*, 2012; Buvat *et al.*, 2011). However, we do not know whether testosterone replacement therapy would improve subsequent erectile response to PDE5Is.

Anabolic Effects on the Skeletal Muscle (Table 2)

Testosterone is a potent anabolic hormone which increases skeletal muscle mass, maximal voluntary muscle strength, and muscle power in healthy men (Bhasin *et al.*, 1996, 2001). Testosterone replacement also increases fat-free mass and maximal voluntary strength, and decrease fat mass in healthy hypogonadal men, HIV-infected men with weight loss, and in men with chronic obstructive lung disease or end stage renal disease (Bhasin *et al.*, 1997, 2000, 2005a; Grinspoon *et al.*, 1998; Casaburi *et al.*, 2004; Wang *et al.*, 2000). Randomized clinical trials in middle-aged and older men with low or low-normal testosterone concentrations have also demonstrated greater gains in lean body mass and grip strength with testosterone administration compared with placebo (Bhasin *et al.*, 2005b; Snyder *et al.*, 1999; Page *et al.*, 2005; Kenny *et al.*, 2001, 2002; Blackman *et al.*, 2002; Basaria *et al.*, 2010; Travison *et al.*, 2011; Srinivas-Shankar *et al.*, 2010; Storer *et al.*, 2008, 2017; Nair *et al.*, 2006; Emmelot-Vonk *et al.*, 2008; Liverman and Blazer, 2004). The loss of fat mass induced by testosterone therapy is distributed evenly between appendicular and trunk compartments and within each of those compartments between superficial subcutaneous, and deep intramuscular, and visceral fat compartments (Woodhouse *et al.*, 2004).

In spite of a clear demonstration of the gains in muscle mass and strength by testosterone administration, the effects of testosterone replacement on performance-based measures of physical function have been inconsistent across studies. In older men with mobility limitation, testosterone replacement improves VO_{2peak} (Storer *et al.*, 2016), self-reported function, and some performance-based measures of physical function that are more closely related to lower extremity muscle strength and power, such as stair climbing speed and power (Basaria *et al.*, 2010; Travison *et al.*, 2011; Storer *et al.*, 2008). However, testosterone's treatment effect on 6-min walking speed and distance in randomized trials has been small, and its clinical meaningfulness remains unclear (Snyder *et al.*, 2016).

Testosterone-induced increase in muscle strength is related to the gains in muscle mass; testosterone does not improve the specific force of the muscle (Storer *et al.*, 2003). Thus, testosterone administration does not improve the contractile properties of the skeletal muscle. Testosterone administration induces muscle fiber hypertrophy and increases the cross-sectional area of both type 1 and type 2 skeletal muscle fibers; testosterone does not change the absolute number of type 1 or type 2 muscle fibers or their relative proportion (Sinha-Hikim

Table 2 Effects of testosterone on the skeletal muscle

Effects on skeletal muscle histomorphology

- Hypertrophy of type 1 and 2 muscle fibers
- Increased mitochondrial mass and quality
- Increased number of myonuclei and satellite cells

Effects on body composition

- Increase in whole body, appendicular, and trunk lean mass
- Decrease in whole body, subcutaneous, intermuscular, and abdominal fat

Effects on measures of skeletal muscle performance

- Increased maximal voluntary muscle strength in both upper and lower extremity muscle groups
- Increased muscle power
- No change in specific force

Effects on aerobic performance

- Attenuation of age-related decline in VO_{2peak}

Effects on self-reported and performance-based measures of physical function

- Improvements in self-reported function, including perception of walking ability
- Improved stair climbing speed and power
- Small improvement in 6-min walking distance

et al., 2002). Testosterone increases the number of myonuclei and muscle satellite cells, but it does not alter the myonuclear domain (Sinha-Hikim *et al.*, 2003). Thus, the primary action of testosterone is to induce skeletal muscle fiber hypertrophy by increasing the number of satellite cells.

Testosterone promotes the differentiation of mesenchymal progenitor cells into the myogenic lineage and inhibits their differentiation into the adipogenic lineage (Bhasin *et al.*, 2003; Sinha-Hikim *et al.*, 2006; Singh *et al.*, 2003, 2006). In addition, testosterone has been postulated to improve net muscle protein balance by stimulating muscle protein synthesis, decreasing muscle protein degradation, and improving the reutilization of amino acids (Ferrando *et al.*, 2002; Urban *et al.*, 1995). Testosterone stimulates circulating growth hormone secretion and IGF-1 levels although circulating IGF-1 is not obligatory for mediating testosterone's effects on the skeletal muscle (Serra *et al.*, 2011).

Testosterone's anabolic effects on the skeletal muscle are mediated largely through the classical AR and are blocked by AR antagonists. Testosterone's binding to AR leads to a conformational change in AR, enables it to associate with other co-regulators, including β catenin, stabilizing the latter (Singh *et al.*, 2009). The liganded AR along with β catenin and other associated chaperon proteins translocate into the nucleus and binds to the transcription factor TCF-4 (**Fig. 2**) (Singh *et al.*, 2009). The quaternary complex then binds specific DNA regions to regulate the transcription of Wnt-target genes including follistatin, which blocks the action of several members of the TGF β family (Singh *et al.*, 2009; Braga *et al.*, 2012), including myostatin and activins (**Fig. 2**). Follistatin plays an important role in mediating the anabolic effects of testosterone on the muscle; blocking follistatin action abrogates testosterone's effects on myogenic differentiation (Braga *et al.*, 2012). The administration of recombinant follistatin (rFst) increases muscle mass in mice, but has no effect on prostate mass (Jasuja *et al.*, 2014). Microarray analysis of mRNAs from prostate and levator ani of castrated male mice treated with vehicle, testosterone, or rFst revealed that testosterone and rFst shared the regulation of many transcripts in levator ani; however, in prostate, 593 transcripts in several growth-promoting pathways were differentially expressed after testosterone treatment, while rFst showed a negligible effect with only nine transcripts differentially expressed. Among pathways that were differentially responsive to testosterone in prostate, we identified ornithine decarboxylase (Odc1), an enzyme in polyamine biosynthesis, as a testosterone-responsive gene that is unresponsive to rFst (Jasuja *et al.*, 2014). When we administered testosterone with and without α-difluoromethylornithine (DFMO), an Odc1 inhibitor, to castrated mice, DFMO selectively blocked testosterone's effects on prostate, but did not affect testosterone's anabolic effects on the muscle (Jasuja *et al.*, 2014). Thus, co-administration of testosterone and an Odc1 inhibitor offers a novel therapeutic strategy for prostate-sparing anabolic therapy (Jasuja *et al.*, 2014).

Testosterone's Effects on Erythropoiesis

Testosterone administration increases hemoglobin, hematocrit, and red cell mass (Coviello *et al.*, 2008; Calof *et al.*, 2005), and has been used to treat anemia of inflammation and chronic kidney disease. Testosterone also is efficacious in correcting unexplained anemia of aging (Roy *et al.*, 2017) and inflammation (Guo *et al.*, 2016). The erythropoietic effects of testosterone are related to testosterone dose, circulating testosterone concentrations, and age (Coviello *et al.*, 2008). Older men experience greater increments in hemoglobin and hematocrit than younger men, after adjusting for testosterone dose (Coviello *et al.*, 2008). Erythrocytosis is the

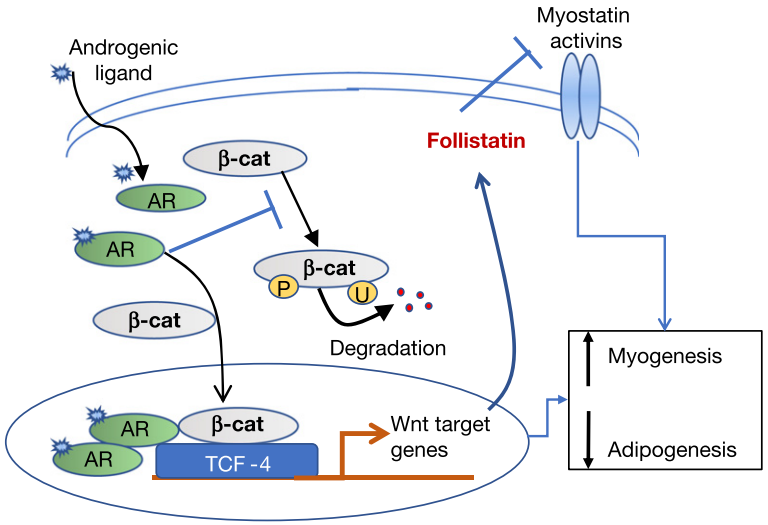

Fig. 2 The molecular mechanisms by which testosterone regulates the differentiation of mesenchymal progenitor cells through activation of Wnt signaling. The effects of testosterone on mesenchymal progenitor cell differentiation are mediated largely through the activation of Wnt signaling. After ligand binding, the androgen receptor undergoes conformation change and binds to beta catenin, stabilizing the latter, and preventing its degradation. The AR-beta catenin complex which may include additional chaperone proteins translocated into the nucleus and after binding TCF-4 regulates a number of Wnt-target genes, including follistatin, which blocks the action of a number of TGF β family members, such as myostatin and activins. Follistatin plays an important role in mediating the regulation of mesenchymal progenitor cell differentiation into the myogenic lineage and inhibiting their differentiation into adipogenic lineage.

most frequent adverse event noted in testosterone trials, and is an important dose-limiting adverse effect of testosterone therapy (Calof *et al.*, 2005).

The mechanisms by which testosterone increases hemoglobin and hematocrit are incompletely understood. Testosterone stimulates iron-dependent erythropoiesis through multiple mechanisms (Guo *et al.*, 2013). Testosterone increases iron availability and iron incorporation into the red cells by inhibiting hepcidin (Guo *et al.*, 2013; Bachman et al., 2010, 2014). Testosterone inhibits hepcidin transcription through its interaction with BMP-Smad signaling (Guo *et al.*, 2013). Testosterone administration promotes the association of liganded-AR with Smad1 and Smad4 to reduce their binding to BMP-response elements in the hepcidin promoter in the liver (Guo *et al.*, 2013). Testosterone increases erythropoietin production and resets the erythropoietin to hemoglobin set point (Bachman *et al.*, 2014). In older mice, testosterone may also improve red cell survival (Guo *et al.*, 2014).

Effects on Bone Metabolism

Sex steroids are important contributors to sex differences in bone mass and strength, and fracture risk (Vanderschueren *et al.*, 2014; Khosla *et al.*, 1998; Riggs *et al.*, 2004). Men have greater bone mass and strength than women in part because of the greater cortical bone acquisition during puberty (Vanderschueren *et al.*, 2014). Testosterone increases bone mass and strength through multiple pathways either by binding to the AR itself or indirectly through its conversion to estradiol 17β and signaling through the estrogen receptor α (ERα) (Finkelstein *et al.*, 2016; Vanderschueren *et al.*, 2014). AR is expressed in the osteoblastic and osteoclastic lineages, and by chondrocytes (see Vanderschueren *et al.*, 2014 for an excellent review of this topic, Kasperk *et al.*, 1989). Testosterone promotes apoptosis of osteoclasts and exerts antiapoptotic effects on osteoblasts and osteocytes (Kousteni *et al.*, 2001). Testosterone regulates the differentiation of bone progenitor cells into osteoblasts and osteoclasts and reduces the number of bone remodeling cycles (Khosla *et al.*, 1998; Kasperk *et al.*, 1990). Testosterone directly stimulates periosteal bone formation; additionally, genetic studies suggest that testosterone also regulates trabecular bone via AR in osteoblasts and osteocytes, independent of its aromatization to estradiol (Callewaert *et al.*, 2009; Moverare *et al.*, 2003; Venken *et al.*, 2006). Thus, AR actions, independent of its aromatization, contribute to greater periosteal bone formation and enhanced trabecular bone development during puberty in male mice (Callewaert *et al.*, 2009; Moverare *et al.*, 2003; Venken *et al.*, 2006). Estradiol acting via ERα in osteoblast lineage cells regulates cortical as well as the trabecular bone mass (Vanderschueren *et al.*, 2014; Bord *et al.*, 2001; Syed *et al.*, 2011; Venken *et al.*, 2006). Activation of ERα signaling, the dominant mediator of estrogen actions in bone, stimulates periosteal and trabecular bone formation and inhibits endocortical and trabecular bone resorption in female mice (Vanderschueren *et al.*, 2014; Bord *et al.*, 2001; Syed *et al.*, 2011; Venken *et al.*, 2006). Thus, both AR and ERα actions play a role in male cortical and trabecular peak bone mass acquisition (Vanderschueren *et al.*, 2014; Kousteni *et al.*, 2001; Kasperk *et al.*, 1990; Callewaert *et al.*, 2009; Moverare *et al.*, 2003; Venken *et al.*, 2006; Bord *et al.*, 2001; Syed *et al.*, 2011).

Testosterone also regulates bone mass and bone strength indirectly through its anabolic effects on muscle mass and muscle strength (Vanderschueren *et al.*, 2014). Increase in muscle mass and strength may also reduce fall propensity and reduce fracture risk.

Testosterone replacement therapy in severely hypogonadal improves areal bone mineral density (aBMD) as well as volumetric BMD (vBMD) (Snyder *et al.*, 2000; Katznelson *et al.*, 1996; Benito *et al.*, 2005; Zhang *et al.*, 2008; Al Mukaddam *et al.*, 2014). Testosterone treatment of hypogonadal men improves trabecular architecture and estimated bone strength (Zhang *et al.*, 2008; Al Mukaddam *et al.*, 2014; Snyder *et al.*, 2017). Meta-analyses of randomized trials of testosterone in older men with low or low normal levels have reported improvements in vertebral aBMD (Tracz *et al.*, 2006). In community-dwelling older men, 65 years or older, who had unequivocally low testosterone levels and symptoms of low libido, mobility problems or low vitality, testosterone treatment for 1 year increased vBMD of trabecular bone in the spine more than placebo and increased estimated bone strength of trabecular bone in the spine more than placebo (Snyder *et al.*, 2017). Testosterone also significantly increased whole bone vBMD and strength of the spine and trabecular and whole bone vBMD and strength of the hip (Snyder *et al.*, 2017). Testosterone's efficacy in reducing fracture risk in men with osteoporosis has not been studied.

Effects on Mood and Depressive Symptoms

In the TTrials, testosterone treatment had a small positive effect on mood and depressive symptoms (Snyder *et al.*, 2016). The magnitude of each of these effects was small and their clinical meaningfulness remains unclear.

Multiple lines of evidence support the hypothesis that a major subset of persistent depressive disorder (PDD) in men—defined as mid-life-onset, low-grade, chronic depression—might be responsive to testosterone replacement (Seidman, 2003; Almeida *et al.*, 2008). Epidemiological studies have demonstrated an association between lower testosterone levels and chronic low-grade depression (Seidman *et al.*, 2002; Markianos *et al.*, 2007; Shores *et al.*, 2004). Testosterone, whether administered alone or as an augmentation strategy in conjunction with traditional antidepressant therapy, tends to be ineffective in men with major depressive disorder (MDD) (Seidman *et al.*, 2001, 2005; Orengo *et al.*, 2005; Pope *et al.*, 2010). However preliminary evidence suggests that testosterone therapy can improve depressive symptoms in men with low-grade, late-onset depression (Seidman *et al.*, 2009; Shores *et al.*, 2009; Bloch *et al.*, 1999). These data suggest that testosterone replacement might be efficacious as a treatment for this subgroup of men with late-onset, low-grade PDD (previously referred to as dysthymia in DSM-4), but not in men with MDD.

Testosterone's Effects on Reproductive and Nonreproductive Behaviors (Table 3)

Testosterone regulates the expression of many sexually dimorphic reproductive behaviors in rodents and higher mammals, including mounting and ejaculation (Fielder *et al.*, 1989; Bhasin *et al.*, 1988; Muller, 2017). Testosterone also influences territorial scent marking, and promotes aggression and competition among males of many mammalian species at the time of mate selection (Fielder *et al.*, 1989; Steensland *et al.*, 2005; Fischer *et al.*, 2007; Carrillo *et al.*, 2011; Le Greves *et al.*, 1997). Androgens induce both offensive and defensive behaviors in rats, hamsters, and nonhuman primates, and activate the associated signaling molecules and their receptors (Steensland *et al.*, 2005; Fischer *et al.*, 2007; Carrillo *et al.*, 2011; Le Greves *et al.*, 1997). Rats treated with androgens exhibit enhanced dominant behavior in a competition test compared to controls (Steensland *et al.*, 2005; Fischer *et al.*, 2007; Carrillo *et al.*, 2011; Le Greves *et al.*, 1997; Henderson *et al.*, 2006; Kindlundh *et al.*, 2003; Schwartzer and Melloni, 2010; Breuer *et al.*, 2001). The brain pathways associated with aggression include neural circuits that utilize signaling by excitatory amino acid systems (e.g., glutamate, GABA), and monoaminergic and peptidergic neurotransmitters (Fischer *et al.*, 2007; Carrillo *et al.*, 2011; Le Greves *et al.*, 1997; Henderson *et al.*, 2006; Kindlundh *et al.*, 2003; Schwartzer and Melloni, 2010). The key brain regions involved in aggressive behavior include the anterior hypothalamus, periaqueductal gray, and amygdaloid nuclei, particularly the central and medial amygdala. Androgens enhance ß endorphin in the VTA and may thereby activate the brain reward system (see Wood, 2008 for a review). Rats and mice display conditioned place preference to testosterone and male hamsters will self-administer testosterone to the point of death (Wood, 2008; Arnedo *et al.*, 2000). Interestingly, the opioid antagonist naltrexone can block testosterone self-administration in hamsters (Johansson *et al.*, 2010). These observations, combined with others, suggest that opioidergic mechanisms may be involved in the hedonic pathway to androgen dependence (Wood, 2008; Arnedo *et al.*, 2000; Johansson *et al.*, 2010).

Table 3 Effects of testosterone on reproductive and nonreproductive behaviors in mammals

Reproductive behaviors
• Promotes mounting and ejaculation, reduces latency to mount, and ejaculate and post-ejaculatory interval
• Promotes competition and aggression among males of many mammalian species
Nonreproductive behaviors
• Promotes territorial scent marking
• Induces offensive and defensive behaviors
• Enhances dominant behavior
• Conditioned place preference for testosterone

Testosterone Effects on Cognition

Sex differences between men and women in several domains of cognition have supported the hypothesis that testosterone exerts domain-specific effects on cognition (Maccoby and Jacklin, 1974). Although there are substantial interindividual differences in performance, as a group, on average, men perform better than women in tests of visuospatial cognition and women perform better than men in tests of verbal memory and verbal fluency (Maccoby and Jacklin, 1974; Shute *et al.*, 1983). Women with congenital adrenal hyperplasia with high testosterone levels score higher on tests of spatial cognition and individuals with androgen insensitivity syndrome perform worse on tests of spatial cognition than age- and sex-matched controls (Maccoby and Jacklin, 1974).

Androgen receptors are expressed in specific brain regions (Simerly *et al.*, 1990), and some organizational effects of testosterone on brain are mediated through AR-mediated mechanisms (Lustig, 1994). Androgens stimulate neurite arborization, facilitating intercellular communication (Lustig, 1994). Testosterone may also exert nongenomic effects and affect neurotransmitters such as serotonin, dopamine, acetylcholine, and calcium signaling (Fernández-Balsells *et al.*, 2010; Khaw and Barrett-Connor, 1992). The nongenomic effects of testosterone are less well characterized than genomic effects. Testosterone also is aromatized to estradiol within brain regions, and some effects of testosterone may be mediated through estrogen receptor. Testosterone's organizational effects on reproductive and nonreproductive behaviors during critical development windows of sexual differentiation have been well characterized in preclinical models.

Intervention trials have provided inconsistent data on the effects of testosterone therapy on verbal memory and visual-spatial cognition (Cherrier *et al.*, 2001). In older men with low testosterone levels, testosterone treatment compared with placebo did not improve delayed paragraph recall, visual memory, spatial ability, executive function, subjective memory complaints, or immediate paragraph recall (Resnick *et al.*, 2017). Testosterone also did not improve measures of cognition in older men with minimal cognitive impairment (Resnick *et al.*, 2017). It is not known whether testosterone can prevent progression from minimal cognitive impairment to dementia and retard the development of Alzheimer disease.

Testosterone's Effects on Metabolic and Cardiovascular Risk

We do not know whether testosterone replacement increases the risk of major adverse cardiovascular events in hypogonadal men (Liverman and Blazer, 2004; FDA, 2017; EMA, 2018). Testosterone exerts a number of physiologic effects, some of which may be beneficial and some potentially deleterious to the cardiovascular system (**Table 4**) (Liverman and Blazer, 2004). Testosterone acts a vasodilator by inhibition of L-type calcium channels, resulting in increased coronary and penile blood flow; testosterone acts like the dihydropyridine calcium channel blockers to reduce calcium influx into vascular smooth muscle, thereby promoting vasodilation (Scragg *et al.*, 2004). Testosterone decreases whole body, subcutaneous and intra-abdominal fat (Bhasin *et al.*, 2001; Woodhouse *et al.*, 2004); it reduces vascular reactivity and improves endothelial function; and, it shortens QTc intervals (Gagliano-Jucá *et al.*, 2017).

The potential deleterious effects of testosterone that could increase the risk of cardiovascular events, include the increase in hematocrit (Calof *et al.*, 2005; Fernández-Balsells *et al.*, 2010), a small but significant reduction in plasma HDL cholesterol (Fernández-Balsells *et al.*, 2010), induction of platelet aggregation presumably via stimulation of thromboxane A2, and sodium and water retention, which could contribute to edema formation or exacerbation of preexisting heart failure. In preclinical models, testosterone promotes smooth muscle proliferation and increases VCAM expression. Testosterone increases the levels of both prothrombotic as well as antithrombotic factors.

Testosterone has been shown to retard atherosclerosis in some preclinical models but not in others. Testosterone induces myocardial hypertrophy in some mouse strains, but not in others. Some randomized trials have reported increase in blood pressure, but these data are not consistent across trials.

The effects of testosterone on insulin sensitivity also are inconsistent across trials. Low total, but not free, testosterone levels have been associated with central adiposity, insulin resistance and diabetes risk (Khaw and Barrett-Connor, 1992; Haffner, 1996; Makhsida *et al.*, 2005; Muller *et al.*, 2004). Total testosterone concentrations are highly correlated with SHBG concentrations and SHBG concentrations are independently associated with the risk of diabetes and metabolic syndrome (Ding *et al.*, 2009; Bhasin *et al.*, 2011). In fact, free testosterone levels are either not associated or only weakly associated with metabolic risk (Bhasin *et al.*, 2011). In models of severe androgen deficiency, such as in men receiving androgen deprivation therapy, or men experimentally rendered hypogonadal by administration of GnRH agonists or antagonists, there is an acute worsening of insulin sensitivity (Pitteloud *et al.*, 2005). However, the effects of testosterone on measures of insulin sensitivity in intervention trials have been inconsistent in RCTs (Singh *et al.*, 2002).

The relationship of testosterone and coronary artery disease and cardiovascular events in cross-sectional and prospective cohort studies has been inconsistent (Ohlsson *et al.*, 2011; Srinath *et al.*, 2015; Khazai *et al.*, 2016; Corona *et al.*, 2011). Some epidemiologic studies have reported a negative relationship between testosterone concentrations and common carotid artery intima-media thickness, a measure of subclinical atherosclerosis (Srinath *et al.*, 2015; Khazai *et al.*, 2016).

Low total testosterone levels have been associated with higher risk of all-cause mortality, especially cardiovascular mortality (Araujo *et al.*, 2011). Epidemiological studies can only show association but cannot prove causality; reverse causality cannot be excluded. It is possible that testosterone is a marker of health, and those who are higher risk of dying have lower testosterone levels.

The number of venous thromboembolic events in randomized trials of testosterone have been too few to permit strong inferences. Some retrospective analyses have suggested an increased risk of venous thromboembolism during the first few months

Table 4 Potential beneficial and deleterious physiologic effects of testosterone on the cardiovascular system

Potentially beneficial effects

- Increased coronary blood flow
- Decreased whole body and abdominal fat mass
- Improved vascular reactivity
- Reduced QTc interval

Potentially deleterious effects

- Increased hematocrit
- Lowering of HDL cholesterol
- Increased platelet aggregation
- Increased vascular smooth muscle proliferation
- Salt and water retention

Inconclusive or inconsistent effects

- Myocardial hypertrophy in some strains but not others
- Retards early atherogenesis in some animal models but not in others
- Increases the circulating levels of both prothrombotic and antithrombotic factors
- No consistent increase in blood pressure in randomized trials

of testosterone therapy (Baillargeon *et al.*, 2015; Martinez *et al.*, 2016). Most cases of venous and arterial thrombotic events have occurred in men with preexisting hypercoagulable condition (Glueck *et al.*, 2016).

Several retrospective analyses of the association of testosterone administration with mortality and cardiovascular events have been published, but these studies have yielded conflicting results (Vigen, 2013; Sharma *et al.*, 2015; Anderson *et al.*, 2016; Muraleedharan *et al.*, 2013; Cheetham *et al.*, 2017; Borst *et al.*, 2014; Alexander *et al.*, 2017). These retrospective analyses of electronic medical records suffer from many limitations: the inclusion of heterogeneous study populations; differences in the duration of intervention; the use of variable definitions; and a lack of prospective ascertainment of cardiovascular outcomes (Vigen, 2013; Sharma *et al.*, 2015; Anderson *et al.*, 2016; Muraleedharan *et al.*, 2013; Cheetham *et al.*, 2017; Borst *et al.*, 2014; Alexander *et al.*, 2017). Treatments indications, treatment regimens, on-treatment testosterone levels and exposure differed among studies (Vigen, 2013; Sharma *et al.*, 2015; Anderson *et al.*, 2016; Muraleedharan *et al.*, 2013; Cheetham *et al.*, 2017; Borst *et al.*, 2014; Alexander *et al.*, 2017). They also suffered from a potential for residual confounding in that the patients assigned to testosterone therapy differed from comparators in baseline cardiovascular risk factors. A number of meta-analyses have examined the association between testosterone replacement and cardiovascular events, major cardiovascular events, and death in RCTs (e.g., Alexander *et al.*, 2017). Most of these meta-analyses have not shown a statistically significant association between testosterone and cardiovascular events, major cardiovascular events, or deaths (Alexander *et al.*, 2017). These meta-analyses are limited by the heterogeneity of randomized trials included in these analyses (Kloner *et al.*, 2016). The trials also were heterogeneous with respect to eligibility criteria, testosterone dose and formulation, and intervention durations. The variable quality of adverse event recording in clinical trials has been well-documented, and was particularly apparent in these trials. The small size of many trials and the inclusion of pilot studies with very small sample sizes was another constraint. Cardiovascular outcomes were not prespecified, they were often defined post-hoc, and were of varying clinical significance. The major cardiovascular events were not adjudicated, not specified prospectively, and the total number of major cardiovascular events was too small to draw strong inferences.

There are no published randomized trials that were long enough or large enough to determine the effects of testosterone therapy on CV events. In two RCTs in middle-aged and older men, testosterone replacement therapy did not affect the rate of atherosclerosis progression assessed using common carotid artery intima-media thickness or coronary artery calcium scores (Basaria *et al.*, 2015; Budoff *et al.*, 2017). In the TTrials, testosterone treatment was associated with a greater increase in the volume of noncalcified plaque volume (Budoff *et al.*, 2017).

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Hormone Replacement Therapy in Men☆

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Glossary

Androgens Androgens are steroid hormone that bind to the androgen receptor to exert actions on the male reproductive tract. Androgens are required for pubertal development, male secondary sex characteristics development and fertility.

Male hypogonadism Male hypogonadism is men with testosterone deficiency and disruption of spermatogenesis

Introduction

Over the past several decades, there have been progress in the field of testosterone (T) replacement therapy with many new formulations, routes of administration and delivery systems. Currently, T treatment is indicated for symptomatic male hypogonadism (Bhasin *et al.*, 2010) (*Note*: New guidelines will be published by the Endocrine Society in 2018). This article will review criteria for T replacement, benefits, potential adverse effects, and current treatment modalities. Recent controversies and issues concerning long-term cardiovascular and prostate safety, the relative benefits and risks of treating older men with androgen deficiency will be briefly addressed.

Increasing Trends of T Prescriptions

The primary indication of T therapy is for the treatment of male hypogonadism. The T prescribing trends have increased about three fold in the United States (US) from 2001 to 2011 (Baillargeon *et al.*, 2013).This trend is common to many countries in the developed world (Gabrielsen *et al.*, 2016, Handelsman, 2013). The rising trend of T prescription is associated with studies reporting that about 25% of the men treated either did not have serum T measured or did not meet the criteria for treatment recommended by the Endocrine Society before start of T treatment (Baillargeon *et al.*, 2013). The rise in initiation of T treatment may be related to televised direct-to-consumer marketing in the US (Layton *et al.*, 2017). Because of the possible "overuse of T" in men (Gabrielsen *et al.*, 2016), the US. Food and Drug Administration (FDA) examined the indications for T treatment. They concluded that the principle use of T replacement therapy remains for men with "classic" hypogonadism that is caused by specific well-recognized medical conditions. They questioned the need to replace T in older men with low serum T levels who lack a distinct, well-recognized cause of hypogonadism because there is no definitive evidence that increasing serum T concentration in older men is beneficial and safe (Nguyen *et al.*, 2015). In contrast to this opinion, a placebo controlled double blinded interventional set of studies in 790 men over age 65 supported by the National Institutes of Health in the US (the T trials) demonstrated significant benefits to sexual activity and desire, bone mineral density, anemia and modest improvements in mood. This 1 year study was not powered in in size nor duration for the above major cardiovascular and prostate events (Snyder *et al.*, 2016, Snyder *et al.*, 2018). Physicians encountering men with symptomatic low T concentration need to discuss and weigh the benefits and risks and allow the patient to participate in the decision of T replacement.

Criteria for Androgen Replacement and Diagnosis of Male Hypogonadism

The guidelines from The Endocrine Society and other professional societies recommend that the diagnosis of T deficiency should be based on persistently low serum T level and signs and symptoms consistent with T deficiency (Bhasin *et al.*, 2010). Common more specific signs and symptoms of T deficiency are decrease in libido, erectile function, muscle mass and strength, bone mineral density, and hair (face, body); presence of gynecomastia, and small testes. Other less specific symptoms include lack of energy; increase in visceral fat; sleep disturbances; and negative mood such as irritability, nervousness, and inability to concentrate.

In addition to symptoms and signs, the diagnosis of male androgen deficiency can be confirmed by measuring a total serum T level. Serum T should be measured in the morning, between 7 and 10 am, when T levels are highest—a reflection its diurnal pattern/variation. The diurnal variation is much less pronounced in middle aged and older men (Bremner *et al.*, 1983, Diver *et al.*,

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Table 1 Benefits and risks of T replacement

Benefits	*Risks*
• Improve sexual function	• Acne, oily skin
• Maintain secondary sex characters	• Decrease testis volume
• Increase bone mass	• Suppress spermatogenesis
• Increase muscle mass and strength	• Erythrocytosis
• Decrease visceral adiposity	• Weight gain, fluid retention
• Increase hematocrit/hemoglobin in unexplained anemia	• Decrease HDL, increase LDL: HDL ratio
• Improve mood	• Gynecomastia (with some preparations)
• Improve physical activity and balance?	• Sleep apnea?
• Reduce insulin resistance, fasting glucose, hemoglobin A1C?	• Prostate dysfunction (benign prostate hyperplasia, prostate cancer)?
• Coronary vasodilation, improve heart failure.	• Increased cardiovascular disease risk?
• Decrease cardiovascular disease risk	

2003). A subnormal T level is more easily noted during a time when normal T levels should be high. It is also recommended that blood should also be drawn while fasting as glucose ingestion decreases the serum T concentration. If the levels of T are near the lower limit of the reference range, measurement should then be repeated to achieve greater diagnostic confidence. Studies have shown that 30% of men with an initial low T level would have serum T within the normal range on repeat measurement (Brambilla *et al.*, 2007). The preferred reliable method for T assessment is by liquid chromatography–tandem mass spectrometry (LC–MS/MS) though many immunoassays have been recently harmonized against a standard LC–MS/MS method yielding more accurate results (Rosner *et al.*, 2013). Using a standardized T measurement, the reference range of serum T in large populations of adult men from Europe and US have recently been established (19–39 years, 264–916 ng/dL) (Travison *et al.*, 2017). In patients with T levels near the lower limit of normal or when suspicion of aberrant sex hormone binding globulin, measuring free T level, preferably by equilibrium dialysis method or estimation by formulae may provide additional information. It should be noted that the commonly available free T analogue immunoassays provide no additional information than total T and are not useful in clinical diagnosis of hypogonadism (Goldman *et al.*, 2017). Despite this report the adjunctive use of free T measurements to assist in the diagnosis of men with borderline T measurements is recommended by some experts.

Once T deficiency has been established, primary (testicular/gonadal) and secondary (hypothalamic–pituitary) hypogonadism can be distinguished by obtaining serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels. Low FSH and LH levels suggest secondary hypogonadism and serum prolactin and MRI of the sella may discover the cause of hypogonadism.

Routine screen men for T deficiency in the general population for hypogonadism is not recommended due to the incidence of high false positives. In addition, studies have shown minimal benefits in treating asymptomatic men with a low T level. Low T levels also occur among men suffering from an acute illness or using certain medications, such as opioids and glucocorticoids, that suppresses T concentrations. In these men withdrawal of the medication or recovery from an acute illness may restore serum T to the normal range.

Benefits and Risks of Testosterone Treatment (Table 1)

T replacement therapy has been shown to be beneficial in men diagnosed with symptomatic hypogonadism (Ceponis *et al.*, 2017). The consistent main benefit is improvement in sexual function, increase in lean and bone mass and decrease in fat mass. Moderate to strong improvement in sexual function is reported in younger (Isidori *et al.*, 2005a, Wang *et al.*, 2004) and mild to moderate in older men (Legros *et al.*, 2009, Snyder *et al.*, 2016). Sexual desire and activity are increased which is often followed by improvement in erectile dysfunction. Consistent improvement in lean mass and decrease in fat mass and visceral adiposity are associated with T replacement in both younger (Isidori *et al.*, 2005b, Wang *et al.*, 2000) and older hypogonadal men (Page *et al.*, 2005, Srinivas-Shankar *et al.*, 2010). In some of these studies improvement in muscle strength are also seen but sometimes this improvement is not translated to improvement in physical activity (Srinivas-Shankar *et al.*, 2010, Snyder *et al.*, 2016). T treatment is associated with increase in bone mineral density and bone strength (Wang *et al.*, 2004, Amory *et al.*, 2004, Isidori *et al.*, 2005b, Snyder *et al.*, 2017) however T treatment has not been studied to demonstrate a reduced fracture rate. Improvement of mood and energy are reported in some studies and the improvement (Snyder *et al.*, 2016, Basaria *et al.*, 2015, Steidle *et al.*, 2003, Wang *et al.*, 2000) (**Table 1**). Though small studies suggest that T treatment may improve cognition (Cherrier *et al.*, 2001), a recent large placebo controlled trial showed that T replacement did not improve cognitive ability in older men with memory impairment compared to placebo group (Resnick *et al.*, 2017).

It is well known that T causes erythrocytosis in a small but significant proportion of men which may be a limiting factor in T replacement specially in older men (Coviello *et al.*, 2008). T stimulates the bone marrow but more recently shown to be related to reduced hepcidin, increased iron in cells and elevated hemoglobin (Guo *et al.*, 2013). Recently in a placebo-controlled trial in older hypogonadal men, T treatment increased hemoglobin in men with unexplained anemia such that at the end of 12 months treatment over 50% were no longer anemic (Roy *et al.*, 2017). On the other hand, elevated hematocrit and hemoglobin levels are

putative risks for hyper-viscosity and thromboembolism. Some studies have shown that T treatment reduces insulin resistance and improve glucose levels in men with metabolic syndrome and type 2 diabetes but these studies are not conclusive (Jones *et al.*, 2011, Kalinchenko *et al.*, 2010). Other studies reported improvement of congestive heart failure and decrease in angina symptoms after T treatment in small number of subjects. These data have to be confirmed in larger placebo controlled studies (Malkin *et al.*, 2006, Malkin *et al.*, 2004).

Testosterone replacement therapy causes a decrease in HDL-cholesterol which may be associated with a small decrease in total cholesterol (Fernandez-Balsells *et al.*, 2010). The decrease in HDL-cholesterol appeared to be less with transdermal T delivery systems. The significance of reduced HDL-cholesterol levels on cardiovascular disease risk in men treated with T is not clear. In addition to the concentration of HDL-cholesterol, the density of HDL particles and the metabolism of HDL are important factors.

Testosterone administration does not cause benign prostatic hyperplasia or cause prostate cancer. Prostate cancer is an androgen dependent cancer and may grow with T treatment. However a metaanalysis showed no increase in prostate disease after T replacement (Fernandez-Balsells *et al.*, 2010). Recent data showed that once the prostate cancer has been treated and the patient has clinical cure, T therapy can be considered if the patient has symptomatic androgen deficiency without adversely affecting overall or cancer specific mortality (Kaplan *et al.*, 2014).

Currently, there is inconclusive evidence to support a definitive connection between T replacement therapy and cardiovascular morbidity (Kloner *et al.*, 2016). Past epidemiological studies suggest that low T concentrations are related to increase in all cause and cardiovascular mortality (Shores *et al.*, 2006, Khaw *et al.*, 2007). A placebo controlled trial of T replacement in older frail men reported increased cardiovascular adverse events in the T treated compared to the placebo group (Basaria *et al.*, 2010). A similar study on T replacement in older frail men showed improved physical function but did not find increased cardiovascular adverse events (Srinivas-Shankar *et al.*, 2010).Retrospective cohort studies and metaanalyses of controlled trials yielded controversial conclusion. Two large cohort studies found a significant increase in cardiovascular events associated with T use (Vigen *et al.*, 2013, Finkle *et al.*, 2014), whereas two others found a significant reduction in all-cause mortality (Shores *et al.*, 2012, Muraleedharan *et al.*, 2013). Another study found no significant change in the risk of myocardial infarction among men treated with T (Baillargeon *et al.*, 2014). The metaanalyses also had conflicting findings—one reported an increased risk of broadly defined cardiovascular-related adverse events among T users (Xu *et al.*, 2013); the other reported no additional risk of major adverse cardiovascular events (Corona *et al.*, 2014). Although the limitations and potential confounders or biases in these studies preclude a clear conclusion regarding the role of T therapy in adverse cardiovascular outcomes, a possible association cannot be overlooked. The US Food and Drug Administration (FDA) recommended that a well-designed, randomized, controlled study of T treatment in T deficient men involving over 6000 men be conducted to specifically evaluate cardiovascular outcomes. This study is underway sponsored by pharmaceutical companies.

Contraindications to Testosterone Treatment

Absolute contraindications to androgen replacement therapy are untreated or active androgen-dependent tumors, such as prostate and male breast cancer, and elevated hematocrit or hemoglobin level (hematocrit $>54\%$ or hemoglobin >17 g/L). Androgen treatment may cause fluid retention, and may precipitate or aggravate heart failure or sleep apnea. Patients with symptoms or signs of moderate or of severe congestive heart failure or sleep apnea should be treated and controlled before initiating androgen replacement therapy (Bhasin *et al.*, 2010).

While there is minimal evidence to implicate T in the development or aggravation of benign prostate hyperplasia (BPH), if a patient has significant lower urinary tract obstructive symptoms due to BPH then the symptoms should be controlled before instituting T replacement. If the patient is considering having a family, then androgen treatment may not be the best therapeutic choice, alternative treatment with human chorionic gonadotropin or antiestrogens may be considered if the hypothalamic–pituitary testis axis is intact and the patient does not have primary testicular failure.

Precautions Before Initiation and Monitoring of T Treatment

Before initiation of T treatment, men >40 years of age and older should have a digital rectal examination and a serum PSA performed to assess prostate abnormalities. In patients with palpable prostate nodule, induration, or PSA $>$ 4 ng/mL or PSA $<$ 3 ng/mL in men at high risk of prostate cancer, such as African Americans or men with first-degree relatives with prostate cancer, a referral to a urologist before start of T treatment is appropriate. Men who had lower urinary obstructive symptoms, obstructive sleep area and congestive heart failure should be treated for their medical conditions before initiation of T treatment. A baseline complete blood count, liver function tests and lipid panel should be available before start of T treatment.

Current Testosterone Treatment Options (Table 2)

The patient with T deficiency has many options for T replacement. The formulations currently available are listed in **Table 2**. All these delivery systems have proven efficacy in clinical trials. Thus, the selection of a therapeutic regimen should be based on

Table 2 Available testosterone preparations

Formulation	*Preparation*	*Regimen*	*Advantages*	*Disadvantages*
Intramuscular: intermediate duration	Testosterone enanthate or testosterone cypionate	100–200 mg q 2 weeks IM; 50–100 mg q 1 week SC	Most available and affordable	Fluctuating hormone levels Higher risk of increase hemoglobin and hematocrit Rare postinjection cough
Intramuscular: long-acting	Testosterone undecanoate	750 mg initially 750 mg at week 6 750 mg q10 week	Most convenient (longer injection intervals)	Injection site pain Postinjection cough (pulmonary oil microembolism?)
Oral (not in US)	Testosterone undecanoate	40–80 mg twice or three times/day	Convenient	Low serum T concentrations before next dose
Transdermal	1%	50–100 mg/day	Stable T levels	Expensive
• Gel • Solution • Patch	1.62% 2% 2% 2–4 mg/patch	20.25–81 mg/day 10–70 mg/day 30–120 mg/day 2–8 mg/day	No transfer to others	Transference to others on close skin contact Skin irritation, rash
Transmucosal	Buccal testosterone Intranasal testosterone	30 mg twice per day 5,5 mg/nostril 3 times/day	Stable T levels Stable T levels	Gum irritation minor Dislodgement from gum Multiple dosing required per day
Subcutaneous implants	Testosterone pellets (US 75 mg, non-US 100–200 mg)	150–750 mg every 3–6 months 400–1200 mg every 3–6 months	Stable T levels for 3–4 months	Minor surgical procedure Risk of pellet extrusion, infection, fibrosis

patient's preference in relation to pharmacokinetics, convenience, treatment burden, and cost. The decision is made by the patient with the physician providing the available information and discussing the pros and cons of each preparation. It has been proposed that initiation of T replacement in older men should start with a short acting gel rather than an injectable lasting 10 weeks' duration. The rationale is that if an adverse reaction occurs, for example, elevation of PSA, the gel can be stopped and androgen action withdrawn quickly. The development of selective androgen receptor modulators has not been directed for T replacement for hypogonadism but for other indications such as sarcopenia and physical dysfunction of aging or associated with chronic medical illness. These may be steroidal or nonsteroidal drugs and may have prostate growth sparing effect. However because many of these compounds are modified androgens (Kumar *et al.*, 1992) or novel nonsteroidal structures (Dalton *et al.*, 2013), they would not be aromatized (Attardi *et al.*, 2008) and may not have the positive effects of T on bone mass and fat mass due to the conversion to estradiol.

Monitoring Testosterone Replacement

The goal of T therapy in hypogonadal men is to restore the serum T to mid normal range and alleviate symptoms. Thus, serum T level should be measured after drug administration based on the pharmacokinetic characteristics of the specific preparation for dose adjustments. Since serum T levels are maintained in a relatively steady state by transdermal gels (Swerdloff *et al.*, 2000) and lotions, T levels can be measured at any time after the levels have plateaued, usually about a week after initial application. In older men, considerable within-individual variation of serum T occurred when serum T was measured on different days. Serum levels of T 8–12 h after application of transdermal T gel appeared to best reflect average serum T over 24 h (Swerdloff *et al.*, 2015).

The injectable T preparations, T enanthate and cypionate after an IM injection peaks at 2–3 days and troughs at 10–14 days. Serum T should be measured before the next injection to ensure trough levels are near the lower end of the reference range for adult men, and that there is no accumulation of T. Serum T should be periodically measured at day 7 to ensure that serum T levels are within normal limits. These T esters have been administered as a subcutaneous injection every week and serum T levels are more stable. Once a stable dose is determined, frequent measurement of serum T levels is generally not necessary unless dose adjustments are made. With T undecanoate injections, blood should be drawn prior to the next injection in order to establish the timing of future injections.

Since administration of T may unmask histological prostate cancers by increasing serum PSA levels, measuring PSA early after treatment initiation, for example, 1 to 3 months, is recommended. Thereafter, PSA should be checked on a yearly basis according to the urological practice applicable to each man.

Hemoglobin and hematocrit should be checked at 3 months and then after each dose adjustment followed by yearly intervals. Subjects whose hemoglobin level is high before treatment should be monitored more carefully. Following practice guideline, a yearly liver function test and lipid profile may be performed.

Conclusion

Androgen replacement therapy should be considered for all hypogonadal men with persistently low serum T levels and symptoms of T deficiency who meet the criteria for replacement. T replacement therapy should not be initiated in those with contra-indications to androgens use. The goal of T therapy is to restore serum T to mid normal range and alleviate symptoms. Therefore, it is important to have a baseline evaluation and laboratory results for serial monitoring of therapeutic response, adverse effects, and adherence. T replacement has shown in many studies to improve sexual function, energy and mood, increase muscle mass and strength, as well as bone mineral density. With numerous T preparations available and many more in development, selecting an androgen regimen can be tailored to each patient's individual needs and preferences. Selective androgen receptor modulators may have the potential of optimizing beneficial effects while minimizing potential adverse effects. With care, androgens can be used efficaciously and with minimal potential side effects.

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Male Hormonal Contraception☆

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Glossary

Azoospermia The absence of sperm in the ejaculate.
Gonadotropins The pituitary hormones, luteinizing hormone and follicle-stimulating hormone, which regulate spermatogenesis in males.
Oligozoospermia The presence of ≤ 3 million sperm/mL in the ejaculate.
Severe oligozoospermia The presence of ≤ 1 million sperm/mL in the ejaculate.

Introduction

In the United States, male contraceptives account for nearly one-third of all contraceptive use (Daniels *et al.*, 2015); worldwide, men represent approximately one-quarter of all contraceptive users (Ross and Hardee, 2016). Male contraceptives include methods of pregnancy prevention that men use directly or that require their cooperation for correct and consistent use. These methods include vasectomy, condoms, withdrawal, and fertility awareness. While vasectomy is one of the safest and most effective methods of contraception, it is essentially irreversible. The other reversible methods of male contraception, if used over the long-term have a relatively high failure rate, with unintended pregnancies occurring among 13%–20% of users over the course of a year (Sundaram *et al.*, 2017). As men may be invested in decisions to both plan for and prevent pregnancy, and as some couples may not be able to rely on more effective, reversible methods of female contraception, alternative options are needed. From a public health standpoint, the widespread use of effective methods of contraception by reproductive-age men and women would help to prevent pervasively high rates of unintended pregnancy worldwide, estimated at $>40\%$ of pregnancies (Singh *et al.*, 2010), as well as decrease the global population's currently unsustainable rate of growth. Specifically, alternative contraceptive options for men would provide a model for engaging men in sharing the burden of pregnancy prevention that has been disproportionately shouldered by women throughout human history. While a new method of male contraception has yet to be approved for widespread use, clinical trials of hormonal methods have received the most attention for their demonstrated efficacy and acceptability. This article will focus on the development of hormonal male contraception.

Mechanism of Action

The primary mechanism of action of hormonal male contraception is the suppression of spermatogenesis, thereby preventing the development of mature spermatozoa, and fertilization and pregnancy in the female partner. Spermatogenesis is regulated by the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both directly and indirectly (see **Fig. 1**). Spermatogenesis occurs within the seminiferous tubules in the testis, which house Sertoli cells and the germ cells. Sertoli cells are stimulated by FSH to facilitate the development and differentiation of spermatogonial stem cells into spermatozoa. Leydig cells, circumscribing the seminiferous tubule, are stimulated by LH to produce and maintain the critical concentration of intratesticular testosterone (T) required to initiate and complete spermatogenesis. There is crosstalk between Sertoli and Leydig cells. The secretion of gonadotropins by the pituitary requires the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus; therefore, either GnRH deficiency, nonpulsatile stimulation, or exogenous administration of T, progestins and estradiol can inhibit the pituitary secretion of gonadotropins, leading to the downregulation of Sertoli cell function and Leydig cell-mediated T production in the testis. The decreased production of T in the testis results in decreased serum concentrations of T. Symptoms and signs of hypogonadism (e.g., sexual dysfunction, fatigue, loss of muscle mass, infertility) can result if serum systemic T levels are not maintained at physiologic levels with exogenously administered T or other androgens. Inhibitors of the hypothalamic–pituitary–gonadal axis are shown in **Fig. 1**. The suppression of the hypothalamic–pituitary–gonadal axis for the purposes of hormonal male contraception thus entails the use of hormonally active agents to both suppress intratesticular T and

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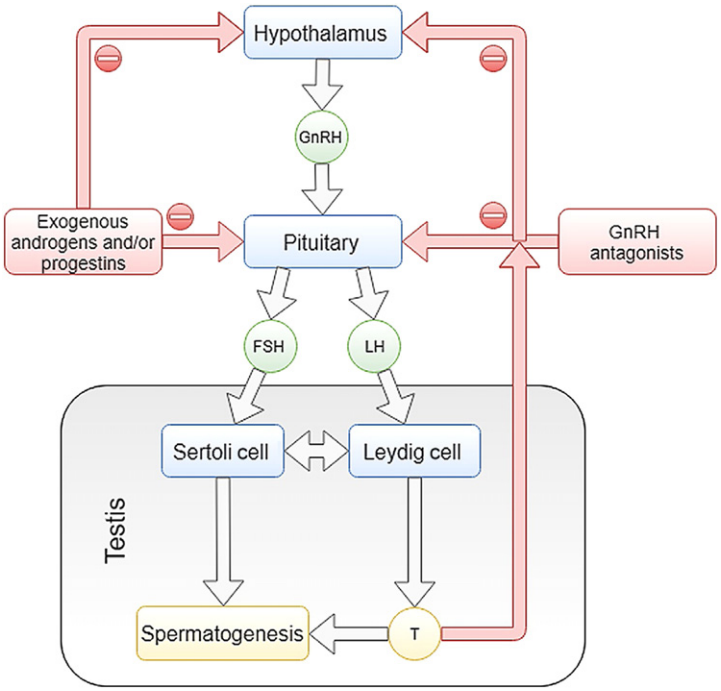

Fig. 1 The endocrinology of spermatogenesis and hormonal male contraception. The *gray arrows* show the physiologic pathway promoting spermatogenesis with the *red arrows* showing inhibitory influences. Endogenous T acts via negative feedback pathways to inhibit both GnRH release from the hypothalamus and gonadotropin (LH and FSH) release from the pituitary, leading to low intratesticular T levels. Exogenous androgens and/or progestins, as well as GnRH analogues reversibly manipulate this physiological response. *GnRH*, gonadotropin-releasing hormone; *LH*, luteinizing hormone; *FSH*, follicle-stimulating hormone; *T*, testosterone.

sperm production, while maintaining serum systemic levels of T to avoid hypogonadal side effects. This can be accomplished by androgen-only regimens and androgen combinations with other agents, such as progestins or GnRH antagonists (Wang and Swerdloff, 2010; Wang *et al.*, 2016).

Hormonal Male Contraceptive Regimens: Androgen Alone

Unmodified testosterone (T), as produced physiologically from the testis, was an expected, initial candidate androgen for use as hormonal male contraception. Ingested orally, unmodified T is well-absorbed, however it is rapidly degraded by first-pass metabolism in the liver. Novel, modified oral androgens are currently being studied in clinical trials (Ayoub *et al.*, 2017; Surampudi *et al.*, 2014). Unlike the earlier 17-alkylated androgens, these newer androgens have no apparent effect on liver function. Transdermal delivery of T by gels and lotions forms a subcutaneous reservoir, which delivers a steady release of T to the body. Transdermal T preparations are combined with a progestin to allow more rapid suppression of spermatogenesis (Ilani *et al.*, 2012). Testosterone implants have also been used for hormonal contraception but are more efficacious when administered with an injectable progestin (Turner *et al.*, 2003b). Intramuscular, depot preparations of T for hormonal male contraception, such as T enanthate or T undecanoate, are esterified by adding a fatty acid side chain to the T molecule to form T esters. Esterification reduces hepatic breakdown and the lipid soluble esters form a depot after IM injection prolonging their duration of therapeutic action (World Health Organization Task Force on Methods For the Regulation of Male Fertility, 1990; World Health Organization Task Force on the Regulation of Male Fertility, 1996; Gu *et al.*, 2009). The following sections will limit the review to preparations that were used specifically in contraceptive efficacy trials where pregnancy in the female sexual partner was the primary endpoint for the study (**Table 1**).

Testosterone Enanthate Injections

Testosterone enanthate (TE) is an esterified version of testosterone with a half-life of 4.5 days. In comparison, unmodified T has a half-life of 10 min (Nieschlag *et al.*, 2012). TE has been used for T replacement for hypogonadal men for many years with a good safety profile. Following many small studies demonstrating the efficacy of TE administered as intramuscular (IM) injections suppression of sperm output to very low levels (Patanelli, 1978), TE was used in a multicenter contraceptive efficacy trial where men received weekly IM injections of TE 200 mg for 12 months following confirmation of azoospermia. Among 271 healthy, fertile men, 157 completed the suppression phase of the trial and became azoospermic. The mean latency time until azoospermia

Table 1 Contraceptive efficacy in clinical trials of male hormonal contraception

Contraceptive efficacy trial reference	*Androgen*	*Progestin*	*# Enrolled*	*Sperm conc. threshold required for efficacy (million/mL)*	*# Reaching threshold/# Completing suppression (%)*	*# Completing efficacy/# Entering efficacy (%)*	*Expected length of efficacy phase (months)*	*Exposure to pregnancy risk (years)*	*# Participants with sperm rebound during efficacy*	*# Pregnancies (# per 100 person-years; 95%CI[a])*
World Health Organization Task Force on Methods For the Regulation of Male Fertility, 1990, International)[b]	IM TE 200 mg/week	–	271	0	157/225 (69.8%)	119/157 (75.8%)	12	123.8	11	1 (0.8; 0.0–4.5)
World Health Organization Task Force on Methods For the Regulation of Male Fertility, 1996, International)[c]	IM TE 200 mg/week	–	399	Initial: <5 Revised: <3	349/357 (97.8%)	209/349 (59.9%)	12	279.9	4	4 (1.4; 0.4–3.7)
McLachlan et al. (2000, Australia)[d,e]	T implants 800 mg or 1200 mg/13 weeks	–	36	<1	21/29 (72.4%)	12/18 (66.7%)	3–16	17.8	4	0
Gu et al. (2003, China)[f]	IM TU 1000 mg loading 500 mg/month	–	308	<3	299/305 (98.0%)	280/296 (94.6%)	6	143	6	1 (2.3; 0.5–4.2)
Gu et al. (2009, China)	IM TU 1000 mg loading, 500 mg/month	–	1045	<1	855/898 (95.2%)	733/855 (85.7%)	24	1554.1	10	9 (1.1; 0.4–1.8)
Turner et al. (2003b, Australia)[g]	T implants 800 mg/6 versus 4 months	IM DMPA		300 mg/3 months	55	<1	51/53 (96.4%)	25/51 (49.0%)	12	35.5
4 (T/6 months) 0 (T/4 months)	0									
Behre et al. (2016, International)[h]	TU 1000 mg/8 weeks	NETE		200 mg/8 weeks	320	<1	274/283 (96.8%)	111/266 (41.7%)	12	183.5
6	4 (2.2; 0.8–5.8)									

[a]This is the Pearl Index, a commonly indicator of contraceptive efficacy by comparing failure rate.
[b]Participants in this trail entered the efficacy phase upon achieving two sperm concentrations showing azoospermia, no spermatozoa in ejaculate.
[c]Participants in this trial entered the efficacy phase upon achieving two sperm concentrations of <5 million/mL for three consecutive samples. Given early findings of five pregnancies with three occurring among participants with a sperm concentration >4 million/mL, the threshold required to enter the efficacy phase was reduced to <3 million/mL.
[d]The proportion of participants observed to reach the threshold for efficacy is low by design to additionally test the suppressive effect of finasteride, a 5α-reductase inhibitor.
[e]The efficacy phase of this trial included one participant who received a single dose of oral finasteride 5 mg daily for 3 months along with an additional T implant 800 mg.
[f]The single pregnancy observed during the efficacy phase was attributed to sperm rebound.
[g]Participants in this trial initially received T implant every 6 months; however, failure to maintain eugonadal T levels prompted replacements every 4 months.
[h]This trial was terminated early due to the frequency of side effects reported by participants.
IM, intramuscular; *T*, testosterone; *TE*, testosterone enanthate; *TU*, testosterone undecanoate; *DMPA*, depot medroxyprogesterone acetate; *NETE*, norethisterone enanthate.

was approximately 120 days. During the efficacy phase, a single pregnancy was observed following 1486 months of cumulative exposure in 119 couples, giving a Pearl index of 0.8 per 100 person-years (95% CI: 0.02–4.5) (World Health Organization Task Force on Methods For the Regulation of Male Fertility, 1990). The Pearl index is a commonly used measure of contraceptive efficacy, calculated using the number of pregnancies, the number of couples at risk of pregnancy, and the time during which they are using the method. For comparison, Pearl indices for marketed oral contraceptives for women range between 0.29 and 2.86 per 100 person-years (Trussell and Portman, 2013). The favorable results of this trial of azoospermia led to a second international, multicenter trial where entry into the efficacy phase was allowed for men achieving oligozoospermia (initially arbitrarily defined as $\leq$5 million/mL ejaculate). Among 399 couples, 349 completed the suppression phase and demonstrated sperm concentrations of $\leq$5 million/mL. These couples entered the efficacy phase and over 180 person-years of exposure, 5 pregnancies were observed. As three of the pregnancies occurred among couples where the man's sperm concentration was $>$4 million/mL, the threshold for entry into efficacy phase was reduced to $\leq$3 million/mL. Following this change in entry criteria for 49.5 person-years, the trial noted 4 pregnancies, none of which occurred to men with azoospermia. The mean latency time until reaching the revised definition of oligozoospermia ($\leq$3 million/mL) was 68 days. The overall Pearl index for the trial was 1.4 (95% CI: 0.4–3.7) per 100 person-years, which was again comparable to hormonal contraceptive methods used by women. Median time to recovery of normal sperm concentration was 112 days (World Health Organization Task Force on Methods For the Regulation of Male Fertility, 1996). These proof of concept studies demonstrated that hormonal suppression of spermatogenesis to azoospermia was feasible for nearly 70% of participants, rising beyond 95% when suppressing to oligozoospermic levels (**Table 1**). Further, suppression to these levels ensured contraceptive efficacy and was reversible to the normal range of sperm concentrations in 98% of participants completing follow-up. However, this approach had two drawbacks: (1) unsatisfactory pharmacokinetics of TE injections resulted in widely fluctuating levels of T and the need for frequent, weekly injections; (2) high doses of TE were utilized to induce and maintain adequate suppression of spermatogenesis, causing supraphysiologic levels of T, which may have impacted the incidence of side effects and overall acceptability of the preparation. Side effects encountered in these studies were likely androgen-related, including acne, weight gain, behavioral effects, lowered high-density lipoprotein cholesterol (HDL-C), and increased hematopoiesis. For these reasons, longer acting T esters and alternative administration strategies were sought.

Testosterone Implants

Following the success of the WHO's TE trials, T implants were proposed as a long-acting preparation to avoid the frequent injections and associated high peaks and troughs of intramuscular TE injections. T implants are small cylindrical pellets containing crystalline T that are inserted into the subcutaneous tissue of the lower abdominal wall under local anesthesia. T implants were able to suppress sperm counts to a degree comparable to TE injections while maintaining a lower average serum T levels, such that androgenic side effects were less frequent than those seen in the TE injection trials (Handelsman *et al.*, 1996, 1992). In a multi-dose efficacy trial conducted in Australia, T implants 800 mg (4 $\times$ 200 mg) were administered to 36 men, 17 (47%) of whom reached sperm concentrations of $<$1 million/mL within 6 months. Eight men who were unable to suppress adequately via T implants at 800 mg doses were given T 1200 mg (6 pellets), 5 of whom then reached sperm concentrations of $<$1 million/mL within 3 months. Sixteen couples entered the efficacy phase where no pregnancies were reported following 214 months of exposure. Sperm concentrations returned to normal values on average within 10 months and only one man complained of acne from the 1200 mg group. While those who maintained sperm concentrations of $\leq$1 million/mL of ejaculate did not experience pregnancies, several men still could not be suppressed even at T 1200 mg, suggesting that methods with T alone might not be sufficient. Additionally, acceptability of this method may be dampened by the need for minor surgical procedural insertion and the incidence of implant extrusion, which occurred in 10% of insertions (McLachlan *et al.*, 2000).

Testosterone Undecanoate (TU) Injections

TU is partially absorbed by the intestinal lymphatics when given orally; it has low bioavailability and requires twice or thrice daily dosing, making it a poor candidate for an oral contraceptive. However, its preparation in tea seed oil (in China) and castor oil (in Germany) for use as an IM injection has yielded longer-lasting T depots with more favorable pharmacokinetics than TE. A Phase II contraceptive efficacy trial of IM TU injections was conducted in China, enrolling 308 men who received an initial loading dose of TU 1000 mg IM, followed by TU 500 mg at monthly intervals until they reached severe oligozoospermia (defined as $\leq$3 million/mL). Only 9 men were unable to achieve levels sufficient to enter the efficacy phase (failure of suppression in 2.9% of participants). No pregnancies occurred among the 296 couples who entered the 6-month efficacy phase and maintained adequately low sperm concentrations over a 143 person-year length of exposure. The only pregnancy observed during the efficacy phase was in the case of a man who demonstrated sperm rebound, where sperm concentration rose above the threshold for contraceptive efficacy after suppression. The mean length of time required for achieving azoospermia was 108 days (Gu *et al.*, 2003). Of note, the link between sperm rebound—seen in 2.3% of men in the efficacy phase—and pregnancy in this study, as predicted by concentrations between 1.1 and 3 million/mL led to recommendations to decrease the threshold for contraceptive efficacy to $\leq$1 million/mL. These recommendations were made with the recognition that future trials and practical, marketed methods could not require users to obtain more frequent confirmatory semen analyses to detect sperm rebound after suppression to severe oligozoospermia was attained. Decreasing the threshold for contraceptive efficacy to $\leq$1 million/mL thus may provide a wider margin for user error or method

insufficiency and would be expected to prevent incidental pregnancy (Aaltonen *et al.*, 2007). The subsequent Phase III contraceptive efficacy trial, using the same regimen thus defined the criterion for entry into the efficacy phase at ≤1 million/mL. The trial recruited 1045 Chinese men, 855 of whom entered the 24-month efficacy phase after reaching the contraceptive threshold (failure of suppression in 4.8% under the new criteria). 733 couples completed the study, giving a total of 1554 person-years of contraceptive exposure, during which nine pregnancies were observed, six of which could be attributed to sperm rebound concentrations between 2 and 8 million/mL—a failure rate of 1.1% at 24 months. Sperm rebound during the treatment phases of the study occurred in 1.3% of participants. The most frequent adverse events reported were injection site discomfort, acne, coughing after the injection, changes in mood and behavior, and skin rashes or facial swelling. After stopping the injections, all but two of the participants' sperm concentrations returned to the fertile reference range; recovery to normal values required 182 days on average (Gu *et al.*, 2009).

Current Developments With Androgens

The National Institutes of Health is developing several modified androgens that are bioavailable when administered by mouth where preclinical studies showed no hepatotoxicity. These androgenic compounds also bind to the progesterone receptor with transactivator activities. These compounds are promising for use as single-agent hormonal male contraceptives that possess both androgenic and progestational activity (Attardi *et al.*, 2011, 2006). Some of these new modified androgens are in phase 1 clinical trials and preliminary results demonstrate dose-dependent pharmacokinetic profiles and gonadotropin/T suppressive activity, as well as good safety and tolerability (Ayoub *et al.*, 2017; Surampudi *et al.*, 2014).

Ethnic Variations in Androgen Alone Regimens

While the contraceptive efficacy trials of TU injections provided substantial evidence of both efficacy and acceptability, as indicated by continuation rates beyond 85% of participants beyond 2 years (Gu *et al.*, 2009), the trials were conducted solely on Chinese men and their results may not translate to non-Asian or Caucasian users. The WHO-sponsored international efficacy trials of TE injections observed substantial ethnic disparities in the effectiveness of male hormonal contraceptive preparations using androgens alone where azoospermia was achieved by 91% of Chinese men compared to 60% of non-Asian men. The effect of T on lipids and liver function enzymes also varied by ethnicity in these studies (Ilani *et al.*, 2011). The need for preparations that could provide suppression across all ethnic groups and increase rates of suppression to azoospermia or oligozoospermia prompted studies that combined androgens with other hormonally active, gonadotropin-suppressing compounds such as progestins and GnRH antagonists.

Combined Hormonal Male Contraceptive Regimens: Androgens and GnRH Antagonists or Progestins

Gonadotropin-releasing hormone (GnRH) antagonists and exogenous progestins can inhibit gonadotropin secretion, reduce systemic T levels, and suppress spermatogenesis in men. Several different progestin or GnRH antagonist and androgen combinations have been studied.

GnRH Antagonists

GnRH antagonists are synthetic analogs of GnRH that bind to the GnRH receptors in the pituitary, rapidly suppressing the secretion of gonadotropins. Suppression is sustained throughout the time during which GnRH antagonists are administered and reversible within 1–2 weeks when treatment is stopped. These characteristics led to attempts at combining GnRH antagonists with T to increase the proportion of men achieving azoospermia, noting more complete suppression with the combination than with T alone (Bagatell *et al.*, 1993, 1995). Furthermore, when the GnRH antagonist, Nal-Glu, was used to initiate suppression, this suppression could then be maintained with the regular administration of T alone (Swerdloff *et al.*, 1998). Although this combination was found to have contraceptive potential, GnRH antagonists have a short half-life and limited oral bioavailability, warranting the need for frequent subcutaneous injections. Additionally, they are expensive and are associated with local, histamine-like skin irritation at the injection sites, making the preparation unlikely to be an acceptable one. Orally bioavailable or long-acting depot preparations of potent GnRH antagonists that are in development may have a place in male hormonal contraception in the future.

Progestins

The combined administration of T with a progestin can suppress gonadotropins to near undetectable levels and more completely suppress spermatogenesis to concentrations ≤1 million/mL than preparations using androgens alone. Multiple progestins, commonly used in hormonal contraceptive preparations for women, have thus been tested in combination with T preparations. However, the results of trials combining individual progestins in combination with T have varied, likely due to the unique properties of the various progestins used, but also possibly linked to different regimens of T administration among trials.

Cyproterone acetate (CPA) is a unique progestin for its direct antiandrogenic activity via concurrent blockade of T at the receptor level along with gonadotropin suppression as a progestin at the hypothalamus/pituitary. Its combination as an oral pill taken daily with weekly IM injections of TE, while able to achieve azoospermia in 100% of participants in a small trial ($n = 10$) and at half the time compared to men receiving TE alone, however was associated with dose-dependent decreases in hemoglobin and body weight (Meriggiola *et al.*, 1996; Wang and Yeung, 1980). As these effects were likely related to CPA's antiandrogenic properties, use of this combination has been limited. Levonorgestrel (LNG), given its safety profile when used for hormonal contraceptive among women, was extensively studied with a variety of androgens to determine its potential for male hormonal contraception. In one trial of sperm suppression combining weekly IM injections of TE with daily oral administration of LNG 500 μg, more rapid and complete sperm suppression was achieved than with TE alone—9.9 versus 15.3 weeks and 67% versus 33% azoospermia, respectively (Bebb *et al.*, 1996). Further study with lower doses of LNG (250 and 125 μg) did not compromise sperm suppression rates, however the trials noted dose-dependent decreases in levels of HDL cholesterol and weight gain (Anawalt *et al.*, 1999). Similar findings of complete azoospermia hindered by decreasing HDL cholesterol levels were noted in trials combining TE with desogestrel (DSG), an oral third-generation progestin with potent progestational activity and low androgenicity (Wu *et al.*, 1999). While the clinical significance of metabolic effects, such as the decrease in HDL, associated with the use of oral progestins for male hormonal contraception is unclear, concern that the chronic depression of HDL cholesterol levels may be associated with increased risk of cardiovascular disease has dampened interest in these combinations. However, preparations that could decrease overall exposure to supraphysiologic serum T levels and their associated risks without impacting contraceptive efficacy, such as long-acting or parenteral methods, remain promising.

Depot medroxyprogesterone acetate (DMPA) was combined with T implants in a study aimed at developing a method of male hormonal contraception that could circumvent the metabolic effects thought to be linked to daily progestin administration, with the added benefit of not requiring frequent administration. DMPA added to a suboptimal T implant dose of 800 mg could produce more effective suppression than T implants given at a 1200 mg dose (Handelsman *et al.*, 1996). A contraceptive clinical efficacy trial was then conducted in Australia where 55 men were enrolled, receiving T implants at 800 mg doses every 6 months and IM injections of DMPA 300 mg every 3 months. While the trial had to be amended to increase the frequency of T implant administration to every 4 months to avoid sperm rebound and symptomatic androgen deficiency, the participants received a median of 12 months of treatment without switches between treatment intervals. No pregnancies resulted following 426 person-months of exposure among 51 couples; azoospermia was maintained by nearly all participants through the efficacy phase of the trial. Time to suppression and the proportion of participants who were able to achieve azoospermia were improved markedly with the addition of DMPA. The effects were reversible. Unlike the trials combining T with LNG, CPA, or DSG, no significant weight changes or metabolic effects resulted. Lethargy and sexual dysfunction resulting from androgen deficiency seen among participants in the 6-month T implant interval were not seen when giving T implants at 4-month intervals (Turner *et al.*, 2003a). The limitation of DMPA, however, is its long duration of action such that recovery of the hypothalamic–pituitary–testis axis and spermatogenesis may be prolonged.

Complementing efforts to develop long-acting parenteral progestin and T combinations, one study examined the contraceptive potential of the subdermal etonogestrel (ENG) implant. ENG is the active metabolite of DSG and has been used with documented safety in the female contraceptive implants, Implanon® and Nexplanon®. In this study, 28 men were randomized to receive one or two ENG 68 mg implants in combination with T implants 400 mg, with azoospermia achieved in the majority of participants and without significant changes in HDL cholesterol levels (Anderson *et al.*, 2002). Inconsistent suppression among participants receiving a single ENG implant, however, warranted further development of preparations that provide higher doses of ENG within a single implant. A large multicenter trial sponsored by two pharmaceutical companies combined high and low dose ENG implants with TU IM at two doses. The study enrolled 354 men and 89% of men achieved a sperm concentration of $\leq$I million/mL at 4 months and suppression was maintained in 91% of men at 42/44 weeks of treatment. Recovery to normal sperm concentration took about 17 weeks. This study was unique in that a placebo was embedded in the different arms to evaluate possible adverse events. When compared to the placebo group, men in the active groups reported more weight gain, acne, sweating and libido changes. This study confirmed the efficacy of this androgen–progestin combination, but suggested that optimization may be achieved with changing the dose or the mode of delivery of the steroids (Mommers *et al.*, 2008).

More recently in 2008, the WHO, along with the Contraceptive Research and Development Program (CONRAD), led a late Phase II international contraceptive efficacy trial of norethisterone enanthate (NETE) 200 mg and TU 1000 mg IM injections. NETE is a depot progestogen administered via intramuscular injection and then converted to its metabolically active form, norethisterone. The trial recruited 320 men to receive injections every 8 weeks; 274 achieved severe oligozoospermia and entered a 12-month efficacy phase, during which 4 pregnancies were observed, giving a Pearl index of 2.18 per 100 person-years (95% CI 0.82–5.80). While six men exhibited sperm rebound during the efficacy phase, they were not linked to pregnancies. There was no difference in the rates of sperm suppression by ethnic group and adequate suppression was achieved in over 95% of men, thus highlighting the role of adding progestins to androgen preparations in overcoming the limitations of androgen-only methods (Behre *et al.*, 2016). The most common adverse events were acne ($n = 147$), increased libido ($n = 122$), injection site pain ($n = 74$), and emotional disorders ($n = 54$). While the frequency of adverse events led the WHO's Human Reproduction Program Research Project Review Panel to recommend halting further injections before the planned end of the study, the majority of adverse events were considered mild and $>$80% of participants reported willingness to use this method of contraception at their final visit (Behre *et al.*, 2016). It should be noted that most of the adverse events were reported in one Asian center. Moreover, because TU was administered without the loading

dose commonly utilized in clinical practice to rapidly raise serum T levels to the eugonadal range, there may have been times during the earlier part of the study where serum T levels were below the reference range and likelier to incur side effects.

The latest development in male contraceptive progestins is Nestorone® (NES), a 19-nor-progesterone-derived progestin with selective affinity for the progesterone receptor to the exclusion of androgenic, estrogenic, and minimal glucocorticoid activity at therapeutic doses (Kumar *et al.*, 2000; Sitruk-Ware *et al.*, 2003). Consequently, NES is not expected to be associated with undesirable side effects seen with the use of other androgenic progestins, such as weight gain or mood changes. NES gel (8 or 12 mg), when applied daily transdermally with T gel (100 mg T in 1% T gel), has been shown to induce severe oligozoospermia among >88% of users while maintaining normal serum total and free T concentrations. Adverse effects were minimal with the exception of mild to moderate acne in 21% of men, an effect thought to be related to the dose of T used in the study (Ilani *et al.*, 2012). The combination of NES and T into a single topical gel with reduced volume and lower T dose is expected to simplify application and improve adherence. This has been shown to suppress gonadotropins to a level expected to inhibit spermatogenesis as in the previous study (Mahabadi *et al.*, 2009). In 2018, the National Institute of Child Health and Human Development (NICHD) will conduct an international Phase 2b trial examining the contraceptive efficacy of daily applications of this combined NES/T transdermal gel in 450 couples. Following a suppression phase to confirm contraceptive effect (<1 million/mL) among male participants, couples will rely solely on the contraceptive gel for 12 months for the prevention of pregnancy. This study will incorporate an acceptability component.

Safety and Potential Side Effects of Hormonal Male Contraception

Studies on hormonal male contraceptive preparations in men began in the 1970s and to date, androgen and androgen plus progestin combinations have been relatively safe and well tolerated. Yet, no method of hormonal male contraception has been approved for clinical use. Relatively few clinical trials have been conducted with sufficiently large numbers of participants over a prolonged time period. Consequently, data that would accurately inform the incidence of side effects and rare adverse events associated with hormonal male contraceptive use are limited. The requisite safety data would not be available until a product is marketed for general use in men, after which postmarketing studies can be performed to monitor long term, off-target effects. However, the shared physiology and hormonal treatment of hypogonadal men with androgen replacement provides a model for anticipating similar side effects that might arise when using hormonal preparations to concurrently suppress intratesticular T production and optimize serum T levels. The side effects that are associated with the use of androgens for replacement therapy in symptomatic hypogonadal men are well-known, however their manifestation may depend on variations in dosing and routes of administration. Common side effects include acne, oiliness of the skin, weight gain, mood changes including depression, and fluctuations in libido. Laboratory-based changes include reductions in high-density lipoprotein (HDL) cholesterol and increases in hemoglobin and hematocrit. The adverse effects of progestins on men are not known, as they have not previously been administered to men clinically nor have their effects been characterized separately from those of androgens. Potential adverse effects have been identified in some contraceptive clinical trials, however they may be minimized by optimizing the doses of androgen and progestin to balance the suppression of spermatogenesis against potential side effects in future trials. These dose-finding studies were lacking before some of the larger contraceptive efficacy studies begun.

With respect to long-term side effects, a systematic review and meta-analysis of T replacement therapy in adult men did not find any significant effects on the incidence of prostatic or urologic adverse events. While it is unlikely that T replacement increases the risk of prostatic hyperplasia or cancer, none of the included studies examined exposures beyond 3 years (Fernandez-Balsells *et al.*, 2010), for which reason long-term effects on the prostate are still unknown. With respect to cardiovascular risk, results from a cohort study based on a healthcare database noted increased cardiovascular disease risk among men who were 65 years old or older, but not in younger men without preexisting heart disease (Finkle *et al.*, 2014). Analyses of such healthcare databases however, may not reflect clinical practice because a substantial number did not meet guidelines for starting T replacement (Baillargeon *et al.*, 2015). Several epidemiological studies (Vigen *et al.*, 2013; Shores *et al.*, 2012) and meta-analyses of randomized controlled trials (Corona *et al.*, 2016; Xu *et al.*, 2013) highlighted the controversy over the risk of major acute coronary events (MACE) and stroke among men with low T concentrations who receive replacement therapy. Nevertheless, the United States Food and Drug Administration and experts in the field recognize the inadequate amount of evidence and the need for long-term, adequately powered safety trials (Nguyen *et al.*, 2015) and recommend a longer term study to specifically address whether T treatment will increase the long-term risk of cardiovascular disease. Such a study is in progress.

Potential Noncontraceptive Benefits

Women who use hormonal methods of contraception receive several noncontraceptive benefits, not limited to the prevention of acne, menstrual irregularities, anemia, benign breast disease, ovarian cysts, and gynecologic cancers (Mishell, 1982; Kaunitz, 2002). While not the primary outcome of interest in hormonal male contraceptive trials, noncontraceptive benefits may similarly be expected to arise among some men. Based on trials of T replacement therapy, these men may expect to experience some increases in sexual desire, erectile function, energy, and mood, as well as hemoglobin, lean body mass, muscle strength, and bone density (Corona *et al.*, 2014; Isidori *et al.*, 2005a, b).

Summary and Conclusions

Despite numerous developments in hormonal contraceptives for women, including long-acting reversible methods such as the subdermal implant and intrauterine device, rates of unintended pregnancy remain persistently elevated. As data from the United States' National Survey of Family Growth indicate high rates of dissatisfaction leading to contraceptive discontinuation among couples (Moreau *et al.*, 2007), and as women are disproportionately burdened with the responsibility and risk, as well as the loss of resources associated with contraceptive use (Kimport, 2017), the availability of a novel male contraceptive could have far-reaching public health and sociocultural benefits. Yet while hormonal male contraceptives are one of the most studied methods that may be closest to being marketed, several barriers may prevent their widespread uptake.

Logistically, the use of any male hormonal contraceptive requires confirmation of sufficient suppression of spermatogenesis for contraceptive efficacy, thereby adding both an additional step for the user, as well as uncertainty about its efficacy at the individual level. In clinical trials of various male hormonal contraceptive preparations, the majority of participants could achieve sufficient suppression. However, a minority of men were unable to reach at least severe oligozoospermia within a reasonable timeframe, thereby limiting the potential impact of hormonal preparations studied to date. Additionally, even if a man intends to use a male hormonal contraceptive, he may have to wait as long as 60 to 70 days to achieve suppression of the hypothalamic–pituitary-–testicular axis and spermatogenesis. Nevertheless, a precedent for contraceptive latency and need for confirmation is already seen among vasectomy users who have to wait at least 2–4 months before confirming azoospermia via postvasectomy semen analysis (Sharlip *et al.*, 2012). This lag time after vas occlusion reflects the duration of time needed to turnover preexisting spermatozoa (Misell *et al.*, 2006) and for the mature spermatozoa to traverse the reproductive tract. Further research will be needed to develop methods with latency periods as short as those seen in women's methods—women who initiate hormonal methods need only wait 7 days for contraceptive protection (Curtis, 2016). As male hormonal contraceptives do not provide protection from sexually transmitted infections, they will likely be adopted by monogamous couples. In this context, the choice of contraceptive method used will likely be planned and coordinated such that the obstacles mentioned above are unlikely to deter potential users.

Public-funded research has reached a stage where the realization of male hormonal contraception is within reach. Further progress will be dependent upon public outreach to increase awareness and support of male contraceptive development efforts, which may be strengthened by efforts to engage men in family planning and reproductive responsibility. A change in culture and attitudes that provides men with reproductive choices via the promise of novel male contraceptives may attract investments from the pharmaceutical industry.

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Estrogen and the Male☆

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Glossary

Adrenarche A period that begins prior to puberty, at 7 or 8 years of age, when plasma concentrations of adrenal androgens begin to rise.

Aromatization The conversion of testosterone to estradiol (E_2) and of androstenedione to estrone (E_1) in the tissues that contain the aromatase enzyme.

Gynecomastia A benign proliferation of the glandular tissue in the male breast (histological definition); the presence of firm palpable tissue extending concentrically from the nipple (clinical definition).

Introduction

With new scientific advances during recent years, estrogen can no longer be regarded as a "female hormone." Our understanding of the role of estrogen in male physiology was fundamentally challenged when a man with a null mutation of estrogen receptor-α (ERα) was first described in 1994. The major shift in thinking regarding estrogen action in males was further fueled by the descriptions of men with inactivating mutations of the aromatase gene, the development of knockout mouse models with defects of the estrogen receptor (ERKO) and the aromatase gene (ArKO), the discovery of a second estrogen receptor, ERβ, and their numerous isoforms. This article provides an overview of the role of estrogen in male physiology and pathophysiology.

Estrogen Physiology in the Male

Adult males produce about 100 times more testosterone than estrogen under normal circumstances. The total production rates of estradiol (E_2) and estrone (E_1) are 45 and 66 μg/day (160 and 220 nmol/day), respectively, with only about 15% of E_2 secreted directly by the testis. Consequently, in a healthy young adult male, approximately 85% of circulating E_2 and all of E_1 are derived from peripheral conversion of testosterone and androstenedione by aromatization. Once androgens are converted to estrogens, the process is irreversible. The *CYP19* gene, located on chromosome 15q21, encodes the human P450 aromatase enzyme. The expression of the *CYP19* gene is regulated by tissue-specific promoters. It is expressed in the placenta, ovary, testis, prostate, liver, bone, heart, brain, muscle, hair follicles, breast, and (most important) adipose tissue where most of peripheral aromatization takes place.

Aromatase activity in peripheral tissues tends to increase with age and body size. Mouse models have shown that obesity-associated white adipose tissue inflammation increases aromatase gene expression (Polari *et al.*, 2015). In an obese male man, it was found that subcutaneous adipose tissue cell size rather than aromatase expression is related to low T (Bekaert *et al.*, 2015). Men over 50 years of age have a 50% increase in total E_2 with little change in free E_2 in part due to elevated levels of sex hormone-binding globulin (SHBG). Opposite to ageing, obesity is associated with a significantly lower SHBG (Cooper *et al.*, 2015). Increased adipose tissue leads to lower T levels that maintain deposition of visceral fat and may lead to a chronic hypogonadal state, called the hypogonadal–obesity cycle (Cohen, 1999, 2001). Data on E_2 levels in obese men have been inconsistent across studies, as some reported increased E_2 levels (Schneider *et al.*, 1979; Zumoff *et al.*, 1981), while others reported no changes or even lower levels (Abate *et al.*, 2002; Colangelo *et al.*, 2009; Dhindsa *et al.*, 2011).

Pituitary secretion of luteinizing hormone (LH) controls secretion of E_2 and testosterone from the testis. E_2 and testosterone, in turn, provide negative feedback at the level of the hypothalamus and anterior pituitary to regulate gonadotropin secretion. Similarly to testosterone, testicular E_2 secretion is stimulated whenever plasma LH/human chorionic gonadotropin (hCG) levels are elevated. Elevated levels of estrogen, either through endogenous or exogenous route, can reduce testicular testosterone secretion through suppression of LH release. Furthermore, excess estrogen stimulates SHBG synthesis in the liver, resulting in increased bound testosterone with a reduction in plasma-free testosterone.

Two estrogen receptor (ER) subtypes have been identified: ERα and ERβ. ERα has at least three different isoforms, and ERβ has at least five different isoforms (Li *et al.*, 2015). ERβ is far more widely distributed in the body than ERα. ERs are expressed at many

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Table 1 Estrogen actions

Onset and duration of action	*Estrogen actions*	*Receptors*	*Location*	*Mode of action*
Rapid and short acting (occurs within seconds)	Nongenomic	GPR30	Cell membrane	Changes in ionic transport through cell surface
Slower (hours) and more stable	Genomic	ERα ERβ	Cell nucleus	Gene expression

ERα, estrogen receptor alpha; *ERβ*, estrogen receptor beta; *GPR30*, G protein-coupled receptor.
Modified after Rochira, V. et al. (2016). Estrogens and male reproduction. MDText.com Inc.

of the sites where aromatase is also expressed, suggesting that many target tissues are locally producing estrogen. Evidence indicate that ERα and ERβ are expressed in bone, adipose tissue, prostate, cells of the immune system, thymus, pituitary gland, heart, the reproductive system, and various regions of the brain.

Both ER subtypes belong to the nuclear receptor superfamily and mediate the classical pathway, however they exhibit different ligand specificity and transcriptional activity (**Table 1**). ERβ has a weaker transcriptional activity compared to ERα. Moreover, co-expression of ERα and ERβ in a cell through complex mechanisms may result in antagonistic effects (Heldring *et al.*, 2007; Rochira *et al.*, 2016). In vitro studies have also shown rapid estrogen effects with short latency between administration of estrogens and their biological effect. These effects are realized through nongenomic estrogen action and are mediated by a cell-surface G protein-coupled receptor (GPR30). There are data to suggest that a monomeric portion of ERα is translocated from the nucleus to the membrane (Hammes and Levin, 2007, 2011).

Data on differential effects of each ER subtype on various systems are discussed in the respective sections below.

Roles of Estrogen in Male Physiology

Lessons From Males With Estrogen Deficiency States

Descriptions of a man with estrogen resistance and a number of men with aromatase deficiency provide insights into our understanding of estrogen action in the male. In 1994, Smith and colleagues described a 28-year-old man with estrogen resistance due to an autosomal recessive missense mutation in exon 2 of ERα, resulting in a complete loss of protein function. He had eunuchoid skeletal proportions with a height of 204 cm, delayed bone age (15 years), open epiphyses, persistent linear growth, and accelerated bone turnover with marked osteopenia. Biochemical testing revealed normal serum testosterone and elevated circulating levels of follicle-stimulating hormone (FSH), LH, E_2, and E_1.

Subsequently, from 1995 to 2017, fifteen unrelated cases of aromatase deficiency secondary to distinct autosomal recessive missense mutations of the *CYP19* gene in men have been reported: twelve adult men, one adolescent and two children (**Table 2**).

Similarly to the man with estrogen resistance, the aromatase-deficient men (24–44 years of age) all had a tall stature (height of 182–204 cm) with continued linear growth, delayed skeletal maturation (bone age of 14.0–18.0 years), open epiphyses, and evidence of severe osteopenia (**Table 3**). Biochemical studies revealed normal or elevated levels of testosterone, LH, and FSH, and showed very low levels of circulating E_2 and E_1 (**Table 4**).

When the ERα-negative man was treated with high doses of estrogen, there were no clinical, hormonal, or metabolic changes. In contrast, the aromatase-deficient men responded dramatically to relatively low doses of estrogen, resulting in correction of the described hormonal abnormalities without side effects such as gynecomastia (**Table 5**).

Observations in estrogen-resistant and aromatase-deficient men have redefined our view of estrogen action and the male skeleton. Other findings from these human models, including the role of estrogen in lipid/carbohydrate metabolism and spermatogenesis, are discussed in subsequent sections of this article.

Estrogen and the Male Skeleton

Despite normal or elevated levels of testosterone, men with impaired estrogen synthesis or action all had delayed epiphyseal closure, severe osteopenia, and evidence of increased bone turnover (**Table 3**). Although these men had normal pubertal onset and normal male secondary sexual characteristics, they did not experience the classic growth spurt associated with puberty. Estrogen stimulates pubertal growth spurt in both sexes by enhancing growth hormone secretion during puberty (Frank, 2003; Rochira *et al.*, 2015). Contrary to prior belief, epiphyseal fusion in males is not mediated by testosterone; rather, it is mediated by estrogen (Vanderschueren *et al.*, 2014). With estrogen treatment, despite declining levels of testosterone, all of the men had complete epiphyseal fusion within 6–9 months and had a progressive increase in bone mass. In one man, after 3 years of continuous estrogen therapy, bone mass increased by 20.7% in the lumbar spine, 15.7% in the femoral neck, and 12.9% in the distal radius (Bilezikian *et al.*, 1998). Thus, androgen alone is clearly not sufficient to promote normal skeletal growth and maturation. Both androgen and estrogen are required for accrual of optimal peak bone mass in men. Estrogen appears to increase bone mass by exerting anabolic as well as antiresorptive effects. While exact mechanisms are not fully known, there is sufficient proof that ERα directly affects osteoblasts, osteocytes, osteoclasts, and chondrocytes, while AR primarily works on osteoblasts and osteocytes.

Evidence from mouse knockout models (α-ERKO, β-ERKO, and ArKO mice) has also contributed to our understanding of estrogen and the male skeleton during recent years. Deletion of the ERα or aromatase gene in the male mouse results in osteopenia with increased

Table 2 Genetic impairments in men with estrogen deficiency states

Patient	*Inheritance*	*Publications*	*Mutation*	*Age (years)*
ERα resistant	Autosomal recessive	Smith *et al.* (1994)	ERα gene (missense)	28
Aromatase deficiency #1		Morishima *et al.* (1995)	Homozygous single base change at BP 1123 (C + T) in exon IX of the CYP19 gene, a	24
Aromatase deficiency #2		Carani *et al.* (1997)	A single G→A mutation at BP 1094 in exon 9 of the CYP19 gene	38
Aromatase deficiency #3		Herrmann *et al.* (2002)	A C to A substitution in intron V, at position-3 of the splicing acceptor site before exon VI of the CYP19 gene	27
Aromatase deficiency #4		Maffei *et al.* (2004)	Homozygous point mutation at the last nucleotide of exon V (G3A) inactivating CYP19 gene	29
Aromatase deficiency #5		Maffei *et al.* (2007)	Heterozygous mutations ibp380 (T→G) in exon IV and at BP 1124 (G→A) in exon IX inactivating CYP19A1 gene	25
Aromatase deficiency #6		Lanfranco *et al.* (2008)	Heterozygosity due to 23 bp deletion in exon IV and a point mutation in the first nucleotide of intron IX of the CYP19A1 gene	26.8
Aromatase deficiency #7		Baykan *et al.* (2013) and Pignatti *et al.* (2013)	Homozygous R375H guanine-adenine (G-A) mutation in CYP19A1 gene	27
Aromatase deficiency #8		Pignatti *et al.* (2013)	Homozygous for a point mutation in the first nucleotide of intron 3 (IVS3 + 1G>T) in CYP19 gene	26
Aromatase deficiency #9			Homozygous mutation in exon IV (c.434 G>A) leading to Arg to Gln substitution at position 115 (p.R115Q) in CYP19 gene	44
Aromatase deficiency #10				29
Aromatase deficiency #11		Chen *et al.* (2015)	Heterozygous *CYP19A1* mutations (Y81C and L451P) (missense)—partial loss of the enzymatic activity of aromatase	24
Aromatase deficiency #12		MiedLich *et al.* (2016)	Homozygous c.628G>A mutation in exon 5 CYP19 gene	25
Aromatase deficiency #13 weeks		Deladoëy *et al.* (1999)	Homozygote 1—BP (C) deletion in exon 5—CYP19 gene (missense) in CYP19 gene	4
Aromatase deficiency #14		Bouillon *et al.* (2004)	Homozygosity for a C-base deletion in exon 5 of the CYP19 gene	16.4
Aromatase deficiency #15	Bouchoucha *et al.* (2014)	point mutation of CYP19A1 at c.575 G>A in exon 5—nearly a complete loss of aromatase activity	15 months	

BP, base pair.

markers of bone remodeling. In contrast, the skeleton of the ERβ knockout male mouse is phenotypically identical to that of the wild type. It appears that estrogen mediates its effects on the bone primarily through ERα, although both ERα and ERβ are expressed in the bone.

Estrogen is clearly critical for bone mass acquisition in the growing skeleton. However, the inherited states of impaired estrogen synthesis or action do not clearly reflect the role of estrogen in maintaining bone density in the mature adult skeleton. Additional data from nearly all of the cross-sectional observational studies looking at the relationships between sex steroid levels and bone mineral density (BMD) show that E_2, especially the non-SHBG-bound E_2, has a better correlation with BMD than do either total or free testosterone levels (Araujo *et al.*, 2008; Paller *et al.*, 2009; Woo *et al.*, 2012; Slemenda *et al.*, 1997).

In a prospective study, it was found that the BMD in young men (20–40 years of age), when compared with that in elderly men (60–90 years of age), was most closely correlated with E_2 levels (Khosla *et al.*, 2001). In particular, the decreased bioavailable E_2 levels in elderly men appear to be a good predictor of age-related bone loss (Khosla *et al.*, 1998). This relationship was further investigated by giving elderly men physiological hormone replacement after pretreatment with a gonadotropin-releasing hormone (GnRH) agonist and an aromatase inhibitor. Estrogen alone was far more effective than testosterone alone in preventing an increase in bone resorption markers (Falahati-Nini *et al.*, 2000). Estrogen and testosterone combined have positive synergistic effects on bone remodeling. Based on the data, it was estimated that estrogen is responsible for about 70% of the total effect of sex steroids on bone resorption, whereas testosterone (in the absence of conversion to estradiol) accounts for no more than 30% of the effect. Another study reported that when elderly men were treated with an aromatase inhibitor for 9 weeks, they had a significant increase in bone resorption markers and a decrease in bone formation markers when compared with baseline (Taxel *et al.*, 2001). Therefore, a subset of elderly men with low bioavailable E_2 are the most susceptible ones to age-related bone loss similar to the increased risk of bone loss seen in postmenopausal women. In a more recent hallmark study by Finkelstein *et al.*, 189 healthy younger men with GnRH agonist induced hypogonadism were given graded doses of T with or without aromatase inhibition to prevent conversion to E_2 (Finkelstein *et al.*, 2016). Low E_2 levels were consistent with greater bone resorption despite the T levels. It was also shown that E_2 deficiency primarily affected cortical bone due to increased cortical porosity but had minimal

Table 3 Effects of estrogen deficiency states on skeleton

Patient	*Age (years)*	*Stature (cm)*	*Bone age (years)*	*Bone mineral density*
ERα resistant	28	204	15	Severe osteopenia
Aromatase deficiency #1	24	204	14	Severe osteopenia
Aromatase deficiency #2	38	187	14.8	Severe osteopenia
Aromatase deficiency #3	27	197	16.5	Severe osteopenia
Aromatase deficiency #4	29	183.5	15	Osteopenia
Aromatase deficiency #5	25	191.8	15.3	Osteopenia at ultradistal forearm (T-score −1.8); osteoporosis of the forearm (T-score −3.7)
Aromatase deficiency #6	26.8	193	15.5	Osteopenia (T-score −3.0)
Aromatase deficiency #7	27	187	15	Osteoporosis—lumbar BMD (T-score −4.1), femoral BMD (T-score −1.6)
Aromatase deficiency #8	26	187	14	Lumbar BMD (T-score −3.4) femoral BMD (T-score −2.4)
Aromatase deficiency #9	44	185	17	Osteoporosis—lumbar BMD (T-score −3.0)
Aromatase deficiency #10	29	197	15	Osteoporosis—lumbar BMD (T-score −4.1)
Aromatase deficiency #11	24	182.5	16–18	Osteopenia
Aromatase deficiency #12	25	180	15	Osteoporosis—spine Z-score −2.6, right femoral neck Z-score −2.8
Aromatase deficiency #13	16.4	172	12	Osteopenia

BMD, bone mineral density.

effects on trabecular structure. Animal models have also shown E_2 affects cortical bone, while effects on trabecular bone are mostly exerted through AR (Ucer *et al.*, 2015; Khosla, 2015; Notini *et al.*, 2007).

Effects of Estrogen on Metabolism

Despite differences of total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels in estrogen-resistant and aromatase-deficient men, serum concentrations of high-density lipoprotein (HDL) cholesterol are uniformly low. The low HDL cholesterol is likely due to low estrogen and unopposed androgen action as with E_2 therapy levels of HDL cholesterol and other lipids improved.

Estrogen deficiency is associated with propensity of developing insulin resistance (**Table 5**). The estrogen-resistant man had axillary *acanthosis nigricans*, a cutaneous marker of insulin resistance, in addition to elevated glycosylated hemoglobin. Similar features were observed in majority of men with aromatase deficiency: 13 of the adult men were overweight and nine had impaired glucose tolerance of varying degree. Most of the impairments resolved with estrogen replacement, with a notable exception of the estrogen-resistant man.

Similar results were shown in healthy men with induced hypogonadism: a decrease in E_2 through blocked aromatization lead to an increase in adipose tissue that was independent of T, while androgens were responsible for lean mass and other muscle parameters (Finkelstein *et al.*, 2013).

There is evidence to suggest that severe estrogen and testosterone ratio imbalance increases insulin resistance in estrogen-deficient men, as well as in rodent models (Mauvais-Jarvis, 2011). Aromatase deficient men are not homogenous, however, as in a patient, described by Chen *et al.*, treatment with estradiol improved glucose levels—but not insulin resistance (Chen *et al.*, 2015).

Data on estrogen effects on insulin sensitivity remains inconsistent in studies done in humans of both sexes, as well as animal models. It seems that favorable estrogen effect depends on a tight physiological concentration of E_2. This theory is supported by findings that supraphysiological levels of E_2 or overstimulation of ER, increase insulin resistance through hyperinsulinemia or a reduction in total glucose transporter 4 (GLUT4) expression (Nadal *et al.*, 2009; Barros *et al.*, 2008).

ERα is more abundant than ERβ in many glucoregulatory tissues. Activation of ERα increases glucose uptake into skeletal muscle when insulin is present; meanwhile activating ERβ alone or both ERs did not show beneficial effect (Gorres *et al.*, 2011). Homozygous ERβ deletion failed to produce insulin resistance in mice (Ohlsson *et al.*, 2000); yet there is evidence that ERβ, despite limited expression, promotes insulin resistance, especially when ERα action is reduced (Mauvais-Jarvis *et al.*, 2013).

Table 4 Reproductive effects of estrogen deficiency states in men

Patient	*Age at diagnosis*	*Testicular size*	*Cryptorchism*	*FSH/LH*	*Testosterone*	*Estradiol*	*Sperm analysis*
ERα resistant	28 years	Normal (20–25 mL)	No	Very high 33/ 37 mIU/ mL	Normal 15.4 nmol/L	Very high 437 pmol/L	25 × 10^6/mL; 18% viable
Aromatase deficiency #1	24 years	Large (>25 mL)	No	Very high 28.3/ 26.1 mIU/ mL	Very high 69.9 nmol/L	Very low <26 pmol/L	N/A
Aromatase deficiency #2	38 years	Small (8 mL)	No	High/normal 13.6/ 8.9 IU/L	Normal 13.5 nmol/L	Undetectable<37 pmol/ L	1 × 10^6/mL; 100% immotile spermatozoa
Aromatase deficiency #3	27 years	Normal (13–14 mL)	No	High/normal 11/ 6.0 mIU/ mL	High 31.2 nmol/L	Low <73 pmol/L	17.4 × 10^6/mL; 10% motility; 55% vitality
Aromatase deficiency #4	29 years	Right 10 mL, left 11 mL (in the inguinal canal)	Bilateral cryptorchidism	High/normal 20/7 mIU/ mL	Low normal 9.37 nmol/L	Undetectable <5.5 pmol/L	Biopsy—total germ cell depletion. Refused semen analysis
Aromatase deficiency #5	25 years	Normal (15 and 14 mL)	No	High/normal 10.12/ 6.75 IU/L	Normal TT 20.2 nmol/L	Undetectable <18 pmol/L	Biopsy –13 mature spermatids per tubule (severe oligospermia)
Aromatase deficiency #6	26.8 years	Normal (16 and 21 mL)	Right cryptorchidism	High/normal 12.5/ 3.1 IU/L	Normal TT 29.1 mmol/L, FT 147.7 pmol/ L	Undetectable	70 × 10^6/mL, 45% motility
Aromatase deficiency #7	27 years	Normal (>20 mL)	No	Normal 18.81/ 8.26 mIU/ mL	Normal 28.4 nmol/L	Undetectable <73 pmol/L	56 × 10^6/mL, 40% motility
Aromatase deficiency #8	26 years	Normal (20 mL)	No	Normal 13.4/ 5.4 mIU/ mL	Normal 23 nmol/L	Undetectable <73 pmol/L	N/A
Aromatase deficiency #9	44 years	Normal (>20 mL)	No	High/normal 18.0/ 5.3 mIU/ mL	Normal 14.2 nmol/L	Undetectable <37 pmol/L	N/A
Aromatase deficiency #10	29 years	Normal (>20 mL)	No	Normal 2.57/ 7.6 mIU/ mL	Normal 18 nmol/L	Undetectable <73 pmol/L	N/A
Aromatase deficiency #11	24 years	Normal (15 mL)	No	Normal 14.4/ 4.8 IU/L	Normal 20.5 nmol/L	Undetectable	129 × 10^6/mL; vitality 97%
Aromatase deficiency #12	25 years	Normal (20 mL)	No	Normal 4.8/ 5.5 mIU/ mL	Normal 30.2 nmol/L	Very low 26 pmol/L	N/A
Aromatase deficiency #13	2 weeks	Normal	No	@2 m/o Normal 3.9/ 12.5 IU/l	High to normal FT 182–44.8 pmol/ L	Low to undetectable 44 —<18 pmol/L	N/A
Aromatase deficiency #14	16.4 years	Normal	No	Normal 8/ 8 mU/mL	High TT 45.3 nmol/L FT 1.46 nmol/L	Undetectable	N/A

Table 4 Continued

Patient	*Age at diagnosis*	*Testicular size*	*Cryptorchism*	*FSH/LH*	*Testosterone*	*Estradiol*	*Sperm analysis*
Aromatase deficiency #15	15 months	@6 y/o intrascrotal—normal (20 × 10 mm)	Bilateral cryptorchidism	@4 y/o Low <0.4/1 mIU/mL	@4 y/o 0.3 nmol/L	N/A	N/A

TT, Total testosterone; *FT*, Free testosterone; *N/A*, Data not available.

Table 5 Metabolic profiles of men with estrogen deficiency states

Patient	*BMI*	*Insulin resistence*	*Insulin*	*Glucose tolerance*	*HbA1c (%)*	*DM*	*Estrogen therapy*
ERα resistant	30.5	Acanthosis nigricans	Hyperinsulinemia	Impaired	9.5 (n. 4.4–8.8)	No[a]	N/A
Aromatase deficiency #1	32.4	No	Hyperinsulinemia	Fasting glucose—normal	7.4 (n. 5.1–8.5)	No[a]	N/A
Aromatase deficiency #2	27.5	No	Normal	Normal	N/A	No	Slight improvement in fasting insulin levels at 6 months.
Aromatase deficiency #3	30.9	↑HOMA-IR	Hyperinsulinemia	Normal	N/A	No	Improved glucose metabolism at 3 and 6 months HOMA-IR intact
Aromatase deficiency #4 Glucose metabolism and Acanthosis nigricans improved	27.7	Acanthosis nigricans[b], skin tags	Hyperinsulinemia	Impaired	8.3	T2 DM	
Aromatase deficiency #5	35.8	Acanthosis nigricans; ↑HOMA-IR	Hyperinsulinemia	Normal	5.5	No	N/A
Aromatase deficiency #6	29.3	↑HOMA-IR	Hyperinsulinemia	Normal	5.6	No	Hyperinsulinemia and insulin resistance improved
Aromatase deficiency #7	25.7	Acanthosis nigricans	ULN	ULN	N/A	No	N/A
Aromatase deficiency #8	30.6	Acanthosis nigricans	N/A	Impaired	N/A	No	N/A
Aromatase deficiency #9 N/A	27.4	Acanthosis nigricans	N/A	Impaired	N/A	T2 DM	
Aromatase deficiency #10	30.9	Acanthosis nigricans	N/A	Impaired	N/A	No	N/A
Aromatase deficiency #11	26.5	Acanthosis nigricans	Hyperinsulinemia	Impaired	6.9 (n. 4.5–6.3)	No[a]	Improved glycemic levels, insulin resistance worsened
Aromatase deficiency #12	17.5	Acanthosis nigricans	Normal	Normal	4.8	No	N/A

[a]HbA1c within current diabetic levels.

[b]Patient AD#4 developed Acanthosis nigricans at the end of high-dose testosterone treatment.

HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; *ULN*, Upper limit of normal range; *N/A*, data not available.

Direct estrogen actions in glucose homeostasis and energy metabolism mechanisms remain to be fully understood as it is still unclear what tissues and which ERs are targets of estrogen action in insulin resistance.

Estrogen Effect on Male Cardiovascular System

Both ERs are widely expressed within cardiovascular tissues and have multiple effects. It seems that quantity and density of ERs depend on gender, age, estrogen levels, and concomitant diseases. For example, it is well known that more ERs are expressed in female arteries (Nakamura *et al.*, 2005). On the other hand, it was shown that estradiol can be produced locally in the male vasculature and stimulate ERs of endothelial and vascular smooth muscle cells (Vitale *et al.*, 2010).

Hypertension

Both animal models and human studies show that ERβ is important in regulating arterial tone and blood pressure (Zhu *et al.*, 2002; Hodges *et al.*, 2000). ERβ-deficient mice were shown to be hypertensive (Zhu *et al.*, 2002; Pelzer *et al.*, 2005); additionally, administration of ERβ agonist at a dose which did not activate ERα resulted in blood pressure-lowering effect (Arias-loza *et al.*, 2008). Estrogen can also cause arterial vasodilation through nongenomic pathway by activating endothelial cell nitric oxide synthase (Paper *et al.*, 2006).

Atherosclerosis

Endothelial dysfunction is an early contributor to developing atherosclerosis. Low-dose estrogen replacement improved endothelial function and diffuse atherosclerosis in men with aromatase deficiency (Carani *et al.*, 1997; Smith *et al.*, 1994; Chen *et al.*, 2015). Moreover, men who were treated with aromatase inhibitors due to prostate cancer, showed reversible endothelial dysfunction (Mendelsohn and Rosano, 2003; Lew *et al.*, 2003). In line with these findings, defective mutations in estrogen synthesis or ER expression were associated with premature development of atherosclerosis due to reduced endothelial function in men (Vitale *et al.*, 2010).

Ischaemia

Estrogen replacement therapy has been attributed to increased risk of myocardial infarction and death in men (Coronary Drug Project Research Group, 1974). However, recent data has shown potentially beneficial estrogen effects in cellular ischemia models, possibly through reduction in intracellular calcium and sodium during metabolic inhibition, as demonstrated in male mice cardiac myocytes (Sugishita *et al.*, 2001).

Estrogen and the Prostate

Benign prostatic hypertrophy (BPH) is usually found in men over 50 years of age. Estrogen concentration in the stroma increases with age with contributions from peripheral sources and from aromatization of androgen within the stroma. Estrogen can induce squamous epithelial metaplasia and can stimulate growth of the fibromuscular stroma, resulting in BPH. Estrogen also induces synthesis of insulin-like growth factor-1 (IGF-1), which acts to prime the response of epithelium to androgen. Therefore, estrogen can increase epithelial proliferation indirectly by enhancing androgen action.

By increasing the levels of circulating SHBG, estrogen can exert an inhibitory effect on the prostate by reducing the amount of free testosterone available for the androgenic stimulation of prostate. The decrease in the plasma concentration of testosterone is further mediated by the negative feedback action of estrogen on LH secretion. Therefore, in contrast to its growth-promoting effect in BPH, estrogen in pharmacological amounts can reduce the size and function of the epithelial cells of the prostate and has been a form of therapy in prostate cancer. Alternatively, estrogen may exert antiandrogen effects within the tumor cells, or it may be directly inhibitory to the tumor cells.

Epidemiological studies demonstrate geographical differences in incidence rates of prostate cancer, with a 15-fold greater incidence in the United States than in Japan and a 60-fold greater incidence in the United States than in Shanghai, China (Hsing *et al.*, 2000). The geographical differences have been attributed to dietary factors such as soy (phytoestrogens), which may be protective against the development of prostate cancer, in addition to genetic factors (Zhang *et al.*, 2016). The effects of phytoestrogen on BPH are not completely understood, but there are some evidence showing an inhibitory effect on proliferation (Nicholson and Ricke, 2011).

Within the prostate, ERα is located primarily in the stromal cells, whereas ERβ is present in the epithelial cells and seem to exert antagonistic effects. Stimulation of ERα induces proliferation (Wada-Hiraike *et al.*, 2006a; Omoto *et al.*, 2005), which has been related to chronic prostatitis, benign prostate hyperplasia, carcinogenesis, and progression to cancer (Prins and Korach, 2009). Conversely, ERβ seems to inhibit proliferation and promote differentiation (Wada-Hiraike *et al.*, 2006b; Warner and Gustafsson, 2010). Most studies reported decline of ERβ in localized prostate cancer with higher Gleason scores (Leav *et al.*, 2001; Horvath *et al.*, 2001). In support to this data, overexpression of ERβ showed a tumor suppressing effect and was associated with longer patient survival while controlling cell growth, cell cycle progression and apoptosis (Pinton *et al.*, 2010). Mice models suggest that beneficial effect on prostate through ERβ might be caused by downregulating androgen receptor expression (Weihua *et al.*, 2001). Potential therapeutic effects of ERβ-selective agonists need to be further explored.

Estrogen and Central Nervous System

A number of studies have shown estrogen to have neuroprotective, antioxidative, and antiinflammatory effects in the brain (Brann *et al.*, 2007; Fiocchetti *et al.*, 2012; Simpkins *et al.*, 2010; Kramer *et al.*, 2004). Estrogens are involved in various cognitive functions such as learning and memory; effect on physiological and behavioral endpoints including social behavior, cognition, and neurodevelopmental processes has been shown (Brann *et al.*, 2007; Craig *et al.*, 2008; Craig and Murphy, 2007a,b). While estrogens are important for certain aspects of memory, however, estradiol treatment failed to improve short or long-term memory in elderly men (Matousek and Sherwin, 2010).

Through regulation of dopaminergic, serotonergic, and cholinergic neurons, estrogens are involved in several neurotransmitter systems (Koehler *et al.*, 2005). Recent findings suggest that estrogens can work as neuromodulators by themselves locally at the site of synthesis—for example, estrogen effects in the synapse include synaptogenesis, neurotransmission, and synaptic plasticity (Badalà *et al.*, 2014).

There is evidence of de novo estrogen synthesis from cholesterol in the brain; the major site for this seem to be the neurons in the hippocampus (Fester *et al.*, 2011; Badalà *et al.*, 2014). Despite such synthesis, the main source of estrogens in the male brain still comes through local aromatization of testosterone (Badalà *et al.*, 2014). Local aromatization of testosterone leads to dynamic fluctuations in estrogen concentrations that are associated with probably nongenomic effects on various physiological and behavioral effects including social behavior and cognition (Chalovich and Eisenberg, 2005).

Neurodegeneration

Research has shown that estrogens are involved in prevention or slowing of neurodegenerative processes, which may be significant in Alzheimer's and Parkinson's diseases, multiple sclerosis, stroke, and traumatic brain injury (Luchetti *et al.*, 2014; Badalà *et al.*, 2014; Bourque *et al.*, 2015; Suzuki *et al.*, 2009; Tiwari-Woodruff *et al.*, 2007a, b). Both ERs are related to neuroprotective effect in brain through various mechanisms, including antiapoptotic protein-mediated signaling cascades and promotion of mitochondrial viability via regulation of calcium signaling (Nilsen and Brinton, 2004; Simpkins and Dykens, 2008; Zhao and Brinton, 2007; Zhao *et al.*, 2004). It has been suggested that the mechanisms and timing of effects through ERα and ERβ may differ as in mice models of ischemic brain injury and experimental autoimmune encephalomyelitis (an animal model for multiple sclerosis) ERα are induced earlier than ERβ (Tiwari-Woodruff *et al.*, 2007a, b; Tiwari-Woodruff and Voskuhl, 2009; Gillies and McArthur, 2010).

Estrogen-Excess State in the Male

The major clinical signs of estrogen excess in males are gynecomastia, testicular atrophy, erectile dysfunction, and infertility. Estrogen-secreting tumors and other endogenous or exogenous sources of estrogen can induce inhibition of testicular function by inhibiting gonadotropin secretion (**Table 6**).

Gynecomastia

The crucial factor in the development of gynecomastia from any cause may not the absolute level of estrogen but rather the ratio of estrogen to testosterone; the higher the ratio, the greater the likelihood of developing gynecomastia. **Table 6** summarizes the various causes of gynecomastia.

Physiological gynecomastia occurs during the newborn period, during adolescence, and with advanced age. Breast enlargement occurs in approximately 60%–90% of newborns due to stimulation by placental estrogen, and it usually resolves within a few weeks. Pubertal gynecomastia usually has an onset between 10 and 12 years of age and peaks at approximately 13–14 years of age. It affects 30%–40% of adolescent boys, and in the majority of cases it resolves by 17 years of age. The high plasma ratio of E_2 to testosterone in boys with pubertal gynecomastia is likely the result of normal aromatase activity in the testis and the extraglandular tissues before maximum production of testosterone is achieved. The prevalence of gynecomastia in older men ranges from 40% to 65% (Mathur and Braunstein, 1997; Braunstein, 2007); these may include misdiagnosed pseudogynecomastia cases, however prevalence of pseudogynecomastia has shown to be relatively low at around 5% (Dickson, 2012). With advanced age, the increased ratio of estrogen to testosterone favors feminization. However, in elderly men, it is a diagnosis of exclusion given that many elderly patients take medication or have medical problems that may contribute to breast enlargement.

Pathological gynecomastia can be due to a variety of causes, resulting in an alteration of the estrogen/testosterone ratio (**Table 6**). Gynecomastia can be seen in men with androgen deficiency or resistance. When testosterone production or action is compromised, elevated plasma gonadotropin levels further alter the plasma estrogen/testosterone ratio by stimulating the testis to produce more estrogen. Likewise, men with testicular tumors or bronchogenic carcinoma have increased testicular estrogen production largely from the stimulating effects of hCG produced by the tumor. Liver disease, starvation, thyrotoxicosis, and adrenocortical tumors are conditions where increased amounts of aromatizable androgen (i.e., androstenedione) are produced and gynecomastia can occur due to increased availability of this substrate for extraglandular aromatization.

Drugs that alter the ratio of estrogen to androgen at the level of the breast can cause gynecomastia (**Table 6**). There also have been reports of gynecomastia in children who ingested dairy or meat products from estrogen-injected and pregnant cows (Maruyama *et al.*, 2010; Andersson and Skakkebaek, 1999). As mentioned previously, gonadotropin-like substance such as hCG may cause gynecomastia by increasing testicular estrogen synthesis. In addition, drugs that impair testosterone synthesis or action can also cause gynecomastia. Approximately 50% of the men who receive high-dose spironolactone (150 mg/day) develop gynecomastia due to inhibition of both testosterone synthesis and testosterone action (Prisant and Chin, 2005). Spironolactone at low doses (50 mg/day) may cause gynecomastia, although less commonly, by blocking androgen binding to the receptor, with less effect on testosterone synthesis.

Estrogen and the Male Reproductive System

FSH and androgens are known to be important in the regulation of spermatogenesis. There is evidence to suggest that estrogen also plays an important role in spermatogenesis and male fertility. On a cellular level, estrogen may play a role in controlling

Table 6 Causes of gynecomastia

Physiological gynecomastia
Newborn
Adolescence
Elderly
Pathological gynecomastia
Testosterone deficiency
Hypergonadotropic hypogonadism
Klinefelter syndrome
Enzyme defect of testosterone synthesis (17β-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase)
Secondary testicular failure (viral orchitis, trauma, infiltrative disease, renal failure)
Hypogonadotropic hypogonadism (pituitary or hypothalamic causes)
Androgen resistance
Hyperprolactinemia
Estrogen overproduction
Testicular tumors
hCG-secreting tumors (bronchogenic carcinoma)
Adrenocortical adenoma or carcinoma
Severe liver disease
Hyperthyroidism
Recovery from malnourishment
Excess aromatase activity (hereditary)
Drug induced
Estrogens or estrogen agonists (digitalis, phytoestrogens, estrogen creams, diethylstilbestrol)
Clomiphene, gonadotropins
Aromatizable androgens
Antiandrogens or inhibitors of androgen synthesis (spironolactone, ketoconazole, flutamide, metronidazole, cimetidine, etomidate, cyproterone)
Drugs that cause elevated prolactin (phenothiazines, haloperidol, methyldopa, reserpine, metoclopropamide, tricyclic antidepressants)
Others (ranitidine, omeprazole, alkylating agents, cisplatin, busulfan, isoniazid, penicillamine, captopril, enalapril, amiodarone, nifedipine, verapamil, diazepam, phenytoin, amphetamines, heroin, marijuana, alcohol)
Idiopathic gynecomastia

proliferation, development, and function of the Leydig, Sertoli, and germ cells. Estrogen appears to be important in the development and maintenance of the efferent ductules and epididymides. It was shown that in rats the efferent ductule contains the highest expression of ERα in the male tract (Hess *et al.*, 1997). Estrogen effects in efferent ductule have been shown through genetic disruption in ERαKO mouse (Couse *et al.*, 2001; Dupont *et al.*, 2000) or ERs inhibition by antiestrogen (Oliveira *et al.*, 2001, 2002). Estrogen regulates efferent ductule fluid reabsorption by altering the expression of several major ion transporters and aquaporin (AQP) water channels (Fisher *et al.*, 1998; Zhou *et al.*, 2001). Published reports on AQP hormonal regulation have been controversial, but most likely both estrogen and androgen have role in regulating fluid reabsorption in efferent ductule. In rat models AQP-9 is modulated by estrogen in epithelium of the efferent ductule and by 5α-dihydrotestosterone (DHT) in the initial segment of the epididymis, while neither estrogens nor androgens showed effect on regulating AQP-1 (Oliveira *et al.*, 2005). Moreover, regulation of AQP varies among species: in tropical bat neither estradiol nor androgens had any effect on AQP-9 (Oliveira *et al.*, 2013). Meanwhile, in the marine sea bream AQP-1ab was activated by estrogens in proliferating spermatogonia (Boj *et al.*, 2015). More data is needed to better understand AQP hormonal regulation.

Men with estrogen deficiency due to a defect in estrogen action or synthesis usually have abnormal spermatogenesis (**Table 4**). Conversely, men with either exogenous or endogenous estrogen excess can experience azoospermia. The ratio of testosterone to E_2 is perhaps more important in spermatogenesis and fertility than is the absolute level of estrogen. Detailed data on reproductive system hormone levels in men with congenital estrogen deficiency are shown in **Table 4**.

Increased environmental exposure to estrogen in males during fetal development may also have detrimental effects on development and function of the male reproductive system. Pregnant women who took diethylstilbestrol (DES) during pregnancy from 1945 to 1971 had male offspring with a significantly higher incidence of disorders of the reproductive tract, decreased sperm counts, and decreased sperm activity (Reed and Fenton, 2013).

Male Breast Cancer

Breast cancer is responsible for less than 1% of all cancer deaths in men (Giordano *et al.*, 2004). The ratio of female breast cancer to male breast cancer is 100:1 in Caucasians and 70:1 in African Americans. A higher incidence is reported in Jewish men and in men living in a stretch of Bantu-speaking countries in central Africa. Other risk factors include a family history of breast cancer, history of exogenous estrogen exposure, increased circulating endogenous estrogen from chronic liver disease, testicular pathology, and prior chest wall

irradiation (Fentiman *et al.*, 2006). The greatest risk factor for developing male breast cancer is Klinefelter syndrome. The risk in affected men is about 50-fold higher than in men with normal genotype. The risk of male breast cancer appears to be associated with conditions with relative estrogen excess and/or lack of androgen, resulting in an altered ratio of estrogen to testosterone.

Conclusion

During recent years, further knowledge on some of the critical roles played by estrogen in the male has been gained, however, we have just begun to understand estrogen action in men. Sex hormones, including estrogen, through complex regulatory networks and numerous receptors exert diverse effects throughout the body. Further research is needed to clarify exact mechanisms by which estrogen mediates these effects in male physiology.

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Gynecomastia☆

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Glossary

Androgens A steroid, such as testosterone or dihydrotestosterone, which controls the development and maintenance of masculine characteristics.

Aromatase An enzyme complex present in the gonads, fat, liver, skin, and other tissues that convert estrogen precursors such as testosterone or androstenedione into estrogens.

Estrogens Any of several natural or synthetic substances formed by the ovary, placenta, testis, or certain plants that stimulate the female secondary sex characteristics, exert systemic effects such as the growth and maturation of long bones, and are used to treat disorders due to estrogen deficiency and to ameliorate cancers of the breast and prostate.

Gonadotropin A hormone that stimulates the growth and activity of the gonads, especially any of several pituitary hormones that stimulate the function of the ovaries and testes. The pituitary gonadotropins include luteinizing hormone (LH), which in the males stimulates testicular testosterone production and follicle-stimulating hormone (FSH), which stimulates spermatogenesis.

Human chorionic gonadotropin (hCG) A glycoprotein that is produced by the placenta, is excreted in the urine of pregnant women, and acts to stimulate ovarian secretion of the estrogen and progesterone that are required to maintain the conceptus. Production of hCG by tumors or administration of the hormone to males results in stimulation of testicular testosterone and estrogen production.

Sex hormone-binding globulin (SHBG) A high affinity, low capacity sex steroid hormone binding protein which circulates in the blood. Because of the tight binding of the hormones, most of the steroid hormones bound to SHBG are unavailable to target tissues.

Introduction

Gynecomastia represents a benign enlargement of the male breast glandular tissue and is a common clinical finding found in normal individuals without any underlying pathological disorder, but it also may represent a clinical manifestation of a disease. It is the result of an imbalance between estrogen and androgen action at the breast tissue level.

Sources of Androgens and Estrogens in Males

Approximately 95% of the major androgen in men, testosterone, is secreted by the testes, with the other 5% being derived from the adrenal gland production of androstenedione, a weaker androgen, which is converted to testosterone in peripheral tissues. The two major estrogens in males are estradiol and estrone. In contrast to testosterone, only 15% of estradiol and less than 5% of estrone are directly secreted by the testes. The major source of these estrogens is the conversion of androgens to estrogens in extraglandular tissues, including the liver, fat, and muscle. Thus, most of the circulating estradiol is derived from testosterone, and most of the circulating estrone is derived from androstenedione. The enzyme responsible for the conversion of androgens to estrogens is aromatase or estrogen synthetase, which is actually an enzymatic complex that has tissue-specific expression (Braunstein, 1993, 1999).

Both testosterone and estrogens circulate in the bloodstream primarily bound to serum proteins, especially sex hormone-binding globulin (SHBG). When bound to SHBG, the hormones are not available to enter cells. Only the free or unbound component can enter cells and bind with their respective receptors and thereby exert their biological action.

Because gynecomastia is the result of an estrogen/androgen imbalance, it can result from an increase in the production of serum estrogen, a decrease in the production of testosterone, alterations in the concentration of bioavailable estrogen or testosterone, problems with the androgen receptor in androgen target tissues, or an increase in the sensitivity of breast tissue to estrogens.

Prevalence

The age distribution of gynecomastia shows three distinct peaks. The first occurs during infancy, where 60%–90% of all newborns develop palpable breast tissue owing to the transfer of maternal estrogen to the fetus through the placenta. This transient

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enlargement of the breast tissue resolves within several weeks. Puberty represents the second time period where gynecomastia is frequently found. The prevalence figures of pubertal gynecomastia in various studies show wide variation, ranging from 4% to 69%. A reasonable estimate is that approximately a third of boys will develop some degree of clinically apparent gynecomastia during puberty. Gynecomastia generally has its onset between 10 and 12 years of age and peaks between 13 and 14 years of age. The gynecomastia usually involutes within 18 months and is completely resolved by 16–17 years of age in most male adolescents. The final peak of gynecomastia is during adulthood, when up to two-thirds of males between 50 and 80 years of age are found to have some degree of gynecomastia when carefully examined.

Pathological Causes of Gynecomastia

Table 1 outlines the broad categories of causes of gynecomastia. Gynecomastia may be found either in patients with germ cell tumors of the testicle, which secrete human chorionic gonadotropin (hCG), or in patients with Sertoli cell or Leydig cell tumors, which secrete excessive quantities of estrogens. Feminizing adrenocortical adenomas or carcinomas also either directly produce high quantities of estrogens or cause hyperestrogenemia through excessive production of estrogen precursors, such as androstenedione, that are aromatized to estrogens. Some tumors, such as giant cell carcinomas of lung, hepatoblastomas, and gastric or renal carcinomas, may secrete hCG, which stimulates the testes to produce excessive quantities of estrogen, resulting in gynecomastia.

Primary hypogonadism, which is due to a testicular pathology, results in a lowering of testosterone and, in some instances, an elevation of estrogen concentrations. A mild degree of testicular failure can be found in some men as part of the aging process. This may be accompanied by gynecomastia, which in the past has been termed "involutional gynecomastia." Secondary hypogonadism, which is due to a defect in the pituitary or hypothalamus and results in a loss of appropriate gonadotropin stimulation of the testes, is a rare cause of gynecomastia, which results from a decrease in testosterone while estrogens are still produced from adrenal steroid hormone precursors.

Androgen insensitivity from a defect in the intracellular androgen receptor leads to an inability of androgens to act at the target tissues. This results in unopposed estrogen activity and is a rare cause of gynecomastia. Some males with hyperthyroidism from Graves' disease may develop gynecomastia due to an increase in the concentration of SHBG, which binds testosterone more avidly than it binds estrogen, leading to a greater lowering of the free, biologically active testosterone level relative to the free, biologically active estradiol level.

As noted previously, the majority of circulating estrogens in males are derived from peripheral extraglandular aromatization of estrogen precursors. Increased aromatization is found in a variety of clinical situations, including aging, obesity, hyperthyroidism, liver disease, congenital adrenal hyperplasia, and Klinefelter syndrome, and as a primary defect with excessive aromatase activity from persistence of an unregulated fetal aromatase enzyme (Braunstein, 1999).

A number of drugs are also associated with gynecomastia, and the broad groups are listed in **Table 2**. However, many of the individual drugs that have been associated with gynecomastia are based on anecdotal reports or small, uncontrolled series of patients. A strong relationship between the occurrence of gynecomastia and medication use has been shown for androgens and anabolic steroids, estrogens and estrogen agonists, antiandrogens such as flutamide, the antibiotic ketoconazole, cimetidine, spironolactone, and alkalating agents. Drugs with fair evidence of an association with gynecomastia in adults include first and second generation antipsychotics, calcium channel blockers, omeprazole, HIV drugs, alkylating agents, alcohol, and opioids. Anabolic steroid use in adolescents and adults may also be associated with gynecomastia. All of the other drugs reported to be associated with gynecomastia currently fall in the poor level of evidence category (Deepender and Braunstein, 2012). There are a number of other sources of estrogen that occasionally cause gynecomastia. These include the use of over-the-counter phytoestrogens and androstenedione used by athletes. In addition, there may be occupational exposure and percutaneous absorption from antibalding creams or a sexual partner's use of an estrogen-containing vaginal cream.

Table 1 Pathological causes of gynecomastia

- Tumors
  - Testes
  - Adrenal
  - Other
- Hypogonadism
- Androgen insensitivity
- Hyperthyroidism
- Enhanced aromatization
- Drugs

Table 2 Drugs associated with gynecomastia

- Hormones
- Antiandrogens
- Antiulcer drugs
- Cancer chemotherapeutic drugs
- Cardiovascular drugs
  - Antihypertensives
  - Digitoxin
  - Spironolactone
- Phytoestrogens
- Psychoactive drugs

Evaluation of the Patient With Gynecomastia

The first question that must be answered is whether the breast enlargement is gynecomastia. The proper way in which to examine the male breast is to have the patient lie down with arms extended over or behind the head and for the examiner to take his or her thumb and forefinger and place them spread apart over the patient's breasts with the nipple centered in between. When the two digits are gently moved toward the nipple, gynecomastia will be detected as a firm or rubbery, mobile, round mound of tissue that arises concentrically from beneath the nipple and areola (Braunstein, 2007). The two most important conditions that should be differentiated from gynecomastia are pseudogynecomastia, which represents fatty enlargement of the breast without glandular proliferation, and breast carcinoma. With pseudogynecomastia, there will be no mound of tissue felt as the fingers close in on the nipple. Some of the features of breast carcinoma that distinguish it from gynecomastia include an eccentric location; mass that is hard, firm, and possibly fixed to underlying tissues; skin dimpling; retraction or crusting of the nipple or a frank nipple discharge; and the presence of axillary lymphadenopathy.

Once it is determined that the patient indeed has gynecomastia, the next question is whether medications are involved. A very careful history concerning medications, herb and vitamin intake, illicit drug use, and possible sources of environmental exposure to estrogens should be taken. If the patient is taking a medication known to be associated with gynecomastia, it should be stopped and the patient should be reexamined in 1 month.

The next question is whether the patient is pubertal. Because pubertal gynecomastia occurs frequently and is self-limited, its presence requires only reassurance that gynecomastia is a normal part of the pubertal process as well as a follow-up at 6 months or longer if the gynecomastia persists. Various epidemiological studies have noted that pubertal gynecomastia may persist in 1.3%–20% of males (Nydick *et al.*, 1961; Georgiadis *et al.*, 1994).

Another important question concerns whether the gynecomastia is of recent onset or is painful or tender. Gynecomastia of any cause undergoes the same pattern of histological evolution. The "florid phase" is found during the first 6 months. The breast ducts proliferate and exhibit epithelial hyperplasia, and there is an increase in the periductal and stromal constructive tissue, increased vascularity, and a substantial degree of periductal edema. Between 6 and 12 months is a transitional phase. The "involutional phase" is seen in patients with long-standing gynecomastia that has been present for 1 year or longer. There is a marked reduction in the epithelial proliferation and increased stromal hyalinization, dilation of the ducts, and fibrosis. The presence of pain or tenderness indicates that the patient is in the florid phase and that the gynecomastia has been of relatively recent onset. It is also during this phase that medical therapy directed toward the gynecomastia is most likely to be effective. Once the tissue has entered the inactive or fibrotic stage, medical therapy is unlikely to be beneficial.

The patient should undergo a full physical examination, with particular attention given to the breast exam as described previously, the thyroid for signs of hyperthyroidism, an abdominal examination for signs of cirrhosis or adrenocortical masses, and a careful testicular examination, evaluating for the presence of masses and consistency, especially looking for the reduced size or decreased consistency consistent with hypogonadism.

If the patient is not in the pubertal age group and is not ingesting medications known to be associated with gynecomastia, laboratory investigation should include measurements of hCG, luteinizing hormone (LH), estradiol, and testosterone. An elevated hCG should lead to a testicular ultrasound, looking for a mass that would be compatible with a germ cell tumor. A normal ultrasound suggests that the patient has either an extragonadal germ cell tumor or an hCG-secreting nontrophoblastic neoplasm, which should be evaluated through additional imaging studies. An elevated LH and a decreased testosterone are compatible with primary hypogonadism, whereas a low LH and a low testosterone suggest secondary hypogonadism. In that instance, a serum prolactin should be measured to evaluate the patient for a prolactin-secreting pituitary tumor. If the prolactin is normal, the serum concentration of androstenedione and estrone should be determined. A normal or low level suggests secondary hypogonadism, whereas elevation of these hormones is indicative of the rare 17-ketosteroid reductase enzyme deficiency.

An elevated LH and an elevated testosterone are compatible with either hyperthyroidism or androgen resistance due to an androgen receptor defect. These can be discriminated through measurements of thyroid function tests, including free T-4 and thyroid-stimulating hormone (TSH). An elevated thyroxine (T4) and a suppressed TSH are diagnostic of hyperthyroidism, whereas normal thyroid function tests indicate that the patient has androgen resistance.

A testicular ultrasound should be carried out in patients who have an elevation of the estradiol concentration and a decreased or normal LH level. A testicular mass in this setting is indicative of a Leydig or Sertoli cell tumor. If the testicular ultrasound is normal, an adrenal CT scan or MRI should be performed, looking for an adrenal neoplasm. A normal result from that exam suggests that the patient has increased extraglandular aromatase activity. If all results are normal, it is likely that the patient has idiopathic gynecomastia with enhanced sensitivity of the breast tissue to normal amounts of estrogen or that the inciting factor that initiated the gynecomastia has been corrected.

Treatment

The indication for therapy includes anxiety, embarrassment, and pain. It should be noted that approximately 85% of patients who have gynecomastia of recent onset will undergo a spontaneous resolution, whereas patients with long-standing gynecomastia will have an approximately 10% chance of spontaneous resolution. Also, as noted previously, long-standing gynecomastia is less likely to respond to medical therapy than is gynecomastia of recent onset due to the presence of fibrosis in the breast tissue when the gynecomastia has been present for more than 1 year.

There are several therapies that have been tried for gynecomastia. Although there are many anecdotal reports of improvement on various therapies, there are few well-designed studies that have critically examined the different therapies. Many of the studies have been hampered by small numbers and lack of placebo controls. The medical therapies that have been tried include administration of testosterone and its metabolites, danazol (an impeded androgen with weak androgenic and progestational activity), estrogen receptor antagonists such as clomiphene citrate and tamoxifen, and aromatase inhibitors such as testolactone and anastrazole. The best studied of these therapies is tamoxifen, which does appear to be superior to placebo in regard to reduction of breast pain and tenderness as well as breast size. With the exception of testosterone treatment of hypogonadal males, the use of all of these drugs to treat gynecomastia is considered to be an "off label" use (Gruntmanis and Braunstein, 2001).

In a patient who has long-standing gynecomastia or who fails to have an adequate response to medical therapy, surgical removal of the breast glandular tissue should be considered. There are a number of approaches and methods that include sharp excision, liposuction, or a combination of these methods. The approach that is most commonly used currently involves a periareolar incision, a sharp excision of the breast glandular tissue, and liposuction for contouring the breasts. Complications of the procedures include nipple-areolar numbness, hematomas, seromas, scarring, inverted nipples, nipple necrosis, and depressive deformities of the chest wall as well as inadequate tissue removal along with redevelopment of the gynecomastia.

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Relevant Website

http://www.hormone.org/questions-and-answers/2011/gynecomastia
The Hormone and Health Network sponsored by the Endocrine Society.

Male Sexual Dysfunction☆

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Glossary

Erectile dysfunction The inability to achieve penile erection sufficient for satisfactory sexual activity.
Impotence The inability to achieve penile erection.
Libido Conscious or unconscious sexual desire.
Orgasm The acme or climax of the sexual act.
Sexual dysfunction Disturbances in sexual desire and/or in the psychophysiological changes associated with the sexual response cycle in men and women.

Introduction

Sexual behavior is influenced by three types of forces: biological, psychological, and social. At the biological level, the primary function of sex is reproduction. Sexual functions are regulated by nerves and hormones and are sustained by the circulatory, muscular, and other systems. Psychologically, sex consists of a variety of behaviors and relationships aimed at erotic pleasure and affection. At the societal level, sex pervades numerous facets of life. Sexual activity is given meaning as humans experience its psychological and social contexts.

Sexual activity in a man involves four processes: desire, arousal (attaining and maintaining an erection), consummation (ejaculation and orgasm), and fertilization. Significant difficulty in any of these processes can lead to sexual dysfunction. In men, sexual dysfunction most often denotes erectile dysfunction (ED). ED may be defined as difficulty in obtaining or maintaining a penile erection sufficient to permit satisfactory sexual activity.

In a national survey, 31% of men 18–59 years of age reported that they had sexual dysfunction (Laumann *et al.*, 1999). Moderate or severe ED was reported by 21% of men 30–79 years of age in a community survey in the Boston area (Kupelian *et al.*, 2008). The Massachusetts Male Aging Study (MMAS) has demonstrated that 52% of men between the ages of 40–70 have some degree of ED and by the time a man is in his 50's, he has about a one in two chance of having some form of ED and this prevalence increases by about 10% for every increase in decade (Feldman *et al.*, 1994; Kubin *et al.*, 2003). These data demonstrate that a significant proportion of men as they age will begin to suffer from ED.

Anatomy and Physiology of Penile Erection

Because of its anatomy, the penis can rapidly engorge with blood, increase dramatically in size, and develop rigidity. Within the penis are two paired corpora cavernosa and a corpus spongiosum. The corpus spongiosum, which surrounds the urethra, terminates in the glans penis. A thick fibrous sheath, the tunica albuginea, encases the paired cavernosal tissue, which consists of interconnecting lacunar spaces. Trabeculae form the walls of the lacunae and are composed of thick bundles of smooth muscle, fibroblasts, collagen and elastin. This arrangement enables very high intracavernosal pressure generation and penile rigidity when outflow of blood from the cavernosa is reduced and the lacunae are engorged.

When flaccid, the smooth muscle cells of the corpora cavernosa and its associated arteries are in a state of contraction. Relaxation of these smooth muscles (arterial and cavernosal) increases blood flow into the corpora cavernosa. The increased blood volume coming into the cavernosal spaces raises the intracorporal pressure which then passively compresses the egressing venules shut against the more rigid tunica albuginea. This mechanism of veno-occlusion restricts the outflow of blood from the penis during an erection. After ejaculation or cessation of the erotic stimuli, the smooth muscle in the arteries and lacunar spaces contracts in response to a decrease in neural stimulation. As such, inflow of blood is reduced, venous outflow from the corporal spaces resumes, and the penis returns to a flaccid state.

Neuromuscular Influence

Increased arterial flow causing the penile erection is controlled by the autonomic nervous system. Peripheral innervation consists of sympathetic nerves arising from the T11 to L2 and parasympathetic and somatic nerves arising from the S2 to S4. They reach the

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pelvic plexus through hypogastric and pudendal nerves. The glans penis is rich in nerve endings that convey touch, pain, temperature, and vibration. Although neural centers in the lumbosacral spinal cord can cause erection in response to penile stimulation, erections usually are stimulated by olfactory, auditory, visual, and other sensory inputs. These inputs are perceived centrally by the frontal, temporal, parietal, or occipital lobe. Furthermore, they may be stimulated by memory or fantasy. Signals are sent to the visceral brain, to the rhinencephalon, and subsequently to the spinal cord erection center at the level of S2–S4. In a reflexogenic erection, stimulation of the penis leads to transmission of an afferent signal via the pudendal nerve to the sacral erection center. An efferent signal is then transmitted from the sacral erection center via the inferior hypogastric plexus to the cavernous nerves which lead to smooth muscle relaxation.

During the process of an erection that is initiated and maintained by persistent stimulation from the cavernosal nerves, ejaculation will occur when the pudendal nerve via the somatic pathway, stimulates contraction of the pelvic floor muscles, the bulbospongiosus and ischiocavernosus muscles. These muscular events lead to the most rigid phase of an erection and it is during this phase that suprasystolic pressure in the corpora cavernosa ensues. During ejaculation, there is rhythmic contraction of the pelvic floor muscles after semen is deposited into the posterior urethra and then transmitted through the urethra. This is associated with the pleasurable sensation of an orgasm.

On a molecular level, several neurotransmitters are involved in penile erection. Histochemical studies indicate that the corpora cavernosa are rich in adrenergic fibers, acetylcholinesterase-containing (probably cholinergic) nerves, and nerves that are immunoreactive to vasoactive intestinal polypeptide (VIP) and neuropeptide Y. The main neurotransmitter responsible for an erection, however, is nitric oxide which is synthesized from L-arginine by neuronal nitric oxide synthase (nNOS) within the axons of the cavernosal nerve (Ignarro *et al.*, 1990; Rajfer *et al.*, 1992). Nitric oxide from the nerve diffuses passively into the underlying corporal smooth muscle and then activates soluble guanylyl cyclase in the cytoplasm of the smooth muscle cells to form cyclic guanosine monophosphate (cGMP). cGMP then activates certain proteins which open ion channels leading to a decrease in intracellular calcium resulting in smooth muscle relaxation. cGMP is normally degraded by the phosphodiesterase-5 enzyme located in the cytoplasm of the corporal smooth muscle cells, and pharmacological inhibition of this specific enzyme is effective in potentiating corporal smooth muscle relaxation and as such, the erectile process.

Endocrine Influence

In humans, testosterone and dihydrotestosterone (DHT) play critical roles during embryogenesis of the testes, epididymis, vas deferens, seminal vesicles, prostate, and external genitalia. Sex steroids are implicated in imprinting gender identification on the central nervous system. Development of accessory glands, testes, external genitalia, and secondary sex characteristics at puberty depends on normal circulating androgen levels and on target androgen tissue responsiveness. In addition, testosterone mediates the development and maintenance of libido and erectile function. Androgens exert a direct effect on penile tissue to maintain erectile function and androgen deficiency produces a metabolic and structural imbalance in the corpus cavernosum.

With aging, serum total and free testosterone levels fall and sex hormone-binding globulin (SHBG) levels increase (Feldman *et al.*, 1994). Comorbid medical conditions, certain medications, and pituitary function can also influence testosterone levels.

Etiology of Erectile Dysfunction

Vascular

ED frequently is due to vascular dysfunction. Altered blood flow to and from the penis is thought to be the most frequent organic cause of ED. The former is thought to be due to either atherosclerosis and/or arteriosclerosis, while the latter is due to incomplete relaxation of the trabecular smooth muscle. When this latter state occurs, the high intracorporal pressure necessary to achieve compression of the subtunical emissary veins is not achieved and this leads to a "venous leak," which, depending on its severity, can lead to either an inability to attain a rigid erection or very commonly in its earliest stages to maintain an erection once it is achieved. Incomplete smooth muscle relaxation can result from either an intrinsic smooth muscle dysfunction or simple aging or disease induced apoptosis of the smooth muscle itself. The most common associations with arteriogenic impotence include diabetes mellitus, hypertension, hyperlipidemia, cigarette smoking, perineal or pelvic trauma, and pelvic irradiation or surgery. The most common cause of smooth muscle dysfunction is simply aging related apoptosis of the smooth muscle itself.

Diabetes Mellitus

The risk of ED in men with diabetes mellitus is three and a half times more than in men without diabetes with the prevalence being about 53% in diabetic men (Kouidrat *et al.*, 2017). In men with diabetes, ED progresses gradually, and other symptoms and signs of autonomic neuropathy are frequently present. ED is more common in men with severe vascular or neurological complications. ED in diabetic men occurs at an earlier age and is more prevalent than in men without diabetes. Diabetic ED consists of a complex interplay between a variety of factors but can be simplified into neurogenic, hormonal, vascular, and smooth muscle dysfunction. There is evidence to suggest that diabetic men have a lower degree of smooth muscle relaxation mediated by both the

parasympathetic nervous system and endothelial factors (Saenz de Tejada *et al.*, 1989). Animal models have demonstrated selective apoptosis leading to decreased neuronal NOS and nitric oxide (Vernet *et al.*, 1995). The risk for hypogonadism is increased in diabetics compared to nondiabetics. The Hypogonadism in Males Study demonstrated that 50% of diabetic men above the age of 45 are hypogonadal (Mulligan *et al.*, 2006).

Hypertension

Men with hypertension frequently report difficulty in initiating or maintaining erections that are adequate for sexual intercourse. Sometimes this could be due to a side effect of antihypertensive medication. The reported incidence of erectile failure is 14% with propranolol, 9% with hydrochlorothiazide, and 1%–41% with clonidine. However, the more plausible explanation is that hypertension, like ED, also stems from an underlying dysfunction of the vascular smooth muscle, a cell common to both the arterial system as well as the corpora cavernosa (Clavijo *et al.*, 2014). Untreated hypertension is independently associated with increased ED as well as a myriad of health problems and therefore it is imperative that patients work with a primary care physician to help control their blood pressure despite the possible side effects from antihypertensive medications.

Obesity

Central obesity is associated with both hypogonadism and erectile dysfunction. In the European Male Aging Study there was a significant positive correlation between obesity and severity of erectile dysfunction (Corona *et al.*, 2010). Other studies have demonstrated that lifestyle changes to improve obesity lead to an improvement in ED when both conditions coexist (Esposito *et al.*, 2004; Esposito *et al.*, 2009). The presence of ED in an obese individual should be used by clinicians to convince an individual to make lifestyle changes with an improvement/resolution of their ED as a reward.

Neurogenic Impotence

Disorders affecting the spinal cord or the peripheral efferent autonomic fibers to the penis cause partial or complete ED. This may be due to afferent limb interruption of reflexogenic erections as well as to interruption of tactile perception, with projections to supraspinal centers that may be important in maintaining psychogenic erections. Although the reflexogenic erectile mechanism is preserved in men with supra-sacral spinal lesions, the erections usually are poorly maintained without constant tactile stimulation.

Common neurological disorders associated with ED include spinal cord injury, multiple sclerosis, and peripheral neuropathy due to diabetes mellitus or alcoholism. In addition, surgical procedures (e.g., radical prostatectomy, cystoprostatectomy, proctocolectomy) may disrupt the autonomic nerve supply to the corporal bodies. Even in nerve sparing procedures there is evidence that the period of neuropraxia before recovery may lead to smooth muscle atrophy and apoptosis and replacement of the smooth muscle cells by fibrosis (Iacono *et al.*, 2005). This is due to an increase in the concentration of pro-fibrotic substances (Rambhatla *et al.*, 2008) and explains on a scientific basis the rationale for the long-term use of phosphodiesterase 5 inhibitors, recently reported to have antifibrotic and antiapoptotic properties, in the setting of penile rehabilitation after pelvic surgery (Ferrini *et al.*, 2006).

In patients with spinal cord injury, preservation of erectile function is largely dependent on the completeness and level of the spinal lesion. Although 75% of patients with spinal cord injury have some capacity to have penile erections, the erections are only adequate for penetration without assistance in about 25% (Wyndaele *et al.*, 1986).

Endocrine Impotence

Androgen Deficiency

Androgen deficiency is a widely recognized cause of sexual dysfunction. Diminished libido, reduced ejaculate volume, and ED commonly accompany androgen deficiency that develops after puberty. Specific signs and symptoms of hypogonadism are eunuchoidism, low libido, decreased spontaneous erections, and decreased bone mineral density (Bhasin *et al.*, 2010a). According to the Endocrine Society, values below 300 ng/dL (10.4 nmol/L) have a greater likelihood of leading to symptoms (Bhasin *et al.*, 2010a). The Baltimore Longitudinal Aging Study demonstrated that 20% of men in their 60's and 50% of men in their 80's are hypogonadal as measured by a serum testosterone level less than 325 ng/dL (11.3 nmol/L) (Harman *et al.*, 2001). The MMAS also shows that there is a decline in serum testosterone of 1.6% per year as men age (Feldman *et al.*, 1994).

Many patients with ED and testosterone deficiency have pituitary or adult onset hypogonadism. Pituitary and hypothalamic imaging is recommended as well as checking a prolactin level when the morning testosterone level is less than 150 ng/dL (5.2 nmol/L) (Dean *et al.*, 2016). On average, older patients are less likely to experience an improvement in erections due to comorbid medical conditions that cause ED when the condition is treated.

Hyperprolactinemia

ED is common in men with pituitary tumors. 76% of patients with a tumor in the region of the sella turcica reported decreased or absent libido or potency. These patients often have subnormal serum testosterone levels. Prolactin levels are more than 50 μg/L in most patients with prolactinomas. Normalizing minimal hyperprolactinemia does not ameliorate ED. Prolactin levels more than 50 ng/mL (normal 2–18 ng/mL for males) should alert the physician to evaluate medications and thyroid, liver, and renal function as a possible cause for hyperprolactinemia. Studies performing routine measurement of serum prolactin found a low yield of prolactinomas (<1% of patients). Measuring the morning prolactin level is recommended in the setting of a testosterone level less than 300 ng/dL (10.4 nmol/L) and an inappropriately low/normal luteinizing hormone (LH) level or when gynecomastia is present.

Diagnostic Workup of Erectile Dysfunction

The evaluation of patients with ED begins with a thorough history and physical examination. Initial questions should focus on the onset, progression, and duration of the symptoms. The presence of nocturnal erections and the ability to attain erections with a different partner may help to differentiate psychogenic causes from organic causes. It also is important to assess libido, which may indicate androgen deficiency, depression, or excessive stress. The medical history is essential to assess comorbidities commonly associated with ED (especially diabetes, hypertension, peripheral vascular disease, neurological disorders, and coronary artery disease). Because the use of certain drugs is associated with ED, a detailed and complete medication history should be taken. Recent changes in social status, alcohol consumption, and cigarette smoking should be noted.

The physical examination is essential. It should focus on the cardiovascular, endocrine, neurological, and genitourinary systems. Blood pressure, palpation of peripheral pulses, and auscultation for abdominal and femoral bruits should be assessed. Special attention to signs of androgen deficiency (e.g., decreased body and facial hair, gynecomastia, testicular atrophy or decreased testicular consistency, regression in prostate size) must be undertaken. The penis should be examined for fibrous plaques (Peyronie's disease), and the rectal exam should assess the sphincter tone, the bulbocavernosus reflex and the prostate gland.

Appropriate laboratory testing should complement the history and physical examination. Patients should be screened for diabetes as well as for liver and renal disease. Early morning serum testosterone levels should be checked as well as thyroid-stimulating hormone (TSH) if there are signs suggesting thyroid disease.

Nocturnal penile tumescence testing (NPT) and duplex ultrasonography in conjunction with intracavernosal administration of vasoactive agents may be indicated in select patients. Patients with ED and normal NPT usually have psychogenic impotence; whereas abnormal NPT in conjunction with normal sleep usually indicates an organic cause (vascular or neurological). These tests should be performed in centers where there are facilities and expertise to perform and interpret them. If a venous leak is suspected, dynamic infusion cavernosometry can be employed to make a diagnosis. A CT cavernosogram can be completed to determine where the venous leak is occurring, and if it is a localized leak, it may be amenable to surgical correction although long-term results of such surgeries are suboptimal.

Treatment

There are many treatment considerations and options for men with ED. The goal of treatment is to restore the capacity to acquire and sustain an erection. Treatment should address the cause of ED. Patients should be asked to avoid cigarette smoking, excessive alcohol consumption, and drug abuse. Changes in medication, if possible, should be tried as medication may contribute to ED. The major drugs that induce ED are thiazide, beta-blockers, and some antidepressants. Angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB) generally are associated with the least sexual side effects and can be tried in those men with concomitant hypertension.

Lifestyle modification with diet, exercise, and weight loss, having good glycemic control, works well in preventing ED in diabetic men, but is not a good treatment option once ED has set in. The Look AHEAD (Action for Health in Diabetes) Trial demonstrated that once diabetic ED has started, lifestyle modification does not effectively treat ED (Wing *et al.*, 2010). These men are often dependent on medical or surgical therapy for their ED.

Psychosexual Therapy

When depression and excessive anxiety are associated with ED, psychosexual counseling is indicated. When it is selected appropriately, the success rate for psychosexual therapy can reach 70%. Furthermore, some patients with organic ED can benefit from sex therapy because it reduces anxiety and sexual inhibition. Yohimbine, an alpha-2-adrenergic receptor–blocker, may be effective in some men with psychogenic ED. In a double-blind, placebo-controlled study that evaluated men with psychogenic ED, it was found that 37% of men treated with yohimbine responded to treatment (Riley *et al.*, 1989). However, other studies have not found yohimbine to be beneficial. Nonetheless, an appointment with a practitioner specializing in psychosexual therapy is prudent when psychogenic ED is suspected.

Hormonal Therapy

Testosterone replacement therapy is the treatment of choice for men with hypogonadism. According to the endocrine society guidelines, to make the diagnosis of hypogonadism one needs to have symptoms/signs of hypogonadism as well as biochemical confirmation of a low testosterone level (Bhasin *et al.*, 2010b). There are various formulations that testosterone is available in such as transdermal gel and patches, oral pills, buccal tablets, intramuscular injections, and subcutaneous pellets. Prior to starting testosterone replacement therapy a baseline testosterone, estradiol, PSA, and hematocrit should be obtained. A baseline prostate exam should also be preformed. After starting testosterone replacement therapy labs should be checked at 3 and 6 months and then annually (Bhasin *et al.*, 2010a).

Testosterone replacement therapy has received bad press regarding possible cardiovascular side effects. However, on close inspection of these studies, it is determined that there are significant deficits in study design and the ability to draw conclusions from these studies. Several studies have suggested that testosterone replacement therapy in hypogonadal men leads to better cardiovascular status and overall health (Khaw *et al.*, 2007). Research is finding that even in men with prostate cancer, where testosterone replacement was once contraindicated, it may be safe and can significantly improve quality of life (Kacker *et al.*, 2016).

The other problem that surrounds testosterone replacement therapy is the improper diagnosis and monitoring while on testosterone replacement therapy. A study by Baillargeon and colleagues found that there has been a threefold increase in testosterone prescriptions over the past 15 years, only 51% of men started on testosterone replacement had a diagnosis of hypogonadism, and 25% of men never had a testosterone level checked prior to starting testosterone therapy (Baillargeon *et al.*, 2013). Increasingly, nonspecific symptoms of hypogonadism are being used to make to make the diagnosis of hypogonadism, often times without any biochemical confirmation. Due to improper diagnosis of hypogonadism and the steroid abuse epidemic, the United States Food and Drug Administration has recommended against the use of testosterone to treat age related hypogonadism.

If it is thought that sexual symptoms are due to low testosterone levels, then a 3–6 month trial of testosterone can be started. Data from studies suggest that if an improvement is to occur in the hypogonadal symptom one is treating, that improvement should occur in the first 3 months of treatment. (Wang *et al.*, 2004) If there is no improvement after such a 3 month trial of testosterone replacement therapy, a work up for other causes of sexual dysfunction should be carried out.

Hyperprolactinemia may decrease libido by suppressing gonadotropin-releasing hormone (GnRH), resulting in reduced LH and testosterone levels. Treatment of hyperprolactinemia with a dopamine agonist or surgical removal of a pituitary tumor may restore testosterone levels and potency.

Medical Treatment

After excluding conditions treatable with specific therapies, the clinician should consider treatment with a type 5-phosphodiesterase inhibitor (PDE5i). These drugs provide effective oral therapy for a wide range of conditions leading to ED. PDE5i work by inhibiting the phosphodiesterase type 5 enzyme, resulting in increased intracellular cGMP. cGMP facilitates smooth muscle relaxation of both the arteries to the penis and the trabeculae of the corpora cavernosa. The use of PDE5 inhibitors to treat ED results in about a 70% success rate. Each of the PDE5 inhibitors should be used according to their pharmacokinetic profile. For example, the short half-life of sildenafil, vardenafil, avanafil and udenafil dictate that it should be taken ½ to 1 h before planned intercourse and preferably at least 2 h after a meal since food can inhibit its absorption from the stomach. Tadalafil, a PDE5i with a much longer half life of about 18 h is not subject to the absorption issues of the other PDE5 inhibitors. Its maximum efficacy occurs about 2 to 3 h after taking the pill but its effect can last much longer than the other PDE5 inhibitors. In addition, because tadalafil accumulates in the tissues, daily low doses of the drug can be used to obviate a timing issue with the initiation of sexual activity. Regardless of the PDE5 inhibitor being used, logic dictates that one should ideally begin treatment with a low dose and titrate up to the maximum dose depending on efficacy versus side effects. All PDE5 inhibitors are absolutely contraindicated in patients taking nitrates. Some level of exercise tolerance should be documented prior to initiation of therapy with PDE5i; otherwise a treadmill test should be considered for patients at risk for cardiac disease. Common side effects include headaches, flushing, rhinitis, and visual changes. It is considered a therapeutic failure of these medications when one has failed a trial of at least two different PDE5i on three separate occasions for each medication.

Intracavernosal injections are an effective way of delivering medication directly to the smooth muscle in the penis. This treatment involves injecting vasoactive drugs (e.g., alprostadil, papaverine, phentolamine, atropine) that induce relaxation of the smooth muscles. They work by increasing cyclic AMP and cyclic GMP concentrations within the smooth muscle cell. Intracavernosal injection therapy is especially effective in men with neurogenic impairments such as spinal cord injury and diabetes mellitus. The major side effects are penile pain and priapism. Patients should be counseled on proper injection techniques as improper technique can lead to scarring within the corporal tissue.

Alprostadil also is available for intraurethral application (Medicated Urethral System for Erection [MUSE]). In a placebo-controlled study, 65% of patients treated with MUSE successfully completed intercourse compared with 19% of men treated with placebo. However, this efficacy rate has not been replicated in unselected populations of men with ED. MUSE is usually not as effective as intracavernosal injection therapy.

Medical Devices

Vacuum devices increase the volume of blood in the penis by creating a vacuum around the penis and pulling blood into the corpora cavernosa. After the penis is engorged, a constriction ring is placed at the base of the penis to prevent venous outflow. Once the constriction ring is removed, venous outflow resumes and this leads to loss of the erection. The constriction ring should not be left on for more than 25 min. The induced erection sometimes is sufficient for vaginal penetration and sexual intercourse. Adverse events include pain, bruising, and retrograde ejaculation. Vacuum constriction devices represent an alternative for patients who cannot tolerate type 5 phosphodiesterase inhibitors or who do not wish to use intracavernosal injection.

Surgery

Surgical therapy is offered to patients who are refractory to other less invasive treatments. Vascular reconstruction is sometimes indicated in young patients with a history of pelvic trauma. Skepticism exists regarding other vascular reconstruction or venous ligation surgeries. Penile prostheses are a viable option for patients who cannot be treated medically. There are two kinds of penile prostheses: the semirigid prosthesis and the inflatable prosthesis. The inflatable prosthesis is better accepted and in general is associated with higher satisfaction rates. It also is associated with more frequent mechanical failure than are the semirigid devices. The average life expectancy for the inflatable device is about 10–15 years. If these devices fail, they can be removed and a new device placed.

Stem Cell Therapy

Current treatments for ED focus on a temporary solution to the problem with the exception of placement of a penile prosthesis, however this creates a life long dependence on a device that is subject to potential mechanical failure. Studies are underway with the use of stem cells as a possibility to reverse the pathology seen in ED. There has been some evidence in animal studies to suggest there is benefit to stem cell intracavernosal injection either by growth factor expression, cell engraftment, or cell incorporation (Albersen *et al.*, 2013). We are still awaiting data from clinical trials in humans. Stem cells have the potential to treat ED associated with aging, diabetes, post-prostatectomy, and peyronie's disease.

Ejaculatory Dysfunction

Ejaculatory dysfunction includes problems related to semen transport. Semen consists of sperm and seminal fluid as well as prostatic secretions. These conditions can have implications for reproductive function as well as psychological distress. Ejaculation is the discharge of semen from the male reproductive tract and occurs in conjunction with orgasm. After sufficient stimulation is achieved, under the control of the sympathetic nervous system, emission of sperm and seminal fluid occurs into the posterior urethra. With contraction of the bulbospongiosus muscle, propulsion of semen out of the urethra occurs in the second phase of ejaculation.

Retrograde ejaculation refers to semen going into the bladder instead of out through the urethral meatus. This occurs when the bladder neck muscles do not contract in the propulsion phase of ejaculation. Failure of emission results in no semen being deposited into the posterior urethra. These can be seen in men with neurologic conditions such as multiple sclerosis or the neuropathy from diabetes or as a side effect of medications such as alpha blockers. Treatment consists of correcting reversible causes or a trial of alpha agonists. Anorgasmia refers to the inability to achieve sensation of orgasm. Without an orgasm semen transport will usually not occur although a patient may have an ejaculation but not experience an orgasm. Anejaculation refers to no antegrade or retrograde ejaculation and is usually due to failure of emission or anorgasmia (Alwaal *et al.*, 2015).

Premature ejaculation is when ejaculation occurs before it is desired and can be psychologically distressing. It is particularly troublesome with an intra-vaginal latency time of less than 30 s. It can be either chronic or acquired and can be treated with penile desensitization, topical lidocaine, or serotonin selective reuptake inhibitors (SSRI). Delayed ejaculation is the opposite effect and can be caused as a side effect of certain medications such as SSRI's. Treatment focuses on correcting reversible causes and psychosexual counseling if needed.

Conclusion

Male sexual function is a complex interplay of psychological, neurogenic, vascular, and hormonal factors which leads to an erection and culminates in the transport of sperm out of the urethra with ejaculation. An interruption anywhere along this process leads to male sexual dysfunction. Pathophysiology of each disorder guides the treatment options for men who suffer from these conditions. Current studies are focusing on stem cells to help improve erectile function. Better treatment options are also needed in men with ejaculatory problems. Even though we have made a significant amount of progress so far, future research studies are needed to help improve treatment options in men suffering from male sexual dysfunction.

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Androgens and Benign Prostatic Hyperplasia☆

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Glossary

5-Alpha reductase (5AR) A rate limiting enzyme that converts testosterone to the more potent metabolite dihydrotestosterone (DHT). There are three isoforms of which type 2 is present in the prostate. This enzyme is the target for 5-alpha-reductase inhibitors (5AR-Is) which reduce intratissue DHT levels and AR-mediated activities, thus reducing prostate size and function.

Androgen receptor Steroid hormone group of nuclear receptors which have a dual function, acting as both intracellularly located receptors and as ligand-activated transcription factors. They play a role in prostate development and hyperplasia.

Bladder outlet obstruction (BOO) A symptom caused by an increase in detrusor pressure and reduced flow rate, that may have multiple etiologies.

Cytokines Small secreted proteins released by cells that act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). There are both pro-inflammatory cytokines and anti-inflammatory cytokines.

Chemokines Cytokines that stimulate recruitment of leukocytes, hence play a role in inflammation.

Direct rectal examination (DRE) An internal examination of the rectum performed by a physician or other healthcare professional to assess for abnormalities in the prostate, rectum or fecal abnormalities (in the case of gastrointestinal bleeding).

Lower urinary tract symptoms (LUTS) A symptom complex caused by lower urinary tract dysfunction consisting of obstructive and irritative symptoms. It is associated with, but not pathognomonic of, benign prostatic hyperplasia.

Prostate-specific antigen A normal product of the prostate that is secreted via prostatic ducts during semen emission. It may be elevated in serum, under pathologic conditions, such as prostate cancer, infection, or benign prostatic hyperplasia.

Transition zone The part of the prostate most likely to be pathologically enlarged due to benign prostatic hyperplasia. It includes the periurethral part of the prostate and is therefore involved in outflow resistance to the bladder.

Transrectal ultrasound (TRUS) An imaging modality used to evaluate the size and structure of the prostate. It is usually used to help guide prostate biopsies.

Urogenital sinus Present in the embryo and forms both urinary and reproductive organs. The upper part forms the *future urinary bladder*, while the middle and lower part forms the definitive urogenital sinus and, from it, the pelvic and phallic parts of the urethra.

The Role of Androgens in Normal Prostate Development

The Prostate

The prostate is an accessory male reproductive gland that resides in the true anatomical pelvis and forms the proximal part of the male urethra. The embryological development of the prostate gland is dependent on androgens for reciprocal growth of both epithelium and mesenchyme (undifferentiated connective tissue). In the adult prostate, androgens continue to play a role in prostate tissue homeostasis. The most common prostate pathologies are prostate cancer and benign prostatic hyperplasia (BPH); whether androgens and estrogens play a causal role in the development of these conditions is unknown. The only firmly established risk factors for BPH are increasing age, genetics and male gender (with functional testes). BPH has been traditionally associated with lower urinary tract symptoms (LUTS) which may be obstructive, irritative or both. Medical pharmacotherapy for BPH is focused upon both symptom improvement and growth inhibition.

Anatomy

The adult prostate is located below the bladder and surrounds the upper third of the male urethra. It weighs around 20g at 20 years of age and is composed of branching ducts of pseudostratified columnar epithelium surrounded by fibromuscular stroma (Toivanen and Shen, 2017).

Historically the prostate was divided into three lobes based on anatomic studies from the 1900s (Lowsley, 1915) until the McNeal model, which is now widely accepted, was proposed in the 1970s. John McNeal (McNeal, 1984) divided the prostate gland into four zones that are anatomically and histologically distinct and have pathological and histological correlations. McNeal

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noted that the prostate, when examined from different planes (coronal, transverse and longitudinal), demonstrated unique patterns, distinct from the traditional three lobes.

The four prostate zones are: peripheral, central, transitional and anterior. The peripheral zone forms the postero-inferior aspect of the gland and includes approximately 70% of the prostatic volume. This is where the majority of prostate adenocarcinomas occur. The central zone comprises approximately 25% of the prostate volume, contains the ejaculatory ducts, and is the zone that is most commonly affected by inflammatory processes such as prostatitis. The transitional zone represents only 5% of the total prostatic volume, and is where BPH occurs along with nearly 25% of prostatic adenocarcinomas. The transitional zone consists of two lateral prostate lobes together with the periurethral glands. Finally, the smallest zone by volume, the anterior zone, is mostly fibromuscular without glandular structures.

At the cellular level, the surrounding stroma serves to optimize the micro-environment for the epithelial compartment and appears to play a role in gland homeostasis in both the healthy state and during regeneration (Schauer and Rowley, 2011). The mechanisms underlying zonal predilection for different pathologies are unclear. However, gene expression profiling of each zone has revealed specific differences between the gene products that modulate cell-cell and stromal-epithelial interactions (Van Der Heul-Nieuwenhuijsen *et al.*, 2006).

Androgens and the embryology of the urogenital tract and the prostate

At conception, the mammalian embryo has the potential to develop into either female or male phenotype depending on the interplay of four critical structures: the mesonephric (Wolffian) and Müllerian ducts, the urogenital sinus (UGS) and the fetal gonad. Androgens play an integral role in the development, growth and differentiation of the prostate inducing reciprocal mesenchymal-epithelial interactions. Prostate organogenesis has been conceptually divided into four stages: sexual dimorphism, prostate budding, branching morphogenesis and epithelial differentiation.

The prostate arises from the primitive UGS, which is a caudal extension of the hindgut and derived from endoderm (Seifert *et al.*, 2008). Prior to 8 weeks gestation, the UGS and hindgut form a single excretory tract at the embryonic cloaca, which then separates into the urogenital and anorectal tracts, in a cranial to caudal direction. The UGS displays sexual dimorphism, depending on levels of circulating testosterone, with the ability to form either the prostate in males, or upper part of the vagina in females. Male embryos synthesize testosterone in the Leydig cells of the testes from the 9th week post conception (Siiteri and Wilson, 1974). Once testosterone reaches the UGS, it is converted into its more potent metabolite dihydrotestosterone (DHT) by the enzyme 5α-reductase (5AR) (Ekman, 2000). DHT binds to androgen receptor (AR), resulting in downstream nuclear signaling cascades.

During very early embryogenesis, ARs are expressed only in the mesenchyme, not on the epithelial cells. At around 10 weeks, the activation of stromal ARs stimulates growth factors and induces growth in adjacent prostatic epithelial cells resulting in prostate budding at the orifice of the paramesonephric ducts.

In the third stage of prostate organogenesis or branching morphogenesis, epithelial buds from the UGS grow into the surrounding mesenchyme, then elongate and split into a tree-like network of branches with terminal tips. These terminal tips eventually give rise to the epithelial duct system in a process providing the final size and shape of the adult prostate (Risbridger *et al.*, 2005). This process leads to the formation of the different prostatic zones within an initially uni-lobular organ (Timms, 2008). In the final stage, the solid epithelial cords become canalized to form the ductal lumen and cells undergo differentiation to give rise to functional glandular epithelium.

Histology of the prostate

The adult prostate consists of stromal and epithelial elements. The epithelium is organized as glandular acini that secrete into a lumen which then converges upon a duct, and into the urethra. There are three major differentiated cell types.

The luminal epithelial compartment is composed of androgen dependent cuboidal or columnar cells which produce PSA and acid phosphatase. Below the luminal cell layer are basal cells in one or two layers, which are attached to the basement membrane (Kurita *et al.*, 2004; McNeal, 1981; McNeal, 1988). They range from small, flattened cells with condensed chromatin and small amounts of cytoplasm to cuboidal-like cells with an increased cytoplasm and more open-appearing chromatin. These basal cells are less differentiated than secretory epithelial cells and contain no secretory products such as prostate-specific antigen (PSA). The epithelial compartment also contains specific stem cells, and rare bipotent cells, with the ability to differentiate into basal or luminal cells (Barron and Rowley, 2012; Frank and Miranti, 2013).

Of note, the prostate gland has the greatest number of neuroendocrine glands (NE) of all the genitourinary organs in the male, and expresses diverse neurosecretory products including chromogranin A (CGA) and B, serotonin, calcitonin family of peptides, thyroid-stimulating hormone-like peptide and somatostatin. It is thought that prostatic NE cells are involved in the regulation, secretion, differentiation, and proliferation of prostatic secretory and basal cells via exocrine, endocrine, paracrine, and autocrine mechanisms (Schauer and Rowley, 2011). NE cells are found in the epithelial luminal compartment and tend to be more prevalent in the major ducts compared to the acinar tissue.

Finally, the stroma is comprised of AR -positive smooth muscle cells, immune cells, nerves and blood vessels. The basal lamina surrounds the epithelium and forms a barrier between the epithelium and the stroma.

Physiology of the prostate and contribution to male reproduction

The adult prostate is an accessory reproductive gland that plays an important role in male fertility. During ejaculation alpha-adrenergic stimulation results in transport of the seminal fluid containing sperm from the ampulla of the vas deferens into the

posterior urethra. Secretions from the prostate contribute about 30% of the seminal fluid volume, the remainder contributed directly from the seminal vesicles and bulbourethral glands. Enzymes provided in prostatic secretions liquefy the seminiferous fluid to maximize sperm motility. Sperm motility is achieved via the coordinated action of the components of the two main fluids in the human seminal plasma: prostatic fluid which contains kallikreins, a specific subfamily of serine proteases, including prostate specific antigen (PSA) (Kalinska *et al.*, 2016), and semenogelin, a protein involved in the formation of a gel matrix that encases ejaculated spermatozoa secreted by the seminal vesicles (Verze *et al.*, 2016). Together with coordinated release of zinc and citrate (Costello and Franklin, 2016), the co-secretion of these fluids markedly impacts sperm motility and the final stages of spermiation (the maturation of spermatids into mature, motile spermatozoa). Prostatic fluid also increases the pH of the vaginal canal promoting increased sperm viability (Rey *et al.*, 2013; Drabovich *et al.*, 2014).

The role of androgens in adult prostate physiology

Androgens, including testosterone (T) and its more potent metabolite, DHT, play a vital role in prostate physiology. The testes produce greater than 95% of endogenous androgens in men, with the remainder produced as androstenedione by the adrenal cortex. This small amount of non-testicular androgen has a minimal impact on prostate function in physiologically normal males (Partin *et al.*, 1991). Circulating testosterone is taken up by the prostatic epithelial and stromal cells and converted to DHT by the rate limiting enzyme 5AR; type 2). There are three isoforms of 5AR (types 1–3) with type 2 predominantly expressed in the prostate whereas type 1 is the dominant isoform in hair follicles.

Both T and DHT mediate their action via binding to the androgen receptor (AR). The AR belongs to the steroid hormone group of nuclear receptors which include the estrogen receptors (ER), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR) (Tsai and O'malley, 1994). The AR is one of 48 human nuclear receptors, a subfamily of receptors that have a dual function, acting as both intracellularly located receptors and as ligand-activated transcription factors (Tan *et al.*, 2015). DHT has a greater binding affinity and a slower rate of dissociation from the AR compared to testosterone, and also upregulates AR synthesis and reduces AR turnover (Swerdloff *et al.*, 2017). The use of 5 alpha-reductase inhibitors (5AR-Is) reduces intratissue DHT levels and AR-mediated activities, thus reducing prostate size and function. These inhibitors have been used to reduce prostate hypertrophy and the symptoms of lower urinary tract obstruction in benign prostate hypertrophy (BPH) (see below).

In contrast to the prostate, there is very little conversion of T to DHT in the testes, thus circulating concentrations of DHT are much lower than T in healthy men. Circulating DHT has little impact on prostate growth. Despite a gradual decline of serum T with aging, intra-prostatic DHT concentrations in elderly men remain similar to those of young men (Bartsch *et al.*, 2002), likely because intra-prostatic 5AR remains active. In fact, intra-prostatic androgens have been shown to be independent of serum levels (Page *et al.*, 2006) and circulating androgen levels are not necessarily reflected within the prostate (Page *et al.*, 2011). Moreover, recent data indicates that DHT can be synthesized in the prostate from substrates other than T in what is termed the "backdoor" pathway and via the intracrine reverse synthesis pathway (Swerdloff *et al.*, 2017). Overall, intra-prostatic DHT is a critical component for maintenance of prostate physiology, but circulating testosterone levels are not a surrogate marker for the intra-prostatic androgen milieu under normal physiologic conditions.

The Role of Androgens in Benign Prostatic Hyperplasia (BPH)

The human prostate gland is one of the only internal organs that continue to enlarge throughout development, during adolescence and throughout adulthood. BPH involves hyperplasia of the of the stromal cell compartment and to a lesser extent, the glandular epithelium of the transitional zone of the prostate gland. Normal male aging is associated with gradual prostate enlargement, with the average prostate increasing from 25–30 g for men in their 40s, to 30–45 g for men in their 60s (Roehrborn, 2005).

BPH is a histological diagnosis, but clinical manifestations occur most commonly after significant prostatic enlargement that impairs bladder emptying, and results in LUTS. LUTS symptoms are divided into two categories; obstructive and irritative. Among the obstructive symptoms are hesitancy, straining, weak flow, prolonged voiding, partial or complete urinary retention, and, ultimately, overflow incontinence. The irritative symptoms consist of frequency, urgency with urge incontinence, nocturia, and painful urination, as well as small voided volumes.

The relationship between BPH, prostatic enlargement, and LUTS is complex. Not all men with BPH develop benign prostatic enlargement (BPE), significant LUTS or compression of the urethra with compromised urinary flow and bladder outlet obstruction (BOO). Other men who do not have histological BPH will develop LUTS due to prostate enlargement or other pathology (Emberton *et al.*, 2008), including prostatitis, prostate cancer, seminal vesical outlet obstruction (urethral stricture, bladder neck sclerosis), or bladder pathology (carcinoma in situ, inflammation and bladder calculi). Thus, clinically significant BPH is defined by the presence of LUTS; however, LUTS can occur without BPH.

Incidence and Prevalence

Clinically significant BPH has negative effects on quality of life (QOL), and represents a considerable health problem for older men. The overall prevalence of BPH has been estimated as 10%, with an overall incidence rate of 15 per 1000 man-years,

increasing with age (3 per 1000 at age 45–49 years, to 38 per 1000 at 75–79 years). Fifty percent of males exhibit some BPH symptoms between ages 51–60 years. Seventy percent of males present with BPH by the age of 70, and incidence increases to eighty percent by the age of 85 (Verhamme *et al.*, 2002). Both increased age and prostate volume increase the risk of symptomatic BPH. In the Olmsted county study (Jacobsen *et al.*, 1999), a retrospective cohort study, 2115 participants 40–79 years old underwent a voiding study and completed the American Urological Association Symptom Index (AUA-SI). A 25% random subsample underwent transrectal sonographic (TRUS) imaging of the prostate to determine prostate volume and measurement of serum PSA. During follow up, 167 men were treated for BPH, yielding an overall incidence of 16/1000 person-years. As expected, there was a strong age-related increase in risk for BPH treatment (3.3/1000 person-years for men 40–49 versus 30/1000 person-years for those >70 years old or older). Men with moderate-severe depressed peak urinary flow rates, enlarged prostate (greater than 30 mL) or elevated serum PSA (>=1.4 ng/mL) had a fourfold increased risk of BPH treatment than those who did not and an increased risk of acute urinary retention. Consistent with these results, a meta-analysis of three large multi-national trials (Marberger *et al.*, 2000) demonstrated the 2-year incidence of spontaneous acute urinary retention was higher in men with enlarged prostates (prostate volume > or = 40 mL) and higher PSA levels.

Risk Factors for BPH

Age and prostate size are the best characterized risk factors for BPH, but hormones likely play some role. It appears that the alteration in the androgen-estrogen ratio may also contribute, especially in the context of metabolic syndrome (MetS) and likely related to obesity (Tyagi *et al.*, 2018), which is also a risk factor. Hyperlipidemia is also associated with an increased risk of BPH (Shih *et al.*, 2018).

Additional risk factors include a positive family history, as early BPH may occur as a familial disease. (Sanda *et al.*, 1994). Increased physical activity, causing decreased vascular tone and oxidative stress (Vuichoud and Loughlin, 2015) decreases the risk of BPH. Alcohol taken in moderation also is associated with reduced risk of BPH (Roehrborn, 2005).

Pathophysiology

The mechanisms underlying the pathogenesis of BPH are complex and likely involve an age-related interplay of chronic inflammation, hormone dysregulation and abnormal wound repair (Schauer and Rowley, 2011).

Inflammation and wound repair

The development of BPH is closely related to an inflammatory microenvironment. The involvement of immune cells is a significant component of the disease. The stroma of patients with BPH produces various chemokines that attract these immune system cells (specifically monocytes, B and T lymphocytes) within the prostatic tissue. These cells produce various cytokines (IFN-c and IL-17) which stimulate the production of chemokines by stromal cells and these appear to contribute to hyperplasia and the development of BPH (Ricke *et al.*, 2011).

It is still unclear whether the development of BPH is due to preexisting inflammation causing hyperplasia or if prostate hyperplasia itself promotes inflammation. Studies have shown that patients with prostatitis have higher chances of developing BPH/LUTS. Chronic inflammation, which generally follows an acute inflammatory process caused by infectious agents, appears to be hormonal or metabolic abnormalities (La Vignera *et al.*, 2016), which may drive the inflammatory process. Of note, an inflammatory prostate phenotype with tissue remodeling has been demonstrated in male rabbits with low serum T and high E2, and MetS (Corona *et al.*, 2014; Vignozzi *et al.*, 2012). This has led to the idea that hypogonadism may play an important role in the development of prostate inflammation. Contributors to the development of BPH in the context of MetS include insulin resistance, pelvic atherosclerosis, and local inflammation. Increased insulin levels are associated with stimulation of the IGF-1 receptor, higher IGF-1 levels, and lower IGF-1 binding. There is also higher cytosolic-free calcium in smooth muscle and neural cells, activation of the sympathetic nervous system and increase in the prostatic smooth muscle tone. Atherosclerosis is associated with ischemia of the prostate and bladder tissues (La Vignera *et al.*, 2016). MetS itself is also involved with the production of key inflammatory cytokines (Rohrmann *et al.*, 2005; Gacci *et al.*, 2015; Lotti *et al.*, 2014; Vignozzi *et al.*, 2014).

Studies have also shown both the stroma and epithelial compartments in BPH contain reactive myofibroblasts, typical of the wound repair of inflammation-induced fibroses, and in cancers such as in the lung, liver and kidney (Schauer and Rowley, 2011). Transforming growth factor-β (TGFβ), appears to play a role in the differentiation of fibroblasts into myofibroblasts and remodeling of the stromal cells and is therefore regarded as a key inducer in the reorganization of pathogenic prostatic stromal cells (Sampson *et al.*, 2013). Other growth factors mainly secreted by stromal cells act in an autocrine or paracrine manner to maintain prostate cellular homeostasis. These growth factors include insulin-like growth factor (IGF) and fibroblast growth factors (FGFs). Alterations of these interactions can modify the balance between cell proliferation and death leading to the development of BPH (Vignozzi *et al.*, 2014).

The role of androgens

Androgen exposure during puberty is required for growth and development of the prostate, specifically DHT (see above). This is clearly demonstrated by the phenotypic changes resulting from mutations in the 5alpha-reductase-2 gene (5AR-2). Affected 46XY

individuals have high normal to elevated plasma testosterone levels with decreased DHT concentrations and elevated testosterone/DHT ratios. The prostate in adulthood is small and rudimentary, and facial and body hair is absent or decreased. Neither BPH nor prostate cancer have been reported in these patients. (Imperato-McGinley, 2002). Similarly, the prostate is small in mice with 5alpha-reductase-2 gene deletions or treated with specific 5AR-Is (Zhu and Imperato-McGinley, 2009).

The role of androgens in the development of BPH, following normal prostate development, is unclear. Firstly, serum testosterone concentrations decline with age, whilst the incidence of BPH increases (Harman *et al.*, 2001; Lenzi *et al.*, 2009). Secondly, studies have shown no correlation between prostate size and circulating testosterone levels (Roehrborn, 2008; Vignozzi *et al.*, 2014). Finally studies in older men receiving exogenous testosterone replacement therapy, resulting in elevated serum DHT levels and normal T levels, have shown minimal increases in prostate size, no impact on prostate androgen concentrations, and no known increased risk of prostate disease or LUTS (Swerdloff *et al.*, 2017).

However, the potency of 5AR-Is in reducing prostate hypertrophy and LUTS provides evidence for a role for androgens in maintaining BPH. It is important to note that 5AR-Is act on the intra-prostatic hormonal milieu reducing intra-prostatic DHT. Circulating DHT, which is much less than intra-prostatic levels, is unlikely to play a role in the pathogenesis of BPH (Vignozzi *et al.*, 2014). In addition, it appears that stromal cell AR, rather than the epithelial cell AR, promotes the development of BPH via modulation of stromal-epithelial cell interactions in both normal and abnormal prostate development (Emery *et al.*, 2007; Wen *et al.*, 2015). In addition, suppression of AR in transgenic mice that spontaneously develop BPH as a result of prolactin (PRL) overexpression has suggested that PRL may contribute to stromal cell proliferation (Lai *et al.*, 2013).

The role of estrogen

Although the prostate is commonly thought of as an androgen target tissue, estrogen also appears to play a role in both normal prostate physiology and in the development of BPH. In men, the major circulating estrogen is formed from aromatization of testosterone (via CYP19/aromatase) into estradiol-17β (E2) (Nicholson and Ricke, 2011). As men age they develop an increased estrogen to androgen ratio (due to decline in serum androgens and lesser decrease in E2) (Gray *et al.*, 1991; Roberts *et al.*, 2004) which can coincide with the development of BPH. E2 is produced in fat and, to a lesser extent, muscle in men, with only 20% secreted by Leydig cells in the testes.

E2 plays important roles in bone health, lipid metabolism and in the brain in men. However, serum levels of E2 do not necessarily reflect prostate concentrations of E2 (Vermeulen *et al.*, 2002). It has been postulated that local prostatic E2 production may influence local estrogen regulated processes and may be involved in prostatic hyperplasia (Ellem and Risbridger, 2009). Estradiol acts via two receptors, ERalpha and ERBeta, both of which are expressed in the human prostate but have differing functions. Both ERs have proliferative and antiproliferative effects on prostate cells, via multiple mechanisms including apoptosis, aromatase expression and paracrine regulation via prostaglandin E2 (Ho and Habib, 2011). Finally, estrogen may be enriched in the stroma of men with BPH, further supporting a role for E2 in the pathophysiology of BPH, although definitive data is still lacking.

Like endogenous estrogens, exogenous estrogens such as phytoestrogens, (polyphenols, flavonoids, and isoflavonoids), selective estrogen receptor modulators (SERMs), and endocrine disruptors (bisphenol-A and insecticides) may also affect estrogen action in men (Nicholson and Ricke, 2011). SERMs are compounds which modulate the activity of estrogen receptors (ERs) and may have agonist or antagonist effects in tissues, depending on the individual SERMs. The utility of SERMs in BPH remains to be evaluated but therapeutic SERMs are a promising treatment option for BPH. In contrast, exposure to BPA, DES, or E2 in developing rodents causes increased prostate susceptibility to adult-onset of dysplasia and hormonal carcinogenesis (Macey and Raynor, 2016). Although the role of BPA and other environmental estrogens in BPH has not been fully determined in humans, the effects of endocrine disruptors are likely to be observed in estrogen target tissues such as the prostate.

Clinical Evaluation

Symptomatic BPH is characterized by lower urinary tract symptoms (LUTS) that include both voiding (obstructive) symptoms and storage (irritative) symptoms. Given the difficulties in accurately diagnosing BPH in men presenting with LUTS, an initial evaluation recommended by the "American Urological Association (AUA) Guidelines on the Management of Benign Prostatic Hyperplasia (http://www.auanet.org/guidelines/bph.cfm) includes a clinical history, use of a validated questionnaire to assess symptoms, a physical examination with digital rectal examination (DRE), urinalysis and serum PSA measurement (Kevin *et al.*, 2014).

Scoring systems

There are several scoring systems currently available to quantify symptoms of BPH. The American Urologic Association (AUA) Symptom Index is widely used and consists of seven questions that assess emptying, frequency, intermittency, urgency, weak stream and straining with each graded with a score of 0-5. The International Prostate Symptom Score (IPSS) is a modification of the AUA Symptom Index adding a single question assessing the quality of life or bother score based on the patient's perception of the problem (Barry *et al.*, 1992). The questionnaire is self-administered and elicits a response score ranging from 0 to 35 points. Men scoring from 0 to 7 points are classified as not or mildly symptomatic, those scoring between 8 and 18 points as moderately symptomatic, and those scoring 19 points or greater as severely symptomatic (Roehrborn, 2005).

Progression of BPH

Given the natural history of BPH, studies have sought to identify those men at greatest risk of progression. These factors include worsening of symptoms, reduction of urinary flow rate, increase in prostate volume (PV), and outcomes such as acute urinary retention and the need for surgery either for acute urinary retention or symptomatic relief (Emberton *et al.*, 2008).

Prostate volume (PV) is perhaps the most extensively studied of the risk factors for BPH progression. Men with a PV > 30 mL are more likely to suffer moderate-to-severe symptoms, decreased flow rates, and AUR compared with men with PV < 30 mL (Anderson *et al.*, 2001). An enlarged prostate is also predictive of the need for surgical intervention. Data from the placebo arm of the Proscar Long-term Efficacy and Safety Study (PLESS) demonstrated that PSA is a strong predictor of prostate size, one that is likely to increase in size, as well as the risk of developing LUTS, poor urinary flow, acute urinary retention and/or BPH-related surgery (Roehrborn *et al.*, 1999).

Management of BPH

The initial management for BPH includes watchful waiting or medical therapy, especially in those patients with mild or moderate symptoms and no indications for surgical intervention. Treatment is often undertaken on an elective basis for such patients. Those men who develop complications (including AUR, recurrent UTIs, bladder calculi, hematuria and renal impairment) require treatment. A range of treatment options are available and should be individualized depending on symptoms, success rates, possible complications and patient preference. The standard of care for BPH includes medical and surgical treatment.

Medical therapy

First line medical therapy for men with moderate to severe LUTS secondary to BPH are alpha-blockers, including the second generation drugs terazosin and doxazosin and third generation alfuzosin and amulosin. These agents act on the alpha-1 adrenergic receptors present throughout the smooth muscle of the male urinary tract (including the stroma of the prostate), and at the level of the spinal cord ganglia and nerve terminals, and may also have extra-prostatic effects (Nitti, 2005). These agents relax the smooth muscle within the bladder neck and prostate, reducing the obstruction to urinary flow. They are most effective for men with prostate size of <30 mL (Djavan *et al.*, 2004), and reach maximal effectiveness within a few weeks of initiating therapy (Michel and Vrydag, 2006). The most common side effects, which are reduced in the more uroselective drugs (which are the later generation compounds), include light-headedness, dizziness, headache, nasal congestion and retrograde ejaculation. Despite their slightly different side effect profiles, all four second and third generation alpha-blockers have similar efficacy (Roehrborn, 2005)

While alpha-blockers are effective at improving LUTS for many patients, they do not affect prostate growth or reduce the risk of structural BPH complications. In contrast, 5AR-Is reduce prostate size, via reduction of intra-prostatic DHT, which can result in improved urinary flow rates. 5-ARIs can prevent progression of LUTS secondary to BPH and they reduce the risk of urinary retention and future prostate-related surgery. They are appropriate and effective treatment alternatives for men with LUTS secondary to BPH with known or suspected prostate enlargement.

The two 5AR-Is in clinical use are finasteride and dutasteride. Finasteride is specific for 5AR2 while dutasteride inhibits both 5AR1 and 5AR2. Both appear to be similar in terms of clinical efficacy, however, in contrast to alpha-blockers, maximal efficacy is achieved after 3–6 months of therapy. Side effects may include erectile and ejaculatory dysfunction and reduced libido. It should also be noted that 5-ARIs reduce PSA levels by approximately half, so a baseline measurement at commencement is recommended for individuals electing to use PSA for prostate cancer screening or surveillance (Blankstein *et al.*, 2016).

The combination of an α-blocker and a 5AR-I (combination therapy) is an appropriate and effective treatment for patients with LUTS associated with prostate enlargement based on volume measurement, PSA level (as an indicator of volume), and/or prostate enlargement on digital rectal examination. In early studies of one-year duration or less, combination therapy proved equal to alpha blocker therapy in efficacy and safety, but superior to 5-ARI therapy alone (Kirby *et al.*, 2003; Lepor *et al.*, 1996). However, the Medical Therapy of Prostate Symptoms (MTOPS) Study demonstrated that in the long term, among men with larger prostates, combination therapy is superior to either α-blocker or 5-ARI therapy alone in preventing progression and improving symptoms (McConnell *et al.*, 2003).

A second major combination therapy study was the slightly larger (4000 men) international, four-year, Combination of Avodart and Tamsulosin trial (CombAT) comparing tamsulosin, dutasteride and a combination of both (Roehrborn *et al.*, 2008). The primary endpoints were similar to the MTOPS Study and included progression to urinary retention and need for prostate surgery as well as symptom progression. Combination therapy was shown to be superior to either monotherapy, consistent with the MTOPS study findings.

Phosphodiesterase type 5 Inhibitors (PDE5-Is) have been shown to improve symptoms in some patients with BPH/LUTS, despite being initially approved for erectile dysfunction (Brock *et al.*, 2013; Carson *et al.*, 2014). PDE5-Is increase intracellular cyclic guanine monophosphate (cGMP), causing nitric oxide (NO) mediated reduction in smooth muscle tone in the prostate, detrusor muscle and urethra. There is, however, no effect on prostate size (Blankstein *et al.*, 2016). In terms of efficacy, tadalafil was shown to be as effective as the alpha-blocker tamulosin, with the added benefit of reducing ED (Oelke *et al.*, 2012). A recent small study showed additional administration of tadalafil to patients with LUTS with poor responses to alpha-1 blockers improved both LUTS/BPH symptoms as well as sexual function (Hayashi *et al.*, 2016). Side effects include headache, flushing, back pain and dyspepsia, and PDE5s are contraindicated in patients taking nitrates.

Some complementary and alternative therapies (CAM) have been used for symptoms of BPH. Phytotherapy refers to common plant extracts and herbs as treatment modalities, such as *Seronoa repens* (saw palmetto) and *Pyogeum africanum* (African plum).

However, the active ingredients and therapeutic dosages are unknown for most extracts and supplements, the mechanism of action is poorly understood, and the safety profile and efficacy has not been adequately studied with randomized clinical trials. A meta-analysis (Tacklind *et al.*, 2012) concluded that at double and triple doses, *Seronoa repens* did not improve urinary flow measures or prostate size in men with lower urinary tract symptoms consistent with BPH.

Surgical treatment

Surgery is recommended for those men who fail medical treatment or who develop complications of BPH. In 2015 there were 12.2 million men who were actively managed for BPH/LUTS. 54% of those men had medical management and only 1.1% had surgery or procedure (Amerson, 2015).

There are a now a number of surgical options available including minimally invasive procedures, traditional and newer transurethral approaches using electrosurgical and laser techniques, as well as robotic, open and laparoscopic techniques.

Minimally invasive surgical therapy includes transurethral needle ablation of the prostate (TUNA), transurethral microwave therapy (TUMA), or interstitial laser coagulation. Although these procedures improve flow rates and quality of life (QOL), they lack long term effectiveness when compared to transurethral resection (TURP), and do not play an important role in the long term management of LUTS secondary to BPH (Macey and Raynor, 2016).

The gold-standard treatment for BPH is transurethral resection of the prostate (TURP) which relieves obstruction by removing hyperplastic tissue in the transition zone. In most instances, the surgical procedure requires approximately 60 minutes and most patients are observed for $<=24$ hours in the hospital, dependent upon the risk for bleeding and obstruction. Various different energies have been used for cautery including traditional monopolar and the more popular bipolar electrocautery. Transurethral incision (TUIP) is usually an outpatient procedure that is suitable for men with moderate to severe LUTS and smaller prostates (<30ml). IT is associated with fewer complications but increased need for follow-up secondary procedures (Macey and Raynor, 2016).

Newer laser therapies such as GreenLight Photo Vaporisation (PVP) and Holmium laser enucleation (HoLEP) have improved the traditional TURP, by reducing length of hospital stay, need to stop anticoagulation and faster return to work (Tholomier *et al.*, 2015). Laser therapies are also associated with fewer complications such as bleeding, sexual dysfunction (retrograde ejaculation and erectile dysfunction), UTIs and urinary incontinence (Eltabey *et al.*, 2010).

Open prostatectomy is associated with increased complications when compared to TURP and is usually reserved for men unresponsive to medical therapy, those with very large volume prostates (>80 mL), and those with associated bladder pathology, such as bladder stones or diverticula. Open prostatectomy has excellent long term results with approximately 98% of patients experiencing relief of symptoms and increases in flow rate (Macey and Raynor, 2016), although there are surgical risks including sexual dysfunction and incontinence. Several approaches have been described including robotic, laparoscopic and open prostatectomy (Ferretti and Phillips, 2015). Overall surgery improves symptoms and maximum urinary flow rate (Qmax) but symptoms (particularly urinary incontinence) may persist in up to 15%–20% of men require postsurgical interventions for BPH. Such interventions include detrusor instability (DOA) and periprocedural damage to the urethra (Macey and Raynor, 2016).

Conclusion

The prostate is an accessory male reproductive organ. BPH is the result of hyperplasia of the transitional zone of the prostate, involving the stroma, to a greater extent than the epithelial compartment. The majority of men will develop BPH with advanced age, however not all men will develop LUTS. BPH with LUTS can adversely impact quality of life.

Androgens (testosterone and DHT) play a crucial role in the development and maintenance of the prostate gland micro-environment. The role of androgens in the pathophysiology of BPH in unclear, although it is likely a permissive one. Inflammation appears to drive hyperplasia as men with prostatitis are at increased risk for developing BPH, and hypogonadism may help drive inflammation. Estrogen likely plays a role in promoting prostate hyperplasia, alone and also within the altered estrogen/androgen ratio that occurs with aging. Overall, the pathogenesis of BPH is complex and likely involves an age related interplay of chronic inflammation, hormone dysregulation and abnormal wound repair.

Medical management is targeted towards relief of LUTS. There are various surgical options for men ranging from minimally invasive to open prostatectomy; TURP is the gold standard.

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Prostate Cancer☆

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Glossary

Digital rectal exam (DRE) Part of the physical exam in which a gloved, lubricated index finger is inserted into the rectum. The prostate is then inspected for asymmetry, the presence of nodular areas. These findings are considered abnormal and should lead to biopsy to evaluate for the presence of cancer. Occult fecal blood testing and palpation for rectal masses should also be performed.

Gleason grade A pathologic evaluation based on the glandular pattern of prostate cancer as examined at relatively low-power magnification. The predominant and secondary patterns of glands within the tumor are identified and each assigned a grade of 1–5, with 1 being the least aggressive and 5 being the most aggressive pattern. The two grades are added together to provide a Gleason sum score of 2–10 that reflects the aggressiveness of the tumor.

Magnetic resonance imaging (MRI) An imaging modality used to evaluate the prostate.

Peripheral zone The part of the prostate most likely to contain cancer. Most of the peripheral zone is located in the posterior part of the prostate gland, making it accessible to the transrectal approach for procedures.

Prostate biopsy Systematic removal of small amounts of tissue from the prostate that should be done with transrectal ultrasound guidance. Tissue is examined microscopically for the presence of prostate cancer.

Prostate-specific antigen (PSA) A serum marker for the presence of prostate cancer. It is normally produced by the prostate and secreted via prostatic ducts during semen emission. Under pathologic conditions, including prostate cancer, it may be elevated in serum.

Transrectal ultrasound An imaging modality to evaluate for abnormal structures within the prostate and to help guide prostate biopsies.

Pathophysiology

Most prostate cancers are adenocarcinomas that derive from glandular tissue within the prostate and typically progress locally before becoming metastatic. Other prostate tumors (<2%) include intraductal carcinoma (a variant of prostate cancer with more limited response to hormonal manipulation and radiation therapy), carcinosarcomas, squamous cell carcinoma, and urothelial carcinoma involving the prostatic urethra with or without stromal invasion (Nelson *et al.*, 2003).

Epidemiology

Prostate cancer is the second most common cancer diagnosed in men in the United States. Detection of prostate cancer cases peaked in 1995, when there were approximately 244,000 new cases and 44,000 deaths from the disease (Parker *et al.*, 1996). Subsequently, the number of cases detected per year has decreased. There will be an estimated 164,690 new cases detected in 2018, with approximately 29,430 deaths (Siegel *et al.*, 2018). The decrease in death rates is associated with the introduction of the prostate-specific antigen (PSA) test, but a causal relationship between application of PSA and decreased death rate has not been proven. Nearly all cancers are diagnosed in men between ages 45 and 89. The exact cause of prostate cancer is not well delineated, although evidence for both genetic and environmental factors exists.

Etiology

Both genetic and environmental factors affect the risk of development of prostate cancer. Twin studies have suggested that prostate cancer has a greater genetic basis than any other common neoplasm. This is reflected by the familial risk of prostate cancer development that is related to the number of men in the family affected by prostate cancer and the age of detection of tumor. For

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men who have a first-degree relative with prostate cancer detected before age 50, the risk of detection of prostate cancer increases significantly (Carter *et al.*, 1992; Gomella *et al.*, 2001; Kicinski *et al.*, 2011).

The development of familial prostate cancer (defined as the presence of three or more first-degree relatives with disease) is associated with a locus on the long arm of chromosome 1 (HPC-1). Familial prostate cancer affects less than 10% of men with this disease, but it is common (>50% of men affected) for those individuals detected with disease at younger than 53 years of age (Carter *et al.*, 1992). Several other loci have been genetically linked to the risk of development of prostate cancer, including alterations in the androgen receptor gene (Alvarez-Cubero *et al.*, 2013).

The presence of genetic factors affecting the risk of prostate cancer is also supported by data on the incidence of prostate cancer in different racial groups. The prostate cancer incidence rate is highest for men of African American descent (149/100,000 person-years), with intermediate rates for US Caucasian men (107/100,000) and lower rates for men of Asian descent (39/100,000 for Japanese men and 28/100,000 for Chinese men). For Asian men who migrate to the United States, the rate of prostate cancer increases dramatically, although it remains lower than that observed for US white males (Lichtensztajn *et al.*, 2014). Even when corrected for stage of detection and access to medical care, black men with prostate cancer tend to have lower survival rates than white men with the same disease status.

The increase in prostate cancer incidence for men who migrate from Japan to the United States suggests the presence of environmental factors, especially dietary risk factors, in the development of prostate cancer (Loeb *et al.*, 2015). However, there is controversy regarding the role of dietary factors in the development of prostate cancer. Several studies have suggested that dietary fat, especially fat from red meat, appears to be an important risk factor for the development of this disease (Sonn *et al.*, 2005). Other dietary elements may have an acute effect on PSA, a marker for prostate cancer growth. Lycopenes (a substance derived from tomato with antioxidant activity) or soy protein (a phytoestrogen) have apparent antitumoral effects in men with prostate cancer (Rowles *et al.*, 2017). It is not clear whether soy protein acts directly or only through its estrogenic activity against prostate cancer.

Hormonal factors are critical for the development of prostate cancer. Testosterone is necessary for prostate epithelium to grow, and early prostate cancer has been shown to be androgen dependent. There is no evidence that testosterone replacement in hypogonadal men induces the development of prostate cancer or transition from a localized to a more invasive cancer (Morgentaler *et al.*, 2011).

Diagnosis

Detection of Prostate Cancer

Suspicion of the presence of cancer

An abnormal DRE of the prostate or an elevated PSA blood test level suggest the presence of prostate cancer. An abnormal exam includes the presence of nodular areas of the prostate or an asymmetric prostate gland. The presence of either of these factors is an indication for prostate biopsy in men who would benefit from the diagnosis of prostate cancer (men with a >10-year life expectancy and the possibility of localized disease or those with symptoms of metastatic disease).

Prostate-Specific Antigen

Prostate specific antigen (PSA), first described in 1981, is a glycoprotein that is produced by prostate tissue and liquefies coagulated semen (Nadji *et al.*, 1981). PSA is normally secreted outside of the body in urine or semen. If PSA backs up into the body and is present at elevated levels in the blood, then an abnormal condition is present within the prostate. This abnormality may be caused by trauma to the prostate (such as occurs after biopsy or cystoscopy), infection, benign enlargement, or prostate cancer. Therefore, an elevated PSA level does not diagnose the presence of prostate cancer, and prostate biopsy is required to confirm a clinical suspicion of cancer (Adhyam and Gupta, 2012).

Since PSA levels tend to increase with prostatic enlargement, PSA can be normalized to total prostate volume using the index of PSA (ng/mL)/prostate volume (cc). Prostatic enlargement tends to occur with age; therefore, it is possible to assume increased prostatic size as men age. This approach allows assessment of age-specific PSA levels. For men younger than 50 years of age, PSA levels should be 0–2.5 ng/mL. For men 50–59 years of age, normal PSA levels are 0–3.5 ng/mL. For men 60–69 years of age, PSA should be 0–4.5 ng/mL. For men 70–79 years old, PSA levels of 0–6.5 ng/mL are considered normal (Catalona *et al.*, 1994; Loeb *et al.*, 2006).

The specificity of the PSA blood test has been improved by the use of a derivation of the PSA. PSA may exist free in serum or bound to large proteins such as α_1-antichymotrypsin inhibitor. The percentage of free PSA is a measurement of the proportion of PSA protein that is free in the bloodstream versus that which is bound to other proteins. Men with low PSA levels (<1.0 ng/mL) rarely have cancer, and the vast majority of men with markedly elevated PSA levels (>30 ng/mL) have cancer. The percentage of free PSA is useful for determining whether biopsies are needed for men with a PSA between 3 and 10 ng/mL. Prostate cancers tend to release PSA into the bloodstream that is bound to proteins, whereas benign prostatic tissue tends to release more free PSA. Therefore, men with a low percentage of free PSA (<25% free) are more likely to have prostate cancer than men with elevated percentage free levels in the presence of an abnormal serum PSA level (between 3 and 10 ng/mL) (Adhyam and Gupta, 2012; Catalona, 1996).

Prostate cancer screening

PSA based prostate cancer screening has resulted in the majority of men diagnosed with this condition having organ confined, nonmetastatic disease. However, prostate cancer screening has been controversial due to concerns of overtreatment of less aggressive disease and the typically slow time for disease progression in some patients. This limits the ability to demonstrate benefit of screening in cohorts that have not been study over a 15- to 20-year period of time. The United States Preventive Services Task Force (USPSTF) initially recommended against prostate cancer screening with PSA in 2012 due to concerns of overtreatment resulting in increased morbidity with no improvement with prostate cancer mortality (Moyer and U.S. Preventive Services Task Force, 2012). In 2017 the USPSTF revised their initial opinion and in a draft recommendation suggested PSA screening may be beneficial in men between the ages of 55–69 years (U.S. Preventive Services Task Force, 2018). They gave no recommendations in men under 55 and recommended against screening in men 70 years and older. The American Urologic Association recommends the following for PSA based screening: They do not recommend PSA screening in men under 40 years old and in men age 40–54 years old with average risk. Men aged 55–59 may benefit from PSA screening and recommends "shared decision-making." Men over 70 years old or in men with less than 10–15 years of life expectancy are advised not recommended to have PSA screening for prostate cancer (Carter *et al.*, 2013).

The two largest studies that have looked at prostate cancer screening are the European Randomized Study of Screening for Prostate Cancer (ERSPC) and the Prostate, Lung, Colorectal, and Ovarian Cancer Screening (PLCO) trials. The ERSPC trial involved seven countries in Europe and essentially demonstrated that PSA screening reduced the mortality by 21%, but this benefit is seen greater than 10 years (Schroder *et al.*, 2014). The PLCO trial was run in the United States and demonstrated no significant difference in men who were screened compared to men who were not screened (Andriole *et al.*, 2012). A major limitation and critique of the PCLO trial however is that over half of the men in the control arm were already screened.

Other tests for prostate cancer detection

Adjunctive tests used to quantify the risk of prostate cancer with men who have indeterminant PSA levels or elevated levels with prior negative biopsies have been applied. These include PCA-3, a urine test for detection of RNA from the noncoding gene, PCA3, performed after prostatic massage, and 4kScore, a blood test that evaluates a variety of forms of PSA and other proteins, and also taking into account patient characteristics (age, prior biopsy history). 4kScore results are reported as the % risk of the patient having "clinically significant" (larger or more aggressive prostate cancer) (Sartori and Chan, 2014).

Imaging of prostate cancer

The use of ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI), PET or other forms of nuclear scintigraphy (bone scan) may be indicated for detection of the location of cancer within the prostate or for evaluation of the extent of disease. US is used as an adjunctive imaging technique during prostate needle biopsy to direct random ("systematic") biopsies, but it has poor performance in localizing sites of prostate cancer inside the gland. CT and nuclear scintigraphy is predominately focused on staging of more aggressive or "higher risk" cancers (e.g., those associated with higher PSA) after a diagnosis has been made (Rifkin *et al.*, 1990). There has been great interest recently in prostate MRI as it can be used for better prediagnosis localization of disease, staging, surgical planning, radiation therapy planning, and the detection of local recurrence after treatment. The American Urologic Association and the Society of Abdominal Radiology recommend prostate MRI's use in men with elevated PSA and a prior negative prostate biopsy (Rosenkrantz *et al.*, 2016). Prostate MRI is increasing being used to help guide therapy, with some trials suggesting it may be useful as an adjunct to PSA screening by helping determine men who need biopsy. Prostate MRI generally detects larger and more aggressive tumors. PET scanning based on detection of PSMA (prostate surface membrane antigen) isolation has been suggested to provide detection of disease in some men where standard scanning or radionuclide scintigraphy is unable to identify sites of disease (Bouchelouche and Choyke, 2016).

Prostate Biopsy

Prostate cancer is detected after patients undergo biopsies of the prostate gland. Historically this is done via a transrectal approach using ultrasound guidance, although transperineal approaches are increasingly used as well. Transrectal ultrasound (TRUS) is used to evaluate prostate size and to ensure that biopsies have an adequate distribution of samples throughout the prostate gland. Typically, at least 12 core biopsies are done sampling the medial and lateral sides of the base, middle, and apex of the prostate on each side. Additional samples of the anterior (transitional zone) can be done as well. Although TRUS is used to guide the biopsies and many tumors are hypoechoic, there is no consistent diagnostic appearance of cancers on ultrasound (Harvey *et al.*, 2012).

Prostate MRI is also commonly used in further evaluating men with persistently elevated PSA levels and a negative biopsy. Lesions that are identified on the prostate MRI are then targeted to increase the accuracy of a prostate biopsy. MR Fusion biopsy machines use a computer which incorporates prostate MRI with real time US images allowing the surgeon to specifically target the area of concern seen on the MRI. This technique improves the accuracy of prostate cancer detection, particularly cancers that need to be treated (Kasivisvanathan *et al.*, 2018; Ahmed *et al.*, 2017).

Latent Versus Clinical Prostate Cancer

More than 90% of prostate cancers are adenocarcinomas. They are present as microscopic lesions in the majority of men older than age 50. These microscopic (latent) tumors cannot generally be detected by standard biopsies and are detectable only at autopsy. Latent tumors progress into clinically detectable prostate cancer in approximately 10% of all men. The mechanisms by which latent prostate cancer progresses to clinically significant and detectable tumors are poorly understood. All racial groups have similar rates of latent cancer, but the rate of progression to clinically significant cancer and death differs greatly. Therefore, some genetic factors appear to act by accelerating the rate of transformation of latent prostatic tumors into clinically significant tumors.

PSA-based screening for prostate cancer does not significantly increase the frequency of detection of small, presumably latent and clinically insignificant cancers. Therefore, it is inaccurate to state that a cancer found on biopsy is unimportant. Longitudinal series of men from Sweden (where prostate cancer management is usually expectant) who have prostate cancer and who live 10 years or more have shown that the majority who are not provided initial treatment die of the disease (Popiolek *et al.*, 2013).

Precursors of Prostate Cancer

There is no in situ carcinoma of the prostate that progresses to an invasive tumor. However, high-grade (grade II or III) prostatic intraepithelial neoplasia (PIN) is associated with invasive carcinomas. For men with high-grade PIN in the absence of an invasive carcinoma on prostate biopsies, a subsequent diagnosis of invasive cancer is made in at least 30%–50%. Low-grade PIN (PIN I) is of no clinical significance. It is not associated with subsequent detection or development of prostate cancer (Bostwick and Cheng, 2012).

Cancer Location and Volume

Based on the site of detection of early palpable and nonpalpable tumors, most (85%) prostate cancers develop out of the peripheral part of the prostate, primarily in the posterior part of the gland. At the time of detection, prostate cancers are usually multifocal, with an average of seven sites observed on serial sectioning of the entire gland. Cancers less than 0.2 cm^3 in volume and of low grade (Gleason sum <7) are rarely aggressive and are most commonly found in autopsy series or in prostates removed for benign prostatic hypertrophy (BPH). Cancers less than 4 cm^3 in volume rarely invade the seminal vesicles or metastasize to pelvic lymph nodes (Epstein *et al.*, 1994).

Grade

Historically prostate cancer was graded using the Gleason system (Gleason, 1966). This approach evaluated the glandular pattern of the tumor as examined at relatively low-power magnification. Cytologic features of the tumor are not considered in the Gleason grading system. The predominant and secondary patterns of glands within the tumor are identified and each is assigned a grade of 1–5, with 1 being the least aggressive (most differentiated) and 5 being the most aggressive (least differentiated) pattern. The two grades are added together to provide a Gleason sum score of 2–10. Gleason score is an important prognostic factor of the progression of prostate cancer and are used to stratify risk with lower risk prostate cancer's being Gleason score 6, intermediate risk prostate cancer being Gleason score 7, and high-risk prostate cancer being Gleason score 8–10. The Gleason grading system was recently replaced with the grade group (GG) system which GG 1 (Gleason score ≤ 6), GG 2 (Gleason score $3 + 4 = 7$), GG 3 (Gleason score $4 + 3 = 7$), GG 4 (Gleason score 8), and GG 5 (Gleason scores 9–10) (Epstein *et al.*, 2016a, b).

Staging

Prostate cancers are staged using the T, N, and M classification system. Localized tumors include clinical T1 or T2 tumors (N0M0), whereas clinical T3 (N0M0) tumors are considered locally advanced, and any nodal or metastatic involvement constitutes systemic prostate cancer. Clinical staging is primarily performed by DRE. The most common stage at which prostate cancers are detected is T1c (a nonpalpable lesion detected on biopsy evaluation of an elevated PSA). Nodal involvement may be detected with computed tomography (CT) of the pelvis, whereas metastases to bone (the next most common site) is primarily performed by radionuclide bone scan. For most men with localized disease (T1c, Gleason sum score 5–6, or PSA <10), there is a $<2\%$ risk of finding metastatic disease on bone scan or CT scan. MRI is not currently recommended in the staging of prostate cancer (Zaorsky *et al.*, 2012).

Treatment

Treatment of prostate cancer is generally limited to those men with a life expectancy >10 years or men with symptomatic metastatic disease. Therefore, it is possible to discuss treatment options for prostate cancer based on the stage of disease diagnosed.

Localized Disease

The goal of treatment of clinically localized disease is the eradication of all local tumor. Despite apparently effective treatment, some men with clinically localized disease may have micrometastatic disease that limits local treatment to a less than 100% success rate. Nevertheless, the majority of men with clinically localized disease are potentially curable with effective treatment.

Watchful Waiting

Many men with prostate cancer have long-term survival without aggressive treatment, this is particularly true for men with Gleason score 6 (Grade Group 1). Alternatively, for men with Gleason grade 8–10 tumors (Grade group 3, 4, 5), the 5-year death rate from prostate cancer without initial treatment may approach 50%. These observations suggest that watchful waiting may be very appropriate for patients with a life expectancy of <10 years and well-differentiated tumors. Only men with expected life expectancy greater than 10 years or symptomatic disease should be considered for treatment. Watchful waiting is reserved for men with major health issues and/or short life in which treatment is avoided to prevent its associated morbidity. Watchful waiting differs from active surveillance in its end point with watchful waiting only initiating treatment when a patient becomes symptomatic from prostate cancer, whereas with active surveillance, definitive curative treatment is delayed, but may still be offered with evidence of disease progression. For most men with more aggressive tumors and longer life expectancy, effective local treatment may be helpful to avoid death from prostate cancer. For younger men with prostate cancer, treatment may be critical to avoid death from this disease (Holmberg *et al.*, 2002; Loeb *et al.*, 2017; Wilt *et al.*, 2012).

Active Surveillance

Active surveillance (AS) is where one carefully monitors localized, low risk prostate cancer with repeated PSA, exams, and prostate biopsies in the hopes of delaying definitive treatment without compromising oncologic outcomes. The benefit of AS is that it helps men avoid the side effects (impotence and incontinence) of treatment for men with limited chance of benefiting from local treatment. AS has been seen to be safe in men with low risk, low volume prostate cancer, with up to 55% of men avoiding treatment and its associated side effects at 15 years. The largest randomized controlled study, the ProtecT trial evaluated 1643 men with localized prostate cancer to either active surveillance, radical prostatectomy, or radiation therapy and found no significant difference in prostate-cancer mortality at 10 years (Hamdy *et al.*, 2016). Indications to stop AS are rising PSA, increasing GG on repeat biopsy, increasing volume of cancer on repeat biopsy, or patient preference.

Radiation Therapy

Prostate cancer can be treated with radiation therapy. Radiation may be delivered to the prostate using external beam radiation therapy (EBRT), which is commonly applied using a three-dimensional conformal technique or brachytherapy delivered by seeds placed directly into the prostate gland, or a combination of both treatments. Radiation therapy can be given with or without androgen deprivation therapy depending on the overall risk of prostate cancer. The effectiveness of radiation therapy is limited by the relative insensitivity of prostate cancer to radiation. With increasing radiation doses, there is a lower risk of subsequent positive biopsies. When radiation doses of 60–70 Gy are delivered to the prostate, positive biopsies occur in 30%–40% of treated patients. These results are not acceptable for long-term cure, although short-term control of cancer may be provided. Only when doses higher than 81 Gy are applied do positive biopsy rates decline below 10%. This dose of radiation is difficult to apply because the small intestine, bladder, and rectum are all highly sensitive to radiation damage. Differences between EBRT and brachytherapy are delivery and dose. EBRT is using giving in fractionated doses over the course of 8–9 weeks to enable total dose of approximately 80 Gy (Mayles *et al.*, 2004; Sydes *et al.*, 2004). There are evolving techniques which allow higher fractioned doses with EBRT targeting that is based on imaging the prostate which allows a higher dose delivery which can thus shorten the treatment time. Brachytherapy is given in a single session by implanting radioactive iodine-125 or palladium-103. The dose delivered with iodine-125 is 145 Gy and 125 Gy for palladium-103 (Goy *et al.*, 2016). More recently, SBRT (short-course external beam radiation therapy) has been applied with similar effectiveness and risks of standard EBRT. The observation of locally persistent prostate cancer (on biopsy) after attempted definitive radiation provides a possible explanation for the slightly lower 10-year disease-free rates observed after radiation therapy compared to results obtained with surgical therapy. However, the difference in survival after treatment of clinically localized disease with different approaches may be due to patient selection biases for treatment approach rather than the effectiveness of the treatment. Selection biases in treatment arms can be controlled to some degree by categorization of disease status based on PSA, clinical stage, and Gleason grade. Unfortunately, disease status has to be categorized to allow comparison of different treatment regimens because randomized trials of treatment modalities for localized prostate cancer have not been performed.

Surgical Treatment

Surgical prostatectomy has been used as a treatment for prostate cancer since 1904. However, successful early results were dependent on the detection of small-volume cancers, which was rare. In addition, a poor understanding of pelvic anatomy resulted in high complication rates (impotence and infection) after surgical prostatectomy. Furthermore, the surgical margins of resection around the prostate are limited by the apposition of the prostate to the bladder and sphincteric muscles as well as the rectum.

Significant advances in the surgical treatment of prostate cancer depended on an improved understanding of the anatomic relationship of the prostate to the nerves that provide erectile function as well as the relationship of the prostate to the sphincteric muscles. These observations were made possible because of the anatomic observation of the relationship of blood vessels to the prostate. With better control of blood loss, identification and preservation of urethral sphincteric muscles and the neurovascular bundles containing the nerves responsible for erectile function became possible (Lepor *et al.*, 1985). Nerve sparing radical prostatectomy (Eggleston and Walsh, 1985) allows preservation of erectile function for 50%–80% of men after radical prostatectomy at selected centers, with incontinence rates of 2%–10%. The rate of complications is related in large part to the age of the patient. Other improvements in surgical techniques include laparoscopic and robotic assisted laparoscopic radical prostatectomy have led to shorter hospitalization times and fewer surgical complications without compromising oncologic outcomes (Lepor, 2005).

Despite the potential risks of surgical therapy, prostate cancer appears to be better controlled with surgery than radiation therapy in most series of patients with 10–15 years of follow-up (Wallis *et al.*, 2016). Thus, radical prostatectomy is the primary choice for treatment of localized prostate cancer for most men who have a life expectancy >10 years and who are candidates for a major surgical operation.

Ablative Therapies

Prostate cancer identification with imaging has continued to improve, especially with prostate MRI. The improvement of detection has led to great interest in the management of prostate cancer with ablation in which the identified region of prostate cancer is treated and the remaining prostate is not treated. Ablative techniques include cryoablation, high intensity focused ultrasound (HIFU), focused laser ablation (FLA), photodynamic therapy (PDT), and irreversible electroporation (IRE) (Chaussy and Thuroff, 2017; Gao *et al.*, 2016; Moore *et al.*, 2009; Scheltema *et al.*, 2017; Sullivan and Crawford, 2009). These techniques may provide similar oncologic outcomes for men with localized disease with an improved side effect treatment profile.

Metastatic Disease

Systemic prostate cancer may involve a wide spectrum of disease, from biochemically detected cancer detectable only on PSA blood test with no evidence of lesions on bone scan or CT scan to symptomatic, extensive metastatic lesions easily seen on bone scan with extensive replacement of bone marrow by disease. The initial treatment of systemic prostate cancer has changed little since the 1940s, when it was demonstrated that hormonal therapy with androgen deprivation can result in dramatic antitumor responses for men with disabling metastatic prostate cancer.

Hormonal Therapy

Androgen withdrawal, by either castration or medical therapy, can result in dramatic responses of PSA levels and clinical disease in the vast majority of men with advanced prostate cancer. Medical therapy usually involves administration of gonadotropin-releasing hormone (GnRH) agonists, which with tonic administration results in ablation of luteinizing hormone (LH) secretion by the pituitary and castrate levels of testosterone production by the testes. GnRH is normally released in a pulsatile fashion from the hypothalamus, in which it directly acts on the pulsatile release of LH (and follicle-stimulating hormone) from the pituitary. Tonic high-level GnRH stimulation of the pituitary (in distinction to pulsatile stimulation) results in a bimodal effect on LH secretion. Tonic pituitary stimulation (as provided by long-acting, potent GnRH agonists) causes an initial surge in LH secretion with increased testosterone, maximally noted approximately 1 week after GnRH agonist treatment. This tonic stimulation results in downregulation of GnRH receptors on the gonadotrope cells of the pituitary so that the pituitary becomes unresponsive to GnRH and LH secretion stops. The net effect of GnRH agonist treatment is an initial increase in testosterone levels (a flare effect), followed by suppression of LH with a decrease in testosterone to castrate levels within 1 month of continued treatment. No flare effect should be seen with repeated doses of an effective GnRH agonist, usually given as a depot injection or implant.

Obstruction of androgen action by competitive androgen receptor blockers can prevent the action of testosterone and other androgens on prostate cancer cells (Lepor and Shore, 2012).

Prostate cancer is remarkably androgen dependent and will usually respond to androgen deprivation for a median duration of 18 months in men with extensive prostate cancer. For some men who are treated with androgen deprivation for a prolonged period of time subsequently have progression of disease, as measured by increased PSA levels which is called castrate resistant

prostate cancer (CRPC). Cessation of the antiandrogens will result in a decrease in PSA levels for many patients. However, further treatment with antiandrogen therapy can be given as well. Antiandrogen therapy includes abiraterone (an androgen synthesis inhibitor) or enzalutamide (an antagonist of the androgen receptor) (Izumi *et al.*, 2017; Evans *et al.*, 2016; Beer *et al.*, 2014). Addition of second-line hormonal therapy can result in substantial benefits in progression-free and overall survival with limited side effects. Since abiraterone results in decreased glucocorticoid production as well as a risk of mineralocorticoid excess, patients are typically cotreated with oral prednisone 5 mg bid during abiraterone therapy (Fizazi *et al.*, 2017).

Chemotherapy

Docetaxel, an antimicrotubular chemotherapy drug, that is FDA approved in CRPC (Zhu *et al.*, 2010). It has been shown to improve survival in men with symptomatic, metastatic prostate cancer in two randomized controlled trials (Petrylak *et al.*, 2004; Tannock *et al.*, 2004). Carbazitaxel, another antimicrotubular chemotherapy drug is FDA approved from men with CRPC who have failed docetaxel therapy (Eisenberger *et al.*, 2017). Several studies have suggested that men with high risk advanced prostate cancer (e.g., those with visceral metastases or high-volume disease) may benefit from combined treatment with docetaxel at the time of initiation of androgen deprivation therapy (Sweeney *et al.*, 2015).

Immunotherapy

Sipuleucel-T is an immunotherapy that is FDA approved in the management of CRPC. It is a vaccine against prostate cancer cells after a patient's white blood cells have been trained to target a ligand specific to prostate cancer (prostatic acid phosphatase-granulocyte macrophage colony- stimulating factor (PAP-GM-CSF recombinant fusion protein). Treating men with Sipuleucel-T has been shown in a multicenter, randomized control trial to have a 22% reduction in mortality (Kantoff *et al.*, 2010).

Radiation Therapy in Metastatic Disease

Metastatic lesions typically respond to either spot radiation therapy of 8 Gy or systemic radiation directed to bony lesions, such as with intravenous injection of radium-223 dichloride, strontium-89, or samarium-153. Relief of pain occurs in 60%–80% of patients with local bone pain from metastatic prostate cancer. For men with solitary symptomatic deposits, local radiation is most appropriate, whereas patients with multiple sites of symptomatic involvement are best treated with systemic administration of strontium-89. Radium-223 dichloride is FDA approved for treatment of patients with painful castrate resistant prostate cancer bony metastasis (Saad *et al.*, 2016).

Conclusion

Prostate cancer is a significant health concern for nearly one in nine men in the United States. The management of prostate cancer is evolving as patient risk stratification has improved with some men being able to be safely monitored with active surveillance, while others can be more appropriately treated with definitive surgical or radiation therapy. Mainstay treatment of localized prostate cancer is surgery or radiation, however ablative therapies will likely become an integral component in the management of localized prostate cancer. Hormonal therapy is the mainstay of systemic therapy, but it is not a curative therapy as most tumors develop cell lines whose growth is androgen independent. Effective systemic therapies for prostate cancer are evolving and now include immunotherapy. Further understanding of the pathophysiology of prostate cancer is progressing rapidly, in conjunction with improved methods of early detection and improved local treatments.

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Insulin-Like Peptide 3 (INSL3)

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Introduction

Insulin-like peptide 3 (INSL3), previously called both Leydig insulin-like peptide (Ley-IL) and relaxin-like factor (RLF), was initially discovered in the early 1990s as a testis-specific gene transcript in boar (Adham *et al.*, 1993) and independently in mouse (Pusch *et al.*, 1996). Subsequently, INSL3 has been detected at the mRNA and protein level in the steroidogenic Leydig cells of the testes of all mature mammals studied, including humans (**Fig. 1**; Ivell *et al.*, 1997; Ivell and Anand-Ivell, 2009). Although initially named to link it with the insulin-like growth factors, IGF1 and IGF2, it is structurally and functionally more closely related to the relaxin family of insulin-like peptide hormones, which includes besides the ovarian H2-relaxin, H1-relaxin, relaxin-3, INSL4, INSL5, and INSL6 (Bathgate *et al.*, 2006).

Structure and Evolution of INSL3

INSL3 is produced in Leydig cells as a typical secreted peptide hormone, the mRNA encoding a 16–20 kD pre-pro-peptide which is cotranslationally transported first to the lumen of the endoplasmic reticulum with accompanying loss of the initial N-terminal signal peptide. It is then transported via the Golgi apparatus to secretory vesicles in preparation for secretion, which appears to be accompanied by further cleavage of two internal furin-like proteolytic cleavage sites and the ordering of three cysteine bridges. The final product is an approximately 6 kD A–B heterodimer with sulfhydryl bridges connecting the A and B chains together and a single internal sulfhydryl bridge determining the conformation of the A chain. The connecting C-peptide appears to be lost. Much of this pathway has been determined by analogy to insulin and relaxin processing. In only two species, bull and boar, has the structure of INSL3 actually been determined from the circulation in vivo (Bullesbach and Schwabe, 2002; Minagawa *et al.*, 2012). Moreover in both species, there is probably a large proportion of immunoreactive INSL3 present as the unprocessed B–C–A pro-form, which appears to be equally bioactive with the A–B heterodimer (Minagawa *et al.*, 2012). Certainly, when extracted from testicular tissue and subjected to western blotting the 12–18 kD pro-form predominates (Hombach-Klonisch *et al.*, 2004).

There is no information to date as to whether INSL3 is secreted via the so-called regulated pathway inside cells, or via a constitutive unregulated pathway. The former would imply that secretion of peptide would be dependent upon both synthesis (transcription and translation) as well as a separate process of secretory granule release, as for many pulsatile peptides, whereas the latter would exclude the second process. In support of the constitutive pathway are the observations firstly, that INSL3 secreted into the bloodstream appears only to be dependent upon the number of Leydig cells and their differentiation status, and is acutely independent of the influence of the hypothalamic–pituitary–gonadal (HPG) axis (Ivell and Anand-Ivell, 2009). Secondly, in the human, INSL3 does not follow any kind of diurnal rhythm, unlike testosterone secreted from the same cells (Chong *et al.*, 2015). It should be noted that INSL3 is produced by those cells which make testosterone and where the cytoplasm is almost completely

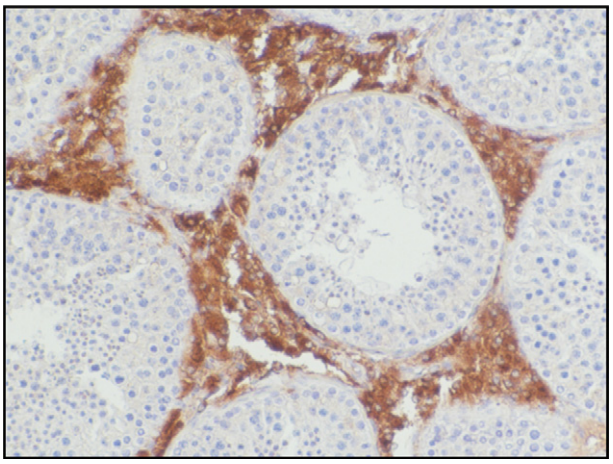

Fig. 1 INSL3 immunohistochemistry of the healthy human testis. The Leydig cells are specifically stained (*brown*) using a polyclonal antiserum which was raised against the INSL3 pro-form (Ivell *et al.*, 1997). Cell nuclei are counterstained blue using hematoxylin.

dominated by the smooth endoplasmic reticulum essential for steroidogenesis. Nevertheless, immunohistochemistry indeed shows considerable specific INSL3 staining in the cytoplasm of Leydig cells, particularly within what appears to be the Golgi apparatus (Balvers *et al.*, 1998).

Most members of the relaxin-like family of peptide hormones belong to what have been called the group of "neohormones" (Anand-Ivell *et al.*, 2013a). These are endocrine molecules which have evolved to address the physiological needs of becoming a mammal. They are hormones which specifically regulate the uniquely mammalian processes such as internal fertilization, implantation and placentation, birth, and lactation, as well as associated physiological adjustments such as osmotic balance during pregnancy. In males, this required prolonged sperm storage in the epididymis at a cooler temperature, and appropriate mating behavior. The former led to the evolution of a scrotum and the requirement for testes to relocate from a peri-renal position to the inguinal region. Studies in knockout mice show that one of the primary functions of INSL3 is to regulate the first transabdominal phase of testicular descent during gestation (Nef and Parada, 1999; Zimmermann *et al.*, 1999) (see below).

However, neohormones like INSL3 needed to evolve from something else. The presence of INSL3 in lower vertebrates such as fish suggests that INSL3 may have had an earlier role as a local autocrine/paracrine factor regulating testis function, including spermatogenesis (Assis *et al.*, 2016). This role is still evident in modern mammals (see below).

The INSL3 Gene

There is a single autosomal gene in the mammalian genome encoding INSL3 (human chromosome 19 p13.11). Altogether the gene encompasses approximately 5000 nucleotides, with the functional gene comprising two exons and a single intron (**Fig. 2**). Data from RNA-Seq and RT_PCR experiments suggest there may be minor alternative splice variants involving additional sequences from within the single intron (Zarreh Hoshyari Khah *et al.*, 1999; Truong *et al.*, 2003; https://www-ncbi-nlm-nih-gov; gene ID 3640). Surprisingly, the INSL3 gene is very close in the genome to the JAK3 (janus kinase 3) gene and in some species, such as the mouse, is actually collocated within a downstream intron of that gene (Koskimies *et al.*, 1997). The INSL3 gene

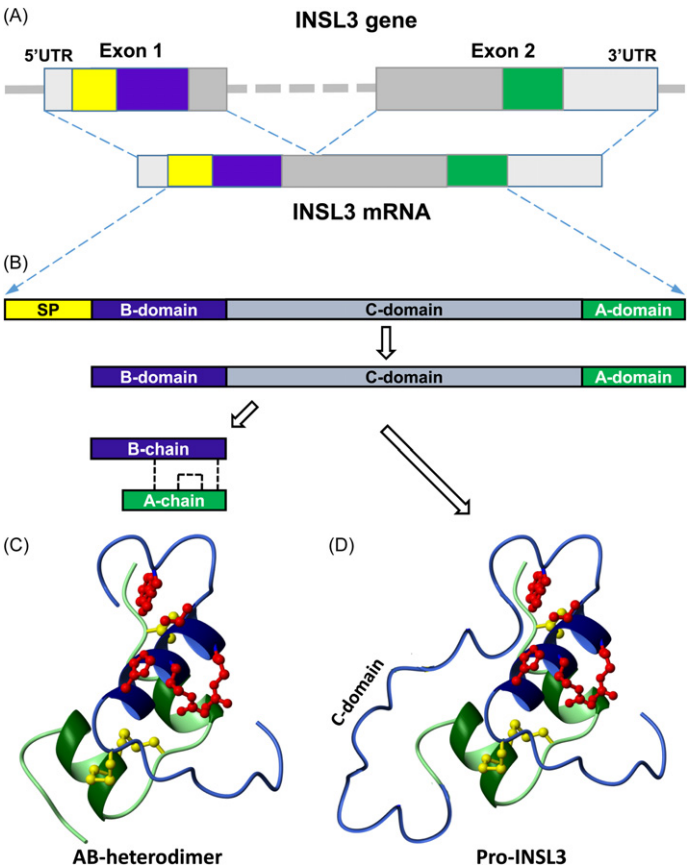

Fig. 2 Scheme to show the structure of the INSL3 gene (A), its derived mRNA, and the primary and secondary translation products (B–D). The preproform first loses its signal peptide (SP) as it is cotranslationally moved to the lumen of the endoplasmic reticulum; the resulting pro-form (pro-INSL3; D) is transported to the Golgi apparatus and thence to secretory vesicles, where it may or may not be cleaved further, with the elimination of the C (connecting) domain, to give rise to the AB-heterodimer (C). Modified after Ivell, R., Wade, J.D., Anand-Ivell, R. (2013). INSL3 as a biomarker of Leydig cell functionality. *Biology of Reproduction* 88, 147.

evidently arose by an ancient gene duplication of the relaxin gene which is still located close to the DMRT1 sex determination locus (human chr. 9p24; Ivell and Grutzner, 2009), and interestingly has maintained some of the original synteny, since the relaxin gene is still located close to the JAK2 gene (Dai *et al.*, 2017a). The proximity to the JAK3 gene also implies that it is likely that all regulatory elements governing the expression of the INSL3 gene are within the confines of only a few thousand nucleotides upstream and downstream of the INSL3 gene, where it does not overlap with JAK3 sequences, since the latter has a quite different pattern of expression.

The regulation of the INSL3 gene has been studied for human, mouse, rat, and bovine sequences (Koskimies *et al.*, 1997; Sadeghian *et al.*, 2005; Tremblay *et al.*, 2009; Dai *et al.*, 2017b). All have in common that the immediate upstream promoter region of about 1000 bp shows the presence of 2 or 3 responsive elements recognizing steroidogenic factor 1 (SF1). Moreover, where it has been studied, one or more of these *cis*-acting SF1 elements is indeed responsible for the activity of the INSL3 gene promoter (Koskimies *et al.*, 1997; Sadeghian *et al.*, 2005; Tremblay *et al.*, 2009; Dai *et al.*, 2017b). The INSL3 gene is thus, like many genes expressed in steroidogenic cells, governed by the interaction of the transcription SF1, or a paralogue like Nur77, with the upstream gene promoter region. Such transcription factors, besides providing cell-type specific activation can also act as platforms for the input from other signaling pathways, either by interaction with other transcription factors or by phosphorylation (Ivell *et al.*, 2014a). There is evidence to suggest that INSL3 may also be regulated by steroid hormones, acting via their specific receptors, although there are no specific steroid receptor responsive elements at least within the immediate region of the INSL3 gene. Using promoter-reporter constructs in mouse Leydig cells, estrogens appear to inhibit and androgens stimulate INSL3 gene expression (Lague and Tremblay, 2009; Tremblay *et al.*, 2009). Studies with other SF1-regulated genes suggest that steroid hormones can have their effect by exploiting nonclassical pathways where the activated steroid receptors do not themselves interact directly with the promoter region (Ivell *et al.*, 2014a).

RXFP2—The INSL3 Receptor

The receptor for INSL3, now known as relaxin family peptide receptor 2 (RXFP2) was the first receptor to be identified for any of the members of the relaxin-like family of peptide hormones. Originally referred to as Great, later as LGR8, and then renamed RXFP2, it was found as a genomic mutation causing cryptorchidism by a failure of the first transabdominal phase of testicular descent, with a very similar phenotype to that caused by deletion of the INSL3 gene (Gorlov *et al.*, 2002; Kumagai *et al.*, 2002). It was later shown that INSL3 is the only ligand able to activate RXFP2, and that INSL3 appears unable to activate any other receptor (Bogatcheva *et al.*, 2003), thus forming a unique ligand-receptor pair. What was truly surprising was that RXFP2 belongs to the group of G-protein coupled receptors with a typical 7-transmembrane region, and thus completely different from the receptors for insulin or the IGFs, which are membrane-linked tyrosine kinase receptors with only a single transmembrane domain. Subsequently, the receptor for relaxin was identified as RXFP1 (previously LGR7; Hsu *et al.*, 2002), as well as those for relaxin-3 and INSL5, RXFP3 and RXFP4, respectively (Bathgate *et al.*, 2006). RXFP1 and RXFP2 are unusual in having very large N-terminal extracellular domains, unlike RXFP3 and RXFP4. These extracellular domains comprise several different modules; at the N-terminus is an LDLa module, this is followed by 10 leucine-rich-repeat structures and then a hinge region before joining the first of the transmembrane domains (**Fig. 3**). Extensive molecular pharmacological studies have now shown that primary binding of INSL3 occurs via its B-chain to different parts of the leucine-rich repeats (Halls *et al.*, 2015). This induces a conformational change which moves the extracellular hormone receptor complex such that contact is made between INSL3 and some of the extracellular loops of the 7-transmembrane domain. At the same time the N-terminal LDLa module is able to induce activation of the receptor and consequently coupling to an intracellular G-protein (Kong *et al.*, 2014). Although there is ligand binding, lack of the LDLa module prevents receptor activation (Kong *et al.*, 2014).

As its name suggests, as a member of the GPCR superfamily, ligand bound RXFP2 leads to activation of an intracellular G-protein. In transfected cell systems where RXFP2 is overexpressed, this is the stimulatory G-protein, $G\alpha_s$ (Heng *et al.*, 2008), activation of which leads to the production of cAMP by adenylyl cyclase. There are few natural cell systems which express functional RXFP2. In bovine ovarian theca cells and also myometrial cells, INSL3 has also been shown to elicit an increase in cAMP production (Dai *et al.*, 2017a), and in Leydig cells INSL3 has been shown to cause an increase in testosterone production (Pathirana *et al.*, 2012), which is also considered a downstream consequence of cAMP production. However, INSL3 has been suggested to activate not $G\alpha_s$, but $G_{i/0}$ via RXFP2 expressed in oocytes (Kawamura *et al.*, 2004).

Considerable work has been done to identify and characterize potential INSL3 agonists and antagonists. For full bioactivity it is essential that the B-chain which interacts with the RXFP2 leucine-rich repeat region maintains the correct conformation. This is dependent upon the correct shape induced by the cysteine-dependent interaction with the more rigid A-chain, which is constrained by an internal cysteine bond. Hence simply scrambling the cysteine bonds, for example, by heating, is sufficient to completely inactivate INSL3. A constrained B-chain dimer has been shown to be an effective antagonist (Shabanpoor *et al.*, 2011), and several amino acids can be removed from the C- and N-termini of the A- and B-chains without jeopardizing activity (Tregear *et al.*, 2009). That these terminal amino acids are less important is also shown by the bioactivity of the pro-form where retention of the C-domain appears to have no quantitative effect on RXFP2 activation (Luo *et al.*, 2009), and can in fact be shortened considerably without loss of activity.

The gene encoding RXFP2 (human chromosome 13q13.1) is relatively large comprising 18 exons (**Fig. 3**A). Most of these encode the extracellular N-terminal domain of the receptor, where each of the 10 leucine-rich repeats is encoded by a separate

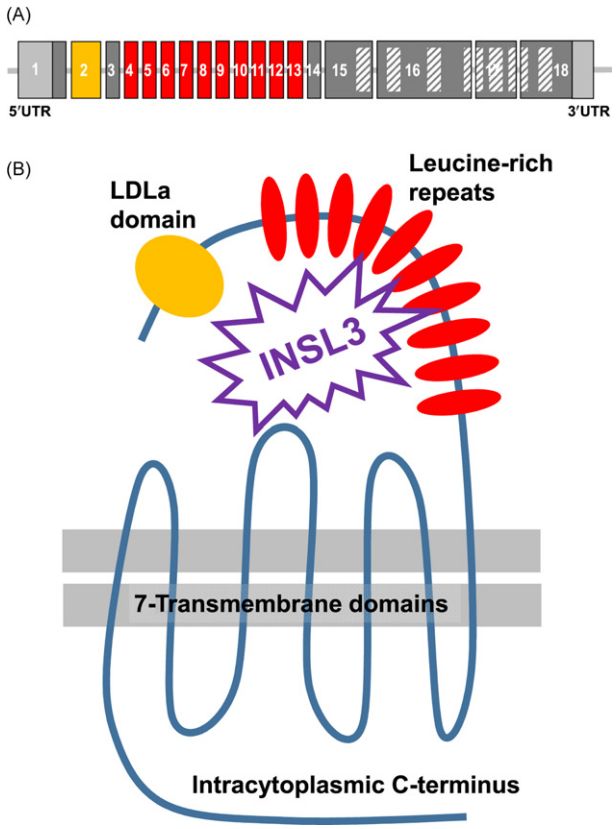

Fig. 3 Scheme to show the exon structure of the RXFP2 gene (A). The regions encoding membrane-spanning domains are indicated by cross-hatching. (B) Simplified structure of the RXFP2 receptor protein. *Yellow* and *red* colors indicate the LDLa and leucine-rich repeat domains, respectively.

short exon. RNA analysis has shown that the RXFP2 gene in different species can be expressed with multiple splice variants, many of which involve the differential splicing of exons 4–13 which encode the leucine-rich repeats (Muda *et al.*, 2005; Anand-Ivell *et al.*, 2006a; Heng *et al.*, 2008; Hanna *et al.*, 2010; Dai *et al.*, 2017a). Mostly such splice variants do not lead to frame-shifts and new stop codons, but merely produce a protein lacking one or more of the leucine-rich repeats. Whether these are expressed and functional is not known, though it seems likely that the binding of INSL3 will be compromised.

RXFP2 transcripts have been identified in several cell-types and tissues, though in few of these has its functionality been verified. Best studied are the RXFP2 receptors on the fetal gubernaculum, particularly in rodents (Kubota *et al.*, 2002; Yuan *et al.*, 2010; Kaftanovskaya *et al.*, 2011; Barthold *et al.*, 2014). These are responsible for the thickening of the gubernacular ligament which retains the fetal testes in the inguinal region during the first phase of testicular descent while the other abdominal organs are growing in an antero-dorsal direction. INSL3 has been shown to induce gubernacular cell growth also in culture (Kubota *et al.*, 2002). It is believed that expression of the RXFP2 gene in the gubernaculum is dependent on androgens produced by the fetal testis (Yuan *et al.*, 2010), though no studies have examined the regulatory elements involved at a molecular level. Within the testes, RXFP2 has been identified at both mRNA and protein level in both humans and rodents within pre- and postmeiotic germ cells and within the Leydig cells themselves, and no other cell types (Anand-Ivell *et al.*, 2006a; Feng *et al.*, 2007; Minagawa *et al.*, 2012). Interestingly, within the ovary, RXFP2 is expressed in the corresponding female cells, namely the oocytes and in the theca interna cells of growing ovarian follicles (Hanna *et al.*, 2010; Dai *et al.*, 2017a). RXFP2 expression has also been identified at the transcript level in bone cells (Ferlin *et al.*, 2008, 2011), brain (Sedaghat *et al.*, 2008), thyroid gland (Hombach-Klonisch *et al.*, 2010), and in the glomerular capsules of the kidney (Fu *et al.*, 2006). The role of INSL3 in these cells is considered in later sections.

INSL3 Antibodies and Immunoassays

Because the native INSL3 molecule is highly constrained with a defined three-dimensional structure, only antibodies which have been raised against INSL3 in its native conformation seem able to recognize the peptide when present in blood and other fluid matrices. Such antibodies do not appear to recognize well INSL3-containing moieties in western blots, probably due to antigenic epitopes being obscured during blotting, denaturation of the correct conformation during electrophoresis, and possibly simple loss of small molecules. In contrast, some antibodies have been raised against unconstrained free terminal peptides from the

B-chain, or against the denatured recombinant pro-form; these appear to be effective in immunohistochemistry or on western blots, particularly where these involve a denaturation step such as antigen retrieval. The effectiveness of the antibodies raised against the pro-form is probably due to the antigenicity of the relatively unconstrained C-peptide domain which is still present. A consequence of such observations is that western blots often only indicate the presence of the larger pro-form of INSL3 and not the 6 kD heterodimer. This does not necessarily mean that the latter is absent in vivo. Furthermore, when measuring INSL3 by immunoassay, this does not reveal the molecular form of the INSL3 which might be present as a secreted pro-form, which is similarly bioactive.

Several immunoassays have been developed against INSL3 from various species. Whilst the time-resolved fluorescent immunoassay (TRFIA) has provided consistent and robust values for INSL3 for more than a decade, with measurements usually as singlets in a range of matrices and low coefficients of variation, and detection limits as low as 10 ng L^{-1} (Ivell and Anand-Ivell, 2009), earlier radioimmunoassay and ELISA formats often gave rise to inconsistent data different from the TRFIA. Recent studies by several assays now confirm that adult men have a median INSL3 in their circulation of between 0.7 and 1.2 μg L^{-1}, depending on age, whereas women have much lower levels ranging from undetectable up to about 100 ng L^{-1} (see below). In other male mammals using different assays, circulating INSL3 can be much higher, up to 20 μg L^{-1} (**Table 1**). Interestingly, there appear to be differences in serum concentration also depending upon breed within a species (Anand-Ivell *et al.*, 2009, 2011), implying a genetic element in determining INSL3 production.

INSL3 in the Fetus

The first phase of testicular descent whereby the testes which, like the ovaries, are originally in a perirenal position, are retained in the inguinal region while the kidneys and other abdominal organs grow away in an antero-dorsal direction, is due to a thickening of the ventral gubernacular ligament and simultaneous relaxation of the cranial suspensory ligament. Loss of either INSL3 or its receptor RXFP2 in the fetus leads to a failure of the testes to relocate to the inguinal region (Ivell and Hartung, 2003). Loss of androgen action as in the *tfm* mouse leads to retention of the cranial suspensory ligament and the positioning of the fetal testes somewhere between the kidneys and the inguinal region, since now both ventral and cranial ligaments are competing to position the fetal testes. The second phase of testicular descent which involves the movement of the testis and epididymis from above the inguinal ring in the abdominal cavity through the inguinal canal into the scrotum, appears to require androgens and possibly also the anti-Müllerian hormone (AMH), which is also responsible for the involution of the Müllerian duct in the male fetus. Whether INSL3 and RXFP2 are involved and/or essential for this second phase of testicular descent is not clear (Yuan *et al.*, 2010).

There are important species differences in the timing of testicular descent in the male fetus (Anand-Ivell and Ivell, 2014). Whereas in humans and in ruminants, the first INSL3-dependent phase begins early, during the transition from the first to second

Table 1 Average blood levels of INSL3 in different mammalian species

Species	*Breed*	*Sex*	*Age*	*Mean INSL3 (μg L^{-1})*	*References*
Human		Male	35–40 years	1.29 ± 0.47	Anand-Ivell *et al.* (2006b)
		Male	75–80 years	0.79 ± 0.39	Anand-Ivell *et al.* (2006b)
		Female	Adult	<0.10	Anand-Ivell *et al.* (2013b)
Macaque		Male	Adult	~0.42	Hanna *et al.* (2010)
		Female	Adult	~0.15	Hanna *et al.* (2010)
Rat	Sprague–Dawley	Male	3 months	2.80 ± 0.17	Anand-Ivell *et al.* (2009)
	Sprague–Dawley	Male	3 months	~3.5	Heng *et al.* (2012)
	Sprague–Dawley	Male	>22 months	0.94 ± 0.03	Anand-Ivell *et al.* (2009)
	Sprague–Dawley	Female	3 months	0.08 ± 0.03	Anand-Ivell *et al.* (2009)
	Wistar	Male	3 months	1.50 ± 0.09	Anand-Ivell *et al.* (2009)
	Wistar	Male	>22months	0.76 ± 0.21	Anand-Ivell *et al.* (2009)
	Wistar	Female	>22 months	0.08 ± 0.02	Anand-Ivell *et al.* (2009)
Mouse	CBA	Male	Adult	0.78 ± 0.03	Anand-Ivell *et al.* (2009)
	CBA	Female	Adult	0.05 ± 0.002	Anand-Ivell *et al.* (2009)
Pig	Duroc	Male	Adult	~12.5	Minagawa *et al.* (2012)
	Germ. Landrace	Female	Adult	0.1–0.2	Vernunft *et al.* (2016)
	Germ. Landrace	Male	Fetus	1.5–2.0	Vernunft *et al.* (2016)
Bovine	Japanese Black	Male	Adult	~8.0	Hannan *et al.* (2015)
	Japanese Black	Male	Adult	~18.0	Kawate *et al.* (2011)
	Angus	Male	Fetus	~3.2	Anand-Ivell *et al.* (2011)
	Brahman	Male	Fetus	~2.0	Anand-Ivell *et al.* (2011)
	Brahman/Angus	Female	Adult	~0.2	Anand-Ivell *et al.* (2011)
Ovine	Merino	Female	Adult	0.1–0.2	Anand-Ivell *et al.* (2011)

trimester of gestation, in pigs and dogs this phase occurs somewhat later during mid-gestation, and in rodents this phase does not occur until close to the end of the third trimester. This is important to understand since factors influencing testicular descent in rodents may have quite different phenotypic associations compared to factors affecting testicular descent in humans. Similarly, the sensitive exposure window for impacts on testis function and testis descent in rodents is quite late (70%–80% of gestation) compared to humans (25%–50% of gestation).

Corresponding to the first phase of testicular descent, analysis of human amniotic fluid samples collected at routine amniocentesis shows highest INSL3 concentration from weeks 12–16 of pregnancy, declining thereafter (Anand-Ivell *et al.*, 2008). In contrast, highest concentrations of INSL3 in fetal rat serum occur on days 17 and 19 of gestation (Anand-Ivell and Ivell, 2014). Importantly, for monotocous species such as the human or the cow, with mostly only a single fetus, fetal INSL3 can only be detected in body fluids derived from male fetuses (Anand-Ivell *et al.*, 2008, 2011); in female fetuses, which have no testes, and where the ovaries are hormonally relatively quiescent, INSL3 is consistently below the level of detection. This is not true for polytocous species, such as the pig, with multiple fetuses within a pregnancy, where we have shown that INSL3 is indeed detectable in amniotic and allantoic fluids as well as in fetal serum from female fetuses, albeit at lower levels than in male fetuses (Vernunft *et al.*, 2016). Detailed analysis of fetal location during pregnancy makes it clear that particularly early in gestation INSL3 is being transported from male fetuses to neighboring female fetuses (Vernunft *et al.*, 2016). Whether this has any consequences for the affected female fetuses is not known.

In the bovine, we could show that INSL3 is evidently also crossing the placenta from male fetuses at mid-gestation to cause a significant increase in circulating maternal INSL3 (Anand-Ivell *et al.*, 2011). Since only male fetuses and neither female fetuses nor the pregnant mothers are producing INSL3, this means that INSL3 is an absolutely fetal gender-specific hormone able to influence either placental or maternal physiology if its receptor RXFP2 is expressed. There are numerous reported instances for women of fetal gender impacting on maternal pathology or physiology (Clifton, 2010; Leon-Garcia *et al.*, 2016); while it has often been assumed that this is due to fetal steroid hormones, these are much less fetal gender-specific than is the case for INSL3 (Anand-Ivell *et al.*, 2008). Future research will determine whether fetal INSL3 is indeed part of a fetal gender recognition system. Another aspect of this is that measurement of maternal INSL3, at least in mid bovine gestation, can be used as an effective indicator of fetal gender (Anand-Ivell *et al.*, 2011). Whether this is true for other species has not been studied.

Testis development and descent is proceeding very actively in the transition from first to second trimester in the human. Thus any factor which reflects this organogenetic activity is also acting as an overarching sentinel of fetal wellbeing in this important but analytically obscure phase of human pregnancy. INSL3 can be considered therefore as a significant indicator of pregnancy health and the effect of a range of environmental impacts (Anand-Ivell and Ivell, 2014). In one study it was shown that mid-gestation amniotic INSL3 was significantly higher in women who later became preeclamptic than in controls (Anand-Ivell *et al.*, 2008). In another study, amniotic INSL3 concentration was negatively affected by maternal smoking, unlike testosterone, and was also negatively impacted by maternal exposures to certain phthalate metabolites, known to affect male reproductive parameters in rodent studies (Jensen *et al.*, 2015). Also the ubiquitous endocrine–disrupting chemical PFOS was shown to be negatively correlated with INSL3 in the same large cohort of human amniotic fluid samples (Toft *et al.*, 2016). Due to improvements in assessment of fetal cell DNA analysis, mid-gestation human amniotic fluid is unlikely to continue being accessed routinely in the future. Nevertheless, this does offer one of the few media which can provide considerable endocrine information about fetal wellbeing. Perhaps improved future methodology might be able to revive this valuable diagnostic opportunity.

As an alternative to measuring INSL3 in amniotic fluid, several studies have attempted to make use of cord blood (Bay *et al.*, 2007; Araki *et al.*, 2014; Mitsui *et al.*, 2015; Chevalier *et al.*, 2015; Fenichel *et al.*, 2015). Inevitably, INSL3 concentration is already reduced in venous fetal blood at term of pregnancy, so long after the events of testis development and descent. As anticipated, significant differences in INSL3 concentration can be detected between cord bloods from male and female fetuses. Moreover, independent studies indeed suggest a negative effect at the population level of maternal phthalate or bisphenol A exposure on cord blood INSL3 levels (Araki *et al.*, 2014; Chevalier *et al.*, 2015). One study also showed a negative relationship between cord blood INSL3 and the incidence of idiopathic cryptorchidism (Fenichel *et al.*, 2015). The latter is somewhat surprising, given that the recent large study of Danish amniotic fluids collected at the end of the first trimester of pregnancy failed to show any relationship between amniotic INSL3 concentration and the incidence of cryptorchidism or hypospadias (Jensen *et al.*, 2015).

In the male fetus, INSL3 is made exclusively by the steroidogenic Leydig cells of the fetal testes, and although these appear to have a common origin with cells of the adrenal gland, the latter do not appear to express INSL3, nor do any cells in the ovaries of female fetuses. There have been numerous experimental studies examining the impacts of a range of putative endocrine disrupting chemicals either in pregnant rodents or using explanted human fetal tissues. Early studies exposing pregnant rodents to estrogens, such as estradiol or diethylstilbestrol (DES), confirmed findings from women treated with DES during pregnancy that this steroid at moderately high concentration effectively impacted on testis development leading to cryptorchidism. This was apparently due to a suppression of Leydig cell differentiation and loss of INSL3 expression (Emmen *et al.*, 2000). Other compounds such as phthalates were shown to have a similar effect, at least in rats, leading to what has been named the "phthalate syndrome" (Foster, 2006), with resultant testicular dysgenesis and cryptorchidism, and loss of INSL3 expression in the late gestation male fetus. Studies with human fetal testis xenograft or explant cultures have only partly confirmed this effect of phthalates on fetal Leydig cell function and INSL3 expression (Albert and Jegou, 2014). More recently, it has been shown that exposure to common analgesics such as paracetamol, aspirin or ibuprofen can also impact on Leydig cell differentiation, using such culture systems (Mazaud-Guittot *et al.*, 2013; Ben Maamar *et al.*, 2017). Importantly, it could be shown that the age of the fetal testis (gestational weeks

10–12) was critical in order to see the negative effects (Ben Maamar *et al.*, 2017), younger or older testes failing to respond, again emphasizing the importance of a critical "window of sensitivity."

Considerable research effort in this area has shown that there are differences between rodents and humans in terms of sensitivity to endocrine-disrupting chemicals and in terms of the time windows of exposure. Moreover, often high experimental exposure levels are required before significant effects are evident. In real life, exposures will always be multiple and complex. Importantly, INSL3 has shown itself to be a sensitive and specific biomarker for testis development during these critical phases, both in the human and in rodent studies.

INSL3 in Male Puberty

Following birth and the descent of the testes into the scrotum there is usually a shorter or longer period of relative hormone quiescence in all mammals. In humans, where this period lasts several years, there is usually a short phase, referred to as the "minipuberty" at about 3 months of age. This is associated with an increase in circulating testosterone thought to be important in masculinizing the brain and perhaps finalizing the location of the testes in the scrotum. There appears to be a small increase in circulating INSL3 at this time in most male infants (Bay *et al.*, 2007), although this has not been studied in depth.

The postnatal prepuberal period is typified by an involuted testis with small relatively undifferentiated Leydig cells, which at least in rodents appear to be a product of an independent lineage from the fetal Leydig cells. These Leydig stem cells are located around the seminiferous tubules and are activated to proliferate and then differentiate under the influence of pituitary LH as puberty progresses. Once puberty is complete, there appears to be very little if any further proliferation of Leydig cells, nor is there any evidence of substantial cell death, such that the complement of Leydig cells present in the testes at the end of puberty remains roughly the same throughout life.

Puberty itself is initiated with the increase in GnRH pulsatility and pituitary gonadotrope activity, leading to increased circulating testosterone levels, and accompanying signs of male hormone activity, such as the onset of spermatogenesis, skeletal development and pubic hair. As end-points, the hormones of the hypothalamic–pituitary–gonadal (HPG) axis, particularly during puberty and particularly for testosterone, are very variable with massive diurnal and within-individual variations. In contrast, INSL3 appears to be a very good monitor of pubertal progression in young male mammals (Ivell *et al.*, 2014b). The reason for this is that INSL3 appears to be secreted only by relatively mature Leydig cells and in a constitutive manner, independently of any acute stimulation or effects of the HPG axis (Ivell and Anand-Ivell, 2009). Circulating INSL3 uniquely reflects the number and differentiation status of the Leydig cells in the testes. Consequently, unlike testosterone, it does not show any diurnal variation (Chong *et al.*, 2015), and repeat sampling within an individual appears to be remarkably consistent. In laboratory rats, INSL3 increases to a maximum at around the time of the first appearance of sperm in the ejaculate (42 days) and subsequently declines to an adult level which changes little in ensuing months (Anand-Ivell *et al.*, 2009). This profile is interesting in that it appears to reflect closely the dramatic activity of the HPG axis during pubertal development, followed by the gradual decline in such activity once puberty has been attained and hormones become stabilized during young adulthood. Unfortunately, similarly detailed profiles are not yet available for other species, including humans. Of the studies carried out in humans, most only continued to Tanner stage 5 (about 14–15 years) and thus preclude later events (Wikstrom *et al.*, 2006; Ferlin *et al.*, 2006; Johansen *et al.*, 2014). Such studies also encompass large between-individual variability due to the differences in the ages at which boys enter puberty. Future research will need to explore the origins of such large variation. Evidence from experimental animals strongly suggests that environmental factors, such as maternal exposure to endocrine-disrupting chemicals can alter the pubertal trajectory (Anand-Ivell and Ivell, 2014). Also childhood nutrition and obesity may significantly impact on pubertal progression (Tinggaard *et al.*, 2012).

INSL3, as the only constitutive Leydig cell biomarker, is optimally suited to investigate the normal physiology and pathology of pubertal progression in male mammals and will ideally complement visual Tanner staging or estimates of scrotal size. Not only can it be used to confirm primary or secondary hypogonadism (Trabado *et al.*, 2014), but as a quantitatively sensitive and constitutive biomarker it offers the potential to differentiate a wide range of pubertal pathologies.

Seasonally breeding mammals, such as deer or hamsters appear to pass through sequential phases of testis involution and regeneration, in a day-length dependent manner, not dissimilar to what occurs in puberty. INSL3 immunohistochemistry of the Leydig cells accurately reflects these differentiation changes (Ivell *et al.*, 2003; Hombach-Klonisch *et al.*, 2004), suggesting that circulating INSL3 could be used effectively as a biomarker to monitor these seasonal changes.

INSL3 and the Aging Male

Measurement of INSL3 in men across the lifespan shows that Leydig cell functional capacity (numbers × differentiation status) appears to decline in old age (Anand-Ivell *et al.*, 2006b). Unlike the other Leydig cell product, testosterone, which declines at the population level at approximately 7% per decade, INSL3 declines at closer to 14% per decade. This difference is due to the fact that testosterone feeds back to the HPG axis and is acutely regulated by LH from the pituitary gland; as testosterone decreases, LH secretion is increased in order to compensate the HPG axis. This does not occur for INSL3, which being constitutively expressed is not acutely stimulated by LH and thus not compensated in the same way. Consequently, INSL3 accurately reflects the overall functional capacity of the Leydig cells, also to make testosterone. Multiple correlation analysis shows that INSL3 correlates closely

with total testosterone, and is negatively associated with smoking, as well as with obesity (as BMI) and muscle weakness (Atlantis *et al.*, 2009). Because INSL3 is expressed constitutively and is not subject to the large within-individual variation observed with testosterone, it is likely that INSL3 will prove to be a very useful biomarker for testis function in future aging studies.

A similar decline with age in testicular INSL3 production is also evident in rodents (Paust *et al.*, 2002; Anand-Ivell *et al.*, 2009), so that these can be used effectively to assess testicular changes in response to aging.

INSL3 and Testicular Cancer

There is little information regarding INSL3 in the context of testicular cancer. Whereas Leydig cell hyperplasia indicates an increase in the number of Leydig cells, these do not appear to show much alteration in INSL3 expression within individual cells (Lakis *et al.*, 2017). In contrast, where Leydig cells have formed true tumors, these cells appear to be less differentiated, showing only very little or no immunohistochemical staining for INSL3 (Klonisch *et al.*, 1999; Lakis *et al.*, 2017). However, where there is marked testicular dysgenesis from various causes, Leydig cells may exhibit a broad range of INSL3 expression (Lottrup *et al.*, 2014).

INSL3 in the Female

Although INSL3 has maximal expression in the male, it is nevertheless also expressed at lower levels in the adult female mammal. It is expressed in the ovary at both mRNA and peptide levels, particularly within the steroidogenic theca interna cells of healthy growing antral follicles (Bamberger *et al.*, 1999; Irving-Rodgers *et al.*, 2002). These are the equivalent cells in the female to the Leydig cells in the male. Using the bovine model, it has been shown that theca cell INSL3 expression is essential to promote the formation of androstenedione by theca cells, and that this is controlled in an autocrine/paracrine manner via RXFP2 receptors present on the theca cells themselves, which respond to INSL3 with an elevation of cAMP (Glister *et al.*, 2013; Dai *et al.*, 2017a). In synchronized bovine cycles, INSL3 has been measured in blood in exact correspondence with the growth of antral follicles within a wave (Satchell *et al.*, 2013). Comparable results have been observed for the human, where INSL3 in the blood of healthy young women also appears to follow a pattern representing repeated waves of follicle growth, achieving a maximum concentration of approximately 100 ng L^{-1} (Anand-Ivell *et al.*, 2013b). Moreover, circulating INSL3 concentration correlates significantly with antral follicle count measured by ultrasound (Anand-Ivell *et al.*, 2013b). Assessment of INSL3 in a large population of women attending an infertility clinic indicated that only low ovarian reserve (reduced) or polycystic ovarian syndrome (PCOS; increased) significantly altered the circulating levels of INSL3 (Anand-Ivell *et al.*, 2013b). Whether, INSL3 is directly involved in the etiology of PCOS is not known, since the higher circulating levels of INSL3 may simply reflect the increased theca cell mass in these subjects. Nevertheless a genetic study has shown a significant association between one INSL3 polymorphism and the incidence of PCOS (Shaikh *et al.*, 2016), indirectly suggesting a possible causative interaction. Measurement of INSL3 in follicular fluid from healthy cows (Dai *et al.*, 2017a) and monkeys (Hanna *et al.*, 2010) shows that concentrations are quite high (>100 μg L^{-1}).

In mice, where the INSL3 gene has been inactivated, the females show reduced fertility with a smaller litter size and fewer corpora lutea (and hence fewer follicles) (Spanel-Borowski *et al.*, 2001). Interestingly, not only theca cells have RXFP2 receptors, but also the oocytes themselves in rodents and cows express RXFP2. How these receptors are involved in oocyte function is not yet clear, though preliminary studies in mice suggest that INSL3 may induce increased intracellular $Ca2^{+}$ in the oocytes (Kawamura *et al.*, 2004). Although the follicles appear to be the major source of INSL3 in the ovary, INSL3 mRNA and immunoreactivity have also been detected in corpora lutea from human, monkey, bovine and mouse ovaries (Bathgate *et al.*, 1996; Balvers *et al.*, 1998; Bamberger *et al.*, 1999; Hanna *et al.*, 2010); however, comparison with the patterns of INSL3 in the blood implies that the corpora lutea are probably not contributing to the circulating hormone.

Because INSL3 is produced mostly from growing antral follicles, it cannot be measured in blood from prepuberal girls (Hagen *et al.*, 2015), nor is it detectable in postmenopausal women (Ivell and Anand-Ivell, 2009).

Physiology of INSL3

INSL3 and Bone Metabolism

Mice in which the INSL3 gene has been inactivated or humans carrying a deleterious mutation in the gene for RXFP2 both consistently exhibit a high degree of osteopenia or osteoporosis (Ferlin *et al.*, 2008). Subsequent studies using cultured osteoblast and osteoclasts indicate that INSL3, acting via RXFP2, can directly modulate bone cell metabolism (Ferlin *et al.*, 2011). This is important since it shows that aging or age-related bone pathology might not only be dependent on diminishing steroid production from the testes or ovaries, as is generally conceived, but that the associated loss of circulating INSL3 is also implicated.

INSL3 and Horn Growth in Ruminants

Not unrelated to bone metabolism, genetic studies in ruminants have also shown that the INSL3–RXFP2 system is evidently also involved in horn growth, with particular SNPs significantly associated with larger and stronger horns (Johnston *et al.*, 2013).

INSL3 and the Kidney

The kidney has been identified as also expressing RXFP2 receptors, specifically within the mesangial cells of the developing glomeruli (Fu *et al.*, 2006). Moreover, treatment of primary rat glomerular cells in culture with INSL3 indicates a significant effect on cell growth (Fu *et al.*, 2006). Taken together with an observed altered electrolyte balance in RXFP2-deficient mice (http://www.informatics.jax.org/), this would indicate that INSL3, at least in the male, may be involved in renal function.

Spermatogenesis

RXFP2 receptors are present on pre- and postmeiotic stages of male germ cells in rodents and humans (Kawamura *et al.*, 2004; Anand-Ivell *et al.*, 2006a,b). Whilst studies in mice have been problematic due to the primary phenotype of cryptorchidism, other studies suggest that INSL3 may exert an antiapoptotic or pro-survival influence on male germ cells. In humans subject to a steroidal contraceptive regimen, it was shown that maximum reduction in sperm counts was attained significantly in those men with lowest circulating INSL3 concentration (Amory *et al.*, 2007). Moreover, active immunization of boars using an INSL3 immunogen also indicated a significant increase in apoptotic germ cells in the testes and reduced testis weight (Sagata *et al.*, 2015). Injection of a specific INSL3 antagonist into the testes of rats also leads to a significant reduction in size of the treated testes with notable germ cell apoptosis (Del Borgo *et al.*, 2006). Analysis of INSL3 peptide in the testes of rats and boars shows that, as expected, greatest INSL3 concentrations are found in the immediate neighborhood of the Leydig cells in the interstitial space (200–400 μg L^{-1}), but nevertheless significant amounts of the peptide are also transported across the blood-testis barrier to reach the seminiferous compartment (**Fig. 4**; Anand-Ivell *et al.*, 2009; Minagawa *et al.*, 2012) and hence are able to interact with RXFP2 on both pre- and postmeiotic germ cells.

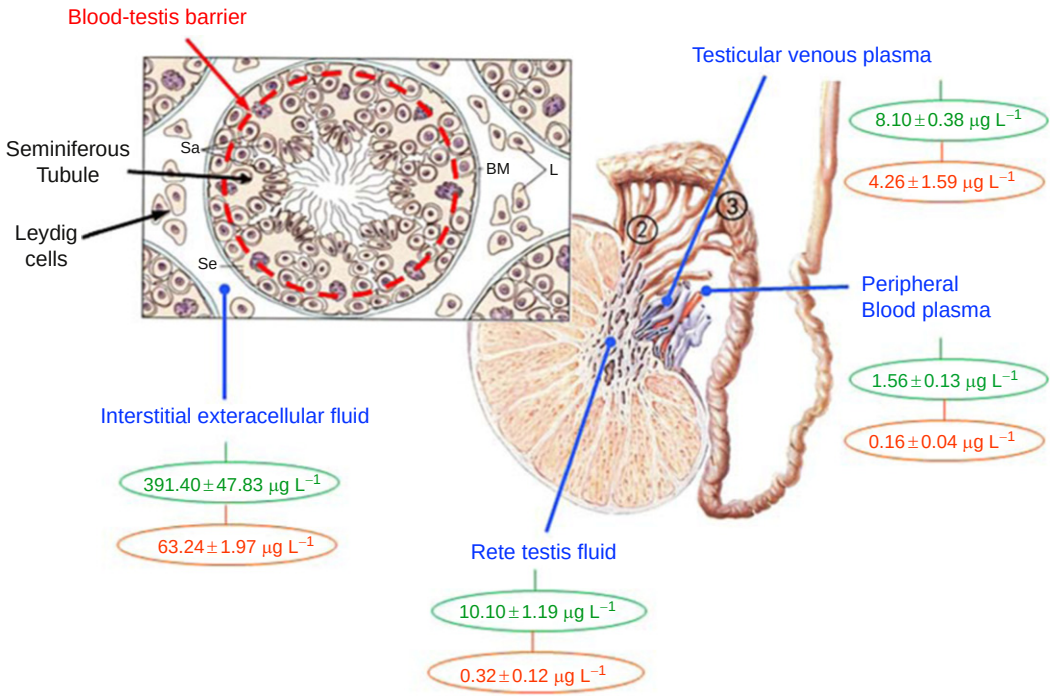

Fig. 4 The dynamics of INSL3 flow within the rat testis. Rat INSL3 was firstly measured (*green* data lozenges) using a species-specific immunoassay in different testicular fluid compartments (as indicated). In a second set of experiments human INSL3 was injected into the interstitial extracellular fluid of the rat testis and its distribution monitored after 30 min using a human INSL3 specific immunoassay (*orange* data lozenges) which did not recognize rat INSL3 at all (for details see Anand-Ivell, R., Heng, K., Hafen, B., Setchell, B., Ivell, R. (2009). Dynamics of INSL3 peptide expression in the rodent testis. *Biology of Reproduction* 81, 480–487, reproduced with permission). The findings clearly show that INSL3 from the interstitial fluid surrounding the Leydig cells is able to be transported not only into the bloodstream but importantly also across the blood-testis barrier to the seminiferous compartment where it can interact with RXFP2 receptors present on male germ cells.

INSL3 and RXFP2 in the Brain

RXFP2 has been described throughout the thalamic region of the rat brain, particularly in those areas of the sensorimotor system responding to dopaminergic innervation (Sedaghat *et al.*, 2008). Moreover, preliminary phenotypic observation suggests mild behavioral impairment and hypoactivity in mice in which the *rxfp2* gene has been inactivated (http://www.informatics.jax.org/), which would support a role for the INSL3–RXFP2 system in the rodent brain. The ligand itself has not been detected in the brain or its regions in mice or bovine by northern hybridization (Pusch *et al.*, 1996; Bathgate *et al.*, 1996), but it is plausible that low levels of peptide may be produced locally within the brain. Whether circulating INSL3 in the blood can cross the blood-brain barrier is not known.

INSL3 and the Adrenal Gland

Although steroidogenic adrenocortical cells appears to share a common embryological origin with at least a subpopulation of fetal Leydig cells, the normal healthy adrenal gland does not express the INSL3 gene in rodents or humans. Nevertheless, certain adrenal pathologies do appear to show INSL3 expression within regions of macronodular adrenal hyperplasia (Lefebvre *et al.*, 2003). Also in a mouse model of pituitary hyperstimulation, caused by gonadectomy, INSL3 can be detected in the neoplastically enlarged adrenal glands (Schillebeeckx *et al.*, 2015).

Other Systems (Thyroid, Mammary, Prostate)

Using a combination of immunohistochemistry and RT-PCR, components of the INSL3–RXFP2 system have also been detected, particularly within the epithelial cells of the thyroid, mammary and prostate glands (Hombach-Klonisch *et al.*, 2000, 2004; Klonisch *et al.*, 2005). Of note is that for the thyroid the system was most expressed in neoplastic and hyperplastic cells, though not in normal tissues (Hombach-Klonisch *et al.*, 2004). The reverse appears to be the case for the mammary gland (Hombach-Klonisch *et al.*, 2000), and for the prostate gland, some carcinoma-derived cell-lines appeared to express both receptor and ligand, as was also suggested by the studies of benign hyperplastic prostate tissue (Klonisch *et al.*, 2005).

Genetics and Variants of INSL3 and RXFP2

Both INSL3 and RXFP2 are autosomally expressed genes. Since they form an essential hormone-receptor system responsible for testicular descent any dominant or homozygous mutation in either gene is likely to lead to cryptorchidism and infertility. Consequently, only mutations are found in the human population which are nonfunctional, recessive, or otherwise spontaneous and extremely rare. For INSL3 there are three common polymorphisms identified, G27A, G126A, G178A, only the last of which leads to a missense mutation (Ala/Thr). However these do not appear to be associated specifically with cryptorchidism and are equally represented in cryptorchids and normal controls (Koskimies *et al.*, 2000; Krausz *et al.*, 2000; Takahashi *et al.*, 2001; Lim *et al.*, 2001; Baker *et al.*, 2002; Feng *et al.*, 2004; Mamoulakis *et al.*, 2014; Huang *et al.*, 2016). Nor does there appear to be any association between these variants and male infertility (Yun *et al.*, 2007). More recently, for a Moroccan population, El Houate *et al.* (2007) identified three new INSL3 variants in 109 cryptorchid men and in none of 270 controls. One of these was in the gene promoter (C-19G), and two were missense mutations in the coding region (G52A, G319A). Of the latter the G52A variant caused a change from valine to methionine and could be shown in vitro to cause a significant reduction in cAMP production using a transfected RXFP2 system (El Houate *et al.*, 2007).

For RXFP2 the situation appears to be similar. Whilst SNPs associated with RXFP2 do turn up as significantly associated in some GWAS studies, for example with temporomandibular disorder in women (Sanders *et al.*, 2017), or with udder conformation traits in cows (Pausch *et al.*, 2016), no variant is consistently and significantly linked to cryptorchidism. Nevertheless, two missense variants (T222P and R223K) both within one of the extracellular leucine-rich repeats, do appear to be suggestively linked to cryptorchidism. In vitro both mutations expressed homozygously in cells appear to compromise membrane expression of the receptor (Bogatcheva *et al.*, 2007). Yet, whereas in an Italian population the T222P mutation associated significantly with cryptorchidism (Ferlin *et al.*, 2009), this was not the case for a Moroccan or a Spanish population (El Houate *et al.*, 2008; Ars *et al.*, 2011). Moreover, for the Italian population in 22 men carrying the T222P mutation 64% had significantly reduced bone mineral density (Ferlin *et al.*, 2008), strongly implying the functionality of this heterozygous missense mutation.

Taken together, and since none of the mutations are occurring homozygously, we can conclude that some of the missense mutations, though rare, appear to be conferring a susceptibility to cryptorchidism, and possibly to other maladies, evidently working together with other genetic and possibly environmental elements. This was emphasized in data from the Italian population where amongst a large population of cryptorchid boys and taking all variants of INSL3 and RXFP2 together, only 7/600 indicated mutations for all subjects at birth, 7/303 where there was persistent unilateral cryptorchidism, 5/120 where there was persistent bilateral cryptorchidism, and none in a control population (Ferlin *et al.*, 2009). Thus while these heterozygous genetic variants individually may not necessarily confer pathology, at a population level they are significant, also, for example, in association with PCOS incidence (Shaikh *et al.*, 2016). Interestingly, in the case of the involvement of RXFP2 with horn growth in

ruminants, it was shown that this represents a rare example of a genetic trade-off between sexual selection for large horns, and natural selection for simple survival (Johnston *et al.*, 2013).

Summary and Outlook

INSL3 as a New Clinical Biomarker

Because of its constitutive expression and low within-individual variation INSL3 is proving to be a valuable new clinical biomarker for the assessment of Leydig cell status, especially in the context of fetal development, male pubertal development, and in male aging. It may also prove important in the differential analysis of ovarian function in women and in PCOS.

INSL3 as a Male-Specific Hormone

Increasingly, INSL3 is being highlighted as a gender-specific hormone, with male-specific functions in the context of testicular descent and cryptorchidism, in spermatogenesis, and possibly in bone metabolism. Nevertheless, within the female, INSL3 at a local, paracrine level has been shown to be an essential component of ovarian steroidogenesis and follicle development.

Knowledge Gaps

There are still substantial gaps in our understanding of INSL3 physiology. For example, during pregnancy, INSL3 appears to be absolutely fetal gender-specific (unlike testosterone) and thus competent to inform both the mother and placenta, and potentially other fetuses of the existence of a male fetus. Could this offer an explanation for the numerous gestational pathologies with a demonstrated link to fetal gender? We still know very little about other physiological systems where the INSL3/RXFP2 system appears to be expressed, for example, in the thyroid gland or in the kidney. Finally, since INSL3 declines markedly with age, and is absent in postmenopausal women, it might be worth investigating whether INSL3 or an analogue should be included as part of any HRT concept, especially considering its potential role in bone metabolism and pathology.

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Further Reading

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Epididymis

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Glossary

Androgen Generic term for an agent, usually a hormone (e.g., testosterone, androsterone), that stimulates the activity of the accessory male sex hormones and the genesis of spermatozoa.

Cryptorchidism The failure of one or both testes to descend into the scrotum, leading to male infertility if the testes are not surgically placed into the scrotum before puberty.

Epididymitis Inflammation of the epididymis.

Testosterone Male sex hormone produced by the Leydig cells within the testis responsible for maintaining male secondary sex characteristics.

Transcription factor Proteins involved in the process of transcribing DNA into messenger RNA by binding to a specific DNA sequence.

Wolffian Duct Eponym for the mesonephric duct. So named after Caspar Friedrich Wolff, who described the ducts in chick embryos.

Basic Development, Structure, and Function

The epididymis originates as a tube/duct from the mesonephros, called the mesonephric duct or the Wolffian duct (also referred to as the nephric duct). As the primitive duct migrates from the rostral end of the embryo to fuse with the cloaca (which will become the bladder), it induces a series of mesonephric tubules from the surrounding mesenchyme. These tubules will eventually form the efferent ducts that connect the testis to the epididymis. During embryonic and postnatal development, there is considerable elongation and coiling such that the epididymis reaches in adulthood approximately 6 m in the human, 3 m in the rat, and 1 m in the mouse. This highly convoluted epididymal duct connects the efferent ductules to the vas deferens. The epididymal epithelium differentiates into a number of cell types (see below) during the postnatal period. In the rat, this postnatal epididymal growth and differentiation is composed of an undifferentiated period between postnatal days 1–15, followed by a differentiation phase between postnatal days 15–44 (when spermatozoa are first observed in the lumen), and finally an expansion period from postnatal day 44 to adulthood (for review see Robaire and Hinton, 2015).

Classically, the epididymis is divided into several regions: initial segment, caput, corpus, and cauda, although some investigators include the initial segment as a part of the caput (**Fig. 1**). With the advent of gene array studies and further exploration of the gross anatomy of rodent epididymides, the epididymis was further subdivided into segments, for example, in the mouse there are 10 segments and in the rat, 19 segments (**Fig. 1**). Comparing regions to segments in the rat: the "initial segment" region corresponds to segments 1–4, the caput corresponds to segments 5–11, the corpus corresponds to segments 12–13 and the cauda corresponds to segments 14–19. Each segment expresses its own repertoire of genes that are unique to that segment and overlap with adjacent segments, for example, in the rat epididymis, the β-defensin gene *Defb42* is only expressed in segment 1, whereas *Defb13* is expressed in segments 8–11 (**Fig. 1**).

Epididymal epithelial cell types that differentiate during the postnatal period include: principal, basal, apical, clear, narrow, and halo cells. Dendritic cells are also found and surround the epithelial cells to varying degrees in each segment. Current studies focus on examining the role(s) of each cell type.

Sperm maturation is a collective term meaning that as spermatozoa progress along the epididymis, they acquire the ability to swim in a forward direction and develop the ability to recognize and fertilize an egg. The exact mechanisms by which sperm maturation occurs are not known although there are some mouse null mutations of certain genes that result in the failure of sperm to undergo maturation. Examples of such genes include *Ros1*, *Dicer1*, *Pten*, *Lgr4*, *Pkd1*, *Foxi1*, members of the β-defensin family, and some members of the *Hox* gene family of transcription factors. Interestingly, either those gene themselves or downstream targets of those genes clearly provide potential targets for a male contraceptive.

In different species, it takes approximately 10–14 days (12 days in man) for the spermatozoa to transit through the epididymis. For most species, spermatozoa are held in a quiescent state by luminal factors and therefore do not propel themselves along the duct. This journey is against an increasing hydrostatic pressure gradient and proceeds even when fluid flow from the testis is prevented by ligation of the efferent ductules. Their transport is achieved by the continuous production and movement of fluid originating from the testis and is augmented by neuromuscular mechanisms that cause rhythmic contractions of the smooth muscle surrounding the epididymal tubule. The epididymal smooth muscle layer increases in both thickness and autonomic innervation as it proceeds from proximal to distal epididymal regions. Neuropeptides (vasopressin, oxytocin, neuropeptide Y, endothelin), prostaglandins (e.g., PGF2α and PGE2), and other factors, are also modulators of epididymal contractions acting on their own receptors that are differentially expressed along the epididymis in various species. The switch from lower scrotal to higher body temperature increases epididymal contractility and significantly accelerates sperm transport through the epididymis;

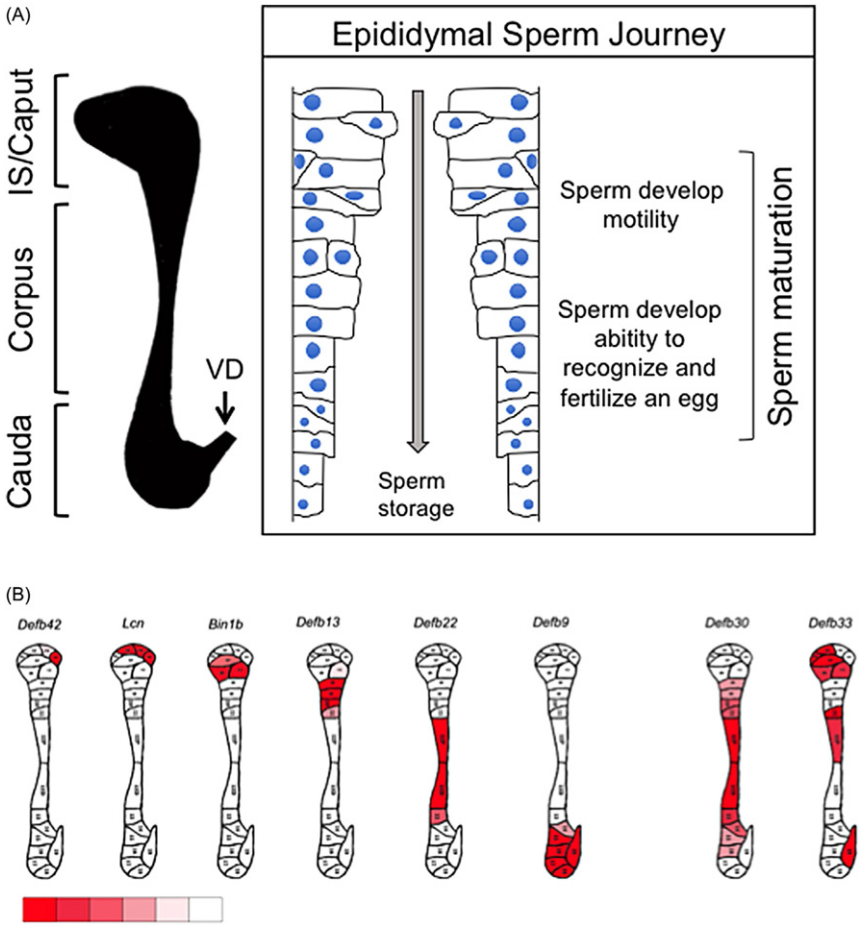

Fig. 1 Classically the epididymis can be divided in different regions: initial segment (IS), caput, corpus, and cauda (A). In the rat, the epididymis can be further subdivided into 19 segments: theIS corresponds to segments 1–4, the caput corresponds to segments 5–11, the corpus corresponds to segments 12–13 and the cauda corresponds to segments 14–19 (Panels A and B). Throughout its length, the epididymal epithelium creates sequential changes in the composition of luminal fluid, e.g. proteins, ions, solutes, and lipids, and forms a barrier to create a unique microenvironment in the lumen, where interactions between epithelial cells and spermatozoa occurs via the luminal fluid (A). These complex interactions are crucial for sperm maturation. Subsequently, spermatozoa acquire the ability for fertilization upon reaching the epididymal cauda, where it is stored until ejaculation (A). The epididymides shown in (B) are a modified figure from Zhang *et al.* (2011) where the expression pattern of lipocalin (*Lcn*) and different β-defensin (*Defb; Defb42, Defb13, Defb22, Defb9, Defb30, Defb33, and Bin1b*) messenger RNAs are used as examples of highly regionalized gene expression pattern along the various segments of the rat epididymis. Original data taken from Jelinsky *et al.* (2007). The color-coded bar below show degree of expression with red being the highest expression. Figures of each epididymis were kindly provided by Prof. Y.L. Zhang, reproduced with the permission of the American Society of Andrology. VD: vas deferens.

thus its dysregulation can have potential deleterious effects on sperm maturation and fertility (for review see Robaire and Hinton, 2015).

Sperm maturation is complete by the time the spermatozoa reach the cauda region where they are then stored before ejaculation (**Fig. 1**). The human epididymis does not have a prominent cauda region; for this reason it has little capacity to store large numbers of spermatozoa, as compared to many other species.

The epididymal luminal fluid environment also provides efficient protection for the transiting and stored spermatozoa against the innate immune system components, harmful xenobiotics, and oxidative stressors (reactive oxygen species, ROS). Tight junctional complexes between the epididymal epithelial cells (collectively referred to as the blood-epididymis barrier) form an important anatomical and physiological barrier that selectively restricts the types of compounds that can enter/exit the epididymal lumen and also establishes a tolerogenic environment for a continuous flow of autoantigenic spermatozoa which are protected from the immune system. Several types of immune cells (dendritic cells, mast cells, macrophages, lymphocytes) and differential epididymal expression of immunoregulatory genes and pathogen-sensing molecules (such as Toll-like receptors) are known to act in concert along the length of the epididymal duct (for review see Rodrigues *et al.*, 2008; Hedger, 2011; Da Silva *et al.*, 2011; Guiton *et al.*, 2013).

The defense mechanisms include the synthesis and secretion of antimicrobial and immunomodulatory proteins (such as cathelidicins and β-defensins), the synthesis and secretion of antioxidant compounds (such as glutathione and taurine) and the

rapid elimination of potential toxicants through the synthesis and secretion of antioxidants (e.g., glutathione, albumin, lactoferrin) and conjugating enzymes (e.g., γ-glutamyl transpeptidase, glutathione-metabolizing enzymes) (for review see Hall *et al.*, 2007; Robaire and Hinton, 2015; Guiton *et al.*, 2013). Because spermatozoa mature in a hyperosmotic environment that is distinctly different in composition from either blood plasma or epididymal interstitial fluid, the epididymal lumen also ensures spermatozoa protection from changes in osmolality that can affect spermatozoal autoregulation of cell volume during epididymal transit (Hinton *et al.*, 1995).

Knockout mice with partial deletion of a cluster of nine β-defensin genes in the chromosome 8 presented sperm dysfunction and male infertility, representing the first in vivo evidence for the importance of β-defensins to male fertility. More recently, the contribution of β-defensins shaping Wolffian duct/epididymal morphogenesis in response to different developmental cues has also been proposed (for review see Dorin, 2015; Ribeiro *et al.*, 2016).

Androgenic Regulation of Epididymal Function

The development and maintenance of the structure and function of the epididymis are dependent on a complex interplay of autocrine, paracrine, lumicrine and endocrine factors (including gonadal and adrenal steroid hormones), among which androgens are the primary regulators. These factors play important roles in the region- and cell-specific expression of epididymal genes (for review see Ribeiro *et al.*, 2016; Silva *et al.*, 2014; Patrao *et al.*, 2009; Murashima *et al.*, 2015a).

Plasma levels of androgens fluctuate throughout male lifespan. Their actions are mediated by the androgen receptor (AR), a steroid-hormone activated transcription factor member of the nuclear receptor superfamily. AR binds both testosterone and its 5α-reduced metabolite dihydrotestosterone (DHT), a more potent androgen.

The epididymis primarily depends on androgens to be fully functional. Disruptions of androgens/AR signaling by changes in either androgen availability and/or AR expression/function (e.g., due to genetic factors and endocrine diseases) can significantly impact epididymal development, structure, and function. Ultimately, malfunction of the epididymis results in male infertility (O'Hara *et al.*, 2011; Murashima *et al.*, 2015a,b).

During prenatal life testosterone, but not DHT, is essential for the stabilization of the Wollfian duct, and then for its morphological differentiation into an epididymis. The dominant role of testosterone ion these events are clinically supported by normal Wolffian duct differentiation in 5α-reductase-deficient patients (which prevents conversion of testosterone to DHT), which confirm that DHT is not present in the Wollfian duct until epididymal differentiation is complete.

Mesenchymal ARs are required for Wolffian duct stabilization, growth (cell proliferation/elongation), and coiling, while epithelial ARs are needed for later development and adult function. Thus, Wolffian duct morphogenesis requires direct androgen action on the mesenchyme to maintain its survival and, through androgen-dependent mesenchyme-derived components, dynamic mesenchyme–epithelium crosstalk that determines the regional specialization of the epithelium. Androgen-targets during these events include gene products such as *inhibin beta A* (*Inhba*), growth factors (e.g., EGF, FGFs), *Wnt5a*, *Dusp6*, *Hoxa10*, among others (see review by Shaw and Renfree, 2014; Murashima *et al.*, 2015a,b).

From later stages of postnatal development to adulthood, AR expression in the epididymal epithelium is in greater abundance than in the stromal cells. In the adult epididymis, androgen levels are higher in the proximal than in the distal regions, one of the main determinants of the highly regionalized pattern of gene expression along this tissue. The spatio-temporal AR gene expression pattern in the adult epididymis of several species is widely known. Postnatally, differential expression of steroid 5alpha-reductases (types 1 and 2) in epithelial cells along the epididymis can reduce testosterone to DHT. The androgen concentration in the epididymal lumen is several-fold higher than that found in the circulation, and the ratio of testosterone to DHT declines as one progresses down the epididymal duct (for review, see Smith *et al.*, 2013; Robaire and Hinton, 2015).

Androgens modulate epididymal function via direct binding of activated AR to putative androgen response elements present in the promoter regions of androgen-dependent epididymal genes (classical genomic androgen signaling; within minutes to hours), via direct association with other transcription factors (such as AP1 and NFKB), or via rapid responses through membrane-bound AR involving intracellular kinase signaling pathways (e.g., MAPKs) that can lead to phosphorylation of AR itself and other growth and transcription factors (not prevented by inhibiting transcription or translation; within seconds to minutes). This latter AR-induced signaling pathway, referred to as nongenomic androgen action may, in turn, ultimately modulate nuclear gene transcription (Robaire and Hamzeh, 2011). In the human epididymis, small noncoding RNAs (called microRNAs) have been identified as factors contributing to epididymal region-specific gene expression profiles. At the posttranscriptional level, microRNAs also influence epididymal postnatal development in an androgen-dependent manner, revealing additional mechanisms by which AR/androgen signaling mechanisms can be attained in the epididymis (Belleannee *et al.*, 2012; Zhang *et al.*, 2010).

Androgen ablation by surgical castration (orchiectomy) of adult males results in a decrease of epididymal weight, associated with apoptosis of epididymal epithelial principal cells and dedifferentiation of the caput epididymal epithelium. The luminal diameter and epithelial cell height decrease, while the intertubular stroma increases, mainly in principal epithelial cells that are particularly more sensitive to androgen levels than other epididymal cell types. Most of these castration-induced changes in the caput, corpus and cauda epididymis can be readily restored to normal (or near normal) by androgen administration. However, experimental efferent duct ligation (which prevents testicular components from entering the epididymis) indicates that both circulating testosterone and other testicular factors are necessary to maintain normal morphology and function of the proximal epididymis, especially the initial segment. Administration of antiandrogens, such as flutamide, to adult or pubescent animals

results in an accelerated sperm transit time through the epididymis, impairment of sperm motility and decreased ability of the cauda epididymis to store sperm (for review see Murashima *et al.*, 2015a; Robaire and Hinton, 2015).

Expression of nuclear AR in interstitial/peritubular cells of multiple species suggests that extratubular cells in the epididymis are also modulated by androgens, possibly contributing to tubule development and function. Paracrine relationships between epididymal epithelium and other adjacent cell types are also observed in adult animals as well (Tomsig *et al.*, 2006; Jimenez-Trejo *et al.*, 2007).

Estrogens in the Epididymis

Estrogens are pivotal in many aspects of male physiology, as demonstrated by the clinical consequences of estrogen-deficient and estrogen-excess states in men. The two classic estrogen receptor subtypes α (ESR1) and β (ESR2) are members of the nuclear receptor superfamily and expressed along the epididymis, mainly in the proximal regions, from different species. The G protein-coupled estrogen receptor-1 (called GPER-1) is also detected in this tissue. Targeted disruption of ESR1 and ESR2 ($Esr1^{-/-}$, $Esr2^{-/-}$, $Esr1^{-/-}$, $/Esr2^{-/-}$) and aromatase enzyme ($Cyp19^{-/-}$) impairs spermatogenesis, steroidogenesis, and male fertility. Animals treated with the antiestrogen ICI 182,780 also display similar outcomes, demonstrating the crucial role of estrogen for male reproductive function. Ablation of estrogen action in the efferent ductules results in a dramatic reduction of the fluid uptake capacity of the ductular epithelium, with consequent infertility. The effects of withdrawing estrogen from the epididymis are less understood. The presence of aromatase in epididymal spermatozoa has also been demonstrated in several species, including human. This enzyme is localized to cytoplasmic droplets, with decreasing presence as spermatozoa are transported along the epididymis (Hess *et al.*, 2011; Hess, 2014; Pereira *et al.*, 2014).

Glucocorticoids in the Epididymis

Glucocorticoids (cortisol in human and corticosterone in rodents) are a class of adrenal steroid hormones known to have complex roles in the male reproductive tract. Their actions are mediated by the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors. Ligand-occupied GR induces or represses the transcription of several genes by direct binding to DNA response elements and/or by physically associating with other transcription factors.

GR is expressed in the adult rat epididymis. In vivo administration of dexamethasone (synthetic glucocorticoid highly selective for GR) induces ligand-dependent GR nuclear translocation and altered glucocorticoid-responsive gene expression (e.g., inflammatory cytokines) in adult rat epididymis, indicating that GR is fully active in this tissue. Observation of heterologous regulation of epididymal AR and GR expression changes by glucocorticoid and androgen plasma levels, respectively, supports the hypothesis that functional interactions between these two receptors and their ligands occur in this tissue (for review see Silva *et al.*, 2011).

Glucocorticoids may play a role in both absorptive and secretory activities of epididymal epithelial cells (Waddell *et al.*, 2003; Sharp *et al.*, 2007), and in overall epididymal zinc and copper metabolism as well (Nair *et al.*, 1998).

Glucocorticoid ablation by bilateral adrenalectomy (surgical removal of adrenal glands) in rats demonstrated that systemic levels of glucocorticoids are also physiologically required for the maintenance of testicular spermatogenesis and the adequate number and quality of spermatozoa stored in the caudal epididymal regions (Silva *et al.*, 2014). The data emphasize the importance to control hormonal replacement therapy in adrenalectomy patients, especially in the individuals at reproductive age; either under- or over-titrating hormone replacement levels may adversely affect their fertility.

Aldosterone and Epididymis

The rat epididymis responds to the principal mineralocorticoid hormone aldosterone and also to aldosterone antagonists. Their actions are mediated by the mineralocorticoid receptor (MR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors. Although MRs have been localized in epididymal epithelial cells, the specific role aldosterone in influencing epididymal fluid movement remains to be determined. In clear cells, the presence of MR indicate their potential ability to regulate water transport from the epididymal lumen in vivo (for review see Robaire and Hinton, 2015).

Diseases of the Epididymis

There are human clinical issues that result from failure of the Wolffian duct to develop into a fully functional epididymis. Epididymal disjunction is the failure of the mesonephric tubules either to fully form the efferent ducts and/or to fail connect to the testis. This occurs in approximately 2%–4% of the general population but is seen in 25% of adult males that were cryptorchid at birth. This can occur either unilaterally or bilaterally, with varying degrees of male infertility depending upon severity of the disjunction.

Epididymal diseases encompass epididymitis (inflammation of the epididymis), torsion (often associated with testicular torsion; epididymal torsion itself is rare), as well as benign and malignant lesions. Among these pathologies, epididymitis is currently the most common and malignant lesions of the epididymis the most rare.

Benign lesions include cysts, spermatoceles, granulomas, cystadenomas (e.g., seen in von Hippel–Landau syndrome patients), adenomatoid tumors, and leiomyomas. Malignant lesions include lymphomas and, rarely, metastases from the testis, prostate, and kidney. It is estimated that the incidence of epididymal tumors comprises no more than 0.03% of all male cancers, and the mechanism(s) by which the epididymis protects itself from tumorigenesis is unclear.

Disorders in AR/Androgen Signaling and the Epididymis

Severe mutations that completely disrupt the AR activity in XY males can also be associated with the absence of epididymis/vas deferens, whereas patients with milder mutations that allow residual AR activation display normal Wolffian duct development (see review by Shaw and Renfree, 2014). Consistent with this clinical observation, Wolffian duct derivatives are absent in AR knockout (ARKO) mice (Welsh *et al.*, 2009).

Disorders in androgen synthesis and/or action have also been linked to atypical development of the male reproductive tract, including the epididymis. At least 50 different congenital abnormalities of urogenital structures are recognized by the medical term of disorder of sex development (DSD). Recently, it has been proposed that reproductive problems such as primary hypogonadism (testicular failure to produce testosterone), cryptorchidism, hypospadias, testicular germ cell cancer and low sperm count have a common origin in fetal life as a result of impaired testosterone synthesis or action during the masculinization programming window (more recently hypothesized to form the testicular dysgenesis syndrome, TDS). In the rat, TDS occurs between embryonic ages of E15.5 and E17.5, which would most closely correspond to 8–14 weeks of gestation in the human. In addition to genetic causes (e.g., AR mutations), environmental exposure of fetal testes to androgenic and antiandrogenic endocrine disrupting chemicals, including estrogens, is hypothesized as an important factor in TDS susceptibility. Thus, androgen disturbances during this period have the potential to lead to clinical pathologies with long-lasting consequences throughout life (see Welsh *et al.*, 2008 and reviews by van den Driesche *et al.*, 2017; Skakkebaek *et al.*, 2016).

Epididymitis

Epididymitis represents the most frequent cause of scrotal pain and inflammation. It is commonly diagnosed in the investigation of reproductive tract health and infertility factors, representing the fifth most common urologic diagnosed in men of ages 18–50 years. Both sexually transmitted infections (e.g., *Neisseria gonorrhoeae* and *Chlamydia trachomatis*) and typical uropathogens (e.g., Gram-negative bacteria) are of etiological relevance. Epididymitis is clinically relevant since it may induce acute/temporary epididymal dysfunction (symptoms lasting less than 6 weeks) that can ultimately lead to chronic/persistent infertility. Most patients still receive inadequate early diagnosis and therapy, representing a serious threat to men's health, especially in reproductive age. Pathogen detection is an indication for antibiotic therapy aimed at eradicating the pathogen; this treatment, however, does not always prevent permanent sperm parameter abnormalities/infertility, presumably due to the induction of persistent immunopathological processes in the genital tract. In the epididymis, sperm functionality can be compromised by direct damage from the pathogen or indirectly via damage from inflammation-associated molecules such as proinflammatory cytokines or ROS (Pilatz *et al.*, 2015; Michel *et al.*, 2015).

Escherichia coli and *Enterococcus* spp. are among the most prevalent pathogens involved in epididymitis. They commonly reach and colonize the epididymis by ascending through the urethra. The clinical course of epididymitis and its impact on fertility has been studied from animal models that mimic the clinical condition. The in vivo retrograde injection of bacteria, or their pathogen-associated molecular pattern (PAMP) components, into the vas deferens lumen in rats and mice renders a progressively dysfunctional epididymis displaying morphological changes that include epithelial and smooth muscle damage, ductal obstruction, and fibrotic remodeling that are mainly observed in the distal region of the epididymis. Steps toward a mechanistic understanding of acute and chronic experimental epididymitis and its impact on epididymal function in rodents have been used to help identify early diagnosis and targets for adjuvant therapy as tools to diminish the detrimental consequences of chronic lesions that can lead to irreversible loss of fertility and chronic scrotal pain (Turner *et al.*, 2011; Michel *et al.*, 2015; Silva *et al.*, 2018; Schagdarurengin *et al.*, 2016). Clinical epididymitis can also result from non-infectious conditions such as trauma, reflux of urine into the ejaculatory ducts, or idiopathic etiologies (Tracy and Costabile, 2009).

Obstructive Azoospermia

Clinically, in cases of obstructive azoospermia or congenital vas agenesis, spermatozoa can also be aspirated from the epididymis and inseminated in vitro. The fertilization rates with spermatozoa retrieved from different regions of the epididymis vary, with the highest being from cells collected in the most distal regions. After in vitro fertilization (IVF) and transfer, pregnancies have also been reported with spermatozoa recovered from the corpus and proximal caput epididymis. However, the consequences of either pathological conditions of obstructive azoospermia or the use of immature spermatozoa to fertilization remain under investigation.

Epididymal-Related Male Infertility

As reviewed by Turner (2011), there are over a dozen genes or gene products expressed in the epididymis whose dysfunction either significantly reduced or completely eliminated male fertility in a variety of species. The mouse and rat present 75 genes and 110 genes, respectively, that are transcribed *only* in the epididymis (Johnston *et al.*, 2005; Jelinsky *et al.*, 2007), and in the mouse, 35 of those 75 genes transcribe protein products with the signal sequences for secreted proteins (Dean *et al.*, 2008), indicating the unique intraluminal environment required for sperm maturation and storage. Thus, these data confirm how vital the epididymis is to male fertility. Conversely, this information is also used in the development of male contraceptive based on inhibition of epididymal function.

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Fertility in Men With Spermatogenesis Abnormalities☆

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Glossary

ART Assisted reproductive technology.
Azoospermia Absence of sperm in semen.
Gonadotoxins Agents or chemicals toxic to the gonads or testicles.
ICSI Intra-cytoplasmic sperm injection.
Infertility Inability to achieve pregnancy after 1 year of unprotected intercourse.
IVF In vitro fertilization.
Oligozoospermia Reduced sperm concentration (<15 million mL).
Spermatogenesis Orderly process whereby immature diploid germ cells pass through two successive meiotic divisions and subsequent cellular remodeling to become haploid spermatozoa.
Varicocele Dilated testicular veins caused by reflux of blood into these veins.

Definition

It is estimated that 10%–15% of couples suffer from infertility and that male factor infertility, by itself or in combination with female causes, is responsible for approximately 50% of these cases (Practice Committee of American Society for Reproductive Medicine, 2012). Male factor infertility can be due to a primary spermatogenesis defect (primary testicular failure), genital duct obstruction (posttesticular failure) or hormonal deficiency (pretesticular failure). Men with primary spermatogenesis abnormalities fit onto a spectrum. At one end of the spectrum are subfertile men with a mild degree of spermatogenic impairment characterized by hypospermatogenesis on testicular biopsy and subnormal semen parameters (reduced sperm concentration, motility, and/or morphology) on semen analysis. At the other end of the spectrum are men with end-stage testicular failure characterized by azoospermia on semen analysis and complete germ cell aplasia or Sertoli cell-only pattern on testicular histology. As such, the evaluation, treatment and prognosis of men with spermatogenic failure can vary depending on the severity of the infertility.

Evaluation

History and Physical Examination

The evaluation of infertile men with spermatogenic failure should begin with a thorough history. The history should include documentation of duration of infertility and any previous fertility treatments. Evaluation of the female partner is important to rule out any significant female factors (e.g., anovulation, tubal obstruction). One must inquire about sexual history including the timing and mechanics of intercourse and use of lubricants. Knowledge of childhood and pubertal development is critical to uncover conditions such as cryptorchidism and delayed pubertal development. A positive family history may suggest a hereditary form of infertility (e.g., cystic fibrosis gene mutations are associated with CBAVD—congenital absence of the vas deferens). A medical history is important to rule out systemic illnesses (such as renal failure or hemochromatosis) which may be associated with impairment of spermatogenesis. A surgical history focuses on prior history of abdominal, pelvic or scrotal surgery (to rule-out iatrogenic genital duct injury). Infections can contribute to infertility by causing scarring and obstruction of the genital ducts (e.g., gonorrhea, chlamydia) or impairing sperm function through the elaboration of spermatoxic cytokines (e.g., prostatitis). Environmental gonadotoxins (e.g., radiation and industrial chemicals) can affect testicular function and fertility (Evenson and Wixon, 2005; Kenkel *et al.*, 2001; Sengupta and Banerjee, 2013). Moreover, lifestyle factors such as smoking and scrotal hyperthermia (hot baths) can be detrimental to sperm function (Rao *et al.*, 2016; Evenson *et al.*, 2000; Kunzle *et al.*, 2003; Richthoff *et al.*, 2008; Sailer *et al.*, 1997; Vine, 1996; Vine *et al.*, 1996; Li *et al.*, 2011; Virtanen *et al.*, 2012). Specific lifestyle measures, such as, minimizing testicular hyperthermia can improve sperm quality (Jung *et al.*, 2001).

☆*Change History*: May 2018. Katherine Rotker and Mark Sigman updated the sections: III Evaluation (A History and Physical Examination, B Semen Analysis, C Hormonal Evaluation, D Imaging Studies, E Hormonal Evaluation, F Genetic Evaluation, G Testicular Biopsy), IV Management (A General Recommendations, B Medical Therapy for Spermatogenic Failure, C Surgical Therapy for Spermatogenic Failure, D Assisted Reproduction). They have updated the ASRM practice guidelines reference to reflect the latest addition and have added several recent references to the reference list.

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Medications can influence testicular function and sperm production through one of several mechanisms. Hormonal agents such as androgens (frequently used by bodybuilders), estrogens or antiandrogens can impair normal testicular function by interfering with the hormonal control of spermatogenesis (Meriggiola *et al.*, 1998). Chemotherapeutic agents (e.g., alkylating agents) interfere with spermatogenesis and cause germ cell depletion and reduced sperm production. Sperm-specific toxicants exert their detrimental effects at the posttesticular level (e.g., calcium channel blockers).

The physical examination may provide additional information regarding the etiology of spermatogenic failure. A more female body habitus and hair distribution provide evidence of lack of androgenization which could indicate conditions such as hypogonadism or Klinefelter syndrome. If hyperprolactinemia is suspected, examination of visual fields is important to rule-out a pituitary adenoma. Examination of the abdomen and pelvis is important to rule-out surgical scars and/or hepato-splenomegaly. The penile exam should focus on the assessment of penile length (indicative of normal development) and position of the urethral meatus (for normal deposition of semen). While prostate exam may enable one to assess prostate size, firmness, tenderness it rarely is useful in the diagnosis of causes of infertility due to cysts (congenital, ejaculatory duct), which may be associated with ejaculatory duct obstruction.

The scrotal exam should be performed in a warm examining room to promote relaxation of the scrotum. It is important to record the position (scrotal, inguinal), volume (normal 15–25 mL) and consistency (normally firm) of the testis. Normally, more than 70% of the testis volume is from germ cells alone. Therefore, a soft and/or small testis is indicative of abnormal spermatogenesis. The diagnosis of a varicocele is generally made on physical examination. The detection of a varicocele is greatly facilitated by examining the patient standing. Varicoceles are graded as follows: grade I—palpable only with Valsalva, grade II—palpable without Valsalva, grade III—visible (Dubin and Amelar, 1978). The presence or absence of the vas deferens can be confirmed on physical examination.

Semen Analysis

The semen analysis is the cornerstone of male infertility evaluation. At least two semen samples collected after 2–3 days of abstinence should be analyzed. These can be collected by masturbation or by intercourse using special semen collecting condoms that do not contain spermatocides. The sample should be evaluated within 1 h of production and should be kept at room or body temperature during transport if collected at home (Jarow *et al.*, 2010). Assessment of semen volume is important in that it is indicative of seminal vesicle function (70% of the semen volume is of seminal vesicle origin) and patency of the ducts distal to this gland (ejaculatory ducts). In azoospermic men, a low semen volume may be due to an incomplete sample collection (a common problem), ejaculatory duct obstruction, seminal vesicle agenesis or hypoplasia (associated with congenital absence of the vas deferens), testosterone deficiency, retrograde ejaculation or lack of emission (lack of propulsion of sperm into the posterior urethra). Seminal pH is important to assess in low volume azoospermia as pH less than 7.0 suggests lack of seminal vesicle secretions as is found with CBAVD or ejaculatory duct obstruction, but not with retrograde ejaculation.

Sperm concentration is an indicator of quantitative spermatogenesis and testicular function. The 2010 WHO semen analysis manual has set the lower threshold of the fertile reference range sperm concentration at 15 million sperm per mL (this value represents the lower 5th centile of a population of fertile men) (Cooper *et al.*, 2010). However, spontaneous pregnancies are possible in couples in whom the man's sperm concentration is under 15 million sperm per mL (since 5% of the fertile population fall into this range). Percent sperm motility and sperm morphology are indicators of qualitative spermatogenesis. According to the 2010 WHO semen analysis manual, the percent progressive motility should be greater than or equal to 32%, total motility greater than or equal to 40%, and the percentage of sperm with normal morphology (by strict criteria) should be greater than or equal to 4% (these values represent the lower 5th centile) (Cooper *et al.*, 2010). Although each of the conventional semen parameters (sperm concentration, motility, and morphology) may help to differentiate infertile from fertile men, none of these parameters alone is a powerful discriminator as there is significant overlap between semen parameters of infertile and fertile men). The more parameters that are below the reference range limits, the more likely male infertility is present (Guzick *et al.*, 2001). Indeed, some infertile men have normal semen parameters and are said to have unexplained infertility. Moreover, conventional semen parameters may exhibit a high degree of variability from sample to sample and, as such, there is often a need for at least two analyses.

Specialized sperm function tests have been developed in the hope of better discriminating fertile from infertile men to better predict fertility potential (in vivo and in vitro) than conventional semen parameters. The most promising and well-studied tests of sperm function are the sperm DNA integrity assays (Collins *et al.*, 2008; Simon *et al.*, 2010; Zini and Sigman, 2009). Several assays exist to measure sperm DNA and chromatin damage. However, a significant amount of controversy exists regarding the utility of these tests due lack of standardization of assay protocols and thresholds causing suboptimal sensitivity and specificity. At this time, the Practice Committee of the American Society for Reproductive Medicine and the European Society for Human Reproduction and Embryology have concluded that current data doesn't support its routine use (Practice Committee of the American Society for Reproductive Medicine, 2013; Barratt *et al.* 2010). However, some find it useful in certain situations such as repeated miscarriages whereas its use for determining decisions between IUI, IVF, ICSI, and testicular sperm instead of ejaculated sperm require further studies.

Hormonal Evaluation

Hormonal evaluation is indicated if there is an abnormally low sperm concentration (<10 million/mL), impaired sexual function or clinical findings suggestive of endocrinopathy (Jarow *et al.*, 2010). The initial hormonal evaluation should include

measurement of serum FSH and testosterone (T) levels (drawn in the morning). An abnormal FSH or T should prompt further evaluation with serum LH and prolactin determination. Many men with abnormal spermatogenesis have a normal serum FSH but an elevated FSH clearly indicates an abnormality in spermatogenesis. While many commercial labs have reference ranges for FSH of 18 mIU/mL or higher, most men with normal spermatogenesis have FSH values of less than 5–7.6 (Myers *et al.*, 2009; Schoor *et al.*, 2002). As such these men with high FSH and small testes are not candidates for surgical reconstruction and do not need diagnostic testicular biopsies. However, men with elevated serum FSH may harbor small foci of active spermatogenesis amidst a histologic background that is predominantly characterized by maturation arrest or Sertoli cell only pattern. In these men, sperm may be retrieved through microdissection testicular sperm extraction (micro-TESE) for use in conjunction with IVF/ICSI. Sperm has been found with this technique in this population even when a conventional biopsy showed no sperm (Ramasamy and Schlegel, 2007).

Low serum FSH, T, and LH is suggestive of hypogonadotropic hypogonadism with secondary spermatogenic failure. In these men, a prolactin level is indicated and if elevated should prompt pituitary imaging to rule out a pituitary lesion. These men will often respond to hormonal replacement therapy (in the form of intra-muscular or subcutaneous hCG and FSH or pulsatile subcutaneous GnRH) (Dwyer *et al.*, 2013).

A normal hormonal evaluation is suggestive of normal, active spermatogenesis but does not rule out spermatogenic failure.

Imaging Studies

Scrotal/testicular ultrasound may be used in select cases for assessment of testicular or epididymal masses and presence or absence of varicoceles when physical examination is insufficient. While trans-rectal ultrasound (TRUS) is primarily used to assess prostate anatomy, in infertile men, a TRUS is also used to evaluate the seminal vesicles and ejaculatory ducts when ejaculatory duct obstruction is suspected. At the time of surgical reconstruction, vasography may be performed to confirm and localize ductal obstruction.

Genetic Evaluation

It has been found that severe male factor infertility may be caused by genetic defects and that these may be transmitted to the male's offspring through assisted reproduction. In many cases, this only causes the same problem in the next generation (infertility in a male child), but some genetic defects may cause other diseases, miscarriage, or even early death. As such, appropriate genetic testing and counseling are an essential part of the infertility evaluation in patients with a genetic etiology of infertility. The available clinical genetic tests for male-factor infertility include the karyotype, Y-microdeletion analysis, and evaluation for cystic fibrosis mutations.

Karyotype

The prevalence of chromosomal abnormalities found in the general population is less than 1% (Berger, 1975). It is reported that the prevalence of chromosomal abnormalities in men with severe spermatogenic deficiency is up to 14% (Van Assche *et al.*, 1996). Klinefelter syndrome (47,XXY) is the most common chromosomal abnormality, observed in about 10% of azoospermic men (Becker *et al.*, 1966). Chromosome abnormalities described in infertile men include 47,XYY; 46,XX; translocations; duplications; deletions; and peri-centric inversions. Karyotypic abnormalities are identified in 10%–15% of azoospermic men with sex chromosomal abnormalities dominating. In men with oligospermia, the prevalence of chromosomal abnormalities is about 5%. Sex chromosome abnormalities are seen in 1%–2% while autosome abnormalities constitute the other 3%. In clinical practice, a karyotype is recommended in infertile men with severe oligospermia (<5 million sperm per mL) and nonobstructive azoospermia (Jarow *et al.*, 2010). Of importance, abnormalities such as balanced translocations may become unbalanced translocations during meiosis of spermatogenesis, potentially causing greater abnormalities in offspring (e.g., miscarriage, early postnatal mortality and birth malformations). Clearly, the karyotype analysis is important given the serious reproductive consequences of transferring an abnormal chromosomal complement to the offspring.

Y-chromosome microdeletion

Approximately 10% of men with azoospermia or severe oligospermia (less than 5 million sperm per mL) have a small (micro) deletion of the long arm of the Y chromosome, in an area known as the AZF (azoospermia factor) with subregions AZFa, AZFb, and AZFc (Stahl *et al.*, 2010). Several important spermatogenesis genes reside within this deleted region of the Y chromosome and, as such, explain the observed phenotype associated with Y-microdeletions. It does not appear that men with this condition have any other physical problems apart from the infertility. There is evidence to show that men with a Y-microdeletion will pass on the genetic abnormality to their male offspring through IVF/ICSI (Oates *et al.*, 2002; Stahl *et al.*, 2010). Genetic counseling and Y-microdeletion testing is recommended in infertile men with severe oligozoospermia (<5 million sperm per mL) and non-obstructive azoospermia. Men with NOA (nonobstructive azoospermia) and a complete microdeletion of subregions AZFa and/or AZFb have a very poor prognosis (very low or absent probability of finding sperm with attempts at testicular sperm retrieval while over 50% of those with AZFc deletions have successful testicular sperm retrieval (Hopps *et al.*, 2003; Stahl *et al.*, 2010).

The cystic fibrosis gene and male infertility

The Cystic Fibrosis Trans-membrane Conductance Regulator (CFTR) gene is located on chromosome seven. Over 1700 different mutations of the cystic fibrosis (CF) gene have been reported to date and mutations in this gene are associated with cystic fibrosis and absence of vas deferens. Men with congenital bilateral absence of the vas deferens (CBAVD) can have mutations in the CFTR gene approximately 60%–70% of the time with no other clinical symptoms of cystic fibrosis (Ferlin *et al.*, 2007). In the Caucasian population, the carrier frequency of these autosomal recessive mutations is approximately 4%. CF point mutations along with the 5T variation in intron 8 of the CFTR gene (a polythyimidine sequence containing only 5 bases instead of 7 or 9 in intron 8) are associated with congenital bilateral absence of the vas deferens (CBAVD) in 80% of patients with CBAVD (Mak *et al.*, 1999). However, CF mutations have not been associated with testicular failure or nonobstructive azoospermia (Mak *et al.*, 1999). CF mutations can result in a defective chloride channel and this is associated with production of thick respiratory and GI secretions, leading to the CF phenotype. In male infertility, CF generally results in obstruction of the excurrent duct system due to absence of the vas deferens, resulting in obstructive azoospermia with low volume, acidic semen samples. Genetic counseling and CFTR mutation and 5T testing is recommended in infertile men with congenital bilateral absence of the vas deferens. In addition, since carrier status is common and if present in the female partner, could result in offspring having clinical cystic fibrosis, the male's partner should also undergo testing for CFTR mutations and the presence 5T polymorphism. (Cuppens and Cassiman, 2004).

Testicular Biopsy

Because FSH is a sensitive indicator of the quality of spermatogenesis, a diagnostic testicular biopsy is rarely performed unless done in conjunction with testicular sperm retrieval. Men with prior vasectomy and men with CBAVD generally have normal spermatogenesis and a testis biopsy is not usually indicated. Men with serum FSH above 5–7.6 mIU/mL will likely have non-obstructive azoospermia and a pure diagnostic testis biopsy is not indicated, as it is unlikely to indicate normal spermatogenesis. Thus the diagnostic biopsy will usually not affect management in most patients.

With the advent of advanced assisted reproductive technologies (ARTs), such as intracytoplasmic sperm injection (ICSI), testicular biopsy has more commonly been performed as a therapeutic instead of a diagnostic procedure. In approximately 50% men with NOA have rare foci of normal spermatogenesis in the testis. The sperm may be surgically retrieved and used for ICSI (Schlegel and Li, 1998). Of note, in men for whom diagnostic testicular biopsy does not show spermatogenesis, sperm may still be found through micro-TESE (Ramasamy and Schlegel, 2007).

Management

General Recommendations

Prior to initiating therapy (medical or surgical) for the treatment of male infertility, efforts should be made to eliminate any potential gonadotoxins (agents toxic to the gonads or testicles) and adverse lifestyle factors. A number of medications are known to have adverse effects on sperm production. These medications include hormonal agents (e.g., testosterone or its derivatives), antineoplastic agents (chemotherapeutic drugs) and other miscellaneous drugs. Alpha blockers used for treatment of enlarged prostates may cause impairment of ejaculation. The clinician should review medications with online databases to determine potential gonadotoxicity. Cigarette smoking is associated with decreased sperm counts and sperm function (Vine *et al.*, 1996; Li *et al.*, 2011; Jensen *et al.*, 2014; Virtanen *et al.*, 2012). Similarly, excessive alcohol consumption (binge drinking) has a detrimental effect on sperm production in the testis. Proper nutrition is also important to maintain adequate sperm production. Finally, chronic exposure to excessive heat (hyperthermia) is detrimental to normal sperm production in the testis.

Medical Therapy for Spermatogenic Failure

Nonspecific therapy

In general, nonspecific or empiric therapy for male infertility is ineffective and should not be considered first line therapy unless studies demonstrate clear efficacy and a low risk of complications. However, a number of small, randomized controlled trials have shown that antioxidant supplements and selective estrogen receptor modulators (such as clomiphene citrate) can improve sperm parameters in some men with idiopathic infertility (Chua *et al.*, 2013; Showell *et al.*, 2011). However, most men do not respond, and it is yet not possible to select those patients that may benefit from empiric therapy. Alternatively, for most couples with idiopathic male infertility and spermatogenic failure, assisted reproduction technologies (ART) can be effective and are more effective options. Some couples choose empiric therapy rather than ART due to financial or religious considerations.

Specific therapy

In general, specific medical therapy (targeted to specific pathology, e.g., hormonal replacement for hypogonadotropic hypogonadism) will be effective in improving spermatogenesis and semen quality. A small subset ($<5\%$) of infertile men with spermatogenic failure suffers from endocrine infertility, one of the most effectively treated forms of infertility. Specific causes of

secondary testicular failure include hypogonadotropic hypogonadism, hyperprolactinemia, hypothyroidism, and congenital adrenal hyperplasia.

Hypogonadotropic hypogonadism can be congenital (Kallman syndrome) or acquired (secondary to a pituitary adenoma, pituitary infarction, radiotherapy or idiopathic-unknown). The standard treatment involves replacement of deficient hormones. Typically, patients are treated with intra-muscular or subcutaneous human chorionic gonadotropin (hCG, 500–2000 IU, 2–3 times per week) and adding follicle stimulating hormone (FSH, 150 IU, 2–3 times per week) after several months (Foresta *et al.*, 2000, 2007). Alternatively, pulsatile gonadotropin releasing hormone (GnRH, 2–40 mg, every 2 h by pump) can be administered (Dwyer *et al.*, 2013) but it is more expensive. Generally, this form of treatment results in increased testicular volume and improved semen quality with many men experiencing a return of normal fertility potential.

Hyperprolactinemia can be secondary to a pituitary tumor (generally benign), hypothyroidism, liver disease, medications (antidepressants, cimetidine) or can be idiopathic. Typically, patients are treated with cabergoline (1 mg per week, taken twice per week) rather than bromocriptine due to a better side effect profile. With large tumors, transphenoidal resection of the pituitary adenoma may be recommended.

Hypothyroidism or hyperthyroidism may be associated with depressed sperm production. Treatment is thyroid hormone replacement or ablation/suppression of the hyperactive thyroid, respectively. While this leads to improved sperm production and return of fertility, it is rare for patients with undiagnosed and untreated thyroid disorders to present with infertility such that general screening of all infertile men is not warranted.

Congenital adrenal hyperplasia is a condition caused by a congenital enzyme deficiency leading to suppressed cortisol and aldosterone levels. These patients are treated with steroid replacement and generally this leads to improved sperm production and return of fertility.

Surgical Therapy for Spermatogenic Failure

Varicocelectomy

Varicocelectomy is the only male infertility surgery specifically designed to improve spermatogenesis. Varicocelectomy is indicated in men with a clinical varicocele, abnormal semen parameters and couple infertility (Cortes-Gutierrez *et al.*, 2008). This is based on the demonstration that varicocele is associated with a progressive decline in testicular function (Damsgaard *et al.*, 2016) and that repair of varicocele can improve spermatogenesis. Varicocelectomy is also indicated in men with a clinical varicocele and testicular pain and in the child or adolescent with a clinical varicocele and decreased ipsilateral testicular volume (greater than 4 mL difference between the right and left testis) (Schlegel and Goldstein, 2011).

A large number of studies have evaluated the outcome of varicocelectomy on fertility parameters and most of these studies have demonstrated an improvement in semen quality and pregnancy rates. Overall, varicocelectomy results in a significant improvement in semen parameters and pregnancy rates after varicocelectomy are in the range of 20%–60% (Schlesinger *et al.*, 1994). However, the bulk of the outcome data on varicocelectomy come from uncontrolled or poorly designed controlled studies. Nonetheless, several independent meta-analyses of the (few) randomized trials on varicocelectomy and pregnancy suggest that varicocelectomy increases the probability of natural pregnancy (Baazeem *et al.*, 2011; Kroese *et al.*, 2012).

While studies have indicated that there may be some benefit in repairing varicoceles in infertile men with azoospermia and clinical varicocele (Kim *et al.*, 1999; Matthews *et al.*, 1998) this remains controversial. Although significant improvement in semen quality (appearance of sperm in the semen) is reported in approximately 20% of these men, a clinically significant outcome (with spontaneous pregnancy) is reported in less than 10% of these cases. Preoperative testicular biopsy is predictive of outcome in these cases (Kim *et al.*, 1999). Only men with mature spermatids or spermatozoa on testicular biopsy had a good outcome (appearance of sperm in the semen). Men with maturation arrest or Sertoli cell only pattern on testicular biopsy remained azoospermic postoperatively. In addition, many men who develop sperm in the semen, revert to azoospermia and still require testicular sperm retrieval and ICSI (Schlegel and Kaufmann, 2004).

Surgical sperm retrieval

The advent of advanced assisted reproduction has made it possible for men with spermatogenic failure to father children. In that subset of men with spermatogenic failure and azoospermia, only testicular surgical sperm retrieval coupled with ARTs will allow these men to father children. Although a standard open testicular biopsy can be used to extract testicular sperm in men with spermatogenic failure, microdissection testicular sperm extraction (micro-TESE) has been advocated as the gold-standard approach for sperm retrieval in men with nonobstructive azoospermia. In men with NOA (nonobstructive azoospermia), the probability of finding sperm using micro-TESE is in the range of 40%–60% (Dabaja and Schlegel, 2013). With the testicle opened, the operating microscope enables the surgeon to identify and specifically extract the most dilated seminiferous tubules (indicative of active spermatogenesis) while thinner tubules (indicative of germ cell aplasia) are spared. As such, micro-TESE allows for maximal sperm recovery and minimal tissue extraction. Micro-TESE has been used successfully in azoospermic men with a history of cryptorchidism, prior chemotherapy for cancer and Klinefelter syndrome (Dabaja and Schlegel, 2013). Testicular spermatozoa can then be used in IVF/ICSI with pregnancy rates in the range of 30%–45%.

Assisted Reproduction

The use of assisted reproductive technologies (ARTs) is indicated for couples in which the man suffers from idiopathic spermatogenic failure. ART is also indicated for couples in which the man has a specific cause for the spermatogenic failure but has not responded to conventional therapy (e.g., varicocelectomy for varicocele) or has chosen ART instead of conventional therapy.

In men with a mild degree of spermatogenic failure and borderline semen parameters, three to six cycles of sperm washing with intra-uterine insemination (IUI) combined with ovulation induction may be attempted first (after consideration of female partner factors). IUI is a minimally invasive ART, however, pregnancy rates are in the range of 5%–15% per cycle and are highly dependent on the concentration of motile sperm (the minimum threshold for effective IUI is 3–5 million motile sperm) (Verhulst *et al.*, 2006; Miller *et al.*, 2002).

In those couples where the man has severe oligozoospermia or nonobstructive azoospermia, only the most advanced ART (IVF/ICSI) will allow these couples to achieve a pregnancy. In men with nonobstructive azoospermia, a surgical testicular sperm retrieval procedure is necessary to obtain spermatozoa (see above). IVF/ICSI is also indicated in those couples that have failed to achieve a pregnancy through IUI. The success rates with advanced ART (e.g., IVF/ICSI) continue to improve, with the most recent final live birth per cycle being cited at 41% for women <35% and 24% for women 35–40 (SART National Summary Report, 2014).

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Genetics of Male Infertility

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Glossary

ART Assisted reproductive techniques.
Asthenozoospermia Reduced sperm motility (≤32% spermatozoa with progressive motility).
Azoospermia Absence of spermatozoa in the ejaculate.
Couple infertility Inability to achieve pregnancy after 1 year of unprotected intercourse.
Cryptozoospermia Identification of spermatozoa only in the sediment of the semen post-centrifugation.
Exome Part of the genome consisting of exons that code information for protein synthesis.
ICSI Intracytoplasmic sperm injection, injection of a single spermatozoa in the oocyte's cytoplasm.
Karyotype The number, size, and shape of chromosomes in an organism.
Moderate oligozoospermia Reduced sperm concentration (<10 million spermatozoa/mL).
Oligozoospermia Reduced sperm count (≤15 million spermatozoa/ml or ≤39 million spermatozoa/ejaculate).
Severe oligozoospermia Reduced sperm concentration (<5 million spermatozoa/mL).
Teratozoospermia Reduced number of spermatozoa with normal shape and size (<5%).

Introduction

Couple infertility is an emerging issue in Western countries, involving about 15% of couples (WHO, 2010). Male reproductive anomalies are present in about half of cases, either as the main reason or as a cofactor for couple infertility (Krausz, 2011). Male infertility is a multifactorial complex pathological condition with highly heterogeneous phenotypic representations, from the complete absence of spermatozoa in the testis to distinct alterations of sperm quality. Recently, male reproductive dysfunction has been classified into four etiologic categories: (i) hypothalamic–pituitary axis dysfunction; (ii) quantitative alterations of spermatogenesis; (iii) qualitative alterations of spermatogenesis; and (iv) ductal obstruction/dysfunction (Tournaye *et al.*, 2017). Genetic factors play an important role in each of these categories, with the highest prevalence in azoospermic men. Indication for genetic testing varies according to the etiology and semen phenotype. For instance, in cases of quantitative alterations of spermatogenesis (severe oligozoospermia/azoospermia), karyotype and Y chromosome microdeletion analysis are routinely performed. In the remaining three etiologic categories and in the case of a specific disease condition, mutation screening in specific candidate genes are indicated. Genetic screening is a diagnostic tool not only in male infertility, but also for pharmacogenetics purposes in relationship with follicle-stimulating hormone (FSH) treatment of idiopathic infertile men.

Chromosomal Anomalies in Oligo/Azoospermia

Karyotype Anomalies

Overview

Chromosomal abnormalities may affect the number or the structure of chromosomes. Their frequency reaches the highest value (15%–16%) in Non-Obstructive Azoospermia (NOA), showing a progressive decline with increasing sperm count (Jungwirth *et al.*, 2012) (**Fig. 1**). Klinefelter syndrome (KS) represents the main genetic cause for NOA, whereas structural autosomal anomalies (translocations/inversions) are relatively more frequent in oligozoospermic men (Jungwirth *et al.*, 2012; Krausz, 2011).

Who should be tested?

The most widely accepted cut-off for karyotype is moderate oligozoospermia. Additional indications for karyotype analysis are family history for recurrent abortions, malformations, mental retardation, or infertility.

Klinefelter syndrome

The most common sex chromosome aneuploidy in humans is the KS, which may manifest with different chromosomal constitution: 47, XXY or mosaic 46, XY/47, XXY, or higher-grade sex chromosomal aneuploidy, that is, 48, XXXY, 49, XXXXY, etc. Although its incidence is high (1:660 in live births and 1:300 in spontaneous abortion), the disease is often undiagnosed due to the large phenotypic variability of the disease. The only constant finding in affected individuals is the presence of small, firm testes due to hyalinization of seminiferous tubules. Azoospermia is present in over 90% of patients, while the remaining semen phenotype can be crypto/severe oligozoospermia (mainly in mosaic cases of KS). After puberty, the large majority of these patients

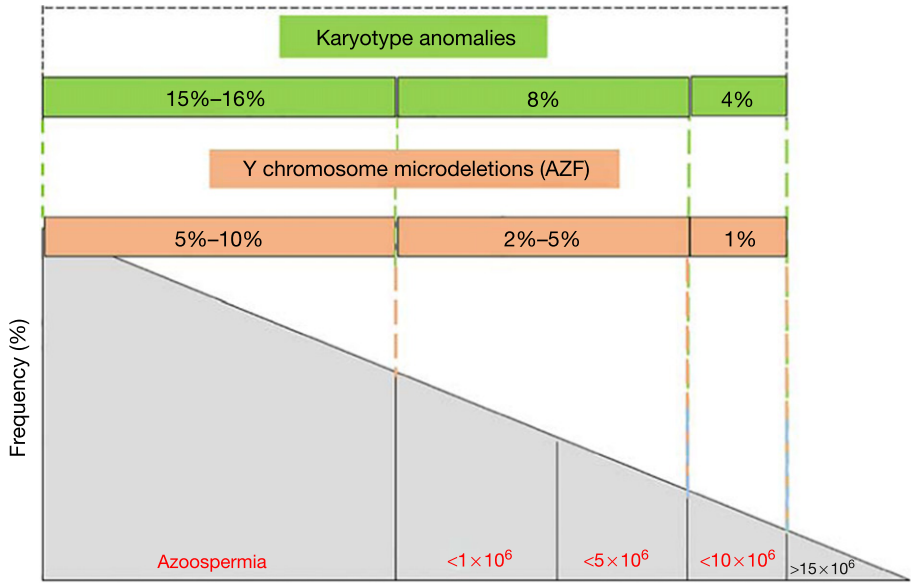

Fig. 1 Frequency of karyotype anomalies and Y chromosome microdeletions (AZF) in quantitative spermatogenic disturbances (from azoospermia to oligozoospermia).

present signs of androgen deficiency; these signs range from hypogonadism with gynecomastia and eunuchoid body proportions to variable levels of undervirilization (Aksglaede and Juul, 2013). In addition to reproductive/sexual dysfunctions, KS patients present higher morbidity for a series of diseases such as metabolic syndrome, autoimmune diseases, venous thromboembolism, and cognitive/psychiatric disturbances (Calogero *et al.*, 2017).

Management

The testicular sperm retrieval from Micro-dissection Testicular Sperm Extraction (micro-TESE) may detect residual foci of active spermatogenesis in the testes of azoospermic 47, XXY adult males (Dabaja and Schlegel, 2013). The average testicular sperm recovery rate is 50%, and spermatozoa can be used for the conception of a biological child through Intracytoplasmic Sperm Injection (ICSI) (Corona *et al.*, 2017). An early diagnosis is relevant for preserving fertility, since germ cell loss is progressive with age and micro-TESE has a higher recovery rate under the age of 30 years (Rohayem *et al.*, 2015). The early diagnosis of KS is relevant not only for fertility preservation but also for the prevention of health complications due to associated general health problems. Preventive measures include androgen replacement therapy, lifestyle changes, metformin, and statins according to the presence/absence of metabolic syndrome.

Translocations/inversions

The most frequent include Robertsonian translocations, inversions, and reciprocal translocations. In 60% of all cases of Robertsonian translocations, a (13;14)-translocation is found. The Robertsonian translocations carriers are healthy, but this abnormality is often found in oligozoospermic patients (about nine times higher in infertile men than in healthy controls) and is rarely observed in azoospermic subjects (Vincent *et al.*, 2002).

Management

Structural anomalies, especially Robertsonian translocations, might become unbalanced in offspring, increasing the risk of aneuploidy and imprinting diseases. For this reason, in cases of assisted reproduction, Preimplantation Genetic Diagnosis (PGD) is advised in order to ensure the transfer of embryos with normal chromosomal constitution.

Y Chromosome Microdeletions

Overview

Following the identification of de novo deletions in azoospermic men, Tiepolo and Zuffardi (1976) suggested the existence of Azoospermia Factor (AZF) region(s) on the long arm of the Y chromosome (Yq). Greater than 20 years later, Vogt *et al.* (1996) and Skaletsky *et al.* (2003) finally defined the AZF regions at the molecular level and identified the gene content of these regions. There are five fragile sites on the Yq leading to different deletions patterns, designated as AZFa, AZFb, AZFb + c (with two different breakpoints), and AZFc deletions (Krausz and Casamonti, 2017). The large majority of clinically relevant microdeletions removes completely one or more of the AZF regions, and occurs rarely in the general male population (1:4000), whereas they occur in 5%–10% of idiopathic NOA patients and in 2%–5% of severe oligozoospermic ones (**Fig. 1**) (Krausz *et al.*, 2014).

There is a strict genotype/phenotype correlation for the complete AZFa and AZFb deletions, that is, the first type of deletion inevitably leads to Sertoli cell-only syndrome (SCOS) while the second causes SCOS or spermatogenetic arrest. On the other hand, the rare partial AZFa and partial AZFb deletions are associated with residual sperm production. The complete AZFc deletions are associated with a variable semen phenotype ranging from severe oligozoospermia (mainly below 2 million spermatozoa/mL) to azoospermia (Krausz and Casamonti, 2017). Among the different partial AZFc deletions, the "gr/g deletion" confers on average a twofold increased risk for oligozoospermia due to the reduced AZFc gene dosage (Krausz and Casamonti, 2017). The phenotypic expression of the deletion may vary in different ethnic groups, depending on the Y chromosome background. In the Caucasian population, the highest risk (OR 4.2, CI 2.0–8.8, $P < 0.001$) has been reported in selected idiopathic oligozoospermic men of Italian and Spanish origin (with a frequency of 3.5%) when compared to normozoospermic controls of the same ethnic origin.

Who Should Be Tested?

All patients with severe oligozoospermia or azoospermia.

Management

Testing for AZF deletions in azoospermic men has a prognostic value for testicular sperm retrieval. In fact, in cases of complete AZFa and AZFb deletions the chance of finding spermatozoa upon surgical testicular exploration is virtually zero, hence TESE is not recommended in these patients (Krausz *et al.*, 2000). In contrast, the testicular sperm retrieval rate in azoospermic men, carrying *AZFc* microdeletion is on average 50% (Oates *et al.*, 2002). All AZF deletion carriers with residual spermatogenesis (AZFc or partial AZFa, AZFb) will transmit the deletion to male offspring, with consequent impaired spermatogenesis (from severe oligo to azoospermia) in the offspring's adulthood. Regarding the "gr/g deletion," the couple should be aware that the deletion (i.e., a genetic risk factor for impaired sperm production) may become a complete AZFc deletion (i.e., a clear-cut causative factor for spermatogenic impairment) in the next generation (Lu *et al.*, 2009; Zhang *et al.*, 2007).

Monogenic Causes of Specific Infertile Phenotypes

Congenital Hypogonadotropic Hypogonadism (cHH)

Overview

Male infertility can be due to congenital or acquired hypothalamic–pituitary axis dysfunction. Multiple factors affect GnRH neuron migration, GnRH gene expression, GnRH pulse generator, GnRH secretion, GnRH receptor expression, and gonadotropin synthesis and release (Kaprara and Huhtaniemi, 2017). Congenital HH (cHH) is a rare complex genetic disease (incidence of 1:8000 males) with variable expressivity, penetrance, and inheritance. cHH may manifest as an isolated or syndromic condition. Kallmann syndrome refers to hypogonadism associated with anosmia/hyposmia. Developmental anomalies may be present in both Kallmann syndrome and in some normosmic cHH, depending on the gene defect (Boehm *et al.*, 2015). The hypogonadal phenotype ranges from absent puberty (usually associated with cryptorchidism, micropenis, and gynecomastia) to milder forms such as delayed puberty and late-onset HH. cHH presents two peculiar features from a genetic point of view: (i) some of the genes involved in this disease (e.g., *FGFR1*, *PROKR2*) may cause both Kallmann syndrome and normosmic cHH with obvious complexity for genetic counseling; (ii) it does not follow the rules of Mendelian inheritance, since in about 20% of cases there is a digenic/oligogenic inheritance, that is, presence of two heterozygous mutations in two or more cHH genes in the same individual.

To date, 35 candidate genes in cHH have been identified (Boehm *et al.*, 2015; Tournaye *et al.*, 2017), and are implicated either in the development/migration of the GnRH neurons or in the neuroendocrine regulation of GnRH secretion or action (**Table 1**).

Who should be tested?

cHH patients after the exclusion of all secondary forms (pituitary tumors, empty sella, etc).

Management

Hormonal therapies are available for the development of secondary sexual characteristics and induction of fertility. Patients who are not interested in fertility should receive testosterone replacement therapy. Since "reversibility" of the gonadotrophin deficiency after testosterone therapy has been described in about 10%–15% of patients (Dwyer *et al.*, 2016), a periodic suspension of the hormonal replacement therapy is recommended for all cHH patients. The administration of gonadotrophins is indicated when patients desire fertility. In about 80% of cHH patients, spermatogenesis can be induced (Dwyer *et al.*, 2016), and pregnancy obtained either spontaneously or through ART. PGD or prenatal diagnosis should be offered to couples, mainly for syndromic cases.

Table 1 Genes implicated in cHH and subdivided according to their predicted functions

Gene	*Chromosome (GRCh38/hg38 assembly)*	*cHH phenotype*	*Other syndromes*
Embryonic differentiation of GnRH neuron			
HS6ST1	2q14.3	KS; ncHH	–
IL17RD	3p14.3	KS	–
HESX1	3p14.3	KS	CPHD, SOD
SPRY4	5q31.3	KS; ncHH	
FGFR1	8p11.23-p11.22	KS; ncHH	CPHD, SOD, HS, SHFM
FGF17	8p21.3	KS; ncHH	D-WAS
CHD7	8q12.1-q12.2	KS; ncHH	CHARGE
FGF8	10q24.32	KS; ncHH	CPHD
DUSP6	12q21.33	KS; ncHH	—
FLRT3	20p12.1	KS	—
SOX10	22q13.1	KS	WS
Migration of GnRH neuron			
PROK2	3p13	KS; ncHH	
SEMA3A	7q21.11	KS	
SEMA3E	7q21.11	KS	
FEZF1	7q31.32	KS	
CHD7	8q12.1-q12.2	KS; ncHH	CHARGE
NELF (NSMF)	9q34.3	KS; ncHH	
WDR11	10q26.12	KS; ncHH	CPHD
SEMA7A	15q24.1	KS; ncHH	
AXL	19q13.2	KS; ncHH	–
PROKR2	20p12.3	KS; ncHH	CPHD; MGS
KAL1 (ANOS1)	Xp22.3	KS	
Upstream and metabolic regulation of GnRH neuron function			
LEPR	1p31.3	ncHH	–
KISS1	1q32.1	ncHH	–
TACR3	4q24	ncHH	–
OTUD4	4q31.21	ncHH	GHS
PCSK1	5q15	ncHH	–
RNF216	7p22.1	ncHH	GHS
LEP	7q32.1	ncHH	–
TAC3	12q13.3	ncHH	–
DMXL2	15q21.2	ncHH	PEPNS
PNPLA6	19p13.2	ncHH	GHS
KISS1R	19p13.3	ncHH	–
NR0B1	Xp21.2	ncHH	–
GnRH synthesis			
GnRH1	8p21.2	ncHH	–
GnRH activation			
GnRHR	4q13.2	ncHH	–

In each group, cHH candidate genes are reported according to the numerical order of the chromosomes (from chr1 to crh X) to which they are mapping, with associated cHH phenotypes (KS and/or ncHH) and other complex syndromes. *CHARGE*, coloboma, heart defects, atresia of choanae, retardation of growth and/or development, genital and/or urinary defects, ear anomalies or deafness; *CPHD*, combined pituitary hormone deficiency; *CTO*, contributes to oligogenicity; *D-WS*, Dandy-Walker syndrome; *GHS*, Gordon Holmes syndrome; *HS*, Hartsfield syndrome; *MGS*, Morning Glory syndrome; *PEPNS*, polyendocrine deficiencies and polyneuropathies; *SHFM*, split-hand/ft malformation; *SOD*, septo-optic dysplasia; *WS*, Waardenburg syndrome.

Congenital Absence of Vas Deferens (CAVD)

Overview

Approximately 10% of oligo/azoospermia can be attributed to ductal obstruction/dysfunction, which can be congenital or acquired. Among the congenital forms, CAVD is the most frequent and may occur either as an isolated urogenital disorder or as an atypical symptom of cystic fibrosis (CF). It accounts for 1%–2% of infertile men, 4%–17% of azoospermic men, and up to 25% of those with obstructive azoospermia (OA) (Oates and Amos, 1994). It may affect one (CUAVD) or both vas deferens (CBAVD).

CUAVD is a rare condition (1:100 CAVD patients) associated with either oligozoospermia or normozoospermia, depending on the functional competency of the contralateral testis. It is associated with ipsilateral and contralateral seminal vesicle agenesis in 90% and 20% of patients, respectively. It is worth noting that it is associated with renal agenesis in 79% of cases (Lotti and Maggi, 2015).

The CBAVD is associated with bilateral seminal vesicles agenesis in about half of the patients, in which it is characterized by typical semen alterations, such as low semen volume (<1.0 mL) with an acid pH (<7) and absence of spermatozoa (**Fig. 2**A). In contrast to CUAVD, it usually presents with normal kidneys (renal agenesis occurring in 1 out of 10 patients (Schlegel *et al.*, 1996).

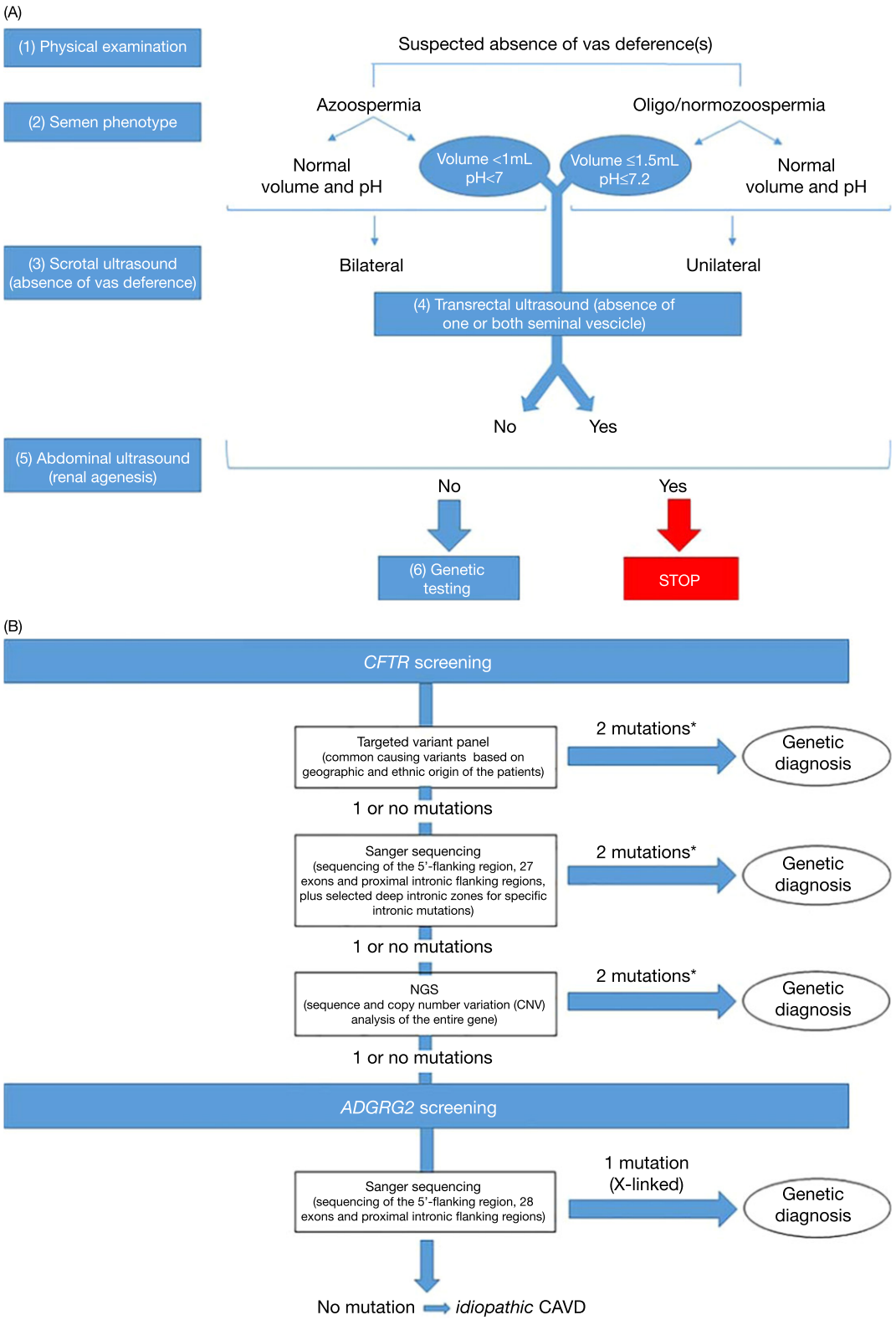

Fig. 2 Flow chart illustrating the diagnostic steps in cases of suspected CAVD. (A) After physical examination, semen analysis (azoospermia or oligo/normozoospermia) is followed by scrotal ultrasound in order to confirm the clinical suspect of bilateral or unilateral absence of vas deferen(s). Transrectal ultrasound is performed when semen analysis is indicative for the bilateral or unilateral absence of seminal vesicle(s). Finally, abdominal ultrasound is performed in order to rule out renal agenesis. (B) *CFTR* screening is the first genetic test in CAVD. The testing is based on an initial targeted variant panel followed by extended Sanger sequencing (in case only one or no mutation is found). Finally, if the patient is wild-type or a carrier of only one mutation, an NGS procedure is recommended. The *ADGRG2* testing should be performed in *CFTR* negative cases. Since *ADGRG2* is an X-linked gene, the presence of only one causative variant in the patient allows the genetic diagnosis of CAVD. The *CFTR* and *ADGRG2*-negative patients are defined as "idiopathic" cases of CAVD. CAVD: congenital absence of vas deferen(s). *Testing of female partner for CFTR.

CAVD is strictly related to *CFTR* gene mutations. >2000 variants have been identified in this gene, and they can be classified in severe and mild mutations depending on their functional effect. The presence of two severe mutations leads to the cystic fibrosis phenotype, whereas two mild mutations or one severe plus a mild mutation are causative for CAVD. Although geographical and ethnic differences have been demonstrated in *CFTR* mutations, the common mutations in CBAVD patients are F508del, 5T, and R117H. The genetic testing is based on three consecutive steps (**Fig. 2**B). After a comprehensive analysis of the *CFTR* gene, in about 20% of cases the origin of CAVD remains unknown. Recently, the *ADGRG2* gene has been identified as a new candidate gene in CBAVD (Patat *et al.*, 2016; Yang *et al.*, 2017). This gene is specifically expressed within the efferent ducts (Obermann *et al.*, 2003), in which most of the testicular fluid carrying immature spermatozoa is reabsorbed. Pathogenic *ADGRG2* variants were reported, accounting for 11%–15% of the CBAVD patients who are *CFTR*-negative (Patat *et al.*, 2016; Yang *et al.*, 2017).

Who should be tested?

Genetic testing of CAVD patients should be preceded by ultrasound scan of the abdominal region in order to exclude renal agenesis (the genetic basis of which is unknown) (Jungwirth *et al.*, 2012) (**Fig. 2**). The female partner of a man with CAVD without congenital kidney anomalies or with CF should be tested prior to assisted reproduction.

Management

Given that the carrier frequency of *CFTR* mutations in subjects with European descent is high (1:25), the *CFTR* panel screening in the female partners is mandatory. If mutations are detected in both partners, the risk of an offspring with CF (or mild forms of CF, depending on the type and combination of mutations) is very high and PGD should be advised to the couple. However, genotype/phenotype correlation is difficult due to different degrees of penetrance of the same mutation in different individuals (Cuppens and Cassiman, 2004).

Partial or Mild Androgen Insensitivity Syndrome (PAIS or MAIS)

Overview

Spermatogenic disturbances in hypoandrogenized men can be due to androgen receptor (AR) dysfunction. The phenotypic spectrum of androgen insensitivity syndromes ranges from complete androgen insensitivity (CAIS; Morris syndrome), characterized by a female phenotype in 46, XY individuals, to partial (PAIS; Reifenstein syndrome) and mild (MAIS) forms, ranging from undervirilized male phenotype with ambiguous genitalia to impaired sperm production with normal male genitalia, respectively (Krausz and Chianese, 2014). The AR gene maps to the X chromosome. Up to now, >1000 *AR* variants have been described, which account for 41% of PAIS cases (regarding the MAIS phenotype, there are no certain estimates in the available mutation databases) (Gottlieb *et al.*, 2012).

Besides pathogenic mutations, two polymorphisms sites in the N-terminal trans-activation domain of the receptor, the polyglutamine tract (CAG)*n* and the polyglycine tract (GGC)*n*, have been proposed as genetic risk factors of male infertility. According to the majority of studies, variations of the GGN repeat length are not associated with infertility (Davis-Dao *et al.*, 2007; Ferlin *et al.*, 2004; Lundin *et al.*, 2003; Rajender *et al.*, 2006; Ruhayel *et al.*, 2004). As far as the polyglutamine tract variation is concerned, the originally described inverse relationship between CAG repeat length and the receptor trans-activation (Davis-Dao *et al.*, 2007; Gao *et al.*, 1996) led to the hypothesis that a longer CAG repeat conferred a higher risk for infertility. This hypothesis has been challenged by novel functional and observational studies reporting that both a longer CAG tract and a shorter CAG tract might have a negative effect on the receptor function, hence an optimal number of CAG repeats is necessary for the highest transcription (Davis-Dao *et al.*, 2012; Nenonen *et al.*, 2011). We can speculate that the optimum range may vary between the genomic and non-genomic actions, and also in different tissues, because the effect of polyQ repeat on transactivation is cell specific, presumably due to distinct profiles of co-regulator proteins (Krausz, 2012). The role of CAG repeats in male infertility is probably more complex than has been previously believed, hence screening for this polymorphism is not indicated in the diagnostic setting.

Who should be tested?

AR mutational analysis is advised only in infertile men with suspected PAIS or MAIS, hence in patients with hypoandrogenization (with or without ambiguous genitalia) with high LH and relatively high testosterone levels.

Monomorphic Teratozoospermia

Overview

The congenital qualitative alterations of spermatogenesis may cause abnormal sperm morphology/motility and some of them can be the consequence of genetic anomalies. Monomorphic teratozoospermia are very rare pathological conditions with autosomal recessive mode of inheritance. Among these disturbances, globozoospermia and macrozoospermia (or sperm macrocephaly) are the most frequent, with a <0.1% and <1% prevalence in the subfertile population, respectively. Both diseases are incompatible with natural conception. Globozoospermia is characterized by round-headed, acrosomeless spermatozoa, which are unable to

fertilize the oocyte. The most frequent and validated genetic cause is the complete deletion of the *DPY19L2* gene (accounting for about 80% of patients) (Ray *et al.*, 2017; Chianese *et al.* 2015). Macrozoospermia is characterized by large-headed and multi-flagellated spermatozoa (Nistal *et al.*, 1977) and is related to *AURKC* gene mutations (90% of patients are homozygous for a deletion of a cytosine in exon 3) (Ray *et al.*, 2017).

Who should be tested?

DPY19L2 testing should be limited to patients with 100% globozoospermia. In cases where a homozygous carrier status for *DPY19L2* mutation is confirmed, the female partner should also be tested for *DPY19L2* deletion. The rationale is the relatively high deletion carrier frequency in the general population (1:85). *AURKC* testing should be recommended in all men affected by macrozoospermia.

Management

In cases of complete globozoospermia due to *DPY19L2* mutations, ICSI is the only therapeutic option. Although ICSI overcomes the absence of acrosin, which serves to spermatozoa to reach the occytes, injected spermatozoa are often unable to activate the oocytes due to the lack of acrosome phospholipase C zeta (Eskandari *et al.*, 2017). In addition, the fertility outcome (pregnancy and live-birth success rates) is relatively poor due to high sperm aneuploidy rates, DNA damage, and epigenetic alterations (Ray *et al.*, 2017).

In cases of macrozoospermia, ICSI is not advised for couples, since *AURKC* mutations are associated with an extremely high aneuploidy rate (in the majority of cases, spermatozoa are tetraploid and thus unable to allow normal embryonic development) (Ray *et al.*, 2017).

Genetic Factors in Idiopathic Oligo/Azoospermia

After a complete diagnostic workup, including the above-described genetic tests, in about 50% of cases of impaired spermatogenesis the etiology remains unknown (Krausz, 2011). These "idiopathic" cases of infertility are likely to be related to yet unidentified genetic/epigenetic and environmental factors. The discovery of the "hidden" genetic factors is relevant for both avoiding unnecessary surgical or empirical treatments and informing the couple undergoing ART about the risk of transmitting genetic alterations to the future offspring.

Different genetic approaches were applied in order to identify the cause of idiopathic infertility. The first one was based on the re-sequencing of candidate spermatogenesis genes (involved in hormonal regulation, cell metabolism/proliferation, and meiosis). These studies have targeted only a few genes in relatively small study populations without obtaining clinically useful results (Krausz *et al.*, 2015; Mitchell *et al.*, 2017). Since at least 2000 genes are involved in spermatogenesis, much expectation was given to "high-throughput" genomic technologies (SNP and CGH arrays) allowing Genome Wide Association Studies (GWAS). Although these approaches led to important advancements in the understanding of a series of complex diseases (Auton *et al.*, 2015; Riggs *et al.*, 2014), GWAS were largely unsuccessful for idiopathic male infertility (Krausz *et al.*, 2015). GWAS based on SNP-arrays identified a few common SNPs with significant but low effect size which may exert a modulating effect on the onset of impaired spermatogenesis, especially if they are present contemporarily in the same individual (Aston *et al.*, 2010; Kosova *et al.*, 2012). Another microarray-based approach is by Comparative Genomic Hybridization array (CGH-arrays), which is used for determining Copy Number Variations (CNVs). In patients with spermatogenic quantitative disturbances, only a few partially overlapping CNVs have been observed in different studies (Krausz *et al.*, 2015 and reference therein). The clinically most promising ones are the *TEX11* intragenic deletion and CNV67 (a deletion which removes between 5.417 and 25.513 kb of DNA mapping to the long arm of the X chromosome in q28) (Yatsenko *et al.*, 2015; Lo Giacco *et al.*, 2014; Shen *et al.*, 2017). These X-linked anomalies may act directly, since no compensatory allele is present in the male genome.

A relevant finding of the array-CGH studies is the presence of a "CNV burden" (especially deletions) in idiopathic infertile patients (Krausz *et al.*, 2012; Lopes *et al.*, 2013; Tuettelmann *et al.*, 2011). A high deletion load may suggest a higher genomic instability in these men, which may have an impact not only on their fertility status but also on their general health. In fact, a CNV burden may well be one of the possible explanations for the higher morbidity and lower life expectancy observed in infertile men in respect to fertile men (Eisenberg *et al.*, 2015; Jensen *et al.*, 2009; Krausz *et al.*, 2012; Salonia *et al.*, 2009).

With the advent of Next Generation Sequencing (NGS), Whole Exome analysis (WES) became feasible and allowed the genetic diagnosis of NOA in consanguineous families (**Table 2**), leading to discovery of 12 novel candidate genes (Arafat *et al.*, 2017; Ayhan *et al.*, 2014; Gershoni *et al.*, 2017; Kherraf *et al.*, 2017; Maor-Sagie *et al.*, 2015; Okutman *et al.*, 2015, 2017; Ramasamy *et al.*, 2015; Tenenbaum-Rakover *et al.*, 2015). Since only homozygous carriers of rare mutations (with $<1\%$ frequency in the general population) in the above 12 genes show azoospermia, the diagnostic value for sporadic NOA cases is questionable. It is highly unlikely that rare mutations can be present in homozygous state in patients with unrelated parents. It remains to be established if the presence of a rare autosomic gene variant in heterozygosis may eventually affect the efficiency of spermatogenesis, that is, be responsible for a milder phenotype (oligozoospermia). Large-scale studies, based on WES of thousands of well-characterized infertile men, are ongoing; the ultimate objective is to provide a comprehensive picture on the genetic determinants of idiopathic oligo/azoospermia.

Table 2 List of genes discovered to be associated with non-obstructive azoospermia (NOA) through Whole Exome Sequencing (WES) studies in consanguineous families

Gene	*Chromosome (GRCh38/hg38 assembly)*	*Associated semen phenotype (s)*	*Genotype in affected individuals*	*References*
DNAH6	2p11.2	NOA	Homozygous	Gershoni *et al.*, 2017
NPAS2	2q11.2	NOA	Homozygous	Ramasamy *et al.*, 2015
SPINK2	4q12	NOA	Homozygous	Kherraf *et al.*, 2017
TEX15	8p12	NOA + SO	Homozygous	Okutman *et al.*, 2015
SYCE1	10q26.3	NOA	Homozygous	Maor-Sagie *et al.*, 2015
TDRD9	14q32.33	NOA + cryptozoospermia	Homozygous	Arafat *et al.*, 2017
MEIOB	16p13.3	NOA	Homozygous	Gershoni *et al.*, 2017
ZMYND15	17p13.2	NOA	Homozygous	Ayhan *et al.*, 2014
TEX14	17q22	NOA	Homozygous	Gershoni *et al.*, 2017
TAF4B	18q11.2	NOA + oligozoospermia	Homozygous	Ayhan *et al.*, 2014
MCM8	20p12.3	NOA	Homozygous	Tenenbaum-Rakover *et al.*, 2015
MAGEB4	Xp21.2	NOA + SO	Hemizygous	Okutman *et al.*, 2017

The genes are reported according to the numerical order of the chromosomes (from chr 1 to chr X) to which they are mapping. Associated semen phenotype, genotype in affected individuals and references are reported for each gene.

SO: Severe oligozoospermia.

Pharmacogenetics in Idiopathic Male Infertility

Single-nucleotide polymorphisms (SNPs) in the genes encoding for the b-subunit of Follicle-Stimulating Hormone (FSHb) and for the Follicle-Stimulating Hormone Receptor (FSHR) have been investigated in relationship with reproductive parameters and with individual responsivity to FSH therapy, opening the way towards a pharmacogenetic approach in idiopathic infertility. One of the major determinants of serum FSH level is the *FSHB*-211G>T in the gene promoter, that is, levels of serum FSH, as well as sperm count, are significantly lower in G/T heterozygous men, and even lower in T/T homozygous carriers (Grigorova *et al.*, 2011). For the *FSHR*, c.-29G>A and 2039A>G (p.Asn680Ser) polymorphisms, homozygous A/A or G/G carriers show a reduced FSH receptor sensitivity with consequent high circulating FSH levels in men with normal sperm count (Grigorova *et al.*, 2014; Tuettelmann *et al.*, 2012).

While FSH therapy together with hCG is a standard treatment to induce fertility in subjects with gonadotrophin deficiency, the use of FSH in normogonadotrophic idiopathic infertile men is a debated issue. According to two meta-analyses, FSH treatment in idiopathic infertile men increases quantitative and qualitative semen parameters and pregnancy rate (Attia *et al.*, 2013; Santi *et al.*, 2015), but only about 50% of patients will respond in terms of improvement of semen parameters (Casamonti *et al.* 2017).

To date, four pharmacogenetic studies have been carried out in relationship with these polymorphisms and all are based on relatively small cohorts (Casamonti *et al.*, 2017; Ferlin *et al.*, 2011; Selice *et al.*, 2011; Simoni *et al.*, 2016). These studies reported a strict correlation between the presence of FSHb (−211G>T) and FSHR (p.Asn680Ser) polymorphisms and response to treatment. In their studies, patients carrying the mutated allele of FSHb and FSHR showed a significantly higher improvement of the "classical" seminal parameters (total sperm count, motility, and morphology) than patients with the "wild type" genotype. On the other hand, Simoni *et al.* (2016) demonstrated that FSH administration is effective in reducing sperm DNA fragmentation in men carrying the combined FSHR p.Asn680Ser homozygous and FSHb −211G>T homozygous genotypes (i.e., "wild type" genotypes). Finally in a recent paper, all three variants (FSHB c.−211G>T, FSHR c.2039A>G, and FSHR c. −29G>A) have been analyzed in relation to total sperm count, total motile sperm count, and hyaluronic acid binding capacity which is a biomarker of sperm maturity (Casamonti *et al.*, 2017). In contrast to the other studies, responsiveness to treatment was independent from *FSHR*/*FSHB* polymorphisms. These controversies clearly indicate that at present, a pre-selection of "responders" to FSH therapy cannot be based on the analysis of these three SNPs. Further large studies are needed to clarify this clinically relevant issue.

Conclusions

Over the past 30 years, besides karyotype analysis, molecular genetic testing became a routine diagnostic tool in male reproductive dysfunction. Currently, genetic diagnosis can be made in about 25% of male infertility providing an important input for clinical decision-making. The polygenic nature of this pathological condition and the relatively high prevalence of unknown etiology suggest that many novel genetic/epigenetic causes are likely to be discovered in the coming years.

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Testicular Tumors

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Glossary

Germ cell neoplasia in situ (GCNIS) The precursor lesion for testicular germ cell tumors that occur in young adults (seminoma and non-seminoma), previously known as carcinoma in situ, GCNIS consists of developmentally arrested immature germ cells with features of pluripotency located inside testicular seminiferous tubules.

Gonadoblastoma A premalignant lesion, similar to GCNIS, usually present in dysgenetic testes or gonads in individuals with disorders of sexual development.

Leydig cells Cells producing testosterone and other hormones located in the interstitial (inter-tubular) compartment of the testicle.

Non-seminoma A name for a group of tumors derived from immature germ cells characterized by morphological heterogeneity, most common in testis, but can also occur in extragonadal locations.

Seminoma A germ cell tumor derived from immature germ cells.

Sertoli cells Cells nurturing germ cells within seminiferous tubules of the testicle.

Teratoma A subtype of non-seminoma, characterized by somatic differentiation, can contain components resembling any tissue type.

Introduction

Testicular cancer can be derived from several cell types of the testicle, but germ cell-derived tumors constitute the vast majority of cases. Testicular germ cell tumors (TGCT) are most common among adolescents and young men, but can also occur in early childhood (infantile TGCT) and in older men (spermatocytic tumor). The focus of this article is on the most common TGCTs of young men, which are derived from germ cell neoplasia in situ (GCNIS), originally described as carcinoma in situ (CIS) of the testis (Skakkebaek, 1972). Testicular tumors can also be derived from somatic cells within the testicle, and are known as Leydig cell tumors and sex cord-stromal neoplasms. Both are relatively rare in adults but are not uncommon in childhood, and will be briefly mentioned because of their endocrine symptoms.

Pathogenesis of TGCT of adolescents and young adults has a strong developmental component and overlaps with other disorders of the male reproductive system, such as cryptorchidism and mild genital malformations, within the proposed testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*, 2001; Rajpert-De Meyts, 2006; Juul *et al.*, 2014). All these disorders, but especially testicular cancer have been increasing in incidence around the world, but etiologic factors have not yet been elucidated.

Clinical management of testicular cancer is mainly handled by urologists and oncologists, but endocrinologists or andrologists have an important part, especially concerning the follow-up of the survivors, which requires treatment of androgen insufficiency and infertility, which are frequent in these patients.

Classification, Histopathology and Molecular Features of Testicular Tumors

Histopathology of testicular tumors is very complex, because of their heterogeneity, pluripotency and ability to transdifferentiate. Primary testicular tumors can be derived from germ cells, from Sertoli cells (or their female equivalents, granulosa cells) and from Leydig cells (grouped together as sex cord/gonadal stromal tumors). In addition, there are other very rare tumors occurring in the testis, e.g. rhabdomyosarcoma. The testicle can also be a site of hematolymphoid malignancies: leukemia (mainly in children) and lymphoma (mainly in adults). Detailed classification and pathology of testicular tumors can be found in the WHO consensus classification (Moch *et al.*, 2016). For the use of non-pathologists, a simplified division of testicular tumors into main types is sufficient, as shown in **Table 1**.

Testicular Germ Cell Tumors Derived From GCNIS

TGCT derived from GCNIS; seminoma and non-seminoma, occur predominantly in young adult men. The precursor, GCNIS, consists of developmentally arrested immature germ cells (gonocytes) that persisted outside of fetal/perinatal life and then underwent malignant transformation (Rajpert-De Meyts, 2006; **Fig. 1**). GCNIS cells resemble morphologically fetal gonocytes, and have a similar gene expression profile, with a high level of expression of embryonic pluripotency factors, such as OCT4, NANOG, TFAP2C, LIN28 (Almstrup *et al.*, 2004; Sonne *et al.*, 2009). GCNIS cells are located inside well-developed seminiferous tubules, usually along the basement membrane, in the place of spermatogonia. Gonadoblastoma is a

Table 1 Simplified division of primary testicular tumors, based on the 2016 WHO classification

Germ cell tumors (GCT)

- ● GCT derived from germ cell neoplasia in situ (GCNIS)
- ○ Precursor lesions: GCNIS, gonadoblastoma
- ○ Seminoma, pure
- ○ Non-seminomatous GCT, pure
  - – Embryonal carcinoma
  - – Yolk sac tumor (post-pubertal type)
  - – Choriocarcinoma and other trophoblastic tumors
  - – Teratoma (post-pubertal type)
- ○ Mixed germ cell tumors
- ● GCT unrelated to GCNIS
- ○ Prepubertal (pediatric) tumors
  - – Teratoma (prepubertal type)
  - – Yolk sac tumor (prepubertal type)
  - – Mixed prepubertal type tumors
- ○ Spermatocytic tumor

Sex cord/gonadal stromal tumors

- ● Leydig cell tumor
- ● Sertoli cell tumor (SCT)
- ○ Malignant SCT
- ○ Large cell calcifying SCT
- ● Sertoli–Leydig cell tumors
- ● Granulosa cell tumors
- ○ Juvenile-type
- ○ Adult-type
- ● Mixed and unclassified sex cord-stromal tumors

Hematolymphoid tumors
Miscellaneous tumors

preinvasive lesion, similar to GCNIS, which occurs predominantly in individuals with disorders of sexual development (DSD), also known as intersex syndrome, for example, in patients with mixed gonadal dysgenesis (45X/46XY). Gonadoblastoma consists of groups of cells, which are embedded in poorly developed tubules, containing also small somatic cells, resembling granulosa cells. GCNIS and gonadoblastoma can be present in the same gonad, and combined lesions are not uncommon in patients with TDS (Jørgensen *et al.*, 2015).

Overt seminoma is most often diagnosed in men 25- to 45-year-old, whereas non-seminomatous tumors occur in younger men, mainly in the age group 17–35 years. Seminoma is a histologically homogeneous tumor consisting of a mass of immature germ cells which are virtually identical to GCNIS cells and resemble fetal gonocytes, with a similar gene expression pattern. In addition, nearly all seminomas, and a large subset of GCNIS cases are characterized by prominent lymphocytic infiltration (Hvarness *et al.*, 2013; Klein *et al.*, 2016). Non-seminomatous tumors are histologically heterogeneous and several subtypes can be present as pure or mixed components (**Table 1**). The undifferentiated form is embryonal carcinoma, which is characterized by a high level of expression of pluripotency factors, such as OCT4, but also SOX2, which is downregulated in human germ cells (Perrett *et al.*, 2008). Embryonal carcinoma readily differentiates into somatic components of any tissue type (teratoma) or into extra-embryonic tissue components (yolk sac and trophoblastic tumors). The mixed tumors contain various components of non-seminoma and seminoma, but classified and clinically treated as non-seminoma, which is a more aggressive TGCT (Moch *et al.*, 2016; Rajpert-De Meyts *et al.*, 2016; Verrill *et al.*, 2017).

The genome of all neoplasms derived from GCNIS is characterized by aneuploidy (near tetraploidy), with a pathognomonic for overt seminomas and non-seminomas amplification of chromosome 12, often as isochromosome 12p (Atkin and Baker, 1982; von Eyben, 2004). The tumors can also contain secondary aberrations caused by gene/transcript fusion (Hoff *et al.*, 2016). Another interesting feature of the genome of TGCTs is a very low in GCNIS and seminomas but variable in non-seminomas DNA-methylation (Smiraglia *et al.*, 2002; Kristensen *et al.*, 2013). In addition to protein-coding genes, TGCTs of young adults, including their precursor, GCNIS, but excluding teratomas, produce and secrete specific micro-RNA (miR) similar to embryonic stem cells. This micro-RNA profile is characterized by a high level of miR-371-3 cluster, miR-302 and miR-367 (Voorhoeve *et al.*, 2006; Palmer *et al.*, 2010; Dieckmann *et al.*, 2012; Novotny *et al.*, 2012).

Specific causative gene mutations have not been identified, except secondary gain-of-function *KIT* mutations (predominantly in seminomas), or *KRAS* (predominantly in non-seminomas) (Tian *et al.*, 1999; McIntyre *et al.*, 2005). However, familial risk of testicular cancer is high in comparison to other type of cancers. Brothers and sons of patients with TGCT have an estimated 8–10-

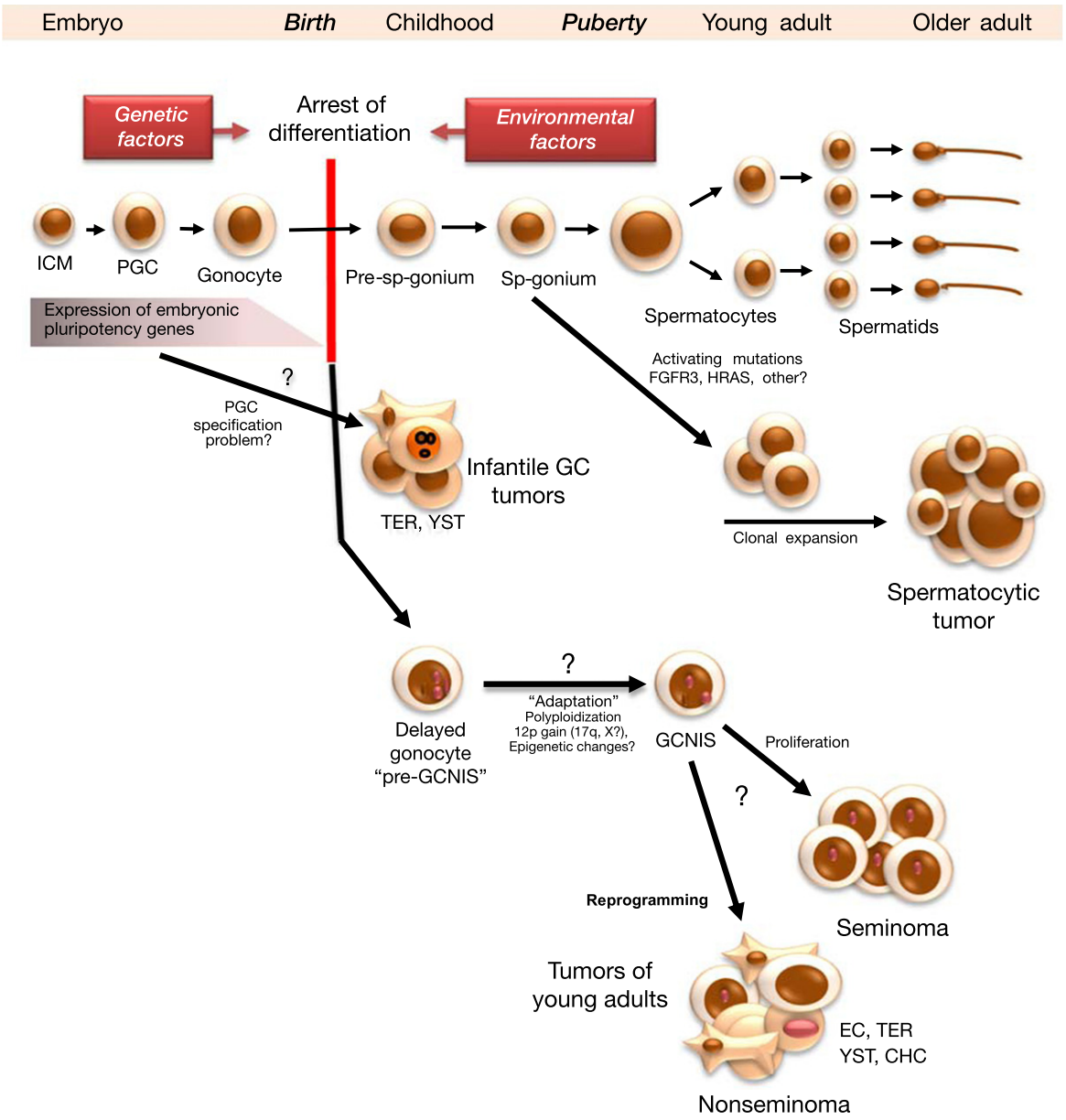

Fig. 1 Schematic histogenesis of testicular germ cell tumors (TGCT) in relation to the normal development of germ cells. Normal germ cell development is shown near the top part of the picture. Rare TGCTs that are unrelated to germ cell neoplasia in situ (GCNIS): infantile germ cell tumors and spermatocytic tumor, are shown in the middle part of the graph. The most common TGCT of young adults (seminoma and non-seminoma) that are derived from fetal germ cells (delayed gonocytes) via the stage of GCNIS are shown in the lower part of the figure. These tumors are likely caused by a combination of genetic and environmental factors acting during early development. Reprinted from Rajpert-De Meyts et al. Lancet 2016, with permission from Elsevier.

fold and 4–6-fold increased risk, respectively (Hemminki and Chen, 2006). This was roughly confirmed by a large study of Nordic twin populations, which estimated the heritability of testicular cancer as more than one third of cases, with about a quarter attributed to shared environment (Mucci *et al.*, 2016).

As mentioned below (in the "Epidemiology of Testicular Cancer" section), predisposition to testicular cancer differs widely in different ethnic groups, with the highest incidence among Caucasians and the lowest among men of African ancestry (Znaor *et al.*, 2014). Several genome-wide association studies (GWAS) have identified a number of possibly predisposing gene variants, some of which have different prevalence among whites and blacks. The strongest genetic markers for an increased risk of TGCT are located within or near the following loci: *KITLG*, *SPRY4*, *DMRT1*, *PRDM14*, *DAZL*, *HPGDS* (Rapley *et al.*, 2009; Kanetsky *et al.*, 2009; Turnbull *et al.*, 2010). Other informative markers are being revealed by meta-analytic GWAS that combine data from very large cohorts (Litchfield *et al.*, 2017; Wang *et al.*, 2017). Interestingly, these predisposing genetic markers represent predominantly

pathways involved in germ cell specification and gonadal development or in DNA repair and telomerase function (Litchfield *et al.*, 2015).

Testicular Germ Cell Tumors Not Associated With GCNIS

In early childhood TGCT can also occur, these tumors comprise two histological types, similar in appearance to the adult types: prepubertal-type yolk sac tumor or mature teratoma (including dermoid cyst) (Oosterhuis and Looijenga, 2005; Moch *et al.*, 2016; **Fig. 1**). The childhood GCTs display also gene expression and micro-RNA profiles similar to the adult equivalents (Palmer *et al.*, 2010; Mosbech *et al.*, 2014; Murray *et al.*, 2015). The etiology and pathogenesis of these tumors is unknown, but the cell of origin is most likely pluripotent primordial germ cell (Murray *et al.*, 2015).

Another rare TGCT, spermatocytic tumor, previously known as spermatocytic seminoma, occurs on the other end of the age spectrum; in older men (40–80 years). This tumor occurs only in testes and is composed of germ cells of three different cell sizes, with phenotypic features and gene expression profile similar to spermatogonia or primary spermatocytes (Looijenga *et al.*, 2006; Lim *et al.*, 2011). Spermatocytic tumors grow from expanding clones of spermatogonia, which underwent genomic changes facilitating their proliferation, such as amplification of chromosome 9 (with *DMRT1* locus), chromosome 20, activating mutations in *FGFR3*, *HRAS*, *NRAS*, or a whole-chromosome aneuploidy (Looijenga *et al.*, 2006; Goriely *et al.*, 2009; Giannoulatou *et al.*, 2017; **Fig. 1**).

Sex-Cord/Gonadal Stromal Tumors (Leydig Cell-, Sertoli Cell- or Granulosa Cell Tumors)

Histopathological division of these rare tumors has been revised in the latest WHO consensus classification (Moch *et al.*, 2016; Idrees *et al.*, 2017). In comparison to TGCTs, Leydig cell tumors, LCT (*leydigioma*) are relatively rare but can occur in all ages, predominantly in adults. In children these tumors can cause precocious puberty, so-called testotoxicosis (Rich and Keating, 2000). LCT resemble morphologically a mass of Leydig cells, and express similar markers. Malignant LCTs have to be distinguished from benign hyperplasia, adenomas and adrenal rest tumors (Lottrup *et al.*, 2015). Histopathological features of malignant LCTs are cytologic atypia with high mitotic activity, angiolymphatic invasion, necrosis, also DNA aneuploidy is consistent with malignant transformation. Molecular background of Leydig cell tumors is not well elucidated. In some malignant LCT, mutations of fumarate hydratase were identified (Carvajal-Carmona *et al.*, 2006). In most childhood cases an activating mutation in luteinizing hormone receptor (*LHCGR*) (Liu *et al.*, 1999; Boot *et al.*, 2011). Precocious puberty, sometimes starting in infancy is caused by excessive gonadotropin-independent production of testosterone and other steroid hormones by the LCT, and the patients appear to be at risk of germ cell neoplasia later in life (Mortensen *et al.*, 2017).

Sertoli cell tumors (SCT) and granulosa cell tumors are mainly diagnosed in children and adolescents and characterized by heterogeneous histopathological features, resembling immature cells of origin. SCT most often form tubular-like structures, while granulosa tumors resemble the ovarian counterparts. These tumors are usually benign. Other, very rare SCT, are grouped into mixed and unclassified category; these are usually unilateral and occasionally malignant (Idrees *et al.*, 2017). SCT are frequently found in association with multiple neoplasm syndromes; Carney complex and Peutz–Jeghers syndrome (Moch *et al.*, 2016). The phenotype of Carney complex includes skin and heart myxomas, skin pigmentation, testicular and adrenal tumors (Carney *et al.*, 1985). The tumors arising in the testes of the Carney patients are usually bilateral and benign, and have a typical histology of are large-cell calcifying SCT (Washecka *et al.*, 2002). Large-cell calcifying SCT can also occur in isolation (Lai *et al.*, 2015). Germline mutations identified in Carney complex most often occur in *PRKAR1A* gene (Kirschner *et al.*, 2000) but other loci on chromosome 2 have also been identified (Stratakis *et al.*, 1996). Tumors that arise in Peutz–Jeghers syndrome are also benign, bilateral and multifocal but are associated with gastrointestinal tumors and often gynecomastia (Hertl *et al.*, 1998). These SCT can be distinguished from those associated with Carney complex by typical intratubular proliferation of lightly eosinophilic cells with prominent basement membrane deposits (Idrees *et al.*, 2017). The genetic cause of Peutz–Jeghers syndrome is a loss-of-function germline *STK11* mutation that encodes for a serine–threonine kinase LKB1, which leads to overexpression of aromatase (Ham *et al.*, 2013).

Epidemiology of Testicular Cancer

This section deals exclusively with testicular germ cell tumors (TGCT) associated with GCNIS, because other types of tumors in the testis are rare and their incidence has not been changing.

The incidence of TGCT has been rising markedly around the world in the second half of the twentieth century, with interesting geographical variability (Chia *et al.*, 2010; Znaor *et al.*, 2014). These tumors are now the most common malignancy among Caucasian young men, but the incidence remains low in Asian and African populations (**Fig. 2**). An interesting exception is a relatively high incidence of TGCT among Maoris of New Zealand (Wilkinson *et al.*, 1994). The incidence trends in testicular cancer are dynamic, with signs of abating in the North-West of Europe, e.g. in Denmark, but dramatically growing rates in the Southern Europe, e.g. in Slovenia or Croatia, and among Hispanic populations of America (Znaor *et al.*, 2014; Ghazarian *et al.*, 2015).

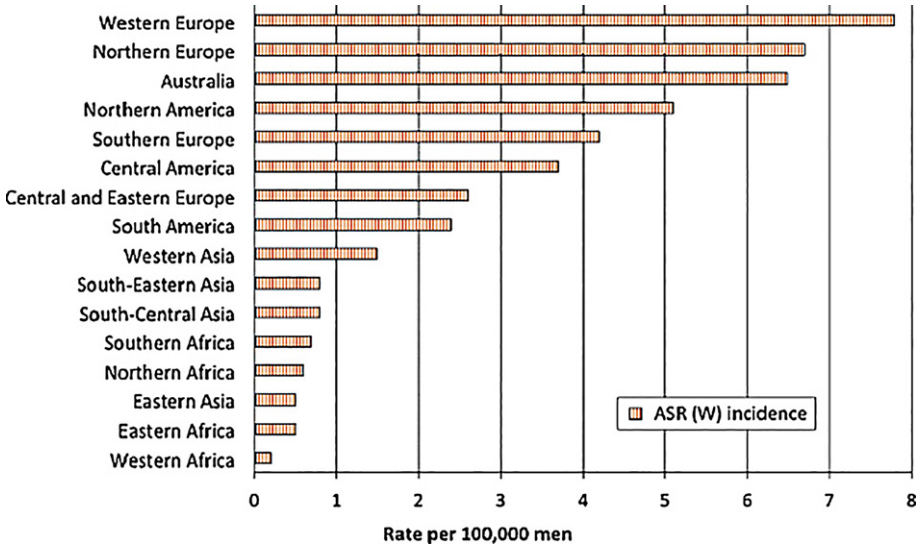

Fig. 2 Incidence rates of testicular cancer in different regions of the world. Age-standardized incidence rates (ASR) for testicular cancer for men of all ages in selected regions and countries of the world, extracted from GLOBOCAN 2008 database. Note that reporting systems varied by country and data quality may have fluctuated between regions. Reprinted from Rosen, A., Jayram, G., Drazer, M., and Eggener, S. E. (2011). Global trends in testicular cancer incidence and mortality. *European Urology* **60**, 374–379, with permission from Elsevier.

Interestingly, the TGCT risk is associated with the calendar year of birth rather than with the age of diagnosis; the so-called birth cohort effect (Bergström *et al.*, 1996).

Individuals with developmental abnormalities of the gonads and sex differentiation (disorders of sex development, DSD) are at high risk of germ cell neoplasia. The risk in DSD individuals varies, but it is greatest in persons with 45,X/46,XY karyotype, and with a partial androgen insensitivity syndrome (Cools *et al.*, 2011; Jørgensen *et al.*, 2015). Cryptorchidism is the most significant risk factor for sporadic testicular cancer (Dieckmann and Pichlmeier, 2004). In addition, inguinal hernia, low birth weight, high maternal age, being born first, late age at puberty, tall stature and poor spermatogenesis were reported associated with a risk of TGCT (Cook *et al.*, 2010; Jacobsen *et al.*, 2000; Lerro *et al.*, 2010; Maule *et al.*, 2012). Increased incidence of cryptorchidism, genital malformations and male infertility at the same time as of testicular cancer was a basis for a hypothesis that these conditions could be etiologically linked within the testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*, 2001). However, not all cases of genital malformations or infertility are a part of TDS; only those that are linked to early development, and without an obvious genetic aberration, are involved (Jørgensen *et al.*, 2010).

Most epidemiological studies are consistent with the early initiation of TGCT derived from GCNIS but it has been difficult to identify causal factors. Some associations with pre-natal and maternal exposures to xenobiotics/endocrine disruptors have been reported. Early studies investigated estrogens, including in utero exposure to diethylstilbestrol, but were not conclusive (Dieckmann and Pichlmeier, 2004; Strohsnitter *et al.*, 2001). Later studies focused on anti-androgenic organochlorine compounds (e.g. DDT) and phthalates, but human data are very scarce. Some evidence was found for higher serum levels of the persistent polychlorinated biphenyls (PCBs) in mothers of men with TGCTs (Hardell *et al.*, 2006), and of DDE in the serum of patients with TGCT (McGlynn *et al.*, 2008; Giannandrea *et al.*, 2013).

Very few risk factors for TGCT that act in adulthood have been identified. The only consistently reported post-pubertal risk factor for TGCT (mainly non-seminoma) is a heavy marijuana use (Daling *et al.*, 2009; Gurney *et al.*, 2015).

Management of Testicular Tumors

Clinical Diagnosis and Tumor Markers

A testicular mass, either felt by the patient, or detected in an ultrasonic investigation of the scrotum is usually the first presentation of testicular cancer. In some patients, testicular cancer can first manifest as metastatic disease, with usually uncharacteristic symptoms, such as lumbar pain, pulmonary symptoms or palpable lymph nodes. In the cases of some sex-cord stromal tumors, the clinical diagnosis can be related to excessive levels of steroid hormones produced by the tumors; androgens in Leydig cell tumors, estrogens or aromatase in Sertoli cell tumors. Accordingly, warning signs requiring careful differential diagnosis are precocious puberty in young boys, or gynecomastia at any age.

The preinvasive stage of TGCT, GCNIS is usually asymptomatic, so diagnosis at this early stage is rare, and most often happens in individuals from high-risk groups, such as DSD, history of cryptorchidism or infertility. Among the infertile men, the risk is much higher in patients with testicular atrophy and/or poor semen quality, and concomitant testicular microlithiasis (Jacobsen

et al., 2000; Rud et al., 2013; Tan et al., 2010). Diagnosis of GCNIS in requires testicular biopsy, which in most cases should be done bilaterally. Recognition of GCNIS requires experience, so immunohistochemical staining for GCNIS markers (e.g. primordial germ cell markers, such as PLAP or OCT4, mentioned above) are mandatory in all cases (Rajpert-De Meyts et al., 2015). In about 5%–8% cases of unilateral TGCT, GCNIS is present in the contralateral testicle, so a biopsy of the remaining testis is advised at the time of orchidectomy for the primary tumor, at least in men at high risk. A detailed description of the testicular biopsy methods and evaluation can be found in several review articles dedicated to this subject (McLachlan et al., 2007; Dieckmann et al., 2011; Rajpert-De Meyts et al., 2017). There are ongoing efforts to replace testicular biopsy for detection of GCNIS by a less invasive method of detection in a semen sample, which can be taken repeatedly. There is already an cytologic detection method, using an automated double-staining assay for alkaline phosphatase and AP2-gamma or OCT4, which are present only in GCNIS/tumor cells in semen, but further improvement of sensitivity is needed for routine use in the clinics (Almstrup et al., 2011).

Detection of incipient or disseminated TGCT, especially non-seminomas, is helped by circulating tumor markers. Taking a blood sample for these markers is obligatory in the clinics, both, at the time of diagnosis and later during the monitoring of treatment. The commonly used markers are beta chorio-gonadotrophin (β-hCG), α-fetoprotein (AFP), and (in specialized centers) lactate dehydrogenase (LDH, LD-1) (Gilligan et al., 2010; Albers et al., 2011). However, many cases of pure seminoma are marker-negative, so a recent discovery of specific micro-RNA secreted by overt TGCT and GCNIS-cells, both in adult men and children, is very promising (Palmer et al., 2010; Dieckmann et al., 2012; Novotny et al., 2012; Gillis et al., 2013).

Treatment of GCNIS and Tumors Confined to the Testis (Stage I)

All adult patients diagnosed with a preinvasive or overt testicular tumor should be offered semen cryopreservation before treatment. Bilateral GCNIS or GCNIS found in the only remaining testicle after removal of the other, is treated by a low-dose scrotal irradiation (Albers et al., 2011). This treatment preserves Leydig cell function but destroys normal germ cells, so radiotherapy may be postponed if the patient wishes to father a child. Chemotherapy should not be used to treat preinvasive lesions, because it often fails to eradicate GCNIS (Kleinschmidt et al., 2009).

Management of unilateral GCNIS and all types of stage I TGCTs is by radical orchiectomy (Albers et al., 2011). Surgical enucleating of small, well delineated tumors should only be considered in benign somatic cell tumors, e.g. LCT or in very large, resistant to treatment adrenal rest tumors. Pure seminomas have a good prognosis, with only 13%–19% of patients relapsing, so the vast majority of patients with stage I seminoma do not require any treatment after the surgery, and are followed by active surveillance (Nichols et al., 2013; Mortensen et al., 2014). Despite a higher relapse rate of 30%, a surveillance strategy is also practiced by most specialized centers in patients with clinical stage I non-seminomas, but some centers use adjuvant therapy with a single course of chemotherapy in selected patients (Nichols et al., 2013; Tandstad et al., 2014). The majority of relapses occur within 2 years but late relapses can occur in some cases, thus individual management and lifetime follow-up is recommended.

Treatment of Disseminated or Relapsed TGCT

The most common management of overt TGCT with disseminated disease includes surgery, radiotherapy and systemic combination chemotherapy with cytotoxic drugs, such as cisplatin, etoposide, bleomycin, vinblastine and methotrexate. It is important to evaluate the risk in each patient, including staging, prognostic factors and careful check of organ function, in order to select the best treatment with the lowest risk for late toxicity (Rajpert-De Meyts et al., 2016). Monitoring of serum markers is obligatory for accurate prognosis and assessment of treatment. Other prognostic factors are based on histopathologic analysis of tumor tissue and include vascular invasion, degree of neovascularization, a copy number of isochromosome 12p, and expression of proliferation markers and adhesion proteins (George et al., 2003; Albers et al., 2011). Based on the prognostic factors the patient is classified into a good, intermediate or poor prognosis, and the chemotherapy regimens differ accordingly (summarized in Oldenburg et al., 2013; Rajpert-De Meyts et al., 2016). The standard first line regimen is BEP (bleomycin, etoposide and cisplatin), administered three or four cycles depending on the patient's risk category. In patients with poor prognosis or resistance to chemotherapy, salvage regimens and complex surgery for residual tumors are needed. Overall the management is difficult, thus it should be carried out in specialized tertiary centers.

Late Effects and Follow-Up

Depending on the type of tumor, patients should be followed according to variable protocols, taking into account not only the possibility of relapse of the malignancy, but also the health issues related to the lack of one or both testicles, such as sub-fertility, hypogonadism, sexual dysfunction, metabolic syndrome and osteoporosis later in life. Patients with TGCT derived from GCNIS have poor spermatogenesis and decreased fertility even before the overt tumor has developed, and in most men there is further deterioration when the tumor grows (Petersen et al., 1999; Rives et al., 2012). In long-term survivors fertility is decreased and signs of Leydig dysfunction are common (Cvancarova et al., 2009; Bandak et al., 2017). Endocrinologic/andrologic follow-up is important, with close monitoring of testosterone levels, because hypogonadism consist a major risk factor for metabolic syndrome (Willemse et al., 2013).

Additional problems in patients treated with radio- or chemotherapy are the increased risks of second cancers, cardiovascular disease, peripheral neuropathy, ototoxicity and hepatotoxicity (Chovanec *et al.*, 2017). Of importance are quality of life issues related to prolonged anxiety and stress, often lower socioeconomic status or unemployment (Skaali *et al.*, 2011; Smith *et al.*, 2016). A growing consensus is that there is a need of individualized treatment to diminish immediate and late side-effects and attention should be given to the reproductive health and quality of life issues.

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Male Oncoinfertility

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Introduction

Oncofertility is a subject that bridges the clinical care and research in oncology and reproductive medicine, with aims to explore and expand options for the reproductive future of cancer survivors and to minimize adverse events in their offspring. The importance of recognizing oncofertility as a discipline is two-folds. First, cancer diagnosis and therapies, from surgeries and radiation therapies to immuno- and chemotherapies, have a significant negative impact on reproductive health. Further, even for young adults and children, at the time of cancer diagnosis, fertility specialists are generally not involved in providing the needed counseling on fertility preservation options and future prospects of fertility risks. It is only after the individual has survived cancer and enters the parenthood-planning state that they seek care from fertility specialists; unfortunately, it is then too late to implement various fertility preservation strategies that could have optimized their chances to be successful in having children.

Indeed, oncofertility is an interdisciplinary subject that demands close collaboration of a multidisciplinary team of health care professionals, ranging from clinicians (including nurses, oncologists, geneticists, reproductive gynecologists and urologists), scientists (including andrologists, embryologists, basic scientists) and to psychosocial experts (including psychologists, social workers, ethicists and legal professionals). In addition to the management of sub- or infertility, oncofertility also incorporates reproductive issues after cancer treatment, such as family planning, complex contraception, hormonal management throughout survivorship, surrogacy, and adoption. The focus of this article will be on the impact of cancer and cancer therapies on male reproductive health and the various options currently available for male fertility preservation.

Epidemiology of Cancer in Boys and Young Men

Common cancers in men that receive most attention in the public media include lung, colon, prostate, skin, and liver cancers. These cancers, however, tend to affect men who have passed the age when most men are concerned about reproductive potential. For boys and young men, the most common cancers include testicular cancer, lymphoma, leukemia, sarcoma, and brain cancers. The incidence of childhood cancer worldwide has been steadily increasing over the past 50 years (American Cancer Society, 2016; Siegel *et al.*, 2017). With an estimated cumulative incidence of 1720 per million, equivalent to a risk of 1 in 581, childhood cancer is indeed one of the leading causes of death among children younger than 15 years of age. According to the recent data from the American Cancer Society, 51,350 boys and men in the United States under the age of 45 will be newly diagnosed with cancer in 2017.

Thanks to tremendous strides in cancer management, including early detection strategies and advances in various treatment modalities such as surgeries, radiation, and combination chemotherapy regimens, the survival rates of many childhood cancers have increased dramatically over the past 40 years (National Cancer Institute, 2009). It is estimated that in North America approximately 1 in 900 of the population aged 20–45 years is a childhood cancer survivor (Bhatia, 2005).

While many of these young cancer survivors can expect a good quality of life, they may also face a series of undesired consequences related to their cancer and cancer therapies. Impairment in reproductive health is a well-known complication of cancer therapy; it occurs in a significant proportion of cancer survivors due to the inhibition of spermatogenesis (spermatotoxicity) of cancer treatments such as chemotherapy and radiation therapy. Many young cancer survivors have not initiated or completed forming a family. Interestingly, surveys indicate that almost 80% of childless cancer survivors report the desire to have children and believe that their experience of surviving cancer will make them better parents (Schover *et al.*, 1999). In a recent study, adolescents prioritized fertility as a top goal after good health (Klosky *et al.*, 2015).

Reproductive Health in Cancer Patients Prior to Cancer Treatment

The reproductive health of cancer patients may be suboptimal even before receiving cancer therapies, as revealed by studies on the sperm density and morphology of prechemotherapy sperm banked samples and on case–control studies of their natural fecundity (Baker *et al.*, 2005; Meseguer *et al.*, 2006; O'Flaherty *et al.*, 2008). This may be due in part to the decline in the physical state (poor nutrition, fever, cachexia, pain, etc.) of the patients from cancer. The psychosocial stress attributed to the cancer diagnosis may play a role in the well-being of the subject. Prolonged periods of sexual abstinence may also contribute to the poor sperm quality before chemotherapy. In testicular cancer, poor sperm profile may be secondary to having only one remaining contralateral noncancerous testis to produce sperm. The remaining noncancerous testis may have compromised reproductive function due to a higher risk of

coexisting intraepithelial germ-cell tumors and abnormal spermatogenesis, both quantitatively and qualitatively (Baker *et al.*, 2005; Dieckmann *et al.*, 2007).

Prior to chemotherapy, 37% of men with testis cancer and 81% of men with Hodgkin lymphoma demonstrate abnormal sperm nuclear chromatin structure despite having normal sperm density and motility (O'Flaherty *et al.*, 2008). Several studies have supported these findings (Ståhl *et al.*, 2009; Rives *et al.*, 2012; Bujan *et al.*, 2013), while some others did not (Ribeiro *et al.*, 2008; Smit *et al.*, 2010). The main concern is that with subsequent cytotoxic cancer therapy, sperm from cancer patients are at risk for further genetic damage.

Cancer Management Strategies

The choice among various cancer management modalities, including surgery, radiation and chemotherapy, depends on the nature and stage of the cancer and the comorbidity of the subject. A combination of these modalities in various orders may be required to achieve optimal cancer control. Complications of each modality also vary. Mechanisms of how each treatment modality may potentially compromise male reproductive health are discussed in this section.

Impact of Surgical Management for Cancer on Male Reproductive Status

The purpose of surgical resection of tumor is to remove the tumor with adequate surgical margins to aim for cure or to debulk the volume of tumor to facilitate the effect of adjuvant therapy with radiation or chemotherapy. A common surgical management for testicular cancer in young males is radical orchiectomy. Removal of one testis affects the total spermatogenic activity in an individual. Men with testicular cancer are at risk of having decreased spermatogenic activity even in the remaining testis. Other surgical managements for cancers in young males may result in damage to the autonomic nervous system required for semen emission leading to infertility despite preservation of spermatogenic function.

Radiation Therapy

Germ cells and somatic cells in testes are prone to damage post radiation. The usual clinical dosage of radiation therapy for cancer ranges from 0.2 to 70 Gy, depending on the nature, stage, and anatomical location of the tumor. A cumulative dosage of 2.5–6 Gy directly to the testes may permanently damage germ cells, leading to prolonged or permanent azoospermia (Clifton and Bremner, 1983). Even for radiation therapy outside the pelvic areas (e.g., paraaortic lymph nodes) with gonadal shielding to reduce the extent of direct gonadal toxicity, the scattering effects of radiation may still contribute to impaired fertility. Such damage to sperm production may be further attributed to damage to cells in the somatic compartment of the testis. Using spermatogonial stem cell (SCC) transplantation in rat, Zhang *et al.* (2007) demonstrated that transplantation of SCCs from irradiated animals into testes of irradiated nude mice permitted differentiation of the donor spermatogonia to spermatozoa. Conversely, transplantation of SCCs from untreated prepubertal rats into irradiated rat testes showed that the donor spermatogonia were able to colonize along the seminiferous tubules, but could not differentiate. Their findings suggest that the defect caused by radiation in the rat testes that hinder spermatogonial differentiation is due to damage to the somatic compartment (Zhang *et al.*, 2007).

Clinically, radiation therapy may have further negative impact on sperm chromatin integrity for men treated with childhood cancers (Romerius *et al.*, 2010) or prostate cancer as adult (Singh *et al.*, 2012). Fluorescence in situ hybridization (FISH) demonstrated an increase in the incidence of sperm aneuploidy on chromosome 18, X and Y in men treated with radiotherapy for testicular seminoma (Le *et al.*, 2014). Taken together, current evidence supports the presence of a significant risk of impairment of the male reproductive status after radiotherapy for cancer.

Chemotherapy

Chemotherapy is generally indicated in advanced and metastatic cancer, although its use in certain cancers, such as germ cell tumors at an early, localized stage, may help to lower the risks of subsequent metastasis. In addition to malignant cells, any rapidly dividing cells, including germ cells at various phases of spermatogenesis, are targets of chemotherapy. Gonadotoxicity of chemotherapy to an individual depends on at least three factors: (1) the nature of the malignancy, which dictates the type of chemotherapeutic agents to be used, (2) the stage of the disease, which dictates the duration and dosages of chemotherapy, (3) host factors, such as the baseline reproductive health of the individual.

Impact of Chemotherapy on Male Reproductive Health: Animal Studies

Using rodents (rats and mice) as models, a large body of evidence has emerged demonstrating that treatment with chemotherapeutic agents usually have dramatic effects on the production of male germ cells (Robaire and Hales, 2003; Anderson and Brinkworth, 2006). Depending on the mechanism by which such agents act on the different phases of spermatogenesis (spermatogonial mitotic cell division, meiosis, or spermiogenesis), consequences can range from complete elimination of germ cells

from the testis, resulting in Sertoli-cell-only syndrome, to no apparent histological effects on spermatogenesis, but functional effects on germ cells (their motility, fertilizing ability, or capacity to produce normal viable offspring). Over the past 20 years, studies on male mediated adverse effects of chemotherapeutic drugs, such as cyclophosphamide (CPA), bleomycin, etoposide, cisplatin, or procarbazine, on fertility and progeny outcome have clearly established some of the underlying molecular mechanisms that result in loss of fertility and altered progeny outcome (Anderson, 2005; Hales *et al.*, 2005; Meistrich, 2009; Downey *et al.*, 2018).

Using CPA or the combination of drugs used for treating testicular cancer (bleomycin, etoposide, and cisplatin, BEP) as model drugs and the rat as the model animal, it has been demonstrated that paternal exposures result in adverse reproductive outcomes that range from increased preimplantation and postimplantation loss or early postnatal death, to growth retardation and congenital malformation; significantly, some of these outcomes are transmitted to subsequent generations (Hales *et al.*, 1992; Downey *et al.*, 2018). It is particularly noteworthy that the action of such drugs on germ cells not only affects the number of germ cells that the testis can produce but also alters markers of chromatin structure (Comet, acridine orange, TUNEL, MBBr, and CMA3 assays, nuclear proteome) in spermatozoa (Delbes *et al.*, 2010). It is clear from animal studies that spermatozoa that have damaged chromatin as a result of paternal drug treatment are capable of fertilizing oocytes (Bieber *et al.*, 2006; Sakkas and Alvarez, 2010; Menezo *et al.*, 2010). These studies have also revealed that the effects of paternal exposure on progeny can be wide ranging. While treatment with BEP caused a decrease in both sperm production and sperm motility, no apparent effects were observed on progeny at the end of gestation, yet postnatal death rates were dramatically increased (Bieber *et al.*, 2006). By contrast, chronic CPA treatment had minimal effects on sperm number and motility, yet a wide range of effects were observed in progeny, ranging from abnormalities at birth to learning deficits as adults and in subsequent generations as well as abnormal reproductive capacity (Auroux *et al.*, 1990; Downey *et al.*, 2018).

The effects of such chemotherapeutic treatments can not only result in DNA breaks and crosslinks but can also cause epigenetic modifications; these include an alteration in DNA methylation profile (Chan *et al.*, 2012) and changes in sperm nuclear proteins (Maselli *et al.*, 2012; Bagheri-Sereshki *et al.*, 2016). Remarkably, proteins implicated in the translational control and post-translational processing of protamine 1 are also significantly elevated 9 weeks post-BEP treatment, suggesting that histone eviction may dictate the DNA availability for protamine binding (Maselli *et al.*, 2013). Males mated to control females 9 weeks after BEP treatment have reduced litter sizes; moreover, the profile of gene expression in the developing testes of their pups is altered (Maselli *et al.*, 2014). Liu *et al.* (2015) reported that exposure of male germ cells to a BEP induces telomere shortening in all stages of rat spermatogenesis. Thus altering epigenetic marks or nuclear proteins in mature spermatozoa impacts on male fecundity, potentially threatening normal progeny development; this raises concerns regarding transgenerational risks of chemotherapy exposure.

Clinical Studies

The assessment of the consequences on progeny outcome of exposure of men to chemotherapeutic drugs presents remarkable challenges. Chemotherapy often results in transient or permanent azoospermia or oligozoospermia in cancer patients (Gandini *et al.*, 2006). Large epidemiological studies, discussed above, have revealed that there is clearly an effect on fertility and time to pregnancy (Green *et al.*, 2010). In addition, the standard semen parameters (sperm number, motility, and morphology, as established by the WHO (World Health Organization, 2010)) are not sufficiently reliable predictors of male fertility (Virro *et al.*, 2004; Payne *et al.*, 2005; O'Flaherty *et al.*, 2008).

Consequently, the focus has shifted in recent years to assessing the nature and quality of chromatin in spermatozoa. In recent comprehensive reviews, Barratt *et al.* (2010) and Zini *et al.* (2014) have outlined our current clinical understanding and uncertainties related to the many assays used to ascertain sperm chromatin quality. Aneuploidy is one of the more striking consequences of anticancer drugs on sperm chromatin quality. Using multicolor-FISH assay in testicular cancer and Hodgkin lymphoma patients before and up to 24 months after the initiation of chemotherapy, Tempest *et al.* (2008) found that at 6 months, all cancer patients showed significantly increased frequencies of XY disomy and nullisomy for chromosomes 13 and 21. Although frequencies of aneuploidy generally declined over time after termination of treatment, increased aneuploidy frequencies persisted in some chromosomes at 24 months.

Using a series of assays that provide complementary information on sperm chromatin structure, for example, extent of single- and double-strand breaks, degree of protamination, cross-linking of sulfhydryl bonds, O'Flaherty *et al.* (2008) have shown that, prior to initiation of chemotherapy, sperm chromatin integrity was poorer in cancer patients than in a control population. After treatment with chemotherapeutics, not only was there the expected decline in sperm production and chromatin quality but also, up to 2 years later, a reduction in spermatozoal chromatin integrity in over 40% of the patients who had a return of spermatogenesis (O'Flaherty *et al.*, 2010). Subsequent multicenter prospective longitudinal studies of Hodgkin and non-Hodgkin lymphomas (Bujan *et al.*, 2014) and testicular germ cell tumors (Bujan *et al.*, 2013) supported these findings.

The negative impact of antineoplastic agents appears to extend beyond the genome in humans as well as it does in animals. It has recently been reported that temozolomide, an oral alkylating agent used for treatment of advanced astrocytoma and melanoma affected sperm quantity and quality (increased aneuploidy rate) as well as epigenome integrity (Heikens *et al.*, 1996). Alteration of spermatozoal DNA methylation profiles has a known association with clinical male infertility with oligozoospermia (Cui *et al.*, 2016). Hypomethylation of normally hypermethylated paternally imprinted loci is associated with neoplasia and other

metabolic and growth defects such as Beckwith–Wiedemann syndrome and disorders in neurodevelopment, cognition and behavior. Further studies are thus required to evaluate if such an imprinting error is corrected after fertilization and the potential extent of transgenerational risks.

Based on the limited studies to date, it is clear that the presence of several cancers in young men results, to varying degrees, in sperm chromatin with reduced integrity. Furthermore, treatment of cancer may cause transient partial or complete loss of spermatozoa. Under some conditions, it is clear that the germ cells that eventually return to repopulate the seminiferous epithelium are still damaged, while under others, they appear to be normal. Whether spermatogonial stem cells (SCC) are able to repair all the damage caused by radiation or chemotherapy or not remains to be established.

Male Fertility Preservation and Restoration Strategies

Sperm Cryopreservation

Sperm cryopreservation or sperm "banking" is currently the only available strategy to preserve male fertility. Ideally, sperm samples should be collected before any cytotoxic cancer therapies, through ejaculation by masturbation after 2 to 4 days of sexual abstinence. Then, sperm samples should be analyzed, frozen, and stored in aliquots in liquid nitrogen for future use. With the advances in and increased access to assisted reproductive technologies (ART's) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), a very low number of living spermatozoa are required to achieve fertilization; therefore, even sperm samples that are far from meeting the semen parameters set by the WHO may still be used to achieve fertilization.

Young men with newly diagnosed cancer that require cytotoxic therapy experience simultaneously complex psychosocial stress (Rosberger *et al.*, 2010). Important factors that influence the decision of cancer patients to bank sperm include: healthcare providers' role in discussing fertility preservation, importance of fatherhood, current fatherhood status, partner/parent influence, attitudes toward survival, complexity of sperm banking, cultural factors, sexual orientation and cost (Achille *et al.*, 2006). In the absence of fees for sperm banking and subsequent storage, young cancer patients are more willing to participate in sperm banking sessions in order to preserve their fertility prior to cancer treatment, despite the fact that they are under significant level of stress and time constrain to begin cancer treatment. The benefit is that sufficient sperm would be available for their future use, potentially leading to a higher chance of procreation success.

Sperm cryopreservation does have its limitations as a fertility preservation strategy. First, only subjects who have entered adolescence, when "spermarche" begins, can have spermatozoa in the semen for cryopreservation. One study of 62 attempts by adolescents to bank sperm before cancer therapy resulted in totally normal semen in only four subjects (Postovsky *et al.*, 2003). Semen procurement by masturbation may not always be feasible among adolescents, even for those who have spermatogenesis. In fact, for cultural and religious reasons, the act of masturbation may be viewed as inappropriate by parents of young adolescent cancer patients (Rosoff and Katsur, 2003). Alternative methods to obtain mature sperms in adolescents using high-frequency penile vibratory stimulus, electroejaculation, or surgical testicular sperm extraction may be required. The need of sedation/anesthesia (for electroejaculation or surgical extraction) may be too invasive for youngsters. Thus, sperm banking is not universally practiced in pediatric-oncology centers, and few adolescent friendly facilities exist.

For preadolescent boys with cancer, there is currently no accepted and proven option for fertility preservation. Early investigators held the view that being prepubertal during anticancer therapy conferred protection against gonadal damage. However, a study evaluating 12 men who survived childhood malignancy revealed that although puberty had progressed apparently normally in all 12, 8 patients were azoospermic, and only 1 had normal semen analysis 2–16.5 years post chemotherapy (Mustieles *et al.*, 1995). In addition, following treatment of Hodgkin lymphoma in childhood, severe germ cell damage was observed in the majority of patients, even 17 years after chemotherapy (Heikens *et al.*, 1996). Evidently, there is no gonadal protection in the prepubertal male against chemotherapy-induced damage (van Casteren *et al.*, 2009). In fact, some investigators believe that prepubertal testes are more vulnerable to the cytotoxic effects of chemotherapy than adult testes (Revel and Revel-Vilk, 2008).

Pharmacological Strategies

The hypothesis that blocking the hypothalamic–pituitary–gonadal axis prior to the initiation of chemotherapy to preserve the nondividing germ cell or SSC population was first proposed by Glode *et al.* (1981). Hormonal manipulation, including the use of exogenous gonadotropin-releasing hormone (GnRH) analogs and steroids (testosterone) to suppress gonadotropin release, has been investigated as a potential fertility preservation strategy. Since cytotoxic treatment acts mainly on rapidly dividing cells, germ cells have been postulated to be less susceptible to cytotoxic effects if hormone treatments are used to render the testes quiescent. This technique has been successful in some rodents (rats, but not mice) (Meistrich and Shetty, 2008); in addition, in rats the extent of the damage of chemotherapeutic agents extends beyond the germ cells to the somatic cells surrounding them (Zhang *et al.*, 2003). Thus far, clinical trials have not shown any benefit of this method (Thomson *et al.*, 2002). Furthermore, this approach would be ineffective for prepubertal children as the proliferation of germ cells in prepubertal primates appears to be gonadotropin independent (Kelnar *et al.*, 2002).

Fertility Restoration With Germ-Cell Transplantation

Stem cells of the male germ line, termed SSCs, exist in the testis prior to birth. Harvesting either SSCs or tissue blocks from testes for cryopreservation before anticancer therapies offers the hope for prepubertal boys with cancer to preserve fertility and form their family in the future (Nagano, 2004). After the patient is cured and is at an appropriate state of maturity, preserved SSCs, or SSCs derived from frozen tissue blocks, could be autotransplanted back to the seminiferous tubules to regenerate complete spermatogenesis. Cryopreservation of testis tissue from prepubertal boys has revealed that germ cells can be preserved (Keros *et al.*, 2007). An important feature of this strategy is that instead of just preserving fertility, it aims to "restore" fertility. This fertility restoration scheme, based on germ cell or tissue transplantation, has been established with mice and other species (Oatley and Brinster, 2008; Schlatt *et al.*, 2009) and is currently under investigation to extend its application to humans.

To date, several groups have reported different cell culture systems designed to maintain and expand human SSCs (e.g., Nagano *et al.*, 2002; Lim *et al.*, 2010; Sadri-Ardekani *et al.*, 2011; Guo *et al.*, 2015). Further, xenotransplantation of cultured human SSCs to immunodeficient mice—a well-acknowledged and the only available assay for functionality of human SSCs—demonstrated their migration to the niche at the basal membrane of the seminiferous tubules, indicating their SSC capabilities (Nagano *et al.*, 2002). Initiation of human spermatogenesis in the host mice has yet to be achieved; nonetheless, the steady progress in the development of male fertility restoration strategy with SCCs in the past two decades gives hope for breakthroughs that will affect clinical practice.

The advances made in culturing SCCs open an opportunity to combine these advances with the exciting germline genomic editing technology to potentially improve clinical outcomes. Several recent reports demonstrated the feasibility of genetic and epigenetic editing in SSC transplantation (Fanslow *et al.*, 2014; Wu *et al.*, 2015; Chapman *et al.*, 2015; Sato *et al.*, 2017). The use of the CRISPR-Cas9 system can successfully repair mutations, for example, the hemoglobin beta gene in β-thalassemia (Xie *et al.*, 2014), the dystrophin gene in Duchenne muscular dystrophy in patient-induced pluripotent stem cells (Li *et al.*, 2015), and the cystic fibrosis transmembrane conductor receptor locus in cultured intestinal stem cells from patients with cystic fibrosis (Schwank *et al.*, 2013). Potentially, identified cancer-inducing genes can be corrected in SCCs harvested in cancer patient during fertility preservation to reduce or eliminate the risk of transmission of cancer to offspring.

While significant progress has been made in this fertility restoration regime in the recent years, several hurdles must be overcome prior to realizing its clinical application. In addition to using a minimally invasive surgical approach to harvest SCCs (particularly when dealing with preadolescent boys) from the testes and proper isolation of the SCC population for cryopreservation, culture, clonal expansion and subsequent transplantation for spermatogenesis reintroduction, the risk of contamination with lingering cancer cells, as in the case of hematological cancers (e.g., leukemia) or metastatic cancers must be reduced to zero. Several groups have reported mixed results in the elimination of cancer cells when harvesting SCCs (Geens *et al.*, 2007; Hermann *et al.*, 2011; Sadri-Ardekani *et al.*, 2014). Additionally, the costs, risks, success rate and efficacy along with the potential well-being of the offspring produced with the complex interplay of various biotechnologies must be considered through rigorous ethical reflection and societal debate, particularly when genomic or epigenomic editing of the SCCs aimed at correcting adverse mutations may soon be a reality.

The Use of Assisted Reproductive Technologies in Cancer Survivors

For the majority of cancer survivors who desire to have children but have poor sperm quantity and quality, ART's with IVF/ICSI are sought to help them to father their own children (Chan *et al.*, 2001). ICSI is generally required when using cryopreserved gametes for reproduction. With regards to the outcomes of ICSI using cryopreserved sperm, the usage rate of cryopreserved sperm is significantly lower among cancer survivors compared to noncancer patients, for example, for infertility treatment, 11% versus 31%, respectively. Nonetheless, the live-birth rate of offspring with ICSI among male cancer survivors was comparable to that of noncancer patients (Hansen *et al.*, 2005). Specifically, the average success rate of achieving parenthood using cryopreserved sperm was 62.1%, which was at least comparable to the infertile patient population: oligospermic and testicular sperm extraction (TESE) patients (40% and 48.6% respectively). This provides evidence that cancer patients can bank sperm as effectively as men banking for infertility reasons.

Health of Offspring of Male Cancer Survivors

Whereas the nature, mechanisms and extents of gamete damage from cytotoxic anticancer therapies are important research questions, for cancer survivors one of the most important clinical questions is the health risks to their offspring after cancer. Several recent studies reported a nonsignificant risk of adverse offspring health outcomes from cancer survivors. Two studies from retrospective cohort analyses of the Childhood Cancer Survivor Study reported no increase in the risk of still birth, neonatal death (Signorello *et al.*, 2010) and congenital anomalies (Signorello *et al.*, 2012) among survivors of childhood cancer survivors who had undergone radiation and chemotherapy. A Danish case-cohort study also reported no increased risks of genetic disease from cancer survivors exposed to childhood/adolescent alkylating chemotherapies or radiation (Winther *et al.*, 2012). Using data collected from 1953 to 2004 from registries (e.g., national cancer, population birth and hospital discharge registries) on close to 7000 offspring of cancer survivors with congenital anomalies and over 35,000 offspring of these survivors' siblings, another recent

study demonstrated no significantly increase in offspring anomaly rates from cancer survivors, regardless of the age when the cancer was diagnosed; however, there was a significantly higher risk of congenital anomalies in the offspring of survivors with cancer diagnosed in earlier period (1955–64, prevalence ratio 2.77, 95% CI 1.26–6.11) (Seppänen *et al.*, 2016).

While messages from these new studies may be reassuring, a few important points must be noted. First, other earlier and contemporary series observed either increased (Magelssen *et al.*, 2008; Chow *et al.*, 2009; Ståhl *et al.*, 2011) or no increased risks (Mueller *et al.*, 2009; Green *et al.*, 2010; Byrne *et al.*, 1998) of congenital malformations in the offspring of cancer survivors. The inconsistency of the results may be in part related to the differences in samples size and power, definitions of outcomes, study designs and selection bias. Further, it should be noted that most of these data focus on outcomes of offspring from natural conceptions rather than with assisted reproduction—which many male cancer survivors may need with fresh or cryopreserved sperm. Indeed, Ståhl *et al.* (2011) reported a significantly higher risk of birth abnormalities in offspring of men with a history of cancer (relative risk 1.17, 95% CI = 1.02–1.31) with both natural conception as well as assisted reproduction. Perhaps most importantly, these data do not address adequately other important reproductive outcomes such as time required to achieve pregnancy, risks of lower number of offspring or rate of miscarriage, particularly early (<20 weeks) miscarriage; these are some of the endpoints that might be predicted to be affected based on the animal studies described above. Studies of offspring of cancer survivors beyond early developmental stages need to be undertaken to determine whether potential alterations in epigenetic marks can lead to behavioral, immunological, reproductive or other deficits in adulthood.

Taken together, cytotoxic cancer therapies not only will have negative impact on the quantitative and qualitative changes in conventional semen parameters and sperm chromatin quality leading potentially to adverse reproductive outcomes: clinical infertility, increased use of assisted reproduction and potential adverse outcomes in offspring. Even for male cancer survivors who manage to achieve live births naturally or via assisted reproduction, the potential risks of adverse outcomes including congenital malformation, genetic diseases, low-birth-weight cannot be completely eliminated. Further large scale prospective longitudinal studies of cancer survivor cohorts and multicenter cancer registry follow-up studies will shed lights on the actual reproductive risks to allow formulation of proper counseling to these young cancer survivors. Meanwhile, precancer treatment fertility preservation counseling is the key to minimize the potential risks of adverse outcomes in the reproductive status of these patients.

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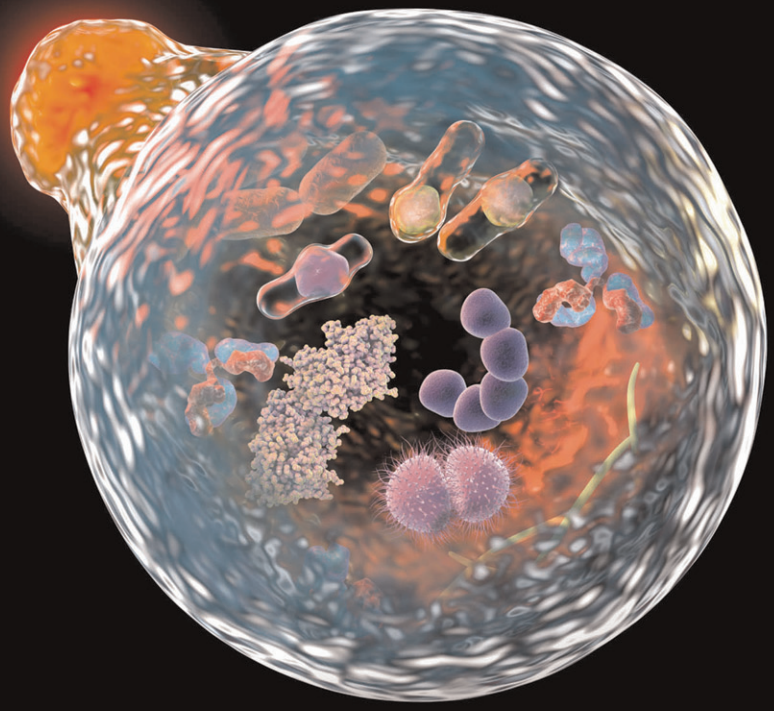
Editors in Chief
Ilpo Huhtaniemi and Luciano Martini



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DEDICATION

Professor Luciano Martini, 1927–2017

The other Editor in Chief of the Encyclopedia, Professor Luciano Martini, passed away on July 13th, 2017. He was an internationally acclaimed authority in the field of endocrinology, in particular neuroendocrinology, a brilliant and imaginative scientist, and an impressive and erudite scholar.

Luciano achieved the venerable age of 90, and his long career was full of outstanding scientific achievements, leadership positions in academia and in scientific societies, academies, and committees.

Luciano received his MD degree from the University of Milan in 1950. He then rapidly progressed through junior academic ranks up to the position of Professor and Chairman of the Department of Pharmacology at the University of Perugia in 1968, and subsequently, in 1972, he returned to his *alma mater*, the University of Milan, as full Professor and Chairman of the Department of Endocrinology, a post he held until 2001. He served in Milan as director of the training and research program entitled Physiology of Reproduction for nearly 20 years and attracted to his team top-class Italian and foreign scientists to address his main research interests of neuroendocrine regulation of reproductive functions.

Scientific severity, ethical integrity, fine perception, and deep farsightedness describe best Luciano's character as a scientist. He created in his institute a scientific research group devoted to experimental endocrinology, which grew over the years in size and visibility and became widely recognized internationally. Luciano published more than 400 peer-reviewed and highly cited papers mainly in the fields of neuroendocrinology, endocrine oncology, physiology of reproduction, and steroid and energy metabolisms.

Luciano was a prolific editor of scientific books and journals, which include the two volumes of *Neuroendocrinology* and the nine biennial volumes of *Frontiers in Neuroendocrinology*. He was Editor in Chief of *Comprehensive Endocrinology* published in 12 volumes and the first Edition of *Encyclopedia of Endocrine Diseases*. He served as President in many national and international scientific societies including the International Society of Neuroendocrinology, the Italian Society of Endocrinology, the International Society of Endocrinology, and the European Federation of Endocrine Societies. For his scientific achievements Luciano received honorary doctorates in the universities of Liège, Santiago de Compostela, Pécs, and Milan, and he was the recipient of numerous scientific awards and invited academy memberships.

Luciano's portrait could not be complete if one forgets to mention his life-time passion for music. He was a well-trained and accomplished pianist, a passionate music listener, and an enthusiastic connoisseur of all types of music. He also was an amateur in visual arts and deeply interested in history.

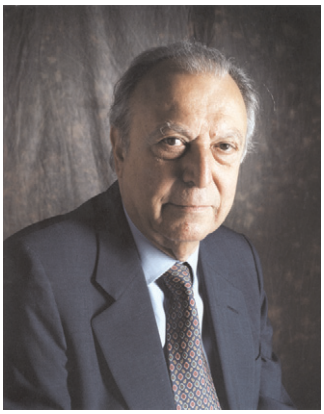
All of us who knew Professor Luciano Martini deeply mourn the loss of a great scientist and friend, the real "Il Maestro", teacher, colleague, and pioneer of modern neuroendocrinology. I trust Luciano would have been proud of this new edition of the Encyclopedia of Endocrine Diseases, and all of us having worked on its production would like to dedicate it to his memory.

Ilpo Huhtaniemi

Editor in Chief
Encyclopedia of Endocrine Diseases, 2nd edition

EDITORS IN CHIEF

Ilpo Huhtaniemi received his MD and PhD at University of Helsinki, Finland, did postdoctoral training in United States (UC San Francisco and NIH, Bethesda), and has been on sabbatical leave in Germany, United States and Scotland. In 1986–2002 he held the post of Professor and Chairman of Physiology at University of Turku, Finland. He moved in 2002 to UK to a Chair in Reproductive Endocrinology at Imperial College London, from which position he retired in 2015. He has received several national and international honors, amongst them a fellowship of The Academy of Medical Sciences, United Kingdom, and a Doctor Honoris Causa at the Medical University Łódź, Poland, and University of Szeged, Hungary. He was the Chief Managing Editor of *Molecular and Cellular Endocrinology* 1999-2017, has served in the Editorial Board of *Endocrinology and Endocrine Reviews* and is/has been the Editor or Editorial Board Member of several other scientific journals (e.g., *European Journal of Endocrinology*, *Clinical Endocrinology*, *Human Reproduction Update*, *Journal of Endocrinology*, *Molecular Human Reproduction*, *Reproduction*, *Asian Journal of Andrology*). He has extensive experience as Official of international scientific organizations (e.g., Past President of International Society of Andrology). His research interests include clinical and basic reproductive endocrinology, in particular the function of gonadotrophins and male reproductive endocrinology. He also has long-term interests in development of male contraception, hormone-dependent cancer, and the endocrinology of aging. He has authored about 700 peer-reviewed research articles and reviews, and his H-factor is 78.



Luciano Martini was born on May 14, 1927, in Milan, Italy. He obtained the degree of Medical Doctor "summa cum laude" on November 24, 1950, from the Faculty of Medicine of the University of Milan, Italy. He was Emeritus Professor of Pharmacology of the University of Perugia, Italy, and Emeritus Professor of Endocrinology of the University of Milan, Italy. He was Doctor Honoris Causa in Medicine of the Universities of Liège, Belgium, Santiago de Compostela, Spain, and Pécs, Hungary, and Doctor Honoris Causa in Biotechnological Sciences of the University of Milan, Italy. He was an author of more than 400 peer-reviewed scientific publications in the fields of endocrinology, neuroendocrinology, pharmacology, physiology of reproduction, steroid biochemistry, and basic oncology. He was elected member of the Accademia Nazionale dei Lincei (Italian National Academy) and of the American Academy of Arts and Sciences (Honorary Foreign Member).

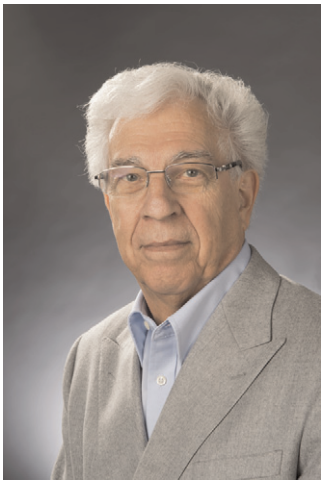
Luciano Martini acted as Editor in Chief of the journal *Frontiers in Neuroendocrinology* from 1990 to 2001, and was a Member of the Editorial Board of *Endocrinology* (Foreign Consulting Editor, 1961–65), as well as of several other speciality journals, such as *Experimental and Clinical Endocrinology*, *Biochemistry*, and *Steroids*. He has acted as Editor of several textbooks (e.g., *Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1966–67, and *Clinical Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1977–82) as well of a series of books under the name *Comprehensive Endocrinology* (13 volumes), Raven Press, New York, 1979–84. He acted as Editor in Chief for the first edition of *Encyclopedia of Endocrine Diseases* (4 volumes), Academic Press-Elsevier, San Diego, 2004.

EDITORIAL BOARD

SECTION EDITORS

Professor **Jean-Jacques Body** has been trained as an endocrinologist and a medical oncologist. He was Head of the Department of Medicine at University Hospital Brugmann in Brussels and Full Professor of Medicine (Internal Medicine) at the Free University of Brussels, (ULB), Brussels, Belgium. He was previously Head of the Internal Medicine Clinic at Institute J. Bordet (Cancer Center of ULB). He has also developed the "Supportive Care Dept" at the same Institute. His particular research interests are osteoporosis and bone metastases. He has a long-standing interest for bone metabolism and turnover in osteoporosis and tumor bone diseases. He has authored or co-authored more than 250 international peer-reviewed papers and he counts more than 200 invited lectures for international meetings.

Felipe F. Casanueva is Professor of Medicine at University of Santiago de Compostela and Head of Department of Endocrinology and Nutrition at University Hospital Santiago. He has been President of the scientific societes, such as: European Federation of Endocrine Societies (EFES), The Pituitary Society, International Society of Endocrinology (ISE) and, Sociedad Española para el Estudio de la Obesidad (SEEDO). Has written more than 50 chapters in international books and published more than 700 papers in international journals. He has received several awards for research at national and international level, such as: Rey Jaime I to the Medical Research, Geoffrey Harris Prize in Neuroendocrinology, Fundación Lilly of Bio-medical Research Clinic, Fundacion Danone – Professional Career – Dr Carlos Martí Hennberg, European Hormone Medal by the European Society for Endocrinology (ESE); he has been named Honorary Doctorate in Medicine of the University of Łódź, Erciyes, and Belgrade, and Honorary Member of the European Society of Endocrinology.



Dr. **Jean-Louis Chiasson** is currently Full Professor of Medicine at the University of Montreal. He is Head of the Research Group on Diabetes and Metabolic Regulation at the Research Center of the Centre hospitalier de l'Université de Montréal (CRCHUM).

Dr. Chiasson obtained his MD at Laval University in Quebec City in 1967. He did his specialty training in Internal Medicine at Laval Univesity and in Endocrinology at McGill University. He then did a research Fellowship in Diabetes at Vanderbilt University in Nashville, Tennessee. In 1974–76 and 1978–80, he was appointed Assistant Professor in the Department of Medicine and Physiology respectively at Vanderbilt University. In 1980, he returned to Montreal as Assistant Professor in the Department of Medicine at the University of Montreal and as Endocrinologist at Hotel-Dieu Hospital, now merged into the Centre hospitalier de l'Université de Montréal.

Dr. Chiasson's research interests include the regulation of carbohydrate metabolism in health and diabetes, as well as the development and evaluation of new strategies for the treatment and prevention of diabetes and its vascular complications. He has contributed over 250 scientific publications and lectures nationally and internationally on various topics on diabetes mellitus, its pathogenesis, its treatment, and its prevention. His scientific contribution puts him in the prestigious club of the 100 most cited publications in the world in the field of diabetes.

Sophie Christin-Maitre received her MD at University of Paris XI and her PhD at University Paris VI, Pierre and Marie Curie, France. She did a postdoctoral training in United States (Massachusetts General Hospital, Harvard University, Boston); she specialized in reproductive medicine. She holds the post of Professor of Endocrinology at University of Sorbonne, Paris, France. She has been the head of the Adult Endocrine Unit, in Hôpital Saint-Antoine, Assistance-Publique Hôpitaux de Paris, since 2011. She is a member of the INSERM research unit UMR S_933, specialized in identifying new genes in reproductive disorders. Her interests include clinical and basic reproductive endocrinology, in particular the management of patients with Turner syndrome, patients with primary ovarian insufficiency, patients with hypogonadisms, and patients with abnormalities of sex development. She has authored approximately 150 peer-reviewed research articles and reviews.

Ulla Feldt-Rasmussen is Professor at Copenhagen University and Chief of Medical Endocrinology, National University Hospital. Her research interests involve the thyroid gland and autoimmunity, as well as pituitary and adrenal dysfunction.

She has published more than 410 papers in peer-reviewed journals on e.g., thyroid hormones and body composition, thyroid autoimmunity and cancer, cytokines as regulators of endocrine cells, influence of thyroid disrupting chemicals on thyroid cells, growth hormone deficiency related to body composition, bone metabolism and other pituitary axes, and transition from adolescent to adult care, as well as several aspects of Fabry disease. In recent years her group has embarked on studies on pituitary function after traumatic brain injury in a nationwide setting, and focusing on diagnostic accuracy of pituitary testing procedures. She has further authored numerous proceedings, textbook chapters, and other publications; as well as organized numerous international meetings and postgraduate courses, and has led several European projects and other collaborations within many areas of endocrinology.

Professor Feldt-Rasmussen reviews for international journals, and is an editorial board member of several endocrine journals. She belongs to many international professional organizations, including the Endocrine Society, ETA, ATA, ENEA, and GRS; she has served as Secretary-Treasurer of ETA and as President of the ETA Cancer Research Network.

Professor Feldt-Rasmussen serves on the advisory boards of several ad hoc endocrine committees, and has received many prestigious prizes including the Mayo Clinic's Haynes Lecturer's Award and ETA's Pinchera Research Prize.

Wouter W. de Herder M.D. Ph.D. (1960) is Professor of Endocrine Oncology at the Erasmus MC in Rotterdam, the Netherlands. In this University Hospital he is chairman of a multidisciplinary group for endocrine oncology (tumorwerkgroep endocriene tumoren) and he is head of the ENETS centre of excellence for neuroendocrine tumors. His major research interests are neuroendocrine and endocrine tumors.

Professor de Herder received his M.D. in 1985 and his Ph.D. in 1990 from the Erasmus University in Rotterdam, the Netherlands.

He is a member of several international and Dutch national societies, such as the Dutch Society for Endocrinology (NVE), the Endocrine Society (USA), the European Society of Endocrinology (ESE), European Neuroendocrine Tumor Society (ENETS) and the North American Neuroendocrine Tumor Society (NANETS). He served as a board member of the Dutch Society for Endocrinology (NVE) (2009–14). He served as chairman (2006–08) and vice-chairman of ENETS (2008–10) (European Neuroendocrine Tumour Society). He is member of the advisory boards of ENETS and NANETS.

Professor de Herder has (co-)published over 400 peer-reviewed papers and book chapters and is a reviewer for many international journals.

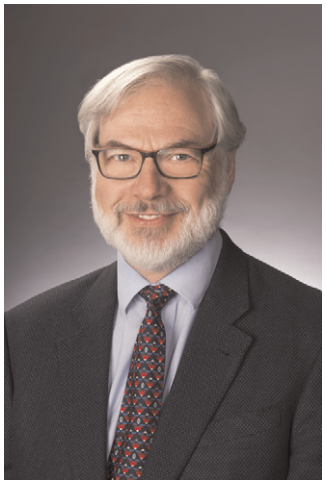
He is a member of the editorial boards of *Neuroendocrinology; Endocrinology, Diabetes & Metabolism Case Reports; Clinical Endocrinology*, and *Endocrine-Related Cancer*.

Professor de Herder has given over 200 invited presentations at Dutch national and international meetings.

Ieuan Hughes is currently Emeritus Professor of Pediatrics at the University of Cambridge and Honorary Consultant Pediatrician at Cambridge University Hospitals NHS Foundation Trust and Cambridge Biomedical Campus. He is the author of more than 300 papers and chapters across the whole range of paediatric endocrinology. His particular expertise is in disorders of sex development for which he coordinated the International Consensus on the approach to the investigation and management of this broad topic. Research interests focus on steroid enzyme deficiencies and molecular mechanisms of androgen action.
Professor Hughes has served on the editorial boards of several journals, including *Clinical Endocrinology*, *Journal of Clinical Endocrinology*, and *Metabolism* and *Archives of Disease in Childhood* where he was also the Associate Editor. He is Past-Secretary and President of the European Society for Pediatric Endocrinology and a recipient of the highest award of the Society, the Andrea Prader Prize. Professor Hughes is a James Spence Medallist of the Royal College of Pediatrics and Child Health for outstanding contributions to paediatric knowledge. He is a Fellow of the Academy of Medical Sciences, a Council Member of the Learned Society of Wales and a Trustee of two charities. The chapter on Disorders of Sex Development in *Williams Textbook of Endocrinology* (now in its 14e) by Hughes and co-authors is considered to be a definitive and up to date regular review of this topic, specific and key to pediatric endocrinology.

Dr. **Gregory Kaltsas** MD FRCP (Lon) is Professor in General Medicine and Endocrinology at the National and Kapodistrian University of Athens, Greece. He was trained in General Medicine in Athens, Greece and London, UK, and in Endocrinology at the Middlesex and St Bartholomew's Hospital, London, UK. He developed a particular interest in neuroendocrinology (pituitary and neuroendocrine tumors) and adrenal physiology and diseases. Upon returning to Greece he established a neuroendocrine network and he is currently running the European Neuroendocrine Tumor Society (ENETS) Center of Excellence at Laiko Hospital in Athens, Greece. He has served as a member of the advisory board of ENETS and of the Executive Committee of the European Neuroendocrine Association (ENEA) and he has been elected in the Executive Committee of the International Society of Endocrinology. He has recently been elected as a representative of the European Society of Endocrinology in the ExCo of the International Society of Endocrinology. He has published more than 300 original papers, reviews, and chapters and serves on editorial boards and as associate editor in several endocrine journals.

Jean-Marc Kaufman obtained his MD and PhD degrees at the Ghent University, Belgium. He was a Senior Postdoctoral Research Fellow (1982–84) in reproductive physiology at the University of Texas Medical School at Houston. He is board certified in Endocrinology and in Nuclear Medicine. In 1985 he joined the staff of the Ghent University Hospital; he headed the department of Endocrinology from 2003 to 2014 and the Laboratory for Hormonology from 1995 to 2014. He was appointed in 1993 Professor of Medicine at the Ghent University (1993) and is past Chair of the Department of Internal Medicine at the Ghent University (2010–14). From October 1st 2014 he is Professor Emeritus at the Ghent University where he is pursuing clinical and research activities. Main research interests are in the assessment, regulation, and action of sex steroids with focus on their role in health, disease, and aging in men, and in osteoporosis in men. He is (co)author of over 300 publications in international peer-reviewed journals.



André Lacroix, MD FCAHS is Professor of Medicine, Division of Endocrinology at Centre hospitalier de l'Université de Montréal (CHUM). His areas of interest include the mechanisms of adrenal Cushing syndrome, primary aldosteronism, adrenal tumorigenesis, the role of aberrant adrenal hormone receptors in adrenal overfunction, as well as new drugs in the therapy of Cushing disease, primary aldosteronism and adrenocortical cancer.
He was trained at the University of Montreal followed by fellowships in Endocrinology and research at Vanderebilt University and National Institutes of Health, USA. He was Chairman of Medicine and Director of Academic Affairs at CHUM. Former President of the Canadian Society of Endocrinology and Metabolism, he is currently chairperson of the International Society of Endocrinology (2016–20), Editor, Adrenal Section of UpToDate and *Encyclopedia of Endocrinology*, Senior Editor of the *European Journal of Endocrinology*. Fellow of the Canadian Academy of Health Sciences since 2008 and Foreign member of the National Academy of Medicine of France since 2016.

Franco Mantero received his MD at the University of Padua, Italy, did postdoctoral training in Switzerland (Clinique Medicale Therapeutique, Hopital Cantonal, University of Geneva) and in United States (University of California, San Francisco), and has been on sabbatical leave in United Kingdom, United States, and France. He held a post of Associate Professor in Medicine at the Institute of Semeiotica Medica, University of Padua (1981–86). In 1986 he moved to the University of Catania to the Chair of Andrology and Endocrinology, in 1992 to the University of Ancona, and in 2000 to the University of Padua to the Chair of Endocrinology and Chief of the Endocrinology Unit of the Department of Medicine. He has received national and international honors, including a Doctor Honoris Causa at the Semmelweis University, Budapest, Hungary.
He has been Editorial Board Member of several scientific journals (e.g., *Clinical Endocrinology*, *Endocrinology*, *Journal of Hypertension*, *Journal of Endocrinology Investigation Steroids*)
He has served as Member of the Council of several international scientific societies (including International Society of Endocrinology, International Aldosterone Conference, Journee Klotz d'Endocrinologie Clinique, ENS@T) and one of the founders of the European Network for the Study of Adrenal Tumors. His research interests include clinical and basic endocrinology of the adrenal gland and endocrinology of hypertension, in particular pathophysiology of mineralocorticoids and primary aldosteronism. He has authored approximately 500 peer–reviewed articles and edited several books and proceedings.

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PREFACE

The first Edition of the *Encyclopedia of Endocrine Diseases* was published in 2004. Because of the enormous development in the field it was found important to produce a completely revised and updated Second Edition of the Encyclopedia. The new Edition is a must-have one-stop reference covering every aspect of the physiological background, pathogenesis, clinical diagnostics, and therapeutic aspects of the wide array of endocrine and related metabolic diseases.

The functional balance of the body (homeostasis) is maintained by two regulatory circuits, i.e., the nervous and the endocrine systems. Among the most complex constructs in the body, the endocrine system comprises a group of glands that secrete hormones directly into the bloodstream, where they reach their specific receptors in other parts of the body, evoking specific intracellular signaling pathways leading to their biological effect. Many classically non-endocrine organs (e.g., the heart) have also turned out to have endocrine functions. The endocrine system maintains and regulates the body's homeostasis by using hormones to control metabolism, temperature, biological cycles, internal fluid volume, reproduction, growth, body composition, and development. The system is a marvel when functioning optimally, i.e., maintaining the body homeostasis. Unfortunately, there is a myriad of ways these processes, actions, and functions can go awry, resulting in various endocrine and metabolic diseases, which form the over-arching theme of the Encyclopedia.

The Encyclopedia is not meant as a primer on the subject of endocrinology, but instead intended to provide a comprehensive reference work on the extensive spectrum of diseases and disorders that can occur within the endocrine and metabolic system. The updated version of this groundbreaking encyclopedia is especially timely, as it covers the dramatic discoveries in the field of endocrinology and metabolism over the past 10 years, particularly with respect to novel diagnostic techniques and treatment approaches. In particular, there have been tremendous advancements in our understanding of the molecular basis of endocrine and metabolic diseases (mutations, epigenetics, signaling), as well as pathogenesis and therapy of the common forms of these diseases (e.g., diabetes, obesity, and endocrine malignancies).

The Encyclopedia offers a unique source of up-to-date information for the physicians and basic scientists working in the field. It is an essential resource for every clinician diagnosing and treating endocrine patients. The Encyclopedia also offers the prime source of information for students of medicine and science around the world, as well as basic research workers in academia, the pharma industry, and in other areas in need of information on endocrinology and metabolism. It also offers useful information for the lay public about normal and abnormal functions of hormones.

The Encyclopedia is intended to serve as a useful and comprehensive source of information spanning the many and varied aspects of the endocrine and metabolic system. The chapters have been written to be accessible to both clinical and nonclinical readers. The articles have been formatted in similar fashion and each is intended as a stand-alone presentation. Each article begins with a glossary list defining key terms that may be unfamiliar to the reader and are important for understanding the article. The body of the article begins with a brief introduction to the subject under discussion, bold headings lead the reader through the text, and figures and tables explain and illuminate most articles. The main text is followed by referenced citations to provide the reader with access to additional information on the topic, and cross-references lead the reader to related entries in the encyclopedia. The relatively short stand-alone articles have allowed us to recruit the best experts available for each topic.

Unlike the first Edition, where the articles were arranged in alphabetical order, the 2nd Edition is arranged in organ-based thematic order, where each organ-based group of diseases is presented as cluster of articles in the first four volumes. The fifth volume is a stand-alone compilation of all articles on pediatric endocrinology. The thematic organization gives the reader a better general view of the coverage of articles on a specific endocrine organ or disease type.

The Second Edition of the Encyclopedia builds of the first edition. Nevertheless, to bring a major reference work with such a broad scope from initial conception to final publication involved a great deal of planning and organization, together with the efforts of innumerable individuals. The authors of the first edition were invited to update their earlier texts. If this was not possible, the Section Editors invited another expert in the topic either to update the previous text or to write a de novo text; the latter happened in most of these cases. Hence, the Second Edition contains to a large extent totally new information, or at least the fluency of all texts has been scrutinized. Furthermore, all manuscripts have undergone peer-review arranged by the Section Editors.

Assembling a large volume of articles with the purpose to cover all essential topics of endocrine diseases posed multiple challenges. Coverage was a significant problem: on one hand some redundancy of the topics was almost impossible to avoid in places while, on the other, there were inevitable gaps. Some of these arose from late cancellations; others from oversights on our part. We can only promise to fill these gaps in future editions. We also note that as can be expected for a large multi-author compilation the individual articles do differ in detail and approach. We considered it more important to allow our experts substantial latitude in deciding how to present their topics than to apply rigid guidelines.

Most of the editing work of the Encyclopedia has been carried out by a highly competent board of 16 Section Editors, each of them internationally renowned experts in their respective field within clinical endocrinology. First, the broadest possible list of topics was compiled, aiming at the best possible coverage. Throughout the editorial process, the Section Editors supervised their subject area of expertise, recommended and corresponded with fellow editors and article contributors, reviewed the manuscripts, and continuously helped to refine the final list of topics. This has made the task of the Editor in Chief easy, mainly entailing the supervision of smooth progress of the project.

The Section Editors and their fields deserve being listed here: *Jean-Jacques Body* (Belgium, bone endocrinology), *Felipe F. Casanueva* (Spain, metabolism and obesity), *Richard N. Clayton* (United Kingdom, pituitary gland), *Jean-Louis Chiasson* (Canada, diabetes), *Sophie Christin-Maitre* (France, female reproduction), *Wouter W. de Herder* (The Netherlands, neuroendocrinology), *Ulla Feldt-Rasmussen* (Denmark, thyroid gland), *Ieuan Hughes* (United Kingdom, pediatric endocrinology), *Gregory Kaltsas*, Greece, and *Martin O. Weickert*, United Kingdom, (gastrointestinal hormones), *Jean-Marc Kaufman* (Belgium, endocrinology of aging), *André Lacroix* (Canada, adrenal cortex), *Franco Mantero* (Italy, adrenal medulla and endocrine hypertension), *Jorma Toppari* (Finland, endocrine disruptors), *Jacquetta Trasler* (Canada, endocrine epigenetics) and *Christina Wang* (United Kingdom, male reproduction).

The Elsevier editorial staff, *Will Smaldon*, *Laura Escalante Santos*, and *Kate Miklaszewska-Gorczyca*, have been of enormous help to the editors at every step during this long project. I admire the professionalism of everyone and am deeply indebted to all for their dedication and hard work to make the Encyclopedia the leading reference book of clinical endocrinology.

The authors of the individual chapters, more than 450 in total, were specifically selected by the Section Editors to represent the best available knowledge on the topic available. They all should be thanked for their dedication and the excellent quality of their contributions.

Ilpo T. Huhtaniemi
Editor in Chief

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Adrenal Cortex; Physiology☆

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Glossary

Adrenocorticotropic hormone or corticotropin (ACTH) ACTH is synthesized from proopiomelanocortin, a 241-amino acid precursor. Usually, ACTH is derived from the pituitary gland to stimulate the adrenal glands through the melanocortin-2 receptor. ACTH is rarely produced ectopically from neuroendocrine tumors (i.e., in ectopic Cushing syndrome), or in the adrenal glands, as seen in primary bilateral macronodular adrenocortical hyperplasia.

Corticotropin-releasing hormone (CRH) CRH is a hypothalamic 41-amino acid peptide that usually stimulates the pituitary gland to release ACTH.

Hypothalamic–pituitary–adrenal (HPA) axis Hypothalamic-releasing factors including CRH are influenced by central nervous system afferents. For instance, stress can trigger CRH release. The main peptide secreted by corticotroph cells when POMC is activated by CRH is ACTH, which subsequently stimulates the adrenal glands to produce and release steroids, including cortisol. Cortisol, on the other hand, can inhibit further release of CRH and ACTH through a tightly regulated negative feedback loop.

Steroids These members of a large family of compounds that are derived from the cyclopentanoperhydrophenanthrene ring structure that consists of three cyclohexane rings and one cyclopentane ring and are produced by the adrenal glands. The nomenclature denotes rings by a letter and the individual carbon atoms by a number. Gonane is the unsaturated 17'carbon ring structure. Estranes are steroids with 18 carbons (C18 steroids) by adding a methyl group at C13. Androstane is a C19 steroid with two methyl groups. Pregnane is a C21 steroid with methyl and ethyl groups.

Introduction

In 1563, Bartholomeo Eustachius, a famous Italian anatomist and artist, was credited for the first full description of the anatomy of the adrenal glands (Miller, 2013a,b). Subsequently in 1849, Thomas Addison, a renowned 19th-century English physician and scientist, described the central physiologic role of the adrenal glands (Miller, 2013a,b). In the 21st century, our growing understanding of adrenal zonation, genetics, and steroidogenesis has improved our understanding of the pathophysiologic states of the adrenal glands (Xing *et al.*, 2015).

The adrenal glands are divided into two major anatomic areas, cortex and medulla. The adrenal cortex is composed of three zones; glomerulosa (ZG), fasciculate (ZF), and reticularis (ZR). The largest zone in humans is the ZF, where glucocorticoids including cortisol are produced (Xing *et al.*, 2015). In general, extracellular volume status is influenced by aldosterone, the hormone of ZG. Adrenal androgens such as dehydroepiandrosterone (DHEA) are primarily produced by the ZR, which begins to grow at approximately age 4, shortly before adrenarche (Xing *et al.*, 2015).

Corticotropin (ACTH), produced from the pituitary gland, is the principal stimulator for cortisol and/or adrenal androgen production (and to a minor extent, aldosterone) (Margioris and Tsatsanis, 2000). Corticotropin is released under stress and other stimuli through hypothalamic corticotropin-releasing hormone (CRH) secretion. Like CRH, arginine-vasopressin (AVP), a hypothalamic hormone, stimulates the pituitary release of ACTH. A tightly regulated feedback loop exists between the hypothalamus (H), pituitary (P), and adrenal glands (A), known as the HPA axis. High peripheral cortisol levels inhibit further release of CRH and corticotropin. Hypothalamic CRH and pituitary corticotropin are suppressed in various conditions (e.g., in conditions of supraphysiologic exogenous glucocorticoid administration), with subsequent atrophy of the adrenal glands due to the lack of corticotropin. The adrenal medulla forms postnatally and exerts effects on the adrenal cortex and vice versa. Adrenomedullary chromaffin cells are intermingled with the adrenal cortex, facilitating interaction between the two layers.

Fetal Adrenal Gland Development

The fetal adrenal cortex plays a critical role in regulating intrauterine homeostasis and the maturation of fetal organ systems that are necessary for extrauterine life. The important mediators for these functions are steroid hormones from the fetal adrenal glands.

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Throughout gestation and postnatally, the fetal adrenal glands undergo morphological and functional changes during its transformation to the adult adrenal gland (Ishimoto *et al.*, 2011; Merke *et al.*, 2006).

Following the formation of the adrenal cortex at the fourth week of human embryonic development, a blastema of undifferentiated cells of mesodermal origin forms from either the medial part of the urogenital ridge or mesoderm (**Fig. 1**) (Xing *et al.*, 2015; Merke *et al.*, 2006). The adrenogonadal primordium cells undergo proliferation and invasion of the underlying mesenchyme that is dependent on the interplay between the transcriptional factors steroidogenic factor 1 (SF1) and DAX1 that ultimately separates from the gonads by day 33 post conception (Xing *et al.*, 2015; Beuschlein *et al.*, 2002; Hammer *et al.*, 1999). Further mesodermal cell proliferation, under the control of fetal corticotropin, forms the first evidence for zonation: a definitive zone (DZ) and a fetal zone (FZ) that arise from the celomic epithelium, while the transitional zone (TZ) originates from the mesonephron and arises from the region of Bowman's capsule. Thus, the progenitor cells of the adrenal cortex stem from a cell lineage that also leads to steroid-secreting cells of the gonads. The FZ consists of large eosinophilic steroid-secreting cells that express high levels of steroid 17α-hydroxylase (CYP17) and DZ consists of cells that do not express CYP17 (Scheys, 2011). The TZ is composed of cells similar to those of the zona fasciculata of the adult adrenal glands (Scheys, 2011).

Several endocrine, paracrine, and autocrine factors influence the steroidogenesis of the fetal adrenal cortex. Between the 8th and 12th embryonic week, sinusoidal vascularization of the glands forms the framework for the zonation of the adult cortex (Scheys, 2011). At gestational week 8, chromaffin cells enter the rudimentary adrenal glands and cluster as discrete islands until day 8 postnatally, before they form a rudimentary adrenal medulla. Cortisol is produced from the rapidly growing FZ at about the 6th week of development, reaching a peak between the 8th and 9th week (Scheys, 2011). Gradually, aldosterone and cortisol are made by DZ and TZ cells, respectively, whereas the FZ, which represents 85% of the cortical volume, produces large amounts of DHEA and DHEA sulfate (DHEAS), that ultimately support estrogen production through the fetal-placental unit. Placental estrogen supports the fetal adrenal glands to synthesize cortisol (Sidiropoulou *et al.*, 2000). By the 9th week, progenitor populations of the adult adrenal cortex encapsulate the adrenal glands, expressing *Nr5a1* and *Gli1*. Migrating neural crest cells forms the adrenal medulla and intermingles with cortical cells of the FZ, attaining a maximum adrenal size by the 4th month. Thereafter, the gradual receding of FZ, and expansion of DZ and TZ, gives rise to the adult ZG and ZF, respectively (Scheys, 2011). After birth, FZ involutes and the corticomedullary junction separates between steroid hormone-producing and catecholamine-secreting cells. A transition zone of primarily fibrous tissue separates the FZ from the remaining gland. By the end of the second year of life, the first evidence of an anatomically distinct ZR appears; however, steroidogenic activity of this zone is not present until the age of 5 years, concomitant with the onset of adrenarche (Nakamura *et al.*, 2009). The adult adrenal cortex likely reaches maturity as early as 8 years of age to as late as after mid-puberty (Nakamura *et al.*, 2009).

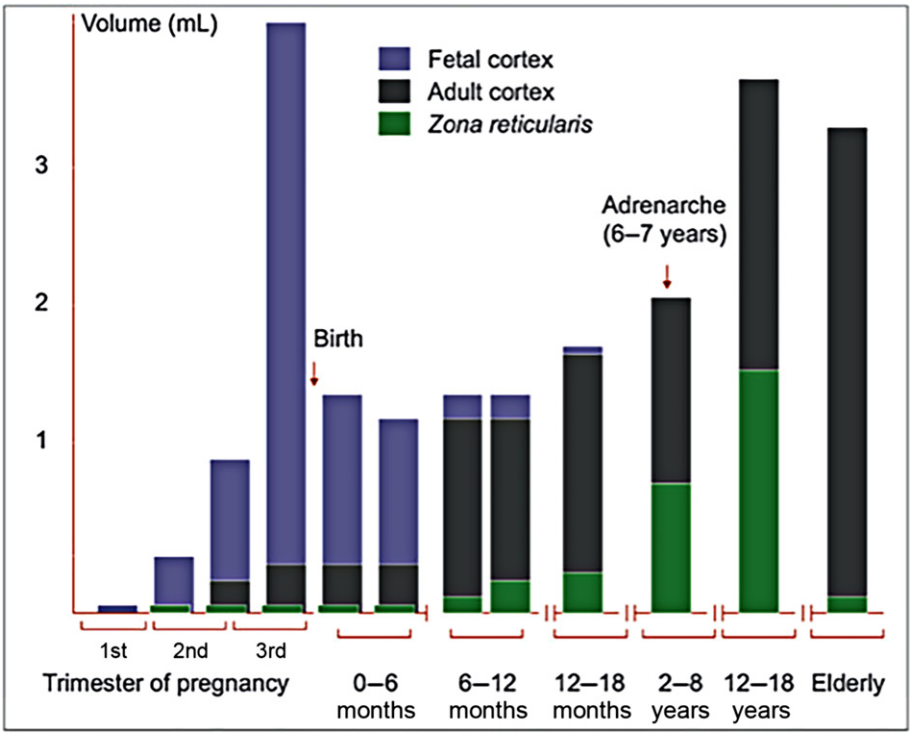

Fig. 1 Fetal and adult adrenal cortex development. Modified from Merke, D. P. *et al.* (2006). The adrenal life cycle: The fetal and adult cortex and the remaining questions. *Journal of Pediatric Endocrinology and Metabolism* **19**, 1299–1302. Elderly defined as age >65 years.

Growth Factors

During the first trimester of pregnancy, human chorionic gonadotropin (HCG) regulates the growth of the fetal adrenal glands. Corticotropin is critical for growth, steroidogenesis, and differentiation of the fetal adrenal glands (Scheys, 2011). Corticotropin becomes the main growth factor after the 5th month of gestation. Corticotropin deficiency leads to increased apoptosis and subsequent atrophy of the adrenal glands, whereas corticotropin excess (e.g., in Cushing syndrome or congenital adrenal hyperplasia) can cause hyperplasia of the adrenal glands. In addition to HCG, corticotropin, and its receptor ACTHR, local growth factors are important for steroidogenesis, growth, and development of the adrenal gland. Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), their receptors, and binding proteins are all expressed in the fetal adrenal glands. IGF-1 amplifies the effect of corticotropin on the adrenal and enhances adrenal steroidogenesis by increasing the activities of 17α, 21-, and 11β-hydroxylase (Scheys, 2011). Similarly, IGF-2 promotes the action of corticotropin. In addition, IGF-2 helps the fetal adrenal to synthesize cortisol and androgen by regulating the enzymes p450scc, p450c17, and 3β-hydroxysteroid dehydrogenase (Scheys, 2011).

Basic fibroblast growth factor is a mitogenic protein and is more effective (stimulating proliferation) on adrenal cells of the DZ than those of the fetal zone. This may lead to hypertrophy of the fetal adrenal glands. EGF and EGFR play an important role in ACTH secretion from the pituitary gland. The TGF-β family of growth factors, including activin, inhibin, and TGF-β_1, are paracrine/autocrine regulators of growth and steroidogenesis in the fetal adrenal cortex. Activin increases corticotropin-stimulated cortisol production but not DHEAS production in fetal zone cells. In the adult adrenal cortex, activin has no effect on growth or steroidogenesis. In fact, activin may lead to apoptosis and involution of the fetal adrenal cortex postnatally. TGF-β_1 appears to decrease fetal and definitive zone cell proliferation and steroidogenesis.

Nuclear Receptors

The proliferation and invasion of adrenogonadal primordium cells are dependent on the interplay between the transcriptional factors SF1, DAX1 and estrogen receptor (ER) (Hanley *et al.*, 2006; Lin *et al.*, 2006). These factors belong to the nuclear receptor superfamily. Members of this family are transcription factors that are important for regulating expression of genes involved in cellular growth control and differentiation. SF-1 is classified as an orphan receptor because its ligand is unknown. The human cDNA sequence of SF-1 is highly homologous ($>95\%$) to murine and bovine sequences. Human adrenal cortex, ovaries, testes, and spleen show high SF-1 mRNA expression. In human placenta, SF-1 is not or only minimally expressed. SF-1 plays an essential role in the organogenesis of the fetal adrenal glands and also in regulating genes that code for steroidogenic enzymes (Hanley *et al.*, 2006). SF-1 stimulates the promoter activities of genes encoding steroidogenic acute regulatory (StAR) protein, the scavenger receptor-type class BI (SR-BI), and the corticotropin receptor. StAR protein is critical in the translocation process of cholesterol from the outer to the inner mitochondrial membrane. In contrast to the adult adrenal glands, the fetal adrenal glands uses low-density lipoprotein (LDL) rather than high-density lipoprotein (HDL) cholesterol as the main source for steroid biosynthesis (Miller, 2013a,b). It appears that SR-BI binds to LDL with high affinity. SF-1 influences the constitutive activity of the human corticotropin receptor gene promoter and regulates steroid hydroxylase enzymes (Miller, 2013a,b). In addition, SF-1 regulates the genes coding for the β-subunit of luteinizing hormone, the α-subunit of the glycoprotein hormones, gonadotropin-releasing hormone receptor, prolactin receptor, oxytocin, Mullerian inhibiting substance, and aromatase (Hammer *et al.*, 1999). Furthermore, SF-1 interacts with other proteins and cofactors.

DAX1 is an orphan nuclear receptor that is highly expressed in the fetal and adult adrenal glands, gonads, ventromedial hypothalamus (VMH), testes, ovaries and the pituitary gonadotropes (Hanley *et al.*, 2006). Together with SF-1, they may coregulate steroidogenesis as well as adrenal and gonadal organogenesis. DAX-1 can block steroidogenesis by inhibiting the activity of StAR and the expression of p450scc and 3β-hydroxysteroid dehydrogenase (Margioris and Tsatsanis, 2000). Estrogens are important in cell differentiation, growth, and function of various tissues. Estrogen receptors are members of the steroid receptor superfamily and mediate the action of estrogens. ERβ is highly expressed in the fetal adrenal gland, in contrast to ERα.

Mice that are knockouts (KO) for Sf1 have complete absence of the adrenal glands, whereas mice KO for Dax1 have developmental adrenal gland defects without adrenal insufficiency (AI) (Scheys, 2011; Miller, 2013a,b). In humans, X-linked DAX1 (mutations in *NR0B1*) defects cause the most common human form of congenital adrenal insufficiency (Lin *et al.*, 2006). These patients are usually 46,XY phenotypic boys and may have hypogonadotropic hypogonadism and a family history of male-only congenital adrenal insufficiency. Additionally, gene deletions at Xp21/22, where the DAX-1 gene is located, may lead to glycerol kinase deficiency, hypogonadotropic hypogonadism, and/or Duchenne's muscular atrophy. Humans with heterozygous *SF1* (coded by the *NR5A1* gene) mutations have adrenal insufficiency and gonadal abnormalities (Zanaria *et al.*, 1994). More recently, patients with isolated adrenal insufficiency and heterozygous *NR5A1* mutations have been described. SF1 gene mutations were also found in patients who also had isolated 46,XY gonadal dysgenesis and have been rarely identified in patients with congenital adrenal insufficiency without evidence of gonadal defects.

Adult Adrenal Cortex

Steroid Biosynthesis and Regulation of Cortisol Production

The normal adult human adrenal glands weighs approximately 5 g. Ninety percent of this weight is contributed by the adrenal cortex, which is composed of three zones (from outside to inside): the ZG, ZF, and the ZR. The adrenal medulla forms postnatally

and is composed of chromaffin cells, some of which may still be intermingled and spread within the adrenal cortex. The adult adrenal cortex produces glucocorticoids, mineralocorticoids, and adrenal androgens (**Fig. 2** and **Table 1**) (Hammer *et al.*, 1999; Miller, 2013a,b). The blood flow in the adrenal glands is centripetal (from outside to inside), which exposes the inner zones and the adrenal medulla to increasing concentrations of adrenal steroids. High cortisol levels in the medulla are needed to induce enzymes for epinephrine biosynthesis. In fact, patients with congenital adrenal hyperplasia have a compromised development and function of the adrenomedullary system due to cortisol deficiency.

Seventy-five percent of the adrenal glands weight is due to the ZF, the largest zone and the one that synthesizes glucocorticoids. The ZF and ZR also produce DHEA and DHEAS, whereas cortisol is primarily produced in the ZF. In contrast to the ZF, the ZR is small and not very involved in adrenal androgen production until adrenarche. The precursor for glucocorticoid production is cholesterol, which is initially converted to pregnenolone in the adrenal cortex. Steroids are derived from the cyclopentanoperhydrophenanthrene four-ring hydrocarbon nucleus, a relatively inert structure. Depending on the presence of several enzymes in the respective adrenal cortex zone, several steroid hormones can then be synthesized. Cytochromes P450 are categorized into two classes: type 1 enzymes that reside in the mitochondria and type 2 enzymes located at the smooth endoplasmic reticulum (**Table 1**) (Miller *et al.*, 2011).

The secretion and synthesis of cortisol are regulated by the hypothalamic–pituitary–adrenal (HPA) axis. Certain stimuli including stress lead to the release of CRH in the hypothalamus. CRH then stimulates corticotropin release from the pituitary gland (**Fig. 3**). Corticotropin binds to corticotropin receptors located on adrenocortical cells and stimulates the release of cortisol through cyclic adenosine monophosphate (cAMP). Cortisol leads to an increase in energy-providing compounds, including glucose, free fatty acids, and free amino acids. As mentioned previously, corticotropin is also growth promoting on the adrenal cortex; that is, continuous stimulation by corticotropin may lead to adrenal hypertrophy, whereas a lack of corticotropin may lead to adrenal atrophy. The HPA axis is very sensitive to exogenous and chronic glucocorticoid exposure, which can easily lead to

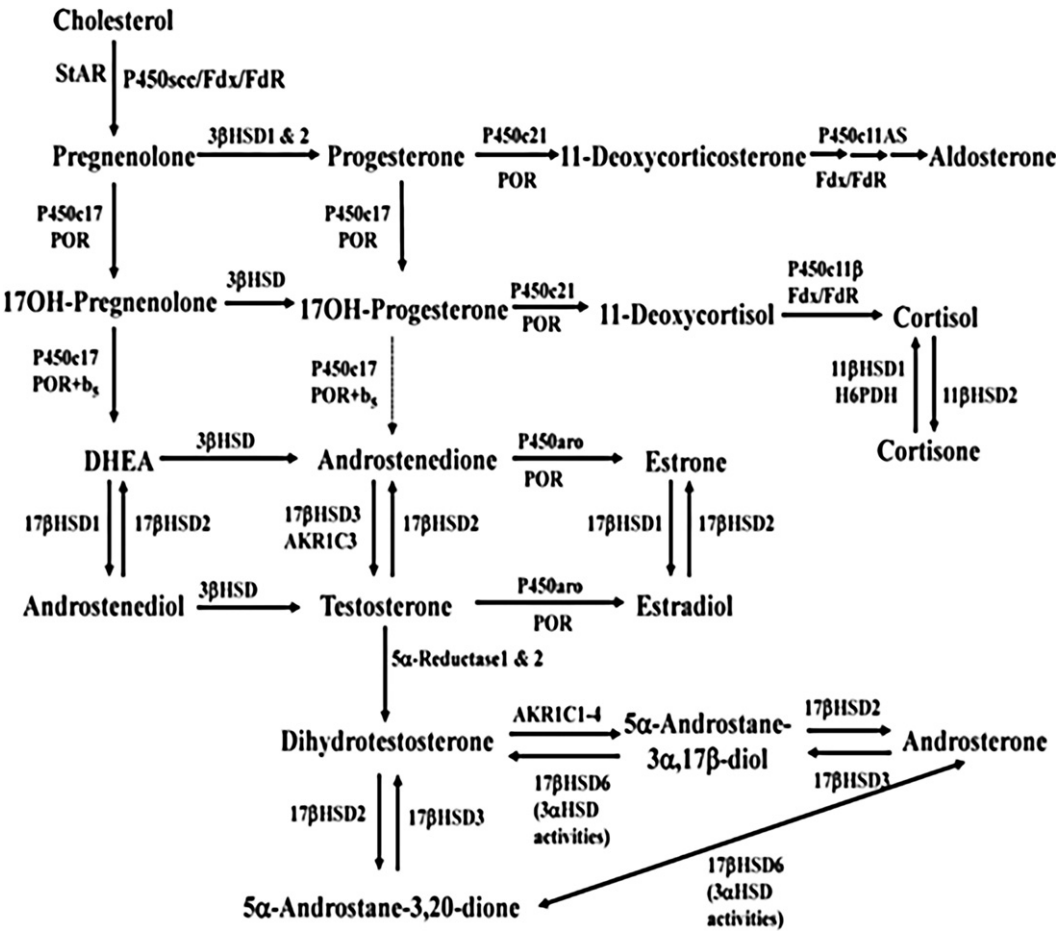

Fig. 2 Adrenal steroidogenesis. In brief, desmolase converts cholesterol to pregnenolone, 3β-OH-steroid dehydrogenase I/II convert pregnenolone to progesterone, 17-OH-pregnenolone converts progesterone to 17-OH-progesterone, P450c11 converts deoxycorticosterone to 18-OH-corticosterone and 11-deoxycortisol to cortisol, etc. Not all intermediate steroids, pathways, and enzymes are shown. Adapted with permission from Miller, W. L. *et al.* (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews* **32**, 81–151. © 2011 by The Endocrine Society.

Table 1 Key human steroidogenic enzymes and cofactor proteins (A) and their location (B).

(A)

Enzyme	*Gene*	*Chromosomal location*
StAR	*STAR*	8p11.2
P450scc	*CYP11A1*	15q23-24
P450c11β	*CYP11B1*	8q21-22
P450c17	*CYP17A1*	10q24.3
P450c21	*CYP21A2*	6p21.1
P450aro	*CYP19A1*	15q21.1
3βHSD1	*HSD3B1*	1p13.1
3βHSD2	*HSD3B2*	1p13.1
5-α-Reductase 1	*SRD5A1*	5p15
5-α-Reductase 2	*SRD5A2*	2p23
SULT2A1	*SULT2A1*	19q13.3

(B)

Endoplasmic reticulum	*Cytoplasm*	*Mitochondria*
11β-HSD I and II	3α-HSD	3β-HSD II
5α-Reductase I and II	17β-HSD V	StAR
17β-HSD I–III	17β-HSD I	Adrenodoxin reductase
3β-HSD II	3β-HSD II	P450c11AS
Cytochrome b_5	StAR	P450c11β
P450 oxidoreductase	Adrenodoxin	P450scc
P450aro		
P450c21		
P450c17		

Modified from Auchus *et al.* The principles, pathways, and enzymes of human steroidogenesis, *Endocrinology* 6th edn. (2010).

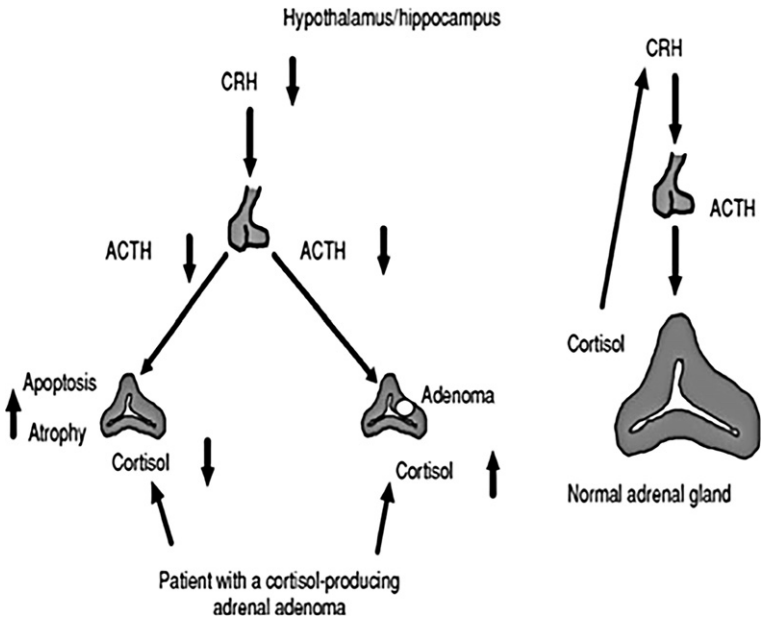

Fig. 3 The HPA axis. Modified from Koch, C.A. and Chrousos, G.P. (2001). Editorial: Is the diminuto/dwarf1 gene involved in physiologic adrenocortical size regulation and tumor formation? *The Journal of Clinical Endocrinology & Metabolism* **86**, 5127–5129.

corticotropin suppression through a negative feedback loop on CRH, and ACTH in corticotroph cells. In normal individuals who are not working in (night) shifts, there is a diurnal variation of cortisol production, with serum cortisol being highest in the morning and lowest at midnight. In patients with Cushing syndrome (hypercortisolism), these normal physiologic circuits are

disturbed. Prolonged (7–48 h) increases in corticotropin leads to an increased synthesis of all the steroidogenic enzymes, especially P450scc, as well as an increased uptake of cholesterol from the circulation (Hanley *et al.*, 2006; Miller *et al.*, 2011). Chronic lack of corticotropin (e.g., through exogenous glucocorticoid administration) leads to adrenal atrophy. Therefore, the exogenous glucocorticoid has to be tapered to allow the pituitary and adrenal glands to recover in order to synthesize normal levels of cortisol on its own. Depending on the level of suppression, this may take weeks or months.

Biosynthesis and Regulation of Aldosterone Production

Aldosterone, the major human mineralocorticoid, is produced in the ZG of the adrenal cortex. Its secretion is stimulated mainly by potassium, angiotensin II (and III) through the renin–angiotensin–aldosterone system and, to a lesser extent, by corticotropin. Chronic infusion of corticotropin stimulates aldosterone secretion for only 24 h. Less potent stimulators of aldosterone secretion are endothelin and serotonin. Also, increases in potassium concentrations stimulate aldosterone production (Hattangady *et al.*, 2012). An increase in serum potassium of 0.1 mmoL/L can elevate plasma aldosterone by 35%. On the other hand, a decrease in serum potassium of 0.3 mmoL/L can reduce plasma aldosterone by 46%. Aldosterone promotes potassium excretion, sodium reabsorption and fluid retention, thereby increasing the extracellular fluid volume. However, after a few days of extracellular fluid expansion by increased aldosterone levels, the individual will be protected from continuous expansion through a so-called "escape" mechanism that denotes attaining a new equilibrium of sodium balance and the formation of a new steady state (Hattangady *et al.*, 2012).

Target tissues of aldosterone, including kidney (distal tubules and cortical collecting ducts), colon, and salivary glands, have mineralocorticoid receptors that bind aldosterone. In the distal nephron, cortisol is a potent agonist at the mineralocorticoid receptor. Among inhibitors of aldosterone secretion are atrial natriuretic peptide (ANP) and dopamine (Hattangady *et al.*, 2012). ANP strongly inhibits stimulated (e.g., low sodium intake) aldosterone secretion, with much less effect on basal (e.g., normal or high sodium intake) activity. Chronic sodium restriction leads to increased activity of aldosterone synthase and a higher content of this enzyme in the ZG. The first steps of aldosterone biosynthesis are identical to those of cortisol biosynthesis (**Fig. 2**). The synthesis of cortisol, however, depends on 17α-hydroxylation of pregnenolone by 17α-hydroxylase (P450c17), which is exclusively expressed in the ZF (Hanley *et al.*, 2006; Miller *et al.*, 2011). On the other hand, aldosterone synthase is normally expressed only in the ZG.

Regulation of Adrenal Androgen Production

At approximately 4 years of age, in both sexes the ZR forms and continues to grow until the mid-20's. After age 40, this zone gradually regresses. Corticotropin and prolactin stimulate adrenal androgen secretion in the fetal adrenal zone (Sidiropoulou *et al.*, 2000). Postnatally, the ZR responds to corticotropin, as exemplified in congenital adrenal hyperplasia in which corticotropin and androgen hypersecretion can occur. During infancy, only small amounts of androgens are secreted, and it is unknown how adrenarche, the time point at which a slight amount of pubic hair develops, is regulated. Seventy percent of circulating testosterone in women with normal menstrual cycles derives from the conversion of adrenal DHEA. The principal androgens secreted by the adrenals are DHEA, DHEAS, androstenedione, and (minimally) testosterone (Nakamura *et al.*, 2009). DHEAS per se has only weak androgenic effects. Peripheral conversion of the aforementioned precursors leads to more potent androgens, such as testosterone and dihydrotestosterone. Major conversion sites include the hair follicles, sebaceous glands, external genitalia, and prostate. Peripheral adipose tissue can convert androgens into estrogens by the highly active enzymes aromatase and 17-ketosteroid reductase. Glucocorticoids stimulate aromatase. Inactivation or degradation of androgens and their metabolites occur at different sites, including the liver and kidneys. Exogenous adrenal androgen administration can suppress gonadotropin secretion. Excess endogenous androgen production can be caused by several conditions, including congenital adrenal hyperplasia, polycystic ovary syndrome and adrenal tumors.

Impact of the Sympathoadrenal System on the Regulation of Adrenocortical Function

Adrenocortical steroid hormones influence the differentiation and hormone production of adrenal chromaffin cells. On the other hand, the sympathoadrenomedullary system modulates diurnal variations of steroidogenesis in the adrenal cortex. The adrenal cortex is innervated by neurons originating in cell bodies within the adrenal medulla and by nerves that have cell bodies outside the adrenal, reaching the cortex via blood vessels. Adrenal chromaffin cells contain many neuropeptides that regulate adrenocortical steroid production in many species. Adrenomedullary cells are found throughout the adrenal cortex, which facilitates the paracrine action of their products. Another avenue for adrenomedullary secretory products reaching the adrenal cortex is the lymphatics.

Conclusion

The adrenal cortex fulfills important and essential functions throughout a persons lifespan. Prenatally, the fetal adrenal cortex is large and consists of the fetal zone, which produces large amounts of DHEAS, a hormone that serves as a precursor for other

androgens. DHEAS is used by the placenta to synthesize estriol and to regulate intrauterine homeostasis as well as maturation of fetal organ systems that are necessary for life after birth. Cortisol and aldosterone, both vital for homeostatic functions, are produced in the adrenal cortex and play important physiological functions in various tissues. Postnatally, the adrenal cortex becomes a three zoned structure with the ZF being the largest zone. All three zones gradually regress during a life span and their dysfunction could lead to serious illness.

See also: Adrenal Cortex; Development. Regulation of POMC and ACTH Secretion. Adrenal Steroidogenesis

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Adrenal Cortex; Development☆

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Glossary

Adrenarche The gonadotropin-independent increase in adrenal androgen production marked by the rise in plasma DHEAS occurring at around 6–9 years of age. Adrenarche corresponds to the emergence of zona reticularis at the corticomedullary junction.

Embryogenesis Differentiation of the fertilized ovum during the period of most rapid development, that is, after the long axis appears until all major structures are represented.

Nuclear receptor Ligand-inducible transcription factor that specifically regulates the expression of target genes involved in metabolism, development, and reproduction.

Pregnancy or gestation The condition of having a developing embryo or fetus in the body. Duration of pregnancy is 266 days in women, 184 days in baboons, 165 days in rhesus monkeys, 145–150 days in sheep, 21 days in rats, and 18.5 days in mice.

Steroid hormones Lipophilic molecules, having a C_{17} ring as the basis of their chemical structure, that freely cross the cell membrane and interact with nuclear receptors.

Transcription factor Protein that directly affects the initiation of transcription of specific genes.

Maturation of the hypothalamic–pituitary–adrenal axis, which is characterized by increased activity during late gestation, is essential for the development of the fetus and plays a critical role in preparing for its transition to extrauterine life. The fetal adrenal cortex synthesizes and secretes androgens, glucocorticoids, and mineralocorticoids. In primates, androgens are necessary for placental conversion to estradiol, a hormone that is crucial for the maintenance of pregnancy, the maturation of the fetal and maternal tissues, and immunosuppression, leading to implantation of the placenta and the fetus. Glucocorticoids are essential for the maturation of brain, lung, liver, gut, kidney, and the adrenal itself. In some species, a surge in fetal glucocorticoid secretion has been suggested as being integral to the cascade of events leading to the onset of parturition. However, premature or abnormal exposure of fetuses or newborns to high levels of glucocorticoids leads, through a programming mechanism, to an increased prevalence of metabolic and cardiovascular disease. This article details both morphological and functional aspects of adrenal cortex development and zonation in humans and rodents. In particular, the role of nuclear receptors and transcription factors in the regulation of adrenocortical organogenesis and steroidogenesis is examined.

Embryogenesis, Development and Growth

Fig. 1 depicts the major milestones of adrenal cortex development in human and mouse.

In Humans

Human adrenal development begins at approximately the 4th week of gestation (28–30 dpc) and continues into adult life. Adrenocortical and gonadal cells derive from a single cell lineage that originates in the celomic epithelium in the notch between the primitive urogenital ridge and the dorsal mesentery, marked by the expression of steroidogenic factor 1 (SF-1, NR5A1) and forming the adrenogonadal primordium (AGP). Five landmark phases have been described:

Condensation of the celomic epithelium forms the AGP (3–4 weeks of gestation).

Proliferation and migration of celomic epithelial cells (weeks 4–6) that stream medially and cranially, accumulating at the cranial end of the mesonephros, forming the adrenal blastema that progressively separates from the mesenchyme and becomes fully encapsulated by a fibrous layer by 52 dpc.

Morphological differentiation of fetal adrenal cortical cells into two distinct zones (weeks 8–10): the fetal zone and the definitive zone. The fetal zone is an inner cluster of large, eosinophilic cells and represents the largest part (80%–90%) of the adrenal cortex. The definitive zone is a thin outer band of small basophilic cells, densely packed, showing structural characteristics of proliferative cells that appear to function as a reservoir of progenitor cells that may populate the remainder of the gland. A third zone, located between the fetal and definitive zones, has been called the transitional zone. By week 30 of gestation, the definitive and transitional zones resemble

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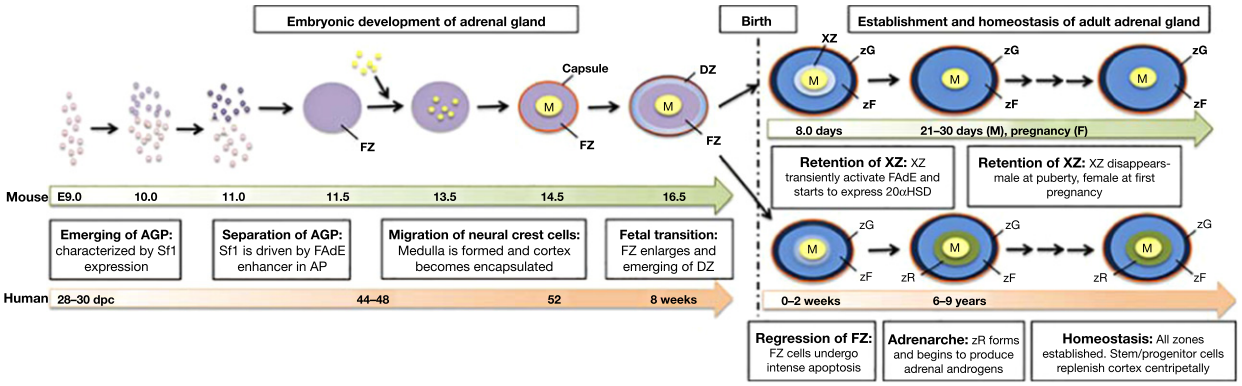

Fig. 1 Developmental and zonation of the adrenal gland. FZ: fetal zone. DZ: definitive zone. XZ: remnant of fetal zone (X-zone). zG: zona Glomerulosa. zF: zona Fasciculata. zR: zona Reticularis. Adapted from Xing, Y., Lerario, A.M., Rainey, W., Hammer, G.D. (2015). Development of adrenal cortex zonation. *Endocrinology and Metabolism Clinics of North America* **44**(*2*): 243–274. DOI: 10.1016/j.ecl.2015.02.001.

the adult zona glomerulosa and the zona fasciculata, respectively. A period of rapid growth begins at approximately week 10 and continues to term. The fetal zone grows by hypertrophy and limited proliferation, whereas growth in the definitive zone occurs mainly by hyperplasia. Several lines of morphological evidence indicate that the human fetal adrenal gland is a dynamic organ, in which proliferating cells located at the periphery migrate, differentiate, and finally undergo senescence in the central part of the gland.

Decline and disappearance of the fetal zone (first 3 postnatal months); by this period, the primate adrenal cortex remodeling involves apoptosis of the fetal zone with a corresponding decrease in adrenal androgen levels and expansion of the preexisting zona glomerulosa and zona fasciculata. Following the involution of the fetal zone, chromaffin elements, derived from the fetal ectoderm, begin to cluster around the central vein. The medulla acquires an adult-like pattern by 12–18 months.

At 6–8 years of age in girls and 7–9 years in boys, emergence of the zona reticularis at the corticomedullary junction, heralds the onset of adrenarche and the return of adrenal androgen production, including DHEA/DHEAS and androstenedione.

Stabilization of the adult zonal pattern (10–20 years of age) with the individualization and homeostatic maintenance of the three distinct cell layers: the outer zona glomerulosa, the central zona fasciculata, and the inner zona reticularis.

In Rodents

In mice, the adrenal gland starts to individualize from the AGP on E11. On E12.5, the cortical cells forming the fetal zone are found intermingled with adrenal medulla sympathoblasts migrating from the neural crest. Encapsulation of the mouse fetal zone by mesenchymal cells from the intermediate mesoderm is achieved by E14.5. Following encapsulation, a second population of cells emerges at the periphery of fetal zone that forms the definitive zone or future adult cortex.

By contrast to humans, the fetal zone in mice does not seem to support any specific steroid hormone production and the individualization of adult adrenocortical layers, zonae glomerulosa and fasciculata, from the definitive zone is completed by birth. Finally, the mouse adrenal cortex does not undergo zona reticularis differentiation but possesses a transient developmental zone between the zona fasciculata and the adrenal medulla, the X-zone. The X-zone becomes histologically distinct at 8–14 dpc and enlarges until 21 dpc, then regresses at sexual maturity in males and during first pregnancy in females. Although its function remains yet unknown, lineage tracing studies clearly identified X-zone as being a remnant of the mouse fetal zone.

Ontogeny of Steroid Biosynthesis

The adrenals synthesize several classes of steroids: androgens, glucocorticoids, and mineralocorticoids. The major human adrenal steroidogenic pathways are summarized in **Fig. 2** Schematically, the process of adrenal steroidogenesis has two major components. The first is quantitative; that is, it regulates how much steroid can be made at a given moment. The second is qualitative; that is, it regulates which particular steroid is made.

First step: Conversion of cholesterol to pregnenolone. This step involves the delivery of the substrate cholesterol to the inner mitochondrial membrane, driven by steroid acute regulatory protein (StAR), and the conversion of cholesterol to pregnenolone, catalyzed by P450 steroid chain cleavage (P450ssc), adrenodoxin, and adrenodoxin reductase.

Second step: Transformation of pregnenolone to active hormones. The coordinate regulation of several enzymes will direct the transformation of pregnenolone toward a given class of steroid hormone—androgens, glucocorticoids, and mineralocorticoids. During adrenal cortex development, the synthesis of these various steroids is not temporally coordinated, is zone-dependent, and is species-specific. **Table 1** summarizes the localization and relative expression of the enzymes involved in fetal adrenal steroid biosynthesis.

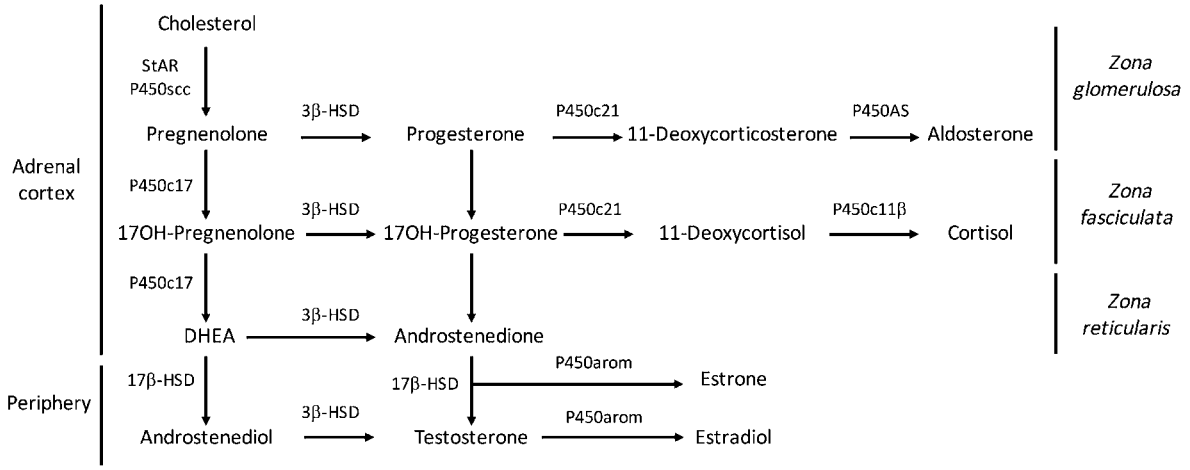

Fig. 2 Schematic view of human adult adrenal and peripheral steroidogenic pathways. StAR, steroid acute regulatory protein; P450ssc, side-chain cleavage enzyme; P450c17, enzyme complex having 17α-hydrolase and 17,20-lyase activities; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c21, 21-hydroxylase; P450c11AS, aldosterone synthase (contains 11β-hydroxylase, 18-hydroxylase, and 18β-hydroxysteroid dehydrogenase activities); P450c11β, 11β-hydroxylase; P450arom, aromatase.

Table 1 Localization and relative expression of the enzymes involved in fetal adrenal steroid biosynthesis

Human	*< Week 25*			*> Week 25*		
	DZ	TZ	FZ	DZ	TZ	FZ
P450ssc	+	+	+	+	+	+
3β-HSD	−	−	−	+ +	+ +	−
P450c17	−	+ + +	+ +	−	+ + +	+ +
P450c21	+ +	+ +	+	+ +	+ +	+
P45011β	−	+	−	+ +	+ + +	+ +
Rodents	*Whole cortex*					
	E16.5	E18.5	E21	P1	P10	P25
P450ssc	+	+ +	+ + +	+	+ + +	+ + +
3β-HSD	+	+ +	+ + +	+	+ +	+ + +
P450c17	+	−	−	−	−	−
P450c21	ND	+	ND	+	+	+ +
P45011β	ND	+	+ +	+	+ +	+ + +

Note: *E*, day of gestation; *P*, postnatal day; *DZ*, definitive zone; *TZ*, transitional zone; *FZ*, fetal zone; *P450ssc*, side-chain cleavage enzyme; *3β-HSD*, 3β-hydroxysteroid dehydrogenase; *P450c17*, enzyme complex having 17α-hydrolase and 17,20-lyase activities; *P450c21*, 21-hydroxylase; *P450c11β*, 11β-hydroxylase; *ND*, not determined.

Androgens

The conversion of cholesterol to pregnenolone is mature in the fetal zone during early pregnancy in humans. High levels of 17α-hydroxylase/17,20-lyase (P450c17) and of its allosteric regulator cytochrome *b*5 (CYB5) favoring lyase activity are present in the fetal zone very early (as soon as 44 days postconception) together with low levels of 3β-HSD. As a consequence, the fetal zone produces dihydroepiandrosterone (DHEA), the bulk of which appears to be secreted as a 3-sulfoconjugate DHEA sulfate (DHEAS) that is formed by the action of DHEA sulfotransferase and the cofactor 3′-phosphoadenosine 5′-phosphosulfonate. DHEAS is used by the placenta for conversion to estrogens. By term, the fetal zone produces approximately 200 mg DHEA per day.

Due to the dramatic postnatal involution of fetal zone, androgen production virtually ceases in the first few months of life and remains very low until the emergence of zona reticularis that marks adrenarche onset. Biochemical adrenarche is defined by the reincrease in circulating adrenal androgen levels leading to serum DHEAS concentrations $\geq$40 µg/dL. This leads to axillary and pubic hair development, adult-like body odor, oily hair and possibly brain maturation.

In rodents, the postnatal adrenals do not express P450c17, do not develop a functional zona reticularis and, thus, do not synthesize DHEA. Interestingly, a functional reticularis-like zone (expressing P450c17, high CYB5, and low 3β-HSD) producing adrenal androgens, can be forced to differentiate in a genetic mouse model where adult cortical cells renewal has been stimulated by constitutive activation of PKA signaling (Dumontet et al., 2018). Thus, the onset of adrenarche seems to depend on the combined effect of PKA and cell turnover that will dictate organ size. The possibility that such a mechanism also occurs in humans to induce adrenarche, remains to be explored.

Glucocorticoids

In Humans

Assays of steroids in cord blood have suggested that human fetus can synthesize cortisol as early as the 10th week of gestation. This hypothesis is supported by clinical and biological observations in patients with congenital adrenal hyperplasia (CAH) caused by deficiency of 21-hydroxylase (P450c21), 3β-hydroxysteroid dehydrogenase (3β-HSD), or 11β-hydrolase (P450c11β). Patients with this disorder have impaired glucocorticoid production with a compensatory increase in pituitary adrenocorticotropic hormone (ACTH) secretion and subsequent stimulation of DHEA synthesis. Interestingly, female infants with CAH have ambiguous external genital development, indicating that, in unaffected female fetuses, cortisol is synthesized by the adrenal by the time of external genital differentiation, a process that is sensitive to androgens and begins at approximately week 10. These observations do not necessarily indicate that the week 10 fetal adrenal is able to synthesize cortisol de novo from cholesterol. Although StAR and P450ssc immunoreactivities are expressed in the cytoplasm of human fetal adrenocortical parenchymal cells of the transitional zone as soon as week 14, in early gestation the fetal adrenal can synthesize cortisol using placental progesterone as substrate, since high levels of progesterone are present in the fetal circulation. P450c21 and P450c11β immunoreactivities have been detected in the transitional zone of human fetal adrenals as soon as weeks 13–14 and P450c17mRNA is present in the transitional zone of week 22 human fetal adrenal. This is consistent with the observation that progesterone infusion into human fetuses between weeks 16 and 18 results in cortisol synthesis. As a consequence, the key enzyme for the production of cortisol de novo from cholesterol during early gestation is 3β-HSD. It has been reported that 3β-HSD immunoreactivity and mRNA could not be detected in human adrenals before weeks 22–24, suggesting that the transitional zone of human fetus adrenals is able to synthesize de novo cortisol beginning at week 24. Similarly, in nonhuman primates, 3β-HSD mRNA is undetectable during early gestation, starts to increase at midgestation, and is further stimulated at late gestation. 3β-HSD protein expression in the transitional zone of adrenals from fetal rhesus monkeys follows a comparable developmental pattern.

In Rodents

Adult mouse and rat adrenal cortex, which lack P450c17, produce mainly corticosterone. P450ssc and adrenodoxin are expressed at midgestation in the rodent adrenal cortex (E15–E16). 3β-HSD mRNA and protein have been detected in the fetal rat adrenal as early as E16; their labeling in zona fasciculata is at a higher level than in the zona glomerulosa at E18. P450c21 and P450c11β immunoreactivity and mRNA are present in the fetal rat adrenal at E18. P450c17mRNA and activity are detectable in the adrenals of mouse fetuses at E12.5, increase in abundance from E12.5 to E14.5, and are then lost between E16.5 and E18.5, suggesting that the fetal mouse adrenal is able to synthesize cortisol during late gestation. Although the ontogeny of StAR expression in the rodent embryonic adrenal remains to be elucidated, it is known that fetal rat adrenals synthesize and secrete corticosterone as early as E13. The corticosterone contents of the fetal rat adrenal are high from E16 to E20 and plasma corticosterone concentrations rise progressively from E16 to E19. This phenomenon occurs following activation of pituitary ACTH and the hypothalamic neuropeptides that control ACTH synthesis and secretion.

During the first 10 days after birth, there is a marked decrease in adrenal corticosterone contents and both basal and stress-induced circulating corticosterone levels. This period has been called the "stress hyporesponsive period" (SHRP). These low-circulating glucocorticoid levels are believed to be essential for normal brain and behavioral development. A decrease in the steroidogenic capacity of the newborn adrenal cortex may account, at least in part, for the SHRP. It has been demonstrated that StAR mRNA and protein are highly expressed in the adrenals at birth, decrease subsequently until P14, and increase thereafter. The level of expression of P450ssc is comparable to that of P1 in adults. At birth, adrenal 3β-HSD activity is low at P1, increases at P10, and remains stable until adulthood. P450c21 activity is low (approximately half of the adult values) on P1 and P10. P450c11β immunoreactivity has been detected in the adrenals of newborn rats and did not change during neonatal development. In P7 rats, P450c11β mRNA is present at high levels only in the zonae fasciculata and reticularis.

Mineralocorticoids

In Humans

Regarding the ontogeny of aldosterone secretion, assays of steroids in cord blood have suggested that human fetus can synthesize aldosterone as early as weeks 16–20. However, in vitro experiments have demonstrated that at midgestation human fetal adrenal tissues do not produce detectable levels of aldosterone, under basal or stimulated conditions. The primary steps in aldosterone synthesis, that is, those driven by StAR, P450ssc, 3β-HSD, and p450c21, are mature in the definitive zone in human fetal adrenals at the end of the midgestation period. However, in human fetal adrenals obtained from second-trimester abortuses, aldosterone synthase (P450c11AS) immunoreactivity is absent in the definitive zone and P450c11AS mRNA is weakly detectable in the whole cortical zone. Similarly, P450c11AS immunoreactivity is absent in the definitive zone of fetal rhesus monkey adrenal until near term. Activation of the late gestation fetal rhesus monkey HPA axis (obtained after treatment with metyrapone, a compound that

inhibits P450c11β) was able to induce P450c11AS expression. Taken together, these data indicate that in the primate fetal adrenal gland the definitive zone has the capacity to synthesize aldosterone, but not until term.

In Rodents

In rats, P450c11AS immunoreactivity is detected in E16 adrenals, in small clusters of cells, dispersed throughout the gland. By E18–E19, the number of P450c11AS-labeled cells increases and these cells become localized in the outer cortex. P1 adrenals have a pattern of P450c11AS staining comparable to that of the adult gland. In P7, rats, P450c11AS mRNA is confined to the subcapsular zona glomerulosa. Aldosterone content in fetal adrenal homogenates increases between E17 and P1 and remains stable thereafter.

Role of Nuclear Receptors and Transcription Factors

The complex cascade of the development and differentiation of the adrenal cortex, from the formation of the urogenital ridge to the zonation of the fetal adrenal, involves several nuclear receptors and transcription factors (**Table 2**).

Table 2 Transcription factors and nuclear receptors involved in adrenal differentiation and steroidogenesis

Transcription factor or nuclear receptor	*First detected on*	*Phenotype of total or conditional[a] mouse KO*	*Genes that are regulated in adrenal cells*
Sf-1	Mouse: E11; Human: E33	Lethal: lacks adrenals and gonads	StAR P450ssc 3β-HSD P450c17 P450c21 P450c11AS P450c11β ACTH-R Dax-1 Angiopoietin 2
Wt1	Mouse: E9	Lethal: lacks adrenals, kidneys, and gonads	Dax-1 Sf-1
Pbx1	Mouse: E10; Human: W7	Lethal: lacks adrenals	ACTH-R P450ssc IGF-I-R Sf1
Cited2	Mouse: E 10; Human: W7	Lethal: lacks adrenals	Sf1
Dax-1	Mouse: E10.5	Lack of X zone regression	StAR P450c17 P450c21 ACTH-R
Wnt4/β-catenin[a]	Mouse: E11.5; Human: E33	Impaired zona glomerulosa development, cortex aplasia	P450c21 P450c11AS Axin2 Lef1
Rspo3[a]	Mouse: E11.5	Impaired zona glomerulosa development, cortex, aplasia	P450c11β P450c11AS Axin2 Shh
Gata-6[a]	Mouse: E14.5; Human: W19	Whole cortex aplasia, ectopic gonadal-like cells	P450scc P450c17 3β-HSD CYB5 SULT2A1

[a]Conditional KO meaning using floxed alleles of the indicated genes that will be recombined by the Cre recombinase specifically in the adrenal cortex.

Note: Sf-1, steroidogenic factor-1; *Wt-1*, Wilm's tumor suppressor gene 1; *Pbx1*, pre-B-cell transcription factor 1; *Cited2*, CBP/p300-interacting transactivator, with Glu/Asp-rich C terminal domain; *Dax-1*, dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1; *WnT4*, wingless-related mouse mammary tumor virus integration 4; *E*, day of gestation; *W*, week of gestation; *StAR*, steroid acute regulatory protein; *P450ssc*, side-chain cleavage enzyme; *P450c17*, enzyme complex having 17α-hydrolase and 17,20-lyase activities; *17β-HSD*, 17β-hydroxysteroid dehydrogenase; *3β-HSD*, 3β-hydroxysteroid dehydrogenase; *P450c21*, 21-hydroxylase; *P450c11AS*, aldosterone synthase; *P450c11β*, 11β-hydroxylase; *ACTH-R*, adrenocorticotropic hormone receptor; *IGF-I R*, insulin-like growth factor type I receptor.

Wt1, Sf-1, and Dax-1

Several transcription factors, such as Wt1 (Wilm's tumor suppressor gene 1), steroidogenic factor-1 (Sf-1), dosage-sensitive sex-reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (Dax-1), Pre-B-cell transcription factor 1 (Pbx1), and CBP/p300-interacting transactivator, with Glu/Asp-rich C terminal domain, 2 (Cited2) play major roles in the development of the adrenal primordium, its functional zonation, and the regulation of fetal adrenal steroidogenic capacities.

Wt-1

Wt1, which encodes a protein having many characteristics of a transcription factor, is crucial for the development of the urogenital ridge. Wt1 is developmentally one of the earliest known genes that specifies kidney, gonadal, and adrenal cell lineages. Mice fetuses knocked out for Wt1 and rescued with a YAC (yeast artificial chromosome) construct spanning the Wt1 locus have adrenal-like structures that are greatly reduced in size and express lower levels of P450ssc mRNA than wild-type mice.

Sf-1

Sf-1, also called Ad4BP or NR5A1, belongs to the orphan receptor class of nuclear receptors. It has been proposed that Sf-1 acts through recruitment of cofactors, including both coactivators (CREB-binding protein, Wt-1, and nuclear receptor coactivator 1) and corepressors (nuclear receptor corepressor, DEAD/H box polypeptide 20, COP9 constitutive photomorphogenic subunit 2, nuclear receptor-interacting protein 1, and Dax-1). In mouse, Sf-1mRNA was detected in the adrenal primordium from E9.5. From E14–E14.5 onward, Sf-1mRNA is restricted to the steroidogenic cells in the cortex. In humans, Sf-1 was expressed as early as E33 in the presumptive adrenal primordium. Newborn Sf-1 knockout mice are devoid of adrenals and die from adrenocortical insufficiency shortly after birth. However, Sf-1 heterozygous mice (*Sf-1*$^{+/-}$) are viable but show adrenal disorganization (reduced adrenal size and hypoplastic zona fasciculata and adrenal medulla), altered adrenal gene expression (increased StAR mRNA and decreased ACTH receptor mRNA), and impaired basal and stress-induced glucocorticoid secretion. Studies of Sf-1 gene mutations in humans demonstrated that Sf-1 regulates adrenal development in a dose-dependent manner. A number of studies have demonstrated that Sf-1 acts a global regulator of the proteins involved in adrenal steroidogenesis (StAR, P450ssc, 3β-HSD, P450c21, P450c11AS, P45011β, and P450c17). In addition, Sf-1 regulates adrenal ACTH sensitivity through a cell-specific modulation of both constitutive and cyclic AMP-induced expression of the ACTH receptor gene. Moreover, Sf-1 post-translational modification by sumoylation also affects its functional capacity that, in turn, alters adrenal development. Indeed, a genetically engineered mouse expressing an unsumoylatable Sf-1 mutant, develops perturbation of cell-fate specification in adrenal cortex characterized by ectopic localization of gonadal cells and persistence of the X-zone (Lee et al., 2011). Sf-1 sumoylation sites mapping at Lysine 119 and 194 are conserved between species but until now no mutation have been described in humans.

Dax-1

Dax-1 is an orphan nuclear receptor that regulates both adrenal development and functional zonation. Mutations in the Dax-1 gene are responsible for X-linked adrenal hypoplasia congenita, an inherited disorder in humans that is characterized by hypoplasia of the fetal adrenal glands with absence of the definitive zone and persistence of the fetal zone. Dax-1 has been postulated to bind to hairpin loops of the StAR promoter and/or to function as an RNA-binding protein. In mouse, it has been shown that Dax-1 is colocalized with Sf-1 in the developing adrenal and that Sf-1 expression precedes or coincides with expression of Dax-1, suggesting that these two molecules could cooperate in regulating adrenal development. In vitro, Dax-1 inhibited Sf-1-induced stimulation of transcription of the adrenal promoter of StAR, P450c17, Dax-1 itself, possibly through interactions with Sf-1, and transcriptional corepressors such as NcoR and Alien, whereas Sf-1 stimulated expression of the Dax-1 promoter. In vivo, male mice with a single Dax-1-deleted allele (Dax-1$^{-/y}$) have normal zona glomerulosa, but the zona fasciculata is less well developed and shows decreased staining for 3β-HSD and delayed X-zone regression. Dax-1$^{-/y}$ animals have increased stress-induced corticosterone secretion and enhanced adrenal responsiveness to exogenous ACTH stimulation, most probably following increased adrenal P450c21 and ACTH receptor expression. Dax-1$^{-/y}$ animals have normal expression of Sf-1 and expression of Dax-1 is maintained in Sf-1$^{+/-}$ mice. The absence of Dax-1 partially reverses adrenal growth defects in Sf-1$^{+/-}$ mice. However, the precise mechanisms governing the interplay between Sf-1 and Dax-1 in regulating adrenal development and steroidogenesis are not well known. Interestingly, sumoylation of Sf-1 increases its physical interaction with the corepressor Dax-1, resulting in the complete inactivation of the fetal zone-restricted Sf-1 enhancer FAdE which ultimately leads, by a yet unexplained mechanism, to postnatal regression of the fetal remnant cortex, the X-zone (Xing et al., 2017). These data are therefore reminiscent to the converging phenotype—partial retention of the X-zone—shared by Dax-1$^{-/y}$ and sumoylation-deficient Sf-1 mice. In human fetuses, Dax-1 is expressed in the adrenal primordium as early as E33. Between W15 and 21 Dax-1 immunoreactivity has been found mainly in the nucleus in the outer definitive zone and in the cytoplasm in the fetal zone, while the density of Dax-1 positive cells decreased from the internal to the external portion of the gland.

Pbx1

Pbx1, an homeodomain protein, is expressed as soon as E10 in the mouse adrenal primordium. Embryos of *Pbx1*$^{-/-}$ mice lack the adrenal glands. This was linked to a decrease in Sf-1 expression, while that of Wt1 was unaffected. Adult Pbx1 haploinsufficient mice have lighter adrenals together with a reduced level of proliferation and of IGF-I receptor. In addition, it has been demonstrated that Pbx1 stimulates ACTH receptor expression, synergistically with Sf-1. Pbx1 was detected in 7 week gestation human fetal adrenal extracts.

Cited2

Cited2 was first detected in the mouse adrenogonadal primordium at E10, with high levels of expression in the adrenal cortical cells at E13.5 and later. *Cited2*$^{-/-}$ embryos showed a lack of expression of Sf-1 in the adrenal primordium at E10.5 and of Dax-1 and Wnt-4 at later stages, resulting in a lack of adrenal development. Embryos heterozygous for Cited2 and Wt1 have strongly reduced adrenal size at E13.5 with decreased Sf-1 mRNA. Cited2 was detected in 7 week gestation human fetal adrenal extracts.

Other Transcription Factors and Signaling Pathways

Rspo/Wnt/β-Catenin

Wnt (wingless-related mouse mammary tumor virus integration) 4, a member of the Wnt family of developmentally regulated signaling molecules, is involved in the development of the adrenal, kidney, pituitary gland, female reproductive system, and mammary gland. Wnt canonical signaling pathway is transduced to the nucleus by β-catenin. Wnt4 is expressed next to the anterior site of the mouse mesonephros on E11.5 and in the developing adrenal cortex from E12.5 onward. Adrenals of Wnt4 knockout mice are morphologically comparable to those of wild-type animals. However, Wnt4 knockout animals have impaired zona glomerulosa differentiation together with reduced adrenal P450c21 and P450 aldosterone synthase mRNA concentrations and decreased aldosterone production. All the above-mentioned observations strongly suggest that Wnt4 and possibly β-catenin are involved in the establishment of the zonated function of the adrenal. Indeed, β-catenin ablation in Sf-1 expressing cells leads, depending of the amount of affected cells, to adrenocortical aplasia that ranges from definitive zone failure in embryos to progressive cortex thinning in aging animals. Similarly, R-spondin 3 (Rspo3), a signal molecule secreted by capsular cells that modulates β-catenin activity in the underlying steroidogenic compartment, is essential to imprint glomerulosa identity and to maintain organ growth by ensuring replenishment of adult cortex (Vidal et al., 2016). Thus, β-catenin activation (at least responding to Wnt4 and Rspo3 signals) is required for definitive zone emergence during development, proper maintenance of adrenocortical progenitor cells in postnatal cortex and glomerulosa zonation. Moreover, Wnt and PKA signaling pathways play essential and complementary roles in functional zonation of the adrenal cortex (Drelon et al., 2016). Gain- and loss-of-function mouse models show that the Rspo3/Wnt4/β-catenin pathway is a driver of glomerulosa identity, a repressor of fasciculata identity and has tumorigenic activity when constitutively activated. Reciprocally, constitutive activation of cAMP/PKA signaling inhibits glomerulosa fate, triggers fasciculata identity, and counteracts β-catenin-induced adrenocortical tumorigenesis.

Sonic Hedgehog

Hedgehog family proteins are secreted molecules that act upon target cells by binding to multiprotein receptor complexes. Both sonic hedgehog (Shh)-expressing cells and Shh-responsive cells are detected in the mouse embryonic adrenal cortex at E12.5 at the periphery of the gland. In adults, Shh produced by partially differentiated cortical cells acts on non-cortical cells of the overlying capsule. Shh mutants adrenals have a thinner capsule and a disproportionately reduced cortex that is well differentiated with appropriate zonation. Thus it appears that Shh signal is important for capsule and cortex growth, but does not regulate cortical differentiation or zonation. This hypothesis is consistent with the observation that corticosterone levels in Shh mutants are normal at birth but strongly reduced in adult animals.

GATA Proteins

GATA proteins are transcription factors that bind to the consensus sequence (A/T)GATA(A/G) in the promoter and enhancer regions of their target genes. GATA-4 is present in human adrenocortical carcinoma and in a transgenic mouse model developing adrenocortical tumors. GATA-4 and GATA-6 are detectable from E14 and week 19 onward in the mouse and human adrenal cortex, respectively. After birth, GATA-4 expression decreases, whereas GATA-6 continues to be expressed. Studies in chimeric mouse embryos demonstrated that GATA-4 is not essential for early adrenocortical differentiation. In contrast, GATA-6 conditional gene deletion in the adrenal cortex (in Sf-1 expressing cells) leads to severe adrenal insufficiency with a deficit in both zona glomerulosa and fasciculata cells, complete absence of the X-zone and ectopic differentiation of gonadal-like steroidogenic cells (Tevosian et al., 2015). These data indiquate that GATA-6 is essential for adrenocortical cells maintenance in developing and postnatal glands and repression of gonadal fate but its loss does not completely preclude adrenocortical formation and

steroidogenesis since active steroidogenic cells expressing the master regulator Sf-1 remain in the hypoplastic glands and sustain viability.

See also: Adrenal Cortex; Physiology. Adrenal Steroidogenesis

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Corticotropin-Releasing Hormone Peptide Family☆

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Glossary

Corticotropes Cells of the anterior lobe of the pituitary gland, which produce adrenocorticotropic hormone and respond to corticotropin-releasing-hormone.

G protein-coupled receptor (GPCR) A polypeptide receptor with seven transmembrane domains. Ligand binding occurs on the extracellular side of the plasma membrane and results in stimulation of G proteins which modulate intracellular signal transduction pathways.

Homeostasis In an organism, homeostasis is any process that acts to actively regulate/maintain a physiological variable within an acceptable range necessary for survival. Examples include the regulation of body temperature, fluid balance, pH, and blood glucose.

Hypothalamic–pituitary–adrenal axis (HPA) axis A major neuroendocrine system involving the hypothalamus, the pituitary gland, and the adrenal glands. The HPA axis functions as the central stress response system by coordinating central nervous system and endocrine responses to physiological or psychological stress.

Homology In biology, the term homolog indicates shared ancestry between a pair of genes or structures in different taxa. Homologous sequences are orthologous if the genes in different species have evolved from a common gene by speciation. Homologous sequences are paralogous if the genes are related by duplication within the genome of a single species. Orthologs tend to retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.

Limbic system A collection of brain structures, including the hippocampus, hypothalamus, amygdala, and prefrontal cortex, that support various functions including emotion, behaviour, motivation, long-term memory, and olfaction. Afferent neural pathways connecting structures of the limbic system to the paraventricular nucleus of the hypothalamus activate CRH neurons and play an important role in modulating the HPA axis and the stress response.

Corticotropin-Releasing Hormone: Driver of the HPA/I Stress Axis

Biological or physiological stress is commonly defined as any event, whether perceived or real, that can disturb the homeostatic state of an organism. The concept of stress was originally developed by Hans Selye (1907–1982) and described in his treatise, "Stress" (Selye, 1950). It was in this work that he defines the "General Adaptation Syndrome" which describes the physiological response to a stressor (e.g., disease, infection, or injury), and involves modulation of both the nervous and endocrine systems. Whereas Selye's work focused mainly on the chronic aspects of stress, it was Walter Cannon (1871 to 1945) and his studies on epinephrine (adrenaline) that outlined the short-term elements of stress and coined the term "fight or flight response." Together, the work performed by these researchers established the foundation of the stress-associated aspects of the sympathetic nervous system. Psychological responses associated with perceived threats arising from environmental novelty, social interaction, and challenge can also induce stress. The contribution of the psychological aspects of stress was later introduced by Robert Sapolsky, leading to our modern definition of the stress response (Sapolsky, 1994).

In vertebrates, maintenance of homeostasis in response to a stressor includes a multifaceted response involving the endocrine, nervous, and immune systems (Chrousos and Gold, 1992; Carrasco and Van de Kar, 2003; Haddad *et al.*, 2002). The primary mediator of the stress response is the hypothalamus–pituitary–adrenal/inter-renal (HPA/I) axis. During periods of stress, the physiological function of the HPA/I axis is to divert energy production to where it is needed most. Typically, this involves increasing sympathetic nervous system activity necessary for defense and survival (e.g., increased cardiovascular activity, respiratory rate, and intermediate metabolism), and reducing maintenance activities associated with the parasympathetic nervous system (e.g., feeding, growth, and development) (Smith and Vale, 2006). The principle anatomical structures that mediate the stress response are the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal gland. In response to a challenge, corticotropin-releasing hormone (CRH) is secreted from parvocellular neuroendocrine cells of the PVN into the hypophyseal portal system, the primary blood supply of the anterior pituitary gland. CRH stimulates corticotropes in the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH) into the bloodstream. In addition to CRH, a number of other factors that modulate ACTH release are also secreted in response to a stress challenge (e.g., arginine vasopressin). The primary target of circulating ACTH is the adrenal cortex, where cells in the zona fasciculata layer are stimulated to synthesize and

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secrete glucocorticoids. Glucocorticoids, such as cortisol in humans (corticosterone in rats, birds, and reptiles), are the downstream effectors of the HPA/I axis and regulate physiological changes throughout the body, primarily through an enhancement of energy metabolism. The HPA/I axis is a negative feedback cycle in which circulating glucocorticoids act back on the hypothalamus and pituitary to suppress CRH and ACTH production (**Fig. 1**).

The evolutionary development of the HPA/I axis in vertebrates has had a major impact on their physiology. It integrated the neural actions of CRH with the glucocorticoid-synthesizing tissues of the periphery by linking a large and mobility-constrained neuropeptide (CRH) with small lipophilic hormones (glucocorticoids) that could traverse all tissues. This interaction allowed the organism's stress-response metabolism to be placed under the control of a single neuropeptide. Additionally, CRH is controlled by both sensory and associative inputs, allowing the HPA/I axis to act as a key stress-perceiving and integrating unit. As a result, the evolution of the HPA/I axis allowed organisms to anticipate a future stressor and to mount a metabolically integrated response to the stimulus.

Discovery of the CRH Family of Peptides

CRH was first isolated from purified sheep hypothalami by Wylie Vale (1941–2012) and colleagues in 1981. It was determined to be 41 amino acid residues in length and capable of stimulating ACTH secretion from cultured anterior pituitary cells (Vale *et al.*, 1982). The discovery of CRH occurred following decades of previous research that suggested the existence of a peptide hormone capable of regulating the release of ACTH from the pituitary gland in vertebrates. Indeed, the concept of "releasing factors" produced in the brain that can regulate pituitary hormone activity can be traced as far back as the early 20th century (see Lovejoy, 2005 for discussion). Together, this lead to the proposal by Harris (1948) that there is a physiological system in which chemical factors from the hypothalamus are released into the hypophyseal portal blood vessels to stimulate the release of pituitary hormones. This gave rise to a further postulation that each pituitary hormone was controlled by a single hypothalamic releasing factor. Attempts to purify the putative ACTH-releasing factor were first independently described by Schally and Saffran (1955) and Guillemin and Rosenberg (1955); however, its low concentration in tissues made it difficult to attain sufficient amounts to solve its structure.

Prior to solving the structure of CRH multiple other studies reported CRH-like peptides in non-mammalian species. In Montecucchi *et al.* (1979) and associates identified the structure of sauvagine (SVG), a 40-amino acid peptide isolated from the skin of a neotropical leaf frog (*Phyllomedusa sauvagei*). The ability of SVG to induce the release of ACTH from the pituitary gland led

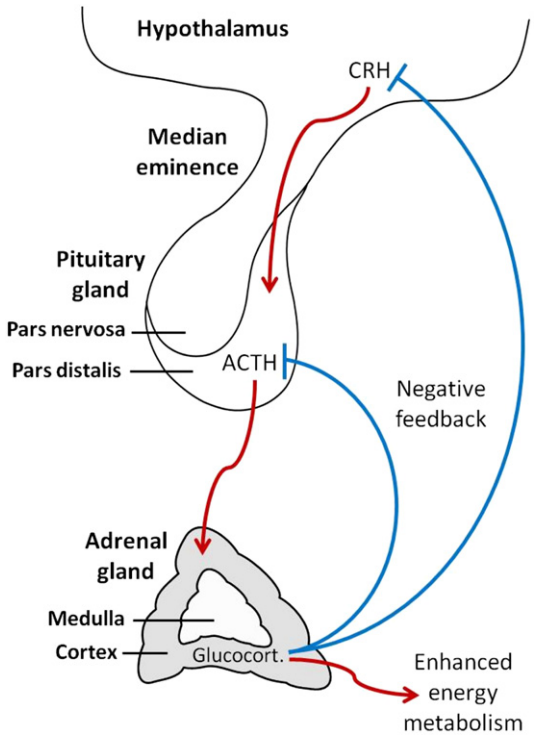

Fig. 1 Mammalian hypothalamic–pituitary–adrenal axis. In mammals, CRH is secreted from the hypothalamus to the median eminence, where it travels via the hypophyseal portal system into the pars distalis of the pituitary gland. In the pituitary gland, CRH stimulates the release of ACTH into the bloodstream. ACTH then acts in the cortex of the adrenal gland to induce production and secretion of glucocorticoids (Glucocort.), which enable enhanced energy metabolism under stress conditions and negatively feedback on CRH and ACTH to reduce their production and release.

to the speculation that SVG was structurally related to the elusive ACTH-releasing factor (Erspamer and Melchiorri, 1980). Following the discovery of CRH, Lederis and colleagues isolated a 41-amino acid peptide from the neurosecretory organ (urophysis) of the white sucker fish (*Catostomus commersoni*) with sequence similarity to CRH (Lederis *et al.*, 1982). They named this neuropeptide urotensin-I (UI; termed "urocortin" (Ucn) in mammals), after its tissue of origin. Both SVG and UI were found to stimulate ACTH release from the rat pituitary gland, although their pharmacological profiles differed from that of CRH (see Lovejoy and Balment, 1999; Lovejoy, 2009 for reviews).

These studies indicated that CRH-like peptides were found in a variety of vertebrate species; however, how they were functionally and genomically related remained unclear at the time. In 1989, the structure of the first insect CRH-like peptide, diuretic hormone (DH), was reported by Kataoka *et al.*, and since then, many CRH-related peptides have been reported in insects (Coast, 1996, 1998; Lovejoy and Jahan, 2006; Cardoso *et al.*, 2014). These additional sequences allowed Vale and his colleagues to determine the structures of two additional CRH-like peptides, which they termed urocortin 2 (Ucn2) and urocortin 3 (Ucn3) (Reyes *et al.*, 2001; Lewis *et al.*, 2001). Simultaneously, Hsu and Hsueh (2001) reported the structure of the same peptides, which they called "stresscopin" and "stresscopin-related peptide." Since these publications, numerous homologous structures have been identified in vertebrates demonstrating that these peptides were physiologically essential to the survival of early vertebrates and, therefore, retained in the genome throughout vertebrate evolution (Cardoso *et al.*, 2014; Endsin *et al.*, 2017).

Expression of CRH and CRH-Like Peptides

In CRH expressing neurons, the CRH gene is first translated into a prepro-CRH peptide and is subsequently cleaved in the rough endoplasmic reticulum to generate a pro-CRH peptide (Itoi *et al.*, 1998; Perone *et al.*, 1998). The pro-CRH peptide is then processed within the trans-Golgi-network and secretory granules to generate the 41 amino acid mature CRH peptide (Itoi *et al.*, 1998; Perone *et al.*, 1998). CRH production is stimulated by a number of different signals including: neurotransmitters: norepineprine, epinephrine serotonin, histamine, and acetylcholine; neuropeptides: arginine vasopressin, angiotensin II, neuropeptide Y, cholecystokinin, activin, and enkephalin; the cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α; and leptin (see Aguilera and Lui, 2012 for review). Transcriptional regulation of the CRH gene involves the cyclic AMP (cAMP)/protein kinase A (PKA)-dependent pathway leading to downstream recruitment of the phosphorylated cAMP response element binding protein (pCREB) by the cAMP response element (CRE) of the CRH promoter (Seasholtz *et al.*, 1988). In addition, CRH transcription requires cAMP-dependent translocation of the CREB co-activator, transducer of regulated CREB activity (TORC; see Aguilera and Lui, 2012 for review). Negative regulation of CRH production is primarily by glucocorticoids, but also by estrogens, and γ-aminobutyric acid (GABA) (Itoi *et al.*, 1998; Orth, 1992; Owens and Nemeroff, 1991; Stephanou *et al.*, 1997; Torpy and Chrousos, 1996). Secretion of CRH is also inhibited by dynorphin, substance P, somatostatin, and galanin (Itoi *et al.*, 1998).

CRH expressing neurons are dispersed throughout the CNS (**Fig. 2**A), with the highest concentration found in the PVN (Swanson *et al.*, 1983). Within the PVN there are three areas that have CRH expressing neurons which project to different parts of the brain. In the anterior and medial-dorsal PVN, axons of parvocellular CRH neurons project to hypophyseal portal capillaries and modulate the stress response. Magnocellular oxytocinergic neurons from the dorsolateral aspect of the PVN have axons that project to the posterior pituitary and release CRH into the peripheral circulation. CRH expressing neurons are also located in the dorsal, medial-ventral and lateral parvicellular areas of the PVN, and they project to the brainstem and spinal cord and regulate the sympathoadrenal system (see Aguilera and Lui, 2012 for review). CRH is also present in the limbic brain region as well as other structures involved in the stress response, including the bed nucleus of the stria terminalis, the central nucleus of the amygdala, locus coeruleus, cerebral cortex, cerebellum, and dorsal root neurons of the spinal cord (Makino *et al.*, 1995). Interestingly, limbic dysfunction and dysregulation of the HPA/I axis are common features of affective disorders, indicating that CRH neurons from these brain regions are involved in these disease states (Herman *et al.*, 2005). CRH expressing neurons are also located in other cells of neural origin including chromaffin cells of the adrenal medulla and sympathetic ganglia of the autonomic nervous system. Immune cells, skin, and cells in the gastrointestinal tract also express CRH (Aguilera and Lui, 2012).

In the mammalian brain, expression of Ucn is concentrated in the Edinger–Westphal nucleus (EW), which appears to be the only brain site where Ucn expression is conserved across species (Vasconcelos *et al.*, 2003). Ucn is also expressed in the lateral superior olivary and supraoptic nuclei, with lower expression levels in cranial nerves, spinal motoneurons and neurons in the forebrain (Bittencourt *et al.*, 1999; **Fig. 2**A). Projections from these neurons input into the PVN, cerebellum, brainstem, and spinal cord indicating that the Ucn system is involved in control of autonomic, endocrine, and sensorimotor functions. In rodents, Ucn2 mRNA expression is primarily in subcortical brain regions, with high expression in the paraventricular, supraoptic, and arcuate nuclei of the hypothalamus, and the locus coeruleus of the rostral pons. In addition, Ucn2 mRNA expression is localized to motor nuclei of the brainstem and the spinal ventral horn (Reyes *et al.*, 2001). Ucn3 expressing neurons are primarily located in the hypothalamus and amygdala, with some expression in the brainstem (Li *et al.*, 2002; **Fig. 2**A).

Evolution of CRH Family of Peptides

The ancestral peptide to the CRH family is not known; however, there is a growing body of evidence which provides clues to its origin. CRH and related peptides exist in a variety of vertebrate and invertebrate species, which indicates that the ancestral

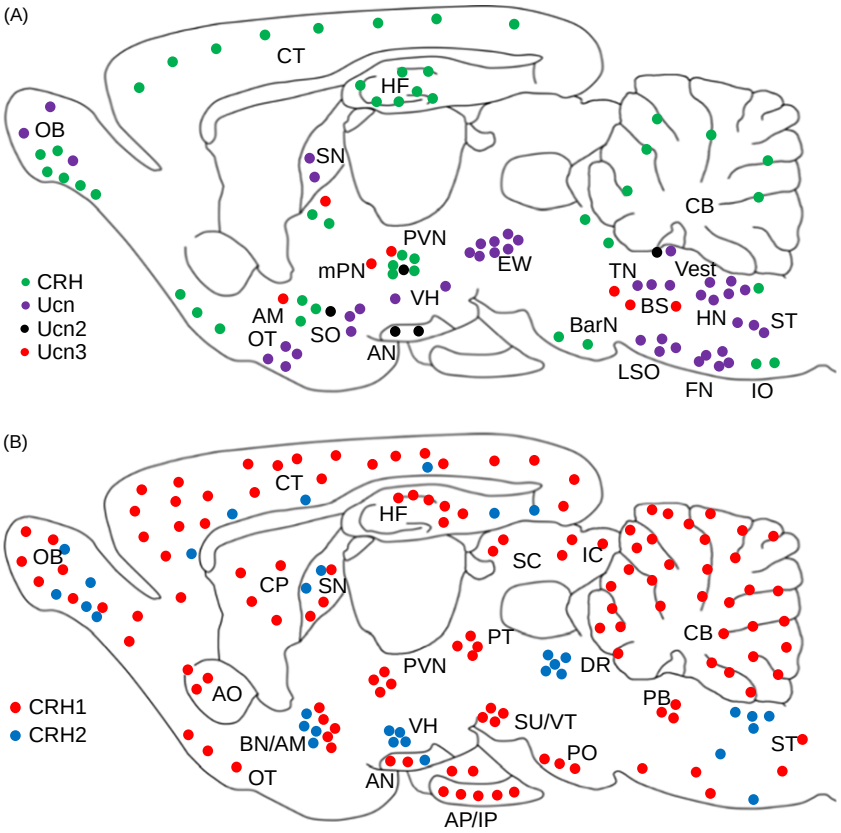

Fig. 2 Neuroanatomical localization of CRH receptors and ligand family member mRNA in rat brain. (A) Schematic of a sagittal section through the rat brain summarizing regions where cell bodies express CRH, Ucn, Ucn2, and Ucn3 mRNA. (B) Schematic of a sagittal section through the rat brain summarizing regions of CRH1 and CRH2 localization. Abbreviations: *AM*, amygdala; *AN*, arcuate nucleus; *AO*, accessory olfactory bulb; *AP*, anterior pituitary gland; *BarN*, Barrington's nucleus; *BN*, bed nucleus; *BS*, brain stem; *CB*, cerebellum; *CP*, caudate putamen; *CT*, cortex; *DR*, dorsal raphe nucleus; *EW*, Edinger–Westphal nucleus; *FN*, facial nucleus; *HF*, hippocampal formation; *HN*, hypoglossal nucleus; *IC*, inferior colliculus; *IO*, inferior olivary complex; *IP*, intermediate lobe of the pituitary gland; *LSO*, lateral superior olivary nucleus; *mPN*, medial preoptic nucleus; *OB*, olfactory bulb; *OT*, optic tract; *PB*, parabrachial nucleus; *PVN*, paraventricular nucleus; *PO*, pontine nuclei; *PT*, pretectum; *SC*, superior colliculus; *SN*, septal nucleus; *SO*, supraoptic nucleus; *ST*, nucleus of the solitary tract; *SU*, substantia nigra; *TN*, motor nucleus of trigeminal nerve; *Vest*, vestibular nuclei; *VH*, ventromedial hypothalamus; *VT*, ventral tegmental area. (A) Figure constructed from Itoi, K., Seaholtz, A. F. and Waton, S. J. (1998). Cellular and extracellular regulatory mechanisms of hypothalamic corticotropin-releasing hormone neurons. *Endocrine Journal* **45**, 13–33; Yan, X., Toth, Z., Schultz, L. et al. (1998). Corticotropin-releasing hormone (CRH)-containing neurons in the immature rat hippocampal formation: Light and electron microscopic features and colocalization with glutamate decarboxylase and parvalbumin. *Hippocampus* **8**, 231–243; Bittencourt, J. C., Vaughan, J., Arias, C. *et al.* (1999). Urocortin expression in rat brain: Evidence against a pervasive relationship of Urocortin-containing projections with targets bearing type 2 CRF receptors. *Journal of Comparative Neurology* **415,** 285–312; Reyes, T. M., Lewis, K., Perrin, M. H., *et al.* (2001). Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proceedings of the National Academy of Sciences* **98,** 2843–2848; Lewis, K., Li, C., Perrin, M. H., *et al.* (2001). Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proceedings of the National Academy of Sciences* **98,** 7570–7575; Cavalcante, J. C., Sita, L. V., Mascaro, et al. (2006). Distribution of urocortin 3 neurons innervating the ventral premammillary nucleus in the rat brain. *Brain Research* **1089,** 116–125. (B) Figure constructed from Lovejoy, D. A., Chang, B. S., Lovejoy, N. R. and del Castillo, J. (2014). Molecular evolution of GPCRs: CRH/CRH receptors. *Journal of molecular endocrinology* **52**, T43–T60.

peptide to the CRH family evolved early in animal evolution, likely between 400 and 500 million years ago. A possible progenitor to the CRH family is the evolutionarily ancient teneurin C-terminal associated peptide (TCAP) family which has structural similarities to CRH, as well as the closest known resemblance in terms of amino acid sequence (Lovejoy *et al.*, 2006; Lovejoy and De Lannoy, 2013). In addition, both CRH and TCAP bind and activate G protein-coupled receptors (GPCRs) to induce their physiological effects. The cognate receptor of the CRH family is the class B/Secretin-like receptor family of GCPRs that also includes glucagon, calcitonin, parathyroid hormone, vasoactive intestinal peptide, and growth hormone releasing hormone receptors. The TCAP and the protein they are derived from, the teneurins, bind to a member of the adhesion class of GPCRs, and play a role in regulation of energy metabolism. Evidence indicates that the secretin family of GPCRs evolved directly from the adhesion GPCRs (Schiöth *et al.*, 2010; Fredriksson *et al.*, 2003), providing an additional evolutionary link between these the CRH and TCAP peptides systems. Furthermore, the adhesion GPCR latrophilin 1 (ADGRL1), a cognate receptor for the teneurins (Silva *et al.*, 2011), was identified based on its ability to bind the black widow spider toxin,

α-latrotoxin, another peptide with structural similarity to the secretin family of peptides (Holz and Habener, 1998). Together this indicates that the earliest ancestor of CRH peptides may have evolved near the base of metazoan evolution and subsequently became essential in the regulation of energy metabolism.

In vertebrates, the CRH family of peptides is composed of four paralogous peptides with two distinct lineages; the CRH and UI (Ucn in mammals and SVG in frogs) lineage, and the Ucn2 and Ucn3 lineage. This agrees with Ohno's (1970) 2R hypothesis which proposes that there were two rounds of genomic duplications at the base of chordate evolution, resulting from a single CRH precursor gene inherited from a protochordate ancestor and leading to the four homologues found in most chordates (Lundin, 1993; Holland and Garcia-Fernandez, 1996). During the first duplication event in chordates, a CRH-like and a Ucn3 peptide were first formed, as evidenced by the presence of both CRH and UI in lampreys (Endsin *et al.*, 2017) and cartilaginous fish (Nock *et al.*, 2011) as well the absence of Ucn2 in lampreys. Then, during the second genome duplication event, the CRH-like peptide split into both CRH and UI (Lovejoy, 2009; Lovejoy and De Lannoy, 2013) while the Ucn3-like gene was unaffected. Subsequently, possibly just before the emergence of the cartilaginous fishes, the Ucn3-like gene split into Ucn2 and Ucn3 as known in extant species (Cardoso *et al.*, 2014; Endsin *et al.*, 2017). Prior to the appearance of the cartilaginous fishes the CRH gene also split into two forms as evidenced by two CRH genes in cartilaginous fish and some teleosts (Nock *et al.*, 2011; Hosono *et al.*, 2015).

Early metazoans had a less complex genome and physiology, and extracellular signaling systems were primarily associated with the coordination of energy metabolism to organismal survival with respect to feeding, digestion, diuresis, and defense. Indeed, in studies conducted primarily in insect models, where generally only a single CRH-like peptide (DH) is present, the earliest function of the peptide family is associated with diuresis and feeding (Kataoka *et al.*, 1989; Audsley *et al.*, 1997; Coast, 1998, 2007). Given the phylogenetic age of the CRH peptides, this is not surprising. Therefore, the formation of the CRH-like peptides in ancestral metazoans may have been selected and conserved through evolution because they acted to regulate cellular and organismal energy acquisition and production for defense against environmental stressors. This has come to be known as the organismal stress response that acts to protect the organism from external and internal environmental challenges.

Mechanisms of CRH Action: Receptors, Ligands and Signaling Pathways

In vertebrates, CRH binds to and activates two distinct receptor isoforms: the CRH receptor 1 (CRH_1) and CRH receptor 2 (CRH_2). It should be noted that the International Union of Basic and Clinical Pharmacology (IUPHAR) has promoted the use of the nomenclature corticotropin-releasing factor 1 (CRF_1) and CRF_2 (Hauger *et al.*, 2003). Both receptors are seven-transmembrane domain GPCRs that, upon ligand binding, activate intracellular signaling cascades. Pharmacological studies of CRH homologues have determined that receptor activation occurs through a two-part process: (1) the C-termini of the CRH family of ligands binds to the extracellular binding pocket of the CRH receptor, and (2) the N-termini interact with the other extracellular loops of the transmembrane domain to activate the intracellular signal cascade (Perrin and Vale, 2002; Grace *et al.*, 2004; Hoare *et al.*, 2004).

In 1993, Vale's team cloned the first CRH receptor, CRH_1, using an expression library from a human corticotropic tumor (Chen *et al.*, 1993). The receptor was reported to be a 415-amino acid protein with seven putative membrane-spanning domains. Further studies determined that the CRH_1 receptor has significant structural variability, differing from 415 to 446 residues depending on the species: human, (Chen *et al.*, 1993; Vita *et al.*, 1993), mouse (Vita *et al.*, 1993), rat (Chang *et al.*, 1993; Perrin *et al.*, 1993), chicken (Yu *et al.*, 1996), frog (Dautzenberg *et al.*, 1997), tree shrew (Palchaudhuri *et al.*, 1998), sheep (Myers *et al.*, 1998), and fish (Arai *et al.*, 2001; Pohl *et al.*, 2001). Binding and functional studies have determined that CRH_1 exhibits a distinct ligand-selective profile, having the highest binding affinity for CRH and Ucn, as well as the Ucn orthologues orthologues UI and SVG

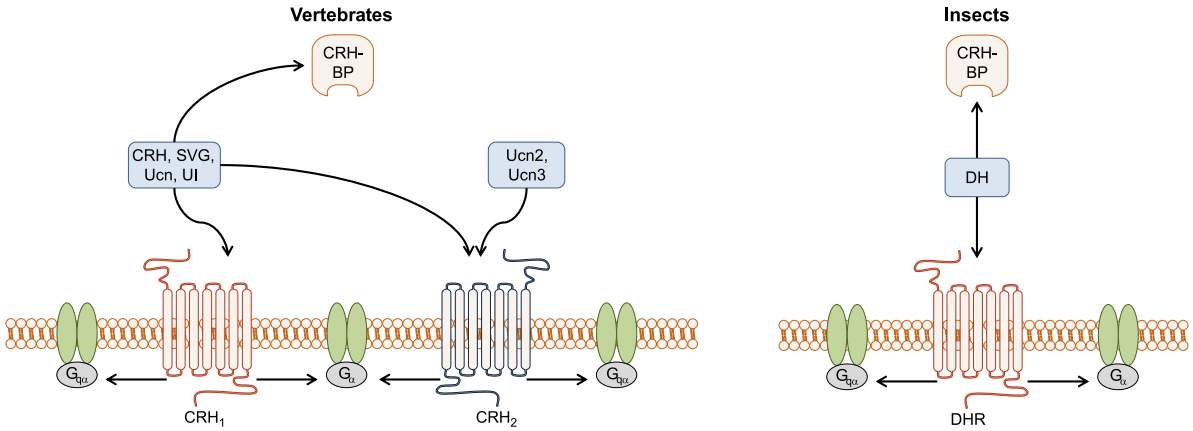

Fig. 3 Scheme of the molecular interaction among CRH ligands, receptors and the binding protein in chordates and insects. See text for discussion.

(Donaldson *et al.*, 1996; Dautzenberg *et al.*, 1997, 2001; Perrin *et al.*, 1999). It has little affinity for Ucn2 or Ucn3 (Hsu and Hsueh, 2001; Lewis *et al.*, 2001; Reyes *et al.*, 2001; **Fig. 3**).

Identification of CRH_2 was reported shortly after the discovery of CRH_1. The two receptors are encoded by different genes and orthologues of these two receptors are about 70%–80% identical to each other at the amino acid level (see Bonfiglio *et al.*, 2011; Lovejoy *et al.*, 2014 for reviews). Structural characterization of CRH_2 has been reported in the rat (Lovenberg *et al.*, 1995) mouse (Kishimoto *et al.*, 1995; Perrin *et al.*, 1995; Stenzel *et al.*, 1995), and human (Liaw *et al.*, 1996). It has a higher binding affinity for UI/Ucn-like peptides than for CRH, and binds to all vertebrate paralogues as well as several synthetic CRH peptide variants (Tellam *et al.*, 2002; Hauger *et al.*, 2006). A key reason for the difference in ligand specificity is that the two receptors exhibit significant differences in their ligand binding sites, having only about 47% sequence similarity (Grammatopoulos, 2012).

An additional component of the CRH system is the secreted CRH-binding protein (CRH-BP). First isolated from human blood plasma, CRH-BP is a 37-kDa N-linked glycoprotein that is structurally unrelated to the CRH receptors and exhibits a high degree of sequence similarity among species. CRH-BP binds both CRH and Ucn with high affinity, as well as other CRH orthologues (Behan *et al.*, 1989; Valverde *et al.*, 2001; Sutton *et al.*, 1995). Orthologues of CRH-BP have been identified in each of the major vertebrate classes, with the exception of Chondrichthyes (cartilaginous fish) and are well established in the insect lineages (Seasholtz *et al.*, 2002; Huising and Flik, 2005). This degree of conservation indicates that CRH-BP evolved early in the evolution of CRH/DH ligand–receptor systems and has become an integral part of the CRH and DH physiology. The role of the binding protein is unclear; however, CRH-BPs have comparable binding affinities to CRH receptors (Sutton *et al.*, 1995) indicating that one function might be to modulate the bioavailability of CRH-like peptides. Generally, the CRH-BP appears to act as a negative regulator of CRH activity (Stinnett *et al.*, 2015).

Similar to other class B/secretin-like GPCRs, CRH receptors have a highly glycosylated external domain and a long N-terminal domain (Attwood and Findlay, 1994; Harmar, 2001; Fredricksson *et al.*, 2003). The receptors in this family are commonly associated with functioning in the regulation of ion and nutrient transport and being involved in the cellular stress response (Harmar, 2001; Fredricksson *et al.*, 2003). The primary signal transduction pathway activated by CRH and the Ucns involve coupling of CRH receptors to the G_s (stimulatory) protein subunit, resulting in activation of the adenylate cyclase-PKA pathway (Chen *et al.*, 1993; Olianas *et al.*, 1995; Hauger *et al.*, 2006) which phosphorylates downstream cytosolic and nuclear targets. There is also substantial evidence of CRH-mediated activation of G_q proteins and other G proteins to stimulate inositol triphosphate (IP_3)- and Ca^{2+}-mediated signal transduction pathways (Hauger *et al.*, 2006; Gutknecht *et al.*, 2010), indicating a capacity for regulation of neuronal excitability and cell–cell communication. Furthermore, CRH receptors have been demonstrated to activate multiple G-alpha (Gα) subunits, including G_s, Go_o, $G_{q/11}$, $Gi_{1/2}$, and G_z (Grammatopoulos *et al.*, 2001).

In general, the action of CRH is associated with cellular and organismal homeostasis with respect to regulation of energy metabolism and the related diuretic requirements of an animal in response to physiological and environmental stress (Lovejoy, 2012; Janssen and Kozicz, 2013; Lovejoy and De Lannoy, 2013). Fundamentally, CRH and the CRH receptor system are associated with activation of the sympathetic arousal system (e.g., increased heart rate and glycogenolysis) and inhibition of the parasympathetic system (e.g., growth, feeding and digestion) in vertebrates. Both of the receptors are expressed in a variety of tissues depending on species and taxa, although a full profile is currently incomplete. CRH_1 is predominantly expressed in the central nervous system (CNS) with high expression in the cortex, cerebellum, hippocampus, amygdala, olfactory bulb, pituitary gland, and spinal cord (Potter *et al.*, 1994; Chalmers *et al.*, 1996; Palchaudhuri *et al.*, 1998; Van Pett *et al.*, 2000; **Fig. 2**B). Due to CRH_1 expression, CRH-mediated signaling also influences several neurotransmitter systems within the CNS, including glutamatergic neurons of cortex and hippocampus, GABAergic cells in the reticular thalamic nucleus, globus pallidus and septum, dopaminergic neurons of the substantia nigra pars compacta and ventral tegmental area, and serotonin-containing (5-HT) neurons of the dorsal and medial raphe nuclei (Refojo *et al.*, 2011). CRH_1 is also found at low levels in peripheral tissues, including the female reproductive tract (Nappi and Rivest, 1995; Kiapekou *et al.*, 2011; Wypior *et al.*, 2011), skin (Slominski *et al.*, 1995, 2001), adrenal gland (Willenberg *et al.*, 2005; Squillacioti *et al.*, 2011) and gastrointestinal (GI) tract (Chatzaki *et al.*, 2004).

CRH_2 is likewise found in many regions of the brain, however its expression is more limited compared to that of CRH_1 (Lovenberg *et al.*, 1995; Perrin *et al.*, 1995; Van Pett *et al.*, 2000). CRH_2 receptors are typically expressed in subcortical regions such as the ventromedial hypothalamus, dorsal raphe nuclei of the midbrain, nucleus of the solitary tract in the hindbrain and various hindbrain nuclei (Bittencourt *et al.*, 1999; **Fig. 2**B). Relative to CRH_1, CRH_2 has lower expression in the cortex; however, it is also found in regions that express CRH1 as well, such as the septal nuclei. In the periphery, CRH_2 is expressed in the heart (Kishimoto *et al.*, 1995; Lovenberg *et al.*, 1995; Perrin *et al.*, 1995; Stenzel *et al.*, 1995), lung (Lovenberg *et al.*, 1995), GI tract (Perrin *et al.*, 1995; Chatzaki *et al.*, 2004), skeletal muscle (Kishimoto *et al.*, 1995), male reproductive system (Perrin *et al.*, 1995), and adrenal gland (Müller *et al.*, 2001).

Dysfunction of the CRH System: Neuropathological Actions of CRH Peptides

The CRH system integrates autonomic and behavioral responses to stress through interactions primarily at the level of the CNS. Therefore, in vertebrates, CRH and Ucn induce organismal responses to stress and environmental novelty such as elevated physiological arousal, defense actions, decreased food intake and reduced reproductive activity. These responses are achieved by CRH_1-mediated activation of the HPA/I axis and sympathetic branches of the stress response. In contrast, CRH_2 has been associated with anxiolytic effects, thus Ucn2 and 3 have been considered anxiolytic peptides as they bind exclusively to this

receptor (Hauger *et al.*, 2006; Ohata and Shibasaki, 2004). Together, the actions of all four peptides and their receptors provide a balanced regulation of the stress response under most situations. Dysregulation of this balance is associated with several human neuropsychiatric conditions.

The CRH system has been implicated in particular with the onset of affective disorders. Affective disorders may be loosely defined as those conditions that affect the mood and well-being of patients. These conditions generally include major depression and anxiety, panic disorder, post-traumatic stress disorder, anorexia nervosa and possibly aspects of bipolar disorder. Conditions of chronic stress can result in hypersecretion of CRH causing the initially beneficial effects of CRH to induce anxiety- and depression-like behaviour (Landgraf, 2006). This is outlined in the CRH hypothesis of depression, which posits that stress-induced increases in secretion of CRH, activation of the HPA axis, and the resulting elevation in glucocorticoid levels are associated with mood and anxiety disorders. Patients with depression have a significantly higher number of CRH-expressing neurons, and higher CRH mRNA levels in neurons in the PVN compared to those without depression (Raadsheer *et al.*, 1994, 1995; Wang *et al.*, 2008). Furthermore, symptoms of depression, including decreased appetite, decreased sexual interest, disrupted sleep, and anxiety, can all be reproduced in experimental animals by the intracerebroventricular injection of CRH (Holsboer, 2001). Conversely, these symptoms can be reduced by CRH_1 receptor antagonists or inhibition of receptor expression. (Lu *et al.*, 2008a,b; Holsboer and Ising, 2008). Additionally, antidepressants have been found to decrease CRH synthesis and the upregulation of glucocorticoid receptor expression, making the HPA system more responsive to feedback inhibition by cortisol (Barden, 1996). CRH levels are also increased in the cerebrospinal fluid (CSF) of depressed patients (Nemeroff *et al.*, 1984), and treatment with antidepressants decreases CRH levels in both healthy volunteers and depressed patients (Heuser *et al.*, 1998).

CRH and the HPA axis are associated with addiction and are activated by drugs of abuse, including opioids, cocaine, alcohol, and nicotine. These drugs activate the HPA axis both when they are first taken and again during acute withdrawal. Repeated cycles of drug taking and withdrawal desensitize the HPA axis, but the consequent exposure to high levels of glucocorticoids can sensitize the CRH system in the amygdala (Imaki *et al.*, 1991; Makino *et al.*, 1994; Swanson and Simmons, 1989). Sensitization of CRH system in the amygdala leads to the withdrawal stress response associated with drug addiction (reviewed in Koob, 2010). Currently, CRH receptor antagonists are being evaluated for use in the clinical treatments of addiction (reviewed in Grammatopoulos and Chrousos, 2002; Zorrilla *et al.*, 2014).

Due to its influence over so many physiological systems, dysregulation of CRH signaling and the HPA axis has also been implicated in anorexia and bulimia nervosa (Laessle *et al.*, 1992; Licinio *et al.*, 1996; Birketvedt *et al.*, 2006, Bailer and Kaye, 2003; Casper, 2006), obesity (reviewed in Arora, 2006; Valassi *et al.*, 2008), insomnia and obstructive sleep apnea (reviewed in Buckley and Schatzberg, 2005), and neurodegenerative disorders such as Alzheimer's disease (Dautzenberg and Hauger, 2002; Dunn and Berridge, 1990; Nemeroff and Owens, 2002). The origin of these pathologies with respect to CRH over- or under-secretion is not understood but appears to involve both genetic and environmental factors that lead to changes in peptide, receptor and binding protein regulation, as well as changes in neurosynaptic regulation and the neurotransmitters that regulate the CRH system.

Summary

CRH is the principle neuropeptide responsible for activation of the organismal stress response in vertebrates. CRH had its evolutionary origins relatively early in the ancestry of animals, and its role became associated with the regulation of the HPA/I axis in the vertebrates. Along with its paralogous peptides, the Ucns, this family is essential for the regulation of the stress response. Dysregulation of this peptide family along with their cognate receptors and binding protein has been implicated in the development of some neuropsychiatric disorders and further elucidation of the physiological functions played by CRH will aid in the development of therapeutics for treating these diseases.

See also: Regulation of POMC and ACTH Secretion. Glucocorticoids and Immunity

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Regulation of POMC and ACTH Secretion

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Tissue Specific Expression of POMC

The pituitary gland comprises the posterior pituitary lobe (~ 1/3 of total gland) that stores and releases arginine vasopressin (AVP, also known as antidiuretic hormone ADH) and oxytocin. These hormones are synthesized in the hypothalamic neurosecretory cells of the paraventricular and supraoptic nucleii and travel downward along axon endings that project into the posterior pituitary lobe. When stimulated by secretagogues, AVP and oxytocin are released into the bloodstream, where they maintain water balance and regulate parturition and breast milk release respectively. The anterior pituitary comprises the remaining 2/3 of the pituitary and contains five distinct hormone-secreting cell types, namely corticotrophs, lactotrophs, somatotrophs, thyrotrophs and gonadotrophs (Nussey and Whitehead, 2001). POMC is predominantly expressed in corticotrophs and melanotrophs of the anterior and intermediate pituitary lobes, and in a few thousand neurons of the hypothalamus (Drouin, 2016). This distinct cell-restricted POMC gene expression is precisely controlled by spatiotemporal coordination of a set of signaling molecules and transcription factors during embryogenesis (Nudi *et al.*, 2005).

Pituitary Organogenesis

In vertebrates, the anterior and the intermediate pituitary lobes (the adenohypophysis) originate from the nonneural surface ectoderm, whereas the posterior pituitary lobe (neurohypophysis) develops from the anterior neural plate (**Fig. 1**A) (Suga *et al.*, 2011). In birds and mammals, the anterior pituitary primordium forms as an invagination of the stomodeal ectoderm (oral ectoderm) at the roof of the oral cavity, which develops initially into Rathke's pouch (**Fig. 1**B) (Zhu *et al.*, 2007). Simultaneously, a part of the adjacent ventral diencephalon, which originates from the neural plate and is destined to become the hypothalamus and the posterior lobe of pituitary, outgrows to form the infundibulum in close proximity to Rathke's pouch (**Fig. 1**B) (Zhu *et al.*, 2007). Thereafter, directed by local growth factors and morphogens, such as Sonic Hedgehog (SHH), Fibroblast Growth Factors (FGFs), Bone Morphogenetic Proteins (BMPs), and Wingless-Type MMTV Integration Site (WNTs) factors, Rathke's pouch thickens and elongates ventrally. The rapid proliferation inside the cranial region of Rathke's pouch gives rise to the progenitors of the hormone-secreting cell types, and together with emerging vascularized mesenchymal cells, the rostral region of Rathke's pouch develops into the anterior pituitary lobe, or the pars distalis. The dorsal part of Rathke's pouch remains less expanded and forms the intermediate pituitary lobe, or pars intermedia (Scully and Rosenfeld, 2002), and following this, Rathke's pouch degenerates to a rudimentary structure and separates from the ventral pharyngeal epithelium (Skowronska-Krawczyk *et al.*, 2016). The continuous outgrowth of the infundibulum leads to the development of the posterior lobe of the pituitary gland (**Fig. 1**C).

Several notochord-derived ectoderm signals are essential for head and pituitary development during early embryogenesis (Cleaver and Krieg, 2001). Surgical disruption of the notochord or deletion of the SHH gene, the major notochord secreted factor, results in malformation of midline structures, cyclopia, and absence of Rathke's pouch (Chiang *et al.*, 1996). Gli1 and Gli2 are the main downstream zinc finger transcription factors of the SHH pathway. Genetic deletion of both Gli1 and Gli2 results in a general defect of midline structures, including complete absence of the pituitary gland (Park *et al.*, 2000). Distinct from SHH's general effects on early embryogenesis, FGF8, and FGF10 have also been shown to play important roles in pituitary development. Deletion of FGF10 or FGF receptor 2 IIIb in mice causes anterior pituitary agenesis (Ohuchi *et al.*, 2000). Deletion of FGF8 is embryonic lethal, and overexpression of FGF8 under the control of αGSU regulatory element led to pituitary hyperplasia with dramatic expansion of the POMC-producing cell lineage accompanied by ectopic Lhx-3 induction (Treier *et al.*, 1998). Interestingly, BMP2 signaling appears to have an opposing action to FGF8 in Rathke's pouch. BMP2 induces expression of Isl-1, a LIM homeodomain transcription factor and αGSU, but suppresses ACTH expression (Ericson *et al.*, 1998). BMP4 signaling is critical for progression of pituitary development, and BMP4 overexpression leads to mild anterior pituitary hyperplasia (Ericson *et al.*, 1998). Notch and WNT signaling are part of an evolutionary conserved mechanism that regulates the maintenance of stem cell fate and differentiation. During early embryogenesis, transient Notch and WNT expression activate the downstream stem cell transcription factors, Hesx1 and β-catenin, which sequentially turn on the pituitary lineage specific transcription factors Prop1 and Pit1, leading to pituitary growth and differentiation into specific pituitary cell subtypes. Prolonged activation of Notch2 blocks terminal differentiation of pituitary cells, such as thyrotrophs and gonadotrophs (Raetzman *et al.*, 2006), emphasizing the role these signals play in both a temporal and spatial manner to direct distinct transcription factor-mediated specific cell lineages (**Fig. 1**D).

Regulators of POMC Lineage Commitment in Pituitary

POMC expressing corticotrophs are the first terminal differentiated lineage to appear in the developing anterior pituitary (E12.5 in the mouse). Several tissue specific transcription factors interact with upstream regulatory sequences of the POMC promoter to

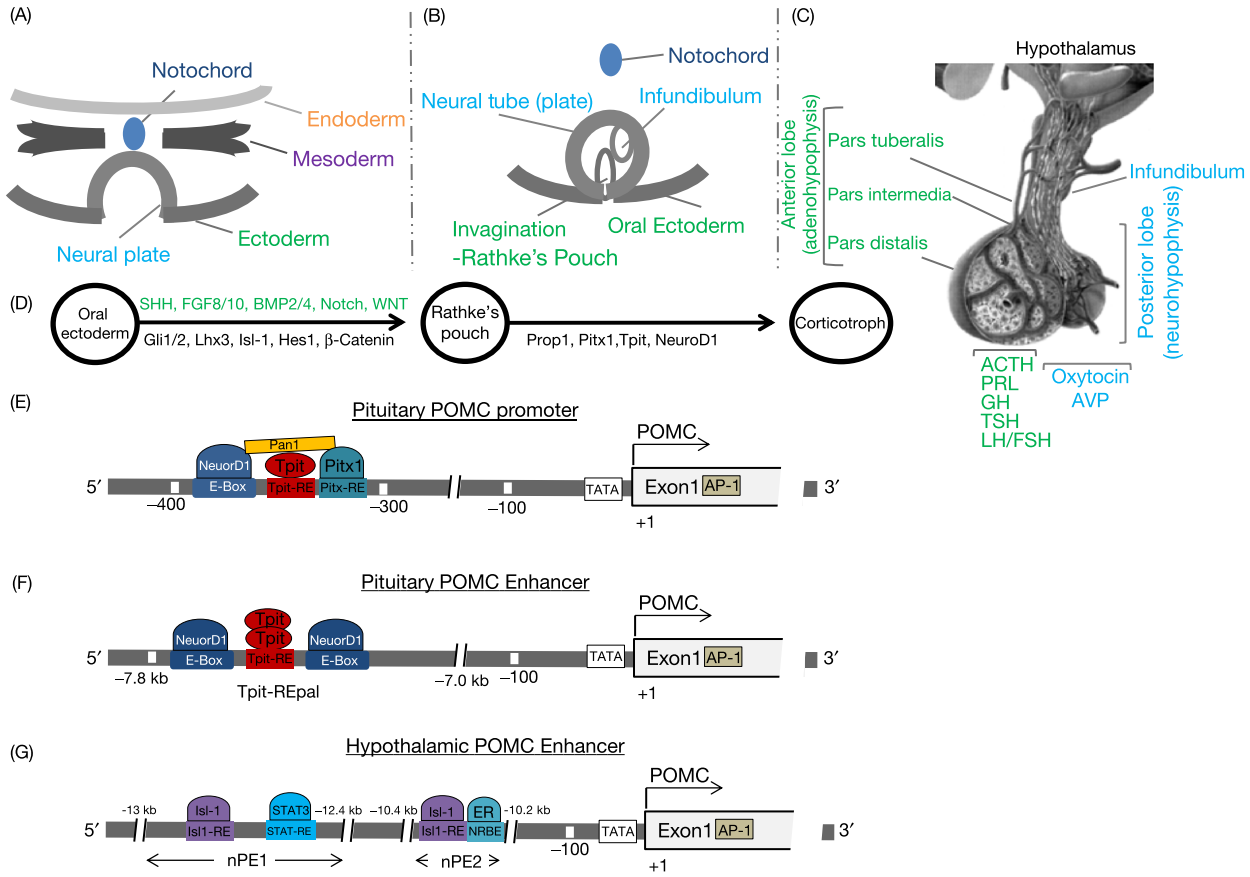

Fig. 1 Schematic of human POMC lineage origin during pituitary development. (A) The anterior and the posterior pituitary lobes originate from nonneural surface ectoderm and the anterior neural plate respectively. (B) The anterior pituitary primordium forms as an invagination of the stomodeal ectoderm at the roof of the oral cavity, which develops initially into Rathke's pouch. The primordium of the posterior lobe of pituitary originates from an outgrowth of the ventral diencephalon to become the infundibulum. (C) Depiction of the anatomic structure of the mature adult pituitary gland showing its cell sub-type secreted hormones from anterior and posterior pituitary. *ACTH*, adrenocorticotropic hormone; *PRL*, prolactin; *GH*, growth hormone; *TSH*, thyroid-stimulating hormone; *LH*, luteinizing hormone; *FSH*, follicle-stimulating hormone; *AVP*, arginine vasopressin. (D) Ontogeny of signaling molecules and stem cell transcription factors that direct pituitary organogenesis in early (A) and late (B) developmental stages. *SHH*, sonic hedgehog; *FGF*, fibroblast growth factor; *BMP*, bone morphogenetic protein; *WNT*, Wingless-type MMTV integration site; *Lhx3*, LIM homeobox 3; *Isl-1*, ISL LIM homeobox 1; *Prop1*, PROP paired-like homeobox 1; *Pitx1*, paired like homeodomain 1; *Tpit*, also known as T-box 19. (E–G) Depiction of transcriptional regulation of corticotroph-lineage commitment during pituitary organogenesis (E: POMC promoter region; F: POMC enhancer region) and hypothalamus organogenesis (G: POMC enhancer region). Tpit-RE, Tpit response element; Pitx-RE, Pitx1 response element; Isl1-RE, Isl-1 response element; STAT-RE, STAT response element; NRBE, nuclear receptor binding element.

precisely control the spatiotemporal programming of corticotroph cell commitment (Jeannotte *et al.*, 1987). For example, Pitx1, a pan-pituitary activator of transcription which belongs to the bicoid-type homeodomain subfamily, is expressed from E8 in the presumptive oral ectoderm when Rathke's pouch initially forms. Pitx1 binds the human POMC promoter at $-307/-299$ (transcription initiation site set as $+1$, **Fig. 1**E) and its interactions with adjacent other trans-elements, including Tpit-RE and *E*-box, are required for pituitary development (Lanctôt *et al.*, 1999). Tpit (also known as Tbx19), a T-box containing transcription factor, exhibits relatively low affinity for its response element (Tpit-RE, at $-318/-312$) on the human POMC promoter, but through a protein-protein interaction with Pitx1 which is already bound to Tpit-RE/Pitx-RE, Tpit is a potent activator of corticotroph POMC gene expression (**Fig. 1**E) (Liu *et al.*, 2001). In contrast to Pitx1 knockout, which only modestly affects POMC lineage development (Lanctôt *et al.*, 1999), Tpit knockout mice almost completely lack pituitary POMC-expressing cells (Budry *et al.*, 2011), and Tpit mutations in humans cause early onset isolated ACTH deficiency (Pulichino *et al.*, 2003). Tpit-RE/Pitx-RE is also bipartite with an *E*-box element located at $-370/-365$, which binds a basic helix-loop-helix (bHLH) transcription factor called NeuorD1 (also known as BETA2) (Lamolet *et al.*, 2004). The synergism of Tpit-RE/Pitx-RE and E-box$_{neuro}$ relies on direct protein interactions between Pitx1 and a NeuorD1 heterodimer partner Pan1. NeuroD1 is essential for specific binding to the E-box$_{neuro}$, and by anchoring to the POMC promoter, the tertiary complex (Tpit/Pitx1/NeuroD1) potentiates POMC transcription (**Fig. 1**E). An additional more responsive Tpit-RE palindrome site flanked by two E-box sites is located in a -7 kb enhancer region of pituitary POMC and binds a Tpit homodimer for activation at this site (**Fig. 1**F). Compared to the Tpit-RE in the proximal promoter, this enhancer is more selectively activated in pituitary corticotrophs compared to melanotrophs, and is independent of Pitx1 binding (**Fig. 1**F) (Langlais

et al., 2011). In summary, these composite regulatory elements coordinate with a variety of cell-specific and pituitary-specific transcription factors to confer corticotroph lineage-restricted POMC expression in the anterior pituitary.

Hypothalamic POMC Expression During Embryogenesis

In parallel, the hypothalamus develops from the adjacent neuroepithelium, and common early transcription factors, such as Hesx1, and local gradients of SHH, BMPs and FGFs, as well as tissue specific hormones generated at later developmental stages orchestrate programming of the pituitary anlagen and the hypothalamic sulcus (Zhu *et al.*, 2007). POMC expression in the ventral diencephalon of the developing hypothalamus is regulated by a −12 kb enhancer rather than the −480 bp proximal promoter involved in pituitary development. The LIM homeodomain transcription factor, Isl-1, binds to two target sequences called nPE1 and nPE2 on the POMC enhancer, and contributes to hypothalamic POMC lineage commitment (**Fig. 1**G) (Drouin, 2016). STAT response element (STAT-RE) and an estrogen receptor binding consensus (NRBE) have also been identified in the nPE1 and nPE2, and act as POMC neuron regulators in hypothalamus (**Fig. 1**G) (Bates *et al.*, 2003; de Souza *et al.*, 2011).

Regulation of POMC Synthesis

POMC Gene Structure

POMC belongs to an ancestral opioid/orphanin gene family, which plays an essential role in pain signaling and reward circuitry. This gene family encodes four prepropeptides, proenkephalin (PENK), prodynorphin (PDYN), pronociceptin/orphanin (PNOC), and POMC (Dores and Baron, 2011; Sundström *et al.*, 2010). They share three common sequence similarities: (a) a set of conserved six cysteine residues located at the N-terminus region (four Cys in POMC, **Fig. 2**A), (b) at least one core opioid (YGGF) or orphanin (FGGF) sequence in the C-terminus of the precursor protein (**Fig. 2**A) and (c) a single intron located between the signal peptide and the subsequent codons (the example of POMC is delineated in **Fig. 2**B). The human POMC gene is located on chromosome 2p23.3, spanning 7999 bp on the reverse strand. It contains three exons (354, 152, and 900 nt respectively) separated by two introns (3705 and 2888 nt respectively, **Fig. 2**B). The 5′-untranslated region (5′-UTR) of POMC mRNA includes the entire Exon 1 and the first 20 nt of the proximal region of Exon 2 (**Fig. 2**B). The remaining sequence of Exon 2 encodes a signal peptide and the first 18 aa of the POMC precursor. A large part of Exon 3 is translated to 223 aa of the POMC peptide and the 3′-UTR covers a 228 bp sequence of the 3′ end of Exon 3 (**Fig. 2**B). The N-terminal 26 aa-signal peptide of the precursor POMC protein is cleaved during maturation, and the remaining 241 aa region is further processed into several peptides in a tissue-specific manner (**Fig. 2**B). In the anterior pituitary lobe, POMC gives rise to several bioactive peptides, which include a 16 kDa N-POMC fragment (also known as Pro-γ-MSH), a joining peptide (JP), ACTH and beta-lipotropin (β-LPH, **Fig. 2**B). In the intermediate lobe of pituitary or hypothalamus, these peptides are further processed to gamma-melanocyte-stimulating hormone (γ-MSH), alpha-MSH (α-MSH), corticotropin-like intermediary peptide (CLIP), gamma-lipotropin (γ-LPH), beta-MSH (β-MSH) and beta-endorphin (β-END, **Fig. 2**B). Very low levels of POMC peptide and mRNA have also been identified in several extrapituitary sites, including the pancreas, stomach, appendix, kidney, testis, prostate, placenta, skin, adipose tissue, skeletal muscle, lymph nodes, brain, adrenal and lung. However the POMC transcript in these tissues is significantly shorter, containing approximately 800 nt derived from Exon 3 but lacking the N-terminus signal peptide for secretion (Clark, 2016). This finding indicates that tissue specific factors are involved in POMC transcriptional regulation.

The Effects of Hypothalamic Neural Peptides on Pituitary POMC

The spatial contiguity between the pituitary and hypothalamus is not only attributable to inter-dependent organogenesis, but involves close central-peripheral communication orchestrated by a plethora of neuropeptides through the portal circulation (Nieman and Loriaux, 1989). Hypothalamic-derived corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) are the most physiologically important stimuli of pituitary ACTH synthesis and secretion. CRH is a 41 aa peptide and its precursor is synthesized in the hypothalamic paraventricular nuclei neurons (PVN) that project to the external layer of the median eminence (ME). There, CRH is released into the hypophyseal portal system by which it reaches the anterior pituitary lobe (**Fig. 3**). CRH neurons also receive innervation from multiple brain regions, including the brainstem and limbic structures, coordinating neuroendocrine, autonomic and peripheral stimuli to transmit signals to the pituitary gland (Papadimitriou and Priftis, 2009). CRH actions on anterior pituitary corticotrophs exclusively rely on the seven transmembrane G protein-coupled CRH receptor-1 (CRH-R1). Upon ligand binding, CRH-R1 couples to G_s, the stimulatory heterotrimeric GTP binding protein that activates adenylate cyclase (AC). Increased cAMP levels and protein kinase A (PKA) activity subsequently induce ACTH synthesis and secretion (**Fig. 3**) (Mason *et al.*, 2002). CRH-mediated actions on POMC transcription are immediate, reaching their peak within 10–30 min, and do not require de novo protein synthesis, indicating these rapid CRH actions are mediated by changes in signaling cascades that alter protein modification or protein interaction. The orphan nuclear receptor Nur77 (also known as nerve growth factor-induced protein IB, NGFI-B) binds to either the POMC promoter Nur responsive element (Nur-RE, located at −397/−377) as a heterodimer with Nur-related 1 (Nurr1), and/or neuron-derived orphan receptor 1 (NOR-1). Nur77 also interacts with Nur factor binding responsive element (NBRE, located at −73/−52) as a homodimer following CRH stimulation. Nur77 undergoes a phosphorylation modification that recruits the steroid receptor co-activator (SRC) to enhance POMC transcription (**Fig. 3**) (Mason *et al.*, 2002). CRH treatment also increases Nur77 and c-Fos mRNA expression which bind

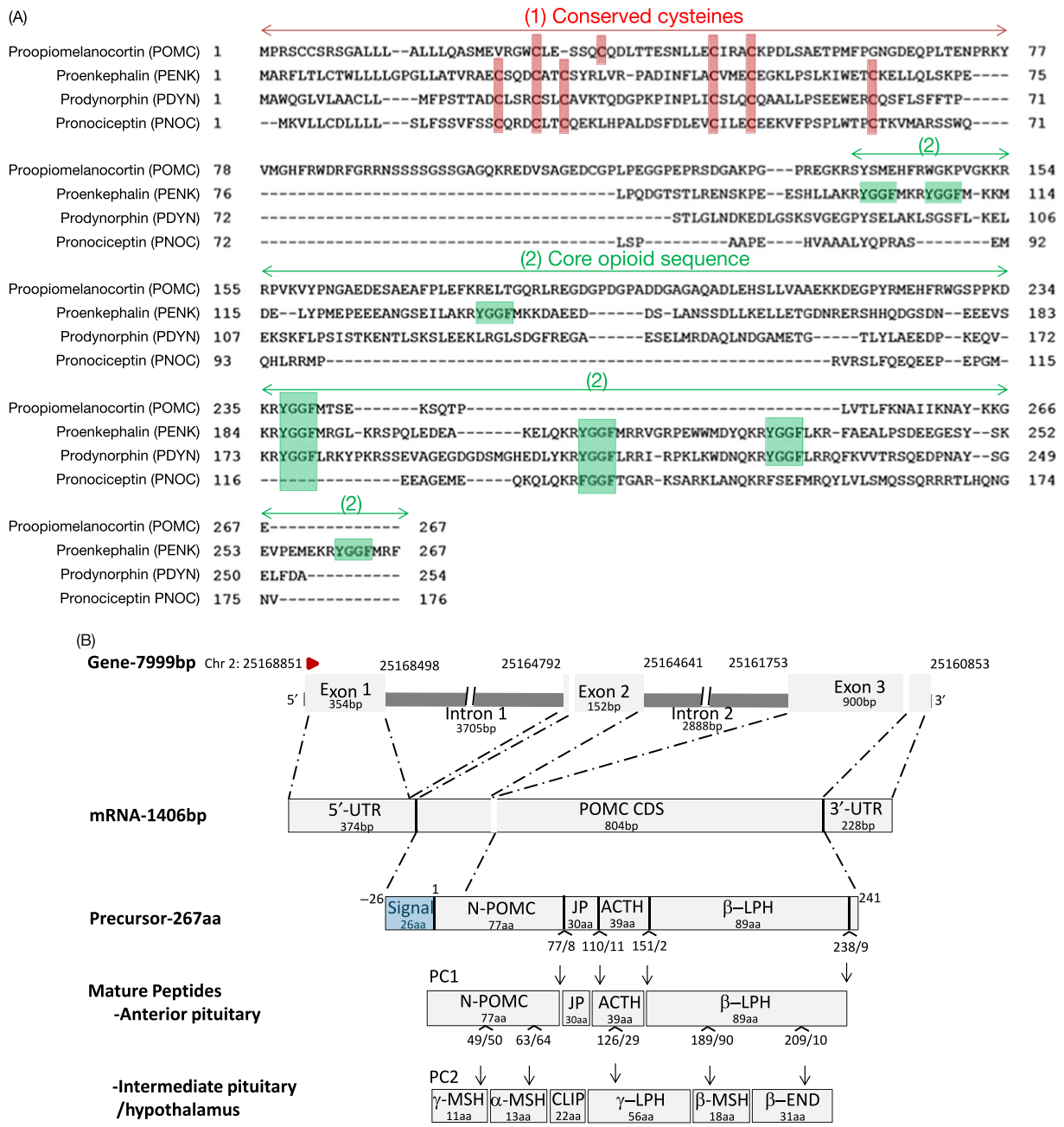

Fig. 2 Gene structure and the bioactive peptides derived from human proopiomelanocortin (POMC) gene. (A) Alignment of precursors for the human opioid gene family, including Proopiomelanocortin (POMC, NP_000930.1), Proenkephalin (PENK, NP_001129162.1), Prodynorphin (PDYN, NP_001177821.1), and Pronociceptin/proorphanin (PNOC, NP_006219.1) depicting their common sequence similarities: (1) Conserved cysteine residues (six Cys in PENK, PDYN, and PNOC; four Cys in POMC) at the N-terminus are highlighted in . (2) Core opioid sequences, including one YGGF core in POMC, seven YGGF cores in PENK, three YGGF cores in PDYN, and one FGGF core in PNOC, are highlighted in . (B) The human POMC gene is located on chromosome 2p23.3, spanning 7999 bp on the reverse strand. Its transcript contains three exons and two introns, and translates a precursor protein of 267 amino acids. The N-terminus 26 aa-signal peptide is cleaved during maturation, and the remaining region is further processed into several peptides in a tissue-specific manner. In the anterior pituitary, POMC gives rise to a 16 k N-POMC (also known as Pro-γ-MSH), joining peptide (JP), ACTH and beta-lipotropin (β-LPH). Whereas in the intermediate pituitary or hypothalamus, these peptides are further processed to generate gamma-melanocyte-stimulating hormone (γ-MSH), alpha-MSH (α-MSH), corticotropin-like intermediary peptide (CLIP), gamma-lipotropin (γ-LPH), beta-MSH (β-MSH) and beta-endorphin (β-END).

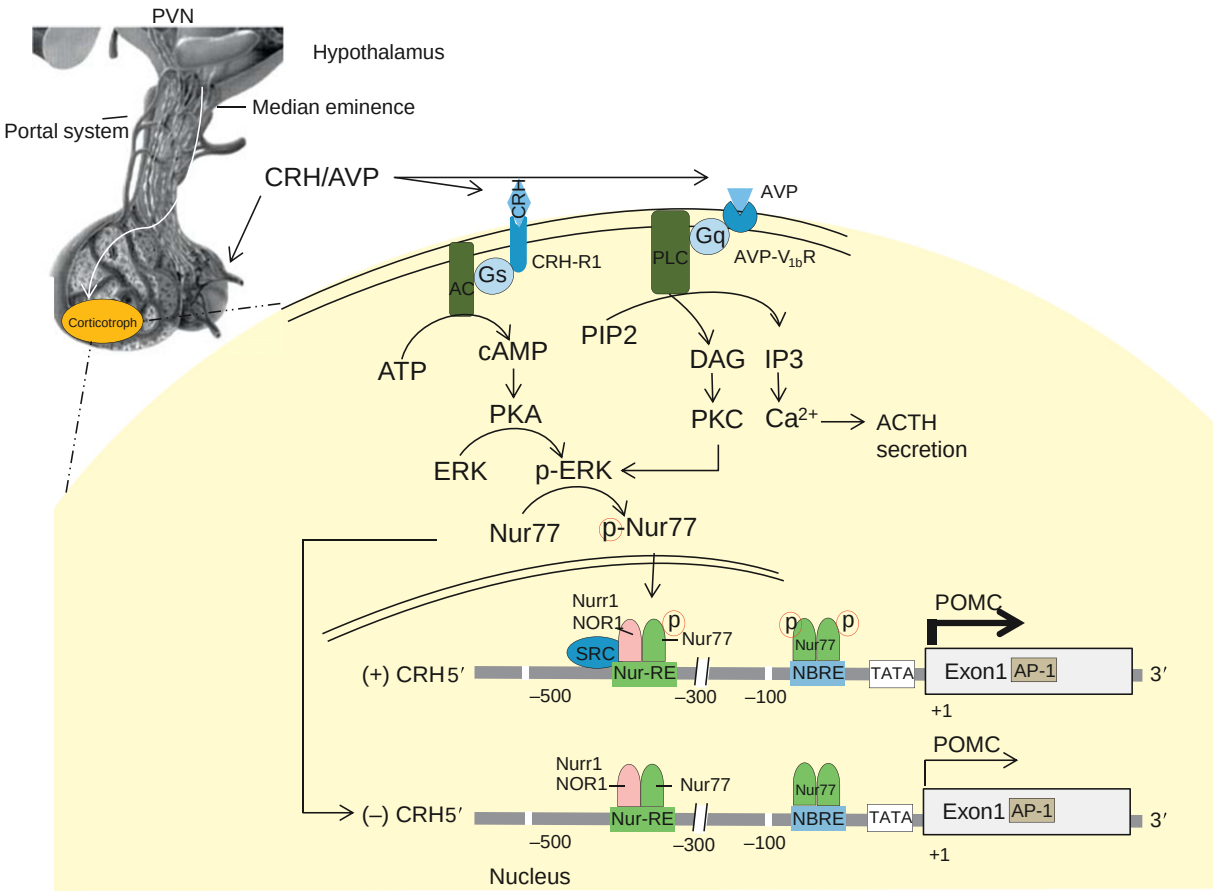

Fig. 3 Schematic summary of some of the key intracellular signaling pathways that regulate pituitary POMC transcription by the hypothalamic derived neural peptides CRH and AVP. By activating cognate receptors, hypothalamic corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) simultaneously activate the protein kinase A (PKA) and protein kinase C (PKC) pathways which in turn phosphorylate Nurr77 to bind Nur-RE and NBRE to activate POMC transcription.

and activate a Nur-RE (− 397/ − 377) and an AP-1 site (+ 41/ + 47 in Exon 1) respectively on the POMC promoter. This is a more delayed CRH response and its occurrence in the intermediate pituitary lobe melanotrophs mediates amphibian camouflage.

AVP (also known as antidiuretic hormone, ADH) is a 9 aa peptide hormone, derived from a 164 aa glycosylated precursor protein, preprovasopressin (Aguilera and Rabadan-Diehl, 2000). Pro-AVP is synthesized in the paraventricular nucleus and supraoptic nuclei magnocellular neurons and travels downward through the infundibulum to the posterior pituitary lobe, where the processed AVP is released into peripheral circulation to regulate fluid homeostasis (Barakat *et al.*, 2006). AVP is also synthesized in the hypothalamic parvocellular neurons, from where AVP is transported to the median eminence (ME), and then travels through the hypophyseal portal system to the anterior pituitary (**Fig. 3**). Corticotrophic cells express AVP-V_{1b} receptor, whose activation synergizes with the CRH action to produce ACTH, but AVP by itself is a weak secretagogue (Castro, 1993). Ligand bound AVP-V_{1b} receptor couples to $G_{q/11}$ to stimulate phospholipase C (PLC), leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), two key intracellular messengers for protein kinase C (PKC) and Ca^{2+} channel activation which mediate ACTH synthesis and secretion (**Fig. 3**) (Christ-Crain and Fenske, 2016). Both the CRH and AVP receptors are subject to dynamic changes, such as receptor-uncoupling, internalization or down-regulation, which can lead to desensitization during repeated or prolonged CRH and/or AVP stimulation. Reversible, this alternating desensitization–resensitization represents a tier of adaptation to chronic stress at the pituitary level, integrating signals from variable CRH and AVP concentrations as well as adrenal derived steroid feedback under different degrees of stress (Aguilera and Rabadan-Diehl, 2000).

Adrenal Glucocorticoid Actions on POMC

Adrenal-derived glucocorticoids (GCs) are the major negative feedback mediators of pituitary, hypothalamic and central hippocampus-derived ACTH secretion (**Fig. 4**) (Papadimitriou and Priftis, 2009), whose acute inhibitory effects occur within minutes through blocking of ACTH secretion rather than synthesis. It is hypothesized that these actions are mediated by GC-induced

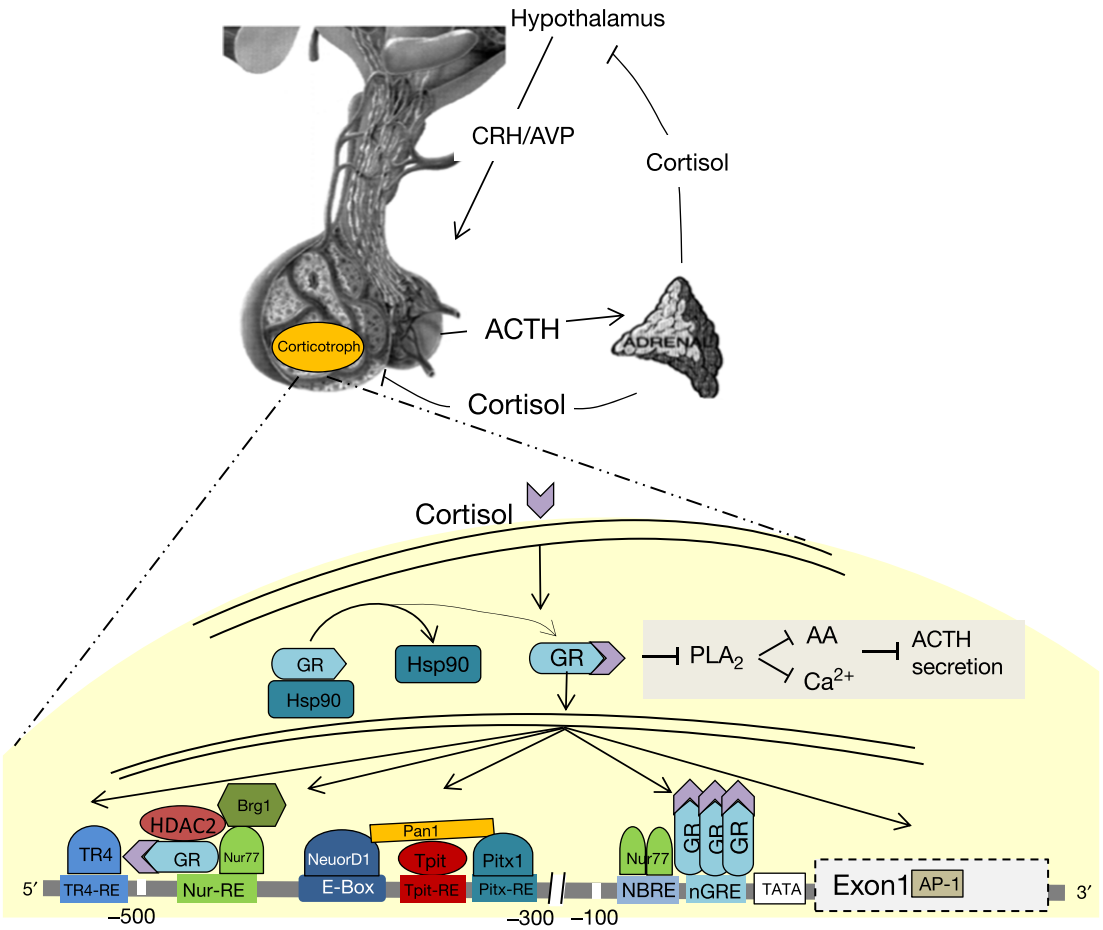

Fig. 4 Schematic diagram of the molecular mechanisms by which glucocorticoids and the glucocorticoid receptor (GR) mediate regulation of the POMC gene. Negative feedback regulation of the HPA axis is exerted by glucocorticoids both at the hypothalamic and pituitary level. It comprises acute inhibitory actions via inhibition of phospholipase A2 and slower inhibitory actions via the nGRE by steric interference of CRH-involved Nur factor binding. Target DNA regions (Nur-RE, NBRE, nGRE, TR4-RE) that modulate GR action on pituitary POMC transcription repression are represented.

inhibition of phospholipase A2 (PLA_2) activity and the resultant reduction in the generation of arachidonic acid (AA) and extracellular Ca^{2+} influx (**Fig. 4**) (Won and Orth, 1994). However, it is GC-mediated inhibition of pituitary ACTH synthesis that constitutes the most important aspect of physiological HPA axis autoregulation (Martini, 2004). Upon ligand binding, the glucocorticoid receptor (GR) undergoes a conformational change, disassociates from its cytoplasmic chaperone Hsp90, and translocates into the nucleus where it transrepresses POMC transcription by binding to the negative glucocorticoid response element (nGRE) as well as interacting with other transcription factors, coactivators and remodelers of the chromatin landscape (**Fig. 4**) (Drouin, 2016). Quite distinct from the inducing GRE which binds monomeric GR, the nGRE recruits three distinct moieties of the GR, and mutation of this GR DNA binding domain causes increased POMC transcription (Giraldi *et al.*, 2011). On the human POMC proximal promoter, the nGRE is located at $-69/-61$, overlapping with the Nur factor binding responsive element (NBRE), which is located at $-73/-52$. Therefore the three GR-ligand complexes sterically interfere with Nur factor binding to the NBRE and blunt CRH stimulated Nur-RE-mediated actions to increase POMC transcription and vice versa, leading to a reciprocally competitive "Yin-Yang" between the GC and CRH response (**Fig. 4**) (Giraldi *et al.*, 2011).

Brahma-related gene 1 (Brg1) is the ATPase component of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex and stably resides on POMC promoter, contributing to POMC basal expression. In the presence of GCs, Brg1 stabilizes the GR-Nur77 interaction to interfere with Nur-RE activation, and also recruits histone deacetylate 2 (HDAC2), causing histone H4 deacetylation and promoter clearance to inhibit POMC transcription (**Fig. 4**) (Bilodeau *et al.*, 2006). Based on these observations, it has been proposed that reduced or mislocated Brg1 and HDAC2 may contribute to the GC resistance that is observed in Cushing's disease (Bilodeau *et al.*, 2006). In addition to directly regulating POMC transcription, Brg1 also functions as a negative cell cycle modulator through repression of Cyclin E (Roussel-Gervais *et al.*, 2010). More recently CABLES1 (Cdk5 and ABL enzyme substrate 1), which is rapidly upregulated following GC treatment, has been demonstrated to mediate the growth inhibitory effect of Dexamethasone (Roussel-Gervais *et al.*, 2016). Although pathogenic germline missense CABLES1 variants (2.2%, 4/181) are uncommon (Hernández-Ramírez *et al.*, 2017), CABLES1 is underexpressed in nearly half of corticotrope adenomas where it correlated strongly with low p27 expression (Roussel-Gervais *et al.*, 2016), suggesting there may be epigenetic

mechanisms reducing corticotroph tumor CABLES1 levels. The role, if any, of CABLES1-mediated POMC regulation is as yet unclear and additional cases from larger CD pedigrees would be beneficial to confirm the findings.

Intact Tpit-RE/Pitx-RE is also required for GC-mediated transrepression since competitive interaction between the coactivators SRCs and GR reduces Tpit-RE/Pitx-RE activation, which also needs SRC for activity (**Fig. 4**). A similar mechanism also operates for antagonism of AP-1 activity by ligand bound GR. Although no canonical cAMP responsive element (CRE) was identified in the human POMC promoter, a phorbol ester responsive element (TRE) located in Exon 1 ($+41/+47$) can be activated by Jun-Jun homodimer or a Jun-Fos heterodimer (**Fig. 4**) (Jenks, 2009). GCs interfere with TRE-mediated POMC activation through multiple mechanisms, including reducing CRH-induced AP-1 DNA binding, inhibiting the CRH-stimulated c-fos mRNA increase (Autelitano, 1994), and affecting upstream kinase (JNK) activation (González *et al.*, 2000). This partially underlies the anti-inflammatory and anti-tumor activities of glucocorticoids. Our own studies recently demonstrated that an orphan nuclear receptor called testicular receptor 4 (TR4, also known as NR2C2) positively regulates POMC gene transcription and ACTH secretion which is in part mediated by an interaction of TR4 with the GR to override GR repressive actions on POMC transcription (**Fig. 4**) (Du *et al.*, 2013; Zhang *et al.*, 2016a,b).

Regulation of POMC by Nuclear Receptors

Nuclear receptors are a large group of transcription factors that are involved in many fundamental biological processes, including development, differentiation, and homeostasis (Willson and Moore, 2002). In addition to Nur transcription factors, GR and TR4 as described above, several members of the thyroid hormone and retinoid X receptor subfamilies have been implicated in the regulation of pituitary ACTH synthesis and secretion (Hashimoto *et al.*, 2011; Heaney *et al.*, 2002; Matsumoto *et al.*, 2009; Páez-Pereda *et al.*, 2001; Saito-Hakoda *et al.*, 2015).

All-trans-retinoic acid (atRA) and 9-*cis* retinoic acid (9-*cis*-RA) are the most biologically potent retinoic acid isomers. However, they have short plasma half-lives (<1 h), and induce their own metabolism. In contrast, 13-*cis*-retinoic acid (13-*cis*-RA) has a half-life >13 h and cannot induce self-metabolism but is less biologically active (Labeur *et al.*, 2009). The retinoic acid receptor (RARα, RARβ, RARγ) and retinoid X receptor (RXRα, RXRβ, RXRγ) are the cogent nuclear receptors of the retinoic acids (Labeur *et al.*, 2009). In the absence of ligand, these retinoid receptors function as transrepressors by binding to specific retinoic-acid response element (RARE) on target genes and recruit nuclear co-repressors, such as nuclear receptor co-repressor (NCOR) and silencing mediator of RAR and thyroid hormone receptor (SMRT). On ligand binding, RARs release from their co-repressors and recruit transcriptional co-activators such as steroid receptor co-activator-1 (SRC-1) to regulate a diverse range of target genes (Labeur *et al.*, 2009). In support of this concept, the synthetic RARα/β agonist Am80 up-regulates POMC gene transcription by increasing NeuroD1 and Tpit expression (Uruno *et al.*, 2014). In apparent contradiction, it has been reported that retinoic acid inhibits cell proliferation and ACTH transcription in ACTH-secreting human pituitary and small cell lung cancer cells through inhibiting AP-1 and Nur77 (Páez-Pereda *et al.*, 2001). Interestingly, the RA actions were not seen in normal pituitary cells due to inhibition by the orphan receptor Chicken Ovalbumin Upstream Promoter-Transcription Factor I (COUP-TFI), which is reportedly absent in corticotroph tumors (Páez-Pereda *et al.*, 2001). Studies in dogs reported retinoic acid treatment led to tumor shrinkage and relief of hypercortisolism (Castillo *et al.*, 2006). Two recent prospective trials in patients with persistent or recurrent hypercortisolism after transsphenoidal surgery showed sustained normalization of 24 h urinary free cortisol in 25% of patients treated with 13-*cis* retinoic acid for between 6 and 12 months (Occhi *et al.*, 2014; Pecori Giraldi *et al.*, 2012; Vilar *et al.*, 2016).

In addition to homo- and heterodimerization between RAR and RXR, RXRs also heterodimerize with other nuclear receptors, and are activated by a wide spectrum of ligands (Evans and Mangelsdorf, 2014). When RXRs homodimerize or heterodimerize with peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR) and pregnane X receptor (PXR), the receptor complex responds to agonists of both RXR and the partner, and these heterodimers are so-called permissive heterodimers. Conversely, when RXR forms a heterodimer with RAR, vitamin D nuclear receptors (VDRs) and/or thyroid receptors (TRs), the complex can only be activated by the agonists of the partners but not that of RXRs, this group of heterodimers being called nonpermissive heterodimers (Saito-Hakoda *et al.*, 2015). These apparent contradictory actions of RAR activation to either stimulate or inhibit POMC transcription could be explained by involvement of an RXR homodimer or permissive heterodimer that mediates mixed RXR/RAR actions. A heterodimer of RXRα/LXRα has also been found to bind at an LXR-RE half site at $-73/-52$ region of the POMC proximal promoter (Matsumoto *et al.*, 2009), and the LXR agonist TO901317 positively regulates POMC transcription.

Our own studies demonstrated that activation of PPAR-γ, which was abundantly expressed in human corticotroph tumor, by the thiazolidinedione compound rosiglitazone inhibited ACTH secretion and growth of human and murine corticotroph tumor cells in vitro and in an in vivo xenograft corticotroph tumor animal model (Heaney *et al.*, 2002). Not dissimilar to RA, Rosiglitazone treatment in a small numbers of patients with Cushing's Disease reported normalized urinary free cortisol in $\sim$30% of treated patients (Ambrosi *et al.*, 2004; Giraldi *et al.*, 2006; Morcos *et al.*, 2007).

Regulation of POMC Processing

Intracellular Trafficking of POMC

The endoplasmic reticulum (ER) is an interconnected bilayer membrane system which manufactures, processes and transports a multitude of proteins and peptides. POMC is soluble and highly charged, and its N-terminal signal peptide directs the newly synthesized precursor

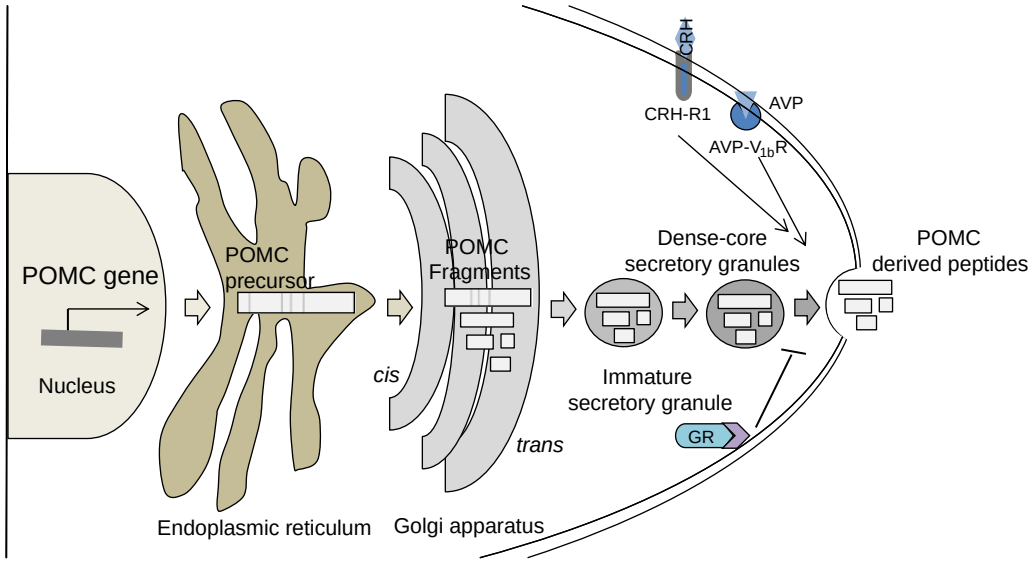

Fig. 5 The posttranslational processing and release of POMC and derived peptides. Schematic depiction of the multiple steps involved in POMC processing and transportation from its site of synthesis in the nucleus to the release of its processed peptides through the regulated secretory pathway.

into the ER lumen. After the POMC protein is properly folded in smooth ER, it enters the adjacent Golgi apparatus (GA), which comprises several membrane-covered cisternae (**Fig. 5**). The Golgi apparatus is polar in both structure and function, and the POMC protein enters the Golgi apparatus through the *cis* face (close to the nucleus and ER) and exits from the *trans* face toward the plasma membrane (**Fig. 5**). When budding off from the *trans* Golgi network (TGN), POMC is sorted into immature secretory granules (ISGs), which ultimately develop into large dense-core secretory granules (DCSGs) containing the processed products of POMC (**Fig. 5**). These are then delivered to the plasma membrane by a regulated secretory pathway to await a secretagogue signal.

Enzymatic Processing of POMC

The sequential processing of POMC involves a spectrum of enzymes, including endoproteases, exopeptidase, *N*-acetyl transferase and peptidyl amidating monoxygenase (Stevens and White, 2010). The initial processing of POMC occurs during transportation to the *trans* Golgi network by prohormone convertase 1 (PC1), which belongs to the subtilisin-like serine protease family (Cawley *et al.*, 2016). This family contains nine proprotein convertases, and PC1 and PC2 are mostly localized within immature and large dense-core secretory granules of neural and endocrine cells, and process most polypeptide prohormones within the regulated secretory pathway (Seidah and Prat, 2012). PC1 is most abundant in the anterior pituitary and similar to POMC, is synthesized in the endoplasmic reticulum as an inactive proprotein that undergoes maturation by initial autocatalytic cleavage followed by an N-terminal cleavage and a tertiary C-terminal truncation step as it transits from the *trans* Golgi network to the secretory vesicles (Chrétien and Mbikay, 2016). PC1 activity is inhibited by its own unprocessed peptide as well as an endogenous inhibitor called ProSAAS. The latter is a granin-like protein that is processed by PC1, but also binds to PC1 to inhibit its enzymatic activity in the early stages of the regulated secretory pathway (Stijnen *et al.*, 2016). PC1 cleaves POMC at dibasic residues, generating three peptide intermediates, namely a 16 kDa N-POMC (1–77), ACTH and β-LPH (**Fig. 2**B) (Chrétien and Mbikay, 2016). PC1 null mice have impaired ACTH production and compensatory accumulation of POMC mRNA, and a patient with mutated PC1 exhibited high levels of partially processed POMC intermediate products and a phenotype that included obesity, impaired glucose homeostasis and orange hair (Chrétien and Mbikay, 2016). PC2 is expressed in the neurointermediate lobe, but not in the anterior lobe, and processes POMC to γ-MSH, α-MSH, CLIP, γ-LPH, β-MSH and β-END (**Fig. 2**B). PC2 null mice lack α-MSH, and accumulate ACTH, ACTH-containing intermediate peptides, POMC, and β-END. These animals exhibit retarded growth and developmental defects due to improper processing of growth hormone and other prohormones, including insulin (Chrétien and Mbikay, 2016). PC2 has an endogenous chaperone, 7B2, that inhibits PC2 in both its unprocessed and processed forms (Stijnen *et al.*, 2016). Similar to PC2 knockout animals, 7B2 null mice develop spontaneous Cushing's disease due to excessive ACTH secretion. In addition to the PC family, several other enzymes process POMC, including Yapsin A, an aspartic protease that cleaves POMC at dibasic residues. Acidic ACTH-converting enzyme (AAVE), a tetrabasic residue-specific enzyme found in the intermediate lobe, is a calcium dependent serine protease that cleaves ACTH at the Arg 17-Arg18 residues to yield ACTH 1–17 and CLIP (Cawley *et al.*, 2016). As a penultimate step in POMC processing, aminopeptidases and carboxylpeptidase E (CPE) remove extending N- and C-terminal lysine and arginine residues from POMC products (Cawley *et al.*, 2016). Additionally, POMC fragments undergo cell specific posttranslational modifications, such as *N*-acetylation and amidation which modulate the stability and activity of the various POMC-derived peptides (Cawley *et al.*, 2016).

Transport and Exocytosis of Secretory Granules

The N-terminus 26 aa signal peptide of the POMC precursor is predicted to act as a "sorting" peptide that binds to sorting receptors of the *trans* Golgi network, potentially carboxylpeptidase E and secretogranin III. Independent of its enzymatic activity, carboxylpeptidase E functions as a sorting receptor by adoption of a transmembrane orientation, interacting through its cytoplasmic tail with a complex of "motor" proteins, including dynactin, and the kinesins 2 and 3. It is proposed that POMC containing secretory granules anchor to and travel along microtubules aided by the high affinity of carboxylpeptidase E for its motor complex (Cawley *et al.*, 2016). Gamma-adductin is a newly defined binding partner of carboxylpeptidase E, and it is proposed that it partners with carboxylpeptidase E to facilitate budding off from the *trans* Golgi network through an interaction with peri-Glogi F-actins (Cawley *et al.*, 2016). Once the regulatory secretory granule vesicles containing POMC-derived peptides have transferred along the microtubule track to the actin cortex close to the plasma membrane, they are then tethered there and docked by the small guanosine triphosphatase (GTPase) Rab proteins and their effectors (Park and Loh, 2008). Rab3D in association with Rab3A, docks the POMC-derived peptides containing vesicles to the plasma membrane (Park and Loh, 2008). The SNARE complex then facilitates the fusion of the vesicles with the plasma membrane. Following an ACTH stimulus, Ca^{2+} influx occurs and activates the Ca^{2+} sensor synaptotagmin, which binds the SNARE complex and triggers membrane fusion and release of vesicular ACTH content (Park and Loh, 2008). Detailed characterization of trafficking and transportation of secretory vesicles is still poorly understood, and the exact molecular mechanisms involved may vary in a tissue and cell specific manner (Park and Loh, 2008).

Concluding Remarks

The human POMC gene and its orthologs are highly conserved across mammalian organisms. POMC first appeared in the vertebrate kingdom ~ 500 million years ago prior to the rise of jawless Agnatha. Since corticotrophin was first purified from pig pituitary extracts in 1950 (Payne *et al.*, 1950), our understanding of the biochemical characteristics and physiological activity of ACTH has vastly improved. However, many details of the physiopathological regulation of POMC remain unknown. For example, plasma ACTH and cortisol exhibits a distinct circadian rhythm in humans and higher mammals with an ~24 h cycle length, peaking between 5 and 8 am with its nadir at 11 pm-3 am (Dickmeis, 2009). Interestingly, this ACTH and cortisol oscillatory profile is disturbed in Cushing's disease's and in disrupted sleep-wake cycles or feeding intervals (Dickmeis, 2009). Further insights into the molecular mechanisms that regulate POMC synthesis and secretion, and its complex interplay with other target organs especially the adrenal cortex will advance our understanding of this fascinating area in endocrinology.

See also: Adrenal Cortex; Physiology. Corticotropin-Releasing Hormone Peptide Family. Stress and Endocrine Physiology

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ACTH, Melanocortin Receptors, and MRAP Accessory Proteins☆

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Nomenclature

1 R: first genome duplication event that occurred in the ancestral agnathan vertebrates.
2 R: second genome duplication event that occurred in the ancestral agnathan vertebrates.
3 R: genome duplication event that occurred in the ancestral ray-finned fishes.

Glossary

ACTH Adrenocorticotropin; polypeptide hormone derived from POMC.
αMSH Alpha-melanocyte stimulating hormone; polypeptide hormone derived from POMC.
βMSH, γMSH, δMSH Polypeptide hormones related to αMSH and derived from POMC.
Endoproteolytic cleavage site Amino acid motifs (i.e., KR, RR, RK, KKRR, RKRR) located within a polypeptide that serve as the cleavage site for either prohormone convertase 1 or 2.
Genome duplication event Complete duplication of an entire genome.
GPCR G protein-coupled receptor.
Local gene duplication Duplication of a gene within a chromosome.
MC1R Melanocortin-1 receptor.
MC2R Melanocortin-2 receptor.
MC3R Melanocortin-3 receptor.
MC4R Melanocortin-4 receptor.
MC5R Melanocortin-5 receptor.
Melanocortin peptide. A polypeptide with the HFRW Motif.
Melanocortin receptor A GPCR with a binding site for the HFRW motif of a melanocortin peptide.
MRAP1 Melanocortin-2 receptor accessory protein 1.
MRAP2 Melanocortin-2 receptor accessory protein 2.
Opioid/Orphanin A polypeptide chemical signal that has the N-terminal motif, YGGF, YGGL, or FGGF that can bind to either an opioid receptor or an orphanin receptor.
POMC Proopiomelanocortin; the common precursor for melanocortin peptides and the opioid peptide β-endorphin.

Introduction

ACTH is one of several melanocortin-related peptides that are derived from the common precursor protein, POMC (Nakanishi *et al.*, 1979). The *pomc* gene appears to have emerged early in the evolution of the chordates, and is a member of the opioid/orphanin gene family (Dores *et al.*, 2002). By the time the gnathostomes (jawed vertebrates) appeared in the fossil record, the organizational plan for the POMC common precursor protein was well established. In addition it is likely that all five melanocortin receptor genes, and the two accessory proteins, melanocortin receptor-2 accessory protein-1 (MRAP1) and melanocortin-2 accessory protein-2 (MRAP2), were also present in the genomes of the ancestral gnathostomes. Before considering the properties of melanocortin peptides, melanocortin receptors, and the MRAP-related accessory proteins, some comments about the transformations that have occurred in chordate genomes may be helpful.

Extant chordates (i.e., members of phylum Chordata) include protochordates such as the tunicates and lancets (*Amphioxus*, genus), the jawless fishes, such as hagfishes and lampreys, and the jawed vertebrates (gnathostomes) such as cartilaginous fishes, bony fishes (ray-finned and lobe-finned), amphibians, reptiles, birds, and mammals (Hedges, 2009). The features that unite all of these organisms include the presence of a dorsal nerve cord, pharyngeal gill slits, and a notochord at some point in development (Buchsbaum *et al.*, 1987). A feature that separates the various extant lineages of chordates is the number of genome duplication events a particular lineage has endured (Ohno *et al.*, 1968; Holland *et al.*, 1994; Lundin, 1993; Kuraku *et al.*, 2009). As shown in **Fig. 1**A, there is evidence that during the radiation of the ancestral jawless fishes two genome duplication events occurred. Hence, while there may have been a single gene in the ancestral protochordates for some trait, there could be, theoretically, four paralogous genes potentially located on separate chromosomes in the genome of the ancestral gnathostomes. During this period of genome expansion the possibility that some paralogous genes might undergo duplication events within a chromosome, or be moved to a different chromosome, or accumulate point mutations that resulted in a pseudogene cannot be dismissed. To add to

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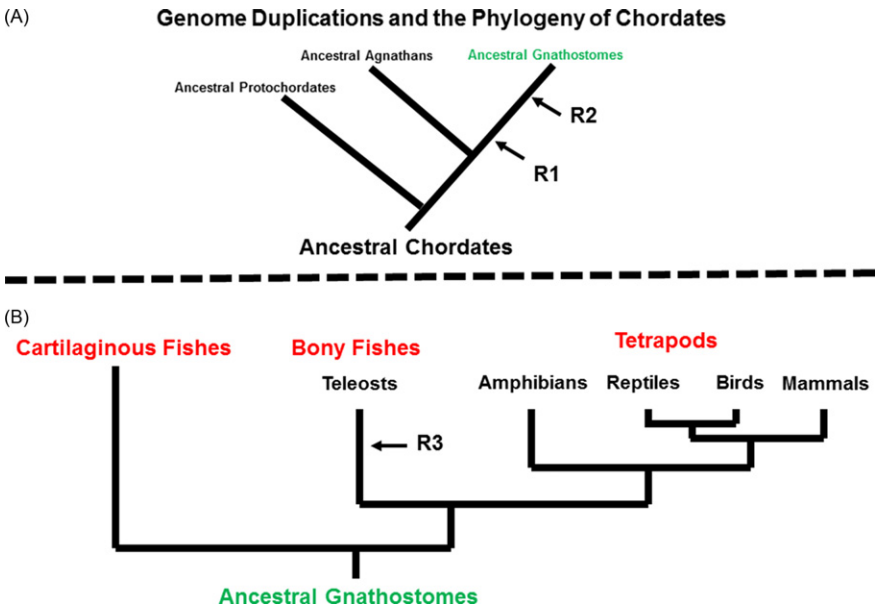

Fig. 1 Chordate phylogeny. (A) The ancestral chordates experienced two whole genome duplication events during the radiation of the ancestral agnathan vertebrates. (B) Within the radiation of the gnathostomes a third genome duplication event occurred in the ancestors to the teleosts. 1R—first genome duplication event; 2R—second genome duplication event; 3R—third genome duplication event.

this complexity, a third genome duplication event occurred at some point in the radiation of the ancestral ray-finned fishes, and as a result modern bony fishes (teleosts) are 3R (Meyer and Van de Peer, 2005).

The implications of chordate genome duplication events can be seen for the opioid/orphanin gene family. This gene family appears to have its origin in the ancestral protochordates, and the *pomc* gene appears to be the result of the 1R duplication event (Sundstrom *et al.*, 2010). However following the 2R event, it appears that a duplicate copy of the *pomc* gene was lost in the genomes of most nonteleost gnathostomes. The organization of gnathostome POMC will be discussed in Section "ACTH and POMC."

With respect to the melanocortin receptors, the current hypothesis is that this gene family arose in the ancestral protochordates (Vastermark and Schiöth, 2011). Hence, the presence of five paralogous melanocortin genes in most gnathostome genomes is consistent with two genome duplication events and one local gene duplication event. In support of this conclusion the *melanocortin-1 receptor* (*mc1R*) gene, the *melanocortin-2 receptor* (*mc2r*) gene, the *melanocortin-3 receptor* (*mc3r*) gene, and the *melanocortin-4 receptor* (*mc4r*) gene are located on separate chromosomes in most gnathostome genomes, and the *melanocortin-5 receptor* (*mc5r*) is usually found on the same chromosome as the *mc2r* gene (Schiöth *et al.*, 2003).

Currently, the *mrap1*gene and the *mrap2* gene have only been found on separate chromosomes of representative 2R organisms such as the extant gnathostomes (Dores, 2016). This observation would suggest that these paralogous genes arose after the 2R genome duplication event, and the ancestral *mrap* gene had its origin in a 1R ancestral jawless vertebrate lineage.

ACTH and POMC

When considering the organization plan for POMC in extant gnathostomes, mammalian POMC serves as a good model (**Fig. 2**A). All POMC orthologs have the sequence of the endogenous opioid peptide, β-endorphin, located at the C-terminal of the precursor. In addition, mammalian POMC orthologs have three melanocortin peptide sequences: an ACTH sequence as well as a γMSH and a βMSH sequence. Note that the sequence of αMSH is embedded in the sequence of ACTH. Each of the melanocortin peptides sequences has the melanocortin core motif, HFRW, and each sequence is flanked by a set of paired basic amino acids that serve as endoproteolytic cleavage sites (Chang *et al.*, 1980). The role of the HFRW motif will be discussed in Section "Melanocortin Receptors." In addition there is a tetrabasic amino acid motif in the ACTH sequence that allows for the generation of αMSH following endoproteolytic cleavage (Eipper and Mains, 1980).

One of the intriguing features of POMC is that this precursor protein can undergo cell specific endoproteolytic cleavage to yield different sets of end products (Seidah and Chrétien, 1999). For example in mammalian corticotropic cells of the anterior pituitary, POMC undergoes endoproteolytic cleavage to yield ACTH and β-endorphin as major end products. Whereas, in mammalian melanotropic cells of the intermediate pituitary, and in selected neurons in the central nervous system POMC undergoes endoproteolytic cleavage to yield the various MSH-sized peptides (i.e., αMSH, βMSH, γMSH), and β-endorphin as major end products (Eipper and Mains, 1980). The implications of this differential posttranslational processing of POMC will be discussed in Section

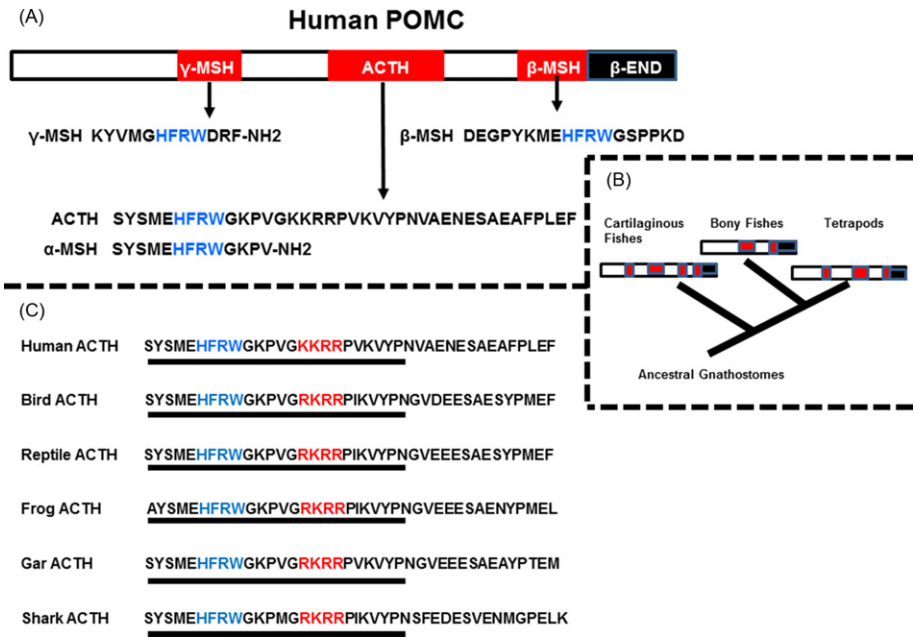

Fig. 2 POMC and ACTH. (A) The organizational plan for POMC is presented in schematic form. The relative positions of γMSH, ACTH, and βMSH within the precursor protein are highlighted in *red*. Below each melanocortin peptide position is the sequence of the human form of the peptide. Note that the HFRW motif in each peptide sequence is highlighted in *blue*. The relative position of the β-endorphin sequence is highlighted in *black*. (B) The phylogeny of cartilaginous fish POMC, Bony Fish POMC, and Tetrapod POMC is presented in this schematic representation. The location of the melanocortin sequences is shown in *red*, and the location of the β-endorphin sequence is shown in *black*. Note the organizational plan for the POMC sequences is very similar for these representative gnathostomes. (C) Comparison of Gnathostome ACTH Sequences. The amino acid sequences for representative gnathostome ACTH orthologs were aligned. The HFRW motif is highlighted in *blue* and the tetrabasic motif is highlighted in *red*. The location of ACTH(1–24) for each sequence is underlined with a *black line*. The following sequences were aligned: Chick (*Gallus gallus* NP_001026269); Alligator (*Alligator mississippiensis* NP_0012745535.1); Frog (*Xenopus tropicalis* NP_0011318.1); Gar (*Lepisosteus osseus* AAB03227.1); Dogfish (*Squalus acanthias* BAA 32606.1).

"Melanocortin Receptors." That said, the differential posttranslational processing of POMC observed in the pituitary of mammals is also seen in the pituitaries of cartilaginous fishes and nonmammalian bony vertebrates (Dores and Lecaude, 2005). In addition, as shown in **Fig. 2**B, the basic organization plan for POMC (i.e., multiple melanocortin peptide sequences and a single β-endorphin sequence) is found in cartilaginous fishes and the bony vertebrates. Subtle variations in this organizational plan are seen in cartilaginous fishes with the presence of an additional melanocortin sequence (δMSH), and in teleosts with the loss of the γMSH sequence (Vallarino *et al.*, 2012). Thus, it would be reasonable to assume that the POMC common precursor in the ancestral gnathostomes also had multiple melanocortin peptide sequences and a β-endorphin sequence, and that in the pituitary of the ancestral gnathostomes the enzymes for performing cell-specific differential posttranslational processing were in place.

Finally, as shown in **Fig. 3**C, gnathostome ACTH sequences have a number of interesting features. Earlier studies had shown that full bioactivity for ACTH only requires the first 24 amino acids of the peptide [ACTH(1–24); **Fig. 3**C, underlined] (Schwyzer, 1977). Within this domain of ACTH, there is 83% primary sequence identity for the ACTH orthologs presented in **Fig. 3**C. In addition, all of the ACTH sequences have the canonical HFRW motif highlighted in *blue*, and all of the ACTH sequences have a tetrabasic amino acid motif (KKRR or RKRR) highlighted in *red*. As noted, in cells of the intermediate pituitary and POMC neurons in the central nervous system, the tetrabasic motif serves as an endoproteolytic cleavage site that results in the generation of αMSH. Hence in these secretory cells ACTH(1–39) serves as a biosynthetic intermediate (Lowry *et al.*, 1977). However, when corticotropic cells of the anterior pituitary secrete ACTH(1–39), the tetrabasic amino acid motif plays an important role in the activation of melanocortin-2 receptors of bony vertebrates (see Section "Melanocortin Receptors"). These two functional roles for the tetrabasic motif of ACTH are quite unique, and it is difficult to identify another chemical signal with similar properties.

Melanocortin Receptors

The melanocortin receptors are G protein-coupled receptors (GPCR) that were initially characterized from mammals in the early 1990's (for review see Cone, 2006), and then later from several species of nonmammalian tetrapods, bony fishes, and a few cartilaginous fishes (Schiöth *et al.*, 2003). The melanocortin receptors are Class 1 (Rhodopsin-like) GPCRs that belong to Subfamily 13 (Attwood and Findlay, 1994). As shown in **Fig. 3**, these receptors have seven transmembrane domains, and when positioned on the plasma membrane, an N-terminal domain faces the extracellular space and a C-terminal domain faces the cytosol. These various domains differ in amino acid sequence to a sufficient degree such that five paralogous genes are clearly

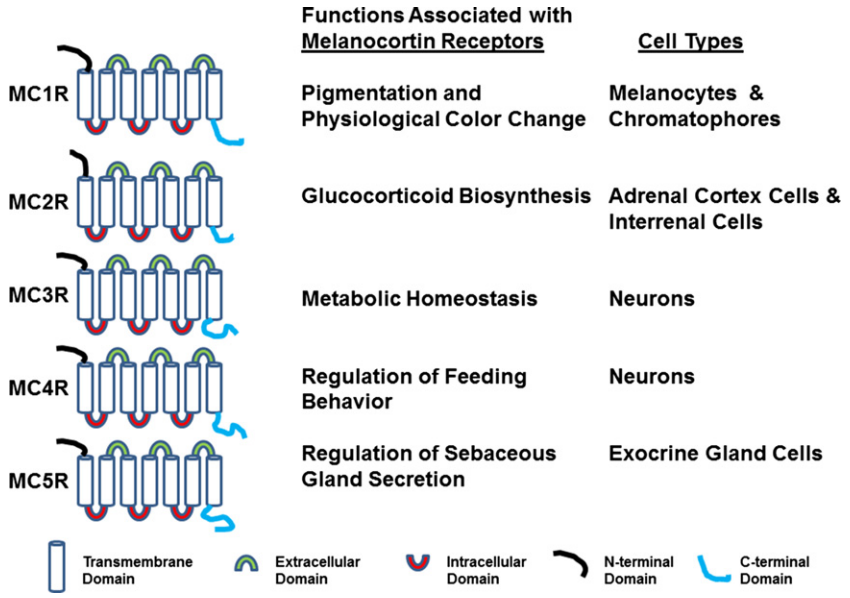

Fig. 3 Melanocortin receptors. This figure presents a schematic representation of the five melanocortin receptors. Some of the functions associated with each receptor are presented, and the cell types associated with these functions are also presented.

present in gnathostome genomes, but these receptors share a number of amino acid motifs which clearly define the melanocortin receptors as a distinct family within Class 1.

The functions that have been studied most extensively for these receptors are listed in **Fig. 3**. However, it is appreciated that members of this receptor family have additional functions and a broader tissue distribution than indicated in **Fig. 3** (Caruso *et al.*, 2014). In this regard, several studies indicate that MC1R can be found on the surface of melanocytes and melanophores, and these cells located in the integument play a role in the regulation of pigmentation in mammals, and physiological color change in several species of amphibians, reptiles, and teleosts, respectively (Cone, 2006; Bagnara *et al.*, 1968; Logan *et al.*, 2006). MC2R can be found on the surface of adrenal cortex cells (mammals, birds, and reptiles), and interrenal cells (amphibians and fishes), and the binding of ACTH to this receptor initiates glucocorticoid biosynthesis (Gallo-Payet and Bastista, 2014). MC3R and MC4R are found on neurons in the central nervous that are involved in the regulation of metabolism and feeding behavior (Abdel-Malek, 2001). In mammals and perhaps other gnathostomes, MC5R has been implicated in the regulation of sebaceous gland secretion (Thiboutot *et al.*, 2000). However, MC5R appears to also play a role in the immune system (Taylor *et al.*, 2006). An objective of this review is to comment on three issues related to melanocortin receptor pharmacology, which have physiological implications: (a) pharmacological features of MC1R, MC3R, MC4R, and MC5R orthologs in teleosts and tetrapods; (b) pharmacological properties of MC2R orthologs in teleosts and tetrapods; and (c) the pharmacological properties of melanocortin receptors in cartilaginous fishes.

Pharmacological Properties of Teleost and Tetrapod MC1R, MC3R, MC4R, and MC5R

From a pharmacological perspective, teleost and tetrapod MC1R, MC3R, MC4R, and MC5R orthologs appear to exhibit the ligand selectivity redundancy that can occur as a result of genome duplications events. There are now several studies which show that following the individual expression of these paralogous melanocortin receptors in various mammalian cell lines, activation of any of these receptors can be achieved following incubation with either ACTH- or MSH-related ligands (Gantz and Fong, 2003; Ling *et al.*, 2004; Dores *et al.*, 2014; Takahashi *et al.*, 2016). What are the implications of these observations from a physiological perspective? Consider that in an amphibian or reptile that utilizes melanophores for physiological color change, the simultaneous release of αMSH, βMSH, and γMSH from cells of the intermediate pituitary will stimulate activation of melanocortin-1 receptors on chromatophores to facilitate physiological color change. However, since these waves of MSH-sized ligands are released into the vascular system, melanocortin-5 receptors positioned on cells of various organs in the periphery could also be activated. While "side effects" are certainly possible when amphibians and reptiles release melanocortin peptides during a physiological color evident, documentation of these "side effects" has not been a focus of these types of studies.

This issue of "cross-talk" between melanocortin receptors in the periphery of gnathostomes may be more relevant following the release of ACTH from corticotropic cells of the anterior pituitary. While the intended target for ACTH when the Hypothalamus/Pituitary/Adrenal-Interrenal system is activated is the melanocortin-2 receptors located on either adrenal cortex cells or interrenal cells (see Section "Pharmacological Properties of Teleost and Tetrapod MC2R"), ACTH could also activate melanocortin-1 receptors and melanocortin-5 receptors located on various cell types in the periphery. For example, ACTH does effect melanin synthesis in mammalian melanocytes in the integument (Carlson *et al.*, 2007), and pigment granule movement within

chromatophores of amphibians (Burgers and Van Oordt, 1962). That said, the physiological effects of ACTH binding to melanocortin-5 receptors on organs in the periphery have received relatively little attention.

In the case of the melanocortin-3 receptor and the melanocortin-4 receptor, the focus shifts to the roles that these receptors play on neurons in the central nervous system. In these circuits presynaptic neurons express the *pomc* gene and following post-translational process of POMC, package MSH-sized end products in regulated secretory vesicles. At the synapse, either melanocortin-3 receptors or melanocortin-4 receptors are positioned on the dendrite of the postsynaptic neuron. In for example mammals, at the synapse αMSH, βMSH, and γMSH will be released in equimolar amounts by the presynaptic neuron thus concentrating the melanocortin signal at the synapse for interaction with the melanocortin receptors on the postsynaptic neuron. The implications for signaling from all three melanocortin peptides interacting with the receptor virtually simultaneously have not been evaluated. The common approach for studying the ligand selectivity of melanocortin-3 and melanocortin-4 receptors is to transiently express these receptors individually in a mammalian cell line, and then stimulate with a single melanocortin peptide.

That said, teleost and tetrapod MC1R, MC3R, MC4R, and MC5R paralogs can respond to ACTH or any of the MSH-sized ligands due to the fact that all melanocortin peptides have the HFRW motif, a motif required for activation of melanocortin receptors, and in these melanocortin receptor paralogs there is a common HFRW binding site. A schematic representation of the HFRW binding site for the human melanocortin-4 receptor is presented in **Fig. 4**A. Pogozheva *et al.* (2005) used a single alanine substitution paradigm to identify critical amino acid positions in human MC4R in transmembrane domains 2, 3, 6, and 7, which form an HFRW binding site near the surface of the receptor. These amino acid positions are rigorously conserved in human MC1R, MC3R, and MC5R (Pogozheva *et al.*, 2005), and these amino acids are also conserved in the MC1R, MC3R, MC4R, and MC5R paralogs of bird, amphibian, and bony fishes (Dores, 2009; Baron *et al.*, 2009). Hence, the presence of this HFRW binding site in teleost and tetrapod MC1R, MC3R, MC4R, and MC5R paralogs accounts for the generic ligand selectivity of these receptors for any melanocortin-related peptide.

Pharmacological Properties of Teleost and Tetrapod MC2R

By contrast, teleost and tetrapod melanocortin-2 receptor orthologs have more stringent ligand selectivity properties. The melanocortin 2 receptor is a critical component of the Hypothalamus/Pituitary/Adrenal axis is mammals, birds, and reptiles, and the

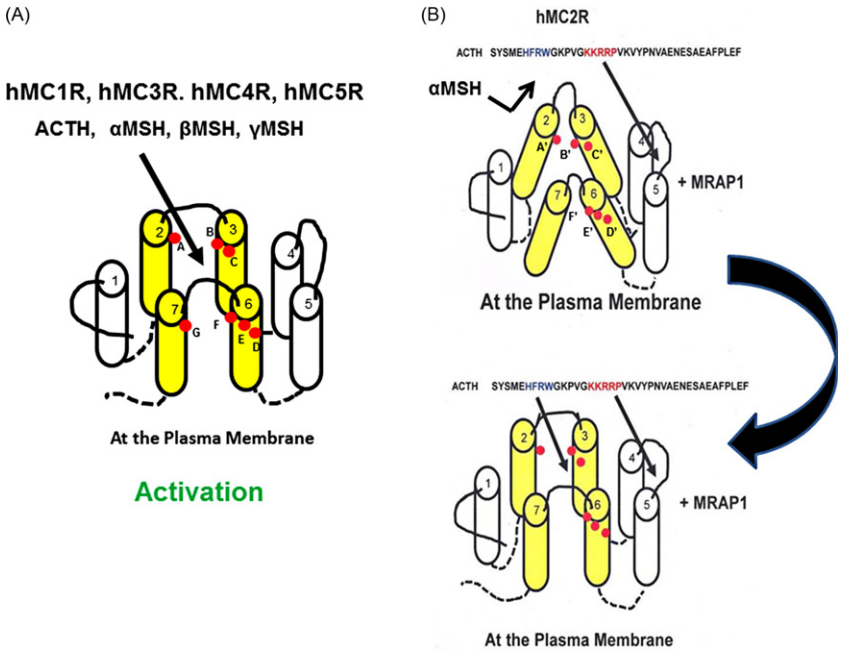

Fig. 4 Proposed mechanisms for activating melanocortin receptors. (A) Human MC4R is presented in a cartoon format, and the critical amino acid positions in the HFRW binding site are based on the work of Pogozheva *et al.* (2005). That study indicated that the following amino acid positions are involved in the αMSH binding event: (A) E^{100} in TM2, (B) D^{122} in TM3, (C) D^{126} in TM3, (D) F^{261} in TM6, (E) H^{264} in TM6, (F) L^{265} in TM6, and (G) L^{288} in TM7. These transmembrane domains form a shallow hydrophobic binding pocket on the extracellular space side of the receptor. The binding of αMSH at this pocket results in the activation of the G protein associated with human MC4R. (B) Human MC2R has six of the seven conserved amino acid position predicted for the HFRW binding site (i.e., A′, B′, C′, D′, E′, and F′). However, αMSH cannot bind to this receptor (Mountjoy et al., 1992). As a result, in this cartoon TM2, TM3, TM6, and TM7 are depicted in a confirmation in which the HFRW binding site is closed. Hence, the proposed mechanism for activation of human MC2R by ACTH(1–24) involves a two-step process. First the tetrabasic motif in ACTH(1–24) binds to a predicted site in the TM4/EC2/TM5 domain (Dores et al., 2016). In this proposed mechanism, binding at the TM4/EC2/TM5 domain would result in a conformation change in the receptor that would open the HFRW binding site, and activation of the G protein associated with human MC2R would occur after this second binding event.

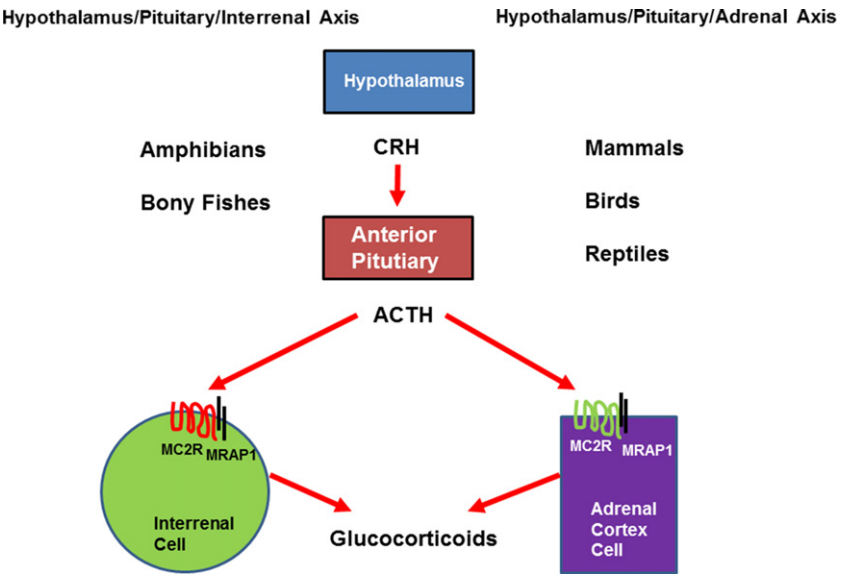

Fig. 5 Overview of the Hypothalamus/Pituitary/Adrenal-Interrenal axis. The Hypothalamus/Pituitary/Adrenal axis (HPA) and the Hypothalamus/Pituitary/Interrenal axis (HPI) follow a common pathway. Sensory information is evaluated by higher brain centers, and as a result, neurons in the hypothalamus release the neuropeptide CRH (corticotropin releasing hormone). CRH binds to receptors on corticotropic cells in the anterior pituitary and ACTH is released. For mammals, birds, and reptiles ACTH will bind to the melanocortin-2 receptor (MC2R) that is associated with the accessory protein, MRAP1 on adrenal cortex cells. This binding event initiates the synthesis and release of glucocorticoids. In amphibians and fishes, ACTH will bind to the melanocortin-2 receptor (MC2R) that is associated with the accessory protein, MRAP1 on interrenal cells to initiate the synthesis and release of glucocorticoids.

Hypothalamus/Pituitary/Interrenal axis of amphibians and bony fishes (**Fig. 5**). The primary objective of the HPA/HPI axis is to restore homeostasis for metabolic processes, the immune system, and behavior within an organism after a stress event through the release and action of glucocorticoids: steroid released from adrenal cortex cells or interrenal cells (Abdel-Malek, 2001).

As summarized by Schwyzer (1977), the "ACTH" receptor on mammalian adrenal cortex cells could be activated by ACTH, but not by αMSH. Mountjoy *et al.* (1992) renamed the human "ACTH" receptor, MC2R, and found that this melanocortin receptor paralog could only be activated by ACTH, but not by αMSH. Subsequent studies on bird, reptile, amphibian, and teleost MC2R orthologs have also showed that these MC2R orthologs could only be activated by ACTH, but not by αMSH (Agulleiro *et al.*, 2010; Davis *et al.*, 2013; Barlock *et al.*, 2014). The paradox in these observations is that six of the seven critical amino acid positions in human MC4R required for αMSH binding are present in human MC2R, and other tetrapod and teleost MC2R orthologs (**Fig. 4**B; Pogozheva *et al.*, 2005; Dores, 2009; Baron *et al.*, 2009). To reconcile these observations, the cartoon presented in **Fig. 4**B would propose that the HFRW binding site on teleost and tetrapod MC2R orthologs is closed prior to a binding event; hence αMSH is blocked from binding to the receptor. However, to account for ACTH's ability to active MC2R orthologs, Schwyzer (1977) proposed that the tetrabasic motif (i.e., $K^{15}K^{16}R^{17}R^{18}$) in human ACTH) binds to another site on the receptor. It is further proposed that this binding event causes a conformation change in the receptor to open the HFRW binding site, and this two-step process results in activation of the G Protein (**Fig. 4**B). Support for this hypothesis comes from alanine substitution experiments in the TM4/EC2/TM5 domain of human MC2R (Chen *et al.*, 2007; Dores *et al.*, 2016). Single alanine substitution at several positions in this domain either lowered or completely blocked activation of human MC2R following an ACTH stimulation event. Definitive binding studies to confirm that the TM4/EC2/TM5 domain in teleost and tetrapod MC2R orthologs is the tetrabasic motif binding site remain to be done. In addition, the caveat to the mechanism presented in **Fig. 4**B is that the MC2R ortholog must form a heterodimer with the accessory protein, MRAP1. Previous studies had shown that when MC2R orthologs were expressed alone in nonadrenal mammalian cell lines, the MC2R ortholog did not traffic to the plasma membrane (Rached *et al.*, 2005). Thus to understand the unique ligand selectivity properties of teleost and tetrapod MC2R orthologs, an understanding of the accessory proteins, MRAP1 and MRAP2, is needed. However, before getting into that discussion some comments on ligand selectivity properties of cartilaginous fish melanocortin receptors should be considered.

Pharmacological Properties of Melanocortin Receptors of Cartilaginous Fishes

As noted in **Fig. 1**B, early in the radiation of the ancestral gnathostomes, the ancestral cartilaginous fishes diverged from the ancestral bony fishes (Sallan and Coates, 2010). Since the ancestral gnathostomes most likely had all five melanocortin receptor genes, this gene family has been diverging in parallel in the cartilaginous fishes lineage and the bony vertebrate lineage with some interesting twists.

The ligand selectivity for various cartilaginous fish melanocortin receptors have been determined for the dogfish (*Squalus acanthias*) MC3R, MC4R, and MC5R (Ringholm *et al.*, 2003; Klovins *et al.*, 2004: Reinick *et al.*, 2012a); the elephant shark

(*Callorhinchus milii*) MC2R and MC3R (Reinick *et al.*, 2012b; Liang *et al.*, 2013); and the Japanese stingray (*Dasyatis akajei*) MC1R, MC2R, MC3R, MC4R, and MC5R (Takahashi *et al.*, 2016). For every cartilaginous fish melanocortin receptor paralog, even elephant shark MC2R and stingray MC2R, the paralog could be activated by either a cartilaginous fish ACTH or cartilaginous fish MSH-sized peptide (i.e., αMSH, Des-Acetyl-αMSH, βMSH, γMSH, or δMSH). In addition, the elephant shark and stingray MC2R orthologs did not require coexpression with an MRAP1 ortholog for functional express in Chinese Hamster Ovary Cells (Reinick *et al.*, 2012b; Takahashi *et al.*, 2016). The study on stingray MC2R is particularly interesting because this receptor, as well as stingray MC5R, is expressed in stingray interrenal cells. Cartilaginous fishes do have a Hypothalamus/Pituitary/Interrenal axis (Gelsleichter, 2004), hence at least for the stingray, the interrenal cells could theoretically be induced to produce glucocorticoids either through the action of ACTH- or the MSH-sized peptides.

While the ligand selectivity properties of cartilaginous fish MC1R, MC3R, MC4R, and MC5R paralogs are not surprising, the ligand selectivity properties of elephant shark MC2R and stingray MC2R are unexpected. Perhaps at this stage it would be appropriate to consider the radiation and properties of the accessory proteins, MRAP1 and MRAP2.

Melanocortin Receptor Accessory Proteins: MRAP1 and MRAP2

The inability to functionally express mammalian MC2R orthologs in nonadrenal mammalian cell lines (Rached *et al.*, 2005) was an enigma until the discovery of the accessory protein, MRAP (Metherell *et al.*, 2005). Not long after the discovery of MRAP, a paralog, MRAP2, was characterized (Chan *et al.*, 2009). For clarity MRAP will be referred to as MRAP1. In **Fig. 6** mouse MRAP1 and MRAP2 are used to illustrate the functional domains in teleost and tetrapod MRAP1 and MRAP2 paralogs. The first feature to note is that both MRAPs form a homodimer in which each monomer is oriented in an antiparallel manner. This orientation is referred to as reverse topology (Sebag and Hinkle, 2009). There is a critical amino acid motif in the N-terminal domain of MRAP1 and MRAP2 that is required for reverse topology (**Fig. 6**). In addition, in MRAP1 orthologs, but not in MRAP2 orthologs, there is another amino acid motif in the N-terminal domain referred to as the activation motif (**Fig. 6**). Alanine substitution in this region of the N-terminal of MRAP1 interferes with activation of teleost and tetrapod MC2R orthologs when the heterodimer forms (Sebag and Hinkle, 2009).

As shown in **Fig. 6**, since MRAP1 forms a homodimer with reverse topology, it was not apparent which activation motif (i.e., extracellular space side or cytosolic side) was making contact with teleost or tetrapod MC2R. However, recently Malik *et al.* (2015) found that the activation motif on the extracellular space side of MRAP1 makes contact with an extracellular domain on MC2R. At present the exact extracellular domain has not been identified. In addition, Sebag and Hinkle (2007) observed that the transmembrane domain of MRAP1 makes contact with a transmembrane domain on MC2R to facilitate trafficking of the receptor from the endoplasmic reticulum to the plasma membrane. Once again the exact transmembrane domain in MC2R interacting with MRAP1 has not been identified.

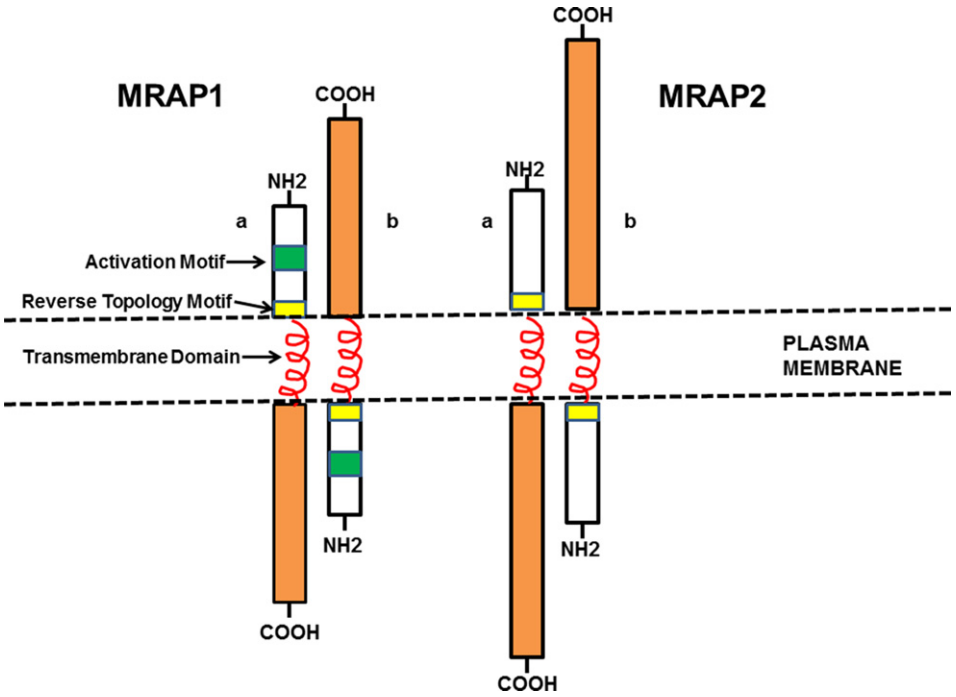

Fig. 6 MRAP1 and MRAP2. This figure provides a cartoon representation of MRAP1 and MRAP2. Mouse MRAP1 and MRAP2 were used as examples. Please note the images are not drawn to scale. Both MRAP1 and MRAP2 form a homodimer with reverse topology. For each homodimer the monomers are presented as (a) and (b). Each monomer has a single transmembrane domain. The C-terminal domain for each monomer is demarcated by an orange box (COOH). In the N-terminal (NH2) of each monomer an amino acid motif is present this is required for reverse topology (*yellow box*). Only in the N-terminal domain of MRAP1 monomer will you find an activation motif (*green box*).

As noted in Section "Introduction", the current hypothesis is that the ancestral *mrap* gene emerged prior to the 2R genome duplication event. As a result, the *mrap1* and *mrap2* genes were most likely present in the genome of the ancestral gnathostomes. Recently an MRAP1 ortholog was detected in the genome of the elephant shark. Thus, it is possible that other cartilaginous fishes also have an MRAP1 ortholog in their genomes. While the role that MRAP1 plays in the trafficking and activation of bony vertebrate MC2R orthologs is now very clear, the possible interaction between cartilaginous fish MRAP1 and cartilaginous fish MC2R orthologs remains to be determined.

Conclusions and Possibilities

When considering the coevolution of melanocortin peptides, melanocortin receptors, and melanocortin receptor accessory proteins it would appear that all three elements originated within phylum Chordata, but most likely not simultaneously. The ancestral *pomc* gene and the ancestral *melanocortin receptor* gene probably arose in a lineage of the protochordates on the line of evolution that gave rise to the jawless fishes. The ancestral *melanocortin receptor accessory protein* gene arose much later in a lineage of ancestral jawless fishes on a line of evolution which gave rise to the gnathostomes. The circumstances that linked the melanocortin peptides to the melanocortin receptors, or the sequence of events which led to the interaction between MRAP1 and the melanocortin-2 receptor, are unknown. However, there are features of this neuroendocrine network which played critical roles in forming these connections.

The interaction between melanocortin peptides and melanocortin receptors hinges on the HFRW motif in the melanocortin peptides, and the emergence of a binding site for this amino acid motif on melanocortin receptors. To retain this relationship, in gnathostomes there have been strong selection pressures to maintain, to a high degree, the primary sequence of the first 24 amino acids in ACTH. As a result of these selection pressures the primary sequence of αMSH is also highly conserved among gnathostomes. In addition, the high degree of primary sequence conservation in ACTH (1–24) helps to explain the unique ligand selectivity of bony vertebrate MC2R orthologs where both the HFRW motif and the tetrabasic amino acid motif (i.e., KKRR or RKRR) are essential for activation. Since the cartilaginous fish MC2R orthologs that have been studied can be activated by either ACTH or αMSH, the role that the tetrabasic amino acid motif in ACTH is playing most have emerged after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes. That said, the phylogeny of the melanocortin-2 receptor appears to be unique as compared to the other melanocortin receptors.

While most gnathostomes have retained all five paralogous melanocortin receptor genes, and each melanocortin receptor has an HFRW binding site, there is a very clear dichotomy between bony vertebrate melanocortin receptors. Whereas MC1R, MC3R, MC4R, and MC5R can all be activated by either ACTH- or MSH-sized ligands, MC2R can only be activated by ACTH. This dichotomy for ligand selectivity is not seen in the cartilaginous fish melanocortin receptors. Clearly something "happened" to MC2R following the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes from their common ancestor. Vastermark and Schiöth (2011) have observed that gnathostome MC3R, MC4R, and MC5R paralogs have maintained a high degree (at least 65%) of primary sequence identity during the radiation of gnathostomes, which would explain the common ligand selectivity properties of these receptors in taxa as diverse as cartilaginous fishes and mammals. By contrast, during the radiation of the gnathostomes, MC2R orthologs have undergone extensive primary sequence variation. For example, stingray MC2R and human MC2R have only 37% primary sequence identity, and even among bony vertebrates the primary sequence identity between gar MC2R and human MC2R is only 55% (Dores, 2016). Given the important role that MC2R plays in the Hypothalamus/Pituitary/Adrenal-Interrenal axis, the degree of primary sequence variable among MC2R orthologs is counterintuitive.

Hence, in order to put the preceding observations into a physiological context, it is important to factor in the pivotal role that MRAP1 plays in the Hypothalamus/Pituitary/Adrenal-Interrenal axis. While the function that MRAP1 played in the interrenal cells of the ancestral gnathostomes is unknown, several studies on extant bony vertebrate MC2R orthologs indicate that the interaction between MRAP1 and MC2R not only makes it possible for MC2R to move from the endoplasmic reticulum to the plasma membrane, but also places MC2R into a unique conformation such that this receptor can only be activated by ACTH, but not by any of the MSH-sized melanocortin peptides (Metherell *et al.*, 2005; Hinkle and Sebag, 2009; Webb and Clark, 2010). The next issue that should be resolved for the bony vertebrate MC2R orthologs is to identify the sites on the receptor that make contact with the activation motif and the trafficking motif in MRAP1. With this information it should be possible to critically analyze the proposed MC2R activation mechanism presented in **Fig. 4**. In addition, since an MRAP1 ortholog has been detected in the genome of the elephant shark (Dores, 2016), studies are need to determine whether MRAP1 plays some supporting role in the activation cartilaginous fish MC2R orthologs.

See also: Adrenal Steroidogenesis

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Further Reading

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Adrenocorticotropic Hormone (ACTH): Physiology and Its Involvement in Pathophysiology☆

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Glossary

Adrenocorticotropic hormone (ACTH) A 39 amino-acid peptide hormone (MW 45,000) that is part of the proopiomelanocortin precursor molecule. It controls the function of the adrenal cortex.

Proopiomelanocortin (POMC) A precursor molecule (MW 28,500) that, in the anterior lobe of the pituitary, is processed to adrenocorticotropic hormone and b-lipotropin and further processed to b-endorphin, an endogenous opioid peptide of 31 amino acids

Biochemistry

Adrenocorticotropic hormone (ACTH) is a 39-aminoacid peptide hormone (MW 45,000) that is part of the proopiomelanocortin (POMC) precursor molecule (MW 28,500). The PMOC gene is located on chromosome 2. Proopiomelanocortin is expressed in the brain, skin, and immune system and in the anterior and intermediate lobes of the pituitary gland. This gene undergoes extensive, tissue-specific, post-translational processing via cleavage by subtilisin-like enzymes known as prohormone convertases. There are eight potential cleavage sites within the polypeptide precursor and, depending on tissue type and the available convertases, processing may yield as many as ten biologically active peptides involved in diverse cellular functions. The encoded protein is synthesized mainly in corticotroph cells of the anterior pituitary where four cleavage sites are used (DiBlasio *et al.*, 1987). Specifically POMC is processed to ACTH and β-lipotropin (β-LPH), which is further processed to β-endorphin, an endogenous opioid peptide of 31 amino acids. (Fig.1). In the intermediate lobe, ACTH is processed to α-melanocyte-stimulating hormone (α-MSH) (ACTH 1–13) and CLIP (ACTH 18–39). In human corticotrophs, POMC is processed predominantly into N-terminal glycopeptide (1–76), joining peptide (JP 79–108), ACTH (112–150) and β-LPH (153–240). Much smaller amounts of α-MSH (112–124), CLIP (130–150), β-endorphin (210–240) and a truncated form of N-terminal glycopeptide (1–61), also known as "big" γ-MSH, are also present. There is no evidence of cleavage after arg (50) and therefore no evidence for the presence of γ-MSH (51–61) in the human pituitary. Whereas the production of distinct POMC peptide derivatives is clearly segregated between the anterior and intermediate lobes of the rodent pituitary evidence suggests that the strict dichotomy between corticotrope and melanotrope POMC processing doesn't extend to human pituitaries. Human POMC is also expressed in several brain sites outside the anterior pituitary predominantly in the arcuate nucleus of the anterior hypothalamus and the nucleus tractus solitarius of the caudal medulla where it generates a range of bioactive peptides, including ACTH, β-lipotropin, and γ-MSH. β-Lipotropin, a fragment with 91 amino acids, contains β-MSH (41–58), γ-LPH (1–58), and β-endorphin which is secreted in equimolar quantities with ACTH. The endogenous opioid peptides α-, β-, and γ- endorphin are derived from POMC and are composed of amino acids 61–76, 61–91, and 61–77, respectively, of β-LPH. In these extrapituitary locations POMC is expressed to a greater extent than in anterior pituitary. In the brain

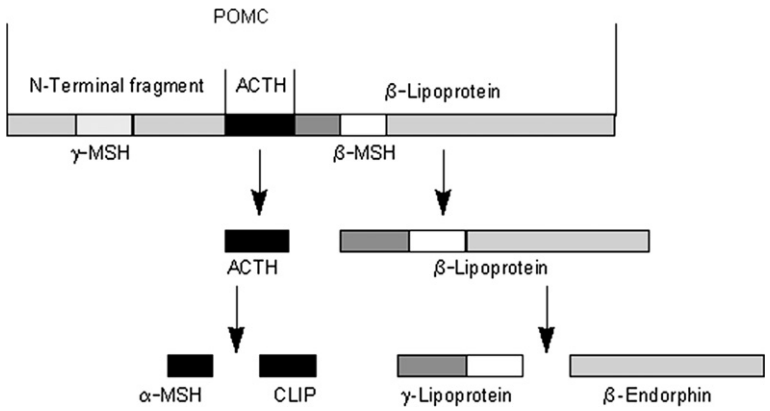

Fig. 1 Enzymatic process of POMC in the pituitary.

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ACTH (112–150) is cleaved to α-MSH (112–124) and CLIP (130–150) so that the amount of α-MSH (112–124) relative to ACTH is 300-fold higher in hypothalamus telencephalon and mesencephalon than it is in anterior pituitary (Bicknell, 2008).

The biological effects of POMC-derived peptides are diverse and are largely mediated through melanocortin (MC) receptors (R), five of which have been described. MC1R, MC2R, and MC5R have established roles in the pigmentation in the skin, adrenal steroidogenesis, and thermoregulation, respectively. However, the functional basis of the role of POMC in appetite and obesity is largely due to interactions between POMC-derived peptides in the brain and the neuroendocrine specific receptors, MC3R and MC4R (Smith and Funder, 1988; Whitfeld *et al.*, 1982).

In other tissues, including the hypothalamus, placenta, and epithelium, all cleavage sites may be used, giving rise to peptides with roles in pain and energy homeostasis, melanocyte stimulation, and immune modulation.

Pathology and Embryology

ACTH is synthesized within the corticotroph cells of the anterior pituitary lobe which represent 15–20% of adenohypophyseal cells. They are most numerous in the midsagittal region of the pituitary (median wedge) but also occur in the lateral wings of the gland. The embryological origin of corticotrophs is the intermediate lobe, but groups of cells migrate during development into regions of the anterior and posterior lobes (Grino *et al.*, 2001). Although the vast majority of ACTH is synthesized in anterior pituitary corticotrophs, it is also expressed in several non pituitary human tissues both within and outside the central nervous system (e.g. infundibular nucleus of the basal hypothalamus, substantia nigra, periventicular grey matter and hippocampus). In humans in addition to sellar pituitary there is a pharyngeal pituitary which is located in either the sphenoid sinus or within the sphenoid bone. It consists of pituitary-like tissue approximately 2–5 mm by 0.2 mm in size. It is connected to the sellar gland by trans-sphenoidal vessels. Only 1–2% of the cells in the pharyngeal pituitary contain immunoreactive ACTH in contrast to approximately 14% of the cells in the sellar pituitary (Favrod-Coune *et al.*, 1986).

Besides ACTH many other neuropeptides have been found to be co-localized in the corticotrophs (e.g. neurophysin, galanin, chromogranin A) although in most cases it is not clear whether this is due to binding or synthesis of the peptide within the cell. Histologically, corticotroph cells are polygonal medium sized to large sized, basophilic and strong PAS positive because of a carbohydrate moiety contained in POMC (Fig.2). In immunostained preparation corticotrophs show strong and diffuse cytoplasm ACTH staining consistent with the numerous secretory granules present at the ultrastructural level. Corticotrophs may also contain other POMC derivatives, including β-LPH, MSH, endorphin and enchepalin. Perinuclear bundles of cytokeratin filaments are also a characteristic feature of ACTH cells. Under conditions of glucocorticoid excess, either exogenous or endogenous, corticotrophs accumulate cytokeratin as a manifestation of Crooke's hyaline change (Puy and Ciocca, 1986; Doniach, 1985).

Measurement of ACTH

As regards assay methodology, comparison between competitive, single-antibody RIA and sandwich, two-site immunometric assays revealed that human plasma contains different ACTH species which can variably affect assay results. In most laboratories, RIA has been superseded by non-competitive radiometric or chemiluminescent assays but, even among the latter, some will yield up to 20% higher

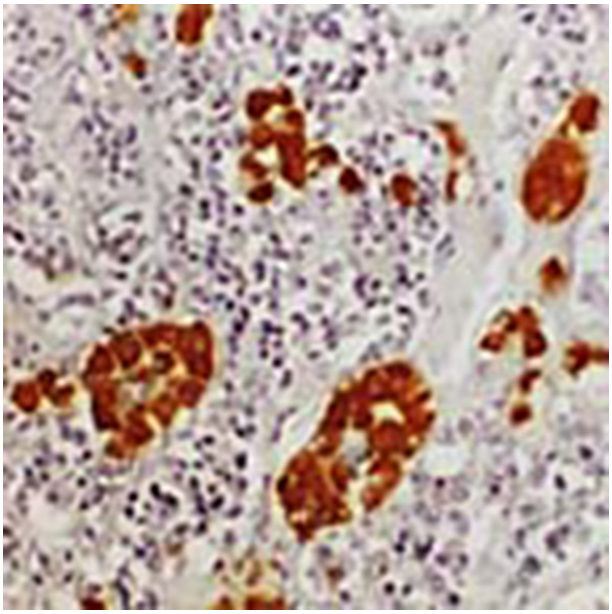

Fig. 2 Corticotroph cells in the anterior pituitary positively stained immunohistochemically with a basophilic staining.

measurements than others. In fact, although correlation between measurements was statistically sound, deviances in the lower assay range may prove clinically significant for certain situations (for example the differential diagnosis of Cushing syndrome). The basal morning concentration of ACTH ranges from 9 to 80 pg ml^{-1}. The episodic secretion of ACTH causes fluctuations in plasma ACTH and cortisol levels. ACTH secretion has a distinct diurnal rhythm, with peak levels in the early morning and the lowest levels at approximately midnight. The interpretation of ACTH values requires a simultaneous cortisol determination. Provided that adrenocortical function is intact, plasma cortisol measurements, from a practical viewpoint, are a reliable index of ACTH secretion (Giraldi and Alberto, 2015) The half-life of ACTH depends on the assay used for its measurement. Bioactive ACTH disappears from the circulation more rapidly (half-life of 3–9 min) than does immunoreactive ACTH (half-life of 7–12 min).

Physiology

Control of ACTH Secretion

The secretion of ACTH is controlled by an inherent diurnal rhythmicity; it is augmented by noxious stimuli that are neurally, hormonally, and biochemically mediated, which is termed stress (open-loop component), and is inhibited by glucocorticoids (closed-loop, negative feedback) (Miller and Tyrell, 1995). In normal circumstances, the daily secretion of ACTH and cortisol is episodic and variable (Jefcoate *et al.*, 1986; Krieger, 1979). The highest burst of activity is observed in the early morning hours. ACTH secretion is characterized by pulsatile release of ACTH from the corticotroph in a burst-like pattern with no interpulse secretion. Fifteen minute sampling reveals approximately 12 ACTH and cortisol pulses over a 24 h period, whereas more frequent 10 min sampling reveals a 40 ACTH pulses in 24 h. Blood ACTH rises by an average of 24 pg ml^{-1} per pulse. Spontaneous ACTH and cortisol pulses correlate highly. It should be stated also that the 24 h pattern of ACTH pulses, but not the cortisol pattern, differs between males and females (Veldhuis *et al.*, 2009).

Diurnal Rhythmicity

The basis of the diurnal rhythm is poorly understood. There is evidence for the involvement of at least three factors in the regulation of the diurnal rhythmicity of ACTH: (1) intrinsic rhythmicity of the secretion of the corticotropin-releasing factor (CRH) as well as vasopressin (AVP); (2) light–dark exposure; and (3) feeding times. Diurnal variation frequently disappears during periods of stress and depression and is also changed by conditions that affect cortisol metabolism (liver disease, chronic renal failure, alcoholism).

Intrinsic Rhythmicity of Hypothalamic CRH and AVP

Tests for the evaluation of the CRH secretion pattern have shown a diurnal intrinsic rhythmicity that persists even in hypophysectomized animals that are deprived of ACTH and glucocorticoid feedback. This hypothalamic rhythmicity appears to be neuronal but not hormonal. On the other hand, the presence of diurnal rhythmicity of ACTH in women during pregnancy when the increased circulating levels of placenta-derived CRH do not show a nychthemeral rhythm strongly suggests that hypothalamic AVP secretion plays a role in this diurnal regulation (Malee and Mellon, 1991). In addition although in situ hybridization studies show that there is a circadian rhythm in CRH expression in the suprachiasmatic nucleus, other reports do not confirm this. Moreover, the circadian rhythm persists despite a continuous infusion of CRH, suggesting that other factors are responsible for the modulation of ACTH pulses, most likely AVP: immunocytochemical studies show a circadian rhythm in AVP expression and transgenic knockout mice for CLOCK gene show a loss in the circadian rhythm in AVP RNA expression (6–10). The circadian CLOCK system consists of central and peripheral components, which are located respectively in the suprachiasmatic nucleus (SCN) of the hypothalamus and virtually all remaining organs and tissues. The SCN acts as a "master" CLOCK under the strong influence of light/dark input from the eyes, whereas the peripheral CLOCK behaves as a "slave", subjugated by the former through as yet unclear mechanisms. Both master and slave CLOCKs share almost the same transcriptional regulatory machinery with coordinated activation/inactivation of a set of transcription factors including the "circadian locomotor output cycle kaput" (Clock), its heterodimer partner "brain-muscle-arnt-like protein 1" (Bmal1). These transcription factors create a negative feedback transcriptional loop through mutual transcriptional activation and repression that ultimately maintains an approximately 24-h oscillation of their gene expression. Findings indicate that CLOCK/BMAL1 is a reverse-phase negative regulator of glucocorticoid action in target tissues, antagonizing the biologic actions of diurnally fluctuating circulating glucocorticoids and providing a local target tissue counter-regulatory feedback loop to the central clock influence on the HPA axis. As a result, tissue sensitivity to glucocorticoids is decreased in the morning (when circulating cortisol concentrations are elevated) and increased in the evening and early night (when cortisol concentrations reach their nadir). In addition to the predictably regular day/night changes in the environment, humans face frequent unforeseen short- and long-term influences, the "stressors". To adapt to these stressful stimuli, they possess another regulatory system, the hypothalamic-pituitary-adrenal (HPA) axis, (the "Stress System"). The circadian clock system and the HPA axis regulate the activity of one another through multilevel interactions to ultimately coordinate homeostasis against the day/night change and various unforeseen random internal and external stressors. Uncoupling of or dysfunction in either system alters internal homeostasis and causes pathologic changes virtually in all organs and tissues, including those responsible for

intermediary metabolism and immunity. Disrupted coupling of cortisol secretion and target tissue sensitivity to glucocorticoids may account for (i) the development of central obesity and the metabolic syndrome in chronically stressed individuals, whose HPA axis circadian rhythm is characterized by blunting of the evening decreases of circulating glucocorticoids, as a result of enhanced input of higher centers upon the hypothalamic paraventricular nucleus secretion of CRH and AVP; and (ii) the increased cardiometabolic risk and increased mortality of rotating shift workers or subjects exposed to frequent jet lag because of traveling across time zones. In addition, given that tissue sensitivity to glucocorticoids is increased in the evening, clinicians should avoid the administration of high doses of glucocorticoids for the treatment of adrenal insufficiency or congenital adrenal hyperplasia at night, because they increase the possibility of glucocorticoid-related side effects (Takahashi *et al.*, 2008; Nader *et al.*, 2009; Chrousos, 2009; Roozendaal *et al.*, 2009; Nicolaides *et al.*, 2014; Kino and Chrousos, 2011; Ko and Takahashi, 2006; Hastings *et al.*, 2007; Kalsbeek *et al.*, 2006).

Light–Dark Cycles

In parallel with cortisol, ACTH levels vary in an endogenous circadian rhythm, reaching a peak between 0600 and 0900 h, declining through the day, to a nadir between 2300 and 0200 h, and beginning to rise again at about 0200–0300 h (Veldhuis *et al.*, 1990) This rhythm usually appears after the first year of life but may not be established until the age of 8 years. Normal children between the ages of 1 year and 16 years do not differ from adults in ACTH, β-endorphin and cortisol responses to CRH, and the responses of boys do not differ from girls. Reversal of the normal asleep–awake patterns, as occurs when an individual moves to a distant time zone, is followed by a corresponding change in the diurnal pattern of ACTH secretion over the course of 2–3 weeks (Desir *et al.*, 1981).

Feeding Cycles

Cortisol is well known to rise after eating but not after parenteral feeding. This has been shown in studies to be provoked by two mechanisms: (i) by direct stimulation of the HPA axis; and (ii) via regeneration of cortisone to cortisol by stimulation of 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1). There appear to be key differences not only between the effects of individual macronutrients, but also between the gut hormones which are released in response to enteral nutrients (Quigley and Yen, 1979; Follenius *et al.*, 1982; Stimson *et al.*, 2014).

Open-Loop Control (Stress)

The open-loop component of ACTH control may be initiated by noxious stimuli of various sorts, all of which represent types of physical or emotional stress, such as pain, fever, trauma, hypoglycemia, hypoxia, surgery, anxiety, and depression. All of these stimuli stimulate the secretion of ACTH via the release of CRH. Corticotropin-releasing hormone and AVP are probably the two major physiologic secretagogues of hypophyseal ACTH. Immunoreactive CRH is found in the human hypothalamus in the paraventricular, supraoptic, and infundibular nuclei and also in the human thalamus, cortex, cerebellum, and pons. However, most human CRH-secreting neurons are located in the anterior portion of the paraventricular nucleus and their nerve endings project to the external layer of the median eminence, where CRH is released into the portal hypophyseal circulation. Besides stimulating POMC transcription and ACTH biogenesis, CRH stimulates the release of ACTH, leading to a biphasic response with the fast release of a pre-synthesized pool of ACTH, and the slower and sustained release of newly synthesised ACTH (DeBold *et al.*, 1983). The same neuronal bodies in the paraventricular nucleus also produce AVP. These AVP neurons are probably the most important in vasopressin control of ACTH release, but the major site of vasopressin neurons is the supraoptic nucleus. These vasopressin neurons are usually of the magnocellular type and most of them project to the neural lobe.

There is strong evidence that both α- and β-adrenergic stimuli, cytokines, other hormones (angiotensin II, oxytocin), VIP, ANP, GH secretagogues (e.g. ghrelin, hexarelin, GHRH, GH releasing peptide2-) opiates, as well as the endocannabinoid system are involved in the regulation of ACTH secretion (Mesiano *et al.*, 1991). During infection, autoimmune processes, or trauma, a complex cascade of events ensues, characterized by fever, circulation of cytokines, and alterations in acute-phase proteins in plasma that are important to initiate, propagate, and terminate host defense mechanisms. In addition, it has been known for several decades that activation of the hypothalamic–pituitary–adrenal (HPA) axis occurs in parallel. It has become apparent that several mediators of inflammation play a major role in this phenomenon. Among all cytokines, three tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 are responsible for most of the stimulation of the HPA axis that is associated with the immune/inflammatory response (Chrousos, 1995; Mastorakos *et al.*, 1993). These three cytokines are produced at inflammatory sites and elsewhere in response to inflammation. Tumor necrosis factor, which has a tumoricidal activity and is responsible for cachexia, is the first to appear in the inflammatory cascade of the events and stimulates both IL-1 and IL-6; similarly, IL-1 stimulates both TNF and IL-6. In contrast, IL-6, which participates in a major fashion in the acute-phase reaction, inhibits the secretion of both of the other cytokines. All three inflammatory cytokines have been shown to activate the HPA axis, that is, ACTH secretion in vivo, alone or in synergy with one another. This effect can be blocked significantly with CRH neutralizing antibodies, glucocorticoids, and prostanoid synthesis inhibitors. When administered to humans, both IL-1 and TNF have significant toxicity, including fever, general malaise, and hypotension, at the doses needed to activate the HPA axis. In the past, it has been demonstrated that IL-6,

with its ability to inhibit the two other inflammatory cytokines and its modest toxicity in experimental animals, was a potent stimulator of the HPA axis in humans, causing an impressively marked and prolonged elevation of plasma ACTH and cortisol when administered either subcutaneously or intravenously. The elevations of ACTH and cortisol attained after stimulation with IL-6 were well above those observed with maximal stimulatory doses of CRH, suggesting that parvocellular AVP and other ACTH secretagogues were also stimulated by this cytokine. In a dose–response study, maximal levels of ACTH were seen at doses at which no peripheral AVP levels were increased. At higher doses, however, IL-6 stimulated peripheral elevations of AVP, indicating that this cytokine might also be able to activate magnocellular AVP secreting neurons. This suggested that IL-6 might be involved in the genesis of the syndrome of inappropriate secretion of antidiuretic hormone, which is observed in the course of infectious or inflammatory diseases or during trauma. It has been shown that IL-6, in patients suffering head trauma (an aseptic inflammatory state) and presenting with syndrome of inappropriate secretion of AVP, is quantitatively correlated with AVP. In addition to their hypothalamic effects, the inflammatory cytokines can apparently directly stimulate pituitary ACTH and adrenal cortisol secretion. This may be related to the chronicity of the elevation of the inflammatory cytokines or may be a dose related phenomenon. It is noteworthy that IL-1 and IL-6 are themselves produced in the anterior pituitary and adrenal glands, where they may have autocrine/paracrine effects (Mastorakos *et al.*, 1994; Gionis *et al.*, 2003; Crofford *et al.*, 1997).

Finally it should be stated that cells of the immune system produce CRH, ACTH and endorphins. Releasing hormones and cytokines interact to stimulate ACTH production from immune cells. A feedback loop exists between immune and neuroendocrine systems. Cytokines released by immune cells stimulate secretion of ACTH and glucocorticoids which are active against infection. Subsequently these hormones suppress synthesis of cytokines (Tanaka *et al.*, 1978).

Closed-Loop Feedback

The negative feedback control of ACTH secretion is mediated by cortisol, which exerts inhibitory effects on both the central nervous system and the pituitary. Negative feedback occurs via three mechanisms: (1) fast feedback, which is sensitive to changes in the levels of circulating cortisol, (2) intermediate feedback, and (3) slow feedback, which is sensitive to the absolute cortisol level. Increased concentrations of glucocorticoids accelerate the progression from fast to slow feedback. Fast feedback occurs within seconds to minutes and involves inhibition of CRH-stimulated ACTH and CRH release, not synthesis, and occurs during the period when plasma glucocorticoids are increasing. It doesn't involve protein synthesis and it appears there is some involvement of the endocannabinoid system (Di *et al.*, 2003). Cortisol given to patients at the start of surgery attenuates the surgery induced ACTH rise, and may be an example of fast feedback. Intermediate feedback is due to inhibition of ACTH release but not synthesis, and may be important after short duration of exposure to glucocorticoids or after non continuous, repeated exposure. It develops after 45–20 min, and maximal inhibition occurs 2–4 h after administration of one dose of glucocorticoid. Unlike ACTH, CRH synthesis as well as release may be affected by intermediate feedback. Slow feedback is most important after long exposure to a moderately high dose of glucocorticoid and is a function of the total dose of glucocorticoids, the glucocorticoid level achieved, and the amount of time since the steroid was given. It occurs after more than 24 h of exposure to glucocorticoids and can persist for days. POMC biosynthesis is inhibited leading to inhibition of basal and stimulated ACTH secretion, and intracellular ACTH decreases implying decreased synthesis. Cortisol modulates the responsiveness of the pituitary. The corticotroph is dependent on CRH stimulation to maintain ACTH secretion while glucocorticoids suppress CRH induced ACTH secretion in vivo and in vitro. On the other hand when endogenous cortisol levels are suppressed by metyrapone, basal ACTH and CRH-induced ACTH release is increased. Glucocorticoid inhibition of ACTH secretion from the corticotroph may recover more quickly than CRH secretion from the hypothalamus. Secondary adrenal insufficiency due to long term glucocorticoid therapy may in part be due to continued suppression of hypothalamic CRH secretion. Adrenalectomized patients on exogenous glucocorticoid therapy have a blunted ACTH response to CRH that normalizes after several CRH boluses, suggesting the lack of stimulation of the corticotroph by the CRH suppresses the ACTH response. On the other hand, corticotrophs of patients recovering from trans-sphenoidal surgery for Cushing's disease are profoundly unresponsive to CRH, which cannot be attributed solely to deficient CRH priming. Glucocorticoids decrease the hypothalamic content of CRH and AVP although the sensitivity of CRH and AVP transcription to glucocorticoid feedback is markedly different. Additionally there is evidence that ACTH can inhibit CRH synthesis, and β-endorphin levels, in patients with Addison's disease or hypopituitarism, but not in the context of normal human subjects, suggesting that ACTH inhibits CRH secretion.

Action of ACTH

The adrenal cortex is the principal target organ for ACTH. ACTH stimulates the synthesis and release of steroids by binding to high-affinity plasma membrane receptors of adrenocortical cells. The ACTH–receptor interaction then activates adenyl cyclase and therefore stimulates the production of intracellular cyclic AMP (cAMP). Once formed the cAMP activates a number of intracellular phosphoprotein kinases that mediate both acute and chronic effects on steroidogenesis.

Acute and Chronic Actions of ACTH

ACTH stimulates the synthesis and release of cortisol within 2–3 min by increasing free cholesterol formation as a consequence of increased cholesterol esterase activity and decreased cholesteryl ester synthetase activity. ACTH rapidly promotes the transport of

cholesterol across the mitochondrial membranes, facilitates the binding of cholesterol to the cytochrome P450scc, and facilitates the release of newly synthesized pregnenolone from the mitochondria. ACTH also stimulates the release of adrenal mineralocorticoids and androgens, as well as the release of various intermediate products. Chronic actions of ACTH are exerted on both adrenal architecture and steroidogenesis. ACTH chronically stimulates low-density lipoprotein (LDL) uptake and metabolism and the synthesis of the LDL receptor and of other factors, so it has tropic effects on all known early steps in steroidogenesis. Chronic effects of ACTH on steroidogenesis occur mainly by promoting the transcription of the genes that encode steroidogenic enzymes and other factors. ACTH increases the transcription of the genes for P450scc, P450c17, P450c21, and P450c11 and stimulates the accumulation of human P450scc mRNA and human P450scc activity. The exact mechanisms of ACTH stimulation of the side-chain cleavage enzyme P450scc remain to be elucidated. In addition to its prominent role in regulating steroidogenesis ACTH has profound trophic effects upon the adrenal. Its role in adrenocortical mitogenesis and hyperplasia is not well understood. ACTH at physiologic concentrations can promote the synthesis of insulin-like growth factor-2 (IGF-2) and also the synthesis of basic fibroblast growth factor and epidermal growth factor, which may act with IGF-2 to stimulate adrenal growth. As far as the presence of ACTH receptor outside the adrenal gland it should be noted that it is also expressed elsewhere in the body, specifically in the osteoblast, which is responsible for making new bone. It has been demonstrated that the response of bone forming cells to ACTH includes production of VEGF, as it does in the adrenal. This response might be important in maintaining osteoblast survival under some conditions (Zhong *et al.*, 2005; Zaidi *et al.*, 2010).

Aging of HPA Axis

Studies in humans and experimental animals have shown evidence that hyperactivity of the HPA axis contributes to neuronal and peripheral deterioration associated with aging. High basal levels of glucocorticoids and loss of circadian rhythm have been associated with greater cognitive decline at a given age. Aging is associated with high basal levels circulating corticosteroids, although there is not always a correlation between plasma ACTH and corticosteroids. In addition, there is also an alteration to the circadian rhythm of the HPA axis, as demonstrated by studies using a feeding-associated circadian rhythm paradigm. This suggests that the aged HPA axis appears to take longer to adjust to changes in circadian rhythm, but such adjustments do not 'stick' as well as compared to the younger HPA axis.

Extensive studies reveal that the effects of age on the HPA axis are modulated by obesity and sex and the type of stress activating the system.

Controversy exists regarding unstressed mean ACTH concentrations, which are reportedly unchanged across the age span of 20–100 years, decreased with age or increased with age. Comorbidities, assay nonuniformities and sampling inconsistencies may, in part, explain the discrepant reports. Similar uncertainty applies to plasma, urinary or salivary (free) cortisol concentrations in aging individuals. Intra-abdominal adipose tissue mass is a major confounder of ACTH and/or cortisol output with high intra-abdominal adipose tissue mass predicting increased sympathetic outflow, cortisol production and cortisol inactivation, and (in women) increased mean ACTH concentrations.

Aging is also associated with increase in expression of 11βHSD1 both in brain and peripheral tissues. Such changes could conceivably expose tissues to elevated levels of glucocorticoids and contribute to an aging process.

The effects of aging on CRH regulation and whether CRH influences the course of aging are still unclear. Studies have reported increased, unchanged or reduced hypothalamic CRH release and expression during aging (Swaab *et al.*, 2005; Ferrari and Magri, 2008; Aguilera, 2011; Kasckow *et al.*, 2001; Tizabi *et al.*, 1992; Lupien *et al.*, 1996; Linkowski *et al.*, 1993; Franz *et al.*, 2010; Cooper, 2008; Holmes *et al.*, 2010; Blichert-Toft and Hummer, 1977; Sherman *et al.*, 1985; Haus *et al.*, 1989; Purnell *et al.*, 2004; Van Cauter *et al.*, 1996; Andrew *et al.*, 1998; Roelfsema *et al.*, 2012).

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Adrenal Steroidogenesis

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Abbreviations

3β-HSD 3β-Hydroxysteroid dehydrogenase
ACBD1 Acyl-CoA binding domain 1
ACTH Adrenocorticotropic hormone
ATAD3 Protein AAA+ ATPase domain 3
ATII Angiotensin II
CAH Congenital adrenal hyperplasia
CMO Corticosterone methyl oxidase deficiency
CYP Cytochrome P450s
DF Definitive zone
DHEA Dehydroepiandrosterone
DHEAS Dehydroepiandrosterone sulfate
DHT Dihydrotestosterone
FZ Fetal zone
HDL High-density lipoprotein
HMG-CoA 3-Hydroxy-3-methylglutaryl-coenzyme A
HSD Hydroxysteroid dehydrogenases
HSL Hormone-sensitive lipase
IMM Inner mitochondrial membrane
KO Knock out
LDL Low density lipoprotein
MR Mineralocorticoid receptor
NADP Nicotinamide adenine dinucleotide phosphate
OMM Outer mitochondrial membrane
PKA cAMP-dependent protein kinase
POR Flavoprotein P450 oxidoreductase
SF Steroidogenic factor
SR-BI Scavenger receptor class BI
STAR Steroidogenic acute regulatory protein
SULT Sulfotransferase
TASK TWIK-related acid-sensitive potassium channels
TSPO Translocator protein
VDAC1 Voltage-dependent anion channel
ZF Zona fasciculata
ZG Zona glomerulosa
ZR Zona reticularis

Introduction

The adrenal gland is a multilayer endocrine organ that impacts the body's physiologic response to stress and immune system through the production of cortisol and water balance and systemic blood pressure via aldosterone. The adrenal gland does not store steroid hormones and the production kinetics of the steroid products vary depending on the stimuli. Cortisol is produced within seconds of hormonal or sympathetic signaling, while aldosterone exhibits a delayed and steady rise that is secondary to the complex renin–angiotensin–aldosterone activating system. The adrenals also produce dehydroepiandrosterone (DHEA) and its sulfonated form DHEA-S, which are the most abundant adrenal steroids, via the sulfotransferase SULT2A1. The adrenal gland is also responsible for a small percentage of the body's testosterone, which is derived from the precursor androstenedione.

The Gonadal and Adrenal Gland Share a Common Developmental Origin

Adrenal and gonadal biogenesis of steroid hormones can be traced to a common developmental origin in the intermediate mesoderm. Early in development, around the 4 week of gestation, the adrenogenital primordium gives rise to the bipotential gonad, a precursor of the testes and ovary (Hanley *et al.*, 2001). Soon after, a group of adrenal progenitors can be identified by their expression of the steroidogenic factor (SF)-1, an essential transcription factor driving the expression of the steroidogenic machinery (Hatano *et al.*, 1996). These cells develop from the dorsal aortal side of the adrenogenital primordium around the 8 week of gestation and expand into a large mass of cells referred to as the definitive zone (DF) (Hammer *et al.*, 2005). In humans, there is an additional layer of inner cells known as the fetal zone (FZ) that express CYP17A1 but lack 3β-hydroxysteroid dehydrogenase (3β-HSD) expression (Mesiano and Jaffe, 1997). Development of the definitive zone is followed by the migration of chromaffin cells, which are of neural crest origin, as well as by encapsulation. Soon after birth, the fetal adrenal gland undergoes cell zonation where chromaffin cells are compacted into a rudimentary medulla and the definitive zone starts to give rise to the outer zona glomerulosa (ZG), the zona fasciculata (ZF), and the inner zona reticularis (ZR). In rodents, there is a transient cell layer known as the X zone that develops between the ZF and ZR. These cells disappear when males reach puberty and after females undergo their first pregnancy (Holmes and Dickson, 1971).

Adrenal Gland Steroidogenesis is Initiated by G-Coupled Receptor Activation or Changes in Extracellular Potassium Levels

In the human adrenal gland, the tissue-specific expression of steroidogenic enzymes produces aldosterone in the ZG, cortisol in the ZF, as well as DHEA and DHEA-S. The cellular mechanisms that initiate steroid biosynthesis are specific to the adrenal cortex layer (**Fig. 1**). In the

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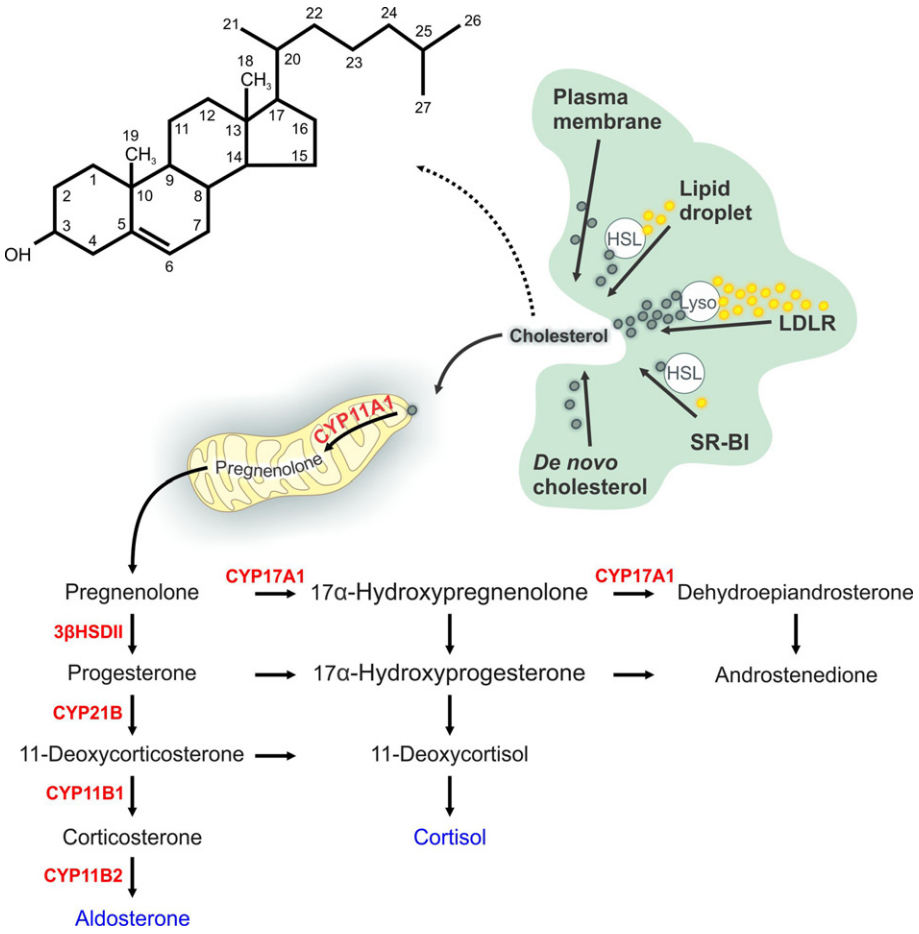

Fig. 1 The steroidogenic pathway. Cholesterol for steroidogenesis is harvested from extra- and intra-cellular sources. LDL-mediated import of cholesterol is the preferred source in humans. HDL (through the SR-BI pathway) and lipid droplets contain esterified cholesterol (*yellow*) that can be used in steroidogenesis after de-esterification (*green*) via hormone-sensitive lipase (HSL). Cholesterol is then imported to the inner mitochondria membrane where CYP11A1 cleaves the side chain of cholesterol. Thereafter, the tissue-specific expression of CYPs enzymes and 3β-HSDII result in the formation of aldosterone, cortisol, and dehydroepiandrosterone. The numbering of the carbons of cholesterol indicate the various reaction sites.

ZG, aldosterone production is stimulated by numerous molecules; however, angiotensin II, potassium, and adrenocorticotropic hormone (ACTH) have the most physiological relevance (Spat and Hunyady, 2004). The ZG expresses the angiotensin II type I receptor, which is part of the renin–angiotensin system that maintains blood pressure and water balance. The ZG also strongly expresses TWIK-related acid-sensitive potassium channels TASK1 and TASK3, two-pore domain potassium channels that detect changes in circulating potassium levels (Bandulik *et al.*, 2010). TASK1, which is also found in the ZF and ZR, appears to be involved in adrenal development since its deletion results in hyperaldosteronism and altered adrenal zonation (Heitzmann *et al.*, 2008). The ACTH receptor is expressed throughout the adrenal cortex and mediates the biosynthesis of glucocorticoids and androgens in the ZF and ZR (Gill, 1976). Ligand-mediated activation of these receptors and changes in extracellular potassium levels stimulate the release of second messengers that initiate cholesterol mobilization and prime the mitochondria for steroidogenesis.

Cholesterol is the Substrate for All Steroid Hormones

Steroid hormones are produced from the common building block, cholesterol (Payne and Hales, 2004). Cholesterol for steroid biosynthesis can be obtained from extracellular or intracellular sources or can be synthesized de novo. The preferred sources of cholesterol are tissue-specific; here we will focus on pathways preferred by the adrenal gland.

Extracellular cholesterol can be imported into the cell by the low-density lipoprotein (LDL) or scavenger receptor class BI (SR-BI) pathways (Kraemer, 2007). The LDL pathway internalizes lipoproteins containing apolipoprotein B or E and transports esterified cholesterol that is then hydrolyzed by liposomal acid lipase into unesterified cholesterol. The free cholesterol can then be used for steroidogenesis or re-esterified and stored in lipid droplets by acyl CoA:cholesterol acyltransferase encoded by the *ACAT1* gene. In humans, dietary cholesterol that is transported via LDL is the main source of cholesterol (Ikonen, 2006). This is contrary

to rodents where the SR-BI pathway mediates the uptake of high-density lipoprotein (HDL)-bound lipids (Connelly and Williams, 2003) and is the main source of cholesterol for adrenal steroid biosynthesis. Esterified cholesterol delivered by this pathway is de-esterified by the hydrolase action of the hormone-sensitive lipase (HSL) encoded by the *LIPE* gene (Kraemer and Shen, 2002). HSL is responsible for 90% of the cholesterol ester hydrolysis activity in the adrenal gland (Kraemer *et al.*, 2004). Lipid droplets also contain esterified cholesterol and require HSL function to provide free cholesterol to sustain steroidogenesis.

De novo cholesterol synthesis starts by combining acetyl CoA and acetoacetyl CoA precursors into 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) in a reaction catalyzed by HMG-CoA synthase. This is followed by the rate-limiting step in de novo cholesterol biosynthesis, the production of mevalonate catalyzed by the enzyme HMG-CoA reductase, which is the target of statin drugs (Lennernas and Fager, 1997). HMG-CoA reductase function is negatively regulated when LDL is in adequate supply and is stimulated by ACTH (Fluck and Miller, 2008). Although de novo cholesterol biosynthesis may help maintain adrenal gland steroidogenesis, studies have shown that lipoprotein-bound cholesterol is the primary source of cholesterol for normal adrenal steroidogenesis (Verschoor-Klootwyk *et al.*, 1982).

The Transport of Cholesterol Into the Mitochondria is the Rate-Limiting Step in Steroid Biosynthesis

The rate-limiting site of steroid biosynthesis occurs during the translocation of cholesterol into the mitochondria where pregnenolone is produced. Upon activation of the cellular mechanism for steroidogenesis, cholesterol transport to the mitochondria is aided by the steroidogenic acute regulatory protein (STAR), encoded as a 37 kDa preprotein by the *STARD1* gene located in Chromosome 8p11. Interestingly, dormant *Star* mRNA is transcribed soon after steroidogenic cell activation through the PKA and ERK kinase signaling pathways (Duarte *et al.*, 2014). STAR is directed into the mitochondria by a cellular targeting sequence, where it is cleaved into an inactive 30 kDa protein (Bose *et al.*, 2002). The presence of STAR is essential for adrenal steroidogenesis since mouse knockout studies and humans with STAR mutations exhibit an inability to produce sufficient levels of steroids (Baquedano *et al.*, 2013).

Other proteins involved in the translocation of cholesterol into the mitochondria are collectively found in a large multiprotein complex (Rone *et al.*, 2014). At the outer mitochondrial membrane (OMM), this complex is composed of the cytoplasmic proteins acyl-CoA binding domain 1 (ACBD1), ACBD3, STAR and cAMP-dependent protein kinase (PKA); the OMM proteins, translocator protein (TSPO) and voltage-dependent anion channel (VDAC); and CYP11A1 located in the inner mitochondria membrane (IMM) (Midzak and Papadopoulos, 2016). Moreover, cholesterol entry into the IMM is also helped by the formation of contact points between the OMM and IMM that increase the hydrophobic medium in which cholesterol travels. The presence in this bioactive complex of the IMM protein AAA+ ATPase domain 3 (ATAD3), which extends through the OMM to the endoplasmic reticulum, forming mitochondria-associated membrane bridges, supports the concept of a protein–protein interaction-mediated transfer of cholesterol across membranes to CYP11A1 in the IMM (Issop *et al.*, 2015). The presence of TSPO seems to be essential for ACTH-induced steroid production, since TSPO knockout studies, mutations in rodents and a human polymorphism impair the rate of adrenal steroid synthesis (Fan *et al.*, 2015; Owen *et al.*, 2017).

The Cytochrome P450s and the Hydroxysteroid Dehydrogenases Catalyze Formation of Steroid Hormones From Cholesterol

Adrenal steroid biosynthesis is carried out by two main families of enzymes, the cytochrome P450s (CYP) and the hydroxysteroid dehydrogenases (HSD). CYP enzymes are identified by their ability to absorb light in their reduced form at 450 nm and the presence of a heme prosthetic group that is critical in mediating the oxygenation of the target protein (Midzak and Papadopoulos, 2016). In humans, 5 out of the 57 genes coding for CYP enzymes play a role in adrenal steroid biosynthesis. CYP11A1, CYP11B1, and CYP11B2 are localized in the mitochondria, while CYP17A1 and CYP21A2 are found in the endoplasmic reticulum. The HSDs can be generally divided into dehydrogenases or reductases. These enzymes need cofactors: NAD+ is used by dehydrogenases to oxidize hydroxysteroids to ketosteroids, and the reductases use NADPH to catalyze the reverse reaction (Agarwal and Auchus, 2005). 3β-HSD, 17HSD3, and 17HSD1 are all involved in steroid biosynthesis. Interestingly, CYP enzymes are coded from a single gene, while the HSDs have many isoforms (gene splicing generates various enzyme versions) or isozymes (many genes code for the same enzyme) that modify their cellular distribution and affinity for cofactors and substrates (Payne and Hales, 2004). Together, the CYP and HSD enzymes cleave and modify cholesterol to yield more oxygenated and hydrophilic cholesterol derivatives that give rise to a group of highly versatile hormones (**Fig. 2**).

CYP11A1 Catalyzes the First Step in Steroid Hormone Formation

CYP11A1 is a 9-exon gene located on Chromosome 15q23 that codes for a highly conserved 56 kDa protein highly expressed in all the layers of the adrenal cortex. The amino terminus contains a 39 amino acid mitochondrial targeting sequence that positions the protein in the inner mitochondrial matrix, where it receives and cleaves the aliphatic side chain of newly translocated cholesterol. Cloning studies where CYP11A1 was directed to other cellular compartments revealed that both mitochondrial positioning and interaction with its microenvironment are essential for the enzyme to function (Black *et al.*, 1994). CYP11A1 is the only enzyme that can carry out this function and is commonly used as a marker to identify steroidogenic cells. CYP11A1 is the slowest enzyme

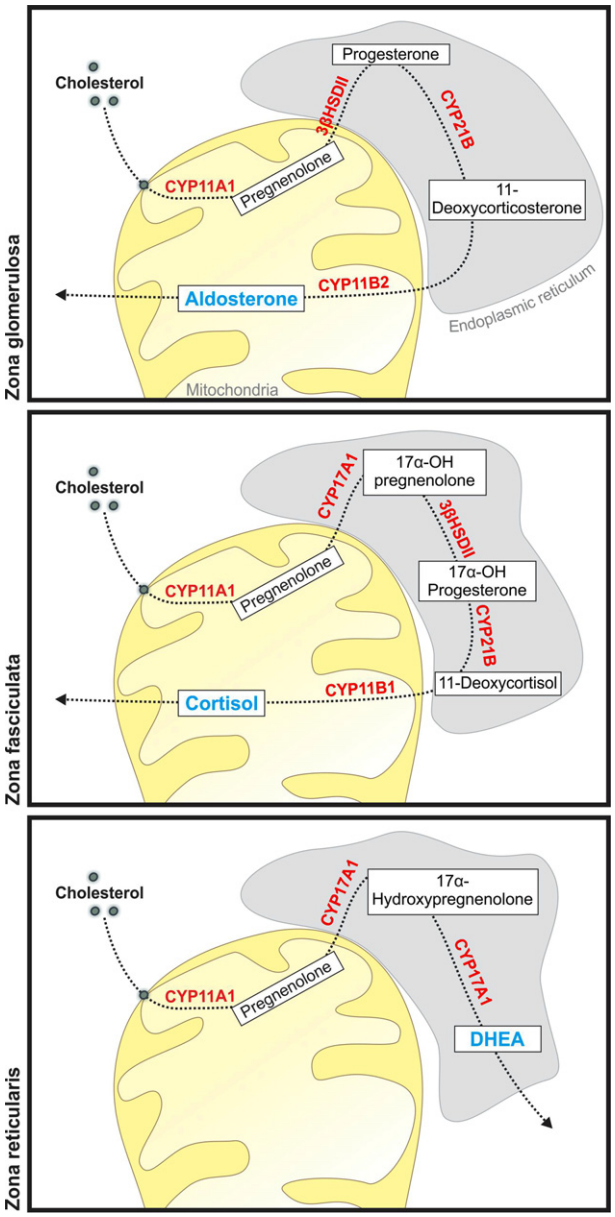

Fig. 2 Steroid production in human in the adrenal cortex. The figure depicts the enzymes, steroid intermediates, and end products at the zona glomerulosa, zona fasciculata, and zona reticularis. DHEA = dehydroepiandrosterone.

of the pathway, with each protein cleaving 6.2 molecules of cholesterol per minute (Kuwada *et al.*, 1991). The slow kinetics and the non-replaceable function of CYP11A1 make this the rate-limiting enzyme in steroidogenesis, and its protein levels have a direct correlation with steroidogenic activity of the cell.

CYP11A1 carries out three oxidation reactions, a C22 and C20 hydroxylation that yields 20,22 hydroxycholesterol followed by a cleavage between the hydroxylation sites, with each of the reactions needing an oxygen and a NADPH molecule. The transfer of electrons between NADPH and CYP11A1 is facilitated by ferredoxin reductase that harvests electrons from NADPH and passes them to ferredoxin, which in turn, shuttles electrons to the ferrous ion of CYP11A1. The resulting products are the C21 steroid pregnenolone and isocaproaldehyde. Isocaproaldehyde is a reactive molecule that is subsequently oxidized to isocaproic acid.

CYP17A1 is the Branching Point of the Adrenal Steroidogenic Pathway

CYP17A1 is on Chromosome 15q23 and codes a 57 kDa enzyme that catalyzes two oxidase reactions resulting in a hydroxylation and a cleavage (lyase activity). The zone-specific expression of CYP17A1 commits it to the production of cortisol, DHEA and

DHEAS in the ZF and ZR, while the absence of CYP17A1 in the ZG leads to aldosterone biosynthesis. Newly formed pregnenolone can either be metabolized to progesterone by 3β-HSD (Δ^4 pathway) or to 17α-hydroxypregnenolone by CYP17A1 (Δ^5 pathway). In humans, the Δ^5 pathway is preferred, while rodents follow the Δ^4 pathway, since they lack adrenal CYP17A1 expression. The species-specific absence of CYP17A1 expression means that rodents produce corticosterone instead of cortisol as their main corticosteroid and lack DHEA and DHEA-S biosynthesis.

CYP17A1 can use pregnenolone or progesterone as a substrate. In the Δ^5 pathway, pregnenolone is first hydroxylated at C17 yielding 17α-hydroxypregnenolone, followed by a cleavage reaction between C17 and C20 that results in C19 DHEA and acetaldehyde. In the Δ^4 pathway, progesterone is converted to 17α-hydroxyprogesterone, and in a follow up lyase reaction, C19 androstenedione is generated, with each reaction consuming oxygen and an NADPH molecule. While the Δ^4 pathway yields C19 androstenedione, it is not a significant source of androgens, since the Δ^5 pathway is more efficient in humans.

CYP17A1 is localized in the endoplasmic reticulum and needs flavoprotein P450 oxidoreductase (POR) to carry out its function. POR is a flavoprotein that moves two electrons from NADPH to CYP17A1. Moreover, the lyase activity of CYP17A1 is promoted by the allosteric cofactor cytochrome *b*5, which is positioned between POR and CYP17A1, and the phosphorylation of CYP17A1 serine residues (Auchus *et al.*, 1998).

3β-Hydroxysteroid Dehydrogenase

3β-HSDII is a 42 kDa protein expressed in the adrenal cortex, ovary, and testes and catalyzes the formation of progesterone from pregnenolone. In addition, 3β-HSDII can take 17α-hydroxypregnenolone and dehydroepiandrosterone from the Δ^5 pathway and convert them to 17α-hydroxyprogesterone and androstenedione, respectively, as previously discussed.

Several isoforms of 3β-HSD exist resulting in variations in tissue protein expression, activity, and affinity for substrates and cofactors. In the human, *HSD3B2* is a four-exon gene located on Chromosome 1p13.1 and expresses 3β-HSD type II in the adrenal cortex. It is important to note that the numeric nomenclature between humans and rodents is not indicative of an orthologue gene, and thus, the orthologue gene in the mouse for human *HSD3B2* is *Hsd3b1*, which codes for 3β-HSDI (Payne and Hales, 2004).

3β-HSDII carries out its reaction in a two-step process. First, the dehydrogenation of 3β-hydroxysteroid uses NAD^+ to generate a 3-keto intermediate and NADH. This is followed by the isomerization of the Δ^5-3-ketosteroid to form Δ^4-3-ketosteroid, where the reduced NADH propels the reaction forward.

Expression of 3β-HSDII in the fetal adrenal gland was reported to start after week 23 of gestation with expression mostly in the transitional zone and the definitive zone (Narasaka *et al.*, 2001). The lack of expression of 3β-HSDII correlates with the inability of fetal cells to produce glucocorticoids or mineralocorticoids during early gestation.

CYP21

CYP21B is a 10-exon gene found on Chromosome 6p21.3 and codes for a 56 kDa protein that is highly expressed in the ZG and ZF. *CYP21A*, which has high homology with *CYP21B*, is also expressed in humans but various deletions and insertions prevent the synthesis of a mature protein (Rodrigues *et al.*, 1987). The mouse has similar duplicated genes but, unlike the human, *Cyp21a* is the gene that expresses a mature protein (Parker *et al.*, 1986).

CYP21 places a hydroxyl group at carbon 21 of progesterone and 17α-hydroxyprogesterone to produce 11-deoxycorticosterone and 11-deoxycortisol, respectively. This reaction consumes oxygen and NADPH. Of clinical interest, most of the congenital adrenal hyperplasia cases are related to defects in CYP21 (Fluck and Miller, 2008).

CYP11B1 and CYP11B2 are Highly Homologous But Carry Independent Reactions

CYP11B1 is located on Chromosome 8q21 and produces a 50 kDa protein expressed mainly in the ZF and ZR. CYP11B1 carries out the 11β-hydroxylation of C11 of 11-deoxycorticosterone and 11-deoxycortisol to synthesize corticosterone and cortisol, respectively.

CYP11B2, aldosterone synthase, is located adjacent to *CYP11B1* on Chromosome 8q21-q22 and codes for a 48.5 kDa protein exclusively expressed in the ZG. This enzyme catalyzes three reactions, taking in 11-deoxycorticosterone and yielding aldosterone, with each of the reactions consuming oxygen, an NADPH molecule, and using the electron transport system (Payne and Hales, 2004). First, 11-deoxycoticosterone is 11β-hydroxylated to corticosterone, followed by a second hydroxylation at C18 to yield 18-hydroxycorticosterone. The third reaction oxidizes C18 resulting in aldosterone. It is important to note that CYP11B2 carries out this reaction only starting from 11-deoxycorticosterone, since studies have shown that corticosterone is not a substrate for this enzyme (Kawamoto *et al.*, 1992). This is reflected in the clinic where patients with CYP11B1 deficiencies produce aldosterone but not cortisol, while patients with CYP11B2 deficiencies, synthetize cortisol but lack aldosterone biosynthesis (Zhang *et al.*, 1995).

CYP11B1 and CYP11B2 use ferredoxin/ferredoxin reductase electron transport and consume oxygen and NADPH during each of the reactions catalyzed. Both enzymes share 95% identity and are directed to the inner mitochondria membrane by a targeting

sequence that is cleaved upon mitochondrial entry (White *et al.*, 1994). Moreover, expression levels of CYP11B1 are significantly higher than CYP11B2. Gene regulation of these enzymes are part of an endocrine feedback loop where ACTH increases CYP11B1 expression, and ATII and potassium levels stimulate CYP11B2 expression (Clyne *et al.*, 1997; Nanba *et al.*, 2015). Glucocorticoids, on the contrary, decrease CYP11B1 expression.

The SULT Enzymes Catalyze the Sulfation of Steroids

Steroid hormones can be sulfonated by the sulfotransferase (SULT) enzyme superfamily. These enzymes are all cytoplasmic, and SULT1A, SULT2A1, and SULT2B1 are known to sulfonate steroids. SULT2A1 is expressed in the adrenal gland and catalyzes the sulfonation of 3β-hydroxyl group of pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone, and androstenedione.

11β-Dehydrogenases are Expressed in Peripheral Tissues and Confer Steroid Selectivity

11β-HSD1 and 11β-HSD2 are two enzymes that depend on the presence of $NADP^+$ or NADPH to carry out their reductase or oxidase function. Both enzymes can catalyze the interconversion between cortisol and cortisone, a less active corticosteroid, with the different kinetics dictated by their affinity for the substrate. 11β-HSD1 has micromolar affinity while 11β-HSD2 has low nanomolar affinity for glucocorticoids (Takeyama *et al.*, 2000). This difference in substrate affinity results in inactivation of glucocorticoids in tissues that express 11β-HSD2 and the mineralocorticoid receptor (MR), which shares a similar affinity for glucocorticoids and mineralocorticoids, and thus, allows for selective binding of aldosterone and 11-deoxycorticosterone. The protective effect of 11β-HSD1 and 11β-HSD2 against glucocorticoids is also seen in the placenta and in fetal tissues, where both enzymes work to inactivate maternal glucocorticoids (Hirasawa *et al.*, 1999). In other tissues, like the liver, were selective MR activation is not desired, the 11β-HSD1 expression is thought to protect against excessive levels of glucocorticoids.

Other Steroidogenic Enzymes

While 17HSD3, 17HSD1, CYP19A1, and 5α-reductase do not play a role directly in adrenal steroid formation, they are involved in steroid biology. 17HSD3 is expressed in the testes and uses DHEA and androstenedione as a substrate to yield androstenediol and testosterone, respectively. CYP19A1, aromatase, is a 58 kDa protein that catalyzes the aromatization of testosterone into 17β-estradiol, which is the main steroid generated by the Leydig cells. Moreover, CYP19A1 can also convert androstenedione to estrone. 5α-Reductase increases the potency of testosterone several folds. This enzyme is expressed in peripheral tissues.

Mutations in Enzymes or Proteins Lead to Steroid Deficiency

Adrenal hyperplasia and lipoid adrenal hyperplasia are two histopathological findings in adrenal diseases arising from genetic errors in the enzymes or proteins involved in steroidogenesis. Congenital adrenal hyperplasia (CAH) groups together various recessive disorders that arise most commonly from steroidogenic enzyme mutations. The reduced enzymatic performance or, in some cases, enzyme inactivation halts the progression of steroid biosynthesis. Since steroidogenesis is tightly regulated, feedback loops triggered by the lack of end-products result in adrenal cortex overstimulation. This increased intracellular signaling is the root of the histopathological findings and is responsible for adrenal hypertrophy and lipid accumulation. Newborns show symptoms soon after birth, where the prompt diagnosis is critical to avoid possible life-threatening complications.

Mutations/deletions in the enzymes and proteins involved in steroidogenesis will have a wide gamut of outcomes depending on the severity of the modification. Moreover, the existence of a back door pathway and other CYPs that may metabolize intermediates to a certain degree may account for detectable levels of steroids even where there are inactivating mutations of enzymes (Auchus, 2004). In addition, steroidogenesis may also be affected by disorders that target the intracellular regulation and movement of cholesterol (Miller, 2017).

Steroidogenic Enzymes

The most frequent cause of CAH is a deficiency of CYP21 (90% of CAH) with an incidence of 1:14,000, followed by 11β-HSD (5% of CAH) with an incidence of $\sim$1:100,000 (Therrell *et al.*, 1998). The lack of CYP21 shunts steroidogenic biosynthesis away from the production of cortisol and aldosterone, which are exclusively produced in the adrenal cortex. The resulting decrease in cortisol triggers a feedback loop that increases the levels of ACTH, which results in the increase production of the androgen precursors DEHA, DHEAS, and androstenedione. Ultimately, the levels of testosterone, and the more potent DHT, increase and contribute to the pathology. In females, this condition is suspected by signs of virilization at birth, while the diagnosis in males maybe delayed until the onset of additional symptoms originating from the loss of cortisol and aldosterone (Hanley and Arlt, 2006). In severe

cases where the levels of aldosterone and corticosterone are below those needed to sustain physiological function, the newborn will exhibit signs and symptoms of salt-wasting accompanied by vomiting, lethargy, and failure to thrive perinatally. Various degrees of symptomatology can be observed in the clinic that correlates with the impact on enzyme kinetics by the mutation (Fluck and Miller, 2008).

CYP11A1 is the first and most common enzyme in all the steroidogenic pathways and any gene mutations lead to congenital adrenal insufficiency. In these patients, ACTH and renin activity are highly elevated, while the levels of adrenal-made steroids are decreased. Clinical findings depend on the severity of the mutations but may include sex reversal in males, severe adrenal failure at birth, or late-onset adrenal failure. While the decrease in adrenal steroid levels were maybe comparable to CAH, adrenal enlargement or lipoid histopathology are not part of the disease (Kim *et al.*, 2008).

Mutations of *CYP11B2* lead to congenital corticosterone methyl oxidase deficiency (CMO). As discussed, CYP11B2 catalyzes three consecutive reactions where the precursor deoxycorticosterone is metabolized to corticosterone, 18-hydroxycorticosterone, and aldosterone. Depending on the *CYP11B2* mutation site, catalytic activity may stop at corticosterone (CMO I) or 18-hydroxycorticosterone (CMO II) (Ulick *et al.*, 1992).

STAR

STAR is thought to bind cholesterol and to facilitate intracellular cholesterol transport (Miller, 2007). Human mutations in *STAR* are associated with lipoid adrenal hyperplasia and various degrees of steroid deficiency (Miller, 2017). Mouse KO models showed viable litters that later died from adrenocortical insufficiency and had adrenal lipid deposits. Interestingly, comparable lipid deposits were less in testes and not observed in the ovary, indicating that STAR may play a preferential role in adrenal steroidogenesis.

TSPO

TSPO is an 18 kDa high-affinity cholesterol- and drug-binding protein located in the outer mitochondria membrane (Papadopoulos *et al.*, 2006). TSPO is part of a protein complex that aids in the translocation of cholesterol into the mitochondria to initiate steroidogenesis. TSPO is the target of various drugs that modulate the import of cholesterol and thus formation of steroids and neurosteroids developed for the treatment of neuropsychiatric and neurological diseases. Changes in the drug binding affinity have been reported in patients with the rs6971 polymorphism (Owen *et al.*, 2012). *TSPO* deletion mutations in rat and its corresponding rs6971 polymorphism in humans alters ACTH-induced plasma corticosteroid concentrations (Owen *et al.*, 2017).

See also: Adrenal Cortex; Physiology. Corticotropin-Releasing Hormone Peptide Family. Stress and Endocrine Physiology

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Overview of Glucocorticoids☆

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Abbreviation

3β-HSD	3β-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
AP-1	Activator protein-1
AVP	Arginine vasopressin
CBG	Corticosteroid-binding globulin
CpG	Cytosine-guanine dinucleotides
CRH	Corticotropin-releasing hormone
CYP	Cytochrome P450
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DOC	Deoxycorticosterone
hGR	Human glucocorticoid receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
HSP	Heat shock proteins
LBD	Ligand-binding domain
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-κB
PI_3K	Phosphatidylinositol 3-kinase
STATs	Signal transducers and activators of transcription

Glossary

Addison disease An endocrine disorder caused by destruction of the adrenal cortex and characterized by a deficiency in aldosterone, cortisol, and sex hormones.

Cushing syndrome An endocrine disorder caused by excessive production of cortisol.

Cholesterol The major fatty steroid alcohol of all vertebrate cells, especially animal fat, blood, liver, nerve tissue, and brain tissue.

Diurnal or circadian pattern Displaying a daily cycle.

Steroidogenesis The biosynthesis or production of steroids through enzymatic pathways.

Introduction

Glucocorticoids are steroid hormones produced by the adrenal glands and secreted into the systemic circulation in an ultradian, circadian and stress-related fashion (Nicolaides *et al.*, 2014, 2017). These molecules regulate a variety of biologic processes, including proliferation, differentiation and programmed cell death (apoptosis), and exert profound influences on many physiologic functions by virtue of their diverse roles in growth, development, and maintenance of basal and stress-related homeostasis (Charmandari *et al.*, 2005). Accumulating evidence suggests that glucocorticoids may alter the methylation status of many cytosine-guanine dinucleotides (CpG) located in the promoter regions of several genes. These glucocorticoid-induced epigenetic alterations are mediated through changes in the expression and/or activity of enzymes involved in the methylation/demethylation process (Zannas and Chrousos, 2017).

Pathways of Steroid Biosynthesis

The adrenal cortex consists of three anatomical zones: the outer *zona glomerulosa*, the intermediate *zona fasciculata*, and the inner *zona reticularis*. The *zona glomerulosa* is responsible for the production of aldosterone, the *zona fasciculata* is responsible for the production of cortisol, and the *zona reticularis* is responsible for the production of adrenal androgens. The adrenal medulla is functionally related to the sympathetic nervous system and secretes epinephrine and norepinephrine both under basal conditions and in response to stress (Stewart, 2003; Miller, 2005).

The glucocorticoid cortisol and the mineralocorticoid aldosterone are synthesized by the adrenal cortex under the control of regulatory systems that largely function independently. All steroid hormones produced by the adrenal cortex are derived from cholesterol. Low-density lipoprotein (LDL)-cholesterol is the major source of cholesterol utilized in adrenal steroidogenesis. Proteolytic and lipolytic enzymes act on LDL to release cholesterol esters for storage in lipid droplets in the adrenal cells (Simpson and Waterman, 1995). In order for the adrenal cortex to synthesize active steroid hormones, a number of changes are required in

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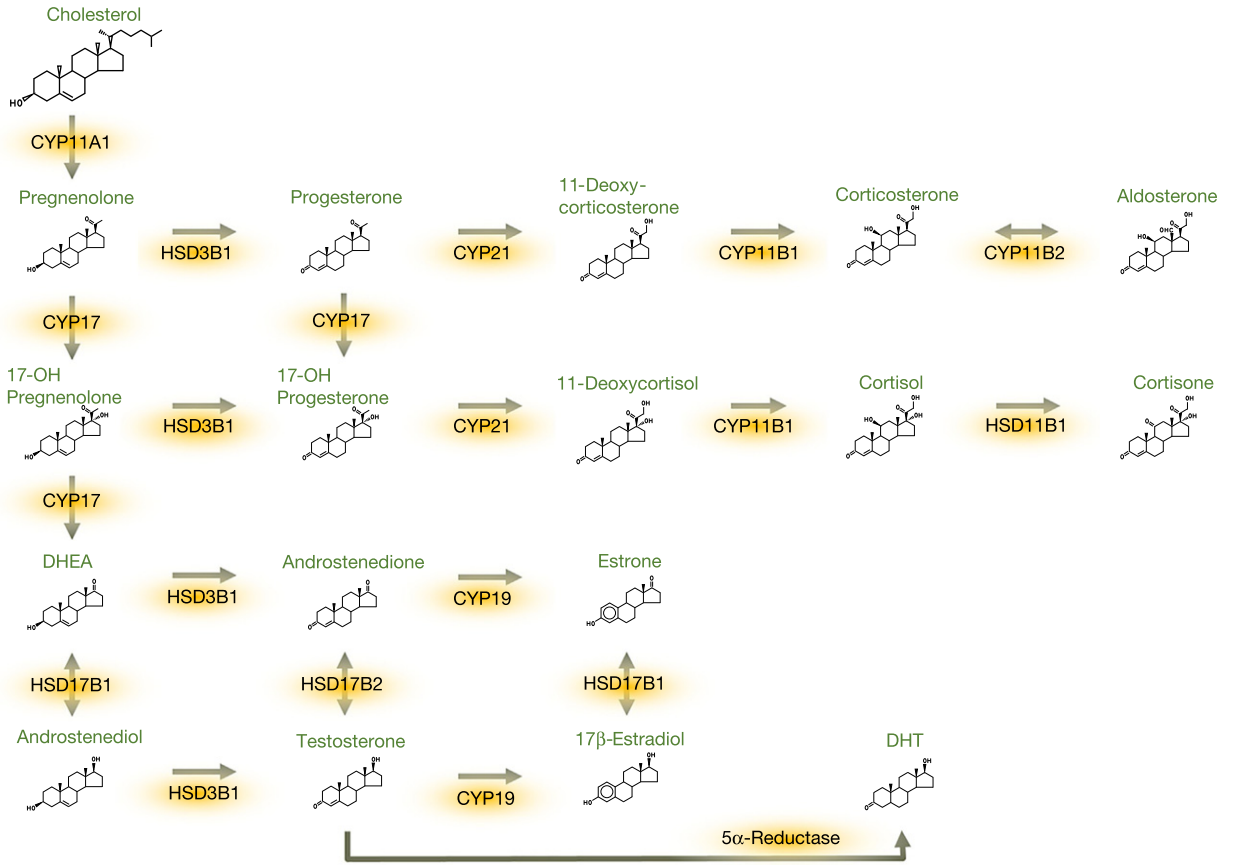

Fig. 1 Schematic representation of adrenal steroidogenesis.

the structure of cholesterol. Several of these reactions are catalyzed by the steroid hydroxylases, which are members of a superfamily of genes known collectively as cytochrome P450 (CYP). Adrenal steroidogenesis follows three distinct routes, which reflect the zonal differences in terms of function and regulation (**Fig. 1**).

The rate-limiting step in steroid biosynthesis is importation of cholesterol from cellular stores to the matrix side of the mitochondrial inner membrane, where the cholesterol side-chain cleavage system is located. This is controlled by the steroidogenic acute regulatory protein, the synthesis of which is increased by trophic stimuli, such as adrenocorticotropic hormone (ACTH) (Simpson and Waterman, 1995; Stocco and Clark, 1996; Arakane *et al.*, 1998). The first enzymatic step in steroid biosynthesis common to all steroidogenic pathways takes place in the mitochondrion and leads to the cleavage of six carbon atoms from the side chain of cholesterol, converting this C-27 compound to the C-21 steroid pregnenolone. This reaction is known as cholesterol side-chain cleavage and is catalyzed by the cytochrome P450 enzyme CYP11A (P450scc, cholesterol desmolase, side-chain cleavage enzyme), which is an integral protein of the inner mitochondrial membrane (Nebert *et al.*, 1991). Pregnenolone, the common precursor for all other steroids, then passes by diffusion from the mitochondrion to the endoplasmic reticulum, where it undergoes further metabolism by several other enzymes.

To synthesize mineralocorticoids in the *zona glomerulosa*, 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum and mitochondria converts pregnenolone to progesterone (Cherradi *et al.*, 1997). The latter is 21-hydroxylated in the endoplasmic reticulum by CYP21 (P450c21, 21-hydroxylase) to produce deoxycorticosterone (DOC). Aldosterone, the most potent 17-deoxysteroid with mineralocorticoid activity, is produced by 11β-hydroxylation of DOC to corticosterone, followed by 18-hydroxylation and 18-oxidation of corticosterone (**Fig. 1**). The final three steps in aldosterone synthesis are accomplished by a single mitochondrial P450 enzyme, CYP11B2 (P450aldo, aldosterone synthase) (White *et al.*, 1994).

To produce cortisol, CYP17 (P450c17, 17α-hydroxylase/17,20-lyase) in the endoplasmic reticulum of the *zona fasciculata* and *zona reticularis* converts pregnenolone to 17α-hydroxypregnenolone (Yanase *et al.*, 1991). 3β-HSD in the *zona fasciculata* utilizes 17α-hydroxypregnenolone as a substrate, producing 17α-hydroxyprogesterone. The latter is 21-hydroxylated by CYP21 to form 11-deoxycortisol, which is further converted to cortisol by CYP11B1 (P450c11, 11β-hydroxylase) in the mitochondria.

In the *zona reticularis* of the adrenal cortex and in the gonads, the 17,20-lyase activity of CYP17 converts 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA), a C-19 steroid and sex steroid precursor. DHEA is further converted by 3β-HSD to androstenedione. In the gonads, androstenedione is reduced by 17β-hydroxysteroid dehydrogenase (Penning, 1997). In pubertal ovaries, aromatase (CYP19, P450c19) can convert androstenedione and testosterone to estrone and estradiol, respectively

(Simpson *et al.*, 1994). Testosterone may be further metabolized to dihydrotestosterone by steroid 5α-reductase in androgen target tissues (Wilson *et al.*, 1993).

Regulation of Cortisol Secretion

Plasma glucocorticoid concentrations are regulated in ways that reflect the varying physiologic needs for the hormones under basal conditions and in response to stress. Cortisol secretion is primarily regulated by ACTH, a 39-amino-acid peptide released by the anterior pituitary (Stewart, 2003; Miller, 2005; Simpson and Waterman, 1995). ACTH is synthesized as part of a higher molecular weight precursor peptide, proopiomelanocortin. ACTH is secreted in regular pulses of variable amplitude over 24 h, with peak concentrations attained in the early morning hours (4:00–8:00 AM), thus forming the basis of the circadian pattern of cortisol secretion (Wallace *et al.*, 1991). The acute action of ACTH is to increase the flux of cholesterol through the steroidogenic pathway, resulting in the rapid production of steroids. ACTH also influences the remaining steps of steroidogenesis as well as the uptake of cholesterol from plasma lipoproteins, thus ensuring a continuous supply of cholesterol to the mitochondria to meet the demands of activated pregnenolone biosynthesis. It also maintains the size of the adrenal glands, stimulates melanocytes, and results in hyperpigmentation when secreted in excess.

Corticotropin-releasing hormone (CRH) is the principal hypothalamic factor that stimulates, synergistically with the arginine vasopressin (AVP), the pituitary production of ACTH (Itoi *et al.*, 1998). Both peptides are produced in the paraventricular nuclei of the hypothalamus, but are also found in other parts of the central nervous system as well as in noncentral locations (Habib *et al.*, 2001). CRH and AVP are secreted in a pulsatile fashion that results in the episodic secretion of ACTH and the circadian variation of cortisol secretion. The magnitude of cortisol response to each ACTH burst remains relatively constant; it is therefore the number of secretory periods, rather than the magnitude of each pulse of CRH/AVP or ACTH, that determines the total daily cortisol secretion. Although CRH increases the amount of ACTH secreted from each responsive corticotroph, vasopressin appears to increase the number of CRH-responsive corticotrophs. In addition to ACTH, other factors may play an important role in the regulation of the adrenal cortex (Ehrhart-Bornstein *et al.*, 1998; Bornstein and Chrousos, 1999).

Cortisol is the primary negative regulator of basal hypothalamic-pituitary-adrenal (HPA) axis activity through negative feedback on ACTH and CRH secretion (Keller-Wood and Dallman, 1984). The negative feedback effects of cortisol are exerted at the level of both the hypothalamus and the pituitary and are mediated by type II corticosteroid receptors. Whether and to what extent direct glucocorticoid feedback on the adrenal cortex itself regulates cortisol synthesis is not clear (Reincke *et al.*, 1998).

Secretion and Metabolism

In normal subjects, the secretion of glucocorticoids follows a diurnal pattern, with peak concentrations observed between 6:00 and 8:00 AM and the lowest concentrations observed at approximately 12:00 AM (Krieger *et al.*, 1971). The cortisol production rate is approximately 12 mg/m^2/day (Esteban and Yergey, 1990). More than 90% of circulating cortisol, and to a lesser extent aldosterone, is bound tightly to corticosteroid-binding globulin (CBG) or transcortin (Brien, 1981). The remaining (10%) of the circulating cortisol is free or loosely bound to albumin. The free and albumin-bound fractions of cortisol represent the biologically active form of the hormone. When plasma cortisol concentrations exceed 20 μg/dL, CBG becomes fully saturated and most of the excess cortisol is biologically active. CBG is synthesized in the liver. Estrogens, thyroid hormones, pregnancy, and oral contraceptives are associated with increased CBG concentrations (Brien, 1981; Mataradze *et al.*, 1992; Stolk *et al.*, 1996), whereas hypercortisolism, hepatic disease, or renal disease results in decreased CBG concentrations. In the presence of an intact HPA axis, alterations in CBG concentrations are likely not to affect circulating free cortisol concentrations.

The primary site of cortisol metabolism in humans is the liver, and a number of cytosolic and microsomal enzymes, including cytochrome P450, 5α/5β-reductase, 3α/3β-oxidoreductase, and 11β-hydroxysteroid dehydrogenase, play an important role in the hepatic metabolism of cortisol (Gower, 1984; Iyer *et al.*, 1990; Draper and Stewart, 2005). The major routes of hepatic metabolism involve A-ring and side-chain reduction followed by conjugation with glucuronic acid and sulfate (Abel *et al.*, 1992). The inactive glucuronide and sulfate metabolites are excreted by the kidneys, whereas less than 1% of cortisol is excreted unchanged in the urine. The metabolic clearance of cortisol, therefore, is influenced primarily by factors altering hepatic clearance and to a much lesser degree by factors affecting renal excretion.

Mechanisms of Glucocorticoid Action

At the cellular level, the actions of glucocorticoids are mediated by an intracellular receptor protein, the glucocorticoid receptor, which functions as a hormone-activated transcription factor that regulates the expression of glucocorticoid target genes (Nicolaides *et al.*, 2010). The human glucocorticoid receptor (hGR) is encoded by the *NR3C1* gene, which is located on chromosome 5 and consists of 10 exons; exon 1 contains non-coding DNA regions, whereas exons 2-9α/9β are transcribed and translated into the four functional domains of the receptor: the immunogenic or N-terminal domain, the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD) (Ramamoorthy and Cidlowski, 2016). The alternative use of exon 9α or 9β produces the

two main hGR isoforms, the hGRα and the hGRβ. In addition, the eight alternative translation initiation sites generate eight distinct receptor isoforms for hGRα and, possibly, for hGRβ (Lu and Cidlowski, 2005; Chrousos and Kino, 2005).

At the cellular and molecular level, the hGRα resides primarily in the cytoplasm and forms a multiprotein complex that consists of heat shock proteins (HSP90, HSP70) and immunophillins (FKBP51 and FKBP52). Upon ligand binding, the receptor undergoes conformational alterations that result in the dissociation of hGRα from the multiprotein complex and subsequent translocation to the nucleus (Chrousos and Kino, 2005). In the nucleus, the ligand-bound hGRα forms homo- or hetero-dimers, which bind onto specific DNA sequences located in regulatory regions of glucocorticoid responsive genes, influencing the transcription of the latter in a positive or negative fashion. In addition to hGRα-mediated induction or repression of target genes, the activated receptor may interact with other transcription factors, including signal transducers and activators of transcription (STATs), the activator protein-1 (AP-1), and the nuclear factor-κB (NF-κB), thereby altering their transcriptional activity (Barnes and Karin, 1997; Karin and Chang, 2001; Didonato *et al.*, 1996).

Glucocorticoids may also induce some effects within a short time frame. These effects occur in cells lacking nucleus, and are not sensitive to transcription/translation inhibitors (Groeneweg *et al.*, 2012). Although the molecular mechanisms underlying the nongenomic glucocorticoid effects have not been elucidated yet, provocative studies have revealed an important role of a membrane-bound GR that activates the mitogen-activated protein kinase (MAPK) and/or the phosphatidylinositol 3-kinase (PI_3K) pathways (Samarasinghe *et al.*, 2012). Further to nongenomic effects, glucocorticoids exert mitochondrial actions through a locally expressed hGR (Psarra and Sekeris, 2011). Interestingly, synthetic glucocorticoids are used for the treatment of several hematologic malignancies, because they cause apoptosis in malignant cells, an effect that occurs in mitochondria (Lee *et al.*, 2013).

Treatment With Glucocorticoids

Natural and synthetic glucocorticoids can be used for both endocrine and nonendocrine disorders. In clinical practice, glucocorticoids are used to establish the diagnosis and cause of Cushing syndrome and in the treatment of adrenal insufficiency and congenital adrenal hyperplasia. Glucocorticoids are also given in pharmacologic doses to treat patients with inflammatory, allergic, or immunologic disorders (Stewart, 2003; Miller, 2005; Simpson and Waterman, 1995). Chronic therapy has many side effects, ranging from suppression of the HPA axis and Cushing syndrome to infections and changes in mental status. Factors that influence both the therapeutic and adverse effects of glucocorticoids include the pharmacokinetic properties of the glucocorticoid, daily dosage, timing of doses during the day, individual differences in steroid metabolism, and the duration of treatment.

Glucocorticoid Replacement Therapy

In deficiency states, physiologic replacement is best achieved with a combination of hydrocortisone and the mineralocorticoid fludrocortisone; hydrocortisone alone does not usually provide sufficient mineralocorticoid activity for complete replacement. In Addison's disease or following adrenalectomy, hydrocortisone at 10–15 mg/m^2 daily by mouth is usually required. This is given in two doses, the larger in the morning and the smaller in the evening, mimicking the normal diurnal rhythm of cortisol secretion. The optimum daily dose is determined on the basis of clinical response (Charmandari *et al.*, 2010). Glucocorticoid therapy is supplemented by fludrocortisone 50–300 μg daily. In acute adrenocortical insufficiency, hydrocortisone is given intravenously (preferably as sodium succinate) at doses of 100 mg every 6–8 h in 0.9% sodium chloride intravenous infusion. In hypopituitarism, glucocorticoids should be given as in adrenocortical insufficiency, but since the production of aldosterone is regulated by the renin–angiotensin system, a mineralocorticoid is not usually required. Additional replacement therapy with levothyroxine and sex hormones should be given as indicated by the pattern of hormone deficiency (Charmandari *et al.*, 2010).

Glucocorticoid Therapy

In comparing the relative potencies of corticosteroids in terms of their anti-inflammatory (glucocorticoid) effects, it should be borne in mind that high glucocorticoid activity in itself is of no advantage unless it is accompanied by relatively low mineralocorticoid activity (Charmandari *et al.*, 2010). The mineralocorticoid activity of fludrocortisone is so high that its anti-inflammatory activity is of no clinical relevance. The equivalent anti-inflammatory doses of corticosteroids are shown in **Table 1**.

The relatively high mineralocorticoid activity of cortisone and hydrocortisone and the resulting fluid retention make them unsuitable for disease suppression on a long-term basis. However, they can be used for adrenal replacement therapy; hydrocortisone is preferred because cortisone requires conversion to hydrocortisone in the liver. Hydrocortisone is used on a short-term basis by intravenous injection for the emergency management of some conditions. The relatively moderate anti-inflammatory potency of hydrocortisone also makes it a useful topical corticosteroid for the management of inflammatory skin conditions because side effects (both topical and systemic) are less marked; cortisone is not active topically (Charmandari *et al.*, 2010).

Prednisolone has predominantly glucocorticoid activity and is the corticosteroid most commonly used by mouth for long-term disease suppression (Charmandari *et al.*, 2010). Betamethasone and dexamethasone have very high glucocorticoid activity but insignificant mineralocorticoid activity. This makes them particularly suitable for high-dose therapy in conditions where fluid

Table 1 Equivalent anti-inflammatory doses of corticosteroids

Prednisolone	5 mg
≡ Betamethasone	750 μg
≡ Cortisone acetate	25 mg
≡ Deflazacort	6 mg
≡ Dexamethasone	750 μg
≡ Hydrocortisone	20 mg
≡ Methylprednisolone	4 mg
≡ Triamcinolone	4 mg

Note: This table takes no account of mineralocorticoid effects, nor does it take account of variations in duration of action.
Reprinted from the British Medical Association and the Royal Pharmaceutical Society of Great Britain (2002). British National Formulary No: 44. Chapter 6, pp. 348–351. London: Pharmaceutical Press.

retention would be a disadvantage. Betamethasone and dexamethasone also have a long duration of action and this, coupled with their lack of mineralocorticoid action, makes them particularly suitable for conditions that require suppression of ACTH secretion (e.g., congenital adrenal hyperplasia). Some esters of betamethasone and beclometasone (beclomethasone) exert a considerably more marked topical effect (e.g., on the skin or the lungs) than when given by mouth; use is made of this to obtain topical effects while minimizing systemic side effects (e.g. for skin applications and asthma inhalations) (Charmandari *et al.*, 2010). Deflazacort is derived from prednisolone and has high glucocorticoid activity (Charmandari *et al.*, 2010).

Side Effects of Glucocorticoids

Overdosage or prolonged use may exaggerate some of the normal physiologic actions of corticosteroids, leading to mineralocorticoid and glucocorticoid side effects. Mineralocorticoid side effects include hypertension, sodium and water retention, and potassium loss. They are most marked with fludrocortisone, but are significant with cortisone, hydrocortisone, corticotropin, and tetracosactide (tetracosactrin). Mineralocorticoid actions are negligible with the high-potency glucocorticoids betamethasone and dexamethasone and occur only slightly with methylprednisolone, prednisolone, and triamcinolone (Charmandari *et al.*, 2010). Glucocorticoid side effects include diabetes and osteoporosis, avascular necrosis of the femoral head, mental disturbances (a serious paranoid state or depression with risk of suicide may be induced, particularly in patients with a history of mental disorder), euphoria, and muscle wasting (proximal myopathy) (**Table 2**). Corticosteroid therapy is also weakly linked with peptic ulceration. High doses of corticosteroids may cause Cushing syndrome, which is usually reversible on withdrawal of treatment, but this must always be gradually tapered to avoid symptoms of acute adrenal insufficiency. In children, administration of corticosteroids may result in suppression of growth. Other complications include increased susceptibility to infection, poor wound healing, and activation of latent granulomatous infections (Charmandari *et al.*, 2010).

Adrenal Suppression

During prolonged therapy with corticosteroids, adrenal atrophy develops and may persist for years after stopping. Abrupt withdrawal after a prolonged period may lead to acute adrenal insufficiency, hypotension, or death. Withdrawal may also be associated with fever, myalgia, arthralgia, rhinitis, conjunctivitis, painful itchy skin nodules, and weight loss (Charmandari *et al.*, 2010).

To compensate for a diminished adrenocortical response caused by prolonged corticosteroid treatment, any significant intercurrent illness, trauma, or surgical procedure requires a temporary increase in corticosteroid dose or, if already stopped, a temporary reintroduction of corticosteroid treatment. Anesthetists must therefore know whether a patient is taking or has been taking a corticosteroid, to avoid a precipitous fall in blood pressure during anesthesia or in the immediate postoperative period. A suitable regimen for corticosteroid replacement, in patients who have taken more than 10 mg prednisolone daily (or equivalent) within 3 months of surgery, is as follows:

- Minor surgery under general anesthesia—usual oral corticosteroid dose on the morning of surgery or hydrocortisone 25–50 mg (usually sodium succinate) intravenously at induction; the usual oral corticosteroid dose is recommenced after surgery.
- Moderate or major surgery—usual oral corticosteroid dose on the morning of surgery and hydrocortisone 25–50 mg intravenously at induction, followed by hydrocortisone 25–50 mg three times a day by intravenous injection for 24 h after moderate surgery or for 48–72 h after major surgery; the usual preoperative oral corticosteroid dose is recommenced on stopping hydrocortisone injections.

Patients on long-term corticosteroid treatment should carry a Steroid Treatment Card, which gives guidance on minimizing risk and provides details of prescriber, drug, dosage and duration of treatment (Charmandari *et al.*, 2010).

Table 2 Effects of Chronic Pharmacologic Use of Glucocorticoids

Endocrine and metabolic
Suppression of HPA axis (adrenal suppression)
Growth failure in children
Carbohydrate intolerance
Hyperinsulinism
Insulin resistance
Abnormal glucose tolerance test
Diabetes mellitus
Cushingoid features
Moon facies, facial plethora
Generalized and truncal obesity
Supraclavicular fat collection
Posterior cervical fat deposition (buffalo hump)
Glucocorticoid-induced acne
Thin and fragile skin, violaceous striae
Impotence, menstrual disorders
Decreased thyroid-stimulating hormone and triiodothyronine
Hypokalemia, metabolic alkalosis
Gastrointestinal
Gastric irritation, peptic ulcer
Acute pancreatitis (rare)
Fatty infiltration of liver (hepatomegaly) (rare)
Hematopoietic
Leukocytosis
Neutrophilia
Increased influx from bone marrow and decreased migration from blood vessels
Monocytopenia
Lymphopenia
Migration from blood vessels to lymphoid tissue
Eosinopenia
Immunologic
Suppression of delayed hypersensitivity
Inhibition of leukocyte and tissue macrophage migration
Inhibition of cytokine secretion/action
Suppression of the primary antigen response
Musculoskeletal
Osteoporosis, spontaneous fractures
Aseptic necrosis of femoral and humoral heads and other bones
Myopathy
Ophthalmologic
Posterior subcapsular cataracts (more common in children)
Elevated intraocular pressure/glaucoma
Neuropsychiatric
Sleep disturbances, insomnia
Euphoria, depression, mania, psychosis
Pseudo-tumor cerebri (benign increase of intracranial pressure)
Cardiovascular
Hypertension
Congestive heart failure in predisposed patients

Infections

Prolonged courses of corticosteroids increase susceptibility to infections and severity of infections; clinical presentation of infections may also be atypical. Serious infections, for example, septicemia and tuberculosis, may reach an advanced stage before being recognized and amebiasis or strongyloidiasis may be activated or exacerbated (they should be excluded before corticosteroid treatment is initiated in those at risk or with suggestive symptoms). Fungal or viral ocular infections may also be exacerbated (Charmandari *et al.*, 2010).

Chickenpox

Unless they have had chickenpox, patients receiving oral or parenteral corticosteroids for purposes other than replacement should be regarded as being at risk of severe chickenpox. Passive immunization with varicella-zoster immunoglobulin is needed for

exposed nonimmune patients receiving systemic corticosteroids or for those who have used them within the previous 3 months; varicella-zoster immunoglobulin should preferably be given within 3 days of exposure and no later than 10 days. Confirmed chickenpox warrants specialist care and urgent treatment. Corticosteroids should not be stopped and dosage may need to be increased. Topical, inhaled, or rectal corticosteroids are less likely to be associated with an increased risk of severe chickenpox (Charmandari *et al.*, 2010).

Measles

Patients taking corticosteroids should be advised to take particular care to avoid exposure to measles and to seek immediate medical advice if exposure occurs. Prophylaxis with intramuscular normal immunoglobulin may be needed (Charmandari *et al.*, 2010).

Administration of Corticosteroids

Whenever possible, local treatment with creams, intra-articular injections, inhalations, eye drops, or enemas should be used in preference to systemic treatment. The suppressive action of a corticosteroid on cortisol secretion is lowest when it is given as a single dose in the morning. In an attempt to reduce HPA axis suppression further, the total dose for 2 days can sometimes be taken as a single dose on alternate days; alternate-day administration has not been very successful in the management of asthma. HPA axis suppression can also be reduced by means of intermittent therapy with short courses. In some conditions, it may be possible to reduce the dose of corticosteroid by adding a small dose of an immunosuppressive drug (Charmandari *et al.*, 2010).

Dosage of corticosteroids varies widely in different diseases and in different patients. If the use of a corticosteroid can save or prolong life, high doses may need to be given, because the complications of therapy are likely to be less serious than the effects of the disease itself. When long-term corticosteroid therapy is used in some chronic diseases, the adverse effects of treatment may become greater than the disabilities caused by the disease. To minimize side effects, the maintenance dose should be kept as low as possible (Charmandari *et al.*, 2010).

Withdrawal of Corticosteroids

A gradual withdrawal of systemic corticosteroids should be considered in those subjects whose disease is unlikely to relapse and who have: (1) recently received repeated courses (particularly if taken for longer than 3 weeks); (2) taken a short course within 1 year of stopping long-term therapy; (3) other possible causes of adrenal suppression; (4) received more than 40 mg daily prednisolone (or equivalent); (5) been given repeat doses in the evening; or (6) received treatment for more than 3 weeks (Charmandari *et al.*, 2010).

Systemic corticosteroids may be stopped abruptly in those whose disease is unlikely to relapse and who have received treatment for 2 weeks or less and who are not included in the patient groups described above. During corticosteroid withdrawal, the dose may be reduced rapidly to physiological doses (equivalent to prednisolone at 5 mg daily) and then reduced more slowly. Assessment of the disease may be needed during withdrawal to ensure that relapse does not occur (Charmandari *et al.*, 2010).

See also: Adrenal Cortex; Physiology. Corticotropin-Releasing Hormone Peptide Family. Stress and Endocrine Physiology

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Stress and Endocrine Physiology☆

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Nomenclature

ACTH	Adrenocorticotropic hormone
AVP	Arginine-vasopressin
BMAL1	Brain–muscle Arnt-like protein 1
Chrono	ChIP-derived repressor of network oscillator
CLOCK	Circadian locomotor output cycle kaput
CRH	Corticotropin-releasing hormone
CRY	Cryptochromes
HPA axis	Hypothalamic–pituitary–adrenal axis
IL-6	Interleukin-6
LC	Locus caeruleus
PER	Periods
PTSD	Posttraumatic stress disorder
SCN	Suprachiasmatic nucleus

Glossary

Autonomic nervous system The part of the nervous system that controls involuntary actions, including the smooth muscles, cardiac muscle, and glands. It is composed of the sympathetic and parasympathetic nervous systems.
Glucocorticoids Any of a group of adrenocortical steroid hormones whose metabolic effects include stimulation of gluconeogenesis, increased catabolism of proteins, and mobilization of free fatty acids; they are also potent inhibitors of the inflammatory reaction.
HPA axis The hypothalamic–pituitary–adrenal axis.

Introduction

Stress is defined as a state of disharmony or threatened homeostasis (Chrousos, 2009). The concepts of stress and homeostasis can be traced back to ancient Greek history; however, the integration of these notions with related physiologic and pathophysiologic mechanisms and their association with health and disease are much more recent (Nicolaides *et al.*, 2015).

This article focuses on the cellular and molecular infrastructure of the physiologic and behavioral adaptive responses to stress and defines the pathophysiologic effects of the dysregulation of the stress response, which may result in vulnerability to several disease entities, such as anxiety, depression, chronic inflammatory processes, and cardiovascular disease.

Physiology of the Stress Response

Life exists by maintaining a complex dynamic equilibrium, or homeostasis, that is constantly challenged by intrinsic or extrinsic adverse forces, the stressors (Chrousos and Gold, 1992; Chrousos, 2009). Under favorable conditions, individuals can be invested in vegetative and pleasurable functions that enhance their emotional and intellectual growth and development and the survival of their species, such as food intake and sex. In contrast, activation of the stress response during threatening situations that are beyond the control of the individual can be associated with dysphoria and eventually emotional or somatic disease (Chrousos, 2009; Nicolaides *et al.*, 2015).

When faced with excessive stress, whether physical or emotional, a subject's adaptive responses attain a relatively stereotypic nonspecific nature, referred to by Hans Selye as "the general adaptation syndrome" (Selye, 1936a, b). The adaptive responses have some specificity toward the stressor that generates them, which, however, is progressively lost as the severity of the stressor increases. During stress, attention is enhanced and the brain focuses on the perceived threat. Cardiac output and respiration are accelerated, catabolism is increased, and blood flow is redirected to provide the highest perfusion and fuel to the aroused brain, heart, and muscles (Chrousos, 1998).

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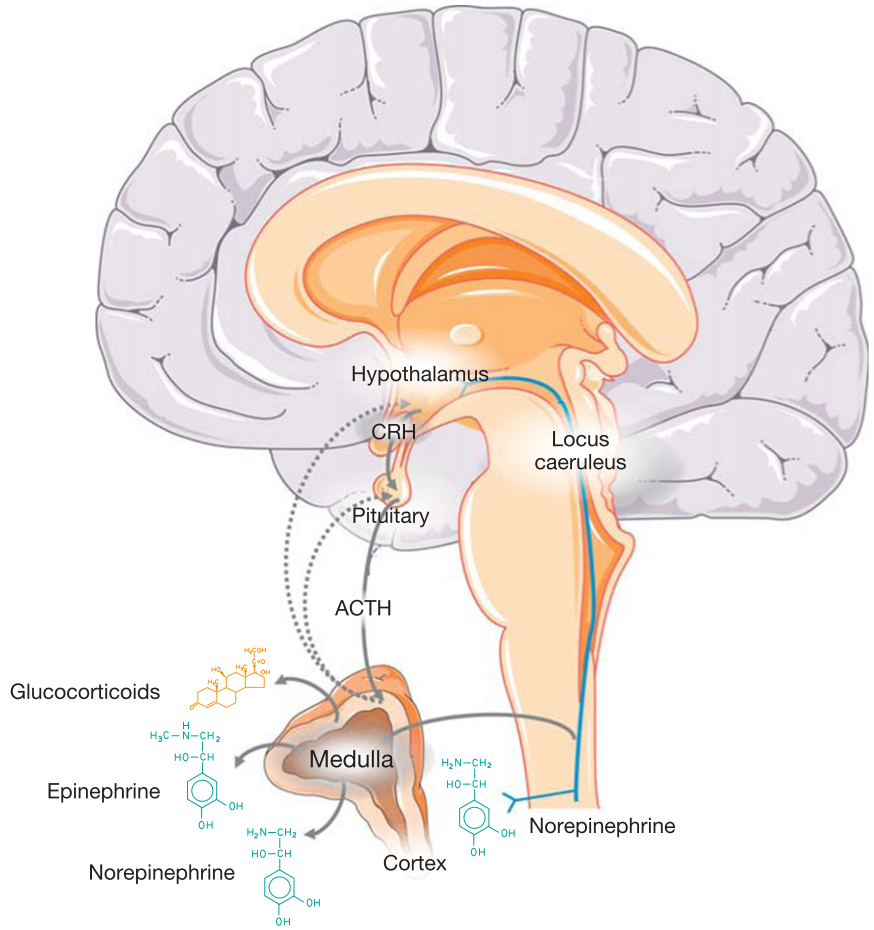

Fig. 1 The stress system consists of the HPA axis and the locus caeruleus/autonomic nervous system. *Solid lines* indicate stimulation; *dashed lines* indicate inhibition. *CRH*: corticotropin-releasing hormone; *ACTH*: adrenocorticotropic hormone. Modified from Chrousos, G.P., 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. New England Journal of Medicine 332, 1351–1362.

The stress system consists of the hypothalamic–pituitary–adrenal (HPA) axis and the locus caeruleus/autonomic nervous system (**Fig. 1**). The central control stations of the stress system are located in the hypothalamus and the brainstem and include the parvocellular corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) neurons of the paraventricular nuclei of the hypothalamus and the locus caeruleus (LC)-norepinephrine (NE) system (central sympathetic system). The hypothalamic–pituitary–adrenal (HPA) axis and the efferent sympathetic/adrenomedullary system represent the effector limbs, via which the brain influences all body organs during exposure to threatening stimuli (**Fig. 1**). The brain also differentially activates a subset of vagal and sacral parasympathetic efferents that mediate the gut responses to stress (Chrousos and Gold, 1992; Nicolaides *et al.*, 2015).

There are mutual interactions of the central stress stations with three higher brain control areas that influence affect and anticipatory phenomena (mesocortical/mesolimbic systems); the initiation, propagation, and termination of stress system activity (amygdala/hippocampus complex) and the setting of the pain sensation (arcuate nucleus) (**Fig. 2**). Several molecules and factors influence substantially the stress system activity (**Fig. 2**).

The Hypothalamic–Pituitary–Adrenal Axis

The hypothalamus controls the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which, in turn, stimulates the secretion by the adrenal cortex of glucocorticoid hormones, mainly cortisol in humans (Tsigos and Chrousos, 1994; Chrousos, 2009). The principal hypothalamic stimulus to the pituitary–adrenal axis is CRH, a 41-amino-acid peptide first isolated in 1981 by Wylie Vale (Vale *et al.*, 1981). AVP is a potent synergistic factor with CRH in stimulating ACTH secretion; however, AVP has little ACTH secretagogue activity by itself (Gillies *et al.*, 1982; Antoni, 1993). Furthermore, it appears that there is a reciprocal positive interaction between CRH and AVP at the level of the hypothalamus, with each neuropeptide stimulating the secretion of the other (Rivier *et al.*, 1984).

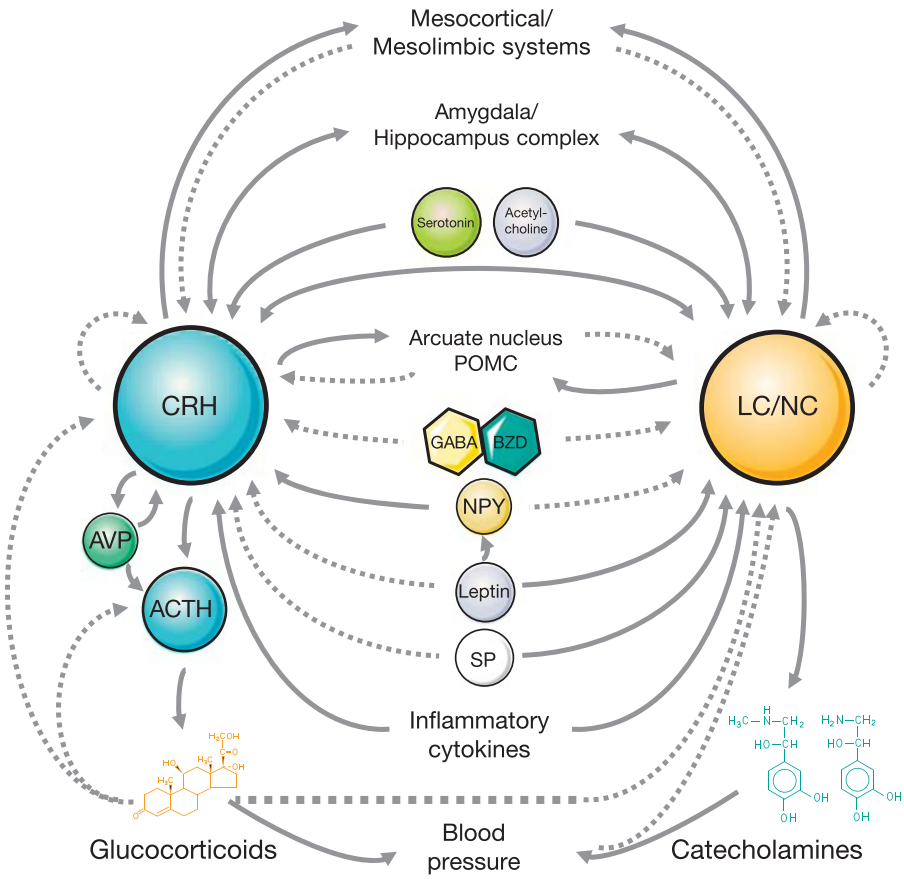

Fig. 2 A simplified schematic representation of the central and peripheral components of the stress system, their functional interrelations, and their relationships to other central systems involved in the stress response. The CRH–AVP neurons and central catecholaminergic neurons of the LC–NE system reciprocally innervate and activate one another. The HPA axis is controlled by several feedback loops that tend to normalize the time-integrated secretion of cortisol, yet glucocorticoids stimulate the fear centers in the amygdala. Activation of the HPA axis leads to suppression of the GH/insulin-like growth factor-I, luteinizing hormone/testosterone/estradiol, and TSH/triiodothyronine axes; activation of the sympathetic system increases interleukin-6 secretion. *Solid lines* indicate stimulation; *dashed lines* indicate inhibition. *BZD*, benzodiazepine; *GABA*, g-aminobutyric acid; *NPY*, neuropeptide Y; *SP*, substance P. Modified from Chrousos, G.P., Gold, P.W., 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. Journal of the American Medical Association 267, 1244–1252.

In nonstressful situations, both CRH and AVP are secreted in the portal system in a circadian, pulsatile fashion, with a frequency of approximately two to three secretory episodes per hour (Engler *et al.*, 1989; Redekopp *et al.*, 1986). Under resting conditions, the amplitude of the CRH and AVP pulses increases in the early morning hours, resulting finally in ACTH and cortisol secretory bursts in the general circulation (Horrocks *et al.*, 1990). These diurnal variations are perturbed by changes in lighting, feeding schedules, and activity, are influenced by age, body mass index (BMI), and gender, and are disrupted by stress (Veldhuis *et al.*, 2011).

During acute stress, the amplitude and synchronization of the CRH and AVP pulsations in the hypophyseal portal system markedly increase, resulting in increases in ACTH and cortisol secretory episodes. Depending on the type of stress, other factors such as AVP of magnocellular neuron origin, angiotensin II, and various cytokines and lipid mediators of inflammation are secreted and act on hypothalamic, pituitary, or adrenal components of the HPA axis, potentiating its activity (Tsigos *et al.*, 2016).

Circulating ACTH is the key regulator of glucocorticoid secretion by the adrenal cortex and participates in aldosterone secretion by the *zona glomerulosa* (Aguilera, 1993). Other hormones or cytokines, either originating from the adrenal medulla or coming from the systemic circulation, as well as neuronal information from the autonomic innervation of the adrenal cortex may also participate in the regulation of cortisol secretion.

Glucocorticoids are the final effectors of the HPA axis and participate in the control of whole-body homeostasis and the organism's response to stress. They play a key regulatory role on the basal activity of the HPA axis and on the termination of the stress response by acting at extrahypothalamic centers, the hypothalamus, and the pituitary gland. The inhibitory glucocorticoid feedback on the ACTH secretory response acts to limit the duration of the total tissue exposure to glucocorticoids, thus minimizing the catabolic, antireproductive, and immunosuppressive effects of these hormones (Nicolaides *et al.*, 2015).

Glucocorticoids exert their effects through their ubiquitous cytoplasmic receptors (Chrousos and Kino, 2005). On ligand binding, the glucocorticoid receptors (GRs) translocate into the nucleus, where they interact as homodimers with specific

glucocorticoid-responsive elements within the DNA to activate appropriate hormone-responsive genes. The activated receptors also inhibit, through protein–protein interactions, other transcription factors, such as c-jun/c-fos, and nuclear factor κB, which are positive regulators of the transcription of several genes involved in the activation and growth of immune and other cells. Furthermore, glucocorticoids change the stability of mRNAs and hence the translation of several glucocorticoid-responsive proteins, as well as the electrical potential of neuronal cells (Nicolaides *et al.*, 2010).

Circulating glucocorticoids in humans have a circadian pattern of secretion, regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus. The zenith of cortisol secretion is reached in the early morning and the nadir, at midnight, with the purpose of helping adjust the body's activities to the regular periodicity of day/night changes. CLOCK (the "circadian locomotor output cycle kaput") and its heterodimer partner BMAL1 (the "brain muscle ARNT-like protein-1") are essential in the formation of the circadian rhythm in man, participating in the physiological adjustment to daily activities, to the regular day/night changes and to unpredictable external or internal challenges (Nicolaides *et al.*, 2014, 2017).

The Autonomic Axes

The autonomic nervous system provides a rapidly responding mechanism to control a wide range of functions. Cardiovascular, respiratory, gastrointestinal, renal, endocrine, and other systems are regulated by the sympathetic nervous system or the parasympathetic system or both (Gilbey and Spyer, 1993). Interestingly, the parasympathetic system may assist sympathetic functions by withdrawing and can antagonize them by increasing its activity.

Sympathetic innervation of peripheral organs is derived from the efferent preganglionic fibers, whose cell bodies lie in the intermediolateral column of the spinal cord. These nerves synapse in the bilateral chains of sympathetic ganglia with postganglionic sympathetic neurons that richly innervate the smooth muscle of the vasculature, the heart, the skeletal muscles, the kidney, the gut, fat, and many other organs (Burnstock and Miller, 1989). The preganglionic neurons are cholinergic, whereas the postganglionic neurons are mostly noradrenergic. The sympathetic system also has a humoral contribution, providing most of the circulating epinephrine and some of the norepinephrine from the adrenal medulla. In addition to the classic neurotransmitters acetylcholine and norepinephrine, both sympathetic and parasympathetic subdivisions of the autonomic nervous system contain several subpopulations of target-selective and neurochemically coded neurons that express a variety of neuropeptides and, in some cases, ATP, nitric oxide, or lipid mediators of inflammation (Benarroch, 1994). Interestingly, CRH, neuropeptide Y, and somatostatin are colocalized in noradrenergic vasoconstrictive neurons. Transmission in sympathetic ganglia is also modulated by neuropeptides released from preganglionic fibers and short interneurons (e.g., enkephalin, neurotensin), as well as from primary afferent collaterals (e.g., substance P) (Elfvin *et al.*, 1993).

Regulation of the Stress Response

The orchestrated interplay of several neurotransmitter systems in the brain underlies the characteristic phenomenology of behavioral, endocrine, autonomic, and immune responses to stress. These transmitters include CRH, AVP, opioid peptides, dopamine, and norepinephrine (Tsigos *et al.*, 2016).

CRH–AVP

Shortly after its isolation, it became apparent that CRH was implicated in other components of the stress response, such as arousal and autonomic activity. Supportive evidence was derived from intracerebroventricular or selective brain administration of CRH in rodents and nonhuman primates, which precipitated several coordinated responses characteristic of stress (Dunn and Berridge, 1990). Moreover, administration of CRH peptide antagonists into selected areas of the brain suppresses many aspects of the stress response. Finally, CRH type 1 receptor knockout mice were shown to have a markedly deficient ability to mount an effective stress response.

CRH and CRH receptors were found in many sites in the brain outside of the hypothalamus, including parts of the limbic system and the central arousal sympathetic systems (LC–sympathetic systems) in the brainstem and spinal cord (Aguilera *et al.*, 1987; De Souza *et al.*, 1985). Stress is a potent general activator of CRH release from the hypothalamus and extra-hypothalamic sites. The mechanisms via which stress stimulates CRH neurons are unclear. Whether CRH or another transmitter (e.g., NE) is upstream in eliciting the neurocircuitry of stress remains to be determined.

CRH-binding sites are also found in various peripheral tissues, such as the adrenal medulla, heart, prostate, gut, liver, kidney, and testes (Tsigos *et al.*, 2016). CRH receptors belong to the G protein-coupled receptor superfamily and CRH binding stimulates the intracellular accumulation of cyclic AMP (Perrin and Vale, 1999). Two distinct CRH receptor subtypes, designated CRH-R1 and CRH-R2, have been characterized, encoded by distinct genes that are differentially expressed (Polymeropoulos *et al.*, 1995; Meyer *et al.*, 1997). CRH-R1 is the most abundant subtype found in the anterior pituitary and is also widely distributed in the brain, the adrenal gland, skin, gastrointestinal tract, ovary and testis (Chen *et al.*, 1993). CRH-R2 receptors are expressed mainly in the peripheral vasculature and the heart, as well as in subcortical structures in the brain (Lovenberg *et al.*, 1995).

Locus Caeruleus–NE System

The locus caeruleus and other noradrenergic cell groups of the medulla and pons are collectively known as the LC–NE system. Brain epinephrine serves globally as an alarm system that decreases neurovegetative functions, such as eating and sleeping, and that contributes to accompanying increases in autonomic and neuroendocrine responses to stress, including HPA axis activation. NE also activates the amygdala, the principal brain locus for fear-related behaviors, and enhances the long-term storage of aversively charged emotional memories in sites such as the hippocampus and striatum.

Reciprocal neural connections exist between the CRH and catecholaminergic neurons (LC–NE neurons) of the central stress system, with CRH and norepinephrine stimulating each other, the latter primarily through α_1-noradrenergic receptors (Valentino *et al.*, 1983; Calogero *et al.*, 1988a, b, c; Kiss and Aguilera, 1992). There is an ultra-short autoregulatory negative feedback loop on the CRH neurons exerted by CRH itself, just as there is a similar loop in the LC–NE neurons, by way of presynaptic CRH and α_2-noradrenergic receptors, respectively (Aghajanian and VanderMaelen, 1982; Calogero *et al.*, 1988a, b, c; Silverman *et al.*, 1989). There is also parallel regulation of both central components of the stress system by other stimulatory and inhibitory neuronal pathways. Several neurotransmitters, including serotonin and acetylcholine, excite CRH and the LC–NE neurons (Calogero *et al.*, 1990; Fuller, 1992). The negative feedback controls include glucocorticoids, γ-aminobutyric acid, corticotropin, and several opioid peptides, which inhibit both CRH and LC–NE neurons (Keller-Wood and Dallman, 1984; Calogero *et al.*, 1988a, b, c; Overton and Fisher, 1989).

Body Systems' Responses to Stress

HPA Axis–Immune System Interactions

It has been known for several decades that stress, whether inflammatory, traumatic, or psychological, is associated with concurrent activation of the HPA axis. In the early 1990s, it also became apparent that cytokines and other humoral mediators of inflammation are potent activators of the central stress response, constituting the afferent limb of a feedback loop through which the immune–inflammatory system and the CNS communicate (Reichlin, 1993; Chrousos, 1995).

All three inflammatory cytokines, tumor necrosis factor α, interleukin-1β (IL-β), and interleukin-6, can cause stimulation of the HPA axis alone or in synergy with one another (Bateman *et al.*, 1989; Akira *et al.*, 1990; Besedovsky and del Rey, 1992). There is evidence to suggest that IL-6, the main endocrine cytokine, plays the major role in the immune stimulation of the axis, especially in chronic inflammatory stress (Mastorakos *et al.*, 1993; Tsigos *et al.*, 1997; Rohleder, 2012).

Some of the activating effects of cytokines on the HPA axis may be exerted indirectly by stimulation of the central catecholaminergic pathways. Also, activation of peripheral nociceptive, somatosensory, and visceral afferent fibers would lead to stimulation of both the catecholaminergic and CRH neuronal systems via ascending spinal pathways (Tsigos *et al.*, 2016). Other inflammatory mediators, such as eicosanoids, platelet-activating factor, and serotonin, may also participate in the activation of the HPA axis, via autocrine/paracrine and/or endocrine effects (Bernardini *et al.*, 1989a, b).

Conversely, activation of the HPA axis has profound inhibitory effects on the inflammatory/immune response because virtually all the components of the immune response are inhibited by cortisol (Chrousos, 2000a, b; Charmandari *et al.*, 2004). Alterations of leukocyte traffic and function, decreases in production of cytokines and mediators of inflammation, and inhibition of the latter's effects on target tissues are among the main immunosuppressive effects of glucocorticoids (Chrousos, 2000a, b; Charmandari *et al.*, 2004).

The efferent sympathetic/adrenomedullary system apparently participates in a major fashion in the interactions of the HPA axis and the immune/inflammatory reaction by being reciprocally connected with the CRH system, by receiving and transmitting humoral and nervous immune signals from the periphery, by densely innervating both primary and secondary lymphoid organs, and by reaching all sites of inflammation via the postganglionic sympathetic neurons (Ottaway and Husband, 1992; Bellinger *et al.*, 1992). When activated during stress, the autonomic system exerts its own direct effects on immune organs, which can be immunosuppressive or both immunopotentiating and antiinflammatory (Hirano *et al.*, 1990; Rohleder, 2012). CRH secreted by postganglionic sympathetic neurons at inflammatory sites has proinflammatory properties (immune CRH) (Karalis *et al.*, 1991); one of its key actions is to degranulate mast cells (Theoharides *et al.*, 1998).

HPA Axis–Clock System Interactions

To adjust their activities to day/night cycles, all organisms have developed a highly sophisticated and conserved system, the circadian (from the Latin "circa diem" meaning "approximately a day") clock system, which generates internal rhythmicity according to light/dark information. This system consists of a central "master" clock located in the SCN of the hypothalamus and the peripheral "slave" clocks expressed in every tissue including the brain minus the SCN (Ko and Takahashi, 2006; Takahashi *et al.*, 2008; Nader *et al.*, 2010). The central clock communicates with peripheral clocks through unknown mechanisms, ultimately regulating many homeostatic systems (Kalsbeek *et al.*, 2006). At the molecular level, the circadian clock system creates a self-oscillating rhythm through transcriptional/translational loops, which are mediated by the heterodimer CLOCK/BMAL1 and

several negative transcription factors, including Periods (PER1, PER2 and PER3) and Cryptochromes (CRY1 and CRY2) (Kondratov *et al.*, 2006; Kiyohara *et al.*, 2006; Padmanabhan *et al.*, 2012).

Accumulating evidence suggests that there are molecular interrelations between the HPA axis and the circadian clock system. In addition to the above-discussed diurnal oscillation of circulating glucocorticoid concentrations, the central clock influences the adrenal cortex response to plasma ACTH via a multisynaptic neuronal pathway (Ishida *et al.*, 2005; Ulrich-Lai *et al.*, 2006). Moreover, in peripheral tissues, the circadian rhythm transcription factor CLOCK acetylates the GR and represses GR-induced transcriptional activity on glucocorticoid-responsive genes (Nader *et al.*, 2009). This Clock-mediated repression of GR transcriptional activity, oscillates in inverse phase to the HPA axis, acting as a target tissue counter regulatory mechanism to the diurnally fluctuating circulating glucocorticoids (Nader *et al.*, 2010; Kino and Chrousos, 2011a, b). Interestingly, a recently identified circadian CLOCK component and BMAL target gene, the *Gm129*, later termed as "Chrono" ("ChIP-derived repressor of network oscillator") was demonstrated to interact with the GR, providing a novel potential link between the circadian clock system and the HPA axis (Goriki *et al.*, 2014; Robinson, 2014).

On the other hand, glucocorticoids may reset the activity of peripheral clocks by phase-shifting the expression of several clock-related genes, including *Pers* (Yamamoto *et al.*, 2005). Indeed, glucocorticoids induce the expression of *Per1* and *Per2* genes, leading to a phase delay of the peripheral clocks, but not the SCN master clock (So *et al.*, 2009). Furthermore, glucocorticoids can regulate local oscillators, both in CNS and peripheral tissues, as demonstrated by rodent studies. Prolonged administration of prednisolone or adrenalectomy influence substantially the expression of a number of clock-related genes (Oster *et al.*, 2017). All these results indicate that there are many reciprocal interactions between the HPA axis and the circadian clock system (Nicolaides *et al.*, 2014; Nicolaides *et al.*, 2017).

HPA Axis–Other Endocrine Axes Interactions

Gonadal, growth and thyroid axes

The systems responsible for reproduction and growth are directly linked to the stress system and both are profoundly inhibited by various components of the HPA axis, the effector of the stress response. Either directly or through arcuate proopiomelanocortin (POMC) neuron β-endorphin, CRH suppresses the gonadotropin-releasing hormone (GnRH) neurons of the arcuate nucleus of the hypothalamus (Tsigos *et al.*, 2016). Glucocorticoids exert inhibitory effects at the levels of the GnRH neuron, the pituitary gonadotroph, and the gonads themselves and render target tissues of sex steroids resistant to these hormones (MacAdams *et al.*, 1986; Rivier *et al.*, 1986; Rabin *et al.*, 1990). Suppression of gonadal function caused by chronic HPA axis activation has been demonstrated in highly trained athletes of both sexes, in ballet dancers, and in individuals sustaining anorexia nervosa or starvation (Kyrou and Tsigos, 2008; Tsigos *et al.*, 2016). Moreover, during inflammatory stress, cytokines suppress reproductive function directly and indirectly by activating hypothalamic secretion of CRH- and POMC-derived peptides, as well as by peripheral elevations of glucocorticoids and inhibition of steroidogenesis at both the ovaries and the testes (Tsigos *et al.*, 1999).

The interaction between CRH and the gonadal axis appears to be bidirectional (Kyrou and Tsigos, 2008). Thus, the presence of estrogen-responsive elements has been demonstrated in the promoter area of the CRH gene, as well as direct stimulatory estrogen effects on CRH gene expression (Vamvakopoulos and Chrousos, 1993). This finding indicates that the CRH gene is a potentially important target of ovarian steroids and a potential mediator of gender-related differences in the stress response and HPA axis activity (Vamvakopoulos and Chrousos, 1994).

The growth axis is also inhibited at many levels during stress. Prolonged activation of the HPA axis with elevations in glucocorticoids leads to suppression of growth hormone (GH) secretion, inhibition of somatomedin C, and other growth hormone effects on their target tissues (Unterman and Phillips, 1985; Dieguez *et al.*, 1988; Burguera *et al.*, 1990). However, acute elevations of growth hormone concentration in plasma may occur at the onset of the stress response or after acute administration of glucocorticoids, presumably through stimulation of the GH gene by glucocorticoids through glucocorticoid-responsive elements in its promoter region (Casanueva *et al.*, 1990). In addition to the direct effects of glucocorticoids, CRH-induced stimulation in hypothalamic somatostatin secretion may also result in GH suppression, providing another potential mechanism for stress-related suppression of GH secretion (Rivier and Vale, 1985).

Similarly, activation of the HPA axis is associated with decreased production of thyroid-stimulating hormone (TSH) and inhibition of the conversion of the relatively inactive thyroxin to the more biologically active triiodothyronine in peripheral tissues (the "euthyroid sick" syndrome) (Duick and Wahner, 1979; Benker *et al.*, 1990; Roelfsema *et al.*, 2009). Both phenomena may be caused by the increased levels of glucocorticoids and may serve to conserve energy during stress. In the case of inflammatory stress, inhibition of TSH secretion may occur in part through the action of cytokines on both the hypothalamus and the pituitary (Torpy *et al.*, 1998).

Metabolism

Glucocorticoids directly inhibit pituitary growth hormone, gonadotropin, and thyrotropin secretion and make the target tissues of sex steroids and growth factors resistant to these hormones. Thus, glucocorticoids antagonize the beneficial actions of GH and sex steroids on fat tissue (lipolysis) and on muscle and bone anabolism (Chrousos, 2000a, b; Kyrou and Tsigos, 2007). Chronic activation of the stress system would be expected to increase visceral adiposity, decrease lean body (muscle and bone) mass, and suppress osteoblastic activity (Chrousos, 2000a, b; Kyrou and Tsigos, 2007; Pervanidou and Chrousos, 2012). Interestingly, the phenotype of central obesity and decreased lean body mass is shared by patients with Cushing syndrome and some patients with

the combined diagnosis of melancholic depression or chronic anxiety disorder and the metabolic syndrome (visceral adiposity, insulin resistance, dyslipidemia, hypertension) or "pseudo-Cushing syndrome."

Because increased hepatic gluconeogenesis is a characteristic feature of the stress response and because glucocorticoids induce insulin resistance, activation of the HPA axis may contribute to the poor control of diabetic patients during periods of emotional stress or concurrently with inflammatory and other diseases (Lloyd *et al.*, 1993).

Obese subjects with psychiatric manifestations ranging from those of melancholic depression to anxiety with perception of "uncontrollable" stress frequently have mild hypercortisolism, whereas carefully screened obese subjects without such manifestations are eucortisolemic (Pasquali *et al.*, 1993; Tamashiro *et al.*, 2011). The former may have stress-induced glucocorticoid-mediated visceral obesity and metabolic syndrome manifestations, which in the extreme may be called a pseudo-Cushing syndrome state that needs to be differentiated from frank Cushing syndrome. Stress-induced hypercortisolism and visceral obesity and their cardiovascular and other sequelae increase the all-cause mortality risk of affected subjects two- to threefold and curtail their life expectancy by several years (Tsigos *et al.*, 2016).

HPA Axis: Pathophysiology

Generally, the stress response with the resultant activation of the HPA axis is meant to be acute or at least of a limited duration. The time-limited nature of this process renders its accompanying antireproductive, antigrowth, catabolic, and immunosuppressive effects temporarily beneficial rather than damaging. In contrast, the chronicity of stress system activation would lead to the syndromal state that Selye described in 1936 (Selye, 1936a, b). Because CRH coordinates behavioral, neuroendocrine and autonomic adaptation during stressful situations, increased and prolonged production of CRH could explain the pathogenesis of the syndrome (Chrousos and Gold, 1992; Chrousos, 2009).

The prototypical example of chronic hyperactivation of the stress system (both the HPA axis and the LC–NE system) is manifested in melancholic depression, with dysphorichyperarousal and relative immunosuppression (Gold *et al.*, 1988a, b). Indeed, cortisol excretion is increased and the plasma ACTH response to exogenous CRH is decreased (Gold and Chrousos, 2002). Hypersecretion of CRH has been observed in depression and suggests that CRH may participate in the initiation or perpetuation of a vicious cycle. Owing to chronically hyperactive stress, patients with melancholic depression may sustain several severe somatic sequelae, such as osteoporosis, features of the metabolic syndrome, varying degrees of atherosclerosis, innate and T helper 1-directed immunosuppression, and certain infectious and neoplastic diseases. When not treated, these patients have a compromised life expectancy curtailed by 15–20 years after suicides are excluded (Gold and Chrousos, 2002).

In addition to melancholic depression, a spectrum of other conditions may be associated with increased and prolonged activation of the HPA axis (**Table 1**), including anorexia nervosa with or without malnutrition, obsessive–compulsive disorder, panic anxiety, chronic active alcoholism, alcohol and narcotic withdrawal, excessive exercising, poorly controlled diabetes mellitus, childhood sexual abuse, and hyperthyroidism (Tsigos *et al.*, 2016).

Another group of states is characterized by hypoactivation of the stress system, rather than sustained activation, in which chronically reduced secretion of CRH may result in pathological hypoarousal (**Table 1**). Patients with atypical, seasonal depression and chronic fatigue syndrome fall into this category (Demitrack *et al.*, 1991; Joseph-Vanderpool *et al.*, 1991). Similarly, patients with fibromyalgia have decreased urinary free-cortisol excretion and frequently complain of fatigue (Griep *et al.*, 1993). Hypothyroid patients also have clear evidence of CRH hyposecretion (Kamilaris *et al.*, 1987).

Table 1 States associated with hyperactivation or hypoactivation of the HPA axis

Increased HPA axis activity	*Decreased HPA axis activity*	*Disrupted HPA axis activity*
Severe chronic disease		
Melancholic depression	Atypical depression	Cushing syndrome
Anorexia nervosa	Seasonal depression	Glucocorticoid deficiency
Obsessive–compulsive disorder	Chronic fatigue syndrome	Glucocorticoid resistance
	Fibromyalgia	
Panic disorder	Hypothyroidism	
Chronic excessive exercise	Adrenal suppression	
Malnutrition	Postglucocorticoid therapy	
Diabetes mellitus	Poststress	
	Nicotine withdrawal	
	Postpartum	
Hyperthyroidism	Menopause	
Central obesity	Rheumatoid arthritis	
Childhood sexual abuse		
Pregnancy		

Modified from Chrousos, G. P. (1992). Regulation and dysregulation of the hypothalamic–pituitary–adrenal axis: The corticotropin releasing hormone perspective. *Endocrinology and Metabolism Clinics of North America* **21,** 833–858.

Withdrawal from smoking has been associated with decreased cortisol and catecholamine secretion (Elgerot, 1978; Puddey *et al.*, 1984). Decreased CRH secretion in the early period of nicotine abstinence could explain the increased appetite and weight gain frequently observed in these patients. In Cushing syndrome, the clinical picture of atypical depression, hyperphagia, weight gain, fatigue, and anergia is consistent with suppression of the CRH neuron by the associated hypercortisolism. The periods after cure of hypercortisolism or following cessation of chronic stress and the postpartum period are also associated with atypical depression, suppressed CRH secretion, and decreased HPA axis activity (Doherty *et al.*, 1990; Gomez *et al.*, 1993).

The pattern of centrally increased CRH secretion, together with decreased cortisol and increased catecholamines in the periphery, is typically represented in posttraumatic stress disorder (PTSD), a stress-related disorder than develops after exposure to traumatic life-events (Pervanidou and Chrousos, 2010). This unique neuroendocrine profile in PTSD suggests an increased cortisol signaling capacity, so that lower cortisol concentrations may suppress more efficiently the HPA axis, thus exerting increased negative feedback. This may lead to decreased exposure of the LC/NE system to the negative actions of cortisol, contributing to increased sympathetic activation. Mechanisms of down-regulation of pituitary CRH receptors secondary to elevated CRH and enhanced negative feedback inhibition of the pituitary to cortisol may also lead to lower peripheral cortisol concentrations in PTSD (Pervanidou and Chrousos, 2010). The important role of stress reactions and, consequently, stress biology in the pathophysiology of posttraumatic stress disorder (PTSD) has been highlighted in the last revision of the Diagnostic and Statistical Manual for Mental Disorders (DSM-5, 2013), where, PTSD is no more classified as an anxiety disorder, but moved into a new class, the "trauma and stressor-related disorders."

It is believed that an excessive HPA axis response to inflammatory stimuli would mimic the stress or hypercortisolemic state and would lead to increased susceptibility of the individual to a host of infectious agents or tumors as a result of T helper-1 suppression, but enhanced resistance to autoimmune/inflammatory disease (Sternberg *et al.*, 1992a,b). In contrast, a defective HPA axis response to such stimuli would reproduce the glucocorticoid-deficient state and would lead to relative resistance to infections and neoplastic disease, but increased susceptibility to autoimmune/inflammatory disease, such as Hashimoto's thyroiditis or rheumatoid arthritis. Thus, an increasing body of evidence suggests that patients with rheumatoid arthritis have a mild form of central hypocortisolism (Chikanza *et al.*, 1992a, b). Dysfunction of the HPA axis may actually play a role in the development or perpetuation of autoimmune disease, rather than being an epiphenomenon. The same rationale may explain the high incidence of autoimmune disease in the period after cure of hypercortisolism, as well as in glucocorticoid underreplaced adrenal insufficiency (Elenkov and Chrousos, 1999).

CRH Receptor as a Promising Therapeutic Target

Antalarmin, a novel CRH receptor type 1 antagonist, decreases the activity of the HPA axis and LC–NE system, suppresses neurogenic inflammation, and blocks CRH-induced skin mast cell degranulation, in addition to blocking the development and expression of conditioned fear and stress-induced colonic hyperfunction (Habib *et al.*, 2000). Chronic administration of antalarmin is not associated with glucocorticoid deficiency and permits HPA axis and LC–NE responses to severe stress (Wong *et al.*, 1999). These data suggest that such CRH antagonists may be useful in human pathologic states, such as melancholic depression and chronic anxiety, associated with chronic hyperactivity of the stress system, along with predictable behavioral, neuroendocrine, metabolic, and immune changes, based on the interrelations outlined above. Conversely, potentiators of CRH secretion and action are needed to treat atypical depression, postpartum depression, and the fibromyalgia/chronic fatigue syndromes, all characterized by low hypothalamic–pituitary–adrenal axis and LC–NE system activity, fatigue, depressive symptomatology, hyperalgesia, and increased immune/inflammatory responses to stimuli (Sajdyk and Gehlert, 2000; Lawrence *et al.*, 2002).

See also: Adrenal Cortex; Physiology. Regulation of POMC and ACTH Secretion

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Hypothalamic–Pituitary–Adrenal Suppression☆

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Abbreviations

ACTH Adrenocorticotropic hormone
AP-1 Activator protein-1
CBG Cortisol binding globulin
CRH Corticotropin-releasing hormone
GREs Glucocorticoid response elements
GWS Glucocorticoid withdrawal syndrome
HPA axis Hypothalamic-pituitary-adrenal axis
NF-κB Nuclear factor-κB
POMC Proopiomelanocortin
STATs Signal transducers and activators of transcription

Glossary

ACTH (adrenocorticotropic hormone) A hormone synthesized and secreted by the anterior pituitary that is primarily responsible for the regulation of cortisol and adrenal androgen secretion.

Adrenal crisis A medical emergency in which the subject is affected by an extreme state of adrenal insufficiency, with symptoms resembling shock or coma; considered to be life-threatening and requiring immediate administration of fluids, electrolytes, glucose, and intravenous glucocorticoids. May occur in situations of extreme stress or after the abrupt cessation of a period of glucocorticoid therapy.

Cushing syndrome A metabolic disorder relating to the chronic oversecretion of adrenal glucocorticoids (mainly cortisol); may be associated with the long-term administration of pharmacological amounts of synthetic glucocorticoids. A condition first described by U.S. neurosurgeon Harvey Cushing in 1912.

HPA axis An anatomical complex consisting of the hypothalamus, the pituitary gland, and the adrenal cortex; ultimately controls the secretion of glucocorticoids from the adrenal cortex and thus plays a key role in various neuroendocrine, behavioral, autonomic, and immune responses to alterations in homeostasis.

Introduction

Glucocorticoids are produced by the cortices of the adrenal glands and secreted into the systemic circulation in a circadian fashion and in response to stressful stimuli (Nicolaides *et al.*, 2014, 2017). These steroid hormones play pivotal roles in the regulation of intermediary metabolism, maintenance of cardiovascular function, stimulation of behavior, and control of the immune inflammatory reaction (Charmandari *et al.*, 2005; Chrousos and Kino, 2005). The major endogenous glucocorticoid in humans is cortisol, the synthetic form of which has traditionally been called hydrocortisone. Cortisone, the 2-keto form of cortisol, was first used therapeutically in the management of rheumatoid arthritis by Hench and coworkers in 1949. Since then, a large number of synthetic compounds with glucocorticoid activity have been developed, and glucocorticoids (administered systemically or in a compartmental fashion) have been used in the therapy of a broad spectrum of nonendocrine and endocrine diseases (Liapi and Chrousos, 1992).

One of the adverse effects of long-term glucocorticoid therapy in supraphysiologic doses is suppression of the hypothalamic–pituitary–adrenal (HPA) axis, which can render the adrenal glands unable to generate sufficient cortisol if glucocorticoid treatment is abruptly stopped, and the patient may develop glucocorticoid deficiency manifestations. The true prevalence of adrenal suppression is not known since physicians usually discontinue high glucocorticoids gradually to allow recovery of the HPA axis (Chrousos *et al.*, 2009). Some of the risk factors for HPA axis suppression are clearly defined, whereas others are less certain (Christy, 1992; Krasner, 1999). Systemic glucocorticoid therapy is more likely to suppress the HPA axis than compartmentalized use of glucocorticoids, with the possible exception of intra-articular steroids. Systemic glucocorticoid potency is also known to correlate with risk for adrenal insufficiency. For this reason, if glucocorticoid dosage is to be reduced, it should be tapered slowly (Chrousos, 2007).

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Physiology of the HPA Axis

The adrenal cortex consists of three anatomic zones: the outer *zona glomerulosa*, the intermediate *zona fasciculata*, and the inner *zona reticularis*. The *zona glomerulosa* is responsible for the production of aldosterone, the *zona fasciculata* for the production of cortisol, and the *zona reticularis* for the production of adrenal androgens (Prigent *et al.*, 2004). Adrenocorticotropic hormone (ACTH), synthesized and secreted by the corticotrophs of the anterior pituitary, is the primary regulator of cortisol and adrenal androgen secretion. Hypothalamic control of ACTH secretion is exerted primarily by corticotropin-releasing hormone (CRH), a 41-amino acid peptide produced by parvocellular neurons of the paraventricular nucleus and secreted into the hypophyseal portal system; Arginine vasopressin produced also by parvocellular neurons of the same nucleus and secreted in the hypophyseal portal system, as well as by magnocellular neurons, exerts a synergistic action on ACTH secretion (Rhen and Cidlowski, 2005).

There are several regulatory negative feedback loops that function to constrain the activity of the HPA axis. Prominent negative feedback loops are those exerted by glucocorticoids on CRH and ACTH secretion (Alves *et al.*, 2008). The adrenal cortisol secretion rate under basal conditions is 12–15 mg/m^2/day. In normal individuals, the highest plasma cortisol levels occur between 6:00 and 8:00 AM and the lowest at approximately midnight (Alves *et al.*, 2008). Cortisol secretion increases two- to fourfold under stress. Plasma cortisol concentrations are elevated during physical and/or emotional stress, including illness, trauma, surgery, and starvation.

In the systemic circulation, 75%–80% of the cortisol is bound to a transport protein, termed transcortin or cortisol binding globulin (CBG). A small percentage (6%) of cortisol circulates as a free hormone, and passes through the cytoplasmic membrane of glucocorticoid target cells (Alves *et al.*, 2008). Subsequently, cortisol binds to an intracellular receptor, the glucocorticoid receptor, which functions as a ligand-induced transcription factor (Nicolaides *et al.*, 2010). Upon cortisol binding, the receptor undergoes conformational changes, translocates to the nucleus, and binds as homo- or hetero-dimer onto specific DNA sequences, the glucocorticoid response elements (GREs), located in regulatory regions of target genes (Nicolaides *et al.*, 2010). Once bound to DNA, the activated receptor induces or suppresses the expression of glucocorticoid responsive genes. Alternatively, glucocorticoid receptor can modulate gene expression by interacting as a monomer with other important transcription factors, such as the activator protein-1 (AP-1), the nuclear factor-κB (NF-κB) and signal transducers and activators of transcription (STATs), providing the molecular basis of anti-growth, anti-inflammatory and immunosuppressant glucocorticoid effects (Barnes and Karin, 1997; Karin and Chang, 2001; Didonato *et al.*, 1996).

Pharmacology of Glucocorticoids

Since the introduction of glucocorticoids in the treatment of rheumatoid arthritis in 1949, intense efforts have been made by science and industry to maximize the beneficial effects and to minimize the side effects of these analogues. Thus, many synthetic compounds with glucocorticoid activity have been manufactured and tested. The pharmacologic differences among these chemicals result from structure alterations of their basic steroid nucleus and its side groups. These changes may affect the bioavailability of these compounds (including their gastrointestinal or parenteral absorption, plasma half-life, and metabolism in the liver, fat, or target tissues) and their abilities to interact with the glucocorticoid receptor and to modulate the transcription of glucocorticoid-responsive genes (Pousseau *et al.*, 1972). In addition, structural modifications diminish the natural cross-reactivity of glucocorticoids with the mineralocorticoid receptor, eliminating their undesirable salt-retaining activity. Other modifications increase glucocorticoids' water solubility for parenteral administration or decrease their water solubility to enhance topical potency (Liapi and Chrousos, 1992; Magiakou and Chrousos, 1996).

Synthetic glucocorticoids are classified into three groups depending on the duration of ACTH suppression that is induced by a standard dose equivalent to 50 mg of prednisone: short action analogues, including hydrocortisone, cortisone, deflazacort, which suppress ACTH for less than 36 h; intermediate action analogues, such as triamcinolone, prednisone, prednisolone, methylprednisolone, suppressing ACTH for approximately 48 h; and prolonged action analogues (e.g., dexamethasone, betamethasone) that suppress ACTH for more than 48 h (Alves *et al.*, 2008). **Table 1** shows the relative glucocorticoid and mineralocorticoid potencies of commonly used systemic glucocorticoids and their approximate plasma and biologic effect half-lives.

Therapeutic Indications

Glucocorticoids may be administered as replacement therapy in patients with primary or secondary adrenal insufficiency, as adrenal suppression therapy in congenital adrenal hyperplasia and glucocorticoid resistance (Magiakou and Chrousos, 1996), and as antiinflammatory or immunosuppressant therapy in a broad range of mostly nonendocrine disorders affecting many different systems (Boumpas *et al.*, 1993). Thus, glucocorticoids are used in endocrine, autoimmune, collagen, renal, gastrointestinal, respiratory, nervous, hematologic, and ophthalmic diseases and are used in the suppression of the host versus graft and graft versus host reaction in cases of organ transplantation. Neoplastic disorders of the lymphoid system, such as leukemia and lymphomas, are also treated with glucocorticoids, along with the appropriate chemotherapy (Rix *et al.*, 2005).

Acute administration of pharmacologic doses of glucocorticoids is necessary in a small number of nonendocrine diseases, such as malignant hyperthermia, and in patients with craniospinal trauma or brain tumors or in those who are undergoing major neurosurgical operations to decrease the temperature and prevent destruction of neural tissue from the local edema and

Table 1 Glucocorticoid equivalencies

	Equivalent dose (mg)	*Glucocorticoid potency*	*Mineralocorticoid potency*	*Plasma half-life (min)*	*Biologic half-life (h)*
Glucocorticoids					
Short acting					
Cortisol	20.0	1.0	2	90	8–12
Cortisone	25.0	0.8	2	80–118	8–12
Intermediate acting					
Prednisone	5.0	4.0	1	60	18–36
Prednisone	5.0	4.0	1	115–200	18–36
Triamcinolone	4.0	5.0	0	30	18–36
Methylprednisolone	4.0	5.0	0	180	18–36
Long acting					
Dexamethasone	0.5	25–50	0	200	36–54
Betamethasone	0.6	25–50	0	300	36–54
Mineralocorticoids					
Aldosterone	–	0.3	300	15–20	8–12
Fluorocortisone	2.0	15.0	150	200	18–36
Desoxycorticosterone acetate	–	0.0	20	70	–

Adapted from Liapi, C. and Chrousos, G. P. (1992). Glucocorticoids. In: Jaffe, S. J., Aranda, J. V. (eds) Pediatric pharmacology, 2nd edn., Philadelphia: WB Saunders Co, pp. 466–475.

inflammatory reaction, respectively. In addition, glucocorticoids have been used in the prevention of the respiratory distress syndrome in the premature neonate, when delivery is anticipated before week 34 of gestation. In this case, treatment of the pregnant woman with 12 mg of betamethasone, followed by 12 mg 18–24 h later, stimulates the production of pulmonary surfactant and the maturation of the fetal lungs (reviewed in Chrousos *et al.*, 2000).

Side Effects

Side effects occur only with supraphysiologic doses of glucocorticoids and not with proper replacement, which is equivalent to 12–15 mg of hydrocortisone/m^2 body surface area/day. Major complications are unlikely for short-term treatment (<2 weeks) with high doses of glucocorticoids, although sleep disturbances and gastric irritation are common complaints, and depression, mania, or psychosis may be infrequently precipitated. On the other hand, many side effects are associated with long-term daily administration of pharmacologic amounts of glucocorticoids (**Table 2**), including the development of varying degrees of Cushing syndrome manifestations during therapy and secondary adrenal insufficiency (adrenal suppression) after discontinuation of treatment. Growth retardation is one of the major side effects of long-term daily glucocorticoid therapy in children (Laue *et al.*, 1989).

Adrenal Suppression

Adrenal suppression is the most common form of tertiary adrenal insufficiency caused by abrupt withdrawal of chronic glucocorticoid administration (Lamberts *et al.*, 1997; Kaltsas and Alexandraki, 2015). This condition also occurs following correction of endogenous hypercortisolism. In adrenal suppression, the suprahypothalamic and hypothalamic nuclei of the HPA axis are incapable of rapidly recovering their normal function when glucocorticoid therapy is discontinued. In sufficiently severe cases of endogenous hypercortisolism, adrenal suppression occurs following removal of a cortisol-secreting adrenal tumor, or after removal of an ACTH-secreting pituitary adenoma, or a tumor secreting ACTH ectopically (Kaltsas and Alexandraki, 2015). The clinical manifestations of adrenal suppression include anorexia, abdominal pain, nausea, vomiting, myalgia, arthralgia, fever, weight loss, orthostatic hypotension, and circulatory collapse (Charmandari *et al.*, 2014). The main electrolytic disturbance that is present in pituitary-adrenal suppression is hyponatremia (Kaltsas and Alexandraki, 2015). Adrenal suppression can be partial or complete (Krasner, 1999). The true prevalence of adrenal suppression is unknown granted that physicians reduce high doses of glucocorticoids, gradually, thereby allowing the recovery of the HPA axis (Chrousos *et al.*, 2000).

Predictors of Glucocorticoid-Induced HPA Axis Suppression

Several predictors of glucocorticoid-induced HPA axis suppression have been discussed. The following are the most important:

1. Type of steroid used and glucocorticoid potency: Glucocorticoid potency (**Table 1**) correlates positively with risk for adrenal insufficiency. Thus, hydrocortisone and cortisone acetate are the least potent and, therefore, least suppressive agents. Prednisone, prednisolone, methylprednisolone, and triamcinolone are moderately suppressive, and dexamethasone suppresses ACTH the longest (Chrousos *et al.*, 2000).

Table 2 Effects of Long-Term Glucocorticoid Therapy

Endocrine and metabolic
Suppression of HPA axis (adrenal suppression)
Growth failure in children
Carbohydrate intolerance
Hyperinsulinemia
Insulin resistance
Abnormal glucose tolerance test
Diabetes mellitus
Cushingoid features
Moon facies, facial plethora
Generalized and truncal obesity
Supraclavicular fat collection
Posterior cervical fat deposition (buffalo hump)
Glucocorticoid-induced acne
Thin and fragile skin, violaceous striae
Impotence, menstrual disorders
Decreased thyroid-stimulating hormone, thyroxine and triiodothyronine
Hypokalemia, metabolic alkalosis
Gastrointestinal system
Gastric irritation, peptic ulcer
Acute pancreatitis (rare)
Fatty infiltration of liver (hepatomegaly) (rare)
Hemopoietic system
Leukocytosis
Neutrophilia
Increased influx from bone marrow and decreased migration from blood vessels
Monocytopenia
Lymphopenia
Migration from blood vessels to lymphoid tissue
Eosinopenia
Immune system
Suppression of delayed hypersensitivity
Inhibition of leukocyte and tissue macrophage migration
Inhibition of cytokine secretion or action
Suppression of the primary antigen response
Musculoskeletal system
Osteoporosis, spontaneous fractures
Aseptic necrosis of femoral and humoral heads and other bones
Myopathy
Ophthalmic
Posterior subcapsular cataracts (more common in children)
Elevated intraocular pressure or glaucoma
Neuropsychiatric disorders
Sleep disturbances, insomnia
Euphoria, depression, mania, psychosis
Cognitive and memory disturbances, dementia
Pseudotumor cerebri (benign increase of intracranial pressure)

Adapted from Laue, L., Kawai, S., Udelsman, R., *et al.* (1989). Glucocorticoid antagonists: Pharmacological attributes of the prototype antiglucocorticoid RU 486. In: Lichtenstein, L. M., Claman, H. & Oronsky, A. (eds.) Antiinflammatory steroid action: Basic and clinical aspects, pp 303–329. New York: Academic Press.

2. Systemic vs. compartmental therapy: Systemic glucocorticoid therapy, especially parenterally, is more likely to suppress the HPA axis than are intra-articular, inhalational, or topical glucocorticoids (Alves *et al.*, 2008; Mortimer *et al.*, 2006; Molimard *et al.*, 2008).
3. Daily therapy: There is evidence that patients are at lower risk for adrenal insufficiency if they can take glucocorticoids on alternate days from the outset or if they can convert to alternate-day therapy before the HPA axis is suppressed (Ackerman and Nolsn, 1968).
4. Once-a-day dosing in the morning or mimicking normal diurnal cortisol rhythms: Since evening doses of glucocorticoids tend to suppress the normal early morning surge of ACTH secretion, it is better, whenever possible, to treat patients with a single

morning dose. Once-a-day dosing is usually feasible for prednisone, triamcinolone, and dexamethasone. The short-acting hydrocortisone and cortisone acetate are usually given twice a day, at waking and at approximately 5 PM. To mimic normal diurnal cortisol rhythms, the morning dose is two-thirds and the afternoon dose is one-third of the total daily dose (Alves *et al.*, 2008; Nichols *et al.*, 1965).

5. Duration and cumulative dose of glucocorticoid treatment: Although traditionally the duration of glucocorticoid therapy and the cumulative dose of glucocorticoid received have been considered as predictive of the likelihood of HPA axis suppression, several studies suggest that they only roughly predict HPA axis suppression (Schlaghecke *et al.*, 1992; Henzen *et al.*, 2000). Adrenal insufficiency is extremely rare in patients treated for 1 week or less (Carella *et al.*, 1993).
6. Cushingoid features: Patients with clinical manifestations suggestive of Cushing syndrome are more likely to have a suppressed HPA axis (Alves *et al.*, 2008; Chrousos *et al.*, 2000).

Perhaps the best predictor of HPA axis suppression is the patient's current glucocorticoid dosage. A strong correlation has been found between prednisone maintenance doses >5 mg/day and a subnormal ACTH stimulation test result. From the practical point of view, patients who receive high doses of glucocorticoids (>20–30 mg hydrocortisone or equivalent per day) (**Table 1**) for more than 2 weeks and patients who have developed overt Cushingoid features are more likely to develop HPA axis suppression (Kaltsas and Alexandraki, 2015). Regarding the timing of drug administration, prednisolone in a dose of 5 mg given at night and 2.5 mg in the morning is more likely to cause HPA axis suppression compared to 2.5 mg at night and 5 mg in the morning (Kaltsas and Alexandraki, 2015). This is because the sensitivity of tissues to glucocorticoids increases in the evening hours (Charmandari *et al.*, 2011).

Acute Adrenal Crisis

Recovery of the HPA axis can take 12 months or longer. Abrupt cessation of glucocorticoid treatment or quick tapering can precipitate an acute glucocorticoid deficiency crisis. Clinical awareness is fundamental to recognize patients with this emergency condition. The main symptoms range from anorexia, fatigue, nausea, vomiting, dyspnea, fever, arthralgia, myalgia, and orthostatic hypotension to dizziness, fainting, and circulatory collapse (Charmandari *et al.*, 2014). Hypoglycemia is occasionally observed in children and very thin adults. The diagnosis is urgent and treatment should consist of immediate administration of fluids, electrolytes, glucose, and parenteral glucocorticoids (Charmandari *et al.*, 2014).

Diagnostic Tests

As previously mentioned, glucocorticoid treatment may not suppress the HPA axis at all, or it may cause central suppression and adrenal gland atrophy of varying degrees (Krasner, 1999). The insulin tolerance test and the metyrapone test have been employed in the diagnosis of adrenal suppression and are quite sensitive. However, the risks involved with both tests do not justify their use when a rapid ACTH stimulation test can distinguish clinically significant adrenal suppression (Chrousos *et al.*, 2000).

To evaluate the adequacy of HPA axis recovery, the rapid Synachten (or high-dose ACTH stimulation test) is most commonly used. An intravenous bolus of 250 μg of corticotropin 1–24 is administered, and cortisol is measured after 30 or 60 min or both. A plasma cortisol concentration $>$ 18–20 μg/dL at these times indicates adequate recovery of the HPA axis (Kamilaris and Chrousos, 1991). This test can also be done intramuscularly. However, if adrenal suppression is of recent onset, adrenals may still respond to 250 mcg of ACTH, because they may have not yet atrophied. In such cases, a modified Synachten test has been recommended in lieu of the standard test. Only 1 μg of corticotropin 1–24 is administered instead of 250 μg (Poon and Smith, 1996; Chrousos *et al.*, 2009). A number of studies have shown that the low-dose Synachten test may be a good test for the diagnosis of secondary/tertiary adrenal insufficiency, however, it is fraught with dosing problems in the clinical setting (reviewed in Magnotti and Shimshi, 2008).

The corticotropin-releasing hormone (CRH) test can be performed to distinguish between secondary and tertiary adrenal insufficiency in patients who receive glucocorticoids for prolonged periods (Gellner *et al.*, 1999). In both forms of adrenal insufficiency, cortisol concentrations are low at baseline and remain low following CRH administration (Kaltsas and Alexandraki, 2015). In secondary adrenal insufficiency, CRH administration results in a little or no ACTH response, whereas in the tertiary form there is a prolonged and exaggerated response of ACTH (Kaltsas and Alexandraki, 2015).

Weaning Patients From Glucocorticoid Therapy

Glucocorticoid withdrawal is indicated when the maximum therapeutic benefit has been achieved, or when inadequate therapeutic benefit has been obtained following an adequate therapeutic trial, or when significant side effects appear and become serious and uncontrollable, including diabetes mellitus, psychosis, severe hypertension and osteoporosis (Chrousos *et al.*, 2000). To avoid the occurrence of adrenal insufficiency, dose tapering is suggested as the best method of glucocorticoid withdrawal. Although there is no consensus on rapid or slow dose tapering of glucocorticoids, the common point is that glucocorticoid withdrawal should never

be abrupt (Alves *et al.*, 2008; Chrousos *et al.*, 2000). Patients receiving any glucocorticoid dose for less than 2 weeks are not likely to develop adrenal suppression and could be advised to discontinue glucocorticoid therapy without tapering, in the case tapering is not needed for the disease under treatment. There is only one possible exception to this, which is the patient who takes frequently "short" steroid courses, such as in asthma therapy (Chrousos *et al.*, 2000). In regimens longer than 2 weeks, the objective is to reduce rapidly the glucocorticoid dose to a physiologic level that is equivalent to 5 mg/day prednisolone. For example, this can be achieved by reducing 2.5 mg every week for a period of few weeks, and then by slower glucocorticoid withdrawal to permit full recovery of the HPA axis (Chrousos *et al.*, 2000).

Glucocorticoid Withdrawal Syndrome

Amatruda *et al.* first defined the steroid withdrawal syndrome as a symptom complex resembling true adrenal insufficiency, with nonspecific symptoms such as weakness, nausea, and arthralgias, occurring in patients who have completed a dosage reduction of glucocorticoid therapy and who respond normally to HPA axis testing (Amatruda *et al.*, 1960). Thus, following cessation of glucocorticoid therapy, patients may suffer from anorexia, myalgia, nausea, emesis, lethargy, headache, fever, skin desquamation, arthralgias, weight loss, and postural hypotension. In addition, they may experience exacerbation of a previously present autoimmune disease (e.g., rheumatoid arthritis, atopic dermatitis, and asthma) or develop a new autoimmune disease (e.g., Hashimoto's thyroiditis and Graves' disease).

The glucocorticoid withdrawal syndrome (GWS) might represent a withdrawal reaction caused by physical dependence on supraphysiologic glucocorticoid concentrations (Hochberg *et al.*, 2003). The pathogenetic mechanisms underlying GWS are not fully understood. Several neuroendocrine mediators and other factors, including vasopressin, CRH, POMC, cytokines and prostaglandins, have been considered as crucial molecules involved in GWS (Hochberg *et al.*, 2003). Interestingly, the syndrome is self-limited and may last approximately 10 months. Patients with GWS should be advised to have a temporary increase in glucocorticoid doses followed by slow tapering to a physiologic dose (Bhattacharyya *et al.*, 2005).

Follow-Up

All patients receiving glucocorticoids for long periods should be informed to increase glucocorticoid doses (supplementation equivalent to 100–150 mg of hydrocortisone) during exposure to major stressors, including surgical procedures, fractures, burns and severe systemic infections. They should also be advised to carry means of identification, such as medical alert bracelet (Kaltsas and Alexandraki, 2015).

See also: Regulation of POMC and ACTH Secretion

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Glucocorticoid Metabolism and Activation

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Introduction

Corticosteroid hormones were first isolated and characterized in the 1930s by Kendall and Reichstein from the adrenal cortex (Mason *et al.*, 1936, 1937; Reichstein, 1936, 1937). In 1949, compound E (subsequently named cortisone) was synthesized and trialed in patients suffering from rheumatoid arthritis and rheumatic fever (Hench *et al.*, 1950). The extraordinary impact that this had in the successful treatment of these conditions lead to a surge of trials using cortisone as a treatment for nearly every disease. These trials lead to the discovery that cortisone was beneficial in adrenal deficiency disorders, inflammatory disorders, and malignancies such as lymphoma but was harmful in infective disease. Two years later, in 1951, compound F (cortisol) was synthesized and it soon became evident that this was the active compound providing some of the first evidence that glucocorticoids (GCs) undergo metabolism in vivo that can impact upon their activity (Hollander *et al.*, 1951). This prompted a new wave of studies investigating GC metabolism in rodents and in humans. Amelung *et al.* (1953) reported that cortisone was converted to cortisol in a number of rodent tissues, but this activity was highest in the liver (Amelung *et al.*, 1953).

This raised the important concept of prereceptor cortisol metabolism as a fundamental regulatory step in GC action. Cortisol, the predominant circulating GC in humans, is mainly bound (>90%) with high affinity to cortisol binding globulin and it is only the "free," unbound circulating cortisol that is available to enter cells. Once within the cell, cortisol can bind the cytosolic GC receptor (GR), translocate to the nucleus, and alter gene transcription. However, prereceptor GC metabolism means that prior to engaging with a relevant steroid hormone receptor, GCs can be metabolized to either enhance or limit their ability to bind and activate the GR.

Prereceptor GC metabolism occurs in many tissues throughout the body, but notably within the liver, kidney, adipose tissue, placenta, colon, and muscle. There are a variety of different enzyme systems that have been identified and they are summarized later (**Fig. 1**).

- 11β-Hydroxysteroid dehydrogenase type 1 and type 2 (11β-HSD1 and 2) interconvert active cortisol and inactive cortisone.
- A-ring reductases (5α-reductase types 1 and 2 (5αR1 and 5αR2) and 5β-reductase (5βR)) metabolize cortisol to 5α- or 5β-dihydrocortisol which is then subsequently metabolized to 5α-tetrahydrocortisol (allo-THF) and 5β-tetrahydrocortisol (THF), respectively, by 3α-hydroxysteroid dehydrogenase. Similarly, cortisone is converted to tetrahydrocortisone (THE). These metabolites are conjugated with glucuronic acid and excreted in the urine.
- 6β-Hydroxylase converts cortisol to 6β-hydroxycortisol and cortisone to 6β-hydroxycortisone.
- 20β-Oxoreductase reduces cortisol to 20β-dihydrocortisol and cortisone to 20β-dihydrocortisone.

11β-Hydroxysteroid Dehydrogenase-Type 1

11β-HSD1 is a bidirectional enzyme which acts as both an oxoreductase converting cortisone to cortisol and as a dehydrogenase inactivating cortisol to cortisone. However, in vivo, it acts mainly as an oxoreductase regenerating cortisol locally principally in metabolically active tissues such as liver, adipose tissue, and muscle (Tomlinson *et al.*, 2004). Within the cell, 11β-HSD1 is located in the endoplasmic reticulum (ER) membrane and its catalytic domain has a luminal orientation (Odermatt *et al.*, 1999). Its oxoreductase activity is critically dependent upon a tightly associated enzyme, hexose-6-phosphate dehydrogenase (H6PDH) that generates the cosubstrate, NADPH, to permit the reduction of cortisone to cortisol. It is the maintenance of the high intra-lumen NADPH/$NADP^+$ ratio which confers the directionality of 11β-HSD1 (Draper *et al.*, 2003). In the absence of H6PDH, 11β-HSD1 acts as dehydrogenase and oxidizes cortisol to cortisone. In humans, 11β-HSD1 is a 292 amino acid protein and is encoded by the gene HSD11B1 found on chromosome 1q32.2 (Tannin *et al.*, 1991).

11β-HSD1 is mainly not only expressed in hepatocytes, adipose stromal cells, gonads, and neuronal brain cells but can also be found in the cardiac myocytes and fibroblasts, spleen, lymph nodes, Peyer's patch, thymus, macrophages, osteoblasts, osteoclasts, corneal epithelium and endothelium, nonpigmented ciliary epithelial cells, surface epithelia and lamina propria, kidney cortex and medulla, pancreatic islets of Langerhans, skin fibroblasts, vascular smooth muscle, skeletal myoblasts and placental syncytiotrophoblast, chorion, and decidua (Tomlinson *et al.*, 2004). There are many factors that regulate its expression. GC, proinflammatory cytokines, peroxisome proliferator-activated receptor γ agonists, and CCAAT/enhancer-binding protein increase expression and activity. Conversely, growth hormone and liver X receptor agonists decrease its expression (Tomlinson *et al.*, 2004). Oestradiol reduces 11β-HSD1 expression in rat liver and kidney but testosterone has no effect (Gomez-Sanchez *et al.*, 2003).

Genetic Defects

Cortisone reductase deficiency and apparent cortisone reductase deficiency

There are two clinical phenotypes associated with impaired oxoreductase activity of 11β-HSD1 that arise as a result of specific genetic defects. Loss of function mutations in the HSD11B1 gene underpins "true" cortisone reductase deficiency (CRD) whereas

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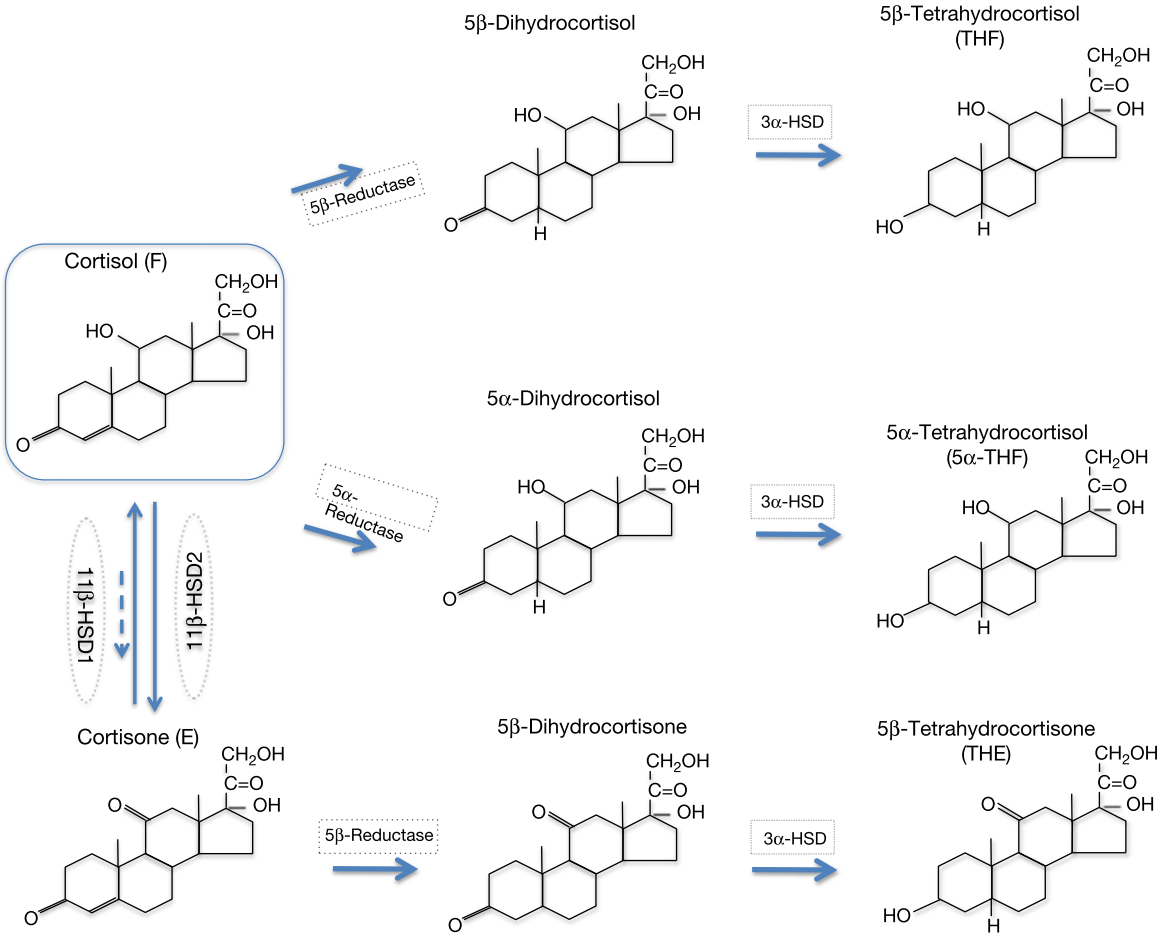

Fig. 1 Summary of cortisol metabolism: Cortisol is metabolized by the enzymes 5α-reductase (to 5α-dihydrocortisol) and 5β-reductase (to 5β-dihydrocortisol). 3α-Hydroxysteroid dehydrogenase (3α-HSD) further metabolizes 5α-dihydrocortisol to 5α-tetrahydrocortisol and 5β-dihydrocortisol to 5β-tetrahydrocortisol. Cortisol and cortisone are interconverted by the isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD2 inactivates cortisol to cortisone, while 11β-HSD1 predominantly reactivates cortisol from inactive cortisone. Cortisone is metabolized by 5β-reductase to 5β-dihydrocortisone, which is further metabolized by 3α-HSD to 5β-tetrahydrocortisone.

mutations in the H6PD gene cause "apparent" CRD (ACRD). ACRD is the more severe form of the condition as the loss of H6PDH activity instigates a decrease of the intralumen NADPH/$NADP^+$ ratio thus favoring the dehydrogenase activity of 11β-HSD1 where cortisol is inactivated to cortisone (Lavery *et al.*, 2008). The cortisol inactivation seen in ACRD induces a compensatory HPA axis activation and ACTH-mediated adrenal GC and androgen secretion. The activation of the HPA axis ensures that circulating cortisol levels are maintained and as a result these patients do not display features that would be indicative of cortisol deficiency. Clinically this condition presents with a phenotype that resembles polycystic ovary syndrome (PCOS) in adult females or as premature adrenarche in children. The urinary steroid profiles show extremely high cortisone with concomitantly low cortisol metabolites consistent with impairment of 11β-HSD1 activity. True CRD is a milder form of the condition; two cases have been reported presenting with premature adrenarche. As might be expected the urinary steroid profile shows a milder abnormality in comparison with ACRD reflecting that both the reductase and dehydrogenase activity of 11β-HSD1 are reduced (Lawson *et al.*, 2011; Lavery *et al.*, 2013). However, as in patients with ACRD there was secondary HPA axis activation and increased androgen secretion driving the clinical phenotype.

HSD11B1 and H6PDH polymorphisms

Several studies have been conducted to investigate possible links between single nucleotide polymorphisms (SNPs) of HSD11B1 and disease. Conditions such as PCOS, Alzheimer's disease, type 2 diabetes, and cardiovascular disease have been investigated but there is currently conflicting data on the association between HSD11B1 SNPs and these diseases (Gathercole *et al.*, 2013). Genetic variations in H6PDH have also been examined in the context of common diseases and associations with specific polymorphisms and multiple sclerosis, carotid intima-medial thickness, and atherosclerosis have been identified (Rahman *et al.*, 2011; Wellcome Trust Case Control *et al.*, 2007; Alcina *et al.*, 2010). Additionally, PCOS has been associated with the rs668832 H6PD SNP as reported in two small case-control studies (San Millan *et al.*, 2005; Martinez-Garcia *et al.*, 2012).

Tissue-Specific Impact of 11β-HSD1 Expression and Activity

Adipose tissue

11β-HSD1 is highly expressed in both white and brown adipose tissues and acts predominantly as an oxoreductase generating active GC cortisol. H6PDH and GRα receptors have been reported to be more highly expressed in omental adipose tissue compared to subcutaneous (SC) and therefore the impact of 11β-HSD1 activity may be more pronounced in this depot. Absolute expression levels of 11β-HSD1 are, however, similar in both omental and SC adipose tissue in humans (Veilleux *et al.*, 2009), but in mice, 11β-HSD1 is most highly expressed in SC adipose tissue (Morton *et al.*, 2004b).

GCs play an important role in adipocyte biology, promoting adipocyte differentiation, and 11β-HSD1 activity has been implicated in both the induction of adipogenesis and the maturation of adipocytes (Bujalska *et al.*, 2008). In addition, GCs promote fatty acid release through lipolysis. 11β-HSD1 inhibition and the consequent decrease in active GC availability decrease lipolysis both in vitro and in vivo (Gathercole *et al.*, 2011; Tomlinson *et al.*, 2007).

Rodent models have contributed significantly to our understanding of the role of 11β-HSD1 in adipose tissue. Perhaps the most important proof-of-concept observation as to the importance of 11β-HSD1 in adipose tissue was provided by Masuzaki *et al.* (2001) who overexpressed 11β-HSD1 in adipose tissue in mice under the aP2 promoter. These transgenic mice developed visceral obesity without changes in circulating GC levels. They did however report an increase in corticosterone (the active GC in rodents) in the portal vein, consistent with putative export of regenerated active GC from visceral adipose tissue. These mice developed many features of the metabolic syndrome including insulin resistance, dyslipidemia, and hypertension. Additionally, Morton *et al.* (2004a) have provided a detailed characterization of the 11β-HSD1 KO mouse model and reported that these animals have lower visceral adipocyte accumulation on high fat diet and resist the development of high fat diet induced insulin resistance and diabetes.

There is strong translational evidence to support the concept that 11β-HSD1 activity and expression are dysregulated in human obesity (Chapman *et al.*, 2013), while circulating GCs levels are unchanged in comparison with lean individuals (Travison *et al.*, 2007). In patients with simple obesity, 11β-HSD1 activity and expression are increased two- to threefold in SC adipose tissue often with a concomitant decrease in hepatic 11β-HSD1 activity (see later). However, in obese individuals with type 2 diabetes, the reduction in hepatic 11β-HSD1 activity is not observed and it has been postulated that this ongoing exposure to locally regenerated cortisol may fuel unchecked hepatic glucose output (Chapman *et al.*, 2013). There are fewer studies that have examined the impact of obesity on visceral 11β-HSD1 activity and expression and the data are more variable. Woods *et al.* (2015) reported a positive correlation between visceral adipose tissue 11β-HSD1 gene expression and BMI but not in SC adipose tissue. They did however report a reduction in HSD11B1 gene expression in SC adipose tissue following weight loss. On the other hand, 11β-HSD1 gene expression in hepatocytes was negatively correlated with BMI which is consistent with previous studies (Woods *et al.*, 2015). These findings are in accordance to the reports by Mlinar *et al.* (2011) and Michailidou *et al.* (2007) who reported that visceral adipose tissue HSD11B1 gene expression correlates with adiposity and adipocyte size. However, in contrast, Goedecke *et al.* (2006) reported that HSD11B1 gene expression in omental adipose tissue did not correlate with obesity and therefore there remain some discrepancies in the published literature.

Liver

11β-HSD1 is highly expressed in the liver and in humans it is mainly located centripetally and found in high levels around the central vein (Ricketts *et al.*, 1998b), and it acts exclusively as an oxoreductase (Jamieson *et al.*, 1995; Ricketts *et al.*, 1998a).

In global 11β-HSD1 KO mice, 11β-HSD1 deficiency has little effect on liver function in the fed state, but in the fasted state there is no rise in PPARα which is consistent with reduced GC action. On refeeding, there is an exaggerated increase in the expression of lipogenic enzymes and a parallel reduction in catabolic enzymes. Therefore, 11β-HSD1 deficiency causes a more favorable lipid profile and improved liver insulin sensitivity (Morton *et al.*, 2001). Mouse models with both liver-specific overexpression and deletion of 11β-HSD1 have also been generated. Overexpression of 11β-HSD1 in liver was achieved by using the apolipoprotein E promoter and these mice developed hypertension, dyslipidemia, and hypertriglyceridemia which lead to hepatic steatosis, but did not progress to steatohepatitis (Paterson *et al.*, 2004). They also became insulin resistant but were not obese. In mice with hepatic 11β-HSD1 deletion, there is no significant change in insulin sensitivity or hepatic or serum lipids. There was, however, an increase in adrenal size, despite circulating corticosterone levels being unchanged, suggestive of an increase in HPA axis activation (Lavery *et al.*, 2012).

As described earlier, in simple obesity, 11β-HSD1 activity in the liver is reduced (as reflected by the analysis of urinary steroid metabolites measured by gas chromatography, mass spectrometry, and the generation of cortisol from oral cortisone); however in patients who also have type 2 diabetes activity is unchanged (Stimson *et al.*, 2011). This is in accordance to the findings reported by Torrecilla *et al.* (2012) who reported that there was higher expression of GR, 11β-HSD1, H6PDH, and PEPCK in hepatocytes of obese patients with metabolic disease compared to obese patients (Torrecilla *et al.*, 2012). However, in the db/db diabetes murine model, 11β-HSD1 and GR expressions are both increased which is consistent with findings from human studies (Deary *et al.*, 2006).

Muscle

11β-HSD1 is present in skeletal muscle but at lower levels when compared to the liver (Whorwood *et al.*, 2001). In human muscle, 11β-HSD1 acts as an oxoreductase, but its role in metabolic disease remains controversial. Whorwood *et al.* (2001) reported that

11β-HSD1 levels did not correlate with adiposity in human muscle ex vivo but Abdallah *et al.* (2005) reported that there was an increased expression in myotubes from obese patients with type 2 diabetes. Similar findings were also shown in obese rodents with diabetes where there were increased levels of 11β-HSD1 mRNA and protein (Zhang *et al.*, 2009a). Morgan *et al.* (2009) showed that altering 11β-HSD1 activity can impact on insulin sensitivity in skeletal muscle in humans and rodent models (Morgan *et al.*, 2009). 11β-HSD1 inhibition in the hyperglycemic KK/Ta jcl mouse induced an increase in skeletal muscle insulin receptor substrate 1 and a decrease in expression of genes involved in lipid metabolism (Gathercole *et al.*, 2013).

More recently it has been postulated that 11β-HSD1 may be involved in age-related sarcopenia. 11β-HSD1 expression in skeletal muscle was increased with age in women and also associated with reduced grip strength, insulin resistance, and an adverse body composition profile (Hassan-Smith *et al.*, 2015).

Pancreatic islets of Langerhans

11β-HSD1 mRNA has been identified in rodent and human pancreatic islets but there has been controversy regarding its exact location with conflicting reports suggesting that it may be expressed in either α- (Swali *et al.*, 2008) or β-cells (Davani *et al.*, 2000).

The relationship between pancreatic 11β-HSD1 activity and metabolic disease has been investigated using several rodent models and increased 11β-HSD1 levels seem to be associated with β-cell failure (Davani *et al.*, 2000). However, the precise relationship between 11β-HSD1 expression and metabolic disease is not clear. In the global 11β-HSD1 KO mouse, there is impairment in the β-cell function, but with improved glucose tolerance (Turban *et al.*, 2012). In a diabetes-prone rodent model where 11β-HSD1 is overexpressed in the pancreas there is enhanced glucose-stimulated insulin secretion (Turban *et al.*, 2012). However, increases in 11β-HSD1 expression in the pancreatic islets of obese ob/ob mice (Ortsater *et al.*, 2005) and Zucker diabetic fatty fa/fa rats (Duplomb *et al.*, 2004) correlate with increasing hyperglycemia. This increase is reversed by troglitazone which inhibits hyperglycemia and hyperlipidemia. In normal mice, high-fat diet causes a compensatory increase in insulin secretion with upregulation of 11β-HSD1, whereas in severe obesity, very high islet 11β-HSD1 levels, and therefore GC regeneration, contribute to β-cell failure (Chapman *et al.*, 2013). In mouse strains that are susceptible to β-cell failure, high-fat diet causes a decrease in 11β-HSD1 expression whereas in mice that are not prone to metabolic disease there is some increase in 11β-HSD1 expression.

Cardiovascular disease

Both 11β-HSD1 and 11β-HSD2 are expressed in the vasculature (Walker *et al.*, 1991). 11β-HSD1 is mainly found in the vascular smooth muscle and is involved in angiogenesis and vascular remodeling (Walker *et al.*, 1991), whereas 11β-HSD2 is located in the endothelium (Hadoke *et al.*, 2001).

GCs inhibit angiogenesis and mice deficient in 11β-HSD1 exhibit increased angiogenesis particularly in cases of myocardial injury (Small *et al.*, 2005). 11β-HSD1 is also expressed in differentiated macrophages and 11β-HSD1 deficiency is associated with acute inflammation that exacerbates atherosclerosis. Hermanowski-Vosatka *et al.* (2005) reported that 11β-HSD1 inhibition reduces cholesterol levels in atherosclerosis-prone ApoE$^{-/-}$ mice. However, Lloyd *et al.* (2009) found that in atherosclerosis susceptible LDL receptor null mice, there was no effect. Therefore, 11β-HSD1's role in atherosclerosis remains controversial and has not been fully elucidated.

Nervous system

11β-HSD1 is expressed in the adult brain with highest expression in the hippocampus, cortex, cerebellum, and anterior pituitary. 11β-HSD2 is also located in the brain but it is expressed at considerably lower levels. 11β-HSD1 mainly acts as an oxoreductase but H6PDH is not frequently coexpressed with 11β-HSD1 in the brain (Gomez-Sanchez *et al.*, 2008). 11β-HSD1 is thought to be involved in cognition; the use of 11β-HSD inhibitors in elderly mice (Sooy *et al.*, 2010) and humans (Sandeep *et al.*, 2004) improves cognitive function. In a transgenic mouse model where 11β-HSD1 was overexpressed in the forebrain, there was evidence of cognitive impairment similar to that observed with aging (see later) (Holmes *et al.*, 2010).

11β-HSD1 is also involved in the regulation of aqueous humor production in the ocular ciliary epithelium and is thought to have a role in regulating aqueous humor production by increasing local cortisol generation (Rauz *et al.*, 2001). Rauz *et al.* (2003) reported that the nonspecific 11β-HSD inhibitor, carbenoxolone, decreased intraocular pressure in patients with ocular hypertension (Rauz *et al.*, 2003).

Dysregulation of 11β-HSD1 in the choroid plexus and arachnoid granulation tissue may be implicated in the development of idiopathic intracranial hypertension (IIH), which is known to be associated with GC excess and with simple obesity. Sinclair *et al.* (2010) reported that the largest fall in intracranial pressure in obese subjects with IIH was observed in those who had the largest decrease in global 11β-HSD1 activity associated with weight loss.

Aging

GC excess shares many phenotypic similarities with the aging phenotype including the development of central fat accumulation, hypertension, hyperlipidemia, insulin resistance, and sarcopenia. With increasing age, there is an increase in plasma cortisol concentrations, dampening of diurnal rhythm, and an increase in evening ACTH and consequent cortisol secretion (Van Cauter *et al.*, 1996). In addition, there is evidence to suggest increased tissue-specific cortisol regeneration through the activity of 11β-HSD1 and this may be an additional important contributory factor to local GC excess. The mechanisms underpinning this increased activity are not completely understood but higher levels of inflammatory cytokines, which upregulate 11β-HSD1, and lower levels of GH and IGF-1, which inhibit 11β-HSD1 activity, maybe important (Gathercole *et al.*, 2013).

Inflammation and immunity

GCs have an important role in modulating inflammatory and immune responses. Alteration of 11β-HSD1 expression and activity has been shown to have an important role in the modulation of the inflammatory response.

Many proinflammatory cytokines, including TNF-alpha and IL1-β, are potent upregulators of 11β-HSD1 activity and expression (Chapman *et al.*, 2013). In addition, IL-4 and IL-13 induce moderate 11β-HSD1 expression in monocytes (Thieringer *et al.*, 2001). 11β-HSD1 is induced during monocyte differentiation to macrophage (Thieringer *et al.*, 2001); lipopolysaccharide stimulates 11β-HSD1 expression in macrophages, which subsequently induces transformation to a proinflammatory M1 phenotype, and also in brain microglia (Martinez *et al.*, 2006; Gottfried-Blackmore *et al.*, 2010). In neutrophils, 11β-HSD1 expression is increased during inflammation, but absent during cellular apoptosis (Coutinho *et al.*, 2011). It is also expressed in mouse CD4 and CD8 positive lymphocytes, B cells, and dendritic cells (Freeman *et al.*, 2005).

11β-HSD1 KO mice have an impaired antiinflammatory response when challenged in models of joint inflammation, peritonitis, and lung inflammation; the inflammatory response was enhanced with a slower recovery rate (Coutinho *et al.*, 2012). In humans, 11β-HSD1 activity has been found to be augmented in rheumatoid arthritis (Hardy *et al.*, 2008) with increased expression in inflammatory bowel disease (Cooper *et al.*, 2011). Specifically, there is higher expression during acute exacerbation compared to remission suggesting that 11β-HSD1 has an active role in acute inflammation (Cooper *et al.*, 2011).

Bone and joint

11β-HSD1 is predominantly expressed in osteoblasts, while 11β-HSD2 is the only isoform found in osteosarcoma cells. Its activity in osteoblasts is induced by proinflammatory cytokines and GC (Cooper *et al.*, 2003). 11β-HSD1 KO mice do not exhibit any changes in bone mass, formation, or resorption (Justesen *et al.*, 2004). Sher *et al.* (2006) reported that in transgenic mice where 11β-HSD2 was overexpressed (with consequent increased local clearance of active GC) in osteoblasts there was a decrease in femoral cortical bone area and Weinstein *et al.* (2010) reported that 11β-HSD2 overexpression in osteoblasts and osteocytes of mice provided an age-related reduction in bone mass.

Using urinary steroid metabolites, Cooper *et al.* (2003) demonstrated that measurements of 11β-HSD1 activity were able to predict the fall in bone formation markers following a 7-day course of prednisolone therapy in healthy male volunteers. Therefore, this has the potential to be utilized in clinical practice, where 11β-HSD1 could predict an individual's susceptibility to GC-induced osteoporosis (Cooper *et al.*, 2003).

Skin and salivary glands

11β-HSD1 and 11β-HSD2 are both expressed in human and mouse skin (Tiganescu *et al.*, 2011; Vukelic *et al.*, 2011; Terao *et al.*, 2011). 11β-HSD1 acts as an oxoreductase and is expressed in the epidermis and dermis (Hennebert *et al.*, 2007) with high expression in keratinocytes (Terao *et al.*, 2011). 11β-HSD1 expression is increased with age and photodamage, and has been suggested to be a key mechanism in the atrophic, age-related skin changes (Tiganescu *et al.*, 2011). GCs are well recognized for their ability to inhibit wound healing. In rodent models, selective inhibition of 11β-HSD1 induced keratinocyte proliferation and enhanced cutaneous wound healing and prevented age-related skin changes (Hennebert *et al.*, 2007). The translation potential of selective 11β-HSD1 inhibitors to improve would healing in clinical studies is yet to be explored.

Selective 11β-HSD1 Inhibitors

Much of the data described earlier has led to the concept that decreasing tissue-specific GC exposure may have the potential to improve metabolic and also cognitive phenotype. On this background, highly potential selective (in that they inhibit 11β-HSD1, but not 11β-HSD2) 11β-HSD1 inhibitors have been developed.

The first selective compound was developed by Biovitrum (BVT2733) and reduced fasting blood glucose, insulin, and cholesterol levels in hyperglycemic rodent models (Barf *et al.*, 2002; Alberts *et al.*, 2002, 2003). Subsequently, many other compounds have been developed by pharmaceutical and biotech companies (including compounds A and 531) and have demonstrated potent metabolic benefits to reduce mesenteric fat mass, hepatic steatosis, improve insulin sensitivity, and reduce hepatic glucose production (Berthiaume *et al.*, 2007a,b; Edgerton *et al.*, 2010).

The first translational study in humans to examine the metabolic impact of these compounds used INCB013739 in patients with type 2 diabetes. After 2 weeks of administration, fasting blood glucose decreased, particularly in those patients with more pronounced hyperglycemia and there was also reduction in LDL cholesterol but no change in HDL cholesterol. In more prolonged 12-week studies in patients already taking metformin, there was modest weight loss and improvement in overall glycemic control as measured by a reduction in glycated hemoglobin (Rosenstock *et al.*, 2010). A further selective 11β-HSD1 inhibitor, MK0916, was trialed in patients with type 2 diabetes. This caused a modest reduction in weight and waist–hip ratio as well as an HbA1c reduction of 0.3% in the 6 mg group. However, there was no alteration in fasting plasma glucose, postprandial glucose or insulin levels. There was also increase in LDL and non-HDL cholesterol (Feig *et al.*, 2011). Other selective 11β-HSD1 inhibitors have been trialed and have mostly shown moderate effectiveness in improving glycemic control and blood pressure in patients with diabetes and metabolic disease (Gathercole *et al.*, 2013). The fact that these improvements were relatively modest (in comparison with other pharmaceutical therapies already available) has limited the subsequent development of this class of drug.

Importantly, in all studies where selective 11β-HSD1 inhibitors have been used and where they have been measured appropriately (including measurements of adrenal androgens and ACTH), there is secondary activation of the HPA axis as a consequence of the decreased cortisol half-life.

11β-HSD1 and GC Excess

There is emerging evidence to suggest that 11β-HSD1 may have a role to regulate the local availability of GCs in conditions of either endogenous or exogenous GC excess. In patients with Cushing's disease, 11β-HSD1 activity as measured by urinary steroid metabolite analysis is increased (Stewart *et al.*, 1995). However, patients have been described who have a functional deficit in 11β-HSD1 activity and it has been postulated that this inability to regenerate active GC within key target tissues may protect those individuals from the development of the classic Cushing's phenotype (Tomlinson *et al.*, 2002; Arai *et al.*, 2008), independent of their circulating GC levels. Similar observations have been made in rodent models with genetic deletion of 11β-HSD1 (Morgan *et al.*, 2014) and translational clinical studies using selective 11β-HSD1 inhibitors in this context are underway. This has potential implications not only for patients with Cushing syndrome but also for the large number of patients, perhaps up to 2%–3% of the population of the United Kingdom and United States, who take regular prescribed GC therapy and are vulnerable to their array of averse metabolic side effects.

11β-Hydroxysteroid Dehydrogenase-Type 2

11β-HSD2 is a unidirectional enzyme that acts solely as a dehydrogenase and is NAD^+ dependent. Its cDNA was first cloned from sheep kidney, followed by human, rat, rabbit, mouse, and other species (Chapman *et al.*, 2013). In humans, it is expressed by the gene HSD11B2 that is located on chromosome 16q22 (Agarwal *et al.*, 1995). Human 11β-HSD2 comprises of 405 amino acids with a molecular mass of about 44 kDa (Chapman *et al.*, 2013). Within cells, it is localized in the ER membrane, similar to 11β-HSD1, most likely via three transmembrane helices (Brown *et al.*, 1996).

11β-HSD2 is located mainly in mineralocorticoid-target tissues including kidney, colon, placenta, sweat, and salivary glands and it is also found in the exocrine pancreas and adrenal cortex. 11β-HSD2 expression does display some interspecies differences in that in humans, it can be found in testis but not in mice or rats (Chapman *et al.*, 2013). 11β-HSD2 appears to have an important role in developmental programming and it is widely expressed in the fetus and placenta (see later) (Chapman *et al.*, 2013).

Genetic Defects: Apparent Mineralocorticoid Excess

Apparent mineralocorticoid excess (AME) is caused by both homozygous and heterozygous mutations in the HSD11B2 gene. Clinical features of this condition consist of hypertension, plasma volume expansion, hypokalemic alkalosis, and a suppressed renin–angiotensin–aldosterone system (Stewart, 1999). This is caused by the absence or reduced activity of 11β-HSD2 in the kidney which consequently reduces the inactivation of cortisol to cortisone in the kidney allowing cortisol to bind and activate the mineralocorticoid receptor (MR) for which it shares the same affinity as the MR endogenous ligand, aldosterone. There are two types of AME; type I is congenital and is caused by a homozygous mutation of HSD11B2 which instigate full or partial loss of enzyme activity and is commonly found in consanguineous families. Type II normally develops in adolescence or early adulthood and is a milder form of the condition. It is also caused by homozygous mutation of the HSD11B2 gene (Palermo *et al.*, 2004).

In mice with a heterozygous HSD11B2 gene disruption, basal blood pressures are normal; however, blood pressure does rise on sodium loading. Interestingly, this effect is reduced by inhibiting the GR instead of the MR by mechanisms which are currently unknown (Bailey *et al.*, 2011). Defects in the HSD11B2 gene resulting in a prolonged cortisol half-life have been linked to the development of essential hypertension (Soro *et al.*, 1995; Walker *et al.*, 1993). With increasing age, there is a decline in 11β-HSD2 activity which is associated with an increase in blood pressure (Henschkowski *et al.*, 2008). HSD11B2 polymorphisms have been associated with hypertension in black men with renal failure but this finding has not been consistent in other studies (Watson *et al.*, 1996; White *et al.*, 2001). HSD11B2 polymorphisms have also been implicated with sodium-sensitive blood pressure changes in white men.

Acquired 11β-HSD2 deficiency through consumption of liquorice roots is well described. Liquorice roots contain *Glycyrrhizin* which is a potent competitive inhibitor of 11β-HSD2 in vivo. It therefore mimics a mild form of AME and patients present clinically with hypertension and hypokalemia and in some severe cases myopathy and cardiac arrhythmia (Palermo *et al.*, 2004). Flavonoid consumption, found in fruit such as grapefruit, also have an inhibitory effect on 11β-HSD2 which can also present in a similar way to excessive licorice consumption (Palermo *et al.*, 2004).

Kidney

As mentioned earlier, 11β-HSD2 is highly expressed in the kidney and specifically in the distal nephron and the cortical collecting duct cells in close proximity to the MR. Here it serves to deactivate cortisol into the inactive cortisone and thus prevent cortisol

from activating the MR (for which it shares the same affinity as its cognate ligand, aldosterone). The importance of 11β-HSD2 in the kidney was proved by Leckie *et al.* (1995) by transfecting distal nephron cells with MR and an MR reporter gene with or without 11β-HSD2. 11β-HSD2 coexpression was able to prevent GC activation of MR and this was reversed by carbenoxolone (a nonselective inhibitor of both 11β-HSD1 and 2) administration (Leckie *et al.*, 1995). Even though cortisol has a much higher affinity for binding to MR than to 11β-HSD2, 11β-HSD2 is still able to deactivate cortisol sufficiently to prevent it from activating the MR. It is not completely understood how this mechanism occurs but it has been suggested that cortisol may preferentially track through intracellular membranes such as the ER and be deactivated by 11β-HSD2 before reaching the cytosolic MR (Odermatt *et al.*, 1999).

Placenta

Total and free cortisol concentrations in the circulation rise during gestation and it is the presence of 11β-HSD2 in the placenta that offers a protective barrier to the fetus from maternal excess GC (through the inactivation of cortisol to cortisone). In humans and rats, placental 11β-HSD2 is found in the syncytiotrophoblast. Nacharaju *et al.* (2004) used the nonselective 11β-HSD inhibitor carbenoxolone to inhibit 11β-HSD2 activity in placental trophoblasts in vitro. This caused an increase in human chorionic gonadotropin secretion with rapid cytotrophoblast differentiation into syncytial trophoblasts, demonstrating the important functional role of 11β-HSD2 in placental development and function (Nacharaju *et al.*, 2004).

Placental 11β-HSD2 expression increases throughout gestation, but activity decreases in the last 2 weeks prior to delivery. This is in accordance with the fact that GCs are important in tissue and organ maturation; therefore 11β-HSD2 is widespread in the fetus prior to mid gestation but is subsequently downregulated to permit GC delivery. 11β-HSD2 activity in the placenta can impact upon fetal growth in humans. Human fetuses with homozygous or compound heterozygous mutations of HSD11B2 have lower birth weights (Dave-Sharma *et al.*, 1998; Kitanaka *et al.*, 1996; Mune *et al.*, 1995). This is in agreement with findings in 11β-HSD2 KO mice that also have decreased birth weights (Holmes *et al.*, 2006). In addition, women who consume large amounts of liquorice, a nonselective 11β-HSD inhibitor while pregnant, have shorter pregnancies and their off-spring have poorer cognitive function which is accompanied with hyperactivity of the HPA axis (Raikkonen *et al.*, 2009, 2010).

Lung

Both 11β-HSD enzymes are expressed in the human lung but specifically 11β-HSD1 is located in the alveoli and 11β-HSD2 in the bronchioles and trachea and vascular epithelium (Suzuki *et al.*, 1998). However, in adult rats, 11β-HSD1 is expressed mainly in the interstitial fibroblasts (Brereton *et al.*, 2001) and 11β-HSD2 is expressed at very low levels (Alikhani-Koopaei *et al.*, 2004).

In fetal lungs, in both humans and rodents, there is high expression of 11β-HSD2 in early to midgestation which is then subsequently downregulated (with 11β-HSD1 upregulation) in late gestation (Chapman *et al.*, 2013) to enhance local GC delivery which is vital for lung maturation.

Colon

11β-HSD1 and 11β-HSD2 are both expressed in the human colonic epithelium and lamina propria (Smith *et al.*, 1996; Whorwood *et al.*, 1994). In rodents, 11β-HSD1 is located in the lamina propria and 11β-HSD2 in the epithelial cells (Whorwood *et al.*, 1994). In humans with inflammatory bowel disease, 11β-HSD1 expression is increased and 11β-HSD2 is decreased (Zbankova *et al.*, 2007). This reciprocal regulation may be mediated by proinflammatory cytokines TNF-α and IL-1 (Kostadinova *et al.*, 2005). 11β-HSD2 inhibition has been reported to have an anticancer effect in colorectal adenocarcinoma. It has been postulated that this is due to the increased GC suppression of COX-2 and PGE_2 (Zhang *et al.*, 2009b).

Skin

In the skin, 11β-HSD2 is mainly located in the sweat glands as it is the main site in the skin that is a mineralocorticoid target (Smith *et al.*, 1996). 11β-HSD2 is also found in the parotid and submandibular salivary glands and thus salivary cortisol could potentially be used clinically as a biomarker of serum free cortisol (Smith *et al.*, 1996).

The A-Ring Reductases

Three different A-ring reductases have been identified and described. These include 5αR1, 5αR2, and 5βR (Russell and Wilson, 1994; Uemura *et al.*, 2008). They are a series of enzymes that have multiple roles to metabolize steroid hormones and bile acids. The 5α-reductases are keys enzymes in the metabolism of testosterone, cortisol, and progesterone (Russell and Wilson, 1994). They metabolize testosterone to 5α-dihydrotestosterone and reduce cortisol to 5α-dihydrocortisol, which is then metabolized further to 5α-tetrahydrocortisol by 3α-hydroxysteroid dehydrogenase. Therefore, 5α-reductases increase androgen but reduce GC availability making it an important regulator enzyme in the activity of steroid hormones. Similarly, 5βR clears cortisol to 5β-dihydrocortisol

(with further downstream conversion to 5β-tetrahydrocortisol), but is also a key regulatory step in the bile acid synthesis pathway. In contrast therefore to the regenerative, GC-enhancing activity of 11β-HSD1, the A-rung reductases clear cortisol and therefore limit GC action.

5α-Reductase Type 1 and Type 2

5αR1 is expressed by gene SRD5A1 which is located on chromosome 5p15.31 in the human genome. The enzyme consists of 259 amino acids and has a molecular weight of 29 kDa. It is found in the liver, nongenital skin, muscle, adipose tissue, and brain. Testosterone is the most well-recognized substrate for this enzyme, even though it has the highest affinity for progesterone. It is thought to be accountable for approximately a third of circulating dihydrotestosterone (Gisleskog *et al.*, 1998; Andersson and Russell, 1990; Normington and Russell, 1992). There are no known mutations of SRD5A1.

5αR2 is expressed by gene SRD5A2 which is located on chromosome 2p23.1 in humans. The enzyme consists of 254 amino acids and has a molecular weight of 28 kDa (Russell and Wilson, 1994). It is mainly expressed in the male reproductive tissues such as prostate, epididymis, and seminal vesicles (Russell and Wilson, 1994). A number of mutations and polymorphisms in SRD5A2 have been identified, some of which cause the clinical phenotype of 46XY disorder of sex development (Samtani *et al.*, 2010). Affected males lack virilization and have poor development of external genitalia due to reduction in the production of the potent androgen, dihydrotestosterone. Conversely, increased dihydrotestosterone production via 5αR2 has been associated with disorders such as polycystic ovarian syndrome, and breast and prostate cancers (Jakimiuk *et al.*, 1999; Labrie *et al.*, 1993).

Tissue Distribution and Function

Both 5αR1 and 5αR2 are expressed in human liver whereas in rodents, only 5αR1 is expressed. Therefore, 5αR1 function in the liver has been investigated using rodent models. 5αR1 KO male mice have a higher incidence of hepatosteatosis and consequently higher risk of liver fibrosis and scarring (Dowman *et al.*, 2013; Livingstone *et al.*, 2015). 5αR1 is also expressed in adipose tissue in both humans and rodents as well as in skeletal muscle (Upreti *et al.*, 2014). It is also found in vascular endothelium and smooth muscle and inhibition of 5αR causes endothelial damage and dysfunction (Campelo *et al.*, 2012). In the kidney, 5αR1 is expressed; however, 5αR2 and 5βR are absent (Quinkler *et al.*, 2003).

There is emerging evidence from translational studies to suggest that 5αR1 and 2 may have a role in the regulation of metabolic phenotype. In patients with and without PCOS, 5αR activity correlates with worsening metabolic phenotype (Blumenfeld *et al.*, 2016; Crowley *et al.*, 2014; Tomlinson *et al.*, 2008a,b; Tsilchorozidou *et al.*, 2003; Vassiliadi *et al.*, 2009). In addition, longitudinal data over a 5-year period suggest that baseline 5αR activity is able to predict the development of future adverse metabolic features including insulin resistance and dysglycemia (Crowley *et al.*, 2014). The precise molecular mechanisms underpinning these observations are currently unknown (Zyirek *et al.*, 1987; Perel *et al.*, 1986).

5α-Reductase Inhibition

5α-reductase inhibitors are traditionally used for their antiandrogen effects and they are commonly prescribed for conditions such as benign prostate hyperplasia and prostate cancer. A very small number of clinical studies have been undertaken in order to investigate their potential implication in steroid metabolism and metabolic phenotype. As mentioned previously, cross-sectional and longitudinal studies have shown that 5α-reductase activity is higher in the presence of high BMI and insulin resistance (Crowley *et al.*, 2014; Tomlinson *et al.*, 2008a,b). Hazlehurst *et al.* (2016) performed a clinical trial in 12 healthy male volunteers who were randomized to receive either a 3-week course of dutasteride (nonselective dual 5αR1 and 5αR2 inhibitor) or finasteride (5αR2 selective inhibitor). They reported that dual 5α-reductase inhibition using dutasteride was associated with increased hepatic lipid accumulation (Hazlehurst *et al.*, 2016). Similar results have also been published by Upreti *et al.* (2014) in which 46 men were randomized to take finasteride, dutasteride, or placebo for 3 months. They demonstrated that dual 5α-reductase inhibition increased insulin resistance and decreased skeletal muscle glucose disposal (Upreti *et al.*, 2014). Taken together, these studies provide some preliminary evidence to suggest that dutasteride may have an adverse impact on the metabolic phenotype, although additional studies are now warranted.

5β-Reductase

5βR is expressed in the liver and at lower levels in the testis (Charbonneau and The, 2001). It metabolizes cortisol and cortisone to 5β-dihydrocortisol and 5β-dihydrocortisone respectively, with conversion to their tetra-hydro metabolites by the activity of 3α-hydroxysteroid dehydrogenase. It also reduces testosterone to the inactive metabolite 5β-dihydrotestosterone. While it is a key protein in clearing GC, mineralocorticoids, and sex steroids, in addition, it is responsible for a key enzymatic step in the bile acid synthetic pathway. It is coded by the gene AKR1D1 which is found on chromosome 7q33 and it is made of 326 amino acids with a molecular weight of 37 kDa. Mutations in this gene cause bile acid deficiency and neonatal cholestatic liver disease which can lead to hepatic failure (Lemonde *et al.*, 2003) although spontaneous recovery into adult life is reported (Palermo *et al.*, 2008). As expected, these patients have a characteristic urinary steroid profile with a profound reduction on 5β-reduced steroids and a mild

Table 1 The impact of 11β-HSD1, 11β-HSD2, and 5αR1/2 on tissues

	11β-HSD1			*11β-HSD2*		*5αR1/2*	
Tissue	*Main enzymatic activity*	*Impact of increased activity/expression*	*Impact of decreased activity/expression*	*Main enzymatic activity*	*Impact of decreased activity/expression*	*Main enzymatic activity*	*Impact of decreased activity/expression*
Adipose	Oxoreductase (activates cortisol from cortisone)	Visceral obesity Increase in active GC in the portal vein Insulin resistance Dyslipidemia Hypertension (Masuzaki et al., 2001)	Decreased visceral obesity Decrease in insulin resistance following HFD (Morton et al., 2004a)				
Liver	Oxoreductase (activates cortisol from cortisone)	Hypertension Insulin resistance Dyslipidemia Hypertriglyceridemia Hepatic steatosis (Paterson et al., 2004)	No change in insulin sensitivity or hepatic or serum lipids Increase in HPA axis activation (Lavery et al., 2012)			Reduces cortisol to 5α-dihydrocortisol (metabolized to 5α-tetrahydrocortisol)	(5αR1) Higher incidence of hepatosteatosis (Dowman et al., 2013; Livingstone et al., 2015)
Muscle	Oxoreductase (activates cortisol from cortisone)	Insulin resistance (Hassan-Smith et al., 2015)	Insulin sensitivity (Morgan et al., 2009)				
Bone	Oxoreductase (activates cortisol from cortisone)		No changes in bone mass, formation or resorption (Justesen et al., 2004)				
Kidney				Dehydrogenase (deactivates cortisol to cortisone) in the distal nephron	Activation of MR by cortisol causing hypertension, hypokalemic alkalosis and suppression of renin–angiotensin–aldosterone system (Stewart, 1999; Palermo et al., 2004). Causes include mutations in the HSD11B2 gene and liquorice consumption		
Placenta				Dehydrogenase (deactivates cortisol to cortisone) in the syncytiotrophoblast		Decreased birth weights (Dave-Sharma et al., 1998; Kitanaka et al., 1996; Mune et al., 1995; Holmes et al., 2006)	

GC, glucocorticoid; *HFD*, high fat diet; *MR*, mineralocorticoid receptor; *AME*, apparent mineralocorticoid excess.

compensatory increase in 5α-reduced metabolites (Palermo *et al.*, 2008). The role of 5βR in the regulation of metabolic phenotype has not been explored.

Conclusion

GC metabolism and activation are important regulatory steps in determining GC action that are independent of circulating GC levels. Regeneration of the active GC cortisol, through the activity of 11β-HSD1 enhances GC action while conversely, 11β-HSD2 and the A-ring reductases clear cortisol and decrease steroid hormone receptor activation. Consequently, dysregulated expression of these enzymes leads to pathological phenotypes such as AME and ACRD. In common conditions, altered tissue-specific expression levels of these enzymes have been implicated in many disease processes, perhaps most notably metabolic disease including insulin resistance, obesity, and type 2 diabetes, but also, aging, cognitive decline, osteoporosis, glaucoma, skin damage, and wound healing (**Table 1**).

While the clinical impact of selective 11β-HSD1 inhibitors has been modest (perhaps too modest for them to be taken forward as standalone therapies in metabolic disease), there is now some evidence that they may have a role in preventing the adverse effects of prescribed steroids although relevant clinical data are yet to emerge (Morgan *et al.*, 2014).

See also: Glucocorticoid Receptor

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Glucocorticoid Receptor☆

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Nomenclature

AF	Activation function domain
AP-1	Activator protein-1
CBP	Cyclic AMP-responsive element-binding protein
CDK	Cyclin-dependent kinase
CLOCK	Circadian locomotor output cycle kaput
DBD	DNA-binding domain
DRIP	Vitamin D receptor-interacting protein
Gas5	Growth Arrest-Specific 5
GR	Glucocorticoid receptor
GREs	Glucocorticoid-response elements
GRIP1	Glucocorticoid receptor-interacting protein 1
hGRα	Human glucocorticoid receptor alpha
hGRβ	Human glucocorticoid receptor beta
HPA axis	Hypothalamic–pituitary–adrenal axis
HSP	Heat shock protein
LBD	Ligand-binding domain
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-κB
NRB	Nuclear receptor-binding domain
NTD	Amino-terminal domain
p/CAF	p300/CBP-associated protein
PI3K	Phosphatidylinositol 3-kinase
SRC1	Steroid receptor coactivator 1
STATs	Signal transducers and activators of transcription
SUMO-1	Small ubiquitin-related modifier-1
TAFIIs	TBP-associated proteins
TBP	TATA-binding protein
TCR	T-cell receptor
TRAP	Thyroid hormone-associated protein complex

Glossary

Coactivator A protein transducing the transcriptional signal of the glucocorticoid receptor to the transcriptional initiation complex consisting of several general transcription factors and RNA polymerase II.

Glucocorticoid receptor-α and -β (GRα and GRβ) Two glucocorticoid receptor isoforms produced by alternative use of exon 9α and 9β.

Nuclear localization signal A specific protein sequence in the glucocorticoid receptor (GR), which mediates the nuclear translocation of the GR.

Protein–protein interaction A mechanism that transduces a signal from one protein to another protein by their direct association.

Introduction

Glucocorticoids regulate a variety of biologic processes and exert profound influences on many physiologic functions by virtue of their diverse roles in growth, development, and maintenance of basal and stress-related homeostasis (Nicolaides and Charmandari, 2017). At the cellular level, their actions are mediated by an intracellular receptor protein, the glucocorticoid receptor (GR), which functions as a hormone-activated transcription factor that regulates the expression of glucocorticoid target genes. The human (h) GR belongs to the superfamily of steroid/thyroid/retinoic acid receptor proteins that function as ligand-dependent transcription factors (Nicolaides *et al.*, 2010).

Structure of *NR3C1* Gene and hGR Protein

The *NR3C1* gene consists of 10 exons and is located on chromosome 5 (**Fig. 1**) (Chrousos and Kino, 2005). The alternative splicing of exon 9 generates two highly homologous receptor isoforms, termed α and β. These are identical through amino acid 727, but then diverge, with hGRα having an additional 50 amino acids and hGRβ having an additional, nonhomologous 15 amino acids. The molecular weights of these receptor isoforms are 97 and 94 kDa, respectively. The hGRα is ubiquitously expressed in almost all human tissues and cells, resides primarily in the cytoplasm, and represents the classical glucocorticoid receptor that functions as ligand-dependent transcription factor (Chrousos and Kino, 2005). The hGRβ is also ubiquitously

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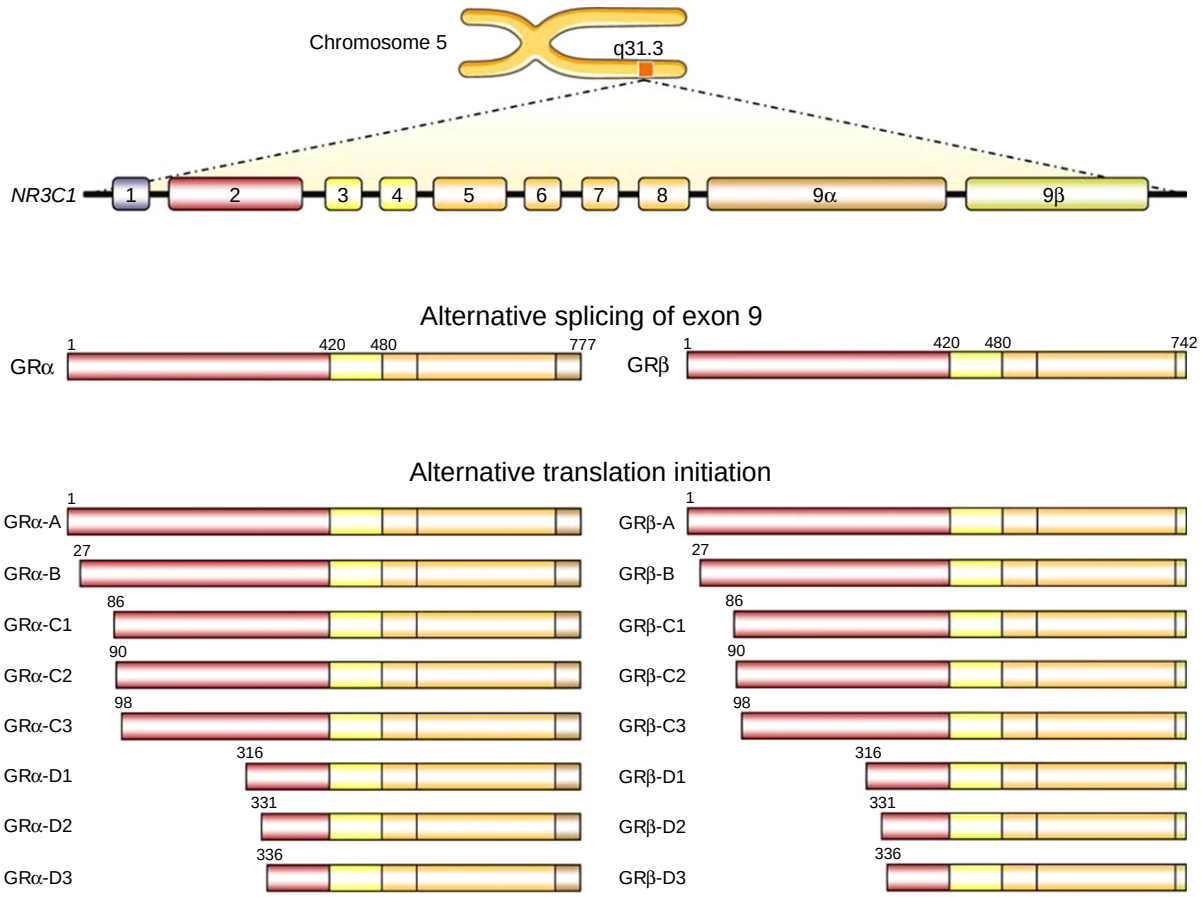

Fig. 1 The *NR3C1* gene is located in chromosome 5 and consists of 10 exons. Exon 1 is an untranslated region, exon 2 codes for the amino-terminal domain, exons 3 and 4 code for the DNA-binding domain, and exons 5–9 code for the hinge region and the ligand-binding domain. The *NR3C1* gene contains two terminal exons 9 (exon 9α and 9β), alternatively spliced to produce the hGRα and hGRβ isoforms. The alternative initiation of GRα mRNA translation may generate eight receptor isoforms (GRα-A, -B, -C1, -C2, -C3, -D1, -D2, and -D3). It is likely that the hGRβ mRNA might also be translated into eight distinct translational receptor isoforms.

expressed in tissues, usually at lower concentrations than hGRα, with the exception of epithelial cells and neutrophils. In contradistinction to hGRα, hGRβ interacts poorly with heat shock proteins (HSPs), resides primarily in the nucleus of cells independently of the presence of ligand, does not bind glucocorticoids or antiglucocorticoids, does not activate glucocorticoid-responsive genes, and can directly influence the transcription rate of a number of target genes (Kino *et al.*, 2009a).

The alternative initiation of GRα mRNA translation may give rise to eight distinct receptor isoforms, termed GRα-A, -B, -C1, -C2,-C3, -D1, -D2, and -D3 (**Fig. 1**) (Lu and Cidlowski, 2005). These isoforms have similar affinity to bind to glucocorticoids and similar capability to bind to GREs in the promoter regions of glucocorticoid-responsive genes (Ramamoorthy and Cidlowski, 2016). However, they have different transcription properties with less than 10% of target genes being commonly regulated by these receptor isoforms (Wu *et al.*, 2013). They also differ in terms of subcellular localization and tissue expression; for example GRα-D is primarily localized in the nucleus, and is expressed in the spleen and bladder. GRα-B and GRα-C reside in the cytoplasm and translocate to the nucleus following ligand binding. GRα-B is mostly expressed in thymus and colon, whereas GRα-C might be found in lung, pancreas and colon (Lu and Cidlowski, 2005). It is likely that the hGRβ mRNA is also translated into eight distinct translational receptor isoforms (**Fig. 1**).

The hGR protein is composed of a poorly conserved amino-terminal domain (NTD), a central, highly conserved DNA-binding domain (DBD), a hinge region, and a carboxyl-terminal, ligand-binding domain (LBD) (Nicolaides *et al.*, 2010). The NTD contains a major transactivation domain, termed activation function (AF)-1, which is located at amino acids 77–262 of hGR. The DBD spans over amino acids 420–480 and contains two zinc-finger motifs through which it binds to specific DNA sequences in the promoter region of target genes, the glucocorticoid-response elements. The hinge region is responsible for the structural flexibility of the receptor, and contains critical lysines that can be acetylated by the "circadian locomotor output cycle kaput" (CLOCK), a core transcription factor involved in the circadian oscillations of gene expression (Nader *et al.*, 2009). The LBD contains a second transactivation domain, AF-2, as well as sequences important for interaction with HSPs, nuclear translocation, and receptor dimerization. The LBD is composed of 12 helical structures and undergoes conformational changes following binding to the ligand.

Molecular Mechanisms of GR Action

Genomic GR Actions

Nucleocytoplasmic shuttling of GR

In the absence of ligand, the hGRα resides primarily in the cytoplasm of cells as part of a large multiprotein complex, which consists of the receptor polypeptide, heat shock proteins (HSP90, HSP70) and immunophillins (FKBP51 and FKBP52) (**Fig. 2**) (Grad and Picard, 2007). These molecules are thought to sequester hGRα in the cytoplasm of cells by maintaining the receptor in a conformation that masks or inactivates its nuclear localization signals (NLSs) (Pratt and Toft, 1997). Upon hormone binding, the receptor undergoes an allosteric change, which results in dissociation from HSPs and immunophillins, unmasking of the NLSs, and exposure of the ligand-binding pocket. In its new conformation, the activated, ligand-bound hGRα translocates into the nucleus, where it binds as homo- or hetero-dimer to glucocorticoid-response elements (GREs) located in the promoter region of target genes (**Fig. 2**) (Chrousos and Kino, 2005; Nicolaides *et al.*, 2010; Nicolaides and Charmandari, 2017). The hGRα then communicates with the basal transcription machinery and regulates the expression of glucocorticoid-responsive genes positively or negatively, depending on the GRE sequence and promoter context (**Fig. 3**). Following hGRα-mediated transcriptional activation or repression, the receptor dissociates from the ligand, and remains in the nucleus for hours or days. The hGRα is then exported from the nucleus to the cytoplasm, poised for a new round of ligand-induced activation (**Fig. 3**). Specific motifs in the DBD of the receptor and the calreticulin export pathway play fundamental role in the nuclear export of hGRα (Nicolaides *et al.*, 2010).

Mechanisms of transcriptional activation by GR

Following binding to GREs, which consist of two 6-nucleotide half sites separated by three nucleotides (GGAACAnnnTGTTCT), the activated hGRα enhances the expression of glucocorticoid-responsive genes by regulating the assembly of a transcriptional preinitiation complex at the promoter region of target genes (Beato, 1989). This is achieved by interaction of the liganded receptor with the basal transcription factors, a group of proteins composed of RNA polymerase II, TATA-binding protein (TBP), and a host of TBP-associated proteins (TAFIIs) (**Fig. 3**). The interaction between the activated receptor and the basal transcription factors is mediated by the coactivators, which are nucleoproteins with chromatin-remodeling activity and other enzymatic activities.

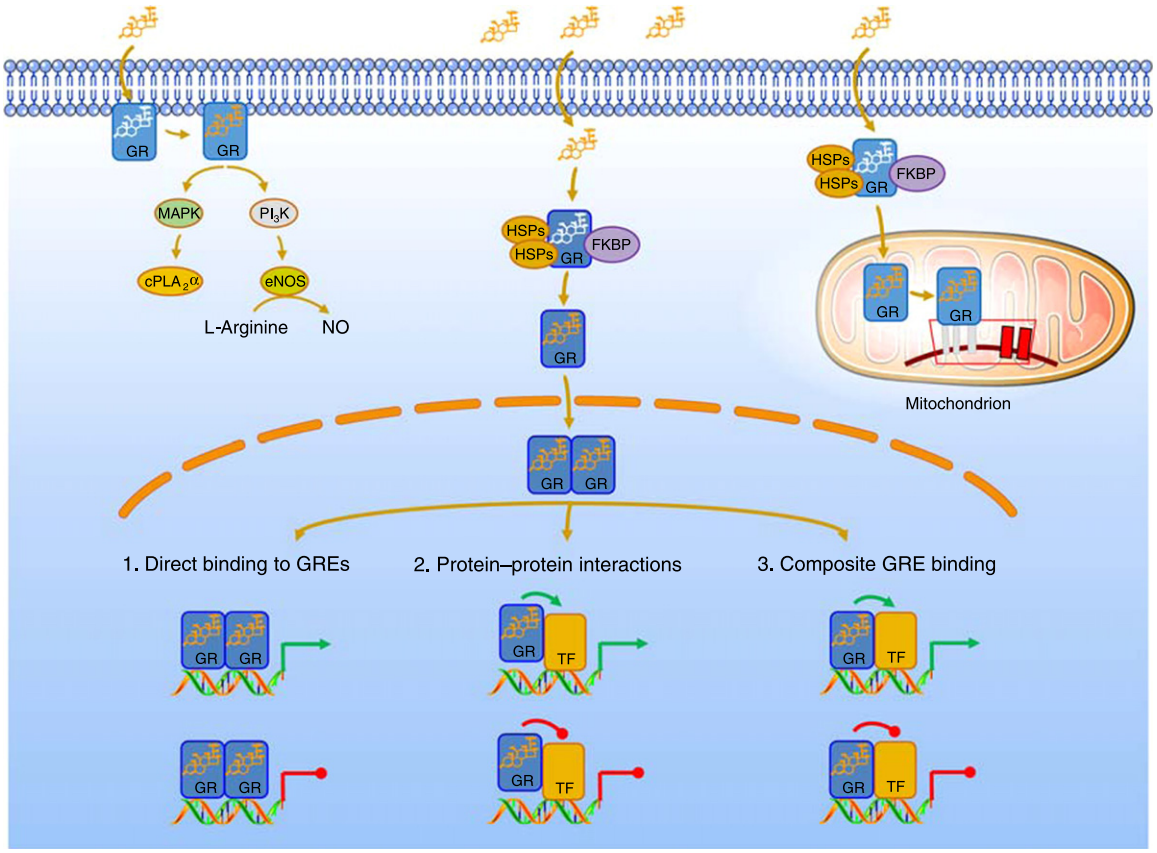

Fig. 2 Nongenomic, genomic and mitochondrial glucocorticoid signal transduction. cPLA2α: cytosolic phospholipase A2 alpha; eNOS: endothelial nitric oxide synthetase; FKBP: immunophillins; GR: glucocorticoid receptor; GREs: glucocorticoid response elements; HSP: heat shock proteins; MAPK: mitogen-activated protein kinases; NO: nitric oxide; PI3K: phosphatidylinositol 3-kinase; TF: transcription factor.

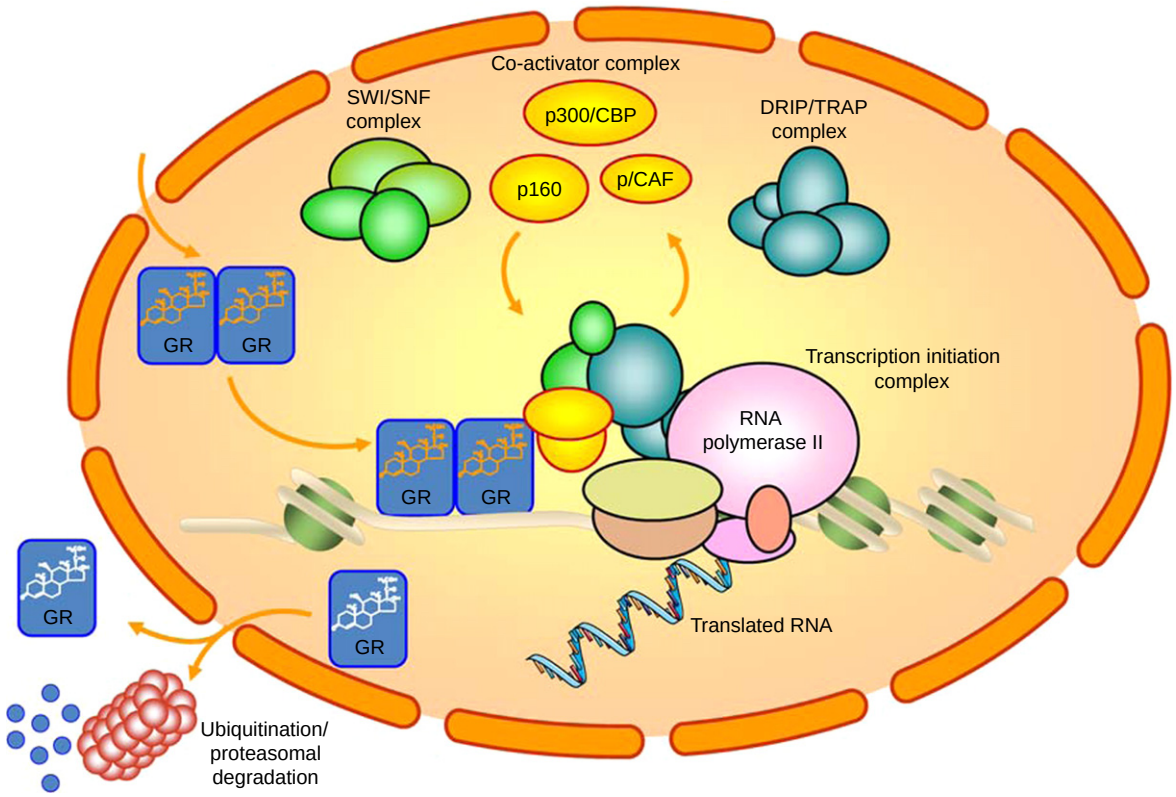

Fig. 3 Schematic representation of the interaction of the glucocorticoid receptor with coactivators and other chromatin modulators. GR: glucocorticoid receptor; p/CAF: p300/CBP-associated protein; CBP: cyclic AMP-responsive element-binding protein; DRIP: vitamin D receptor-interacting protein; SNF: sucrose nonfermenting; SWI: mating-type switching; TRAP: thyroid hormone receptor-associated protein.

Like other transcriptional activators, the hGRα uses its transcriptional activation domains AF-1 and AF-2 as surfaces to recruit chromatin remodeling factors and to interact with the coactivators that link enhancer-bound transcription factors to general transcription factors, thereby initiating transcription (Nicolaides *et al.*, 2010). At least two regions of hGRα possess intrinsic transcriptional activation functions: AF-2, which maps to the carboxyl terminus, is glucocorticoid-dependent, with ligand binding promoting the formation of a surface that permits protein–protein contacts between AF-2 and additional regulatory factors. In contrast, AF-1 is located at the amino terminus of the receptor, is glucocorticoid-independent, and can recruit both positive and negative regulatory factors that differentially regulate hGRα transcriptional enhancement (Nicolaides *et al.*, 2010).

Several families of nuclear hormone receptor coactivators have been described, including the p160 coactivators, such as the steroid receptor coactivator 1 (SRC1) and the glucocorticoid receptor-interacting protein 1 (GRIP1), the p300 and cyclic AMP-responsive element-binding protein (CBP) cointegrators, and the p300/CBP-associated protein (p/CAF) (Auboeuf *et al.*, 2002; McKenna *et al.*, 1999a,b; McKenna and O'Malley, 2002). The p160 coactivators are the first to be tethered to the promoter region of steroid target genes, thus playing a pivotal role in hGRα-mediated transactivation. These coactivators interact directly with both the AF-1 of hGRα through their carboxyl-terminal domain and the AF-2 of hGRα through multiple amphipathic LXXLL signature motifs, which are located in their nuclear receptor-binding domain (NRB) (Heery *et al.*, 1997). They also contain an additional binding site for hGRα, called auxiliary nuclear receptor-interacting domain, which is located between the NRB and the carboxyl-terminal AF-1-binding site. The p160 proteins have intrinsic histone acetyl-transferase activity, which disrupts the DNA nucleosomal interactions at these promoters, allowing the initiation of transcription. Other coactivators that interact with hGRα include the switching/sucrose nonfermenting (SWI/SNF) complex and the newly described chromatin remodeling complex, vitamin D receptor-interacting protein (DRIP)/thyroid hormone-associated protein (TRAP) complex (Heery *et al.*, 1997). The DRIP/TRAP complex interacts with both the AF-2 and AF-1 domains of hGRα via its components DRIP205 and DRIP150, respectively. Through coordinated interactions with AF-1 and AF-2, the coactivators enhance the transmission of signals from the DNA-bound hGRα to the transcriptional initiation machinery, loosen chromatin structure, and facilitate access and/or binding of other transcription factors and transcription initiation components to DNA, leading to full transcriptional activity of ligand-bound hGRα (Auboeuf *et al.*, 2002; McKenna *et al.*, 1999a,b; McKenna and O'Malley, 2002).

Mechanisms of transcriptional repression by GR

The activated hGRα can bind as dimers to negative GREs (nGREs) and suppress gene expression. These DNA elements have the consensus sequence CTCCn0-2GGAGA, which is different from the positive GRE in terms of nucleotide sequence and the number

of nucleotides between the half sites which can range from zero to two (Hudson *et al.*, 2013). Following binding to nGREs, the hGRα recruits corepressors, including NCoR1 and SMRT, and histone deacetylases, ultimately leading to repression of several target genes (Surjit *et al.*, 2011).

Interaction of GR with and actions via other transcription factors

In addition to binding to classic or negative GREs, the hGRα may regulate transcription by physically interacting with other transcription factors. Protein–protein interactions between hGRα and nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and signal transducers and activators of transcription (STATs) result in negative or positive regulation of their responsive genes, mediating many of the antiinflammatory and immunosuppressive effects of glucocorticoids (Barnes and Karin, 1997; Karin and Chang, 2001; Didonato *et al.*, 1996). During this mode of protein–protein interactions, the activated hGRα interacts with the above-mentioned transcription factors without binding to DNA (**Fig. 2**) (Ramamoorthy and Cidlowski, 2016). In addition to tethering, the hGRα may bind to composite DNA elements, which consist of a GRE and a response element for a specific transcription factor, such as AP-1 or the STATs (**Fig. 2**) (Ramamoorthy and Cidlowski, 2016).

Nongenomic GR actions

Glucocorticoids exert some of their numerous effects in a short time frame. These rapid actions can occur in nonnucleated cells and do not require transcription/translation; therefore, they are termed as "nongenomic" and are thought to be mediated by membrane-bound GRs that alter the activity of many kinases, including the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K) (**Fig. 2**) (Groeneweg *et al.*, 2012). Some examples of nongenomic glucocorticoid actions are: (i) the immediate suppression of ACTH release from the pituitary gland by glucocorticoids (Hinz and Hirschelmann, 2000); (ii) the rapid increase of frequency of excitatory postsynaptic potentials in the hippocampus resulting in altered memory function upon acute exposure to glucocorticoids (Karst *et al.*, 2005); (iii) the glucocorticoid-induced vasorelaxation in patients with myocardial or brain ischemia (Hafezi-Moghadam *et al.*, 2002); and (iv) some immunosuppressive glucocorticoid effects through disruption of T-cell receptor (TCR) complex (Löwenberg *et al.*, 2006). The nature of the membrane GRs is under intense investigation (Nicolaides *et al.*, 2017).

Mitochondrial GR actions

Importantly, the GR has been detected in mitochondria, while GREs have been identified within the mitochondrial genome (**Fig. 2**) (Psarra and Sekeris, 2011). The mitochondrial gene expression is influenced directly by mitochondrial GR–GREs interactions, and, indirectly, by nuclear GR–GREs interactions leading to the expression of mitochondrial RNA-processing enzymes, nuclear respiratory factors or mitochondrial transcription factors (reviewed in Nicolaides and Charmandari, 2017).

Factors That Modulate GR Activity

Post-Translational Modifications of GR

Phosphorylation of GR

Following ligand binding, the hGRα is phosphorylated at serine residues, such as S113, S134, S141, S203, S211, S226, and S404 (Oakley and Cidlowski, 2011, 2013; Ramamoorthy and Cidlowski, 2016). The mitogen-activated protein kinase (MAPK) and the cyclin-dependent kinases (CDKs) phosphorylate hGR and modulate its transcriptional activity. MAPK suppresses the activity of hGR in yeast, whereas CDKs stimulate it. JNK, another mitogen-activated kinase, also phosphorylates hGR and suppresses its transcriptional activity. All these kinases phosphorylate hGRα at different sites, indicating that the function of the receptor may be also regulated by other signal transduction pathways through phosphorylation (Oakley and Cidlowski, 2011, 2013; Ramamoorthy and Cidlowski, 2016).

Ubiquitination of GR

Ubiquitination is a posttranslational modification characterized by the attachment of ubiquitin, a 76-amino-acid protein, to specific lysines, thereby marking proteins for proteasomal degradation. The hGRα undergoes ubiquitination at lysine 419, leading to receptor degradation by the 26S proteasome (Oakley and Cidlowski, 2011, 2013; Ramamoorthy and Cidlowski, 2016).

Sumoylation of GR

The addition of a small ubiquitin-related modifier-1 (SUMO-1) to lysine residues is referred to as "sumoylation." The hGRα is sumoylated at lysines 277, 293, and 703. Sumoylation at these residues results in receptor degradation and inhibition of its transcriptional activity in a promoter-specific fashion (Oakley and Cidlowski, 2011, 2013; Ramamoorthy and Cidlowski, 2016).

Acetylation of GR

Acetylation of hGRα occurs in multiple lysine residues at amino acid positions 492–495 (sequence KKTK) in the hinge region of the receptor. Interestingly, the circadian rhythm-generating transcription factor CLOCK acetylates hGRα at several lysines located in the hinge region. This CLOCK-mediated hGRα acetylation attenuates the binding of the GR to classic GREs, ultimately leading to repression of hGRα transcriptional activity (Nader *et al.*, 2009).

Chaperones and Cochaperones

The hGRα forms heterocomplexes with several heat shock proteins, including hsp90, hsp70, hsp56, and possibly hsp23, and the immunophillins FKBP51 and FKBP52, which may affect its transcriptional activity. The hsp90 and receptor-associating protein 46 regulate the transcriptional activity of hGR in a negative fashion (Grad and Picard, 2007).

MicroRNAs and Long Non-Coding RNAs

MicroRNAs are small non-coding RNAs, which are involved in numerous physiologic processes, including cell differentiation and proliferation. A number of microRNAs influence GR signaling, thereby altering tissue sensitivity to glucocorticoids (e.g., miR-433, miR-124, miR-18 etc.) (Wang *et al.*, 2017). Moreover, the "Growth Arrest-Specific 5" (Gas5) long non-coding RNA binds to the DBD of GRα and downregulates the expression of glucocorticoid target genes (Kino *et al.*, 2010). Interestingly, poor responders to glucocorticoids have increased Gas5 levels, indicating that Gas5 might be used as a biomarker of tissue sensitivity to synthetic glucocorticoids (Lucafo *et al.*, 2015).

GR Polymorphisms

A polymorphism is an inheritable genetic variant of a single locus (usually a single nucleotide variation), which can be found in at least 1% of the population. Polymorphisms in the *NR3C1* gene are associated with either glucocorticoid "resistance" or "hypersensitivity" phenotypes (Nicolaides *et al.*, 2010; Oakley and Cidlowski, 2011, 2013; Ramamoorthy and Cidlowski, 2016). Individuals harboring the ER22/23EK polymorphism, an arginine (R) to lysine (K) substitution at amino acid position 23 (R23K) located in exon 2, have a glucocorticoid resistant phenotype, and present with a healthier metabolic profile, better body composition and prolonged survival. On the other hand, carriers of the N363S or *Bcl1* polymorphisms exhibit a glucocorticoid hypersensitivity phenotype characterized by increased body mass index (BMI), insulin resistance and metabolic complications (Nicolaides *et al.*, 2010; Oakley and Cidlowski, 2011, 2013; Ramamoorthy and Cidlowski, 2016).

Natural GR Mutations

Mutations in the *NR3C1* gene may impair one or more of the molecular mechanisms of hGRα function, resulting in alterations in tissue sensitivity to glucocorticoids and the clinical phenotype of Chrousos syndrome (**Fig. 4**) (Nicolaides and Charmandari, 2017). Primary generalized familial or sporadic glucocorticoid resistance is a rare condition characterized by generalized, partial end-organ insensitivity to physiologic glucocorticoid concentrations. Patients have compensatory elevations in circulating adrenocorticotropic hormone and cortisol concentrations and resistance of the hypothalamic–pituitary–adrenal (HPA) axis to dexamethasone suppression, but no clinical evidence of overt hypo- or hypercortisolism. More than 22 sporadic cases with the condition have been reported (**Fig. 4**). Abnormalities of several hGRα characteristics, including cell concentrations, affinity for glucocorticoids, stability, and translocation into the nucleus, have been associated with this condition (Nicolaides and Charmandari, 2017).

In addition to *NR3C1* gene mutations causing Chrousos syndrome, only one mutation has been reported to be associated with primary generalized glucocorticoid hypersensitivity. The hGRαD401H was identified in a patient with obesity, dyslipidemia, and type 2 diabetes (**Fig. 4**) (Charmandari *et al.*, 2008).

Glucocorticoid Receptor Beta

The hGR*β* functions as a dominant-negative inhibitor of hGRα activity and inhibits hGRα-mediated transactivation of many target genes in a dose-dependent manner (Kino *et al.*, 2009b). The mechanism(s) underlying this inhibition has not been fully elucidated, but may involve competition between hGRα and hGR*β* for binding to GREs, formation of hGRα–hGR*β* heterodimers that are transcriptionally inactive, and/or titration or squelching of coactivators needed by hGRα for full transcriptional activity (Bamberger *et al.*, 1995; Charmandari *et al.*, 2005; Kelly *et al.*, 2008; Yudt *et al.*, 2003). The ability of hGR*β* to antagonize the function of hGRα suggests that hGR*β* may play a critical role in regulating target tissue sensitivity to glucocorticoids. Increased expression of hGR*β* has been documented in generalized and tissue-specific glucocorticoid resistance and leads to a reduction in the ability of hGRα to bind to GREs. Therefore, an imbalance in hGRα and hGR*β* expression may underlie the pathogenesis of several clinical conditions associated with glucocorticoid resistance, such as inflammatory diseases (rheumatoid arthritis, systemic

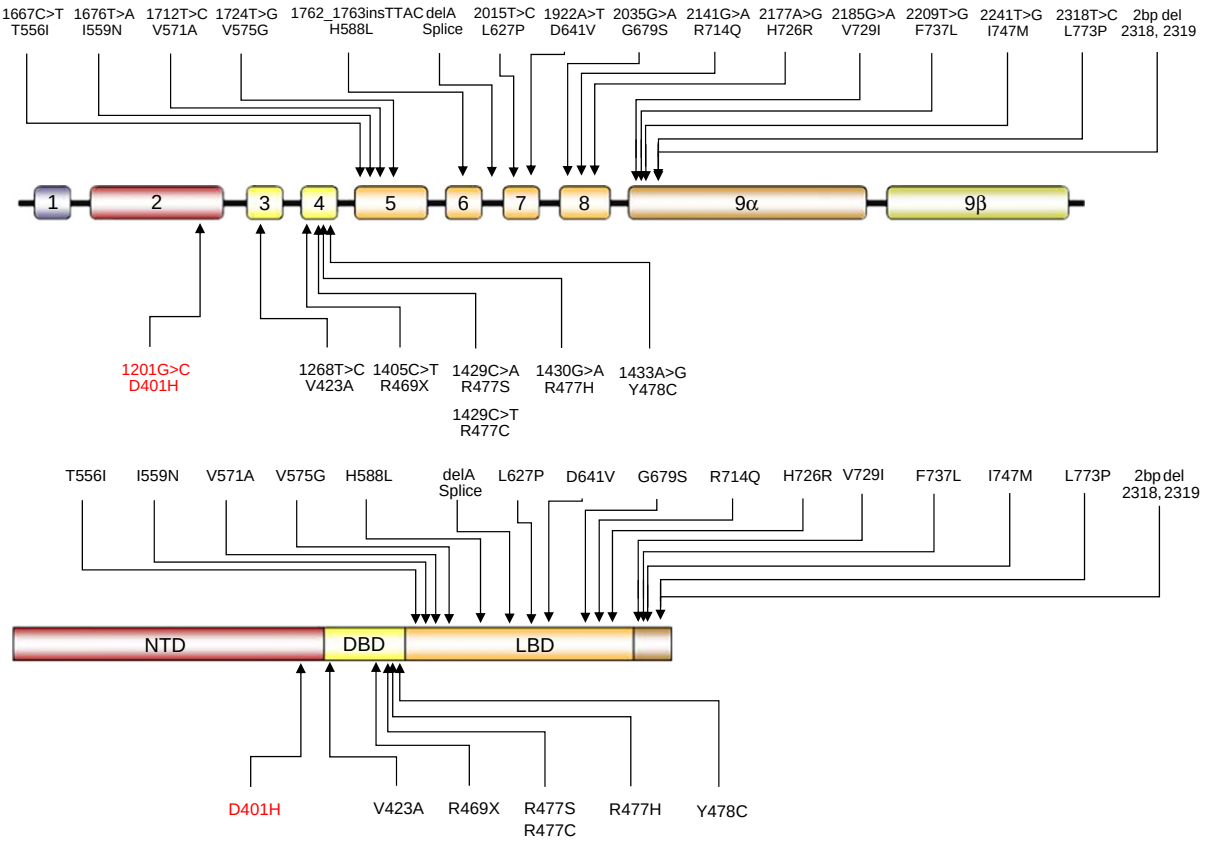

Fig. 4 Location of the known mutations of the *NR3C1* gene causing alterations in tissue sensitivity to glucocorticoids. Mutations indicated by dark color have been associated with primary generalized glucocorticoid resistance or Chrousos syndrome, while the D401H, which is indicated in *red* color, has been associated with primary generalized glucocorticoid hypersensitivity. DBD: DNA-binding domain; LBD: ligand-binding domain; NTD: amino-terminal domain.

lupus erythematosus, and ulcerative colitis), hematologic malignant disorders (acute lymphoblastic leukemia or chronic lymphocytic leukemia), and mood disorders (major depression, schizophrenia). In addition to its important role in inhibiting hGRα transcriptional activity, hGRβ might be involved in insulin signaling, gluconeogenesis, glioma formation and migration of bladder cancer cells (He *et al.*, 2015; Yin *et al.*, 2013; McBeth *et al.*, 2016).

See also: Glucocorticoid Metabolism and Activation. Impact of Glucocorticoid Receptor Polymorphisms on Glucocorticoid Action. Glucocorticoid Resistance Syndromes and States

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Glucocorticoids and Immunity☆

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Glossary

Autoimmune disease A disease that is caused when an individual produces an immune reaction against its own tissues.

Chemokines A subgroup of cytokines that guide cell movement towards a target location.

Cytokines A group of small proteins released from cells that are important in cell signaling and control of the immune system.

Extravasation The movement of leukocytes from capillaries into the tissues surrounding them.

Glucocorticoids Any of a group of adrenocortical steroid hormones whose metabolic effects include stimulation of gluconeogenesis, increased catabolism of proteins, and mobilization of free fatty acids; they are also potent inhibitors of the inflammatory response (allergic response).

Immune system The cells and tissues involved in recognizing and attacking foreign substances in the body.

Immunity A biological condition whereby a body is capable of resisting or overcoming an infection or diseases.

Immunosuppression The process of inhibiting a normal immune response with the use of drugs, biological agents, or chemical agents; commonly used in association with tissue transplantation or to control autoimmune diseases.

Studies carried out over the past 50 years have revealed the broad spectrum of actions of glucocorticoids on the immune system. Glucocorticoids shape almost all aspects of an immune response. Their antiinflammatory effects were recognized shortly after the discovery of glucocorticoids in the 1940s, and they remain a highly effective treatment for acute and chronic inflammation. The potent immunosuppressive effects of synthetic glucocorticoids also underpin their use in autoimmune disease and organ transplantation. This article discusses the mechanisms of glucocorticoid actions on immune cells and provides context to explain the immunosuppressive and anti-inflammatory actions of glucocorticoids.

Glucocorticoids: Receptors and Glucocorticoid Metabolism in Immune Cells

Much of what we know about glucocorticoid effects on the immune system has come from research using synthetic glucocorticoids such as dexamethasone, betamethasone and prednisolone. Synthetic glucocorticoids have greater potency and bioavailability than the physiological glucocorticoids, cortisol and corticosterone. Furthermore, whereas cortisol and corticosterone can bind to mineralocorticoid receptor (MR) as well as to glucocorticoid receptor (GR), most synthetic glucocorticoids preferentially activate GR, with little activity at MR. This is an important consideration when discussing the effects of glucocorticoids on the immune system (Coutinho and Chapman, 2011). Prolonged use of synthetic glucocorticoids suppresses endogenous cortisol/corticosterone production, potentially depriving MR of its ligand in those cells where it acts primarily as a high affinity glucocorticoid receptor. Both are expressed in immune cells: GR is present in most or possibly all immune cells, whereas MR distribution is more restricted (it is not expressed in the thymus for example) (McEwen *et al.*, 1997). The two receptors exert opposing effects in some instances. In macrophages, MR activation promotes a pro-inflammatory macrophage phenotype whereas GR activation generates an anti-inflammatory phenotype (Rickard and Young, 2009). A further complexity comes from glucocorticoid metabolism by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (Chapman *et al.*, 2013b; Coutinho and Chapman, 2011). There are two 11β-hydroxysteroid dehydrogenase isozymes. The type 2 enzyme, 11β-HSD2, which converts cortisol to the intrinsically inert cortisone, is absent from immune cells in healthy adults. However, 11β-HSD1, which does the opposite, converting cortisone to cortisol, is present in many immune cells. Thus, by increasing intracellular cortisol/corticosterone levels in specific cells, 11β-HSD1 can contribute to GR and/or MR activation and help shape the immune response to infection or inflammation (Chapman *et al.*, 2013a). Prednisolone and prednisone are also interconverted by 11β-HSD, but dexamethasone is a poor substrate. Recent studies, particularly those in vivo in mice with genetic alterations in GR, MR or 11β-HSD1, are providing much needed information about the role of physiological glucocorticoids and their receptors in shaping immune responses (Cain and Cidlowski, 2017; Chapman *et al.*, 2013a). A detailed discussion of this is beyond the scope of this article, but the interested reader can learn more from the reviews referenced in the text.

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Glucocorticoids Alter Leukocyte Distribution and Trafficking

Intravenous injection of glucocorticoids or acute cortisol release following stress rapidly and profoundly affects white blood cell (leukocyte) trafficking. Even the diurnal rhythm of glucocorticoid release impacts on blood lymphocyte numbers, with peak glucocorticoid levels corresponding with a diurnal trough in blood lymphocyte numbers and vice versa. Thus, endogenous glucocorticoids exert a tonic control over the immune system (McEwen *et al.*, 1997). The appropriate location of immune cells in different body compartments is vital for immune surveillance as well as for appropriate immune responses. Hence, glucocorticoid effects on immune cell trafficking between the blood, immune organs and other body compartments orchestrate immune responses, ensuring that immune cells are in the right location and ready to combat infection or inflammation. The redistribution of lymphocytes from blood to other body compartments (especially the bone marrow), the increased neutrophilia, and the profound depletion of monocytes, eosinophils and basophils result mainly from glucocorticoid-induced alterations in expression of adhesion molecules—selectins, integrins and members of the immunoglobulin superfamily—on leukocytes and endothelial cells (Besedovsky *et al.*, 2014; Guo *et al.*, 2017; McEwen *et al.*, 1997). These factors act passively, to modulate bone marrow release, peripheral tissue infiltration, and clearance of leukocytes by retaining (or extruding) leukocytes in specific compartments. As well as effects in uninfected or noninflamed conditions, during an ongoing infection or inflammatory response, glucocorticoids potently inhibit the infiltration of neutrophils, eosinophils and monocytes across the endothelium into sites of inflammation, effects that require new protein synthesis. This inhibition of leukocyte extravasation is critical for the anti-inflammatory effects of glucocorticoids and largely results from glucocorticoid inhibition of chemotaxis: glucocorticoids markedly suppress synthesis of a variety of chemokines including the neutrophil chemoattractants, IL-8 and other CXC (cysteine-X-cysteine) chemokines and the eosinophil chemoattractants —CC (cysteine–cysteine) chemokines, such as eotaxin, eotaxin 2, CCL-13/MCP (monocyte chemotactic protein)-4 and RANTES (regulated on activated normal T-cell expressed and secreted) (Franchimont, 2004).

The glucocorticoid-induced neutrophilia, familiar to most clinicians, results from three processes: an increase in neutrophil release from the bone marrow, inhibition of neutrophil extravasation and promotion of the survival of neutrophils (in contrast, glucocorticoids induce apoptosis of eosinophils) (Coutinho and Chapman, 2011; McEwen *et al.*, 1997). However, in the presence of severe hypoxia or pro-inflammatory mediators, glucocorticoids do not enhance neutrophil survival (Marwick *et al.*, 2013) suggesting that their effects are highly dependent on the microenvironment. Inhibition of neutrophil extravasation by glucocorticoids occurs through decreased expression of leukocyte adhesion molecules on endothelial cells, such as *E*-selectin/CD (cluster of differentiation)-62E, InterCellular Adhesion Molecule (ICAM)-1 and vascular cell adehsion molecule (VCAM)-1 (the latter, immunoglobulin superfamily members) and by reducing L-selectin/CD62L (important for neutrophil tethering and rolling along the endothelium) on the surface of neutrophils (Cain and Cidlowski, 2017; Franchimont, 2004; McEwen *et al.*, 1997). However, the immediate action of glucocorticoids on neutrophils is not related to a direct regulation of L-selectin on circulating neutrophils. Instead, glucocorticoids promote shedding of L-selectin from neutrophils, preventing their attachment to endothelial cells. Glucocorticoids also decrease the expression of L-selectin on bone marrow progenitors and on differentiating neutrophils that, then, reach the bloodstream with low L-selectin levels (Franchimont, 2004).

It is still unclear exactly how glucocorticoids increase bone marrow release of neutrophils. Mature neutrophils express CXCR4, the receptor for CXCL12/Stromal cell Derived Factor (SDF)-1α. CXCL12/SDF-1α is highly expressed in the bone marrow, where it retains neutrophils. Granulocyte colony-stimulating factor (G-CSF) indirectly down-regulates the CXCR4/SDF-1α chemokine axis as well as promoting the generation of neutrophils (Furze and Rankin, 2008). There appears to be a complex relationship between glucocorticoids and G-CSF, and G-CSF may be a target for glucocorticoid action (Root and Dale, 1999). During inflammation, chemokines released from the inflamed tissue are also important for recruiting and activating neutrophils and initiating extravasation. As mentioned above, glucocorticoids potently suppress chemokine synthesis. Importantly, whereas glucocorticoids decrease the expression of some chemokine receptors on neutrophils, eosinophils and other leukocytes, they enhance the expression of others, thus orchestrating the migration of specific leukocyte populations during an immune or inflammatory response.

In many immune cells (hematopoietic progenitor cells, specific T cell subsets, blood monocytes and eosinophils) but notably not neutrophils, CXCR4 expression is increased by glucocorticoids, causing these immune cells to be retained by SDF-1α in the bone marrow (Besedovsky *et al.*, 2014). The redirection of T cells and eosinophils to the bone marrow explains their disappearance from the circulation following glucocorticoid administration or stress. Glucocorticoids also prevent the influx of lymphocytes into draining lymph nodes, independently of the classical lymph node homing molecules, L-selectin and CCR7. This blockade of lymphocyte migration to certain lymph nodes may account for glucocorticoid suppression of some lymphocyte responses, for example, the production of antibody in response to antigen presenting cells in lymph nodes. This may also be an important mechanism in glucocorticoid suppression of acute relapse in multiple sclerosis: in an animal model of multiple sclerosis, glucocorticoids redirect T cell migration in a manner dependent upon the CXCR4/CXCL12 pathway, reducing T cell infiltration of the brain parenchyma (Schweingruber *et al.*, 2014).

The flow and movement of monocytes also seems tightly regulated by glucocorticoids via similar mechanisms, namely, through the repression of monocyte and endothelial adhesion molecules and the regulation of chemokines and their receptors.

Innate and Adaptive Immunity

Although the categorical distinctions between the innate and adaptive immune systems have become blurred over the last decade, they remain useful working concepts to understand the effects of glucocorticoids on immunity.

The innate immune response is critical for early defense following infection or injury. It is engaged following activation of pattern recognition receptors (PPRs) by pathogen-associated molecular patterns (PAMPs) or host-derived damage-associated molecular patterns (DAMPs). PPR activation triggers activation of pro-inflammatory pathways via the transcription factors NF-kB, AP-1, C/EBP and others leading to induction of genes encoding cytokines, chemokines, adhesion molecules and enzymes (Newton and Dixit, 2012). Adaptive immunity takes longer to establish. Immune memory is the hallmark of the adaptive immune system, lasting for many years and residing in antigen-specific T and B cells. The innate immune response has not traditionally been considered to provide immune memory. However, this view has been challenged in recent years with the discovery of memory lasting weeks to months in natural killer (NK), innate lymphoid cells (ILCs) and other innate immune cells. This recently described nonspecific type of immune memory occurs in response to foreign insult (e.g., infection or endotoxins) and has been termed "trained immunity" or "innate immune memory" (Netea *et al.*, 2016). Examples of this are the nonspecific protective effects against infections induced by vaccines against measles or tuberculosis. In experimental animals, a widely used model is the attenuated inflammatory response to high dose endotoxin (usually bacterial lipopolysaccharide; LPS) following several days "priming" with low dose LPS (Netea *et al.*, 2016).

The innate and adaptive immune systems can be divided into two types of cell-mediated effector immunity, categorized as type 1 and type 2. This nomenclature, which originally arose from the division of CD4+ T cell immune responses into those that promoted cell mediated (Th1) and humoral (Th2) immunity, has been extended more generally to cover other cell types, including cytotoxic (CD8+) T cells, ILCs and, importantly, macrophages: M1 being classically activated, pro-inflammatory and M2 being alternatively activated, or pro-repair (although in reality, macrophages can adopt a wide variety of phenotypes across a continuum from M1 to M2) (Lappin and Campbell, 2000). Type 1 and type 2 responses can be broadly classified by the cytokines associated with those responses. Thus, type 1 effector responses are pro-inflammatory, providing defense against microorganisms including bacteria and viruses as well as neoplasia, but they are also associated with autoimmune disease. These responses are strongly associated with Interferon (IFN)-γ, IL-12 and IL-18 and traditionally involve CD4+ Th1 (and more recently included, Th17) cells. Type 2 responses promote tissue repair/wound healing and provide defense against extracellular parasites, but are also important in driving allergic and fibrotic disorders. Type 2 responses are associated with IL-4, IL5, and IL-13 and traditionally involve CD4+ Th2 cells (Lappin and Campbell, 2000).

Glucocorticoids affect both the innate and adaptive immune systems. Generally speaking, they inhibit type 1 responses and promote a switch to type 2, thus altering the balance of an innate or adaptive immune response (del Rey and Besedovsky, 2000). Glucocorticoids prevent the overshoot of inflammatory responses under the control of the innate immune response. They also limit the adaptive immune response and prevent the expansion of immune cells with little or no affinity for the antigen that triggers an immune response. Glucocorticoid actions on immune cells, both in the innate and adaptive immune systems, include suppression or stimulation of numerous pathways involving pro-inflammatory or antiinflammatory mediators. They also alter immune cell phenotype and cellular differentiation programs to promote resolution of inflammation and adaption to future insult or infection. Many of these actions are described below, with reference to the cytokines and their receptors that may mediate these actions.

Glucocorticoid Effects on Innate Immune Responses

Endogenous glucocorticoids are vital to prevent the overshoot of an early innate immune response to infection. Adrenal insufficiency can be life-threatening in certain infections. Similarly, in animal models, adrenalectomy sensitizes mice or rats to the lethal effects of endotoxin, a powerful stimulatant of the HPA axis (Besedovsky and Del Rey, 2000; del Rey and Besedovsky, 2000). The stimulation of immune cells—by viral infection, endotoxin, or by neoplastic or damaged cells—triggers the release of IL-1 and other cytokines early during an immune response. These then activate the HPA axis, increasing glucocorticoid levels. Glucocorticoids subsequently prevent an excessive inflammatory reaction by suppressing the synthesis of numerous pro-inflammatory mediators, such as many cytokines (including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-12, IL-13, IL-16, IL-17, IFNγ and TNF-α), prostaglandins, leukotrienes, proteolytic enzymes, reactive oxygen species, and nitric oxide. At the same time, they exert direct actions on immune cells, shaping their function and indeed, the adaptive immune response (see below). In fact, the levels of glucocorticoid achieved during an immune response are enough to inhibit the response to another, unrelated, antigenic stimulus (Besedovsky and del Rey, 2000; del Rey and Besedovsky, 2000). This may be an important mechanism associated with, and that may contribute to, innate immune memory.

Monocytes/macrophages and dendritic cells are key targets of glucocorticoid actions. In mice, knockout of GR in macrophages and/or dendritic cells (DC) increases mortality in models of septic shock, associated with elevated levels of type 1 cytokines (IL-12, IL-1, TNF-α) (Bhattacharyya *et al.*, 2007; Kleiman *et al.*, 2012; Li *et al.*, 2015). This identifies macrophages/DC as the target for the protective effects of endogenous glucocorticoids in preventing the overshoot of the innate immune response. At the same time, glucocorticoids promote the resolution of inflammation by skewing macrophage polarization towards an M2-like (termed M2c) highly phagocytic phenotype, promoting the clearance of microorganisms, apoptotic cells and debris. M2c macrophages show

high expression of the hemoglobin scavenger receptor, CD163 as well as the protein S/Mer tyrosine kinase pathway, important for the engulfment and clearance of apoptotic cells (Martinez *et al.*, 2008). They secrete the antiinflammatory cytokines, IL-10 and transforming growth factor (TGF)-β, important in terminating an inflammatory response as well as inducing tolerance via the adaptive immune system (see below). In monocytes themselves, glucocorticoids induce an activated antiinflammatory state that resembles myeloid suppressor cells, with down-regulation of adhesion related proteins and increased migratory properties. It should be noted though, that many of the effects of glucocorticoids upon immune cells have been characterized in vitro. Given the high context dependence of glucocorticoid action, their effects upon immune cells in vivo are likely to depend upon the prevailing inflammatory and cellular microenvironment. Nevertheless, the suppression of pro-inflammatory cytokine synthesis by glucocorticoids has been clearly demonstrated both in vitro and in vivo. This is mediated by interference with the activity of the master pro-inflammatory transcription factors NF-kB and AP-1 (Cain and Cidlowski, 2017). Glucocorticoids also reduce pro-inflammatory cytokine levels via induction of tristetraprolin, which targets their mRNAs for degradation. Other antiinflammatory effects also involve gene activation by GR (Coutinho and Chapman, 2011). Glucocorticoids induce expression of IL-10, annexin A1, DUSP (DUal-Specificity Phosphatase)-1, GILZ (glucocorticoid-induced leucine zipper) and other proteins that promote the termination and resolution of inflammation. In genetically altered mice, the requirement for these anti-inflammatory proteins for dexamethasone-dependent effects during an inflammatory response depends upon the nature of the inflammation and the specific cell type in question. For example, glucocorticoids fail to suppress sterile peritonitis in mice lacking DUSP-1, demonstrating that DUSP-1 is required for the antiinflammatory effect of glucocorticoids effects in this model (Abraham *et al.*, 2006). In contrast, DUSP-1 knockout mice remain sensitive to the suppressive effects of glucocorticoids in mast cell-dependent anaphylaxis (Maier *et al.*, 2007). As well as the antiinflammatory mediators mentioned above, glucocorticoids also induce the receptors for a number of pro-inflammatory cytokines and chemokines (Cain and Cidlowski, 2017). This is likely to be a mechanism to increase the sensitivity of particular immune cells to these mediators to help these cells to resolve inflammation. Such mechanisms are important in shaping an ongoing immune response, with newly recruited cells differentiating within an altered inflammatory environment. Soluble or decoy receptors that inhibit the inflammatory process are also regulated by glucocorticoids, representing antiinflammatory mechanisms. For example, expression of the decoy receptor IL-1R2, which binds IL-1 without driving its signaling, is increased by glucocorticoids, though the cells responsible are unknown.

Under pro-inflammatory conditions some of the effects of glucocorticoids can be over-ridden, giving rise to apparent glucocorticoid resistance. One mechanism for this is via macrophage migration inhibitory factor (MIF) (Franchimont, 2004; Lerch *et al.*, 2014). MIF is secreted from the pituitary in response to endotoxin challenge or pro-inflammatory cytokines such as TNFα. This may account for the high MIF levels in serum that are associated with a range of chronic inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, lupus and diabetes. MIF can override the antiinflammatory and immunosuppressive actions of glucocorticoids. MIF increases lethality, whereas genetic deletion or therapeutic neutralization confers protection against endotoxemia, acute distress respiratory syndrome, and septic shock. The finding that toll-like receptor (TLR) 4 expression is increased by MIF may explain why MIF-deficient mice are hyporesponsive to LPS. Thus, the notion that glucocorticoids enhance the secretion of a major pro-inflammatory cytokine that counteracts their effects represents a yin/yang mechanism of control of the acute-phase response or septic shock that may become dysregulated in chronic inflammation.

Lipid mediators, specific locally acting molecules derived from phospholipids and polyunsaturated fatty acids, initiate and perpetuate inflammation. Importantly though, certain lipid mediators actively promote resolution and tissue repair pathways. Glucocorticoids suppress the synthesis of eicosanoids, lipid mediators that include prostaglandins and leukotrienes. This is mediated by induction of annexin A1, which suppresses activity of phospholipase A2 thereby reducing the production of arachidonic acid, a key intermediate in eicosanoid biosynthesis (Franchimont, 2004). Glucocorticoids also reduce the generation of prostaglandins by suppressing inducible cyclooxygenase (COX/prostaglandin-endoperoxide synthase)-2, whilst having no effect on the constitutive COX-1 enzyme. Further down the pathway, the synthesis of PGE_2, a major inflammatory mediator, is reduced by glucocorticoid suppression of microsomal PGES (prostaglandin E synthase), an enzyme functionally coupled to COX-2. However, glucocorticoids do not affect cytosolic PGES, which is functionally coupled to COX-1. Thus, whilst glucocorticoids reduce production of prostaglandins and other lipid mediators during inflammation via some routes, synthesis continues via other routes. Glucocorticoids induce 5-lipooxygenase and 5-lipooxygenase-activating protein expression in monocytes and eosinophils, thus diverting arachidonic acid metabolism to leukotriene biosynthesis rather than prostaglandins. They also increase expression of the leukotriene B(4) receptor and activate the lipoxin A(4) receptor on human neutrophils, the latter mediated via annexin A1. This ability of glucocorticoids to suppress eicosanoid biosynthesis yet enhance other aspects of lipid signaling is reminiscent of their diverse effects on cytokines/chemokines and their receptors that redirect cell migration and behavior. Thus, glucocorticoids orchestrate lipid mediators to limit an inflammatory reaction and promote resolution, by altering their synthesis (suppressing or even, perhaps, inducing) and efficacy (cellular sensitivity via specific receptors). Nitric oxide is an important intra- and intercellular signaling molecule in shaping the innate and adaptive immune response, with potential for both detrimental and protective effects. Glucocorticoids suppress the cytokine induction of inducible nitric oxide synthase (iNOS/NOS2) expression, decreasing nitric oxide release by endothelial cells. This inhibition, mediated by the glucocorticoid second-messenger annexin A1, may prevent overshoot of an early endothelial cell-mediated inflammatory reaction (Franchimont, 2004). Glucocorticoids therefore play a vital role in shaping an immune response. The elevation in endogenous glucocorticoids following immune stress or trauma orchestrates the ongoing cellular, cytokine and lipid response to repair the damage caused by the initial injurious stimulus, leading to the resolution of an inflammatory response. The timing is crucial. The use of glucocorticoids in sepsis, for example, is highly controversial and too early (or too high) concentrations may suppress the immune response to sepsis, allowing persistence of the

bacterial infection. Similarly, the failure to engage appropriate resolution mechanisms or persistence of the injurious/infectious stimulus can lead to chronic inflammation. Glucocorticoids play a critical role in shaping the adaptive immune response to infection or other antigens and are important in the balance between immunity and tolerance.

Adaptive Immunity

On first encounter with a pathogen or an antigen, a primary immune response is elicited, depending on the nature of the microorganism or the antigen. Presentation of antigen complexed with MHC class I activates CD8+ cytotoxic T cells whereas antigen presentation with MHC class II activates CD4+ T cells. This leads to the generation of memory cells, highly specific for the inducing antigen, that in turn, mediate the secondary response if the same antigen is encountered subsequently. The cell-mediated response (which can be adoptively transferred by lymphoid cells but not by serum) results in the expansion of antigen-specific cytotoxic CD8+ T cells that kill infected, damaged or cancerous cells. The humoral response (which can be passively transferred by serum from immune individuals) is mediated by B cells and results in the production of high affinity, antigen specific antibodies to eliminate extracellular pathogens and parasites. CD4+ T cells are central to both cell-mediated and humoral immunity: they enhance and maintain CD8+ T cell responses and help B cells make antibody. As well as being important regulators of macrophage function, CD4+ T cells orchestrate immune responses to ensure the right balance between an appropriate magnitude and persistence of an immune response and prevention of autoimmunity.

A key cytokine that plays a pivotal role in the T cell response is IL-2, produced after antigen activation (Liao *et al.*, 2013). It promotes CD8+ T cell (and NK cell) cytotoxic activity and promotes CD4+ cell differentiation into Th1 and Th2 cells, whilst inhibiting differentiation of Th17 cells, the latter implicated in autoimmune and inflammatory disorders. IL-2 is essential for Treg development and maintenance, mediating tolerance and limiting inappropriate immune reactions. Glucocorticoid effects on IL-2 signaling are complex. They suppress IL-2 expression. This prevents excessive expansion and activation of naïve immune cells. On the other hand, glucocorticoids induce IL-2 receptors on activated T cells, amplifying the effect of IL-2 during clonal expansion. In a specific immune response, this suppresses immune cells with little or no affinity for an antigen and favors the clonal expansion of those with high affinity for an antigen (del Rey and Besedovsky, 2000).

Antigen Presentation and Adaptive Immune Response

Dendritic cells (DCs) represent the crucial interplay between innate and adaptive immunity. Following an encounter with microorganisms or antigen, immature tissue-resident DCs avidly capture, process and present antigens, maturing into immunostimulatory cells in the process. Activated DCs then rapidly migrate to draining lymph nodes where they activate T cells, triggering an adaptive immune response.

As they mature into professional antigen-presenting cells (APCs) during their migration, DCs express major histocompatibility complex (MHC) class II and costimulatory molecules to efficiently present the antigen to naive or memory T cells. Crosstalk between T cells and DCs through T-cell receptor(TCR)/MHC II-bound antigen, costimulatory molecules and cytokines allows the development of a T cell immune response and T cell expansion or deletion. When exposed to glucocorticoids during maturation, DCs differentiate into tolerogenic DCs in a manner at least partly dependent upon GILZ. Tolerogenic DCs resemble immature DCs in many respects: they are proficient in antigen uptake but have a poor ability to present antigens and elicit a T cell response (Coutinho and Chapman, 2011). Thus, glucocorticoids prevent MHC class II up-regulation and the expression of the costimulatory molecules, such as Cluster of Differentiation (CD)86 and to some extent CD80, CD40 and the ICAM-1/LFA-1 (intercellular adhesion molecule-1/lymphocyte function associated antigen-1) complex (Franchimont, 2004). Unlike immature DCs however, tolerogenic DCs induce the formation of T regulatory (Treg) cells. In contrast already terminally differentiated mature DCs exposed to glucocorticoids continue to express these molecules due to their relative glucocorticoid resistance. The timing of exposure to glucocorticoids thus appears to be essential during dendritic cell maturation.

The effects of glucocorticoids upon the terminal differentiation of DCs greatly impacts their cytokine secretion profile and their ability to modulate and shape the T cell immune response once it has flared up. The differentiation of $CD4^+$ T cells into T helper 1 (Th1) lymphocytes, which drive cellular immunity, or into Th2 lymphocytes, which drive humoral immunity, depends on the type of antigen encountered and the type of cytokines produced during antigen presentation. Interleukin-12 is a key link between innate and cellular immunity - it is required for CD4+ lymphocyte differentiation into Th1 cells and secretion of Th1 cytokines, such as IFN-γ and TNFα. Genetic disruption of IL-12 signaling in IL-$12^{-/-}$ and IL-$12R^{-/-}$ mice is associated with a defective Th1 immune response. In both mice and humans, the presence of glucocorticoids during the primary immune response is associated with reduced IL-12 production by macrophages or DCs, enhanced Th2 cytokine secretion and decreased Th1 cytokine secretion by $CD4^+$ lymphocytes on secondary stimulation. This effect can be reversed by the addition of exogenous IL-12 to glucocorticoid-treated APCs during the primary stimulation. Glucocorticoids therefore reduce the drive to a Th1 dominated response (Franchimont, 2004). Mice with selective knockout of GR in DCs are highly susceptible to LPS-induced sepsis because of a failure to suppress production of IL-12. Antibody neutralization of IL-12 in these mice prevents hypothermia and death, demonstrating the important role of glucocorticoid-mediated suppression of IL-12 in DCs. IL-12 neutralization also restores the ability of low-dose

LPS to tolerise these mice to subsequent challenge with high dose LPS (innate immune memory). Thus, glucocorticoids promote a DC phenotype that will ultimately generate a Th2 immune response and the secretion of Th2 cytokines.

As well as regulating the T cell response via their effects on DCs, glucocorticoids also exert direct effects on T cells.

Cellular Immune Response

Glucocorticoids affect both CD4+ and CD8+ T cells. Endogenous glucocorticoids attenuate acute graft versus host disease by suppressing the cytotoxic capacity of CD8+ T cells. Mice with specific knockout of GR in T cells develop severe acute graft versus host disease, which is not attenuated by therapeutic administration of glucocorticoids (Theiss-Suennemann *et al.*, 2014). As well as suppressing IL-12 secretion by monocytes/macrophages and DCs, glucocorticoids suppress IL-12Rβ1 and IL-12Rβ2 expression on T cells. They also interfere with IL-12 signaling in T cells by preventing phosphorylation of STAT (signal transducer and activator of transcription)-4 in response to IL-12. In turn, this reduces expression of interferon regulatory factor-1 and other STAT4-induced genes. STAT4-deficient mice are unable to elicit a Th1 immune response. STAT4 may be downstream of COX2: COX2 inhibition suppresses IL-12-induced T cell responses, associated with a block in STAT4 phosphorylation. Importantly, mice with a selective knockout of GR in T cells are unable to suppress COX-2 mediated lethal immune activation and show elevated levels of STAT4 phosphorylation (Brewer *et al.*, 2003). Both the lethal immune activation and the block in STAT4 phosphorylation are rescued by COX2 inhibition. Glucocorticoids also profoundly suppress secretion of the Th1 cytokines IFN-γ and TNFα, lessening NK and T cytotoxic effector functions. Such massive inhibition of the Th1 immune response by pharmacological glucocorticoids causes severe cellular immunodeficiency and impairs defense against intracellular and opportunistic infections.

Humoral Immune Response

Whereas glucocorticoids decrease the secretion of Th2 cytokines on first encounter with an antigen during primary stimulation, they promote Th2 differentiation during secondary stimulation. Independent of monocytes/macrophages and DCs, glucocorticoids prime naive T cells to Th2 commitment during a secondary immune response. The presence of glucocorticoids during primary stimulation promotes IL-10 secretion during secondary stimulation in both naive and memory T cells. Sequential contact with glucocorticoids during the primary and/or secondary immune response may thus influence the pattern of Th2 cytokines. This context-dependent action of glucocorticoids may explain some discrepancies reported in the literature on the regulation of Th2 cytokines by glucocorticoids. Similar to their effects on DCs, stimulation of CD4+ cells in the presence of glucocorticoid induces IL-10 secreting T regulatory (Treg) cells. Treg cells are immunosuppressive and repress the activation, cytokine production and proliferation of both CD4+ and CD8+ T cells. Treg cells themselves secrete high levels of IL-10. Their potent in vivo immunoregulatory properties are protective in experimental models of multiple sclerosis and other models of auto-immunity. The balance of CD4+ T cells between the related Th17 and Treg subsets is important in the pathogenesis of chronic inflammatory disease, including asthma. Glucocorticoids suppress the formation of Th17 cells by reducing production of Th17 promoting cytokines (particularly IL-6) by DCs and by repressing expression of ROR$\gamma\delta$ and STAT3, key transcription factors in Th17 cell differentiation. Thus, by shifting the balance from Th17 to Treg cells, glucocorticoids can help suppress chronic Th2 cell-mediated inflammatory disease. In mice, GR in T cells is needed for pharmacological suppression of Th1 and Th17 cell derived pro-inflammatory cytokines and suppression of antigen-induced arthritis. IL-17$^{-/-}$ mice (but not IFN$\gamma^{-/-}$ mice) are resistant to the therapeutic effects of glucocorticoids, suggesting Th17 cells (rather than Th1) are the likely target (Baschant *et al.*, 2011). The underlying mechanism remains unclear. However, glucocorticoids preferentially inhibit nonactivated lymphocytes but increase the expression of IL-2 receptors on activated lymphocytes. This increases the sensitivity of activated T cells to IL-2, thus preserving IL-2/IL-2R signaling in activated T cells whilst down-regulating it (via reduced IL-2 production) in naïve T cells (del Rey and Besedovsky, 2000). Given the vital roles for IL-2 signaling in clonal expansion as well as tolerance (IL-2 signaling is essential for Treg growth and survival), these effects of glucocorticoids on IL-2/IL-2R signaling may be important in the induction of Treg cells. They certainly contribute an important mechanism to shape T cell immune responses, limiting clonal expansion and ultimately contributing to down-regulation. Dysregulation of these regulatory circuits may contribute to auto-immune disease.

Thus, exposure to endogenous or exogenous glucocorticoids progressively shifts a cellular Th1 immune response to a humoral Th2 immune response. Several mechanisms are likely to be important in the ability of glucocorticoids to induce Th2 lymphocyte differentiation and the humoral immune response. The repression of IL-12 expression in APCs is likely to be one factor. Glucocorticoids also inhibit the transcription factor T-bet, the master regulator of Th1 differentiation (Cain and Cidlowski, 2017). Additionally, although glucocorticoids block IL-12-mediated STAT4 activation, they do not alter IL-4-induced STAT6 phosphorylation, essential for CD4+ Th2 development. These actions allow a Th2 immune response to develop whilst concomitantly suppressing a Th1 response.

The effects of glucocorticoids on B cells and antibody production have been less well studied. Nevertheless, similar to effects on T cells, glucocorticoids restrain B cell proliferation and the early steps in B cell development. GILZ may be at least partly responsible for the effects of glucocorticoids on B cell number: mice deficient in GILZ show exaggerated B cell proliferative responses. Like DCs and effector T cells, B cells become resistant to the inhibitory actions of glucocorticoids as they proceed through the different stages of differentiation and maturation. Surprisingly, the question of how these hormones influence B cell

receptor signaling compared to TCR signaling has not been explored, underscoring the paucity of information concerning glucocorticoid actions on B cells. Generally speaking, therapeutic glucocorticoid administration reduces immunoglobulin (Ig) concentrations. The number of activated B cells (secreting IgM, IgG and IgA) in the spleen inversely correlates with blood glucocorticoid levels (McEwen *et al.*, 1997). However, in vivo administration of glucocorticoids raises IgE serum levels in asthma or atopic patients. Glucocorticoids induce IgE isotype switching, acting synergistically with IL-4, the critical cytokine that induces Th2 differentiation and promotes B cell differentiation. Such IgE isotype switching is dependent on the CD40/CD40L complex since it is not observed in CD40L-deficient patients. In fact, glucocorticoids up-regulate CD40L expression on B cells and this may contribute to their synergistic actions on IgE isotype switching (Franchimont, 2004). Note, however, that the effects of endogenous glucocorticoids may differ from pharmacological treatment; adrenalectomy experiments in animals have shown that endogenous glucocorticoids help mount efficient immunization responses.

Allergy-Mediated Immune Response and Inflammation

The efficacy of glucocorticoids in the treatment of asthma and allergies despite their promotion of a humoral Th2 immune response and IgE secretion poses a paradox. Although glucocorticoids enhance IgE secretion, they strongly suppress allergic inflammation and chemokine-driven tissue infiltration of eosinophils. There may be several targets for the therapeutic benefit of glucocorticoids in these conditions, including mast cells and other innate immune cells. Glucocorticoids deplete tissue mast cells by decreasing expression of stem cell factor, the essential fibroblast-derived mast cell survival factor. Engagement of mast cell FcεRI by specific IgE triggers degranulation and release of preformed inflammatory mediators such as histamine, proteases, cytokines, and lipid mediators responsible for the early phase of type I hypersensitivity manifestations. Glucocorticoids suppress IgE receptor-mediated degranulation and release of inflammatory mediators from mast cells. They interfere with FcεRI signaling by disrupting raf-1/heat shock protein 90 and the subsequent activation of mitogen-activated phosphokinase and phospholipase A2 responsible for the de novo synthesis of arachidonic acid-derived metabolites (Franchimont, 2004). Glucocorticoids prevent the late phase of type 1 hypersensitivity characterized by mast cell activation with de novo synthesis of inflammatory mediators and secondary infiltration of Th2 cells, basophils, and eosinophils. Glucocorticoids also affect other innate immune cells to suppress allergic responses: studies in GR knockout mice suggest that macrophages and neutrophils are the target in contact allergy (Tuckermann *et al.*, 2007).

T Cell Development and Homeostasis

Though supra-pharmacologic doses of glucocorticoids induce T cell apoptosis, endogenous glucocorticoid action can induce T cell survival or apoptosis, depending on the differentiation stage, the degree of T cell activation and the timing of glucocorticoid exposure (before, during, or after activation). The effect of adrenalectomy in promoting thymocyte expansion in animal models is well known. The majority of thymocytes are immature double positive (CD4 + CD8 +) cells. These and some earlier stages in thymocyte differentiation are exquisitely sensitive to glucocorticoid-induced apoptosis. Indeed, the glucocorticoid-sensitivity of T cell progenitors to the pro-apoptotic effects of glucocorticoids is the reason glucocorticoids are the first line treatment in acute lymphoblastic leukemia and other blood cancers. Concomitant TCR signaling and glucocorticoid receptor (GR) signaling promote thymocyte survival, whereas either TCR signaling alone or glucocorticoid alone induces apoptosis (Ashwell *et al.*, 2000). In contrast, single positive (CD4 + or CD8 +) thymocytes are relatively resistant to the pro-apoptotic effects of glucocorticoids. Somewhat puzzlingly, thymic cellularity is normal in $GR^{-/-}$ mice. Studies in mice with T cell selective alteration of GR levels have produced conflicting results on thymic cellularity. Some insight into this paradox comes from animal studies showing that ACTH promotes thymocyte expansion when glucocorticoid levels are low, independently of the pro-apoptotic effects of glucocorticoids on thymocytes (Talaber *et al.*, 2015). The biological relevance of this remains unclear. Interestingly, glucocorticoids up-regulate IL-7Rα, thereby enhancing sensitivity to IL-7, a key cytokine for T cell development. IL-7 potently enhances both thymic-dependent thymocyte differentiation and thymic-independent antigen-dependent peripheral expansion of mature T cells, thereby restoring immunity in T cell-depleted mice. Conversely, deletion of IL-7Rα in mice and humans is associated with a lack of T cells. The positive regulation of IL-7Rα expression by glucocorticoids suggests their strong influence in the maintenance of peripheral T cell homeostasis (Franchimont, 2004).

Concluding Remarks

Glucocorticoid effects on the immune response are complex and depend upon many factors: type of glucocorticoid (synthetic or endogenous), cell type, differentiation and activation state, timing of glucocorticoid exposure and the cytokine milieu. Glucocorticoid effects can be immuno-suppressive or stimulatory and even, seemingly, both at the same time. The outcome of glucocorticoid action during an ongoing inflammatory response is different to that when glucocorticoid is administered prior to immune activation. If glucocorticoids are administered during the course of an immune response, they are less effective immunosuppressants than if administered prior to an immune response. This allows the early steps of an immune response (including

antigen presentation) to proceed, with activated cells resistant to the effects of glucocorticoids, without engaging more cells at later stages, preventing excessive expansion and activation of naïve immune cells.

Thus, glucocorticoids shape the immune response as it progresses, with different effects during the injury/infection and repair/resolution/immune memory phases. Maladaptation can contribute to auto-immune or chronic inflammatory disease. For example, in patients with rheumatoid arthritis, the HPA axis is hypo-reactive given the degree of inflammation and fails to control the auto-immunity and inflammation. Similar HPA axis hypo-reactivity to cytokine activation has been described in animal models of lupus or in susceptibility to experimental arthritis. The great advantage of the clinical use of glucocorticoids in Th1-inflammatory and Th1-autoimmune diseases is obvious because they restrain the inflammatory reaction, prevent tissue destruction, and block Th1-driven immune responses. The relative sparing of innate immunity and antibody responses may explain why glucocorticoids exhibit clinical benefit in certain bacterial infections such as in bacterial meningitis. However, these beneficial effects coexist with deleterious adverse effects. Because of their suppressive actions on Th1 immunity, they increase susceptibility to intracellular and opportunistic infections such as tuberculosis or viral infections. High levels of glucocorticoids may impair early macrophage and neutrophil responses by switching to the resolution phase of inflammation prematurely. The sum total of glucocorticoid action upon a particular immune response is therefore the balance of diverse effects on the many immune cells and inflammatory mediators that comprise a response. A greater understanding of the complexity of these interactions could be useful to enhance ex vivo therapies in auto-immune disease, organ transplants and cancer.

See also: Corticotropin-Releasing Hormone and Inflammation

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Corticotropin-Releasing Hormone and Inflammation☆

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Introduction

The hypothalamic–pituitary–adrenal (HPA) axis is activated during stress and represents one of the major pathways through which the brain regulates the I/I response. Conversely, products of the I/I response influence brain function. In addition, immune cells produce a number of hormones and neuropeptides, such as corticotropin-releasing hormone (CRH) and α-melanocyte-stimulating hormone, which probably act locally as autacoids during both the early and late stages of the I/I process. This locally produced CRH, hereafter called "peripheral CRH," has been also implicated in other physiological functions, such as reproduction (Chrousos, 1995).

Central CRH and the HPA Axis-Peripheral CRH

Sauvagine, urotensin I, urocortin (UCN), and CRH belong to a family of peptides with similar activities. In humans the CRH and UCN genes are located on chromosomes 8 and 2, respectively. Initially, CRH is synthesized as a larger precursor molecule (spanning 191 amino acids in humans) from which it is cleaved at flanking basic amino acid pairs. CRH is a 41-amino-acid peptide and it shows significant interspecies homology at the amino terminal region (Rivier *et al.*, 1982; Vale *et al.*, 1983). CRH is synthesized by parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) and is secreted—along with other adrenocorticotropic hormone (ACTH) secretagogues, such as arginine vasopressin (AVP), cholecystokinin, met-enkephalin, and dynorphin—into the hypophyseal portal blood via projecting axons to the median eminence. The plasma half-life of CRH in humans is 4 min. The secretion of CRH is regulated by inputs from higher centers integrating the effects of the circadian pacemaker, stress, and glucocorticoid negative feedback (acting at pituitary, hypothalamic, and higher levels, such as the hippocampus). There have not been noted any sex or age differences in plasma ACTH or cortisol responses to CRH (Pavlov *et al.*, 1986). However, the corresponding cortisol response is maximized late in the afternoon due to cortisol receptor change of affinity (Grino *et al.*, 1987). ACTH released by CRH leads to the secretion of cortisol and other adrenal steroids. Pituitary adenylate cyclase activating polypeptide (PACAP) increases CRH mRNA levels in the parvocellular region of the PVN of the hypothalamus, suggesting that this polypeptide is involved in the positive regulation of CRH gene expression. Interleukin (IL)-6, produced in the PVN stimulates CRH gene expression both directly and indirectly. Forskolin and PACAP stimulate IL-6 mRNA and protein levels in the hypothalamic cells. IL-6 may be important to sustain the activity of CRH and AVP genes. Estradiol may enhance the activation of CRH gene expression in response to stress (Kageyama and Suda, 2009; Navarra *et al.*, 1991; Vallieres and Rivest, 1999).

Most of the plasma CRH is apparently of nonhypothalamic origin since hypothalamic CRH is rapidly enzymatically decomposed at the pituitary level. Although the contribution of hypothalamic CRH to the total plasma CRH is small, in certain circumstances, such as insulin-induced hypoglycemia, hypothalamic CRH release leads to increments in plasma CRH concentration.

Peripheral CRH has been found in the adrenal medulla, the testes, the ovaries, the cardiovascular system, the gastrointestinal tract, the pancreas, the lung, the spinal cord, the myometrium, the endometrium, and the placenta (Grino *et al.*, 1987; Mastorakos *et al.*, 1996; Ferrari *et al.*, 1995; Petraglia *et al.*, 1992; Fabbri *et al.*, 1990; Baigent, 2001), as well as in diverse inflammatory sites (Mastorakos *et al.*, 1994a; Scopa *et al.*, 1994). Peripheral sensory afferent type C fibers and postganglionic sympathetic nerves also express CRH (Zhao and Karalis, 2002) and have been suggested as an additional source of the immune CRH (Chrousos, 1995). Another peripheral organ where CRH is locally produced is the skin where CRH receptor type 1 (CRH-R1) isoforms are expressed in keratinocytes (Zbytek *et al.*, 2004). Peripheral CRH has the same electrophoretic profile as hypophyseal CRH and the same expression pattern during acute inflammation, as the acute phase reactants, substance P (SP) and TNF, including their down-regulation by glucocorticoids (Karalis *et al.*, 1991). The epithelial cells of human and rodent endometrium produce CRH throughout the menstrual cycle, whereas the stroma needs to undergo decidualization in order to produce CRH. The placenta secretes CRH, leading to an increased maternal plasma CRH concentration during the third trimester of pregnancy. Only in humans, plasma CRH is bound to a high-affinity binding protein (37 kilodaltons) (CRH-BP), with a concomitant reduction in its bioactivity. This protein has been characterized and is expressed in the brain, the pituitary and the peripheral tissues (liver, kidney, spleen) (Guillemin, 2005).

Two types of G protein-coupled CRH receptors, types 1 and 2 (CRH-R1 and CRH-R2, respectively), with seven transmembrane domains each, have been described. They share an approximately 70% homology of their amino acid sequence but exhibit unique

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pharmacologic profiles. They are differentially expressed and appear to mediate selective actions of CRH at different tissues. The affinity that CRH exhibits toward CRH-R1 is 10 times higher than toward CRH-R2 (Perrin and Vale, 1999). CRH-R1 is encoded by human chromosome 17q21 while CRH-R2 is encoded by human chromosome 7p14 (Mastorakos *et al.*, 1993a, 1994b; Vamvakopoulos and Sioutopoulou, 1994). Alternatively nine spliced isoforms α, β, c, d, e, f, g, h and v-1 have been identified for CRH-R1, whereas isoforms α, β, and γ, plus a stomach variant, have been identified for CRH-R2 (Catalano *et al.*, 2003; Johnson *et al.*, 2003; Pisarchik and Slominski, 2001).

CRH-R1α contains 415 amino acids. It mediates CRH and UCN 1 actions and is widely expressed throughout the body. Transcription of all 14 exons results in a CRH-R1 variant (CRH-R1β), a 444-amino acid heptathelial protein receptor with an extended first intracellular loop that exhibits impaired agonist binding and signaling properties. CRH-R1β can be regarded as a "pro-CRH-R1" receptor isoform without knowing its biological actions (Teli *et al.*, 2008). CRH-R1β is expressed in a tissue specific manner and is present at the anterior pituitary (Chen *et al.*, 1993), at reproductive tissues such as myometrium, endometrium and chorion trophoblast cells and mast cells but not in the placenta, adrenal and synovium (Merali *et al.*, 2008). Another CRH-R1 variant is CRH-R1d, containing 14 amino acids missing from the putative seventh transmembrane domain due to exon 13 deletion, a splicing event which is common in the other members of the B1 family of G protein-coupled receptors. In human embryonic kidneys the CRH R1d variant is mainly retained in the cytoplasm in contrast to CRH-R1α, although some cell membrane expression is evident, too. Membrane-expressed CRH-R1d contains an extracellular C terminus. A stomach variant has also been identified for CRH-R2 (Slominski *et al.*, 2001). CRH-R1 expression is highest in the cerebral cortex, striatum, amygdale and cerebellum. On the other hand, CRH-R2 is mostly present in subcortical structures such as the lateral septal nucleus, several nuclei of the hypothalamus and the choroid plexus (Chalmers *et al.*, 1996). CRH receptors mRNA and protein are also detectable in adipose tissue. CRH-R2 expression in fat tissue is comparable with its expression in heart, where the highest expression of CRH-R2 is detected. At the periphery, CRH-R2 is widely expressed in the gastrointestinal tract, the lung, skeletal muscle, arteries and the heart muscle. Indeed, peripherally injected CRH augments gastrointestinal motility, inhibits gastric secretion, lowers blood pressure and affects heart output and decreases inflammatory reactions. CRH-R2 is also expressed in the colon of patients with ulcerative colitis. CRH-R2 downregulation in distal/sigmoid biopsies of ulcerative colitis patients is indicative of change in CRH-R2 signaling associated with the process of inflammation (Chatzaki *et al.*, 2013). Increased serum CRH with decreased lesional skin CRH-R1 gene expression was observed in patients with psoriasis and atopic dermatitis, suggesting their involvement in stress-induced worsening of symptoms (Vasiliadi *et al.*, 2012). Substance P, another factor involved in inflammatory diseases, can stimulate mast cells and increase the expression of functional CRH-R1. CRH induces NK-1 gene expression, explaining CRH-R1 and NK-1 expression in lesional skin of psoriatic patients (Asadi *et al.*, 2012). CRH-R1 is also detected in the ovarian stroma and the theca and in the cumulus oophorus of the graafian follicle (Mastorakos *et al.*, 1993b; Asakura *et al.*, 1997). CRH-R1α is present on both epithelial and stromal cells of the human endometrium (Mastorakos *et al.*, 1996). CRH-R1/CRH-R2 ratio varies according to adipose tissue type. CRH-R1 expression is higher in subcutaneous than in visceral fat in contrast to CRH-R2 expression which is the opposite. Urocortin and stresscopin, ligands of the CRH-R, are also expressed in fat tissue, having metabolic and potent anorexic effects. CRH-R1 and CRH-R2 expression is downregulated by CRH in isolated adipocytes (Seres *et al.*, 2004).

CRH binds to type CRH-R1 of the anterior pituitary corticotrophs, resulting in adenyl cyclase activation and increased intracellular cyclic AMP (cAMP) concentration, cAMP-dependent protein kinase A activation, increased influx of extracellular calcium via L-type calcium channels, and the production of lipoxygenase metabolites of arachidonic acid. The net result is the secretion of ACTH and other proopiomelanocortin (POMC)-derived peptides within a few seconds, whereas increased POMC gene transcription and POMC biosynthesis ensue. The number of CRH-R in corticotroph cells may modulate the ACTH response.

It was shown that the CRH-induced activation of the NF-κB was mediated by two protein kinase systems, PKA and Protein Kinase C (PKC) (Rossant *et al.*, 1999; Sananbenesi *et al.*, 2003). CRH affects the PKC signaling pathway in the pituitary, Leydig cells, adrenals, placenta, immunocytes, myometrium and hippocampus. In human epidermoid cells, CRH induces the activity and the translocation of the conventional PKC isoenzymes (Kiang *et al.*, 1994). It is very interesting that UCN exerts its neuroprotective effect on cultured hippocampal neurons by the PKC pathway via activation of the CRH-R1 receptor (Pedersen *et al.*, 2002). Activation of the PKC pathway may modify the sensitivity to CRH since it affects the number of CRH-Rs (Dieterich and DeSouza, 1996; Dermitzaki *et al.*, 2005). CRH increases the concentration of cytosolic calcium ions in calcium rich and in calcium free media. As a result, the PKA signaling pathway mediates both effects of CRH while on the other hand the PKC signaling pathway mediates only the CRH-induced mobilization of calcium ions from intracellular stores, possibly via P-type calcium ion channel (Kuryshev *et al.*, 1995, 1996; Dermitzaki *et al.*, 2004). CRH-R1α and -R1β exhibit differential responses to PKC-induced phosphorylation. This might represent an important mechanism for functional regulation of CRH signaling in target cells. PKC activation increases internalization of CRH-R1β but not CRH-R1α in a β-arrestin-independent manner, although both CRH-R1 variants are susceptible to homologous desensitization and internalization following treatment with CRH (Marcovic *et al.*, 2006).

The I/I Response

The cellular components of the I/I response consist of circulating nonlymphoid leukocytes and local immune accessory cells. Nonlymphoid leukocytes include the monocytes/macrophages, neutrophils, basophils, and eosinophils. Local immune accessory cells include the endothelial cells, tissue fibroblasts, resident macrophages, and macrophage-related cells, such as Kupffer cells,

type A synovial-lining cells, as well as the basophil-related mast cells. Many substances secreted locally in the inflammatory area by the above-mentioned cells act as autocrine or paracrine regulators and/or mediators of the inflammatory response, as well as endocrine messengers between the inflammatory process and other systems such as the CNS, HPA axis, and peripheral nervous system. These substances include the vasoactive amines, histamine and serotonin, the kallikrein/kinin system, the Hageman factor and other clotting factors, the fibrinolytic system, several components of the complement system, and eosinophil and platelet activators. They also include cytokines (such as tumor necrosis factor α (TNF α), TNFβ, interferon-α, interferon-β, and interferon-γ), interleukin-1 (IL-1) through IL-4 (as well as their binding proteins and natural antagonists), many lipid and glucolipid products of triglyceride metabolism (such as several active products of arachidonic acid, including the endoperoxides, the thromboxanes, prostacyclin, and leukotrienes), and platelet-activating factor. In addition, active oxygen radicals, including nitric oxide and lysosomal constituents such as neutral proteases, participate in the inflammatory response.

The first step in the initiation of the I/I response is the activation of the innate immune system. This nonspecific response serves to locate the injurious agent, restrict the tissue damage and eliminate the harmful agent, initiate the adaptive immune system and determine the path that will be followed (cellular/humoral response). Subsequently, the adaptive immune system is activated by the innate immune responses. As lymphocytes arrive at the inflammatory area, antigen-presenting cells (APCs) such as plasmacytoid dentritic cells, interstitial and Langerhans dentritic cells and astrocytes present infectious agent antigens of macrophages to T cells. This is the ignition signal for the activation of the adaptive immunity, consisting of cellular (T4, T8, NK lymphocytes) and humoral (B lymphocytes, plasma cells, antibodies) immunity. CD4 helper T cells are the regulators of this antigen-specific response. These cells can be subdivided on the basis of cytokines produced in Th1 T cells which promote primarily the cellular/inflammatory immunity and Th2 cells which have a primary role in the regulation of humoral immunity. T-helper cells produce interleukin 17, a cytokine that acts as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation. IL-17 is induced by IL-23 which results in destructive tissue damage in delayed-type reactions. IL-17 acts synergistically with TNF and IL-1.The balance between Th1 and Th2 is important for the homeostasis within the immune system. Glucocorticoids and catecholamines, the stress hormones, have a significant effect on this balance. Glucocorticoids suppress the production of IL-12 by monocytes/macrophages, the main inducer of Th-1 responses (Cao *et al.*, 2005; Elenkov *et al.*, 1996) and thus, they affect the Th-1/Th-2 balance leading to a Th-2 shift. In contrast to catecholamines, GCs also have a direct effect on Th-2 cells by upregulating their IL-4, IL-10 and IL-13 production (Elenkov *et al.*, 1996; Blotta *et al.*, 1997). On the other hand, the two major catecholamines, norepinephrine (NE) and epinephrine potently inhibit the production of IL-12 by APCs thus suppressing the development of Th1 type cells (Cao *et al.*, 2005; Ramierez *et al.*, 1996; Panina-Bordignon *et al.*, 1997).

CRH and the I/I Response

Hypothalamic (central) CRH has been considered to act indirectly in an antiinflammatory fashion, since the end product of the HPA axis' stimulation is cortisol, which has thoroughly studied antiinflammatory actions. Moreover, it is known that among the proinflammatory cytokines, IL-6 is a potent stimulator of the human HPA axis, and a secretagogue of magnocellular AVP (Mastorakos and Ilias, 2006). The administration of IL-6 subcutaneously provokes considerable elevations in plasma ACTH, cortisol, and AVP. It has been shown that IL-6 in head trauma (an aseptic inflammatory state) and a syndrome of inappropriate secretion of antidiuretic hormone is quantitatively correlated with AVP (Gionis *et al.*, 2003; Mastorakos *et al.*, 1993a, 1994c). The latter is also known to be a potent activator of the HPA axis.

However, disturbances in the HPA axis activity affect the I/I response. An excessive HPA response (e.g., a state of stress or relative hypercortisolemia) can increase susceptibility to infectious agents and tumorogenesis but enhance resistance to autoimmune or aseptic inflammatory diseases. On the other hand, a defective HPA axis response (e.g., relative glucocorticoid deficient state) diminishes susceptibility to infections and tumorogenesis but increases susceptibility to autoimmune or aseptic inflammatory diseases. Indeed, these suggestions have been ascertained in Fischer and Lewis rats, two highly inbred strains selected for their resistance (Fischer rats) or susceptibility (Lewis rats) to inflammatory disease. In Lewis rats hypothalamic CRH neurons respond poorly to all neurotransmitters and the overall HPA-axis response to stress is decreased (Chrousos, 1995). Moreover, CRH deficiency disrupts endogenous glucocorticoid production and enhances allergen-induced airway inflammation and lung mechanical dysfunction in CRH knock-out mice. Thus, inherited or acquired CRH deficiency could increase asthma severity in human subjects (Silverman *et al.*, 2004). Hypofunction of the HPA axis was also found in patients with Sjogren syndrome (Johnson *et al.*, 2000) and sarcoidosis (Porter *et al.*, 2003). Interestingly, high plasma levels of cortisol, CRH and ACTH that have been found in centenarians indicate an activation of the entire stress axis, likely counteracting the systemic inflammatory process occurring with age. This activation fits with the hypothesis that lifelong low-intensity stressors activate ancient, hormetic defense mechanisms, favoring healthy aging and longevity (Genedani *et al.*, 2008). On the other hand, the relative hypercortisolemia that accompanies aging has been suggested to result from an age-related HPA axis resilience.

Since CRH is the main stimulator of POMC production from the pituitary, and certain POMC products (like ACTH and β-endorphin) can directly affect the I/I response, it has been hypothesized that CRH itself might be directly involved with the I/I response. The putative role of CRH in the I/I response was further suggested by the presence of CRH-specific binding sites in human lymphocytes secreting POMC-derived peptides (ACTH and β-endorphin). By employing the rat air-pouch model of acute aseptic chemical inflammation, immunoreactive CRH (IrCRH) was localized in the inflammatory tissue by

immunohistochemistry. IrCRH was also found in the cytoplasm of immune accessory cells such as macrophages, endothelial cells surrounding vessels, and tissue fibroblasts. Immunoneutralization studies in vivo with a highly specific anti-CRH polyclonal antiserum resulted in a significant suppression of the inflammatory response, suggesting that peripheral CRH promotes inflammation. The decrease in inflammation by anti-CRH antiserum was similar to that caused by immunoneutralization of TNF (a well-known mediator of the I/I response that was used as a protocol). The effects of the combined administration of anti-CRH and anti-TNF were not additive, indicating that the two antisera might interfere with a common pathway of the inflammatory response. Furthermore, locally produced somatostatin mediates the glucocorticoid antiinflammatory effects at the inflammatory sites, whereas CRH levels at inflammatory sites are lowered in the presence of somatostatin analogues.

The mobility of this "immune" peripheral CRH is similar, as assessed by high-performance liquid chromatography, to that of r/hCRH 1–41 (the form produced by the rat and human hypothalamus as well as by the human placenta). The presence of CRH at peripheral inflammatory sites has also been demonstrated in other animal models of both acute and chronic inflammation. IrCRH was present in inflammatory cells of rat joint tissue with streptococcal cell wall- and adjuvant-induced arthritis. CRH mRNA was present in the inflamed synovial from arthritic rat joints that express specific CRH-binding sites. Furthermore, IrCRH was seen in the synovial-lining cell layers and blood vessels from joints of patients with rheumatoid arthritis (RA) and osteoarthritis, whereas high levels of CRH immunoreactivity were found in the synovial fluids of patients with RA (Crofford *et al.*, 1993). IrCRH was also found in immune accessory cells from uveitic retinas and corpora vitrea from Lewis rats with experimentally induced autoimmune uveitis (Mastorakos *et al.*, 1995a,b). Additionally, the local presence of CRH appears to be of pivotal importance in the process of experimental autoimmune uveoretinitis in rodents, since retinas from immunized B10.A mice treated with anti-CRH antibody showed significantly lower apoptosis and Fas and Fas ligand (FasL) expression than placebo-treated animals. Thus, CRH at inflammatory sites seems to be involved in the activation of the Fas/FasL system.

Whereas central CRH participates in the systemic endocrine inhibition of the I/I reaction, peripheral immune CRH may participate in autocrine/paracrine stimulation of inflammation. The mechanisms of the peripheral CRH-mediated component of the I/I response are still unclear, although these may be mediated by local POMC gene products with known proinflammatory activity and/or by inflammatory cytokines. Another possible source of peripheral immune CRH and other neuropeptides that are important in inflammation (such as substance P and SMS), other than the accessory immune cells, is the primary afferent (sensory) nerves as well as the sympathetic postganglionic neurons. CRH and substance P are depleted in the rat spinal cord and dorsal root ganglia in response to capsaicin, which is toxic to the sensory afferent fibers. Also, IrCRH is present in the intermediolateral sympathetic column as well as the ganglia of the sympathetic chain and, therefore, could contribute to the inflammatory process through the sympathetic postganglionic fibers. Thus, CRH is involved in opposing, and site-specific, pro- and antiinflammatory actions.

Regarding studies on models of septic inflammation, CRH-deficient mice had reduced ileal secretion, histological damage and inflammation in response to clostridium difficile (toxin A). In addition, the content of SP (a sensory neuropeptide with pivotal role in the mediation and amplification of toxin A-induced inflammatory signal) at the inflammatory sites is CRH-dependent. These results revealed the major proinflammatory role of CRH in the pathophysiology of toxin A-mediated inflammatory diarrhea and indicate a SP-linked pathway (Anton *et al.*, 2004). Recent findings support the idea that increased peripheral CRH mediates the enhanced visceral nociception in rats recovered from experimental colitis (La *et al.*, 2008).

Data exist showing that CRH enhances the expression of nitric oxide synthase (NOS III) to promote NO production from CRH-R1α expressing cells. These results confirm a role for CRH-R-mediated responses in regulating vascular changes associated with chronic synovitis (Ralph *et al.*, 2007). Corticotropin-releasing hormone acting through both receptors induces a significant increase of reactive oxygen species (ROS) content, catalase activity and superoxide dismutase activity, accompanied by a simultaneous significant decrease of endothelial NO activity and NO levels as well as a significant increase in nitrotyrosine (peroxynitrite) levels (Gougoura *et al.*, 2010). These data indicate that CRH may act as a regulator of proinflammatory mechanisms inducing adaptation of endothelial cell function to local oxidative stress (Gougoura *et al.*, 2010).

Interestingly, CRH-deficient mice are resistant to experimental autoimmune encephalomyelitis (Karalis *et al.*, 1991). This effect of peripheral CRH is independent of its ability to increase corticosterone production, because adrenalectomized wild-type mice had similar disease course and severity as control mice. Thus, it seems that peripheral CRH exerts a proinflammatory effect in experimental autoimmune encephalomyelitis with a selective increase in Th1-type responses indicating a novel contribution of peripheral CRH to the regulation of Th1-mediated inflammation. These findings might have implications for the treatment of Th1-mediated diseases such as multiple sclerosis (MS), a demyelinating autoimmune disease characterized by inflammation of the central nervous system (Benou *et al.*, 2005). Substantial evidence indicates that stress can precipitate or worsen symptoms of inflammation in multiple sclerosis (MS). However, the exact mechanism of how stress affects MS is not well understood. Karagkouni *et al.*, proposed that neuropeptides secreted under stress, such as CRH and neurotensin, activate microglia and mast cells to release inflammatory molecules, leading to maturation and activation of T17 autoimmune cells, disruption of the blood–brain barrier and T cell entry into the central nervous system, thus promoting brain inflammation and contributing to MS pathology. Reduction of stress and inhibition of these processes by selected flavonoids could provide novel therapeutic approaches (Karagkouni *et al.*, 2013).

Peripheral CRH exerts proinflammatory effects, possibly through mast cell activation. Acute psychological stress induces CRH-dependent mast cell degranulation. In a similar way CRH causes mast cell degranulation in human skin, releasing great amounts of histamine, which appears to be the principal mediator of the vasodilatory effects of CRH in human skin (Wright Ian, 2003). In addition, CRH is synthesized and secreted by human mast cells acting in autocrine and paracrine fashion, especially in allergic

inflammatory disorders exacerbated by stress (Kempuraj *et al.*, 2004). Finally, CRH can induce secretion of VEGF selectively (Mann-Chandler *et al.*, 2005).

Stress participates and worsens not only asthma and atopic dermatitis but also acute coronary syndromes (ACSs) which are associated with coronary inflammation. Activation of coronary mast cells by stress through CRH and other neuropeptides, contributes to coronary inflammation and coronary artery disease. Therefore, inhibition of cardia mast cells may be a novel treatment approach (Alevizos *et al.*, 2014).

Periodontal disease involves inflammation of the gingival tissues, caused by microbial pathogens. Recent research suggests that emotional stress worsens periodontal disease. Papathanasiou *et al.*, proposed that stress-induced CRH secretion stimulates gingival mast cells and other neuropeptides and cytokines as well to secrete proinflammatory molecules that contribute to periodontal pathology. Stress reduction and/or mast cell inhibition may provide additional therapeutic approaches (Papathanasiou *et al.*, 2013).

Septic shock is associated with decreased ACTH synthesis in humans and in rats, which is not compensated by its two natural secretagogues, AVP and CRH. Polito *et al.*, suggested that one underlying mechanism might be increased expression of iNOS in hypothalamic parvocellular neurons (Polito *et al.*, 2011). In septic patients during cardiovascular deregulation, increased levels of POMC derivatives have been found. Majetec *et al.*, found that after CRH administration, heart rate, cardiac index and stroke index increased and the systemic vascular resistance index decreased. Moreover, a positive correlation between ACTH concentration and stroke index as well as an inverse correlation between α-melanocyte stimulating hormone (αMSH) concentration and systemic vascular resistance index was observed. Corticotropin releasing hormone and ACTH may have opposite effects on the blood pressure. Therefore, POMC derivatives seem to have influences on patients hemodynamics during sepsis (Matejec *et al.*, 2011). In patients with septic shock, the upregulation of mHLA-DR expression after CRH infusion is independent of POMC derivatives release. From the positive correlation between plasma concentration of αMSH and mHLA-DR expression they concluded that in this group of patients, the downregulation of mHLA-DR expression is accompanied by the loss of αMSH release by monocytes into the cardiovascular compartment (Matejec *et al.*, 2013).

It has been shown previously that CRH induces NF-κB DNA-binding activity in mouse and human leukocytes. Karalis *et al.*, demonstrated that in the human monocytic P-1 cells, CRH activates the PI3K/Akt and ERK1/2 pathways. CRH-R2 mediates these CRH effects as suggested by their abolishment following treatment with the specific CRH-R2 antagonist, astressin 2B. The CRH-mediated PI3K/Akt activation induces cell survival as suggested by the stimulation of the antiapoptotic factor Bcl-2. ERK1/2 activation results in upregulation of IL-8 expression, an effect inhibited by the CRH-induced activation of PI3K/Akt. These studies demonstrate novel effects of CRH in human monocytes mediated by the activation of PI3K/Akt and they reveal pathway-specific effects of the CRH/CRH-R2 system in chemokine activation and cell survival which is important for the development of new therapeutic agents for inflammatory diseases (Chandras *et al.*, 2009).

CRH in the I/I Phenomena of the Female Reproductive System

Physiological phenomena taking place in the female reproductive tract (such as decidualization and luteolysis) bear characteristics of an aseptic inflammation (Nepomnaschy *et al.*, 2007; Vrekoussis *et al.*, 2010). In addition, pregnancy has been explored from the immunological point of view since it can be considered a semiallograft situation.

CRH and CRH-R have been localized in the ovary in rat and in the ovary, endometrium, myometrium, and placenta in human (Kalantaridou *et al.*, 2003). Further tentative actions of CRH on the female reproductive system have been suggested. Corticotropin releasing hormone R1 receptors are detected in the ovarian stroma and the theca and in the cumulus oophorus of the graafian follicle (Mastorakos *et al.*, 1993b; Asakura *et al.*, 1997). Granulosa cells are devoid of the expression of CRH and CRH-R1 genes and peptides (Asakura *et al.*, 1997). CRH was shown to have an inhibitory effect on ovarian steroidogenesis, mediated through CRH and interleukin-1 receptors, and possibly linked to the processes of follicular atresia and luteolysis. Corticotropin releasing hormone exerts an inhibitory effect on estrogen and progesterone production in human granulosa-lutein cells isolated from the follicular fluid upon oocyte retrieval (Calogero *et al.*, 1996; Ghizzoni *et al.*, 1997; Erden *et al.*, 1998). CRH inhibited LH-stimulated DHEA and androstenedione production in isolated follicular theca cells (Muramatsu *et al.*, 2001). The addition of CRH has an inhibitory effect on in vitro fertilized oocytes, resulting from cultured preantral follicles at all stages of preimplantation embryo development (Dinopoulou *et al.*, 2013). In addition, the presence of CRH in the culture medium inhibits steroidogenesis by preantral mouse follicles cultured in vitro (Dinopoulou *et al.*, 2013). Corticotropin releasing hormone, UCN and CRHR gene expression is higher during the regression of the human corpus luteum than in the earlier stages of the luteal phase (Muramatsu *et al.*, 2001).

Epithelial cells of the human endometrium and differentiated stoma cells also express the CRH gene (Mastorakos *et al.*, 1996; Makrigiannakis *et al.*, 1995a, b). In addition, CRH-R1 is present in both epithelial and stroma cells of human endometrium and myometrium (Di Blasio *et al.*, 1997; Grammatopoulos and Hillhouse, 1998; Grammatopoulos *et al.*, 1999). Endometrial CRH participates in the regulation of intrauterine inflammatory processes such as stroma decidualization, blastocyst implantation and early maternal tolerance (Gravanis *et al.*, 2002). The progesterone-induced decidualization is modulated by locally produced inflammatory factors. Epithelial and stoma CRH affects decidualization of stoma cells by regulating local modulators, that is, prostanoids (PGE2) and cytokines (IL-1 and IL-6). The net effect of its actions is the fine-tuning of the decidualizing effect of progesterone (Zoumakis *et al.*, 2000). Endometriosis is considered as an aseptic inflammatory disease, characterized by the

presence of ectopic endometrium-like tissue. Corticotropin-releasing hormone-R1β and -R2α are expressed in endometriotic sites and they are more strongly expressed in eutopic endometrium of women with endometriosis compared to healthy women endometrium at the mRNA and protein level (Vergetaki *et al.*, 2013). Corticotropin-releasing hormone, UCN, CRH-R1 and -R2 mRNAs and proteins are also more highly expressed in ectopic rather than eutopic endometrium in women with endometriosis. These findings indicate that CRH and UCN might play an immunoregulatory role in endometriotic sites by affecting reproductive functions such as decidualization and implantation in women with endometriosis (Vergetaki *et al.*, 2013).

Locally produced embryonic and endometrial CRH plays a role in both the aseptic inflammatory process of implantation and the antirejection process that protects the fetus from the maternal immune system. Placental trophoblasts express both CRH-R1 and -R2 (Gao *et al.*, 2008). The syncytiocytotrophoblasts of the placenta and the fetal membranes express the CRH -R1α, -R1c, -R1d and -R2β subtypes (Karteris *et al.*, 1998). By contrast to the hypothalamic CRH system, the production of placental CRH is positively regulated by both fetal and maternal GCs (Karalis *et al.*, 1996). Placental CRH production is also modified by estrogen, progesterone and NO which are inhibitory and by a range of neuropeptides which are stimulatory (Smith, 2007). CRH increases estrogen biosynthesis in cultured human placental cells (You *et al.*, 2006).

Early in pregnancy, the implantation sites in rat endometrium contain 3.5-fold higher concentration regions. As pregnancy progresses, the myometrium starts to express CRH-R2α. In addition, at term, the myometrium expresses the CRH -R1c and -R1d subtypes, indicating a possible functional role for these receptor subtypes at the end of pregnancy (Grammatopoulos *et al.*, 1999). CRH-R1 maintains myometrial quiescence whereas CRH-R2 promotes smooth muscle contractility. It has been suggested that CRH of fetal and maternal origin regulates FasL production, thus affecting the invasion process through a local autocrine/paracrine regulatory loop of cytotrophoblast cells and regulating their own apoptosis. CRH decreases FasL expression in embryonic trophoblast and maternal deciduas and promotes apoptosis of activated T lymphocytes. Abnormalities of maternal immune tolerance to the fetal semiallograft, have been implicated in pathological conditions of pregnancy, such as recurrent early miscarriage, preeclampsia, and eclampsia. These conditions are characterized by inflammation in the fetal–maternal interface and/or systemic manifestations. More specifically, it has been proposed that inadequate, CRH-mediated, self-induction of FasL in extravillous trophoblasts might be involved in the pathophysiology of infertility and recurrent fetal resorption or miscarriage. Abortive deciduas contain leukocytes that are positive for FasL and EVTs, which show increased rates of apoptosis and increased expression of Fas, in contrast to normal pregnancies (Minas *et al.*, 2007). Although very little is known regarding regulation of expression of CRH-Rs in intrauterine tissues, it is possible that chronic exposure to elevated levels of placental CRH in preeclampsia or IUGR might downregulate its own receptors (Karteris *et al.*, 2005). In fetuses with fetal growth restriction umbilical cord CRH is also elevated (Madhappan *et al.*, 2003). Interestingly, the observed downregulation of CRH-R in the preeclamptic placenta is not an isolated finding: several hormonal signals that regulate NOS expression and activity have attenuated effects due to downregulation of their respective receptors, including CRH-R1 and -R2. This downregulation results in compromised responses and reduced relaxation in feto-placental vessels from preeclamptic placentas (Vatish *et al.*, 2006).

IL-6 and CRH are both secreted in a pulsatile function during the active phase of term human labor. The time-integrated concentrations of the two hormones are positively correlated, with IL-6 leading CRH secretion (Papatheodorou *et al.*, 2013). It appears, thus, that proinflammatory mediators may be direct and/or indirect promoters of placental CRH release. Furthermore, the secretion of IL-6, which is a myokine, seems to be associated positively with uterine contractility. Additional studies are needed to elucidate the combined effect of inflammation, placental CRH release and/or CRH-Rs in parturition (Papatheodorou *et al.*, 2013). Corticotropin-releasing hormone induces the production of chemokines and cytokines in myometrium at term and subsequently participates in the cascade of inflammation in uterus. The CRH-induced inflammation can lead to activation of the uterus (You *et al.*, 2014).

Pregnancy is known to be a state of transient hypercortisolemia (Mastorakos and Ilias, 2003). Increased levels of unbound placental CRH are responsible for the hypercortisolism of the second half of pregnancy. This hypercortisolism is followed by a transient suppression of hypothalamic CRH secretion in the postpartum period. This has been associated to the depressive states frequently observed in the postpartum period (Vitoratos *et al.*, 2006).

Perspectives

Pyrrolopyrimidine compounds have been developed as CRH receptor antagonists. Antalarmin has been used in investigations of the physiologic central and peripheral roles of CRH in the I/I response and reproductive function. The binding kinetics of antalarmin were determined in competitive displacement binding experiments with homogenates prepared for tissues differentially expressing CRH-R subtypes. In experiments in rats, the in vivo administration of antalarmin significantly antagonized both central and peripheral actions of CRH. In these experiments, the prior administration of antalarmin or neutralizing anti-CRH antibody blocked pituitary CRH receptors and the exogenous or endogenous CRH-induced ACTH release. Confirming the peripheral pro-inflammatory actions of CRH, antalarmin also suppressed the subcutaneous inflammation induced by carrageenan (Webster *et al.*, 1996). Leukocyte concentrations of subcutaneous exudates were reduced by antalarmin compared to vehicle controls. This effect was dose-dependent and was comparable to that of CRH antibody, where suppression of the leukocyte concentration was observed.

In some experiments, antalarmin was administered in rats from day 1 of pregnancy and for the following 10 days. A 50% reduction was observed in the number of implantation sites in animals that received the higher dose of antalarmin. Additionally,

female rats treated with antalarmin showed diminished endometrial FasL expression. Hence, implantation in rats was prevented by antalarmin, which blocked CRH-R1. Antalarmin reduced the inflammatory-like reaction of the endometrium to the invading blastocyst. Consequently, antalarmin and analogous compounds might represent a new class of nonsteroidal inhibitors of pregnancy at its very early stages.

In sheep, hypothalamic CRH stimulates the fetal production of ACTH, which in turn leads to a surge of fetal cortisol secretion that precipitates parturition. A 10-day intravenous infusion of antalarmin in sheep fetuses significantly prolonged gestation. Thus, CRH receptor antagonism in the fetus can also delay parturition.

Astressin B, a nonspecific CRH receptor antagonist is shown to have a different antagonist profile than antalarmin. Astressin B is unlikely to have access to the CNS following systemic administration in contrast to antalarmin which exerts its activity on the CNS. The differences between antalarmin and astressin B may be due either to non-CRH receptor-mediated effects of antalarmin or to a complex interaction of antalarmin effects at both central and peripheral CRH-Rs (Broadbear *et al.*, 2004). Another study demonstrated that antalarmin and astressin exert different effects on prostaglandin biosynthetic enzymes and thereby modulate the output of prostaglandins from placenta which would be important for controlling pregnancy and parturition (Gao *et al.*, 2008). Xiao *et al.*, showed that astressin B treatment accelerates the return to normal cyclicity, by restoring the normal neuroendocrine regulation of gonadotropin secretion. This effect of CRH receptor antagonist happens although astressin B does not blunt the peripheral HPA axis response to the stressor. The ability to restore normal luteal phase places astressin B or any other CRH antagonist in a very important position in the research of a potential therapeutic agent in stress-related endocrine dysfunction, including the functional hypothalamic chronic anovulation syndrome or the persistent inadequate luteal phase syndrome, and therefore in the treatment of infertility (Xiao *et al.*, 2007).

Given the ability of peripheral CRH to degranulate mast cells, CRH-R1 antagonists could be considered for the treatment of allergic conditions such as asthma, eczema, urticaria or even stress-induced brain inflammatory disorders that increase blood–brain-barrier permeability (Theoharides *et al.*, 1998; Esposito *et al.*, 2002). In the GI tract, these compounds open new therapeutic options in the treatment of lower GI inflammatory diseases associated to CRH, such as the chronic inflammatory bowel syndromes, irritable bowel disease and ulcerative colitis (Kawahito *et al.*, 1995; Gravanis and Margioris, 2005). In human endometrium, CRH-R1 antagonists may be used as antiimplantation agents interfering with the inflammatory phenomena taking place during implantation (Gravanis and Margioris, 2005). Administration of antalarmin to early pregnant rats (day 1 of pregnancy) results in a 70% reduction in implantation sites (Makrigiannakis *et al.*, 2001; Chan, 1998). These examples illustrate the potential therapeutic significance of the CRH in regulating inflammatory phenomena without affecting the rest of the immune system.

The therapeutic potential of pyrrolopyrimidine compounds in some forms of inflammation directly mediated by CRH-R1 is evident and hopefully will enhance the understanding of the multitude of roles that CRH plays in I/I reactions. Although the systemic toxicity of this class of compounds has not yet been fully determined, preliminary studies in rats and nonhuman primates have indicated that they are relatively safe.

See also: Glucocorticoids and Immunity

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Adrenal Insufficiency: Etiology and Diagnosis☆

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Nomenclature

3β-HSD	3β-Hydroxysteroid dehydrogenase
ABCD1	ATP-binding cassette, subfamily D, member 1
ACTH	Adrenocorticotropic hormone
APS	Autoimmune polyendocrinopathy syndrome
CRH	Corticotropin-releasing hormone
CYP	Cytochrome P450
NNT	Nicotinamide nucleotide transhydrogenase
StAR	Steroidogenic acute regulatory protein
TRXR2	Thioredoxin reductase 2
SGPL1	Sphingosine-1-phosphate (S1P) lyase 1

Glossary

Cushing syndrome A condition caused by increased adrenocortical secretion of cortisol resulting from adrenocortical hyperplasia or tumor and characterized by central obesity, acne, amenorrhea, hypertension, abdominal pain, and weakness.

HIV infection Human immunodeficiency virus, the causative agent of AIDS.

Thrombosis The formation, development, or presence of a thrombus, or blood clot, in a blood vessel or the heart.

Introduction

Adrenal insufficiency is a life-threatening disorder characterized by impaired production or action of glucocorticoids, mineralocorticoids and/or adrenal androgens (Charmandari *et al.*, 2014). Adrenal insufficiency may result from diseases affecting the adrenal cortex (primary), the pituitary gland (secondary), or the hypothalamus (tertiary) (Arlt and Allolio, 2003). The clinical manifestations of this condition were first described by Thomas Addison in 1855, and include fatigue, weakness, abdominal pain, anorexia, weight loss, salt craving and orthostatic hypotension. In case of primary adrenal insufficiency, patients may also present with the characteristic hyperpigmentation of the skin (Addison, 1855; Løvås and Husebye, 2005). Although rare, the prevalence of primary adrenal insufficiency has been doubled since 1960s (Mason *et al.*, 1968). The most frequent cause of primary adrenal insufficiency in adults is autoimmune adrenalitis, whereas the most common form in children is congenital adrenal hyperplasia (Charmandari *et al.*, 2014). Interestingly, the ever-increasing application of next generation sequencing technologies has enabled us to have a deeper understanding of genetic defects causing adrenal insufficiency (Flück, 2017). This chapter provides a brief overview of the etiology and diagnosis of adrenal insufficiency.

Causes of Adrenal Insufficiency

Primary Adrenal Insufficiency (Table 1)

Autoimmune Adrenalitis

This condition is the most common cause of primary adrenal insufficiency in adults, and results from an autoimmune process that destroys the adrenal cortex. Both humoral and cell-mediated immune mechanisms directed at the adrenal cortex are involved. Antibodies against several steroidogenic enzymes as well as all three zones of the adrenal cortex are detected in 60%–75% of patients with autoimmune primary adrenal insufficiency but only rarely in patients with other causes of adrenal insufficiency or normal subjects (Husebye and Løvås, 2009a, b). Indeed, antibodies that react with 21-hydroxylase are found in approximately 90% of patients with primary adrenal insufficiency (Erichsen *et al.*, 2009). In addition, antibodies against 17α-hydroxylase and cholesterol side-chain cleavage enzyme have been detected in adults with autoimmune adrenalitis (Mitchell and Pearce, 2012). Approximately 50% of patients with autoimmune adrenal insufficiency have one or more other autoimmune endocrine disorders, as occurs in autoimmune polyendocrinopathy syndrome (APS) type 1 or APECED (autoimmune polyendocrinopathy, candidosis, ectodermal dystrophy) syndrome, which is characterized by adrenocortical insufficiency, hypoparathyroidism, chronic

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Table 1 Causes of primary adrenal insufficiency

Disease	*Pathogenetic mechanism*
Autoimmune adrenalitis	
Isolated	Associations with HLA-DR3-DQ2, HLADR4-DQ8, MICA, CTLA-4, PTPN22, CIITA, CLEC16A, Vitamin D receptor
APS type 1 (APECED)	AIRE gene mutations
APS type 2	Associations with HLA-DR3, HLA-DR4, CTLA-4
APS type 4	Associations with HLA-DR3, CTLA-4
Infectious adrenalitis	
Tuberculous adrenalitis	Tuberculosis
AIDS	HIV-1, cytomegalovirus
Fungal adrenalitis	Histoplasmosis, cryptococcosis, coccidiodomycosis
Syphilis	Treponema pallidum
African trypanosomiasis	Trypanosoma brucei
Bilateral adrenal hemorrhage	Meningococcal sepsis (Waterhouse–Friderichsen syndrome), primary antiphospholipid syndrome
Bilateral adrenal metastases	Primarily lung, stomach, breast and colon cancer
Bilateral adrenal infiltration	Primary adrenal lymphoma, amyloidosis, haemochromatosis
Bilateral adrenalectomy	Unresolved Cushing syndrome, bilateral adrenal masses, bilateral pheochromocytoma
Drug-induced adrenal insufficiency	
Anticoagulants (heparin, warfarin), tyrosine kinase inhibitors (sunitinib)	Hemorrhage
Aminoglutethimide	Inhibition of P450 aromatase (CYP19A1)
Trilostane	Inhibition of 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2)
Ketoconazole, fluconazole, etomidate	Inhibition of mitochondrial cytochrome P450-dependent enzymes (e.g., CYP11A1, CYP11B1)
Phenobarbital	Induction of P450-cytochrome enzymes (CYP2B1, CYP2B2), which enhance cortisol metabolism
Phenytoin, rifampin, troglitazone	Induction of P450-cytochrome enzymes (primarily CYP3A4), which enhance cortisol metabolism
Genetic disorders	
Adrenoleukodystrophy or adrenomyeloneuropathy	*ABCD1* and *ABCD2* gene mutations
Congenital adrenal hyperplasia	
21-Hydroxylase deficiency	*CYP21A2* gene mutations
11β-Hydroxylase deficiency	*CYP11B1* gene mutations
3β-Hydroxysteroid dehydrogenase type 2 deficiency	*HSD3B2* gene mutations
17α-Hydroxylase deficiency	*CYP17A1* gene mutations
P450 Oxidoreductase deficiency	*POR* gene mutations
P450 Side-chain cleavage deficiency	*CYP11A1* gene mutations
Congenital lipoid adrenal hyperplasia	*StAR* gene mutations
Smith–Lemli–Opitz syndrome	*DHCR7* gene mutations
Adrenal hypoplasia congenita	
X-linked	*NR0B1* gene mutations
Xp21 contiguous gene syndrome	Deletion of the Duchenne muscular dystrophy, glycerol kinase and NR0B1 genes
SF-1 linked	*NR5A1* gene mutations
IMAGe syndrome	*CDKN1C* gene mutations
Kearns–Sayre syndrome	Mitochondrial DNA deletions
Wolman disease	*LIPA* gene mutations
Sitosterolemia (also known as phytosterolemia)	*ABCG5* and *ABCG8* gene mutations
Familial glucocorticoid deficiency (*FGD, or ACTH insensitivity syndromes*)	
Type 1	*MC2R* gene mutations
Type 2	*MRAP* gene mutations
Variant of FGD	*MCM4* gene mutations
FGC—deficiency of mitochondrial ROS detoxification	*NNT*, *TXNRD2*, *GPX1*, *PRDX3* gene mutations
Primary generalized glucocorticoid resistance or Chrousos syndrome	*NR3C1* gene mutations
Triple A syndrome (Allgrove syndrome)	*AAAS* gene mutations
Sphingosine-1-phosphate lyase 1 deficiency	*SPGL1* gene mutations
Infantil Refsum disease	*PHYH*, *PEX7* gene mutations
Zellweger syndrome	*PEX1* and other *PEX* gene mutations

Modified from Charmandari, E., Nicolaides, N. C. and Chrousos, G. P. (2014). Adrenal insufficiency. Lancet 383, 2152–2167; Nicolaides, N.C., Chrousos, G.P. and Charmandari, E. (2017). Adrenal Insufficiency. In: De Groot L. J., Chrousos G., Dungan K., Feingold, K. R., Grossman A., Hershman, J. M., Koch, C., Korbonits, M., McLachlan, R., New, M., Purnell, J., Rebar, R., Singer, F., Vinik, A., eds. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc., 2000 Flück, C.E. (2017). Mechanisms in Endocrinology: Update on pathogenesis of primary adrenal insufficiency: Beyond steroid enzyme deficiency and autoimmune adrenal destruction. European Journal of Endocrinology 177, R99–R111.

mucocutaneous candidosis, hypoplasia of the dental enamel, and nail dystrophy (Akirav *et al.*, 2011; Michels and Gottlieb, 2010). In APS type 2, patients may present with autoimmune thyroid disease, adrenal insufficiency, with or without diabetes mellitus type 1 (Betterle *et al.*, 2002, 2004; Michels and Gottlieb, 2010). APS type 4 is characterized by autoimmune adrenal insufficiency in association with one or more autoimmune diseases, such as atrophic gastritis, coeliac disease, hypogonadism, vitiligo, pernicious anemia and alopecia, but without thyroid diseases, diabetes mellitus type 1 or other characteristics of APS type 1 or 2 (Betterle *et al.*, 2002, 2004; Michels and Gottlieb, 2010). On the other hand, patients with the more common autoimmune endocrine disorders, such as type 1 diabetes mellitus, chronic autoimmune thyroiditis, or Graves' disease, rarely develop adrenal insufficiency.

Infectious Adrenalitis

Many infectious agents may affect the adrenal gland and result in adrenal insufficiency, including tuberculosis, syphilis, African trypanosomiasis, disseminated fungal infections, HIV infection, and HIV-associated infections, including adrenalitis due to cytomegalovirus and mycobacterium avium complex (Bhatia *et al.*, 1998; Walker *et al.*, 1989; Norbiato *et al.*, 1994).

Hemorrhagic infarction

Bilateral adrenal infarction caused by hemorrhage or adrenal vein thrombosis may lead to adrenal insufficiency (Rao *et al.*, 1989; Rao, 1995). Adrenal hemorrhage has been mostly associated with coagulopathies and the heparin-induced thrombocytopenia syndrome, the primary antiphospholipid syndrome, as well as with meningococcemia (Waterhouse–Friderichsen syndrome) and Pseudomonas aeruginosa infection (Caron *et al.*, 1998).

Drugs

Drugs may cause adrenal insufficiency by inhibiting cortisol biosynthesis, particularly in individuals with limited pituitary and/or adrenal reserve (aminoglutethimide (antiepileptic), etomidate (anesthetic-sedative) (Wagner *et al.*, 1984; Jabre *et al.*, 2009), ketoconazole, fluconazole (antimycotic) (Sonino, 1987) and metyrapone (Schoneshofer and Claus, 1985)), or by accelerating the metabolism of cortisol and most synthetic glucocorticoids following induction of hepatic mixed function oxygenase enzymes (phenytoin, barbiturates, and rifampicin) (Elias and Gwinup, 1980). Moreover, many novel tyrosine kinase-targeting drugs, such as sunitinib, have been shown to cause adrenal dysfunction in animal models (Rock *et al.*, 2007).

Genetic disorders

Several genetic disorders have been identified to cause primary adrenal insufficiency (Reviewed in Flück, 2017). Adrenoleukodystrophy is an X-linked disorder caused by mutations in the ATP-binding cassette, subfamily D, member 1 (*ABCD1*) gene leading to impaired transport of very long-chain fatty acids into peroxisomes and disturbed β-oxidation (Kemp *et al.*, 2012). In patients with adrenoleukodystrophy, these fatty acids are accumulated in many organs, including the adrenal glands, causing dysfunction. In addition to adrenoleukodystrophy, congenital adrenal hyperplasia (CAH) also causes primary adrenal insufficiency due to mutations in genes encoding crucial enzymes for biosynthesis of cortisol in the adrenal cortex. The most frequent form of CAH is classic 21-hydroxylase deficiency, which is caused by *CYP21A2* gene mutations, and characterized by impaired production of glucocorticoids and mineralocorticoids, hyperandrogenism, and dysfunction of the adrenal medulla (Miller and Auchus, 2011; Merke *et al.*, 2000; Charmandari *et al.*, 2002). Other rare forms of CAH have been attributed to mutations in genes encoding 11β-hydroxylase, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 17,20-lyase or P450 oxidoreductase. On the other hand, adrenal hypoplasia congenita has also been demonstrated to cause primary adrenal insufficiency. There are three forms of this condition: (i) the X-linked form, which is caused by *NR0B1* gene mutations; (ii) the Xp21 contiguous gene syndrome, which is attributed to deletion of genes for Duchenne muscular dystrophy, glycerol kinase and *NR0B1*; and (iii) the SF-1 linked form due to *NR5A1* gene mutations (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017). Moreover, many other rare pathologic conditions listed in **Table 1** have been associated with primary adrenal insufficiency, including the Triple A syndrome (Allgrove syndrome), the IMAGe syndrome, the Kearns–Sayre syndrome, the Wolman's disease, and sitosterolemia (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017; Flück, 2017). Furthermore, familial glucocorticoid deficiency or corticotropin insensitivity syndromes have been listed as potential causes of primary adrenal insufficiency. Many of these cases have been associated with mutations in the *MC2R* gene (type 1), *MRAP* gene (type 2), and *MCM4* gene (variant of familial glucocorticoid deficiency) (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017; Flück, 2017). In patients with familial glucocorticoid deficiency syndrome, who do not have any mutations in the above-mentioned genes, the application of next generation sequencing methods revealed novel mutations in the nonclassic steroidogenic acute regulatory protein (*StAR*), *CYP11A1*, nicotinamide nucleotide transhydrogenase (*NNT*), and thioredoxin reductase 2 (*TRXR2*) genes (Reviewed in Flück, 2017). Interestingly, primary adrenal insufficiency was recently associated with steroid resistant nephrotic syndrome, and optionally ichthyosis, cryptorchidism, primary hypothyroidism, neurological anomalies and immunodeficiency. Patients with this syndrome harbored mutations in the gene encoding sphingosine-1-phosphate (S1P) lyase 1 (*SGPL1*) (Prasad *et al.*, 2017; Lovric *et al.*, 2017). Finally, a rare cause of primary adrenal insufficiency is primary generalized glucocorticoid resistance or Chrousos syndrome, which affects almost all tissues, and is characterized by partial insensitivity to glucocorticoids due to mutations in the gene for human glucocorticoid receptor (*NR3C1*) (Nicolaides and Charmandari, 2017).

Secondary and Tertiary Adrenal Insufficiency (Tables 2 and 3)

Secondary adrenal insufficiency may be caused by any disease process that affects the anterior pituitary and interferes with ACTH secretion. The ACTH deficiency may be isolated or occur in association with other pituitary hormone deficits (**Table 2**) (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017). On the other hand, tertiary adrenal insufficiency can be caused by any process that involves the hypothalamus and interferes with CRH secretion (**Table 3**) (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017). The most common causes of tertiary adrenal insufficiency are abrupt cessation of high-dose glucocorticoid therapy and treatment of Cushing syndrome; therefore slow withdrawal of glucocorticoids over 9-–12 months is highly recommended for complete recovery of the glucocorticoid-suppressed hypothalamic-pituitary-adrenal (HPA) axis.

Table 2 Causes of secondary adrenal insufficiency

Disease	*Pathogenetic mechanism*
Space occupying lesions or trauma	
Pituitary tumors (adenomas, cysts, craniopharyngiomas, ependymomas, meningiomas, rarely carcinomas) or trauma (pituitary stalk lesions)	Decreased ACTH secretion
Pituitary surgery or irradiation for pituitary tumors, tumors outside the HPA axis or leukemia	Decreased ACTH secretion
Infections or Infiltrative processes (lymphocytic hypophysitis, hemochromatosis, tuberculosis, meningitis, sarcoidosis, actinomycosis, histiocytosis X, Wegener's granulomatosis)	Decreased ACTH secretion
Pituitary apoplexy	Decreased ACTH secretion
Sheehan syndrome (peripartum pituitary apoplexy and necrosis)	Decreased ACTH secretion
Genetic disorders	
Transcription factors involved in pituitary development	
HESX homeobox 1	HESX1 gene mutations
Orthodentical homeobox 2	OTX2 gene mutations
LIM homeobox 4	LHX4 gene mutations
PROP paired-like homeobox 1	PROP1 gene mutations
SRY (sex-determining region Y) – box 3	SOX3 gene mutations
T-box 19	TBX19 gene mutations
Congentital Proopiomelanocortin (POMC) deficiency	POMC gene mutations
Prader–Willi Syndrome (PWS)	Deletion or silencing of genes in the imprinting center for PWS

Modified from Charmandari, E., Nicolaides, N. C. and Chrousos, G. P. (2014). Adrenal insufficiency. Lancet 383, 2152–2167; Nicolaides, N. C., Chrousos, G. P. and Charmandari, E. (2017). Adrenal insufficiency. In: De Groot, L. J., Chrousos, G., Dungan, K., Feingold, K. R., Grossman, A., Hershman, J. M., Koch, C., Korbonits, M., McLachlan, R., New, M., Purnell, J., Rebar, R., Singer, F., Vinik, A., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000.

Table 3 Causes of Tertiary Adrenal Insufficiency

Disease	*Pathogenetic mechanism*
Space occupying lesions or trauma	
Hypothalamic tumors (craniopharyngiomas or metastasis from lung, breast cancer)	Decreased CRH secretion
Hypothalamic surgery or irradiation for central nervous system or nasopharyngeal tumors	Decreased CRH secretion
Infections or Infiltrative processes (lymphocytic hypophysitis, hemochromatosis, tuberculosis, meningitis, sarcoidosis, actinomycosis, histiocytosis X, Wegener's granulomatosis)	Decreased CRH secretion
Trauma, injury (fracture of skull base)	Decreased CRH secretion
Drug-induced adrenal insufficiency	
Glucocorticoid therapy (systemic or topical) or endogenous glucocorticoid hypersecretion (Cushing syndrome)	Decreased CRH and ACTH secretion
Mifepristone	Tissue resistance to glucocorticoids through impairment of glucocorticoid signal transduction
Antipsychotics (chlorpromazine), antidepressants (imipramine)	Inhibition of glucocorticoid-induced gene transcription
Opioids	Decreased CRH and ACTH secretion
Protease inhibitors	Increased sensitivity to GC feedback

Modified from Charmandari, E., Nicolaides, N. C. and Chrousos, G. P. (2014). Adrenal insufficiency. Lancet 383, 2152–2167; Nicolaides, N. C., Chrousos, G. P. and Charmandari, E. (2017). Adrenal insufficiency. In: De Groot, L. J., Chrousos, G., Dungan, K., Feingold, K. R., Grossman, A., Hershman, J. M., Koch, C., Korbonits, M., McLachlan, R., New, M., Purnell, J., Rebar, R., Singer, F., Vinik, A., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000.

Clinical Manifestations of Adrenal Insufficiency

In most of cases, the onset of adrenal insufficiency is insidious and the diagnosis may be difficult until an illness or other stressors precipitate an adrenal crisis.

Adrenal Crisis

Adrenal crisis or acute adrenal insufficiency may be precipitated by a severe infection, bilateral adrenal infarction or hemorrhage or any stressors. Patients with adrenal crisis may present with shock or other nonspecific symptoms, including abdominal pain, nausea, vomiting, fatigue, weakness, confusion or coma. The main pathogenetic mechanism leading to hypotension in adrenal crisis is mineralocorticoid deficiency; however, glucocorticoid deficiency may also cause hypotension by decreasing the synthesis of renin substrate, reducing the responsiveness of blood vessels to vasoconstrictive hormones, and/or increasing the production and action of vasodilatory hormones (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017).

Chronic Primary Adrenal Insufficiency

The clinical manifestations of chronic primary adrenal insufficiency are fatigue, weakness, general malaise, weight loss, anorexia, abdominal pain, nausea, vomiting, diarrhea, which may alternate with constipation, hypotension, hyperpigmentation, decreased axillary and pubic hair, and loss of libido and amenorrhea in women. Electrolyte abnormalities include hyponatremia, hyperkalemia and metabolic acidosis (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017).

Secondary or Tertiary Adrenal Insufficiency

Patients with secondary or tertiary adrenal insufficiency may present with clinical manifestations similar to those of chronic primary adrenal insufficiency; however, hyperpigmentation of the skin is not present, because plasma ACTH concentrations are not increased. In addition, dehydration and hyperkalemia do not occur in cases of secondary or tertiary adrenal insufficiency, because the synthesis of mineralocorticoids is preserved; therefore hypotension is less prominent. On the other hand, hyponatremia in these patients may be caused by the "inappropriate" increase in vasopressin secretion. Hypoglycemia mostly occurs in secondary adrenal insufficiency because of the concomitant growth hormone insufficiency and in isolated ACTH deficiency. Clinical manifestations of other anterior pituitary hormone deficiency, headache or visual field defects may also occur in cases of pituitary or hypothalamic tumors (Charmandari *et al.*, 2014; Nicolaides *et al.*, 2017).

Diagnosis

The clinical diagnosis of adrenal insufficiency can be confirmed by demonstrating inappropriately low cortisol secretion, determining whether the cortisol deficiency is secondary or primary and, hence, dependent or independent of ACTH deficiency, and detecting the cause of the disorder (Arlt and Allolio, 2003; Neary and Nieman, 2010).

Cortisol Concentrations

The diagnosis of adrenal insufficiency depends on the demonstration of inappropriately low cortisol secretion. Serum cortisol concentrations are normally highest in the early morning hours (06:00 h–08:00 h) and range between 10 and 20 μg/dL (275–555 nmol/L). Serum cortisol concentrations of less than 3 μg/dL (80 nmoL/L) at 08:00 h are strongly suggestive of adrenal insufficiency, whereas values less than 10 μg/dL (275 nmol/L) make the diagnosis likely (Hägg *et al.*, 1987). Basal urinary cortisol and 17-hydroxycorticosteroid excretion is low in patients with severe adrenal insufficiency but may be low-normal in patients with partial adrenal insufficiency. Generally, baseline urinary measurements are not recommended for the diagnosis of adrenal insufficiency.

ACTH, Renin and Aldosterone Concentrations

Inappropriately low serum cortisol concentrations in association with increased plasma ACTH concentrations determined simultaneously are suggestive of primary adrenal insufficiency. The normal concentrations of plasma ACTH concentrations range between 20 and 52 pg/mL (4.5–12 pmol/L). Patients with primary adrenal insufficiency have increased plasma ACTH concentrations, increased concentration or activity of plasma renin, reduced aldosterone concentrations, hyponatremia and hyperkalemia (Oelkers *et al.*, 1992). On the other hand, inappropriately low baseline morning cortisol and ACTH concentrations indicate secondary or tertiary disease. In these cases, patients have normal concentrations of renin and aldosterone (Oelkers *et al.*, 1992). Given that plasma ACTH measurements depend on proper preparation of the sample and may not be readily available, confirmation of the diagnosis requires stimulation of the adrenal glands with exogenous ACTH.

Standard Dose ACTH Stimulation Test

A standard-dose ACTH stimulation test should be performed in all patients suspected to have adrenal insufficiency (Gleeson *et al.*, 2003; Agha *et al.*, 2006; Arlt, 2009; Chrousos *et al.*, 2009). It involves the intravenous administration of 250 mcg synthetic ACTH (1–24) (cosyntropin), which has the full biologic potency of native ACTH(1–39), and subsequent measurement of serum cortisol concentrations at 0, 30, and 60 min post stimulation. The test is normal if serum cortisol concentrations at 30 min are higher than 18–20 mcg/dL (500–550 nmol/L), making the diagnosis of primary adrenal insufficiency and most of the cases of secondary adrenal insufficiency unlikely. It is worth mentioning that with LCMS or more recent assays for cortisol, the normal values tend to be less than with previous immunoassay methods. This test should not be used in cases of recent onset of secondary adrenal insufficiency, because the adrenal glands will still have the ability to normally respond to ACTH stimulation, since they have not yet atrophied (Arlt, 2009; Chrousos *et al.*, 2009). In such cases, a low-dose ACTH stimulation test or an insulin-induced hypoglycemia test may be used to increase the sensitivity.

Low-Dose ACTH Stimulation Test

This test theoretically provides a more sensitive index of adrenocortical responsiveness because it results in physiologic plasma ACTH concentrations. It is performed by measuring serum cortisol concentrations immediately before and $+10$ min, $+15$ min, $+20$ min, $+25$ min, $+30$ min, $+35$ min, $+40$ min and $+45$ min after intravenous injection of cosyntropin in a dose of 1.0 μg (160 mIU) per 1.73 m^2 (Abdu *et al.*, 1999). In normal subjects, this dose leads to a peak plasma ACTH concentration approximately twice that of insulin-induced hypoglycemia (Nye *et al.*, 1999). A value of 18 μg/dL (500 nmoL/L) or more at any time during the test is indicative of normal adrenal function. The advantage of this test is that it can detect partial adrenal insufficiency that may be missed by the standard high-dose test (Rasmuson *et al.*, 1996; Thaler and Blevins, 1998; Kazlauskaite *et al.*, 2008). The low-dose test is also preferred for patients with secondary or tertiary adrenal insufficiency (Mushtaq *et al.*, 2008; Stewart and Clark, 2009).

Prolonged ACTH Stimulation Tests

Prolonged ACTH stimulation tests are rarely performed because the history and physical examination, the CRH test, and/or determination of cortisol and ACTH concentrations in association with the low-dose ACTH test may provide all necessary information. Prolonged stimulation with exogenous ACTH is used to differentiate between primary and secondary or tertiary adrenal insufficiency. In secondary or tertiary adrenal insufficiency, the adrenal glands display cortisol secretory capacity following prolonged stimulation with ACTH, whereas in primary adrenal insufficiency, the adrenal glands are partially or completely destroyed and do not respond to ACTH. During the test, 250 μg of ACTH is administered intravenously as an infusion over 8 h (8-h test) or over 24 h on two (or three) consecutive days (two-day test). Before and after the infusion, serum cortisol, 24-h urinary cortisol and 17-hydroxycorticoid (17-OHCS) concentrations are simultaneously measured (Rose *et al.*, 1970).

Insulin-Induced Hypoglycemia Test

The insulin tolerance test is another choice to confirm the diagnosis of secondary adrenal insufficiency, especially of recent origin. This test investigates the integrity of the HPA axis. For this purpose, insulin is administered at a dose of 0.1–0.15 U/kg to induce hypoglycemia. Serum cortisol concentrations are measured every 30 min for at least 2 h (Chrousos *et al.*, 2009; Grossman, 2010). Importantly, this test should not be performed in patients with cardiovascular disease or a history of seizures.

CRH Stimulation Test

This test is used to differentiate between secondary and tertiary adrenal insufficiency. In both conditions, cortisol concentrations are low at baseline and remain low after CRH. In this test, 1 mcg/kg CRH (up to a maximum of 100 mcg) is administered intravenously. Subsequently, serum cortisol and plasma ACTH concentrations are determined at 0, 15, 30, 45, 60, 90 and 120 min. In patients with secondary adrenal insufficiency, there is little or no ACTH response, whereas in patients with tertiary disease there is an exaggerated and prolonged response of ACTH to CRH stimulation, which is not followed by an appropriate cortisol response (Schulte *et al.*, 1984; Gold *et al.*, 1987).

Autoantibody Screen

More than 90% of patients with autoimmune adrenalitis of recent onset have autoantibodies to the adrenal cortex or against 21-hydroxylase. Moreover, autoantibodies that react against other enzymes involved in the steroidogenic pathway and antibodies to steroid-producing endocrine cells can be detected in some patients (Husebye and Løvås, 2009a, b; Betterle and Morlin, 2011).

Very Long Chain Fatty Acids

When adrenoleukodystrophy is suspected in cases of male patients with primary adrenal insufficiency without any autoantibodies, plasma concentrations of very long chain fatty acids should be determined (Kemp *et al.*, 2012).

Imaging

A computed tomography (CT) scan of the adrenal glands is highly recommended in patients with no associated autoimmune disease and negative autoantibodies. In patients with tuberculous adrenalitis, CT shows initially hyperplasia of the adrenal glands and spotty calcifications as the disease progresses in the late stages. CT also enables the detection of bilateral adrenal lymphoma, adrenal metastases or adrenal infiltration (amyloidosis, sarcoidosis, and hemochromatosis). If secondary or tertiary adrenal insufficiency is suspected, a magnetic resonance imaging (MRI) scan of the pituitary and hypothalamus should be performed to detect any potential disease, including pituitary adenomas, meningiomas, craniopharyngiomas, metastases and infiltration by Langerhans cell histiocytosis, sarcoidosis or other granulomatous diseases (Boland, 2011; Ouyang *et al.*, 2011). It is worth noting that imaging is not required if autoantibodies against the adrenal cortex have been detected.

See also: Steroid Replacement in Adrenal Insufficiency

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Primary Adrenal Hypoplasia and ACTH Resistance Syndromes☆

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Glossary

Hormone insensitivity Inability of the body to respond to the physiological action of a hormone.
Hypothalamic–pituitary–adrenal (HPA) axis The hormonal axis that regulates the production of adrenal steroid hormones, including glucocorticoids, mineralocorticoids and androgens.
Hypoplasia Lack of normal development of an organ or a tissue (atrophy).
Orphan receptor A receptor for which no endogenous ligand has been identified as yet.

Introduction

Steroid hormones, including glucocorticoids, mineralocorticoids, and androgens, are secreted by the cortex of the adrenal glands (Kyrou and Tsigos, 2005; Tsigos and Chrousos, 1994). Cortisol (the main glucocorticoid in humans) is secreted by the cells of the intermediate *zonae fasciculata*, under the tight regulatory control of an endocrine cascade involving corticotropin-releasing hormone (CRH), vasopressin (AVP), and the adrenocorticotropic hormone (ACTH), thus forming the hypothalamic–pituitary–adrenal (HPA) axis. Glucocorticoids constitute the final effectors of the HPA axis and are vital for the maintenance of metabolic, cardiovascular, and immune homeostasis. The mineralocorticoid aldosterone is produced by the outer adrenal *zona glomerulosa* and regulates water and electrolyte homeostasis. The adrenal secretion of aldosterone is under the principal control of the renin–angiotensin axis, while it is only weakly influenced by ACTH. Finally, adrenal androgens with 19 carbon atoms, such as dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), and androstenedione, are secreted by the inner *zona reticularis* and are under the control of ACTH.

A spectrum of genetic defects can disrupt the normal development of the adrenal glands and the HPA axis function, causing adrenal hypoplasia and various forms of adrenal insufficiency presenting in infancy or childhood (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013; Mazilu and McCabe, 2011; Ferraz-de-Souza and Achermann, 2008; Tsigos *et al.*, 1993). The genetic causes of primary/familial adrenal hypoplasia syndromes can be broadly categorized into:

(a) Familial adrenal hypoplasia caused by ACTH resistance syndromes (i.e., familial glucocorticoid deficiency and Triple A syndrome).
(b) Adrenal hypoplasia caused by primary defects in the development of the adrenal glands (i.e., X-linked adrenal hypoplasia congenital caused by DAX1 mutations, primary adrenal hypoplasia caused by steroidogenic factor-1 mutations, and the IMAGe syndrome caused by cyclin-dependent kinase inhibitor 1C gene mutations).

ACTH Resistance Syndromes Causing Familial Adrenal Hypoplasia

Familial Glucocorticoid Deficiency

Familial, isolated, glucocorticoid deficiency (FGD) is an autosomal-recessive disorder which is rare and potentially lethal, manifesting as primary adrenal insufficiency (usually without mineralocorticoid deficiency) (**Table 1**) (Meimaridou *et al.*, 2013; Clark *et al.*, 2009; Chan *et al.*, 2008; Tsiotra *et al.*, 2006; Penhoat *et al.*, 2002). FGD was first described in 1959 (Shepard *et al.*, 1959), and since then it was apparent that affected individuals suffer from a form of hereditary unresponsiveness to ACTH. Children with FGD commonly present within the first 2–3 years of life with hyperpigmentation, recurrent hypoglycemia which can cause convulsions or coma, chronic asthenia, and failure to thrive. In the long term, FGD can lead to neurological complications due to recurrent hypoglycemic episodes and even death, if left undiagnosed/untreated. Tall stature may also be observed in some cases of FGD caused by MC2R mutations (Elias *et al.*, 2000). Biochemically, the affected children exhibit deficient production of cortisol and adrenal androgens, in the presence of markedly elevated ACTH levels. Circulating cortisol levels are undetectable in the majority of these cases and fail to increase with short or prolonged stimulation with pharmacological doses of ACTH. Moreover, the aldosterone and adrenal androgen responses to ACTH are also lost in these patients, suggesting that the underlying unresponsiveness to

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Table 1 ACTH resistance syndromes

ACTH resistance syndrome	*Genetic defect*	*Clinical presentation*
FGD Type 1 (~25% of FGD cases)	MC2R gene mutations	Hyperpigmentation, episodes of recurrent hypoglycemia, lethargy, and muscle weakness, but normal blood pressure (intact renin–angiotensin axis). Tall stature may be observed
FGD Type 2 (~20% of FGD cases)	MRAP gene mutations	Hyperpigmentation, hypoglycemia, lethargy, and muscle weakness, but normal blood pressure (mostly normal production of mineralocorticoids)
FGD variant (5% of FGD cases)	StaR CYP11A1	CLAH with adrenal and gonadal steroid deficiencies (markedly under-androgenized external genitalia). A milder phenotype may be observed (without gonadal derangement) in partial loss of function mutations (non-classical CLAH)
FGD variant form (~5%–10% of FGD cases)	NNT gene mutations	NNT (antioxidant defense) gene defects associated with glucocorticoid deficiency. Extra-adrenal features have been speculated, but not described in humans so far
FGD variant form (DNA repair defect)	MCM4 gene mutations	Short stature, classical natural killer cell deficiency with susceptibility to viral infections. Progressive glucocorticoid deficiency, but with normal mineralocorticoid production. Increased risk of cancer and developmental defects
FGD variant form	TXNRD2 gene	Disrupted redox homeostasis causes primarily glucocorticoid deficiency. Extra-adrenal manifestations may be expected (? dilated cardiomyopathy)
Triple A syndrome (Allgrove syndrome)	AAAS gene mutations (coding the ALADIN protein)	Difficulty in swallowing due to achalasia of the esophagus (achalasia cardia), lack of tears (alacrima), deafness, hyperpigmentation, mental retardation, skin hyperkeratosis and progressive neurodegeneration. Glucocorticoid deficiency may be accompanied by a variable degree of mineralocorticoid deficiency

AAAS: Achalasia-addisonianism (adrenocortical insufficiency)-alacrimia syndrome; *ACTH*: adrenocorticotropic hormone; *ALADIN*: ALacrima-Achalasia-aDrenal Insufficiency-Neurologic disorder; *CLAH*: congenital lipoid adrenal hyperplasia; *FGD*: familial glucocorticoid deficiency; *MC2R*: melanocortin 2 receptor; *MCM4*: mini-chromosome maintenance deficient 4; *MRAP*: melanocortin-2 receptor accessory protein; *NNT*: nicotinamide nucleotide transhydrogenase; *TXNRD2*: thioredoxin reductase 2.

ACTH is generalized. Renin and aldosterone levels, however, are usually normal and exhibit appropriate responses to activation of the renin–angiotensin axis by orthostasis, salt restriction, or furosemide-induced diuresis. Imaging studies of the adrenal glands in affected patients (computed tomography (CT) and magnetic resonance imaging (MRI) scans) show bilateral adrenal hypoplasia with small in size adrenal glands. In addition, histological postmortem studies of the adrenals have revealed extreme atrophy of the inner ACTH-dependent *zonae fasciculata* and *reticularis* which are reduced to a narrow band of fibrous tissue. Contrary, the outer angiotensin II-dependent *zona glomerulosa* is relatively well preserved, indicating that the defect is limited to the ACTH-dependent *zonae* of the adrenal cortex. Treatment of hereditary isolated glucocorticoid deficiency consists of glucocorticoid replacement therapy, which allows the affected subjects to achieve normal growth and development and live an otherwise normal life.

Genetic defects

FGD is a genetically heterogeneous disease, involving primarily mutations in the ACTH receptor (also referred to as the melanocortin 2 receptor (MC2R); approximately 25% of FGD patients) and mutations in the MC2R accessory protein (MRAP; approximately 20% of FGD patients) (**Fig. 1** and **Table 1**) (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013; Mazilu and McCabe, 2011; Clark *et al.*, 2009; Chan *et al.*, 2008; Collares *et al.*, 2008; Tsigos *et al.*, 1993).

The cloning of the ACTH receptor gene in 1992 allowed to precisely study the implication of the ACTH receptor gene in FGD (Tsigos *et al.*, 1993; Clark *et al.*, 1993), as indeed hypothesized since the first description of the syndrome in the late 1950s (Shepard *et al.*, 1959). The ACTH receptor (ACTH/MC2-R) consists of 297 amino acids and is encoded by an intronless gene mapped to the distal end of chromosome 18 (Tsigos *et al.*, 1993; Clark *et al.*, 1993). ACTH/MC2-R belongs to the distinct melanocortin subfamily of the G protein-coupled receptors that couple to G_s and adenylyl cyclase to generate cyclic AMP as a second intracellular messenger. To date, multiple point mutations and frameshift mutations in the ACTH receptor gene (homozygote or compound heterozygote mutations) have been detected in different pedigrees with isolated glucocorticoid deficiency (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013). These mutations are scattered throughout the ACTH receptor molecule and have been shown to affect all aspects of the receptor function (e.g., ligand binding/affinity, signal transduction, trafficking, and cell surface expression) or cause receptor truncation and structural disruption (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013; Hirsch *et al.*, 2011; Chung *et al.*, 2008, 2010; Collares *et al.*, 2008; Tsigos *et al.*, 1993, 1995, 2000; Clark *et al.*, 1993). Interestingly, in one case, compound heterozygosity of a frameshift mutation in the coding region with a single base substitution in the promoter area of the ACTH receptor gene, seemed to have resulted in the clinical manifestation of ACTH resistance (Tsiotra *et al.*, 2006). Mutations in the ACTH receptor appear to account for approximately 25% of FGD patients (also referred to as FGD type 1; **Table 1**) (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013; Mazilu and McCabe, 2011; Clark *et al.*, 2009; Chan *et al.*, 2008; Collares *et al.*, 2008).

The involvement of the MRAP gene in FGD was described in 2005 (Metherell *et al.*, 2005). MRAP is a small single transmembrane accessory protein which is essential for the translocation/trafficking of the ACTH/MC2-R from the endoplasmic reticulum to the cell surface and, thus, for subsequent signaling in response to ACTH (Cerdá-Reverter *et al.*, 2013; Novoselova *et al.*, 2013; Webb and Clark, 2010; Metherell *et al.*, 2005). Mutations of the MRAP gene appear to account for approximately 20%

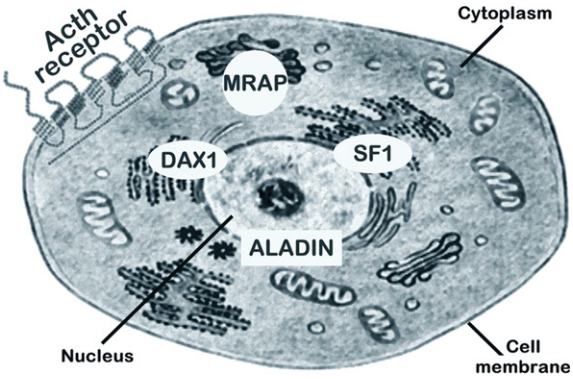

Fig. 1 Key proteins which can cause adrenal hypoplasia when mutated, depicted in an adrenocortical cell secreting glucocorticoids and/or mineralocorticoids. ACTH: adrenocorticotropic hormone; ALADIN: ALacrima-Achalasia-aDrenal Insufficiency-Neurologic disorder, nuclear envelope protein; DAX1: dosage-sensitive sex reversal-adrenal hypoplasia congenita-critical region on the X chromosome gene 1, nuclear receptor protein encoded by the NR0B1 gene; MRAP: melanocortin-2 receptor accessory protein; SF1: steroidogenic factor-1.

of FGD cases (also referred to as FGD type 2; **Table 1**) (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013; Mazilu and McCabe, 2011; Clark *et al.*, 2009; Chan *et al.*, 2008; Collares *et al.*, 2008).

Mutations in the steroidogenic acute regulatory protein (StAR protein; responsible for the transfer of cholesterol across the mitochondrial membrane, and, thus, for initiating the first step of steroidogenesis) and in CYP11A1 (cytochrome P450 monooxygenase; catalyzes the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of steroids) appear to account for approximately another 5% of FGD cases. Mutations in these two enzymes result in both adrenal and gonadal steroid deficiencies, a condition called Congenital Lipoid Adrenal Hyperplasia (CLAH or Lipoid Congenital Adrenal Hyperplasia, LCAH) (**Table 1**) (Meimaridou *et al.*, 2013; Novoselova *et al.*, 2015; Metherell *et al.*, 2009; Flück *et al.*, 2011). Milder phenotypes of adrenal deficiency (without gonadal involvement) have also been described in patients with certain partial loss-of-function mutations of these genes (non-classical CLAH) (Meimaridou *et al.*, 2013; Novoselova *et al.*, 2015; Metherell *et al.*, 2009; Flück *et al.*, 2011).

Another gene implicated in FGD is the nicotinamide nucleotide transhydrogenase gene (NNT: an antioxidant defense gene, encoding a redox-driven proton pump of the inner mitochondrial membrane; NNT mutations account for approximately 5%–10% of FGD cases; **Table 1**) (Meimaridou *et al.*, 2013; Novoselova *et al.*, 2015; Meimaridou *et al.*, 2012; Yamaguchi *et al.*, 2013). The adrenal cortex is very sensitive to reactive oxygen species (ROS), and both steroidogenesis and cell survival depend on adequate NADPH production by the NNT enzyme. Extra-adrenal features have been speculated, given the ubiquitous expression of NNT, but not described in humans so far. Interestingly, oxidative stress is implicated in other forms of primary adrenal failure, such as the Triple A syndrome and X-linked adrenoleukodystrophy (Prasad *et al.*, 2014a). Of note, a homozygous mutation in the thioredoxin reductase 2 (TXNRD2 gene: encoding one of the three thioredoxin reductases, which is mitochondria specific and contributes to the maintenance of redox homeostasis) was recently described in an extended consanguineous Kashmiri kindrend presenting with FGD (**Table 1**) (Prasad *et al.*, 2014b). Glucocorticoid production was primarily affected, as the final step of cortisol production in the mitochondria accounts for nearly 40% of the total electron flow for NAPDH. Extra-adrenal manifestations may also be expected. Indeed, novel mutations in TXNRD2 have been identified in patients with dilated cardiomyopathy (Prasad *et al.*, 2014b; Sibbing *et al.*, 2011), but unfortunately no information was available about their adrenal status.

Recently, mutations in another gene, namely the minichromosome maintenance-deficient 4 (MCM4) gene, have been identified in patients with growth retardation and classical natural killer cell deficiency (susceptibility to viral infections and complications of viral illnesses) (**Table 1**) (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013). Typically these patients have short stature and go on to progressively develop primary adrenal insufficiency characterized by ACTH resistance with isolated glucocorticoid deficiency. Notably, abnormalities in the MCM4 gene, which is important for DNA replication and genome integrity, result in genomic instability and increased incidence of cancer and developmental defects (Charmandari *et al.*, 2014; Meimaridou *et al.*, 2013). Finally, in approximately 40% of all FDG cases the underlying genetic defect remains unknown.

Triple A Syndrome

In addition to hypocortisolism (adrenal insufficiency), a subset of the patients with hereditary unresponsiveness to ACTH also develop alacrima (lack of tears) and achalasia of the esophagus (achalasia cardia; leading to difficulty in swallowing). This constellation of symptoms is known as the Triple A syndrome (alacrima-achalasia-adrenal insufficiency/Addison disease; **Table 1**), which was first described by Allgrove *et al.* (1978) (Allgrove Syndrome). Alacrima is attributed to autonomic dysregulation and structural abnormalities of the lacrimal glands and is probably the earliest and most consistent feature of this syndrome, although achalasia cardia and/or adrenal insufficiency are commonly the presenting manifestations at diagnosis. Low tear production is usually present from early infancy on and may be confirmed by the Schirmer's test. Esophageal dysmotility in affected patients can

be documented by barium swallow and/or endoscopic examination. Of note, in some cases the diagnosis of achalasia may precede the diagnosis of cortisol deficiency by several years. Moreover, affected patients may also develop a varying degree of mineralocorticoid deficiency. Finally, the Triple A syndrome is also frequently (approximately 60% of cases) associated with progressive and variable neurologic impairment, involving both central, peripheral, and autonomic neurons. Neurological defects may include autonomic and peripheral neuropathy, ataxia, mental retardation and cerebellar and neuro-ophthalmological signs, thus, potentially resulting in severely disabling disease (Sarathi and Shah, 2010). Moreover, a number of other features have been described in association with this syndrome, including short stature, skin hyperkeratosis, osteoporosis, xerostomia, pes cavus (a foot deformity that is manifested with a high arch of the foot which does not flatten with weight bearing), and microcephaly (Huebner *et al.*, 1999; Houlden *et al.*, 2002; Kimber *et al.*, 2003; Storr *et al.*, 2009).

Genetic defects

Triple A syndrome is a rare autosomal recessive disorder. Initially, it was proposed that the ACTH receptor may also be defective in the Triple A syndrome; however, there were no mutations found in the entire coding region of this gene in several affected families (Tsigos *et al.*, 1995; Clark and Weber, 1994). Contrary, homozygote or compound heterozygote mutations were found in the AAAS gene on chromosome 12q13 in families with the Triple A syndrome (**Table 1**) (Handschug *et al.*, 2001; Weber *et al.*, 1996). The AAAS gene codes for the WD repeat-containing protein ALADIN (ALacrima-Achalasia-aDrenal Insufficiency-Neurologic disorder; a nuclear pore complex protein located in the nuclear envelope and involved in nucleo-cytoplasmic transport/trafficking—**Fig. 1**) (Sarathi and Shah, 2010; Huebner *et al.*, 2004). More than 60 different mutations have already been described in this gene and appear to account for approximately 90% of the Triple A syndrome cases (Storr *et al.*, 2009). Most of these mutations produce a truncated protein, but missense and point mutations have also been reported. Some Triple-A syndrome patients lack identified mutations in the AAAS gene. Of note, the phenotype/genotype correlation of this syndrome is weak, even between affected siblings, indicating that additional factors may be implicated in its manifestations and progression (Storr *et al.*, 2009; Brooks *et al.*, 2005; Prpic *et al.*, 2003; Houlden *et al.*, 2002; Sandrini *et al.*, 2001).

Primary Adrenal Hypoplasia Syndromes

X-Linked Adrenal Hypoplasia Congenita

X-linked adrenal hypoplasia congenita (X-linked AHC) is a rare disorder which is characterized by primary adrenal insufficiency, usually during early infancy, and hypogonadotropic hypogonadism in adolescence (**Table 2**) (Achermann and Vilain, 2013; Tabarin, 2001; Achermann *et al.*, 1999, 2001; Peter *et al.*, 1998; Sikl, 1948). Most affected patients with X-linked AHC present with acute primary adrenal insufficiency in childhood and more frequently during early infancy (approximately 60% of X-linked AHC cases present at an average age of 3 weeks). However, it should be noted that, a few affected patients with partial forms of X-linked AHC present later in adulthood with delayed-onset adrenal failure or partial hypogonadism. Acute-onset adrenal insufficiency typically presents in male infants with a spectrum of symptoms, including vomiting, dehydration, feeding difficulty or even shock caused by a salt-wasting episode. Indeed, isolated salt loss or recurrent hypoglycemic episodes that can cause seizures may constitute the initial symptoms of X-linked AHC. Acute episodes of stress and/or superimposed illness (e.g., infections), injury or surgery can trigger the onset of adrenal failure in older affected children. In adolescence, affected males typically exhibit delayed or arrested puberty with hypogonadotropic hypogonadism and remain infertile, as administration of human chorionic gonadotropin only stimulates testosterone concentrations into the normal range in most patients, but not spermatogenesis. In these patients pubertal delay is considered related to defective production of hypothalamic gonadotropin-releasing hormone (GnRH) and

Table 2 Primary adrenal hypoplasia syndromes

Primary adrenal hypoplasia syndrome	*Genetic defect*	*Clinical presentation*
X-linked AHC	NR0B1 (DAX1) gene mutations	Acute adrenal insufficiency usually in male infants with salt loss and hypogonadotropic hypogonadism at puberty. Precocious puberty with impaired spermatogenesis has also been reported. Carrier females may occasionally exhibit symptoms of adrenal insufficiency and hypogonadotrophic hypogonadism
SF1 linked	NR5A1 (SF1) (autosomal)	In some cases early adrenal failure is combined with complete 46,XY sex reversal or gonadal dysgenesis. In most cases, milder gonadal phenotypes develop, with or without adrenal failure
IMAGe syndrome	CDKN1C (maternal transmission; imprinted mode of inheritance)	AHC and intrauterine growth restriction, metaphyseal dysplasia, hypercalcemia, and mild genitourinary abnormalities. Growth restriction constitutes the cardinal component of this syndrome

CDKN1C: Cyclin-dependent kinase inhibitor 1C (p57, Kip2); *DAX1*: dosage-sensitive sex reversal-adrenal hypoplasia congenita-critical region on the X chromosome gene 1, nuclear receptor protein encoded by the NR0B1 gene; *NR0B1*: nuclear receptor subfamily 0, group B, member 1; *NR5A1*: nuclear receptor subfamily 5 group A member 1; *SF1*: steroidogenic factor-1; *X-linked AHC*: X-linked adrenal hypoplasia congenita.

impaired responsiveness of pituitary gonadotropes to GnRH. In more than 10% of affected males, bilaterally undescended testes are noted at birth. However, in some cases early pubertal development with pubertal arrest has also been reported. Furthermore, in a few cases carrier females may also exhibit symptoms of adrenal insufficiency or hypogonadotropic hypogonadism (Achermann and Vilain, 2013; Jadhav *et al.*, 2011; Seminara *et al.*, 1999; Merke *et al.*, 1999).

Primary adrenal failure in a male infant in the presence of increased ACTH serum concentrations with low aldosterone and low or normal 17-hydroxyprogesterone serum concentrations is highly suggestive of X-linked AHC (Achermann and Vilain, 2013). Adrenal failure is typically manifested with hyperkalemia, hyponatremia, metabolic acidosis and hypoglycemia and can rapidly lead to death if left undiagnosed and/or untreated with glucocorticoids and mineralocorticoids. Circulating cortisol concentrations during the first weeks of life in these cases can range from very low to even high; however, the latter still remains inappropriately low for a severely sick child (Achermann and Vilain, 2013; Landau *et al.*, 2010). An ACTH test would detect cortisol deficiency in affected patients, while a GnRH test would likely reveal impaired gonadotropin responsiveness (Jadhav *et al.*, 2011).

Genetic defects

X-linked AHC is caused by mutations of the NR0B1 gene (NR0B1 for Nuclear Receptor subfamily 0, group B, member 1; historically known as DAX1 for Dosage-sensitive sex reversal-adrenal hypoplasia congenita-critical region on the X chromosome gene 1—**Fig. 1** and **Table 2**) (Achermann and Vilain, 2013; Lin *et al.*, 2006; Phelan and McCabe, 2001; Tabarin, 2001; Zanaria *et al.*, 1994). The NR0B1 gene is located on the short arm of the X chromosome (Xp21) and encodes a 470 amino-acid protein which has the structure of a transcription factor and is classified as a member of the nuclear receptor superfamily with an unknown ligand (orphan receptor). This orphan nuclear hormone receptor has a novel DNA-binding domain with a unique structure that does not resemble the classic zinc-finger DNA-binding domain of these receptors. Nuclear receptors are transcription factors and appear to play a functional role in the establishment and maintenance of steroidogenic tissues, regulating gene networks which are important for development, homeostasis and reproduction in response to various extracellular and intracellular signals. The DAX1 gene is expressed not only in the adrenal glands, but also in most of the reproductive tissues, including the hypothalamus, the anterior pituitary gonadotropic cells, and the gonads, thus playing a key role in the development and function of both the adrenals and the gonads. The exact molecular mechanisms and biological role of DAX1 have not been fully clarified yet (Achermann and Vilain, 2013; Phelan and McCabe, 2001; Tabarin, 2001).

Multiple different defects in the DAX1 gene have been reported in patients with X-linked AHC, including missense, nonsense, frameshift and splice site mutations, as well as large and small deletions and insertions (Achermann and Vilain, 2013; Lin *et al.*, 2006; Phelan and McCabe, 2001; Tabarin, 2001; Muscatelli *et al.*, 1994). Of note, gross deletions usually occur as a continuous gene deletion including the genes of glycerol kinase and Duchene muscular dystrophy. Finally, it is also noteworthy that a remarkable discrepancy characterizes the genotype/phenotype relationship of X-linked AHC, even within affected families (e.g., the same mutation resulted in two brothers with the complete syndrome, an unaffected grandfather, and a homozygote aunt with hypogonadotropic hypogonadism and normal adrenal function). This genotype/phenotype discrepancy indicates that the clinical presentation and manifestations of this syndrome may be affected by additional genetic or environmental factors (Achermann and Vilain, 2013; Jadhav *et al.*, 2011; Landau *et al.*, 2010; Phelan and McCabe, 2001; Tabarin, 2001).

Primary Adrenal Hypoplasia Caused by Steroidogenic Factor-1 Mutations

Steroidogenic factor-1 (SF1) is an orphan nuclear receptor (**Fig. 1**) which was initially identified as a key factor in the tissue-specific expression of cytochrome P450 steroid hydroxylases that are essential for the synthesis of steroid hormones (Lala *et al.*, 1992). Subsequently, SF1 was recognized as a crucial regulator of multiple genes involved in adrenal and gonadal development, steroidogenesis and reproduction (Parker *et al.*, 2002; Parker and Schimmer, 1997). Indeed, SF1 is encoded by the nuclear receptor subfamily 5 group A member 1 gene (NR5A1; located on chromosome 9q33) and is expressed in both the adrenals and the gonads, being essential for the development and function of these organs and for normal sexual differentiation (Achermann, 2005; Parker *et al.*, 2002; Ozisik *et al.*, 2002; Parker and Schimmer, 1997; Wong *et al.*, 1996). In the adrenals, SF1 not only regulates the levels of cytochrome P450 steroid hydroxylases, but also modulates the expression of the ACTH receptor, 3β-hydroxysteroid dehydrogenase, and steroidogenic acute regulatory protein (StAR). Furthermore, SF1 appears to play multiple roles in the central nervous system (CNS) and is expressed in the pituitary gland and the hypothalamus, contributing to the differentiation of pituitary primordial cells into gonadotropes (Büdefeld *et al.*, 2012; Ramayya *et al.*, 1997).

Genetic defects

SF1 mutations in humans were initially described in patients with 46,XY disorders of sex development (46,XY DSD), testicular dysgenesis and primary adrenal failure (**Table 2**) (Achermann *et al.*, 1999, 2002). The first mutation was reported in a 46,XY phenotypic female that had a de novo heterozygous loss of function missense mutation in exon 3 of one of the SF1 genes (Achermann *et al.*, 1999; Ito *et al.* 2000). This mutation causes substitution of glycine by glutamate at amino acid 35 in the "P" box region of the first zinc-finger of the DNA-binding domain of SF1, hence abolishing its DNA-binding activity and markedly affecting its function. This affected patient presented in the first 2 weeks of life with primary adrenal failure characterized by low circulating cortisol and aldosterone and high ACTH levels. At the age of 10 years, although pituitary gonadotropins responded to GnRH, testosterone did not respond to administration of human chorionic gonadotropin, indicating defective gonadal function. The

karyotype of the affected patient was 46,XY, however, normal Müllerian structures were found at laparotomy and streak-like gonads containing poorly differentiated seminiferous tubules and connective tissue were detected and removed. Treatment of this patient with estradiol gel and cyclical progestogen resulted in breast development, growth of the uterus and regular menstruation.

Furthermore, a second mutation was reported in a phenotypically female infant born to consanguineous parents that exhibited a recessively-inherited homozygous mutation (Achermann *et al.*, 2002). This mutation affected the "A" box of SF1, a region that functions as a secondary DNA-binding domain, hence, resulting in partial loss of function. Notably, three relatives (both parents and the sister) of the affected infant were phenotypically normal despite being heterozygous for the mutation. This infant presented with hyponatremia, hyperkalemia, progressive hypotonia, weight loss and hypoglycemic convulsions. Circulating 17-hydroxyprogesterone levels were low, although the fetal adrenal steroid DHEA-S was detectable. Abdominal CT scanning revealed left adrenal hypoplasia and right adrenal agenesis. Although the karyotype of the affected infant was 46,XY, a uterus was detected on pelvic ultrasound and by MRI.

Finally, a heterozygous SF1 mutation has also been described in a 46,XX female that presented at the age of 14 months with apparently normal ovarian development, primary adrenal failure and seizures after an otitis/tonsillitis episode 1 week earlier (Biason-Lauber and Schoenle, 2000). This genotypically and phenotypically normal girl had a heterozygote G > T transversion in exon 4 of the NR5A1 gene, leading to the missense p.R255L mutation in the SF1. This mutant SF1 protein fails to transactivate target genes potentially due to inability to bind canonical DNA sequences. This mutation in a genotypically female patient suggests that SF1 is not necessary for female gonadal development, despite playing a vital role in the formation of the adrenals in both genders.

More recent studies revealed that heterozygous missense, nonsense or frameshift mutations affecting the DNA-binding domain of the SF1 protein, can lead to 46,XY disorders of sex development (46,XY DSD; e.g., sex reversal, XY gonadal dysgenesis) (Lourenco *et al.*, 2009; Reuter *et al.*, 2007; Correa *et al.*, 2004; Hasegawa *et al.*, 2004; Mallet *et al.*, 2004) or other milder phenotypes, including partial androgen insensitivity (Coutant *et al.*, 2007), hypospadias (Köhler *et al.*, 2008; Lin *et al.*, 2007), bilateral anorchia (Philibert *et al.*, 2010) and primary ovarian insufficiency (Warman *et al.*, 2011; Lourenco *et al.*, 2009), without adrenal failure. Thus, it is evident that SF1 mutations may lead to a relatively wide spectrum of sex development phenotypes, which are only rarely related to primary adrenal insufficiency.

IMAGe Syndrome

An association between AHC and intrauterine growth restriction, metaphyseal dysplasia, and genitourinary abnormalities has been also described (IMAGe syndrome: intrauterine growth retardation—metaphyseal dysplasia—AHC—genital abnormalities), with growth restriction being the cardinal component of this syndrome (**Table 2**) (Achermann and Vilain, 2013; Arboleda *et al.*, 2012; Lin *et al.*, 2006; Vilain *et al.*, 1999). Adrenal crises may develop in the neonatal period, while radiologic identification of metaphyseal dysplasia or delayed endochondral ossification with osteopenia and hypercalcemia/hypercalciuria may help the diagnosis. Growth hormone deficiency represents another common finding in these patients and early replacement therapy could improve linear growth. Dysmorphic craniofacial features include prominent forehead, low-set ears, short nose, short arms and legs, whilst genital abnormalities of variable severity are confined to male patients and include micropenis, hypospadias, and undescended testes.

Genetic defects

The genetic cause of IMAGe syndrome has recently been shown to be mutations in the cyclin-dependent kinase inhibitor 1C (p57, Kip2) gene (CDKN1C gene: located on 11p15; encodes p57 (KIP2) which is a tight-binding inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation; expressed in the brain, eye, heart, lung, skeletal muscle, kidney, pancreas, testis, placenta, and in the subcapsular or developing definitive zone of the adrenals) (**Table 2**) (Arboleda *et al.*, 2012). Of note, de novo mutations or an imprinted mode of inheritance have been revealed by familial analysis, with exclusive maternal transmission of the mutation. Missense mutations in the PCNA-binding domain in the carboxy-terminal region of the CDKN1C gene have been identified (Arboleda *et al.*, 2012; Tan *et al.*, 2006; Milani *et al.*, 2014). Interestingly, IMAGe-associated mutations have been shown to markedly increase the protein stability, suggesting that the reduced-growth phenotype of this syndrome probably derives from CDKN1C gain-of-function due to IMAGe-associated mutations causing increased protein stability (Hamajima *et al.*, 2013). Finally, a novel variant in the CDKN1C has been associated with intrauterine growth restriction, short stature, and early-adulthood-onset diabetes, but without adrenal insufficiency or metaphyseal dysplasia (Kerns *et al.*, 2014).

See also: Adrenal Insufficiency: Etiology and Diagnosis

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Impact of Glucocorticoid Receptor Polymorphisms on Glucocorticoid Action

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Glossary

Cortisol The most important and abundant glucocorticoid in humans.

Glucocorticoids Class of steroid hormones produced by the adrenal glands which can influence glucose homeostasis and various other processes throughout the body.

Glucocorticoid receptor (GR) Intracellular protein that belongs to the nuclear receptor family which regulates the genomic and nongenomic action of glucocorticoids.

Polymorphism A common genetic variation in the general population.

Introduction

The adrenal glands are responsible for producing several classes of steroid hormones. An important part of its end products comprises the glucocorticoids with cortisol being the major player within this class of hormones. Despite the name hinting on effects on the glucose metabolism, glucocorticoids are in fact responsible for various effects throughout the body. It is known that this class of hormones are involved in a wide range of metabolic, cardiovascular, inflammatory, cognitive, and reproductive processes, throughout the lifespan of humans and animals.

The importance of glucocorticoids additionally becomes clear giving the distribution of its receptor, the glucocorticoid receptor (GR), which is expressed in essentially all tissues (Dezso *et al.*, 2008). Moreover, genome-wide analysis revealed hundreds of genes which are in one way or another influenced by glucocorticoids with regard to their expression (Reddy *et al.*, 2009). This all together indicates the importance of glucocorticoids and explains why absence of cortisol, as seen in patients with severe untreated adrenal insufficiency, or GR mutations leading to total inactivation are not compatible with life.

Glucocorticoid Receptor

The first step in the cascade of glucocorticoid action starts with binding to the intracellular GR. The GR is part of the nuclear receptor family and hence has the capacity to regulate the expression of certain genes after binding to its ligand. The gene encoding GR, *NR3C1*, is located on the long arm of chromosome 5 and comprises one noncoding exon (exon 1) followed by eight coding exons (exons 2–9) as shown in **Fig. 1**. Alternative splicing of the last exon yields two isoforms, i.e., GRα and GRβ, with each distinct properties. Three functional domains of the receptor have been identified, which are the N-terminal transactivation domain, the DNA-binding domain, and the C-terminal ligand-binding domain (Gross and Cidlowski, 2008).

In unbound state, GR monomers are coupled to a complex of proteins, especially heat shock proteins, and remain inactive in the cytoplasm (Pratt *et al.*, 2006). Once bound to glucocorticoids, the GR gets released and subsequently translocates into the

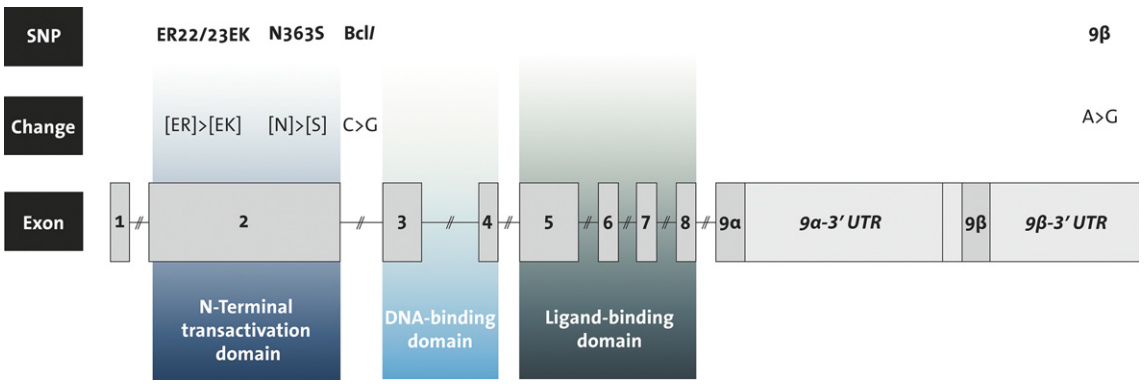

Fig. 1 Glucocorticoid receptor gene with four of its most studied polymorphisms. The letters inside brackets indicate an amino acid change. Abbreviations: *A*, nucleotide adenine; *C*, nucleotide cytosine; *E*, amino acid glutamic acid; *G*, nucleotide guanine; *K*, amino acid lysine; *N*, amino acid asparagine; *R*, amino acid arginine; *S*, amino acid serine; *SNP*, single nucleotide polymorphism; *UTR*, untranslated region.

nucleus. The mode of action on genomic level is carried out by functioning as a transcription factor leading to alterations in gene expression. By binding directly to specific glucocorticoid response elements (GRE), the glucocorticoid-GR complex can activate the transcription of certain genes (transactivating effect), whereas binding on other specific GREs can repress the transcription (transrepressing effect) of the corresponding target gene. In the same manner, transactivation and transrepression can also occur by interacting with other transcription factors, such as NFκB, AP-1, and STAT proteins, and thereby influencing their effect (Reichardt and Schutz, 1998; Necela and Cidlowski, 2004). Another mode of glucocorticoid action occurs outside the nucleus, hence called the nongenomic action, and is rather responsible for the quick response usually seen upon administration of exogenous glucocorticoids (Song and Buttgereit, 2006).

The transactivating effect is usually held accountable for inducing Cushingoid-like metabolic adverse events in glucocorticoid users, whereas the preferable immunosuppressive effects of glucocorticoids are attributed to its transrepressing effect (for example suppression of IL-2, GM-CSF, TNF-*a* transcription) (Barnes, 1998; Newton and Holden, 2007). So far, thousands of polymorphisms of the GR have been indexed (see website dbSNP) of which the majority have been identified in the intronal regions. Some of these lead to alterations in GR effect and have been associated with distinct differences in body composition, cognitive functioning, and effect of glucocorticoid treatment. Four of the most studied variants with their corresponding reference single nucleotide polymorphism (SNP) ID numbers include the Bcl*I* (rs41423247), N363S (rs6195), ER22/23EK (rs6189 and rs6190) and the 9*β* (rs6198) variants. These variants are broadly considered as either "glucocorticoid sensitive" (Bcl*I* and N363S) or as "glucocorticoid resistant" (9*β* and ER22/23EK) polymorphisms.

Bcl*I* (rs41423247) Polymorphism

The Bcl*I* variant is due to a single nucleotide change of C➔G in intron 2. Carriers of this variant have been shown to be more sensitive to glucocorticoids (van Rossum *et al.*, 2003), which is in line with clinical studies showing to varying degrees unfavorable anthropometric and metabolic features in homo- and heterozygous carriers. It is assumed to be the most common functional GR polymorphism with variations in prevalence across ethnicities (Fleury *et al.*, 2003).

N363S (rs6195) Polymorphism

The N363S variant covers an A➔G nucleotide substitution in codon 363 of exon 2 leading to an amino acid change from asparagine (N; codon AAT) to serine (S; codon AGT). In vivo studies suggest this variant to be associated with increased glucocorticoid sensitivity as, for example, carriers had increased cortisol suppression and insulin response after dexamethasone administration in comparison to wild-type subjects (Huizenga *et al.*, 1998). A previous in vitro study also showed the N363S polymorphism to be associated with increased transactivating capacity without alterations in the transrepression action (Russcher *et al.*, 2005a).

ER22/23EK (rs6189 and rs6190) Polymorphism

Another common polymorphism in exon 2 leading to alteration in GR function is the ER22/23EK variant. This variant actually consists of two linked G➔A substitutions in adjacent codons leading to an alteration of codons GAG AGG to GAA AAG. At protein level, there is no change in amino acid resulting from the first variation (codon 22; glutamic acid (E)), whereas the second SNP in codon 23 leads to a change from arginine (R) to lysine (K). Effects of this variant have been found to be in line with a relative glucocorticoid resistance in in vivo and in vitro studies. ER22/23EK carriers had for example a reduced suppression of cortisol levels after dexamethasone administration (van Rossum *et al.*, 2002), and decreased transactivating capacity in bioassays and transfection experiments (Russcher *et al.*, 2005a). Several mechanisms have been proposed regarding the underlying mechanisms including a switch in GR forms due to change in translation start sides, yielding receptors with relatively less transactivating activity (Russcher *et al.*, 2005b).

9*β* (rs6198) Polymorphism

Alternative splicing of the GR transcript yields several different isoforms among which the GR*β* splice variant. In contrast with the active GRα, which can induce transcriptional changes upon activation, the GR*β* variant does not bind glucocorticoids and instead has a dominant-negative inhibitory effect on GR action. The 9*β* variant results from an A➔G nucleotide substitution in exon 9*β* leading to a higher expression and a relatively more stable GR*β* isoform (Derijk *et al.*, 2001). This is thought to give rise to glucocorticoid resistance. Accordingly, expression assays with peripheral blood mononuclear cells of homozygous carriers revealed decreased repression of *IL-2* expression upon dexamethasone addition, whereas no effect was found concerning the transactivating effect (van den Akker *et al.*, 2006).

Body Composition and Cardiometabolic Traits

Excess glucocorticoid exposure and subsequent increased cellular action manifests itself in phenotypic and biochemical changes. Patients with Cushing syndrome who are endogenously secreting exuberant amount of cortisol usually gain weight (especially around the waist) and develop, among others, a moon-face, buffalo-hump, hypertension, glucose intolerance, and dyslipidemia (Nieman, 2015). Similar findings are also present in patients with iatrogenic Cushing syndrome due to glucocorticoid treatment with weight gain reported as the most common adverse event (Curtis *et al.*, 2006). The combination of (central) obesity with metabolic disturbances additionally increases the risk of metabolic syndrome and its sequela as for example diabetes mellitus and cardiovascular diseases. This emphasizes the importance of proper glucocorticoid action. Given the altered glucocorticoid sensitivity of the mentioned GR polymorphisms, previous studies have extensively focused on their associations with cortisol-related body compositional and cardiometabolic features.

Body Mass Index and (Central) Obesity

Most of the studies concerning GR polymorphisms and body composition have been performed for the variants associated with a relative increased glucocorticoid sensitivity. Regarding the Bcl*I* polymorphism, carriers were more likely to have higher abdominal obesity as measured as visceral fat area (Buemann *et al.*, 1997; Ukkola *et al.*, 2001), sagittal diameter (Rosmond *et al.*, 2000), and waist-to-hip ratio (Krishnamurthy *et al.*, 2008; Rosmond *et al.*, 2000; Srivastava *et al.*, 2011), which is in line with the expected central fat accumulation in case of high glucocorticoid action. The association with body mass index (BMI) has been found to be more inconsistent, with studies showing (a tendency towards) a higher BMI (Clement *et al.*, 1996; Rosmond *et al.*, 2000), or no relationship at all (Buemann *et al.*, 1997; Panarelli *et al.*, 1998; Weaver *et al.*, 1992). Several individual studies investigating the link between obesity and N363S found an association with higher BMI (Huizenga *et al.*, 1998; Roussel *et al.*, 2003) and (abdominal) obesity (Lin *et al.*, 2003a; Dobson *et al.*, 2001). Even within a group of patients with severe obesity, female N363S heterozygotes had significantly higher BMI as well as a higher food intake in comparison to women with the wild-type genotype (Di Blasio *et al.*, 2003). Interestingly, an allele-dosage effect was shown in two separate study populations by Lin *et al.* with the homozygous N363S carriers having the highest BMI (Lin *et al.*, 1999b). The previous findings concerning anthropometric measures in relation to the N363S variant were not observed in several other studies with only male participants (Echwald *et al.*, 2001; Rosmond *et al.*, 2001) nor in a meta-analysis (Marti *et al.*, 2006). However, in our recent study we showed an increased metabolic syndrome risk in low-educated subjects and young men with the N363S variant, indicating an interaction with environmental factors (Wester *et al.*, 2016).

With regard to the ER22/23EK polymorphism, we previously demonstrated sex-specific beneficial differences in favor of the carriers. Male adult carriers were shown to be taller, leaner, and have more muscle strength, whereas female carriers tended to have a smaller waist circumference in comparison to noncarriers (van Rossum *et al.*, 2004b). For the other relative glucocorticoid resistant polymorphism, Caucasian female carriers of the 9β variant were also more likely to have lower waist circumference and a lower waist-to-hip ratio (Syed *et al.*, 2006), which are in line with what one would expect in case of decreased glucocorticoid effect. This was not evaluated in another study with elderly subjects (van den Akker *et al.*, 2006), which could perhaps hint at a potential interaction with age. Neither of the studies regarding the 9β polymorphism could show any relation with BMI.

Glucose and Lipid Profile, and Blood Pressure

Disturbances in glucose homeostasis have been described in studies concerning the effect of the Bcl*I* polymorphism. Among obese women, carriers were more likely to have hyperinsulinemia and have a higher homeostatic model assessment (HOMA)-index compared to noncarriers with comparable BMI and waist-to-hip ratio (Weaver *et al.*, 1992). Unfavorable alterations in insulin and glucose were also shown in another obese study population by Srivastava *et al.* who showed higher levels for both measurements in Bcl*I* carriers (Srivastava *et al.*, 2011). Rather similar results were also found in Bcl*I* homozygous patients with Addison's disease, who had a higher HOMA-index, and higher glucose levels after oral glucose tolerance test in comparison to heterozygous carriers and noncarriers (Giordano *et al.*, 2012). The authors showed no differences in duration of the disease or dose of glucocorticoid treatment between the groups, however, the homozygous carriers were on average more centrally obese, which could (additionally) induce metabolic alterations. Moreover, the same carriers were also shown to have higher total cholesterol and triglyceride levels. These findings were nonetheless not observed in other studies without Addison patients (Kuningas *et al.*, 2006; Srivastava *et al.*, 2011). Di Blasio and colleagues had also found no differences in metabolic measures, however, subjects carrying *both* Bcl*I* and N363S polymorphisms did have significantly higher total and LDL-cholesterol levels (Di Blasio *et al.*, 2003). Some studies focusing on N363S found higher triglyceride and cholesterol levels among carriers (Kuningas *et al.*, 2006; Lin *et al.*, 2003b), which persisted after adjustments for important covariates as gender, BMI, and waist-to-hip ratio (Lin *et al.*, 2003b).

As expected, considering the decreased rather than increased glucocorticoid sensitivity, we had earlier found opposite associations in ER22/23EK carriers. This polymorphism was associated with a lower HOMA-index, lower fasting insulin, total, and LDL-cholesterol levels, and also with a relatively lower cortisol suppression after a 1.0 mg dexamethasone suppression test (van Rossum *et al.*, 2002). Moreover, in a follow-up study of elderly men, we also found a better survival in carriers compared to noncarriers (van Rossum *et al.*, 2004a). Other works involving elderly participants reported no differences in metabolic profile

(Mora *et al.*, 2012) or found, in persons aged 85 and older, higher HbA_{1c}-levels in carriers (Kuningas *et al.*, 2006). For the 9β polymorphism, beneficial lipid levels, with lower total cholesterol and higher HDL-cholesterol, have been described in Caucasian male carriers (Syed *et al.*, 2006).

Blood pressure seems to be relatively unaffected by the mentioned polymorphisms giving the fact that no differences were found between noncarriers and carriers of the Bcl*I* (Di Blasio *et al.*, 2003; Syed *et al.*, 2008), N363S (Huizenga *et al.*, 1998; Lin *et al.*, 1999a, 2003a), ER22/23EK (van Rossum *et al.*, 2002, 2004a), or 9β variant (Syed *et al.*, 2006). A higher blood pressure is however previously described in Bcl*I* carriers from Northern India (Srivastava *et al.*, 2011), and likewise in children with the homozygous 9β genotype who also appeared to have an increased heart rate and left ventricular mass (Geelhoed *et al.*, 2011).

Mental Health

Many psychological illnesses show a connection with disturbances in the hypothalamic-pituitary-adrenal (HPA) axis function. This usually reflects in elevated cortisol levels which are additionally resistant to suppression with dexamethasone (Keller *et al.*, 2006). Patients are also frequently found to have increased levels of the corticotropin-releasing hormone (CRH) (Claes, 2004), which stimulates the HPA axis and subsequently leads to the secretion of cortisol. High circulating cortisol levels are known to give rise to anatomical alterations as well as imbalances in neurotransmitter systems (Popoli *et al.*, 2011). Hence, smaller hippocampal volume, cerebral atrophy, and psychological comorbidities such as depression and anxiety disorders, are regularly being observed in patients with Cushing syndrome (Starkman *et al.*, 1992; Bourdeau *et al.*, 2002; Sonino and Fava, 2001). However, the impaired negative feedback regulation in combination with increased CRH levels have also put the focus on possible alterations at GR level in mental disorders (Pariante, 2009).

We previously assessed the association between the glucocorticoid sensitive Bcl*I* variant and major depression disorder (MDD) and found it to be more prevalent among patients with MDD in comparison to controls (van Rossum *et al.*, 2006). Similar findings were also described in other studies with the homozygous Bcl*I* variant being more common in depressed patients (Zobel *et al.*, 2008; Krishnamurthy *et al.*, 2008). With regard to treatment response, we did not find any treatment effect for the Bcl*I* polymorphism (van Rossum *et al.*, 2006). However, Brouwer and colleagues showed that Bcl*I* carriers tended to be more resistant to treatment with paroxetine in comparison to noncarriers (Brouwer *et al.*, 2006), whereas a more recent study showed carriers to have a better response to another SSRI (fluvoxamine) and no difference after treatment with the serotonin-norepinephrine reuptake inhibitor milnacipran (Takahashi *et al.*, 2014).

Regarding the relative glucocorticoid resistant ER22/23EK polymorphism, we previously reported this variant to be associated with beneficial cerebrovascular effects given the findings that elderly carriers had lower risk of dementia and white matter lesions (van Rossum *et al.*, 2008). Additionally, the carriers among depressed inpatients tended towards better results on the divided attention test in comparison to noncarriers (van Rossum *et al.*, 2006). With respect to mood disorders, the ER22/23EK has been linked to presence of MDD (van West *et al.*, 2006), recurrence of depressive disorders (Galecka *et al.*, 2013; van Rossum *et al.*, 2006), but also to a faster treatment response with antidepressants (van Rossum *et al.*, 2006). Interestingly, carriers of either the ER22/23EK or 9β polymorphism were more likely to develop depressive symptoms only in case of childhood adversity (Bet *et al.*, 2009).

Glucocorticoid Treatment Response and Adverse Events

The first synthetic glucocorticoids were produced halfway through the last century and have since then experienced a remarkable development resulting in a variety of glucocorticoid analogous. By modifying the chemical structure, newer variants became available which were more potent in regard to their anti-inflammatory action while the mineralocorticoid effects became less or (nearly) absent (Benedek, 2011). A high antiinflammatory potency warrants higher efficiency in suppression of the inflammatory process, but in general also increases the likelihood of developing glucocorticoid-related side effects such as abdominal obesity, glucose intolerance, and osteoporosis. Moreover, glucocorticoids with high mineralocorticoid potency are more likely to also induce disturbances as hypokalemia as well as edema and hypertension.

Glucocorticoids are nowadays available in a wide variety of local and systemic administration forms which makes it applicable to a wide range of illnesses. We recently showed in a large Dutch population-based cohort study that almost 11% of all participants were using any form of exogenous glucocorticoids (Savas *et al.*, 2017). Highest user rates were especially found for the local administration forms, in particular, the inhaled, nasal, and dermal glucocorticoids. Similar prevalence rates were found in the United States, with one in every seven patients in ambulatory care receiving adrenal corticosteroids (Raofi and Schappert, 2006). Since glucocorticoids are utilized on such a large scale, it becomes even more important to balance between maintaining proper therapeutic response on one hand, and preventing or minimizing undesired side effects on the other hand. GR polymorphisms leading to alterations in glucocorticoid action, either by affecting transactivating and/or transrepressing potency, could theoretically affect this balance.

The relationship between the mentioned GR polymorphisms and glucocorticoid treatment outcomes have been investigated for different medical conditions. An overview of these is given in **Table 1**. In acute lymphoblastic leukemia (ALL), glucocorticoids (especially predniso(lo)ne, and dexamethasone) play an important therapeutic role due to their antiproliferative and apoptotic effects in the leukemic cells (Pui and Evans, 2006). Several studies have assessed the difference in effect of glucocorticoid treatment in the background

Table 1 Overview of clinical studies investigating the relationship between glucocorticoid receptor polymorphisms and glucocorticoid treatment outcomes

Indication	*Reference*	*Study population*	*Type of glucocorticoid treatment*	*GR SNP (s)*[a]	*Relevant observations*
Acute lymphoblastic leukemia	Eipel *et al.* (2013)	346 pediatric ALL patients	Systemic (prednisolone, dexamethasone)	N363S	– Good responders to prednisone; – Better 5-year event-free survival; – Higher prevalence of treatment-related hepatotoxicity and abnormal glucose metabolism
	Felder-Puig *et al.* (2007)	37 pediatric ALL patients	Systemic (prednisolone, dexamethasone)	BclI/ N363S ER22/ 23EK	– No association between polymorphisms and adverse psychological reactions due to glucocorticoid treatment
	Fleury *et al.* (2004)	222 pediatric ALL patients	Systemic (prednisone, dexamethasone, hydrocortisone (IT))	BclI/ N363S ER22/ 23EK	– Decreased overall survival probability in homozygous BclI carriers; – No differences for other variants in regard to event-free, disease-free, and overall survival
	Kaymak Cihan *et al.* (2017)	49 pediatric ALL patients	Systemic (prednisolone, dexamethasone)	BclI/ N363S	– Higher prevalence of Cushingoid-like symptoms, dyspepsia, and depression symptoms during treatment in BclI carriers; – No N363S carriers (Turkish study population)
	Labuda *et al.* (2010)	310 pediatric ALL patients	Systemic (prednisone, dexamethasone, hydrocortisone (IT))	BclI/ 9β	– Lower event-free survival in both variants; – Lower overall survival in BclI homozygotes
	de Ruiter *et al.* (2014)	25 pediatric ALL patients	Systemic (prednisone)	BclI/ N363S ER22/ 23EK 9β	– Increased duration of adrenal insufficiency in homozygous BclI carriers, whereas decreased duration in ER22/23EK carriers
	Tissing *et al.* (2005)	57 pediatric ALL patients	Systemic (prednisone)	BclI/ N363S ER22/ 23EK	– No correlation with in vivo prednisone response, in vitro prednisolone sensitivity, and event-free survival
	Xue *et al.* (2015)	63 pediatric ALL patients	Systemic (prednisone)	BclI/ ER22/ 23EK 9β	– Good responders to prednisone with BclI variant; – No 9β and ER22/23EK carriers (Chinese study population)
Asthma	Keskin *et al.* (2016)	82 asthmatic children with moderate or severe exacerbations	Inhaled (fluticasone propionate)	BclI/	– Better response to inhaled glucocorticoids during asthma exacerbation in homozygous carriers
	Mohamed *et al.* (2015)	40 severe adult asthmatic patients	Systemic (prednisolone)	BclI/	– C allele carriers more frequent among glucocorticoid sensitive patients
	Panek *et al.* (2012)	234 asthmatic adults	Not mentioned	N363S	– Homo- and heterozygous carriers less likely to have uncontrolled asthma
	Tsartsali *et al.* (2012)	62 asthmatic children	Inhaled (budesonide, fluticasone)	BclI/	– Higher basal cortisol levels in heterozygous carriers; – Lower cortisol levels in homozygous carriers (in interaction model with other SNPs)
	Szczepankiewicz *et al.* (2008)	113 asthmatic children	Inhaled (budesonide, fluticasone)	BclI/ N363S ER22/ 23EK	– No association between variants with asthma severity and steroid demand
Duchenne muscular dystrophy	Bonifati *et al.* (2006)	48 pediatric Duchenne patients	Systemic (prednisone, deflazacort)	N363S	– Carriers tended to have a later age at loss of ambulation
Graves' orbitopathy	Vannucchi *et al.* (2014)	43 adult patients with moderate-severe Graves' orbitopathy	Systemic (methylprednisolone)	BclI/ N363S ER22/ 23EK	– No associations with glucocorticoid therapy response or occurrence of related adverse events

Table 1 Continued

Indication	*Reference*	*Study population*	*Type of glucocorticoid treatment*	*GR SNP (s)*[a]	*Relevant observations*
Immune thrombocytopenia	Xuan *et al.* (2014)	473 young and adult immune thrombocytopenic patients	Not mentioned	BclI/ N363S ER22/ 23EK	– No relation between BclI and steroid response; – No N363S and ER22/23EK carriers (Chinese study population)
Inflammatory bowel disease	De Iudicibus *et al.* (2007)	119 young IBD patients (64 CD/55 UC)	Systemic (prednisone)	BclI/ N363S ER22/ 23EK	– Better treatment response in carriers of BclI/ polymorphism; – No differences for other SNPs
	De Iudicibus *et al.* (2011)	154 young IBD patients (82 CD/72 UC)	Systemic (prednisone)	BclI/	– More common in responders and associated with good steroid response
	Krupoves *et al.* (2011)	296 pediatric CD patients	Systemic (prednisone, methylprednisolone, budesonide)	N363S ER22/ 23EK	– ER22/23EK (rs6190) associated with resistance to glucocorticoid treatment
	Maltese *et al.* (2012)	200 young and adult IBD patients (100 CD/100 UC)	Systemic (prednisone)	BclI/ N363S ER22/ 23EK	– No differences between glucocorticoid responders and resisters
	Mwinyi *et al.* (2010)	173 adult IBD patients (84 CD/89 UC)	Not mentioned	N363S ER22/ 22EK	– No association with glucocorticoid therapy outcome
Nephrotic syndrome	Teeninga *et al.* (2014)	113 pediatric NS patients	Systemic (prednisolone)	BclI/ N363S ER22/ 23EK 9β	– BclI carriers tended towards a better therapeutic outcome; – 9β polymorphism associated with steroid dependency and worse therapeutic outcome; – ER22/23EK and N363S not assessed due to low allele frequencies
	Zalewski *et al.* (2008)	118 pediatric NS patients	Systemic (prednisone)	BclI/	– Shorter time to proteinuria resolution in carriers of BclI variant in combination with rs33389 polymorphism (not in single BclI carriers)
Retinal diseases	Gerzenstein *et al.* (2008)	52 elderly patients	Intravitreal (triamcinolone acetonide)	BclI/ N363S ER22/ 23EK	– No association between BclI variant and degree of intraocular pressure change; – N363S and ER22/23EK not included in analyses
Rheumatoid arthritis	Quax *et al.* (2012)	147 female (pregnant) rheumatoid arthritis patients	Systemic (prednisone)	BclI/ N363S ER22/ 23EK 9β	– Glucocorticoid treatment in group of BclI and/or N363S carriers resulted in lower postpartum disease activity scores in comparison to treated group of 9β or 9β with ER22/23EK carriers
Photorefractive keratectomy	Szabo *et al.* (2007)	102 adult patients undergoing photorefractive keratectomy	Ocular (fluorometholone, prednisolone acetate)	BclI/ N363S ER22/ 23EK	– Increased intraocular pressure with prednisolone treatment in N363S carriers; – No effect found for other variants

[a]Only SNPs as discussed in this chapter are mentioned.

Abbreviations: *ALL*, acute lymphoblastic leukemia; *CD*, Crohn's disease; *GR*, glucocorticoid receptor; *IT*, intrathecal; *NS*, nephrotic syndrome; *SNP*, single nucleotide polymorphism; *UC*, ulcerative colitis.

of GR polymorphisms in pediatric ALL patients. A good prednisone response, based on a defined concentration of <1000 leukemic blasts/μL in peripheral blood after treatment, showed no association with the BclI, N363S, and ER22/23EK variants (Tissing *et al.*, 2005). However, another study performing a similar combined treatment of prednisone with methotrexate in Chinese ALL patients showed BclI carriers more likely to be good responders to prednisone (Xue *et al.*, 2015). In contrast, a larger Canadian study comprising more than 300 ALL patients treated with different treatment protocols showed lower event-free survival in BclI carriers, with homozygotes having also a reduced overall survival. The researchers also observed a lower event-free survival in 9β carriers (Labuda *et al.*, 2010). Fleury et al. additionally found homozygous BclI carriers to have a decreased overall survival probability (Fleury *et al.*, 2004). Concerning the N363S variant, carriers were more likely to be good responders to prednisone and have higher event-free survival, but

were also more susceptible to develop hepatotoxicity (31.3% vs. 11.2%) and abnormalities in glucose metabolism (18.8% vs. 3.7%) when compared to noncarriers (Eipel *et al.*, 2013). Kaymak Cihan and colleagues also evaluated steroid-induced side effects in ALL patients and found Bcl*I* carriers to be more prone for developing dyspepsia, depression symptoms, and Cushingoid-like changes (e.g., adiposity, striae, diabetes mellitus) (Kaymak Cihan *et al.*, 2017), which fits with our previously observed associations of a relative glucocorticoid hypersensitivity (van Rossum *et al.*, 2003). Moreover, adrenal insufficiency after treatment with prednisone was found to be of longer duration in homozygous Bcl*I* carriers, whereas the opposite was found in patients with the ER22/23EK polymorphism (de Ruiter *et al.*, 2014). For adverse psychological events due to glucocorticoid treatment, no associations were found with the Bcl*I*, N363S, and ER22/23EK variants (Felder-Puig *et al.*, 2007).

Bronchial asthma is one of the main indications for glucocorticoid treatment, which also becomes clear giving the high prevalence of inhaled glucocorticoid use in the general population (Savas *et al.*, 2017). In asthmatic patients, contrasting results have been described regarding the presence of Bcl*I* variant. Pietras *et al.* found patients with asthma to be more likely to have the Bcl*I* polymorphism (Pietras *et al.*, 2011), whereas another study showed the opposite in severe asthmatic patients (Mohamed *et al.*, 2015). Within the asthmatic group, the latter also found the wild-type genotype to be more common in patients with a relatively good FEV_1 improvement (i.e., $>30\%$) after a two-week prednisolone course. Another study in glucocorticoid-naïve (for at least 3 months) asthmatic children showed heterozygous Bcl*I* carriers to have higher basal cortisol levels both before and after treatment with inhaled glucocorticoids. However, SNP interaction analyses with polymorphisms of other HPA axis related genes (i.e., *CRHR1* and *MC2R*) showed homozygous Bcl*I* carriers to have diminished cortisol levels when compared to wild-type and heterozygous carriers (Tsartsali *et al.*, 2012). Homozygous Bcl*I* carriers have also been described to have a better improvement in FEV_1 (24.2% vs. 7.9%) after treatment for asthma exacerbation in children (Keskin *et al.*, 2016). Regarding the N363S variant, carriers of the G allele were less likely to have uncontrolled asthma which the authors also linked to a better anti-inflammatory steroid response (Panek *et al.*, 2012). However, this association was not observed in another study with asthmatic children (Szczepankiewicz *et al.*, 2008).

Glucocorticoids are also essential in the treatment of inflammatory bowel diseases (IBD). High doses of prednisone are usually given to patients with moderate-to-severe Crohn's disease and ulcerative colitis in order to induce a disease remission. The response to glucocorticoid treatment varies significantly between patients with some showing complete remission to others not responding at all (Faubion *et al.*, 2001). De Iudicibus *et al.* investigated the differences in Bcl*I* genotype between glucocorticoid-dependent and responsive IBD children and found that patients from the latter group, i.e., without need for glucocorticoids 1 year after initial treatment, were more likely to have the Bcl*I* variant (De Iudicibus *et al.*, 2007, 2011). Another Italian study also investigated differences in regard to the Bcl*I* variant between glucocorticoid responders and resisters and did not find any association (Maltese *et al.*, 2012). However, clinical response was only evaluated in the short term with responders defined as achieving remission within one to 2 months. For the N363S and ER22/23EK polymorphisms, no associations were found with clinical response to steroid therapy (De Iudicibus *et al.*, 2007; Maltese *et al.*, 2012; Mwinyi *et al.*, 2010). One study showed the polymorphism rs6190, leading to amino acid change in ER22/23EK carriers, to be associated with resistance to glucocorticoid treatment (Krupoves *et al.*, 2011). However, a meta-analysis on the ER22/23EK, Bcl*I*, and N363S variants could not establish an association between any of the three polymorphisms with glucocorticoid resistance in IBD patients (Chen and Li, 2012).

The steroid response in relationship to GR polymorphisms has also been investigated in childhood nephrotic syndrome patients. Haplotype analysis of three intronic single nucleotide polymorphisms, including the Bcl*I* variant, was performed in 118 steroid treated nephrotic syndrome children. Combination of the Bcl*I* variant with a certain other SNP (rs33389) in the GR gene was more prevalent in patients who reached remission within 7 days after treatment with prednisone in comparison to late responders. However, this was not the case anymore for the Bcl*I* carriers in absence of the rs33389 polymorphism (Zalewski *et al.*, 2008). In another study with Dutch nephrotic syndrome patients, a trend towards a better therapeutic outcome was observed in children with the Bcl*I* haplotype, whereas the 9β variant was related to steroid dependency (52% vs. 25% in noncarriers) and worse therapeutic outcome (Teeninga *et al.*, 2014).

Moreover, the difference in effect of glucocorticoid treatment between the carriers of the mentioned variants has been assessed in female rheumatoid patients before, during, and after pregnancy. Disease activity scores in the postpartum period were significantly lower in treated patients with the Bcl*I* and/or N363S variants when compared to the relatively glucocorticoid resistant polymorphisms (Quax *et al.*, 2012). In regard to Bcl*I*, carrying this variant was not related with response to glucocorticoid treatment in immune thrombocytopenic patients (Xuan *et al.*, 2014) nor in patients with active Graves' orbitopathy (Vannucchi *et al.*, 2014), and was also not associated with change in intraocular pressure after intravitreal injection (Gerzenstein *et al.*, 2008). The N363S polymorphism tended towards beneficial effect in children with Duchenne muscular dystrophy concerning the age at loss of ambulation (Bonifati *et al.*, 2006) and was associated with increased intraocular pressure due to prednisolone droplets after laser eye surgery (photorefractive keratectomy) (Szabo *et al.*, 2007).

In summary, glucocorticoid treatment effects and adverse events seem to be altered to a certain extent in carriers of the relatively glucocorticoid sensitive and resistant GR polymorphisms, depending on disease, duration, and dose of the steroid therapy.

Conclusion

The GR is essential in the pathway of glucocorticoid action. Numerous polymorphisms of the GR have been described, however, only some of these have been extensively investigated. These SNPs can roughly be divided in relatively glucocorticoid sensitive (Bcl*I* and N363S) and glucocorticoid resistant (ER22/23EK and 9β) variants. Both in vivo and in vitro studies have shown differences in carriers

matching expectations of an altered glucocorticoid sensitivity. Not all findings are observed consistently though, which may be attributed to differences in study design, demographic characteristics (e.g., ethnicity, age, sex), and other factors influencing glucocorticoid action. Nevertheless, evidence is mounting that GR polymorphisms may indeed alter the glucocorticoid sensitivity leading to modest changes in somatic and mental characteristics as well as in response to glucocorticoid treatment.

See also: Glucocorticoid Receptor. Glucocorticoid Resistance Syndromes and States

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Relevant Website

https://www.ncbi.nlm.nih.gov/snp—Database of Single Nucleotide Polymorphisms (dbSNP).

Glucocorticoid Resistance Syndromes and States☆

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Nomenclature

AF	Activation function domain
AP-1	Activator protein-1
DBD	DNA-binding domain
GR	Glucocorticoid receptor
GREs	Glucocorticoid-response elements
GRIP1	Glucocorticoid receptor-interacting protein 1
hGRα	Human glucocorticoid receptor alpha
hGRβ	Human glucocorticoid receptor beta
HPA axis	Hypothalamic–pituitary–adrenal axis
HSP	Heat shock protein
LBD	Ligand-binding domain
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-κB
NRB	Nuclear receptor-binding domain
NTD	Amino-terminal domain
PI_3K	Phosphatidylinositol 3-kinase
STATs	Signal transducers and activators of transcription

Glossary

Glucocorticoids Steroid hormones, including cortisol, corticosterone, and cortisone, released by the cortex of the adrenal gland in a circadian fashion or during stress.

Receptor Protein molecules on the cell surface or inside the cell that recognizes a specific ligand or hormone.

Resistance Degree of nonresponsiveness to a particular hormone in the body. Also called insensitivity.

Introduction

Glucocorticoids are steroid hormones synthesized in the adrenal cortex and secreted into the circulation under the control of the hypothalamic–pituitary–adrenal (HPA) axis. These lipid molecules are major effectors of basal and stress-related homeostasis, influencing cardiovascular functions, intermediary metabolism, as well as the quantity, and quality of immune response (Nicolaides and Charmandari, 2017). All these fundamental biologic actions are mediated by specific intracellular receptor proteins, termed *glucocorticoid receptors* (GRs), which function as ligand-activated transcription factors (Nicolaides *et al.*, 2010).

Abnormalities of tissue glucocorticoid sensitivity can be divided into two major groups: resistance and hypersensitivity (Nicolaides *et al.*, 2016b). Complete glucocorticoid resistance is incompatible with life because of severe neonatal respiratory distress syndrome, as demonstrated in an in vivo study in which GR − / − knockout mice died within a few hours after birth. On the other hand, when partial glucocorticoid resistance occurs, the decreased tissue responsiveness to glucocorticoids causes compensatory activation of the HPA axis leading to hypersecretion of adrenocorticotropic hormone (ACTH) by the anterior lobe of pituitary gland (Nicolaides and Charmandari, 2015). The increased plasma ACTH concentrations result in adrenal cortex hypertrophy and increased secretion of cortisol (corticosterone in rodents) and cortisol precursors with mineralocorticoid activity [deoxycorticosterone (DOC) and corticosterone] and adrenal androgens (Nicolaides and Charmandari, 2015).

Primary familial or sporadic generalized glucocorticoid resistance or Chrousos syndrome has been described in 22 cases/kindreds and the molecular mechanisms of resistance in all of these patients have been elucidated using molecular and structural biology methods (reviewed in Nicolaides and Charmandari, 2017).

Molecular Actions of Glucocorticoids

At the cellular level, glucocorticoids carry out their actions through a ~94 kDa intracellular receptor protein, the GR (Nicolaides *et al.*, 2010). Since 1985, when the sequence of GR cDNA was first published, two alternative splicing products of the same gene located on chromosome five have been described: the GRα and the GRβ. The two receptors are highly homologous, differing in just the last 50 and 15 amino acids, respectively (Chrousos and Kino, 2005). Therefore, the first eight exons of the *NR3C1* gene containing the 5′ noncoding and coding sequences are common to both receptor isoform cDNAs, whereas exons 9α and 9β containing the coding and 3′ noncoding sequences are specific for GRα and GRβ, respectively. The GRα and β receptor isoforms

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have different properties in terms of ligand-binding ability, cellular localization and transcriptional activity (Chrousos and Kino, 2005). In addition to these two main isoforms, it was shown that the hGRα mRNA translation might be initiated from eight alternative sites giving rise to eight hGRα isoforms (hGRα-A, hGRα-B, hGRα-C1, hGRα-C2, hGRα-C3, hGRα-D1, hGRα-D2, and hGRα-D3) (Lu and Cidlowski, 2005). We hypothesize that similar molecular mechanisms might exist for the translation of hGR*β* mRNA into eight translational receptor isoforms.

In the absence of the ligand, the hGRα resides primarily in the cytoplasm of cells in a multiprotein complex consisting of the receptor polypeptide, heat shock proteins (HSPs), and immunophillins (**Fig. 1**) (Grad and Picard, 2007). The HSPs are thought to allow proper folding and stabilization of the receptor, maintaining the latter in a high-affinity, hormone-binding friendly state and preventing its interaction with proteins of the importin system, which are involved in the cytoplasm-to-nucleus translocation of many proteins (Pratt and Toft, 1997). Experimental evidence supports a model of constant bidirectional shuttling of the complex in the unliganded state between the cytoplasm and the nucleus. Once the hormone binds, the receptor dissociates from the multiprotein complex and homo- or hetero-dimerizes with another translational or alternative splicing isoform. This complex interacts with the importin system and translocates via the nuclear pore into the nucleus, where it binds to specific DNA sequences located in the promoter regions of glucocorticoid-responsive genes, termed *glucocorticoid response elements* (GREs), influencing the transcription rate of numerous genes in a positive or negative fashion (Chrousos and Kino, 2005; Nicolaides *et al.*, 2010; Nicolaides and Charmandari, 2017). Alternatively, the hGRα can modulate target gene transcription possibly as a monomer via protein–protein interactions with other transcription factors, such as activator protein-1 (AP-1), nuclear factor KB (NF-κB), and signal transducers and activators of transcription (STATs) (**Fig. 1**) (Barnes and Karin, 1997; Karin and Chang, 2001; Didonato *et al.*, 1996).

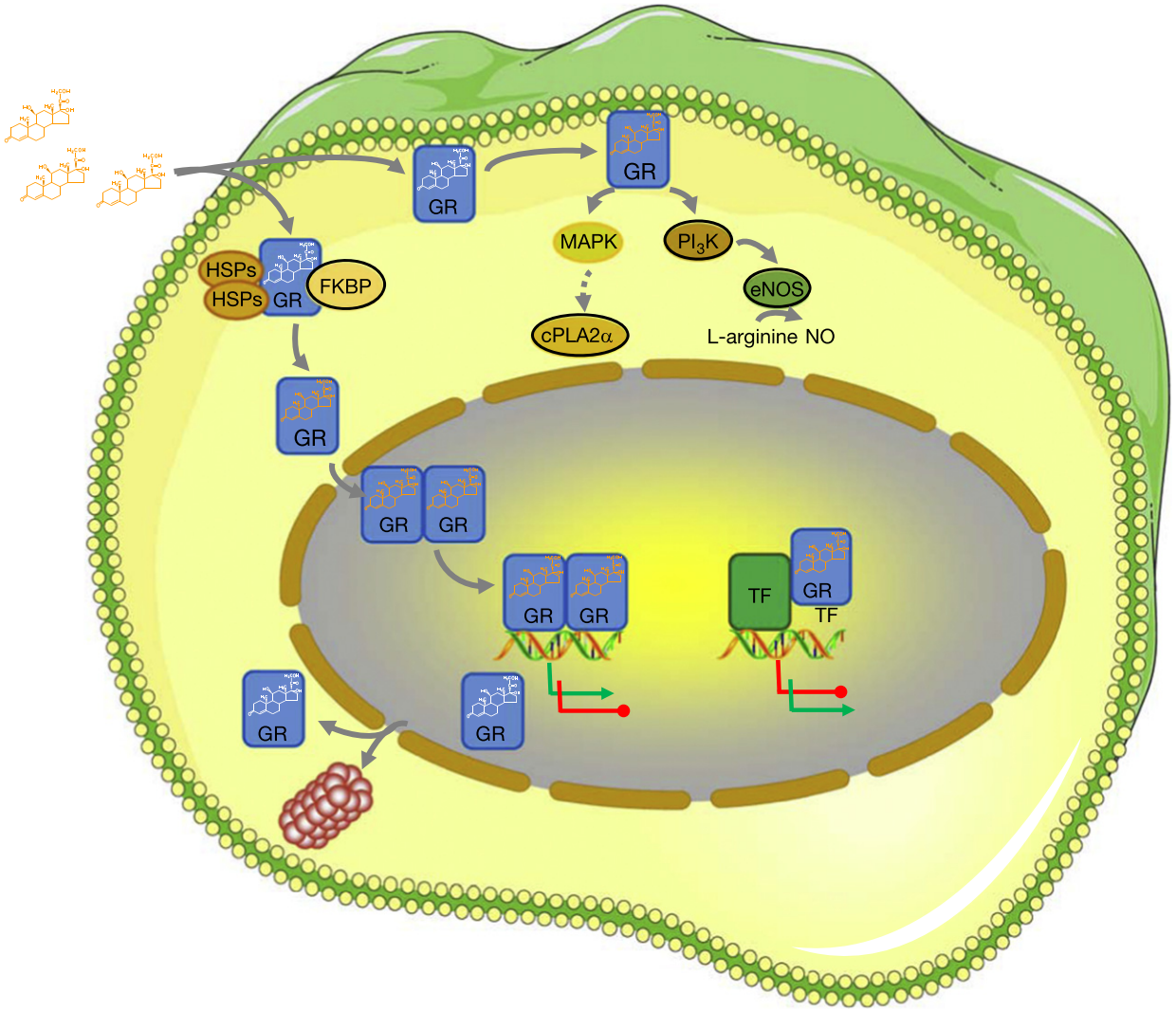

Fig. 1 Genomic and nongenomic actions of glucocorticoids. *cPLA2α*, Cytosolic phospholipase A2 alpha; *eNOS*, endothelial nitric oxide synthetase; *FKBP*, immunophillins; *GR*, glucocorticoid receptor; *HSPs*, heat shock proteins; *MAPK*, mitogen-activated protein kinases; *NO*, nitric oxide; *PI_3K*, phosphatidylinositol 3-kinase; *TF*, transcription factor.

In contrast to hGRα, the hGR*β* is localized in the nucleus of certain types of cells, such as endothelial cells, and functions as a dominant negative regulator of hGRα transcriptional activity (Bamberger *et al.*, 1995). Importantly, the hGR*β* was recently shown to have intrinsic transcriptional activity independently of hGRα (Kino *et al.*, 2009). Moreover, accumulating evidence suggests that this isoform plays an important role in insulin signaling, gluconeogenesis, glioma formation and bladder cancer cells migration (He *et al.*, 2015; Yin *et al.*, 2013; McBeth *et al.*, 2016).

In addition to genomic actions, growing evidence suggests that glucocorticoids can induce nongenomic effects through membrane-bound GRs, which upon ligand-binding, activate specific kinase signaling pathways, such as mitogen-activated protein kinases (MAPKs) or phosphatidylinositol 3-kinase (PI_3K) (**Fig. 1**) (Groeneweg *et al.*, 2012). Apart from their short time frame, these effects are pharmacologically resistant to transcriptional or translational inhibitors and can occur in nonnucleated cells, such as red blood cells (Stellato, 2004).

Molecular Mechanisms of Glucocorticoid Resistance

The molecular basis of glucocorticoid resistance has been ascribed to mutations in the *NR3C1* gene that impair one or more of the molecular mechanisms of GR function, thus altering tissue sensitivity to glucocorticoids (**Fig. 2**) (Chrousos, 2011; Charmandari, 2012; Charmandari *et al.*, 2013; Nicolaides *et al.*, 2014b). Inactivating mutations within the DNA- and ligand-binding domains, as well as a 4 bp mutation at the 3′ boundary of the *NR3C1* gene, have been identified and functionally characterized (**Table 1**).

In 1976, Vingerhoeds *et al.* described the first two patients, a father and a son, with long-term "hypercortisolism" not associated with clinical manifestations of Cushing syndrome (Vingerhoeds *et al.*, 1976). In 1982, Chrousos *et al.* demonstrated that these patients had abnormal glucocorticoid receptor properties and suggested that they suffered from glucocorticoid resistance (Chrousos *et al.* 1982). In 1991, the Chrousos group described the molecular mechanism underlying the disease in this family: the propositus was a homozygote for a single non-conservative point mutation, replacing aspartate at amino acid position 641 with valine, in the ligand-binding domain of the GR (Hurley *et al.*, 1991). This mutation reduced glucocorticoid receptor binding affinity for dexamethasone by threefold and caused loss of transactivation activity on the mouse mammary tumor virus (MMTV) promoter (Hurley *et al.*, 1991).

The second family was described in 1993 by the Chrousos group as well; the proposita of this family was a young woman with manifestations of hyperandrogenism (Karl *et al.*, 1993). Molecular analysis showed a 4 bp deletion at the 3′ boundary of exon 6, removing a donor splice site. This resulted in complete ablation of expression of one of the GR alleles associated with a 50% decrease in GR protein in the affected members of the family (Karl *et al.*, 1993).

The propositus of the third kindred, a boy with peripheral precocious puberty, had a single homozygotic point mutation at amino acid 729 (valine to isoleucine) in the ligand-binding domain, which reduced both the affinity and the transactivation activity of the GR (Malchoff *et al.*, 1993). Moreover, the hGRαV729I showed delayed nuclear translocation following dexamethasone binding, and impaired interaction with the glucocorticoid receptor-interacting protein (GRIP)1 coactivator (Malchoff *et al.*, 1993; Charmandari *et al.*, 2004).

In 1996, another interesting, sporadic case of a man with a history of infertility, hypertension, and 5- to 10-fold elevation of urinary free-cortisol levels was described by the Chrousos group (Karl *et al.*, 1996). This patient had a de novo, germ-line heterozygotic mutation at amino acid 559 (isoleucine to asparagine) in the ligand-binding domain, at the hinge region of the GR. This receptor had a two- to threefold more potent dominant negative activity than GR*β* on the wild-type receptor and was expressed at a 1:1 ratio with the normal GRα in the patient's cells. Interestingly, this mutant receptor prevented translocation of the normal receptor into the nucleus, an effect that would be overcome at very high dexamethasone concentrations (Karl *et al.*, 1996;

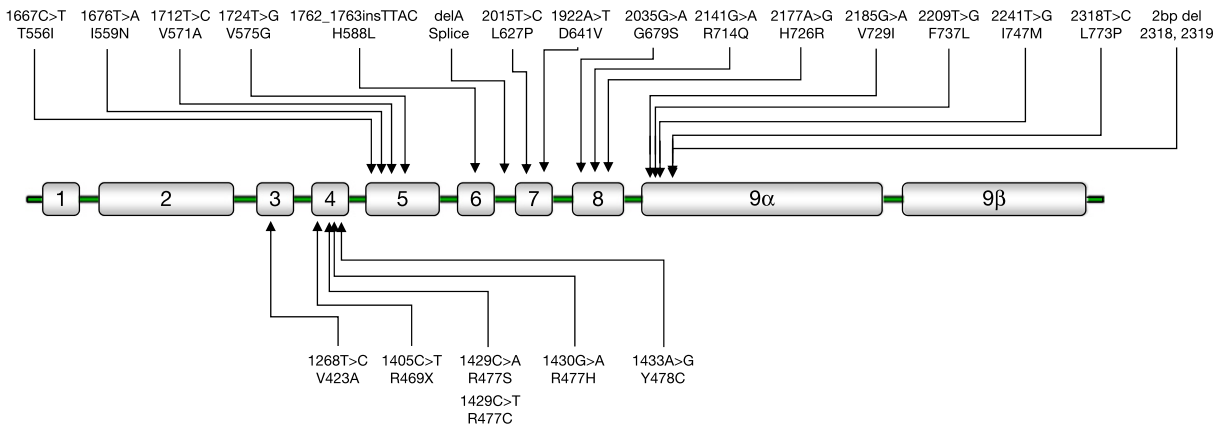

Fig. 2 Schematic representation of the known mutations of the *NR3C1* gene causing Chrousos syndrome. Mutations in the upper panel are located in the LBD of the receptor, while mutations in the lower panel are located in the DBD of the receptor.

Table 1 Mutations of the human glucocorticoid receptor gene causing Chrousos syndrome

References	*Mutation position*		*Molecular mechanisms*	*Genotype*	*Phenotype*
	cDNA	*Amino acid*			
Chrousos *et al.* (1982) Hurley *et al.* (1991) Charmandari *et al.* (2004)	1922 (A→T)	641 (D→V)	Transactivation ↓ Affinity for ligand ↓ (×3) Nuclear translocation: 22 min Abnormal interaction with GRIP1	Homozygous	Hypertension Hypokalemic alkalosis
Karl *et al.* (1993)	4 bp deletion in exon-intron 6		hGRα number: 50% of control Inactivation of the affected allele	Heterozygous	Hirsutism Male-pattern hair-loss Menstrual irregularities
Malchoff *et al.* (1993) Charmandari *et al.* (2004)	2185 (G→A)	729 (V→I)	Transactivation ↓ Affinity for ligand ↓ (×2) Nuclear translocation: 120 min Abnormal interaction with GRIP1	Homozygous	Precocious puberty Hyperandrogenism
Karl *et al.* (1996) Kino *et al.* (2001) Charmandari *et al.* (2004)	1676 (T→A)	559 (I→N)	Transactivation ↓ Decrease in hGR binding sites Transdominance (+) Nuclear translocation: 180 Abnormal interaction with GRIP1	Heterozygous	Hypertension Oligospermia Infertility
Ruiz *et al.* (2001) Charmandari *et al.* (2006)	1430 (G→A)	477 (R→H)	Transactivation ↓ No DNA binding Nuclear translocation: 20 min	Heterozygous	Hirsutism Fatigue Hypertension
Ruiz *et al.* (2001) Charmandari *et al.* (2006)	2035 (G→A)	679 (G→S)	Transactivation ↓ Affinity for ligand ↓ (×2) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Hirsutism Fatigue Hypertension
Mendonca *et al.* (2002) Charmandari *et al.* (2004)	1712 (T→C)	571 (V→A)	Transactivation ↓ Affinity for ligand ↓ (×6) Nuclear translocation: 25 min Abnormal interaction with GRIP1	Homozygous	Ambiguous genitalia Hypertension Hypokalemia Hyperandrogenism
Vottero *et al.* (2002) Charmandari *et al.* (2004)	2241 (T→G)	747 (I→M)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Cystic acne Hirsutism Oligo-amenorrhea
Charmandari *et al.* (2005a,b)	2318 (T→C)	773 (L→P)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×2.6) Nuclear translocation: 30 min Abnormal interaction with GRIP1	Heterozygous	Fatigue Anxiety Acne Hirsutism Hypertension
Charmandari *et al.* (2007)	2209 (T→C)	737 (F→L)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×1.5) Nuclear translocation: 180 min	Heterozygous	Hypertension Hypokalemia
McMahon *et al.* (2010)	2 bp deletion 773 at nt 2318-9		Transactivation ↓ Affinity for ligand: absent No suppression of IL-6	Homozygous	Hypoglycemia Fatigability with feeding Hypertension
Nader *et al.* (2010)	2141 (G→A)	714 (R→Q)	Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hypoglycemia Hypokalemia Hypertension Mild clitoromegaly Advanced bone age Precocious pubarche
Bouligand *et al.* (2010)	1405 (C→T)	469 (R→X)	Transactivation ↓ Ligand-binding sites ↓ No DNA binding No nuclear translocation	Heterozygous	Adrenal hyperplasia Hypertension Hypokalemia
Zhu *et al.* (2011) Nicolaides *et al.* (2016a,b)	1667 (G→T)	556 (T→I)	Transactivation ↓ Transrepression ↑ Affinity for ligand ↓ Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Adrenal incidentaloma

(*Continued*)

Table 1 Continued

References	Mutation position		Molecular mechanisms	Genotype	Phenotype
	cDNA	Amino acid			
Roberts *et al.* (2013)	1268 (T→C)	423 (V→A)	Transactivation ↓ Affinity for ligand: N No DNA binding Nuclear translocation: 35 min Interaction with GRIP1: N	Heterozygous	Fatigue Anxiety Hypertension
Nicolaides *et al.* (2014a,b)	1724 (T→G)	575 (V→G)	Transactivation ↓ Transrepression ↑ Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Melanoma Asymptomatic daughters
Nicolaides *et al.* (2015)	2177 (A→G)	726 (H→R)	Transactivation ↓ Transrepression ↓ Affinity for ligand ↓ (×2) Nuclear translocation ↓ Abnormal interaction with GRIP1	Heterozygous	Hirsutism, acne Alopecia, anxiety Fatigue Irregular menstrual cycles
Velayos *et al.* (2016)	1429 (C→T)	477 (R→C)	Not studied yet	Heterozygous	Mild hirsutism Asymptomatic mother
Velayos *et al.* (2016)	1762_1763insTTAC	588 (H→L*5)	Not studied yet	Heterozygous	Hirsutism, anxiety Chronic fatigue
Vitellius *et al.* (2016)	1429 (C→A)	477 (R→S)	No transactivation Affinity for ligand: N No DNA binding Nuclear translocation ↓	Heterozygous	Adrenal incidentaloma
Vitellius *et al.* (2016)	1433 (A→G)	478 (Y→C)	Transactivation ↓ Affinity for ligand: N DNA binding ↓ Nuclear translocation ↓	Heterozygous	Adrenal incidentaloma
Vitellius *et al.* (2016)	2015 (T→C)	672 (L→P)	No transactivation No affinity for ligand No DNA binding No nuclear translocation	Heterozygous	Adrenal incidentaloma

Kino *et al.*, 2001; Charmandari *et al.*, 2004). Later this patient developed Cushing disease due to an ACTH-secreting pituitary adenoma.

Since then, a few novel cases of generalized glucocorticoid resistance have been attributed to inactivating point mutations in the *NR3C1* gene (**Table 1**). Our group recently identified three novel heterozygous *NR3C1* mutations and proceeded to the functional characterization of these gene defects (Roberts *et al.*, 2013; Nicolaides *et al.*, 2014a, 2015). The first patient, a 9-year old boy with a history of anxiety, fatigue and hypertension, harbored a mutation in the *NR3C1* gene, which resulted in substitution of valine (V) by alanine (A) at amino acid position 423 in the DNA-binding domain (DBD) of the receptor (Roberts *et al.*, 2013). Compared to the wild-type receptor, the hGRαV423A showed reduced transcriptional activity due to decreased ability to bind to the promoter regions of glucocorticoid-responsive genes, and required longer time to fully complete nuclear translocation following activation (Roberts *et al.*, 2013). Structural biology studies demonstrated that the substitution of the hydrophobic valine by alanine permits diffusion of water molecules into the ion-binding region of the mutant receptor, ultimately leading to reduced DNA-binding of the mutant receptor (Roberts *et al.*, 2013).

The second *NR3C1* mutation that was recently reported by our group was found in a 70-year-old man and his two daughters who had increased urinary free cortisol excretion without any symptoms or signs suggestive of Cushing syndrome (Nicolaides *et al.*, 2014a). All of them carried a point mutation in the *NR3C1* gene that caused a substitution of valine (V) by glycine (G) at amino acid 575 in the LBD of the receptor. The hGRαV575G displayed lower affinity for dexamethasone, demonstrated reduced transcriptional activity, had a 2.5-fold delay in nuclear translocation, and interacted with the GRIP1 coactivator mostly through its AF1 domain, since the substitution of valine by glycine at this amino acid position led to the loss of two noncovalent bonds formed between the valine of the wild-type receptor and the LXXLL motif of the GRIP1 coactivator (Nicolaides *et al.*, 2014a).

We lastly described a 30-year-old woman with a long-lasting history of hirsutism, acne, alopecia, anxiety, fatigue and irregular menstrual cycles, who harbored an inactivating *NR3C1* mutation that resulted in histidine (H) to arginine (R) substitution at amino acid position 726 in the LBD of the receptor (Nicolaides *et al.*, 2015). The mutant receptor hGRαH726R causes generalized glucocorticoid resistance through several molecular mechanisms, such as reduced transactivation, decreased affinity for the ligand, delayed nuclear translocation, and interaction with the GRIP1 coactivator through its AF1 domain (Nicolaides *et al.*, 2015).

Structural biology studies demonstrated that the H726R mutation resulted in a structural shift in the rigidity of helix 10 in the LBD of the receptor, leading to reduced flexibility and decreased affinity of the defective receptor for the ligand (Nicolaides *et al.*, 2015).

We also investigated the molecular mechanism of action of the mutant receptor hGRαT556I, which was identified in a 56-year old man with an adrenal incidentaloma (Zhu *et al.*, 2011; Nicolaides *et al.*, 2016a). We demonstrated that the hGRαT556I caused tissue resistance to glucocorticoids through decreased affinity for dexamethasone, delay in nuclear translocation following dexamethasone-induced activation, and impaired interaction with the GRIP1 coactivator, ultimately causing reduced ability to transactivate glucocorticoid-responsive genes (Nicolaides *et al.*, 2016a). The T556I mutation caused a disruption of the hydrogen bond between threonine with the =O group of P637 backbone, leading to decreased ligand binding of the mutant receptor and abnormal interaction with the GRIP1 coactivator (Nicolaides *et al.*, 2016a).

Velayos and collaborators identified recently two novel point mutations in the *NR3C1* gene in three patients (Velayos *et al.*, 2016). The first mutation was a cytosine to thymine substitution (1429C→T) in exon 4, causing an arginine to cysteine (R→C) substitution at amino acid position 477 in the DBD of the receptor (Velayos *et al.*, 2016). The second *NR3C1* genetic defect was an insertion of four bases between nucleotides 1762 and 1763, which caused a substitution of four amino acid residues at positions 588–591, leading to a truncated protein (Velayos *et al.*, 2016).

Vitellius *et al.* described three patients with adrenal incidentalomas and elevated glucocorticoid concentrations without any stigmata of Cushing syndrome (Vitellius *et al.*, 2016). The R477S and the Y478C mutations were located in the DBD, whereas the L672P was found in the LBD of the receptor. In vitro studies showed that the mutant receptors hGRαR477S and hGRαY478C displayed decreased ability to transactivate glucocorticoid target genes, reduced ability to bind to DNA and a significant delay in nuclear translocation following ligand-binding. In terms of structural biology, the hGRαR477S lost two hydrogen bonds with GREs, whereas the hGRαY478C displayed impaired interaction with neighboring amino acids (Vitellius *et al.*, 2016). The hGRαL672P was not able to influence glucocorticoid target genes, had reduced ligand binding and maintained exclusively its cytoplasmic localization. Structural biology assays showed that the hGRαL672P caused a conformational change of helix 8 leading to glucocorticoid resistance (Vitellius *et al.*, 2016).

The importance of GRβ under physiologic conditions is controversial, but it has been proposed that in pathologic situations, such as glucocorticoid resistance or hypersensitivity, it might play a pathophysiologic role. Taking into consideration the finding that increasing amounts of GRβ produce a dose-dependent decrease in wild-type GRα transcriptional activity, an imbalance in the expression of these two isoforms might determine an altered sensitivity to glucocorticoids. Supporting a possible role of GRβ in glucocorticoid sensitivity, a genetically determined imbalance in the expression of the glucocorticoid receptor isoforms was observed in cultured lymphocytes from a patient with congenital generalized glucocorticoid resistance and chronic leukemia (Shahidi *et al.*, 1999). In this patient, a low GRα to GRβ ratio was found compared to a group of normal controls, possibly explaining the glucocorticoid resistance, since no abnormalities in the sequence of the entire cDNA or in individual exons of this patient's gene were found (Shahidi *et al.*, 1999). In keeping with these findings, significantly higher hGRβ expression levels are associated with glucocorticoid resistance in numerous inflammatory disorders, including rheumatoid arthritis, systemic lupus erythematosus, asthma, and ulcerative colitis, as well as in several mood disorders, such as major depression, schizophrenia (Lewis-Tuffin and Cidlowski, 2006; Ramamoorthy and Cidlowski, 2016).

Animal models of systemic glucocorticoid resistance, such as New World primates, including squirrel monkeys, marmosets and owl monkeys, have been described (Chrousos *et al.*, 1983, 1986). These animals have total plasma cortisol levels that are 7–20 times higher than in humans or other Old World primates, whereas the concentration, affinity, and predicted amino acid sequence of their GRs are similar to those of the human receptor. Interestingly, these animals exhibit resistance to a variety of other steroid/sterol hormones, including estrogens, progesterone, androgens, aldosterone, and vitamin D (Chrousos *et al.*, 1983, 1986). Immunoreactivity of both isoforms of the GR has been found in Epstein-Barr virus-transformed B lymphocytes from marmosets, with the β-isoform being ~10 times overexpressed compared to the corresponding human cells. An altered splicing pattern of the GR pre-mRNA or differential rates of mRNA translation, mRNA degradation, and/or GR protein degradation might contribute to the steroid resistance of these animals. Alternatively, these animals may have decreased coactivator activity and/or increased corepressor activity, leading to their "pansteroid" resistance (Chrousos *et al.*, 1986).

Two sisters with manifestations of glucocorticoid resistance were described by New and collaborators (1999). Their evaluation revealed resistance not only to glucocorticoids, but also to mineralocorticoids and androgens; however, they displayed no resistance to vitamin D or thyroid hormones. The diagnosis of these patients was multiple, partial steroid resistance (New *et al.*, 1999). The New World primate physiologic and biochemical syndrome and the two pathologic human multiple steroid resistance syndrome cases are the first conditions in which a defective steroid receptor coregulator has been suggested to be responsible for an altered clinical and/or biochemical picture.

Clinical Manifestations of Glucocorticoid Resistance

Patients with the primary glucocorticoid resistance syndrome have compensatory elevations in circulating cortisol and ACTH concentrations, which maintain circadian rhythmicity and appropriate responsiveness to stressors, albeit at higher hormone concentrations (Charmandari, 2011, 2012; Charmandari *et al.*, 2013; Nicolaides and Charmandari, 2015; Nicolaides *et al.*, 2016b). They also have resistance of their HPA axis to dexamethasone suppression, but no clinical evidence of overt hypo- or

hypercortisolism. The excess of ACTH results in increased production of adrenal steroids with mineralocorticoid and/or androgenic activity (Charmandari, 2011, 2012; Charmandari, *et al.* 2013; Nicolaides and Charmandari, 2015; Nicolaides *et al.*, 2016b).

The clinical spectrum of this disease is quite broad, ranging from completely asymptomatic to mild to severe symptomatic conditions. A large number of subjects may be asymptomatic displaying biochemical alterations only. Clinical manifestations due to the excess of mineralocorticoids (including cortisol itself), acting on the intact mineralocorticoid receptor of the patients, are hypertension with or without hypokalemic alkalosis (Charmandari, 2011, 2012; Charmandari, *et al.* 2013; Nicolaides and Charmandari, 2015; Nicolaides *et al.*, 2016b). The increased amounts of androgens, on the other hand, lead to acne, hirsutism, male pattern baldness, menstrual irregularities (oligoamenorrhea), oligoanovulation, and infertility in women. In children, early and excessive prepubertal adrenal androgen secretion has been associated with ambiguous genitalia and peripheral precocious puberty. In adult men, oligospermia and infertility have been observed, possibly as the result of interference with follicle-stimulating hormone feedback regulation by the excessive adrenal androgens or by the ACTH-induced intratesticular growth of adrenal rests, which may occur as they do in classic and "late-onset" congenital adrenal hyperplasia. Because of the excessive secretion of adrenal androgens and decreased glucocorticoid effects, bone mass density is usually high-normal to elevated in patients with glucocorticoid resistance, in contrast to patients with Cushing syndrome, in whom osteoporosis is observed (Charmandari, 2011, 2012; Charmandari *et al.*, 2013; Nicolaides and Charmandari, 2015; Nicolaides *et al.*, 2016b).

Diagnostic Approach

The hallmark of the diagnostic evaluation of glucocorticoid resistance is increased 24-h serum cortisol concentrations and 24-h urinary free-cortisol (UFC) excretion without Cushing syndrome clinical stigmata (Charmandari, 2011, 2012; Charmandari *et al.*, 2013; Nicolaides and Charmandari, 2015; Nicolaides *et al.*, 2016b). Importantly, patients may have significant variations in the 24-h serum cortisol concentrations and UFC excretion because of variations in the impairment of glucocorticoid signal transduction. Serum cortisol concentrations may be up to sevenfold higher than the highest value of its normal range, while the 24-h UFC excretion may be up to 50-fold higher when compared with the upper normal range (Nicolaides and Charmandari, 2015). Despite cortisol excess, plasma ACTH concentration is normal or high. The circadian rhythm of cortisol and its responsiveness to stress are intact in patients with glucocorticoid resistance who are also resistant to single or multiple doses of dexamethasone.

The dexamethasone suppression test remains one of the most useful tools to evaluate the responsiveness of the HPA axis to exogenous glucocorticoids, as well as to determine the appropriate pharmacologic dose to be administered when treatment will be commenced. Therefore, dexamethasone at increasing concentrations (0.3, 0.6, 1.0, 1.5, 2.0, 2.5, 3.0 mg) are administered per os at midnight every other day, and serum cortisol and dexamethasone concentrations are determined at 08:00 h the following morning. To effectively suppress serum cortisol concentrations by 50%, dexamethasone should be administered at a dose up to 7.5-fold higher than that required to achieve the same degree of HPA axis suppression in normal subjects (Nicolaides and Charmandari, 2015).

Therapeutic Management

Asymptomatic, normotensive subjects with primary glucocorticoid resistance do not require any treatment. In contrast, patients with symptomatic generalized glucocorticoid resistance are treated with high, individualized doses of oral mineralocorticoid-sparing synthetic glucocorticoids, such as dexamethasone (1–3 mg given once daily at night) to effectively activate the mutant and/or wild-type hGRα, and suppress the endogenous secretion of ACTH (Charmandari, 2011, 2012; Charmandari *et al.*, 2013; Nicolaides and Charmandari, 2015; Nicolaides *et al.*, 2016b).

Hypertensive patients should receive the smallest dose that lowers the serum concentration of cortisol and other mineralocorticoids and corrects electrolyte abnormalities. Hirsute patients should be treated with doses able to reduce the androgen excess.

Untreated patients have no risk of adrenal insufficiency and do not need extra doses of dexamethasone in particularly stressful situations, such as surgery and illness. In contrast, patients undergoing chronic treatment should receive the appropriate glucocorticoid coverage.

See also: Glucocorticoid Receptor. Impact of Glucocorticoid Receptor Polymorphisms on Glucocorticoid Action

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X-Linked Adrenoleukodystrophy/Adrenomyeloneuropathy☆

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Glossary

Leukodystrophy A heritable and progressive disorder of the cerebral white matter.

Peroxisome A membrane-enclosed organelle characterized by the presence of catalase and an enzyme system for fatty acid β-oxidation.

Very-long-chain fatty acid A fatty acid that is 22 or more carbons long.

Clinical Features

Patients with X-ALD are free of symptoms in infancy and early childhood. This is in contrast to neonatal adrenoleukodystrophy, which is a distinctly different disease that bears similarity to X-ALD in its name and some biochemical features. Neonatal adrenoleukodystrophy will not be discussed here.

Childhood Cerebral ALD

The most severe X-ALD phenotype, referred to as childhood cerebral ALD (CCALD), constitutes approximately one-third of the cases. CCALD is marked by prominent central nervous system demyelination that usually develops between 5 and 12 years of age. Affected boys initially present with subtle cognitive symptoms of inattentiveness, declining school performance, emotional lability, staring spells, or behavioral changes. The diagnosis is often delayed, however, until more overt neurologic symptoms appear, such as gait abnormalities, declining vision, deteriorating handwriting, or rarely a seizure. Patients show a rapidly progressive downhill course, leading to blindness, loss of speech, feeding problems, and a vegetative state within 2–5 years. From this point, time of survival time is variable. Magnetic resonance imaging (MRI) of the brain typically shows white matter disease involving the parieto-occipital lobes with a periventricular distribution (Fig. 1A). White matter disease can be seen on MRI prior to the onset of neurologic symptoms and it typically progresses in the early phase of the disease along with symptoms. The presence

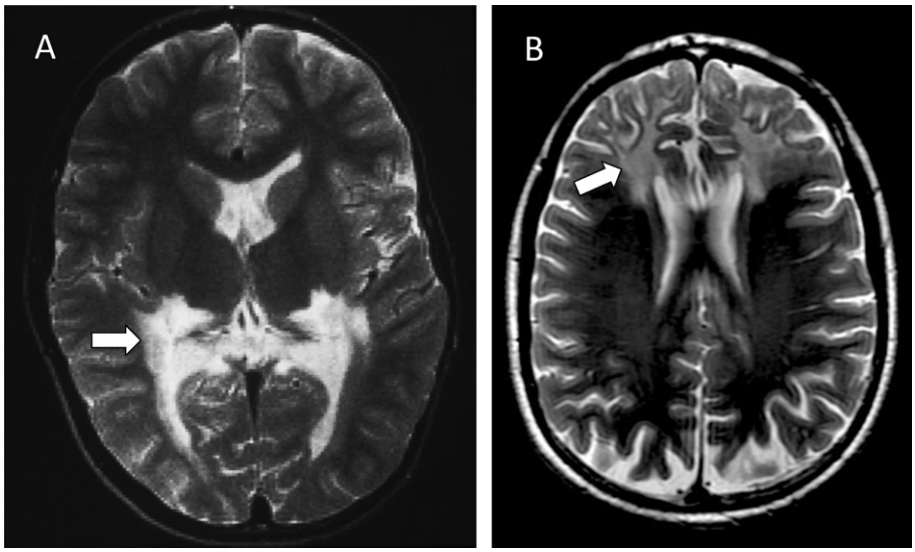

Fig. 1 Brain MRI of X-ALD patients with the typical parieto-occipital white matter disease (A) or a more frontal involvement (B). Arrows point to regions of white matter disease on T2-weighted images.

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of gadolinium-enhancing lesions on MRI is a characteristic finding early in the disease. Patients with prominent hyperactivity and behavioral abnormalities may have a more frontal lobe involvement (Fig. 1B). Visual-evoked potentials and brainstem auditory-evoked responses (BAERs) are usually abnormal.

Adrenomyeloneuropathy

Adrenomyeloneuropathy (AMN) accounts for at least one-half of all X-ALD cases. It has its neurologic onset usually in the second to fifth decade of life and is dominated by a slowly progressive spinal cord involvement with spastic paraparesis and peripheral neuropathy. Neurologic symptoms are insidious at onset. Leg stiffness on awakening in the morning is a frequent early symptom of the spastic paraparesis that ultimately ensues. In time, patients develop a worsening gait, leg scissoring, urinary retention, and impotence as the neurologic disease progresses. Hyperreflexia in the lower extremities with a positive Babinski sign is elicited in all patients. Peripheral neuropathy is manifest in the lower extremities with relative sparing of the hands. Patients show an abnormal wear pattern to their shoes. In most cases, the brain MRI is normal at the time of initial diagnosis, but approximately 50% of AMN patients ultimately develop cerebral white matter involvement and carry a worse prognosis. MRI of the spine shows non-specific atrophy. Somatosensory-evoked potentials and BAERs are often abnormal and nerve conduction velocities in the lower extremities may be delayed. As the neurologic disease progresses over the course of 10–30 years, cerebral involvement becomes more apparent in many AMN patients and signs of subcortical dementia supervene. Unrelated to the neurologic or adrenal disease, AMN patients frequently develop premature thinning of their scalp hair.

Other Forms of X-ALD

In addition to CCALD and AMN, there are several less common ALD phenotypes. Some males have adolescent or adult-onset forms of cerebral X-ALD, with a rapid progression of neurologic disease similar to that seen in CCALD. A small number (5%) of X-ALD patients exhibit Addison's disease only and lack neurologic symptoms. By screening families of X-ALD probands, a significant proportion of asymptomatic males who carry the gene, but lack neurologic or adrenal symptoms have been identified. Most of the males who are beyond 12 years of age are destined to develop AMN or other neurologic variants as they get older.

Although males express the full spectrum of symptoms, as many as 65% of female heterozygotes ultimately develop mild spastic paraparesis, similar to AMN males, in the fourth to sixth decades of life. Most of these show subtle signs of neurologic disease, such as hyperreflexia and decreased vibratory sensation in the lower extremities. The neurologic involvement tends to progress very slowly over decades. Some females will develop spastic paraparesis as severe as that seen in affected men. However, female heterozygotes rarely develop adrenal insufficiency.

A perplexing observation is the lack of consistency in the X-ALD phenotypes within families. Approximately one-half of X-ALD kindreds have male patients affected with CCALD and AMN, even though these patients share the same ALD mutation. Furthermore, identical twins have been reported to show divergent phenotypes. This argues strongly for the presence of other genetic, epigenetic or environmental factors that modify the clinical expression of X-ALD. Genetic studies implicate the existence of one or more modifier genes, which have yet to be convincingly identified.

Adrenal Insufficiency and Testicular Dysfunction

Adrenal insufficiency is a characteristic finding of X-ALD that may develop years prior to onset of neurologic disease or afterwards. There is no apparent correlation between the neurologic disease and adrenal symptoms. At the time of diagnosis, approximately 30% of CCALD patients have a history of adrenal symptoms, such as multiple episodes of hyponatremia and dehydration, prolonged recovery from general anesthesia or other stress, or rarely hypoglycemia. At least 80% of patients will exhibit frank adrenal insufficiency or diminished adrenal reserve on provocative testing. Hyperpigmentation may be present, although it is often missing at the time CCALD is diagnosed.

Approximately 30–50% of AMN men have normal adrenal function at the time of diagnosis. In those with adrenal insufficiency, hyperpigmentation is a common sign and some patients relate a lifelong history of resistance to sunburn. The adrenal insufficiency may be heralded by psychiatric problems, electrolyte disturbances, or overt Addisonian crises.

In addition to the adrenal disease, AMN patients often show diminished testicular function with decreased testosterone levels in combination with elevated luteinizing hormone and follicle-stimulating hormone levels. Nevertheless, many AMN patients have fathered children prior to the onset of neurologic symptoms or early in its course.

Genetic Defect

X-ALD is inherited as an X-linked trait with variable expressivity. It is caused by mutations in the *ABCD1* gene on Xq28. The gene spans 26 kb and is composed of 10 exons that code for a 745-amino-acid protein (ALDP), which is localized to the peroxisomal membrane. ALDP is a member of the ATP-binding cassette (ABC) family of membrane transport proteins and shows homology to several other ABC proteins that are located within peroxisomal membranes, including ALDR and PMP70. ALDP is a half-

transporter that is active when it forms a dimer with itself, ALDR, or other ABC half-transporter proteins. The ALDP protein is necessary for transport of VLCFA-CoA esters into peroxisomes where they are broken down by β-oxidation.

Almost 700 mutations have been found in the ALD gene, including missense mutations, deletions, insertions, and splicing defects. Most of these mutations result in the absence or severe reduction of immunologically detectable ALDP. It is notable that there is no correlation between the type of mutation and the associated X-ALD phenotype or the severity of the VLCFA oxidative defect as measured in cultured skin fibroblasts. Approximately 4% of male probands represent new mutations.

ABCD1 is subject to X-inactivation and female heterozygous carriers for the mutant gene are functionally mosaic for cells that express either the mutant gene or the wild-type gene. Owing to the random nature of X-inactivation, the nervous tissues of ALD heterozygotes may be composed of a preponderance of functionally mutant cells, which is probably responsible for their neurologic symptoms.

Biochemical Abnormalities

Patients with X-ALD have abnormal VLCFA metabolism. Free VLCFAs are converted to metabolically active VLCFA-CoA esters by action of fatty acyl-CoA synthetase (FATP4), which is localized in peroxisomes, mitochondria and endoplasmic reticulum. VLCFA-CoAs are metabolic substrates for synthesis of various lipids in the endoplasmic reticulum. Degradation of VLCFA-CoA occurs in peroxisomes and requires ALDP for transport across the peroxisomal membrane where they are degraded by β-oxidation enzymes. Mutant ALDP prevents VLCFA-CoA esters from entering the peroxisome. As a consequence, VLCFA-CoAs are diverted into alternate pathways in the endoplasmic reticulum for biosynthesis of other lipids, including phospholipids, cholesterol esters, and sphingolipids.

In almost all tissues of X-ALD patients, fatty acid analyses of total lipids reveal abnormal elevations in saturated VLCFAs that are longer than 22 carbons. In the adrenal cortex and brain, saturated VLFAs (C24:0 and C26:0) may account for over 50% of the fatty acid composition of cholesterol esters, whereas VLCFA in cholesterol esters are almost undetectable in normal controls. VLCFA elevations are seen in other lipid fractions as well. In plasma from X-ALD patients, C26:0 levels in total lipids are increased up to sixfold, whereas X-ALD erythrocytes generally show a lesser twofold accumulation. In cultured skin fibroblasts grown from X-ALD patients, total C26:0 levels are elevated about sixfold over normal controls, which provides a diagnostically useful marker for this disease.

Lipid species of lyso-phosphatidylcholine (lyso-PC) comprised of saturated VLCFAs, such as C24:0 or C26:0, have recently been demonstrated to accumulate in X-ALD. Lyso-PCs containing VLCFAs are a minor component of total phospholipids in all tissues, but may be pathologically important in X-ALD due to their unique biological effects.

Pathogenesis of X-ALD

Neurologic Disease

The pathogenesis of endocrine and neurologic disease in X-ALD is complex and poorly understood. Pathologic mechanisms must account for the extraordinarily diverse clinical phenotypes and the intrafamilial variation in symptoms. Nevertheless, an excess of saturated VLCFAs is the cardinal biochemical feature of X-ALD that underlies all aspects of disease mechanisms (Fig. 2). Although the extent of lipid abnormality and the types of VLCFA-containing lipids vary from one tissue to another, the vulnerable organs in X-ALD are those that show the greatest accumulation of VLCFAs. In electron micrographs, these lipids are manifest as structurally observable lamellar lipid inclusions in adrenal cortical cells, Leydig cells, Schwann cells, and brain macrophages.

VLCFA-containing lipids are very hydrophobic and have a great propensity to partition into membranes. Free VLCFAs, especially saturated ones, are toxic when added to cultured cells including oligodendrocytes, astrocytes and neurons, whereas shorter chain fatty acids (i.e. C16:0) do not elicit this effect. Cells exposed to VLCFA develop mitochondrial depolarization and abnormal intracellular calcium homeostasis, but it is unclear whether these changes are a result of free VLCFAs, which are a minor component in X-ALD, or VLCFA-containing lipids.

The major neuropathologic differences between cerebral forms of X-ALD and pure AMN are the active cerebral inflammatory demyelination observed in the former and the prominent axonal degeneration observed in the latter. The brain in CCALD exhibits decreased myelinated axons and oligodendrocytes, reactive astrocytosis, sudanophilic lipid deposits in macrophages, and a striking perivascular lymphocytic infiltration. Both T and B cell lymphocytes are present. Inflammatory mediators, such as tumor necrosis factor-α and interleukin-1, are produced in regions of active demyelination, which is associated with localized endothelial damage and disruption of the blood–brain barrier.

Although VLCFA levels in plasma and cultured fibroblast are equivalent in patients with CCALD and AMN, there is evidence for a relatively greater amount of VLCFA in autopsied brain from CCALD than AMN. This same trend is seen in cultured oligodendrocytes derived from induced pluripotent stem cells grown from CCALD and AMN patients. Lipid analyses of select regions of autopsied CCALD brain show that the fatty acid abnormality precedes areas of demyelination, suggesting a link between VLCFA and inflammatory demyelination.

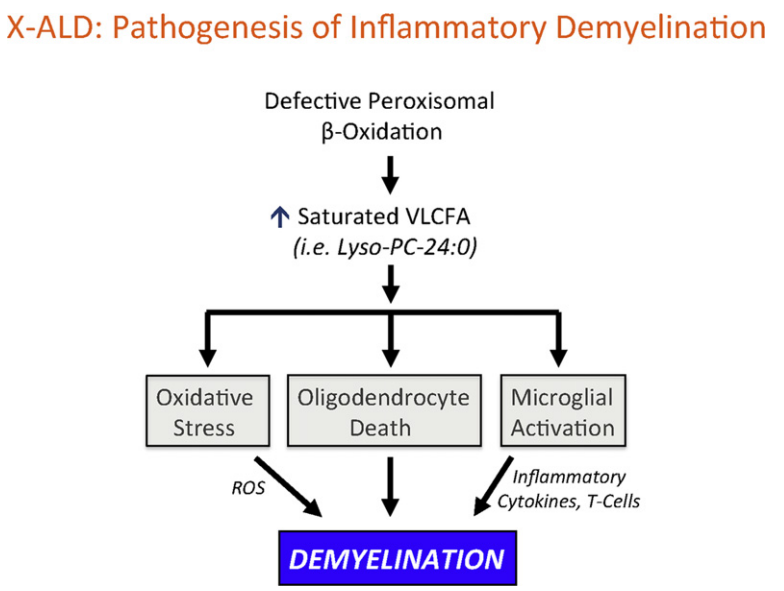

Fig. 2 Likely pathogenic mechanisms for cerebral demyelination in ALD.

The particular lipid species that contain VLCFA may be critical for the inflammatory reaction in CCALD brain. VLCFA accumulation seems to initiate in phosphatidylcholine and later spreads to other lipids as demyelination proceeds. Actively demyelinating lesions show the highest level of cholesterol esters containing VLCFAs, but these lipids are unlikely to elicit an antigenic or inflammatory response. In contrast, animal studies demonstrate that intracerebral injection of C24:0-lyso-PC into mouse brain leads to microglial activation and apoptosis, whereas C16:0-lyso-PC does not have a similar effect. This raises the possibility that VLCFA-lyso-PC lipids are critically involved in initiating cerebral inflammation (Fig. 2). In addition, certain myelin proteins in X-ALD, particularly proteolipid protein, have been shown to be acylated with VLCFAs, which could also contribute to the underlying myelin abnormalities. Irrespective of the mechanism, the inflammatory demyelinating lesions in the brain of X-ALD are not seen in other lipidoses associated with white matter disease, implicating VLCFA accumulation in this process.

In contrast to the cerebral forms of X-ALD, AMN seems to lack an immune component to the neurologic disease. In AMN, the spinal cord is mainly affected with loss of myelinated axons in the ascending and descending tracts. The peripheral nerve is also affected. This dying back axonopathy is characteristic of AMN, but the mechanism is still unclear. A mouse *Abcd1* knockout model for X-ALD develops an AMN phenotype after 18 months without evidence of cerebral inflammation and demyelination. Studies have revealed prominent mitochondrial dysfunction and depletion in the spinal cord, which leads to impaired energy formation and the release of toxic reactive oxygen species (ROS). Oxidative damage to the mouse spinal cord is seen many months prior to onset of a spastic gait. Treatment of the affected animals with antioxidant drugs dramatically reduces the oxidative damage and improves the gait symptoms. These animal studies implicate VLCFA-dependent mitochondrial damage as a causative factor resulting in oxidative stress in the pathogenesis of the myelopathy (Fig. 2).

Endocrine Disease

VLCFA accumulation in adrenocortical cells is thought to be solely responsible for the adrenal gland dysfunction, but more than one pathogenic mechanism may be in play. Addition of saturated VLCFAs to cultured adrenocortical cells has been shown to blunt adrenocorticotropic hormone (ACTH) receptor responsiveness, probably by increasing plasma membrane microviscosity. In addition, cholesterol esters containing VLCFAs, which accumulate in the adrenal gland, are poor substrates for hydrolyzing enzymes to generate free cholesterol precursors for steroidogenesis. In time, adrenocortical cells become ballooned and striated from lipid accumulation and then die. In contrast to autoimmune forms of Addison's disease, anti-adrenal antibodies are missing in X-ALD. Whether analogous pathogenic mechanisms occur in Leydig cells and are responsible for testicular dysfunction is not known.

Diagnosis

The diagnosis of X-ALD may be challenging because of its rare occurrence and striking clinical variation. Identification of X-ALD is often delayed until symptoms involving the nervous system and adrenal gland appear together, a condition that is lacking in a significant proportion of patients at the time of initial presentation. AMN males and symptomatic heterozygote females are therefore frequently misdiagnosed as having multiple sclerosis or familial spastic paraparesis. In boys with neurologic symptoms,

however, the diagnosis of CCALD is more readily suspected owing to the characteristic distribution of white matter disease on brain MRI.

Because neurologic symptoms can be missing or develop many years after the onset of adrenal disease, X-ALD should be suspected in any male with isolated primary adrenal insufficiency. In adults, adrenal insufficiency is often caused by other etiologies, but a significant proportion of boys who present with isolated Addison's disease in childhood have X-ALD. In those who initially present with neurologic symptoms, however, adrenal disease might be suspected only after a diagnostic work-up for leukodystrophy has been initiated. In these patients, a 60 min ACTH stimulation test will usually detect adrenal involvement. Measurement of a morning serum cortisol concentration alone is not an adequate screening test, because basal cortisol levels may be maintained in the face of diminished adrenal reserve. However, plasma ACTH is usually elevated before hypocortisolemia develops, so both cortisol and ACTH should be measured together.

Owning to its wide clinical variation, the diagnosis of X-ALD is critically dependent on laboratory confirmation. The most convenient diagnostic test is measurement of VLCFAs in plasma, which is noninvasive, relatively inexpensive, and detects abnormal elevations of VLCFAs in patients long before symptoms develop, even at the time of birth. Affected patients accumulate C26:0 and C24:0, but have normal C22:0. The C26:0/C22:0 and C24:0/C22:0 ratios are particularly useful for discriminating X-ALD patients from non-ALD controls. In males, there are very few conditions, such as a ketogenic diet, that give rise to false-positive results and false-negative results are uncommon. In circumstances where the plasma VLCFA results are equivocal, fatty acids can be measured in cultured skin fibroblasts grown from a patient. Female carriers for X-ALD tend to show intermediate elevations in plasma VLCFAs, but interpretation of a normal test result is problematic because plasma VLCFAs are elevated in only 85% of carriers. A normal result, therefore, does not completely eliminate the possibility that the female is carrying the X-ALD gene. DNA testing is more reliable for heterozygote detection if the mutation is known in the family. The lack of a common mutation in the X-ALD gene hampers the development of simple DNA screening tests for routine diagnosis.

Newborn screening for X-ALD has recently been initiated in the United States. By detecting elevated levels of C26:0-lyso-PC in a dried blood spot using liquid chromatography-tandem mass spectrometry, newborn infants with X-ALD can be readily discriminated from unaffected controls. The ability to identify all newborn infants with X-ALD will have major impact on diagnosis, disease surveillance and therapy in the future.

The diagnosis of X-ALD in a family has profound implications for genetic counseling, disease prevention and outcomes. Family studies indicate that only 4% of the X-ALD probands represent new mutations. It is therefore important to screen at-risk family members by measuring plasma VLCFA or by mutation analysis. This frequently leads to the identification of asymptomatic males who should be monitored for the appearance of symptoms even though it is not yet possible to predict which X-ALD phenotype a presymptomatic male may later develop.

Prenatal diagnosis affords the ability to prevent X-ALD in at-risk families. Affected fetuses can be identified by DNA analysis or by measuring VLCFA content of chorionic villi cells obtained at 8.5–10 weeks gestation and in amniocytes obtained during the second trimester.

Preimplantation genetic diagnosis of X-ALD has also been accomplished. After in vitro fertilization, DNA from a single blastomere is removed from an early embryo and amplified by PCR to detect unaffected embryos. Non-ALD embryos are transferred into the mother with subsequent delivery of unaffected infants.

Management

All X-ALD patients should be monitored for the onset of adrenal insufficiency. In presymptomatic X-ALD boys and those lacking adrenal insufficiency at the time of neurologic diagnosis, adrenal function should be tested at least yearly by measuring plasma ACTH in combination with a morning serum cortisol. The adrenal insufficiency in X-ALD is easily treated with hormone replacement, but it has no effect on the progression of neurologic disease. Hydrocortisone is typically used alone or in combination with fludrocortisone, depending on the severity of mineralocorticoid deficiency. The glucocorticoid dosage should be increased in response to stresses, such as febrile illnesses and general anesthesia. No therapy has been found to prevent or reverse the adrenal insufficiency.

Therapeutic options for the serious neurologic symptoms of X-ALD have thankfully emerged. Allogeneic hematopoietic stem cell transplantation (HSCT) has been shown to stabilize the neurologic disease in CCALD. To receive clinical benefit, however, patients need to be transplanted at the earliest sign of neurologic involvement when white matter changes first appear on brain MRI or only cognitive symptoms are predominant. With HSCT, it is assumed that the genetically normal transplanted stem cells migrate into the brain and alter the inflammatory demyelination by differentiating into microglial cells, which are metabolically normal and not activated to produce inflammatory molecules. The clinical response requires a period of 12–18 months during which the neurologic symptoms may still progress. Presymptomatic X-ALD boys should therefore receive brain MRI every 6–12 months to detect the onset of white matter disease at its earliest appearance. Given the known risks of HSCT, the procedure is not recommended for asymptomatic or AMN patients.

Gene therapy has recently been applied to several boys with early CCALD symptoms. Using an *ex vivo* gene therapy approach, bone marrow was harvested and the normal *ABCD1* gene was transferred into the patient's own hematopoietic stem cells using a Lentivirus vector, and subsequently infused back into the patient. In the initial small cohort of boys receiving gene therapy, the outcome appears to be similar to allogeneic HSCT without any graft-vs-host complications.

Other therapeutic approaches are either ineffective or less robust. Dietary restriction of saturated VLCFA has no effect on the disease, probably because endogenous fatty acid synthesis is the major source of VLCFA. In contrast, VLCFA restriction together with dietary supplementation with monounsaturated fatty acids (Lorenzo's oil) normalizes plasma C26:0 by inhibiting saturated VLCFA synthesis. This approach has no significant clinical impact once neurologic symptoms develop, probably because fatty acids in the brain are not readily altered. In presymptomatic boys, however, the Lorenzo's oil diet has been reported to delay the onset of neurologic disease and uncontrolled studies suggest that it may decrease the rate of neurologic deterioration in AMN men. Rigorous controlled investigations, however, are needed to confirm these conclusions. Attempts to modify the inflammatory reaction in the brain of CCALD boys with immunosuppressive drugs, gamma globulin or β-interferon have been unsuccessful. Furthermore, pharmacologic approaches to increase the peroxisomal VLCFA-oxidizing activity using 4-phenylbutyrate or statin drugs, which upregulate expression of ABC-half transporters and restore VLCFA oxidation in cultured cells, have not been clinically effective.

See also: Adrenal Insufficiency: Etiology and Diagnosis

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Steroid Replacement in Adrenal Insufficiency

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Abbreviations

11βHSD 11β-hydroxysteroid dehydrogenase
ACTH Adrenocorticotrophic hormone
AI Adrenal insufficiency
BMD Bone mineral density
BSA Body surface area
CAH Congenital adrenal hyperplasia
CBG Cortisol binding globulin
CRH Corticotrophin releasing hormone
GCR Glucocorticoid receptor
HCeq Hydrocortisone equivalent
HPA Hypothalamic–pituitary–adrenal (HPA)
HRQoL Health related quality of life
MCR Mineralocorticoid receptor
QoL Quality of life
SCN Suprachiasmatic nucleus

Background

Thomas Addison first described adrenal insufficiency (AI) in 1855. The condition was universally fatal until the 1930s, when adrenal glands extracts were recognized to reverse the features of AI. Cortisone was isolated in 1935 (Mason *et al.*, 1936) and in 1948 hydrocortisone, the pharmacological equivalent of cortisol, was synthesized for the first time (Benedek, 2011). Despite 70 years of experience in the treatment of AI, patients continue to experience an impaired quality of life and an excess of cardiovascular, metabolic, and bone related morbidity. The relative risk of death for AI patients is more than double that of the background population (Bensing *et al.*, 2008; Bergthorsdottir *et al.*, 2006; Erichsen *et al.*, 2009). Patients with non-functioning pituitary adenoma and adrenal insufficiency on doses greater than 20 mg hydrocortisone equivalent (HCeq) every day have a higher mortality when compared to similar patients on doses <20 mg day^{-1} (Hammarstrand *et al.*, 2017). It is likely that these poor outcomes in part reflect the difficulty in replicating physiological cortisol levels using current glucocorticoid medications (Porter *et al.*, 2017). Here we review the physiology of cortisol, current protocols for cortisol replacement, the treatment of adrenal crisis and new technologies in development.

The Hypothalamic–Pituitary–Adrenal Axis

Serum cortisol levels are regulated by the hypothalamus in response to various neuronal and humeral inputs, stimulated by changes in the internal and external environment. The hypothalamic–pituitary–adrenal (HPA) axis is an example of a classical feedback loop. Corticotrophin releasing hormone (CRH) and arginine vasopressin release from the paraventricular nucleus of the hypothalamus stimulate release of adrenocorticotrophic hormone (ACTH) from corticotrophs in the anterior lobe of the pituitary which, in turn, stimulate cortisol synthesis and rhythmic release from the zona fasciculata of the adrenal gland. Cortisol is secreted by diffusion into the peripheral circulation, and then exerts an inhibitory effect on CRH secretion from the hypothalamus and ACTH secretion from the pituitary. Studies of healthy adults and children report cortisol secretion rates of 6.3 mg m^{-2} body surface area (BSA)/day (range 5.1–9.3) in adults and 8.0 mg m^{-2} BSA/day (range 5.3–12.0) in children (Peters *et al.*, 2013). Cortisol secretion shows a circadian rhythm, regulated by the central pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN rhythm has an approximate period of 24.2 h, and requires a daily resetting via the daily light/dark photoperiod to maintain a 24 h rhythm (Dunlap, 1999). Cortisol concentrations peak shortly after waking and decline through the day. A nadir is reached at midnight, and cortisol concentrations start to rise again from 02.00 to 04.00 (Debono *et al.*, 2009; Peters *et al.*, 2013). The circadian rhythm of cortisol secretion can be observed from the age of 2 months (Porter *et al.*, 2017). Cortisol secretion is also stimulated in response to physical and psychological stress, with the magnitude of change in cortisol concentration being related to the nature, intensity, and duration of stress. This "stress response" is superimposed on the normal circadian profile.

Adrenal Insufficiency

The causes of AI are classified according to the point at which the HPA axis is disrupted: Disorders of the adrenal are described as "primary AI," those of the pituitary as "secondary AI" and those of the hypothalamus, "tertiary AI." Causes of AI are detailed in **Table 1**. In childhood, congenital adrenal hyperplasia (CAH) accounts for most causes of primary AI, while autoimmune disease is the commonest cause of acquired primary AI. In adults secondary AI is more common than primary AI and is most frequently due to benign pituitary adenomas. Tertiary AI due to exogenous steroid administration is increasingly recognized and may be much more common than both primary and secondary AI as glucocorticoids remain widely prescribed antiinflammatory agents (Fardet *et al.*, 2011).

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Table 1 Causes of adrenal insufficiency

Primary adrenal insufficiency

Defects of steroid biosynthesis

- Congenital adrenal hyperplasia: 21 hydroxylase deficiency, 11B hydroxylase deficiency, 3B hydroxysteroid dehydrogenase deficiency, 17 hydroxylase deficiency, P450 oxidoreductase deficiency
- Congenital lipoid adrenal hyperplasia
- P450 side chain cleavage syndrome
- Aldosterone synthase deficiency
- Cortisone reductase deficiency
- Apparent cortisone reductase deficiency

Adrenal dysgenesis

- X linked adrenal hypoplasia congenital
- IMAGe syndrome
- Pallister–Hall syndrome
- Pseudotrisomy 13
- Galloway–Mowat syndrome

ACTH resistance

- Familial Glucocorticoid Deficiency
- DNA repair defect
- Triple A syndrome

Cholesterol synthesis disorders

- Wolman disease
- Smith–Lemli–Opitz disease
- A beta liporoteinaemia
- Familial hypercholesterolaemia

Metabolic disorders: peroxismal defects

- X Linked adrenoleukodystrophy
- Neonatal adrenoleukodystrophy
- Infantile Refsum disease
- Zellweger syndrome

Autoimmune disorders

- Isolated autoimmune adrenalitis
- Autoimmune Polyglandular syndromes type 1, 2, and 4

Acquired defects

- Haemorrhage
- Infections
- Surgery/trauma
- Infiltration
- Drugs

Secondary adrenal insufficiency

Congenital

- Pituitary aplasia/hypoplasia
- Pituitary stalk interruption syndrome
- Isolated ACTH deficiency
- Proprotein convertase 1

Acquired

- Steroid withdrawal after prolonged administration or endogenous overproduction
- Tumor, e.g., pituitary macroadenomas
- Radiation therapy
- Lymphocytic hypophysitis

Tertiary adrenal insufficiency

Congenital

- Septo-optic dysplasia
- CRH deficiency

Table 1 Continued

Acquired
• Steroid withdrawal after prolonged administration or endogenous overproduction
• Trauma
• Radiation therapy
• Surgery
• Tumors, e.g., craniopharyngioma
• Infiltrative disease
• Drugs such as opiates

Glucocorticoids

Glucocorticoids are steroid hormones with a common structure, incorporating the delta-4,3-keto-11-beta,17-alpha,21-trihydroxyl configuration, which is essential for glucocorticoid activity. Modification of this basic structure alters glucocorticoid activity and pharmacokinetics. Cortisol circulates bound to cortisol binding globulin (CBG) which has high affinity and low capacity and albumin that has low affinity and high capacity. Only 5% of circulating cortisol is unbound or "free" (Lewis *et al.*, 2005) and is able to interact with the glucocorticoid receptor (GCR). The degree of protein binding for other, synthetic glucocorticoids is variable, with most only binding weakly to albumin and others circulating as free hormones. Prednisolone has approximately 60% of the CBG binding affinity of cortisol, prednisone 5%, and dexamethasone only 1% (Migeon *et al.*, 1959; Pugeat *et al.*, 1981).

Two isoforms of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) regulate glucocorticoid activity at the target tissue level. The inactive hormone, cortisone, and synthetic glucocorticoid prednisone, are converted to the active hormones, cortisol and prednisolone, by 11βHSD type 1. Polymorphisms for 11βHSD type 1 have been implicated to explain the higher tendency for certain individuals to develop metabolic complications from glucocorticoids (Molnar *et al.*, 2016). In tissues where the mineralocorticoid receptor is expressed, including salivary glands, the bowel, kidney and placenta, cortisol and prednisolone are inactivated by conversion to cortisone and prednisone by 11βHSD type 2. This process of inactivation protects the mineralocorticoid receptor from activation by glucocorticoids, which has equal affinity for cortisol and aldosterone. Synthetic glucocorticoids differ in their affinity for these two isoforms of 11βHSD. Dexamethasone, which is fluorinated at the 6-alpha or 9-alpha position, is protected from inactivation by 11βHSD type 2, while prednisolone, which has a double bond between positions 1 and 2, is inactivated more effectively than cortisol by 11βHSD type 2.

Glucocorticoids signal through the GCR which is expressed by nearly every cell in the body, while mineralocorticoid receptor (MCR) expression is restricted to the limbic-forebrain, where activation promotes attention, memory retrieval and appraisal and drives expression of fear and aggression, and tissues that influence Na^+/K^+ balance, including the distal renal tubule, parotid glands, colon, sweat glands and the nucleus tractus solitarii and circumventricular organs of the brain.

Activation of the GCR by glucocorticoid binding induces or suppresses the activation of target genes, which together comprise 10%–20% of the human genome (Oakley and Cidlowski, 2013). A wide range of physiological processes are regulated by glucocorticoids, including intermediate metabolism, growth, cognition, reproduction and immunity and glucocorticoids play an essential role in homeostasis. A single gene encodes the GCR, however a number of different GCR isoforms arise from alternative splicing and alternative translation, and various posttranslational modifications. Differential expression of GCR isoforms, in part, determines cellular responses to glucocorticoid binding (Oakley and Cidlowski, 2013). GCR polymorphisms are associated with increased weight gain and may influence the dose needed for replacement (Molnar *et al.*, 2016). The mechanism of action of glucocorticoids through binding the GCR are genomic through transactivation and transrepression of genes, nongenomic independent of gene transcription and at very high dose possibly through nonspecific interactions with cell membranes (Buttgereit *et al.*, 2004).

Glucocorticoids are metabolized primarily in the liver, through reduction, oxidation or hydroxylation and the products of these reactions are conjugated with sulfate or glucuronic acid to make them water soluble, ready for elimination in the urine. Hepatic metabolism of glucocorticoids is influenced by a number of factors, including concomitant medications. Drugs inhibiting CYP 3A4 increase glucocorticoid concentrations whilst enzyme inducers decrease glucocorticoid levels (**Table 2**). Glucocorticoid metabolism is also increased in patients with cystic fibrosis (Dove *et al.*, 1992) and hyperthyroidism (Frey *et al.*, 1988), and patients with these diagnoses may require higher or more frequent dosing.

Glucocorticoid Treatment of Adrenal Insufficiency

The goal of cortisol replacement therapy in patients with AI is to ameliorate the symptoms of cortisol deficiency, prevent adrenal crisis, restore normal quality of life, and avoid the adverse effects of glucocorticoid excess. In current practice, five medications are commonly used: Hydrocortisone, cortisone, prednisolone, prednisone, and dexamethasone. The choice of glucocorticoid medication, dose and frequency of administration is the focus of some considerable clinical research, driven by a desire to improve

Table 2 Medications that interact with glucocorticoid metabolism

Class of drug: examples	*Mechanism*	*Effect*
Anticonvulsants	Induction of CYP-3A	Accelerated clearance and reduced effect
• Carbamazepine • Phenobarbitone • Phenytoin • Primidone		
Antibiotics		
• Rifampicin • Rifabutin		
Antibiotics	Inhibition of CYP-3A	Reduced clearance, enhanced effect
• Erythromycin		
Antifungals		
• Itraconazole • Ketoconazole		
Anti-virals		
• Atazanavir		
Estrogens		
HIV treatment boosting agents:		
• Cobicistat • Ritonavir		
Immunsuppressives:		
• Cyclosporin • Tacrolimus • Everolimus		
Oral Antacids	Reduced absorption possible	Variable effects

clinical outcomes. Hydrocortisone as the native hormone is first choice in clinical guidelines (Bornstein *et al.*, 2016), but requires twice or thrice daily administration and doesn't replace the circadian cortisol rhythm. Prednisolone has its advocates because of price and only requiring twice daily administration (Amin *et al.*, 2014), and dexamethasone has the potential for once daily administration.

Metabolic syndrome and glucocorticoid choice: All glucocorticoid therapies given in excess will result in the features of Cushing syndrome that include the metabolic syndrome. In a cohort of nearly 200 adult patients with CAH, the prevalence of obesity was reported to be 40%, of hypercholesterolemia, 46%, and insulin resistance 29% (Arlt *et al.*, 2010; Han *et al.*, 2013a). This was felt primarily to relate to glucocorticoid therapy. Patients treated with prednisolone are reported to have a worse lipid profile than those treated with hydrocortisone (Han *et al.*, 2013b; Quinkler *et al.*, 2017), and insulin resistance occurred more commonly in those treated with dexamethasone (Han *et al.*, 2013a). Patients with secondary and tertiary AI may be more difficult to study; it can be difficult to distinguish the adverse effects of glucocorticoid replacement therapy from the effect of the primary pathology and related endocrinopathies, in particular untreated growth hormone deficiency. Patients with hypopituitarism are at increased risk of developing the metabolic syndrome, and hydrocortisone doses in excess of 20 mg day^{-1} are associated with an increased risk of dyslipidaemia (Filipsson *et al.*, 2006). Following preprandial administration of glucocorticoids, serum insulin and glucose are elevated, even in the presence of acceptable cortisol concentrations (al-Shoumer *et al.*, 1995). Higher doses of hydrocortisone are associated with higher systolic and diastolic blood pressures (Werumeus Buning *et al.*, 2016b) in patients with secondary AI.

Quality of Life (QoL) and glucocorticoid treatment regimen: QoL and glucocorticoid treatment regimen has been studied in a diverse population of 1250 patients, recruited through patient organizations from across the world (Forss *et al.*, 2012). In this cohort, 84% reported they had primary AI, 11% secondary and the remaining patients were uncertain. 74% of patients were treated with hydrocortisone, 11% with prednisolone and the remaining patients with cortisone. Health related QoL was reported to be adversely affected by AI in 87% of patients with secondary AI and 60% of patients with primary AI. The likelihood of reporting an impaired QoL was inversely related to the frequency of glucocorticoid doses, and patients treated with hydrocortisone were less likely to report impaired QoL than those treated with prednisolone, prednisone or cortisone. In patients with CAH, QoL is also reported to be poorer in patients treated with prednisolone or dexamethasone than in those treated with hydrocortisone (Han *et al.*, 2013b), and in obese patients and those with insulin resistance (Arlt *et al.*, 2010). In patients treated with hydrocortisone, QoL has been related to total daily dose, with those treated with higher doses having a poorer QoL than those in whom doses are

lower (Ragnarsson *et al.*, 2014). The challenge in interpreting these studies is that patients with a poor QoL may be the patients who have their dose increased or transfer to other glucocorticoids.

Bone mineral density (BMD): BMD in patients with AI, BMD is lower than the background population (Løvås *et al.*, 2009), and is inversely related to glucocorticoid dose (Zimmermann *et al.*, 2009). Nearly 50% of adult patients with CAH are reported to have osteopenia or osteoporosis (Han *et al.*, 2013b). Total glucocorticoid exposure during childhood and adolescence has been associated with the risk and severity of impaired BMD in adult life in previous cohorts (Paula *et al.*, 2008; Zimmermann *et al.*, 2009). Prednisolone treatment is associated with lower BMD than treatment with hydrocortisone, and normal BMD has been reported in patients with primary AI treated with hydrocortisone in doses of 15 mg m^{-2} day^{-1} for CAH and 12 mg m^{-2} day^{-1} for primary AI of other causes (Koetz *et al.*, 2012). Reductions in hydrocortisone doses in patients treated with higher doses have been associated with improvements in bone health (Schulz *et al.*, 2016). Treatment with dexamethasone has been associated with lower rates of bone turnover than hydrocortisone in biochemical studies of bone turnover markers (Suliman *et al.*, 2003).

Childhood: data describing long term outcomes in pediatric patients come from cohorts of patients with CAH, as other causes of AI in childhood are rare. These patients are at increased risk of obesity, insulin resistance, elevated leptin levels, dyslipidaemia, and impaired glucose metabolism (Völkl *et al.*, 2006). The severity of this adverse metabolic profile has been related to the total hydrocortisone dose and the duration of treatment (Zimmermann *et al.*, 2010). Working memory is poorer in children with CAH than in their unaffected relatives and quality of life is also reported to be reduced (Browne *et al.*, 2015). Boys and girls are equally affected suggesting that this is not simply related to androgen excess in girls and associated disorders of sex development. As noted above, the doses of hydrocortisone used in the treatment of CAH are higher than those used in other causes of AI in childhood, and long term outcomes in other groups of patients may be better.

Glucocorticoid Treatment Regimens

Hydrocortisone and cortisone are widely recommended as the glucocorticoids of first choice in clinical guidelines (Bornstein *et al.*, 2016), because they are of lower potency than the synthetic glucocorticoids and may be associated with fewer adverse effects. Doses can be titrated in smaller increments and adequacy of treatment assessed clinically. It is recommended that the use of the synthetic glucocorticoids prednisolone and prednisone is restricted to patients in whom symptoms of cortisol deficiency are not adequately controlled with hydrocortisone or cortisone, or where adherence to multiple daily dose regimens is poor (Bornstein *et al.*, 2016). The use of dexamethasone is discouraged because of the risk of excess glucocorticoid exposure. Data from the EU-AIR observational study suggest that these recommendations are adhered to in European clinical practice. In this cohort of nearly 1500 patients treated in the UK, Germany, the Netherlands, and Sweden, 92% of patients were treated with hydrocortisone or cortisone, while 5% were treated with prednisolone and only 0.1% were treated with dexamethasone (Murray *et al.*, 2017). In childhood, little work has been done to critically compare the effectiveness and side effects of treatment with different glucocorticoids. In a small pilot study of nine prepubertal children with CAH, ACTH, androstenedione and 17-hydroxyprogesterone (17-OHP) were lower during treatment with dexamethasone (0.3 mg m^{-2} day^{-1}), and higher during treatment with prednisone (3 mg m^{-2} day^{-1}), than during treatment with hydrocortisone (15 mg m^{-2} day^{-1}) (Nebesio *et al.*, 2016). In a study of children followed to final adult height, treatment with prednisolone was associated with a lower final adult height than treatment with hydrocortisone. Hydrocortisone treated patients receiving doses in excess of 20 mg m^{-2} day^{-1} at the start of puberty were significantly shorter than those treated with lower doses (Bonfig *et al.*, 2007). Prednisolone is generally accepted to be more potent than hydrocortisone. The conversion of prednisone to prednisolone in childhood is unreliable as 11β hydroxysteroid dehydrogenase type 1 activity is less predictable than during adult life (Wiegand *et al.*, 2007).

Hydrocortisone

The plasma half-life of hydrocortisone is only 90 min, requiring the administration of multiple doses to achieve adequate cortisol concentrations throughout the waking hours. Unless the patient wakes to take an early morning dose before rising in the morning, cortisol concentrations are subphysiological on waking, and overnight hypoglycaemia has been reported in patients with Addison's disease, treated with hydrocortisone (Meyer *et al.*, 2012; Petersen *et al.*, 2015). In a pharmacokinetic study of 13 different hydrocortisone regimes in 50 subjects with AI 50%–80% of patients were over- or undertreated at different times of the day (Simon *et al.*, 2010).

Current guidelines recommend hydrocortisone doses for adult patients are 15–25 mg day^{-1} and for cortisone, 20–35 mg (Bornstein *et al.*, 2016), given in two to three divided doses. These treatment guidelines are based on a number of studies that have estimated cortisol secretion rates, and investigated the effect of different dosing regimens on biochemical parameters, bone health and health related QoL (HRQoL). However, most of these studies are of small patient populations, and the observation periods are too brief to enable meaningful application of study findings to long term dosing regimens. It is also important to recognize that the pharmacokinetics of hydrocortisone show marked inter-individual variability (Simon *et al.*, 2010), even after intravenous administration (Jung *et al.*, 2014). Individual dosing regimens should be titrated according to symptoms whilst on treatment:

Lethargy, headache, nausea, poor concentration, weight loss and in children, inadequate weight gain etc., and cortisol excess: rapid weight gain, hypertension, peripheral oedema, and poor sleep. In CAH treatment should be adapted to patient requirements at the stage of life; essentially supraphysiological doses when regulation of hyperandrogenism is crucial and using physiological doses in adults and the elderly when symptomatic control is not vital but prevention of glucocorticoid side effects is a priority.

The bioavailability of hydrocortisone is nearly 100%, and cortisol concentrations rise rapidly from 30 min following an oral dose (Hindmarsh, 2014; Jung *et al.*, 2014; Werumeus Buning *et al.*, 2017). Cortisone requires activation to the active drug, hydrocortisone, and the onset of action is slightly slower. When plasma cortisol concentrations approach 550 nmol L^{-1}, the binding capacity of CBG is exceeded, free cortisol concentrations rise and then fall rapidly as free cortisol is filtered in the urine (Tunn *et al.*, 1992). At these levels cortisol clearance increases lowering the half-life of the steroid. As a consequence of this combination of rapid absorption followed by rapid elimination, patients may experience wide fluctuations in cortisol concentrations from supra-physiological levels shortly after a dose of hydrocortisone, followed by troughs when cortisol concentrations may be undetectable (Charmandari *et al.*, 2001a, b, Maguire *et al.*, 2007). In eight dexamethasone suppressed healthy volunteers, cortisol concentrations ranged from 943 to 1419 nmol L^{-1} 1 h following an oral dose of 20 mg (Jung *et al.*, 2014), well in excess of spontaneous cortisol peaks reported in healthy individuals (Debono *et al.*, 2009; Whitaker *et al.*, 2014).

Smaller doses of hydrocortisone, given more frequently, have the potential to give a more physiological cortisol profile than once or twice daily dosing. When cortisol profiles and wellbeing were studied in patients with adrenal insufficiency, treatment with three daily doses was superior, to twice daily doses, and was favored by most patients (Groves *et al.*, 1988). Improvements in cortisol and ACTH profiles were reported when cortisone was given thrice rather than twice daily (Laureti *et al.*, 2003). In a double blind, cross over, placebo controlled study, comparing two to four times daily dosing regimens, patients stated a preference for the four times daily dosing regimen (Ekman *et al.*, 2012). Nevertheless, it is also important to recognize that adherence to treatment for chronic disease is inversely related to the number of medication doses prescribed per day (Coleman *et al.*, 2012), and for some patients twice daily dosing is more practicable.

In patients in whom hydrocortisone is prescribed twice daily, two thirds of the dose is prescribed in the morning and one third in the afternoon. Three times daily dosing is distributed so that the largest dose of the day is given upon waking, the second dose after lunch and the final, smallest dose of the day in the early evening, not less than 4–6 h before bed as later dosing may have an adverse effect on sleep and insulin sensitivity (Plat *et al.*, 1999). Dose adjustment for body size may be beneficial in adult patients, as doses derived from either BSA or weight are more likely to result in cortisol concentrations within the normal range, than treatment with fixed 10 mg doses (Mah *et al.*, 2004). While patients are reported to favor weight based and more frequent dosing, improved clinical outcomes in the longer term have yet to be reported.

A variable dose dependent effect between glucocorticoid dose and HRQoL has also been reported. In a double-blind cross-over randomized controlled of weight based dosing regimens in patient with secondary AI, HRQoL was superior during higher, compared to lower, dose treatment (0.2–0.3 vs. 0.4–0.6 mg kg^{-1} body weight day^{-1}) (Werumeus Buning *et al.*, 2016a). In an observational study, authors reported poorer HRQoL in patients treated with more than 30 mg day^{-1} (Bleicken *et al.*, 2010). In a second observational study of patients with secondary AI, higher doses were also associated with a poorer HRQoL, and mortality almost doubled in patients treated with doses in excess of 20 mg (Hamarstrand et al., 2017), but did not differ from the background population in those treated with lower doses. A causative relationship between higher hydrocortisone dose and these adverse outcomes has not been demonstrated. In observational studies, it is possible that hydrocortisone doses are increased to treat symptoms of AI that arise from other causes. While recommendations can be made regarding hydrocortisone doses, it will always be important to consider how individual variability in glucocorticoid sensitivity and metabolism may require titration of doses against symptoms of cortisol deficiency and excess, and serum cortisol concentrations.

Despite these data, favoring lower compared to higher daily doses, the authors of a recent systematic review and metaanalysis found no evidence of benefit from low total daily doses of hydrocortisone (20 mg vs. 30 mg), or increased frequency of dosing (Al Nofal *et al.*, 2017). They did, however, report improved QoL in patients treated using subcutaneous hydrocortisone infusions and modified release hydrocortisone.

The use of stress hydrocortisone dosing in AI has been debated as cortisol levels naturally rise during stress such as exercise. During and after intense and moderate intensity exercise, blood glucose and adrenaline concentrations are lower in patients with AI than in healthy controls, and aerobic capacity and stamina is also reported to be significantly reduced (Green-Golan *et al.*, 2007; Simunkova *et al.*, 2016; Weise *et al.*, 2004). In a double blind, placebo controlled trial, a 10 mg dose of hydrocortisone taken 1 h before intensive exercise had no effect on exercise performance, metabolic or endocrine parameters or sense of well-being, although cortisol concentrations were double those recorded in healthy control subjects (Simunkova *et al.*, 2016). Similar data have been reported in patients with CAH during high and moderate intensity exercise (Weise *et al.*, 2004), and preexercise doses of hydrocortisone are not currently recommended.

Hydrocortisone therapy in childhood

In pediatric practice, hydrocortisone and cortisone doses are always derived from measurements of body size, generally body surface area. Cortisone can be used with caution, as 11βHSD type 1 concentrations are variable in childhood and estimates of dose equivalency for hydrocortisone quoted from the adult literature may be unreliable (Wiegand *et al.*, 2007).

In patients with AI due to causes other than CAH, hydrocortisone doses of 8 mg m^{-2} day^{-1}, given in three daily doses, are recommended. Doses are titrated against clinical features of adrenal insufficiency or glucocorticoid excess as in adult practice, and monitoring growth and weight gain provides additional, important information.

In children with CAH, higher doses of hydrocortisone are required to achieve adequate suppression of ACTH drive to the adrenal. Doses of 10–15 mg m^{-2} day^{-1} are usual, but doses as high as 25 mg m^{-2} day^{-1} may be required for some patients (Clayton *et al.*, 2002). In these patients, rapid growth is likely to result from increased androgen secretion in patients treated with hydrocortisone doses that are insufficient to suppress ACTH drive to the adrenal, while slow growth is likely to indicate treatment with doses that are excessively high. Final adult height in patients with CAH has been related to corticosteroid exposure during infancy and adolescence, with a loss in final adult height of 0.4 cm for every 1 mg m^{-2} day^{-1} increase in dose (Sarafoglou *et al.*, 2014). Doses not exceeding 20 mg m^{-2} day^{-1} in infancy and 17.5 mg m^{-2} day^{-1} in older children are recommended to minimize the adverse effect on growth (Silva *et al.*, 1997).

Prednisolone

Treatment with prednisolone is recommended for adult patients in whom the symptoms of adrenal insufficiency cannot be treated adequately with hydrocortisone, and those in whom adherence to the three times a day, hydrocortisone treatment regimen is difficult. Another use for prednisolone could be in those who are unable to take their hydrocortisone tablet for a long period such as during prolonged fasting. Prednisolone is absorbed rapidly following oral administration and concentrations peak within the first hour (English *et al.*, 1975). Like hydrocortisone, the plasma half-life of prednisolone is reported to show marked inter-individual variability, even after intravenous administration, and ranges from 120 to 360 min (English *et al.*, 1975; Al-Habet and Rogers, 1980). The longer half-life results in a smoother profile of glucocorticoid exposure, enabling once daily dosing. The problem with this is that there is a tendency to bind longer to the glucocorticoid receptor with slower dissociation, preventing the latter from exchanging rapidly with glucocorticoid response elements in DNA and slowing down gene cycling. This may alter body physiology and increase the risk of side effects. On the other hand the effect of hydrocortisone is faster with rapid redistribution of the receptor-response element on completing a glucocorticoid response (Stavreva *et al.*, 2009). Recommended doses are either as a daily dose of 4 or 5 mg at 07:00 h or as a twice daily dose of 3 mg and 2 mg or 3 mg and 1 mg at 07:00 h and 14:00 h, respectively (Husebye *et al.*, 2014). Doses up to 6 mg day^{-1} may be used in CAH. In some countries prednisone is used. This has a plasma half-life of 60 min and, similar to cortisone, is inactive. It must be reduced by 11βHSD Type 1 in the liver to the active prednisolone.

Studies of the effect of glucocorticoids on ACTH suppression, indicate that prednisolone has approximately four times the biological activity of hydrocortisone (Khalid *et al.*, 1982) despite having only 1.7-fold higher trans-activating activity of the GCR (Grossmann *et al.*, 2004). This discrepancy may be accounted for, in part, by the relatively higher concentrations of free hormone, as prednisolone has only 50% of the affinity for CBG as hydrocortisone, and a higher re-activation rate from the inactive form, prednisone (Diederich *et al.*, 2002). The growth suppressive effect has been reported to be 15-fold that of hydrocortisone (Punthakee *et al.*, 2003), while the immunosuppressive effects are reported to be approximately fourfold that of hydrocortisone (British National Formulary, 2017). The spectrum of potencies of prednisolone on systems regulated by glucocorticoids suggest the determinants of the synthetic glucocorticoid activity are not simply dose related, but are regulated by differential effects on glucocorticoid target genes.

Prednisolone is used rarely in childhood, as its high potency increases the risk of clinical features of glucocorticoid excess, in particular poor growth (Punthakee *et al.*, 2003). It may be helpful in some adolescent patients, nearing completion of growth in whom adherence to a three times daily dosing regimen required for hydrocortisone is particularly difficult. For patients with CAH, the recommended doses are 2–4 mg m^{-2} day^{-1}, given once daily (Clayton *et al.*, 2002) or twice daily, and in adolescents with adrenal insufficiency from other causes, lower doses are likely to be sufficient. Irrespective of being classified as an intermediate acting glucocorticoid the use of prednisolone in patients with CAH does not control morning androgen levels with hormonal escape occurring after 10 h (Debono *et al.*, 2015).

A modified release formulation of prednisolone with a delayed release mechanism, Lodotra, has been produced as an attempt to try and provide more physiological replacement. By administering this drug before sleeping at 22:00 h patients with adrenal insufficiency showed a better quality of life with less complaints and fatigue when compared to taking prednisolone on waking, highlighting the importance of early morning glucocorticoid exposure (Langenheim *et al.*, 2013). Randomized placebo controlled studies are necessary to confirm the benefits of this formulation in patients with adrenal insufficiency.

Dexamethasone

The use of dexamethasone as a cortisol replacement therapy is discouraged in both adult and pediatric practice. The plasma half-life is around 200 min and the biologic half-life varies between 36 and 54 h. The potency, estimated by antiinflammatory effect, is 30 times that of hydrocortisone though the antigrowth effect may be up to 80 times higher (Finkielstain *et al.*, 2012). Dose titration is difficult because of the potency of dexamethasone, and the risk of Cushingoid side effects is high (Clayton *et al.*, 2002; Husebye *et al.*, 2014). Adult replacement dose are 0.25–0.75 mg day^{-1} (Bornstein *et al.*, 2016), and for children doses of 0.25/0.375 mg m^{-2} day^{-1} have been used (Clayton *et al.*, 2002). Although better control of androgens is achievable with dexamethasone and so the drug may be useful for a short period to improve fertility in women and reduce testicular adrenal rest tumor size in males it is associated with a high risk of metabolic complications such as insulin resistance (Han *et al.*, 2013a). In addition in CAH androgens escape control after around 16 h (Debono *et al.*, 2015).

Subcutaneous Hydrocortisone Infusion

The use of insulin pumps to administer hydrocortisone subcutaneously, offers the opportunity to reproduce the physiological, diurnal profile of cortisol. Infusion sets and cannula need to be re-sited every 3 days, and patients need to learn how to increase hydrocortisone infusion rates during periods of ill health. Despite considerable advances in pump technology, technical problems still occur frequently, and site infections are not uncommon (Ross *et al.*, 2015, 2016). Improved QoL has been reported in open label pump studies or case reviews, together with a reduction in the frequency of hospital admissions (Björnsdottir *et al.*, 2015; Khanna *et al.*, 2015). Improved cortisol and nocturnal glucose profiles are described in adults with Addison's disease, without deterioration in insulin sensitivity (Björnsdottir *et al.*, 2015). However, the only blinded randomized controlled trial failed to demonstrate superior QoL during hydrocortisone infusion therapy compared to treatment with oral hydrocortisone in Addison's patients with preexisting good quality of life (Gagliardi *et al.*, 2014). In children and adults with CAH, androgen profiles have been reported to improve during subcutaneous hydrocortisone therapy (Bryan *et al.*, 2009; Nella *et al.*, 2016; Tuli *et al.*, 2011), and this may be particularly helpful during puberty, when altered hydrocortisone pharmacokinetics and poor adherence make treatment particularly challenging (Charmandari *et al.*, 2001a,b). They may also be useful in patients with gastric side effects or fast metabolizers. In patients with AI subcutaneous hydrocortisone infusions achieved ACTH and cortisol levels similar to normal circadian levels (Oakley and Cidlowski, 2013). While these studies demonstrate that hydrocortisone infusion therapy is safe, enables physiological cortisol profiles, improved biochemistry and possibly QoL, there is no evidence that these changes translate into improvements in the long term morbidity and mortality of AI, and the issue of health economics has yet to be considered.

Modified Release Hydrocortisone Formulations

Two modified release (MR) hydrocortisone formulations have been developed or are in development Plenadren and Chronocort. Plenadren (Shire Plc. Jersey) is a modified release hydrocortisone with an outer coating layer that provides an immediate release of the drug and an extended release core. Plenadren provides a more extended serum profile of cortisol compared to immediate release Hydrocortisone. In adults a single morning dose of Plenadren gives similar cortisol exposure to a thrice daily regime of immediate release hydrocortisone although Plenadren tends to provide higher levels of cortisol in the late morning and lower in the late evening than a conventional regime and overall has approximately 20% less bioavailability (Johannsson *et al.*, 2012). Studies in adult patients reported promising results, with improvements in metabolic profiles, weight, and blood pressure (Quinkler *et al.*, 2015). Beneficial effects are also reported for QoL (Bergthorsdottir *et al.*, 2015; Giordano *et al.*, 2016). For the treatment of CAH, Plenadren is unlikely to control excess androgens as the overnight rise in cortisol is not replicated, and nocturnal dosing of Plenadren would expose patients to high levels of cortisol throughout the night during the quiescent period of the cortisol circadian rhythm.

Chronocort (Diurnal Ltd. UK) a product under development is a modified release hydrocortisone, but differs from Plenadren in having a delayed and sustained absorption profile rather than an immediate and sustained release profile (Porter *et al.*, 2017). Chronocort aims to replace physiological cortisol levels by dosing at morning and night such that the night time dose provides release of hydrocortisone in the early hours of the morning providing a prewaking rise in cortisol levels and a cortisol profile similar to physiological cortisol levels (Whitaker *et al.*, 2014). In CAH patients, Chronocort given twice daily last thing at night and first thing in the morning gave superior control of androgens both at night and in the day compared with standard hydrocortisone given three times daily (Jones *et al.*, 2017; Mallappa *et al.*, 2015).

Management of Adrenal Crisis

Adrenal crisis is a life threatening condition which requires immediate treatment. In a large, prospective study, 8.3 episodes of adrenal crisis/100 patient years were reported, with an associated mortality rate of 0.5/100 patient years (Hahner *et al.*, 2015). Patients with CAH use sick day rules 171 times during their life and attend hospital for an adrenal crisis 11 times (Hummel *et al.*, 2016). Patient education regarding possible precipitants, the need to increase glucocorticoid doses during periods of stress and prompt recognition of the early features of adrenal crisis is essential. All patients are encouraged to wear or carry identification, alerting health care professionals of the diagnosis of AI to enable prompt treatment in an emergency setting.

Patients with primary adrenal insufficiency are most likely to be affected, but during periods of stress, particularly during vomiting or diarrheal illness when oral glucocorticoid absorption is unreliable and fluid and electrolyte losses may be high, patients with secondary or tertiary AI may also be affected. Other common precipitants include flu like illnesses, and in children, respiratory tract infections. The introduction of new medications that alter glucocorticoid clearance (**Table 2**) may also precipitate adrenal crisis.

The biochemical features of adrenal crisis include hyponatraemia and hyperkalaemia in patients with mineralocorticoid deficiency with a high urea. Patients with secondary and tertiary AI are likely to have normal mineralocorticoid activity, but hyponatraemia may be present due to vasopressin secretion. Hypoglycaemia is more common in children than in adults.

The treatment of adrenal crisis is based on expert opinion, and to date no clinical trials have been performed to determine the optimal glucocorticoid dosing regimen in either adults or children. In 2016, the Endocrine Society, in collaboration with the European Society of Endocrinology and the American Society for Clinical Chemistry published treatment recommendations

Table 3 Treatment of adrenal crisis

Hydrocortisone therapy	*Intravenous fluid*
Adults 100mg IV bolus *Followed by* 200 mg day^{-1} as a continuous infusion for 24 h *Followed by* 100 mg day^{-1} as a continuous infusion Children 50–100 mg m^{-2} IV bolus *Followed by* 50–100 mg m^{-2} day^{-1} as boluses given every 6 h	Adults 1000 mL isotonic saline or 5% dextrose in isotonic saline over first hour *Followed by* Continuous infusion of isotonic saline, adjusted according to patient need Children Rapid bolus of isotonic saline 20 mL kg^{-1}, to be repeated up to 60 mL kg^{-1} within first hour for shock Hypoglycaemia: 0.5–1.0 g dextrose or 2–4 mL kg^{-1} of 25% dextrose (maximum single dose 25 g) infused at a rate of 2–3 mL h or 5–10 mL kg^{-1} 10% dextrose for patients <12 years old.

detailed in **Table 3**. Hydrocortisone is always used for the emergency treatment of adrenal crisis as it has a mineralocorticoid effect at the high doses prescribed in this scenario, as 11βHSD type 2 is saturated at high cortisol concentrations.

Cortisol concentrations have been measured in eight healthy volunteers, following dexamethasone suppression of the HPA, after the administration of 50 mg hydrocortisone intravenously. In these subjects cortisol concentrations exceeded 2000 nmol L^{-1} 30 min after the dose had been given, and ranged from 400 to 900 nmol L^{-1} 5 h following a dose (Jung *et al.*, 2014), suggesting that it may be safe to use lower doses of hydrocortisone during acute illness.

Management of Intercurrent Illness and Surgical Stress

Early management of intercurrent illness and surgical stress should avoid progression to acute adrenal crisis in most patients. The cortisol response to stress reflects the intensity, duration and nature of the stressor, and hydrocortisone doses titrated against the severity of the stress are recommended.

While there is little doubt that severe stress requires additional glucocorticoid, the threshold at which doses should be increased is uncertain. An effect of general anesthesia on cortisol concentrations has been studied in children and adults. Approximately a quarter of children undergoing general anesthesia for elective imaging demonstrated a stress response (Hsu *et al.*, 2012; Rains *et al.*, 2009), although a reduction in cortisol concentrations was also reported in a third and was not associated with an increased risk of adverse events (Rains *et al.*, 2009). A study of adult patients undergoing thyroidectomy or parathyroidectomy, described a cortisol stress response during reversal of anesthesia, endotrachial extubation, and the early recovery period, but not during the surgical procedure (Udelsman *et al.*, 1987).

Studies of the cortisol response to minor surgery, for example, inguinal hernia repair in adults (Chernow *et al.*, 1987), and elective urological procedures in healthy children (Taylor and Auchus, 2013) report that, for these minimally invasive procedures, there is no significant cortisol response. Adult patients undergoing moderately (e.g., cholecystectomy) or highly (e.g., colectomy) stressful procedures mounted a stress response within an hour of surgery, which persisted in some for up to 24 h, with a return to baseline values in all patients within 5 days (Chernow *et al.*, 1987). During childhood febrile illnesses cortisol levels are reported to be three to five times higher than baseline levels, with the magnitude of change reflecting the severity of the illness (Nickels and Moore, 1989).

Glucocorticoid dosing regimens draw on data reported in studies measuring cortisol secretion rates during surgery. In a review article published in 1994, data from seven studies were integrated (Salem *et al.*, 1994). For minor surgery, cortisol secretion rates were estimated to be 25 mg day^{-1} hydrocortisone equivalent, for moderate surgery, 50–75 mg day^{-1} and for major surgical stress, 100–150 mg day^{-1}. Recommended doses are higher than these, recognizing that there are currently no data to demonstrate that these doses have an adverse effect, or that lower doses are safe. The recommendations of the Endocrine Society, in collaboration with the European Society of Endocrinology and the American Society for Clinical Chemistry (Bornstein *et al.*, 2016) are given in **Table 4**.

Conclusion

Patients with adrenal insufficiency have a higher mortality rate compared to the normal population and are at risk of multiple complications, including metabolic disturbances, a poor quality of life and adrenal crises. Conventional glucocorticoid replacement since development in mid-20th century has had a positive impact on the lives of these patients but remains inadequate. No

Table 4 Glucocorticoid management of intercurrent illness and surgical stress

Home management of pyrexial illness
Adult and pediatric patients
• Temperature $>38^{\circ}C$: hydrocortisone doses double • Temperature $>39^{\circ}C$: hydrocortisone doses tripled
Plus increased consumption of electrolyte containing fluids
Intercurrant Illness: oral hydrocortisone not tolerated Adult patients: 100 mg hydrocortisone S.C. or I.M. Pediatric patients: 50 mg m^{-2} I.M. *or* • Infants 25 mg • School age children 50 mg • Adolescents 100 mg
Minor to moderate surgery Adult patients: 25–75 mg day^{-1} hydrocortisone Pediatric: 50 mg m^{-2} I.M. or normal replacement doses doubled or tripled
Major surgery/trauma/delivery or critical care[a] Adult patients: 100 mg I.V. bolus followed by 200 mg day^{-1} for 24 h, followed by 100 mg day^{-1} Pediatric patients: 50 mg m^{-2} I.V. followed by 50–100 mg m^{-2} day^{-1} every 6 h *plus* weight appropriate fluids (5% dextrose and 0.2 or 0.45% NaCl)

[a]Illness requiring intensive care.
S.C., subcutaneous; I.M., intramuscular; I.V., intravascular.

specific biomarkers for glucocorticoids are available to aid management. Weight adjusted hydrocortisone dosing in adults or BSA based in children, administered three times daily, seem the best options resulting in least variability with potential for less side effects.

Oral modified release hydrocortisone formulations and subcutaneous hydrocortisone infusion pumps that mirror the physiological cortisol circadian rhythm are promising but long term studies assessing metabolic outcomes, quality of life and mortality are still awaited. Patient education to avoid adrenal crises remains fundamental and should be strengthened at each clinic visit. Identifying diagnostic and therapeutic strategies based on pharmacogenetics or biochemical markers to individualize patient treatment could in the future offer physicians novel tools to upgrade and modernize the management of adrenal insufficiency.

See also: Adrenal Insufficiency: Etiology and Diagnosis

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Management of Adult Patients With Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency

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Nomenclature

21OHD	21-hydroxylase deficiency
BMI	Body mass index
BP	Blood pressure
CAH	Congenital adrenal hyperplasia
cIMT	Carotid intima-media thickness
CV risk	Cardiovascular risk
DEX	Dexamethasone
GC	Glucocorticoid
HC	Hydrocortisone
MC	Mineralocorticoid
SV	Simple-virilizing form
SW	Salt-wasting form
TART	Testicular adrenal rest tumor
UK	United Kingdom

Introduction

Congenital adrenal hyperplasia (CAH MIM 201910) corresponds to a group of inherited autosomal recessive disorders that arise from defective steroidogenesis and results from a deficiency in one or several of the enzymes of cortisol biosynthesis.

Deficiency of the 21-hydroxylase enzyme is the most common form of CAH, accounting for >95% of the cases and is one of the most common known autosomal recessive disorders. CAH due to 21-hydroxylase deficiency (21OHD) is the result of deletions or deleterious mutations in the active gene CYP21 (Auchus, 2015). There are many mutations of the CYP21 gene identified so far, which cause of varying degrees of impairment of 21-hydroxylase activity (New *et al.*, 2013). Most patients are compound heterozygotes. The clinical phenotype, related to the less severe mutated allele, is classified as classic for the severe form, or nonclassic. Classic CAH encompasses salt-wasting (SW) or simple-virilizing (SV) forms, depending on the degree of aldosterone deficiency. Hormonal treatment is based on cortisol and, when necessary, aldosterone substitution. Its role is to reduce the excessive ACTH production and consequently the increased androgen production by the adrenal gland, to ensure normal fertility, and to avoid the long-term consequences of GC use. The physiological circadian rhythm of cortisol cannot be mimicked with traditional oral GC, and the doses needed to suppress the androgens are usually higher than those needed for substitution (Auchus, 2015; New *et al.*, 2013; Han *et al.*, 2014).

In this article, we decided to focus on the new findings published on CAH over the last years. An exhaustive PubMed research has been performed with the terms "congenital adrenal hyperplasia," "21 hydroxylase deficiency." We excluded all the data that were dealing with nonclassic CAH and childhood outcomes of CAH, with female fertility and prenatal treatment, in order to focus on studies dealing with classic CAH in adults. We also chose to go further into four themes: cardiovascular risk and mortality, male fertility, bone mineral density and treatment management, since most of the recent publications concern those topics.

Metabolic and Cardiovascular (CV) Risk

Cohorts of adults with CAH due to 21OHD from Europe (Arlt *et al.*, 2010; Bachelot *et al.*, 2015; Falhammar *et al.*, 2015) and the United States (Finkielstain *et al.*, 2012) have been published in the recent years and have shown an increased risk for metabolic disorders (Han *et al.*, 2014; Falhammar *et al.*, 2011; Falhammar and Thorén, 2012). Overweight and obesity have been reported in adult patients with CAH. In the cross-sectional study conducted in the United Kingdom, BMI was found to be higher in CAH patients than in the general population (Arlt *et al.*, 2010). However, in the American cross-sectional study and in two French cohorts, prevalence of obesity was similar to that found in the general population (Bachelot *et al.*, 2015; Bouvattier *et al.*, 2015). More recently, repartition of adipose tissue was studied in CAH adolescents and young adults, and showed increased abdominal adiposity, with a higher proportion of proinflammatory visceral adipose tissue in CAH patients (Kim *et al.*, 2015). Metabolic syndrome was observed in nearly 20% of CAH adults in the NIH cohort (Finkielstain *et al.*, 2012). Blood pressure (BP) control in children and adult CAH patients has been investigated by several independent groups, with some studies reporting normal resting (Mooij *et al.*, 2010; Ubertini *et al.*, 2009; Sartorato *et al.*, 2007; Falhammar *et al.*, 2007) and 24 h BP profile (Marra *et al.*, 2015) and others reporting a slight increase systolic BP (Arlt *et al.*, 2010; Cutler and Laue, 1990; De Silva *et al.*, 2004; Völkl *et al.*, 2006; Nermoen *et al.*, 2012; Mooij *et al.*, 2011; Roche *et al.*, 2003). In a recent epidemiological Swedish registry study, increased frequency of hypertension in individuals with CAH has been shown, but when analyzing the different subgroups, only SV females had increased blood pressure (Falhammar *et al.*, 2015). This was in accordance with a recent study showing that adult males with classic CAH have a rather low BP compared with healthy men (Bouvattier *et al.*, 2015). Dyslipidemia in individuals with CAH has

been reported in some studies. In the epidemiological Swedish registry study, an increased rate of dyslipidemia was found, especially in males with null genotype (Falhammar *et al.*, 2015).

In this intricate scenario, it is reasonable to expect a high CV risk profile in CAH patients. However, cardiovascular morbidity and mortality are not easy to bring out in this population, as very few of the studied patients are older than 50. Carotid intima-media thickness (cIMT) is a marker of early, subclinical atherosclerotic change, that is correlated to the risk for coronary artery disease and stroke (Stein *et al.*, 2008). While increased cIMT has been shown in CAH adults in one study (Ubertini *et al.*, 2009), further studies in children or adolescent CAH patients showed normal cIMT compared to a body mass index (BMI)-matched cohort (Sartorato *et al.*, 2007; Falhammar *et al.*, 2007) or increased cIMT compared to controls, but linked to higher BMI and unfavorable metabolic parameters (Marra *et al.*, 2015; Cutler and Laue, 1990; De Silva *et al.*, 2004). Recently, we reported the complex interactions between gonadotropins and steroid hormones on the duration of ventricular repolarization. We found that CAH QT interval duration was shorter in women with CAH than in control women (Abehsira *et al.*, 2016). These findings and their clinical impact have to be further examined. The association between endothelial dysfunction, cIMT progression, hormonal imbalance, treatment of CAH, and CVD events, will be important to figure out in this at-risk cohort.

The rate of CV events among adults with CAH is beginning to be characterized (Falhammar *et al.*, 2015). The long-term outcomes in CAH patients were studied using the Swedish national CAH registry. The mean age of death was lower in CAH patients (41.2 $\pm$ 26.9 vs. 47.7 $\pm$ 27.7 years ($P < 0.001$)). The hazard ratio of death was 2.3 (1.2–4.3) in males and 3.5 (2.0–6.0) in females. The causes of death were adrenal crisis (42%), cardiovascular diseases (32%), cancer (16%), and suicide (10%) (Falhammar *et al.*, 2014). Interestingly, the same team analyzed CV and metabolic morbidity in CAH patients (Falhammar *et al.*, 2015). This study showed an increase in both CV and metabolic disorders (OR 3.9; 95% CI [3.1–5.0]), and CV disease (OR, 2.7; 95% CI [1.9–3.9]). Separate analyses of the individual diseases showed higher frequencies of hypertension, diabetes, obstructive sleep apnea, dyslipidemia and atrial fibrillation in CAH patients. Obesity was consistently increased in all subgroups. However, the nonobese patients with CAH were similarly affected as the entire CAH cohort. Increased frequency of venous thromboembolic events was also reported. This should be further studied to determine if, as reported in both Cushing syndrome and GC use, there is a higher risk of venous thromboembolism due to a state of hypercoagulability, which should lead to more frequent use of thrombosis prophylaxis in this population.

CAH is therefore associated with higher CV risk factors and probably with excess CV and metabolic morbidity. Some subgroups of patients seem to be more affected. Regular follow-up is needed, along with lifestyle interventions, to limit the onset of weight gain and obesity, to screen for diabetes, other metabolic disorders and CV risk factors. Close monitoring of GC doses is important. Further studies on larger cohorts are necessary to better clarify the mechanisms leading to metabolic and CV abnormalities, and to precise the respective roles of androgen and lifelong GC treatment.

Male Fertility

Male patients with CAH may present impaired gonadic function and infertility. It appears that adult males with CAH face a dual problem. Adrenal steroid overproduction, especially androgen and progesterone, might interfere with FSH and LH production, resulting in gonadotropic deficiency. In addition, testicular adrenal rest tumors (TARTs) may become hypertrophic under chronic ACTH stimulation and influence both endocrine and exocrine testicular functions (Reisch *et al.*, 2010).

TARTs are rare and benign testicular tumors, first defined in 1940 by L. Wilkins. They have usually been reported in classic CAH and, even if it is still controversial, presence of TART would be sometimes also detected in nonclassic CAH (Falhammar and Thorén, 2012). TARTs have been identified with a prevalence of 30%–95% depending on age and modality of diagnosis, that is, palpation or ultrasound (Bouvattier *et al.*, 2015; Pierre *et al.*, 2012). The prevalence of TARTs increases with age, after onset of puberty (Claahsen-van der Grinten *et al.*, 2014; Aycan *et al.*, 2013). TARTs volume and prevalence seem higher in patients with SW form compared with those in the SV form (Pierre *et al.*, 2012; Reisch *et al.*, 2009; Yu *et al.*, 2015). Because of their central localization in testis, TARTs are difficult to palpate especially if they measure <2 cm. Therefore, imaging plays an important role. Ultrasonography should be the method of first choice for detection and follow-up of these lesions, because it is the cheapest and quickest imaging technique (Stikkelbroeck *et al.*, 2003). Magnetic resonance imaging (MRI) has not been completely approved yet. But, in particular cases (like surgery treatment), MRI could be recommended because it details mapping and shows lesion margins optimally (Stikkelbroeck *et al.*, 2003; Ozisik *et al.*, 2017).

The pathogenesis of TARTs could result from proliferation of aberrant adrenal-like tissue. Its cells of origin may be adrenal cortical rest cells, hilar Leydig cells or pluripotential cells of the testes (Engels *et al.*, 2017). In CAH disease, the elevated ACTH level stimulates such remnant adrenal cells leading the development of this testicular adrenal rest tumors. However, some studies report the development of TARTs despite good hormonal control, suggesting that undertreatment is not the only cause for their growth (Chihaoui *et al.*, 2016; Reisch *et al.*, 2013). Indeed, a recent study about molecular characterization of TARTs has shown that these tumors have multiple steroidogenic properties, including the expression of adrenal cortex and typical Leydig cell markers (Smeets *et al.*, 2015).

TARTs are most often responsible for impaired spermatogenesis. In patients with and without TARTs, inhibin B levels differ significantly and there are higher total sperm counts and concentration in patients without TARTs (Bouvattier *et al.*, 2015). In a large cohort of 164 male CAH patients who underwent testicular assessment with ultrasound, 71 had a sperm analysis. Seventy percent of the patients had severe oligospermia or azoospermia when TARTs were found versus only 3.6% when they were not

(Bouvattier *et al.*, 2015). Because of their central localization near the rete testis, TARTs can lead to compression of the seminiferous tubules that may finally lead to obstructive azoospermia and irreversible damage of the surrounding testicular tissue (Reisch *et al.*, 2009). They also can destroy and replace healthy testicular tissue. A decreased tubular diameter and presence of peritubular fibrosis and tubular hyalinization were reported in testicular biopsies of man CAH patients with TARTs and infertility history (Claahsen-van der Grinten *et al.*, 2008). The profile of the gonadotropic–testicular axis in these patients will primarily show testicular failure and eventually reveal endocrine and exocrine testicular dysfunctions (Bry-Gauillard *et al.*, 2014).

Fertility preservation is a key management goal in TART. Treatment options for male patients with TARTs are still limited and mainly based on a good hormonal control with GC (Yu *et al.*, 2015). Intensified glucocorticoid treatment is recommended as a first step treatment. However, in some patients, treatment is poorly tolerated, and the medical response is disappointing. Sparing surgical approach can be proposed for important tumors and steroid unresponsive masses or in testicular pain situation but there are no data on fertility preservation. Recently, Bry-Gaillard *et al.* have shown that mitotane could restore fertility in CAH patients with TARTs (Bry-Gauillard *et al.*, 2014). After 8 months of treatment, gonadotropins levels, inhibin B and sperm counts have improved, and on the other hand, size of TARTs has shrunk.

Prevention has a real important place in the management of male CAH fertility. A systematic ultrasound evaluation is recommended at puberty to detect lesions at an early stage. A semen analysis should also be realized as soon as possible, and the question of systematic sperm cryopreservation seems fully justified (King *et al.*, 2016).

Reduced fertility in CAH men can also be secondary to hypogonadotropic hypogonadism due to poor hormonal control with increased adrenal androgens and progesterone, leading to an increase in estrogen levels by aromatization. A recent case report has demonstrated the restoration of fertility by gonadotropin replacement in a CAH man (Rohayem *et al.*, 2014). The patient had hypogonadotropic azoospermia and TARTs due to untreated SV form. A treatment with gonadotropin replacement permitted to obtain after 21 months a stable low sperm concentration with good sperm motility, enabling the couple to have a healthy girl.

Besides these somatic causes of impaired fertility in CAH males, there might be aspects of psychosocial adaptation and sexual well-being. Very few data have been reported on sexual satisfaction in CAH males. Two recent studies have shown that fewer CAH patients than controls had an active sexual life and that they had fewer lifetime partners (Falhammar and Thorén, 2012; Falhammar *et al.*, 2014). In a Swedish cohort of 30 CAH males, erectile dysfunction was found in about half of these patients (Falhammar *et al.*, 2014) as was described in the study from Dudzińska *et al.* A sexual well-being study of 20 CAH males has revealed impairments in sexual drive, erections and ejaculations (Dudzińska *et al.*, 2014). Poor control disease was associated with a reduced sexual drive. However, in the recent Swedish follow-up study described previously, although the reason is unknown, men were more often married than controls (OR 1.6 (1.0–2.5)) but, as the CAH women, they had less biological children than controls (OR 0.4 (0.2–0.6)) (Strandqvist *et al.*, 2014). Further studies are needed to properly assess these psychosocial outcomes, to improve the care given to the patients.

Bone Mineral Density

Bone mineral density alteration has been an understandable concern for adult patients with CAH and who receive chronically glucocorticoids (Ogilvie *et al.*, 2006). Corticosteroid therapy is known to inhibit osteoblastic activity, which potentially leads to decreased bone density (Guo *et al.*, 1996); similarly, markers of bone formation such as osteocalcin, are reported to be low in adult CAH. However, the potential impact of chronic GC therapy on BMD is difficult to precisely determine due to the young age of patients enrolled in some studies, leading therefore to conflicting results. Most reports fail to show decreased BMD in young patients, contrarily to studies enrolling older patients (Mora *et al.*, 1996; Cameron *et al.*, 1995; Bachelot *et al.*, 2007; Chakhtoura *et al.*, 2008). Although increased BMI might protect against low BMD, this has not been found in CAH patients. However, all these studies provide compelling evidence that the lowest possible GC dose should be used in the treatment of CAH, and lower-dose and short-acting GC may be sufficient in the middle-aged and elderly female when osteoporosis becomes, more than fertility, an important concern (Merke, 2008). Osteoporosis prophylaxis such as physical activity and calcium and Vitamin D supplementation should be implemented at a young age. Screening dual-energy x-ray absorptiometry (DXA) should be performed in CAH adults.

Treatment of Classic CAH in Adults

Treatment in classic 21OHD is necessary to compensate for GC and MC deficiencies but also to correct adrenal androgen excess. Ideally, the treatment should be monitored in order to avoid iatrogenic comorbidities and to enable a good quality of life (Reisch, 2015). However, this goal is not reached up to now, since increased comorbidities and mortality are reported in patients with CAH (Bachelot *et al.*, 2017).

GC substitution is available since the 1950s. Although this treatment has notably changed the prognosis of children with CAH, it has remained the only medical solution for the last decades and has failed to meet all of the needs for the patient. Indeed, contrarily to primary adrenal insufficiency, the aim of GC treatment is not only to compensate for the deficient hormone but also to blunt the nocturnal ACTH secretion, which is the major driver of adrenal androgen production. Since cortisol is secreted mainly in the morning and, reaches a peak between 6 and 8 am, most oral GC regimens are proposed with at least half or 2/3 of the global dose in the morning. Up until now, whatever regimen used, the dilemma has persisted between using the physiological HC, well

tolerated but with a poor control of androgen secretion or the long acting prednisone, prednisolone or dexamethasone (DEX), with a higher risk of side effects. Unfortunately, an adequate androgen secretion is difficult to achieve without a high dose of GC, therefore leading to side effects in relation to hypercorticism. Recently a new slow release HC formula (Plenadren) has become available and another one (Chronocort) is under current investigation. In addition, besides the GC approach, non-GC approaches are under current development, such as the use of molecules interfering with CRH function and nonselective adrenal steroidogenesis blockade (Turcu and Auchus, 2016) (**Fig. 1**).

New Glucocorticoid Approaches

The first molecule is a dual-release HC which was developed for once-daily morning administration in patients with primary adrenal insufficiency (Plenadren, ViroPharma-Shire) (Johannsson *et al.*, 2009). It is a modified-release HC with an outer coating layer that provides an immediate release of the drug and an extended-release core. It provides a more extended serum profile of cortisol compared with immediate-release HC. In adults, a single morning dose of Plenadren gives similar cortisol exposure to a thrice-daily regimen of immediate-release HC. Preliminary studies in patients with primary adrenal insufficiency and CAH have demonstrated that this new formula compared with HC regimen, improves body weight, systolic and diastolic blood pressure, and glucose metabolism (Johannsson *et al.*, 2012; Quinkler *et al.*, 2015). This molecule also provides a more circadian-based serum cortisol profile in patients with primary adrenal insufficiency. Unfortunately, there are no data currently available regarding hormonal control in patients with CAH. However, in the latter case, this molecule is unlikely to control excess androgens as the overnight rise in cortisol is not replicated, and evening administration of Plenadren would expose patients to high levels of cortisol during the night.

The second molecule, Chronocort, is under current development by Diurnal (United Kingdom) (Debono *et al.*, 2015). This molecule is a modified-release HC, but it differs from Plenadren by having a delayed and sustained absorption profile rather than an immediate- and sustained-release profile. Chronocort aims at replacing physiological cortisol concentrations by dosing at

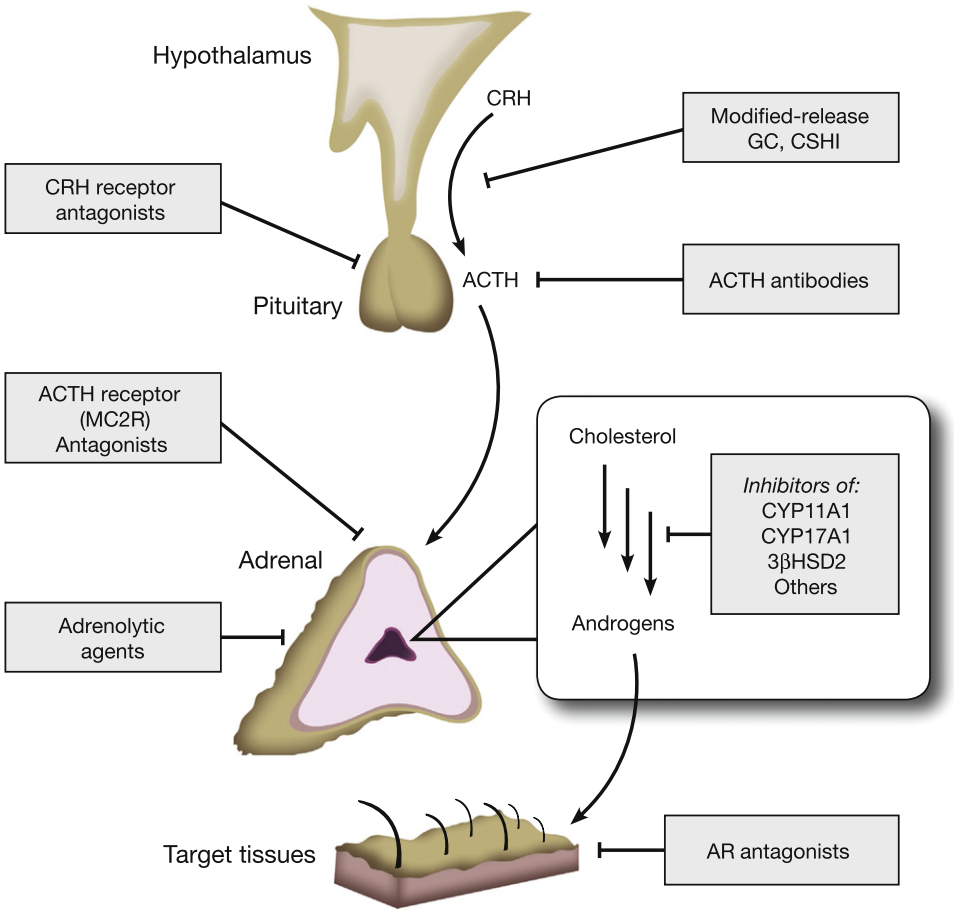

Fig. 1 Potential therapeutic targets in congenital adrenal hyperplasia. *3βHSD2*, 3β-hydroxysteroid dehydrogenase type 2; *ACTH*, adrenocorticotropin hormone; *AR*, androgen receptor; *CRH*, corticotropin-releasing hormone; *CSHI*, continuous subcutaneous hydrocortisone infusion; *CYP11A1*, cholesterol side chain cleavage enzyme; *CYP17A1*, 17α hydroxylase/17,20 lyase; *GC*, glucocorticoids.

morning and night such that the night dose provides release of HC in the early hours of the morning providing a prewaking rise in cortisol levels. In a first Phase II, open label study, Chronocort has shown its ability to decrease the 8 am 17OHP level; however, androgen levels rise in the afternoon with once-daily dosing, suggesting that an additional morning dose of GC is needed (Verma *et al.*, 2010). Another phase 2 study on 16 patients was designed to evaluate the efficiency of a double dose of Chronocort (10 mg at 7 am and 20 mg at 11 pm). It showed that after 6 months of treatment, the mean androstenedione and 17OHP levels were diminished compared with those observed under classical GC therapies (Mallappa *et al.*, 2015). More recently, Chronocort has been shown to reduce the 17OHP and alternative pathway metabolite excretion, also observed in CAH patients by gas chromatography–mass spectrometry, to near-normal levels, more consistently than other GC preparations (Jones *et al.*, 2017). All these preliminary data need to be confirmed also on clinical parameters and in larger populations.

Before the development of these new molecules, the use of parenteral GC infusion had been discussed. The reproduction of cortisol circadian rhythm permitted to bring 17OHP and ACTH levels closer to physiological values compared to conventional therapies (Merza *et al.*, 2006). More recently, a continuous subcutaneous HC infusion was found to be more efficient than conventional GC in a randomized trial on 33 patients with primary adrenal insufficiency. Doses of HC were adjusted depending on salivary cortisol. In this study, ACTH levels and cortisol profiles were respectively lower and more physiological than those observed under conventional oral GC therapy. However, the impact of this treatment on quality of life remains a matter of debate (Oksnes *et al.*, 2014; Gagliardi *et al.*, 2014). Another approach has been proposed to improve the dynamics of HC infusions. In healthy volunteers, intrinsic adrenal function was blocked by DEX, whereas HC was permanently infused subcutaneously (Russell *et al.*, 2014). Cortisol and ACTH were measured every 10 and 60 min, leading to the replication of the circadian rhythmicity. A recent phase 2 trial on the use of sub cutaneous hydrocortisone pump in eight CAH patients with poor control has been published (Nella *et al.*, 2016). In this study, subcutaneous hydrocortisone pump approximated physiologic cortisol secretion. Six months treatment resulted in improved adrenal steroid control and had positive effects on quality of life. All these studies may be considered as interesting or even more promising; however they also underline the difficulty in managing the patients daily (Russell *et al.*, 2014). The cost of these pumps, the potential dysfunction and local irritation, may also be considered as limiting factors. The long-term effect, tolerance and acceptation of this treatment will require further studies.

Nonglucocorticoid Approaches

The most radical treatment is the surgical removal of both adrenal glands. This approach must be carefully discussed since it can induce the development of TARTs in men and less frequently retroperitoneal adrenal rest tumors in women (Tiosano *et al.*, 2010; Crocker *et al.*, 2012). Chemical adrenalectomy may be obtained using Mitotane, an adrenolytic agent for which the mechanisms of action remain poorly understood. Recent evidences focus on apoptotic effects at adrenocortical level reducing activity of the respiratory chain complex and inducing mitochondrial fragmentation, leading to programmed cell death (Gentilin *et al.*, 2014; Hescot *et al.*, 2013). Mitotane also modulates the expression of several genes involved in steroid hormone biosynthesis (Hescot *et al.*, 2013). In addition, the lack of LH increase following the decrease in free testosterone suggests that Mitotane may have a toxic effect on the testes and also on the pituitary by reducing viability of gonadotroph cell lines through activated apoptosis (Gentilin *et al.*, 2014).

Two novel approaches are under current investigation: the development of androgen biosynthesis inhibitors and the development of ACTH and CRH receptor antagonists.

The development of androgen biosynthesis inhibitors is based on the necessity of decreasing secretion and action of androgens since, as previously mentioned, most of the GC treatments are unable to induce such blockade. Abiraterone is an inhibitor of CYP17A1, an enzyme necessary for androgen synthesis (**Fig. 1**). In men treated for prostate cancer, abiraterone acetate has proven its efficiency in decreasing testosterone levels (Ryan *et al.*, 2015). A recent phase I study in CAH women permitted to observe, after 6 days of treatment, a decrease by 2/3 of the androstenedione level when the administered dose was 100 mg/day. When the dose was increased up to 250 mg/day, the androstenedione level was completely normalized after 6 days of treatment. However, this approach does not compensate for the adrenal insufficiency, and therefore requires the adjunction of a GC, and in some patients MC treatment (Auchus *et al.*, 2014). This treatment cannot be used in male patients, as it blocks all androgen synthesis.

Since ACTH is a key factor in controlling adrenal function, the opportunity of interfering with the corticotrope axis has been proposed. ACTH is the only known naturally occurring agonist for its receptor. The high degree of ligand specificity suggests that antagonism of its receptor could provide a useful therapeutic approach and at least an investigational tool. Different experimental models, in animals, may provide new insights in this potential new approach (Clark *et al.*, 2016).

Besides ACTH, CRH is at the hypothalamic level, the key factor inducing, after a specific binding to CRH receptor type 1, ACTH secretion. Any antagonism to this receptor could be a potential therapeutic strategy. This approach has been recently explored with a selective CRH receptor type 1 antagonist NBI-77860. In a single blind, placebo-controlled study, eight patients with CAH have received a fixed dose of 300 or 600 mg, each period of treatment being separated by a 3 weeks washout time; treatment was prescribed at 10 pm and ACTH and 17OHP were measured sequentially during the day after drug administration. There was a reduction of ACTH by a mean of 43% and 41%, under 300 and 600 mg respectively compared with placebo, whereas 17OHP was reduced by a mean of 0.7% and 27% under the same treatments (Turcu *et al.*, 2016). These promising data provide a rationale for ongoing experimental studies using CRH receptor antagonist, without forgetting that this approach does not compensate for cortisol and aldosterone deficiencies.

Table 1 Management of adult patients with classic CAH

Monitoring of the efficacy of glucocorticoid replacement therapy
● Early-morning serum concentrations of 17-OHP, Δ4-androstenedione, total testosterone, 30 to 60 min after drug intake
● SHBG approximately every six to 12 months
● Diurnal 17OHP curve with dried blood spots if available
● Weight gain and clinical signs of cortisol and androgen excess
Monitoring of the efficacy of mineralocorticoid replacement
● Blood pressure
● Plasma electrolytes
● Early-morning plasma renin activity concentration
Periodic measurements and/or monitoring of the following:
● Weight
● Lipid profile
● Blood pressure
● Glycemia
● Bone mineral density
Assessment of male gonadic function and fertility
● Testicular adrenal rest by ultrasonography
● Sperm analysis
● Fertility preservation
● Hormonal measurements: total testosterone, LH, FSH
Genetic counseling
Psychological support

Conclusion

Recent years have brought new insights in the description of CAH comorbidities especially in the CV and fertility areas (**Table 1**). In both cases this description suggests the need for new therapeutic approaches. After decades of relatively stagnant progress, advances are now noted. Besides improved GC delivery systems, oral or parenteral, new GC approaches are under current elevation such as inhibitors of androgen biosynthetic enzymes or CRH receptor antagonists. All these approaches may have pros and cons. In all cases, larger trials to determine the outcomes and safety profiles are needed, in adults as well as in children.

See also: Adrenal Insufficiency: Etiology and Diagnosis. Steroid Replacement in Adrenal Insufficiency

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Cushing Syndrome; Screening and Differential Diagnosis

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Abbreviations

ACTH	Adrenocorticotropic hormone or corticotropin
ARMC5	Armadillo Repeat Containing 5 gene
BMAH	Bilateral macronodular adrenal hyperplasia
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid-binding globulin
CRH	Corticotropin releasing hormone
CS	Cushing syndrome
CT scan	Computed tomography scan
DST	Dexamethasone suppression test
FDG PET scan	Fluorodeoxyglucose-positron emission tomography scan
GNAS	Stimulatory G-protein alpha subunit
GPCR	G-protein coupled receptor
HDDST	High dose dexamethasone suppression tests
HPLC	High performance liquid chromatography
iMAD	Isolated micronodular adrenocortical disease
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LNSC	Late night salivary cortisol
MC2R	Melanocortin 2 receptor (ACTH receptor)
MRI	Magnetic resonance imaging
PET	Positron emission tomography
PKA	Protein kinase A
PPNAD	Primary pigmented nodular adrenal dysplasia
PRKACA	Protein kinase A catalytic subunit α
SPECT	Single-photon emission tomography
UFC	Urinary free cortisol in 24 h collection

Glossary

ARMC5 Was recently identified as a gene whose germline and somatic mutations cause 25%–50% of BMAH; it is thought to be a tumor suppressor gene but its precise function is still unknown.

BMAH Primary bilateral macronodular adrenal hyperplasia is a rare cause of Cushing syndrome.

Cortisol secreting adrenal adenoma The most frequent cause of ACTH-independent Cushing syndrome, in which a unilateral adrenocortical tumor produces excess cortisol.

Cushing disease The most frequent etiology of Cushing syndrome, in which a pituitary corticotroph tumor produces excess ACTH.

PPNAD Primary pigmented nodular adrenal dysplasia is a rare cause of bilateral micronodular adrenal hyperplasia that can be familial and associated with other tumors of the Carney complex.

Introduction

Cushing syndrome (CS) patients often constitute a challenge for clinicians who suspect and wish to confirm hypercortisolism, identify its specific etiology and achieve optimal treatment, which generally involves removal of a causative tumor(s) (Pendleton *et al.*, 2010; Nieman *et al.*, 2008). The diagnosis of CS is considered to be one of the most difficult to establish in endocrinology. This rare and highly morbid condition often requires several months or years before proper diagnosis and effective therapy are achieved.

There is a wide range of severity of cortisol excess in endogenous CS from clinically almost silent, cyclical, mild to rapid onset severe variants (Arnaldi *et al.*, 2003; Biller *et al.*, 2008; Nieman *et al.*, 2008; Lacroix *et al.*, 2015). The variable biology of the different tumors responsible for CS explains the imperfect reliability of biochemical and radiological investigations, in particular in the initial phases or mild forms of the syndrome (Arnaldi *et al.*, 2003; Nieman *et al.*, 2008; Lacroix *et al.*, 2015). The numerous caveats to diagnosis of CS together with its rarity emphasize the need for the eventual referral to expert teams that have a solid experience in CS. We review here the recent progress on the initial screening and identification of the various etiologies of CS. Other chapters of this Encyclopedia review the molecular mechanisms, specific investigation and therapies of the various specific etiologies of CS.

Epidemiology and Etiologies

As CS is a rare condition, its precise incidence and prevalence has not been well established. Its incidence was 0.2–5.0/million/year and its prevalence between 39 and 79/million individuals in various studied populations (Etxabe and Vazquez, 1994; Lindholm *et al.*, 2001; Steffensen *et al.*, 2010; Bolland *et al.*, 2011; Valassi *et al.*, 2011). A Danish population studied during 11-years yielded

an incidence of CS (nonmalignant disease) of 2.3/million/year; the median age was 41.4 year (range, 3.6–77.7) with a female: male ratio of 3:1 (Lindholm *et al.*, 2001). Using a retrospective private health insurance claims database (16 million insured lives per year from all regions of the United States), CS incidence rates was estimated to be of 39.5–48.6 per million person-years, a much higher rate than reported for European populations (Broder *et al.*, 2015); ascertainment of diagnosis was not however as stringent as in the previous European studies. CS incidence was higher in women than men, with rates of 61.5–77.4 for women, compared to 15.6–17.2 for men (Broder *et al.*, 2015). Some studies identified higher, but variable prevalence of CS in patients with uncontrolled diabetes type II, hypertension or early onset osteoporosis (Taniguchi *et al.*, 2008; Murakami *et al.*, 2010; Chiodini *et al.*, 2007; Gagliardi *et al.*, 2010; Terzolo *et al.*, 2012; Krarup and Hagen, 2012).

Endogenous CS is caused by ACTH-dependent or ACTH-independent disorders (**Table 1**) (Lacroix *et al.*, 2015). The most frequent cause is excess secretion of ACTH either by a pituitary corticotroph adenoma (Cushing's disease (CD)), or less frequently from extra-pituitary (i.e., "ectopic") benign or malignant tumors, which are primarily neuroendocrine tumors (NET) (Lacroix *et al.*, 2015). Up to 20% of the ACTH-secreting ectopic tumors remain occult for many years (Ilias *et al.*, 2005; Isidori *et al.*, 2006). Rarely, the responsible tumor (medullary thyroid carcinoma or pheochromocytoma) secretes either corticotropin-releasing hormone (CRH) alone, which stimulates excess pituitary ACTH secretion, or both CRH and ACTH (Lacroix *et al.*, 2015).

The prevalence of the most frequent etiology, CD, was 39.1 per million inhabitants (incidence 1.2–2.4 newly diagnosed cases/million persons per year) in previous European studies (Lindholm *et al.*, 2001; Etxabe and Vazquez, 1994). The prevalence of CD was higher (62.1/million) in an Icelandic population, with a significant female predominance (52.8 vs. 9.3/million for men) (Agustsson *et al.*, 2015). In a recent Danish pediatric age population study, the annual incidence of CS was 0.89 cases/million population with a median age at the time of diagnosis of 13.8 years (interquartile range: 10.5–18.2 years), 58% were female and 70% had a corticotroph pituitary adenoma (Holst *et al.*, 2017). The average age at diagnosis in other pediatric CD series was similar at 13 years (symptom onset 10.6 years) (Kanter *et al.*, 2005; Lonser *et al.*, 2013). Symptom initiation to diagnosis averages 2–3 years in pediatric and adult cases (Zillio *et al.*, 2014). Before puberty, the ratio of female-to-male cases is similar (1:1) (Kanter *et al.*, 2005; Lonser *et al.*, 2013).

Cortisol excess from unilateral adrenocortical adenomas or carcinomas suppresses ACTH levels, leading to their characterization as "ACTH-independent." These tumors account for approximately 20% of CS cases (Arnaldi *et al.*, 2003; Biller *et al.*, 2008; Nieman *et al.*, 2008; Lacroix *et al.*, 2015). Infrequently, CS is caused by primary bilateral macronodular adrenal hyperplasia (BMAH), or primary pigmented nodular adrenocortical disease (PPNAD) and its nonpigmented variant, isolated micronodular adrenocortical disease (iMAD) (Lacroix, 2009; Stratakis, 2008; Doppman *et al.*, 1989). As 10%–15% of adrenal incidentalomas are bilateral, BMAH with modest cortisol secretion is more frequent, but its precise prevalence is unknown (Lacroix, 2009, BMAH chapter in this Encyclopedia). Similarly, 5%–30% of unilateral incidentalomas are associated with modest cortisol secretion that rarely progresses to overt clinical CS as the cases with overt Cushing syndrome frequently carry *PRKACA* mutations (Chiodini, 2011; Fassnacht *et al.*, 2016; Beuschlein *et al.*, 2014).

Clinical Symptoms and Initial Screening

The wide distribution of glucocorticoid receptors throughout the body explains the varied and multiple clinical manifestations of CS that include morphological changes (weight gain, central obesity, buffalo hump, etc.); neuropsychiatric (insomnia, cognitive impairment, depression and irritability) and dermatological (easy bruising, facial plethora, purple striae, acne, hirsutism) disorders; immunosuppression leading to vulnerability to infections; cardiovascular (hypertension, atherosclerosis), metabolic (hypokalemia, glucose intolerance, diabetes mellitus, and dyslipidemia), musculoskeletal (proximal muscle weakness, osteoporosis), and sexual/reproductive complications (hypogonadism, hirsutism) (Arnaldi *et al.*, 2003; Biller *et al.*, 2008; Nieman *et al.*, 2008; Lacroix *et al.*, 2015; Pivonello *et al.*, 2015, 2016). This heterogeneity makes the clinical diagnosis of CS challenging, considering that many features of this condition are nonspecific and highly prevalent among the general population, such as high blood pressure, abdominal obesity, diabetes, osteoporosis, and altered phenotypic characteristics (Nieman *et al.*, 2008; Pivonello *et al.*, 2016).

Screening is recommended in individuals in whom CS manifestations are most likely: patients with unusual age-related features (e.g., hypertension in young adults), with multiple and progressive manifestations, children with decreasing growth velocity and increasing weight, and patients with adrenal incidentaloma (Nieman *et al.*, 2008). Multiple and progressive features suggestive of CS might not be all be present, but some more specific ones, such as catabolic features and central obesity justify screening (Nieman *et al.*, 2008; Guignat and Bertherat, 2010); patients with unexplained severe features, such as resistant hypertension and osteoporosis, regardless of age, require screening. Proximal muscle weakness, wide purple striae, and in children, diminished growth, appear more specific to CS, but are usually found in cases with higher or longer lasting hypercortisolism (Nieman *et al.*, 2008; Pecori Giraldi *et al.*, 2003). The use of exogenous glucocorticoids should be excluded before biochemical screening. As CS is a rare condition, routine screening in patients with morbid obesity, type 2 diabetes or in those with hypertension is unwarranted unless progressive new symptoms have accumulated relatively recently (Nieman *et al.*, 2008).

Biochemical evaluation of CS exploits the cardinal features of the increased endogenous production of cortisol: abnormal feedback of cortisol excess on the pituitary–adrenal axis, and the loss of the normal cortisol circadian rhythm (Lacroix *et al.*, 2015; Lonser *et al.*, 2017).

Table 1 Causes of endogenous Cushing syndrome

	Proportion (%)	*Age (peak)*	*Female:male*	*Features*
ACTH-dependent	70–80	–	–	–
Cushing's disease	60–70	–	–	–
Corticotroph adenoma	60–70	3rd–4th decades	3–5:1	Roughly 50% nonvisible on MRI
Corticotroph hyperplasia	Very rare	–	–	–
Ectopic ACTH[a]	5–10	–	–	–
Malignant neuroendocrine tumors	About 4	5th–6th decades	0.6–1:1	Might have very high ACTH
Benign neuroendocrine tumors	About 6	3rd–4th decades	–	Might respond to dexamethasone, CRH, desmopressin
Occult neuroendocrine tumors	About 2	–	–	–
Ectopic CRH	Very rare	–	–	Causes pituitary corticotroph hyperplasia
ACTH-independent	20–30	–	–	–
Unilateral adrenal	–	–	–	–
Adenoma	10–22	4th–5th decades	4–8:1	Most pure cortisol secretion
Carcinoma	5–7	1st, 5th–6th decades	1.5–3:1	Mixed cortisol and androgen frequent
Bilateral adrenal	1–2	–	–	–
Bilateral macronodular adrenal hyperplasia[b]	<2	5th–6th decades	2–3:1	Modest cortisol secretion compared with size; raised steroid precursors; might have combined androgen and mineralocorticoid cosecretion
Aberrant G-protein-coupled receptors	–	–	–	–
Autocrine ACTH production	–	–	–	–
Sporadic or familial (ARMC5)	–	–	–	–
Bilateral micronodular adrenal hyperplasias	<2	–	–	Adrenal size often normal
Primary pigmented nodular adrenocortical disease	Rare	lst–3rd decades	0.5:1 <12 years 2:1 >12 years	Frequent paradoxical increase of urine free cortisol with Liddle's oral dexamethasone suppression test
Isolated or familial with Carney complex	Rare	lst–3rd decades	–	–
Isolated micronodular adrenocortical disease	Very rare	Infants	–	Nonpigmented adrenal micronodules
Primary bimorphic adrenocortical disease	Very rare	Infants	–	–
McCune–Albright syndrome	Rare	Infants (<6 months)	1:1	Internodular adrenal atrophy
Bilateral adenomas or carcinomas	Rare	4th–5th decades	2–4:1	–

[a]Most frequent sources of ectopic ACTH syndromes are small cell lung carcinoma and neuroendocrine tumors of lung, thymus, and pancreas. Less frequent causes include medullary thyroid carcinoma, gastrinoma, pheochromocytoma, prostate carcinoma, and several others.

[b]In bilateral macronodular adrenal hyperplasia tissues, autocrine and paracrine ACTH might be produced and contribute to cortisol secretion. If confirmed by in vivo studies, the ACTH-independent classification will need to be modified in the future.

ACTH, Adrenocorticotropic hormone; CRH, corticotropin-releasing hormone.

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Three screening tests for the diagnosis of CS are recommended in the clinical practice guidelines of the Endocrine Society: late night salivary cortisol, 24-h urine free cortisol (UFC) and the 1 mg overnight (or 2-day 2 mg) dexamethasone suppression test (**Table 1**) (Nieman *et al.*, 2008; Lacroix *et al.*, 2015; Sharma *et al.*, 2015). To optimize sensitivity, despite decreased specificity, it is recommended to use the upper limit of the reference range as a cutoff for normal basal cortisol values; the assay sensitivity should allow for measurement of a serum cortisol below 50 nmol/L (1.8 μg/dL) the morning after 1 mg dexamethasone administration, which is the upper threshold for a normal response (Nieman *et al.*, 2008).

Other guidelines such as the French "National Diagnosis and Treatment Guideline (NDTG) for Cushing's syndrome" recommend screening for CS not only in those with unusual features based on age, but also if there are features with atypical severity, such as resistant hypertension, or unexplained osteoporosis (Guignat and Bertherat, 2010). The French NDTG does not consider the 2 mg 48-h DST as appropriate for first-line testing due to the difficulty in its implementation in the outpatient setting. It is therefore recommended as a potential second-line test, only following referral to an endocrinologist. Furthermore, the French disagree with the Endocrine Society guideline's suggestion that the desmopressin test be restricted to the research setting; the NDTG placed greater weight on emerging evidence from Europe that the desmopressin test may have better diagnostic accuracy than the Dex-corticotropin-releasing hormone (CRH), particularly in mild cases of hypercortisolism when the differential diagnosis includes only CD and pseudo-Cushing's states (Nieman *et al.*, 2008; Guignat and Bertherat, 2010).

CS diagnosis is highly probable in patients with a high pretest positive probability who have two abnormal screening tests (Nieman *et al.*, 2008). Patients that present cyclic hypercortisolism or nonconsistent screening tests responses may require additional testing. It is indicated to repeat tests, perform alternate tests, and/or await progression in patients with only mild features of CS (low pretest probability) and mildly abnormal test results (Nieman *et al.*, 2008; Lacroix *et al.*, 2015). Confirmation of hypercortisolism benefits from frequent midnight salivary cortisol and UFC measurements over a long period, sometimes over years (Atkinson and Mullan, 2011; Graham *et al.*, 2013). In a series of 152 patients with confirmed CD, the intrapatient coefficient of variation of UFC was 52% (95% CI: 48–56) between four basal values (Petersenn *et al.*, 2014).

Urinary Free Cortisol

UFC is an index of circulating free (biologically active) cortisol (de Castro and Moreira, 2007; Newell-Price *et al.*, 1998; Nieman *et al.*, 2008). Unlike plasma cortisol, which measures total cortisol (bound and unbound), factors that modulate corticosteroid-binding globulin (CBG) concentrations do not affect UFC results. When plasma cortisol exceeds the maximal binding capacity of CBG (approximately 690 nmol/L), plasma free cortisol and UFC increase more rapidly than cortisol production, and become sensitive indicators of hypercortisolism. UFC values greater than fourfold normal are rare except in CS (Nieman *et al.*, 2008; de Castro and Moreira, 2007; Newell-Price *et al.*, 1998). Lesser values are found either in CS or pseudo-CS. Up to 11% of patients with CS may have a normal UFC in one of four collections (Nieman *et al.*, 2008; Petersenn *et al.*, 2014; Ceccato *et al.*, 2015). When UFC is measured by immunoassays, the upper limit of normal in various assays ranges up to 220–330 nmol/day (80–120 μg/day) and often the antibodies will cross-react with various metabolites of cortisol and some exogenous glucocorticoids, which are measured as "cortisol." When separation of urinary glucocorticoids and cortisol metabolites is achieved by HPLC (or more recently by structure-based liquid chromatography tandem mass spectrometry (LC-MS/MS)), the normal range of UFC is usually $<$140–170 nmol/day. For the detection of CS, the sensitivity of UFC by HPLC is 95%–100% and its specificity is 94%–98% (Newell-Price *et al.*, 1998; Arnaldi *et al.*, 2003; Lacroix *et al.*, 2015) and by LCMS/MS of 97% and 91%, respectively (Ceccato *et al.*, 2015). Due to the variability of cortisol secretion from day to day, three 24-h urine specimens should be collected in order to increase the diagnostic yield. Urinary creatinine and volume also should be measured to verify the adequacy of the collection. If glomerular filtration rate is less than 30 mL/min, UFC is decreased, and may be normal despite the presence of CS (Nieman *et al.*, 2008).

Plasma Cortisol Variability and Midnight Plasma Cortisol

Several factors modify cortisol binding globulin (CBG) and consequently alter total cortisol concentrations. Predictably, oral estrogen administration (at higher doses in contraceptives) and pregnancy increase serum CBG and increase total cortisol values. The utility of plasma cortisol measurements for the diagnosis of CS rests on the loss or blunting of the normal circadian cortisol nadir. Healthy individuals who consistently sleep at the same time of day have a nadir cortisol that is tightly entrained to sleep onset (Krieger *et al.*, 1971). By contrast, although patients with CS often have awakening serum cortisol concentrations within or above the normal range, they lose the normal diurnal nadir, as the tumors secrete throughout the day even if in a pulsatile way. In hospitalized CS patients compared to healthy individuals, plasma cortisol was above 50 nmol/L, when sleeping at midnight, with sensitivity and a specificity of 100% (Newell-Price *et al.*, 1995). Midnight plasma cortisol was greater than 207 nmol/L (7.5 μg/dL) in awake patients with CS; this value had a sensitivity of 96% and a specificity of 100% in differentiating CS from pseudo-Cushing's states (Papanicolaou *et al.*, 1998). However serum cortisol measurements are not practical to obtain as most patients are not admitted to hospital for investigation.

Late Night Salivary Cortisol (LNSC)

Cortisol concentration in saliva correlates highly with free plasma cortisol, is independent of salivary flow rates, and is stable for up to 1 week in samples at room temperature (Raff, 2009). LNSC obtained at 23:00 can distinguish normal individuals from patients with CS (Raff, 2013). Diagnostic ranges vary between studies, due to the different assays and comparison groups used to set cutoff points (de Castro and Moreira, 2007). Salivary cortisol can be measured by immunoassay or liquid chromatography–tandem mass spectrometry (LC/MS-MS). Given the lower sensitivity of LC/MS-MS (as it may not measure cortisol metabolites/precursors), immunoassay remains a frequently utilized methodology for Cushing syndrome screening (Gafni *et al.*, 2000; Raff, 2013).

Education of the patient on optimal timing of specimen collection in relation to their normal sleep–wake cycle, as well as avoiding exciting or stressful experiences on the evening of the test, are important. False-positive results also can result from contamination of the saliva sample with blood from tooth brushing. Recent reports suggest that increased age and comorbidities of diabetes and hypertension are associated with increased values (Liu *et al.*, 2005; Raff *et al.*, 1999). However, overall, ease of collection, stability at room temperature, and a greater than 90% sensitivity and specificity make it a highly useful test, especially in outpatients, children, and in assessment of cyclic Cushing syndrome (de Castro and Moreira, 2007; Raff, 2013).

Low-Doses Dexamethasone Suppression Tests (DST)

The low dose DST is used to differentiate CS patients from those who do not have CS (**Fig. 1**); it is also the most sensitive screening test to identify modest cortisol secretion from adrenal incidentalomas (Nieman *et al.*, 2008; Fassnacht *et al.*, 2016). The overnight low dose DST (1 mg orally between 23:00–midnight) excludes with high probability the presence of CS when the 08:00–09:00 plasma cortisol on the following morning is below 50 nmol/L (1.8 μg/dL) and the patient does not have cyclical CS (Nieman *et al.*, 2008); rare patients with mild CS may suppress with 1-mg but not with 0.5-mg overnight DST (Liddle, 1960; Findling *et al.*, 2004). However, its specificity is limited, due to misclassification of patients with increased CBG, acute and chronic illness or pseudo-CS. If the next day 8:00 am serum cortisol is >275 nmol/L (10 μg/dL), the patient has a high probability of having CS. Between 50 and 275 nmol/L (1.8–10 μg/dL) the result is equivocal and further testing should be performed (Nieman *et al.*, 2008; Sharma *et al.*, 2015).

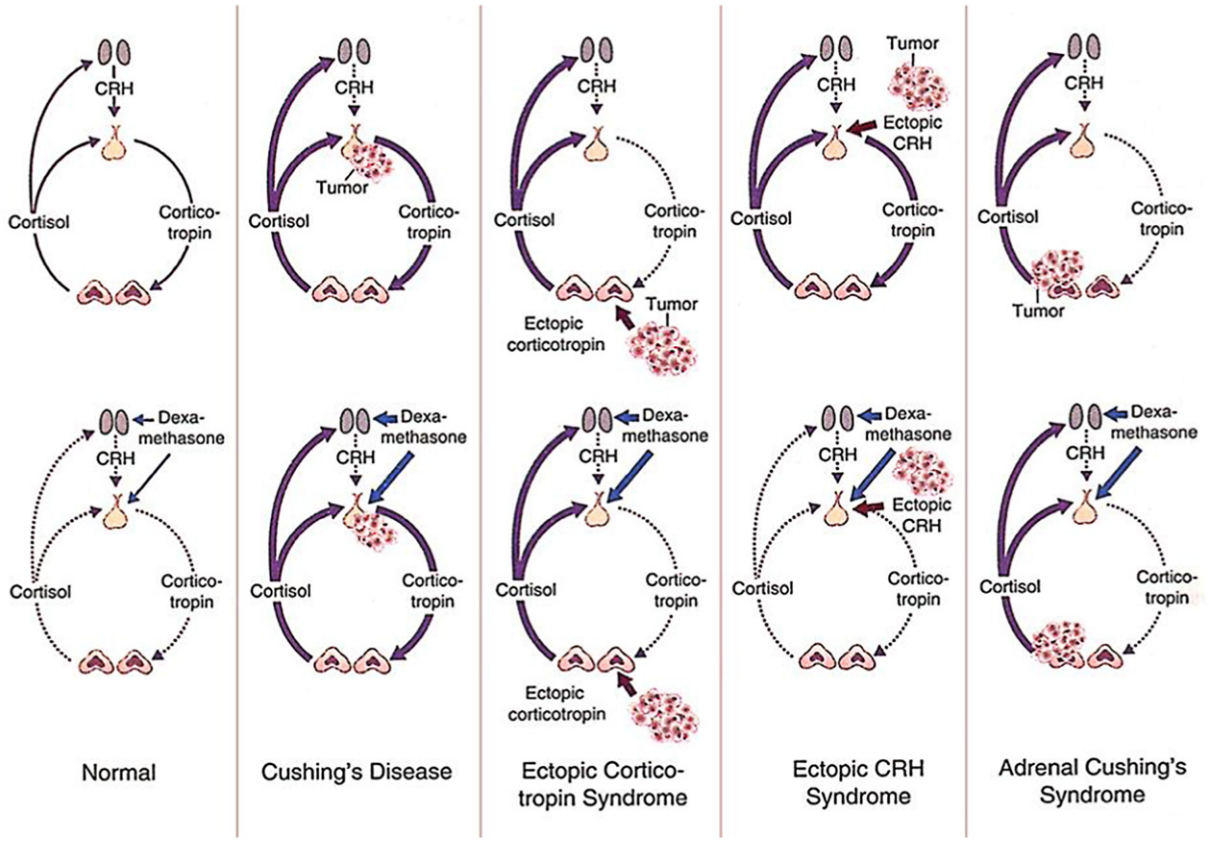

Fig. 1 Differential regulation of the hypothalamic–pituitary–adrenal axis in normal subjects and in the various etiologies of Cushing syndrome. In normal subjects, stimulation of the hypothalamus by central nervous system centers regulates the secretion of CRH which in turn increases ACTH secretion. ACTH stimulates adrenal secretion of cortisol which, as exogenous low dose dexamethasone (lower panel), inhibits the secretion of both CRH and ACTH. In Cushing's disease, excess ACTH originating from the corticotroph tumor is partially resistant to excess glucocorticoid and high dose dexamethasone suppression, while CRH and normal corticotropes are suppressed. Ectopic ACTH secretion from malignant tumors is usually autonomous from CRH and dexamethasone regulation, while ectopic ACTH originating from up to 50% of benign NET can be suppressed partially by high dose dexamethasone or respond to exogenous CRH or desmopressin similar to Cushing disease. In adrenal Cushing syndrome caused by unilateral adrenal tumors, ACTH is suppressed by excess cortisol and is not modified by CRH or high doses of dexamethasone. In bilateral macronodular adrenal hyperplasia, cortisol can be regulated by the ligands of various aberrant hormone receptors (GPCR) stimulating paracrine ACTH production by adrenal hyperplasia cells acting on the ACTH receptor (MC2R); however, circulating ACTH levels remain low. Normal hormone secretion is shown by lines, suppressed secretion by thinner or dotted lines, and hypersecretion by thick lines. Reproduced with permission from Lacroix, A., Feelders, R.A., Stratakis, C.E. and Niemann, L.K. (2015). Cushing's syndrome. *Lancet* **386**, 913–927.

In the 2-day low dose test, dexamethasone 0.5 mg is taken orally at 6-h intervals for eight doses. Urine is collected for UFC on two baseline days and during dexamethasone administration. A normal response consists of a urinary free cortisol <27 nmol (10 μg) per 24 h on the second day of dexamethasone administration (Nieman *et al.*, 1986); plasma cortisol should be <50 nmol/L (1.8 μg/dL) on the third morning.

Interfering conditions causing an apparent lack of suppression following dexamethasone include: decreased dexamethasone absorption, drugs enhancing CYP3A4 hepatic dexamethasone metabolism (barbiturates, phenytoin, carbamazepine, rifampin, meprobamate, methaqualone), increased concentration of CBG (estrogen treatment, pregnancy) and pseudo-CS. Plasma dexamethasone levels can be determined at the time of the cortisol blood-draw and should be between 20 and 51 nmol/L (Newell-Price *et al.*, 1998; Nieman *et al.*, 2008).

Dexamethasone-CRH Test

This test developed at NIH was shown to be particularly helpful in distinguishing CS from pseudo-CS (Yanovski *et al.*, 1993). The rationale was that patients with pseudo-CS have chronically elevated centrally stimulated CRH levels and do not respond to additional exogenous CRH administration, under dexamethasone suppression, as opposed to Cushing's disease patients who have suppressed endogenous CRH levels and fail to suppress normally to dexamethasone. Dexamethasone is taken orally starting at 12:00 (0.5 mg every 6 h) for 48 h and ovine CRH (1 μg/kg) is administered iv at 8:00 (2 h after the last dose of dexamethasone). The plasma cortisol value 15 min after CRH increased above 38 nmol/L (1.4 μg/dL) in patients with CS, but remained suppressed in normal individuals and in patients with pseudo-CS. This combined test yielded a sensitivity of 98%–100% and a specificity of 88%–100% (Yanovski *et al.*, 1993, 1998). Other groups reported that the addition of CRH test did not provide added information to the conduct of the 2 days low dose DST (Gatta *et al.*, 2007; Pecori Giraldi *et al.*, 2007a, b). Several drugs utilized by patients undergoing investigation for CS can alter interpretation of Dex-CRH test (Valassi *et al.*, 2009). In patients with anorexia nervosa, several patients did not suppress morning cortisol levels after 2 days low dose DST, but did not increase cortisol following CRH administration; this stresses the importance of examining cortisol levels not only at the 15 min time point following CRH administration (Duclos *et al.*, 1999). Desmopressin was also found to be able to distinguish between CD and pseudo-Cushing's states (Tirabassi *et al.*, 2010; Pecori Giraldi *et al.*, 2007a, b). However, the exact diagnostic accuracy of these tests and the optimal cutoffs for diagnosis need further evaluation (Nieman *et al.*, 2008). A prospective study of 73 patients in Netherlands with clinical features of CS and an abnormal DST or UFC result reported a positive predictive value of 100% and a negative predictive value of 90% for the Dex-CRH test (sensitivity 94%, specificity 100%) using a 15-min post-CRH cortisol cutoff of 3.2 μg/dL (87 nmol/L) (Alwani *et al.*, 2014).

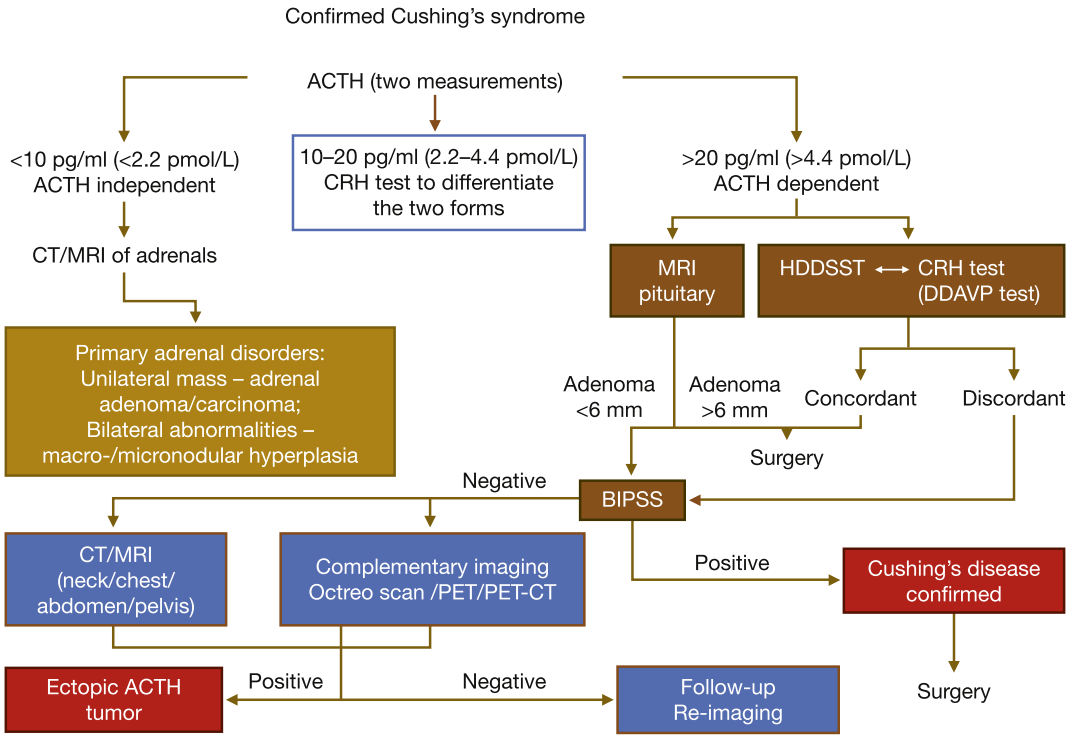

Fig. 2 Clinical decision making flow chart for the differential diagnosis of confirmed Cushing syndrome of different etiologies. Reproduced with permission from Lacroix, A., Feelders, R.A., Stratakis, C.E. and Niemann, L.K. (2015). Cushing's syndrome. *Lancet* **386**, 913–927.

Patients with minimal CS features, abnormal screening tests and no tumoral cause are referred as having "Pseudo-Cushing syndrome." Various conditions associated with mild hypercortisolism should be considered in the differential diagnosis. Generally, hypercortisolism resolves as these conditions are treated or remit (Findling and Raff, 2017).

Determining the Etiology of Confirmed CS

Once CS diagnosis is established, the cause must be determined to effect a specific treatment. Cortisol excess, apart from cyclic or minimal hypercortisolism, leads to CRH and ACTH suppression from normal cells (**Fig. 2**) (Lacroix *et al.*, 2015). Thus, the first step in the differential diagnosis of CS is measurement of plasma ACTH in the basal state, preferably on two different mornings (Arnaldi *et al.*, 2003; Lacroix *et al.*, 2015).

CS With Suppressed ACTH

A decreased plasma ACTH concentration (<5 pg/mL or 1.1 pmol/L) in a patient with overt endogenous hypercortisolism indicates an adrenal cause (Lacroix *et al.*, 2015) (**Figs. 1** and **2**). Intermediate ACTH values between 5 and 15 pg/mL (1.1–3.3 pmol/L) are less clear, but if CS is moderate to severe, it is probably ACTH-dependent. When cortisol secretion is modest or cyclic, ACTH suppression may be incomplete; a CRH stimulation test is recommended to evaluate ACTH responsiveness (Newell-Price *et al.*, 1998; Lacroix *et al.*, 2015). If ACTH is <1.1 pmol/L, thin-section CT images of the adrenal glands usually identifies its cause; unilateral adenoma is the most common (Arnaldi *et al.*, 2003; Lacroix *et al.*, 2015). If a suspicious lesion (by morphology or large size) is found, or if DHEAS levels are elevated, the possibility of adrenal carcinoma is high; FDG-PET scan and MRI may help to establish this diagnosis (Else *et al.*, 2014).

In bilateral micronodular hyperplasias, the adrenal size is not enlarged, but occupied by several bead-like small nodules on high resolution CT (Doppman *et al.*, 1989). In PPNAD, cortisol is usually poorly stimulated by exogenous ACTH and patients respond frequently with a paradoxical UFC increase during sequential low and high doses dexamethasone tests as a consequence of overexpression of glucocorticoid receptors in the micronodules (Stratakis *et al.*, 1999; Bourdeau *et al.*, 2003).

In BMAH, plasma 17-OH-progesterone or urinary 17-OH-corticosteroids are proportionally more elevated than urinary free cortisol as a result of reduced activity of several steroidogenic enzymes in the tumor cells with accumulation of steroid precursors (Antonini *et al.*, 2006; Hsiao *et al.*, 2009; Libe *et al.*, 2010). Screening for aberrant GPCR are helpful to confirm BMAH with overt or subclinical primary CS (Hsiao *et al.*, 2009; Libe *et al.*, 2010; Hofland *et al.*, 2013; El Ghorayeb *et al.*, 2015). A genetic cause of BMAH should be sought using analysis for germline *ARMC5* mutations (Drougat *et al.*, 2015; Espiard *et al.*, 2015; Fragoso *et al.*, 2015); if the index case does not carry an *ARMC5* mutation, family screening can be performed, searching for excess plasma morning cortisol following the 1 mg overnight DST; those with cortisol responses >50 nmol/L (1.8 μg/dL) should undergo adrenal imaging (Lacroix, 2009; Lacroix *et al.*, 2015).

ACTH-Dependent CS

Pituitary imaging

Pituitary imaging should be performed only after biochemical confirmation of ACTH-dependent CS (Fig. 3). T1-weighted SE MRI with Gadolinium contrast identifies pituitary tumors in $\sim$50% of patients with CD (Patronas *et al.*, 2003; Newell-Price *et al.*, 1998; Lacroix *et al.*, 2015). Approximately 10% of healthy individuals have incidental pituitary lesions up to 6 mm diameter on MRI (Hall *et al.*, 1994; Buurman and Saeger, 2006). As a result, the finding of a pituitary lesion $\leq$6 mm does not reliably indicate that CD is the cause of CS. Other MRI techniques may have better sensitivity: spoiled gradient recalled acquisition in the steady state technique (SPGR) had better sensitivity for detection of microadenomas than T1 SE imaging in adults (80% vs. 49%) and children (68% vs. 29%) (Patronas *et al.*, 2003; Leães *et al.*, 2009; Batista *et al.*, 2005; Kasaliwal *et al.*, 2013).

Noninvasive testing

In contrast to most ectopic ACTH tumors, most CD adenomas maintain sensitivity to CRH stimulation and suppression with high dose dexamethasone test (Newell-Price *et al.*, 1998; Arnaldi *et al.*, 2003; Nieman *et al.*, 2008). Results are heterogeneous using human versus ovine CRH, different ACTH assays and different criteria for ACTH and cortisol response (Tirabassi *et al.*, 2011). The two largest studies found for the ACTH response to CRH a sensitivity of 93% (oCRH) and 70% (hCRH), respectively, and a specificity of 100% (Nieman *et al.*, 1993; Newell-Price *et al.*, 2002). For the cortisol response, sensitivity was 91% and 85% and specificity 88% and 100% respectively to detect CD.

Intravenous administration of 10 μg desmopressin (a preferential V2-vasopressin receptor agonist) increases ACTH secretion in 80%–90% of corticotroph adenomas via activation of vasopressin (AVPR2, AVPR1B) receptors; normal subjects only rarely increase ACTH secretion after desmopressin (Arnaldi *et al.*, 2003; Tsagarakis *et al.*, 2002; Tirabassi *et al.*, 2011). However, about 20% of ectopic ACTH secreting tumors express those receptors and respond to desmopressin, so the test cannot distinguish the ACTH source (Tsagarakis *et al.*, 2002). An increase in serum cortisol of 33% or more or a plasma ACTH increase of 30% or more is

considered as a positive response. Desmopressin is less expensive than CRH and can be used in performing petrosal sinus sampling and in the postoperative assessment of these patients (Tsagarakis *et al.*, 2002).

Various protocols with oral or intravenous high dose dexamethasone suppression tests (HDDST) evaluate CS etiology (Newell-Price *et al.*, 1998; Lacroix, 2017) UTD; the most frequently used are the oral 2-day 8 mg HDDST and the 8 mg overnight test using a >50% suppression of cortisol levels as indicative of pituitary-dependent CS. The diagnostic performance of HDDST alone is poor because 20%–30% of patients with ectopic ACTH (mainly bronchial carcinoids) suppress cortisol <50% of baseline and up 20%–30% of patients with CD fail to suppress cortisol levels >50% (Ilias *et al.*, 2005; Isidori *et al.*, 2006; Aron *et al.*, 1997). Combining CRH and HDDST tests increases specificity so that a positive response to both tests indicates CD; however, discordant results of CRH test and HDDST, with only one test positive, are found in up to 65% of patients with CD (Isidori *et al.*, 2003; Nieman *et al.*, 1986).

Petrosal sinus sampling (BIPSS)

BIPSS is the "gold standard" test to identify a pituitary versus ectopic source of ACTH, with sensitivity and specificity of ~95% (Arnaldi *et al.*, 2003; Nieman *et al.*, 2008; Lacroix *et al.*, 2015). A pituitary source results in a central to peripheral gradient >2 before and >3 after CRH or desmopressin (Fig. 3). Falsely positive results can occur in ectopic ACTH patients with cyclic hypercortisolism or mild hypercortisolism with insufficient suppression of normal corticotropes. False negative sampling may result from abnormal venous drainage or inability to cannulate the veins (Doppman *et al.*, 1999). Measurement of prolactin (to normalize ACTH values) in cases without a gradient can confirm successful catheterization (Sharma *et al.*, 2011). In 396 patients, all 10 with falsely negative results (none with positive results) had peak petrosal sinus ACTH values of <400 pg/mL (Wind *et al.*, 2013). BIPSS has limited value in identifying tumor location within the pituitary; a side-to-side gradient of >1.4 correctly identified tumor side in 69% of lateral adenomas (Wind *et al.*, 2013).

Source of ectopic ACTH/CRH production

Thin-cut multislice CT and/or MRI of thorax and abdomen and scintigraphic studies localize tumors in 70%–90% of ectopic ACTH cases (Ilias *et al.*, 2005; Isidori *et al.*, 2006). NET express somatostatin receptors, and may be identified with ^{111}In-pentreotide scintigraphy (octreoscan), with a sensitivity ranging from 25% to 80% depending on dosing and use of single-photon emission tomography (SPECT) (Tsagarakis *et al.*, 2003). Hypercortisolism can suppress tumoral somatostatin subtype 2 receptor levels leading to false negative results; negative octreoscan can become positive after medical control of hypercortisolism (de Bruin *et al.*, 2012). ^{111}In-pentreotide SPECT/CT fusion imaging improves tumor localization (Wong *et al.*, 2010). 18-Fluorodeoxyglucose (FDG) positron emission tomography (PET) has limited diagnostic yield for low grade NET. [18*F*]l-3,4-dihydroxyphenylalanine (F-DOPA)-PET and 11C-5-hydroxy-tryptophan (11C-5-HTP)-PET have higher sensitivity to detect NET, but no large series have been published with ectopic ACTH (Wong *et al.*, 2010; Pacak *et al.*, 2004; Koopmans *et al.*, 2006; Zemskova *et al.*, 2010; Nikolaou *et al.*, 2010; Jagannathan *et al.*, 2009).

Pediatric CS

Approximately 10% of CS cases occur in children, with a pubertal female:male predominance; in very young children a male to female predominance may exist (Stratakis, 2012). Before age 7, adrenal causes of CS (adenoma, carcinoma or bilateral hyperplasia) are most common while CD accounts for approximately 75% of CS after that age (Savage *et al.*, 2007; Stratakis, 2012). Overall, primary adrenal etiologies account for ~15% of CS in childhood. Adrenocortical neoplasms represent 0.6% of all childhood tumors and CS is present in one third of pediatric adrenal cancers; most patients present under age 5 and there is a female: male predominance. Micronodular bilateral hyperplasias (PBAD, PPNAD and iMAD) are a more frequent cause of CS in children than in adults (Stratakis, 2012). Ectopic ACTH accounts for <1% of CS in adolescents; neuroblastomas and ganglioneuromatous tumors may exceptionally cause ectopic ACTH in young infants.

The most common symptom of pediatric CS is weight gain, but a unique feature is the effect on height gain: the combination of weight gain and decreased height velocity is pathognomonic for CS in children. Other manifestations include facial plethora, headaches, hypertension, hirsutism, virilization, amenorrhea or delayed sexual development. Acne, violaceous striae, bruising and acanthosis nigricans are also common. The investigation and treatment of adult and pediatric CS are similar (Savage *et al.*, 2007; Stratakis, 2012).

Cyclical CS

The evaluation of patients with cyclical Cushing syndrome can be a major diagnostic challenge, in part due to its frequency, which can reach 15% of cases (Alexandraki and Grossman, 2011; Atkinson and Mullan, 2011). Cyclical CS has been reported from all causes of the syndrome (Mullan *et al.*, 2007). Regardless of age, PPNAD and iMAD often present with cyclical CS (Stratakis, 2012). Confirmation of hypercortisolism benefits from frequent midnight salivary cortisol and UFC levels over a long period, sometimes over years (Atkinson and Mullan, 2011; Graham *et al.*, 2013). Recently, the utility of capillary (hair) cortisol has been evaluated with the goal of establishing a temporal characterization of the cortisol secretion in cyclic Cushing syndrome (Manenschijn *et al.*, 2012).

CS in Pregnancy

Pregnancy occurs rarely in CS, probably because hypercortisolism inhibits ovulation. When present, it increases risk of fetal abortion, perinatal death, premature birth, intrauterine growth retardation, hypertension, diabetes, and preeclampsia (Lindsay *et al.*, 2005; Lekarev and New, 2011; Bronstein *et al.*, 2015; Caimari *et al.*, 2017). Diagnosis is complicated by the overlap of UFC levels between CS patients and normal pregnant women. Additionally, the etiology of CS in women who become pregnant is most frequently adrenal in origin (50%–60% of the cases), while in nonpregnant population pituitary-dependent Cushing's disease (CD) is responsible for 70% of the cases (Lindsay *et al.*, 2005; Abdelmannan and Aron, 2011; Bronstein *et al.*, 2015). The reason for this difference is not known; however, it has been suggested that in CD there is hypersecretion of both cortisol and androgens, impairing fertility, while in CS of adrenal origin hypersecretion is almost exclusively of cortisol with minimal androgen production (Buescher *et al.*, 1992). In primary adrenal causes of CS during pregnancy, ACTH levels are not uniformly suppressed, as CRH and ACTH of placental origin are not regulated identically to the ACTH of pituitary origin (Lindsay *et al.*, 2005; Abdelmannan and Aron, 2011; Bronstein *et al.*, 2015). Exacerbation or transient CS during pregnancy can regress partially postpartum when aberrant LHCG receptors are expressed in unilateral adenomas or BMAH (Lacroix *et al.*, 1999; Lacroix *et al.*, 2010; Plöckinger *et al.*, 2017); as the placenta produces many hormones, regulation by other placental factors could explain adrenal CS etiologies during pregnancy. TSS or adrenalectomy are preferred treatment except perhaps late in the third trimester where metyrapone can be used cautiously; remission may not improve the fetal prognosis (Lindsay *et al.*, 2005; Lekarev and New, 2011; Bronstein *et al.*, 2015; Caimari *et al.*, 2017).

Conclusions

Patients with CS require complex and often repeated investigations by a multidisciplinary expert team to identify the cause of their syndrome. This is the essential initial step which will then require specific surgery or medical therapy to hopefully result in remission, with requirement of monitoring for possible recurrence, insure adequate hormonal replacement and to treat the psychological and multiorgan consequences of the previous exposure to excess glucocorticoids which have been reviewed recently elsewhere and in other chapters of this Encyclopedia (Nieman *et al.*, 2015; Pivonello *et al.*, 2015; Lacroix *et al.*, 2015).

See also: ACTH-Secreting Pituitary Tumors

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Molecular Genetics of Cushing Disease

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Abbreviations

11β-HSD	11-β-Hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
ACTHR	Adrenocorticotropic hormone receptor
AIP	Aryl hydrocarbon receptor-interacting protein
AKT	v-akt murine thymoma viral oncogene homolog 1
BMP4	Bone morphogenetic protein 4
Brg1	Brahma-related gene 1
CABLES1	CDK5 and ABL1 enzyme substrate 1
CCND2	Cyclin D2
CCNE1	Cyclin E
CD	Cushing disease
CDH23	Cadherin-related 23
CDK	Cyclin-dependent kinase
CDK4	Cyclin-depentent kinase subunit 4
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CNC	Carney complex
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor 1
DKC1	Dyskeratosis congenital
DRD2	Dopamine receptor 2
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
ERBB2	v-erb-b2 erytrhoblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-regulated protein kinase
FGF	Fibroblast-growth factor
FGFR	Fibroblast-growth factors receptor
FIPA	Familial isolated pituitary adenomas
GNAS	Guanine nucleotide binding protein, alpha stimulating
GPR101	Orphan G protein-coupled receptor
GR	Glucocorticoid receptor
Gs	Stimulatory G protein
HDAC2	Histone deacetylase
HSP90	90 kDa heat shock protein
IL-6	Interleukin-6
LGALS3	Lectin galactoside-binding soluble 3
LIF	Leukemia inhibitory factor
MAS	McCune–Albright syndrome
MEK	Mitogen activated protein kinase
MEN	Multiple endocrine neoplasia
MGMT	O6-methylguanine DNA methyltransferase
miRNAs	microRNAs
NR0B1	Nuclear receptor subfamily 0 group B member 1
NR3C1	Nuclear receptor subfamily 3 group C member 1
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
POMC	Proopiomelanocortin
PRKAR1A	Protein kinase c-AMP dependent type1 regulatory subunit α
PRKCD	Protein kinase C-δ
PTTG	Pituitary tumor transforming gene
RPS13	Ribosomal protein S13
SDH	Succinate dehydrogenase
SHH	Sonic hedgehog
SSTR	Somatostatin receptor
TIMP2	Metalloproteinase inhibitor 2
TR4	Testicular orphan receptor 4
TSC	Tuberous sclerosis complex
USP8	Ubiquitin-specific protease 8
V<n>R	Vasopressin receptor <n>
ZNF676	Zinc-finger 676

Introduction

Cushing disease (CD) is a severe condition caused by the chronic exposure to excessive endogenous glucocorticoids triggered by ACTH-secreting pituitary tumor (corticotropinoma). It is characterized by multiple complications resulting in increased morbidity and mortality and severe impairment of quality of life (Newell-Price *et al.*, 2006; Dekkers *et al.*, 2007). It is a rare disease, with an annual incidence of 1.2–1.7 cases per million (Lindholm *et al.*, 2001). Corticotropinomas are usually sporadic tumors and only rarely they occur as part of genetic familial syndromes. The molecular basis of Cushing disease was unclear until recently, when next generation sequencing revealed a single somatic mutational hotspot in the ubiquitin-specific protease 8 (*USP8*) gene in almost half of the corticotropinoma cases (Reincke *et al.*, 2015; Ma *et al.*, 2015).

The Pathophysiology of Corticotroph Tumor Cells

The corticotroph function is tightly regulated by stimulatory and inhibitory signals from the hypothalamus and the negative regulatory glucocorticoid feedback loop from the adrenal gland.

Hypothalamic Regulation

Corticotrophin-releasing hormone (CRH) is the main corticotroph trophic factor that stimulates *POMC* transcription and ACTH synthesis. Its receptors (CRHR1 and 2) are coupled to the Gs protein that triggers cAMP production and downstream cAMP/PKA signaling cascade. CRHR1 is overexpressed in corticotropinomas compared to silent corticotropinomas and nonfunctioning pituitary tumors (De Keyzer *et al.*, 1998; Tateno *et al.*, 2007).

Vasopressin is another hypothalamic factor which, synergistically with CRH, stimulates ACTH release. Vasopressin receptors also belong to the G protein couple receptor superfamily. The vasopressin receptor V3R is abundant in corticotroph tumors but no mutations were detected in the coding region of the gene (de Keyzer *et al.*, 1996; Dahia *et al.*, 1996). Corticotroph tumors also express high levels of vasopressin receptor subtype 1b (VPR1b) and respond to the synthetic VPR1 ligand desmopressin by increasing their proliferation in vitro (Luque *et al.*, 2013).

Hypothalamic peptides that exert inhibitory action on corticotroph function include dopamine and somatostatin. Dopamine receptor D2 (DRD2, also known as D2DR and D2R) is found in the majority of corticopinomas and treatment with dopamine agonists inhibit ACTH secretion in vitro and suppress cortisol levels in patients with Cushing disease (Pivonello *et al.*, 2004). Corticotropinomas also express somatostatin receptor (SSTR) 5 and -in lesser extent-2 and 3 (Miller *et al.*, 1995). Excessive glucocorticoid stimulation suppresses *SSTR2* transcription probably accounting for the reduced SSTR2 levels (de Bruin *et al.*, 2009); SSTR2 expression is recovered after restoring cortisol levels to physiological levels (van der Pas *et al.*, 2013). Glucocorticoids do not affect SSTR5 and DRD2 expression and more than half of cases co-express DRD2 and SSTR5 (Hofland *et al.*, 2005; de Bruin *et al.*, 2009). To date use of SSTR5 targeting somatostatin analogs alone or in combination with the DRD2 agonist cabergoline is the only approved tumor-targeted pharmacological therapy available for the management of patients with Cushing disease (Feelders *et al.*, 2010).

Glucocorticoid Resistance

One of the hallmarks of Cushing disease is the resistance to the negative glucocorticoid feedback (Nieman *et al.*, 2008). Mutations in the *NR3C1* gene that encodes for the glucocorticoid receptor are extremely rare in corticotroph tumors (Karl *et al.*, 1996a,b; Dahia *et al.*, 1997). One study suggested loss of heterozygosity at the GR gene locus may contribute to glucocorticoid resistance (Huizenga *et al.*, 1998).

The local glucocorticoid action is regulated by the 11-β-hydroxysteroid dehydrogenases: the isoenzyme type 1 (11β-HSD1) metabolizes cortisone to cortisol whereas the isoenzyme type 2 (11β-HSD2) inactivates cortisol to cortisone. Corticotropinomas express high levels of 11β-HSD2, suggesting that increased inactivation of cortisol to cortisone may represent another molecular mechanism of intratumor glucocorticoids resistance (Korbonits *et al.*, 2001).

The transcriptional repression of *POMC* depends on transcriptional co-regulators like Brahma-related gene 1 (Brg1) and histone deacetylase 2 (HDAC2) (Bilodeau *et al.*, 2006). Brg1 and HDAC2 levels are dramatically decreased in corticotropinomas, which may hinder the ability of glucocorticoids to repress *POMC* transcription, resulting in glucocorticoid resistance (Bilodeau *et al.*, 2006). In contrast corticotropinomas show overexpression of testicular receptor 4 (TR4 nuclear receptor subfamily 2, group C, member 2) that blocks the GR transcriptional repressor activity resulting in increased *POMC* transcription, ACTH secretion and tumor growth in vitro and in vivo (Du *et al.*, 2013; Zhang *et al.*, 2016b).

A recent study has highlighted the importance of the heat shock protein 90 (HSP90) in promoting glucocorticoid resistance in corticotropinomas (Riebold *et al.*, 2015). HSP90 is a chaperone protein that regulates GR function influencing the correct folding of the ligand-binding domain and controlling its translocation to the nucleus. HSP90 was found to be overexpressed in corticotropinomas and to impair the ability of glucocorticoids to repress *POMC* transcription. Inhibition of HSP90 specifically at the c-terminal with silibilin restored glucocorticoid sensitivity in ATt20 cells and displayed potent antiproliferative and antisecretory action, improving clinical features of CD, in a mouse corticotroph model (Riebold *et al.*, 2015).

Mechanisms of Tumorigenesis

Corticotroph tumors present with aberrant expression of proteins crucial in cell cycle control, proliferation, growth, and differentiation.

Cell Cycle

Cell cycle is strictly controlled and fine-tuned by the complex interaction between cyclins and cyclins depended kinases (CDKs), which promote cell cycle progression, and CDK inhibitors, which arrest cellular proliferation (Sherr and Roberts, 1999). Cell cycle pathways are deregulated in all pituitary tumor types and as we will see below some specifically in corticotropinomas.

Cyclin D—INK4 cell cycle regulatory module

Cyclin D1 is encoded by the *CCND1* gene. It binds to CDK4 that phosphorylates and inactivates retinoblastoma (Rb), thereby promoting G1/S cell cycle progression. Cyclin D1 overexpression is observed mainly in nonfunctioning and aggressive pituitary tumors and is not a frequent event in corticotropinomas (Jordan *et al.*, 2000).

The cyclin D/CDK4/6 action is inhibited by the INK4 family of CDK inhibitors (p16, p18, and p19). p16/INK4 is encoded by the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene. It specifically binds to CDK4/6 and prevents it from phosphorylating Rb, arresting the cells at G1. Undetectable or lower levels of p16 were documented in all pituitary tumors, including corticotropinomas (Woloschak *et al.*, 1996; Seemann *et al.*, 2001).

Cyclin E—Cip/Kip cell cycle regulatory module

Cyclin E is encoded by the *CCNE1* gene. It associates with the CDK2 that hyperphosphorylates Rb at the late G1/S phase. Cyclin E overexpression is predominant in corticotroph tumors (Jordan *et al.*, 2000).

The cyclin E/CDK2 function is inhibited by the Cip/Kip family of CDK inhibitors (p21, p27, and p57). p27/Kip1 is encoded by the cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene. It binds to CDK2 causing cell cycle arrest at the late G1 phase. p27/kip1 protein is dramatically reduced specifically in corticotroph tumors (Lidhar *et al.*, 1999). No changes were described at mRNA level indicating a posttranscriptional deregulatory event. However factors that regulate p27 translation, such as dyskeratosis congenita (DKC1)/Dyskerin, ribosomal protein S13 (RPS13), miR221 and miR222, are not deregulated in corticotropinomas (Martins *et al.*, 2016).

Cyclin E overexpression and p27 protein loss are concomitantly observed in corticotroph tumors (Roussel-Gervais *et al.*, 2016). A feasible explanation is that the cyclin E-bound CDK2 phosphorylates and marks p27/Kip1 for proteasomal degradation. Cyclin E is transcriptionally suppressed by Brg1, therefore the decreased Brg1 expression observed in corticotroph tumors may allow for excessive cyclin E expression that leads to p27 degradation and protein loss and aberrant cell cycle progression. These observations provide with a common pathogenetic mechanism responsible for the glucocorticoid resistance and cell cycle deregulation observed in corticotroph tumors.

CABLES 1

CABLES1 (CDK5 and ABL1 enzyme substrate 1) is a tumor suppressor gene, which inactivates several CDK causing cell cycle arrest. In corticotroph cells it is activated in response to glucocorticoids, playing an important role in the physiological negative feedback. CABLES 1 immunoreactivity is absent/dramatically reduced in in more than half corticotropinoma cases (Roussel-Gervais *et al.*, 2016). CABLES1 stabilizes members of the Cip/Kip family of CDK inhibitors and its loss of expression has been correlated with p27/kip1 loss in corticotroph tumor cells.

Screening a cohort of 146 pediatric and 35 adult CD patients for *CABLES1* mutations revealed four germline missense variants in four female patients, but no loss-of-heterozygosity (Hernández-Ramírez *et al.*, 2017). All patients harboring mutations had large corticotropinomas with high Ki-67 proliferation index and a difficult to manage disease. In vitro studies demonstrated that corticotroph cells harboring CABLES1 mutations are not sensitive to glucocorticoid negative feedback.

PTTG

The pituitary tumor transforming gene (PTTG) encodes for securin that regulates sister chromatid separation during anaphase. In addition it stimulates the expression of growth factors such as fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) and *CMYC* oncogene. PTTG is overexpressed in pituitary tumors including corticotropinomas (Zhang *et al.*, 1999). PTTG overexpression is accompanied by increased proliferation index Ki-67, and both are associated with tumor aggressiveness and increased risk of recurrence (Filippella *et al.*, 2006).

Growth Factors and Cytokines

The anterior pituitary gland is under the regulation of trophic and inhibitory growth factors, cytokines and developmental factors and their receptors that are intrinsically expressed and finetune hormone production and cell proliferation.

EGF

Epidermal growth factor (EGF) is a major mitogenic and differentiation factor that regulates pituitary hormone secretion and cell proliferation. The EGF–EGFR system is of particular importance in corticotroph pathophysiology. EGF is a trophic factor for corticotroph cells and stimulates ACTH synthesis to similar extent as CRH (Theodoropoulou *et al.*, 2015 and references therein). EGFR is highly expressed in corticotropinomas where it correlates negatively with p27/Kip1 immunoreactivity (Kontogeorgos *et al.*, 1996; Theodoropoulou *et al.*, 2004). Small molecule EGFR inhibitors effectively suppressed corticotroph tumor growth and ACTH synthesis and ameliorated Cushing disease in xenograft animal models (Theodoropoulou *et al.*, 2004; Fukuoka, 2015; Fukuoka *et al.*, 2011).

EGFR activates two major signaling pathways, the mitogenic MEK/ERK, and the survival PI3K/Akt. Enhanced Akt signaling has been documented in pituitary tumors including corticotropinomas (Muşat *et al.*, 2005). In contrast there is evidence for a special role of the MEK/ERK pathway in regulating ACTH synthesis downstream to EGFR in corticotropinoma cells (Reincke *et al.*, 2015). This is further highlighted by the observations of remarkable reduction in corticotroph tumor growth and ACTH secretion in vitro and in vivo after treatment with the specific MEK-162 small molecule inhibitor (Zhang *et al.*, 2016a).

FGF

Fibroblast-growth factors (FGFs) play an important role in several processes such as mitogenesis, differentiation, angiogenesis, and tumorigenesis. The normal anterior pituitary gland expresses FGFR1, FGFR2, FGFR3, and low levels of FGFR4. In contrast corticotropinomas overexpress FGFR4 (Brito *et al.*, 2010) and functional *FGFR4* polymorphisms were linked either to silent macrocorticotropinomas (R388) or to small hormonally active tumors (G388). The FGFR-G388 polymorphism has been associated to lower postoperative remission rate in patients with Cushing disease (Nakano-Tateno *et al.*, 2014).

Cytokines

Interleukin 2 (IL-2) and its receptor are expressed in corticotropinoma cells were they exert an autocrine–paracrine loop that regulates cell proliferation (Arzt *et al.*, 1992).

IL-6 is potent stimulator of POMC transcription and ACTH secretion from corticotroph tumor cell cultures (Pereda *et al.*, 2000).

Leukemia inhibitory factor (LIF) is a pleiotropic immune cytokine, which plays an important role in the hypothalamo–pituitary–adrenal response to inflammation. LIF enhances *POMC* transcription in vitro in synergy with CRH (Bousquet *et al.*, 1997; Ray *et al.*, 1996). LIF knockout mice show low *Pomc* transcript levels and reduced adrenal response to stress, that is reversed by administration of exogenous murine LIF (Chesnokova *et al.*, 1998). LIF overexpression in transgenic animals induces corticotroph hyperplasia, hypercortisolism, and Cushingoid features (Yano *et al.*, 1998). No mutations on the gene encoding for the LIF receptor were found in the seven corticotropinomas analyzed (Heutling *et al.*, 2004).

BMP4

Bone morphogenetic protein 4 (BMP4) is member of the transforming growth factor-beta superfamily. BMP4 inhibits *POMC* transcription and secretion. BMP4 expression is decreased in corticotropinomas and nude mice inoculated with corticotroph tumor cells transfected with a dominant-negative form of the BMP4 or with a BMP4 inhibitor present with increased tumorigenesis reinforcing its role as a corticotroph tumor suppressor (Giacomini *et al.*, 2006). BMP4 mediates the antiproliferative and antisecretory action of pharmaceuticals with potential for the treatment of Cushing disease such as retinoic acid and somatostatin analogs (Giacomini *et al.*, 2006; Tsukamoto *et al.*, 2010).

Sonic hedgehog

Sonic hedgehog (Shh) signaling is crucial for early pituitary differentiation, but in the adult pituitary its expression is restricted to the corticotroph cells where it crosstalks with CRH to regulate ACTH secretion (Vila *et al.*, 2005a) Shh restrains corticotroph cell proliferation and its expression is lost in corticotropinomas indicating that it may act as a tumor suppressor (Vila *et al.*, 2005b).

Results From Transcriptomics Studies

Transcriptomic analysis of 12 corticotropinomas showed that the invasive corticotropinomas displayed different expression profile compared to noninvasive ones. Highlighted genes include *CCND2* coding for cyclin D2, and Zinc-finger 676 (*ZNF676*) that are increased in invasive corticopinomas, whereas death-associated protein kinase 1 (*DAPK1*) and metalloproteinase inhibitor 2 (*TIMP2*) were decreased (de Araújo *et al.*, 2017).

MicroRNAs

MicroRNAs (miRNAs) are noncoding RNA transcripts that silence gene expression posttranslationally and are subsequently involved in several biological processes such as proliferation, differentiation, and apoptosis. miRNAS have been detected in normal pituitary tissue and in pituitary tumors. Corticotropinomas show downregulation of the miR-let7a, -15a, -16, -21, -141, -143, -145, and -150 compared to the normal pituitary tissues (Amaral *et al.*, 2009). In contrast, miR-26a, -30, -122 and, -493 are significantly upregulated, with the last two being increased in corticotroph carcinomas compared to nonmetastatic tumors (Bottoni *et al.*, 2007; Stilling *et al.*, 2010; Gentilin *et al.*, 2013).

miR-26a suppresses protein kinase C delta (PRKCD), a serine-threonine kinase that regulates cell cycle, cellular proliferation, differentiation, and apoptosis. Knocking down *PRKCD* in corticotroph cells increased *POMC* transcription and ACTH secretion as well as cell viability (Gentilin *et al.*, 2013).

miR-493 regulates, among others, the expression of lectin galactoside-binding soluble 3 (*LGALS3*) gene that encodes for the galectin 3 protein, which is overexpressed in corticotropinomas where it correlates with tumor aggressiveness (Riss *et al.*, 2003; Stilling *et al.*, 2010).

Genetic Syndromes Associated With Cushing Disease

Corticotropinomas are usually sporadic tumors and only rarely described in the context of familial and other genetic syndromes.

MEN1

Multiple endocrine neoplasia type 1 (MEN1) is caused by an inactivating mutation of the *MEN1* suppressor gene that encodes for menin, a transcriptional cofactor that regulates the expression of genes encoding for cell cycle proteins, such as p27/kip1 and CDK4. MEN 1 syndrome is typically characterized by primary hyperparathyroidism, neuroendocrine tumors, and pituitary tumors. Corticotropinomas are rarely found in MEN1 patients and can be macro- or microadenomas (Thakker *et al.*, 2012; Vergès *et al.*, 2002; Simonds *et al.*, 2012). Early onset Cushing disease was described in two siblings with MEN1 syndrome (Matsuzaki *et al.*, 2004). Nevertheless corticotropinomas are rare in pediatric MEN1 patients (Rix *et al.*, 2004; Stratakis *et al.*, 2010).

MEN2

Multiple endocrine neoplasia type 2 (MEN2) is an autosomal dominant syndrome caused by an activating mutation of the proto-oncogene RET (Hofstra *et al.*, 1994). MEN2a syndrome presents with parathyroid adenomas, medullary thyroid carcinomas and pheocromocytomas, while MEN2b with medullary thyroid cancer, pheochromocytoma, multiple mucosal neuromas, intestinal ganglioneuromas, and marfanoid habitus. Cushing disease is extremely rare with only two cases reported to date, one in a MEN2A and the other in a MEN2B patient, both due to microcorticotropinoma (Kasturi *et al.*, 2017; Naziat *et al.*, 2013).

MEN4

Multiple endocrine neoplasia type 4 (MEN4) is an extremely rare MEN1-like syndrome caused by germline loss-of-function mutations in the *CDKN1B* gene that encodes for the cell cycle inhibitor p27/Kip1 (Pellegata *et al.*, 2006). A germline *CDKN1B* mutation was found in a female patient presenting with primary hyperparathyroidism, cervical neuroendocrine carcinoma and Cushing disease due to a corticotropinoma (Georgitsi *et al.*, 2007b). Nevertheless, no mutations were found in the *CDKN1B* gene in neither adult nor pediatric Cushing disease patients (Dahia *et al.*, 1998; Igreja *et al.*, 2009; Stratakis *et al.*, 2010). In contrast, a p27 rs2066827 (V109G) polymorphism was found to associate with the incidence of corticotropinomas in a large cohort of 447 patients presenting with various endocrine tumors (Sekiya *et al.*, 2014).

McCune–Albright Syndrome

McCune–Albright syndrome (MAS) is caused by a mosaicism of activating somatic mutations in the *GNAS1* oncogene (guanine nucleotide binding protein, alpha stimulating) that encodes for the alpha subunit of the stimulatory Gs protein. Clinical characteristics include polyostotic fibrous dysplasia, *café-au-lait* skin pigmentation and hyperfunction of endocrine glands including the adrenals. Hypercortisolism might occur in these patients, mostly as adrenal Cushing syndrome due to adrenal hyperplasia or adenoma. A somatic mutation in the codon Q227 of the *GNAS1* gene was identified in two unrelated patients with Cushing disease (Williamson *et al.*, 1995). Similarly a pediatric patient with Cushing disease was found to carry a somatic mutation at *GNAS1* R201 (Riminucci *et al.*, 2002).

Carney Complex

Carney complex (CNC) is caused by inactivating mutations in the PRKAR1A gene encoding for the 1 alpha regulatory subunit of the c-AMP dependent protein kinase A (PKA) in 70% of the cases (Kirschner *et al.*, 2000). CNC is characterized by myxomas, spotty skin pigmentation, schwannomas, and endocrine tumors. Adrenal hyperplasia is the sole cause of hypercortisolism in CNC (Correa *et al.*, 2015). However, very recently, the first case of a CNC patient with ACTH-dependent hypercortisolism due to a corticotropinoma was reported; the patient had a de novo heterozygous germline mutation in exon 2 of the *PRKAR1A* gene and the wild-type allele was partially lost in the tumor indicative of loss of heterozygosity (Kiefer *et al.*, 2017).

FIPA

Non syndromic-familial isolated pituitary adenomas (FIPA) are pituitary tumors in family members. Genes potentially associated with FIPA include *AIP*, *CDKN1B*, *MEN1*, *PRKAR1A*, *SDHA*, *SDHB*, *SDHC*, and *SDHD*. Mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene are found in around 20% of FIPA families (Tichomirowa *et al.*, 2011). Cushing disease is very rarely encountered in FIPA (Georgitsi *et al.*, 2007a). A germline AIP mutation was found in a 50 years old patient with family history of pituitary disease presenting with an aggressive macrocorticotropinoma (Dinesen *et al.*, 2015). Screening of 44 sporadic corticotropinomas revealed germline AIP mutations in only three patients (Cazabat *et al.*, 2012). Similarly germline AIP mutation was found in only one pediatric Cushing disease patient (Stratakis *et al.*, 2010).

TSC

Tuberous sclerosis complex (TSC) is caused by germline mutations in the *TSC1* (hamartin) or *TSC2* (tuberin) tumor suppressor genes genes and is characterized by the development of multiple hamartomas in several organs. TSC1/2 dimers inhibit mTOR, a

major regulator of cell growth and metabolism. There are two documented cases of TSC patients presenting with Cushing disease to date, one adult and one pediatric (Nandagopal *et al.*, 2007; Tigas *et al.*, 2005).

DICER

DICER syndrome is caused by heterozygous germline mutations in the *DICER1* gene, which encodes for a cytoplasmic endoribonuclease type III that cleaves precursor microRNAs into short, mature miRNAs. It is a pleiotropic early-onset tumor syndrome that includes pleuropulmonary blastoma, cystic nephroma, ovarian Sertoli-Leydig cell tumors, sarcoma of the uterine cervix, ciliary body medulloepithelioma, pineo-blastoma, pituitary blastoma, and thyroid cancer. Pituitary blastoma is a very rare pituitary tumor that bears embryonic tissue characteristics and occurs in infants (Scheithauer *et al.*, 2008). The most common presentation of pituitary blastomas is severe Cushing disease that may turn lethal as observed in the few cases of infants with germline and somatic *DICER1* mutations (Scheithauer *et al.*, 2008, 2012; Wildi-Runge *et al.*, 2011; Sahakitrungruang *et al.*, 2014; De Kock *et al.*, 2014).

Other

Patients with congenital adrenal hyperplasia (CAH) secondary to mutations in the 21-hydroxylase enzyme (CYP21A2) show long-term impairment of glucocorticoid secretion secondary to 21-hydroxylase deficiency that could lead to the development of secondary and ultimately tertiary hypercortisolism by chronic stimulation of the hypothalamic–pituitary axis; however very few cases of Cushing disease were reported in CAH (Haase *et al.*, 2011).

A patient with X-linked congenital adrenal hypoplasia, a rare genetic disorder that is caused by mutation or deletion in the *NR0B1* gene encoding for the orphan nuclear receptor DAX1, presented with hyperpigmentation and elevated ACTH levels, which were not suppressed by steroid substitution, due to an invasive large ACTH-producing tumor (De Menis *et al.*, 2005). Histological examination of the pituitary tumor mass excluded the possibility of hyperplasia.

Duplications in the *GPR101* gene were found to be responsible of X-linked acrogigantism (X-LAG) (Trivellin *et al.*, 2014). *GPR101* encodes for an orphan G protein-coupled receptor that activates the cAMP cascade and is involved in the hypothalamic regulation of energy homeostasis and pituitary hormone secretion. Recently the possible role of *GPR101* in the development of corticotropinomas has been investigated in a cohort of pediatric Cushing disease, but neither mutations nor deregulations in transcript levels were found (Trivellin *et al.*, 2016).

Somatic Genetic Events

Proto-oncogenes and tumor suppressor genes that are frequently mutated in other cancers are very rarely reported in corticotropinomas. Mutations in the TP53 gene were found in a handful of ACTH-scecreting carcinomas and invasive corticotropinomas (Tanizaki *et al.*, 2007; Kawashima *et al.*, 2009).

USP8

Screening a limited set of corticotropinomas with whole exome sequencing led to the identification of a single somatic mutational hotspot in the gene encoding for ubiquitin-specific peptidase 8 (*USP8*) (Reincke *et al.*, 2015; Ma *et al.*, 2015). Subsequent studies showed that somatic USP8 mutations are found in 35%–60% of Cushing disease tumors (Perez-Rivas *et al.*, 2015; Hayashi *et al.*, 2016). Somatic *USP8* mutations were also found in 13 out of 42 pediatric Cushing disease cases (Faucz *et al.*, 2017). An independent whole genome sequencing effort identified *USP8* mutations exclusively in half corticotropinomas, but in no other pituitary tumor subtype (Song *et al.*, 2016). In contrast, no *USP8* mutations were found in ectopic ACTH-secreting neuroendocrine tumors (Perez-Rivas *et al.*, 2017).

The *USP8* gene encodes for a deubiquitinase that rescues tyrosine kinase receptors, such as EGFR, from proteasomal degradation and secures their activation (Mizuno *et al.*, 2005). The *Usp8* knockout is embryonic lethal and conditional knockout adult mice die because of liver failure; both cases present with dramatic reduction or absence of growth factor tyrosine kinase receptors (Niendorf *et al.*, 2007).

All *USP8* mutations identified so far in Cushing disease are somatic heterozygous single point mutations, while no germline mutations have been reported. The mutational hotspot is located in exon 14 and affects the USP8 protein region that binds to 14-3-3 proteins. Loss of 14-3-3 binding renders USP8 susceptible to proteolytic cleavage and allows the formation of a C-terminal fragment that possesses high catalytic capacity. Indeed the USP8 mutants displayed higher deubiquitinase activity and effectively deubiquitinated EGFR potentiating EGF-induced Erk1/2 signaling in vitro (Reincke *et al.*, 2015). Corticotroph tumor cells overexpressing USP8 mutants showed higher EGFR-induced *Pomc* transcription and ACTH synthesis. This effect was Erk1/2 dependent and concentrated on the AP1 binding site of the *Pomc* promoter (Reincke *et al.*, 2015). Studies in human Cushing tumors showed that those with somatic *USP8* mutations show higher *POMC* transcript levels compared to the wild type ones (Hayashi

et al., 2016). In contrast there is controversy regarding EGFR expression in *USP8* mutation positive Cushing disease tumors, with one study showing overexpression and the other finding no significant differences (Ma *et al.*, 2015; Hayashi *et al.*, 2016).

Patients with *USP8* mutation positive corticotropinomas are more frequently female (Perez-Rivas *et al.*, 2015). An interesting and with potential therapeutic implications observation is that *USP8* mutant corticotropinomas have higher levels of somatostatin receptor 5 (SSTR5) and O6-methylguanine DNA methyltransferase (MGMT), indicating that they may respond favorably to SSTR5-targeting somatostatin analogs and temozolomide (Hayashi *et al.*, 2016). Finally USP8 itself constitutes an attractive pharmaceutical target; a small molecule USP8 inhibitor displayed antisecretory and antiproliferation action in immortalized murine corticotroph tumor cells (Jian *et al.*, 2016).

Other

A somatic mutation in the *PIK3CA* gene encoding for the PI3K α subunit was reported in a noninvasive microcorticotropinoma (Murat *et al.*, 2012). *PIK3CA* mutations are rare in pituitary tumors and the few cases described were mostly aggressive tumors (Lin *et al.*, 2009).

Whole exome sequencing on familial and sporadic pituitary tumors revealed a germ-line missense mutation in the cadherin-related 23 (*CDH23*) gene that regulates cell–cell adhesion in all pituitary tumor subtypes, including sporadic corticotropinomas (4 out of 20) (Zhang *et al.*, 2017).

Conclusions

Corticotroph tumorigenesis is marked by posttranscriptional/translational deregulations in cell cycle proteins, growth factors, cytokines and their receptors as well as transcriptional co-regulators. The recent discovery of somatic driver mutations in the *USP8* gene represents an important progress to clarify genetic alterations underlying Cushing disease. The fact that so far *USP8* mutations have been detected exclusively in corticotropinomas underline a specificity to signaling cascades influenced by this deubiquitinase. The implications in clinical care and therapeutic management are subject of ongoing studies as is the search for driver mutations in the Cushing disease tumors with intact *USP8* gene.

See also: ACTH-Secreting Pituitary Tumors. Cushing Syndrome; Screening and Differential Diagnosis

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Pituitary Surgery for Cushing Disease

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Glossary

ACTH Adrenocorticotropic hormone.
CRH Corticotroph releasing hormone.
IPSS Inferior petrosal sinus sampling.

Introduction

Cushing Syndrome (CS) is caused by chronically elevated levels of systemic glucocorticoids. This leads to a constellation of symptoms including weight gain with centripetal fat distribution, hypertension, irritability, excess hair growth, irregular menses, decreased glucose tolerance, impaired immunity, osteoporosis, and increased risk of cardiac events (Boscaro and Arnaldi, 2009; Raff, 2015; Lonser *et al.*, 2017). While exogenous administration of steroids is the most common cause of CS overall, Cushing Disease (CD), or overproduction of adrenocorticotropic hormone (ACTH) by a pituitary adenoma, is the most common cause of endogenous CS, responsible for 70%–80% of cases. Other endogenous causes include an ectopic ACTH-producing tumor, often carcinoid, or overproduction of cortisol by an adrenal adenoma.

Diagnosis of Cushing Disease

Epidemiology

CS involves a spectrum of clinical sequelae from excess of corticosteroid exposure that was first defined by Harvey Cushing over a century ago. While CD is the most common noniatrogenic cause of CS, the disease itself is overall fairly rare, with an average incidence around 1.2–2.4 cases per million inhabitants per year (Daniel and Newell-Price, 2015; Hall *et al.*, 1994). The disease is typically diagnosed around 3 years after the onset of illness (Zilio *et al.*, 2014; Lonser *et al.*, 2013). The mean age at diagnosis is in the fourth decade of life. The disease is more common in women, with studies showing three to six times the prevalence compared to men (Lonser *et al.*, 2017; Zilio *et al.*, 2014).

Clinical Features

There are a number of characteristic features of patients who present for evaluation of possible CS/CD. The excess cortisol leads to central adiposity and fat deposits in face, supraclavicular, and posterior cervical spine regions, hyperglycemia, frequent ecchymosis, dark striae, excess sweating, proximal muscle weakness, edema, hypokalemia, thromboembolic events, psychiatric disorders like depression with cognitive impairment, and atypical infections. Of these symptoms, hypertension and abnormal glucose metabolism are the major predictors of morbidity and mortality in untreated disease. The overall mortality ratio for patients with CD compared to death rate in the general population is reported to be around 1.9–4.8, with the majority of deaths due to vascular disease (Yaneva *et al.*, 2013; Lonser *et al.*, 2017). The mortality rate is significantly greater in women aged 45–70 compared to men.

Cushing Disease Physiology

In addition to the clinical findings, evaluation of suspected CD also includes biochemical testing to confirm ACTH-dependent hypercortisolemia, followed by localization of the source (Daniel and Newell-Price, 2015). As discussed in other chapters, normal physiologic conditions of the hypothalamic–anterior pituitary–adrenal axis leads to a peak in cortisol levels in early morning and nadir late at night. Circulating cortisol provides physiological control of this endocrine axis by its negative feedback inhibition of corticotropin-releasing hormone (CRH) secretion by the hypothalamus and ACTH secretion by the pituitary. CS occurs when an excess of circulating cortisol disrupts the normal physiologic feedback mechanisms, either from an excess of ACTH (ACTH-dependent CS) or from an excess of cortisol secreted by the adrenal glands (ACTH-independent CS).

CD represents an ACTH-dependent elevation of cortisol caused by a pituitary adenoma that produces excess ACTH. As a result, the adrenal glands secrete an excess of cortisol, which then increases the negative feedback inhibition on CRH secretion by the hypothalamus. However, the ACTH-secreting adenoma is relatively resistant to excess cortisol and so biochemical testing reveals paradoxically elevated, or "nonsuppressed," levels of ACTH in addition to elevated levels of circulating cortisol.

Biochemical Testing for Diagnosis of Cushing Disease

CS is first determined by confirming excess of circulating cortisol, which can be done through various methods. Abnormal levels can be assessed by evaluating the amount of cortisol excreted in the urine over a 24-h period (urine free cortisol [UFC]), measurement of late-night (11 PM) salivary cortisol (LNSC), or low-dose dexamethasone suppression testing (DST) with overnight 1-mg DST or 48-h 2 mg DST. Established clinical guidelines recommend confirming excess endogenous cortisol by at least two of these screening tests (Nieman *et al.*, 2008).

Once CS is confirmed, additional testing is needed to identify the underlying cause. This first involves determining whether the hypercortisolemia is ACTH-dependent or ACTH-independent, and then whether the ACTH source is central or ectopic. Patients with ACTH-dependent CS most often have an underlying pituitary corticotroph adenoma (CD), but may sometimes have an ectopic tumor secreting either ACTH or, very rarely, CRH.

Once an ACTH-dependent hypercortisolemia is identified, the next step involves identification of the source of excess ACTH. This can be done biochemically, with a high-dose (8 mg) DST, or radiographically, with a pituitary-protocol MRI. With the high dose DST, cortisol secretion is suppressed in approximately 80% of CD patients, but cortisol levels in the majority of ectopic ACTH-secreting tumors typically do not suppress. This test has 81%–82% sensitivity and 67%–79% specificity for CD. A peripheral CRH stimulation test can be helpful in some patients, especially where intermediate levels of ACTH are identified, with the belief that corticotroph cells of the pituitary adenoma are more likely to respond to CRH stimulation than ectopic ACTH-secreting tumors. The CRH stimulation test has 70% to 93% sensitivity and 88% specificity for CD (Lonser *et al.*, 2017).

MRI

A pituitary MRI is sometimes diagnostic, but an adenoma is only visible in 40%–60% of CD cases (Jagannathan *et al.*, 2009; Ciric *et al.*, 2012; Hall *et al.*, 1994). When visible, the vast majority (90%) of pituitary corticotroph tumors are microadenomas. A high-resolution (1- to 1.5-mm slice thickness) MRI using a pituitary protocol enhances detection of ACTH-secreting pituitary tumors, with a sellar mass seen in 60%–70% of CD patients. It should be noted, however, that incidental sellar hypodensities of small size (<10 mm) are present in approximately 10% of individuals in the general population. Therefore, the presence of a pituitary abnormality on MRI is not always the source of ACTH excess.

Inferior Petrosal Sinus Sampling

When there is no obvious pituitary abnormality on MRI but clinical suspicion for CD is high, inferior petrosal sinus sampling (IPSS) is a useful technique for confirming a pituitary source before proceeding with transsphenoidal surgery. Tumors ≥ 6 mm have been associated with 96% specificity for CD, so the author's group typically pursues IPSS for tumors smaller than this (Yogi-Morren *et al.*, 2015). However, a study of 501 patients with surgically-confirmed ACTH-secreting adenomas showed that MRIs with obvious lesion ≥ 4 mm had a greater positive predictive value for laterality of lesion compared to IPSS (86% vs. 72%)(Wind *et al.*, 2013). Generally, IPSS is useful for predicting ACTH-secreting lesions for patients with MRI findings <7 mm, and MRI abnormalities ≥ 4 mm may help in guiding surgical approach for tumor localization.

The IPSS procedure involves inserting catheters in the inferior petrosal veins bilaterally and sampling ACTH levels before and after the administration of corticotropin-releasing hormone (CRH) or desmopressin (Deipolyi *et al.*, 2017; Giraldi *et al.*, 2015; Machado *et al.*, 2007). Central levels of ACTH are compared to peripheral venous levels of ACTH at the same time points, and, to confirm a central source of excess ACTH the ratio of central to peripheral ACTH should exceed 3:1 after CRH stimulation. Confirmation of hypercortisolemia at the time of the procedure is a prerequisite for accurate testing (Giraldi *et al.*, 2015). While this test is considered the gold standard for distinguishing between pituitary and ectopic sources, it requires considerable expertise to be performed safely and reliably. In rare cases it has been associated with thrombotic and neurologic complications. Anomalous venous drainage or incorrect sampling can limit the diagnostic performance of this test, thus, it is imperative that both the venous anatomy and accurate catheter positioning in the inferior petrosal sinuses be verified before and after specimen collection to ensure appropriate sampling. Measuring serum prolactin level in specimens may also help improve the diagnostic accuracy of this test, as elevated ratio of central to peripheral prolactin in cases of anomalous venous drainage can decrease the rate of false negative IPSS (Findling *et al.*, 2004; Sharma *et al.*, 2011).

Ectopic Sources

In cases where patients with ACTH-dependent CS are suspected of having an ectopic source, imaging studies of their chest, abdomen, and pelvis is recommended. Typically this involves a computed tomography (CT) study, although MRI, positron emission tomography (PET) in combination with CT, and/or whole-body scintigraphy using indium-111–labeled pentetreotide may also be useful. Ectopic sources of ACTH are typically small and may not be detected despite extensive imaging examinations in up to 19% of patients. There are additional laboratory tests that can be helpful in localizing an occult ectopic tumor, including tests for serum CRH, calcitonin, chromogranin A, fractionated plasma metanephrines, and 24-h urine 5-hydroxyindoleacetic acid (Lonser *et al.*, 2017).

Surgical Management of Cushing Disease

Surgery is considered first-line treatment option for CD, with the goal of adenomectomy to eradicate the ACTH-secreting lesion. Remission rates range from 65% to greater than 90%, with higher rates of remission achieved by more experienced neurosurgeons (Valassi *et al.*, 2010; Aranda *et al.*, 2015). Recurrence rates range from 10% to 35% (Hammer *et al.*, 2004; Swearingen *et al.*, 1999). Surgical options include microscopic approaches via either sublabial or endonasal exposure, as well as the endonasal endoscopic approach. The endonasal approach has become the more common of the microscopic approaches because of the decreased incisional morbidity, although some centers utilize the sublabial approach. Recurrences may be treated with repeat transsphenoidal surgery, as well as radiosurgery or radiation therapy with interim medical therapy until radiation therapy takes effect. There are now newer pharmacologic options as well. Refractory cases requiring urgent remission may be considered for bilateral adrenalectomy.

Microscopic

The microscopic, compared to the endoscopic approach, offers the advantage of a binocular stereoscopic view with superior optics. After induction of general anesthesia, the patient is positioned supine with the head on a gel headrest or in 3-point fixation. Navigation is provided by either fluoroscopy or CT- and/or MRI-guided stereotaxy.

In the endonasal approach, the posterior septal mucosa is infiltrated with local anesthetic containing epinephrine, and incised opposite the middle turbinate. The mucosa is lifted with a periosteal elevator, and the posterior septum fractured. A piece of the bony septum may be retained for later use. A self-retaining nasal speculum provides retraction as the anterior wall of the sphenoid sinus is drilled to provide sufficient room for later insertion and manipulation of appropriately sized curettes.

The sella is identified, and opened in the midline. A laser Doppler probe may be used to help identify the position of the cavernous carotid arteries, especially in cases of medially located cavernous sinuses. The dura is coagulated with a bipolar and sharply opened. The gland is hemisected and explored for adenoma. Tumor fragments are dissected free and delivered from the gland using an assortment of ring curettes. Because approximately 90% of tumors in CD are microadenomas, care should be taken to obtain sufficient specimen for histopathological analysis. The surface of the pituitary gland is closely inspected for residual fragments of adenoma. If no obvious tumor is visualized, a systematic exploration is undertaken. Serial frozen-section biopsies are often useful. If these remain negative, some advocate for hemi-hypophysectomy on the side predicted by the preoperative bilateral IPSS (Utz *et al.*, 2005).

Following resection, hemostasis is obtained. There are a number of techniques to reconstruct the sella. The authors employ fat harvested from a separate abdominal incision, which is then covered with a piece of previously harvested septal bone, titanium mesh, or other synthetic plug. If cerebrospinal fluid (CSF) leak is suspected, a layer of fibrin glue or other sealant may be applied. The nasal mucosal flap is reapproximated, and the nasal passage inspected before completion of the procedure.

Endoscopic

The endoscopic approach offers the advantage of a wider field of view and the capacity to inspect regions of the sella with angled fiber bundles. In this technique, a nasal speculum is not required. A 0 degree 4-mm endoscope is typically used during the sellar exposure. The ostia of the sphenoid sinuses are identified bilaterally. The sphenoid ostia are widened inferiorly and medially with care taken to preserve the posterior septal branch of the sphenopalatine artery, in case a nasoseptal flap is necessary for CSF leak repair. The goal of exposure is to achieve a sphenoidotomy that extends from floor to roof of the sphenoid sinus and laterally to bilateral superior turbinates. At this point, the approach to the pituitary gland and tumor resection is similar to the microscopic technique. During tumor dissection, the endoscope can be affixed to the operating table with an articulating arm or held by the assistant, allowing the surgeon to use conventional bimanual techniques. In the case of CSF leak, a nasoseptal flap may be harvested for a vascularized flap repair of the sella or a free mucosal graft may be harvested from nasal floor or middle turbinate.

Recent improvements in endoscope technology have provided much clearer high-definition visualization, including 4K resolution. Three-dimensional endoscopes are also growing in use and improving in quality. (Barkhoudarian *et al.*, 2013; Tabaee *et al.*, 2009) Several centers have reported remission rates similar to those of microscopic approaches, especially in cases of macroadenomas. (Wagenmakers *et al.*, 2013; Netea-Maier *et al.*, 2006)

Outcomes After Surgery

Predictors of Remission

Several factors have been linked to likelihood of successful surgical outcome, including size of tumor, invasiveness of tumor, and identification of tumor on pre-operative work-up and intraoperatively.

Tumor size

Most studies show an improved rate of remission with microadenomas (Swearingen *et al.*, 1999; Rollin *et al.*, 2007; Honegger *et al.*, 2012; Hofmann *et al.*, 2008; Fomekong *et al.*, 2009; Ammini *et al.*, 2011). Larger macroadenomas may have worse outcomes, perhaps because larger tumors are more likely to be invasive (Meij *et al.*, 2002; Cannavò *et al.*, 2003).

Tumor invasion

Patients with presence of tumor invasion are less likely to achieve remission after transsphenoidal surgery (Rees *et al.*, 2002; De Tommasi *et al.*, 2005). Invasion of the cavernous sinus and dura or suprasellar tumor extension results consistently in a higher incidence of persistent disease (Hammer *et al.*, 2004). Remission is unlikely even in cases where tumor seems to be removed from the cavernous sinus given the likelihood of dural involvement (Lonser *et al.*, 2012).

Tumor identification

Studies indicate that identification of adenoma on preoperative MRI and intraoperative histopathology have also been linked to improved chance of remission (Esposito *et al.*, 2006; Chee *et al.*, 2001; Fomekong *et al.*, 2009; Shimon *et al.*, 2002). Intraoperative identification of an adenoma is an important positive prognostic factor, and allows selective adenomectomy to be performed rather than a potentially more morbid procedure such as partial or total hypophysectomy (Hofmann *et al.*, 2008; Chee *et al.*, 2001; Nakane *et al.*, 1987; Prevedello *et al.*, 2008). The use of intraoperative frozen sections can be useful and, in one study, the use of preoperative MRI and bilateral IPSS for localization along with intraoperative frozen sections achieved 100% remission in 18 patients (Lim *et al.*, 2011). Systematic exploration of the gland can lead to identification of MRI-invisible adenomas (Oldfield and Vortmeyer, 2006). Intraoperative ultrasonography has been reported to increase intraoperative adenoma localization and yield higher remission rates (Watson *et al.*, 1998).

Tumor Pseudocapsule

Adenomas may sometimes create a "pseudocapsule" of surrounding fibrous tissue and compressed gland that, when present, may be an aide to tumor dissection and successful removal. This technique yields excellent results in experienced hands, with initial remission of 96.6%, increasing to 100% following early repeat surgery for initial treatment failures, with a recurrence of only 2.3% at a mean of 7 years (Jagannathan *et al.*, 2009; Oldfield and Vortmeyer, 2006; Lonser *et al.*, 2013).

Biochemical remission

Several biochemical parameters have been proposed to define remission, although a perfect predictor of long-term remission has not yet been identified. A number of parameters have been used to assess remission. Potential early predictors include low fasting serum cortisol (< 2 μg/dL), low 24 h urine free cortisol levels (below 20 μg/24 h), low serum ACTH (<5 pg/mL), or low midnight salivary cortisol within the first week after surgery (Lonser *et al.*, 2017; Carrasco *et al.*, 2008; Lindsay *et al.*, 2011; Lonser *et al.*, 2013). One study found 97% sustained remission in patients with serum cortisol less than 5 mg/dL within the first two postoperative days, although follow-up was brief at 33 months (Acebes *et al.*, 2007). More recent data suggests that morning serum cortisol level less than 1 μg/dL predicts remission with a positive predictive value (PPV) of 96% (Lonser *et al.*, 2017). Currently, based on this data, a consensus statement from clinicians experienced in management of CD recommends immediate reevaluation of patients with persistent serum cortisol levels greater than 5 μg/dL, and careful observation of those patients with cortisol levels between 2 and 5 μg/dL (Biller *et al.*, 2008).

Volume-outcome effect

While database studies indicate a volume-outcome effect, whereby surgeons that perform a higher volume of pituitary surgery have reduced morbidity and mortality rates, the contribution of surgical experience to surgical success in CD is difficult to document (Ciric *et al.*, 1997; Barker *et al.*, 2003). While many studies showing no changes in remission outcomes over a surgeon's career, several others reporting improvement with experience over time (Rees *et al.*, 2002; Yap *et al.*, 2002; Chee *et al.*, 2001; Hofmann *et al.*, 2008).

Predictors of Recurrence

Despite initial surgical cure of CD, many patients may develop recurrence of disease months or even years following initial surgery. Due to this variable timeframe of recurrence, the overall rate is difficult to determine because studies report differing lengths of follow-up. Overall, recurrent disease appears in 3%–22% of patients at a mean of 1.75–9.6 years (Pereira *et al.*, 2003; Prevedello *et al.*, 2008; Yap *et al.*, 2002; Rees *et al.*, 2002; Rollin *et al.*, 2007; Shimon *et al.*, 2002; Atkinson *et al.*, 2005; Wagenmakers *et al.*, 2013; Invitti *et al.*, 1999; Hammer *et al.*, 2004; Swearingen *et al.*, 1999). Recurrence continues to increase over time with long-term follow-up (Hammer *et al.*, 2004; Invitti *et al.*, 1999; Atkinson *et al.*, 2005; Prevedello *et al.*, 2008; Patil *et al.*, 2008a; Pereira *et al.*, 2003). For example, one study showed recurrence of 0.5%, 6.7%, 20.8%, and 25.5% at 1, 2, 3, and 5 years, respectively, and ultimate recurrence of 46% in patients followed for at least 5–13 years. Undetectable postoperative cortisol does not exclude the chance of future recurrence, and in one study where postoperative cortisol predicted remission at 6 months, it was not related to long-term remission (Atkinson *et al.*, 2005; Yap *et al.*, 2002; Pereira *et al.*, 2003). Other studies have also shown that elevated

postoperative ACTH as well as increased cortisol and ACTH levels following CRH stimulation have been related to recurrence (Sheth *et al.*, 2012; Lindsay *et al.*, 2011; Invitti *et al.*, 1999)

Recurrent tumors likely are caused by growth of residual microscopic tumor remnant from the initial surgery (Nakane *et al.*, 1987; Dickerman and Oldfield, 2002). Recurrence rates may be a function of tumor size, with macroadenomas recurring more frequently as a results of increased invasiveness (Swearingen *et al.*, 1999). However, a small study showed excellent long-term remission rates in patients with macroadenomas, and several studies have shown no difference in recurrence rates based on size (Yap *et al.*, 2002; Tritos *et al.*, 2011). Undetected dural invasion may contribute to unresected tumor tissue, and dural invasion increases with adenoma size, patient age, and male gender (Meij *et al.*, 2002; Dickerman and Oldfield, 2002).

The Role of Reoperation

Initial transsphenoidal surgery for CD has a higher likehood of successful remission compared to repeat surgery performed for treatment failure or recurrence, and subsequent surgeries may be associated with higher rate of complication. (Swearingen *et al.*, 1999; Patil *et al.*, 2008b; Hofmann *et al.*, 2008; Esposito *et al.*, 2006; Rollin *et al.*, 2007). Nonetheless, studies specifically examining the scenario of repeat surgery after initially unsuccessful procedures have found remission rates of 62%–87.5%. Repeat surgery should therefore be considered an option for refractory cases when initially remission was not achieved (Shimon *et al.*, 2002; Nakane *et al.*, 1987; Prevedello *et al.*, 2008).

Complications

Overall, the transsphenoidal procedure has a relatively low rates of mortality, with range of 0%–1.9%, and low rates of serious morbidity, ranging1.8%–15%. A nationwide study of inpatient data from transsphenoidal surgery for CD found in-hospital mortality of 0.7%, overall adverse outcomes, including death, of 2.9%, and morbidity of 42.1%*** (Patil *et al.*, 2007). Patients with CD who undergo transsphenoidal surgery may have higher immediate and delayed complication rates than patients with other pituitary tumors who undergo this operation, perhaps because of the higher incidence of medical comorbidities in the CD population and the effects of cortisol withdrawal (Aulinas *et al.*, 2012; Sheth *et al.*, 2012). Patients who are successfully surgically treated have improved long-term mortality risk, whereas those with persistent disease have increased mortality (Swearingen *et al.*, 1999; Hammer *et al.*, 2004).

Endocrine

Postoperative adrenal insufficiency is an anticipated sequela after a successful corticotroph adenomectomy. In CD, the normal corticotroph cells of the pituitary gland are inhibited by the hypersecretion of ACTH by the adenoma and the consequent increase in cortisol. If the tumor is successfully removed, patients will require glucocorticoid replacement for on average 6–18 months following surgery until their normal hypothalamic–pituitary–adrenal axis is restored. It is debated whether routine postoperative institution of steroid replacement is advisable; some centers withhold replacement until after inpatient testing is completed, while others begin low dose decadron (0.5 mg QD) immediately, so that outpatient testing can be safely performed without the risk of symptomatic adrenal insufficiency (Tritos *et al.*, 2011).

Deficiency of other pituitary hormones may also occur after transsphenoidal for CD, but this is highly dependent on the aggressiveness of surgery required. Deficiency of at least one pituitary hormone is reported in 8.6%–53% of patients, with lower rates in selective adenomectomy and increasing rates with extensive exploration, approaching 100% for subtotal or total hypophysectomy. The rate of hypopituitarism may increase with repeat surgeries (Friedman *et al.*, 1989; Rollin *et al.*, 2007).

Diabetes insipidus (DI) is a common postoperative endocrinopathy, and results from trauma to the posterior gland. It can occur transiently, lasting for days to weeks postoperatively, in 6%–75% of cases, whereas permanent DI is rare and occurs in 1%–15% of cases. Patients with DI require hormonal replacement with desmopressin (Semple and Laws, 1999; Ciric *et al.*, 1997; Swearingen *et al.*, 1999; Hammer *et al.*, 2004; Chee *et al.*, 2001; Fomekong *et al.*, 2009). Patients may also experience postoperative syndrome of inappropriate antidiuretic hormone (SIADH), usually within 7–14 days postop, and therefore all patients should be monitored for hyponatremia in the early postoperative period.

Neurologic

Neurologic complications are rare, but most often involve cranial nerve dysfunction. The most serious of these involves damage to the optic apparatus, which can cause vision loss. This is rare in surgery for CD, given that the vast majority of CD tumors are small and intrasellar. Other cranial nerve injuries typically arise from manipulation in cavernous sinus exploration. Cranial nerve palsies may be transient or permanent. The overall risk of carotid artery injury after transsphenoidal surgery has been reported to range from 0% to 2.5%; this is also rare for intrasellar microadenomas (Ciric *et al.*, 1997; De Tommasi *et al.*, 2005).

Cerebrospinal fluid rhinorrhea

Cerebrospinal fluid (CSF) may be encountered intra-operatively at a rate that ranges from 20% to 72%, and typically is repaired intraoperatively with fat graft packing, reconstruction of the sella with cartilage or titanium, dural sealant, or a vascularized or free

muscosal graft (Jakimovski *et al.*, 2014). CSF rhinorrhea discovered postoperatively requires prompt intervention, either though a lumbar drain with CSF diversion or by re-exploration for operative repair.

Infection

Recurrent and chronic sinus infection may occur in as many as 1.5%–8.5% of patients (Jagannathan *et al.*, 2009; Ciric *et al.*, 1997). Antibiotics are the first line of treatment, but many patients may require surgical drainage to achieve cure (Batra *et al.*, 2005). Meningitis is rare and is often associated with a persistent CSF leak. Incidence of postoperative meningitis is reported at 0.4%–7.9% (Hammer *et al.*, 2004; Ciric *et al.*, 1997).

Nasal/sinus

Postoperative epistaxis is an uncommon risk in the weeks following surgery. Occasionally, arterial bleeding from the septal branch of the spheno-palatine artery may be severe enough to require nasal packing, vessel cauterization, or endovascular vessel sacrifice. Severe epistaxis is rare, occurring in 0.4%–3.4% of patients (Swearingen *et al.*, 1999; Esposito *et al.*, 2006; Ciric *et al.*, 1997; Semple and Laws, 1999). Nasal morbidity also includes septal perforations and crusting.

Thromboembolic

Hypercortisolism is associated with a hypercoagulable state. There is a higher risk of venous thrombosis in patients with CD compared to patients undergoing transsphenoidal surgery without CD. It is important that patients receive appropriate deep vein thrombosis (DVT) prophylaxis, such as sequential compression devices and/or subcutaneous low molecular weight heparin. DVT or pulmonary embolism has been reported in 1%–6% of cases (Fomekong *et al.*, 2009; Rees *et al.*, 2002). Before treatment, patients with CD have an incidence of thromboembolic disease of 14.1 per 1000 person years. CD patients have a higher postoperative rate of thromboembolic events than patients undergoing pituitary surgery for nonfunctioning adenomas (van der Pas *et al.*, 2013; Stuijver *et al.*, 2011), and the advisability of routine postoperative anticoagulation has been debated. Although no evidence-based guidelines exist for thromboembolic prevention in the perioperative period he author's group, the author's group uses sequential compression devices during the immediate preoperative, intraoperative and postoperative periods. For mobile patients, early ambulation within hours of surgery is encouraged. For less mobile patients or those that will need extended postoperative recovery at a rehabilitation facility, chemoprophylaxis in the form of subcutaneous low molecular weight heparin may be used.

Conclusion

Given the significantly increased mortality and morbidity of untreated CD, it is imperative to diagnose the disease and treat expeditiously. Despite the advent of alternative medical therapy, surgery remains first line treatment. Overall, surgical management of CD yields high remission rates and low complication rates following either microscopic or endoscopic transsphenoidal surgery. Reoperation may be considered for recurrences of if the initial procedure is unsuccessful.

See also: ACTH-Secreting Pituitary Tumors

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Evaluation and Follow-Up of Patients With Cushing Disease After Pituitary Surgery

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Abbreviations

ACTH	Corticotrophin
CD	Cushing disease
CDDT	Combined dexamethasone-desmopressin test
CRH	Corticotrophin-releasing hormone
DST	Dexamethasone suppression testing
LNSC	Late-night salivary cortisol
UFC	Urinary free cortisol

Introduction

Cushing disease (CD) results from inappropriate ACTH secretion by a corticotroph pituitary tumor (almost always a benign adenoma), leading to a state of chronic hypercortisolism. CD is a severe condition responsible for numerous comorbidities and increased mortality (Feelders *et al.*, 2012).

The treatment goals in CD are to selectively remove the pituitary adenoma, thereby inducing long-term normalization of the hypothalamic–pituitary–adrenal (HPA) axis and, consequently, reversing the clinical features of Cushing syndrome while preserving normal pituitary function (Nieman *et al.*, 2015). Transsphenoidal surgery is the only therapeutic option that can achieve these goals and offer definitive cure with minor side effects. When performed rapidly after diagnosis, pituitary surgery has been shown to abrogate the increased mortality resulting from the chronic cortisol excess (Clayton *et al.*, 2016). Many factors influence the outcome of pituitary surgery, including gender, adenoma size, dural invasion, localization on preoperative imaging, intraoperative tumor visualization, and histological confirmation of a corticotroph adenoma (Pivonello *et al.*, 2015).

Pituitary surgery for CD is notoriously difficult, owing to the usually very small size of corticotroph adenomas and frequent bleeding in the operative field which can mask the pituitary gland. It is therefore highly recommended to address patients to an experienced neurosurgeon who treats a high volume of patients with CD. This helps to ensure successful resection of the corticotroph adenoma and reduces the potential complications of pituitary surgery (Nieman *et al.*, 2015; Petersenn *et al.*, 2015). However, even in the best conditions, the initial surgical cure rate for CD is only 65%–80% for patients with ACTH-secreting corticotroph microadenomas, and lower for patients with macroadenomas (see **Table 1**). Furthermore, pituitary surgery may be followed by a recurrence even after many years of apparent cure. It is therefore recommended to use the term postoperative remission, as a definitive cure can only be declared after the absence of recurrence several decades after initial surgery. Given the frequency of recurrences, life-long follow-up is recommended for patients operated on for CD (**Fig. 1**).

The following measures are crucial in the postoperative period:

- To precisely assess the status of the HPA axis, in order to diagnose surgical failure/remission and, ideally, to evaluate the individual risk of recurrence;
- To evaluate the rest of pituitary function and to compensate for any hormonal secretory deficits;
- To plan personalized patient follow-up.

The best criteria for defining remission versus ongoing disease in the postoperative period are controversial, and so are the most reliable prognostic factors for sustained remission. Given the rarity of CD, the natural history of recurrences following postoperative remission is poorly documented, and the best strategy for detecting early recurrences is also controversial (Nieman *et al.*, 2015). The following section discusses the modalities of postoperative evaluation and follow-up, in view of the current literature and the experience of our center. We limit this discussion to adult patients.

Initial Postoperative Evaluation

Establishing postoperative remission from CD is crucial but challenging. Several approaches have been used to evaluate postoperative HPA axis function.

It is generally accepted that the diagnosis of remission cannot rely solely on clinical findings. The time required for the symptoms of Cushing syndrome to subside may range from a few weeks to several years, even in case of a sustained cure (Sippel *et al.*, 2008). Furthermore, although biochemical remission is associated with clinical improvement, comorbidities such as obesity, metabolic syndrome, cognitive impairments and psychiatric disorders may not fully resolve (Ragnarsson and Johannsson, 2013).

Although it is well accepted that the diagnosis of remission requires biochemical criteria, the ideal protocol and criteria are controversial, for several reasons. Firstly, many biochemical tests, with various normal ranges, have been used to characterize remission (Petersenn *et al.*, 2015) (**Table 1**), and treatment statistics are obviously dependent on the criteria used to define outcome. Furthermore,

Table 1 Selection of surgical series of Cushing disease involving at least 60 operated patients per center with long-term follow-up

Author, year	*Mean follow-up (months, range)*	*Number of patients*	*Postoperative remission (%)*	*Biological criterion of early postoperative remission (≤ 6 months)*	*Recurrence (%)*	*Mean time to recurrence (months, range)*
Sonino *et al.* (1996)	87.6 (24–216)	103	76.7	UFC ≤ 248 nmol/day (*N*: 55–331), normal LDDST	25.9	NA
Blevins Jr *et al.* (1998)	55.5 (8–164)	96	85.0	Morning SC (≤ 5 μg/dL), UFC (≤ 15μg/day)	15.8	32.5 (8–142)
Barbetta *et al.* (2001)	57.5 (12–252)	68	89.7	Low/normal morning SC, normal UFC, normal DST	21.0	36.1 (8–84)
Yap *et al.* (2002)	92 (6–348)	89	68.5	Morning SC (≤ 1.8 μg/dL)	11.5	36.3 (6–60)
Chen *et al.* (2003)	60 (NA)	162	79.0	1 mg DST (SC ≤ 3 μg/dL)	6.6	27 (6–48)
Hammer *et al.* (2004)	133.2 (7.2–288)	289	82.0	Morning SC (≤ 5 μg/dL) and 1 mg DST (≤ 5 μg/dL) OR normal UFC, normal morning SC at 6 months	9.0	58.8 (13.2–133)
Atkinson *et al.* (2005)	115.2 (12–252)	63	71.4	Low/normal UFC, normal 4 mg DST	22.2	62.4 (12–108)
Hofmann *et al.* (2008)	72.3 (3–300)	426	68.5	2 mg DST (SC ≤ 2 μg/dL)	5.6	122.3 (74–278)
Carrasco *et al.* (2008)	45 (6–123)	68	74.0	Morning SC (≤ 5 μg/dL)/subnormal response to metyrapone test OR MSC ≤ 7.5 μg/dL or normal UFC (≤ 90 μg/day) and 4 mg DST (SC ≤ 1.8 μg/dL) at 6 months	14.3	51 (9–90)
Patil *et al.* (2008)	45 (6–166)	215	85.6	Normal UFC	17.4	39 (3–134)
Alwani *et al.* (2010)	84 (7–121)	79	65.0	1 mg DST(SC ≤ 1.8 μg/dL), normal UFC	20.0	7.5 (7–121)
Bou Khalil *et al.* (2011)	50.4 (7–99)	127	79.5	Hypocortisolism or [normal UFC, MSC (≤ 7.5 μg/dL) and LNSC (≤ 2 ng/mL)]	20.8	47.5 (3–149)
Ciric *et al.* (2012)	68.9 (6–396)	136	83.4	Morning SC (≤ 5.3 μg/dL)	9.7	72.9 (12–176)
Hassan-Smith *et al.* (2012)	55 (20.4–116.4)	72	83.0	Morning SC (≤ 1.8 μg/dL)	13.0	25 (16–37)
Alexandraki *et al.* (2013)	184 (72–432)	131	65.6	Morning SC (≤ 1.8 μg/dL)/mean daily SC 150–300 nmol/L	24.4	63.1 (NA)
Lambert *et al.* (2013)	75.6 (1–360)	346	75.9	Morning SC (≤ 5 μg/dL)/normal/low UFC	10.8	70 (14–345)
Wagenmakers *et al.* (2013)	71 (5–164)	86	72.1	Morning SC (≤ 1.8 μg/dL) OR 1 mg DST (SC ≤ 1.8 μg/dL)	16.0	42 (10–98)
Costenaro *et al.* (2014)	72 (31.2–105.6)	101	80.2	Normal UFC (≤ 90 μg/day), 1 mg DST (SC ≤ 3 μg/dL)	8.1	46.8 (21.6–66)
Dimopoulou *et al.* (2014)	79 (6–252)	120	71.0	1 mg DST (SC ≤ 5 μg/dL), normal UFC	34.0	54 (5–205)
Amlashi *et al.* (2015)	53.5 (5.8–155)	89	89.0	Morning SC (≤ 5 μg/dL) and normal UFC	28.0	21.7 (3.1–54)
Chandler *et al.* (2016)	80.4 (12–348)	276	80.0	Hypocortisolism or normal morning SC, normal UFC	17.0	48 (NA)
Keskin *et al.* (2017)	90 (60–120)	82	72.3	Morning SC (≤ 2 μg/dL) OR normal LNSC, normal UFC, 1 mg DST (SC ≤ 1.8 μg/dL)	30.3	NA
Johnston *et al.* (2017)	52 (12–118)	101	83.0	Morning SC (≤ 3 μg/dL) or normal UFC, LNSC and LDSST	6.0	NA
Le Marc'hadour *et al.* (2015)	52 (18–180)	67	–	Hypocortisolism or normal circadian rhythm of SC, normal UFC, 1 mg DST (SC ≤ 1.8 μg/dL)	16.4	36 (3–100)
Lindsay *et al.* (2011)	126 (12–274)	331	–	Morning SC (≤ 5 μg/dL)/normal UFC (≤ 20 μg/day)	12	50.4 (12–156)
			Mean = 77.4 ± 7.2		Mean = 16.6 ± 7.8	Mean = 47.3 ± 22.8

LDDST: low dose dexamethasone suppression test, 1 mg DST: 1 mg dexamethasone suppression test; DST: suppression test with unknown dose of dexamethasone; SC: serum cortisol.

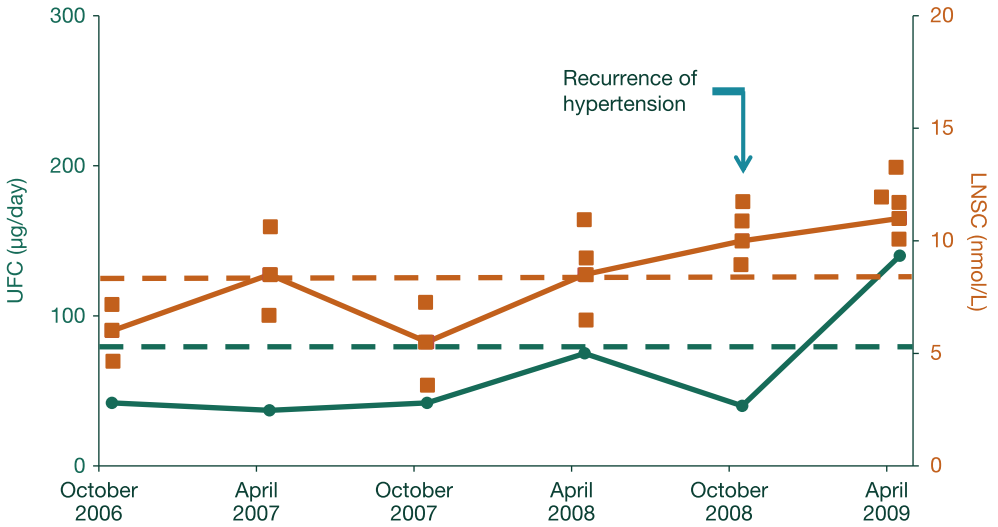

Fig. 1 A typical example of follow-up and postoperative recurrence in a patient with Cushing disease. Note that the increase in the mean value of LNSC values (*squares*) occurs before the increase in UFC measured at the same period. October 2006 correspond to the recovery of a normal HPA axis function following a 8 months period of postoperative corticotropic insufficiency (issued from Danet-Lamasou *et al.*, 2015). LNSC: late-night salivary cortisol; UFC: 24 h urinary free cortisol. Dashed lines represent the upper limit of normal range (ULN) for UFC (80 µg/day) and home assay of LNSC (8 mmol/L).

most published studies concern retrospective series comparing limited numbers of procedures. Follow-up is often short and the number of patients limited. As there is no agreement on the definition of remission or surgical failure, it is difficult to distinguish between recurrence following true remission versus progression in disease intensity following incomplete cure (e.g., persistence of milder disease) and to compare management strategies used in different clinical studies. This may explain the wide variations in reported remission rates, which ranged between 52% and 97% (mean 77%) and between 25% and 100% (mean 78%) in two recent meta-analyses (Petersenn *et al.*, 2015; Pivonello *et al.*, 2015) as well as our own analysis of the literature (**Table 1**).

From a pathophysiological perspective, CRH and normal corticotroph cells are suppressed by the chronic hypercortisolism in CD. It is therefore expected that the plasma cortisol concentration will be very low or undetectable after complete resection of the ACTH-producing tumor. Accordingly, measurement of morning plasma cortisol, alone or together with other clinical or biological parameters such as the plasma ACTH concentration, the 24 h urine free cortisol level (UFC) and cortisol suppression in response to dexamethasone administration (DST), has been the most widely used criterion. Remission is generally defined as a low morning serum cortisol concentration within 7–10 days following pituitary surgery. However, a variety of morning cortisol thresholds have been used, ranging from "undetectable" to "below the upper limit of the normal range of the assay" (**Table 1**). Evidently, less stringent cortisol thresholds lead to higher remission rates (Petersenn *et al.*, 2015). While some authors claim that a postoperative cortisol concentration below 50 nmol/L is associated with sustained remission with 100% specificity, the Endocrine Society considers remission to be associated with a cortisol concentration below 138 nmol/L (Nieman *et al.*, 2015). The use of additional tests such as the 24 h UFC and DST does not significantly influence the estimation of the remission rate in patients with low morning plasma cortisol concentrations (Petersenn *et al.*, 2015). More recently, a few studies have suggested that a low midnight or late-night salivary cortisol concentration (LNSC) has roughly equivalent performance for diagnosing remission as a combination of various biochemical tests including morning serum cortisol (Carrasco *et al.*, 2008; Amlashi *et al.*, 2015).

Although it is recommended to evaluate HPA axis function within 7–10 days following pituitary surgery, remission may occur later in a subset of patients. In a series of 56 patients cured by surgery, 6 patients with serum cortisol concentrations >138 nmol/L within 2 weeks after surgery had values below 50 nmol/L 3 months after surgery (Pereira *et al.*, 2003). Similarly, in a multicenter study involving 620 patients, 5.6% of those who had early elevated or normal UFC levels experience a delayed and persistent cortisol decline 38 $\pm$ 50 days after surgery (Valassi *et al.*, 2010). Delayed necrosis of residual corticotroph adenoma cells after the surgical insult is suspected, but the pathophysiology of these delayed responses is unknown. Be as it may, these observations suggest that the HPA axis should be reevaluated after several weeks following surgery before drawing definite conclusions, particularly in patients with eucortisolism. This attitude must be reconciled with recent data showing that the duration of exposure to excess cortisol is a determining factor for the postcure persistence of comorbidities associated with Cushing syndrome, suggesting that hypercortisolism should be corrected as rapidly as possible (Ragnarsson and Johannsson, 2013; Nieman *et al.*, 2015). It should be noted that delayed remission occurs in a minority of patients.

In accordance with the recommendations of the Endocrine Society (Nieman *et al.*, 2015), we classify patients' status during the immediate postoperative period as follows:

- patients with nonsuppressed morning plasma cortisol and who should have complementary investigations such as midnight salivary cortisol, 24 h UFC and 1 mg DST. These investigations will differentiate patients with persistent CD (surgical failure) who require further treatment from patients with eucortisolism who can be safely monitored without treatment and reevaluated at 3-month intervals.
- patients with hypocortisolism who are classified as being in remission. In these patients, hydrocortisone replacement therapy should be started, as well as education for treatment of adrenal insufficiency and prevention of acute adrenal crises. Although this topic is outside the scope of this article, patients should also be informed of the so-called "glucocorticoid withdrawal syndrome," which includes nonspecific disorders such as fatigue, anorexia, myalgias and arthralgias, and symptoms of depression, which undermine their quality of life. It usually persists for several months and is marginally improved by an increase in the hydrocortisone dosage.

The above-mentioned classification based on the morning plasma cortisol concentration assumes that normal corticotroph cells are suppressed by chronic hypercortisolism. This may no longer be the case when patients receive cortisol-lowering drugs preoperatively. Indeed, prolonged preoperative cortisol normalization may avoid early hypoadrenalism in cured patients and may therefore confound the interpretation of immediate postoperative findings. A small retrospective series of 16 patients in whom hypercortisolism was "controlled" by steroidogenesis inhibitors for a median of 139 days (21–161 days) suggests that preoperative cortisol-suppressive therapy does not necessarily reactivate dormant healthy corticotroph cells and that patients still may experience transient hypocortisolism following successful surgery (van den Bosch *et al.*, 2014). Complementary studies are needed to conclude on this point, as well as on the overall benefit of presurgical medical preparation of patients with CD. The same issue of absence of postoperative corticotropic insufficiency may arise in patients with cyclical Cushing disease. When the results of usual investigations may be misleading or ambiguous, the use of LNSC, a sensitive maker of hypercortisolism, is recommended (Nieman *et al.*, 2015).

The immediate postoperative period must also include an evaluation of the adverse consequences of pituitary surgery itself. Nonspecific adverse effects include hemorrhage, cerebral fluid leakage, meningitis, deep vein thrombosis and electrolyte disturbances. Serum sodium and fluid intake should be assessed several times during the first 2 weeks to detect hyponatremia and diabetes insipidus. Hyponatremia, which seems to be more frequent than after pituitary surgery for other pituitary tumors, may be secondary to inappropriate secretion of antidiuretic hormone (a probable consequence of SIADH), but also to the overhydration associated with adrenal insufficiency in patients not receiving glucocorticoid replacement therapy. Transient diabetes insipidus may occur in about one-quarter of patients but usually resolves over a 3- to 5-day period (Pivonello *et al.*, 2015). Deep vein thrombosis and pulmonary thromboembolism may be more frequent after pituitary surgery for Cushing disease, and anticoagulation with low-molecular-weight heparin during the postoperative period may be warranted (Stuijver *et al.*, 2011). Hypopituitarism, a common complication of pituitary surgery, is clearly correlated with the aggressiveness and extent of surgery. The typical small size of corticotroph adenomas, requiring extensive exploration/dissection of the pituitary gland and frequent hemihypophysectomy or subtotal hypophysectomy rather than selective adenomectomy, explains the higher frequency of hypopituitarism following surgery for Cushing disease than for other pituitary adenomas. Importantly, fewer complications are reported in centers with extensive experience (Petersenn *et al.*, 2015). In a recent meta-analysis, the mean frequency of hypopituitarism was 6.6%, 20.2% and 80.2% in patients who had undergone adenomectomy, hemi-hypophysectomy and total hypophysectomy, respectively (Pivonello *et al.*, 2015). Evaluation of pituitary secretion in the early postoperative period is complex, however, and the results may be difficult to interpret because chronic hypercortisolism itself inhibits the gonadotropic and thyrotropic axis as well as GH secretion. This suppressive action may take several weeks to resolve after remission of Cushing syndrome. Extensive clinical and biological evaluations should ideally be performed after 2–3 months, and the Endocrine Society recommends focusing on thyroid function during the early postoperative period, based on a comparison of free T4 concentrations obtained within 1 week after surgery with preoperative levels to identify hypothyroidism (Nieman *et al.*, 2015).

Pituitary MRI is often poorly contributive in the diagnostic work-up of CD and should not be used to diagnose remission. MRI is recommended within 2–3 months following surgery in order to serve as a new baseline in case of recurrence (Nieman *et al.*, 2015).

Recurrence of Cushing Disease

A major challenge in the treatment of Cushing disease is that, despite initial remission, CD recurs in a significant number of patients. In a recent meta-analysis of 17 studies using biochemical criteria, alone or combined with clinical endpoints, the mean recurrence rate was 15.2% but ranged from 5.0% to 47.4% (Petersenn *et al.*, 2015)! The mean time to recurrence was 50.8 months, with a range of 3–158 months. In our own analysis of 25 published studies (Sonino *et al.*, 1996; Blevins *et al.*, 1998; Barbetta *et al.*, 2001; Yap *et al.*, 2002; Chen *et al.*, 2003; Hammer *et al.*, 2004; Atkinson *et al.*, 2005; Carrasco *et al.*, 2008; Hofmann *et al.*, 2008; Patil *et al.*, 2008; Alwani *et al.*, 2010; Bou Khalil *et al.*, 2011; Lindsay *et al.*, 2011; Ciric *et al.*, 2012; Hassan-Smith *et al.*, 2012; Alexandraki *et al.*, 2013; Lambert *et al.*, 2013; Wagenmakers *et al.*, 2013; Costenaro *et al.*, 2014; Dimopoulou *et al.*, 2014; Amlashi *et al.*, 2015; Le Marc'hadour *et al.*, 2015; Chandler *et al.*, 2016; Johnston *et al.*, 2017; Keskin *et al.*, 2017) (**Table 1**), those including at least 60 patients operated on by the same team with lengthy follow-up (mean 78 months; range: 6–432) and using acceptable biological criteria for remission and recurrence reported roughly the same results: the mean recurrence rate was 18.5%

(range: 6%–44%) with a mean time to recurrence of 47.3 months (range: 3–345). The risk of recurrence increases with long-term follow-up (Pereira *et al.*, 2003; Patil *et al.*, 2008; Ciric *et al.*, 2012; Alexandraki *et al.*, 2013), some recurrences being observed even 20 years after surgery. However, most recurrences are seen during the 15 years following surgery. This points to variability in the aggressiveness of corticotroph adenomas: aggressive adenomas would recur early while less aggressive tumors would recur later. Interestingly, a recent meta-analysis of surgical series showed the highest recurrence rates in studies where 10 patients or fewer were operated on per year of study duration, while the lowest rates were seen when 31–40 patients were operated on per year (Petersenn *et al.*, 2015). These data form the basis of the Endocrine Society's recommendation that CD patients should be operated on by an expert surgeon and receive life-long follow-up after successful surgery (Nieman *et al.*, 2015).

Early Prediction of Recurrences of Cushing Disease

Bearing in mind that only a minority of patients will relapse, and that life-long follow-up is needed, a number of teams have tried to identify postoperative predictive factors of recurrence in patients experiencing remission, the ultimate goals being to stratify patients according to their ultimate risk of recurrence and to define groups at high and low risk for whom the necessary duration and frequency of follow-up examinations may differ.

Early postoperative corticotropic insufficiency is found to be a protective factor for recurrence in many but not all studies (Alexandraki *et al.*, 2013; Vassiliadi *et al.*, 2016). For example, in the large series reported by Patil *et al.* (2008), the actuarial recurrence rate at 5 years in patients who had postoperative serum cortisol >2 μg/dL was 28.5%, compared to 20.6% in patients who had serum cortisol ≤2 μg/dL. In the study by Chandler *et al.* (2016), 13% of patients with corticotropic insufficiency (morning plasma cortisol ≤3 μg/dL) recurred during follow-up, compared to 50% of eucortisolic patients (morning plasma cortisol and UFC within the normal range). Some authors have claimed that the probability of relapse correlates negatively with the morning cortisol concentration (e.g., low vs. very low concentrations) (Le Marc'hadour *et al.*, 2015) while others have not found this difference (Lindsay *et al.*, 2011). Obviously, although being a favorable prognostic factor, corticotropic insufficiency is not an absolute criterion for definitive cure. The reverse is also true for postoperative eucortisolism, that does not always identify patients with a residual tumor leading always to late recurrence (Bochicchio *et al.*, 1995). In addition, several groups have shown that the time to recurrence is longer in patients with postoperative corticotropic insufficiency (Bou Khalil *et al.*, 2011; Keskin *et al.*, 2017), a finding which suggests that a smaller number corticotroph adenomatous cells remain in these patients.

Late-night salivary cortisol (LNSC) has been shown to be useful tool for diagnosing mild Cushing disease, such as Cushing syndrome with normal UFC (Elias *et al.*, 2014; Raff, 2015). However, early postoperative LNSC (obtained within 3 months) was not found to predict the risk of recurrence.

The duration of corticotropic insufficiency and the need for hydrocortisone supplementation was the only significant predictor of recurrence in a recent UK study. Recovery within 6 months, 1 year and 2 years had a decreasing positive predictive value for recurrence of 64%, 61% and 59%, respectively (Alexandraki *et al.*, 2013) and no patients with corticotropic insufficiency lasting for >3 years experienced a recurrence. Although the duration of corticotropic insufficiency is probably related to the duration and intensity of Cushing syndrome, as well as to individual susceptibility to excess glucocorticoids, a similar prognostic value was found in a multicenter study (Bochicchio *et al.*, 1995).

Several groups have suggested that more accurate evaluation of the degree of postoperative hypocortisolism than that provided by a single measurement of morning plasma cortisol might be a better predictor of long-term remission. This was the rationale for evaluating pituitary and adrenal responses to the CRH stimulation test. In the largest prospective cohort published to date, with a median follow-up of 11 years, a plasma ACTH concentration ≥ 18 pg/mL in response to CRH during the 10 days following surgery was associated with a higher rate of recurrence in patients with low postoperative plasma cortisol concentrations (Lindsay *et al.*, 2011). However, there was such a large overlap with the response of patients who did not experience recurrence that CRH-stimulated cortisol and ACTH values had no added benefit for recurrence prediction over early basal cortisol testing.

The rationale underlying the use of the desmopressin test in the postsurgical evaluation of CD patients is based on evidence that ACTH and cortisol increments following desmopressin injection are secondary to the stimulation of V2 receptors, and that these receptors are expressed and upregulated only in adenomatous corticotroph cells, whereas normal corticotrophs only express V3 receptors, for which desmopressin is a weak agonist. A "paradoxical" response to desmopressin would therefore indicate the persistence of adenomatous corticotrophs aberrantly expressing V2 receptors that may induce late CD recurrence. A prerequisite and limitation of this test is that it would be informative only in patients with a positive response before surgery, which is the case of 70%–80% of patients with Cushing disease. Three studies have examined the prognostic value of desmopressin testing in the early postoperative period in selected patients exhibiting a positive response to desmopressin prior to surgery and who experienced postoperative remission (Romanholi *et al.*, 2008; Losa *et al.*, 2009; Vassiliadi *et al.*, 2016). Overall, the authors found that a positive response of ACTH or cortisol in the early postoperative period was associated with an increased risk of recurrence. However, the specificity and predictive value of the test were found to be low in two studies (Romanholi *et al.*, 2008; Losa *et al.*, 2009) while sensitivity for recurrence was 97% in one study (Vassiliadi *et al.*, 2016). The use of various criteria to define remission and the response to the desmopressin test may explain the discrepancies between these results. Further studies are thus needed to assess the usefulness of the desmopressin test in this setting.

A small proportion of individuals without Cushing disease (mostly obese subjects) may have a mild response to desmopressin, which might thus limit the specificity of the desmopressin stimulation test. It has therefore been suggested that 1 mg of

dexamethasone, administered at midnight prior to the injection of desmopressin at 0800 h (combined dexamethasone-desmopressin test or CDDT), may increase the specificity of the desmopressin test by suppressing normal corticotroph cell activity (Le Marc'hadour *et al.*, 2015). The performance of the CDDT test at 3–6 months after surgery has been compared to that of the desmopressin test and postsurgical morning plasma for the prediction of subsequent recurrence (Le Marc'hadour *et al.*, 2015). The CDDT had a negative predictive value of 93% (meaning that a lack of response may predict sustained remission), while its positive predictive value was only 50%. However, the study involved limited follow-up (3 years) and the patients were not tested preoperatively to exclude nonresponders, thus compromising the performance of the test for recurrence prediction. Overall, the results of this study were rather disappointing, as the CDDT test did not give more insights than the postoperative cortisol level.

In conclusion, there is currently no gold-standard predictor of long-term outcome after surgical treatment of CD that allows initial stratification according to a patient's risk of recurrence, or individualized follow-up in terms of duration and frequency. Life-long follow-up thus remains necessary (Nieman *et al.*, 2015). Although postoperative hypocortisolism does not eliminate the possibility of a recurrence, profound and prolonged postoperative corticotropic insufficiency is found to be a positive prognostic factor in the majority of studies, as well as the lack of recurrence during the 10 years following surgery. Follow-up might therefore be less rigorous for individuals who meet these conditions. Special attention should be paid to patients with postoperative eucortisolism, although this does not inevitably predict a recurrence. Further studies are needed to determine the utility of the desmopressin test.

Follow-Up and Early Diagnosis of Recurrences of Cushing Disease

Glucocorticoid replacement with hydrocortisone at 10–12 mg/m^2/day in two or three divided daily doses, with the first dose taken immediately after waking and the last not after 1700 h, is recommended for patients with postoperative corticotropic insufficiency. Hydrocortisone is preferred to other synthetic glucocorticoids with a longer half-life, as the latter may favor some degree of hypercortisolism, induce HPA axis suppression, and delay recovery of the HPA axis. There is no consensus protocol for assessing recovery of the HPA axis. According to the Endocrine Society (Nieman *et al.*, 2015), the morning plasma cortisol concentration should be measured before the morning intake of hydrocortisone, every 3 months, followed by a short synacthen ACTH stimulation test when the morning cortisol concentration is $\geq$200 nmol/L. A baseline or stimulated concentration of 450 nmol/L or higher in modern assays almost rules out the risk of acute adrenal insufficiency and authorizes the cessation of hydrocortisone replacement. A variety of hydrocortisone tapering and discontinuation strategies are used, including abrupt discontinuation when the function of the HPA axis has recovered, or tapering the dose at fixed intervals (Ragnarsson and Johannsson, 2013).

The cessation of corticotropic insufficiency raises the question as to whether the patient has achieved physiological recovery of the HPA axis or if the cortisol elevation is driven by tumoral corticotrophs indicating disease recurrence. In this perspective, our usual practice is to perform a complete evaluation of the HPA axis at the time of recovery, including at least two 24 h urinary UFC, two LNSC or midnight plasma cortisol, and a 1 mg DST to assess the precise status of the HPA axis and to serve as a new baseline in case of subsequent recurrence. The importance of this evaluation is illustrated in the study by Estrada *et al.* (2001). In a retrospective evaluation of a limited number of patients, these authors showed that patients exhibiting complete normalization of HPA axis function following a transient phase of hypocortisolism (including full suppression following the 1 mg DST, a normal diurnal cortisol rhythm (assessed by midnight plasma cortisol concentration), and a normal response to insulin-induced hypoglycemia) had a very low risk of recurrence as compared to patients with some abnormalities of these parameters (Estrada *et al.*, 2001). Importantly, the two groups of patients with different prognosis had similar morning plasma cortisol and UFC values at the time of the evaluation.

Many strategies have been proposed for the long-term surveillance pattern. However, the variety of test protocols and markers used, the limited number of patients involved in most studies, and the lack of prospective comparative protocols hamper attempts to define an optimal management strategy. It is important to inform the patient that life-long follow-up is necessary, including at least a yearly mandatory examination during the first 10–15 years.

Obviously, follow-up should rely on both clinical and biological investigations. Clinical follow-up is mandatory, as some comorbidities may persist despite biochemical remission or even cure (Ragnarsson and Johannsson, 2013). The clinician should be aware that some comorbidities may take several months or years to resolve (Sippel *et al.*, 2008). It is important to evaluate and treat the long-term sequelae of chronic hypercortisolism to reduce morbidity, improve QOL, and reduce the long-term excess mortality associated with CD (Dekkers *et al.*, 2007; Clayton *et al.*, 2016). The spontaneous clinical course of recurrences following transient remission is poorly documented in the literature. In this author's experience, despite extensive clinical experience in Cushing syndrome, regular monitoring usually identifies the recurrence of biochemical abnormalities before that of clinical symptoms (Danet-Lamasou *et al.*, 2015). Obviously, it is very important for the clinician to note his or her precise findings. Given the multiple clinical symptoms of Cushing syndrome, the development of a pertinent clinical score would be highly useful. Some patients who are exquisitely sensitive to glucocorticoids may experience early recurrence of a particular symptom such as hypertension or hyperglycemia. Elsewhere, some patients are intimately persuaded that their disease has recurred, based on psychological perception of their state during the phase of hypercortisolism. Further biochemical evaluation is warranted in such cases.

Classical biological follow-up relies on the tools used for the diagnosis of Cushing syndrome, such as 24 h UFC and overnight DST. However, several studies suggest that the increase in 24 h UFC is a late marker of CD recurrence and that LNSC, which has proven its usefulness in the diagnosis of mild Cushing syndrome, may be more sensitive for diagnosing recurrences at an early stage. We studied 36 patients in surgical remission of CD after a follow-up of 69.2 $\pm$ 10.6 months and who had successive

measurements of LNSC as well as an extensive biochemical evaluation within 3 months before or after saliva samples (24 h UFC, in-patient midnight plasma cortisol measurement, 1 mg DST, desmopressin test) (Danet-Lamasou *et al.*, 2015). Patients were considered to be in remission when all investigations were normal, and in early-stage recurrence when they had at least two abnormal results, no clinical evidence of recurrence, and a normal or mildly increased UFC (<2 ULN). Interestingly, UFC was normal in 61% of patients at the time of early-stage recurrence, and was mildly elevated ($<2 \times$ ULN) in only 39% of patients, a finding which illustrates that an unequivocal increase in UFC is a late marker of recurrence. In contrast, LNSC was as efficient for depicting early recurrences and correlated with the combined results of the multiple endocrine tests requiring hospitalization. However, owing to the large interindividual variability in LNSC at this period of the disease, three to four saliva samples and a revised threshold were necessary to ensure optimal diagnostic performance. Similarly, in the study by Bou Khalil *et al.* (2011), involving 21 patients with recurrence among a cohort of 101 patients, the increase in midnight cortisol occurred after a mean of 38.2 months, as compared to 50.6 months for the UFC elevation, and LNSC was abnormally elevated in all the patients while UFC was pathological in only 47% of these cases. Similarly, two studies (Carrasco *et al.*, 2008; Amlashi *et al.*, 2015), including a follow-up study in a large cohort of patients (Amlashi *et al.*, 2015), concluded that LNSC identified recurrences with a diagnostic accuracy superior to that of 24 h UFC. These results therefore favor the use of LNSC, a simple and noninvasive test, for the follow-up of patients in remission from CD. Unfortunately, no comparison with the results of the 1 mg DST alone was performed.

Stimulation testing has also been proposed to diagnose recurrences at an early stage. In a retrospective study (Bou Khalil *et al.*, 2011), the desmopressin and CRH tests were positive in respectively 85% and 93% of patients, and a positive response to either test preceded the increase in midnight cortisol or UFC in respectively 71% and 64% of patients. This suggests that a positive response to the desmopressin and/or CRH test occurs early during recurrence, being followed by an increase in midnight cortisol, while UFC elevation occurs at a later stage. Unfortunately, no comparison with the 1 mg DST was made in this series. Another limitation of this study is that the CRH and desmopressin tests were not performed in the group of patients with maintained remission (Bou Khalil *et al.*, 2011). This is important, as a positive CRH response may also be physiological and associated with normalization of HPA axis function. The desmopressin test and above-mentioned CDDT (see the section "Early Prediction of Recurrences of Cushing Disease") have also been evaluated by Castinetti *et al.* for earlier recurrence detection than with conventional hormonal tests (Le Marc'hadour *et al.*, 2015). The results were rather disappointing, however: the desmopressin and CDDT tests had positive predictive values of respectively 20% and 44% and negative predictive values of 72% and 100%. These results suggest that negative tests predict long-term remission more reliably than positive tests detect early recurrence. The limited number of patients studied and the relatively short follow-up hamper definitive conclusions on the usefulness of these investigations, which cannot currently be recommended for general use.

In summary, current knowledge supports life-long follow-up for patients in remission from CD. Individualized follow-up is still difficult to define, owing to problems in accurately stratifying patients at a low versus a high risk of recurrence. In the opinion of the author, surgery in an expert center, followed by a very low early postoperative cortisol level and prolonged corticotropic insufficiency would favor a less rigorous (albeit long-term) follow-up schedule with yearly examinations, while more stringent follow-up (every 6 months) would be preferable for other patients. Similarly, follow-up could be lightened (but not interrupted) after 10–15 years without recurrence. The predictive role of dynamic testing such as the desmopressin test deserves further prospective and comparative studies in large cohorts with lengthy follow-up. Recent publications favor clinical examination and repeated LNSC to diagnose recurrences at an early stage. The results of LNSC screening should be confirmed with accurate clinical examination and other biological investigations commonly used for the diagnosis of Cushing syndrome, although these may be pathological at a later stage. When LNSC is not available, the 1 mg DST test could be proposed for patients without interfering conditions, while 24 h UFC may detect later-stage recurrence. This raises questions as to the optimal treatment for patients experiencing early recurrence with a normal UFC. In a small retrospective "common practice in clinical care" study of 12 patients with mild recurrences diagnosed with the LNSC but a normal UFC (Carroll *et al.*, 2016), a significant clinical improvement was observed in 7 patients after various therapeutic approaches (repeat surgery, pharmacological treatment, or bilateral adrenalectomy). Systematic studies are needed to determine the optimal timing and modalities of therapeutic intervention for recurrent CD.

In conclusion, the best follow-up strategy for early diagnosis of CD recurrence after surgery, as well as well as the clinical effects and benefits/risks of treatment for mild hypercortisolism at the time of early recurrence, is listed in the *Future Directions and Recommended Research* of the 2015 clinical practice guidelines of the Endocrine Society for the treatment of Cushing syndrome (Nieman *et al.*, 2015).

See also: Pituitary Surgery for Cushing Disease

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Medical Therapy of Hypercortisolism

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Introduction

Cushing syndrome (CS) is characterized by chronic overproduction of cortisol by the adrenal glands. This chronic state of hypercortisolism results in multisystem morbidity and an increased mortality (Dekkers *et al.*, 2007; Clayton *et al.*, 2011; Newell-Price, 2016). CS is traditionally divided into adrenocorticotropin (ACTH)-dependent CS and ACTH-independent CS (Lacroix *et al.*, 2015). ACTH-dependent CS which accounts for approximately 80% of cases, is most frequently caused by an ACTH-producing pituitary adenoma (≈70%) and, more rarely, by ectopic ACTH production (≈10%). ACTH-independent CS is caused by neoplasia of the adrenal gland. This involves most frequently a benign unilateral adrenal adenoma and more rarely bilateral micro- or macronodular adrenal hyperplasia or a cortisol producing adrenal carcinoma (Lacroix *et al.*, 2015).

The morbidity of CS includes major changes in body composition, alterations in other endocrine systems, important neuropsychiatric disturbances, clustering of cardiovascular risk factors and an increased thromboembolic risk (Lacroix *et al.*, 2015). Mortality is increased in untreated or suboptimally treated CS with cardiovascular disease as leading cause of death (Plotz *et al.*, 1952; Dekkers *et al.*, 2007; Clayton *et al.*, 2011; Newell-Price, 2016). In order to reverse morbidity and mortality in CS, treatment should aim to completely normalize cortisol production. The first-line treatment of all forms of CS is surgery. However, other treatment modalities including medical therapy, radiotherapy and bilateral adrenalectomy are necessary when surgery is not successful or not indicated in case of a high surgical risk or metastatic disease (Nieman *et al.*, 2015). This article will focus on medical therapy for CS with discussion of indications for medical therapy, the currently available drugs and recent developments in medical therapy. Because most studies on medical therapy have been performed in patients with Cushing disease (CD) the emphasis will be on drug therapy for corticotroph pituitary adenomas.

Indications for Medical Therapy in CS

There are subgroups of CS patients in whom medical therapy is (potentially) indicated: (1) in patients with CD medical therapy can be given after unsuccessful pituitary surgery, whether or not as bridging therapy during the period before radiotherapy becomes effective. In some CD patients primary medical therapy can be considered, for example, patients with a high surgical risk or patients with a nonvisible adenoma. Preoperative cortisol-lowering therapy is applied in some centers aiming to improve patient's condition and morbidity (e.g., diabetes, hypertension) and to prevent bleeding tendency during operation. However, to date there is no evidence yet that pretreatment reduces complications and improves surgical outcome; (2) patients with metastatic disease due to an ACTH producing neuroendocrine tumor or an cortisol producing adrenal carcinoma in whom surgery is not an option; (3) adrenal CS patients who are awaiting surgery or who have bilateral disease; and (4) patients with all forms of CS who present with acute complications of (severe) cortisol excess, for example. psychosis, severe hypertension and (opportunistic) infections (Feelders and Hofland, 2013).

Drug Categories for Medical Treatment of CS

Drugs for treatment of CS can be divided into three categories: (1) adrenal blocking drugs that suppress adrenal cortisol production via inhibition of steroidogenic enzymes; (2) pituitary-targeting drugs that inhibit ACTH production by the corticotroph adenoma; and (3) glucocorticoid receptor (GR) antagonists which block the GR and counteract the effects of cortisol at tissue level (Feelders and Hofland, 2013). The efficacy and side effects of the various drugs (**Table 1**.) as well as recently developed compounds within these drug categories will be discussed in the following sections.

Adrenal Blocking Drugs

Adrenal blocking drugs interfere with steroidogenic enzymes at different steps in the pathway of adrenal steroid hormone production. Ketoconazole and metyrapone are most widely used for this purpose (Daniel *et al.*, 2015). Ketoconazole suppresses many steroidogenic enzymes whereas metyrapone mainly inhibits the last step in cortisol synthesis by targeting 11-β-hydroxylase. The efficacy of both drugs in CS is comparable with normalization of cortisol production in approximately 50%–70% of patients, although efficacy data can only be obtained from retrospective studies (Castinetti *et al.*, 2014; Daniel *et al.*, 2015). Main side effects of ketoconazole are gastrointestinal complaints, hepatotoxicity and male hypogonadism, whereas metyrapone can cause gastro-

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Table 1 Drugs for treatment of Cushing syndrome: dosage and side effects

Category	*Drug*	*Dosage*	*Main side-effects*
Adrenal blocking drugs	Ketoconazole	400–1600 mg/day	Hepatotoxicity, gynecomastia, gastrointestinal complaints, male hypogonadism
	Metyrapone	0.5–4.5 g/day	Dizziness, rash, gastrointestinal discomfort, worsening of hypertension, acne and hirsutism
	Mitotane	3–5 g/day	Gynecomastia, hepatotoxicity, hypercholesterolemia, prolonged bleeding time, gastrointestinal complaints, dizziness, ataxia, confusion, dysarthria, disturbed memory
	Etomidate	0.1–0.3 mg/kg/h	Nephrotoxicity
Pituitary targeting drugs	Cabergoline	0.5–7 mg/week	Headache, dizziness, gastrointestinal discomfort
	Pasireotide sc Pasireotide LAR	750–2400 μg/day 10–30 mg/month	Hyperglycemia, gastrointestinal complaints, cholelithiasis
Glucocorticoid receptor antagonists	Mifepristone	300–1200 mg/day	Hypokalemia, worsening of hypertension, clinical adrenal insufficiency, endometrial hyperplasia, gastrointestinal complaints

intestinal upset, and less frequently worsening of hirsutism and mineralocorticoid effects (hypertension, hypokalemia, and edema) (Daniel *et al.*, 2015).

Mitotane also inhibits multiple steroidogenic enzymes and has adrenolytic effects. For these properties it is mainly used for the treatment of adrenal carcinoma, aiming to suppress both steroid hormone production and cell growth (Baudry *et al.*, 2012). Considering the serious gastrointestinal, neurological and metabolic side effects it is not widely used for other forms of CS. Etomidate is an anesthetic drug that potently inhibits 11-β-hydroxylase and can only be administrated in an intensive care setting. It might, however, be useful in CS patients with life-threatening complications of severe hypercortisolism (Daniel *et al.*, 2015).

Two new adrenal blocking drugs are currently under investigation. Osilodrostat (LCI699) is a potent inhibitor of 11-β-hydroxylase and two pilot studies showed an efficacy, in terms of normalization of 24 h urinary cortisol excretion (UFC), between 79% and 92% (Bertagna *et al.*, 2014; Fleseriu *et al.*, 2016). Osilodrostat was generally well tolerated, main side effects were gastrointestinal complaints, hirsutism and hypocortisolism. Efficacy and safety of osilodrostat are currently evaluated in large multicenter trials. Ketoconazole is a racemic mixture of two enantiomers (2S,4R and 2R,4S). Levoketoconazole (COR-003) is the single 2S,4R enantiomer which may have two advantages over ketoconazole, that is, it may more potently inhibit steroidogenic enzymes and it seems to be less hepatotoxic (Rotstein *et al.*, 1992; Schwartz *et al.*, 2008; Thieroff-Ekerdt and Mould, 2016). Also levoketoconazole is studied in an ongoing large multicenter trial.

Adrenal blocking drugs can be effective in all forms of CS. All patients treated with these drugs should be carefully monitored, clinically and biochemically, for development of hypocortisolism.

Within the entity of adrenal CS the identification of G-protein coupled receptors (GPCR) and intra-adrenal ACTH production in bilateral macronodular adrenal hyperplasia (BMAH) offers new potential targets for medical treatment. BMAH often over-express eutopic or ectopic GPCR, for example, the receptors for luteinizing hormone, glucose-dependent insulinotropic peptide and vasopressin, with aberrant regulation of cortisol production by their ligands (El Ghorayeb *et al.*, 2015). Pharmacological treatment with blockade of these receptors or with inhibition of endogenous ligand production can successfully control cortisol production in selected patients (El Ghorayeb *et al.*, 2015). Recently it was shown that ACTH can be produced locally in BMAH tissue and can stimulate cortisol production in an autocrine or paracrine manner (Louiset *et al.*, 2013). ACTH receptor antagonists are in development (Halem *et al.*, 2016) and may be a medical treatment option for BMAH in the future.

Pituitary-Targeting Drugs

Pituitary-targeting drugs aim to inhibit ACTH production by the corticotroph adenoma. Somatostatin and dopamine receptors are both GPCR expressed by corticotroph tumors and in particular somatostatin receptor subtype 5 (SST5) and dopamine receptor subtype 2 (DA2) are important targets for medical therapy for CD (Feelders and Hofland, 2013).

Pasireotide is a somatostatin analog with a high affinity for SST5 and has been evaluated for treatment of CD in several clinical trials. In a randomized phase III clinical trial 162 patients with CD, were treated with subcutaneous (sc) pasireotide at a dose of 600 μg and 900 μg bid (Colao *et al.*, 2012). On average, UFC decreased by 48% after 6 months and UFC levels completely normalized in 15% and 26% of patients treated with lower and higher dose, respectively. Parallel with the decrease in UFC, clinical symptoms improved like body weight and blood pressure as well as quality of life. Interestingly, corticotroph tumor volume decreased in a subset of patients (Colao *et al.*, 2012). Most important side effect of pasireotide is induction or worsening of hyperglycemia via inhibition of incretin and insulin secretion (Henry *et al.*, 2013). A recent 12 months randomized trial evaluated the efficacy of long-acting pasireotide in 150 patients. With a monthly dose of both 10 and 30 mg pasireotide, normalization of

UFC was achieved in approximately 40% of patients after 7 months (Lacroix *et al.*, 2018). Pasireotide is officially approved for treatment of patients with CD for whom surgery is not an option.

Cabergoline is a potent DA2 agonist and several retrospective studies indicate that cabergoline can induce biochemical remission in approximately 30% of patients, although escapes do occur at the long term (Pivonello *et al.*, 2009; Godbout *et al.*, 2010; Ferriere *et al.*, 2017). Most important side effects of cabergoline include headache, nausea and postural hypotension.

Next to drugs targeting GPCR, the nuclear receptor ligand retinoic acid can inhibit ACTH secretion by corticotroph adenomas. Two small studies showed that retinoic acid can normalize UFC excretion in a subset of patients (Pecori Giraldi *et al.*, 2012; Vilar *et al.*, 2016).

An apart entity within CD includes aggressive corticotroph tumors that show invasive growth. The chemotherapeutic drug temozolomide can be useful in these patients to stabilize or reduce tumor size and to decrease ACTH secretion (Raverot *et al.*, 2012).

Several new potential targets in corticotroph tumor cells for medical treatment have been identified like the epidermal growth factor receptor (EGFR) (Fukuoka *et al.*, 2011), cyclin-dependent kinases (CDK) (Liu *et al.*, 2015) and heat shock protein 90 (HSP90) (Riebold *et al.*, 2015). Corticotroph tumor cells express the EGFR and ligand binding results in increased ACTH secretion in vitro (Fukuoka *et al.*, 2011). Recently it was shown that in approximately one third of corticotroph adenomas a mutation is present in the gene encoding for the deubiquitinase USP8 (Perez-Rivas *et al.*, 2015; Reincke *et al.*, 2015). Due to this mutation the activity of USP8 is enhanced leading to increased deubiquitination of the EGFR which prevents lysosomal degradation. As a result, EGFR signaling is increased in these corticotroph adenomas (Perez-Rivas *et al.*, 2015). The tyrosine kinase inhibitor gefitinib targets the EGFR and was demonstrated to inhibit ACTH secretion and tumor growth in vitro (Fukuoka *et al.*, 2011) and its efficacy in vivo is currently evaluated in a clinical study.

In corticotroph tumor cells CDK are involved in regulation of cell growth and expression of proopiomelanocortin (POMC). R-roscovitine is an CDK inhibitor and in experimental animal models this compound suppresses cell growth and POMC expression (Liu *et al.*, 2011, 2015). R-roscovitine is currently investigated in patients with CD.

Recently it was shown that HSP90 expression is increased in corticotroph tumor cells (Riebold *et al.*, 2015). HSP90 can capture the GR, preventing binding to its ligand. Because only the GR-glucocorticoid complex can exert transrepressive effects on gene (e.g., POMC) transcription, increased HSP expression is thought to cause glucocorticoid resistance. Inhibitors of HSP90 suppressed cortisol production and corticotroph cell proliferation in in vitro- and experimental animal models (Riebold *et al.*, 2015; Sugiyama *et al.*, 2015). To date, no clinical studies have been performed with HSP90 inhibitors in CD.

Glucocorticoid Receptor Antagonists

GR antagonists counteract the effects of cortisol at tissue level by blocking the GR. At present, mifepristone is the only GR antagonist available for clinical use. Mifepristone has a rapid onset of action and can be useful in patients who present with acute complications, for example, psychosis, of (severe) cortisol excess (Castinetti *et al.*, 2012). Mifepristone was demonstrated to improve blood pressure and glycemic regulation in patients with CS (Fleseriu *et al.*, 2012) and is approved in the US for treatment of CS patients with dysregulated diabetes mellitus who are no candidates for surgery. Currently no biochemical parameter is available to adjust the dose of mifepristone. Consequently, patients are at risk to develop clinical adrenal insufficiency in case of overtreatment. Furthermore, mifepristone interferes with negative feedback via the GR on the corticotroph adenoma which can result in increased ACTH and concomitantly increased cortisol production. Increased cortisol levels, in turn, can induce or worsen mineralocorticoid effects, that is, hypokalemia, hypertension and edema. Mifepristone not only antagonizes the GR but also the progesterone receptor. Therefore, during chronic treatment female patients should be monitored for development of endometrial hyperplasia (Castinetti *et al.*, 2012).

CORT125134 (relacorilant) is a new GR antagonist that selectively binds to the GR without anti-progesterone receptor effects (Hunt *et al.*, 2017). This new compound is currently evaluated in a phase II study (NCT02804750).

Combination Therapy

Patients with moderate to severe hypercortisolism usually need medical combination therapy to achieve complete biochemical remission. One study showed that a combination of ketoconazole, metyrapone and mitotane was effective in patients with severe, complicated CS and could prevent a rescue bilateral adrenalectomy (Kamenicky *et al.*, 2011). Combining drugs may also have synergistic effects. For instance, simultaneous targeting of SST5 and DA2 may result in enhanced signaling (Rocheville *et al.*, 2000). Two studies show that stepwise addition of cabergoline to pasireotide almost doubled the number of patients with controlled cortisol production (Feelders *et al.*, 2010, 2017). Combination of drugs may also allow for lower dosages of individual compounds with a potentially lower chance on developing side effects. For instance, combination of cabergoline and low dose ketoconazole was well tolerated and effective in a subset of patients (Vilar *et al.*, 2010; Barbot *et al.*, 2014).

Concluding Remarks

Medical therapy is an important treatment modality in patients with CS for whom surgery is not an option. Drugs to treat CS can be categorized into adrenal blocking drugs, pituitary targeting drugs and glucocorticoid receptor antagonists. In each patient medical therapy should be given in a tailor-made approach taking several factors into account including patient characteristics, severity of hypercortisolism, drug efficacy and (potential) side effects and drug availability and costs. It should be emphasized that treatment should completely normalize cortisol production in order to reverse morbidity and mortality. For this purpose medical combination therapy is often required.

See also: ACTH-Secreting Pituitary Tumors. Cushing Syndrome; Screening and Differential Diagnosis. Evaluation and Follow-Up of Patients With Cushing Disease After Pituitary Surgery

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Radiation Therapy in Patients With Cushing Disease

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Overview

Cushing disease (CD) is associated with substantial morbidity and mortality, which can be mitigated by achieving prompt control of hypercortisolism (Cushing, 1932, 1969; Nieman *et al.*, 2015). The initial treatment of choice in most patients with CD is transsphenoidal pituitary surgery (TSS), performed by an experienced pituitary neurosurgeon (Nieman *et al.*, 2015; Swearingen *et al.*, 1999). However, persistent or recurrent CD may occur in about 10%–20% and 20%–30% of patients, respectively, thus necessitating additional therapies (Swearingen *et al.*, 1999; Tritos *et al.*, 2011; Patil *et al.*, 2008). A second TSS can be an effective option in controlling cortisol excess in approximately 50%–60% of patients (Nieman *et al.*, 2015).

Radiation therapy to the sella is often recommended as adjunctive treatment in patients with CD who have active disease after TSS (Loeffler and Shih, 2011; Pashtan *et al.*, 2014). In rare cases, radiation therapy can be used as primary therapy in patients who are not surgical candidates. Radiation therapy is effective in preventing tumor growth and controlling hypercortisolism in the majority of treated patients, but requires time to take effect, generally ranging between several months to a few years (Loeffler and Shih, 2011; Pashtan *et al.*, 2014). In the interim, treated patients require medical therapy to control hypercortisolism (Tritos and Biller, 2014).

The aims of the present article are to review radiation therapy modalities used to treat CD and discuss patient outcomes with regards to efficacy and safety. To retrieve articles used in this review, computerized literature searches were conducted using the terms "Cushing disease" or "Cushing syndrome," "radiosurgery," "radiation therapy," "radiotherapy." Pertinent articles were retrieved and considered for inclusion in this article, based on authors' judgment.

Radiation Therapy Modalities and Techniques

Radiation therapy to the sella has been conventionally administered in fractions (typically 45–54 Gy in 25–30 sessions, delivering 1.8–2 Gy per fraction) (Loeffler and Shih, 2011). Using conventional techniques, dose fractionation is required to minimize radiation-induced injury to off-target structures, including the optic chiasm, other cranial nerves, the cochlea and the brain. Although effective, this approach is somewhat inconvenient to patients, as it necessitates daily (weekday) visits over a 5–6 week period.

Stereotactic radiation therapy techniques, also known as stereotactic radiosurgery (SRS), were developed to deliver a single, high dose of radiation (typically ranging between 12 and 35 Gy) to the tissue target in an extremely precise and accurate manner that minimizes radiation exposure to surrounding healthy tissues (Loeffler and Shih, 2011). Dose fractionation is also possible using stereotactic treatment modalities. Stereotactic techniques include the Gamma Knife™ (Elekta, Stockholm, Sweden) and linear accelerator-based treatment options (including the Cyberknife™ (Accuray, Sunnyvale, CA)), each of which deliver photon-based radiation therapy. Other stereotactic techniques employ proton beams or helium beams, which deliver positively charged particles.

Advanced methods in therapy planning and radiation beam shaping are required to achieve high tissue selectivity, which maximizes delivery of radiation to the intended target while minimizing exposure of healthy structures (Pashtan *et al.*, 2014). In addition, the physical properties of charged particle beams (made up of protons or helium nuclei) are instrumental in achieving selective delivery of radiation to target tissues. Charged particle beams deliver most of their energy at a predetermined tissue depth, known as the "Bragg peak," which can be designed to deposit almost the entire particle energy within the target area with essentially zero exit dose, thus minimizing radiation exposure to healthy tissues (Pashtan *et al.*, 2014).

The obvious advantage of SRS over conventional radiation therapy is greater convenience for patients, who typically receive this type of radiation therapy in a single treatment session. It also appears that endocrine remission may occur sooner after SRS in comparison with conventional radiation therapy (based on comparisons with historic data) (Sheehan *et al.*, 2005). Stereotactic techniques optimize radiation delivery to the tumor (target tissue) while sparing healthy, radiosensitive structures, including the optic chiasm, other cranial nerves, the cochlea or the brain. This selectivity would be predicted to translate into a more favorable long-term safety profile. However, large studies of long duration are needed to establish this conjecture with certainty.

Treatment Planning and Delivery

The choice between treatment modalities used to deliver radiation therapy obviously depends on local equipment availability. Proton beam facilities are very expensive to set up and maintain, which has limited the use of this technique to a relatively small

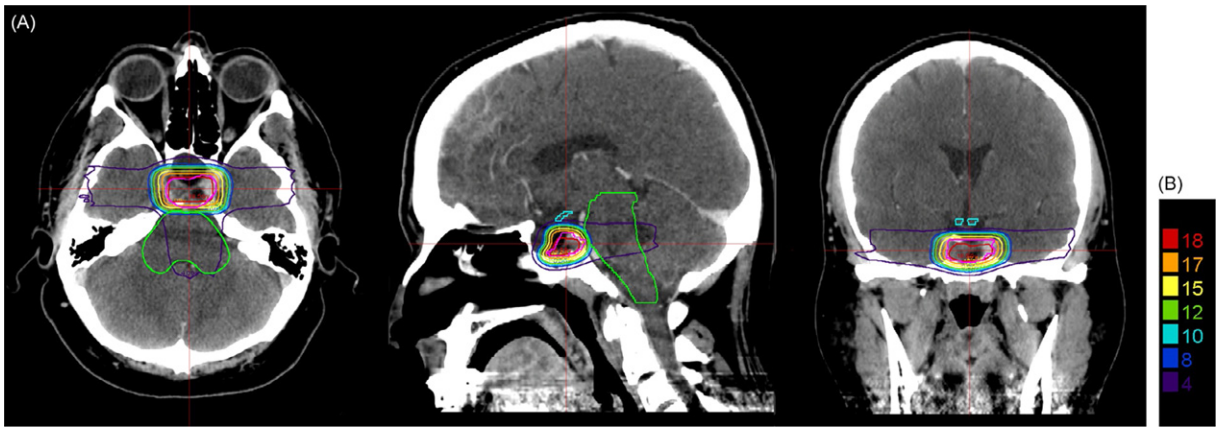

Fig. 1 Stereotactic proton beam radiation therapy plan in a patient with Cushing's disease (panel A). Color codes for isodose lines are shown in legend (panel B).

number of centers. Whenever possible, use of stereotactic techniques is advisable because of the potential advantages of these treatment modalities, as outlined above.

Using SRS, it is advisable to limit the radiation dose to the chiasm below 8–10 Gy, which is crucial in order to minimize the risk of optic neuropathy (Mayo *et al.*, 2010). However, administration of radiation therapy in a single fraction (SRS) is not always possible, depending on tumor size, geometry and its proximity to radiosensitive structures (such as the optic apparatus). In general, patients with pituitary adenomas larger than 3 cm or those that are closer than 3–5 mm to the optic chiasm are not good candidates for single dose therapy (i.e., SRS) because of increased risk of complications (Pashtan *et al.*, 2014). These patients may still be treated with fractionated therapy, which is considered to be safer and may also be delivered via stereotactic techniques. Use of a fraction size of 1.8–2 Gy per day is associated with a lower risk of optic neuropathy in comparison with a higher dose per fraction (Mayo *et al.*, 2010).

During treatment planning, high resolution, cross-sectional imaging data are used to identify the target and establish a treatment plan by means of dedicated software. An example of a treatment plan depicting isodose radiation curves in the case of a patient receiving proton beam radiation therapy to the sella is shown in **Fig. 1**.

Accurate patient positioning is critical in patients receiving SRS. A thermoplastic face mask is created to help position and immobilize the patient's head during conventional radiation therapy. A higher degree of precision in head positioning is required during SRS and is achieved with a variety of methods, including use of a positioning frame secured to the skull by means of implanted pins (fiducial markers), a custom-made bite piece and/or high-precision image guidance (Pashtan *et al.*, 2014).

In patients receiving SRS using Gamma Knife™, the head is immobilized in a metal helmet that bears >200 collimated bores that focus radiation emanating from cobalt 60 sources to the target (Loeffler and Shih, 2011; Pashtan *et al.*, 2014). Use of multiple non-coplanar radiation beams helps optimize target selectivity in patients receiving Gamma Knife™ or proton beam therapy. In patients treated with the Cyberknife™, the treatment gantry rotates around the patient's head to deliver radiation beams from different angles during the session.

Outcomes of Patients With Cushing Disease Who Received Radiation Therapy

As already noted, radiation therapy to the sella is generally recommended as adjunctive therapy in patients who have persistent or recurrent hypercortisolism after undergoing TSS and are not candidates for additional pituitary surgery (Loeffler and Shih, 2011; Nieman *et al.*, 2015; Pashtan *et al.*, 2014). Other candidates for radiation therapy include patients with CD who have residual or enlarging corticotroph tumors after TSS (Loeffler and Shih, 2011; Nieman *et al.*, 2015; Pashtan *et al.*, 2014). Patients who receive radiation therapy require medical therapy to control hypercortisolism until the salutary effects of radiation therapy occur (usually for a period ranging between several months to several years) (Tritos and Biller, 2014).

Several retrospective or prospective studies have reported on the outcomes of patients with CD who underwent radiation therapy (**Table 1**) (Littley *et al.*, 1990; Levy *et al.*, 1991; Murayama *et al.*, 1992; Tsang *et al.*, 1996; Estrada *et al.*, 1997; Witt *et al.*, 1998, Laws and Vance, 1999; Sheehan *et al.*, 2000; Kobayashi *et al.*, 2002; Jane *et al.*, 2003; Devin *et al.*, 2004; Colin *et al.*, 2005; Castinetti *et al.*, 2007; Jagannathan *et al.*, 2007; Minniti *et al.*, 2007; Petit *et al.*, 2008; Wan *et al.*, 2009; Sheehan *et al.*, 2013; Budyal *et al.*, 2014; Wilson *et al.*, 2014; Wattson *et al.*, 2014; Marek *et al.*, 2015; Cohen-Inbar *et al.*, 2016; Mehta *et al.*, 2017). These case series presented the outcomes of patients treated with a single treatment modality (either conventional radiation therapy or various SRS options) and did not include a patient control group. Tumor control, defined as either lesion stability or regression, was achieved in 83%–100% of patients after radiation therapy. Biochemical control of hypercortisolism, which was most often defined as normal 24 h urine free cortisol with or without normal plasma ACTH levels (in the absence of concurrent medical

Table 1 Effectiveness of radiation therapy in patients with Cushing disease[a]

Study first author, year	*Number of patients treated*	*Treatment modality*	*Radiation dose (median or mean) (Gy)*	*Follow-up in months (median or mean)*	*Biochemical control (% patients)*[d]	*Tumor control (% patients)*[e]
Littley et al. (1990)	24	Conventional	20[b]	94.8	46	NR
Levy *et al.* (1991)	64	Proton or helium beam	150[b]	NR	86	NR
Murayama *et al.* (1992)	20	Conventional	54[b]	90.5	55	100
Tsang *et al.* (1996)	29	Conventional	45[b]	87.6	56	96
Estrada *et al.* (1997)	30	Conventional	50[b]	42	83	100
Witt *et al.* (1998)	25	Gamma knife	38[b]	32	28	94
Laws and Vance (1999)	50	Gamma knife	NR	NR	58	NR
Sheehan *et al.* (2000)	43	Gamma knife	47[b]	44	63	100
Kobayashi *et al.* (2002)	20	Gamma knife	49[b]	64	35	100
Jane *et al.* (2003)	45	Gamma knife	15[c]	NR	73	NR
Devin *et al.* (2004)	35	Linear accelerator	33.7[b]	42	49	91
Colin *et al.* (2005)	40	Gamma knife	NR	54.7	43	NR
Castinetti *et al.* (2007)	40	Gamma knife	29.5[c]	54.7	42.5	NR
Jagannathan *et al.* (2007)	90	Gamma knife	49[b]	45	54	95
Minniti *et al.* (2007)	40	Conventional	45[b]	108	84	93
Petit *et al.* (2008)	33	Proton beam	22.2[b]	62	52	94
Wan *et al.* (2009)	68	Gamma knife	19 (microadenomas); 25 (macroadenomas)[c]	60–90 [range]	28	90
Sheehan *et al.* (2013)	96	Gamma knife	22[c]	48	70	98
Budyal *et al.* (2014)	20	Conventional	45[c]	37.5	75	95
Wilson *et al.* (2014)	36	Linear accelerator	20[c]	66	22	83
Wattson *et al.* (2014)	74	Proton beam	20[c]	52	54	98
Marek *et al.* (2015)	26	Gamma knife	28[c]	98.5	81	92
Cohen-Inbar *et al.* (2016)	36	Gamma knife	25[c]	159.5	81	93
Mehta *et al.* (2017)	278	Gamma knife	23.7[c]	67	80	95

[a]Including studies of 20 or more patients that were published since 1990.
[b]Maximal dose.
[c]Tumor margin dose.
[d]Biochemical control was variably defined (most commonly as normal 24 h urine free cortisol).
[e]Tumor control was defined as lack of progression (that is, either stability or regression); NR: not reported.

therapy) or, less often, as normal cortisol response on 1 mg dexamethasone suppression testing, was variably reported in 22%–86% of patients after radiation therapy in different series. There are no published data on late night salivary cortisol levels in patients with CD who received radiation therapy. Based on historical comparisons, it appears that the interval between

administration of radiation therapy and achievement of control of cortisol excess may be shorter in patients who received SRS than those who were administered conventional fractionated radiation therapy (Sheehan *et al.*, 2005). However, published studies directly comparing two treatment modalities in a head to head manner are lacking.

Some data have suggested that endocrine remission is delayed or decreased in subgroups of patients with CD who have been receiving concurrent medical therapy at the time radiation therapy has been administered (Sheehan *et al.*, 2013; Castinetti *et al.*, 2007). These findings have raised the possibility that concomitant medical therapy may blunt the effects of radiation therapy to the sella. On the basis of these data, it has been suggested that medical therapy be withdrawn for several weeks before administration of radiation therapy to the sella. However, allocation to concurrent medical therapy was not random in these studies. It is possible that patients who received medical therapy at the time radiation therapy was administered had more severe hypercortisolism at baseline. Thus, confounding by indication is another possible explanation for these observations. As a corollary, additional research is needed to fully elucidate this issue.

Recurrence of hypercortisolism may occur in a minority of patients who achieved endocrine remission after the administration of radiation therapy. In one study of 96 patients who received SRS (Gamma knife™), remission occurred in 70% at a median time interval of 16.6 months. Subsequently, biochemical recurrence developed in 15 patients at a median time interval of 38 months (Sheehan *et al.*, 2013). In a recent multicenter study of 278 patients with CD, recurrence of hypercortisolism developed in 18% of patients who had initially achieved remission (Mehta *et al.*, 2017). Clearly, all patients with CD require lifelong follow-up in order to establish long-term outcomes.

Safety

Patients who have received radiation therapy to the sella are at long-term (likely lifelong) risk of anterior hypopituitarism (Pashtan *et al.*, 2014). In these patients, the normal pituitary gland is very close to the tumor target, thus imparting a substantial risk of radiation-induced damage to anterior pituitary function. The risk of anterior hypopituitarism appears to be comparable in patients who have received either conventional radiation therapy or SRS (Pashtan *et al.*, 2014). Between 20% and 60% of patients who received radiation therapy developed one or more anterior pituitary hormone deficits at 5 years after treatment in different series (Estrada *et al.*, 1997; Minniti *et al.*, 2007; Sheehan *et al.*, 2005). Up to 85% of patients developed evidence of anterior hypopituitarism at 15 years after radiation therapy (Minniti *et al.*, 2007).

Radiation-induced optic neuropathy or other cranial neuropathies may also occur in 1%–3% of patients (Brada *et al.*, 1993; Sheehan *et al.*, 2005; Mehta *et al.*, 2017). Proper treatment planning aimed at limiting radiation exposure to the radiosensitive chiasm and optic nerves can help minimize the risk of optic neuropathy. Specifically, maintaining a dose limit to the optic chiasm of $<$8–10 Gy during SRS or 45–55 Gy (administered in 1.8–2 Gy fractions) in patients receiving fractionated therapy helps minimize the risk to vision (Mayo *et al.*, 2010).

Conventional radiation therapy has been associated with a delayed risk of stroke, presumed to occur as a long-term consequence of irradiation of the major blood vessels in the circle of Willis and/or internal carotid arteries. Such cerebrovascular events have been reported in 4% of patients at 5 years after conventional fractionated radiation therapy, with cumulative risk rising further over time (Brada *et al.*, 1999). It is hoped that SRS and proton beam radiation therapy will prove to be associated with a lower risk of stroke; data regarding this endpoint are not yet available.

Other infrequent risks of radiation therapy include temporal lobe necrosis (rare) and secondary tumor formation (including meningiomas and sarcomas, reported in 1%–3% of patients at 20 years after radiation therapy) (Minniti *et al.*, 2005; Sheehan *et al.*, 2005). Thus far, these events have only been reported in patients who have received conventional fractionated radiation therapy but have not yet been described after SRS. With the advent of modern planning techniques, stereotactic approaches and proton beam radiation therapy, it is anticipated that the risks of these events will prove to be extremely low in the modern era. However, long-term studies of adequate size are needed in order to reliably estimate the risk of such delayed and infrequent potential adverse outcomes.

Summary

Radiation therapy is an effective treatment option in CD. It is generally recommended for patients who have persistent or recurrent hypercortisolism and/or residual or growing tumor after undergoing TSS, and are not considered candidates for additional pituitary surgery. Since there is a latency period after the administration of radiation therapy until hypercortisolism is controlled, medical treatment is required as a "bridge" intervention.

The advent of modern treatment planning techniques, SRS and proton therapy helps minimize untoward radiation exposure of the normal brain and other radiosensitive structures, including the optic apparatus. All patients who received radiation therapy to the sella are at lifelong risk of anterior hypopituitarism and possibly of neurologic sequelae, and require long-term follow-up.

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See also: ACTH-Secreting Pituitary Tumors. Evaluation and Follow-Up of Patients With Cushing Disease After Pituitary Surgery

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Bilateral Adrenalectomy for Cushing Disease

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Introduction

Cushing disease is a rare condition due to an adrenocorticotropin hormone (ACTH)-secreting pituitary adenoma, leading to hypercortisolism. A chronic or acute increase in the cortisol level leads to a high number of clinical signs, hypertension, diabetes, osteoporosis, and depression. This explains why an optimal management of this condition is mandatory. Bilateral adrenalectomy is now still considered as the ultimate therapeutic option in Cushing disease, probably because the "primum non nocere" paradigm is always considered by physicians who prefer to avoid adrenal insufficiency (Lacroix *et al.*, 2015). This is why the majority of endocrinologists now prefer to use long-term medical treatments aimed at controlling cortisol hypersecretion: such treatments, as we shall discuss later, have their own drawbacks. The aim of this review is to clarify the potential roles of bilateral adrenalectomy in the management of Cushing disease.

Since the first description of bilateral adrenalectomy in 1936 (Young, 1936), several technical advances have been done, the most important being laparoscopy about 25 years ago. Gagner *et al.* reported their experience of laparoscopic adrenalectomy in three patients, one with unilateral adrenal adenoma, one with a pheochromocytoma, and the last with failed transsphenoidal surgery leading to bilateral adrenalectomy. In this latter case, the operative time was 3.5 h for both adrenalectomies, and the patient could be discharged on the third postoperative day, receiving supplementation by hydrocortisone and fludrocortisone (Gagner *et al.*, 1992). The original approach was slightly modified by posterior retroperitoneoscopy or robot-assisted bilateral adrenalectomy (Lan *et al.*, 2015; Raffaelli *et al.*, 2014), but the major principles remained the same. The side effects are rarely severe with a mortality $<3\%$, and an overall morbidity of $<20\%$ (Reincke *et al.*, 2015; Ritzel *et al.*, 2013). The per-operative complications are more frequently due to the hypercortisolic state, when still present at the time of surgery, than the surgery per se.

Merits and Pitfalls of Bilateral Adrenalectomy in the Context of Insidious Cushing Disease

The Natural History and Management of Cushing Disease

Cushing disease is one of the most challenging diseases for endocrinologists. Most of the time, the diagnosis will be confirmed only after several years of general complaints, including weight gain, depression, fatigue, badly controlled hypertension, diabetes, etc. This progressively evolving hypercortisolic state will be named classical Cushing disease in this section, by opposition with rapidly evolving hypercortisolic states, named severe hypercortisolism in the following section.

In classical Cushing disease, transsphenoidal surgery is considered as the first-line treatment: in experienced hands, it can lead to remission in up to 80% cases (Nieman *et al.*, 2015). However, even in patients with reassuring postoperative predictive factors, recurrence can be observed in up to 30% of patients (Patil *et al.*, 2008). As Cushing disease is frequently due to micro or picoadenoma (and surgery can sometimes be performed in patients with negative pituitary MRI), it is expected that the majority of patients will present recurrence and negative magnetic resonance imaging (MRI), excluding the possibility to perform radiation techniques. At that stage, the pituitary staff usually have to decide between lifelong medical treatment, transient medical therapy while awaiting for radiological signs of tumor recurrence, or bilateral adrenalectomy. Medical treatment will not be detailed as it is not the aim of this article. Endocrinologists should, however, keep in mind the difficulties of ascertaining eucortisolism in patients treated with drugs lowering cortisol or ACTH: as there is currently no perfect biological marker of eucortisolism, it is indeed difficult to be sure that such patients will not present mild hyper or hypocortisolism on a long-term basis, leading to long-term comorbidities (Petersenn *et al.*, 2014). Such treatments can also present more or less severe side effects (liver enzymes increase, worsening of hypertension or hypokalemia, diabetes, etc.) that require a close follow-up. The treatments cannot cure the patient, and escape after initial control can be observed in up to 10%–20% of cases. Altogether, these treatments usually allow control of hypersecretion in roughly 50% of cases. This rate has to be compared with the efficacy of bilateral adrenalectomy, and the expected side effect of the surgical procedure, that is, adrenal insufficiency.

Control of Hypercortisolism by Bilateral Adrenalectomy and the Risk of Recurrence

While one should expect 100% cure after bilateral adrenalectomy for Cushing disease, the overall efficacy is probably a little lower, as few cases of recurrence have been reported in four different studies. However, it is difficult to determine whether these "recurrences" were true clinical recurrences, or only slightly higher than expected levels of cortisol. Nagesser *et al.* reported that 27% of 44 patients treated by bilateral adrenalectomy had early morning cortisol levels >50 nmol/L, in favor of persistent adrenal tissue; however, only 4.5% presented significant clinical recurrence (Nagesser *et al.*, 2000). These numbers were close to the ones reported by three other studies, in which the risk of recurrence was close to 2% (Chalmers *et al.*, 1981; Chow *et al.*, 2008; Kemink *et al.*, 1992). Most of the time, the recurrence will be due to incomplete first surgery, with recurrence happening a few years after

Table 1 Recurrence of adrenal remnants after bilateral adrenalectomy reported in the literature

First author, year	*Patients*	*Persistent adrenal tissue*	*Significant clinical recurrence*	*Time to recurrence*	*Subsequent treatment*
Chow *et al.* (2008)	68	NA	2 (2.9%)	Not cured immediately after surgery	Follow-up without treatment
Nagesser *et al.* (2000)	44	12 (27.3%)	2 (4.5%)	Early relapse	Additional surgery allowing cure ($n = 1$), follow-up without treatment ($n = 1$)
Kemink *et al.* (1992)	50	9 (18%)	1 (2%)	9 years	Transsphenoidal surgery
Chalmers *et al.* (1981)	5[a]	5	4	1, 2, 3, and 7 years after the surgery	Recurrence of Cushing's disease, pituitary radiotherapy, 5 months and 5 years later ($n = 2$); pituitary radiotherapy ($n = 2$); still in remission 4 years after glucocorticoid withdrawal ($n = 1$)
Pooled data (95% confidence interval)	162	23% (15–32%)	3.8% (1.4–7.3%)	—	—

[a]Five isolated case reports from five different centers (from Guerin *et al.*, 2016).

the procedure, because of the persistent stimulation of the adrenal tissue by ACTH. In rare cases, recurrence (or failed surgery) will be due to ectopic adrenal tissue. From a practical viewpoint, recurrence should be looked for in a patient presenting with persistent signs of cortisol overexposure, despite progressively lowering the dose of hydrocortisone, especially if postsurgical cortisol level is superior to 50 nmol/L. The main studies reporting this risk of recurrence are summarized in **Table 1** (from Guerin *et al.*, 2016).

The Risk of Nelson Syndrome After Bilateral Adrenalectomy

Nelson syndrome was first reported in 1958: skin hyperpigmentation, increased ACTH, and pituitary tumor were shown in a woman who had been treated by bilateral adrenalectomy a few years earlier. These three symptoms now represent the definition of Nelson syndrome, which might be due to either the loss of negative feedback of cortisol on the production of corticotropin releasing hormone (CRH) and ACTH, or independent tumor progression (Azad *et al.*, 2015). The possibility of developing Nelson syndrome has long been considered the major restraint for treating patients with bilateral adrenalectomy. This is likely because prior to introduction of MRI, pituitary imaging was not truly effective (cranial X-ray); the diagnosis of Nelson syndrome was made when the situation was already critic. This also led to prophylactic pituitary radiotherapy in patients treated with bilateral adrenalectomy. Novel imaging modalities with pituitary MRI allowed a close follow-up of patients after bilateral adrenalectomy, making unnecessary systematic pituitary radiotherapy (Assie *et al.*, 2007). Nelson syndrome prevalence is thought to be between 10% and 50% of patients treated by bilateral adrenalectomy for Cushing disease. The risk increases when an adenoma remnant is visualized before bilateral adrenalectomy, and when ACTH levels increase considerably during the first year after bilateral adrenalectomy. When Nelson syndrome is diagnosed, several therapeutic options can be used: transsphenoidal surgery, radiation techniques, cabergoline, or Pasireotide. Temozolomide has also been used successfully in a rapidly progressive pituitary remnant (Azad *et al.*, 2015). As previously mentioned, performing regular imaging allows the identification and rapid treatment of a progression in size of the remnant. As no large series has ever been reported showing the superiority of these different therapeutic options, the decision will have to be taken by expert pituitary staff.

The Adrenal Insufficiency

While the mortality due to adrenal insufficiency is now very low, it can still be an issue despite the education of patients. An overall mortality rate of 1% was found in the patients followed in a German center, where they received a minimal education for dose change in stress situations; the risk of adrenal crises was evaluated at 8–9 per 100 patients per year (Hahner *et al.*, 2015). This emphasizes the need for a clear and repeated education of patients after bilateral adrenalectomy. Other than mortality, the lack of physiological replacement of cortisol nycthemeral rhythm by hydrocortisone intake is also an issue: as stated by the specific AddiQoL, patients with adrenal insufficiency have complaints due to chronic over- or under-dose and the lack of biological parameters to allow an optimal dose planning during the various periods of a day (Husebye *et al.*, 2014).

Is Eucortisolism Necessary Before Bilateral Adrenalectomy?

The per-operative improvement of patients operated on while in eucortisolism rather than hypercortisolism is still a matter of controversy. Only one study tried to determine whether a pre-surgical medical treatment (4 months with steroidogenesis

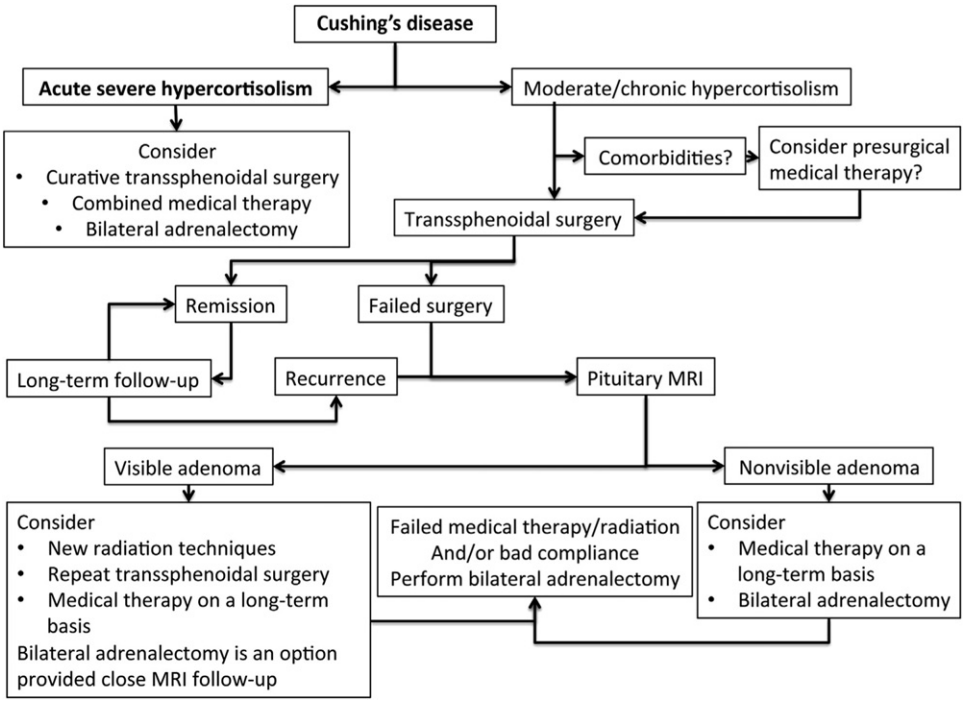

Fig. 1 Suggested therapeutic algorithm in Cushing disease. From Guerin, C., Taieb, D., Treglia, G., Brue, T., Lacroix, A., Sebag, F. and Castinetti, F. (2016). Bilateral adrenalectomy in the 21st century: When to use it for hypercortisolism? *Endocrine-Related Cancer*, **23**, R131–R142.

inhibitors before transsphenoidal surgery) allowed improvement of the general condition of the patients: the results were unclear, with seemingly a long-term improvement of hypertension and a decreased risk of thrombo-embolic events in patients with pretreatment (Valassi *et al.*, 2012). For this latter point, the number of events in the study was very low, and large-scale studies will be necessary to ascertain this conclusion. In a review by Ritzel *et al.*, the mortality and morbidity after bilateral adrenalectomy were comparable whether the patients were pretreated or not (1% vs. 2% mortality, 19% vs. 21% morbidity) (Ritzel *et al.*, 2013). In patients with classical Cushing disease, trying to obtain eucortisolism will not worsen the general condition of the patient: it thus can be tried, but should not delay the surgical procedure too much.

Current Roles of Bilateral Adrenalectomy in the Therapeutic Algorithm of Classic Cushing Disease

The first-line treatment of Cushing disease is transsphenoidal surgery. It is usually recommended even in patients with a negative pituitary MRI. However, the risks of recurrence remain very high (up to 15%–30%), even in patients with the best predictive factors (such as immediate postsurgical corticotroph deficiency). Usually, at the time of recurrence, transsphenoidal surgery is performed only in patients with an obvious adenoma remnant. In that case, if surgery is contra-indicated or technically impossible, radiation techniques can also be performed, as they will lead to cure in roughly 50% of cases. For these patients, the delay to remission can be prolonged, requiring a medical treatment to control hypercortisolism while waiting for the maximal efficacy of radiation techniques.

Bilateral adrenalectomy should thus be considered in any situation when transsphenoidal surgery is contra-indicated or when the pharmacological treatment is not effective (including the patients waiting for the efficacy of radiation techniques, but for whom medical treatment cannot control hypercortisolism). It should be noted that some patients might prefer a radical treatment rather that a lifelong intake of medical treatments, and this is also a point to consider, provided the patient is aware of the need for a prolonged substitutive treatment.

When a bilateral adrenalectomy is performed, one should keep in mind the risk of Nelson's syndrome: the ACTH level should be closely monitored, and a pituitary MRI be performed during the first year after bilateral adrenalectomy (though the frequency to perform pituitary MRI remains to be determined, particularly in patients with negative MRI despite biological recurrence). Prolonged clinical, biological, and imaging surveillance is required for these patients. Current management of Cushing disease is summarized in **Fig. 1** (from Guerin *et al.*, 2016).

The Specific Case of Bilateral Adrenalectomy in Severe Cushing Disease

Severe hypercortisolism is rarely due to Cushing disease: it is most frequently due to ectopic ACTH secretion or adrenal carcinoma. However, severe hypercortisolism is a life-threatening condition for which bilateral adrenalectomy is always considered. The aim is

Table 2 Results of fast-acting pharmacological treatments in severe hypercortisolism

Author, year	*Patients*	*Treatment*	*Theoretical onset of action*	*Dose*	*Short-term antisecretory efficacy*	*Adrenal insufficiency*	*Short-term side effects*
Corcuff *et al.* (2015)	14 EAS	Ke + Me	2–7 days	400–1200 mg/d (Ke) + 0.5–4 g/d (Me)	73% normal UFC	14%	Nausea and vomiting Hypokalemia Increased liver enzymes
Kamenicky *et al.* (2011)	7 EAS – 3 CD	Ke + Me + Mi	1–3 days	400–1200 mg/d (Ke) + 3–4.5 g/d (Me) +3.5 g/d (Mi)	63% normal UFC	36%	Nausea and vomiting Hypokalemia Increased liver enzymes
Castinetti *et al.* (2009)	8 ACTH dependent	Mifepristone	48 h				400–2000 mg/d
		Improvement of clinical signs in all the patient, no biological follow-up available	14% (no biological evidence available)	Hypertension Hypokalemia			
Preda *et al.* (2012)	12 case reports	Etomidate	12–24 h	0.03–0.3 mg/kg/h	83% normal UFC	Depending on the dose/sedation level	Hemolysis, thrombophlebitis, pain at the injection site, nephrotoxicity

to decrease rapidly the levels of cortisol to avoid severe comorbidities (such as severe depression, pulmonary embolism, acute respiratory distress, uncontrolled hypertension or diabetes, and severe hypokalemia) or even death. Over the last few years, several studies showed interesting results on the association of steroidogenesis inhibitors, and this led to the questioning of the remaining roles of bilateral adrenalectomy in such patients. It should be noted that all these studies were retrospective, and were not aimed at comparing one approach (medical treatments) with the other (bilateral adrenalectomy). The main association reported up to now was based on ketoconazole and metyrapone, two highly effective adrenal steroidogenesis inhibitors. A large retrospective series as a single treatment reported an antisecretory efficacy in 50%–80% cases (Castinetti *et al.*, 2014; Daniel *et al.*, 2015). It was thus expected that the association of both would allow a rapid control of hypercortisolism in the majority of cases. In one of these two studies, mitotane was given at the same time, with the aim of allowing a long-term medical control (after withdrawal of ketoconazole and metyrapone, as mitotane is a slow-acting drug). While 24 patients were reported in these 2 studies, only 3 were presenting with Cushing disease. Ketoconazole and metyrapone were given at high doses. Roughly 70% of the patients were finally controlled: what was impressive was the decline of cortisol levels, with a dramatic decrease of urinary free cortisol in the first few days. Tolerance was good, provided a close monitoring of liver enzymes, potassium, and blood pressure was performed. Adrenal insufficiency was reported in 25% of the patients (Corcuff *et al.*, 2015; Kamenicky *et al.*, 2011).

Other drugs are also known to decrease rapidly the effects of hypercortisolism. Mifepristone is a glucocorticoid receptor antagonist. We reported about 10 years ago its rapid efficacy in patients with severe psychosis induced by hypercortisolism (Castinetti *et al.*, 2009). The drug is now marketed in the United States in the management of hypercortisolism, and it could be considered in these critical situations. One should keep in mind, however, that the blockade of glucocorticoid receptors is also present in the pituitary, thus leading to increased ACTH and cortisol levels, making it impossible to monitor the biochemical control of the efficacy of the drug. Moreover, increased cortisol levels can also lead to a pseudo-hyperaldosteronism, with a worsening of hypokalemia and hypertension. The use of mifepristone should thus be reserved to highly experienced teams (Fleseriu *et al.*, 2012).

Finally, etomidate, a rapidly effective adrenal steroidogenesis inhibitor, has also been used as a treatment of severe hypercortisolism. It is a drug that is difficult to use as it has to be administered in an intensive care unit (where it can also be used to induce anesthesia). Literature data showed a good efficacy in 12 patients with severe hypercortisolism and inability to take medications orally (Preda *et al.*, 2012).

To summarize, given the high efficacy of the drugs, the good tolerance, and the state of high frailty in which these patients can be at the time of initial diagnosis, one should thus always consider a trial with medical treatments in the aim of improving the general condition of the patient before surgery (whatever the surgery would be). The use of these treatments in these critical conditions should be reserved to highly experienced teams.

Main studies reporting the outcome of patients treated by adrenal steroidogenesis inhibitors for severe hypercortisolism are summarized in **Table 2** (from Guerin *et al.*, 2016).

Conclusions and Perspectives

Bilateral adrenalectomy is a rapidly acting treatment for Cushing disease. It leads to another disease, adrenal insufficiency, that needs proper management and follow-up. However, it is not yet clear whether other approaches always lead to eucortisolism: new radiation techniques lead to hypopituitarism in 20%–30% of cases; medical treatment improves hypercortisolism, but there is still a gap between normal urinary free cortisol (UFC) and real eucortisolism. Future studies should be aimed at defining whether metabolic parameters and quality of life are different in patients on a long-term medical treatment, and patients with a permanent adrenal insufficiency. In the second case, it is also likely that quality of life should be improved by new formulations of hydrocortisone. The final conclusion is that one should never consider that bilateral adrenalectomy is an obsolete treatment of Cushing disease; it might even become more important in the therapeutic algorithms in the future.

See also: ACTH-Secreting Pituitary Tumors. Evaluation and Follow-Up of Patients With Cushing Disease After Pituitary Surgery

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Cushing Syndrome—Unilateral Adrenal Adenoma

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Introduction

Cushing syndrome is characterized by signs and symptoms caused by chronic glucocorticoid hypersecretion. Despite the major advances that have been made during the last years, the diagnosis and the treatment of Cushing syndrome still represent a major challenge among physicians. Cushing syndrome is a life-threatening disease, if left untreated. An early diagnosis and a prompt appropriate treatment are therefore mandatory. Cushing syndrome may be either ACTH-dependent (the most frequent form) or ACTH-independent. This article is focused on the most common pathological condition among ACTH-independent causes of Cushing syndrome, the unilateral adrenocortical adenomas.

Epidemiology and Clinical Presentation

Cushing syndrome due to unilateral adrenocortical adenomas is a rare clinical condition. Among the ACTH-independent forms of hypercortisolism, which represents 15% of cases of endogenous Cushing syndrome, it is the most frequent pathological condition, followed by adrenocortical carcinomas and bilateral micro- and macronodular hyperplasia. The highest reported prevalence is 18.7% in a large series of patients with Cushing syndrome (Invitti *et al.*, 1999). The incidence of endogenous Cushing syndrome has been estimated between 0.7 and 2.4/million/year (Sharma *et al.*, 2015). The incidence of the disease peaks between the third and the fifth decades of life (Bertagna and Orth, 1981) and a strong preponderance of female sex has been reported in unilateral adenomas associated with Cushing syndrome (Pikkarainen *et al.*, 1999). Although this pathological condition has been considered rare in population-based studies, the exact prevalence and incidence is difficult to be estimated and may be higher than previously thought. Studies investigating cohorts of patients with uncontrolled diabetes have shown a prevalence of Cushing syndrome between 2% and 3% (Leibowitz *et al.*, 1996; Catargi *et al.*, 2003), even though not confirmed in other studies (Mullan *et al.*, 2010; Reimondo *et al.*, 2007; Newsome *et al.*, 2008; Gagliardu *et al.*, 2010). However, a systematic screening for Cushing syndrome in all patients with diabetes is not currently recommended.

The clinical presentation of Cushing syndrome is characterized by typical signs and symptoms related to prolonged cortisol exposure. The recognition of Cushing syndrome may be extremely difficult, because some features of the syndrome are unspecific and may be common in a large number of individuals. Sarcopenic obesity, hypertension, alteration of the bone mineral density, altered glucose metabolism, or depression, in isolation, do not necessarily characterize the clinical picture of Cushing syndrome. However, the simultaneous presence of those features or other comorbidities potentially associated with hypercortisolism raises the suspicion of Cushing syndrome. According to the results of a recent study, the ability of the physicians in distinguishing the clinical picture of Cushing syndrome from that of the so-called pseudo-Cushing syndrome have changed during the last 20 years, mainly because of the increasing knowledge of the clinical phenotype of hypercortisolism, leading to an early recognition of the disease (Schneider *et al.*, 2012). The typical clinical picture of Cushing syndrome is characterized by severe comorbidities and adverse events, due to the direct effects of hypercortisolism. The glucocorticoid receptor is expressed among almost all tissues of the human body and the effects of cortisol hypersecretion involve many organ systems. Classic signs and symptoms include easy bruising, facial plethora, proximal muscle weakness, and striae rubrae (Nieman *et al.*, 2008). Hypertension is diagnosed in up to 80% of the patients, with loss of nocturnal dipping. Drug-resistant hypertension is encountered in 17% of the cases (Magiakou *et al.*, 2006; Arnaldi *et al.*, 2004; Zacharieva *et al.*, 2004). Alterations of glucose and lipid metabolism are also common findings in patients with Cushing syndrome and diabetes is diagnosed in 22%–47% of the cases. Other clinical manifestations include centripetal obesity, due to increased visceral fat accumulation, oligo/amenorrhea, osteoporosis, mood disorders (e.g., depression or psychosis in severe hypercortisolism), hypercoagulable state, and recurrent infections. The clinical picture of patients affected by Cushing syndrome due to unilateral adrenocortical adenomas is characterized by elevated cortisol levels without increase in androgen secretion, as shown by reduction in DHEA-sulfate levels. The reduced androgen secretion is caused by alteration of the electron transport system due to decreased activity of P450 oxidoreductase and its cofactor cytochrome *b*5, leading to reduced 17,20 lyase activity of the enzyme P450c17 (Sakai *et al.*, 1994). Additionally, the suppression of ACTH secretion observed in patient with Cushing syndrome due to adrenocortical adenomas results in a reduced stimulation of adjacent and contralateral adrenal cortex, eventually leading to reduced androgen production. This specific pattern of androgen reduction is in sharp contrast with the one observed in patients with adrenocortical carcinomas associated with Cushing syndrome, who show often hirsutism and hyperandrogenemia.

Those comorbidities cause a severe impairment of the cardiovascular profile of patients with Cushing syndrome, with an increased incidence of cardiovascular events and mortality, if left untreated (Trementino *et al.*, 2010; Lindholm *et al.*, 2001). Moreover, there is an increased rate of severe infectious complications, increased incidence of osteoporotic fractures, and psychiatric disorders (Arnaldi *et al.*, 2012; Colao *et al.*, 2012).

Highlights on Diagnosis

Patients presenting the typical clinical features described above should be screened by hormonal testing to rule out Cushing syndrome. Moreover, patients with unusual findings for their age, such as osteoporosis or hypertension in young adults, or severe comorbidities potentially associated with hypercortisolism should undergo a hormonal screening. After exclusion of iatrogenic Cushing syndrome by an accurate anamnesis, patients should undergo one of the three first-line screening tests: overnight 1 mg dexamethasone suppression test (DST), late-night salivary cortisol, or 24-h urinary free cortisol (UFC) (Nieman *et al.*, 2008). Cushing syndrome is diagnosed in the presence of at least two different clearly altered tests. Cortisol levels after 1 mg DST <50 nmol L^{-1} (1.8 mcg dL^{-1}) are considered adequately suppressed and exclude Cushing syndrome. UFC levels should be clearly elevated (e.g., threefold above the upper limit of normal) to have a reliable diagnostic power in identifying Cushing syndrome. Once Cushing syndrome is diagnosed, hormonal tests and imaging should be performed to investigate the cause of hypercortisolism. Plasma morning ACTH concentration <1.1 pmol L^{-1} (5 pg mL^{-1}) provides evidence of ACTH-independent hypercortisolism and requires thin-section computed tomography (CT) scan of the abdomen, to investigate the morphology of the adrenal gland. Adrenocortical adenomas are identified in the presence of regular margins, maximum diameter usually <4 cm, and homogeneity of the lesion. The majority of adrenocortical adenomas (70%) contains significant amount of intracellular lipids (Boland *et al.*, 2008; Garrett *et al.*, 2016). Attenuation values ≤ 10 Hounsfield units (HU) on unenhanced CT identify lipid-rich adenomas with 98% specificity and 71% sensitivity (Boland *et al.*, 1998). Unenhanced images followed by venous and delayed phases (70–80 s and 15 min after iodine contrast administration, respectively) provide a better discriminant power in distinguish adenomas from nonadenomatous adrenal lesions (Boland *et al.*, 2008; Korobkin *et al.*, 1998; Caoili *et al.*, 2000). This technique is also useful for the identification of lipid-poor adrenal adenomas, which are typically characterized by high attenuation values at unenhanced CT scan (>10 HU). Adrenocortical adenomas have a faster contrast washout at 15 min than other types of adrenal lesions, including malignant tumors, which usually show poor contrast washout due to capillary leakage (Pena *et al.*, 2000). FDG-PET or FDG-PET/CT scan may be useful in difficult cases, as an aid in distinguishing adrenal adenomas from small adrenocortical carcinomas, with 97% sensitivity and 91% specificity (Boland *et al.*, 2011).

Pathophysiology

Genetic and Molecular Background

The genetic and molecular background of Cushing syndrome due to adrenocortical tumors has remained largely unknown until recently. Lately, several studies have thoroughly investigated the pathogenetic mechanisms of those tumor entities, thanks to the application of next-generation sequencing techniques. Therefore, it has been possible to unravel the molecular mechanisms underlying tumor progression and cortisol production in more than half of the cases. One of the major intracellular pathways involved in the pathogenesis of adrenocortical tumors associated with hypercortisolism is the cAMP/PKA pathway. However, several other intracellular signaling pathways seem to play a role in a nonnegligible proportion of tumors.

The cAMP/PKA pathway in adrenocortical cells

The cyclic AMP (cAMP) signaling pathway is a pivotal regulator of metabolism, cell proliferation, differentiation, and apoptosis in endocrine tissues. The discovery of this intracellular signaling pathway has been a milestone in cell biology, as highlighted by the assignment of Nobel prizes to Earl Sutherland for the discovery of cAMP (1971), Edmond Fischer and Edwin Krebs for the discovery of PKA (1992) and Alfred Gilman and Martin Rodbell for the discovery of G proteins (1994) (Chin *et al.*, 2002). One of the major effectors of cAMP is protein kinase A (PKA), which phosphorylates several cytosolic and nuclear substrates, including transcription factors of the CREB/ATF family (Rosenberg *et al.*, 2002). cAMP levels are tightly balanced in production and degradation by adenylyl cyclases and phosphodiesterases (PDEs), respectively (Hannah-Shmouni *et al.*, 2016). The second messengers cAMP and PKA are key regulators of almost all cellular functions and mediate the effects of several hormones and neurotransmitters via G protein-coupled receptors (GPCRs).

Under normal conditions, ACTH controls activity and proliferation of adrenocortical cells through the melanocortin 2 receptor (MC2R), a GPCR highly expressed on the surface of those cells. Upon binding to the MC2R, the stimulatory Gs protein leads to the activation of the adenylyl cyclase, which, in turn, increases the intracellular concentration of cAMP. The resulting stimulation of PKA ultimately leads to increased glucocorticoid production. PKA is a serine/threonine kinase and a typical model of allosteric regulation (Taylor *et al.*, 2012). In its inactive form, the PKA holoenzyme forms a tetramer made by a dimer of two regulatory (R) and two catalytic (C) subunits (Taylor *et al.*, 2012). PKA has two isoforms (I and II) depending on which type of R subunits bind the C subunits (16, 17 Weigand Sci Rep). There are three isoforms of C subunits (Cα, Cβ, Cγ) and four of R subunits (RIα, RIβ, RIIα, RIIβ), each coded by different genes (Taylor *et al.*, 2012). Additionally, the human protein kinase X (PrKX) can associate with the R subunits of PKA (Diskar *et al.*, 2010). The R subunits form homo- or heterodimers through docking and dimerization domains (Taylor *et al.*, 2012; Taskén *et al.*, 1993), which are also involved in binding the A Kinase-Anchoring Proteins, tethering different PKA isoforms to specific subcellular structures (Taylor *et al.*, 2012). In the inactive state, an inhibitory sequence of the R subunit occupies the active site cleft of the corresponding C subunit, impeding the access of PKA substrates by acting as a tethered substrate or pseudo-substrate. The binding of two cAMP molecules to each R subunit generates a conformational change that

allows the release of the C subunit, which, in turn, is able to phosphorylate cytosolic and nuclear targets (Kim *et al.*, 2005). The C subunit activation in adrenocortical cells activates the glucocorticoid synthesis machinery regulating the fast as well as the slow long-term steroid production, by inducing transcription of steroidogenic enzymes and genes involved in cell replication (Gallo-Payet and Payet, 2003).

Several genetic alterations in components of the cAMP/PKA pathway point toward the pivotal role of this intracellular signaling in inducing tumor progression and cortisol hypersecretion in several familial cases of adrenocortical hyperplasia, as well as in sporadic adrenocortical tumors (Calebiro *et al.*, 2015).

Genetic alterations in adrenal Cushing syndrome

PRKACA *somatic mutations*

Whole exome sequencing studies performed in sporadic cortisol-producing adrenocortical adenomas have highlighted several somatic mutations in *PRKACA*, the gene encoding the Cα subunit of PKA (Beuschlein *et al.*, 2014; Cao *et al.*, 2014; Goh *et al.*, 2014; Sato *et al.*, 2014). Replication studies using Sanger sequencing have confirmed the results of the exome sequencing (Di Dalmazi et al., 2014a; Nakajima *et al.*, 2014; Thiel *et al.*, 2015; Li *et al.*, 2016). The prevalence of *PRKACA* somatic mutations identified in studies published up to now are summarized in **Fig. 1**. Somatic mutations in *PRKACA* are found in 23%–66% of patients with Cushing syndrome due to sporadic adrenocortical tumors, with a mean pooled prevalence of 47% in all series published up to now (Di Dalmazi and Beuschlein, 2017). The studies reporting results of the genetic analysis of sporadic adrenocortical tumors and the list of the specific mutations are shown in **Table 1**. *PRKACA* somatic mutations have been identified selectively in adrenocortical unilateral adenomas associated with clinically-manifest Cushing syndrome, whereas they have been identified only in a small proportion of patients with subclinical hypercortisolism (0%–12%, mean pooled prevalence 5%). All somatic mutations are clustered in a hot-spot region of the coding sequence of *PRKACA*. The most common alteration is a missense mutation leading to the substitution of a leucine residue at position 206 with an arginine in Cα subunit (p.L206R). Additional somatic alterations identified in sporadic adrenocortical tumors associated with Cushing syndrome include mutations leading to the insertion of a valine between the amino acids 200 and 201 (p.C200_G201insV), the insertion of a tryptophan between the amino acids 199 and 200 (p.L199_C200insW), and the substitution of the serine in position 213 with an arginine, in addition to a large amino acid insertion (p.S213R + p.L212_K214insI-I-L-R).

Mechanism of PKA activation by PRKACA somatic mutations

All the amino acid changes caused by *PRKACA* somatic mutations are located on the surface of the Cα subunit, at the interface with the R subunit of PKA. The solved X-ray crystal structure of the mouse RIIβ–Cα-holoenzyme (Kim *et al.*, 2005) showed that somatic *PRKACA* mutations may interfere with the association between Cα subunit and R subunit, resulting in a constitutive activation of the former (Beuschlein *et al.*, 2014; Di Dalmazi et al., 2014a; Calebiro *et al.*, 2014). The in silico 3D reconstruction of the crystal structure of the mouse RIIβ–Cα-holoenzyme in wild type and mutants is depicted in **Fig. 2**.

L206 is part of the active site cleft of the Cα subunit of PKA. It takes part in the formation of a hydrophobic pocket that is responsible for substrate recognition, binding, and interaction with the inhibitory sequence of the R subunit. The substitution of L206 with a bulky and positively charged amino acid like arginine leads to steric hindrance between the side chain of R206 and residues V115 and Y228 of the R subunit (Beuschlein *et al.*, 2014; Calebiro *et al.*, 2014).

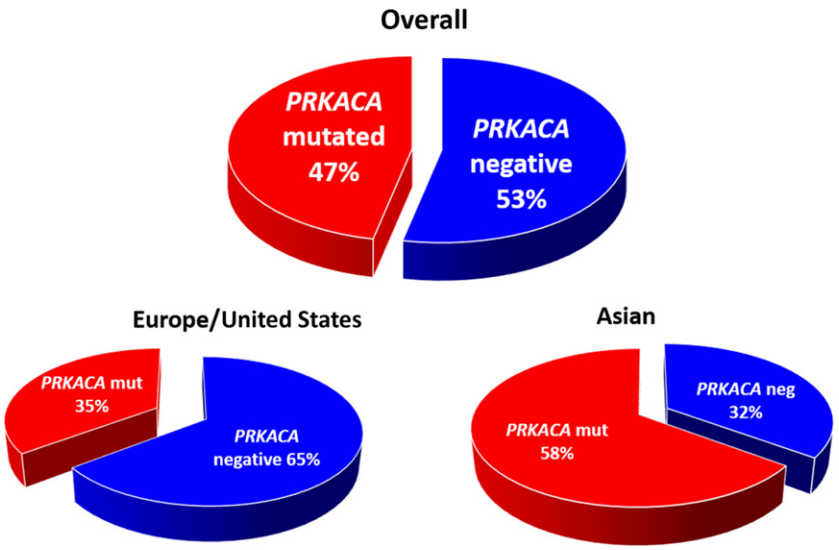

Fig. 1 Prevalence of PRKACA somatic mutations in all series published until 2017.

Table 1 Prevalence of *PRKACA* somatic mutations and amino acid changes in sporadic unilateral adrenocortical tumors associated with Cushing's syndrome

Reference	*Definition of Cushing's syndrome*	*Prevalence of* PRKACA *mutations*	*Amino acid change*
Beuschlein *et al.* (2014)	Three abnormal tests or clinical signs/symptoms of Cushing's syndrome + two abnormal tests among: – elevated urinary free cortisol – increased midnight cortisol – ACTH <2.2 pmol L^{-1} – cortisol after 1 mg DST >138 nmol L^{-1}	22/59 (37%)[a]	p.L206R
Cao *et al.* (2014)	According to Endocrine Society guidelines	57/87 (65.5%)[b]	p.L206R
Sato *et al.* (2014)	According to Endocrine Society guidelines	33/55 (60%)[c]	p.L206R
Goh *et al.* (2014)	According to Endocrine Society guidelines	10/29 (35%)[d]	p.L206R
Di Dalmazi *et al.* (2014a)	Three abnormal tests or clinical signs/symptoms of Cushing's syndrome + two abnormal tests among: – elevated urinary free cortisol – increased midnight cortisol – ACTH <2.2 pmol L^{-1} – cortisol after 1 mg DST >138 nmol L^{-1}	22/64 (34%)	p.L206R (18/22) p.C200_G201insV (3/22)[f] p.S213R + p.L212_K214insI-I-L-R (1/22)[f]
Nakajima *et al.* (2014)	According to Endocrine Society guidelines	3/13 (23%)	p.L206R
Thiel *et al.* (2015)	Three abnormal tests or clinical signs/symptoms of Cushing's syndrome + two abnormal tests among: – elevated urinary free cortisol – increased midnight cortisol – ACTH <2.2 pmol L^{-1} – cortisol after 1–2 mg DST >138 nmol L^{-1}	11/32 (34%)[e]	p.L206R
Li *et al.* (2016)	Three abnormal tests or clinical signs/symptoms of Cushing's syndrome + two abnormal tests among: – UFC >500 nmol L^{-1} – MSC >166 nmol L^{-1} – ACTH <1.1 pmol L^{-1} – cortisol after 4 mg DST >50 nmol L^{-1}	21/40 (52.5%)	p.L206R (21/23) p.C200_G201insV (2/23)

[a]8/10 by exome sequencing.
[b]27/39 by exome sequencing.
[c]4/8 by exome sequencing; 1 not known if CS or SH.
[d]3/6 by exome sequencing.
[e]6 patients already included in the series reported by Goh *et al.*
[f]Evidence for pathogenetic role of mutation from in silico analysis.
DST: Dexamethasone suppression test.

L199 and C200 are close to T198, which is part of the so-called activation loop. This region is parallel to the inhibitory sequence of the R subunit and is phosphorylated during the synthesis of the Cα subunit (Taylor *et al.*, 2012). Residues G201 and L199 are involved in main-chain hydrogen bonding with residues V115 and A117 of the R subunit. The insertion of an amino acid in this region interferes with the interaction between the R and Cα subunits (Beuschlein *et al.*, 2014; Calebiro *et al.*, 2014).

3D in silico reconstruction of p.S213R + p.L212_K214insI-I-L-R predicted a functional role also for this variant. Even though this region is not part of any direct contact site between Cα and R subunits, this mutation may interfere with the formation of a stable PKA holoenzyme. L212 is located on the surface of the Cα subunit in a "tip"-like structure that protrudes to the R subunit. The insertion of several amino acids in this region leads to an enlargement of this tip, impairing the association between Cα and R subunits (Di Dalmazi et al., 2014a).

Functional analyses have been performed for the most frequent mutation, the p.L206R, and for the variant p.C200_G201insV. In vitro experiments performed by coimmunoprecipitation assays, chromatography, and real-time fluorescence resonance energy

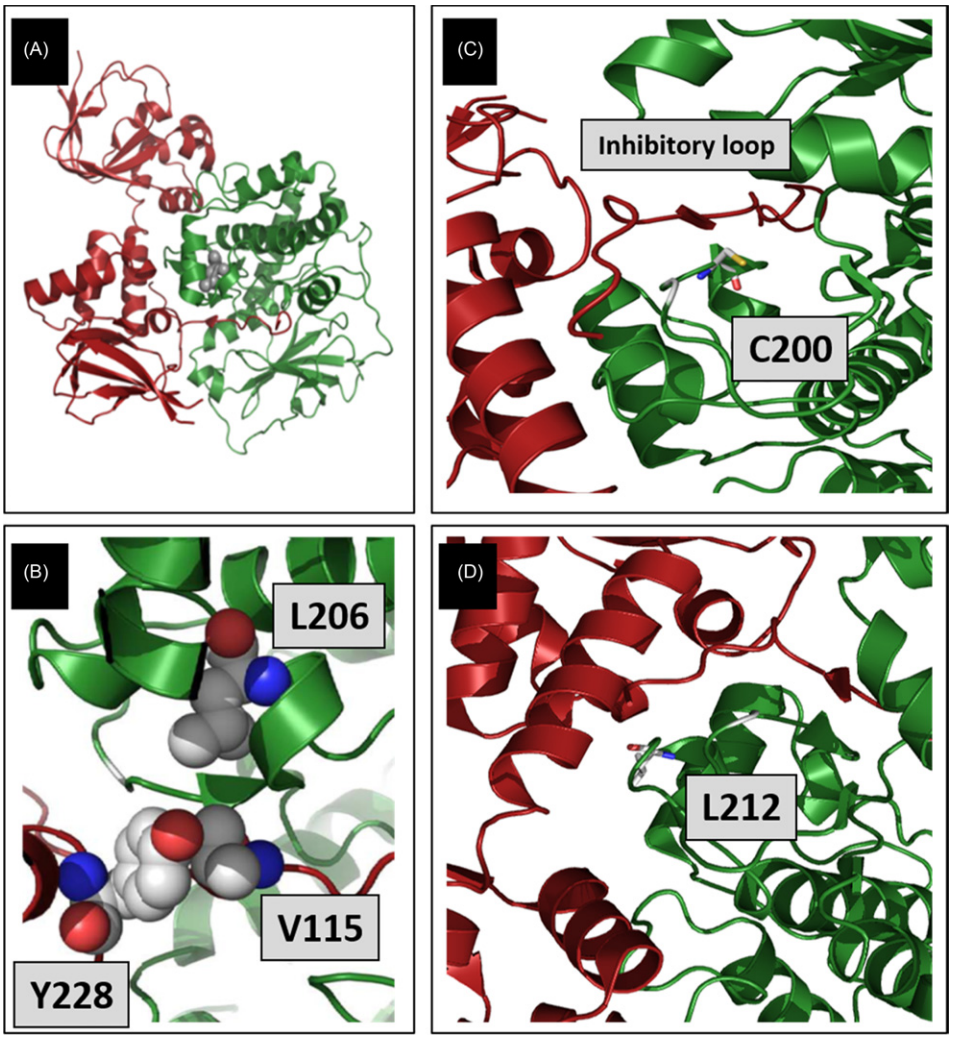

Fig. 2 3D–in silico reconstruction of the mouse RIIβ–Cα-holoenzyme with analysis of the amino acid changes in the catalytic subunit of PKA. (A) Wild-type PKA assembly with the regulatory subunit in *red* and the catalytic subunit in *green*. Leucine 206 is depicted in *gray*. The remaining boxes show the enlargement of the image in A, showing the position of leucine 206 (B), cysteine 200 (C), and leucine 212.

transfer (FRET) measurements showed that both mutations abolished the association between Cα mutants and the R subunits. When compared to wild-type Cα subunit, mutant cells showed higher PKA activity, which was independent of the cAMP concentration. The maximal PKA activity of both mutants was also not different from that of the wild-type Cα subunit. According to the results of FRET experiments in living cells, both mutations impaired the formation of a stable PKA holoenzyme, causing a loss of regulation by cAMP (Calebiro *et al.*, 2014). All these experiments provide an explanation for the constitutive activation of PKA caused by *PRKACA* somatic mutations in adrenocortical tumors associated with Cushing syndrome.

Other somatic mutations

Several studies have identified different somatic alterations in components of the cAMP/PKA pathway, supporting the primary role of this intracellular signaling in cortisol hypersecretion and tumor progression (Lodish and Stratakis, 2016).

One of the first genetic aberrations was found in patients affected by adrenal hyperplasia in McCune–Albright syndrome, which is characterized by bone fibrous dysplasia, café au-lait spots on the skin, and involvement of several endocrine glands. Some patients also develop Cushing syndrome due to adrenal hyperplasia. Genetic analysis revealed mosaic, activating mutations of *GNAS*, the gene encoding the Gαs protein (Weinstein *et al.*, 1991). Additional genetic defects were found in patients with primary pigmented nodular adrenocortical disease in the context of Carney complex, characterized by cutaneous and neuronal tumors, cardiac myxomas, and pigmented lesions of skin and mucosae. Mutations in the gene encoding the RIα subunit of PKA (*PRKAR1A*) have been considered as a causative factor of the syndrome, by reducing the expression of the RIα subunits or impairing its association with Cα subunits (Kirschner *et al.*, 2000). Inactivating mutations in the two genes encoding phosphodiesterases 8 and 11A (*PDE8* and *PDE11A*) have been found in patients with adrenal hyperplasia (Horvath *et al.*, 2006; Rothenbuhler *et al.*, 2012).

The analysis of sporadic adenomas associated with Cushing syndrome has also revealed somatic mutations of *GNAS* (Cao *et al.*, 2014; Goh *et al.*, 2014; Sato *et al.*, 2014; Ronchi *et al.*, 2016, Fragoso *et al.*, 2003), *PDE8B*, and *PDE11A* (Rothenbuhler *et al.*, 2012; Hannah-Shmouni *et al.*, 2016). Somatic loss of 17q22–24 have been found in 23% of adrenocortical adenomas (Bertherat *et al.*, 2003) and inactivating mutations of the *PRKAR1A* gene were identified in a few number of sporadic tumors (49 Calebiro EJE). *PRKAR1A* mutations were predicted to cause a prematurely terminated R1α subunit and were considered as pathogenetic.

Apart from all alterations of the cAMP/PKA pathway described in sporadic adrenocortical tumors so far, with somatic mutations of *PRKACA* as the most frequent event, several different intracellular pathways have been identified as potentially pathogenetic for tumor progression and cortisol hypersecretion. Alterations of components of the Wnt/β-catenin pathway have been described in one fourth of the patients with adrenocortical adenomas. The most common alterations are somatic mutations of *CTNNB1*, the gene encoding β-catenin (Tissier *et al.*, 2005; Thiel *et al.*, 2015; Ronchi *et al.*, 2016). A specific analysis performed by exome sequencing revealed a prevalence of 18% of *CTNNB1* somatic mutations in adrenocortical adenomas associated with Cushing syndrome (Ronchi *et al.*, 2016). Most of *CTNNB1* mutations occur in a hot spot region, leading to amino acid substitutions in the serine in position 45 with a proline or a phenylalanine residue (p.S45P and p.S45F). These amino acid changes cause a constitutive activation of the Wnt/β-catenin pathway, a well-known intracellular signaling involved in cell proliferation in benign and malignant adrenocortical tumors. Besides the cAMP/PKA pathway and the Wnt/β-catenin pathway, the genetic background of sporadic adrenocortical tumors associated with Cushing syndrome is highly heterogeneous. The analysis of *PRKACA* wild-type tumors performed by exome sequencing revealed several somatic "private" mutations in different genes, without recurrent events (Ronchi *et al.*, 2016). However, the cluster analysis performed by combining mutational status and gene expression data revealed that mutations of components of the intracellular Ca^{2+}-signaling might play a role in adrenocortical tumorigenesis and cortisol hypersecretion. However, functional data proving the pathogenetic consequences of these variants are still lacking.

Aberrant receptors in adrenal Cushing syndrome

Hypercortisolism in adrenocortical adenomas may be caused by aberrant receptors in a nonnegligible proportion of patients. Aberrant receptors, usually GPCR, may be ectopic receptors that are not significantly expressed in the normal zona fasciculata or eutopic receptors that are highly expressed in adrenocortical tumors. Ectopic receptors include those for glucose-dependent insulinotropic polypeptide (or gastric inhibitory polypeptide; GIP), vasopressin (V_2–V_3 vasopressin receptors), serotonin, glucagon, and β-adrenergic receptors, whereas V1-vasopressin receptor and LH/HCG receptor are listed among eutopic receptors associated with hypercortisolism (El Ghorayeb *et al.*, 2015). Aberrant receptors have been found in up to 50% of the patients with unilateral adrenocortical adenomas associated with hypercortisolism, even though the prevalence of this disorder is higher in patients with subclinical hypercortisolism. Recent findings have shown that the increased GIP receptor expression in adrenocortical adenomas associated with food-dependent Cushing syndrome is driven by monoallelic overexpression of the *GIPR* gene, caused by chromosome rearrangements and other genetic alterations of the *GIPR* regulatory regions (Lecoq *et al.*, 2017).

Genotype–Phenotype Correlation

Keeping into account the deleterious effects of *PRKACA* somatic mutations on Cα subunit, which is constitutively active, the phenotype of patients carrying the mutations is expected to be more severe than that of subjects without mutations. Patients carrying somatic *PRKACA* mutations have a severe hypercortisolism, as demonstrated by the higher cortisol levels after DST than in wild-type patients. Moreover, 24-h free cortisol and midnight serum and salivary cortisol are higher in *PRKACA*-mutated patients (Beuschlein *et al.*, 2014, Sato *et al.*, 2014, Di Dalmazi et al., 2014a, Thiel *et al.*, 2015, Li *et al.*, 2016). PKA activity has been shown to play a role also in cell proliferation. Interestingly, although patients carrying *PRKACA* mutations have a higher cortisol secretion rate, the tumor size is smaller than their wild-type counterpart. The increased PKA activity associated with a smaller tumor size has been also observed in patients carrying *PRKAR1A* inactivating mutations (Bertherat *et al.*, 2003). The association between increased PKA activity and lower cell proliferation rate is still unexplained. Early studies have shown that the imbalance of RI/RII ratio in adrenocortical cells regulates apoptosis and stimulates, when increased, or inhibit, when decreased, cell proliferation (Mantovani *et al.*, 2008). This mechanism might explain the lower proliferation rate observed in *PRKAR1A* inactivating mutations. A recent study has also demonstrated that tumors carrying *PRKACA* mutations have reduced expression of the RIIβ subunit (Weigand *et al.*, 2017). Nevertheless, although this mechanism may provide a pathogenetic explanation for the reduced cell proliferation in cAMP/PKA altered tumors, the smaller size of tumors carrying *PRKACA* mutations might also be explained by the severity of the hormonal phenotype in patients with the mutations, who come earlier to medical attention, leading to timely clinical recognition and tumor removal. The younger age of patients with somatic *PRKACA* mutations strengthens this hypothesis. The prevalence of *PRKACA* mutations has shown some variability according to the different cohorts of patients (**Fig. 1**). Data from cohort of patients in Europe and United States highlight a mean pooled prevalence of 35%, consistent among all studies. The prevalence of *PRKACA* somatic mutations in patients form Asian regions is higher, being $>50\%$ in all but one study, with a mean pooled prevalence of 59% (Di Dalmazi and Beuschlein, 2017). The reason for this discrepancy is currently unknown and there is no evidence of genetic, environmental, or other factors that could explain the higher rate of *PRKACA* somatic mutations in Asian cohorts of patients. However, the possibility of a referral or enrollment bias among different cohorts of patients has not been investigated. Somatic

mutations in *PRKACA* are more frequent in females than in males. The reason for this specific gender distribution is not completely understood, and no studies have addressed this issue so far.

According to the results of the aforementioned studies, patients with somatic *PRKACA* mutations are mostly females, at young age, with severe hypercortisolism and full-blown Cushing syndrome, with relatively small adrenocortical tumors. This specific clinical picture seems to be common to all patients bearing mutations of components of the cAMP/PKA pathway. On the contrary, the phenotype of patients carrying somatic mutations of components of the Wnt/β-catenin pathway, mainly *CTNNB1*, is in strong opposition and it is characterized by large tumors, in older patients affected by Cushing syndrome with low clinical expression. The clinical phenotype of patients with genetic alterations involving pathways other than cAMP/PKA or Wnt/β-catenin (notably, alterations of Ca^{2+} signaling) is not associated with any specific characteristic (Ronchi *et al.*, 2016).

In summary, the clinical spectrum of Cushing syndrome due to adrenocortical adenomas is highly variable among patients. However, some phenotypic characteristics are associated with specific genetic and molecular background.

Treatment

Surgical Approach

The treatment of Cushing syndrome due to unilateral adrenal adenomas is surgical. The removal of the adenoma is virtually curative in all patients, considering that those tumors are monoclonal and that specific molecular events occurring at a somatic level have been identified as a causative factor for tumor proliferation and cortisol hypersecretion.

Firstly introduced in the early 1990s, laparoscopic surgery has become the recommended approach, thanks to lower rate of complications, shorter hospital stay, faster return to normal activity, and reduced postoperative pain than traditional open adrenalectomy (Shen *et al.*, 2005). Laparoscopic adrenalectomy may be performed through either a trans-peritoneal or a retro-peritoneal approach, depending on the experience of the surgeon. Robot-assisted laparoscopic adrenalectomy was firstly described in the late 1990s. Since then, this technique has shown safety and efficacy similar to laparoscopic adrenalectomy and it has slowly gained in popularity, thanks to the potential advantage of shorter length of hospitalization and less blood loss during surgical intervention (Brandao *et al.*, 2014). However, the higher cost represents the main limitation to the application of this technique. Stress-dose glucocorticoid supplementation (e.g. 100 mg hydrocortisone intravenously every 8 h) should be given in the perio-perative period and tapered postoperatively.

Post-Operative Adrenal Insufficiency

Adrenalectomy in unilateral adrenocortical adenomas associated with Cushing syndrome invariably results in adrenocortical insufficiency, requiring glucocorticoid replacement therapy. The adrenal insufficiency is caused by the suppression of CRH-producing cells in the hypothalamus, the inhibition of the secretion of stored ACTH, and the reduced transcription of the POMC gene, as a consequence of the chronic glucocorticoid excess.

In all studies published since 1981, the mean pooled prevalence of adrenal insufficiency after adrenalectomy is 99.7% (100% in all but one study that reported a prevalence of 80%) (Di Dalmazi et al., 2014b). The recommended dose of glucocorticoids as a replacement therapy is 10–12 $mg^{-1}\ m^{-2}\ day^{-1}$ of hydrocortisone given twice or thrice daily (Nieman *et al.*, 2015). The first dose should be given as soon as the patient wakes up. Other synthetic glucocorticoids may be given at the lowest replacement dose when hydrocortisone is not available. Glucocorticoid replacement therapy should be tapered according to the results of hormonal testing and the clinical judgment until hypothalamus–pituitary–adrenal (HPA) axis recovers its normal activity.

The mean prevalence of recovery of the HPA axis in patients with adrenal Cushing syndrome reported in the main series published up to now is 92%. Seven studies reported a total mean prevalence of 8% of patients who did not recover eucortisolism in a period of 69–103 months (Di Dalmazi et al., 2014b). There is no standardized definition of recovery of HPA axis after adrenalectomy, leading to high heterogeneity among studies. Most of the studies did not perform any hormonal test to define recovery, relying on clinical evaluation of the patients, whereas one-fourth of the studies performed short Synacthen test (250 mcg), with cut-off for normal response of cortisol after 60 min at 500 or 550 nmol L^{-1}, or morning cortisol >165 nmol L^{-1}. The most recent guidelines suggest to investigate the HPA axis by assessing morning cortisol level every 3 months, followed by an ACTH stimulation test when cortisol level is $\geq 7.4\ \mu g^{-1}\ dL^{-1}$ (200 nmol L^{-1}). Recovery can be considered when baseline or stimulated cortisol levels are $\geq 18\ \mu g^{-1}\ dL^{-1}$ (500 nmol L^{-1}).

The mean time to recovery a normal HPA axis function after adrenalectomy for Cushing syndrome in patients with unilateral adrenocortical adenomas is 18 months (range 1–30 months in the main series published up to now) (Di Dalmazi et al., 2014b; Berr *et al.*, 2015). In a study comparing different subtypes of hypercortisolism, patients with adrenal Cushing syndrome showed the longer time of HPA axis recovery, followed by patients with Cushing disease and those with ectopic Cushing syndrome, the latter showing the fastest return to eucortisolism despite similar dose of glucocorticoid replacement therapy (Berr *et al.*, 2015).

The recovery of HPA axis function is a stepwise process that involves the hypothalamus and the pituitary in the very early postsurgical period and adrenocortical cells in a later time. The phases of recovery of adrenocortical function in patients under-going adrenalectomy for Cushing syndrome due to unilateral adenomas can be summarized in an early phase of hypothalamic and adrenal insufficiency, followed by the recovery of the ACTH secretion, which rises to supernormal levels, and the subsequent

restoration of normal glucocorticoid secretion (Graber *et al.*, 1965; Bertagna and Orth, 1981; Gordon *et al.*, 1987; Klose *et al.*, 2004). This specific pattern of recovery seems to be independent of the cause of hypercortisolism and it was also observed in patients with iatrogenic Cushing syndrome after withdrawal of glucocorticoid treatment. The low expression of ACTH-receptor mRNA observed in peritumoral adrenocortical cells of patients operated for Cushing syndrome might be involved in the process of HPA axis recovery (Imai *et al.*, 2001).

Several patients suffer from glucocorticoid withdrawal syndrome, despite glucocorticoid replacement therapy. The clinical picture of the syndrome is characterized by symptoms like anorexia, nausea, weight loss, fatigue, myalgias, and psychiatric disorders (Dorn *et al.*, 1997) that can improve after a temporary increase in the dosage of glucocorticoid replacement therapy. However, no evidence has proven that supra-physiological doses of glucocorticoids in the early postoperative period might limit the onset of glucocorticoid withdrawal syndrome.

Patients undergoing unilateral adrenalectomy for sporadic adrenal adenomas associated with Cushing syndrome should be considered as cured by surgery, once the diagnosis has been confirmed by pathological examination.

Conclusion

Unilateral adrenocortical adenomas are the most frequent pathological condition among ACTH-independent forms of Cushing syndrome. During the last years, the pathogenesis of sporadic adrenocortical tumors associated with the syndrome has been clarified for most of the cases. Thanks to the application of next generation sequencing techniques, *PRKACA* somatic mutations could have been identified as the most common pathogenetic cause, occurring in more than half of the patients with Cushing syndrome. The discovery of the molecular events leading to tumors formations and cortisol hypersecretion has broadened the understanding of the pathogenesis of the disease, with future implications for diagnosis and treatment strategies. Laparoscopic adrenalectomy represents the best treatment option for patients affected by Cushing syndrome due to unilateral adrenocortical adenomas. The surgical treatment is invariably followed by adrenal insufficiency, which requires glucocorticoid replacement therapy for a variable period. Postoperative hormonal assessment should be performed at regular intervals to assess HPA axis function and avoid unnecessary glucocorticoid treatment. Once the diagnosis of unilateral adrenocortical adenoma is confirmed by histology, the patients should be considered as cured and no hormonal reassessment is required.

See also: Cushing Syndrome; Screening and Differential Diagnosis

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Somatic β-Catenin Gene Mutations and WNT Signaling in Adrenal Tumors

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Abbreviations

ACC Adrenocortical cancer
AA Adrenocortical adenoma
APA Aldosterone producing adenoma
APC Adenomatous polyposis coli
ARMC5 Armadillo repeat containing 5 (*ARMC5*)
CK1α Casein kinase1α
CTNNB1 Catenin beta 1 or beta-catenin gene
Dvl Disheveled
FAP Familial adenomatous polyposis
GnRH Gonadotropin-releasing hormone
GSK3 *β* Glycogen synthase kinase3*β*
LDL Low-density lipoprotein
LEF Lymphoid enhancer-binding factor
LHCG Luteinizing hormone chorionic gonadotropin
NGS Next-generation sequencing
PKA Protein kinase A
PPNAD Primary pigmented nodular adrenal disease
TCF T-cell specific factor
TLE-1 Transducin likeenhancer-1
TP53 Tumor protein 53
WNT Wingless

Dysregulation of canonical Wnt/beta-catenin signaling has been associated with various diseases and cancers (Zhan *et al.*, 2017). Wnt/beta-catenin pathway plays a role on both embryonic development of the adrenal cortex and in maintenance of the adult normal adrenal glands (Kim *et al.*, 2008). Moreover, in the last decade Wnt/beta-catenin signaling was recognized as one of the major key player in adrenocortical tumorigenesis (Mazzuco *et al.*, 2012) which is the focus of this article.

Wnt Signaling Pathway

Wnt signaling is characterized by two distinct cascades: the noncanonical and the canonical pathways. The canonical Wnt pathway named Wnt/beta-catenin operates via beta-catenin protein. The two other noncanonical pathways include the planar cell polarity pathway and the Wnt/calcium pathway and both do not require beta-catenin protein. This article focuses on the involvement of the canonical Wnt/beta-catenin signaling in adrenocortical tumorigenesis.

In the absence of a Wnt signal, cytoplasmic beta-catenin is associated with a destruction complex composed of APC, axin, and 2 serine/threonine kinases: the casein kinase1 (CK1) and the glycogen synthase kinase3*β* (GSK3 *β*) (MacDonald *et al.*, 2009). CK1 and GSK3*β* phosphorylate the N-terminal of beta-catenin. First, CK1 phosphorylates Ser45 which allow the GSK3 *β*-sequential phosphorylation of Thr41, Ser37, and Ser 33 (Liu *et al.*, 2002). Phosphorylated beta-catenin is recognized by the E3 ubiquitin ligase beta-TrCP which lead to its ubiquitination and proteasomal degradation maintaining low cytoplasmic level of beta-catenin (Kimelman and Xu, 2006).

However, the pathway is activated when the secreted Wnt ligands bind to their receptor complex including the frizzled receptor and its coreceptor the low-density lipoprotein (LDL) receptor LRP5/6. This binding leads to phosphorylation of disheveled (Dvl) which in turn recruit Axin and GSK3 adjacent to the plasma membrane, thus preventing the formation of the destruction complex. The membrane sequestration of the destruction complex prevents phosphorylation and degradation of beta-catenin allowing beta-catenin to dissociate and accumulate in the cytoplasm and translocate to the nucleus.

In the nucleus, beta-catenin will bind to T-cell specific factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors to activate transcription of WNT target genes. In the absence of nuclear beta-catenin, TCF/LEF interact with the transcriptional corepressor transducin likeenhancer-1 (TLE-1), thus preventing beta-catenin target gene expression (**Fig. 1**) (Brantjes *et al.*, 2001).

Beta-catenin is composed of three distinct domains: the N-terminal domain, the C-terminal domain and the central armadillo domain repeats which bind various partners including Axin, APC and transcriptions factors. The C-terminal domain serves as a binding factor for several complexes promoting beta-catenin-mediated transcription while phosphorylation of the N-terminal domain leads to degradation of beta-catenin. Most mutations affecting the *CTNNB1* gene in adrenocortical tumors and in other nonadrenal cancers are localized in the exon 3 of the gene corresponding to beta-catenin N-terminal which harbors the phosphorylation sites for CK1 and GSK-3b. Thus *CTNNB1*mutations lead to disruption of the beta-catenin destruction complex allowing beta-catenin accumulation and Wnt activation (Dar *et al.*, 2017).

Beta-Catenin Mutations in Adrenocortical Adenomas and Carcinomas

One of the first evidence that Wnt/beta-catenin signaling was involved in the development of adrenal tumors was the identification of mutations in the beta-catenin gene itself. In 2005, we and others described for the first time the presence of beta-catenin

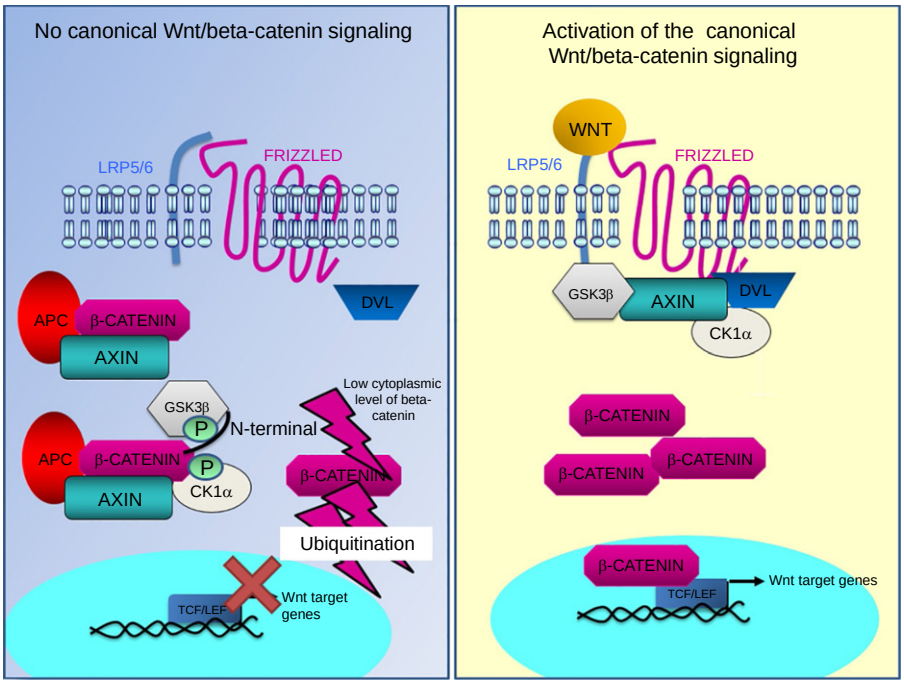

Fig. 1 Schematic representative of the canonical Wnt/beta-catenin signaling pathway. *Left panel*: *In the absence of a Wnt signal*, beta-catenin is associated with a destruction complex composed of APC, axin and two kinases; Casein Kinase1α (CK1α) and glycogen synthase kinase3β (GSK3 β). The kinases phosphorylate beta-catenin making it available for ubiquitination and maintaining its level low in the cytoplasm. *Right panel*: *The pathway is activated* when a Wnt ligand binds its frizzled receptor and its coreceptor LRP5/6. This complex associates with the disheveled protein that will prevent the formation of the destruction complex, avoiding beta-catenin phosphorylation and allowing beta-catenin to dissociate and accumulate in the cytoplasm and translocate to the nucleus. In the nucleus, beta-catenin will bind the T-cell factor (TCF)/Lymphoid enhancer factor LEF) transcription factors to activate Wnt target genes.

mutations in adrenocortical adenomas (AA) and adrenocortical carcinomas (ACC) (Tadjine *et al.*, 2005; Tissier *et al.*, 2005; Tadjine *et al.*, 2008a). Mutations of serine/theronine amino acids avoid beta-catenin degradation and leads to its cytoplasmic and nuclear accumulation and ultimately to upregulation of Wnt activity.

Since 2005, 7 large cohorts of patients with adrenocortical adenomas and/or carcinomas were screened for the presence of somatic *CTNNB1* mutations using direct sequencing (Tadjine *et al.*, 2005; Tadjine *et al.*, 2008a; Tissier *et al.*, 2005; Masi *et al.*, 2009; Gaujoux *et al.*, 2011; Bonnet *et al.*, 2011; Thiel *et al.*, 2015; Rubin *et al.*, 2016). Overall the prevalence of somatic *CTNNB1* mutations is of 25% in AA and ACC which is a high prevalence compared to other cancers such as colon cancer or breast cancer where the prevalence of *CTNNB1* mutations is of 4,7% and 0,2% respectively (Zhan *et al.*, 2017). Please note that these studies on adrenocortical tumors included mainly nonsecreting or cortisol-secreting tumors. **Table 1** summarizes the results of these seven studies.

Mutations in Other Components of the Wnt Signaling Pathway in Adrenocortical Tumors

An important observation is that beta-catenin protein accumulation is more frequent than *CTNNB1* mutations in adrenocortical adenomas and even more in adrenocortical carcinomas. For example, two studies found a prevalence of *CTNNB1* mutations of 27% (Tissier *et al.*, 2005) and 36% (Bonnet *et al.*, 2011) in adrenocortical adenomas, while beta-catenin protein accumulation was present with a prevalence of 39% and 59% respectively (Tissier *et al.*, 2005; Bonnet *et al.*, 2011). Similarly, *CTNNB1* mutations were found in 30,7% (Tissier *et al.*, 2005) and 18,4% (Gaujoux *et al.*, 2011) of ACC but beta-catenin accumulation was observed more frequently with a prevalence of 84,6% (Tissier *et al.*, 2005) and 44,8% (Gaujoux *et al.*, 2011). This suggests that defects in other components of the Wnt signaling pathway may lead to Wnt/beta-catenin pathway activation in adrenocortical tumorigenesis.

APC (adenomatous polyposis coli) which is part of the beta-catenin destruction complex was a candidate gene of the pathway. Inherited mutations of the *APC* gene are found in familial adenomatous polyposis (FAP). FAP is an autosomal dominant disease in which hundreds of colorectal adenomas develop early in life and may lead to colorectal cancer. Inactivation of the second allele of the *APC* gene conducts to stabilization of beta-catenin constitutively by impairing activity of the destruction complex. The incidence of adrenocortical tumors is increased in patients with FAP being two to four times more prevalent than in the general population (Marchesa *et al.*, 1997; Smith *et al.*, 2000). In addition, a germline *APC* mutation was found in a patient with BMAH (Hsiao *et al.*, 2009). Based on this data it was logical to exclude *APC* mutations in adrenocortical tumors. However, so far only

Table 1 Summary of larger cohorts of patients that were screened using direct sequencing for the presence of somatic *CTNNB1* mutations

	AA	*ACC*
Tissier et al. (2005)[a]	26,9% (7/26)	30,8% (4/13)
Tadjine et al. (2008a)	15% (5/33)	0% (0/4)
Masi et al. (2009)	19,5% (8/41)	20% (3/15)
Gaujoux et al. (2011)	NA	18,4% (9/49)
Bonnet et al. (2011)[a]	36% (36/100)	NA
Thiel et al. (2015)[a]	23,1% (12/52)	NA
Rubin et al. (2016)	18,3% (13/71)	20,1% (5/24)

[a]Excluding aldosterone-producing adenomas.
AA, Adrenocortical adenoma; ACC, Adrenocortical carcinoma.

silent *APC* mutations and polymorphisms were found in sporadic adrenocortical tumors (Gaujoux *et al.*, 2010). One other candidate was the Wnt signaling inhibitor *AXIN2* gene. AXIN2 inactivation leads to Wnt activation. The *AXIN2* in-frame heterozygous 12 bp deletion c2013_2024del12 was found in two adrenocortical adenomas and two carcinomas in two different studies (Chapman *et al.*, 2011; Guimier *et al.*, 2013). However, the significance of this variant remains to be determined.

Wnt Signaling in Adrenocortical Hyperplasia

In addition to adenomas and carcinomas, we described previously *CTNNB1* mutations in 2 out of 18 patients with primary pigmented nodular adrenal disease (PPNAD) (Tadjine *et al.*, 2008b) a form of bilateral micronodular adrenal hyperplasia. In both cases the mutations occurred in large adenomas that had formed in the background of PPNAD. Immunohistochemistry showed nuclear accumulation of beta-catenin in cells of the adenoma tissue whereas no nuclear immunoreactivity was detected in adjacent PPNAD nodular cells (Tadjine *et al.*, 2008b). Similarly, Gaujoux *et al.*, demonstrated beta-catenin accumulation in nine cases of PPNAD (Gaujoux *et al.*, 2008). Moreover, *CTNNB1* somatic activating mutations were found in the macronodule of two of the five macronodular PPNADs studied in this report (Gaujoux *et al.*, 2008).

Using microarray analysis, we found that Wnt signaling related genes were aberrantly expressed in bilateral macronodular adrenal hyperplasia (BMAH) however no *CTNNB1* mutations were described so far in BMAH (Bourdeau *et al.*, 2004). Recently, germline mutations in the Armadillo Repeat Containing 5 (*ARMC5*) gene were identified in sporadic and familial cases of BMAH (Assie *et al.*, 2013; Alencar *et al.*, 2014; Bourdeau *et al.*, 2016). In most cases an additional somatic *ARMC5* mutations were found in the BMAH adrenal tissue supporting the tumor suppressor role of *ARMC5*. Interestingly, ARMC5 contains an amino acid tandemly repeated sequence motif as in the mammalian armadillo homolog beta-catenin protein. Very recently, mouse models of ARMC5 deficiency were generated (Berthon *et al.*, 2017; Hu *et al.*, 2017) and lead to hypercorticosteroniemia at older age. Adrenocortical tissue analysis of the older ARMC5 deficient mice showed abnormal activation of the Wnt/beta-catenin signaling pathway in a subset of zona fasciculate cells (Berthon *et al.*, 2017).

Phenotype of Patients With Adrenal Tumors and Wnt Activation

In addition, to provide a better understanding on the molecular events leading to the development of adrenal tumors, Wnt signaling was described as associated to a more aggressive phenotype in patients with ACC (Gaujoux *et al.*, 2011). The presence of beta-catenin nuclear staining by immunohistochemistry and *CTNNB1* mutations were both significantly associated with decreased survival and disease free survival (Gaujoux *et al.*, 2011). These results were verified in two independent cohorts of ACC patients; the Cohin-COMETE cohort and the German-ENSAT cohort.

Regarding adrenocortical producing adenomas, in 2011, Bonnet *et al.*, studied a cohort of 100 tumors (Bonnet *et al.*, 2011). They found that tumors with *CTNNB1* mutations were predominantly nonsecreting and had larger size and weight compared to wild-type tumors suggesting less tumoral differentiation (Bonnet *et al.*, 2011).

This data was confirmed more recently in a study performed by the ENSAT study group where the genetic landscape of 99 adrenocortical cortisol-secreting or nonsecreting adenomas without the recurrent activating L206R mutation in the catalytic subunit of the cAMP-dependent protein kinase A (PKA) was determined using next generation whole exome sequencing (Ronchi *et al.*, 2016). In this study, the most frequent alterations were *CTNNB1* missense mutations localized at the amino acid 45 in the gene with a prevalence of 18% in patients with Cushing syndrome, 54% in patients with subclinical Cushing syndrome and 52% in patients with endocrine inactive tumors supporting Bonnet's data (Bonnet *et al.*, 2011). Thus *CTNNB1* mutations are more frequent in tumors with less differentiation. Moreover, this last study showed that mutations in the catalytic subunit of Protein kinase A (PKA) occur mutually exclusive to *CTNNB1* mutation (Ronchi *et al.*, 2016).

Beta-Catenin Mutations in Aldosterone-Producing Adenomas

Although most earlier studies on beta-catenin mutations focused on cortisol-secreting and nonsecreting tumors, few cases of aldosterone-producing tumors were reported in initial cohorts that were studied. In 2008, we described a 69-year old woman with primary aldosteronism where a somatic *CTNNB1* mutation was found in her aldosterone-producing adenoma (Tadjine *et al.*, 2008a). In 2009, Masi *et al.*, described two patients with aldosterone-producing adenomas and one patient with aldosterone-producing ACC with somatic *CTNNB1* mutations in their tumors (Masi *et al.*, 2009). In 2014, very important data was reported by a French team who found that constitutive activation of WNT/beta-catenin signaling was present in 70% of a cohort of 47 aldosterone-producing adenomas (APA) using immunohistochemistry (Berthon *et al.*, 2014). Although, no *CTNNB1* mutations were found in this study of 26 aldosterone-producing adenomas they demonstrated that decreased expression of the Wnt inhibitor SFRP2 may contribute to deregulation of Wnt signaling and APA development (Berthon *et al.*, 2014). In 2015, Teo *et al.*, reported the cases of three women with primary aldosteronism harboring *CTNNB1* mutations in their adenomas (Teo *et al.*, 2015). Two patients developed primary aldosteronism during pregnancy and one after menopause. In this study, the authors concluded to an association between somatic *CTNNB1* mutations in their adenomas and pregnancy or menopause due to high expression of LHCG receptors and GnRH receptors compared to other aldosterone-producing adenomas (Teo *et al.*, 2015). However, more recently, two large cohorts of patients with primary aldosteronism that were screened for the presence of *CTNNB1* mutations showed that the *CTNNB1* mutations may be found not only in females but in males as well. Although, no somatic *CTNNB1* mutations were found in initial studies (Boulkroun *et al.*, 2011; Berthon *et al.*, 2014), more recently somatic *CTNNB1* mutations were found in 2 out of 99 (prevalence of 2,1%) (Scholl *et al.*, 2015), 10 out of 198 (5, 1%) (Akerstrom *et al.*, 2016) and, 8 out 219 (3,7%) patients with primary aldosteronism (Wu *et al.*, 2017). All the patients reported so far in the literature with primary aldosteronism and somatic *CTNNB1* mutations are described in **Table 2**.

In Vitro Studies of Wnt-Signaling in Adrenocortical Cancer Cell Lines and In Vivo Mouse Models

The first evidence of a causal role of beta-catenin in the development of adrenal tumors was demonstrated by Lalli's group (Doghman *et al.*, 2008). They showed that the inhibitor of the T cell factor-beta-catenin complex PKF115–584 dose-dependently inhibits the human adrenocortical cancer cell line H295R proliferation (by decreasing the percentage of H295R cells and

Table 2 Summary of all cases of primary aldosteronism with somatic beta-catenin mutations described so far which include 19 females and 7 males with a mean age of 51-year old

	Sex	*Age (yo)*	CTNNB1 *mutations*
Tadjine *et al.* (2008a)	F	69	26,995 del 271 bp
Masi *et al.* (2009)	M	69	S45P
	F	66	Ala43_Glu53del
Scholl *et al.* (2015)	F	24	S45P
	F	65	A39Efs*3
Teo *et al.* (2015)	F	34	Ser33Cys
	F	26	p.Ser45Phe
	F	52	Gly34Arg
Akerstrom *et al.* (2016)	F	39	S45P
	F	51	S45P
	F	51	S45P
	F	26	S45F
	M	35	T41A
	M	66	S45P
	M	30	S45P
	M	34	S45P
	F	76	S45P
	F	32	S45P
Wu *et al.* (2017)	F	50	S45F
	F	54	S45P
	F	51	S45P
	F	86	S45P
	M	62	S45P
	F	58	S45P
	F	62	S45F
	M	67	S45F

M: Male, F: Female, yo: year old, *CTNNB1*: beta-catenin gene.

increasing the % of apoptotic cells) (Doghman *et al.*, 2008). Furthermore, In 2010, Berthon *et al.*, showed that constitutive activation of beta-catenin in the adrenal cortex of transgenic mice leads to hyperplasia of the adrenal glands at 5 and 10 months with increase of adrenal size at 10 months. At 17 months, the mice developed primary hyperaldosteronism and their adrenal glands had malignant characteristics in 30% of cases (Berthon *et al.*, 2010).

Using microarray analysis we compared the gene expression profile of AA harboring *CTNNB1* mutations and wild-type AA (Durand *et al.*, 2011). We demonstrated up-regulation of *ISM1* (isthmin1, zebrafish homolog), *RALBP1* (RalAbinding protein 1), and *PDE2A* (phosphodiesterase 2A,cGMP-stimulated) in adrenocortical tumors with *CTNNB1* mutations compared with either WT adenomas or normal adrenal glands (Durand *et al.*, 2011). While *PHYHIP* (phytanoyl-CoA2-hydroxylase-interacting protein) was under expressed in tumors with *CTNNB1* mutations compared with normal adrenal tissue. mRNA expression and protein levels of *RALBP1*, *PDE2A*, and *ENC1* were decreased in a dose-dependent manner in the human adrenocortical cell line H295R which harbor a *CTNNB1* mutation after treatment with both antagonists of TCF/beta-catenin complex PKF115–584 and PNU74654 (Durand *et al.*, 2011).

Next-Generation Sequencing of Adrenal Tumors and Wnt/Beta-Catenin Signaling

In the last 3 years, the use of next generation sequencing including exome sequencing allowed to confirm the prevalence of *CTNNB1* mutations but also to identify new players of the Wnt/beta catenin signaling pathway. Genomic characterization of 122 ACC by Assié *et al.*, revealed that the most altered pathway in ACC was the Wnt/beta-catenin pathway with *CTNNB1* mutations being present in 16% of cases, and *APC* mutations in 2% of cases (Assie *et al.*, 2014). Moreover, homozygous deletions of the *ZNRF3* gene was found in 21% of tumors. *ZNRF3* encodes a cell surface transmembrane E3 ubiquitin ligase that acts as a negative feedback regulator of Wnt/beta-catenin signaling by promoting the degradation of the LRP and Frizzled receptors. *ZNRF3* mutations lead to loss of its negative feedback and activation of Wnt/beta-catenin signaling such as *CTNNB1* gene mutations but at a lesser degree. In other recent studies of ACC using NGS/exome sequencing, *CTNNB1* mutations were found in 9,8% (Juhlin *et al.*, 2015) and 16% (Zheng *et al.*, 2016) of ACC cases and *ZNRF3* mutations in 11,1% (Goh *et al.*, 2014), 9,8% (Juhlin *et al.*, 2015), and 16% (Zheng *et al.*, 2016) of ACCs. Similarly, a study combining exome sequencing and sanger sequencing approaches of 71 pediatric adrenocortical tumors demonstrated the presence of beta-catenin mutations in 13 out of 71 tumors with a prevalence of 18% but no *ZNRF3* mutations were reported in this pediatric series (Pinto *et al.*, 2015). *CTNNB1* mutations ($n = 13$) were detected only in tumors with wild-type germline *TP53* and not in those with constitutional *TP53* mutations (Pinto *et al.*, 2015).

Inhibition of Wnt Signaling Pathway as Potential Therapy

Taking into account the major role of Wnt signaling in adrenocortical tumors, Wnt components targets are of great interest for new treatments of ACC. Inactivation of Wnt signaling pathway could be achieved by various ways including (1) targeting extracellular signaling molecules with monoclonal antibodies for example, (2) preventing the FZD/Dvl interaction (3) stabilizing the destruction complex (4) increasing beta-catenin proteasomal degradation or (5) preventing the interaction between beta-catenin and its cofactors in the nucleus (Pez *et al.*, 2013). A wide variety of drugs inhibiting the Wnt pathway are currently used in preclinical studies or clinical trials for various cancers such as colon, breast or pancreatic cancers but none in in the treatment of ACC yet (Katoh and Katoh, 2017). Further studies are needed to explore the potential efficiency of these inhibitors for the treatment of patients with ACC for whom the actual options of treatment are limited and the overall survival remains poor.

See also: Adrenal Incidentalomas. Adrenocortical Carcinoma: Diagnosis and Therapy. Cushing Syndrome—Unilateral Adrenal Adenoma

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Adrenocortical Tumors and gsp Mutations

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Introduction

Heterotrimeric G-proteins are molecular switches that activate intracellular signaling cascades in response to the activation of G-protein-coupled receptors (GPCRs) by extracellular stimuli (Oldham and Hamm, 2008). These proteins are composed of α, β, and γ subunits, and their function depends on the ability of the G-protein α subunit (Gα) to cycle between an inactive conformation (GDP-bound) primed for interaction with an activated receptor and an active conformation (GTP-bound) that can modulate the activity of downstream effector proteins. The α subunit is a GTPase, which can be stimulated by the receptor to release GDP, bind GTP, and create an active state. Several α subunit types act directly on effector molecules to modulate their activity in specific manner: αs activates adenylate cyclases, αi inhibits adenylate cyclases, and αq activates phospholipase C isozymes (Hamm, 1998). (See **Table 1**.)

The GNAS gene encodes the α subunit of stimulatory G-proteins (GNAS, OMIM 139320) and is located on chromosome 20q13.32 (Weinstein *et al.*, 2004). Somatic gene point mutations are also termed *gsp* mutations and determine the loss of intrinsic α subunit GTPase activity, leading to subsequent constitutive activation of adenylate cyclase (Antonini *et al.*, 2004; Brown *et al.*, 2010).

gsp mutations are involved in the pathogenesis of McCune–Albright syndrome (MAS), which is classically defined by the clinical triad of bone fibrous dysplasia, *café au lait* skin macules, and gonadotropin-independent precocious puberty, in addition to other endocrine disorders. MAS is the first disease that has been linked to cAMP pathway signaling and Cushing syndrome due to excess cortisol production from the fetal adrenal gland, which primarily effects children in their first years of life (Dumitrescu and Collins, 2008).

Patients with MAS have the *gsp* mutation in a mosaic pattern with varying degrees of tissue involvement, ranging from a single site to a widespread distribution (Happle, 1986).

Naturally occurring mutations in codons 201 and 227, which alter GTPase activity in the *GNAS* gene, have been described in autonomous hormone-producing tumors. Mutations involving the substitution of arginine for cysteine; histidine; and, rarely, serine at codon 201 or arginine for glutamine at codon 227 were first described in GH-producing pituitary tumors (Landis *et al.*, 1989).

cAMP/PKA Signaling in Adrenocortical Cells

Metabolism, cell proliferation, steroidogenesis, and cortisol biosynthesis in the adrenal cortex are regulated predominantly by the cAMP/protein kinase A (PKA) signaling pathway (Almeida *et al.*, 2012; Bimpaki *et al.*, 2009; Bourdeau *et al.*, 2006). PKA exerts its functions via its effector, cAMP, by phosphorylating transcription factors of the cyclic AMP response element-binding (CREB) protein family. In normal adrenal cells, this pathway is activated through the binding of adrenocorticotropic hormone (ACTH) to the melanocortin 2 receptor (MC2R), a G-protein-coupled receptor (GPCR) that is highly expressed in adrenocortical cells, leading to increased levels of intracellular cAMP and subsequent activation of PKA (Bertherat *et al.*, 2003; Lefebvre *et al.*, 2015).

PKA is composed of four subunits, two catalytic and two regulatory, that are bound together when the pathway is not stimulated. As a result of pathway activation by hormones or neurotransmitter agonists of GPCRs, cAMP binds to the regulatory subunits of PKA. This binding induces the release of the catalytic subunits, which translocate to the nucleus in order to phosphorylate transcription factors. In the adrenal cortex, this process induces the transcription of steroidogenic enzymes and cortisol biosynthesis (Almeida and Stratakis, 2011; Bourdeau *et al.*, 2006).

Genetic alterations in the cAMP/PKA pathway have been reported in adrenocortical cells that lead to constitutive activation of this pathway and adrenal gland overstimulation, leading to tumor development and excessive hormone secretion (Louiset *et al.*, 2017).

Several genetic defects in cAMP/PKA that maintain pathway activation have been associated with adrenal disorders: ectopic expression of G-protein-coupled receptors (Lacroix *et al.*, 2001; Assie *et al.*, 2010), activating mutations in the gene encoding the $G\alpha_s$ protein (*GNAS*) (Landis *et al.*, 1989; Levine *et al.*, 1986; Weinstein *et al.*, 2004), activating mutations in *MC2R* (Almeida *et al.*, 2014), inactivating mutations in the gene encoding the RIα subunit of PKA (*PRKAR1A*) (Almeida *et al.*, 2012; Bimpaki *et al.*, 2009; Bourdeau *et al.*, 2006), inactivating mutations in the genes encoding two phosphodiesterases (PDE8 and PDE11A) (Horvath *et al.*, 2006; Horvath *et al.*, 2008), and activating mutations in the gene encoding the Cα subunit of PKA (PRKACA) (Calebiro *et al.*, 2014).

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Table 1 Percentage of *gsp* mutations in a variety of tumors

Type of tumors	*Gsp mutations (%)*	*References*
McCune–Albright syndrome	100	Weinstein *et al.* (1991)
Corticotroph adenomas	4.4–40	Lyons et al. (1990), Hosoi *et al.* (1993)
Primary macronodular adrenocortical hyperplasia (PMAH)	3, 2–9, 6	Fragoso *et al.* (1998) and Hsiao *et al.* (2009)
Adrenal aldosterone-producing adenomas	6	Nakajima *et al.* (2016)
Cortisol-producing adrenocortical adenomas	11	Ronchi *et al.* (2016)
Ovarian Leydig cell tumors	66, 6	Fragoso *et al.* (1998)
Granulosa cell tumors of the ovary	~30	Hsiao *et al.* (2009)
Thyroid cancer	~10	Suarez *et al.* (1991)

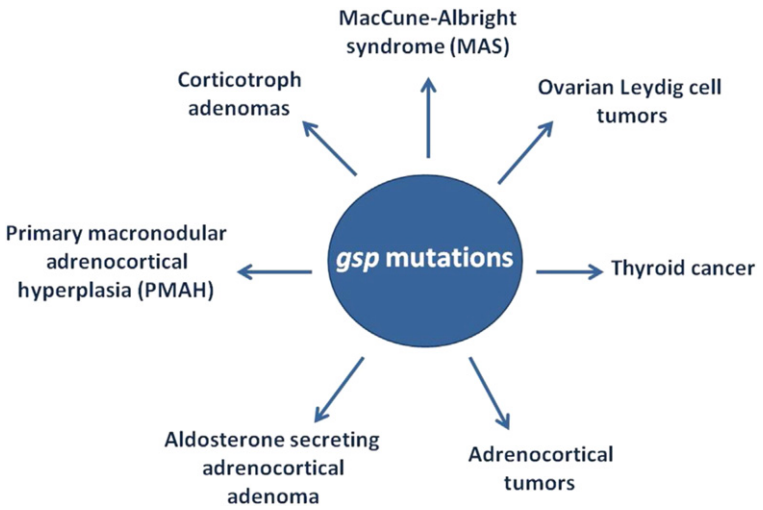

Fig. 1 *gsp* mutations associated with a variety of endocrine disorders.

Nevertheless, the molecular events described earlier are unable to explain all cases of adrenocortical disorders and the molecular pathogenesis of Cushing syndrome, as understanding the pathophysiology of adrenal tumors remains a challenge for the scientific community.

gsp Mutations in Adrenocortical Tumors

Although the pathogenesis of adrenocortical adenomas has not been completely elucidated, the genetic understanding of sporadic adrenocortical tumors has become clearer in the previous decades. Somatic mutations of genes encoding components of the cAMP/PKA pathway (*GNAS*, *PRKAR1A*, and *PDE8B*) and β-catenin (*CTNNB1*) have been reported in a small subset of cortisol-secreting adrenocortical tumors (Durand *et al.*, 2011).

gsp mutations have also been described in several tumors, such as corticotroph adenomas, thyroid cancer, ovarian and Leydig cell tumors, primary macronodular adrenocortical hyperplasia (PMAH) (Antonini *et al.*, 2004; Fragoso *et al.*, 2003; Hsiao *et al.*, 2009), and in rare cases cortisol- and aldosterone-secreting adrenocortical adenomas (**Fig. 1**). All of these described cases were outside of the classical context of McCune–Albright syndrome.

The first description of the involvement of *gsp* mutations in adrenal tumors was made by Yoshimoto *et al.* in 1993. This study systemically screened Gsα mutations in 197 human endocrine tumors, including pituitary, thyroid, parathyroid, endocrine pancreatic, and (cortex and medulla) adrenal tumors. The authors identified a unique female patient who was 29 years old and had primary aldosteronism associated with a somatic *gsp* mutation in an aldosterone-secreting adrenocortical adenoma, suggesting that when the renin–aldosterone system is suppressed, aldosteronomas become more sensitive to corticotropin stimulation. Accordingly, corticotrophins could be stimulated by Gsα-mediated cAMP production. The study hypothesized that *gsp* mutations may constitutively stimulate aldosterone synthesis via the zona glomerulosa by transmitting a constitutive signal through Gs-mediated cAMP production, which could play an important role in the tumorigenesis of aldosterone-secreting adenomas (Yoshimoto *et al.*, 1993).

Following the study by Yoshimoto *et al.*, several studies have detected the presence of *gsp* mutations in adrenal tumors, but none could explain their presence alongside the mechanism that leads to tumor formation and hormone hypersecretion. In 2000, Bugalho *et al.* described the presence of a mutation in codon 201 (CGT to TGT) in a patient with Cushing syndrome due to a

functioning adrenal adenoma that also expressed aberrant LH receptor (Bugalho *et al.*, 2000), Another patient with concomitant aberrant LH receptor expression and low-level autonomous cortisol secretion, formerly called "subclinical" Cushing syndrome, was found to have a heterozygous single-nucleotide substitution in the adrenocortical adenoma tissue, which altered codon 227 (Q227H) of the *GNAS* gene (Kobayashi *et al.*, 2000).

In 2004, *gsp* mutation was identified in one patient with ACTH-independent Cushing syndrome (Dall'Asta *et al.*, 2004). In 2013, the presence of genetic alterations was investigated in a series of 10 ACTH-independent Cushing syndrome cases due to adrenocortical cortisol-secreting adenomas. A *gsp* mutation was identified in only one case (Libé and Bertherat, 2005).

The first study to suggest that an activating mutation of *GNAS* associated with epigenetic alterations could lead to tumorigenesis was published by Sidhu *et al.* in 2013 (Sidhu *et al.*, 2013). This study demonstrated the presence of the *GNAS*-activating mutation (p.R201C) in a pediatric adrenocortical tumor. The tumor showed the following morphological features: areas of necrosis, microcytic degeneration, and venous and capsular microinvasion. The tumor tissue also presented an abnormal allele-specific hypomethylation of the *KCNQ1OT1* gene involved in Beckwith–Wiedemann syndrome (Sidhu *et al.*, 2013). It is known that somatic mutations in these genes may constitutively activate the cAMP/PKA signaling pathway, leading to cellular hyperproliferation that may result in genomic instability and epigenetic alterations to provide favorable conditions for the genesis of ACTs. The authors postulated that this activating mutation of the *GNAS* gene was the inciting event leading to hypomethylation of *KCNQ1OT1* (Sidhu *et al.*, 2013), which was already associated with tumorigenesis in other contexts (Sidhu *et al.*, 2013; Libé *et al.*, 2005; Sato *et al.*, 2014).

More recently, somatic mutations of *PRKACA* (encoding the catalytic subunit of protein kinase A) have been identified in more than one-third of the patients with Cushing syndrome due to sporadic adrenocortical adenomas. However, the molecular pathogenesis of the majority of sporadic adrenocortical tumors remained unclear (Bourdeau *et al.*, 2006; Libé *et al.*, 2005).

In a cohort of 65 adrenocortical tumors and matched normal specimens, a whole-exome sequencing study demonstrated that *PRKACA* was mutated in more than 50% of the tumor samples ($n = 34$; 28 confirmed as somatic), resulting in an identical c.T617G mutation predicted to cause the replacement of a leucine with an arginine at amino acid position 206 (p.L206R). This modification prevents binding to the inhibitory R subunits and leads to constitutive cAMP-independent activation of PKA. Furthermore, *GNAS* mutations (p.R201C) were found in 11 cases (6 confirmed as somatic). Mutations in *PRKAR1A*, *PDE11A*, *PDE8B*, and *ARMC5* were also investigated, but none were identified. Apparently, *PRKACA* and *GNAS* mutations in adrenocortical cells play an important role in the overt production of cortisol, as together they accounted for as many as 70% of the patient cohort in that study, almost exclusively in patients with clinical Cushing syndrome. Furthermore, *PRKACA*-mutated adenomas presented higher cortisol levels in the 1 mg overnight dexamethasone test and a smaller size when compared with wild type. Altogether, these results strengthen the hypothesis that mutations in these genes are related to the clinical features of these patients (Sato *et al.*, 2014).

Previous studies have demonstrated that mutations in *GNAS* and *PRKACA* are mutually exclusive and appear to be associated with small tumors, younger age at presentation, and overt Cushing syndrome (Sato *et al.*, 2014; Goh *et al.*, 2014).

In opposition to what has been observed in humans and mice, *GNAS* mutations in dogs seem more prevalent than *PRKACA*, as they were observed in 14 out of 44 cortisol-secreting adrenocortical tumors, whereas no functional mutations were found in *PRKACA*. This result, supported by the fact that *PRKACA* mutations have never been observed in dogs, suggests a difference in the molecular origin of the cAMP–PKA activation between these species (Kool *et al.*, 2013).

Recently, studies using next-generation sequencing have reported mutations in GNAS in an extensive variety of tumors, including parathyroid cancer, hepatocellular carcinoma, colon cancer, and pancreatic tumors. Next-generation whole-exome sequencing was performed by Ronchi *et al.*, in 2016, to detect the presence of *gsp* mutations in 11% of analyzed cortisol-producing adrenocortical adenomas (Ronchi *et al.*, 2016). *GSP* mutations have been identified in around 4.0% of the 9486 tumor sequences deposited in the COSMIC database of 185 somatic mutations in human cancer, making it one of the most frequently mutated G-proteins in cancers (Nakajima *et al.*, 2016; Fragoso *et al.*, 2003; Yoshimoto *et al.*, 1993).

De Martino *et al.* used hot spot gene sequencing and Sanger sequencing to identify a GNAS mutation in one adrenocortical carcinoma (ACC) case from a cohort of 40 ACC patients. This mutation was found in a patient presenting with isolated Cushing syndrome (stage III ACC (ENSAT), Weiss score = 6) and no clinical evidence of fibrous dysplasia (De Martino *et al.*, 2013). Juhlin *et al.* used whole-exome sequencing to detect 966 nonsynonymous somatic mutations in an ACC cohort, including 40 tumors with a mean of 16 mutations per sample and 1 tumor with more than 100 mutations. They also identified one sample with a mutation in *GNAS* (Arg201His) (Juhlin *et al.*, 2015).

Nakajima *et al.* recently detected *gsp* mutations in aldosterone-producing adenomas (APAs) with autonomous cortisol secretion. Interestingly, these mutations may induce the secretion of either cortisol and aldosterone or cortisol alone. The authors were the first to identify the *GNAS* mutation p.R201C in 2 out of 33 APAs, both of which presented with mild autonomous cortisol secretion as detected by nonsuppressed levels of cortisol after a 1 mg overnight dexamethasone test. In addition, they observed that in APAs harboring *KCNJ5* mutations, the presence of *GNAS* mutations was mutually exclusive (Nakajima *et al.*, 2016).

In summary, the presence of *gsp* mutations could affect both zonas fasciculata and glomerulosa, leading to either tumors that cosecrete aldosterone and cortisol or tumors that secrete cortisol alone. However, further studies are required to establish the mechanism by which *GNAS* mutations in these tumors cause hormone overproduction and cell proliferation.

Conclusion

Genetic analyses and a better understanding of the molecular pathogenesis of sporadic cortisol-secreting adenomas confirm that the cAMP/PKA signaling pathway plays an essential role in adrenocortical physiology and pathophysiology. Constitutive activating mutations of *GNAS* may cause cortisol-/aldosterone-secreting adenomas through the stimulation of both adrenocortical cell function and proliferation. Studies in this field have provided insight into the development of adrenal hormonal autonomy and may provide the basis for novel advances in the diagnosis and the treatment of Cushing syndrome due to adrenal disorders.

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See also: Cushing Syndrome; Screening and Differential Diagnosis. Primary Bilateral Macronodular Adrenal Hyperplasia

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PPNAD, Carney Complex, and Other Micronodular Adrenal Hyperplasia

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Nomenclature

cAMP	Cyclic adenosine monophosphate
CS	Cushing syndrome
CNC	Carney complex
iMAD	Isolated micronodular adrenocortical disease
MRI	Magnetic resonance imagery
PDE	Phosphodiesterases
PKA	Protein Kinase A
PPNAD	Primary pigmented nodular adrenal disease
PRKACA	Protein kinase cAMP-dependant catalytic subunit alpha
PRKAR1A	Protein kinase cAMP-dependant regulatory subunit type 1 alpha
US	Ultrasound

Introduction

The Carney complex (CNC) was first described in Carney *et al.* (1985). J. Aidain Carney and co. identified at the Mayo Clinic four cases of a quite unusual form of adrenal hyperplasia with micronodules and pigmentation leading to adrenal Cushing syndrome (CS) that he has named the primary pigmented nodular adrenal disease (PPNAD). After studying the history of these patients and additional patients presenting with PPNAD, he described the association of "myxomas, spotty pigmentation, and endocrine overactivity" named later Carney complex. Other syndromes previously described as the LAMB (lentigineses, atrial myxoma, mucocutaneous myxoma, blue naevi) (Rhodes *et al.*, 1984) or the NAME (naevi, atrial, myxoma, myxoid neurofibroma, ephelide) (Atherton *et al.*, 1980) are part of the CNC.

The PPNAD is the most frequent endocrine manifestations of the CNC. In the majority of cases, CNC is due to a germline mutation in the gene *PRKAR1A* encoding for the regulatory subunit type I alpha of the protein kinase A (PKA). Mutations of the gene can lead also to isolated PPNAD, that is, without other manifestations of the CNC (Espiard and Bertherat, 2013). Apart from this context, an isolated micronodular adrenocortical disease (iMAD) has been described in few cases related to mutations in the genes encoding for the phosphodiesterases (PDEs) *PDE11A* and *PDE8B* and to duplication of the gene *PRKACA* encoding for the catalytic subunit C-alpha of the PKA. The term of iMAD is preferred to PPNAD since a pigmentation of the micronodules is not constant in these cases (Almeida and Stratakis, 2010). To date, other cases of CNC or iMAD remain unexplained.

Carney Complex

Epidemiology and Clinical Presentations

The CNC is a rare disease. Its prevalence is also unknown (Lodish and Stratakis, 2016). The biggest published cohort included 353 patients from the National Institute Health (Bethesda, United States), the Mayo Clinic (Rochester, United States), and Cochin Hospital (Paris, France) (Bertherat *et al.*, 2009). Female are more often affected than male (Bertherat *et al.*, 2009; Stratakis *et al.*, 2001). There is no ethnic predisposition. Average age at diagnosis is 20-years-old (Stratakis *et al.*, 2001).

The CNC could be considered as an endocrine and nonendocrine multiple neoplasia syndrome (Stratakis *et al.*, 1998). The spectrum of manifestations, their severity and the age at diagnosis differ between the patients, even inside a same family. PPNAD can be the single manifestation of the disease, named in these cases isolated PPNAD. Isolated lentigines and isolated cardiac myxomas have been also described (Bertherat *et al.*, 2009). Diagnostic criteria have been established in 2001 (Stratakis *et al.*, 2001) (**Table 1**).

Cardiac Myxoma

Primary cardiac tumors are rare and cardiac myxomas are the most common of them. They are nonmalignant tumors, but with potential severe cardiovascular complications. Only 3%–10% of the cardiac myxomas are observed as familial cases. The majority of these familial cases occur as a component of CNC. The clinical presentation is variable: patients can be asymptomatic or presenting cardiovascular symptoms including heart failure, dyspnea, palpitations, and angina. Nonspecific constitutional symptoms as fever, asthenia, malaise, arthralgia have been also described. Embolization of tumor fragments or surface thrombi leads to the most dramatic complications: the stroke attack or the systemic infraction (Jain *et al.*, 2015). CNC patients are affected by cardiac myxomas in 32%–53% of cases (Bertherat *et al.*, 2009; Stratakis *et al.*, 2001). The usual locations are the left atrium

Table 1 Diagnostic criteria (established in Stratakis *et al.*, 2001) and their frequency among CNC patients

Major diagnostic criteria for CNC	*Frequency*[a] *(%)*
1. Spotty skin pigmentation (lentiginosis) with typical distribution (lips, conjunctiva and inner or outer canthi, vaginal mucosa)	70–77
2. Myxoma (cutaneous and mucosa)[b]	20–32
3. Cardiac myxoma[b]	32–53
4. Breast myxomatosis[b] or fat-suppressed magnetic resonance imaging findings suggestive of this diagnosis	20
5. Primary pigmented adrenocortical disease[b] or paradoxical positive response of urinary glucocorticosteroid excretion to dexamethasone administration during Liddle's test	26–60
6. Acromegaly due to GH-producing adenoma[b]	10–12
7. Large-cell calcifying Sertoli cell tumor[b] or characteristic calcification on testicular ultrasound	33–41
8. Thyroid carcinoma[b] or multiple, hypoechoic nodules on thyroid ultrasound in a young patient	5–25
9. Psammomatous melanotic schwannomas[b]	8–10
10. Blue nevus, epithelioid blue nevus[b]	50
11. Breast ductal adenoma[b]	20
12. Osteochondromyxoma[b]	2
Supplementary criteria	
1. Affected first degree relative	
2. Inactivating mutation of the *PRKAR1A* gene	

[a]According to (Stratakis *et al.*, 2001) and (Bertherat *et al.*, 2009).
[b]After histological confirmation.
Diagnosis of CNC is made if the patient exhibits two of the major manifestations or one of these and one of the supplemental criteria (Stratakis *et al.*, 2001).

followed by the right atrium, but in CNC, by contrast with sporadic myxomas, they can be located in any chambers and can be multiple (Bertherat *et al.*, 2009; Jain *et al.*, 2015). The median age at the detection of the first myxoma varies between 35 and 50-years-old depending on the series (Bandettini *et al.*, 2016; Bertherat *et al.*, 2009). Indeed, there is a large range in the age at diagnosis and tumor can be diagnosed in pediatric patients as early as 3-years-old. Risk of recurrence is higher in CNC than for sporadic cases. The growth of the tumor can be very fast and appearance of new myxomas can be observed in less than 1 year of time (Bertherat *et al.*, 2009; Jain *et al.*, 2015). Recently, it has been suggested that patients with growth hormone (GH) excess are more prompt to develop cardiac myxomas (Bandettini *et al.*, 2016).

Transthoracic echocardiography is the primary imaging for screening and follow-up of the cardiac myxomas. The tumor appears as an isoechogenic mass compared to cardiac wall (**Fig. 1**A). Transesophageal echocardiography and cardiac magnetic resonance imagery (MRI) are useful complement to better characterize multiple tumors and tumor shape and density. On MRI, the myxoma is heterogeneous, hyperintense in T2 weighted lesion (**Fig. 1**B) (Courcoutsakis *et al.*, 2013; Jain *et al.*, 2015).

The only definitive treatment is surgery. Progress made in this area the last decade improved the morbi-mortality of CNC patients. Historically, these tumors were the main cause of death because of their own complications but also the surgical complications (Espiard and Bertherat, 2013). Diagnosis must be also early and annual follow-up after 4-years-old is suggested (Stratakis *et al.*, 2001).

Skin Manifestations

The most frequent lesions are the spotty skin pigmentation component named lentigines observed in about 70% of patients (Bertherat *et al.*, 2009; Mateus *et al.*, 2008; Stratakis *et al.*, 2001). They are hamartomatous melanocytic lesions appearing typically as brownish to black macules located on the lips, the eyelids, the genital area in female or the fingers (**Fig. 2**A and B). The spots appear during the two first decades of life but can be detectable at birth. Color is more intense after puberty and can fade over time. The distinction with solar pigmented spots becomes also difficult (Espiard and Bertherat, 2013; Mateus *et al.*, 2008). Other pigmented lesions (blue, Spitz, and typical naevi, café-au-lait spots) are present in about 50% of patients. Blue naevi are the second most frequent dermatologic lesions in CNC patients (**Fig. 2**C) (Bertherat *et al.*, 2009; Mateus *et al.*, 2008).

Cutaneous myxomas are benign lesions detected in 20%–33% of patients but frequency is probably underestimated by lack of specialized screening. It is the most specific sign of the disease but differential diagnosis with other lesions as papilloma or cutaneous fibroma can be difficult at clinical examination. Skin biopsy is therefore usually needed to confirm the diagnosis. Appearance varies from small pink papules to large sessile lesions (**Fig. 2**D). Typical locations are the eyelids, the external ear canal and the trunk (Bertherat *et al.*, 2009; Mateus *et al.*, 2008; Stratakis *et al.*, 2001).

No malignant transformation of these lesions have been described and CNC does not seem to predispose to skin carcinomas (Espiard and Bertherat, 2013; Mateus *et al.*, 2008). The identification of the dermatologic lesions is important because they are among the earliest manifestations of the disease and patients with multiple skin manifestations seems to present larger number of others manifestations (Mateus *et al.*, 2008).

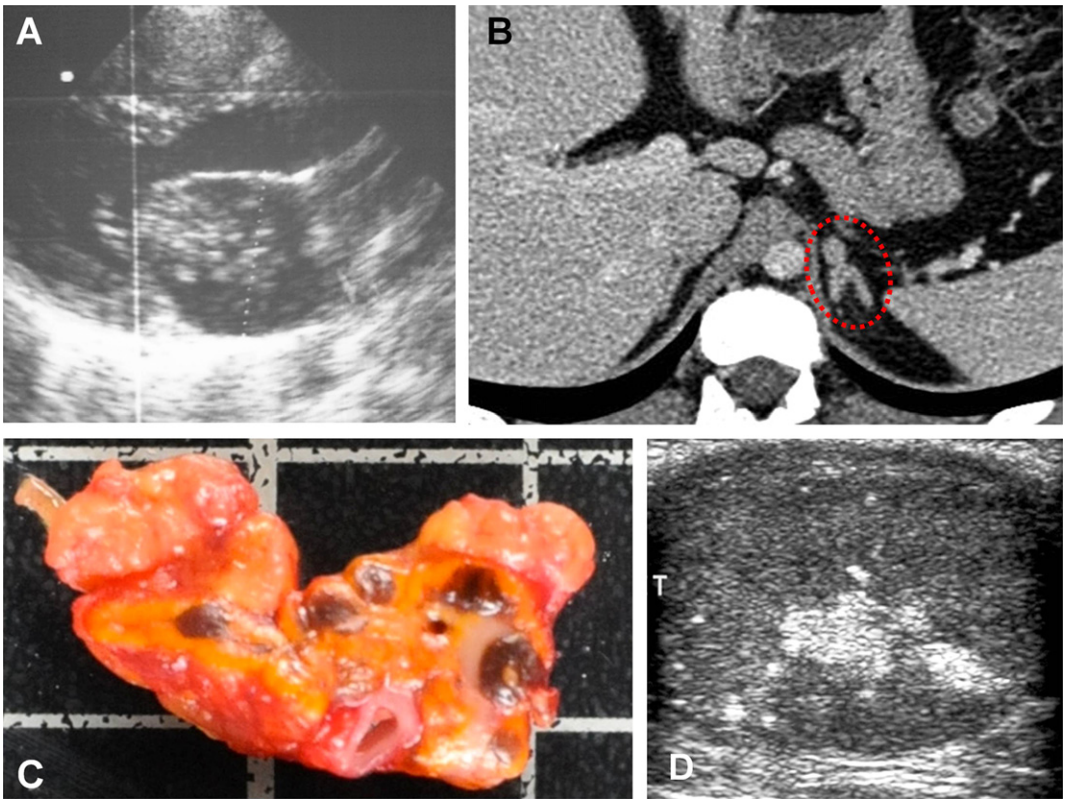

Fig. 1 Radiological and pathological findings in CNC. (A) Typical presentation of a left atrial cardiac myxoma on transthoracic echocardiography. (B) PPNAD appearance on CT-scan: left adrenal hyperplasia with micronodules of the body of the adrenal (*red circle*). (C) Macroscopic presentation of PPNAD: normal size adrenal with multiple pigmented nodules. (D) Multiple microcalcifications typical of LCCST on testicular US.

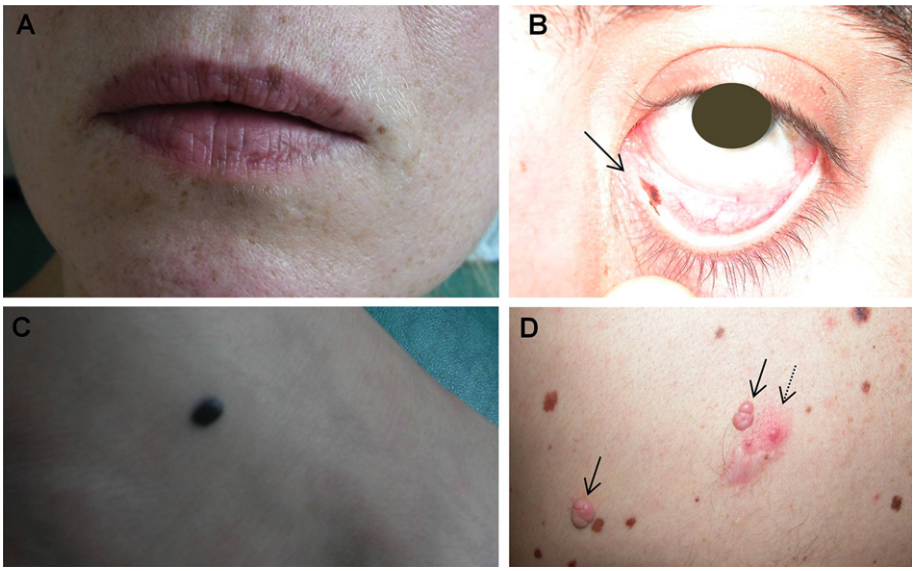

Fig. 2 Skin manifestations of CNC. (A) The perioral lentigines appears as pigmented spots. The presence of several lentigines and the confluency of some of them realize a typical lentiginosis. (B) Periocular lentigines with a typical lentigines of the inferior eyelid (*arrow*). (C) Bleu naevi: *dark blue* nodular lesion. (D) Cutaneous myxomas appearing as sessile nodular lesions (*continue arrows*) or papule (*discontinued arrow*).

PPNAD (Primary Pigmented Nodular Adrenocortical Disease)

PPNAD is the most frequent endocrine manifestations of the CNC reported in 26%–60% of patients (Bertherat *et al.*, 2009; Stratakis *et al.*, 2001). It is isolated for 12% of patients (Bertherat *et al.*, 2009). Some patients developed PPNAD during the first

years of life but the majority are diagnosed during the second and third decade (Stratakis *et al.*, 2001) with a median age evaluated at 35-years-old. After puberty, a female predominance is observed (Bertherat *et al.*, 2009).

The clinical presentation is variable: clinical CS (observed for about half of the patients), subclinical CS or no clinical signs (Lowe *et al.*, 2017). The appearance of the CS can be brutal, insidious or cyclic (Gunther *et al.*, 2004; Lowe *et al.*, 2017). The abnormal laboratory findings vary from an overt ACTH-independent CS to an isolated loss of cortisol suppression after dexamethasone test (Lowe *et al.*, 2017). These biological abnormalities could vary overtime. A paradoxical elevation of urinary free cortisol after Liddle's dexamethasone suppression test (increase of 50% of the basal UFC after administration of 0.5 mg/6 h for 2 days followed by 2 mg/6 h for 2 days) has been reported in 69% of patients with PPNAD (Stratakis *et al.*, 1999). This paradoxical response has been also observed in cortisol-producing adrenocortical adenoma due to somatic mutation of *PRKAR1A* (Bertherat *et al.*, 2003). This paradoxical response has been related to a stimulation of PKA subunits by dexamethasone through a glucocorticoid receptor (GR)-mediated mechanism (Bourdeau *et al.*, 2003; Louiset *et al.*, 2009).

There is no specific computed tomography feature of PPNAD. Imaging most often reveals micronodules or an abnormal contour of the glands but normal imaging or the presence of macronodules are frequent (Groussin *et al.*, 2002a; Vezzosi *et al.*, 2015). The realization of high resolution computed tomography with 3 mm-slice thickness and injection of contrast media is important to detect micronodules which appeared as hypodense spots (Courcoutsakis *et al.*, 2013). Noriodocholesterol scintigraphy shows a bilateral adrenal uptake in accordance to the bilateral nature of the disease, but asymmetrical scintigraphic uptake can be observed in patients with macronodules (Vezzosi *et al.*, 2015).

Pathologically, the glands have a normal size and weight or are slightly enlarged and the adrenal cortex harbors multiple small (<10 mm) yellow to black pigmented nodules around an atrophic cortex (**Fig. 1**C) (Carney *et al.*, 1985; Shenoy *et al.*, 1984; Stratakis *et al.*, 1999). Systematic autopsy shows adrenal lesions in every patient, even those who were asymptomatic (Stratakis *et al.*, 1999).

Recently, two cases of adrenocortical carcinomas were described in patients affected by CNC. In both cases, were observed a cosecretion of androgens and cortisol and rapid occurrence of metastasis and local recurrence (Anselmo *et al.*, 2012; Bertherat, 2012; Morin *et al.*, 2012). One case of androgen secreting benign adenoma has been described (Hofland *et al.*, 2013). It is not clear if these very rare manifestations could be considered as part of the syndrome.

The usual treatment for patients harboring CS is the bilateral adrenalectomy (Powell *et al.*, 2008; Sarkar *et al.*, 1990). Anticortisolic drugs as ketoconazole or Op'DDD have been more rarely used because they are less appropriated for a long-term treatment even if case reports have been described (Campo *et al.*, 2015). Usual follow-up for mutated patients who remain asymptomatic is annual measurement of the UFC (Stratakis *et al.*, 2001) but overnight 1 mg DXM suppression test could be considered now.

Acromegaly and Other Pituitary Manifestations

GH-producing tumor has been described in 10%–12% of patients (Bertherat *et al.*, 2009; Stratakis *et al.*, 2001). Clinical acromegaly is not frequent and does not become often apparent after treatment of CS if associated. On MRI, micro or macroadenomas, unique or multiple can be observed (Kirschner, 2010; Lonser *et al.*, 2017). Beyond these patients, biological dysregulation of the somatotroph axis without anomaly of the pituitary MRI is a frequent feature of CNC patients, especially an absence of suppression of GH after oral glucose tolerance test or a paradoxical increase of GH after TRH stimulation (Courcoutsakis *et al.*, 2013; Pack *et al.*, 2000; Raff *et al.*, 2000; Watson *et al.*, 2000). Mild elevation of PRL may be frequent (Raff *et al.*, 2000) but the occurrence of prolactinoma is rarely described (Kirschner, 2010). Pathological examination after surgery usually shows hyperplasia of the somatotroph compartment plus possibly the lactotroph one (Pack *et al.*, 2000). The adenoma, if present, stains for GH as well as frequently PRL, sometimes alpha subunit and rarely TSH and LH (Kirschner, 2010; Pack *et al.*, 2000). Management of patients with GH-producing tumors has no specificity comparing to non-CNC patients, even if partial or complete hypophysectomy may be required to achieve biochemical remission by surgery. Also, medical treatment as somatostatin analogue or GH antagonist are frequently used (Espiard and Bertherat, 2013; Lonser *et al.*, 2017; Watson *et al.*, 2000).

Thyroid Tumors

The occurrence of multiple thyroid nodules at young age has been described as part of CNC. Prevalence of nodules has been evaluated from 5% to 25% of CNC patients (Bertherat *et al.*, 2009; Stratakis *et al.*, 1997, 2001) but systematic thyroid ultrasound screening can reveal multiple, small, hypoechoic lesions in up to 64% of patients. The thyroid function is usually normal (Stratakis *et al.*, 1997). In case of benign nodules, pathological examination shows follicular adenomas (Stratakis *et al.*, 1997, 2001). CNC is associated with an increased risk to develop thyroid cancer. The prevalence of papillary of vesicular thyroid tumors has been evaluated from 2.5% to 3.8% of patients (Bertherat *et al.*, 2009; Stratakis *et al.*, 1997, 2001). It is therefore suggested to realize a thyroid US at the diagnosis (Stratakis *et al.*, 2001). Follow-up may be as recommended for the general population in function of the initial observation.

Testicular Lesions

Male patients can develop testicular lesions, especially large cell calcifying Sertoli tumors (LCCST) and rarely Leydig tumor or adrenocortical rests (Carney *et al.*, 1985; Stratakis *et al.*, 2001).

LCCST are a particular form of sex cord stromal tumors that is usually bilateral and multifocal (Gourgari *et al.*, 2012; Washecka *et al.*, 2002). Frequency is estimated to 33% to 41% of male (Bertherat *et al.*, 2009; Stratakis *et al.*, 2001). Mean age at diagnosis is in the third decade, but tumors have been observed as early as 4-years-old (Gourgari *et al.*, 2012; Washecka *et al.*, 2002). Most of the time, the lesions are not detectable by physical examination and are asymptomatic. Rarely, macrocalcifications and testis enlargement can be palpated. Because these tumors express aromatase, conversion of androstenedione to estrone in prepubertal males can cause growth acceleration and gynecomastia while conversion of testosterone to estradiol in postpubertal male can cause gynecomastia. Rare malignant forms with occurrence of metastasis have been described (Washecka *et al.*, 2002). Progressive expansion of the tumors can block the seminiferous tubes and participate to a reduction of the fertility. However, an independent decrease of the quality and the quantity of the sperm has been described in CNC male patients (Burton *et al.*, 2006). This might explain in part that in families, the disease tends to be more frequently transmitted by the mother (Stratakis *et al.*, 2001). Testicular US can show bilateral enlargement in testicular volume with or without microcalcifications (Gourgari *et al.*, 2012) (**Fig. 1**D). Medical treatment by antiaromatase or surgical treatment can be proposed (Brown *et al.*, 2007; Crocker *et al.*, 2014). Surgical treatment is only proposed for symptomatic disease (enucleation of tumors or partial orchidectomy) (Washecka *et al.*, 2002).

Leydig tumors or adrenal rest are observed in less than 1% of patients (Stratakis *et al.*, 2001). Cases of recurrence of the CS after bilateral adrenalectomy due to the gonadic rest have been described (Carney *et al.*, 1985).

Ovarian Lesions

Ovarian lesions have been diagnosed in 14% of female patients in a large restrospective study (Bertherat *et al.*, 2009). These are mainly surface-derived epithelial tumor. Systematic pelvic ultrasound show cystic lesions in more than half of the patients (Stratakis *et al.*, 2000). When surgery is needed, serous cystadenomas have been diagnosed but occurrence of ovary carcinoma has been described (Stratakis *et al.*, 2000).

Rarely, benign cystic teratoma (germinal tumor) (Carney *et al.*, 1985; Stratakis *et al.*, 2000) and granulosa tumor (stromal tumor) have been described, the latter causing virilization by testosterone production (Carney and Stratakis, 2011).

Schwannomas

Schwannomas has been observed in 8%–10% of patients (Bertherat *et al.*, 2009; Stratakis *et al.*, 2001). Specific pathological features have been described in CNC patients: pigmentation by melanin, frequent calcifications, and psammomatous body. Because of these characteristics they are named melanocytic psammomatous schwannomas. Usually these are multifocal, mainly located at the paraspinal sympathetic chain but also at the gastrointestinal tract, in bone or skin (Carney and Stratakis, 1998). Paraspinal and bone locations can cause neurological complications and pain respectively (Carney and Stratakis, 1998; Stratakis *et al.*, 2001). Malignancy has been observed (up to 10% of these schwannomas could probably be malignant) with occurrence of lung, liver or brain metastasis. MRI presentation depends of the size and the localization. Most often, they appear as a soft tissue tumor with calcifications and possibly bone osteolysis and osteosclerosis (Courcoutsakis *et al.*, 2013). Surgical treatment can be proposed but could be challenging for some localization and regarding the risk of surgical complications. In case of malignancy and metastasis, medical treatment or radiotherapy are alternative (Mees *et al.*, 2008).

Breast Lesions

Breast lesions are observed in about 20% of female patients (Bertherat *et al.*, 2009; Carney and Toorkey, 1991). However this percentage is likely to represent an underestimation of the true incidence of these lesions because breast imaging (MRI, ultrasound or mammography) was performed in only a small number of the patients of these series. The lesions are possibly palpable and most of the time asymptomatic (Courcoutsakis *et al.*, 1997, 2013).

Breast myxomas are characterized by an accumulation of myxoid material in single lobules, small groups of lobules or large aggregates of lobules resulting in the formation of myxoid fibroadema. Usually, lesions are multiple and bilateral. Male can also been affected (Carney and Toorkey, 1991; Stratakis *et al.*, 2001). Mammography shows opaque well-limited noncalcified lesions. These lesions are usually numerous and referred also as "breast myxomatosis." Chest MRI with inversion recovery sequence shows all the lesions which appeared hyperintense (Courcoutsakis *et al.*, 1997).

Breast ductal adenoma are detected in about 3% of patients (Carney and Toorkey, 1991; Stratakis *et al.*, 2001) and present usually as an unique palpable nodule. Mammography shows a well-limited calcified soft tissue tumor (Courcoutsakis *et al.*, 1997, 2013).

About 1% of female in the series reported in 2001 developed a breast carcinoma. Also a needle aspiration of every suspicious nodules, as the calcified ones, should be realized (Stratakis *et al.*, 2001).

Osteochondromyxomas

The osteochondromyxomas are rare bone lesions corresponding to benign chondrogenic tumors (Carney *et al.*, 2001). Frequency in CNC is estimated to 2% of patients (Stratakis *et al.*, 2001). These tumors have been mainly described in pediatric patients as early as few month of life suggesting that they could be congenital (Carney *et al.*, 2001). The most frequent location is the diaphysis of long bones or the sinus and nasal bones (Golden and Siordia, 2016). These tumors are painless. They are often diagnosed if they cause edema or mass effects as nasal obstruction for ethmoid tumor. There are no uniform radiological features and osteochondromyxomas can appear as lytic or as expansile bone lesions. On spinal MRI, it has been suggested that the vertebral lesions slightly hyperintense on T2 weightening with postcontrast enhancement observed in some CNC patients could correspond to spinal osteochondromyxoma (Courcoutsakis *et al.*, 2013). The pathological diagnosis is difficult: the tumors appeared as a mix bony and cartilaginous, myxoid, and hyalinous osteolytic lesion involving the adjacent soft tissue (Carney *et al.*, 2001; Golden and Siordia, 2016).

Other Lesions

Pancreatic tumors have been described in about 2% of CNC patients and are responsible for 4%–20% cause of death (Bertherat *et al.*, 2009; Carney and Toorkey, 1991). These tumors are mainly pancreatic cystic neoplasms, especially intraductal papillary mucinous neoplasms (IPMN) or pancreatic acinar cell carcinomas (Bertherat *et al.*, 2009; Gaujoux *et al.*, 2011).

Other rare tumors have been reported as malignant colonic or gastric tumors, hepatocellular adenomas or and carcinomas, and one case of retro-peritoneal fibrosing hystocytioma (Stratakis *et al.*, 2001).

Molecular Genetics and Pathophysiology

The cAMP/PKA-Signaling Pathway

The second messenger cAMP plays a major in development, maintenance and secretory activity of several endocrine glands. For instance, stimulation of the cAMP pathway by adrenocorticotropin (ACTH) is essential for adrenal cortex growth, maintenance and glucocorticoid and adrenal androgens production. ACTH stimulates the cAMP pathway after binding to the melanocortin 2 receptor, a 7-transmembrane receptor coupled to Gs protein. The activated Gs protein stimulated the enzyme adenyl cyclase (AC) which assures the synthesis of cAMP. PKA is a heterotetramer formed by two regulatory subunits and two catalytic subunits. In human, four different regulatory subunits (RIalpha, RIbeta, RIIalpha, RIIbeta), and four catalytic subunits (Calpha, Cbeta, Cgamma and PRKX) have been identified. When cAMP binds to the regulatory subunits of PKA, this results in the dissociation from the catalytic subunits. The free catalytic subunits can phosphorylate many cytoplasmic and nuclear targets such as the transcription factor cAMP response binding protein (CREB) involved in steroidogenesis. The cAMP is degraded by the phosphodiesterases which regulate the pathway (**Fig. 3**A).

Identifications of the CNC Gene Locus

Genetic linkage analysis identified two independent loci for CNC: CNC1 located on chromosome 17q22–24 and CNC2 on the 2p16 (Casey *et al.*, 1998; Stratakis *et al.*, 1996). In OMIM (Online Mendelian Inheritance in Man) database, the disease is referred as MIM160980 for CNC1, MIN605244 for CNC2 and MIM610489 for the isolated PPNAD.

The gene located at 17q has been identified in 2000 as *PRKAR1A* (Casey *et al.*, 2000; Kirschner *et al.*, 2000). The haploinsufficiency or the defective protein leads to an overactivation of the PKA signaling pathway by the lost of its inhibitory effect on the catalytic subunit (**Fig. 3**B).

Currently no candidate gene has been found on the CNC2 locus. Chromosomal alterations as an amplification of this loci has been described in different tumors from patients wild-type or mutated for *PRKAR1A* (Matyakhina *et al.*, 2003).

PRKAR1A Defects

The *PRKAR1A* gene contains 10 coding exons. In 2010, 117 different mutations had been reported in 284 unrelated families of various ethnic origins (Horvath *et al.*, 2010) and new mutations continue to be reported. In more than 60% of CNC index cases a *PRKAR1A* defect can be detected, including 80% of CNC familial cases and 37% of CNC sporadic cases (namely without a family history suggestive of CNC). The majority of the mutations in sporadic cases are also de novo (Bertherat *et al.*, 2009). Molecular alterations are point mutations or small deletions involving up to 15 bp (Bertherat *et al.*, 2009; Horvath *et al.*, 2010) although larger gene deletions have been described in rare cases (Horvath *et al.*, 2008a). *PRKAR1A* is a suppressor gene according the Knudson theory: one allele is inactivated by a germline mutation and the second allele is inactivated in the tumor (**Fig. 4**A). Mutations are spread along the exons and their adjacent intronic sequences. Exons 2, 3, 5, 7, and 8 are more frequently involved.

Mutations have been categorized in groups according to their molecular consequences. The first group including 80% of the mutations (nonsense, frame shift or splice variants) leads to a premature stop codon causing degradation of the messenger RNA (mRNA) by a mechanism of Nonsense Mediated mRNA Decay (NMD). In this case no mutant protein is produced. In the tumor,

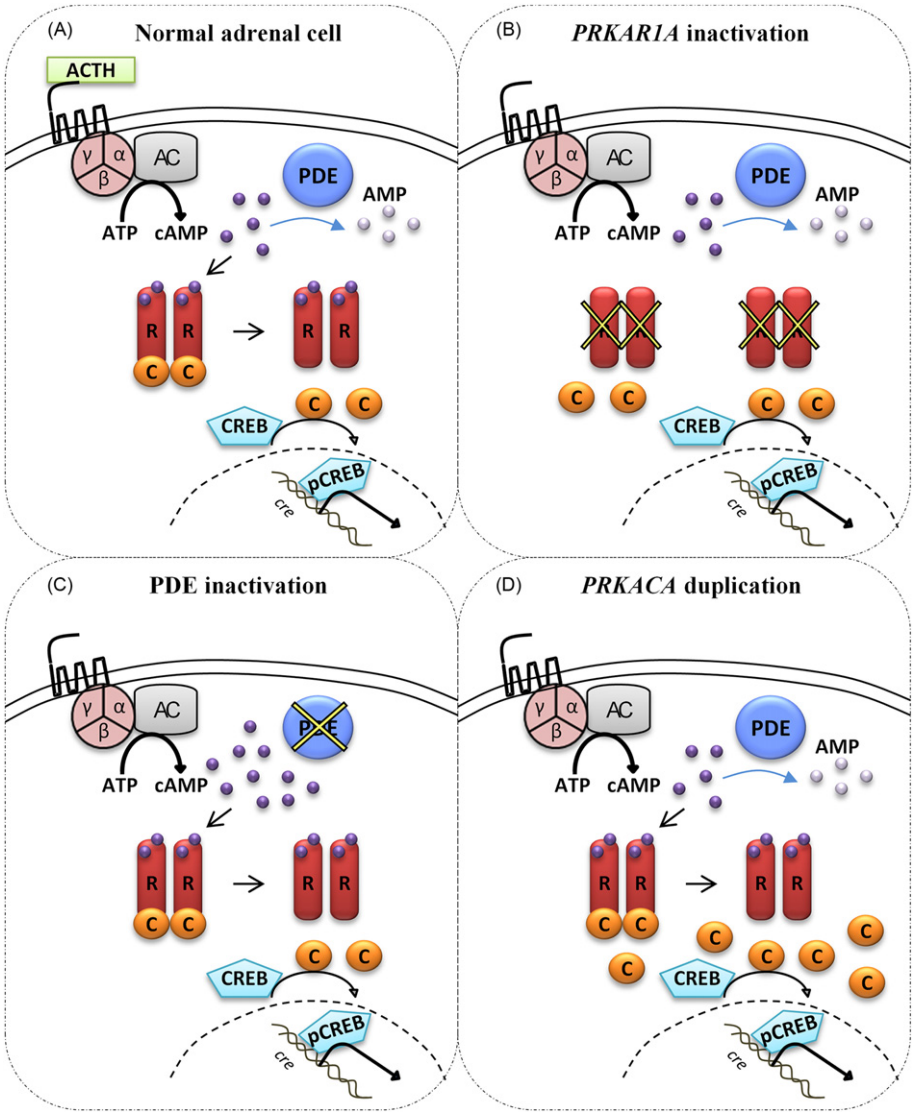

Fig. 3 Mechanism of activation of PKA pathway in micronodular hyperplasia. (A) Normal activation of the cAMP pathways by ACTH. (B) Inactivating mutations of the regulatory subunit I alpha of the PKA (*PRKAR1A*) lead to the dissociation of the catalytic subunits in the absence of cAMP. (C) Inactivation of phosphodiesterase by mutation leads to an accumulation of cAMP and overactivation of the pathway. (D) Duplication of *PRKACA* gene leads to an overexpression of catalytic subunits alpha and increase of its effect on target genes. *ACTH*, Adrenocorticotropic hormone; *AC*, adenylate cyclase; *PKA*, protein kinase A; *PDE*, phosphodiesterase; *R*, regulatory subunits of protein kinase A; *C*, catalytic subunits of protein kinase A; *CREB*, cAMP response binding protein. Adapted from Espiard, S. and Bertherat, J. (2015). The genetics of adrenocortical tumors. *Endocrinology and Metabolism Clinics of North America* **44**, 311–334.

the loss of the other allele is frequently observed. The second group of mutations escapes the NMD and leads to a defective mutant protein with an altered sequence for missense mutations. Alternatively, the protein is either shorter for premature stop codon in the 3′end or large deletion, either longer for frameshift mutation located in the last coding exons. Because these mutant proteins can exert a dominant negative effect over the wild type protein, the somatic allelic loss of the wild type allele is not always required with this group of mutations (Groussin *et al.*, 2002b; Horvath *et al.*, 2010). More recently, for some frameshift mutations located in the last coding exon of the gene, a mechanism of proteasomal degradation of the mutant protein leading to an haploinsufficiency has been described (Patronas *et al.*, 2012) (**Fig. 4**A and B).

Three mutations have been found in more than three pedigrees and are considered as hot spot: c.82C>T (exon 2), c.491_492del (exon 5) and c.709(-7-2)del (intron 7). The two latter were observed in 11 and 14 families respectively (Bertherat *et al.*, 2009). The other mutations are considered as "private," identified in only one or few families.

The overall penetrance of *PRKAR1A* mutations has been evaluated around 95% by the age of 50 years. Only two mutations (c.709(-7-2)del6 and c.1A>G) have an incomplete penetrance (Horvath *et al.*, 2010). Some genotype/phenotype correlations have been established (Bertherat *et al.*, 2009):

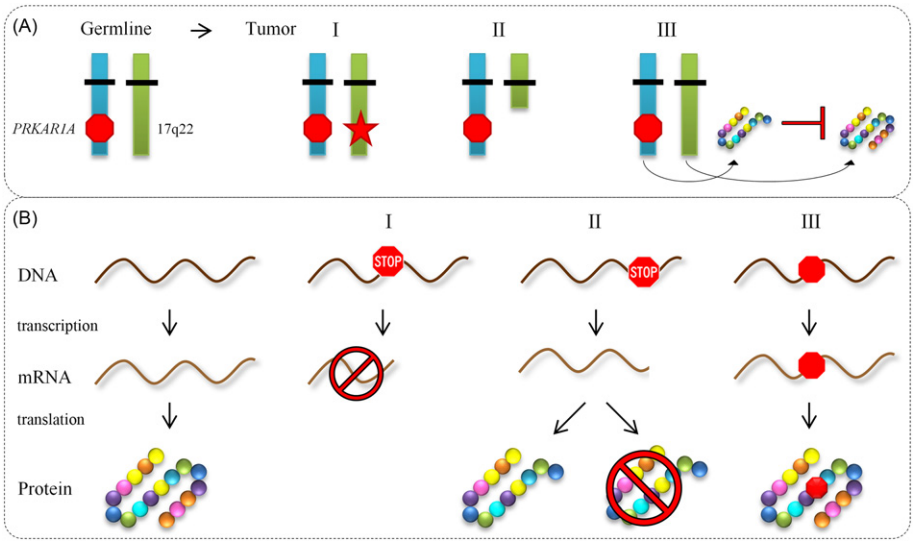

Fig. 4 Mechanism of inactivation of *PRKAR1A*. (A) One mutation (*red octagon*) inactivates one allele at the germline level. In the tumor, either the second allele is inactivated by another mutation (*red star*) (I), either the second allele is lost (II), either the mutant protein inhibits the normal function of the wild-type protein (dominant negative effect) (III). (B) The premature stop codon leads to the degradation of the mRNA (Nonsense mediated decay) (I). A mutation located at the end of the gene can lead to the translation of a defective protein (example a shorter protein for a stop codon) which can be quickly degraded by proteosome. A missense mutation leads to the translation of a defective protein (III) which can also exert a dominant negative effect on the wild-type protein.

- Intronic mutations lead to a less severe phenotype, especially the c.709(-7-2)del6 and c.1A>G mutations that are associated with isolated PPNAD.
- Exonic mutations are more often associated with acromegaly, cardiac myxomas, lentigines, and schwannomas.
- Mutations that escape NMD and lead to the translation of a mutant protein are associated with a higher number of CNC manifestations.
- The hot spot c.491–492delTG is most often associated with cardiac myxomas, lentigines and thyroid tumors.
- Patients without *RKAR1A* mutations have less myxomas at any locations and manifestations appeared latter.
- Recently, it has been suggested that the patients harboring large deletion of the gene may have a severe phenotype (Bataille *et al.*, 2014; Stelmachowska-Banas *et al.*, 2017).

Others Genetic Defects

Phosphodiesterases genes

The phosphodiesterases (PDE) hydrolyze cAMP and/or cGMP to decrease their cellular level. Inactivating germline mutations of *PDE11A* have been found in children with iMAD (Horvath *et al.*, 2006a). In addition, rare single nucleotide polymorphisms leading to a slight decrease of activity of the enzyme predispose Carney patients with *PRKAR1A* mutation to developed a PPNAD and/or a LCCST (Libé *et al.*, 2011). A mutation of the *PDE8B* gene has been described in a patient who developed PPNAD at 2-years-old (**Fig. 3**C) (Horvath *et al.*, 2008b; Rothenbuhler *et al.*, 2012).

PRKACA

Germline duplication of the locus at chromosome 19p containing the *PRKACA* have been described in patients presenting iMAD or isolated PPNAD responsible of an early and severe CS (**Fig. 3**D) (Beuschlein *et al.*, 2014; Lodish *et al.*, 2015).

Mice Models of CNC and Adrenal Hyperplasia

Various transgenic mice models have been developed to study the consequences of the inactivation of *PRKAR1A* on tumors development. *Prkar1a* homozygous knock-out mice died during embryonic development (Amieux *et al.*, 2002). To bypass the embryonic lethality, transgenic mice carrying inducible antisense transgene were established. These mice develop different tumors including thyroid hyperplasia and adenomas, adrenocortical hyperplasia, lymphomas, hepatocellular carcinomas, sarcomas, and others mesenchymal tumors (Amieux *et al.*, 2002). Heterozygous mice survive and develop only schwannomas, thyroid tumors and fibro-osseous bone lesions (Kirschner *et al.*, 2005) meaning that a complete loss a *prkar1a* is necessary for the development of the majority of tumors. Mice with a tissue-specific inactivation of *prkar1a* develop tumors of the targeted tissue, especially pituitary (Yin *et al.*, 2008a), heart (Yin *et al.*, 2008b), thyroid (Pringle *et al.*, 2012), neural crest (Jones *et al.*, 2008), and pancreatic tumors

Table 2 Summary of the murine models of carney complex (inactivation of *PRKAR1A*)

Mouse models	*Phenotype*	*References*
General knock out/down		
Prkar1a–/–	Death at E9.5 No functional heart tube development	Amieux *et al.* (2002)
Prkar1a + / –	Non pigmented schwannomas Fibro-osseous bone lesions Thyroid tumors Liver tumors Reduced fertility for male (sperm abnormality)	Kirschner *et al.* (2005) and Burton *et al.* (2006)
Prkar1a X2AS – / – (*tTA/X2AS* line)	Thyroid follicular hyperplasia and adenomas Adrenocortical hyperplasia Lymphomas Other mesenchymal tumors	Griffin *et al.* (2004)
Tissue-specific knock out		
Adrenal cortex (*Akr1b7*- CRE)	Cushing syndrome Adrenal tumors	Sahut-Barnola *et al.* (2010)
Cardiac (*αMHC*- CRE)	Death at E11.5 to 12.5 Thin-walled, dilated hearts Cardiac myxomatous degeneration	Yin *et al.* (2008b)
Pituitary (*GHRHR*- CRE)	Alterations of the GH axis Pituitary tumors with GH, prolactin, or TSH immunostaining	Yin *et al.* (2008a)
Neural crest precursor (*TEC3KO*)	Schwannomas	Jones *et al.* (2008)
Thyroid (*Tpo*- CRE)	Hyperthyroidism Benign and malignant follicular thyroid neoplasms	Pringle *et al.* (2012)
Pancreas (*Pdx1*-CRE)	Nonfunctioning endocrine or mixed endocrine/acinar cell carcinomas	Saloustros *et al.* (2017)

Adapted from Espiard, S. and Bertherat, J. (2013). Carney complex. *Frontiers of Hormone Research* **41**, 50–62.

(Saloustros *et al.*, 2017) (**Table 2**). Inactivation of *prkar1a* in the adrenal cortex lead to corticosteroids overproduction and a bilateral corticoadrenal hyperplasia caused by improper maintenance and proliferation of fetal adrenal cells (Yu *et al.*, 2012). Transgenic mice with inactivation of *Pde8b* and *Pde11* do not develop adrenal hyperplasia (Szarek and Stratakis, 2014).

Others Signaling Pathway

PRKAR1A haploinsufficiency is a general tumorigenic signal and may need additive effects of other oncogene, inactivation of other tumor suppressors and/or tissue-specific factors. Involvement of other signaling pathways has been also shown in CNC tumors as the activation of the Wnt/β-catenin signaling pathway in PPNAD (Almeida *et al.*, 2012; Horvath *et al.*, 2006b; Tadjine *et al.*, 2008). A cross-talk with the MAPK (mitogen activated protein kinase) signaling pathway (Robinson-White *et al.*, 2006) and the mTOR (mammalian target of rapamycin) pathway (de Joussineau *et al.*, 2014) contributes to the development of the adrenal micronodular hyperplasia. Interestingly, the activation of the mTOR pathway causes autophagic deficiency in *prkar1a* – / – mouse embryonic fibroblasts. Hyperpigmentation in PPNAD nodules is due to an accumulation of lipofuscin and may be a consequence of autophagic deficiency (Mavrakis *et al.*, 2006).

Conclusion

The PPNAD and the iMAD are rare causes of CS in children and young adults. Most of the PPNAD are part of the CNC. The CNC is a rare genetic neoplasia syndrome leading to a large spectrum of endocrine and nonendocrine tumors which has been well described. However, diagnostic difficulties persist. The identification of early manifestations, especially dermatologic lesions, is essential for the diagnosis and the follow-up since there are associated with more severe disease. After identification of a *PRKAR1A* defect in an index case, familial screening can be offered. Screening of all manifestations at the diagnosis of the disease or from 4-years-old in relative harboring *PRKAR1A* defect may prevent complications of life threatening manifestations as cardiac myxomas or malignant tumors. There are no clear recommendations about the follow-up of patients yet. Also, multidisciplinary management and follow-up in reference centers of these patients are important.

See also: Cushing Syndrome; Screening and Differential Diagnosis. Genetics of Familial Forms of Cortisol-Secreting Adrenal Tumors and Hyperplasias

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Genetics of Familial Forms of Cortisol-Secreting Adrenal Tumors and Hyperplasias

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Abbreviations

ACA	Benign unilateral adenomas
ACC	Adrenocortical carcinomas
ACT	Adrenocortical tumors
ACTH	Adrenocorticotropic hormone
AIMAH	ACTH-independent macronodular adrenal hyperplasia
AIMBAD	ACTH-independent massive bilateral adrenal disease
AMAH	Autonomous macronodular adrenal hyperplasia
APC	Adenomatous polyposis coli
ARMC5	Armadillo repeat containing 5
BAH	Bilateral adrenocortical hyperplasia
BWS	Beckwith–Wiedemann syndrome
cAMP	Cyclic adenosine monophosphate
cAMP/PKA	Cyclic AMP/protein kinase A
CDKN1C	Cyclin-dependent kinase inhibitor 1C
CNC	Carney complex
cPPNAD	CNC-associated PPNAD
DMR	Differentially methylated region
FAP	Familial adenomatous polyposis
GIP/GIPR	Gastric inhibitory polypeptide/gastric inhibitory polypeptide receptor; also known as "glucose-dependent insulinotropic polypeptide" and "glucose-dependent insulinotropic polypeptide receptor", respectively
GNAS	Guanine nucleotide binding protein, alpha stimulating
HNPCC	Hereditary nonpolyposis colorectal cancer syndrome
IC	Imprinting center
IGF2	Insulin-like growth factor 2
iMAD	Isolated micronodular adrenocortical disease
iPPNAD	Isolated PPNAD
LFL	Li–Fraumeni-like syndrome
LFS	Li–Fraumeni syndrome
LH/hCG	Luteinizing hormone/human chorionic gonadotropin
LOH	Loss of heterozygosity
LS	Lynch syndrome
MAS	McCune–Albright syndrome
MEN 1	Multiple endocrine neoplasia type 1
MMAD	Massive macronodular adrenocortical disease
MMR	Mismatch repair
MSI	Microsatellite instability
NLS	Nuclear localization signal
PA	Primary aldosteronism
PBAD	Primary bimorphic adrenocortical disease
PBMAH	Primary bilateral macronodular hyperplasia
PDE	Phosphodiesterase
PKA	Protein kinase A
PPNAD	Primary pigmented nodular adrenocortical disease
PRKAR1A	Protein kinase A regulatory subunit 1A
UDP	Uniparental disomy

Introduction

Cortisol-producing adrenocortical tumors (ACT) collectively characterize a wide variety of neoplasms affecting the adrenal cortex. They may be divided into three major categories: benign unilateral adenomas (ACA), bilateral adrenocortical hyperplasias (BAH), and adrenocortical carcinomas (ACC), representing 75%–90%, 10%, $<5\%$ of all adrenocortical neoplasms, respectively (Lodish, 2017). Despite shared signaling pathways, each one of these entities has specific pathophysiologic, secretory, and genetic profiles. For example, the degree of cortisol oversecretion is very variable within these adrenocortical tumors leading to different degrees of severity of Cushing syndrome. Most ACTs occur sporadically; less than 10% of ACTs are due to hereditary conditions, mostly familial tumor syndromes, such as multiple endocrine neoplasia type 1 (MEN 1), Beckwith–Wiedemann syndrome (BWS), familial adenomatous polyposis (FAP), Li–Fraumeni syndrome (LFS), and Lynch syndrome (LS), considered among the 10 most penetrant syndromes (Nagy *et al.*, 2004). Each one of these inherited tumor syndrome is caused by genes (*MEN1*, *APC*, *MMR*, *TP53*, *IGF2*, and *CDKN1C*) that represent different biological pathways (**Table 1**). BAHs, on the other hand, and about half of all ACAs are linked to alterations of the cAMP/PKA pathway (**Table 2**). The discovery of causative genes in the inherited tumor syndromes has led to the identification of molecular defects associated with sporadic ACTs (Libé and Bertherat, 2005). The most recent wider availability of advanced sequencing technologies has greatly enhanced the molecular elucidation of all ACTs. In addition, genetics assisted in the discovery of new entities and, hence, a new classification of BAHs was proposed which incorporated histological and genetic data (Stratakis and Boikos, 2007). The most recent description of *ARMC5* mutations in primary bilateral macronodular adrenocortical hyperplasia (PBMAH) further enhanced our understanding of BAH and changed dramatically the management of these patients (Assié *et al.*, 2013). This article covers these new developments.

Table 1 Genetics of familial tumor syndrome and adrenal lesions associated

Syndrome	*Associated gene*	*Chromosomal location*	*Protein function*	*Prevalence of ACT*	*Type of ACT*	*Cortisol-secreting adrenal tumors (%)*
MEN1	*MEN1*	11q13.1	Transcriptional regulator	Between 9% and 73%	– Adenomas – Nonactive bilateral adrenal (most of them) – PBMAH – ACC	<5%
BWS	– *IGF2* – *DMR1* – *H19* – *CDKN1C* – *DMR2* – *KCNQ1OT1*	IC1 IC2	11p15.5 – Regulate cell growth and differentiation – Growth and development regulatory ncRNAs – Cell cycle – Chromatin regulatory ncRNAs	Up to 20%	– Benign including cysts and adrenals hyperplasia – ACC (1% of cases of BWS children)	Variable level of cortisol
FAS	*APC*	5q21	Wnt/βcatenin pathway	~ 7% and up to 13% in patients with family history	– PBMAH – Nonfunctional cortisol producing adenoma	Five cases reported as presenting an excessive secretion of cortisol
LS	– *MLH1* – *MSH2* – *MSH6* – *PMS2*	– 3p22.2 – 2p21–p16.3 – 2p16.3 – 7p22.1	DNA mismatch repair	Rare (the prevalence of LS in ACC patients is ~3%)	ACC	Variable level of cortisol
LFS	*TP53*	17p13	Maintaining the integrity of the genome "guardian of the genome"	Up to 11.56%	ACC	Variable level of cortisol

MEN1, Multiple endocrine neoplasia type 1; *BWS*, Beckwith–Wiedemann syndrome; *FAS*, familial adenomatous polyposism; *LS*, Lynch syndrome; *LFS*, Li–Fraumeni syndrome; *PBMAH*, primary bilateral macronodular hyperplasia; *ACC*, adrenocortical carcinomas; *IC*, imprinting center.

Table 2 Genetics of familial bilateral adrenal hyperplasia

Adrenal lesions	*Age patients*	*Associated gene*	*Chromosomal location*	*Protein function*	*Cortisol-secreting adrenal tumors (%)*
PPNAD	~10–40 years old	– *PRKAR1A* – Locus 2p16 – *PDE11A* – *PDE8B*	– 17q22–24 – 2q31.2 – 5q13	cAMP/PKA pathway	Variable level of cortisol
PBMAH	~50–60 years old	– *MEN1* – *APC* – *FH* – *PDE11A* – *ARMC5* – *PRKACA*	– 11q13.1 – 5q21 – 1q43 – 2q31.2 – 16p11.2 – 19q13	– Transcriptional regulator – Wnt/b-catenin pathway – Krebs cycle – cAMP/PKA pathway – Indirect or direct role in steroidogenesis, cell cycle, T-cell function, fetal development and cAMP/PKA pathway – cAMP/PKA pathway	2% of overt Cushing syndrome Unknown % of bilateral modest cortisol-secreting incidentalomas

PBMAH, Primary bilateral macronodular hyperplasia; *PPNAD*, primary pigmented nodular adrenocortical disease.

Multiple Endocrine Neoplasia Type 1

Multiple endocrine neoplasia type 1 (MEN 1) or Werner syndrome is caused by germline mutations in the *MEN1* gene. The human *MEN1* gene, which maps to chromosome 11q13.1, encodes for the *menin* protein (a molecule of 610 amino acids with a molecular weight of 68 kDa). The primary structure of menin reveals several domains involved in interaction with its partners and

a nuclear localization signal (NLS) domain influencing its cellular location. The nuclear scaffold protein menin modulates multiple processes related directly or indirectly to gene expression. Menin behaves as a tumor suppressor in endocrine cells, following the well-known Knudson's two hit mechanism. The MEN 1 syndrome is most often familial (approximately 90% of cases have inherited a mutation); very few cases are documented de novo defects. More than 70% of the patients diagnosed with MEN 1 syndrome are carriers of a germline mutation in the *MEN1* gene (Falchetti, 2010; Marini *et al.*, 2009); approximately 10% or more may harbor *MEN1* gene and/or chromosomal deletions that are not detected by simple sequencing. MEN 1 patients have a very high risk of developing multiple tumors involving several endocrine tissues: the parathyroid glands (up to 95% of cases), pancreas (50%–70% of cases), and the pituitary (20%–40% of cases) (Angelousi *et al.*, 2016; Falchetti, 2010; Lodish, 2017). Other tumors include adrenocortical hyperplasia and/or adenomas, and other ACTs, thyroid neoplasms, and lesions in nonendocrine tissues, such as facial angiofibromas, lipomas, meningiomas, and collagenomas. ACTs are found in as many as half of the patients with MEN 1 (a prevalence of 9%–73% has been reported) depending on the age of the patients and screening approaches (Gatta-Cherifi *et al.*, 2012; Goudet *et al.*, 2015; Langer *et al.*, 2002; Schaefer *et al.*, 2008; Skogseid *et al.*, 1995; Waldmann *et al.*, 2007). ACTs in MEN 1 develop either unilaterally or bilaterally and may be adenomas, macronodular hyperplasia, or even carcinoma; however, most adrenal lesions in MEN 1 are bilateral and functionally nonactive adenomas (20%–40%). Less than 5% of adrenal lesions in MEN 1 are cortisol-producing (Gatta-Cherifi *et al.*, 2012; Langer *et al.*, 2002; Schaefer *et al.*, 2008; Skogseid *et al.*, 1995; Waldmann *et al.*, 2007). Interestingly, Thevenon and collaborators reported, from a large cohort constituted of 797 patients from 265 families, that ACTs in patients carrying *MEN1* mutations affecting the JunD-interacting domain have a high heritability (approximately 65%). They established a reversed correlation between the adrenal tumor and the degree of relatedness; meaning that the familial correlation coefficient trends to be reduced between first- and second-degree relatives (0.16 vs. 0.14) and more dramatically for the third-degree relatives (0.04) (Thevenon *et al.*, 2015). This study supports the idea that the adrenal lesions are a common manifestation of MEN1 syndrome but more importantly that their growth depends on an inherited predisposition. Based on recent data from a MEN1 cohort including young patients diagnosed before the age of 21 years, Goudet and collaborators showed that ACC represents 1% of all lesions described. The authors mentioned that first analysis was undertaken in the late 1990s with different testing over the years and, thus, may represent an underestimate (Goudet *et al.*, 2015). Others found no ACTs among patients aged from 6 to 31 (Vannucci *et al.*, 2017); it is possible that ACTs develop later in patients with MEN 1 but when they do develop earlier they are more likely to be malignant (Goudet *et al.*, 2015). In summary, patients with MEN 1 develop ACTs and they may be screened for these; however, aggressive treatments and invasive approaches (i.e., adrenal surgery) should be individualized and reserved for the most likely to be functional and/or malignant.

Beckwith–Wiedemann Syndrome

Beckwith–Wiedemann syndrome (BWS) was first described by Wiedemann in 1964 (Wiedemann, 1964) and Beckwith in 1969. BWS is the most common overgrowth syndrome in infancy, with an incidence similar for female and male estimated of approximately 1 in 13,700 (Angelousi *et al.*, 2016; Pettenati *et al.*, 1986; Thorburn *et al.*, 1970; Weksberg *et al.*, 1993). BWS is characterized by a broad spectrum of symptoms including macrosomia, hemihyperplasia, macroglossia, abdominal wall defects (umbilical hernia, omphalocele, diastasis recti), neonatal hypoglycemia, ear creases, visceromegaly, renal malformations, facial nevus flammeus (Mazzuco *et al.*, 2012; Shuman *et al.*, 1993; Weksberg *et al.*, 1993). BWS results in genetic and epigenetic dysregulations of several growth regulatory genes mapped on chromosome 11p15.5. These genes are under control of imprinting center (IC), leading to a predominant or exclusive expression from either maternal or paternal alleles. Among them, the paternally expressed genes *IGF2* (insulin-like growth factor 2) and *H19* clustered in the IC1 and the maternally expressed genes *CDKN1C* (cyclin-dependent kinase inhibitor 1C or $p57^{KIP2}$) and *KCNQ1OT1* in IC2 are clearly links to BWS. Both IC contain a differentially methylated region DMR1 and DMR2, respectively (Choufani *et al.*, 2010; Weksberg *et al.*, 2003). Genetic alterations in BWS include loss of methylation of the IC2 on the maternal chromosome leading to an increased activity of the *KCNQ1OT1* gene (50%), paternal uniparental disomy (UPD) of 11p15.5 (20%), gain of methylation of the IC1 on the maternal chromosome (5%), mutation of the maternal CDKN1C allele (5%), and duplication, inversion or translocation of the chromosome 11p15.5. In approximately 15% of cases the cause remains unknown (Choufani *et al.*, 2010; Weksberg *et al.*, 2003, 2005). Children with BWS are predisposed to tumor development with an estimated overall risk of 7.5% (range between 4% and 21%), manifesting in the first decade of the life (Rump *et al.*, 2005; Shuman *et al.*, 1993; Weksberg *et al.*, 2010). The more frequent tumors are Wilms tumor, hepatoblastoma, neuroblastoma, rhabdomyosarcoma, and ACTs or rhabdomyosarcoma (Else *et al.*, 2014; MacFarland *et al.*, 2017; Mussa *et al.*, 2016). Most ACTs are benign including cysts and adrenal hyperplasia; they occur in up to 20% of all BWS cases; ACCs are described in 1% of children with BWS (Else, 2012; Lapunzina, 2005). A recent meta-analysis combining BWS cases studied in the last 15 years confirmed the high frequency of ACTs, present in 9.4% of 1370 patients (Mussa *et al.*, 2016). Few of these tumors are cortisol- and/or androgen-producing (Cardinalli *et al.*, 2012; Carney *et al.*, 2012; Hertel *et al.*, 2003; Schofield *et al.*, 1995).

On the other hand, one of the hallmarks of sporadic ACC is the aberrant expression of IGF2, a cell survival factor stimulating cell proliferation but unable to drive the tumorigenesis on its own (Drelon *et al.*, 2012; Guillaud-Bataille *et al.*, 2014). Elevated expression of IGF2 is also the most common molecular change seen in adrenal tumors associated with BWS, resulting from gain of methylation in IC1, duplication of the paternal allele, or some alterations in epigenetic imprinting at 11p15. Recently, aberrant expression of IGF2 were reported in 78% of patients with ACT, confirming the frequent changes in the IGF2 expression related to ACC in BWS (MacFarland *et al.*, 2017). In most cases, occurrence of BWS is sporadic (80%–85%) rather than familial (10%–15%)

(Weksberg *et al.*, 2010). A pathogenic variant is reported in *CDKN1C* gene in 40% of familial cases (Riccio *et al.*, 2009; Shuman *et al.*, 1993). Despite of low expression of CDKN1C regularly reported in ACC, mutations of this gene are not commonly found (Barzon *et al.*, 2001; MacFarland *et al.*, 2017). Interestingly, patients with dysregulation of imprinting center IC1 (IGF2/H19) present fewer mutations in *CDKN1C* gene, which do not seem to influence *IGF2* expression (Brioude *et al.*, 2015). Other familial BWS cases were explained by microdeletions at IC1 and more rarely microduplication at IC2 (De Crescenzo *et al.*, 2011; Shuman *et al.*, 1993). It is unclear what is the percentage of BWS cases that have excessive steroid hormone secretion. However, patients with BWS need to be carefully followed up for lesions of the adrenal in general, ACC development in particular, and the possible link to excessive steroid hormone secretion (Brioude *et al.*, 2013; MacFarland *et al.*, 2017).

Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) represents approximately 1% of all colorectal cancer cases, making it the second multiple colorectal most frequent cause of inherited colorectal cancer (Lv, 2017). A germline mutation and then somatic loss of heterozygosity (LOH) of the *APC* (adenomatous polyposis coli) gene on chromosome 5q21 lead to the development of colorectal cancer in patients with FAP with a nearly 100% penetrance (Waller *et al.*, 2016). APC is a negative regulator of the Wnt/β-catenin signaling pathway, belonging to the β-catenin destruction complex which is composed by axin, CK1, GSK3β. The turnover of the β-catenin is controlled by phosphorylation-dependent proteasome degradation via this destruction complex; Wnt stimulation results in the inactivation of the complex degradation, leading to the accumulation and nuclear translocation of β-catenin. Nuclear β-catenin protein may then carry out its oncogenic transcriptional activity inducing the expression of a number of target genes (Berthon and Stratakis, 2014; Eshghifar *et al.*, 2017).

Patients with FAP develop, at an early age, multiple polyps in the lower gastrointestinal tract which may then lead to colorectal cancer, following the classically described adenoma–carcinoma sequence. These patients are also subject to the formation of other types of cancers such as adrenocortical tumors. More than a century ago, the first case of adrenal tumor associated with FAP was published in 1912 by Devic and Bussy; subsequent cases were mainly discovered at autopsies (Naylor and Gardner, 1981; Robinson *et al.*, 1972). Today, with imaging surveillance of FAP patients we know that the prevalence of adrenal masses is approximately 7% (Marchesa *et al.*, 1997) to 13% (Smith *et al.*, 2000), which is significantly higher than that in the general population (at approximately 3%) (Herrera *et al.*, 1991). Only few genetic defects were identified in *APC* gene in ADTs, in codons 2016–2017, 1542, 1577, 1981, 1061 (Beuschlein *et al.*, 2000; Hosogi *et al.*, 2009; Kartheuser *et al.*, 1999; Rekik *et al.*, 2010; Wakatsuki *et al.*, 1998). Interestingly, codon 1061 is known as frequently mutated in patients with FAP (8%) (González *et al.*, 2005). Nuclear localization of β-catenin is frequently seen in ACTs (Gaujoux *et al.*, 2010). Most patients with FAP have bilateral macronodular adrenal hyperplasia (Hsiao *et al.*, 2009; Kartheuser *et al.*, 1999; Yamakita *et al.*, 1997); few have been described with nonfunctioning ACAs and, rarely, ACC (Beuschlein *et al.*, 2000; Chelaïfa *et al.*, 2003; Naylor and Gardner, 1981; Painter and Jagelman, 1985; Pinés Corrales *et al.*, 2006; Wakatsuki *et al.*, 1998). To our knowledge, only four cases have been reported as presenting with Cushing syndrome due to a cortisol-producing ACA (Beuschlein *et al.*, 2000; Marchesa *et al.*, 1997; Rekik *et al.*, 2010); one patient with FAP presented with an ACA and hyperaldosteronism (Pinés Corrales *et al.*, 2006). In summary, even though ACTs are a rare manifestation of FAP, patients with this condition should be clinically followed for the development of both functioning and nonfunctioning ACTs, as well as the occasional ACC.

Lynch Syndrome

Lynch syndrome (LS), also known as hereditary nonpolyposis colorectal cancer syndrome (HNPCC), represents approximately 3% of all colorectal cancer cases, making it the most frequent cause of inherited colorectal cancer (approximately 15% of all colorectal cancer cases) (Rustgi, 2007; Shawki and Kalady, 2016). LS is an autosomal dominant genetic disorder caused by mutations in *MLH1*, *MSH2*, *MSH6*, and *PMS2* genes, belonging to the Mut-related family of DNA mismatch repair (MMR) genes (Kastrinos and Syngal, 2012). Ninety percent of mutations occurs in the *MLH1* and *MSH2* genes (Peltomäki, 2001). Germline mutations in any one of four genes leads to either microsatellite instability (MSI) or at the loss of expression of the related protein. Patients with LS are predisposed to colorectal cancer development with an estimated overall risk up to 80%, with peak age of incidence between 44 and 61 years (Bhattacharya and McHugh, 2017). Moreover, LS patients have a high risk of developing a carcinoma of endometrium (up to 60% of females), ovary, gastric, small bowel, hepatobiliary system, pancreas, central nervous system, lung adenocarcinoma, sarcoma, and ACC (Bhattacharya and McHugh, 2017; Raymond *et al.*, 2013). Even if ACC is not the most frequent cancer in LS, the prevalence of LS in ACC patients (3%) is equivalent to those from patients with colorectal and endometrial cancer (2%–5%) (Liu *et al.*, 2014; Raymond *et al.*, 2013).

MSH2, *MSH6*, and *MLH1* genes were reported mutated in LS patients with ACC, leading to a loss of expression of one or several MMR proteins analyzed by IHC, similar to other LS-associated-cancer (Berends *et al.*, 2000; Challis *et al.*, 2016; Else *et al.*, 2014; Zheng *et al.*, 2016). In combining studies describing mutations in MMR genes found in LS patients with ACC, MSH2 gene seems more frequently affected by mutations in these patients having a positive family history. It should be noted that ACCs do not demonstrate microsatellite instability unlike colorectal cancer (Berends *et al.*, 2000; Broaddus *et al.*, 2004; Challis *et al.*, 2016; Karamurzin *et al.*, 2012; Medina-Arana *et al.*, 2011). Several reports have linked ACC to Lynch syndrome, and this is further

supported by a more recent study (Challis *et al.*, 2016). Patients with LS should therefore be regularly surveilled clinically for the possible development of ACC (Challis *et al.*, 2016; Medina-Arana *et al.*, 2011).

Li–Fraumeni Syndrome

Li–Fraumeni syndrome or Li–Fraumeni-like syndrome (LFS, LFL) is a rare autosomal dominant syndrome, first described by Li and Fraumeni in 1969. The gene found responsible for Li–Fraumeni syndrome is *TP53*, located on chromosome 17p13. Germline mutations in the *TP53* tumor suppressor gene are harbored by up to 80% of LFS cases, with a high penetrance estimated of at least 80% by age 50 years (Angelousi *et al.*, 2016; Hwang *et al.*, 2003; Nagy *et al.*, 2004). Penetrance was not influenced by genre although the females are a greater risk to be diagnosed with cancer and at a younger age (Hwang *et al.*, 2003). Mutations in the *BRCA2* and *CHEK2* genes have also been reported in LFS; however, patients with these mutations do not seem to develop ACTs (Bachinski *et al.*, 2005; Bell *et al.*, 1999; Evans *et al.*, 2008; Mazzuco *et al.*, 2012).

Most LFS-related tumors are brain and soft tissue sarcomas and osteosarcomas, breast cancer, leukemia, whereas ACC is relatively less frequent (Hisada *et al.*, 1998; Li *et al.*, 1988; Malkin *et al.*, 1990): up to 11.56% of patient with LFS develop ACC, which is, thus, the fourth most common cancer among patients with LFS, according to the p53 database (www-p53.iarc.fr). Other studies show a prevalence of ACC between 6.9% and 9.9% of patients with LFS (Gonzalez *et al.*, 2009; Olivier *et al.*, 2003). ACC in patients with LFS usually presents either early (before the age of 10 years) or later, in the 6th decade of life (Kleihues *et al.*, 1997; Olivier *et al.*, 2003; Wajchenberg *et al.*, 2000; Wasserman *et al.*, 2015); the former is the case with 92% of the patients with ACC and LFS, whereas the latter is true for only about 8% of them (Wasserman *et al.*, 2012). In contrast to Cushing syndrome which appears to be the most frequently presenting manifestation in adults, ACCs in children with LFS usually present with virilization (Michalkiewicz *et al.*, 2004; Sandrini *et al.*, 1997).

In southern Brazil, children exhibit an increased risk for ACC that is 10–15-fold higher than children worldwide (Garritano *et al.*, 2010; Sandrini *et al.*, 1997). This is apparently due to the R337H *TP53* mutation described in more than 90% of these patients (Borges and Ayres, 2015; Ribeiro *et al.*, 2001), a defect that is present within the oligomerization domain of the gene. Most other *TP53* mutations causing ACC are located in the DNA-binding domain of the molecule (Wasserman *et al.*, 2012). Whereas the 175, 245, 248, 249, 273, and 282 codon hotspots predominate in *TP53*-related tumors (Petitjean *et al.*, 2007), Olivier and colleagues also showed hotspots that are specific for ACC at residues 151, 152, 219, and 220 (Olivier *et al.*, 2003; Wasserman *et al.*, 2015). Thus, certain codon mutations seem to be more specific for ACC development (Heinze *et al.*, 2014). Interestingly, Petitjean and colleagues showed that the mean age of disease onset for patients with ACC carrying nonfunctional mutants is earlier, compared to partially or fully functional missense mutants (Petitjean *et al.*, 2007).

Bilateral Adrenocortical Hyperplasia

Bilateral ACTs are further divided into two types: macronodular or micronodular, defined by the size of the associated nodules in each adrenal gland, larger or smaller than 1 cm, respectively. Patients with bilateral tumors represent up to 10% of all patients with adrenal-dependent Cushing syndrome and can occur in sporadic or familial form. To date, no less than six types of bilateral adrenal hyperplasias (BAH) were described, which are distinguished from their histologic and genetic characteristics (Stratakis and Boikos, 2007) (**Table 2**).

Micronodular Bilateral Adrenocortical Hyperplasia

Micronodular BAH may be further distinguished by the presence or absence of pigment in the associated nodules: primary pigmented nodular adrenocortical disease (PPNAD) is associated with pigmentation, whereas idiopathic micronodular adrenocortical disease (iMAD) is not. Most cases of PPNAD are associated with Carney complex (CNC) (cPPNAD, ~90% of PPNAD cases), whereas few are sporadic and isolated (iPPNAD); in addition, familial isolated PPNAD has also been reported very rarely (Bertherat *et al.*, 2009; Pandey *et al.*, 2013; Stratakis and Boikos, 2007). Patients with PPNAD and CNC may present with a variety of other tumors before they present with Cushing syndrome which typically develops in late childhood or young adulthood and is more frequent in females (Groussin *et al.*, 2006; Horvath and Stratakis, 2008). Atypical or cyclical forms of Cushing syndrome are also frequent in these patients (Hofland *et al.*, 2013; Stratakis *et al.*, 1999). CNC patients may also develop cardiac myxomas, thyroid tumors, and schwanomas which can complicate the treatment of Cushing syndrome in these patients (Almeida and Stratakis, 2010; Angelousi *et al.*, 2016). CNC is transmitted in an autosomal dominant manner, involving at least two loci mapped to chromosomes 17q22–24 (CNC1) and 2p16 (CNC2). The causal gene for CNC2 remains unknown; CNC1 is caused by defects inactivating the gene coding for PRKAR1A, the regulatory subunit type 1α (R1a) of PKA (Kirschner *et al.*, 2000; Stratakis, 2002). To date, the *PRKAR1A* mutation database contains approximately 135 mutations; defects in exons 2, 3, and 7 of the gene account for more than half of all (https://prkar1a.nichd.nih.gov/hmdb/prkar1a.html). Approximately 43% of those defects cause frameshift, 25% are nonsense and 11% missense mutations, and 4% are deletions. Functionally, all defects lead to partial (~50%) or complete deficiency of the PRKAR1A mutant protein. Most defects also cause nonsense-mediated decay (NMD) of the mutant

PRKAR1A mRNA (Almeida and Stratakis, 2010; Kirschner *et al.*, 2000; Robinson-White *et al.*, 2003). More than 70% of patients with CNC, and more than 80% of patients with PPNAD have *PRKAR1A* defects, which also have high penetrance that is estimated to be near 98% by age 40 years (Almeida and Stratakis, 2010; Stratakis *et al.*, 2001).

The *PDE11A* gene, mapped on 2q31–35, was found altered in patients with PPNAD or iMAD who did not have *PRKAR1A* or *GNAS* mutations (Horvath *et al.*, 2006a). Deficiency of the protein encoded by the *PDE11A* gene is caused by truncating mutations (2 frameshift and 1 nonsense) or missense substitutions affecting conserved regions of the gene. Although the *PDE11A* gene is highly polymorphic, some inactivating mutations with low-penetrance are related to an inherited predisposition to ACTs (Carney *et al.*, 2010; Horvath *et al.*, 2006b). Interestingly, *PDE11A* variants are also frequent in patients with PPNAD who are carriers of *PRKAR1A* mutations (Libé *et al.*, 2011) suggesting that in at least some patients with PPNAD the development of Cushing syndrome may be due to the coexistence of *PRKAR1A* and *PDE11A* defects. The PDE11A protein is highly present in the adrenal cortex and other tissues, such as the gonads and the prostate. Thus, *PDE11A* genetic variants have been reported in association with testicular germ cell tumors (TGCTs) (Horvath *et al.*, 2009). To date, the contribution of the *PDE11A* gene in the development of sporadic ACTs is not fully understood; *PDE11A* defects appear to act as modifiers cooperating with other genes in predisposition to adrenocortical and other tumors, such as TGCTs (Hannah-Shmouni *et al.*, 2016).

The *PDE8B* gene on chromosome 5q13, another member of the PDE family, was also linked to iMAD and PPNAD (Horvath *et al.*, 2006a; Rothenbuhler *et al.*, 2012). First, a missense substitution (H305P) found in a young girl with Cushing syndrome due to PPNAD, without any symptoms of CNC (Horvath *et al.*, 2006a). Then, several *PDE8B* germline variants were described in association with ACTs including PBMAH, PPNAD, and ACAs (Rothenbuhler *et al.*, 2012). Rothenbuhler *et al.* described two novel damaging variants of the *PDE8B* gene, H391A and P660L, in two patients with PBMAH and a nonsecreting adrenal tumor, respectively. PDE8B expression is positively correlated with higher cortisol production in sporadic cortisol-producing ACAs (Wilmot Roussel *et al.*, 2013). In conclusion, *PDE8B* defects are rare, but like *PDE11A*, they appear to contribute to predisposition to a variety of ACTs.

Primary Bilateral Macronodular Hyperplasia

Genetics of PBMAH between 1964 and 2013

More than 50 years ago, Kirschner *et al.*, described PBMAH (primary bilateral macronodular hyperplasia) for the first time (Kirschner *et al.*, 1964). Previously called autonomous macronodular adrenal hyperplasia (AMAH), ACTH-independent massive bilateral adrenal disease (AIMBAD), giant or huge macronodular disease, AIMAH (ACTH-independent macronodular adrenal hyperplasia) or also MMAD (massive macronodular adrenocortical disease), PBMAH is now the most recently preferred name. This new term eliminates ACTH-independence from the disease's name, since Louiset *et al.* showed ACTH produced by PBMAH cells stimulating local cortisol hypersecretion (Louiset *et al.*, 2013). PBMAH is a benign entity that is seen in less than 2% of endogenous Cushing syndrome cases. It is characterized by multiple nodules in both adrenal glands leading to increased adrenal volume. Its incidence peaks between 50 and 60 years of age (Drougat *et al.*, 2015; Lodish and Stratakis, 2016).

In PBMAH, the cAMP/PKA pathway is activated by the aberrant expression of receptors like those for luteinizing hormone/human chorionic gonadotropin (LH/hCG), vasopressin (V1, V2) or the 5-HT4 serotoninergic and β-adrenergic ones. Likewise, food-dependent Cushing syndrome is due to GIPR overexpression in this variant of PBMAH. These abnormalities are seen in both sporadic and familial cases (Christopoulos *et al.*, 2005; El Ghorayeb *et al.*, 2015; Lacroix *et al.*, 2010; Libé *et al.*, 2010). Familial cases were thought to be infrequent until recently (Hsiao *et al.*, 2009). A major genetic defect causing PBMAH was identified in 2013 (Assié *et al.*, 2013)—see below. The few genes that were identified to predispose to the development of PBMAH prior to 2013, were those affecting cAMP/PKA signaling, such as *GNAS*1 (which increases cAMP); the phosphodiesterases (PDEs), PDE8B and PDE11A that are involved in the regulation of cAMP levels; the ACTH receptor gene (MC2R, melanocortin-2 receptor), as well as others involved in a variety of pathways. These include *MEN1* coding for menin (see above in this article), fumarate hydratase (FH) (De Venanzi *et al.*, 2014), and adenomatosis polyposis coli (APC) (see also above in this article) (Hsiao *et al.*, 2009).

Genetics of PBMAH after 2013

Assié *et al.* identified inactivating mutations of the *ARMC5 (Armadillo repeat containing 5)* gene (Assié *et al.*, 2013) in approximately half of all patients with PBMAH that he studied. The protein encoded by the gene *ARMC5*, located on chromosome 16p11.2, is mostly composed by two domains involved in mediating protein–protein interactions, armadillo and BTB/POZ domain and is involved in numerous cellular mechanism (steroidogenesis, immune system, proliferation, and apoptosis) (Berthon *et al.*, 2017a, b; Cavalcante *et al.*, 2017; Hu *et al.*, 2017). To date, more than 100 mutations affecting *ARMC5* have been identified in many studies from several cohorts composed by people of different origins (French, American, French–Canadian, Australian, Africo-American, Portuguese, Brazilian, Italian, Japanese, Chinese). No hotspot was described among these mutations in this gene and in PBMAH. Indeed, the mutations are uniformly distributed along the gene *ARMC5* without affecting preferentially a specific domain or part of the gene, even if some mutations can be recurrent. More than 40 novel germline and somatic mutations in the *ARMC5* gene were identified in the last 2 years representing a total of 54 germline and 48 somatic exonic mutations, combining all studies published to date (Albiger *et al.*, 2017; Alencar *et al.*, 2014; Assié *et al.*, 2013; Bourdeau *et al.*, 2016; Cavalcante *et al.*, 2017; Correa *et al.*, 2015; Elbelt *et al.*, 2014; Emms *et al.*, 2016; Espiard *et al.*, 2015; Faucz *et al.*, 2014; Gagliardi *et al.*, 2014; Mulatero *et al.*, 2016;

Rego *et al.*, 2017; Suzuki *et al.*, 2015; Yu *et al.*, 2018; Zilbermint *et al.*, 2015). Three additional mutations were identified in tumor DNA but the germline or somatic origin was not determined (Else, 2012; Espiard *et al.*, 2015). Forty-two percentage of these defects are missense mutations, 36% frameshift, 18% nonsense, and 4% deletions. Interestingly, there are differences between germline and somatic mutations. Half of the somatic mutations are frameshift, the remaining equally divided between missense (21.9%) and nonsense ones (21.9%) with only 2% being deletions. Most of germline defects are missense (58%), followed by frameshift (27.6%), nonsense defects (14%), and deletions (4%). These data are from a total of seven studies, representing 58% of all mutations identified to date (Drougat *et al.*, 2015). Patients operated for PBMAH harboring *ARMC5* mutations present with more severe Cushing syndrome than those without *ARMC5* defects (Albiger *et al.*, 2017; Espiard *et al.*, 2015); however many *ARMC5* mutation carriers have only mild cortisol excess or only unilateral lesions when identified (Alencar *et al.*, 2014). ARMC5 defect may also predispose to meningioma and primary aldosteronism (PA) (Gagliardi *et al.*, 2014). Two different cohorts of 56 and 39 patients, respectively, reported PA among *ARMC5* mutation carriers (Mulatero *et al.*, 2016; Zilbermint *et al.*, 2015). Two studies showed *ARMC5* defects predisposing to meningioma within kindreds with PBMAH (Elbelt *et al.*, 2014; Alencar *et al.*, 2014). However, ARMC5 mutations underlie only 25% of all cases of PBMAH; germline amplification of the *PRKACA* gene may be associated with PBMAH in a few cases (Beuschlein *et al.*, 2014; Lodish *et al.*, 2015).

McCune–Albright syndrome and ACTs

MAS is associated with infantile Cushing syndrome (Angelousi *et al.*, 2015). *GNAS1* defects cause PBMAH without necessarily an MAS phenotype in older adults (Fragoso *et al.*, 2003). Cushing syndrome in children with MAS may vary from very severe and often lethal to mild and atypical that eventually burns out; the histology of these lesions is also interesting from simply hyperplasia of fetal adrenal-looking cells to distinct adenomas like in PBMAH (Carney *et al.*, 2011).

Conclusions

Our understanding of the genetics of ACTs has remarkably expanded over the last decade and is constantly evolving. New diseases and previously unknown genetic defects have been described that may lead to new medical treatments for these patients. It is essential that clinicians understand the connections between ACTs and inherited predisposition to other tumors in the context of genetic syndromes that have recently been molecularly elucidated.

See also: Adrenocortical Tumors and gsp Mutations. PPNAD, Carney Complex, and Other Micronodular Adrenal Hyperplasias. Primary Bilateral Macronodular Adrenal Hyperplasia

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Primary Bilateral Macronodular Adrenal Hyperplasia

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Nomenclature

ACTH Adrenocorticotropic hormone or corticotropin
AIMAH ACTH-independent bilateral macronodular adrenal hyperplasia
APC Adenomatous polyposis coli
ARMC5 Armadillo repeat containing 5 gene
BMAH Bilateral macronodular adrenal hyperplasia
cAMP Cyclic adenosine monophosphate
CRH Corticotropin releasing hormone
CS Cushing syndrome
CT scan Computed tomography scan
FDG-PET scan Fluorodeoxyglucose-positron emission tomography scan
GIP Glucose-dependent insulinotropic peptide or gastric inhibitory peptide
GNAS Stimulatory G-protein alpha subunit
GnRH Gonadotropin-releasing hormone
GPCR G-protein coupled receptor
HLRCC Hereditary leiomyomatosis and renal cell cancer
MC2R Melanocortin 2 receptor (ACTH receptor)
MRAP Melanocortin receptor associated protein
MRI Magnetic resonance imaging
NCCAH Nonclassic congenital adrenal hyperplasia
PBMAH Primary BMAH
PDE11A Phosphodiesterase isoform 11A
PKA Protein kinase A
PPNAD Primary pigmented nodular adrenal dysplasia
PRKACA Protein kinase A catalytic subunit α
SNP Single nucleotide polymorphisms
UFC Urinary free cortisol in 24-h collection
WNT Wingless-related integration site

Glossary

ARMC5 ARMC5 was recently identified as a gene in which germline and somatic mutations are causative in 25%–50% of BMAH; it is thought to be a tumor suppressor gene but its precise function is still unknown.

Autocrine/paracrine stimulation Several compounds are able to induce hyperfunction and proliferation of adrenocortical cells. In addition to ACTH, serotonin, vasopressin, and other molecules can stimulate adrenal function in a paracrine way.

Fumarate hydratase Fumarate hydratase (FH) is a mitochondrial enzyme. Rare cases of BMAH were associated with mutations of FH.

Menin Menin is a tumor suppressor protein, known to regulate cell proliferation and differentiation. In MEN-1 mutation carriers, a higher incidence of adrenal nodules or BMAH is reported.

Wnt/β-catenin signaling The Wnt (Wingless-related integration site) signaling pathways are a group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors. In the absence of a Wnt signal, β-catenin interacts with a destruction complex composed by adenomatous polyposis coli (APC), AXIN proteins, and glycogen synthase kinase-3 β (GSK3 β), thus facilitating its phosphorylation by casein kinases 1 alpha (CK1α). The destruction of the phosphorylated complex is mediated by the activation of the ubiquitin pathway. BMAH can occur in patients with familial adenomatous polyposis (FAP).

Introduction

Endogenous Cushing syndrome (CS) is a morbid condition secondary to chronic excess production of cortisol which is secondary to diverse tumors with complex molecular mechanisms (Lacroix *et al.*, 2015). The syndrome is divided as either ACTH-dependent or independent; the majority are ACTH-dependent resulting from excess production of ACTH by pituitary corticotropinomas in 70% of cases (Cushing disease) or less frequently from nonpituitary malignant or benign tumors (ectopic ACTH syndrome) (Lacroix *et al.*, 2015). Approximately 15%–20% of cases are independent of ACTH of pituitary origin, and those are mainly secondary to cortisol-secreting unilateral adenomas or carcinomas (Lacroix *et al.*, 2015). CS rarely (<2%) results from primary bilateral nodular hyperplasias, either bilateral macronodular (nodules larger than 1 cm) adrenal hyperplasia (BMAH or PBMAH—which have replaced the previous term ACTH-independent macronodular adrenal hyperplasia (AIMAH)) or micronodular (nodules <1 cm) hyperplasia including primary pigmented nodular adrenal dysplasia (PPNAD) (Stratakis, 2008; Lacroix, 2009; Lacroix *et al.*, 2015). BMAH with mild or modest cortisol secretion (previously referred to as subclinical) is more frequent than those with overt CS, but its precise prevalence has not been determined in population studies (Lacroix, 2009). As 10%–15% of adrenal incidentalomas found in ~4% of adults are bilateral, a high proportion is secondary to BMAH with modest production of cortisol (Fassnacht *et al.*, 2016; Lacroix, 2009). BMAH needs to be distinguished from ACTH-dependent bilateral macronodular

adrenal hyperplasia secondary to long-term adrenal stimulation in patients with Cushing's disease or ectopic ACTH syndrome (Smals *et al.*, 1984; Lacroix *et al.*, 2015). Despite progressively suppressed ACTH levels in circulation, abnormal cortisol secretion in primary BMAH is frequently regulated by G-protein coupled receptors (GPCR) aberrantly expressed in adrenocortical tissues, which increase cyclic AMP (cAMP) levels and protein kinase A (PKA) activity (Lacroix *et al.*, 2010; El Ghorayeb *et al.*, 2015). BMAH's primary molecular mechanisms are still not completely understood and are heterogeneous, but much progress has been accomplished in recent years (De Venanzi *et al.*, 2014; Fragoso *et al.*, 2015; Drougat *et al.*, 2015), and will be reviewed here with its clinical characteristics and therapy.

Epidemiology

BMAH was first described in 1964 (Kirschner *et al.*, 1964) and in 1994, only 24 cases had been published (Lieberman *et al.*, 1994). Since then, a much larger number of cases has been reported and the characteristics of BMAH have been increasingly delineated (Malchoff and Malchoff, 1996; Swain *et al.*, 1998; Hsiao *et al.*, 2009, Lacroix, 2009; Libe *et al.*, 2010; Hofland *et al.*, 2013). BMAH accounts for $<1\%$ of overt CS etiologies (Lacroix *et al.*, 2015). It more often presents as an incidental radiological finding with underlying modest level of dysregulated cortisol secretion (Lacroix, 2009). BMAH with overt CS is usually diagnosed in the fifth or sixth decade of life, but careful history identifies subtle hypercortisolism symptoms on average of 7.8 years evolution (Malchoff and Malchoff, 1996). Its diagnosis occurs at a later age than other forms of primary bilateral adrenal hyperplasias, in particular compared with PPNAD with an average age of 18 years (Stratakis and Kirschner, 1998). Hypercortisolism in the first years of life is rarely due to BMAH, but can be part of McCune–Albright syndrome (MAS), in which somatic *GNAS* (stimulatory G-protein alpha subunit) postzygotic mutation during embryogenesis generate mosaic diverse organ lesions including adrenal macronodules; the nonmutated internodular adrenal cortex becomes atrophic secondary to suppressed ACTH levels (Kirk *et al.*, 1999).

While BMAH was equally distributed between genders in initial reports, recent publications report a higher female incidence in sporadic cases with a F/M ratio of 2–3:1, while there is an equal sex distribution in familial cases (Malchoff and Malchoff, 1996; Swain *et al.*, 1998; Hsiao *et al.*, 2009; Libe *et al.*, 2010; Hofland *et al.*, 2013; Assié *et al.*, 2013; Louiset *et al.*, 2013; Alencar *et al.*, 2014; Elbelt *et al.*, 2015; Bourdeau *et al.*, 2016).

Imaging in BMAH

On an abdominal CT scan, several bilateral adrenal nodules measuring up to 4–5 cm, of soft tissue density, are present and the internodular adrenal tissue can also be enlarged (Doppman *et al.*, 2000; Malayeri *et al.*, 2013). In some cases, the adrenal glands appear diffusely enlarged but lack distinct nodules. On MRI, T1-weighted images are hypointense relative to the liver and isointense relative to muscle. T2-weighted images tend to be hyperintense relative to the liver (Doppman *et al.*, 1991; Rockall *et al.*, 2004). In contrast, the nodules of patients with chronic ACTH stimulation appear isointense relative to the liver on T2-weighted MR images (Doppman *et al.*, 1991; Rockall *et al.*, 2004). Occasionally, there is an asymmetric development of nodules in BMAH, leading to the erroneous diagnosis of unilateral pathology (N'Diaye *et al.*, 1999; Alencar *et al.*, 2014). Iodine 131-6-β-iodomethyl-19-norcholesterol (NP-59) scintigraphy usually shows bilateral uptake (Doppman *et al.*, 2000). One study reported increased fluorodeoxyglucose-positron emission tomography (FDG-PET) signal in BMAH tissues despite the absence of malignancy; the frequency of such increased FDG-signal uptake remains to be confirmed in further studies (Alencar, *et al.*, 2011; Cavalcante, *et al.*, 2016).

Pathology

By definition, BMAH nodules diameter is >1 cm and can reach 5 cm or more (Doppman *et al.*, 1991). Each gland usually contains several nodules and adrenal size may reach 10–12 cm and 200 g in weight (normal adrenal glands are 4–6 g in weight) (Doppman *et al.*, 1991; Cugini *et al.*, 1989; Cheitlin *et al.*, 1988). The combined adrenal weight is usually above 60 g; the mean combined weight in one series was 132 g (Doppman *et al.*, 1991), which is usually larger than found in patients with Cushing's disease, where it was 22.9 g in a series of 30 patients (Smals *et al.*, 1984). On cut sections, the nodules are yellow due to their high lipid content (Aiba *et al.*, 1991). The nodules are composed of two cell types either with clear cytoplasm (lipid-rich) that form cordon nest-like structures, or with compact cytoplasm (lipid-poor) that form nest- or island-like structures (Aiba *et al.*, 1991; Sasano *et al.*, 1994). Two different subtypes are distinguished: BMAH with atrophic internodular cortex (type 1) and hyperplasia of both nodular and internodular tissue (type 2) (Swain *et al.*, 1998). The histology identifies nodules composed of large clear cells forming cordon nest-like structures staining for 3β-HSD, and smaller compact cells forming island-like structures including P-45017α positive cells (Sasano *et al.*, 1994). The sectorial enzymes distribution may contribute to inefficient steroidogenesis with a low rate of cortisol production not proportional to the large gland size. BMAH is a benign process that has not been shown to acquire a malignant potential or to metastasize (Lacroix, 2009).

Laboratory Findings

As the majority of patients with BMAH present with bilateral incidentalomas, the endocrine evaluation should include the recommended incidentaloma testing: 1 mg overnight dexamethasone suppression test, 24-h urinary metanephrine or plasma free metanephrine levels, aldosterone/renin ratio (if the patient has hypertension) (Lacroix, 2009; Fassnacht *et al.*, 2016). In addition, in cases of bilateral incidentalomas, it is recommended to measure early-morning cortisol and ACTH levels (to rule out adrenal insufficiency from bilateral infiltrative/infectious/metastatic diseases), and serum 17-OH-progesterone levels to rule out nonclassic congenital adrenal hyperplasia due to partial 21-hydroxylase deficiency (Lacroix, 2009; Fassnacht *et al.*, 2016).

BMAH most often secretes mainly cortisol, usually in modest amounts; therefore, the integrated urinary free cortisol (UFC) values will only rarely exceed the normal range in patients with overt CS. More frequently, milder inadequate cortisol production revealed by lack of normal suppression after 1 mg overnight dexamethasone suppression test (cortisol above 50 nmol/L (1.8 mcg/dL)) or the loss of diurnal rhythm of cortisol using late night salivary cortisol will be found (Lacroix, 2009). Some cases secrete aldosterone or estrogens concurrently, and rarely isolated androgens' hypersecretion was found (Wada *et al.*, 2002; Goodarzi *et al.*, 2003; Ghayee *et al.*, 2011). Depending on the level of cortisol hypersecretion, plasma ACTH and its stimulation by CRH will become progressively suppressed (Lacroix, 2009; Hsiao *et al.*, 2009; Libe *et al.*, 2010). In BMAH cells, there is decreased expression of ACTH receptor (melanocortin-2, *MC2R*) and of several steroidogenic enzymes compared with normal adrenal cortex (Antonini *et al.*, 2006; Assié *et al.*, 2013). Because of the large cell mass and despite inefficient steroidogenesis, ACTH administration produces a large increase of cortisol in blood and of its precursors such as plasma 17-OH-progesterone (Libe *et al.*, 2010); urinary 17-OH-corticosteroids (U17OHCS) can be proportionally more elevated than UFC (Hsiao *et al.*, 2009). The progression of cortisol secretion to reach overt CS over time seems highly infrequent, and rare published cases progressed during a follow-up period of 7 years (Ohashi *et al.*, 2001).

Genetic Studies

The bilateral nature of adrenal enlargement suggested that BMAH was caused either by a somatic gene mutation occurring during the early phases of embryogenesis or by an inherited germline mutation (Lacroix *et al.*, 2001; Drougat *et al.*, 2015). Heterogeneous genetic etiologies have been identified in BMAH (**Table 1**): in 16 BMAH cases, three patients had a family history of BMAH, and three other patients without family history were found to carry either germline mutations of *MEN1* (multiple endocrine neoplasia 1), *APC* (adenomatous polyposis coli), or somatic *GNAS1* mutation (Hsiao *et al.*, 2009). In one patient with hereditary leiomyomatosis and renal cell cancer (HLRCC), a germline mutation was identified in fumarate hydratase *FH* gene (Matyakhina *et al.*, 2005); 4 bilateral adrenal lesions were found in 255 cases of HLRCC (Shuch *et al.*, 2013). One of the three familial BMAH cases was a carrier of the R867G *PDE11A* (phosphodiesterase isoform 11A) gene polymorphism (Hsiao *et al.*, 2009). Several *PDE11A* missense variants are more frequent in BMAH (28%) than in controls (7.2%), and cells transfected with the variants had baseline and stimulated cAMP levels higher than cells with wild-type isoform of the gene (Vezzosi *et al.*, 2012).

Several familial cases of BMAH were described recently, with an autosomal dominant pattern of transmission (Mazzuco *et al.*, 2012; Drougat *et al.*, 2015; Fragoso *et al.*, 2015). In series of MEN1 patients, up to 21% presented mostly nonfunctional bilateral adrenal lesions, possibly BMAH (Mazzuco *et al.*, 2012). A retrospective analysis of 715 French MEN1 patients found adrenal lesions in 20% of them; bilateral lesions were present in 12.9% of patients with adrenal nodules but it is unknown how many had BMAH by histology. Most unilatral or bilateral tumors were nonfunctional, but primary aldosteronism was more frequent than in a control group of adrenal incidentalomas (Gatta-Cherifi *et al.*, 2012). The association of BMAH with hyperparathyroidism, insulinomas, and/or pituitary tumors without MEN1 mutation was also reported (Yoshida *et al.*, 2011; Lee *et al.*, 2011; Sato *et al.*, 2006). A few cases of bilateral adrenal nodules have been found in patients with familial adenomatous polyposis (FAP) (Hsiao *et al.*, 2009; Mazzuco *et al.*, 2012).

The most frequent genetic finding in BMAH, recently identified using SNP arrays, linkage analysis, and whole genome or exome sequencing, are germline mutations of armadillo repeat containing 5 gene protein (*ARMC5*) in ~25%–50% of patients with

Table 1 Main genes associated with primary bilateral macronodular adrenal hyperplasia

Gene	*Locus*	*Action*	*Type of mutation*
ARMC5	16p11.2	Causes apoptosis and adrenal cell death	Germline and somatic
MEN-1	11q13	Regulation of cell proliferation and differentiation	Germline
APC	5q12–22	Prevention of β-catenin accumulation	Germline
PDE11A	2q31–35	Catalyzes the hydrolysis of cAMP and cGMP	Germline inactivation
GNAS	20q13.1	Stimulates cAMP production	Somatic during embryogenesis
MC2R	18p11.2	Regulates cortisol production and adrenal growth	Somatic
FH	1q42.1	Involved in Krebs cycle (mitochondria) and amino acids metabolism (cytosol)	Germline

Abbreviations: *ARMC5*, armadillo repeat containing 5 gene; *APC*, adenomatous polyposis coli; *FH*, fumarate hydratase; *GNAS*, stimulatory G-protein alpha subunit; *MC2R*, melanocortin 2 receptor; *MEN1*, multiple endocrine neoplasia 1; *PDE11A*, 11A phosphodiesterase isoform.

Modified with permission from De Venanzi, A., Alencar, G.A., Bourdeau, I., Fragoso, M.C.B.V. and Lacroix, A. (2014). Primary bilateral macronodular adrenal hyperplasia. *Current Opinion in Endocrinology. Diabetes and Obesity* **21**, 177–184.

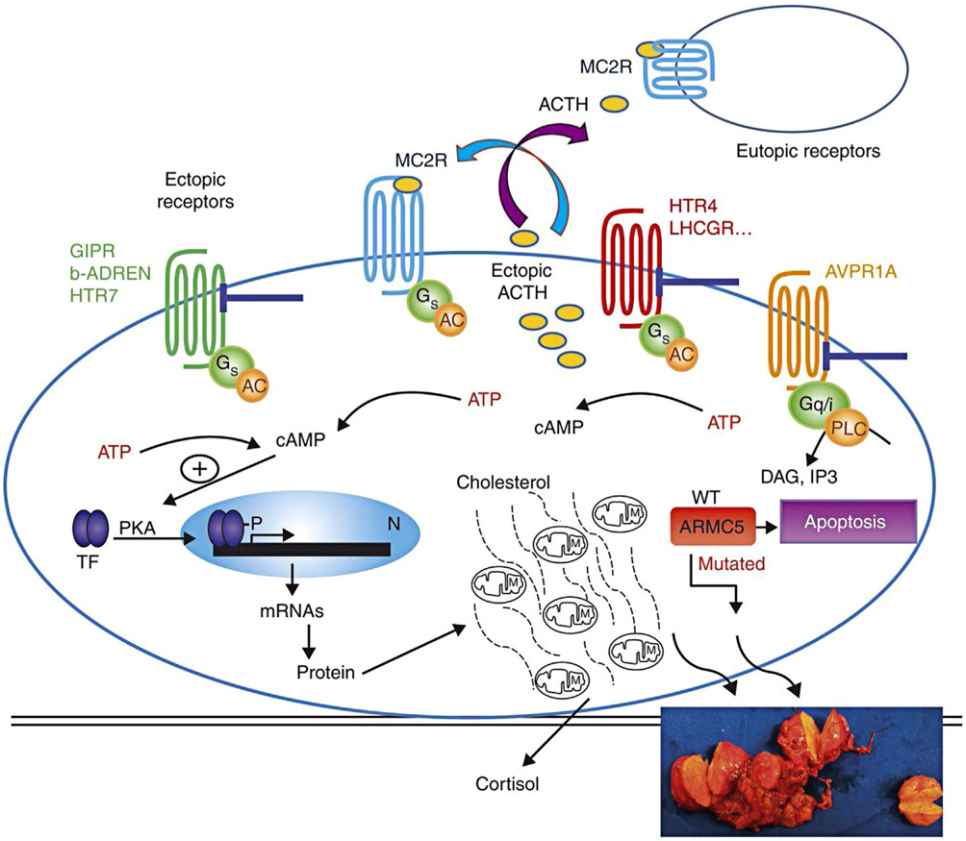

Fig. 1 The main mechanism for BMAH development. The *MC2R* is a G protein-coupled receptor of ACTH, expressed in adrenal zona fasciculata. Abnormal regulation of the adrenal cortex can be mediated by aberrant hormone receptors. Various hormones, including gastric inhibitory peptide (GIP), epinephrine (E), norepinephrine (NE), luteinizing hormone (LH), human chorionic gonadotropin (hCG), serotonin, and vasopressin, can bind to their respective aberrant membrane receptors (ectopic or eutopic) coupled to assorted G proteins (Gs, Gi, and Gq), thereby determining the activation of adenylate cyclase (AC)-mediated signaling pathways by cAMP and protein kinase A (PKA). Ectopic ACTH produced in clusters of BMAH cells acts in autocrine/paracrine regulatory mechanisms on *MC2R* to increase cortisol production after stimulus by the various aberrant G-protein coupled receptors. The activation of this pathway leads to phosphorylation of a number of transcription factors (TFs), culminating in the expression of steroidogenic enzymes during cortisol synthesis in the adrenal glands, as well as hyperplasia. The inactivating mutations of ARMC5 prevent its proapoptotic function probably involved in adrenal hyperplasia; however, this mechanism is unclear. *MC2R*, melanocortin 2 receptor; CYP11A1, cholesterol desmolase; CYP11B1, 11β-hydroxylase; CYP17, 17α-hydroxylase; CYP21A2, 21-hydroxylase; DAG, diacylglycerol; IP3, inositol triphosphate; M, mitochondrion; N, nucleus; PLC, phospholipase C; V1R, V1 vasopressin receptor; 3β-HSD, 3β-hydroxysteroid dehydrogenase; ARMC5, armadillo repeat containing 5 gene protein. Modified with permission from Fragoso, M.C.B.V., Alencar, G.A., Lerario, A.M., Bourdeau, I., Almeida, M.Q. *et al.* (2015). Genetics of primary macronodular adrenal hyperplasia. *Journal of Endocrinology* **224**, R31–R43.

apparently sporadic cases and in familial BMAH (Assié *et al.*, 2013; Alencar *et al.*, 2014; Faucz *et al.*, 2014; Gagliardi *et al.*, 2014; Elbelt *et al.*, 2015; De Venanzi *et al.*, 2014; Drougat *et al.*, 2015; Espiard *et al.*, 2015; Fragoso, *et al.*, 2015; Albiger *et al.*, 2017) (**Table 1** and **Fig. 1**). They were mostly frameshift and nonsense mutations leading to *ARMC5*'s loss of function. Interestingly, 6 out of 11 (54%) first degree relatives of 7 probands carried a germline *ARMC5* mutation; 5 of these showed at least one nodule on CT scan, coupled with increased cortisol secretion in 3 cases (Assié *et al.*, 2013). In the first large BMAH family studied, a heterozygous germline variant in the *ARMC5* gene (p.Leu365Pro) was identified in all 16 affected Brazilian family members, as well as other mutations in two of three other families (Alencar *et al.*, 2014). Interestingly, only two mutation carriers had overt CS and the majority had subclinical disease and one carrier had no manifestations despite being 72 years old. In addition, in one-third of the affected individuals, only unilateral adrenal lesion was present as progression of the full-blown disease, requires many years and the occurence of additional somatic mutations to develop several macronodules. This raises the question of the prevalence of the *ARMC5* mutation in apparently unilateral incidentalomas in the general population. In fact, screening of sporadic cases of patients with bilateral incidentalomas revealed a low frequency (1 out of 39 patients) of *ARMC5* mutation (Emms *et al.*, 2016). The index cases operated for CS with *ARMC5* mutations presented more severe CS than cases operated for CS without *ARMC5* mutation; carrier patients had larger adrenals glands on imaging with a higher number of nodules (Faucz *et al.*, 2014; Espiard *et al.*, 2015).

The search for *ARMC5* or eventually other responsible genes should allow familial screening through a simple blood test to select who should undergo further evaluations (Lacroix, 2013; Fragoso *et al.*, 2015; Drougat *et al.*, 2015). Further family studies will be useful to clarify the clinical penetrance of *ARMC5* mutations.

ARMC5 is located at 16p11.2 and its function was unknown. It codes for a 935-amino acids protein and contains an approximate 40-amino acid long tandemly repeated sequence motif; similar repeats are found in the mammalian armadillo homolog β-catenin and the APC tumor suppressor protein (Berthon and Stratakis, 2014). It behaves as a tumor suppressor gene and causes apoptosis when transfected in H295 adrenal carcinoma cell lines (Assié *et al.*, 2013). *ARMC5* inactivation decreased the expression of *MC2R* and steroidogenic enzymes (Assié *et al.*, 2013). As it is a tumor suppression gene, a second hit is needed for disease development: an additional different somatic *ARMC5* mutation, deletion, or loss of heterozygosity was found in each adrenal macronodule examined and appears important in accelerating the proliferation of affected cells (Assié *et al.*, 2013; Alencar *et al.*, 2014). In internodular hyperplasia, only germline mutation was present (Assié *et al.*, 2013). As BMAH patients usually develop several nodules, germline-independent and distinct somatic *ARMC5* mutations were identified in each one of the macronodules; in one extreme example, 1 germline and 15 different somatic mutations were found in a patient with BMAH (Correa *et al.*, 2015). Thus, *ARMC5* mutations may be associated with very high mutability and genetic heterogeneity of the other allele (Correa *et al.*, 2015). Previous studies identified that beyond a common event such as ectopic glucose-dependent insulinotropic peptide (GIP) receptors or germline *ARMC5* mutation in diffuse hyperplasia, several somatic genetic events occurred in the different macronodules (Lampron *et al.*, 2006; Almeida *et al.*, 2011); thus second somatic *ARMC5* or other second hits may contribute to the progression of macronodules. A germline deletion in exon 1–5 of *ARMC5* gene rather than mutation of *ARMC5* was reported in a family presenting with vasopressin responsive SCS and BMAH (Suzuki *et al.*, 2015). In addition, DNA sequencing from the right and left adrenal nodules and peripheral blood of the son revealed the presence of another germline: missense mutation in *ARMC5* exon 3 (p.P347S) (Suzuki *et al.*, 2015).

The *ARMC5* gene is widely expressed in various normal human tissues including the brain and pituitary gland (Berthon *et al.*, 2017a), supporting the theory that mutations may be responsible for additional pathologies beyond BMAH. This was confirmed by the association of meningiomas in patients with BMAH (Lee *et al.*, 2005; Alencar *et al.*, 2014) with distinct somatic *ARMC5* mutation in meningiomas found in a patient with familial BMAH carrying a distinct germline mutation (Elbelt *et al.*, 2015).

Two models of *ARMC5* gene knockout (KO) in mice were published recently (Hu *et al.*, 2017). In the first model, Hu and collaborators showed that *ARMC5* is involved in T-cell immune response through an important role for T-cell proliferation and differentiation demonstrating that *ARMC5*'s function is not limited to the adrenal cortex. KO mice presented normal adrenal gland histology and serum glucocorticoid levels at a young age (less than age 12 months) but older (>15 months) KO mice showed enlarged adrenal glands with adrenal hyperplasia, and their serum glucocorticoid levels were significantly increased compared with wild-type mice (Hu *et al.*, 2017).

A second mouse model of *ARMC5* inactivation was developed bearing *LoxP* sites, to allow tissue-specific inactivation (Berthon *et al.*, 2017b). *ARMC5* KO (KO) mice (*ARMC5* −/−) died during early embryonic development, demonstrating an essential role of *ARMC5* during gastrulation. The heterozygote mouse (*ARMC5* +/−), developed hypocorticosteronism at least in part due to a downregulation of some steroidogenic enzymes (*star*, *cyp11a1*, *cyp21*) at 12 months of age. This was associated with a decrease of PKA catalytic subunit α (Cα) expression both at the RNA and protein levels that were also seen in human patients with BMAH and *ARMC5* defects. However, this was transient, as corticosterone levels normalized later, followed by the development of hypercorticosteronemia in one-third of the mice at 18 months of age, which was associated with increases in PKA and Cα expression. Adrenocortical tissue analysis from *ARMC5* +/− mice at 18 months showed an abnormal activation of the Wnt/β-catenin signaling pathway in a subset of zona fasciculata cells (Berthon *et al.*, 2017a,b).

Pathophysiology

The pathophysiology of BMAH is complex, as the adrenal process of growth and hypersecretion appears to be explained through different mechanisms (**Fig. 1**). A large number of alterations implicate activation of the cAMP pathway, alterations of PKA subunits expression and activity, with frequent participation of aberrant GPCR and, in rare cases, activating mutations of *MC2R* and *GNAS* (De Joussineau *et al.*, 2012; Swords *et al.*, 2004; Fragoso *et al.*, 2003). A growing interest concerns the local production of hormones by the steroidogenic cells of BMAH generating autocrine/paracrine regulation of cortisol production (Lefebvre *et al.*, 2013).

Aberrant GPCR

Systematic studies of patients affected by BMAH with subclinical or overt CS showed that 77%–87% of patients had aberrant cortisol response to at least one stimulation and usually to several (Hsiao *et al.*, 2009; Libe *et al.*, 2010; Hofland *et al.*, 2013; El Ghorayeb *et al.*, 2015). Vasopressin (V1) and serotonin (HT4) agonists were the most frequent causes of aberrant responses, but receptors for vasopressin (V2 or 3), serotonin (HT7), GIP, catecholamines, luteinizing hormone/human chorionic gonadotropin, and angiotensin can also be activated (Hsiao *et al.*, 2009; Libe *et al.*, 2010; Hofland *et al.*, 2013; Lacroix *et al.*, 2010; El Ghorayeb *et al.*, 2015). The overall prevalence and magnitude of aberrant cortisol increase were similar in patients with subclinical and overt CS (El Ghorayeb *et al.*, 2015). When comparing *in vitro* data to *in vivo* testing, some discrepancies are found, suggesting that the criterion of 25%–50% serum cortisol elevation to establish the partial/complete responsiveness should be re-evaluated, at least when V1R and HT4R are tested (Hofland *et al.*, 2013). A transcriptome study discovered further GPCR overexpressed in BMAH

including for motilin (MLNR), g-aminobutyric acid (GABBR1), and α2 adrenergic receptor (ADRA2A) (Assié *et al.*, 2010). Detailed studies to correlate the aberrant GPCR to specific genetic causes have not been conducted yet; however, aberrant response to upright posture and serotonin agonists but none to GIP were found in *ARMC5*-mutated patients (Espiard *et al.*, 2015).

Specific aberrant GPCR including vasopressin, β-adrenergic, and HT4 receptors was found in all members from several families with BMAH, either alone or in combination, while different aberrant receptors were found in some members of one large Brazilian family (Lee *et al.*, 2005; Vezzosi *et al.*, 2007; Alencar *et al.*, 2014).

The question of whether the expression of aberrant GPCR plays an initiating role or is a secondary event following a proliferative imitating event remains controversial. Transient hypercortisolism and adrenal hyperplasia due to aberrant LH/CG receptors during pregnancies with BMAH and overt CS occurring only after sustained increase of LH secretion after menopause is an interesting model to examine this question (Lacroix *et al.*, 1999). Detailed studies were conducted recently in a 22-year-old woman who presented with transient bilateral adrenal hyperplasia and severe CS during two pregnancies: *in vivo* and *in vitro* studies in resected adrenals demonstrated that hCG could stimulate the transformation of progenitor subcapsular adrenal LHCG receptor expressing cells into hyperplastic adrenocortical cells, increasing their steroidogenesis under HCG stimulation (Plöckinger *et al.*, 2017). Thus, in this case, the aberrant expression of LHCGR was an early event which persisted even when transient adrenal hyperplasia regressed between pregnancies.

The molecular mechanisms underlying the aberrant expression of GPCR in adrenocortical tissues are still mostly unknown. In a recent study of adrenal tissues from 14 patients with GIP-dependent adrenal CS and 1 patient with GIP-dependent aldosteronism, *GIPR* expression in all 3 unilateral adenomas and 11 BMAH samples occurred through transcriptional activation of a single allele of the *GIPR* gene (Lecoq *et al.*, 2017). No abnormality was detected in proximal *GIPR* promoter methylation, but somatic duplications in the chromosome region 19q13.32 containing the *GIPR* locus in the adrenocortical lesions was found in resected adrenal from 3 patients. In 2 adenoma samples, the duplicated 19q13.32 region was rearranged with other chromosome regions, whereas a single tissue sample with BMAH had 19q duplication only. Juxtaposition with *cis*-acting regulatory sequences such as glucocorticoid response elements in the newly identified genomic environment was driving abnormal expression of the translocated *GIPR* allele in cells of one adenoma. The specific molecular mechanism responsible for the monoallelic overexpression of *GIPR* in the other cases of GIP-dependent Cushing's BMAH remains to be identified; however, this study indicated for the first time that in some cases, the aberrant GPCR expression can be the direct consequence of a genetic initiating event (Lecoq *et al.*, 2017).

Paracrine Production of ACTH in Bilateral Macronodular Adrenal Hyperplasia

Ectopic ACTH production was previously reported in steroidogenic adrenocortical cells in a few cases of BMAH (Mazzuco *et al.*, 2007; Iwata *et al.*, 2012). In a more extensive study, proopiomelanocortin (POMC) mRNA was detected in 26 BMAH samples analyzed; moderate/intense immunostaining for ACTH were detectable in all but 1 sample, which did not express prohormone convertase 1 (Louiset *et al.*, 2013). No ACTH staining was present in normal cortex or primary cortisol-secreting adenomas (Louiset *et al.*, 2013). ACTH secretion was demonstrated by a gradient of ACTH in adrenal vein samples in 2 BMAH patients (Louiset *et al.*, 2013); ACTH levels remained low in their blood samples and adrenal secretion was estimated to be 50 times less than in normal pituitary gland (Mazzuco *et al.*, 2007). Perfused BMAH cells secreted ACTH in pulses followed by cortisol secretion (Louiset *et al.*, 2013). ACTH secretion was not regulated by CRH, dexamethasone, or the GR antagonist mifepristone. In contrast, tissues expressing aberrant GPCR released ACTH and cortisol during perifusion with GIP, serotonin, or hCG; ACTH receptor antagonists cortistatin and corticotropin 7-38 inhibited cortisol secretion induced by aberrant ligands by 40% in these tissues (Louiset *et al.*, 2013). Thus, cortisol production is controlled both by aberrant GPCR and ACTH produced within the adrenocortical tissue, amplifying the effect of the aberrant receptors ligands (**Fig. 1**). The co-expression in the ACTH-positive cells of insulin-like 3, a Leydig and luteal-cell marker, suggests an abnormal tissue differentiation event occuring during embryogenesis in common gonadal–adrenal progenitor (Louiset *et al.*, 2013). Other studies indicate that BMAH tissues may also produce serotonin, vasopressin, glucagon, and other factors that suggest further paracrine regulatory loops of cortisol secretion and cell proliferation (Lefebvre *et al.*, 2013). The confirmation that paracrine adrenal production of ACTH is central in cortisol regulation of BMAH will necessitate clinical studies to examine whether new drugs which would block the ACTH (*MC2R*) receptor can reverse the cortisol excess of affected patients (Lacroix, 2013).

The ectopic expression of POMC and synthesis of ACTH was recently confirmed in BMAH primary cultures (Cavalcante *et al.*, 2017); the mRNA expression of POMC of prohormone convertase type 1 (PCSK1), of ACTH synthesis, and of aberrant GPCR were maintained in primary cultures of cells with or without *ARMC5* mutations. Interestingly, stimulation of the cells by ACTH increased POMC expression and ACTH secretion and as found in normal pituitary corticotroph cells, transcriptional activity of the POMC promoter is dependent on PKA-mediated regulation of calcium channels (Cavalcante *et al.*, 2017).

Other Molecular Mechanisms

The *MC2R* activation is the first step of cAMP/PKA downstream pathway activation, and every step may be affected (**Fig. 1**) (De Joussineau *et al.*, 2012). Rare cases of mutation resulting in *MC2R* activation have been reported (Swords *et al.*, 2004). The chronic *in vitro* administration of ACTH increases *MC2R* expression in adrenal cells, while acute stimulation causes internalization of the receptor (De Joussineau *et al.*, 2012; Hofland *et al.*, 2012). In recent years, two *MC2R* associated trafficking proteins called MRAP

and MRAP2 were identified. They exert regulatory function on *MC2R*: MRAP positively affects trafficking of *MC2R* to cell surface and its signaling transduction, while MRAP2 has opposite functions; they are in turn regulated by some intracellular signaling, particularly cAMP (Hofland *et al.*, 2012). *MC2R* function would thus be submitted to various signals from the aberrant GPCRs and by the paracrine release of ACTH. The decrease in *MC2R* levels in BMAH is not related to any changes in MRAP or MRAP2, but could be related to *ARMC5* mutation (Assié *et al.*, 2013; Hofland *et al.*, 2012). Recently, in one case of BMAH, a germline copy number gain of a chromosome 19 region which includes the protein kinase A (*PRKACA*) gene was found in a patient with BMAH resulting in increased tissue enzyme activity (Beuschlein *et al.*, 2014). In contrast, in unilateral adrenal adenomas secreting high amounts of cortisol, somatic mutations in the catalytic subunit of PRKACA were found in up to 50% of cases; the mutation inactivates the site where the negative regulatory subunit RIIβ should bind, resulting in increased PKA activity and constitutive cortisol secretion (Beuschlein *et al.*, 2014).

Therapy

Steroidogenesis inhibitors such as ketoconazole or metyrapone can be efficient options in acute situations or before adrenalectomy (Lacroix, 2009; van der Pas *et al.*, 2012).

Specific Medical Therapy

BMAH with aberrant GPCR can benefit from specific medical therapies. Long-term control of CS was achieved with beta-blockers or leuprolide acetate when β-adrenergic or LH/CG receptors were functional (Lacroix *et al.*, 1997, 2010; El Ghorayeb *et al.*, 2015; Mazzuco *et al.*, 2009). In catecholamine-dependent CS and BMAH, beta-adrenergic receptor antagonists can be effective in the long-term control of hypercortisolism (Lacroix *et al.*, 1997; Mazzuco *et al.*, 2009; Bourdeau *et al.*, 2016; Miyamura *et al.*, 2003). Administration of the long-acting gonadotropin-releasing hormone (GnRH) agonist, leuprolide acetate, resulted in suppression of endogenous LH and normalization of cortisol production (Lacroix *et al.*, 1999). In a 59-year-old woman with androgen-secreting BMAH resulting in virilization, aberrant LH/hCG receptors were identified in one resected adrenal gland. Suppression of endogenous LH with leuprolide acetate normalized androgen secretion from the contralateral adrenal, thereby avoiding bilateral adrenalectomy (Goodarzi *et al.*, 2003).

Only short-term control of GIP-dependent BMAH was possible using octreotide or pasireotide, following desensitization of somatostatin receptors in GIP-secreting duodenal cells (Preumont *et al.*, 2011; El Ghorayeb *et al.*, 2015). No clinically effective antagonists for the frequent vasopressin V1 or serotonin HT4 receptors are currently available (El Ghorayeb *et al.*, 2015).

Surgical Therapy

Bilateral laparoscopic adrenalectomy (BA) was initially suggested as the main treatment for BMAH (Lacroix, 2009; Karapanou *et al.*, 2013; Ritzel *et al.*, 2013; Aggarwal *et al.*, 2013; Dalvi *et al.*, 2012). In 45 BA or BMAH, no surgery-related death was noted (Ritzel *et al.*, 2013). Since BA requires lifelong steroid replacement and may induce an adrenal insufficiency crisis, unilateral adrenalectomy (UA) was proposed in selected cases (Lacroix, 2009; Xu *et al.*, 2013; Kobayashi *et al.*, 2012). Remission was achieved by UA in patients with mild CS (Kobayashi *et al.*, 2012). A study reported a 93% success rate from UA with a median follow-up of 69 months; two patients experienced transient symptoms of hypocortisolism (Xu *et al.*, 2013). UFC <2 times the upper limit of normal and marked asymmetry of adrenal enlargement are the best predictors of UA effectiveness (Lacroix, 2009).

In a French study, 15 BMAH patients with moderate CS were treated by UA; resection of the larger gland led to hypercortisolism remission after 3 months in all 15 patients and a low risk of recurrence (2 of 15 [13%]) after 7–9 years of follow-up (Debillon *et al.*, 2015). Six patients had transient hypothalamic–pituitary–adrenal (HPA) insufficiency after UA. Thus, careful postoperative evaluation of the HPA axis should be performed, and glucocorticoids replacement should be provided if needed (Debillon *et al.*, 2015). Glucocorticoids are then tapered as the axis recuperates over the following months.

NP-59 scintigraphy was used rarely to lateralize the source of cortisol, but is no longer available in many countries (Wong *et al.*, 2010). Adrenal venous sampling to select which adrenal gland to remove plays a marginal role and standardization for data interpretation are lacking. In the largest study in 10 patients, an adrenal/peripheral vein ratio ≥ 6.5 was consistent with cortisol hypersecretion and a ratio ≤ 2.0 was in favor of bilateral source of cortisol (Martins *et al.*, 2012).

Conclusion

The molecular and genetic findings identified recently should enable early diagnoses and the identification of new targets to develop personalized pharmaceutical therapy. It is likely that other genes implicated in the development of BMAH will be identified. Genetic screening should allow identification of mutation carriers. Longitudinal follow-up of larger cohorts of patients in an international collaborative effort should define the natural history and indications for intervention therapies in these patients.

See also: Cushing Syndrome; Screening and Differential Diagnosis

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Adrenal Incidentalomas☆

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Glossary

Adrenal incidentaloma Incidentally detected adrenal tumor.

Adrenocortical carcinoma (ACC) Malignant tumor of the adrenal glands.

Chemical shift MRI (CSI) Identifies benign adrenal adenomas with high lipid content by a typical signal intensity loss on chemical shift imaging relative to the liver.

Computerized tomography (CT) Series of detailed pictures of areas inside the body taken from different angles; the pictures are created by a computer linked to an X-ray machine.

Fine needle aspiration cytology (FNA) A method to aspirate tumor cells by a fine needle, which is punctured in the tissue of interest.

Magnetic resonance imaging (MRI) Procedure in which a magnet linked to a computer is used to create detailed pictures of areas (imaging) inside the body.

Definition and Clinical Presentation

Adrenal masses are among the most prevalent human tumors and are frequently detected unexpectedly by an imaging study performed for reasons unrelated to any suspect of adrenal diseases (such as abdominal, or back pain, or kidney stone) (Terzolo *et al.*, 2011; Fassnacht *et al.*, 2016). The widespread use of diagnostic ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) resulted in the frequent discovery of incidental adrenal masses (Chidiac and Aron, 1997). Such masses are commonly defined as "adrenal incidentalomas" and represent a public health challenge because they are increasingly recognized in current medical practice. The term adrenal incidentaloma is an 'umbrella' definition encompassing a spectrum of different pathological entities that share the same path of discovery (National Institutes of Health, 2002; Terzolo *et al.*, 2011; Fassnacht *et al.*, 2016).

Based on autopsy studies the prevalence of adrenal incidentalomas is around 2%, ranging from 1% to 8.7%. Radiological studies report an increasing frequency parallel to advancing age, from around 3% in the age of 50 years up to 10% in the elderly (Fassnacht *et al.*, 2016). Most of these tumors are small adrenal adenomas. The etiology of adrenal incidentalomas includes mostly benign non-secreting tumors, however a number of lesions are malignant or can lead to hormonal diseases (pheochromocytoma, aldosterone producing adenoma, and cortisol producing adenoma). The frequency varies in the context of different studies; however, overestimation of malignant and secreting tumors id likely in surgical series (**Table 1**), (Kloos *et al.*, 1995; Latronico and Chrousos, 1997; Mantero *et al.*, 2000).

Up to 20% of patients bearing an incidental adrenal adenoma present the so-called subclinical Cushing syndrome (Reincke, 2000). It is a highly debated condition as to its diagnostic definition, clinical relevance and management. Ascertainment of subclinical Cushing syndrome should stand on three criteria: first, the adrenal incidentaloma has radiologic characteristics of an adrenal adenoma; second, the patient does not present any specific Cushingoid sign; third, the endocrine work-up shows at least partial autonomous (pituitary ACTH-independent) cortisol secretion (Reincke, 2000; Terzolo *et al.*, 2012). This condition very rarely progresses into a full-blown Cushing syndrome over a prolonged follow-up. However, studies have shown that this low-grade cortisol excess may be associated with various comorbidities (hypertension, type 2 diabetes mellitus, obesity, dyslipidemia, and osteoporosis) (Barzon *et al.*, 1999; Morelli *et al.*, 2014; Reincke, 2000). Therefore, the term subclinical may be equivocal because the condition may well have clinical consequences and for these reasons in the recent European Society of Endocrinology (ESE) guidelines it was decided to avoid the term "subclinical Cushing syndrome" and use instead the term "autonomous cortisol secretion" (Fassnacht *et al.*, 2016).

Clinical Questions

Adrenal incidentalomas raise challenging questions for both physicians and their patients and represent one of the leading reasons for seeking endocrinological consultation. In a patient with an adrenal incidentaloma, the following issues have to be considered:

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This article is an update of Martin Reincke, Incidentaloma, Adrenal, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004.

Table 1 Epidemiology of different types of adrenal incidentaloma

Type of tumor	*Average (%)*	*Range*
Clinical studies including patients with adrenal mass		
Adenoma	80	33–96
Nonfunctioning	75	71–84
Cortisol secreting	12	1.0–29
Aldosterone secreting	2.5	1.6–3.3
Pheochromocytoma	7.0	1.5–14
Carcinoma	8.0	1.2–11
Metastasis	5.0	0–18
Surgical studies		
Adenoma	55	49–69
Nonfunctioning	69	52–75
Cortisol secreting	10	1.0–15
Aldosterone secreting	6.0	2.0–7.0
Pheochromocytoma	10	11–23
Carcinoma	11	1.2–12
Myelolipoma	8.0	7.0–15
Cyst	5.0	4.0–22
Ganglioneuroma	4.0	0–8.0
Metastasis	7.0	0–21

Table adapted according to Terzolo M., Stigliano A., Chiodini I., *et al.* (2011). AME position statement on adrenal incidentaloma. *European Journal of Endocrinology* 164, 851–870.

- How to assess the risk of malignancy?
- How to assess hormone excess?
- Who should have surgical treatment?
- What is the appropriate follow-up for patients treated conservatively?

Diagnostic Approach

The patient with an adrenal incidentaloma requires a complete history and careful physical examination, which may sometimes detect Cushingoid signs missed at first evaluation, a biochemical workup, and (possibly) additional radiological studies. The diagnostic dilemma is to avoid missing potentially dangerous conditions (i.e., adrenocortical carcinoma, pheochromocytoma) avoiding overdiagnosis and overtreatment.

Assessment of the Risk of Malignancy

The probability of malignancy is influenced by several factors, including young age (portending increased risk of adrenocortical cancer), history of extra-adrenal cancer and constitutional symptoms (low-grade fever, weight loss, malaise). Among patients with a history of cancer, up to 25% of incidentalomas are metastases. In contrast, in non-cancer patients, $>75\%$ of adrenal incidentalomas are benign. ACC is an extremely rare tumor, with an estimated annual incidence of 0.5–2 cases per million population. The prevalence of ACC in incidentaloma patients is related to mass size. In surgical series, ACC accounts for 2% of tumors <4.0 cm, 6% of tumors between 4.1 and 6.0 cm, and 25% of tumors >6.0 cm. Although size is classically viewed as a strong predictor of malignancy, other imaging characteristics should be considered to distinguish between benign and malignant lesions (National Institutes of Health, 2002; Kloos *et al.*, 1995; Terzolo *et al.*, 2011). Although limited data are available and no randomized study comparing imaging tests have been performed, non-contrast CT is the first line imaging investigation. The most consistent support to the diagnosis of a benign adrenal adenoma is the presence of a ≤ 4 cm homogenous mass with smooth borders and lipid-rich, hypodense texture, defined by an attenuation value of <10 Hounsfield units (HU) on a non-contrast CT. This parameter has a high sensitivity ranging from 91% to 100% but low specificity, because 20%–30% of benign adrenal adenomas are lipid poor, thus showing mass density >10 HU. In this case, the mass is considered of indeterminate nature and second-line imaging tests are needed to define the diagnosis. The ESE guidelines suggest three possible alternative tests: (1) CT with delayed contrast media washout with an absolute washout $>60\%$ or a relative washout $>40\%$ that are indicative of a benign lesion; (2) chemical shift MRI with a loss of signal intensity on out-phase imaging that is indicative of a benign lesion; (3) FDG-PET/CT with mass uptake less than liver that is indicative of a benign lesion. The available literature does not allow concluding which test has superior diagnostic performance (Boland *et al.*, 1998; Dinnes *et al.*, 2016).

In patients with adrenal incidentalomas who have no history of extra-adrenal malignancy, fine needle aspiration (FNA) has no proven efficacy. FNA is not free of side effects and may lead to pneumothorax, frank retroperitoneal bleeding, or needle track

metastasis in the case of ACC. In patients with a history of malignancy and with a lesion not conclusively characterized as benign by imaging, FNA is a valuable tool (sensitivity and specificity of approximately 90%) and should be performed to exclude the presence of adrenal metastasis if this information may alter management. Pheochromocytoma should always be ruled out prior to FNA because of the risk of hypertensive crises or even death (Bancos *et al.*, 2016a).

Endocrine Work-Up

In non-surgical series roughly 20% of all incidentalomas are functional. Several studies have shown that the risk of subclinical endocrine activity increases with size. Lesions smaller than 1 cm are generally nonfunctional. However, the prevalence of hormone excess increases to 40% in lesions that are 6 cm or larger (Fassnacht *et al.*, 2016).

All subjects with an incidentally discovered adrenal mass should be screened for both catecholamine overproduction and hypercortisolism, with the exception of patients with adrenal masses whose imaging characteristics are typical for myelolipoma or adrenal cyst. Primary hyperaldosteronism should be considered only in hypertensive and/or hypokalemic patients. Using the strictest inclusion criteria and the purest definition of incidentaloma, which imply the lack of the more specific signs of hypercortisolism, will reduce the proportion of secretory tumors and will virtually eliminate the possibility of overt Cushing syndrome. Moreover, sex hormones and steroid precursors should be determined in patients with clinical or imaging features suggestive of adrenocortical carcinoma (Ross and Aron, 1990; Terzolo *et al.*, 2011; Fassnacht *et al.*, 2016).

A major challenge is that Cushing syndrome includes a spectrum of clinical presentations that is difficult to sort out in different categories. The heterogeneity of the clinical phenotype mainly depends on the variability of cortisol secretion that is distributed continuously from apparently non-functioning adrenal adenomas to overtly cortisol-producing adenomas. Categorization of Cushing syndrome is also influenced by clinical experience, because physicians who have less expertise might overlook (mild) signs of hypercortisolism. For these reasons, demonstration of mild partially autonomous cortisol secretion is extremely difficult in practice.

According to the ESE guidelines, the best mean to uncover autonomous cortisol secretion is the 1 mg overnight dexamethasone suppression test. It is recommended to consider the test results as a continuous rather than categorical variable; thus, cortisol levels <1.8 μg/dL (50 nmol/L) exclude an autonomous cortisol secretion. Cortisol values between 1.8 and 5 μg/dL (51–138 nmol/L) are considered as evidence of "possible autonomous secretion," while cortisol levels ≥ 5 μg/dL (138 nmol/L) are diagnostic of "autonomous cortisol secretion." In these two categories an extended biochemical assessment should be undertaken (i.e., ACTH, 24 h urinary free cortisol, late-night salivary cortisol), in particular in younger patients and in patients presenting potential cortisol-related comorbidities, who may benefit of the surgical treatment. In fact, several studies (although not all) have demonstrated that patients with impaired suppression of cortisol after 1 mg dexamethasone test have a higher risk of type 2 diabetes, hypertension, cardiovascular events and vertebral fractures. Although only few studies are available that include a low number of events, the risk of mortality seems to increase in parallel to increasingly higher cortisol levels after 1 mg dexamethasone (Fassnacht *et al.*, 2016).

In hypertensive patients, serum potassium and a plasma aldosterone/plasma renin ratio should be determined to evaluate primary aldosteronism. A normal potassium concentration does not exclude primary hyperaldosteronism given that the majority of patients are normokaliemic under random conditions. The reader is referred to the Endocrine Society Guidelines on primary aldosteronism for further details on screening and confirmation of diagnosis (Funder *et al.*, 2016).

Pheochromocytoma among adrenal incidentalomas is found in approximately 5% of patients. Most patients do not show the typical clinical symptoms such as tachycardia, sweating, and headache, and up to 40% are completely asymptomatic. In all patients with adrenal incidentalomas, fractionated metanephrines should be measured in urine (sensitivity 97%) or free metanephrines in plasma (sensitivity 99%) (Fassnacht *et al.*, 2016). Normal results rule out pheochromocytoma, while an elevation of more than threefold above the reference interval establishes the diagnosis. False-positive results should be considered in patients with equivocal elevation of plasma, or urinary normetanephrine. In these subjects, measurements should be repeated in the absence of possible interfering conditions (Fassnacht *et al.*, 2016; Lenders *et al.*, 2014).

Therapeutic Considerations

It is overall accepted that adrenal tumors leading to clinically significant hormone excess (primary aldosteronism, pheochromocytoma, overt Cushing syndrome) or radiological suspicion of malignancy should be surgically removed. More difficult is to define which subjects with benign imaging studies and non-functioning adrenal masses may require surgery. The systematic review of several papers reported a very low estimated risk for developing malignancy (0.2%) or overt Cushing syndrome (0.3%). The estimated risk for developing autonomous cortisol secretion without signs of overt Cushing syndrome is up to 11%, but the clinical meaning of this finding is uncertain. Due to increased risk of malignancy in larger masses, it is accepted to consider surgery in patients with adrenal masses larger than 4 cm, although non-functioning and with benign imaging characteristics (Terzolo *et al.*, 2011; Fassnacht *et al.*, 2016).

The introduction of laparoscopic adrenalectomy has significantly reduced surgical-related morbidity (5%–10%) and mortality ($<1\%$). However, widening surgical indications, even with a safe procedure, may lead to increased morbidity. It has to be emphasized that this operation may be performed in specialized centers with experienced laparoscopic surgeons (Fassnacht *et al.*, 2016).

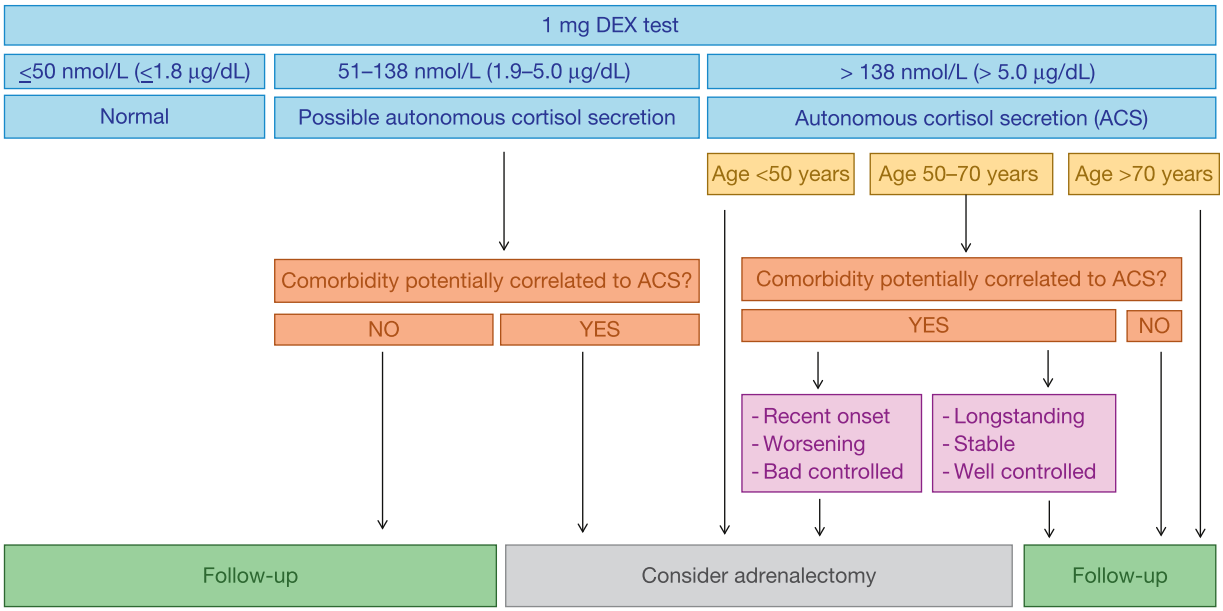

Fig. 1 Proposed assessment and management of "autonomous cortisol secretion" in patients with adrenal incidentalomas. Figure adapted according Terzolo M., Pia A. and Reimondo G. (2012). Subclinical Cushing syndrome: Definition and management. *Clinical Endocrinology* **76**, 12–18; Fassnacht M., Arlt W., Bancos I., *et al.* (2016). Management of adrenal incidentalomas: European Society of Endocrinology Clinical Practice Guideline in collaboration with the European Network for the Study of Adrenal Tumors. *European Journal of Endocrinology* **175**, G1–G34.

Treatment of Autonomous Cortisol Secretion

It remains doubtful whether all patients with autonomous cortisol secretion may benefit from adrenal surgery. There are some data indicating the improvement of comorbidities potentially related to cortisol excess, such as obesity, hypertension, hyperglycemia, and dyslipidemia with surgery. However, there are no long-term data on mortality and cardiovascular events (Bancos *et al.*, 2016b; Fassnacht *et al.*, 2016). Thus, the ESE guidelines suggest to individualize the therapeutic approach and consider surgery in younger patients, in patients with a higher degree of cortisol excess, or in patients with comorbidities, particularly if there are worsening clinical features because of suboptimal control despite adequate medical therapy. (see **Fig. 1**, near here) (Terzolo *et al.*, 2012; Fassnacht *et al.*, 2016).

Follow-Up in Patients Not Undergoing Adrenal Surgery

How to organize follow-up remains a most controversial issue. The ESE guidelines recommend that homogeneous ≤ 4 cm mass with density ≤ 10 HU on non-contrast CT do not need any further imaging for diagnosis or follow-up. In masses >4 cm, it could be reasonable to repeat CT or MRI 6–12 months after the initial study to exclude significant growth. In patients who experience enlargement of the lesion by $>20\%$, in addition to a 5 mm increase of the maximum diameter, surgical resection should be considered (Fassnacht *et al.*, 2016).

There is also limited utility in repeating endocrine tests and patients with a normal hormonal work-up at the initial evaluation should avoid repeated hormonal assessment unless new clinical signs of endocrine activity appear, or in presence of worsening comorbidities. In patients with non-suppressed cortisol levels after 1 mg dexamethasone test at initial evaluation, an annual assessment for cortisol excess and comorbidities may be considered to detect a worsening course. In some cases, surgical treatment should be reconsidered. There are few data on the optimal duration of follow-up and different studies proposed follow-up periods ranging from 2 to 5 years (Fassnacht *et al.*, 2016).

See also: Adrenocortical Carcinoma: Diagnosis and Therapy. Cushing Syndrome; Screening and Differential Diagnosis

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Adrenocortical Carcinoma: Diagnosis and Therapy

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Glossary

Adrenal incidentaloma Incidentally detected adrenal tumor.
Adrenocortical carcinoma (ACC) Malignant tumor of the adrenal glands.
Chemical shift MRI (CSI) Identifies benign adrenal adenomas with high lipid content by a typical signal intensity loss on chemical shift imaging relative to the liver.
Computerized tomography (CT) Series of detailed pictures of areas inside the body taken from different angles; the pictures are created by a computer linked to an X-ray machine.
Fine needle aspiration cytology (FNA) A method to aspirate tumor cells by a fine needle, which is punctured in the tissue of interest.
Magnetic resonance imaging (MRI) Procedure in which a magnet linked to a computer is used to create detailed pictures of areas (imaging) inside the body.

Diagnosis

Hormonal Assessment

Adrenocortical carcinoma (ACC) has the propensity to produce and secrete steroids; thus, in all patients with a (suspected) ACC signs and symptoms of cortisol excess, androgen excess in women, mineralocorticoid excess, and estrogen excess in men should be actively searched for with careful history and physical examination (Allolio and Fassnacht, 2006). About 50%–60% of patients with ACC will show clinical and biochemical evidence of steroid excess, mainly hypercortisolism and/or hyperandrogenism. Concomitant secretion of different steroids is a hallmark of ACC (Fassnacht and Allolio, 2009). Steroid secretion may be clinically silent (i.e., subclinical cortisol excess) and should therefore be excluded even in asymptomatic patients. Since ACC have an inefficient steroid factory due to decreased expression of several steroidogenic enzymes (Fassnacht *et al.*, 2011), excessive amount of adrenal steroid precursors can be detected in urine by gas chromatography/mass spectrometry or LC-MS in tumors classified as nonfunctioning with conventional diagnostic methods. Therefore, the so-called steroidobolomic approach may find evidence of steroid oversecretion in the majority of patients and may represent a powerful technique in the differential diagnosis between benign and malignant adrenal tumors, and in the early detection of ACC recurrence (Arlt *et al.*, 2011).

A detailed endocrine assessment has to be performed preoperatively since it may allow: (i) to establish the adrenocortical origin of a tumor excluding other differential diagnoses (i.e., lymphoma, sarcoma) if steroid secretion is present; (ii) to suspect the malignant potential of an adrenal tumor (i.e., estradiol excess in males, high concentration of androgens or steroid precursors); (iii) to predict the risk of life-threatening postoperative adrenal insufficiency in patients with cortisol secreting tumors; (iv) to make available tumor markers in the postoperative follow-up to assess persistence or recurrence of disease (Fassnacht and Allolio, 2009; Fassnacht *et al.*, 2011).

Cortisol excess should be demonstrated by measurement of 24 h urinary free cortisol (UFC) excretion and eventually by measurement of late-night salivary cortisol and the 1 mg overnight dexamethasone suppression test (1 mg DST), which has the highest sensitivity (95% with a threshold of 1.8 μg/dL) particularly for the detection of milder forms (Nieman *et al.*, 2008; Terzolo *et al.*, 2012). ACTH levels that are undetectable, or at the lower end of the normal range, confirm the condition of excess cortisol secretion. Aldosterone-producing carcinoma is rare and hypokalemia found in ACC patients is usually due to severe cortisol excess. However, screening for hyperaldosteronism is recommended in all hypertensive and/or hypokalemic patients with an adrenal mass, by measuring plasma aldosterone or precursors and plasma renin activity (PRA), or direct renin concentration (PRC) (Terzolo *et al.*, 2011). Hypersecretion of sexual steroids is frequently observed in ACC patients, with increased levels of plasma 17-OH progesterone, androstenedione and DHEAS, leading to increased plasma testosterone in females. Estrogens hypersecretion, though rare, should be ascertained in males (particularly when presenting gynecomastia) and postmenopausal females (Zeiger *et al.*, 2009).

Radiological Assessment

Imaging has a prominent role in diagnosis of ACC, staging of the disease, and monitoring of tumor response to treatment. On cross sectional imaging, morphological features suggesting that an adrenal mass may be an ACC include tumor heterogeneity (due to necrosis or hemorrhage), lobulated shape, irregular margins, calcifications and a large size. None of these features can be considered as a definitive sign of malignancy, with the exception of venous thrombus, infiltration of surrounding structures, or metastatic spread to lymph nodes or distant organs (Fishman *et al.*, 1987; Zhang *et al.*, 2012).

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The probability that an adrenal mass be an ACC is correlated to its size; however, since ACC is infrequent among adrenal incidentalomas, the posttest probability associated with any tumor size remains low (Terzolo *et al.*, 2012). Recent findings from the SEER database confirmed that an increased tumor size correlates with a higher likelihood of malignancy. The analysis of data on 192 ACCs presenting with localized disease showed that a tumor size of 4 cm had a sensitivity of 96% and specificity of 52% for diagnosing ACC (Kebebew *et al.*, 2006), a figure very close to that observed in the Italian survey on adrenal incidentaloma (Mantero *et al.*, 2000).

Although no randomized study comparing imaging tests has been performed, noncontrast CT is generally considered as the first-line imaging test. The most consistent support to the diagnosis of a benign adrenal adenoma is the presence of a ≤ 4 cm homogenous mass, with smooth borders and lipid-rich, hypodense texture, defined by an attenuation value of <10 Hounsfield units (HU) on a noncontrast CT. This parameter has a high sensitivity ranging from 91% to 100% but low specificity, because 20%–30% of benign adrenal adenomas are lipid poor, thus showing mass density $>$ 10 HU (Vassiliadi and Tsagarakis, 2011). In this case, the mass is considered of indeterminate nature and second-line imaging tests are needed to define the diagnosis. The ESE guidelines on management of adrenal incidentalomas suggest three possible alternative tests: (1) CT with delayed contrast media washout with an absolute washout $>60\%$ or a relative washout $>40\%$ indicative of a benign lesion; (2) chemical shift MRI with a loss of signal intensity on out-phase imaging that is indicative of a benign lesion; (3) FDG PET/CT with mass uptake less than liver indicative of a benign lesion (Fassnacht *et al.*, 2016). The available literature does not allow concluding which test has superior diagnostic performance. Enhanced CT is extremely useful to assess organ invasion and metastatic spread. Metastases from ACC generally involve lungs, liver, regional and para-aortic lymph nodes and bones. Enhanced CT is the technique of choice for ACC staging and assessment of response to treatment, while MRI is generally preferred for assessment of vascular involvement by tumor thrombus (Chiche *et al.*, 2006). Fine needle aspiration (FNA) has no established role in securing the diagnosis, if not in case of unresectable ACC, when it can serve to inform further management (Fassnacht *et al.*, 2016).

FDG PET may be useful in ACC staging; however, practice varies as to its standard use in this setting. In a recent study, FDG PET/CT was complementary to total body CT in detection of metastatic sites of disease (Leboulleux *et al.*, 2006). Research from our group showed the utility of FDG PET/CT as a second-line test in the postoperative surveillance of ACC patients following CT findings of a potential recurrence. The greater specificity of FDG PET/CT was particularly useful in ruling out suspected ACC recurrences found by CT and results of the test had an impact on patient management (Ardito *et al.*, 2015).

Staging classification

Several staging systems have been used in the past, but in the last years most studies in the field have employed the system developed by the European Network for the Study of Adrenal Tumors (ENSAT). The ENSAT staging system represents an improvement over previous classifications because it is based on the analysis of disease-specific survival curves for each stage obtained in larger series (**Table 1**). The ENSAT staging system allows a more precise prognostic differentiation among stages, which in the original study showed a progressively reduced 5-year survival of 81%, 61%, 50%, and 13%, from stage 1 to stage 4, respectively (Fassnacht *et al.*, 2009). In this system, tumor infiltration in surrounding tissues, tumor thrombus in caval or renal vein, and/or positive lymph nodes define stage III, whereas the presence of distant metastasis is the only criteria for stage IV (Fassnacht *et al.*, 2009). Comparative studies confirmed that the ENSAT staging system has better prognostic accuracy for ACC-specific survival than previous classifications (Lughezzani *et al.*, 2010; Berruti et al., 2012a).

Pathological Assessment

The Weiss score is the cornerstone of pathological diagnosis and may have also a prognostic stratification power (**Table 2**). Weiss score includes nine criteria of proliferation, nuclear abnormality and tumor extension (Weiss *et al.*, 1989). A Weiss score of 0–2 defines benign adrenal tumors, while tumors with a Weiss score ≥ 3 are considered malignant. Tumors with a Weiss score of 2 or 3 may eventually display an undetermined behavior. A correct assessment of this morphological score is strictly dependent on individual expertise and an easier standardization is urgently needed. Intraindividual variability in readings of Weiss score has been pointed out, particularly for the parameters of venous invasion and sinusoidal invasion (Tissier *et al.*, 2012).

Table 1 Staging system for ACC proposed by the European Network for the Study of Adrenal Tumors (ENSAT) (Fassnacht *et al.*, 2009)

Stage	
I	T1, N0, M0
II	T2, N0, M0
III	T1–T2, N1, M0; T3–T4, N0–N1, M0
IV	Any T, any N, M1

T1, tumor ≤ 5 cm; T2, tumor >5 cm; T3, tumor infiltration into surrounding tissue; T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein; N0, no positive lymph nodes; N1, positive lymph node(s); M0, no distant metastases; M1, presence of distant metastasis.

Table 2 The Weiss score (Weiss *et al.*, 1989)

Nuclear atypia
Atypical mitoses
Mitotic rate > 5 in 50 HPF
Character of cytoplasma
Architecture of tumor cells
Necrosis
Invasion of venous structure
Invasion of sinusoidal structure
Invasion of the capsule of tumor

It is matter of debate if Weiss score as a whole may predict prognosis (Morimoto *et al.*, 2008; Volante *et al.*, 2009; Papotti *et al.*, 2011). One component of the Weiss score, mitotic activity, has been found to be the most significant determinant of survival. High mitotic activity was found to be a predictor of poor outcome either in localized or metastatic ACC (van Slooten *et al.*, 1985; Tissier *et al.*, 2012). Also the presence of tumor necrosis and atypical mitotic figures have been associated with poor prognosis and advanced disease stage (Stojadinovic *et al.*, 2002; Assié *et al.*, 2007; Volante *et al.*, 2009), although they were less powerful predictors. In patients with stage IV ACC, multivariate analysis identified high mitotic index (>20 per 50 HPF) and the number of organs involved as the major factors influencing prognosis (Assié *et al.*, 2007). A recent pathological study showed that it was possible to stratify prognosis on the basis of stage and mitotic index of the primary tumor. Stage III/IV and mitotic index >9 per 50 HPF qualified the worst prognosis group (Volante *et al.*, 2009). These findings support the concept that a grading system based on mitotic count might help in the prognostic stratification of patients (Giordano, 2011).

Since the Weiss score is difficult to apply, subjective and time consuming, despite several attempts of revision and implementation (Aubert *et al.*, 2002; Stojadinovic *et al.*, 2002), Duregon et al. have introduced a new method, the "reticulin algorithm." This method identifies malignancy through detection of an altered reticulin framework evaluated using a specific staining associated with one out of three parameters among necrosis, high mitotic rate and vascular invasion (Duregon *et al.*, 2013). This method shows a better accuracy and higher reproducibility than the classic Weiss score among different pathologists (Duregon *et al.*, 2013) and may also represent a valid tool for identification of specific ACC variants, such as pediatric, oncocytic, myxoid, and sarcomatoid tumors. Further studies are needed to confirm its value for securing the diagnosis of ACC.

Therapy

Adjuvant Treatment Concepts

The need to consider adjuvant therapy in ACC is derived from the observation that up to 80% of patients show loco-regional recurrence or distant metastases after an apparent complete surgical excision. Despite en bloc, complete resection of tumor in patients without evidence of metastatic disease, the 5-year survival rate is only approximately 50% (Bellantone *et al.*, 1997; Schulick and Brennan, 1999; Wajchenberg *et al.*, 2000; Vaughan, 2004). It is plainly evident that these findings make a strong case in favor of the use of adjuvant therapy in ACC patients; however, this therapeutic option for patients with stage I-III ACC following radical surgery remains debated. Mitotane is the only drug approved by international pharmaceutical agencies for treatment of advanced ACC but its use in adjuvant setting remains matter of heavy debate, and its in this setting is off-label in many countries (Huang and Fojo, 2008; Wängberg *et al.*, 2010).

Mitotane

Although randomized, controlled trials on the use of adjuvant mitotane in ACC patients following radical surgery are still unavailable, a large retrospective case–control study reported that patients treated with adjuvant mitotane had a significantly longer recurrence-free survival (RFS) and overall survival (OS), compared with two independent groups of patients left untreated following surgery (Terzolo *et al.*, 2007). Recently, the same group has updated the follow-up of these cohorts of patients with almost 10 years of additional observation, confirming that adjuvant mitotane treatment is associated with a significant benefit in terms of RFS regardless of the hormone secretory status (Berruti *et al.*, 2017). Advantage on OS is less evident but this may be explained by different treatment of ACC recurrence between groups. Despite its retrospective nature, this study remains the most informative piece of evidence on the topic and represents a reference for decision making in ACC patients. Strengths of the study are the inclusion of contemporary groups of matched patients, who were allocated to treatment or follow-up based on the treatment policy the center. Conversely, in many studies patients with unfavorable characteristics were more likely selected for adjuvant mitotane, thus introducing a bias. An example of this may be found in a recent study reporting a multicenter, retrospective analysis on 207 ACC patients, showing that adjuvant mitotane was associated with decreased RFS and OS. However, 42% of the patients treated with mitotane had stage IV ACC and, indeed, chemotherapy was frequently associated to mitotane therapy (Postlewait *et al.*, 2016). A retrospective study from the University of Michigan confirms the finding that adjuvant mitotane

treatment is associated with a significantly improved RFS although it failed to prolong significantly OS (Else *et al.*, 2014). The lack of effect on OS may be explained with the short follow-up (25.6 months).

Despite controversy on this issue, there is general agreement on the adjuvant use of mitotane following surgical removal of ACC in high-risk patients. The condition of high risk of recurrence has been defined as stage III, or Ki-67 $>$ 10%, or Rx-R1 resection by a panel of international experts (Berruti et al., 2012b). For low-risk patients, who are characterized by stage I or II, R0 resection and Ki-67 $\leq$ 10%, adjuvant mitotane therapy is not mandatory. An international, multicentric, prospective, randomized trial (ADIUVO trial) is currently enrolling low-risk ACC patients, who are randomized to mitotane or observation, in order to definitely establish the effectiveness of adjuvant mitotane in this cohort. A recent retrospective analysis demonstrated that blood mitotane concentrations $\geq$14 mg/L were associated with a prolonged RFS in patients treated with adjuvant mitotane following macroscopically radical surgery (Terzolo *et al.*, 2013). Thus, maintenance of target mitotane concentration may represent a predictor of response to adjuvant treatment. It is common practice in expert centers to monitor regularly blood mitotane concentrations during treatment and to target levels of 14–20 mg/L (Terzolo *et al.*, 2014). There is no consensus on how to start treatment: the ESMO guidelines (Berruti et al., 2012b) recommend that mitotane therapy should be administered following a high-dose regimen with the aim of reaching a daily dose of 6 g/daily rather soon and then adjust the dose according to tolerability and mitotane levels. However, in our personal practice we start treatment at lower doses because they are better tolerated and less patients have to discontinue treatment (Terzolo *et al.*, 2014). Duration of adjuvant mitotane therapy has not been definitively established, but it is reasonable to continue therapy for at least 2 years, because this is the period when most of ACC recurrences are detected. In our practice, we have currently extended treatment till to 3–5 years, if tolerated.

The most common unwanted effects are gastrointestinal manifestations that appear early in the course of treatment, independently on mitotane levels (Faggiano *et al.*, 2006). Diarrhea and nausea are particularly frequent and can be managed with temporary dose reduction and supportive therapy. Elevated γ-glutamyl-transferase levels are also frequently observed but are not actually troublesome unless values are exceedingly elevated. Clinically significant liver toxicity is characterized by a marked increase in transaminases and bilirubin, but is infrequently observed in the absence of predisposing conditions (Terzolo *et al.*, 2014). Central neurologic toxicity (cerebellar symptoms, disturbed cognitive performance) is more closely associated with elevated mitotane concentrations (20 mg/L) but subtler symptoms, such as memory impairment or attention deficit, may be observed in some patients even at lower drug concentrations (Terzolo and Berruti, 2008) In this context, monitoring of circulating mitotane levels may be useful to tailor individually the therapy and limit side effects thus attaining better compliance to treatment. The implementation of blood mitotane monitoring, through a service provided by the company distributing Lysodren® in Europe (Lysosafe, www.lysodren-europe.com), has rendered the use of this drug more feasible because it is possible to some extent to anticipate and prevent toxicity. In our current practice, measurement of circulating mitotane concentration has become mandatory for a proper management of patients with ACC. A general measure to deal with mitotane toxicity is a step down to the previously tolerated dose, or temporary drug withdrawal in the event of severe manifestations. However, well-informed and motivated patients are able to cope with side effects and maintain compliance to treatment. To accomplish this task, it is important to establish a close patient–physician relationship to induce and maintain adherence to treatment. Patients seek advice frequently, also because their local physicians are unfamiliar with mitotane use and its attendant complications, and it is necessary to give a timely counseling to keep patients on treatment.

Because of the adrenolytic effect of mitotane, all patients should receive glucocorticoid replacement to prevent adrenal insufficiency. Steroid doses are typically higher than in Addison's disease, due to an enhanced metabolic clearance rate of glucocorticoids induced by mitotane (Arlt *et al.*, 2011; Fassnacht *et al.*, 2011; Stigliano *et al.*, 2015). An inadequate treatment of adrenal insufficiency increases mitotane-related toxicity, particularly gastrointestinal side effects, and reduces tolerance (Daffara *et al.*, 2008). Mineralocorticoid supplementation is not mandatory in all patients because the zona glomerulosa is partly spared by the toxic effect of mitotane (Daffara *et al.*, 2008). Moreover, mitotane affects thyroid and gonadal function by mechanisms that are still to be completely elucidated. Mitotane administration is associated with low FT4 levels without a compensatory rise in TSH, an effect that becomes apparent early in the course of treatment. This prompts thyroxin replacement, even if the benefit of this measure may be difficult to appreciate (Daffara *et al.*, 2008; Terzolo and Berruti, 2008). In women, gonadal function is usually preserved and most female patients have regular cycles unless PRL levels are significantly increased (Daffara *et al.*, 2008; Terzolo and Berruti, 2008; Fassnacht *et al.*, 2011) due to a weak estrogen-like action of mitotane (Nader *et al.*, 2006). Conversely, in men mitotane treatment causes sexual dysfunction as a late but common unwanted effect, due to inhibition of testosterone secretion. Sex steroid replacement may become necessary to treat hypogonadism in some patients but may worsen gynecomastia (Daffara *et al.*, 2008; Terzolo and Berruti, 2008; Fassnacht *et al.*, 2011). Mitotane use is associated with increasing levels of LDL and HDL cholesterol, and triglycerides (Allolio and Fassnacht, 2006). However, the value of introducing statins remains uncertain although patients may be worried about their lipid levels. The decision to use antilipid drugs, which may further complicate supportive therapy and is not exempt from potential toxicity, should be carefully though at considering patient life expectancy.

Radiotherapy and chemotherapy

In a retrospective analysis from the United States, adjuvant radiotherapy was reported to decrease of 4.7 times the risk of local failure compared with surgery alone (Sabolch *et al.*, 2011). In a retrospective analysis from the German ACC Registry, radiotherapy in an adjuvant setting resulted in a significant better 5-year RFS, but did not affect OS and disease-free survival (Fassnacht *et al.*, 2006). However, no difference between surgery plus radiotherapy and surgery alone was found in another retrospective study done in the United States (Habra *et al.*, 2013). A review of the literature concluded that adjuvant radiotherapy should be considered in

patients with incomplete, or R1 resection, or Rx resection, who are at high risk for local recurrence (Polat *et al.*, 2009). A total dose of >40 Gy with single fractions of 1.8–2 Gy should be administered. However, prospective investigations are required and no definitive conclusions are available at the moment.

As far as chemotherapy is concerned, limited data are available. A recent paper published data on 3982 ACC patients from the National US Cancer Data Base (NCDB), revealing that adjuvant chemotherapy was performed in 10% of cases. By comparing these subjects with those treated with surgery only, OS was not different, while no RFS analysis was reported (Bilimoria *et al.*, 2008). Anecdotal cases reported a more favorable outcome after an adjuvant etoposide—cisplatin based chemotherapy (Keskin *et al.*, 2013). A phase II clinical trial reported that the combination of mitotane plus streptozotocin was effective in an adjuvant setting. However, the study design does not allow discriminating the relative merits of the two drugs (Khan *et al.*, 2000).

Treatment for Advanced Disease

About 50% of newly diagnosed ACC patients present with metastatic or unresectable disease (Berruti et al., 2012b). Moreover, despite initial complete resection of ACC, up to 70%–80% of patients are destined to develop recurrent or metastatic disease (Berruti et al., 2012b; Terzolo *et al.*, 2014). The prognosis of patients with advanced/metastatic ACC is generally poor but it is heterogeneous and long-term survivors have been described (Terzolo *et al.*, 2014; Libé *et al.*, 2015). The management of these patients is mainly centered on systemic therapy including mitotane alone or mitotane in combination with chemotherapy. The standard chemotherapy regimen for advanced ACC is EDP (etoposide, doxorubicin and cisplatin) plus mitotane that was initially tested in two patients (Berruti *et al.*, 1992), its feasibility was further assessed in seven patients (Pia *et al.*, 1995), then the activity was evaluated in a multicenter prospective phase II study conducted in Italy (Berruti et al., 1998, 2005). Finally its efficacy was compared against the combination of streptozotocin and mitotane in a prospective randomized phase III clinical trial conducted worldwide (Fassnacht *et al.*, 2012). Three hundred and four patients were prospectively enrolled in about 6 years. Patients with disease progression to the first-line treatment received the alternate regimen. EDP-M was superior to Sz-M both in terms of disease response rate and progression-free survival (PFS). Analysis of OS also favored patients initially randomized to receive EDP-M but due to the attenuating effect of the cross over to EDP-M of patients randomized to the Sz-M at disease progression, the difference just failed to attain statistical significance. In addition to systemic therapy also local regional therapies, that is, surgery (Bellantone *et al.*, 1997; Deschamps *et al.*, 2014), radiofrequency ablation (RFA) (Wood *et al.*, 2003; Deschamps *et al.*, 2014), and chemoembolization (Wong *et al.*, 2017) can be taken into consideration in a selected patient population. Moreover, in patients, who have contraindications to EDP or poor performance status, either cisplatin or carboplatin administered as single agents could be reasonable options.

In the modern approach to advanced/metastatic patients a careful evaluation of prognostic factors should be performed and the most appropriate treatment strategy should be prescribed accordingly (Baudin, 2015). Finally, the morbidity caused by ACC and the prognosis derives not only from the spread of malignant cells into other organs but also from the consequences of hormone excess. Consequently, the goals of treatment in ACC include both control of tumor growth and mitigation of the effects derived from hormone excess in patients with hormone secreting ACC.

Prognostic factors

In a recent study of the ENSAT network, which analyzed 444 patients with advanced stage III to IV ACC, the stage was redefined by a new modified ENSAT (mENSAT) classification in which the presence of N positive moved from stage III to IV and the number of tumor organs showed a major prognostic role together with four additional parameters (GRAS parameters) (Libé *et al.*, 2015). The GRAS parameters consist in grading (G) assessed by Ki67, which is defined favorable if <20%, primary R0 resection status performed (R favorable), age < 50 years (A favorable) and absence of symptoms at diagnosis (either related to cortisol hypersecretion or tumor mass) (S favorable). GRAS parameters are classified as pejorative in case of: grading as defined by Ki67 > 20% and/or primary R1–2 resection status. By combining mENSAT stage with GRAS parameters, four different prognostic subgroup were defined: group 1) mENSAT stage III and GRAS either favorable or unfavorable: 5 years survival 50%; group 2) mENSAT stage III and GRAS pejorative or stage III-IV A-B (3 or less tumor organs involved) and GRAS favorable: 5 years 25%–50%; group 3) mENSAT stage IV A-B and GRAS unfavorable: 5 years 10%–25%; group 4) mENSAT stage IV C (>3 tumor organs involved) or stage IV A-B and GRAS pejorative: 5 years <10% (Baudin, 2015).

Treatment of patients with limited metastatic spread and more indolent disease

Patients with oligo-metastatic disease with favorable prognostic factor as assessed by GRAS and/or a relatively long disease-free interval from previous surgery (i.e., 12 months or more) represents a minority of advanced/metastatic ACC. These patients have a relative long survival perspective and may not benefit from an aggressive systemic treatment such as the EDP-M regimen. Therefore, single agent mitotane could be a reasonable option. The treatment of these patients also includes loco-regional approaches. Surgery of primary and or metastases can be recommended if a complete resection (R0) is achievable. Surgery of multiple metastases is considered on a case-by-case basis and should be performed mainly in patients with favorable prognostic factors, sustained disease response to systemic therapy, and long-term R0 resection expectations. In patients who are not candidates for surgery, percutaneous image-guided RFA is a locally effective treatment and chemoembolization is another possibility to

treat liver metastases. RFA in combination with surgical resection may allow better disease control in the setting of limited disease (Wood *et al.*, 2003; Deschamps *et al.*, 2014; Wong *et al.*, 2017).

Tumor debulking generally offers little benefit, however surgery of primary disease in newly diagnosed patients with oligometastatic disease and limited extra-adrenal tumor volume can be performed in case of good response to systemic therapy. It should be noted that the efficacy of local regional therapies in the management of such patients has never been assessed in a randomized prospective clinical trial, so we cannot exclude that the long-term benefit obtained in some cases can be ascribed to a patient selection. In the author opinion, the long-term benefit is due at least in part to the efficacy of systemic therapy; therefore, it is recommended that all local regional approaches should be used in combination with systemic therapy, mitotane in particular.

Management of metastatic patients with poor prognostic features

The majority of metastatic ACC patients have poor prognostic features, that is, two or more organ involved and/or unfavorable/pejorative GRAS parameters. For these patients, chemotherapy with EDP-M regimen represents the treatment of choice. In case of painful metastasis, palliative radiotherapy is an option, especially in bone lesions. Due to the latency of mitotane to attain the therapeutic range, the drug administered alone is not indicated in the management of patients with clinical evidence of fast growing tumors.

Metastatic ACC submitted to EDP-M regimen have a survival perspective of 18 months as demonstrated by the results of the FIRM-ACT trial (Fassnacht *et al.*, 2012). However, 15% of patients are alive after 5 years. In terms of progression-free survival, 50% of patients submitted to EDP-M showed disease progression after 5 months, and 25% of patients were free from progression after 12 months, and 15% after 2 years. In addition, few patients were still alive and free from progression after 5 years (Fassnacht *et al.*, 2012). These data show that the efficacy of chemotherapy plus mitotane is overall modest but a small subset of patients are destined to obtain a long-term disease control. The identification of factors that may predict chemotherapy efficacy is very important to select patients destined to benefit from this aggressive strategy and address nonresponding patients to experimental therapies. In a recently published paper, our group has demonstrated that the expression of topoisomerase II was associated with EDP-M efficacy (Roca *et al.*, 2017). These data need confirmation. It should be noted, however, that EDP is usually administered for a maximum of 6–8 cycles while mitotane is usually maintained till progression. It is possible that cytotoxic chemotherapy is useful to attain rapid tumor shrinkage but the long-term efficacy observed in some cases could be attributed to the mitotane maintenance. If this is true, predictive factors of mitotane efficacy are needed. Human cytochrome P450 2B6 (CYP2B6) (D'avolio *et al.*, 2013) and CYP2W1 (Ronchi *et al.*, 2014) that are involved in mitotane metabolism and may activate mitotane in the adrenocortical tissue, respectively, or ribonucleotide reductase large subunit 1 (RRM1) gene expression (Volante *et al.*, 2012) are promising predictive factors of mitotane efficacy.

Management of hormone secreting tumors

Patients with metastatic ACC that exhibits excess steroid secretion should be treated with steroidogenic inhibitors to ameliorate their effects (hypertension, hyperglycemia, hypokalemia, and muscle atrophy). The management of hormone excess in patients with metastatic ACC is often challenging. The presence of Cushing syndrome may consistently increase the toxicity of chemotherapy since it is associated with immune depression that may favor infections particularly in the neutropenia phase. Therefore, a rapid control of hormone hypersecretion is mandatory. Mitotane has both antisecretive and antiproliferative activity; however, the slow onset of mitotane activity is a main limitation for the management of Cushing syndrome (Terzolo *et al.*, 2014). Faster drug in lowering the serum cortisol levels are needed. Ketoconazole is more rapid than mitotane in controlling Cushing syndrome (Kamenický *et al.*, 2011) but it requires several weeks and its clinical employment is hampered by the hepatic toxicity. Metyrapone is an adrenolytic agent targeting the 11-beta-hydroxylase. In a recently published experience by our group, metyrapone was associated upfront to the EDP-M regimen and this combination was very well tolerated and led to a rapid control of Cushing syndrome induced by cortisol secreting ACC (Claps *et al.*, 2017).

Second-line therapies

The results of second-line therapy in patients with disease progression to platinum-containing regimens plus mitotane were as a whole modest. The association of Gemcitabine to metronomic capecitabine showed a limited activity in a prospective multicenter phase II trial conducted in Italy (Sperone *et al.*, 2010). Results have been confirmed a series of patients treated in a real world practice both in Germany and in Italy (Henning *et al.*, 2017). This regimen still remains an option as second-line therapy.

Several small phase II trials have tested the efficacy of molecular agents targeting EGFR, angiogenesis, IGFR, and mTOR pathways. These treatments administered in pretreated patients either alone or in combination with chemotherapy, or with other molecular target agents obtained poor results (Naing *et al.*, 2011; Berruti et al., 2012b; Kroiss *et al.*, 2012). In a multicenter randomized phase III trial involving most referenced centers in Europe and United States, the drug Linsitinib (OSI-906), an orally available IGFR inhibitor failed to demonstrate a superiority over placebo in terms of both progression-free and overall survival in advanced pretreated ACC patients (Fassnacht *et al.*, 2015). Also modern immunotherapy failed to show efficacy in advanced ACC. In a phase 1b cohort (NCT01772004), 50 patients with metastatic ACC and prior platinum-based therapy received avelumab at 10 mg/kg IV Q2W until progression. Only two patients (5%) attained a disease response while PFS was 5.5 and 1.5 months in patients with PDL-1 positive and negative ACC patients, respectively (Le Tourneau *et al.*, 2017).

Concluding Remarks

ACC is generally associated with a grim prognosis; however, there is significant heterogeneity in clinical presentation and biological behavior. Thus, improving our capability of predicting tumor prognosis and its response to drugs will translate in better patient outcome and quality of life.

Despite recent advancements in our understanding of molecular biology of ACC, current treatment protocols are often disappointing. Mitotane remains the cornerstone of treatment and novel data in favor of adjunctive mitotane have been published. The level of evidence remains low in the absence of prospective studies; however, most experts recommend postoperative adjuvant mitotane in patients at high risk of recurrence.

In advanced ACC, the chemotherapy regimen EDP in association with mitotane has been established as the reference treatment after publication of the first controlled study in this rare tumor. Targeted therapies have overall failed to demonstrate sufficient activity although we cannot abandon a vigorous search for most active and less toxic therapies.

See also: Adrenal Incidentalomas. Adrenocortical Carcinoma: Genetics and Molecular Markers

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Adrenocortical Carcinoma: Genetics and Molecular Markers

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Introduction

During the last decade, major breakthrough occurred in the field of ACC, owing to new pan-genomic techniques. A consistent molecular classification of ACC emerged, with strong prognostic impact. The aim is to review this classification, and discuss its clinical impact.

Germline Genetics

Several germline alterations have been reported. The most prominent is TP53—encoding the tumor suppressor p53. Historically, ACC was first reported as part of the Li–Fraumeni syndrome in 1969 (Li and Fraumeni, 1969; Li *et al.*, 1988), and later linked to TP53 mutations in 1990 (Malkin *et al.*, 1990). In terms of prevalence, childhood ACC must be distinguished from adult ACC. Indeed, in children, up to 90% (Wagner *et al.*, 1994; Pinto *et al.*, 2015) of cases are related to TP53 germline mutations. The most spectacular situation is observed in the region of Parana, a South state of Brazil. There, a founder mutation of TP53 (Ribeiro *et al.*, 2001)—R337H—reaches a prevalence as high as 0.3% (Custódio *et al.*, 2013), responsible for an unusually high annual incidence of ACC in this particular region—up to 4 per million children under 15 years (Sandrini *et al.*, 1997). On the opposite germline mutations of TP53 in adults is much lower, reaching less than 5% of patients (Herrmann *et al.*, 2012; Raymond *et al.*, 2013a).

Another constitutive germline alteration is observed in the 11p15 locus. This locus encompasses the IGF2 and CDKN1C genes, submitted to parental imprinting. This locus is altered most often through uniparental disomy, with loss of the maternal allele, responsible for the Beckwith–Wiedemann syndrome (Fraumeni and Miller, 1967; Henry *et al.*, 1989; Wilkin *et al.*, 2000). Childhood ACC is part of this syndrome.

DNA damage repair genes have recently emerged as important germline players in ACC. Mutations in mismatch repair (MMR) genes originally reported in the Lynch syndrome—including MSH2, MLH1, and MSH6 mutations—have been identified in up to 3% of patients (Raymond *et al.*, 2013b). More recently, mutations in MUTYH—encoding a protein responsible for base excision repair—was reported in ACC patients, responsible for a specific molecular phenotype of hypermutation with a specific nucleotide signature (Pilati *et al.*, 2017). Germline mutation in BRCA2—a key player of homologous recombination mediated repair—was also described in ACC (El Ghorayeb *et al.*, 2016).

Anecdotal reports mentioned the occurrence of ACC in patients carrying several other mutations, including genes responsible for endocrine tumors and hyperplasias—including MEN1 (Waldmann *et al.*, 2007), PRKAR1A (Anselmo *et al.*, 2012), CYP21A2 (Hayashi *et al.*, 2013), or SDH genes (Else *et al.*, 2017), or in known tumor suppressor genes—including APC (Gaujoux *et al.*, 2010) or NF1 (Wagner *et al.*, 2005).

Should we today screen for germline predisposition in patients? In children definitely TP53 mutations should be screened systematically (Else and Rodriguez-Galindo, 2016). In adults, some authors propose a systematic screening of germline predisposition in a panel of genes, including TP53 and Lynch syndrome related genes, taking advantage of the new targeted NGS technologies. With such a strategy, up to 10% of adult ACC patients have a germline predisposition (Petr and Else, 2016).

Somatic Genetics

Candidate gene approaches were first used to identify somatic genetic alterations, targeting first the known germline predisposing genes. Thus TP53 was screened, identifying somatic mutations in up to 25% of sporadic ACC (Reincke *et al.*, 1994; Ohgaki *et al.*, 1993).

Somatic alterations of the 11p15 locus have also been identified in sporadic ACC, reaching almost 90% of cases (Gicquel *et al.*, 1994; Gicquel *et al.*, 1997).

Another common gene altered in ACC is CTNNB1, encoding the beta-catenin. Somatic mutations of CTNNB1 are observed in up to 25% of sporadic ACC (Tissier *et al.*, 2005; Gaujoux *et al.*, 2008). These are activating mutations, activating the Wnt/beta-catenin pathway.

More recently new technologies such as exome sequencing and SNP arrays systematically screened for somatic gene alterations (Pinto *et al.*, 2015; Assié *et al.*, 2014a; Zheng *et al.*, 2016; Juhlin *et al.*, 2015). A new prevalent gene came out, ZNRF3, encoding for

E3-ubiquitin Ligase (Assié *et al.*, 2014a; Zheng *et al.*, 2016; Juhlin *et al.*, 2015). This gene is known as a negative regulator of the Wnt/beta-catenin pathway, through regulation of Wnt receptor turnover (Hao *et al.*, 2012). ZNRF3 alterations—mainly homozygous deletions—are observed in up to 20% of ACC. ZNRF3 mutations are mutually exclusive with CTNNB1 mutations (Assié *et al.*, 2014a; Zheng *et al.*, 2016; Juhlin *et al.*, 2015).

These pan-genomic screens provided a list of approximately 20 genes recurrently altered in sporadic ACC, including cell-cycle-related genes (TP53, CDKN2A, RB1, CDK4), Wnt/beta-catenin related genes (CTNNB1, ZNRF3, APC), telomere maintenance related genes (TERT, TERF2, ATRX, DAXX), along with other genes such as MEN1, NF1 or MMR genes. These studies also reported a large list of genes with private somatic mutations, the interpretation of which is challenging.

Somatic Genomics

Beyond genes alterations, the recent advent of genomic technologies—including transcriptome, mirNome, methylome, and genome profiling-opened the way to a comprehensive molecular picture of ACC. Especially two pangenomic characterizations were published, one from ENSAT consortium (Assié *et al.*, 2014a), the other from TCGA (Zheng *et al.*, 2016).

Numerous transcriptome studies compared ACC and adrenocortical adenomas, providing a benign signature enriched in steroidogenic differentiation genes for adenomas, and a proliferation-related signature in cancer (Assié *et al.*, 2014b). IGF2 gene, located in 11p15, appeared as one of the most highly expressed genes in a majority of ACC (Giordano *et al.*, 2003). The most striking transcriptome feature comes from the unsupervised classification of ACC (de Reyniès *et al.*, 2009; Giordano *et al.*, 2009). Indeed two major groups emerged, with striking differences in terms of survival, with one group named C1A with a poor outcome, and the other called C1B with a much better outcome. Though tumor grades and stages are higher in C1A compared to C1B, distributions of these two strong prognostic parameters overlap between the 2 groups, raising the interest of a molecular classification in a prognostic aim. This two-group classification was more recently further granulated into subgroups: C1A was divided into "steroid high—proliferation high", "steroid low—proliferation high", and "steroid high—proliferation low" subgroups, and C1B was affiliated to the "steroid low—proliferation low" subgroup, along with a small subset of "steroid high—proliferation low" subgroup (**Fig. 1**). Steroid-low subgroups are characterized by a high expression of immunity related genes (Zheng *et al.*, 2016).

MiRNome profiling was also performed by several teams in ACC, especially in comparison with adenomas. Variable lists of miRNAs were identified as differential. The most robust one is probably the mir483-5p overexpression in ACC, a miRNA encoded within the IGF2 locus on 11p15 (Soon *et al.*, 2009; Özata *et al.*, 2011; Patterson *et al.*, 2011; Duregon *et al.*, 2014; Chabre *et al.*, 2013; Feinmesser *et al.*, 2015). Within ACC, different subgroups have emerged, more or less consistent between studies, potentially owing to technical variability. Further studies will probably conciliate these apparent discrepancies.

Methylome profiling reported hypomethylation of ACC compared to adenomas, due to global hypomethylation of CpGs in intergenic regions, as observed in a majority of cancers. In contrast, focusing specifically on CpGs in CpG islands—corresponding to genomic regions enriched with at least 50% of CpG, generally observed in the promoter regions of genes—a subset of ACC appeared as globally hypermethylated (Rechache *et al.*, 2012; Fonseca *et al.*, 2012; Barreau *et al.*, 2013). Interestingly hypermethylation in CpG island is strongly associated with a poor survival (Barreau *et al.*, 2013).

Chromosome alteration profiling reported differences between ACC and adenomas, mainly in terms of numbers of alterations. Within ACC, a subset displays loss of heterozygosity (LOH) extended to half or more of the genome, reflecting a loss of one DNA copy for a majority of chromosomes—referred to as hypodiploidy (Assié *et al.*, 2014a; Zheng *et al.*, 2016; Ronchi *et al.*, 2013). These tumors can remain hypodiploid, or replicate the remaining genome either once, twice or more, generating chromosome profiles paradoxically associating LOH and normal or even high DNA copy numbers. These extended LOH profiles have been called "chromosomal" profiles. Another subset of ACC displays numerous anarchic chromosomal losses and gains, and has been referred to as "noisy". A third and more limited subset of ACC has limited chromosomal alterations, and is referred to as "quiet" (**Fig. 2**). These distinct genome profiles are associated with distinct

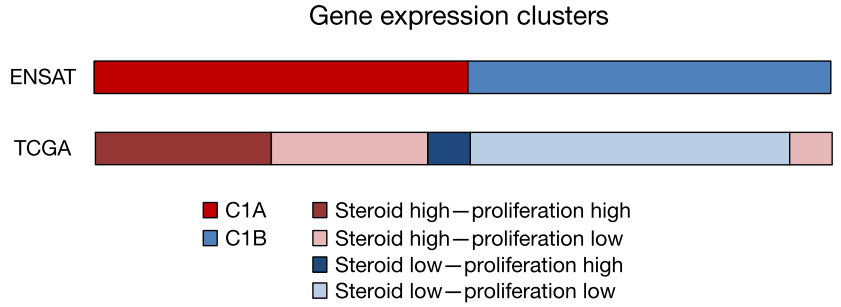
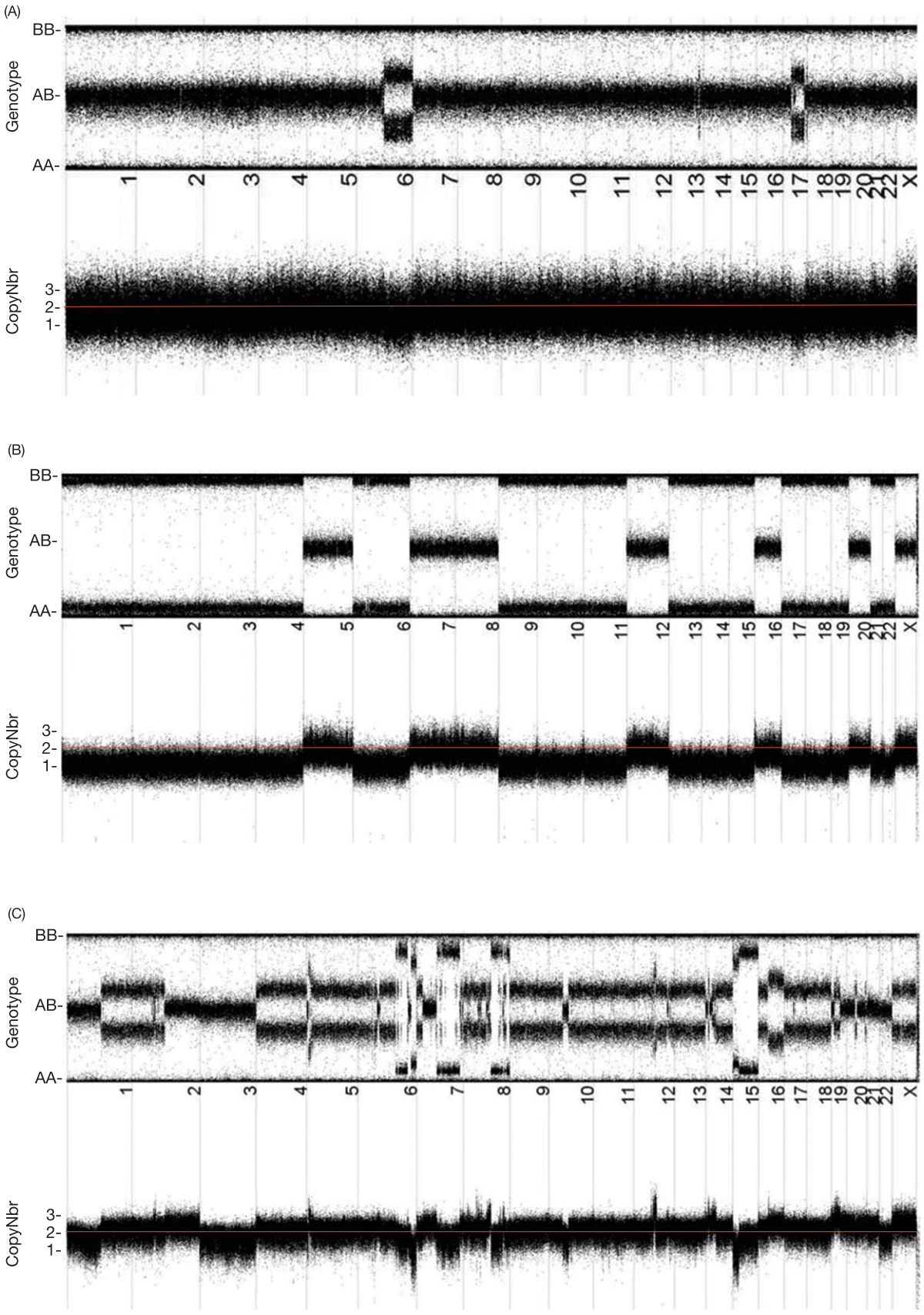


Fig. 1 Gene expression clusters from ENSAT and TCGA studies.


(A)
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X
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outcomes. Especially "noisy" ACC are associated with a worse prognosis. In addition ACC with polyploidy seem also more aggressive (Zheng *et al.*, 2016).

Combination of these various genetic and genomic alterations into a single classification generated a global classification of ACC in three groups. A first subgroup associates C1A transcriptome profile, CpG island hypermethylation, a "noisy" genome profile, and an accumulation of mutations in cell-cycle related and Wnt-beta-catenin related genes. In terms of clinical significance, this subgroup is associated with an obviously poor outcome. On the opposite, a subgroup of ACC associate a C1B transcriptome profile, along with no CpG island hypermethylation, and "chromosomal" or "quiet" genome profiles. This subgroup is associated with a much better outcome. Finally an intermediate subgroup of ACC, characterized with a C1A transcriptome along with one or two additional pejorative molecular alterations, shows an intermediate prognostic (**Fig. 3**).

Prognostic Molecular Markers

Historically molecular markers were developed in the 1990s, targeting 17p13 region—locus of TP53- and 11p15 region—locus of IGF2. Loss of heterozygosity (LOH) in these regions were shown to be associated with a shorter disease-free survival (Gicquel *et al.*, 2001), probably reflecting the malignant nature of adrenal tumors harboring these features. Along with 11p15 genome alterations, IGF2 overexpression was also identified as a malignancy feature.

More recent genomic classifications also provide new generations of markers. The general strategy is to try and recapitulate pan-genomic information into targeted molecular measures, for obvious cost and efficiency reasons. A first approach was the design of targeted gene expression signatures in ACC recapitulating the transcriptome prognostic information. Two pairs of gene differential expression have been proposed—BUB1B/PINK1 and DLGAP5/PINK1—combining one gene high and one gene low in C1A compared to C1B (**Fig. 3**). These combinations have shown significant association with disease-free and overall survival, in two independent and distinct cohorts (de Reyniès *et al.*, 2009; Fragoso *et al.*, 2012). Interestingly survival prognosis remained significant after stratification on tumor stage.

More recently targeted measures of DNA methylation in ACC was proposed as a molecular prognostic marker, reflecting methylome classification (**Fig. 3**). Methylation measured in CpG islands of four genes—PICARD, PAX5, PAX6, and GSTP1—were associated with disease-free and overall survival. Prognostic value remained significant in multivariate models including both tumor stage and tumor grade (Jouinot *et al.*, 2017).

Several miRNAs measured in ACC have shown some potential prognostic value (Cherradi, 2015), and validation in independent cohorts is awaited. miRNAs have also opened the perspective of liquid biopsies, with molecular markers measurable in blood. Indeed, circulating miRNAs associated with ACC prognosis have been identified (Chabre *et al.*, 2013; Patel *et al.*, 2013; Szabó *et al.*, 2014; Salvianti *et al.*, 2017). Larger validation cohorts are expected in the coming years, to define the potential value in clinical use.

A last development for liquid biopsy focused on cell-free circulating tumor DNA (ctDNA). Two recent studies demonstrated that ctDNA is detectable in some ACC patients, but not in all (Creemers *et al.*, 2017; Garinet *et al.*, 2017). In addition, ctDNA may reflect tumor burden evolution (Garinet *et al.*, 2017). It is not clear now how sensitive detection is, nor whether ctDNA detection is relevant in terms of prognostic value.

Conclusion and Perspectives

After a couple of decades using candidate genes-based approaches, the recent advent of pan-genomic techniques has boosted molecular characterization of ACC in the recent years. Now is time for post-genomic development of molecular markers suitable for clinical routine. This effort is ongoing, and relies on multicentric networks to reach the critical size for including patients with such a rare disease. At the present time, a strong molecular prognostic signature seems to emerge, with an expected clinical benefit of better determining the risk of recurrence after a complete surgery. How these molecular measures will concretely be performed in clinical setting remains to be determined.

Finally, up to now the impact of these massive molecular information on therapeutic aspects has been quite limited, despite the urgent need for new efficient treatments. A better prognostic stratification of patients may help therapeutic decision, especially for orienting the need for—and potentially the type of—adjuvant therapy after complete surgery. Hopefully in the coming years research will focus more on markers predicting response to treatment, and optimistically will identify efficient therapeutic targets?

Fig. 2 Chromosome alterations profiles determined by SNP array analysis.The upper part of each panel represents the proportion of the two investigated alleles "A" and "B" and determines the genotype (homozygous AA/BB or heterozygous AB) at each locus. Chromosomes are separated with vertical lines and numbered in ascending order. The lower part of each panel represents the copy number (Nbr). Red horizontal lines correspond to normal copy number (diploid). "Quiet" profile (A) is characterized by few number of large alterations. "Chromosomal profile" (B) shows copy-loss loss of heterozygosity (LOH) in several chromosome arms. "Noisy" (C) profile displays numerous chromosome breaks, with many anarchic losses and gains.

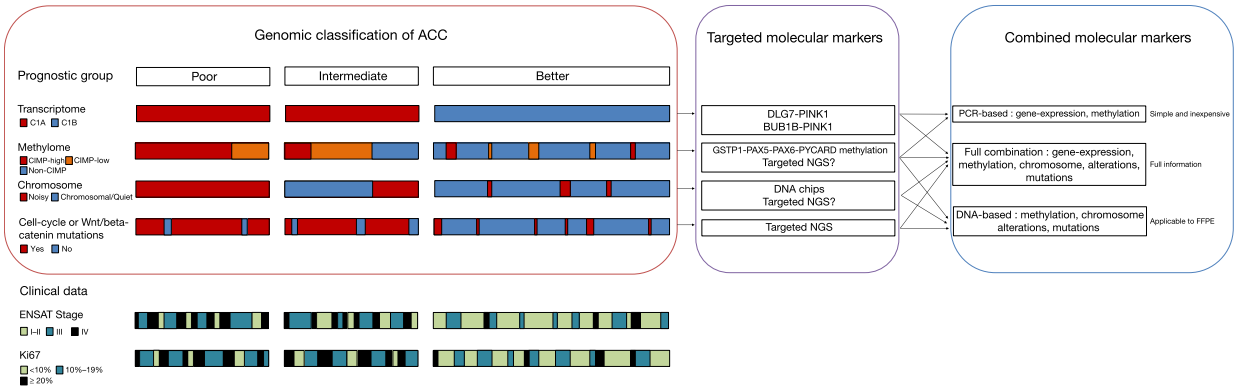

Fig. 3 Pan-genomic classification of ACC and molecular prognostic markers.

See also: Adrenocortical Carcinoma: Diagnosis and Therapy

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Adrenal Surgery

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Indications for Adrenalectomy

Nonfunctioning Adrenal Mass

Nonfunctioning adrenal masses are usually found incidentally and are rarely found due to locoregional symptoms. Adrenal incidentalomas are defined as lesions >1 cm detected on imaging studies performed for unrelated conditions. The prevalence of adrenal incidentalomas on abdominal imaging increases with age and is 1% or less in patients below 40 years of age versus up to 6% in patients in their seventh decade of life (Abecassis *et al.*, 1985). The main indication for adrenalectomy in patients with nonfunctioning adrenal tumors is the risk of malignancy. A malignant nonfunctioning adrenal mass may be due to adrenocortical carcinoma (ACC) or an adrenal metastasis from another primary.

Primary nonfunctioning adrenocortical carcinoma versus adenoma

The assessment of an adrenal mass to distinguish between a primary ACC versus adenoma is important when considering adrenalectomy for nonfunctioning adrenal masses and when selecting the surgical approach and technique. One of the most predictive factors for malignancy is tumor size. The risk of malignancy increases with tumor size and is 1%–2.9% for tumors <4 cm and 78% for tumors 8 cm and greater (Sturgeon *et al.*, 2006).

Although tumor size on imaging is an important predictive variable for malignancy, other radiologic features on computed tomography (CT) and/or magnetic resonance imaging (MRI) and functional imaging with 18F-FDG positron emission tomography (PET) CT are also helpful for evaluating the risk of malignancy in patients with a nonfunctioning adrenal mass. Also, several benign lesions that do not require adrenalectomy have pathognomonic imaging features that allow for active surveillance in patients with these types of tumors (unless they are symptomatic) (**Table 1** and **Fig. 1**). Features associated with a higher risk of malignancy on anatomic imaging studies include irregular margins, tumor heterogeneity, >10 Hounsfield units (HU), and the presence of necrosis or calcifications (**Fig. 2**) (Zhang *et al.*, 2012; Petersenn *et al.*, 2015). On MRI, malignant lesions are usually isointense compared to the liver on T1 and hyperintense on T2 (**Fig. 3**). ACC have higher ^{18}F-FDG PET/CT SUV as compared to adrenocortical adenomas (ACA). A maximum tumor SUV >4.1 and SUVmax of tumor-to-liver ratio >1.5 has a sensitivity of 86.7% and specificity of 75%, respectively, in differentiating ACA from ACC (**Fig. 4**) (Guerin *et al.*, 2017; Boland *et al.*, 2011).

The decision to perform an adrenalectomy for a nonfunctional adrenal mass is made on a case-by-case basis. When the risk of malignancy is high based on the imaging characteristics described, adrenalectomy is warranted regardless of tumor size or the patient's age. The threshold for adrenalectomy should be low in patients younger than 30 years old with even a small adrenal mass because adrenal incidentalomas are rare in this age group. On the other hand, in an older age group, adrenalectomy is recommended for lesions >6 cm, though some older patients with large adrenal masses and nonworrisome imaging features and comorbidities could have active surveillance (Arnold *et al.*, 2003).

Nonfunctioning adrenal mass suspected to be a metastasis

The most common primary tumor sites that spread to the adrenal gland are lung, melanoma, renal cell carcinoma, and gastrointestinal adenocarcinomas. Less common ones include breast cancer and sarcoma. In cases with indeterminate previous biopsy results or where the primary site is not amenable to biopsy, adrenalectomy may be indicated to establish the diagnosis. When performing adrenalectomy to achieve no evidence of disease, patient selection is important. Patients may benefit from adrenalectomy if the metastasis is isolated to the adrenal gland or in the presence of oligometastatic disease, where curative surgical resection of the primary tumor along with other metastatic sites is performed. The median overall survival after adrenalectomy for adrenal metastasis is 24–46 months with a 5-year survival rate of 22.5%–38% (Marangos *et al.*, 2009; Howell *et al.*, 2013). Factors associated with longer survival time in patients who have adrenal metastasectomy include synchronous metastasis (diagnosed within 6 months of treatment of the primary disease site), R0 resection (negative gross and microscopic margin), and primary tumor site (renal cell carcinoma and colon cancer as compared to melanoma and nonsmall cell lung carcinoma) (Vazquez *et al.*, 2012; Muth *et al.*, 2010). Retrospective studies suggest that laparoscopic (lateral or retroperitoneal approach) adrenalectomy may have similar outcomes to open adrenal metastasectomy in terms of tumor margin status, rate of local recurrence, and rate of port site metastasis/recurrence (Sarela *et al.*, 2003; Heniford *et al.*, 1999).

Table 1 Differential diagnosis of adrenal incidentaloma and imaging characteristics

Differential diagnosis (% of total cases)	*Imaging features (Allen and Francis, 2015)*
Adrenal cortex adenoma	
Nonfunctional (60%–90%)	CT: HU <10;[a]
Functional (2%–20%)	Absolute contrast washout (AW) $\geq 60\%$;
Cushing syndrome	Relative contrast washout (RW) $\geq 40\%$;[b]
Conn syndrome	
Adrenocortical carcinoma	Large
	Heterogeneous
	Irregular margins
	Areas of necrosis and calcifications
	AW $< 60\%$
	RW $< 40\%$
Adrenal medulla	
Pheochromocytoma (1%–10%)	Heterogeneous
	Hypervascular with arterial enhancement
	AW $< 60\%$
	RW $< 40\%$
	Hyperintense on T2-weighted MRI
Metastasis (10%–75%)	Nonspecific
Cyst (4%–22%)	Thin-walled
	Homogeneous
	Fluid attenuations
Hematoma/hemorrhage (0%–5%)	Acute: round high attenuation mass
	Chronic: dense calcifications, pseudocyst with peripheral calcifications
Myelolipoma (5%–15%)	Presence of macroscopic fat
Ganglioneuroma (0%–6%)	CT: HU < 40
	Punctate or discrete calcification
	MR: T2 increase signal intensity
Tumors from adjacent organs ($<1\%$)	

[a]Hounsfield unit.

[b]Absolute contrast washout (AW) = 100 [(ICT–DCT)/(ICT–UCT)]; relative contrast washout (RW) = 100 [(ICT–DCT)/ICT] (I = initial, D = delayed, U = unenhanced).

Functioning Adrenal Masses

Functioning adrenal masses may hypersecrete catecholamine and their metabolites (pheochromocytoma), aldosterone causing primary hyperaldosteronism (PA) (Conn syndrome), cortisol causing overt or subclinical hypercortisolemia (adrenal Cushing syndrome), and less commonly, androgens (virilizing/feminizing tumors) or multiple hormones. Functioning adrenal masses may be malignant in patients with pheochromocytoma, adrenal Cushing syndrome (over 40% of ACCs are functional), and virilizing/feminizing syndromes (Ayala-Ramirez *et al.*, 2013; Luton *et al.*, 1990). Patients with each type of functional adrenal mass require attention to specific perioperative management issues to ensure optimal patient outcome (covered at the end of the article).

Pheochromocytoma/abdominal paragangliomas

Pheochromocytomas and paragangliomas (PC/PGL) are present in 0.2%–0.6% of patients with hypertension and account for 4% of adrenal incidentalomas (Darr *et al.*, 2017). Patients may present with symptoms of headache, anxiety, diaphoresis, and palpitations, or they may be asymptomatic. The diagnosis of PC is established by biochemical testing for plasma-free metanephrines, or urinary fractionated metanephrines, which have a sensitivity of 97%–100% and a specificity of 69%–89% (Lenders *et al.*, 2002). Chromogranin A may also be elevated and is a useful marker in biochemically silent tumors. PGLs are extra-adrenal tumors that may or may not hypersecrete catecholamines and their metabolites. The surgical management of abdominal paragangliomas is included in this article. Once the diagnosis has been established, anatomic and functional imaging are used for localization and to evaluate for multiple tumor sites or metastases.

The only curative treatment for functional PC/PGL is surgical resection and, if it is left untreated, patients have significant morbidity/mortality from catecholamine oversecretion, which includes diabetes and cardiovascular events such as malignant hypertension, stroke, and heart failure (Stolk *et al.*, 2013). The extent of surgical resection and approach is dictated by the risk of malignancy, presence of synchronous disease/multiple primary tumor sites, and risk of developing metachronous primary PC/PGL. Malignant PC/PGL is defined by the presence of gross locoregional invasion or metastatic disease in tissue where chromaffin cells are not present and include lymph nodes, bone, lung, and liver (Fliedner *et al.*, 2010).

Germline and somatic mutations in up to 15 genes have been reported in patients with PC/PGL (Gupta and Pacak, 2017). Germline mutations in susceptibility genes for PC/PGL have been shown to have genotype–phenotype associations, and knowledge of this information is important in the surgical management of patients with PC/PGL. Patients with germline *RET*, *VHL*, or *NF1* mutations are at low risk for having or developing metastatic disease, whereas patients with

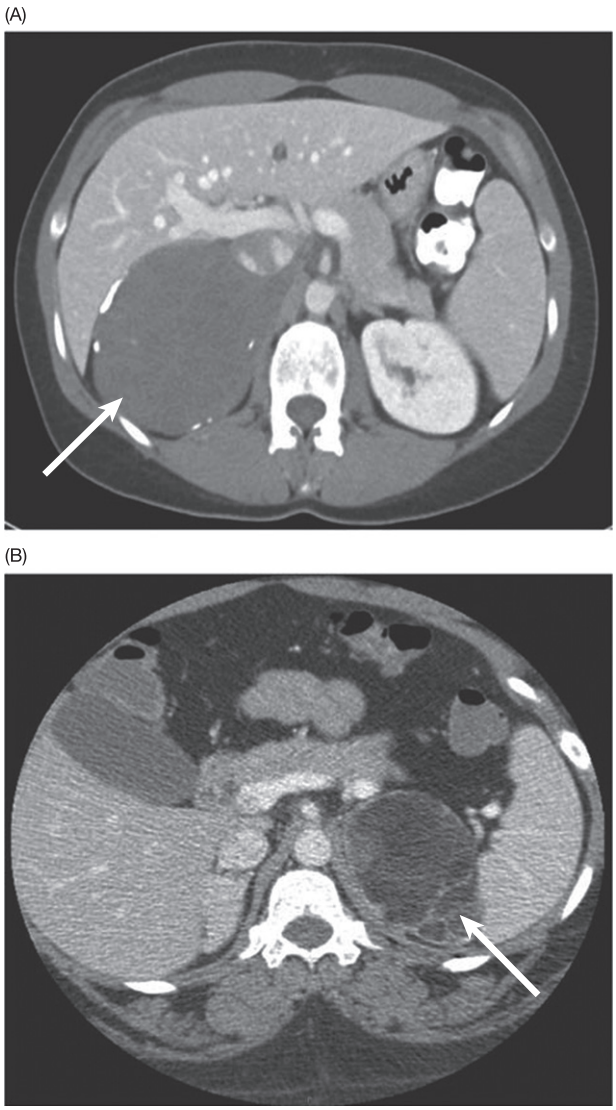

Fig. 1 Computed tomography (CT) of a ganglioneuroma (A) and myelolipoma (B). CT with intravenous contrast of a ganglioneuroma of the right adrenal gland. The tumor has a low Hounsfield unit on contrast and is isointense with the muscle, and it has peripheral fine calcifications. CT with intravenous contrast showing a myelolipoma of the *left* adrenal gland. The mass is hypointense and isointense with the peritoneal fat and has interspersed higher attenuation tissue.

germline mutations in *SDHB*, *FH*, and *MAX* have a higher risk of metastatic disease, which may be up to 90% in patients with the *SDHB* germline mutation (Matro *et al.*, 2013; Castro-Vega *et al.*, 2014). Patients with *RET*, *VHL*, and *NF1* germline mutations have a high risk of having synchronous or metachronous bilateral PC (**Fig. 5**); therefore, given the low risk of malignancy, adrenal-sparing adrenalectomy has been advocated in patients harboring these mutations in order to avoid the need for life-long steroid replacement as well as Addisonian crisis (Castinetti *et al.*, 2014; Aufforth *et al.*, 2015). Germline mutations in susceptibility genes are present in over 40% of patients with PC/PGL, and approximately a quarter of these patients may present with no family history of PC/PGL. Thus, all patients with PC/PGL should have preoperative genetic testing for susceptibility genes and genetic counseling, as this information could optimize surgical management. The development of next-generation sequencing platforms to test for germline mutations in PC/PGL susceptibility genes is becoming widely available and cost-effective; therefore, it should be performed preoperatively in patients with PC/PGL to allow for precision adrenal surgery.

Functional imaging studies including FDOPA PET/CT, F-dopamine, MIBG, and ^{18}F FDG PET/CT have been used in patients with PC/PGL to detect sites of multifocal or metastatic disease, which is present in up to 90% of patients with the germline *SDHB* mutation. ^{18}F FDG PET/CT is currently the recommended functional imaging modality in patients with PC/PGL (Lenders *et al.*, 2014). Although functional imaging is recommended both when malignant disease is suspected and in patients with germline *SDHB* mutation, all patients with PC/PGL may benefit from preoperative imaging with at least one functional imaging study.

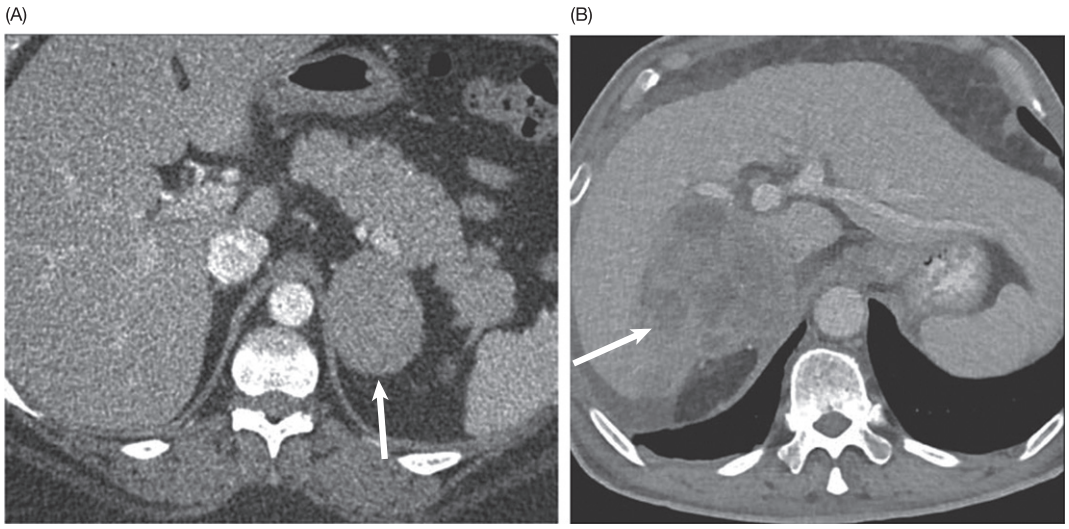

Fig. 2 Computed tomography (CT) of an adrenocortical tumor with typical features for an adenoma (A) and carcinoma (B). CT scan with intravenous contrast of a left adrenocortical adenoma on histology shows a well-circumscribed tumor with a Hounsfield unit of <10 U, no necrosis, and no calcification in (A). CT scan with contrast in (B) shows an adrenocortical carcinoma in the *right* adrenal gland with invasion into the *right* lobe of the liver and abutting the *right* portal vein that is also invading the inferior vena cava. There is necrosis and irregular margins.

Janssen *et al.* compared ^{68}Ga DOTATATE, a newer functional imaging modality, to anatomic and other functional imaging modalities and showed its superior detection rates (Janssen *et al.*, 2016).

Patients with PC/PGL should have alpha blockade therapy with phenoxybenzamine or doxazosin for 7–14 days before their operation. The target blood pressure readings are systolic <140 and diastolic <80 mmHg. If tachycardia develops and the blood pressure is adequately controlled, then beta blocker therapy should be instituted. Alternative agents for better blood pressure control include calcium channel blocker or tyrosine hydroxylase inhibitors (Pacak, 2007; Wachtel *et al.*, 2015). Prolonged exposure to excess catecholamines can cause contraction of the intravascular volume; therefore, hydration should be initiated when the patient starts on alpha blockade therapy as well as intravenously 24 h prior to the operation. Intraoperative manipulation of the tumor should be minimized to avoid intraoperative hemodynamic instability. The anesthesiologist should be prepared to treat hypertensive crisis with phentolamine, sodium nitroprusside, or nicardipine. Continuous blood pressure monitoring with an arterial line is imperative, and a central venous line for fluid and drug administration should be considered in all patients with PC/PGL.

At the time of the operation, once the tumor is removed or when the main adrenal vein is ligated and divided, patients may develop significant hypotension. Thus, close communication with the anesthesiologist is imperative during the operation to allow a coordinated management plan. Patients may require significant fluid resuscitation to maintain their blood pressure, and a central venous line is helpful for accurately determining the patient's fluid status. If the patient has persistent hypotension after adequate fluid resuscitation is established, then norepinephrine or dopamine agonists may be required to maintain a normal blood pressure. Blood glucose should also be measured in the postoperative period until stable glucose levels are observed, as patients may experience hypoglycemia due to the sudden drop in catecholamine levels (Chen *et al.*, 2014).

Primary Hyperaldosteronism

PA is diagnosed in 11% of patients with hypertension (Rossi *et al.*, 2006). PA is associated with cardiovascular morbidity that is worse than essential hypertension, even when having similar blood pressure levels (Milliez *et al.*, 2005). Patients should be screened for PA if they have persistent hypertension ($>150/100$); require more than three antihypertensive medications ($>140/90$); have hypokalemia; or have hypertension with an adrenal mass, sleep apnea, or family history of hypertension or stroke prior to the age of 40. The diagnosis of PA is established by an aldosterone level >15 ng/dL and an aldosterone-to-renin ratio >20 (Young, 2007). Some patients with an aldosterone level of 9–16 may still have PA (Mosso *et al.*, 2003). Confirmatory testing with a saline suppression test, oral sodium loading test, fludrocortisone suppression test, or a captopril challenge test are recommended (Funder *et al.*, 2016).

In 65%–70% of PA cases, PA is due to bilateral adrenal hyperplasia, for which medical management is the treatment of choice. If medical management fails and patients develop severe metabolic complications, bilateral adrenalectomy may be considered—though it is rarely necessary. PA is due to ACA, unilateral hyperplasia with ACA in 30%–35% of patients, and rarely due to isolated unilateral hyperplasia. Typically, patients with unilateral disease are referred for adrenalectomy unless there is a contraindication (Funder *et al.*, 2016). Other rarer causes of PA include ACC, familial hyperaldosteronism (Type I–III), and ectopic aldosterone-producing tumor. Type I familial hyperaldosteronism is due to the *CYP11B1* (11-β-hydroxylase) promoter region, which is fused

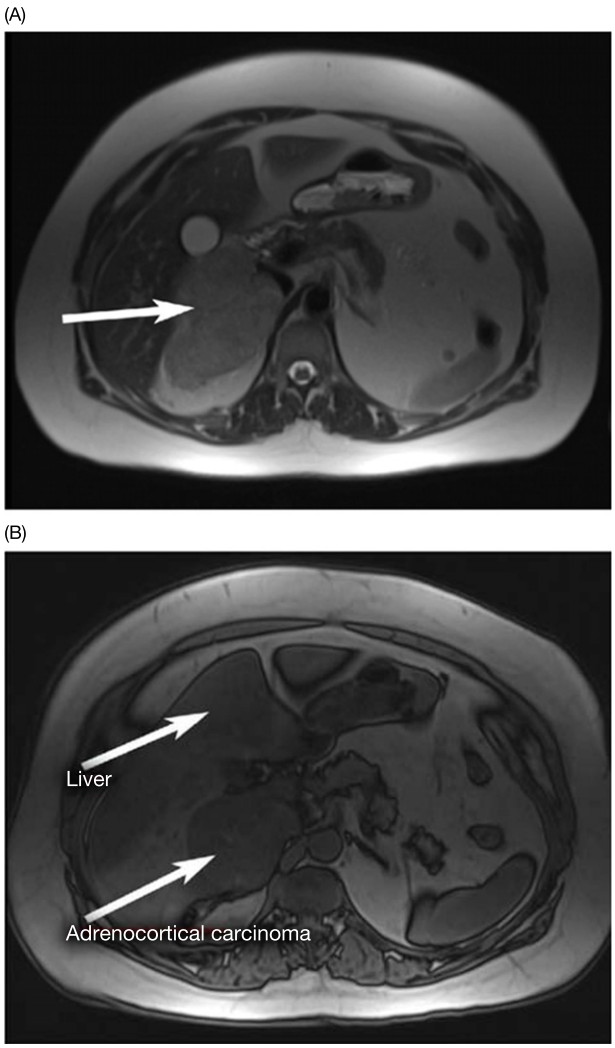

Fig. 3 Magnetic resonance imaging (MRI) features of an adrenocortical carcinoma. (A) Adrenocortical carcinoma enhancing on T2-weighted image shows higher intensity than the liver. (B) T1-weighted imaging of adrenocortical carcinoma showing similar intensity to the liver.

to *CYP11B2* (aldosterone synthase gene), resulting in a CYP11B1/2 chimera that results in ACTH-dependent aldosterone hypersecretion. Thus, glucocorticoid therapy is effective, and patients do not require adrenalectomy. The cause of Type II familial hyperaldosteronism is unknown, but patients may be treated medically or with adrenalectomy. Lastly, type III familial hyperaldosteronism is due to a *KCNJ5* germline mutation, and patients with uncontrolled metabolic complications with medical therapy may rarely require bilateral adrenalectomy (**Fig. 6**).

Once the biochemical diagnosis of PA is made—and if the patient is interested and is a candidate for adrenalectomy—then anatomic imaging with CT or MRI should be performed. It is impossible to differentiate an aldosterone-producing adenoma from a nonfunctioning adenoma based on imaging characteristics, so patients who are either older than 35 years of age, have bilateral adrenal lesions, or have normal bilateral adrenal glands should undergo adrenal vein sampling (AVS) to lateralize the disease and to differentiate unilateral from bilateral disease (Mathur *et al.*, 2010). Many criteria for AVS to distinguish between bilateral and unilateral PA have been used and have shown variable accuracy (Webb *et al.*, 2012). The most accurate criterion is using ACTH stimulation to determine proper catheter positioning: cortisol [adrenal]/cortisol [periphery] >5.0 and to determine lateralization: aldosterone/cortisol [A/C] dominant: A/C nondominant $>4{:}1$. AVS in patients with PA ensures they have unilateral disease and are likely to benefit from an adrenalectomy, and it helps avoid wrong-side surgery in the case where there are bilateral normal or abnormal adrenal glands.

Stringent blood pressure control should be pursued prior to adrenalectomy, and potassium levels should be repleted to normal. The surgeon should personally review the results of AVS as well as imaging studies to make sure the operation is being performed on the correct side.

Postoperative patients should be monitored for mineralocorticoid insufficiency and hyperkalemia, though in most patients it is self-limited. Factors associated with a higher risk of hyperkalemia include older age (>50 years), increased duration of

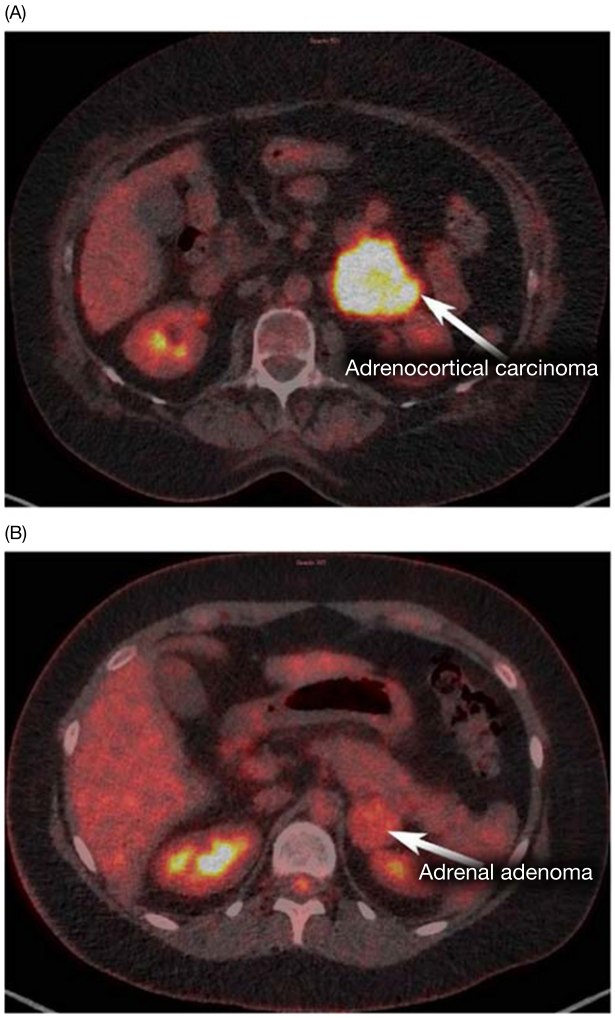

Fig. 4 ^{18}F-FDG PET/CT scan of an adrenocortical carcinoma (A) and adrenocortical adenoma (B). *Arrow* showing tumor with a high SUVmax in the left adrenal gland in (A) consistent with an adrenocortical carcinoma and arrow showing low SUVmax in the *left* adrenal mass in (B) consistent with an adrenocortical adenoma.

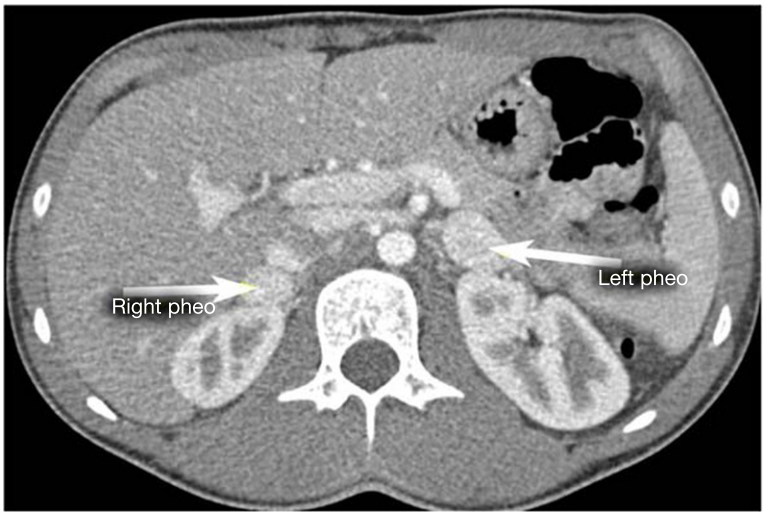

Fig. 5 Computed tomography with intravenous contrast shows enhancing bilateral pheochromocytomas in a patient with MEN2A due to a germline *RET* mutation in codon 634.

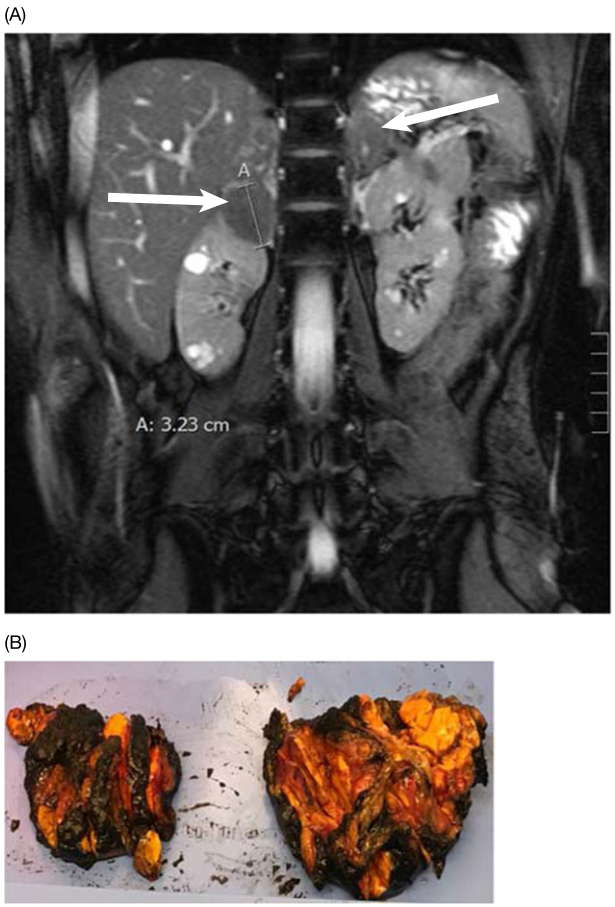

Fig. 6 Bilateral adrenal hyperplasia in a 24-year-old woman with a germline *KCNJ5* mutation who required bilateral laparoscopic adrenalectomy for uncontrolled hypertension and chronic renal failure. (A) Coronal section of MRI showing bilateral large adrenocortical tumors consistent with macronodular disease. (B) Picture of resected gross specimen after inking.

hypertension (>10 year), and renal insufficiency (Tahir *et al.*, 2016). Hypertension may improve over a period of up to 1 year, but it does not always resolve. An PA resolution score has been developed to provide a precise outcome measure with respect to several clinical endpoint variables after adrenalectomy for unilateral PA. Patients older than 55 years, males, patients with a body mass index >25, patients requiring three or more antihypertensive medications, and patients with a history of hypertension for more than 6 years are associated with a lower rate of hypertension resolution (<25%) (Aronova *et al.*, 2014). However, improvement in hypertension is achieved in 98% of patients as defined either by better control on antihypertensive medication than before adrenalectomy or by requiring a reduced number of antihypertensive agents. In addition to better blood pressure control, some patients with PA who have adrenalectomy will see cardiac and renal dysfunction reversal, and some report improvements in quality of life 3–6 months after adrenalectomy (Funder *et al.*, 2016).

Cushing Syndrome

Cushing syndrome is due to hypercortisolism presenting with typical physical features such as abdominal striae, facial plethora, proximal myopathy, weight gain, and decrease in growth in pediatric patients, to name a few. Patients may also present with metabolic complications and behavioral abnormalities. Patients with incidentally found adrenal mass, metabolic complications not expected for their age, and pediatric patients with growth stunting and obesity should be tested for hypercortisolism. Cushing's syndrome may be either ACTH-dependent (excess ACTH secreted by a pituitary tumor or ectopic tumor) or ACTH-independent, caused by hypersecretion of cortisol from the adrenal gland. In 80% of the cases, it is the result of an ACA (**Fig. 7**), followed by ACC in 15% of the cases and finally by bilateral micro/macronodular adrenocortical hyperplasia. The initial diagnostic evaluation includes midnight salivary cortisol, a low-dose dexamethasone suppression test, and 24-h urine-free cortisol. Exogenous hypercortisolism should be ruled out, and the tests should be repeated to confirm the diagnosis (Nieman *et al.*, 2008).

Lack of typical physical exam findings such as purple striae, truncal obesity, or cervical fat pat does not exclude a state of hypercortisolism. The condition of excess cortisol without physical characteristics is defined as Subclinical Cushing syndrome (SCS). These patients often have metabolic complications of excess cortisol such as hypertension, obesity, and diabetes mellitus

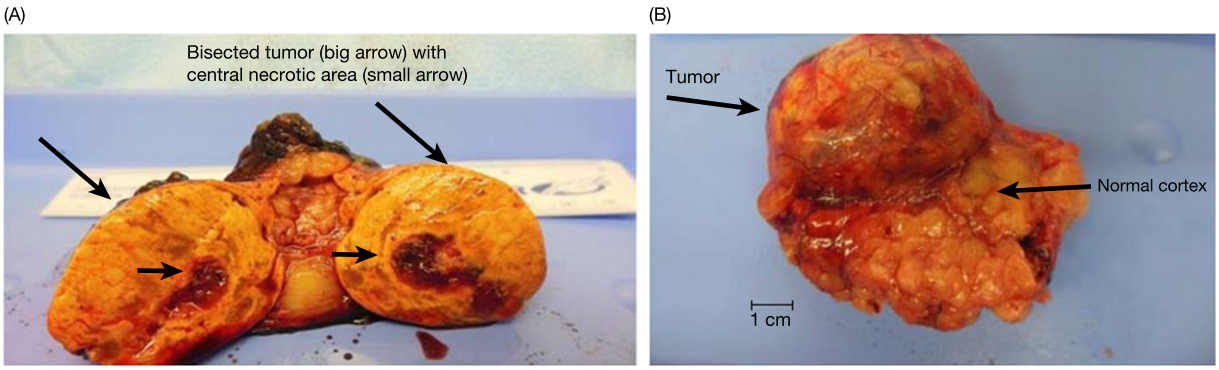

Fig. 7 An adrenocortical cortisol-secreting adenoma in a patient with adrenal Cushing syndrome. (A) Bisected adrenocortical adenoma. (B) Adrenocortical adenoma and adjacent normal cortex.

type 2, but the diagnostic criteria for SCS vary, and there is no "gold standard" diagnostic tool to confirm SCS (Chiodini, 2011). There is no consensus on the optimal management of SCS, but adrenalectomy is beneficial in many patients and is associated with improved blood pressure, reversal of obesity, and better glucose control (Chiodini *et al.*, 2010; Chiodini, 2011; Iacobone *et al.*, 2015).

Patients with Cushing syndrome (ACTH-independent and those who fail primary treatment for ACTH-dependent tumors) benefit from adrenalectomy. The benefits of adrenalectomy in patients with Cushing syndrome include improvements in hypertension, diabetes, and quality of life, as well as improvements in physical characteristics such as weight gain, proximal muscle wasting, skin changes (striae, easy bruising, darkening, thinning), and level of anxiety and depression (Neychev *et al.*, 2015; Brunt *et al.*, 2001; Thompson *et al.*, 2007).

Perioperative preparation in patients with Cushing syndrome includes postoperative administration of stress dose hydrocortisone of 100 mg with a rapid (24 h) taper postoperatively. Surgeons should be aware that prolonged hypercortisolemia makes the tissues friable and prone to injury with minimal manipulation, so they should be handled with extra caution. The risk of venous thromboembolism is also up to tenfold higher, so we continue deep vein thrombosis and pulmonary emboli prophylaxis for a total of 28 days and start prophylaxis the day before the operation (Small *et al.*, 1983; van der Pas *et al.*, 2013; Tirosh *et al.*, 2017). The need for continued steroid replacement until recovery of hypothalamus–pituitary axis (HPA) should be evaluated. Some centers perform an immediate postoperative cosyntropin stimulation test and, if the unstimulated cortisol level is normal and the stimulated cortisol level is ≥ 18 μm/dL, then the patient may be discharged without steroid replacement. Otherwise, it should be rechecked every 3–6 months (Benhammou *et al.*, 2010). Almost all patients experience adrenal insufficiency, and the average recovery time of the HPA axis is 11.2 months (Di Dalmazi *et al.*, 2014).

Surgical Approach

There are multiple surgical approaches and extents of adrenalectomy that can be utilized in patients requiring adrenalectomy, but the optimal procedure should be dictated by the individual patient characteristics, functional status of the tumor, imaging and biochemical features, and the genetic predisposition. Most functional benign tumors can be addressed using a minimally invasive approach, whereas large tumors suspicious for malignancy or tumors that are locally invasive should be resected using an open surgical approach. Both surgical approaches should be considered as a continuum in a patient requiring an adrenalectomy.

Currently, minimally invasive adrenalectomy is regarded as the gold standard surgical approach for the management of benign adrenal disorders, although there are no prospective randomized trials comparing this technique with open adrenalectomy. Minimally invasive adrenalectomy as compared to open adrenalectomy is associated with less blood loss, earlier patient mobility, decreased length of hospital stay, faster return to regular activity, and decreased morbidity, including pulmonary, renal, and cardiac complications (Prinz, 1995; Thompson *et al.*, 1997). Since 2000, robotic technology has been introduced into laparoscopic clinical practice (Gagner *et al.*, 1997; Lombardi *et al.*, 2008; Lezoche *et al.*, 2008; Bonjer *et al.*, 2000). The robotic system has been used for adrenalectomy but does not appear to offer patient or surgeon-specific benefits as compared to laparoscopic adrenalectomy. Below, each surgical approach is discussed with the proper patient population defined for each surgical approach as well as the extent of adrenalectomy.

Open Adrenalectomy

Several open approaches have been used for adrenalectomy using a subcostal, thoracoabdominal, midline, or posterior flank incision (**Fig. 8**). In 1889, Thornton first performed the transperitoneal approach (Thornton, 1890). The flank approach was described by Charles Mayo in 1927 (Mayo, 1927), and in 1936, Young, from Johns Hopkins, reported the first posterior approach

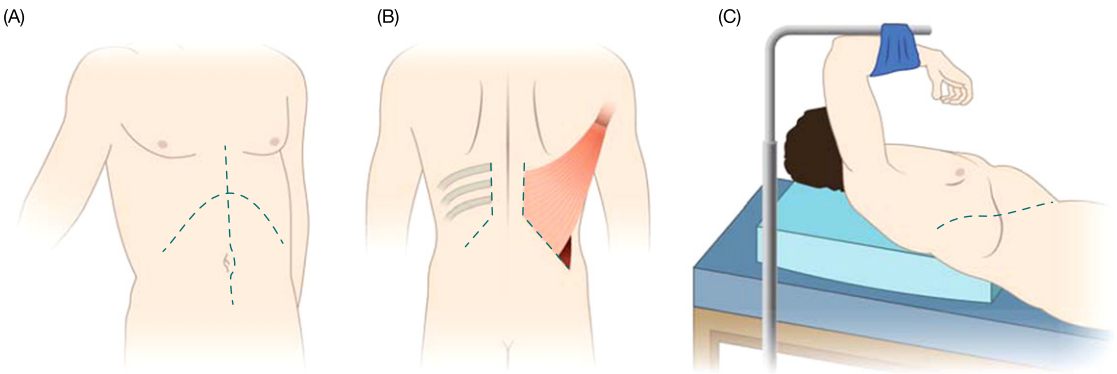

Fig. 8 Open adrenalectomy approaches. (A) Incision for an anterior open approach through either a bilateral subcostal incision or a midline incision. (B) Incision for a posterior approach. (C) Incision for a thoracoabdominal incision with a bump when required.

Table 2 Surgical approach and indications

Open adrenalectomy	*Laparoscopic transperitoneal*	*Retroperitoneal*	*Partial adrenalectomy*
Radiologic/clinical signs of malignancy:[a]	No malignant potential		
– Local invasion – Regional lymphadenopathy			
Large tumor size		History of multiple abdominal surgeries	Genetic predisposition for metachronous or synchronous pheochromocytoma in contralateral gland as in patients with germline *RET*, *VHL*, and *NF1* mutations
Contraindication for laparoscopic surgery	Surgeons' skills level	Surgeons' skills level	Adrenal remnant >30% of normal adrenal gland

[a]Role of laparoscopic adrenalectomy is controversial.

(**Table 2**) (Young, 1936). An open adrenalectomy should be performed for known ACC and metastatic/malignant PC/PGL or when the likelihood of malignancy is high, such as in large tumors (>6 cm) (Zeiger *et al.*, 2009), a tumor with suspicious features for local invasion, or locoregional disease or recurrence.

In the open transperitoneal approach (**Figs. 8** and **9**), a subcostal or midline incision is usually used, and a thoracoabdominal incision is used for a tumor invading the diaphragm or a vena cava tumor thrombus extending into the supra or intrahepatic cava. For a right adrenalectomy, the falciform and triangular ligament of the liver are divided, and the right hepatic lobe is mobilized medially to allow exposure of the inferior vena cava (IVC) and the adrenal gland in the retroperitoneum. If further exposure is necessary, the liver can be completely mobilized by releasing the coronary ligaments. Small veins from the IVC can be ligated and divided to help with mobilization. Vascular control is imperative in cases of large or invasive tumors or when there is invasion of IVC with tumor thrombus. An extended Kocher maneuver is performed to expose the infra hepatic IVC. Once the suprahepatic IVC and the infrahepatic IVC (preferably above the renal veins, if technically feasible) are controlled, dissection of the right border of the IVC is then carried out by incising the peritoneal reflection along the right border of the IVC all the way up to the diaphragm. Care should be taken not to injure the right hepatic vein. When thrombosis into the right atrium is anticipated, adrenalectomy should be performed under veno-venous bypass or cardiopulmonary bypass (**Fig. 10**). If IVC tumor thrombus is suspected, a venotomy is performed with extraction of the tumor thrombus. In rare instances of IVC invasion, resection of the IVC and an interposition graft may be necessary but should be done on veno-venous or cardiopulmonary bypass.

Dissection along the right border of the IVC leads to the identification of the right adrenal vein, which is then ligated and divided. The arterial supply of the right adrenal gland is controlled and divided as it is encountered along this dissection (it usually consists of three main arterial branches, from the right renal artery, the aorta, and the right inferior phrenic artery). Care should be taken not to injure any accessory or superior branches of the right renal artery. The dissection along the right border of the IVC is then carried further posteriorly until reaching the quadratus lumborum muscle and then inferiorly until reaching the right renal hilum. In the case of a locally invasive tumor, it may be necessary to resect a portion of the diaphragm to achieve negative margins, and this can be repaired by simple primary repair or with a Gortex™ patch.

On the left side, the adrenal tumor is exposed by medial rotation of the spleen, pancreas, and left colon. Care should be taken to avoid any injury to the splenic vessels and pancreatic tail during the dissection. If invasion into the renal hilum is anticipated, then the renal vascular pedicle should be controlled before resection. If the diaphragm is resected, either a primary closure or Gore-

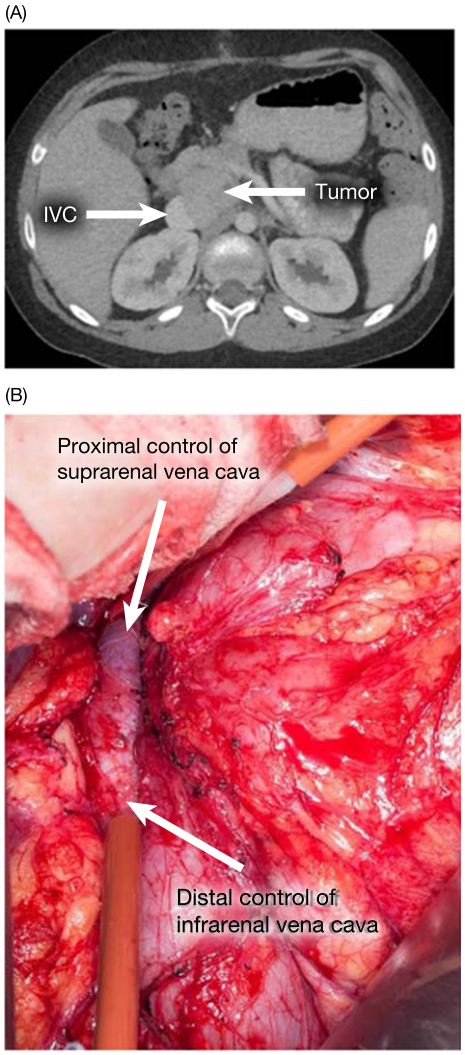

Fig. 9 Open resection of an aortocaval paraganglioma invading the inferior vena cava (IVC). (A) Computed tomography scan of tumor invading IVC. (B) Intraoperative image after extended Kocher maneuver, medial mobilization of right colon with proximal and distal control of the IVC.

Tex patch graft can be used for repair along with an angled chest tube left above the diaphragm. If the tumor is locally invasive or if the goal is to achieve negative margins for malignant tumors, it may be necessary to perform en-bloc resection with distal pancreatectomy, splenectomy, and or partial/complete nephrectomy (**Fig. 11**).

In patients with a lesion highly suspicious for a malignant adrenal neoplasm based on preoperative imaging studies or intraoperative assessment, it is imperative that the surgical resection achieve negative margins (R0 resection), as the completeness of the initial surgery is a major prognostic factor (Schulick and Brennan, 1999; Nilubol *et al.*, 2016). In stage I ACC, a wide margin, including the surrounding retroperitoneal fat and lymphatics, is necessary. In stage II or III, complete removal of the tumor en-bloc with invaded nearby organs is essential (Nilubol *et al.*, 2016). Lymph node involvement is an important prognostic factor, and lymph node metastasis is present in approximately 20% of patients with ACC (Gaujoux and Brennan, 2012). However, the role of prophylactic lymphadenectomy in patients with ACC is unclear. A multicenter, retrospective analysis from Germany showed a significantly lower rate of tumor recurrence and disease-related mortality in patients with ACC who had lymphadenopathy (Reibetanz *et al.*, 2012). However, a study on the surveillance, epidemiology, and end results database showed no survival benefit in 8% of patients with ACC who had lymphadenectomy (Nilubol *et al.*, 2016). Other follow-up studies have found that lymphadenectomy in patients with ACC is associated with better overall survival (Gerry *et al.*, 2016).

Minimally Invasive Adrenalectomy

Numerous studies have shown the safety and feasibility of minimally invasive adrenalectomy, which has become the gold standard operation for functioning and nonfunctioning benign and small-to medium-sized adrenal tumors (Gagner *et al.*, 1997; Kebebew *et al.*, 2001). The most frequently used approach is the lateral transperitoneal laparoscopic adrenalectomy,

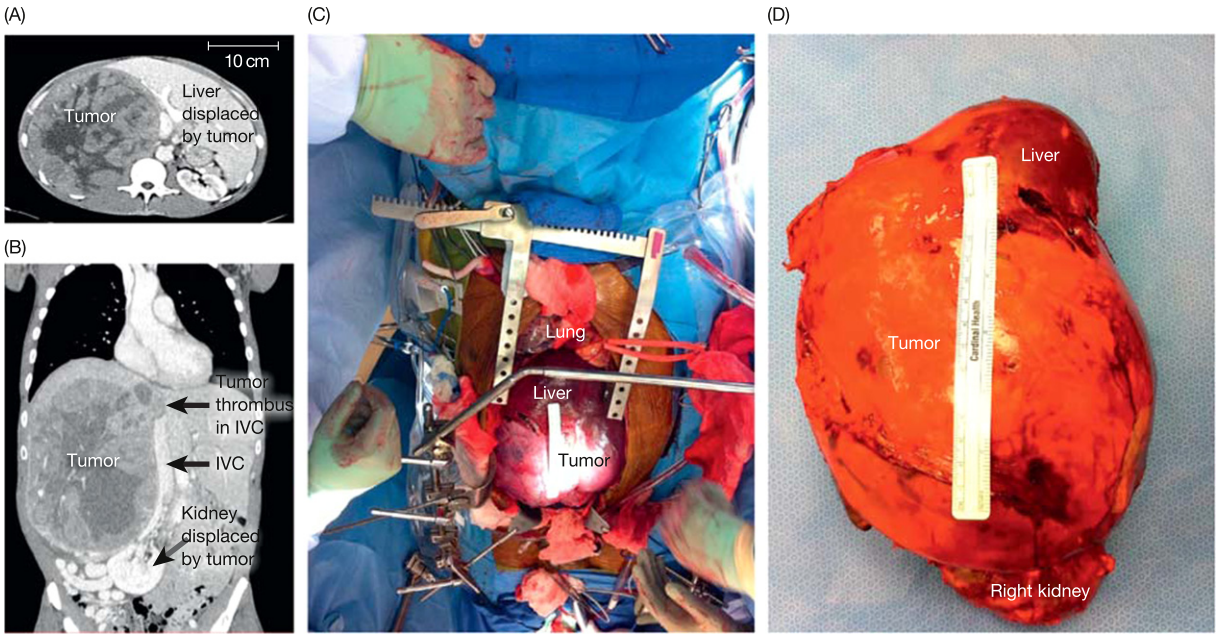

Fig. 10 En-bloc resection of a right adrenocortical carcinoma with tumor thrombus extending into the right atrium on cardiopulmonary bypass. (A and B) Computed tomography of right adrenocortical carcinoma displacing the kidney and invading the IVC with tumor thrombus. (C) Intraoperative image of a combined midline abdominal incision and median sternotomy with tumor in situ. (D) Tumor specimen after resection.

with the patient in the lateral decubitus position (Lombardi *et al.*, 2008). Other techniques include the anterior transperitoneal technique (Lezoche *et al.*, 2008) and retroperitoneal approach, which consists of either a posterolateral or a true posterior approach. Posterior retroperitoneoscopic adrenalectomy (PRA) has gained some popularity in certain regions and was first described by Mercan (Mercan *et al.*, 1995) and popularized by Walz *et al.* (2001). There are some advantages and disadvantages to each minimally invasive adrenalectomy approach. Meta-analyses of retrospective and randomized controlled trials have shown no superiority to either approach (Nigri *et al.*, 2013; Constantinides *et al.*, 2012). Recently, in a randomized controlled trial, PRA was shown to be superior in terms of shorter surgery duration, lower blood loss, lower postoperative pain, faster recovery, improved cost-effectiveness, and lower risk of surgical access site herniation as compared to laparoscopic transperitoneal adrenalectomy (Barczynski *et al.*, 2014). Further studies are needed to objectively evaluate these techniques, excluding selection bias and bias related to differences in surgeons' experiences and clinically meaningful patient-related outcome variables.

In the transperitoneal approach, the patient is placed in a lateral decubitus position with anterior superior iliac spine aligned with the point of flexion on the operating room table, which is flexed (**Fig. 12**). The peritoneal cavity is entered one finger breadth below the costal margin in the midclavicular line. For a right adrenalectomy, four trocars are placed between the *midaxillary* line and midclavicular line approximately two finger breadths apart. The triangular ligament is divided, and the right lobe of the liver is medially mobilized. A snake, fan, or paddle retractor is placed through the medial-most port to create the working space. The peritoneum and Gerota's fascia are opened lateral to the IVC from the top down. Care must be taken to avoid manipulating or grasping the tumor: this is known as the "no touch technique." The right adrenal vein is identified between the IVC and the gland and is clipped and divided (**Fig. 13**). A plane between the gland and the kidney is created, and the posterior attachments of the gland to the quadratus lumborum muscle are divided. The adrenal gland is freed posteriorly all the way up to the diaphragm. The specimen is removed through the lateral port site.

For the left adrenalectomy, three trocars are inserted from the midclavicular line to the midaxillary line, two finger breadths apart (**Fig. 14**). The peritoneum is incised 1 cm lateral to the edge of the spleen. The peritoneum is opened all the way up to the diaphragm until the gastric fundus is visualized (**Fig. 15**). The spleen is retracted medially by gravity, and the dissection is carried down posteriorly over the quadratus Lumborum muscle. The inferior phrenic vein is traced from the diaphragm until it joins the adrenal vein before emptying into the left renal vein. Once the left adrenal vein is identified, it is clipped and divided. The inferior aspect of the gland is retracted anteriorly and superiorly and divided off the posterior muscles (**Fig. 16**). Care must be taken to avoid injury to the left renal vein. Once the gland is completely detached, hemostasis is secured, and then the specimen is removed from the lateral-most port site.

The posterior/retroperitoneal approach (PRA) is most useful in patients who have previously undergone abdominal surgery and in patients who require bilateral adrenalectomy to avoid patient repositioning. The primary contraindication to this approach is a large-sized tumor due to the limited working space available in the retroperitoneum. The PRA may be contraindicated in patients with copious retroperitoneal fat, as it makes identifying the adrenal gland and tumor difficult.

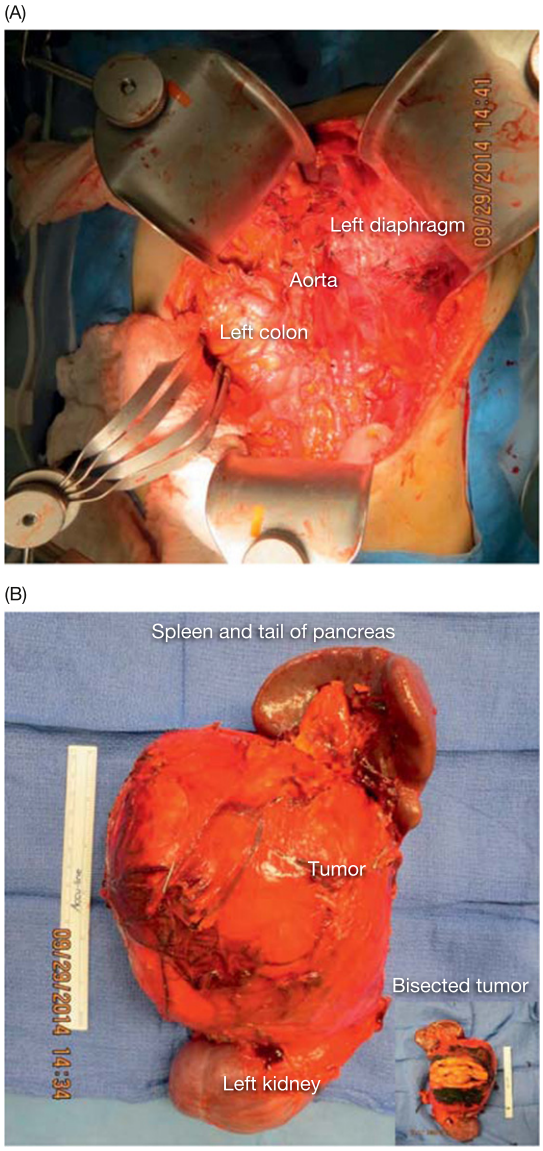

Fig. 11 Open en-bloc resection of a left adrenocortical carcinoma with tail of pancreas, spleen, and left kidney and regional lymphadenectomy through a left subcostal incision. (A) Intraoperative image of resection bed. (B) Resected gross specimen.

For the PRA, the patient is placed in a prone jackknife position (**Fig. 17**). A trocar is placed 1.5 cm below the tip of the 12th rib. The working space is created by blunt digital dissection of the retroperitoneal fat. Then, a 5-mm lateral trocar is inserted 4–5 cm from the initial one, and a 10-mm trocar is inserted medially 2–3 cm at the same level as the first port under finger guidance. A 10-mm balloon blunt trocar is inserted, and insufflation with carbon dioxide of up to 20 mmHg is initiated. The gland is dissected free from the diaphragm and the superior pole of the kidney. Then it is mobilized medially and inferiorly. The adrenal vein is clipped and divided (**Fig. 17**). The remaining attachments are dissected free laterally and superiorly, and the specimen is extracted (**Fig. 17**).

Laparoscopic Adrenal-Sparing Adrenalectomy

In order to avoid lifelong steroid replacement and to minimize the risk of Addisonian crisis, adrenal-sparing adrenalectomy should be considered in patients who have PC and have a low risk of malignancy but have bilateral PC or are at risk of metachronous PC, such as patients with MEN2, VHL, or NF1 (Castinetti *et al.*, 2014). The goal of adrenal-sparing adrenalectomy is to ensure the removal of all PC while leaving an adequate adrenal remnant. Large PC along with the presence of several smaller tumors in the same gland could preclude this approach, as generally at least one-quarter to one-third of a normal gland is needed to maintain adequate adrenocortical function (Brauckhoff *et al.*, 2003). An intraoperative laparoscopic ultrasound is helpful for identifying smaller tumors and for defining the margin of resection. The adrenal gland

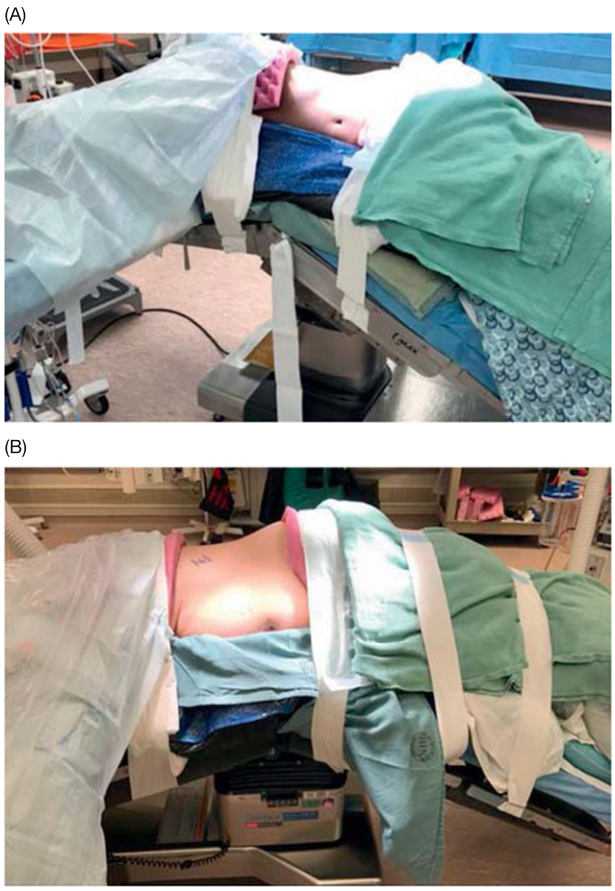

Fig. 12 Patient position for a lateral laparoscopic adrenalectomy. Image shows a patient positioned for a *left* lateral laparoscopic adrenalectomy. (A and B) Patient is placed in a lateral decubitus position with anterior superior iliac spine aligned with the point of bed flexion.

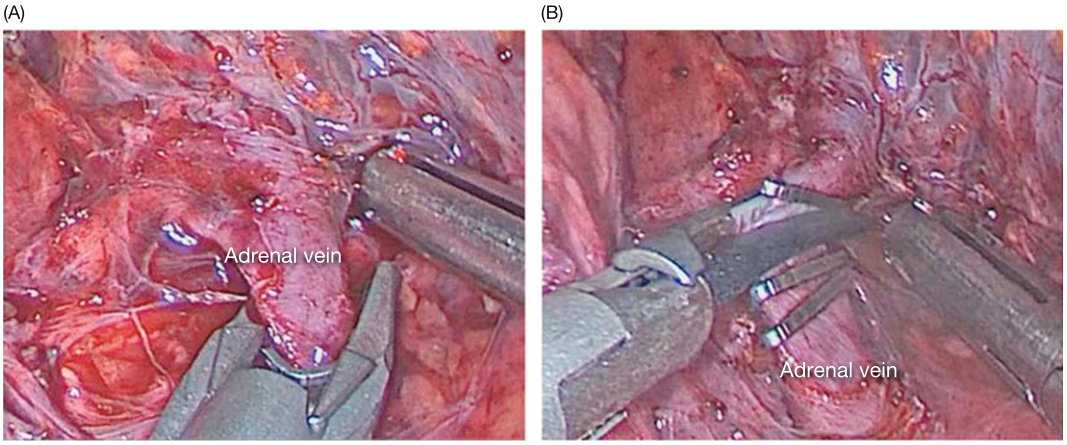

Fig. 13 Intraoperative image of *right* adrenal gland and adrenal vein draining into the inferior vena cava. (A) Clip being placed adjacent to the inferior vena cava with grasper pushing the adrenal gland away from the vena cava. (B) The adrenal vein after the distal and proximal vein is clipped (two clips are placed on the remnant side).

parenchyma is transected using electrocautery, Harmonic®, and/or LigaSure® scissors (**Fig. 18**). Preservation of the main adrenal vein is not necessary to have sufficient cortical function but is preferable if technically feasible (Walz *et al.*, 1998). The disadvantage of adrenal-sparing adrenalectomy is the risk of recurrent PC, which has been reported to be 3% at a median follow-up of 6–13 years in patients with MEN2 and 9% at a median follow-up of 17 years in patients with VHL (Castinetti *et al.*, 2014; Aufforth *et al.*, 2015).

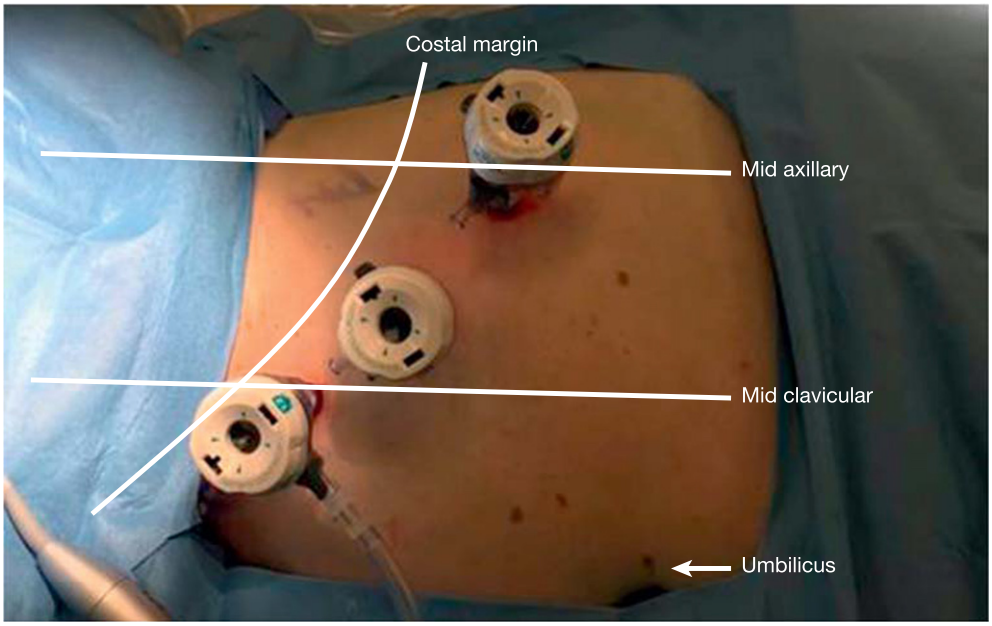

Fig. 14 Trocar positions for a left lateral laparoscopic adrenalectomy. Image shows three trocars placed for a laparoscopic *left* adrenalectomy. The trocars are inserted from the midclavicular line to the midaxillary line, two finger breadths apart.

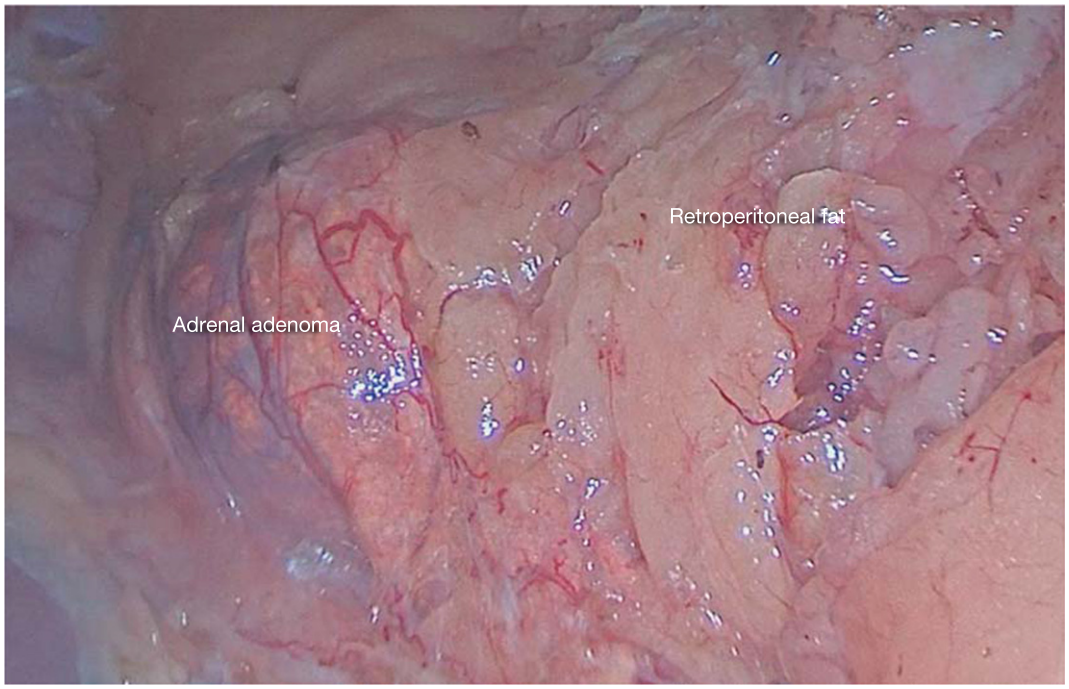

Fig. 15 Intraoperative laparoscopic image of an adrenocortical cortisol-secreting adenoma surrounded by retroperitoneal fat in a patient undergoing a left adrenalectomy for Cushing syndrome.

Robotic Adrenalectomy

Robotic adrenalectomy has been proven to be feasible and safe. It may offer the surgeon greater dexterity and a more ergonomic procedure than other minimally invasive adrenalectomies; however, to date, no study has shown this approach to be superior. A meta-analysis of 1162 patients including only one randomized controlled trial comparing laparoscopic versus robotic adrenalectomy reported no significant difference for intraoperative complications, postoperative complications, mortality, conversion to

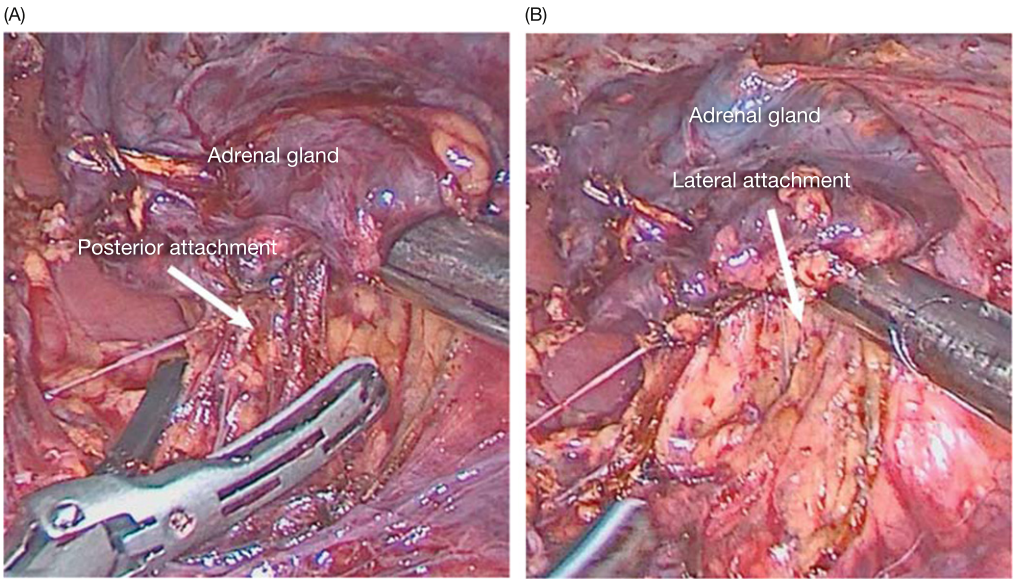

Fig. 16 Intraoperative laparoscopic image of completing an adrenalectomy. (A and B) Dissection and freeing of the remaining lateral and posterior attachments of the adrenal gland.

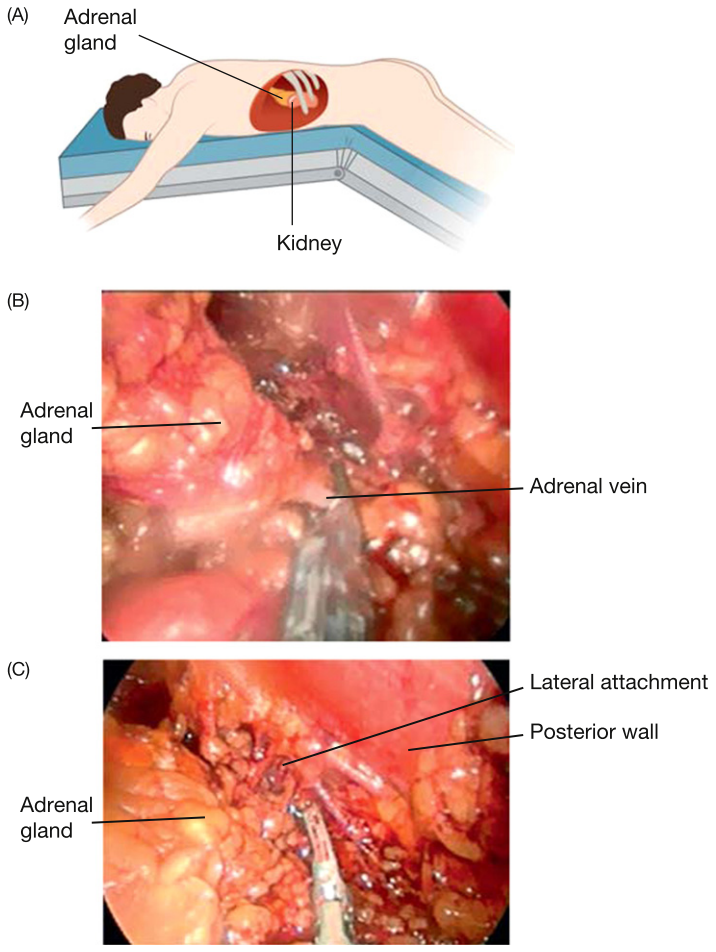

Fig. 17 Adrenalectomy using the posterior retroperitoneal approach. (A) Patient positioning in prone jackknife position. (B) A clip being placed on the adrenal vein. (C) Freeing of the lateral attachments of the adrenal gland.

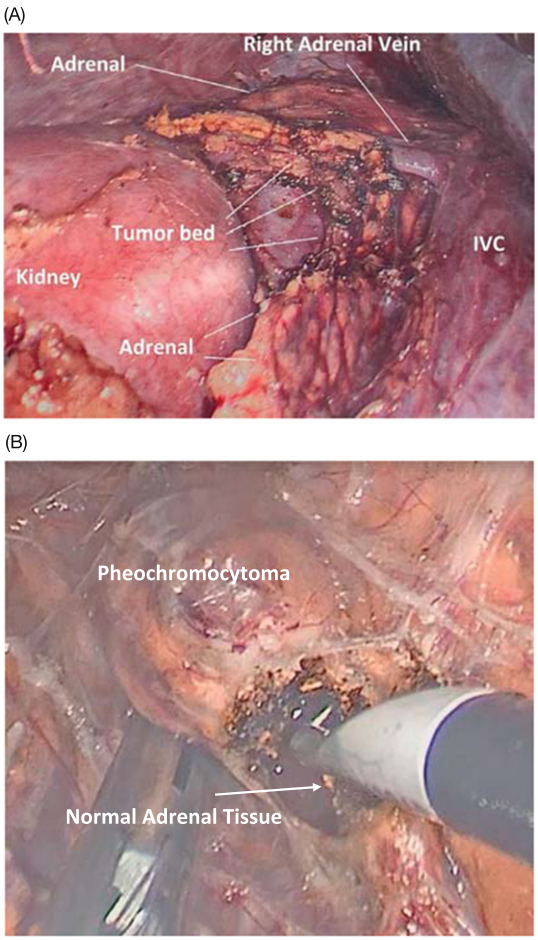

Fig. 18 Cortical-sparing resection of a pheochromocytoma in a patient with a germline *RET* mutation.

laparotomy or laparoscopy, and blood loss (Economopoulos *et al.*, 2017) Most centers do not use robotic adrenalectomy, given the unclear benefit for patients and the cost of the procedure.

Summary

The evaluation of adrenal lesions, which includes interpreting laboratory work-up and anatomic and functional imaging as well as incorporating genetic information, requires a multidisciplinary approach. New knowledge on the genetics of adrenal neoplasms will likely continue to impact the surgical management of patients with adrenal neoplasms, including the indications for adrenalectomy and the approach and extent of adrenalectomy in patients with adrenal Cushing syndrome, PC/PGL, and PA. The surgeon's experience and technical expertise is important for optimal patient outcome. In patients with functioning adrenal neoplasms, adrenalectomy has a great and often instantaneous impact on the patients' health and well-being, which is gratifying for both patients and providers.

See also: Bilateral Adrenalectomy for Cushing Disease. Cushing Syndrome; Screening and Differential Diagnosis. Cushing Syndrome—Unilateral Adrenal Adenoma

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Long-Term Complications of Hypercortisolism

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Introduction

Chronic endogenous hypercortisolism is responsible for a rare and severe endocrine disease named Cushing syndrome (CS). CS can be classified in ACTH-dependent and ACTH-independent forms. The formers are due to the presence of ACTH-secreting pituitary tumors (70% of cases), named Cushing disease (CD), or ACTH-secreting or CRH-secreting ectopic tumors (10% of cases), named ectopic CS (ECS), whereas the latter are due to the presence of cortisol-secreting adrenal lesions, including adrenal hyperplasia, adenoma or carcinoma (20% of cases), named adrenal CS (ACS) (Lacroix *et al.*, 2015; Pivonello *et al.*, 2017, 2016a, 2015a, 2008). The estimated prevalence of CS is 40 cases per million people and the estimated incidence is 0.7–2.4 cases per million people per year, mainly affecting females aged between 40 and 60 years (Lacroix *et al.*, 2015; Pivonello *et al.*, 2017, 2016a, 2015a, 2008). The clinical picture of CS is variable, resulting from a cohort of different signs, including weight gain, moon face, facial plethora, buffalo hump, supraclavicular and dorsal fat pads, purple striae, easy bruising, skin thinning, proximal myopathy, hirsutism, acne and alopecia, as well as symptoms, mainly including asthenia, fatigue and mood disorders (Lacroix *et al.*, 2015; Pivonello *et al.*, 2017, 2016a, 2015a, 2008). This clinical picture is complicated by several comorbidities including metabolic syndrome, which is characterized by visceral obesity, impairment of glucose metabolism, dyslipidemia and systemic arterial hypertension (SAH) and it is strictly associated with cardiovascular diseases, including vascular atherosclerosis and cardiac damage. These comorbidities, together with thromboembolism, contribute to increase the cardiovascular risk in CS patients. Additional clinical complications include musculoskeletal diseases, such as osteoporosis, skeletal fractures and myopathy; immune disorders characterized by increased susceptibility to infections, and possibly complicated by sepsis; neuropsychiatric diseases, such as impairment of cognitive function, depression, anxiety and bipolar disorders; impairment of gonadal function with consequent infertility or sexual disturbances (Lacroix *et al.*, 2015; Pivonello *et al.*, 2017, 2016a, 2015a, 2008). These clinical complications negatively impact on quality of life (QoL) and increase the mortality, mainly due to cardiovascular events and sepsis. Therefore, a prompt screening, a confirmatory diagnosis and an effective multidisciplinary therapeutic approach are mandatory in the attempt to improve clinical picture, morbidity, QoL and mortality (Lacroix *et al.*, 2015; Pivonello *et al.*, 2017, 2016a, 2015a, 2008). Nowadays, the first line treatment for all CS forms still remains surgery, whereas radiotherapy and medical therapy represent a second choice (Nieman *et al.*, 2015; Pivonello *et al.*, 2015a).

Metabolic Syndrome

Metabolic syndrome (MS) is a chronic and systemic syndrome defined by the presence of abdominal visceral obesity (measured by elevated waist circumference) plus two of the following factors: elevated blood pressure, fasting glucose or triglycerides or reduced HDL cholesterol (Alberti *et al.*, 2006; Ford, 2005). Chronic hypercortisolism induces visceral obesity, hypertension, impairment of glucose metabolism and dyslipidemia causing a specific form of MS in about 50%–63% of patients (Barahona *et al.*, 2009a; Giordano *et al.*, 2014), apparently without significant differences in men and women (Giordano *et al.*, 2014). The mechanisms leading to this metabolic disorder are complex and probably only partially understood, although several direct or indirect effects of glucocorticoids (GCs) on liver, skeletal muscle, adipose tissue, pancreas and the central nervous system have been indicated (Pivonello *et al.*, 2016a). GCs act on the liver stimulating gluconeogenesis, hepatic glucose output, lipoprotein secretion and fatty acid synthesis, inducing hepatic insulin resistance and liver steatosis (Pivonello *et al.*, 2016a; Wang, 2005). These effects contribute to the abnormal glucose and lipid metabolism observed in CS (Pivonello *et al.*, 2016a). The effects of GCs on skeletal muscle include an increased lipid oxidation and a reduced translocation of GLUT4, which contribute to the generation of a peripheral insulin resistance (Pivonello *et al.*, 2016a, 2010). In adipose tissue, the GCs stimulate the lipolysis and the differentiation of preadipocyte to adipocyte, whereas they reduce the amino acids uptake, the GLUT4 translocation and the lipogenesis. These effects contribute to generate peripheral insulin resistance and fat accumulation (Lee *et al.*, 2014; Pivonello *et al.*, 2016a). Additionally there are evidences that GCs also inhibit the insulin secretion from the pancreas and stimulate the appetite, which also contribute to the abnormal glucose and lipid metabolism observed in CS (Pivonello *et al.*, 2016a, 2010; Tataranni *et al.*, 1996) (**Fig. 1**).

After CS remission, the prevalence of MS has been reported to be significantly lower than in patients with active CS (16% vs. 50%) (Giordano *et al.*, 2014); however, the effects of hypercortisolism resolution after surgery or medical treatment on the prevalence of MS, considered as the presence of all the criteria to full-fit this diagnosis, as well as the effect of specific intervention to treat MS or the influence of possible hormonal deficits associated with CS have been scantly investigated. Conversely, more data have been reported on the presence and the outcome of the different disorders that compose the MS. Hypertension is one of the

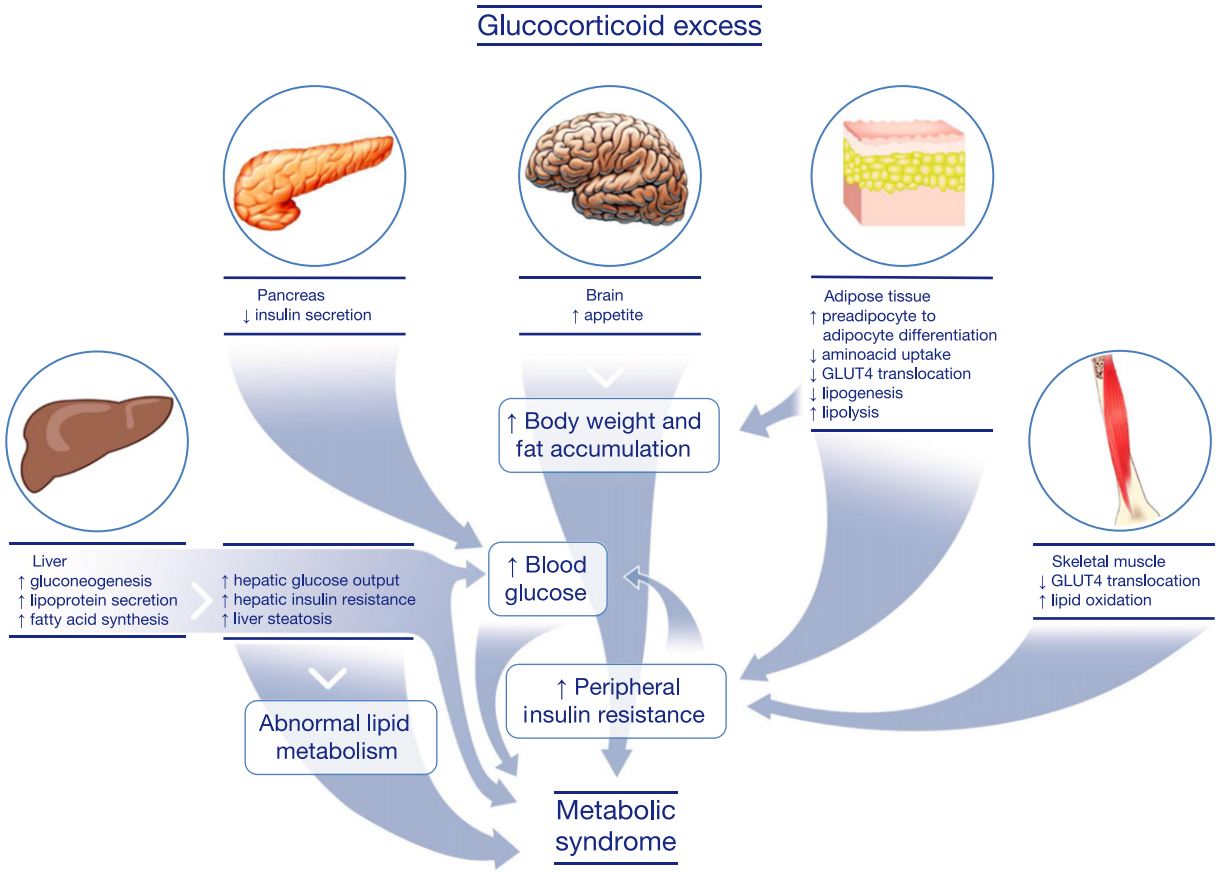

Fig. 1 Metabolic alterations in CS.

components of MS, but because it is largely involved in the pathogenesis of cardiovascular diseases associated with CS, it has been extensively described in the paragraph dedicated to cardiovascular alterations.

Visceral Obesity

Visceral obesity is currently considered a prerequisite in the diagnosis of MS (Alberti *et al.*, 2006; Ford, 2005) and is considered an important risk factor for obesity-related diseases in the general population (Klein *et al.*, 2007). The pathological increase in the body mass index (BMI) is one of the most common feature of CS (57%–100% of patients). Particularly, obesity and overweight have been reported in 25%–100% and 33%–48% respectively of patients with active CS (Colao *et al.*, 2000; Faggiano *et al.*, 2003b; Giordano *et al.*, 2011; Mancini *et al.*, 2004; Pivonello *et al.*, 2016a, 2008, 2005). The above mentioned direct effects of GCs responsible for insulin resistance and MS are also involved in the mechanisms leading to preferential visceral fat accumulation (Bonora, 2006; Chanson and Salenave, 2010; Pivonello *et al.*, 2016a, 2010). A differential expression in visceral versus subcutaneous adipose tissue of 11β-hydroxysteroid dehydrogenase type1 (11β-HSD1), an enzyme converting inactive cortisone to active cortisol, might influence the local availability of cortisol, contributing to the abdominal fat distribution (Lee *et al.*, 2014; Pivonello *et al.*, 2016a). Recently, it has been reported that also a differential expression of different GCs receptor types (type 1 and 2) in visceral versus subcutaneous adipose tissue, might play a role in preferential visceral fat accumulation, determining a differential response to GCs in the two different adipose tissues (Lee *et al.*, 2014; Pivonello *et al.*, 2016a). The visceral adipose tissue in patients with CS has been reported to be structurally and functionally different from normal subjects. Particularly, an increased lipogenesis has been described in patients with Cushing's disease (CD) compared to obese controls (Galton and Wilson, 1972; Pivonello *et al.*, 2016a), whereas enlarged abdominal fat cells, elevated lipoprotein lipase activity and low lipolytic capacity has been described in women with CS compared with normal women (Lee *et al.*, 2014; Pivonello *et al.*, 2016a). Adipose tissue secretes adipokines, proteins with multiple functions, that contribute to cardiovascular disease in patients with MS (Nakamura *et al.*, 2014) and seem to play a role in cardiovascular disease also in CS patients (Valassi *et al.*, 2012; Wagenmakers *et al.*, 2015).

The presence of visceral obesity in patients with active CD and CS have been documented by the presence of increased waist to hip-ratio or waist circumference, markers of visceral obesity (Faggiano *et al.*, 2003b; Giordano *et al.*, 2011; Pivonello *et al.*, 2016a), and confirmed also in studies using whole body magnetic resonance imaging (Geer *et al.*, 2010). The duration of hypercortisolism correlates with the presence of obesity (Mancini *et al.*, 2004). In CD patients waist to hip-ratio correlates with glucose, insulin and

blood pressure levels, suggesting a pivotal role of visceral obesity in determining MS in CS (Faggiano *et al.*, 2003b). The prevalence of obesity seems similar in women and men, but a higher BMI has been reported in female as compared with male CS patients (Giordano *et al.*, 2014; Pecori Giraldi *et al.*, 2003). One or even five years after surgical hypercortisolism resolution, obesity has been reported to be significantly improved, but not normalized (Colao *et al.*, 1999; Faggiano *et al.*, 2003b; Giordano *et al.*, 2011). However, an improvement in waist to hip-ratio or waist circumference has been reported (Faggiano *et al.*, 2003b; Giordano *et al.*, 2011). Hypercortisolism control with medical treatment can also ameliorate obesity in patients with CS (Pivonello *et al.*, 2016a). Among pituitary directed drugs, treatment with cabergoline in CD patients has been associated with an amelioration of waist to hip-ratio already after 3 months of treatment and an improvement also in BMI after 24 months (Pivonello *et al.*, 2009). More recently, treatment with pasireotide has been reported to improve weight, BMI and waist circumference already after 6 months of treatment (Pivonello *et al.*, 2014a). Among other drugs the inhibitors of steroidogenesis ketoconazole and mitotane and the GC receptor antagonist mifepristone have also been reported to ameliorate weight excess in CS patients (Baudry *et al.*, 2012; Katznelson *et al.*, 2014; Moncet *et al.*, 2007). However the long-term effects of surgical or medical hypercortisolism resolution on obesity in CS deserve further investigation. Several hormonal deficits can be potentially associated with CS and they or their suboptimal treatments might play a role in obesity, both during the active and remission phases of disease, but these effects have been scantly investigated (Pivonello *et al.*, 2016a). Patients with CS probably deserve a specific nutritional regimen to improve their metabolic profile, but studies on the effects of diet or other interventions specifically targeting obesity as complication of CS both during the active and remission phases of disease are missing. These interventions, together with a tailored approach to the hormonal deficits, might play an important role in the long-term management and outcome of CS patients.

Impairment of Glucose Metabolism

Impairment of glucose metabolism is also frequent (27%–87%) in patients with CS. Particularly, diabetes has been described in 11%–47%; impaired glucose tolerance in 7%–64% and impaired fasting glucose in 6%–14% of patients (Etxabe and Vazquez, 1994; Faggiano *et al.*, 2003a; Giordano *et al.*, 2014, 2011; Pivonello *et al.*, 2016a). In patients with CS the impairment of glucose metabolism has been ascribed to the increased gluconeogenesis and the presence of insulin resistance associated with the inability of beta-pancreatic cells to adequately compensate the insulin resistance (Pivonello *et al.*, 2016a,b; Giordano *et al.*, 2014). Additionally, hypercortisolism stimulates proteolysis and lipolysis increasing levels of amino acids and fatty acids respectively, which contribute to impair insulin sensitivity (Pivonello *et al.*, 2010). The prevalence of diabetes or impaired glucose tolerance in patients with CS has been reported to be higher than BMI-matched controls (Giordano *et al.*, 2011) and patients with CD have higher glucose and insulin levels after glucose loading than BMI-matched controls (Faggiano *et al.*, 2003b) suggesting that the excess in body weight can contribute to the impairment of glucose metabolism in CS, but it is not the only determinant factor (Pivonello *et al.*, 2016a). The prevalence of glucose abnormalities seems similar in women and men (Pecori Giraldi *et al.*, 2003), whereas a higher prevalence of diabetes has been reported in patients with ectopic CS as compared with other CS etiologies (Valassi *et al.*, 2011). The high frequency of abnormalities in glucose metabolism in CS patients suggests the need of specific investigation in all patients with CS. This evaluation should include the measurement of fasting glucose and glycated hemoglobin in all CS patients, and the oral glucose tolerance test in patients without overt diabetes. These investigations should be repeated during the follow-up of CS patients and they should contribute to drive specific interventions aimed at glycemic control. One or even 5 years after surgical hypercortisolism resolution, glucose abnormalities has been reported to be significantly improved, but not normalized (Colao *et al.*, 1999; Faggiano *et al.*, 2003b; Giordano *et al.*, 2011; Pivonello *et al.*, 2007). One year after surgical hypercortisolism resolution for adrenal CS or CD, a significant reduction in the prevalence of impaired glucose tolerance was observed only in adrenal CS (Giordano *et al.*, 2011), suggesting that in this subgroup of patients abnormal glucose metabolism might recover faster than in others. In most cases, medical treatment is associated with an improvement of glucose metabolism, with the exception of pasireotide. Particularly, cabergoline seems capable to improve insulin sensitivity and the prevalence of glucose abnormalities (Pivonello *et al.*, 2009), whereas pasireotide has been reported to worsen glycemic control in CD patients, particularly in those with preexisting alterations of glucose metabolism (Colao *et al.*, 2012; Pivonello *et al.*, 2014a). This adverse event of pasireotide is likely related to a direct inhibition of pancreatic insulin and gastrointestinal incretin secretion (Henry *et al.*, 2013). Therefore, in patients on pasireotide treatment, glucose metabolism should be monitored and glycemic control should be obtained by the introduction of metformin and staged treatment intensification with a dipeptidyl peptidase-4 inhibitor, or a glucagon-like peptide 1 receptor agonist or the initiation of insulin, as required (Colao *et al.*, 2014). Among other drugs, the inhibitors of steroidogenesis and the GC receptor antagonist mifepristone have been reported to ameliorate glucose metabolism in CS patients (Castinetti *et al.*, 2009, 2014; Fleseriu *et al.*, 2012; Pivonello *et al.*, 2016a; Pivonello *et al.*, 2015a). Several hormonal deficits can be potentially associated with CS and they or their suboptimal treatments might affect glucose metabolism, both during the active and remission phases of disease, but these effects have been scantly investigated (Pivonello *et al.*, 2016a). Therefore, a tailored approach to the treatment of hormonal deficits might play an important role in the long-term management and outcome of CS patients. Taking into account the central role of insulin resistance in the pathogenesis of glucose abnormalities in CS, the use of insulin sensitizers might be useful in patients with this type of complication; however, in patients with a more difficult glycemic control other specific treatments, including the use of insulin, can be required (Pivonello *et al.*, 2016a). These interventions, together with an appropriate nutritional approach and a prolonged follow-up of glucose parameters, both during the active and remission phases of disease, might play an important role in the long-term management and outcome of CS patients.

Dyslipidemia

Dyslipidemia in patients with CS has been poorly investigated and a variable prevalence has been described (12%–72%) (Bolland *et al.*, 2011; Faggiano *et al.*, 2003a; Giordano *et al.*, 2014, 2011; Pivonello *et al.*, 2016a). Several GCs effects involved in the pathogenesis of the MS associated with CS can contribute to determine lipid abnormalities in CS patients, particularly the direct and indirect cortisol actions on lipolysis, free fatty acid and very low density lipoprotein cholesterol (VLDL) production (Arnaldi *et al.*, 2010). Common findings are elevated total and low density lipoprotein cholesterol (LDL) and triglycerides levels and reduced high density lipoprotein cholesterol (HDL) levels (Chanson and Salenave, 2010; Colao *et al.*, 1999; De Leo *et al.*, 2010; Faggiano *et al.*, 2003b; Giordano *et al.*, 2011; Mancini *et al.*, 2004). Similar abnormalities in lipid profile have been described in patients with CD or adrenal CS (Giordano *et al.*, 2011). An abnormal lipid profile could contribute to the increased cardiovascular risk in CS, therefore it is advisable to screen for the presence of these metabolic disorders all patients with CS, by performing a complete lipid profile evaluation. One or even 5 years after surgical hypercortisolism resolution, the lipid profile has been reported to be significantly improved, but not normalized (Colao *et al.*, 1999; Faggiano *et al.*, 2003b; Giordano *et al.*, 2011). Particularly total- and LDL-cholesterol were higher than age- and sex matched controls, but not than BMI-matched controls, suggesting a role of obesity in the persistence of these metabolic abnormalities (Pivonello *et al.*, 2016a). The effect of medical treatment on lipid profile has been scantly investigated. Pasireotide seems to positively affect lipid profile, reducing total- and LDL-cholesterol in treated patients (Pivonello *et al.*, 2014a). By contrast mitotane increases total- and LDL-cholesterol, which generally is associated with a rise of HDL-cholesterol and is managed with the addition of statins not metabolized by CYP3A4 (e.g., pravastatin, rosuvastatin) when LDL/HDL cholesterol consistently increases during the follow-up (Berruti *et al.*, 2012). The presence of hormonal deficits or their suboptimal treatments can potentially affect lipid profile, both during the active and remission phases of disease, but these effects have been scantly investigated (Pivonello *et al.*, 2016a). The role of optimal management with specific intervention to correct lipid profile in CS patients and the consequence on long-term management and outcome of CS patients deserve further investigation.

Cardiovascular Complications

CS is characterized by a wide range of cardiovascular complications, including systemic arterial hypertension (SAH), morphologic and functional heart and vessels alterations as well as thrombotic diathesis, which all compete in increasing the cardiovascular mortality (Pivonello *et al.*, 2016a) (**Fig. 2**).

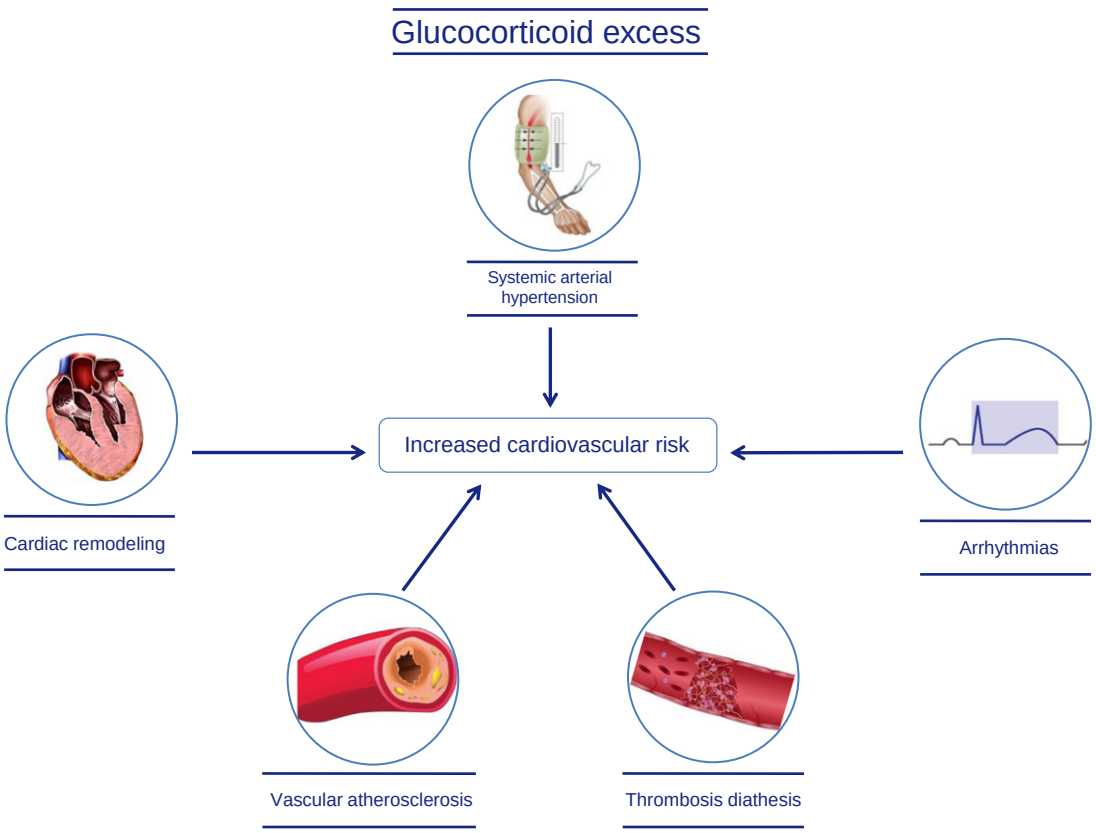

Fig. 2 Cardiovascular alterations in CS.

Systemic Arterial Hypertension (SAH)

SAH, according to the new American Heart Association (AHA) guidelines, is defined as systolic blood pressure levels $\geq$130 mmHg or diastolic blood pressure levels $\geq$80 mmHg (Whelton *et al.*, 2017). However, it is noteworthy that most clinical studies focusing on SAH in CS, published nowadays, defined SAH as systolic blood pressure levels $\geq$140 mmHg or diastolic blood pressure levels $\geq$90 mmHg, according to the previous AHA guidelines (Chobanian *et al.*, 2003). The pathogenesis of SAH in CS is still not completely understood and involves a large number of mechanisms including renin–angiotensin system (RAS), mineralocorticoid (MC) receptors hyperactivation, sympathetic nervous system and vasoregulation dysfunction (Isidori *et al.*, 2015a; Pivonello *et al.*, 2016a). RAS is the most widely studied system; the increased angiotensinogen liver synthesis and the increased angiotensin II receptors (type 1A) expression, induced by chronic hypercortisolism, seem to be the main two factors underlying RAS involvement in SAH development (Isidori *et al.*, 2015a; Pivonello *et al.*, 2016a; Ritchie *et al.*, 1990; Saruta *et al.*, 1986; Shibata *et al.*, 1995; Yasuda *et al.*, 1994). MC receptors hyperactivation is an additional important mechanism involved in the SAH pathogenesis. In physiological conditions, MC receptors, mainly expressed in kidneys and able to bind MC and cortisol with the same affinity, usually maintain their specificity because of the transformation of intracellular active cortisol to inactive cortisone by 11β-hydroxysteroid-dehydrogenase type 2 (11βHSD2) enzyme, expressed in the renal cortex (Fuller and Young, 2005; Isidori *et al.*, 2015a; Quinkler and Stewart, 2003). In case of CS, the protective 11βHSD2 activity is overwhelmed by hypercortisolism, leading to a spill-over effect of cortisol on MC receptors, that along with the proper GC receptors activation, cause the epithelial sodium channel (ENaC) hyperactivation and glomerular hyperfiltration, with a consequent sodium retention and hypervolemia, all contributing to SAH development (Bailey *et al.*, 2009; Heaney *et al.*, 1999; Isidori *et al.*, 2015a). Furthermore, the additional role of sympathetic nervous system in SAH development is still controversial; indeed, a possible, but not confirmed, enhanced vasoconstriction response to the sympathetic mediators is described (Heaney *et al.*, 1999; Isidori *et al.*, 2015a; Ritchie *et al.*, 1990). The last major pathogenetic role in SAH development is played by vasoregulation dysfunction, characterized by increased vasoconstriction tone due to a rise of vasoconstrictor endothelin- 1 (ET-1) circulating level, enhanced response to vasoconstrictor factors because of the downregulation of vascular sodium–calcium exchanger, and reduced synthesis of vasodilators, including nitric oxide, prostaglandins, prostacyclins and kallikrein-kinin system compounds (Axelrod, 1983; Kirilov *et al.*, 2003; Isidori *et al.*, 2015a; Radomski *et al.*, 1990; Shimamoto *et al.*, 1995; Simmons *et al.*, 1996; Smith and Smith, 1994; Wallerath *et al.*, 1999).

SAH in CS, usually characterized by the loss of physiological nocturnal blood pressure decrease (nondipper profile), showed a prevalence of 25%–93% (Pivonello *et al.*, 2016a). Noteworthy, no significant prevalence differences were reported among genders or etiologies, although a positive relation was described between SAH onset and hypercortisolism duration (Isidori *et al.*, 2015a; Mancini *et al.*, 2004; Pivonello *et al.*, 2016a). The long-term hypercortisolism resolution after surgery is usually associated with an improvement of SAH, even if a persistence in 25%–54% of patients was reported (Pivonello *et al.*, 2016a). Beyond surgical approach, medical therapy also demonstrated a proper efficacy on SAH control. Considering the pituitary directed drugs, cabergoline improved the blood pressure levels in 33%–100% of CD responsive patients (Ferriere *et al.*, 2017; Pivonello *et al.*, 2009) whereas daily pasireotide induced a reduction in systolic and diastolic blood pressure of $-$6.1 mmHg and $-$3.7 mmHg, as well as of $-$11.3 mmHg and $-$7.2 mmHg, after 12 and 24 months of treatment, respectively (Colao *et al.*, 2012; Pivonello *et al.*, 2014a; Schopohl *et al.*, 2015). Similarly, monthly pasireotide induced a reduction in systolic and diastolic blood pressure of $-$4.8 mmHg and $-$3.2 mmHg, after 12 months of treatment (Lacroix *et al.*, 2017). Among the adrenal directed drugs, ketoconazole and mitotane, improved the blood pressure levels in 40%–80% (Castinetti *et al.*, 2014; Moncet *et al.*, 2007; Nieman, 2002) and 63% (Donadille *et al.*, 2010; Lee *et al.*, 2014; Pivonello *et al.*, 2016a) of patients, respectively; whereas, metyrapone and newer compound osilodrostat, inducing an increase of aldosterone precursor levels, showed a potential paradoxical hypertensive effect (Bertagna *et al.*, 2014; Fleseriu *et al.*, 2016; Verhelst *et al.*, 1991). The GC receptor antagonist mifepristone improved blood pressure levels in 38%–50% of patients, although a potential hypertensive effect cannot be excluded because of increased cortisol levels due to the drug action, with consequent spill-over effect on MC receptors (Castinetti *et al.*, 2012; Fleseriu *et al.*, 2012). The impact of hormonal deficits potentially associated with CS and they or their suboptimal treatments on SAH requires further investigation, even if it is largely known that a suboptimal replacement therapy can affect the blood pressure levels and contributes to a poor cardiovascular outcome (Pivonello *et al.*, 2016a; Johannsson and Ragnarsson, 2014).

A specific antihypertensive algorithm was elaborated for CS patients, considering the mechanisms involved in SAH pathogenesis. According with this algorithm, the initial treatment should include compounds such as angiotensin I-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB), which also exert protective effects on heart and kidney. If the treatment goal is not achieved, potassium levels should be accounted; in case of hypokalemia, mineralocorticoid antagonists, such as spironolactone or eplerenone, should be used, considering also the potential positive effect on heart failure; in case of normal potassium levels, calcium antagonists should be preferred, considering also the potential effects in the prevention of stroke and atherosclerosis. Moreover, when treatment goal is not achieved, a third compound should be started, preferring nitric-oxide donors and α-blockers. At last, if the blood pressure levels are not normalized, an additional treatment with β-blockers or diuretics should be considered. These latter compounds are not usually preferred because of demonstrated low efficacy and frequent contraindications occurrence, such as diabetes mellitus and obstructive sleep apnea syndrome (OSAS) for β-blockers, or hypokalemia, hyperuricemia and nephrolitiasis for diuretics (Isidori *et al.*, 2015a).

No recommendations regarding the long-term monitoring of SAHare available, but the assessment of blood pressure levels every 3–6 months and eventually, the execution of a 24 h ambulatory blood pressure measurement (24 h ABPM) in case of uncontrolled SAH, may be a valid clinical suggestion.

Cardiac Damage

Cardiac damage is characterized by left ventricular hypertrophy, concentric remodeling and myocardial fibrosis, in CS. The cardiac damage pathogenesis mainly involves two different mechanisms, both related to hypercortisolism: (1) the enhanced cardiac noradrenaline and angiontensin II response; (2) the MC or GC cardiac receptors hyperactivation. However, the hypercortisolism-induced SAH and MS can also play an important additional role in cardiac damage (Ainscough *et al.*, 2009; Isidori *et al.*, 2015a; Mihailidou *et al.*, 2009; Muiesan *et al.*, 2003; Ritchie *et al.*, 1987; Sudhir *et al.*, 1989; Toja *et al.*, 2012). Cardiac morphologic damage usually turns into functional alterations, indeed, systolic and diastolic dysfunction, including reduced left ventricular systolic performance and change of diastolic filling toward abnormal relaxation pattern, were observed at echocardiography (Muiesan *et al.*, 2003; Toja *et al.*, 2012). Recent cardiac magnetic resonance findings also reported a reduced biventricular systolic performance and a reduced left atrial ejection fraction (Kamenický *et al.*, 2014). All these features contribute to explain the higher risk of heart failure (hazard ratio: 6) and acute myocardial infarction (hazard ratio: 2.1) reported in CS patients when compared to matched controls (Isidori *et al.*, 2015a). Furthermore, ventricular hypertrophy together with hypokalemia may be also responsible for QT interval prolongation in CS patients. QT interval prolongation seems to be more pronounced in males than females, suggesting an additional pathogenetic role played by testosterone deficiency in CS male patients (Pecori Giraldi *et al.*, 2011). Considering the importance of cardiac hypertrophy, occurring in 24%–42% of CS patients (Kamenický *et al.*, 2014), an echocardiography should be performed in all patients at CS diagnosis. The hypercortisolism resolution seems to improve, and sometimes normalize, the cardiac alterations: indeed, a reduction of left ventricular mass index and relative wall thickness, an improvement of left and right ventricular systolic ejection fractions, a normalization of left ventricular diastolic function as well as an improvement of left atrial ejection, were reported in CS patients, mainly after surgery (Kamenický *et al.*, 2014; Pereira *et al.*, 2010a; Toja *et al.*, 2012). The impact of hormonal deficits potentially associated with CS and they or their suboptimal treatments on cardiac damage persistence requires further investigation (Pivonello *et al.*, 2016a).

In patients with active persistent disease, a specific treatment with aldosterone antagonists could be useful to control the cardiac alterations (Mihailidou *et al.*, 2009), as well as an adequate treatment of SAH, especially with ACEi (Isidori *et al.*, 2015a). A yearly echocardiography execution may be a valid clinical suggestion to monitor the cardiac damage progression.

Vessels Damage

Vessels damage, is characterized by atherosclerosis and vascular remodeling hypertrophy, including increased intima-media thickness (IMT), wall thickness, wall fibrosis and media to lumen ratio (Rizzoni *et al.*, 2006). The pathogenesis of atherosclerosis and vascular remodeling involves multiple variables, already described in MS, including SAH, insulin resistance and dyslipidemia, as well as additional factors due to hypercortisolism, such as smooth cells MC receptors hyperactivation, increased levels of vascular endothelial growth factor (VEGF), an important angiogenic factor, and thrombotic diathesis (Albiger *et al.*, 2006; Geer *et al.*, 2010; Molnar *et al.*, 2008; Pivonello *et al.*, 2016a; Zacharieva *et al.*, 2004). In CS patients, the additional presence of endothelial dysfunction was also reported. This functional vascular alteration is characterized by impaired microvascular reactivity, related to SAH, and smooth cell dysfunction, related to hyperinsulinemia and hybrid insulin/IGF-1 receptors formation (Bender *et al.*, 2013; Gatenby and Kearney, 2010; Prázný *et al.*, 2008). The carotid and aortic arteries IMT increase, and the consequent premature atherosclerotic lesion onset, results in arised susceptibility to develop cardiovascular events, particularly stroke and acute myocardial infarction, showing an hazard ratio of 4.5 and 2.1 compared to matched healthy controls, respectively (Albiger *et al.*, 2006; Dekkers *et al.*, 2013; Isidori *et al.*, 2015a); therefore a carotid echo-Doppler should be performed at CS onset. One or even five years after radiotherapic or surgical hypercortisolism resolution, carotid IMT has been reported to be significantly improved, but not normalized because of partial persistence of some risk factors such as SAH or inflammatory state (Colao *et al.*, 1999; Faggiano *et al.*, 2003b). The effects of medical therapies on vessels damage have been scantly investigated. The effect of GCs replacement therapy after hypercortisolism resolution should be further investigated, however, it is noteworthy that in studies comparing hypopituitaric patients with ACTH deficiency in GCs replacement therapy and hypopituitaric patients without replacement therapy because of ACTH sufficiency, the cardiovascular morbidity is similar, resulting in a stroke and coronary heart disease prevalence of 2.1% and 4.3% in both groups, respectively (Filipsson *et al.*, 2006; Pivonello *et al.*, 2016a). No specific treatment recommendation are available to reduce the cardiovascular morbidity in CS, but an adequate control of risk factors including SAH, insulin resistance and secondary diabetes, dyslipidemia and thrombotic diathesis, is strictly suggested. Lastly, considering the persistence of increased risk of cardiovascular events after hypercortisolism resolution, an yearly carotid echo-Doppler execution also after CS remission, may be a valid clinical suggestion.

Thrombotic Diathesis

Thrombotic diathesis, resulting from an imbalance among prothrombotic and fibrinolytic factors, in favor of hypercoagulative state, is typical of CS and competes in determining the increased cardiovascular risk (**Table 1**). The pathogenesis of thrombotic diathesis, due to increased abdominal fat mass, chronic endothelial damage, atherosclerosis and direct effect of hypercortisolism (Mertens and Van Gaal, 2002; Pivonello *et al.*, 2016a; Targher *et al.*, 2010; Van der Pas *et al.*, 2013), involves multiple factors including the hyperactivation of coagulative cascade and the reduced fibrinolytic activity. The hyperactivation of coagulative cascade is due to the increased production and activation of prothrombotic factors, including Factor VIII (Boscaro *et al.*, 2002; Casonato *et al.*, 1999; Kastelan *et al.*, 2009; Ikkala *et al.*, 1985; Patrassi *et al.*, 1992; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013,

Table 1 Pathological alterations involved in thrombotic diathesis

Thrombotic diathesis
Coagulative cascade hyperactivation
↑Factor VIII
↑von Willebrand Factor antigen
↑von Willebrand Factor:ristocetin cofactor
↑Fibrinogen
Fibrinolytic activity reduction
↑Plasminogen activator inhibitor type I
↑Thrombin-activatable fibrinolysis inhibitor
↑α2 antiplasmin

2012), fibrinogen (Ambrosi *et al.*, 2000; Boscaro *et al.*, 2002; Erem *et al.*, 2009; Manetti *et al.*, 2010; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2012, 2013), von Willebrand Factor (vWF) antigen (Boscaro *et al.*, 2002; Casonato *et al.*, 1999; Ikkala *et al.*, 1985; Patrassi *et al.*, 1992; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013), and vWF:ristocetin cofactor (Boscaro *et al.*, 2002; Casonato *et al.*, 1999; Ikkala *et al.*, 1985; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013), which all compete in determining hypercoagulability, platelet adhesion to subendothelium and platelet aggregation increase, resulting in a shortening of activated partial thromboplastin time (aPTT) (Boscaro *et al.*, 2002; Casonato *et al.*, 1999; Erem *et al.*, 2009; Kastelan *et al.*, 2009; Manetti *et al.*, 2010; Patrassi *et al.*, 1992; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013, 2012). The reduced fibrinolytic activity is due to the increased levels of plasminogen activator inhibitor type I (PAI-I) (Boscaro *et al.*, 2002; Erem *et al.*, 2009; Kastelan *et al.*, 2009; Manetti *et al.*, 2010; Patrassi *et al.*, 1992; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013, 2012), thrombin-activatable fibrinolysis inhibitor (TAFI) (Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013, 2012) and α2 antiplasmin (Manetti *et al.*, 2010; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013, 2012) which all compete in determining inhibition of tissue plasminogen activator (tPA)-induced plasmin formation, reduced tPA and plasminogen binding to fibrin, and plasmin-induced fibrin degradation, resulting in increase of clot lysis time (CLT) (Boscaro *et al.*, 2002; Patrassi *et al.*, 1992; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013, 2012). Additionally, also inhibitors of fibrin formation, including antithrombin III, protein C and protein S, are often increased, probably due to a compensatory effect (Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013). The thrombotic diathesis is reflected by a >10-fold increased risk of venous thromboembolism (VTE) occurrence, with an incidence of 6%–20% (Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013). After surgical hypercortisolism resolution, a normalization of vWF concentration, Factor VIII and vWF:ristocetin cofactor activity, as well as an increased of aPTT and fibrinolytic activity with reduced levels of PAI-I and α2 antiplasmin were observed, even if hemostasis was not fully normalized 1 year later in CS patients (Casonato *et al.*, 1999; Manetti *et al.*, 2010; Pivonello *et al.*, 2016a; Van der Pas *et al.*, 2013), probably due to the persistence of abdominal fat mass (Van der Pas *et al.*, 2013). Noteworthy, when biochemical remission was obtained with medical therapy using pasireotide, cabergoline and ketoconazole, in mono- or combined therapy, the hemostasis did not normalize 12 weeks later, indeed prothrombotic factors and fibrinolytic inhibitors remained elevated (Van der Pas *et al.*, 2013, 2012); however further studies are needed to clarify the long-term effects of medical therapy. Nowadays, no studies focused on the hemostasis alterations and GCs replacement therapy, are available (Isidori *et al.*, 2015b). Moreover, considering the high risk of VTE in active phase and within 3 months following surgery, because of proinflammatory rebound related to cortisol levels fall, thromboprophylaxis should be considered for all CS patients, at least from diagnosis until to 4 weeks after surgery (Van der Pas *et al.*, 2013; Nieman *et al.*, 2015).

Musculoskeletal Diseases

Musculoskeletal diseases include the damage of skeletal system and skeletal muscle; this damage are responsible not only of the generalized weakness and fatigue, but also for the increased risk of fractures and consequent disability that significantly contribute to the impairment of the quality of life affecting patients with CS.

Skeletal Damage

Skeletal damage has been reported in 64%–100% of patients with CS (skeletal fractures in 11%–76%; osteoporosis in 22%–57%; osteopenia in 40%–78%) (Bolland *et al.*, 2011; Di Somma *et al.*, 2003, 2002; Kawamata *et al.*, 2008; Minetto *et al.*, 2004; Ohmori *et al.*, 2003; Pivonello *et al.*, 2016a; Tauchmanova *et al.*, 2007, 2006; Valassi *et al.*, 2011). GCs affect bone metabolism both directly uncoupling bone turnover, and indirectly altering pituitary hormone secretion, calcium balance and muscle strength (Canalis *et al.*, 2007; Pivonello *et al.*, 2016a; Seibel *et al.*, 2013; Weinstein, 2011). Particularly, GCs induce an imbalance between bone formation and bone reabsorption inhibiting osteoblast cell differentiation (attributed to inactivation of the Wnt/β-catenin signaling pathway, induction of nuclear factors of the CCAAT enhancer binding protein family and peroxisome proliferator-activated receptor gamma type 2) and function (impaired type I collagen synthesis, that reduces the bone matrix available for

mineralization), promoting osteoblast and osteocyte apoptosis (activating caspase 3), prolonging osteoclast life span and promoting osteoclastic activity (modulating receptor activator of nuclear factor kappa-B ligand and osteoprotegerin production in osteoblasts) (Canalis *et al.*, 2007; Pivonello *et al.*, 2016a; Seibel *et al.*, 2013). The decreased number of osteocytes induces bone microarchitectural alterations, reducing bone surface turnover in response to mechanical forces (Canalis *et al.*, 2007; Pivonello *et al.*, 2016a). A role of some polymorphism in GC receptor or higher 11β-HSD1 activity in determining a different predisposition to GCs induced bone damage, has also been suggested (Canalis *et al.*, 2007; Pivonello *et al.*, 2016a; Seibel *et al.*, 2013) (**Fig. 3**). Prompt diagnosis of CS seems to be crucial to reduce skeletal complications, as supported by the evidence that low-energy fractures occurs, particularly within the 2–3 years before disease recognition and treatment of CS (Gatenby and Kearney, 2010; Vestergaard *et al.*, 2002). A potential negative influence of disease severity or adrenal androgen suppression on bone status in CS has been suggested by the evidence that some studies reported an higher prevalence of vertebral fractures in ectopic CS than in CD (Tauchmanova *et al.*, 2006; Valassi *et al.*, 2011) and an higher prevalence of osteoporosis in patients with adrenal CS as compared with those with CD (Minetto *et al.*, 2004; Ohmori *et al.*, 2003), although these data are still controversial (Pivonello *et al.*, 2016a). Testosterone deficiency could have a negative impact on bone status in male CS, as suggested by the reported higher prevalence of osteoporosis and vertebral fractures in male than female patients (Pecori Giraldi *et al.*, 2003; Pivonello *et al.*, 2016a; Valassi *et al.*, 2011). Conversely, the negative effects of GCs on bone seem to overcome the estrogenic bone protection in female CS patients because similar prevalence of fractures were described in amenorrhoeic and eumenorrhoeic women (Pivonello *et al.*, 2016a; Tauchmanova *et al.*, 2007, 2006). The frequent impairment of bone status suggests that in all patients with active CS a careful medical interview for the presence of anamnestic report or symptoms suggestive of traumatic or nontraumatic fractures should be conducted and all patients should be screened and followed for the presence of osteoporosis, osteopenia or fractures thought appropriated methodologies such as dual-energy X-ray absorptiometry and X-ray scan. Bone damage seems to be reversible after surgical hypercortisolism resolution although the time to complete bone recovery can be relatively long and variable (Pivonello *et al.*, 2016a). Several studies report an amelioration of bone mineral density (Bolland *et al.*, 2011; Di Somma *et al.*, 2003; Futo *et al.*, 2008; Kawamata *et al.*, 2008; Kristo *et al.*, 2006), which is generally slower in the femoral neck than the lumbar spine (Futo *et al.*, 2008; Kawamata *et al.*, 2008; Kristo *et al.*, 2006). In some cases of CS, an improvement in bone status has been observed already 3 months after surgery (Kawamata *et al.*, 2008), but generally several months seem necessary to observe the normalization

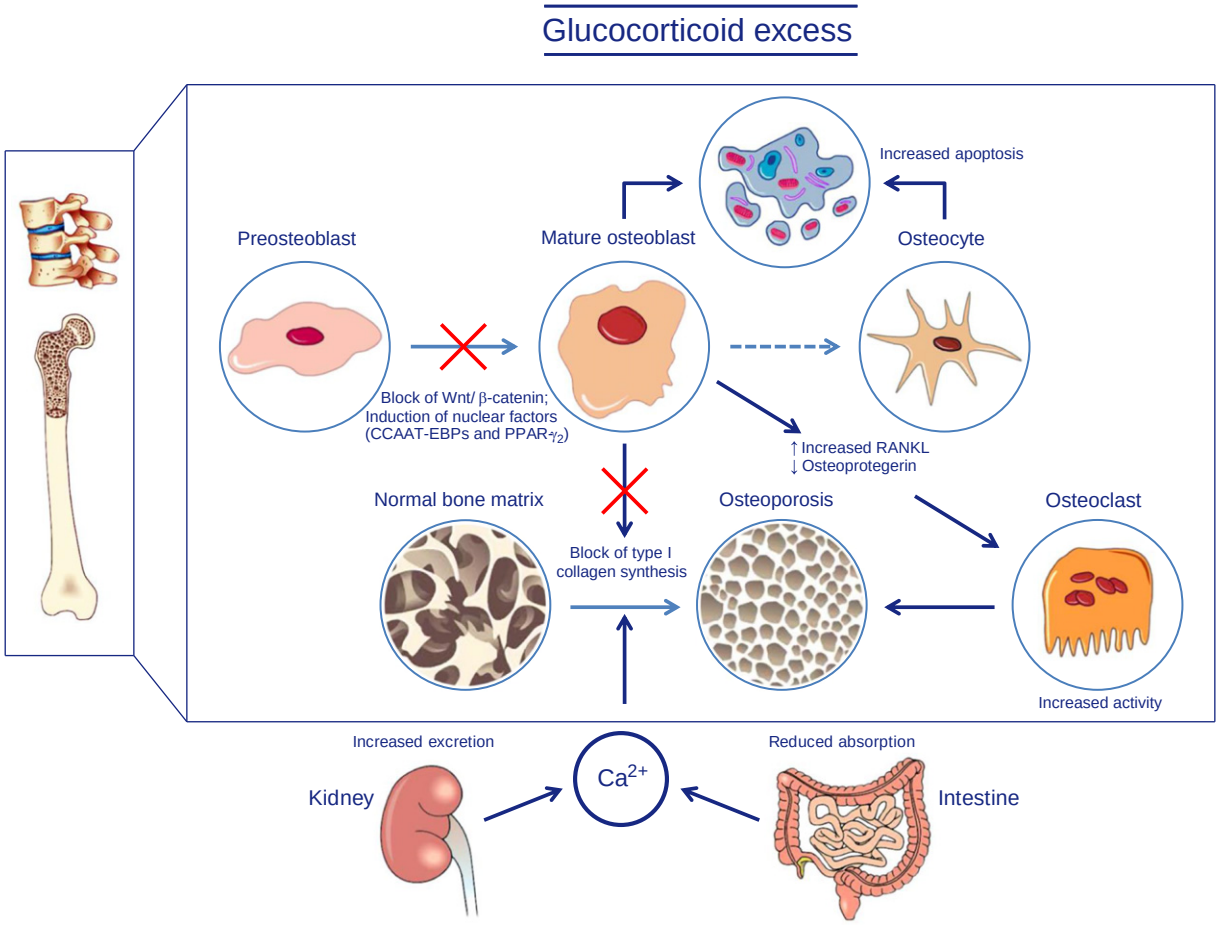

Fig. 3 Bone alterations in CS.

of lumbar spine and femoral neck bone mineral density in CS patients (Kristo *et al.*, 2006). After remission, a greater increase in bone mineral density has been reported in men as compared with women (Futo *et al.*, 2008). The effect of medical treatment on skeletal damage has been scantly investigated. In small cohorts of patients treated with ketoconazole controversial effects on bone status have been reported (Castinetti *et al.*, 2008; Di Somma *et al.*, 1998; Luisetto *et al.*, 2001), whereas the association of ketoconazole with alendronate seems to ameliorate bone status already after 6 months of treatment (Di Somma *et al.*, 1998). The presence of hormonal deficiencies or their suboptimal treatments can potentially affect bone status, during both the active and remission phases of disease. Particularly, sex and GC over-replacement might influence the time to bone recovery as suggested by the evidence that in female patients with CS after surgical remission since long time, the duration of GCs replacement was negatively correlated with lumbar spine bone mineral density (Barahona *et al.*, 2009a,b; Pivonello *et al.*, 2016a). Specific guidelines for the treatment of skeletal damage in patients with CS are still missing, although a stratification of patients in subgroups according with CS etiology, age, gonadal status, presence of fractures and expected time for hypercortisolism resolution, has been recently suggested (Scillitani *et al.*, 2014). According with this stratification, active bone therapy with bisphosphonates, teriparatide, or denosumab should be reserved to patients with an higher risk of skeletal damage (severe hypercortisolism, prevalent hip or vertebral fractures, older than 70 years), whereas in patients with a lower risk (absence of fractures, pre-menopausal women, men younger than 50 years) calcium and vitamin D supplementation should be used (Pivonello *et al.*, 2016a; Scillitani *et al.*, 2014). Considering the high prevalence of skeletal damage in patients with CS and the long time to recover after treatment, a long-term follow-up of bone status should be performed also after disease remission. In all CS patients the requirement of calcium and vitamin D and a careful tailoring of hormonal replacement treatments should be considered.

Muscle Damage

Muscle damage has been reported in 42%–83% of patients with CS (Pecori Giraldi *et al.*, 2003; Pivonello *et al.*, 2016a, 2008). GCs excess induces type 2 muscle fiber atrophy impairing skeletal muscle structure and function. These effects are mediated by both antianabolic and catabolic actions of GCs (Minetto *et al.*, 2011; Schakman *et al.*, 2013) (**Table 2**). Particularly, the antianabolic actions include the inhibition of aminoacid uptake; the repression of the insulin-like growth factor 1 (IGF-1)-activated mechanistic target of rapamycin pathway, and the inhibition of myogenesis by myogenin downregulation, whereas the catabolic actions are mainly represented by the stimulation of proteolysis, particularly through the activation of the ubiquitin–proteosome system (Minetto *et al.*, 2011; Pivonello *et al.*, 2016a; Schakman *et al.*, 2013). Additional mechanisms might include the impairment in mitochondrial function and sarcolemmal excitability and the alteration of local growth factors such as the inhibition of IGF-1 and the stimulation of myostatin (Minetto *et al.*, 2011; Pivonello *et al.*, 2016a; Schakman *et al.*, 2013).

In CS myopathy, the proximal part of the lower limbs is more severely affected (Minetto *et al.*, 2011). In subgroups analysis muscle damage seems to be more frequent in ectopic CS and in male patients (Pecori Giraldi *et al.*, 2003; Valassi *et al.*, 2011). The resolution of myopathy after surgical remission seems to take months. Additionally, musculoskeletal pain and acute bilateral carpal tunnel syndrome can be part of a withdrawal syndrome after surgical remission. However, the effects of surgical or medical hypercortisolism resolution, as well as the effects of hormonal deficiencies and their treatment on muscle damage in CS deserve further investigation (Pivonello *et al.*, 2016a). Further investigation are also required to define how diagnose and follow-up muscle damage in CS patients and to understand if the use of anabolic factors or physical activity programs can be helpful in the management of this complication in CS patients (Minetto *et al.*, 2011).

Immunological Disorders

CS is associated with a wide range of immunological alterations, in both innate and adaptive immune system, resulting in increased risk of infections and sepsis, particularly in disease active phase, and autoimmune disorders, particularly in disease

Table 2 Pathological alterations involved in muscle damage

Muscle damage
Antianabolic effects
Aminoacids uptake inhibition
IGF1-activated rapamycin pathway repression
Catabolic effects
Proteolysis stimulation
Additional effects
Mitochondrial function impairment
Sarcolemmal excitability impairment
IGF1 inhibition
Myostatin stimulation

Table 3 Pathological alterations involved in immunological disorders

Immunological disorders
Innate immune system
Proinflammatory cytokines reduction
Macrophages maturation reduction
Adaptive immune system
Lymphopenia
Th1 cellular immunity decrease
Th2 humoral immunity increase

remission phase (**Table 3**). Infections and sepsis represent one of the most important causes of mortality in CS patients (Pivonello *et al.*, 2016a).

Hypercortisolism induces an immunodepression state, altering the cellular response to infections of innate immune system, through the inhibition of antigen presentation by dendritic cells, the reduction of natural killer and neutrophil cells action, the decreased production of eosinophil and monocyte cells, as well as the impaired maturation of macrophage cells (Da Mota *et al.*, 2011; Fareau and Vassilopoulou-Sellin, 2007; Pivonello *et al.*, 2016a). Additionally, hypercortisolism also alters the humoral response to infections of innate immune system, through the reduced expression of inflammatory cytokines, complement factors, as well as the lymphocytes proliferation impairment (Da Mota *et al.*, 2011; Fareau and Vassilopoulou-Sellin, 2007; Pivonello *et al.*, 2016a). At last, hypercortisolism also affects the response to infections of adaptive immune system, through the reduced T- and B-cells maturation and proliferation (Da Mota *et al.*, 2011; Fareau and Vassilopoulou-Sellin, 2007; Pivonello *et al.*, 2016a). Particularly, a decreased ratio of circulating CD4 + /CD8 + was described, as well as an imbalance among the subclass of T-helper (Th) cells, with a decrease in Th1 cellular immunity and an increase in Th2 humoral immunity, in favor of the latter (Da Mota *et al.*, 2011; Fareau and Vassilopoulou-Sellin, 2007; Pivonello *et al.*, 2016a). Th1 cells produce a wide range of cytokines which induce B-cells to release IgG antibodies capable to opsonize virus, bacterium and fungus, favoring their phagocytosis and elimination, whereas Th2 cells produce cytokines which induce B-cells to release IgE antibodies capable to opsonize parasites, but also responsible for the immune response activation in presence of allergens. This phenomenon explains the increased risk of infections in CS patients, particularly the increased risk of opportunistic infections during the active phase of disease, and together with the phenomenon of proinflammatory rebound, it contributes to explain the presence of autoimmune diseases in these patients, particularly during the remission phase of disease (Da Mota *et al.*, 2011; Fareau and Vassilopoulou-Sellin, 2007; Pivonello *et al.*, 2016a). Opportunistic infections are very common in CS, reaching a prevalence of 21%–51% of cases, and positively related to hypercortisolism duration and severity, as well as to ectopic etiology (Broder *et al.*, 2015; Fareau and Vassilopoulou-Sellin, 2007; Pivonello *et al.*, 2016a). Additionally, the infection prevalence further increased 1 year before (hazard ratio 5.7) and 3 months after surgery approaches (hazard ratio 38.2) (Gatenby and Kearney, 2010). The most common forms of pathogens, associated with a severe and prolonged infective disease, are represented by bacteria, including *Staphylococcus* spp., *Streptococcus* spp., *Listeria* spp., *Nocardia* spp., Enterobacter, Legionella; viruses, including herpes simplex, herpes zoster, cytomegalovirus, adenovirus, influenza virus, Epstein-Barr virus; and fungi, including *Candida* spp., *Aspergillus* spp., *Cryptococcus* spp., *Pneumocystis jirovecii* (Fareau and Vassilopoulou-Sellin, 2007; Lionakis and Kontoyiannis, 2003; Pivonello *et al.*, 2016a; Rizwan *et al.*, 2014). Considering that hypercortisolism may induce an asymptomatic course of infective disease, hemocrome should be periodically assessed in all CS patients. The infection treatment should be chosen according to the pathogen, considering that the treatment response is also related to hypercortisolism control. In patients with very severe hypercortisolism, such as plasma cortisol level higher than 2500 nmol/L, a prophylaxis with cotrimoxazole should be considered in order to avoid *Pneumocystis jirovecii* infection (Pivonello *et al.*, 2016a). The impact of GC replacement therapy on infection risk in remission phase has been scantly investigated, although it is widely known that in case of uncontrolled hypocortisolism, infections represent one of the main causes of mortality (Chabre *et al.*, 2017). Autoimmune disorders are also very common in CS, showing a prevalence of 0%–20% in active phase of disease and reaching a prevalence of 60% after hypercortisolism resolution (Pivonello *et al.*, 2016a). The most common autoimmune diseases are represented by thyroiditis and celiac disease (Colao *et al.*, 2000; Da Mota *et al.*, 2011; Pivonello *et al.*, 2016a; Takasu *et al.*, 1993, 1990). For this reason, although autoimmune alterations should be further investigated, a screening for thyroiditis and celiac disease, in all CS patients within 6 months after hypercortisolism resolution might be useful (Pivonello *et al.*, 2016a).

Neuropsychiatric Disorders

Neuropsychiatric disorders are one of the most serious and disabling complications of CS and should be always considered during the current management of CS, both in the active phase of disease and after disease remission (Pereira *et al.*, 2010a,b; Pivonello *et al.*, 2016a, 2015a; Sonino and Fava, 2001). Indeed, chronic brain exposure to hypercortisolism is able to cause deep structural changes in various cerebral areas, mainly in the hippocampus, the amygdala and the prefrontal cortex, namely limbic system, in which GC receptors have a pleiotropic distribution, and are considered to be structural modulators (De Kloet *et al.*, 1998).

Particularly, hippocampus plays a key role in learning, memory, spatial abilities and emotional behaviors, whereas prefrontal cortex is involved in executive functions, memory and emotional responses. Therefore, it is not surprising that hypercortisolism can lead to structural and functional central nervous system changes, mainly consisting in brain atrophy (De Kloet *et al.*, 1998). The mechanisms by which hypercortisolism induces brain atrophy are largely unknown, although four theories have been suggested (Bourdeau *et al.*, 2002; De Kloet *et al.*, 1998; Jacobs *et al.*, 2000; Patil *et al.*, 2007; Pivonello *et al.*, 2015a,b; Simmons *et al.*, 2000). The decrease of glucose uptake: according with this first theory, GCs induce brain damage by reducing brain glucose utilization, as supported by evidence of a generalized reduction in cerebral glucose metabolism in all brain areas of CD patients (Patil *et al.*, 2007). The increase of excitatory aminoacids is responsible of "toxic" effects on nervous cells: GCs increase the release or enhance the effects of excitatory aminoacids, such as glutamate, which cause dendritic cell atrophy, particularly in the hippocampus (Patil *et al.*, 2007). The inhibition of "long-term potentiation," that is believed to be the mechanism behind learning processes and memory formation, is responsible of cognitive deficits: GCs reduce the synthesis of neurotrophic factors, mainly nerve growth factor-b and brain-derived neurotrophic factor, which through a presynaptic mechanism inhibit the long-term potentiation (Patil *et al.*, 2007). Additionally, this neurotrophic factor reduction can be per se responsible of brain atrophy (Patil *et al.*, 2007). Lastly, hypercortisolism may suppress neurogenesis in the dentate gyrus, which determines hippocampus volume loss (Bourdeau *et al.*, 2002; Jacobs *et al.*, 2000; Patil *et al.*, 2007; Simmons *et al.*, 2000). All these mechanisms seem to explain the GC-induced brain and mainly hippocampus damage, responsible for neuropsychiatric disorders. The atrophy of the prefrontal cortex (Patil *et al.*, 2007) and the suppression of neurogenesis in the dentate gyrus (Jacobs *et al.*, 2000) have been specifically identified as crucial pathophysiological events for depression (**Fig. 4**).

Although CS has repeatedly been related to different neuropsychiatric disorders, frequently literature detected only the presence or absence of symptoms rather than providing a complete major diagnosis through structured interviews and not many articles have addressed the specific issue of prevalence of the different neuropsychiatric disorders associated with CS, except for depression. A few studies have reported the presence of anxiety disorders using specific diagnostic criteria.

Major depression, the neuropsychiatric disorder better explored in CS patients, represents the most severe and life-threatening disorder associated with CS with all domains, including mood, affect, vegetative, and cognitive functions, possibly compromised. Its prevalence is reported to be about 50%–86% (Cohen, 1980; Dorn *et al.*, 1997, 1995; Haskett, 1985; Hudson *et al.*, 1987; Jeffcoate *et al.*, 1979; Kelly, 1996; Kelly *et al.*, 1996; Loosen *et al.*, 1992; Pereira *et al.*, 2010b; Pivonello *et al.*, 2015a,b; Sharma *et al.*, 2015; Sonino and Fava, 2001; Sonino *et al.*, 1998, 1993; Starkman, 2013; Starkman *et al.*, 2001, 1986, 1981). Different degrees of severity were observed, ranging from latent to very severe melancholic forms, also associated with suicidal thoughts and attempts (5%–17%) (Cohen, 1980; Haskett, 1985; Jeffcoate *et al.*, 1979). Early manifestations might be represented by irritability, insomnia and decreased libido (Starkman, 2013; Starkman *et al.*, 1981). Episodic exacerbations at irregular intervals without cyclicity, usually 1–2 days a week, rarely more than 3 days is the most common major depression "pattern." In up to 86% of CS patients depression is associated with anxiety disorders, irritability, anger, "over-reactivity and hypersensitivity to stimuli," 74% of CS patients show depressed mood, often characterized by "oversentimentality," increased feeling of crying, short spells of sadness,

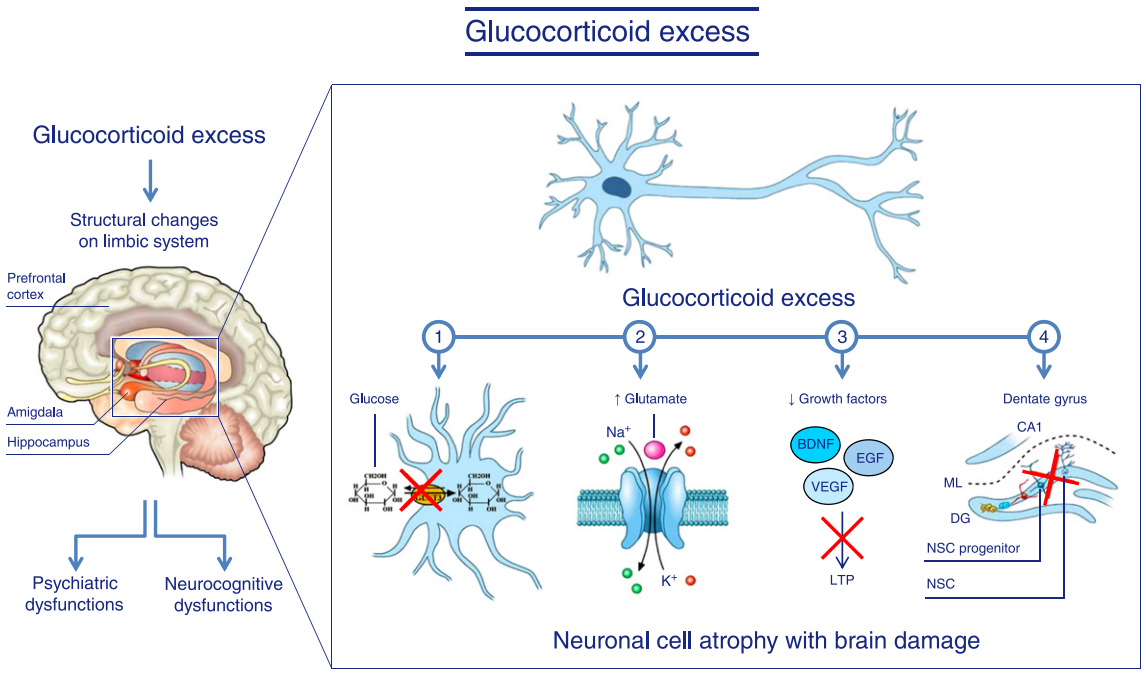

Fig. 4 Neuropsychiatric alterations in CS. *LTP*, long-term potentiation; *BDNF*, brain-derived neurotrophic factor; *VEGF*, vascular endothelial growth factor; *EGF*, Epidermal growth factor; *CA1*, Cornu Ammonis 1; *ML*, molecular layer; *DG*, dentate gyrus; *NSC*, neural stem cells.

constant hopelessness, social withdrawal with discomfort in large groups, intermittent inability to experience pleasure, rarely persistent anhedonia, and less often self-accusatory or irrational guilt (Starkman, 2013; Starkman *et al.*, 1981). Anhedonia with inability to experience pleasure is infrequent (Starkman, 2013; Starkman *et al.*, 1981). Also vegetative functions have been frequently reported impaired: fatigue in almost all patients and a decreased libido in about two-thirds; increased or decreased appetite in about 34% and 20% respectively, and sleep disturbances with insomnia and alterations in the frequency and type of dreams in 29%–69% and about 33%, respectively (Haskett, 1985; Starkman *et al.*, 1981).

Interestingly, focusing on 162 CD patients, major depression was reported significantly associated with female gender, older ages, higher urinary cortisol levels, more severe clinical conditions and undetectable pituitary tumor (Sonino *et al.*, 1998). No differences were reported among CS etiologies: hypercortisolism itself appears to be the cause.

Generalized anxiety and panic disorders have also been reported in a significant percentage of 53%–79% of CS patients, more frequently described in the chronic and advanced stage of active hypercortisolism. Also bipolar disorders, including maniac and hypomanic episodes, have been observed in about 30% of CS patients and may represent also an early manifestation of CS (Cohen, 1980; Haskett, 1985; Hudson *et al.*, 1987; Loosen *et al.*, 1992; Starkman, 2013; Starkman *et al.*, 1981).

Chronic hypercortisolism is also associated with neurocognitive dysfunctions reported in about two-thirds of CS patients, with variable degrees from mild to severe, including impairment of memory in 83% of CS patients, with difficulties in processing new information and forgetfulness of information, impaired concentration in 66% of CS patients, with mind-wandering when reading, watching television and during the course of conversations, inattention, distractibility, difficulties with reasoning ability and comprehension, impairment in verbal learning and language performance, visual and spatial abilities (Haskett, 1985; Starkman, 2013; Starkman *et al.*, 2001, 1981; Whelan *et al.*, 1980).

To date, the issue of whether CS remission may completely resolve neuropsychiatric disorders remains controversial. Several studies demonstrated a significant improvement after hypercortisolism resolution in CS patients treated with surgery, radiotherapy or medical treatment, with a progressive, although slow, improvement in brain atrophy (Dorn *et al.*, 1997; Howland, 2013; Jeffcoate *et al.*, 1979; Sonino *et al.*, 2007, 2006; Starkman *et al.*, 2001, 1986; Whelan *et al.*, 1980; Wolkowitz and Reus, 1999). However, cortisol levels normalization was associated with partial reversal of structural and functional changes observed in several cerebral areas involved during the active phase of hypercortisolism, mainly in limbic system and in frontal and prefrontal cortex, suggesting that neuropsychiatric improvement may be incomplete (Andela *et al.*, 2015; Dorn and Cerrone, 2000; Forget *et al.*, 2002; Howland, 2013; Ragnarsson and Johannsson, 2013; Ragnarsson *et al.*, 2012; Resmini *et al.*, 2012; Sonino *et al.*, 2007, 2006; Sonino and Fava, 2001; Tiemensma *et al.*, 2010a,b; Wolkowitz and Reus, 1999). Even occasionally an increase in the frequency of panic attacks and suicidal ideation was observed, probably due to the relative GC deficiency and unrestrained increase in catecholamines (Dorn *et al.*, 1997). Neurocognitive features slightly and not always significantly improve after remission, remaining impaired compared to controls (Dorn and Cerrone, 2000; Forget *et al.*, 2002). Memory, learning and concentration are reported to be impaired even after 10 years remission (Ragnarsson *et al.*, 2012;Tiemensma *et al.*, 2010b). A proper neuropsychiatric diagnosis, not easy to establish, is essential to choose the most suitable treatment. Firstly, it should be considered that some symptoms might not necessarily imply a clinical diagnosis (Santos *et al.*, 2017). Different instruments can be used for the evaluation of neuropsychiatric symptoms and disorders: clinical interviews and screening tools, mainly self-reported questionnaires, may be used to decide if a more complete neuropsychiatric evaluation is necessary. Major depression, anxiety and panic disorders may be diagnosed according to Diagnostic and Statistical Manual of Mental Disorders (Santos *et al.*, 2017).

The first therapeutic recommendation is a prompt hypercortisolism treatment, according to guidelines, before prescribing psychotropic drugs (Resmini *et al.*, 2012; Santos *et al.*, 2017). Indeed, patients may not respond properly to psychotropic drugs until cortisol normalizes. GC lowering drugs, mainly ketoconazole, metyrapone or aminoglutethimide, can also lead to an improvement in psychiatric symptoms, especially depression, in patients with a more severe CS (Santos *et al.*, 2017). Psychotropic drug treatment is recommended especially for moderate or severe symptoms or in patients with a past history of major depressive disorder prior to CS. In CS patients, selective serotonin reuptake inhibitors (SSRI) are now the most commonly prescribed group of antidepressants; low-dose clonazepam may be used for treating anxiety, whereas clozapine and mifepristone, alone and/or combined with etomidate, risperidone, aripiprazole, olanzapine or quetiapine, can be useful in case of severe psychotic symptoms, acute psychotic crises and psychotic depression (Santos *et al.*, 2017). Because cortisol normalization may take a long time, patient and family information, psychotherapy and psychoeducation are essential (Pivonello *et al.*, 2015b; Santos *et al.*, 2017). Once hypercortisolism normalization is achieved, reassessing neuropsychiatric symptoms is essential for a proper management. Also after CS remission, SSRI are the most commonly prescribed group of antidepressants, whereas short-term benzodiazepine treatment may be considered in patients with moderate to severe anxiety symptoms (Santos *et al.*, 2017). All these evidence highlight the importance of a long-term follow-up and careful periodic investigation of neuropsychiatric symptoms in CS patients, also after hypercortisolism resolution.

Reproductive and Sexual Disorders

Reproductive and sexual disorders are one of the most common complications of CS that should be considered during the disease management, both in females and males (Pivonello *et al.*, 2016a). Hypercortisolism can lead to reproductive dysfunction: GCs inhibit the release of gonadotropin-releasing hormone (GnRH), leading to a decline in circulating luteinizing hormone (LH) levels, resulting in hypogonadotropic hypogonadism (Whirledge and Cidlowski, 2010). Moreover, the localization of GC

receptors in testis and ovary suggests a direct influence of GCs on reproductive function at the gonadal level, through inhibition of steroid hormone production or GC-induced apoptosis (Whirledge and Cidlowski, 2010). Therefore, in CS a decline in estrogens concentration in females and testosterone concentration in males is not surprising and clearly associated with the occurrence of fertility impairment, although fertility impairment may also be a consequence of metabolic CS comorbidities, mainly including visceral obesity, and decrease in libido and sexual activity (24%–90%), which is more prevalent in males than in females (Pivonello *et al.*, 2016a; Valassi *et al.*, 2011; Whirledge and Cidlowski, 2010).

In CS females, menstrual irregularities, including oligomenorrhea, amenorrhea and polymenorrhea, were reported as common findings (43%–80%), more frequently observed in CD than in ACS (Pivonello *et al.*, 2016a; Valassi *et al.*, 2011). Beyond menstrual irregularities, hirsutism, acne, obesity and insulin resistance frequently characterize the CS clinical picture, rendering challenging the differential diagnosis with polycystic ovary syndrome (PCOS), the most common endocrine disorder causing female infertility. Particularly, a high prevalence of CS females, >50%, was reported to be erroneously diagnosed and treated for PCOS (Brzana *et al.*, 2014). Therefore, a differential diagnosis is required, aimed at excluding the possible presence of endogenous hypercortisolism, and at avoiding the serious clinical outcomes associated with a missing CS diagnosis. However, it should be also considered that CS and PCOS may coexist, with few distinctive features: in the ovaries of CS patients, the number of primordial follicles is reduced, fibrosis and a volumetric decrease is observed and the cortical stromal hyperplasia and luteinization is absent (Iannaccone *et al.*, 1959; Kaltsas *et al.*, 2000).

The influence of hypercortisolism on reproductive axis in CS females is also testified by the rarity of pregnancy in patients with active CS. The etiology of CS in females who become pregnant is most frequently adrenal (Caimari *et al.*, 2017). The past obstetrical history of CS females is clearly worse in comparison to the general population, as well as the frequency of spontaneous abortion (34.8%) and fetal loss (10.1%), respectively 2-fold and 10-fold higher than in healthy subjects (Caimari *et al.*, 2017). Females with CS who become pregnant are at higher risk of developing gestational diabetes mellitus (25%), hypertension (68%), preeclampsia (14%), wound infection and risk of death (2%) compared with healthy pregnancies (Pivonello *et al.*, 2016a). Risks also affect the fetus, which is at higher risk of prematurity (43%), intra uterine growth retardation (21%), spontaneous abortion or intrauterine death (5%), and several other more rare complications, including infections, hypoglycemia or respiratory distress (Caimari *et al.*, 2017; Pivonello *et al.*, 2016a). After CS remission, patients normalize both maternal and fetal risks, becoming similar to healthy pregnancies: these evidence highlight that the gonadal status impairment in CS is reversible and that pregnancy should be discouraged in the presence of active hypercortisolism, given the increased incidence of both maternal and fetal complications (Caimari *et al.*, 2017). This latter recommendation is reinforced by the limited number of cases described in literature that precludes any definitive conclusion on the optimal management for CS during pregnancy, which is usually individualized for each patient depending on the cause, the stage of pregnancy and the severity of hypercortisolism (Caimari *et al.*, 2017). Neurosurgery has been recommended as a first choice treatment, since surgical risks are lower than those of wait-and-see management (Pivonello *et al.*, 2014b). It appears safer in the second trimester due to the lower risk of fetal and maternal complications (Caimari *et al.*, 2017; Pivonello *et al.*, 2014b). As alternative treatment, when surgery is contraindicated, or initially after diagnosis for symptomatic control, different drugs have been used in a significant number of pregnant patients, with metyrapone being the most commonly used drug and generally well tolerated (Caimari *et al.*, 2017; Pivonello *et al.*, 2016a,b). Hypogonadism hormone replacement therapy in active CS females is not suggested, due to the high thromboembolic and cardiovascular risk; however, it should be considered after CS remission in case of irreversible gonadal status impairment (Pivonello *et al.*, 2016a).

In CS males, hypogonadism was reported in 50%–75% of cases, mainly consisting in loss of libido, erectile dysfunction and oligospermia (Pivonello *et al.*, 2016a). An historical histological examination of testes in CS patients revealed disorganization of the seminal epithelium, sloughing of immature elements, hypospermatogenesis, seminiferous tubular thickening and fibrosis (Gabrilove *et al.*, 1974). Maturation arrest, advanced tubular atrophy, fibrosis and decreased Leydig cells number were observed only in severe and untreated hypercortisolism cases (Gabrilove *et al.*, 1974). Although hypogonadism reversibility has been frequently reported after CS remission, with a spontaneous normalization of testosterone levels, no data are available on semen quality, rendering uncertain the reversibility of testicular damage. Hypogonadism hormone replacement therapy is suggested in CS males without normal testosterone levels within 3 months after hypercortisolism treatment (Pivonello *et al.*, 2016a).

Mortality

CS is a severe endocrine disease characterized by a large series of systemic, particularly metabolic and cardiovascular comorbidities, which cause an increased mortality ratio in active phase and, probably, also in remission phase of disease, when compared to general population mortality rate (Pivonello *et al.*, 2016a).

The main mortality causes vary according to CS etiology, including cardiovascular diseases, sepsis and suicide in case of CD and benign ACS, neoplastic progression and pulmonary thromboembolism in case of malignant ACS as well as neoplastic progression and sepsis in ECS (Pivonello *et al.*, 2016a).

The reported overall standardized mortality ratio (SMR) is 0.98–9.3 in CD, 1.14–12 in benign ACS, up to 48 in malignant ACS and 13.3–68.5 in ECS (Pivonello *et al.*, 2016a). Discordant data are available about mortality in cured CS patients, indeed, an unchanged (SMR = 0.31–1.8) (Dekkers *et al.*, 2007; Hammer *et al.*, 2004; Lindholm *et al.*, 2001; Yaneva *et al.*, 2013), or increased (SMR = 2.47–8.3) (Bolland *et al.*, 2011; Clayton *et al.*, 2011; Hassan-Smith *et al.*, 2012; Ntali *et al.*, 2013) mortality rate was

observed. However in studies considering only the subgroup of patients with persistent or recurrent disease, the increased mortality rate (SMR = 2.4–16) (Clayton *et al.*, 2011; Dekkers *et al.*, 2007; Hammer *et al.*, 2004; Lindholm *et al.*, 2001; Ntali *et al.*, 2013; Yaneva *et al.*, 2013) was confirmed.

Recently, three important literature metaanalysis, reported an increased mortality ratio in overall CD patients (SMR = 1.84) (Graversen *et al.*, 2012); a similar (SMR = 1.2) (Graversen *et al.*, 2012), or increased (SMR = 1.61–2.5) (Clayton *et al.*, 2016; Van Haalan *et al.*, 2015) mortality rate in cured patients; and an increased mortality rate in uncured patients (SMR = 3.3–4.6) (Graversen *et al.*, 2012; Van Haalan *et al.*, 2015). Lastly, different positive predictive factors were identified, including older age at diagnosis, presence and duration of disease, etiology, comorbidities occurrence (particularly hypertension and diabetes mellitus 2), as well as the number of treatments received and the presence of GCs replacement therapy after the cure (Bolland *et al.*, 2011; Etxabe and Vazquez, 1994; Gabrilove *et al.*, 1974; Pivonello *et al.*, 2016a; Yaneva *et al.*, 2013). A possible predictive role of gender has also been suggested, but further investigation are still required, because discordant and not definitive data are available (Etxabe and Vazquez, 1994; Yaneva *et al.*, 2013). Nowadays, further studies are needed to have definitive data and to eliminate confounding factors as well as the heterogeneity of different studies (Pivonello *et al.*, 2016c).

Conclusions

Chronic endogenous hypercortisolism is responsible for a rare and severe endocrine disease named CS, complicated by several comorbidities, mainly including metabolic syndrome, characterized by visceral obesity, glucose metabolism impairment, dyslipidemia and SAH, strictly associated with cardiovascular diseases, characterized by vascular atherosclerosis and cardiac damage. These comorbidities, together with thromboembolism, contribute to increase cardiovascular risk. Additional clinical complications include musculoskeletal diseases, such as osteoporosis, skeletal fractures and myopathy; immune disorders, characterized by increased susceptibility to infections, sepsis and autoimmune disorders; neuropsychiatric diseases, such as impairment of cognitive function, depression, anxiety and bipolar disorders; impairment of gonadal function, with consequent infertility and sexual disturbances. Disease duration and hypercortisolism severity appear to have a negative impact on comorbidities, with consequent QoL impairment and mortality risk increase. Therefore, a prompt screening, a confirmatory diagnosis and an effective multidisciplinary therapeutic approach are mandatory to improve clinical picture, reducing morbidity and mortality.

Several studies have explored whether comorbidities persist after hypercortisolism resolution, although the persistence of an increased mortality risk remains debated. Metabolic syndrome, cardiovascular alterations, myopathy, immune disorders and neuropsychiatric dysfunctions can persist even after remission, requiring a challenging long-term management. Conversely, skeletal damage, reproductive and sexual disorders seem to be reversible, although the time to complete recovery can be relatively long and variable.

In conclusion, prospective studies, aimed at better clarifying the potential persistence of comorbidities and increased mortality risk after hypercortisolism resolution will be helpful to improve CS management.

See also: Cushing Syndrome; Screening and Differential Diagnosis

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Effects of Glucocorticoids on the Brain

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Introduction

Physiological Regulation of Glucocorticoid Secretion and Action

Glucocorticoids, cortisol in the human and cortisone in rodents, are synthesized in the adrenal cortex and secreted under the control of the hypothalamus–pituitary–adrenal (HPA) axis. Corticotrophin releasing hormone (CRH), secreted from parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus, stimulates the pituitary to release adrenocorticotropin (ACTH) after cleavage from the pro-opiomelanocortin precursor. Subsequently, activation of ACTH receptors in the adrenal cortex leads to the synthesis and secretion of glucocorticoids. The secretion of ACTH and cortisol is characterized by a circadian rhythm with highest secretion in the early morning and a gradual decrease during the day reaching its nadir around midnight (Roelfsema *et al.*, 2016; Young *et al.*, 2004). In addition, ACTH and cortisol secretion is characterized by a pulsatile, ultradian rhythm with the biological purpose. This ultradian rhythmicity seems to be an intrinsic characteristic of the pituitary-adrenocortical feedforward-feedback loop, as can be evoked by constant infusion of CRH in the rat (Walker *et al.*, 2012). It has been argued that such dynamics also allow constant responsiveness of the system. Glucocorticoids act via two nuclear receptor types: the mineralocorticoid (MR) and the glucocorticoid receptor (GR) (de Kloet *et al.*, 2005). Availability to bind to the receptor is regulated via expression of the 11-beta-hydroxydehydrogenases type 1 and 2 (11-beta HSD1 and -2) different tissues (Draper and Stewart, 2005), and via co-activators and repressors (Zalachoras *et al.*, 2016). The regulation of the basal, spontaneous activity of the HPA axis, occurs by so-called negative glucocorticoid feedback at the level of the anterior pituitary and hypothalamus (de Kloet *et al.*, 2005). In addition to the well-known genomic actions of glucocorticoids, recent evidence suggests that also rapid, nongenomic effects of glucocorticoids are mediated via lower affinity MR and GR variants localized in the cell membrane (Jiang *et al.*, 2014; Karst *et al.*, 2005). This, so-called fast negative-feedback control of glucocorticoid action, appears to be mediated by another pleiotropic physiological system: the endocannabinoid system. Endocannabinoids play a pivotal role in the control of glucocorticoid action, via modulation of the excitatory action of glutamate on CRH neurons in the PVN (Evanson *et al.*, 2010). Glutamate activation is a crucial step in the activation of the HPA axis, and the inhibition of glutamate release appears to be specifically mediated by cannabinoids in the hypothalamic PVN.

The Brain as Target of Glucocorticoids: From the Evolutionary Perspective of the Stress Response

The brain is the major target for glucocorticoids orchestrating the stress response. Evolution has provided us with powerful tools to ensure survival, and an adequate response to a stressor in this respect is fundamental. A normal stress response is a prerequisite for a normal behavioral and metabolic adaptation to the stressor. When an individual is exposed to a stressor, the response is characterized by stimulation of the sympathetic nervous system (leading to catecholamine release) and activation of the hypothalamus–pituitary–adrenal (HPA) axis. Cortisol, or corticosterone in the rodent, is the main mediator of the adrenocortical stress response that ultimately serves only one purpose: to induce the required behavioral and metabolic adaptations enabling the individual to adequately cope with the stressor. Thus, activation of the HPA axis, and, consequently, increased cortisol secretion is fundamental for modeling the stress response (de Kloet *et al.*, 2005).

The regulation of stress-induced HPA activation, occurs, like nonstressed/basal HPA axis activity, by negative glucocorticoid feedback at the level of the anterior pituitary and hypothalamus. In clinical endocrinology, this negative feedback action exerted at the pituitary by synthetic glucocorticoids is exploited in the diagnostic workup and subsequent treatment of primary and secondary adrenal insufficiency. However, this clinical model of the HPA axis actually is a truncated model from a biological perspective, because higher centers, including brain stem catecholamines, modulate CRH production by the hypothalamus and limbic brain structures such as the amygdala (McCall *et al.*, 2015). This activation is of paramount importance in the responses to psychological stressors, which trigger emotional arousal and require cognitive operations for coping and storing the experience in the memory for future use. Glucocorticoids exert a strong feedback and feedforward action on these limbic forebrain areas (Laugero *et al.*, 2002). Two nuclear receptor types mediate this action exerted by these steroids: the mineralocorticoid (MR) and the glucocorticoid receptor (GR) (de Kloet *et al.*, 2005). Dysregulation of the activity of the HPA axis occurs when the glucocorticoid response is either inadequate, or too extreme and prolonged. This aberrant glucocorticoid response to stressors can have deleterious consequences for the organism. The inability to effectively terminate the stress response may lead to continued hypersecretion of glucocorticoids, which eventually leads to wear and tear of tissues and organs with an increased risk for metabolic and cardiovascular diseases, compromised immune responses, and psychopathology. Alternatively, an inadequate cortisol response is unable to restrain the initial stress reactions, as is the case for instance in inflammatory disorders and autoimmune diseases.

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The Regulation of Emotion and Cognition by the HPA Axis (for Coping and Storing Experience in the Memory for Future Use)

It is well established that the action of cortisol in the central nervous system is mediated by two steroid receptors, the mineralocorticoid (MR) and glucocorticoid receptor (GR). In the principal neurons of the hippocampus both MR and GR are expressed, and mediate opposite effects on neuronal excitability. This has led to the MR—GR hypothesis in which balanced activation is key for optimal control of emotion and cognition that is regulated by the limbic system. However, MR and GR may also cooperate, and there are also GR effects that are not necessarily counterbalanced by MR. A central role of cortisol action in regulating mood and cognition is supported—and was initially indicated—by high MR and GR expression especially in the hippocampus, amygdala, and prefrontal cortex (Datson *et al.*, 2001, de Kloet *et al.*, 2000). Basal levels of cortisol activate MR even before the onset of stressors, given their high affinity. MRs stimulate neuronal excitation and in this way, determine the initial defense against the stressor, a finding that translates to vulnerability and resilience to psychiatric disease (Klok *et al.*, 2011). MRs at the neuronal membrane mediate rapid (within minutes) effects of cortisol through nongenomic but ill-understood mechanisms. These effects require considerable higher cortisol levels, and thus MR acts here as a sensor for stress-induced cortisol. The effects are thought to support the initial response to stress, and sometimes prime later genomic effects (Karst *et al.*, 2005). In contrast, stress-induced activation of GR coordinates the recovery, processing of information, and storage of the experience in the memory through reduction of neuronal excitation.

In a general sense, these effects on excitability affect the overall activity of brain regions and circuits in ways that bias emotional and behavioral responses toward more likely survival in response to immediate threat (e.g., by increasing likelihood of habitual rather than goal-directed responses, Sousa and Almeida, 2012).

These roles of MR and GR in the response to a "model" acute and transient stressor are well understood and make for a satisfactory scenario. Importantly, cortisol levels may also be elevated for much longer periods of time, the face of more chronic stressors (be it disease of psychosocial threats and challenges). Even in the case of stressors that last for a number of hours, GRs role may also change from mainly "containing" the stress response to ongoing support of the response. Transient changes in excitability can be consolidated through changes in synaptic density, dendritic spine turnover, reorganization of dendritic morphology and long-term changes in the activity of (neurotransmitter) signaling activity. In these cases, mildly elevated trough levels may also bias receptor activation toward the MR (Meijer *et al.*, 1997). It is still incompletely understood how different brain regions get functionally reorganized in chronic stress, how MR and GR play a role in this, and what the trade-off is between chronic adaptation (or "allostatic" change) and risk for brain disease.

The effects of cortisol on different brain structures do not only depend on (free) plasma hormone concentrations and intracellular receptor levels. In the brain stem nucleus of the solitary tract, the enzyme 11-beta OH-dehydrogenase (11-HSD) type 2 inactivates cortisol. In these cells aldosterone has exclusive access to the MR, to regulate blood pressure but also affect higher brain areas in relation to for example, salt hunger. In the hippocampus, the 11-HSD type 1 is present, which regenerates cortisol from inactive cortisone, leading to stronger activation of MR and also GR in this area. Inhibition of the type I HSD has been suggested to ameliorate cognitive decline during age-related cumulative overexposure to cortisol (Chapman *et al.*, 2013; Sandeep *et al.*, 2004).

Expression of MR and GR can vary as a consequence of genetic variation (van Leeuwen *et al.*, 2011), epigenetically mediated early life programming effects (Turecki and Meaney, 2016), and regulation during adult life. Because of MRs activation even at basal concentrations of cortisol, regulation of receptor amount is an important level of regulation. However, receptor regulation of expression is also a relevant variable for GR, and long-term changes in receptor expression after early life stress have been suggested to have significant functional consequences (Green *et al.*, 2011). Next to expression levels, the receptors are subject to post-translational modifications, such as specific phosphorylations, that modulate receptor activity of stability.

MR and GR by themselves primarily induce changes in *responsiveness* of neurons and other cells (de Kloet, 2014). Transcriptional changes will only lead to immediate effects in case of regulation of factors that are rate limiting for ongoing processes. For example, corticosterone affects the ongoing turnover of dendritic spines, the microstructures that form the attachment point for synaptic inputs. More obvious examples from the clinic are steroid effects on ongoing processes such as bone turnover and liver metabolic processes. Thus, while a steroid psychosis may occur in rare occasions (Judd *et al.*, 2014), in a typical situation increased activation of MR and GR in absence of stimuli has no obvious immediate effects. However, the response to (threating or rewarding) stimuli will be affected by changes in cortisol exposure.

In case of acute stressors, MR and GR act in conjunction with central stress-responsive transmitters such as noradrenalin, corticotrophin releasing hormone (CRH), and urocortins. For example, noradrenalin and glucocorticoid hormones interact in the amygdala and hippocampus with glutamatergic transmission to facilitation of memory consolidation (Roozendaal and McGaugh, 2011). The increase in GR occupancy that occurs in the hippocampus after 20–30 min after an acute stressor strengthens hippocampal memory consolidation processes, as a logical part of adaptation to a stressor that allows anticipation and optimal coping for reoccurrences of the same stressor. Conversely, activation of brain GRs is detrimental to *retrieval* of stored memories, when the increase in cortisol is out of context and not relevant to the actual memory content. This context-dependence of GR action is likely linked to the negative consequences of chronic exposure to high levels of cortisol, as the actual signaling function of the receptor is lost in such settings (de Kloet *et al.*, 1999).

The context dependence of MR and GR action is also present at the level of the intracellular signaling cross-talk. For example, brain-derived neurotropic factor (BDNF) can increase GR phosphorylation in the hypothalamus, which in turn potentiates GR

effects on gene expression (Lambert *et al.*, 2013). Likewise, a prior history of stressful circumstances led to a dramatic change in the genes that were regulated in the rat hippocampus upon treatment with a single dose of corticosterone (Qin *et al.*, 2004). Thus, present state and individual history jointly determine both the nature and the magnitude of the response to corticosteroids. While in many cases similar outcomes of cortisol exposure occur, the mechanisms of action of MR and GR, and their placement in brain signaling events, explain why the eventual outcome of acute and chronic exposure can be so different between individuals.

Animal Models of HPA Axis Disturbances

As a rule of thumb, the effects of glucocorticoids under acute stress conditions tend to be adaptive, while chronic or excessive exposure is detrimental to brain function. The particular components of time, pattern and level of brain exposure have best studied in laboratory animals, although human stem cell derived neuronal cultures may add substantially to our knowledge in the near future (de Kloet *et al.*, 2009). Classic models of glucocorticoid exposure include treatment via implanted pellets and drinking water. Recent developments include the generation of adrenal Cushing's disease in mice, and it will be interesting to see how the brain changes in these naturalistic disease models (Leccia *et al.*, 2016).

Such studies—in absence of stressors—have revealed many effects of corticosteroids on the "ongoing" processes mentioned in the previous paragraph. These studies have emphasized that chronic hypercortisolemia has substantial effects on the morphology of neurons and size of brain areas, including shrinking of dendrites of the principal cells in the CA3 area, and effects on adult neurogenesis in the dentate gyrus.

Apart from overall cortisol exposure, the pattern of exposure over the day is an important factor. For example, increased levels of cortisol that have been observed during the trough of the circadian rhythm have been associated with major depression. Animal studies have allowed to show relevance of the pattern of corticosterone exposure via treatment with low, constant levels of corticosterone, which leads to suppression of the endogenous secretion at the time of the circadian peak. This regimen ensures flattened diurnal rhythms in absence of overt hypercorticism (Akana *et al.*, 1992; Meijer *et al.*, 1997; Sarabdjitsingh *et al.*, 2010b). Even the *ultradian* rhythm of hourly glucocorticoid secretory bursts seems to be relevant, as its abolishment in rats, led to marked effects on behavioral and endocrine stress responsiveness that correlated with changes in neuronal activation in the amygdala (Sarabdjitsingh *et al.*, 2010a).

Underexposure to corticosteroid (and the option for controlled substitution) can be achieved via adrenalectomy, but this is of course a complex manipulation that also leads to depletion of mineralocorticoids, adrenal androgens, and epinephrine. A more elegant approach creates a state of selective central hypocorticism by treatment with a low dose of dexamethasone (Karssen *et al.*, 2005). Dexamethasone strongly suppresses ACTH secretion at the level of the pituitary, but at low doses do not penetrate into the brain leaving brain GRs and MRs underactivated (de Kloet *et al.*, 1975; Meijer *et al.*, 1998). This is the approach that was used to demonstrate the importance of glucocorticoid rhythmicity for the plasticity of dendritic spines. Circadian glucocorticoid peaks allowed the formation dendritic spines, while troughs were required for stabilizing newly formed spines, which are important for long-term memory retention (Liston *et al.*, 2013).

As mentioned before, many potential effects of corticosteroids will go undetected in animal models that are not appropriately challenged. In this respect, region specific manipulation of has yielded interesting information. Mice that are devoid of GR specifically in dopamine sensitive neurons have a much lower response to cocaine (Ambroggi *et al.*, 2009).

More recently the relationship between glucocorticoids, reward and addiction was emphasized in a study where the GR antagonist mifepristone was efficacious both in a rat model of alcohol abuse and in a group of addicted human subjects (Vendruscolo *et al.*, 2015). Of note, studies using receptor antagonists or cortisol-lowering agents (Sooy *et al.*, 2015) in specific disease models can point to involvement of cortisol in pathogenic processes, even in situations without an obvious or dominant stress-related component.

Human Models for the Effects of Glucocorticoids on Neuropsychological Function and the Central Regulation of Metabolism

Cushing Syndrome

Cushing syndrome is a rare endocrine disorder characterized by long-term exposure to elevated glucocorticoid levels. The most common cause of endogenous Cushing syndrome is an ACTH secreting pituitary adenoma (70% of cases); other causes include ectopic ACTH secretion (mostly bronchial carcinoids), autonomous cortisol hypersecretion secondary to an adrenal adenoma/carcinoma, or adrenal hyperplasia. Cushing syndrome can also be induced by long-term administration of supra-physiological doses of synthetic corticosteroids, as is prescribed in clinical practice for a variety of inflammatory conditions and autoimmune diseases. This is a highly prevalent condition, and is called exogenous Cushing syndrome. Exogenous Cushing syndrome is insufficiently recognized in routine clinical practice, especially in the milder cases.

Regardless the underlying cause, and in accordance with the earlier described biological effects of glucocorticoids, the vast majority of patients with Cushing syndrome have both physical and psychological morbidity (Crespo *et al.*, 2015, van Aken *et al.*, 2005). In a United States population based study, the prevalence of all features of the stress response, like hypertension, increased

insulin resistance and diabetes, major depression, and osteoporosis was much higher in patients with Cushing disease than in controls that had a similar prevalence of overweight and obesity (Feelders *et al.*, 2012). In accordance, a Danish epidemiological study demonstrated a 2–6 increased relative risk for specific cardiovascular morbidity (venous thromboembolism, myocardial infarction, stroke, heart failure) and threefold increased relative risk for fractures in patients with Cushing syndrome (Dekkers *et al.*, 2013).

When neurocognitive function (that includes cognition, mood, and personality), is assessed in patients with Cushing syndrome, virtually all patients are affected during active or uncontrolled disease, and psychopathology is also often observed (Santos *et al.*, 2017). The frequency of psychiatric symptoms in active Cushing syndrome was reported starting in the early 1980s, demonstrating that symptoms like irritability, depressed mood, and anxiety were present in the majority of the patients (Starkman and Schteingart, 1981). In accordance, depression was present in $>50\%$ of patients in a large cohort of patients with Cushing disease reported by Sonino and colleagues, and was significantly associated with older age, female sex, higher pretreatment urinary cortisol levels, a more severe clinical condition, and no pituitary adenoma on pituitary imaging (Sonino and Fava, 2001). Intriguingly, an increased overall psychiatric disability score was associated with increased cortisol secretion. In addition, patients with active Cushing syndrome report cognitive impairments, like memory problems and lack of concentration. Thus, the most common comorbid disorder is major depression, and a severe clinical presentation of Cushing often also includes depression (though to a lesser extent, mania and anxiety disorders have also been reported). These observations are in line with the pivotal evolutionary role ascribed to cortisol in the control of mood and behavior. Because limbic structures like the hippocampus and the prefrontal cortex are rich in glucocorticoid-receptors, these clinical observations suggest that these structures are particularly vulnerable to the cortisol excess as is present in Cushing syndrome.

The limited numbers of patients that have been reported after treatment indicate that a significant improvement occurs within the first year after treatment (Pereira *et al.*, 2010). In addition, reduction of glucocorticoid synthesis or action, either with metyrapone, ketoconazole, or mifepristone, rather than treatment with antidepressant drugs, is generally successful in relieving depressive symptoms, as well as other disabling symptoms (Pereira *et al.*, 2010). Thus, following successful correction of cortisol excess, both physical and psychiatric signs and symptoms improve substantially. In the long-term, however, it now becomes evident from an accumulating number of studies that patients do not completely return to their premorbid level of functioning. These studies demonstrated residual physical and psychopathological morbidity despite long-term biochemical remission (Dorn *et al.*, 1997; Milian *et al.*, 2014; Tiemensma *et al.*, 2010a). In addition, patients with long-term remission of CD reported persistent impairments in cognitive functioning (Resmini *et al.*, 2012; Tiemensma *et al.*, 2010b) and a reduced quality of life (van Aken *et al.*, 2005). To which extent psychopathology still affects general well-being after long-term cure of CS is still, however, not clear.

An emerging topic of interest in this respect is the relation between glucocorticoid excess and changes in brain structure and function, and consequently, its relationship with neuropsychological dysfunction.

The first observations in the human indicating that long-term exposure to elevated glucocorticoids may affect the brain were reported by Lupien *et al.* (1998). In that particular study, exposure to prolonged elevated cortisol levels in aged humans lead to reduced hippocampal volumes as well as memory deficits (when compared to controls with normal cortisol levels). In later studies, however (in healthy young men), a larger hippocampal volume associated with a greater cortisol response both in a social stress test (Trier social stress test) and in the cortisol awakening response, questioning the relevance of the former finding in aged individuals for younger individuals (Pruessner *et al.*, 2007). Many psychiatric diseases, like major depressive and bipolar disorder have been linked to alterations in the HPA axis (Antonijevic, 2006; Belvederi Murri *et al.*, 2016), and GC receptor polymorphisms that alter glucocorticoid sensitivity have been associated with depression (reviewed in reference Spijker and van Rossum, 2009). In addition, other studies in patients with psychiatric diseases indicate that limbic structure volumes, like the hippocampus and the amygdala, are smaller (Harrisberger *et al.*, 2015; Malykhin and Coupland, 2015), though these changes may also be associated with brain aging and interact with the progression of the disorder (Alves *et al.*, 2014).

The effects of Cushing syndrome on the brain, reflecting long-term excessive overexposure to endogenous cortisol, were recently reported in a systematic review (Andela *et al.*, 2013). This review systematically evaluated all studies in patients with active and remitted Cushing disease or syndrome using MRI ($n = 19$). These studies demonstrated that structural abnormalities in the gray matter were present in patients with active disease, that were characterized by smaller hippocampal volumes, enlarged ventricles, and cerebral atrophy (see also reference Burkhardt *et al.*, 2015). In addition, functional changes occurred, characterized by alterations in neurochemical concentrations and functional activity. Intriguingly, the reversibility of structural and neurochemical alterations after correction of cortisol excess was incomplete, even when patients were evaluated after long-term remission. The structural alterations after long-term remission included smaller gray matter volumes of the anterior cingulate cortex, greater gray matter volume of the left posterior lobe of the cerebellum (Andela *et al.*, 2015) (see also **Fig. 1**), and widespread reductions in white matter integrity (Pires *et al.*, 2015; van der Werff *et al.*, 2014). Long-lasting functional alterations included increased resting state functional connectivity between the limbic network and the subgenual subregion of the anterior cingulate cortex (van der Werff *et al.*, 2015), and altered neural processing of emotional faces (Bas-Hoogendam *et al.*, 2015). Some findings as obtained using MRI were related to the severity of the cortisol excess, and others also to neuropsychological functioning (as reflected by mood, cognition, and emotional functioning), and quality of life. This points toward persistent changes in brain function after previous exposure to hypercortisolism. In agreement with these findings, the first prospective study found persistent negative effects after 36 months on attention, executive performance and nonverbal memory in 18 patients treated for Cushing syndrome (Forget *et al.*, 2016).

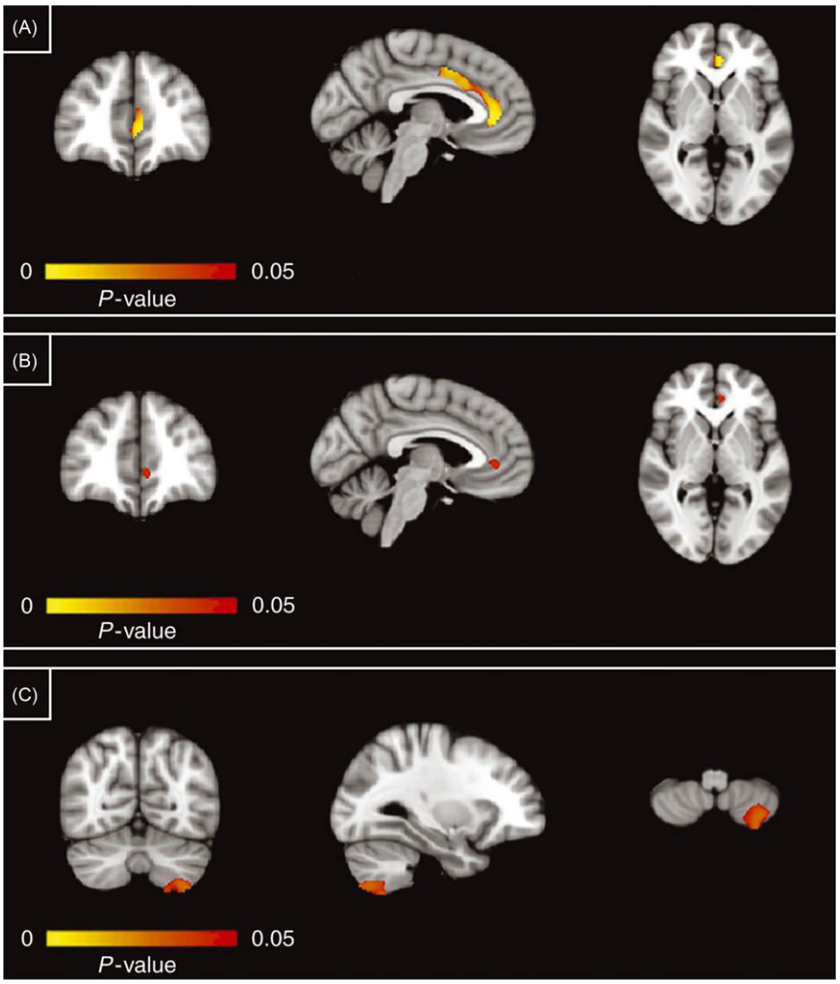

Fig. 1 VBM analysis results. (A) Results of regions of interest analysis, with lesser *gray* matter volumes in patients than that in controls ($P < .05$; 617 voxels, 2 mm isotropic). (B) Results of whole brain analysis with lesser *gray* matter volumes in patients than that in controls ($P < .05$; 37 voxels, 2 mm isotropic). (C) Results of whole brain analysis with lesser *gray* matter volumes in patients than that in controls ($P < .05$; 323 voxels, 2 mm isotropic). Effects are presented on the MNI-152 1 mm standard brain at a threshold of $P < .05$. Coordinates are $x = -4$, $y = 42$, and $z = 0$ for (A and B) and $x = -29$, $y = -66$, and $z = -56$ for (C). The left hemisphere corresponds with the right side of the image.

Adrenal Insufficiency

Not only glucocorticoid excess but also adrenal insufficiency per se, by definition, will result in impaired stress responsiveness. In the human, this is best exemplified by the clinical application of the insulin tolerance test that is considered the golden standard for the diagnosis of adrenal insufficiency. The test is based upon induction of the stress response by insulin-induced hypoglycemia, that from an evolutionary perspective is one of the most potent physiological stressors because severe hypoglycemia is potentially lethal. In accordance, the response to severe hypoglycemia is characterized both by a sympathetic noradrenergic response (tachycardia, agitation, sweating, etc.) and by activation of the HPA axis with resultant stimulation of cortisol secretion. Patients with adrenal insufficiency (regardless the cause) are not able to secrete sufficient cortisol after hypoglycemia (and thus fail this test). The subsequent metabolic and behavioral adaptations orchestrated by cortisol via the mineralo and glucocorticoid receptor, are not, or insufficiently induced. Thus, by definition, these patients exhibit impaired stress responsiveness. Intriguingly, even patients with adrenal insufficiency that were on long-term stable hydrocortisone replacement reported impairments in quality of life (Aulinas and Webb, 2014; Bancos *et al.*, 2015; Tiemensma *et al.*, 2014).

Cognitive function in patients with adrenal insufficiency on hydrocortisone replacement has been reported only in seven studies involving a total of 195 patients (Harbeck *et al.*, 2009; Henry *et al.*, 2014; Klement *et al.*, 2009; Schultebraucks *et al.*, 2016, Tiemensma *et al.*, 2016; Tytherleigh *et al.*, 2004; Werumeus Buning *et al.*, 2015). These studies indicate that mild cognitive deficits may persist, especially in memory and executive functioning tasks. Intriguingly, patients performed better on concentration and attentional tasks when compared with controls (Tiemensma *et al.*, 2016), and cognitive function was neither affected by the dose

used (high vs. low daily dose) (Werumeus Buning *et al.*, 2015), nor by postponement of the first daily dose by a few hours (Tiemensma *et al.*, 2016).

Neurocognitive functioning, besides cognition, also includes mood and personality. Patients with adrenal insufficiency may present solely with psychiatric manifestations (Anglin *et al.*, 2006; Pavlovic and Sivakumar, 2011) and epidemiological studies indicate that patients with adrenal insufficiency may be at increased risk of developing severe affective disorders. When hospitalized patients with Addison's disease were compared to hospitalized patients with osteoarthritis the former had a more than two times greater rate of affective disorders and 1.7 times greater rate of depressive disorders (Thomsen *et al.*, 2006). In the Leiden cohort more psychosocial morbidity (irritability and somatic arousal) were observed in the presence of impairments in quality of life when patients with adrenal insufficiency were compared with controls. Patients and controls did not differ regarding maladaptive personality traits, however, the daily hydrocortisone dose proved to be strongly associated both with the prevalence of maladaptive personality traits and with depression (Tiemensma *et al.*, 2014).

Patients Using Glucocorticoids

Glucocorticoids are frequently prescribed for various conditions like chronic obstructive pulmonary diseases and autoimmune diseases to inhibit the inflammatory response. Soon after their introduction in the 1950s, the first cases were reported on severe neuropsychiatric manifestations after the initiation of glucocorticoid therapy (Manzini, 1958; Piguet, 1958). In agreement with the studies in endogenous CS reported by Sonino and colleagues, more than 50% of patients exposed to glucocorticoids for >3 months developed neuropsychiatric symptoms/manifestations (Fardet *et al.*, 2007). A recent review beautifully summarized the topic of the adverse neuropsychological consequences of glucocorticoid therapy (Judd *et al.*, 2014). The acute and long-term effects on both mood and cognition have been studied in prospective studies, and the severe neuropsychiatric effects in case studies and with the use of epidemiological databases (Fardet *et al.*, 2012). The observed rates and spectrum of manifestations of depression, anxiety disorders, and cognitive dysfunction are similar to those observed in endogenous Cushing syndrome, and exemplifies that glucocorticoids can induce the same neuropsychological phenotype (in predisposed individuals). The most prominent risk factors identified were gender (male patients being more prone to develop mania and delirium, and female patients being more prone for depression), a past history for psychiatric disorders, and the initial daily glucocorticoid dose (in general above 40 mg of prednisone daily equivalent). Finally, withdrawal from long-term glucocorticoid therapy also increases the risk for severe psychiatric manifestations. Again, a past history of psychiatric disease, but also the use of long-acting glucocorticoids (especially dexamethasone) increased the risk for depression and delirium following discontinuation of glucocorticoid therapy (Fardet *et al.*, 2013).

Summary and Conclusions

The evolutionary pivotal role of glucocorticoids is reflected by the widespread expression of both receptors for glucocorticoids in the brain. Glucocorticoids play a key role in the control of neuropsychological functioning, which is exemplified by the evolutionary conserved control of behavior in the "fight or flight response". In accordance, both animal and human models of uncontrolled, (and therefore abnormal) exposure to glucocorticoids show impaired stress responsiveness, alterations in feeding behavior, cognitive dysfunction, and a broad spectrum of neuropsychiatric disorders, ranging from severe depression and anxiety disorders to acute psychosis and delirium. The fact that the same phenotype can be induced by exogenous glucocorticoid administration proves the causal role of glucocorticoids per se on neurocognitive and neuropsychiatric functioning. Finally, it now becomes clear that these effects on the brain may be long-lasting and even may not be completely reversible, because cognitive dysfunction and maladaptive personality traits persist in the presence of altered coping strategies and affected illness perceptions despite long term optimal treatment. This implies that long-term care for both patients with pituitary and adrenal disorders and patients using glucocorticoids should incorporate self-management interventions that help to improve health perception and quality of life.

See also: Cushing Syndrome; Screening and Differential Diagnosis

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Hypertension; Overview☆

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Glossary

Autonomic nervous system A network of nerves that carry information from the central nervous system (brain and spinal cord) to nearly all internal organ systems; this nerve system can operate with minimal, if any, conscious effort and functions to maintain internal homeostasis.

Cardiac output The volume of blood pumped by the left ventricle of the heart in 1 min; cardiac output is a measure of the pumping action of the heart.

Cardiovascular system The organ system that includes the heart and all of the blood vessels—arteries, veins, and capillaries; it serves to supply all parts of the body with nutrients and to transport waste products for elimination.

Diastolic pressure The minimum arterial blood pressure measured in large arteries during the rest phase (termed diastole) of the heart; this pressure, measured in millimeters of mercury (mmHg), is largely determined by the rate of outflow of blood from the arterial vascular compartment into the capillaries.

Neurotransmitters and neurohormones Chemicals made by and stored in nerve cells that, on release by a process termed exocytosis, carry information from one nerve cell to another or from a nerve cell to a target cell (e.g., smooth muscle cell); neurotransmitters travel very short distances, whereas neurohormones enter the bloodstream and may travel throughout the body.

Systolic pressure The maximum arterial blood pressure (mmHg) measured in large arteries during contraction of the left ventricle and ejection of blood into the arterial compartment; this pressure is determined by the contractile functions of the left ventricle of the heart and the arterioles and compliance of the large arteries.

Total systemic vascular resistance The resistance of the arterial compartment to the flow of blood being pumped by the left ventricle; the resistance comes from the state of contraction of vascular smooth muscle cells located in the terminal arterioles.

Blood Pressure: Definition, Physiological Importance, and Characterization

Definition

Blood pressure is simply the pressure of the liquid within blood vessels. This article focuses on excessive blood pressure within the arterial blood vessel network, or arterial compartment, that is, the network of blood vessels that carry blood away from the left ventricle of the heart toward all tissues except the lungs, which receives blood from the right ventricle. Most measurements yield values that reflect arterial blood pressure (ABP) distributed across this entire arterial compartment and therefore are measures of "systemic" ABP. Likewise, systemic arterial hypertension refers to a chronic excessive elevation of systemic ABP across the entire arterial blood vessel compartment. At a first approximation, systemic ABP levels are determined by several factors: (1) the force of blood being ejected into the arterial compartment by contraction of the left ventricle of the heart; (2) the rate of flow of blood out from the arterial compartment into the capillaries of tissues that is controlled by "resistance elements" in the smallest arteries, termed terminal arterioles; (3) total blood volume; and (4) tension generated by the walls of the largest blood vessels (termed conduit arteries) in resisting the pulse of blood ejected into the arteries by the heart. Therefore, the origins of systemic hypertension may be multifactorial.

Systemic Vascular Compartments

All multicellular organisms, from the most primitive to humans, have one key requirement for survival: a functional circulatory pathway whereby all of the organism's cells may be reached so that nutrients and critical gases (e.g., oxygen) may be distributed and waste products (e.g., carbon dioxide) may be removed. Evolution has matched complex multicellular organisms with comparably complex circulatory systems. In mammals, blood, containing both cells and dissolved materials, circulates continuously through an extensive closed network of tubes (blood vessels). This vascular system actually consists of three separate but connected sections, or compartments: arterial (arteries), capillary, and venous (veins).

The arterial compartment carries blood away from the heart, beginning with the aorta, which branches to the carotid arteries (subserving the head and brain) and to the descending aorta, which extends in an ever-branching manner to all other organ systems and tissues. The arterial system is a high-pressure circuit that allows all organ systems an equitable portion of the arterial

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blood. Large arteries leaving the heart (termed conduit arteries) receive the full pressure and flow of the pulse of blood pumped (or ejected) from the left ventricle with each contraction of the heart. Conduit arteries serve as "feeder vessels" to an increasing number of smaller arterial vessels ultimately connecting with the capillary compartment within organ systems. The capillaries are the simplest blood vessels designed to facilitate exchange of nutrients and cellular waste between tissues and blood. Arterial pressure is highest in conduit vessels (aorta and carotid) and declines steadily as the arterial vessels become smaller with the lowest arterial pressure at the junction with the capillary network. Conduit arteries also serve to dampen the large "pressure pulse" generated by contraction of the left ventricle, and function to smooth out the pressure wave that spreads from the heart to the smallest blood vessels. One way this dampening and smoothing is accomplished is through the elasticity of the conduit vessel wall. This elasticity allows "capture" of a portion of the energy of the pulse of blood ejected by the heart into the arterial compartment. As the pressure wave progresses away from the heart, the conduit arterial walls relax and return the energy to the blood, supporting the arterial pressure while the heart is not contracting. This process is like the rebound of a stretched rubber band of a slingshot that exerts a force on the object being propelled (here blood), a process termed the "windkessel effect." The windkessel effect of the conduit arteries is related directly to the elasticity (termed compliance) of the arterial wall. When conduit arteries lose their elasticity, systolic hypertension, a disorder common in the elderly, may result.

Arterial blood vessels are complex consisting of multiple cell types and several discrete layers. In immediate contact with blood is a single cell-thick lining of endothelial cells with underlying associated connective tissue proteins. This layer is termed the "intima" of the vessel. Arterial endothelial cells serve many functions, including local release of chemicals that may relax or contract the vessel wall and regulation of hemostasis and clot formation. The intima is very important with many major functions including the following: (1) reduce viscosity and turbulence of flowing blood; (2) provide a source of chemicals that regulate thrombosis or clot formation; and (3) release chemical mediators that affect the state of contraction or relaxation of underlying smooth muscle cells. The middle layer of the arterial vessel wall is termed the "media" and consists primarily of multiple layers of contractile smooth muscle cells that can directly and indirectly affect local arterial pressure or vessel compliance. The state of contraction (termed "tone") of the smooth muscle cells imparts strength to the wall to resist the ABP within the lumen of the vessel, and determines the resistance to flow of blood through the vessels. At the junction of the smallest arteries (arterioles) with the capillary compartment, the smooth muscle cells provide "resistance elements" that determine the rate of flow of blood out of the arterial compartment into the capillary network. Total systemic arterial resistance (TSVR) is a measure of the average tone (contraction state) of all the resistance elements of the arterial compartment. The outer layer of the artery wall is the "adventitia," consisting of connective tissue cells such as fibroblasts, elastic and connective tissue proteins, nerve endings of the sympathetic branch of the autonomic nervous system (ANS), and other cell types that vary according to the size of the vessel and the organ system. Large conduit arterial blood vessels also contain a microvascular blood network within the adventitia that serves to insure all cells within the adventitia get perfused with oxygenated blood. Chronic hypertension results in cellular and functional changes within all three layers of arterial blood vessels and constitutes part of the hypertension syndrome.

The capillary network constitutes the smallest blood vessels in all three compartments of the vascular system. These vessels consist of only a single-cell layer of endothelial cells with associated connective tissue proteins, and no media or adventitia. This structure permits rapid diffusion of materials, in both directions, between the blood within the vessel and fluids in the adjoining tissue. This vascular compartment is primarily a circuit for material and cellular exchange between the blood and tissues of the organ system. For this reason, it has significant importance in maintaining the amount of fluid outside of the vascular compartments and in tissues. An imbalance in fluid exchange, due to altered control of fluid dynamics between the arterial and venous compartments, contributes to tissue fluid retention in individuals with compromised heart function as well as in uncontrolled hypertension. Although antihypertensive drugs are not designed to work directly on the capillaries, many of these drugs do affect capillary functioning indirectly through improved fluid dynamics and enhanced elimination of retained fluids within tissues.

The third compartment is the network of vessels, termed the venous compartment, which collects the fluid output from the capillary compartment. The venous compartment commences at the outflow from the capillary compartment and extends to the right atrium of the heart. There are two functions of this compartment that directly influence arterial pressure. First, by collecting the blood from the capillary compartment and returning it to the heart (termed "venous return"), this insures that the heart has the available blood both to pump into the arterial circuit and to pump to the lungs for gas exchange. Secondly, the veins serve as a major reservoir of blood for the body. Large veins are termed "capacitance vessels" because they have the capacity to expand and hold large amounts of blood. When needed, contraction of the capacitance vessels (through contraction of venous smooth muscle) allows the recruitment of blood back into the arterial and capillary compartments. This is accomplished by contraction or relaxation of venous smooth muscle in large part through the activity of the sympathetic branch of the ANS. The venous compartment is a mirror image of the arterial compartment with small veins, highly ramified, joining into successively larger vessels. Veins are complex vessels, like arteries, and consist of an endothelial cell lining, medial smooth muscle, nerves, and multiple types of cells; however, the venous system is a low-pressure circuit, so the media and adventitia of the vessel wall are thinner than arteries.

There are other vascular compartments that are unique to the organ system they serve and which will not be discussed in detail in this article. Among such vascular compartments are the pulmonary and cerebral vascular compartments. Blood vessels that connect the heart and lung constitute the pulmonary vascular compartment, whereas arteries from the left ventricle of the heart to all of the organ systems (except the lungs) carry highly oxygenated blood, and veins returning to the right atrium of the heart carry low-oxygenated blood from the tissues, the converse exists in the pulmonary vascular compartment. The pulmonary artery, from the right ventricle of the heart to the lungs, carries venous blood of low oxygen content, whereas the pulmonary vein, from the lungs to the left atrium of the heart, carries highly

oxygenated blood. Pulmonary blood pressure normally is a low-pressure system; however, similar to the arterial pressure compartment, pulmonary hypertension is a condition of excess blood pressure in the pulmonary artery from the heart to the lung. This can affect the right pumping chambers of the heart and fluid exchange in the lungs. Pulmonary hypertension differs in both cause and treatment from systemic arterial hypertension, the subject of this article. For this reason, this article does not deal with this form of hypertension. Likewise the cerebral vascular compartment is also unique. While delivering blood to brain tissue (parenchyma), it also has other important functions. First, it is autoregulatory, that is, cerebral vasculature responds to changes of pressure within the compartment so as to maintain constant blood flow to brain tissues. Secondly, its structure and function are designed to dampen the systolic arterial pressure pulse so the brain parenchyma does not feel the full force of the pulse pressure wave—it has high smooth muscle tone. Headaches can be one symptom of relaxing the inherent tone of cerebral vasculature smooth muscle. Thirdly, much of the cerebral vascular compartment is structurally designed to be selective to the passage into the brain parenchyma of blood-borne biological materials as well as chemicals, proteins, and cells. This physical structure acts like a selective molecular and cellular sieve and is termed the "blood brain barrier." However, there are brain areas which function by monitoring blood-borne materials so are partially selective or without a blood brain barrier.

Many organ systems have unique aspects to their intraorgan vascular system. Very relevant to hypertension is the kidney vascular compartment which allows that organ to control both total body electrolytes (particularly sodium and potassium) and water. Through control of these two aspects of volume homeostasis, systemic blood pressures are also directly controlled. Abnormal functioning of ion and volume homeostasis by the kidney underlies many aspects of systemic hypertension.

ABP Gradient

Blood pressure is highest in the large conduit arteries, which leave the left ventricle of the heart, and decreases steadily through the arterial, capillary, and venous compartments; the largest drop in pressure occurs on the arterial side. Maintenance of this decreasing gradient of pressure is essential to ensure adequate blood delivery to all tissues, to optimize fluid and material exchange in the capillary network, and to deliver the blood back to the right side of the heart. A disease process that interferes with any of these three functions will invariably result in a form of arterial hypertension.

Determinants of Levels of Arterial Pressure

Arterial pressure derives from the pumping action of the left ventricle of the heart; therefore, the level of arterial pressure at any point in the arterial vascular compartment reflects functioning of the left ventricle. During each contraction of the left ventricle (termed systole), the highest systemic pressure generated within the arteries is termed the "systolic pressure." When the left ventricle stops contracting, the heart valve controlling outflow from the left ventricle into the aorta closes and the left ventricle relaxes and refills (between beats). This phase of the heart is termed diastole. During diastole the arterial pressure drops as the arterial blood rapidly flows out of the arterial compartment into the capillaries. The lowest arterial pressure during this rest phase of the left ventricle is termed the "diastolic pressure." The rate of drop of arterial pressure is primarily controlled by the terminal resistance arterioles, located at the junction of artery to the capillaries, which meter the rate of outflow of blood from the arteries. A second factor is the interbeat interval, the time between contractions of the left ventricle, the heart rate (HR). At constant arteriole resistance, increasing HR may increase apparent diastolic pressure since there is less time for blood to leave the arterial compartment. A third factor is the rebound of the conduit arteries, the windkessel energy effect that sustains the arterial pressure during diastole. Diastolic pressure also is indirectly determined by the systolic pressure in that an increase in systolic pressure leads to a higher starting point from which the arterial pressure may descend between contractions. This leads to a higher diastolic pressure starting point. In a normally functioning and contracting heart, the lowest systemic arterial pressure level is reached just prior to the next contraction. Thus, systolic pressure reflects multiple contributions—the action of the heart, resistance to outflow from the arterial compartment, and the windkessel effect.

The pressure difference between systolic and diastolic pressure is termed the "pulse pressure." Pulse pressure, which is sensed by the blood vessel elements, has recently been deemed a potential contributor to the development of both systemic arterial hypertension and arterial wall damage contributory to atherosclerosis.

Arterial pressure is influenced by many factors. These include age, gender, body weight, level of physical conditioning, current physical activity, and behaviors of all kinds, for example, stress, eating, drinking, and exercise. Arterial pressure can also be influenced by many agents, both prescription and over-the-counter drugs, herbal products, caffeine-loaded energy drinks, psychoactive drugs, and drugs of abuse. Further, arterial pressure varies continuously with variations caused by changes in heart beat-to-beat intervals, periods of rest and sleep, as well as levels of psychological stress.

Quantifying Arterial Pressure

Human systemic arterial pressure is typically measured with an occlusive device (cuff) placed on one arm. When arterial pressure is measured in this manner, two values are derived: an upper value and a lower value are quoted, for example, systolic pressure of 120 over diastolic pressure of 80. The units of pressure are traditionally expressed as "millimeters of mercury" (mmHg) based on the traditional use of mercury manometers to measure blood pressures. A more recent unit is "millibars." An average between these two extremes is occasionally used, namely mean arterial pressure (MAP), which is an approximate average pressure over the

period of one systole and the trailing diastole. A discussion of MAP provides insight into what determines arterial pressure. Mathematically, MAP is defined by the following relationship involving cardiac output (CO) of the heart and TSVR of the arterial vascular compartment: MAP = CO × TSVR. TSVR is the sum total resistance to the flow of blood out from the arterial compartment into the capillary network, and reflects the summary of the actions of all the terminal arterioles. CO is the amount of blood (in liters) pumped by the left ventricle of the heart over a full minute. The force of contraction of the left ventricle, the HR, the amount of blood ejected from the left ventricle chamber during each contraction, and the stroke volume (SV) determine this volume of blood. The latter results from venous return and by the resistance encountered when the heart pumps the blood into the arterial circuit. Because capacitance veins influence venous return, changes in both blood volume and the degree of constriction of venous smooth muscle influence the low blood pressure in the veins and the amount of blood returned to the heart. Because CO is defined by volume of blood ejected by the left ventricle with each beat and by HR, arterial pressure is determined by SV, HR, and TSVR.

$$\text{MAP} = \text{CO} \times \text{TSVR} = \text{SV} \times \text{HR} \times \text{TSVR}$$

Therefore, the two single numbers, systolic and diastolic pressures, are influenced and determined by many organ systems and cardiovascular components.

Within all organisms arterial pressure control is the resultant of exchange of information through the actions of both nervous systems and chemical mediators. The major nervous system that regulates and sets arterial pressure is the ANS, which works in an integrated fashion with the central nervous system (CNS), which constitutes the brain and spinal cord. There are two identifiable "branches" of the ANS—termed sympathetic and parasympathetic—that work together to control arterial pressure. In some respects the sympathetic system is viewed as being the "excitatory" branch while the parasympathetic is the "inhibitory" branch. The actions of these two separate nervous networks have been viewed as working in opposition to each other so as to affect cardiovascular functions, including arterial pressure. However, it is more accurate to view the two systems as being highly integrated and working cooperatively in a coordinated fashion to achieve the immediate goal of the organ system, namely, to respond to demands of the organism. What is the ultimate goal? It is to permit the organism to survive by allowing each organ system to meet the immediate and long-term needs of the whole organism. Transient changes in arterial pressure, high and low, occur numerous times per day. Hypertension is a chronic, frequent or continuous high arterial pressure. If systemic arterial pressure is high, the first question that should be asked is why, and potentially what need of the organism might this high blood pressure be satisfying? Of course, the pathophysiological origin of the hypertension might reside in an abnormally functioning ANS control mechanism. Appreciation of this concept is important to understand the importance of blood pressure dynamics and how to treat chronic hypertension.

For example, the sympathetic system is considered the "stress-responsive" branch of the ANS because it alters organ system functions to optimize an organism's response to a stressor, a stress that can arise externally or internally. The parasympathetic system is considered the "vegetative" branch of the ANS, regulating the most primitive and essential biological actions necessary for mere survival of the organism and the species. An organism (human or animal) can live for a long time without the sympathetic system, but not without the parasympathetic system. The sympathetic system (1) can increase HR and force of contraction thereby increasing systolic pressure; (2) can increase tone of the smooth muscle in the terminal arterioles thereby increasing systemic vascular resistance and decreasing the rate of outflow of blood from the arterial compartment between heart beats—raising both systolic and diastolic pressures; (3) stimulate release of chemicals from the kidney and adrenal glands that are important for control of blood volume, blood electrolytes, and contraction or relaxation of smooth muscle in the arteries and arterioles, affecting both pressures; and (4) control a myriad of additional functions from metabolism, to functioning of eyes, to sexual functions. However, one of the most important functions of the sympathetic system is facilitation of the dynamic shifting of blood flow among organ systems in order to meet the needs of the tissues, and ultimately the organism as a whole. Each organ system gets a fraction of total CO; however, during some functions (e.g., "flight-or-fight" responses) one organ system might need more for survival of the organism. The CNS through a selective increase in sympathetic nerve activity to particular organ systems accomplishes this "shunting" of blood to organ systems that need more blood.

In contrast, the parasympathetic system controls many organ systems so as to maintain normal homeostasis in the absence of stress. For example, the parasympathetic system slows HR and decreases cardiac function leading to a decrease in CO, increases gastrointestinal activity and secretion to aid digestion, facilitates elimination of waste products from the body, protects the lungs from inhaling toxic chemicals and substances, protects the retina from excessive light, and facilitates vision at short distances. Both the sympathetic and parasympathetic branches of the ANS project from the CNS to the heart; however, only the sympathetic system sends nerve projections to blood vessels. To summarize, in blood vessels the sympathetic system increases smooth muscle tone, increasing TSVR and decreasing rate of outflow of blood from the arterial compartment, and on the venous side contributes to increasing venous return. The ANS originates in the CNS and links brain areas important in coordinating cardiovascular and respiratory functions (brainstem) with areas that are important for both primitive and complex behaviors, even cognition. Under normal nonstressful situations each organism behavior or action of an individual requires an appropriate and selective ANS response; otherwise, the organism could not perform the desired action. For example, "fear" generally increases sympathetic activity and diminishes parasympathetic activity. Yet, although both fear from an external threat and fear derived from an internally perceived cognitive threat may result in activation of a sympathetic response (e.g., increased HR), the totalities of specific changes in autonomic functioning are not the same. Thus, one cannot generalize and say that all fear responses will have the same effect on the cardiovascular system; some may be

more demanding or even more detrimental than others. The relationships between behavior and normal or abnormal cardiovascular functioning are being elucidated with new molecular and cellular technologies allowing studies to define coordination of behaviors with observed cardiovascular responses. Such studies comprise an area of psychophysiological investigation termed "behavior–autonomic coupling" (Printz *et al.*, 2003). That such coupling is dictated by genes, and thus becomes controlled partly by inheritance, has been established through studies both within our laboratory and by other investigators. One basic question is whether an individual may inherit genes that lead to aberrant behavior–autonomic coupling and thereby be more susceptible to developing hypertension, or other cardiovascular or organ system disorders, as a consequence of behavioral or cognitive stress (Alemayehu *et al.*, 2002). A second fundamental question is whether and how abnormal cardiovascular responses might feed back into determining an individual's behavior.

Systemic arterial pressure varies over 24 h in a diurnal rhythm that is generally higher during the awake/day period and lower during the rest/sleep period. Generally an individual's arterial pressure decreases from a high, during the awake or active period, to a low during the sleep or rest period. Such an individual is classified as a "dipper." Some individuals, including some human hypertensive subjects, exhibit a failure to "dip" and are termed "nondippers." The implications of nondipping to chronic hypertension remain an area of investigation. Sleep periods and resting states are also important to normal arterial pressure as well as to behavioral states. This may be another example of the importance of behavior–autonomic coupling.

The endocrine system also has direct and indirect effects in determining the levels of systemic arterial pressure (Buijs *et al.*, 2003). Steroids, both of gonadal and adrenal cortex origins, exert direct influences on cellular components of the arterial compartment, including smooth muscle and endothelial cells, on kidney functions that relates to retention of sodium and water and on actions of the heart. Endocrine systems are also linked with control of diurnal rhythms and will directly influence the CNS including cognitive areas. Given that every behavior must have an appropriate and coordinated autonomic and cardiovascular response, it is clear that subtle endocrine-mediated changes in behavior, when exerted over an extended period, may have profound effects on the level of systemic arterial pressure.

Hypertension as a Disorder in Humans

As discussed above, arterial pressure while easy to measure in humans is a very complex trait influenced directly and indirectly by many factors among which are functioning of the heart, resistance to the outflow of blood from the arterial compartment, state of constriction of veins, total volume of the system, behavior–autonomic coupling, and endocrine state. For this reason, the pathophysiology leading to human hypertension could involve many different factors or causes. This complexity underlies the concept that most forms of chronic arterial hypertension reflect a "syndrome" rather than a single complex disorder. It is believed that nearly 80% of human hypertension, termed essential hypertension (EHT), is of unknown etiology but with a strong genetic component. EHT is considered to be the consequence of multifactorial and multigene contributions likely exacerbated and enhanced by gene–environment interactions. However, what further denotes this disorder as a syndrome is that evidence from genetic animal studies argues that the major contributory factor or factors that sustain the hypertension vary with progression of the hypertension over time. Early stages of EHT reflect excessively heightened sympathetic nervous system activity, the origins of which remain controversial. Since EHT has major genetic origins, it may originate from "susceptibility genes" that predispose the individual to an elevated arterial pressure. Over time, early primary symptoms (see below) lead to loss of homeostatic control mechanisms, in part arising from remodeling of organ systems in response to the high pressure along with direct organ system damage. These secondary factors begin to add their contributions to the causation or sustainment of the elevated arterial pressure. It is established that chronic hypertension is a risk factor for other cardiovascular disorders, which also have strong genetic determinants, such as diabetes, kidney failure, blood vessel disorders including atherosclerosis, and heart function abnormalities. These secondary disorders may then further complicate and potentially change the etiology of the hypertension. Thus, the "cause" of the EHT inherently varies with progression and duration of the hypertension syndrome. This is one reason why treatment must be designed to stem progression of the hypertension and to return arterial pressure to "normative" levels based on age, gender, and physical condition (Printz *et al.*, 2003).

Failure and Resetting of Homeostatic Mechanisms

Chronic systemic hypertension is a disorder of regulation of arterial pressure. Numerous integrated control mechanisms function to keep arterial pressure constant (Ciriello and de Olivera, 2002). For example, if TSVR increases and leads to increased arterial pressure, CO is reflexively decreased through homeostatic mechanisms that increase parasympathetic activity to slow HR and reduce contractility. This reflex is known as the high-pressure baroreceptor reflex. Likewise, if CO drops (e.g., due to a sudden loss of blood), there is a homeostatic increase in sympathetic activity to blood vessels, resulting in strong constriction of the smooth muscle in resistance arterioles and thereby a concomitant increase in TSVR so as to maintain diastolic pressure. This response is critical because aberrant low diastolic pressure threatens venous return and the ability of the left ventricle of the heart to supply blood into the arterial vascular compartment. These homeostatic regulatory systems operate continuously and dynamically on a beat-by-beat basis. Chronic arterial hypertension results in a loss of homeostasis through a physiological process of "resetting," the consequence of which is that the higher level of arterial pressure is maintained and becomes the "new set point."

One organ system important to determining arterial pressure and a component of this resetting process is the kidney that functions to maintain systemic (total body) sodium and volume homeostasis. There are forms of hypertension that arise from abnormal kidney function. Elimination of sodium and water requires appropriate arterial perfusion pressure to the kidneys, as well as properly functioning intrakidney filtration and reabsorption mechanisms. With chronic and long-standing elevated arterial pressure the kidney structurally remodels which contributes to the loss in homeostasis. The consequence is the retention of sodium (salt) and correspondingly, water, which elevates volume and further raises arterial pressure. Elevating systemic arterial pressure may initially correct this failure to excrete salt and water; however, this also may result in further loss of kidney function. Thus, the resetting process becomes part of the hypertension syndrome! This becomes a challenge as well in lowering arterial pressure in hypertensive subjects by drugs. As pressure is lowered, kidney perfusion pressure may be inadequate and the net effect is retention of sodium and water. This requires treatment with another class of drugs known as diuretics which function by enhancing sodium and water elimination by the kidney.

Chronic hypertension also leads to a compensatory thickening of the media smooth muscle layer of arteries to resist the increased pressure within the vessel. However, this thickening of the media also causes the lumen of the vessel to become smaller in diameter which raises resistance to blood flow (TSVR), which by the above equation directly affects blood pressure. If it is not reversed in time, this thickened lumen leads to a resetting of the basal state of resistance to flow. Thus, structural remodeling of the blood vessel becomes a determinant of the level of systemic arterial pressure. High arterial pressure over years also can result in a decrease of conduit vessel compliance and a failure to dampen out pulse pressure. Structural changes also take place in the heart, termed left ventricular hypertrophy, to increase pumping strength against the high conduit arterial pressure, which can progress to heart failure if the hypertension is not treated.

Arterial Pressure: A Quantitative Trait

Systemic arterial pressure is termed a "quantitative trait" because it is a measurable trait and because it shows continuous variation; there is not a single value for normal arterial pressure. In addition to reflecting age, gender, body mass, and potentially ethnic (genetic) origins of the individual, it is also a trait that changes continuously depending on an individual's behavior. Therefore, a value for normal systemic arterial pressure must be defined from studies of large populations of individuals with multiple measurements taken of each individual in the population, and under stringently controlled conditions. Further, the population individuals must be "equivalent"; for example one cannot combine children and adults in a population study to arrive at normal values since a child's blood pressures are generally lower than adults, and normal arterial pressure increases systematically with age of the individual, even after adulthood. Generally, the rate of increase of arterial pressures with age is steeper for those with hypertension than for those with normotension. Several studies have presented evidence that hypertension may exist even in young children, and how to define and treat such subjects remains a controversial issue. Quantitative traits, such as arterial pressure, are determined largely by the genes of the individual. This means that arterial pressures, and causes of hypertension, likely vary among diverse ethnic populations. With genetic mapping, investigators are beginning to dissect the role of genes in determining "normalcy."

Population studies provide a distribution of values of arterial pressures, even for subjects considered as having normal arterial pressure, with some individuals on the extreme low end and others on the extreme high end. An important and controversial discussion is whether the extremes in a normal population should be considered abnormal or normal. Are there definable "absolute" low and high values of arterial pressure that warrant clinical intervention and treatment (This issue is discussed further below in the section on JNC7 and JNC8). Rat and mouse studies also describe a range of arterial pressure values even among individuals identical in their genetic makeup. From many years population studies of young to middle-aged healthy adults, a set of values for "normal average" have become accepted starting points, namely 120 mmHg systolic and 80 mmHg diastolic. These values are, at best, approximations for the population of adults as a whole. To derive a position on what is normal and abnormal pressure and to get a consensus on how and when to treat individuals, several national commissions have evaluated population and clinical studies of clinicians and researchers. However, controversies remain following these commission recommendations.

It must be appreciated that an individual's pressure may be higher or lower than the normative average due to environmental factors. This is especially the case in the early stages of hypertension when other elements of the hypertension syndrome are not yet evident. Many factors must be considered in deciding "normalcy." Gender: arterial pressures of normally cycling females are generally lower than those of age-matched males or postmenopausal females. Body weight: blood pressure is influenced by body mass (increased body mass generally leads to increased pressures) and by physical conditioning. Behaviors: nonpharmacological treatments such as weight loss of 5%–10% or behavioral modification have had dramatic effects in lowering apparent hypertension to the normal values even in subjects who have been under drug treatment for years. Further, anxiety can have a profound effect in raising arterial pressure, especially systolic. For example, a well-described phenomenon termed "white coat hypertension" is an elevated arterial pressure whenever pressure is measured in a physician's office or clinical setting. This form of apparent hypertension likely reflects an over-reactive cardiovascular response to the stress or anxiety associated with the environment. White coat hypertension often disappears when the pressure is taken in a less forbidding environment, such as the residence of the individual. The use of 24 h ambulatory monitoring of arterial pressure can be used to exclude white coat hypertension and to identify extremes of arterial pressure over a typical 24 h activity day. All these, and other influences, including genetics and epigenetics, lead to "biological variability." For this reason, a thorough patient history and physical examination with appropriate and repeated blood pressure testing are essential in defining the disorder of systemic arterial hypertension.

What Defines Systemic Arterial Hypertension

When does an individual become classified as having hypertension? Based on years of studies and measurements of young, healthy adults, a generally accepted position is that systolic pressures should not exceed 140 mmHg and diastolic pressures should not exceed 90 mmHg. Critical to this definition is that arterial pressures be measured several times, over weeks, in both the physician's office and if possible a less anxious environment, and all potentially extenuating circumstances or influences be defined and excluded, to the extent possible. For many years a high diastolic pressure was a cause of major concern by the physician since it likely reflected structural remodeling of the blood vessel wall and resistance arterioles. Thus, the structural remodeling would reflect chronic or frequent episodic elevations of arterial pressure over the 24 h period. On the other hand, high systolic pressure in the office could reflect "stress" or anxiety elevation and was deemed of lesser diagnostic significance. However, more recently concerns about systolic pressure have taken the forefront, especially when diastolic pressures are "normal." The challenge is to understand the origins of the values since high systolic pressure could be compensation to high diastolic pressure, and not the primary origin of the hypertension. One may visualize this concept as systolic pressure "riding on the back" of diastolic pressure. Recall that systolic pressure is determined largely by the action of the heart and vessel compliance. In contrast, diastolic pressure is determined largely by systemic vascular resistance that controls the rate of flow of the blood out of the arterial compartment between left ventricular contractions.

Joint National Committee Reports

In 2003 the National Heart Lung and Blood Institute issued the Seventh Report of the Joint National Committee (JNC) on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, titled the JNC7 Report. The JNC7 Report argued that healthy adults should have target average (awake) systolic/diastolic pressures not exceeding 140/90 mmHg, whereas that of individuals with diabetes or chronic kidney disease should not exceed 130/80 mmHg. The JNC7 Report concluded that for individuals over 50 years of age, a systolic pressure over 140 mmHg is a more important cardiovascular risk factor than diastolic pressure. Certainly, loss of conduit artery compliance raises systolic pressure and this is much more common in an aging population. However, could lowering systolic pressure in some elderly individuals below 140 mmHg raise the potential of cerebrovascular or behavioral consequences due to underperfusion of the brain, and is there a gender component to this risk? Additionally, the JNC7 Report recommended that individuals with systolic pressures of 120–139 mmHg or diastolic pressures of 80–89 mmHg be considered as being "prehypertensive" and therefore strong candidates for lifestyle-promoting activities to prevent future cardiovascular risks. This replaced the prior concept of borderline hypertension that had, for years, extended the decision point for treatment to 90–94 mmHg. These recommendations were based on population studies showing increased risk of developing hypertension for systolic/diastolic pressures over 115/75 mmHg. The JNC7 Report also recommended treatment goals and regimens for subjects with uncomplicated hypertension (i.e., without diabetes or chronic kidney disorders).

In 2014 the Eighth JNC Report was issued which did not change or redefine the definition of hypertension or prehypertension, but did recommend new thresholds for when pharmacological therapies should be added onto lifestyle modifications. There is little disagreement that a sustained diastolic pressure exceeding 90 mmHg or a repeatable systolic pressure over 140 mmHg warrants appropriate therapeutic intervention—both pharmacological and nonpharmacological. The question of intervention becomes a physician's decision based on the individual's clinical and behavior/body mass historical parameters history, and should in this genetic era factor in gender, ethnicity, familial history of hypertension or cardiovascular disorders, diet, and environment. The rapid and facile movement of individuals from one part of the world to another clearly shows that nation's environment (and diet) change risk factors and hypertension susceptibility. Within a decade it is likely that both genetic maps and biomarkers will begin to redefine hypertension and the hypertension syndrome. Ultimately, it is likely that personalized treatments of EHT and other forms of arterial hypertension will become available based on genetics and biological markers of syndrome progression.

In 2017, in place of another Joint National Committee set of guidelines on clinical practice for hypertension and associated cardiovascular disorders, writing and evaluative committees of the American College of Cardiology (ACC) and the American Heart Association (AHA) combined with committees from other stakeholder groups to draft an extensive set of clinical practice recommendations and guidelines for cardiovascular disorders primarily for adults with definable hypertension (Whelton *et al.*, 2017). The overall intent was that it be applicable to patients with risk factors that associate with hypertension and/or complications of hypertension. One objective of this new coordinated effort was to publish updates of the guidelines on a 6-year cycle. A second objective was to try and address issues that have been somewhat controversial such as recommendations on salt intake, blood pressure levels that define hypertension, when to institute pharmacotherapy versus behavioral modification to lower systemic arterial pressure, the effect of body weight and obesity on arterial pressure, etc. The effort involved numerous ACC/AHA committees evaluating published clinical studies and basic science reports related to hypertension. The effort sought to address questions that had remained controversial after JNC7 and JNC8, and to integrate into clinical practice guidelines new findings that might advance the clinical approach to the treatment of patients with complex cardiovascular disorders. In effect this effort relates directly to the objective of advancing personalized medicine as well as the advances being made in the genetics of hypertension and other cardiovascular disorders.

The ACC/AHA Guidelines also modified the categorization of hypertension based on age and levels of systolic and/or diastolic arterial pressures *provided* arterial pressure is measured in the correct manner, as recommended. Specifically, the Guidelines

recommend that blood pressure in patients be measured on two separate occasions, using the appropriate instrumentation, such as cuff size, and under conditions that mitigate environmental or behavioral influences on the nervous system control of arterial pressure. In adults, the ACC/AHA Guidelines define "normal" adult systolic blood pressure as being less than 120 mmHg *AND* diastolic as being less than 80 mmHg. The Guidelines further define an "elevated" systolic arterial pressure as being 120–129 mmHg *AND* diastolic being less than 80 mmHg. Further, "Hypertension" is subdefined within two stages: stage 1 hypertension as systolic being 130–139 mmHg *OR* diastolic being 80–89 mmHg, while stage 2 hypertension as having a systolic pressure as *EQUAL TO OR* greater than 140 mmHg *OR* diastolic being *EQUAL TO OR* greater than 90 mmHg.

How Is Hypertension Diagnosed

Hypertension has been termed the "silent killer" because it often does not present with obvious symptoms. Although some believe that headaches and rapid heartbeat are indicators of hypertension, these symptoms are not most commonly associated with the disorder. However, frequent headaches should be discussed with your physician as it might relate to circulatory anomalies. Annual physical examinations, which include repeat blood pressure measurements, are most important to identify chronically elevated blood pressure; however, home or drug store testing can also permit a subject to track their blood pressures and HR—provided the instrumentation is reliable. This is especially important to subjects with overt anxieties who might present with white-coat hypertension. If individuals use store instruments, they should check their pressures at different sites to exclude instrument problems. Ophthalmologists and opticians may discover hypertensive individuals while examining the retina (the back inner surface of the eye). The retina is highly vascularized, and chronic untreated hypertension damages the retinal vascular bed (a damage that can be observed on examination). However, atherosclerosis and diabetes may cause similar changes in the retina, and such conditions must be excluded.

Identification of hypertension during routine physical examination should be rigorous and replicated. A single finding of elevated diastolic or systolic pressure does not constitute sufficient proof to initiate therapy, whereas dismissal of borderline readings of heightened arterial pressure without follow-up also places the patient at risk for future disease. Accurate measurement of arterial pressure is not trivial. Nearly all methods of measurement employ a form of the sphygmomanometer technique, an indirect method using the cuff occlusion. This involves measurement of the external pressure required to occlude, totally or in part, blood flow in a readily accessible artery such as the brachial artery of the upper arm. The procedure involves wrapping a "correctly sized" pressure cuff around the upper arm and inflating it to transiently stop the flow of blood through the major artery. The resumption of blood flow as the pressure is lowered is tracked. In the traditional method using a stethoscope, two characteristic sounds (termed Korotkoff sounds) are monitored. The first is the start of flow into the vessel as the cuff pressure is lowered and defines systolic (maximal) pressure while the second is when no flow sounds are present and indicates no restriction on flow, defining the diastolic (minimal) pressure. It is critical that the cuff fits the individual; too small a cuff may give an anomalously high value, whereas too large a cuff may give a falsely low value. Arterial pressure is measured with the subject sitting and, most importantly, with the cuff and occluded hand/arm being at the level of the heart. Pressures may also be taken with the subject standing (termed upright) or when the individual is in a lying-down (termed supine) position. Measurements should be taken in a calming environment to minimize stress and anxiety effects. Although automated cuff arterial pressure measuring devices found in pharmacies and food stores may be quite reliable, they cannot be used to exclude or diagnose hypertension. Repeated measurements are necessary to confirm suspected hypertension and some physicians utilize 24 h ambulatory monitoring especially if they suspect highly variable pressures over the 24 h period.

Forms of Arterial Hypertension

The most common form of human hypertension, EHT, is believed to have a strong genetic component and its origins are complex, multigenic, that is, arising from different genes in different individuals, and multifactorial. Some investigators believe that EHT reflects a variety of forms of hypertension, each due to a different abnormality of arterial pressure and/or volume regulation. EHT, being a disorder of genetic origins, explains why there is a strong sensitivity to environmental factors including salt-dependent or obesity-dependent forms of hypertension. Genetic hypertension in humans (and rodents) is believed to be the consequence of the inheritance of multiple genes that predisposes the individual (or organism) to develop the disorder, usually after puberty. EHT is age-dependent in symptom expression; however, studies indicate that elevated arterial pressures may be observed even at very early ages, leading to increased efforts to evaluate arterial pressure in children (Anyaegbu and Dharmidharka, 2014).

A smaller percentage of individuals (estimated at 10%–25%) have a form of hypertension that is attributable to a known or identifiable cause, most often of endocrine-related origins. This form of hypertension is termed "secondary hypertension." Secondary hypertension may also have a significant genetic component to disorder expression. Such genes may be directly involved (causative genes) or secondarily involved (susceptibility genes). Renovascular hypertension, believed to be responsible for 5%–10% of all human hypertension, is caused by a pathophysiological process that affects kidney blood flow and function and that results in activation of a peptide hormonal system, the renin–angiotensin system. Renovascular hypertension may be caused by a tumor or growth that impedes and restricts blood flow to the kidney. Removal of the tumor may reverse the hypertension provided that the elevated arterial pressure was not of sufficient duration to lead to irreversible changes in the kidney blood vessels structure. Chronic secondary hypertension will also trigger the hypertension syndrome, the consequence of which is that correcting the initiating causes does not reverse the hypertension and pharmacological or nonpharmacological treatments need to be

implemented. Another potential cause of secondary hypertension is a rare tumor of the adrenal medulla, a pheochromocytoma, which episodically releases into the blood stream large quantities of the neurohormones epinephrine and norepinephrine, along with other adrenal medullary mediators. Such patients exhibit episodes of very high arterial pressure. Again, removal of the tumor before it metastasizes may reverse the hypertension if irreversible changes in the arteries or kidney have not occurred.

Hypertension may also result from taking drugs that exert their direct actions on the kidney, arterial or venous smooth muscle, or endocrine systems. For example, oral contraceptive-induced hypertension became widespread shortly after the birth control pill was released for general use. Changing the composition of the pill greatly reduced the incidence of this drug-induced hypertension. This form of secondary hypertension has been attributed to sex steroid induction of the synthesis by the liver of angiotensinogen, the protein precursor of the angiotensin peptide. Angiotensin functions to promote salt and water conservation and retention, as well as increased contraction of arterial smooth muscle. Excessive formation of angiotensin may result in many actions, including increased synthesis and release of the steroid aldosterone from the adrenal cortex that directly drives salt and water retention, heightened TSVR, and blunting of the high-pressure baroreceptor reflex. Another form of secondary hypertension strikes some women during the second and third trimesters of pregnancy and poses significant risks for both the fetus and the mother. Although the causes of pregnancy-induced hypertension remain controversial, this too is a secondary form of hypertension because it generally reverses following delivery. There is evidence that these latter forms of secondary hypertension may also be under some gene influences. Lastly, there is an extreme form of hypertension termed malignant hypertension. This is a term used for patients who show extremely high and frequently irreversible hypertension. This term does not imply a cancerous origin to the disorder; rather, patients with malignant hypertension are those who have had long-standing, untreated hypertension or who fail to respond to drug therapy and combinations of antihypertensive drugs. Malignant hypertension is often associated with severe kidney failure and constitutes a medical emergency necessitating bold drug treatment to lower the blood pressure and prevent further organ damage and heart failure.

Genetic Predisposition to Hypertension

As discussed above, human EHT has a genetic origin; that is, the potential for developing hypertension may be passed from one generation to another within a family. This means that there are hypertensive-prone individuals. However, because there is also a strong influence of environment on many genes that might increase susceptibility to hypertension, it is not clear whether all hypertensive-prone individuals will eventually become hypertensive. In fact, there is evidence from animal and human studies that hypertension may be significantly delayed in onset or even prevented by appropriate control of the environment, diet, lifestyle, and exercise/activity. Remember that hypertension develops with age, so changes in lifestyle that delay the development of elevated arterial pressure will likely prevent its occurrence. Is it possible that earlier generations were hypertensive prone but that their lifestyle prevented the disorder from appearing until they were quite elderly? An interesting study conducted nearly 20 years ago with a strain of rat that spontaneously develops hypertension at 6–12 weeks of age (due to inheritance of hypertension genes) showed that if the animals were raised from birth in a stress-free environment under controlled dietary conditions, the rate of development of hypertension was greatly delayed and nearly prevented. Environmental effects also influence the state of the endocrine system, and altered hormonal secretions may be coupled to the rate of onset and progression of arterial hypertension.

Potential Causes of EHT

EHT is a multigene disorder with strong contributions from environmental factors on disease development. Discovering the cause is complicated by the many factors that determine the level of arterial pressure. A complete discussion of these factors is beyond the scope of this article; however, a brief introduction is warranted because therapy is directed at one or more of these possible causative mechanisms.

There is ample evidence from both animal and human studies that an elevation of activity of the sympathetic branch of the ANS is observed in all forms of hypertension. This system is critical to arterial pressure regulation and nearly all the effective antihypertensive drugs in use today interfere, directly or indirectly, with sympathetic activity or its physiological effects. Physiologically, if the sympathetic branch of the ANS is blocked with drugs, individuals show a loss of arterial pressure control in going from a supine position to an upright one (termed postural hypotension). In addition to effects on the heart and blood vessels, the sympathetic system is important in autonomic regulation of glucose and fat metabolism, coupling hypertension with diabetes, atherosclerosis, and obesity. Perhaps most important, the sympathetic branch is a major mechanism for stimulating secretion of the proteolytic enzyme renin into the blood by the kidney. Renin enzymatically releases an inactive angiotensin peptide from a precursor protein, angiotensinogen, which undergoes enzymatic conversion by an enzyme known as ACE, to a family of biologically active peptides of which angiotensin II is the major active peptide hormone. Angiotensin II promotes salt and water retention, will directly increase TSVR and arterial pressure, and is also a potent inhibitor of the high-pressure baroreceptor reflex, which would normally reduce sympathetic activity if arterial pressure became too high. Blockade of the receptor for angiotensin II or its enzymatic formation constitutes a class of drugs very important to the therapy of almost all forms of arterial hypertension.

In addition to autonomic control, regulation of systemic arterial pressure is achieved through electrolyte (e.g., sodium, potassium, chloride) and water homeostasis. Endocrine mechanisms are central to volume control mechanisms and operate both

within the CNS and in peripheral organ systems. A very common finding with established EHT and many forms of secondary hypertension are abnormalities in kidney function that lead to abnormal retention of sodium and, with it, water. Adrenal cortex steroid hormones, such as aldosterone, and vasopressin secreted by the pituitary, are also involved in salt and water homeostasis. Restriction of sodium (or salt) intake can lower arterial pressure in most hypertensive patients, yet severe restriction may be counterproductive because too little sodium intake actually activates the renin and angiotensin systems.

There is strong epidemiological evidence that excessive dietary salt intake elevates population averages of arterial pressure, and many studies have demonstrated a positive relationship between the amount of salt consumed in the diet and the incidence of hypertension. Yet we now know from human and rodent genetic studies that salt intake and the effect of salt on arterial pressure are also genetically controlled. We have found in rodent genetic studies that genes that control salt sensitivity of arterial pressure are inherited independently of genes for hypertension, at least in this rodent genetic hypertension model. Therefore, salt sensitivity of arterial pressure is an inherited trait; however, coinheritance of genes for salt sensitivity with one or more genes predisposing to hypertension may exacerbate the disorder and likely leads to onset of hypertension at an earlier age.

As discussed above, kidney function is central to sodium-volume homeostasis and through this mechanism has a very important role in normal homeostasis of systemic arterial pressure. However, there is also crosstalk between the kidneys and the CNS in this process, through nervous system pathways. The kidney communicates information on its functioning to the CNS (via the spinal cord to the brainstem) through sensory nerves, termed kidney afferents, and receives action signals via motor nerves, termed efferent, as well as from CNS-controlled hormonal (chemical) mediators. The role of the kidney afferents in normal and aberrant arterial pressure control has received much study over the past three to four decades. Recent clinical trials are investigating the effect of interrupting kidney–CNS communications as a mechanism of controlling hypertension, especially in subjects with a form of salt-sensitive hypertension resistant to pharmacological control. This kidney–brain communication should be appreciated as another mechanism for homeostatic control of volume and arterial pressure.

An attractive theory is that repeated exposure to stress in everyday life activities may result in abnormal cardiovascular responses in genetically predisposed, hypertensive-prone individuals. This would constitute a gene—environment interaction as a potential cause or contributor to EHT. It is possible that repeated stress leads to irreversible changes in arterial blood vessels and endocrine mechanisms resulting in a sustained elevation of TSVR and ultimately to hypertension. Behavioral stressors have been shown to result in adjustments in CO, and repeated changes in blood pressure due to stress may cause a resetting of the baroreceptor reflex associated with hypertension. There is genetic evidence that supports the effects of stress on increases in blood pressure. Individuals with family histories of hypertension exhibit heightened cardiovascular responses to both physiological and psychological stressors compared with those with no family histories of hypertension. Although it may be intuitive to expect that repeated exposure to stressful situations would have some adverse cardiovascular effects, there is no evidence supporting a "hypertensive personality."

The potentially deleterious effects of mental stress on the cardiovascular system may reflect an impaired "coping" mechanism. A failure to habituate to, or cope with, stress appears to pose an inherent risk factor in the development of hypertension; however, it remains uncertain whether it merely exacerbates the hypertension, increases its rate of development, or increases the risk of hypertension-induced organ system damage. Finally, the majority of research suggests that stress-induced increases in arterial pressure and changes in CO that lead to the development of hypertension, over time, are the result of increased activity of the sympathetic nervous system. One reason why it is difficult to resolve the role of stress in the development of hypertension is the complex interplay of multiple endocrine mechanisms that regulate arterial pressure and blood volume. There is also a complex genetic component in both physiological and psychological coping or adjustment to stress that has not been resolved.

Not only arterial pressure and HR but also metabolic factors (Cao, 2014), such as blood glucose, insulin, other neurohormones, and endocrine secretions, exhibit inherent diurnal rhythms. Some hypertensive individuals (and genetic hypertensive rats) exhibit abnormal diurnal arterial pressure and HR rhythms (nondippers). This abnormal rhythm has been associated with evidence of cardiovascular damage. However, unpublished animal genetic studies suggest that dipping or nondipping is an "independent factor" from susceptibility to exhibit hypertension. In other words, separate genetic influences determine both traits —diurnal variations and genetic hypertension; independent inheritance leads to subjects showing both traits. The indirect effect of abnormal diurnal endocrine and metabolic mechanisms could also tie with and have a direct role in the complex hypertension syndrome in some subjects.

Therapy of Hypertension

When to initiate therapy for suspected arterial hypertension and which type of therapy to implement first have been quite controversial. Therapy takes the form of either antihypertensive drugs (i.e., pharmacological therapy) or nonpharmacological approaches such as behavior and lifestyle modification. For secondary forms of hypertension drug therapy, along with surgery as needed, may be the treatment choice. However, with EHT whether to treat or not depends on the classification of severity and the presence or absence of other risk factors (e.g., diabetes, atherosclerosis, obesity, heart condition). Borderline or prehypertension should always be addressed first by behavior and lifestyle modification before pharmacotherapy. Mild to moderate hypertension warrants both nondrug and drug therapy. The primary goal of all therapeutic approaches must be to lower the excessive arterial pressures into the normal range for the individual's population norm, or achieve a pressure as low as possible consistent with patient acceptability and compliance. All therapeutic approaches must be individualized to the patient.

Among nonpharmacological approaches are weight reduction, restriction of salt intake, stress reduction including meditation, lifestyle changes, exercise, potentially acupuncture, discontinuance of alcohol intake, and cessation of smoking. For some individuals, these nonpharmacological therapeutic approaches may be all that is needed. For example, three decades ago it was shown that a relatively small reduction in body weight (less than 10%) markedly lowered arterial pressure in EHT subjects allowing a reduction or even total discontinuance of antihypertensive drugs in some patients. Weight reduction achieves two immediate goals: reduction of the workload placed on the heart and reduction in total blood volume. Stress reduction lowers sympathetic drive, thereby lowering activation of neurohormones and decreasing heart stimulation. Nondrug therapies such as stress reduction may take several months before evidence of their effectiveness is clear. For this reason, patients are usually placed on limited antihypertensive drug therapy to assist in lowering the blood pressure more quickly. Whether or not this is necessary depends on the individual patient.

The JNC7 and JNC8 Reports differ somewhat in their recommendations for treatment approaches. While both have similar treatment goals (targets for optimum arterial pressure control), they differ somewhat in treatment approaches when the disorder is confounded by other factors such as diabetes, kidney disease, and heart problems. Pharmacotherapy of hypertension has always employed what is termed a "stepped care approach." Specifically, this approach starts with trying a single drug to control the hypertension and adding or substituting additional drugs to get to the target goal of arterial pressure. The JNC7 recommended starting with a diuretic while JNC8 provides several potential starting first-line agents: diuretics; calcium channel blockers; angiotensin converting enzyme inhibitors (ACEI); or angiotensin receptor blockers (ARB). Due to the baroreflex resetting phenomenon (discussed above), almost every first line antihypertensive drug necessitates co-addition of a diuretic. Again, this is because the increased arterial pressure was necessary to adequately blood-perfuse the kidneys and allow for optimum sodium and water excretion. When increasing the dumping of sodium and water, the function of a diuretic, there may be excessive potassium wasting as well. This can lead to adverse cardiovascular effects and some physicians prefer to use a diuretic that is "potassium sparing," that is, does not lose blood potassium ion along with the sodium ion and water. Popular first-line drugs today are beta-adrenergic blockers (which block cardiac stimulation or decrease sympathetic nervous system activity) and angiotensin system blockers (ACEI or ARB). For both classes of antihypertensive agents a diuretic must be added for long-standing hypertension so as to control against sodium/water retention. To get further insight into pharmacological agents useful to treat arterial hypertension, access the JNC7 and JNC8 Reports that are available via the Internet.

It is generally considered that, with time (years), patients will progress to either combinations of drugs or the stronger third-line agents. However, many investigators believe that there has been inadequate attention to combining nondrug methods with pharmacotherapy. Reliance on pharmacotherapy without strong efforts to promote concurrent nondrug therapies has demonstrated that progression is inevitable.

Conclusion

Hypertension is a disorder of regulation of systemic arterial pressure and constitutes a complex syndrome, not a single-entity disorder. EHT and many forms of secondary hypertension have major genetic elements that dictate predisposition to the disorder. Early recognition of abnormal regulation of arterial pressure is essential if progress to additional cardiovascular damage is to be arrested. All therapeutic approaches must focus on slowing progression of the disorder and returning, if possible, the arterial pressure to population-defined normative limits. However, what is normal arterial pressure for one individual may be abnormal for another, or conversely, what is abnormal for one individual may be normal for another. Arterial pressure permits organisms to function and survive. It must be understood that hypertension may be the result of efforts by the body to satisfy the cardiovascular and autonomic requirements of the individual. Reversing hypertension without addressing the origins of the elevated arterial pressure may be therapeutically inadequate and potentially detrimental. Until more is known about the genes that regulate cardiovascular and autonomic coupling, the approach to the treatment of hypertension must be to slow progression of the disorder. Thus, treatment of hypertension involves an ongoing series of decisions and evaluations by the physician based on the individual patient. Continued interaction and communication among patient, physician, and health professional are essential to the prevention of the damaging effects of hypertension. In the case of hypertension, as with many diseases or disorders, prevention remains the preferred first step in treatment.

See also: Endocrine Hypertension. Hypertension and Target Organ Damage. Neurogenic Hypertension. Renal Hypertension. Resistant Hypertension

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Neurogenic Hypertension☆

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Autonomic Cardiovascular Control

Several are the hypothesis for the autonomic dysfunction occurring in the hypertensive state. The first is related to the impairment of the baroreflex, that is, of a major restraining mechanism of parasympathetic tone. The impaired parasympathetic modulation of the heart by the arterial baroreceptors is documented by the less responses to the vasoactive drug technique or by the spontaneous baroreflex sequence technique. This impairment does not involve the sympathetic component of the baroreflex function (Grassi, 2010). However this do not mean that reflex cardiovascular regulation may not be involved in the adrenergic overdrive. Several data have observed that a resetting of baroreflex toward elevated blood pressure levels is present in hypertension thus indicating a preservation of its influence rather than a suppression of the increased sympathetic activity (Grassi *et al.*, 1998). Second is the observation that the sympathoinhibitoy influences of cardiopulmonary receptors, which control circulating blood volume and release of vasoactive substances such as atrial natriuretic peptides, vasopressin and renin, is reduced in the hypertensive state (Grassi, 2010). The third hypothesis claims that the sympathetic activation and the parasympathetic inhibition seen in hypertension depend on the hyperinsulinemia and the related insulin resistance which are frequently ($>40\%$) present in the hypertensive state (Scherrer and Sartori, 1997). It is also evident the opposite, that is sympathetic activation may cause insulin resistance. This is supported by the evidence of a greater sympathetic activation when conditions characterized by insulin resistance, such as obesity and complicated obesity, are associated with hypertension (Seravalle and Grassi, 2017).

Adrenergic Tone and Target Organ Damage

Different techniques to investigate the adrenergic tone have clearly shown an increased norepinephrine spillover in the kidney and in the heart in young borderline hypertensives. This condition and the pre-hypertensive state are also characterized by an increased sympathetic nerve traffic directly recorded to the skeletal muscle (Seravalle *et al.*, 2015). This hyperadrenergic activation undergoes a progressive and further potentiation with the increase in blood pressure values and with the increase in age.

Metabolic alterations associated with an increase in insulin resistance (Seravalle and Grassi, 2016), alterations in dipping profile, presence of white coat and masked hypertension, are all characterized by a sympathetic overdrive.

The hypertension-related increase in adrenergic outflow appears to be peculiar to the hypertensive state of essential nature. This is documented by the evidence that the secondary forms of high blood pressure elevation caused by primary hyperaldosteronism, renal arterial stenosis or by adrenal pheochromocytoma appear not to be characterized by an elevated sympathetic outflow (Grassi *et al.*, 1998) (**Fig. 1**).

Several evidences have clearly shown that the sympathetic activation is able to promote cardiac and vascular alterations and to impair renal function. The increase in left ventricular hypertrophy (Grassi *et al.*, 2009) and the development and progression of arterial wall hypertrophy and remodeling (Heagerty, 1997) not only are able to maintain the blood pressure elevation but also contribute to the increase in morbidity and mortality rate described in untreated hypertension.

☆*Change History*: December 2017. G. Seravalle, G. Grassi and G. Mancia included two new paragraphs on "autonomic cardiovascular control" after "cardiac and neural mechanisms" and "adrenergic tone and target organ damage" at the end of the chapter. The last new paragraph included also a new Figure.

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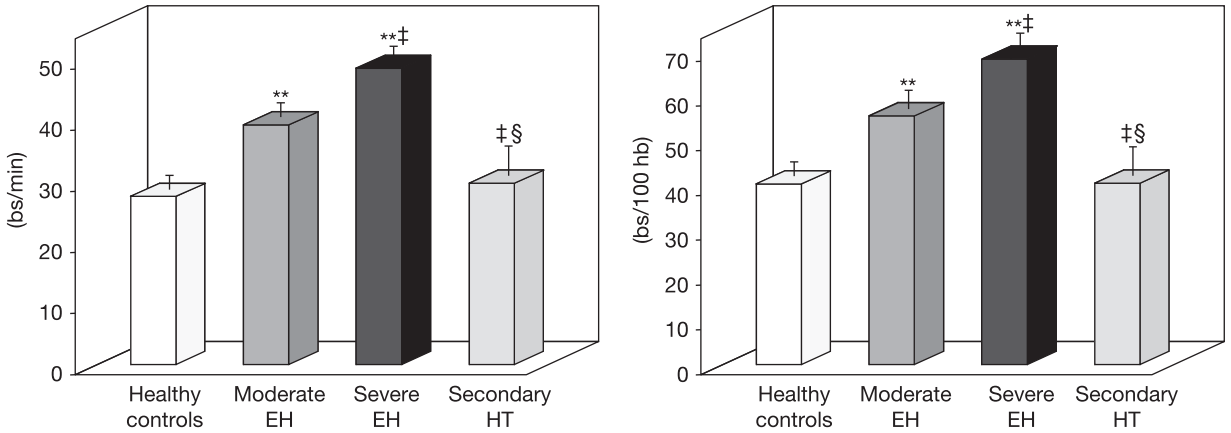

Fig. 1 Muscle sympathetic nerve activity (MSNA) expressed as burst incidence over time (*left panel*) or corrected for heart rate values (*right panel*) in healthy controls, moderate essential hypertensives, severe essential hypertensives and secondary hypertensive patients. Data are expressed as mean ± SEM. **P*, .05; ***P*, .01 versus controls; †*P*, .05; ‡*P*, .01 versus moderate EH; §*P*, .05 versus severe EH. Figure obtained from data of Grassi, G., Cattaneo, B.M., Seravalle, G., Lanfranchi, A. and Mancia, G. (1998). Baroreflex control of sympathetic nerve activity in essential and secondary hypertension. *Hypertension* **81**, 68–72.

See also: Adrenergic Mechanisms. Baroreceptor Responses. Hypertension; Overview. Novel Insights in ß-Adrenergic Receptor Signaling

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Renal Hypertension

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Abbreviations

CKD	Chronic kidney disease
DM	Diabetes mellitus
ECF	Extracellular fluid
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
GWAS	Genome-wide association studies
RAS	Renin–angiotensin system
THP	Uromodulin or Tamm–Horsfall glycoprotein
UMOD	Uromodulin gene

Introduction

Arterial hypertension and cardiovascular disease have been identified both as a cause and as a consequence of chronic kidney disease (CKD) (Anon, 1996; Mailloux and Levey, 1998; Walker, 1993). Even today hypertension is the major single more important risk factor for CKD (defined as glomerular filtration—GFR—rate less than 60 mL/min) (U.S. Renal Data System, USRDS, 2017).

Epidemiology

The specific aspect of epidemiology of CKD in relation to hypertension. At least 30% of adult individuals can be diagnosed as hypertensive (Kearney `*et al.*, 2005), also if classification criteria vary somehow according to differences in guidelines (Mancia *et al.*, 2013; Whelton *et al.*, 2017). Whatever the case, the prevalence of hypertension is higher among patients with CKD. This is of particular clinical relevance since CKD must be considered as part of the rising worldwide burden of noncommunicable disease as hypertension, diabetes mellitus (DM), and obesity. Moreover hypertension, DM, and obesity are strong risk factors for CKD itself. While the rate of hypertension control is discouraging, since less that 50% of hypertensives have controlled blood pressure (BP) with treatment (Pereira *et al.*, 2009; Crim *et al.*, 2012), the rate of hypertension increases with the severity of renal insufficiency (Coresh *et al.*, 2001). As GFR decreases, the prevalence of hypertension rises significantly (**Table 1**).

To conclude, CKD prevalence is relevant to hypertension because hypertension is extremely common in CKD patients. As GFR decreases the prevalence of hypertension rises significantly.

Hypertensive Nephrosclerosis

Also if the precise cause of hypertension is not clear for the majority of affected individuals, the idea that the kidney plays a role in hypertension dates back almost 200 years. Among the first who shed some light on the relationship between kidney and hypertension, in the 19th century, Richard Bright (Jay, 2000) found an association between abnormalities of urine composition (albuminuria), of urine production (dropsy) or kidney macroscopic appearance (contracted kidneys) with hard pulse and

Table 1 CKD stage definitions and prevalence of hypertension

	CKD stage[a]		*% HBP*
eGFR (mL/min/1.73 m^2)	≥90 normal and high	G0	23.3%
	≥90 normal and high + *markers*	G1	35.8%
	89–60 mild reduction	G2	48.1%
	60–30 moderate–severe reduction	G3	59.9%
	30–15 <15 severe reduction—kidney failure	G4–G5	84.1%

[a]CKD stage: A patient is said to have chronic kidney disease (CKD) if abnormalities of kidney function or structure are present for more than 3 months. The definition of CKD includes all individuals with markers of kidney damage or those with an eGFR of less than 60 mL/min/1.73 m^2 on at least two occasions 90 days apart (with or without markers of kidney damage).

According to one recent NHANES survey. Prevalence of hypertension also varies with the cause of CKD. Strong association with hypertension in presence of renal artery stenosis (93%), diabetic nephropathy (87%), and polycystic kidney disease (74%) (Crim *et al.*, 2012).

Markers of kidney disease may include albuminuria (ACR >3 mg/mmol), hematuria (or presumed or confirmed renal origin), electrolyte abnormalities due to tubular disorders, renal histological abnormalities, structural abnormalities detected by imaging (e.g., polycystic kidneys, reflux nephropathy) or a history of kidney transplantation.

CKD classification. CKD is classified based on the GFR and the level of proteinuria.

hypertrophied hearth as in the case of long lasting hypertension (Bright, 1836). Shortly after, besides confirming the association of increased arterial pulse tension, hypertrophy of the left ventricle with contracted, sclerotic kidneys, Toynbee and Johnson reported also that there was hypertrophy of the arterial walls and obstruction of systemic capillaries in the microdissections of the diseased kidneys (Toinbee, 1843; Johnson, 1868). In 1856, Ludwig Traube argued that cardiac hypertrophy in Bright's disease was due to increased pressure within the arterial system, renal disease being always the prime mover (Traube, 1856).

Only a century later the importance of the kidney in the pathogenesis of hypertension was unquestionably acknowledged, though leaving unresolved the question whether hypertension is cause or consequence of kidney disease. Indeed, the kidney is unique among the target organs of hypertension as it both suffers damage and contributes to its pathophysiology through several different pathways. The observation was synthetized in a seminal clinical paper by Chasis and Baldwin who pointed clearly in the sixties to the frequent association of hypertension and intrinsic renal diseases as well as to the relationship between hypertension and the occurrence of major renal artery disease and/or specific renal functional abnormalities as adrenal hyperplasia or chronic glomerulonephritis (Chasis and Baldwin, 1966).

Today hypertensive nephrosclerosis or glomerulosclerosis is considered a major cause of CKD or of end-stage renal disease (ESRD). Though more frequent in individuals of African origin, they are present in all ethnicities. While the terms themselves remain an easy escape that indicates our limited ability to classify the kind of renal insufficiency (potentially also an underlying neglected chronic glomerulonephritis complicated by hypertension), metabolic derangements, as obesity, oxidative stress, dyslipidemia, and atherosclerosis are often confounding factors as well as precipitating cofactors. Some sort of accelerated aging of the renal microvascular bed and glomerular sclerosis are a fairly common morphologic finding, with arteriolar intimal thickening (in more severe cases arteriosclerosis or hyalinosis), explaining the association hypertension (Glassock and Rule, 2016). Together with the vascular and glomerular, an at least equally severe tubulointerstitial damage occurs, with tubular atrophy and interstitial fibrosis due to loss of peritubular capillaries (Nangaku, 2006).

Pathophysiologic Considerations

A century after Bright's observation, Harry Goldblatt demonstrated that he could induce severe hypertension in dogs by obstructing one of the renal arteries (Goldblatt *et al.*, 1934), while the conclusive achievement was provided by Arthur C Guyton, demonstrating that the kidney affects BP regulating extracellular fluid volume, argued that chronic hypertension could not occur in the absence of impairment of renal handling of sodium. Guyton showed that any acute rise in BP resulted in brisk increase in renal sodium excretion and normalization of BP unless sodium excretion was hampered by renal ablation (e.g., as in the Goldblatt model), or by angiotensin or aldosterone infusion (Guyton *et al.*, 1980; Hall, 2003). Conversely when sodium excretion was hampered, BP increase was first mediated by extracellular fluid (ECF) volume expansion, slight reduction of total peripheral resistance, and reflex increase of cardiac output. Later ECF volume and cardiac output tended to normalize and high BP resulted from high peripheral resistance, substantially indistinguishable from high BP observed in essential hypertension (Guyton, 1990).

Though the exact nature of renal defect or defects responsible for inappropriate sodium excretion, or of factors that mediate the subsequent rise in peripheral resistance, remain unclear in essential hypertension, Guyton introduced the concept of pressure–natriuresis, meaning that when BP increases from any cause, renal perfusion pressure increases as well, with a resulting increase of sodium and water excretion. Based on the substantial capacity for a normal kidney to excrete sodium, this BP-tempering mechanism has sufficient gain to limit intravascular volume and thereby to lower BP. Guyton demonstrated that the system works in response to several tested experimental hypotheses as increased heart rate and peripheral resistance (Guyton, 1991). Guyton's model *imposes* that some change of the pressure–natriuresis response must happen to perpetuate a chronic elevation in intraarterial pressure in such a way that the equilibrium point for salt and water excretion is shifted to a higher level of arterial BP (Guyton, 1990). A rightward shift and less steep slope of the pressure–natriuresis function could account for the primary mechanism leading to Na/volume retention (**Fig. 1**).

While Guyton did most of his experimental observations on computer simulations and on nonhuman mammals, some indirect evidences on the primacy of the kidney in the pathogenesis of hypertension are available also for humans. At least two family history studies tell us that kidney transplant recipients of a kidney from a normotensive family need less antihypertensive treatment and have lower BP than the recipients of a kidney from a hypertensive family (Guidi *et al.*, 1996) or see their BP treated after transplantation (Curtis *et al.*, 1983) while one pathology study on victims of car accidents that were otherwise healthy besides their BP demonstrated that those with high BP had fewer nephrons at autopsy than those with normal BP (Keller *et al.*, 2003).

Severity of Renal Damage and Salt Sensitivity

While it is relatively easy to assign to the kidney a causal role in hypertension when gross morphological damage is evident, it is less intuitive to accept it when such damage is minimal or even absent. Experiments performed inducing hypertension with the "two-kidney, one-clip maneuver," where only one kidney is clipped and the other is left in site, untouched provide the best example for it. BP is mildly increased and often returns to normal with time. Hypertension is initiated by the activation of the renin–angiotensin system from the clipped, ischemic kidney and removal of the clip may rapidly normalize BP. However, if the clip is kept in place for enough time, some damage may have occurred in the unclipped kidney, as it has been perfused at high BP.

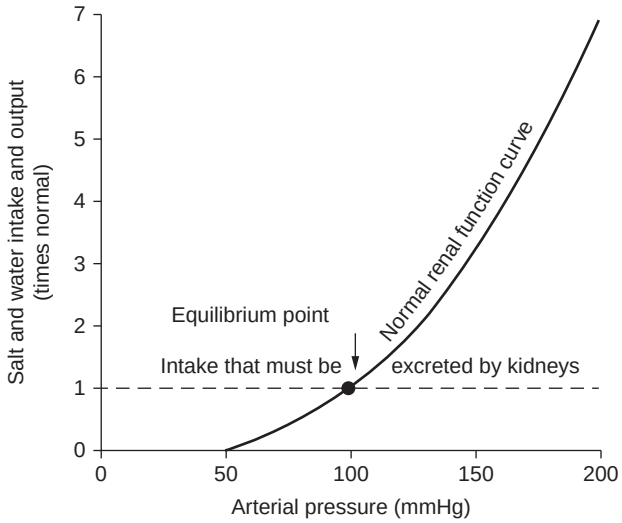

Fig. 1 Equilibration of the normal renal function curve with the salt and water intake. Point labeled "equilibrium point," where the curve crosses the line, gives pressure level at which kidney-fluid mechanism will control arterial pressure. From Guyton, A. C. (1990). The surprising kidney-fluid mechanism for pressure control—Its infinite gain! *Hypertension* **16**, 725–730.

On histological examination the lesions are very mild indeed, but are enough to cause a form of salt sensitive hypertension and similar to those obtained experimentally after angiotensin infusion (Johnson *et al.*, 2002).

Salt Intake, Kidney and Blood Pressure

This ability of the kidney to tolerate a sodium load declines with age, so that even small increases in salt intake may lead to a rise in BP at later ages.

Though short-term effects of change of salt intake on BP are evident in hypertensive and prehypertensive subjects, some still debate the long-term effects on stable BP and on cardiovascular prevention debated (Cappuccio, 2016; Graudal, 2016). However, not only general opinion but also current WHO recommendations propose a strong action for reducing salt intake to about half actual value (Beaglehole *et al.*, 2011), in order to reduce BP and cardiovascular and renal risk. The reason for the persistence of the debate against generalized measures of salt reductions can be found in the large interindividual differences in the response to salt restriction, as in **Fig. 2**. The figure reports an old, but crucial experiment that showed how large can be the heterogeneity of the response to such maneuver in individuals. In order to maintain optimal environmental homogeneity, healthy, normotensive free-living volunteers participated. The study investigated the effect on BP of the reduction of salt intake for 3 months from an average intake of sodium of 150 mmol/day (which is standard for a western diet) to less than 75, which approximates the level now proposed by WHO. Results indicated significant decrease in both systolic and diastolic BP, however with such a large inter-individual variability that at least 10% of the participants experienced BP increase and another 25% did not see substantial BP change (Miller *et al.*, 1987). The experiment tells us not only that BP can be reduced lowering salt intake also in normotensives, but that the response to reduced salt intake is largely heterogeneous. Indeed, lowering sodium intake has larger pressor effect on individuals of African ancestry, middle and older-aged subjects (Luft *et al.*, 1983) and those with already high BP values, with DM or with CKD. Notably, categories that share blunted RAS (He *et al.*, 2001).

The heterogeneity in BP response to change in salt intake is defined salt-sensitivity, classifying those that respond with BP change to change in salt intake or to Na load or depletion as salt-sensitive and those that do not despond as salt-resistant. The detection of salt-sensitivity has substantial clinical relevance, not only because salt-sensitivity is an important risk factor for cardiovascular and renal disease (Weinberger *et al.*, 2001), but also for the simple, intuitive factor that salt-sensitive hypertensives can reduce substantially their BP reducing salt intake.

Renal Sodium Handling Along the Nephron and Blood Pressure Regulation

Sodium homeostasis depends on the intrinsic capacity of the kidney to produce glomerular ultrafiltrate and on its ability to reabsorb salt at different levels along the nephron. Due to their complex regulation, the renal epithelial sodium channels (ENaC) are a major player of sodium balance. Located in the cortical collecting duct, they are under renin–angiotensin–aldosterone system control. The complex interplay of the system depends from the fact that Angiotensin II is produced as a response to decreased renal oxygen tension or decreased perfusion pressure. Angiotensin II production aims to bring back BP to its normal value, causing vasoconstriction, increasing sympathetic nerve activity and myocardial contractility. Angiotensin II also directly stimulates

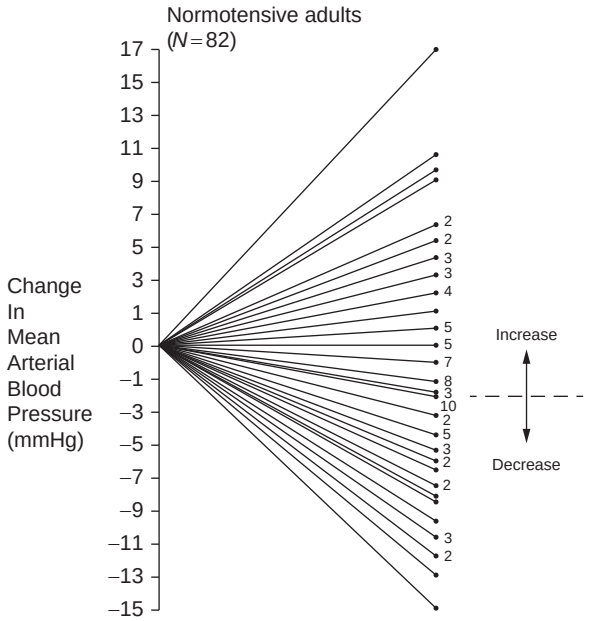

Fig. 2 Change in blood pressure with sodium restriction for 3 months from the average Caucasian diet of 150 mmoles Na day to 75 mmoles Na day in 82 adults. Displayed are the differences between the mean BP before staring diet and after and diet intervention for each individual. Overall BP decreased slightly but significant ($P < .01$) with sodium restriction. Older individuals were more likely to respond to sodium restriction. It is evident that the individual BP responses were heterogeneous, with increases in pressure observed in some subjects. Notably, sodium restriction in all normotensive adults *may not* be innocuous. From Miller, J. Z., Weinberger, M. H., Daugherty, S. A., Fineberg, N. S., Christian, J. C., Grim and C. E. (1987). Heterogeneity of blood pressure response to dietary sodium restriction in normotensive adults. *Journal of Chronic Disease* **40**, 245–250.

aldosterone production that induces the synthesis of ENaC subunits that assemble in channels in the plasma membrane or by increasing the probability that the channels are open permitting the passage (reabsorption) of sodium (Fuller and Young, 2005).

As renal sodium handling plays a crucial role in BP regulation, the systems involved in sodium handling and the underlying genetic mechanisms have been object of research for many years for understanding their roles in sodium homeostasis and salt sensitivity.

While there is no doubt that a positive salt balance plays a fundamental role in renal hypertension, other factors as the activation of the renin–angiotensin system (RAS) (Rassler, 2010), increased production of endothelin (Elijovich and Laffer, 2002), and endogenous digitalis-like substance (Blaustein *et al.*, 2009); reduced generation of vasodilators such as nitric oxide (Raij, 1999) and kinins (Mamenko *et al.*, 2014).

Family studies estimated heritability of salt-sensitive phenotype from moderate to high (Gu *et al.*, 2007; Svetkey *et al.*, 1996), with large difference between different studies, probably due at least in part to differences in ethnicities. Whatever the case, elucidation of the genetic contribution to BP salt sensitivity provides important information on how polymorphism in the genes controlling sodium reabsorption interact with sodium intake influencing individual BP value. Dissecting Mendelian forms of BP variation where mutations in single genes impart large effects on BP helped to identify fundamental pathways which could affect BP in humans (**Fig. 3**; Lifton *et al.*, 2001). While the figure summarizes only the diseases of Na reabsorption inherited in Mendelian forms (hypertensive disorders in *red* and hypotensive disorders in *blue*), a large number of proteins performing or controlling renal Na transport or other proteins controlling hormone synthesis or release affecting sodium transport became candidates for genomic research since they took part to the same physiological pathway. The list of candidate genes associate to hypertension is very long but provided however inconsistent results (Basson *et al.*, 2012; Singh *et al.*, 2016) except for the face that the vast majority of the positive findings was pointing to renal mechanisms.

The picture does not seem having been improved by genome-wide association studies (GWAS) (Padmanabhan *et al.*, 2017). In spite of a tremendous effort spent by large consortia involving tents of groups worldwide and typing thousands of individuals along the genome, also in this case, despite the increasing pace of discovery of variants associated with BP, CKD, and HTN, the utility of the discoveries seems limited, with the sole exception of the UMOD gene.

Uromodulin Rediscovery

Uromodulin or Tamm–Horsfall glycoprotein (THP) (Tamm and Horsfall, 1952) is a glycoprotein well known to nephrologists. It can be found in abundance in the urines of all mammals and is part of the matrix of renal stones. What seems important is that it is the most abundant protein excreted in urine, low urinary levels of uromodulin suggest predisposition to renal stone formation,

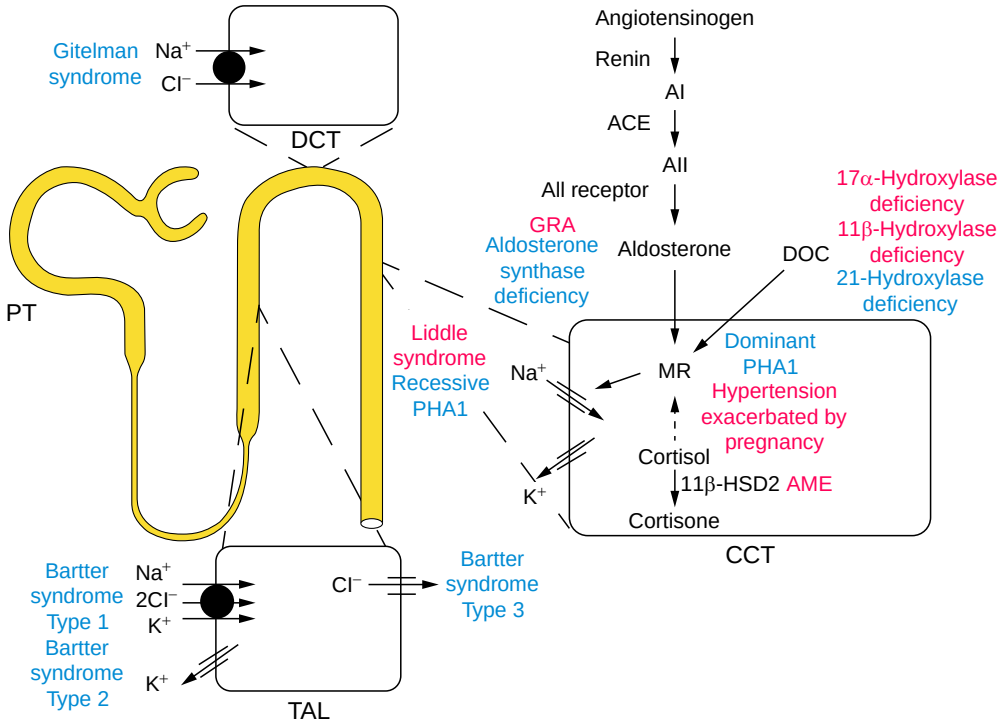

Fig. 3 Schematic representation of a nephron. The molecular pathways mediating NaCl reabsorption in individual renal cells in the thick ascending limb of the loop of Henle (TAL), distal convoluted tubule (DCT), and the cortical collecting tubule (CCT) are indicated, along with the pathway of the renin–angiotensin system, the major regulator of renal salt reabsorption. Inherited diseases affecting these pathways are indicated, with hypertensive disorders in *red* and hypotensive disorders in *blue*. *AI*, angiotensin I; *ACE*, angiotensin converting enzyme; *AII*, angiotensin II; *MR*, mineralocorticoid receptor; *GRA*, glucocorticoid-remediable aldosteronism; *PHA1*, pseudohypoaldosteronism, type-1; *AME*, apparent mineralocorticoid excess; *11 HSD2*, 11-hydroxysteroid dehydrogenase-2; *DOC*, deoxy-corticosterone; and *PT*, proximal tubule. From Lifton, R. P., Gharavi, A. G. and Geller, D. S. (2001). Molecular mechanisms of human hypertension. *Cell* **104**, 545–556.

uromodulin binds to *Escherichia coli* and seems to mitigate renal bacterial infections. It is encoded in humans by the UMOD gene. More importantly uromodulin regulates the activity of the sodium–potassium–chloride transporter (Mutig *et al.*, 2011) and of the renal outer medullary potassium channel (ROMK) (Renigunta *et al.*, 2011), the two main ion transporters involved in NaCl reabsorption by the thick ascending limb of Henle's loop, crucial for sodium reabsorption. In 2009 UMOD locus was associated to CKD (Köttgen *et al.*, 2009) and in 2010 to HTN (Padmanabhan *et al.*, 2010). Susceptibility variants detected in the UMOD gene are located in the promoter of the gene, have a high frequency in the general population and confer increased risk for CKD and for hypertension (between 15% and 20%). Transgenic mice overexpressing UMOD increase THP renal excretion, have BP higher and more sensitive to furosemide than controls (Trudu *et al.*, 2013), while UMOD knockout mice have low BP and are salt-resistant (Graham *et al.*, 2014). Interestingly, also humans carrying the risk allele in homozygous form were, like transgenic rodents more sensitive to furosemide (had prompt natriuretic response and reduced their BP more than the rest of the sample) (Trudu *et al.*, 2013).

See also: Salt-Sensitivity of Blood Pressure. Diuretics

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Endocrine Hypertension☆

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Glossary

Atherosclerosis A disease of the intima of the arterial wall characterized by smooth muscle migration and proliferation with extracellular lipid deposition.

Atrial natriuretic peptide A polypeptide synthesized, stored, and released by atrial myocytes in response to expended extracellular volume.

Leptin A hormone that helps regulate the energy balance, mainly secreted by adipocytes.

Microalbuminuria The ratio of albumin to creatinine of more than 30 μg/mg but less than 300 μg/mg that is detected by sensitive radioimmune assay but not by conventional urine dipstick.

Natriuresis The process of excreting sodium in the urine.

Plasminogen (tissue) activator A thrombolytic factor that activates plasminogen to plasmin.

Plasminogen activator inhibitor-1 A substance produced by visceral and omental fat cells that inhibits tissue plasminogen activator; elevated in persons with diabetes and cardiometabolic syndrome.

Von Hippel–Lindau disease Hemangiomas of the retina, cerebellum, brainstem, and spinal cord as well as pancreatic cysts, adenomas, and carcinomas; autosomal dominant inheritance, with 20% developing pheochromocytomas.

Introduction

Hypertension represents one the single most important preventable contributing factor to morbidity and mortality worldwide (James *et al.*, 2014). Multiple endocrine disorders present with HTN as the initial clinical sign and early recognition can lead to successful medical or surgical treatments that in some cases are curative (Young *et al.*, 2017). This article explores the most frequent endocrine causes of HTN. Understanding the relationships between different hormonal axis and their impact on regulation of blood pressure is important to the diagnosis and management of HTN.

Obesity-Induced Hypertension

Obesity is a major public health burden in the United States and more than 300,000 deaths each year are attributable to obesity or overweight (Flegal *et al.*, 2010). The relationship between obesity and HTN was first shown in the Framingham Heart Study where the prevalence of HTN with obesity accounted for over 60% of incident HTN (Kannel *et al.*, 1967). Furthermore, it has been demonstrated that the prevalence of HTN increases in relation to body mass index (BMI) (Brown *et al.*, 2000; Shihab *et al.*, 2012).

Potential mechanisms contributing to HTN in patients with obesity include inappropriate activation of the renin–angiotensin–aldosterone system (RAAS), increased sympathetic nervous system (SNS) activity, insulin resistance, impaired pressure natriuresis, structural changes in the kidneys that will eventually lead to chronic kidney disease (CKD) and further potentiate HTN (**Table 1**) (Demarco *et al.*, 2014; Hall *et al.*, 2015).

Obese individuals have been shown to have mild-to-moderate increase in circulating renin levels, angiotensinogen, angiotensin-converting enzyme (ACE), angiotensin II (Ang II), and aldosterone (Engeli and Sharma, 2001). The effects of obesity-induced RAAS activation was further demonstrated in obese individuals who lost 5% of their body weight, which resulted in decreased angiotensinogen, renin, ACE activity, and aldosterone levels (Engeli *et al.*, 2005). Despite sodium retention, volume expansion and HTN, the RAAS remains active in obesity-induced HTN.

Obesity is associated with activation of the SNS and with baroreflex dysfunction. SNS activity is increased in the setting of excess body weight, with obese individuals noted to have increased renal SNS activity compared to nonobese counterparts, as well as increased cardiac SNS activity in obese individuals with HTN compared to normotensive obese people (Rumantir *et al.*, 1999). Several factors have been suggested to promote obesity-associated HTN by activating the SNS, including hyperinsulinemia, hyperleptinemia, RAAS activation (via Ang II), baroreflex dysfunction, and obstructive sleep apnea (OSA) (Hall *et al.*, 2010; Lohmeier and Iliescu, 2013). Of note, OSA activates the SNS independently of obesity-related mechanisms (Narkiewicz *et al.*, 1998; Goodfriend and Calhoun, 2004). A study of both lean and obese hypertensives has demonstrated that increased SNS activity

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Table 1 Metabolic and cardiovascular risk factors associated with insulin resistance and/or visceral obesity

- Increased systolic and diastolic blood pressure
- Hyperinsulinemia/insulin resistance
- Endothelial dysfunction
- Low high-density lipoprotein cholesterol levels
- High triglyceride levels
- Increased apolipoprotein B levels
- Small, dense low-density lipoprotein levels
- Increased fibrinogen levels
- Increased plasminogen activator inhibitor-1 levels
- Hypercoagulability (increased PAI-1/plasminogen activator ratio)
- Increased blood viscosity
- Increased C-reactive protein and other inflammatory markers
- Microalbuminuria
- Absent nocturnal dipping of blood pressure and heart rate
- Increased uric acid levels
- Increased left ventricular hypertrophy
- Premature atherosclerosis (coronary artery disease and stroke)

may be an important factor in the development and maintenance of HTN in obesity, by showing that blood pressure is more sensitive to alpha- and beta-adrenergic blockade in obese than in lean hypertensive patients (Wofford *et al.*, 2001).

Insulin resistance and compensatory hyperinsulinemia are present in the majority of hypertensive individuals, and constitutes a common pathophysiologic feature linking obesity, glucose intolerance, and HTN (Modan *et al.*, 1985). Additionally, when hypertensive patients, whether obese or of normal body weight, are compared with age- and weight-matched normotensive control subjects, a heightened plasma insulin response to a glucose challenge is consistently found (DeFronzo and Ferrannini, 1991). Hyperinsulinemia may not be a major cause of obesity-induced HTN; however, insulin resistance contributes to HTN by other mechanisms including inflammation, oxidative stress resulting from abnormal glucose and lipid metabolism, activation of the SNS, renal sodium retention, altered membrane cation transport, vascular smooth muscle growth, remodeling, and vasoconstriction (McFarlane *et al.*, 2001; Demarco *et al.*, 2014).

The role of visceral adiposity and ectopic accumulation of fat in the kidney in obesity has been discussed previously. This is associated with increases in intrarenal pressure, impaired pressure natriuresis, in addition to renal sodium retention and volume expansion (Hall *et al.*, 2014). Increased tubular sodium reabsorption is closely linked to activation of the SNS and RAAS, and possible changes in intrarenal physical forces due to medullary compression by excess visceral adiposity. Obesity causes renal injury and functional nephron loss, contributing to elevated blood, further renal injury and therefore initiating a vicious cycle of further pressure increase and renal tissue damage (Aneja *et al.*, 2004).

Of interest is the progression to a chronic hypertensive state in obese individuals that appears to be preceded by a loss of nocturnal blood pressure dipping in the absence of elevated daytime blood pressure (Lurbe *et al.*, 2008; Demarco *et al.*, 2013). Although, the exact mechanisms for this nondipping pattern of blood pressure remain to be fully clarified, it has been proposed that insulin resistance, autonomic nervous system dysfunction, increased SNS activity, and increased inflammation may be contributing factors (Demarco *et al.*, 2013; Dangardt *et al.*, 2011).

The pathophysiology of obesity-related HTN remains complex, with continuous accumulation of hormonal, environmental, and genetic factors that contribute to its pathogenesis. As the prevalence of obesity continues to increase, the effect of obesity on HTN and related cardiovascular, renal, and metabolic disorders is becoming ever more important.

Diabetes Mellitus

HTN is present in more than half the patients diagnosed with diabetes mellitus (DM), regardless of type, and contributes significantly to both microvascular and macrovascular disease (Sowers *et al.*, 2001, 2011; Sowers, 2013; Stamler *et al.*, 1993). The risk for cardiovascular disease (CVD) is fourfold higher in patients with both DM and HTN compared with the normotensive nondiabetic controls (Stamler *et al.*, 1993; Hu *et al.*, 2007). Recent studies conducted in the Framingham population cohort with DM (Kivimäki *et al.*, 2010) indicate that the presence of HTN in these participants was a resulting risk factor for CVD (Chen *et al.*, 2011).

Features of HTN in diabetic patients include increased sensitivity to salt and volume expansion; accelerated atherosclerosis, which lead to premature increased stiffness of larger arteries and results in isolated systolic HTN at a relatively younger age; and loss of nocturnal dips in blood pressure and heart rate (Sowers, 2013; Kario, 2007). All of these characteristics are associated with

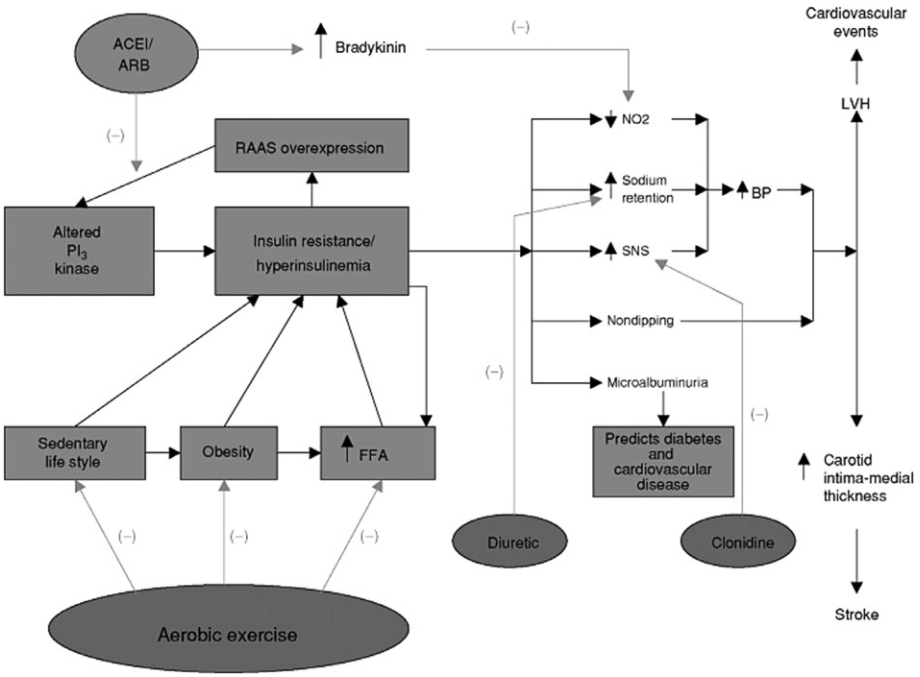

Fig. 1 Insulin resistance and cardiovascular disease. RAS, renin–angiotensin–aldosterone system; FFA, free fatty acids; BP, blood pressure; SNS, sympathetic nervous system; LVH, left ventricular hypertrophy; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; NO2, nitric oxide. Minus sign (–) indicates inhibition of pathological process.

microalbuminuria in type 2 diabetics (**Fig. 1**). Furthermore, DM and HTN share several pathophysiologic mechanisms, including inappropriate RAAs activation, excessive production of reactive oxygen species (ROS), inflammation, impaired insulin-mediated vasodilatation, increased sympathetic nervous system (SNS) activation, dysfunctional innate and adaptive immune responses, and abnormal renal processing of sodium (Sowers *et al.*, 2011; Sowers, 2013; Lastra *et al.*, 2014).

Appropriate control of blood pressure in patients with DM is important for preventing and delaying both microvascular and macrovascular complications (UK Prospective Diabetes Study Group, 1998; Hansson *et al.*, 1998). Data from landmark trials such as the United Kingdom Prospective Diabetes Study (UKPDS), Hypertension Optimal Treatment (HOT), Systolic Hypertension in the Elderly (SHEP), Systolic Hypertension in Europe (Syst-Eur), and The Appropriate Blood Pressure Control in Diabetes (ABCD) demonstrated that strict blood pressure control is beneficial in hypertensive patients with DM (Hansson *et al.*, 1998; Tuomilehto *et al.*, 1999; Curb *et al.*, 1996; Schrier *et al.*, 1996; Cushman *et al.*, 2010). Overall, these studies showed significant benefits of blood pressure management, including reductions in macrovascular diseases, including MI, sudden death, stroke, and peripheral vascular disease. Although specific targets for blood pressure are still controversial, control of HTN in the setting of DM is strongly supported by current evidence showing the critical impact that blood pressure has on CVD in diabetic individuals.

Primary Hyperaldosteronism

The incidence of primary hyperaldosteronism (PA) in individuals with HTN is 5%–10% (Mulatero *et al.*, 2004; Rossi *et al.*, 2006) and it reaches up to 20% in resistant HTN (Calhoun *et al.*, 2002; Eide *et al.*, 2004). Early diagnosis results in reduced cardiovascular morbidity and mortality. Aldosterone causes cardiac and vascular fibrosis in animals, an action prevented by mineralocorticoid receptor (MR) blockade (Lastra *et al.*, 2008; Stas *et al.*, 2007). Aldosterone antagonists improve endothelial dysfunction and decrease hospitalization, symptoms, and mortality from progressive CHF and other heart-related deaths. Eplerenone, a selective MR blocker, displays similar results and has fewer incidence of side effects, in particular related to antiandrogenic actions.

The mechanism of HTN can be attributed to excessive mineralocorticoid action, which leads to an increase in the number of epithelial sodium channels (ENaC) that reabsorb sodium in the collecting duct of the kidney. HTN is caused by sodium and water retention by the kidney, which leads to expansion of the extracellular fluid compartment. Direct effects of aldosterone on the central nervous system may contribute to HTN as well (Leenen *et al.*, 2010).

Screening for PA should be considered in all patients with refractory or severe HTN, with spontaneous hypokalemia or diuretic provoked low serum potassium levels requiring excessive potassium supplements. Importantly, approximately 61%–70% of all PA cases have normal potassium. Therefore, utilizing the association of severe HTN and hypokalemia as an indication to work up patients for PA is a poor screening method.

The ratio of aldosterone to plasma–renin activity (A/R) is a good screening test (Funder *et al.*, 2016), but may be limited due to confounding factors including: posture, dietary sodium intake, plasma potassium level, and medication. False-positive results with A/R ratio may occur in cases of renal impairment due to suppression by sodium retention, and aldosterone may be increased by the associated hyperkalemia (McKenna *et al.*, 1991). Beta-blockers, methyldopa, and clonidine may suppress renin and give false-positive results. Drugs that stimulate renin, such as diuretics, ACEIs, and dihydropyridine calcium channel blockers, falsely lower the A/R. False-negative results may occur with severe sodium restriction, pregnancy, and malignant HTN due to excessive renin, and may also occur in patients with hypokalemia, which should always be corrected prior to biochemical testing for PA (Stowasser *et al.*, 2010). Verapamil and hydralazine have little effect, whereas prazosin has no effect on A/R. Although there are no golden standard test recommended, the diagnosis should be confirmed by a high 24-h urinary aldosterone on a high'sodium diet, acute saline loading, captopril challenge, or fludrocortisone suppression (Funder *et al.*, 2008).

Once the diagnosis is established, it is necessary to distinguish between a unilateral and bilateral adenoma. Computed topography (CT) of the adrenals is the initial imaging modality and, if surgical intervention is desired, it should be followed by adrenal venous sampling for localization (Funder *et al.*, 2016). A solitary tumor greater than 1 cm in a young patient is consistent with unilateral aldosterone producing adenoma and usually constitutes an indication for surgery. A nondiagnostic CT or an adrenal mass less than 1 cm requires bilateral adrenal vein sampling to demonstrate and lateralize an adenoma, especially because CT scans miss 50% of adenomas and can incorrectly lateralize the mass. Lateralization based on an elevated aldosterone/cortisol ratio associated with contralateral suppression is more sensitive and specific than imaging and is an indication for unilateral adrenalectomy. For nonlateralization, or if sampling is not available, spironolactone is the treatment of choice.

Of the enzyme abnormalities that cause congenital adrenal hyperplasia (CAH), the two that are more frequently associated to HTN are the deficiencies of 11β-hydroxylase (CYP11B1) and 17α-hydroxylase (CYP17A1).

11β-Hydroxylase Deficiency(S)

Approximately 5%–8% of CAH cases are due to CYP11B1 deficiency (Merke and Bornstein, 2005). In cases of CYP11B1 deficiency, production of both cortisol and corticosterone is inhibited, and enhanced adrenocorticotropic hormone (ACTH) secretion further increases the production of cortisol precursors, in particular deoxycorticosterone (DOC) and 11-deoxycortisol (S), as well as androgens. Plasma renin activity (PRA) and aldosterone are both suppressed. Hypokalemia leading to muscle weakness and cramping is seen in a minority of patients. The severe homozygous classic form is common in Jewish populations (mostly Sephardic) from Morocco (White *et al.*, 1991), and at least 40 mutations have been identified with varying degrees of severity (New *et al.*, 2005). Signs of androgen excess are demonstrated in both sexes, and female infants are typically masculinized. Accelerated skeletal growth and short stature with acne and premature adrenarche are usually clinically manifest. Most patients have mild-to-moderate HTN. Diagnosis is established by demonstrating an increase in basal and/or ACTH-stimulated serum DOC, S, and adrenal androgens or elevated levels of urinary metabolites, tetrahydro-DOC and tetrahydro-S, and 17-ketosteroids in a 24-h urine collection. Steroid replacement suppresses ACTH levels, subsequently lowering both mineralocorticoid and adrenal androgen production. Potassium-sparing diuretics, such as spironolactone and amiloride, may be needed to control persistent HTN.

17α-Hydroxylase Deficiency

Delayed puberty or lack of development of secondary sexual characteristics or primary amenorrhea differentiates CYP17A1 deficiency from CYP11B1 deficiency, which might not be apparent until the expected onset of puberty. Prepubertal females appear to be normal but may later fail to develop expected normal sexual characteristics. Genetic males are phenotypically female, with a blind-ending vagina, inguinal testes, or undescended testes, or they may present with genital ambiguity. Although cortisol is not produced, adrenal insufficiency does not develop because corticosterone, which has glucocorticoid activity, is still produced in sufficient amounts. DOC is produced in excess, resulting in low-renin HTN and hypokalemia. Goals of treatment are to minimize mineralocorticoid excess, and can be achieved with the use of glucocorticoids to suppress ACTH stimulation, or spironolactone (Peter *et al.*, 1993; Mantero *et al.*, 1995).

Glucocorticoid-Remediable Aldosteronism

Glucocorticoid-remediable aldosteronism (GRA), also called dexamethasone-suppressible hyperaldosteronism or familial hyperaldosteronism type 1, was first described by Sutherland and colleagues in 1966 (Halperin and Dluhy, 2011). Inherited in an autosomal dominant fashion, GRA results from a chimeric gene fusion, where the 5′-promotor region of the 11b-hydroxylase gene (regulated by ACTH) fuses with the coding sequences of the aldosterone synthase gene. Consequently, the aldosterone production is abnormally under the control of ACTH (Lifton *et al.*, 1992a,b). This results in aldosteronism with diurnal elevations of aldosterone and responds to lowering ACTH with glucocorticoids (Vaidya and Dluhy, 2000). GRA is a mineralocorticoid excess condition, characterized by low PRA with an A/R ratio greater than 30, HTN, and spontaneous hypokalemia. Since blood pressure elevation ranges from moderate to severe and patients have normal plasma potassium levels, GRA can be mistaken as essential

HTN. However, although seldom seen GRA can be associated with an increased prevalence of early, and often fatal, cerebrovascular hemorrhage.

The diagnosis is supported by improved blood pressure following a dexamethasone suppression testing (DST), which also shows a decrease in aldosterone to nearly undetectable levels, thus demonstrating the dependency of this condition on production of ACTH. A plasma aldosterone of less than 4 ng/dL post-DST is considered sensitive and specific for diagnosis. Although suppression can also occur with aldosterone-producing adenomas (APAs) and bilateral adrenal hyperplasia (BAH), aldosterone usually falls to a lesser degree in these conditions. Furthermore, elevated 18-hydroxy and 18-oxy cortisol in the urine of patients with GRA will help to distinguish it from APA and BAH. However, sometimes these urinary metabolites are also elevated with APA. Sustained ACTH stimulation may cause an initial rise in aldosterone followed by a decline in normal persons and patients with APA and BAH, whereas in GRA the rise in aldosterone is sustained, similar to that seen with CAH. However, in contrast to CAH, patients with GRA show a normal cortisol and 17-hydroxy urinary corticosteroid response to ACTH. Since the biochemical tests used for diagnosis are subject to numerous pitfalls, genetic testing is essential for confirmation of GRA.

Although glucocorticoid suppression is the treatment of choice, normalization of blood pressure might not always occur. Linear growth should be monitored in children, and manifestations of Cushing syndrome should be avoided. Furthermore, hypoaldosteronism may occur with hypotension. Spironolactone is also effective as monotherapy; amiloride and triamterene may be used as alternatives.

Liddle Syndrome

In 1963, G.W. Liddle described a syndrome of HTN, increased potassium excretion, metabolic alkalosis, decreased sodium excretion, and suppressed plasma aldosterone concentrations (Botero-Velez *et al.*, 1994). The mechanism is related to an abnormal increase in the function of the sodium channel located in the collecting tubule, also known as the epithelial sodium channel (ENaC) or the amiloride-sensitive sodium channel. The disorder is inherited in an autosomal dominant pattern and it is the result of a gain of function mutation on chromosome 16p12 that encode the beta and gamma subunits of the ENaC, also called SCNN1B and SCNN1G (Devonald and Karet, 2004; Hansson *et al.*, 1995; Snyder *et al.*, 1995). The defective ENaC cannot bind an intracellular ubiquitin protein ligase (Nedd4) that normally removes them from the cell surface (Goulet *et al.*, 1998; Abriel *et al.*, 1999), which explains why patients do not respond to aldosterone antagonists.

HTN with hypokalemia and lack of response to spironolactone is usually a giveaway for the diagnosis. Triamterene, amiloride along with salt restriction can correct hypokalemia and HTN (Young *et al.*, 2017). Renin is suppressed by a volume-expanded state with atrophy of the juxtaglomerular apparatus. It has been observed that HTN, hypokalemia, and metabolic alkalosis can be ameliorated by renal transplantation. The absence of edema may be attributed to aldosterone escape due to suppressed sodium reabsorption in other parts of the nephron and to enhanced release of atrial natriuretic peptide (ANP) in response to volume expansion. Thus, this is a salt-retaining, volume-expanded, genetic form of HTN (Botero-Velez *et al.*, 1994).

Pseudohypoaldosteronism Type 2 (Gordon Syndrome)

In 1970, R.D. Gordon described a congenital renal tubule defect that includes HTN and severe hyperkalemia, in association with suppressed renin and aldosterone, which was completely reversed with dietary sodium restriction (Gordon *et al.*, 1970). This syndrome is characterized by hyperkalemia despite normal renal glomerular filtration and HTN with variable findings of hyperchloremia, metabolic acidosis, and suppressed plasma renin (Mansfield *et al.*, 1997). The underlying genetic abnormalities of Type 2 pseudohypoaldosteronism are due to gain-of-function mutation in the serine-threonine kinases WNK1 and WNK4, which localize to the distal nephron enhancing sodium chloride reabsorption (Wilson *et al.*, 2001; Choate *et al.*, 2003; Kahle *et al.*, 2003). Additionally, recently identified mutations in Type 2 pseudohypoaldosteronism patients include Kelch-like 3 (KLHL3) and Cullin 3 (CUL3) genes, which are components of Cullin/RING E3 ligase complexes (CRLs) that ubiquitinate substrates bound to Kelch propeller domains, that localize in the distal convoluted tubule of the nephron, suggesting a mechanistic link between *KLHL3*/*CUL3* mutations, increased Na–Cl reabsorption, and HTN (Boyden *et al.*, 2012). Thiazide diuretics correct the physiologic abnormalities associated with Type 2 pseudo hyperaldosteronism, suggesting that increased Na–Cl cotransporter activity is likely to be the common pathogenic mechanism (Boyden *et al.*, 2012).

Syndrome of Apparent Mineralocorticoid Excess

The syndrome of apparent mineralocorticoid excess (AME), is an autosomal recessive form of low-renin HTN that classically presents in infancy and childhood with hypernatremia, hypokalemia, metabolic alkalosis, and low-aldosterone. In some cases, patients also present with diabetes insipidus, hypercalciuria, nephrocalcinosis, and chronic kidney disease (Morineau *et al.*, 2006; Bockenhauer and Bichet, 2013). The first patient was identified in the early 1970s, and by the late 1970s a handful of patients with similar features of AME were identified and their clinical presentation was termed "apparent mineralocorticoid excess" (Werder *et al.*, 1974; New *et al.*, 1977; Ulick *et al.*, 1979). Cortisol is converted to its metabolite, cortisone, which has no mineralocorticoid

activity, by a reversible kidney enzyme, type 2 11β-hydroxycorticosteroid dehydrogenase (11βHSD2). Once 11βHSD2 was identified, investigators found mutations in the encoding gene, HSD11b2, and these were identified in patients with AME (Mune *et al.*, 1995; Wilson *et al.*, 1995; Dave-Sharma *et al.*, 1998; Morineau *et al.*, 2006; Alzahrani *et al.*, 2014). 11βHSD2 is a gate-keeping enzyme that protects the mineralocorticoid receptor (MR) from activation by glucocorticoids. A decreased activity of 11βHSD2 caused by genetic mutations or inhibition, by agents such as glycyrrhizic acid from licorice root and its semisynthetic analogue carbenoxolone, can induce HTN mediated by the excess activation of the MR by cortisol, which results in sodium and fluid retention (via over-activation of epithelial sodium channel in the distal nephron), hypokalemia, metabolic alkalosis, low renin, and low aldosterone. The diagnosis of AME is based on measuring the ratio of urinary free cortisol to cortisone and their respective metabolites tetrahydrocortisol, allotetrahydrocortisol, and tetrahydrocortisone (Morineau *et al.*, 2006; Antonelli *et al.*, 2014). Treatment of AME is directed toward reducing endogenous cortisol production with dexamethasone or blockade of mineralocorticoid effects by inhibiting the MR via spironolactone or eplerenone or inhibition of ENaC with amiloride (Morineau *et al.*, 2006; Alzahrani *et al.*, 2014). There are reports that kidney transplantation may be curative (Palermo *et al.*, 1998; Khattab *et al.*, 2014).

Cushing Syndrome and Hypertension

In Cushing syndrome 75%–80% of patients present with HTN (Sacerdote *et al.*, 2005; Baid and Nieman, 2004). The pathogenesis of glucocorticoid induced HTN is complex and involves multiple mechanisms including intrinsic mineralocorticoid activity, activation of the RAAS, enhancement of cardiovascular inotropic and pressor activity of vasoactive substances (e.g., catecholamines and or/vasopressin and angiotensin II) and suppression of the vasodilatory systems (NO synthase, prostacyclin, and kinine/kallikrein systems). These phenomena ultimately result in upregulating cardiac output, peripheral vascular resistance, and plasma volume (Magiakou *et al.*, 2006). Although cortisol and aldosterone have equal binding affinity to the MR, 11β-HSD, by locally transforming cortisol to cortisone, prevents it from binding to the mineralocorticoid receptor in the nephron-collecting tubule. Excess cortisol may overwhelm this enzyme and bind to MR (Ulick *et al.*, 1992; Walker *et al.*, 1991). Indeed, aldosterone and renin levels are low in Cushing's HTN, suggesting an apparent mineralocorticoid overactivity that causes sodium and volume retention. Despite this, hypokalemia, metabolic alkalosis, and edema are more often associated with ectopic secretion of ACTH, due to its very high levels. Elevated levels of corticosterone and deoxycorticosterone associated with ectopic ACTH production may also contribute. Since ANP and decreased sodium reabsorption occur with volume expansion, thereby causing aldosterone (in this case mineralocorticoid) escape, the amount of sodium being exchanged is increased. This may explain the presence of normal potassium levels and the lack of edema seen with Cushing syndrome. Nonetheless, plasma volume is expanded with salt intake, although not to the point of causing edema.

Screening for Cushing's starts with a careful history taking and appropriate physical exam in the search for evidence of clinical features. A biochemical profile should be established by initial screening with at least two positive tests, such as measurement of 24-h urinary free cortisol, overnight low dose (1 mg) dexamethasone suppression test or measuring late night salivary cortisol (Nieman *et al.*, 2008). After identifying clinical and biochemical evidence of Cushing syndrome, it is imperative to determine its dependence on ACTH before further investigations with imaging for localization. ACTH-dependent Cushing syndrome represents about 80% and consists of Cushing disease (CD)—the most common—a pituitary corticotroph secreting adenoma, and extrapituitary or ectopic tumors secreting ACTH comprised of occult or neuroendocrine tumors (Newell-Price *et al.*, 2006; Biller *et al.*, 2008). ACTH-independent—about 20% of cases of Cushing's, include primary unilateral adrenal adenomas or carcinomas that suppresses ACTH and rarely primary bilateral macronodular adrenal hyperplasia or primary pigmented nodular adrenocortical disease (Lacroix, 2009).

Pituitary MRI and confirmation with inferior petrosal sinus sampling (IPSS) is indicated in CD. Adrenal tumors producing cortisol could be evaluated with adrenal imaging and confirmed with adrenal vein sampling.

Treatment of the HTN should be directed at the primary source of excess cortisol: ectopic/pituitary ACTH, adrenal, or exogenous. Calcium channel blockers or drugs that improve endothelial NO production, such as ACEIs and ARBs, may be used until a final diagnosis is reached.

Secondary Hyperaldosteronism and Renovascular Hypertension

Secondary hyperaldosteronism may be an appropriate physiological response to hypovolemia, renal ischemia, or low perfusion state in context of liver or heart failure the latter not being associated with HTN. However, renovascular HTN (RVH) in context of renal artery stenosis represent a noncompensatory and detrimental pathophysiological process where systemic blood pressure raises in response to renal hypoperfusion. A vast majority is attributed to atherosclerosis which increases with age but a small percentage is related to fibromuscular dysplasia, particularly young or middle-aged women. Although 14%–33% of patients subjected to angiograms have renal artery stenosis, only a 1%–5% of patients with HTN have RVH.

Clinical features of RVH due to occlusive pathology varies from malignant HTN to flash pulmonary edema to acute reversible or permanent kidney injury. Particularly important for clinicians is HTN before age 30, malignant HTN, increase creatinine more

than 30% after treatment with ACEI or ARB, unexplained pulmonary edema or asymmetric kidney on imaging with sudden loss of function.

Once suspected, duplex ultrasound is an inexpensive noninvasive diagnostic tool. Other diagnostic modalities include magnetic resonance imaging (MRI), magnetic resonance angiography (MRA), computed tomography (CT), computed tomography–angiography, scintigraphy, and captopril-enhanced renography or scintigraphy. Selective use of renal MRA can avoid invasive, potentially nephrotoxic conventional angiography in high-risk patients. Biochemical evidence of secondary aldosterone excess including plasma aldosterone and plasma renin activity lack specificity, however, renal vein renin sampling which allows localization of the culprit has been helpful in predicting effectiveness of revascularization or nephrectomy (Goupil *et al.*, 2015). Randomized controlled trials show limited benefits in revascularization for atherosclerotic renal artery stenosis (Herrmann *et al.*, 2015), but each case should be evaluated and treated in a personalized fashion depending on the clinical scenario. Gill and colleagues reported improved blood pressure in a majority of resistant cases with recovery of renal function post stenting (Gill and Fowler, 2003). Ischemic nephropathy is best treated before the onset of renal failure. Revascularization with surgery or stenting should be considered with creatinine less than 2 mg/dL, bilateral renal artery stenosis, absence of proteinuria, and/or evidence of end-organ injury.

Severe HTN with secondary hyperaldosteronism can occur in patients with systemic sclerosis due to renal artery vasospasm and subsequent hyperreninemia. Nifedipine has led to normalization of BP, renin, and aldosterone. Secondary hyperaldosteronism and HTN from renin-secreting juxtaglomerular cell tumors can present in young adults. Surgical treatment results in prompt normalization of BP, and a calcium channel blockade may control blood pressure until surgery. Renal cell carcinoma rarely causes hyperreninemia or secondary hyperaldosteronism.

Pheochromocytoma

Pheochromocytoma and paraganglioma (PPGL) are tumors derived from chromaffin cell of the adrenal medulla or paravertebral ganglia of the sympathetic chain. Paroxysmal HTN is the most common sign, present in 95% of cases due to excess catecholamines and is the result of increased peripheral resistance (Calhoun *et al.*, 2008). Norepinephrine activates α_1 receptors found on peripheral smooth muscle arteries and veins, producing vasoconstriction with an increase in systemic pressure, and has a positive inotropic effect. Norepinephrine acts as a negative feedback via α_2 receptors which are located on the presynaptic terminal of postganglionic sympathetic neurons by inhibiting further release of norepinephrine. β_1 receptor is stimulated by both epinephrine and norepinephrine with positive inotropic and chronotropic effect on cardiomyocytes and heart pacemaker respectively; it also stimulates renin production and increases blood pressure via RAAS. Epinephrine acts on β_2 receptor inducing vasodilatation of muscular arteries and incites the release of norepinephrine from the sympathetic ganglia (Manger and Gifford, 1996). High levels of dopamine can activate α and β_1 receptors.

PPGL can be life threatening and thus early recognition is critical. HTN can present with paroxysmal episodes or sustained, the prevalence of PPGL in patients with sustained HTN is 20–60 per 10,000 persons (Manger, 2006; Mansmann *et al.*, 2004). A crisis can be associated with signs and symptoms of myocardial infarction, supraventricular tachycardia, or CHF. Sudden elevations in arterial pressure do not correlate with the levels of plasma catecholamines. Even in patients with persistent elevations of BP, symptoms may be paroxysmal; the classic symptoms include headache, diaphoresis, and palpitations that can last from minutes to an hour. Other symptoms may include pallor, nausea, tremor, anxiety, abdominal pain, chest pain, dyspnea, and (rarely) flushing. Frequently, an orthostatic drop in blood pressure is observed because of relative intravascular volume depletion. A patient may be normotensive between episodes. Sometimes, paroxysms may be absent, mimicking essential HTN. Approximately 10% are bilateral, extra-adrenal (paragangliomas), familial, multiple (other than bilateral), or malignant.

Diagnosing PPGL requires a high clinical index of suspicion, biochemical profile is necessary to confirm the diagnosis: adrenal pheochromocytomas primarily make epinephrine or a combination of norepinephrine and epinephrine, whereas extra-adrenal PPGL produce norepinephrine predominantly and small amounts of dopamine due to lack of the enzyme phenylethanolamine-*N*-methyl transferase (PNMT) required in norepinephrine metabolism. Of critical importance is the technique for blood sample collection when measuring plasma free metanephrines. To avoid false positive results, the patient should rest in supine position for at least 30 min after the insertion of the indwelling cannula. If not feasible, a 24-h urine collection for fractioned metanephrines can be used.

The urine fractionated metanephrines quantified by mass spectrometry yields a 97% sensitivity and a 91% specificity (Perry *et al.*, 2007). Free plasma metanephrines alone have a specificity of 96% and a sensitivity of 97% for the familial form of PPGL. Although the sensitivity remains high for the sporadic form, the specificity drops to 82%. Combining plasma metanephrines with urinary metanephrines increases the specificity to 89%. Intermediate values require further testing. A clonidine suppression test can be used to distinguish true-positive from false-positive borderline patients when measuring normetanephrine with a low false-negative rate (Eisenhofer *et al.*, 2003).

Once biochemical diagnosis is established, next step is imaging and localization. According to 2014 Endocrine Society guidelines, CT is the first-choice imaging modality with an 88%–100% sensitivity (Lenders *et al.*, 2014). Important features include large size (>3 cm), more than 10 Hounsfield Units, $<50\%$ washout at 10-min, heterogeneous appearance with areas of necrosis with some calcifications, solid, or cystic regions. A T2-weighted image of a PCC has a characteristically hyperintense image in comparison with that of the liver. MRI is preferred for skull base and neck PPLG with sensitivity between 90% and 95% (Gimenez-

Roqueplo *et al.*, 2013). Scintigraphy with ^{123}I-metaiodobenzylguanidine (MIBG) is used in metastatic disease as a functional imaging prior to radiotherapy treatment with ^{131}I-MIBG. 18F-FDG PET/CT scanning is recommended in patients with metastatic PPGLs and is preferred over ^{123}I-MIBG scintigraphy (Lenders *et al.*, 2014).

Treatment involves avoidance of hypertensive episodes during surgery and postsurgery. The nonselective alpha blocker phenoxybenzamine and a high sodium di*et al*lows for volume repletion, but is associated with significant reflex tachycardia and orthostasis and may prolong postsurgical hypotension. After volume repletion, selective alpha$_1$ blockers such as prazosin have a shorter duration of action and do not cause reflex tachycardia. Alpha$_1$ blockers can still be associated with postoperative hypotension as well as mask the hypotension associated with the removal of the tumor. The latter is used by surgeons as an indication of complete tumor removal. Boutros and colleagues suggest use of alpha blockers and CCBs, as CCBs seldom cause orthostasis or hypotension and are safe to use in normotensive patients. They also prevent catecholamine-induced spasm of the coronary arteries (Boutros *et al.*, 1990). By decreasing the norepinephrine-induced increases of calcium entry into the VSMCs, CCBs can prevent the hypertensive response in PPGL. Beta-blockers should be used only after alpha blockade. An acute hypertensive crisis can be treated by sodium nitroprusside and a beta$_1$-selective blocker can control supraventricular or reflex tachycardia. Esmolol is preferred for its rapid onset and short duration. Definitive treatment is surgical and laparoscopic approach is preferred (Pacak, 2007).

Primary Hyperparathyroidism

Primary hyperparathyroidism is the most common cause of hypercalcemia and has been associated with an increase frequency of HTN (Richards *et al.*, 1988). However, the exact etiology of how hyperparathyroidism-induced HTN remains unclear as there is no direct correlation with elevated parathyroid hormone or calcium levels. Furthermore, this is compounded by inconsistencies in studies where patients with primary hyperparathyroidism and HTN who underwent parathyroidectomy showed decreased, increased, or no change in blood pressure (Heyliger *et al.*, 2009; Rydberg *et al.*, 2010; Richards *et al.*, 1988). Although the relationship between hyperparathyroidism and HTN remain unclear, recent studies have shown that mild hyperparathyroidism may cause endothelial dysfunction, increased vascular stiffness, and increased carotid intimal thickening (Walker and Silverberg, 2008; Rubin *et al.*, 2005; Smith *et al.*, 2000; Walker *et al.*, 2009). Additionally, observational studies have suggested a relationship between parathyroid hormone and the RAAS, with more recent studies showing that PTH enhances secretion of renin and aldosterone (Brown *et al.*, 2014; Vaidya *et al.*, 2015). Further work remains to elucidate the etiology by which parathyroid hormone may induce clinically relevant changes in blood pressure.

Acromegaly

Cardiovascular disease is the primary cause of mortality in acromegalic patients, which is characterized by HTN, left ventricular hypertrophy, and cardiomyopathy. The cardiomyopathy of acromegaly has been contributed to both HTN and acromegaly itself (López-Velasco *et al.*, 1997). HTN is reported to occur in up to 40% of patients with acromegaly and is associated with sodium retention and extracellular volume expansion (Berg *et al.*, 2010; Terzolo *et al.*, 1999). Although the exact etiology of HTN in acromegalic patients remains unclear, more recent studies have shown that growth hormone and insulin-like growth factor 1 (IGF-1) act on the kidney to increase sodium and fluid retention, without major contributions of the RAAS or antinatriuretic peptides. Growth hormone and IGF-1 most likely act together to stimulate ENaC-dependent transepithelial sodium transport in the distal nephron, independent of aldosterone, as demonstrated in an acromegalic model of rats (which have increased ENaC activity and normal-to-low aldosterone levels) and in a small cohort of acromegalic patients who had an increased natriuretic response to amiloride but a reduced response to furosemide (Kamenicky *et al.*, 2008; Kamenický *et al.*, 2014). HTN in acromegaly is most effectively treated by removing the etiology of excess growth hormone. However, in cases where surgical cure is not possible, hypertensive patients with acromegaly respond well to diuretic therapy.

Hypothyroidism

Hypothyroid patients have predilection for HTN threefold compared to general population, and 1% of patients with diastolic HTN have hypothyroidism (Streeten *et al.*, 1988). Treatment with thyroxine contributes to reductions in blood pressure. Hypothyroidism is also associated with an increase in cholesterol, triglycerides, homocysteine, and total cholesterol/HDL ratio as well as with accelerated atherosclerosis and coronary artery disease (Nyirenda *et al.*, 2005). Subclinical hypothyroidism is also associated with HTN and lipid disorders. In one study, treatment of hypothyroidism improved coronary insufficiency, with relapse on therapeutic withdrawal. The increase in blood pressure may be due, in part, to increased vascular resistance and aortic stiffness. A correlation of blood pressure reduction with an associated decrease in aortic stiffness occurred with thyroxine treatment in hypothyroid patients (Kalra *et al.*, 2016). Stimulation of adrenal function by augmenting PRA, with an increase in aldosterone and cortisol, occurs in hypothyroidism. This also is reduced with thyroxine treatment (Barreto-Chaves *et al.*, 2010; Marcisz *et al.*, 2011).

Hyperthyroidism

A decreased total peripheral resistance occurs in hyperthyroidism. HTN associated with an overactive thyroid is mainly systolic and is due to increased cardiac output. This may be the result of an increased number of beta adrenergic receptors in the heart making it more sensitive to catecholamines (Nyirenda *et al.*, 2005). Treatment with beta adrenergic receptor blockers can control many of the symptoms of hyperthyroidism, including elevated blood pressure. Furthermore, treatment of the hyperthyroid state leads to a decrease in or complete control of blood pressure in those who are younger and without essential HTN (Danzi and Klein, 2003). Another cardiovascular effect of thyrotoxicosis, is the development of LVH, which was prevented by valsartan, suggesting that the RAAS plays a role (Tamer *et al.*, 2005).

See also: Mineralocorticoid Excess Syndromes. Pheochromocytoma and Paraganglioma Syndromes

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Resistant Hypertension

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Introduction

Resistant hypertension (RH) is defined as the blood pressure (BP) of a hypertensive patient that is elevated above target level in spite of the use of three antihypertensive drugs of different classes, one of which must be a diuretic (Calhoun *et al.*, 2008). All antihypertensive agents must be administered at optimal doses and with the correct dosing frequency. The term RH also applies to patients with BP controlled below the target level when the patient is taking four or more antihypertensive agents. The importance of RH is that patients with this disorder have increased risk for target organ damage, morbidity and mortality in spite of ongoing antihypertensive drug therapy (Calhoun *et al.*, 2008). Patients with RH also have increased prevalence of secondary forms of hypertension, most of which are endocrine disorders, compared to hypertensive patients without drug resistance.

Recommendations for new BP targets have been published in the 2017 American College of Cardiology/American Heart Association Clinical Practice Guideline for the Prevention, Detection, Evaluation and Management of High Blood Pressure in Adults (Whelton *et al.*, 2017). In this guideline, the BP goal of treatment is reduced from its previous level of $<140/90$ to $<130/80$ mmHg. Thus, RH is now defined as BP $\geq$ 130/80 mmHg in the presence of three or more antihypertensive agents (Whelton *et al.*, 2017). The prevalence of RH (using the 140/90 mmHg cut point) has been estimated at approximately 13% of the hypertensive population (Persell, 2011). Because the new hypertension guideline reduces the threshold for initiating antihypertensive agents from $\geq 140/90$ to $\geq 130/80$ mmHg in patients with cardiovascular disease (CVD) and in those with increased CVD risk [10-year atherosclerotic CVD (ASCVD) risk $\geq 10\%$ according to the American Heart Association Pooled Cohort Equations (2)] as well as reduces the goal of therapy, the 2017 guideline recommendations (Whelton *et al.*, 2017) are expected to increase the prevalence of RH. In this article, I will outline the sequential steps necessary to diagnose, evaluate and manage RH.

Excluding Pseudoresistance

Before diagnosing RH, several important factors must be taken into account. These include inaccurate BP measurement, medication nonadherence and the "white coat" effect, each of which interfere with the diagnosis of true RH. The term "pseudoresistance" refers to patients with apparent treatment RH but presence of one or more of these interfering factors.

Errors of office BP measurement can account for the misdiagnosis of RH. The 2017 ACC/AHA guideline (Whelton *et al.*, 2017) emphasizes the proper methods to insure accurate BP measurement, including the preparation of the patient, environmental conditions, cuff size and technique of BP measurement (Whelton *et al.*, 2017). Because of BP variability, it is critical to obtain an average of at least two BP readings on two separate occasions before making a diagnosis of treatment resistance. Thus, inaccurate BP measurement must be excluded before making the diagnosis of RH.

Nonadherence in taking prescribed antihypertensive medications also must be excluded before diagnosing RH. Indeed, nonadherence is highly prevalent in patients with RH (Hameed *et al.*, 2016). The prevalence of nonadherence is underscored by observations that one-quarter of patients newly initiated on antihypertensive therapy fail to fill their initial prescriptions; that during the first year of treatment the average patient has possession of antihypertensive medication only half of the time; and that only 20% of patients have sufficient adherence to achieve the benefits observed in clinical trials (Gwadry-Sridhar *et al.*, 2013). Identification of medication nonadherence has proven difficult in clinical practice. Indirect methods such as pill counts, self-report and prescription refill data are simple, inexpensive and widely employed but can be manipulated to overestimate adherence. Direct methods such as observed drug ingestion and urine or blood measurement of drug or metabolite levels are more accurate, but are relatively expensive and do not perfectly estimate the level of adherence. Since all methods have limitations, the best assessment of adherence should likely involve a combination of approaches (Berra *et al.*, 2016).

The "white coat" effect also must be excluded before diagnosing true RH. The white coat effect is defined as office BP above goal but out-of-office BP levels, measured preferably by ambulatory BP monitoring (ABPM) or, if ABPM is unavailable, by home BP monitoring (HBPM), at or below target in a patient taking ≥ 3 antihypertensive drugs. The risk of CVD complications in patients with the white coat effect similar to that in patients with controlled hypertension (Mancia *et al.*, 2013; Franklin *et al.*, 2016). Because intensifying antihypertensive therapy in this setting would not be warranted, out-of-office BP monitoring should be performed on every patient with apparent RH to exclude the white coat effect.

Identifying and Reversing Contributing Lifestyle Factors

Lifestyle factors contribute greatly to treatment resistance. These include obesity, physical inactivity, high sodium intake and excessive alcohol consumption. In several large cohort studies, body mass index ≥ 30 kg/m^2 has been associated with a high prevalence of RH (approximately twofold above patients with BMI below this level) (Egan *et al.*, 2011; de la Sierra *et al.*, 2012; Sim

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et al., 2013). Although it is clear that weight loss can reduce BP in hypertension, the impact of weight loss on BP in RH has not specifically been studied. However, it is generally recommended that, while the best goal is ideal body weight, weight loss should be directed towards at least a 1-kg reduction in body weight for most adults who are overweight, with the expectation of about 1 mmHg BP reduction for every 1-kg reduction in body weight (Whelton *et al.*, 2017). Dietary sodium excess has been strongly implicated in the pathophysiology of RH, as marked reduction of BP has been observed in patients with RH in response to low sodium diet (Pimenta *et al.*, 2009). The optimal goal is <1500 mg sodium/day, but a practical approach consists of at least a 1000-mg/day reduction in most adults (Whelton *et al.*, 2017). While evidence is still lacking on the role of physical inactivity in RH, there is no question about its importance to lower BP in hypertension. Current recommendations are for 150 minutes of structured exercise per week (Whelton *et al.*, 2017). Excessive alcohol intake is also associated with hypertension, and the recommendations are limitation to one standard drink for women and two or less for men (Whelton *et al.*, 2017). Importantly, the BP reduction expected from each of these individual lifestyle changes is additive.

Discontinuing or Minimizing Interfering Substances

Several classes of pharmacologic agents can increase BP and contribute to the development of treatment resistance. These include non-steroidal anti-inflammatory agents (NSAIDs), oral contraceptives and hormone replacement therapy, sympathomimetic amines, glucocorticoids and mineralocorticoids, immunosuppressive agents, recombinant erythropoietin, tyrosine kinase inhibitors, cocaine, amphetamines and antidepressants. Among these agents, perhaps the most important in terms of frequency of use in medical practice are NSAIDs. NSAIDs may interfere with the BP lowering effect of diuretics, angiotensin converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), and beta-blockers (White, 2007). However, they do not usually interfere with the antihypertensive effect of calcium channel blockers (CCBs) (White, 2007). BP effects from NSAIDs are due to cyclooxygenase (COX) inhibition, but selective COX-2 inhibitors have less BP raising effects than general COX inhibitors (Ruschitzka *et al.*, 2017). With each class of potentially interfering agent, it is important to discontinue or at least minimize the dose of these drugs in a trial-and-see approach to determine their effect on BP.

Screening for Secondary Causes of Hypertension

Patients with RH should be screened for secondary causes of hypertension, almost all of which are endocrine in nature. The frequency if secondary hypertension is markedly increased in drug resistant patients compared with those with controlled hypertension.

Primary aldosteronism is the most common form of secondary hypertension with a prevalence of about 20% in RH (Calhoun, 2013). The disorder includes hypertension due to volume expansion and sympathetic nervous system activation, hypokalemia, metabolic alkalosis, and advanced cardiovascular and renal disease. Due to the toxic effects of aldosterone on the heart and blood vessels, primary aldosteronism is associated with a major increase in CVD and CVD events compared to that observed with primary hypertension, including stroke, myocardial infarction and atrial fibrillation (Milliez *et al.*, 2005). Screening for primary aldosteronism should be conducted using the plasma aldosterone concentration (PAC) to plasma renin activity (PRA) ratio (ARR) from a blood sample obtained in the morning with the patient seated for at least 30 minutes prior to sampling (Funder *et al.*, 2016). A positive screening test entails ARR ≥ 30, or ≥ 20 if PAC is ≥ 16 ng/dL (Funder *et al.*, 2016). Prior to the test, hypokalemia which decreases aldosterone secretion, should be corrected and agents that markedly affect the renin-angiotensin-aldosterone system, especially spironolactone and eplerenone, should be withdrawn for at least 5 weeks (Funder *et al.*, 2016). Beta-blockers, central α_2 receptor agonists and renin inhibitors suppress PRA and ACEIs, ARBs and non'potassium sparing diuretics increase PRA, thus potentially affecting ARR. If the initial test results suggest that any of these medications may be interfering, they can be selectively withdrawn 1 or 2 weeks prior to testing. During withdrawal, BP may be controlled by agents that do not influence the renin-angiotensin-aldosterone system, such as the α_2 receptor antagonists (preferably doxazosin), hydralazine and/or slow-release verapamil. If the screening test is positive, the patient is usually referred to an endocrinologist or hypertension specialist for further workup and treatment.

Renal parenchymal disease is both a cause and complication of poorly controlled hypertension. Screening is by serum creatinine and estimated glomerular filtration rate (eGFR). Treatment resistance is in large part related to sodium and fluid retention and intravascular volume expansion. Thus, special attention should be focused on dietary sodium restriction in combination with diuretic therapy that evolves from thiazide-like agents to loop diuretics as renal function declines.

Renal artery stenosis is one of the most common causes of drug treatment resistance. Most cases are due to ASCVD, but renal artery stenosis can also occur due to fibromuscular dysplasia, renal dissection or infarction, Takayasu's arteritis, radiation fibrosis or aortic endovascular stent graphs. Screening is with renal Duplex Doppler ultrasound or magnetic resonance or computerized tomographic arteriography. Moderate degrees of renal vascular hypertension can be managed with medical therapy, especially with agents that block the renin–angiotensin system such as ACEIs or ARBs (Raman *et al.*, 2016).

Pheochromocytoma/paraganglioma is a chromaffin cell tumor that induces hypertension by secretion of catecholamines. The disorder is suggested by a variety of symptoms including paroxysmal hypertension associated with headache, palpitations, pallor and piloerection. Because of the high morbidity and mortality of these tumors, the usual 3-year delay in diagnosis and that at least

1/3 are inherited, it is essential to consider the diagnosis in any patient with RH. The screening test of choice is measurement of either circulating free metanephrines or fractionated urinary metanephrines (Eisenhofer *et al.*, 2003). Only after biochemical evidence of pheochromocytoma has been obtained, imaging should be pursued with either abdominal CT or MRI scanning. Patients who screen positively for pheochromocytoma/paraganglioma should be referred to an endocrinologist for further workup and preparation for surgery (Lenders *et al.*, 2014).

Cushing syndrome, caused by increased glucocorticoid exposure from exogenous or endogenous sources, is a relatively uncommon cause of hypertension. The syndrome is characterized by a constellation of classic symptoms (e.g., mood disorders, menstrual irregularity and muscular weakness) and signs (e.g., central weight gain, abdominal striae, hirsutism, dorsal and supraclavicular fat, and cutaneous fragility) that suggest the diagnosis. Screening for Cushing syndrome includes multiple 24-hour urine cortisol measurements and the overnight dexamethasone suppression test (Nieman *et al.*, 2008).

Other uncommon or rare causes of secondary hypertension include coarctation of the aorta, hypo- and hyperthyroidism, primary hyperparathyroidism, congenital adrenal hyperplasia, other mineralocorticoid excess syndromes and acromegaly.

Managing Resistant Hypertension

Management of RH first entails introducing or modifying lifestyle interventions, including weight loss, dietary sodium restriction, dietary potassium supplementation, heart-healthy diet such as the Dietary Approaches to Stop Hypertension (DASH) diet, exercise and alcohol restriction (Whelton *et al.*, 2017).

If the patient is adherent to three antihypertensive agents of different classes at maximum or maximally tolerated doses and BP is uncontrolled, diuretic therapy should be maximized. This includes substitution of a thiazide-like diuretic (i.e., chlorthalidone or indapamide) for a thiazide diuretic (e.g., hydrochlorothiazide). Recent studies have demonstrated a 7–8 mmHg BP reduction with thiazide-like diuretics over thiazide diuretics (Roush & Sica, 2016). If the BP remains out of control, a mineralocorticoid receptor antagonist (MRA) (i.e., spironolactone or eplerenone) should be added. Recent randomized trials have demonstrated the superiority of spironolactone over other antihypertensive agents as add-on drugs in RH (Williams *et al.*, 2015). With the substitution of a thiazide-like diuretic and addition of a MRA, the vast majority of patients with RH will have their BP controlled.

Beyond this management step, there is very little or no randomized clinical trial evidence for the efficacy of specific drug additions. Among other possibilities, addition of a beta-blocker or alpha-beta blocker would be reasonable given that sympathetic nervous system activity may be increased by the hypertensive process itself or by the other pharmacologic agents administered. Further considerations include addition of a vasodilator such as hydralazine, which must be taken a minimum of three times daily accompanied by a beta-blocker to block vasodilator-induced reflex tachycardia. Ultimately, if BP is still not able to be controlled, referral to a hypertension specialist is recommended.

See also: Antiadrenergic Agents. Calcium Channel Blockers. Diuretics. Endocrine Hypertension. Renal Hypertension

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Hypertension and Target Organ Damage

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Introduction

Hypertension-induced mortality and morbidity is produced through the impact of elevated blood pressure on the heart, the brain, the small and large arterial vessels and the kidney. Evaluation of early target organ damage (TOD) in these organs is an important step in a risk stratification strategy to reduce cardiovascular and renal damage. The European Society of Hypertension/European Society of Cardiology (ESH/ESC) Guidelines 2013 (Mancia *et al.*, 2013) underscored the convenience of assessing target organ damage for global risk stratification and of repeating TOD assessment during the follow-up.

A panel of TOD was included in the ESH-ESC guidelines in 2013. Some TOD, such as the ankle/brachial index or the presence of estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2, indicates advanced organ damage, while others are not yet routinely available. Based on availability, cost and clinical significance, the evaluation of left ventricular hypertrophy (LVH) by electrocardiography and possibly by echocardiography, the measurement of urinary albumin excretion and glomerular filtration rate are the minimal recommended among all TOD.

Several studies have shown that the regression of asymptomatic TOD induced by antihypertensive treatment reflects the treatment-induced reduction of morbid and fatal cardiovascular (CV) events. Therefore, the evaluation of TOD during treatment may offer valuable information on whether patients are more or less effectively protected by the treatment strategies adopted.

The effect of aldosterone on the development and regression of target organ damage has been studied in patients with adrenal gland disease, in particular with primary aldosteronism, and the results are reported and discussed.

Heart

Electrocardiographic LVH is a powerful marker of CV morbidity/mortality in the general population as well as in different clinical settings (Agabiti-Rosei *et al.*, 2006). In hypertensive patients LVH may predict the occurrence of CV events, including myocardial infarction, stroke sudden death and heart failure (Angeli *et al.*, 2015; Vakili *et al.*, 2001). The incidence of atrial fibrillation and of renal events, such as creatinine doubling, eGFR <30 mL/min/1.73 m^2 or the need for end-stage renal disease replacement, are also higher in the presence of LVH (Tsioufis *et al.*, 2010).

BP values, mainly measured during 24 h monitoring, and body mass index (BMI) are strictly associated to LVH development (Peer *et al.*, 2013). In addition metabolic syndrome, diabetes mellitus (Wijkman *et al.*, 2012), hyperuricemia and chronic neurohormonal activation may further influence LVH development; the risk of incident LVH is particular relevant among women (**Fig. 1**).

Available data suggest that in patients with secondary hypertension due to hyperaldosteronism LV mass (LVM) is higher; conflicting results have been published on changes of geometric adaptation of the left ventricle, showing an increase in relative wall thickness in some patients, while in other groups of primary aldosteronism (PA) patients an increase in LV diameter was observed. Denolle *et al.* (1993) found that LVM index in increased to a greater extent in patients with primary aldosteronism and adrenal adenoma, as compared to patients with pheochromocitoma or renovascular hypertension.

Muiesan *et al.* (2008) have hypothesized that in primary aldosteronism the increase in LVM may be the consequence of excess aldosterone, leading to an increase in LVM inappropriate for demographic characteristics and hemodynamic load; in fact patients with primary aldosteronism, even in the absence of LVH, had a greater prevalence of inappropriate LVM.

Marker of OD	*Prognostic value*	*Change detection sensitivity*	*Time for evaluating changes*
LVH (electrocardiogram)	++++	+	>6 months
LVH (echocardiography)	++++	++	>6 months
Albuminuria	++	+++	Weeks–months
Estimated glomerular filtration rate (eGFR)	(+)	++	Years
Pulse wave velocity (PWV)	(+)	+++	Weeks–months
Ankle brachial index (ABI)	−	+	−
Carotid wall thickness	−	−/+	>1 year

Fig. 1 Prognostic value of changes during treatment of markers of asymptomatic organ damage. *LVH*, left ventricular hypertrophy. Modified from Mancia, G. (2013). ESH/ESC guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *European Heart Journal* **34** (28), 2159–2219.

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In PA (Rossi *et al.*, 2002) and more recently in pheochromocytoma patients (Ferreira *et al.*, 2016), a diffuse cardiac fibrosis has been documented, leading to both systolic and diastolic functions impairment.

Treatment with antihypertensive drugs is associated with a significant reduction in ECG LVH and in echocardiographic LVM. The magnitude of the decrease is related to the baseline LVM and to changes in clinic BP; the correlation between changes of LVM and changes in BP is more significant when average 24 h BP is considered (Mancia *et al.*, 1997).

Among all classes of antihypertensive drugs, ACE-inhibitors, angiotensin receptor blockers and calcium antagonists seem to be more effective as compared with beta-blockers (Fagard *et al.*, 2009). In most studies, however, patients were not receiving monotherapy but a combination of drugs (usually with a diuretic). The choice of a specific class of antihypertensive drugs seems to be less relevant then the adequate and long-term BP control.

A normalization of LVM is not always possible and seems to be more difficult to obtain in women (Devereux *et al.*, 2004), obese or diabetic patients (De Simone *et al.*, 2013), elderly subjects with isolated systolic hypertension (Mancusi *et al.*, 2014) or patients with coronary artery disease, despite effective BP reduction. A normalization of LV geometry is possible during antihypertensive treatment and may have relevant prognostic significance (Muiesan *et al.*, 2004).

Regression of ECG-graphic LVH assessed by voltage and strain criteria may be induced by treatment (Levy *et al.*, 1994; Mathew *et al.*, 2001; Okin, 2003; Wachtell *et al.*, 2007; Okin, 2009; Prineas, 2001), and large changes in ECG voltage and strain results in improved prognosis (Bang *et al.*, 2014; Okin *et al.*, 2004). Changes in echocardiographically LVH induced by treatment reflect the effects on cardiovascular events; however a residual risk may be observed in patients with LVH regression, in whom LVM remains higher, although in the normal range, than in patients with persistently normal LVM (Angeli *et al.*, 2015). Changes in echocardiographic LVM and in renal function may independently predict the occurrence of cardiovascular events (Salvetti *et al.*, 2012).

During antihypertensive treatment the modifications of LV geometry, of left atrial size, of midwall fractional shortening and of diastolic dysfunction parameters have been also shown to be associated with the incidence of cardiovascular events, independently of LVM change (Kurt *et al.*, 2009; Muiesan *et al.*, 2004; Wachtell *et al.*, 2010).

In patients with PA surgical or medical treatment are both effective in reducing LV mass (Catena *et al.*, 2012).

Vascular Damage

Carotid arteries

The thickness of intima-media complex in the carotid arterial wall may be measured noninvasively by high resolution ultrasound, according to a validated method. Carotid intima-media thickness (C-IMT) is the most widely accepted non-invasive marker of subclinical atherosclerosis (Bots *et al.*, 2003; Lorenz *et al.*, 2007; Touboul *et al.*, 2004), and the threshold of >0.9 mm is considered a risk of future myocardial infarction and/or cerebrovascular disease (Bots *et al.*, 1997; Salonen and Salonen, 1991; Chambless *et al.*, 1997; Hodis, 1998; Lorenz *et al.*, 2007; Den Ruijter *et al.*, 2012). The use of Radiofrequency C-IMT measurements has been recently shown to have an additional stratification power for coronary artery disease, in addition to the Framingham risk score (O'Leary *et al.*, 1999).

The presence of plaques and plaque morphology may be assessed by ultrasound. More recently plaque volume assessment by three-dimensional reconstruction of ultrasound or nuclear magnetic resonance images has been proposed to better evaluate atherosclerotic lesions changes, and to stratify patients' risk (Moreo *et al.*, 2015; Wannarong *et al.*, 2013). Traditional risk factors, including aging, male sex, being overweight or obese, elevated blood pressure, diabetes, smoking, are all positively associated with an increase in carotid IMT in observational and epidemiological studies. Hypertension, and particularly high systolic BP values, seems to have the greatest effect on IMT (Van Engelen *et al.*, 2014); patients with metabolic syndrome have higher IMT than patients with individual metabolic risk factors.

Carotid IMT has also been found to be associated with preclinical cardiovascular alterations, in the heart, in the brain, in the kidney, and in the lower limb arteries.

Double blind randomized trials have shown that different classes of antihypertensive drugs may have a more or less marked effect on carotid IMT and plaque progression. Compared with no-treatment, diuretics/+ beta-blockers or ACE inhibitors, CCBs attenuate the rate of progression of carotid intima-media thickening (Zanchetti *et al.*, 2002). Few studies have shown a lower thickness of intima-media during treatment with angiotensin II antagonists in respect with patients treated with betablockers (Wang *et al.*, 2006).

The odds ratio for all fatal and nonfatal cardiovascular events in trials comparing active treatment with placebo reached statistical significance ($P = 0.007$).

Comparative studies assessing the effect of statins on IMT progression have demonstrated a beneficial effect on common carotid mean IMT; the effect was greater in the setting of secondary prevention versus primary prevention, in younger patients versus older patients and in studies with a greater proportion of male patients (Kang *et al.*, 2004; Agabiti-Rosei, 2006). Plaque volume was reduced to a greater extent with the long term treatment with angiotensin II blocker in respect to the betablocker was demonstrated by noninvasive 3D plaque measurement (Stumpe *et al.*, 2007). A significant change in 3D plaque volume was also observed during short term treatment with a high dose of statin in a small group of 20 patients (Huang *et al.*, 2013), while no significant changes in plaque composition were observed after 4 years of treatment with either lacidipine or atenolol (Paliotti *et al.*, 2005).

It has not been demonstrated whether a decrease of IMT progression may be associated with a reduction of cardiovascular events and an improvement in prognosis (Zanchetti *et al.*, 2009; Costanzo *et al.*, 2010).

Changes in plaque volume and composition characteristics seem to have additional prognostic significance (Van Engelen *et al.*, 2014).

Aortic stiffness

In more recent years it has become possible to evaluate the vascular aging process by the measurement of arterial stiffness with non-invasive technique (Nilsson *et al.*, 2013). Regional and local arterial stiffness can be measured noninvasively, at various sites along the arterial tree, based on direct measurements of parameters strongly linked to wall stiffness. By arterial tonometry carotidofemoral (aortic) pulse wave velocity (PWV) may be measured and the threshold of 10 m/s indicates the presence of increased large artery stiffening (Mancia *et al.*, 2013). Nuclear magnetic resonance may be the most appropriate method for the evaluation of less superficial vessels (Laurent *et al.*, 2006; Mancia *et al.*, 2013; Van Bortel *et al.*, 2012).

The importance of carotidofemoral (aortic) PWV as a sub-clinical organ damage for a more accurate and precise CV risk stratification has been underscored by the 2013 ESH/ESC guidelines. Several studies have documented a correlation between arterial stiffness measures (in particular aortic PWV) and aging, arterial hypertension, diabetes mellitus, metabolic syndrome, hypercholesterolemia and even markers of chronic inflammation processes (Laurent *et al.*, 2006).

A large number of publications and several reviews have reported the various pathophysiological conditions associated with increased arterial stiffness and wave reflections, including arterial hypertension and in particular isolated systolic hypertension (Asmar, 2015; Mitchell *et al.*, 2003). Arterial stiffness is associated with target organ damage including LVH (Asmar, 1995), micro-albuminuria, carotid intima-media thickening, endothelial dysfunction and microvascular alterations (Muiesan *et al.*, 2013)).

Aging and BP are the main determinants of vascular stiffness, inducing a reduced synthesis and an increased degradation of elastin, in association with an increased synthesis and reduced degradation of type 1 and type 3 collagen (Cliff, 1970); calcification of the vessel wall may also occur, as frequently observed in elderly subjects and in hypertensive patients.

In recent years numerous studies have reported an association between alterations of arterial stiffness or of pulsatile hemodynamic parameters and the occurrence of CV events (Vlachopoulos *et al.*, 2010). The increase in cfPWV has been shown to predict all-cause mortality, CV mortality, fatal and non-fatal coronary artery events, fatal stroke in the general population (Mattace-Raso *et al.*, 2006; Shokawa *et al.*, 2005; Willum-Hansen *et al.*, 2006) and in different groups of patients, with arterial hypertension (Laurent *et al.*, 2001; Laurent *et al.*, 2003), diabetes mellitus (Cruickshank *et al.*, 2002), end stage renal disease (Blacher *et al.*, 1999; Shoji *et al.*, 2001), elderly patients (Meaume *et al.*, 2001; Sutton-Tyrrell *et al.*, 2005). Most importantly the prognostic significance of PWV is independent of brachial BP values, of major CV risk factors, and of the Framingham risk score (Boutouyrie *et al.*, 2002), indicating that aortic stiffness has a better predictive value than each of the classical risk factors (Ben-Shlomo *et al.*, 2014).

Data about prognostic significance of arterial stiffness measured at other arterial sites are less consistent. Carotid stiffness was predictive of CV events in a small number of patients with ESRD (Pannier *et al.*, 2005) or following renal transplantation (Barenbrock *et al.*, 2002) and in a large prospective cohort with a high prevalence of insulin resistance (Van Sloten *et al.*, 2014), while no predictive value was demonstrated in a larger number of patients with manifest arterial disease (Dijk *et al.*, 2005).

Data on changes in PWV and occurrence of CV events are still limited. Guerin *et al.* (2001) have demonstrated that in end stage renal disease patients the persistence of elevated cfPWV was associated with a worse survival as compared to patients with a decrease of cfPWV, independently of brachial BP changes. It remains to be demonstrated in hypertensive patients at lower CV risk that a reduction or anormalization of arterial stiffness is effective in reducing CV events beyond brachial BP reduction.

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The reduction of BP per se may induce a decrease of cfPWV (Ong *et al.*, 2011). Several non-pharmacological measures, such as exercise training, weight loss, low to moderate sodium diet, moderate alcohol consumption, and dietary supplements may have a beneficial effect on changes in arterial stiffness.

Among antihypertensive classes of drugs, diuretics, beta blockers, ACE inhibitors, angiotensin II type 1 receptor blocker (AT1 blockers), and calcium channel antagonists are able to reduce arterial stiffness (Ghiadoni *et al.*, 2009; Laurent *et al.*, 2006).

Some studies have also shown that a non-dihydropiridinic calcium-antagonist (acute administration) or an ACE-inhibitor (Asmar *et al.*, 2001; Tropeano *et al.*, 2006) or an angiotensin II receptor blocker (Laurent and Boutouyrie, 2014) are able to reduce arterial stiffness and/or wave reflections independently of the reduction in brachial BP.

A decrease in arterial stiffness has been induced in congestive heart failure patients by ACE inhibitors, nitrates, and aldosterone antagonists, statins, and antidiabetic drugs. The additional BP independent effect of different pharmacological interventions remains to be further evaluated.

Microvascular structure

Essential hypertension is associated with the presence of structural alterations in the microcirculation (Mulvany and Aalkjaer, 1990; Schiffrin, 2004b), that may be also the result of adaptive mechanisms to the increased blood pressure load. Since structural alterations might have hemodynamic consequences, their evaluation represent an important target, also in terms of cardiovascular risk stratification (Izzard *et al.*, 2005; Aalkjaer *et al.*, 1989).

The mechanisms underlying the development of microvascular structural alterations are only partially elucidated. The extent of structural alterations in subcutaneous small resistance arteries is particularly pronounced in hypertensive patients with type 2 diabetes mellitus (Endemann *et al.*, 2004; Rizzoni *et al.*, 2001) or obesity (Grassi *et al.*, 2010; De Ciuceis *et al.*, 2011), suggesting that the association of several cardiovascular risk factors may have a synergistic, deleterious effect on the microcirculation.

The increase of media to lumen ratio of small resistance arteries, taken from a mesenteric or gluteal region subcutaneous tissue biopsy, was related to the incidence of cardiovascular events in 3 different studies (Rizzoni *et al.*, 2003; De Ciuceis *et al.*, 2007; Mathiassen *et al.*, 2007) including patients at high or moderate global CV risk. The media to lumen ratio was significantly associated to the occurrence of cardiovascular events, independently of other CV risk factors, strongly indicating a relevant prognostic significance of small resistance artery structural alterations in a high risk population. In addition Buus *et al.* (2013) have demonstrated that changes of small resistance arteries structure during antihypertensive treatment may have a prognostic role. Antihypertensive treatment may induce the reversal of increased media to lumen ratio and inhibitors of the RAS (including ACE-inhibitors, angiotensin II receptor antagonists, aliskiren) and calcium antagonist are more effective in this regard (Agabiti-Rosei *et al.*, 2009).

Other new techniques for evaluation of microvascular morphology in the retinal vasculature are presently used or under clinical investigation, representing a promising and interesting future perspective (Ritt *et al.*, 2008; Koch *et al.*, 2014).

Endothelial dysfunction, oxidative stress, chronic inflammation and fibrosis may be responsible for vascular damage in patients with secondary hypertension, in particular when the renin angiotensin aldosterone system is activated (Rizzoni *et al.*, 1998). Aldosterone may induce vascular smooth muscle cells hypertrophy and hyperplasia (Schiffrin, 2004c) leading to an increase in the media to lumen ratio of small resistance arteries (Rizzoni *et al.*, 1996) and may alter extracellular matrix composition, stimulating collagen deposition and fibroblasts proliferation (Schiffrin, 2004a).

Vascular changes and aldosterone

Rizzoni *et al.* have demonstrated that in patients with renovascular hypertension and in those with PA, the media to lumen ratio of small resistance arteries was increased, according to hypertrophic or eutrophic remodeling, with a narrower diameter and an increased stiffness of media layer, due to excessive collagen III content (Rizzoni *et al.*, 1998; Rizzoni *et al.*, 2006). The effect of aldosterone could be mediated, at least in part, by endotelin-1, as suggested by Schiffrin *et al.* (1997).

Several studies have evaluated large arteries changes in patients with primary aldosteronism, in order to assess the effect of aldosterone beyond blood pressure induced damage. Changes in aortic stiffness, carotid intima-media thickness or carotid plaque have been measured, with conflicting results.

Rossi *et al.* have observed that carotid IMT and the number of plaques are greater in patients with renovascular hypertension, but not in those with primary aldosteronism, as compared with essential hypertensive patients (Rossi *et al.*, 1993). Similar results were obtained by Rizzoni *et al.* (1998) and more recently confirmed by Muiesan (2015). Turchi *et al.* have observed in patients with PA a higher CV risk, due to a greater prevalence of hyperglycemia, metabolic syndrome and LVH, but similar IMT and arterial peripheral disease before and after medical or surgical treatment (Turchi *et al.*, 2014). On the opposite an increase in IMT was reported by some authors (Holaj *et al.*, 2007; Bernini *et al.*, 2008), when comparing patients with PA and essential hypertensives and/or normotensive subjects.

In some other studies, conducted in a small number of patients an unfavorable effect of aldosterone on large arteries structure and function was observed. Štrauch *et al.* (2006) and Bernini *et al.* (2008) have observed an increase in cfPWV and of augmentation index (AI) in patients with PA as compared with normotensives and essential hypertensives. Mark *et al.* (2014) report similar findings, obtained with nuclear magnetic resonance measurements of aortic distensibility. Park *et al.* observed a significant correlation between heart-femoral PWV and plasma aldosterone levels, and a significant increase in heart-femoral PWV but not of peripheal PWV (brachial-ankle PWV) in PA as compared to essential hypertensives (Park *et al.*, 2007). More recently Rosa *et al.* (2012) reported a significantly higher cfPWV and femoro-tibial PWV in PA patients as compared to essential hypertensives.

Chang *et al.* (2014) suggested that levels of serum potassium may exert a role on aortic stiffness, based on a correlation observed between serum potassium concentration and augmentation index.

On the opposite no significant differences cf. PWV were observed by Tsioufis *et al.* (2008) between essential hypertension and PA patients.

More recently Fujii *et al.* have not found significant differences between patients with adrenal bilateral hyperplasia and essential hypertensive patients as related to endothelial function (evaluated by flow mediated vasodilation), cardio-ankle vascular index (CAVI), IMT and coronary artery calcium score (Fujii, 2016).

Only one study has investigated the role of endothelial progenitor cells (EPC) in PA patients, showing a reduced number of circulating EPC with a significant increase 6 months after adrenalectomy or medical treatment with spironolactone (Wu *et al.*, 2011).

The effect of surgical adrenalectomy on cardiovascular alterations has been analyzed in different studies, suggesting a favorable effect of adrenalectomy on blood pressure control, possibly restoring normal vascular structure. In one study (Strauch *et al.*, 2008) a significant decrease in aortic stiffness was observed after adrenalectomy, but not during medical treatment with spironolactone. Other studies (**Table 1**) have addressed this point, with conflicting results.

Table 1 Main studies evaluating vascular target organ damage in primary aldosteronism patients

Authors study	*Publication year*	*PA patients (N)*	*IMT*	*Plaque prevalence*	*Cf PWV*	*Peripheral PWV*	*AI*	*FMD*
Rossi	1993	17	=	=				
Rizzoni	1998	14	=					
Nishizaka	2004	36						↓
Strauch	2006	36			↑		↑	
Park	2007	53			↑	=		
Holaj	2007	33	↑					
Tsioufis	2008	17			=			
Bernini	2008	23	↑		↑	↑	↑	
Wu	2011	113			↑			
Lin	2012	20	↑		↑	↑		
Rosa	2012	49			↑	↑		
Mark	2014	14			↑		↑	
Turchi	2014	102	=					
Chang	2015	37					↑	
Fujii	2016	47	=					=

IMT, carotid intima-media thickness; *PWV*, pulse wave velocity; *AI*, augmentation index; *FMD*, flow mediated vasodilatation.

Renal TOD

According to the 2013 ESH/ESC Guidelines the assessment of serum creatinine and the calculation of estimated glomerular filtration rate (eGFR) is recommended to assess renal excretory function (Mancia *et al.*, 2013; Sternlicht and Bakris, 2017), and urinary albumin excretion is considered a biomarker of early renal damage. Both measurements are low-cost and easy to perform. Urinary albumin excretion and eGFR should be measured both in hypertensive patients, in order to better stratify cardiovascular risk (Mancia *et al.*, 2013; Viazzi *et al.*, 2016).

Albuminuria

The increase in blood pressure values, but also other risk factors, such as insulin resistance, salt sensitivity, central obesity, smoke, atherogenic lipid profile, have been found to be associated to microalbuminuria. In addition the presence of albuminuria is usually related to other signs of extra-renal organ damage such as left ventricular hypertrophy, carotid atherosclerosis and biomarkers of vascular endothelium damage (Sternlicht and Bakris, 2017). Moreover a relationship between even modest increase in albuminuria and cardiovascular morbidity and mortality (Currie and Delles, 2013) has been observed, leading to the estimation of microalbuminuria as an integrated marker of cardiovascular risk.

As shown by Redon *et al.* albuminuria reaches normal values in about 50% of patients, during antihypertensive treatment, while in about 10% of them a progression towards more severe proteinuria occurs (Redon and Martinez, 2012; Pascual *et al.* 2005). Poor blood pressure control and a progressive increase in glucose plasma levels are the main determinants of a new development of microalbuminuria during treatment. However, since a reliable change in albuminuria requires a regression or an increase of more than 50% of the initial value, some caution should be taken when evaluating albuminuria changes during treatment. The variability in microalbuminuria changes evaluation may be reduced by the calculation of the average value of two different measurements performed in different days.

A recent meta-analysis of trials evaluating blood pressure targets with respect to proteinuria progression has shown that a more aggressive target (<130 mmHg vs. <140 mmHg) is associated with a lower prevalence of albuminuria (Lv *et al.*, 2013; Viazzi *et al.*, 2016).

Some randomized clinical trials have suggested that drugs acting on the renin–angiotensin–aldosterone system (RAAS) are more effective in reducing albuminuria in respect to placebo in individuals with diabetic or nondiabetic nephropathy and with cardiovascular disease 104 (Pascual *et al.*, 2005). The association of two RAAS blockers is not recommended, when a combination treatment is needed.

In patients with resistant hypertension, treated with ACE inhibitor or ARB monotherapy, the addition of a mineral corticoid receptor antagonist was associated with a further reduction of albuminuria, possibly because of the greater efficacy in home BP reduction.

The reduction of albuminuria during treatment may favorably affect cardiovascular and renal prognosis (Redon and Martinez, 2012).

Glomerular filtration rate

Glomerular filtration declines with increasing age, and high blood pressure values, diabetes, and dyslipidemia represent the main factors accelerating the decrease glomerular filtration rate over time (Sternlicht and Bakris, 2017), when all diseases producing a direct renal damage are excluded.

The diagnosis of chronic kidney disease (CKD) corresponds to the presence of eGFR values below 60 mL/min/1.73 m^2. CKD is observed in a significant proportion of hypertensive patients, mainly in older individuals, in women and in diabetics. A lower eGFR is associated with a higher risk for CV events (Sternlicht and Bakris, 2017) but few data have demonstrated that changes in eGFR may influence cardiovascular prognosis in hypertensive patients.

The adequate and long term control of BP values during antihypertensive treatment favors the preservation of renal function and delays progression to end-stage renal disease in hypertensive patients with or without chronic nephropathy, although a slight increase in serum creatinine, due the transient renal hypoperfusion, may occur immediately after starting antihypertensive treatment. Blood pressure targets with respect to CKD progression have shown that a more aggressive target (<130 mmHg vs. <140 mmHg) is not associated with a reduction in eGFR (Viazzi *et al.*, 2016; Lv *et al.*, 2013). Recent data from the SPRINT study (Systolic Blood Pressure Intervention Trial) have shown that in hypertensive non diabetic patients with CKD who achieved during treatment a value of unattended blood pressure lower than 120 mmHg the risk of cardiovascular events was lower (SPRINT Research Group *et al.*, 2015).

In patients with even mild degree of CKD a combination treatment of a RAAS blocker with other classes of antihypertensive drugs is usually needed to reach adequate BP control. In the ACCOMPLISH study the association of an ACE-inhibitor and a calcium antagonist was more effective in preventing the progression to creatinine doubling and/or to the development of end stage renal disease, as compared with the combination of an ACE-inhibitor and a diuretic (Jamerson *et al.*, 2008).

An excessive amount of aldosterone may induce an increase in glomerular filtration rate and an increase in renal perfusion and albuminuria independently of BP values.

In patients with PA the presence of renal disease has been investigated with conflicting results. A modest level of renal parenchymal damage has been observed at renal biopsies by Danforth *et al.* (1977). According to Catena *et al.* (2007) CKD (i.e. eGFR <60 mL/min/1.73 m^2) was found in 7% of a relatively large number of PA patients ($n = 56$). In the multicenter PAPY study the prevalence of microalbuminuria was twice greater in PA patients (around 30%) as compared to essential hypertensives (around 15%) (Rossi *et al.*, 2006); in patients with adrenal adenoma the amount of albuminuria is even higher.

In patients with PA higher values of both eGFR and albuminuria have been observed, suggesting a condition of glomerular hyperfiltration, caused by sodium retention and volume expansion induced by aldosterone excess (Sechi *et al.*, 2009). Albuminuria and eGFR were significantly increased in PA patients as compared to essential hypertensive patients and normotensive subjects (Catena *et al.*, 2007) and after adrenalectomy, but not during medical treatment, these changes were significantly reduced (Ribstein *et al.*, 2005). On the opposite medical treatment was as effective as adrenalectomy in another group of PA patients (Sechi *et al.*, 2006).

In the German registry of PA patients, Reincke *et al.* have observed an increased prevalence of patients with serum creatinine values >1.25 mg/dL in PA (29%) as compared to essential hypertensives (10%). In addition after adrenalectomy, but also after medical treatment with spironolactone, eGFR was reduced and serum creatinine values were increased (Reincke *et al.*, 2009).

In conclusion it seems that surgical or medical treatment may reduce renal hyperfiltration induced by the aldosterone excess, revealing the presence of renal dysfunction (Ribstein *et al.*, 2005; Sechi *et al.*, 2006; Reincke *et al.*, 2009).

Moreover a higher prevalence of renal cysts was reported in PA patients (44%) as compared to essential hypertensives (25%) by Torres *et al.* (1990). During a long period of follow-up after adrenalectomy or medical treatment no changes in the number and dimensions of renal cysts was found, suggesting that both therapeutic solutions may be effective in halting the development and or progression of renal cysts (Novello *et al.*, 2007).

Conclusions

ESH/ESC guidelines 2013 recommend the measurement of serum creatinine, urine albumin excretion and electrocardiographic LVH in all patients with hypertension both at baseline and during treatment. In addition echocardiographic left ventricular mass, ultrasonic carotid wall thickness, aortic PWV and ankle-brachial index measurements should be considered to better stratify the cardiovascular risk.

In the future, more effort should be made to identify which combination of markers to measure, at what time points, in which patients, and with which consequence for a better clinical use of TOD during antihypertensive treatment.

See also: Hypertension; Overview. Stroke

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Stroke☆

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Glossary

Lacunar syndrome Set of signs and symptoms indicating a stroke that is related to disease of a single small perforating artery.

Pituitary apoplexy Term for an ischemic or hemorrhagic stroke of the pituitary gland.

Introduction

According to the World Health Organization (WHO), a stroke constitutes the sudden occurrence of signs and symptoms of a focal disorder in the brain (paresis, sensory loss, ataxia, or disturbance of higher cortical functions) for which there is no other cause than a vascular one. The WHO requires the signs and symptoms to be present for at least 24 h. If signs and symptoms clear completely within 24 h, the term "TIA" (transient ischemic attack) is used.

In practice, the World Health Organization's (WHO) definitions of stroke and TIA (transient ischemic attack) would not allow the diagnosis of stroke during the first 24 h. It should be noted that in the vast majority of TIAs (where signs and symptoms clear completely within 24 h), the signs and symptoms clear within 30–60 min. Any syndrome lasting longer than 60 min is much more likely to become a stroke than a TIA. Strokes are ischemic in approximately 80% of patients and are hemorrhagic in 20%.

Diabetes Mellitus and Other Disorders of Glucose Metabolism

The one endocrinological disorder that stands out because of its intricate associations with stroke is diabetes mellitus. All types of associations with stroke occur in the case of diabetes mellitus.

First, diabetes mellitus is an important risk factor for stroke, independent of other risk factors such as hypertension and age. The risk of ischemic stroke is increased two- to fourfold in patients with diabetes mellitus. Approximately 15% of patients with diabetes mellitus will eventually have a fatal stroke. Conversely, approximately 20% of patients with stroke have diabetes mellitus. Diabetes mellitus is more strongly related to stroke in the presence of hypertension and hypercholesterolemia. In patients with such risk factors, small vessel disease is especially likely. Such small vessel disease accounts for approximately 20% of strokes. Strokes due to small vessel disease are typified by lacunar syndromes (which lack cortical symptomatology and manifest as pure motor, pure sensory, sensorimotor, ataxic hemiparesis or dysarthria clumsy hand syndromes). Imaging shows lacunar strokes that have a subcortical distribution and are smaller than 1 cm in diameter. The pathological finding in small vessel disease is lipo-hyalinosis of small penetrating arteries. Retinopathy and autonomic neuropathy are other markers of an increased risk of stroke in diabetic patients. The pathogenesis of stroke in patients with diabetes mellitus involves increased fibrinogen, factor V and VII levels, increased hematocrit, platelet aggregation and adhesion, decreased red blood cell deformability, decreased fibrinolytic activity, and increased release of B thromboglobulin. Unfortunately, tight glycemic control in diabetic patients has not been shown to reduce the risk of stroke (as either a primary or a secondary preventive measure), although it does reduce the risk of many other mostly microvascular complications. However, the intensive concurrent treatment of various risk factors for cardiovascular disease, including diabetes in a study by Gaede and colleagues was shown to decrease the risk of stroke. In patients with myocardial infarction, treatment of hyperglycemia (blood sugar levels >11 mmol L^{-1}) was shown to decrease mortality. Although nonfatal stroke was not the primary end point, and intensive treatment was simultaneously targeted at a number of risk factors such as diabetes, there were only 3 nonfatal strokes in the intensive treatment group and 11 in the control group. However, the specific contribution of glycemic control to this overall risk reduction is not clear.

Second, diabetes mellitus and acute hyperglycemia are related to the outcome in a complex way. Hyperglycemia at the onset of stroke is associated with a poorer outcome of the presenting stroke if it is cortical yet a better outcome when the stroke is subcortical. These observations have been made in animal and clinical data. The detrimental effects of hyperglycemia on cortical strokes have been hypothesized to be due to the larger penumbral areas in cortical compared to subcortical strokes. The mechanisms leading to the increased vulnerability of these penumbral zones (=hypoperfused but viable border zones surrounding brain infarction) are not clearly understood, but include increasing tissue acidosis secondary to anaerobic glycolysis, increased blood–brain barrier permeability, and vascular changes. Animal studies have shown convincingly that hyperglycemia, as

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compared with euglycemia, increases the extent of ischemic damage in rats and monkeys. However, in focal ischemia models, this effect was shown only for reperfused brain tissue, albeit less consistently. The negative effects of hyperglycemia on outcome of brain ischemia are probably mediated through increased lactic acidosis and increased release of excitatory amino acids (glutamate in particular) that may contribute to neuronal cell death (excitotoxicity), exaggeration of edema formation, blood–brain barrier disruption, and hemorrhagic transformations. In humans, the data underlining a causal relation between hyperglycemia and stroke are still only circumstantial. This is explained partly by the intricate relations between the pathogenesis of hyperglycemia and the evolution and type of the stroke.

Reperfused brain tissue may be especially vulnerable to hyperglycemia, whereas anoxic tissue may even benefit from hyperglycemia. It has been shown that the ischemic penumbra in stroke patients, represented as a mismatch between perfusion and diffusion on magnetic resonance imaging (MRI) (**Fig. 1**), is more likely to progress to infarction when the patient had hyperglycemia at presentation. Also, hyperglycemia levels were related to lactate levels, which in turn were independently related to salvage of mismatch tissue. In a previous study, a poorer outcome of strokes had been reported when hyperglycemia occurred in nonlacunar strokes that may show reperfusion but not in lacunar strokes with little or no reperfusion. A special vulnerability of reperfused brain tissue to hyperglycemia is also suggested by the experiences with thrombolysis in stroke patients. In the first trial showing the benefit of thrombolysis in acute stroke patients, the negative effects of hyperglycemia on outcome of thrombolysis were also reported. Further studies have shown that it was specifically in patients with reperfusion after tissue plasminogen activator (tPA) that hyperglycemia was associated with poor outcome. In these patients, there is a relation with hemorrhagic transformation of the infarct. Interestingly, diabetes mellitus has actually been reported to carry a decreased risk of primary intracerebral haemorrhage, so it seems less likely that diabetic vasculopathy explains the increased incidence of hemorrhagic transformation in thrombolyzed strokes. The fact that stroke type determines the association between hyperglycemia and stroke outcome is also underlined by the fact that, in hemorrhagic strokes, hyperglycemia is not related to a poorer outcome in both nondiabetic and diabetic patients.

It seems that acute hyperglycemia may be more strongly related to poor outcome than is hyperglycemia due to diabetes mellitus. In a large meta-analysis, hyperglycemia was found to have a relative risk of mortality after ischemic stroke of 3.1 in nondiabetic patients and only 1.3 in diabetic patients. In conclusion, the literature suggests a causal relation between hyperglycemia and stroke outcome rather than hyperglycemia as a paraphenomenon of strokes with more severe outcome. It seems sensible to withhold intravenous fluids containing glucose during the acute phase of a cortical stroke, and hyperglycemia should be controlled with the usual measures, especially when blood sugar levels higher than 16 mmol L^{-1} are measured. However,

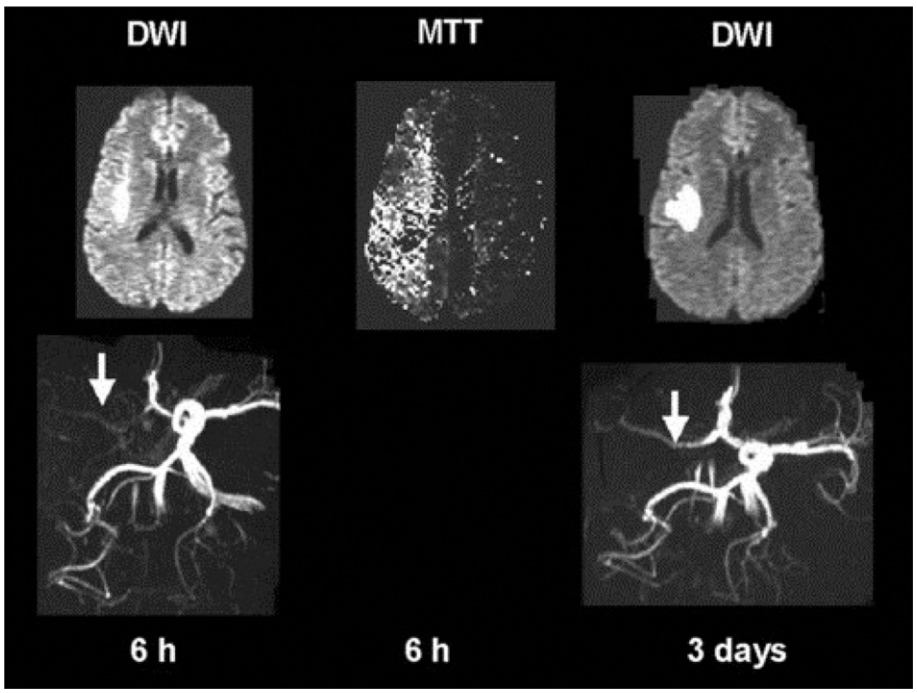

Fig. 1 Diffusion weighted imaging (DWI) sequence 6 h after the onset of a stroke showing a circumscribed area of high signal indicating disturbed diffusion. This represents infarction of the brain. The magnetic resonance (MR) angiography after 6 h shows absent flow in the right middle cerebral and carotid artery. The mean transit time (MTT) MR shows hypoperfusion of the total right middle cerebral artery territory. The area of hypoperfusion on the MMT MR minus the area of disturbed diffusion on the DWI MR is generally considered to be the penumbra. The DWI MR after 3 days shows that the hypoperfused area has not progressed to infarction. The MR angiography shows reperfusion of the right middle cerebral artery through the circle of Willis. Courtesy of Geoffrey Donnan, National Stroke Research Institute.

whether tight control of hyperglycemia should be the goal in patients with stroke cannot be determined with certainty. A British trial was stopped due to slow recruitment but failed to show any significant effect of acute treatment of hyperglycemia on the outcome of stroke. In a recent meta-analysis, intensive glucose lowering had no effect on outcome/mortality of stroke.

Hyperglycemia occurs in 20–50% of stroke patients. One-third of these hyperglycemic patients were known to have diabetes mellitus. In another third, the hyperglycemia is the initial presentation of *de novo* diabetes, demonstrated by an elevation of glycosylated hemoglobin (HbA1c) levels. In the remainder (with a normal HbA1c at the time of hyperglycemia), the hyperglycemia is considered to be a result of the stroke, but the mechanism for this is unclear. Although a general stress response may lead to hyperglycemia, this is probably not the predominant cause in patients with a stroke. Other parameters of the stress response, such as levels of catecholamines, have been shown not to be related to blood sugar levels after stroke. Because the effects of focal ischemic events on neurotransmitter release at a distance from the focal ischemia can operate in the entire ipsilateral hemisphere of the stroke, the neuroendocrine axis may well be influenced by these alterations in neuronal excitation. Alternatively, the focal ischemic brain may mediate hyperglycemia directly through as yet unclarified mechanisms. If this were to occur preferentially in inadequately reperfused brain, the association of hyperglycemia and poor outcome of stroke would represent a paraphenomenon and not a causal relationship.

Although hyperglycemia will rarely be confused with stroke, the differential diagnosis between stroke and hypoglycemia can be problematic. The blood sugar level in the patient presenting with coma or focal neurological deficit is obviously of crucial importance. If it is normal, hypoglycemia is unlikely. However, in the case of focal neurological deficit, the hypoglycemia may have been corrected by the patient's oral glucose intake before the neurological signs and symptoms resolve. When the patient is comatose, established hypoglycemia will be the likely cause. More difficulties arise when the patient presents with hypoglycemia and focal neurological deficits. Hypoglycemia can occasionally present with focal neurological deficit without any accompanying symptoms of hypoglycemia. Correction of blood glucose levels will generally lead to a rapid recovery of the deficit, but excluding a transient ischemic event might be impossible in these cases. In patients with previous strokes, hypoglycemia reproduces or worsens the previous deficit. When there are accompanying signs and symptoms of hypoglycemia, the balance will definitely shift toward hypoglycemia as a cause of the neurological deficit.

Thyroid Disease

Both hyperthyroidism and hypothyroidism can contribute to a cardioembolic source for stroke. The most frequent event is hyperthyroidism leading to atrial fibrillation, a factor that generally leads to a fivefold increase in risk of subsequent stroke and also a factor that, if it cannot be cured, leads to anticoagulation. Hypothyroidism can affect cardiac function and, thereby, the risk of intracardiac thrombus formation. Graves–Basedow disease is rarely associated with cerebral vasculitis, an entity also referred to as Hashimoto's encephalopathy. In a large cohort study of 47,573 subjects subclinical hypothyroidism was related to an increased risk of stroke only in subjects younger than 65 years.

Thyroid disease has not been firmly established as affecting the outcome of strokes. However, in general, a range of systemic diseases affect the outcome of stroke, and it seems appropriate to diagnose and treat thyroid disease promptly in patients in these circumstances.

Strokes have not been shown to lead to thyroid disease.

Thyrotoxicosis may feature in the differential diagnosis of stroke. Delirium and coma may be a feature of both, but the fever, tachycardia, hypotension, vomiting, and diarrhea should point toward thyrotoxicosis. Hyperthyroidism has been reported to lead to isolated corticospinal tract dysfunction, the mechanism of which is not known. Hypothyroidism may present with limb and gait ataxia as signs of cerebellar dysfunction. The onset is often more gradual than in strokes.

Parathyroid Disease

Hyperparathyroidism has been shown to contribute significantly albeit weakly to overall cardiovascular risk. Hyperparathyroidism contributes to hypertension. Patients with osteoporosis and compensatory hyperparathyroidism may have an increased risk of stroke due to this contribution. Hypoparathyroidism has not been identified as a risk factor for stroke, but it is related to MELAS syndrome. MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke) is a mitochondrial disorder related to a point mutation in the transfer RNALeu(UUR) gene. It is typified by mitochondrial (ragged red fiber) myopathy, epilepsy, lactate acidosis, and stroke-like episodes. Frequently, this syndrome is associated with hypothalamo–pituitary axis dysfunction, diabetes mellitus, and/or hyperthyroidism.

Parathyroid dysfunction has not been shown to affect the outcome of stroke or to be the result of stroke.

Hypercalcemia, whether as a result of hyperparathyroidism or secondary to malignancy, may be confused with a stroke when a patient presents with confusion, nausea, and vomiting. In hypercalcemia, there is fatigue, anorexia, constipation, increased urination, a short QT interval, and generally a serum calcium level higher than 2.9 mmol L^{-1} (11.5 mg dL^{-1}).

Hypothalamo–Pituitary Axis Disease

Hypopituitarism is associated with an increased risk of stroke, reflected in an excess mortality rate for cerebrovascular disease of 2.4. Because of the complexity of hormonal and metabolic disturbances in hypopituitarism, the causes of this excess mortality are

poorly defined. A prothrombotic tendency due to growth hormone deficiency (which tends to persist even when hormones are adequately substituted), Cushing syndrome, and secondary hyperthyroidism are among the factors that may be implied in the pathogenesis of strokes. In a population of pituitary adenomas this excess risk of stroke was also shown. It was shown not to be related to radiation therapy of the pituitary region.

Pituitary disease has not been shown to affect the outcome of stroke, although it may affect the chances of recurrence of stroke.

Pituitary apoplexy is the term that refers to a stroke in the pituitary gland. Such strokes can be either ischemic or hemorrhagic and are often complications of pituitary adenomas or the surgery or radiotherapy thereof. The condition is probably underdiagnosed given that the pathological correlate of apoplexy has a prevalence of 1–3% in autopsy studies. The clinical presentation is highly variable but should be suspected in any patient with severe headache, visual field defects, ophthalmoplegia, and/or altered mental status. In some cases, there is only headache at first. This headache is accompanied by nausea, vomiting, nuchal rigidity, fever, stupor, and coma (when blood and necrotic tissue leak and cause the features of subarachnoid hemorrhage or aseptic meningitis). In tumorous apoplexy, the destruction of the pituitary in most cases leads to hypopituitarism because most underlying adenomas are endocrinologically silent. In cases of endocrinologically active adenomas, there can be spontaneous resolution of preexisting endocrinopathy after apoplexy. The slowly evolving expansion of adenomas rarely leads to cranial nerve deficit because the nerves slowly lengthen in response to this expansion. In apoplexy (**Fig. 2**), there is sudden compression of cranial nerves due to hemorrhage or infarction and swelling. Compression of the optic nerve leads to visual field defects and decreased visual acuity, whereas compression of nerves III, IV, and VI leads to oculomotor disturbances. Compression of the trigeminal nerve may result in facial paresthesias and absent corneal reflex. Compression of the cavernous sinus may result in proptosis and eyelid edema. Finally, there can be Horner syndrome due to compression of the sympathetic chain and hyperpyrexia, and there can be diabetes insipidus or SIADH (syndrome of inappropriate antidiuretic hormone) when the hypothalamus is compressed.

The radiological diagnosis can be made on computed tomography (CT) if the stroke is hemorrhagic or if the adenoma is sufficiently large. The investigation of choice is MRI, which shows hemorrhage and underlying adenomas in great detail (**Fig. 3**). Infarction can be difficult to diagnose on routine magnetic resonance sequences. Diffusion-weighted images greatly increase the accuracy of the diagnosis of pituitary infarction. Peripheral enhancement of intrasellar masses can be another less specific sign. The hypothalamic–pituitary unit can be tested as outlined elsewhere. Pregnant women have a special predisposition to apoplexy due to the prothrombotic state related to pregnancy (and the resulting 12-fold increase in risk of stroke) and the dramatic enlargement of the pituitary gland due to the proliferation of prolactin-secreting cells. The classical features of postpartum pituitary apoplexy are absence of lactation, persistent amenorrhea, and lethargy. Other predisposing factors to pituitary apoplexy include bleeding disorders, anticoagulation, upper respiratory tract infections, trauma, carotid angiography, Cushing's disease, diabetes mellitus,

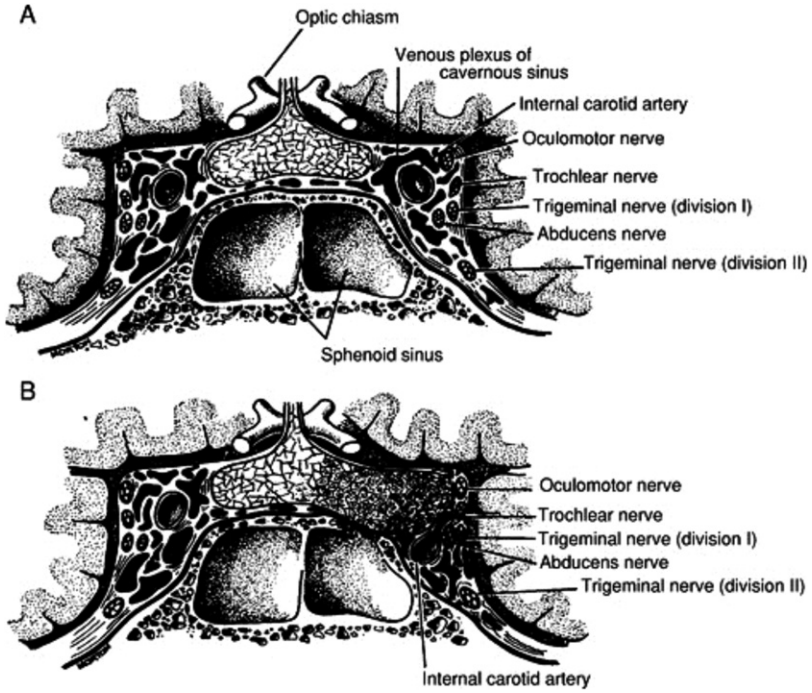

Fig. 2 (A) Posterior coronal view showing anatomical relationship of pituitary gland to optic chiasm superiorly, sphenoid sinus inferiorly, and cavernous sinus laterally. (B) Mechanism of acute compression of structures within cavernous sinus from sudden expansion of pituitary adenoma due to hemorrhage or infarction and edema. Note that the further the tumor has eroded the floor of sella turcica prior to apoplectic episode, the more likely it is that multiple structures within the cavernous sinus will be involved. Reproduced from Reid, R., Quigley, M., and Yen, S. (1985). Pituitary apoplexy. *Archives of Neurology* 42, 712–719.

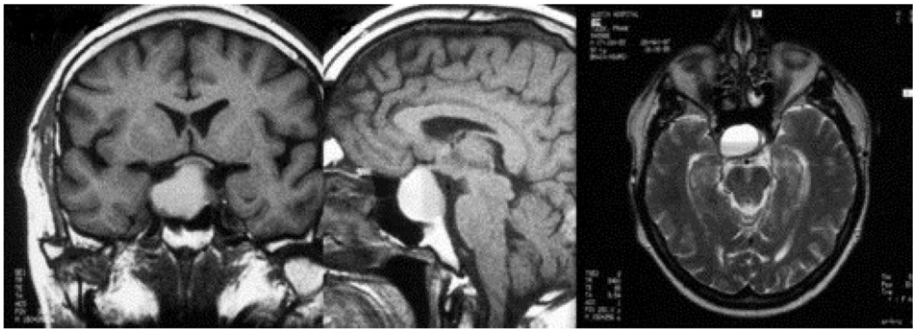

Fig. 3 Axial and sagittal T1-weighted and transverse T2-weighted MRIs showing a large hemorrhage into the pituitary gland. Courtesy of G. Fitt.

adrenalectomy, atherosclerosis, sickle cell trait, and acromegaly. Surgical intervention is advocated in the majority of patients, with radiotherapy a less likely option.

Less catastrophic is the effect of nonpituitary stroke on the hypothalamic–pituitary–adrenal (HPA) axis. Stroke is associated with increased activity of the HPA, manifested particularly by hypercortisolism. The normal regulation of cortisol secretion by adrenocorticotropic hormone (ACTH) is disturbed after stroke, a process in which cytokines seem to be implicated. Such a pathological HPA axis may exhibit an overriding function on established risk factors for cardiovascular disease, diabetes mellitus, and stroke such as abdominal obesity, hypertension, cholesterol, and triglycerides. Whether there is a causal relation between stroke and increased HPA axis activity needs to be further elucidated given that depression, anxiety, alcohol consumption, and smoking all have been shown to have similar effects. Also, changes in growth hormone, prolactin, and thyrotropin response to thyrotropin-releasing hormone have been reported, and some of these changes may be a consequence of the hypersensitive HPA axis. They may explain some of the insulin resistance after stroke. It has been postulated that stroke in the caudate nucleus interrupts neurotransmitter pathways involved in the control of secretion of gonadotropins. Although hypercortisolism and some of the other disturbances have been related to disorientation and levels of motor impairment, the clinical relevance of many of the changes remains uncertain. In general, stroke leads to an increase in antidiuretic hormone (ADH) levels. However, this usually does not lead to hyponatremia. Patients with subarachnoid hemorrhage are especially likely to develop a syndrome of inappropriate ADH secretion (SIADH). However, it is important to distinguish SIADH from cerebral salt wasting, which is the more likely explanation for hyponatremia in patients with subarachnoid hemorrhage.

Adrenal Gland Disease

The intermittent hypertension in pheochromocytoma increases the risk of stroke. In some cases, the pheochromocytoma is actually not identified until the patients present with a stroke. Also, Cushing's disease has been postulated to have an increased risk of stroke due to hypercortisolism or to the effects of treatment of Cushing's disease such as external pituitary irradiation and post-treatment hypopituitarism. There are indications that stroke leads to mild relative adrenal insufficiency.

See also: Hypertension and Target Organ Damage

Further Reading

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Normetanephrine and Metanephrine☆

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Glossary

Catecholamines Include norepinephrine, epinephrine and dopamine and exert their biological activity on adrenergic receptors.
Metanephrines *O*-methylated metabolites of norepinephrine and epinephrine and have no biological activity.
Catechol-*O*-methyl transferase (COMT) Is the enzyme responsible for the conversion of catecholamines into metanephrines. Metanephrines are sensitive biomarkers for the presence of chromaffin cell tumors such as pheochromocytoma and paraganglioma.
Pheochromocytoma and paraganglioma Tumors originating in the adrenal medulla and in the sympathetic ganglia respectively. They produce an excess of catecholamines and metanephrines

Introduction

The metanephrines normetanephrine (NMN) and metanephrine (MN) are *O*-methylated metabolites of the catecholamines norepinephrine (NE) and epinephrine (E). The common basic structure of both molecules is the methylated catechol ring (3-methoxy 4-hydroxy phenyl) in conjunction with an ethylamine group (Fig. 1).

In humans, the physiological enantiomers are L-NMN and L-MN. Metanephrine has a higher molecular weight (197) because, in contrast to NMN (183), it contains a methylated amino group. Metanephrines are not biologically active. The metanephrines provide markers of the extraneuronal metabolism of the parent catecholamines. In clinical medicine, they also provide useful diagnostic tools for patients suspected of having a tumor of chromaffin cells such as pheochromocytoma and paraganglioma. Both metabolites can be measured in plasma and urine. Measurement of conjugated metanephrines in urine is a long-standing and well-established technique. The emergence of a reliable test for plasma-free metanephrines and urinary free metanephrines has fostered a more extensive assessment of the metabolism of catecholamines.

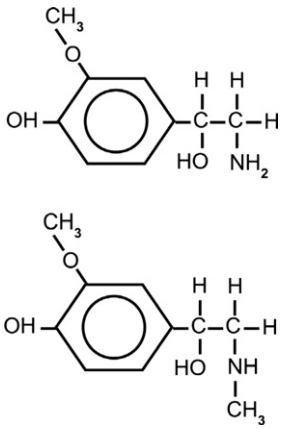

Fig. 1 The chemical structure of normetanephrine (*upper panel*) and metanephrine (*lower panel*).

☆*Change History:* October 2017. JWM Lenders and G Eisenhofer replaced Figs. 1 and 2 by more updated ones and an extra figure on the structure of metanephrines has been added. In the paragraph on Kinetics and metabolism, a short section has been added on the upper reference limits of plasma free metanephrines as related to age. At the end of the paragraph on Kinetics and metabolism, a short section has been added on the seasonal variations in plasma free metanephrines. In the paragraph on Measurement of metanephrines, three short sections have been added on the use of measurements of plasma and urinary metanephrines by the LC-MS/MS assay and made a few textual changes and one reference was added.

This article is an update of Jacques W.M. Lenders and Graeme Eisenhofer, Normetanephrine and Metanephrine, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 387-390.

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Source of Metanephrines

The exclusive source of metanephrines is the parent catecholamines NE and E. Catecholamines are biological active amines that play a pivotal role in the regulation of neuroendocrine and cardiovascular function (Lenders and Eisenhofer, 2014). NE functions as a neurotransmitter in the sympathetic nervous system, whereas E is a hormone released from the adrenal medulla. Sympathetic nerves release NE by exocytosis from neuronal vesicular stores into the synaptic cleft. NE leaking from the intraneuronal vesicular stores is converted to 3,4-dihydroxyphenylglycol (DHPG) by the mitochondrial-bound enzyme monoamine oxidase (MAO) (Eisenhofer *et al.*, 2004). Approximately 90% of the NE released into sympathetic neuroeffector junctions is recaptured by neuronal NE transporters (uptake-1) (Eisenhofer, 2001). Some of this is also deaminated by MAO to DHPG, but most is sequestered into storage vesicles for re-release. Of the approximately 10% of neuronally released NE that escapes reuptake, approximately half is removed by extraneuronal uptake (uptake-2), leaving the other half to reach the systemic circulation. After extraneuronal uptake, the NE is metabolized by catechol-*O*-methyltransferase (COMT) to NMN and then by MAO to 3-methoxy-4-hydroxyphenylglycol (MHPG) (Eisenhofer *et al.*, 2004). A small amount of NMN is not metabolized by MAO and enters the bloodstream. Smaller amounts of NMN are formed from extraneuronal metabolism of NE that escapes into the blood stream. Extraneuronal uptake and metabolism accounts for approximately 20% of the total turnover of NE, but only a small proportion of this is represented by circulating and urinary levels of NMN (Eisenhofer *et al.*, 1995b).

COMT is not located in neurons but is found in nonneuronal tissues, such as liver, muscles, and kidneys. An additional important location of COMT is the adrenal medulla, in which the enzyme is responsible for the conversion of E to MN and that of NE to NMN. Circulating E is also subject to uptake-2, but $<10\%$ of MN is derived from this source. $>90\%$ of circulating MN and 23%–40% of circulating NMN are derived from *O*-methylation of E and NE within adrenal medullary chromaffin cells (Eisenhofer *et al.*, 1995a; Eisenhofer *et al.*, 1995b). The importance of the adrenal medulla for production of MN and NMN reflects the fact that COMT is predominantly present in this tissue as the membrane-bound enzyme, an isoform of COMT with particularly high affinity for catecholamines. Therefore, plasma metanephrines are specific markers for extraneuronal and intra-adrenal metabolism of catecholamines, whereas plasma DHPG is a specific marker for neuronal metabolism of catecholamines. From a quantitative standpoint, the neuronal pathway of metabolism greatly predominates over extraneuronal and intra-adrenal pathways for metabolism of NE, whereas the extraneuronal and intra-adrenal pathways are more important for metabolism of E.

Kinetics and Metabolism of Metanephrines

Metanephrines are present in plasma and urine in the free and conjugated form. In plasma, the levels of free plus conjugated metanephrines are approximately 20-fold higher than the levels of the free (unconjugated) metanephrines (Lenders *et al.*, 1995; Pamporaki *et al.*, 2013). In human urine, most metanephrines ($>80\%$) are in the conjugated form. Conjugation of metanephrines by the specific sulfotransferase isoenzyme, SULT1A3, represents an important route of NMN and MN metabolism (Eisenhofer *et al.*, 2001). This enzyme is located mainly in extraneuronal tissue of the mesenteric organs, particularly the wall of the gut. These conjugates are excreted by the kidneys. Other routes of metabolism of free metanephrines involve their conversion by MAO to MHPG. MHPG is further oxidized in the liver by alcohol dehydrogenase to the final end product of catecholamine metabolism, vanillylmandelic acid (**Fig. 2**) (Goldstein *et al.*, 2003).

Plasma levels of free metanephrines in healthy subjects under resting baseline conditions average approximately 0.60 nmol L^{-1} for NMN and 0.30 nmol L^{-1} for MN (Lenders *et al.*, 2002). In contrast to plasma levels of MN, those of NMN increase with age

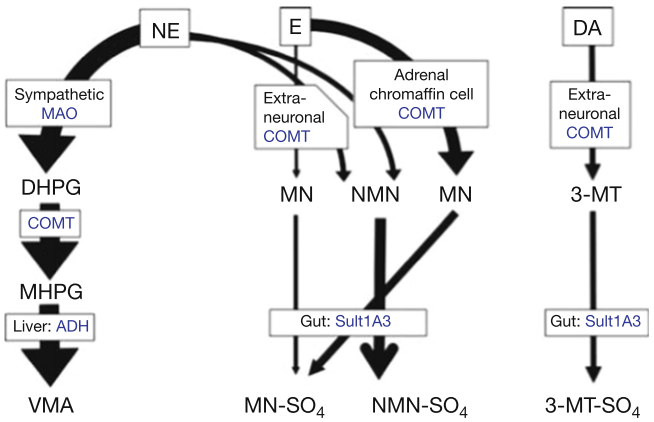

Fig. 2 Diagram showing the major metabolic pathways of norepinephrine (NE), epinephrine (E) and dopamine (DA). The main metabolic pathway for NE is deamination by monoamine oxidase (MAO) within sympathetic nerves to dihydroxyphenylglycol (DHPG) which is the basis for the final metabolite vanillylmandelic acid (VMA). A small part of NE is converted by catechol-*O*-methyltransferase (COMT) extraneuronally and intra-adrenally into normetanephrine (NMN). Epinephrine is mainly converted in the adrenal medulla to metanephrine (MN). Extraneuronal conversion of E contributes to a minor degree to MN formation. Dopamine is *O*-methylated by COMT to 3-methoxytyramine (3-MT). Both metanephrines and 3-MT are conjugated by gut sulphotransferase type 1A3 (Sult1A3) to sulphates ($-SO_4$).

while plasma metanephrine is 30% higher in female than in male subjects. The upper reference limit of plasma MN is 0.45 nmol L^{-1} while that of NMN increases from 0.47 below the age of 18 years to 1.05 nmol L^{-1} over the age of 60 years (Eisenhofer *et al.*, 2013). The plasma half-lives of free (unconjugated) metanephrines are similar to those of catecholamines (approximate 3 or 4 min) (Eisenhofer and Lenders, 2012; Eisenhofer *et al.*, 1995b). The plasma clearances of free NMN and MN are approximately 1.5 L min^{-1}, slightly lower than that of catecholamines (2 L min^{-1}). In contrast, the plasma half-lives of conjugated NMN and MN are much longer (>60 min) and their circulatory clearances are much lower (0.1 L min^{-1}). Since the circulatory clearance of conjugated metanephrines is determined almost exclusively by their extraction by the kidneys, plasma levels of conjugated metanephrines do not always reflect production of free metanephrines. For example, plasma levels of conjugated metanephrines are substantially increased in patients with renal failure, whereas those of free metanephrines are only mildly affected (Eisenhofer *et al.*, 2005).

Plasma levels of metanephrines increase during activation of the sympathetic nervous and/or adrenomedullary hormonal systems. However, increases in plasma levels of NMN and MN above baseline levels are relatively less than those of their parent catecholamines (Eisenhofer *et al.*, 1995a). Even during intense adrenomedullary stimulation induced by hypoglycemia, plasma MN levels increase only 3-fold, in contrast to a 25-fold increase in plasma E levels (Eisenhofer *et al.*, 1998). During less intense stressful conditions, such as mental stress, the responses of catecholamines are proportionally much greater than those of metanephrines. Consequently, metanephrines are inferior to catecholamines for gauging stress responses. Recently it was shown that there is a seasonal variation in plasma NMN levels with higher levels in colder than warmer seasons. This reflects partly an increased sympathetic activity associated with a lower ambient temperature (Pamporaki *et al.*, 2014).

Measurement of Metanephrines

Measurements of metanephrines in urine have been available for clinical purposes since the early 1960s (Manu and Runge, 1984). Historically, the term total metanephrines was coined to indicate the combined measurement of both NMN and MN by early spectrophotometric assays that did not allow separate measurement of NMN and MN. The development of high pressure liquid chromatography (HPLC) techniques has allowed separate measurement of both amines, hence the term fractionated metanephrines. However, urinary measurements of NMN and MN are usually carried out after subjecting samples to acid hydrolysis or enzymatic deconjugation with sulfatase, which liberates the free from conjugated metanephrines. These measurements therefore reflect levels of both the free and conjugated forms of NMN and MN. Specific measurements of urinary free metanephrines has become feasible with the advent of liquid chromatography with mass spectrometric detection (LC-MS/MS) (Boyle *et al.*, 2007).

In plasma, metanephrines can be measured as free metanephrines or as free plus conjugated metanephrines by HPLC with electrochemical detection (ED) (**Fig. 3**) or by LC-MS/MS (Lagerstedt *et al.*, 2004; Lenders *et al.*, 1993; Peitzsch *et al.*, 2015). LC-MS/

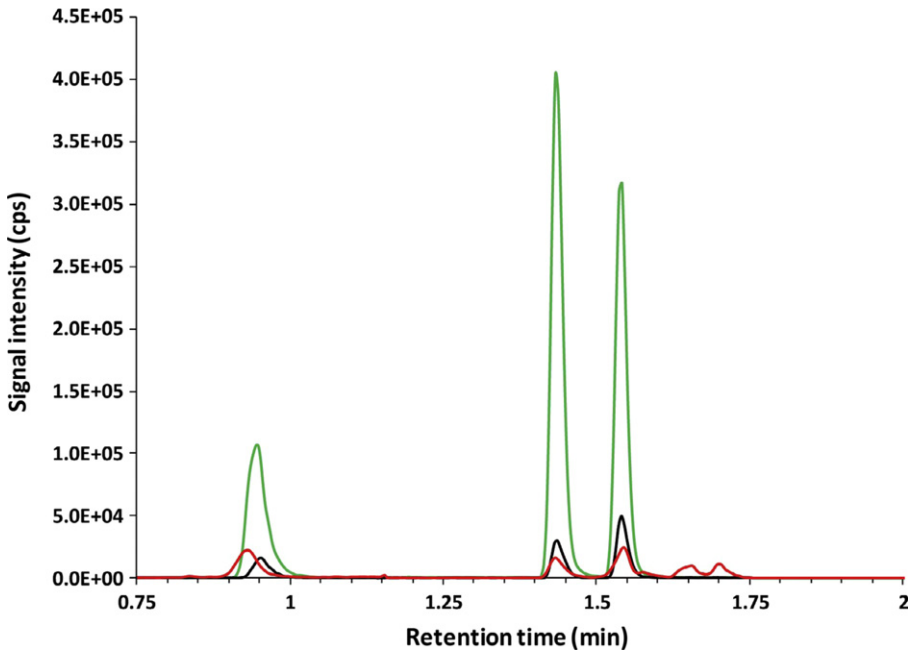

Fig. 3 Peaks of normetanephrine (NMN) at a retention time of 0.9 min and of metanephrine (MN) at a retention time of 1.4 min. The green curve represents the internal standard (500 pg mL^{-1} each), the black line represents the calibrator (50 pg mL^{-1} each) and the red line represents a sample of healthy subject (NMN: 99 pg mL^{-1} and MN 24 pg mL^{-1}). The third peak at the retention time 1.53 min is methoxytyramine, the *O*-methylated metabolite of dopamine.

MS is nowadays the preferred assay method since it provides a higher specificity and suffers from less analytical interference of drugs than HPLC-ED. In addition, it is more cost-effective. In view of the much lower plasma concentrations of free metanephrines as compared to the conjugated metanephrines, the required analytical sensitivity is much higher for free metanephrines. Many factors should be considered as potential sources of falsely high or falsely low test results. Despite the fact that plasma metanephrines are less sensitive than plasma catecholamines to sympathoadrenal excitation, sympathoadrenal excitation must still be considered a potential cause of falsely high test results (Lenders *et al.*, 2002). Analytical interference from drugs depends on the type of assay and is usually more frequent for HPLC-ED than LC-MS/MS assays. Pharmacokinetic interference, resulting in falsely-positive results of plasma or urinary metanephrines can be caused by several drugs such as tricyclic antidepressants and sympathomimetics (Lenders *et al.*, 2014). Conversely, COMT inhibitors and central sympatholytic drugs may lower NMN and MN levels. Despite the availability of high specific assays, caution for drug interference remains warranted in the interpretation of test results.

Application of Measurement of Metanephrines

The development of plasma metanephrines has enabled investigators to obtain more detailed and comprehensive insights into the metabolism of catecholamines. This has led to several promising applications. First, measurement of plasma free metanephrines provides a test for the diagnosis of pheochromocytoma with the highest sensitivity (Lenders *et al.*, 2002). The abundant presence of COMT in pheochromocytoma tumor tissue is responsible for a continuous intratumoral conversion of catecholamines to metanephrines (Eisenhofer *et al.*, 2004). These metanephrines diffuse to the circulation, independently of the release of catecholamines by the tumor. >94% of the increased circulating levels of NMN and MN in patients with pheochromocytoma is derived from catecholamine metabolism within tumor cells (Crout and Sjoerdsma, 1964; Eisenhofer *et al.*, 1998). Thus, only small increases in NMN and MN levels indicate metabolism of catecholamines after their secretion by the tumor into the circulation. This largely explains why plasma free metanephrines have an approximately 100% sensitivity. Elevations of plasma metanephrines in patients with pheochromocytoma show larger relative increases above normal levels compared to the parent catecholamines. This also contributes to the higher diagnostic sensitivity of measurements of free metanephrines over catecholamines (Lenders *et al.*, 2002). The continuous production of metanephrines within the tumor tissue also explains why levels of plasma metanephrines are less apt to increase compared to plasma catecholamines during paroxysmal catecholamine release from a pheochromocytoma or during surgical manipulation of the tumor.

Other applications of plasma free metanephrines include a role in the assessment of COMT activity. In conjunction with measurements of plasma DHPG levels, measurements of free NMN can also be used to assess the activity of MAO: An increased NMN/DHPG ratio indicates inhibition of MAO and can also be used to establish MAO-deficiency states (Lenders *et al.*, 1996). Finally, since plasma free MN is almost completely derived from intramedullary conversion from adrenomedullary E, plasma MN levels can serve as a marker of the adrenomedullary E stores (Merke *et al.*, 2000).

See also: Pheochromocytoma/Paraganglioma: Diagnosis and Treatment

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Adrenergic Mechanisms☆

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Glossary

Glucosensitive, thermosensitive, and barosensitive fibers These words refer to sympathetic nerve fibers that respond to alterations in blood glucose, temperature and blood pressure respectively.

Introduction

The autonomic nervous system regulates many bodily functions. It is a classical control system with an input function, provided by afferents from various parts of the body, a central processor and output responses mediated by parasympathetic and sympathetic fibers as well as the adrenal medulla. Parasympathetic fibers can be afferent and efferent; sympathetic fibers are always efferent. The sympathetic system, in conjunction with its central nuclei and the adrenal medulla, is also known as the adrenergic system (AS). The AS comprises all neurons which use norepinephrine or epinephrine as transmitter substance.

The central part of the AS consists of nuclei located, amongst others, in the cerebral cortex, the hypothalamus, the periaqueductal gray of the midbrain, the ventrolateral pons with the area postrema at the floor of the fourth ventricle, the nucleus tractus solitarii (NTS), the caudal ventrolateral medulla (CVLM), the rostral ventrolateral medulla (RVLM) and the intermediolateral (IML) region of the T1–T5 spinal cord. The hypothalamus and the NTS are the main sites where autonomic functions such as the regulation of metabolism, body temperature and blood pressure are integrated.

The efferent AS consists of preganglionic neurons originating in the thoracolumbar area of the spinal cord and their postganglionic connections. Functionally, these neurons show some differentiation in the sense that when the sympathetic system is activated, this does not always produce the same effector response but one which is required to restore the primary functional disturbance.

Acetylcholine and the catecholamines norepinephrine (NE) and epinephrine (EPI) serve as the main neurotransmitters within the AS. Acetylcholine is the neurotransmitter at preganglionic sites in both sympathetic and efferent parasympathetic fibers. It is also the neurotransmitter in postganglionic parasympathetic neurons and at the interface between sympathetic fibers and the adrenal medulla. NE acts as transmitter in postganglionic sympathetic fibers, which are also known as adrenergic neurons. EPI is synthesized only in adrenal medullary cells.

At the level of the target organs the neurotransmitters react with specific receptors which can be classified as alpha-1, alpha-2, beta-1, beta-2 and beta-3 adrenoceptors.

Central Adrenergic Pathways

Adrenergic influences on neuroendocrine/autonomic function originate in NE-synthesizing cell groups referred to as A1, A2, and A5, located in the CVLM, the NTS, and the ventrolateral pons, respectively on the one hand and in EPI-synthesizing neurons that are found in the C1, C2, and C3 groups, located in the RVLM, the NTS, and the rostral dorsomedial medulla on the other (Jänig, 2008). The A2/C2 neurons are innervated by primary visceroceptive afferents carried by the vagal and the glossopharyngeal nerves that convey information from mechano- and chemoreceptors related to cardiovascular, pulmonary, and gastrointestinal systems. Neurons in the NTS that receive visceral afferents in turn project to A1 and C1 neurons. Descending projections from C1 cells to the spinal cord innervate sympathetic preganglionic neurons and so can directly activate catecholamine-mediated responses to visceral changes. Ascending projections from A1/C1 and A2/C2 cells travel through the ventral adrenergic bundle and terminate in several limbic nuclei and hypothalamic areas. AS terminals are found in the ventromedial, arcuate, dorsomedial, and lateral hypothalamic nuclei, and a particularly dense innervation reaches the paraventricular hypothalamus (PVH). Neurons in these nuclei (particularly in the paraventricular and lateral hypothalamic area) are in turn connected to (1) the pituitary (e.g., driving pituitary–adrenal and pituitary–thyroid activities), (2) mesencephalic and prosencephalic limbic nuclei (thereby influencing the expression of behavioral, learning/memory, and emotional responses), (3) premotor and preganglionic autonomic neurons (thereby controlling autonomic function), and (4) A1/C1/A2/C2 neurons (where they can modulate visceral reflexes mediated by these cells). Other hypothalamic, hypophysiotropic (e.g. somatostatin, luteinizing hormone-releasing hormone) and

☆*Change History*: December 2017. Peter W de Leeuw and Abraham A Kroon have completely rewritten the text.

This chapter is an update of José Marino-Neto, Marcelo Sabi and Marta Aparecida Paschoalini, Adrenergic Mechanisms, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 106–111.

neurosecretory (arginine vasopressin and oxytocin) neurons are also innervated by NE/EPI terminals. It is also important to point out that although terminals from A6 (the locus ceruleus) NE-synthesizing neurons are ubiquitous in the central nervous system, these neurons have limited direct input to the hypothalamic circuitry. The locus ceruleus receives a major, essentially inhibitory innervation from EPI- and NE-producing medullary neurons, through which the latter gain access to a "brain-wide web" of NE-mediated modulation of cognitive and arousal functions as well as to indirect influences on neuroendocrine mechanisms through prosencephalic limbic nuclei.

Abundant evidence from experimental studies has shown that several adrenergic cell types are sensitive to local changes in glucose levels with a fall in glucose provoking eating and a rise in glucose. These neurons, therefore, are in a perfect position to mediate metabolic and behavioral responses to central (or systemic) changes in glucose levels and/or energy status.

A1/C1 and A2/C2 cells are also activated by other stressful situations such as hemorrhage, immune challenge, noise and restraint. In each case, activation of medullary adrenergic systems occurs either directly through visceral afferents or after processing of the stressful stimulus at a higher level. Thus, these circuits may be of importance under different conditions of stress and energy demand.

The PVH is by far the most sensitive area with respect to feeding-related AS activity. Feeding responses are mediated by central alpha-2 adrenoceptors, whereas activation of central alpha-1 adrenoceptors suppresses food intake. As far as adrenergic systems are concerned, the lateral hypothalamic nuclei mainly have a hypophagic effect, mediated by central beta-2 adrenoceptors.

Another AS circuitry that is of importance is the one which is related to control of the cardiovascular system. Again, available information rests primarily on observations in animals. Cardiovascular effector responses use either fast transmitters (such as glutamate) or slow transmitters (such as NE). Ascending noradrenergic neurons (classified as A1–A7) are found mainly in the locus ceruleus; most descending fibers stem from A5 and A6 + A7 neurons. EPI-using neurons of the C1 type are most prevalent in the RVLM while C2 neurons are located close to the NTS (Korner, 2007). Numerous experiments have been performed to pinpoint which structures in the brain are responsible for which cardiovascular responses. By and large, the RVLM as well as hypothalamic nuclei and several other centers which synapse in RVLM neurons determine resting sympathetic tone.

Although it is virtually impossible to study central adrenergic systems in man, an interesting observation comes from Australian investigators who sampled blood simultaneously from both jugular veins in patients with essential hypertension. Using a sophisticated technique, they were able to measure NE spillover from nerve endings into the blood. As only one of the two jugular veins drains blood from the hindbrain and hypothalamus, the NE spillover which the investigators measured in this vessel, is thought to reflect the activity of central adrenergic neurons in these areas. The data show that there is a close relationship between central noradrenergic neuron activity and peripheral sympathetic activity (Ferrier *et al.*, 1993).

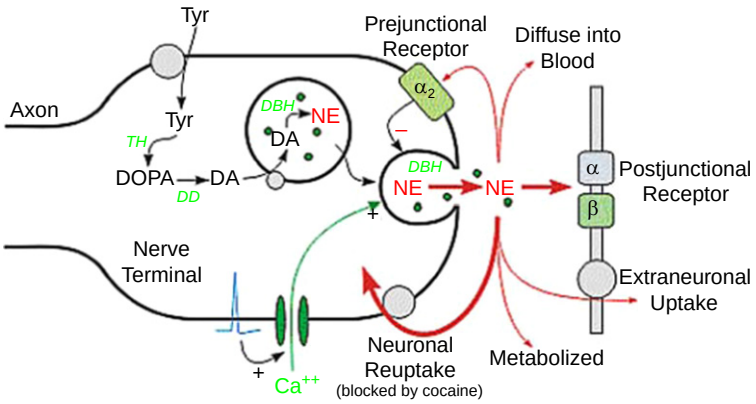
Peripheral Adrenergic Neurotransmission

The information carried forward through preganglionic parasympathetic and sympathetic fibers is first relayed to postganglionic fibers and then via a neuroeffector junction to specific target organs. The adrenal medulla is directly innervated by fibers from the IML nucleus of the thoracolumbar segments of the spinal cord.

The neurotransmitter NE is synthesized in the axoplasm from tyrosine which is actively transported into the nerve. The enzyme tyrosine hydroxylase which converts tyrosine into DOPA is the rate-limiting step in the biosynthesis of NE and EPI. Cytoplasmic enzymes convert DOPA to dopamine and this, in turn, is converted to NE by dopamine beta-hydroxylase (DBH). In adrenal medullary cells NE is further converted to EPI by the enzyme phenylethanolamine-*N*-methyltransferase (PNMT). In the nerve terminals NE is kept in storage vesicles which fuse with the plasma membrane and liberate their NE by exocytosis as soon as an action potential has caused an influx of calcium into the cell.

When NE is released from the nerve terminals, its fate and effect are determined by a complex array of mechanisms. First, only a part of the neurotransmitter activates the adrenoceptors in the membrane of the postsynaptic cell as synaptically released NE is subject to active re-uptake into the nerve terminal (uptake 1) by the norepinephrine transporter (NET), a process which can be blocked by cocaine and a variety of antidepressant drugs. Second, the NE that penetrates the target cell (uptake 2) is rapidly inactivated by monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT). A third mechanism that is important is the potential of NE to stimulate presynaptic alpha-2 receptors which inhibit further exocytotic release of the neurotransmitter and beta-adrenoceptors (presumably of the beta-2 subtype) which enhance its release. Finally, recent research has shown that several different mediators are co-transmitted at the same time (Macarthur *et al.*, 2011). Indeed, neuropeptide Y (NPY) and adenosine triphosphate (ATP) are co-localized with NE in the storage vesicles and are released at the same time as the "classical" neurotransmitter NE. Both substances activate postsynaptic receptors which lead to amplification of the effects of NE. In addition, they may inhibit their own release as well as that of NE through activation of presynaptic receptors on the nerve terminal (**Fig. 1**).

The most powerful mechanism of the body to regulate the effects of adrenergic stimulation, is by re-uptake 1. This is exemplified by the fact that inhibitors of this process can markedly potentiate the adrenergic symptoms while inhibitors of the uptake-2 mechanism have little effect. Moreover, there is evidence to suggest that prejunctional modulation of NE is more dependent upon the endogenously released NE than on its co-transmitters (Macarthur *et al.*, 2011). Apart from these sympathetic mediators, several other substances such as angiotensin II, nitric oxide, endothelin and prostaglandins may



Tyr = tyrosine; TH = tyrosine hydroxylase; DD = DOPA decarboxylase;
DA = dopamine; DBH = dopamine β-hydroxylase; NE = norepinephrine

Fig. 1 Schematic representation of adrenergic neurotransmission at effector sites. Reproduced with permission from: http://cvpharmacology.com.

modulate the degree of neurotransmission. All these modulating factors make that the final response to an adrenergic stimulus is difficult to predict.

Sympathetic Efferents

The sympathetic nervous system can be viewed upon as a highly organized and differentiated system which transmits the final "order" that comes from the central nuclei, after they have integrated all afferent information, to the relevant effector organs. Neurophysiological studies in various species, including man, indicate that sympathetic neurons exhibit substantial differentiation with respect to their target cells and thus, their function (Janig and Habler, 2003). In other words: when a certain physiological function gets disturbed, the autonomic response, mediated through the sympathetic fibers, will be specifically directed towards the variable that needs to be regulated and will, in general, not affect other systems. Based on the final physiological responses, the system functionally consists of three main types of fibers: those that are involved in metabolic regulation (glucosensitive efferents), in the regulation of body temperature (thermosensitive efferents) and in the regulation of the cardiovascular system (barosensitive efferents).

Glucosensitive Efferent Adrenergic Mechanisms

Glucosensitive efferents are involved in the autonomic response to hypoglycemia and physical exercise or a state of negative energy balance. Data obtained in experimental animals suggest that NPY-containing neurons in the hypothalamus, particularly in the arcuate and paraventricular nuclei, can sense changes in the concentrations of blood glucose and insulin. In addition, glucosensing elements have been identified in the area postrema and the NTS. When a fall in blood glucose is perceived, downstream integrative networks and motor neurons activate the adrenergic system and stimulate the adrenal medulla to release EPI. Through sympathetic nerve fibers, the central neurons also stimulate the release of growth hormone and corticotrophin-releasing hormone which, in turn, causes the anterior pituitary to secrete more ACTH. Finally, the autonomic system stimulates the liver to enhance gluconeogenesis and pancreatic cells to increase glucagon and reduce insulin output (Thorp and Schlaich, 2015; Watts and Donovan, 2010).

Insulin can bind to specific insulin receptors on central NPY-neurons and in doing so, may exert an inhibitory effect on appetite and energy expenditure. Conversely, a lack of insulin signaling in these NPY-neurons leads to increased energy stores and an obese phenotype (Loh *et al.*, 2017). Although very little is known about these mechanisms in man, a post-mortem study showed that in patients with type II diabetes NPY immunoreactivity is increased in the arcuate nucleus of the hypothalamus (Saderi *et al.*, 2012). This seems to suggest that in type II diabetes, the NPY neurons act as if glucose levels are lower than normal. It could explain also why diabetic patients have an ongoing increased sympathetic tone with all its metabolic and vascular consequences (Thorp and Schlaich, 2015).

Whereas central NPY neurons also play a role in appetite and eating behavior, it is largely unknown how and to what extent adrenergic mechanisms are involved in humans.

When hypoglycemia occurs (e.g. after too much insulin has been administered), it is primarily the adrenal medulla that is activated (by the glucosensitive system) with a relatively mild response of the other components of the adrenergic system. Accordingly, plasma levels of EPI rise far more than those of NE.

Thermosensitive Efferent Adrenergic Mechanisms

The maintenance of body temperature during thermal challenges is of paramount importance for survival. Within the brain, the hypothalamic preoptic area (POA) serves as the main site of thermoregulation. Thermal threats to the body include changes in both ambient temperature and in internal temperature due to e.g. exercise or illness. Again, most of our understanding of thermoregulation is based on experiments in animals but there are good reasons to believe that the situation is not much different in humans.

Exposure of the body to cold activates two basic mechanisms, both of which are mediated by the sympathetic system: cutaneous vasoconstriction to prevent heat loss and thermogenesis (production of heat) by brown adipose tissue (Morrison, 2016). Constriction of skin vessels by adrenergic fibers is accompanied by visceral arterial and venous dilation to increase core blood flow. Stimulation of brown adipose tissue leads to an increase in energy expenditure due to enhanced oxidation of fatty acids and glucose. Thermogenesis, therefore, is determined largely by the degree of adrenergic outflow to brown adipose tissue and the degree of NE binding to beta-3 adrenoceptors on the cell membrane of brown adipocytes. Although the brown fat was initially seen as a thermoregulatory organ in rodents and small mammals only, it is now known that this thermoregulatory effector system is also important in humans (van Marken Lichtenbelt *et al.*, 2009). Except by cold, thermosensitive efferents are also activated by, for instance, emotional stimuli and hyperventilation.

Basically, opposite reactions occur during heat stress, although the mechanism of cutaneous vasodilation is less well understood (Smith and Johnson, 2016). At first sight, one would think that the sweating response to heat is also adrenergic in nature. While this response certainly involves sympathetic fibers, these are cholinergic rather than adrenergic.

Barosensitive Efferent Adrenergic Mechanisms

By far the largest part of the sympathetic system is concerned with the control of the cardiovascular system. Although an adequate tissue perfusion is the most important aim of circulatory regulation, it is the mean arterial pressure (MAP) that mainly determines this tissue perfusion. From that perspective, it is not too surprising that systemic blood pressure is the primary regulated variable. Cardiac output (CO) and total peripheral vascular resistance (TPVR) together determine MAP according to the formula: $MAP = CO \times TPVR$. Besides these purely hemodynamic factors, the filling of the arterial system is also of importance (Guyton, 1980). Sympathetic fibers run to blood vessels and the heart to cause vasoconstriction and an increase in cardiac performance respectively. In addition, they innervate the kidney at several levels to promote, for instance, renin release and tubular reabsorption of sodium and water.

The afferent information that these peripheral adrenergic fibers respond to mainly comes from baroreceptors which, in fact, are more like mechanoreceptors because they do not measure the pressure but rather arterial distension. Arterial (high-pressure) baroreceptors are located in the carotid sinus area and in the aortic arch. Low-pressure baroreceptors can be found in large systemic veins and at several places in the cardiopulmonary circulation, including the right atrium and ventricle of the heart. Via the glossopharyngeal and vagal nerves, the information from the baroreceptors reaches the central nuclei which integrate the information from the various parts of the body. These nuclei are located in the NTS, the CVLM and the RVLM.

As a short-term feedback loop, the baroreceptor reflex is the primary homeostatic system to compensate for sudden changes in systemic blood pressure. Its most important task is to prevent a fall in blood pressure which may occur, for instance, during standing or because of blood loss or forced vasodilation. Under normal conditions, the arterial baroreceptors exert a tonic inhibitory influence on sympathetic outflow via central alpha-2 adrenoceptors. In fact, the NTS stimulates the CVLM and this nucleus, in turn, inhibits sympathetic outflow from the RVLM (**Fig. 2**). However, when blood pressure at the level of the carotid baroreceptors falls (either because systemic pressure is too low or because of a sudden reduction in blood flow through the carotid artery), the stretch on the baroreceptors diminishes. Their firing rate decreases (so-called unloading of the receptors) and the inhibitory effect on sympathetic tone wanes off. The ensuing increase in adrenergic tone leads to arteriolar and venular vasoconstriction, increased cardiac contractility and tachycardia. The venular constriction will increase venous return (preload) to the heart, while arteriolar vasoconstriction will raise resistance and hence blood pressure. As pointed out by Guyton, the gain of the baroreceptor reflex is less than zero, meaning that this reflex does not fully restore the change in pressure (Guyton, 1980). This makes sense because then adrenergic stimulation will continue until the challenging threat is over. Otherwise, blood pressure would fall again as soon as it would have reached its original value. The opposite chain of events occurs in case the pressure rises, e.g. when an endogenous signal such as stimulation of the renin–angiotensin system increases vascular resistance.

Stimulation or inhibition of the adrenergic system is paralleled by inhibition or stimulation respectively of the cholinergic system. Thus, there always exists a delicate balance between the activities of the sympathetic and parasympathetic system.

It should be stressed that barosensitive efferent activity is not solely dependent upon the input from the arterial baroreceptors in the carotid as similar influences come from baroreceptors in the aortic arch. Moreover, barosensitive efferent output is also modified by the activity of the low-pressure receptors. The role of the latter is primarily to maintain volume control.

Finally, chemoreceptors, located also in the carotid body and the aortic arch, may influence cardiovascular adrenergic activity. For instance, when partial oxygen pressure in arterial blood falls, the firing rate of chemoreceptors will be augmented and this, in turn, will raise adrenergic activity to the cardiovascular system. Although the afferents also project onto the NTS, signals are relayed from the NTS directly into the RVLM, thus bypassing the inhibitory influence of the CVLM.

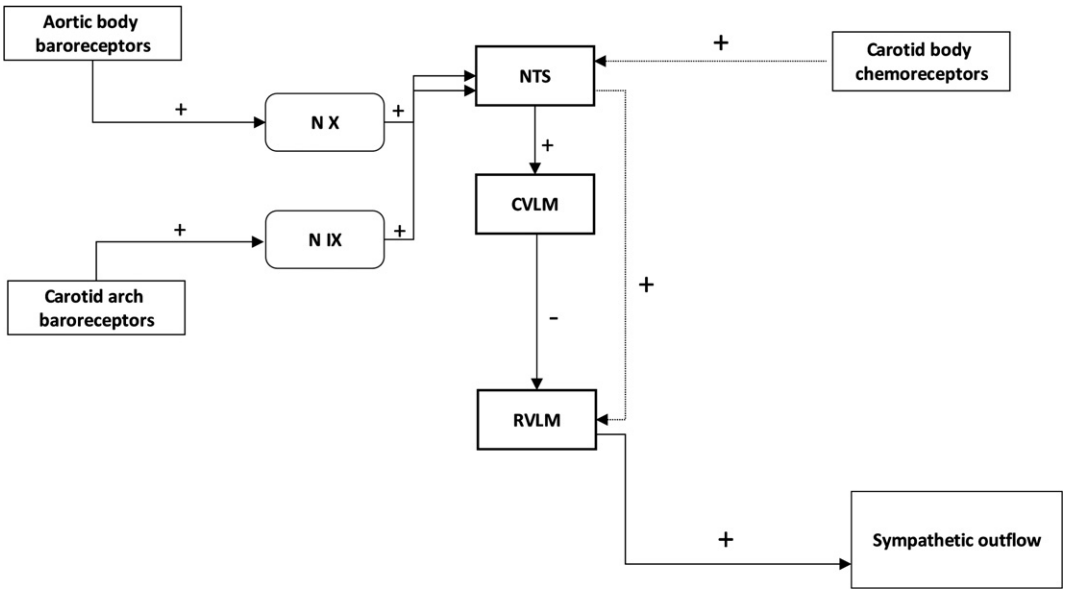


Fig. 2 Baroreflex control of the circulation. *N*, nervus; *NTS*, nucleus tractus solitarii; *CVLM*, caudal ventrolateral medulla; *RVLM*, rostral ventrolateral medulla. For explanation: see text.

Clinical Significance of Adrenergic Mechanisms

It will come as no surprise that the adrenergic system with its vast array of functions plays a significant role in various disease processes. First, the adrenergic system is needed to maintain homeostasis during periods of stress to the body. When an individual is confronted with a serious drop in pressure or even circulatory shock due to, for instance, hypovolemia (massive blood loss), reduced cardiac output (myocardial infarction) or redistribution of blood volume (sepsis), activation of the barosensitive adrenergic efferents is necessary to restore blood pressure and peripheral perfusion. Usually, the sympathetic activation will also cause tachycardia, increased transpiration and a number of other symptoms such as pupillary dilation and agitation. In the case of hypovolemia or myocardial infarction, cutaneous vasoconstriction and increased sweating are usually apparent and the patient feels cold. However, septic shock is often associated with cutaneous vasodilation and less perspiration and the patient feels warm. This points towards some degree of selective activation of the three efferent adrenergic pathways. Also, during severe surgical stress there may be a slight increase in blood glucose which is probably mediated, at least in part, by glucosensitive efferents.

While in many instances adrenergic compensation works well to help the individual survive, a problem may arise when the insult is so strong that adrenergic activation (causing vasoconstriction) comes into conflict with the process of autoregulation which strives to vasodilation when perfusion pressure falls (Guyton, 1980). It will depend upon the severity of disease how tissue perfusion will then be affected. Also, In the case of heart failure, the adrenergic overdrive may be so overwhelming that the heart will no longer be able to cope with the high afterload (due to the massive vasoconstrictor response). Subsequently, a vicious cycle starts in which the heart increasingly fails with an ultimately fatal outcome.

Whereas the adrenergic system is meant to serve a protective role in disease states, there are situations where the system itself is part of a disease process. Logically, there are two ways the adrenergic system can be involved in pathological processes. Either its activity is too low or too high. In both cases the disturbance may be due to a primary defect in the sympathetic fibers or to an otherwise adequate response to a pathological afferent signal. In the following, a few examples will be presented to illustrate these possibilities. In nearly all cases an abnormal blood pressure is the presenting symptom.

Insufficient Adrenergic Activity

The "simplest" form of adrenergic dysfunction is due to neurodegenerative disease in which various parts of the autonomic system may be affected separately or collectively. This condition is called pure autonomic failure (PAF) and it is caused by abnormal accumulation of the protein alpha-synuclein in nerve cells. When the central nervous system is involved as well, patients may have Parkinson's disease, multiple system atrophy or Lewy body dementia. Some of the other causes of adrenergic dysfunction may be diabetic neuropathy, auto-immune disease, amyloidosis and intoxications.

The most conspicuous symptom of adrenergic failure is orthostatic hypotension, i.e. an excessive fall in blood pressure upon standing. In true PAF, the orthostatic fall in pressure does occur without any increase in heart rate, reflecting the defective innervation of the heart. There is, however, another syndrome which is characterized by only a tendency to orthostatic hypotension but with an excessive heart rate. This syndrome is called postural orthostatic tachycardia syndrome (POTS). Probably, the

accelerated heart rate response is compensatory so that blood pressure falls less or not at all during standing. Whether POTS is a precursor or a less severe variant of PAF, is currently unknown.

Other features of PAF are anhidrosis (impaired sweating) with resultant heat intolerance, urine retention, constipation, incontinence and impotence. All these symptoms can be easily explained on the basis of (reduced) sympathetic activity. For reasons that are not completely understood, patients with PAF often have supine hypertension.

Because EPI is a main effector hormone in glucose homeostasis, one would also expect some degree of hypoglycemia to occur when the adrenergic system fails. However, while blood sugar levels may indeed be slightly lower in patients with autonomic failure, the other important hormones (insulin and glucagon) are able to keep glucose levels within acceptable limits.

In patients with diabetes mellitus, in particularly those who are being treated with insulin, the problem of hypoglycemia unawareness (HU) may develop. This means that the patient does not feel the symptoms of hypoglycemia because the sympathoadrenal response is attenuated or absent. This does not necessarily mean that the adrenergic system itself is at fault but it is a condition in which the central monitoring of glucose and several other putative mechanisms play a role. However, the exact nature of the abnormality remains enigmatic. Because (HU) usually develops only after the patient has gone through various episodes of hypoglycemia, one hypothesis states that the autonomic system gets exhausted when it has been activated so often after periods of hypoglycemia.

In some cases, adrenergic failure is due to an impaired or even absent ability to synthesize NE. This is the case in the rare syndrome of DBH-deficiency in which dopamine cannot be metabolized to NE. Patients with this disorder have all the signs of adrenergic failure but their sweating response is normal because the sympathetic cholinergic fibers can still work properly.

Finally, patients with adrenocortical insufficiency from whatever cause tend to have lower EPI levels than controls indicating that the adrenal part of the adrenergic system is less active. In these patients, functions which are dependent on adrenergic nerve fibers remain intact though.

Excessive Adrenergic Activity

The role of sympathetic overdrive in disease states is far less clear than deficient adrenergic activity. Of course, signs and symptoms of increased adrenergic activity are most noticeable during periods of physical or mental stress but that could be considered an adequate reaction. There exists an erratic syndrome, called "paroxysmal sympathetic hyperactivity," the etiology of which is unknown but which seems to preferentially affect people who sustained traumatic brain injury (Perkes *et al.*, 2010). So far, there is still no consensus about the significance of this syndrome that bears resemblance to, for instance, malignant hypothermia or neuroleptic malignant syndrome.

A particular situation where excessive adrenergic activity is apparent, is that of baroreceptor failure (Robertson *et al.*, 1993). This afflicts mostly patients who had extensive neck surgery and radiation treatment for cancers of the neck and in whom afferent nerve fibers from the carotid body have been damaged. In addition, paragangliomas, trauma to the neck and carotid calcifications in the carotid body area have been implicated as potential causes. Due to the loss of afferent signals, the brain no longer exerts its tonic inhibitory influence on sympathetic outflow which then increases considerably. This produces a volatile type of hypertension that may be paroxysmal and be accompanied by tachycardia. The disorder may be difficult to differentiate from pheochromocytoma, a tumor of the adrenal medulla, that causes similar symptoms.

Baroreceptor failure usually is nonselective, meaning that efferent parasympathetic activity is impaired as well. Occasionally, however, the efferent parasympathetic fibers are spared (selective baroreceptor failure). When the latter is the case, hypotension and bradycardia are more common symptoms.

Essential hypertension is yet another condition in which excess sympathetic activity is thought to have pathogenetic significance. Indeed, many studies support the concept that a dysbalance between parasympathetic activity (supposedly reduced) and sympathetic activity (supposedly increased) underlies the development of essential hypertension but the exact nature of this dysbalance is still not well understood. Studies using the technique of measuring NE spillover have shown that the increase in sympathetic outflow is not uniform but preferentially affects the heart and the kidneys and is associated with higher renin levels, particularly in younger patients (Esler *et al.*, 1989). It is unclear whether the defect is primary or not, whether it originates in the central nuclei of the brain or not and to what extent the baroreceptor system is involved.

Other conditions in which adrenergic activity has been found to be increased include type II diabetes, even in the very early stages of this disease, obesity and the metabolic syndrome, chronic kidney disease, ischemic heart disease, obstructive sleep apnea and preeclampsia.

Although it is beyond the scope of this article to discuss all therapeutic potentials, basically there are two ways to reduce excessive sympathetic activity: with ant-adrenergic drugs or with devices which electrically or mechanically stimulate the baroreceptor area. As far as drugs is concerned, one has the possibility to stimulate central alpha-2 adrenoceptors with, for instance, clonidine or methyldopa. This stimulation leads to a reduction in sympathetic outflow. Another possibility is ganglionic blockade but this is not selective for sympathetic fibers. Finally, one can block the peripheral alpha- and beta-adrenoceptors on the target organs. Due to unacceptable side-effects, centrally acting drugs and ganglion blockers are hardly used anymore these days.

Another method to reduce sympathetic outflow is to electrically stimulate the baroreceptors and, by doing so, to elicit an enhanced firing response through afferent fibers which, in turn, will shift the balance between sympathetic and parasympathetic activity towards the latter. As shown in **Fig. 3**, continued stimulation over a period of several years leads to a sustained fall in blood pressure without any signs of "exhaustion" (de Leeuw *et al.*, 2017). Recently, a variant form of "barostimulation" was successfully implemented, namely the insertion of an intra-carotid stent which alters the geometry of the vessel (Spiering *et al.*, 2017).

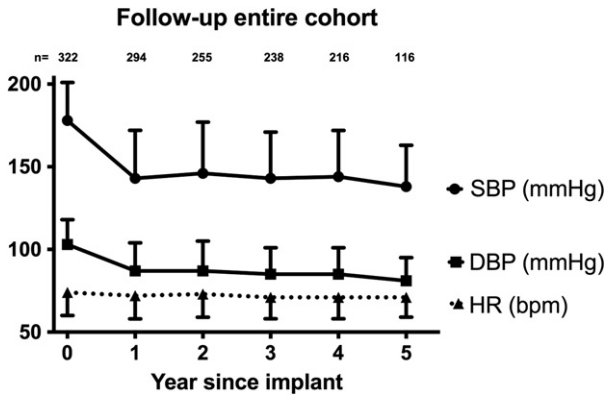


Fig. 3 Sustained reduction in blood pressure over a 5-year period in hypertensive patients who have been treated with electrical stimulation of the baroreceptor area. Reproduced from de Leeuw, P. W., et al. (2017). Sustained reduction of blood pressure with baroreceptor activation therapy: Results of the 6-year open follow-up. *Hypertension* **69**(5), 836–843.

Conclusions

Adrenergic mechanisms are important for a variety of physiological functions. Although the main targets of the sympathetic system are the heart and the vasculature, the system is also crucially involved in metabolic activities and thermocontrol. Both a reduced and an increased activity of the sympathetic efferents may lead to disease states. Recent developments have made it possible to modulate sympathetic activity by means of device-based stimulation of the baroreceptor area.

See also: Baroreceptor Responses. Novel Insights in ß-Adrenergic Receptor Signaling

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Novel Insights in β-Adrenergic Receptor Signaling☆

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Introduction

The adrenergic receptors (ARs) are part of a large family of G-protein-coupled receptors (GPCR) which trigger the intracellular signaling in response to several stimuli, including light, odorants, neurotransmitters, lipids, and various peptides and proteins (Marinissen and Gutkind, 2001). GPCR activation promotes interaction with heterotrimeric guanosine triphosphate-binding proteins (G-proteins), resulting in guanosine diphosphate/guanosine triphosphate exchange and subsequent dissociation of the α and $\beta\gamma$ subunits. These subunits regulate the activity of multiple effector proteins, including adenylyl cyclases, phospholipases, phosphodiesterases, ion channels, and phosphatidylinositol 3-kinase. Thus, GPCRs regulate several key biologic functions including cellular proliferation, cell differentiation, survival, sensory perception, neurotransmission, chemotaxis, development (Sorriento *et al.*, 2011).

The ARs family includes α and β receptors which mediate physiological responses to catecholamines. βARs are vasodilators which are divided into three subtypes: β_1AR is found at its highest levels in the heart, β_2AR is distributed extensively throughout the body and β_3AR is mainly expressed in the white and brown adipose tissue (Liggett, 2002; Weitl and Seifert, 2008; **Table 1**). αARs mainly induce vasoconstriction and include three α_1 receptor subtypes ($\alpha_{1\alpha}$ AR, $\alpha_{1\beta}$ AR, α_{1D} AR) and three α_2 receptor subtypes ($\alpha_{2\alpha}$ AR, $\alpha_{2\beta}$ AR, α_{2C} AR) which differ in their localization and responsiveness to drugs (Molinoff, 1984; **Table 1**).

Adrenergic Receptors From the Discovery to the 2012 Nobel Prize

For several decades, it was a mystery how cells could sense and interact with their environments. Given the notion that hormones, like adrenalin, had several effects on humans (an increase of blood pressure and heart beats), it started to take hold the idea that cell surfaces could contain something that interacted with hormones. In 1948 the American pharmacologist Raymond Ahlquist was the first to identify two types of receptors for adrenaline, that he called α and β receptors based on the different abilities of ligands to stimulate several physiological processes (Ahlquist, 1948). This was the proof that cellular receptors existed on plasma membrane even if their structure and function remained obscured for decades. Then, the development of new techniques allowed the study of ARs structure and actions. Robert Lefkowitz was the first to develop the radioligand binding method to study the β-adrenergic receptor (Mukherjee *et al.*, 1975) and the α-adrenergic receptor (Williams and Lefkowitz, 1976). He used the radioactivity of ^{125}I to trace cellular receptors by attaching the radioactive iodine isotope to various hormones. This technique allowed to study the regulation of the receptors in response to several factors (Stiles *et al.*, 1984), to discover unknown receptor subtypes (Lorenz *et al.*, 1990) and to better understand the mechanisms of actions of such receptors. By this technique, Lefkowitz team identified a new membrane component which was part of the agonist/receptor complex, the guanine nucleotide regulatory protein (G protein) (Kent *et al.*, 1980). Then, Lefkowitz team extracted the receptor from its hiding place in the cell wall to study how it works (Cerione *et al.*, 1984). The next big step in the knowledge of adrenergic receptors was the isolation of the gene coding for the β-adrenergic receptor from gigantic human genome which was performed in the lab of Prof. Kobilka (Kobilka *et al.*, 1987). This led to the notion that the receptor was similar to one in the eye that captures light. Thus, it started to realize that a complete family of receptors should exist that have a similar structure and act in the same manner. From these great discoveries, the puzzle has been assembled piece-by-piece till obtaining a detailed knowledge about GPCRs functions and regulation. In 2011 Kobilka and his team captured an image of the β-adrenergic receptor at the exact moment that it is activated by ligand binding and sends a signal into the cell (Rasmussen *et al.*, 2011a,b). For their contribution to the study of GPCRs, in 2012 Lefkowitz and Kobilka were awarded the Nobel Prize in Chemistry.

New Findings in Canonic ARs Signaling

The canonic pathway of activation of adrenergic receptors is based on structural changes promoted by agonist binding, which allow their coupling to heterotrimeric G proteins. Subsequent activation and dissociation of G proteins lead to the generation of second messengers and cellular responses (Whalen *et al.*, 2011; Lefkowitz and Shenoy, 2005; Shukla *et al.*, 2014a). It has been demonstrated that the binding of agonists to adrenergic receptors promotes a desensitization process via phosphorylation by the G

☆*Change History*: December 2017. Daniela Sorriento and Guido Iaccarino completely updated the text, summarizing the information of the previous version of the article in Table 1 and added two tables and three figures.

This chapter is an update of Linda F. Hayward, Patrick J. Mueller and Eileen M. Hasser, Adrenergic Receptors, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 112–115.

 doi:10.1016/B978-0-12-801238-3.95888-4

Table 1 The main characteristics of adrenergic receptors subtypes

Type of receptor	*Localization*	*Signaling*	*Physiological responses*
β1 AR	Cardiac and adipose tissue, cerebral cortex, caudate, and cerebellum	• Elevation of intracellular cyclic AMP • Calcium reuptake into the sarcoplasmic reticulum	• Positive inotropic effect • Lusitropic effect
β2 AR	Smooth muscle, liver, human white cells, cardiac tissue cerebral cortex, caudate, and cerebellum	• Elevation of intracellular cyclic AMP • Calcium reuptake into the sarcoplasmic reticulum • Phosphatidylinositol 3′ kinase pathway	• Positive inotropic effect • Lusitropic effect • Anti-apoptotic mechanisms
β3 AR	White and brown adipose cells as well as in skeletal muscle	• Coupling to both stimulatory and inhibitory G proteins and activation of nitric oxide synthase • Activates a mitochondrial uncoupling protein • Reduction of leptin expression	• Left ventricular dysfunction • Thermogenesis • Antiobesity and antidiabetic properties
α1 AR	Vasculature, heart, lung, kidney, liver, and brain	• Coupling to Gq/11 and Activation of phospholipase C	• Control of vascular tone
α2 AR	Presynaptically and postsynaptically	• Inhibition of Ca^{2+} channels, activation of K^{+} channels, activation of phospholipase C, increased Ca^{2+} release from intracellular sources, and stimulation of mitogen-activated protein kinase	• Neurotransmitter release, control of vascular tone, regulation of renin release, inhibition of insulin secretion, inhibition of lipolysis, and platelet aggregation • Vasoconstriction peripherally and hypotension centrally

Receptors are classified based on their localization, signaling and physiological effects.

protein-coupled receptor kinases (GRKs) with the involvement of β-arrestins (Kohout and Lefkowitz, 2003). GRK-phosphorylated receptors recruit β-arrestins, which compete with G protein coupling and desensitize the G protein signaling response (Shenoy and Lefkowitz, 2003a). Moreover, β-arrestins also promotes receptors clathrin-dependent endocytosis (Goodman *et al.*, 1996).

The αARs are GPCRs that couple to $G_{\alpha q}$ protein that is a primary activator of phospholipase C (PLC). This latter, in turn, promotes the cleavage of the inositol substrate phosphatidyl-inositol 4,5 bisphosphate (PIP2) to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Then, DAG and IP3 promote the activation of protein kinase C (PKC). The α_1AR subtype can also activate specific adenylate cyclases (AC) leading to an increase in cAMP levels. DAG and cAMP are second messengers that affect several cell signaling pathways and responses.

Agonist-activated βARs couple to the stimulatory G protein, G_s, leading to the activation of adenylyl cyclase and the accumulation of cAMP (Dixon *et al.*, 1986; Emorine *et al.*, 1989). The increase of cAMP induces the activation of protein kinase A (PKA) which in turn phosphorylates several substrates to activate specific intracellular signaling. Among these latters, it has been shown that the activation of the axis β_2AR/PKA regulates the synthesis of catecholamines in the endothelium (Sorriento *et al.*, 2012). It is a new interesting finding in the field of βARs-dependent regulation of vascular function even if the pathophysiological role of endothelial catecholamines is still unknown. Recent studies indicate that under certain conditions βAR, and particularly β_2AR, can also couple to G_i (Asano *et al.*, 1984; Buxton *et al.*, 1987; Chaudhry *et al.*, 1994; Gauthier *et al.*, 1996; Xiao *et al.*, 1995). Indeed, the β_2AR itself become a PKA substrate. The PKA-dependent phosphorylation of the third intracellular loop of the β_2AR increases the affinity of the receptor for G_i protein (Okamoto *et al.*, 1991; Zamah *et al.*, 2002). G_i activation inhibits adenylyl cyclase activity, leading to the reduction of cAMP levels and to the increase of "non-cAMP dependent signaling," such as the activation of the extracellular signal-regulated kinases ERK1/2 and PI_3K (Baillie *et al.*, 2003; Jalali *et al.*, 1998; Luttrell and Lefkowitz, 2002; Nagao *et al.*, 1999; Xiao, 2001). Several factors have been identified which are able to regulate membrane protein structure and activity. Among them, lipids seem to have a key role in the regulation of adrenergic receptor activation since they act as direct allosteric modulators of receptor activity (Dawaliby *et al.*, 2016). Specifically, phosphatidylglycerol markedly favored agonist binding and facilitated receptor activation, whereas phosphatidylethanolamine favored antagonist binding and stabilized the inactive state of the receptor (Dawaliby *et al.*, 2016). Protein post-translational modifications also affect ARs activation and signaling. In this context, it has been recently shown that N-glycosylation of β_2AR regulates receptor function by affecting its dimerization (Li *et al.*, 2017). Furthermore, new phosphorylation sites on β_1AR have been identified in mouse heart which affect receptor function (Hayashi *et al.*, 2017). In particular, the phosphorylation at Ser274, Ser280, and Ser462 have been found to be enhanced in response to stimulation with agonists (Hayashi *et al.*, 2017).

The "Biased Signaling"

Classically, adrenergic receptors activate G protein-dependent signaling pathways in response to ligands stimuli. For many decades, these receptors were thought to possess just a simple "on/off" status which was dependent on the type of ligand (the agonist

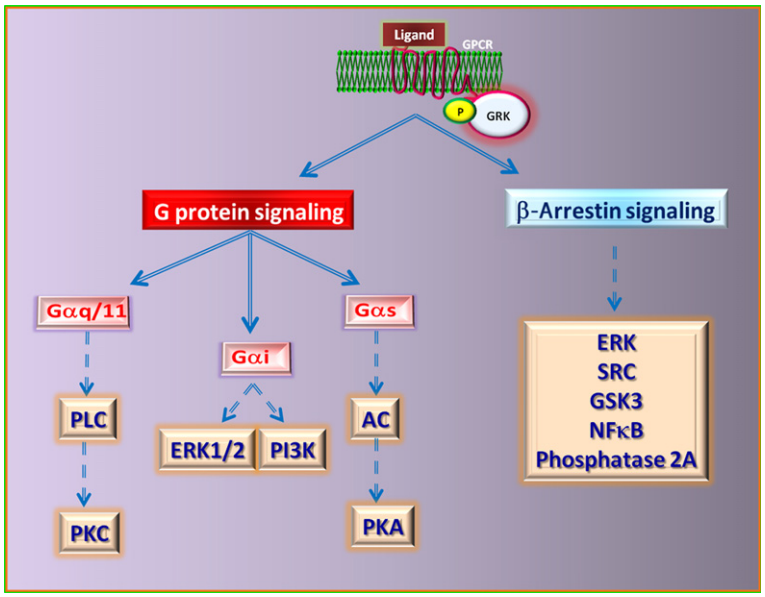

Fig. 1 GPCR signaling. In response to ligands, GPCR can signal through G protein or β-arrestin. Traditionally, adrenergic receptors ligands signal through both mechanisms. However, there are specific ligands, known as "biased agonists," which are able to induce the conformational state of the receptor that selectively activates one of the two signaling pathways. Reproduced from Bologna, Z., Teoh, J. P., Bayoumi, A. S., Tang, Y., Kim, I. (2017). Biased G protein-coupled receptor signaling: New player in modulating physiology and pathology. *Biomolecules & Therapeutics* **25** (1), 12–25.

turns on and the antagonist turns off the signal). In the last decade, however, it has been shown that several GPCR, including β_2AR, can signal independently from G protein activation, through the GRK/β-arrestin pathway (**Fig. 1**).

Traditionally, adrenergic receptors agonists and antagonists signal through both mechanisms. However, some ligands are able to favor one mechanism over the other and are known as "biased agonists." These ligands can induce the conformational state of the receptor that selectively activates one of the two signaling pathways (Liu *et al.*, 2012).

β_2AR ligands, such as the β-blocker carvedilol (Shenoy and Lefkowitz, 2011) and the agonist isoetharine (Drake *et al.*, 2008), have been shown to promote differing degrees of signaling in G-protein and arrestin pathways (Urban *et al.*, 2007). Indeed, carvedilol, a β_1/β2-AR–blocker used for the treatment of pathological cardiac diseases (including high blood pressure), is now recognized as a "biased agonist" that is able to stabilize a β-AR conformation promoting the uncoupling to G_s and to induce the β-arrestin-mediated signaling (Wisler *et al.*, 2007). In the same manner, the β_1-AR-blocker nebivolol is also considered a β-arrestin biased agonist (Erickson *et al.*, 2013).

The study of the complexes of the β_2-adrenergic receptor with various ligands suggests that the cytoplasmic ends of helices VI and VII adopt two major conformational states. Different ligands affect the conformational equilibrium involving helices VI and VII (Liu *et al.*, 2012). In particular, ligands that induce G protein signaling active state of helix VI whereas β-arrestin-biased ligands predominantly promote the conformational states of helix VII (Liu *et al.*, 2012).

βAR-Dependent β-Arrestin Signaling

β-arrestins are ubiquitously expressed proteins that were first described for their role in desensitizing G protein-coupled receptors (GPCRs). Indeed, β-arrestins are recruited to receptors in response to agonist-induced phosphorylation by GRKs (Krasel *et al.*, 2005). Besides their ability to disrupt receptor/G-protein signaling, these proteins are now recognized as multifunctional adapter proteins that regulate several cellular functions. In fact, arrestins confer novel GPCR-signaling capacity by recruiting protein kinase, phosphatase, phosphodiesterase, and ubiquitin ligase activity into the receptor complexes with proteins (Shukla *et al.*, 2014a).

The β_2AR-β-arrestin 1 complex has been studied in detail by means of electron microscopy, hydrogen-deuterium exchange mass spectrometry and chemical cross-linking (Shukla *et al.*, 2014b). β-arrestin seems to trigger a biphasic mechanism to engage the receptor, involving two different interactions, in the carboxy-terminus of the receptor and in the seven-transmembrane core (**Fig. 2**; Shukla *et al.*, 2014b). These interactions should preclude receptor engagement of G-protein heterotrimers, thereby blocking classical GPCR signaling.

β-arrestins recruited to agonist-activated and phosphorylated receptors can promote several G protein-independent signaling pathways, such as ERK, c-Src, GSK3, protein phosphatase 2A, and NFκB signaling (Luttrell and Miller, 2013). Actually, β-arrestins exert a triple function on GPCR which includes the regulation of receptors desensitization, endocytosis, and signaling (Shenoy and Lefkowitz, 2011).

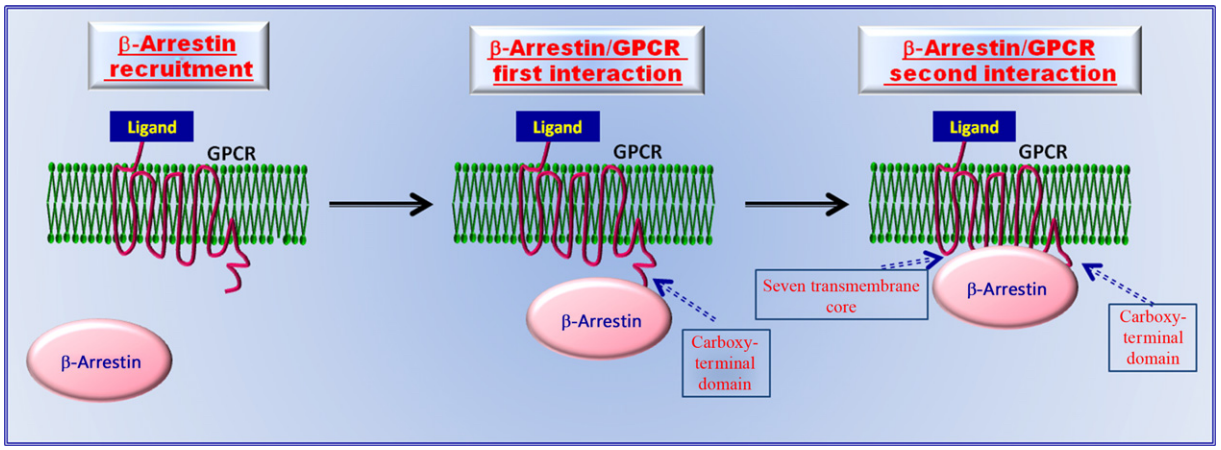

Fig. 2 β-arrestin/GPCR interaction. The interaction of β-arrestin with GPCR involves two separate sets of interactions, the first in the carboxy-terminus of the receptor and the second in the seven-transmembrane core (see *white arrows*). These interactions should preclude receptor engagement of G-protein heterotrimers, thereby blocking classical GPCR signaling. Adapted from Shukla, A. K., Westfield, G. H., Xiao, K., Reis, R. I., Huang, L. Y., Tripathi-Shukla, P., Qian, J., Li, S., Blanc, A., Oleskie, A. N., Dosey, A. M., Su, M., Liang, C. R., Gu, L. L., Shan, J. M., Chen, X., Hanna, R., Choi, M., Yao, X. J., Klink, B. U., Kahsai, A. W., Sidhu, S. S., Koide, S., Penczek, P. A., Kossiakoff, A. A., Woods, V. L. Jr., Kobilka, B. K., Skiniotis, G. and Lefkowitz, R. J. (2014). Visualization of arrestin recruitment by a G-protein-coupled receptor. *Nature* **512**, 218–222.

β-arrestins is able to promote ERK1/2 activation by inducing Raf-1/MEK1 pathway and sequestering ERK in the cytosol (Luttrell *et al.*, 2001). This precludes ERK-mediated transcription and prolongs ERK signaling. Similarly, arrestin 3 can scaffold JNK1/2 favoring their phosphorylation by the kinases MKK4 and MKK7 (Kook *et al.*, 2013).

β- arrestins can directly bind to Src family kinases and recruit them to an agonist-occupied receptor. The stimulation of β2 adrenergic receptors triggers the assembly of a protein complex containing activated Src, β-arrestin and the receptor (Luttrell and Lefkowitz, 2002). Such complex affects several GPCR-dependent mechanisms, such as dynamin phosphorylation (Miller *et al.*, 2000), ERK activation (Defea *et al.*, 2000), neutrophil degranulation (Barlic *et al.*, 2000). Moreover, it has been shown that both β-arrestin 1 and 2 are able to interact with IκBα, the inhibitor of the transcription factor NFκB, and inhibit NFκB signaling (Witherow *et al.*, 2004).

The Role of Calcium in β-Adrenergic Receptors Signaling

It is known that adrenergic receptors are able to regulate intracellular calcium flux. Indeed, in some cell types, β2AR activation has been linked to the mobilization of Ca^{2+} from intracellular stores through the actions of cAMP on PKA (Betzenhauser *et al.*, 2009; **Fig. 3**). In animal models of heart failure, the stoichiometry and function of the RyR2 macromolecular complex are altered due to hyperphosphorylation by PKA, promoting Ca^{2+} influx (Reiken *et al.*, 2001). The treatment with β blockers reverses PKA hyperphosphorylation of RyR2, restores the stoichiometry of the RyR2 macromolecular complex, and normalizes the single-channel function (Reiken *et al.*, 2001). Recently, it has been shown in vitro that β2AR regulates Ca^{2+} mobilization from intracellular stores also through the exchange protein activated by cAMP (EPAC) (Pereira *et al.*, 2012). This latter leads to the activation of phospholipase C and the opening of inositol trisphosphate (InsP3) receptors (**Fig. 3**; Galaz-Montoya *et al.*, 2017), without involving members of the canonical β2AR signaling cascade (Galaz-Montoya *et al.*, 2017).

Targeting β-Adrenergic Receptors

To date, GPCRs, including adrenergic receptors, represent one of the largest therapeutic targets in clinical medicine from cancer to cardiovascular diseases (Lappano and Maggiolini, 2011; Pacher *et al.*, 2006). In fact, adrenergic receptors ligands account for almost 40% of approved drugs, such as α- and β-blockers and β-agonist (Chalmers and Behan, 2002).

Commercial Drugs

Among available therapeutic ligand of ARs, beta-blockers are categorized as "selective" or "non-selective" based upon whether they block only beta-1 receptors, that are predominantly present in cardiac muscle, or also beta-2 receptors, mainly found in bronchial and smooth muscles (**Table 2**).

β-blockers, such as metoprolol, bisoprolol, and carvedilol, antagonize the effects of catecholamines on beta-adrenergic receptors leading to a reduction in the contractile force and the rate of contraction, to the inhibition of renin release from the

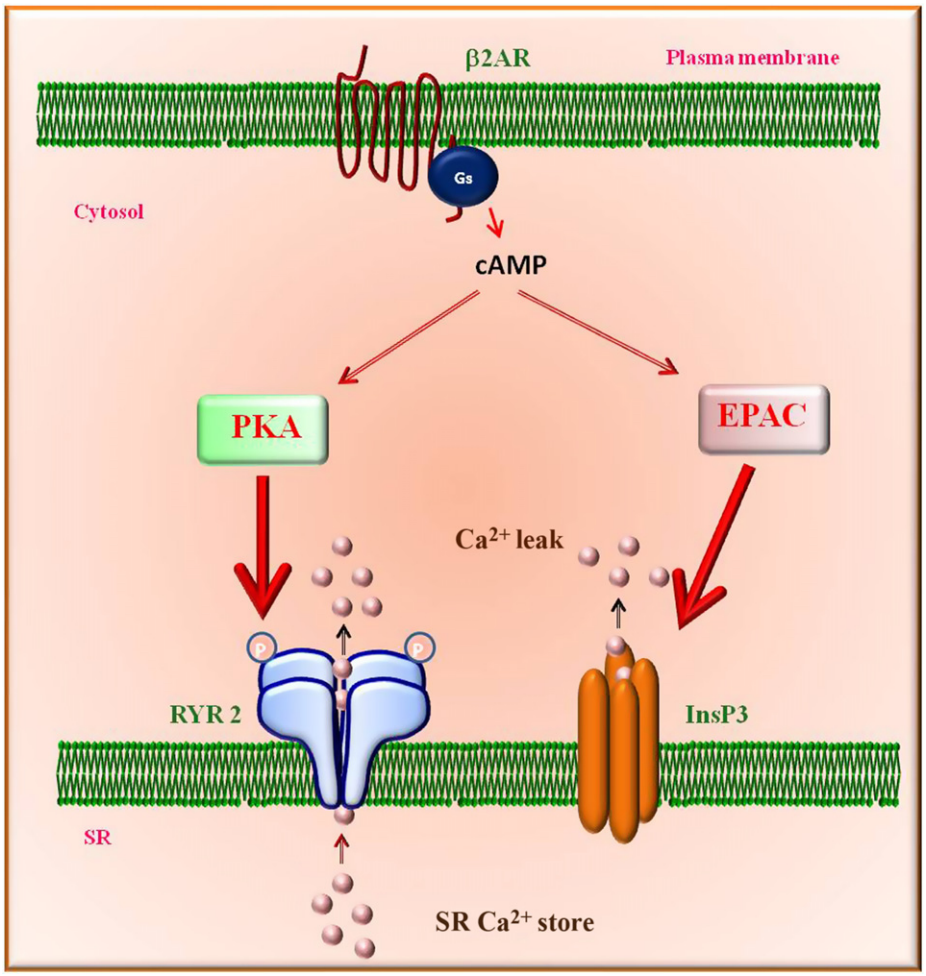

Fig. 3 βAR and calcium flux. Adrenergic receptors activates PKA which in turn phosphorylates RyR2 promoting sarcoplamic reticulum (SR) Ca^{2+} leak. This leads to a depletion of SR Ca^{2+} stores and a reduction of EC coupling. Reproduced from Reiken, S., Gaburjakova, M., Gaburjakova, J., He Kl, K. L., Prieto, A., Becker, E., Yi Gh, G. H., Wang, J., Burkhoff, D. and Marks, A. R. (2001). Beta-adrenergic receptor blockers restore cardiac calcium release channel (ryanodine receptor) structure and function in heart failure. *Circulation* **104**, 2843–2848; Galaz-Montoya, M., Wright, S. J., Rodriguez, G. J., Lichtarge, O., and Wensel, T. G. (2017). Beta-2 adrenergic receptor activation mobilizes intracellular calcium via a non-canonical cAMP-independent signaling pathway. *Journal of Biological Chemistry* **292** (24), 9967–9974; Pereira, L., Ruiz-Hurtado, G., Morel, E., Laurent, A. C., Metrich, M., Dominguez-Rodriguez, A., Lauton-Santos, S., Lucas, A., Benitah, J. P., Bers, D. M., Lezoualc, H. F., and Gomez A. M. (2012). Epac enhances excitation-transcription coupling in cardiac myocytes. *Journal of Molecular and Cellular Cardiology* **52**, 283–291.

kidney and to a decrease in blood pressure. These drugs are actually used for the treatment of hypertension, ischemic heart disease, myocardial infarction, arrhythmias, congestive heart failure.

Nebivolol has the highest β_1-receptor affinity among β-blockers. It has been demonstrated that this drug also has antioxidant properties and regulate endothelial functions by promoting the activity of the endothelial nitric oxide synthase (Mason *et al.*, 2005).

Some drugs, such as carvedilol, are able to block both $\alpha 1$ and β_1/β_2 receptors. Thus, carvedilol is mainly used for the treatment of hypertension but it is also effective in congestive heart failure. Indeed, besides the inhibition of adrenergic receptors signaling, this drug is able to reduce the production of reactive oxygen species (Cardinale *et al.*, 2015; Spallarossa *et al.*, 2004).

$\alpha 1$-receptor blockers, such as prazosin, are commonly used for the treatment of hypertension since they reduce blood pressure and also have diuretic effects.

Clonidine, methyldopa, guanabenz, and guanfacine are $alpha_2$ receptors agonists which decrease sympathetic outflow to the heart and blood vessels. Among them, clonidine is the most widely used drug in this class.

Besides the commercially available drugs, the new findings on the ability of adrenergic receptors to signal via both G protein and non-G protein effectors has prompted the search for new ligands that can "bias" downstream signaling in favor of one selective signaling. Thus, the increase of knowledge on ARs biased signaling will be helpful for designing drugs with higher specificity and effectiveness than the commercially available ones.

Table 2 List of beta blockers that are commonly used in clinic

Drug name	*Generation*	*Selectivity on βAR*	*Selectivity on αAR*	*Other properties*	*Biased agonist*
Propanolol	First	Non-selective	–	–	–
Esmolol	Second	Selective	–	–	–
Atenolol	Second	Selective	–	–	–
Metoprolol	Second	Selective	–	–	–
Nebivolol	Third	Selective	–	NO-vasodilation and Antioxidant properties	Yes (Mason et al., 2005)
Labetalol	Third	Non-selective	a1AR	–	–
Carvedilol	Third	Non-selective	a1AR	Antioxidant properties	Yes (Cardinale et al., 2015; Spallarossa et al., 2004)

Several selective and non-selective βAR drugs are commonly used for the treatment of hypertension and cardiovascular diseases. Among them, carvedilol and nebiovilol are considered biased agonists giving their ability to activate G protein independent signaling.

Potential Molecular Targets

Beyond the above described available class of drugs which are able to target ARs and block adrenergic signaling, several peptides have been identified that affects ARs activation and could become useful target for drugs. Among them, GRK2 and β-arrestins are the most suitable since they act upstream of ARs signaling. Different strategies have been put in place to inhibit GRK2 in cardiovascular diseases both in vivo (Raake *et al.*, 2013; Casey *et al.*, 2010; Schumacher *et al.*, 2015) and in vitro (Lorenz *et al.*, 2003; Mayer *et al.*, 2008; Sorriento *et al.*, 2015) which are based on different molecular mechanisms from the inhibition of the kinase catalytic activity to the regulation of its shuttling within the cell (Sorriento *et al.*, 2016). GRK2 inhibition is commonly achieved through the viral mediated expression of βARKct, which resembles the carboxy-terminal domain of GRK2 that is responsible for its translocation to the plasma membrane and its binding to Gβγ. It has been shown that the long-term expression of βARKct induced a significant amelioration cardiacfunction in animal models of heart failure and myocardial infarction (Raake *et al.*, 2013). Recently, an alternative approach has been suggested based on the synthesis of small molecules which selectively inhibit GRK2 activity. Small molecules has the advantage to autonomously cross the plasma membrane, thus avoiding the immune response and cytotoxicity often associated with viral use. Several promising compounds that bind to the Gβγ binding site of GRK2 have been identified (Anis *et al.*, 2004; Gomez-Monterrey *et al.*, 2014) which effectively restores cardiac function in animal models of pathology (Sorriento *et al.*, 2015). Similarly, β-arrestins appears to be promising targets for therapeutics, given their multiple functions within GPCR activation and signaling. It has been shown that their effects are regulated by several protein post-translational modifications. Indeed, β-arrestins dephosphorylation (Lin *et al.*, 2002), ubiquitination (Shenoy and Lefkowitz, 2003b) and nitrosylation (Ozawa *et al.*, 2008) affect receptors internalization and trafficking. Thus, targeting these modifications could be an useful strategy to regulate AR signaling.

See also: Adrenergic Mechanisms. Baroreceptor Responses

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Baroreceptor Responses

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Glossary

Bradycardia Decreased heart rate.
Catecholamines A distinctive chemical structure common to epinephrine and norepinephrine.
Hypotension Reduced arterial blood pressure.
Hypovolemia Reduced blood volume.
Tachycardia Increased heart rate.

Introduction

Given the importance of the cardiovascular system in maintaining adequate perfusion of tissues, it is not surprising that elaborate mechanisms have evolved to promote the stability of arterial blood pressure and blood volume, which are essential for maintaining tissue perfusion. These mechanisms include well-known physiological responses that restore blood pressure and volume after blood loss with or without accompanying decreases in arterial blood pressure, including autonomic reflexes, endocrine secretions, and ingestive behaviors motivated by thirst and salt appetite.

Animals must be able to detect changes in blood pressure and volume for any of these compensatory responses to occur. Insight into the location of the receptors, and their properties, comes from a consideration of how changes in blood volume and pressure affect the cardiovascular system. After a moderate hemorrhage, for example, the blood loss is not distributed uniformly throughout the cardiovascular system but instead is borne almost entirely on the venous side of the circulation. The veins, which are very distensible, collapse around the remaining blood and thereby help to reduce the impact of the blood loss on venous blood pressure. Together with an appropriate increase in heart rate, arterial blood volume and pressure undergo little change. Thus, it makes sense that receptors are situated to monitor blood volume on the venous side of the circulation. In contrast, after severe hemorrhage the effects of blood loss may extend to the arterial side of the circulation and reduce arterial blood volume and pressure. Thus, it also makes sense that additional receptors are situated to monitor blood pressure on the arterial side of the circulation. Indeed, as arterial blood pressure is a key determinant of tissue perfusion, this parameter is sensitively monitored and tightly regulated. The receptors that monitor the stretch of venous and arterial blood vessels are known to be located at key sites in the cardiovascular system. On the venous side, the most important site is at the junction of the inferior vena cava and the right atrium. On the arterial side there are two key sites, the aortic arch through which arterial blood leaving the heart is carried and the carotid arteries through which blood flows to the brain.

The receptors in question are cells called 'baroreceptors', which literally means that they are responsive to changes in pressure. The sensory endings of these cells are mechanoreceptors, located on the outside walls of the blood vessels and cardiac atria, which respond to changes in the conformation of the vessel wall, much like other stretch receptors on the outside walls of the stomach and bladder detect the fullness of those organs. Thus, following a moderate hemorrhage, the loss in blood volume from the venous side decreases venous pressure and stretch of the right atrium and vena cava thereby decreasing the firing rate of these sensory nerves. Following a more severe hemorrhage, blood pressure in the proximal aorta and carotid sinus decrease, leading to decreased distention of these blood vessels and thus decreased activity of the aortic and carotid sinus baroreceptors. Three additional points regarding baroreceptors should be noted. First, although this chapter is written from the perspective of decreases in blood volume, any changes in stretch, increased or decreased, whatever the cause, will be detected. Second, baroreceptors, like other stretch receptors, adapt to chronic stretch, and so baroreceptor activity does not simply reflect the pressure but it also is affected by the rate of change in pressure. Third, other circulating substances (e.g., hormones) or locally released signaling molecules acting on baroreceptors can influence their activity as well.

Responses

Neural afferents from these low- and high-pressure baroreceptors project as part of the ninth and tenth cranial nerves to the nucleus of the solitary tract (NST) in the caudal brain stem. From there, complex neural circuitry enables the variety of responses that occur in response to decreases in blood volume or arterial blood pressure (see **Fig. 1**). Among the most prominent responses are the following.

Cardiac Reflexes

Changes in heart rate and cardiac contractility (and therefore cardiac output) help to compensate for changes in blood volume and pressure. Thus, for example, an increase in heart rate and cardiac contractility occurs during hypovolemia or hypotension, which is

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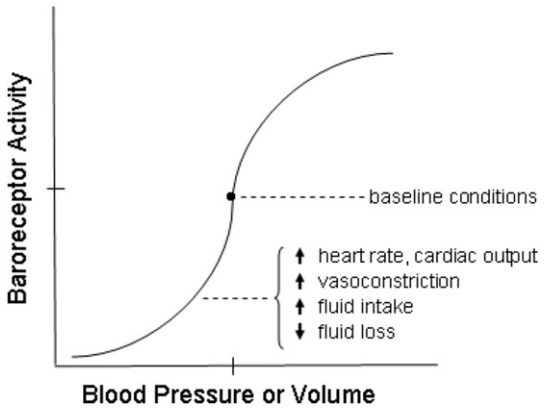

Fig. 1 Baroreceptor-evoked responses to hypovolemia and hypotension. The activity of veno-atrial baroreceptors and arterial baroreceptors are related to changes in blood volume and blood pressure, respectively. Neural and endocrine baroreceptor reflex responses to decreases in blood volume or blood pressure produce increases in heart rate and cardiac output, increases in vasoconstriction, and decreases in fluid excretion by the kidneys. Additionally, baroreceptor input to the brain elicits ingestive behaviors to increase fluid body fluid volume. In general, increases in blood volume and blood pressure elicit baroreceptor-evoked responses that are the converse of those seen with decreases in these parameters.

one of the well known 'baroreceptor reflexes'. This response appears to reflect the three autonomic effects that result from baroreceptor input to the NST. The first involves the control of cardiac sympathetic neural activity, which accelerates heart rate and increases cardiac contractility when stimulated during volume contraction or decreases in arterial pressure. The second is the converse inhibitory effect on parasympathetic neural activity, which also causes tachycardia. The third involves stimulation of the adrenal medulla and the release into the systemic circulation of epinephrine, which has a prominent effect on beta-adrenergic receptors on the heart and thereby further stimulates heart rate and cardiac contractility. The multiple controls of heart rate ensure that these responses are rapid and precise.

Vasoconstriction

Blood vessels are also responsive to sympathetic neural input. During hemorrhage, norepinephrine is released from sympathetic nerve fibers and binds to alpha-adrenergic receptors located on vascular smooth muscle to cause vasoconstriction. The constriction of blood vessels raises peripheral resistance and blood pressure. At the same time, constriction of some arterioles versus others permits the cardiovascular system to maintain blood flow to critical organs. Activation of sympathetic neural input to veins causes venoconstriction, thereby increasing venous return of blood to the heart and cardiac output.

Renin-Angiotensin System

Increased sympathoadrenal activity, brought about by decreased arterial blood pressure and or blood volume, stimulates renin secretion from the kidneys, which begins a cascade of biochemical events that result in the generation of the peptide hormone angiotensin II, the most potent pressor agent available to the animal in response to blood loss. Briefly, renin is a proteolytic enzyme that is synthesized in renal juxtaglomerular cells and released in response to increased activation of beta-adrenergic receptors on these cells, either by sympathetic nerves or by circulating epinephrine, by decreased renal sodium concentration, or by a direct action of large decreases of renal arterial perfusion pressure. Renin acts by cleaving angiotensinogen, a large protein synthesized in the liver and always present in the circulation, to produce a 10-amino acid protein (angiotensin I) that then quickly loses two more amino acids through the action of an enzyme that converts angiotensin I to angiotensin II. This enzyme, angiotensin converting enzyme or ACE, is heavily concentrated in the lung capillaries, an arrangement that allows the hormone to be delivered into the arterial blood that leaves the heart. Circulating angiotensin II acts directly on angiotensin II receptors in vascular smooth muscle to support blood pressure. In addition, it acts in the adrenal cortex to stimulate secretion of the mineralocorticoid aldosterone, which promotes urinary Na^+ retention (and, secondarily, fluid retention) as well as urinary K^+ excretion (which is especially useful when blood loss is associated with tissue damage). Angiotensin II also acts on angiotensin receptors in the brain, in particular in the subfornical organ (SFO). The SFO, which is situated dorsal to the third cerebral ventricle, lacks a blood–brain barrier and therefore can detect variations in blood levels of the hormone. Neural circuits from the SFO then appear to stimulate central systems that increase sympathoadrenal activity. They also stimulate secretion of vasopressin, another hormone with pressor properties, from the posterior lobe of the pituitary gland.

Vasopressin

The release of pituitary vasopressin (also called antidiuretic hormone) is stimulated by neural pathways connecting the NST to the supraoptic and paraventricular nuclei of the hypothalamus, in response to decreased baroreceptor input during hemorrhage. Whereas low physiological blood levels of vasopressin (1–10 picomolar) have prominent effects on water retention by the kidneys, vasopressin release stimulated by decreased blood volume or arterial blood pressure results in much higher levels of vasopressin (>25 picomolar) and at these high levels vasopressin causes constriction of arteriolar smooth muscle. Thus, in response to hemorrhage, vasopressin causes vasoconstriction, thereby helping to maintain arterial blood pressure. Pituitary secretion of the other neurohypophyseal hormone, oxytocin, parallels the secretion of vasopressin in response to these stimuli. The contribution of oxytocin to the regulation of blood volume and arterial blood pressure is presently unclear, though studies in rats revealed that it constituted yet another significant stimulus of renin secretion.

Pituitary-Adrenal Axis

Neural projections from the NST to the paraventricular nuclei of the hypothalamus also stimulate release of corticotrophic releasing factor (CRF), which elicits secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH, in turn, stimulates the adrenal cortex to increase its synthesis and release of two steroid hormones, aldosterone (mentioned above) and the glucocorticoid cortisol. Cortisol improves vascular reactivity to the catecholamines and other general responses to stress.

Ingestive Behavior

In addition to these physiological responses, hypovolemia is known to stimulate thirst and, in rats and some other species (but not in human subjects), salt appetite. Remarkably, the resultant ingestion of water and NaCl solution occurs in amounts equivalent to a fluid mixture that is isotonic to plasma. Baroreceptor input to the brain stem is presumed to provide the main signal of thirst under hypovolemic conditions, although angiotensin II is a known dipsogen and presumably contributes stimulation as well. The signal of salt appetite is less clear and presently is controversial, although an important role of angiotensin II seems apparent. Thirst (but not salt appetite) also is stimulated by arterial hypotension in rats, but the induced water intake is mediated exclusively by angiotensin II, and baroreceptors do not appear to provide a direct neural signal.

Summary

Many adaptive responses occur during hypovolemia and arterial hypotension that appear to involve signals that originate in the activity of cardiovascular baroreceptors. These responses include several chemical agents that independently provide vasoconstriction and support of arterial blood pressure, hormone secretions that promote urinary conservation of water and Na^+, and the sensations of thirst and salt appetite that lead to water and NaCl consumption, respectively. The precise stimulus of each response is not always certain, but it may involve neural signals, blood-borne signals, or both. This redundancy of stimuli is a common feature of homeostatic regulatory systems, which is adaptive because it allows compensation for damage- or disease-induced impairments of individual components of the system. On the other hand, this redundancy makes it very difficult for investigators to identify all the contributing signals and effector systems that participate in homeostasis, and their importance relative to one another.

Although this description has focused on the baroreceptor responses seen after reductions in blood volume and pressure, it is important to recognize that many, but not all, of these same variables are influenced by increases in blood volume and pressure. For example, hypervolemia or high blood pressure causes a reflexive bradycardia through low and high-pressure receptors, respectively. Other baroreceptor responses to hypervolemia and hypertension are generally analogous although some inconsistencies are evident. Thus, vasopressin secretion is decreased when blood volume is expanded though not when blood pressure is elevated. Conversely, thirst is decreased when arterial blood pressure is elevated acutely though not when blood volume is expanded. In short, baroreceptors mediate a variety of adaptive autonomic, endocrine, and behavioral responses both to decreases and increases in blood volume and pressure.

See also: Adrenergic Mechanisms. Novel Insights in ß-Adrenergic Receptor Signaling

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Antiadrenergic Agents☆

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Glossary

Adrenergic nervous system The sympathetic part of the autonomous nervous system, where the neurotransmitters (norepinephrine and epinephrine) are adrenergic.

Cholinergic nervous system The parasympathetic part of the autonomic nervous system, where the neurotransmitter (acetylcholine) is cholinergic.

Pre/postganglionic Anatomical position of the sympathetic neuron with respect to the ganglion.

Pre/postsynaptic Anatomical position of receptors with respect to the synapse/nerve ending.

Synapse Anatomic/functional structure allowing the transition of nerve activity from the nerve ending (postganglionic) through the synaptic cleft and toward the target organ (blood vessel, heart, etc.).

Antiadrenergic agents are drugs that suppress the activity of the sympathetic (adrenergic) nervous system, targeting the cardiovascular system and other organs.

Introduction

The autonomic nervous system is responsible for the homeostasis/regulation of several internal organs, tissues, and metabolic systems. Well-known examples of such targets of the autonomic nerves are the cardiovascular system (heart and blood vessels), the gastrointestinal system (stomach, intestine, biliary duct, etc.), the respiratory tract (lungs and airways), and glucose/lipid metabolism. The autonomic nervous system is subdivided into the sympathetic and parasympathetic systems. The sympathetic or adrenergic system is activated with the purpose of adapting the individual organism for "flight, fright, or fight"—that is, the short-term availability and release of energy and alertness. In contrast, the parasympathetic nervous system facilitates the storage of energy and metabolic components to allow the release of energy in a situation of flight, fright, or fight. As such, the sympathetic and parasympathetic systems counteract each other's activities in a sort of "yin-yang" balance. Activation of the sympathetic nervous system implies the stimulation of the cardiovascular system, thus increasing heart rate, cardiac output, cardiac contractility, and blood pressure. During sympathetic stimulation, the activity of the gastrointestinal system is attenuated. Stimulation of the parasympathetic system implies the activation of the digestive system, particularly the gastrointestinal tract, with the aim of facilitating the storage of energy. During parasympathetic activation, the cardiovascular system's activity is reduced. Both the sympathetic and parasympathetic systems are subject to modulation by the central nervous system, particularly the brainstem. However, higher (cortical) brain centers can also modulate both subgroups of the peripheral autonomic nervous system. Several diseases are associated with dysregulation of either subgroup of the autonomic nervous system. Relevant examples of this pathophysiological involvement particularly concern the sympathetic nervous system, which is known to be overactive in important cardiovascular derangements or diseases, such as hypertension, congestive heart failure, or cardiac tachyarrhythmia. For this reason, a vast body of research has accumulated with the aim of finding drugs that can attenuate the hyperactivity of the sympathetic nervous system. Drugs with such activity are called antiadrenergic agents.

Sympathetic (Adrenergic) Nervous System and Its Receptors

The brainstem, consisting predominantly of the pons/medullary region, controls the peripheral sympathetic nervous system (Fig. 1). The peripheral sympathetic nervous system contains a kind of relay station called the sympathetic ganglion. Transmission in the ganglion is performed by the neurotransmitter acetylcholine (ACh), which targets the nicotine-like AChN receptors. Peripheral sympathetic neurons are subdivided into pre- and postganglionic types according to their anatomical position with respect to the ganglion. The sympathetic activity is transferred from the postganglionic neuron to the target organ (e.g., the heart or a blood vessel). The anatomical Transition structure required for this process is called the synapse (Fig. 2). The process of humoral neurotransmission within the synapse occurs as follows: The neurotransmitter norepinephrine (NE; also called noradrenaline) is

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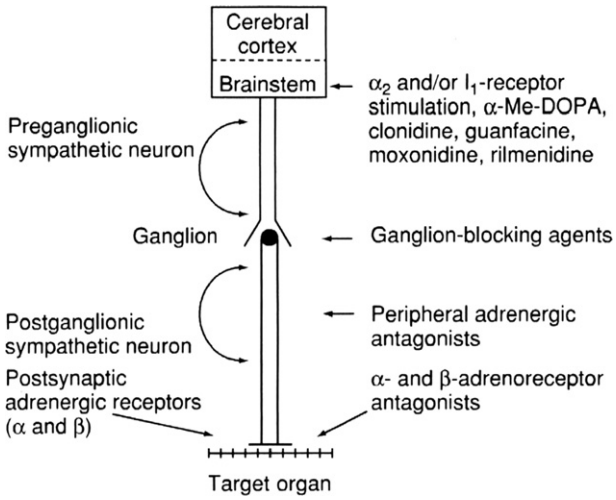

Fig. 1 Schematic representation of the sympathetic (adrenergic) nervous system. Adrenergic structures and receptors in the brainstem modulate the activity of die peripheral sympathetic nervous system. The targets of all relevant antiadrenergic drugs are shown. Virtually every structure/receptor of the system can be manipulated selectively by drugs.

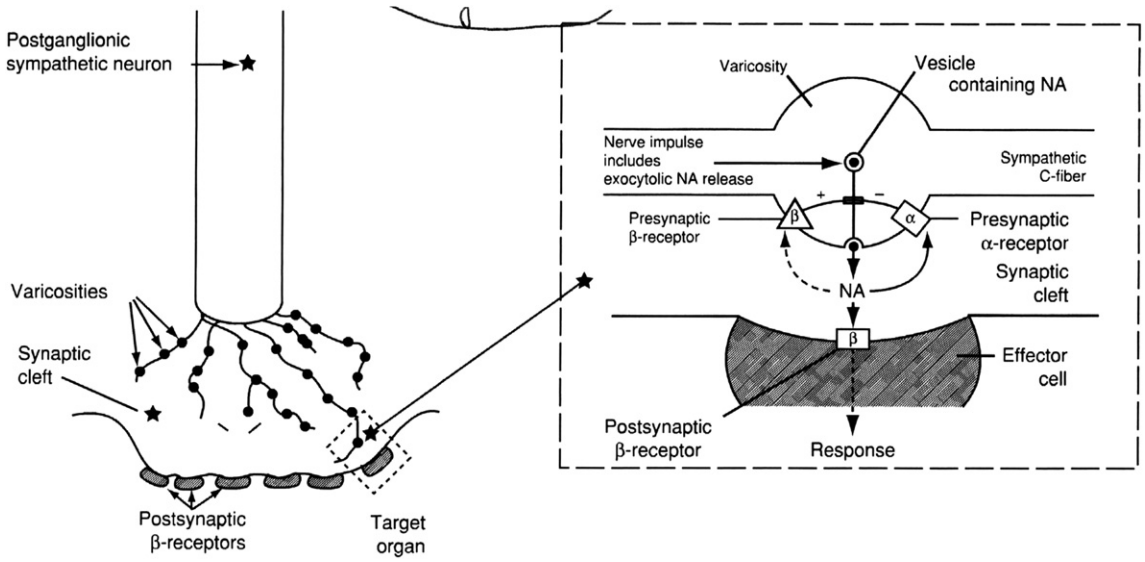

Fig. 2 Schematic representation of a sympathetic (adrenergic) synapse. Nerve activity releases the endogenous neurotransmitter from the varicosities at the nerve endings. Stimulation of postsynaptic α-adrenoceptors causes a physiological/pharmacological effect (e.g., vasoconstriction). Blockade of the α-adrenoceptor (postsynaptic) counteracts the effect of norepinephrine. Stimulation of presynaptic α-adrenoceptors inhibits the release of the neurotransmitter. Conversely, presynaptic α-receptor blockade stimulates the release of the neurotransmitter.

released from the postsynaptic nerve ending, diffuses through the synaptic cleft, and then binds to the receptors in the target organ. The receptor thus activated mediates a physiological/functional process (e.g., constriction of a blood vessel) or an increase in cardiac frequency or contractility. The receptor in the target organ is activated by the endogenous neurotransmitter NE but also by synthetic drugs, such as methoxamine, phenylephrine, and isoprenaline, that imitate the effects of NE. These activator drugs are called sympathomimetic agents. They are agonists with respect to the receptor. Antagonists are chemical compounds that block the stimulating effect of NE (the neurotransmitter) and that of sympathomimetic agents. The antagonist or blocker binds to the receptor, thus preventing the stimulating effect of the endogenous neurotransmitter NE. In the sympathetic nervous system, the receptor antagonists are also called antiadrenergic agents. The sympathetic or adrenergic receptors are subdivided into a variety of subtypes. α-Adrenoceptors are predominantly located in blood vessels but also in the brainstem. 3-Adrenoceptors are mainly present in various structures of the heart but also in the bronchi. In a later stage, there is a further subdivision into α_1/α_2 and $\beta_1/\beta_2/\beta_3$. The functional roles of the most important α- and β-adrenoceptors are summarized in **Tables 1** and **2**. The tables also note the effects of stimulation by an agonist and the effects of blockade by an antagonist.

Table 1 Functional effects of the stimulation/blockade of α-adrenoceptors by an agonist and an antagonist

Receptor	*Stimulation by an agonist causes*	*Blockade by an antagonist (blocker) causes*
α_{1A}	Vasoconstriction	Vasodilation
α_{1A} (prostate)	Contraction	Smooth muscle relaxation
α_2 (postsynaptic) vascular	Vasoconstriction	Vasodilatation
α_2 (presynaptic)	NE release↓	NE release↑
α_2 (brainstem)	Sympathoinhibition Blood pressure↓	

Table 2 Functional effects of the stimulation/blockade of β-adrenoceptors by an agonist and an antagonist

Receptor	*Stimulation by an agonist causes*	*Blockade by an antagonist causes*
β_1 (cardiac)	Heart rate↑ Contractility↑ A–V conduction↑	Heart rate↓ Contractility↓ A-V conduction↓
β_2 (vascular)	Vasodilatation	Vasoconstriction
β_2 (bronchi)	Bronchodilatation	Bronchoconstriction
β_2 (metabolic)	Blood glucose↑	Blood glucose↓
β_3 (metabolic)	Lipolysis—thermogenesis in brown fat	Antilipolysis—less thermogenesis
β_3 (cardiovascular)	Less oxidative stress—vasodilatation	Oxidative stress—vasoconstriction

The α- and β-adrenoceptors in the brainstem deserve special attention. These receptors in the pons/medulla region are involved in the central nervous regulation of the cardiovascular system, particularly that of blood pressure. The receptors belong to the α_2 subpopulation. Their stimulation by an agonist causes sympathoinhibition in the periphery, resulting in a reduced release of NE from the nerve endings and a decrease in blood pressure. These central α_2-adrenoceptors are also the target of centrally acting antihypertensives, such as clonidine and α–methyl-DOPA (through its active metabolite α-methylnorepinephrine).

Antiadrenergic Agents

As illustrated in Fig. 1, virtually any structure or receptor of the sympathetic nervous system can be modulated more or less selectively by antiadrenergic drugs. Several of these drugs play a role (or have played a role) as therapeutic agents in the treatment of cardiovascular or respiratory diseases.

α-Adrenoceptor Antagonists (α-Blockers)

Competitive inhibition of α-adrenoceptors by appropriate antagonists predominantly causes vasodilatation and a reduction of blood pressure, particularly in hypertensive patients. Doxazosin and prazosin are the prototypes of α_1-blockers. They are selective for the postsynaptic α_1-adrenoceptor subtype: This means that presynaptic α_2-adrenoceptors are not blocked, thus preventing the enhanced release of NE from the sympathetic nerve endings (Fig. 2). Nonselective, older α_1-blockers, such as phentolamine or phenoxybenzamine, are no longer used because they are poorly tolerated.

Doxazosin and prazosin are moderately effective antihypertensive drugs. Orthostatic hypotension, and sometimes headache, and reflex tachycardia are well-known adverse reactions, which are caused by vasodilation (also in the venous blood vessels). A few newer α-adrenoceptor antagonists display a certain degree of selectivity for the α_{1A}-receptor in the smooth muscle of the prostate. These agents cause relaxation of this smooth muscle tissue and hence facilitate urinary flow in patients with benign prostate hyperplasia (BPH). Alfuzosin, terazosin, and tamsulosin are examples of such agents. Silodosin is an α-blocker more uroselective and therefore may induce less postural hypotension, but ejaculation disfunction may be even more frequent.

β-Adrenoceptor Antagonists (β-Blockers)

β-Adrenoceptor antagonists are competitive inhibitors of the effects of endogenous catecholamines (norepinephrine and epinephrine) at the level of β-adrenoceptors in various tissues. In clinical medicine, they are widely used in the treatment of

hypertension, angina pectoris, supraventricular tachyarrhythmias, congestive heart failure, and as secondary prevention following myocardial infarction. Their main therapeutic activity is to counteract the detrimental effects of catecholamines released by the hyperactive sympathetic nervous system. Well-known adverse reactions are bronchoconstriction, cold hands and feet (caused by peripheral vasoconstriction), and sleep disturbances. Patients with COPD may receive β-blocker treatment, but careful titration and the use of β_1-selective blockers is advised. The therapeutic actions are all mediated by the blockade of β_1-adrenoceptors, whereas blockade of β_2-adrenoceptors is the cause of most of the side effects. For this reason, β_1-selective blockers are preferable. Atenolol, bisoprolol, and metoprolol are examples of β_1-selective blockers. Newer β-blockers, such as carvedilol and nebivolol, contain an additional vasodilator component that may offer a hemodynamic and metabolic (i.e., less or no interference with insulin sensitivity and lipid metabolism) advantage. Among vasodilating β-blockers, the mechanism of action for arteriolar dilatation is different. Carvedilol acts through blockade of the α_1-receptors while Nebivolol increases availability of endothelial-derived NO through stimulation of nitric oxide synthase (eNOS) and possibly is also β_3-adrenoceptor agonist.

Postganglionic Neuron Blockers

The electrical membrane activity in postganglionic neurons can be blocked (via the inhibitory influx of Na + ions) by drugs such as guanethidine, cyclazenine, and bretylium. Although they are effective blood pressure-lowering drugs, these agents have been abandoned because of their poor tolerability.

Ganglion Blocking Drugs (Ganglioplegic Agents)

These agents are competitive blockers of the AChN receptors in the sympathetic and also the parasympathetic ganglia. Ganglionic blockade in the sympathetic ganglia accounts for their effective lowering of blood pressure, particularly in hypertensive patients. Pentamethonium, hexamethonium, and trimethaphan are examples of ganglioplegic agents. These agents have been abandoned as therapeutics because of their low tolerability, which is partly due to the blockade of the parasympathetic ganglia.

Rauwolfia Alkaloids

These older drugs have lost their importance as therapeutics because of their poor tolerability. Reserpine and related alkaloids (from Rauwolfia serpentina) destroy the storage sites (granules) of norepinephrine in the sympathetic nerve endings and hence impair the function of the sympathetic system. Although reasonably effective blood pressure lowering agents, their adverse reactions preclude their use in the treatment of hypertension.

Centrally Acting Antihypertensives

α-Adrenoceptors present in the pontomedullary region of the brainstem (e.g., the nucleus tractus solitarii and the vasomotor center) will cause peripheral sympathoinhibition when stimulated with endogenous norepinephrine and also centrally acting antihypertensives. Clonidine, guanfacine, and α-methyl-DOPA (through its active metabolite α-methylnoradrenaline) are α_2-adrenoceptor agonists and, accordingly, cause peripheral sympathoinhibition and the reduction of elevated blood pressure in hypertensive patients. The centrally acting drugs are effective antihypertensives, and their mode of action appears to be attractive on hemodynamic grounds. Their adverse reactions are unpleasant (sedation, dry mouth, and male sexual impotence). These side effects are also mediated by α_2-adrenoceptors (although not located in the brainstem) and thus it is virtually impossible to develop drugs with the same mode of action as clonidine, etc., but with a better profile of adverse reactions. Imidazoline (I1) receptors in the brain (in the ventrolateral medulla) have been identified as targets for centrally acting drugs. Stimulation of central I1 receptors also causes peripheral sympathoinhibition and lowering of blood pressure. Newer drugs causing this effect are moxonidine and rilmenidine, which display lower affinity for α_2-adrenoceptors than clonidine or α-methyl-DOPA. It is hoped that these newer agents display a better tolerability.

Therapeutic Use of Antiadrenergic Drugs

Essential Hypertension

A subtle association between essential hypertension and hyperactivity of the sympathetic nervous system has been repeatedly demonstrated. On the other hand, the treatment of hypertension was initially based on the use of various antiadrenergic drugs, which seems a rational approach. Other types of drugs have been introduced, such as diuretics, calcium antagonists, and suppressants of the renin–angiotensin system (ACE inhibitors and AT 1 blockers). β-Blockers continue to be widely used in antihypertensive treatment, and this approach is supported by numerous clinical trials and several meta-analyses. α-Blockers, particularly doxazosin, have obtained a moderate position in antihypertensive treatment, which is not clearly supported by evidence on an epidemiological scale. Centrally acting agents such as clonidine and α-methyl-DOPA have been largely abandoned

because of their poor tolerability. This approach may be somewhat improved by the introduction of the imidazoline agonists (moxonidine and rilmenidine), but the clinical follow-up for the use of these agents is insufficient. α-Methyl-DOPA is still one of the drugs that still may be used for the treatment of hypertension in pregnancy. Rauwolfia alkaloids, postganglionic neuron blockers, and ganglioplegic agents have been abandoned because of their poor tolerability.

Angina Pectoris

Stable angina pectoris is routinely treated with a variety of anti-ischemic drugs in addition to invasive interventions for revascularization, such as balloon dilatation of a stenosis, or cardiac surgery. Nitroglycerin and other nitrates are used for the treatment of acute symptoms. β-Blockers are first choice for long-term treatment, unless contraindications preclude the use of these drugs. The reduction in heart rate is the major basis of the anti-ischemic action of β-blockers, thus reducing myocardial oxygen consumption. Calcium antagonists, long-acting nitrates, low-dose aspirin, and a statin are usually included in the treatment schedule.

Secondary Prevention After Myocardial Infarction

Recurrent infarction and/or sudden death after myocardial infarction (MI) are major risks in post-MI patients. β-Blockers have been demonstrated to be significantly protective against these risks, probably as a result of their antiarrhythmic activity, which protects against the toxic effects of endogenous catecholamines released by the sympathetic system.

Supraventricular Tachyarrhythmia

The antisympathetic effects of β-blockers explain their antiarrhythmic potency in the treatment of supraventricular tachyarrhythmia. Sotalol, frequently used as an antiarrhythmic agent, is a β-blocker with additional antiarrhythmic potency because of its class III (Vaughan-Williams' classification) activity. β-blockers are indicated in order to reduce an high ventricular rate in patients with atrial fibrillation. They may also prevent new onset or recurrent atrial fibrillation in patients with heart failure, after myocardial infarction and following cardiac surgery.

Congestive Heart Failure

β-Blockers are recognized as beneficial therapeutics in the management of congestive heart failure (CHF). CHF is usually accompanied by sympathetic overactivity, which is detrimental for the decompensated heart. β-Blockers counteract this process. Inotropic activity of the β-blockers that is too negative can be avoided if these agents are initiated in clinically stable patients at a low dose and gradually uptitrated to the maximum tolerated dose. Beneficial activity in CHF has been demonstrated for carvedilol, bisoprolol, metoprolol and nebivolol. β-blockers have been shown to be able of reducing mortality and hospitalization for heart failure in symptomatic patients with reduced ejection fraction, despite treatment with an ACEI and a diuretic in most cases. There is no evidence so far that they may improve prognosis in patients with heart failure and ejection fraction above 50% or/and atrial fibrillation.

Glaucoma Simplex

In patients with glaucoma simplex (open-angle glaucoma), intraocular pressure can be lowered by the topical application of β-blockers such as timolol. The therapeutic activity is based on reduction of the production of aqueous humor.

Benign Prostate Hyperplasia

The moderately beneficial effect of α_{1A}-blockers in the symptomatic treatment of BPH was mentioned previously. Drugs used for this purpose are alfuzosin, terazosin, tamsulosin and silodosin. Hypotension is a side effect of these agents, probably less with silodosin.

See also: Adrenergic Mechanisms. Baroreceptor Responses. Novel Insights in ß-Adrenergic Receptor Signaling

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Pheochromocytoma and Paraganglioma Syndromes☆

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Introduction

Catecholamine-producing tumors arise from adrenal medulla (pheochromocytoma (PHEO) or adrenal paraganglioma) or paraganglia (paraganglioma (PGL) or extra-adrenal pheochromocytoma). The last associates with either sympathetic or parasympathetic paraganglia – each having unique anatomic (location) and functional (secretory) characteristics. Sympathetic PGL (sPGL) are secretory and follow sympathetic ganglia, widely distributed from skull base down along paravertebral sympathetic chain, while parasympathetic PGL are mostly nonsecretory and usually found in the head and neck area along the branches of glossopharyngeal and vagus nerves (head and neck PGL – HNPGL). PHEO/PGL is a rare disease – its prevalence is low (0.1–0.6%) in patients with hypertension. On the other hand, because of the high prevalence of hypertension, as well as lack of specific symptoms and signs and, as in case of HNPGL, lack of any functional sign other than local effect of the tumor, disease has to be suspected and worked up in a very large number of patients. To further complicate this situation, PHEO/PGL presents with mainly nonspecific and at times surprisingly vague symptoms and signs, so unless thought about, it could be easily missed and progress to potentially preventable but rather devastating complications.

Conceptual Shift

Recent decades propelled our knowledge in PHEO/PGL field to a qualitatively different level. These developments significantly changed both diagnostic and management strategies, but rather than displace, integrated them into main conceptual approach to PHEO/PGL as part of neuroendocrine neoplasia. These also deepened our understanding of pathophysiology of PHEO/PGL and overall tumorigenesis. We will discuss below these conceptual shifts based on recent discoveries and the current stand of the PHEO/PGL field.

Definition – "Who Is Who"

Definition of adrenal tumors as pheochromocytomas and their paragangial counterparts as paragangliomas is rather a historical nod of respect to initial chromium salt staining of pheochromocytomas that generated the original name of the tumor. It was logical to divide them into adrenal and extra-adrenal pheochromocytomas, combining the idea that historical positive staining of the tumorous tissue correlates closely with its secretory activity. Definition of the disease at this point was one of catecholamine-producing tumors arising from chromaffin (chromium salt affine) cells. What is known now as HNPGLs were called glomus tumors and thought to be unrelated to PHEO, treated by surgeons mostly for local effects and seldom seen by endocrinologists. It is not surprising that our current definition of this group of tumors differs dramatically from both initial "catecholamine-producing neoplasms" and more updated – "tumors bearing catecholamine-metabolizing cells" – based on phenomenon of intracellular methylation of catecholamine compounds, currently used for biochemical diagnosis of the disease (see below). Based on identical histology, it is more universal and relies on the fact that PHEO/PGL are tumors arising from single embryonic origin (neural crest), whenever it is intra- (medulla) or extra-adrenal (paraganglia), sympathetic or parasympathetic, secretory or nonseretory, benign or malignant.

Etiology – "The Not so Much Rule of 10 Anymore"

PHEO/PGL was usually seen as mostly sporadic disease with only rare – around 10% – hereditary causes and this area is probably the most significant of recent changes in our understanding of the disease. We know now that 30–40% of PHEO/PGL are associated with inherited mutations and additional 14% of sporadic tumors are found to have somatic mutations. As the number of additional hereditary forms of PHEO/PGL continues to grow, we know now of at least 13 susceptibility genes, associated with PHEO/PGL and the whole spectrum of hereditary can be divided into two large clusters: pseudoxypoxia and kinase signaling pathways (**Tables 1** and **2**). While the last pathway is relatively common in current oncology, the pseudohypoxia-driven tumorigenesis is unique and empowers us to deeper understand events and processes that lead to tumor formation – important for both diagnosis and treatment not only of these particular tumors, but very possibly many others. Syndromatic classification of genetically determined tumors had dynamic history and, in addition division by signaling pathways could also be classified historically as follows:

☆*Change History*: 2014. Vitaly Kantorovich and Karel Pacak updated the text and references.

This article is an update of Karel Pacak, Graeme Eisenhofer, Christian A. Koch, Pheochromocytoma, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 594-596.

Table 1 PHEO/PGL with hypoxic transcriptional signature

Gene	*Age*	*Tumor*	*Biochemistry*	*Malignancy*	*Additional features*
VHL	30s	PHEO	NE or NE/DA	Low	CCRC, retinal hemangiomas, CNS hemangioblastomas
SDHA	27–77	HNPGL, sPGL, PHEO	Not clear	Not clear	CCRC, GIST, pituitary adenoma
SDHB	30s	sPGL	NE or DA	High	CCRC, GIST, pituitary adenoma, neuroblastoma, pulmonary chondroma
SDHC	40s	HNPGL	NE or NS	Low	CCRC
SDHD	30s	HNPGL or sPGL	DA or NS	Low	CCRC, GIST, pituitary adenoma, pulmonary chondroma
SDHAF2	30s	HNPGL	Not clear	Low	
HIF2α	17–35	HNPGL	NE	Low	Polycythemia, somatostatinoma

Table 2 PHEO/PGL with kinase receptor transcriptional signature

Gene	*Age*	*TumorBiochemistry*	*Malignancy*	*Additional features*
RET	30s	PHEO E or E/NE	Low	MTC, HPT, marphanoid habitus, mucosal ganglioneuromas
NF1	40s	PHEO E or E/NE	Low	Café-au-lait spots, neurofibromas, MTC, carcinoid, neural sheath tumors
TMEM127	40s	PHEO E	Low	
MAX	30s	PHEO E/NE	Moderate	
HRAS	31–76	PHEO E or NE	Low	

Abbreviations: CCRC, Clear cell renal carcinoma; E, epinephrine; NE, norepinephrine; DA, Dopamine; GIST, Gastrointestinal stromal tumor; NS, nonsecretory; MTC, medullary thyroid cancer; HPT, hyperparathyroidism.

"The good old trio"

von Hippel–Lindau syndrome

Described over 100 years ago, the type 2 of this syndrome associates with predisposition to several tumors, apart from pheochromocytomas: hemangioblastomas, retinal angiomas, clear cell renal carcinomas, renal and pancreatic cysts, pancreatic tumors and epididymal cystadenomas. The mechanism of tumorigenesis is probably one of the best understood in current oncology and is carried through interaction of the von Hippel-Lindau (VHL) protein and hypoxia-inducible factor (HIF). In normoxic conditions, HIF is hydroxylated, which opens a binding site for VHL protein. The last possesses an E3 ubiquitin ligase activity, thus "marking" HIF for ubiquitination/degradation. In physiologic/pathologic hypoxia, HIF1α overexpression induces angiogenesis to promoted increased glucose supply. With mutated and nonfunctional VHL protein, the overexpression of HIF1α is accompanied by significant shift towards expression of HIF2α, which adds anti-apoptotic effect and increased cellular proliferation through upregulated cyclin D1, which results in tumorigenesis. Undegraded HIF1α forms heterodimer with HIF1β, becoming a transcription factor able of direct activation of VEGF and erythropoietin production and interaction with mTOR. Pheochromocytoma in VHL is usually adrenal, frequently bilateral and mostly benign. Because of low expression of phenylethanolamine N-methyltransferase (PMNT) in VHL pheochromocytomas, secretory profile consists almost exclusively of norepinephrine.

Multiple endocrine neoplasia type 2

Multiple Endocrine Neoplasia type 2 (MEN2) is an autosomal dominant syndrome related to the mutation in *RET* proto-oncogene. The last encodes for tyrosine kinase receptor that regulates cell proliferation and apoptosis. MEN2 is characterized by combination of medullary thyroid cancer in most of the patients, risk of pheochromocytoma later in the course of the disease in about half of the patients and either hyperparathyroidism (MEN2A) or mucosal ganglioneuromas and marphanoid habitus (MEN2B). Pheochromocytomas in this syndrome are also adrenal, often bilateral, rarely malignant (unless developed in childhood) and display epinephric secretory profile.

von Recklinghausen's disease (Neurofibromatosis type 1)

von Recklinghausen's disease represents an autosomal dominant disorder related to mutation in the neurofibromatosis type 1 tumor suppressor gene, which controls cellular growth and differentiation through inhibition of RAS signaling cascade and mTOR kinase pathway. Pheochromocytomas are rare in these patients, who usually display following findings: café-au-lait spots, cutaneous or plexiform neurofibromas, iris hamartomas, optic nerve gliomas, and extensive freckling. When found, pheochromocytomas are of epinephric secretory profile, adrenal and mostly benign.

The paraganglioma syndromes

The history of paraganglioma syndromes shows that even in very recent history new clinical syndromes are described and uncover very exciting pathological processes. It was in early 1970's when first association between high altitude and carotid body tumors was described. Later four distinct clinical PHEO/PGL syndromes strongly believed to be hereditary were described and awaited their genetic conformation. In meantime, pathophysiologic background of these conditions started to emerge, based on the decreased activity of the succinate dehydrogenase (SDH) in some of these tumors. Finally, genetic cause of these syndromes was discovered, starting with the D subunit of the complex (SDHD), further through all subunits. Although uncovering actual complex mutations remains to be extraordinary achievement in the history of hereditary PHEO/PGL, it is the physiologic phenomenon that makes this discovery many times more exciting. SDH is an enzyme complex, located on the inner mitochondrial membrane. This complex plays dual role – as complex II of the mitochondrial electron transport chain and as an enzyme that oxidases succinate to fumarate within citric acid cycle. The complex is assembled from 4 independent units – SDHA, SDHB, SDHC and SDHD – each encoded by respective gene. Mutation of either, as well as associated SDHAF2 – accessory protein that flavinates SDHA and thus maintains its proper functional activity, results accumulation of succinate and reactive oxygen free radicals, which stabilize HIFα (see above).

Type 1 (SDHD)

Tumors with SDHD mutations associate with HNPGL, often multifocal, bilateral and recurrent. On the other hand, the rate of malignancy is low. While most of tumors are nonsecretory, these that do secrete show dopaminergic and norepinephric secretory prolife.

Type 2 (SDHAF2)

Patients with SDHAF2 mutation-associated disease usually develop benign multiple nonsecretory HNPGL.

Type 3 (SDHC)

These mutations predispose to formation of HNPGL, usually nonsecretory and, while nonmalignant, can often be multifocal.

Type 4 (SDHB)

Best studied, this mutation associates with mostly sPGL, although PHEO and HNPGL also occur. Secretory profile represents dopamine and norepinephrine, while nonsecreting tumors also occur. This condition occurs in younger patients and has significantly increased rate of malignancy.

SDHA

The last of the SDH complex mutations causing PHEO/PGL to be discovered, this association is very rare – only half dozen patients have been described till now, show variable penetrance, appears as both PHEO and PGL, showing mostly norepinephric or nonsecretory profile.

The newbies

Several additional gene mutations-associated PHEO/PGL have been recently discovered, thus further expanding out understanding of the tumor formation processes.

Transmembrane protein (TMEM) 127

Patient with TMEM127 mutation-related disease develop adrenal epinephrine secreting PHEO, although several cases of extra-adrenal tumors were also described. The disease is frequently bilateral and benign. TMEM127 protein was suggested to play role in intracellular protein trafficking, as well as associates with components of mTOR signaling cascade.

Myc-associated factor X (MAX)

Pheochromocytomas in patients with MAX gene mutation are often bilateral, at times metastatic and show both epinephric and norepinephric secretory profile. Several sPGL were also described. The protein is involved in the regulation of cellular proliferation, differentiation and apoptosis.

PHD2

There is a very rare association between mutation in the PHD2 gene and PHEO/PGL. The prolyl-hydroxylases domain (PHD) proteins hydroxylate propyl residues at the oxygen dependent degradation domain of HIFα, thus allowing binding of the VHL protein and further degradation of HIFα. Mutation of PHD2 associates with overproduction of erythropoietin that leads to Chuvash polycythemia. It was also described in one patient with polycythemia and recurrent PGL.

H-RAS

There were only few patients described with H-RAS mutation-related PHEO/PHL – secretory profile is epinephric. The protein represents part of the same signaling pathway, affected in patients with MEN2 and NF1 mutations.

HIF2α

Gain-of-function mutations of HIF2α were recently described to associate with PHEO/PGL with or without polycythemia and somatostatinomas, showing norepinephric secretory profile. In context of this mutation, the Pacak-Zhuang syndrome includes multiple PHEO/PGL and duodenal somatostatinomas associated with polycythemia in females only.

Carney triad and diad (Carney–Stratakis syndrome)

Patients are found to have PHEO/PGL with (triad) or without (diad) pulmonary chondromas, and while in triad no single gene was found to be responsible for the disease, in the diad majority shows mutations in one of the SDHx genes, suggesting complex genetic cause of these clinical syndromes.

Others

Mutations of isocitrate dehydrogenase (ID) and fumarate hydratase (FH), which like SDH complex, are part of the citric acid cycle were found to associate with rear cases of PHEO/PGL, in addition to glioblastoma multiforme (ID) and renal cell cancer (FH). Mutation of KIF1Bβ was found in rare cases of PGEO and neuroblastomas, possibly through its' function of apoptosis regulation.

Genetic testing

Genetic testing for familial PHEO/PGL is indicated in all cases of disease that presents in young patients, patients with family history suggestive of PHEO/PGL or components of combined syndromes, patients with multiple or metastatic disease. Currently, mutations in *SDHx* (subunits A, B, C, D), *SDHAF2*, *MAX*, *RET*, *TMEM127* and *VHL* are available as a combined panel, providing with high 98.7% sensitivity for detecting mutations associated with PHEO/PGL.

Clinical Presentation

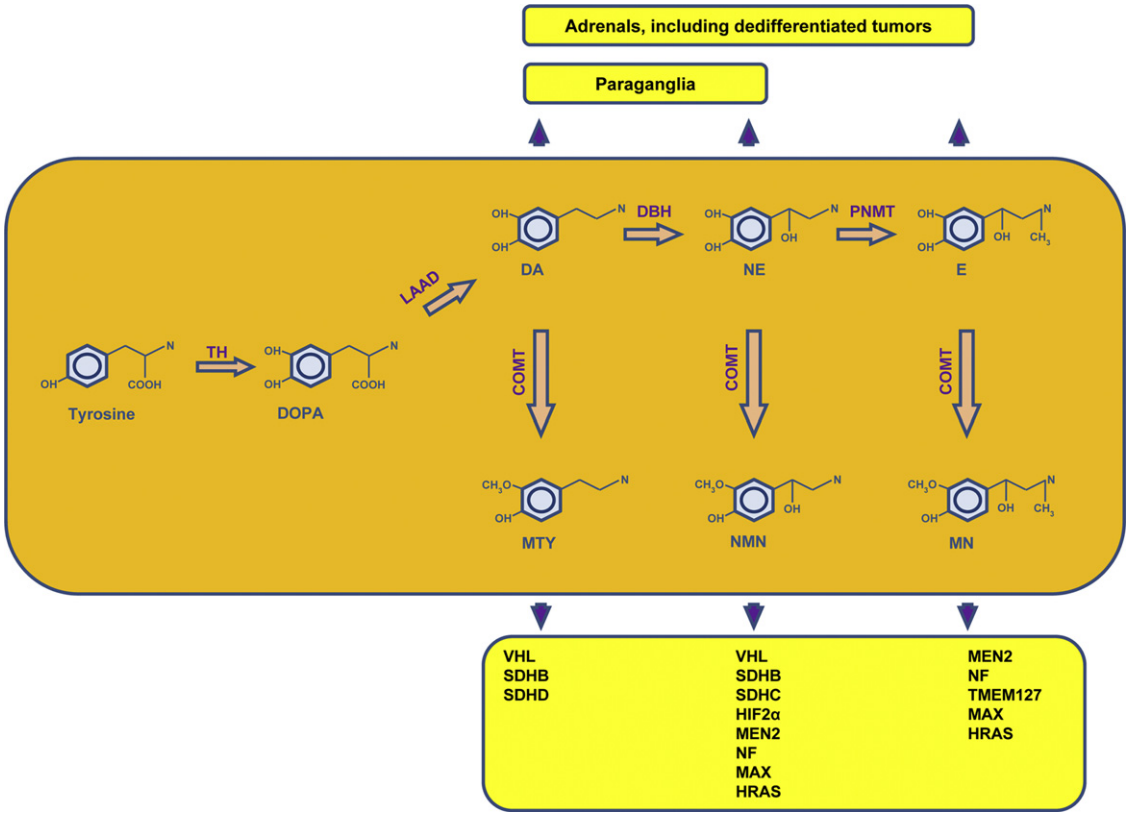
Clinical presentation of PHEO/PGL relates to several different processes – acute/paroxysmal hypercatecholaminemia, chronic hypercatecholaminemia with end organ effects, existence of co-secretory substances, existence of other syndromic features and local effects. There is significant genotype-biochemical phenotype correlation in cases of mutation-related disease (discussed above). There are also significant differences in clinical presentations related to the unique secretory profile of the tumor. Episodic secretion of catecholamines, especially with predominance of norepinephrine, will associate with the "classic PHEO attack" – paleness, tachycardia, paroxysmal hypertension, anxiety, palpitations and post-attack dermal hyperemia, diaphoresis and severe fatigue. About a third of cases present with persistent hypertension and mask well under the rest of the idiopathic hypertension patients' pool. Patients with epinephric and dopamine-secreting profiles, on the other hand, present with hypotension and orthostatic complains, resulted from peripheral vasodilatatory effect of these compounds. Long standing hypertension will afterall result in loss of vascular elasticity and cardiac hypertrophy. Of particular importance are rare cases of PHEO crises, resulting in severe malignant hypertension, strokes, acute heart attacks, arrhythmias and multiorgan failure. Some of these happen acutely during significant stress events, like unrelated surgeries in relatively healthy individuals. PHEO/PGL earned its famous nickname of the "Great Masquerader" also for range of nonspecific symptoms like leukocytosis, hypercalcemia, hyperglycemia, constipation, megacolon, hematuria, nausea and vomiting etc. Additional signs and symptoms could also relate to the co-secretory substances like ACTH, PTHrP, vasopressin, calcitonin etc.

Diagnostic Approach

The mainstay of the diagnostic approach changed significantly in means, but remains same in concept: a]. as a rare disease, PHEO/PGL has to be suspected – based on symptoms or signs, family history or clinical findings, b]. clinical suspicion has to be proven biochemically, supporting the existence of PHEO/PGL, c]. tumor/tumors are to be localized. The sequence of events has significantly changed over recent decades – it became "the rule out" rather than "rule in" diagnoses, because of majority workups carried for imaging or surgical incidentalomas, suggestive of disease because of location or histologic findings.

Biochemistry

Demonstration of biochemical hypercatecholaminemia represents biochemical proof of existing PHEO/PGL. Although still correct, this statement had been significantly modified over recent decades. Catecholamines – epinephrine, norepinephrine and dopamine are secreted episodically and show relatively short half-life. Majority of norepinephrine is synthesized in sympathetic neurons and in case of significant stress acute systemic spill-over can reach significant values. Adrenal medulla, on the other hand, possesses significantly higher amount of phenylethanolamine-N-methyltransferase (**Fig. 1**), which converts norepinephrine to epinephrine. One of recent changes in concept relies on the fact that less differentiated and more malignant tumors also express less "advanced" catecholamine-producing enzymatic machinery and drop synthesis at earlier stages, ending up secreting precursors, including dopamine, rather than more advanced end products – nor- and epinephrine. This finding can be used not only as diagnostic but also as prognostic sign. Another important concept is acceptance of the fact that HNPGL are



Adrenals, including dedifferentiated tumors
Paraganglia
DA
DBH
NE
PNMT
E
LAAD
TH
Tyrosine
DOPA
COMT
COMT
COMT
MTY
NMN
MN
VHL
SDHB
SDHD
VHL
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HIF2α
MEN2
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norepinephrine analogue has high affinity to the norepinephrine transporter, which allows intracellular transfer and accumulation of ^{123}I- or ^{131}I- tracer within catecholamine-producing tumor. Positron emission tomography (PET) technology uses both standard glucose-based tracer, as well as PHEO/PGL-specific catecholamine-based tracers (DOPA and dopamine). The fact that some PHEO/PGL express somatostatin receptors is used in somatostatin receptor imaging – octreoscan.

Fig. 1 Catecholamine synthesis, metabolism and secretion in different PHEO/PGL syndromes. Short arrows define active secretion or spill-over of catecholamines and their metabolites from chromaffin cell of PHEO/PGL into systemic circulation. Abbreviations: DOPA, dihydroxyphenylalanine; DA, dopamine; NE, norepinephrine; E, epinephrine; MTY, methoxythyramine; NMN, normetanephrine; MN, metanephrine; TH, tyrosine hydroxylase; LAAD, aromatic *L*-amino acid decarboxylase; DBH, dopamine β-hydroxylase; PMNT, phenylethanolamine *N*-methyltransferase; COMT, catechol-*O*-methyltransferase. For abbreviations of clinical syndromes please see text.

Management

Definitive therapy of PHEO/PGL remains to be surgical resection. Medical therapy plays crucial role is stabilization of acutely decompensated or significantly symptomatic patient in the initial phase of management, preoperative preparation of the patient to assure uneventful surgery, symptomatic treatment of recurrent or inoperable disease. The mainstay consists of initial α-adrenergic blockade with additional β-blockade as needed. Phenoxybenzamine – is an irreversible long acting oral α-adrenergic antagonist and is still widely used because of efficacy and extended safety profile. It does cause longer postoperative hypotension, when compared with shorter acting selective α_1-adrenergic antagonist like doxazosin, prazosin and terazosin. β-blockade is initiated after achieving full α-adrenergic blockade and is used to prevent excessive tachycardia. Combined α- and β-adrenergic blockers – labetalol and carvedilol could have unfavorable ration between α- and β- blocking components and should be used with caution or after completed α-adrenergic blockade. Methyrosine "pre-occupies" tyrosine hydroxylase with production of inactive L-catecholamines and is used as additional therapy in cases of severe hypercatecholaminemia. Different groups have successfully used calcium channel blockers, especially nicardipine. In cases of hypertensive crises or during surgery, IV therapy is carried out with either sodium nitroprusside or phentolamine. After surgery, patients are followed up for disease recurrence, especially in cases of familial syndromes prone to recurrent or malignant disease (SDHB).

Summary

Our knowledge and understanding of PHEO/PGL had significantly changed in recent decades. While the disease is famously called "The Great Masquerader" for its' varying and at times diagnostically challenging clinical appearances, recent changes in our understanding of pathophysiology and genetics could suggest at least some modification in the nickname, maybe between "The Metamorphic Masquerader" or just "The Shapeshifter".

See also: Malignant Pheochromocytoma. Pheochromocytoma/Paraganglioma: Diagnosis and Treatment. Pheochromocytoma/Paraganglioma: Management, Genetics, and Follow-up

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Pheochromocytoma/Paraganglioma: Diagnosis and Treatment

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Introduction

The terms pheochromocytoma (PHEO) and paraganglioma (PGL) refer to a broad spectrum of clinical conditions that have as their common denominator the origin from a pathological (neoplastic) proliferation of neural crest cells.

Clinical presentation and evolution of the disease is extremely variable in relation to several factors, such as the secretive functional characteristics of the cells involved, the primitive site of neoplastic proliferation and the tendency to form remote metastases, the presence of a genetic predisposition that may affect the expression of particular phenotypes.

PHEO/PGL is sometimes a complex diagnostic and therapeutic challenge, but the main difficulty is probably the fact that it is a rare condition that is met on very few occasions by most physicians and therefore may remain unrecognized. A diagnostic algorithm is outlined iGn **Fig. 1**.

A good knowledge of the polymorphic expressions of this disease should result in clinical suspicion, which can be confirmed or excluded reliably in most cases. Similarly, the therapeutic management of PHEO/PGL is well established in the overwhelming majority of cases, provided it is entrusted to experienced medical staff.

Clinical Presentation

The presence of a PHEO/PGL can be suspected in different clinical settings.

Signs and Symptoms Related to Catecholamine Excess

The clinical picture of PHEO/PGL is particularly variable and mainly reflects the effects of excessive adrenaline and noradrenaline production (see Manger, 2006). The amount and mode of release of these hormones by the tumor is therefore of paramount importance. In some circumstances a PHEO/PGL is asymptomatic, that is, its manifestations are modest and scarcely perceived by the patient; in fact, not a few PHEO/PGLs are detected incidentally or at the autopsy table. In other cases, presentation may be dramatic, with major complications such as acute myocardial infarction, stroke, fatal arrhythmias or acute aortic dissection.

More than half of patients with PHEO/PGL experience paroxysmal crises. Their frequency varies from occasional to multiple times and generally increases with tumor progression. Sometimes there are identifiable trigger factors, which may include (i) the Valsalva maneuver or any movement that exerts mechanical compression on the tumor mass; (ii) the ingestion of foods containing tyramine (brown cheese, some red wines) or synephrine (orange juice); (iii) the intake of certain drugs (opiates, tricyclic antidepressants, ACTH or steroids, …).

The duration of the crisis varies from a few minutes to several hours and can increase over time. Paroxysmal symptoms are variable, but the clinical picture is fairly constant in the same patient.

In most cases the crisis is reported by tachycardia, followed by headache, sweating, anxiety, tremors, nausea, vomiting, abdominal or thoracic pain, paresthesia, asthenia, dyspnea, in various association. The intensity of the symptoms may increase with the progression of the disease.

Hypertension can manifest itself with a real crisis (~25%) or as a crisis overlapping with a stable hypertension (~25%). Body temperature may slightly increase during the crisis and the patient may experience arrhythmias and/or electrocardiographic alterations.

In patients who do not experience a crisis, or in intercritical phases, patients may chronically manifest the same type of symptoms. Stable hypertension is present in more than half of the patients, often accompanied by significant pressure variability and orthostatic hypotension. Symptoms and signs related to increased metabolic activity (heat intolerance, weight loss, hyperglycemia) are sometimes present. The production of different peptides may be responsible for atypical clinical manifestations (hypercalcemia, Cushing syndrome, …).

Local Effect of Tumor Mass

Occasionally PHEO/PGL may present a palpable abdominal mass, whose compression can trigger a crisis. On the other hand, the presence of a painless mass in the upper part of the neck, below the angle of the jaw, is the typical presentation of carotid body PGLs, whereas pulsatile tinnitus and conductive hearing loss are features of jugulo-tympanic PGLs. In addition, head and neck

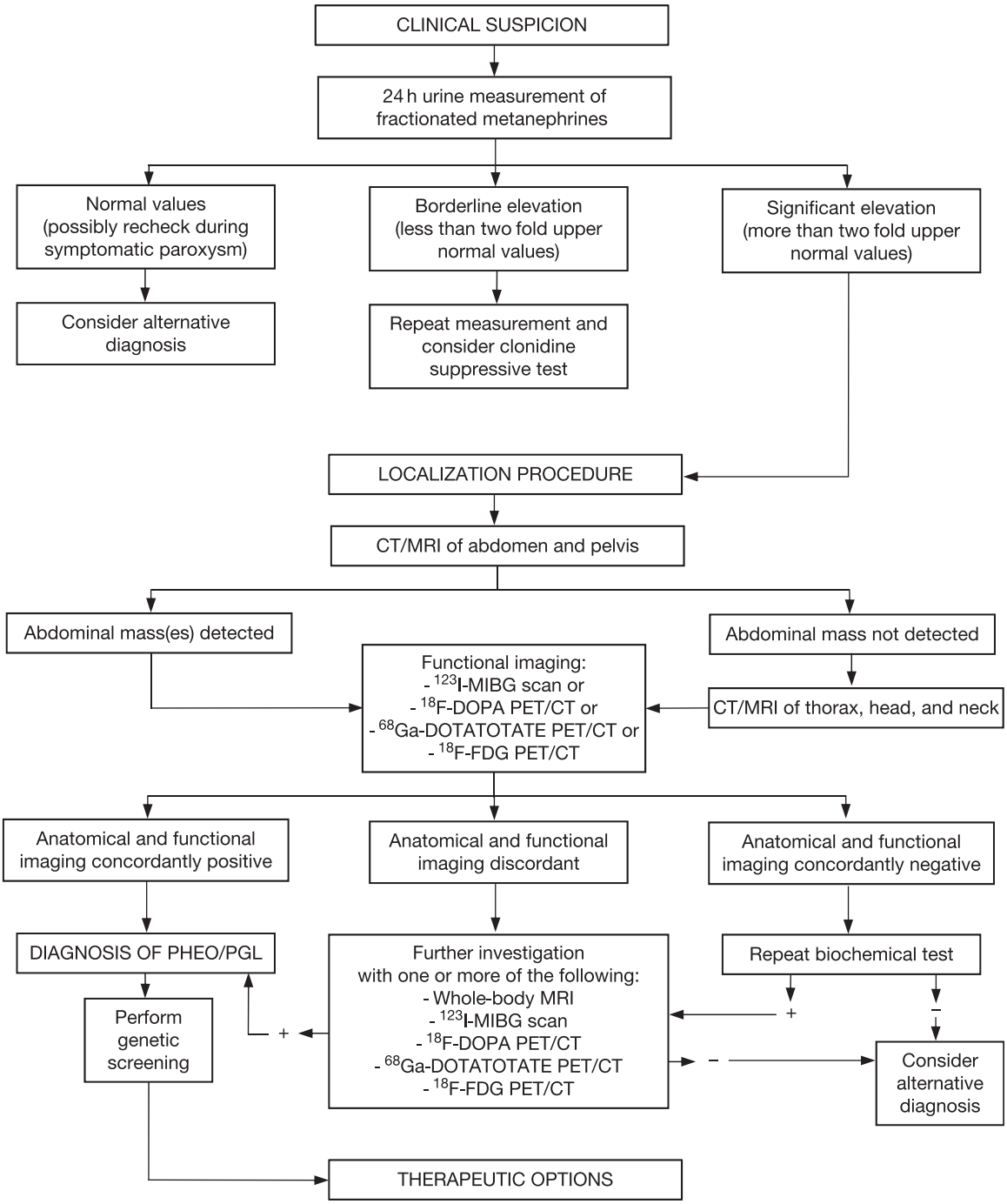

Fig. 1 Diagnostic algorithm of PHEO/PGL.

PGLs may cause several signs and symptoms related to the compression of lower cranial nerves VII, IX, X, XI, and XII (facial nerve paralysis, dizziness, hoarseness, Horner syndrome, dysphagia).

Incidentaloma

An adrenal mass can be incidentally detected when performing imaging techniques [ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography with 18F-2-deoxy-D-glucose (FDG-PET)] not specifically addressed to detect adrenal disease. The prevalence of adrenal incidentalomas varies among studies, ranging from less than 1% up to 4% of abdominal CT scans, and in 1.5%–14% of the cases these masses proved to be PHEOs. More rarely, PGL can be diagnosed as incidentally detected extraadrenal mass.

Associated Syndromic Conditions

A few genetically determined syndromes (including multiple endocrine neoplasia type 2, neurofibromatosis type 1, von Hippel-Lindau, Carney Stratakis dyad) may include PHEO/PGLs, and the presence of clinical features suggestive of these syndromes (Table 1) must prompt the search for the presence of such tumors.

Asymptomatic Carriers of Genetic Predisposition to PHEO/PGL

Many genes have been associated to the pathogenesis of PHEO/PGL (see "Genetic predisposition") and the prevalence of pathogenetic germline mutations among PHEO/PGL patients exceeds 30%. Such a prevalence justifies a systematic genetic screening of *all* patients (irrespective of any evidence of positive family history and/or syndromic presentation), which in turn leads to the detection of a number of relatives carrying the same mutation identified in the proband. Although the penetrance of genetic susceptibility may be rather low, these subjects must undergo clinical evaluation for early detection of PHEO/PGL and possibly associated syndromic lesions.

Biochemical Screening

After the clinical suspicion was formed, diagnosis of PHEO/PGL relies on measurement of urinary and plasma fractionated metanephrines (*o*-methylated metabolites of catecholamines) and catecholamines. The optimal use of these biochemical tests is still matter of debate; however, measurement of urinary fractionated metanephrines in a 24-h urine collection seems to be a

Table 1 Clinical conditions possibly associated to syndromic PHEO/PGLs

Clinical feature	*Syndrome (gene) involved*
Goiter, medullary thyroid carcinoma	Multiple endocrine neoplasia type 2 (RET)
Hirschsprung's disease	Multiple endocrine neoplasia type 2 (RET)
Hyperparathyroidism, nephrolithiasis	Multiple endocrine neoplasia type 2A (RET)
Cutaneous lichen amyloidosis	Multiple endocrine neoplasia type 2A (RET)
Mucosal neuromas	Multiple endocrine neoplasia type 2B (RET)
Ectropion	Multiple endocrine neoplasia type 2B (RET)
Marfanoid habitus	Multiple endocrine neoplasia type 2B (RET)
Scoliosis	Multiple endocrine neoplasia type 2B (RET)
Cafe au lait spots	Neurofibromatosis type 1 (NF1)
Muco-cutaneous neurofibromas	Neurofibromatosis type 1 (NF1)
Plexiform neurofibromas	Neurofibromatosis type 1 (NF1)
Axillary and/or inguinal freckling	Neurofibromatosis type 1 (NF1)
Central nervous system gliomas	Neurofibromatosis type 1 (NF1)
Renal artery stenosis	Neurofibromatosis type 1 (NF1)
Tibial dysplasia	Neurofibromatosis type 1 (NF1)
Sphenoid wing dysplasia	Neurofibromatosis type 1 (NF1)
Clear cells renal carcinoma	von Hippel–Lindau syndrome (VHL); Familial paraganglioma (SDHB)
Gastrointestinal stromal tumor	Carney–Stratakis syndrome (SDHB)
Renal cysts	von Hippel–Lindau syndrome (VHL)
Pancreatic tumor	von Hippel–Lindau syndrome (VHL)
Pancreatic cysts	von Hippel–Lindau syndrome (VHL)
Cerebral, spinal, retinal hemangioblastomas	von Hippel–Lindau syndrome (VHL)
Iris hamartomas (Lisch nodules)	von Hippel–Lindau syndrome (VHL)
Endolymphatic sac tumors of the middle ear	von Hippel–Lindau syndrome (VHL)

reasonable compromise between sensitivity and specificity, is less technical demanding and more widely available than corresponding plasma assay. Plasma fractionated metanephrines are reported to achieve the highest sensitivity, at the expense of slightly reduced specificity; this test is probably appropriate when ruling out PHEO/PGL is the main goal (i.e., subjects with familial predisposition), but its systematic adoption may cause unnecessary recourse to imaging techniques due to false positive findings. To improve specificity, it is necessary to withdraw any pharmacological treatment potentially interfering with biochemical assay (see Lenders *et al.*, 2014 for details).

In case of metanephrines level only slightly elevated, the assay can be repeated after administration of a central inhibitor of sympathetic output, such as clonidine (at our Institution we repeat a 24 h urine collection 48–72 h after the application of a 5 mg clonidine transdermal patch) before proceeding with subsequent diagnostic steps. In case of intermittent symptoms (and catecholamine secretion) urine sampling during or immediately after a crisis may be of some help.

Other tests, such as plasma and urinary fractionated catecholamines, urinary vanillylmandelic acid, plasma chromogranin A, or neuropeptide Y, have less diagnostic accuracy than plasma or urinary fractionated metanephrines.

Imaging Techniques

Imaging techniques for locating the tumor are primarily CT or MRI of the abdomen and pelvis. Overall, they have similar sensitivity in identifying adrenal PHEO; CT has superior spatial resolution but MRI could better identify true pheochromocytomas (hyperintensity on T2 weighted image) and is probably superior in the localization of head and neck PGL. The specificity of the two techniques is similar, but not very high (50%–70%) for the large number of non-catecholaminogenic incidentalomas.

Functional imaging techniques have greatly improved the diagnostic accuracy of PHEO/PGL (see Crona *et al.*, 2017; Lenders *et al.*, 2014). Beside the time-honored scintigraphy with ^{131}I- or ^{123}I-metaiodobenzylguanidine (MIBG), newer PET/CT techniques have shown excellent results in the localization of these tumors. Among them, 18F-dihydroxyphenylalanine (^{18}F-DOPA) positron emission tomography/computed tomography (PET/CT) and ^{68}Ga-DOTATATE/DOTATOC/DOTANOC PET/CT. Current evidence suggests that ^{68}Ga-DOTATATE PET/CT provides excellent and precise diagnostic localization of PGLs but may be inferior to ^{18}F-FDOPA PET/CT in the detection PHEOs. ^{18}F-FDG PET/CT should be used for assessment of metastatic PGLs.

Therapeutic Approach

Once diagnosed with pheochromocytoma, there is an indication of its surgical removal, except for special circumstances that make it necessary to postpone, or contraindicate, the intervention (recent acute coronary syndrome, third trimester pregnancy, concomitant morbid conditions, non-resectable neoplasms, and/or metastatic).

In any case, medical treatment should be initiated immediately to counteract the deleterious effects of excessive circulating catecholamines, including the restoration of adequate volumes (reduced by chronic vasoconstriction) (see Lenders *et al.*, 2014).

The alpha-blocker phenoxybenzamine is still considered a drug of choice by many experts but not available in some countries. However, alpha1-selective blockers (prazosin, doxazosin, and the like) are likely to be equally effective.

Beta-blockers, preferably beta1-selective, may be associated for tachycardia or arrhythmia control, but only with the presence of an appropriate alpha-block (to prevent possible hypertensive crisis of vasodilating effect mediated by beta2-adrenergic).

If necessary, other drugs (e.g., calcium antagonists) may be added to control the pressure values. Medical treatment should be continued for at least 2 weeks in order to minimize the risk associated with anesthesia and surgery but can be maintained indefinitely according to clinical indications.

The laparoscopic technique, associated with lesser perioperative pain, reduced hospitalization, and minor complications, is considered surgical treatment of choice, except in the case of multiple, large, or malignant pheochromocytomas.

Intraoperative treatment of hypertensive crisis, arrhythmias or post-tumor removal hypotension requires an experienced anesthesiologist team. Symptoms disappear after tumor removal; in particular, blood pressure normalize in most patients and the persistence of hypertension could be interpreted as expression of associated primary hypertension or, alternatively, as incomplete removal of tumor mass or the presence of other unrecognized localizations.

Postoperative metanephrines control should be performed a few weeks after intervention; in addition, considering the relatively high percentage of recurrences even after a few years (about 15%), an indefinite follow-up must be initiated, which involves annual urinary or plasma metanephrines measurement. There is no general consensus on indications and timing of imaging surveillance (see Crona *et al.*, 2017; Lenders *et al.*, 2014): in most patients it is not needed unless urinary or plasma metanephrines become elevated. Imaging or other targeted follow-up investigations may be indicated in the case of PGL patients who never presented catecholamine/metanephrine elevation prior to surgical intervention and of potentially syndromic patients.

Malignant PHEO

The percentage of malignant pheochromocytomas, characterized by the presence of local invasion of the tissues surrounding the lesion or the presence of metastases (mainly bones, liver, lymph nodes, and lungs) ranges between 5% and 10% and in these cases

the survival at 5 years falls from 95% to less than 50%. The use of debulking surgery in order to improve survival and/or reduce symptoms is recommended by many experts but with little supportive evidence.

Medical treatment of malignant PHEO/PGLs includes adrenergic receptors blockade, the administration of chemotherapeutic agents (a scheme comprising cyclophosphamide, vincristine, and dacarbazine has shown only partial responses, at best) and the use of ^{131}I-MIBG therapeutic doses (up to 800 mCi and beyond) in the case that the tumor maintains the absorption capacity of such radioligand. According to some preliminary data, in malignant PHEO/PGLs that (hyper)express somatostatin receptors, the administration of somatostatin analogs or a radiotherapeutic approach based on peptide receptor radionuclide therapy (PRRT) with 177Lu-DOTATATE may result in improved survival.

Tyrosine kinase inhibitors (sunitinib, sorafenib, imatinib), VEGF (thalidomide) or mTOR (everolimus) inhibitors are under active study in controlled trials. It should be considered that improving knowledge of tumor driving germline or somatic mutations in PHEO/PGL (see below) might boost targeted therapeutic approach.

In any case, the clinician should be aware that all these treatments are at best palliative and their use should be considered with due attention to patient's quality of life.

Genetic Predisposition

Mutations in more than 25 genes have been associated to the pathogenesis of PHEO/PGL and at least 15 of them have been found as germline mutations underlying familial and/or inheritable conditions (see Crona *et al.*, 2017; NGS in PPGL (NGSnPPGL) Study Group *et al.*, 2017 for details). Many recent studies have shown a total prevalence of pathogenetic germline mutations in PHEO/PGL patients above 30%.

Accordingly, many experts suggest that a systematic screening of genetic predisposition due to germline mutations is indicated for several reasons in all PHEO/PGL patients, independent of positive familial or syndromic features. Beside of the very high prevalence (probably the highest found in any type of human tumor), the identification (or exclusion) of mutations of genes responsible for syndrome forms can lead to the diagnosis of concomitant morbid conditions otherwise unplanned (i.e., avoid unnecessary monitoring of the time of the same). It should also be considered that some genetically determined forms of pheochromocytoma, and in particular those associated with mutations in the SDHB gene, present a high risk of recurrence and/or malignancy, which should be carefully considered at the time of diagnosis or during the subsequent follow-up. Last but not least, the identification of a genetic predisposition in the patient should be followed by a thorough and extensive search of the same mutation in first degree relatives, equally exposed to the risk of developing the disease.

The genetic heterogeneity of the disease makes impractical the adoption of traditional technologies in the search of pathogenetic mutations; accordingly, the implementation of targeted next generation sequencing technology has become the standard for genetic screening of PHEO/PGL (see NGS in PPGL (NGSnPPGL) Study Group *et al.*, 2017).

See also: Malignant Pheochromocytoma. Pheochromocytoma and Paraganglioma Syndromes. Pheochromocytoma/Paraganglioma: Management, Genetics, and Follow-up

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Malignant Pheochromocytoma

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Nomenclature

^{177}Lu-DOTA-TATE	Lutetium-177-DOTA^{0}-Tyr^{3}-octreotate
^{90}Y-DOTA-TOC	Yttrium-90-DOTATOC
CgA	Chromogranin A
CT	Computed tomography
DA	Dopamine
DOPA	Dihydroxyphenylalanine
DOTATOC	DOTA^{0}-D-Phe^{1}-Tyr^{3}-octreotide
ETA/ETB	Endothelin receptor type A/type B
F-FDG	PET with Fluoro-labeled fluoro-deoxy-glucose
FH	Fumarate hydratase
GIST	Gastrointestinal stromal tumors
HIF2α	Hypoxia inducible factor 2 alpha
HNPGLs	Head and Neck PGLs
HRAS	H-RAS proto-oncogene
hTERT	Human telomerase reverse transcriptase
IDH1	Isocitrate dehydrogenase 1
In-penteotride	Indium-11-DTPA-octreotide
KIF1Bβ	Beta form of the Kinesin Family Member1B
MDH2	Malate dehydrogenase 2
MEN2	Multiple endocrine neoplasia type 2
MIBG	Metaiodobenzylguanidine
MRI	Magnetic resonance imaging
NF1	Neurofibromatosis type 1
NGS	Next generation sequencing
PASS	Pheochromocytoma of the Adrenal gland Scales Score
PCCs	Pheochromocytomas
PET	Positron emission tomography
PGLs	Paragangliomas
PHD	Prolyl hydroxylase
PHD2	Prolyl hydroxylase domain 2
SDH	Succinate dehydrogenase
sPGLs	Secreting paragangliomas
VEGF	Vascular endothelial growth factor
VHL	von Hippel–Lindau disease

Glossary

Antiangiogenic therapy Drugs that stop tumors developing their own blood vassels.
Catecholamines Hormones produced by the chromaffin cells of the sympathetic nervous system.
Computerized tomography X-ray instrumental diagnostic procedure in which a three-dimensional image of body structures is constructed by computer from a series of plane-sectional images made along an axis.
Debulking surgery Surgery reduction of as much of the tumor's volume as possible.
Laparoscopic surgery Surgical technique that provides the assistance of a video camera and several thin instruments placed in the abdominal cavity through small incisions in the abdominal wall.
Laparotomic surgery Surgical procedure involving a large incision through the abdominal wall to gain access into the abdominal cavity.
Magnetic resonance imaging Instrumental diagnostic procedure which employs radio-frequency waves and intense magnetic fields to excite atoms in the body under evaluation.
Malignant Presence of metastases (cromaffin cells in tissues where are usually absent).
Metanephrines Metabolites of cathecholamines created by action of catechol-*O*-methyl transferase.
Next generation sequencing (NGS) Platforms performing sequencing of millions of small fragments of DNA in parallel.
Oncoproteins Proteins encoded by oncogenes which if mutated, play a role in the tumors development.
Pheochromocytoma Sympathetic tumor arising from the adrenal medulla.
Paraganglioma Parasympatetic or sympathetic tumors localized out side the adrenal medulla.
PASS score Pheochromocytoma of the Adrenal Gland Scaled Score. Score used to identify tumors with high (>6) or low (<4) risk of aggressive behavior. Not yet used in the clinical practice.
Radiometabolic therapy Administration of a radionuclide whose radiation will destroy cells that have selectively accumulated the labeled substance.
Radiotherapy External treatment of neoplastic disease by using X-rays or gamma-rays to impair the proliferation of malignant cells by decreasing the rate of mitosis or impairing DNA synthesis.
Scintigraphy Nuclear medicine diagnostic procedure consisting of the administration of a radionuclide with affinity for tissue of interest, followed by recording the distribution of the radioactivity with a stationary or scanning external scintillation camera.
Vanylmandelic acid End-stage metabolite of catecholamines created by action of catechol-*O*-methyl transferase and monoamine oxidase.

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Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare tumors of neuroendocrine origin, deriving from neural crest cells. They are located in sympathetic and parasympathetic paraganglia. Mostly of these tumors (about 80%–85%) arise in the adrenal medulla and are named pheochromocytomas (PCCs), whereas 15%–20% are located in extra-adrenal chromaffin tissue in the abdomen and the thorax and are referred to as secreting paragangliomas (sPGLs). PCCs and sPGLs are catecholamine-producing tumors. At variance with these latter, PGLs in the head and neck region, are parasympathetic in origin, do not usually release catecholamines and are named Head and Neck PGLs (HNPGLs) (Lenders *et al.*, 2005).

PCCs and PGLs are mostly sporadic, but recent data have demonstrated a high prevalence of hereditary forms (approximately 35%) (Pacak *et al.*, 2007). Hereditary forms are more frequent in young patients whereas sporadic forms are usually diagnosed in patients older than 40–50 years.

Malignant PCCs/PGLs are extremely rare. Their prevalence, as estimated in USA in 2002, is 93 cases per 400 million persons (Welander *et al.*, 2011). At present, no reliable marker of malignancy has been found. Malignancy is diagnosed by the presence of metastases, tumor spread in tissues where chromaffin cells are normally absent such as lymph nodes, liver, lungs, and bones.

The percentage of malignancy varies among the different tumors: it is $<5\%$ in HNPGLs, about 10% in PCCs and 30% or more in sPGLs, depending also on the genetic background (see below) (Brown *et al.*, 1999; Chrisoulidou *et al.*, 2007; Lee *et al.*, 2002).

Genetic Aspects

Until 2000, only 10% of PGLs were considered familial. They were part of syndromic diseases such as the von Hippel–Lindau disease (VHL), due to germ line mutations in tumor-suppressor gene *VHL* (Latif *et al.*, 1993; Walther *et al.*, 1999), multiple endocrine neoplasia type 2 (MEN2) caused by mutations of the proto-oncogene *RET* (Eng *et al.*, 1994a,b, 1996; Neumann *et al.* 1993, 1994) and neurofibromatosis type 1 (NF1), due to mutations in the tumor-suppressor gene *NF1* (White *et al.*, 1991).

More recently, the discovery of new susceptibility genes has increased the frequency of the familial forms to about 35% (Neumann *et al.*, 2002). These genes include those encoding the four subunits (A, B, C, D) of the succinate dehydrogenase (SDH) (Astuti *et al.*, 2001; Baysal *et al.*, 2000; Burnichon *et al.*, 2010; Niemann and Muller, 2000), the *SDHAF2* gene, which is responsible for the flavination of the SDHA subunit (Hao *et al.*, 2009), the *TMEM127* (Qin *et al.*, 2010) and *MAX* (Comino-Méndez *et al.*, 2011) genes, both mainly related to bilateral PCCs and the more recently discovered genes such as the one encoding fumarate hydratase (FH), the gene encoding the Beta form of the Kinesin Family Member1B (KIF1Bβ), that encoding the prolyl hydroxylase domain 2 (PHD2) and that encoding the malate dehydrogenase 2 (MDH2). Germ line mutations in *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes are responsible for the occurrence of syndromes named PGL5, PGL4, PGL3, PGL1, and PGL2, respectively; these syndromes are characterized by the occurrence of both sympathetic (PCC/sPGL) and parasympathetic (HNPGL) tumors (Mannelli *et al.*, 2009) but also, although much more rarely, by other solid neoplasms as gastrointestinal stromal tumors (GIST), renal cell carcinomas and pituitary adenomas (Mannelli *et al.*, 2018). Notably, *SDHB* mutations are generally associated with higher morbidity and mortality than mutations in the other SDHx genes (Gimenez-Roqueplo *et al.*, 2003). A recent meta-analysis of some studies involving *SDHB* mutated patients has highlighted that 31% of their tumors were malignant (Welander *et al.*, 2011).

Finally, an additional 15% of paragangliomas have been demonstrated to harbor somatic mutations in some of the above mentioned susceptibility genes and in other genes like those encoding the isocitrate dehydrogenase 1 (IDH1), the hypoxia inducible factor 2 alpha (HIF2α), the H-RAS proto-oncogene (HRAS) (Dahia, 2017).

Extensive genetic screening has divided sporadic as well as hereditary PGLs in two main clusters associated to different signaling pathways (Dahia *et al.*, 2005): cluster 1 contains *VHL*-, *SDHx*-, and FH-mutated tumors and is associated with angiogenesis, hypoxia, reduced oxidative response (Favier *et al.*, 2009) and aberrant VEGF signaling, leading to abnormal hypoxia inducible factor (HIF) activation and overexpression of angiogenic factors. Cluster 1 tumors also express a hypermethylated profile, while cluster 2 contains all *RET*- and *NF1*-mutated tumors and is associated with abnormal activation of kinase signaling pathways, such as RAS/RAF/MAPK and PI3K/AKT/mTOR (Califano *et al.*, 2000; Johannessen *et al.*, 2005, 2008; Martin *et al.*, 1990) leading to abnormal cell growth and reduced apoptosis; also *TMEM127* (Qin *et al.*, 2010) and *MAX* (Comino-Méndez *et al.*, 2011) mutated tumors have been associated to activation of mTOR signaling pathway. These data have highlighted the molecular defects in PGLs and might be of help in the development of new effective molecular-targeted therapies.

The genotype/phenotype correlation has been suggested in the recent past to facilitating a genetic testing algorithm based on clinical features as a guide for a more quick and cost-effective genetic screening (Mannelli *et al.*, 2009). More recently, the introduction of the next generation sequencing (NGS) which permits the simultaneous screening of all the susceptibility genes, has made the use of the algorithm obsolete. Nevertheless, the genotype/phenotype correlation is still important as one of the tools permitting the interpretation of the pathogenic significance of the many variants revealed by the NGS (Toledo *et al.*, 2017).

Clinical Aspects

PGLs are often referred to as the great mimics because of their extremely variable clinical picture. Such variability is strongly related to the biology of these tumors which can express different catecholamine biosynthetic enzymes and may release other different

vasoactive substances, such as atrial natriuretic peptide, neuropeptide Y, adrenomedullin, or ACTH (Bravo, 1994). Moreover, some clinical manifestations may be related to mass effect or to the involvement of other organs in the syndromic forms.

Generally, malignant PGLs have a clinical presentation similar to benign tumors. Hypertension is the most common feature: it often has a paroxysmal nature but it may also be continuous or intermittent. Hypertensive crises present with headache, palpitations, and diaphoresis, but other signs or symptoms such as dyspnea, nausea, weakness, arrhythmias or mood disturbances are reported. Moreover metabolic effects such as glucose intolerance or diabetes may be present (Lenders *et al.*, 2005). Sometimes malignant PGLs may have a subclinical picture: in fact, due to their less-differentiated nature, they generally secrete noradrenaline and/or dopamine determining milder cardiovascular manifestations (Mannelli *et al.*, 2012). However, at variance with the benign forms, malignant PGLs may cause "systemic" symptoms such as anorexia, fatigue and weight loss or manifestation related to metastatic spread of the disease (i.e., pain in bone metastasis). The most commons metastatic sites for malignant PGLs are local lymph node, bones, liver, and lung.

The most frequent causes of morbidity and mortality in patients with PGLs, are represented by cardiovascular complications such as sudden death, myocardial infarction, heart failure, and stroke. Patients affected by malignant PGLs with an indolent course and progressive increase of circulating noradrenaline suffer from severe constipation which sometimes turns to be lethal (Thosani *et al.*, 2015).

HNPGLs are usually clinically silent. Only when they reach a large volume they can determine symptoms related to mass effect and compression of the adjacent structures. Large HNPGL appear as a palpable neck mass and may cause, dysphagia, tinnitus, or cranial nerve palsies. (Erickson *et al.*, 2001).

Biochemical Diagnosis

The biochemical diagnosis of PGL is based on laboratory assays demonstrating an increase of catecholamines or their metabolites in plasma or urine. The recommended screening test is the measurement of plasma free-metanephrines or urine-deconjugated differential metanephrines (Lenders *et al.*, 2002). In fact, several studies have evidenced the higher sensitivity of metanephrines (ranging around 98%–99%) in comparison with other analytes such as plasma/urine catecholamines or vanilmandelic acid (Davidson, 2002; Grossman *et al.*, 2006). The higher sensitivity of metanephrines is mainly due to their longer half-life and to their non-episodic production by the tumor where catecholamines are continuously converted to metanephrines by the high methyltransferase activity which characterizes the chromaffin tissue (Eisenhofer *et al.*, 1998). The release of catecholamines is influenced by tumor location. In fact, adrenaline is produced only by PCCs, while sPGL do not. Generally the biochemical phenotype is not useful in differentiating malignant from benign PGLs. However malignant PCCs produce mainly noradrenaline (Rao *et al.*, 2000) and in some circumstances they predominantly or exclusively produce dopamine as a consequence of a less-differentiated catecholamine biosynthetic pathway (Brouwers *et al.*, 2006). Therefore in case of large noradrenaline releasing PGLs and/or in the presence of high plasma levels of dopamine or its metabolite methoxytyramine, the risk of malignant tumors should be considered (Grossman *et al.*, 2006; van der Harst *et al.*, 2002).

Neuroendocrine markers such as plasma chromogranin A (CgA) or neuron-specific enolase does not show high sensitivity in biochemical diagnosis of PGLs, and especially CgA often rise false positive results due to concomitant disease (i.e., liver or kidney failure) or therapy with drugs increasing gastrin plasma levels (i.e., proton pump inhibitors) (Algeciras-Schimnich *et al.*, 2008). However, malignant PGLs are generally associated to high plasma levels of these markers (Grossman *et al.*, 2006) and therefore their evaluation may be useful as a surrogate marker to confirm the diagnosis as well as to monitor the response to treatment in the follow up and/or to evidence the recurrence of the disease.

Anatomical and Functional Imaging

Among anatomical imaging tools, computed tomography (CT) or magnetic resonance imaging (MRI) are very useful in the localization of PGLs. CT shows a sensitivity of 77%–98% and a specificity of 29%–92% in detecting adrenal or extra-adrenal tumors. Diagnostic accuracy is slightly better (sensitivity 90%–100% and specificity 50%–100%) for MRI, especially for the localization of extra-adrenal disease (Ilias and Pacak, 2004). Furthermore MRI with diffusion-weighted imaging may also be used to detect especially lymph node and liver metastases (Takano *et al.*, 2008). On the contrary, ultrasound imaging has a limited diagnostic value, but it may have a relevant role in the detection of HNPGLs (Blake *et al.*, 2004; Ilias and Pacak, 2004).

On MRI PGLs usually show high signal on T2-weighted imaging and strong enhancement after contrast-agent administration because are highly vascularized tumors with a high intracellular water content and frequent intratumoral cystic lesions. However large tumors as well as malignant lesion often contain hemorrhagic and/or necrotic areas, which may reduce the signal intensity on T2-weighted images (Mannelli *et al.*, 2010).

In malignant PGLs, nuclear medicine procedures, named "functional imaging," are generally recommended after anatomical detection. These techniques use specific tracers which may be concentrated by chromaffin tissue. Functional imaging permit to perform a whole-body study, providing a better evaluation of extra-adrenal PGLs as well as of multiple tumors and or metastatic localizations (Shulkin *et al.*, 2006). ^{131}I or ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy represents the first-line functional imaging in the evaluation of patients with PGLs. MIBG has a chemical structure similar to norepinephrine and is concentrated in

chromaffin tissue, via a transporter normally expressed in most of chromaffin cells, responsible for catecholamine uptake (Shulkin *et al.*, 2006). ^{123}I-MIBG has better physical properties than ^{131}I-MIBG and it's superior in terms of quality of images and diagnostic accuracy (sensitivity of 83%–100% vs. 77%–90%) (Ilias and Pacak, 2004). However the sensitivity of ^{123}I-MIBG-scintigraphy may be lower in malignant PGLs, especially in patients with dopamine-secreting tumors which usually do not uptake MIBG, or in SDHB mutation carriers, characterized by a high risk of malignancy (Dubois and Gray, 2005).

In patients with a strong suspect of PGL and negative MIBG scintigraphy, other nuclear medicine techniques may be considered. As MIBG, radiolabeled dopamine (DA) or dihydroxyphenylalanine (DOPA) are transported into chromaffin cells, providing the possibility to use them as tracers in positron emission tomography (PET) imaging. PET with 6-[^{18}F]-fluoro-DA shows a better sensitivity than ^{131}I-MIBG in detecting metastatic PCCs (Ilias *et al.*, 2003), whereas PET with 6-[^{18}F]-fluoroDOPA is superior in imaging sPGLs and HNPGLs (Hoegerle *et al.*, 2002). However, as for MIBG, the sensitivity of these tracers is low in PGLs associated with *SDHB* mutations. The preferred functional imaging modality for staging *SDHB*-related metastatic PGLs is PET with ^{18}F-labeled fluoro-deoxy-glucose (^{18}F-FDG). This technique permits to identify glucose-avid metastatic sites, also the MIBG-negative ones (Mamede *et al.*, 2006), showing a sensitivity close to 100% (Timmers *et al.*, 2007).

Chromaffin cells may express somatostatin receptor type 2, 3, and 5. Therefore radiolabeled somatostatin analogues may be used in order to localized these tumors. Indium-11-DTPA-octreotide (^{111}In-penteotride) is the tracer most commonly used; it may be especially useful in localizing extra-adrenal disease, MIBG-negative metastatic as well as malignant PCCs sites with a sensitivity near to 90% (Ilias and Pacak, 2004; Telischi *et al.*, 2000). On the contrary its sensitivity in benign PCCs is limited. Somatostatin analogues labeled with gallium-68 [^{68}Ga-DOTATOC (DOTA^0-D-Phe^1-Tyr^3-octreotide)] can be used in PET imaging. This functional imaging has shown a better sensitivity than ^{18}F-labeled fluoro-deoxy-glucose (^{18}F-FDG) in detecting malignant PCCs and sPGLs (Buchmann *et al.*, 2007; Hofmann *et al.*, 2001; Kowalski *et al.*, 2003). Finally PET imaging with ^{11}C-hydroxyephedrine has provided high diagnostic accuracy (sensitivity 92% and specificity 100%) in the detection of PGLs, but an higher number of patients have to be studied in order to draw conclusions on its usefulness (Trampal *et al.*, 2004).

Pathology

Unfortunately, at present, no reliable markers of malignancy are available. Malignancy is diagnosed by the presence of metastases. Generally extra-adrenal PGLs, larger than 5 cm, with necrotic areas and high cellularity have a superior risk to be malignant, but still now on the basis of histopathological findings, it remains difficult to predict whether an apparently benign PGL will develop in a malignant tumor.

On the basis of some histological features (i.e., invasion, growth patterns, cytologic appearance as well as mitotic activity), several scoring systems have been proposed to establish the risk of malignancy (Kimura *et al.*, 2005; Linnoila *et al.*, 1990; Thompson, 2002). Among them, the most utilized is "Pheochromocytoma of the Adrenal gland Scales Score (PASS)," proposed by Thompson on 2002. **Table 1** reports items and values used to calculate the score. A score <4 or >6 suggests benign or malignant lesions respectively, whereas a value between 4 and 6 is associated to an intermediate risk.

Some molecular markers can offer additional information (Chrisoulidou *et al.*, 2007). For example inhibin/activin β-subunit is expressed in the normal medulla as well as in benign PGLs, while is almost absent in malignant tumors; neuropeptide Y mRNA is expressed in all benign PGLs and to a lesser extent in the malignant counterpart; on the contrary cyclooxygenase-2, N-cadherin, as well as gene encoding vascular endothelial growth factor (VEGF), endothelin receptor type A (ETA) and type B (ETB), are overexpressed in malignant PGLs. Telomerase seem to be closely related to malignancy: the human telomerase reverse transcriptase (hTERT) promoter has been associated with a number of malignant tumors including PCCs and PGLs (Dwight *et al.*, 2017).

Also the Ki-67 nuclear antigen proliferative index may be useful in predicting malignancy because a value higher than 3%–6% is most commonly found in malignant tumors (Brown *et al.*, 1999; Liu *et al.*, 2004).

Table 1 Pheochromocytoma of the adrenal gland scoring scale (PASS) (Hao *et al.*, 2009)

Items	*Value*
Nuclear hyperchromasia	1
Profound nuclear pleomorphism	1
Capsular invasion	1
Vascular invasion	1
Extension into adipose tissue	2
Atypical mitotic figures	2
>3 of 10 mitotic figures high-power field	2
Tumor cell spindling	2
Cellular monotony	2
High cellularity	2
Central or confluent tumor necrosis	2
Large nests or diffuse growth ($>10\%$ of tumor volume)	2
Total	20

Novel biomarkers are more recently being identified by micro-RNA expression profiling studies. Micro-RNA are small single-strand (~22 bp), non-protein coding RNA fragments, which are able to negatively regulate protein expression by either cleavage or translational repression of mRNA (Engels and Hutvagner, 2006). MicroRNA expression is tissue specific, and it has been demonstrated to be altered in several other human tumors. It has been demonstrated that, in PCCs and PGLs, a reduced expression of specific mRNAs, which are involved in proliferation and apoptosis, such as miR-51a and miR-16 and an increased expression of miR-483-5p are predictive of malignancy (Meyer-Rochow *et al.*, 2010).

However as none of the available molecular markers, as well as histological findings predict malignant development unequivocally, after the removal of PCC/PGL, a follow up of the patient is recommended in order to reveal early disease recurrence.

Therapy

To date, no effective therapy is available to cure malignant PCC/PGL. All the available therapeutic options are aimed at improve patient's survival, slowing the progression of the disease, or at limiting the effects of the catecholamines released by the tumor, improving the patient's quality of life.

Often, patients affected by a malignant PCC/PGL show an indolent course of the disease and maintain a good quality of life. In these cases a prudent "wait and see" behavior should be adopted, reserving more aggressive therapeutic options in case of disease progression.

Medical Therapy

Medical therapy is directed to reduce the negative effects caused by the excess of catecholamines released by the tumor, especially on the cardiovascular system and on the gut. Cardiovascular complications include myocardial ischemia or insufficiency, stroke, sudden death. Alpha blockers as phenoxybenzamine or doxazosin are the drugs of choice. Beta blockers may be added to protect the myocardium but only after alpha blocking therapy has been started. Alpha-methyl-paratyrosine is an other medical option: it blocks the catecholamine synthesis acting both on the tumor tissue and on the sympathetic system thus causing side effects like postural hypotension. At the gut level, catecholamine excess causes a constipation that sometimes is a so severe to be lethal. Constipation should be recognized and promptly treated using laxatives, either stimulant or osmotic, stool softeners, lubricants (Thosani *et al.*, 2015).

Surgery

Surgery, which is the treatment of choice for benign PCC/PGL, may have a role also in the treatment of malignant PCC/PGL. The main purpose of surgical treatment is represented by the removal of the primary tumor and, if possible, the resection of local recurrences and, more rarely, distant metastases. The overall 5-year survival rate of patients with malignant PGLs varies between 34% and 60% and may depend on the sites of the metastatic lesions. In fact, a worse prognosis has been evidenced in patients with liver or lung metastases (<5 years) compared with that of patients with only bone lesions (Pacak *et al.*, 2007).

Generally, the recommended surgical approach to benign adrenal or extra-adrenal tumor is "laparoscopic," but in case of large PCC/PGLs as well as in the presence of high risk of malignancy a laparotomic approach should be considered (Brauckhoff *et al.*, 2004).

Usually surgery alone is rarely curative in the presence of malignant PGLs, but also in case of extensive disease surgical debulking is indicated. In fact, it may permit to reduce clinical manifestations related to catecholamine secretion and it may improve the response to other therapeutic approaches, such radiometabolic therapy. In patients with multiple liver metastases not susceptible to surgical resection, arterial embolization or chemoembolization as well as radiofrequency ablation should be considered (Maithel and Fong, 2009).

It should be remembered that each patient affected by a chromaffin tumor, either benign or malignant, must be preoperatively treated with alpha blockers and fluid administration in order to avoid surgical (i.e., hypertensive crisis, arrhythmias) and/or postsurgical complications (i.e., hypotension) (Lenders *et al.*, 2005).

Radiometabolic Treatment and External Radiotherapy

In patients with metastatic disease and unresectable lesions, radionuclide treatment with beta-emitting isotopes coupled with MIBG or somatostatin analogues should be considered. The evidence of significant radioisotope uptake on diagnostic scintigraphy with ^{123}I-MIBG or ^{131}I-MIBG (>1% uptake of the injected dose) is important in order to select patients who can benefit from this treatment. The treatment may be administered by single or fractionated doses with a cumulative dosage varying between 200 and 1400 (Kaltsas *et al.*, 2003, 2005). A better response is achieved in patients with limited metastatic burden and in those with soft-tissue metastases compared to patients with bone metastases. The therapy with ^{131}I-MIBG is generally well tolerated, especially at low doses. The main side effects include nausea, moderate bone marrow suppression (transient leucopenia and

thrombocytopenia) and moderate hepatic and renal toxicity. Severe bone marrow toxicity (associated with high-dose regimen) is rarely seen. In some cases (10%) also symptoms related to catecholamine release from the irradiated tissue (headache, palpitations, sweating) may appear.

Unfortunately, treatment with ^{131}I-MIBG is rarely curative and other forms of therapy need to be considered.

In patients with metastatic, MIBG-negative PGLs or in those who do not respond to treatment with ^{131}I-MIBG, therapy with radiolabelled somatostatin analogues may be considered. As for ^{131}I-MIBG, the evidence of significant radioisotope uptake on diagnostic scintigraphy with ^{111}In-pentetreotide or especially on PET using ^{68}Ga-DOTA-TOC provides high accuracy in selecting patients (Mamede *et al.*, 2006). Radiolabelled somatostatin analogues are generally used in patients with gastroenteropancreatic neuroendocrine tumors and even if their efficacy is lower in patients with malignant PGLs, they may represent an alternative option for the treatment of surgically incurable PGLs (Forrer *et al.*, 2008). They may determine tumor shrinkage thus reducing catecholamine release. The most commonly used compounds are Yttrium-90-DOTATOC (^{90}Y-DOTA-TOC) and Lutetium-177-DOTA^{0}-Tyr^{3}-octreotate (^{177}Lu-DOTA-TATE) (Kaltsas *et al.*, 2005; Kwekkeboom *et al.*, 2005, 2008). The main side effects associated to these compounds are represented by leucopenia and thrombocytopenia.

In some cases a combined treatment with radiolabelled MIBG and radiolabelled somatostatin analogues may be considered. These two treatments may have a synergistic effect, permitting the use of lower doses of both radionuclides, limiting side effects, particularly bone marrow toxicity.

Although malignant PGLs are relatively radio-resistant, external radiotherapy may be considered especially in patients with bone metastases in order to reduce chronic pain or symptoms related to local compression. During external radiotherapy the patients have to be monitored because the radio-induced damage of the lesion can induce massive catecholamine secretion, thus inducing hypertensive crises (Teno *et al.*, 1996).

Targeted Therapy

The limited effect elicited by chemo- and radionuclide therapy in improving survival highlights the need for novel targeted therapies. The identification of the main PCC/PGL susceptibility genes as well as the understanding of the specific molecular pathways activated by their mutations and responsible for malignancy, provide the bases for the development of multiple molecular-targeted therapy. Genetic profiling has grouped, both benign and malignant PGLs, into two distinct clusters (1 and 2) characterized by different molecular pathways leading to oncogenesis. Moreover, malignant PCCs seem to overexpress HSP90, a molecular chaperone that plays an important role in molecular stability, maintaining the folding and conformation of multiple oncoproteins that play a role in malignant phenotype (Banerji, 2009; Powers and Workman, 2006).

Antiangiogenetic drugs seem to be more appropriate for patients affected by cluster 1 PCCs. Accordingly, sunitinib, a receptor tyrosine kinase inhibitor acting on several targets (VEGF, PDGF, and c-KIT), with strong antiangiogenic and antitumor activity, has been used in the treatment of malignant PCCs, with good results (Canu *et al.*, 2017; Hahn *et al.*, 2009; Jimenez *et al.*, 2009; Joshua *et al.*, 2009; Park *et al.*, 2009). Imatinib, another tyrosine kinase inhibitor already used for hematologic and GIST, has not been found effective in the treatment of malignant PCCs (Gross *et al.*, 2006). Nevertheless, these observations need to be confirmed in larger cohorts of patients and drug effectiveness need to be tested against placebo treatment. Several clinical trials are currently ongoing and will hopefully enable such validations. These trials include the FIRSTMAPPP study, a randomized double-blind phase II international multicenter study that is evaluating the efficacy of sunitinib versus placebo in patients with progressive malignant PGLs [http://clinicaltrials.gov/ct2/show/NCT01371201], and two nonrandomized phase II studies that are evaluating the response to sunitinib [https://clinicaltrials.gov/ct2/show/NCT00843037] and axitinib [https://clinicaltrials.gov/ct2/show/NCT01967576].

Thalidomide, by targeting VEGF and basic fibroblast growth factor, is another antiangiogenic agent. It has been used in combination with temozolamide in patients with metastatic carcinoid, PCC or pancreatic neuroendocrine tumors. These latter seem to be more responsive to this treatment but a positive response was also found in one of the three treated patients with malignant PCC (Kulke *et al.*, 2006).

The HIF1a inhibitors are molecular targeted drugs interfering with HIF hypoxia-driven transcription pathway thus decreasing HIF activity directly. These molecules have shown marked antineoplastic activity in human tumor xenografts in mice and seem to be promising also for malignant PGLs, although conclusive data are missing (Semenza, 2007; Welsh *et al.*, 2003, 2004).

Activators of prolyl hydroxylase (PHD) (such as ERBB2 inhibitors) are on evaluation as promising antitumoral agents. These molecules, by activating the PHD, increase HIF hydroxylation, and promote its degradation, thus reducing the expression of some angiogenic factors (Choi *et al.*, 2008; Temes *et al.*, 2005).

A dysfunction in PI3/Akt/mTOR pathway increases cell proliferation, angiogenesis and decreased apoptosis, thus potentiating malignant transformation (Druce *et al.*, 2009); in vitro studies have involved this pathway in the pathogenesis of malignant neuroendocrine tumors, including PCCs. mTOR inhibitor everolimus (RAD001) has been evaluated in malignant PGLs, but all patients experienced disease progression (Chrisoulidou *et al.*, 2007; Druce *et al.*, 2009). Maybe the low efficacy is due to a compensatory activation of PI3K/AKT and ERK in response to mTOR inhibition, so a specific novel dual PI3k/mTOR inhibitor (NVP-BEZ235) might offer a novel therapeutic approach (Nölting and Grossman, 2012). However the therapeutic response to mTOR inhibitors in patients with malignant PGLs remains to be tested in a clinical trial.

Due to overexpression of HSP90 in malignant PCCs (Boltze *et al.*, 2003a,b), inhibition of its pathway could represent a future therapeutic challenge for the treatment of malignant PCCs. Specific HSP90 inhibitors have been developed and seem promising in

other malignancies (i.e., breast cancer) (Modi *et al*., 2007, 2011); however, at present, no specific trials are currently carried out in patients with malignant PGLs.

Conclusions

Malignant PCC/PGL are very rare and so far incurable tumors. Their clinical course is variable, ranging from aggressive to indolent. There are many risk factors suggesting a potential malignancy but, in the absence of a reliable biological marker, malignancy is proved only by the presence of metastases. Metastatic spread is often found years after the removal of a supposedly benign tumor and therefore studies comparing benign and malignant PCC/PGL are difficult and deserve a long clinical follow up of patients.

Genetic studies of the last 15 years have highlighted the pathways responsible for PCC/PGL development and suggested new targeted therapeutic approaches whose effectiveness has still to be confirmed by large collaborative international studies.

As a whole, at present, malignant PCC/PGL has to be considered an orphan disease.

See also: Pheochromocytoma/Paraganglioma: Diagnosis and Treatment. Pheochromocytoma/Paraganglioma: Management, Genetics, and Follow-up

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Pheochromocytoma/Paraganglioma: Management, Genetics, and Follow-up

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Introduction

Paraganglioma and pheochromocytoma (PPGL) are neuroendocrine tumors arising from extra-adrenal paraganglia (head and neck paragangliomas and thoracic-abdominal or pelvic sympathetic paragangliomas) and the adrenal medulla, respectively (Lloyd *et al.*, 2017). PPGL is a rare disease (low prevalence, estimated, from autopsy series, at 5–10 per 1000 inhabitants and at 0.2%–0.6% in hypertension patients). The first case of pheochromocytoma to be described was published in 1884 and concerned an 18-year-old woman who died from malignant hypertension. Bilateral adrenal tumors and thyroid gland enlargement, corresponding to bilateral pheochromocytoma and medullary thyroid carcinoma, were detected on autopsy. More than a century later, a diagnosis of multiple endocrine neoplasia type 2 was finally made for this patient, with the identification of a germline mutation of the *RET* gene in her descendants. The princeps case of pheochromocytoma was, thus, genetically determined (Neumann *et al.*, 2007). The management of patients with paraganglioma or pheochromocytoma has greatly benefited from advances in biology and imaging technologies, and from new genetic insight. A dozen new PPGL susceptibility genes have been discovered since the start of the 21st century. A germline mutation in a known susceptibility gene is found in about 40% of cases. The management and follow-up of mutation-carriers depends on the susceptibility gene mutated (Lenders *et al.*, 2014; Favier *et al.*, 2014).

Classical Management of PPGL

Clinical Presentation

PPGL usually has a nonspecific clinical presentation. The time from the onset of the first symptoms to PPGL diagnosis has been estimated at about 3 years, on average (Amar *et al.*, 2005b). The circumstances in which PPGL diagnosis should be considered are listed in **Table 1**. Symptoms may reflect catecholamine production (paroxysmal or permanent hypertension, sweating, tachycardia, headaches, etc.) for pheochromocytoma or catecholamine-producing paraganglioma, or may be caused by the tumor burden (tumoral mass, tinnitus, hearing loss or deafness, etc.) for head and neck paraganglioma. At least 10% of PPGL are diagnosed incidentally or after severe complications, such as adrenergic cardiomyopathy.

Biochemical Testing

The process for PPGL diagnosis is outlined in **Fig. 1**. Briefly, biochemical testing is the first step in PPGL diagnosis. It should include measurements of plasma free metanephrine concentration, with the patient remaining in a supine position for at least 30 min before blood sampling, or urinary fractionated metanephrine determinations on a 24-h urine sample, with the completeness of urinary collection checked by the determination of urinary creatinine concentration. Metanephrines are the methylated metabolites of catecholamines. Both these tests have a sensitivity and specificity for PPGL diagnosis of more than 95% (Lenders *et al.*, 2014). The European Society of Endocrinology clinical practice guidelines recommend assays of 3-methoxytyramine and chromogranin A for the detection of dopamine-producing or nonfunctional PPGL (Plouin *et al.*, 2016).

Table 1 Clinical circumstances of pheochromocytoma and/or paraganglioma diagnosis

Resistant hypertension (SBP $\geq$140 mmHg or DBP $\geq$90 mmHg in a patient treated with at least a diuretic and two other different types of antihypertensive drug) or orthostatic hypotension
Catecholamine crisis presenting as Takotsubo cardiomyopathy, stroke, heart attack, multiple-organ failure or malignant hypertension
Headaches and/or tachycardia and/or sweating
Blood pressure lability recorded on 24-h ambulatory blood pressure monitoring or during surgery
Diabetes + hypertension in a patient under 50 years of age with a body mass index $<$25
Pulsatile tumor mass
Hypoacousia, deafness, tinnitus, dysphagia, cranial nerve paralysis
Incidentaloma
Behavioral disorders and/or schooling difficulties in children, polyuria and polydipsia, unexplained weight loss, gastrointestinal disorders
Clinical screening in a patient with a germline mutation of a pheochromocytoma/paraganglioma (PPGL) susceptibility gene
Clinical screening of at-risk relatives for mutations of PPGL susceptibility genes

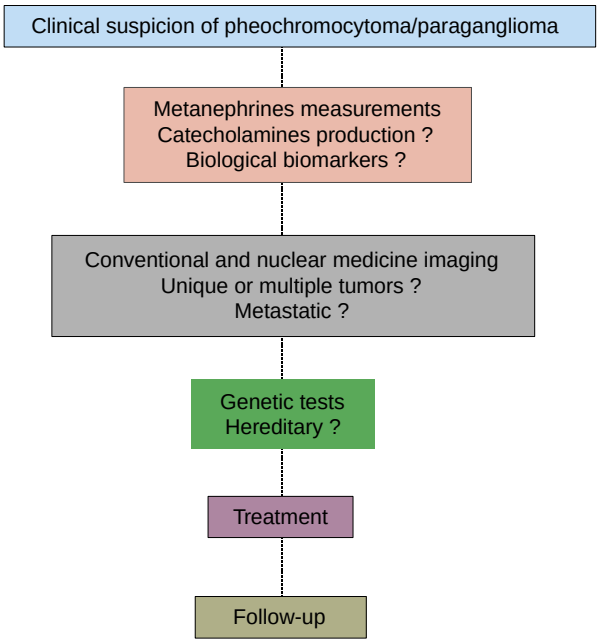

Fig. 1 The different steps in the diagnosis and management of pheochromocytoma and paraganglioma.

Imaging

All patients with positive results for a biochemical test should undergo imaging, to screen for and locate PPGL, and to detect multiple PPGL and/or metastases, if present. The principal conventional imaging method recommended for this purpose is CT-scan with contrast staining, due to its high sensitivity for PPGL diagnosis, which has been estimated at 90%–100%. MRI is indicated in children, pregnant women, patients with allergy to CT contrast agents and patients known to have a germline mutation (Lenders *et al.*, 2014). However, for head and neck PGL, the imaging method of choice is contrast-enhanced angio-MRI (Gimenez-Roqueplo *et al.*, 2013; Gravel *et al.*, 2016). Additional functional imaging examinations can be performed for patients with PGL or suspected metastatic disease. Positron emission tomography (PET) combined with computed tomography (CT) scanning has supplanted ^{123}I-metaiodobenzylguanidine (mIBG) and ^{111}In-pentetreotide (octreoscan) scintigraphy. ^{18}Fluoro-2-deoxy-D-glucose (^{18}F-FDG) PET/CT is the leading nuclear medicine examination for these patients, due to its high sensitivity for the diagnosis of both pheochromocytoma and paraganglioma, and its ability to detect metastases (Timmers *et al.*, 2012). New tracers, such as ^{18}fluorodihydroxyphenylalanine (^{18}F-DOPA) and ^{68}gallium-DOTA (0)-Tyr(3)-octreotate (^{68}Ga-DOTATATE), seem to provide added value, particularly for the assessment of genetically determined forms (see below) (Castinetti *et al.*, 2015).

Genetic Testing

The third main element in the PPGL diagnosis pathway is genetic testing, because the identification of a germline mutation modifies patient management and follow-up (as described below). About 40% of patients with PPGL carry a germline mutation in a known PPGL susceptibility gene. International and European guidelines therefore recommend genetic testing for all affected patients. Genetic counseling should be available to patients before and after testing. The international next-generation sequencing (NGS) in PPGL Study Group, which comprised of experts in PPGLs from 10 separate institutions representing 8 countries, has stated that molecular genetic testing should be performed by specialized and accredited laboratories using NGS technology, which is highly suitable for the screening of all PPGL susceptibility genes identified to date (NGS in PPGL (NGSnPPGL) Study Group *et al.*, 2017).

Treatment

Single apparently benign tumors

The treatment of choice is surgery. However, medical preparation is indicated before surgery, to prevent perioperative cardiovascular complications due to the unpredictable release of catecholamines during resection of the tumor mass. Treatment with alpha-adrenergic blockers should be initiated 7–15 days before surgery. The coadministration of beta-blockers is also indicated, to control heart rate and blood pressure. Moreover, a high-sodium diet and high levels of fluid intake can reduce the risk of severe hypotension after tumor removal. Laparoscopic resection is usually performed for pheochromocytomas of less than 6 cm in diameter. Open resection is restricted to the largest tumors and cases of head and neck paraganglioma (Lenders *et al.*, 2014).

Metastatic PPGL

The presence of metastases, defined as the presence of chromaffin tissue in a nonchromaffin organ (mostly the lymph nodes, bones, liver, and lungs), remains the only definitive criterion for malignant PPGL according to the most recent World Health Organization (WHO) classification of endocrine tumors (Lloyd *et al.*, 2017). At least 10% of PPGL are malignant (Amar *et al.*, 2016). The prognosis is unfavorable, with a median 5-year survival of less than 50% in published studies. Metastases may be diagnosed at presentation or during follow-up and, as mentioned above, ^{18}F-FDG-PET/CT is superior to ^{123}I-mIBG scintigraphy for the detection of metastases. Nevertheless, ^{123}I-mIBG scintigraphy is still used to determine whether targeted radiotherapy with ^{131}I-mIBG is possible, and magnetic resonance imaging of the liver and bones is indicated for the staging and follow-up of metastases. Therapeutic options should be discussed with multidisciplinary teams at expert centers. The therapeutic arsenal for metastatic PPGL includes surgery, interventional radiology, external or targeted radiotherapy, chemotherapy, and targeted molecular therapies (Baudin *et al.*, 2014).

Follow-Up

The risk of new events after complete PPGL resection was recently estimated at about 10%, on average, over the first 5 years of follow-up, in the PPGL cohort (701 patients) of the European Network for the Study of Adrenal Tumors (ENS@T). It has, therefore, been suggested that patients should be followed for at least 10 years. Patients at high risk, such as those young at diagnosis, those with genetic and/or a syndromic disease, a large tumor and/or a paraganglioma, would benefit from life-long annual follow-up, because the risk of recurrence exceeds 20% in such patients. The recommended monitoring methods are blood pressure determination, annual biochemical tests, and thoraco-abdomino-pelvic MRI every 1–2 years (Plouin *et al.*, 2016).

Genetics of PPGL

The "10%" Rule

Before 2000, it was widely thought that about 10% of PPGL were malignant, 10% could be diagnosed during infancy, 10% were multiple, and 10% would appear in patients with a familial history of PPGL. Three genes, *NF1*, *VHL*, and *RET*, had been identified by classical genetic linkage studies (**Fig. 2**). These three genes cause three syndromic diseases (neurofibromatosis type 1, von Hippel-Lindau disease, and multiple endocrine neoplasia type 2). The diagnosis of these diseases in the context of a first PPGL should lead to screening for other syndromic lesions and strict follow-up of all organs at risk.

Neurofibromatosis type 1

Germline mutations of the *NF1* tumor suppressor gene cause neurofibromatosis type 1 (NF1) or von Recklinghausen disease, an almost fully penetrant autosomal dominant syndromic disease. NF1 is generally easy to diagnose in adults, on the basis of the presence of at least two of the seven clinical diagnosis criteria defined by the National Institutes of Health consensus development conference statement of July 1987 (**Table 2**) (Anon, 1988). The risk of PPGL is less than 5%, but the penetrance of the disease is about 1/3000 (Friedman, 1999). In NF1, PPGL usually takes the form of an epinephrine-producing pheochromocytoma diagnosed in patients in their thirties (Bausch *et al.*, 2006).

Von Hippel-Lindau disease

Germline mutations of the *VHL* tumor suppressor gene cause von Hippel-Lindau disease, an autosomal dominant syndromic disease with an incidence of about 1/36,000. Two types have been defined: type 1 (without PPGL) and type 2 (with PPGL)

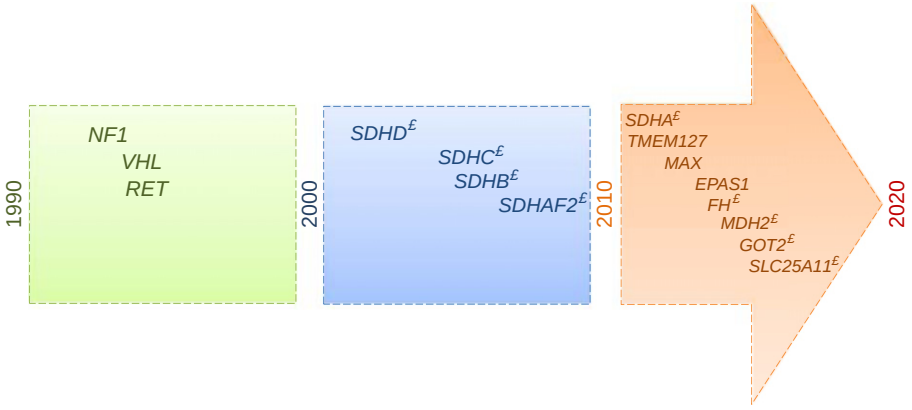

Fig. 2 Timeline of the identification of the main PPGL susceptibility genes (£ indicates TCA cycle genes encoding enzymes or transporters).

Table 2 Diagnostic criteria for neurofibromatosis type 1

Six or more "café-au-lait" spots with a largest diameter exceeding 5 mm in prepubertal individuals or with a largest diameter exceeding 15 mm in postpubertal individuals
Two or more neurofibromas of any type or one plexiform neurofibroma
Freckling in the axillary or inguinal region
Optic glioma
Two or more Lisch nodules (iris hamartomas)
A distinctive osseous lesion, such as sphenoid dysplasia, or thinning of a long bone cortex with or without pseudoarthrosis
A first-degree relative (parent, sibling or offspring) with neurofibromatosis

Table 3 Main syndromic features associated with PPGL in von Hippel-Lindau disease

Retinal hemangioblastoma
Central nervous system hemangioblastoma
Clear-cell renal-cell carcinomas and/or kidney cysts
Pancreatic neuroendocrine tumors or cysts
Endolymphatic sac tumors
Epididymal cystadenoma in men or cystadenoma of the broad ligament in women

Table 4 Main syndromic features associated with pheochromocytoma in multiple endocrine neoplasia type 2

Main syndromic features associated with pheochromocytoma in multiple endocrine neoplasia type 2A
Medullary thyroid carcinoma
Primary hyperparathyroidism
Main syndromic features associated with pheochromocytoma in multiple endocrine neoplasia type 2B
Medullary thyroid carcinoma
Marfanoid appearance
Mucosal neuromas of the tongue, lip, palate
Intestinal ganglioneuromatosis

(Gossage *et al.*, 2015). The various other disorders that can occur in patients with this syndrome are summarized in **Table 3**. In von Hippel-Lindau disease, PPGL is usually diagnosed early, during infancy/adolescence, or in young adults. Tumors may be multiple or bilateral and most produce norepinephrine (Amar *et al.*, 2005a).

Multiple endocrine neoplasia type 2

Gain-of-function germline mutations of the *RET* proto-oncogene cause multiple endocrine neoplasia type 2 (MEN2), an autosomal dominant syndromic disease that affects 1/30,000 individuals. The disease is caused by hot-spot mutations specifically located in several codons of exons 8, 10, 11, 13, 14, 15, and 16. Two clinical types (type 2A and 2B) have been defined and the phenotypic characteristics of these two clinical types are reported in **Table 4**. The American Thyroid Association (ATA) revised its guidelines in 2015 and defined three ATA risk categories for medullary thyroid carcinoma (MTC) caused by *RET* mutations: "highest risk," (HST) "high risk," (H) and "moderate risk" (MOD). The ATA-HST category includes patients with MEN2B and the *RET* codon M918T mutation (in such cases, prophylactic thyroidectomy is recommended before the age of 1 year, to prevent the risk of metastatic MTC). The ATA-H category includes patients with *RET* codon C634 mutations or *RET* codon A883F mutation (in such cases, prophylactic thyroidectomy is recommended before the age of 5 years). The ATA-MOD category includes patients with *RET* codon mutations other than M918T, C634, and A883F. In MEN2, PPGL are diagnosed in 50% of affected patients as single or bilateral pheochromocytomas producing epinephrine, of early onset (before the age of 30 years) (Wells *et al.*, 2015).

SDHx Genes

Our understanding of the genetics of PPGL has progressed considerably since the turn of the century (see **Fig. 2**), with the demonstration that germline mutations of *SDHD*, SDHC, *SDHB*, and then *SDHAF2* and *SDHA* are responsible for hereditary PPGL (Favier *et al.*, 2014). All the *SDHx* genes are tumor suppressor genes encoding the succinate dehydrogenase enzyme. They follow an autosomal dominant mode of inheritance, associated with maternal genomic imprinting for carriers of *SDHD* and *SDHAF2* mutations. As a result, only patients with mutations of the paternal allele of these genes are likely to develop PPGL. Large

phenotype–genotype studies have shown that multiple head and neck PGL is suggestive of *SDHD* mutation, whereas thoracic-abdominal or pelvic PPGL producing dopamine or norepinephrine indicate *SDHB* mutation (Burnichon *et al.*, 2009; Lenders *et al.*, 2014). Nevertheless, multiple PPGL can emerge at sites distant from the initial PPGL in *SDHx* mutation carriers. Thus, thoracic-abdominal or pelvic PGL can be diagnosed in carriers of *SDHD* mutations and head and neck PGL can be detected in carriers of *SDHB* mutations (Gimenez-Roqueplo *et al.*, 2013). Penetrance is incomplete, but higher in individuals carrying *SDHD* mutations than in those carrying *SDHB* mutations. Germline mutations of *SDHC*, *SDHA*, and *SDHAF2* are rarer, but can be identified in patients with PPGL at various sites. It has been shown that mutations of the *SDHB* gene are associated with a high risk of malignancy (Gimenez-Roqueplo *et al.*, 2003) and a poor prognosis in patients with metastatic PPGL (Amar *et al.*, 2007). Finally, rare gastrointestinal stromal tumors (GIST) and kidney cancers caused by *SDHx* mutations have also been reported.

New PPGL Susceptibility Genes Revealed by Massively Parallel Sequencing Technology

Sanger sequencing, based on chain termination techniques performed on capillary sequencing machines, has recently been replaced by next-generation sequencing (NGS) methods, which facilitate massively parallel sequencing at low cost (Toledo and Dahia, 2015). Since 2010, new PPGL susceptibility genes have frequently been identified by the whole-exome sequencing of constitutional and tumoral DNA from one or a few affected patients (see **Fig. 2**). Each of these new PPGL genes accounts for less than 3% of all inherited forms of PPGL. *TMEM127* and *MAX* germline mutations were the first to be identified in patients with a familial presentation (Qin *et al.*, 2010; Comino-Méndez *et al.*, 2011). Nevertheless, *TMEM127* mutations can be found in patients with apparently sporadic epinephrine- and norepinephrine-producing PPGL, whereas *MAX* mutations are usually diagnosed in younger patients (about 30 years old) with norepinephrine-producing PPGL (Abermil *et al.*, 2012; Burnichon *et al.*, 2012).

Germline mutations of *FH* (Letouzé *et al.*, 2013), *MDH2* (Cascón *et al.*, 2015), *GOT2* (Remacha *et al.*, 2017), and *SLC25A11* (Buffet *et al.*, 2018) have been found in patients with norepinephrine-producing PPGL, which was malignant in some cases. *FH* is the only one of these genes for which mutations are known to cause a syndromic disease characterized by the association of cutaneous and uterine leiomyomas and, in 15% of cases, aggressive renal cell carcinoma (papillary type 2).

Two genes have been found to display somatic mutations and a low level of constitutional mosaicism in patients. The first, *EPAS1*, has been found mutated in patients with PPGL, polycythemia and/or somatostatinoma, and in patients with apparently sporadic PPGL (Zhuang *et al.*, 2012; Buffet *et al.*, 2014). The second, *H3F3A*, has been found mutated in a very small number of patients with PPGL and giant-cell tumors of the bone (Toledo *et al.*, 2016). These mutations, identified in DNA extracted from tumoral tissue, can arise at a postzygotic stage and deep sequencing should be performed to check for their presence in the germline, to determine the risk of the disease occurring in the patient's descendants.

Finally, germline mutations of *PHD2* (or *EGLN1*), *KIF1Bβ*, and *MERTK* have been identified in a very small number of patients with a predisposition to pheochromocytoma and polycythemia (Ladroue *et al.*, 2008), neuroblastoma and ganglioneuroma (Schlisio *et al.*, 2008), or medullary thyroid carcinoma (Toledo *et al.*, 2016), respectively, and a germline mutation of the *IDH3B* gene was recently reported in a single patient with head and neck PPGL (Remacha *et al.*, 2017). However, the contributions of all these genes, including *H3F3A*, to the genetics of PPGL, should be confirmed in additional studies.

The large number of PPGL susceptibility genes reported to date justifies the recommendation of NGS for genetic testing for PPGL. The PPGL gene panels targeted by NGS should include at least the *FH*, *MAX*, *NF1*, *RET*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *TMEM127*, and *VHL* genes (NGS in PPGL (NGSnPPGL) Study Group *et al.*, 2017).

Particularities of the Diagnosis and Follow-Up of Inherited PPGL

Personalized Management

Hereditary PPGL is a very rare disease that is likely to recur or to develop into other lesions or metastases. For the best possible outcome, a personalized approach is currently recommended, developed for individual patients by a multidisciplinary team from a specialist center with appropriate expertise (Lenders *et al.*, 2014).

Molecular Classification of PPGL

The comprehensive multiomics profiling studies on large PPGL collections carried out by the COMETE and ENSAT networks in Europe, and by The Cancer Genome Atlas (TCGA) in the United States identified well-defined PPGL subtypes based on mutational status and strongly associated with specific tumorigenic pathways. Unsupervised hierarchical clustering separates PPGL into two main clusters: a "pseudohypoxic" (cluster 1) and a "MAP kinase signaling" cluster (cluster 2). Cluster 1 comprises two different subtypes: cluster 1A and 1B. Cluster 1A contains all tumors due to mutations of genes (*SDHx*, *FH*, *MDH2*, *SLC25A11*, *GOT2*) encoding proteins of the tricarboxylic acid (TCA) or Krebs cycle. Briefly, the inactivation of these enzymes or transporters unbalances the succinate/oxoglutarate ratio in the cell, leading to the inhibition of prolyl hydroxylases, and DNA and histone demethylases. Both these phenomena lead to inappropriate angiogenesis in presence of a normal oxygen concentration in the cell, and the occurrence of an epithelial–mesenchymal transition favoring oncogenesis. Cluster 1B consists principally of PPGL caused by germline or tumoral *VHL* mutations. Like mutations of TCA cycle enzyme genes, *VHL* mutations impair the normal degradation

of the VHL protein by prolyl hydroxylases, leading to HIF protein stabilization and constitutive activation of the hypoxic-angiogenic pathway. All other PPGL of genetic origin belong to cluster 2, and are due to mutations of genes involved in MAP kinase and/or mTOR/AKT signaling (Dahia, 2014). Somatic mutations of *NF1*, *RET*, *MET*, and *H-RAS* have been found in sporadic PPGL classified into cluster 2 (Burnichon *et al.*, 2016). Physicians need to be aware of the biological differences between the two categories of PPGL, and should take these differences into account to ensure the correct management of their patients.

Biochemical Testing

The first step in the processing of catecholamines is the conversion of tyrosine into dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. DOPA decarboxylase then converts DOPA into dopamine, dopamine beta-hydroxylase converts dopamine into norepinephrine and, finally, phenylethanolamine *N*-methyltransferase (PNMT) catalyzes the production of epinephrine from norepinephrine (**Fig. 3**). Studies of the transcriptome and methylome have shown that the expression of *PNMT* is downregulated in cluster 1-related PPGL, due to the hypermethylation of its promoter (Letouzé *et al.*, 2013). Consequently, PPGL due to mutations of TCA cycle or *VHL* genes cannot produce epinephrine. Thus, in cases of PPGL producing norepinephrine or dopamine, mutations of one of the TCA cycle genes or of *VHL*, should be suspected. By contrast, tumors with mutations of *NF1*, *RET*, or *TMEM127* can process catecholamines to their end product, epinephrine, and may be revealed by a severe catecholamine crisis.

Imaging

The European Association of Nuclear Medicine, the Society of Nuclear Medicine, the Endocrine Society and the European Society of Endocrinology have proposed guidelines and clinical algorithms for the imaging of PPGL according to genetic status (Taïeb *et al.*, 2012). It has been clearly demonstrated that (^{18}F-FDG) PET/CT is the best first-line imaging technique for patients with *SDHB* mutations, because *SDHB*-related primary PPGL and metastases are characterized by a high glucose uptake (Timmers *et al.*, 2007). For somatostatin receptor imaging, the prospective multicenter PGL.EVA study established in 2013 that a combination of ^{111}In-pentreotide scintigraphy with thoraco-abdomino-pelvic computed tomography and head and neck magnetic angiography had a sensitivity of 91.7% with local assessment, or of 98.6% with expert centralized image assessment, for the diagnosis of *SDHx*-related PPGL (Gimenez-Roqueplo *et al.*, 2013). The clinical approval and availability of new tracers differ between countries, but recent studies have suggested that ^{18}F-DOPA and ^{68}Ga-DOTATATE PET/CT are more sensitive than conventional imaging methods and should be used, in particular, for complete tumor staging before therapy (surgery or radiotherapy) in patients with an SDHx-related PPGL (Marzola *et al.*, 2014; Jha *et al.*, 2017).

Treatment

The main features of genetically determined forms are the risk of recurrence or of a new PPGL at the same site or in a different area, the risk of malignancy, particularly in patients with mutations of genes encoding TCA cycle proteins and the risk of other syndromic lesions. For all these reasons a multidisciplinary team should tailor the treatment to the patient.

Single PPGL

Surgery, performed by a specialist anesthetic and surgical team, is usually indicated in patients with a genetically determined PPGL present as a first, single, catecholamine-producing tumor.

Decisions about surgery for head and neck PGL depend mostly on tumor location, tumor burden, and the risk of neurological deficits after surgery. Radiotherapy (external, proton beam therapy or gamma knife radiosurgery) is an alternative approach.

Metastatic PPGL

Most PPGL are caused by a germline or somatic mutation in a single PPGL susceptibility gene. Thus, PPGL patients with hereditary metastatic disease should benefit from precision medicine based on the molecular genomic profiles of their tumors. For example, a large proportion of metastatic PPGL belong to the "pseudohypoxic" cluster 1, and randomized trials are currently underway to

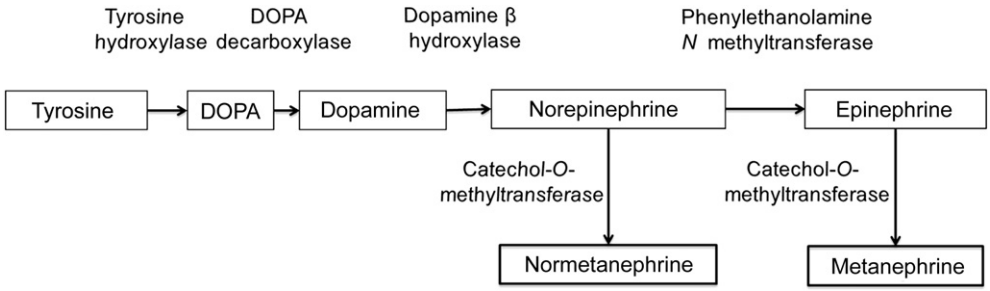

Fig. 3 Synthesis and metabolism of catecholamines.

assess antiangiogenic therapies for progressive malignant PPGL. A retrospective study recently reported that temozolamide was effective against SDHB-related metastatic PPGL, due to silencing of the *O*-6-methylguanine-DNA methyltransferase (MGMT), which normally repairs the DNA damage induced by alkylating chemotherapy, by hypermethylation of the promoter of *MGMT* gene (Hadoux *et al.*, 2014). PPGL genetic testing and molecular profiling would be expected to favor the development of personalized medicine for PPGL in routine practice, potentially improving the prognosis of genetically determined metastatic PPGL (Favier *et al.*, 2014).

Follow-Up

Life-long annual follow-up

Patients with a genetic form of PPGL have a 17% risk of a new event. Life-long annual follow-up is recommended and should include at least blood pressure determination and biochemical testing (Plouin *et al.*, 2016). Cervical and thoraco-abdomino-pelvic MRI should be performed regularly for the detection of nonsecreting PPGL. Imaging methods involving the use of ionizing radiation should be limited during follow-up and restricted to screening for new tumors suspected or detected on the basis of biochemical analysis and/or magnetic resonance imaging (Lenders *et al.*, 2014; Plouin *et al.*, 2016). A disease-specific surveillance program should also be carried out.

Screening and follow-up of relatives

Evidence for a genetically determined PPGL in an index case should lead to all first relatives at risk (taking into account the specific model of inheritance and age at diagnosis) being offered presymptomatic genetic testing, with specific screening for the familial mutation in DNA extracted from leukocytes. Genetic counseling should be offered to all subjects at risk, both before and after testing. Positive genetic tests should lead to the organization of screening to detect small tumors and/or other syndromic lesions and the implementation of specific follow-up (**Table 5**).

Table 5 First screening and life-long follow-up of mutation carriers

Genes	*First screening and life-long follow-up of mutation carriers*
NF1	– Physical examination with blood pressure monitoring every year – Annual metanephrines (plasma free or fractionated urinary) determination – Annual ophthalmologic examination – If patient has hypertension or abnormal metanephrines levels: abdominal MRI (or CT scan)
RET	– Physical examination with blood pressure monitoring every year – Annual metanephrines (plasma free or fractionated urinary) determination – Annual plasma calcitonin determination – Abdominal MRI (or CT scan) at first screening and then, only if hypertension or abnormal metanephrines levels occur during follow-up
VHL	– Annual physical examination with blood pressure monitoring – Annual metanephrines (plasma free or fractionated urinary) determination – Annual ophthalmologic examination – Abdominal-pelvic MRI every 2 years and renal ultrasound every other year – MRI scan of head + spine every 2 years
TCA cycle	– Annual physical examination (+ gynecological and dermatological examination, for *FH* mutation carriers only) with blood pressure monitoring – Annual metanephrines (plasma free or fractionated urinary) determination – At first screening: head and neck plus thoracic-abdominal-pelvic contrast MRI or CT – At first screening: ^{18}F-FDG PET/CT, especially for patients at risk of malignancy, such as *SDHB* or *FH* mutation carriers, or ^{18}F-F DOPA PET/CT – Whole-body MRI every year or 2 years during follow-up
TMEM127 *MAX*	– Annual physical examination with blood pressure monitoring – Annual metanephrines (plasma free or fractionated urinary) determination – At first screening: head and neck plus thoracic-abdominal-pelvic contrast MRI or CT – Whole-body MRI every year or 2 years during follow-up
HIF2A	– Annual physical examination with blood pressure monitoring – Annual hemoglobin determination – Annual metanephrines (plasma free or fractionated urinary) determination – At first screening: head and neck plus thoracic-abdominal-pelvic contrast MRI or CT – Whole-body MRI every year or 2 years during follow-up

Conclusion

Our understanding of PPGL and the management of this disease have been radically modified by new insight into the genetics of PPGL obtained over the last 20 years. PPGL is now considered to be the neuroendocrine tumor most strongly affected by genetics. PPGL management is going to shift from classical approaches to personalized medicine tailored to the patient and driven by the genetic status and molecular profile of the tumor. Patients with PPGL should be managed by specialized multidisciplinary expert centers endowed with the different skills required to deal with the rarity and complexity of the different subtypes of this disease.

See also: Pheochromocytoma and Paraganglioma Syndromes. Pheochromocytoma/Paraganglioma: Diagnosis and Treatment

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Renin and Prorenin

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Abbreviations

11 β-HSD2 11 β-hydroxysteroid dehydrogenase
ACE Angiotensin converting enzyme
AI Angiotensin I
AII Angiotensin II
Ang 1–7 Angiotensin 1–7
AT 1-AT2 Angiotensin II receptors
HRP Handle regional peptide
JGA Juxtaglomerular apparatus
JGC Juxtaglomerular cells
PRA Plasma renin activity
PRC Plasma renin concentration
PRR (Pro)renin receptors
RAS Renin–angiotensin–system
RVH Renovascular hypertension
SNS Sympathetic nervous system

History

In 1898 the Finnish physiologist Robert Tigerstedt and his Swedish assistant Gustav Bergman observed that the injection of rabbit kidney extracts in the jugular vein of rabbits induced marked elevation in blood pressure. They attributed this pressor effect to a renal substance that for brevity they decided to name "renin." However in the following years several investigators failed to reproduce their findings that thereafter were neglected to the point that in Tigerstedt's obituary the discovery of renin was not even mentioned among his scientific achievements. However some 30 years later Volhard and Hessel reported their experiments in which normal dogs injected with blood from ischemic kidney developed hypertension. In 1927 John Loesch was the first to propose the mechanisms whereby renal ischemia may raise blood pressure. In the early 1930s, Harry Goldblatt developed if famous canine model of hypertension by means of renal ischemia, a prototype of human renovascular hypertension, fueling the interest toward the kidney as a source of pressor agents. In the following two decades all the components of the renin–angiotensin–system (RAS) were purified and sequenced setting the stage for the opening of one the most exciting and rewarding area of basic and clinical research in medical history.

Components and Functions of the RAS

Renin, an aspartyl protease, is the first rate limiting step of the RAS in that it catalyzes the conversion of angiotensinogen, the renin substrate produced and secreted by the liver, to a decapeptide called angiotensin I (AI). AI has no biological action by itself but is the substrate of another endothelial cell-associated peptidase, ubiquitous in the body but primarily localized in pulmonary vasculature, called angiotensin converting enzyme (ACE). ACE cleaving the terminal dipeptide of AI generates angiotensin II (AII) the octapeptide final effector of the system (Sparks *et al.*, 2014) (**Fig. 1**). An isoform of angiotensin converting enzyme called ACE2 catalyzes the conversion of AI to the eptapeptide angiotensin 1–7 (ANG1–7). Angiotensin II actions are exerted through the binding to a family of surface trans-membrane receptors widely distributed in the body, the best known for their effects being the type 1 and type 2 angiotensin II receptors (AT 1 and AT2) whereas ANG1–7 binds to surface MAS receptors. Activation of AT1 results in contraction of vascular smooth muscle cells with increase in peripheral vascular resistance and elevation in blood pressure. These effects are partially counterbalanced by AT2 and ANG1–7 mediated vasodilation. In addition to the regulation of vascular tone AII stimulates sodium reabsorption directly in the distal convoluted tubules of the nephron and, indirectly, augmenting the production from the adrenal cortex of aldosterone, the most potent mineralocorticoid hormone that regulates the sodium–potassium exchange in the collecting tubules. Also, AII enhances the activity of peripheral sympathetic nervous system (SNS) and, interacting with vasopressin, regulates thirst perception in the central nervous system. Because of all these interconnected actions the RAS is indisputably considered a major player in the regulation of blood pressure, sodium balance and body fluid homeostasis.

The scientific and clinical interest for RAS increased exponentially in the last decades because of the overwhelming amount of pathophysiological studies supporting the notion that an excessive activation of RAS via proinflammatory and prothrombotic actions has a mechanistic role in causing cardiovascular and renal damages beyond and above those induced by blood pressure elevation. This knowledge was further reinforced by the results of many randomized controlled studies proving the beneficial effects of RAS blockade on morbidity and mortality in highly prevalent and ominous pathological conditions such as primary and secondary hypertension, diabetes, coronary artery disease, heart failure and chronic kidney disease.

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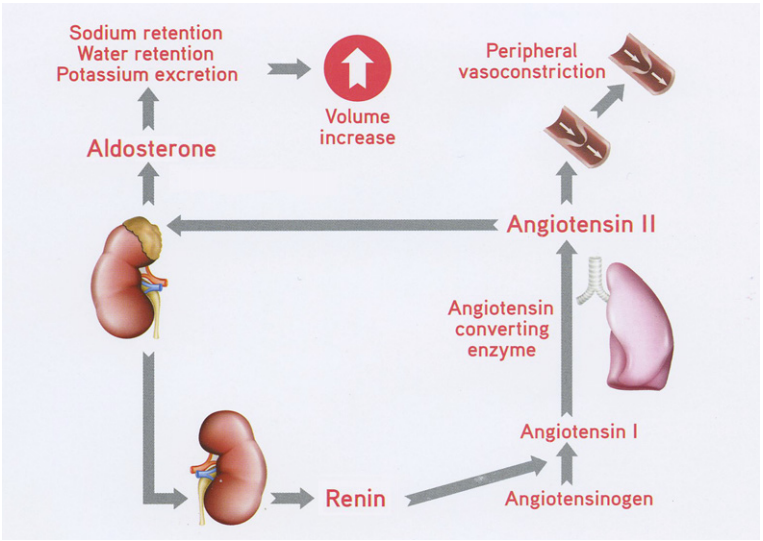

Fig. 1 Schematic illustration of the renin–angiotensin–aldosterone system.

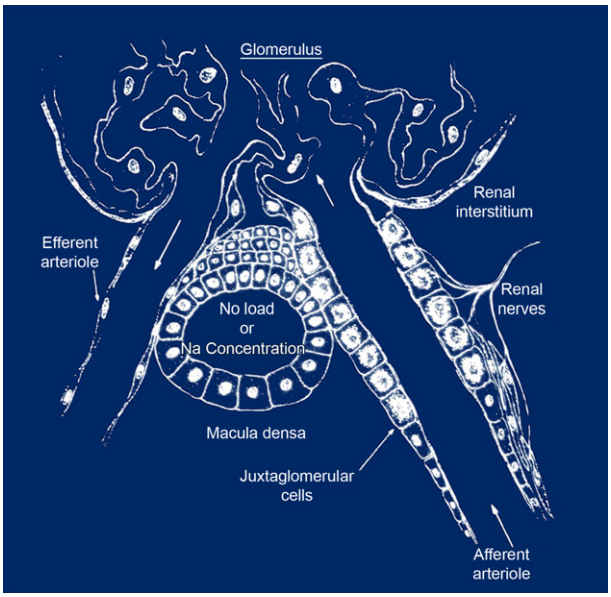

Fig. 2 Schematic illustration of the juxtaglomerular apparatus.

Site of Prorenin–Renin Production and Processing

The kidney is the only source of circulating renin since after bilateral nephrectomy renin is undetectable in blood whereas prorenin, the renin precursor, is also produced by numerous organs including the adrenal gland, testis, placenta and eye. In the kidney renin is synthetized by the juxtaglomerular cell (JGC), specialized epithelioid cells located along the length of the afferent arterioles just at the entrance of the glomerulus. JGC are part of the juxtaglomerular apparatus (JGA) in close contact with the macula densa cells of the ascending limb of the upper nephron that also have the capacity of producing renin (**Fig. 2**). In human JGC the primary transcript of the renin gene located in chromosome 1 is preprorenin that is converted to prorenin in the endoplasmic reticulum. Following glycosylation in the Golgi apparatus prorenin is either secreted by a constitutive pathway or is proteolytically converted to active renin. Active renin is stored in cytoplasmic vesicles ready to be released in response to regulatory stimuli. Cyclic AMP and calcium are the key factors in the intracellular regulation of renin exocytosis with counterbalancing effects in that cyclic AMP stimulates renin release whereas calcium, at variance with other secretory cells, inhibits it. The explanation recently proposed to explain this "calcium paradox" is that in JGC calcium accelerates cyclic AMP degradation via the activation of a calcium calmodulin dependent phosphodiesterase 1C (Ortiz-Capisano *et al.*, 2009).

Regulation of Renin Secretion

Renin secretion and synthesis are dissociated in time in that the secretion in response to an acute stimulus occurs in minutes whereas the increase in synthesis requires hours to take place. However these different time-course of response are unlikely to become a problem because the intracellular stores of renin are large enough to cope even with intense and prolonged stimuli. Also, under conditions of intense homeostatic stress such as profound dehydration, sodium depletion, blood loss, low pressure or prolonged pharmacological blockade of the RAS, the JGC increase in number (hyperplasia) to provide enough renin to reestablish blood pressure and volume. Whenever the disequilibrium persists other cells of JGA, like the vascular pericytes and mesangial cells, are recruited to synthetize renin reassuming the phenotype of fetal life (Gomez and Sequeira-Lopez, 2016).

Renin secretion is under the control of a number of factors (Kurtz, 2011). A fast rise in systemic blood pressure inhibits renin secretion via the direct effect of increased perfusion pressure on preglomerular vessels while circulating renin increases within minutes after a sharp fall in pressure. Augmented intake of dietary sodium chloride with attendant increase in urinary sodium excretion suppresses renin secretion from the macula densa cells that, vice versa, are stimulated by sodium depletion. Any increase in circulating catecholamines, either released from nerve endings or from adrenal medulla cause an increase in renin secretion through the activation of beta 1 adreno-receptors. In man even mild sympathetic stimulation such as that occurring in response to assumption of upright posture doubles plasma renin within 15 min (Morganti *et al.*, 1979). Sympathetic discharge of renal nerves also affects JGC cells number that is halved by renal denervation. Renin secretion is in itself controlled by a short-loop negative feed-back mechanism exerted by AII through the AT1 located in the JGC. Finally renin production is controlled by numerous humoral factors of endothelial (nitric oxide, prostaglandins) and cardiac (natriuretic peptides) origin.

(Pro)Renin Receptors

Plasma concentration of prorenin is on average 10-fold higher than that of active renin and in some body fluids such as the amniotic fluid or the vitreous fluid of patients with diabetic retinopathy even 100-fold higher (Campbell, 2008). However prorenin is enzymatically inactive because a 43 amino acid N-terminal peptide (prosegment) obliterates the pocket where angiotensinogen is cleaved to generate AI. Inside the JGC prorenin is proteolytically converted to active renin but there is no evidence of such conversion in plasma or extrarenal tissues. Thus it was unclear how prorenin could contribute to AII production. However 15 years ago the seminal paper by Nguyen *et al.* (2002) demonstrated the existence of specific (pro)renin receptors (PRR) expressed in the kidney, heart, brain, liver, coronary vessels, and placenta. Moreover these authors reported that upon binding to PRR prorenin undergoes a conformational change that spatially removing the prosegment exposes the catalytic pocket of renin making possible the local formation of AII. In addition they showed that the (pro)renin binding to PRR activates the intracellular signaling pathways in a parallel but distinct way from that of circulating AII. These findings triggered a decade of intense research aimed at clarifying whether and how PRR have a pathophysiological meaning. To this aim a peptide antagonist of the "handle" region of the prosegment (HRP) was developed to assess the effects of preventing the binding of prorenin and renin to PRR. Overall studies with HRP in diabetic and hypertensive animal models yielded conflicting results but it became clear that the micromolar concentration of HRP used in these investigations were well above those reached by (pro)renin at tissue level even in extreme pathological conditions. In last years the research on this topic turned to examine the possible actions of PRR not related to those of RAS. Preliminary results suggest that PRR may participate in embryonic development interacting with the β catenin signaling pathway, in the intracellular acid–base regulation via the action on the V-ATPase system and in the lipoprotein and energy metabolism. The forefront advances in PRR were recently reviewed (Sun *et al.*, 2017).

Growth Defects Associated With Genetic Manipulations of RAS

A large body of evidence from animal studies suggests that RAS is important also for the development during fetal life. Indeed genetic manipulations of RAS lead to severe growth defects in several organs and particularly in the kidney. In mice deletion of the renin gene causes dilation of the renal pelvis, interstitial fibrosis and arteriolar hypertrophy. Similar renal phenotypes were described in mice deficient of angiotensinogen, ACE and angiotensin receptors. These animals also have hypotension and, if males, reduced fertility. Mice lacking ACE2 display reduced cardiac contractility that makes them prone to heart failure while mice lacking the adrenal aldosterone receptors become hyponatremic and hypotensive. In general all these animals survive shortly after birth. In line with these observations it has been repeatedly reported that sheep treated with RAS blockers have a significantly higher rate of abortion than controls sheep. Accordingly guidelines recommend not to treat pregnant women with RAS antagonists, particularly in the early stages of gestation (Mancia *et al.*, 2013).

Also the genetic alterations of PRR are associated with severe malformations or dysfunctions. PRR deletion in mice causes heart failure and loss of lysosomes in smooth muscle cells. In addition PRR knockout is associated with reduction of lipoprotein receptor protein and impaired adipogenesis. Studies in man are limited. However hyperexpression of PRR was found in kidney biopsy samples from patients with chronic kidney disease and in the adipose tissue of insulin resistant obese women.

How to Measure Renin and Prorenin

For more than half a century renin was measured as plasma renin activity (PRA) as a substitute of AII because the very low blood concentration of this short peptide (in the order of few picograms/mL) made its quantification with radioimmunoassay (RIA) quite difficult. PRA instead exploits the capacity of renin to generate, during an incubation step carried out at 37°C and pH 5.7 –6.0, sufficient amounts of AI to be quantified with RIA. Moreover increasing the duration of the incubation step from the conventional 3 h up to 18 h makes the PRA assay suitable to determine the renin levels also when its circulating concentrations are extremely low (Sealey *et al.*, 2005). The basic assumption for using PRA as an index of RAS activity "in vivo" is that angiotensinogen concentration is not altered to the point of changing the kinetics of the renin–renin substrate reaction. This may occur in patients with advanced liver insufficiency, a disease in which the production of angiotensinogen is reduced and in pregnancy where angiotensinogen is markedly augmented. Except in these conditions and provided subjects are not on treatment with drugs interfering with RAS (see below), PRA values are quite strictly correlated with those of AII. Values of PRA, being an enzymatic assay, are expressed in ng AI mL^{-1} h^{-1}.

However PRA is a complex method with several drawbacks, the most relevant being the poor reproducibility among laboratories. The availability of monoclonal antibodies raised against specific epitopes of the renin molecules has made possible the direct quantification of plasma renin as mass concentration (PRC) with immunometric or immunochemiluminescent methods (Perschel *et al.*, 2004). In brief plasma samples are incubated with a capture antibody against both prorenin and renin and with a second antibody specific for active renin conjugated with a radioactive or acridinium tracer. After separation of the" sandwich" immunocomplexes with streptavidin magnetic beads followed by washing of the unbound tracer the radioactive counts or the light signal generated by the addition of a trigger solution are directly proportional to the concentration of renin. Values of PRC are usually expressed in mU mL^{-1} or pg mL^{-1}. These new assays are calibrated against the International Standard Reference Preparation of Renin and their reproducibility among laboratories is greater than that of PRA. (Morganti *et al.*, 1995). In normal conditions PRA and PRC are well correlated except in the very low range. Both PRA and PRC assay can be used to measure prorenin. Prorenin values are calculated subtracting those obtained after the exposure of plasma to the procedures of prorenin activation (irreversible proteolysis with trypsin or reversible unfolding of prosegment with acid or prolonged cold exposure) to those measured before these procedures.

When to Measure Renin

Evaluation of plasma renin is advisable whenever patients have clinical signs suggestive of secondary forms of hypertension (young age, abdominal vascular bruits, low serum potassium, worsening of renal function particularly if during treatment with RAS antagonists, advanced atherosclerosis, kidney asymmetry). In patients with renovascular hypertension (RVH) due to a fibromuscular dysplasia or to an atherosclerotic stenosis of the renal arteries (RAS), usually greater than 80% of vessel diameter, JGC are stimulated to increase renin secretion by the reduction in renal blood flow. In RVH the vascular and cardiac damages caused by the increments in plasma renin and plasma aldosterone contribute to aggravate those due to high blood pressure. However high renin values are not exclusive of RVH. Indeed hyperreninemia is found also in patients with malignant hypertension and in about 20% of those with benign essential hypertension. A high renin profile in relation to 24 h sodium excretion is also found in patients with JGC tumor. At variance with RVH in these rare cases also prorenin is very high as well as in other forms of renal or extrarenal cancers. In JGC tumor the imaging of renal arteries excludes the presence of RAS while the measurement of PRA or PRC in blood collected from the renal veins confirms the lateralization of renin secretion from the suspected side. A similar pattern of renin secretion can be seen in patients with renal infarction where the ischemic process mimics the effects of a tight RAS. High renin levels can also be found in patients with catecholamine secreting tumors (pheochromocytoma and paragangliomas) and in immunological diseases associated with renal vasculitis.

On the other side of the spectrum renin secretion is suppressed and not responsive to physical or pharmacological stimuli in patients with primary aldosteronism. In these cases JGC are silenced by the expansion of body fluids caused by the excess of aldosterone production that can be due either to adrenal hyperplasia or to an adenoma. Recent guidelines recommend the evaluation of aldosterone to renin ratio (ARR) as firs step for the diagnosis of primary aldosteronism (Funder *et al.*, 2016). Suppressed renin and high aldosterone are also observed in the less frequent cases of familial ACTH dependent aldosteronism. A clinical condition that mimics primary aldosteronism but is characterized by very low levels of renin and aldosterone is the apparent mineralocorticoid excess (AME) syndrome. This syndrome is due to mutations in the gene that encodes for 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the enzyme that metabolizes cortisol to inactive cortisone thus preventing cortisol binding to mineralocorticoid receptors (MR). Because of this mutation inactive forms of 11β-HSD2 are produced allowing the binding of cortisol to MR that, in turn, results in increase sodium and water reabsorption in the kidney, volume expansion, hypertension and suppression of renin and aldosterone secretion. AME syndrome is usually diagnosed in early childhood being associated with low birth weight and growth retardation. In adults the alterations in renin and aldosterone profiles seen in AME syndrome are reproduced by prolonged ingestion of licorice because the glycyrrhizinic acid contained in licorice is a potent inhibitor of 11-β-HSD2. Liddle syndrome is also associated with hypertension and renin and aldosterone suppression, in this case due to mutation of a subunit of the epithelial sodium channel of distal renal tubules, resulting in increased sodium reabsorption

and potassium loss. Spirolactone has little effect in patients with Liddle syndrome whereas they respond well to amiloride or triamterene.

How Drugs Interfere With RAS

The majority of drugs commonly used for treatment of cardiovascular and renal diseases profoundly interfere with renin secretion and their effects often take several weeks to wear off. Thus when assessing RAS activity ideally all medications should be discontinued at least for a fortnight. RAS antagonists blocking AII formation (renin and ACE inhibitors) or its action at receptor level (ARBs) increase renin secretion interrupting the negative short-loop feed-back exerted by AII on JGC. It is important to recall that during treatment with ACEs or ARBs PRA and PRC increase in parallel whereas diverge during treatment with renin inhibitors because PRA measuring the enzymatic activity of renin is reduced by these drugs whereas PRC measuring the concentration of renin molecules is increased. These changes in opposite directions of PRA and PRC are useful to assess whether the blockade of AII production is actually achieved. It is also worth mentioning that during treatment with any of the RAS antagonists plasma aldosterone should decrease as a consequence of the abolished AII stimulation. However, quite unexpectedly, it was found over and over that during treatment with all RAS antagonists, even when combined and irrespective of the doses, in about 50% of patients PAC levels return toward the baseline values and, occasionally, even above those (Bomback and Klemmer, 2007). This phenomenon often referred to as "aldosterone escape" or "aldosterone breakthrough" has clinical relevance in that it may explain why in some patients RAS antagonists are less efficacious than expected.

Loop and distal tubule diuretics as well as aldosterone receptor antagonists stimulate renin secretion through the increased sodium load to the macula densa cells while direct vasodilators like hydralazine stimulate JGC to increase renin release via the fall in systemic and preglomerular blood pressure and the attendant reflex increase in SNS. Among the vasodilators the calcium channel blockers and the alpha adrenergic receptors blockers are the only compounds with little, if any, effect on renin secretion and for this reason are preferentially used in patients who cannot discontinue treatment when undergoing plasma renin measurement.

Blockers of the $\beta 1$ adrenergic receptors (BB) as well as central sympatholytic agents like clonidine markedly suppress renin secretion abolishing the nerve mediated component of its release. The degree of the suppressive effect of BB depends on the partial agonistic action of each compound. It is important to recall that discontinuation of BB is particularly important when assessing the aldosterone to renin ratio (ARR) because these drugs suppress renin to a greater extent than aldosterone causing falsely high ARR values and potentially misleading diagnosis of primary aldosteronism.

See also: Angiotensinogen and Angiotensins. Kininases. Tissue Ace–Angiotensin–AT1 Receptor Axis and Repair in the Heart. Hyperreninemia. Interference With the Renin–Angiotensin System (RAS): Classical Inhibitors and Novel Approaches

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Angiotensinogen and Angiotensins

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Abbreviations

AGT	Angiotensinogen
ACE	Angiotensin converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
ASO	Antisense oligonucleotides
AT1R, AT2R	Angiotensin II type 1 and type 2 receptors, respectively
DPP III	Dipeptidyl peptidase III
MAPK	Mitogen-activated protein kinase
MLDAD	Mononuclear leukocyte-derived aspartate decarboxylase
MrgD	Mas-related G-protein–coupled receptor, member D
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
RAS	Renin–angiotensin system
SHP-1	Src homology (SH) 2 domain-containing tyrosine phosphatase-1
SNP	Single nucleotide polymorphism

Glossary

Case-control association studies A population-based genetic approach able to compare the allele/genotype distribution of a certain DNA variant in cases versus controls. The prevalence of a variant nucleotide within the DNA sequence in diseased individuals compared to unaffected subjects can help to establish the role of a certain gene into the pathogenesis of a selected disease.

Chromosome 1q32.1 It indicates that the renin gene maps on chromosome 1, on the long (q) arm of the chromosome in a region designated q32.1.

Chromosome 1q42.2 It indicates that the AGT gene maps on chromosome 1, on the long (q) arm of the chromosome in a region designated q42.2.

***Cis*-acting DNA regulatory elements (CRE)** Are regions of noncoding DNA which regulate the transcription of nearby genes. CREs are found in the vicinity of the genes that they regulate. CREs typically regulate gene transcription by functioning as binding sites for transcription factors.

Genetic polymorphisms The term refers to a variation of the standard DNA sequence of a gene, that is, a single base substitution. The gene sequence variation can be used, as a gene marker, for population-based genetic investigations.

hypoAGT mouse model A mouse model carrying a whole body reduction of AGT obtained by placing 3 LoxP sites encompassing exon 2 of the mouse AGT gene and a neo cassette in intron 2.

In vitro site-mutagenesis study By using this method, a mutation can be created at any specific site in a gene whose wild-type sequence is already known. As a result, the effects of specific amino acid changes in a protein can be learned, relative to the protein from the wild-type gene. The resulting structural changes may then be linked to observed changes in function, stability, and/or activity of the protein.

Mice with human AGT and renin transgenes A genetically modified mouse model overexpressing both human AGT and renin genes.

Renovascular hypertension A secondary form of hypertension explained by an atherosclerotic plaque causing a stenosis of the main renal artery or of one of its branches. The resulting renal ischemia activates the RAS with consequent excess of renin, Ang II, and aldosterone production.

Whole-body AGT-deficient mice A genetically modified mouse model carrying a whole body AGT gene deletion (AGT knock-out).

Renin–Angiotensin System Components

The renin–angiotensin system (RAS) represents an important endocrine system in view of its multiple regulatory properties on sodium and water homeostasis as well as on the functions of heart and blood vessels (Ferrario, 1990). Besides its hormonal actions, the RAS has also paracrine and intracrine functions within tissues in multiple organs. Renin, the rate limiting enzyme of the cascade, is a highly specific endopeptidase that generates angiotensin I (Ang I) from the cleavage of angiotensinogen (AGT). Ang I, in turn, is the substrate for angiotensin converting enzyme (ACE) (a kininase II enzyme) to generate angiotensin II (Ang II) (Ferrario, 1990). The renin gene is localized on the human chromosome 1q32.1. It encodes a 406 amino acids long peptide, called prorenin. A 43 amino acids long prosegment covers the cleavage site of the molecule making prorenin inactive. Prorenin becomes activated when the prosegment is cleaved by proteases such as trypsin, kallikrein, cathepsin B, D, and G (irreversible activation) (Sealey and Rubattu, 1989; Pitarresi *et al.*, 1992). On the other hand, both low temperature and low pH lead to a reversible activation of prorenin (Pitarresi *et al.*, 1992). Most importantly, the discovery of the renin receptor led to the observation that prorenin becomes reversibly activated by the binding to the receptor (Jan Danser *et al.*, 2007). Renin is highly expressed in the juxtaglomerular apparatus of the kidneys, where it is stimulated by the β-adrenergic system, by reduced sodium delivery to the

macula densa and by increased cAMP levels (Persson, 2003). Higher blood pressure levels, high sodium concentrations, as well as elevated levels of Ang II and of calcium concentration inhibit the renin release. Apart from the kidney, the renin gene is expressed in the heart, brain, adrenal glands, and the reproductive organs where it exerts local regulatory functions (Persson, 2003). The renin released from the kidneys circulates in the body.

The classical view of the RAS includes prorenin/renin/AGT/Ang I/ACE/Ang II/AT1R-AT2R, with AT2R playing opposing effects to those exerted by AT1R. In fact, AT2R activation leads to vasorelaxation, as opposed to AT1R-induced vasoconstriction (Ferrario, 1990). Importantly, a cross-talk between the two Ang II receptors has been documented (Gigante *et al.*, 1997; De Paolis *et al.*, 1999).

Our knowledge on the RAS has been progressively increased over the years with the discovery of important additional components, such as AGT and derived angiotensins, with additional novel functions in selected tissues.

Angiotensinogen

The binding of renin to the renin receptor increases the catalytic activity of renin on AGT. The latter is the only substrate of renin and is mostly synthetized, in a constitutive manner, from the liver (Lynch and Peach, 1991; Nakagawa *et al.*, 2007; Campbell and Habener, 1986). Renin is a negative regulator, whereas Ang II is a positive regulator of liver AGT synthesis. Multiple putative *cis*-acting DNA regulatory elements, including glucocorticoids, estrogens, and acute phase responsive elements, are located within a region of 1 kb that is immediate upstream of the human AGT gene (Fukamizu *et al.*, 1990; Tewksbury *et al.*, 1978). As a consequence, dexamethasone and estrogens are positive regulators of AGT synthesis in the liver. In fact, plasma AGT concentrations increase during glucocorticoid administration, during pregnancy, and upon synthetic estrogen administration with oral contraceptives (Deschepper, 1994). Although structurally resembling a serin protease inhibitor (SERPIN), AGT is a noninhibitory SERPIN. The gene is located on human chromosome 1q42.2 and includes five exons and four introns (Isa *et al.*, 1990; Gaillard *et al.*, 1989). Human AGT has 485 amino acids, including a 33 amino acids long signal peptide. The 10 N-terminal amino acids are cleaved by renin to provide Ang I, which is the source for an array of active angiotensin peptides (see section later). Although the sequence of AGT protein is highly variable among species, the sequence of the 10-N-terminal amino acids corresponding to Ang I is relatively conserved (Streatfeild-James *et al.*, 1998). Human AGT protein contains four putative sites for N-linked glycosylation (Asn-X-Ser/Thr): Asn14, Asn137, Asn271, and Asn295. In vitro site-mutagenesis studies demonstrated that all four sites can be glycosylated, with preference at Asn14 and Asn271. Asn14 is close to the renin cleavage site (Leu10–Val11), and its glycosylation has been demonstrated to lower the affinity of AGT for renin (Gimenez-Roqueplo *et al.*, 1998). Interestingly, an intermolecular disulfide bond at positions 18 and 138 of human AGT (Cys18–Cys137 in the mice protein) is very much conserved across species and is predicted to expose the N-terminus of AGT by a redox-dependent mechanism (Zhou *et al.*, 2010). Renin cleavage efficacy can also be facilitated by interactions with domains beyond the N-terminal amino acids of AGT. In addition, the cleavage of AGT by renin is highly species specific.

The removal of Ang I from AGT leaves a protein termed des(AngI)AGT which exerts direct biological effects on renal function, blood–brain barrier, angiogenesis, adipose expansion, and liver steatosis (Lu *et al.*, 2016a; Wu *et al.*, 2011).

Genetic manipulations helped to clarify the roles of AGT. Whole-body AGT-deficient mice show low neonatal survival rate, impaired growth and renal development, and low blood pressure levels. Interestingly, mice with human AGT and renin transgenes also have less body weight gain compared with their wild-type controls, although these mice have increased AGT and renin expression, resulting in increased Ang II production. Therefore, the lean phenotype of these two mouse models cannot be explained by changes of Ang II production (Massiera *et al.*, 2001).

Interestingly, a whole-body reduction of AGT (hypoAGT mouse model) did not compromise neonatal survival rate, general growth, and kidney development. Unexpectedly, these mice have diminished body weight gain and liver steatosis when they are fed a diet that has a fat content (42% kcal from saturated fat) similar to the average dietary composition of Western nations (Lu *et al.*, 2016b).

The effects of AGT inhibition have been also explored using antisense oligonucleotides (ASO). ASO against AGT reduces intact AGT and des(AngI)AGT leading to reductions of total AGT (Wu *et al.*, 2011). Inhibition of AGT not only reduces blood pressure levels and atherosclerosis but also diminishes body weight gain. In agreement with AGT inhibition, renin inhibition reduces blood pressure and atherosclerosis; however, it has no effect on diet-induced obesity. These findings support the concept that AGT has both Ang II-dependent and -independent functions. The latter are yet to be understood.

Mouse studies have demonstrated that hepatocyte-derived AGT is a major functional component, with all studies reporting pronounced decrease in blood pressure in the model of hepatocyte-specific AGT depletion (Wu *et al.*, 2011). Moreover, mice with hepatocyte-specific, but not kidney-specific, depletion of AGT have diminished AGT accumulation in kidney proximal convoluted tubules (Wu *et al.*, 2011). These findings provide direct evidence that hepatocytes are the primary source for AGT production and functions in vivo.

Adipocytes can also synthetize AGT (Jones *et al.*, 1997). To determine the role of adipocyte-derived AGT, adipocyte-specific deletion of AGT was produced in mice with an initial evidence of reduction in plasma concentrations by 24%–28%, accompanied by reductions of blood pressure in the absence of effects on body weight, fat mass, adipocyte size, or glucose homeostasis (Jones *et al.*, 1997). Since no consistent findings were obtained in other studies, it is concluded that the adipocyte-derived AGT has modest or no discernable effects on obesity and blood pressure in mice.

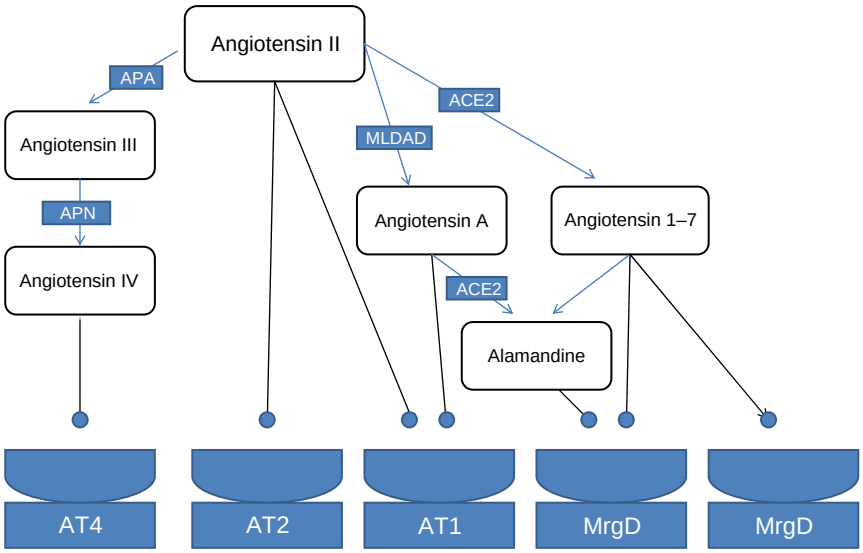

Fig. 1 Schematic representation of Ang II processing. Ang III is produced by aminopeptidase A (APA), and subsequently Ang IV is produced by aminopeptidase N (APN); Ang (1–7) is released by ACE2. Both Ang (1–7) and angiotensin A are precursor peptides of alamandine. The main receptors mediating the effects of the known angiotensins are represented.

Interestingly, the AGT gene has been explored in humans in relation with hypertension, obesity, and atherosclerosis through the investigation of genetic polymorphisms (single nucleotide polymorphisms, SNPs) in case-control association studies. In fact, genetic association studies have shown a significant association of M235T gene variant (that is related with plasma AGT concentrations (Corvol *et al.*, 1997)) with obesity in female hypertensive patients of different populations, although this variant failed to display any correlation in males (Procopciuc *et al.*, 2010; Cooper *et al.*, 1997). Controversial findings were also reported with regard to plasma AGT levels and obesity (Wu *et al.*, 2011). These inconsistent results support the experimental evidence that adipose-derived AGT is not a major contributor to AGT-mediated body weight changes. Two missense mutations, T174M and M235T, and multiple single-nucleotide polymorphisms, including A(-6)G and A(-20)C in the promoter region of the AGT gene, have been investigated in hypertension. However, inconsistent findings were obtained for all mentioned SNPs in association with hypertension (Jeunemaitre *et al.*, 1992; Larson *et al.*, 2000; Province *et al.*, 2000). The AGT gene has also been investigated in patients with atherosclerotic diseases. While a few studies failed to define an association of M235T with atherosclerosis, most studies demonstrated that this polymorphism was associated with atherosclerosis in different populations (Sethi *et al.*, 2003; Katsuya *et al.*, 1995). In addition to M235T, few studies have also reported that T174M, another polymorphism in exon 2 of the AGT gene, is related with risk factors or prevalence of coronary artery disease (Gardemann *et al.*, 1999). In conclusion, the causal relationship, if true, between SNPs in AGT gene and hypertension, atherosclerosis, or obesity remains to be established and the underlying mechanisms have still to be explained. Several confounding and limiting factors including small sample size, population heterogeneity, environmental and culture/ethic differences, multiple disease states, and complex interactions within the gene or with many other genes may complicate the interpretation of these AGT polymorphism studies.

Angiotensins

The cleavage of AGT by renin produces the decapeptide Ang I which is then cleaved by ACE1 to form the octapeptide Ang II, the most potent vasopressor of the RAS (Ferrario, 1990). This peptide, in turn, can be cleaved by both ACE2 to form angiotensin-(1–7) [Ang (1–7)] and by aminopeptidase A to form Ang III (angiotensin 2–8) (Ferrario *et al.*, 2010). The latter can be further processed by aminopeptidase N to obtain the exapeptide Ang IV (angiotensin 3–8) (Ahmad and Ward, 1990). ACE2 has approximately 400-fold less affinity to Ang I than to Ang II. Therefore, Ang II is the major substrate for Ang-(1–7) synthesis (Ferrario, 1990; Donoghue *et al.*, 2000; Tipnis *et al.*, 2000; Vickers *et al.*, 2002). ACE2 can also form Ang-(1–7) less efficiently through hydrolysis of Ang I to Ang-(1–9) with subsequent Ang-(1–7) formation (Vickers *et al.*, 2002; **Fig. 1**).

As a result, we now recognize the existence of four functional angiotensins: Ang II, Ang III, Ang IV, Ang-(1–7) (see **Table 1** and **Fig. 1**). Among them, Ang II, as part of the classic AGT/renin/ACE/Ang II/AT1R, is critical to maintain physiological cardiovascular, blood pressure, and renal homeostasis by the RAS. In fact, a balance between production and degradation of Ang II is of fundamental relevance to avoid cardiovascular, hypertensive, and kidney diseases. Moreover, a potential role of renin as a biomarker in cardiovascular diseases has been proposed (Volpe *et al.*, 2012).

Functions of the four angiotensins are mediated by four distinct receptors: AT1, AT2, AT4/RAP, Mas1.

Both Ang II and Ang III can bind and act through AT1 and AT2 receptors (AT1R, AT2R). However, Ang III has a preferential binding to AT2 receptor, although with a lower affinity than Ang II (Yatabe *et al.*, 2011; Kemp *et al.*, 2012). Interestingly, an AT3 receptor was reportedly cloned but it remains to be recognized (Unger *et al.*, 1996).

Table 1 Sequence of all known angiotensins

Angiotensin I	DRVYIHPFHL
Angiotensin II	DRVYIHPF
Angiotensin III	RVYIHPF
Angiotensin IV	VYIHPF
Ang-(1–7)	DRVYIHP
Alamandine	ARVYIHP
Angiotensin A	ARVYIHPF
Angioprotectin	PQVYIHPF

On the other hand, Ang IV acts on AT4/RAP (Chai *et al.*, 2004). The Mas1 is the receptor of Ang-(1–7) (Santos *et al.*, 2003). All receptors, but Mas1, are G-protein coupled receptors with seven transmembrane domains (Bader *et al.*, 2014). The AT4/RAP receptor is a transmembrane enzyme insulin-regulated membrane aminopeptidase (Chai *et al.*, 2004).

The AT1R signals through the calcium/inositol triphosphate pathway (Li *et al.*, 2006). The AT2R signals through the mitogen-activated protein kinase 1 (MAPK-1), the SHP-1, and protein phosphatase 2A that modulate the cyclic guanosine monophosphate/nitric oxide pathway (Kemp *et al.*, 2014; Carey, 2017). The AT4/RAP signals through an increase of intracellular Ca^{2+} concentration, the modulation of MAPKs, the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and production of cGMP (Albiston *et al.*, 2001). The signaling pathway of Mas1 is not yet fully understood.

The AT1R mediates vasoconstriction, thirst, release of vasopressin and of aldosterone, renal sodium reabsorption (with consequent increase of blood pressure levels), hypertrophy, proliferation and fibrosis, inflammation, angiogenesis, vascular aging, and atherosclerosis (Ferrario, 1990; Li *et al.*, 2006; Volpe *et al.*, 1995).

The AT2R mediates vasodilation, antiproliferative, antihypertrophic, antifibrotic, and antithrombotic effects (Carey, 2017; Albiston *et al.*, 2001; Matavelli and Siragy, 2015), and therefore plays opposing effects to AT1R. AT2R is mostly embryonic, with a decrease of its expression in adults where it is confined to certain organs such as the kidneys (Matavelli and Siragy, 2015). The binding of Ang III with AT2R leads to a rise of blood pressure, aldosterone release in adrenal cortex, renal and mesenteric vasoconstrictor effects that are less potent than those obtained with Ang II (Chai *et al.*, 2004). Of note, a series of studies support the hypothesis that Ang III is the predominant ligand for proximal tubule AT2 receptors and it induces natriuresis (Yang *et al.*, 2015). Interestingly, the Ang III/AT2R-dependent natriuresis may play a role in the presence of AT1R blockade (Simões e Silva *et al.*, 2013).

The Ang IV/AT4/RAP axis mediates NF-κB activation, interleukin 6 induction, and tumor necrosis factor α induction. In the brain, Ang IV has been shown to markedly enhance learning and memory in normal rodents and reverse memory deficits observed in animal models of amnesia (Chai *et al.*, 2004). In the kidney, Ang IV has been demonstrated to mediate a number of effects including increasing renal cortical blood flow and decreasing Na^+ transport in isolated renal proximal tubules (Chai *et al.*, 2004).

Finally, Mas1 receptor mediates vasodilation, endothelial function, antihypertrophic, antifibrotic, and antithrombotic properties of Ang-(1–7) (Simões e Silva *et al.*, 2013). The great functional relevance of Ang-(1–7) through the Mas receptor has been understood both in physiology and pathology. In fact, following its discovery, it is now proposed that, in addition to the ACE/Ang II/AT1 receptor branch, the RAS possesses a counter-regulatory axis composed by ACE2, Ang-(1–7) and the Mas receptor (Simões e Silva *et al.*, 2013). Whereas Ang II induces vasoconstriction, cardiac hypertrophy, renal vasoconstriction, aldosterone biosynthesis and release, anti-natriuresis, activation of the sympathetic nerve activity the ACE2/Ang-(1–7)/Mas exerts opposing effects and it antagonizes the deleterious effects of Ang II (Zhuo *et al.*, 2013). Thus, the net biological functions of the RAS may result from the balance between the arm of ACE/Ang II/AT1R and that of ACE2/Ang-(1–7)/Mas.

Activation of the ACE2/Ang-(1–7)/Mas axis decreases inflammation and fibrogenesis in diverse models of human diseases, and it exerts a protective role in experimental models of renal diseases (Zhuo *et al.*, 2013). The renoprotective effects of Ang-(1–7) seem to involve the vasodilation of afferent arterioles, an increase of renal blood flow, the modulation of oxidative stress, the production of nitric oxide and prostaglandins, the leukocyte influx and activation, and fibrosis in renal tissues (Pinheiro *et al.*, 2009; Li *et al.*, 2009). The few data provided by human studies also indicate a beneficial role for the activation of this alternative RAS axis in patients with renal diseases (Zhuo *et al.*, 2013). Finally, the beneficial effects of both ACEi and ARBs in renal diseases might involve, at least in part, the elevation of plasma Ang-(1–7) levels. AVE 0991 is a nonpeptide Ang-(1–7) agonist and it stimulates the Mas receptor to counteract the pathophysiological effects of AT1R activation (Santos and Ferreira, 2006; Rodrigues-Machado *et al.*, 2013). Moreover, A779 is a nonpeptide agonist and it inhibits the signaling of Mas producing unfavorable effects (Savergnini *et al.*, 2010).

Dipeptidyl peptidase III (DPP) III is a zinc-dependent aminopeptidase and acts to preferentially cleave two amino acids (dipeptide residues) from the N-terminus of few oligopeptides including Ang II and its metabolites Ang III and Ang IV (Pang *et al.*, 2016). Degradation of the vasopressor peptide Ang II by DPP III leads to the same cardiovascular, blood pressure, and renal protective effects induced by ACE inhibitors and AT1R blockers.

More recently, novel angiotensin-related peptides were discovered.

Alamandine is a heptapeptide derived from the catalytic hydrolysis of the octapeptide Ala1-Ang II (Ang A) by human ACE2 and structurally similar to Ang-(1–7), except that aspartic acid in position 1 of Ang-(1–7) is substituted with alanine in position 1 of alamandine (Lautner *et al.*, 2013). Alamandine can be generated from Ang-(1–7) in the rat heart and it circulates in human blood with increased levels in nephropathic patients (Lautner *et al.*, 2013).

Unlike Ang (1–7), which binds and activates the Mas receptor, alamandine binds and activates the MrgD receptor (Tetzner *et al.*, 2016). It exerts vasodepressor and antifibrotic effects in rats, although more studies are needed to assess its true role in physiology and pathology within the cardiovascular system.

A novel endogenous Ang II-like octapeptide, angioprotectin, was recently discovered. Compared with the sequence of Ang II, angioprotectin substitutes Asp1 and Arg2 in Ang II with Pro1-Glu2 raising the possibility that angioprotectin may be derived from Ang II (Jankowski *et al.*, 2011). Angioprotectin may represent an additional agonist for the Mas receptor. However, very few studies measured its levels in rodent and human plasma and tissues. Moreover, the molecular, biochemical and physiological, or pathological roles and therapeutic implications of angioprotectin remain to be further investigated.

Of note, kallikrein can cleave AGT within the heart to produce a dodecapeptide (Ang 1–12). Thereafter, chymase either produced in cardiac myocytes or released by mast cells during ischemia reperfusion/injury (I/R) or increased oxidative stress generates Ang II intracellularly from Ang-(1-12) (Ahmad *et al.*, 2011, 2013).

Assessment of RAS Activity in Physiology and Pathology

The changes of the RAS activity in the body still represent a valid diagnostic tool in human pathologies, with particular regard to the secondary forms of hypertension related to excess renin production (renovascular hypertension). The measurement of plasma renin activity (PRA) determines the amount of Ang I generated from AGT per unit time during incubation at 37°C of plasma samples in vitro (PRA is expressed as ng Ang I/mL/h). In contrast, the plasma renin concentration is the result of an immunoradiometric assay and it does not relate to the activity of renin. Plasma levels of aldosterone, the hormone stimulated by Ang II from the adrenal glands, can be measured by an Elisa kit and they can integrate information obtained with the PRA values.

Antisera raised against *N*-acetylated angiotensin (Ang) II and *N*-acetylated Ang III analogues were used to develop radioimmunoassays (Lawrence *et al.*, 1990). However, they do not offer a practical procedure for clinical purposes in order to measure plasma angiotensins in human samples.

Ang-(1–7) can be measured in plasma and urine samples collected in healthy subjects and in patients with hypertension (Simões e Silva *et al.*, 2013). Levels of Ang-(1–7) are modulated by changes in blood pressure, extracellular volume, sodium intake, and renal function. Low levels of Ang-(1–7) are associated with hypertension and cardiac hypertrophy (Simões e Silva *et al.*, 2013).

Therapeutic Implications of Angiotensins

Blockade of Ang II. This is a well-established and largely used therapeutic approach that has been introduced several years ago in clinical practice. The blockade of Ang II can be achieved by both the ACE inhibition and the AT1R antagonism with specific molecules (sartan). Their use in treating several cardiovascular diseases, with particular regard to hypertension, ischemic heart disease, heart failure, has significantly reduced major adverse cardiovascular events and mortality (Mentz *et al.*, 2013).

Stimulation of AT2R. Firstly, it has been shown that AT2R contributes to the vascular effects of the AT1R antagonists (Gigante *et al.*, 1998; Cosentino *et al.*, 2005). Due to the known beneficial effects of AT2R, through the stimulation of selected angiotensins, this approach promises to be helpful for treating human cardiovascular diseases (Volpe *et al.*, 2003). Two AT2R agonists are available. CGP42112A, a peptide agonist, induces renal natriuretic responses via AT2R-mediated NO/cGMP signaling, lowers blood pressure in SHR, and inhibits VEGF-induced migration in human endothelial cells (Kumagai *et al.*, 1993). A nonpeptide AT2R agonist, compound 21, has been developed to treat pulmonary fibrosis, hypertension, renal failure, and acute ischemic stroke (Unger and Dahlöf, 2010). The available evidence suggests that this nonpeptide AT2R agonist is not an optimal antihypertensive agent but, through its antifibrotic and antiinflammatory actions, may help to prevent and treat hypertensive-related TOD (Steckelings and Unger, 2012).

Ang-(1–7) as a therapeutic agent. Due to its favorable effects, it is expected that this peptide could help in antagonizing the harmful effects of Ang II. In this regard, a mimetic of Ang-(1–7), the nonpeptide compound AVE0991, appears to behave as a Mas agonist. In vitro studies revealed that it was able to induce NO release from endothelial cells (Santos and Ferreira, 2006). In vivo, AVE0991 exerted protection from postischemic heart failure in rats, prevented diabetes-induced cardiovascular dysfunction, inhibited atherogenesis in ApoE-knockout mice, and attenuated cardiac hypertrophy (Rodrigues-Machado *et al.*, 2013). However, this compound has never been tested in humans.

See also: Renin and Prorenin. Kininases. Hypertension and the Renin–Angiotensin–Aldosterone System. Hyperreninemia

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Kininases

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Glossary

Autocrine A hormone that affects the action of the cell that produced it.

Paracrine A hormone that affects the function of a cell that is near but different from the one that produced it.

Kininases are enzymes that cleave peptide bonds in the kinins, bradykinin (BK) and kallidin (Lys-BK). Kininases can be classified according to their structure or, more importantly, according to their function. Thus, the first distinction should be whether the enzyme cleaves a kinin *in vivo* or whether its activity has been characterized only *in vitro*. This has not been uniformly established for all enzymes. Since Lys-BK has nine peptide bonds and BK has eight peptide bonds, many enzymes ranging from serine proteases to metallopeptidases can hydrolyze one or more of the bonds.

Introduction

The term "kininases" is a catch-all term. By 1970, a list of reports on kininases in extracts, tissues, and cells had spread over three pages in the Handbook of Experimental Pharmacology.

BK and Lys-BK are products of the hydrolysis of plasma kininogen by kallikreins as a result of a cascading process. This involves the initial activation of plasma prokallikrein, which releases BK from kininogen, or of tissue prokallikrein, which in turn usually releases Lys-BK. Kinins are agonists of the so-called BK B_2 receptor. This is a seven-transmembrane, G protein-coupled receptor activated by kinins, provided that they have the intact Phe^8-Arg^9 C-terminus. The group of enzymes that inactivates kinins by cleaving the Phe^8-Arg^9 bond, and thereby releasing a single arginine, is collectively called kininase I. However, the discovery of a second kinin receptor showed that this issue is much more complex. This second receptor, B_1, reacts with kinins lacking the C-terminal Arg (des-Arg derivatives). Consequently, kininase I-type enzymes inactivate kinins only as ligands of the B_2 receptor, whereupon they become active agonists of the B_1 receptor.

In contrast to the widely distributed and constitutively expressed B_2 receptor, B_1 is usually induced only under noxious or inflammatory conditions by endotoxins, cytokines, and other agents. B_1 is also a seventransmembrane domain G protein-linked receptor but shares only 36% homology with the B_2 receptor.

Enzymes belonging to a second group of kininases are called collectively kininase II. These enzymes act as peptidyl dipeptidases because, instead of releasing a single amino acid from a kinin, they release the Cterminal dipeptide (Phe-Arg) and thereby completely inactivate it. Angiotensin (Ang) I-converting enzyme (ACE) and neprilysin (NEP, neutral endopeptidase 24.11) are important members of this group.

Kininase I (Carboxypeptidase N)

Historically, carboxypeptidase N (CPN) was the first kininase characterized. Shortly after the synthesis of BK, an enzyme that released the C-terminal Arg was found in human plasma. CPN activity was studied in the plasma of several hundred patients; it was elevated in pregnancy but was below normal in patients with cirrhosis of the liver. CPN is synthesized and released from the liver into the circulation, where it is sustained at a relatively high level (10^7 M). This constitutive activity is probably more important for hydrolyzing substrates other than kinins, such as anaphylatoxins.

The circulating enzyme is a complex protein consisting of two heterodimers of high- and low-molecularweight subunits (83 and 50 kDa, respectively), held together by noncovalent forces to form a tetramer. The high-molecular-weight regulatory subunit is heavily glycosylated (36%), whereas the low-molecular-weight subunit contains the active center with a $Zn^{2þ}$-binding site. The regulatory high-molecularweight subunit can be an allosteric modifier of the active center of the low-molecular-weight subunit. It keeps the 50 kDa subunit in the circulation and protects this sensitive protein against inactivation at 37°C.

CPN is also an anaphylatoxin inactivator because it cleaves the C-terminal Arg of C3a, C4a, and C5a, but their rates of hydrolysis differ. The Gly-Arg bond of C5a is cleaved approximately 10 times slower than the Ala-Arg terminus of C3a.

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Human CPN is considered to be life-sustaining, since no individuals whose blood completely lacks this enzyme are known. For example, inhibition of CPN activity in guinea pigs resulted in sudden death after complement activation when the generated anaphylatoxins were not inactivated.

In humans, low enzymatic activity may be important in the protamine-reversal syndrome. Protamine, when given to neutralize the effects of heparin after extracorporeal circulation, can trigger a catastrophic reaction: pulmonary vasoconstriction, bronchoconstriction, and systemic hypotension. Protamine is a potent inhibitor of this carboxypeptidase (CP) and can also stimulate the generation of kinins and anaphylatoxins; consequently, the inhibition of anaphylatoxin or kinin inactivation may contribute to the protamine reversal syndrome.

Plasma CPN activity may be an aid in the diagnosis of myocardial infarction where the blood level of creatine kinase (CK) released from heart muscle is measured. CPN cleaves the C-terminal lysine residue from either the M or B subunit of CK to yield des-Lys MM or MB isoforms when separated electrophoretically. A variation in blood carboxypeptidase N activity may affect this CK isoform ratio.

Although CPN cleaves the C-terminal Arg of kinins (see Fig. 1), it preferentially hydrolyzes C-terminal Lys, an important function in the regulation of plasminogenactivation. CPNcanreduceplasminogen binding to cells because C-terminal lysine residues on cell surface proteins are plasminogen "receptors". As bound plasminogen is more efficiently activated, CPN effectively lowers plasminogen binding to cells and thereby influences plasminogen activation.

No individuals have been reported to completely lack CPN but a 65-year-old patient and his sister had only 20% of normal activity. He suffered from weekly occurrence of angioedema. Other members of the family had angioedema, urticaria, hay fever, and asthma, but only slightly depressed CPN levels. An analysis of the 50 kDa subunit genomic DNA from the 65-year-old patient revealed a frameshift mutation in exon 1 that was not present in any of 128 normal controls.

Carboxypeptidase U

Carboxypeptidase U (CPU; thrombin activatable fibrinolysis inhibitor, TAFI; plasma CPB; CPR; EC 3.4.17.20) is another B-type CP synthesized in the liver and secreted into the blood. In contrast to CPN, it is normally in plasma as an inactive proenzyme of 60 kDa bound to plasminogen. CPU is proteolytically activated during coagulation to the active 35 kDa form by the thrombin–thrombomodulin complex, but it is unstable and rapidly inactivated at 37°C or by further proteolytic cleavage. The primary role of CPU is to down-regulate plasminogen binding and activation by cleaving C-terminal Lys residues from cell surface proteins and in fibrin clots.

Regarding the potential function of CPU as a kininase, one of the first groups to identify the enzyme utilized bradykinin as a substrate and studies of the purified enzyme confirmed these findings. However, the reported K_m of bradykinin is extraordinarily high (10 mM), even though it was lower than that of all other substrates (K_m ¼63–290 mM). This brings into question the physiological relevance of CPU as a kininase, although serum degrades bradykinin fivefold faster than can be accounted for only by CPN. Because CPU is normally a proenzyme and is unstable after activation, CPU may be a significant kininase only in certain circumstances, such as shortly after initiation of the coagulation cascade.

Carboxypeptidase M

Early studies indicated that tissues and urine contained CPN-like activity, but it was assumed that the 50 kDa active subunit would enter organs and attach to plasma membranes or be excreted. When the active CP that cleaved basic C-terminal amino acids was

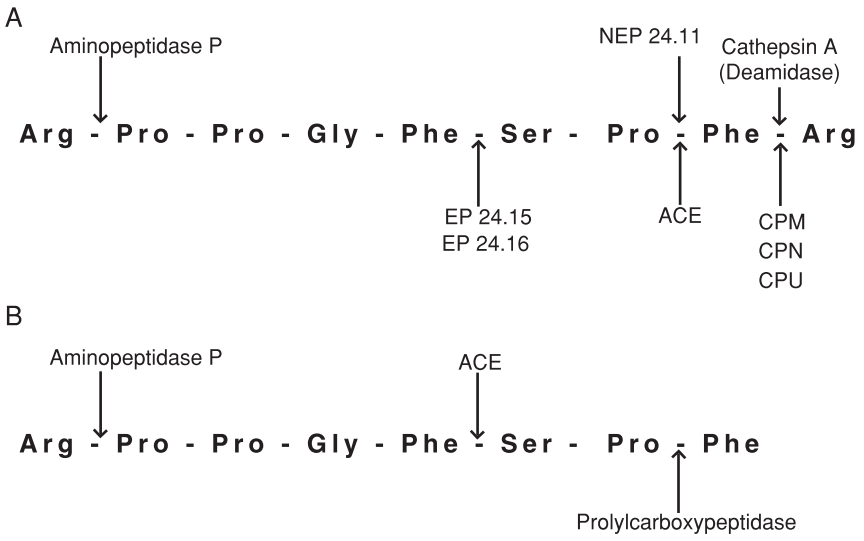

Fig. 1 Bonds hydrolyzed in bradykinin (A) and des-Arg^9-bradykinin (B) by peptidases.

purified from human urine and from placental membrane fractions, it became clear that this was not the case. Purification, cloning, and engineering of recombinant enzyme revealed that this enzyme had a different specificity and structure than those of CPN. Owing to its membrane attachment, it was called carboxypeptidase M (CPM).

CPM is a glycoprotein (23% carbohydrate by weight) with a molecular weight of 62 kDa anchored to the plasma membrane via a glycosylphosphatidylinositol tail. CPM is present in many tissues including kidney, lung, placenta, brain, intestine, peripheral nerves, and blood vessels and in cultured cells such as endothelial cells, fibroblasts, and Madin-Darby canine kidney cells. It is a differentiation-dependent cell surface antigen on white blood cells. The enzyme can be released from the membrane and soluble CPM is found in various body fluids including amniotic fluid, seminal plasma, and urine.

CPM has a pH optimum in the neutral range (6.5–7.5) ideally suited to cleaving peptides at the cell surface. There are no known endogenous inhibitors of the enzyme; thus, it is considered to be constitutively active on the cell surface. Tested with a variety of synthetic and endogenous peptides, CPM cleaved C-terminal arginine in preference over lysine, but the penultimate amino acid residue can also dramatically affect the rate of hydrolysis. The naturally occurring peptide substrates of CPM include bradykinin, $Arg^6$and Lys^6-enkephalins, dynorphin A_{1-13}, and epidermal growth factor (EGF). Of the substrates tested, bradykinin has the lowest K_m (16 mM) and the second highest specificity constant (k_{cat}/K_m).

As a widely distributed ectoenzyme, CPM can participate in a variety of processes, such as control of peptide hormone activity at the cell surface and degradation of extracellular proteins and peptides. It may also carry out the second step in prohormone processing, (i.e., removal of C-terminal Arg or Lys residues from peptides released from prohormones by convertases), if and when incompletely processed peptides are released from secretory granules. Because many peptides work in an autocrine or a paracrine fashion, the location of CPM on the plasma membrane near peptide receptors makes it well suited to control their activities locally.

Within the kinin system, CPM can either inactivate or alter the specificity of kinin peptides by cleaving the C-terminal Arg^9 as mentioned above for CPN. Because CPM is localized on the cell surface, close to the B1 and B2 receptors, it is well suited to this important role. Indeed, it has been shown that CPM-like activity on the surface of human lung microvascular endothelial cells was up-regulated approximately twofold by treatment with interleukin-1β and interferon-γ; these conditions increased B_1 receptor responses approximately fourfold. When BK stimulated these cells, the resulting nitric oxide release was prolonged and enhanced because CPM converted the peptide to des-Arg^9-BK to activate the B_1 receptor. These effects were then blocked by a CP inhibitor or by a B_1 receptor antagonist.

CPM efficiently removes the C-terminal Arg from EGF and may also cleave other growth factors that contain a C-terminal Arg or Lys [e.g., other EGF-like peptides, nerve growth factor (NGF), amphiregulin, hepatocyte growth factor, erythropoietin, macrophage-stimulating protein]. Although the role of the C-terminal basic residue of these factors has not been investigated in detail for most of them, a C-terminal Arg residue is required for association of EGF and NGF with their respective binding proteins.

The role of the up- or down-regulation of CPM in the differentiation of monocytes to macrophages and of B_1 lymphocytes is unknown, but a subject to be investigated further. The released free Arg, as a substrate for the inducible nitric oxide synthase, may provide more nitric oxide in inflammation, possibly just as the related enzyme carboxypeptidase D does in a mouse macrophage cell line.

In the lung, the presence of CPM on type I cells, which constitute 93% of the total alveolar surface, indicates that it may have protective functions here. This enzyme is also readily mobilized from the cell surface. The pulmonary synthesis or release of CPM may be up-regulated in disease states as the enzyme levels in bronchoalveolar lavage fluids were elevated almost fivefold in patients with pneumocystic or bacterial pneumonia or lung cancer.

The functions of CPM in other locations have not been explored. For example, in the placenta it may protect the fetus from maternally derived peptides. CPM in central nervous system (CNS) myelin and in Schwann cells in peripheral nerves may participate in the growth or maintenance of neurons.

Kininase II

Kininase II (ACE)-type activity, the enzymatic release of Phe^8-Arg^9 from BK, was detected first with a *Clostridium histolyticum* preparation and then by an enzyme separated from a membrane-enriched fraction (microsomal) of homogenized kidney and also from human plasma. Originally, ACE was discovered by Skeggs and colleagues in the 1950s in horse plasma. It converted the inactive Ang I decapeptide released by renin – called at that time hypertensin or angiotonin – to the active vasoconstrictor octapeptide Ang II.

After the discovery of kininase II in 1965–1967, it was reported in 1970–1971 that the same enzyme cleaves both BK and Ang I by releasing Phe-Arg from BK and His-Leu from Ang I; thus, it is a peptidyl dipeptide hydrolase called peptidyl dipeptidase I. The conclusion that the two activities were due to the same protein was not readily accepted because different amounts of Cl ions are needed to cleave Ang I or BK. The lack of chloride inhibited Ang I conversion by 93%, but the hydrolysis of BK was reduced by only approximately 50%. The discovery of two independent active site domains of ACE led to the finding that some substrates and inhibitors react preferentially with just one of the two active sites, with different Cl requirements.

The K_m of BK is much lower than that of Ang I (0.1–1 μM versus 10–50 μM), which, taken with the turnover number (k_{cat}), indicates a higher specificity constant with BK than with Ang I for human ACE; generally, ACE (kininase II) is considered to be the most important human kininase.

In addition to Ang I and BK, ACE cleaves a variety of bioactive peptides *in vitro*. Thus, beyond hydrolyzing C-terminal dipeptides, it released *in vitro* Cterminal tripeptides, e.g., from des-Arg^9-BK, protected C-terminal tripeptides [e.g., from substance P, luteinizing hormone-releasing hormone (LHRH)], protected C-terminal dipeptide (e.g., substance P), and even protected

N-terminal tripeptide (e.g., of LHRH). Compared to other ACE substrates, it is likely that the Arg^6-Phe^7 of Met^5-enkephalin-Arg^6-Phe^7 is split off with the highest turnover number by the N-domain active center of ACE. The N-domain of ACE hydrolyzes *in vivo* the tetrapeptide Ac-Ser-Asp-Lys-Pro, which is involved in the control of hematopoietic stem cell proliferation by preventing their recruitment into S phase.

After molecular cloning of the somatic form of ACE, two homologous domains were identified, the N- and C-domains; this was taken as an indication for the duplication of an ancestral gene. The germinal, testicular form has only one domain. The highest (89%) sequence similarity of the two domains of human ACE occurs around the active sites. The catalytic sites contain the canonical zinc-binding motif HEXXH. There is a high level of sequence similarity, 80%–90%, in both the nucleotide and amino acid sequences, between the somatic mammalian ACE cDNA sequences cloned from different species.

ACE is an ectoenzyme anchored to the plasma membrane by a C-terminal transmembrane domain; most of it is exposed at the extracellular surface of the cell. There are two ACE isoforms; the somatic form of approximately 150–180 kDa is in endothelial, epithelial, and neuronal cells and the smaller isoform (90–110 kDa) is present in germinal cells. Germinal ACE contains 732 amino acids, compared to 1306 in somatic ACE, and corresponds to the C-domain of somatic ACE with a single active site. Germinal ACE has the same hydrophobic transmembrane peptide and cytosolic domains as somatic ACE. The inactivation of the ACE gene in the homozygous male mouse leads to reduced fertility attributed to inactivation of the germinal form of ACE. Somatic ACE is highly expressed, for example, in the plasma membrane of vascular endothelial cells in the lung oriented to metabolize circulating substrates. ACE concentration is probably even higher on the microvilli of the brush borders of absorptive epithelia of the small intestine, placenta, and kidney proximal tubules. T lymphocytes and fibroblasts also contain ACE. In brain, ACE is highly expressed in the choroid plexus, which may be the source of ACE in cerebrospinal fluid, and in ependyma, subfornical organ, basal ganglia (caudate putamen and globus pallidus), substantia nigra, and pituitary. Neuronal ACE and the endothelial and epithelial forms of the enzyme have the same specificities and are not distinct isoenzymes.

Soluble ACE is found in many biological fluids such as serum, seminal, amniotic, and cerebrospinal fluids, released from cell membranes by a class of enzymes called secretases.

The high carbohydrate content of both ACE domains has frustrated attempts to crystallize the enzyme. Finally, in 2003, the crystallization of the human testicular ACE, and thus, the C-domain of ACE without the hydrophobic transmembrane and intracellular domains, was reported. The enzyme was complexed with the inhibitor lisinopril. Although the first orally active ACE inhibitor, captopril, was synthesized on the basis of an assumed similarity to carboxypeptidase A, the structure of the crystallized enzyme resembled instead the other kininase II-type enzyme NEP, despite the lack of sequence homology. The active site is at the bottom of a groove in ACE; this may explain the rather limited size of the peptides that ACE can hydrolyze, consisting usually of less than a dozen amino acids.

Because both CPN and ACE are metallopeptidases, in initial experiments some inhibitors of BK breakdown by CPN, such as metal sequestering agents, inhibited both enzymes. Of other ACE inhibitors, the structures of two snake venom peptides that potentiated BK were reported at a meeting in 1969. The pentapeptide BPP_{5a} from *Bothrops jararaca* had a single C-terminal proline, whereas the undecapeptide from *Agkistrodon halys blomhoffii* had a C-terminal Pro-Pro sequence. A synthetic nonapeptide, teprotide – found in snake venom originally – also had C-terminal ProPro sequence and it became the first ACE inhibitor to be tested clinically. When this ACE inhibitor was given intravenously to patients, it not only lowered the elevated blood pressure, but was also beneficial in congestive heart failure.

The studies on kininase inhibition and BK potentiation led to the synthesis of ACE inhibitors that are given orally to millions of patients to treat hypertension and a variety of clinical conditions in which the effects are not related to lowering the blood pressure. These conditions range from congestive heart failure to diabetic nephropathy. ACE inhibitors lower the mortality and morbidity after heart failure and they even reduce its incidence. Many laboratory and clinical findings attributed at least part of the results with ACE inhibitors to enhancing the effects of BK. In addition to being involved in BK metabolism and potentiating the effects of B_2 agonists via ACE, some of the ACE inhibitors can directly activate the B_1 receptor of des-Arg-BK followed by the release of nitric oxide, in the absence of the peptide ligand in cultured endothelial cells.

The Angiotensin Metabolites

Some of the Ang metabolites, enzymatic breakdown products (see **Fig. 2**), are active on their own. Some of them mimic Ang II, whereas others antagonize the effects of Ang II. Two of them are mentioned here because they potentiate BK, probably by interacting with ACE, but not necessarily acting as inhibitors only.

Ang (1–7) (des-Phe^8-Ang II) is a substrate of the Ndomain, but an inhibitor of the C-domain of ACE. Another enzymatic product of Ang I hydrolysis, Ang (1–9) (des-Leu^{10}-Ang I), inhibits both domains of ACE; both Ang (1–7) and Ang (1–9) can enhance BK's effect on the B_2 receptor.

Serine Carboxypeptidases

A serine carboxypeptidase-type enzyme, called deamidase, cathepsin A, lysosomal protective protein, or lysosomal CPA, also cleaves BK at the Phe^8-Arg^9 bond as shown *in vitro*. This enzyme has an acid pH optimum for CP and esterase activities, but deamidates amidated C-terminal amino acids (e.g., substance P) at neutral pH.

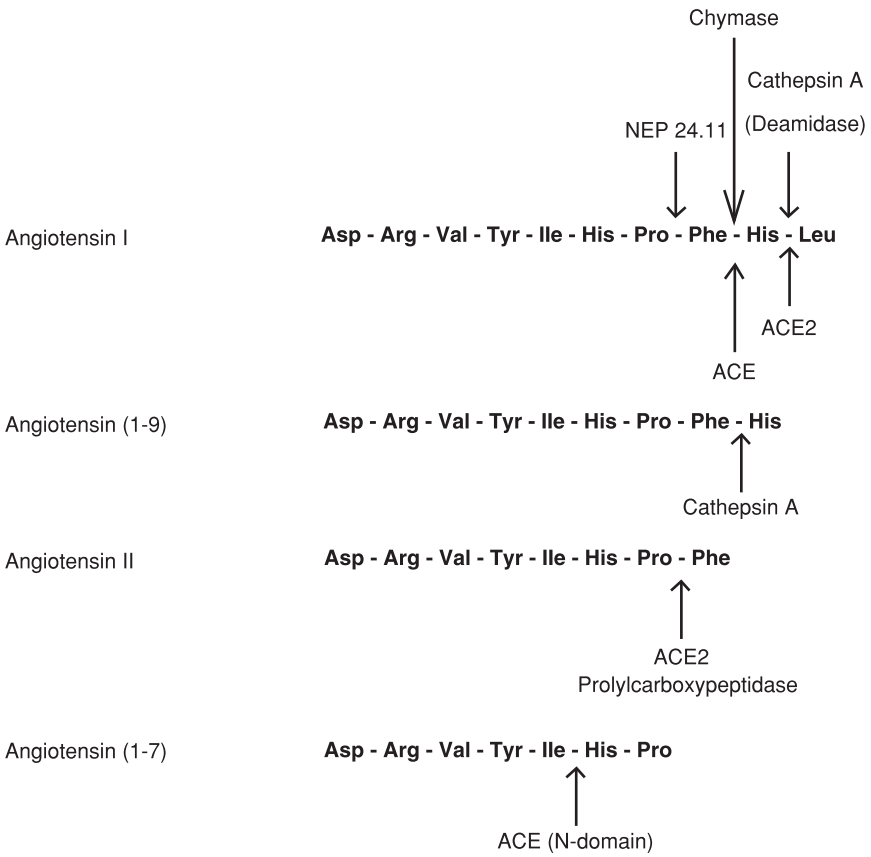

Fig. 2 Peptide bonds hydrolyzed in angiotensins by peptidases.

Another serine CP, the lysosomal Pro-X CP (prolylcarboxypeptidase or angiotensinase C), inactivates desArg^9-BK, the ligand of the B_1 BK receptor, at the Pro^7-Phe^8 bond (see **Fig. 1**). Prolylcarboxypeptidase occurs in abundance in neutrophils; this may have some bearing on B_1 receptor activity, as it is upregulated by agents promoting inflammation. Thus, this enzyme may be an inactivator of the B_1 receptor agonists. The same bond is also hydrolyzed in Ang II both by Pro-X-CP and by an enzyme called ACE2 with sequence similarities to ACE, but it is not inhibited by ACE inhibitors (**Fig. 2**).

Aminopeptidases

Aminopeptidases have two different functions in kinin metabolism. A blood-borne aminopeptidase, present also in tissues, converts Lys-BK to BK by cleaving the Lys^0-Arg^1 bond. A second aminopeptidase, called aminopeptidaseP,butoriginallyreferredtoas "prolidase", is presentinerythrocytes,kidney,lung,andothertissues.It inactivates BK by cleaving at Arg^1-Pro^2. Rat tissues have the-highestconcentrationsofthisenzyme;humantissues apparently express much less aminopeptidase P.

In contrast to ACE and NEP, which are bound by a transmembrane peptide as type I and type II transmembrane enzymes, aminopeptidase P is membranebound via a glycosylphosphatidylinositol anchor. Its molecular weight is approximately 90–95 kDa. As with some other peptidases, it forms dimers or even oligomers. Aminopeptidase P, a zinc metallopeptidase, can be activated by Mn^{2+} with some substrates and is inhibited by chelating agents. Other inhibitors include sulfhydryl compounds. Interestingly, many ACE inhibitors also inhibit aminopeptidase P, although with a higher K_i (in the micromolar range) and Mn^{2+} enhances this inhibition. This is likely to be due to the presence of Pro or Pro-like structures in the ACE inhibitors and to the effective zinc-binding moieties.

Neutral Endopeptidase 24.11

Neutral endopeptidase 24.11 (neprilysin, NEP) is a zinc metallopeptidase with a single active site containing the HEXXH sequence to bind Zn^{2+}. The enzyme is a single-chain protein of 742 amino acids and, as a type 2 membrane protein, is bound via an uncleaved N-terminal signal peptide.

NEP is distributed widely; its expression in vascular endothelial cells is low, but epithelial cells, especially in microvillar structures, are rich in NEP. Its presence in the CNS has been investigated in detail because neuropeptides are among its substrates.

The relevance of the high concentration of NEP in the male genital tract, especially in prostate gland, is not known. NEP under the name "common acute lymphoblastic leukemia antigen" (CALLA or CD10) is present in lymphoblasts and also in neutrophils. Of the solid tumors, NEP is highly expressed in malignant liver cells of rats and humans.

NEP cleaves peptides at the N-termini of hydrophobic amino acids and as such it is a second kininase II-type enzyme that releases the C-terminal Phe^8Arg^9 of BK. It was first discovered as an endopeptidase that cleaves the B-chain of insulin, but its substrates include enkephalins, endothelin, atrial natriuretic peptide, substance P, and a chemotactic peptide.

Purified human NEP was used to establish the kinetics of hydrolysis of BK. The k_{cat} for BK is higher with NEP than with ACE but because of the much higher K_m (120 mM versus 0.2 mM) the specificity constant, k_{cat}/K_m, of NEP is lower (40 μM^{-1} min^{-1} versus 3667 μM^{-1} min^{-1}).

The NEP level in circulating blood plasma is normally low, but increases 60- to 80-fold in adult respiratory distress syndrome with septic pneumonia. Inhibition of NEP in rat bronchial epithelium markedly enhances bronchoconstriction induced by substance P and BK. BK-induced bronchoconstriction in asthmatic patients is augmented by NEP inhibitors. Indirect actions of BK are also influenced by NEP inhibition, because when BK releases substance P from nerve endings, it is subsequently cleaved by NEP.

Microvascular leakage in guinea pig airways and enhanced myocardial blood flow after sensory nerve stimulation in the rat heart were potentiated by NEP inhibitors, possibly through blocking BK inactivation.

An NEP inhibitor has been used to successfully treat young boys suffering from acute watery diarrhea. The results were attributed to blocking the breakdown of endogenous enkephalins acting on δ-opiate receptors.

Because of the increased reactivity to the combined administration of inhibitors of two peptidases, several compounds that inhibit both NEP and ACE, or ACE and aminopeptidase, have become available. Extensive reports deal with the clinical and laboratory experimental results with the dual ACE and NEP inhibitors, for example, with omapatrilat (Vanlev).

Endothelin-converting enzyme, an NEP-related peptidase with some sequence similarity, also cleaved BK at the Pro^7-Phe^8 bond, albeit with a K_m approaching 1 mM.

Endopeptidases 24.15 and 24.16

Endopeptidase 24.15 (EP24.15; thimet oligopeptidase) and endopeptidase 24.16 (EP24.16; neurolysin) are closely related (80% similarity and 63% identity in amino acid sequences) zinc metalloenzymes that contain the HEXXH zinc-binding motif. They are active atneutral pH and cleave a number of biologically active peptides *in vitro*. Both enzymes are abundant in brain and have been purified from rat brain homogenates.

EP24.15 is a 78 kDa protein containing 687 amino acid residues and is expressed at highest levels in brain and testis. When EP24.15 was purified from rat brain, it was determined that the enzyme was identical to two other enzymes named earlier, endopeptidase A, originally described as a kininase, and Pz-peptidase, which cleaved a synthetic substrate of collagenase. EP24.15 is activated by low concentrations of thiols, which convert an inactive multimer, with a blocked catalytic site, to an active monomer by disruption of intermolecular disulfide bridges. EP24.16 does not have these –S–S– bridges.

Tissues such as spleen, liver, kidney, lung, adrenal, and thyroid also contain EP24.15 activity. Although the enzyme apparently lacks both a signal sequence and a membrane-binding motif, an estimated 10%–25% of activity is associated with the plasma membrane fraction of a number of tissues.

EP24.16 is a 704-amino-acid protein that, like EP24.15, is present in both plasma membraneassociated (10%–20% of activity) and soluble forms. In addition, EP24.16 is found in mitochondria. After EP24.16 was isolated from rat brain and sequenced, its identity to some other proteins, including a soluble Ang II-binding protein from porcine liver and a microsomal endopeptidase from rabbit liver, was revealed. The enzyme has also been purified from rat ileum and kidney and a variant, called EP24.16B, has been isolated from rat testis.

Both enzymes share most of their natural substrates and preferentially cleave them at the carboxyl side of hydrophobic amino acids. BK is hydrolyzed at the Phe^5-Ser^6 bond. Other peptides,such as AngI, opioids, gonadotropin-releasing hormone, substance P, and neurotensin, are also cleaved *in vitro* by both of the enzymes. These substances are hydrolyzed at the same bond by both enzymes, except for neurotensin, which is inactivated at Arg^8-Arg^9 by EP24.15 and at the Pro^{10}-Tyr^{11} bond by EP24.16.

Because of the predominantly cytosolic nature of these two enzymes, their roles as kininases are not clear. With the advent of specific, biologically stable enzyme inhibitors, investigators found that the enzymes can be kininases. An inhibitor of both EP24.15 and EP24.16 potentiated the increase in permeability of pial (brain surface) microvasculature induced by BK *in vivo*. This suggests that one or both enzymes can moderate BK's effect on the blood–brain barrier, possibly in cases of inflammation.

The formation of BK 1–5 in the medium of cultured bovine aortic endothelial cells after incubation with BK was inhibited 45% by an EP24.16 inhibitor and 18% by an EP24.15 inhibitor. Thus, even a transiently extracellular location of these enzymes on endothelial cells would allow them to function as kininases in the vasculature.

Epilogue

The very short half-life of kinins *in vivo* (seconds) pointed to the importance of their enzymatic metabolism but the acceptance of this conclusion took decades. It also follows that limited conclusions can be deduced from measuring circulating kinin levels, as

kinin's actions are more likely paracrine or autocrine in nature. Only a long time after the discovery of the "original" kininase, CPN, were some of its functions in the body, for example, on plasminogen activation, clarified.

The induction of so-called Chagas' heart disease by *Trypanosoma cruzi* has been found to be promoted by BK in mice. It was suggested that changes in kininase levels can affect parasite infectivity and thus the outcome of the disease.

ACE inhibitors, which have become widely used, originated from studies performed *in vitro*. The obvious consequence of the seemingly opposite, dual actions of kininase II or ACE on BK and Ang I was that its inhibitors block the release of Ang II while they can enhance the effect of BK. Because related peptidases cleave different active peptides, compounds that inhibit both ACE and NEP when tested clinically could block Ang I, BK, and atrial natriuretic peptide hydrolysis.

See also: Angiotensinogen and Angiotensins. Renin and Prorenin

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Tissue Ace–Angiotensin–AT_1 Receptor Axis and Repair in the Heart☆

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Introduction

The circulating renin–angiotensin–aldosterone system (cRAAS) is integral to maintaining cardiovascular homeostasis (Chappell *et al.*, 2000; Erdös, 2001). Pharmacologic inhibition of angiotensin-converting enzyme (ACE) and antagonism of angiotensin (Ang) II and aldosterone (ALDO) cognate receptor binding have proven effective strategies in the management of resistant hypertension and heart failure. They further have provided important insights into the pivotal contribution of cRAAS activation to the pathophysiologic expression of these disorders (Francis *et al.*, 1990; Pitt *et al.*, 1999; Rouleau *et al.*, 1993; Swedberg *et al.*, 1990; Weber, 2001; Weber and Brilla, 1991). ACE inhibition leads to risk reduction for morbid and mortal events, including the progressive nature of heart failure (The SOLVD Investigators, 1991, 1992). More recently, a tissue fibrogenic ACE–angiotensin–AT_1 receptor axis has been recognized together with a counterregulatory fibrolytic ACE2/Ang1–7/MasR axis. The role of each in regulating repair in diverse tissues has received considerable interest (De Mello, 2017; Hammer *et al.*, in press; Kim *et al.*, 2016; Madro *et al.*, 2016; Morales *et al.*, 2016; Pawlik *et al.*, 2016; Rivoli *et al.*, 2016; Slamkova *et al.*, 2016; Wang *et al.*, 2012; Weber *et al.*, 2013). Herein, we focus on these fibrogenic and fibrolytic axes as they relate to the diseased heart.

Tissue Fibrogenic Ace/ANGII/AT_1R Axis

The number of ACE transcripts is increased in tissue homogenates prepared from the explanted failing human heart (Studer *et al.*, 1994). ACE and TGF–β_1 expression are each increased in myocardium obtained from patients undergoing aortic valve replacement for aortic stenosis, where they correlate with the extent of fibrosis (Hein *et al.*, 2003). In autopsied hearts, ACE and AngII are present in the fibrous tissue that contributes to human aortic sclerosis (O'Brien *et al.*, 2002). ACE-dependent AngII formation is increased in the infarcted myocardium (Ihara *et al.*, 2000), while the expression of angiotensinogen, renin, ACE, and AngII receptor genes is upregulated in cardiomyocytes in response to injury (Anversa *et al.*, 2000).

Temporal and spatial responses in autoradiographic ACE binding have been assessed in a rat model of MI, where other forms of injury are also present including the pericardium, kidneys, and incised skin. Each tissue serves as positive control in the analysis of ACE and tissue repair. Only low-density ACE binding is present in normal rat myocardium, where renin mRNA expression is not found (Sun *et al.*, 2001). High-density ACE binding and renin mRNA expression, on the other hand, appear at the site of MI and are coincident with the appearance of scar tissue. As scar tissue forms, the density of renin expression and ACE binding, AT_1R, TGF–β_1, and type I collagen (see panels A and F, **Fig. 1**) increase progressively. A large transmural MI is associated with high-density ACE binding and the appearance of fibrosis at sites remote to the infarct, including the noninfarcted left ventricle (LV), interventricular septum, and right ventricle (RV), where the appearance of fibrosis is directly related to the extent of infarction (Smits *et al.*, 1992; van Krimpen *et al.*, 1991; Volders *et al.*, 1993). With a large infarction, infarcted and noninfarcted LV are involved by fibrosis tissue.

Noninfarct-related sites of injury serve to further address the relationship between the appearance of ACE and repair. Sham operation includes manual handling of the heart leading to inflammation and subsequent pericardial fibrosis. Silk ligature placement around the left coronary artery or into skin for closure, a foreign-body fibrosis, appears at each site. At each site high-density autoradiographic ACE binding is temporally and spatially concordant with fibrous tissue formation. Angiotensinogen, renin, ACE, and TGF–β_1 mRNA levels are increased in the rat RV after monocrotaline-induced pulmonary injury (Park *et al.*, 2001).

Nonischemic models of cardiac myocyte necrosis also have been examined. They include endogenous release of catecholamines that accompanies AngII infusion from implanted minipump (Ratajska *et al.*, 1994) or exogenous administration of isoproterenol (Benjamin *et al.*, 1989) and chronic (4 weeks) administration of aldosterone by minipump in uninephrectomized rats on a 1% salt diet (Campbell *et al.*, 1993; Darrow and Miller, 1942). At each site of nonischemic myocyte loss, and irrespective of its etiologic basis, the temporal and spatial appearance of high-density ACE binding is coincident with the deposition of fibrous tissue (Sun and Weber, 1996a; Sun *et al.*, 1993).

Thus irrespective of the etiologic basis of injury, the tissue involved, or the presence of ischemic versus nonischemic repair, ACE binding is coincident with fibrous tissue formation. Furthermore, high-density ACE binding to be spatially concordant with marked autoradiographic AngII and TGF–β recepto-binding densities and type I collagen expression at these sites (**Fig. 1**) (Weber *et al.*, 2013). Collectively, these findings implicate the fibrogenic ACE/AngII/AT_1R axis as a common signaling pathway involved in repair (Weber, 1997a).

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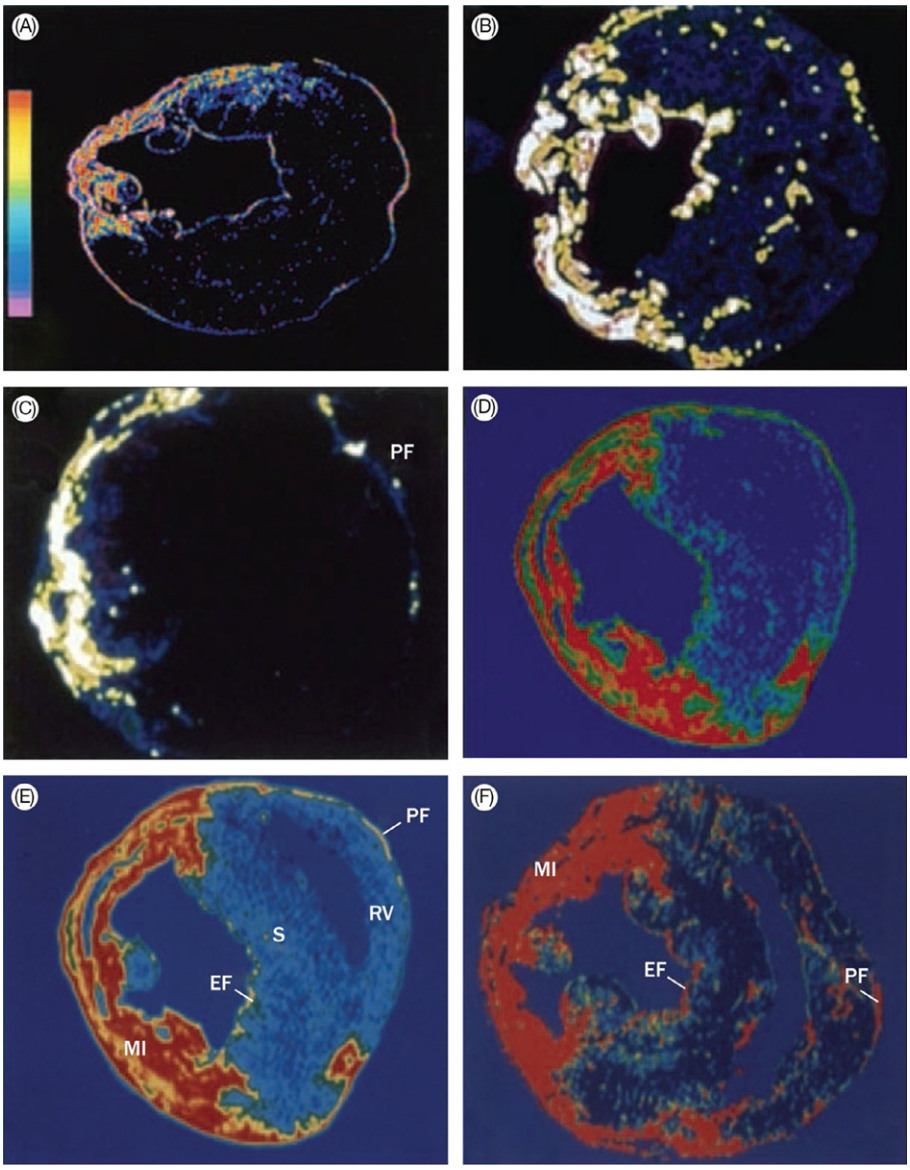

Fig. 1 Fibrous tissue is found at, and remote to, the site of injury 4 weeks after MI. Soluble signals generated by the "secretome" at the site of injury are capable of traversing the interstitial space to remote sites, including the interventricular septum and right ventricle, where they provoke interstitial and adventitial fibroblasts to produce collagen. Additionally, circulating signals might be transported to remote sites via the coronary circulation. mRNA expression of (A) renin, (B) angiotensin-converting enzyme, (C) the angiotensin II type 1 receptor, (D) transforming growth factor β1, and (E) type I collagen are shown. (F) Picrosirius red staining of fibrillar collagen. Notably, endocardial and pericardial fibrosis are also sites of high expression of angiotensin-converting enzyme and angiotensin II type 1 receptor. Abbreviations: *EF*, endocardial fibrosis; *MI*, myocardial infarction; *PF*, pericardial fibrosis; *RV*, right ventricle; *S*, interventricular septum. Panel (A) reprinted from Sun, Y., Zhang, J., Zhang, J.Q., Weber, K.T. (2001). Renin expression at sites of repair in the infarcted rat heart. *Journal of Molecular and Cellular Cardiology* **33**, 995–1003. Panel; (B) reproduced from Sun, Y., Weber, K.T. (1994). Angiotensin II receptor binding following myocardial infarction in the rat. *Cardiovascular Research* **28**, 1623–1628. Panel; (C) reproduced from Sun, Y., Cleutjens, J.P.M., Diaz-Arias, A.A., Weber, K.T. (1994). Cardiac angiotensin converting enzyme and myocardial fibrosis in the rat. *Cardiovascular Research* **28**, 1423–1432. Panel (F) reproduced from Weber, K.T. (1997). Extracellular matrix remodeling in heart failure. A role for de novo angiotensin II generation. *Circulation* **96**, 4065–4082.

Cells Expressing Ace/Angii/AT_1R

Cells expressing ACE in normal rat heart include endothelial cells lining atrial and ventricular valve leaflet surfaces; and fibroblast-like cells residing within leaflet matrix also termed myoFb (Filip *et al.*, 1986; Katwa *et al.*, 1995; Messier *et al.*, 1994). The identity and temporal response in renin expression of ACE-positive cells seen at and remote to the infarct site include macrophages that invade the infarct site and myoFb (Falkenhahn *et al.*, 1995; Sun and Weber, 1996b; Sun *et al.*, 2001). Within 24 h of MI, macrophages appear at the interface between viable and necrotic myocardium; by day 3, stromal fibroblasts coaggregate with

macrophage clusters bordering on the infarct. Thereafter, fibroblast differentiation follows resulting in α-SMA positive myoFb phenotype that then proliferate and migrate into the site of necrosis during the remainder of week 1. A combination of cell growth with spatial control of growth and fibrillar collagen assembly governs rebuilding of infarcted tissue. Macrophages and myoFb found at the infarct site also express AngII receptors, TGF–β_1, and its receptors (Sun *et al.*, 2001).

Beyond day 14, only myoFb and endothelial cells are renin and ACE positive coincident with the gradual disappearance of macrophages via apoptosis. Persistent renin expression and high-density ACE and AngII receptor binding are present at the infarct site for 8 weeks of more postMI (Lefroy *et al.*, 1996; Sun *et al.*, 2001). This is primarily due to α-SMA positive myoFb, which remain in infarct scar tissue for prolonged periods of time. In the infarcted human heart these myoFb persist at the infarct site for years (Willems *et al.*, 1994).

MyoFb have considerable phenotypic and functional diversity (Sappino *et al.*, 1988; Skalli *et al.*, 1989). Immunolabeling with α-SMA, vimentin, and desmin defines their phenotype at the infarct site. Fibroblast-like cells express vimentin (V). ACE-labeled fibroblasts found in the infarct scar and involved in the expression of fibrillar collagen mRNA are positive for cytoskeletal proteins α-SMA and V. These VA-positive myoFb are instrumental to tissue repair including wound contraction. They likewise are found in the connective tissue that comprises endocardial fibrosis, pericardial fibrosis, renal infarction, and sites of foreign-body fibrosis. Unlike incised skin, where myoFb contribute to tissue repair and then progressively disappear through apoptosis at week 4 (Desmouliére *et al.*, 1995), the VA phenotype found at the infarct site remain for prolonged periods (Sun and Weber, 1996b).

In vitro emulsion autoradiography identifies α-SMA-positive myoFb as expressing AngII receptors (**Fig. 2**) (Sun and Weber, 1996c). Together with displacement studies, using an AT_1 receptor antagonist, losartan, or AT_2 receptor antagonist, PD123177, the great majority of receptors in the infarcted rat heart are of the AT_1 subtype (Lefroy *et al.*, 1996; Passier *et al.*, 1996; Sun and Weber, 1994, 1996c; Sun *et al.*, 1994). MyoFb found at sites of microscopic scarring involving both infarcted and noninfarcted tissue also express mRNA for the fibrogenic cytokine TGF–β_1 and TGF–β receptors (Sun *et al.*, 1998). This has implicated locally produced AngII in regulating collagen turnover, which has been further suggested by losartan in attenuating fibrous tissue formation at and remote to MI (Sun *et al.*, 1998).

ACE/ANGII/AT_1R and Cardiac Repair

High-density ACE binding in tissue repair implies marked ACE activity at these sites. ACE activity has been examined in the failing, infarcted, and noninfarcted human hearts (Hokimoto *et al.*, 1995). Infarct tissue ACE activity exceeds that of control tissue several fold, and the extent of activity is related to the severity of tissue damage. In rat heart tissue homogenates prepared from sites remote to a large transmural anterior MI, ACE activity is increased and the extent to which substrate conversion is increased correlates with infarct size (Hirsch *et al.*, 1991).

A paradigm of tissue repair has been proposed in which ACE and local AngII of the myoFb secretome are integral to the orderly and sequential nature of repair that eventuates in fibrosis (Weber, 1997b). ACE is involved in the de novo generation of AngII within granulation tissue that forms at sites of injury. The first component to local AngII generation is provided by macrophages. In an autocrine manner, it regulates expression of the fibrogenic cytokine TGF–β_1 that determines phenotype conversion of coaggregating stromal fibroblasts. VA positive myoFb generate AngII whose autocrine induction of TGF–β_1 regulates collagen

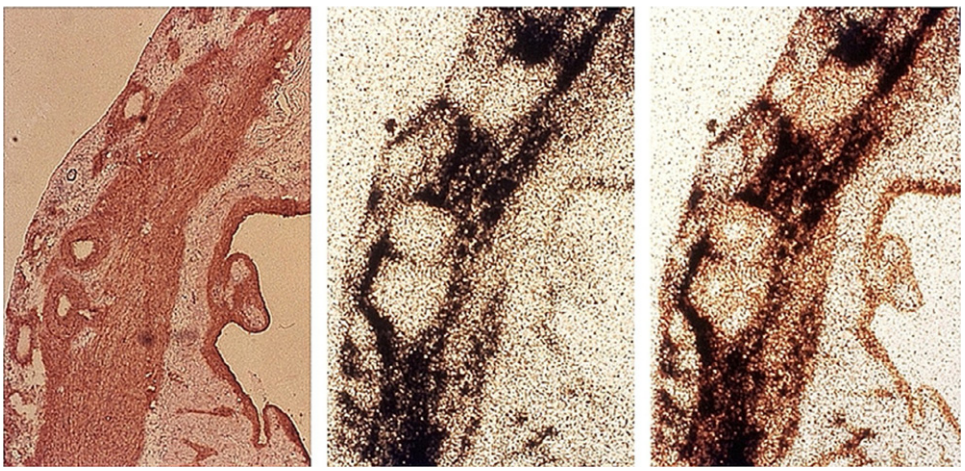

Fig. 2 Colocalization of α-SMA-positive myofibroblasts (left panel) expressing AT_1R receptors (middle panel) at a site of fibrosis and their overlay (right panel). Reproduced from Sun, Y., Weber, K.T. (1996). Cells expressing angiotensin II receptors in fibrous tissue of rat heart. *Cardiovascular Research* **31**, 518–525.

turnover at sites of fibrosis, including infarcted and noninfarcted myocardium. AngII generation at the infarct site by the myoFb secretome is related to the extent of the myoFb response and accordingly the degree of myocyte necrosis and subsequent healing. An extensive transmural MI and accompanying inflammatory cell response generates a large amount of AngII, which reaches remote sites via its diffusion through tissue fluid to promote fibrosis. Accordingly, activation of fibrogenesis is greatest at sites closest to the anterior MI (e.g., interventricular septum) and less so at more remote sites (e.g., RV). Expression of type I and III collagens is greater and persists longer in the septum compared to RV following left coronary artery ligation (Cleutjens *et al.*, 1995).

Fibrous tissue formation at sites remote to MI and the myoFb secretome is also influenced by pharmacologic interventions. Perindopril, given 1 wk. after MI, attenuates the endocardial fibrosis that appears in the nonnecrotic segment of the LV (Michel *et al.*, 1988). Captopril, commenced at the time of coronary artery ligation, attenuates the expected fibrosis of noninfarcted LV and RV (Bélichard *et al.*, 1994; van Krimpen *et al.*, 1991) and proliferation of fibroblasts and endothelial cells that appears at remote sites 1 and 2 weeks following MI (van Krimpen *et al.*, 1991).

Losartan, begun on day 1 after coronary artery ligation and in a dose that reduced AT_1 receptor binding by 50%, reduces infarct scar area (CdC *et al.*, 1997). Moreover, the expected rise in tissue AngII concentration found at the infarct site 3 weeks postcoronary artery ligation is markedly attenuated by an AT_1 receptor antagonist, introduced on postoperative day 1 (Yamagishi *et al.*, 1993). These findings raise the prospect that the number of myoFb or their AngII-generating activity per cell at sites of repair is influenced by AngII. Losartan also prevents fibrosis at remote sites (CdC *et al.*, 1997; Schieffer *et al.*, 1994; Smits *et al.*, 1992).

These cardioprotective effects of ACE inhibition or AT_1 receptor antagonism are not confined to the infarcted heart. The appearance of fibrosis in diverse organs with experimentally induced or naturally occurring tissue injury, without cRAAS activation. These include pericardial fibrosis postpericardiotomy (Sun and Weber, 1994), tubulointerstitial fibrosis associated with unilateral ureteral obstruction (Ishidoya *et al.*, 1995, 1996; Kaneto *et al.*, 1994; Morrissey *et al.*, 1996; Pimentel *et al.*, 1993, 1995; Yanagisawa *et al.*, 1990), toxic nephropathy (Cohen *et al.*, 1994; Diamond and Anderson, 1990; Lafayette *et al.*, 1993), remnant kidney (Anderson *et al.*, 1986; Ikoma *et al.*, 1991; Shibouta *et al.*, 1996; Tanaka *et al.*, 1995) or renal injury following irradiation (Juncos *et al.*, 1993), cardiovascular and glomerulosclerosis that appear in stroke-prone spontaneously hypertensive rats (Kim *et al.*, 1994, 1995; Nakamura *et al.*, 1994, 1996), interstitial pulmonary fibrosis that follows irradiation (Ward *et al.*, 1989, 1990, 1992) or monocrotaline administration (Molteni *et al.*, 1985), and subcutaneous pouch tissue in response to croton oil (Sun *et al.*, 1997). Attenuation of fibrous tissue formation by these interventions in diverse organs with various forms of injury supports the importance of AngII generated by the myoFb secretome in promoting fibrosis.

CounterRegulatory ACE2/Ang1–7/MasR in Repair

The myoFb RAS has expanded with the identification of a novel axis of ACE2, Ang1–7, and the G protein-coupled receptor, Mas (Ferrario *et al.*, 2016). This fibrolytic axis counterregulates the deleterious fibrogenic ACE/AngII/AT_1 axis and therefore could be invoked to regress cardiac fibrosis (**Fig. 3**). ACEI inhibitors and AT_1Ra upregulate the ACE2/Ang1–7/MasR axis in the hypertensive heart (Ferrario *et al.*, 2005,b), while inhibition of ACE2/Ang1–7/MasR axis exacerbates fibrosis (Liu *et al.*, 2012; Trask *et al.*, 2010; Zhong *et al.*, 2011); Ang1–7 overexpression prevents fibrosis (de Almeida *et al.*, 2015; Mercure *et al.*, 2008; Santiago *et al.*, 2010).

The regulation of tissue AngII involves ACE2, a homologue of ACE, which hydrolyzes AngII into Ang1–7, a pentopeptide, whose biologic activity is expressed via binding to its Mas receptor. The ACE2/Ang1–7/Mas axis is in equilibrium with the ACE/AngII/AT_1R axis, whereas Ang1–7 is counterregulatory to AngII, and therefore is cardioprotective. Ang1–7 formation is dependent

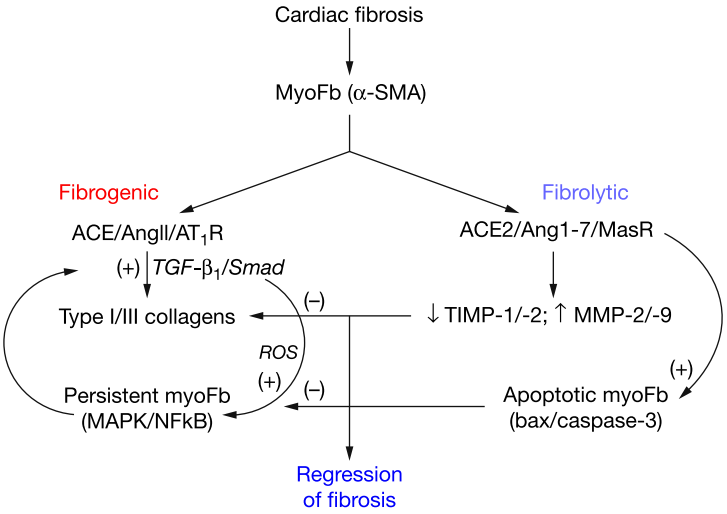

Fig. 3 Competing fibrogenic and fibrolytic axes represented by ACE/AngII/AT_1R and ACE2/Ang1–7/MasR, respectively.

on ACE2 and AngII as substrates (Keidar *et al.*, 2007). Increased levels of cardiac ACE2 and Ang1–7 forming activity are found in the failing heart, where this ectoenzyme is localized to macrophages, endothelial and vascular smooth muscle cells, fibroblasts, and myocytes (Burrell *et al.*, 2005; Zisman *et al.*, 2003). ACE2 activity is insensitive to ACE inhibitors; on the other hand, AT_1R antagonists increase cardiac ACE2 expression (Sukumaran *et al.*, 2011; Takeda *et al.*, 2007).

Targeted disruption or ACE2 overexpression has identified its significance in cardiac remodeling. Loss of ACE2 augments maladaptive AngII-based remodeling following either MI or LV pressure overloading, including ventricular dilation and infarct expansion, together with upregulation and activation of tissue matrix metalloproteinase, accompanied by disruption to fibrillar collagen which could be blocked by AT_1R antagonist (Kassiri *et al.*, 2009). Overexpression of ACE2 and upregulated Ang1–7 attenuate pathologic remodeling which follows MI and occurs in diabetic cardiomyopathy (Dong *et al.*, 2012; Zhao *et al.*, 2010), whereas chronic inhibition of ACE2 with tissue AngII accumulation is pathologic with fibrosis (Trask *et al.*, 2010).

Thus, increased ACE2 and Ang1–7 formation are counterregulatory to augmented tissue AngII formation found at sites of cardiac fibrosis and are cardioprotective by attenuating AngII-based maladaptive remodeling in diverse pathologic states. By raising cardiac ACE2 expression, AT_1R antagonists offer a unique salutary perspective in cardioprotection. Their use in combination with agents activating ACE2 may offer yet another dual strategy in preventing ongoing adverse remodeling in the failing heart.

Summary and Conclusion

The fibrogenic ACE/AngII/AT_1R axis represents a generalized response of multiple tissues evoked by injury and the need for repair. It serves to regulate local concentrations of AngII as a mediator of repair in diverse tissues, including the heart. MyoFb-bound ACE is integral to de novo generation of AngII that modulates expression of TGF–β_1 and whose auto-/paracrine properties regulate collagen turnover at sites of fibrous tissue formation that appear in response to various forms of cardiac injury, including infarction, pericarditis, and foreign-body implant. Persistent myoFb, resistant to apoptosis, and their expression of ACE at sites of injury contribute to a sustained metabolic activity, or secretome, that can account for a progressive fibrosis. Ongoing adverse structural remodeling by fibrous tissue eventuates in the progressive nature of chronic cardiac failure. The counterregulatory fibrolytic ACE2/Ang1–7/Mas axis of the myoFb secretome opposes this fibrogenic axis and may come to prove itself an effective cardioprotective strategy.

See also: Aldosteronism in Congestive Heart Failure. Renin and Prorenin

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Hypertension and the Renin–Angiotensin–Aldosterone System☆

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Glossary

Aldosterone A steroid hormone from the adrenal cortex that primarily functions to regulate sodium potassium, and chloride metabolism.

Peptide bond The bond formed between the carboxyl group of the amino acid and the amino group of another amino acid; an amide linkage joining amino acids to form peptides.

Proteolytic enzyme An enzyme that catalyzes the hydrolysis of peptide bonds in protein.

Introduction

In 1898, Tigerstedt and Bergmann discovered renin when they injected rabbit kidney extract into rabbits and observed an increase in blood pressure. In the following decades, the existence of the vasopressor substance was a subject of some controversy, and in 1934, Goldblatt rediscovered the importance of renin for blood pressure regulation and showed a relationship between renin secretion and renal ischemia. A few years later, in 1937, Blalock and Levy discussed a link between renin secretion and renal perfusion pressure, and renal baroreceptors were postulated. The enzymatic properties of renin were described by Page and Helmer and by Braun-Menendez et al. At the same time, Page et al. identified the renin substrate angiotensinogen as a plasma protein and interpreted the RAAS as a functional unit. In the mid-1950s, Skeggs et al. identified angiotensin-converting enzyme (ACE) in lungs and discovered that ACE catalyzes the conversion of angiotensin I (ANG I) to angiotensin II (ANG II), which mediates the actions of the RAAS. In 1958, Gross postulated a physiological link between the RAAS and aldosterone secretion in the zona glomeruloza of the adrenal gland, demonstrating that ANG II stimulates the secretion of aldosterone, which subsequently enhances sodium retention. Local RAASs—meaning that all components necessary for ANG II synthesis (angiotensinogen, renin, ACE) are expressed in one tissue—have been postulated in various organs such as the heart, vascular wall, kidney, adrenal gland, brain, and skin. Local actions of ANG II seem to comprise not just cardiovascular effects but also the control of cell proliferation and differentiation, thus taking part in mechanisms of tissue repair and remodeling.

The Renin Molecule

Renin is a proteolytic enzyme belonging to the aspartyl protease family. Differing from species to species, the molecular weight of renin varies between 37,000 and 42,000 Da. The active form of renin consists of two polypeptide chains linked by a disulfide bridge to form a bilobal structure with one active site. Renin shows an extremely high substrate specificity for angiotensinogen, the only known endogenous substrate for renin.

Renin Genes

Renin genes derived from various species, including humans, rats, and mice, have been cloned and sequenced. The human renin gene is located on the q42 band of chromosome 1 and spans 12,000 bp. The activity of the renin promoter is rather weak. It is regulated by multiple weak regulatory elements within the first 1301 bp of the 5′-flanking region, a classic silencer element within the first intron (Intron A) of the gene, and strong enhancer located far upstream (bp -5777 to -5552 in humans). There is accumulating evidence that renin expression is regulated according to the "variegation concept," meaning that transcription of a single gene is either switched on or switched off, but not gradually modified. A more or less pronounced expression of renin within a tissue would consequently be the result of more or less "switched-on" renin-transcribing cells.

Additional data show mechanisms for a posttranscriptional control of renin synthesis. These mechanisms seem to involve the stabilization of renin mRNA by the binding of specific proteins, whose expression is stimulated by cyclic AMP.

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Molecular genetics studies did not find and evidence of an association between renin gene expression and hypertension.

Biosynthesis, Processing, and Expression of Renin

Translation of renin genes yields a precursor molecule, preprorenin, which consists of 340 amino acids. Preprorenin is processed to enzymatically inactive prorenin (MW 57,000). Prorenin can be activated in vitro by acidification or prolonged cooling, as well as by exposure to neutral serine proteases (e.g., trypsin, plasmin, kallikrein) or acid proteases (e.g., pepsin, cathepsin D). More than 80% of the total circulating renin is prorenin.

Renin is expressed in a complex tissue-specific and developmentally specific pattern. Early in development, renin is abundant in smooth muscle cells in the intrarenal arteries. During development, however, renin expression is progressively more reduced to the "classical" place of synthesis, the juxtaglomerular apparatus, where it is produced and stored in granules.

Very strong, chronic stimuli, however, are able to reestablish a cell's ability to synthetize renin. In the heart, for example, where renin mRNA is normally absent, conditions known to increase renin synthesis in the kidney (such as sodium depletion for at least a week or chronic ACE inhibition) lead to a massive rise in cardiac renin mRNA expression. The same phenomenon (called "recruitment") was observed in afferent arteriolar cells extending further upstream of the juxtaglomerular apparatus, which usually show no renin mRNA expression.

Mechanisms Controlling Renin Release

Renin is synthesized, stored, and secreted into the renal arterial circulation by the granular juxtaglomerular cells that lie in the walls of the afferent arterioles as they enter the glomeruli.

Effective stimuli for renin release and thereby for the induction of the renin–angiotensin–aldosterone cascade are sodium deficiency, hypovolemia, a significant decrease in arterial blood pressure, and increased sodium concentration in the distal tubule (tubuloglomerular feedback).

The secretion of renin from juxtaglomerular cells is controlled by three pathways, as follows:

The first intrarenal mechanism controlling renin release is called the macula densa pathway. A specialized segment of the early distal tubule, the macula densa segment, comes into direct contact with the afferent and efferent arterioles of its own nephron. A change in NaCl reabsorption by the macula densa results in the transmission of chemical signals to nearby juxtaglomerular cells, which modify the release of renin; the increase in NaCl flux across the macula densa inhibits the release of renin and the decrease stimulates the release of renin. The other chemical signals mediating the macula dense pathway involve adenosine, prostaglandins, and nitric oxide.

The second mechanism controlling renin release is called the intrarenal baroreceptor pathway. In 1934, Goldblatt showed that it was possible to produce persistent hypertension in dogs by constricting the renal arteries. Later, the concept of a "baroreceptor mechanism for renin secretion" was formulated by Skinner. Increases and decreases in blood pressure in the preglomerular vessels inhibit and stimulate renin release, respectively. The underlying mechanism is believed to be a reduction in the tension within the wall of the afferent arteriole.

The third mechanism is called the β-adrenergic receptor pathway. It has long been known that an increase in sympathetic activity influences renin release. The studies of Kirchheim and related investigations have clearly established that an increase in sympathetic tone to the kidney stimulates renin release via the activation of β_1-adrenergic receptors on juxtaglomerular cells.

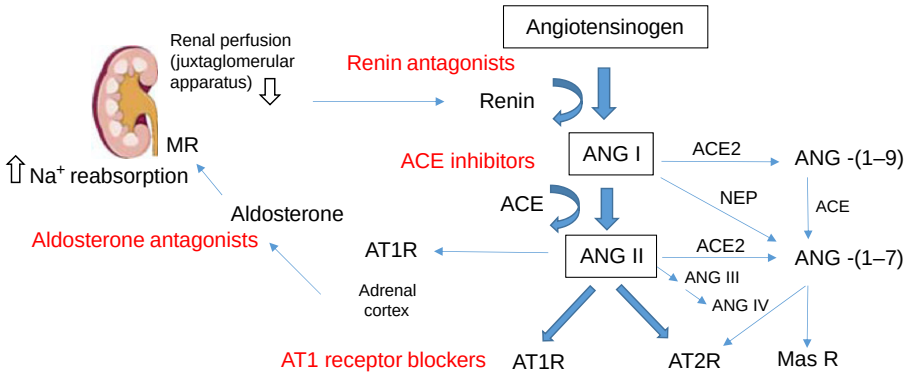

Fig. 1 Drugs affecting the renin–angiotensin–aldosterone system. *ANG*, angiotensin; *AT1R*, angiotensin AT1 receptor; *AT2*, angiotensin AT2 receptor; *ACE*, angiotensin converting enzyme; *MR*, mineralocorticoid receptor; *NEP*, neprilisin; *Mas R*, Mas receptor.

Renin Actions

Renin acts on angiotensinogen, splitting off a decapeptide, angiotensin I, from the N-terminal end of the protein (Fig. 1). ANG I is acted on by a second proteolytic enzyme, ACE, that removes two aminoacids to form the highly active octapeptide, ANG II. The removal of one more amino acids yields the heptapeptide, Angiotensin III (ANG III). ANG II and ANG III induce the release of the mineralocorticoid aldosterone, the most important hormone for sodium balance, from the adrenal cortex.

The Substrate for Renin: Angiotensinogen

Angiotensinogen, an α-globulin, is a precursor molecule for angiotensin II and the substrate for renin. Angiotensinogen is synthesized and secreted mainly by the liver and is found in the α-globulin fraction of plasma. Moreover, it is also found in diverse tissues expressing local RAASs. Its synthesis is stimulated by glucocorticoids, thyroid hormone, estrogens, and ANG II. Increases in angiotensinogen levels are associated with essential hypertension. Moreover, a linkage to essential hypertension was found in regions within or close to angiotensinogen gene. Transgenic mice expressing the rat angiotensinogen gene are hypertensive and mice lacking angiotensinogen gene are hypotensive.

Angiotensin-Converting Enzyme

ACE is the second enzyme (Fig. 1) in the cascade of ANG II synthesis, converting the inactive decapeptide, ANG I, to the potent octapeptide pressure hormone, ANG II. ACE is a nonspecific peptidase that can cleave C-terminal dipeptides from various peptides (dipeptidyl carboxypeptidase). The enzyme kininase II is identical to ACE and contributes to the activation of kinins, such as bradykinin, and other potent vasodilator peptides. ACE is primarily localized on the luminal side of the vascular endothelium. The lung, which has a vast surface area of vascular endothelium, is rich in ACE. Additionally, ACE is present in other organs including kidney, heart, brain, striated muscle, and skin, as it is a part of local RAASs.

Angiotensin converting enzyme 2 (ACE2) is a negative regulator of the RAAS, counterbalancing the multiple functions of ACE (Fig. 1). ACE2 converts the decapeptide, angiotensin I, to angiotensin ANG (1–9), which is converted by ACE to a shorter peptide, ANG (1–7). Alternatively, Ang (1–7) can also be formed directly from ANG I via neutral endopeptidase (NEP, neprilysin).

Ang (1–7) evokes a range of acute central and peripheral effects such as vasodilatation, inhibition of VSMC proliferation, and inhibition of vasopressin release. Furthermore, Ang (1–7) is an endogenous ligand for the Mas receptor, a seven transmembrane domain G-protein-coupled receptor sharing a 31% sequence identity with the AT2 receptor.

Angiotensin II and Its Receptors

ANG II, an octapeptide hormone, is the main active component of the RAAS. It contributes to the regulation of blood pressure, plasma volume (via aldosterone-regulated sodium excretion), and sympathetic nervous activity. Moreover, it is involved in such diverse effects as proliferation, differentiation, regeneration, and apoptosis. The multiple actions of ANG II are mediated via specific, highly complex intracellular signaling pathways that are stimulated following an initial binding of the peptide to its cell surface receptors. The major actions of ANG II are mediated by two subtypes of G protein-coupled angiotensin receptors, the angiotensin receptor type 1 (AT1) and the angiotensin type 2 (AT2) receptors (Fig. 1), which are seven-transmembrane glycoproteins with only 32%–34% sequence homology.

The AT1 Receptor

The AT1 receptor is involved in the classical physiological actions of ANG II: regulation of blood pressure, electrolyte and water balance, thirst, hormone secretion, and renal function. The AT1 receptor belongs to the G protein-coupled receptor superfamily and typically activates phospholipase C through the heterotrimeric Gq protein.

AT1 receptors are present in the human vasculature, lung, liver, brain, kidney, adrenal gland, skin, and endometrium.

The classical actions of AT1 receptors include the following: generalized vasoconstriction; increased release of noradrenaline from sympathetic nerve terminals, reinforcing vasoconstriction and increasing the rate and force of the heart; stimulation of the proximal tubular reabsorption of sodium ions; secretion of aldosterone from the adrenal cortex; and cell growth in the cardiac left ventricle and in the arterial wall.

ANG II via the AT1 receptor furthermore controls cellular growth, adhesion, migration, and intercellular matrix deposition, influencing chronic adaptive changes in vascular and cardiac growth, remodeling, repair, and atherosclerosis. In vascular smooth muscle cells and in endothelial cells, ANG II via its AT1 receptor stimulates phospholipase A_2 activity, leading to the release of arachidonic acid and eicosanoids, which influence vascular and renal mechanisms important in the regulation of blood pressure and cell growth.

ANG II via the AT1 receptor increases the activity of NAD(P)H oxidase thereby stimulating reactive oxygen species (ROS) and nitrogen formation in the vessel wall. ROS products such as superoxide and H_2O_2 may activate mitogen-activated protein kinases, tyrosine kinases, phosphatases, calcium channels, and redox-sensitive transcription factors. Activation of these signaling pathways results in cell growth and expression of proinflammatory genes. The ROS cause also lipid peroxidation and generation of various vasoconstricting molecules such as F2 isoprostans.

The expression of the AT1 receptors is altered by various pathophysiological conditions: renovascular hypertension, myocardial infarction, ventricular hypertrophy, and bilateral nephrectomy. Overexpression of the vascular AT1 receptor can be observed in hypercholesteremic men. These findings may help to explain why hypercholesterolemia is frequently associated with hypertension and why blockade of the RAAS attenuates the progression of atherosclerosis. The downregulation of AT1 receptors in sepsis is the main reason for the attenuated responsiveness of blood pressure and of aldosterone formation to ANG II and, therefore, may contribute to the characteristic septic shock.

The AT2 Receptor

The AT2 receptor is widely expressed in fetal tissues, whereas its expression is dramatically decreased after birth, being restricted to a few organs such as brain, adrenal, heart, kidney, myometrium, skin, and ovary. Although the AT1 receptor is dominant in the adult organism, an increase of AT2 receptor expression has been observed in pathological conditions, such as vascular injury, myocardial infarction, congestive heart failure, renal failure, brain ischemia, and sciatic or optic nerve transection.

The AT2 receptor exerts a protective effect against an overstimulation of AT1 receptors by counteracting AT1 receptor-mediated actions; for example, whereas the AT1 receptor stimulates cell proliferation, the AT2 receptor has an antiproliferative effect and promotes cell differentiation. Moreover, the AT2 receptor activation inhibits sympathetic activity and promotes antiinflammation. Inhibition of this receptor enhances the immediate left ventricular growth response to ANG II.

The AT2 receptor is a seven transmembrane domain G-coupled receptor that acts via several intracellular signaling pathways such as nitric oxide (NO)/cyclic GMP activation, inhibition of mitogen-activated protein kinases (MAPKs) by protein phosphatases, phospholipase A2 stimulation or disruption of AT1 receptor signaling by AT1–AT2 receptor heterodimerization.

The AT2 receptor stimulates the production of NO and, subsequently, the second messenger cGMP. cGMP, in turn, mediates vasodilation, natriuresis, and antigrowth by activating cGMP-dependent protein kinase. The AT2 receptor also inhibits renin biosynthesis and ANG II formation via nitric NO/cGMP dependent mechanism. Moreover, antigrowth, antifibrotic and antiinflammatory features of this receptor might contribute to blood pressure lowering and prevent remodeling in hypertension. Whereas acute vasodilator role of AT2 receptor is well described, chronic decrease of blood pressure seems to be minimal after AT2 receptor stimulation. Nevertheless, the AT2 receptor is known to be important in the prevention of vascular remodeling.

Recently, two selective AT2 receptor agonists (Compound 21 by Vicore Pharma, Sweden and MOR107 by Lanthio Pharma, Netherlands) have been developed and tested in Phase I trials demonstrating their safety in humans.

Further knowledge about the tissue-protective effects of the AT2 receptor might lead to new therapies in the future and should be confirmed by clinical studies.

Aldosterone

The mineralocorticoid aldosterone is produced in the zona glomerulosa of adrenal cortex. Its main action is the resorption of Na^+ (Cl^- and H_2O follow) in exchange for K^+ in the distal tubules of the kidney. Low plasma sodium or high plasma potassium concentrations affect the zona glomerulosa cells of the adrenal directly, stimulating aldosterone release. Aldosterone secretion is also controlled indirectly by the juxtaglomerular apparatus, which is sensitive to the composition of the fluid in distal tubule. A decrease in the sodium chloride concentrations of the filtrate is sensed by macula densa cells, which stimulate renin release. This leads—as illustrated above—to the formation of ANG II, which in turn, via AT1 receptors, stimulates the synthesis and release of aldosterone by the adrenal cortex. Moreover, in the process of aldosterone synthesis, angiotensins (ANG II and ANG III) regulate the corticosterone methyloxidase I and II enzymes, which catalyze the hydroxylation and aldehyde formation at C-18 of corticosterone.

In the kidneys, in the late distal tubule and collecting duct, aldosterone binds to cytoplasmatic mineralocorticoid receptors (MR), which migrate to the nucleus and initiate DNA transcription, translation, and production of proteins, which activate Na^+ and K^+ channels and increase the synthesis of Na^+/K^+ ATPase and production of ATP. As a result, the reabsorption of Na^+ (and the subsequent reabsorption of Cl^- and H_2O) and the secretion of K^+ and H^+ are increased. Na^+ reabsorption increases the osmolarity of extracellular fluids. This stimulates the hypothalamic osmoreceptor to release vasopressin from the posterior pituitary. Vasopressin leads to enhanced free-water reabsorption in the collecting duct, which expands extracellular volume and reduces plasma osmolarity. Vasopressin also acts on other tissues. In the vasculature, vasopressin increases intracellular Ca^{2+} and potentiates vasopressor responses.

The effects of aldosterone on blood pressure regulation extend beyond increased intravascular fluid retention and volume overload. Aldosterone modulates vascular tone by upregulation of the AT1 receptor, by limiting bioavailability of endothelial NO,

and by increasing pressor responses to catecholamines. Moreover, aldosterone excess activates inflammation and oxidative stress, decreases fibrinolytic activity, and promotes vascular hypertrophy.

All these cellular pathways regulated by aldosterone via the MR, and ANG II via its AT1 receptor can reinforce each other.

Drugs Affecting the RAAS

Renin Inhibitors

Since renin catalyzes the first and rate-limiting step of the RAAS cascade, interruption of the generation of ANG II by renin inhibitors at this highly specific initial step of the cascade has long been a therapeutic goal. The inhibition of renin would have the advantage of preventing RAAS synthesis without concomitant accumulation of other peptides, such as kinins or substance P, as in ACE inhibitor treatment, and it would have an advantage of the absence ANG II overexpression and stimulation of other ANG II receptor subtypes as in AT1 receptor antagonist treatment. Despite these theoretical advantages, the lack of oral availability, low efficacy, and high costs of development have thus far prevented renin inhibitors from becoming successful drugs. However, potent nonpeptidic inhibitor of renin, Aliskiren, with acceptable oral bioavailability, has been synthesized. Aliskiren has been shown to decrease in dose-dependent manner plasma renin activity, ANG I and ANG II levels in healthy volunteers, and blood pressure in hypertensive patients. Moreover, in comparison with antihypertensive treatments including AT1 receptor blockade, a β-blocker and calcium channel antagonist, Aliskiren was shown to have equally potent blood pressure lowering effects and antiatherosclerotic effects. On the other hand, large clinical trials, which investigated the combination of Aliskiren with other RAAS blockers, failed to show the expected outcomes or resulted with an increased incidence of adverse effects. In diabetic patients at high risk of developing cardiovascular and renal complications, dual RAAS blockade had no further benefit in preventing end-organ damage.

ACE Inhibitors

ACE inhibitors occupy the angiotensin-converting enzyme as false substrates, thus preventing the conversion of ANG I to ANG II and resulting in reduced levels of circulating and tissue ANG II. They also block the degradation of bradykinin and other vasodilatory peptides, which may have potential benefits in cardiovascular disease. ACE inhibitors affect capacitance and resistance vessels, reduce cardiac load as well as arterial pressure, improve endothelial function, and reduce left ventricular hypertrophy.

ACE inhibitors are well recognized as an important therapeutic step to control blood pressure in hypertensive patients and to reduce morbidity and mortality in patients with hypertension, congestive heart failure, or diabetes mellitus. They appear to possess unique cardioprotective benefits, even when used in patients without high blood pressure or left ventricular dysfunction. ACE inhibitors promote collateral vessel development and improve prognosis in patients who have had coronary revascularization procedure. ACE inhibitors are also effective in the management of chronic renal diseases to delay the progression of renal failure.

AT1 Receptor Antagonists

The "sartan" family comprises a rather new group of pharmaceuticals (losartan, valsartan, candesartan, irbesartan, telmisartan, eprosartan, azilsartan), which all act as antagonists on the AT1 receptor. AT1 receptor antagonists are specific for the RAAS, selective for the AT1 receptor, and act independently of the ANG II synthesis pathway, allowing a more selective blockade of the AT1-mediated effects of ANG II compared with ACE inhibitors. However, by inhibiting the AT1 receptor-mediated negative feedback of ANG II on renin release in the kidney, these drugs may evoke an overstimulation of the AT2 receptor by enhancing ANG II levels in the plasma. An AT2 receptor-mediated increase in the production of vasodilators (nitric oxide, cGMP, prostaglandins) as well as the antigrowth features of this receptor might contribute to a further decrease in blood pressure and prevent hypertrophy and remodeling. AT1 receptor antagonists do not inhibit the degradation of kinins and cough is not a frequent side effect.

The AT1 receptor antagonists are widely used as antihypertensive agents, especially in patients with type 2 diabetes or ACE inhibitor intolerance.

Aldosterone Antagonists

The mineralocorticoid hormone aldosterone is a product of the RAAS that contributes to the development of hypertension and myocardial hypertrophy and has a potential to cause edema through sodium and water retention. Spironolactone and its metabolite canrenone are antagonists of the aldosterone receptor and attenuate the effects of the hormone. Spironolactone is used for the treatment of hypertension, primary aldosteronism, and peripheral edema associated with heart failure.

Monotherapy with spironolactone was shown to be effective in patients with low-renin essential hypertension. Moreover, a recent clinical trial demonstrated that spironolactone was the most effective add-on drug for the treatment of resistant hypertension. However, spironolactone is associated with progestational and antiandrogenic side effects, such as gynecomastia and impotence, as a result of its binding to other steroid receptors.

Eplerenone is the first agent of a new class of drugs known as selective aldosterone receptor antagonists, which provide effective and well-tolerated blood pressure reduction. Because eplerenone has little affinity for androgen and progesterone receptors, it produces fewer steroid-like effects (such as gynecomastia in men) than spironolactone. Unfortunately, eplerenone features a reduced potency (40-fold lower affinity for MR in comparison with spironolactone). Therefore, despite of its better tolerability over spironolactone, the indication for hypertension is not recognized except in the presence of intolerance to spironolactone. Hyperkaliemia is a serious dose-related adverse effect of both spironolactone and eplerenone.

Other nonsteroidal aldosterone antagonists are being developed. Finerenone has greater affinity to the MR than eplerenone. Nevertheless, finerenone does not significantly influence systolic blood pressure.

An alternative approach is to inhibit aldosterone synthesis by selective inhibitors of aldosterone synthase. Such compound, LC1699, has been tested in patients with resistant hypertension and primary hyperaldosteronism. Due to lack of selectivity, LC1699 at higher doses also inhibits 11-beta-hydroxylase, which regulates cortisol synthesis. Thus, more selective substances will have to be developed.

Other Drugs Influencing Renin Release

Several drugs, which do not directly interfere with the cascade of ANG II synthesis, indirectly modify the rate of renin synthesis or release. Loop diuretics stimulate renin release by blocking the reabsorption of NaCl at the macula densa. Antihypertensive drugs in general increase renin release by decreasing arterial blood pressure, which in turn activates baroreceptors. Sympatholytic drugs and β-adrenoreceptor antagonists decrease renin release by blocking the β-adrenoreceptor pathway, leading to a diminished sympathetic tone in the kidney.

See also: Angiotensinogen and Angiotensins. Hyperreninemia. Interference With the Renin–Angiotensin System (RAS): Classical Inhibitors and Novel Approaches. Kininases. Renal Vein Renin Measurement as a Diagnostic Tool. Renin and Prorenin. Tissue Ace–Angiotensin–AT1 Receptor Axis and Repair in the Heart

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Hyperreninemia☆

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Glossary

Primary reninism A rare, purely renin-dependent and surgically curable form of hypertension, usually caused by a juxtaglomerular cell tumor.

Renal artery stenosis A reduction of the diameter of a renal artery by 60% or more.

Renin An aspartyl protease produced by juxtaglomerular cells in the kidney. Renin is essential for the formation of angiotensin II, which is a key hormone in blood pressure regulation and in maintaining sodium, potassium and water homeostasis.

Definition

The renin-angiotensin system [RAS] is a cascade of proteins and peptides – angiotensinogen, renin, angiotensin converting enzyme [ACE], and angiotensins – that leads to the formation of angiotensin [Ang] II, the active hormone. Ang II is a vasoconstrictor and stimulates the adrenal release of aldosterone. Aldosterone in turn enhances sodium reabsorption and potassium secretion in the distal nephron. Vasoconstriction and sodium retention increase blood pressure [BP]. Hyperreninemia therefore includes a variety of conditions, mostly with hypertension, and frequently associated with potassium wasting and hypokalemia.

Assaying the Activity of the Renin-Angiotensin System

Biochemical tests aim to assess whether plasma levels of the RAS components are higher than expected for posture, sodium intake and age. Physiological tests measure the BP and hormonal responses to the acute administration of ACE inhibitors or Ang receptor antagonists.

Human active renin is an aspartyl protease of 340 amino acids and 40 000 Dalton molecular weight with two N-glycosylation sites. It is secreted by the renal juxtaglomerular granular epithelioid cells. It is synthesized in the cells as prorenin and activated by cleavage of an N-terminal prosegment of 43 amino acids. Prorenin or the inactive form of renin is also secreted into the blood. Total plasma renin therefore consists of prorenin and activated renin and the plasma prorenin/active renin ratio is around 9.

Biochemical Techniques

Angiotensinogen and ACE are not usually rate-limiting for Ang II formation, and plasma Ang II concentration is difficult to measure. The method for assaying renin can be indirect, involving the measurement of the enzymatic activity of the mature form of active renin through the *in vitro* production of Ang I. Ang I is produced by incubating plasma in standardized conditions of time, pH and temperature in presence of various inhibitors and is measured by a radioimmunoassay. The result, plasma renin activity [PRA], which describes the work of the enzyme, is generally expressed in ng $ml^{-1}h^{-1}$ Ang I. The plasma active renin concentration [PRC], which corresponds to the concentration of the renin molecules in plasma and is expressed in mU l^{-1}, can also be measured directly by use of an immunometric or a chemoluminescence assay. In physiological conditions, and in the presence of high angiotensinogen concentrations, PRC and PRA are well correlated.

Conditions for Valid Assessment of Renin Levels

Great care should be taken to draw blood and centrifuge the samples at room temperature to avoid cryoactivation of inactive prorenin into active renin by unfolding of the prosegment masking the active site, therefore artifactually increasing renin levels. The RAS is finely tuned by posture and sodium balance, and is affected by age, genetic factors and ethnicity. Walking for one hour increases the renin levels two-fold. Renin secretion is stimulated by sodium depletion and suppressed by sodium loading. In patients on a liberal sodium diet, the renin-sodium profile is based on a nomogram relating renin levels to urinary sodium excretion. Alternatively, patients are instructed to ingest 75 to 150 mmol sodium/day and sodium excretion is determined over a 24-h period to confirm that sodium output is within this range. Renin levels are not markedly affected by variations in sodium

☆*Change History:* January 2015. P-F Plouin, L Amar, and M Azizi updated the text and references.

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intake within these limits. Plasma concentrations of renin are markedly higher in children than in adults. In normal adults, they fall by half between the ages of 30 and 70 years. Patient values should therefore be compared with those of age-matched normal subjects studied in the same conditions. Most antihypertensive agents alter renin release either directly or through counter-regulations in response to BP decrease or suppression of renin-Ang II negative feed-back. Renin release is increased by diuretics, ACE inhibitors, Ang receptor antagonists, renin inhibitors, mineralocorticoid receptor antagonists and vasodilators, and decreased by ß-blockers and centrally-acting agents. Treatment with these drugs should be stopped 2–6 weeks before assessing renin levels, although this may be difficult in patients with severe hypertension. In such cases, alpha-blockers and non-dihydropyridine calcium channel blockers may be continued because they have little effect on renin release.

Acute Renin-Suppression Tests

Acute renin-suppression tests interrupt the RAS, showing that this system is involved in the control of hypertension. The ACE inhibitor captopril is generally administered orally due to its rapid onset of action (20 min) and maximum action (1–3 h). BP should consistently decrease in patients with renin-dependent hypertension. However, this test is no more used because it is not sensitive and specific enough and could mistakenly restrict the use of RAS blockers to patients with a high renin profile, even though the majority of patients with a high cardiovascular risk in whom it is justified to administer an ACE inhibitor have an average to low renin profile.

When is it Useful to Measure Renin? (Table 1)

In patients with essential hypertension, plasma renin determination may help physicians to select the most effective first-line treatment. Patients in the highest renin index quartile show the greatest response to RAS blockers and ß-blockers and those in the lowest quartile respond better to diuretics and CCBs. However, considering the fluctuations in renin levels in individual patients and the relatively high cost, the therapeutic value of measuring PRA or PRC during routine investigation of mild to moderate essential hypertension remains limited.

In contrast, renin should be measured in patients prone to secondary hypertension. Such patients can be identified by their medical history, signs and symptoms, by the presence of hypokalemia, chronic kidney disease, abdominal bruit, or asymmetrical kidneys. Renin should also be measured in the small proportion of patients with severe untreated hypertension or in whom hypertension is resistant to at least a triple therapy including a diuretic, all administered at an optimal and maximally tolerated dosage. In such cases, an unsuspected form of secondary hypertension or a pronounced drug-induced stimulation of the RAS may be found. Such findings may indicate that additional etiologic evaluation is required or that an ACE inhibitor, an Ang receptor antagonist or a ß-blocker should be added to the treatment regimen.

Renin-Dependent Forms of Hypertension (Table 2)

Primary Hypertension

About one in six patients with essential hypertension have high renin levels. The purpose and efficiency of screening for high renin hypertension in this setting is discussed above. Most patients with malignant hypertension have high renin levels. The renin concentration is very high in patients with hyponatremic hypertensive syndrome, leading to hyperangiotensinemia with thirst,

Table 1 When to measure renin?

Setting	*Aim*	*Comment*
Mild or moderate untreated hypertension	Determination of renin subgroup for prognostic or therapeutic purposes	Classification of individual patients into low or high renin subgroups probably not cost-effective in this setting
Severe untreated hypertension (diastolic BP consistently above 110 mm Hg) and/or hypokalemic hypertension	Guidance for subsequent etiologic investigations	High renin levels suggest that CT-angiography, magnetic resonance angiography, or Duplex Doppler sonography should be performed, whereas low renin levels suggest adrenal investigations
Drug-resistant hypertension	Guidance for antihypertensive treatment adaptation	Discontinue current treatment (calcium channel antagonists and α-blockers may be continued) and determine plasma renin levels. High renin levels suggest that a ß-blocker, an ACE inhibitor or an angiotensin II receptor antagonist should be added to the previous treatment regimen
Hypertension with renal failure	Guidance for antihypertensive treatment adaptation	Low or normal renin levels suggest that the daily dose of loop diuretic should be increased, high renin levels suggest that an ACE inhibitor should be added
Hypertension with renal artery stenosis or unilateral small kidney or kidney tumor	Evaluation of asymmetrical renin secretion before nephrectomy or renal artery angioplasty	A RVR ratio $\geq$ 1.5 is generally associated with a favorable BP outcome following nephrectomy or renal artery angioplasty

Table 2 Etiology, presentation and key findings in renin-dependent hypertension.

Underlying disease	*Usual presentation*	*Key tests and findings*
Primary hypertension		
Benign	Non specific. 10–15% of patients with primary hypertension belong to the high renin subgroup	Larger than average response to ß-blockers or ACE inhibitors
Accelerated or malignant	Headache, thirst, weight loss, diastolic BP usually $>$140 mm Hg with hypokalemia, chronic kidney disease, proteinuria	Funduscopy: hemorrhages, exudates, papilledema. An underlying renal disease is present in $\approx$50% of cases
Renal ischemia		
Renal artery stenosis	Recent, progressive and/or severe hypertension, hypertension in young females (fibromuscular dysplasia) or in patients with angina or arteritis (atherosclerosis), hypertension with ACE inhibitor-induced rise in plasma creatinine	Renal angiogram: renovascular (curable) hypertension is probably present if reduction in artery diameter (i) exceeds 75% or (ii) is between 50 and 75% with a positive captopril test, a lateralized captopril renography or a RVR ratio $\geq$1.5
Renal infarction	Hyponatremic hypertensive syndrome: lumbar pain and hematuria followed by acute hypertension, polyuria, hypokalemia, hyponatremia and very high renin concentrations	Segmental renal infarction is detected by CT scan and its cause is determined by renal angiogram (embolism, thrombosis, renal artery dissection or occlusion)
Polyarteritis nodosa	Systemic necrotizing vasculitis	Widespread microaneurysms on renal angiogram
Systemic scleroderma	Skin thickening, Raynaud's phenomenon, progressive hypertension and chronic kidney disease	Clinical presentation. ACE inhibitors greatly improve scleroderma crisis
Renal tumors and cysts		
Juxtaglomerular cell tumor	Severe hypokalemic hypertension with very high prorenin and active renin levels	Normal renal angiogram with a small cortical tumor on CT scan
Other renal renin-producing tumors	Hypertension with renal cell carcinoma	Primary reninism is present and BP returns to normal following nephrectomy in about 5% of cases
Compressive cysts and tumors	Hypertension with ultrasound scan evidence of a large cyst or tumor	Improvement in BP following cyst drainage or tumor resection may be expected if RVR ratio exceeds 1.5
Polycystic kidney	Family history of renal cysts, hypertension and chronic kidney disease	Ultrasound scan evidence of $>$3 bilateral cysts in a person with a positive family history
Unilateral non-vascular small kidneys	Hypertension with asymmetrical kidneys and frequently a history of recurrent urinary tract infection	Pyelographic or ultrasound scan evidence of pyelonephritic scarring, reflux nephropathy and/or segmental hypoplasia
Extrarenal tumors		
Pheochromocytoma	Paroxysmal hypertension with vasomotor symptoms	High urinary catecholamine metabolites, adrenal tumor
Ectopic primary reninism	Malignant tumor with hypertension and/or hypokalemia	Rare cases of lung, liver, pancreas or ovary cancers

polyuria and hyponatremia, and secondary aldosteronism with potassium wasting and hypokalemia. Malignant hypertension may be primary or, as in 45% of cases, the consequence of an underlying renal or adrenal disease.

Renal Artery Stenosis

Renal ischemia associated with renal artery stenosis [RAS] is the most frequent condition occurring with renin-dependent hypertension. About 80% of stenoses are due to atherosclerosis, usually in patients over the age of 50 years. Ten percent are due to fibromuscular dysplasia which is observed mostly in female patients. The gold-standard procedure for diagnosing RAS is catheter angiography. For screening purposes, this invasive procedure can be replaced by CT-angiography, magnetic resonance angiography, or renal Duplex ultrasound. These imaging procedures estimate the frequency of RAS at 1% in unselected patients with hypertension, and 10–30% in patients with drug-resistant hypertension or with atherosclerosis elsewhere.

The standard for defining renovascular hypertension is a favorable BP outcome following revascularization. This definition is retrospective and the actual frequency of the condition is low because hypertension reversal is dependent on several parameters such as stenosis etiology, patient's age, parenchymal consequences of hypertension, and the feasibility of revascularization. In patients with atherosclerotic RAS, randomized trials comparing percutaneous revascularization plus medication to medication alone reported no improvement in BP or renal function following angioplasty (generally with stenting). In patients with fibromuscular dysplasia, retrospective series reported a hypertension cure rate (normal BP without medication) of 30–50%.

Considering the risks associated with percutaneous angioplasty or renal artery surgery, several attempts have been made in the past to design tests to select RAS patients who have truly renin-dependent hypertension. In such patients, the captopril test is expected to induce a sharp decrease in BP. The captopril test, with concurrent determination of plasma renin levels, is predicted to induce a homeostatic increase in renin secretion from the stenotic kidney. Although the captopril test has been analyzed in

numerous patients at risk of having renovascular hypertension, a complete analysis is not possible because of multiple inconsistencies in patient selection, standards, test procedures, and cutoff points. This test has now been abandoned.

Simultaneous determination of renin in both renal veins has also been largely used in the past to predict BP outcome following revascularization in patients with unilateral RAS. If the renal vein renin [RVR] ratio (i.e. the ratio between the RVR levels on the stenotic and non-stenotic sides) exceeded 1.5, the stenosis was assumed to cause renin-dependent hypertension amenable to angioplastic or surgical cure. The test could be performed after acute oral captopril administration. Post-captopril renal scintigraphy was a less invasive alternative to RVR determination and was more accurate than the captopril-renin test especially in unilateral RAS. Considering the poor outcome of revascularization in patients with atherosclerotic RAS, performing the captopril test, the determination of RVR ratio or captopril scintigraphy is no longer recommended. RVR determinations should only be performed in complex cases such as branch stenoses or renovascular disease associated with focal renal parenchymal infarction. In patients with fibromuscular dysplasia, defining the precise degree of stenosis may be challenging especially in patients with multifocal string-of-beads type of stenosis.

Other Conditions Associated With Renal Ischemia

A few conditions characterized by chronic or acute intra-renal ischemia may also induce renin-dependent hypertension. These include systemic diseases such as polyarteritis nodosa and scleroderma, in which BP and renal outcomes are greatly improved by ACE inhibitors, and segmental renal infarction. Segmental renal infarction may result from renal embolism, renal artery thrombosis or dissection, or *in situ* thrombosis in cases with coagulation disorders. It usually presents as an abrupt-onset malignant hypertension with hyponatremic hypertensive syndrome. The condition may revert spontaneously to normotension because the ischemic renal tissue, which releases large amounts of renin, may subsequently turn into a silent renal scar.

Renal Tumors and Cysts

Primary reninism is a very rare, purely renin-dependent form of hypertension. It may be caused by juxtaglomerular cell tumors, malignant kidney tumors (nephroblastoma, adenocarcinoma), epithelial or soft tissue tumors. Primary reninism is associated with very high PRC and by the absence of RAS and intra-renal ischemia as determined by renal angiography. A small, hypodense renal cortical tumor (juxtaglomerular cell tumor) or a larger renal or extrarenal tumor is then detected by CT scan. BP usually drops during ACE inhibition and hypertension can be cured by tumor resection, although it may recur if metastases develop in exceptional malignant cases. In the 43 reported cases of juxtaglomerular cell tumors, hypertension was severe and associated with hypokalemia. PRA or PRC was markedly elevated in all cases. CT scan revealed small renal tumors (diameter 25 [8–50] mm).

Some large non-renin-secreting cysts and tumors may be found in cases of renin-dependent hypertension as the mechanism of renin stimulation is renal artery compression. In selected cases, with a lateralized RVR ratio, cyst drainage or tumor resection may improve or cure hypertension.

Unilateral Non-Vascular Small Kidneys

Pyelonephritic scarring associated with urinary tract infection and vesicoureteric reflux can cause childhood hypertension and progressive degradation of renal function. Renin is often high and has been shown to be the cause of hypertension. High renin levels may also occur in cases of renal hypoplasia. In cases with a very small unilateral kidney and a lateralized RVR ratio, unilateral nephrectomy may improve hypertension.

Extrarenal Tumors

The high BP levels associated with pheochromocytoma are caused by high plasma catecholamine concentrations both directly, through stimulation of vascular alpha-adrenergic receptors, and indirectly, through renin activation mediated by the beta-adrenergic stimulation of juxtaglomerular cells and renal vasoconstriction. Consequently, ACE inhibition may be used to control BP before surgery.

As mentioned above, rare cases of primary reninism may be due to extrarenal tumors.

Hyperreninemia With Normal Blood Pressure Levels

In most conditions with normal BP levels, hyperreninemia is a homeostatic response to reduced renal perfusion pressure or plasma flow. These conditions include dehydration, hemorrhage, diuretic use or pharmacological vasodilatation, cardiac failure, and reduced plasma volume due to hypoproteinemia in patients with nephrotic syndrome or kidney failure. These conditions have numerous signs and symptoms that cannot be listed in this chapter, and the assessment of the RAS has no added diagnostic value. Renin levels are related to survival in patients with cardiac failure, high levels being associated with a poor prognosis. The

prognostic value of atrial and brain natriuretic peptides is higher than that of renin, however, meaning that the latter is rarely used to assess prognosis.

Bartter syndrome, or inherited hypokalemic metabolic alkalosis, is an autosomal recessive disorder due to inactivating mutations of NKCC2 gene which codes for the Na-2Cl-K channel at the level of the Henle's loop which is characterized by salt wasting, insensitivity to the vasoconstrictive effects of Ang II, and consequently high renin levels with normal BP, hyperaldosteronism and hypokalemia. Gitelman syndrome is another salt loosing tubulopathy due to inactivating mutations of the NCC gene which encodes for the NaCl cotransporter at the level of the distal tubule in which patients have hypomagnesemia and hypocalciuria. Recent advances in the field of molecular genetics have demonstrated that this disease is caused by four genetically distinct abnormalities that result from mutations in renal electrolyte transporters and channels.

See also: Hypertension and the Renin–Angiotensin–Aldosterone System. Interference With the Renin–Angiotensin System (RAS): Classical Inhibitors and Novel Approaches. Renal Vein Renin Measurement as a Diagnostic Tool

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Renal Vein Renin Measurement as a Diagnostic Tool☆

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Abbreviations

Ang	Angiotensin
DRC	Direct (active) renin concentration
PRA	Plasma renin activity
RAO	Renal artery occlusion
RPT	Renin-producing tumors
RVH	Reno-vascular hypertension
RVR	Renal vein renin
RVR	Renal vein renin ratio

Definition

The measurement of renin in the renal vein (RVR) blood has been a powerful tool to understand the pathophysiology of renovascular hypertension (RVH). Experience has shown that it is clinically useful for diagnosing this condition as well as for identifying renin-producing tumors.

Introduction

The first to report an understanding of the phenomenon by which renal ischemia can raise blood pressure was Johan Loesch, a physician born in 1897 in a province of the Austro-Hungarian Empire, emigrated to the United States. In 1927 Loesch published in the *Archives of Pathology* a preliminary report of his studies on hypertension induced by renal ischemia, which he had performed in the Physiatric Institute in Morristown, New Jersey (Glodny and Glodny, 2006). Loesch's findings and conclusions were eventually published in an article titled "A contribution to Experimental Nephritis and to Arterial Hypertension" in a German-language journal, *Zentralblatt für Innere Medizin*, in February 1933, 1 year before Harry Goldblatt's most famous work (see later). After publishing few other papers out of the field of hypertension, Loesch moved to private practice and left research and there were no other scientific traces of his work. His seminal work was totally neglected by the scientific community and rapidly forgotten.

In 1932 at the Academy of Medicine in Cleveland, Oh, Harry Goldblatt presented the results of several experiments that he and his colleagues had been performing during the previous 4 years by inducing renal ischemia in dogs (Goldblatt *et al.*, 1934). Apparently Goldblatt and his coworkers had no knowledge of Loesch's prior work. Goldblatt and colleagues had invented a clip to constrict the renal artery and succeeded in inducing persistent elevation of blood pressure by clipping one renal artery. These results were eventually published in the Journal of Experimental Medicine in 1934. Peyton Rous, who was later awarded the Nobel Prize, on behalf of his co-Editor-in-Chief fully recognized the importance of the manuscript they had received and in the letter of acceptance wrote: *It is a pleasure for us as editors to receive a paper of such large significance, so admirably written*.

This article is one of the most highly cited in the medical literature and stimulated more medical research than any single experiment in medical history. The experiment described by Goldblatt and colleagues not only demonstrated that a factor released by the ischemic kidney entails one of the most likely mechanisms by which persistent arterial hypertension occurs, but also indicated that the ischemia of to one kidney is a sufficient condition for the production of persistently elevated BP.

An enormous number of studies have thereafter proved beyond doubts the concept that renin, which had been discovered in Scandinavia about 40 years before Goldblatt's studies, is a major mediator of hypertension induced by renal ischemia has been. This has resulted into a huge number of procedures worldwide aimed at correcting arterial hypertension through relief of renal ischemia and renin over secretion, including surgical revascularization, percutaneous transluminal renal angioplasty (PTRA) and stenting (Harden *et al.*, 1997; Radermacher *et al.*, 2001; Van Jaarsveld *et al.*, 2000). Nonetheless, strangely enough, Goldblatt did not receive the Nobel Prize in Medicine.

Pathophysiologic Principles

The induction of renal ischemia is followed within few minutes by renin release, both within the kidney and in the renal vein blood. In the kidney, locally generated Ang II serves mainly to constrict the postglomerular (efferent) arterioles to maintain glomerular pressure and thus glomerular filtration rate despite the fall in renal perfusion pressure.

Micro-perfusion preparations in vivo have in fact unequivocally confirmed that Ang II is a more potent vasoconstrictor of the efferent than of the afferent arteriole and that this difference is mediated by nitric oxide (NO)- and prostaglandins (PG)-induced modulation of Ang II-vasoconstriction in the afferent arteriole. The vasoconstrictor effect of Ang II on the efferent arteriole is

☆*Change History:* January 2018. Gian Paolo Rossi made some minor updates to the text.

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known to be modulated by PG produced upstream in the glomerulus and, therefore, the intrarenal generation of Ang II and its interactions with renal autacoids are key determinants of glomerular hemodynamics and glomerular filtration rate (Arima *et al.*, 2003).

The fall of renal perfusion pressure, either induced experimentally or caused by a renal artery stenosis, determines an initial release of renin, which is followed by activation of renin synthesis in cell phenotypes, such as the vascular smooth cells in the afferent (preglomerular) arterioles wall, which, under physiological conditions, do not synthesize renin. The recruitment of these cells explains why both renin synthesis and renal renin content are markedly increased after induction of renal ischemia. As time goes, the increase of systemic arterial pressure triggered by enhanced Ang II generation, along with the stimulation of sodium reabsorption resulting from a direct tubular effect of Ang II and from stimulation of aldosterone synthesis and secretion, brings renin secretion back toward values that can be seen as normal, but in reality are inappropriately high for the prevailing level of blood pressure and volume status (Vaughan *et al.*, 1973).

Ang II also exerts strong additional effects in that it induces an increase of wall-to-lumen ration and media cross-sectional area of small (100–300 μm lumen diameter) arterioles, for example, hypertrophic remodeling, which, enhances the pressor effect of Ang II at no energy cost, the so-called Folkow's structural amplifier.

Because of this time course the chances of detecting an activated renin angiotensin system and high plasma renin are, from the clinical standpoint, high when the patient is seen soon after a given renal artery obstructive lesion has become hemodynamically relevant, but wanes off afterwards.(Lüscher *et al.*, 1986).

Accordingly, it can be easily understood why at the time of diagnosis up to one third of the patients with renovascular hypertension (RVH) do not have high plasma renin, both in the peripheral blood and in vein blood from the affected side, unless there is marked persistent renal ischemia as it occurs in those with total occlusion of a renal artery (Rossi *et al.*, 1997; Pickering *et al.*, 1986; Marks and Maxwell, 1975). For these reasons, after the initial enthusiasms the measurement of plasma renin in peripheral plasma and in renal vein blood as a tool to diagnose renal ischemia and to predict the outcome of revascularization has lost ground, even though it still has an important place in the diagnostic work-up of at least two conditions, total renal artery occlusion and renin-producing tumors.

Methodology

Patients Selection

Renal vein renin measurement (RVR) is an invasive procedure, albeit minimally so, which means that patients should be selected for this test based on high degree of suspicion (pretest probability) of RVH, which can be established by using the criteria of Mann and Pickering or recommendations from updated guidelines such as those from American Heart Association (Whelton *et al.*, 2017).

Patients Preparations for RVR Measurements

Multiple conditions and drugs can affect RVR levels (**Table 1**); therefore, adequate preparation of the patient is mandatory in order to achieve the best diagnostic accuracy of this invasive test. The patient should be on a normal sodium intake, as verified by measuring 24-h urinary sodium excretion on the day of the test. Furthermore, since renin secretion is controlled by the sympathetic nervous system, which undergo a circadian pattern of activity, ideally RVR studies should be performed in the morning,

Table 1 Preparations of the patient for RVR studies

1. Put on a normal Na intake (about 6.3 g NaCl or 130 mmol/day) for 1 week.
2. Measure 24-h Na^+/K^+ excretion on the day of the RVR study.
3. Perform the test in the morning between 8 and 12 a.m.
4. Keep the patient supine for at least 1 h prior and during the test.
5. Avoid any stress by explaining the procedure and use benzodiazepines.
6. Control blood pressure with a long acting calcium entry blocker and/or doxazosin.
7. Withdraw mineralocorticoid receptor antagonists (spironolactone, canrenone and potassium canrenoate and eplerenone) at least 6 weeks before the RVR study.
8. Withdraw the following drugs for at least 3 weeks prior to RVR study:
   - diuretics*
   - β-blockers
   - direct vasodilators (minoxidil, hydralazine)
   - ACE—inhibitors
   - AT-1 receptor antagonists (ARBs, sartans)

between 8.00 and 12.00 a.m., after the patient has been fasting overnight and kept lying quietly in the supine position for at least 1 h before the test.

The drugs that alter renin-secretion and, therefore, must be withdrawn for at least 3 weeks prior to the RVR testing are listed in **Table 1**.

Use of a long-acting calcium entry blocker, such as verapamil or a long-acting dihydropyridine compound, alone or combined with doxazosin, if necessary to achieve a decent blood pressure control before and during the test, can be allowed as these agents under chronic conditions negligibly affect renin secretion.

Catheter Placement

A previous angio computed tomography with renal vein anatomy reconstruction should be performed before RVR studies, as it can guide the interventionist in accomplishing selective catheterization of the renal veins and their branches, besides allowing identification of the underlying renal or renovascular disease.

Several factors (listed in **Table 2**), can concur to determine inaccurate results of RVR studies, thus increasing the number of false negative and positive results. Utmost care should, therefore, be exercised in obtaining blood selectively from the renal vein, a task which can be a difficult undertaking, because on venography blood flow is toward the catheter lumen, often leaving uncertainties as to exact positioning of the catheter's tip. Accordingly, it is not uncommon to draw diluted renal blood, for example, blood not draining solely from the kidney. Additional problems relate to the fact that (a) multiple renal veins are present in a substantial proportion (20%–28%) of patients on the right side and in much lower (1%–3%) proportion on the left side, thus setting the stage for collection of less "pure" renal vein blood on the right than on the left side; (b) the right renal vein is shorter than the left renal vein thus exposing more commonly to admixture of vena cava blood on the right than on the left side; (c) plasma drawn from the lower pole vein might not identify an upper pole (segmental) ischemia and vice versa.

Nonsimultaneous Sampling

The use of a single catheter for the sampling of both renal veins can expose to the risk of detecting a factitious gradient between the kidneys or sites of sampling if the interventionist is not fast enough, particularly if an abrupt change in renin secretion occurs between the time of sampling on different sides/sites. Therefore, bilateral catheterization with simultaneous blood sampling is advisable unless the time elapsing between sampling from the two sides can be kept within 5 min. Furthermore, it is necessary to avoid any maneuver that can increase abruptly renin secretion, such as stressful stimuli, or administration of drugs (**Table 1**).

Assay Variability

The measurement of plasma renin, as plasma renin activity, shows some variability (ranging between 8% and 20%), even in the same laboratory and in experienced hands, which should be kept in mind in interpreting the results of RVR. However, it has been estimated that RVR ratio greater than 1.4 would almost never occur simply on the basis of within assay variability.

With the newer chemiluminescent automated assays of immunoreactive active renin there are several advantages and less variability (Rossi *et al.*, 2016) mainly because these methods do not depend on the assumption that the generation of Ang I occurs

Table 2 Factors responsible for failure of RVR determinations to predict response to revascularization accurately

Mix-up of Samples
Problems Related to renal lesions
– Bilateral disease
– Branch or segmental disease
Problems related to renal vein catheterization
– Renal vein not catheterized
– Multiple renal veins
– Dilution of renal vein blood
● Short renal vein on the right
● Gonadal vein enters renal vein on the left
Problems related to renin secretion
– Blunted secretion (usually due to beta-blockers or non-steroidal antiinflammatory drugs)
– Declining secretion (elderly patients, long standing renal artery stenosis)
Nonsimultaneous sampling
Assay variability

with a zero order kinetic as the renin substrate (angiotensinogen) is present in large excess. In fact, this assumption may not be verified if renin production is very high as in total renal artery occlusion or renin-producing tumours. Moreover, the direct assay of active immunoreactive renin assay provides a measurement of the concentration (not the activity) of active renin (DRC) and therefore is, because of this, to be preferred when measuring high or very high renin values as typically occurs in RVR studies in patients with total renal artery occlusion and renin-producing tumors.

Summary of Recommendations

In summary, to gain maximal diagnostic information from RVR studies protocols should take into consideration the principles listed in **Table 3**.Criteria for Interpretation of Results of RVR Measurements

The results of RVR studies can be interpreted by using the four different indexes reported in **Table 4**.

According to studies performed at Cornell in the last decades of the last centuries, (Vaughan *et al.*, 1973; Pickering *et al.*, 1986) if a patient with unilateral hemodynamically relevant renal artery stenosis is seen during the first stage of the disease, for example, when renin is high, the renal venous-arterial renin difference relative to arterial levels from the affected (Visch-Viivc)/Viivc kidney should range between 0.24 and 0.50 and values >0.50 would indicate reduced renal blood flow.

Conversely, values of the renal venous-arterial renin difference relative to arterial levels from the unaffected (Vctl-Viivc)/Viivc kidney close to zero would indicate absent renin secretion, for example, contralateral suppression. According to these premises, a value of [(Visch-Viivc)/Viivc] + [(Vctl-Viivc)/Viivc] <0.50 should suggest either incorrect sampling or a segmental disease, and therefore the RVR study should be repeated with segmental venous sampling.

Some Critical Considerations on Use of Indexes Derived From RVR Measurements

These important studies have provided the framework for the proper interpretation of RVR studies. However, there are several situations in which this simple scheme cannot be applied, such as those with bilateral hemodynamically relevant renal artery obstruction and those with segmental renal artery stenoses. Furthermore, as discussed below, the usefulness of the (Visch-Viivc)/Viivc] and the (Vctl-Viivc)/Viivc indexes did not seem to provide better diagnostic information over the simpler RVR ratio (RVRR).

To date only one study from our group has prospectively investigated by up-to-date statistical procedures, such as the receiver operator characteristics (ROC) curve analysis, in relatively large populations of patients, the usefulness of the indexes derived from the measurement of RVR (Rossi *et al.*, 1997). Until then, this lack of information had generated some confusion as regards the optimal cutoff values. As an example, even the empirically derived most popular cut-off value of 1.5 for the RVRR was never tested for its accuracy with the ROC curve analysis. Furthermore, only limited information existed on the usefulness of RVR measurements in patients with total renal artery occlusion and with bilateral renal artery stenoses. Thus, it remained unclear which of these

Table 3 Recommendations for RVR studies

1. Meticulous labeling of specimen
2. Placement of catheter's tip selectively (deeply) into the renal vein
3. Collection of segmental venous blood, when clinically indicated
4. Performance of the test during steady state renin secretion
5. Simultaneous renal vein sampling (use of separate catheters)
6. Proper consideration to renin assay variability in interpretation of results
7. Preference should be given to DRC assays
8. Frequent interaction between clinician and interventionist

Table 4 Indices derived from RVR studies

A. Indices of lateralization of renin secretion

- RVRR = the ratio of affected and unaffected side, for example, the DRC value in renal vein blood from the ischemic kidney over the DRC value in renal vein of blood from the contralateral kidney
- (Visch-Viivc)/Viivc = the renal venous-arterial DRC difference relative to arterial[a] levels from the affected kidney

B. Indices of contralateral suppression of renin secretion

- Vctl/Viivc = the ratio of unaffected side and infrarenal inferior vena cava blood, for example, the DRC value in renal vein of blood from the contralateral kidney over the DRC value in blood from the inferior vena cava
- (Vctl-Viivc)/Viivc = The simultaneous renal venous-arterial[a] DRC difference relative to arterial levels from the unaffected kidney

[a]The values measured in the infrarenal inferior vena cava blood (Viivc) are usually taken as surrogate of the level in arterial blood. DRC = direct renin (active) concentration.

indexes furnished the best diagnostic accuracy and at which cut-off values the optimal combination of sensitivity and false positive rate can be accomplished.

Clinical Relevance

The RVR measurement was introduced in the clinical arena three decades ago to demonstrate a unilateral overproduction of renin and thus to establish a pathophysiological link between ischemia-triggered activation of renin synthesis, and high BP. The usefulness of RVR for predicting the blood pressure response to angioplasty or stenting has been confirmed also recently. Nonetheless, most investigators would now support the concept that lateralization of renin secretion is not a prerequisite for cure of hypertension upon revascularization. There is, indeed, a general consensus among experts that RVH can only be diagnosed retrospectively, for example, upon demonstration of either normalization of a significant fall of BP values after correction of renal ischemia, due to the lack of accuracy of all tests that have been proposed over the years to predict the outcome of renal revascularization. As regards RVR, this might be due to the fact that at least one third of patients with proven RVH do not have evidence of an activated renin-angiotensin system at the time of diagnosis.

Although some large clinical trials, including the CORAL and the ASTRAL, (ASTRAL Investigators *et al.*, 2009; Cooper *et al.*, 2014) would support the contention that endovascular treatment by means of PTRA plus stenting does not offer substantial advantages in the patients with atherosclerotic renal artery stenoses who do not have compelling indications for revascularization, (Hirsch *et al.*, 2006) the results of a meta-analysis indicate that endovascular treatment is superior to medical treatment at least for control of diastolic blood pressure and lowering the need for antihypertensive drugs (Caielli *et al.*, 2015).

Thus, currently PTRA and stenting should still be used as effective alternative to surgery for the treatment of renovascular hypertension (RVH), because they imply substantially lower risks and minor costs, as compared to surgery, and can be performed on the same occasion of diagnostic angiography. Furthermore, they can be safe and effective even in patients for whom surgery is deemed to be contraindicated, such as those with atherosclerotic ostial renal artery stenosis who can be at high risk due to a widespread atherosclerotic involvement.

Avoidance of RVR measurements could result into saving of time and money due to cutting of catheters and assay costs and therefore, which explains why RVR measurement has largely been abandoned.

Yet there are patients in whom this issue is still relevant, such as those with unilateral small kidney due to total renal artery occlusion and those with renin-producing tumors.

In the former patients nephrectomy remains the best therapeutic option and RVR measurement might be useful to pose the indication to surgery, as discussed below. In the latter patients, a clear-cut gradient of renin secretion can be the only clue to the identification of a small tumor. These tumors can be quite small and therefore may escape detection with CT, MR and even with angiography. Thus, in patients with biochemical evidence of secondary (renin-dependent) aldosteronism and no renal artery stenoses RVR studies with segmental sampling is mandatory.

Use of RVR Data in Clinical Practice

We have investigated several relevant issues concerning RVR by applying systematically the test over the years in patients undergoing digital subtraction angiography, because of a high pretest probability of RVH, with the specific aims of establishing the diagnostic accuracy of the indexes listed in **Table 4**. We focused our attention on the subsets of RVH patients with and without total renal artery occlusion and with unilateral and bilateral renal artery disease (Rossi *et al.*, 1997). Based on this study, answers to the following questions can be put forward.

Which is the Best Index Derived From RVR Measurements?

The RVRR, for example, the ratio between PRA in the ischemic over the contralateral site appears to be the index that provides the best discrimination between patients with and without RVH. The ratio (Visch-Viivc)/Viivc, albeit on average significantly different between patients with and without RVH, shows a greater overlap of values (Rossi *et al.*, 1997). When we examined the usefulness of the RVRR index for diagnosing RVH by ROC curve analysis we found that the area under the ROC curve was significantly higher than for the identity line. Thus, the RVRR furnishes an incremental gain for the diagnosis of RVH. In contrast, the two indexes of contralateral suppression (Vctl-Viivc)/Viivc and Vctl/A) do not provide a satisfactory discrimination between patients with and without RVH.

Can RVRR Allow Identification of RVH Due to Total Renal Artery Occlusion?

A significant proportion of RVH patients that are referred today to hypertension clinics can have a totally occluded renal artery and practically all of them have markedly elevated renin levels both in the peripheral blood, but much more so in the renal vein plasma of the affected side. This translates into a significantly higher ratio of the affected over unaffected side (RVRR), compared to the patients with stenotic (nonoccluded) RVH and to the patients without RVH (Rossi *et al.*, 1997). Thus, unambiguous evidence

of renin lateralization is strongly suggestive of a totally occluded artery and, therefore, RVR could be useful not only for distinguishing vascular (occluded) from nonvascular unilateral small kidney, an information also attainable with echo-color Doppler assessment of intrarenal artery blood flow, but also to establish the indication to nephrectomy.

Can RVRR Allow Identification of RVH Without Total Renal Artery Occlusion

The RVRR offers an incremental diagnostic value to diagnose RVH, but this might be mostly due to the contribution of the RVH patients with total renal artery occlusion. By examining this hypothesis, we could show a clear-cut shift to the left of the ROC curve, which unequivocally confirmed the usefulness of RVRR for diagnosing RVH due total renal artery occlusion. Of note, when patients with angiographic evidence of renal artery occlusion were excluded, there was a shift to the right of the ROC curve, indicating a decrease of the AUC. Thus, RVR identify far more accurately RVH with than without renal artery occlusion (Rossi *et al.*, 1997).

Can RVRR Allow Identification of RVH in Patients With Bilateral Renal Artery Lesions?

We investigated this question with the ROC curve analysis in the subsets of patients with unilateral and bilateral renovascular disease. We found that RVRR, as well as the other indexes, were more accurate in the former than in the latter patients (Rossi *et al.*, 1997). As mentioned above, even for bilateral renal artery lesions, this was particularly true for the identification of RVH caused by total occlusion of the renal artery. When patients without total occlusion of the renal artery were excluded, the AUC of the ROC curve did not differ significantly from the identity line for all indexes. Thus, this finding indicates that in the presence of bilateral lesions lateralization of renin secretion is detectable by RVR measurements only in the presence of a total renal artery occlusion.

What Is the Best Cutoff Value of the RVRR?

At the ROC curve and Youden index analysis we could find that the optimal cutoff value, for example, the value providing the best tradeoff between sensitivity and specificity, for the identification of RVH was 1.55. However, even in a selected population with a high prevalence of RVH this cutoff value corresponded to a low sensitivity (54%) and a relatively high false positive rate (15%) (Rossi *et al.*, 1997).

This finding was at variance with what discovered for identification of RVH due total renal artery occlusion. In this case the RVRR cutoff value that warranted the best combination of sensitivity (87%) and false positive rate (22%) was 1.70, for example, higher than that for identification of RVH. It can, therefore, be concluded that in patients with RVH due to total renal artery occlusion: (a) the RVR measurements can provide a conclusive diagnosis in the vast majority of the cases; (b) a higher cut-off value is needed to optimize diagnostic accuracy of RVR measurements-derived indexes.

Even with such cutoff value, which is higher than the commonly used 1.5 value, the false positive rate is 22%. This high rate is certainly not reassuring from the clinical standpoint and mandates some caution, particularly in the presence of RVRR close to but just above this value.

Since the detection of a unilateral small kidney at an abdominal ultrasound in hypertensive patients is not uncommon, these results are of importance for the following reason. The patients with a unilateral small kidney due to renal artery occlusion pose a special challenge from the therapeutic standpoint because, surgical revascularization can be preferred to nephrectomy, in that it provides a preservation of renal function when undertaken in cases with angiographically demonstrated distal reconstitution of the vessels and an evident nephrogram. However, even in these cases revascularization is often unsuccessful, because of coexistence of marked obliterative changes of the intrarenal vascular bed, and leads to a high rate of secondary nephrectomy. On the other hand, primary nephrectomy normalizes blood pressure in the vast majority of the patients with a unilateral small kidney due to total renal artery occlusion. Thus, prior knowledge of the RVR measurements can help to establish the indication for nephrectomy.

RVR in the Diagnosis of Renin-Producing Tumors

Renin-producing tumors (RPTs) are held to be rare as less than 160 cases have been reported in the literature since the first reported by Richmond (1964). These patients usually present with stage III and/or malignant hypertension, (Rossi *et al.*, 1993) but cases with milder phenotypes have been reported recently (Maiolino *et al.*, 2018). RPTs typically have high renin and hypokalemia due to secondary aldosteronism, but renin can be underestimated because of angiotensinogen consumption when using the PRA method and therefore it is possible that RPTs are much more common than usually held.

In line with this contention, at our institution after the replacement of the PRA with the DRC assay for measuring plasma renin we have diagnosed two RPTs in 6 months, which compares with two cases in the previous 30 years.

In both cases a first imaging (with CT and MR) was held to be inconclusive, and the diagnosis was made only after subsegmental RVR studies demonstrated a clear-cut increase of renin secretion from a given portion of the kidney. *Re*-evaluation of the CT then permitted to locate a 10 and 8 mm size tumor in the first and the second case, respectively.

Thus, RVR studies are fundamental to diagnose and locate these usually small tumors.

Conclusions

RVR measurements are most useful for identifying patients with a small unilateral kidney due to total renal artery occlusion and with renin-producing tumors. At variance, RVR measurements are of limited value for deciding on whether to undertake revascularization of a kidney with renal artery stenosis. Since nephrectomy provides the best chances of cure of hypertension in patients with a totally occluded renal artery, RVR measurements are useful for deciding on the indication for nephrectomy. Therefore, they should be performed whenever the clinical picture is suggestive of a small unilateral kidney due to total renal artery occlusion and/or of a renin producing tumor.

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See also: Hyperreninemia. Hypertension and the Renin–Angiotensin–Aldosterone System

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Interference With the Renin–Angiotensin System (RAS): Classical Inhibitors and Novel Approaches

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Classical RAS Blockers: Single Versus Combined Blockade

The renin–angiotensin system (RAS) is a major player in cardiovascular homeostasis, with its end-product angiotensin II inducing vasoconstriction, water and salt retention, aldosterone synthesis, and growth and remodeling. Not surprisingly given these effects, blockers of the RAS are now widely used for the treatment of cardiovascular and renal diseases. Currently, three types of such blockers are available for clinical application: renin inhibitors, angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 (AT_1) receptor antagonists (ARBs), blocking, respectively, angiotensin I generation from angiotensinogen by renin, angiotensin II generation from angiotensin I by ACE, and the above-mentioned effects of angiotensin II mediated via its major receptor, the AT_1 receptor (**Fig. 1**). Although originally it was thought that these drugs interfered with angiotensin II in the circulation, it is now well-established that the beneficial effects of these drugs are largely due to blockade of the generation or action of angiotensin II at tissue sites (Danser, 2003). According to this concept, angiotensin II is generated at tissue sites rather than in circulating blood. Multiple lines of evidence support this idea (Campbell *et al.*, 1993; Klotz *et al.*, 2005; van Kats *et al.*, 2005, 2001). Remarkably however, although several RAS components are locally expressed (e.g., ACE, AT_1 and angiotensin II type 2 (AT_2) receptors) (Batenburg *et al.*, 2004; Tom *et al.*, 2003; van Esch *et al.*, 2005), thereby allowing such local activity, renin and angiotensinogen are not (Danser *et al.*, 1994, 1997). Thus, the renin required for local angiotensin production is sequestered from the circulation, that is, is kidney-derived, and the angiotensinogen is derived from the major, if not the only, angiotensinogen-synthesizing organ in the body, that is, the liver. Indeed, current data do not support a contribution of nonhepatic angiotensinogen, for example, in the kidney or adipocytes (Koizumi *et al.*, 2016; Matsusaka *et al.*, 2012).

Renal renin release is inhibited by angiotensin II through activation of juxtaglomerular AT_1 receptors, and thus, when blocking angiotensin II production or AT_1 receptors (with ACE inhibitors and ARBs, respectively), the kidneys will release more renin, to counteract the effect of the RAS blocker. As a consequence of the rise in renin release, plasma renin activity (PRA) will increase, sometimes to levels that are several-fold above baseline. A rise in PRA will of course result in a parallel

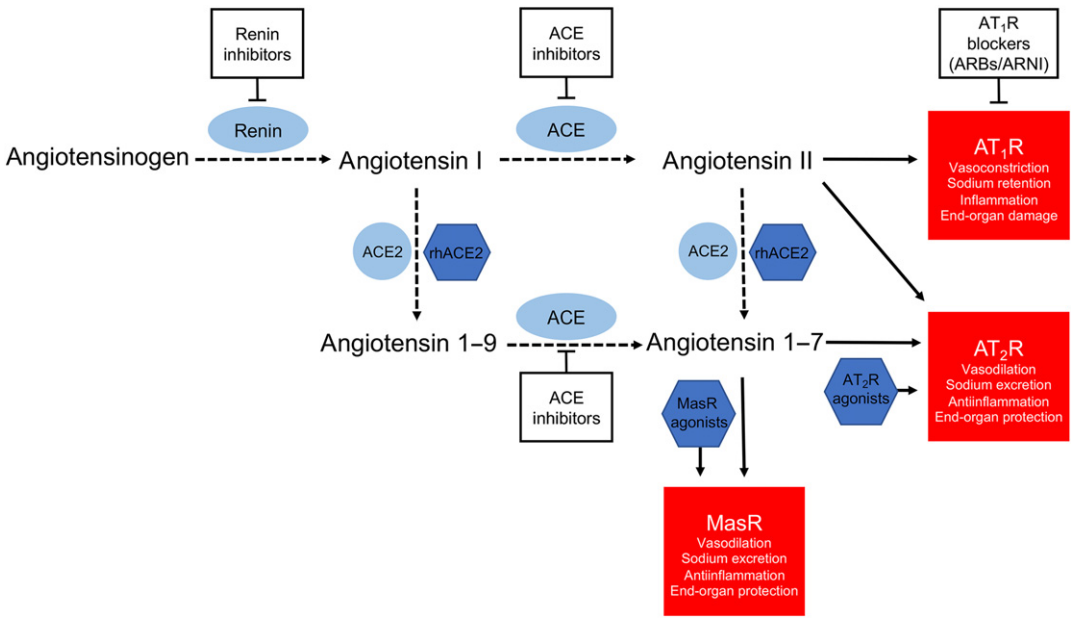

Fig. 1 Components of the renin–angiotensin system and main classes of pharmacological activators and inhibitors of the system. Angiotensin-converting enzyme (ACE); angiotensin-converting enzyme 2 (ACE2); angiotensin receptor blockers (ARBs); angiotensin receptor-neprilysin inhibitor, ARNI; angiotensin II type 1 receptor, AT_1R; angiotensin II type 2 receptor, AT_2R; recombinant human angiotensin-converting enzyme 2, rhACE2; Mas receptor, MasR.

rise in angiotensin I (Mooser *et al.*, 1990). During AT_1 receptor blockade, this rise in angiotensin I will lead to a similar rise in angiotensin II, whereas during ACE inhibition, the rise in angiotensin II will obviously be more modest. Depending on the degree of ACE inhibition however, angiotensin II levels may still return to or even rise above baseline (Campbell *et al.*, 1993; van Kats *et al.*, 2000). For instance, inhibiting ACE by 80% requires a fivefold rise in PRA to allow angiotensin II levels to return to normal.

Thus, plasma and tissue angiotensin II levels will not only rise during AT_1 receptor blockade, but often also following prolonged ACE inhibition ("angiotensin II escape"). During AT_1 receptor blockade, the rise in angiotensin II may result in AT_2 receptor stimulation. Since AT_2 receptors exert opposite effects as compared to AT_1 receptors (e.g., vasodilation) (Batenburg *et al.*, 2004), such AT_2 receptor stimulation has been proposed to contribute to the beneficial effects of AT_1 receptor blockers (Liu *et al.*, 1997). Nevertheless, under pathological conditions, AT_2 receptors may also induce constrictor responses (Verdonk *et al.*, 2015).

The rise in angiotensin II during ACE inhibition will result in activation of both AT_1 and AT_2 receptors. This activation probably occurs less efficiently than normal, since the majority of ACE is now inhibited (van Kats *et al.*, 2005). Normally, ACE generates angiotensin II in a highly efficient manner, in close proximity of AT_1 receptors (Saris *et al.*, 2002; Tom *et al.*, 2003). Consequently, little angiotensin II needs to be generated to obtain maximum (regional) AT_1 receptor stimulation. During chronic ACE inhibition, the increase in angiotensin I generation still allows angiotensin II generation by noninhibited ACE (possibly de novo synthesized ACE) or non-ACE converting enzymes like chymase (Tom *et al.*, 2003; Wei *et al.*, 1999). However, the latter type of angiotensin II generation is less likely to result in a high level of regional AT_1 receptor stimulation. In particular, angiotensin II generated by chymase (which is localized in the adventitia) will be subject to rapid metabolism in the interstitial space on its way to AT_1 receptors (de Lannoy *et al.*, 2001). Moreover, evidence for chymase-dependent angiotensin II generation in humans exclusively comes from studies in homogenized tissues, and whether chymase truly has any role in vivo remains to be proven.

Renin, unlike ACE, has only one substrate. As it is responsible for the first and rate-limiting step of the RAS cascade, without this enzyme (e.g., after a nephrectomy or in renin knockout animals) there are no angiotensins (Danser *et al.*, 1994). Thus, renin inhibitors, unlike ACE inhibitors and AT_1 receptor antagonists, will suppress both angiotensin I and II. As always during RAS blockade, this suppression of angiotensin II formation will result in a rise in renin. Due to the presence of the renin inhibitor however, such a rise in renin will not result in a rise in PRA, and this renin rise can therefore not be demonstrated by measuring the enzymatic (i.e., angiotensin I-generating) activity of renin. Therefore, to confirm this rise, renin must be measured directly, for example, with an immunoradiometric assay (Danser and Deinum, 2005). Importantly, renin rises during renin inhibition were found to be substantial, and increases of >300-fold have been noted (Balcarek *et al.*, 2014).

RAS inhibitors are potent antihypertensive drugs, with established cardiac and renal protective effects, related to and beyond their blood pressure lowering effect. These effects depend, at least in part, on their capacity to suppress aldosterone. Yet, following prolonged blockade, aldosterone levels often return to normal, due to the fact that angiotensin II is not the only aldosterone-regulating hormone, that is, other regulators (like K^+ and adrenocorticotropic hormone) now upregulate aldosterone synthesis. Based on their favorable renal outcomes, ACE inhibitors or ARBs are currently recommended as first-line treatment for patients with chronic kidney disease (CKD) (James *et al.*, 2014). However, effects on cardiovascular outcome and mortality are similar to that of other classes of antihypertensive drugs (Palmer *et al.*, 2015). Moreover, progression toward end stage renal disease is attenuated, but not prevented. Given the multiple feedback loops within the RAS, always allowing angiotensin II to restore its levels and/or effects, the idea has come up to use several RAS blockers in combination, for example in heart failure. There is a risk that such approaches may ultimately lead to RAS annihilation with adverse consequences such as hypotension, renal dysfunction and hyperkalemia (Danser and van den Meiracker, 2015; de Boer *et al.*, 2012). Important questions are therefore: what degree of RAS blockade should be achieved, and do we really need more than one agent for this? Theoretically, increasing the dose of a single RAS blocker should enable the desired degree of RAS blockade to be achieved, unless this dose increase results in adverse effects. For example, ACE inhibition can cause a rise in bradykinin levels, resulting in a dry cough. Continuous AT_2 receptor stimulation (as occurring during AT_1 receptor blockade) may not be beneficial in all patients. To what degree a several hundred-fold rise in renin is harmful is unknown. Increasing the dose of a single drug to an excessive level may therefore not be ideal; a better approach is to combine two or more RAS blockers at lower doses. Careful monitoring of blood pressure, renal function and hyperkalemia is required to determine the optimal number of RAS blockers for an individual patient (Nussberger and Bohlender, 2013). A worse outcome is to be expected in patients with low blood pressure and/or estimated glomerular filtration rate before the start of treatment, due to the fact the RAS is essential to preserve renal function and glomerular filtration. The kidneys will do everything possible to keep these in the normal range, including massive upregulation of renin during RAS blockade. This mechanism is referred to as the "nephrocentric" reaction to RAS blockade in patients with heart failure (Packer, 1987), and may ultimately even result in extrarenal angiotensin II upregulation.

In summary, the beneficial effects of RAS blockade are unequivocal, but the optimal degree of RAS blockade is not identical in each patient. Optimal RAS blockade may require more than one blocker, but simultaneously too intensive blockade of the RAS can be deleterious for the kidney, especially if renal function is already compromised when an additional RAS blocker is added. To solve this problem, either novel RAS-interfering agents might be considered (not blocking the classic renin/angiotensin II/AT_1 receptor axis), or the additional interference with hormonal systems beyond the RAS, like the atrial natriuretic system.

Novel RAS Interference: AT_2 Receptor and ACE2/Angiotensin-(1–7)/Mas Receptor Axis

AT_2 Receptor

The AT_2 receptor opposes all of the classical actions of the RAS mediated via the AT_1 receptor (**Fig. 1**). Preclinical studies have demonstrated that the AT_2 receptor exerts vasodilatory, natriuretic, antifibrotic, antiproliferative and antiinflammatory effects (Kaschina *et al.*, 2017; Mirabito Colafella *et al.*, 2016; Wang *et al.*, 2017). Expression of the AT_2 receptor is highest during fetal life and declines shortly after birth (Kaschina *et al.*, 2017). In general, females have greater expression of the AT_2 receptor as the gene encoding the AT_2 receptor is located on the X chromosome and the female sex hormones, estrogen and relaxin increase expression of the AT_2 receptor (Mirabito Colafella *et al.*, 2016). A polymorphism in the gene encoding the AT_2 receptor is associated with hypertension. During pathological situations including cardiac failure, cardiac fibrosis, renal failure, diabetes and atherosclerosis, expression of the AT_2 receptor may be upregulated (Kaschina *et al.*, 2017), suggesting that the AT_2 receptor plays a protective role in cardiovascular and renal diseases.

Compound 21 (C21)

Originally described in 2004, Compound 21 (C21) is the first selective nonpeptide AT_2 receptor agonist (Wan *et al.*, 2004). C21 is orally and systemically active and is ~4000-fold more selective for the AT_2 receptor than the AT_1 receptor (Bosnyak *et al.*, 2011). Cardiovascular, renal and neurological protective effects of C21 have been reported in various disease models, including stroke, obesity, ischemia–reperfusion injury, pulmonary hypertension, diabetic nephropathy and renal toxicity (Matavelli and Siragy, 2015). Similarly, C21 exerts protective cardiovascular and renal effects in genetic models of cardiovascular disease including the spontaneously hypertensive rat (SHR) and the stroke-prone-SHR (Kaschina *et al.*, 2017). C21 produces dose-dependent relaxation in isolated vessels from mice, rats and humans which is blocked by AT_2 receptor antagonism, although at high doses C21 can have off-target effects (Verdonk *et al.*, 2012). In vivo, C21-mediated vasodilation is usually only apparent in the presence of an ARB (Matavelli and Siragy, 2015). However, it was recently reported that chronic C21 treatment abolishes angiotensin II-induced hypertension in female rats without concomitant AT_1 receptor blockade (Kemp *et al.*, 2016). Within the kidney, C21 has potent diuretic and natriuretic effects which have been observed in normotensive, hypertensive and diabetic rodents (Kaschina *et al.*, 2017). AT_2 receptor stimulation with C21 reduces renal vascular resistance and enhances renal blood flow, urinary sodium and fractional sodium excretions without altering glomerular filtration rate. Moreover, the natriuretic effect of C21 involves changes in sodium'hydrogen exchanger 3 (NHE3) and Na^+/K^+-ATPase within the proximal tubule (Kemp *et al.*, 2016). C21 does not cross the blood–brain barrier. However, central AT_2 receptor stimulation with C21 inhibits sympathetic outflow, with decreases reported in renal sympathetic nerve activity and urinary norepinephrine levels in normotensive and hypertensive rats (Brouwers *et al.*, 2015; Gao *et al.*, 2014). Centrally administered C21 also reduced baroreflex sensitivity in rats with heart failure (Gao *et al.*, 2014). C21 has potent antifibrotic effects. Depending on the tissue, C21 elicits its antifibrotic effects via changes in transforming growth factor β1 (TGFβ1), matrix metalloproteinases, the NO/cGMP pathway and immune mediated mechanisms (Wang *et al.*, 2017). The antiinflammatory effects of C21 are mediated by changes in macrophage differentiation and infiltration, inhibition of the nuclear factor-κBα (NFκB) pathway, which modulates transcription of many cytokines, chemokines and vascular adhesion molecules including tumor necrosis factor-α, interleukin-1β and interleukin-6 and alterations in T-cell populations which are implicated in the pathogenesis of hypertension and cardiovascular and renal diseases (Kaschina *et al.*, 2017).

Interestingly, the majority of the protective effects of AT_2 receptor stimulation with C21 are blood pressure-independent, suggesting that C21 may not be suitable as an antihypertensive medication. However, C21 may be beneficial for end-organ protection in combination with established antihypertensive therapies due to its strong antifibrotic and antiinflammatory effects. Moreover, C21 may be a novel therapy for diseases where fibrosis and/or inflammation are significant aspects of the pathology. C21 has been granted orphan drug status for idiopathic pulmonary fibrosis and has completed stage 1 clinical trials to assess safety, tolerability, pharmacokinetics and pharmacodynamics in healthy men (Steckelings *et al.*, 2017) and in group of men with a compromised metabolic situation (body mass index of 30–35 and waist-to-hip ratio above 0.9; results are yet to be released).

Other AT_2 receptor agonists in development

Two novel AT_2 receptor agonists are currently undergoing phase 1 clinical trials. MP-157 (Mitsubishi Tanabe Pharma Corporation, Japan) is being developed as an antihypertensive therapy. MOR107 (formerly LP2; Lanthio Pharma, The Netherlands) has been reported to reduce alveolar septum and arterial wall thickness, and pulmonary inflammation in neonatal rats (Wagenaar *et al.*, 2013), and is being developed as an antifibrotic agent.

ACE2/Angiotensin-(1–7)/Mas Receptor Axis

The angiotensin-converting enzyme 2 (ACE2)/angiotensin-(1–7)/Mas receptor axis is an endogenous counter-current pathway to the classical RAS pathway (**Fig. 1**). ACE2 controls the pressor/depressor balance of the RAS by (i) converting angiotensin I to angiotensin-(1–9), limiting the amount of substrate available to generate angiotensin II, (ii) converting angiotensin II to angiotensin-(1–7), limiting angiotensin II stimulation of the AT_1 receptor and (iii) generation of angiotensin-(1–7), which is able to bind to its own receptor, Mas receptor or the AT_2 receptor, to oppose the pressor actions of the AT_1 receptor (**Fig. 1**) (Patel *et al.*, 2016). It should be noted that other enzymes can produce angiotensin-(1–7) from angiotensin I and II including neutral

endopeptidase and prolyl-endopeptidase (Santos, 2014). Once synthesized, angiotensin-(1–7) is rapidly broken down into inactive fragments via aminopeptidases and ACE (Santos, 2014).

The ACE2/angiotensin-(1–7)/Mas receptor axis has been shown to play protective roles in numerous disease models including heart failure, diabetic nephropathy, hypertension, liver disease and metabolic syndrome (Bernardi *et al.*, 2016; Moreira de Macedo *et al.*, 2014; Patel *et al.*, 2016; Rentzsch *et al.*, 2008; Ye *et al.*, 2006). In humans, ACE2 mRNA is upregulated in patients with heart failure. The protective effects of the ACE2/angiotensin-(1–7)/Mas receptor axis are primarily mediated by reductions in angiotensin II and proinflammatory cytokine release and inhibition of signaling pathways involved in tissue fibrosis (Rodrigues Prestes *et al.*, 2017). Similar to the AT_2 receptor, expression of the ACE2/angiotensin-(1–7)/Mas receptor axis is greater in females than in males (Mirabito Colafella *et al.*, 2016). The gene encoding ACE2 is located on the X chromosome and ACE2 polymorphism is associated with hypertension (Patel *et al.*, 2014). Furthermore, in women, the response to ACEi is influenced by ACE2 polymorphisms (Chen *et al.*, 2016). While there are numerous pharmacological strategies to enhance the ACE2/angiotensin-(1–7)/Mas receptor axis, including ACE2 supplementation/activators, angiotensin-(1–7) analogs and protection of angiotensin-(1–7) (encapsulated proteins) (Tamargo and Tamargo, 2017), the majority of these are limited to preclinical applications or have been withdrawn from clinical development.

Human recombinant ACE2 (rhACE2)

Preclinical studies have demonstrated that recombinant human ACE2 (rhACE2) has a half-life of ~8.5 h in mice (Wysocki *et al.*, 2010) and that rhACE2 exerts beneficial effects in murine models of cardiac hypertrophy, myocardial fibrosis and cardiac dysfunction (Zhong *et al.*, 2010). Furthermore, in male mice, rhACE2 was able to prevent angiotensin II-induced hypertension (Wysocki *et al.*, 2010). This effect was primarily attributed to circulating ACE2 activity and the lowering of plasma angiotensin II rather than the associated increase in plasma angiotensin-(1–7) (Wysocki *et al.*, 2010). Furthermore, in a model of diabetic nephropathy, rhACE2 reduced tubulointerstitial fibrosis and albuminuria and normalized blood pressure (Oudit *et al.*, 2010). Aside from its effects in cardiovascular and renal diseases, rhACE2 may be a novel therapy for acute lung injury. ACE2 deficient mice develop severe lung injury, which is ameliorated by treatment with rhACE2 (Gu *et al.*, 2016). Similarly, systemic administration of rhACE2 improved pulmonary hemodynamics and oxygenation in a lipopolysaccharide-induced model of acute respiratory distress syndrome in piglets (Treml *et al.*, 2010).

In 2013, the pharmacokinetic and pharmacodynamics characteristics of rhACE2 were first described in healthy men and women (Haschke *et al.*, 2013). The half-life of rhACE2 was ~10 h and in both men and women, rhACE2 reduced plasma angiotensin II and increased plasma angiotensin-(1–7) (Haschke *et al.*, 2013). This effect was apparent within 30 min of administration of rhACE2 and persisted for 24 h. Moreover, these effects were mediated without alterations in blood pressure or heart rate (Haschke *et al.*, 2013). However, it is important to note that this study was not powered to detect acute differences in physiology, rather, it was a proof of principle study. In 2017, a second clinical study with rhACE2 was reported in patients with acute pulmonary injury (Khan *et al.*, 2017). In this study, rhACE2 favorably altered the circulating RAS profile, reducing plasma angiotensin II and increasing angiotensin-(1–7) levels. However, rhACE2 did not improve physiological or clinical measures of acute respiratory distress syndrome. Consistent with the in vivo data, ex vivo treatment with rhACE2 effectively reduced angiotensin II levels and increased angiotensin-(1–9) and angiotensin-(1–7) levels in plasma and cardiac tissue samples collected from heart failure patients (Basu *et al.*, 2017). Collectively, these findings suggest that rhACE2 may be a promising drug for treatment of patients with intolerance to classical RAS inhibitors or in diseases where circulating angiotensin II is elevated.

Mas receptor agonists

Angiotensin-(1–7) has a short plasma half-life and is rapidly degraded in the gastrointestinal tract when given orally. The combination of hydroxylpropyl-β-cyclodextrin (HPβCD) with angiotensin-(1–7) (HPβCD/angiotensin-(1–7)) protects angiotensin-(1–7) from enzymatic degradation allowing angiotensin-(1–7) to be administered orally. Chronic oral administration of HPβCD/angiotensin-(1–7) lowered blood pressure and reduced markers of fibrosis (TGFβ1 and collagen type I) in rats following ischemia–reperfusion injury (Marques *et al.*, 2012). Moreover, HPβCD/angiotensin-(1–7) has been shown to have antiinflammatory effects in a model of atherosclerosis (Fraga-Silva *et al.*, 2014), and improved insulin sensitivity in a model of type 2 diabetes (Santos *et al.*, 2014). In humans, the HPβCD/angiotensin-(1–7) formulation allows the absorption of angiotensin-(1–7), and is safe and well-tolerated. Future clinical trials are now needed with HPβCD/angiotensin-(1–7) to determine its efficacy as a novel treatment for cardiovascular and renal diseases.

Interference With Systems Beyond the RAS: The Natriuretic Peptide System

Like the RAS, the natriuretic peptide system primarily regulates salt, fluid and pressure homeostasis (Zois *et al.*, 2014). In this respect, it can be perceived as an endogenous counterbalance of the RAS. Main effector molecules include A-type (atrial), B-type (brain) and C-type natriuretic peptide, as well as urodilatin. Via their second messenger cyclic guanosine 3′5′monophosphate (cGMP), natriuretic peptides not only counter the RAS by stimulating natriuresis, diuresis and vasodilation (Wong *et al.*, 2017), but also by directly inhibiting renin secretion (Kurtz *et al.*, 1986). Furthermore, cGMP improves myocardial relaxation and reduces cardiac hypertrophy (Tsai and Kass, 2009). Reinforcement of the natriuretic peptide system is therefore a rational and promising strategy to lower blood pressure and prevent complications in patients with essential hypertension.

Natriuretic peptides are degraded by neprilysin (neutral endopeptidase; NEP). Therefore, in the past, NEP inhibitors have been pursued as a potential new class of antihypertensive drugs. However, NEP degrades many enzymes, among which not only vasodilators (e.g., natriuretic peptides, bradykinin and adrenomedullin), but also potent vasoconstrictors (e.g., angiotensin II and endothelin-1) (**Fig. 2**). Hence, monotherapy with NEP inhibition can even raise blood pressure, as was shown in healthy individuals (Ando *et al.*, 1995). Consequently, subsequent strategies involved prolonging activation of the natriuretic peptide system by NEP inhibition on top of RAS blockade. This combination would hold the beneficial effects of increasing natriuretic peptides, while preemptively countering the harmful effects of a NEP inhibitor-induced rise in angiotensin II. The combination of ACE/NEP inhibition was attempted first, and successfully lowered blood pressure. However, both ACE and NEP inactivate bradykinin. As a consequence, dual inhibition often caused angioedema due to increases in bradykinin, prompting discontinuation of drug development (Kostis *et al.*, 2004; Packer *et al.*, 2002). Hence, a better approach is to combine an ARB with a NEP Inhibitor (ARNI). Still, the dose of the NEP inhibitor in this combination remains critical: when dosed too high, endothelin-1 levels start to increase, resulting in a rise in blood pressure (Roksnoer *et al.*, 2015).

Randomized controlled clinical trials demonstrated that ARNI is superior to the ARB valsartan in the treatment of patients with essential hypertension (Ruilope *et al.*, 2010), and also to the ACEi enalapril in patients with heart failure with reduced ejection fraction, in whom this treatment reduced hospitalization and mortality related to progression of disease (McMurray *et al.*, 2014). This led to the current registration of ARNI for this patient population (Ponikowski *et al.*, 2016). While simple RAS blockade is known to improve survival in patients with heart failure with reduced ejection fraction, it has little or no effect in patients with heart failure with preserved ejection fraction (Zheng *et al.*, 2018). However, a phase II trial in this patient population demonstrated a greater, albeit transient, reduction in N-terminal-proBNP at 12 weeks of treatment with ARNI, when compared to valsartan (Solomon *et al.*, 2012). Although this difference was no longer significant at 36 weeks of treatment, at this time point, ARNI superiorly reduced left atrial width and volume, echocardiographic indicators of diastolic dysfunction. Hence, the effect of ARNI on mortality in patients with heart failure with preserved ejection fraction is of great interest and currently under evaluation in a phase III clinical trial (Solomon *et al.*, 2017).

Interestingly, a post-hoc analysis revealed that estimated glomerular filtration rate was better preserved in patients with heart failure after ARNI, when compared to ARB (Voors *et al.*, 2015). In contrast, evaluation of urinary albumin-to-creatinine ratio

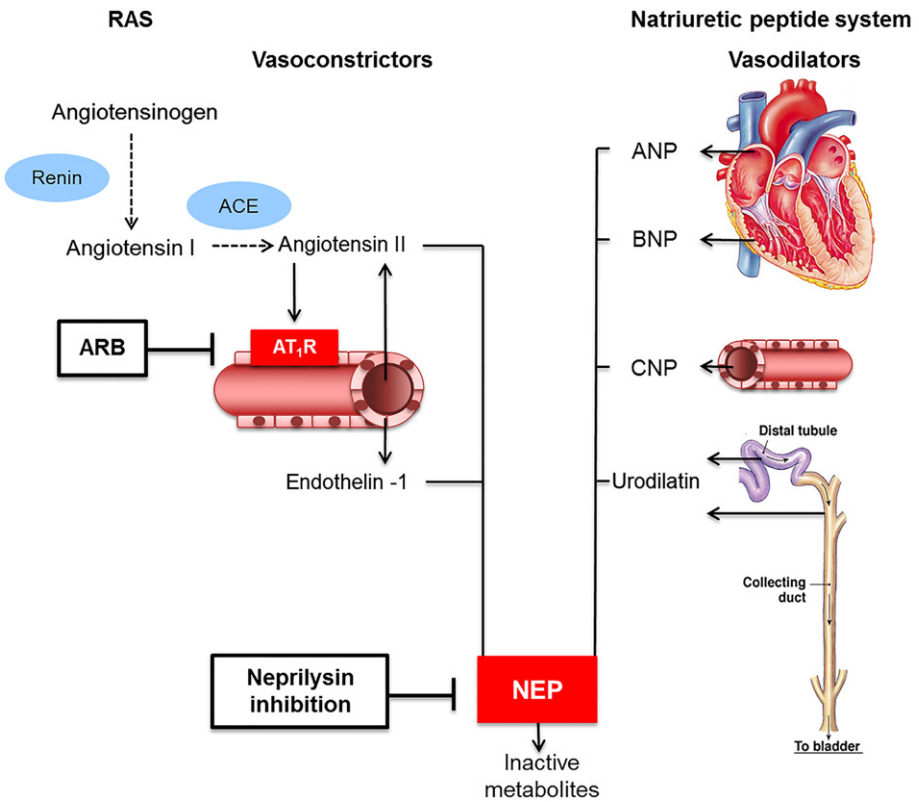

Fig. 2 The angiotensin II type 1 (AT_1) receptor-neprilysin (NEP) inhibition ("ARNI") concept. NEP degrades vasodilators (e.g., A-type (atrial) natriuretic peptide (ANP) produced by atrial myocytes, B-type (brain) natriuretic peptide (BNP) produced by ventricular myocytes, C-type natriuretic peptide (CNP) produced by endothelial cells, and urodilatin produced by the distal convoluted tubule and the collecting duct) as well as vasoconstrictors (e.g., angiotensin II and endothelin-1 produced by endothelial cells) into inactive metabolites. Dual NEP inhibition and AT_1 receptor blockade (ARB) would result in sustaining the beneficial effects of upregulated natriuretic peptides, while not suffering from the harmful consequences of the upregulation of angiotensin II that will simultaneously occur. RAS, renin–angiotensin system.

demonstrated a small but significant increase after ARNI. However, baseline albuminuria was very low in these patients. Furthermore, ARNI treatment did reduce proteinuria in patients with CKD, particularly in those with macroalbuminuria (Ito *et al.*, 2015). Indeed, in rats with severe diabetic, hypertensive kidney damage, greater beneficial effects on proteinuria and glomerular sclerosis were observed after ARNI, when compared to ARB, despite a similar effect of these treatments on blood pressure (Roksnoer *et al.*, 2016). This suggests that ARNI exerts a renoprotective effect, independent of its antihypertensive properties. Possibly, this relates to improvements in glycemic regulation, since ARNI lowered hemoglobin A1c values in patients with heart failure and diabetes (Seferovic *et al.*, 2017), and increased insulin sensitivity in patients with hypertension (Jordan *et al.*, 2017). Most of the pathways proposed to mediate kidney damage due to increased blood glucose levels hold activation of the profibrotic agent TGFβ1 in common as an intermediary step (Ziyadeh, 2004). In addition to a reduction in TGFβ1 upregulation directly related to lower blood glucose levels, ARNI treatment could prevent nuclear localization of TGFβ1 due to augmented half-life of atrial natriuretic peptide (Li *et al.*, 2008), and thereby counteract the effects of TGFβ1 activation at the postreceptor level. The safety and efficacy of ARNI in patients with CKD are currently being studied in a phase III clinical trial (HARP, 2017).

Conclusion

Classical RAS blockers are well-established drugs for the treatment of cardiovascular and renal diseases. Contrary to what was originally thought, more blockade (e.g., by using multiple blockers at the same time) is not necessarily better, and rather results in more side effects. Thus, we either need novel approaches to interfere with the RAS (e.g., stimulate the protective receptors AT_2 and Mas), or combine RAS blockade with drugs that additionally stimulate other, protective hormonal system, like NEP inhibitors. An example of the latter is the ARNI approach, which is now successfully applied in heart failure.

See also: Angiotensinogen and Angiotensins. Hyperreninemia. Hypertension and the Renin–Angiotensin–Aldosterone System. Renin and Prorenin

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Aldosterone; Synthesis and Metabolism

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Glossary

Aldosterone A steroid hormone synthesized in the zona glomerulosa of the adrenal cortex; the major mineralocorticoid in humans.

Mineralocorticoid Corticosteroid hormone secreted by the adrenal gland and exerting its function through the mineralocorticoid receptor; also referred to as a hormone that affects water and electrolyte homeostasis.

Renin It is a key enzyme of the renin–angiotensin–aldosterone system (RAAS), cleaving angiotensinogen to angiotensin I and thereby having an impact on the synthesis rate of the effector hormone of the RAAS, angiotensin II. The RAAS is a hormone system that plays a central role in the control of sodium excretion, fluid volume, and vascular tone, thus controlling blood pressure.

StAR steroidogenic acute regulatory protein Is a transport protein for cholesterol into the mitochondria, a rate-limiting step in the synthesis of steroids.

HSD3B2-3β-hydroxysteroid dehydrogenase δ5-δ4 isomerase (HSD3B2) belongs to the family of oxidoreductases and is an enzyme that catalyzes the formation of progesterone from pregnenolone.

Cytochromes P450 Are proteins of the superfamily containing heme as a cofactor. In the adrenals they are terminal oxidase enzymes in electron transfer chains with NADPH. Hydroxylation reactions use CYP450 enzymes.

Aldosterone synthase CYP11B2 is a member of the cytochrome P450 family of enzymes. The protein only expresses in the zona glomerulosa cells of the adrenal cortex. It catalyzes 11-hydroxylation of deoxycorticosterone (DOC) to corticosterone then 18-hydroxylation and oxidation of that group to the C-18 aldehyde.

Calcium signaling Is required to maintain intracellular concentrations of calcium. Transfer of calcium ions from the extracellular to the intracellular compartment in both directions alters membrane potential of the cell. Calcium ions can be released from intracellular stores, imported, and exited through membrane ion channels. The activities and expression of StAR and CYP11B2 are stimulated at higher calcium concentrations in the mitochondria.

Introduction

The outer layer of the adrenal cortex, the zona glomerulosa, is responsible for the synthesis of aldosterone, although new sites are now recognized, notably adipose, cardiac, and vascular tissues. The glomerulosa cells, when highlighted through specific protein expression, are now seen as small clusters under the capsule rather than what used to be described as a discrete layer. This zona glomerulosa lacks expression of the cytochrome 17α-hydroxylase which is important for cortisol synthesis in the zona fasciculata. The protein also expresses 17,20-lyase activity leading from steroids with 21 carbon atoms to male sex hormones with 19 carbon atoms. Cytochrome P450 enzymes are important in steroidogenesis; these are proteins of about 500 amino acids with a heme group that absorb light at 450 nm (as their name implies) in their reduced states complexed with carbon monoxide. The proteins are denoted with the prefix CYP and the genes are in italics, hence *CYP*. The mechanisms covered in this article have been examined at the molecular genetic level in the search for the basis of primary aldosteronism (see Bandulik, 2017).

Cholesterol

Aldosterone, like other steroid hormones, is synthesized from cholesterol. Most of the cholesterol derives through a receptor-mediated endocytosis uptake of low-density lipoprotein (LDL) into vesicles that fuse with lysosomes. Cholesterol can be esterified by acyl-CoA cholesterol acyl transferase (ACAT) and stored in lipid droplets. Hormone sensitive lipase (HPL) will liberate free cholesterol. There is intracellular trafficking of cholesterol between touching membranes and by cholesterol binding proteins. Steroidogenic acute regulatory protein (StAR) facilitates the transfer of cholesterol from the outer to the inner mitochondrial membrane. Several related proteins called StAR-related lipid transfer proteins (START) are likely involved in intracellular cholesterol transfer. Within the START family, there are a number of START domain proteins (StARD) of which StARD4 and 5 are likely involved in cholesterol transfer. The mechanisms of cholesterol transfer in adrenal cells are not well understood and further debate is not needed in this review. Further discussion can be found in a comprehensive review on the initial steps of steroid synthesis (Miller, 2017). Some clinical disorders (Smith–Lemli–Opitz syndrome, Wolman disease, Nieman–Pick C syndrome (NPC)) have given clues for the involvement of other cholesterol transfer steps like NPC1 and NPC2, SNARE (soluble NSF attachment protein receptor) and oxysterol binding protein. Other proteins may be involved in cholesterol movement into the mitochondria. The benzodiazepine receptor

(translocator protein TSPO) and its ligand, the AT-binding cassette subfamily D (ABCD1) and sterol carrier protein 2 (SCP2), may act as a docking site for StAR but more research is required to delineate all the players in cholesterol transport.

The side chain of the 27-carbon cholesterol is reduced in length to the 21-carbon pregnenolone in mitochondria, which is converted to progesterone and deoxycorticosterone (DOC) in the endoplasmic reticulum. The final steps from DOC to aldosterone are achieved with one mitochondrial enzyme, aldosterone synthase (Fig. 1). Three of these reactions are performed by cytochromes with different cofactor needs.

Pregnenolone

Steroidogenic acute regulatory protein increases the transfer of cholesterol into the mitochondrial inner membrane where a cytochrome P450 for side chain cleavage (scc) enzyme is located. The conversion of cholesterol to pregnenolone involves 22-hydroxylation of cholesterol, 20-hydroxylation of 22R–hydroxycholesterol, and cleavage of the C20 to C22 bond with release of isocaproaldehyde and pregnenolone. Cytochrome P40 scc (desmolase) requires electrons from a flavoprotein called adrenodoxin reductase, an iron sulfur protein called ferredoxin and NADPH. The expression of scc requires the action of the zinc-finger transcription factor, steroidogenic factor 1 (SF1).

Progesterone and Deoxycorticosterone

In the endoplasmic reticulum of the zona glomerulosa cells, pregnenolone is converted to progesterone with oxidation of the 3β-hydroxyl to a ketone and switch in the double bond in the B-ring to the A-ring by 3β-hydroxysteroid dehydrogenase and Δ5–Δ4 isomerase (shift in the double bond from C5 to C4). NAD is the cofactor for this enzyme. A microsomal P450 enzyme (CYP21A2 or CYP21B) catalyzes the C-21 hydroxylation of progesterone to DOC. This enzyme receives electrons from NADPH via a flavoprotein called cytochrome P450 (POR). In evolution, the gene has become duplicated (CYP21A1P or CYP21A), although mutations in the second gene prevent transcription of active protein. Exchange between the two genes is common which is the basis for some cases of 21-hydroxylase deficiency.

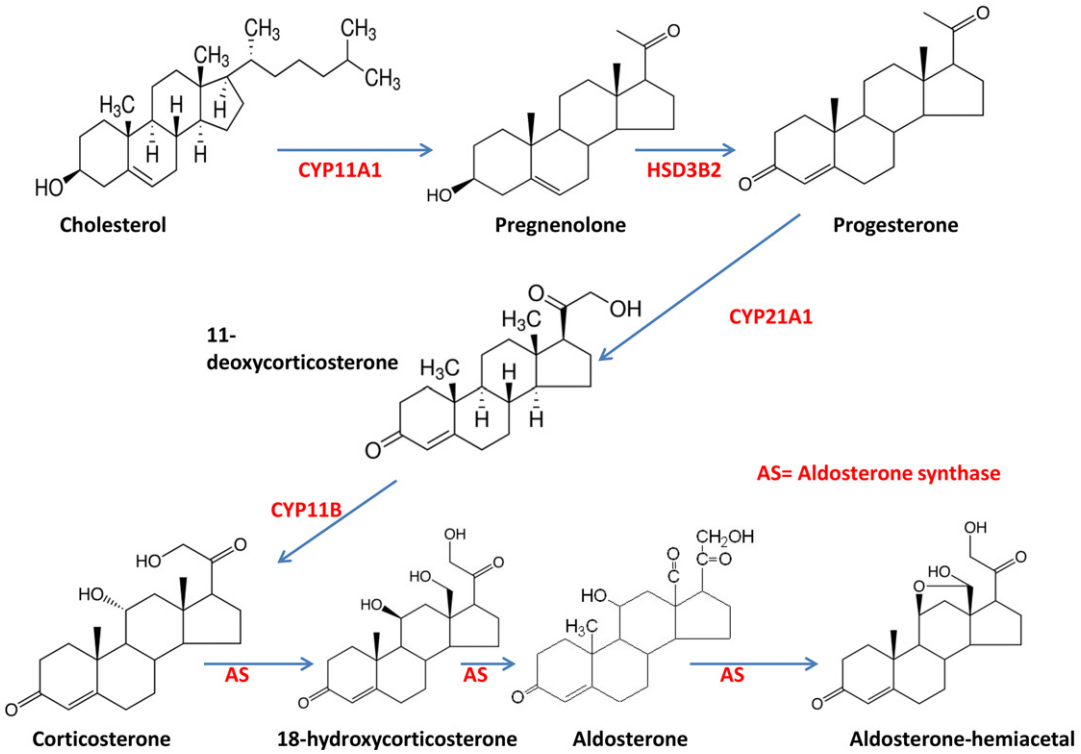

Fig. 1 Aldosterone biosynthesis.

Aldosterone via Corticosterone

The actions of a mitochondrial enzyme are required to complete the synthesis of aldosterone. CYP11B1 is active in the zona glomerulosa and the fasciculata to introduce a 11β-hydroxyl group to DOC or 11-deoxycortisol, respectively, yielding corticosterone or cortisol. In the zona glomerulosa, CYP11B2 (aldosterone synthase) catalyzes 11 hydroxylation, 18-hydroxylation, and oxidation to a C-18 aldehyde (corticosterone methyl oxidase). Both enzymes use ferredoxin, ferredoxin reductase to transfer electrons from NADPH. SF1 and other transcription factors such as NURR1 and NGF1B are needed. This is the late rate-limiting step of aldosterone synthesis.

Regulation of Aldosterone Production

Many factors are involved in the regulation of aldosterone production (summarized in Fig. 2). Aldosterone synthesis is mainly controlled by changes of the membrane potential and calcium homeostasis. The activities and expression of StAR and CYP11B2 are stimulated at higher calcium concentrations in the mitochondria. The cytosolic concentrations of glomerulosa cells are about 100–200 nmol, whereas the extracellular calcium concentration is 1–2 mmol. The sodium/calcium exchanger (NCX) and plasma calcium ATPases (PMCA) export calcium to maintain the resting low calcium concentration. Calcium influx is controlled by store-operated channel (SOC) and two voltage-activated calcium channels (CaV) which are activated by depolarization of the cell membrane. T type CaV is partially open at resting potential, but the L-type CaV is closed at resting potential and opened at higher levels of membrane depolarization. The membrane potential and cytosolic calcium concentrations depend on the actions of

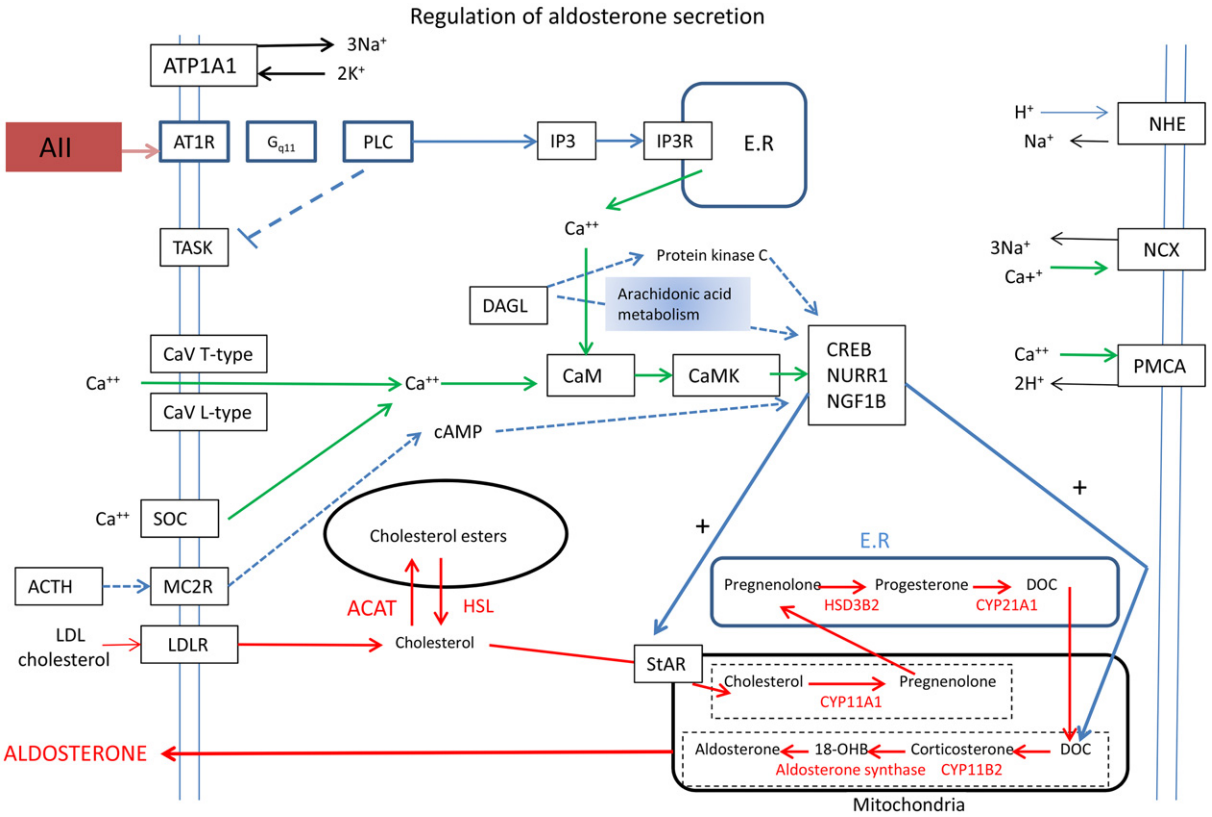

Fig. 2 Control of aldosterone synthesis by angiotensin I through calmodulin and calcium signaling pathways. *AII*, angiotensin II; *AT1R*, angiotensin II receptor type 1; *ATPA1*, sodium/potassium ATPase; *G $_{q/11}$*, guanine protein subunit alpha 11; *PLC*, phospholipase C; *IP1*, inositol triphosphate; *IP3R*, inositol triphosphate receptor; *DAGL*, diacylglycerol; *CaV-T*, voltage-dependent Ca channel type T; *CaV-L*, voltage-dependent calcium channel type L; *CAM*, calmodulin; *CAMK*, calmodulin kinase; *CREB*, cAMP responsive element binding protein 3; NURR1 nuclear receptor subfamily 4 member 2; *NGF1B*, nerve growth factor 1B like receptor; *SOC*, calcium release-activated calcium channel protein; *TASK Potassium*, two pore domain channel subfamily K member 3 (KCN3); *LDLR*, low-density lipoprotein receptor; *NHE*, sodium/hydrogen exchanger; *NCX*, sodium/calcium exchanger; *PMCA*, plasma membrane calcium ATPase; *ER*, endoplasmic reticulum; *ACT*, Acyl-CoA cholesterol transferase; *HSL*, hormone sensitive lipase; *HSD3B2*, 3β-hydroxysteroid dehydrogenase and Δ5-Δ4-isomerase; *CYP21A1*, 21-hydroxylase; *CYP11A1*, 11β-hydroxylase type 1; *CYP11B2*, 11β-hydroxylase type 2; *AS*, aldosterone synthase; *StAR*, steroidogenic acute regulatory protein; *DOC*, 11-deoxycorticosterone; *ACTH*, adrenocorticotrophic hormone; *MC2R*, melanocortin 2 receptor; *cAMP*, cyclic AMP.

potassium channels (TASK or KCN), calcium channels (CaV), sodium/potassium exchangers (ATP1A1), sodium/calcium exchanger (NCX), sodium/hydrogen exchanger (NHE), and plasma membrane calcium ATPases (PMCA). The specific contributions of these pathways to calcium signaling in glomerulosa cells are largely unknown. More detail can be found in a 2014 review by Bollag.

Angiotensin II

Angiotensin II (AII) in the human binds to a membrane AII receptor type 1 (AT1R). The activated receptor in turn couples to G-protein $G_{q/11}$ and this activates phospholipase C (PLCβ) to stimulate inositol triphosphate (IP3) production and diacylglycerol which through specific receptors in the ER generates a calcium release signal from the ER storage site. An α-subunit of the G-protein, with intrinsic GTPase activity, dissociates from the β-subunit. A diacylglycerol lipase releases arachidonic acid and this is converted to further prostaglandin metabolites (HETE) that increase cytosolic calcium concentration, mitogen-activated protein kinase (MAPK), phospholipase C, and cAMP response element binding (CREB). AII depolarizes the cell membrane and inhibits the TASK potassium channels that maintain resting potassium concentrations. AII also inhibits the NA/K ATPase (ATP1A1). Depolarization of the membrane also activates voltage-dependent calcium channels (CaV). The increase in the cytosolic Ca^{2+} concentrations promotes binding of calcium to activate calmodulin which then stimulates calmodulin-dependent kinases (CAM) which in turn triggers cellular responses such as stimulation of protein kinase C, mitochondrial production of NADPH, activated transcription factors CREB, NURR1, and NGF1B, activation of StAR by phosphorylation, and expression of CYP11B2 through cAMP-dependent response elements in the 5′ region of the *CYP11B2* gene. A low sodium diet leads to upregulation of AT1R levels which affects a physiological response to increase aldosterone production.

Angiotensin II can also bind to angiotensin II receptor type 2 (AT2R). This protein has 34% sequence homology to AT1R and expression widely in fetal tissues then restricted to brain, adrenal, heart, kidney, myometrium, skin, and ovary. Expression has been increased in pathological conditions such as vascular injury, myocardial infarction, renal failure, and brain ischemia. The mechanisms of action are not clear but Gi protein coupling, activation of serine/threonine phosphatase PP2A, phosphotyrosine phosphatase, phospholipase, release of bradykinin, and nitric oxide have been reported. Cyclic GMP mediates actions of nitric oxide leading to vasodilatation and natriuresis.

Potassium and ACTH

In addition to the renin–angiotensin system which is the major regulatory mechanism, adrenocorticotrophic hormone (ACTH) provides a transient control mechanism. The plasma potassium concentration also influences aldosterone synthesis. A small increase in extracellular K^+, when potassium efflux is prevented from efflux through the G-protein-activated inward rectifier potassium channel 4 (GIRK4), causes membrane depolarization increasing intracellular calcium concentrations which increases expression of genes for aldosterone synthesis. Conversely hypokalemia inhibits aldosterone synthesis. Potassium acts through several signaling steps (TASK, ATP1A1).

Adrenocorticotrophic hormone binds to the melanocortin receptor MC2R to stimulate cyclic AMP production via the heterotrimeric G-protein G_s which activates protein kinase A and cAMP-dependent protein kinase which increases hormone sensitive lipase as well as increasing StAR and aldosterone synthase gene expression via NURR1 and NGF1B.

Calcium Homeostasis

From the preceding discussion, aldosterone secretion will be clearly influenced by calcium homeostasis. The plasma-ionized calcium concentration is regulated to within very narrow limits (1.3–1.5 mmol/L). This is achieved by both the parafollicular cells of the thyroid gland, and the parathyroid glands sensing the concentration of calcium ions in the blood flowing through them. When the concentration rises the thyroid gland increases the secretion of calcitonin into the blood. At the same time the parathyroid glands reduce their rate of parathyroid hormone (PTH) secretion into the blood. The resulting high levels of calcitonin in the blood stimulate the skeleton to remove calcium from the blood plasma, and deposit it as bone. The reduced levels of PTH inhibit removal of calcium from the skeleton. The low levels of PTH have several other effects: loss of calcium in the urine increases, and loss of phosphate ions via that route is inhibited. Phosphate ions will therefore be retained in the plasma where they form insoluble salts with calcium ions, thereby removing them from the ionized calcium pool in the blood. The low levels of PTH also inhibit the formation of calcitriol (1,25 dihydroxyvitamin D_3) from cholecalciferol (vitamin D_3) by the kidneys. The reduction in the blood calcitriol concentration acts (comparatively slowly) on the epithelial enterocytes of the duodenum inhibiting their ability to absorb calcium from the intestinal contents. The low calcitriol levels also act on bone causing the osteoclasts to release fewer calcium ions into the blood plasma.

When the plasma-ionized calcium level is low the opposite happens. Calcitonin secretion is inhibited and PTH secretion is stimulated, resulting in calcium being removed from bone to correct the plasma calcium level. The high plasma PTH levels inhibit calcium loss in the urine and stimulate the excretion of phosphate ions by that route. The kidneys secrete calcitriol, which

enhances calcium absorption from the intestinal contents into the blood, by stimulating the production of calbindin. The PTH-stimulated production of calcitriol also causes calcium to be released from bone into the blood, by the release of RANK1 (a cytokine) from the osteoblasts which increases the bone resorptive activity by the osteoclasts, although these are relatively slow processes. Hence, regulation of the plasma-ionized calcium level primarily involves rapid movements of calcium into or out of the skeleton in the short term. Longer term regulation is achieved by regulating the amount of calcium absorbed from the gut or lost via the feces. Studies in animals and humans have shown that vitamin D decreases activity of the R–A system by suppressing expression of the renin gene via a cis-DNA element in renin production. This is independent of calcium metabolism.

Actions of Aldosterone

Aldosterone controls blood pressure through blood volume and sodium homeostasis. Aldosterone acts at the distal nephron, salivary glands, and colon. A target cell is entered by passive diffusion of aldosterone, and then reacts with the mineralocorticoid receptor (MR) which is complexed with a dimer of heat shock protein and other proteins such as cyclophilin and FKBP52. This complex dissociates on aldosterone binding and as a homodimer translocates to the nucleus, where they bind to response elements of aldosterone sensitive genes. The transcribed proteins then stimulate the synthesis and activity of sodium channels and pumps to influence sodium transport inward. This is covered elsewhere.

An essential aspect of the MR is the colocalization of an enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD3B2) that inactivates cortisol to cortisone preventing any effect of cortisol on the MR. This is covered in detail elsewhere.

The Renin–Angiotensin–Aldosterone System

In the juxtaglomerular apparatus of the kidney, low plasma sodium concentrations or low renal blood flow stimulates release into the circulation of renin from prorenin. Renin, a proteolytic enzyme, then acts on angiotensinogen to free a 10-amino acid angiotensin I (once considered inert). Angiotensinogen is synthesized in the liver. A further two amino acids are cleaved, at several sites including the lung, from AI to produce AII by angiotensin converting enzyme (ACE). AII has two actions, firstly as the most potent vasoconstrictor but secondly at the adrenal cortex it binds to the angiotensin receptor to stimulate aldosterone secretion as described in detail earlier.

The Renin–Angiotensin–and Other Angiotensins–Aldosterone System

In recent years, our understanding of the RAAS has been extended with the knowledge of the generation of angiotensin peptides downstream of AII. [A(1–8)] is a product of ACE action and is then converted to A(1–7) by the angiotensin converting enzyme ACE2 through the identification of A(1–7) and the enzyme for this conversion, ACE2. This enzyme can also convert AI [A(1 − 10)] to A(1–9)]. The A(1–7) binds to a mas receptor (MrgD-R) that leads to inhibition of MAPK, stimulation of cellular phosphatase, inhibition of cyclooxygenase 2 (COX2), and facilitation of nitric oxide release. A(1 − 7) is implicated in the pathogenesis of cardiovascular diseases. A(1 − 8) acts at the AT2R. A peptide A(1 − 12) has also been discovered as a carboxyl-terminally extended AI with valine and isoleucine. A(1 − 12) is present in plasma and tissues including aorta and kidney. A(1 − 12) is converted to AII by chymase in the heart. The conversion of A1 to A(1–12) has been proven in rat aorta experiments. AII can also be converted to AIII [(A(2–8)] and AIV [A(3–8)] by aminopeptidases A (APA) and N (APN), respectively. AIV can be further cleaved to A(5–8) by endopeptidase and A(5–7) by carboxypeptidase (CPP). AIV inhibits the insulin receptor aminopeptidase (IRAP). The AIV/IRAP axis induces the release of nitric oxide and promotes antiinflammatory and antifibrotic effects. Dipeptidyl peptidase III (DPP III) cleaves two amino acids from the N-terminus of AIV and A(3 − 7) to give A(5 − 8) and A(5 − 7) oligopeptides. Angiotensin A is a newly characterized peptide related to AII with Ala instead of Asp in the first position of the sequence generated by aspartate decarboxylase. Alamandine is A(1–7) with alanine instead of aspartate in the first amino acid position that binds to a novel G-protein-coupled receptor Mas (MrgD). Angioprotectin is like AII with Pro and Glu instead of Asp and Arg. Angioprotectin has high affinity for the mas receptor and has vasorelaxing properties. There is evidence for an intrarenal renin angiotensin system (RAS) as a local feed-forward system for augmentation of intrarenal generation or action of RAS that plays a role in pathogenesis of hypertension and renal disease. There are several components such as PRR, Wnt/β-catenin signaling, and PEG2/EP4 as positive elements and Klotho, VDR, and LXR in the negative arm. These discoveries open potential for the development of new drugs.

The RAAS is continually being revised and at August 2017 there are now five axes identified:

- the classical angiotensin-renin–ACE–AII–AT1R-aldosterone axis
- the prorenin receptor (PRR)–mitogen activated protein (MAP)–kinase pathway
- the AIII-APN-AIV-IRAP-AT4 receptor axis
- the AII-APA-AIII -AT2R-NO-cGMP axis where ATR2 is the angiotensin II receptor type 2, NO is nitric oxide, and cGMP is cyclic GMP
- the AI-AII-ACE2/ang(1–7)-mas receptor axis

The first three axes are powerful vasopressor systems, whereas the others are vasodepressor and cardiorenal protective axes. The effects have been described as Devil/Angel or Yang/Yin of vasoactive systems. The action of angiotensin II through ATR2 is independent of aldosterone.

The idea that AII is the only active peptide of the RAAS is outdated because angiotensinogen is the source of several peptides with new biological actions in adrenal, cardiac, renal, and brain tissues and our understanding of their roles in pathophysiological states is incomplete.

Catabolism and Excretion of Mineralocorticosteroids

Hormones circulating in plasma are inactivated by metabolism primarily in the liver. Conjugation with glucuronic or sulfuric acid also in the liver is important to increase the solubility of steroids prior to renal extraction and excretion in urine.

There are two major pathways of steroid catabolism in the liver:

(1) Reduction of the double bond at C-4 with accompanying reduction of the 3-keto group to a secondary alcohol group—add four hydrogens (tetrahydro products). 5α and 5β-reductases are involved—androst-4-enes to 5-androstanes (dihydro-) followed by 3α-hydroxysteroid dehydrogenase.
(2) Reduction of the 20-keto group to a secondary alcohol group (if after tetrahydro steroid formation gives hexahydro product). 20α and 20β reductases.

Oxidative cleavage of the side chain is confined to 17-hydroxy C21 steroids. Hydroxylation of the aldosterone nucleus has been demonstrated as for other steroids at C2α, 6β, 19 but not 1,7,15,16. 21-Deoxy and 19-nor aldosterone have been identified.

Aldosterone metabolites are conjugated and excreted mainly as glucuronides but also as sulfates at the C3 and C21 positions.

Hepatic Catabolism of Mineralocorticosteroids

Reduction of the "A"-ring to form a 3α-hydroxy-5β-tetrahydro metabolite is the major fate of aldosterone (Fig. 3), DOC, and 18-hydroxy DOC.

In man, the principal metabolites of corticosterone belong to the 3α,5α pregnane series which contrasts with the predominance of 3α,5β pregnane metabolites of aldosterone and DOC as well as progesterone and cortisol. Normally, corticosterone metabolites retain the hydroxyl at C-11, but 18-hydroxy 11-dehydro tetrahydrocorticosterone (tetrahydroCompound A) is the principal metabolite of 18-hydroxy corticosterone. Dihydro reduced metabolites of both corticosterone (20α- and β) and, 21-hydroxy-4-pregnene-3,11, 20-trione (20β only) were identified after ingestion of a large dose of the hormone. Pregnane-3α,11β,20,21-tetrols (hexahydrocorticosterone, 5β- and 5α-) were identified in the urine of a healthy man also following a pharmacological dose of the hormone. The 5α/5β ratio was about 2 and the conversion rate was at least 10% in the first 24 h urine collection, making these metabolites as significant as tetrahydro corticosterone. In the absence of suitable reference compounds, the stereochemistry at C-20 was not established.

Aldosterone in blood and urine exists primarily as 11–18 hemiacetal, so the C-11 hydroxyl is not normally available for oxidation. Although 11-dehydroaldosterone (21-hydroxy-4-pregnene 3,11,20-trione-18-al) has been shown to be an effective

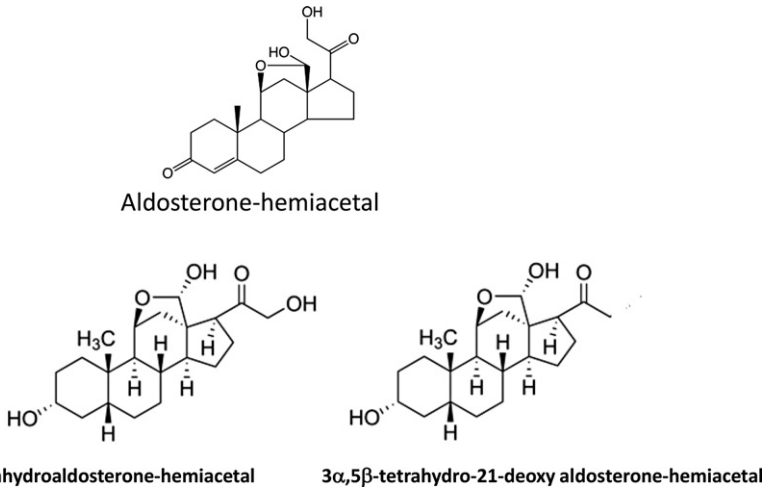

Fig. 3 Metabolites of aldosterone.

precursor in vitro for aldosterone biosynthesis by rabbit adrenal tissue, no aldosterone metabolites with a carbonyl function at C-11 have been positively characterized. Primarily on the basis of its infrared spectrum, 21-hydroxy-4-pregnene-3,11,20-trion-18-al was identified in urine, but this has never been confirmed. 5-Dihydroaldosterone and the 3α, 5α, and 3β epimers of tetrahydroaldosterone were also characterized by synthesis, degradation, and infrared spectroscopy, but the urinary excretion of each represented less than 1% of the original dose. Improved syntheses and identification using nuclear magnetic resonance spectroscopy enabled the characterization of 3α,5β-tetrahydroaldosterone-3-β-glucosiduronic acid after isolation from a 12 h urine collection using ion-exchange chromatography. The recovery was 34% of oral dose which is in good agreement with other reported figures. 3α,5β-tetrahydroaldosterone is inactive but 3α,5α,-tetrahydroaldosterone possesses GLAF activity, potently inhibiting HSD11B2 but not HSD11B1. The excretion of 3α,5α-tetrahydroaldosterone has not been measured and this would be an interesting experiment in sodium deprivation and certain categories of hypertension.

Conjugation and Urinary Excretion

The above reactions serve to inactivate the steroid hormones but before excretion, the polarity of these metabolites is increased by conjugation. Glucuronide conjugates at C-3 and C21 are more readily excreted in the urine than sulfate esters which are also formed. A unique conjugate of aldosterone occurs in human urine as a highly polar metabolite from which free aldosterone can be liberated by hydrolysis at pH1. The structure was confirmed, by degradation and synthesis, as the C-18-β-D-glucosiduronate of aldosterone hemiacetal. This metabolite is produced primarily in the kidney although an increased excretion of aldosterone-18-glucosiduronate in the third trimester of pregnancy has been attributed to metabolism in other organs. The synthesis of aldosterone-21-sulfate preceded the detection and measurement of this compound in urine. Approximately 0.7% of injected aldosterone was recovered in the sulfate fraction of a 48 h urine collection. 6β-hydroxycorticosterone was found to be excreted in urine as the free steroid and equally as its C-21 sulfate ester. This was a minor product but may become important if 6β-hydroxylase enzyme in human liver is induced by certain drugs.

In addition to these conjugates produced during hepatic catabolism of free steroid hormones, the adrenal gland itself secrets steroid C-21 sulfates, particularly in the newborn period. A comparison of the fate of ^{3}H–corticosterone sulfate with ^{14}C–corticosterone injected simultaneously indicated that the conjugate was rapidly metabolized by adults to polar urinary metabolites without hydrolysis, whereas most of the free corticosterone was converted to glucuronide-conjugated tetrahydro-derivatives. This metabolism may be important in pregnancy and the newborn period because the fetal adrenal secretes large amounts of corticosterone sulfate.

Biliary Excretion and Metabolism in the Intestine

After intravenous injection of radiolabeled aldosterone, 90% of the activity can be recovered from the urine. The recovery of activity after injection of other mineralocorticosteroids is much lower, typical values being 79% for corticosterone, about 45% for DOC and about 50% for 18-hydroxy DOC. A comparison of the fate of radiolabeled corticosterone with a separate study of cortisol injected intravenously into the same patients and volunteer indicated major difference in the metabolism of these hormones. The rate of disappearance from plasma of free corticosterone was faster than for cortisol, yet in a 2-day collection of urine less corticosterone metabolites were excreted than cortisol metabolites.

In two patients undergoing cholecystectomy, bile was collected from a T-tube previously inserted at surgery for cholecystitis. The cumulative excretion of activity after corticosterone administration in the first 12 h bile collection reached 20% and 31% of the labeled dose, although no further change was noted. A similar proportion of radioactivity was found in bile after intravenous administration of DOC. From a 16% recovery of a dose of 18-hydroxy-DOC in bile from a patient with incomplete biliary fistula as a guide it was estimated that for a normal bile flow the true excretion could account for 24%–39% of the total dose.

A comparison of steroid metabolism in vitro and in vivo using germ-free and conventional rats demonstrated extensive metabolism of steroid hormones and their metabolites by intestinal bacteria. Subsequently bacteria isolated from the human intestine have been shown in culture to reduce hydroxyl groups in steroids at the 16α- and 21-positions. Since no 21-dehydroxylating enzymes have been reported in human tissues, it must be concluded that the earlier isolation of pregnanediol and 11-oxo-pregnanediol from urine after the administration of DOC and 21-hydroxy-4-pregnene-3,11,20-trione, respectively, was due to bacterial metabolism of biliary steroids which had been readsorbed from the intestine. 11-oxo-pregnanolone and allo-pregnanolone (Fig. 4) may inhibit 11β-hydroxysteroid dehydrogenase type 2 and thus act as glycerrhitinic acid like compounds (GALF's) as proposed recently by Morris. Thirty-three percent of an intravenous radioactive dose of DOC has been recovered from feces, but this is not a significant excretory route for other mineralocorticosteroids since they are effectively reabsorbed from the intestine. The predominant isomers of the steroids both remaining in the free steroid fractions of feces and reabsorbed from the intestine have a 3α,5β, or 3β,5α configuration which indicates that the bacteria also possess reductase and isomerase activities.

Among the steroid conjugates excreted in bile tetrahydro DOC was identified as a mono-and-di-sulfate whilst 5α (and β) tetrahydrocorticosterone and 3α, 21-dihydroxy-5β (and α) -pregnane-11,20-dione were excreted only as monosulfates. Bacteria in the intestine have been shown to possess sulfohydrolase activity so the freed metabolites may be reabsorbed into the pool of neutral metabolites.

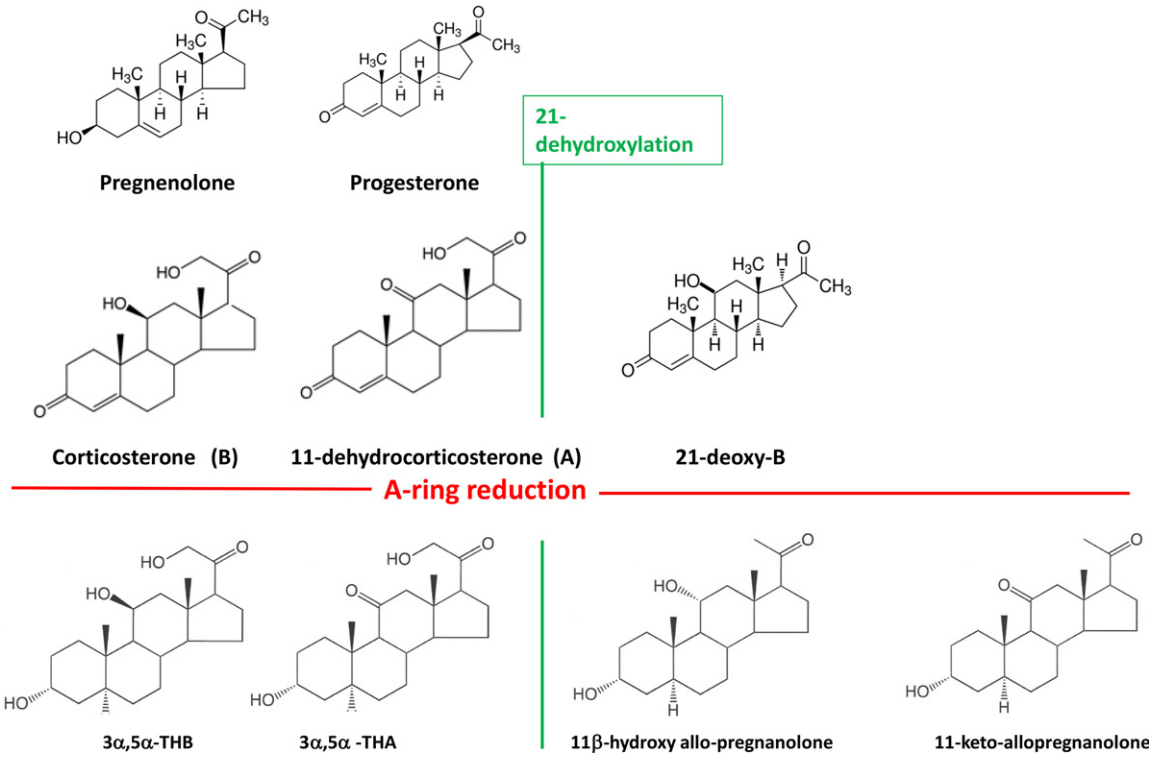

Fig. 4 21-Deoxysteroids that are GALF's.

Two bicyclic acetal metabolites of tetrahydroaldosterone were characterized and formed in each case by reduction of the C-20 carbonyl to a secondary hydroxyl group which then reacts with the 11–18 hemiacetal with the elimination of water. The minor acetal metabolite, representing 1.4% of the oral dose of aldosterone-21-acetate, can be regarded as a product of hexahydroaldosterone which itself was not identified. A 21-deoxy metabolite of tetrahydroaldosterone was characterized as the hemiacetal of 3α, 11β-dihydroxy-5β-pregnane-20-one-18-al, and the second acetal (8% of the dose) was a ketal of this metabolite justified by the general observation that 21-deoxy compounds formed by the bacterial metabolism of 21-hydroxy 20-keto precursors had always been recovered in urine as the 20-alcohols. The structures were confirmed by synthesis of the two compounds. This suggests that tetrahydroaldosterone may also be excreted in bile and reabsorbed from the intestine after bacterial 21-dehydroxylation. No 21-deoxy metabolites of 18 hydroxy DOC or its tetrahydro metabolites have yet been identified in urine or feces.

The neutral corticosteroid metabolites described so far may still represent 70% or less of the total radio activity recovered in urine. However, with improved methods for steroid extraction (e.g., Amberlite XAD-2 and solid phase extraction), it has become possible to recover from aqueous solution some further activity not extracted with organic solvents and, for example, an additional 5%–30% of the total metabolites of cortisol can now be accounted in polar fractions of steroids acids with a 20-oic acid group. An enzyme that oxidizes corticosteroids at position 21 has been purified from human liver obtained at post-mortem but the activity with 17-deoxy C21 steroid substrates is considerably lower than for cortisol and evidence in vivo suggests that acidic metabolite formation from DOC may account for only 0.5%–2% of the hormone, although by measuring the transfer of radiolabel from [21-^{3}H]– DOC into body water. The oxidation of DOC to an acid may be from 2.3% to 8.2%. Some of this activity can probably be attributed to bacterial metabolism of DOC in the intestine since loss of tritium has been demonstrated when [21-^{3}H]-DOC was incubated with human fecal flora. The investigations of steroid acids are in their infancy and the biological activity and influence of disease on these metabolic changes has still to be evaluated. The possibility must also be considered that nonsteroidal acidic or neutral metabolites might be excreted after degradation of the steroid nucleus.

See also: Aldosterone; Action and Function

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Aldosterone; Action and Function

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Glossary

Aldosterone A steroid hormone secreted from the adrenal cortex that primarily controls sodium and potassium transport in the kidney and colon.

Epithelial sodium channel (ENaC) A protein complex that spans the cell membrane to create a pore through which sodium can selectively pass into the cell down an electrochemical gradient.

Mineralocorticoid The name given to corticosteroid hormones that control electrolyte (sodium and potassium) transport. The principal mineralocorticoid is aldosterone.

Renin–angiotensin–aldosterone system (RAAS) A signaling cascade that is involved in the maintenance of blood pressure through control of vascular tone and salt balance.

Introduction

The adrenal steroid, aldosterone is the primary "mineralocorticoid" in many species; it was first isolated by Simpson *et al.* (1953). Aldosterone, or more specifically aldosterone synthase, appeared in evolution with the emergence of terrestrial life and the consequent need to conserve sodium. The primary function of aldosterone, as implied by the term "mineralocorticoid," is to conserve sodium and with that water (Fuller and Young, 2014). This role in sodium homeostasis is tightly coupled to roles in potassium and hydrogen ion homeostasis.

The receptor for aldosterone, the mineralocorticoid receptor (MR), first appeared in cartilaginous fish preceding the appearance of aldosterone synthase by ~80 million years ago (Baker and Katsu, 2017). The MR also binds other steroids, most notably cortisol. Given that cortisol circulates at much higher levels than aldosterone, the MR is arguably functionally only a receptor for aldosterone in those tissues that preclude cortisol binding to the MR through the action of 11β hydroxysteroid dehydrogenase type 2 (HSD2). HSD2 converts cortisol (or corticosterone in rodents) to its receptor inactive metabolite cortisone (11 deoxycorticosterone in rodents). The MR is therefore effectively a "glucocorticoid receptor" in tissues that do not contain HSD2. In this article we therefore focus on those tissues in which aldosterone is acting via "its" receptor by virtue of coexpression of HSD2. It should be noted that in states of aldosterone excess, such as seen in aldosterone-producing adenoma, MR that are unprotected by HSD2 will also be activated by aldosterone with a series of adverse consequences.

Control of Aldosterone Secretion

Aldosterone maintains sodium and potassium homeostasis through a series of endocrine feedback loops. The renin–angiotensin—aldosterone system (RAAS) responds to decreases in volume status to increase sodium retention and vascular tone, thereby increasing available volume (sodium) and decreasing the "space" to be filled (vascular contraction). Volume status is sensed by the renal juxtaglomerular (JG) cells; they release renin, an aspartyl protease, in response to a decrease in tubular flow, to process angiotensinogen to angiotensin I decapeptide, which in turn is subject to further proteolysis by angiotensin-converting enzyme to yield angiotensin II. Angiotensin II mediates both arms of the response being a potent vasoconstrictor and the primary stimulus of aldosterone synthesis in the glomerulosa cells of the adrenal cortex. Aldosterone then acts on the distal nephron to promote sodium reabsorption. This signaling pathway from the JG cells to the sodium transporting epithelia is the RAAS.

The feedback loop involved with potassium homeostasis operates in parallel with, and overlaps, those for sodium. Aldosterone increases potassium secretion in the distal nephron to lower serum potassium levels; these in turn feedback on the glomerulosa cells to decrease aldosterone synthesis. The reverse is true in that increased serum potassium levels stimulate aldosterone synthesis. Aldosterone also modulates acid–base balance by increasing the exchange of hydrogen ions for sodium. Therefore, the net effect of an increase in aldosterone levels, as may result from an aldosterone-producing tumor (Conn syndrome) or exogenous mineralocorticoid administration (e.g., 9α-fludrocortisone), is sodium resorption with consequent volume expansion, hypertension, suppression of plasma renin activity, hypokalemia, and metabolic alkalosis.

Aldosterone synthesis is also positively regulated by adrenocorticotropic hormone (ACTH), and subject to negative regulation by atrial natriuretic peptide, consistent with its role in promoting natriuresis; and dopamine.

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Mineralocorticoid Receptors

The actions of aldosterone are mediated, arguably exclusively, by the MR, a ligand-dependent transcription factor and member of the nuclear receptor superfamily. A putative G-protein-coupled membrane receptor GRP30 has been reported to respond to aldosterone, but its physiological relevance remains to be determined. Haploinsufficiency of the classic MR is associated with salt-wasting in the condition of pseudohypoaldosteronism type 1 and in transgenic mice rendered null for the MR profound neonatal salt-wasting is observed. The MR is described in detail in a separate chapter. Although the MR is widely expressed across a range of tissues, the most abundant expression is observed in traditional mineralocorticoid responsive tissues, the distal nephron, distal colon, and the salivary glands, where abundant HSD2 coexpression is also observed. In the central nervous system MR expression is largely independent of HSD2 expression with very high levels in subregions such as the hippocampus. In most of these CNS regions, the MR is thought to be acting as a cortisol/corticosterone receptor rather than an aldosterone receptor, however, in discrete nuclei associated with salt-appetite MR and HSD2 coexpression are observed (de Kloet and Joëls, 2017).

Mechanisms of the Regulation of Sodium Transport

Aldosterone-mediated transepithelial sodium flux primarily occurs in the cortical collecting ducts and to a lesser extent, in distal convoluted tubules of the nephron and in the distal sigmoid of the colon. In each case, there is a decreasing, distal to proximal, gradient of MR expression. As expected with a nuclear receptor, the temporal pattern of the aldosterone response has a lag period of 30–60 min, followed by an early phase in which preexisting pumps and channels are utilized with a late-phase at 3–6 h. In the late-phase, the number of pumps and channels increases and, with longer exposure, morphological changes are also observed. This time course is consistent with a primarily genomic response, which is to say that ligand-mediated activation of the MR results in the transcriptional regulation of the genes whose encoded proteins either mediate sodium transport per se or modulate components of the transport pathway. Although aldosterone accounts for only a small percentage ($\sim$2%) of the sodium reabsorbed in the nephron, the aldosterone-responsive regions in the nephron and indeed bowel are effectively the final arbiter of urinary sodium excretion; this critical role is reflected in the number of monogenetic syndromes of hyper- and hypotension in which the etiologic mutation involves a component of the aldosterone-responsive sodium transport pathway.

In the classic model of aldosterone-induced vectorial sodium transport across a polarized epithelium, sodium entry at the apical membrane is through an amiloride-sensitive electrogenic sodium channel with efflux mediated by an energy-dependent sodium pump at the basolateral membrane. These mechanisms are best reflected in the predominant cell type of the collecting duct, the principal cells (Shibata, 2017).

Epithelial Sodium Channel

The epithelial sodium channel (ENaC) is central to the aldosterone response (Soundararajan *et al.*, 2012). It consists of three homologous subunits (α, β, and γ) each characterized by two transmembrane domains with intracellular N- and C-termini. All three subunits are required for maximal amiloride-sensitive sodium transport. They form a heterotetromeric complex composed of 2α subunits and one each of the β and γ subunits. The ENaC subunits are members of the DEG/ENaC superfamily of sodium channel genes which are relatively conserved across evolution. The central role of ENaC is reflected both in Liddle syndrome, where activation, secondary to a mutation in the ENaC β or γ subunits results in increased sodium retention and hypertension, or in a subtype of PHA1 where an inactivating mutation in the subunits results in a salt-losing syndrome. Characterization of the mutations in Liddle syndrome identified a motif, proline–proline–proline–X-tyrosine (PY) in the C-terminus of the subunits which is disrupted in all cases. ENaC is a relatively short-lived protein that is ubiquitinated on residues in the N terminus of the α and γ but not β subunits; the PY motif interacts with Nedd4-2, a ubiquitin protein-ligase, whose role is to target the channels for proteosomal degradation. Although there is evidence that ENaC subunit gene expression is regulated by aldosterone this is tissue specific with both β- and γENaC subunit mRNA levels being increased in the colon by aldosterone but not in the renal cortex, whereas an increase in αENaC mRNA levels is seen in the inner medulla. Overall, it would seem that aldosterone can increase ENaC synthesis (at least in the late-phase), an effect in the early phase (i.e., a primary effect) is not a feature at least in the distal nephron. Aldosterone does however increase the expression of the serine, threonine kinase, serum and glucocorticoid-regulated kinase-1 (sgk-1), with a time course consistent with an effect on transcription. Sgk-1 directly interacts with Nedd4-2 to block its binding of the ENaC and, as a consequence, slows ENaC degradation. The primary acute effect of aldosterone is thus to increase the number of channels in the apical membrane by reducing their efflux from the plasma membrane. The glucocorticoid-induced leucine zipper protein, which is also aldosterone-induced, acts to repress ERK-signaling, a negative regulator of ENaC, as well as directly interacting with Nedd 4-2. A further aldosterone-induced protein, connector enhancer of kinase repressor of Ras3 (CNK3), serves as a scaffold protein in the assembly of the ENaC-regulatory complex.

Sgk-1 is also involved in the regulation of ENaCα-subunit gene expression through relief of Dotla-Af9-mediated transcriptional repression. Nedd4-2 is regulated by Usp2-45, a deubiquitinylation enzyme which is itself regulated by aldosterone. Sgk-1 requires phosphorylation by the phosphatidylinositol 3-kinase (PI 3-kinase) pathway which may integrate signaling from membrane-

associated receptors such as the insulin receptor. PI 3-kinase may be activated by small monomeric G proteins including K-ras 2A which has been identified as an aldosterone-induced gene.

Aldosterone also increases the open probability of the ENaC complexes in the plasma membrane through the action of serine protease such as prostasin and kallikrein which cleave the extracellular loop between the two transmembrane domains of the subunits.

NA^+/K^+-ATPase

Active extrusion of sodium from the cell to maintain intracellular sodium homeostasis reflects sodium pump activity in the basolateral cell membrane. Na^+/K^+-ATPase activity is increased in a number of epithelia by aldosterone. Na^+/K^+-ATPase α and β subunit gene expression is significantly increased in the late-phase in response to aldosterone. Na^+/K^+-ATPase activity is exquisitely sensitive to intracellular sodium concentrations; in cortical tubules, the early Na^+/K^+-ATPase response to aldosterone is blocked by amiloride, suggesting that the increased activity is secondary to the sodium influx at the apical membrane. In the late-phase of the aldosterone response, levels of Na^+/K^+-ATPase mRNA, protein, and activity are all increased. Channel-inducing factor (CHIF) is a member of the FXYD family of small transmembrane proteins that includes the γ subunit of Na^+/K^+-ATPase. CHIF gene expression is upregulated in the distal colon in response to aldosterone. CHIF increases the affinity of Na^+/K^+-ATPase for sodium which suggests that the early aldosterone-induced increase in Na^+/K^+-ATPase activity is, at least in part, mediated by CHIF.

Potassium and Hydrogen Ion Transport

Potassium flux occurs in transporting epithelia in response to aldosterone as a result of Na^+/K^+-ATPase-mediated exchange at the basolateral membrane, with the resulting electrochemical gradient favoring potassium excretion. Sgk-1 has been reported to increase the apical membrane channel density of ROMK, a potassium channel, through direct phosphorylation, through Nedd4-2 in the principal cells and also via the WNK (with-no-lysine-kinases) kinases in the distal DCT which also expresses the MR. Also present in the collecting duct are intercalated cells with subtypes α, β, and non-α/non-β. Intercalated cells have a well-recognized role in acid–base homeostasis, and also in potassium homeostasis. Regulation of potassium homeostasis by aldosterone, independent of its effects on sodium transport, and Na^+K^+-ATPase-mediated basolateral membrane exchange of sodium and potassium occurs in the β-intercalated cells. They express the Cl/HCO_3 exchanger, pendrin on the apical plasma membrane, and H^+-ATPase at the basolateral plasma membrane such that increased activity will promote potassium retention. A recent study has suggested that the response of the β-intercalated cell to aldosterone is modulated by phosphorylation of a specific serine in the ligand-binding domain of the MR (serine 843 in the human MR) which then precludes aldosterone binding. Diminished plasma potassium levels promote dephosphorylation of this serine allowing aldosterone-induced increases in both pendrin and H^+-ATPase. Dephosphorylation was also described in response to angiotensin II; this has been suggested to provide a mechanism by which the angiotensin-stimulated, aldosterone-mediated response to volume depletion can result in sodium reabsorption with limited potassium depletion.

As for potassium, aldosterone also has effects on proton excretion in addition to the cation exchange for sodium across the epithelium to maintain electroneutrality in the principal cells. The targets for this effect are carbonic anhydrase-rich cells, particularly the α-intercalated cells within the outer medullary collecting ducts. Aldosterone-induced transport across the apical membrane is through an H^+-ATPase activity, coupled with increased activity of the basolateral Cl/HCO_3 exchanger.

Aldosterone and Nonepithelial Tissues

The last decade has seen significant insights into the nature of the response in other nonepithelial tissues. In some tissues such as the vasculature and specific regions of the central nervous system where HSD2 is coexpressed with the MR, the response is indeed to aldosterone. In other tissues where the MR is expressed absent of HSD2, the biology is that of cortisol activation rather than of aldosterone action, except perhaps in states of aldosterone excess. Several studies have identified aldosterone-induced genes in the heart and the central nervous system, although their full physiological significance remains to be determined. Studies in transgenic mice using tissue-specific deletion of the MR are starting to clarify the specific roles and mechanisms of signaling through the MR in these tissues.

Vascular integrity and homeostasis

As noted earlier, a primary role for MR action is to regulate sodium excretion and resorption in the kidney and thus extracellular volume and cardiovascular homeostasis. Outside of the renal epithelia, the MR has a number other of physiological actions in response to aldosterone that also contribute to cardiovascular homeostasis. This is best defined for the cardiovascular response to hypovolemic shock. In this setting, poor renal perfusion increases renin release and activation of the RAAS. The elevated serum levels of aldosterone act to preserve (defend) circulating plasma volume via the aforementioned renal mechanisms of sodium and

water resorption in the distal nephron but also via two other key mechanisms. Aldosterone acting via the MR in vascular smooth muscle cells and endothelial cells enhances vascular reactivity and constriction in response to vasoactive peptides, thereby increasing total peripheral resistance. Aldosterone also acts on MR in cardiomyocytes to regulate increase chronotropic and ionotropic actions of the myocardium and hence cardiac output. These, largely transient, physiological responses to aldosterone action serve to also reduce aldosterone levels to baseline once homeostasis is restored.

In addition to the peripheral actions of aldosterone, it is well known that aldosterone can also regulate blood pressure when acting at MR located in the CNS. The continuous intracerebroventricular infusion of aldosterone at levels that are without effect when administered subcutaneously produces significant increases in resting blood pressure. Although the MR is expressed numerous locations including in the hippocampus, amygdala, lateral septum, and hypothalamus where the maintenance of normal blood pressure as well as ACTH release, arousal and fluid osmolality equilibrium are regulated largely by glucocorticoids, aldosterone signaling via the MR can also interact with specific signaling systems in the CNS to fine tune sympathetic outflow and salt sensitivity/appetite, and thus have a profound effect on blood pressure control in physiology and disease.

As discussed earlier, the MR has a number of ways to regulate cell function from canonical transcription of target genes to rapid actions on noncanonical pathways such as second messenger systems. Similarly, nonrenal actions of aldosterone in the heart, vasculature, and brain are driven by both transcriptional regulation of genes encoding ion channels (e.g., L-type, T-type calcium channels), sodium and calcium exchangers (e.g., sodium hydrogen exchanger 1; sodium calcium exchanger), vasoactive peptides (e.g., endothelin, nitric oxide), and their receptors (e.g., endothelin 1 receptor; angiotensin receptor 1). The MR can also regulate cellular function in these tissues via numerous noncanonical pathways including rapid phosphorylation events and interaction with cell signaling intermediates (Feldman and Gros, 2013; Ong and Young, 2017). This will be discussed in further detail in the pathophysiological section later.

Aldosterone Action and Function: Other Tissues

Although the MR is expressed in many cell types that regulate homeostasis in the cardiovascular/renovascular system it is also found in an increasing number of tissues unrelated to renal/cardiac function. In these tissues, a new biology is being described that involves many aldosterone-independent effects that presumably indicate that cortisol is the primary ligand in these tissues, for example, adipose tissue both white and brown, and immune cells. However, this does not preclude a role for aldosterone when serum levels are elevated as in primary aldosteronism.

Tissues where ion transport is tightly regulated to control fluid volume and ion concentrations can be regulated by aldosterone. The salivary glands express MR and HSD2 and control ion transport in a manner not dissimilar to the distal nephron. Similarly, HSD2 and MR also colocalize in many cell types of the respiratory tract; in the tracheal and bronchial glands, ciliated bronchial epithelial cells and in type II alveolar epithelial cells, indicating important roles for mineralocorticoid action in the lung.

The ear is another less-appreciated site for aldosterone action that also expresses HSD2, indicating aldosterone-dependent MR signaling in the endolymphatic sac and cochlear in particular. Clinical and experimental studies suggest that aldosterone can restore cochlear auditory function as well as prednisolone, in a manner not related to immune function but via aldosterone-dependent control of ENaC and osmotic pressure of the endolymph in the luminal fluid of the inner ear. Indeed, a low-salt diet has been the main treatment modality for Ménière's disease since the 1930s and is proposed to serve to increase aldosterone levels to increase endolymph absorption in the inner ear.

The MR is also found in several ocular cell types where it is also coexpressed with HSD2. Retinal cone cells, pigment epithelium, epithelium of ciliary body, iris, cornea, and lens all express the MR. In the eye, the neuroretina in particular is mineralocorticoid-sensitive and aldosterone control of hydration of the healthy retina through regulation of ion/water channels expression is a physiological response. Acute aldosterone administration upregulates expression of the ENaC α-subunit, the potassium channel Kir4.1 and aquaporin 4 (AQP4) a water channel to induce retinal swelling and activation of glial cells located in the retina. The importance of this MR signaling in the eye is also demonstrated by the condition of central serous chorioretinopathy (CSCR), a vision-threatening eye disease. CSCR is characterized by dilation and leakage of choroid vessels beneath the retina to cause fluid accumulation and retinal detachment, which is exacerbated by glucocorticoids and involves MR-dependent activation of the endothelial vasodilatory potassium channel, KCa2.3. MR antagonist (MRA) are very effective at reversing this disorder. Whether they are effective in retinopathy associated with diabetic vascular changes is less clear.

The skin is also a tissue in which the function of the MR is incompletely understood. Aldosterone and MRA can modulate elastin and collagen deposition in human skin fibroblasts via regulation of IGF-I signaling and may have therapeutic value for the treatment of damaged skin with reduced volume and elasticity particularly as a therapy for disfiguring dermal lesions to prevent their relapse. Aldosterone is also important in the conservation of sodium in the sweat gland. The MR is also located in the eccrine ducts of sweat glands where the activity of HSD2 has been demonstrated and indicates aldosterone-dependent regulation. Other types of sweat glands (sebaceous and apocrine) do not appear to expression MR and HSD2.

In contrast, epidermal barrier formation in mice in which the MR is overexpressed occurs early and is associated with reduced epidermal atrophy, increased keratinocyte apoptosis, and premature eye opening. Whereas overexpression in the adult leads to alopecia and hair follicle dysfunction and cysts while other areas of skin remained normal. Consistent with these data, MR epidermal knockout mice exhibited increased keratinocyte proliferation and differentiation and showed resistance to glucocorticoid-induced epidermal thinning. However, loss of epidermal MR causes mice to be more sensitive to skin damage and

inflammation. Thus epidermal MR in adult skin regulates homeostasis with a nonredundant role in mediating glucocorticoid actions. Aging-like skin changes in the metabolic syndrome may also be mediated by MR signaling. MR expression is higher in aged skin and in skin from animals subjected to the metabolic syndrome. Given that aging is associated with increased oxidative stress and inflammatory pathway activation, common mediators of aging and metabolic syndrome, it may be that topical MR blockade with spironolactone could be a useful therapy to reverse the progressive tissue damage in this and other pathologies that involve similar etiologies.

In the last decade, a role for aldosterone signaling in the function of adipose tissue has been proposed and supported by a series of experimental and clinical studies. The original observations of a MR-dependent mechanism in adipose depots arose from MR over expressing mice who showed increased brown fat depots that resulted in a "buffalo-like" hump in the mid-scapula space. Cell culture studies subsequently identified aldosterone-mediated regulation of both white and brown adipocytes. MR activation by aldosterone or glucocorticoids is required for expansion and differentiation and, in the mature cell, aldosterone promotes proinflammatory capacity within the cells as well as dysfunction of lipolytic and glucose handling pathways. Given the absence of HSD2 in these white adipose cells, it is likely that under normal conditions, the MR responses to glucocorticoids in most part. However, in the setting primary aldosteronism, MR activation is increasingly recognized as a contributing factor to the onset of metabolic disease in these patients (Manrique *et al.*, 2014). MR signaling in brown adipose cells also regulates proliferation, as evidenced by the expanded brown adipose intrascapular depot in MR plus mice, but more importantly, it also plays a role in regulating brown adipocyte metabolism and nonshivering thermogenesis. Specifically, the use of MRA in experimental animals fed a high fat diet upregulates brown adipocyte-specific transcripts and markedly increased protein levels of uncoupling protein 1. Moreover, these features were also detected in visceral and inguinal fat depots in response to MRA, indicating a transition of white adipose tissue to brown. Underlying mechanisms involved MR-dependent control of autophagic rate. In brown adipose tissue, the MR is similarly unprotected by HSD2, suggesting that glucocorticoids may have a role in regulation of brown adipose tissue, similar to white, via a second receptor signaling mechanism. In the setting of elevated aldosterone it is likely that the adipose receptor is another mediator of tissue and/or systemic pathological changes.

CNS and Salt Appetite

As noted earlier, increasing renal sodium retention is required to restore salt balance and fluid volume homeostasis. Integral to this response is the control of salt appetite in specific regions of the brain that in turn regulates sympathetic outflow to the kidney and vasopressin release, responses that are also tightly controlled by aldosterone. The MR is widely expressed in the brain in regions that include the choroid plexus, hippocampus, specific hypothalamic nuclei, the amygdala, circumventricular organs, brain stem, cerebellum, and cortex. Notably, HSD2 expression has been detected in the nucleus tractus solitarius (NTS) and hindbrain where it is proposed to control aldosterone actions on sodium appetite. Sodium deprivation and salt intake activate separate neuronal subpopulations in the NTS which relays efferent signals via the parabrachial complex in an aldosterone-dependent manner. It is proposed that in some brain regions aldosterone activates signaling mechanisms that are analogous to those in the kidney and colon (e.g., SGK1, NEDD4-2, ENaC) to mediate these responses (Geerling and Loewy, 2009). The MR may also influence "gustatory salt sensing" in the tongue, which is a key requirement for increasing salt intake. Thus, mineralocorticoids appear to induce and regulate similar molecular pathways in multiple tissues in the behavioral/cardiorenal/sensory processes involved in sodium appetite. Given that renal salt retention and increased sodium appetite can, for example, be maladaptive, aldosterone acting at one or more functional sites can sustain the pathophysiology of salt-sensitive hypertension and chronic heart failure. Of note, reduced HSD2 activity in the brain does not inherently cause hypertension, but instead promotes salt appetite and the transition from salt resistance to sensitivity. Data suggest that HSD2-positive neurons (i.e., NTS) integrate salt appetite and the blood pressure response to dietary sodium through a MR-dependent pathway.

The MR has a range of other, nonsodium-related effects in brain regions where cortisol/corticosterone plays a more prominent role given the absence of HSD2. Receptors located in limbic brain regions including hippocampal neurons respond to very low levels of cortisol/corticosterone and aldosterone. Here, the function of the MR is to stabilize neuronal transmission and integrity of the dentate gyrus. In the presence of stress or at the circadian rise in hormone levels, high concentrations of corticosterone also activate GR, which play a role in the maintenance of homeostasis. Coordinated responses for the two corticosteroid receptors is thus essential for regulation of the stress response and behavioral adaptation (Gomez-Sanchez, 2014).

Aldosterone and the MR in Pathophysiological Signaling

The sodium-retaining, potassium-wasting actions of aldosterone were known before aldosterone was isolated in 1953. The first MRA was developed in the few years following aldosterone's discovery and was proposed for clinical use as a potassium sparing diuretic for the control of hypertension. The pressor effect of elevated or continuous aldosterone was thus well recognized as a pathophysiological action of the hormone. In 1956, Jerome W. Conn was the first to describe a patient with uncontrolled hypertension, muscle weakness, and electrolyte imbalance that was due to autonomous aldosterone production by an adrenal cortical tumor. Conn syndrome, together with other mechanisms of autonomous aldosterone production termed "primary aldosteroneism," is now recognized as a potentially curable primary cause of hypertension that may affect up to ~10% of resistant

hypertensive patients. Primary aldosteronism now encompasses many subtypes and is discussed in full detail in the accompanying chapters.

Despite primary aldosteronism being a well-recognized risk factor for cardiovascular and renal disease, it was not until 1992 that experimental studies investigating the pathophysiology of elevated aldosterone renewed research efforts into understanding the action of aldosterone on the cardiovascular system. Experimental animals given exogenous aldosterone and 1% saline solution to drink develop hypertension, cardiac hypertrophy, and fibrosis that recapitulate the tissue inflammation and remodeling observed clinically in patients with heart failure (Lother and Hein, 2016). Many of the pathological processes involved were first described decades earlier by Hans Selye who described the histological changes of the "general adaptation syndrome" in response to high levels of exogenous deoxycorticosterone administration. However, the "granuloma tissue formation, fibrosis and dysfunction of peripheral organs" were ascribed to glucocorticoid actions of deoxycorticosterone (a naturally occurring mineralocorticoid) given that aldosterone was yet to be discovered. These early studies raise an important point that the inflammatory and fibrotic processes and gene expression changes that follow increased MR signaling are to some extent similar between tissues. For example, cardiomyocytes respond to increased aldosterone by regulating a program of gene expression and protein regulation that promotes cardiac hypertrophy, electrical signaling disturbances, and expression of inflammation and chemotactic proteins (Kritis *et al.*, 2016). In the kidney, markers of podocyte injury and glomerular dysfunction and hypertrophy follow aldosterone signaling and regulation of profibrotic markers, chemoattractant factors, and a coordinated program of inflammation by stromal and infiltrating immune cell populations. MR blockade is thus protective against the progression of tissue fibrosis in a range of tissues including heart, kidney, liver, vessel wall, and lung.

There are many pathological actions of the MR that have been described for an increasing number of tissues where the action of aldosterone-mediated MR signaling is on the stromal cells of the organ—podocytes, cardiomyocytes, lung epithelial, adipocytes, etc. Many of these pathological actions are covered in the accompanying sections in the current issue of this Encyclopedia.

Summary

Therefore aldosterone action in epithelial cells results in MR-mediated increased apical sodium influx through the ENaC with a coupled export of sodium at the basolateral membrane by sodium–potassium ATPase, an energy-dependent process. Given that the MR is expressed in a range of other tissues some of which are also involved in electrolyte transport and many other that are not, that is, cardiovascular homeostasis, aldosterone action clearly encompasses a broad range of physiological and pathophysiological roles. It is of course important to keep in mind that in tissues where HSD2 is not coexpressed, the physiological role of the MR may primarily be to mediate glucocorticoid signaling, but in situations of aldosterone excess their inappropriate activation may mediate adverse consequences, which contribute to the pathophysiology of conditions including primary aldosteronism (Conn syndrome) and cardiovascular disease.

See also: Aldosterone; Synthesis and Metabolism. Regulation of Potassium Homeostasis

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Aldosterone Receptors☆

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Glossary

Aldosterone A steroid hormone synthesized in the zona glomerulosa of the adrenal cortex; the major mineralocorticoid hormone in mammals.
Mineralocorticoid Corticosteroid hormone secreted by the adrenal gland or synthetic compound exerting its function through binding to and activation of the mineralocorticoid receptor (MR), also referred to as ligand that regulates water and electrolyte balance.

Introduction

The aldosterone receptor, also referred to as the mineralocorticoid receptor (MR), is a member of the nuclear receptor superfamily (*NR3C2*) that acts as a ligand-dependent transcription factor mediating mineralocorticoid effects on a large variety of target cells. These include epithelial cells of the kidney and colon but also nonepithelial cells in the cardiovascular and central nervous systems, adipose tissues, immune cells, for instance. Alterations of the mineralocorticoid signaling are involved in several pathophysiological conditions in humans. Aldosterone exerts multiple actions through genomic and nongenomic mechanisms. This article will mainly focus on genomic actions which are the best characterized and probably the most prominent in terms of general physiology.

Mechanism of Aldosterone Action

Aldosterone and glucocorticoids (cortisol in humans and corticosterone in rodents) bind to MR with the same affinity (K_d in the nanomolar range). However, the mineralocorticoid selectivity is partly ensured by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in epithelial tissues, which metabolizes 11β-hydroxysteroids into 11 keto-derivatives such as cortisone, which exhibits no affinity for MR, thus preventing permanent occupancy of the MR by the more prevalent glucocorticoid hormones (Funder *et al.*, 1988; Edwards *et al.*, 1988). **Fig. 1** illustrates the mechanism of aldosterone action in a typical, polarized epithelial principal cell of the distal nephron. Aldosterone penetrates cells by passive diffusion and binds to the MR. In the absence of its ligand, the receptor is mainly located in the cytoplasm and is complexed with various chaperon proteins such as the heat shock protein 90 (hsp90), the heat shock protein 70 (hsp70), but also with other proteins including immunophilins such as cyclophilin (Cyp40), and FKBP52 in a hetero-oligomeric complex. These receptor-associated proteins dissociate from the MR upon aldosterone binding, thus inducing conformational changes of the MR. In the nucleus, the hormone-receptor complexes bind mostly as homodimers to specific DNA sequences, which have been identified as mineralocorticoid response elements (MREs), and have been located in the regulatory regions of aldosterone-sensitive genes. The consensus sequence of these MREs has recently been characterized as a 15-nucleotide-sequence AGtACAgxatGTtCt consisting of partial inverted direct repeat as identified by chromatin immunoprecipitation assays followed by DNA sequencing (ChIP-Seq) (Le Billan *et al.*, 2015). Thereafter, MR interacts, in a cyclic, sequential and/or combinatorial manner, with several transcriptional coregulators and some basal transcription factors or components of the machinery to enhance transcriptional activation (initiation and elongation) and to facilitate chromatin remodeling involving histone acetylation/methylation. In epithelial tissues such as kidney and colon, MR stimulates expression of ion transporters: the amiloride-sensitive epithelial Na^+ channel (ENaC), located at the apical membrane, and the basolateral Na^+, K^+-ATPase pump. These channels or transporters are involved in the unidirectional transepithelial sodium transport from the lumen to the interstitium. The best studied aldosterone-regulated gene in epithelial tissues is the serum- and glucocorticoid-regulated kinase (sgk1), which phosphorylates the ubiquitin ligase Nedd4-2, which in turn controls the retrieval of the subunits of ENaC from the apical membrane of the cell. Several other aldosterone-target genes have been identified by high throughput techniques (SAGE or ChIP-sequencing) such as the small monomeric GTP-binding protein Kirsten Ras (Ki-Ras), the glucocorticoid-induced leucine zipper protein (GILZ), the serine/threonine kinase KS-WNK1 (with no lysine K kinase), the N-myc down-regulated gene 2 (NDRG2), which also seem to

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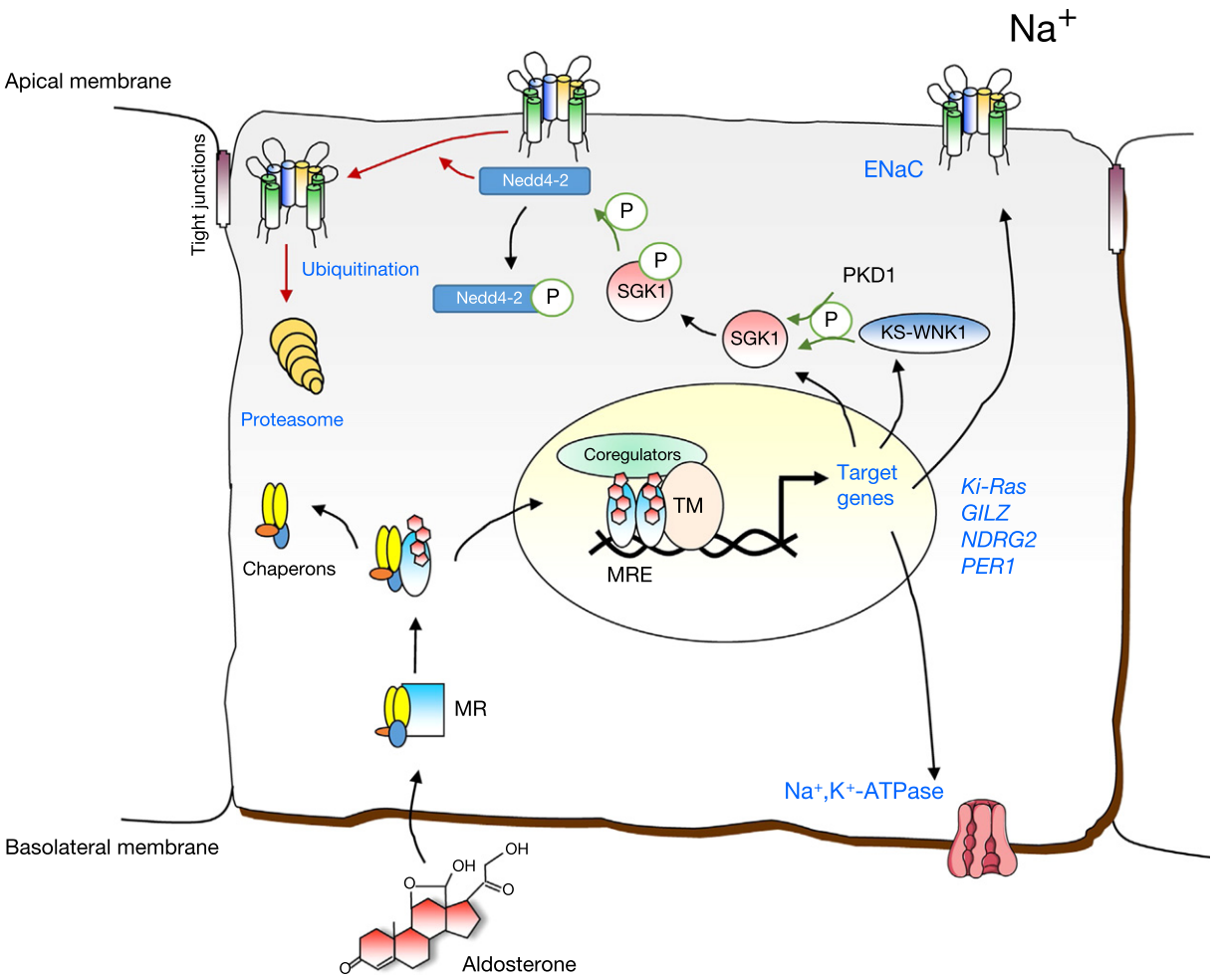

Fig. 1 Schematic representation of aldosterone action in a renal polarized epithelial cell. Aldosterone enters the cell and binds the cytoplasmic MR, which is translocated into the nucleus. MR interacts with mineralocorticoid response elements (MRE), recruits various transcriptional coregulators and some basal transcription factors or components of the machinery to enhance transcriptional activation, and thus modulates expression of aldosterone target genes. At the apical membrane, ENaC (epithelial sodium channel) constitutes the rate-limiting step of apical Na^+ entry. Na^+ is then extruded into the basolateral space by the Na^+/K^+-ATPase pump, the activity of which is enhanced by aldosterone. In the absence of aldosterone, ENaC proteins interact with Nedd4-2, an ubiquitin-ligase, responsible for ENaC degradation by the proteasome. *Sgk1* (serum and glucocorticoid-regulated kinase) is a key aldosterone-regulated target gene that plays a central role in Na^+ reabsorption. Upon aldosterone exposure, activated-PDK1 phosphorylates Nedd4-2, which in turn dissociates from ENaC, increasing its apical membrane abundance. Other aldosterone-induced genes included *Ki-Ras*, kidney specific *KS-WNK1* (serine/threonine kinase With No K), *GILZ* (glucocorticoid-induced leucine zipper), *NDRG2* (N-myc down-stream regulated gene 2) and *PER1* (period circadian regulator 1).

play important roles during the early phase of aldosterone responses in the nephron (Viengchareun *et al.*, 2007). Of particular interest, the *PER1* gene has recently been identified as another aldosterone-target gene in the kidney, suggesting that aldosterone may also regulate the rhythmicity of renal sodium reabsorption since *PER1* belongs to the circadian clock gene family. Beyond this classical model of aldosterone action, recent data obtained from ChIP experiments revealed that MR can also indirectly bind to recognition motifs for other transcription factors (FOX, EGR1, AP1, PAX5), suggesting functional interactions of MR with other transcription factors through tethering mechanisms, thus enabling to modulate target gene expression (Le Billan *et al.*, 2015).

Structure of the MR

The year 2017 marks the 30th anniversary of the cloning of the human MR cDNA by Ron Evans's group (Arriza *et al.*, 1987). This achievement enabled the definition and the characterization of distinct functional domains, which are common to other members of the nuclear receptor superfamily: first, the transactivation domain or amino terminal region (N-Terminal Domain, NTD) is the longest domain (602 amino acids) among nuclear receptor family members and shares less than 15% homology with its closely related receptor, the glucocorticoid receptor encoded by the *NR3C1* gene. However, more than 85% of this MR NTD domain is

highly conserved among all known mammalian species, suggesting that it contains specific and important functional motifs. Indeed, this NTD harbors two ligand-independent activation functions: AF1a located in amino acid (aa) 1–167 and AF1b spanning aa 445–602 and presumably an inhibitory transactivation region between aa 167 and 437 (Tallec *et al.*, 2003). This NTD was also shown to interact specifically with coregulators capable of modulating aldosterone-activated MR transcription. Next, the highly conserved DNA-binding domain (DBD) is responsible for the specific interaction with MREs located in the regulatory regions of aldosterone target genes. This short (<70 aa) hydrophilic domain, rich in cysteine residues, has a rigid structure composed of two zinc finger structures. The P box offers the interacting contact with the half-site of the response element and the D box is responsible for weak dimerization interface with another MR monomer. A nuclear export signal has also been identified between the two zinc fingers, and a weak ligand-independent nuclear localization signal, NSL1, has been shown to be located next to the C-terminal site of the DBD. Finally, the ligand-binding domain (LBD), which is 250 aa long and is separated from the DBD by a hydrophilic proline-rich hinge region, is responsible for ligand binding and contains a ligand-dependent nuclear localization signal (NLS2), multiple contact sites for hsp90 interaction, and a ligand-dependent activating function AF2 domain. This highly structured domain is composed of 11 α-helices (H1–H12, H2 is somehow absent) and two antiparallel β strands on which the steroid hormone lies. It is interesting to note that the mineralocorticoid selectivity of aldosterone action within target cells, beyond the 11β-HSD2 enzyme, is also ensured by an intrinsic property of MR, which is able to discriminate between aldosterone and glucocorticoids since dissociation rates (k_{-1}) are much faster for glucocorticoids than for aldosterone. Moreover, it has been shown that the aldosterone–MR complex presumably adopts structural conformation somehow different from that of the glucocorticoid–MR complex, thereby allowing distinct interaction between the N-terminal domain and the LBD to occur. This leads to the recruitment of specific coactivators resulting in a highly specific transcriptional response (Farman and Rafestin-Oblin, 2001). It has also recently been proposed that specific cyclical and dynamic interaction of MR with DNA, as a function of the nature of the MR-bound ligand, is responsible for distinct transcriptional signature.

Site of MR Expression

Cell-specific MR expression was initially mostly characterized in polarized cells of sodium-transporting epithelia: distal parts of the nephron (distal convoluted tubule, connecting tubule and cortical collecting tubule), small intestine and colon, pneumocytes, salivary and sweat glands, keratinocytes and hair follicles, eye and inner ear (Viengchareun *et al.*, 2007). However, it is now well established that MR expression is also detected in nonepithelial cells or tissues such as neurons of the hippocampus and hypothalamus, cardiomyocytes, endothelial cells, adipocytes, vascular smooth muscle cells, liver, leukocytes, macrophages, uterus and gonads where it is principally activated by cortisol, owing to the absence of 11β-HSD2 expression. In parallel, MR also demonstrates a tissue-specific developmental expression pattern. Indeed, it has been shown that, while MR (kidney, heart, brain and lung), is expressed in the fetus starting from mid gestation (E18 in mice, 20–25 gestational weeks in humans), its expression is

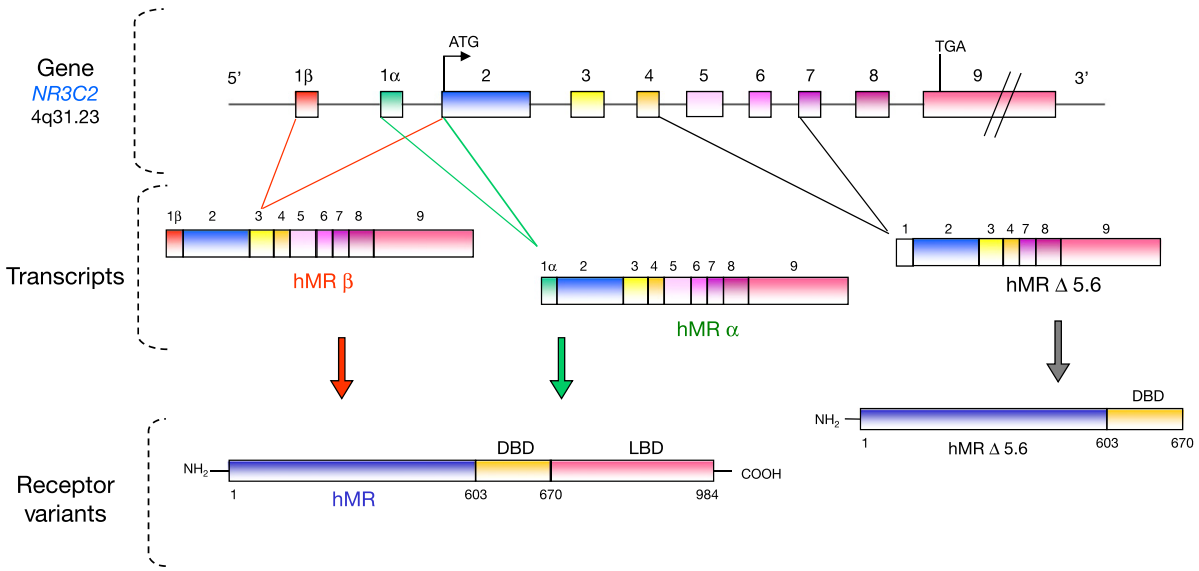

Fig. 2 Schematic illustration of the human *NR3C2* structure. *NR3C2* gene, transcripts, receptor variants, and functional domains are depicted. The human *NR3C2* gene is composed of 10 exons, including two untranslated first exons (1α and 1β).The AUG translational initiation start codon is located 2 bp after the beginning of exon 2, while the stop codon is located in exon 9. Multiple mRNA isoforms generated by alternative transcription or splicing events are translated into various protein variants (i.e., the human MR α, β and Δ 5.6 variants). The receptor is comprised of distinct functional domains: the N-terminal domain (NTD), the DNA binding domain (DBD) and the C-terminal domain (LBD).

temporarily downregulated during the perinatal period in a tissue-specific manner, with no renal, cardiac or neuronal MR expression, at variance with a maintained pulmonary MR expression (Martinerie *et al.*, 2009, 2013).

Regulation of MR Expression

The *NR3C2* gene, which encodes for the human MR, is localized to the q31.2 region of chromosome 4 and spans approximately 450 kb. This gene is composed of 10 exons: exon 2 encodes for the NTD of the receptor and the two small exons 3 and 4 encode for the two zinc fingers of the DBD, whereas the last 5 exons encode for the LBD of the receptor. However, the two first exons, referred to as exon 1α and exon 1β, are not translated (**Fig. 2**). Alternative transcription of these two 5′-untranslated exons generates two mRNA isoforms, referred to as hMRα and hMRβ, which give rise to the same translation product (Zennaro *et al.*, 1995). Interestingly, several other MR splice variants, lacking one (Δ6 MR) or two exons (Δ5,6 MR), have been identified so far and such splice variants seem to play major roles in modulating receptor function (Zennaro *et al.*, 2001; Lema *et al.*, 2017a).

At the Transcriptional Level

The *NR3C2* gene expression is controlled by two alternative promoters which differ in terms of their basal activity as well as their hormonal regulation. Experiments in transgenic mice have shown distinct tissue-specific usage and activity of these two hMR regulatory regions in vivo. The proximal P1 promoter corresponding to the 5′-flanking region of exon 1α is a relatively strong promoter that is transcriptionally active in all aldosterone target tissues, whereas the distal P2 promoter flanking exon 1β is weaker and has a more restricted pattern of expression, notably in the central nervous system; thus, it is presumably activated during specific developmental stages or physiological situations (Le Menuet *et al.*, 2000).

At the Posttranscriptional Level

The stability of MR transcripts is also regulated at the posttranscriptional level. This is particularly true in the kidney where renal MR expression was recently shown to be modulated by large variations of extracellular tonicity, prevailing in the nephron (Viengchareun *et al.*, 2009). Indeed, hypertonicity (500 mOsm/L) was shown to greatly decrease renal MR levels by recruiting the RNA binding protein (RBP) Tis11b (tetradecanoyl phorbol acetate inducible sequence 11b), which physically interacts with MR transcript 3′-untranslated region (3′-UTR), thus modulating its mRNA turnover in response to osmotic stress (Viengchareun *et al.*, 2014). Conversely, renal MR transcript and protein levels were shown to increase under hypotonicity (150 mOsm/L) by recruiting HuR (human antigen R), another RBP member of the Hu family. Under hypotonic stress, HuR behaves as a novel posttranscriptional regulator of MR expression. HuR is specifically and rapidly exported to the cytoplasm of renal cells upon hypotonic stress, where it interacts with MR 3′-UTR to stabilize and increase MR levels, thereby modulating MR signaling (Lema *et al.*, 2017b). During this past decade, microRNA (miRNA) have also emerged as new class of posttranscriptional regulators. Indeed, binding of these miRNA in the 3′-UTR of a target transcript may affect its stability or translation (Bartel, 2004). Interestingly, we and other groups have recently identified several putative miRNA binding sites in MR 3′-UTR, suggesting that miRNA may also modulate MR expression at the posttranscriptional level. Taken together, RNA Binding Protein and miRNA seem to be of particular importance in the control of MR expression, presumably during the prenatal period and notably at birth, where renal MR expression is downregulated, accounting for the transient physiological renal resistance to aldosterone observed in the early neonatal period (Martinerie *et al.*, 2009). Of note, several studies reported that MR protein may also be submitted to posttranslational modifications (PTM) such as phosphorylation, sumoylation or ubiquitinylation (Viengchareun *et al.*, 2007). These PTM did not seem to modulate per se MR expression but rather might alter MR transcriptional activity or bioavailability in cells.

Biological Functions

Mineralocorticoid signaling is primarily implicated in the maintenance of water and salt homeostasis by regulating sodium reabsorption and potassium excretion across tight epithelia. As such, it plays a key role in the control of blood pressure, and in turn, the dysregulated aldosterone secretion and/or mineralocorticoid action are involved in many human diseases such as hypertension, heart failure and chronic kidney diseases associated with increased activation or upregulated mineralocorticoid signaling, while dehydration and sodium loss are related to downregulated or defective mineralocorticoid signaling. In none-pithelial target cells, mineralocorticoid signaling has been linked to various physiological processes, such as memorization and learning, stress response, neuroprotection and regulation of sodium appetite (hippocampus), thermogenesis and differentiation of preadipocytes into mature adipocytes (adipose tissue) and cardiac remodeling.

MR in (Patho)Physiology

Numerous studies have demonstrated a direct relationship between mineralocorticoid signaling dysregulation and human pathologies, mostly in cardio-vascular and kidney diseases. This has led to the emergence of several classes of

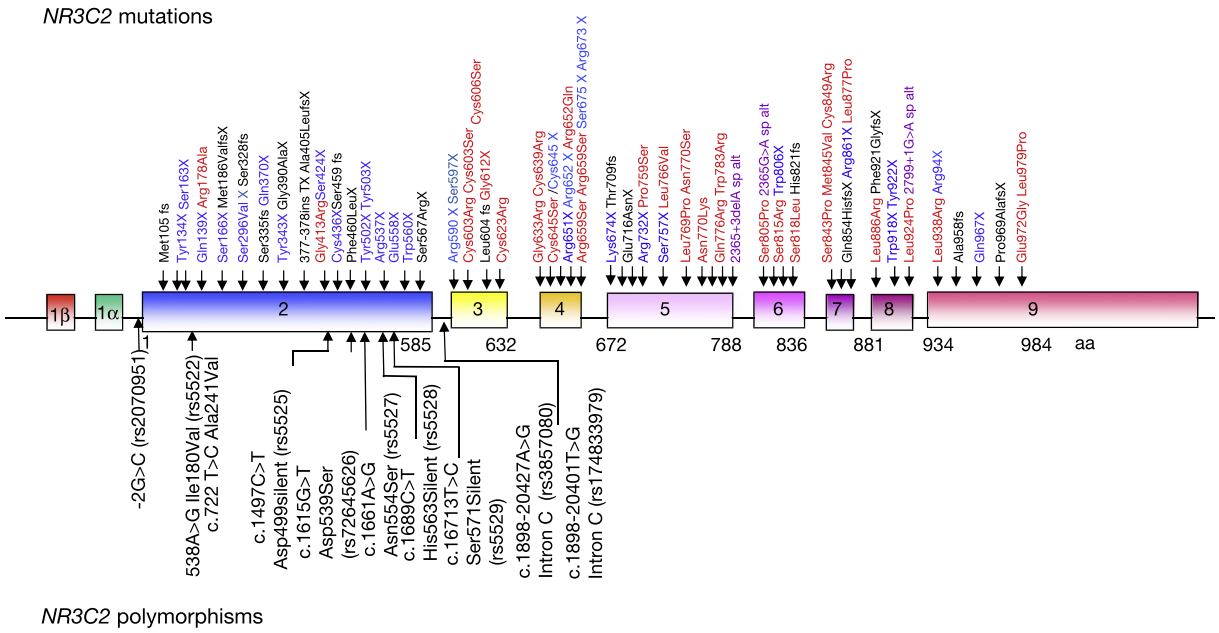

Fig. 3 Reported MR mutations and their localization on the human *NR3C2* gene (as to Jan. 2018). Schematic representation of *NR3C2* gene and its genomic structure (10 exons, from 1β to 9) encoding for various functional domains of the MR protein. Color code of the reported *NR3C2* mutations: *Black* frame shift (fs) mutations associated with premature codon stop. *Red* missense mutations corresponding to a specific aa substitution at a particular position onto a functional domain of the MR protein; NTD, N-terminal domain (1–585 aa encoded by exon 2); DBD: DNA binding domain (586–671 aa encoded by exons 3 and 4); LBD: ligand-binding domain (672–984 aa, encoded by exons 5 to 9), *Violet*, splice variation mutants, *Blue* nonsense mutations with introduction of a stop codon. Localization of some *NR3C2* polymorphisms: most of them are located in exon 2, while others are located in intron B or C. Rs indicates the # single nucleotide polymorphism.

antimineralocorticoid compounds in order to prevent or cure deleterious effects of MR activation. In humans, major clinical trials (RALES study, EPHESUS study, EMPHASIS-HF study, REMINDER study) demonstrated a beneficial effect of antimineralocorticoid drugs (spironolactone, eplerenone) in reducing morbidity and mortality of patients with severe cardiac failure (Pitt *et al.*, 1999; Zannad *et al.*, 2011) or after acute myocardial infarction (Pitt *et al.*, 2003; Montalescot *et al.*, 2014). However, these antagonists induce a nonnegligible risk of hyperkalemia, which has led to the generation of new nonsteroidal antimineralocorticoids (finerenone, esaxerenone) (Arai *et al.*, 2015) that are presently being evaluated in phase-III clinical trials (Kolkhof and Bärfacker, 2017). There is now also clear evidence that genetic alterations of the *NR3C2* gene are associated with human diseases. The first MR mutations were found in patients with autosomal dominant or sporadic pseudohypoaldosteronism type I, an inherited disorder characterized by renal salt wasting during infancy and associated with failure to thrive, hyponatremia, hyperkalemia, and high plasma aldosterone levels, clinical and biological features that are consistent with aldosterone resistance (Geller *et al.*, 1998). These heterozygous frameshift, nonsense, or missense mutations are located within different functional domains of the MR and in turn, differentially impair its function, generally resulting in receptor inactivity (**Fig. 3**). Aside from these mutations, to date 380 polymorphisms have been identified in the *NR3C2* gene (Lek *et al.*, 2016) that may be implicated in variation in sensitivity to salt, blood pressure, stress response or depression. Indeed, these polymorphisms have been linked to differential levels of MR expression and transactivation capacity (Zennaro and Fernandes-Rosa, 2017). Conversely, only one gain of function mutation in the MR has been reported to date, in a family with severe early-onset hypertension that was exacerbated by pregnancy (Geller *et al.*, 2000). The mutation, a substitution of leucine for serine at codon 810, lies within the LBD and has been shown to drastically modify the steroid specificity of the receptor. Indeed, further experiments demonstrated a constitutive MR activation in the absence of ligand. In addition, progesterone, spironolactone, and even cortisone were able to fully activate the mutant receptor, consistent with the clinical presentation of gestational hypertension. Interestingly, various genetically engineered animals mimicking human diseases have proven to be useful in terms of analyzing the in vivo function of the MR. *Nr3c2* gene inactivation achieved by homologous recombination in mice or by the use of a RNA interference strategy in rat led to knockout animals developing symptoms of pseudohypoaldosteronism (Berger *et al.*, 1998; Lim *et al.*, 2008), that can be rescued by intra-peritoneal injections of NaCl (Bleich *et al.*, 1999). In contrast, hMR overexpressing transgenic mice exhibited specific alterations in renal and cardiac function (Le Menuet *et al.*, 2001; Ouvrard-Pascaud *et al.*, 2005). Several cell-specific MR knockdown or overexpressing animals, notably in adipose tissue (Adipo-MORE) presenting with metabolic syndrome (Nguyen Dinh Cat *et al.*, 2016) have been generated. These animal models constitute attractive new experimental systems to further explore the widespread and pleiotropic function of aldosterone receptors in vivo and to decipher the molecular and cellular events underlying the aldosterone signaling pathway.

See also: Mineralocorticoid Receptor Antagonists

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Regulation of Potassium Homeostasis☆

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Glossary

Extracellular fluid The fluid found between the cells in a tissue.

Homeostasis Maintenance of a stable and balanced environment in the body.

Intracellular fluid The fluid within the tissue cells, constituting approximately 30%–40% of the body weight.

Potassium The major intracellular cation (symbol K (kalium), atomic weight 39.0).

Potassium, deficiency A condition due to decreased dietary intake of potassium, as in starvation, or due to gastrointestinal loss in diarrhea, chronic laxative abuse, vomiting, gastric suction, or bowel diversion.

Potassium, dietary Potassium or potassium compounds used in foods or as foods.

Introduction

Chemistry

Potassium is the eighth most abundant element in the Earth's crust. Its abundance is estimated to be about 2.0%–2.5%. It is a soft, silvery-white with an atomic mass of 39.1 Da. Potassium is present in only one oxidation state ($+1$). It is a powerful reducing agent that is easily oxidized. Potassium is an extremely active metal that reacts violently with oxygen in water and air. Because of its high reactivity, potassium is not found free in nature but only as salts. Potassium compounds have good water solubility.

Biochemical Aspects

Potassium is the most abundant exchangeable cation in the body. As the major intracellular solute, potassium plays a relevant role in modulating cell volume and osmolality. Moreover, it has an important role in several metabolic processes. The total body content of potassium is about 40–55 mmol/kg body weight (Preuss and Clouatre, 2012), corresponding to 3–4 mol (110–150 g) for a 70-kg adult. In infants and children, the potassium body content (expressed per kg/body weight) is similar to that reported for adults (Fomon *et al.*, 1982; Butte *et al.*, 2000). Approximately 98% of the total body potassium content is found within cells (particularly muscle cells) at a concentration of 140–150 mmol/L; the concentration of the remaining 2% contained in the extracellular fluid is 3.5–5.5 mmol/L. Therefore, a gradient exists for the diffusion of potassium from the intracellular to the extracellular fluid, whereas a reverse gradient exists for sodium, which is present at a high concentration extracellularly and at a low concentration intracellularly (Bailey *et al.*, 2014; Gumz *et al.*, 2015). The maintenance of the large concentration gradient between intracellular and extracellular potassium is of paramount importance for electrolyte and fluid homeostasis, regulating neural transmission, muscle contraction, and vascular tone (Bailey *et al.*, 2014; Gumz *et al.*, 2015).

Passive transport of potassium occurs via intracellular and paracellular pathways. The intracellular transport mechanism involves potassium channels. The term potassium channels covers different families of membrane proteins found in both excitable and nonexcitable cells. More than 90 genes coding for principal subunit of potassium channels were identified in the human genome. The common characteristic of the channels is that they have "gates" which open or close in response to specific stimuli, such as voltage, ATP, ionic calcium concentration, hormones, and neurotransmitters. Various stimuli sometimes act together on a channel. Potassium channels exhibit great diversity and may be divided into four main groups: voltage-gated potassium channels (Kv channels); calcium-activated (KCa) channels, including big conductance (BK), intermediate conductance (IK), and small conductance (SK) channels; eukaryotic inward-rectifying potassium channels (Kir channels), and two-pore potassium channels (K2p channels) (Tian *et al.*, 2014). Different types of potassium channels have been implicated in functions such as salivary secretion, bile and gastric acid secretion, protein digestion and absorption, insulin secretion, carbohydrate digestion and absorption, and taste transduction.

Since diffusion occurs along concentration gradients, potassium is actively pumped into the cell from the extracellular fluid by Na^+/K^+-ATPase, also called sodium–potassium pump, which reestablishes the gradients, by moving sodium out and potassium in. Na^+/K^+-ATPase, present on the membrane of almost all cell types, is critical to maintain potassium homeostasis close to its setpoint (Palmer, 2015).

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In the mammalian system, the defense against sustained potassium derangements results from a complex balance among intake, excretion, and the distribution between the intracellular and extracellular spaces. Whereas extrarenal mechanisms that modulate potassium concentration inside and outside the cells are the first line of defense, the kidney is the final effector of long-term defense.

Distribution of Potassium Between the Body Compartments

Potassium homeostasis is finely regulated, so that wide variations in dietary potassium intake produce, in healthy individuals, only small changes in the total body potassium content and in the plasma potassium concentration. After being almost completely absorbed in the gut, potassium is distributed widely throughout the body; of a total potassium intake of 50–100 mmol/day, all but a few millimoles are excreted in the urine (only 5% is excreted in stool and sweat) (Agarwal *et al.*, 1994; Bailey *et al.*, 2014). Therefore, loss of potassium through urine, stool, and sweat generally matches potassium intake, and potassium balance is maintained. Two major physiological mechanisms cooperate to achieve this end, namely, extrarenal mechanisms that control the tissue uptake and distribution of the electrolyte, and a renal tubular system that regulates potassium excretion as a function of acute and chronic changes in potassium intake (Gumz *et al.*, 2015).

Although only a small fraction of total body potassium in humans is in the extracellular space, minor changes in extracellular potassium concentration can be life-threatening (Pepin and Shields, 2012). Therefore, plasma potassium concentration must be maintained close to its setpoint. Cells act as an important storage pool for potassium, alternatively taking up excess potassium or releasing potassium, and operate to maintain its plasma concentration within the narrow limits allowed for physiologic function.

Extrarenal Regulation of Potassium Balance

Potassium can be actively transported into and can diffuse out of all cells. Extracellular potassium concentration is therefore regulated by cell membranes that act as effector organs, by buffering rapid changes in plasma potassium concentration, such as those occurring during the absorptive period. This system is highly effective, particularly in protecting against sudden changes in potassium concentration. As an example, within 60 min of an intravenous load of 0.5 mmol/kg of KCl, only 41% appears in the urine, but plasma potassium rises by no more than 0.6 mmol/L, showing the crucial role of intracellular potassium pool in buffering the extracellular load (Williams *et al.*, 1984).

The maintenance of the distribution of potassium across cells is largely dependent on the activity of the Na^+/K^+-ATPase, which expends energy to pump sodium out of the cell and potassium into the cell. Virtually every cell in the body uses this mechanism to maintain a high intracellular potassium concentration and a low extracellular potassium concentration (Palmer, 2015).

There are several regulatory factors that control the distribution of potassium within body fluid compartments (**Table 1**). Among them, hormones play a prominent role. Insulin drives potassium into cells independent of its hypoglycemic effect, possibly increasing Na^+/K^+-ATPase activity (DeFronzo *et al.*, 1980; Cohen *et al.*, 1991). Also, β-agonists, such as epinephrine, promote rapid and profuse potassium movement from the extracellular to the intracellular fluid by cyclic AMP-dependent activation of the Na^+/K^+-ATPase (DeFronzo *et al.*, 1983; Katz *et al.*, 1984). Both insulin release and epinephrine release increase

Table 1 Determinants of transcellular potassium distribution

Lower plasma potassium concentration
Hormones
Insulin
β-Adrenergic agonists
Aldosterone
Metabolic alkalosis/respiratory alkalosis
Increase plasma potassium concentration
Cell destruction (hemolysis, crushing)
Hormones
α-Adrenergic agonists
Glucagon
Hyperchloremic acidosis
Plasma hyperosmolality
Drugs
β2-Antagonists
Angiotensin-converting enzyme inhibitors
Digitalis
Prostaglandin synthesis inhibitors
Succinylcholine

after meals, thus facilitating the movement of the ingested potassium to the intracellular space and preventing meal-related hyperkalemia.

Hyperkalemia stimulates adrenal aldosterone secretion, which not only affects the renal excretion but also favors a shift of potassium to the intracellular space (Rabinowitz, 1996). Apart from hormonal factors, there are other extrarenal mechanisms that control the distribution of potassium within the body compartments. Changes in the acid–base status can lead to changes in plasma potassium concentration. In particular, a decrease in blood pH causes hydrogen ions to enter cells. Consequently, potassium exits cells in exchange for hydrogen entering. Alkalemia generally induces the opposite response (Lee Hamm *et al.*, 2013). An acute rise in plasma osmolality causes an abrupt increase in plasma potassium, most likely due to the rapid exit of water from the cells, accompanied by an increased amount of potassium leaving the cell (Rastegar, 1990). Finally, several drugs (**Table 1**) could significantly affect extrarenal potassium homeostasis.

Renal Regulation of Potassium Balance

About 90% of dietary potassium is readily absorbed, mainly in the small intestine, mostly through passive mechanisms in response to electrochemical gradients and in proportion to the actual load (Agarwal *et al.*, 1994; Bailey *et al.*, 2014). Circulating potassium concentrations remains relatively stable, even in the presence of wide variations in daily potassium intake, due the rapid cellular uptake of potassium entering the body after meals. This process, defined as extrarenal or internal potassium balance, has been discussed in the previous section and is under the control of the factors reported in **Table 1**.

The long-term potassium homeostasis is regulated mainly by the kidney, which ultimately controls plasma potassium concentration as well as total body potassium. Approximately 90% of the daily K intake (40–100 mEq) is excreted in the urine, the balance between dietary intake and renal excretion being the main regulator of body potassium content. During conditions of neutral potassium balance, the daily intake of potassium is equal to the daily excretion rate, primarily in the urine. The remaining part of dietary potassium is eliminated in the feces and, to a lesser extent, in the sweat (Bailey *et al.*, 2014).

Potassium is freely filtered by the glomerulus. In healthy adults, the rate of potassium filtration by the glomerular capillaries is 756 mmol/day, considering a glomerular filtration rate of 180 L/day multiplied by a plasma potassium concentration of 4.2 mmol/L (Guyton and Hall, 2006). Although there is a complex process of potassium reabsorption and secretion by the nephron from the proximal tubule through the loop of Henle, the bulk of filtered potassium load is reabsorbed in the proximal tubule and loop of Henle, so that $<10\%$ of the filtered load reaches the distal nephron. Beyond the early distal tubule, the tubule may modulate potassium delivery by either reabsorbing or secreting it, through both transcellular and paracellular pathways. Hence, urinary potassium excretion is ultimately regulated by changes in potassium secretion at the distal tubule and the collecting duct under the influence of a number of modulators (Giebisch and Wang, 2010; **Table 2**).

Table 2 Determinants of renal potassium excretion

Lower potassium excretion
Decreased intracellular potassium concentration
Decreased dietary intake
Acidosis
Unfavorable electrical or chemical profile
Reduction of lumenal electronegativity
Reduction of distal urine flow
Reduction of distal sodium delivery or reabsorption
Kidney function
Decreased tubular filtration rate
Tubular damage
Diuretics
Inhibitors of the apical sodium channel (amiloride, triamterene)
Inhibitors of aldosterone
Increase potassium excretion
Increased intracellular potassium concentration
Increased dietary intake
Alkalosis
Aldosterone
Favorable electrical or chemical profile
Increase in lumenal electronegativity
Increase in distal urine flow
Increase in distal sodium delivery
Low lumenal chloride concentration
Loop diuretics (furosemide)

It should be considered that under most circumstances, the kidney primarily works to excrete (rather than to reabsorb) potassium to maintain potassium balance against variation in potassium intake from dietary or other sources. Under normal conditions, when ample potassium is consumed in the diet, there is an addition of potassium along the distal nephron; in the presence of a very high potassium intake, the distal nephron effects net secretion, whereas it effects net potassium reabsorption only in the presence of potassium restriction (Unwin *et al.*, 1994). Cells in the connecting and collecting tubules respond to a number of signals by modulating their rate of potassium secretion over a wide range of values. The Na^+/K^+-ATPase located in the basolateral membranes of the tubules provides the driving force for potassium secretion by pumping potassium into the cells. This enzyme system responds to changes in extracellular potassium concentration, changes in extracellular pH, and changes in mineralocorticoid hormone secretion (O'Neil, 1990).

First, the potassium load absorbed with a meal is the most important determinant of potassium secretion, because it is perceived as a stress upon the organism, which requires immediate response, through the involvement of multiple organs, including the gut, kidney, and the muscle and liver (Boyd-Shiwarski and Subramanya, 2017). Second, the feedback control of aldosterone secretion by the zona glomerulosa of the adrenal cortex is needed for regulation of potassium excretion. Aldosterone secretion is under the strict control of two stimuli, that is, the plasma concentration of angiotensin II and the plasma concentration of potassium itself. Aldosterone, which stimulates Na^+/K^+-ATPase, promotes potassium secretion by increasing basolateral potassium entry into the cells and intracellular potassium concentration (O'Neil, 1990). This in turn will favor the delivery of potassium into the renal tubule lumen down its concentration gradient, thereby stimulating potassium secretion. Glucocorticoids also affect potassium secretion primarily by increasing tubule fluid flow rate (Adam *et al.*, 1984). Acid–base changes acutely affect secretion. Alkalosis induces greater potassium secretion at the distal tubule by moving potassium into the cells and acidosis moves potassium out of cells as hydrogen ions move in. The urine flow rate itself affects potassium secretion; with diuresis, increased amounts of fluid and salts move to the distal tubule, enhancing potassium excretion (Giebisch, 1998). Finally, a number of drugs can affect renal potassium handling by a variety of different mechanisms (Howes, 1995). For instance, loop diuretics, such as furosemide, in addition to increasing flow rate to the distal nephron, inhibit potassium reabsorption by the thick ascending limb of the loop of Henle. In contrast, the so-called potassium-sparing diuretics amiloride and triamterene block the apical sodium channel in the late distal tubule and collecting duct. The blockade of the apical sodium channel increases sodium excretion without affecting potassium secretion. Finally, aldosterone antagonists (e.g., spironolactone) also increase sodium excretion without an increase in potassium excretion.

More recently, it has been reported that renal potassium excretion has also a circadian rhythm independent of food. The circadian rhythm, which is regulated by a central clock in the brain, is transmitted to circadian clocks in the tubule cells responsible for variations in potassium excretion. As a result, potassium excretion is enhanced during the daylight phase and reduced during the night time phase (Gumz and Rabinowitz, 2013).

Nutritional and Metabolic Aspects

Potassium is largely present in many foods, particularly fruits, vegetables, whole grains, legumes, and nuts, typically in the form of organic (gluconate and citrate) salts, or, to a lower extent, of inorganic (bicarbonate and phosphate) salts. Potassium is also found in milk and dairy products and in some types of meat. Substantial potassium losses may occur during food processing and cooking. Estimates of the potassium content in different foods can be found in publicly available repositories (e.g., https://ndb.nal.usda.gov/ndb/nutrients/).

The total, nondiscretionary intake of potassium varies among different populations. In the International Collaborative Survey of Electrolyte Excretion and Blood Pressure, which analyzed 24 h sodium and potassium urinary excretion in over 10,000 individuals from 32 countries, the average potassium intake ranged from 25 mmol/day (American blacks in Goodman, Wisconsin, United States) to 87 mmol/day (Xingu Indians, Brazil) (Intersalt Cooperative Research Group, 1988). A few isolated populations participating in this worldwide survey were found to consume much greater amounts of potassium and smaller amounts of sodium than people living in industrialized countries, their sodium/potassium ratio being similar to that estimated by anthropologists for our hunter–gatherer ancestors (Western diet >3, primitive diets 0.5 or below) (Intersalt Cooperative Research Group, 1988; Frassetto *et al.*, 2009). Potassium intake has been reported to be associated with several health outcomes, particularly cardiovascular endpoints. High dietary potassium intake may protect against developing hypertension and improve blood pressure control in patients with hypertension, while inadequate potassium intake may increase blood pressure (Aburto *et al.*, 2013). Furthermore, there is consistent evidence from observational cohort studies that potassium intakes below 3500 mg (90 mmol)/day are associated with a higher risk of stroke (Vinceti *et al.*, 2016). Thus, to prevent the possible consequences of insufficient potassium intake, international bodies such as WHO (WHO, 2012) and EFSA (EFSA, 2016) considered a potassium intake of at least 90 mmol/day (3500 mg/day) adequate for healthy adults.

As discussed earlier, major sustained increases in plasma potassium concentration are almost never caused by dietary changes, because the homeostatic mechanisms are highly efficient in healthy individuals. In response to a large oral potassium load, the excess potassium is partly stored in the large cellular compartment and partly excreted in the urine in a matter of a few hours. Thus, hyperkalemia may develop only in the presence of severe pathological conditions that impair the activity of these defense mechanisms (e.g., renal failure, adrenal insufficiency) (Lehnhardt and Kemper, 2011). On the other hand, the control of potassium homeostasis is less effective in preventing the hypokalemia caused by a subnormal potassium intake, particularly in the

presence of a concomitant high'sodium diet, which induces an obligatory potassium loss. In fact, severe potassium depletion may occur when habitual potassium intake is <25 mmol/day (Young *et al.*, 1995). In particular, two conditions may often lead to potassium depletion. One is the "protein-modified fast," which may be adopted by severely obese patients in the early phase of a weight-reducing program (Lin *et al.*, 1997), and the other is chronic alcoholism, due to the large amount of "empty calories" ingested from alcohol (Elisaf *et al.*, 2002).

For the reasons just discussed, the collection of accurate information about dietary potassium intake is of primary importance. As powerful homeostatic mechanisms act to minimize changes in plasma potassium, the measurement of plasma potassium concentration is of limited value in estimating the adequacy of potassium intake. Several approaches based on dietary assessment methods have been proposed. The personal recall of food consumption is the least reliable method; history records are slightly better, although their accuracy may be hampered by incomplete record-keeping, variability in the potassium content of foods, and errors in estimation of quantity (Freedman *et al.*, 2015). The most accurate single technique for estimation of dietary intake of nutrients, including potassium, is the collection and analysis of duplicate food samples; unfortunately, this method is expensive and not applicable to population studies. For everyday purposes, the measurement of urinary potassium excretion provides a reasonably good estimate of dietary intake (Tasevska *et al.*, 2006). Its use is based on the assumption that at least 90% of the potassium ingested is eliminated through the renal route. Well-recognized limitations of this method include the relatively high day-to-day intraindividual variability in potassium intake (and thus potassium excretion), and also the concomitant variability in sodium intake, which in turn influences potassium excretion (Siani *et al.*, 1989). To overcome this problem, a certain number of 24 h urine collections are requested if a reasonably good estimate of an individual's habitual potassium intake is to be obtained. Despite these limitations, the measurement of urinary excretion is as yet the simplest and least expensive way to assess dietary sodium and potassium intakes in population studies as well as in clinical studies.

See also: Aldosterone; Action and Function

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Salt-Sensitivity of Blood Pressure

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Abbreviations

20-HETE	20-hydroxyeicosatetraenoic acid
ABPM	Ambulatory blood pressure monitoring
ANPs	Atrial natriuretic peptides
BP	Blood pressure
EETs	Epoxyeicosatrienoic acids
NO	Nitric oxide
OW	Overweight
RAAS	Renin–angiotensin–aldosterone system
SNS	Sympathetic nervous system
WHO	World Health Organization

Glossary

Salt-sensitivity of blood pressure The interindividual difference in the blood pressure response to changes in salt intake.

Introduction

Dietary salt intake plays a key role for cardiovascular health (Aburto *et al.*, 2013). A huge amount of animal experimental investigation, epidemiological studies and controlled clinical trials support the strong relationship existing between salt intake and blood pressure (BP) (He and MacGregor, 2009; He *et al.*, 2013). In most countries worldwide the habitual average sodium intake largely exceeds the physiological needs and the recommended adequate intake determined on the basis of scientific evidence (He and MacGregor, 2009; World Health Organization, 2012). In fact, the majority of individuals experience a decrease in BP when reducing salt intake for a sufficiently prolonged period of time (Sacks *et al.*, 2001). The so-called salt-sensitivity of BP actually refers to the interindividual difference in the BP response to a given change in salt intake. Based on these responses, it has become popular to identify, within a given population sample, individuals (or patients) who are defined either salt-sensitive or salt-resistant using "arbitrary" cut-offs (Weinberger *et al.*, 1993; Galletti *et al.*, 1997a, b). Actually, the BP response to changes in salt intake exquisitely behaves as a continuous variable with a simil-Gaussian distribution (Weinberger *et al.*, 1986; He *et al.*, 2001; Sullivan, 1991), not allowing to detect any distinct subpopulation (He *et al.*, 2009). This notwithstanding, salt-sensitivity of BP is of interest and of potential clinical importance as it is associated with several cardio-metabolic risk factors (Galletti *et al.*, 1997b; Strazzullo *et al.*, 2006) and even with a higher risk of premature death (Weinberger *et al.*, 2001). Therefore, scope of this short review will be (1) to focus on the main factors that determine the individual BP salt-sensitivity, including inherited and acquired factors or medical conditions which are at least partly modifiable; (2) to briefly discuss the methods commonly adopted to assess BP salt-sensitivity either as research tools or in clinical settings.

Determinants of Blood Pressure Salt-Sensitivity

Genetic Factors

Rare monogenic forms of hypertension are paradigmatic expressions of the influence of genetic factors on BP salt-sensitivity, in which mutations in a single gene, encoding a molecule involved in sodium transport through the tubular epithelium, lead to an excess in renal tubular sodium reabsorption (Luft, 2002). Typical examples are the mutations in the aldosterone synthase/11β-hydroxylase genes (glucocorticoid-remediable aldosteronism), in the renal-specific isoform of the 11β-hydroxysteroid dehydrogenase gene (apparent mineralocorticoid excess syndrome), and in the (β or γ subunits) amiloride sensitive epithelial sodium channel, as reported in the Liddle syndrome. All these alterations affect the renal-pressure natriuresis curve and cause salt-sensitive hypertension (Luft 2002; Luft *et al.*, 1988).

In addition to these monogenic forms of hypertension, a large number of genetic polymorphisms have been associated with a high rate of hypertension (Strazzullo and Galletti, 2007), among which those affecting the α-adducin molecule (Bianchi *et al.*, 2005; Manunta *et al.*, 1999), the glucagon receptor (Strazzullo *et al.*, 2001a), the G-protein beta-3 subunit (Pamies-Andreu *et al.*, 2003), the adrenergic receptors (Svetkey *et al.*, 2011; Pojoga *et al.*, 2006), the renal–angiotensin–aldosterone system (Siani *et al.*, 2004), the renal isoenzyme of 11beta-hydroxysteroid dehydrogenase (Agarwal *et al.*, 2000; Lovati *et al.*, 1999), the nitric oxide (NO) production (Hoffmann *et al.*, 2005; Dengel *et al.*, 2007), the natriuretic peptides (Arora *et al.*, 2013), and the arachidonic acid metabolites (Laffer *et al.*, 2008; Williams *et al.*, 2011).

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Physiological Aspects Affecting Blood Pressure Salt-Sensitivity

There is ample evidence that aging is associated with increasing BP response to high salt intake (Weinberger and Fineberg, 1991; He *et al.*, 2009). One of the reasons for this could be the progressive loss of functional nephrons and in turn the decline in renal function occurring at a rate of approximately 10% per decade of life after age 40 (Dunnill and Halley, 1973; Hall *et al.*, 1989). This condition is further amplified by hypertension, diabetes or coexisting renal disease (Johnson *et al.*, 2005; Weinberger 1996). Ethnic group comparisons showed that black individuals have a higher BP response to changes in salt intake than whites (Weinberger *et al.*, 1982), independently of baseline BP (Vollmer *et al.*, 2001; Aburto *et al.*, 2013). Few data are available about comparisons between other ethnic groups (He *et al.*, 2009).

Also, little evidence is available about intergender comparisons. The results of the Dash-trial indeed suggested that women may have greater BP salt-sensitivity than men (Vollmer *et al.*, 2001). Notably, salt-sensitivity of BP is highly variable in women due to changes in circulating levels of sex hormones. Data on young women showed that estrogens may protect them toward the unfavorable effect of salt on BP by affecting the pressure–natriuresis relationship (Pechere-Bertschi and Burnier, 2004). In fact, the pressure–natriuresis curve was shown to move to the right in postmenopausal women (Pechere-Bertschi and Burnier, 2004) and other studies pointed to an association between the age-related loss of estrogens and the development of salt-sensitive hypertension (Weinberger and Fineberg, 1991).

A number of physiological mechanisms that regulate the cardiovascular adaptation to salt intake may affect the BP salt-sensitivity. A major role in this regard is played by the renin–angiotensin–aldosterone system (RAAS) (Hall, 1986). During high salt intake, suppression of the RAAS should allow quantitative excretion of sodium and water with maintenance of a neutral balance and without elevation in BP. RAAS suppression however may be blunted in obese individuals and in type 2 diabetic patients, thus increasing BP salt-sensitivity. On the other hand, the fall in BP following reduction of salt intake may depend on the compensatory increase in RAAS activation which may markedly differ among individuals (He *et al.*, 2001; Yatabe *et al.*, 2010).

Aldosterone affects the pressure–natriuresis relationship to an extent similar to that observed for Angiotensin II. Excess aldosterone secretion leads to higher BP salt-sensitivity: indeed, a moderate rise in plasma aldosterone provokes marked hypertension when salt intake is normal or high, while during low salt intake the effect on BP is modest (Guyton, 1980; Hall *et al.*, 1984; de Paula *et al.*, 2004).

A number of studies suggest that impaired NO production may contribute to sodium balance regulation and may play a role in the pathogenesis of salt-sensitive hypertension (Granger and Alexander, 2000; Sanders, 2004). NO reduction may be a consequence of endothelial dysfunction and contribute to BP salt-sensitivity by inhibition of the vasodilatory response to salt loading (Campese *et al.*, 1997). Several lines of evidence indicate that also the endothelin system could play a role in salt-sensitivity of BP: for instance, urinary endothelin-1 is negatively associated with BP while it is positively associated with sodium excretion during a salt load. One study showed that salt-sensitive hypertensive patients had lower levels of the peptide than salt-resistant patients (Hoffman *et al.*, 1994), supporting its possible pathogenetic role, in keeping with the results from experimental studies in animal models (Kassab *et al.*, 1997, 1998).

Also cytochrome P450-derived metabolites of arachidonic acid, in particular 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs), may have a role in BP salt-sensitivity through their effects on renal pressure natriuresis (Roman, 2002). 20-HETE inhibits sodium reabsorption at the proximal tubule (Quigley *et al.*, 2000; Schwartzman *et al.*, 1985) and at the thick ascending loop of Henle (Wang *et al.*, 1996). On a study in hypertensive individuals receiving a salt load, a correlation between urinary excretion of 20-HETE and sodium excretion in salt-resistant hypertensives was showed, while no association with BP was found. Conversely, no correlation between urinary 20-HETE and sodium excretion was detected in salt-sensitive individuals, while positive correlation with BP was found (Laffer *et al.*, 2003). These findings indicate a possible defect in the ability of 20-HETE to inhibit sodium reabsorption in salt-sensitive patients during salt-loading, leading to a shift in the pressure natriuresis curve and to an increase in BP.

In addition to the vasodilator effect, EETs may affect BP salt-sensitivity by inhibition of sodium transport at the proximal tubule (Harris *et al.*, 1990) and collecting duct (Sakairi *et al.*, 1995). This activity could contribute to the adaptation to a salt diet. Indeed, during salt restriction, EETs were found to be lower in plasma and urine of salt-sensitive compared with salt-resistant subjects (Gilbert *et al.*, 2013).

Contrasting results were reported concerning the relationship between atrial natriuretic peptides (ANPs) and salt-sensitive hypertension (Jin *et al.*, 1992; Campese *et al.*, 1996). Some studies reported a decrease in plasma ANP levels during high salt intake (Campese *et al.*, 1996; Lieb *et al.*, 2011), whereas they showed no difference in the ANP response to high salt intake between salt-sensitive and salt-resistant individuals (de la Sierra *et al.*, 1996).

Last but not least, the sympathetic nervous system (SNS) is involved in BP salt-sensitivity. Salt-sensitive subjects were reported to have a greater plasma catecholamine responses to salt depletion than salt-resistant hypertensive individuals due to sympathetic stimulation by the decrease in BP (Laffer *et al.*, 2006; Elijovich *et al.*, 2001). Moreover, salt-sensitive hypertensive patients provided a higher BP response to exogenous norepinephrine than salt-resistant patients, suggesting substantial differences in norepinephrine metabolism between salt-sensitive and salt-resistant hypertensive patients (Campese *et al.*, 1993). This response was also detected in salt-sensitive normotensive individuals during high salt intake (Sharma *et al.*, 1992).

Salt-Sensitivity of Blood Pressure in Relation to Specific Clinical Conditions

A few clinical conditions are associated with higher BP salt-sensitivity. Among these, overweight (OW) and obesity are definitely the most common ones (Galletti *et al.*, 1997b; Strazzullo *et al.*, 2003). An elegant demonstration of the impact of OW on BP salt-sensitivity was provided by an intervention study in obese and nonobese adolescents by Rocchini *et al.* (1989): on a low-salt diet,

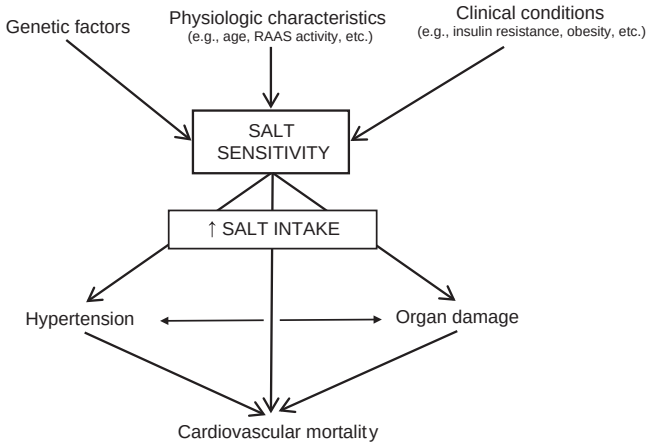

Fig. 1 Mechanisms associated with the heterogeneity in salt-sensitivity of blood pressure. *RAAS*, renin–angiotensin–aldosterone system.

obese and lean adolescents had similar BP, but upon switching to a high-salt consumption, the obese participants developed a much greater increase in BP, arguing for an altered pressure-natriuresis relationship. Moreover, after partial correction of OW through caloric restriction and increased physical exercise, BP salt-sensitivity was reduced and the BP-natriuresis relationship turned out similar to that of normal weight adolescents. Similar alterations of BP salt-sensitivity were described by Strazzullo *et al.* in patients with metabolic syndrome (Strazzullo *et al.*, 2006), confirmed later by the results of the GENSALT study that showed a greater BP response to dietary salt restriction in subjects with metabolic syndrome (Chen *et al.*, 2009). Indeed, both abdominal adiposity (Strazzullo *et al.*, 2001b) and metabolic syndrome (Strazzullo *et al.*, 2006) are associated with an altered rate of proximal tubular sodium reabsorption, which is at least partially explained by the effects of hyperinsulinaemia (Barbato *et al.*, 2004) and hyperleptinaemia (Barba *et al.*, 2003), both common conditions in these subjects.

There is also experimental and clinical evidence of a direct relationship between BP salt-sensitivity and insulin resistance. Thus, in essential hypertensive patients with normal glucose tolerance, insulin-stimulated whole-body glucose uptake was negatively associated with BP salt-sensitivity, independently of age and body mass index (Galletti *et al.*, 1997b). Moreover, BP salt-sensitivity may also be affected by insulin effects through modulation of endothelial relaxation (Bragulat *et al.*, 2001) (**Fig. 1**).

The Assessment of Blood Pressure Salt-Sensitivity

Several ways to measure BP salt-sensitivity have been explored and more or less extensively investigated, however no reliable marker of BP salt-sensitivity has been detected. The gold standard for the evaluation of BP salt-sensitivity should be represented by the BP response to moderate reduction of salt intake maintained over a period of several weeks. However, poor patients' compliance is a serious limitation. To overcome this problem, the assessment of BP salt-sensitivity has most often been made using short-term tests applying huge differences in salt intake for only a few days. Unfortunately, most of the tests have proved inaccurate, poorly reproducible, often costly and not applicable in real life conditions. A widely used protocol for the assessment of BP salt-sensitivity in clinical investigation is the Grim-Weinberger test, which evaluates the BP response to a rapid volume expansion and contraction protocol applied in sequence (Weinberger *et al.*, 1993). BP salt-sensitivity is defined as a 10-mmHg or higher change in mean BP from the level assessed after a 4-h infusion of 2 L of saline solution compared to the level measured the morning after 1 day of a low (10 mmol) sodium diet and administration of three doses of diuretic (Furosemide). This protocol has undergone several reproducibility and comparability tests versus the BP response to dietary salt restriction (Galletti *et al.*, 1997a; Strazzullo *et al.*, 2000) with unsatisfactory results.

More recently, ambulatory blood pressure monitoring (ABPM) has been proposed as another possible way to clinically assess BP salt-sensitivity (Castiglioni *et al.*, 2011). The main study result was that the subjects with greater salt-sensitivity based on the BP response to one-week sodium chloride restriction exhibited an altered circadian BP pattern, with higher night-time BP and heart rate levels, possibly explained as a neurohormonal response to sodium and water retention occurring during daytime. Although of interest, the size of the correlation coefficients was definitely too small to support the possibility of a practical application and, once again, the index of salt-sensitivity used as a reference standard was based on the response to short-term severe reduction in salt intake rather than to moderate long-term salt intake reduction.

Further studies should be carried out for the accurate assessment of the BP salt-sensitivity phenotype in clinical practice, and to overcome the arbitrary stratification of BP salt-sensitivity.

Salt- Sensitivity of Blood Pressure and Cardiovascular Risk

Several studies suggest that BP salt-sensitivity is associated with higher rates of organ injury and cardiovascular events (Bihorac *et al.*, 2000, Morimoto *et al.*, 1997, Weinberger *et al.*, 2001). A longitudinal study showed that BP salt-sensitivity was associated

with higher cardiovascular risk, independently of BP and smoking habits (Morimoto *et al.*, 1997). Another prospective study with 27 year follow-up indicated a higher mortality rate from all causes, both in hypertensive and normotensive subjects, with higher BP salt-sensitivity (Weinberger *et al.*, 2001).

An observational study on normotensive and apparently healthy men with different BP salt-sensitivity showed that the incidence of hypertension, over 15 years, was significantly higher among subjects with a greater degree of BP salt-sensitivity at baseline compared with subjects with lower BP salt-sensitivity (Barba *et al.*, 2007). BP salt-sensitivity was also associated with a higher prevalence of organ damage (e.g. left ventricular hypertrophy, renal dysfunction, etc.) clustered with hyperinsulinaemia, hyperlipidaemia, and evidence of endothelial dysfunction, in untreated hypertensive patients (Bihorac *et al.*, 2000). Likewise, patients with higher BP salt-sensitivity had reduced flow-mediated vasodilation and endothelial dysfunction (Liu *et al.*, 2012).

Conclusions and Perspectives

In summary, a large body of evidence from experimental, clinical and epidemiological studies supports a role for BP salt-sensitivity as predictor of cardiovascular morbidity and mortality. In addition to the genetic and demographic factors, several metabolic and neurohormonal conditions affect BP salt-sensitivity, including excess body weight and its related alterations (i.e. hyperinsulinemia and insulin resistance, hyperleptinemia, over-activity of the RAAS and of the SNS, etc.): most of these conditions are in turn associated with altered renal sodium handling.

In consideration that the majority of subjects in any given population experience a decrease in BP in response to gradual, long-term and moderate dietary salt intake reduction, a population strategy aiming at gradual salt intake reduction as indicated by WHO and the United Nations (World Health Organization, 2013) should be pursued.

See also: Endocrine Hypertension. Hypertension; Overview. Neurogenic Hypertension. Renal Hypertension

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Endogenous Ouabain☆

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Glossary

Allele Any of two or more alternative forms of a gene occupying the same chromosomal locus.

Hypertension A condition of persistently high arterial blood pressure; in adults, this is usually identified as a blood pressure reading that exceeds 140/90 mmHg. The condition may be associated with other disorders (secondary hypertension) or may not have a single identifiable cause (essential or primary hypertension). Individuals affected by hypertension are at risk for heart disease, kidney failure, and stroke.

Ouabain Hygroscopic white plates that are soluble in water and alcohol and used in medicine.

Introduction

Cardiac glycosides have been among the most frequently used classes of drugs for the treatment of heart failure and arrhythmias. It is generally accepted that cardiac glycosides exert their cardiotonic action by binding to the extracellularly exposed recognition sites on the Na^+,K^+-ATPase, an integral membrane protein that establishes the electrochemical gradient of Na^+ and K^+ across the plasma membrane. The phylogenic conservation of isoform-specific regions involved in digitalis binding within the Na^+,K^+-ATPase in distantly related species has led to the proposal that an endogenous counterpart to the plant cardiac glycoside might exist in mammals as well and that the Na^+,K^+-ATPase might represent its functional receptor. The cardiotonic steroid–binding site of the Na,K-ATPase is often called the ouabain-binding site, as this is the cardiotonic steroid most often used in the laboratory. This binding site is highly conserved in diverse organisms (from *Drosophila* to rodent and sheep and to human). The ouabain sensitivity of the Na,K-ATPase is determined by the α subunit.

The Endogenous Na^+,K^+-ATPase Inhibitor: Background Research

Experimental evidence substantiated the presence of several candidates for the mammalian Na^+,K^+-ATPase inhibitor. In particular, the development of volume-expanded conditions in animals was demonstrated to be associated with the appearance in the circulation of an endogenous substance able to inhibit cell membrane sodium transport. These studies documented the existence of a "natriuretic hormone" involved in the control of sodium homeostasis. It has been proposed that an increase in the extracellular fluid volume might be a stimulus for the release of a humoral substance able to counterbalance the increased renal sodium and water reabsorption through its ability to inhibit renal Na^+,K^+-ATPase, the key enzyme involved in renal tubular sodium reabsorptive processes. Moreover, by acting as a physiological regulator of the Na^+–K^+ pump in regions other than the kidney (such as the neurovascular system), the endogenous inhibitor might cause peripheral vasoconstriction, mediated by the increase in intracellular sodium and calcium, leading to a sustained rise in blood pressure (Blaustein, 2014; Hamlyn and Blaustein, 2016).

Many research groups have pursued extensive studies aimed at characterizing the putative humoral substance(s) by isolating the compound(s) under physiological and pathological conditions (hypertension, pregnancy, neonate). Consistent findings over the years indicate that mammalian plasma, urine, and tissues indeed contain distinguishable biologically active candidate inhibitors of the Na^+,K^+-ATPase.

Among the putative endogenous inhibitors, a compound that resembles most of the functional properties of the plant-derived cardiac glycoside has been identified and chemically and functionally characterized. In the human circulation and bovine hypothalamus, Hamlyn's and Haupert's groups recognized this novel steroidal isomer of the plant glycoside ouabain in which the location of two or more steroidal hydroxyl groups may differ. An endogenous compound, showing similarities to ouabain in terms of high-performance liquid chromatography retention time and cross-reactivity with a specific anti-ouabain antibody, was purified from hypertensive rats of the Milan hypertensive strain (MHS) and human plasma (Zhao *et al.*, 1995).

Several other research groups were able to recognize in mammalian plasma and tissues an inhibitor that was indistinguishable from plant ouabain by multiple biochemical and physiological criteria, although the chemical characterization was not completed. Experimental evidence has documented the presence in mammals of several endogenous steroids structurally related to the bufodienolides (as marinobufagenin).

☆*Change History*: February, 2018. P Manunta and M Simonini have updated text and references.

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The existence of distinct endogenous inhibitors of the Na^+,K^+-ATPase is in accordance with the hypothesis that they may be functionally involved as tissue-specific regulators of the different Na^+,K^+-ATPase isoforms and thus they may be independently regulated following particular stimuli. This evidence indicates that such endogenous regulators of the Na^+,K^+-ATPase may play distinct pathophysiological roles.

The Pressor Mechanism of the Endogenous Ouabain

For many years, the authors have been involved in the attempt to elucidate the molecular mechanisms leading to hypertension. The strategy has included studies of renal function and cellular, biochemical, and molecular characterization of the MHS rats, considered a suitable model for at least a subgroup of hypertensive patients. Common genetic–molecular mechanisms underlying the disease have been identified in both species. Increased circulating levels of endogenous ouabain (EO), considered the mammalian counterpart of plant ouabain, and mutations of the gene coding for the cytoskeletal protein adducin have been demonstrated to correlate with increased renal tubular sodium reabsorption and hypertension in both MHS rats and humans. Experimental evidence has suggested that Hexogen (plant) ouabain itself may act as a pressor agent in vivo, since the prolonged infusion of low doses of ouabain into normotensive rats (OS), induces a sustained and reversible form of hypertension. An increased expression and increased activity of renal Na^+,K^+-ATPase have been documented both in vivo, in OS and in MHS rats, and in rat renal cultured cells chronically exposed to nanomolar concentrations of ouabain or in cells transfected with the MHS adducin genetic variant (Manunta *et al.*, 1994). Interestingly, additional data have further investigated the mechanism underlying the increased surface expression of the Na^+,K^+-ATPase induced by both ouabain and adducin. Indeed, it was identify a slower recycling of the protein from the plasma membrane (due to a reduced rate of internalization through the clathrin-coated vesicles during endocytosis) in presence of both conditions (increase of ouabain level and mutated adducin variant). Furthermore, in vitro studies have confirmed that adducin directly interacts with Na^+,K^+-ATPase and that the mutated adducin variant interacts with the enzyme at significantly lower concentrations than the wild-type adducin. Therefore, the overexpression of the renal Na^+–K^+ pump, most probably resulting from a tighter anchoring of the enzyme to the cytoskeletal proteins, appears to be a common biochemical alteration present in both the ouabain- and adducin-dependent forms of hypertension (Lanzani *et al.*, 2010).

At first glance, these findings seem to be inconsistent with the traditional view postulated for the "endogenous Na^+,K^+-ATPase inhibitor," the so-called natriuretic hypothesis, supporting the proposal that the expansion of extracellular volume might induce the release of an endogenous hormone able to promote natriuresis and diuresis. According to this "*old*" hypothesis, a reduction, rather than an increase, in renal Na^+,K^+-ATPase activity would be expected in association with high levels of EO. In order to better clarify this issue, the relationship between EO and changes in sodium balance in both rats and essential hypertensive patients has been investigated and it was demonstrated that an acute and chronic restriction of salt intake, but not acute salt loading, is responsible for a significant rise in plasma EO in both MHS rats and humans (Tripodi *et al.*, 2009).

Collectively, findings in rat and human indicate that EO does not behave like a natriuretic hormone in vivo, but rather EO may be activated under conditions of salt and water reduction. These findings raise the possibility that EO participates in the conservation of body sodium and thus it may be involved in the reestablishment of the hydro-saline homeostatic equilibrium, mediated through its ability to enhance renal Na^+,K^+-ATPase activity. Studies support a unique role played by renal Na^+,K^+-ATPase in the pathophysiological action of EO (Hamlyn and Manunta, 2015).

Since it has been demonstrated that adducin is directly involved in renal Na^+,K^+-ATPase modulation also, the question of whether adducin polymorphism might affect EO response in the adaptation to a low-salt diet has been raised. Indeed, a chronic low-salt diet significantly increased plasma EO levels in hypertensive patients carrying the mutated Trp-460 allele in the ADD1 gene locus, but not in Gly-460 subjects, thus counteracting the hypotensive effect of the low-salt diet. Similarly, an increased EO was shown in MHS rats and in the congenic rat strain NA (obtained by introgressing the MHS ADD1 locus into the normotensive genetic background) but not in normotensive rats. No modifications of plasma EO following consumption of a high-salt diet have been observed in either rats or humans. Data support the hypothesis that the adducin genotype might predict changes in plasma EO levels under salt restriction conditions (Hamlyn and Blaustein, 2013).

Another study has further explored the relationship between EO and blood pressure in the general population and several new findings were reported: (1) the carriers of the mutated α-adducin Trp allele show higher plasma levels than the homozygotic Gly/Gly individuals; (2) EO plasma levels positively correlate with urinary potassium excretion; and (3) there is a significant interaction between EO and urinary sodium excretion in relation to blood pressure (Manunta *et al.*, 2008).

EO appears to behave like a positive regulator of blood pressure when a low-salt diet is followed, whereas EO may act as a compensatory hormone preventing salt-induced high blood pressure when salt intake is elevated. Data indicate that a complex, although not fully elucidated, interrelationship does exist between EO and genetics in the homeostatic regulation of blood pressure in response to changes in salt intake.

Endogenous Ouabain as a Risk Factor for Cardiac and Renal Complications

EO and CV Disease

Data have indicated that EO, in addition to directly influencing blood pressure, is involved in the development of cardiac complications (hypertrophy, heart failure, myocardial infarction) associated with hypertension, as shown in rat models (OS and

MHS rats) and in hypertensive subjects, studied at different stages of the disease (Manunta *et al.*, 1999; Pavlovic, 2014; Pierdomenico *et al.*, 2001). In approximately 50% of patients with uncomplicated essential hypertension, plasma EO levels are increased and positively correlate with left ventricular mass index and stroke volume and negatively correlate with heart rate (Manunta *et al.*, 2011). Moreover, may authors identified a relationship between increased circulating EO and cardiomyopathy; in particular, in the advanced phases of cardiac pathology, plasma EO levels are inversely correlated with stroke volume and are inversely correlated with the ejection fraction. Finally, endogenous cardiotonic steroids (CTS), and in particular endogenous ouabain, have been shown to contribute to pro-hypertrophic and pro-fibrotic cell signaling (Bagrov and Shapiro, 2008).

These findings suggest that EO may play a novel and direct role in vivo as a pro-hypertrophic hormone and thus may affect cardiovascular function and structure, by functioning in cardiac remodeling, which contributes to an increased risk of morbid events. Studies carried out in cultured cells have indeed provided the sequence of the ouabain-induced intracellular events leading to hypertrophic stimuli. The findings described in these reports support the notion that ouabain behaves like a growth-promoting hormone by acting as a signal transducer. By binding to the Na^+,K^+-ATPase, considered the only known functional receptor for digitalis, ouabain activates a complex intracellular signaling cascade, triggered by a tyrosine kinase-activated pathway, via epidermal growth factor receptor transactivation, that finally leads to the transcription of growth-related genes. The molecular mechanism leading to organ hypertrophy in vivo in OS rats has been hypothesized to occur through the activation of an intracellular signaling pathway similar to that described in cultured cells. Preliminary data are in line with the hypothesis that in vivo ouabain activates an intracellular signaling cascade within highly specialized membrane subdomain (Hamlyn and Blaustein, 2016).

EO and Renal Disease

The clearance of cardiac glycosides is one significant determinant of their circulating levels. Although digoxin and digitoxin are cleared primarily by the liver, clearance of EO is mediated to a large extent by the kidneys. Plasma EO rises with the progression of chronic kidney failure (CKF) (Hamlyn and Manunta, 2015). Moreover, with the advent of end-stage failure and near-zero urinary excretion, plasma EO rises substantially into the low nano-molar range. This scenario, analogous to unwanted and uncontrollable digitalization, is worrisome in terms of the potential for undesirable cardiac and vasopressor effects. Indeed, among patients with ESRD in dialysis, plasma EO was strongly associated with left ventricular mass and geometry (Stella *et al.*, 2008). This association was independent of arterial pressure and other well-established determinants of left ventricular mass. Thus, in ESRD, left ventricular hypertrophy and high cardiovascular risk, including that from hypertension, represent additional multifactorial problems potentially related to EO.

In addition to raising BP, the elevated EO in kidney failure may directly damage podocytes. In rats, the chronic elevation of circulating ouabain reduced creatinine clearance, increased urinary protein excretion, and reduced the expression of podocyte nephrin, a selective podocyte marker protein. This last finding was replicated ex vivo by incubating podocyte primary cell cultures with low-dose ouabain.

The observation that ouabain foments kidney damage in the rat suggested a possible role of EO in acute kidney injury (AKI). Indeed, circulating EO rises with an interesting time course during the induction of anesthesia in patients about to undergo elective cardiac surgery. Recently, a significant association has been reported of preoperative EO levels with adverse renal outcomes in cardiac surgery patients and with mortality in critically-ill patients.

In a observational study, the preoperative plasma levels of EO were measured in more than 800 patients admitted for elective cardiac. An increase in incidence of AKI (2.8% vs. 8.3% vs. 20.3%) and ICU stay with each incremental preoperative EO tertile was observed (Bignami *et al.*, 2013). Moreover, the preoperative EO value was added to a different clinical AKI predictive model and resulted in a significant improvement of risk prediction power (AUC of AKI from 0.79 to 0.84) (Simonini *et al.*, 2014). Finally, post-operative EO levels were also associated with a higher mortality rate after cardiac surgery (Simonini *et al.*, 2015).

In this way, EO was identify as a potentially valuable biomarker of individual susceptibility to the development of mild to severe AKI after cardiac surgery. Indeed, EO from one side it is responsible of the initial nephrinuria and of glomerular damage, and, on the other side, it modulates the basolateral Na–K-ATPase, that is known to be involved in tubular ischemic damage.

Endogenous Ouabain as a Target for a Novel Therapeutic Approach

As already mentioned, more than one-third of patients with EH, mostly all patients with CKF, and a large portion of patients with heart failure have increased circulating levels of EO. In this way the pathophysiological implication of EO in hypertension and its associated cardiac and renal complications has led to an interest in developing a novel class of antihypertensive agents for use as antagonistic drugs that can correct selectively the genetic–molecular mechanisms involved in EO and adducin in both rat and human hypertension. Following this original physio-pathological idea, the authors' research group developed a new digitoxigenin derivative compound: *rostafuroxin*. It was selected for its ability to lower blood pressure in the experimental and genetic strains of OS and MHS rats, at oral doses of micrograms/kg/day, and to normalize the biochemical alteration, namely, the up-regulation of renal Na^+,K^+-ATPase activity, in both rat models (Ferrari *et al.*, 1998).

Interestingly, rostafuroxin displays an antihypertensive activity in other rat models, such as the deoxycorticosterone acetate salt rat and the reduced-renal-mass hypertensive rat, both characterized by volume expansion, low renin levels, and increased EO levels. The selectivity of the antihypertensive effect of rostafuroxin is sustained by the absence of activity in normotensive control rats and in spontaneous hypertensive rats (SHR) rats, a model in which EO, adducin polymorphism, and renal Na^+,K^+-ATPase seem not to be involved in the etiology of the disease. Finally, preliminary data suggest that the compound, at oral doses of 7–10 μg/kg/day, is able to revert in vivo in OS rats the ouabain-induced hypertrophic activity (Ferrandi *et al.*, 2010).

However, in a trial of unselected patients with EH (i.e., in which 50%–70% of the patients would not have elevated EO), rostafuroxin had no overall impact on BP. Instead, the BP-lowering activity of rostafuroxin depended on genomic variation in factors related to the synthesis and clearance of EO and to cytoskeletal polymorphisms. In rostafuroxin-sensitive patients, the systolic BP declined 14 mmHg after 4 weeks of treatment relative to controls the gene variants encode enzymes in steroid biosynthesis, transmembrane EO (ouabain) transport, and the cell cytoskeleton. This combination of gene variants occurred in 23% of the 196 (never treated) hypertensives in the study (Lanzani *et al.*, 2010).

Data regarding the impact of rostafuroxin in treating heart failure and the hypertension in patients with end-stage kidney failure are not available. In the latter settings, circulating EO levels are considerably higher than in EH, and dramatically larger doses of rostafuroxin than those used in prior trials may be needed to demonstrate efficacy.

The pharmacological profile of rostafuroxin suggests that it may represent the prototype of a novel class of antihypertensive drugs that is devoid of undesired cardiac and hormonal effects, which are typical of digitalis and diuretics. The compound does not interfere either in vivo or in vitro with a panel of receptors involved in blood pressure regulation or hormonal homeostasis and it has no intrinsic cardiac inotropic or arrhythmogenic activity.

Conclusion

This evidence reinforces the concept that high EO levels and adducin polymorphism are associated with an increased risk of developing hypertension and organ complications. Therefore, particularly in the case of complex multifactorial diseases, such as essential hypertension, a tailored approach should be pursued in order to treat individual patients carrying specific pathogenetic mechanisms.

Rostafuroxin may be considered a safe drug of great relevance for the therapy of those hypertensive patients in whom increased EO levels and adducin polymorphism exert a causal role.

See also: Salt-Sensitivity of Blood Pressure

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Atrial Natriuretic Factor and the Family of Natriuretic Peptides☆

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Introduction

The atrial natriuretic factor family of natriuretic peptides (NPs) consists of three members: atrial natriuretic factor (ANF or ANP), brain natriuretic peptide or B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (de Bold and Flynn, 1983; Flynn *et al.*, 1983; Kojima *et al.*, 1990; Sudoh *et al.*, 1988). These polypeptide hormones play central roles in maintaining fluid and electrolyte balance and circulatory homeostasis. In mammals, the bulk of ANF and BNP is produced and secreted by the cardiac muscle cell (cardiomyocyte). CNP is produced mainly by the vascular endothelium and the brain (Morel and Heisler, 1988; Mukoyama *et al.*, 1991; Suga *et al.*, 1992).

Physiology, Structure, Biosynthesis, and Regulation of ANF

Physiological Actions of ANF

In 1981 it was demonstrated that injections of heart atrial extracts into rats gave rise to pronounced diuresis, natriuresis, and lowered blood pressure (de Bold *et al.*, 1981). Within a short time, a peptide with the same biological properties as the crude atrial extracts was isolated and its amino acid sequence was identified (Flynn *et al.*, 1983). This peptide, known as atrial natriuretic factor (ANF) or atrial natriuretic peptide (ANP), firmly established the heart as an endocrine organ and heralded a new era of research on the control and maintenance of blood pressure, blood volume, and vascular tone. ANF is a potent natriuretic and diuretic agent, owing these properties to both its renal hemodynamic actions and its direct tubular actions (McGrath *et al.*, 2005). By simultaneous dilation of afferent arterioles and constriction of efferent arterioles, ANF increases the glomerular filtration rate and filtration fraction. ANF will directly inhibit water reabsorption by the renal cortical collecting duct and contributes to inhibition of $Na+$ reabsorption by the renal inner medullary collecting duct (Melo *et al.*, 2000). ANF also has profound effects on the cardiovascular system. Acute administration of ANF causes a fall in arterial pressure due to a reduction in cardiac output mediated by a decreased preload and vascular resistance (Lang *et al.*, 1987; Loutzenhiser *et al.*, 1988). The decreased preload is believed to be a consequence of venodilation and reduction of the intravascular volume. Furthermore, ANF also reduces sympathetic tone by inhibiting arterial baroreceptor response and suppression of catecholamine release from autonomic nerve endings (Atchison and Ackermann, 1990; Butler *et al.*, 1994).

The renin–angiotensin–aldosterone system (RAAS) is also a target for ANF. ANF will directly reduce renin secretion, which has the cascade effect of lowering circulating levels of angiotensin II (Ang II), a potent vasoconstrictor and stimulator of aldosterone (Struthers *et al.*, 1986). ANF can also directly inhibit aldosterone synthesis and secretion from the glomerulosa cells of the adrenal cortex (Brenner *et al.*, 1990; Burger, 2005; Franco-Saenz *et al.*, 1989). The central actions of ANF include inhibition of secretion of vasopressin and of salt and water intake (Inoue *et al.*, 2001). ANF also possesses important antimitotic actions on vascular endothelial cells, smooth muscle cells, and cardiac fibroblasts (Calderone *et al.*, 1998; Kapoun *et al.*, 2004; Tamura *et al.*, 2000).

Structure and Biosynthesis of ANF

The mRNA is approximately 1 kb long and encodes a preproANF that contains between 149 and 153 amino acids depending on the species (Flynn *et al.*, 1985; Lewicki *et al.*, 1986; Nakao *et al.*, 1992) (**Fig. 1**). The human amino acid sequence shares strong homology with peptides from other species including rat, mouse, and pig (Potter *et al.*, 2009). In the human atria, ANF is stored mainly within organelles referred to as specific atrial granules as a prohormone called proANF1–126 (Thibault *et al.*, 1987). Subsequent processing releases into the bloodstream the biologically active hormone, ANF99–126. Normal human plasma concentrations of ANF are approximately 6 fmol mL^{-1}. The biological half-life of ANF99–126 is very short (less than 5 min) (Biollaz *et al.*, 1987; Yandle *et al.*, 1986).

All members of the ANF family of natriuretic peptides share a common central ring structure formed by a disulfide bridge (positions 105 and 121 for ANF). Disruption of this 17-member ring leads to a loss of biological activity (Hirata *et al.*, 1988) (**Fig. 2**).

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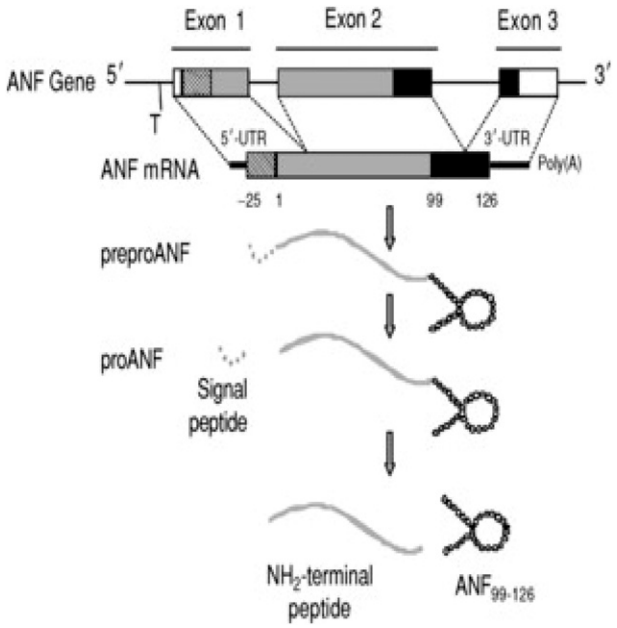

Fig. 1 Structure of the gene and biosynthetic pathway of human ANF. *Solid black* sections are those that code for the mature ANF99–126. *Gray* sections code for NH2-terminal fragments and the striped section codes for the signal peptide. Numbers indicate the amino acid position relative to the sequence encoded in proANF. T indicates the location of the TATAAA-box. *UTR*, untranslated region.

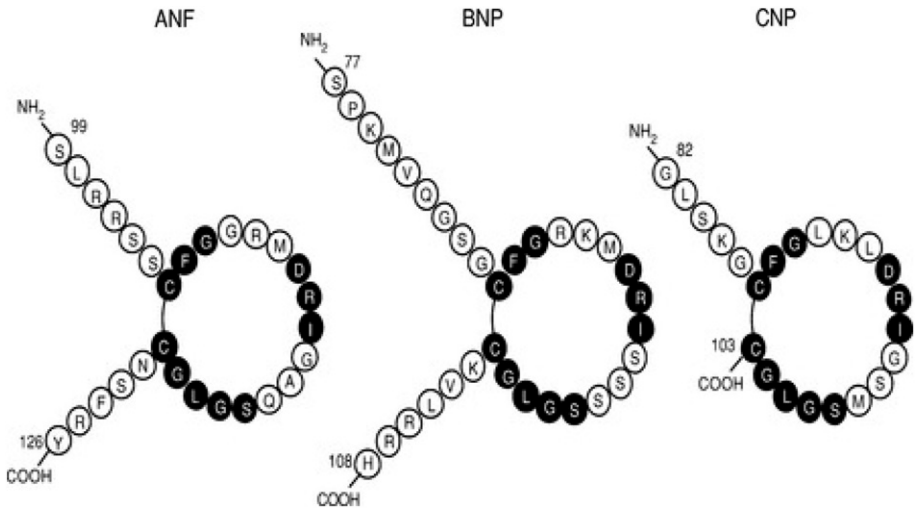

Fig. 2 The biologically active forms of the natriuretic peptides. Amino acids that are shared by all members of the natriuretic peptide family are identified by filled circles. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

ANF Gene Regulation

The ANF gene is mapped to chromosome 1 in humans, chromosome 4 in mouse, and chromosome 5 in rats (Argentin *et al.*, 1985; Nemer *et al.*, 1984). Structurally, the ANF gene is similar in all mammals and consists of three exons and two introns (**Fig. 1**).

Tissue-specific and developmental regulation of ANF gene expression

Atrial expression of ANF rises continuously during fetal and postnatal development to comparatively high levels (1%–3% of total atrial mRNA), whereas ventricular expression of ANF, although quite high during fetal development, falls quickly after birth (1% of atrial levels) (de Bold and Bruneau, 2000). Extracardiac (aortic arch, hypothalamus, pituitary, and kidney) sites of synthesis have also been documented, but the major site of ANF production remains the atria (Lim *et al.*, 1990; Morel and Heisler, 1988). In adulthood, ANF expression by the heart can be markedly increased by mechanical stretch of the cardiomyocyte and/or by neuroendocrine hormones (Ogawa *et al.*, 1999).

The 5′ flanking region of the ANF gene contain cis-acting elements to which various transcription factors are known to bind and thus regulate the level of genetic expression. There are two GATA-4 binding sites in the ANF gene promoter (Charron *et al.*, 1999), and patients with heterozygous GATA-4 mutations shows various congenital heart diseases (Clark *et al.*, 2006), suggesting that GATA-4 is essential for normal heart development. GATA-4 is also involved in the regulation of cardiac hypertrophy and ANF gene expression (Kessler-Icekson *et al.*, 2002; Liang *et al.*, 2001a). The AP-1 transcription factor and the products of two proto-oncogenes, c-fos, and c-jun, bind to AP-1/CRE-like binding sites in ANF gene (Rosenzweig and Seidman, 1991). C-fos and c-jun have been shown to regulate ANF gene expression associated with cardiac hypertrophy (Kovacic-Milivojevic *et al.*, 1996). Two CArG boxes are other binding sites in the ANF gene that bind serum response factor and play important roles in cardiovascular development and diseases (Miano, 2010). Nkx2.5 is an essential transcription factor for cardiac development as mutation of Nkx2.5 accounts for 4% of congenital heart diseases (Clark *et al.*, 2006). Two Nkx2.5 response elements, which are the binding site of Nkx2.5, can be seen in the ANF promoter (Small and Krieg, 2004). Tbx5, another important transcription factor, binds to the T-box binding elements found in the ANF promoter (Bruneau *et al.*, 2001; Hiroi *et al.*, 2001). Tbx5 and Nkx2.5 synergistically activate ANF gene transcription and mutation of Tbx5 results in various cardiac defect and conduction abnormalities (Basson *et al.*, 1997).

Acute mechanical stretch for the regulation of ANF gene expression

ANF is continuously released from the atria of the heart, but appropriate mechanical stretch can increase the rate of ANF secretion (de Bold *et al.*, 1996;Ogawa *et al.*, 1999). In acute experiments with isolated atrial preparations, increases in atrial wall stretch are met with corresponding increases in ANF secretion with no change in ANF gene expression (Bruneau *et al.*, 1997). Despite continued mechanical stimulation, the acute increase in ANF release eventually decays to baseline values within minutes. The precise mechanism underlying mechanotransduction of stretch-induced ANF release remains to be elucidated. However, there is clear evidence that it is mediated by Gi protein signaling (Bensimon *et al.*, 2004).

Neuroendocrine hormones or agents for the regulation of ANF gene expression

Numerous neuroendocrine agents can also increase ANF gene expression and release. α1-Adrenergic agonists (e.g., phenylephrine) and vasoactive peptides, such as endothelin-1 (ET-1) and Ang II, are potent stimulators of ANF gene expression and release (Bruneau *et al.*, 1996, 1997; Bruneau and de Bold, 1994). The vasoconstrictor ET-1 is synthesized and released by the endothelial and mesothelial cells. It is believed that ET-1 can act in a paracrine fashion in vivo to modulate ANF gene expression and release (Bianciotti and de Bold, 2000; Kassab *et al.*, 1998). Ang II, an effector molecule of RAAS, is a potent vasoconstrictor as well as a growth factor for cardiomyocytes (Bianciotti and de Bold, 2002; van Wamel *et al.*, 2001).

Ang II, ET-1, and catecholamines bind to the heterotrimeric G protein heptahelical transmembrane family of receptors. In general, activation of the receptor leads to the recruitment of secondary effector molecules that activate cytosolic and/or nuclear substrates. In particular, Ang II, ET-1, and phenylephrine will rapidly stimulate protein kinase C and the mitogen-activated protein kinase, p44/42 (ERK), which in turn will activate trans-acting factors responsible for the increase in ANF expression (Takahashi *et al.*, 2003; Ueyama *et al.*, 2000). These include the immediate-early genes (IEGs), such as the c-fos and c-jun heterodimer, that can bind to activator protein-1 (AP-1) consensus elements on the 5′-flanking region of the ANF promoter (Kovacic-Milivojevic and Gardner, 1993). Hormonal ligands for nuclear receptors, such as glucocorticoids, have also been shown to upregulate ANF gene expression and release (Kanda *et al.*, 1989). IL-1beta and TNF alpha are powerful stimulants of ANF gene expression and secretion (Ma *et al.*, 2004, 2005). Cardiotrophin I, a member of the interleukin-6 superfamily of cytokines, has been shown to upregulate ANF gene expression (Hamanaka *et al.*, 2000).

Chronic hemodynamic overload and changes in ANF expression

Under chronic hemodynamic overload, mature terminally differentiated cardiomyocytes will respond with cellular hypertrophy to normalize wall stress (Ogawa *et al.*, 1996). Importantly, ANF gene expression and release are markedly increased not only from the atrial cardiomyocytes, but also from the ventricular cardiomyocytes by the hypertrophic stimuli (Yokota *et al.*, 1995). This can translate into a 100-fold increase in ANF plasma concentrations in certain pathophysiological conditions such as chronic congestive heart failure (Cohn, 1990). Hypertrophic stimuli, such as prolonged myocardial stretch or humoral growth factors, will activate the IEGs, such as c-myc, c-fos, and/or c-jun (Bruneau and de Bold, 1994). This is followed by activation of the characteristic embryonic gene program seen during cardiac development. Molecularly, this gene program accounts for the re-expression of ANF, BNP, β-myosin heavy chain, and skeletal α-actin in the hypertrophic adult ventricle (Ogawa *et al.*, 1996). The precise mechanisms responsible for the chronically increased ventricular ANF gene expression and secretion have not been fully elucidated. In vivo experiments have demonstrated that there may be two components responsible for these changes in natriuretic peptide expression: one that is dependent on hemodynamic load (i.e., mechanical stretch) and one that is load independent (i.e., direct effects of humoral growth factors, such as ET-1 and Ang II, on cardiomyocytes) (Ogawa *et al.*, 1996).

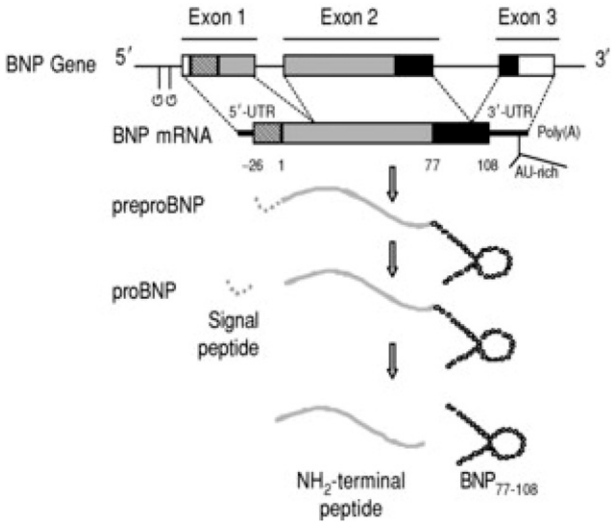

Fig. 3 Structure of the gene and biosynthetic pathway of human BNP. *Solid black* sections are those that code for the mature BNP77–108. *Gray* section codes for the NH2-terminal fragment and the striped section codes for the signal peptide. Numbers describe the amino acid position relative to the sequence encoded in proBNP. G's indicate the location of the GATAAA sequences (TATAAA-box). *UTR*, untranslated region.

Physiology, Structure, Biosynthesis, and Regulation of BNP

Physiological Actions of BNP

Brain natriuretic peptide was originally isolated from porcine brain (Sudoh *et al.*, 1988). However, it was later discovered that the highest concentrations of this peptide were found in the heart. BNP, like ANF, is a potent natriuretic, diuretic, vasorelaxant, antimitotic factor as well as an antagonist of the renin–angiotensin–aldosterone axis (Burger, 2005; Dhingra *et al.*, 2002; Mukoyama *et al.*, 1991; Vanderheyden *et al.*, 2004). Of interest, however, BNP may have functions not associated with ANF. BNP has been shown to have antifibrotic properties (Tamura *et al.*, 2000). This effect may be particularly important in cardiovascular diseases in which cardiac fibrosis contributes to the progression to heart failure (Segura *et al.*, 2014). In addition, BNP has immunomodulatory properties (de Bold *et al.*, 2010).

Structure and Biosynthesis of BNP

BNP is synthesized as a 121- to 134-amino-acid preprohormone (**Fig. 3**). In humans, subsequent processing releases a mature biologically active 32-amino-acid carboxy-terminal fragment (Kambayashi *et al.*, 1990). Unlike ANF or CNP, the amino acid sequence of BNP is not as highly conserved and may differ by as much as 50% between species (Potter *et al.*, 2009). The plasma half-life of BNP is approximately 20 min, which is approximately six times longer than that of ANF. The normal circulating BNP level in humans is approximately 0.9 fmol/mL (Mukoyama *et al.*, 1991).

Regulation of BNP Gene Expression

The BNP gene is mapped to chromosome 1 in human and to chromosome 4 in mouse (Yang-Feng *et al.*, 1985; Ogawa *et al.*, 1994). Structurally the BNP gene is similar to the ANF gene in that it is composed of three exons and two introns. However, there are important differences between the regulation of BNP and ANF at the transcriptional and posttranscriptional levels. Hemodynamic overload increases both atrial and ventricular expression of BNP (and ANF) dramatically (Benvenuti *et al.*, 1997; Yokota *et al.*, 1995). In pathological states such as chronic congestive heart failure, the plasma BNP level can increase 1000-fold; Mukoyama *et al.*, 1991.

The 5′ flanking region of the BNP genes have GATA-4 binding sites and AP-1/CRE-like binding sites, which are also seen in the ANF gene (Marttila *et al.*, 2001; Sawada *et al.*, 2010). As mentioned above for ANF, these sites are essential for normal heart development. GATA-4 is also involved in pathological conditions because the elevation of BNP genes in response to AII, ET-1, isoproterenol, and stretch are mediated by GATA-4 (Clark *et al.*, 2006; Grepin *et al.*, 1994; He *et al.*, 2002). The human BNP promoter contains two M-CAT elements and members of the transcriptional enhancer factor-1 (TEF-1) family binds to them (Yoshida, 2008). Mutation of this sequence significantly depresses basal and phenylephrine (PE)-induced gene expression of rat BNP (Thuerauf *et al.*, 1994). Three shear stress-responsiveness elements (SSRE) are found in BNP promoter. Cyclic mechanical stretch increased in NF-κB binding to the SSRE, and mutation of the SSRE or co-transfection with constitutive suppressor of NF-κB activity caused a maximum of 40% decrease in strain-activated BNP (Liang *et al.*, 2001b).

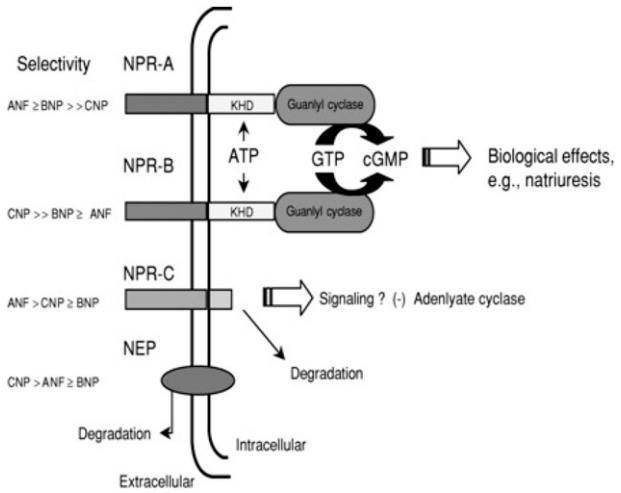

Fig. 4 The natriuretic peptide receptors. Binding of ANF and BNP to NPR-A and CNP to NPR-B stimulates intrinsic guanylyl cyclase activity of the receptor. The transduction of ligand binding to cGMP generation is ATP dependent and requires participation from the KHD portion of the receptor. *NPR-A*, natriuretic peptide receptor-A; *NPR-B*, natriuretic peptide receptor-B; *NPR-C*, natriuretic peptide receptor-C; *KHD*, kinase-like homology domain; *NEP*, neutral endopeptidase 24.11.

In response to certain hypertrophic stimuli, BNP mRNA is rapidly upregulated in a protein synthesis-independent manner with quick turnover (Bruneau *et al.*, 1997). These features are characteristic of an IEG. In support of this, the BNP 3′-untranslated region contains many AU-rich elements that may confer instability to the BNP transcript (mRNA t1/2 ~60 min) not found in ANF mRNA, which is quite stable (Hanford *et al.*, 1994). Interestingly, BNP mRNA stability can be enhanced after treatment with phenylephrine. In addition to neurohumors such as ET-1 and phenylephrine, BNP gene expression and secretion can also be upregulated by pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor α (TNFα) (Liang *et al.*, 2001b). In summary, the significant increase in BNP expression during overload may be due a combination of specific transcriptional and posttranscriptional mechanisms.

Physiology, Structure, Biosynthesis, and Regulation of CNP

Physiological Actions of CNP

CNP is found in the cerebellum, hypothalamus, anterior pituitary, kidney, and the vascular endothelial cells but not in the heart (Komatsu *et al.*, 1991; Nishimura *et al.*, 1998; Sudoh *et al.*, 1990; Ueda *et al.*, 1991). The central actions of CNP include antagonizing angiotensin II-mediated increases in vasopressin, which in turn decreases salt and water intake (Nishimura *et al.*, 1998). The natriuretic activity of CNP is only approximately 1% of that of ANF. CNP has potent anti-growth properties on vascular smooth muscle cells, suggesting that CNP may be an important regulator of blood vessels (Furuya *et al.*, 1991).

Structure and Biosynthesis of CNP

CNP is the most conserved of all natriuretic peptides, with 90% homology observed among human, mouse, pig, and rat (Potter *et al.*, 2009). Produced from a 126-amino-acid preprohormone, the bioactive hormone, unlike ANF or BNP, is only 22 amino acids long, because CNP has no carboxy-terminal tail (Barr *et al.*, 1996; Del Ry *et al.*, 2006) (**Fig. 2**).

Regulation of CNP Gene Expression

The gene for CNP is located on chromosome 2 in humans, in contrast to the ANF and BNP genes, which are located on chromosome 1 in humans (Ogawa *et al.*, 1994). The mouse CNP gene consists of least two exons and one intron. The 5′-flanking region of CNP contains numerous cis-acting regulatory elements including dinucleotide CA repeats, cyclic AMP-response element-like, nuclear factor κB, and shear stress recognition sites (Anand-Srivastava, 2005; Sellitti *et al.*, 2011). Cytokines, such as transforming growth factor-β and TNFα, can upregulate CNP (Mendonca *et al.*, 2010).

Natriuretic Peptide Receptors

The biological actions of the natriuretic peptides are mediated through association with specific high-affinity receptors on the surface of target cells and the generation of cyclic guanosine monophosphate (cGMP) (Lowe *et al.*, 1989; Maack *et al.*, 1993; Schulz *et al.*, 1989) (**Fig. 4**). There are three natriuretic peptide receptors (NPR-A, NPR-B, and NPR-C). NPR-A and NPR-B are linked to guanylate cyclase and, on activation of the receptor, cGMP is formed (Gerzer *et al.*, 1985). cGMP targets may include cGMP-dependent protein kinases and cGMP-gated ion channels (Lincoln and Cornwell, 1993). NPR-A binds both ANF and BNP, with preference for ANF. NPR-B binds CNP with far less preference for either ANF or BNP (Koller *et al.*, 1991). NPR-C is the clearance receptor and binds CNP with slightly greater affinity than ANF or BNP (Anand-Srivastava and Trachte, 1993; Murthy and Makhlouf, 1999). Circulating natriuretic peptides are also inactivated by neutral endopeptidases present within renal tubular and vascular cells (Hashimoto *et al.*, 1994).

Natriuretic Peptides as Biomarkers

Because plasma ANF and BNP are increased in various pathological conditions such as heart failure, myocardial infarction, hypertension, left ventricular hypertrophy, and pulmonary hypertension (Buckley *et al.*, 1993; Cody *et al.*, 1986; Cowie *et al.*, 1997; Foy *et al.*, 1995; Nagaya *et al.*, 1998a,b; Nishikimi *et al.*, 1996; Ramos and de Bold, 2006), plasma levels of ANF and BNP have proven useful for the diagnosis and prognosis of these diseases. In addition to ANF99-126 and BNP77-108, the biologically inactive N-terminal fragments, NT-proANP and NT-proBNP also circulate in plasma. Since the N-terminal prohormones half-lives are longer than those of the C-terminal prohormones (Pemberton *et al.*, 2000), in some cases such as mild heart failure or asymptomatic left ventricular dysfunction, the determination of plasma levels of NT-proBNP has better diagnostic ability than BNP (Steiner and Guglin, 2008).

Natriuretic Peptides as Therapeutic Agents

Because ANF and BNP possess diuretic, natriuretic and hypotensive activities, ANF and BNP have been used in the treatment of various cardiovascular conditions. Infusion of carperitide, a recombinant form of human ANF has proven to be effective in patients with acute heart failure and acute myocardial infarction (Kitakaze *et al.*, 2007; Nomura *et al.*, 2008; Sackner-Bernstein *et al.*, 2005). Efficacy of low-dose administration of carperitide for acute heart failure is in progress as a multicenter, randomized, open-label, controlled study (Nagai *et al.*, 2017).

The effectiveness and safety of nesiritide, a recombinant form of human BNP has been questioned. Analysis of five randomized studies demonstrated that administration of nesiritide to acutely decompensated heart failure patients significantly increased the risk of worsening renal function compared to the control group (Sackner-Bernstein *et al.*, 2005). In another study in which nesiritide was administered to acute heart failure patients, no significant changes regarding death rate, rehospitalization rate, and renal function were observed, and hence the authors did not recommend the routine use of nesiritide to acute heart failure patients (O'Connor *et al.*, 2011).

Seventy-two hours of intravenous infusion of recombinant human BNP to ST-elevation myocardial infarction not only corrected cardiac dysfunction and decreased serum level of NT-pro BNP in the course of infusion, but also reduced cardiac dilatation and improved left ventricular ejection fraction 3 month later in comparison with baseline and control group (Gong *et al.*, 2015). A BELIEVE II study, a randomized, double-blind, placebo-controlled trial of 72 h intravenous infusion of nesiritide in humans with first time ST-elevation acute myocardial infarction, is in progress for the assessment of prevention of adverse left ventricular remodeling and preservation of left ventricular function (Sangaralingham *et al.*, 2013).

Recently Sacubitril/Valsartan, a novel angiotensin receptor-neprilysin inhibitor, was approved for the treatment of heart failure with reduced ejection fraction. Although neprilysin inhibition alone did not exert a significant effect because neprilysin inhibition activates RAAS (Richards *et al.*, 1992), combination of neprilysin inhibition and angiotensin inhibition revealed greater blood pressure reduction in patients with hypertension (Kario *et al.*, 2014; Ruilope *et al.*, 2010), and reduced all-cause mortality in heart failure patients compared with valsartan or enalapril alone (McMurray *et al.*, 2014; Solomon *et al.*, 2012).

Natriuretic Peptides in Other Diseases

Natriuretic Peptides in Inflammation

Plasma BNP but not ANF levels increased significantly during rejection episode, and successful treatment of the rejection resulted in a decrease of BNP plasma levels to values observed prior to acute rejection (Masters *et al.*, 1999). Others also have reported an association between plasma BNP and cardia cellular rejection suggesting that plasma BNP is a plasma marker for detecting heart transplant rejection (Almenar *et al.*, 2002; Arnau-Vives *et al.*, 2004; Hammerer-Lercher *et al.*, 2005; Ogawa *et al.*, 2005). Plasma samples taken from rejecting and nonrejecting patients were analyzed by cytokine array (Meirovich *et al.*, 2008). Regulated on

Activation, Normal T Expressed and Secreted (RANTES), Neutrophil Activating Protein-2 (NAP-2), and Insulin Growth Factor Binding Protein-1 (IGFBP-1) had significant correlations with BNP plasma levels during acute allograft rejection as diagnosed by endomyocardial biopsy suggesting that these cytokines may be involved in the rejection of transplanted heart.

Plasma BNP and NT-proBNP are increased in rheumatoid arthritis patients without a history of cardiac disease (Armstrong *et al.*, 2010; Solus *et al.*, 2008). A prospective case-control study revealed that high BNP levels in rheumatoid arthritis are related to inflammation but not to left ventricular abnormalities (George *et al.*, 2014).

Plasma ANF or BNP levels are increased in sepsis and in septic shock patients (Chua and Kang-Hoe, 2004; Nikolaou *et al.*, 2007; Shor *et al.*, 2006; Witthaut *et al.*, 2003). Administration of LPS, the main bacterial component responsible for the immune response to Gram negative bacterial infection, to healthy men increased plasma NT-proBNP without changing heart rate and blood pressure (Vila *et al.*, 2008). In LPS-treated rats, both plasma ANF and BNP levels increased, and gene expression of MCP-1, MMP-8, TIMP-1 CINC-1, TNFα, ICAM-1, and MIP-3 were correlated with gene expression of BNP suggesting that these cytokines might be involved in the plasma BNP elevation in sepsis patients (Ogawa and de Bold, 2012). Because ANF and BNP appear related to many cytokines, plasma levels of these hormones could become good biomarkers of inflammatory diseases other than those mentioned above.

Natriuretic Peptides in Metabolic Disorders

There is increasing evidence of the involvement of NPs in the pathophysiology of metabolic diseases. Recently inverse relation between circulating NPs levels and bodyweight, insulin resistance and type 2 diabetes has been reported (Das *et al.*, 2005; Khan *et al.*, 2011; Olsen *et al.*, 2005; Wang *et al.*, 2004). NPR-C, identified in human adipose tissue, is increased in adipose tissue of obese hypertensive patients compared to nonobese and normotensive individuals (Dessi-Fulgheri *et al.*, 1997). It is also reported that NPR-C expression in adipose tissue is induced by insulin (Nakatsuji *et al.*, 2010). NPs exert metabolic effects in numerous organs. In human adipose tissue, NPs enhance lipolysis as well as the secretion of adiponectin which has anti-atherosclerotic and anti-insulin resistance effects (Birkenfeld *et al.*, 2012; Sengenes *et al.*, 2000). Treating a human-derived adipose cell line with ANF results in the uncoupling of cellular respiration as well as "browning" of white adipose tissue, suggesting that NPs induce thermogenic programs in adipose tissue (Bordicchia *et al.*, 2012; Collins, 2014). Administration of ANF to healthy individuals increased lipolysis, lipid oxidation, energy expenditure (Birkenfeld *et al.*, 2008). Parts of beneficial effects of exercise and low calorie diet on metabolic disorders such as type 2 diabetes or metabolic syndrome may be related to the increase activity of NPs because physical activity alone or low calorie diet are reported to increase plasma ANF and BNP levels or augment NPR-A expression in skeletal muscle cells (Engeli *et al.*, 2012).

Natriuretic Peptides in Cancer

Recently many cancer cells including melanoma, lung, ovarian, and prostate cancers are reported to express NPR-A gene (Kong *et al.*, 2008; Vesely *et al.*, 2005). In the in vitro experiment, ANF decreased the number up to 97% of pancreatic, breast, colon, kidney, prostate, ovarian, and lung carcinoma cells. Administration of ANF to athymic mice bearing human pancreatic adenocarcinomas results in elimination of 80% of the carcinomas (Vesely *et al.*, 2007). The anticancer mechanisms of ANF may be related to RAS—mitogen-activated protein kinase kinase (MEK 1/2)—extracellular signal-related kinase (ERK 1/2) cascade, vascular endothelial growth factor (VEGF), β-catenin, WNT, JNK, and STAT pathways (Vesely *et al.*, 2007). Mean serum levels of ANF in breast cancer patients were significantly elevated compared to control group and the metastatic breast cancer patients showed significant high ANF levels compared to nonmetastatic group (Houssen *et al.*, 2017). The results of these studies suggest that NPs have the potential to become the therapeutic agents or biomarkers of cancers.

Summary

The ANF family of NPs play important roles in regulating blood pressure, extracellular fluid homeostasis, and cardiovascular growth under normal physiological conditions and in diseased states. NPs defend against excess salt and water retention, promote vascular relaxation, inhibit RAAS, inhibit sympathetic outflow, and have potent antimitotic properties. In many pathophysiological cardiovascular conditions, ANF and particularly BNP plasma levels have become important diagnostic and prognostic tools. ANF, BNP, and the combination of angiotensin and neutral endopeptidase inhibition have shown remarkable results for the treatment of cardiovascular diseases such as heart failure and acute myocardial infarction. Recently, extra-cardiovascular roles of NPs systems have been focused on, including the effect of NPs on inflammation, metabolic disorders and cancers, and some of these studies have revealed promising results suggesting that NPs possess potential for becoming biomarkers and therapeutic agents in these fields.

See also: Aldosterone; Action and Function. Aldosteronism in Congestive Heart Failure

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Mineralocorticoids and Mineralocorticoid Excess Syndromes: Pathophysiology

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Introduction

In setting the scene it may be useful to define 'mineralocorticoid'. Historically, mineralocorticoid was a primarily effector definition—a steroid hormone secreted from the adrenal cortex in response to sodium deficiency/volume depletion, to restore the status quo by increasing transepithelial retention of sodium in the kidney. This definition was based on studies with deoxycorticosterone (DOC), the initial 'classic' mineralocorticoid, and was complemented decades later by the isolation of electrocortin (by chromatography) and demonstration of its natriferic action. Electrocortin was rapidly renamed aldosterone when its structure was established, in recognition of the unique aldehyde group at carbon 18 instead of the otherwise universal methyl group.

It is more than 60 years since the isolation and characterization of aldosterone, and the hormone remains the central pillar of fluid and electrolyte homeostasis. That said, over that time the context in which such homeostasis is achieved has broadened very widely—from mineralocorticoid receptors (MR) to additional sites of mineralocorticoid action, from selective co-expression of the 'protective' enzyme 11βhydroxysteroid dehydrogenase to a variety of clinical situations which might be described as mineralocorticoid excess syndromes. In this complex context aldosterone still stands as the physiologic mineralocorticoid in terrestrial vertebrates, and as such a necessarily brief overview is in order.

Aldosterone

In evolutionary terms aldosterone is a uniquely terrestrial hormone, first found in lungfish—which, as their name implies, were the transition species from an obligate aqueous environment to the land: they still can be found in South Africa, South America, Australia (and Japanese pet shops). Aldosterone is secreted in response to sodium deficiency, potassium loading, circulating volume depletion and—least well defined—stress: the secretagogues are angiotensin11, elevation of plasma $[K+]$ and—acutely, but not chronically—ACTH. The enzyme aldosterone synthase—also called CYP11B2—is responsible in most species for the conversion of the substrate 18-hydroxyDOC to aldosterone in a three step process. The genes encoding CYP11B2 and CYP11B1 (responsible for glucocorticoid synthesis) lie sequentially on chromosome 8q24. Some species (e.g., bovine) express a single CYP11B enzyme, closer to CYP11B2 than CYP11B1 in sequence, and responsible for both activities: determinants of functional zonation in such species are currently unclear.

In species with both CYP11B1 and CYP11B2 expression of the latter is confined to the outermost layer, the zona glomerulosa of the adrenal gland. In many experimental animals (e.g., rats, mice) the width of the zona glomerulosa varies with the sodium status of the animal—thin and sometimes tenuous in sodium loading, regular and thicker in sodium deficiency. In human adrenals a similar pattern has been presumed, recently challenged by the demonstration—in some but not all adults, and not in subjects under 20—that CYP11B2 expression is confined to isolated and scattered aldosterone producing cell clusters (APCC). Some of these APCC, from apparently normal glands removed from renal transplant donors, harbor mutations found in aldosterone producing adenomas, as will be discussed subsequently. The reasons why some adult adrenals show APCC and others not, and why not all APCC express mutations otherwise responsible for hyperaldosteronism, remain to be established.

Circulating aldosterone levels are normally very low, to some extent reflecting the common sodium-replete diet in most countries. These 'normal' levels are $\sim$3 orders of magnitude lower than those of cortisol; in addition most current assays overestimate levels at the lower end of the 'normal' range. The published ranges are very wide— 4–21 ng/dl in Rochester, 3-31 ng/dl in Ancona, a spread of values unlikely to reflect differences in daily salt intake, plasma $[K+]$ or variable sensitivity to aldosterone. African-Americans are on average more sensitive to aldosterone (lower baseline levels, more marked blood pressure responses to fludrocortisone, with no difference in sodium/potassium daily excretion) but are unlikely contributors to the 5–10 fold ranges noted above.

To some extent offsetting the $\sim$1000-fold difference in plasma concentrations of aldosterone and cortisol are the differences in plasma binding. About half the circulating aldosterone is bound with relatively low affinity to albumin; in contrast, only $\sim$5% of the circulating cortisol is free, the rest bound to albumin and much higher affinity transcortin (cortisol binding protein: CBG). One corollary of this is that the plasma half-time for aldosterone ($\sim$12min) is much lower than that of cortisol ($\sim$30 min). Another difference between the two is the shape of the nocturnal rise in steroid levels, much faster for aldosterone (and decaying faster) than for cortisol, the acuteness of the aldosterone response perhaps reflecting the volume shifts from systemic to pulmonary circulation upon recumbency. There are many more facets of aldosterone physiology that need to be taken into consideration, but this is perhaps better done in the context of its action on target tissues, primarily via mineralocorticoid receptors (MR).

Mineralocorticoid Receptors

Twenty years after the isolation and characterization of aldosterone the receptors whereby they act were demonstrated by incubating kidney slices from adrenalectomized rats with [3H] aldosterone alone or with excess non-radioactive aldosterone, and showing displaceable binding of tracer aldosterone to high affinity sites. Initially these were termed Type 1 corticosteroid receptors, to distinguish them from Type 2, [3H]-dexamethasone–binding sites in various glucocorticoid target tissues. Even though the Type 1/Type 2 nomenclature is non-descriptive, and was rapidly replaced by the familiar mineralocorticoid and glucocorticoid receptor (MR, GR) terminology, it is in fact more precise, in that MR have an affinity for physiologic glucocorticoids 10–30 fold higher than do GR. They were also shown to be what had been previously termed 'corticosterone-preferring' receptors in the hippocampus, distinct from pituitary [3H]-dexamethasone-binding pituitary GR. Imperfect as they are, we are stuck with the terms MR and GR.

Both MR and GR are primarily ligand-dependent nuclear transactivating proteins with effects mediated via DNA-directed, RNA-mediated protein synthesis. In humans there are family of 48 nuclear receptors (steroid/thyroid/retinoid/lipid-binding/orphan, the last without or with as yet undiscovered ligands); in mice there are 49. Within this superfamily MR/GR/progesterone receptors (PR)/ androgen receptors (AR) form a closely knit sub-family, sharing ~90% identity in their DNA-binding sites and ~50% in their steroid-binding sites. They all derive from a single ancestral gene, with MR the first to have branched off from this common ancestor, followed by GR, and then PR/AR. MR are found in both bony (e.g., trout, zebrafish) and cartilaginous (e.g., shark, rays) fish; their delineation as a receptor, presumably for cortisol, thus antedates that of the emergence of aldosterone millions of years later in lungfish. The presumption that the cognate ligand for MR in fish was cortisol is based not only on contemporary studies on fish, but also on the finding that MR in humans and experimental animals have the same high affinity for cortisol as for aldosterone; they are truly promiscuous in that this same high affinity extends to corticosterone, deoxycorticosterone and progesterone.

For several decades deoxycorticosterone was the recognized mineralocorticoid, as previously noted. Its physiologic role as a mineralocorticoid is minimal, for at least three reasons. First, its plasma levels are low, usually similar to those of aldosterone. Second, it is ~98% plasma bound, and experimentally only 2–3% as potent a mineralocorticoid as aldosterone. Finally, the major driver of deoxycorticosterone biosynthesis is ACTH, so that it is essentially out of the homeostatic control (low sodium, high potassium) loop triggering a mineralocorticoid response, in contrast with aldosterone.

Pathophysiologically, however, deoxycorticosterone may play a major mineralocorticoid role, in at least two circumstances. One is that of the ectopic secretion of ACTH, which is unconstrained by the normal feedback controls on pituitary ACTH secretion; the other is in patients with defects in 11β oxidation, where levels of deoxycorticosterone and 11-deoxycortisol rise in response to compromised feedback regulation of ACTH secretion. That progesterone—which is a mineralocorticoid receptor antagonist—does have effects via MR is seen in pregnancy, when plasma aldosterone levels rise to levels 3–10 times baseline to counter the MR antagonist action of the very high progesterone levels. The first (spironolactone, canrenone) and second (eplerenone) generation MR antagonists are variously elaborated progesterone derivatives, and drospirenone used as an oral contraceptive is a very potent MR antagonist.

Aldosterone Specifically Activates Epithelial Mineralocorticoid Receptors: The Role of 11βhydroxysteroid Dehydrogenase Type 2

An obvious corollary of the 1000-fold (total)/100-fold (free) plasma concentrations of cortisol (corticosterone in rats and mice) is the question of how aldosterone can specifically activate epithelial MR in the face of the equivalent affinity and far higher concentrations of the physiologic glucocorticoids. This enigma appears to have been solved, with one caveat, by the finding of co-expression with MR of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) in epithelia, and its ability to metabolize cortisol into cortisone (which does not bind to MR). Aldosterone is not a substrate for 11βHSD2, in that its hydroxyl group at carbon 11 cyclizes with the very reactive (and unique) aldehyde group at carbon 18, to form an 11,18 complex (and sometimes an 11, 18, 20 complex): together these forms constitute ~99% of circulating aldosterone.

These studies from almost 3 decades ago are still interpreted as evidence that 11βHSD2 action does not allow cortisol to *occupy* MR in the principal cells of the distal renal tubule, an interpretation which appears to be buttressed by the unrestrained mineralocorticoid action of cortisol when the enzyme is congenitally absent or impaired (in the condition known as apparent mineralocorticoid excess) or blocked by glycyrrhizic/glycyrrhetinic acid in licorice abuse. Inconveniently, however, studies on adrenalectomized rats showed that what 11βHSD2 did was to reduce intracellular corticosterone levels by an order of magnitude —levels, however, still ~10-fold higher than those of aldosterone. What this suggests is that most principal cell MR are normally *occupied but not activated* by normal levels of glucocorticoids. The ten-fold increase in sensitivity in the Kagawa bioassay when rats are adrenalectomized is in accordance with this scenario.

How then, can it be that when 11βHSD2 is operating to metabolize cortisol MR occupied by cortisol are inactive, but when the enzyme is deficient or blocked cortisol can mimic aldosterone and act as a mineralocorticoid? There really can be no question that the metabolism of cortisol by the enzyme is so complete that it can not occupy the receptors. The intracellular concentrations of cortisol are (presumably, like plasma free levels) ~100 times higher than those of aldosterone. To reduce them to 'noise' in the system, let us say they need to be reduced to ~10% of those of aldosterone: reduced only to equality, for instance, would constitute a very unfortunate inappropriate signal. What this requires is that 999 of every 1000 cortisol molecules would need to

be metabolized to cortisone, in an organ that receives 20–25% of the cardiac output and through which the entire blood volume passes every 5 min.

So how does 11βHSD2 block the mineralocorticoid activity of cortisol mineralocorticoid receptor complex? The answer appears to lie in NAD, the commonly disregarded obligate co-substrate for 11βHSD2. For every molecule of cortisol converted to cortisone, one molecule of NAD is converted to NADH. Levels of NAD vary between cell compartments, but under resting conditions are $\sim$600-fold those of NADH. This disparity means that major changes in NADH concentrations—say 100-fold — are possible with minimal change in substrate levels: in other nuclear receptor systems, increased NADH levels have been shown to act as a brake on their transactivational activity.

Cortisol as an MR Agonist

That this effect reflects changes in redox state has been shown in studies on cardiomyocytes (which do not express 11βHSD2). In patch clamp studies on rabbit cardiomyocytes, cortisol normally acts as an aldosterone antagonist. When, however, the cardiomyocytes are perfused with oxidized glutathione (GSSG) to mimic redox change, cortisol becomes a mineralocorticoid receptor agonist, mimicking aldosterone. Studies using Lagendorf preparations of rat hearts confirmed and extended this effect of cortisol. Under ischemia-reperfusion conditions, aldosterone increased the infarct size/area at risk, an effect blocked by spironolactone. Under such conditions (tissue damage, reactive oxygen species generation, redox change) cortisol proved equivalent to aldosterone in aggravating infarct size/area at risk: its effect was blocked by spironolactone, but not by the GR, PR antagonist RU486.

Cortisol is thus a bivalent steroid: an antagonist of MR under 'normal' conditions, but an agonist absent 11βHSD2 in the kidney, or in tissue damage in other organs. In one cell type, however, it appears an unreserved mineralocorticoid receptor agonist. The intercalated cells in the renal tubule do not express 11βHSD2, but do express mineralocorticoid receptors. Normally these receptors cannot bind steroids, in that they are phosphorylated at serine 843 in the ligand binding site. In conditions of sodium deficiency, however, these S^{843} mineralocorticoid receptors are dephosphorylated by angiotensin, and thus able to bind ligand, with cortisol proving to be as active as a mineralocorticoid as aldosterone in promoting Na^+ and Cl^- transport. This, incidentally, answers the conundrum of the different effects of aldosterone synthase and mineralocorticoid receptor gene deletion in mice. MRKO is essentially lethal: CYP11B2 $-/-$ mice have low BP, but survive under low salt conditions, presumably due to corticosterone acting as a mineralocorticoid receptor agonist in renal intercalated cells.

Finally, it should be noted that non-epithelial unprotected MR are cortisol-occupied, and have a life of their own, independent of aldosterone, in tissues such as the hippocampus and cardiomyocyte.

Rapid Effects of Aldosterone

While the classic effects of aldosterone on its target tissue involve DNA-directed RNA-mediated protein synthesis, there is clear evidence for rapid aldosterone effects. Most but probably not all of the acute non-genomic effects of aldosterone are mediated via classical MR: the most salient of these is the rapid secretion and action of aldosterone in response to orthostasis. Experimentally, aldosterone can be shown in vitro to activate the membrane receptor GPER, so called despite its affinity for estradiol being many orders of magnitude lower than circulating estradiol concentrations. Conversely, in some studies it appears to have a very, very high affinity ($K_d \sim 10^{12}$) for aldosterone, with that for other corticosteroids to be determined: its (patho) physiologic significance remains to be established.

Mineralocorticoid Excess Syndromes

The first two cases of successful unilateral adrenalectomy for hypertension and hypokalemia were published in the Polish literature in 1953. Jerome Conn reported his findings in the following year: he had the twin advantages of aldosterone having been isolated and characterized and publishing in English. Primary aldosteronism (hyperaldosteronism, Conn Syndrome: PA) is so-called in that the overproduction of hormone may be driven not by the classic secretagogues but by circumstances intrinsic to the adrenal cortex. This appears to be certainly the case for the minority of cases, those caused by a unilateral aldosterone producing adenoma (APA): whether it is also the case for bilateral adrenal hyperplasia (BAH) is moot, given the possibility that the process may be driven by external input (e.g., leptin, AT_1 R antibodies). In both cases the hypersecretion of aldosterone is outside the recognized feedback control—renin and angiotensin are suppressed, and plasma [K+] low normal or in the hypokalemic range. Secondary aldosteronism is a physiologic response to a low intravascular volume, as occurs in ascites.

Although Jerome Conn predicted that PA may be the cause of elevated blood pressure in $\sim$20% of hypertensives, for many years it was thought (and taught) that PA was rare ($<1\%$) and responsible for a mild form of hypertension, both of which we now know not to be true. At that time diagnosis required obligate hypokalemia: we now know this to be the case in only half of the patients with APA, the more florid form of PA, and fewer than 20% of patients with BAH. Today screening of at-risk hypertensives (30–100% in various guidelines) is by determination of the plasma aldosterone to renin ratio (ARR), followed by one or more of half a dozen confirmatory tests. Currently the prevalence of PA is reckoned as 5–13% of hypertensives, the range largely

representing variation between laboratories in renin and aldosterone assays, and the 'cut-offs' used to distinguish PA from so-called low renin hypertension. The unfortunate prolixity in confirmatory/exclusion testing notwithstanding, once the diagnosis of PA is made the pathway is straightforward albeit taxing.

Patients undergo a CT scan, ostensibly to exclude the very rare aldosterone-secreting carcinoma, actually to find (or not) an adenoma, thus to help in defining the source of the hyperaldosteronism. In patients able and willing to possibly undergo surgery lateralization is by bilateral adrenal venous sampling, to determine whether one or both glands are responsible. Patients who do not undergo adrenal venous sampling, and those who do not lateralize, are treated with a mineralocorticoid receptor antagonist, preferably at low dose, plus amiloride/triamterene and conventional antihypertensives as required: surprisingly, a lowered salt intake appears uncommon as a recommendation. If the patient lateralizes on adrenal venous sampling the recommended pathway is unilateral laparoscopic adrenalectomy, which essentially always cures the hyperaldosteronism, and restores the elevated blood pressure to normal in over half the patients, reducing it in the remainder. In the latter it would be sensible to include a mineralocorticoid receptor antagonist with the conventional antihypertensives, given that normal levels of cortisol mimic the vasoconstrictor effect of aldosterone under conditions of tissue damage: such is the monist view of spironolactone as purely 'an aldosterone antagonist' that this is never considered.

The past decade has seen a remarkable surge in both interest and advances in primary aldosteronism. A landmark study from Paris reported a much higher cardiovascular risk profile (4.2 fold higher risk of stroke, 6.5 fold higher risk of non-fatal myocardial infarct, 12.1 fold higher risk of atrial fibrillation) in patients with PA than in age-, sex-, and BP-matched essential hypertensives. Guidelines for the management of PA were published by the Endocrine Society in 2008, the Japanese Endocrine Society in 2011, and a revised version by the Endocrine Society in 2016. What is remarkable in the Guidelines is the thinness of what would be conventionally recognized as a true evidence base, rather than an (inevitable) reliance on consensus re best practice. This is at its most stark, and least impressive, in the plethora of confirmatory/exclusion tests currently in use. Continuing use of one or another test reflects history, familiarity and fears that a 'broken series' may affect acceptance for publication. Currently an inexpensive, rapid, physiologically appropriate seated saline suppression test is being trialed against the laborious 4-day fludrocortisone suppression test, often considered the 'gold standard'. Even if the very encouraging results are confirmed, it will take a revolution in the field to have it uniformly adopted.

Somatic and Germline Mutations

One revolution in the field has been the finding that more than half—and still counting—of APA carry a somatic mutation responsible for over secretion of aldosterone and (less well established) adenoma formation. The prismatic study reported that 8/22 APA were positive for one of two point mutations in the gene encoding KCNJ5, a subunit of the Kir3.4 potassium channel. The mutation-bearing adrenals were larger than wild type, more florid in terms of plasma aldosterone to renin ratio, more common in women and occurred at a younger age. A subsequent study on over 300 patient samples from the ENS@T archive confirmed and extended the initial observations, with two exceptions: the relative frequency of the two mutations was reversed, and no difference in size of APA was found between the two groups. Subsequently there have been described a series of different point mutations in KCNJ5, and a variety of less common somatic mutations in ATPase (ATP1A1, ATP2B3), calcium channels (CACNA1D, CACNA1H) and the intracellular protein catenin beta 1(CTNNB1). At the time of writing mutation-bearing APA constitute 50–60% of all APA in most series, with higher levels in Japan, China and Korea; whether this represents a real ethnic difference or particularities of patient selection remains to be established.

In addition to somatic mutations, germline mutations are responsible for a relatively small percentage of PA. Familiar hyperaldosteronism Type 1 (FH-1) was first described almost 50 years ago, is rare, and reflects a chimeric gene comprising the 3′ end of CYP11B1 with the 5′ end of CYP11B2. The chimeric gene is expressed ectopically throughout the zona fasciculata and is continually ACTH responsive: FH-I is diagnosed by long PCR, and treated by the minimum doses of dexamethasone required to moderate ACTH release. FH-II is PA in two or more first degree (parent–child, sibling-sibling) family members, appears to be heritable with a genetic locus around chromosome 7P22 in several families, with no specific gene shown responsible; this constitutes the minority of cases of FH-II, and whether the remainder are truly heritable remains moot. FH-III is caused by germline mutations in KCNJ5, and a possible FH-IV by germline mutations in CACNA1D. All of these are rare, and if florid need to be treated by bilateral adrenalectomy and replacement therapy.

Public Health Issues

What is slowly being recognized is that primary aldosteronism is a major public health issue, given its higher, blood-pressure independent cardiovascular risk profile. There are two main reasons for this. First is the possible upward revision of its prevalence, from 5 to 13% of hypertension to ~50%. Second is the fact that in no country are more than 1% of hypertensives ever screened for PA, let alone diagnosed and appropriately treated. Reflecting capacity constraints and the relatively high costs of the diagnostic and therapeutic workup, patient referrals from primary care physicians are uncommon, and so the current estimates of 5–13% understandably constitute the most florid cases, the 'low hanging fruit'. In addition the current work up pathway ignores the acute stimulatory effect of ACTH on aldosterone secretion, increasingly obviously to its detriment. A prediction can be confidently made,

once a series of largely ignored historical studies are acknowledged, and recent studies from Athens repeated and validated, that inappropriate aldosterone levels for sodium status will be gradually recognized as responsible at least in part for BP elevation in 50% of 'essential' hypertensives.

Whether the prevalence of PA is 10% or 50%, the fact remains that fewer than 1% of hypertensives are ever screened for PA. The 2008 Endocrine Society Guidelines recommended ~30% of hypertensives, the Japanese Guidelines 100%, and the 2016 Endocrine Society Guidelines ~50%. All these are counsels of perfection, impossible to put into effect in the foreseeable future. In contrast, MR antagonists have been shown to be safe and efficacious in essential hypertension, and specific in lowering BP and cardiac hypertrophy when added to standard therapy in low renin hypertension. They are similarly specifically efficacious when added to current therapy (3 conventional agents, including a diuretic) in so-called resistant hypertension—and game-changing in primary aldosteronism.

In studies on diabetic hypertensives spironolactone 6.25 mg (one quarter of the current smallest tablet) added to existing therapy significantly lowered BP and halved urinary albumin to creatine ratio over 3 months. *Primum non nocere*: but one day perhaps we will see a 6.25 mg spironolactone (or canrenone) tablet recommended for inclusion in first line therapy for hypertension. This would be a breathtakingly effective public health measure—but don't hold your breath waiting for it to happen.

Acknowledgment

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See also: Aldosterone; Action and Function. Aldosterone Receptors. Apparent Mineralocorticoid Excess. Liddle Syndrome. Mineralocorticoid Excess Syndromes. Primary Aldosteronism; Epidemiology and Screening

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Mineralocorticoids and Mineralocorticoid Excess Syndromes: Clinical Aspects☆

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Introduction

Classic mineralocorticoid-induced hypertension develops due to increased distal renal tubular sodium reabsorption, leading to raised body sodium content and plasma volume expansion. In addition, increased urinary potassium and hydrogen ion secretion leads to a reduction in body potassium content, metabolic alkalosis. Finally, increased plasma volume and sodium causes suppression of renin production by the juxtaglomerular cells of the kidney (and correspondingly low levels of angiotensin II).

Autonomous and excessive production of aldosterone, the most potent and prevalent of the human mineralocorticoid hormones, is now accepted to be the commonest cause of secondary hypertension. Although the incidence of primary aldosteronism (PA) varies according to the population studied and diagnostic methods employed, the most robust recent clinical studies consistently suggest that PA accounts for 5%–15% of cases of hypertension within European populations (Rossi *et al.*, 2006). Moreover, it is increasingly recognized that aldosterone excess is associated with disproportionate cardiovascular morbidity further highlighting the importance of accurate diagnosis.

In addition to aldosterone excess, there are a number of other (less common) causes of mineralocorticoid hypertension that will also be considered within this article. In order to enhance the understanding of aldosterone excess as well as these rarer mineralocorticoid excess syndromes, however, it is first necessary to revise mineralocorticoid synthesis, regulation and action as outlined below.

Mineralocorticoid Biosynthesis and Regulation

Aldosterone is produced from cholesterol by a series of hydroxylation and dehydrogenation reactions within the outermost zona glomerulosa of the adrenal cortex (Fig. 1). Aldosterone synthase (encoded by the gene) carries out two unique biosynthetic

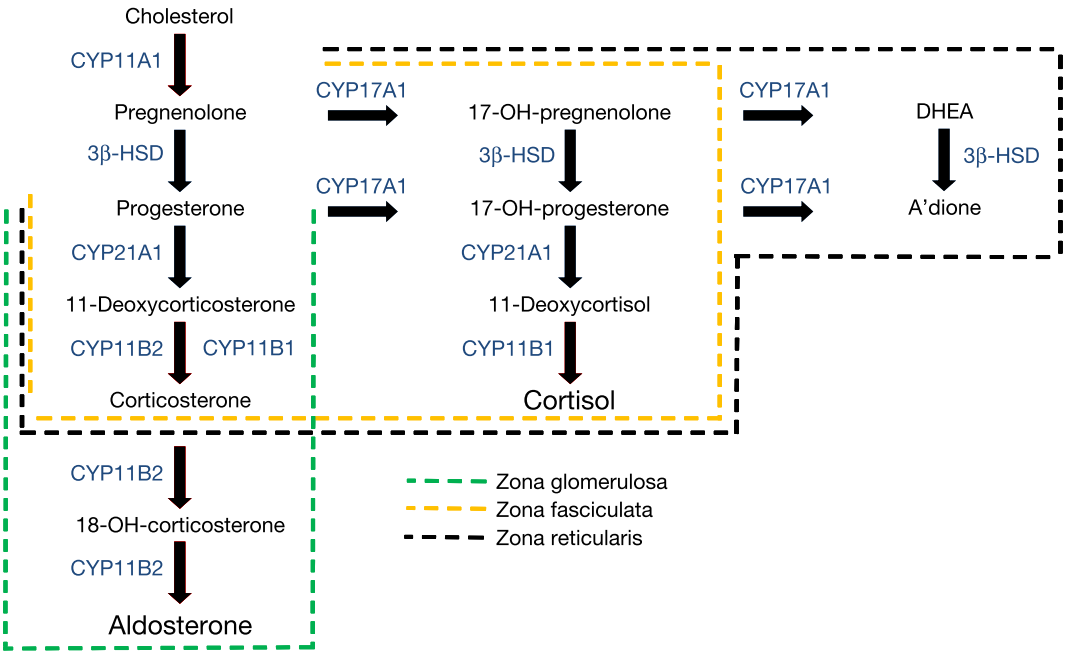

Fig. 1 Corticosteroid biosynthesis within the adrenal cortex. *DHEA*, dehydroandrosterone; *A'dione*, androstenedione.

☆*Change History*: March 2018. E Marie Freel has altered and updated sections (1) Extra section of prevalence of PA, (2) New section on genetic mutations in APA, (3) New section of familial aldosteronism, (4) Section on diagnosis rewritten to include the update American Endocrine Society Clinical Practice Guideline, (5) New section detailing the excess cardiovascular morbidity with aldosterone excess, (6) New section on Gordon Syndrome. Table 1 is new and Table 2 is updated, Figs. 1 and 2 are updated, Figs. 3–6 are new.

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reactions to generate aldosterone (Rainey, 1999). In brief, corticosterone undergoes hydroxylation to form 18-hydroxycorticosterone which, following a second hydroxylation reaction, is spontaneously dehydrated to form aldosterone. Aldosterone synthase also converts the mineralocorticoid precursor, 11-deoxycorticosterone to corticosterone in zona glomerulosa cells.

In the adjacent zona fasciculata of the adrenal cortex, the 11β-hydroxylase enzyme converts 11-deoxycortisol to cortisol and 11-deoxycorticosterone to corticosterone. 11β-Hydroxylase is encoded by the *CYP11B1* gene which is highly homologous to *CYP11B2* (Mornet *et al.*, 1989).

The renin–angiotensin system is the principal regulator of aldosterone production (**Fig. 2**; Connell and Davies, 2005). Loss of sodium (and reduced plasma volume) leads to reduced renal perfusion and activation of the renin–angiotensin system whereas reduced plasma volume suppresses it. Potassium is also a powerful direct stimulus to aldosterone secretion; very small increments (insufficient to cause a significant rise in plasma concentration) raise aldosterone secretion rate. In normal subjects, ACTH can acutely provoke aldosterone release, but chronic pharmacological doses of ACTH given over several days results in suppression of aldosterone synthesis by poorly understood mechanisms.

Mineralocorticoid Action

The mineralocorticoid receptor belongs to the nuclear receptor superfamily of proteins and consists of an N-terminal domain, a DNA-binding domain, and a C-terminal ligand-binding domain (Arriza *et al.*, 1987). Aldosterone binds to this latter domain and causes a conformational change to the mineralocorticoid receptor, whereupon it translocates to the cell nucleus. The ligand–receptor complex interacts directly with hormone response elements of aldosterone-responsive genes in order to activate or repress relevant gene transcription (the so-called "classical genomic effect" of aldosterone) (**Fig. 3**).

The most important physiological action of aldosterone is to increase the activity of the epithelial sodium channel (ENaC) in the apical membrane of the distal convoluted tubule of the nephron (Rossier *et al.*, 1994). This leads to reabsorption of sodium in the kidney and other epithelial sites at the expense of potassium and hydrogen ions (Horisberger and Diezi, 1983). Hydrogen ion excretion by the kidney in the distal nephron is also regulated by aldosterone via an effect on the activity of the ATP-dependent apical hydrogen ion pump and parallel regulation of the basolateral membrane Cl^-/HCO_3^- exchanger (Hays, 1992).

The net effect of aldosterone on the renal tubule is therefore to promote sodium retention at the expense of potassium and also to promote hydrogen ion excretion by the kidney. This explains the clinical features observed in classic cases of autonomous aldosterone excess, that is, plasma hypokalemia, alkalosis, an increased exchangeable sodium content, and low total body potassium.

The 11-β Hydroxysteroid Dehydrogenase System

Aldosterone and cortisol have equal affinities for the mineralocorticoid receptor. Given that cortisol is found at much higher levels in plasma (up to 1000-fold in comparison to aldosterone levels) it would be expected that the vast majority of these receptors

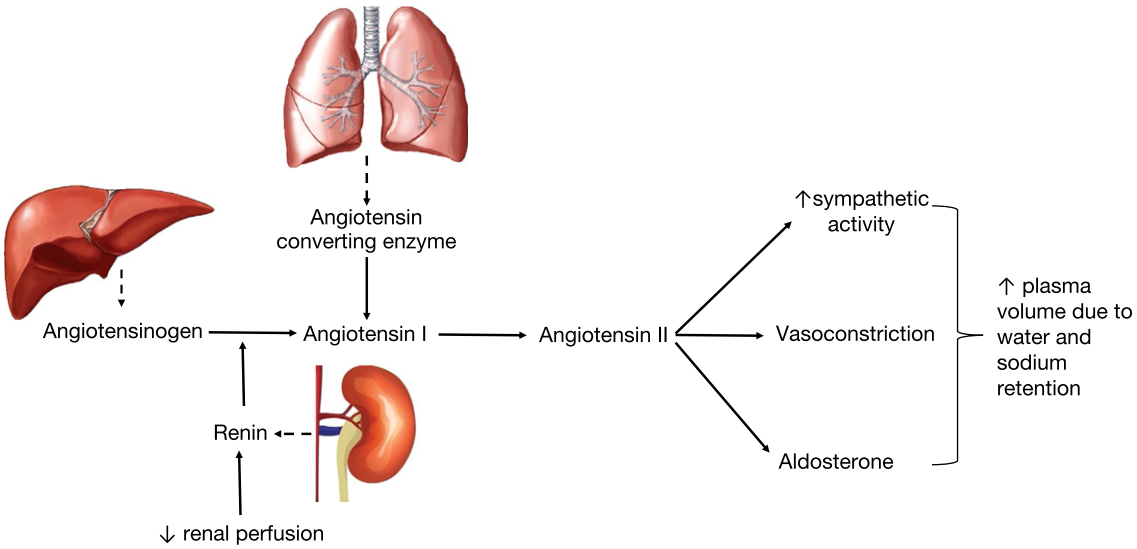

Fig. 2 The renin angiotensin system and the influence of angiotensin II on blood pressure. Renin is produced from the cleavage of pro-renin by the juxtaglomerular apparatus of the kidney in response to reduced circulating plasma volume or sodium. Circulating plasma renin then cuts a short, 10 amino acid long, peptide angiotensinogen (synthesized by the liver) to create angiotensin I. Angiotensin I is then converted, by the removal of two amino acids, to form an octapeptide known as angiotensin II, by the angiotensin-converting enzyme (ACE) found in the capillary endothelial cells especially in the lungs and the epithelial cells of the kidneys. Angiotensin II is a potent vaso-active peptide that causes arteriolar vasoconstriction as well as stimulating aldosterone biosynthesis by the adrenal cortex.

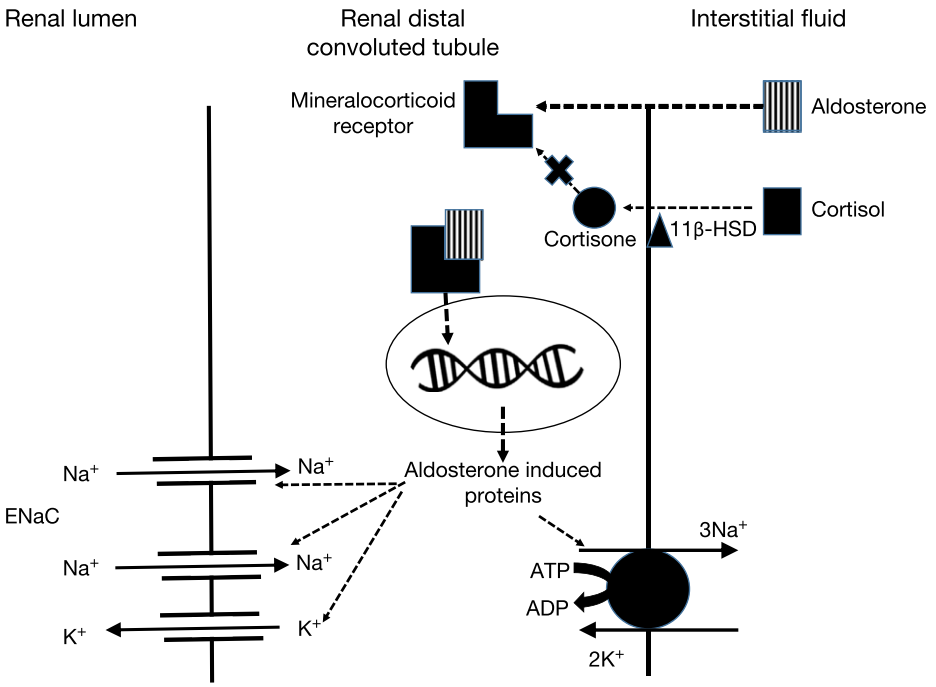

Fig. 3 Mechanism of action of aldosterone in epithelial cells. Aldosterone binds to mineralocorticoid receptor (MR) and leads to alteration in gene transcription by direct binding of the receptor/ligand complex to hormone responsive elements in relevant genes. The MR is protected from activation by cortisol by the 11β-hydroxysteroid dehydrogenase enzyme which converts cortisol to cortisone which has no affinity for the MR. The major effect of aldosterone is to increase availability of ENaC on the apical membrane of the distal convoluted tubule as well as increase activity of the NA-K ATPase pump. Thus, the net effect is increased renal reabsorption of sodium with increased potassium excretion. ENaC, epithelial sodium channel; 11β-HSD2,11β-hydroxysteroid dehydrogenase.

would be expected to be occupied and activated by glucocorticoid. This does not occur as a consequence of the activity of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), which acts as a gatekeeper to prevent the mineralocorticoid receptor being overwhelmed by much higher available levels of cortisol (**Fig. 3**) (Funder *et al.*, 1988). The type 2 isoform of this enzyme is found in the renal distal nephron as well as other aldosterone-sensitive target tissues (colon, salivary glands, and placenta) and converts cortisol to its inactive metabolite, cortisone, which has no affinity for the mineralocorticoid receptor (Edwards *et al.*, 1988). Whenever protection of the mineralocorticoid receptor is compromised by impairment of 11β-HSD2 activity cortisol causes widespread activation of the mineralocorticoid receptor leading to mineralocorticoid hypertension. The clinical consequences of this are described later in this article.

Mineralocorticoid Hypertension

As a result of an understanding of mineralocorticoid biosynthesis and action, mineralocorticoid excess syndromes can be thought of under the following headings:

1. Activation of the mineralocorticoid receptor by excess levels of its normal agonist, aldosterone (primary aldosteronism)
2. Activation of the mineralocorticoid receptor by an alternative ligand (either cortisol in the Syndrome of Inappropriate Mineralocorticoid Excess or mineralocorticoid precursors in rare forms of Congenital Adrenal Hyperplasia)
3. Abnormality within the mineralocorticoid receptor altering sensitivity to other agonists (progesterone induced hypertension)
4. Abnormality within renal ion channels resulting in inappropriate activation (Liddle syndrome and Gordon Syndrome)

Activation of the Mineralocorticoid Receptor by Excess Levels of Its Normal Agonist, Aldosterone-Primary Aldosteronism (PA)

As outlined previously, PA is the commonest cause of secondary hypertension. The current accepted prevalence rates are strongly supported by a seminal Italian study in a large series of unselected patients with hypertension. Using careful confirmatory tests for the disorder, this study reported a prevalence of 11% for primary aldosteronism in such a cohort, with 4.8% of subjects harboring an aldosterone-producing adenoma (Rossi *et al.*, 2006). Another comprehensive study in Greece of patients with resistant hypertension reported an incidence of 11.3% (Douma *et al.*, 2008). Thus, the recently reported frequency rates are consistent with

a previous multicenter retrospective review where more widespread use of the plasma aldosterone to renin ratio (ARR) as a screening test in hypertensive patients resulted in a marked increase (up to sixfold) in the annual detection rate of PA as well as in the proportion of hypertensive patients in whom PA was detected (1%–2% before and 5%–10% after screening) (Mulatero *et al.*, 2004). This apparent increased PA prevalence can mainly be accounted for by increasing recognition and earlier diagnosis of more subtle cases of aldosterone excess through use of the aldosterone to renin ratio as a screening tool (see below).

PA presents with hypertension, which is often severe and resistant to "conventional" antihypertensive therapy. Although hypokalemia had historically been considered to be a prominent clinical feature less than half of all patients have demonstrably low plasma potassium levels (Funder *et al.*, 2016; Mulatero *et al.*, 2004).

Causes of Primary Aldosteronism

Sporadic primary aldosteronism

The relative prevalence of causes of primary aldosteronism varies by geographical location, but in all cases the vast majority are sporadic. In Caucasian populations, the most common cause of sporadic PA is bilateral adrenal hyperplasia, accounting for approximately 60% while an aldosterone-producing adenoma (APA) consists of 35% of patients with PA; unilateral micronodular hyperplasia (caused by micronodular or macronodular hyperplasia of the zona glomerulosa of predominantly one adrenal gland) is an uncommon cause. In contrast, in a Japanese cohort, APA accounts for the majority of primary aldosteronism cases (around 80%) (Omura *et al.*, 2004). Aldosterone-producing adrenal carcinoma is an extremely rare cause of aldosterone excess associated with a poor prognosis.

The precise etiology of these conditions remains largely unknown, and whether APAs arise from nodular adrenal glands, or whether the two conditions are independent is still debated. It has previously been shown that tissue around the resected adenoma differs from normal adrenal tissue and has undergone remodeling with reduced vascularization and zona glomerulosa hyperplasia (Boulkroun *et al.*, 2010). However, a comparison of the transcriptome of tissue adjacent to APA, APA tissue itself and normal adrenal tissue suggests that it this is not an intermediate step in the formation of APAs.

Genetic mutations in APA

Major advances have been made during the past 6 years in understanding the pathophysiology of aldosterone production in patients with PA with the discovery that somatic mutations are found in up to 50% of patients with APA; the most prevalent of these are summarized in **Table 1**. The breakthrough was the identification of somatic mutations in the potassium channel GIRK4 (encoded by *KCNJ5*) in APA and the simultaneous discovery, by the same authors, of a germline mutation within this gene responsible for familial hyperaldosteronism type III (Choi *et al.*, 2011). This was followed by the identification of further somatic mutations in APAs in two ATPases (Na^+/K^+-ATPase 1 and Ca^{2+}-ATPase3,encoded by *ATP1A1* and *ATP2B3*, respectively) and in a subunit of an L-type voltage-gated Ca^{2+}-channel, Cav1.3 (encoded by *CACNA1D*) (Beuschlein *et al.*, 2013; Scholl *et al.*, 2013; Azizan *et al.*, 2013).

Somatic mutations in *KCNJ5* appear to be present in approximately 40% of patients with APAs (Boulkroun *et al.*, 2012; Azizan *et al.*, 2012; Åkerström *et al.*, 2012; Monticone *et al.*, 2012) (**Table 1**). However, the prevalence of such mutations seems to vary according to geographical location with one Chinese study reporting the presence of KCNJ5 mutations in 77% of all tested APA (Zheng *et al.*, 2015). All mutations identified so far affect the selectivity filter of the potassium channel GIRK4 (*KCNJ5*) producing increased sodium conductance and cell depolarization, leading to calcium entry into glomerulosa cells resulting in aldosterone production and cell proliferation.

In one of the first studies to estimate mutation frequency, *KCNJ5* sequencing was performed on somatic (APA, $n = 380$) and peripheral (APA, $n = 344$; bilateral adrenal hyperplasia, $n = 174$) DNA of patients with primary aldosteronism (Boulkroun *et al.*, 2012). Somatic *KCNJ5* mutations (G151R or L168R) were found in 34% (129 of 380) of APAs. They were significantly more common in women (49%) than men (19%, $P < 0.001$) and associated with higher preoperative aldosterone levels. Germline *KCNJ5* mutations were not found in patients with bilateral adrenal hyperplasia. Similar genotype and phenotypic findings have been reported in multiple subsequent studies of APA.

Other somatic mutations in *ATP1A1*, *ATP2B3*, and *CACNA1D* have also subsequently been identified; albeit at a lower frequency than *KCNJ5* mutations. In a study using exome sequencing of 308 APA, somatic mutations of *ATP1A*1 (encoding an Na^+/K^+ ATPase alpha subunit) were found in 16 (5.2%) and of *ATP2B3* (encoding a Ca^{2+} ATPase) in 5 (1.6%) (Beuschlein *et al.*, 2013). Similar results were noted in a subsequent study of 112 APA (Williams *et al.*, 2014).APA harboring these mutations were more common in mean and associated with increased plasma aldosterone concentrations, and lower potassium concentrations compared with mutation-negative cases (Beuschlein *et al.*, 2013).

Additional somatic APA mutations have been identified in *CACNA1D*, encoding a voltage-gated calcium channel. In one study, such mutations were identified in 11% percent of APA (Scholl *et al.*, 2013); patients carrying these mutations had smaller tumors and were older than those with KCNJ5 mutations. In two cases with early onset of PA, de novo germline mutations of *CACNA1D* were identified and associated with complex, severe neurologic and neuromuscular abnormalities (cerebral palsy, seizures, athetosis, spastic quadriplegia).

Table 1 Summary of most significant APA mutations and their relative frequencies

Gene	*Reference*	*Number of APA*	*% somatic mutations*	*Mutations identified*
KCNJ5	Choi *et al.*, 2011	22	41	Gly151Arg Leu168Arg
KCNJ5	Boulkroun *et al.*, 2012	380	35	Gly151Arg Leu168Arg
KCNJ5	Åkerström *et al.*, 2012	351	47	Gly151Arg Leu168Arg E145Q
KCNJ5	Azizan *et al.*, 2012	73	41	Gly151Arg Leu168Arg Ile157del
KCNJ5	Monticone *et al.*, 2012	47	38	Gly151Arg Leu168Arg
KCNJ5	Mulatero *et al.*, 2012	46 (familial PA)	7	Gly151Arg Leu168Arg Thr158Ala
KCNJ5	Williams *et al.*, 2014	112	39.3	Trp126Arg novel mutation
KCNJ5	Zheng *et al.*, 2015	168	77	Gly151Arg Leu168Arg Thr158Ala Thr148-Arg149ins
ATP1A1	Beuschlein *et al.*, 2013	308	5.2	Leu104Arg Val332Gly Phe100_Leu deletion
ATP2B3	Beuschlein *et al.*, 2013	308	1.6	Leu415/Val426 deletion
CACNA1D	Scholl *et al.*, 2013	43	11	Gly403Arg Ile770Met Gly403Arg Gly403Asp Ile770Met
ATP1A1	Zheng *et al.*, 2015	168	2.8	Leu104Arg Met102-Leu103 del

Inherited forms of PA

Familial syndromes are a rare cause of PA (so-called "familial hyperaldosteronism; FHA"). However, as is often the case with Mendelian disease, understanding their etiology gives insight into the control and regulation of aldosterone in both health and disease.

FHA1 (glucocorticoid remediable aldosteronism; GRA)

This is an autosomal dominant monogenic disorder caused by a hybrid gene comprising the regulatory element of 11β-hydroxylase, which catalyzes the final steps of cortisol synthesis and is normally expressed in the zona fasciculata, and the coding region of aldosterone synthase, which catalyzes the final steps of aldosterone production in the zona glomerulosa (**Fig. 1**). This leads to mineralocorticoid hypertension that typically presents in young adults (Lifton *et al.*, 1992). Aldosterone is produced in response to ACTH rather than its usual trophins of potassium and angiotensin II, but importantly, the chimeric gene is expressed ectopically in the fasciculata, which allows the gene product inappropriate access to greater quantities of 11-deoxycorticosterone as a substrate for excessive aldosterone production.

The diagnosis of glucocorticoid-remediable hyperaldosteronism should be suspected in any patient who presents with early onset of PA and/or a positive family history of aldosterone excess. Genetic testing using molecular biologic techniques to detect the chimeric gene is now preferred over dexamethasone suppression testing for making the diagnosis of GRA. Given its relative rarity, the genetic screen should be performed only on selected patients. Indications include primary aldosteronism patients with onset at a young age (e.g., $<$20 years), or a family history of primary aldosteronism or of stroke at a young age (e.g., $<$40 years).

Treatment of GRA can be with either low dose oral glucocorticoid therapy (to suppress ACTH mediated aldosterone excess) or mineralocorticoid receptor antagonists.

FHA2

FHA2 is also an autosomal dominant disorder presenting as PA with a positive family history. While the gene defect causing FHA2 has not been discovered, linkage analysis of affected families have suggested an association between markers within cytogenetic band 7p22 and this phenotype (Lafferty *et al.*, 2000). It is currently diagnosed if patients have at least two relatives with hyperaldosteronism and screening for FHA1 (GRA) is negative.

FHA3 (KCNJ5)

FHA3 has recently been characterized in a single affected family where three members had severe, early onset hypertension and massive bilateral adrenal hyperplasia (Choi *et al.*, 2011). The discovery that germ line mutations in the *KCNJ5* gene lead to autosomal dominant hyperaldosteronism has provided a clear genetic cause of this condition in a small cohort of patients. More importantly, as discussed above, it has provided novel mechanistic insights into the normal control of aldosterone and possible pathogenic mechanism in patients with nonfamilial APAs.

Diagnosis of Primary Aldosteronism

As PA is a relatively common secondary cause of hypertension, with excess cardiovascular mortality compared to hypertensive controls and specific management issues, there is a clear case for identification of PA within hypertensive cohorts. However, the diagnosis of PA can be obtuse, as there are few specific clinical symptoms, and the biochemical tests can be obscured by confounding factors and technical factors. The Endocrine Society's recently published updated guideline (Funder *et al.*, 2016) suggests screening for PA using simultaneous measurements of plasma renin and aldosterone (to give a ratio, the ARR) in patients with moderate to severe or resistant hypertension, those with hypokalemia (spontaneous or in response to diuretic treatment), those with an adrenal incidentaloma and those with a family history of PA. If this initial test is positive then confirmatory testing followed by the subtype diagnosis should proceed. Their approach to diagnosis is summarized in **Fig. 4**.

Screening test

The initial screening test of ARR can be performed without the need for stopping antihypertensive medication if patients have severe hypertension (with the exceptions of spironolactone, eplerenone and amiloride), as these agents have predictable effects on renin and aldosterone measurements (**Table 2**). The ARR should be measured in the context of an unrestricted sodium intake (which should not require adjustment from normal for the majority of patients). Previously plasma renin activity (PRA) was measured using a radioimmunoassay. More recently, the measurement of plasma renin concentration (PRC) has been adopted in many centers as this method is less technically demanding although this assay in general is less sensitive, particularly at lower concentrations (Dorrian *et al.*, 2010). Therefore, adding cut-off of minimum plasma aldosterone concentration (>15 ng/dL or 410 mmol/L) before further confirmatory testing has been proposed to minimize false positive results. This recommendation, however, this is not universally accepted (Dorrian *et al.*, 2010; Funder *et al.*, 2016). The cut-off values for aldosterone and ARR are dependent on local assays and conditions: an aldosterone (pmol/L)/PRC (microIU/mL) ratio of >30, aldosterone (pmol/L)/PRA (ng/mL/h) ratio of >750 are commonly used.

Confirmatory test

Levels of ARR above a threshold require further confirmatory testing for the diagnosis of PA. The exception to this is when there is clear PA (spontaneous hypokalemia with plasma renin below detection levels and plasma aldosterone above 20 ng/dL (550 pmol/L)) when confirmatory testing is unnecessary.

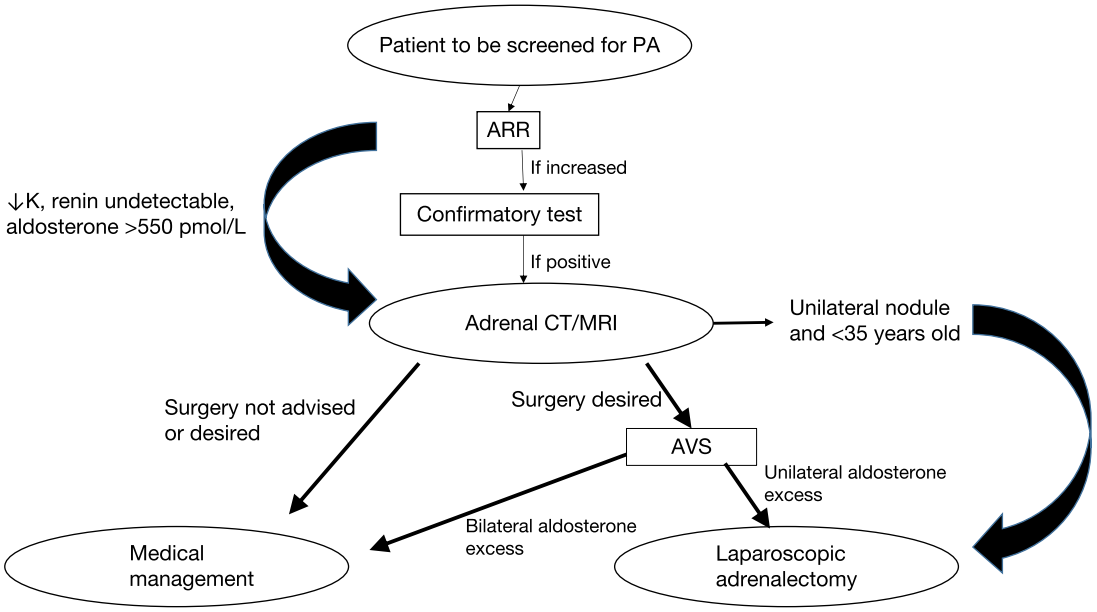

Fig. 4 Diagnostic algorithm for primary aldosteronism. *AVS*, adrenal vein sampling; PA, primary aldosteronism; ARR, aldosterone to renin ratio.

Table 2 Influence of commonly used antihypertensive agents on the renin–angiotensin–aldosterone axis

Medication	*Plasma aldosterone*	*Plasma renin*	*ARR*
Beta-blocker	Reduced	Significantly low	Increased
Loop or thiazide diuretics	No effect or increase	Significantly increased	Reduced
Potassium-sparing diuretics (spironolactone/amiloride)	Increase	Significantly increased	Reduced
ACE inhibitors/ARB	Reduced	Significantly increased	Reduced
Ca channel blockers (dihydropyridine)	No effect or reduced	Increased	Reduced

ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blockers.

The purpose of the confirmatory test is to confirm autonomous secretion of aldosterone which is freed from control by its conventional trophins. There are a number of ways this can be investigated, and the choice of which confirmatory test is a matter of local preference/expertise; no "gold standard" currently exists. It is recommended that, for confirmatory testing, medications with significant effects on the renin–angiotensin–aldosterone axis be avoided (**Table 2**).

Oral sodium loading test

This test requires an increase in oral sodium intake (with sodium chloride tablet if necessary) to >200 mmol (6 g) per day, for 3 days. As with all confirmatory tests, it is important to ensure plasma potassium remains normal as hypokalemia can lead to a false suppression of plasma aldosterone. A 24 h urine aldosterone is measured after 3 days. Autonomous secretion of aldosterone is confirmed if urinary aldosterone excretion exceeds 33 nmol/day (12 μg/24 h). This test is of limited value in patients with renal disease, as aldosterone 18-oxo-glucuronide is a renal metabolite, and its excretion may not rise in patients with renal disease. However, the main limitation of this test is mainly due to the difficulties encountered in organizing 24 h urine collections in most patients.

Intravenous saline infusion test

In this test, 2 L of 0.9% sodium chloride solution are infused over 4 h into the recumbent patient with monitoring of blood pressure and heart rate throughout. After 4 h, plasma aldosterone is measured; levels <139 pmol/L (5 ng/dL) make PA less likely, values between 139 and 277 pmol/L (5 and 10 ng/dL) are indeterminate and levels >277 pmol/L (10 ng/dL) make a diagnosis of PA highly likely. The main disadvantage of this test is the volume of intravenous fluid infused, which may be contraindicated in patients with cardiac failure as well as ongoing debate over the optimal postsaline infusion aldosterone level used to confirm aldosterone excess.

Fludrocortisone suppression test

Fludrocortisone acetate (0.1 mg every 6 h) is administered for 4 days and in addition, the oral sodium intake maintained at an intake of >200 mmol per day using sodium chloride supplementation. Plasma renin should be suppressed and the upright aldosterone in the morning of day 4 should be suppressed to <166 pmol/L (6 ng/dL). While the fludrocortisone suppression test is considered by some to be the most sensitive of the confirmatory tests, it has several limitations which mean its use should be restricted to centers with expertise and facilities to cope with complications (such as hypokalemia and cardiac dysrhythmia). In light of this, most centers undertake this test as an inpatient, which has cost and resource implications.

Captopril challenge

The captopril challenge consists of measurement of plasma renin and aldosterone before and 2 h after a single dose of captopril (25 mg). This would normally be expected to suppress aldosterone by >30% but, in PA, aldosterone will remain high. However, this test has been reported to be less sensitive than salt suppression methods and as such it is now rarely used.

Subtype characterization: Further tests

Only once autonomous secretion of aldosterone has been confirmed should there be an attempt to differentiate between APA and bilateral forms of aldosterone excess. Further imaging is important in order to exclude an aldosterone secreting carcinoma as well as to inform management, which is clearly different depending on the underlying cause of hyperaldosteronism. Lateralizing the site of excess aldosterone production is the next step, and this requires adrenal vein sampling.

Imaging

The preferred imaging modality is computerized tomography (CT) which demonstrates superior spatial resolution over MRI. On the other hand, MRI does not involve radiation exposure which, to some patients, may be more advantageous.

An APA is usually a small (<2 cm) lipid-rich tumor with a typical appearance on CT, corresponding to <10 Hounsfield units with a rapid wash-out phase following the administration of contrast agent. Adrenal hyperplasia can consist of either micro (<1 cm) or macro (>1 cm) adenomas, or a combination of the two (**Fig. 5**).

Importantly, in the majority of cases, CT alone should not be used to distinguish between unilateral disease, amenable to adrenalectomy, and bilateral or idiopathic disease, which should be managed medically. For example, nonfunctioning adrenal

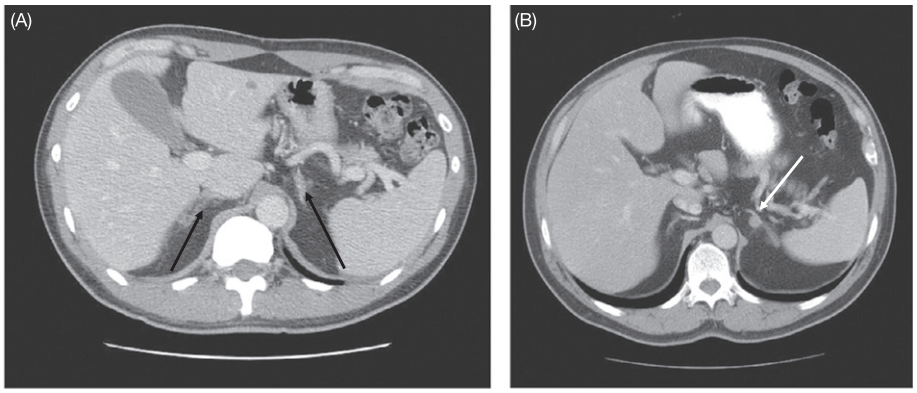

Fig. 5 CT scan of adrenals demonstrating bilateral adrenal hyperplasia (A, *black arrows*) and a unilateral adrenal adenoma (B, *white arrow*).

adenomas are relatively common in older patients and radiologically indistinguishable from APAs. Moreover, small APAs may not be seen on CT and the incorrect diagnosis of idiopathic hyperaldosteronism reached, and conversely, areas that look like microadenomas may in fact represent area of hyperplasia. Consequently, a more accurate method of lateralization of aldosterone secretion is required in order to avoid inappropriate surgery in patients with bilateral disease or withholding curative surgery to patients who may benefit. The exception to this sequence of investigations is in patients under the age of 35, in whom an adrenal adenoma is identified of >1 cm. In these patients, it would be reasonable to proceed to adrenalectomy without further investigation in accordance with The Endocrine Society Clinical Practice Guideline (**Fig. 4**; Funder *et al.*, 2016).

Lateralizing aldosterone secretion

The gold standard investigation to confirm aldosterone excess is adrenal vein sampling (AVS). This is a technically demanding procedure which is difficult to access outwith tertiary referral centers and, as such, is likely to be performed less frequently than is clinically indicated. This notion was supported by the Adrenal Vein Sampling International Study (AVIS which explored AVS practice in 20 centers from Asia, Australia, United States and continental Europe (Rossi *et al.*, 2012). This important study confirmed that AVS was under-utilized with significant variation in techniques and interpretation of results and therefore highlighted a requirement for definitive guidance on the use and interpretation of AVS in identifying unilateral aldosterone excess. This requirement was subsequently addressed by the publication of an expert consensus statement on the use of AVS, based on data collected in the AVIS study, by Rossi and coauthors published in 2014 (Rossi *et al.*, 2014). This now provides a convenient reference to allow standardization of AVS techniques and protocols as well as in interpretation of results.

While there is no doubt that AVS remains a technically challenging procedure, success can be directly correlated with clinical experience. In the AVIS study, 2604 AVS procedures were performed by seven radiologists, with an average of 2.6 radiologists performing the procedure at each center. Unsurprisingly, the complication rate was inversely proportional to the number of radiologists performing AVS at each center (Rossi *et al.*, 2012).

Given these difficulties, the prospect of another tool to differentiate unilateral, surgically curable disease from bilateral disease is appealing. The use of 11^{C}-Metomidate as a radiotracer in positron emission tomography (PET-CT) could be a useful in this situation. Previous data have demonstrated sensitivity and specificity of 76% and 87% using this technique (Burton *et al.*, 2012) which is noninvasive and requires less technical expertise. The use of 11C-Metomidate PET would currently be limited to centers with access to a cyclotron, but this promising diagnostic test may be used more frequently in the future.

Treatment of Primary Aldosteronism

Surgical management

In suitable patients with unilateral disease, laparoscopic (when possible) surgical resection of the affected adrenal gland is the treatment of choice. Adrenalectomy offers the possibility of curing hyperaldosteronism, although it is important to be aware that not all patients achieve complete remission of hypertension. This has been illustrated most recently in a very careful study examining surgical outcomes after adrenalectomy for PA in 12 centers of excellence in 9 countries (Williams *et al.*, 2017). Using a set of criteria agreed in advance by a cohort of international experts, it was reported that surgery resulted in cure of hypertension in 259/705 (37%) of cases; although this varied between centers. An additional 334/705 (47%) of patients demonstrated partial success with reduction in blood pressure or antihypertensive use after surgery. Factors which were associated with cure were found to be younger age, fewer antihypertensives preoperatively and female sex. Interestingly, while clinical cure was only seen in one third of patients, complete biochemical resolution of aldosterone excess was found in 94% which illustrates that primary hypertension and PA often coexist.

Medical therapies

In patients with bilateral disease, or those with APA and unsuitable or unwilling for a surgical procedure, medical therapy with aldosterone antagonists is the mainstay of therapy. Spironolactone is an effective mineralocorticoid antagonist, although it lacks specificity and acts as an antagonist for the androgen and progesterone receptor. These properties account for the side effects which can limit its use, particularly at high doses (gynecomastia and erectile dysfunction in males and menstrual irregularity in women). Eplerenone is more specific for the mineralocorticoid receptor but is less potent, requiring higher doses. Both should be started at low dose and titrated slowly with monitoring of plasma potassium. Once aldosterone action is appropriately suppressed, plasma renin should rise, and this can be a helpful way to monitor the titration regime. If adequate control of hypertension is not achieved with mineralocorticoid antagonists alone, amiloride is a relatively effective additional antihypertensive through its action on blocking the epithelial sodium in the distal convoluted tubule. Calcium channel blockers and diuretics are also reasonable to use in combination with MR antagonists, however, unless plasma renin (and angiotensin II) is released from the inhibitory effects of excessive action of aldosterone, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) are unlikely to improve blood pressure control.

Importance of targeting aldosterone excess

Animal studies in stroke prone hypertensive rats have demonstrated that aldosterone (and salt) results in vascular damage and fibrosis which can be prevented by small doses of spironolactone that do not lower blood pressure (Rocha *et al.*, 1998, 2000). Subsequently, a variety of clinical studies have demonstrated that aldosterone exerts adverse cardiovascular consequences which cannot be attributed to hypertension alone. For instance, a landmark observational study in 2005 comparing cardiovascular events in subjects with PA with those with primary hypertension demonstrated a marked increase in stroke, myocardial infarction and atrial fibrillation in PA patients despite similar severity and duration of hypertension (Milliez *et al.*, 2005). A number of subsequent studies have demonstrated similar results (Savard *et al.*, 2013).

Taken together, both animal and clinical studies suggest that targeting of aldosterone excess (by surgical or medical means) as well as lowering of blood pressure is an effective treatment strategy to reduce cardiovascular morbidity in PA. It has been demonstrated that both mineralocorticoid-receptor blockade and adrenalectomy are effective in reversing adverse target organ damage seen in aldosterone excess (Catena *et al.*, 2008; Sechi *et al.*, 2010).

Activation of the Mineralocorticoid Receptor by an Alternative Ligand

Syndrome of Inappropriate Mineralocorticoid Excess

Apparent mineralocorticoid excess (AME) is a rare syndrome of hypertension and hypokalemia associated with suppression of plasma renin activity and low plasma concentrations of aldosterone and other known mineralocorticoids (Stewart *et al.*, 1988). In this autosomal recessive syndrome, 11β-HSD2 activity (**Fig. 6**) is reduced or absent such that cortisol overwhelms the mineralocorticoid receptor leading to cortisol-mediated mineralocorticoid hypertension.

AME usually presents in childhood with failure to thrive, short stature, significant hypertension, and hypokalemia. Biochemical diagnosis of AME can be made by measuring the ratio of cortisol (compound F) to cortisone (compound E) as indicated by the ratios of their tetrahydro (allo)-urinary metabolites (THF + alloTHF:THE) (Palermo *et al.*, 1996). In AME, however, urinary free cortisone excretion is extremely low compared to normal subjects, leading to an increased THF + alloTHF:THE ratio in urine.

Milder cases of AME, so-called "type II apparent mineralocorticoid excess" with isolated hypertension and normal or low-normal potassium have been described in Italian patients (Li *et al.*, 1998). In these subjects, a homozygous mutation in the 11β-HSD2 gene (R279C) has been identified which causes a reduction but does not completely ablate 11β-HSD2 activity. Therefore, AME comprises a spectrum of mineralocorticoid hypertension with a good correlation between enzyme activity and clinical phenotype.

The most effective treatment of AME is amiloride, although high doses (up to 40 mg daily) can be required in order to be effective. Mineralocorticoid receptor antagonists can also be used but their safety and tolerability may be limited by the relatively high doses required to competitively antagonize the agonist effects of cortisol in this circumstance. Dexamethasone has also been

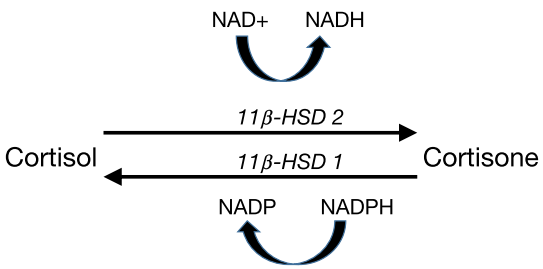

Fig. 6 The 11β-hydroxysteroid dehydrogenase system.

used but its therapeutic benefit is similarly limited by the need to give doses sufficiently high to inhibit endogenous cortisol production, exposing patients to unwanted glucocorticoid side effects.

Deficiency of 11β-HSD and consequent mineralocorticoid hypertension can also occur due to ingestion of liquorice or carbenoxolone (previously used for the treatment of peptic ulcer disease). The active component of liquorice is glycyrrhizic acid and its hydrolytic product glycyrrhetinic acid, which have been shown to inhibit the activity of 11β-HSD2 in the renal tubule allowing cortisol-driven mineralocorticoid hypertension (Stewart *et al.*, 1987). Carbenoxolone is a semisynthetic hemisuccinate derivative of glycyrrhetinic acid and has its effect through a mechanism analogous to that of liquorice.

Congenital Adrenal Hyperplasia

Other mineralocorticoids rarely circulate in sufficient levels to cause hypertension. For example, the aldosterone precursor deoxycorticosterone, which also binds and activates the mineralocorticoid receptor, circulates at concentrations around 2% of those of aldosterone and so, under normal circumstances, does not contribute to electrolyte and blood pressure regulation.

However, excessive plasma levels of deoxycorticosterone can be found in patients with rare inborn errors of adrenal steroid synthesis due to defective 17α-hydroxylase or 11β-hydroxylase activity (**Fig. 1**). In both of these circumstances, low plasma cortisol leads to increased ACTH drive to the adrenal and chronic excess of corticosteroid precursors including deoxycorticosterone (White, 1996). In turn, sodium retention causes suppression of renin release and, as a consequence, aldosterone levels are generally low. Most cases present shortly after birth or in early childhood. While both conditions result in early onset mineralocorticoid hypertension and glucocorticoid deficiency, there are important differences in the associated symptoms. For instance, adrenal androgens are produced in excessive amounts in patients with 11β-hydroxylase deficiency, leading to virilization of female subjects. In contrast, 17-hydroxylase deficiency results in an inability to synthesize sex hormones, with the result that affected males fail to develop normal masculine external genitalia, while females fail to progress through adrenarche or puberty. Diagnosis of either condition is confirmed by measurement of corticosteroid metabolite excretion in the urine.

Abnormality Within the Mineralocorticoid Receptor Altering Sensitivity to Other Agonists (Progesterone Induced Hypertension)

This rare disorder is characterized by constitutive activation of the mineralocorticoid receptor as well as an alteration in receptor sensitivity (Geller *et al.*, 2000). An autosomal dominant mis-sense mutation in the MR gene (S810L) means that the receptor is able to be occupied by a range of steroids, including those that have not undergone 21 hydroxylation, for example, progesterone. Subjects with this mutation develop early onset of severe hypertension with suppression of aldosterone and plasma renin. As progesterone levels increase by up to 100-fold in pregnancy, carriers of the S810L tend to develop severe pregnancy-associated hypertension.

Abnormality Within Renal Ion Channels Resulting in Inappropriate Activation

Liddle Syndrome

This is a rare autosomal dominant condition which presents with significant hypertension in childhood, arising from increased activity of the epithelial sodium channel (ENaC). In common with PA, patients have hypokalemia with a metabolic alkalosis and low plasma renin but in this disorder plasma aldosterone concentrations are also low. The genetic abnormality lies on chromosome 16 leading to "gain of function mutations" within the β- and γ-subunits of ENaC (Shimkets *et al.*, 1994; Rossi *et al.*, 2008). These mutations prevent the interaction of these subunits with intracellular ubiquitin protein ligase (Nedd4) that normally removes the luminal sodium channel from the cell surface. This results in constitutive activity of the ENaC in the cortical collecting duct and a failure to downregulate in response to volume expansion-mediated reduction in renin secretion and aldosterone. Importantly, in this condition, spironolactone is not a useful treatment because activation of ENaC is independent of the mineralocorticoid receptor. However, the ENaC is amiloride-sensitive and this is the treatment of choice in these patients.

Gordon Syndrome (Pseudohypoaldosteronism Type 2)

Pseudohypoaldosteronism Type 2 or Gordon syndrome is characterized by hypertension, hyperkalemia, metabolic acidosis, normal renal function, low or low-normal plasma renin activity and normal or elevated aldosterone concentrations (Gordon, 1986). In addition, patients with this autosomal dominant condition are extremely sensitive to thiazide diuretics suggesting a gain of function mutation in the thiazide-sensitive sodium–chloride transporter in the distal convoluted tubule. However, the mutation lies not within the gene encoding the transporter itself, but rather on chromosomes 12 and 17 each of which encode a With No Lysine Kinase (WNK). WNK1 and 4 localize to the distal convoluted tubule and cortical collecting duct and are involved in the regulation of the sodium–chloride transporter acting via a kinase cascade, leading to loss of inhibition of the sodium chloride transporter and increased expression at the apical surface (Wilson *et al.*, 2001). WNK4 normally inhibits the thiazide-sensitive

Na–Cl cotransporter of the distal nephron; thus somatic mutations increase its activity, leading to thiazide-sensitive hypertension. As a result, systolic and diastolic blood pressure fall significantly after treatment with low dose thiazide diuretic such as hydrochlorothiazide (Mayan *et al.*, 2002). The mechanism of hypertension in subjects with WNK1 mutations is less clear.

Summary

Primary aldosteronism is easily the commonest secondary cause of hypertension. Whilst universal screening for this condition among subjects with hypertension is not yet advocated, its prevalence and relative ease of screening mean it should be considered far more commonly and earlier in hypertensive cohorts (especially if young, presenting with resistant hypertension, presenting with spontaneous or diuretic-induced hypokalemia). Earlier identification of PA means that more targeted treatment (and even cure) can be offered; this is particularly relevant given strong evidence demonstrating blood pressure independent adverse effects of aldosterone excess.

However, it should be noted that PA is not the sole cause of mineralocorticoid hypertension, although other causes, as demonstrated in this article, are extremely rare. Whatever, the cause, baseline measurement of plasma renin and aldosterone can be used as a starting point to guide further investigations. If both are low, then this should trigger a search for more obscure causes of mineralocorticoid hypertension.

See also: Adrenal Venous Sampling for Primary Aldosteronism. Aldosterone-Producing Adenomas; Genetics. Endocrine Hypertension. Genetics of Familial Hyperaldosteronism. Primary Aldosteronism; Diagnosis and Treatment. Primary Aldosteronism; Epidemiology and Screening

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Primary Aldosteronism; Epidemiology and Screening

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Abbreviations

ACE	Angiotensin converting enzyme
AngII	Angiotensin II
APA	Aldosterone-producing adenoma
ARB	Angiotensin receptor blocker
ARR	Aldosterone/renin ratio
BAH	Bilateral adrenal hyperplasia
CCB	Calcium-channel blocker
DRC	Direct renin concentration
FST	Fludrocortisone suppression test
PA	Primary aldosteronism
PRA	Plasma renin activity

Epidemiology

Primary aldosteronism (PA) is currently considered to be the most common secondary endocrine form of hypertension. This represents a marked change in understanding of the prevalence of PA which, throughout the 1970s to early 1990s, was considered to be rare (accounting for $<1\%$ of patients with hypertension) and not worth looking for in the absence of hypokalemia. During the preceding decade, Conn had strongly argued that PA was much more common, having recognized that patients with PA (including those with aldosterone-producing adenoma, APA) need not be hypokalemic (Conn, 1964) and was able to report that 14 of 45 patients diagnosed by his group as having PA and found to have adrenal cortical tumors at the time of surgery were normokalemic preoperatively (Conn, 1966) and that 13 (93%) of these demonstrated either cure or improvement of hypertension postoperatively. Despite these observations, Conn's proposal that normokalemic PA may be a common cause of hypertension was generally rejected by the medical community of that time.

A major advance in the ability to detect patients with PA was the application of the plasma aldosterone/renin ratio (ARR) as a screening test, first reported on in 1981 by Hiramatsu *et al.* (1981). By measuring the ARR in 348 "unselected" hypertensives, these workers were able to detect and remove APAs from 9 (2.6%) patients, only 3 of whom were hypokalemic. Since they used a very high diagnostic cut-off for the ARR, and relied on adrenal venography and scintigraphy to confirm the diagnosis of APA, it is highly probable that they would have missed patients with relatively small APAs, and all with bilateral adrenal hyperplasia (BAH), meaning that the true prevalence of PA was probably much higher than the figure of 2.6% they reported.

From the early 1990s, reports began to emerge of even higher detection rates for PA resulting from the use of the ARR and its wider application among hypertensive populations. At the Greenslopes Hospital Hypertension Unit in Brisbane, Australia, adoption of the policy to apply the ARR to all referred hypertensives, and not just those with hypokalemia or resistant hypertension, led to a tenfold increase in the detection rate of PA (confirmed in each case by fludrocortisone suppression testing [FST]) (Gordon *et al.*, 1992b, 1994a; Stowasser and Gordon, 2004). The proportion who were hypokalemic fell from two-thirds to just one-fifth, the rest being normokalemic and therefore masquerading as "essential hypertension" (Gordon *et al.*, 1992b, 1994a; Stowasser and Gordon, 2004).

What is the prevalence of PA among the hypertensive population? While rates vary across series according to the population screened and the diagnostic methods and cut-offs used, the majority of studies since the 1990s have reported prevalence rates between 5% and 15%, with most patients being normokalemic (Fardella *et al.*, 2000; Gordon *et al.*, 1993, 1994b; Lim *et al.*, 2000; Loh *et al.*, 2000; Mulatero *et al.*, 2004; Nishikawa *et al.*, 2007; Rossi *et al.*, 2006; Young, 1997). In the earliest of these, 6 (12%) of 52 respondents to advertisements seeking volunteers for antihypertensive drug trials and who were screened by ARR testing were found to have PA (confirmed by FST) (Gordon *et al.*, 1993); and (2) PA was confirmed in 19 (8.5%) of 199 consecutively referred normokalemic hypertensive patients who underwent ARR testing (Gordon *et al.*, 1994b). Although the patients in the studies were "selected," similar prevalence rates were reported among relatively unselected hypertensive patients (e.g., those randomly chosen for ARR testing from a general practice database in the United Kingdom; Lim *et al.*, 1999). Such prevalence rates make PA probably the most common specifically treatable and sometimes curable cause of hypertension. In resistant hypertensive cohorts, the prevalence rates appear to be even higher and at least 20% (Calhoun *et al.*, 2002; Eide *et al.*, 2004; Gallay *et al.*, 2001; Strauch *et al.*, 2003). Not unexpectedly, this new understanding has led to a marked increase in the number of ARR tests ordered by treating physicians. There is therefore currently a critical need to better understand the strengths and weaknesses of the ARR, the factors that can complicate interpretation of results, and the steps that can be taken to maximize its performance as a means of screening for PA.

Screening

The diagnostic workup for PA involves (1) screening, (2) confirmatory testing, and (3) determining the subtype of PA. Unlike screening, procedures utilized for confirmatory testing and subtype differentiation are relatively invasive, time-consuming, and

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expensive. Screening of at-risk populations for the possible presence of PA is therefore important as it serves to minimize the number of patients who should then be considered for the subsequent steps of diagnostic assessment.

Who Should Be Screened?

An Endocrine Society Guideline has recommended screening for all but the mildest forms of hypertension, and for patients with hypertension and spontaneous or diuretic induced hypokalemia, hypertension and adrenal incidentaloma, hypertension and sleep apnea, and (in recognition of familial forms) hypertension and a family history of early onset hypertension or cerebrovascular accident at a young age (<40 years) and all hypertensive first degree relatives of patients with PA (Funder *et al.*, 2016).

Could an argument be made for screening all hypertensives? While the majority of authorities would not support this view, proponents point out that: (1) PA is common among the hypertensive population, (2) ARR testing is relatively inexpensive, (3) early diagnosis may be preferable to waiting until hypertension becomes severe and resistant because the degree of benefit achieved from specific treatment of PA (including chance of cure of hypertension following unilateral adrenalectomy for unilateral forms of PA) appears to be inversely related to duration of hypertension at the time of treatment institution (Citton *et al.*, 2015; Gockel *et al.*, 2007; Lumachi *et al.*, 2005; Wachtel *et al.*, 2014; Zarnegar *et al.*, 2008; Zhang *et al.*, 2013), and (4) screening patients before they are commenced on antihypertensive medications avoids potentially confounding effects of treatment on plasma renin and aldosterone levels which could lead to false-positive or -negative ARR results.

Choice of Screening Test

Once relied upon as the main "trigger" for further workup, it is now well established that the demonstration of hypokalemia by measurement of plasma potassium lacks sufficient sensitivity to serve as a sole means of screening for PA. Furthermore, unless great care is taken to avoid factitious rises in potassium due to release from muscle and blood cells during collection, the presence of hypokalemia can sometimes be missed. This involves (1) avoiding fist clenching and releasing the tourniquet after venepuncture has been achieved, (2) waiting (at least 10 s) before withdrawing blood, (3) using a syringe and needle rather than a vacuumed sample container to permit slow and careful blood withdrawal and discharge down the side of the opened sample tube, (4) using lithium heparin (i.e., plasma) rather than plain (serum) tubes so that clot formation (which is associated with release of potassium) does not occur, and (5) separating plasma from cells within 30 min of collection.

Reliance on frankly elevated plasma aldosterone levels also lacks sensitivity for PA. In many patients, levels lie within the wide normal range (Rutherford *et al.*, 1998; Stowasser and Gordon, 2004; Stowasser *et al.*, 2003), but could be viewed as "inappropriately normal" in the face of suppression of the renin which, in individuals without PA, should result in plasma aldosterone levels that are frankly low. Raised plasma aldosterone levels also lack specificity as they may result from raised renin/AngII (secondary hyperaldosteronism), as in renovascular forms of hypertension or in patients receiving diuretic agents.

Plasma renin levels, measured by plasma renin activity (PRA) or direct active renin concentration (DRC), are almost always suppressed in PA. They are therefore superior to plasma potassium and aldosterone in terms of sensitivity. Levels can be normal however in patients who habitually ingest a low sodium diet or are on medications such as diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), or dihydropyridine forms of calcium-channel blockers (CCBs) which can result in renin production. Rarer causes of "false normal" renin levels include concomitant "accelerated" or malignant hypertension (Beevers *et al.*, 1976; Kaplan, 1963; Murphy *et al.*, 1985), or renovascular hypertension (Stowasser *et al.*, 1993).

While a suppressed renin is highly sensitive for PA, it lacks specificity. Hence, reliance on plasma renin alone to screen for PA would lead to frequent false-positive results. Situations (other than PA) in which renin levels may be suppressed include (1) treatment with medications (including beta-adrenoceptor blockers, clonidine, or alpha-methyldopa) which reduce beta-sympathetic stimulation of renin release (Ahmed *et al.*, 2010; Buhler *et al.*, 1972; Gordon *et al.*, 1992a; Oparil, 1982); (2) treatment with nonsteroidal antiinflammatory agents which promote salt-retention (Mitnick *et al.*, 1980); (3) high dietary sodium intake (Gordon, 1995); (4) advanced age (Crane and Harris, 1976; Luo *et al.*, 2016); (5) chronic renal impairment, in which renal renin-producing capacity is reduced and salt-retention contributes to renin suppression (McKenna *et al.*, 1991); and (6) the presence of other salt-dependent, low-renin forms of hypertension of either known (listed in **Table 1**) or unknown cause (Arai and Chrousos, 1995); Geller *et al.*, 2000; Gordon *et al.*, 1970; Irony *et al.*, 1987; Kater and Biglieri, 1994; Stewart, 1996; Walker *et al.*, 1992; Warnock, 2001; White, 2001; Wilson *et al.*, 2001).

Unlike in PA, chronic suppression of renin/angiotensin II (AngII) leads to suppression of aldosterone and hence the ARR is normal in all of the low-renin forms of hypertension listed in **Table 1** with the exception of familial hyperkalemic hypertension, in which elevated plasma potassium levels prevent aldosterone suppression. Because of this, the ARR is more specific than isolated renin measurements while at the same time being much more sensitive than plasma potassium or isolated measurement of plasma aldosterone. As a result, it has become the preferred method of screening for PA.

Despite its superiority over other currently available methods of screening, the ARR can be affected by numerous physiological and pharmacological factors, leading to the potential for false positives and negative results (see what follows). Accordingly, although such factors can often be controlled or at least taken into account when interpreting the ARR, the ratio should still be regarded as a screening test only. Furthermore, given the greater complexity and invasiveness associated with subsequent steps in

Table 1 Low-renin forms of hypertension (other than PA) of known cause

Condition	*Cause/mechanism*	*ARR*	*References*
Liddle syndrome	Activating mutations of ENaC subunits causing excessive distal renal tubular sodium retention	Normal	Warnock (2001)
Syndrome of apparent mineralocorticoid excess	Mutations in gene encoding, or inhibitors (e.g., carbenoxolone or licorice) of, 11β-HSD2, resulting in lack of "inactivation" of cortisol, which thereby gains access to and activates the MR	Normal	Stewart (1996)
DOC-secreting tumors	Excessive DOC (has intrinsic mineralocorticoid activity)	Normal	Irony *et al.* (1987)
Hypertensive forms of CAH	Mutations in gene encoding either 11β-hydroxylase or 17α-hydroxylase leading to reduced cortisol production, hence increased ACTH secretion and, in turn, increased production of DOC	Normal	Kater and Biglieri (1994) and White (2001)
Primary glucocorticoid resistance	Mutations in gene encoding the GR leading to increased ACTH secretion and, in turn, increased production of DOC	Normal	Arai and Chrousos (1995)
Ectopic ACTH syndrome	Excess ACTH leads to DOC excess and "overload" of 11β-HSD2 by very high levels of cortisol	Normal	Walker *et al.* (1992)
Activating mutations of MR	Leads to constitutive activation of MR and anomalous activation by other ligands (e.g., high concentrations of progesterone during pregnancy and spironolactone)	Normal	Geller *et al.* (2000)
Familial hyperkalemic hypertension	Mutations in *WNK1*, *WNK4*, *CUL3* and *KLHL3* which are thought to lead to activation of NCC and excessive sodium and potassium retention in the distal renal tubule	Normal or high	Boyden *et al.* (2012), Glover *et al.* (2014), Gordon *et al.* (1970), and Wilson *et al.* (2001)

ENaC, epithelial sodium channel; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; DOC, deoxycorticosterone; CAH, congenital adrenal hyperplasia; ACTH, adrenocorticotrophic hormone; MR, mineralocorticoid receptor; NCC, sodium chloride cotransporter.

diagnostic workup for PA, it is advisable to measure the ARR more than once before deciding whether or not to go on to confirmatory testing to definitively confirm or exclude PA.

In addition to an elevated ARR, some investigators require the absolute plasma aldosterone concentration to be above a minimum level (e.g., 15 ng dL^{-1} or 410 pmol L^{-1}) before a screening test is considered positive. This is because the ARR is highly dependent on renin (Montori *et al.*, 2001), and therefore, in the presence of very low-renin levels (e.g., at PRA values of $\leq$0.1 ng ml^{-1} h^{-1} or DRC $<$2 mU L^{-1}), the ARR can be elevated even when plasma aldosterone is also very low (e.g., 4 ng dL^{-1} or 120 pmol L^{-1}) and clearly not consistent with PA. Others argue that this approach would lead to many patients with PA (including some with APA and hence potentially curable by surgery) being missed because their plasma aldosterone concentrations fall below this cut-off level (Stowasser and Gordon, 2004). The risk of missing APA at plasma aldosterone levels of $<$10 ng dL^{-1} ($<$277 pmol L^{-1}) is probably low. In such situations, however, if the decision is made not to proceed to confirmatory testing, it would seem prudent to at least continue to follow the patient and periodically repeat the ARR and consider, from time to time, further diagnostic workup depending on the clinical scenario and patient's wishes.

Factors That Affect the ARR

Posture

In normal subjects, plasma aldosterone rises in response to the assumption of upright posture (Tuck *et al.*, 1975). It is likely that this is due primarily to the associated translocation of blood into the lower limbs, which in turn results in (1) an increase in renin, released in response to both a fall in renal perfusion pressure and an increase in sympathetic output and beta-adrenergic receptor stimulation (Gordon, 1995), and (2) a reduction in metabolic clearance of aldosterone due to reduced hepatic blood flow (Balikian *et al.*, 1968; Nowaczynski *et al.*, 1977). Most patients with PA similarly demonstrate a rise in plasma aldosterone in response to upright posture. Such patients have been termed "angiotensin II-responsive" (AngII-R), meaning that their aldosterone levels rise in response to rising renin-AngII and thus to upright posture, and includes all patients with AngII-R APA, which make up 20%–50% of APAs, and most (at least 70%) with BAH (Espiner *et al.*, 2003; Gordon *et al.*, 1987; Mulatero *et al.*, 2008; Stowasser *et al.*, 2003). AngII-unresponsive (AngII-U) forms of PA, in which plasma aldosterone fails to rise in response to upright posture, include AngII-U APA, familial hyperaldosteronism type I, and the remaining up to 30% of BAH (Espiner *et al.*, 2003; Gordon *et al.*, 1987; Mulatero *et al.*, 2008; Stowasser *et al.*, 2003). However, despite the failure of aldosterone to rise in those subjects, upright levels in AngII-U PA are similar to those of patients with AngII-R forms (whose recumbent levels are usually much lower). Taking

all the above into account, performance of the ARR in the upright position is less likely to miss patients with PA, and most centers therefore use a midmorning upright sample for ARR testing, usually while seated for 5–15 min.

Time of day

Upright posture tends to have a greater stimulatory effect on renin and aldosterone in the early morning than in the afternoon (Gordon *et al.*, 1966). Hence, levels obtained midmorning from seated patients will tend to be higher than those measured in the afternoon (Gordon, 2004; Vagnucci *et al.*, 1974). Furthermore, plasma aldosterone levels in PA (in which levels are chronically suppressed) are strongly influenced by ACTH (Kem *et al.*, 1973), levels of peak at around 0800 h and fall rapidly thereafter as part of its normal circadian rhythm (Ney *et al.*, 1963). As a result, ARR levels in patients with PA are more likely to be elevated during the morning rather than in the afternoon (Gordon, 2004).

Age

In the elderly, renin levels fall in association with gradually falling renal function (Crane and Harris, 1976), while the fall in aldosterone is less marked. This leads to rising ARR levels and the possibility of false positives (Luo *et al.*, 2016).

Gender

Administration of exogenous estrogen stimulates production of the renin substrate angiotensinogen (Derkx *et al.*, 1986; Goldhaber *et al.*, 1984). The resulting rising angiotensin levels lead, by negative feedback on the juxtaglomerular apparatus, to falls in renin release and therefore in DRC. However, because PRA measures the generation of angiotensin I by the action of renin (levels of which are reduced) on angiotensinogen (levels elevated), the net effect on PRA is minimal (Fischer *et al.*, 2002). Falling DRC will tend to raise ARR, whereas stable PRA will not. Progesterone, secreted during the luteal phase of an ovulatory menstrual cycle, has mineralocorticoid-antagonist activity (Oelkers, 1996) and can cause natriuresis, leading to a reduction in plasma volume and a compensatory increase in plasma renin and aldosterone. Higher levels of estrogen and progesterone in women, and fluctuations of these levels during the menstrual cycle, thus have the potential to lead to differences in ARR levels between the genders and between different menstrual phases (Ahmed *et al.*, 2011b; Fommei *et al.*, 2008; Pizzolo *et al.*, 2010).

ARRs in females have been reported to be significantly higher than those of males (Ahmed *et al.*, 2011b) and, in another study (Pizzolo *et al.*, 2010), elevated ARR (using DRC) was more prevalent in hypertensive women than men (13.6% vs. 2.3%), but seldom associated with confirmed PA. In females, ARR levels are the lowest (and closest to those of males) during the menses and follicular phase and highest during the luteal phase, during which false positives can occur, but only if DRC (and not PRA) is used to measure renin (Ahmed *et al.*, 2011b). These observations suggest that PRA may be preferable to DRC in the determination of ARR and new reference ranges for ARR may be required which take into account gender and sex hormone levels (Ahmed *et al.*, 2011b).

Dietary sodium intake

Habitual dietary salt restriction raises renin and aldosterone levels, and because the ARR is more dependent on renin than aldosterone (Montori *et al.*, 2001), has the potential to lower the ARR and cause false negatives in patients with PA (Baudrand *et al.*, 2016; Gordon, 1995; Stowasser *et al.*, 2001). On the other hand, occasional false positives might arise in patients without PA who consume very large amounts of salt (Gordon, 1995; Stowasser *et al.*, 2001).

Plasma potassium level

Potassium is a potent chronic regulator of aldosterone secretion. Severe, uncorrected hypokalemia can lower aldosterone secretion in PA (Cain *et al.*, 1972) and therefore has the potential to cause false-negative ratios (Gordon, 1995; Stowasser *et al.*, 2001). Although it is less likely that this could also occur with milder hypokalemia, it is probably safest not to exclude PA on the basis of a normal ARR until it has been measured after correcting plasma potassium with supplemental slow-release potassium chloride tablets.

Medications potentially causing false-positive ratios

Treatment with beta-adrenergic blocking medications, which inhibit beta-adrenoceptor-mediated stimulation of renin production by JG cells (Gordon *et al.*, 1992a; Oparil, 1982), raise the ARR (Ahmed *et al.*, 2010). Methyldopa (Oparil, 1982) and clonidine (Manhem *et al.*, 1982) have a similar effect on renin by reducing central sympathetic outflow. Nonsteroidal antiinflammatory agents not only suppress renin levels by inducing renal sodium and water retention and suppressing renal prostaglandins which normally stimulate renin release, but also promote retention of potassium, which leads to stimulation of aldosterone production and further elevation of the ARR (Mitnick *et al.*, 1980).

Patients receiving oral contraceptive agents or hormone replacement therapy containing estrogen may demonstrate falsely elevated ratios when measurements of DRC are used rather than PRA (Ahmed *et al.*, 2011c, 2017a). This is presumably because the increased hepatic production of angiotensinogen, induced by estrogen, results in increased negative feedback by angiotensin suppressing active renin production (Derkx *et al.*, 1986; Goldhaber *et al.*, 1984; Steingold *et al.*, 1991). This usually prevents PRA from rising significantly but will lead to suppressed DRC and increased aldosterone/DRC ratio. Use of PRA may be preferable to DRC when screening women for PA by ARR measurement without ceasing estrogen-containing agents.

Table 2 Medications and the ARR

Medications that cause false-positive ARR	*Medications that cause false-negative ARR*	*Medications that have relatively little effect on ARR*
Beta-adrenergic blockers	Potassium-sparing diuretics	Verapamil SR
alpha-Methyldopa	Potassium-wasting diuretics	Hydralazine
Clonidine	ACE inhibitors	Prazosin, doxazosin, terazosin
NSAIDs	ARBs	Moxonidine
E2-containing OCPs and HRT[a]	Dihydropyridine CCBs	
Renin inhibitors[b]	SSRI antidepressants	
	Renin inhibitors[a]	

[a]Only if renin is measured as DRC.
[b]Only if renin is measured as PRA.
ARR, aldosterone/renin ratio; NSAIDs, nonsteroidal antiinflammatory drugs; E2, estrogen; OCPs, oral contraceptive preparations; HRT, hormone replacement therapy; ACE, angiotensin converting enzyme; ARBs, angiotensin receptor blockers; CCBs, calcium-channel blockers; SR, slow-release; SSRI, selective serotonin receptor uptake inhibitor.

Medications potentially causing false-negative ratios

False negatives may be encountered in patients taking medications that stimulate renin production. Examples include diuretics, both potassium-wasting and potassium-sparing (such as spironolactone, eplerenone, amiloride, and triamterene) (Gordon, 1995; Young, 1997), which induce volume contraction and sympathetic nervous system stimulation. Potassium-wasting diuretics (such as thiazides), by lowering plasma potassium levels, may also result in reduced aldosterone secretion. Dihydropyridine CCBs stimulate renin (Brown and Hopper, 1999; Mulatero *et al.*, 2002), probably through reflex sympathetic stimulation induced by falling blood pressure and natriuretic effects, and can reduce aldosterone production by interfering with intracellular, calcium-dependent steps in biosynthesis (Anderson *et al.*, 1986). ACE inhibitors (Mulatero *et al.*, 2002) and ARBs (Mulatero *et al.*, 2002) reduce negative feedback of AngII on renin production and may inhibit aldosterone production in patients with AngII-R forms of PA (Atkinson *et al.*, 1980).

Antidepressants are frequently prescribed agents, including among patients with hypertension. Administration of the selective serotonin uptake inhibitors (SSRIs) sertraline and escitalopram was recently reported to be associated with rises in aldosterone, PRA, and DRC and, because renin rose more than aldosterone, falls in the ARR (Ahmed *et al.*, 2011a), raising the potential for SSRIs to be associated with false-negative ARRs in patients with PA.

Renin inhibitors

Renin inhibitors have complex effects on renin levels, which depend on how renin is measured (Campbell *et al.*, 2009), being likely to raise the ARR (and cause false positives) if renin is measured as PRA, and lower it (causing false negatives) if measured as DRC (**Table 2**).

Presence of impaired renal function

Renin levels tend to fall in patients with chronic kidney disease (CKD), presumably because of both reduced renin secretory mass and salt and water retention. Furthermore, potassium retention due to CKD may lead to hyperkalemia and thereby elevate aldosterone. These influences on renin and aldosterone may result in false-positive ratios (McKenna *et al.*, 1991).

Effects of coexisting and other hypertensive conditions

Coexistence (with PA) of conditions in which previously suppressed renin is released from suppression, such as pregnancy (Gordon and Tunny, 1982), malignant hypertension (Beevers *et al.*, 1976; Kaplan, 1963; Murphy *et al.*, 1985), and renal artery stenosis (Stowasser *et al.*, 1993), may cause ARRs to become falsely negative.

False-positive ratios occur in the syndrome of familial hyperkalemia and hypertension, in which a primary defect in distal renal tubular function appears to result in upregulation of the sodium chloride cotransporter, leading to excessive resorption not only of sodium (leading to hypertension and renin suppression) but also of potassium (causing chronic hyperkalemia and thereby counteracting suppression of aldosterone) (Gordon *et al.*, 1970).

Aldosterone and Renin Assays

Accurate measurement of both aldosterone and renin is required for reliable ARR results, making highly reproducible assays essential. Reliable aldosterone quantification is also critical during subsequent confirmatory testing (to definitively confirm or exclude PA) and adrenal venous sampling (used to differentiate unilateral forms, potentially curable by unilateral adrenalectomy, from bilateral forms which are usually managed medically with agents that block aldosterone action) (Gordon, 1995; Stowasser *et al.*, 2001).

Concerns have been raised about currently available immunoassay methodology (Schirpenbach *et al.*, 2006; Stowasser and Gordon, 2006) and have led to development of high-throughput mass spectrometric methods of measuring aldosterone which demonstrate excellent reliability and reproducibility (Taylor *et al.*, 2009). As the required methodology becomes more price-competitive and widely available, it is likely that the use of this approach will gradually move from highly specialized to more general clinical service laboratories, with the potential to improve diagnostic accuracy and optimize clinical outcomes.

Faster, more convenient methods of measuring DRC (Ferrari *et al.*, 2004; Gordon, 2004) using immunometric techniques and automated machinery have rapidly been adopted in large, busy laboratories in recent years and have evolved to include measurement of aldosterone in the same sample (Burrello *et al.*, 2016), but uncertainty exists about their reliability (Gordon, 2004), particularly at the lower end of the reference range. Efforts are underway to develop and validate high-throughput assays of angiotensin I (PRA) and AngII using mass spectrometry and results are awaited with great interest.

An Approach to the Measurement of the Aldosterone/Renin Ratio

A. Prior to ARR measurement
   1. Correct hypokalemia (if present) with oral slow-release potassium tablets. To determine if hypokalemia is present, measure plasma potassium in blood collected slowly using a syringe and needle, avoiding fist clenching during collection, waiting at least 10 s after torniquet released (if used to achieve needle insertion), and ensuring separation of plasma from cells within 30 min of collection.
   2. Encourage a liberal (rather than restricted) dietary sodium intake.
   3. Where possible, withdraw medications which are known to significantly affect the ARR:
      a. Beta-blockers, clonidine, methyldopa, nonsteroidal antiinflammatory drugs (cause false positives)—for at least 2 weeks
      b. ACE inhibitors, ARBs, dihydropyridine calcium blockers (cause false negatives)—for at least 2 weeks
      c. Diuretics including spironolactone, eplerenone, and amiloride (cause false negatives)—for at least 4 weeks

      N.B. Ceasing medications in nonhospitalized patients to attain washout is not without risk and sometimes potentially hazardous. Although complete cessation of interfering anithypertensives can be achieved safely in mildly hypertensive patients who are seen frequently, it is more often necessary to commence in their place a relatively renin-neutral drug alone or in combination (see (A) (4)).
   4. Where necessary, commence other antihypertensive medications which have lesser effects on the ARR (verapamil slow-release ± hydralazine, prazosin or doxazosin, moxonidine; Ahmed *et al.*, 2017b) in order to maintain hypertension control.
   5. Estrogen-containing oral contraceptive agents may cause false-positive ARRs when DRC (rather than PRA) is measured. Do not withdraw unless confident of alternative, effective contraception.

B. Blood collection
   1. Midmorning, after patient ambulant for at least 2 h, seated for 5–15 min
   2. Careful collection of blood sample avoiding stasis and hemolysis [see (A) (1)]

C. Factors to consider when interpreting results
   1. Age—in patients over 65 years, renin can be lowered more than aldosterone, leading to a raised ARR
   2. Gender (ARR higher in females) and phase of menstrual cycle (false positives may occur during luteal phase, but only if calculated using DRC rather than PRA)
   3. Time of day, posture, and length of time in that posture, recent dietary sodium intake
   4. All medications—in cases where a potentially interfering medication cannot be withdrawn, useful information can still be obtained by taking into account its known effects. For example, a raised ratio in patients receiving a diuretic, ACE inhibitor, ARB, dihydropyridine calcium blocker, or SSRI antidepressant would make PA very likely, whereas a normal ARR in the presence of beta-blocker treatment or an estrogen-containing oral contraceptive agent or hormone replacement therapy would make the diagnosis very unlikely.
   5. Method of blood collection including any difficulty
   6. Potassium level
   7. Level of creatinine (false-positive ARR can occur in CKD).

Because of the innate variability of both aldosterone and renin, and the implications for further management (including more invasive diagnostic workup) if the ARR is deemed to be positive, it is important that management decisions not be based on a single ratio. The ratio should be repeated as required until confident that the ARR is raised or otherwise, meanwhile adjusting medications and conditions of collection if indicated.

See also: Adrenal Venous Sampling for Primary Aldosteronism. Aldosterone-Producing Adenomas; Genetics. Genetics of Familial Hyperaldosteronism. Mineralocorticoid Excess Syndromes. Primary Aldosteronism; Diagnosis and Treatment

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Primary Aldosteronism; Diagnosis and Treatment

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Introduction

Primary aldosteronism (PA) is a common form of endocrine hypertension in which aldosterone production is inappropriate and at least partially autonomous of the renin–angiotensin system. The inappropriate production of aldosterone results in sodium retention and suppression of the secretion of renin. PA is commonly caused by an adrenal adenoma (APA) or bilateral hyperplasia (IHA) of the adrenal zona glomerulosa, less common forms include: unilateral adrenal hyperplasia, aldosterone-producing carcinoma, ectopic aldosterone-producing tumors and the familiar forms of hyperaldosteronism (Type I-II-III-IV).

Epidemiology

PA was previously believed to account for $<1\%$ of hypertensive patients. In addition, hypokalemia was considered a prerequisite for pursuing diagnostic tests for PA. However, several studies applying the plasma aldosterone(PAC)/plasma renin activity (PRA) ratio (ARR) as a screening test among both hypokalemic and normokalemic hypertensives have reported a much higher prevalence of this disease, with PA accounting for up to 12% of hypertensive patients of whom only a minority of patients with PA (9%–37%) had hypokalemia (Mulatero *et al.*, 2004). Thus, normokalemic hypertension constitutes the most common presentation of the disease, with hypokalemia probably present in only the more severe cases (Rossi *et al.*, 2006).

Therefore, PA could be the most common identifiable, specifically treatable and potentially curable form of hypertension. It has been suggested that it is only worthwhile to perform diagnostic workup for PA in hypokalemic patients in the belief that the great majority of patients with normokalemic PA have bilateral adrenal hyperplasia (BAH) rather than aldosterone-producing adenoma (APA) and are therefore rarely surgically curable (Young, 2007) but even in APA around 50% of patients was found to be normokalemic (Rossi *et al.*, 2006; Käyser *et al.*, 2016).

Pathophysiology

Elevated plasma aldosterone levels promote excessive preservation of sodium, by increasing the number of open sodium channels in the cell's luminal membrane of the cortical collecting tubules at the expense of hydrogen and potassium loss in the lumen. Sodium retention promotes water retention and an expansion in extracellular volume, resulting in hypertension and suppression of plasma renin, but not in oedema due to the so-called aldosterone escape. This phenomenon, not yet completely understood, is due in part to the increase of atrial natriuretic peptide (ANP), changes in the Na-Cl cotransporter in the distal tubule and to pressure_natriuresis and leads to a new steady state, in which the extracellular fluid volume is increased but stable (Pedrinelli *et al.*, 1988).

Hypokalemia can be absent or present in different grades, sometimes with metabolic alkalosis, mostly depending on the degree and duration of aldosterone excess. Also for plasma potassium concentration there is a mechanism of stabilization at lower level unless some other factor is added, such as the use of diuretic therapy.

Increased levels of aldosterone have also deleterious effects on the cardiovascular system, partly independent on its effect on BP and potassium, including left ventricular hypertrophy, myocardial fibrosis, increased carotid intima-media thickness and endothelial dysfunction (Savard *et al.*, 2013).

Genetics of Primary Aldosteronism

Somatic mutations of APA have been described in the last few years in around 50% of the tumors, constitutively altering the function of potassium and calcium channels and ion pumps. This topic is specifically discussed in an other chapter of the Encyclopedia.

Familiar forms of primary aldosteronism (FH) are also known: FH type I (glucocorticoid-remediable aldosteronism (GRA), Type II (familiar occurrence of APA or IHA, due germline mutation of CLCN2 chloride channel gene), Type III (germline mutations in the KCNJ5 potassium channel), FH IV (germline mutations in CACNA1H gene) (Williams *et al.*, 2016; Zennaro *et al.*, 2018).

This topic is extensively covered in an other chapter of this Encyclopedia.

Diagnosis

Primary aldosteronism has made it into the headlines over the last decade mainly due to the notion that it might be more frequent than previously assumed. Thus previous diagnosis rates would not reflect the entirety of a very rare disease but perhaps only the "tip of iceberg," with many others also affected, but currently undiagnosed. With this perceived paradigm change came a new attitude towards screening for primary aldosteronism (Funder *et al.*, 2008). While we previously aimed to establish this diagnosis mainly in patients presenting with hypokalaemic hypertension, more and more physicians took to actively screening their hypertensive patients for the presence of primary hyperaldosteronism.

The Endocrine Society USA published clinical practice guidelines for the diagnosis and treatment of patients with primary hyperaldosteronism in 2008 (Funder *et al.*, 2008) and a more updated version in 2016 (Funder *et al.*, 2016). Systematic reviews of available evidence were used to formulate the key treatment and prevention recommendations. Those guidelines are the most used consensus criteria for the management, diagnosis and treatment of primary aldosteronism. However, there remains a number of unresolved issues, which unfortunately require more of a detour guide and cannot be easily addressed by a straight forward guideline.

Clinical Aspects

Clinical features might be more severe in patients with APA than in the other forms of PA. Moreover, patients with APA are usually younger than those with IHA, with age at presentation typically being <50 years.

Hypertension is often substantially elevated, but PA may be present also in Stage I and II, and occasionally in normotensives. On the other end, PA is relatively more frequent in patients with resistant hypertension (Funder *et al.*, 2016).

Hypokalemia is present in about half of patients with APA and less frequently in patients with IHA. When severe, symptoms or signs related to metabolic alkalosis, such as tetany or positivity of Chvostek or Troussea sign, symptoms or signs related to myopathy, such as weakness, or to nephrogenic diabetes insipidus, such as polyuria and nocturia, can be present.

In addition, cardiovascular/renal complications such as atrial fibrillation, myocardial infarction, stroke and chronic kidney disease may occur with relative more frequency than in age-BP matched essential hypertensives.

Case Detection

The guidelines recommend case detection of primary aldosteronism "in higher risk groups of hypertensive patients and those with hypokalemia by determining the aldosterone-renin ratio under standard conditions."

The guidelines panel clearly defined the patients perceived at higher risk of hyperaldosteronism and this is important to avoid mass screening for primary hyperaldosteronism in all hypertensive patients.

However, if one looks carefully to this indications it becomes evident that quite a large number of patients are expected to be screened, and this may have some impact on the cost/benefit ratio.

They recommend to determine "the aldosterone-renin ratio under standard conditions," but those are difficult to obtain (Funder *et al.*, 2016; Stowasser and Gordon, 2004). The table reported in the guidelines outlines only "a suggested approach" to measurement of the ARR. The major problem is that patients who are screened for primary aldosteronism may suffer from moderate to severe hypertension and may need to be on antihypertensive drugs which could interfere both with aldosterone and renin (Funder *et al.*, 2016; Mulatero *et al.*, 2002). The guidelines recommend to correct hypokalemia and to remove for at least 2 weeks those drugs which could cause false-positive or false-negative ARR. This comes with significant costs as this approach will require a major monitoring effort in a severely hypertensive patients, and a certain degree of risk. In such case, indeed the switch to a few drugs with minimal effects on ARR is recommended (verapamil, hydralazine, doxazosin, terazosin).

There remains uncertainty, however, about the effects of some of these agents on the ARR. For example, although Mulatero *et al.* (2002) found β-blockers to lead to an elevation in the ARR in some patients with PA, Young (2007) has argued that β-adrenoceptor blockers, despite inducing renin suppression, are not likely to lead to false-positive ARR values because aldosterone levels fall in parallel. Further evaluation of the effects of these drugs in hypertensive patients without PA is required to settle this issue (Gallay *et al.*, 2001).

Moreover, the effects of many other commonly used medications, such as oral contraceptive agents and antidepressants, have received little attention. Because estrogen-containing preparations induce angiotensinogen production by the liver, the resultant rise in angiotensin II levels chronically inhibits renal renin secretion by a negative feedback mechanism. Consequently, plasma renin concentration, measured as "direct active renin" (DRC), would be expected to fall in patients taking estrogen preparations, and there is therefore the potential that the ARR could become elevated if renin is measured in this way. Whether this could be avoided by using plasma renin activity (PRA; which normally shows little change and if anything a rise rather than a fall) remains uncertain as well as whether the phase of the menstrual cycle needs to be taken into account when considering ARR testing.

Furthermore the newly introduced renin inhibitors have complex effects on renin levels, which depend critically on how renin is measured. Put in simplistic terms, these agents are likely to raise the ARR (and cause false-positives) if renin is measured as PRA and to lower it (causing false-negatives) if measured as DRC.

Once measured the ARR the diagnosis is not confirmed. A substantial problem, not really clarified by the guidelines, is to define a corrected cut-off level for the ARR. In the guidelines there is a "substantial variability in cutoff values" for the ARR, and not all researchers agree. Lack of uniformity in diagnostic protocols and assay methods for ARR measurement has been associated with substantial variability in cutoff values used by different groups. Most groups, however, use cutoffs of 20–40 when using PRA as ng/mL/h and aldosterone as ng/dL, when testing is performed in the morning on a seated ambulatory patient.

Certainly it should be accept that the likelihood of a false-positive ARR becomes greater when renin levels are very low. A related issue are the assays that actually where used to measure aldosterone and specifically renin. Most centers currently measure plasma renin activity, but the measurement of direct renin concentrations (DRC), which has the potential to be more accurate and less cumbersome, is becoming more popular, both with regard to accessibility and methodology. On the other side, given the variability in immunometric assays for plasma aldosterone, the move to tandem mass spectrometry for its measurement is almost mandatory.

Case Confirmation

Although The Endocrine Society guidelines were clear in their recommendation that confirmatory testing be performed to definitively confirm or exclude PAL in patients with elevated ARR results, the choice of test remains a matter of debate (Giacchetti *et al.*, 2006). The guidelines enumerate four confirmatory test to definitively confirm or exclude the diagnosis including: oral sodium loading, saline infusion (SIT), fludrocortisone suppression (FST), and captopril challenge (CCT). These four tests are in common use, and there is currently insufficient direct evidence to recommend one over the others. Rossi *et al.* (2007) found SIT and CCT to have similar, but only moderate, accuracy for confirming PAL. By contrast, Mulatero *et al.* (2007) found CCT to be misleading in four of the 11 patients in whom PAL had been confirmed (in six) and excluded (in five) by both FST and SIT. Proponents of the fludrocortisone suppression testing argue that it is the most sensitive approach, being more "physiological" than SIT, and it avoids the problems associated with 24-h urinary aldosterone estimation required for the oral salt loading test used at several major institutions. However, it is also the most labor-intensive and requires admission to hospital for several days, making it the least practical.

Although it is acknowledged that these tests may differ in terms of sensitivity, specificity, and reliability, the choice of confirmatory test is commonly determined by considerations of cost, patient compliance, laboratory routine, and local expertise. The search for the ideal confirmatory test is still on. Indeed, two additional confirmatory tests have been recently suggested. The first is the dexamethasone-enhanced FST, whereby roughly 30% of referred hypertensives show PAC values above the range found in normotensive controls (Funder *et al.*, 2016). The second is a seated variance of the SIT (usually performed with the patient recumbent) which has been sown to have a remarkable agreement with the reliable but less practicable FST (Ahmed *et al.*, 2014).

Another matter of debate is whether it is necessary to perform a confirmatory test really in al patient. For example a young patient with severe hypokalemic hypertension, unequivocal ARR and an unilateral adrenal nodule >1 cm can go straight to surgery as the likelihood that his nodule represents an endocrine inactive adenoma is extremely low. However, we certainly have to take a different view when looking at older patients as the likelihood of a nonfunctioning adrenal adenoma is 3% in 40-year-olds and even 10% in 70-year-olds (Mansmann *et al.*, 2004).

Subtype Classification

As mentioned by the guideline panel also all other published dates agreed that all patients with primary aldosteronism should undergo adrenal computed tomography (CT) as the initial study in subtype testing and to exclude large masses that may represent adrenocortical carcinoma.

However adrenal CT has several limitations. Small APAs may be interpreted incorrectly by the radiologist as IHA on the basis of CT findings of bilateral nodularity or normal-appearing adrenals. Moreover, apparent adrenal microadenomas may actually represent areas of hyperplasia, and unilateral adrenalectomy would be inappropriate. In addition, nonfunctioning unilateral adrenal macroadenomas are not uncommon, especially in older patients (>40 years) and are indistinguishable from APAs on CT. Unilateral UAH (unilateral adrenal hyperplasia) may be visible on CT, or the UAH adrenal may appear normal on CT.

In several studies, in which patients with PAL were evaluated with both CT and adrenal venous sampling (AVS) CT resulted to be accurate in only about 50% of patients. Thus about ¼ of the patients would have been incorrectly excluded from surgery and an other ¼ might have had inappropriate surgery (Rossi *et al.*, 2008a; Mansmann *et al.*, 2004; Rossi *et al.*, 2001; Young *et al.*, 2004).

NMR has no clearcut advantage over CT, except for less irradiation in case of prolonged follow-up.

However CT (or NMR) may be useful in detecting larger tumors with malignant potential, as well as in showing even small tumors (but at least >1 cm) in young patients with marked PAL, thus possibly avoiding the need of AVS in such cases, as well for localizing adrenal veins in order to aid the cannulation during AVS.

In most of the other cases, AVS is essential to direct appropriate therapy in patients with PAL who seek a potential surgical cure.

The guidelines in fact made firm recommendations about the need for AVS for confident differentiation of unilateral from bilateral forms of PA. This insistence on the currently existing "gold standard" approach was based on well-documented limitations in reliability associated with computed tomography (Young *et al.*, 2004; Kempers *et al.*, 2009) and other approaches, but it recognized the need for an experienced, dedicated radiologist to ensure optimal cannulation success rates. A new innovation

designed to improve rates of cannulation success has been the real-time estimation of adrenal and peripheral venous cortisol levels by rapid assay techniques, permitting calculation of adrenal/peripheral venous cortisol gradients (upon which success or otherwise is based) at the time of the procedure (Mengozzi *et al.*, 2007). Debate exists as to the value of ACTH stimulation, which has been used by several groups to maximize adrenal/peripheral venous cortisol gradients, reduce fluctuations in steroid secretion resulting from changes in endogenous ACTH levels during nonsequential AVS, and stimulate aldosterone production by APAs and thus avoid sampling during a relatively quiescent period of secretion (El Ghorayeb *et al.*, 2016; Young *et al.*, 2004). Rossi *et al.* (2008b), however, in a recently reported study examining the effects of different currently used ACTH stimulation protocols, found that the higher doses resulted in a higher proportion of samples that would be regarded as "successful," but they also had the potential to result in incorrect lateralization of aldosterone overproduction. Further sources of debate that remain to be addressed and clarified include the lack of standardization of criteria used for defining success of cannulation (with cortisol gradient cutoffs varying from 1.1 unstimulated to over 5.0 with ACTH stimulation) and for defining lateralization (criteria that variably compare aldosterone/cortisol ratios between side and peripheral, or between one side and other side, with a range of cutoff ratios) (Rossi *et al.*, 2014; Young and Stanson, 2009).

Maybe it should be made careful use of AVS in patients who are suitable and willing to undergo surgery and after stratifying by age. Some form of patient stratification is required, possibly firstly identifying which patients should proceed to surgery set against those who can be managed on effective medical therapy with mineralocorticoid receptor antagonists. The use of AVS must be justified on a case-by-case basis, asking how it will improve patient care and outcome, and be undertaken in centers of excellence to achieve optimal sensitivity.

For a detailed discussion on the role of AVS in making the distinction between unilateral and bilateral adrenal disease please refer to the specific chapter on this Encyclopedia.

Other less reliable tools to distinguish between APA and IHA (Funder *et al.*, 2016; Kempers *et al.*, 2009; Phillips *et al.*, 2000):

- The postural response of plasma aldosterone, which is almost always present in IHA and may be absent in APA (except in the so-called Angiotensin II sensitive adenoma).
- The levels of 18 OH corticosterone,18 OH cortisol and 18 Oxo cortisol, which are significantly higher in patients with APA.
- The scintigraphy with radiolabeled iodocholesterol or nor cholesterol (controllare) under dex suppression.
- The C11 Metomidate PET-CT. This is a promising imaging technique, especially if more specific tracers for aldosterone synthase will be available in the next future.

Treatment

Unilateral adrenalectomy is the treatment of choice in patients with APA (or in more general term in patients who lateralize on AVS), resulting in normalization of the serum potassium in 100% of those who were hypokalemic preoperatively, and cure of hypertension in about 50% of the cases and significant improvement in most of the remainder, allowing a reduction of antihypertensive medications. Laparoscopic adrenalectomy has supplanted open adrenalectomy, due to lower intra- and postoperative risks and faster recovery. Partial adrenalectomy (removing of the adenoma leaving the remaining adrenal intact) is not indicated because of the frequent presence of clusters of aldosterone-producing cells within the apparently normal adjacent adrenal (Funder *et al.*, 2016; Williams *et al.*, 2017; Duncan *et al.*, 2000).

If surgery is declined or the patient is not an appropriate candidate for surgery due to coexisting medical conditions, long-term medical therapy with mineralocorticoid receptor antagonists (MRA) can be successfully undertaken.

In patients undergoing surgery, if already treated with MRA and/or K supplements, the treatment should be discontinued and other antihypertensive agents should be decrease. In addition to BP, also serum K and creatinine should also be carefully monitored, since transient hyperkalemia and increased creatinine may occur, especially in patients with marked preexisting renal damage (Chiang *et al.*, 2013).

Patients with IHA (or demonstrating bilateral aldosterone production on AVS) should be treated chronically with mineralocorticoid receptor antagonists (MRA: spironolactone or canrenone/potassium canrenoate). Relatively low doses (25–50 mg/daily) can significantly reduce blood pressure and normalize serum potassium, if hypokalemia preexists, although the effect may not be immediate (Funder *et al.*, 2016).

At these dosages side-effects (gynecomastia, menstrual irregularities, and reduced libido) are infrequent, but may be still present. In that case, eplerenone, a more selective antagonist with less affinity for sex steroid receptors, can be used, but an higher dosage and a t.i.d. schedule may be needed to obtain a full effect (Parthasarathy *et al.*, 2011). As an alternative, the sodium channel blocking agent amiloride (5–10 mg/daily) may also be administered (Griffing *et al.*, 1982). In a number of cases, however, the addition of conventional antihypertensive agents such as Ca-channel blockers, alpha-antagonists or even ACE inhibitors/ARBs or thiazide diuretics might be necessary to fully control blood pressure.

In the genetic form FH-I hypertension should first tentatively treated with low dose dexamethasone but spironolactone/amiloride may be needed in combination or substitution in case of Cushingoid side effects.

Finally, it should be stressed that the purpose of treatment of PA is not only aimed at controlling hypertension and *K* values but also to reduce the excess cardiovascular risks and renal toxicity which are at least in part due to a direct effect of aldosterone. Several studies have shown that adrenalectomy and medical therapy with MRA are both very effective not only in reducing BP but

also in decreasing the occurrence of cardiovascular events. However, at short term the surgical approach seems to have a more rapid effect in reducing the LVH, and at long term its efficacy in reducing the incidence of atrial fibrillation appears also to be greater (Catena *et al.*, 2008; Sechi *et al.*, 2006; Rossi *et al.*, 2013; Mulatero *et al.*, 2013; Marzano *et al.*, 2015).

See also: Adrenal Venous Sampling for Primary Aldosteronism. Aldosterone-Producing Adenomas; Genetics. Genetics of Familial Hyperaldosteronism. Mineralocorticoid Excess Syndromes. Primary Aldosteronism; Epidemiology and Screening

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Adrenal Venous Sampling for Primary Aldosteronism

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Abbreviations

APA	Aldosterone-producing adenoma
ARB	Angiotensin II type 1 receptors blockers
AVIS	Adrenal Vein Sampling International Study
AVS	Adrenal vein sampling
CT	Computed tomography
IHA	Idiopathic hyperaldosteronism, bilateral idiopathic hyperplasia
IVC	Inferior vena cava
MR	Mineralocorticoid receptor
PA	Primary aldosteronism
PAC	Plasma aldosterone concentration
PCC	Plasma cortisol concentration

Introduction

Compelling evidence indicates that primary aldosteronism (PA) constitutes the most common endocrine form of hypertension: it is highly prevalent among patients with drug-resistant hypertension (Douma *et al.*, 2008), and may be diagnosed in more than 11% of the patients referred to the specialized hypertension centers (Rossi *et al.*, 2006a). Albeit usually unrecognized, because it is not searched for, PA is also quite common, in the unselected hypertensive patient population seen by general practitioners (Olivieri *et al.*, 2004). As PA carries an increased risk of damage to target organs of high blood pressure with ensuing cardio-renal complications (Milliez *et al.*, 2005; Rossi *et al.*, 2013), early identification of affected patients followed by an early institution of specific treatment are key measures for prevention of cardiovascular events and reversal of cardiovascular damage (Rossi *et al.*, 2013; Reincke *et al.*, 2012).

Most PA patients have either bilateral idiopathic hyperplasia (IHA), which is treated with life-long mineralocorticoid receptor (MR) blockade (with spironolactone, canrenone, potassium canrenoate, or eplerenone), or a unilateral aldosterone-producing adenoma (APA), which is optimally treated with unilateral adrenalectomy (Rossi *et al.*, 2013). Hence, the distinction between the cases of PA due to IHA and those due to an APA is essential for selecting the most appropriate treatment (Funder *et al.*, 2008; Rossi, 2011a). To this aim the current clinical practice guidelines advocate use of adrenal vein sampling (AVS) (Funder *et al.*, 2008; Nishikawa *et al.*, 2011), a procedure that was introduced for the first time by Masoni in 1957 (Masoni, 1957). Theoretically the concept behind this test is very simple: to demonstrate that the excessive aldosterone production is caused by one adrenal and not the other by measuring plasma aldosterone concentration (PAC) and plasma cortisol concentration (PCC) in adrenal vein blood. However, as the procedure is technically demanding and many factors can confound the interpretation of results, it was soon realized that this is not a simple undertaking and that erroneous conclusions may lead to remove the wrong adrenal or to undertake unnecessary adrenalectomy For example, besides a number of potential confounders unrelated to the blood sampling per se, large factitious gradients of aldosterone can be generated by the collection of blood closer or farther from the adrenal, by the pulsatile pattern of secretion of aldosterone, by sampling accessory hepatic veins on the right side or phrenic vein on the left side. Thus, even though AVS is a straightforward diagnostic test (Davidson *et al.*, 1975; Rossi, 2007), it remains markedly underutilized worldwide even at major referral centers and even in patients with an unambiguous indication to the procedure, as shown by a recent large survey, the Adrenal Vein Sampling International Study (AVIS) (Rossi *et al.*, 2012). This underutilization is likely due to multiple misconceptions, mostly based on anecdotal experiences and/or on retrospective observational studies (Rossi, 2007), that AVS is technically challenging, invasive, risky, and not always necessary, despite abundant evidence to the contrary (Rossi *et al.*, 2012). Furthermore, the lack of accepted standards for the performance of AVS and of established criteria for interpretation of its results creates additional hesitancy for appropriate use in many patients with PA. Therefore, notwithstanding the guidelines recommendations (Funder *et al.*, 2008; Nishikawa *et al.*, 2011), too many PA patients are denied curative adrenalectomy, or undergo removal of a functionally normal adrenal gland because of the lack of demonstration of lateralized aldosterone excess (Funder *et al.*, 2008; Rossi *et al.*, 2012; Magill *et al.*, 2001).

Recently a panel of internationally recognized experts, which was assembled with the aim of providing updated practical suggestions on how to select the patients for AVS, how to perform the procedure, and on how to interpret its results, published a consensus statement. The publication of this document was followed by several studies, which have provided a good deal of novel data that are useful to further refine multiple issues concerning the performance and interpretation of AVS for the diagnosis of surgically curable subtypes of PA. The purpose of this chapter is, therefore, to update the information on the clinical use of AVS moving from the aforementioned consensus statement (Rossi *et al.*, 2014), and on data published afterward.

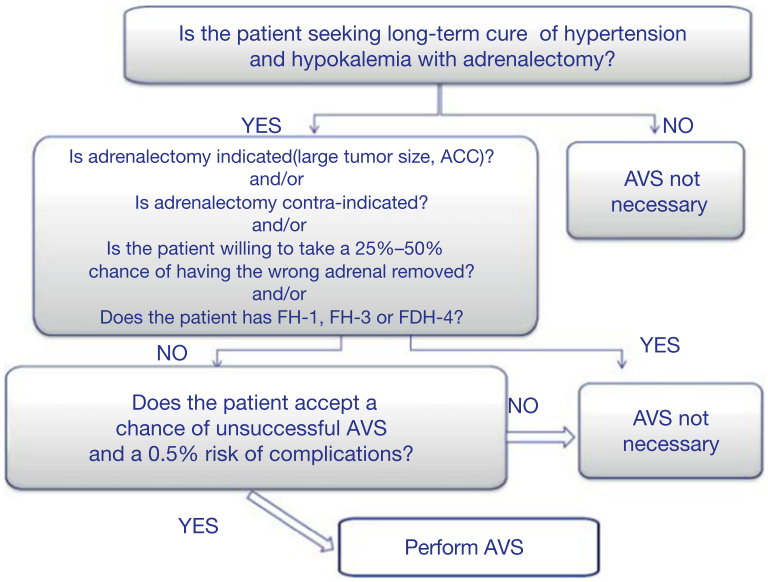

Fig. 1 The flow chart shows the algorithm to be used to select the patients for AVS.

Selection of Patients for Adrenal Venous Sampling

Appropriate selection of the patients is essential beforehand, because AVS is an invasive and expensive test (**Fig. 1**). To this end it is key to remember that an unequivocal biochemical diagnosis of PA must be made before considering AVS, because AVS is aimed at subtyping of PA and not at confirming this diagnosis. Multiple studies have in fact shown that the accuracy of imaging tests, adrenal computed tomography (CT), and magnetic resonance imaging (MRI) in localizing the source of aldosterone excess is poor as aldosterone-producing microadenoma and the majority of bilateral lesions are CT-and MR-undetectable (Kempers *et al.*, 2009). Therefore, in line with international experience that AVS is key to distinguish between unilateral and bilateral aldosterone excess both the U.S. Endocrine Society and the Japan Endocrine Society guidelines recommend AVS in all patients who have the diagnosis of PA and who want to pursue surgical cure (Funder *et al.*, 2008; Nishikawa *et al.*, 2011; Kempers *et al.*, 2009).

As a prerequisite for adrenalectomy, AVS is not indicated when either the patient prefers life-long medical treatment, or the physician considers the risks of surgery to outweigh the benefits—for example, because of the patient's age or general conditions contraindicate general anesthesia and/or surgery (**Fig. 1**). AVS is also not generally required in a patient with PA if surgery is already mandated by the size of the adenoma and/or other radiological features suspicious of adrenocortical carcinoma.

On the premise that nonfunctioning adrenocortical adenoma (so called "incidentaloma") is rare in young people, another subgroup in which AVS might not be needed can include patients <35 years of age with a florid PA—as evidenced by spontaneous hypokalemia, very high plasma aldosterone concentration (PAC), and very low active renin—and a clear-cut unilateral cortical adenoma with a seemingly normal contralateral adrenal gland on imaging (Kupers *et al.*, 2012). However, unilateral aldosterone excess from a small CT-undetectable APA in the adrenal gland contralateral to a CT-detectable adrenal mass cannot be identified without performing AVS, even in such patients. Moreover, bilateral aldosterone secretion, which occurs in those with IHA or familial hyperaldosteronism type I, III, and IV (FH-I, FH-III, FH-IV), and can coexist with incidentaloma, cannot be excluded without AVS. Nonetheless, as the patients with FH usually have bilateral aldosterone excess, AVS is generally not indicated if FH has been genetically proven.

Prediction of Outcome as a Guide for Selecting Patients

In municipalities where health resources are limited, a selection strategy is necessary, as both AVS and the surgical approach are simply not feasible for the multitude of PA patients (Funder, 2012). To this end it is worth remembering that several studies have documented preoperative characteristics associated with cure of hypertension following unilateral adrenalectomy. For example, surgical cure of hypertension has been associated with: young age (Harris *et al.*, 2003; Pang *et al.*, 2007), shorter duration of hypertension (e.g., <5–10 years); fewer antihypertensive medications (e.g., ≤ 2), higher preoperative blood pressure; preoperative normal renal function; body mass index ≤ 25 kg/m^2, female gender, lack of a family history of hypertension (Wang *et al.*, 2000; Sawka *et al.*, 2001), and no evidence of vascular remodeling (Rossi *et al.*, 2008a). These features should be considered when discussing with the patient realistic expectations of surgical outcomes and evaluating the risk benefit ratio. If selected based on AVS results nearly all patients with unilateral PA benefit from the surgical approach, as evidenced by biochemical cure of the aldosteronism and improved hypertension control even if hypertension is not cured (Wang *et al.*, 2000; Sawka *et al.*, 2001;

Zarnegar *et al.*, 2008) and by long-term regression of left ventricular hypertrophy (Rossi *et al.*, 2013). Realistically, however, in most public healthcare systems, priority should be given to young women, who are the most likely to be cured and gain the most in life-years off treatment, and to all the patients with resistant hypertension (or antihypertensive drug intolerance), whose absolute risk of CV complications is the highest.

Preparation of the Patient and Performance of AVS

Performing AVS requires an interdisciplinary team and exploitation of standard operational procedures, including preparation of the patient for the procedure and standardization of the conditions for its performance, as key steps to the success. The following recommendations are based on knowledge of the factors that influence technical success and accuracy and that might confound interpretation of its results.

When preparing the patient for AVS it is generally neither necessary nor advised to withdraw completely the antihypertensive treatment; however, careful adjustment of the antihypertensive agents before and during AVS is fundamental. Peripheral α_1-adrenergic receptor blockers (e.g., doxazosin mesylate, prazosin hydrochloride, and terazosin hydrochloride), and/or the long-acting dihydropyridine or nondihydropyridine calcium-channel blockers (verapamil) are recommended as these agents negligibly affect renin secretion (Rossi, 2011b). In stage 3 and/or drug-resistant hypertensive patients, successful AVS can be achieved notwithstanding the multiple agents that are necessary for minimizing risks of severe and/or uncontrolled blood pressure control. Thus, if angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics, and beta-adrenergic blockers need to be used, AVS can be performed in these patients as long as renin is suppressed. If the patient lateralizes the result is usually valid regardless of medications or serum potassium status; however, one should have to be concerned that the test might not be valid if AVS does not lateralize in patients treated with these agents. Thus, the AVS results should be interpreted with caution, because the feasibility of using these agents during AVS has not been proven, keeping in mind that when renin is not suppressed, aldosterone secretion can be stimulated in the nonculprit adrenal gland (see also later).

Consensus exists that in patients previously on MR antagonists or amiloride, these agents should be withdrawn (for MR antagonists at least 6 weeks, probably less for amiloride) before AVS, and this entails a small clinical risk in patients with resistant hypertension. MR antagonists should be avoided because they have the potential to allow a rise in renin secretion, which can stimulate aldosterone secretion from the unaffected side, thus minimizing the lateralization. Therefore, some centers use plasma renin measurement to decide whether to perform AVS without withdrawing, or downtitrating, the MR antagonists: the finding of low renin (e.g., a direct renin <10 mIU/L or a PRA <0.60 ng/mL/h) as evidence for unlikely stimulation of the contralateral adrenal cortex at a level sufficient to confuse interpretation of lateralization, and were able to use an independent measure of lateralization.

Some centers conduct AVS in outpatients, in which case time should be allowed for the patient to rest in the supine position for at least 1 h prior to AVS (Rossi, 2007; Daunt, 2005). We prefer to perform AVS with a 1-day hospital stay because complications can occur even after 12–24 h. AVS is best performed in the morning to avoid false-negative results, which can be due to diurnal fluctuation in ACTH having a more variable effect on many APAs than on the contralateral adrenal. Hypokalemia, if present, can blunt differences between sides because it decreases aldosterone secretion, and may potentially mask a unilateral aldosterone production. Thus, it should be corrected with oral or intravenous potassium supplements before AVS.

Catheterization of the left adrenal vein is achieved in almost 100% of the cases if the catheter's tip is positioned either at or distal to the orifice of the left inferior phrenic vein to avoid sample dilution from nonadrenal venous blood. In contrast, that of the right adrenal vein is much more difficult owing to its small size and its draining directly into the inferior vena cava at various angles or directly into a small accessory hepatic vein (Davidson *et al.*, 1975; Daunt, 2005). As prior knowledge of the right adrenal vein anatomy can facilitate catheterization in difficult cases (Miotto *et al.*, 2009), CT is important before AVS not only for detecting adrenocortical nodules but also for identifying the right adrenal vein and delineate its anatomy (in venous phase), including position and relationship to surrounding structures. This is also because in about 10% of the patients, the success rate of AVS achieving in selectivity can be affected by dilution from accessory vein blood flow. Prior identification on CT of the right adrenal vein draining into accessory hepatic vein in these cases allows selective cannulation with suitable catheters of the right adrenal vein, instead of the common trunk made by accessory hepatic vein and right adrenal vein or of small independent accessory hepatic veins instead of the right adrenal vein (Rossi, 2011b; Miotto *et al.*, 2009; Matsuura *et al.*, 2008).

Minimizing Stress During AVS

Emotional and pain-related stress, which activates the hypothalamic pituitary adrenal axis with ensuing ACTH-induced cortisol release from both adrenal glands, can confound interpretation of AVS results, as it might lower the PAC to PCC ratio, and thus obscure lateralization to the culprit adrenal. Stress was recently shown to affect the selectivity index (SI), and the ratio of PCC in an adrenal vein and in the infra-adrenal inferior vena cava. This study showed that a stress reaction: (i) occurs in most patients when starting AVS; (ii) waned rapidly, for example, over 15 min; (iii) increases the SI on both sides at the beginning of the procedure (Seccia *et al.*, 2012).

A more recent study simulating sequential AVS showed that this stress reaction influences also the LI values, particularly in patients who do not have APA, thus leading to a factitious diagnosis of lateralization (Rossitto *et al.*, 2017). Therefore, unless sequential catheterization can be performed within 5 min on both sides, which can be challenging in many cases, particularly if there are anatomical variations, bilaterally simultaneous catheterization should be preferred, and precautions to minimize stress should be systematically exploited (Rossi *et al.*, 2012; Seccia *et al.*, 2012).

To the latter goal, measures to be adopted both before and during AVS entail explanation of the procedure to the patient, reassurance by the doctor and nurses, and use of benzodiazepines and local anesthesia before venipuncture. Allowing the patient to rest quietly for at least 15 min before the blood sampling in a friendly environment with psychological assistance can also be useful but, at variance with this, the AVIS study showed that only one of the 20 participating centers used systematically some of these measures (Rossi *et al.*, 2012). Additional measures aimed at either overriding stress effects by maximally stimulating cortisol release from both adrenals, or minimizing stress effects on the PAC to PCC ratio by simultaneously sampling from both adrenal veins are discussed later.

In summary, available evidence indicates that a stress reaction can affect both the SI and LI, and therefore that stress minimization measures should be taken when starting AVS.

Pharmacologic Stimulation During AVS

Stimulation with a continuous infusion of synthetic ACTH (cosyntropin) (50 μg/h started 30 min before sampling) or a bolus (250 μg i.v.) during AVS was introduced in 1979 and is currently used at many centers (Weinberger *et al.*, 1979; Young *et al.*, 2004). There are three reasons for using this stimulation: (i) enhancing the PCC gradient between the adrenal vein and the inferior vena cava, and thus increasing the SI values and confidence of successful sampling; (ii) reducing stress-induced fluctuations in cortisol and aldosterone secretion during sequential AVS; (iii) increasing aldosterone secretion from APA (Weinberger *et al.*, 1979; Young *et al.*, 2004).

Because of the clear-cut increase of success in demonstrating bilateral selectivity, the administration of cosyntropin has become a standard procedure even despite no proof for increased success in ascertaining lateralization.

To assess the value of pharmacologic stimulation during AVS original papers that investigated patients with PA, before and after exposure to pharmacologic agents, were reviewed in terms of impact of cosyntropin on the selectivity index (SI), the lateralization index (LI, see later), and/or of the decision to perform adrenalectomy. Potentially relevant reports were identified that mostly used cosyntropin. These papers are briefly summarized below. Seccia *et al.* showed that high-dose (250 μg i.v. as a bolus) and intermediate-dose (50 μg/h) cosyntropin increased the percentage of patients with selective studies (using 1.10 as cutoff for SI), while the very low dose (250 pg i.v.) was totally ineffective. The increase of the SI occurred at the cost of a reduced accuracy of the LI, which pointed to the incorrect side in 3% and 13% with high-dose and intermediate-dose cosyntropin, respectively (Seccia *et al.*, 2009). Three studies have confirmed that cosyntropin administration, either as bolus or continuous infusion, increased the SI and thus the successful adrenal veins cannulation rate (Nakamura *et al.*, 2011). At the same time the LI was unaffected by either mode of cosyntropin administration using a high LI cutoff value. By no means these studies provided evidence that cosyntropin stimulation improves decision-making in PA, as there were no AVS-independent success criteria. Cosyntropin was also found to decrease the LI, albeit only in subsequently operated patients (Satoh *et al.*, 2007), or even to invert the LI, thus pointing to the wrong adrenal (Rossi *et al.*, 2008b), leading the authors to not recommend systematic cosyntropin stimulation during AVS. Another study on bilaterally simultaneous AVS with cosyntropin stimulation showed also an increase in bilaterally successful catheterization from 21% to 44%. However, in this study neither the effects of cosyntropin on LI nor the rationale for adrenalectomy could be assessed since most patients did not have a successful AVS.

At variance with all these findings and the theoretical expectations, Mathur *et al.* reported that the SI fell after cosyntropin on both sides, and that the LI values remained unchanged in the cases that lateralized on the right, but increased in the cases that lateralized to the left: overall the proportion of patients that could be defined as lateralized increased only slightly (from 95% to 98%). This study was unique in that it provided no evidence that cosyntropin stimulation improves the assessment of selectivity (Mathur *et al.*, 2010). In a more recent study of 47 patients, 85% of which had successful bilateral catheterization, Wolley *et al.* confirmed the highly significant increase of the SI with cosyntropin both on the right and the left sides; however, only 31 of 40 patients had the same diagnosis pre- and postcosyntropin. Moreover, among those with an inconclusive diagnosis precosyntropin only 50% were diagnosed as unilateral postcosyntropin and in 6% of all patients the diagnosis changed (Wolley *et al.*, 2016).

Two further studies using cosyntropin were either too small (Tanemoto *et al.*, 2009), or only reported the precosyntropin SI and LI values (Carr *et al.*, 2004), thus precluding any conclusions in favor or against use of this stimulation. Likewise, the studies with metoclopramide, a mixed DA_2 dopaminergic antagonist and serotoninergic agonist (Rossitto *et al.*, 2018) that increases aldosterone but not cortisol release, showed that this agent does not increase the PCC-based SI, and the LI to the culprit adrenal, but can be useful to unmask factitious contralateral suppression in non APA cases (Wu *et al.*, 2001).

In a more recent study we used the relative aldosterone secretion index (RASI) (**Table 1**), a way to assess aldosterone secretion by each adrenal gland, and also measured the melanocortin type 2 Receptor (MC2R) mRNA in APA and the normal adrenal cortex (Rossitto *et al.*, 2018). Besides conforming that cosyntropin increases the SI, we could provide key information for a better understanding of the effect of cosyntropin as we found that it lowers significantly the RASI, which explains the previously reported decrease, or even inversion of the LI. The fact that compared to cortisol cosyntropin stimulates less potently aldosterone, along

Table 1 Indexes currently used for the interpretation of AVS results

	Formula	*Meaning*
Selectivity index (SI)	$\frac{PCC_{side}}{PCC_{IVC}}$	Values above the cut-off confirm that the sample was obtained in the adrenal vein
Relative secretion index (RASI)	$\frac{(PAC_{side}/PCC_{side})}{(PAC_{IVC}/PCC_{IVC})}$	Estimates aldosterone secretion of each of adrenal gland relative to cortisol
Contralateral suppression index (CLSI)	$\frac{(PAC_{nondominant}/PCC_{nondominant})}{(PAC_{IVC}/PCC_{IVC})}$	Values below the cut-off (generally 1.00) indicate suppression of aldosterone secretion in the contralateral gland
Lateralization index (LI)	$\frac{(PAC_{dominant}/PCC_{dominant})}{(PAC_{nondominant}/PCC_{nondominant})}$	Values above the cut-off indicate lateralized aldosterone excess

PCC, plasma cortisol concentration; *PAC*, plasma aldosterone concentration. Dominant, side with higher PAC; Nondominant, contralateral side.

with the finding that the MC2R is less expressed in APA than in the normal adrenal cortex, provided a straightforward explanation for the fall of the LI and thus for the confounding effect of cosyntropin in identifying the culprit adrenal.

In summary, the bulk of the data indicate that cosyntropin increases the SI, thus facilitating the ascertainment of selective catheterization, which explains why in the AVIS study centers that systematically used cosyntropin were found to use higher cutoff values for the SI than centers that used baseline (unstimulated) values. However, because there was no conclusive evidence on whether use of cosyntropin stimulation is associated with improved or worsened outcome, defined as remission of hypertension and hypokalemia as endpoints, these major referral centers were almost equally split into those that use and those that do not use cosyntropin stimulation (Rossi *et al.*, 2012). In the absence of definitive data, the following suggestions are provided: (i) if cosyntropin stimulation is not used, then bilateral simultaneous AVS should be performed; (ii) if cosyntropin stimulation is used, then higher SI and LI values are indicated; (iii) cosyntropin could be used only at centers that show a low success rate in achieving successful bilateral catheterization and do not have access to measurement of androstenedione instead of PCC; (iv) in the APA patient with less florid biochemical phenotype cosyntropin likely invert lateralization to the nonculprit adrenal.

Bilaterally Simultaneous or Catheterizations Should Be Preferred

The AVIS study showed that almost two-thirds of the centers used the sequential technique with cosyntropin stimulation, while the rest used the bilaterally simultaneous technique with no stimulation (Rossi *et al.*, 2012). This finding could be anticipated since when cortisol secretion is maximally stimulated during cosyntropin infusion, the time difference between blood sampling from one side and the other is irrelevant, at least for the assessment of selectivity.

Given that secretion of aldosterone is pulsatile and highly variable there are chances of creating artificial gradients between the adrenals when using the sequential blood sampling technique, particularly if the interventionist is not proficient and fast enough. Undoubtedly bilaterally simultaneous AVS can avoid this (Rossi *et al.*, 2008b), and therefore if AVS is performed without cosyntropin stimulation the simultaneity of blood sampling is crucial and should be systematically used. The results of a recent study where bilaterally simultaneous AVS was systematically used at different time points, for example, at time -15, for example, when starting the procedure, and at time 0 for example, 15 min after, support this conclusion. Simulation of sequential AVS by combining the samples obtained on each side at the different time points was compared with the simultaneously obtained samples at time 0. The latter furnished a more accurate identification of lateralization than those obtained sequentially, which, moreover, produced a higher chance for creating factitious lateralization to the last sampled size, regardless of it being right or left. This bias was particularly evident in the cases without on APA, likely because of the waning of the waning of the aforementioned stress reaction (Rossitto *et al.*, 2017).

Assessment of Successful Catheterization

In the early years of AVS retrograde injection of contrast medium in the adrenal vein was used to confirm the success of catheterization and to visualize the abnormal vascular tree that can be a feature of an APA. This carries an increased risk of adrenal vein rupture, and should not be done (Rossi, 2007; Daunt, 2005), because with improved adrenal gland imaging by CT and MR, adrenal venography is no longer needed. Nevertheless, injection of a small amount of dye with a gentle pressure is still used to visualize the adrenal vein, and thus confirms the correct positioning of the catheter's tip inside the adrenal vein.

For the left side, the tip of the catheter should be placed distal to the orifice of the left inferior phrenic vein. For the right side, adrenal vein should be distinguished from accessory hepatic vein and when the right adrenal vein drains into accessory hepatic vein, the tip of the catheter should be confirmed to be located in the right adrenal vein instead of hepatic venous tributaries by injection of a single amount of contrast just before and after blood collection. The most popular technique to confirm the success of selective adrenal vein catheterization entails calculation of the ratio of concentrations of a hormone from an adrenal vein and the infra-adrenal inferior vena cava or a peripheral vein, defined as selectivity index (SI, **Table 1**) (Rossi *et al.*, 2008b), based on the theoretical premise that this hormone is exclusively made in the adrenal cortex and, is not overproduced in APA. Therefore, the finding of a concentration gradient between a blood sample in a vein draining the adrenal cortex and the IVC, or a peripheral vein,

is a proxy for the correct placement of the catheter's tip into the adrenal vein. To this aim the hormone most widely used is cortisol, because of its high rate of production and easy assay although attempts to use epinephrine (Freel *et al.*, 2010), metanephrine, and also chromogranin A have been made (Seccia *et al.*, 2012). While the use of SI might seem straightforward, and is being used to assess selectivity in most major international referral centers, the AVIS study showed that some of these centers still analyze their results using absolute hormonal values without prior assessment of the selectivity and correction for the degree of sample dilution, a practice that is no longer acceptable and should be abandoned.

In a recent study we used 17 α—OH progesterone and androstenedione, two steroids that have a higher step-up than cortisol between the adrenal vein and the peripheral blood to determine selectivity in a series of AVS samples judged to be nonselective by using PCC. The SI values calculated using 17 α—OH progesterone and androstenedione were on average 1.6-fold and 12-fold, respectively, higher than those based on PCC (Ceolotto *et al.*, 2017). Thus, this study provided conclusive evidence that these steroids, and particularly androstenedione, should be used instead of PCC.

Some general considerations need, however, to be made as there was a considerable variability in the SI cutoff values used (Rossi *et al.*, 2012): the cutoffs are lower at centers that perform AVS under unstimulated conditions than at those that use cosyntropin stimulation, which is not surprising given the considerations made above. It is altogether intuitive that the higher the SI cutoff values chosen to establish selectivity, the lower the proportion of AVS studies that could be defined as bilaterally selective and vice versa: too restrictive criteria lead to exclude a proportion of successful studies from diagnostic use, while, conversely, too permissive SI cutoffs could limit the diagnostic accuracy of AVS. Moreover, as the SI increases, so does the confidence of the interpretation. In some cases where unilateral aldosterone production is extremely high, a low SI can give the correct interpretation, but in cases when the production is modest it may lead to the wrong conclusion. As the need for a trade-off between too restrictive and too permissive cutoffs was clearly shown (Rossi *et al.*, 2008b), and then repeatedly confirmed (Seccia *et al.*, 2009; Mulatero *et al.*, 2008) these considerations need to be kept in mind rather than adhering strictly to a specific value. It has also to be considered that cutoffs depend on the accuracy (within-assay coefficient of variation, CV) with which hormones can be measured in that specific laboratory. For instance, considering PCC, if the CV is above or around 10%, low SI cutoffs, for example 1.1, are unusable, while if the assay CV is less than 10% a SI cutoff of 1.1 may be a reasonable choice (Rossi *et al.*, 2001, 2006b).

When using PCC for the SI calculation, use of an SI >2.0 for AVS performed under unstimulated conditions, and >3.0 during cosyntropin stimulation are reasonable options according to the AVIS study (Rossi *et al.*, 2012). However, the choice of the SI cutoff should be center-dependent and based on the accuracy with which the laboratory can measure PCC as mentioned above. The caveat to be made is, however, that while an unambiguous diagnosis of APA should be used as reference and guide in validating the findings and selecting the cutoffs, several studies were biased by a circular type of reasoning in that they used the AVS results to validate the diagnosis and justify the choice of their cutoff values (Seccia *et al.*, 2009; Rossi *et al.*, 2001).

In summary, use of absolute hormonal concentrations should be discouraged and AVS studies that are not bilaterally successful should not be used to establish lateralization as prior verification of bilateral selectivity is a prerequisite to the use of the data for diagnostic purposes (**Fig. 2**). Nonetheless, recognizing the fact that many studies are not bilaterally selective and/or can provide equivocal results, a following section has been devoted to the clinical decision-making in these difficult cases.

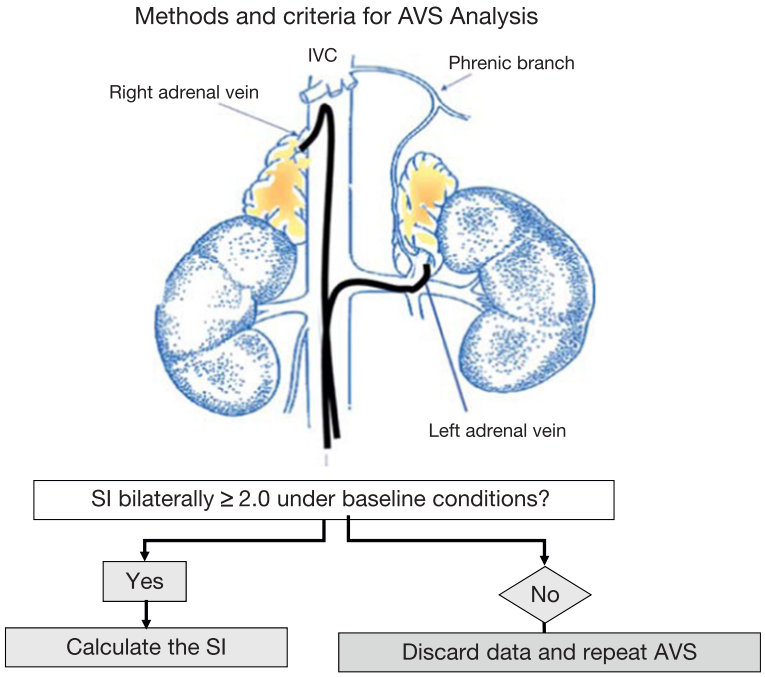

Fig. 2 Methods and interpretation of AVS: only bilaterally selective studies should be used for diagnostic purposes.

Intraprocedural PCC Assay

Given that the hormonal data are available after AVS, and therefore allow judging on the achieved selectivity only retrospectively, some centers have exploited use of extemporaneous, for example, during AVS, PCC measurement. This has the advantage of furnishing to the radiologist the immediate feedback on whether selective blood sampling from each adrenal vein was achieved and to permit further attempts of before removing the catheters, if selective catheterization was not initially obtained, thus avoiding the need for a further catheterization (Mengozzi *et al.*, 2007; Rossi *et al.*, 2011). Unfortunately, this approach that can improve the success rate, particularly during the radiologist's learning curve, is feasible only at centers where PCC can be measured rapidly, which implies a suitable logistic organization and a dedicated laboratory technician standing by Auchus *et al.* (2009) and Betz *et al.* (2011). Thus far, the usefulness of the rapid intraprocedural cortisol assay for increasing the success rate of AVS has been supported mainly by observational studies (Mengozzi *et al.*, 2007; Rossi *et al.*, 2011; Auchus *et al.*, 2009), and one randomized study carried out in Japan (Yoneda *et al.*, 2016), which, however, was performed in some centers that had scant or no experience in performing AVS where, therefore, the observed improvement in the success rate could be anticipated. The hypothesis that the rapid intraprocedural assay of cortisol, and androstenedione, can be useful will therefore be tested prospectively in the Intra-Procedural Cortisol Assay During Adrenal Vein Sampling: Rationale and Design of a Randomized Study I-Padua (Cesari *et al.*, 2017).

Interpretation of AVS and Assessment of Lateralization

The operational diagnostic index to assess the lateralization of aldosterone hypersecretion is the LI, which is calculated as the higher (dominant) over the lower (nondominant) adrenal vein PAC/PCC ratio (**Table 1**). PCC in adrenal venous blood is used for correction of the adrenal aldosterone levels because of inevitable dilution of the samples by nonadrenal blood, which occurs even if efforts are made to avoid blood dilution from accessory hepatic vein and inferior phrenic vein as discussed above. As mentioned above, PCC will probably be replaced by androstenedione for the assessment of selectivity. Therefore, as it is likely that as the LI will make use of this steroid in the future optimal cutoffs should be recalculated.

Ideally, evidence for optimal diagnostic accuracy of LI cutoffs should come from prospective studies in patients undergoing unilateral adrenalectomy regardless of the AVS results, and in which the different LI are thereafter linked to postsurgical cure of hyperaldosteronism rather than only high blood pressure, which is a composite phenotype. Unfortunately, no such prospective studies are available (Funder *et al.*, 2008); the only randomized study in this area performed with the aim to compare on AVS-based with a CT-based strategy for PA patients subtyping (Mengozzi *et al.*, 2007), unfortunately was totally underpowered to furnish any solid information and used a suboptimal AVS technique, for example, sequential blood sampling and cosyntropin stimulation (Funder and Rossi, 2016).

Observational data from the AVIS study showed that most referral centers use arbitrarily chosen LI cut-offs with values that ranged between 2.0 and 4.0 depending on cosyntropin stimulation, higher cutoffs being selected after cosyntropin stimulation (Rossi *et al.*, 2012). At present, an LI of 2.0 for unstimulated AVS and of 4.0 during cosyntropin stimulation are probably the best criteria to document lateralization of aldosterone excess, with the caveat that given the lack of prospective randomized studies, information is only based on observational studies, which show that outcome in terms of cure/improvement of blood pressure is similar for LI values varying between 2.0 and 5.0 (Young *et al.*, 2004; Mathur *et al.*, 2010; Ishidoya *et al.*, 2011; Nwariaku *et al.*, 2006). In fact, a recent study comparing 10 different LI criteria to determine lateralization (Mulatero *et al.*, 2010) found no significant differences in clinical outcome for all criteria: the most accurate criterion for correctly identifying lateralization was an LI >2.0 in combination with an SI >2.0 without cosyntropin, and >4.0 during cosyntropin stimulation.

It is worth considering that the choice of more restrictive (higher) cutoffs, with and without stimulation, undoubtedly leads to selection of a population with a higher chance of being cured with adrenalectomy. Precluding the chances of cure to some potentially curable patients, who have LI below these high cutoffs, is the obvious drawback of this.

To allow use of AVS studies that were not bilaterally selective, in which the LI cannot be calculated, attempts to use a contralateral aldosterone suppression index (PAC/PCC ratio of the nondominant adrenal vein less than the peripheral PAC/PCC ratio, Table 2) as an additional criterion to assess lateralization have been made (Wolley *et al.*, 2015), based on the theoretical premise that the secretion of aldosterone should be suppressed from the side contralateral to an APA. This interesting approach has, however, not been sufficiently validated thus far.

The following considerations need to be made: to assess the accuracy of AVS criteria the outcome after adrenalectomy, defined as normalization of both aldosterone and renin, which consistently occurs after adrenalectomy (Rossi *et al.*, 2013; Quillo *et al.*, 2011) (and not as fall of blood pressure, which may not occur if the patient has concomitant essential hypertension and/or vascular remodeling, (Funder, 2012), should be used. Instead only few studies have used this outcome as reference. Moreover, verification of postadrenalectomy outcome is obviously impossible in the patients who were not operated because they show no lateralization of aldosterone secretion at AVS (Rossi *et al.*, 2006a; Graham *et al.*, 2012). These caveats explain why the quality of evidence is low in this area.

Decision-Making in Equivocal AVS Results

In the AVIS study the most commonly used cutoff values for AVS performed with cosyntropin stimulation were SI of >3.0–5.0 for successful sampling, and an LI of >4.0 for lateralization, with no centers using LI cutoff <2.0 (Rossi *et al.*, 2012). For studies without cosyntropin, these cutoff values were generally lower by a value of 2, but the decision-making principles were the same. Consequently, AVS studies with cosyntropin are considered equivocal when the LI is 2.0–4.0, and the study is technically unsuccessful when one or both SI values are <3.0.

But Is This Conclusion Right? Should All of These Patients Be Managed Medically?

As the "true" result will never be known in the cases not submitted to unilateral adrenalectomy, accuracy of AVS is particularly hard to assess, for the reasons mentioned above and also because false negatives are rarely reinvestigated; the aldosterone/renin ratio falls when any adrenal is removed and, moreover, persisting hypertension can be blamed on many factors. Thus, only persistent hypokalemia with suppressed renin and high aldosterone after adrenalectomy are regarded as definitive evidence of incorrect lateralization. On the other hand, it has to be considered that because APAs are mostly benign, and many patients can be controlled medically, false negatives are held to be less serious than false positives, which led to suggest use of generally high diagnostic thresholds as appropriate. Ambiguous cases can often be resolved by repeating the AVS. If medical control of blood pressure and hypokalemia is poor, the benefit/risk from surgery is increased, and therefore the clinician should lower the threshold for recommending surgery.

Safety and Management of Complications

It is advised that AVS be performed in the specialized referral centers with sufficient throughput and expertise. However, the limited number of specialized centers that can perform for this technically demanding procedure results in missed opportunities for optimal surgical management (Rege *et al.*, 2016), training programs and certification of proficiency in performing AVS for radiologists should be implemented.

The most common and major complication of AVS includes adrenal vein rupture (Daunt, 2005), a complication that is only occasionally followed by complete and permanent adrenal insufficiency, but can be curative if occurring in the adrenal gland harboring the APA. Clinically, adrenal vein rupture is characterized by onset of persistent pain during or after catheterization, which increases in intensity and requires large doses of analgesics over 24–48 h (Daunt, 2005). Confirmation of the diagnosis, by CT and/or MRI, and careful monitoring of vital signs should be applied. The complications usually resolve with conservative treatment (Monticone *et al.*, 2016) and do not carry any sequelae, although they can render subsequent laparoscopic adrenalectomy more difficult due to extensive retroperitoneal adhesions.

Early studies suggested a wide range of complication rates varying between less than 0.2% and 13% (Daunt, 2005; Young *et al.*, 2004; Betz *et al.*, 2011; Vonend *et al.*, 2011). Compared to those higher rate of complications reported in the aforementioned AVIS study, the rate of adrenal vein rupture was 0.61, only at major referral centers (Rossi *et al.*, 2012). This decrease is likely attributable, at least in part, to avoiding routine adrenal venography and minimizing the injection volume for anatomical confirmation of the adrenal vein catheterization. Complications are more common in the right than left adrenal vein, mainly because of the anatomical diversity and complexity. While they do not seem to depend on the methods of catheterization, such as sequential or bilateral simultaneous, and the use of cosyntropin stimulation (Rossi *et al.*, 2012), it was found that they differ significantly even among major referral centers. Regression analysis demonstrated that adrenal vein rupture was inversely related to the number of AVS performed by each radiologist and the number of AVS performed per centers, thus clearly indicating that the complication rate depends on the expertise of the radiologist and the experience of each center (Rossi *et al.*, 2012).

Conclusions

AVS should serve as the "gold standard" diagnostic test for the subtyping of PA because of its high diagnostic accuracy and the very low rate of complications. With few exceptions, this procedure should be systematically used before referring a PA patient PA to the surgeon. Following the suggestions that are herein summarized will render AVS a rewarding diagnostic test both for the doctor and, more importantly, for the patient.

See also: Primary Aldosteronism; Diagnosis and Treatment

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Genetics of Familial Hyperaldosteronism

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Glossary

Next generation sequencing A DNA sequencing technology which performs sequencing of millions of small fragments of DNA in parallel.

Introduction

Primary aldosteronism (PA) is a group of disorders comprising both sporadic and familial forms and characterized by aldosterone overproduction by the adrenal glands, at least partially independent from its physiological regulators, angiotensin II, ACTH, and plasma K^+.

The recent introduction of affordable large scale methods of gene sequencing (e.g., next generation sequencing) has had a profound impact on mutation discovery in both sporadic and familial PA. Four forms of familial hyperaldosteronism (FH-I to FH-IV) (Monticone *et al.*, 2015a) and one form of genetic, but non familial, PA (Scholl *et al.*, 2015) (**Table 1**) have been reported and will be reviewed in detail.

Familial Hyperaldosteronism Type I

FH-I, also known as glucocorticoid remediable aldosteronism (GRA), was first described in 1966 in a father and son as a clinical syndrome characterized by hyperaldosteronism, which could be suppressed by the administration of dexamethasone (Sutherland *et al.*, 1966). Moreover, affected patients displayed high levels of the so-called "hybrid steroids" 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF) (Gomez-Sanchez *et al.* 1988; Ulick *et al.*, 1990), which suggested the hypothesis that FH-I could represent a disorder of the adrenal transitional zone, where it is likely that 17α-hydroxylase and aldosterone synthase coexists (Gomez-Sanchez *et al.*, 1988).

The molecular basis of FH-I remained unknown until 1992, when Lifton *et al.* identified the chimeric *CYP11B1/CYP11B2* gene as the genetic determinant of this disorder (Lifton *et al.*, 1992). *CYP11B1* and *CYP11B2* genes, encoding for 11β-hydroxylase and aldosterone synthase respectively, are located on 8q24 and share a 95% homology in the coding region. 11β-hydroxylase catalyzes the conversion of 11-doxycortisol to cortisol, while aldosterone synthase can catalyze three different reactions: 11β-hydroxylation of DOC to corticosterone (B) 18-hydroxylation of B to 18OHB, and, finally, 18-oxidation of 18OHB to aldosterone.

The chimeric *CYP11B1/CYP11B2* gene results from an asymmetrical crossing over between the two genes and comprises sequences derived from *CYP11B1* at the 5′ end (including ACTH regulatory sequences) and sequences from *CYP11B2* at the 3′ end (Pascoe *et al.*, 1992) (**Fig. 1**, panel A). The hybrid enzyme retains the 18-oxidase activity of CYP11B2 and can therefore produce aldosterone under ACTH control. In addition, it is expressed through the entire adrenal cortex (including the adrenal *zona fasciculata*, where 17α-hydroxylase is expressed) determining the abnormal production of the hybrid steroids (Pascoe and Curnow, 1995) (**Fig. 1**, panel B). Further studies demonstrated that the point of recombination is always comprised between intron 2 and exon 4 (Dluhy and Lifton, 1995) and Gly288 and Ala320 are the two residues indispensable for 18-hydroxylase and 18-oxitase activity respectively (Curnow *et al.*, 1997).

Originally, the diagnosis of FH-I was made on a clinical basis and relied exclusively on the complete suppression of aldosterone production after a 2–4 days dexamethasone suppression test (Liddle and Grant, 1960), together with the hybrid steroids

Table 1 Familial forms of primary aldosteronism

Familial hyperaldosteronism			
Type	Molecular basis	Prevalence	Treatment
Familial hyperaldosteronism, type 1 (or glucocorticoid remediable aldosteronism, GRA)	Chimeric *CYP11B1/CYP11B2* gene	<1% of PA	Dexamethasone, MR antagonists
Familial hyperaldosteronism, type 2	Unknown (possibly heterogeneous)	5% of PA	Unilateral adrenalectomy or MR antagonists
Familial hyperaldosteronism, type 3	*KCNJ5* mutations	Rare (12 affected families reported so far)	Bilateral adrenalectomy or MR antagonists
Familial hyperaldosteronism, type 4	*CACNA1H* mutations	Very rare	MR antagonists
PASNA syndrome	*CACNA1D* mutations	Very rare (two affected subjects)	CCBs, MR antagonists

PA, Primary aldosteronism; *MR*, mineralocorticoid receptor; CCBs, calcium channel blockers.

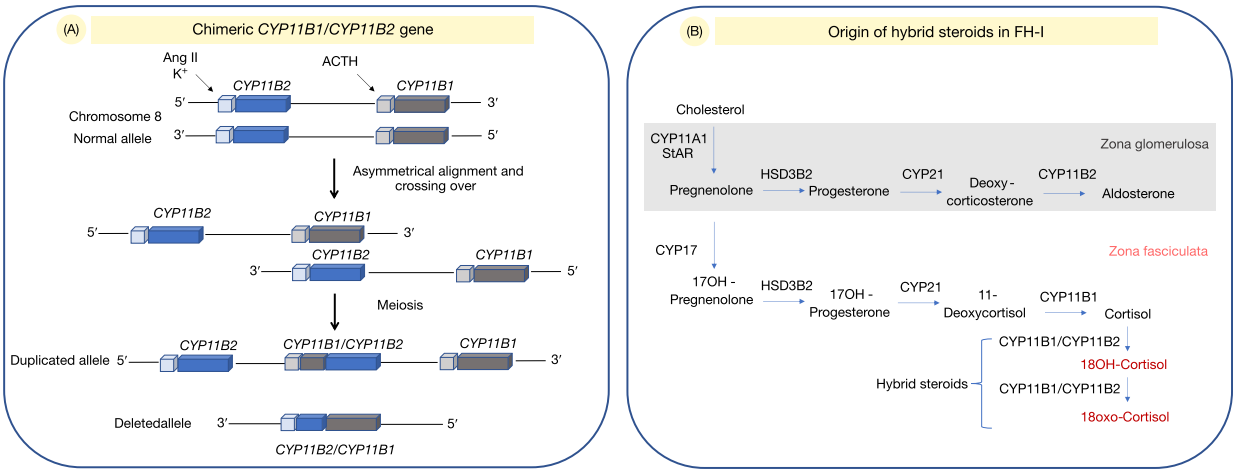

Fig. 1 (Panel A) Asymmetrical crossing over between *CYP11B1* and *CYP11B2* genes on chromosome 8, leading to the chimeric *CYP11B1/CYP11B2* gene in FH-I. (Panel B) Adrenal steroidogenesis in *zona glomerulosa* and *zona fasciculata*, showing the origin of the hybrid steroids in FH-I.

measurement. However, both tests, lacking specificity for FH-I diagnosis, have been replaced by the long PCR amplification of the chimeric gene (Stowasser *et al.*, 1995; Mulatero *et al.*, 1998a). It has infact been shown that a variable proportion of patients with sporadic aldosterone producing adenomas (APAs) show dexamethasone suppressible aldosterone production (Mulatero *et al.*, 1998b) and can produce a certain amount of hybrid steroids (Mulatero *et al.*, 2012a; Williams *et al.*, 2016). Moreover, extremely high levels of hybrid steroids have also been reported in one family affected by FH-III (Geller *et al.*, 2008).

According to the 2016 Endocrine Society guideline (Funder *et al.*, 2016), genetic testing for FH-I should be performed in all patients with the onset of PA earlier than 20 years of age and in those who have a family history of PA or of strokes at young age (<40 years). However, due to the lack of invasiveness and the undoubted benefit of an early diagnosis together with the requirement of a specific therapy, some authors suggest to systematically test all PA patients (Stowasser and Gordon, 2004; Mulatero *et al.*, 2010). Hypertensive children should also be considered for FH-I screening since it has been shown to be relatively frequent in this subgroup of patients (3.1% in untreated hypertensive Chilean children 4–16 years old) (Aglony *et al.*, 2011). In agreement with the data of Aglony *et al.*, the long-PCR test should be performed in all pediatric patients with low PRA and normal/high aldosterone (and, thus, high ARR) and normal/low potassium levels (Aglony *et al.*, 2011; Mulatero *et al.*, 2011a).

The clinical phenotype of FH-I is extremely heterogeneous and varies widely across affected families or even between members of the same pedigree (Fallo *et al.*, 2004), ranging from resistant hypokalemic hypertension to asymptomatic normotension (Mulatero *et al.*, 2002; Fardella *et al.*, 2000). The possible mechanisms underlying such a wide variation in clinical phenotype have been extensively investigated. Both the site of the crossing over (Pascoe *et al.*, 1992) and the Na^+ intake (Stowasser *et al.*, 2000) were not shown to play a role; an inverse relationship between urinary kallikrein levels and blood pressure in patients affected by FH-I was demonstrated in some families (Dluhy and Lifton, 1995), but not in an Italian pedigree (Mulatero *et al.*, 2002). Similarly, a significant correlation between the levels of the hybrid steroids 18OHF and 18oxoF) levels (as an index of the hybrid enzyme activity) was observed in some families (Mulatero *et al.*, 2002; Wyckoff *et al.*, 2000; Stowasser *et al.*, 2000) but not in others (Dluhy and Lifton, 1995).

With respect to target organ damage, normotensive FH-I affected patients display concentric remodeling and reduced diastolic left ventricular function compared with age- and sex-matched normotensive controls, indicating that aldosterone excess is associated with organ damage at least in part independent from the effects on blood pressure (Stowasser *et al.*, 2005).

FH-I affected patients also display an increase cerebrovascular morbidity and mortality at young age (Litchfield *et al.*, 1998). A large retrospective study including 27 GRA pedigrees, for a total of 167 affected patients, showed that a cerebrovascular complication occurred in 48% of the pedigrees and in 11% of patients with GRA, mainly hemorrhagic strokes, related intracranial aneurysms rupture. The mean age at the occurrence of the event was 32 years. It has been hypothesized that early onset of high blood pressure levels and/or the inappropriate amount of aldosterone during vascular development could determine the formation of cerebral aneurysms (Litchfield *et al.*, 1998). Interestingly, pregnant women affected by FH-I display an increased prevalence of pregnancy-aggravated hypertension but not of preeclampsia (Wyckoff *et al.*, 2000).

Glucocorticoids, which inhibits ACTH-driven aldosterone production, are the cornerstone of FH-I treatment and should be administered at the minimum dose necessary to control blood pressure to avoid iatrogenic Cushing syndrome. According to the ES Guideline (Funder *et al.*, 2016) synthetic glucocorticoids that are longer acting than hydrocortisone, such as dexamethasone or prednisone should be preferred and administered at bed time to suppress the morning ACTH peak. If glucocorticoid administration is not sufficient to control blood pressure a MR antagonist can be added. In case of affected children, in whom there might be concerns related to growth retardation and the antiandrogenic effects of glucocorticoids and spironolactone respectively, eplerenone may be considered the first-line therapy (Funder *et al.*, 2016).

Familial Hyperaldosteronism Type II

Familial hyperaldosteronism type II (FH-II) is an inheritable form of PA transmitted as an autosomal dominant disorder in most of the affected families and not associated to the presence of the chimeric *CYP11B1/CYP11B2* gene. This condition was described for the first time by Gordon *et al.* (1991) at the Greenslopes Hospital Hypertension Unit in Brisbane (Australia). Six relatives from three different families were diagnosed with PA (either APA or BAH) and since none of these patients was positive to the dexamethasone suppression test, the authors named this condition FH-II, to distinguish it from FH-I (Stowasser *et al.*, 1992). In one of the families, two second cousins presented with hypertension and hypokalemia at age of 31 and 29 years, both underwent excision of a left APA and the disease was cured. The second family presented two affected members: a 37-year-old female with hypertension and spontaneous hypokalemia and her 63-year-old father affected by poorly controlled hypertension. Both were diagnosed with APA and underwent surgery. The last family displayed a 46 year-old female with bilateral adrenal hyperplasia (BAH) and her 25 year-old hypertensive daughter; both were hypertensive and hypokalemic (Gordon *et al.*, 1991).

The genetic basis of the disease is still unknown. The study of potential genetic abnormalities causing FH-II has been based on both candidate gene approach and genome-wide search. The involvement of many genes has been excluded: *CYP11B2* (Torpy *et al.*, 1998), the gene coding for the angiotensin II receptor type 1 (Klemm *et al.*, 1995), the locus MEN1 (Stowasser and Gordon, 2001) and the gene coding for oncosuppressor p53 (Ballantine *et al.*, 1996). In 2000, through linkage studies, the FH-II causing gene was mapped to chromosome 7p22 (LOD score 3.26) (Lafferty *et al.*, 2000) and subsequent studies confirmed this locus (LOD score 4.61) (So *et al.*, 2005). Afterwards, the linkage has been demonstrated also in other families belonging to three different continents (Australia, Europe and South America) (Sukor *et al.*, 2008). Sequencing analysis has excluded the involvement of several genes within 7p22, including *PRKAR1B* (Elphinstone *et al.*, 2004), *RbaK*, *PMS2*, and *GNA12* (Jeske *et al.*, 2008). However, for two affected Australian families the linkage with locus 7p22 could not be demonstrated (So *et al.*, 2005) and the role of 7p22 is debated because of the genetic heterogeneity of the families. Moreover, in a recent European multicentric study (Mulatero *et al.*, 2012b) including 21 families with suspected FH-II, one family resulted to be affected by FH-III and in two APAs removed from patients belonging to two different families, a somatic *KCNJ5* mutation was identified, indicating that FH-II is very likely to be a heterogeneous disease.

Since the molecular basis remains unknown, the diagnosis of FH-II is clinical and requires two or more family first-degree members affected by PA together with the exclusion of FH-I and FH-III through long-PCR for chimeric gene *CYP11B1/CYP11B2* and *KCNJ5* sequencing, respectively (Mulatero *et al.*, 2011b; Stowasser *et al.*, 2000).

Clinical, biochemical and morphological features of patients affected by FH-II are indistinguishable from sporadic PA (Mulatero *et al.*, 2011b). In the PATOGEN study, including 27 FH-II patients from 12 different families, 92% of patients presented with arterial hypertension and hypokalemia was present in 35% of the patients (50% of index cases and 18% of relatives with PA). Morphologically patients presented with APA in nearly 18% of cases, in other cases they had bilateral hyperplasia (Mulatero *et al.*, 2011b).

It has been shown in two large series of PA patients from Italy (Mulatero *et al.*, 2011b) and Australia (Stowasser *et al.*, 2011), that FH-II is at least five times more common than FH-I and accounts for around 6% of PA patients. However, the prevalence could be even higher because patients who present with low-renin hypertension could evolve in overt PA over time (Mulatero *et al.*, 2011b). Due to its relatively high prevalence, the Endocrine Society guideline recommend that all hypertensive first-degree relatives of patients with PA should undergo screening test (Funder *et al.*, 2016). Thus, the identification of FH-II in the relatives of PA patients is fundamental in order to establish an appropriate therapy, surgical with unilateral adrenalectomy or medical with mineralocorticoid receptor antagonists (Mulatero *et al.*, 2011b).

Familial Hyperaldosteronism Type III

Familial hyperaldosteronism type III (FH-III) is an inheritable autosomal dominant disorder caused by a germline mutation in the *KCNJ5* gene.

FH-III has been described as a distinct clinical entity by Geller *et al.* (2008) in an American family affected by a non-glucocorticoid-suppressible form of familial hyperaldosteronism. The index case of this family was previously reported by Therien *et al.* (1958) as affected by severe hyperaldosteronism and hypokalemia associated with arterial hypertension, polyuria, polydipsia, and myalgia since childhood. Geller *et al.* (2008) described the biochemical and clinical phenotype of the two daughters of the index case, which presented at the age of 7 and 4 respectively, because of severe resistant arterial hypertension (188/140 mmHg and 148/114 mmHg respectively) and profound hypokalemia (< 2 mEq/L). Furthermore, the two patients showed markedly increased levels of 18-hydroxycortisol, 18-oxocortisol, deoxycorticosterone, and 18-hydroxycorticosterone) (Geller *et al.*, 2008). Both the father and the two daughters required bilateral adrenalectomy in order to normalize aldosterone and potassium levels and to control hypertension (Therien *et al.*, 1958; Geller *et al.*, 2008). Adrenal glands showed bilateral hyperplasia and subverted histological architecture of the adrenal cortex (Gomez-Sanchez *et al.*, 2017). Recently, an immunohistochemical and immunofluorescence study described the distribution of the main steroidogenic enzymes (CYP11B1, CYP11B2, and CYP17) in the adrenal cortex of the glands removed from the two girls. The results showed an unexpected co-expression of CYP11B2 and CYP11B1, CYP11B2 and CYP17 or even all the three enzymes in some cells (Gomez-Sanchez *et al.*, 2017).

Initially, causative mutations remained unknown despite the DNA sequencing of candidate genes. The genetic basis of this disorder has been uncovered through the application of the next generation sequencing, which allowed the identification of the germline p.T158A *KCNJ5* mutation in the affected members (Choi *et al.*, 2011).

KCNJ5 is located on chromosome 11q24 and encodes for GIRK4 (also known as Kir3.4), an inward rectifying potassium channel. This channel forms homotetramer and heterotetramer with GIRK1, encoded by *KCNJ3* gene (Corey and Clapham, 1998). *KCNJ5* is a widely expressed gene and, among the adrenal gland, GIRK4 is expressed in the *zona glomerulosa* and in the outer part of *zona fasciculata* and it shows heterogeneous expression in APA (Choi *et al.*, 2011; Monticone *et al.*, 2012). Physiologically, GIRK4 contribute to maintain a state of hyperpolarization of the cell through a potassium outflow. All the mutations described are localized near or within the selectivity filter of the channel, a highly conserved domain across species (**Fig. 2**). The consequent alteration of the filter causes the loss of ion selectivity, sodium influx and depolarization of the cell membrane which activates voltage-dependent calcium channels. (Oki *et al.*, 2012; Monticone *et al.*, 2015b). The increase in intracellular Ca^{2+} concentration stimulates *CYP11B2* transcription through the transcriptional factor *NR4A2* (Monticone *et al.*, 2012), which finally results in an autonomous and excessive aldosterone production (Oki *et al.*, 2012) (**Fig. 3**).

After the identification of the first mutation (p.T158A) by Choi *et al.*, additional 5 *KCNJ5* mutation and 11 FH-III families have been described. In vitro studies conducted on HAC15 adrenocortical cells demonstrated that p.Y152C substitution caused a milder electrophysiological effect compared to other KCNJ5 mutations (Monticone *et al.*, 2013). Whereas, p.G151E substitution caused cell lethality due to a massive sodium influx and the consequent osmotic shock. However the surviving cells can produce large amount of aldosterone (Scholl *et al.*, 2012; Mulatero *et al.*, 2012b): these results explain the normal size of the adrenals and the less severe clinical phenotype in comparison with patients with other mutations.

According to the Endocrine Society guideline, the diagnosis of FH-III should be ruled out in all patients presenting with PA at very young age (Funder *et al.*, 2016), through *KCNJ5* sequencing of DNA obtained from a peripheral blood sample.

Initially the diagnosis was based on the particular clinical features displayed by the family described by Geller *et al.* (2008), but it became evident that these characteristics are not present in all FH-III families. Clinically, the majority of the patients displayed an early onset of PA, hypokalemia, polyuria, polydipsia, and resistant hypertension. However it is important to underline that the clinical phenotype is influenced by the type of *KCNJ5* mutation.

In addition to p.T158A (Choi *et al.*, 2011; Geller *et al.*, 2008; Therien *et al.*, 1958) five other germline mutations (p.G151E, p. G151R, p.I157S, p.Y152C, and p.E145Q) have been identified.

The p.G151E mutation has been reported in three different families, affecting a total of seven patients (Scholl *et al.*, 2012; Mulatero *et al.*, 2012a; Mussa *et al.*, 2012; Greco *et al.*, 1982; Bartter and Biglieri, 1958), who were all diagnosed with PA because of high aldosterone levels and suppressed renin activity. The affected members of the family from Italy showed mildly increased levels of the hybrid steroids within the range of sporadic PA (Mulatero *et al.*, 2012a,b) and they did not show any changes in aldosterone after dexamethasone suppression test. All patient except two showed severe hypertension and low potassium levels with onset in early childhood, which were well controlled by pharmacological therapy with spironolactone and amiloride (only two patients underwent bilateral adrenalectomy) (Scholl *et al.*, 2012). Moreover, none of the affected patients showed adrenal hyperplasia at adrenal imaging.

The p.G151R substitution (Scholl *et al.*, 2012; Adachi *et al.*, 2014; Monticone *et al.*, 2017) has been identified in six patients from four different families. The clinical phenotype was similar to that observed in patients carrying the p.G151E mutation, with early onset of the disease but in this case PA was progressive. Only in the patient reported by Adachi *et al.* (2014) hypertension and

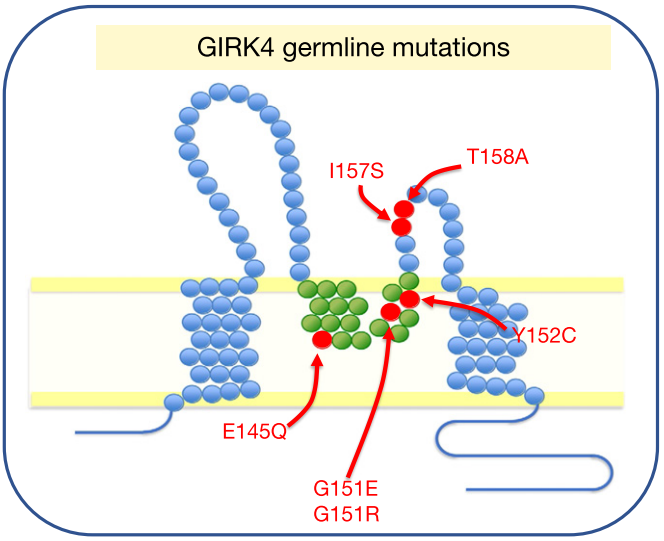

Fig. 2 GIRK4 structure and summary of the germline mutations leading to FH-III. All the mutations are located near or within the selectivity filter of the channel (represented in *green*).

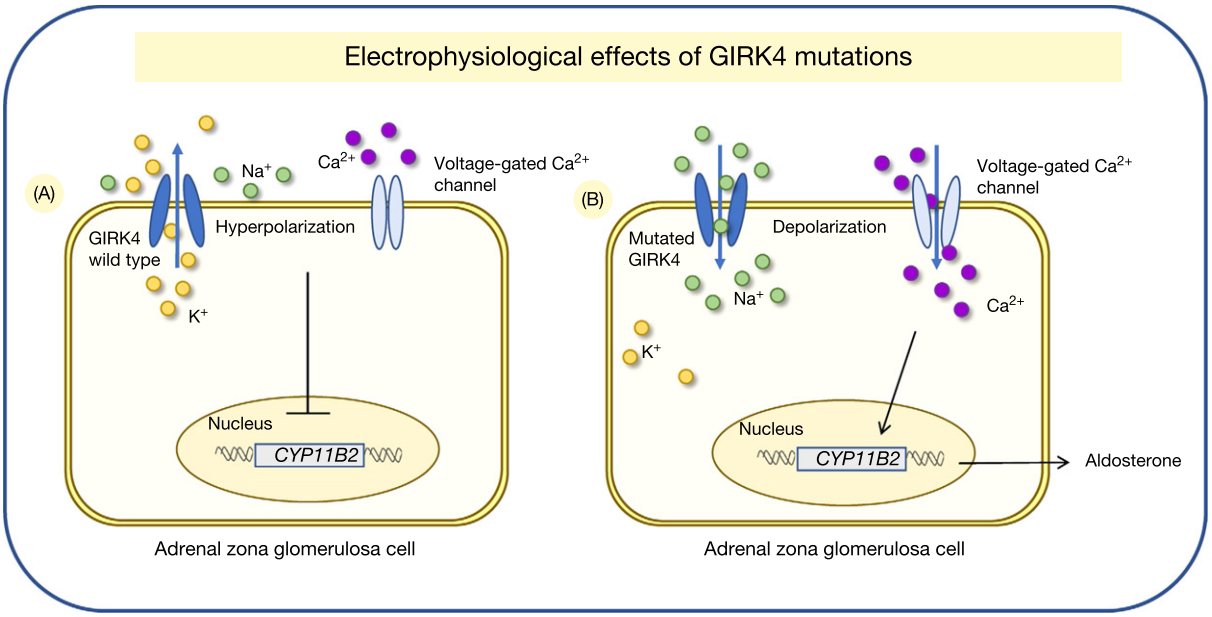

Fig. 3 (Panel A) In adrenal zona glomerulosa cells, GIRK4 wild-type is responsible for K^+ outflow and the cell membrane is maintained in a hyperpolarized state. (Panel B) The mutations, located in proximity of the selectivity filter of the channel result in loss of ion selectivity, Na^+, cell membrane depolarization, opening of voltage-gated Ca^{2+} channels and finally autonomous aldosterone overproduction.

hypokalemia were controlled by spironolactone and adrenal imaging was normal (Adachi *et al.*, 2014). All the other patients required bilateral adrenalectomy in order to control blood pressure, hypokalemia and symptoms mimicking diabetes insipidus. Moreover, histological analysis confirmed nodular hyperplasia in one patient and diffuse hyperplasia of adrenal cortex of three out of four patients who underwent surgery.

The p.I157S mutation (Charmandari *et al.*, 2012) has been identified in two members of a single family displaying resistant hypertension, marked hypokalemia and severe adrenal enlargement which required bilateral adrenalectomy.

The p.Y152C mutation (Monticone *et al.*, 2013) was reported in an African-American woman who was diagnosed with arterial hypertension when she was 48 year old. She had left adrenalectomy on the basis of adrenal CT scanning, but the intervention was not curative and serum aldosterone levels did not normalize.

Finally, the p.E154Q substitution (Monticone *et al.*, 2015b; Tong *et al.*, 2016) has been reported in two patients from two different families: a Caucasian girl who presented with severe PA requiring bilateral adrenalectomy and a Chinese boy who was diagnosed with PA and Cushing syndrome and had left adrenalectomy.

This condition is rare and its prevalence has not been evaluated in large population; Mulatero *et al.* (2012a,b) reported a 0.3% prevalence among patients with PA and 8% prevalence among familial forms.

Familial Hypertension Hyperaldosteronism Type IV

FH4 is a familial form of primary aldosteronism due to germline mutations in the *CACNA1H* gene, encoding a T-type calcium channel, Cav3.2. *CACNA1H* is expressed in kidney, liver, heart, brain (Cribbs *et al.*, 1998) and it is the second most expressed Ca^{2+} channel in the adrenal zona glomerulosa (Scholl *et al.*, 2013), where it is activated at small depolarizing potentials (Perez-Reyes 2003). Cav3.2 is composed by four homologous repeats (I-IV) and each of them has six transmembrane segments (S1–S6).

Using whole exome sequencing in patients with hypertension and onset of PA by the age of 10 years), a novel germline heterozygous *CACNA1H* mutation (p.Met1549Val) has been identified in five unrelated subjects (Scholl *et al.*, 2013). The index cases carrying the p.Met1549Val substitution showed early onset hypertension and PA with normal appearing adrenal glands at adrenal CT scanning, without any other distinctive clinical or biochemical feature. Targeted sequencing of *CACNA1H* in first- and second-degree relatives of the index cases, identified five more subjects carrying the p.Met1549Val substitution, among four families (in one case it was a de novo mutation). The substitution segregated with the disease in one kindred, but, in two other families, two mutation carriers did not display the PA phenotype and were normotensives, indicating an incomplete penetrance. Histological examination of an adrenal gland removed from a FH-IV patient (because of difficult to control hypertension) revealed microscopic hyperplasia of the zona glomerulosa with micronodular invasion of the capsule (Scholl *et al.*, 2013; Korah and Scholl, 2015).

Following the original finding by Scholl *et al.*, four other germline *CACNA1H* mutations have been identified in PA patients (p.Met1549Ile, p.Ser196Leu, p.Pro2038Leu, and p.Val1951Glu) (Daniil *et al.*, 2016). The index case carrying the p.Met1549Ile

showed early onset PA with mild mental retardation at the age of 7 and a diagnosis of multiplex developmental disorder was made 3 years later. The patients carrying the p.Ser196Leu, the p.Pro2083Leu, and the p.Val1951Glu were diagnosed with arterial hypertension between the age of 32 and 48, in two cases there was a familial history of hypertension and PA and in one case (p.Val1951Glu) the patient was affected by apparently sporadic APA (Daniil *et al.*, 2016). Adrenal CT scanning was normal in patients carrying the p.Met1549Ile and in two patients carrying the p.Pro2083Leu mutations; the index case carrying the p.Ser196Leu showed bilateral nodules at adrenal CT scanning; one patient carrying the p.Pro2083Leu mutation had left adrenal hyperplasia and the patient carrying the p.Val1951Glu mutation had a single 8 mm left adrenal nodule (Daniil *et al.*, 2016).

Under physiological conditions, Cav3.2 is activated in response to physiological small changes in aldosterone, angiotensin II and potassium serum levels and the activation threshold is -60 mV, followed by a very fast inactivation. Met1549 lies in a conserved position in the S6 helix of repeat 3 (Marksteiner *et al.*, 2001) and functional studies on the paralogous CACNA1G showed that it regulates channel inactivation. Electrophysiological studies on HEK293T cells demonstrated that p.Met1549Val Cav3.2 showed loss of normal inactivation together with a shift of activation to more hyperpolarized potentials (Scholl *et al.*, 2013). To examine these electrophysiological effect on aldosterone production, the p.Met1549Val channel was expressed in the adrenocortical cell lines HAC15 (Reimer *et al.*, 2016). As expected, overexpression of the mutant Cav3.2 resulted into a sevenfold increase in aldosterone levels compared with vector-transfected cells and this effect was mediated by the increased expression of *CYP11B2*, thereby linking the presence of this mutation to the clinical PA phenotype (Reimer *et al.*, 2016).

In conclusion, *CACNA1H* seems to represent a susceptibility gene that may predispose to PA development, which in turn could present with a wide range of clinical phenotypes (Daniil *et al.*, 2016).

PASNA Syndrome

PASNA (primary aldosteronism with seizures and neurologic abnormalities) is a new mendelian syndrome characterized by primary aldosteronism and neurological manifestations, such as seizures and neuromuscular disease. The molecular basis of PASNA is represented by gain of function mutations in the *CACNA1D* gene (Scholl *et al.*, 2013), which encodes the $\alpha 1$ subunit of a L-type voltage gated calcium channel, Cav 1.3, expressed in adrenal glomerulosa cells, heart and neurons (Baig *et al.*, 2011). Cav 1.3 is formed by four repeated domains with six transmembrane segments (S1–S6) and a membrane associated loop between S5 and S6, which line the channel pore. In normal quiescent adrenal glomerulosa cells, the cell membrane is maintained in a hyperpolarized state, preventing the opening of voltage-gated calcium channels. Mutations in *CACNA1D* alter the cytoplasmatic end of the S6 segment of domains I and II, causing channel activation at less depolarized potentials and altered channel inactivation. As a consequence, the channel opens at more hyperpolarized potentials, closer to the glomerulosa resting potentials, causing an increase in intracellular calcium concentration. Increased calcium influx in glomerulosa cells leads to aldosterone production and cell proliferation, while neurological manifestations are due to abnormal neuronal calcium signaling (Scholl *et al.*, 2013, 2015).

CACANA1D (somatic) mutations have been identified through exome sequencing studies in sporadic APA (Azizan *et al.*, 2013; Scholl *et al.*, 2013; Monticone *et al.*, 2015a) and, subsequently, the Gly403Asp and the Ile770Met substitution have been found as germline mutations in two individuals affected by severe hypertension and neurological manifestations. Both the index cases had healthy parents, demonstrating that de novo germline mutations occurred (Scholl *et al.*, 2013).

The affected children manifested early onset severe hypertension, hypokalemia and seizures. The first patient is a female of European ancestry who suffered from severe hypertension at birth (119/78 mmHg), biventricular hypertrophy, ventricular septal defect, pulmonary hypertension and second degree heart block. Laboratory testing revealed high aldosterone levels, (128.6 ng/dL), low PRA (0.78 ng/mL/h) and hypokalemia. At 7 months the patient had her first generalized tonic-clonic seizures. Other neurological manifestations include apparent cerebral palsy, cortical blindness, neuromuscular abnormalities. Interestingly, blood pressure was normalized by the calcium channel blocker amlodipine and ventricular hypertrophy regressed (Scholl *et al.*, 2013, 2015).

The second patient is an African American female, with cerebral palsy, spastic quadriplegia, mild athetosis, severe generalized intellectual disability, complex and partial seizures at birth. She developed severe hypertension (132/90 mmHg) at the age of 5 years. The diagnosis of primary aldosteronism was made on the basis of hypokalemia, metabolic alkalosis, high serum aldosterone levels (36 ng/dL) and suppressed PRA with elevated ARR. Echocardiography demonstrated mild left ventricular hypertrophy. The patient was treated with clonidine and then spironolactone. CT scan shows normal adrenals.

Different mutations lead to variable calcium conductance, resulting in variable cell lethality. Individuals with mutations causing cell lethality do not develop adrenal hyperplasia (Scholl *et al.*, 2013).

The response of the first patient to a calcium channel blocker may lead to the possibility of a specific treatment for individuals affected by APAs and *CACNA1D* mutations. These drugs are weak antagonists of wild type Cav 1.3, but more specific inhibitors could represent a possible treatment of individuals with *CACNA1D* mutations (Scholl *et al.*, 2013).

Conclusions

The recent advances in the genetics of familial hyperaldosteronism have provided new insight in the molecular mechanisms responsible for autonomous aldosterone overproduction. The identification of a familial form of PA is of outmost importance, in order to establish an early diagnosis, plan a close follow-up of the affected subjects and promptly start a targeted therapy, in order to prevent the cardio- and cerebrovascular complications associated to aldosterone excess.

See also: Adrenal Venous Sampling for Primary Aldosteronism. Aldosterone-Producing Adenomas; Genetics. Mineralocorticoid Excess Syndromes. Primary Aldosteronism; Diagnosis and Treatment. Primary Aldosteronism; Epidemiology and Screening

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Aldosterone-Producing Adenomas; Genetics

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Mutations in K + Channel in Aldosterone-Producing Adenoma

In 2011, using exomes sequencing Choi *et al.* in about 34% of 22 aldosterone-producing adenoma (APA) discovered mutations in the selectivity filter of the KCNJ5 gene, which codes for the Kir3.4 K^+ channel (Choi *et al.*, 2011). This channel is highly expressed in the aldosterone-producing cells of the zona glomerulosa (ZG) and plays a key role in maintaining cell hyperpolarized (Choi *et al.*, 2011) (**Fig. 1**, Panels A and B). The Authors identified two recurrent functional variants (G151R and L168R) in this highly conserved region of the K^+ channel, which allows the exit of K^+ from the cells in a selective fashion, which means with exclusion of other ions. Using cloning techniques and site-directed mutagenesis they could show that mutant-transfected cells in vitro exhibit perturbation in both size and charge of the selectivity filter, resulting in permeability to Na^+, Na^+ influx, and membrane depolarization (**Fig. 2**, Panel A).

After this first report several other mutations were identified, both in the same "hot spot" region of the gene and also outside this region (for review see Zennaro *et al.*, 2017). All were found to impair the selectivity of the filter for K^+ and to allow Na^+ influx, leading to ZG cell membrane depolarization, opening of T type Ca^{2+} channels, Ca^{2+} influx and activation of aldosterone synthesis and secretion. A further novel somatic mutation consisting of a 3 bp insertion (446insAAC) outside of the selectivity filter, thus differing from all previously known mutations, which are located in the selectivity filter, was identified by our group (Kuppusamy *et al.*, 2014). The phenotype associated with this mutation featured severe drug-resistant hypertension that had been previously complicated by an ischemic stroke, and evidence of prominent target organ damage, including left ventricular hypertrophy. At the cellular level this mutation behaved like the other known mutations in that caused increased permeability for Na^+, cell membrane depolarization, and increased Ca^{2+} influx. However, with use of specific inhibitors we could show that this occurred not only via voltage-dependent T Type Ca^{2+} channels opening, but also via the Na^+/Ca^{2+} exchanger working in the reverse mode, for example, Na^+ out and Ca^{2+} in. These changes resulted in overexpression of the aldosterone synthase (CYP11B2) gene and constitutive over-production of aldosterone by the tumor. A different mutation in the same region (445-446insGAA) was thereafter found in a cohort of Chinese APA patients that exhibited a very high prevalence (77%) of KCNJ5 mutations (Zheng *et al.*, 2015). Thus, it seems that the selectivity of the pore can be affected also by mutations located outside of the filter region through mechanisms discussed in details in our paper (Kuppusamy *et al.*, 2014).

Why these mutations occur in this region of the gene is unclear; similarly, unclear is why these KCNJ5 mutations are found to be more common in patients from Asia (Japan and China) than in other continents. In fact, the overall prevalence of KCNJ5 mutations was 43% (range = 12%–80%), but in studies done in Europe, USA, and Australia (35%) their rate was lower ($P < 0.003$) than in Japan and China (63%), as reported in a large meta-analysis (Lenzini *et al.*, 2015).

The latter also showed that the phenotypic features of APA patients with KCNJ5 mutations comprised more pronounced hyperaldosteronism, young age, female gender and larger tumors, but no significant differences in blood pressure and serum K^+. Hence, the latter clinical features do not help in identifying KCNJ5-mutated APA patients.

Effect of Somatic Mutations in K + Channel on Cell Growth

Notwithstanding all these novel discoveries, it remains unclear whether these mutations play any mechanistic role in adenoma growth. This is because, when transfected with mutated KCNJ5 (T158A) channels, adrenocortical cells did show a decreased, rather the hypothesized increase of rate proliferation compared to the wild type cells (Oki *et al.*, 2012). Moreover, cells with mutations in the same codon, the G151R and the G151E mutation, showed an altogether different lethality, which was higher in the former than in the latter and in wild type cells, and was shown to depend on the enhanced conductance for Na^+ (Scholl *et al.*, 2012). It was contended that this latter finding might explain why this mutation did not associate with signs of adrenal hyperplasia and translated in a milder form of familial hyperaldosteronism (Scholl *et al.*, 2012; Mulatero *et al.*, 2012) that responded well to antihypertensive therapy.

The discrepancy between the antiproliferative effect of KCNJ5 mutations, due to the high intracellular concentrations of Na^+ and Ca^{2+}, and the development of the adenoma was suggested to be explained by the observation that APA cells are protected against Ca^{2+}-induced cell death by the calcium-sensor protein Visinin-like 1 (VSNL1). Consistently with this hypothesis VSNL1 would seem to be more expressed in KCNJ5 mutated than in wild type APAs; moreover, its silencing rendered H295R cells sensitive to Ca^{2+}-induced apoptosis (Williams *et al.*, 2012).

Mutations in ATP1A1 and ATP2B3 Genes

In Caucasians, only a minority of APA show KCNJ5 mutations, which seem to predominate in zona–fasciculata-like than in zona glomerulosa-like APA cells (Azizan *et al.*, 2012). Hence, a search for further candidate genes by exome sequencing in non

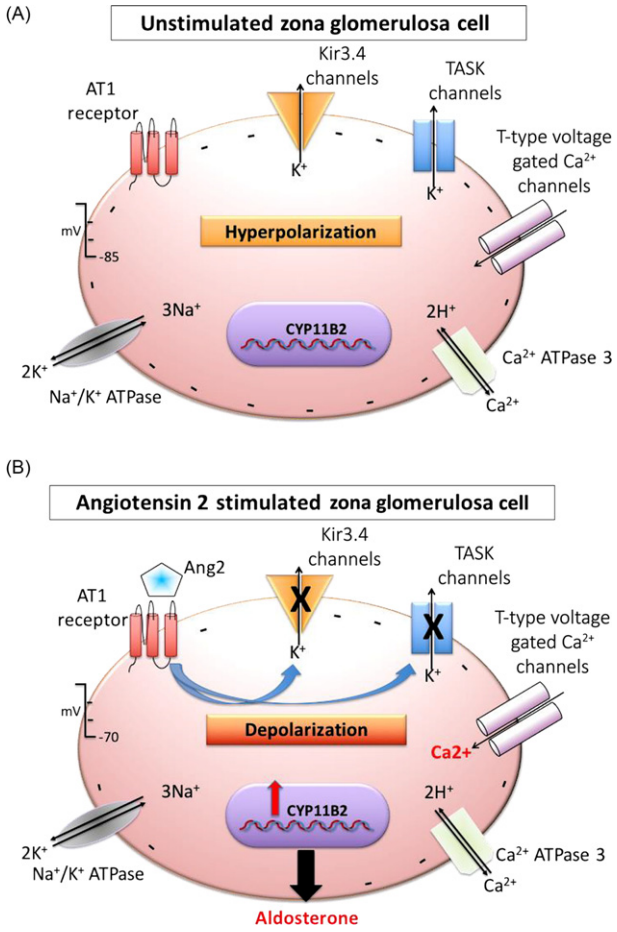

Fig. 1 *Regulation of aldosterone production in normal adrenocortical zona glomerulosa (ZG) cells and effects of aldosterone secretagogue.* Under normal condition, ZG cells have a resting potential of about −85 mV, which means that they are hyperpolarized, through the activity of the TASK and Kir K^+ channels. This hyperpolarization state maintains voltage gated T-type Ca^{2+} channels in a low open state probability. Therefore, the ZG cells do not produce aldosterone (*Panel A*). In the presence of an aldosterone secretagogue stimulus, such as Angiotensin 2, the activity of the TASK and Kir K^+ channels is blunted and the cell is depolarized. This opens the voltage gated T-type Ca^{2+} channels with an increase of intracellular Ca^{2+} and the stimulation of aldosterone biosynthesis (*Panel B*).

KCNJ5-mutated APA composed of zona glomerulosa cells led to the discovery of mutations in ATP1A1 and ATP2B3 genes (Azizan *et al.*, 2013; Beuschlein *et al.*, 2013; Scholl *et al.*, 2013).

ATP1A1, located on chromosome 1p21, encodes the alpha-1 subunit of the Na^+/K^+-transporting ATPase; the ATB2B3 gene, located on chromosome Xq28, encodes the plasma membrane Ca^{2+}-transporting ATPase 3. The former regulates the resting membrane potential by exchanging three cytoplasmic Na^+ ions for two extracellular K^+; the latter is involved in Ca^{2+} homeostasis by exchanging one cytosolic Ca^{2+} with two H^+ (**Fig. 1**, Panels A and B).

Loss-of-function variants of the Na^+/K^+ ATPase gene ATP1A1 were found in 5.2% of APA (Beuschlein *et al.*, 2013). In vitro models of these mutations exhibit abnormal leak currents of H^+ leading to intracellular acidification (L104R, V332G) and/or of Na + ions (L104R, V332G, G99R) (**Fig. 2**, Panel A) (Stindl *et al.*, 2015). Mutations of the Ca^{2+}-ATPase gene ATP2B3 were discovered in 1.6% of APA and reported to increase intracellular Ca^{2+} concentration (**Fig. 2**, Panel D) (Beuschlein *et al.*, 2013).

Mutations in CACNA1D Gene

Gain-of-function somatic mutations of the CACNA1D gene, coding for the voltage-gated calcium channel Cav1.3, were reported in 5%–9% of APA (Azizan *et al.*, 2013; Scholl *et al.*, 2013; Fernandes-Rosa *et al.*, 2014). These mutations cause Cav1.3 activation at less depolarized potentials with ensuing increased Ca^{2+} influx (**Fig. 2**, Panel C). According to two small studies, APA carrying these mutations would be mainly composed of zona glomerulosa cells (Tan *et al.*, 2017; Monticone *et al.*, 2015), but larger studies are obviously necessary.

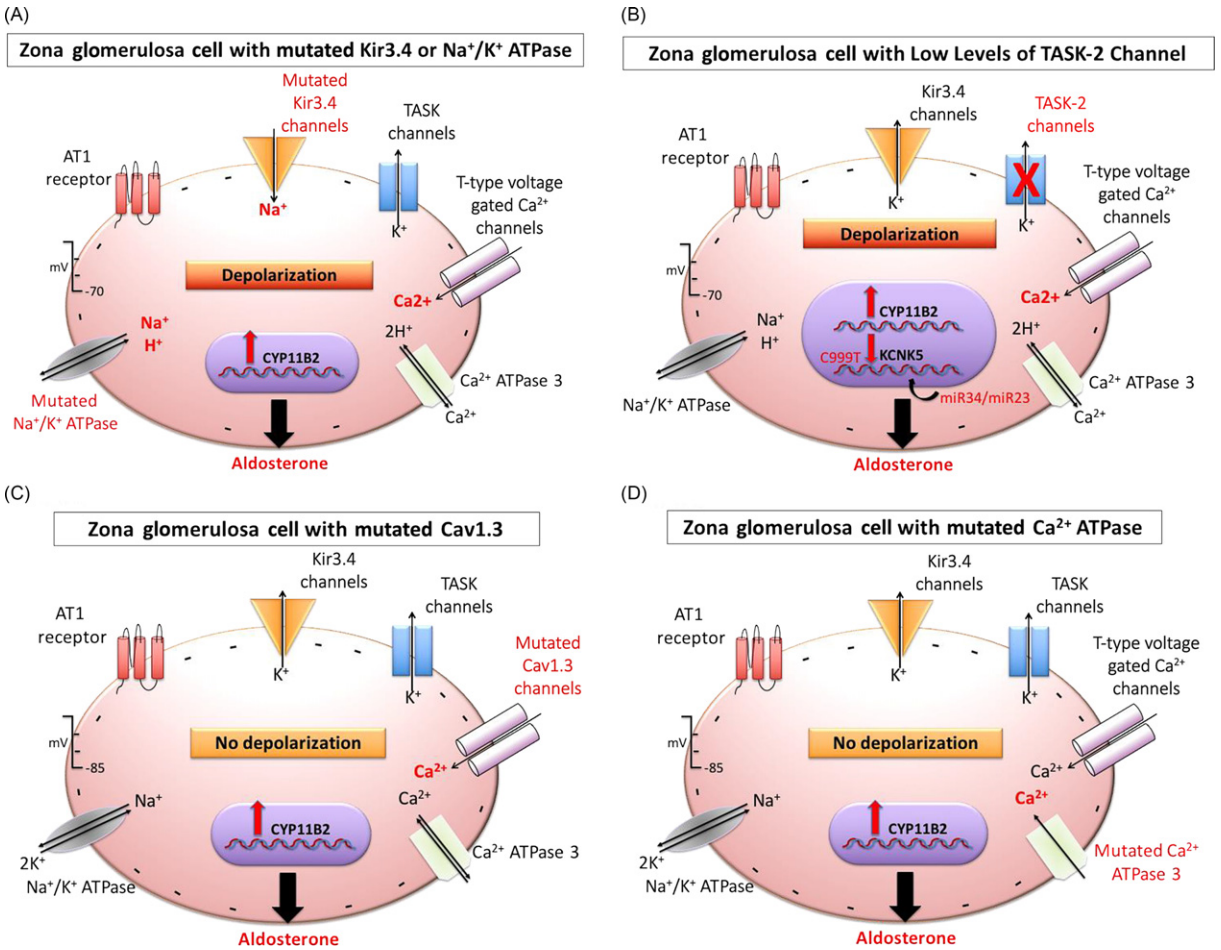

Fig. 2 *Genetic causes of aldosteronism in aldosterone-producing adenoma (APA).* In a percentage of sporadic APA (12%–80%) mutations lead to depolarization of the cell, with ensuing entry of Ca^{2+} via Voltage Gated T-type Ca^{2+} channels and autonomous production of aldosterone. This can occur as a result of Kir3.4 (KCNJ5) mutations, which cause a loss of selectivity for K^+ and Na^+ influx, or of functional mutations in the Na^+/K^+ ATPase leading to abnormal leak currents of H^+ and/or of Na^+ and increased intracellular acidification. (*Panel A*). The TASK-2 channel is expressed at very low levels as compared to normal adrenocortical tissues in all APA, due to multiple molecular mechanisms, including degradation of TASK-2 RNA by the miR23 and miR34 in about 25% of the cases and functional mutations in the promoter region of the TASK-2 gene in another 25% of the cases (*Panel B*). Panels C and D illustrate two additional genetic causes of aldosteronism in APA in which intracellular Ca^{2+} levels are increased without changes in the membrane potential as the cell remain hyperpolarized, either because of the presence of mutated Cav1.3 (*Panel C*), or because of Ca^{2+} ATPase 3 (*Panel D*), in both cases with ensuing augmented Ca^{2+} influx and subsequent stimulation of aldosterone synthesis.

Genetic Heterogeneity of APA

The recent development of a specific antibody able to discriminate aldosterone synthase from the highly homologous 11β hydroxylase (Gomez-Sanchez *et al.*, 2014) has made possible to achieve a more precise immunochemical characterization of the APA. This led to the discovery that nodules expressing the aldosterone synthase and coexisting in the same explanted adrenal gland can express two different KCNJ5 mutations (Dekkers *et al.*, 2014), and/or mutations in KCNJ5 and CACNA1D (Fernandes-Rosa *et al.*, 2015). Moreover, up to seven different mutations in KCNJ5 and CACNA1D were detected in macro-dissected nodules in CT cross-sectional image–negative cases (Yamazaki *et al.*, 2016).

Role of the Twik Related Acid Sensitive K^+ (TASK) Channels in Primary Aldosteronism

The crucial role of K^+ in regulating aldosterone secretion confirmed by the studies described above stands also on the well-established notion that zona glomerulosa cells are exquisitely sensitive to extracellular K^+ in that small elevations in serum K^+, even within the physiological range, are known to increase cell sensitivity to angiotensin II and other aldosterone secretagogue stimuli (**Fig. 1**, Panels A and B) (Spat and Hunyady, 2004). Hence, K^+ represents a key modulator of aldosterone secretion not

only due to its direct effects, but also because of its permissive role on the action of other agents that stimulate aldosterone secretion. Compelling evidences indicate that K^+ channels activity sets the resting membrane potential of aldosterone-producing zona glomerulosa cells and that the intrinsic oscillations of these cells, due to Cav3.2 Ca^{2+} currents, could explain the remarkable sensitivity of aldosterone production in vivo to small incremental changes (0.1 mEq/L) in plasma K^+ (Hu *et al.*, 2012).

Three TASK channels (TASK-1, 2 and 3) are primarily expressed in the adrenal gland (Bayliss and Barrett, 2008). They are coded by the KCNK genes and form active channels through homo- and hetero-dimerization, and show persistent activity at negative membrane potentials, generating background, or "leak," K^+ currents (**Fig. 1**, Panel A).

Of note, the TASK channels are considered to be essential for maintaining a negative resting membrane potential, and therefore for keeping the T-type-calcium channels closed, thus preventing Ca^{2+} influx. Mice knocked-out for both the TASK-1 and TASK-3 genes developed a phenotype resembling human PA in that it features sodium-dependent hypertension with low plasma renin and K^+ and hyperaldosteronism (Davies *et al.*, 2008). The mice with homozygous knock-out of only one channel generated two distinct forms of hyperaldosteronism: the TASK-1 knock-out caused a severe hyperaldosteronism with low-renin hypertension, and a defective adrenocortical zonation. These alterations were glucocorticoid-remediable, albeit only in females (Heitzmann *et al.*, 2008), thus resembling familial hyperaldosteronism type 1 (FH-1) (Lifton *et al.*, 1992). At variance, the TASK-3 knock-out caused a low-renin, salt-sensitive hypertension (Penton *et al.*, 2012; Bandulik *et al.*, 2013; Guagliardo *et al.*, 2012).

In humans, TASK channels are expressed in both the normal adrenal cortex and in APA tissues (Nogueira *et al.*, 2010), but we found the TASK-2 gene KCNK5 to be consistently under-expressed in APAs at both the transcript (**Fig. 2**, panel B) and the protein levels, as compared to the normal human adrenal cortex (Azizan *et al.*, 2012), a finding confirmed in a larger group of consecutive APAs with a low (23%) rate of KCNJ5 mutations (Lenzini *et al.*, 2014). This consistent under-representation in APA than in the normal adrenal cortex was an unexpected and striking finding given the known marked molecular heterogeneity of APAs (Lenzini *et al.*, 2007), but was, however, reported also in the public expression profiles of the GEO Profile Database on human normal adrenal cortex and APA, (Profile 1: GDS3912/219615_s_at; Profile 2: GDS2860/219615_s_at).

Therefore, the blunted expression of this channel can be a common molecular hallmark of these tumors. To prove this hypothesis a dominant-negative mutant of TASK-2 was stably transfected in H295R cells, an approach that allowed to show the involvement of TASK-2 channel in the regulation of aldosterone production in humans: compared to cells transfected with an empty vector, those carrying the dominant negative mutant exhibited a higher CYP11B2 and STAR expression and an increased production of aldosterone. By integrating the transcriptome analysis with microRNA profiling and using a site-directed mutagenesis technique combined to a reporter gene assay it was proven that two microRNAs (hsa-miR-23 and hsa-miR-34a), which were highly expressed in a percentage of APA but not in the zona glomerulosa surrounding the tumor, bind to the 3′ UTR of the TASK-2 gene and modulate its expression. Therefore, an up-regulation of the hsa-miR-23 and hsa-miR-34a microRNAs could lead to enhanced degradation of the TASK-2 channels, and thereby to cell membrane depolarization, with ensuing increased of Ca^{2+} influx and stimulation of steroidogenesis (**Fig. 2**, Panel B).

Since the under-expression of TASK-2 could be explained by enhanced expression of hsa-miR-23 and hsa-miR-34a in only 25% of APA, we investigated in a further study if variations in the promoter sequence of KCNK5 gene can lead to a low expression of TASK-2 in the remaining two third of APA (Lenzini *et al.*, 2017). To this end, the promoter region of KCNK5 was sequenced in APAs ($n = 76$), primary hypertensive patients ($n = 98$) and 20 years-old healthy volunteers ($n = 71$), searching for variants that could affect the expression of this channel. We found TASK-2 promoter mutations in another 25% of the APA: C999T in 6.6%, G595A in 5.3%, G36A in 5.3%, and C562T, Gins468, G265C, C1247T, G1140T and C1399T in 1.3% (each). The C999T mutation was detected in only one of the 98 primary hypertensive patients, but mutations were also in 12% of volunteers: four carried the C999T, three G1288C, one the G1140T mutation and one the 468ins.

The effect of C999T mutation was investigated in H295R cells using reporter vectors with the mutated or the wild-type (WT) TASK-2 promoters. TASK-2 gene expression was decreased by 31% (± 18, $P = 0.01$) in mutated, as compared to WT APA. Likewise, in transfected H295R cells, the C999T mutation decreased TASK-2 transcriptional activity by 35% (normalized luciferase signal fold change: 0.65 ± 0.25 $P < 0.001$).

Thus, mutations in the promoter region of the TASK-2 gene can account for the low expression in 25% of APA (**Fig. 2**, Panel B). After 16 years follow-up none of the healthy subjects developed hypertension or PA, indicating that either the predisposition carried by these gene variants require a very long time and/or may become apparent only late in life and/or other gene variants, yet unknown, are necessary for APA to develop. These variants could act as predisposing factors that, when associated with other genetics variations acquired by the adrenal gland, for example, somatic mutations in other genes involved in the autonomous production of aldosterone, and/or with environmental factors, can eventually concur to the development of PA in a relevant proportion of the cases.

Mutations of the WNT/β-Catenin Pathway

The role of WNT/β-catenin signaling in APA was also considered since transgenic mice with specific activation of β-catenin in the adrenal cortex showed increased aldosterone production due to aberrant adrenal cortex zonation (Berthon *et al.*, 2010). Canonical Wnt signaling has a key role in the development of the adrenal cortex by regulating the translocation of β-catenin to the nucleus, where it controls key gene expression programs through interaction with transcription factors (El Wakil and Lalli, 2011). A constitutive activation of WNT/β-catenin signaling was found in 70% of APA; it was not associated with mutations in the β-catenin

1 gene (CTNNB1), but with low levels of SFRP2 (Secreted frizzled related protein 2), a WNT signaling inhibitor (Berthon *et al.*, 2014). In a cohort of 198 APAs, somatic CTNNB1 mutations were found in 5.1% of the tumors and to be mutually exclusive from the other mutations in KCNJ5, ATP1A1, ATP2B3 and CACNA1D. All known mutations occurred on serine/threonine residues in the GSK3β binding domain in exon 3, suggesting activation of WNT signaling (Åkerström *et al.*, 2016).

According to another study on 219 APA patients, CTNNB1 mutations were found in 8 subjects (3.7%) 6 of whom women. Clinically, these patients had higher risk of postadrenalectomy residual hypertension as compared to the other APA patients, while molecularly they have heterogeneous expression of CYP11B1 and CYP11B2, and of the gonadal receptors LHCGR and GnRHR (Wu *et al.*, 2017). Thus, CTNNB1 mutations and activation of the WNT pathway in APAs seem to be more related to mechanisms causing tumor development, as suggested by the two hit model (Lalli *et al.*, 2016), rather than aldosterone overproduction (Zennaro *et al.*, 2017).

Other Genes Changes

Recently an over-expression of Calneuron -1, a Ca^{2+} binding protein of the endoplasmic reticulum, was reported in APA and found to be associated with increased endoplasmic Ca^{2+} and CYP11B2 expression (Kobuke *et al.*, 2018). As what matters for aldosterone biosynthesis is not endoplasmic Ca^{2+} content, but rather mitochondrial Ca^{2+} content, how this increased endoplasmic Ca^{2+} can lead to increased mitochondrial Ca^{2+} and whether the mitochondrial Ca^{2+} uniporter is involved needs further research.

Another gene seemingly over-expressed in APA is the CXCR4, a chemokine receptor, which was described to be up-regulated in about two thirds of a relatively small series of APA and to correlate with CYP11B2 expression (Heinze *et al.*, 2018). The relevance of this may be that a ligand for this receptor could be used in a PET MR imaging to detect APA noninvasively, which means without performing adrenal vein sampling (AVS). However, the limited data in support of this suggest extreme caution before this strategy can be proposed to replace AVS (Heinze *et al.*, 2018).

Conclusions

There is little doubt that there are no other fields of human hypertension where the progresses in understanding the molecular mechanisms have been so terrific as in primary aldosteronism in the last decades. The detection of expression changes of channels regulating K^+ and Ca^{2+} content has opened a new avenue in the knowledge of mechanisms regulating aldosterone secretion with implications for the development of pharmacological target treatment. The recent report that L168R and G151R KCNJ5 mutated cells are specifically sensitive to inhibition with macrolides and their derivatives (Scholl *et al.*, 2017), followed by our demonstration that clarithromycin blunts aldosterone synthesis and secretion in APA cells ex vivo (Caroccia *et al.*, 2017) testifies the clinical implications of these discoveries that open the way to in vivo studies in the M.A.P.A. trial (Maiolino *et al.*, 2018).

See also: Adrenal Venous Sampling for Primary Aldosteronism. Genetics of Familial Hyperaldosteronism. Mineralocorticoid Excess Syndromes. Primary Aldosteronism; Diagnosis and Treatment. Primary Aldosteronism; Epidemiology and Screening

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Apparent Mineralocorticoid Excess

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Background

Apparent mineralocorticoid excess (AME) is caused by impairment of the enzyme 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). AME is rare; worldwide less than 100 cases have been reported, typically characterized by early onset of hypertension with hypokalemia, metabolic alkalosis, low renin activity, and low aldosterone level. The first patient, described by Maria I. New and colleagues (New *et al.*, 1977; Ulick *et al.*, 1977), was a 3-year old Zuni Indian girl with the history of failure to thrive, low renin-aldosterone hypertension, hypokalemia, cardiomegaly who responded well to a salt restricted diet, and spironolactone. The condition was termed AME because the excess mineralocorticoid was not found. Later in 1979, Ulick and colleagues proposed that the cause of AME related to a defect in peripheral metabolism of cortisol to cortisone (Ulick *et al.*, 1979). In 1983, New and colleagues demonstrated that hypertension and hypokalemia could be induced in AME patients by infusing variable doses of cortisol; this discovery led to our understanding that cortisol plays a major role as a potent mineralocorticoid in such cases (Oberfield *et al.*, 1983). In 1988, Stewart and colleagues found that 11β-HSD2 deficiency of the kidney is the main pathological cause of this syndrome with excessive cortisol action at the mineralocorticoid receptor (MR) (Stewart *et al.*, 1988). Today we have a clear understanding of pathophysiology and the genetic molecular basis of AME.

Pathophysiology and Molecular/Genetic Basis of AME

Cortisol is a biologically active glucocorticoid which is essential for life. At a prereceptor and cellular level, glucocorticoid action is regulated mainly by 11β-hydroxysteroid dehydrogenase (11β-HSD) isoenzymes and the corticosteroid receptors, glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). The tissue-specific expression of 11 β-HSD's and GR/MR play an important role in regulation of the mineralocorticoid and glucocorticoid effects. In humans, two 11β-HSD isozymes have been described, 11 β-HSD-1 and 11β-HSD2, that interconvert active cortisol and inactive cortisone. 11β-HSD1 is a bidirectional enzyme capable of both 11-oxo reductase and dehydrogenase activity dependent on the available cofactor ($NADP/H^+$). The predominant reaction is the oxoreductase pathway critically dependent on high NADPH concentrations generated by hexose-6-phosphate dehydrogenase (H6PDH) within the endoplasmic reticulum. 11β-HSD1 is found in liver, lung, gonads, pituitary, brain, adipose, bone, muscle, and stromal tissues where activation of cortisol from cortisone facilitates cortisol action via the GR. On the contrary, 11 β-HSD2 is a high affinity nicotinamide adenine dinucleotide (NAD) dependent dehydrogenase that inactivates cortisol to cortisone. Expressed predominantly in mineralocorticoid responsive tissues, such as distal nephron, colon, and salivary glands protects the MR from cortisol (Chapman *et al.*, 2013). Circulating cortisol concentrations are 1000-fold higher than the native MR agonist aldosterone; inactivation of cortisol by 11β-HSD2 enables normal specificity for the MR to be maintained (**Fig. 1**).

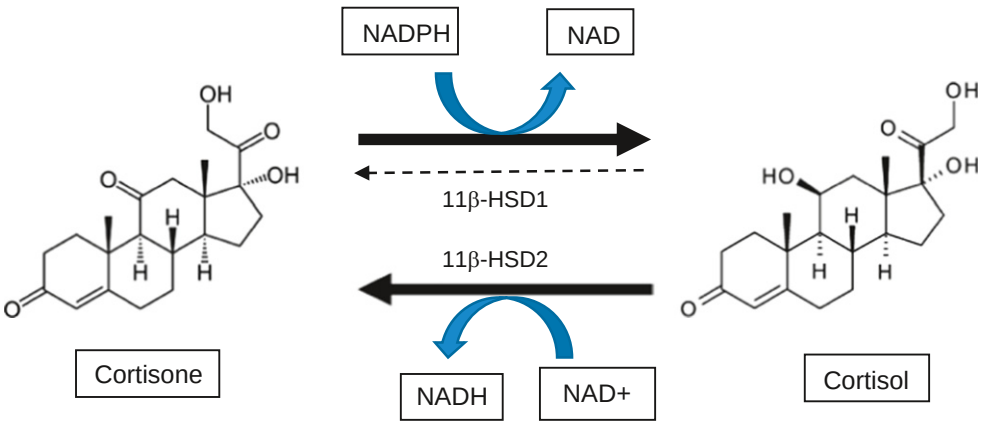

Fig. 1 Biochemical properties of 11β-hydroxysteroid dehydrogenases (11β-HSDs). Type 1 11β-HSD exhibits both oxoreductase (cortisone to cortisol) and dehydrogenase activities (cortisol to cortisone) in vitro, but in vivo it mainly functions as an oxoreductase. The type 2 enzyme exhibits only dehydrogenase activity (cortisol to cortisone).

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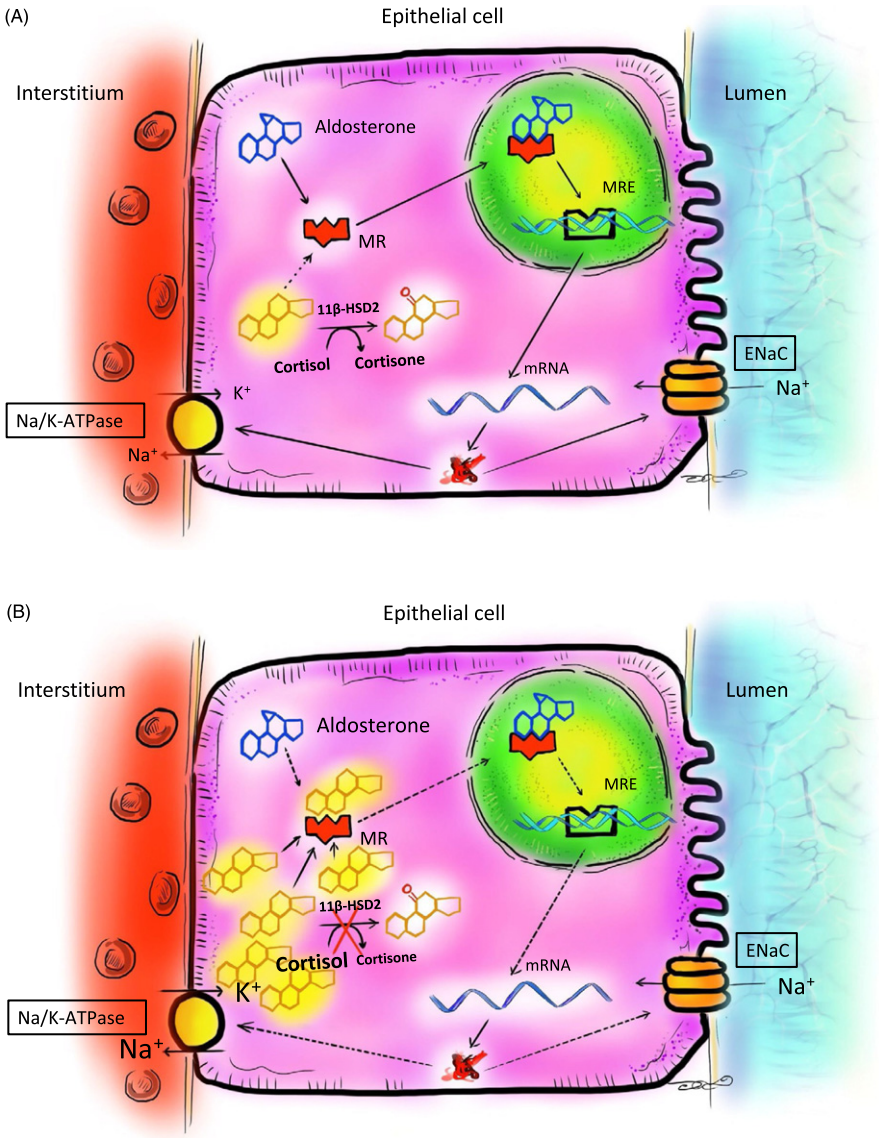

Fig. 2 A renal cortical collecting epithelial cell. (A) Normal physiology showing inactivation of cortisol by 11β-HSD2 and aldosterone binding to MR. (B) In states of 11β-HSD2 deficiency cortisol gains access to the MR. Illustrator Dr. Wisitsak Pakdee, Department of Radiology, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand.

Mineralocorticoids (aldosterone) play an essential role in intravascular volume control and electrolyte homeostasis through their action at the distal nephron (White, 1994). Indeed the classical well-known effect of MR activation by aldosterone is predominantly in the kidney, especially the renal distal tubules and cortical collecting ducts, called mineralocorticoid responsive tissues. MR activation by aldosterone leads to amiloride-sensitive sodium channel (ENaC) and Na^+–K^+-ATPase activation resulting in increased intravascular volume by Na^+ influx, K^+, and H^+ efflux (Fig. 2A). In vitro, aldosterone and cortisol have the same binding affinity for the MR, however in normal physiology cortisol cannot bind to the MR due to 11 β-HSD-2 inactivation which acts as a main protective role for cortisol-binding MR. Excessive amounts of aldosterone or cortisol, hypersensitivity of MR or decreased activity 11 β-HSD-2 enzyme result in Na^+ retention and urinary K^+ and H^+ loss with mineralocorticoid hypertension, characterized by hypertension and hypokalemic metabolic alkalosis.

In summary the pathogenesis of AME is impairment of 11β-HSD2 enzyme activity leading to cortisol-mediated mineralocorticoid activation of the MR, resulting in hypertension, hypokalemia, and metabolic alkalosis (Fig. 2B).

Inherited Form of 11β-HSD2

We now have a clear understanding of the genetic basis for AME. AME is a rare autosomal recessive condition with loss-of-function mutations of the HSD11B2 gene located on the long arm of chromosome 16 (16q22.1). The HSD11B2 gene is approximately

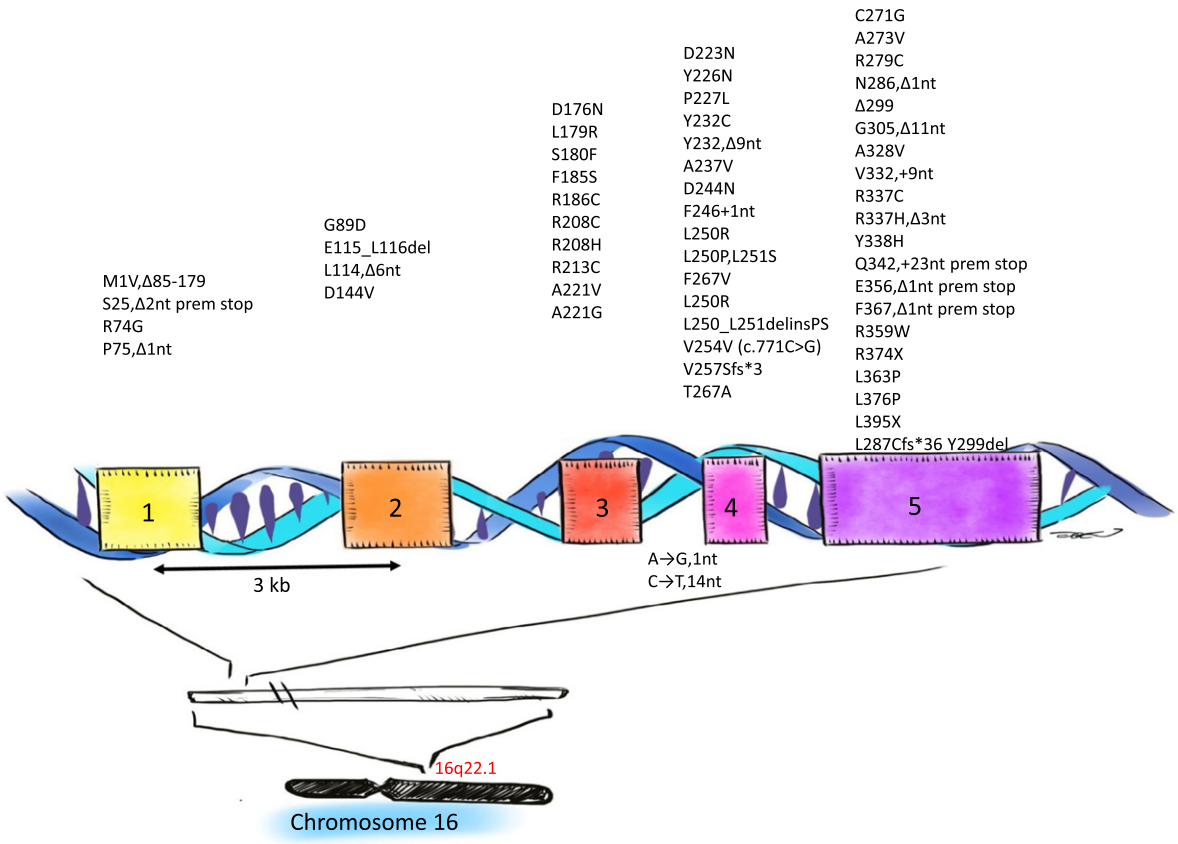

Fig. 3 HSD11β2 gene and the mutations identified in patients with AME. Illustrator Dr. Wisitsak Pakdee, Department of Radiology, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand.

6.2 kb length and contains five exons (Agarwal *et al.*, 1995). First detailed in the mid 1990s by Perrin White and Paul Stewart's group, **Fig. 3** summarizes the mutations reported to date in patients with AME. There is a close correlation between severity of mutation and the severity of diseases. More severe mutations result in reduced enzyme expression, earlier onset and more severe symptoms. Indeed patients with heterozygous mutation may present with a mild degree of late onset hypertension undifferentiated from essential hypertension. Epigenetic mechanisms such as promotor methylation or DNA methylation have been proposed as a contributor to the degree of 11β-HSD2 enzyme insufficiency and phenotypic expression (Alikhani-Koopaei *et al.*, 2004; Friso *et al.*, 2008; Pizzolo *et al.*, 2015). A family history of consanguinity is a part of autosomal recessive inheritance which is commonly found in some ethnic groups such as Iranian, Omani, and Native American (Dave-Sharma *et al.*, 1998; Quinkler *et al.*, 2004; Razzaghy-Azar *et al.*, 2017; Mune *et al.*, 1995; White *et al.*, 1997).

Acquired Deficiency of 11β-HSD2

An "acquired" form of AME is seen following excessive ingestion of 11β-HSD2 inhibitors such as liquorice. Liquorice contains glycyrrhetinic acid, glycyrrhizic acid and its hydrolysis product GE, both of which are potent competitive inhibitors of 11 β-HSD2 (Stewart *et al.*, 1987; Armanini *et al.*, 1983). The amount of liquorice that can produce hypokalemia was reported to be as low as 150 mg confectionery glycyrrhizic acid per week (Cumming *et al.*, 1980). In Stewart's seminal Lancet paper, 200 g daily of liquorice containing 580 mg of glycyrrhizic acid induced salt retention, hypertension, and hypokalemia. This was reversible after cessation of liquorice. Carbenoxolone was used to treat patients with peptic ulceration in the 1970–80s. As a hemi-succinate derivative of glycyrrhetinic acid it too caused severe mineralocorticoid excess via inhibition of 11β-HSD2. More recently flavonoids found in grapefruit juice can also cause a mild mineralocorticoid excess state. It is good clinical practice in all hypertensive patients to enquire about liquorice intake.

Clinical Characteristics

The common presentation of AME is childhood onset of hypertension with hypokalemia and metabolic alkalosis. The pediatric history of moderate intrauterine growth retardation, postnatal failure to thrive, or short stature are the presenting symptoms of

severe phenotypes. Nephrocalcinosis, nephrogenic diabetes insipidus, myopathy, rhabdomyolysis, and abnormal ECG may also occur, attributed to chronic prolonged hypokalemia. The homozygous mutations present with typical mineralocorticoid hypertension in the young. In youth, heterozygous mutations usually have a normal phenotype (Lavery *et al.*, 2003; Morineau *et al.*, 2006). The consequence of chronic severe hypertension causes target organ damage such as hypertensive retinopathy, chronic renal failure, cerebrovascular accident, and cardiovascular disease, and may lead to decreased life-expectancy in severe untreated patients. Milder forms of AME present with late onset, low renin hypertension which responds well to low salt diet and spironolactone.

Diagnostic Methods

Diagnosis of AME and other 11β-HSD2 deficient states is based on clinical characteristics of early onset of hypertension, hypokalemic metabolic alkalosis with low plasma aldosterone and renin activity. Biochemically urinary deficiency of 11β-HSD2 results in a high urinary cortisol/cortisone ratio, prolonged cortisol half-life, and reduced urine cortisone metabolism excretion (**Fig. 4**A). The assessment can be performed by measuring a 24-h urinary collection analyzed by gas chromatography/mass spectrometry for cortisol and cortisone metabolites and free cortisol/cortisone (**Fig. 4**B). The confirmation for inherited AME is genetic testing of the HSD11B2 gene.

Differential Diagnosis

Mineralocorticoid hypertension with low renin activity is usually caused by aldosterone excess. In patients where aldosterone is also suppressed other causes include Liddle syndrome, Cushing syndrome (both exogenous and endogenous), and activating mutations of the mineralocorticoid receptor and glucocorticoid resistance. Ectopic ACTH Cushing syndrome is another cause of mineralocorticoid excess with the history of rapid onset of low renin hypertension with profound hypokalemia. Here the high cortisol secretion rate swamps the renal 11β-HSD2 enzyme system. Liddle syndrome is a cause of monogenic endocrine hypertension that should be mentioned owing to the same clinical features as AME, early-onset severe hypertension, hypokalemia, metabolic alkalosis with strong family history of hypertension caused by hyperactivity of ENaC in renal epithelial cells. The genetic

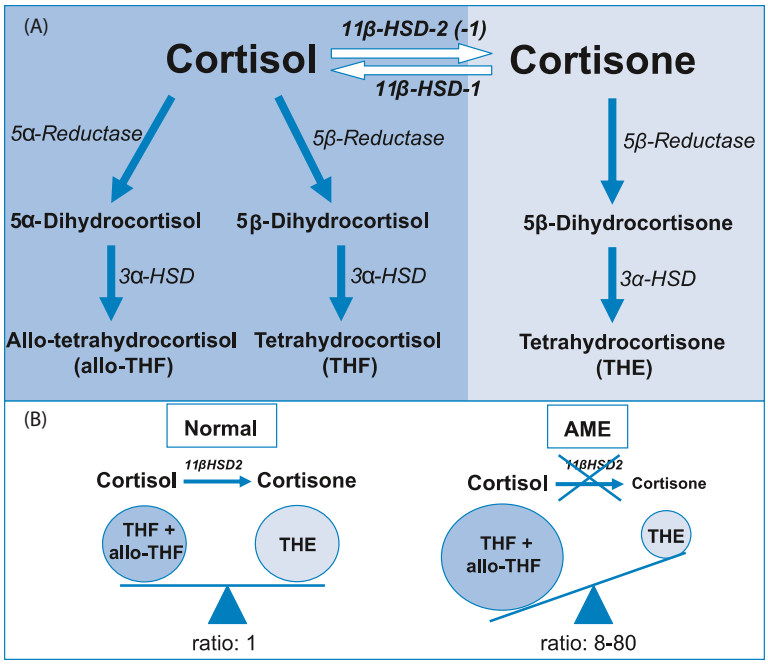

Fig. 4 (A) Schematic depiction of the enzymatic activity involved in glucocorticoid metabolism. Cortisol and cortisone are interconverted by 11β-(11β-HSD's). In the liver, 5- and 5 β-reductases and 3-hydroxysteroid dehydrogenases (HSDs) convert cortisol to 5-tetrahydrocortisol (allo-THF) and 5β-tetrahydrocortisol (THF) and convert cortisone to tetrahydrocortisone (THE). (B) In normal subjects urinary excretion of cortisol metabolites (THF and allo-THF) compared to the cortisone metabolite (THE) is equivalent, resulting in allo-THF, and THF/THE ratio of 1. Inactivation of 11β-HSD2 in apparent mineralocorticoid excess (AME) patients results in a grossly increased urinary excretion of cortisol compounds, whereas THE is dramatically reduced, resulting in a high [THF + allo-THF]/THE ratio. The urinary free cortisol/urinary free cortisone is also dramatically increased. Modified from (2006). *Best Practice & Research Clinical Endocrinology & Metabolism* ***20***(3), 341.

defect is an autosomal dominant disorder with mutations in the genes coding for ENAC at chromosome 16 p that responds well to amiloride that blocks the ENAC activity.

Treatment

The aim of treatment is to maintain normal blood pressure and serum potassium levels. Uncontrolled hypertension and profound hypokalemia causes target-organ damage especially for the brain, heart, retina, and kidneys.

1. Mineralocorticoid receptor antagonists
   1.1 Spironolactone
   Effectiveness of long-term outcome after spironolactone treatment has been shown with normalization of blood pressure, reversal of hypertensive retinopathy, left ventricular hypertrophy and nephrocalcinosis (Dave-Sharma *et al.*, 1998) and decreased morbidity and mortality. There are some concerns about antiandrogenic effects of spironolactone in male patients. The recommended dose is 2–12.5 mg/kg/day.
   1.2 Eplerenone is not approved for use in childhood, but may be given as an alternative.
2. Dexamethasone
   Cortisol is the offending mineralocorticoid in AME. Dexamethasone suppresses endogenous cortisol production by inhibiting the hypothalamic pituitary axis (HPA). Dexamethasone 1.5–2 mg/day as a starting dose followed by 0.5 mg/day is successful in normalizing blood pressure in many patients. There are some controversial issues for long-term treatment because some patients have worsening BP from dexamethasone treatment owing to dexamethasone binding to MR (New *et al.*, 1977; Lan and Baxter, 1982). Other adverse effects are steroid overdose or iatrogenic Cushing syndrome.
3. Low salt diet
4. Renal transplantation
   Patients are reported who have undergone renal transplantation. Disease cure and decreased morbidity, after transplantation, is documented (Razzaghy-Azar *et al.*, 2017; Palermo *et al.*, 2000; Khattab *et al.*, 2014).

Prognosis

The life-threatening condition from hypokalemia is cardiac arrest. Long-term complications of AME without treatment are dissecting aortic aneurysm, left ventricular hypertrophy, cerebrovascular diseases, and end stage renal disease. Long-term treatment with controlled hypertension results in a good prognosis. All of the evidence demonstrates that early detection, tight blood pressure control, and normalized potassium levels are important factors for these patients.

Conclusions

AME is an inherited form of hypertension caused by impaired 11B-HSD2 activity. Clinical clues are early onset of low renin-aldosterone hypertension with hypokalemia. The first recommended screening tools should be urine cortisone/cortisol metabolites with a positive result leading to genetic testing of the 11βHSD2 gene. Therapeutic goal is to maintain blood pressure, normalize serum potassium levels, and prevent long-term morbidity and mortality. Dexamethasone in doses to suppress endogenous cortisol secretion, spironolactone, and salt-restrictive diets are effective treatments.

See also: Mineralocorticoid Excess Syndromes

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Licorice and 11β-Hydroxysteroid Dehydrogenase

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Abbreviations

11HSD 11β-hydroxysteroid dehydrogenase
DOC Deoxycorticosterone
GA Glycyrrhetinic acid
GR Glucocorticoid receptors
MNL Mononuclear leukocytes
MR Mineralocorticoid receptors
PCOS Polycystic ovary syndrome
THE Tetrahydrocortisone
THF Tetrahydrocortisol

Glossary

Addison's disease Pathological condition characterized by primary adrenal insufficiency, mainly due to autoimmunitary cause, adrenal surgery, some drugs (like for example mitotane, and ketoconazole), severe bleeding involving both adrenal glands, or infections (especially tuberculosis, and AIDS).

Aldosterone Steroid hormone synthetized by the zona glomerulosa of the adrenal cortex. It is the main mineralocorticoid hormone, involved in the regulation of electrolyte and fluid balance.

Androstenedione Steroid hormone synthetized by adrenal zona fasciculata and reticularis and by ovarian stroma. It is a week androgen and an intermediate precursor of testosterone and estrogens.

Apoptosis Biological process of programmed cell death that occurs in multicellular organisms.

Carbenoxolone Derivate of glycyrrhetinic acid. It is used as antiulcerative and antiinflammatory treatment.

Cortisol Steroid hormone synthetized by the zona fasciculata of the adrenal cortex. It is the main glucocorticoid, involved in several functions in response to stress, inflammation, and energy metabolism.

Deoxycorticosterone Steroid hormone synthetized by the adrenal cortex. It is the precursor of aldosterone and possesses mineralocorticoid activity.

Diamide Chemical compound used as thiol-specific oxidant agent.

Diogenes Laertius Greek historian lived in the third century BC. He was the author of "Lives and Opinions of Eminent Philosophers," a biography of ancient Greek philosophers.

Dioscoride Greek physician, pharmacologist, botanist, lived in the first century BC. He was the author of the "De Materia Medica," an encyclopedia about herbal medicine, widely read for more than 1500 years.

Flavonoids Class of plant and fungus secondary metabolites. They are the most important plant pigments for fruits, vegetables, and flower coloration. There are several groups of flavonoids, with different biological and pharmacological properties, such as antiinflammatory, antioxidant, antibacterial, anticancer, and estrogen-like activity.

Glabridin Chemical compound of licorice root. It is an isoflavane, with antiinflammatory, antioxidant and estrogen-like activity. It is also a natural skin lightener, inhibiting the production of melanin.

Glycyrrhetinic acid Chemical compound of licorice root and derivate of glycyrrhizic acid. It possesses different biological and pharmacological properties, in common with its precursor and it also regulates the activity of 11 beta-hydroxysteroid dehydrogenase.

Glycyrrhizic acid One of the main constituents of licorice and precursor of glycyrrhetinic acid. It possesses a wide range of biological and pharmacological activities, including antiinflammatory and immune regulatory actions, antiviral and anticancer effects, inhibition of hepatic apoptosis and necrosis.

Heraclitus Pre-Socratic Greek philosopher lived in the sixth century BC. His famous aphorism "panta rhei," which means "everything flows," well characterized his thought about a continuous change as fundamental essence of the universe.

Insulin resistance Pathological condition characterized by decreased insulin sensitivity due to an alteration of insulin receptors or at the level of postreceptor signaling pathways. It causes a compensatory hyperinsulinemia to keep the concentration of serum glucose within the normal range. This condition can precede the development of diabetes and it is usually associated to obesity, sedentary lifestyle, hypertension, and many pathological disorders (such as polycystic ovary syndrome, and Cushing syndrome).

Naringenine Component of grapefruit, able to block the enzyme responsible of the activation of cortisone in cortisol.

Plinius Roman natural philosopher, naval and army commander of the early Roman Empire, lived in the first century BC. He was the author of the "Naturalis Historia," considered an editorial model for encyclopedias.

Polycystic ovary syndrome The most common gynecological endocrine disease, affecting about 15% of women in reproductive age. It is mainly characterized by hyperandrogenism, chronic oligo-anovulation and polycystic ovaries. It is also associated with different cardio-metabolic disturbances, in particular insulin-resistance, obesity, diabetes, dyslipidemia, and hypertension.

Pseudohyperaldosteronism Pathological condition characterized by hypertension and hypokalemia. It mimics hyperaldosteronism, but it is associated with low renin and aldosterone levels. It can be due to dietary (such as licorice)

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or genetic causes (like dysregulation of the epithelial sodium channel).

Receptor Protein molecule that receives chemical signals (ligands) and causes different specific biological response. It can be transmembrane and receives extracellular ligands or intracellular (cytoplasmic or nuclear receptor).

Spironolactone Steroid compound primarily used to treat resistant hypertension and fluid retention due to heart or liver failure, and kidney disease. It is also used as anti-androgen both for women (with acne and/or hirsutism) and for transgender males as hormone therapy. Its pleiotropic action is due to the block of mineralocorticoid and androgen receptors and to some estrogen and progesterone-like effects.

Theophrastus Greek philosopher succeeded to Aristotle in the Peripatetic school and lived in the third-fourth century BC. He is considered the "father of botany," for his work on plants.

11 Beta-hydroxysteroid dehydrogenase Enzyme responsible of the conversion of inert cortisone in active cortisol (type 1), and vice versa (type 2), regulating the interaction of glucocorticoids to the steroid receptors.

Licorice Characteristics

Licorice is a perennial plant, native to Mediterranean area and Asia Minor. Its genus is glycyrrhiza that accounts of 20 species. The most common species in Europe are glygyrrhiza glabra and echinata. Licorice has been studied since long time for its pleiotropic biological and endocrine properties.

The main constituents are triterpene saponins whose principal component is glycyrrhizic acid. Glycyrrhizic acid can be hydrolyzed into 18beta-glycyrrhetinic acid (GA) and two molecules of D-glucoronic acid. Other important constituents are flavonoids that account of 1%–24% of the root weight dependent on the species. The most important flavanoid is glabridin that has estrogen-like effect.

The 2500 Years Long Story of Licorice

Licorice was one of the most widely known medicinal remedies in the ancient history (Armanini *et al.*, 2002). The root had several properties cited by ancient philosophers, botanists and doctors that reported its use for stomach disturbances, cough, female sterility and wounds healing. Licorice medicinal use was known by Assyrians, Chinese, Hindu, Egyptians from around 2500 BC. In the third-fourth century BC Theophrastus reported that licorice root has the property of quenching thirst if one holds it in the mouth (Theophrastus, 1961). He also reported that the Scythians were able to survive 12 days in the desert without drinking water, because they chewed licorice root. Dioscorides in the first century BC gave the plant its botanical name (glukos = sweet, riza = root) and reported that the troops of Alexander the Great were eating licorice roots in order to allay their thirst in situations of water scarcity (Dioscorides, 1958).

In the first century BC, Plinius recommended licorice root to clear the voice and to alleviate thirst and hunger (Plinius, 1987). He mentioned that Roman legionnaires could march up to 10 days without eating or drinking because the licorice properties helped to increase strength and limit hunger. He claimed that licorice was also useful for female sterility.

During the middle age, licorice was popular in Arab medicine and in Avicenna's Canon licorice is reported as a remedy for several diseases (Fiore *et al.*, 2005).

From the middle age until now, the medicinal properties of licorice are known and actually studied to better understand their mechanisms of action.

It is surprising that most of these observations of ancient philosophers, botanists and/or doctors have been now confirmed and validated by scientific studies.

Endocrine and Not Endocrine Effect of Licorice

The most studied side effects of prolonged ingestion of licorice are hypertension and hypokalemia, due to the block of 11 beta-hydroxysteroid dehydrogenase type 2 (11HSD-2). More recently scientific studies have been focused on other endocrine beneficial effect of licorice or of some of its components, such as antiandrogen and estrogen-like effects and as reducer of fat mass. Licorice is also effective as antiviral compound stimulating the secretion of interferon gamma in T cells. Actually it is possible to study not only the extract of licorice root, but also the effect of single components evaluating their individual implications.

Mineralocorticoid Effect and the 11 Beta-Hydroxysteroid Dehydrogenase Enzymatic System

Mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) have a similar structure and they can be bound both by mineralocorticoids and glucocorticoids. The affinity of aldosterone and cortisol for MR is quite similar and considering that the

plasma concentration of cortisol is about thousand fold higher than that of aldosterone, one could derive that MR are only bound by glucocorticoids. In mineralocorticoid target tissues the preferential binding of aldosterone to MR is allowed by the presence in the cytoplasm of the enzyme 11HSD-2 that inactivates cortisol to cortisone (Funder *et al.*, 1988). In tissues lacking 11HSD-2 the MR is occupied by cortisol and in particular in the hippocampus the binding of cortisol to MR is involved in the regulation of circadian rhythm of ACTH (Armanini, 1994).

The interconversion of pharmacologically active cortisol and inactive cortisone is accomplished by two independent 11HSD that exhibit tissue-specific expression (Edwards *et al.*, 1988). Type I isoenzyme (11HSD-1) is expressed at high levels in the liver, where it catalyzes both the dehydrogenation and the reverse reductase reaction, and utilizes NADP+ or NADPH as cofactors. 11HSD-1 is also expressed in central nervous system and in adipose tissue, where it has a reductase function. 11HSD-1 has been characterized also in mononuclear leukocytes (MNL), where it probably modulates the inflammatory and autoimmune reaction (Fiore *et al.*, 2009).

11HSD-2 has a high affinity for related steroids and catalyzes only dehydrogenation utilizing NAD+ as a cofactor. It is mainly expressed in classical target tissues for aldosterone as kidney, salivary and sweet glands, colon, but it was also described in placenta, in some brain areas and in vessels. The enzymatic reaction is synthetically schematized in **Fig. 1**.

When the enzyme is blocked for a genetic or acquired deficiency, the mineralocorticoid effect of cortisol is evidenced by a clinical picture of pseudohyperaldosteronism and by the biochemical evaluation of urinary cortisol to cortisone ratio (Sabbadin and Armanini, 2016). The increased ratio of 5alpha and 5beta reduced metabolites of cortisol and cortisone is another consequence of altered 11HSD activity and therefore the increase of urinary ratio of tetrahydrocortisol + allo-tetrahydrocortisol to tetrahydrocortisone (THF + alloTHF/THE) is an index of impaired activity of 11HSD-2 (White *et al.*, 1997).

Licorice: The Block of 11HSD-2 and the Direct Effect on MR

In 1946 Revers firstly correlated the water retention and the urinary loss of potassium to the licorice ingestion (Revers, 1946). These effects were ascribed to the deoxycorticosterone (DOC)-like activity of licorice, being aldosterone still not discovered.

Following the discovery of aldosterone in 1953, licorice became an important matter of research after the report of hypertension and hypokalemia in subjects eating high amounts of the root or of its preparations.

In 1968 Conn defined the licorice-induced hypertension as a condition of pseudoaldosteronism, characterized by the presence of clinical signs and symptoms of hyperaldosteronism, but with suppression of renin and aldosterone levels (Conn *et al.*, 1968). A lot of reports were published about licorice-induced pseudohyperaldosteronism and actually the prevalence of this disease is sharply reduced due to the knowledge by the doctors and the patients about this risk (Penninkilampi *et al.*, 2017).

Pseudohyperaldosteronism syndrome is not only due to pure extracts of the roots, but it has also been reported during assumption of laxatives (Scali *et al.*, 1990), chewing, tobacco and beverages, where licorice was added for its specific effect or to ameliorate the taste.

The daily amount of licorice responsible for the side effects varies individually and it usually accounts to about 10 g a day of the pure preparation commercially available. However, it depends on individual characteristics of the patient and on the ability to hydrolyze glycyrrhizic acid to GA. In some cases licorice can produce a marked water retention, but not hypertension. Moreover

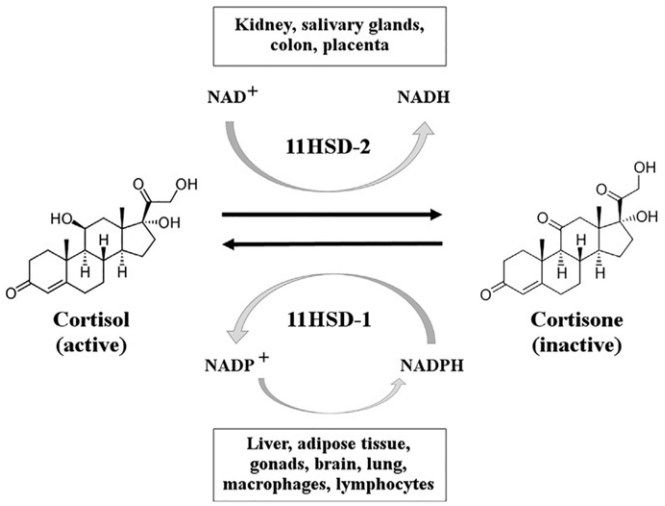

Fig. 1 Intercorversion of cortisol and cortisone by 11HSD. 11HSD-2 is a NAD-dependent dehydrogenase enzyme (that converts active cortisol into inactive cortisone); it is mainly expressed in the kidney, salivary glands, colon, and placenta. 11HSD-1 is a NADPH-dependent reductase (that converts cortisone into cortisol) and a dehydrogenase enzyme too; it is expressed in the liver, adipose tissue, gonads, brain, lung, macrophages, and lymphocytes.

the ingestion of high amounts of licorice could not produce pseudohyperaldosteronism in some subjects, while in other cases the disease could be evident after few days from the beginning of licorice intake at reasonable amounts.

The mineralocorticoid-like effect of licorice usually disappears after 2–3 weeks after the suspension of licorice ingestion in most of the cases, but some patients can also show persistent hypertension (Schambelan, 1994). In some severe cases with marked hypokalemia the treatment with aldosterone receptor blockers is needed to avoid general and in particular cardiovascular complications (Armanini *et al.*, 2014).

The diagnosis is made by the clinical history and eventually by the biochemical evaluation of hypokalemia and suppressed levels of renin and aldosterone. Confirmation derives for the recovery after withdrawal of licorice ingestion.

Therefore it is mandatory to perform an accurate history of all patients with hypertension particularly when potassium is reduced. The specific question about intake of pure licorice or preparations containing the substance is very important to avoid the prescription of exams not necessary, as for example the morphological evaluation of adrenals.

The same pattern of pseudohyperaldosteronism was also subsequently reported for carbenoxolone, a hemisuccinate of GA, used for gastritis and gastric ulcer. Prolonged intake of the drug leads to hypertension, hypokalemia and suppression of renin and aldosterone levels, as shown by in vivo studies in adrenalectomized rats treated with dexamethasone (Armanini *et al.*, 1982). The mechanism of action of carbenoxolone is related to the block of 11HSD-2 and to the direct binding to MR (Armanini *et al.*, 1989a).

It is worth of note that also prolonged ingestion of high amounts of grapefruit can produce a pseudohyperaldosteronism, caused by the component naringenine that blocks 11HSD-2, and the combination of grapefruit and licorice has a synergic effect (Palermo *et al.*, 2003).

When MR was discovered some studies demonstrated a low affinity of GA in kidney-slices MR (Ulmann *et al.*, 1975), in kidney cytosol (Armanini *et al.*, 1983) and later in intact mononuclear leukocytes (MNL) (Armanini *et al.*, 1989b). GA is able to bind to MR with a lower affinity than aldosterone (about 1:10,000 in kidney cytosol and 1:3000 in intact MNL), while carbenoxolone has an affinity of 1:13,000 for kidney MR (Armanini *et al.*, 1982; Armanini *et al.*, 1989a). Despite the low affinity, the chronic ingestion of pure licorice can produce high plasma concentrations of GA consistent with a direct effect at the level of MR (Armanini *et al.*, 1989b). The direct action has been demonstrated measuring the plasma MR active materials in plasma of subjects taking licorice. The study showed an increased binding of plasma mineralocorticoid active substances in adrenalectomized rat kidney cytosol (Armanini *et al.*, 1996).

The mechanism of the reversible block of 11HSD-2 by licorice could be related to a direct enzyme inhibition, to the enzyme saturation with high plasma concentrations of GA, or to a rearrangement of the enzyme structure following the interaction with GA.

In 1978 Epstein reported that cortisol was increased during licorice intake (Epstein *et al.*, 1978). The block of 11HSD-2 by licorice reduces the conversion of cortisol in cortisone in the classical mineralocorticoid target tissues, increasing the urinary cortisol to cortisone ratio.

In 1987 Stewart found that GA and carbenoxolone also inhibit 11HSD-1 (Stewart *et al.*, 1987). The enzyme is particularly expressed in adipose tissue and liver, but it has been found in other tissues as gonads, brain, lung, macrophages and lymphocytes. 11HSD-1 activates circulating cortisone to cortisol and a partial block of the enzyme could be implicated in some cases of low renin essential hypertension: the decreased cortisol availability due to the block of 11HSD-1 increases ACTH to normalize plasma cortisol values. This slight increase of ACTH could assess at higher levels of ACTH-dependent mineralocorticoids, as for example DOC and corticosterone or other minor mineralocorticoids, causing a pseudohyperaldosteronism (Davies *et al.*, 2009).

Licorice can also affect other enzymatic systems as 5beta-reductase in liver (Tamura *et al.*, 1979). This enzyme plays an important role in the regulation of the metabolism of cortisol and aldosterone in the liver. The inhibition of the enzyme leads to a reduction of corticosteroid clearance and to an increase of the biologic half-life of cortisol. Therefore, the increase of cortisol during licorice ingestion seems to be related to the block of 11HSD-2 and 5beta-reductase, while the effect on 11HSD-1 seems to be less relevant.

Licorice also binds to plasma binding proteins, as sex hormone binding globulin, corticosteroid binding globulin (Tamaya *et al.*, 1986) and albumin, displacing the physiological binders. By this mechanism the free fraction of the related steroids can be higher thus interfering in the clinical picture and in the mineralocorticoid, glucocorticoid and sex hormone concentration and function.

All these interactions between GA and steroid receptors, binding proteins, 11HSD and other enzymes have several clinical implications and are involved in the synergic increase of the mineralocorticoid effector mechanism.

All these studies confirm the observation of Theophrastus and other ancient authors about the effect of licorice of quenching thirst in situation of water scarcity. Theophrastus's observation was utilized during the Second World War for acclimating the American soldiers destined to Africa. These soldiers were exercised in a warm humid ambient taking DOC for resisting to the hot and humid climate of the African desert. This kind of adaption is the same used by the Roman soldiers and the Scythians, who ingested licorice roots to resist to climate adversities (Fiore *et al.*, 2006).

An interesting observation was also suggested about the death of Heraclitus. Diogenes Laertius reported that Heraclitus retired in the desert in Asia eating only roots and he became dropsy and subsequently dead. This condition could be related to the ingestion of licorice roots which grow everywhere in that region (Fiore *et al.*, 2008b).

Aldosterone Licorice and Inflammation

From the late 1980 aldosterone was studied as a potent proinflammatory hormone involved in atherosclerosis, fibrosis, and cardiovascular and cerebral risk (Pitt *et al.*, 1999). The administration of aldosterone receptor blockers was able to prevent these effects even in patients with normal aldosterone concentration, demonstrating that the inflammatory effect is not only caused by the high plasma aldosterone concentration, but also to different factors related to the individual ability of aldosterone to bind to MR and GR (Armanini *et al.*, 2014).

This new area of investigation of aldosterone was already hypothesized and validated in 1953, before the discovery of aldosterone, by Selye, who considered DOC having an inflammatory effect opposite to the antiinflammatory effect of cortisol (Selye, 1953). This concept was later lost and aldosterone was studied for several decades only as involved in the electrolyte balance and hypertension or in primary aldosteronism.

From the 1980 the inflammatory and profibrotic effect of aldosterone was studied and aldosterone became a dangerous hormone for its side effects at the level of heart and vessels. In the recent years the prescription of aldosterone receptor blockers became quite mandatory not only in patients with resistant but also with essential hypertension (Pitt *et al.*, 1999; Armanini *et al.*, 2014).

After the characterization of MR in MNL (Armanini *et al.*, 1985) and the demonstration of an effect of aldosterone on the regulation of intracellular electrolytes via genomic and nongenomic mechanisms (Wehling *et al.*, 1987; Wehling and Kuhls, 1989), studies have shown that GA is able to mimic the effect of aldosterone in MNL, leading to regulation of intracellular electrolytes (Armanini *et al.*, 1989b) and activation of oxidative stress (Calò *et al.*, 2004). In fact, incubation of MNL with a concentration of GA related to its affinity for MR had the same effect of the incubation with aldosterone, increasing the expression of two oxidative stress-related proteins, PAI-1 and p22 (phox). Both the effect of aldosterone and GA are reversed by coincubation with canrenone, the active principle of spironolactone (Calò *et al.*, 2004). These in vitro experiments confirmed also the importance of a direct effect of aldosterone at the level of MR independent from 11HSD-2.

Possible interactions between genomic and nongenomic effects of aldosterone and GA were also hypothesized and validated by in vivo and in vitro studies on plasma membrane of erythrocytes. Incubation of red cells with aldosterone induced well-characterized cell-membrane alterations (Bordin *et al.*, 2013), while GA prevented diamide induced band 3 Tyr-phosphorylation in a dose-dependent manner, showing high resistance to proteolysis (Fiore *et al.*, 2008a). From these studies, GA is proposed to strengthen membrane integrity against both oxidative and proteolytic damage. These studies have focused on the opposite effect of GA and aldosterone on plasma membrane of erythrocytes: high concentrations of aldosterone have negative effects reducing the life and increasing the apoptosis of erythrocytes, while GA has an opposite effect. It is interesting to note that canrenone is able to block the effect of aldosterone also in these anucleated cells, in agreement with a nongenomic effect of aldosterone mediated by MR (Bordin *et al.*, 2016). Maybe the direct effect at the level of plasma membrane of GA is due to a mechanism different than the binding to cytoplasmatic MR.

This effect on MNL and erythrocytes validates the concept that GA has an inflammatory effect due to the increased mineralocorticoid activity, but also an antiinflammatory effect due the action on plasma membrane.

All these studies confirm the ancient observation of a beneficial effect of licorice in ameliorating wounds and as antiinflammatory.

Glucocorticoid and Mineralocorticoid Effects

In 1951, before the discovery of cortisol, Groen reported a beneficial effect of licorice in Addison's disease (Groen *et al.*, 1951). Some affected patients were using spontaneously the licorice root, experiencing an amelioration of the symptoms and the clinical picture allowing a survival to the disease. In 1953 Borst demonstrated that licorice did not act directly but enhances the effect of even low concentrations of cortisol, producing better results on the survival of patients with Addison's disease (Borst *et al.*, 1953). In 1978 Epstein reported that cortisol was increased during licorice ingestion (Epstein *et al.*, 1978).

The effect of licorice in Addison's disease seems to be due to the block of 11HSD-2, increasing the effect of the small amount of cortisol secreted by the affected adrenals. Based on our studies, a direct effect of GA also via GR could be implicated, considering the low affinity of GA for GR and the high amount of GA measured in serum after long time consumption (Armanini *et al.*, 1989b). From all these reports GA has both a glucocorticoid-like action by binding to GR and blocking 11HSD-2 and an antiglucocorticoid action in tissues harboring 11HSD-1.

11HSD-1 is highly expressed in fat and therefore licorice can have a role in the modulation of fat mass. A previuos experiment evaluated the effect of topical application of a cream containing GA in the thickness of subcutaneous thigh fat: after 1 month the circumference of the leg and the fat thickness (measured by ultrasound analysis) was significantly reduced with the application of the cream (Armanini *et al.*, 1989b). This effect is probably due to the block of 11HSD-1 by GA in fat, thus decreasing the incorporation of triglycerides into the adipocytes mediated by cortisol. The same experiment showed that after 2 months of topical application the values of renin and aldosterone were not suppressed and blood pressure was unchanged, probably due to the low vascularization of fat and to a direct effect on the volume of adipocytes mediated by the reduced availability of cortisol. Moreover, in subjects eating licorice fat mass measured by bioelectrical impedance analysis was reduced, but weight did not decrease. This discrepancy could be explained by the fact that licorice reduces the fat mass by interacting with 11HSD-1 and increases the water reabsorption by interacting with 11HSD-2.

Licorice can have different effects at different levels depending on 11HDS-1 and −2, 5beta-reductase and the relative affinity for MR and GR. Obesity is often related to the genetic hyperactivity of 11HSD-1. Stewarts reported a higher 11HSD-1 expression in the visceral fat tissue (Stewart *et al.*, 1987). This situation has been called Cushing syndrome of the adipose tissue.

Licorice or carbenoxolone treatment could be useful in reducing local and visceral fat but in all cases the water retention can masque the reduced fat tissue.

Medical Uses of the Mineralocorticoid-, Glucocorticoid- and Antiglucocorticoid-Like Properties of Licorice

Licorice has a positive effect in patients with orthostatic hypotension linked to primary and secondary hypoaldosteronism. As already reported, it can be used in patients with Addison's disease when fludrocortisone in not available, having a mineralocorticoid-like action. In diabetic patients with severe microangiopathy the sclerosis of the juxtaglomerular apparatus can limit renin secretion particularly in orthostatic position, inducing a secondary hypoaldosteronism and orthostatic hypotension, which can be ameliorated by the administration of licorice or fludrocortisone (Basso *et al.*, 1994).

In patients on hemodialysis, Farese *et al.* reported that inhibition of the enzyme 11HSD-2 by GA reduces serum potassium levels and the frequency of hyperkalemia through the enhanced intestinal potassium loss (Farese, 2009). This finding could be an important tool to maintain predialysis serum potassium concentrations within safe limits in some dialysis patients at risk of hyperkalemic arrhythmias.

Licorice is also used in the treatment of patients with polycystic ovary syndrome (PCOS) in association with spironolactone (Armanini *et al.*, 2007; Armanini *et al.*, 2016). The daily amount of pure commercial licorice extract is about 5 g in fractioned doses associated with 100 mg of spironolactone.

Spironolactone is a blocker of MR, but it also acts as an antiandrogen: the drug binds to androgen receptor as an antagonist, blocks 17-hydroxysteroid dehydrogenase at the level of adrenals and gonads 5alfa-reductase, activates aromatase and increases sex hormone binding globulin (Sabbadin *et al.*, 2016). The association with licorice is very useful in the treatment of PCOS, since it reduces the side effects related to the diuretic activity and hyperkalemia of spironolactone; moreover, it potentiates the antiandrogen effect of spironolactone, reducing secretion of testosterone and androstenedione by blocking the 17 hydroxysteroid dehydrogenase and 17–20 liase (Takeuchi *et al.*, 1991; Armanini *et al.*, 1999). These patients show a systemic chronic inflammatory state and an increased cardiovascular risk, partially linked to increased aldosterone levels and aldosterone to renin ratio compared with healthy controls (Armanini *et al.*, 2012): this combined treatment could prevent and reduce this risk due to the antiinflammatory effect of both spironolactone and GA. Finally, the intake of licorice has also a positive action on the ovulatory rate due to its estrogen-like effect, especially in PCOS patients who want to get pregnant (Armanini *et al.*, 2007).

Licorice is effective in the chronic fatigue syndrome and its flavonoid constituent glabridine can be useful for reducing the menopause related affects of estrogen depletion (Baschetti, 1995).

Other beneficial effects of licorice are its use as antiviral remedy, stimulating beta interferon secretion by T lymphocytes, and as antimycotic, blocking the growth of vaginal candida (Fiore *et al.*, 2005).

Conclusive Remarks

Licorice is still a fascinating plant and an interesting topic of research, considering its multiple effects. Many reports have shown that licorice have an antibacterial, antiviral and antimycotic effect. In addition to the well-known hypertensive and antiinflammatory properties, licorice is very useful in some endocrine and gynecological disorders as antiandrogen and estrogen-like agent, and to blunt the potassium increase and/or hypotensive effect due to some drugs such as spironolactone in normotensive patients. The complex interaction of GA with 11HSD-1 and 11HSD-2 could be also useful in reducing fat mass by topical application and by oral administration.

Synthetic derivatives without mineralocorticoid-like properties have been recently developed, such as deglycyrrhizinated licorice, which seems to find a safe long-term application in gastroenterology, virology and in protection of atherosclerosis.

Its millennial use and the actual interest related to the beneficial and side effects of licorice are the reason for which a lot of research work is still in progress in many laboratories.

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See also: Mineralocorticoid Excess Syndromes

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Liddle Syndrome

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Glossary

Acid-sensing ion channels (ASICs) H^+-activated sodium channels.

Nedd4-2 A member of the Nedd4 (neural precursor cell expressed developmentally downregulated protein 4) family of the ubiquitin-protein ligases which ubiquitinates ENaC.

SCNN1A The gene encoding αENaC subunit.

SCNN1B The gene encoding βENaC subunit.

SCNN1G The gene encoding ɣENaC subunit.

Serum and glucocorticoid regulated kinase 1 (SGK1) An aldosterone-induced serine/threonine kinase.

Introduction

In 1963, Grant W. Liddle, a physician at Vanderbilt University (US-TN), reported on a family in which some members had severe hypertension and low plasma K^+ levels. The proband, a 16-year-old girl, and her adolescent brother, had hypertension, high urinary K^+ excretion, hypokalemic metabolic alkalosis, and negligibl
e urinary aldosterone excretion. Moreover, aldosterone administration resulted in a prompt decrease in urinary Na^+ excretion, which was consistent with the maintenance of the renal response to aldosterone. Spironolactone was ineffective on renal Na^+ and K^+ excretion. Conversely, triamterene, together with dietary sodium restriction, decreased urinary K^+ excretion, increased Na^+ excretion, and corrected both hypertension and hypokalemia. Proband's mother and grandmother had been hypertensive and died prematurely (Liddle *et al.*, 1963). Liddle hypothesized that the syndrome was a familial "disorder in which the renal tubules transport ions with such abnormal facility that the end result simulates that of a mineralocorticoid excess," but molecular mechanisms responsible for the disorder remained elusive for a long time.

About 30 years after the seminal work by Liddle, David G. Warnock, a nephrologist at the University of Alabama at Birmingham, serendipitously found that the proband reported by Liddle had developed end-stage renal disease and had successfully undergone deceased-donor kidney transplantation. This provided the opportunity to study the proband's extended pedigree, resulting in the finding of 18 family members suffering from Liddle syndrome (LS), with an autosomal-dominant inheritance pattern (Botero-Velez *et al.*, 1994). Around the same time, the subunits of the epithelial sodium channel (ENaC) were cloned and characterized by Cecilia Canessa and Bernard Rossier at the University of Lausanne, Switzerland (Canessa *et al.*, 1993, 1994b). Eventually, the collaboration between Warnock's group, Bernard Rossier's group, and Richard P. Lifton's group at Yale University led to the discovery of the genetic basis of LS. Genetic linkage analysis in the extended pedigree revisited by Warnock showed the complete linkage of LS with the locus encoding the β subunit of ENaC (Shimkets *et al.*, 1994). In addition, functional expression analysis in the *Xenopus laevis* oocyte system demonstrated an increase in the activity of the mutant compared with the wild-type ENaC (Schild *et al.*, 1995). Later, a gain-of-function mutation of the ɣ subunit of ENaC was identified in another LS kindred (Hansson *et al.*, 1995a).

Afterward, genetic testing of other LS kindreds identified several other mutations of either β or ɣ subunit of ENaC.

Lastly, a gain-of-function mutation of the α subunit of ENaC has recently been identified in a kindred with LS (Salih *et al.*, 2017).

Definition

LS is a rare hereditary condition, transmitted with an autosomal-dominant mode of inheritance, due to gain-of-function mutations in either *SCNN1A* or *SCNN1B* or *SCNN1G* genes, which encode the three ENaC subunits (α, β, γ). Clinical features include early-onset salt-sensitive hypertension, increased incidence of premature cardiovascular events, hypokalemic metabolic alkalosis, suppression of both renin and aldosterone secretion, and unresponsiveness to mineralocorticoid receptor antagonists, as opposed to ENaC blockers responsiveness (i.e., amiloride and triamterene) (Liddle *et al.*, 1963; Botero-Velez *et al.*, 1994; Warnock, 2001). Since the main clinical and biochemical features of LS, such as hypertension, hypokalemic metabolic alkalosis, and suppressed plasma renin activity, are typical of primary aldosteronism, LS has also been termed "pseudoaldosteronism," as aldosterone secretion is reduced in LS. This term, however, is inappropriate since it could be extended to any other form of Mendelian low-renin, low-aldosterone hypertension.

The Epithelium Sodium Channel and its Regulation

So far identified causative mutations of LS lead to constitutive activation of ENaC, a member of the epithelial sodium channel/degenerin family of cation-selective ion channels, which also includes the acid-sensing ion channels (ASICs). ASICs are H^+-

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activated sodium channels composed of different subunits assembled as trimeric structures, mainly expressed in the central and peripheral nervous system where they are involved in mechanoreception, chemoreception, and nociception. They share <20% identity with the ENaC subunits (Hanukoglu, 2017). ENaC is a heteromeric protein composed of three homologous subunits, α, β, and γ, sharing around 30%–40% sequence identity. Each subunit is encoded by a specific gene (*SCNN1A*, *SCNN1B*, and *SCNN1G*, respectively) containing 13 exons. *SCNN1A* is located on 12p13.31, while *SCNN1B* and *SCNN1G* reside within a 400-kb fragment on 16p13-p12. Each ENaC subunit consists of two transmembrane domains (TM1 and TM2), a large extracellular loop, and short cytoplasmic amino and carboxyl termini (Canessa *et al.*, 1993, 1994a,b) (**Fig. 1**).

Despite the lack of direct data about the three-dimensional structure of ENaC, it is proposed as a heterotrimer (1α:1β:1γ) according to the high-resolution crystallographic structure of ASIC-1 (Kashlan and Kleyman, 2011; Rossier *et al.*, 2015). Based on this homology model, the three subunits would be arranged around a central ion-pore, lined by the TM2 domains of the subunits. ENaC is highly selective for Na^+ and Li^+ ions, which are smaller than K^+, and is blocked by amiloride and triamterene, which share a common binding site proximal to TM2 domain and interact competitively with Na^+ ions. The specificity of ENaC for Na^+ depends on the size of the selectivity filter, which allows only the passage of the smallest ions (Na^+ and Li^+) and accounts for a Na^+-to-K^+ selectivity ratio between 100 and 1000. A molecular model based on the site-directed mutagenesis of ENaC subunits hypothesizes that the selectivity filter is formed by nine amino acid residues to which each subunit contributes a conserved sequence of three residues, differing between subunits (Gly560-Ser561-Ser562 for the α subunit, Gly531-Gly532-Ser533 for the β subunit, and Ser540-Cys541-Ser542 for the γ subunit) (Hanukoglu, 2017).

ENaC is expressed mostly in salt absorbing epithelia, that is, in the kidney, colon, lung, sweat, and salivary glands, but also in blood vessels, brain, and taste receptors of the anterior tongue (Kellenberger and Schild, 2015). In contrast to ASICs, ENaC is constitutively active, since it is neither ligand- nor voltage-gated (Hanukoglu, 2017).

In the kidney, ENaC is expressed in the apical (i.e., luminal) membrane of the principal cells of the aldosterone-sensitive-distal nephron (ASDN), which comprises the late distal convolute tubule (DCT2), the connecting tubule (CNT), and the entire collecting duct (CD). ENaC mediates the rate-limiting step in Na^+ absorption in the ASDN. Although ASDN reabsorbs <5% of the filtered load of Na^+, ENaCs are the final Na^+ transporters along the nephron, and their activity is finely regulated by hormones and local factors (**Fig. 2**). As a result, ASDN may reabsorb from zero to nearly 100% of the Na^+ entering the ASDN to precisely match the Na^+ excretion to the daily Na^+ intake (Kellenberger and Schild, 2015; Rossier *et al.*, 2013; Palmer *et al.*, 2012).

ENaCs at the apical membrane fluctuate between an open and a closed state, with an average open probability (Po) of 0.5 (Palmer *et al.*, 2012). The activity of ENaC, as assessed by electrophysiologic measurement of the amiloride-sensitive Na^+ current in cell expression systems, depends on Po and on the number (density) of channels expressed at the apical membrane of ASDN principal cells. The latter is determined by the balance between the rate of transfer of ENaC from intracellular sites to the apical membrane and the rate of ENaC internalization from the apical membrane. Proteolytic cleavage of ENaC is one of the mechanisms affecting the ENaC gating and Po. Indeed, the α and γ subunits exhibit either a high molecular weight form (uncleaved) or a lower molecular form (cleaved), and ENaC is fully active only in its cleaved state. The shift from the uncleaved to the cleaved form is the result of two cleavages by serine-proteases, which release imbedded inhibitory peptides within the extracellular domains of α and γ subunits. Furin, a serine-protease residing in the Golgi apparatus, cleaves the α subunit twice, releasing the inhibitory fragment of the subunit, and cleaves the γ subunit only once. The release of the inhibitory tract of the γ subunit requires a second cleavage by a membrane-bound serine-protease acting on the ENaC already inserted in the apical membrane (Shi *et al.*, 2013).

Aldosterone is the main regulator of ENaC, by interfering with the transcription, the redistribution of ENaC subunits from intracellular sites to the apical membrane, the retrieval of ENaC from the apical membrane, and the Po of the channels. Indeed, aldosterone induces *SCNN1A* transcription with a resulting increase in the expression of the α subunit, associated with the

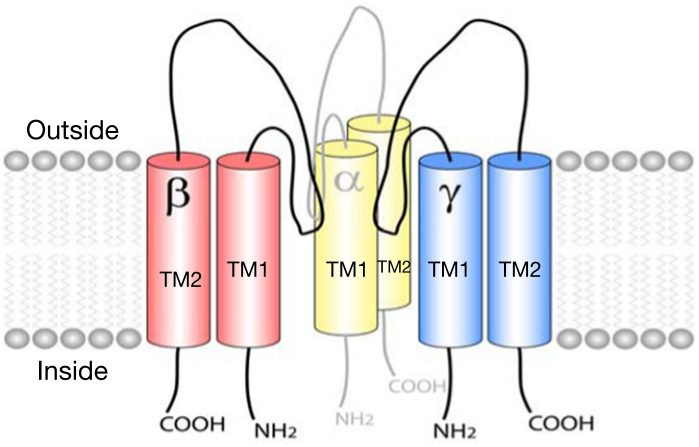

Fig. 1 Composition of the ENaC expressed on the luminal plasma membrane of ASDN.

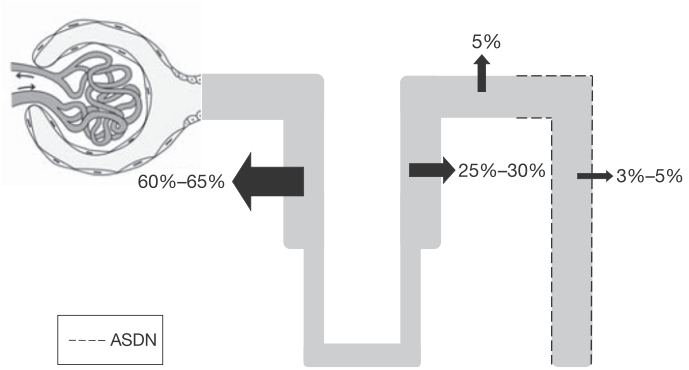

Fig. 2 Percent reabsorption of filtered Na^+ in the different segments of the nephron.

assembly of the whole ENaC. In addition, aldosterone induces the expression of other genes involved in regulating posttranslational modifications of ENaC subunits which result in an increase in both the number and the Po of ENaCs expressed at the apical membrane. In particular, aldosterone-induced serine/threonine kinase SGK1 increases ENaC activity through various pathways. In fact, SGK1 inhibits the removal of ENaC from the apical membrane by phosphorylating the ubiquitin ligase Nedd4-2 (see the next section). In addition, SGK1 directly phosphorylates the carboxyl terminus of the α-subunit of ENaC, resulting in increased Po of apical membrane ENaCs. Moreover, SGK1 also plays a role in promoting ENaC trafficking from intracellular compartments to the apical membrane (Kellenberger and Schild, 2015; Lou *et al.*, 2016).

Aldosterone regulates ENaC activity mainly in the collecting duct, while ENaC activity in the most proximal parts of ASDN (DCT2 and CNT) is partly aldosterone independent (Nesterov *et al.*, 2012).

Genetics and Molecular Mechanisms Responsible For LS

Thirty different LS-causing heterozygous mutations have been reported to date, occurring in familial or sporadic cases (Cui *et al.*, 2017; Salih *et al.*, 2017). Apart from αENaC C479R, βENaC R563Q, and γENaC N530S mutations (Salih *et al.*, 2017; Jones *et al.*, 2011; Hiltunen *et al.*, 2002), all other mutations cluster within exon 13 of either SCNN1B or SCNN1G, and consist of frameshift or nonsense or missense mutations that delete or alter a proline-rich PY motif, corresponding to the conserved sequence PPPXY (where X is any amino acid) in the cytoplasmic C-terminus of the β or γ subunit.

Only one αENaC mutation (C479R) responsible for LS has been reported so far (Salih *et al.*, 2017). Of the other 29 described mutations, 6 concern the γ-subunit. Of these, five cause deletion of the PY motif and one is a missense mutation (γ-N530S) located in the TM2 domain of the subunit, far from the PY motif (Hiltunen *et al.*, 2002; Hansson *et al.*, 1995a; Yamashita *et al.*, 2001; Wang *et al.*, 2007; Shi *et al.*, 2010; Wang *et al.*, 2015). Of the 23 β-subunit mutations, 12 cause deletion of the PY motif, 10 alter the sequence of the PY motif, and 1 (R563Q) is a missense mutation altering a sequence proximal to PY motif (Wang *et al.*, 2015; Shimkets *et al.*, 1994; Jeunemaitre *et al.*, 1997; Jackson *et al.*, 1998; Inoue *et al.*, 1998a,b; Melander *et al.*, 1998; Kyuma *et al.*, 2001; Nakano *et al.*, 2002; Hansson *et al.*, 1995b; Tamura *et al.*, 1996; Uehara *et al.*, 1998; Furuhashi *et al.*, 2005; Freundlich and Ludwig, 2005; Wang *et al.*, 2006; Rossi *et al.*, 2008; Sawathiparnich *et al.*, 2009; Gao *et al.*, 2013; Yang *et al.*, 2015; Cui *et al.*, 2017; Jones *et al.*, 2011).

No explanation has been proposed yet as to why differences in both frequency and mutation type between α, β, and γ subunits occur. Indeed, most mutations affect βENaC, and missense mutations altering the sequence of the PY motif exclusively concern this subunit (**Table 1**).

The PY motif plays a fundamental role in the internalization of ENaCs. PY motifs of all three ENaC subunits are the recognition site for the ubiquitin-protein ligase Nedd4-2, which binds these motifs and ubiquitinates N-terminal lysine groups of the α and γ subunits. Ubiquitination targets the ENaC for internalization and degradation by the lysosomal and proteasomal pathways, thus decreasing the number of ENaC expressed in the apical membrane (Staub *et al.*, 1997). In addition, ENaC ubiquitination decreases proteolytic cleavage of the channel, thus reducing the ENaC Po. Mutations causing deletion or alteration of the PY motif prevent Nedd4-2 binding to ENaC. As a consequence, internalization/degradation of ENaC fails to occur, resulting in an increased density of ENaCs at the apical membrane of the ASDN. Moreover, the inhibition of ENaC ubiquitination increases the fraction of cleaved ENaCs at the luminal membrane, thus increasing channel Po (Knight *et al.*, 2006). Although supported by abundant data, the above paradigm has recently been challenged by findings in genetically engineered mice with selective deletion of Nedd4-2 in kidney tubules. In this model, Nedd4-2 deletion leads to ENaC accumulation in the intracellular ENaC pool, but not at the plasma membrane, as expected. These results suggest that LS mutations might inhibit the Nedd4-2 mediated degradation of ENaC inside the cell and not the retrieval of ENaC from the cell surface (Ronzaud and Staub, 2014). Therefore, these findings suggest that the defective ubiquitination of ENaC by Nedd4-2 may not fully explain LS.

The inhibitory effect of LS mutations on Nedd4-2-mediated ENaC ubiquitination would be similar to one of the mechanisms mediating the aldosterone regulation of ENaC. Aldosterone induces the expression of SGK1 that phosphorylates Nedd4-2 on

Table 1 LS mutations altering the PY motif (^{616}PPPXY^{620}) of βENaC

Reference	*Amino acid residues and codons of the PY motif of βENaC*					
Shimkets *et al.* (1994)	Wild-type PY	Pro616 CCG	Pro617 CCC	Pro618 CCC	Asn619 AAC	Tyr620 TAT
Gao *et al.* (2013)	P616L mutant	*Leu CTG*	Pro617 CCC	Pro618 CCC	Asn619 AAC	Tyr620 TAT
Rossi *et al.* (2008)	P617L mutant	Pro616 CCG	*Leu CTC*	Pro618 CCC	Asn619 AAC	Tyr620 TAT
Inoue *et al.* (1998a,b)	P617S mutant	Pro616 CCG	*Ser TCC*	Pro618 CCC	Asn619 AAC	Tyr620 TAT
Sawathiparnich *et al.* (2009)	P617H mutant	Pro616 CCG	*His CAC*	Pro618 CCC	Asn619 AAC	Tyr620 TAT
Hansson *et al.* (1995b)	P618L mutant	Pro616 CCG	Pro617 CCC	*Leu CTC*	Asn619 AAC	Tyr620 TAT
Freundlich and Ludwig (2005)	P618H mutant	Pro616 CCG	Pro617 CCC	*His CAC*	Asn619 AAC	Tyr620 TAT
Uehara *et al.* (1998)	P618S mutant	Pro616 CCG	Pro617 CCC	*Ser TCC*	Asn619 AAC	Tyr620 TAT
Furuhashi *et al.* (2005)	P618R mutant	Pro616 CCG	Pro617 CCC	*Arg CGC*	Asn619 AAC	Tyr620 TAT
Tamura *et al.* (1996)	Y620H mutant	Pro616 CCG	Pro617 CCC	Pro618 CCC	Asn619 AAC	*His CAT*
Yang *et al.* (2015)	Y620L mutant	Pro616 CCG	Pro617 CCC	Pro618 CCC	*Gln619 CAA*	*Leu CTA*

Codon numbers correspond to the positions in *Homo sapiens*. In the literature, human β subunit mutations are sometimes numbered according to the sequence of *Mus musculus*, which is shifted down by two positions. The first nine mutations are missense mutations, while the last one is a frameshift mutation due to insertion of an additional cytosine between codons 618 and 619, which changes Tyr620 to Leu620 and introduces a stop codon at 621. Substituent amino acid residue and mutated codon for each mutation are indicated in italics.

serine residues, causing the binding of Nedd4–2 to 14-3-3 adaptor proteins that prevent Nedd4-2 interaction with ENaC, thus decreasing ENaC ubiquitination (Kamynina and Staub, 2002).

A transgenic LS mouse model carrying the same mutation (βENaC R566stop) identified in the original kindred observed by Liddle reproduces the human LS phenotype and exhibits an increase in both cell surface density and activity of ENaC in ASDN, which is consistent with the results of functional expression of LS mutations in heterologous cell systems (Rossier *et al.*, 2013; Pradervand *et al.*, 1999; Nesterov *et al.*, 2016). On the basis of the LS mouse model, the primary site of increased ENaC expression and activity corresponds to the most proximal parts of the ASDN, that is DCT2 and CNT, where ENaC activity is largely aldosterone independent. Moreover, LS mice exhibit increased responsiveness to aldosterone in the distal part of ASDN, which accounts for the persistence of residual ENaC expression and activity in the cortical CD, notwithstanding the low plasma levels of aldosterone that characterize LS. Thus, the failure to downregulate ENaC activity in the CD in response to enhanced Na^+ reabsorption in the proximal parts of ASDN, even when aldosterone level is maximally decreased, may contribute to Na^+ retention and salt-sensitive hypertension (Nesterov *et al.*, 2016; Dahlmann *et al.*, 2003). Furthermore, the maintenance of aldosterone responsiveness confirms the clinical observation by Liddle that aldosterone administration leads to a decrease in sodium excretion (Liddle *et al.*, 1963).

The three missense mutations altering sequences far from the PY motif (γENaC N530S, βENaC R563Q, and αENaC C479R) increase ENaC activity less than mutations which delete or alter the PY motif. Indeed, expression of either the mutant γENaC N530S or αENaC C479R in *Xenopus* oocytes demonstrated a twofold increase in ENaC activity, while mutations which delete or alter the PY motif increase the amiloride-sensitive sodium current from three to more than eightfold compared with the wild-type ENaC. In fact, both γENaC N530S and αENaC C479R mutations increase the channel Po, without changing the cell surface expression of ENaC (Hiltunen *et al.*, 2002; Salih *et al.*, 2017). As to the βENaC R563Q mutation, it may cause hypertension in affected subjects, but is associated with the full-blown LS phenotype only in a minority of patients (Jones *et al.*, 2011).

Interestingly, LS is the mirror image of the autosomal recessive form of pseudohypoaldosteronism type-1 (PHA-1), which is caused by loss-of-function mutations of either SCNN1A or SCNN1B or SCNN1G, leading to defective ENaC. The clinical picture of PHA-1 actually encompasses salt wasting, volume depletion, low blood pressure, metabolic acidosis, hyperkalemia, and high levels of plasma renin and aldosterone (Chang *et al.*, 1996). Thus, these two rare human diseases clearly confirm the fundamental role of ENaC in regulating electrolyte balance and blood pressure.

Pathophysiology of LS

In LS, the increased ENaC activity in the apical surface of ASDN leads to a greater Na^+ reabsorption, plasma volume expansion, high blood pressure, and consequent decrease in renin and aldosterone secretion. In addition, the increased Na^+ reabsorption through ENaC generates a transepithelial lumen-negative voltage that drives K^+ secretion across ROMK channels in the apical membrane of the principal cells of ASDN and facilitates the proton secretion by the proton pump in the intercalated cells of ASDN, resulting in hypokalemic metabolic alkalosis (**Fig. 3**).

Thus, LS should be regarded as a tubulopathy. Indeed, the proband reported by Liddle developed end-stage renal disease afterward, and 26 years after the original report she underwent deceased-donor kidney transplantation, which led to the normalization of plasma potassium level and improvement of hypertension (Botero-Velez *et al.*, 1994). Correction of hypokalemia and hypertension after kidney transplantation was also found in another patient with clinical features consistent with LS (Noblins *et al.*, 1992). However, emerging evidence suggests that in addition to renal Na^+ retention other factors contribute to hypertension in LS: ENaC may play an important role in BP regulation or dysregulation not only by virtue of its activity in the kidney, but also in the brain and in the vasculature. In the brain, ENaC is expressed in cardiovascular regulatory centers within the hypothalamus and

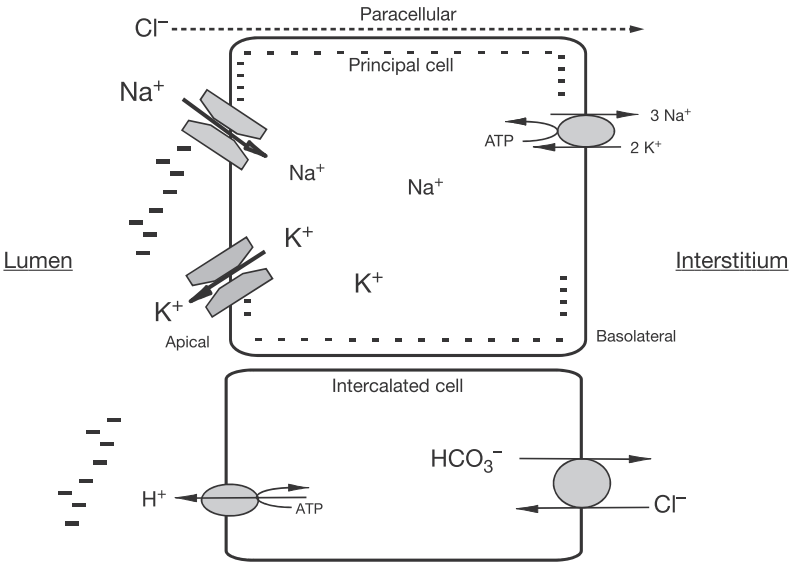

Fig. 3 Handling of Na^+ and K^+ in the cortical collecting duct (CCD). ENaC expressed in the cells of CCD allows Na^+ transport from the lumen into the cell along a favorable electrochemical gradient. Reabsorbed Na^+ is extruded from the principal cell of the CCD into the interstitium through Na^+/K^+ ATPase, and from the interstitium to peritubular capillaries via Starling forces. Both Na^+ flow through ENaC and Na^+ transport through Na^+/K^+ ATPase are electrogenic, and generate a lumen-negative transepithelial voltage, which leads to K^+ diffusion through K^+ channels (ROMK) from the cell to the lumen and facilitates the active H^+ secretion into the lumen associated with HCO_3^- generation in the intercalated cell.

in the choroid plexus epithelium. The increased ENaC expression in cardiovascular regulatory centers in the brain has been associated to sympathetic hyperactivity and hypertension in rat models of salt-sensitive hypertension (Fujita and Fujita, 2013).

In the Nedd4-2 knockout mouse, which may be considered an animal model of Liddle syndrome, increased expression of ENaC has been found in both choroid plexus and neurons. Moreover, intracerebroventricular (IVC) infusion of sodium-rich cerebrospinal fluid (CSF) caused an increase in blood pressure threefold higher than in wild-type animals, and this response was abolished by IVC infusion of an ENaC blocker. Therefore, it has been suggested that the increased ENaC expression in the brain may play a role in the hypertensive response to a high-salt diet through an increase in CSF Na^+ concentration and enhanced responsiveness to CSF sodium (Van Huysse *et al.*, 2012).

As to the role of ENaC in the vasculature, endothelial cells express amiloride-sensitive ENaCs. In contrast to the tight epithelium of ASDN, the endothelium is a leaky barrier, where the ENaCs do not play a role in Na^+ and H_2O transport, which instead is driven by Starling forces. At this level, ENaC is involved in regulating the mechanical properties of endothelial cells. Indeed, an increased expression of ENaC in the luminal membrane of the endothelial cells causes an increase in endothelial stiffness, which in turn decreases nitric oxide (NO) release. An increased endothelial ENaC expression causing endothelial stiffness, which is reversed by amiloride, has been observed in ex vivo vascular preparations from the LS mouse model carrying the βENaC R566stop mutation. Therefore, in LS the increased expression of endothelial ENaC may result in endothelial dysfunction, increase in vascular tone and blood pressure (Jeggle *et al.*, 2013).

These findings challenge the prevailing theory on the common pathways mediating the development of hypertension in the Mendelian human forms of salt-sensitive hypertension. In fact, on the basis of the classic Guyton theory, the expansion of intravascular volume due to the increased renal reabsorption of salt, with the resulting increase in cardiac output, would be the unique necessary and sufficient requirement for the initiation of salt-dependent hypertension. Increased peripheral resistance would contribute to hypertension only in the phase of stabilization of high blood pressure, through the autoregulation of tissue blood flow, which reestablishes a normal cardiac output at the cost of long-term persistence of hypertension resulting from the increase in systemic vascular resistance. In contrast with this prevailing theory, abnormalities in vascular function, resulting from increased expression of ENaC in the brain and in the vessels, would also be at play in both the initiation and stabilization of hypertension in LS, and presumably in other forms of salt-sensitive hypertension (Kurtz *et al.*, 2015).

This emerging paradigm is not at odds with the reversal of hypertension and hypokalemia after kidney transplantation in LS patients, as both Na^+ retention and vascular dysfunction are required for the development of salt-sensitive hypertension. Renal transplantation would normalize renal Na^+ and K^+ handling, with the correction of hypertension and hypokalemia as a result. However, endothelial dysfunction would not be reversed, with the possible persistence of an increased risk of cardiovascular events.

Lastly, ENaC is expressed in the taste bud cells of the anterior tongue, where it serves as the sodium taste receptor, activated by salt and inhibited by amiloride. Moreover, engineered mice lacking ENaC in taste bud cells lose any appetite for salt (Chandrashekar *et al.*, 2010). Even in nonhuman primates, an amiloride-sensitive component has been demonstrated in taste responses to salt stimuli (Hellekant *et al.*, 1997). In humans, the effects of amiloride in the perception of saltiness are controversial (Stahler *et al.*, 2008; Halpern,

1998; Heck *et al.*, 1984). Therefore, in LS patients an increased expression of ENaC in taste receptors could hypothetically lead to attraction to sodium salt, thus leading to high dietary sodium intake, which could contribute to hypertension.

Considering the emerging evidence that an increased expression and activity of ENaC in LS involves not only the ASDN but also the vasculature, the brain, and salt taste receptors, the canonic theory that LS exclusively represents a tubulopathy evidently turns out to be incomplete.

Epidemiological and Clinical Features of LS

LS occurs worldwide, with no ethnic or sex predilection, and is classified by Orphanet as a "rare disease", with a roughly estimated prevalence of $<1/10^6$ within the global population. Actually, no more than about 100 kindreds or sporadic cases have been reported so far, and most of them as case reports (Cui *et al.*, 2017). Though LS is considered a very rare condition, its prevalence in large cohorts of hypertensives has not been investigated so far. However, in a small cohort of 330 Chinese hypertensives aged between 14 and 40 years, LS was diagnosed and confirmed by genetic testing in five patients carrying different ENaC mutations, which corresponds to a prevalence of 1.52% (Halpern, 1998). In another Chinese cohort of 766 subjects with early-onset hypertension, 7 unrelated patients (0.91%) were diagnosed with genetically confirmed LS, with a prevalence of 1.72% among 407 patients with a diagnosis of hypertension before the age of 30 (Liu *et al.*, 2017). Genetic screening of the probands' relatives identified further patients with LS (12 and 10, respectively) in these study cohorts. These findings suggest that the prevalence of LS may not be as low as is commonly believed.

Probands with LS are most often diagnosed between the ages of 10 and 30 years, but some cases have been reported with an earlier onset, at the age of 4, 5, and 8, respectively (Freundlich and Ludwig, 2005; Ciechanowicz *et al.*, 2005; Polfus *et al.*, 2016). However, the diagnosis of LS in the affected relatives of LS probands is commonly delayed, with an interval between the ascertainment of hypertension and LS diagnosis ranging from years to decades, suggesting that LS is currently underdiagnosed and undertreated. Probands' family history often shows early-onset hypertension and premature cardiovascular events in several members across multiple generations of either maternal or paternal lineage, which is consistent with an autosomal-dominant inheritance pattern (**Fig. 4**).

The full-blown LS phenotype includes early-onset salt-sensitive hypertension, increased urinary K^+ excretion, hypokalemic metabolic alkalosis, suppression of both renin and aldosterone secretion, and unresponsiveness to mineralocorticoid receptor antagonists, as opposed to the response to ENaC blockers.

LS is associated with an increased risk of premature stroke, myocardial infarction, and sudden death. These complications are commonly attributed to longstanding misdiagnosed and undertreated hypertension. Indeed, early-onset hypertension is in itself associated with a greater cardiovascular risk than in those with late-onset hypertension, regardless of the cause of hypertension (Niiranen *et al.*, 2017). However, endothelial dysfunction due to increased expression of mutant ENaC in the endothelial cells might contribute as well. Moderate-to-severe hypokalemia (serum K^+ <3 mmol/L) may cause serious complications due to its effects on myocardium and skeletal muscle (**Table 2**).

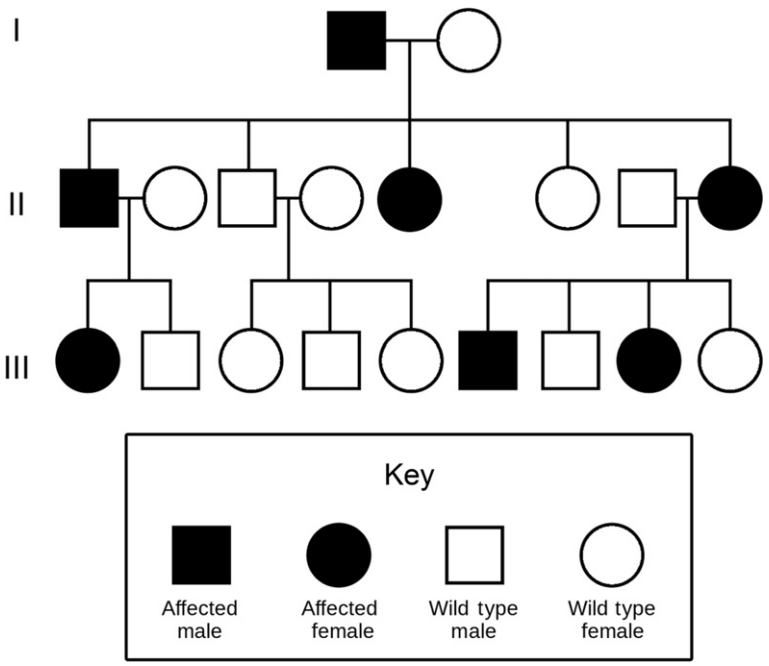

Fig. 4 Autosomal-dominant inheritance pattern.

Actually, LS exhibits remarkable phenotypic variability in age at presentation, degree of hypertension, and plasma potassium concentration. Hypertension may range from mild to severe and plasma potassium from very low to completely normal levels among patients within the same pedigree or unrelated patients with the same mutation, presumably as a result of both allelic variants of other genes and different dietary habits concerning sodium and potassium intake (Botero-Velez *et al.*, 1994; Hiltunen *et al.*, 2002; Tamura *et al.*, 1996; Rossi *et al.*, 2011). The variability of plasma K^+ concentration is reminiscent of other conditions characterized by increased ENaC activity, in primis primary aldosteronism. Indeed, screening of hypertensives for primary aldosteronism regardless of the plasma potassium values has impressively increased the prevalence of this condition from $<1\%$ to $>10\%$ among hypertensives, and showed that the majority of patients with primary aldosteronism are actually normokalemic (Rossi *et al.*, 2006). By contrast, a diagnosis of LS is currently considered exclusively in hypokalemic hypertensives, while normokalemic patients with LS are identified only following the genetic screening of the relatives of hypokalemic probands. Thus, the current approach may overlook many normokalemic patients with LS.

On the contrary, reduced plasma levels of renin associated with either low or normal values of plasma aldosterone are a constant finding in LS patients.

Diagnosis of LS

LS should be considered in all patients with early-onset hypertension, regardless of plasma K^+ concentration, even in the absence of a family history of early-onset hypertension, since de novo mutations may be responsible for sporadic cases of LS. The basal biochemical and hormonal alterations associated with LS are indicated in **Table 3**.

The following functional tests may confirm the increased activity of ENaC.

Cosyntropin (ACTH 1–24) stimulation test. The test is performed in the morning on the fasting patient. Blood samples for plasma aldosterone are taken before and 30 min after intravenous injection of Cosyntropin [ACTH 1–24] 250 μg. In LS patients, the aldosterone response is blunted or absent.

Transmucosal nasal potential difference. Since ENaC is also present in the nasal epithelium, its activity may be quantified by transmucosal electrical potential difference. Higher basal lumen-negative potential difference and greater change in potential difference after topical amiloride application have been observed in few related patients with LS compared with controls (Baker *et al.*, 1998). This test, which explores the ENaC activity "in vivo", has not been performed in other LS kindreds, thus leaving its diagnostic accuracy undefined.

Table 2 Clinical manifestations of moderate-to-severe hypokalemia

Neuromuscular system	Cramp Myalgia Paresthesia Weakness Paralysis Rhabdomyolysis
Cardiovascular system	EKG changes • prominent U waves • flattened or inverted T waves • S-T segment depression • T- and U-wave fusion (pseudo-prolonged QT interval) Arrhythmias • Ectopic supraventricular and ventricular beats • Atrial tachycardia with or without atrioventricular block • Ventricular tachycardia • Ventricular fibrillation • Torsades de pointes Sudden death
Kidney	Polyuria (acquired nephrogenic diabetes insipidus) Hypokalemic tubulo-interstitial nephropathy
Gastrointestinal tract	Constipation Paralytic ileus
Endocrine system	Insulin resistance Impaired insulin release

Table 3 Biochemical and hormonal features of Liddle syndrome

Plasma [K^+]	Low or normal
Plasma renin	Low
Plasma aldosterone	Low or normal
Plasma [HCO_3^-]	Increased or normal
Urinary K^+/24 h[a]	High or normal
Urinary aldosterone/24 h	Low
Urinary aldosterone-to'potassium ratio (ng/mmol)[b]	<60

[a]In the case of hypokalemic LS, a potassium excretion >30 mmol/24 h indicates an increased secretion of K^+ in the ASDN.
[b]A reduced ratio reflects an increased K^+ secretion resulting from an increased sodium reabsorption independent of the aldosterone drive.

However, both basal and functional tests cannot discriminate LS from other Mendelian forms of low-renin, low-aldosterone hypertension, also characterized by an increased ENaC activity independent of the aldosterone drive.

Comparison of the responses to spironolactone and triamterene. LS patients exhibit unresponsiveness to mineralocorticoid receptor antagonists, as opposed to the efficacy of ENaC blockers in correcting hypokalemia and reducing blood pressure. A 3-week trial of spironolactone 100 mg/day does not have effects on both plasma potassium and blood pressure. In contrast, a 3-week trial of either triamterene 100 mg/day or amiloride 10 mg/day corrects hypokalemia (at least partially) and reduces blood pressure. This test actually discriminates LS from other low-renin, low-aldosterone forms of hypertension. However, it is time-consuming, and not appropriate in the case of normokalemic LS patients.

DNA sequence analysis. Direct sequencing of the PCR-amplified final exons of both SCNN1B and SCNN1G is the most accurate tool to identify a causal mutation of LS. Since most mutations causing LS are clustered in a short DNA sequence, screening by direct sequencing of the exon 13 of both SCNN1B and SCNN1G is relatively simple, rapid, and feasible. In the event that no mutation of either SCNN1B or SCNN1G is found, genotyping SCNN1A may be needed, since a causative mutation responsible for LS has been recently identified (Salih *et al.*, 2017).

Restriction fragment length polymorphism (RFLP). Some LS mutations may create a restriction site for specific endonucleases which cut DNA within their recognition sequences (**Fig. 5**).

In this case, the enzymatic cleavage of exon 13 generates DNA fragments only in the mutant allele (**Fig. 6**). RFLP may represent a more rapid way to genetically screen the proband's relatives.

In view of the autosomal-dominant inheritance pattern of LS, each child has a 50% chance of inheriting the disease from an affected parent. Therefore, genetic testing of the offspring of affected patients is advisable, even in asymptomatic children or adolescents, since they may later develop the LS phenotype. Indeed, the finding of a LS mutation in asymptomatic children or adolescents warrants for a continued surveillance and appropriate lifestyle changes recommendations (such as dietary sodium restriction) aimed at delaying the clinical onset of LS.

Functional expression of the mutant ENaC. The genetic discovery of a novel ENaC mutation in patients with the LS phenotype does not necessarily imply its causal role. The conclusive proof of the mutation's pathogenicity comes from studying the activity of the mutant ENaC in expression systems, of which the *X. laevis* oocytes expression system is the most standardized. It consists of the injection of the mutant and the wild ENaC cRNA in oocytes, followed by the electrophysiological measurement of the amiloride-sensitive Na^+ current in the oocytes expressing mutant ENaCs compared with those expressing wild-type channels. However, these techniques are available only in few research laboratories.

Differential diagnosis. LS must be differentiated from other Mendelian forms of low-renin, low-aldosterone hypertension. While some of them exhibit a clearly specific clinical phenotype, mild forms of familial apparent mineralocorticoid excess syndrome (AMES) may be indistinguishable from LS. The autosomal-dominant inheritance pattern may help distinguish LS from AMES, which is an autosomal recessive disorder. As mentioned, however, LS may affect even patients without a family history of early-onset hypertension, since de novo mutations may occur (Uehara *et al.*, 1998; Yamashita *et al.*, 2001; Nakano *et al.*, 2002). In contrast to LS, AMES patients respond to both mineralocorticoid receptor antagonists and ENaC blockers. A key diagnostic marker of AMES is the increased urinary ratio of free cortisol to cortisone, or of reduced metabolites of cortisol to reduced metabolites of cortisone ([THF + αTHF]/THE), which definitively differentiates it from LS.

Therapy

Both hypertension and hypokalemia are corrected in most patients with LS by the use of an ENaC blocker associated with dietary sodium restriction.

ENaC blockers. Both triamterene and amiloride bind a specific site at the extracellular entry of the ENaC pore, where they block Na^+ flow into the cell. In the kidney, ENaC inhibition results in increased Na^+ excretion and decreased K^+ excretion, which normalize plasma volume, and plasma K^+ level. In addition, blocking the ENaCs expressed in the brain and vasculature might contribute to the correction of hypertension and reduction of cardiovascular risk.

Triamterene is a pteridine derivative (**Fig. 7**). Daily doses ranging from 100 to 300 mg are usually effective.

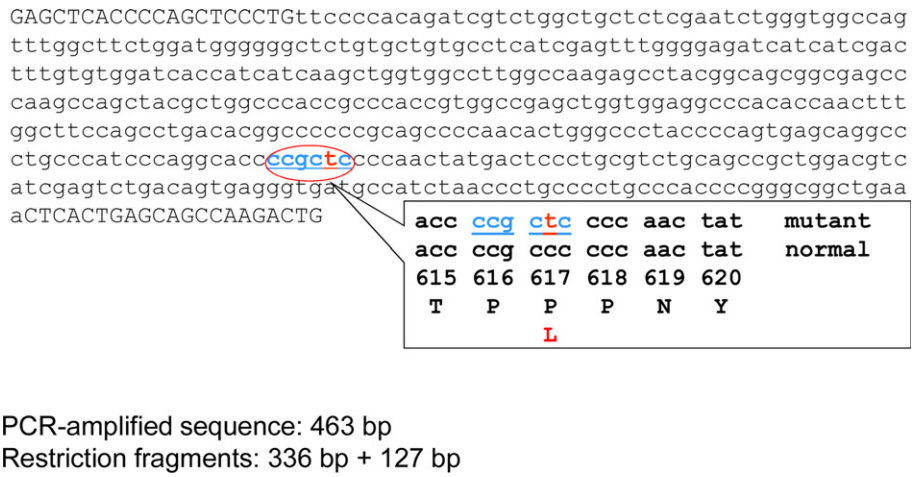

Fig. 5 Recognition site (ccgctc) for the *Bsr*BI endonuclease within the exon 13 of the mutant *SCNN1B* (βP617L). The BsrBI endonuclease recognizes a sequence containing the mutated codon 617 (ctc), resulting in the cleavage of the 463 bp exon 13 of *SCNN1B* into two fragments of 336 and 127 bp.

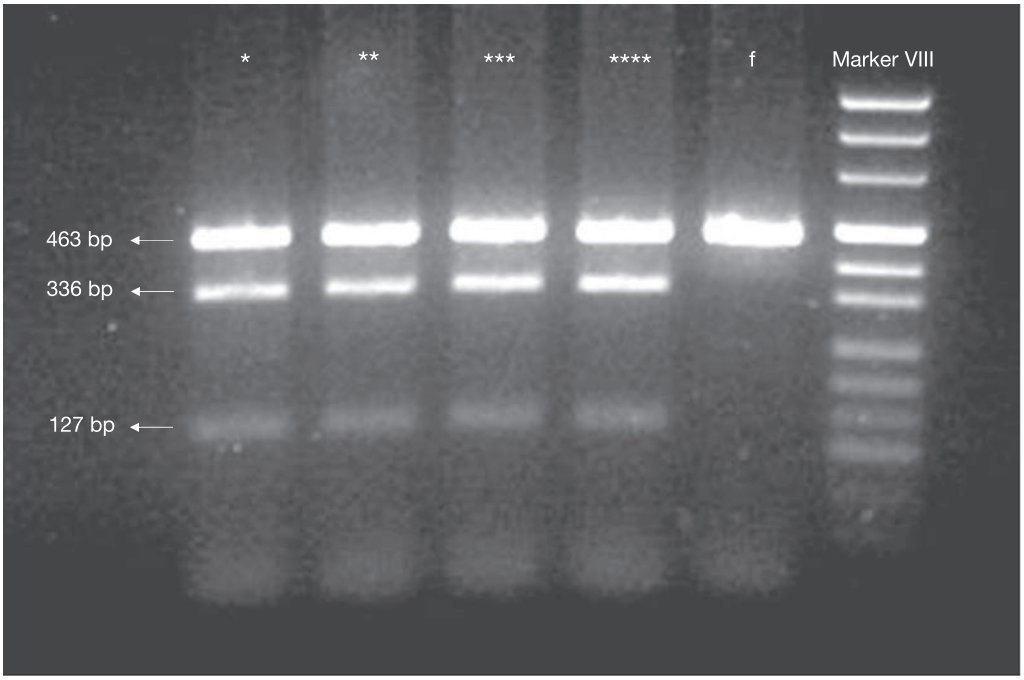

Fig. 6 Restriction fragment length polymorphism (gel electrophoresis) in a kindred carrying the βENaC P617L mutation. A band corresponding to the wild-type allele (463 bp) is present in all subjects. The bands of 336 and 127 bp (resulting from the enzymatic cleavage of the mutant 463 bp allele) are present only in the affected members of the family. *, proband; **, proband's mother; ***, proband's maternal uncle; ****, proband's grandmother; f, proband's father.

Amiloride, an aminopyrazine derivative, is an open-chain analog of triamterene (**Fig. 7**). Its potency is much greater than that of triamterene, and effective daily doses vary from 10 to 30 mg.

The dose of either triamterene or amiloride needs to be titrated based on the effects on serum potassium level and blood pressure values. In addition, the use of an ENaC blocker should be associated with dietary sodium restriction, since these drugs compete with Na^+ ions at the level of the ENaC pore. Therefore, an excessive dietary intake of salt, through an increase in sodium delivery to ASDN, limits the effects of ENaC blockers and maintains both plasma volume expansion and renal potassium wasting.

Both plasma renin activity and plasma aldosterone levels may remain low after several months of treatment with an ENaC blocker, despite the normalization of BP and serum K^+. Indeed, chronic volume expansion caused by the activation of ENaC might lead to a sort of "disuse atrophy" of juxtaglomerular cells in the kidney and glomerulosa cells in the adrenal cortex.

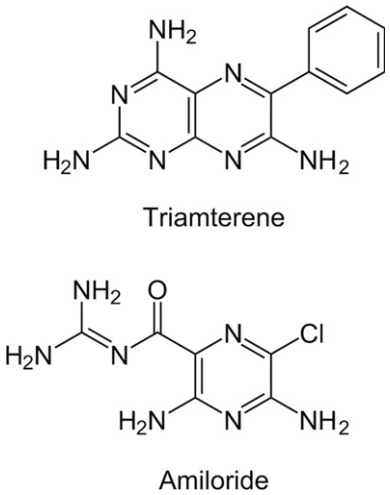

Fig. 7 Chemical structure of triamterene and amiloride.

Amiloride or triamterene alone are often adequate to normalize blood pressure and plasma K^+ concentration. However, in several LS patients, conventional antihypertensive drugs (such as calcium-channel blockers) need to be added for achieving optimal blood pressure control.

Treatment of moderate–severe hypokalemia associated with LS. Severe and symptomatic hypokalemia may require oral or intravenous potassium replacement. Potassium chloride must be preferred to other K^+ salts, since both K^+ and Cl^- administration contribute to correct the coexisting metabolic alkalosis.

Treatment of LS during pregnancy. Hypertension in pregnancy is a major cause of maternal, fetal, and neonatal morbidity and mortality. However, although LS affects women of childbearing age, the optimal management of hypertensive pregnant women with LS is unclear and entails several challenges. Commonly recommended antihypertensive drugs in pregnancy (such as methyldopa, sustained-release nifedipine, and labetalol) may be ineffective in patients with LS. Moreover, diuretics are discouraged in pregnancy, since the sodium retention and the resulting increase in maternal plasma volume, progressively occurring throughout normal pregnancy, ensure an optimal uteroplacental perfusion. Indeed, pharmacological blockade of ENaC in a late pregnant rat prevents sodium retention and maternal volume expansion, with fetal growth restriction as a result (West *et al.*, 2014). On the other hand, in the setting of LS, the volume expansion associated with pregnancy would be excessive and responsible for hypertension, given the basal constitutive activation of ENaC in ASDN.

No randomized trial evaluating the safety profile of ENaC blockers during gestation has been performed to date.

Triamterene is a folic acid antagonist, and its use in pregnancy, at least in the first trimester, may be associated with an increased risk of congenital malformations, such as neural-tube defects, cardiovascular defects, and oral clefts (Hernandez-Diaz *et al.*, 2000). Therefore, its use is not advisable in pregnancy, especially in the first trimester.

With regard to amiloride, teratogenicity studies in rabbits and mice revealed no evidence of harm to the fetus, although a decrease in rat pup growth was observed at doses five or more times the expected maximum dose for humans.

Only two case reports concerning the use of amiloride in pregnant patients with LS have been reported to date. In both cases, amiloride, which was administered from the 25th and 18th gestational week respectively, proved to be effective with no maternal or fetal adverse effects (Caretto *et al.*, 2014; Awadalla *et al.*, 2017). Moreover, amiloride has also been used in pregnant women with either primary aldosteronism or Gitelman/Bartter syndromes, at daily doses ranging from 15 to 30 mg, started at a gestational age from 1 to 17 weeks, with no maternal or fetal adverse effects (Al-Ali *et al.*, 2007; Krysiak *et al.*, 2012; Morton, 2015; Mathen *et al.*, 2013; Deruelle *et al.*, 2004). Increasing doses of amiloride may be needed to control blood pressure as gestational age advances, possibly due to the progressive increase in the expression of mutant ENaCs. After delivery, the dose of amiloride should be reevaluated in relation to possible and excessive decrease in blood pressure. Although it is not known whether amiloride is excreted in human milk, in rats the drug is excreted in milk in concentrations higher than those found in blood. Because of potential adverse effects in nursing infants, a decision should be made whether to discontinue nursing or to discontinue amiloride, taking into account the importance of the drug to the mother.

Conclusions

Several reasons warrant a more extensive search and earlier diagnosis of LS in hypertensive patients. Prevalence of LS may be higher than that commonly perceived. Patients with misdiagnosed LS are at high risk of premature cardiovascular events. Lastly, ENaC blockers together with dietary salt restriction are impressively efficient in both reversing the clinical manifestations and preventing severe complications.

See also: Mineralocorticoid Excess Syndromes

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Secondary Hyperaldosteronism

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Definition

Secondary hyperaldosteronism is defined as an increased aldosterone production resulting from extra-adrenal stimulation of adrenal gland (Corry and Tuck, 1995; Kaplan and Victor, 2015; Schrier, 2011; Young *et al.*, 2017).

Background and Pathogenesis

Extra-adrenal stimulus for increased aldosterone production can be a single factor or an interplay of a number of regulatory factors. Secondary hyperaldosteronism might be a physiological and pathophysiological consequence of reduced "effective" arterial blood volume arising from a circulatory disorder such a heart failure or severe hypertension, leading to renal hypoperfusion and to augmented renin secretion. As a consequence of renin-angiotensin system activation, angiotensin II, together with known modulators, increases aldosterone secretion. In some of these clinical conditions, for example, electrolyte depletion from vomiting or diuretic use, hyperaldosteronism should be regarded more as compensatory effect, lessening when the triggering factor is relieved (Corry and Tuck, 1995; Kaplan and Victor, 2015; Schrier, 2011; Young *et al.*, 2017).

Renovascular Hypertension

Renovascualar hypertension (RVH) is one of the most frequent causes of secondary hypertension. Renal artery stenosis (RAS) the most frequent cause of RVH leads not only to the development of hypertension, but also results in ischemic nephropathy and chronic kidney disease. De Mast and Beutler reviewed the atherosclerotic RAS and estimated the following prevalence of atherosclerotic RAS in various populations: hypertension and diabetes (20%), coronary angiography (10.5%), coronary angiography in hypertensive patients (18%), coronary angiography and suspected renovascular disease (17%), heart failure (54%), peripheral vascular disease (25%), abdominal aortic aneurysm (31%) and end-stage renal disease (41%). However, the existence of RAS does not inevitably leads to RVH. Therefore, these two terms—RAS and RVH—cannot be paralleled. RVH is much less common than is RAS. Prevalence of RVH is estimated as less than 1% of general hypertensive population. In a study of Anderson *et al.*, which included 4429 hypertensive patients, RVH was found in 3.1% of patients. RVH is more frequent in patients with resistant hypertension and malignant hypertension, as well as in elderly which are characterized by higher incidence of atherosclerotic RAS. In a study of Morganti *et al.* the prevalence of RVH was 6.4% in patients with atherosclerotic RAS and 31.3% in patients with RAS caused by fibromuscular dysplasia (FMD) (Kaplan and Victor, 2015; de Mast and Beutler, 2009; Giavarini *et al.*, 2013; Dzielinska *et al.*, 2007; Anderson *et al.*, 1994; Hansen *et al.*, 2002).

The role of the renin-angiotensin-aldosterone system in the pathogenesis of RVH in patients with atherosclerotic RAS is widely accepted. In the early stage of RVH, significant renal ischemia causes marked rise in renin secretion. This further leads to the activation of the renin-angiotensin-aldosterone system. In spite of subsequent hyperaldosteronism, blood volume remains unchanged because of natriuresis in the contralateral kidney. In the next stage, hypertension induced nephrosclerosis, enhancing kidney failure, leads to a progressive expansion of blood volume, subsequently reducing renin secretion. However, renin and angiotensin II are still inappropriately elevated in relation to blood volume expansion (Kaplan and Victor, 2015; de Mast and Beutler, 2009; Giavarini *et al.*, 2013).

It should be noted, that in addition to the activation of the renin-angiotensin-aldosterone system, several other factors and mechanisms contribute to the elevation of blood pressure and progression of kidney failure in RVH. These include increased sympathetic nervous system activity and increased secretion of endothelin-1. Recently it has been also postulated that natural history of atherosclerotic RAS consists of three stages: (1) reduction of blood flow is compensated by a reduction in GFR what preserves renal tissue oxygenation (2) advancing RAS causes renal cortical hypoxia which in turn induces production of inflammatory cytokines and fibrotic pathways, which may result in (3) irreversible damage to the microvasculature (pruning of renal microvessels) (Chrysochou *et al.*, 2012; Textor and Lerman, 2013; Gloviczki *et al.*, 2010; Saad *et al.*, 2013).

When comparing with atherosclerotic RAS, not all pathophysiological mechanisms causing hypertension described above can be extrapolated to patients with FMD related RAS. Recent data indicate, that in patients with renal FMD pathogenesis of hypertension caused by RAS is complex. On one hand it is believed that decrease in renal blood flow is stimulating the renin-angiotensin system—particularly in the early stage of the disease—and result in increased production of angiotensin II. It has been documented in a small group of FMD patients that successful renal angioplasty followed by decrease in BP and subsequent down-regulation of the renin-angiotensin system may decrease oxidative stress resulting in bioavailability of nitric oxide and inhibition of production of angiotensin II (Higashi *et al.*, 2002; Petruzzelli *et al.*, 2016).

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On the other hand the recent findings of van Twist *et al.* argue against the hypothesis that FMD induces hypertension via similar pathophysiological mechanism as in atherosclerotic RAS since renal blood flow was significantly higher in FMD as compared with atherosclerotic RAS. In contrast to patients with unilateral atherosclerotic RAS lateralization in renin secretion was not found in unilateral FMD and systemic and local renin secretion was lower in FMD as compared with atherosclerotic RAS (van Twist *et al.*, 2017).

The most common cause of RAS is atherosclerotic ($\approx$85%), FMD is the second most common cause ($\approx$15%). Clinical signs of RVH include resistant hypertension, deterioration of renal function or, rarely, flash pulmonary edema. **Table 1** details clinical situations raising suspicion of RVH (Young *et al.*, 2017; Mancia *et al.*, 2013).

In patients with a clinical suspicion of RAS, doppler ultrasonography is the first-line imaging modality to screen for significant RAS ($\geq$60% stenosis). The sensitivity and specificity of peak systolic velocity in the main renal artery for RAS has been estimated, respectively, as 85% and 92%. Multidetector computed tomography angiography and magnetic resonance angiography for detection of RAS showed equally high sensitivities (64%–100% and 94%–97%) and specificities (92%–98% and 85%–93%). However digital subtraction angiography (DSA) remains the gold standard for the diagnosis of RAS (Aboyans *et al.*, 2018).

Treatment strategies for RAS have been recently summarized in the European Society of Cardiology (ESC) 2017 guidelines (Aboyans *et al.*, 2018):

- Angiotensin converting enzyme inhibitors or angiotensin receptor blockers are recommended for treatment of hypertension in patients with unilateral RAS (class I, level B recommendation);
- Those drugs can be considered also in bilateral severe RASS or in patients with stenosis in a single functioning kidney, if well tolerated and under close supervision (class IIb, level B recommendation);
- Calcium channel blockers, beta-blockers and diuretics are recommended for treatment of hypertension in patients with RAS (class I, level C recommendation);
- In atherosclerotic RAS routine revascularization is not recommended (class III, level A recommendation);
- In FMD RAS in case of hypertension and/or signs of renal impairment, balloon angioplasty with bailout stenting should be considered (class IIa, level C recommendation);
- In selected patients with RAS and unexplained recurrent congestive heart failure or sudden pulmonary edema, balloon angioplasty, with or without stenting, may be considered (class IIb, level C recommendation);
- Surgical revascularization should be considered in patients with indication for revascularization in case of complex anatomy of the renal arteries, after a failed endovascular procedure or during open aortic surgery (class IIa, level B recommendation).

Malignant Hypertension

Malignant hypertension (MHT), also known as accelerated-malignant hypertension or malignant phase hypertension is defined clinically as high blood pressure associated with bilateral retinal flame-shaped hemorrhages, exudates or cotton wool spots, with or without papilledema. It is the most severe form of hypertension. It has been also proposed to replace the term "malignant hypertension" with "hypertensive crisis with retinopathy." Since the presence of retinopathy may allow other target organs to be included, making the description of this type of hypertensive emergency more accurate (Kaplan and Victor, 2015; Mancia *et al.*, 2013; van den Born *et al.*, 2011; Januszewicz *et al.*, 2016).

MHT is associated with failure of blood pressure autoregulation and develops when the mean arterial pressure (diastolic blood pressure + 1/3 of the difference between systolic and diastolic blood pressure) reaches a critical level of 150 mmHg, as reported in experimental animals. Fibrinoid necrosis appears in the arterial walls, which may be caused by vasoactive factor(s) or may be a nonspecific consequence of very high BP (Kaplan and Victor, 2015; Januszewicz *et al.*, 2016).

Possible pathophysiological mechanisms for the development of MHT have been proposed. These include: rapidly increasing blood pressure, pressure diuresis and natriuresis, severe renal vasoconstriction and ischemia. Moreover, activation of the renin-

Table 1 Clinical situation raising suspicion of renovascular hypertension or renal artery stenosis (Mancia et al., 2013)

Clinical situation raising suspicion of renovascular hypertension	
Atherosclerotic renal artery stenosis	*Fibromuscular dysplasia*
Flash pulmonary edema	Early onset HT (especially in women)
	FMD in other vascular beds or history of spontaneous artery dissection
	HT of abrupt onset,
	Worsening or increasingly difficult to treat HT;
	Resistant or malignant HT;
	Abdominal bruit, bruits over other arteries
	Rapid deterioration in renal function (spontaneous or in response to RAA blockers).
	Hypokaliemia

HT—hypertension; RAAS—renin–angiotensin–aldosterone system.

angiotensin-aldosterone system, microangiopathy, hemolytic anemia and development of retinopathy are found in MHT. The vascular lesions of MHT consist of fibrinoid necrosis and myointimal proliferation (Januszewicz *et al.*, 2016).

Various symptoms and complications may accompany MHT, the most typical being microangiopathic lesions or renal failure. In some patients, the presenting manifestation may be an acute oliguric renal failure. In patients with MHT many features of renal dysfunction, including microalbuminuria, proteinuria, may be also present. About half of patients with MHT may have hypokalemia, reflecting secondary aldosteronism from increased renin secretion induced by intrarenal ischemia. Hyponatremia is also common (Kaplan and Victor, 2015; Januszewicz *et al.*, 2016).

Since MHT is a hypertensive emergency, patients with MHT should receive immediate antihypertensive treatment, under continuous supervision, preferably in an intensive care setting, due to a high risk of renal failure, stroke, myocardial infarction and heart failure (Januszewicz *et al.*, 2016).

The 2013 Guidelines on hypertension from the European Society of Hypertension (ESH) and ESC recommend treatment based on agents that can be administered by intravenous infusion and titrated according to response, lowering blood pressure gradually, avoiding abrupt falls in BP and excessive hypotension (Mancia *et al.*, 2013).

Juxtaglomerular Cell Tumor

In 1967, Robertson *et al.* first described the tumor derived from the juxtaglomerular apparatus producing renin. The case report presented a 16-year–old patient with malignant hypertension, hypokalemia and increased urinary potassium excretion. Intraoperative examination revealed a small tumor in the left kidney. It consisted of juxtaglomerular apparatus cells. Nephrectomy resulted in the normalization of blood pressure and serum potassium concentration (Robertson *et al.*, 1967).

The term "primary reninism" for renin secreting tumors (reninomas) was introduced by J. Conn in the early 1970s. Primary reninism is a very rare form of hypertension with a potentially removable cause. In the last half-century 89 cases were described, and according to other authors—119 cases. Renin-secreting tumors provide a rare opportunity to study the renin-angiotensin-aldosterone system in hypertension; as a source of large amount of renin-secreting cells, they were used to obtain prorenin, renin, their polyclonal antibodies and monoclonal cell cultures as well as for genetic tests. Now, instead of the term primary reninism, the term juxtaglomerular cell tumor is more frequently used (Wong *et al.*, 2008; Beevers *et al.*, 2008; Corvol *et al.*, 1988; Prejbisz *et al.*, 2014).

The clinical picture of juxtaglomerular cell tumor is usually dominated by severe hypertension. Hypokalemia, remarkably increased renin activity or renin plasma concentration and elevated serum aldosterone levels (secondary hyperaldosteronism) are characteristic biochemical abnormalities. Among the imaging methods, computer tomography and magnetic resonance imaging enable a precise location of a tumor in the kidney. Renal vein catheterization might be an useful further diagnostic steps, however in some cases it fails to show gradient of renin concentration between renal veins. Functional imaging techniques are seldom applied in patients with juxtaglomerular cell tumor however it has been reported that they might be useful. This is important for the choice of surgical strategy. Surgical removal of the tumor leads to normalization of blood pressure in most cases (Wong *et al.*, 2008; Beevers *et al.*, 2008; Corvol *et al.*, 1988; Prejbisz *et al.*, 2014).

Congestive Heart Failure

Cardiac failure is related with activation of the renin-angiotensin-aldosterone system. This system's components act to compensate for decreased cardiac output, stabilize the circulation and expand extracellular fluid volume. Resulting from persistence stimulation of the renin-angiotensin-aldosterone system, serum aldosterone level is increased in patient with heart failure. This have deleterious effects on cardiovascular system and kidneys (Masoumi *et al.*, 2015; Januszewicz *et al.*, 2016; Robertson *et al.*, 1967).

Cirrhosis and Nephrotic Syndrome

Likewise as in heart failure, there is variable stimulation of the renin-angiotensin system and the activation of aldosterone secretion, depending on severity, preceding treatment and type of organ failure in cirrhosis and nephrotic syndrome. Both activation of the renin–angiotensin–aldosterone system and sympathetic nervous system, shown in several and experimental studies, lead to increased aldosterone secretion. In cirrhosis the increased aldosterone levels are related to sodium loading and delayed clearance. Also in nephrotic syndrome, aldosterone exerts salt-retaining effects on the distal nephron. It is a site implicated in formation of nephrotic edema. Therefore increased aldosterone activity might be an explanation for observed salt retention (Schrier, 2011; Palmer *et al.*, 2013; Soi and Yee, 2017).

Other Conditions

Secondary aldosteronism might be also associated with a few other conditions (Corry and Tuck, 1995):

- Pregnancy—activation of the renin-angiotensin-aldosterone system might occur early in pregnancy. It is related to the decrease of systemic vascular resistance, in spite of the increase in blood volume. However the increase of aldosterone secretion might be counterbalanced by increased progesterone levels, which has antimineralocorticoid effects.
- Estrogen treatment—estrogen treatment leads to increased renin and angiotensin II production, and subsequently to stimulation of aldosterone secretion.
- Bartter syndrome and Gitelman syndrome—hereditary renal tubular disorders characterized by chronic hypokalemia with renal K+ wasting, metabolic alkalosis, renal salt wasting with low to normal BP. These results in secondary increased renin secretion and secondary hyperaldosteronism. Bartter's syndrome is secondary to defective reabsorption of NaCl in the loop of Henle. Gitelman's syndrome is characterized by defective reabsorption of NaCl in the distal convoluted tubule segments (Lin *et al.*, 2010).

See also: Aldosteronism in Congestive Heart Failure. Hyperreninemia. Hypertension and the Renin–Angiotensin–Aldosterone System

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Aldosteronism in Congestive Heart Failure☆

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Introduction

The clinical syndrome, congestive heart failure (CHF), comprises a constellation of symptoms and signs whose origins arise from renal salt and water retention mediated by effector hormones attendant with an activated renin–angiotensin–aldosterone system (RAAS) (Weber, 2001). RAAS activation occurs in response to reductions in renal blood flow and the subsequent release of renin by juxtaglomerular cells. A fall in renal blood flow may accompany heart failure-reduced ejection fraction (HFrEF) or heart failure with preserved ejection fraction (HFpEF). An index of ventricular systolic function, EF does not predict cardiac output, renal blood flow, and therefore the presence or absence of RAAS activation with salt and water retention, and hence EF does not predict the clinical severity of chronic heart failure and is not a marker of CHF (Weber, 2001). In recognition of the importance of neurohormonal activation in determining morbid and mortal events in patients having CHF, its pharmacologic management has come to include inhibitors of angiotensin II and aldosterone hormone formation and their receptor ligand binding, as well as that of the adrenergic nervous system and its effector hormones.

The aldosteronism of RAAS activation has other deleterious systemic consequences, wherein a cycle of chronic homeostatic responses gone awry begets dyshomeostasis (Kamalov *et al.*, 2010). These adverse effects include secondary hyperparathyroidism (SHPT) with abnormal elevations in plasma parathyroid hormone (PTH) that occur in response to the appearance of ionized hypocalcemia which accompanies increased urinary and fecal calcium excretion; and a pathologic remodeling of the cardiovasculature including the heart, arteriolar circulation, and kidneys.

Herein, we focus on these pathologic consequences of aldosteronism in CHF and where aldosteronism is defined as inappropriate elevations in plasma aldosterone relative to dietary Na^+ intake. We finally review the results of clinical trials involving the efficacy of aldosterone receptor blockade in patients with heart failure.

Excretory Ca^{2+} and Mg^{2+} Losses in Aldosteronism

In human studies, Horton and Biglieri (1962) identified the urinary wasting of Mg^{2+} and K^+ that accompanied an infusion of aldosterone and which was then abrogated by cotreatment with its receptor antagonist spironolactone (see **Fig. 1**). These findings underscored Conn's explanation of why hypomagnesemia and hypokalemia represented several of the cardinal metabolic features of primary aldosteronism (Conn, 1963a,b).

In metabolic studies conducted in 8-week-old male Sprague–Dawley rats, urinary and fecal excretion of Ca^{2+} and Mg^{2+} were monitored in response to a 4-week aldosterone/salt treatment. Marked excretory wasting of these cations occurred with milligram quantities lost in stool and microgram quantities in urine (**Fig. 2**) (Chhokar *et al.*, 2004, 2005). The losses of Ca^{2+} and Mg^{2+} led to the appearance of ionized hypocalcemia and hypomagnesemia (**Fig. 3**) and as expected, this invoked the Ca^{2+}-sensing receptor of the parathyroid glands to elaborate PTH (Chhokar *et al.*, 2005). The ensuing SHPT was responsible for bone resorption with a marked loss in bone mineral density and flexor strength (Chhokar *et al.*, 2004). In a paradoxical manner, however, increased plasma PTH promoted intracellular Ca^{2+} overload and induction of oxidative stress in diverse cells, including cardiac myocytes and peripheral blood mononuclear cells (Chhokar *et al.*, 2005).

SHPT in Aldosteronism

Resnick *et al.* (1985) and Resnick (1986) suggested that a calcium-regulating hormone would determine intracellular Ca^{2+} overloading and the cellular deposition of Ca^{2+} in low-renin hypertension. Rossi *et al.* (1995) would identify the role of PTH and SHPT that accompanied primary aldosteronism and which was corrected by adrenal gland surgery or spironolactone treatment.

The role of PTH as the circulating hormone responsible for intracellular Ca^{2+} overload in aldosteronism was therefore implicated by the marked urinary and fecal wasting of Ca^{2+} with ionized hypocalcemia. The ensuing SHPT accounted for a fall in bone mineral density and increased the risk of atraumatic fractures in patients with CHF (vide infra). Massry *et al.* previously identified PTH as the Ca^{2+}-regulating hormone involved in the Ca^{2+} "intoxication" of SHPT that accompanies chronic renal

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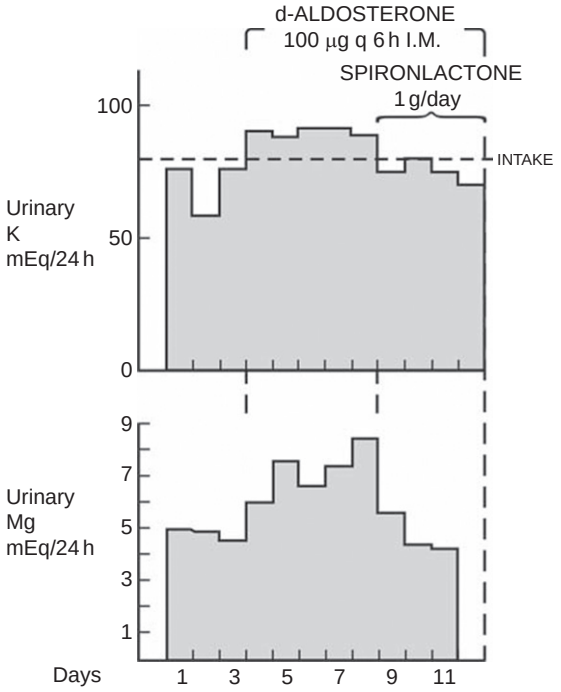

Fig. 1 Urinary K^+ and Mg^{2+} excretion in a patient with bilateral adrenalectomy before and during aldosterone treatment (without and then with oral spironolactone coadministration). Reproduced with permission from Horton, R. and Biglieri, E. G. (1962). Effect of aldosterone on the metabolism of magnesium. *The Journal of Clinical Endocrinology & Metabolism* **22**, 1187–1192.

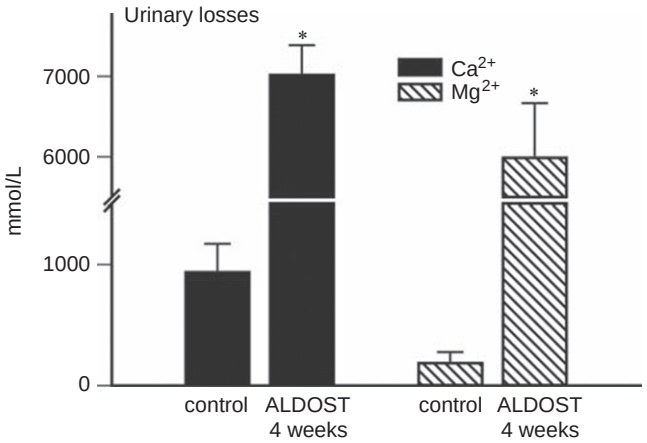

Fig. 2 Urinary Ca^{2+} and Mg^{2+} excretion during a 4-weeks aldosterone/salt treatment (ALDOST) compared to controls. $^*P < 0.05$ vs. control. Adapted from Chhokar, V.S., Sun, Y., Bhattacharya, S.K., Ahokas, R.A., Myers, L.K., Xing, Z., Smith, R.A., Gerling, I.C., and Weber, K.T. (2004). *American Journal of Physiology, Heart and Circulatory Physiology* 287, H2023–H2026.

failure (Chhokar *et al.*, 2005; Massry and Smogorzewski, 1994; Fujita and Palmieri, 2000; Vidal *et al.*, 2006). The rise in cytosolic free $[Ca^{2+}]_i$ includes mitochondrial $[Ca^{2+}]_m$ where there occurs an induction of oxidative stress coupled to an enhanced opening potential of their inner membrane permeability transition pore leading to mitochondrial swelling and destruction. Microscopic myocyte necrosis follows with their replacement by scarring (Cheema *et al.*, 2011; Shahbaz *et al.*, 2011).

Hence, chronic elevations in plasma aldosterone, inappropriate for dietary Na^+ intake, represent a homeostatic response gone awry leading to dyshomeostasis with SHPT accounting for myocyte Ca^{2+} overload, necrosis, and myocardial scarring.

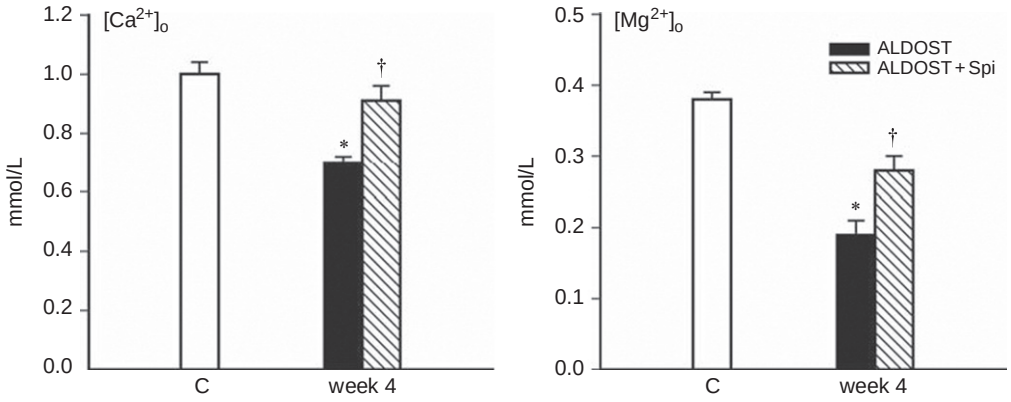

Fig. 3 Compared to controls a fall in plasma ionized $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ accompanied a 4-week aldosterone/salt treatment (ALDOST) and which was prevented with spironolactone (Spi). $^*P < 0.05$ vs. control, $^\dagger P < 0.05$ vs. ALDOST. Adapted from Chhokar, V.S., Sun, Y., Bhattacharya, S.K., Ahokas, R.A., Myers, L.K., Xing, Z., Smith, R.A., Gerling, I.C., and Weber, K.T. (2005). Hyperparathyroidism and the calcium paradox of aldosteronism *Circulation* 111, 871–878.

SHPT and Congestive Heart Failure

Abnormal elevations in plasma PTH (>65 pg/mL) were found in consecutively admitted, African-American (AA) patients hospitalized with CHF and HFrEF and who were found to have ionized hypocalcemia (Khouzam *et al.*, 2006; LaGuardia *et al.*, 2006). SHPT would subsequently be documented in a larger AA cohort consecutively hospitalized with decompensated HFrEF and in whom reduced creatinine clearance could not be incriminated as a causal factor (Alsafwah *et al.*, 2008). In patients with intermittent episodes of RAAS activation and CHF, abnormal elevations in plasma PTH range between 150 and 300 pg/mL as contrasted to higher PTH levels of 600–800 pg/mL seen in patients with persistent chronic renal failure. Normal PTH levels (<65 pg/mL) were found in ambulatory AA with compensated HFrEF having comparable EF to their cohorts with decompensated failure, but in whom RAAS activation was absent as was the case in patients with cardiovascular disease, but no heart failure, as well as in healthy AA volunteers. Reduced Ca^{2+} stores due to vitamin D deficiency could predispose to SHPT; however, serum 25OHD levels were reduced (<30 ng/mL) in the entire AA cohort with and without heart failure (Alsafwah *et al.*, 2008).

The association between SHPT in CHF has now been widely recognized. Elevations in plasma PTH predict the need for hospitalization in patients with symptomatic HFrEF and those with advanced symptomatic HFpEF, where RAAS activation is expected in each case (Alsafwah *et al.*, 2008; Lee *et al.*, 1994; Shane *et al.*, 1997; Schmid and Kiowski, 1998; Ogino *et al.*, 2002; Zittermann *et al.*, 2003, 2011; Garakyaraghi *et al.*, 2010; Sugimoto *et al.*, 2009; Altay *et al.*, 2012a,b; Schierbeck *et al.*, 2011). The combination of elevated plasma PTH and brain natriuretic peptide, each surrogates of RAAS activation, confers a poor prognosis in patients with decompensated failure (Gruson *et al.*, 2015).

Factors contributing to the appearance of SHPT in heart failure include hypovitaminosis D with reduced sunlight exposure and senescent skin with its reduced capacity to generate vitamin D; obesity; lactose intolerance with avoidance of dairy products rich in Ca^{2+}; and high dietary Na^+ and loop diuretic, each of which promotes enhanced urinary Ca^{2+} excretion.

PTH is a cardiovascular risk factor for all-cause and cardiovascular mortality and events in patients with heart failure independent of renal function or age (Schierbeck *et al.*, 2011), and this includes a community-based cohort of elderly men and women (Pilz *et al.*, 2010; Hagström *et al.*, 2009, 2010; Björkman *et al.*, 2009). The risk of sudden cardiac death is increased among persons having higher PTH with vitamin D deficiency (Deo *et al.*, 2011).

SHPT reduces bone mineral density and predisposes to atraumatic fractures, particularly of the hip and where the elevation in PTH is related to the severity of RAAS activation and hence symptomatic failure (Lee *et al.*, 1994; Shane *et al.*, 1997; Schmid and Kiowski, 1998; Terrovitis *et al.*, 2012; van Diepen *et al.*, 2008). In men with heart failure treated with spironolactone, an aldosterone receptor antagonist for 6 months or more, the risk of atraumatic fracture was reduced (Carbone *et al.*, 2008).

Aldosteronism and Cardiovascular Remodeling

An adverse structural remodeling of the heart and vasculature accompanies aldosteronism and where an adverse accumulation of fibrous tissue disrupts tissue architecture and organ function (Brilla *et al.*, 1990; Sun *et al.*, 1997). The causality of the cardiac fibrosis in aldosteronism would remain uncertain for some time. Considering arterial hypertension and/or hypertrophy as responsible factors for such remodeling, the pressure-overloaded, hypertrophied left ventricle would only be affected, while the normotensive, nonhypertrophied right ventricle would be spared. Contrariwise, coronary blood flow reaches the entire heart. Hence, the coronary circulation containing a responsible circulating substance would promote fibrosis of the entire heart. Fibrosis was found to involve the right and left atria and ventricles to thereby implicate a circulating substance (Brilla *et al.*, 1990; Sun *et al.*,

1997). Cotreatment with spironolactone prevented SHPT and fibrosis, and this held true when it was given in either a low, nondepressor dose, which did not prevent hypertension or hypertrophy, or a high, pressor dose which achieved these end points (Brilla *et al.*, 1993; Brilla and Weber, 1992). Subsequent studies would identify elevations in plasma PTH as this substance. Overall findings would implicate a cardioprotective role for spironolactone. Moreover, they paved the way for the evaluation of this mineralocorticoid receptor antagonist (MRA) in the overall management of heart failure in placebo-controlled clinical trials.

Aldosterone Receptor Blockade

Heart Failure-Reduced Ejection Fraction (HFrEF). The RALES trial was conducted in patients with advanced symptomatic heart failure (NYHA Class III and IV) receiving an ACE inhibitor and diuretic, together with low-dose spironolactone (12.5–50 mg) compared to placebo (Pitt *et al.*, 1999). All primary and secondary end points were achieved within 3 months of initiating spironolactone treatment with a 30% risk reduction in overall and cardiac mortality, progression of heart failure, cardiac hospitalizations, and sudden cardiac death. In patients randomized to spironolactone serologic markers of collagen synthesis were reduced to suggest the prevention of cardiac fibrosis (Zannad *et al.*, 2000).

Placebo-controlled trials with another receptor antagonist, eplerenone, followed. EPHESUS addressed mortality and morbidity benefit among patients having HFrEF soon after their acute myocardial infarction (Pitt *et al.*, 2003). Combined with an ACE inhibitor and β-blocker, cardiovascular events, including mortality from sudden cardiac death and morbidity with hospitalization, were reduced with low-dose eplerenone. The EMPHASIS trial (Zannad *et al.*, 2011) demonstrated the combination of eplerenone with an ACE inhibitor, and β-blocker reduced the risk of death and hospitalization in patients having HFrEF with mild symptomatic heart failure (NYHA Class II).

Heart Failure-Preserved Ejection Fraction (HFpEF). HFpEF represents an ever increasing population of patients with symptomatic heart failure, who often are hypertensive elderly women and symptomatic African-Americans having evidence of RAAS activation with aldosteronism (Dunlay *et al.*, 2012; Bishu *et al.*, 2012). A randomized, double-blind, placebo-controlled trial with spironolactone (TOPCAT) in the management of symptomatic patients with HFpEF was conducted in six countries. Overall results suggested primary outcome with death from cardiovascular causes, aborted cardiac arrests, or heart failure-related hospitalizations were not reduced (Pitt *et al.*, 2014). However, in a posthoc analysis important differences were found between patients enrolled in the Americas (Argentina, Brazil, Canada, and United States) versus Russia/Georgia (Pfeffer *et al.*, 2015). Spironolactone significantly reduced the composite primary outcome in the Americas (Pfeffer and Braunwald, 2016). In addition, plasma canrenone, a metabolite of spironolactone, could not be detected in one-third of patients enrolled in Russia and Georgia (Mehra and Lindenfeld, 2016).

Summary and Conclusions

In patients with heart failure, the addition of spironolactone or eplerenone to an ACE inhibitor and β-adrenergic receptor antagonist has proven survival benefits with reduced morbid events. Through their interference with cognate receptor binding, these MRA mitigate against the pathophysiologic consequences of inappropriate (relative to dietary Na^+) elevations in plasma aldosterone that occur in CHF, including SHPT and adverse cardiac remodeling (see **Fig. 4**). Cardioprotective properties of these

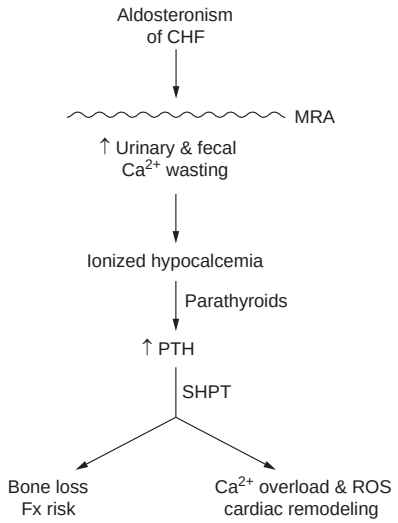

Fig. 4 A summary of pathophysiologic events that accompany aldosteronism in CHF. See text.

agents are multiple. We considered several examples herein. Aldosteronism is now recognized as an important pathophysiologic feature of CHF.

See also: Hyperreninemia. Interference With the Renin–Angiotensin System (RAS): Classical Inhibitors and Novel Approaches

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Mineralocorticoid Receptor Antagonists

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Glossary

Ligand The hormone or drug which binds to the receptor.

MRA Mineralocorticoid receptor antagonist.

PA Primary aldosteronism.

Background

Most children at some stage in their upbringing are asked—usually by the teasing uncle—"which came first: the chicken or the egg?" The same conundrum might be asked of the title of this article, although the answer is very clear: the antagonist (spironolactone) was developed a decade before the first glimmerings of knowledge about the mineralocorticoid receptor, and three decades before it was cloned and sequenced. This was unusual: today a candidate receptor is discovered, a ligand (or ligands) sought, and finally potential antagonists identified, often by sequencing hundreds of thousands of compounds.

The contrast with the development of spironolactone could not be starker. Aldosterone was isolated and characterized in 1953, and recognized to be the major player in renal salt and water metabolism. Aldosterone normally circulates at very low (picomolar) levels, but is 3–10 fold elevated in pregnancy. On the presumption that this reflected a feedback response to the very high progesterone levels somehow blunting the effects of aldosterone in the kidney—subsequently confirmed in animal studies—a series of progesterone derivatives were tested at G.D. Searle in the eponymous Kagawa assay (Kagawa *et al.*, 1957).

Thus was born spironolactone, the first and still by far the most clinically used mineralocorticoid receptor antagonist (MRA). It bears more than traces of its ancestry, in that it retains a measure of progesterone receptor (PR) agonist activity, so that it can interfere with the normal menstrual cycle in premenopausal women, and very occasionally cause breast pain (mastodynia) in women in general. More often, and of more clinical consequence, is that it also has not inconsiderable antagonist activity at androgen receptors (AR). Its affinity for AR is ~one fifth that for mineralocorticoid receptors, and clinically spironolactone may produce dose-related AR-mediated side effects, including male breast enlargement (gynecomastia) and erectile dysfunction.

From the vantage point of 60 years on it is appropriate to begin any account of MRAs with a brief summary of the state of the relevant art in terms of MR. This will be followed by consideration of the current role of MRAs in heart failure, primary aldosteronism, resistant hypertension, low renin hypertension and a miscellany of less common cardiovascular situations. The article will conclude with a brief summary of current potential advances in the MRA field, beyond spironolactone and the second-generation antagonist eplerenone.

Mineralocorticoid Receptors

In this section, and subsequently, MR will be used for both the singular and plural (like sheep). MR are one of a 48 (human)/49 (mouse) member family of nuclear transactivating factors, including receptors for steroid and thyroid hormones, retinoids, lipids and xenobiotics; there remain the so-called orphan receptors, for which no ligand has been found. MR, GR (glucocorticoid receptors), AR and PR share close homology, >50% in the ligand-binding domain, 90% in their DNA binding domain. This subfamily of receptors share a common ancestor in evolution; notably, MR were the first of the four to branch off the ancestral "corticoid receptor," followed by GR, then PR, and finally AR (Kassahn *et al.*, 2011).

Classical, unequivocal MR first appeared in cartilaginous (rays, sharks) and then in bony fish. Millions of years later, the first creature to make aldosterone was lungfish, as its name testifies the species transitioning from an obligate aqueous environment to life on the land. It is important to remember this sequence, in that most people unhesitatingly regard aldosterone as the primary or cognate MR ligand. The primary cognate ligand, as far as it can be established, was cortisol; also, often overlooked, is that ≥90% of the MR in the human body are constitutively occupied by cortisol—the acknowledged and very important role of aldosterone in sodium and water homeostasis notwithstanding.

The issue is that MR are promiscuous. They have equivalent, very high affinity for cortisol, corticosterone, deoxycorticosterone, aldosterone and progesterone. As we have seen, progesterone is an MR antagonist on the basis that aldosterone (and deoxycorticosterone) define agonist activity. The physiologic glucocorticoids circulate at ~1000-fold higher total levels (cortisol, human; corticosterone, rat and mouse) than aldosterone, on a normal salt intake. The obvious question, then, is how does aldosterone ever get to occupy epithelial MR, and to do what we know it does, which is increase transepithelial transport of sodium, and with it water.

Part of the answer is that although the differences in total levels is 1000-fold, the glucocorticoids are much more (~95%) bound in plasma than is aldosterone (~50%), so that the difference in free levels is "only" ~100-fold. What confers specificity on

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aldosterone in terms of activating epithelial MR (plus those in the vascular wall, and the nucleus tractus solitarius of the brain) is the coexpression at high levels of the enzyme llβHydroxysteroid dehydrogenase (llβHSD2), which converts cortisol to receptor inactive cortisone, and the essential co-substrate NAD (nicotinamide adenine dinucleotide) to NADH.

What was originally thought (Edwards *et al.*, 1988; Funder *et al.*, 1988), and unfortunately is still often being taught, is that this process denies cortisol occupancy of renal MR: certainly, if llβHSD2 is congenitally deficient, or blocked (e.g. in liquorice overindulgence) cortisol is a potent agonist in renal MR. In fact, aldosterone "works" by occupying (and activating) ~10% of the MR in a tubular principal cell; the other ~90% are *occupied but normally not activated* by cortisol. What llβHSD2 does is to debulk intracellular levels to 10-fold, rather than 100-fold those of aldosterone, in the process generating relatively high levels of NADH. It is probable, though not yet proven, that such levels of NADH inactivate MR-cortisol complexes, so that aldosterone can act via the remaining 10%. Harking back to the Kagawa bioassay, prior adrenalectomy shifts the urinary Na^+/K^+ well to the left of that in intact animals maintained on a high salt intake—presumably reflecting all renal MR being unoccupied and thus available to aldosterone.

For terrestrial animals, then, the emergence of aldosterone as a major regulatory hormone acting via a legacy MR (really a very high affinity glucocorticoid receptor) raises a series of questions, few of which have been addressed, and fewer answered. Given the 48 or 49 nuclear transactivating factors that have evolved, one more which was aldosterone-specific would not appear too much of a stretch. Maybe the abruptness of the transition to land was such that the co-expression at high levels of an existing member of an ancient enzyme family, but the twin specificity-conferring mechanisms—debulking and cortisol-MR inactivation by NADH—seem the long way around. This does not appear to be an example of intelligent design, but there it is; it's what we have.

It does not stop there, at least for cortisol. One of the early findings in the MR field is that they are widely expressed, not just in epithelia (kidney/colon/sweat glands/salivary glands), plus blood vessel wall and the nucleus tractus solitarius, but elsewhere. In the elsewhere category are the hippocampus, where MR are expressed at high abundance, and similarly in cardiomyocytes: in these and other non-epithelial tissues MR are not "protected" by llβHSD2, and thus overwhelmingly constitutively occupied by normal circulating levels of glucocorticoids.

In terms of normal physiology very little is known about what effects essentially saturating levels of glucocorticoids have via MR in such tissues. There have been a number of in vitro studies using more or less physiological concentrations of aldosterone on dispersed cardiomyocytes, for example, with the effects shown taken as evidence for a role for aldosterone via cardiac MR in, for example, hypertension or heart failure. The failure to include 100-fold higher concentrations of glucocorticoid with the administered aldosterone makes such studies worthless and, unfortunately, misleading.

Where the cardiomyocyte MR are activated by cortisol is under conditions of tissue damage, reactive oxygen species (ROS) generation and change in intracellular redox status. Under such conditions cortisol becomes an MR agonist, mimicking the effects of aldosterone in in vitro studies. In occlusion-reperfusion studies using the Langendorf perfused heart preparation nanomolar aldosterone increases area-at-risk and infarct size. An identical effect is seen with nanomolar cortisol, blocked by spironolactone but not the GR, PR antagonist mifepristone, clear evidence for a cortisol action via unprotected MR in conditions of tissue damage (Mihailidou *et al.*, 2009).

This of course has major implications for what steroid is activating cardiomyocyte MR in hypertension and heart failure. In the prismatic RALES trial (Pitt *et al.*, 1999) (of spironolactone at low dose added to standard of care) plasma aldosterone levels were in the low normal range: even almost two decades later many authors continue to consider aldosterone as the culprit, with fine disregard for steroids (and science). In the circumstances of tissue damage normal circulating levels of cortisol overwhelmingly occupy MR in the damaged cardiomyocyte, and are thus responsible for the ongoing cardiovascular damage. This can be ameliorated by low-dose MRA: after 18 months the RALES trial was stopped, because the group receiving spironolactone (average 26 mg/day) showed 30% lower mortality and 35% fewer hospitalizations, than the matched control group.

RALES presented two conundrums. One, which is how such a low normal concentration of aldosterone produces such major effects, is solved: it's cortisol in the damaged heart that is the culprit. The second enigma is how can such a low dose of spironolactone have such marked effects? Spironolactone itself has a relatively short half-life, being rapidly converted into active metabolites which have half-lives of 18–24 h. What this means in practice is that effector MRA levels take 8–10 days to reach steady state; the modest daily dose belies steadily increasing plasma concentrations over the first week or so of administration.

The extended pharmacokinetic profile helps, but is not the complete answer: the answer is that spironolactone (and incidentally), eplerenone, of which more later) is not just a blocker, in the normally accepted sense of sitting in the receptor and nothing else can get in, but what is called an "inverse agonist." An inverse agonist is not just a bung, but has a life of its own once it is receptor-bound. In the same Langendorf occlusion/reperfusion model, in the absence of any other added steroid, spironolactone at very low dose reduced infarct size and area-at-risk. For good measure, against the possibility of residual steroids, the same result was found in adrenalectomized rats (Ji *et al.*, 2013).

Given the very low concentrations of aldosterone, in the kidney the pharmacokinetics may be enough to keep aldosterone at bay, so that the classical notion of an MRA as "blocker" may hold. This cannot be the case for the cardiomyocyte, where spironolactone binding to only a percentage of MR exerts its inverse agonist activity to counter the agonist action of much higher concentrations of cortisol than those of aldosterone.

Mineralocorticoid Receptor Antagonists: Congestive Heart Failure

1999 Marked the year that cardiologists recognized aldosterone from among the miasma of "neurohormonal" factors, given the results of the RALES trial. Never mind that it was the wrong MR agonist: what it did was to cement a place for low-dose MRA administration in progressive cardiac failure. The patients under study were selected: normal renal function, modest if any comorbidities. The results were celebrated, over-interpreted and inappropriate patients (much reduced renal function, continued potassium supplements) admitted to emergency departments for hyperkalemia. Now, almost 20 years later, it would be fair to say that spironolactone has been accepted at a low dose as normal practice in the management of progressive, New York Heart Association Stage lll systolic heart failure.

There followed two trials with eplerenone, different not only from one another but also for obvious reasons from RALES. Eplerenone is a considerably less potent MRA in vitro, but much less plasma bound: in vivo, as a single dose it is 50%–60% as potent. Unlike spironolactone eplerenone has no active metabolites, and its plasma half-life is of the order of 4 h, so that even with twice daily dosing levels in plasma do not materially increase over time, unlike those after spironolactone.

The first of these trials (Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study—EPHESUS) was a double-blind, placebo-controlled study on a total of 6632 patients with left ventricular dysfunction after myocardial infarction (Pitt *et al.*, 2003). Eplerenone (25 mg/day) was on average begun a week post MI, and reduced all-cause mortality by 15% after a mean follow-up of 16 months, with its beneficial effect clear 30 days after randomization. The long-term benefit at first sight appears to be ~half that in the RALES trial, a difference that no longer holds when patients with comparably reduced ejection fractions are compared.

The second trial (eplerenone in patients with systolic heart failure and mild symptoms—EMPHASIS-HF) randomized 2737 patients with milder (New York Heart Association Class ll) systolic (ejection fraction $\leq$35%) heart failure to eplerenone (up to 50 mg/day) or placebo (Zannad *et al.*, 2011). After a mean follow-up of 21 months eplerenone lowered mortality by 19% and the risk of hospitalization by 36%. Taken with the data from RALES, the EMPHASIS trial shows that low dose MRA should be an integral component of the drug treatment of systolic heart failure, progressive and mild, with a reduced ejection fraction and due attention paid to plasma $[K^+]$ levels.

The third major trial subsequent to RALES reverted to spironolactone as the MRA (Pitt *et al.*, 2014). The trial was funded by NIH, rather than by pharma, and randomized 3445 patients with diastolic failure and a preserved ejection fraction to spironolactone 15–45 mg daily or placebo. Logged as "spironolactone for heart failure with preserved ejection fraction," it was given as a label rather than as an acronym "TOPCAT." The trial was a disaster, in that whereas in patients from North and South America the expected outcomes were seen, they were nullified by the results from Russia and Georgia, where most randomized patients appeared not to be in heart failure. Subsequently investigators have suggested that despite the overall failure there were sufficient indications from the western hemisphere to use low dose MRA in at least a selection of patients with diastolic heart failure.

Mineralocorticoid Receptor Antagonists: Primary Aldosteronism

Given that the driver of elevated blood pressure and cardiovascular damage in primary aldosteronism (PA) is excessive production of aldosterone, there is a prima facie and essentially universally accepted case for the use of MRAs as cornerstone therapy in bilateral disease. What needs to be borne in mind, in accepting this judgment, are two things. First, in remote areas where populations have very limited access to salt aldosterone levels can be sky-high, above those seen in many cases of PA, with no elevation of blood pressure or cardiovascular damage: aldosterone alone is not the culprit in PA.

The elevated aldosterone levels in chronic sodium deficiency are homeostatic: what causes hypertension and cardiovascular damage is aldosterone levels inappropriate for salt status. To cause hypertension and cardiovascular damage aldosterone needs to operate in a milieu of higher-than-appropriate sodium levels. The second thing to be borne in mind is that an MRA, in promoting natriuresis and an elevation in plasma $[K^+]$, may stimulate the adrenal cortex to produce more aldosterone. The salient example, as previously noted, is pregnancy, when the production of progesterone from the placenta sets in train a 3–10 fold elevation of aldosterone levels from the normal adrenal cortex. Although this level of reflex stimulation is rarely seen with the doses currently recommended for spironolactone in PA (or with eplerenone), increases in plasma aldosterone are not uncommon with higher spironolactone dosage.

There are currently three MRAs used in primary aldosteronism due to bilateral adrenal hyperplasia (BAH)—spironolactone, eplerenone and in Europe canrenone (an active and long-lived spironolactone metabolite). Whereas spironolactone used to be prescribed at high doses, up to 400 mg/day in BAH, today arguably best practice in such patients is to start with as little as 12.5 mg/day, plus an ENaC directed natriuretic agent such as amiloride or triamterene, plus standard antihypertensives if and as required to normalize blood pressure. If clinically indicated, 25 mg/day may be used, but very preferably no higher dose. The reason for using as low a dose as effective is neither economy—spironolactone is very cheap—nor faint-heartedness: it is to minimize the risk of gynecomastia and of exacerbating erectile dysfunction in men, thus minimizing the percentage of non-compliant patients. Non-compliance is a particular problem for patients with PA, as their cardiovascular risk profiles are very significantly higher than those in age-, sex-, and blood pressure matched essential hypertensives (Milliez *et al.*, 2005).

Canrenone is reputed to be less potent in terms of androgen antagonist activity, which can only be the case if at least one of the two long-lived metabolites (7α-thiomethyl spironolactone and 6β, 7α-thiomethyl spironolactone) have equivalently more

androgen receptor activity: it is also not widely available, except in Europe. The third currently available MRA is eplerenone, increasingly in use in patients intolerant of the side effects of spironolactone. There are multiple issues complicating its use, as follows. Although it out of patent, it is difficult and expensive to synthesize, and generics do not yet appear to be widely available. It is expensive for patients, except in Japan where it is a quarter of the global price, and elsewhere not widely recompensable. In terms of efficacy, head-to-head studies have differed markedly, with for example one finding it markedly inferior to spironolactone (Parthasarathy *et al.*, 2011) and another (improbably) as effective at the same dose levels as spironolactone (Karagiannis *et al.*, 2008).

The other group of patients for whom MRAs are currently neglected but should be included in their therapeutic regimen are those with biochemical cure (normal plasma $[K^+]$/aldosterone to renin ratio) after unilateral adrenalectomy for an aldosterone producing adenoma but who remain hypertensive. In these subjects with partial remission of PA the residual hypertension is commonly ascribed to vascular damage and/or underlying essential hypertension. A minority of such patients show post-operatively low plasma aldosterone levels, presumably due to inhibition of aldosterone synthesis in the contralateral adrenal, which resolves over weeks or months.

In those who have, or regain, normal plasma aldosterone levels it would make no sense to include an MRA if we are talking—as many cardiologists still are—about "aldosterone receptors." One of the characteristics of an MR is that it has, as we have seen, equivalent high affinity for aldosterone and cortisol, and that in tissue damage cortisol mimics aldosterone. There are convincing data that this is the case: there are also convincing data that eplerenone, as monotherapy, is a very effective anti-hypertensive in essential hypertensives with normal renin, aldosterone and plasma potassium levels (Levy *et al.*, 2004). In laboratory studies, addition of eplerenone from weeks 5 to 8 in DOC/salt hypertensive rats reversed the cardiac fibrosis and level of vascular inflammatory markers to zero-time control levels (Young and Funder, 2004). Whether the residual elevated blood pressure in partial clinical remission after unilateral adrenalectomy for APA represents underlying essential hypertension or vascular damage there is an excellent, if to date unappreciated, case for a low-dose MRA to be included in post-operative care when aldosterone levels are normal.

Mineralocorticoid Receptor Antagonists: Resistant Hypertension

Resistant hypertension is defined as persistent blood pressure elevation over 140/90 mmHg on three conventional anti-hypertensives including a diuretic, or blood pressure requiring ≥ 4 agents to be brought below 140/90 mmHg. Although earlier estimates of its frequency were put at up to 20% of hypertensives, this figure needs to be discounted reflecting suboptimal compliance, with the true figure around $\sim 12\%$. For over a decade it has become clear that MRAs—spironolactone and eplerenone—included in therapy clearly lower blood pressure; more recently, it has been established that MRAs lower blood reassure more than pharmacologic comparators (bisoprolol, furosemide, doxazosin) and similarly are clearly more effective than renal denervation (Fadl Elmula *et al.*, 2017; Sinnott *et al.*, 2017).

When patients with resistant hypertension are screened, 15%–20% are shown to have primary aldosteronism. What is striking is that the hypertensive response to MRA inclusion does not differ between those with frank primary aldosteronism and those without. It is thus tempting to infer that once again the current cut-offs for the diagnosis of primary aldosteronism may be too strict, and that in many if not all patients with resistant hypertension inappropriate aldosterone secretion is at least in part responsible for the stubborn elevation of blood pressure. Whether or not this is the case, it is now widely accepted that the therapeutic regime in resistant hypertension should include a low dose MRA.

Mineralocorticoid Receptor Antagonists: Low Renin Hypertension

It is similarly becoming clear that many subjects with so-called low renin hypertension—a description, not a diagnosis—may also be in fact be suffering from primary aldosteronism as the victims of straitened cut-offs and definitions. A recent modest but prismatic study (Ori *et al.*, 2013) from Israel very clearly made this point. The authors compared outcomes in 24 patients with established primary aldosteronism (seven with an adenoma on imaging), and 24 of 39 patients sequentially diagnosed with low renin hypertension; the diagnosis was made on the basis of a positive aldosterone to renin ratio, but a plasma aldosterone of <14.5 ng/dL. All 48 patients were treated with low dose MRA (spironolactone 46, eplerenone 2), and monitored at 1 and then 3 years. The results were very substantial lowering of blood pressure, and a similarly substantial fall in left ventricular mass index. The most salient finding was that there were no differences in response to low dose MRA between primary aldosteronism and low renin hypertension patients. It can be anticipated with a fair degree of confidence that many if not most patients with "low renin hypertension" are victims of cut-offs set too high, and in fact suffer from primary aldosteronism.

Mineralocorticoid Receptor Antagonists: Additional Roles?

The previous sections addressed MRAs in three currently accepted areas (heart failure, primary aldosteronism, resistant hypertension) and in low renin hypertension, knocking on the door for over 40 years and finally gaining traction. This section deals with

more or less likely additions to the canon, the first and arguably the most urgent of which is pulmonary hypertension/fibrosis. Again, there is a history going back half a century ago on the use of spironolactone in what was then termed cor pulmonale: much more recently, the focus has been on preclinical studies on efficacy. In models of pulmonary hypertension in mice (hypoxia for prevention, monocrotaline for both prevention and treatment) spironolactone as a preventive measure attenuated a series of adverse effects; more potentially clinically relevant, in established pulmonary hypertension, spironolactone decreased right ventricular systolic pressure and pulmonary vascular resistance (Preston *et al.*, 2013). Parallel studies on rodent models using bleomycin-induced pulmonary fibrosis showed both spironolactone and eplerenone attenuated pulmonary injury and fibrosis (Ji *et al.*, 2013). Given the clinical difficulty in managing pulmonary hypertension and fibrosis, a major well designed clinical trial of low dose spironolactone added to standard of care would be in order.

Two elegant studies from Turkey have demonstrated another major indication for the use of spironolactone. Anthracycline group chemotherapeutic agents are used post-surgery in breast cancer patients. In the first study (Akpek *et al.*, 2015), 83 patients on chemotherapy were randomized to spironolactone or control, and their left ventricular ejection fraction (LVEF) determined. In the control group LVEF fell from 67.7 ± 6.3 to 53.6 ± 6.8 ($P < 0.001$); in the spironolactone group the equivalent values were 67.0 ± 6.1 to 65.7 ± 7.4 (ns): no surprise, the difference between groups was highly significant ($P < 0.001$). The second study (Yavas *et al.*, 2017) complemented the first, using rats to study the known cardiovascular toxicity when trastuzumab and radiotherapy are combined. Chronic treatment with spironolactone significantly lowered both cardiac fibrosis and TGFβ expression over trastuzumab and radiotherapy alone.

Mineralocorticoid Receptor Antagonists: The Next Generation?

None of the currently available MRAs are optimal. Spironolactone is a prodrug with a half-life of $\sim$2 h, and three active metabolites, one of which (canrenone) is marketed as such in Europe. Spironolactone is a potent MRA, but not specific, given its PR agonist and AR antagonist activity. A third, relatively recent second generation MRA is eplerenone, which in vivo is only $\sim$half as potent as spironolactone, and as previously noted has a much shorter half-life. It has the advantage of selectivity, which means it has negligible side effects, but the disadvantage of being difficult to synthesize, and expensive except in Japan. What is often termed a side effect of MRAs is in fact an obligate effect, that of elevation of plasma $[K^+]$. In this regard the shorter half-life ($\sim$4 h) of eplerenone, plus the absence of active metabolites, softens its impact in terms of plasma $[K^+]$ elevation compared with spironolactone. Finally, in terms of generation 1 and 2 MRAs, the trials (and tribulations) of the search for potent, selective MRAs, to replace spironolactone are covered in detail in an excellent recent historical paper (Kolkhof and Bärfacker, 2017), as is the development of the ultimately ill-fated drosperinone, marketed as a progestin-only oral contraceptive, with retained progestational/anti-androgen activity and MRA activity 8-fold that of spironolactone.

Third generation MRAs have been defined as non-steroidal (as synthetic chemists wish), as potent as spironolactone, as selective as eplerenone, with a half-life consistent with once daily dosing, easy to manufacture and with a long patent life; fourth generation MRAs as all of the above, plus tubule-sparing. The latter desideratum reflects clinician fear of hyperkalemia, an obligate side effect of current MRAs. What needs to be borne in mind is that MRA-induced natriuresis is pivotal for its efficacy in hyperaldosteronism: a generation four partial agonist, i.e. tubule-sparing, would be potentially dangerous in PA: as previously noted, in chronic sodium deficiency very high aldosterone levels do not cause hypertension or cardiovascular damage. Given the much higher circulating levels of cortisol, and is equivalent high affinity for MR, it is an open question whether its "agonist" activity in tissue damage is equivalently sodium dependent. The development of generation 4 MRAs to address cardiac, vascular renal or for example hepatic inflammation and fibrosis is crucially dependent on an answer, as yet not forthcoming, to that question.

There are several non-steroidal agents currently in late stage clinical trials. Mitsubishi Tanabe Pharma has conducted a Phase ll trial of *apararenone* in Eastern Europe and Japan for diabetic nephropathy, and has begun a Phase ll trial in patients with non-alcoholic steatohepatitis (NASH) in Japan. Daiichi Sankyo out-licensed *esaxerenone* from Exelisis, a compound which is orders of magnitude more potent an MRA than spironolactone, and significantly more selective than even eplerenone. Two different Phase ll studies have been undertaken in Japanese patients, the first in type 2 diabetes and microalbuminuria, the second a safety and efficacy study in hypertension; more recently the company has announced a Phase lll study against eplerenone in essential hypertension.

The most advanced non-steroidal MRA is *finerenone* from Bayer. This nonsteroidal is more potent as an MRA than spironolactone, and more selective than eplerenone: it is also inactive when tested against 65 other receptors/ion channels. Like spironolactone, it is an inverse agonist as well as a competitor for MR binding. It is substantially less lipophilic, and more polar, than steroidal MRAs; when compared with 3H-eplerenone and 3H-spironolactone, 3H-finerenone is more or less equally distributed between heart and kidney, in contrast to the predominant renal uptake of the steroidal MRAs. This is not a very persuasive basis for possible cardiac selectivity; the rats were not adrenalectomized, and the extent of non-specific binding in the two tissues not determined: it is free, not total concentration, of ligand which determines receptor uptake.

Clinically, in patients with chronic heart failure and kidney disease, finerenone was at least as effective as spironolactone, once daily at one fifth the dose; in addition it produced lower increases in plasma$[K^+]$, less frank hyperkalemia and a lower incidence of worsening renal function. Five Phase ll trials in heart failure plus chronic kidney disease and/or diabetes have shown that neither hyperkalemia nor reduced kidney function limit its use. In patients with type 2 diabetes and diabetic kidney disease a dose-ranging

study on over 800 subjects showed significant reductions in albuminuria progressively with dose (7.5, 10, 15, 20 mg/day) after 90 days of treatment. Currently finerenone is thus being investigated at daily doses of 10–20 mg in two large Phase lll outcome trials in patients with diabetic kidney disease. For an agent thought possibly to be more cardiac-specific, the choice of target for the Phase lll studies is interesting.

Without raising any false hopes, it appears that all three non-steroidal MRAs may prove very useful as treatment for diabetic nephropathy (all three), non-alcoholic steatohepatitis (aparenone), essential hypertension (esaxerenone) and heart failure (finerenone). It is likely that there will be subtle differences between these agents: it is interesting that none of the companies involved appear to have considered a trial in primary aldosteronism.

Mineralocorticoid Receptor Antagonists: A Final Word

Not long after spironolactone was introduced into clinical practice, in addition to its PR- and AR-mediated side effects, there appeared a series of studies which resulted in a black box label on Aldactone (the marketing name for spironolactone). In chronic toxicity studies in rats it appeared to be tumorigenic, leading to a decline in prescription rates. If biological effects can be categorized across four domains—physiology, pharmacology, toxicology and strength of materials (more commonly an engineering term) the doses used in rat studies were toward the latter end—e.g. 100 mg/kg/day for 104 weeks, producing benign hepatocellular, thyroid and testicular adenomas. Despite no tumorigenic or carcinogenic findings in dog and monkey studies, in further rat studies with potassium canrenoate (30/90/270 mg/kg/day) for 52 weeks >50% of the 270 mg/kg/day group died with a spectrum of neoplasia. Based on these findings the UK licensing authority directed manufacturers to stop recommending spironolactone for hypertension and idiopathic oedema.

A recent study (Mackenzie *et al.*, 2017), therefore, must come as a welcome relief to this "the sky is falling" series of messages, in two ways. The study covered 74,272 patients exposed to spironolactone between 1986 and 2013 matched 1:2 with unexposed controls. The outcomes were prespecified as primary (first incidence of ovarian, endometrial, pancreatic, colorectal, prostate, renal cell, pharyngeal and thyroid cancer, plus myelomonoblastic leukemias) or secondary (27 other types of cancer). The results are that there clearly is no evidence for an increased risk of any cancer associated with the use of spironolactone. What there was, however, was strong evidence for a significantly lower risk of prostate cancer in men (hazard ratio 0.69:95% confidence limits 0.60–0.80). The authors conclude that "the possible mechanisms and clinical implications merit further investigation," which is clearly the case. It may well reflect the anti-androgenic activity of spironolactone, but we would do well not to rule out effects via PR, expressed in various organs in men, role(s) totally unknown.

What it also means is that the litany of warning prospective patients of the undesirable side effects of spironolactone, there would appear to be one countervailing—and ultimately more important—side effect, that of protection against prostate cancer.

See also: Aldosterone Receptors. Aldosteronism in Congestive Heart Failure. Diuretics. Primary Aldosteronism; Diagnosis and Treatment

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Diuretics

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Glossary

Antiporter A membrane protein that actively transports two or more different molecules or ions at once across a phospholipid membrane in opposite directions.

Cotransporter Membrane transport proteins that couple the favorable movement of one molecule with its concentration gradient and unfavorable movement of another molecule against its concentration gradient. They include antiporters and symporters.

Symporter A membrane protein that transports two compounds simultaneously across a cell membrane in the same direction, one compound being transported down a concentration gradient and the other against a gradient.

Introduction: A Brief History of Diuretics

Diuretics (from the ancient greek word *διουρέω*: to urinate) are therapeutic agents that increase the urinary excretion of sodium and water. They act by decreasing sodium reabsorption at different sites in the nephron. By inducing a negative fluid balance, diuretics find application in different clinical conditions, from high blood pressure to edematous states.

Diuretic properties of specific plants were known since the dawn of medicine: ancient Egyptians used to administer mixtures of diuretic compounds, such as garlic, onion, parsley, juniper and dill, to induce water loss (Nunn, 2002) and Hippocrates also described celery and asparagus as diuretics (Petrovska, 2012). Similarly, diuretic properties of olive, grapes and ivy were known to the ancient Romans (Melillo, 1994). Under the name of cider mixture, an infusion of cider, juniper, mustard seed, ginger, horseradish and parsley-root was still recommended against dropsy in the late 18th century (Biddle, 1889). Among other herbs, digitalis had been discovered as a diuretic agent in 1775 (Withering, 1785), but at that time its inexplicable effectiveness on some, but not all, cases of edema, together with its toxic, occasionally fatal properties, led to the forsaking of the plant for medical use. 40–50 years later, a first distinction between cardiac and renal dropsy was introduced, but the use of digitalis as cardiac stimulator and diuretic in congestive heart failure had to await the middle of the 19th century, when the first animal studies tested the biological effects of digitalis on the heart (Seldin and Giebisch, 1997). Still, its action on the kidney were yet to be elucidated. It was not until the early 20th century, in fact, that the role of the kidney in the pathophysiology of fluid overload and in the functioning of diuretics began to be understood.

In the second half of the 19th century and throughout the first decades of the 20th, the pillar of diuretic therapy was represented by xanthines (Berglund *et al.*, 1935). Caffeine was the first xanthine used for therapeutic purposes, followed by the more powerful, long-lasting, cocoa-derivative theobromine (Richards and Plant, 1922). In 1902 the first synthetic xanthine, theophylline, was developed, followed a few years later by aminophylline. These diuretics revealed particularly effective to sustain diuresis when a decrease in glomerular filtration rate after other strategies was observed (Cragoe, 1983).

Mercury and its compounds represented an additional treatment against edema since the 16th century, following the experience of Paracelsus (Pagel, 1982). In particular, inorganic mercuric compounds were effective diuretics, due to increased chloride excretion, and they were routinely mixed with diuretic herbs, especially digitalis, for further mobilization of water. However, they gradually earned disapproval as a consequence of their toxicity, and it was only after the introduction of the less harmful organomercurials for the treatment of syphilis the 20th century, and the discovery of their effectiveness as powerful injectable diuretics, that these compounds were reintroduced into clinical practice as a mainstay of the treatment of edema at least until the 1950s (Eknoyan and Martínez-Maldonado, 1986). At that time, following the casual discovery of the rheological properties of sulfanilamide and the subsequent demonstration of its action as a carbonic anhydrase inhibitor, the first synthetic diuretics of this class—acetazolamide in 1954, chlorothiazide in 1957—were introduced in clinical medicine (Cragoe, 1983). Soon after the introduction of sulfonamyl diuretics, the progressive understanding of the functioning of the nephron led to the development of loop and potassium-sparing diuretics around the late 1950s to the early 1960s (Maren, 1987). A new era for diuretics had begun, based on the progressive elucidation of the role of the kidney in the management of fluid and ions.

Overview of Electrolytes Transport in the Nephron

To properly discern the mechanism of action of diuretics, it is first imperative to review the renal transport of electrolytes and its regulation. These processes are among the finest in nature and are made possible by the peculiar anatomy of the kidney, supremely condensed in its functional unit, the nephron. Minutely unraveled through the extent of the renal parenchyma, the nephron is composed of the glomerulus, the proximal and distal convoluted tubules (PCT, DCT), and the connecting tubule (CT), all hosted

in the cortex; and the loop of Henle, made of the thin descending and ascending limbs (tDL, tAL) and the thick ascending limb (TAL), which deepens into the medulla. Each tubular section of the nephron has specific properties in terms of permeability to ions and water, and so constitutes the target for a given class of diuretics. In fact, while all the sodium-transporting cells express Na^+/K^+-ATPase pumps in their basolateral membrane, with the duplicate function of returning reabsorbed sodium to the bloodstream and maintaining its intracellular concentration at relatively low levels, the driving force for sodium mobilization changes throughout the tubule, and so does the permeability to water.

Whilst liberally permeable to water, three different mechanisms mediate the sodium reabsorption in the PCT: the electroneutral Na^+/H^+ antiport, the electrogenic Na^+/solute cotransport, and the passive paracellular NaCl absorption (Seldin and Giebisch, 1997; Weinstein, 2013). Thanks to those systems, the reabsorption of the filtered sodium occurs for the greatest extent (around 60%–65%) at the level of the PCT. Such high potential is finely regulated by three key factors: the glomerulotubular balance, consisting in stimulated ions reabsorption following an increase in glomerular filtration rate to prevent massive salt wasting; the cellular pH, which modulates the enzyme activity in response to metabolic disturbances or potassium imbalances; and the effective arterial volume, with salt and water reabsorption representing a primary mechanism to maintain adequate blood pressure (Seldin and Giebisch, 1997).

The progressive change in the composition of the glomerular ultrafiltrate at the level of the loop of Henle exploits the selective permeability of its segments, which also explains the progressive hypertonicity of the interstitium in the medulla and the phenomenon of the countercurrent multiplication. In fact, water permeability is relatively high in the tDL due to the presence of aquaporins, whilst that of solutes is relatively low. As opposite, the tAL has a very low water permeability secondary to the lack of aquaporins, but an extreme passive permeability to salts. In the TAL, which is also impermeable to water, active transport of the intraluminal ions by the Na^+–K^+–$2Cl^-$ cotransporter generates the osmotic gradient that sustains water flow from the tDL into the interstitial space. The consequent selective, passive water absorption at the level of the tDL determines the isotonicity with the interstitial space of the luminal fluid as it advances along the tubule and its subsequent hypotonicity following passive reabsorption of NaCl along the tAL. Thus, water reabsorption in the tDL following active solute reabsorption in the TAL represents the real driving force for passive solute fluxes afterwards. The active Na^+–K^+–$2Cl^-$ cotransport at the level of the TAL is regulated by neurohormonal mechanisms and the glomerulotubular balance. About 20% of the filtered sodium is reabsorbed at the loop of Henle (Alpern *et al.*, 2012; Seldin and Giebisch, 1997).

In the DCT, sodium reabsorption partially mirrors that occurring in the PCT and involving Na^+/H^+ antiport; additional mechanisms include the NaCl coupled cotransport and the electrogenic transcellular Na^+ absorption that also occurs in the CT. As a specific target for mineralocorticoid hormones and vasopressin, the latter is responsible for an additional 3%–5% reabsorption of sodium and is a primary site of potassium transport. Conversely, downregulators of Na^+ absorption in the CT include prostaglandin E2 and bradykinin (Alpern *et al.*, 2012; Seldin and Giebisch, 1997).

The ion exchanges throughout the nephron also encompass other electrolytes, such as calcium and magnesium. We will examine in more detail their involvement when reviewing the single diuretics involved in their mobilization.

Classification and Mechanisms of Action of Diuretics

Based on the site in the nephron where they exert their activity, diuretics are grouped into five classes:

1. Carbonic anhydrase inhibitors;
2. Osmotic diuretics;
3. Loop diuretics;
4. Thiazide diuretics and related compounds;
5. Potassium-sparing diuretics.

The first one acts on the PCT, the second also on the loop of Henle, while the last three at the level of the TAL of the loop of Henle, the DCT-CT, and the aldosterone-sensitive principal cells in the CT, respectively. Vasopressin receptor antagonists, or aquaretics, act as antidiuretic hormone inhibitors and induce a selective water diuresis; they will be examined in a separate section.

Carbonic Anhydrase Inhibitors

The only diuretic of this family still in use in clinical practice is acetazolamide. It acts as a non-competitive inhibitor of the enzyme carbonic anhydrase (CA), which mediates the reversible formation of water and carbon dioxide from carbonic acid in the PCT. The enzyme contributes to the absorption of $NaHCO_3$ and the generation of a Cl^- gradient throughout the PCT that facilitates the passive NaCl absorption in its distal portion. Therefore, the inhibition of CA at that level results in decreased availability of H^+ for the Na^+/H^+ exchanger, with reduced $NaHCO_3$ absorption, and increased NaCl loss after lack of Cl^- gradient formation (Leaf *et al.*, 1954). The net diuresis produced by acetazolamide, however, is residual, mostly because the excess fluid delivered out of the PCT is reclaimed further down the nephron, particularly in the loop of Henle. In addition, a collateral consequence of its action is alkaline diuresis, and the subsequent metabolic acidosis further attenuates its diuretic effect (Leaf *et al.*, 1954).

As a consequence, the clinical uses of acetazolamide are restricted to a few, mainly non-diuretic effects, including the treatment of glaucoma, the CA at the level of the ciliary processes being inhibited, with subsequent reduction of intraocular pressure (Anon, 1955); high altitude illnesses, where it offsets hyperventilation-induced respiratory alkalosis, thus allowing chemoreceptors to better respond to hypoxic stimuli at altitude (Leaf and Goldfarb, 2007; Swenson, 2014); and familial periodic paralysis, where it favors the exchange of Na^+ with H^+ rather than K^+ (Resnick *et al.*, 1968). The main clinical application of acetazolamide as a diuretic remains that in edematous patients with metabolic alkalosis, where its bicarbonate-wasting effects tend to restore acid-base balance (Preisig *et al.*, 1987). This effect may be particularly important in patients with hypercapnic chronic lung disease, especially when posthypercapnic metabolic alkalosis develops and additional diuresis is required. In this case, acetazolamide was found to enhance $NaHCO_3$ excretion and to improve respiratory acidosis (Bear *et al.*, 1977; Miller and Berns, 1977), with shortened ventilator dependence in some (Banga and Khilnani, 2009), but not all (Faisy *et al.*, 2010) studies.

The impact of CA inhibitors on Ca^{++} transport is minimal: in fact, the reduced Ca^{++} reabsorption in the PCT is counter-balanced in the DCT, following increased distal delivery of HCO_3^-.

Acetazolamide can be given orally or intravenous twice a day, since its action lasts about 12 h due to tolerance induced by respiratory compensation for the metabolic acidosis (Wile, 2012).

Osmotic Diuretics

These drugs are filtrable, but non reabsorbable sugar alcohols that owe their diuretic action to the property of attracting water into the lumen of the tubule rather than acting on cellular sodium transport. As such, every solute that is excessively filtered can exert the same action: examples are glucose after severe hyperglycemia, or radiocontrast agents (Seldin and Giebisch, 1997). This class of diuretics is represented by mannitol, a low molecular weight (182 g/mol) isomer of sorbitol (Shawkat *et al.*, 2012). Once filtered, it drives a relative water diuresis in the PCT and the loop of Henle, with consequent increased urinary flow and only modest Na^+ excretion, but intense distal K^+ secretion (Seldin and Giebisch, 1997). Additional actions of mannitol include the induction of renal release of prostaglandins and the scavenging of free-radicals (Seldin and Giebisch, 1997). Based on these properties, mannitol was proposed as a renal protective agent against the risk of developing renal failure, like after cardiac and vascular surgery, renal transplantation, and in case of jaundice and rhabdomyolysis (Shawkat *et al.*, 2012). However, given the inconsistency of results in this field, together with the number of side effects potentially related to the use of mannitol (acute decompensation and pulmonary congestion in patients with heart failure; metabolic acidosis; electrolyte imbalance with hypernatraemia and hypokalaemia), its clinical application is substantially restricted to the treatment of cerebral edema and raised intracranial pressure, based on its immediate effect on plasma expansion, which leads to improved cerebral microvascular flow and oxygenation, and its delayed osmotic effect, which draws water from the cerebral extracellular space into the vasculature, provided the blood–brain barrier is intact (Shawkat *et al.*, 2012). Other uses are, in fact, empiric.

Loop Diuretics

The most powerful diuretics in use are referred to as the loop diuretics, since their site of action is substantially represented by the TAL of the loop of Henle, or high ceiling diuretics, based on their almost linear dose-effect curve as saluretics (Burg *et al.*, 1973). In fact, by competing for the chloride site on the Na^+–K^+–$2Cl^-$ cotransporter, which mediates the greatest NaCl reabsorption, they can induce the excretion of approximately 20%–25% of filtered sodium (Rose, 1991; Wile, 2012). This group includes furosemide, bumetanide, torsemide and ethacrynic acid.

The Na^+–K^+–$2Cl^-$ symporter, located in the TAL and the macula densa cells in the early DCT, is an electroneutral transporter whose action is driven by the low intracellular sodium concentration following its primary active extrusion by the basolateral Na^+/K^+-ATPase pump. The Cl^- gradient is maintained by the ion outflux through the basolateral chloride channel (CLCN), and, together with impermeability to water, the NaCl reabsorption determines urinary dilution and represent the first step for countercurrent multiplication, which is in turn essential for upstream urine concentration. The parallel recycling of K^+ back to the tubular lumen through the ATP-dependent renal outer medullary potassium channel ensures a virtually perpetual reserve of K^+ for coupled Na^+ transportation. In addition, the voltage difference generated by the basolateral Na^+/K^+-ATPase drives the paracellular reabsorption of calcium and magnesium.

In the presence of loop diuretics, the Cl^--binding site gets inactivated, with consequent NaCl loss. In the attempt to retrieve some Na^+, aldosterone-sensitive cells in the CD would exchange it for potassium, which would be wasted in urine. In addition, the transepithelial voltage difference being abolished, calcium and magnesium would be lost as well (Friedman, 1988). Consequent risks of loop diuretics misuse are, therefore, represented by hyponatraemia and possibly some hypokalaemia, hypocalcaemia, hypomagnesaemia, and metabolic alkalosis due to effective volume contraction. The risk of kidney stones and/or nephrocalcinosis after loop diuretics have been primarily reported in premature infants (Hufnagle *et al.*, 1982). The real measure of loop diuretic-induced hypercalciuria depends on the volume depletion secondary to their action: the greatest the latter, less pronounced the former. A clinical condition that mimics side effects of loop diuretics is the Bartter syndrome in its classic form, which develops after mutations in the gene for the symporter.

Among the loop diuretics, the one with uricosuric properties, that is ethacrynic acid, fell into disfavor after its pronounced side-effects of nausea and ototoxicity due to inhibition of an isoform of the Na^+–K^+–$2Cl^-$ cotransporter in the inner ear. The other

loop diuretics decrease urate loss by increasing its net reabsorption and reducing its secretion and, together with thiazides, represent independent risk factors for incident and recurrent gout (Choi *et al.*, 2005; Hunter *et al.*, 2006).

Thiazide Diuretics and Related Compounds

Historically, it was the search for a more powerful AC inhibitor that led to the development of the first thiazide diuretic, chlorothiazide. Accidentally, the latter was found to increase the excretion of NaCl rather than HCO_3^-, and it became subsequently clear that its major site of action was the DCT (Ernst and Moser, 2009). Under the name of thiazide diuretics are, therefore, included those primarily acting on the distal tubule, even though thiazide-type diuretics, namely chlorothiazide and hydrochlorothiazide, present structural differences compared to the thiazide-like compounds, such as metolazone, chlorthalidone and indapamide (Ernst and Moser, 2009).

Thiazide diuretics act by inhibiting the electroneutral Na^+/Cl^- symport located in the DCT after competitively binding its chloride site. The basolateral Na^+/K^+-ATPase represents, once again, the driving force for Na^+ reabsorption. The sulfamyl group on the benzene ring confers to some molecules of this class a limited action as AC inhibitors, but their net diuretic effect is residual, given the subsequent water reabsorption in the loop of Henle. Given the small extent at which Na^+ is reabsorbed at the level of the DCT (3%-5% of the filtered Na^+), the natriuretic effect of thiazide diuretics is limited, with a rapidly flattening dose-effect curve, so that they are also indicated as low ceiling diuretics. As a consequence, their clinical application is primarily represented by the treatment of hypertension, rather than that of edematous states.

Thiazides can also increase potassium and hydrogen losses in the DCT, potentially resulting in hypokalemia and metabolic alkalosis (Lyon and Degraff, 1964). The mechanisms underlying potassium wasting are double and indirect. The first is mediated by the activation of Na^+/K^+-ATPase in the DCT after increased urinary NaCl availability following the inhibition of sodium-chloride symporter. The second involves the activation, secondary to volume depletion, of the renin–angiotensin–aldosterone system, which again boosts Na^+/K^+-ATPase at the level of the CT.

Just as Bartter syndrome and loop diuretics, there is a clinical condition that mimics thiazides-related toxicity, namely Gitelman syndrome (Simon *et al.*, 1996). Also known as familial hypokalemia-hypomagnesemia, or hypomagnesemia-hypokalemia with hypocalciuria, it is a rare, autosomal recessive genetic disorder caused by mutations in the SLC12A3 gene, encoding the thiazide-sensitive NaCl cotransporter and featuring clinical characteristics which are similar to thiazide adverse effects (Simon *et al.*, 1996).

The DCT being the major site of active calcium reabsorption in the nephron, another effect of thiazide therapy is increased Ca^{++} reabsorption, which is thought to be mediated by thiazide-induced hyperpolarization secondary to Na^+ blockade, with consequent activation of voltage-gated Ca^{++} channels, as well as by increased activity of the basolateral Na^+/Ca^{++} exchanger (Costanzo, 1985). Thus, thiazides reveal useful in the management of idiopathic hypercalciuria and related nephrolithiasis (Gesek and Friedman, 1992). The parallel downregulation of the epithelial Mg^{++} channel may explain the observed hypomagnesemia after chronic thiazide use (Nijenhuis *et al.*, 2005).

As anticipated, another metabolic effect of thiazides is hyperuricemia (Healey *et al.*, 1959; Rosch and Sturman, 1962) and the risk of acute gout (Choi *et al.*, 2005; Hunter *et al.*, 2006). Thiazides exploit the organic anion transporter 1 (OAT1) on the basolateral membrane of the PCT to enter the tubular cells, and the apical urate anion exchanger, OAT4, to be released in the tubular fluid. This steps occurs in exchange with urate and drives its reabsorption (Hagos *et al.*, 2007). In addition, by upregulating the Na^+/H^+ exchanger in the PCT, they stimulate pH-dependent OAT4 activity for further urate reabsorption. The inhibition of the luminal multidrug resistance protein 4 also reduces secretion of urate (El-Sheikh *et al.*, 2008).

Additional metabolic effects reported for thiazides include dose-dependent hyperlipidemic (Grimm *et al.*, 1981) and hyperglycemic (Goldner *et al.*, 1960; Zhang and Zhao, 2016) effects. The first, however, is rarely seen at therapeutic doses and its clinical implications are controversial: in fact, it was proven to translate into worst clinical outcomes in some (Zanchetti *et al.*, 2004), but not all (ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group. The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial, 2002; Grimm *et al.*, 1981; Kasiske *et al.*, 1995) studies. Thiazide-induced hyperglycemia is thought to develop after potassium depletion, which, in turn, inhibits insulin release from pancreatic beta cells (Barzilay *et al.*, 2006). In support of this hypothesis is the fact that potassium restoration reverses this effect, unlike what seen with the use of other drugs of common use in hypertension (Rapoport and Hurd, 1964; Shafi *et al.*, 2008; Zillich *et al.*, 2006). The long-term impact of thiazide-induced hyperglycemia or diabetes seems, so far, to be residual (Barzilay *et al.*, 2012; Brown *et al.*, 2016). Recent evidence suggests that thiazide-like diuretics are superior to thiazide-type diuretics in the treatment of hypertension in terms of both efficacy and safety, given the lower incidence of hypokalemia and hyponatremia and the neutral glycolipid profile (Liang *et al.*, 2017; Messerli and Bangalore, 2011).

Potassium-Sparing Diuretics

This category includes two groups of drugs with different mechanism of action, sharing the property to reduce potassium excretion at the level of the aldosterone-sensitive sodium channels in the principal cells of the CT, and consisting in the direct inhibitors of the sodium channel (amiloride and triamterene), and the steroidal (spironolactone, eplerenone) and nonsteroidal (e.g., finerenone, apararenone, esaxerenone) mineralocorticoid receptor antagonists (MRAs) (Horisberger and Giebisch, 1987; Kolkhof and

Bärfacker, 2017). This class of diuretics has relatively weak natriuretic power, leading to the excretion of approximately 1%–2% of filtered sodium (Rose, 1991). Therefore, their main use in clinical practice occurs in combination with loop or thiazide diuretics to decrease potassium loss, rather than increase that of sodium (Hropot *et al.*, 1985).

Amiloride and triamterene determine direct inhibition of the Na^+/K^+ channel, thus blocking the reabsorption of sodium occurring at the expenses of potassium. The same action is observed after the use of the antibiotics trimethoprim and pentamidine (Kleyman *et al.*, 1995; Velázquez *et al.*, 1993). Amiloride is safer than triamterene, which is a potential nephrotoxic agent: in fact, crystalluria and cast/stones formation, with the potential risk of acute renal failure, have been reported after its use (Carr *et al.*, 1990; Farge *et al.*, 1986; Sica and Gehr, 1989). An effective clinical application of amiloride occurs in the treatment of polyuria and polydipsia due to lithium-induced nephrogenic diabetes insipidus. Lithium enters the principal cells of the CT through the same sodium channels targeted by amiloride and interferes with the ability of ADH to increase water permeability: the blockade of sodium channels decreases lithium entry into the tubular cells, thus reverting the concentrating defect (Grünfeld and Rossier, 2009; Trepiccione and Christensen, 2010). Amiloride and triamterene also exert a magnesium-sparing activity by reducing its excretion (Devane and Ryan, 1981). Reabsorption of Ca^{++} is also favored, likely as a consequence of cellular hyperpolarization.

MRAs originate from the search for aldosterone antagonists after this molecule was acknowledged as the mainstay of the regulation of Na^+/K^+ transportation. These drugs competitively inhibit the mineralocorticoid receptor that regulates both the number of apical sodium channels and that of active basolateral Na^+/K^+ pumps in the CT (Horisberger and Giebisch, 1987). Both steroidal and non-steroidal MRAs also appear to reduce proteinuria when used alone or in combination with an ACE inhibitor or an ARB in both type 1 and type 2 diabetes (Bakris *et al.*, 2015; Epstein *et al.*, 2006; Mehdi *et al.*, 2009; Rachmani *et al.*, 2004). In addition, there is evidence of beneficial effects of MR blockade in attenuating chlorthalidone-induced sympathetic activation and insulin resistance in humans, independent of blood pressure reduction (Raheja *et al.*, 2012), and also in reducing aldosterone-induced metabolic abnormalities, although not all of these effects (e.g., hyperglycemia, dyslipidemia) are MR-mediated (Baudrand *et al.*, 2016). Primary and secondary (e.g., heart failure, cirrhosis) hyperaldosteronism are specific clinical indications for the use of MRAs antagonists.

The first steroidal MRA to be developed about 50 years ago was spironolactone (Cella and Kagawa, 1957; Cella and Tweit, 1959), a pharmacologically active prodrug. Beside as a diuretic, spironolactone found enthusiastic clinical application in those conditions where the exploitment of its anti-inflammatory, anti-fibrotic properties could be warranted, such as in heart failure (Pitt *et al.*, 1999; Selye, 1955, 1960). However, it also presents undesirable, potentially harmful effects, from painful gynecomastia and menstrual irregularity, secondary to its binding to androgen and progesterone receptors, to possibly life-threatening hyperkalemia, especially among patients with reduced kidney function (Kolkhof and Bärfacker, 2017). Several molecules have been tested with the aim to develop safer alternatives to spironolactone, one being represented by eplerenone, which overcomes the endocrine side effects of spironolactone thanks to its less affinity for androgen and progesterone receptors (Kolkhof and Bärfacker, 2017). Eplerenone is, however, also less powerful as MRA than spironolactone (Garthwaite and McMahon, 2004). A few years after spironolactone was introduced into commerce, the use of its active metabolite canrenone was tested in humans (Kolkhof and Bärfacker, 2017). Together with potassium canrenoate, which is the only MRA clinically available for parenteral administration, canrenone also exerts lower antiandrogen activity.

Boosted by the challenge of identifying and targeting collateral aldosterone-mediated pathways of therapeutic relevance, basic research of the last decades focused on the development of novel non-steroidal MRAs with safer physicochemical profile and reduced incidence of adverse effects. This approach drove the synthesis of at least three molecules, namely apararenone, esaxerenone and finerenone, now tested in late stage clinical trials in patients with nonalcoholic steatohepatitis (apararenone), in those with hypertension or in the presence of type 2 diabetes mellitus and microalbuminuria (esaxerenone), and in individuals with heart failure plus chronic kidney disease and/or diabetes or patients with diabetic kidney disease (finerenone) (Kolkhof and Bärfacker, 2017). The latter is at the most advanced stages of investigation, and it appears as a more selective, efficient and powerful MRA than spironolactone and eplerenone. Its higher polarity allows a balanced distribution into cardiac and kidney tissues, likely explaining the more efficient reduction in cardiac hypertrophy, pro-B-type natriuretic peptide (pro-BNP) and proteinuria than eplerenone and spironolactone, with marginal effects on blood pressure and significantly lower incidence of hyperkalemia and renal failure (Kolkhof *et al.*, 2015; Naegele *et al.*, 2016; Pitt *et al.*, 2013). Lower mortality and less hospitalization for cardiovascular events occurred in patients treated with finerenone compared to eplerenone in a phase IIb trial on 1066 heart failure patients with reduced ejection fraction and concomitant type-2 diabetes mellitus and/or chronic kidney disease (Filippatos *et al.*, 2016). The results of these ongoing trials will better define the future of old and new MRAs.

Special Diuretics

In contrast with the described molecules, which interfere with electrolytes (namely sodium) and water reabsorption, vasopressin receptor antagonists (VPA), or aquaretics, act as antidiuretic hormone (ADH) inhibitors and induce a selective water diuresis. As such, they found application in specific clinical conditions, including the treatment of hyponatremia in the syndrome of inappropriate ADH secretion (SIADH) and in heart failure, and autosomal dominant polycystic kidney disease (ADPKD).

Vasopressin (ADH) acts through three different receptors: V1a, mainly located in blood vessels and liver, responsible for vasoconstriction and glucose metabolism; V1b, which mediates adrenocorticotropic hormone (ACTH) release from the pituitary gland; and V2, located in the basolateral membrane of the CT cells, primarily involved in the antidiuretic response (Greenberg and

Verbalis, 2006). Orally available VPAs, like tolvaptan, satavaptan, and lixivaptan, are selective for the V2 receptor, while conivaptan, the one that is available for injectable use, acts on both the V2 and V1a receptors. By blocking V2 receptors, VPAs prevent recruitment of aquaporin water channels to luminal cell membrane in CT and so promote selective water diuresis, while V1a blockade may improve systemic hemodynamics by decreasing afterload (Greenberg and Verbalis, 2006). Only tolvaptan and conivaptan are currently marketed.

In ADPKD, tolvaptan demonstrated to slow the progression of the disease, with lower decline in glomerular filtration rate (Ong, 2018; Torres *et al.*, 2017). Among patients with heart failure and symptomatic hyponatremia, short-term treatment with vaptans produces a water diuresis and raises the serum sodium concentration, with potential collateral benefit on mental status (Schrier *et al.*, 2006; Udelson *et al.*, 2001). However, no improvement in long-term cardiovascular or renal outcomes was observed in patients with heart failure after the use of vaptans (Inomata *et al.*, 2017; Konstam *et al.*, 2007; Schrier *et al.*, 2006).

The main adverse effects reported after the use of vaptans include thirst, dry mouth, fatigue, pollakiuria, polyuria, and polydipsia (Berl *et al.*, 2010). Tolvaptan should not be used in liver disease/cirrhosis for the risk of acute liver failure and death (Torres *et al.*, 2012), and clinicians must be warned of the risk of irreversible neurologic injury following excessively rapid correction of hyponatremia (Schrier *et al.*, 2006). Caution is recommended for the use of conivaptan in cirrhotic patients, given the risk of adverse hemodynamic effects and of increased variceal bleeding (Greenberg and Verbalis, 2006).

Conclusions

Centuries after the first, intuitive applications in medicine of diuretic compounds, the show offered by this class of drugs is far from over. The multiplicity of mechanisms involved in electrolytes and fluid balance, together with the invaluable resources deriving from advances in genetics, foretell further progress in this field, with the ultimate goal being represented by the patient-tailored, genetic-guided approach.

See also: Hypertension; Overview. Mineralocorticoid Receptor Antagonists. Neurogenic Hypertension. Renal Hypertension

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Calcium Channel Blockers

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General Pharmacology

Calcium-channel blockers (CCBs) or calcium antagonists bind to L-type calcium channels located on the vascular smooth muscle, cardiac myocytes, and cardiac nodal tissue (sinoatrial and atrioventricular nodes) reducing the influx of calcium into muscle cells, leading to relaxation of smooth muscle and cardiac muscular tissue. In cardiac nodal tissue, L-type calcium channels regulate pacemaker currents and phase 0 of the action potentials. Therefore, CCBs cause vasodilation, decrease myocardial inotropism, decrease heart rate and decrease conduction velocity within the heart (negative dromotropy), particularly at the atrioventricular node (**Fig. 1**) (Goodman and Gilman's, 2012).

Therapeutic Indications

CCBs are used to treat hypertension, angina and arrhythmias (Goodman and Gilman's, 2012).

Hypertension

By causing vascular smooth muscle relaxation, CCBs decrease systemic vascular resistance, which lowers arterial blood pressure. These drugs primarily affect arterial resistance vessels, with only minimal effects on venous capacitance vessels (Goodman and Gilman's, 2012).

Angina

The antianginal effects of CCBs are derived from their vasodilator and cardiodepressant actions. Systemic vasodilation reduces arterial pressure, which reduces ventricular afterload (wall stress) thereby decreasing oxygen demand. The more cardioselective CCBs (verapamil and diltiazem) decrease heart rate and contractility, which leads to a reduction in myocardial oxygen demand, which makes them excellent antianginal drugs. CCBs can also dilate coronary arteries and prevent or reverse coronary vasospasm (as occurs in Prinzmetal's variant angina), thereby increasing oxygen supply to the myocardium (Goodman and Gilman's, 2012).

Arrhythmias

The antiarrhythmic properties (Class IV antiarrhythmics) of CCBs are related to their ability to decrease the firing rate of aberrant pacemaker sites within the heart, but more importantly are related to their ability to decrease conduction velocity and prolong

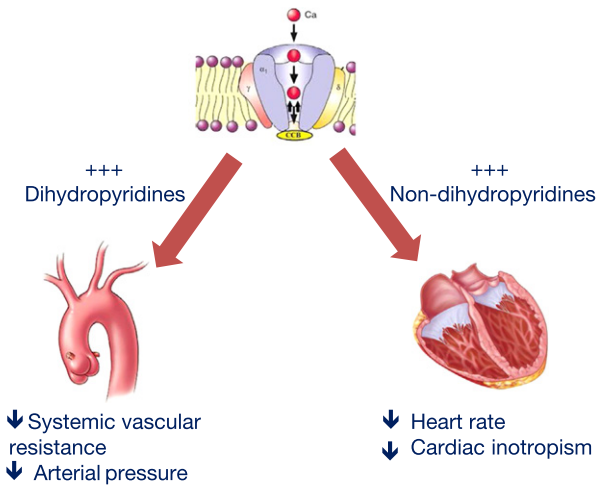

Fig. 1 Main mechanisms of action of calcium channel blockers.

repolarization, especially at the atrioventricular node. This latter action at the atrioventricular node helps to block reentry mechanisms, which can cause supraventricular tachycardia (Goodman and Gilman's, 2012).

Different Classes of Calcium-Channel Blockers

There are three chemical classes of CCBs. They differ not only in their basic chemical structure, but also in their relative selectivity toward cardiac versus vascular L-type calcium channels (**Fig. 1**). The most smooth muscle selective class of CCBs are the dihydropyridines. Because of their high vascular selectivity, these drugs are primarily used to reduce systemic vascular resistance and arterial pressure, and therefore are used to treat hypertension. Extended release formulations or long-acting compounds are used to treat angina and are particularly effecting for vasospastic angina; however, their powerful systemic vasodilator and pressure lowering effects can lead to reflex cardiac stimulation (tachycardia and increased inotropy), which can offset the beneficial effects of afterload reduction on myocardial oxygen demand.

Dihydropyridines include the following specific drugs:

- Amlodipine
- Felodipine
- Isradipine
- Nicardipine
- Nifedipine
- Nimodipine
- Nitrendipine
- Lercanidipine
- Barnidipine
- Lacidipine

Non-dihydropyridines, of which there are only two currently used clinically, comprise the other two classes of CCBs.

Verapamil (phenylalkylamine class), is relatively selective for the myocardium, and is less effective as a systemic vasodilator drug. This drug has a very important role in treating angina (by reducing myocardial oxygen demand and reversing coronary vasospasm) and arrhythmias.

Diltiazem (benzothiazepine class) is intermediate between verapamil and dihydropyridines in its selectivity for vascular calcium channels. By having both cardiac depressant and vasodilator actions, diltiazem is able to reduce arterial pressure without producing the same degree of reflex cardiac stimulation caused by dihydropyridines.

Side Effects and Contraindications

Dihydropyridine CCBs can cause flushing, headache, excessive hypotension, edema and reflex tachycardia. Baroreceptor reflex activation of sympathetic nerves and lack of direct negative cardiac effects can make dihydropyridines a less desirable choice for stable angina than diltiazem, verapamil or beta-blockers. Long-acting dihydropyridines (e.g., extended release nifedipine, amlodipine) have been shown to be safer anti-hypertensive drugs, in part, because of reduced reflex responses. This characteristic also makes them more suitable for angina than short-acting dihydropyridines. The cardiac selective, non-dihydropyridine CCBs can cause excessive bradycardia, impaired electrical conduction (e.g., atrioventricular nodal block), and depressed contractility. Therefore, patients having preexistent bradycardia, conduction defects, or heart failure caused by systolic dysfunction should not be given CCBs, especially the cardiac selective, non-dihydropyridines. CCBs, especially non-dihydropyridines, should not be administered to patients being treated with a beta-blocker because beta-blockers also depress cardiac electrical and mechanical activity and therefore the addition of a CCB augments the effects of beta-blockade (Goodman and Gilman's, 2012).

Cardiovascular Effects of Calcium Channel Blockers

CCBs, especially of the dihydropyridine sub-class, are potent and effective antihypertensive drugs. In the VALUE trial (Julius *et al.*, 2004), performed in high risk patients with essential hypertension, the head-to-head comparison between amlodipine and the angiotensin receptor blocker (ARB) valsartan, demonstrated that after six-month treatment the CCB was significantly more effective in reducing blood pressure values as compared to the ARB (4.3 and 2.5 mmHg for systolic and diastolic blood pressure, respectively). This difference has determined, at the end of the study, a significant reduction in myocardial infarction and a reduction in stroke (very close to be statistically significant) determined by amlodipine as compared to valsartan (**Fig. 2**).

CCBs blockers are very effective on the regression of left ventricular hypertrophy and the prevention of atherosclerosis.

Concerning the left ventricular hypertrophy, common knowledge considers the drugs blocking the renin-angiotensin system as the most potent in determining the regression of this important target organ damage. In contrast the meta analysis from Klingbeil *et al.* (2003) demonstrates that CCBs have the same efficacy as compared to ACE-inhibitors and ARBs.

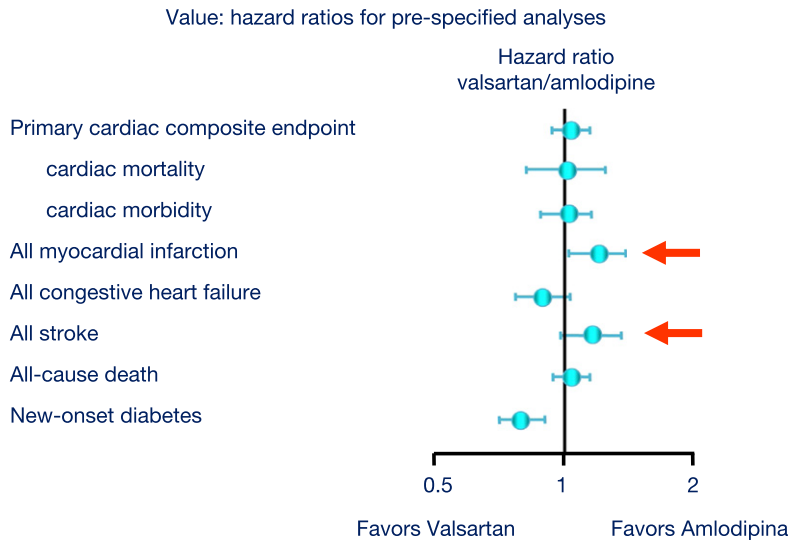

Fig. 2 In the VALUE study, although the effect of Amlodipine and Valsartan was similar on the composite end-point, the CCB significantly reduced the incidence of myocardial infarction as compared to the angiotensin receptor blocker. In addition there was a greater tendency of amlodipine to reduce stroke as compared to valsartan which resulted to be very close to the statistical significance. Adapted from Julius, S., Kjeldsen, S. E., Weber, M., *et al.* (2004). Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine: The VALUE randomised trial. *Lancet 363*, 2022–2031.

Moreover CCBs are highly effective in vascular protection, with a specific effect on the prevention of atherosclerosis. Thus, an early event in the atherosclerosis disease process is endothelial dysfunction, which promotes a constellation of processes that contribute to plaque development, including vasoconstriction, thrombosis, inflammation, oxidation and proliferation. In the healthy endothelium, nitric oxide (NO) protects the vessel wall not only by inducing vasodilation, but also through various anti-inflammatory benefits including scavenging of superoxide, inhibition of platelet aggregation, reduced hyper adhesiveness of leukocytes and interference with platelet aggregation (Flammer *et al.*, 2012). Essential hypertension is associated with endothelial dysfunction, which involves enhanced production of oxygen free radicals, that can destroy NO and reduce its availability, and release of endothelium-derived contracting factors including prostanoids and ET-1. In presence of impaired NO availability, a hyperpolarizing factor (EDHF) accounts for endothelium-dependent relaxations (Flammer *et al.*, 2012). It is worth noting that endothelial dysfunction is associated with increased incidence of atherosclerosis and cardiovascular events. In addition, the reversing of endothelial dysfunction can improve the prognosis of essential hypertensive patients, independently from blood pressure control (Flammer *et al.*, 2012). Thus a correct and modern approach to hypertensive patients needs an adequate blood pressure control associated to ancillary properties on organ damage, including restoration of endothelial function and prevention of cardiovascular events (Ghiadoni *et al.*, 2012). However, antihypertensive drugs show contrasting effects in terms of improvement or restoration of endothelial function (Ghiadoni *et al.*, 2012).

CCBs are among the most powerful compounds to interfere with endothelial dysfunction and therefore they can prevent the development of atherosclerosis, an effect mainly mediated by the strong antioxidant activity which characterizes this drug class. Available literature indicates that CCBs act preferentially on endothelial dysfunction at the level of microcirculation, while ACE-inhibitors are very active at the level of large arteries. The effect exerted by CCBs is mediated by strong antioxidant properties which cause a restoration of NO availability (Ghiadoni *et al.*, 2012).

In addition, CCBs are also effective in reversing vascular remodeling in small arteries, one of the most important mechanisms determining the increase in peripheral vascular resistances in hypertensive patients (Schiffrin *et al.*, 2002). Worth of noting is that this effect is independent from blood pressure reduction since it is not observed with other antihypertensive drug classes, including diuretic of beta-blockers (Schiffrin *et al.*, 2002).

Taken together, all this line of evidence supports the antiatherosclerotic properties of CCBs, a finding which is strongly supported by controlled clinical trials.

VHAS (Verapamil in Hypertension and Atherosclerosis Study) (Zanchetti *et al.*, 1998) Study first demonstrated the superiority of the CCB verapamil, as compare to the diuretic chlorthalidone, to inhibit coronary atherosclerosis. Moreover, a subgroup of patients enrolled in the INSIGHT (Intervention as a Goal in the Hypertension Treatment) (Simon *et al.*, 2001) study and ELSA (European Lacidipine Study on Atherosclerosis) (Zanchetti *et al.*, 2002) study, demonstrated that nifedipine or lacidipine, respectively, significantly reduced the progression of carotid artery intima-media thickness as compare to the diuretic hydrochlorothiazide or the beta-blocker atenolol. All the above reported trials were conducted in patients with essential hypertension.

It is worth noting that similar results were observed in patients with coronary artery disease. INTACT (International Nifedipine Trial on Antiatherosclerotic Therapy) study (Lichtlen *et al.*, 1990) demonstrated that nifedipine can inhibit the formation of new atherosclerotic plaques during a 3-year period of observation. In the PREVENT (Prospective Randomized Evaluation of the

Vascular Effects of Norvasc Trial) (Pitt *et al.*, 2000), amlodipine slowed the progression of carotid artery early atherosclerosis while in the CAPARES (coronary angioplasty amlodipine restenosis study) (Jorgensen *et al.*, 2000) the same compound significantly reduced the incidence of re-stenosis after coronary revascularization (4-month follow up).

Finally, in a sub study of CAMELOT (comparison of amlodipine vs. enalapril to limit occurrence of thrombosis) (Nissen *et al.*, 2004), in 274 patients coronary progression of atherosclerosis was assessed by IVUS at baseline and at the end of the study. As compared to baseline, IVUS revealed a significant progression of atherosclerosis in the placebo group ($P < .001$), a tendency to progression in the enalapril group ($P = .08$), but a lack of progression in the amlodipine group ($P = 0.31$).

Thus the well documented antiatherosclerotic effect of CCBs is further reinforced by a meta analysis which demonstrates that this drug class is superior, as compared to ACE-inhibitors, on this specific end-point (Wang *et al.*, 2006).

Effects of Calcium Channel Blockers on Clinical Hard End Points

Controlled clinical trials demonstrate that CCBs have a preferential effect on stroke. This issue is specifically addressed in a meta analysis (Turnbull *et al.*, 2003) demonstrating that CCBs are superior to ACE-inhibitors or beta-blocker/diuretic combination in preventing cerebrovascular disease (**Fig. 3**). Moreover it is worth of mentioning a meta regression from Verdecchia *et al.* (2005) which evaluates the relationship between blood pressure reduction exerted by ACE-Inhibitor or CCBs and coronary artery disease or cerebrovascular disease. While CCBs reduce the incidence of coronary artery disease exclusively by blood pressure reduction, ACE-inhibitor show that part of the beneficial effect (around 38%) is pressure independent. In contrast, concerning the stroke, the beneficial effect of ACE-inhibitors is totally dependent on blood pressure reduction, while around 34% of effect of CCBs is pressure independent.

Thus, it is conceivable that CCBs might be the first choice drug class for the prevention of cerebrovascular disease.

Does an evidence exist supporting the possibility to use CCBs in high risk patients, including patients with coronary artery disease?

Best demonstration that CCBs are effective in patients with coronary ischemic disease (with or without hypertension) derives from the results of CAMELOT study (Nissen *et al.*, 2004). In 1991 patients with documented coronary disease, the effectiveness of 24-month treatment with amlodipine (10 mg/daily), enalapril (20 mg/daily) or placebo was compared. Primary end point was a composite of cardiovascular death, non fatal myocardial infarction, non fatal cardiac arrest, coronary revascularization, angina pectoris hospitalization, heart failure hospitalization, fatal and non fatal stroke, TIA or new diagnosis of peripheral arteriopathy. Patients received concomitant treatment including beta-blockers, statins and antiplatelet drugs.

Incidence of cardiovascular events was 23.1% in the placebo group, 16.6% in the amlodipine group ($P = .003$) and 20.2% in the enalapril group ($P = .16$). Thus while enalapril did not show any superiority as compared to placebo, only amlodipine determined a statistically significant 31% reduction of cardiovascular events.

Thus CCBs are effective drugs in all patients with cardiovascular disease.

Endocrine Effects of Calcium Channel Blockers

A number of dihydropyridine CCBs possess a mineralocorticoid receptor antagonist activity. Nimodipine and felodipine in particular compete with aldosterone binding to the mineralocorticoid receptor "ligand-binding domain" (LBD) with a similar

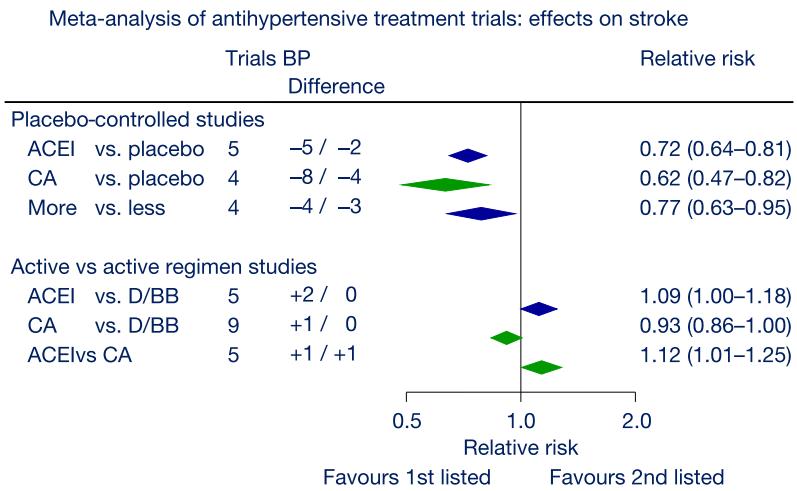

Fig. 3 This meta-analysis shows that CCBs are superior to ACE-inhibitors or to the combination beta-blockers/diuretics in reducing the incidence of stroke. Adapted from Turnbull, F., Blood Pressure Lowering Treatment Trialists' Collaboration. (2003). Effects of different blood-pressure-lowering regimens on major cardiovascular events: Results of prospectively-designed overviews of randomised trials. *Lancet 362*, 1527–1535.

potency as compared to eplerenone (Dietz *et al.*, 2008) (**Fig. 4**). In particular, dihydropyridine CCBs block aldosterone-induced recruitment of coactivators, and inhibit aldosterone-induced gene expression. Among dihydropyridines, a complete inhibition is elicited by nimodipine, felodipine, and nitrendipine, while amlodipine reduced aldosterone activation by only 50%. Some earlier studies suggest also that some dihydropyridine CCBs, such as efonidipine and benidipine (Imagawa *et al.*, 2006), are able to inhibit aldosterone production in in human adrenocortical cell line through blockade of T-type voltage-dependent calcium channels. Radiolabeled aldosterone binding assay suggests that benidipine directly binds to the mineralocorticoid receptor and excludes aldosterone binding. Benidipine showed more potent activity than the other calcium channel blockers, efonidipine, amlodipine, or azelnidipine, but it is less effective than nimodipine (Kosaka *et al.*, 2010). MR antagonist activity of CCBs is restricted to the dihydropyridine class: the non-dihydropyridine CCBs have no effect on MR (Dietz *et al.*, 2008).

Furthermore, the MR antagonist activity of CCBs is not due to the indirect effect by inhibition of L-type calcium channels (Kosaka *et al.*, 2010). In this framework, some authors argue that the beneficial effect of nimodipine on cerebral ischemia and stroke might be related to MR inhibition in the brain rather that to the effect on calcium channels (Dietz *et al.*, 2008).

Genetic studies confirmed the interrelationship between T-type calcium channel receptors and aldosterone signaling. A recurrent gain-of-function mutation in a T-type calcium channel, CACNA1H(M1549V) was identified as the cause of a novel Mendelian disorder characterized by early-onset primary aldosteronism and hypertension. When this mutation is transfected in the aldosterone-producing adrenocortical cancer cell line H295R and its subclone HAC15, aldosterone synthesis raised by seven times and was abrogated by the T-channel blocker antagonist mibrefadil (Reimer *et al.*, 2016).

On this basis, in the last years a new class of dihydropyridine-derived non-steroidal mineralocorticoid antagonists (MRAs) has been discovered. Indeed, very recently new molecules have been patented in the dihydropyridines family with mineralocorticoid receptor antagonist properties and they are designated as third-generation MRAs (Arhancet *et al.*, 2010). The first potent and selective MR antagonist of this class that was investigated was BR-4628. BR-4628 is a nonsteroidal mineralocorticoid receptor

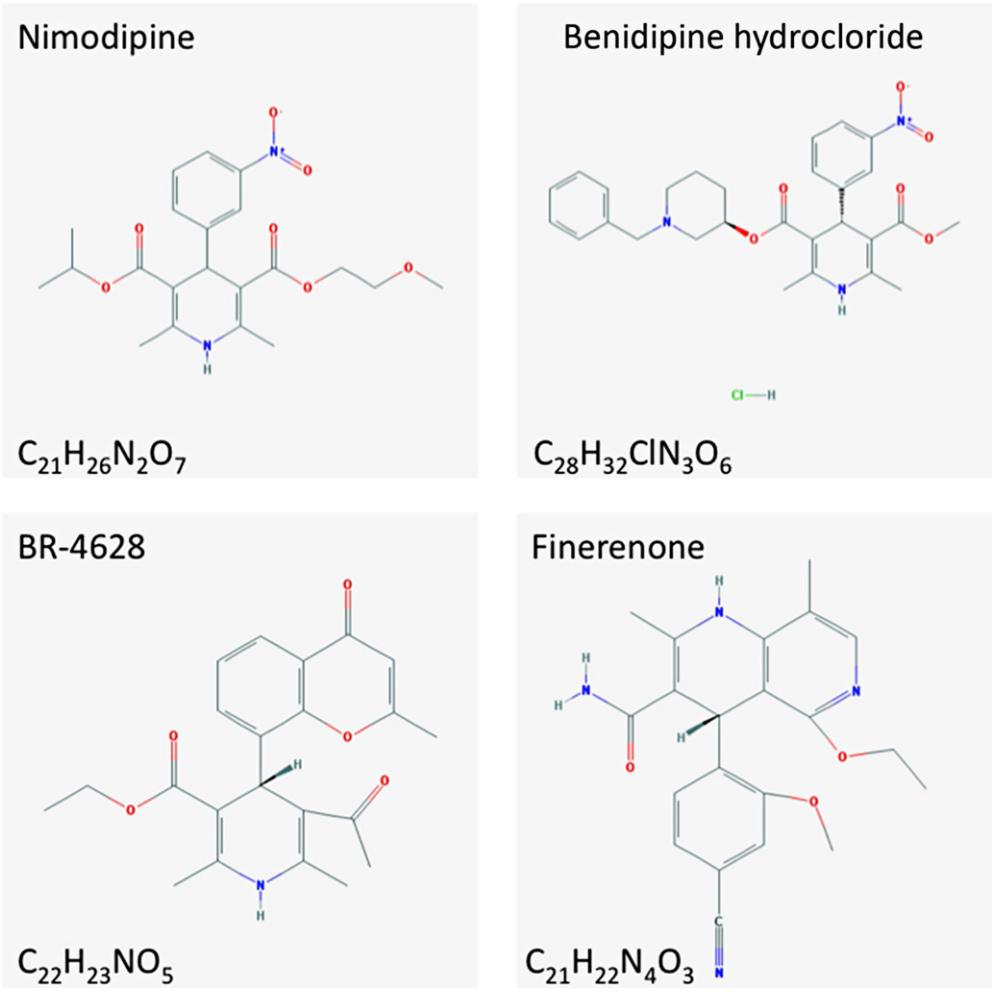

Fig. 4 Chemical structure of old (nimodipine, benidipine) and novel (BR-4628, finerenone) calcium channel blockers with mineralocorticoid receptor antagonist properties.

antagonist, which shares a similar efficacy with spironolactone as mineralocorticoid receptor inhibitor, but has no effect on the other steroidal receptors and on the L-type calcium channel (Fagart *et al.*, 2010).

Although BR-4628 has a strong binding activity to the MR, the MR-ligand complex is highly unstable. This precludes recruitment of transcriptional co-modulators, thereby disabling proper transcription and exerting a global inhibitor effect (Fagart *et al.*, 2010). In vitro studies showed that this compound has a 160-fold larger selectivity for the MR than for the androgen receptor, thus contrasting to an only 3-fold selectivity of spironolactone. Thus, this class is not expected to cause gynecomastia, a well-known adverse side effect of spironolactone. The potency to block the MR is similar to that of spironolactone, with a half-maximally inhibitory concentration (IC50) of 28 and 24 nM, respectively: therefore it is 40 times more potent than eplerenone (IC50: 990 nM). In contrast, this prototype of this class of MR antagonists has very weak calcium channel blocking activity, as compared to conventional dihydropyridine CCBs such as nitrendipine (Fagart *et al.*, 2010).

These seminal findings led to the synthesis of new molecules, such as finerenone (BAY 94-8662), which is substantially more potent and selective than spironolactone. For which data from randomized clinical trials are also available (Barfacker *et al.*, 2012). After several experimental animal studies, this drug has been tested in a randomized controlled trial for safety and efficacy (Pitt *et al.*, 2013). This trial recruited patients with heart failure with reduced EF and moderate chronic kidney disease randomized to: finerenone at different dosages, spironolactone or placebo. Finerenone decreased biomarkers of heart failure to a similar degree as spironolactone and was associated with less hyperkalemia and less renal function worsening than spironolactone.

These encouraging results have been tested in a number of larger phase 2 studies in patients with heart failure (ARTS-HF study) (Filippatos *et al.*, 2016) and patients with type 2 diabetes mellitus with diabetic nephropathy (ARTS-DN) (Bakris *et al.*, 2015).

The MinerAlocorticoid Receptor antagonist Tolerability Study-Heart Failure (ARTS-HF), enrolled 1066 patients with worsening heart failure and reduced ejection fraction and chronic kidney disease and/or diabetes mellitus, randomized to finerenone at different dosages or eplerenone. In this ARTS-HF, a reduction of $>30\%$ in baseline NT-proBNP (the primary endpoint, was achieved by finerenone at all dosages.

Cumulative event rates of the composite clinical endpoint (death from any cause, cardiovascular hospitalization, or emergency presentation for worsening chronic heart failure at 90 days) were significantly improved in the finerenone arms in comparison to eplerenone arm, when full doses were used. This trial also confirmed the neutral effect on BP and a similar incidence of hyperkalemia compared with eplerenone (Filippatos *et al.*, 2016).

In the ARTS-DN study, 823 patients with diabetic nephropathy treated with RAS-blockers and micro- or macroalbuminuria were randomized to finerenone from 1.25 to 20 mg/die or placebo. Finerenone treatment resulted in an improvement in the urinary albumin-creatinine ratio after 90 days (the primary outcome variable) in comparison to placebo, with a fairly good safety profile in terms of incidence of hyperkalemia (maximum 3.2% and GFR reduction (comparable to placebo).

None of the different doses tested induced a significant decrease in systolic BP in any of these studies. This different profile in comparison to classical MR antagonists might be attributed to the fact that Bay 94-8862 has a lower tissue distribution in the kidney as compared to the heart and constitutes an asset in patients with heart failure. An other possibility is the inability to cross the blood–brain barrier (at variance with steroidal MR antagonists) and act on cerebral MR, which are believed to play a major role in the control of blood pressure (Gomez-Sanchez and Gomez-Sanchez, 2012). On the other hand, for the same reason this drug is less suitable as an antihypertensive drug in the setting of essential hypertension or primary hyperaldosteronism. Indeed, no ongoing trials are present to date on clinicaltrials.gov entering as search terms “aldosteronism” and “finerenone” or “BAY 94–8862”.

Conclusions

CEBs can be considered a corner-stone of cardiovascular treatment. Especially in patients with hypertension these drugs are even underused in respect of their potential beneficial effect not only on blood pressure lowering but also on target organ damage and cardiovascular events, including stroke and coronary artery disease.

Concerning their activity on the endocrine system dihydropyridine CCBs possess a mineralocorticoid receptor antagonist activity as well a possible inhibitory effect on the aldosterone secretion which seems to be dependent on blockade of t-type calcium channels. This effect might be potential relevant in cardiovascular medicine and ongoing studies will tell us in the next future their possible application. In addition, these properties have disclosed a new line of research leading to the development of new molecules with should potentially act in a more specific and potent way on the mineralcorticoid system.

See also: Endocrine Hypertension. Hypertension; Overview. Neurogenic Hypertension. Renal Hypertension

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Pseudohypoaldosteronism Type 1

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Introduction

Pseudohypoaldosteronism type 1 (PHA1) is a rare pediatric disease characterized by a resistance to mineralocorticoid hormones. PHA1 is due to abnormalities of the mineralocorticoid receptor (MR) and the epithelial amiloride-sensitive sodium channel ENaC, molecules that play a crucial role in the regulation of water and electrolyte balance mediated by aldosterone. In neonates, the correct action of the mineralocorticoid axis is required as the immature tubular function in this period may impair the regulation of water and electrolyte balance by the kidneys. Moreover, prematurity, infections and, also, the physiological partial resistance to aldosterone in the newborn may contribute for the impaired regulation of electrolyte balance dependent of aldosterone.

The original case was reported by Cheek and Perry in 1958, who described an infant with severe salt wasting and failure to thrive without any renal or adrenal abnormalities. In general, patients exhibit severe salt wasting in the neonatal period, with hyponatremia, hyperkalemia and metabolic acidosis despite extremely high levels of plasma renin and aldosterone (Riepe, 2009). Classically, two different forms of PHA1 have been described, a renal mild form and a severe generalized form (Hanukoglu, 1991) with different genetic alterations described. Additionally, a secondary form of PHA1 associated with renal abnormalities was also described (Rodríguez-Soriano *et al.*, 1983). Moreover some studies have shown intermediate forms of PHA1 with an intermediary phenotype between the renal and the generalized forms.

Aldosterone Function in the Distal Nephron

The knowledge of the physiological role of aldosterone in the distal nephron, regulating volume and electrolyte homeostasis, is fundamental to understand the clinical consequences of the mineralocorticoid resistance. Aldosterone acts in a region of the nephron that includes the late distal convoluted tubule (DCT), the connecting tubule (CNT) and the collecting duct (CD), named aldosterone-sensitive distal nephron (ASDN). ASDN express the aldosterone receptor MR, the final effector of aldosterone in epithelial tissues (the sodium channel ENaC), and the 11-β hydroxysteroid dehydrogenase type 2, enzyme responsible for mineralocorticoid selectivity (Loffing and Kaissling, 2003; Farman and Rafestin-Oblin, 2001). Only 5%–10% of the filtered Na^+ load occur in ASDN, however, these nephron segments are responsible for the fine-tuning of the renal excretion of Na^+ (Campean *et al.*, 2001; Loffing *et al.*, 2001). In ASDN cells, sodium reabsorption is mediated at the apical cell membrane by ENaC (Biner *et al.*, 2002; Loffing and Kaissling, 2003; Reilly and Ellison, 2000). The sodium transport across the basolateral membrane to the interstitial fluid is mediated via the ubiquitous Na^+-K^+-ATPase, generating driving force for Na^+ transport. The sodium transport via the Na^+-K^+-ATPase generates large ion concentration gradients that drive potassium and hydrogen ion secretion (Reilly and Ellison, 2000). In the ASDN, apical K^+ channels as the renal outer medullary potassium channel (ROMK1 or Kir1.1) are involved in K^+ secretion (Wang *et al.*, 2010).

Aldosterone exerts its actions by binding to its receptor MR. After aldosterone binding MR will dilerize dimerization and translocate to the nuclei, where it will bind to regulatory regions of hormone responsive genes. This will increase the expression of aldosterone target genes, including the epithelial sodium channel ENaC, the Na^+–K^+-ATPase, and several genes involved in the transepithelial sodium transport. Aldosterone increases mRNA levels of the serum- and glucocorticoid regulated kinase 1 (sgk1), which phosphorylates Nedd4-2, protein responsible for ENaC ubiquitination. Phosphorylated Nedd4-2 is no more able to interact with ENaC, decreasing its retrieval and degradation, and increasing its localization to the apical membrane (Bhalla and Hallows, 2008). MR also increases ENaC expression in membrane surface and activity by regulating the transcription of the deubiquitylating enzyme Usp2-45 (Verrey *et al.*, 2008). An additional mechanism leading to increased ENaC expression on the cell membrane is the stimulation of glucocorticoid-induced leucine zipper protein (GILZ) expression by the MR, leading to the inhibition of the extracellular signal–regulated kinase (ERK) (Soundararajan *et al.*, 2005). MR seems also to play a role on K^+ secretion by stimulating sgk1 expression. Sgk1 could increase the export of ROMK channels from the endoplasmic reticulum, and suppress the inhibitory effect of serine/threonine protein kinase WNK4 on ROMK channels (Ring *et al.*, 2007; Vallon *et al.*, 2005; Wang *et al.*, 2010).

Renal PHA1

Clinical Features

Renal PHA1 (MIM#177735), also called autosomal dominant PHA1, is a mild and the most frequent form of PHA1, with a prevalence of $\sim$1 per 80,000 newborns (Zennaro and Fernandes-Rosa, 2017). Familial cases with an autosomal dominant inheritance and sporadic cases were described. The phenotypic expression is restricted to the kidney, with patients exhibiting a salt

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losing syndrome in the neonatal period, with different degrees of weight loss, failure to thrive, vomiting and dehydration. Biological analysis is characterized by hyponatremia, hyperkalemia, metabolic acidosis, inappropriately high urinary sodium excretion, and low urinary potassium excretion. Despite the clinical and biological findings compatible with decreased aldosterone, affected neonates exhibit high plasma and urinary aldosterone and high plasma renin levels, confirming the mineralocorticoid resistance (Zennaro and Lombes, 2004). The symptoms usually improve in early childhood (18–24 months), with plasma aldosterone remaining high into adulthood (Zennaro *et al.*, 2012). The explanation for this clinical improvement includes kidney maturation, access to dietary salt and tubulo-glomerular feedback regulation. Phenotypic variability is observed within the PHA1 families, ranging from subjects with severe salt loss in the neonatal period to asymptomatic subjects. Interestingly, adult patients with renal PHA1 and MR mutations have no adverse cardiovascular outcome, but rather an improved diastolic left ventricular function, suggesting suggests that the cardiovascular consequences of aldosterone excess require full MR signaling (Escoubet *et al.*, 2013).

Pathophysiology

The genetic defect underlying the development of renal PHA1 was elucidated in 1998 by Geller *et al.* The authors identified heterozygous mutations in the *NR3C2* gene (coding for the MR) in four dominant and one sporadic cases of PHA1 (Geller *et al.*, 1998). Since this description, more than 70 heterozygous *NR3C2* mutations were described in patients with renal PHA1 (Zennaro and Fernandes-Rosa, 2017). Interestingly, in about 30% of cases, no MR mutations were identified.

NR3C2 spans over ~450 kB on chromosome 4p31.23 and is composed of 10 exons (Zennaro *et al.*, 1995). MR is a nuclear receptor and acts as a transcription factor, regulating the transcription by binding to specific hormone elements located in regulatory regions of target genes. The human MR is composed of 984 amino acids and possess three separate domains: N-terminal, DNA binding and ligand binding domains (Viengchareun *et al.*, 2007). The N-terminal domain (NTD), encoded by exon 2 of *NR3C2* contains an autonomous activation function (AF-1), supports recruitment of specific coregulators responsible for higher selectivity at the transcriptional level, and is a site of post-translational modifications that modulate its transcriptional activity (Pascual-Le Tallec and Lombes, 2005). The DNA binding domain (DBD), encoded by exons 3 and 4 of *NR3C2*, is composed of two zinc-finger involved in DNA binding and receptor dimerization (Luisi *et al.*, 1991). The ligand binding domain (LBD), encoded by exons 5–9 of *NR3C2* is involved in ligand binding and in the interaction with heat shock proteins and transcriptional coactivators (Couette *et al.*, 1996).

Mutations leading to renal PHA1 are always heterozygous and are located in all exons of the *NR3C2* gene. To date, no missense mutations were reported in exon 2, suggesting that missense mutations located in the N-terminal domain do not sufficiently affect MR function. *NR3C2* mutations occur at high frequency in patients with familial autosomal dominant and in sporadic PHA1 (Pujo *et al.*, 2007). The pathogenic mechanism of PHA1 in patients with heterozygous MR mutations is dependent on the type of mutation, comprising haploinsufficiency but also dominant negative effects on the wild type receptor (Riepe *et al.*, 2006; Sartorato *et al.*, 2003, 2004a). MR mutations differentially affect individual gene expression in a promoter-dependent manner (Fernandes-Rosa *et al.*, 2011; Sartorato *et al.*, 2004b). The same mutation may induce complete functional loss of transcriptional activity on one target promoter, while retaining a partial transcriptional activity on another gene. In this case, the phenotype of PHA1 may be modulated, in terms of target gene expression, not only by the quantitative extent of functional reduction but also by the specific qualitative impact of MR function over target genes (Fernandes-Rosa *et al.*, 2011). The clinical severity of renal PHA1, however, is not correlated with a particular MR genotype (Riepe, 2009). This could be explained by adaptive and compensatory mechanisms occurring in the distal part of the nephron.

Generalized PHA1

Generalized PHA1 (MIM #264350), is a severe, autosomal recessive, form of PHA1. It is characterized by salt wasting from multiple organs, including kidney, distal colon, and the salivary and sweat glands (Bosson *et al.*, 1986; Oberfield *et al.*, 1979; Savage *et al.*, 1982). Patients exhibit with severe dehydration, vomiting and failure to thrive in the neonatal period and the clinical course may be complicated by cardiac dysrhythmias, collapse, shock or cardiac arrest (Speiser *et al.*, 1986). Biological analysis is characterized by severe hyperkalemia, high plasma aldosterone and high plasma renin levels, confirming the mineralocorticoid resistance. The presence of elevated sweat and salivary Na^+ and Cl^- and absent nasal amiloride-sensitive Na^+ transport corroborate the systemic mineralocorticoid unresponsiveness (Hanukoglu, 1991; Thomas *et al.*, 2002). Signs of systemic salt loss may include a respiratory syndrome characterized by persistent rhinorrhea of clear liquid, congestion, tachypnea, wheezing, fever and recurrent pulmonary infections, due to their reduced capacity to absorb liquid from airway surfaces (Kerem *et al.*, 1999; Schaedel *et al.*, 1999). Cutaneous lesions similar to those appearing in miliaria rubra, associated to inflammation and damage in the eccrine structures, are due to high concentration of sweat salt (Martin *et al.*, 2005; Hanukoglu *et al.*, 2017). Cholelithiasis and salt loss from the Meibomian glands were also reported in children with PHA1 (Akkurt *et al.*, 1997; Kuhnle, 1997).

Early diagnosis is critical to survival in generalized PHA1 (Belot *et al.*, 2008). In contrast to renal PHA1, patients with generalized PHA1 suffer from recurrent life-threatening episodes of salt loss in childhood (Adachi *et al.*, 2001; Hanukoglu *et al.*, 2008). Interestingly, the evaluation of patients with systemic PHA1 throughout adolescence and early adulthood showed gradual normalization of urinary

Na/K ratios with age (Hanukoglu *et al.*, 2008). In the same way, the patients with near normal lives on a life-long high salt diet, and even PHA1 improvement with cessation of salt supplementation were described (Adachi *et al.*, 2010; Edelheit *et al.*, 2010).

Pathophysiology

Systemic PHA1 is caused by mutations in *SCNN1A*, *SCNN1B*, and *SCNN1G* genes coding for the ENaC subunits α, β and γ, respectively. *SCNN1A* is located on chromosome 12p13.3, whereas *SCNN1B* and *SCNN1G* are located within 400 kb on chromosome 16p12.2-p13.11. The three genes are comprised of 13 exons spanning over 17 kb. Parts of exons 2 and 13 encode the transmembrane regions, and exons 3–13 code for the extracellular loop (Saxena *et al.*, 1998; Thomas *et al.*, 1996; Ludwig *et al.*, 1998). After the association of the locus of ENaC subunits on chromosomes 12 and 16 with generalized PHA1 (Strautnieks *et al.*, 1996b), Chang *et al.* identified mutations in *SCNN1A* and *SCNN1B* in PHA1 patients from Arabian and Iranian Jewish ethnicity (Chang *et al.*, 1996). A mutation affecting *SCNN1G* was described in three families originating from the Indian subcontinent showing linkage to chromosome 16p (Strautnieks *et al.*, 1996a). Different homozygous or compound heterozygous inactivating mutations of ENaC subunits, as well as a large deletion in the promoter region of βENaC, were subsequently reported (Adachi *et al.*, 2001; Bonny *et al.*, 2002; Edelheit *et al.*, 2005; Kerem *et al.*, 1999; Riepe *et al.*, 2009; Saxena *et al.*, 2002; Schaedel *et al.*, 1999). ENaC mutations are in majority homozygous, mostly in consanguineous families, although compound heterozygous mutations can occur in non-consanguineous pedigrees. In contrast to the renal PHA1, ENaC mutations are identified in all subjects investigated.

ENaC constitutes the rate-limiting step for sodium reabsorption in ASDN of the kidney, the distal colon, salivary and sweat glands, and the lung (Canessa *et al.*, 1994). The three ENaC subunits act together to confer the channel's low Na^+ conductance, and its high selectivity for Na^+ and amiloride. While in animal models, the inactivation of one ENaC subunit is associated to specific phenotypes (Hummler *et al.*, 1996; McDonald *et al.*, 1999; Barker *et al.*, 1998), in humans the genotype–phenotype correlations are not well established. Hanukoglu *et al.* evaluated the reported distinct growth and puberty phenotypes in PHA1 patients depending on the degree of functional ENaC impairment (Hanukoglu *et al.*, 2008).

In the lungs, ENaC contributes for removal of fluid from the alveolar space by regulating the transepithelial alveolar sodium transport (Hummler and Planes, 2010). Accordingly, ENaC plays a role on the pathogenesis of pulmonary diseases such as cystic fibrosis and respiratory distress syndrome in preterm infants. In cystic fibrosis, ENaC activity is increased, contributing to airway mucus dehydration and decreased mucociliary transport (Boucher, 2007). In contrast, in systemic PHA1, the absence of ENaC activity results in increased airway surface liquid (Kerem *et al.*, 1999).

Secondary PHA1 and Intermediate Forms

Secondary PHA1 is characterized by transient mineralocorticoid resistance in infants with less than 7 months of age, associated with malformations in the urinary tract and/or urinary tract infections (Bulchmann *et al.*, 2001; Rodríguez-Soriano *et al.*, 1983; Watanabe, 2003). The phenotype is less severe in older infants than in neonates suggesting a pathophysiologic role for the tubular immaturity (Belot *et al.*, 2008). Other pathophysiological hypothesis is the increase in cytokines (mainly the transforming growth factor beta-1, -TGF-β1) in response to the urinary tract obstruction, resulting in the down regulation of MR (Klahr, 2000; Furness *et al.*, 1999; Kuhnle *et al.*, 1993; Bogdanovic *et al.*, 2009). Diagnosis of secondary PHA1 includes hyponatremia, hyperkalemia and metabolic acidosis in the presence of high levels of plasma aldosterone and renin, associated to abnormalities in urine culture and/or ultrasound examination, allowing the differentiation from genetic forms. Transient PHA1 was also observed secondary to congenital jejunal membrane (Nissen *et al.*, 2017), after resection of the ileum and colon in an adult patient (Vantyghem *et al.*, 1999), and in patients treated with immunosuppressants (Deppe *et al.*, 2002).

Recent studies challenged the classical classification of PHA1 in two distinct entities. These reports showed a continuum in the PHA1 phenotype between the severe generalized and the mild renal PHA1. While homozygote carriers of a αENaC mutation exhibit a generalized PHA1 phenotype, heterozygote carriers may exhibit a sub-clinical PHA1 phenotype only with increased sweat sodium and chloride concentrations (Riepe *et al.*, 2009). Moreover, cases of mild forms of PHA1 associated with homozygous ENaC mutations were also described (Dirlewanger *et al.*, 2011). Finally, a patient with a severe PHA1 phenotype was shown to carry two *NR3C2* mutations, each inherited from one parent (Hubert *et al.*, 2011). The gravity of the salt wasting syndrome was similar to the generalized PHA1 despite the MR mutation which is associated to the mild renal PHA1.

Differential Diagnosis

Differential diagnosis of PHA1 comprises diseases leading to renal salt wasting in the neonatal period, mainly congenital adrenal hyperplasia (CAH) and isolated deficiency in aldosterone synthase (**Table 1**). Classic CAH due to 21-hydroxylase (21OH) deficiency with salt loss is the main differential diagnosis of PHA1. Classic CAH-21OH is an autosomal recessive disease with an estimated prevalence of 1:10,000–1:20,000. Defective 21OH-hydroxylation results in decreased glucocorticoid and mineralocorticoid synthesis and elevated precursors, most notably 17-hydroxyprogesterone (17OHP), and concomitant ACTH-stimulated androgen production. It is characterized by hyponatremia and hyperkalemia in the neonatal period, with different degrees of

Table 1 Pseudohypoaldosteronism type 1 differential diagnosis

	Renal PHA1	*Generalized PHA1*	*Secondary PHA1*	*21-Hydroxylase CAH*	*3βHSD2 CAH*	*Isolated aldosterone deficiency*
Clinical phenotype	Different degrees of failure to thrive and dehydration. Improvement with age	Severe dehydration, failure to thrive, pulmonary and skin phenotype. Lifelong episodes of salt wasting	Failure to thrive and dehydration associated to urinary tract malformation and infection	Severe salt wasting syndrome in classic form. Ambiguous genitalia in females	Salt wasting associated with hypovirilization in males and virilization in females	Variant degrees of dehydration and vomiting
Inheritance	Autosomal dominant Sporadic	Autosomal recessive	Sporadic	Autosomal recessive	Autosomal recessive	Autosomal recessive Autosomal dominant with mixed penetrance
Genes	*NR3C2*	*SCNN1A*, *SCNN1B*, and *SCNN1G*		*CYP21A2*	*3βHSD2*	*CYP11B2*
Biochemical findings	Hyponatremia Hyperkalemia Metabolic acidosis Increased aldosterone Increased renin	Severe hyponatremia Hyperkalemia Metabolic acidosis Increased aldosterone Increased renin	Hyponatremia Hyperkalemia Metabolic acidosis Increased aldosterone Increased renin	Hyponatremia Hyperkalemia Hypoglycemia Metabolic acidosis Decreased aldosterone Decreased cortisol Increased ACTH Increased 17-OH Progesterone	Hyponatremia Hyperkalemia Hypoglycemia Metabolic acidosis Decreased aldosterone Decreased cortisol Increased ACTH Increased 17-OH Pregnenolone	Hyponatremia Hyperkalemia Metabolic acidosis Decreased aldosterone Increased renin
Treatment	Salt supplementation in early childhood	Lifelong salt supplementation, ion resins	Salt supplementation. Correction of urinary tract malformation and/or infection	Glucocorticoid and mineralocorticoid replacement. Salt supplementation	Glucocorticoid and mineralocorticoid replacement. Salt supplementation	Mineralocorticoid replacement

genitalia virilization in female neonates (DSD 46,XX) (New, 2003; Bizzarri *et al.*, 2016; El-Maouche *et al.*, 2017). Complete clinical examination to exclude the presence of ambiguous genitalia and measure of 17OHP are crucial for differentiate CAH-21OHD from PHA1. CAH due to 3β-hydroxysteroid dehydrogenase deficiency (3βHSD2) is a rare form of CAH responsible by salt wasting in the neonatal period. Abnormal 3βHSD2 activity leads to decreased in aldosterone, cortisol and androstenedione synthesis and an increase in DHEA production. Neonates affected exhibit hyponatremia and hyperkalemia associated to underdeveloped 46,XY genitalia (DSD 46, XY), and less frequent a virilization in 46,XX. The diagnosis is confirmed by high levels of the precursor 17-OH pregnenolone (New, 2003; El-Maouche *et al.*, 2017). Isolated aldosterone deficiency is a salt wasting disease due to a defect in the final steps of aldosterone biosynthesis caused by mutations in *CYP11B2* (coding for the aldosterone-synthase). Signs of aldosterone deficiency may appear at a few days or weeks of age but some patients are only diagnosed in the early childhood. Diagnosis is confirmed by presence of increased deoxycorticosterone, undetectable levels of aldosterone in the subtype I of the disease, and elevation of the ratio of 18-hydroxycorticosterone to aldosterone in patients with isolated aldosterone deficiency type II (Ulick *et al.*, 1992; White, 2004). Finally, patients with antenatal Bartter syndrome, an autosomal recessive disease caused by mutations in the potassium channel ROMK, may present with hyperkalemia in the first week of life associated with increased renin and aldosterone (Finer *et al.*, 2003). Usually, the hyperkalemia is transient and patients will develop hypokalemia and metabolic alkalosis.

Treatment

Treatment of PHA1 is based in the correction of water, electrolytes and acid–base disturbances. In the acute phase, salt supplementation, hydration and correction of hyperkalemia and acidosis hyperkalemia may be associated with the administration of fludrocortisone (mineralocorticoid) and hydrocortisone (glucocorticoid) while performing the differential diagnosis with CAH. Extracellular volume expansion and ion exchange resins may be required to normalize sodium and potassium levels. Recognize a salt wasting syndrome is important for all pediatricians and physicians in neonatal care and pediatric emergency services; adequate volume expansion, salt supplementation and steroid replacement are crucial in this death risk condition.

After the acute phase and confirmation of PHA1 diagnosis, salt supplementation is the basis of the treatment. In renal PHA1 the amount of salt supplementation depends on the severity of the phenotype, but usually 3–20 mEq/kg/d of Na^+ are able to

remediate the salt loss. Clinical and biochemical improvement are rapidly achieved, with catch-up on growth and development, and electrolytes normalization. Renal PHA1 improves with age and salt supplementation may be discontinued in the majority of patients after 18–24 months of age. Older children are asymptomatic, with normal growth and development, and electrolytes remaining in normal ranges. Some children, however, may exhibit growth on the lower percentiles of the growth curve (Loomba-Albrecht *et al.*, 2010). Symptoms and signs of secondary PHA1 usually improve with the surgical correction of the underlying urinary tract structural abnormalities and treatment of urinary tract infection. In the acute phase and before treatment of the urinary tract disease, patients with secondary PHA1 may need medical care similar to patients with renal PHA1.

Treatment of generalized PHA1 is specific for each patient and is usually based in high doses of sodium supplementation (20–50 mEq/kg/d), associated with ion resins and low K^+ diet to reduce potassium levels. The supplementation of high doses of sodium is difficult and requires tube feeding in some cases. So far, no specific drug has been described, but the use of glucocorticoid or indomethacin seems to be beneficial for some patients (Mathew *et al.*, 1993). Recent preliminary in vitro studies showed that tumor necrosis factor (TNF) activates ENaC current through its lectin-like (TIP) domain, and that peptides mimicking the TIP domain are capable to activate wild-type and mutant ENaC (Willam *et al.*, 2017). These data indicates that these peptides could represent a strategy to treat generalized PHA, however, further studies are necessary to evaluate in vivo efficacy and safety of these peptides. The treatment is needed throughout life but the sodium supplementation may decrease with age to 8–20 g NaCl/day. Specific symptomatic treatment is required for pulmonary and dermatological phenotype.

See also: Regulation of Potassium Homeostasis

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Hyporeninemic Hypoaldosteronism☆

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Introduction

Hyporeinemic hypoaldosteronism (HH), often used interchangeably with type IV renal tubular acidosis (RTA), is a syndrome of diminished renin secretion and resultant decreased aldosterone release. Basal levels of plasma renin activity (PRA) and aldosterone concentration are either low or normal but fail to respond to stimulatory testing (Soriano, 2002). Classically the syndrome is associated with renal dysfunction, most often secondary to long-standing diabetes, but can be present in any disorder that causes tubulointerstitial kidney injury and subsequent damage to the juxta-glomerular apparatus (JGA), the site of renin production.

The most prominent feature is a chronic mild or masked hyperkalemia that can become severe in or chronic kidney injury or when drugs antagonizing the renin–angiotensin–aldosterone system (RAAS) are added. Such medications include angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB), direct renin inhibitors (DRI), and aldosterone antagonists. Symptoms in HH, while rare, are usually due to hyperkalemia and include myopathic weakness, various tachyarrhythmias and bradyarrhythmias and, in severe cases, cardiac arrest and death (Medford-Davis and Rafique, 2014). The pathogenesis of HH and its relationship to hyperkalemia is complex and has multiple contributing factors including underlying diabetic nephropathy, diabetic autonomic neuropathy, hyperglycemia, and medications that act to inhibit the RAAS pathway (Sousa *et al.*, 2016). This last cause is most relevant as many of these drugs are indicated in patients with chronic kidney disease, diabetes and cardiovascular disease as these agents carry with them a significant morbidity and mortality benefit.

Pathogenesis of HH

The human adrenal cortex secretes several steroids including aldosterone which has predominantly mineralocorticoid properties. Aldosterone acts on the distal tubules and collecting duct of the kidney to promote sodium (Na^+) reabsorption and potassium (K^+) and hydrogen proton (H^+) excretion. Under most physiologic conditions, the RAAS is the main regulator of aldosterone secretion. Additionally, changes in the serum sodium content, the effective plasma volume, the sodium chloride concentration sensed by the macula densa, serum potassium levels and the autonomic nervous system can all play a role in aldosterone regulation (Sousa *et al.*, 2016).

Mechanisms of Hyporeninemia

Potential factors in the etiology of HH include inadequate renin and aldosterone production, failure of the autonomic nervous system or resistance to aldosterone action in the principal cells of the distal nephron. Although renin insufficiency plays the predominant role in HH, these other factors can also contribute to this syndrome (Sousa *et al.*, 2016). As HH occurs predominantly in diabetic patients with CKD, destruction of the JGA, in diabetic nephropathy likely via hyalinization of the afferent arteriole (Villoria *et al.*, 1988), reduces renin synthesis. High concentrations of the inactive renin precursor "big renin" (prorenin) have been reported in diabetic patients (Davis and Freeman, 1976) associated with low circulating PRA levels. These findings point to an impaired conversion of prorenin (big renin) to active renin in this disorder.

Sympathetic nerve terminals are found on the JGA and catecholamines, particularly epinephrine, can stimulate renin secretion (Sparks *et al.*, 2014). Loss of SNS activity, as in diabetic autonomic neuropathy, can contribute to the hyporeninemia in HH. Additionally, volume expansion due to sodium retention and chronic mild hyperkalemia as seen in CKD associated with diabetic nephropathy (DN) could further suppress renin release via tubuloglomerular feedback in the macula densa (Persson *et al.*, 2013). Some degree of prostaglandin deficiency or failure of the renal arteriole to respond to PGE in HH could also contribute to renin deficiency (Phelps *et al.*, 1980).

Regulation via Sodium

The secretion of pre-made renin from the JGA cells, located adjacent to the renal glomerulus and afferent arteriole, in response to decreased plasma volume results in angiotensin I synthesis, conversion to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II has a host of roles in the body including increasing vascular tone and release of aldosterone from the adrenal cortex

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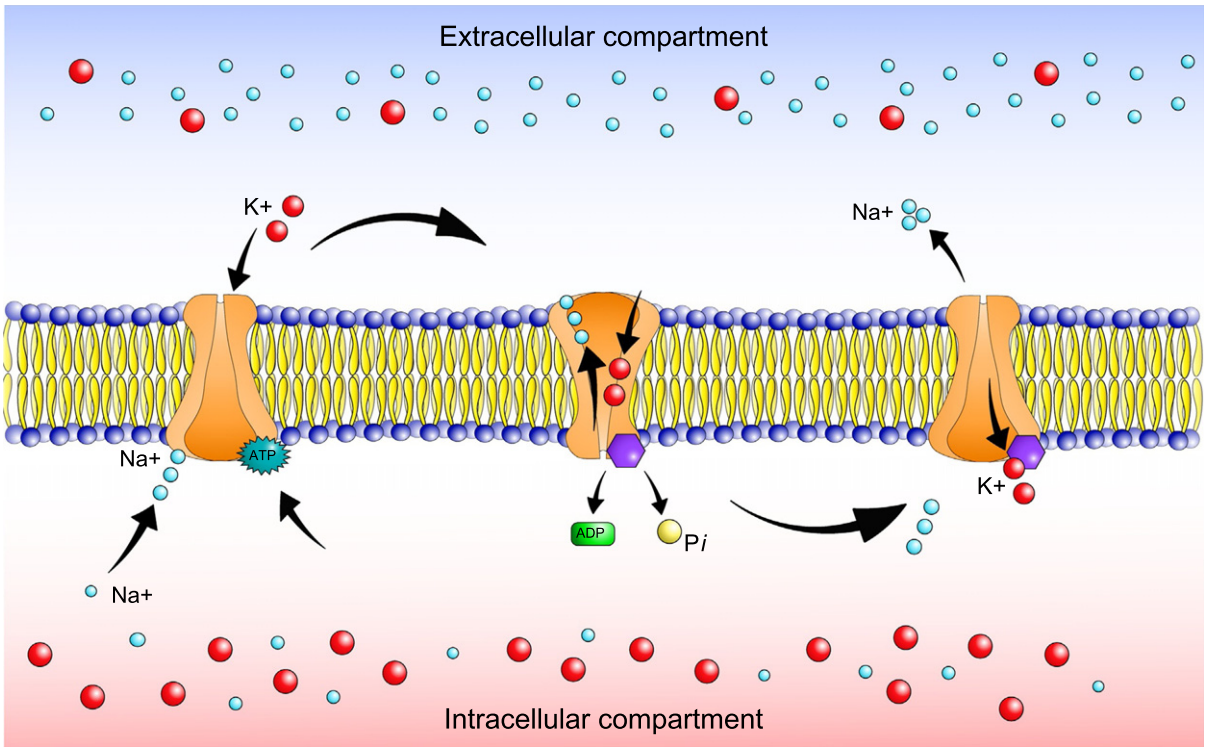

Fig. 1 The effects and regulatory mechanisms of the renin–angiotensin–aldosterone system.

resulting in retention of sodium from the kidney and restoration of circulating blood volume. (**Fig. 1** insert) This process occurs as a feedback loop; volume expansion has the opposite effect, resulting in suppression of renin and thus reduced aldosterone. A related mechanism is release of renin via the macula densa, an organ located near the distal tubule and collecting ducts of the kidney (Sparks *et al.*, 2014). In response to increased tubular sodium in the distal tubule, either due to increased filtration at the glomerulus, decreased resorption or both, the macula densa releases mediators to the JGA causing suppression of renin, constriction of afferent arterioles and dilation of efferent arterioles in the glomerulus of the kidney. This mechanism, termed tubuloglomerular feedback, effectively decreases glomerular filtration rate (GFR) and is thought to allow for rapid regulation of the GFR to prevent hyperfiltration injury (Fu *et al.*, 2012; Persson *et al.*, 2013).

Regulation via Serum Potassium

The other major contributor to aldosterone regulation is the potassium ion, which in the extracellular space, is tightly regulated (Palmer, 2004). Potassium stimulates aldosterone secretion directly at the adrenal gland; this regulation is independent of the RAAS or changes in effective circulating volume (Bollag, 2014). Even minute increases in plasma potassium stimulate aldosterone secretion to promote renal potassium excretion. Potassium depletion has the opposite effect, lowering aldosterone secretion to retain potassium ions and maintain cellular homeostasis.

In HH, potassium excretion via the renal tubules is deficient and subsequently a hyperchloremic metabolic acidosis is present due to multiple reasons including failure of hydrogen ion excretion due to deficient ammonium formation by the collecting duct, changes in the handling of ammonia in the renal medulla and transcellular shift of hydrogen protons out of cells to maintain normokalemia. Further contributing factors to the metabolic acidosis in HH can be acute kidney injury and chronic kidney disease. Because distal nephron acidification is still intact, urine pH is variable but often normal (Reddy, 2011).

Generally, patients with diabetes have higher serum potassium levels than nondiabetics due to a variety of reasons including transcellular shift of potassium, a higher potassium and lower sodium diets in diabetes, antihypertensive agents (beta-blockers, ACEi, ARBs, aldosterone, and renin antagonists) and underling diabetic nephropathy. Chronic hyperkalemia is associated with increased mortality in diabetes and chronic kidney disease (Collins *et al.*, 2017) and generally insulin resistance portends a higher serum potassium and worse diabetes control (Kim *et al.*, 2015). Potassium deficiency occurs less frequently in diabetes and is associated with low serum magnesium, insulin administration or chronic diarrhea states secondary to diabetic complications (Liamis, 2014). Hypokalemia impairs insulin secretion in the islet cells of the pancreas and induces further glucose intolerance perpetuating a vicious cycle of worsening insulin resistance and blood sugar control (Palmer and Clegg, 2015).

Potassium is tightly regulated in the body, with 98% located within the intracellular compartment and 2% in the extracellular space (see **Fig. 2**). Approximately 90% of potassium excretion occurs via the kidneys with gut excretion making up just 10%

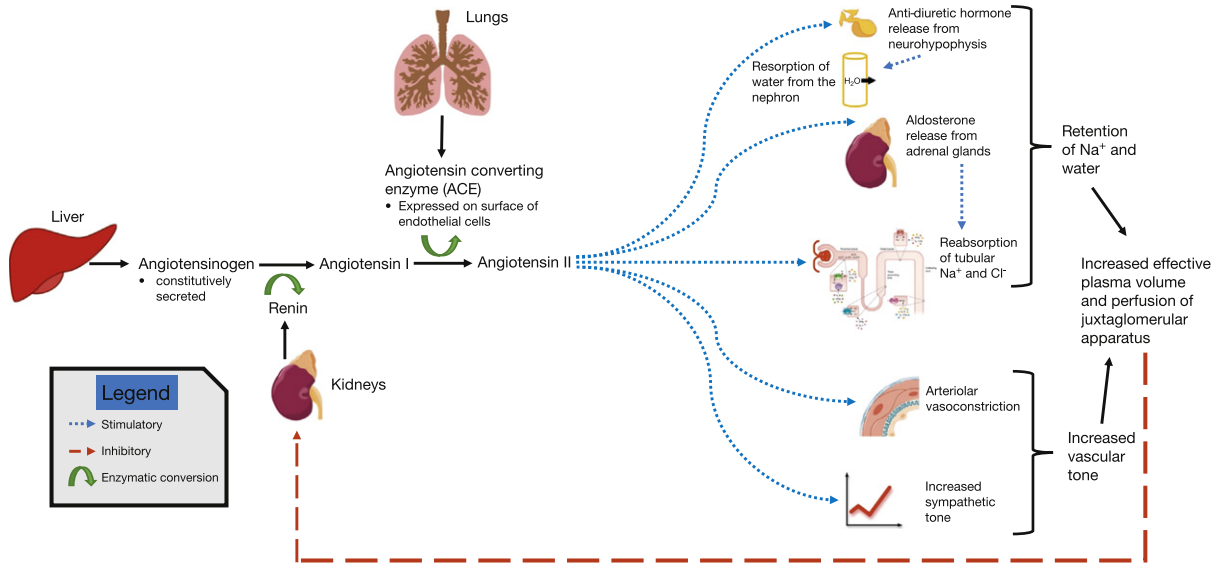

Fig. 2 Na^+–K^+ ATPase. The K^+ gradient in the intracellular space is maintained through active transport. 2 K^+ and 3 Na^+ are transported against their respective electrochemical gradient across cell membranes using energy generated by the cleavage of ATP into ADP and P_i.

however in chronic kidney disease and end-stage renal disease (ESRD) this gut mechanism can account for up to 25% of total potassium excretion (Tuck *et al.*, 1986). Cellular potassium shifts are mediated by Na–K ATPase pumps in response to a variety of endogenous and exogenous signals including, mineralocorticoids, ADH and catecholamines. Filtered potassium from the glomerulus is generally reabsorbed in the proximal tubule and the ascending limb of the loop of Henle. Potassium excretion into urine chiefly occurs in the distal convoluted tubule and cortical collecting duct (CCD) of the kidney. Aldosterone acts directly on the basolateral pumps on the CCD Na–K ATPases to move three sodium ions out in exchange for two potassium ions into the cell. Aldosterone also directly stimulates the Na–K ATPase increasing luminal membrane permeability to sodium and potassium and stimulates the opening of sodium channels in the principal cells of the CCD increasing sodium reabsorption. Sodium poor tubular fluid generates an electrochemical gradient that promotes excretion of potassium via channels on the luminal membrane side (Medford-Davis and Rafique, 2014).

Mechanisms of Metabolic Acidosis

In HH, decreased aldosterone synthesis and release results in impaired potassium excretion and hyperkalemia. Compounded with other mechanisms responsible for hyperkalemia in diabetes, this mineralocorticoid deficiency results in an elevated serum potassium. The chronic elevation of potassium is thought to play a pivotal role in the development of the mild non-anion gap metabolic acidosis that has historically been noted as part of the HH syndrome. The chief mechanism of hydrogen ion excretion and physiologic pH maintenance is renal ammoniagenesis at the proximal convoluted tubule (PCT) of the kidney. Ammonia (NH_3) is generated in the PCT from the amino acid glutamine, produced by the liver. Ammonia is then transferred in the thick ascending loop of Henle into the renal medullary interstitum and subsequently secreted into the CCD. Hydrogen protons generated by the daily acid load of metabolism are also excreted into the CCD by the intercalated cells in a process that is dependent on aldosterone. These protons combine with ammonia to generate ammonium (NH_4^+) which is excreted in the urine (Yaxley and Pirrone, 2016).

Chronic hyperkalemia impairs the generation of ammonia in the PCT though the exact cause is yet to be elucidated (Weiner and Verlander, 2013). Furthermore, elevated serum potassium induces H–K ATPases to exchange potassium ions for hydrogen protons to maintain normokalemia in the plasma. In this regulatory mechanism the body preferentially maintains normal serum potassium levels for a mild excess of hydrogen protons and hence acidemia. Finally, any underlying chronic kidney disease results in decreased ability to excrete generated ammonium and decreased excretion of phosphoric acids (Kraut and Madias, 2016). Thus numerous mechanisms in HH generate the classic findings of hyperkalemia and metabolic acidosis.

Additional Mechanisms in Aldosterone Regulation

Several other minor contributors to the control of aldosterone secretion are thought to be independent of the RAAS and serum potassium. These include baroreceptors in arterial walls of the carotid arteries and aorta, adrenocorticotrophic hormone (ACTH) secretion from the pituitary and innervation by the sympathetic nervous system (SNS). Baroreceptors sense mean arterial pressure and regulate aldosterone to maintain adequate blood volume (Bollag, 2014). The SNS similarly regulates aldosterone release in

response to a whole host of stimuli including emotional states, pain and posture. Prostaglandin E (PGE) is thought to regulate aldosterone by inducing variation of flow in the afferent arteriole and also directly inducing steroidogenesis in the zona glomerulosa (Tanaka and Kako, 1980). Additionally, PGE is also thought to directly stimulate renin release from the JGA (Adam and Wright, 2016). Finally ACTH, which predominantly is involved in cortisol release from the adrenal cortex, also plays a minor role in aldosterone release. ACTH deficiency can result in diminished aldosterone output which, in addition to cortisol deficiency, is thought to contribute to the hemodynamic instability in adrenal crises (Charmandari *et al.*, 2014).

Clinical

Hyperkalemia (serum $K^+ > 5.0$ mEq L^{-1}) is a common metabolic derangement noted in up to 3.3% of all hospitalized patients and is itself an ominous predictor of mortality and morbidity (Khanagavi *et al.*, 2014). When seen in the setting of CKD secondary to DN, it is often due to undiagnosed HH (Villoria *et al.*, 1988). The incidence of HH in diabetes is not certain, likely due to under recognition and subtle presentations however, in one small retrospective study, up to 42% of the patients admitted with diabetic nephropathy and hyperkalemia were noted to have some degree of hypoaldosteronism based on urine and serum potassium values (Haas *et al.*, 2013).

Clinical Presentations

There are two chief situations that strongly suggest HH in the context of diabetic nephropathy or any other chronic disease of the renal interstitum. The first, and likely most common, is asymptomatic hyperkalemia noted in a patient with comorbid diabetes and nephropathy; often the elevated potassium precludes initiation of medications such as ACEis and ARBs that can further worsen hyperkalemia. While most patients have only mild increases in serum potassium, some can present with life-threatening elevations and hemodynamic instability due to more profound mineralocorticoid deficiency (Palacio and Hsiao, 2012). The second common presentation is a dramatic elevation in serum potassium when using a medication that interferes with the RAAS (ACEi, ARB, DRI) or agents that perturb potassium homeostasis (beta-blockers, heparin, NSAIDs, bactrim). These situations often demand cessation of the medication to prevent life-threatening hyperkalemia.

Despite the low aldosterone levels in HH, handling of sodium and volume is retained, with most patients maintaining low-normal serum sodium levels and normal volume status. Indeed, patients can present with actual volume overload if kidney function is significantly compromised. These observations suggest that some degree of aldosterone release is still present in HH to maintain cardiovascular integrity. Other homeostatic mechanisms, including SNS, cortisol and anti-diuretic hormone (ADH) are also involved in preventing profound natriuresis and hemodynamic collapse in HH.

Hyperkalemia in Hyporeninemic Hypoaldosteronism

The clinical effects of hyperkalemia are manifold though, as noted, most often in HH patients with this condition present asymptomatically. The degree of potassium elevation is less a predictor of the severity of clinical response than the rapidity its increase; hence chronic hyperkalemia is much better tolerated than acute owing to numerous homeostatic mechanisms. The chief symptoms that patients report include muscle weakness (including ascending paralysis) and heart palpitations (Palmer and Clegg, 2015).

Skeletal muscle tissue is an enormous reservoir of intracellular potassium which is sensitive to changes in serum potassium. Decreases in the potential across the cell membrane results in muscle weakness (Viera, 2015). The muscle weakness generally resolves after correction of the hyperkalemia. Several classes of cardiac cells are affected by changes in serum potassium as well. As with skeletal muscle, hyperkalemia results in a decreased potential across the cardiac myocyte that generates areas of automaticity, most often in the ventricles and the atria. The electrocardiogram (ECG) findings show premature ventricular and atrial contractions.

The effect of elevated potassium on the conduction system of the heart can be dramatic and life-threatening. While there is no true serum potassium level that corresponds to ECG findings it is generally accepted that levels over 6 mEq L^{-1} cause changes in cardiac conduction. Patients with multiple comorbidities, particularly pre-existing cardiac conduction disease, can have changes at lower serum levels. The first finding on the ECG is peaked T-waves followed by P-wave flattening, PR-interval prolongation, widening of the QRS complex, and sine waves as the potassium progressively increases (Medford-Davis and Rafique, 2014). These changes are harbingers of inadequately perfusing rhythms and can lead to cardiovascular collapse and death. Bradycardia is an often underrecognized symptom of hyperkalemia, particularly among dialysis patients, and can present without any other classic ECG changes (Mohanlal *et al.*, 2013). Hyperkalemia can also exacerbate underlying congestive heart failure and unmask propensities for cardiac tachyarrhythmias including ventricular tachyarrhythmias and atrial fibrillation and flutter. It should be noted that the absolute serum potassium value itself is a poor predictor of clinical outcomes as patients with underlying heart or kidney dysfunction can have adverse outcomes at even mildly elevated potassium levels.

Metabolic Acidosis in Hyporeninemic Hypoaldosteronism

The metabolic acidosis in HH is generally chronic, mild (serum bicarbonate values between 15 and 20 mEq L^{-1}) and is compounded by underlying renal failure and hyperkalemia (Karet, 2009). The prevalence of acidosis in HH is not known however serum bicarbonate values are below 22 mEq L^{-1} in 2.3%–13% of stage III (GFR: 59–30 mL min^{-1}) and 19%–37% of stage IV (GFR: 30–15 mL min^{-1}) of CKD patients, suggesting multiple compounding etiologies for the acidosis. The metabolic acidosis in HH is generally non-anion gap while in CKD without HH there is generally a high-gap acidosis. Additionally, the degree of hyperkalemia is more severe than would be expected for the decrease in GFR (Arai and Chrousos, 2000). Patients are rarely symptomatic from the acidosis alone, however chronically acidosis can have several deleterious long-term health consequences including increased catabolism of muscle protein leading to muscle wasting and a negative nitrogen balance which both contribute to declining renal function (Chen and Abramowitz, 2014). The other significant consequence is metabolic bone disease, a protean entity that includes osteoporosis, osteomalacia and renal osteodystrophy. These factors contribute to increased debility and medical fragility, particularly in the elderly, and are both markers for increased morbidity and mortality (Kraut and Madias, 2016).

Differential Diagnosis

HH can be seen in disorders other than diabetic nephropathy though the exact incidence overall is difficult to determine. Conversely, conditions that affect aldosterone synthesis or prevent the effect of aldosterone without perturbing renin secretion can be indistinguishable clinically from HH. Finally, rare inherited disorders of the function of the aldosterone receptor or of aldosterone synthesis can present with HH-like features however tend to present only in very young and otherwise healthy children.

Disorders of the Renal Interstitium

HH has been described in patients with systemic lupus erythematosus (SLE) suffering from advanced nephritis (Porteous *et al.*, 2011) however this is rare, as most SLE patients present with a distal type I RTA. Patients suffering from human immunodeficiency virus (HIV) can also have hyperkalemia with metabolic acidosis, however the causes can be manifold including adrenalitis from viremia, type I RTA and HH. Generally, the virus has a predilection for the glomerulus and classically causes a "collapsing" focal segmental glomerulosclerosis pattern on pathology. Additionally, multiple HIV antiretroviral medications can induce acidosis and cause tubulointerstitial nephritis predisposing the patient to HH (Wyatt *et al.*, 2008). Trimethoprim, a component of the antibiotic bactrim, has been known to cause a reversible type IV RTA usually in the setting of a preexisting secondary insult to the synthesis of aldosterone such as sickle cell nephropathy (Nath and Hebbel, 2015).

Any condition that affects the JGA or upstream regulators of renin synthesis and excretion can give rise to HH. It has been noted in other cases of intraparenchymal renal injury including chronic tubulointerstitial disease (Quiroga and Garcia de Vinuesa, 2013), Sjogren's disease (Onozaki *et al.*, 2002), amyloidosis (Takemoto *et al.*, 2008), Alport syndrome (Tkacova *et al.*, 1993), venomous snake bites, (Karunarathne *et al.*, 2013), analgesic nephropathy (Perazella, 2005), calcineurin inhibitor nephropathy (Schmoyer *et al.*, 2017), and obstructive nephropathy (Pelleya *et al.*, 1983). (see **Table 1**).

Isolated Deficiencies of Aldosterone

Hypoaldosteronism without hyporeninemia suggests an underlying disorder of the adrenal gland. Primary adrenal insufficiency (PAI) can present with hyperkalemia and metabolic acidosis, however often other clinical and biochemical features are present including diffuse abdominal pain, nausea and vomiting, hyperpigmentation and cardiovascular collapse due to the concomitant lack of glucocorticoids. Furthermore, the causes of PAI are distinct and include autoimmune adrenalitis, inherited disorders, infiltrative diseases, metastatic cancers, granulomatous fungal infections and viremia with HIV and cytomegalovirus (CMV). Insufficiency adrenocorticotrophic hormone production from the pituitary or hypothalamus rarely results in clinical features of aldosterone deficiency due to other mechanisms stimulating aldosterone secretion. Critical illness due to any cause can suppress the hypothalamic–pituitary–adrenal (HPA) axis and cause what is termed "relative" adrenal insufficiency (Charmandari *et al.*, 2014). In these cases renin secretion is usually increased as part of the feedback look to induce aldosterone secretion.

Inherited Disorders of Aldosterone

Perturbation of aldosterone synthesis or resistance to aldosterone action are primarily inherited disorders in the synthesis of aldosterone or resistance to its action. A classic example is congenital hypoaldosteronism, known as the Visser–Cost syndrome, which is due to a mutation in aldosterone synthetase, the final enzyme for aldosterone synthesis in the adrenal gland. Afflicted children generally present at a very young age with salt-wasting, growth retardation and failure to thrive; renin levels are usually elevated (Peter and Sippell, 1996). Pseudohypoaldosteronism includes in its penumbra a heterogenous group of disorders that affect either the mineralocorticoid receptor or sodium channels in the distal tubule and collecting duct. These generally have a

Table 1 Causes of hyporeninemic hypoaldosteronism, hyperreinemic hypoaldosteronism and aldosterone resistance

Hyporeninemic hypoaldosteronism	*Hyperreninemic hypoaldosteronism*	*Aldosterone resistance*
Medications	Adrenal insufficiency	Pseudohypoaldosteronism (PHA) Type I
Cyclooxygenase inhibitors	Infectious adrenalitis	Renal PHA Type I
Heparinoids	*CMV, HIV*	Systemic PHA Type I
Renin antagonists	*Histoplasmosis*	Aldosterone antagonists
Antiretrovirals	*Waterhouse–Friderichsen*	Synthetic progestins
Calcineurin inhibitor	*Tuberculosis*	
Nephropathy		
β-Blockers	Primary adrenal insufficiency	
Paraproteinemia	*Isolated autoimmune adrenalitis*	
Amyloidosis	*Multiple autoimmune endocrinopathies*	
Myeloma	Secondary adrenal insufficiency (ACTH Deficiency)	
Sickle cell anemia	Infiltrative disease	
Analgesic neuropathy	*Sarcoidosis*	
Chronic tubulointerstitial Disease	*Hemochromatosis*	
Lupus nephritis	Medications	
Sjogren's disease	ACEi and ARBs	
Diabetic nephropathy	Trimethoprim	
Chronic urinary tract obstruction	Chronic steroid usage	
HIV nephropathy	Inherited disorders	
Diabetic autonomic neuropathy	Congenital adrenal hyperplasia	
	Congenital hyperreninemic	
Venomous snake bites	Hypoaldosteronism (Visser–Cost syndrome)	

more benign course and present at adolescence or later with unexplained hypertension, hyperkalemia and metabolic acidosis (Scheinman, 2009). Generally, in these inherited disorders renin levels are elevated as part of the feedback loop and as a group these are termed conditions of hyperreninemic hypoaldosteronism.

Iatrogenic Causes of Hypoaldosteronism

Medications that affect the RAAS can produce the signs and symptoms of HH. Most notable among these are the ACEi and ARBs, aldosterone and renin antagonists that directly interfere with some intermediate portion of the RAAS. Other notable medications include beta-blockers which disturb the SNS regulation of renin (Seifarth *et al.*, 2002), non-steroidal anti-inflammatory drugs (NSAIDs) which decrease renal prostaglandin via cyclooxygenase inhibition (Perazella, 2005) and heparin, which suppresses endogenous aldosterone production directly (Bengalorkar *et al.*, 2011). In clinical practice hyperkalemia with metabolic acidosis can develop when these medications are used, however, they can obfuscate the diagnosis of HH and, when used in individuals with an underlying propensity to HH, can cause the metabolic disturbances noted. Thus, HH has a great many imitators, both clinically and biochemically, and care should be taken when diagnosing the disorder as treatments are quite different for each of the disorders.

Diagnosis

As the name suggests hyporeninemic hypoaldosteronism is a disorder of insufficient renin and aldosterone release. The most salient biochemical features, hyperkalemia with or without a mild non-anion gap metabolic acidosis in the setting of mild renal insufficiency, is a common clinical scenario. HH should be considered when the serum potassium values are higher than expected for the decrease in GFR particularly in a patient with diabetic nephropathy.

Urine Studies

Urinalysis including pH and specific gravity, urine electrolyte values, microscopic examination and urine culture should be obtained. Underling tubulointerstitial disease can present with casts and active urine sediment suggests a glomerular lesion. Urine culture should be obtained to rule out an organism which would confound the results of urine indices because of its metabolic byproducts.

The urine pH is useful in differentiating HH from distal type I RTA as in the latter urine pH will be alkalotic (defined as pH $>$ 5.3) regardless of the degree of acidosis whereas in HH the urine pH can be variable but most often is normal (pH $<$ 5.3) (Reddy, 2011). An alkali load with sodium bicarbonate will increase the urine pH in HH transiently to $>$5.3 but not in type I RTA.

Caution should be taken in utilizing urine pH solely as the basis for diagnosis of RTA as volume depletion and urease-splitting organisms in the urine can dramatically alter this value (Yaxley and Pirrone, 2016).

Serum Aldosterone and Renin Values

Values of aldosterone concentration and plasma renin activity (PRA) should be measured to determine the underlying function of the adrenal glands and the JGA respectively. PRA is generally measured rather than renin concentration, though recent advances have suggested that the actual renin concentration is a more accurate measurement of whole body renin (Lonati *et al.*, 2014). Both values should be obtained from a midmorning, volume replete and upright patient as secretion of aldosterone appears to follow a circadian pattern and recumbent positioning can alter the renal perfusion and cause specious values.

Provocative Testing

In HH the ratio of the aldosterone concentration to the PRA is generally preserved but the values of both renin and aldosterone are low, though they can be normal (Villoria *et al.*, 1988). In contrast, in hypoaldosteronism without hyporeninemia, the JGA apparatus and feedback loop are preserved and the renin level is elevated while the aldosterone value will be low. (see **Table 2**) If clinical suspicion is high and laboratory values are non-diagnostic then stimulatory testing should be performed. A loop diuretic, most often furosemide, is administered to determine response to falling tubular sodium levels. Loop diuretics promote natriuresis, that is they increase tubular sodium values, and also block the sodium sensing receptors on the macula densa, effectively inducing activation of the RAAS. Aldosterone levels will not increase appropriately if HH is present (Reddy, 2011).

ACTH stimulation testing can also be used to determine whether hypoaldosteronism is present as the trophic hormone induces acute adrenal release of both cortisol and aldosterone. A blunted response to ACTH is indicative of aldosterone insufficiency (Dluhy, 1974). This latter test cannot distinguish between a primary adrenal defect in aldosterone (such as PAI) and low aldosterone levels due to hyporeninemia. Examination of the entire HPA axis should be considered if there is any suspicion of primary or secondary adrenal cortisol insufficiency.

Treatment

Treatment for HH is contingent on the long term deleterious consequences of chronic hyperkalemia and metabolic acidosis as the mineralocorticoid deficiency in HH rarely causes salt-wasting or cardiovascular collapse. Furthermore, many medications indicated for patients with diabetes, hypertension and chronic kidney disease, conditions commonly associated with HH, directly affect the RAAS and can worsen underlying hyperkalemia. This situation mandates either careful dose titration or discontinuation of these medications which is a dilemma as they have significant morbidity and mortality benefit in this population (Palmer, 2004).

Acute Treatment

In those few patients who do develop signs and symptoms of severe mineralocorticoid deficiency such as syncope, orthostatic hypotension or severe hyperkalemia, acute treatment is necessary. The most pressing issue is the patient's cardiovascular status and the elevated potassium and an isotonic crystalloid fluid, ideally normal saline, should be administered in boluses repeatedly until blood pressure is restored. Isotonic fluids that contain potassium, such as lactated Ringer's solution and colloids should not be used. Empiric so called “stress dose steroids” should be administered; generally, hydrocortisone is used as it has both mineralocorticoid and glucocorticoid activity. An ECG should be performed to evaluate for changes in the electrical architecture of the heart and empiric calcium gluconate or calcium chloride should be administered intravenously (IV) if ECG changes are present. The cardiac rate and rhythm should be continuously monitored during treatment.

The two methods of lowering the serum potassium are promoting excretion from the body and inducing transcellular shift into the cell; the latter method is employed first as it is rapid. Insulin and glucose and a nebulized beta-agonist, such as albuterol, are

Table 2 Expected changes in plasma renin activity, aldosterone concentration, presence of metabolic acidosis, serum potassium values and response to stimulatory testing in each category of aldosterone derangement

	Plasma renin activity (PRA)	*Aldosterone concentration*	*Metabolic acidosis*	*Serum potassium*	*Response to ACTH stimulation*	*Response to furosemide load*
Hyporeninemic hypoaldosteronism	↓ or –	↓ or –	≈	Mildly ↑	– PAC – PRA	– PAC – PRA
Hyperreninemic hypoaldosteronism	↑	↓	~	Mildly ↑ or –	– PAC ↑ PRA	– PAC ↑ PRA
Aldosterone resistance	↑	↑	~	Mildly ↑ or –	↑ PAC ↑ PRA	↑ PAC ↑ PRA

↑, elevated; ↓, lowered; –, normal; ~, variable; ≈, variable (present in approximately 50%).

given to stimulate uptake of potassium into cells. Potassium excretion is promoted by use of oral cation exchange resins, such as polystyrene sulfonate, via the stool. Kaliuresis is induced with a loop diuretic. In cases where potassium is severely elevated and the above methods cannot be employed due to comorbid conditions, acute dialysis is employed. Intravenous bicarbonate therapy has fallen out of favor as a method to shift potassium into cells because of concerns over its efficacy however in cases of metabolic acidosis associated with hyperkalemia, it is still a consideration (Viera, 2015).

If a patient who is symptomatic from HH but does not have life-threatening metabolic disturbances, as with postural syncope or with hyperkalemia, mineralocorticoid replacement such as fludrocortisone can be used. Indeed, patients have been described with asymptomatic severe hyperkalemia or orthostasis who respond well to this treatment (Lee *et al.*, 2009; Polsky *et al.*, 1993; Pelleya *et al.*, 1983). Caution should be used as most patients with HH have some degree of renal impairment and use of a mineralocorticoid agent could exacerbate sodium retention, worsening hypertension and volume overload.

RAAS Medications in HH

While the hyperkalemia in HH is generally mild use of a medication that affects the RAAS can cause rapid and life-threatening increases in serum potassium values. Furthermore, up to 10% of patients must discontinue an ACEi or ARB because of subsequent hyperkalemia (Palmer, 2004). Withholding these medications or discontinuing them brings up a dilemma for clinicians as these medications have a proven mortality benefit in CKD, with or out without diabetes. Furthermore, patients with comorbid coronary artery disease and congestive heart failure, common attendants to both CKD and diabetes, also benefit enormously from ACEi and ARBs (Molnar *et al.*, 2014). These medications improve the degree of proteinuria, decrease GFR preventing hyperfiltration injury and help prevent adverse cardiac remodeling after myocardial ischemia (Mcmurray *et al.*, 2003; Molnar *et al.*, 2014). Addition of an aldosterone antagonist to this regimen further provides a mortality benefit, particularly in heart failure after myocardial infarction (Hostetter, 2003).

When evaluating a patient with HH at risk for hyperkalemia who would benefit from addition of these medications it is useful to eliminate dietary sources of potassium including any herbal supplements. Elimination of other drugs that could affect renin secretion or induce hyperkalemia should be undertaken. NSAIDs are a frequent, often overlooked, cause of hyperkalemia (Zimran *et al.*, 1985). Consideration should be given to adding a loop diuretic which has potent kaliuretic properties and would be a counterweight to the hyperkalemia associated with ACEi, ARBs and aldosterone antagonists.

In HH RAAS inhibitors should be initiated at very low doses and only up titrated gradually to allow maximal therapeutic doses to be reached (Palmer, 2004). Frequent monitoring of all electrolytes and renal function during dose adjustments has been associated with decreased frequency of hyperkalemia (Eschmann *et al.*, 2013). The use of cation resins such as polystyrene sulfonate should be discouraged as they promote osmotic diarrhea and can cause intestinal injury and ischemia (Mcgowan *et al.*, 2009).

Treatment of Metabolic Acidosis

If the metabolic acidosis in HH is of concern then addition of sodium bicarbonate to treat both the acidosis and help lower serum potassium levels can be successful. No official values cutoff values are established for when to start treatment of acidosis in HH or for goal serum bicarbonate levels to attain. The guidelines established for CKD (Kraut and Madias, 2016) recommend initiating treatment at a serum bicarbonate level $< 23\ \mathrm{mg\,dL^{-1}}$. Care should be taken as sodium bicarbonate can provide a significant sodium load to the patient and, in the setting of underlying CKD, can provoke or worsen hypervolemia.

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See also: Regulation of Potassium Homeostasis

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Bartter and Gitelman Syndromes

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Nomenclature

AT1R Angiotensin II type 1 receptor
BP Blood pressure
MLC Myosin light chain
MYPT-1 Myosin phosphatase target protein-1
p63RhoGEF p63 Rho guanine nucleotide exchange factor
PLCβ1 Phospholipase C β1
RGS-2 Regulator of G protein signaling-2

Introduction

In 1962 Frederic C. Bartter and then in 1966 Hillel J. Gitelman described two instances of salt-losing tubulopathy characterized by hypokalemia, metabolic alkalosis, and activation of the renin–angiotensin–aldosterone system (RAAS) notwithstanding normotension or hypotension (Bartter et al., 1962; Gitelman *et al.*, 1966). Further clinical investigations allowed the detection of some subtypes of these familial congenital diseases with different manifestations and severity (Simon *et al.*, 1996a, 1997; Gamba, 2005).

Bartter syndrome (BS) and Gitelman syndrome (GS) have specific symptoms onset timing: however, classic BS reveals in childhood or early adolescence can also have a neonatal form, while GS has a less severe clinical presentation and appears in adolescence or adulthood.

BS typical clinical features are muscle weakness, anorexia, polydipsia, polyuria, failure to thrive, and growth retardation. Hallmarks of this syndrome are salt-wasting, high plasma level of prostaglandins, and hyperprostaglandinuria. The electrolyte abnormalities of BS are similar to those induced by treatment with furosemide or other drugs that inhibit the Na–K–2Cl cotransporter of the thick ascending limb (TAL) of Henle's loop. GS, on the other hand, is shown through salt-craving, muscle weakness, fatigue, and cramps with electrolyte imbalance associated with the side effects of thiazide diuretics treatment acting on the distal convoluted tubule (DCT) (Naesens *et al.*, 2004; Favero *et al.*, 2011) (see **Fig. 1**).

Both syndromes are cataloged in the registry of inherited rare Mendelian diseases (Online Mendelian Inherited In Man®), with respective codes identifying the subtypes. The exact prevalence of these disorders is unknown, although for BS it is estimated to be 1 patient affected in 1 million people worldwide, and for GS 1 subject affected in 40,000, making it one of the most frequently inherited renal tubular disorders (Blanchard *et al.*, 2017).

BS and GS have, as common etiology, mutations in genes that encode for cotransporters or channels involved in the trafficking of electrolyte in the nephrons. The effects of these mutations are nonfunctional proteins, with ensuing salt-wasting and activation of counterbalancing systems as the RAAS.

Notwithstanding elevated plasma levels of renin, aldosterone, and angiotensin II, BS and GS subjects present with normotension or hypotension. This has prompted a great deal of interest in the research of the molecular mechanisms involved in blood pressure (BP) regulation (Calò *et al.*, 2014a).

Molecular Basis

Bartter Syndrome

It was found that electrolyte abnormalities of BS are similar to those induced by the treatment with furosemide or other drugs that inhibit the Na–K–2Cl cotransporter (NKCC2) of the TAL, and this prompted investigations on the gene SLC12A1 that encodes for the NKCC2 (Simon *et al.*, 1996a). Indeed, in affected patients, as a consequence of the mutations there is a loss of cotransporter function, which induces Na^+ and K^+ wasting in the TAL. NKCC2 mutation classifies BS type I (OMIM® #601678). In particular, the consequences of nonfunctional NKCC2 involve systemic alterations from fetal gestation onwards, with severe salt-wasting hypokalemic metabolic alkalosis, hypercalciuria, and hyperprostaglandinuria. Prostaglandin E2 excess is associated with fever, severe dehydration, hypocalcemia, hyponatremia, and subsequent development of nephrocalcinosis and osteopenia in infants.

Other mutations were found in genes involved in the regulation of NKCC2 activity. In particular, alterations in the KCNJ1 (Kir1.1) encoding for the apical ATP-sensitive K channel (renal outer medullary potassium channel, ROMK) identify BS type II (OMIM® #241200). The ROMK is an ATP-sensitive channel which recycles K^+ from the cell back into the lumen; as a consequence, the falling luminal K^+ shuts down NKCC2 activity and results in salt-wasting (Simon *et al.*, 1996b). A nonfunctional ROMK gives a pseudohypoaldosteronism type I phenotype-like for the transient hyperkalemia in the antenatal form. Mutations affecting the KCNJ1 gene cause the production of erroneous proteins which compromise

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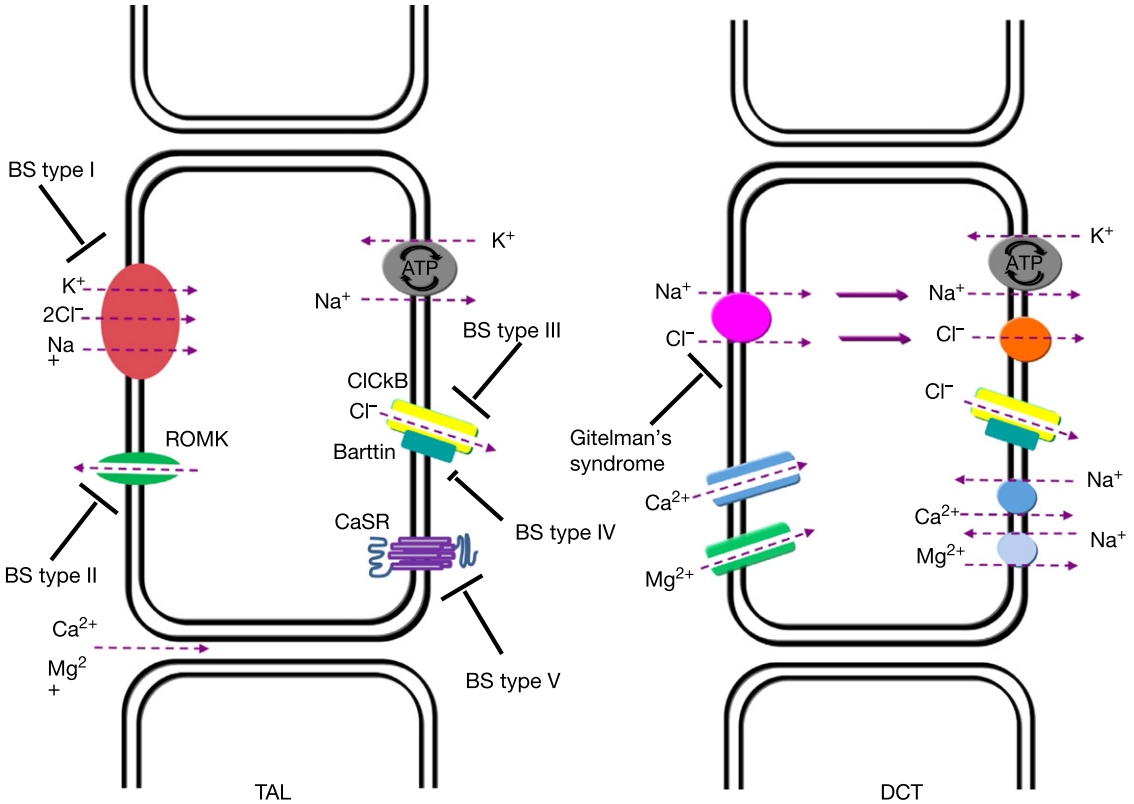

Fig. 1 Sites of genetic defects of BS type I–V and GS. *TAL*: Thick ascending limb of Henle's loop; *DCT*: distal convoluted tubule.

endoplasmic reticulum (ER) folding and are targeted to the ER-associated degradation (Finer *et al.*, 2003; O'Donnell *et al.*, 2017).

Alterations of the chloride channel CLCNKb (which mediates Cl^- reabsorption) might induce intracellular chloride accumulation, thus inhibiting NKCC2 activity. As a clinical outcome, mutations in CLCNKb in affected patients result in salt-wasting and hypovolemia, identifying BS type III (OMIM® #607364) (Simon *et al.*, 1997). CLCNKb requires an essential β-subunit barttin encoded by the BSND gene to function properly. Some mutations affecting the barttin interacting motifs led to significantly impaired protein, as well as causing mutations in the dimerization sites and in the selectivity filter, resulting in a severe phenotype of the disease. Other mutations in the alpha-helices display a milder form which can go undetected until adulthood. (Cheng *et al.*, 2017).

The protein barttin (BSND) is required for the location not only of the basolateral membrane CLCNKb but also for the isoform CLCNKa. As in BS type III, the consequence of mutations in this protein is an accumulation of chloride, which affects NCCK2 activity once more with salt-wasting (Naesens *et al.*, 2004). The characteristic BS type IVa (OMIM® #602522) phenotype appears in an antenatal form causing polyhydramnios, premature birth, and all other BS typical features, as well as sensorineural deafness in infants. In addition to mutations affecting the barttin protein, BS type IVb (OMIM® #613090) is characterized by digenic mutations (CLCKa and CLCKb) that inactivate all the 4 alleles of the 2 genes. In the inner ear, the highly expressed CLCKa contributes to maintaining the high K^+ ion concentration in the endolymph, which is necessary for normal hearing. As a result, an ion imbalance induces nerve deafness and a defect in the sensory-neural transduction of sounds (Miyamura *et al.*, 2003).

The functional characterization of barttin mutations established different mechanisms for impaired function, including: decreased number of coupled CLCNKb/barttin; decreased insertion of CLCNKb/barttin in the basolateral membrane and in the apical membrane; and impaired anion currents and CLCNKb being prevented from activating. An overview on the functional effects of the different mutations demonstrates that the deleterious effects on barttin impact the diversity of phenotypes (Janssen *et al.*, 2009). Indeed, notwithstanding the early manifestations of the disease, with premature birth and severe salt and water loss in the perinatal period, few cases have been reported of late diagnosis of BS type IV. This is probably due to a less severe phenotype with hidden or mild symptoms which led to the condition being unrecognized (Heilberg *et al.*, 2015).

Another phenotype described, BS type V (OMIM® #300971), is due to mutations in the Ca^{2+}-sensing receptor (CaSR). In the parathyroid, CaSR controls the response of the gland to the extracellular Ca^{2+} levels and thus hormone release; in the kidney, in the basolateral membrane of the TAL, it acts as an inhibitor for the reabsorption of Ca^{2+}. CaSR is a pleiotropic receptor regulating

several intracellular signaling pathways, including calcium mobilization and intracellular calcium oscillation. The structure of this receptor comprises an N-terminal domain and a long (~600 aa) extracellular domain, seven transmembrane domains, and a C-terminal intracellular domain. The long extracellular portion is fundamental not only for the binding of the receptor with its agonists, but also for its activation via dimerization. The binding of Ca^{2+} to the extracellular loops promotes a conformational change, which reflects on the transmembrane portions with ensuing modification of the intracellular C-terminus. The latter triggers different downstream pathways including activation of th phospholipase C pathway, extracellular signal-regulated kinases 1/2 (ERK1/2), and Rho kinase pathway, cyclic adenosine monophosphate (cAMP), essentially via regulation of four G proteins ($G_{q/11}$, $G_{i/o}$, G_s, and $G_{12/13}$) (Zhang *et al.*, 2016). Mutations in specific portions of the protein induce either loss or gain of function of the receptor. Experimental studies in rats showed that the activation of CaSR by high concentrations of extracellular calcium ions inhibits the activity of ROMK, which is fundamental to preserve positive luminal potential in TAL driving paracellular reabsorption of calcium and magnesium. Its inhibition by calcium via CaSR is expected to increase urinary excretion of these cations; therefore, gain-of-function mutations induce hypocalcemia that could develop into BS type V and autosomal dominant hypocalcemia (Brown and MacLeod, 2001; Pearce *et al.*, 1996).

Gitelman Syndrome

GS (OMIM® #263800) is the hypocalciuric variant of BS. Indeed, GS is characterized by the co-existence of not only hypokalemia, hypomagnesaemia, and activation of RAAS, but specifically hypocalciuria. Marked symptoms are muscle weakness, cramps, joint pain, polydipsia, polyuria, hypotension, salt craving, dizziness, and a prolongation of QT interval on electrocardiogram (which represents the depolarization and repolarization of the ventricles and causes arrhythmia) (Bettinelli *et al.*, 2002; Scognamiglio *et al.*, 2008). Calcium pyrophosphate (CPP) crystal deposition or chondrocalcinosis associated with arthropathy can also be observed (Favero *et al.*, 2011). In GS the electrolyte imbalance is associated with the side effects of thiazide diuretics. The target of this type of diuretics is the sodium-chloride cotransporter (NCC) present in the DCT. The cloning and characterization of the gene encoding this cotransporter, the SLC12A3 gene, in GS indicated that several mutations affect the NCC causing loss of function of the cotransporter with renal sodium wasting and triggering adaptive mechanisms in the kidney, including aldosterone-driven increased excretion of K+ in exchange for Na^+.

NCC is a member of the big class of solute carrier proteins (SLC) family, comprising 12 of the electroneutral cation-chloride cotransporters, and it is encoded by the gene SLC12A3 (Hediger *et al.*, 2004). SLC12A3 shares a highly conserved amino acid sequence with other members of the family, which features 12 transmembrane portions harboring a central hydrophobic domain and 2 cytosolic terminal ends (NH_2 and COOH) (Wang *et al.*, 2015). The central region seems to be highly specific for ion translocation and for thiazide binding, particularly the 7th and 8th domains, which entail the external loops fundamental for glycosylation sites (Wang *et al.*, 2015; Moreno *et al.*, 2006). Approximately 400 mutations are described that affect the cotransporter and cause GS. Most of them (~250) are missense mutations (see the human gene mutation database at www.hgmd.cf.ac.uk) and are spread along the whole protein. >50 mutations have been reported only in Asian populations (Wang *et al.*, 2015). The Na^+ transport in the early portion of the DCT is electroneutral considering the cotransport of Na^+ and Cl^- through NCC, while in the late portion of DCT, the epithelial sodium channel (ENaC) gives rise to a negative charge in the tubular lumen for its electrogenic transport of Na^+. As a counterpart, to restore a neutral charge in the lumen, potassium secretion is enhanced through ROMK channels or intercalated cells (Loffing and Kaissling, 2003). NCC loss-of-function mutations lead to Na^+ dissipation, which in turn stimulates a gradient for water to exit, whereas ENaC over-activates to restore the Na^+ intake. Finally, to replace the ionic imbalance in the tubular lumen, ROMK drives K^+ out, leading to hypokalemia.

Clinical Characteristics and Diagnosis

BS presents early in childhood with severe failure to thrive and mental or growth retardation. The antenatal form is severe and characterized by mother polyhydramnios, leading to premature delivery. Occasionally, recurrent episodes of severe dehydration and electrolyte imbalance can lead to a fatal outcome. These manifestations may be misleading in premature babies, thus hiding the renal dysfunction and making it difficult to identify the syndrome.

Polyuria and nicturia are a result of the tubule defect and the inefficiency to reabsorb sodium with increased urine volume. If not recognized, this can lead to dehydration with ensuing vomiting, fever, and failure to thrive. The loss of high volume of fluids should lead to low BP, nevertheless, BS patients usually have normal to low BP values. Indeed, there is a compensatory activation of the RAAS, which maintains the vascular resistance and the fluid balance. Typically, biochemical levels of aldosterone and angiotensin II are high; indeed, pseudohypoaldosteronism type I (PHA1) could be misconceived in BS type 2, especially for hyponatremia, transient hyperkalemia, and muscle weakness, but the high plasma renin should be a discriminating factor for its low level in PHA1 conversely to BS and GS.

Hypokalemia resulting from both ROMK mutations and as a compensatory effect of Na and Cl reabsorption drives the metabolic alkalosis. K^+ depletion itself increases bicarbonate reabsorption in the proximal tubule and stimulates ammonia production, which in turn increases urinary acid excretion.

K^+ is also important for the muscle function and contraction for its potential regulation of the cell, along with Mg. When Mg is attached to ATP, actin is released from myosin and therefore the muscle relaxes. In the absence of Mg, the binding of Ca to ATP extends the contraction, causing cramps and tetany.

Nephrocalcinosis is also a clinical feature of the syndrome, and presents with calcification in the renal parenchyma, especially in the tubular epithelium, due to high calcium excreted in the urine.

Calcium imbalance is present in GS as arthropathy associated with CPP crystal deposition or chondrocalcinosis (CC). In addition, GS shares with BS muscle weakness, cramps, limited sport endurance, paresthesia, abdominal pain, and in young patients growth retardation, pubertal delay, and short stature. In adults are reported dizziness, vertigo, polyuria, nocturia, joint pain, and palpitations with long QT interval. Nevertheless, GS is mostly asymptomatic in youth and is less severe when detected in early adulthood. Biochemical criteria for a suspected diagnosis of GS include: documented chronic hypokalemia (<3.5 mmoL/L) concomitant with inappropriate renal potassium wasting (spot urine, potassium creatinine ratio >2.0 mmol/mmol (>18 mmol/g)), in the absence of potassium-lowering drugs; metabolic alkalosis; hypomagnesemia (<0.7 mmoL/L (<1.70 mg/dL); inappropriate renal magnesium wasting (fractional excretion of magnesium >4%); hypocalciuria (spot urine, calcium-creatinine ratio <0.2 mmol/mmol (<0.07 mg/mg) in adults, with normal ranges of calcium-creatinine ratio being different in children with high renin (activity or plasma levels); fractional excretion of chloride >0.5%; normal or low BP; and normal renal ultrasound with an absence of nephrocalcinosis or renal abnormalities. If plasma electrolyte levels are normal or close to normal in a patient taking either or both of potassium or magnesium supplements, these supplements should be stopped for at least 48 h in order to attempt to unmask the abnormalities. Plasma and urine samples should be obtained concomitantly (Blanchard *et al.*, 2017).

A mixed BS-GS phenotype has also been reported, in which BS type III, caused by a mutation in the CLCNKB gene, has clinical and biochemical features common to GS as the co-existence of hypomagnesemia and hypocalciuria. This probably reflects the wide distribution of CLCNKb in the DCT and a possibly involvement of NCC for the physiologic electrolyte balance (Jeck *et al.*, 2000).

Both BS (type 1–4) and GS are autosomal recessive diseases, except for BS type V, which is autosomal dominant. The genetic test should therefore be performed to obtain specific diagnosis and to screen healthy carriers. Indeed, from heterozygous asymptomatic carriers, the risk of producing offspring with the full-blown disease is 25%. The condition to display BS or GS is to have both alleles affected by a mutation or affected by two different mutations.

Treatment

The current therapy for both syndromes is the correction and improvement of electrolyte abnormalities, which is effective in ameliorating the quality of life for patients. Different opinions exist with regard to the dietary salt intake. On the one hand it may enhance the K waste, thus inducing severe hypokalemia; on the other hand, since BS and GS are sodium wasting diseases, this suggests that it would be obvious to consume more NaCl.

In the antenatal form of BS, nonsteroidal antiinflammatory drugs can be used to counteract the excess of prostaglandin E.

In the presence of hypomagnesemia, magnesium supplementation should be considered first, because magnesium repletion will facilitate potassium repletion and reduce the risk of tetany and other complications.

In cases of persistent, symptomatic hypokalemia when supplements are not sufficient despite adherence or when side effects are unacceptable or both, the use of potassium-sparing diuretics, renin angiotensin system blockers, or nonsteroidal antiinflammatory drugs (such as indomethacin), or a combination of these have been proposed. The potassium-sparing diuretics amiloride, spironolactone, potassium canrenoate, and eplerenone can be useful, both to increase serum potassium levels in patients resistant to supplements and to treat magnesium depletion that is worsened by elevated aldosterone levels. The use of renin angiotensin system inhibitors (angiotensin-converting-enzyme inhibitors and angiotensin receptor blockers) has been reported occasionally in the treatment of GS. These drugs also aggravate renal sodium wasting and increase the risk of symptomatic hypovolemia; they should be stopped in cases of acute, salt-losing complications, such as vomiting or diarrhea. Prostaglandin synthase inhibitors such as indomethacin are rarely used in GS, because urinary prostaglandin E2 levels in GS are usually normal. (Blanchard *et al.*, 2017).

Vascular Tone Control: The Angiotensin II Signaling

Peculiar characteristics in the pathophysiology of BS and GS may provide useful insights to understand the mechanisms involved in the control and regulation of vascular tone and BP in humans.

Mostly through the hormone Ang II pathway, RAAS signaling drives increased peripheral resistance and hypertension in healthy individuals (Ravarotto *et al.*, 2015).

Extensive studies from our laboratory provided compelling evidences that in BS and GS Ang II signaling is blunted despite higher level of the hormone and a normal Ang II receptor number and affinity (Calò et al., 2014a). This suggests an interruption of Ang II signaling at postreceptor level or very close to the central switch controlling Ang II signals. The activation of mechanisms involved in the reduction of the BP, despite stimulation of the RAAS, makes BS and GS a "mirror image" of hypertension and a human model of an endogenous antagonism of Ang II signaling via its receptor AT1R.

Understanding the mechanisms involved upon Ang II receptors' stimulation could help to identify novel potential targets of therapy in diseases such as hypertension in which treatments consist in the direct inhibition of Ang II synthesis or activity. Indeed, current treatment of hypertension entails ACE inhibitors, Ang II AT1R blockers, and direct renin inhibitors. Of note, these treatments have the potential to go far beyond BP reduction: evidence from the most important trials on these drugs (EUTOPIA, VIOS, MORE, OLIVUS) demonstrated a reduction of markers of inflammation and improvement of cardiovascular risks, alongside the slowed progression of kidney diseases. This evidence correlates with a complex Ang II activity, which has a clearly pleiotropic nature confirmed by the presence of numerous enzymes, peptides, and receptors involved in its downstream pathways (Nguyen Dinh Cat and Touyz, 2011; Crowley and Coffman, 2012).

Short-Term Ang II Signaling

The multi-effects function of Ang II is exerted through its short- and long-term signaling. The specific intracellular pathways of the short-term signaling are mediated by monomeric and heterotrimeric G proteins and phospholipase Cβ (PLCβ), leading to the release of intracellular messengers inositol trisphosphate (IP3) and Ca^{2+}, the generation of superoxide (O_2^-), and the activation of protein kinase C (PKC) with ensuing vascular smooth muscle contraction (Ravarotto *et al.*, 2015). Noteworthy is a counter-balancing process that is represented by the nitric oxide (NO) system, which has vasodilatory and antiproliferative activity. NO is released by endothelial NO synthase (eNOS) and is negatively regulated by PKC; therefore, the net effect of Ang II on vascular function and structure is the result of the balance of signaling molecules, oxidative stress, and gasomessengers as nitric oxide (Clementi, 1998; Dzau, 2001). Ang II promotes short-term effects by acting directly on its receptors on the cells' membranes (Touyz and Schiffrin, 2000). The binding of Ang II with its heterotrimeric G-protein coupled receptors promotes the increase of free intracellular Ca^{2+} concentration and the activation of the RhoA/Rho kinase pathway with subsequent vasoconstriction. The Ang II–AT1R complex couples with PLCβ via heterotrimeric Gq and Gi proteins and promotes activation of PKC and phosphorylation of the regulatory chain of myosin II. The Ang II–AT1R complex couples first to PLCβ1 via heterotrimeric Gαq/11$\beta\gamma$ and Gαq/12$\beta\gamma$, then to PLCγ via tyrosine kinase. The α subunit of Gi protein transduces the Ang II signal to blunt adenylyl cyclase activity with ensuing decreased formation of the vasodilatory cAMP. In addition, the activation of the monomeric G-protein RhoA and its effector Rho kinase modulates the phosphorylation state of the regulatory chain of myosin II, mainly through inhibition of the myosin phosphatase target protein-1 (MYPT-1). By this mechanism, Ang II induces smooth muscle contraction and increased peripheral resistance.

To elucidate further, this paradoxical picture present in BS and GS of elevated RAAS and low BP is fundamental to investigating the single proteins involved in this signaling. In fact, BS and GS have decreased gene and protein expression of the α subunit of Gq protein and blunted downstream intracellular events that promote Ca^{2+} release and PKC activation (Calò et al., 2001, 2002, 2014a). In addition, in these patients the evaluation of the NO system via the endothelial nitric oxide synthase mRNA levels, urinary excretion of NO metabolites, and NO mediated vasodilation showed a significant increase of eNOS expression (Calò, 2006; Calò *et al.*, 1999), an increased urinary excretion of NO metabolites, which positively correlated with urinary excretion of cyclic guanosine monophosphate (cGMP), the NO second messenger (Calò, 2006; Calò *et al.*, 1995, 1996), and an increased NO mediated vasodilation compared with hypertensive patients (Calò et al., 2008a). In addition to these characteristics, which also underlie an antioxidant potential in these patients, the augmented expression of heme oxygenase 1 (HO-1) (Calò *et al.*, 1998, 2003), establishes an antioxidant and antiinflammatory potential.

The activity of G protein-coupled receptors (GPCR) is regulated by a tool of proteins that control the integration of information and subsequent downstream signaling. Regulators of G protein signaling (RGS) proteins are deputed to this purpose (Heximer and Blumer, 2007). RGS proteins can regulate many different effector proteins acting as GTPase-activating proteins for Gα subunits and also competitively inhibit Gα binding to PLC (Zhong and Neubig, 2001). Both NO and its second messenger, cGMP, increase the RGS-2 guanosine triphosphatase activity of Gq protein, which leads to dephosphorylation of myosin light chain and induces vascular smooth muscle cell relaxation. Collectively, these findings suggest that RGS-2 is central for the vasorelaxing activity of NO (Tang *et al.*, 2003). The relevance of RGS proteins in hypertension has been demonstrated in RGS-2 knockout mice, which showed persistent vasoconstriction and hypertension (Tang *et al.*, 2003). The characteristic normotension or hypotension of BS and GS, upon Ang II stimulation, suggests that abnormalities in the GPCR complex and in its regulator RGS-2 might be involved (Mehta and Griendling, 2007). Patients with BS and GS have, in fact, an increased gene and protein expression of RGS-2 (Calò *et al.*, 2004), which is the opposite of the decrease seen in hypertensive patients (Semplicini *et al.*, 2006; Calò *et al.*, 2004). The increased RGS-2 expression in BS and GS patients may explain their downregulation of Gq protein signaling (Calò et al., 2001, 2002) and the reduced peripheral resistance, vascular hyporeactivity and normotension or hypotension that are typical of these syndromes. Silencing RGS-2 in Bartter's and Gitelman's patients' fibroblasts, in fact, produces effects (Calò *et al.*, 2008b) that coincide with the results in knockout mice and humans (Tang *et al.*, 2003; Semplicini *et al.*, 2006; Heximer *et al.*, 2003).

The Rho A activation induced after Ang II binding with AT1R is mediated by some guanine nucleotide exchange factors (GEF) as p63RhoGEF and p115RhoGEF essentially via binding of the a subunit of Gq protein to activate RhoA and its effector Rho kinase. This leads to vascular contraction, cell proliferation, and cardiovascular remodeling. The impaired Ang II-dependent contraction and proliferation of rat aortic smooth muscle cells after p63RhoGEF depletion and the

blunted endothelin-1-induced portal vein contractile force upon p63RhoGEF silencing support the view that abnormalities of these RhoGEFs contribute to increased activation of RhoA/Rho kinase pathway in hypertension. In BS and GS patients, RhoA/Rho kinase pathway downregulation is accompanied by reduced mRNA and protein expression not only of Ang-II-induced p115RhoGEF, but also of p63RhoGEF. Moreover, these patients have decreased phosphorylation of MYPT-1, a marker of Rho kinase activity (Loirand *et al.*, 2006), which conversely is increased in hypertensive patients (Calò *et al.*, 2014b).

Long-Term Ang II Signaling

Long-term Ang II signaling changes the cell oxidative states inducing cardiovascular and renal remodeling in hypertension, atherosclerosis, and heart and kidney failure (Mehta and Griendling, 2007; Touyz, 2003; Griendling and FitzGerald, 2003).

Oxidative stress is due to an imbalance between oxidant and antioxidant agents and the loss of redox homeostasis due to increased pro-oxidant and pro-thrombotic activities causes harmful effects, in terms of free radicals and nitroxidative stress. NAD(P)H oxidases, Xantine oxidases, P-450 monoxygenases, lipoxygenases, and cycloxygenases are all sources of reactive oxygen species. All these enzymes produce a superoxide anion (O_2^-), which is an intermediate product of oxygen reduction. It reacts rapidly with other groups of molecules to induce high reactive compounds as hydroxyl radical (OH^-) and peroxynitrite ($OONO^-$). The main oxidative stress inducer is NAD(P)H oxidase (Nox), which catalyzes the production of superoxide from oxygen and NAD(P)H (Griendling *et al.*, 2000). Noxs are characterized by a catalytic subunit and a little $p22^{phox}$ subunit that is able to form the heterodimeric cytochrome b_{558}. The association between $p22^{phox}$ and Nox induces phosphorylation cascades that involve cytosolic subunits translations to the membrane and conformational changes in both the enzyme and the cellular membrane (Griendling *et al.*, 2000). The generated O_2^- initiates the production of free radicals and reactive oxidants, which are involved in atherosclerotic lesions and cardiovascular and renal remodeling (Griendling and FitzGerald, 2003; Griendling *et al.*, 2000). In BS and GS patients, the response of the NAD(P)H oxidase to Ang II is reduced. Ang II stimulation produces in these patients a reduced oxidative state in terms of decreased $p22^{phox}$ gene expression (Calò *et al.*, 2003). The decrease of the oxidant potential was related to a decrease in the $OONO^-$ and to an increase in the HO-1 gene expression, as antioxidant enzyme (Calò *et al.*, 2003).

In addition, BS and GS show a reduced susceptibility of the low density lipoprotein (LDL) to oxidation. Oxidated LDL is deeply involved in the progression of the atherosclerotic lesions both in the cardiovascular and renal systems, and the increased NO production in these patients also has a role in the protection of LDL from oxidation (Calò *et al.*, 1998). Furthermore, the reduced production of O_2^- results, in these patients, in an increased bioavailability of NO. In BS and GS the atherothrombogenic factor plasminogen activator inhibitor-1 (PAI-1) is also reduced upon the Ang II challenge (Pagnin *et al.*, 2004). Ang II stimulation promotes long-term fibrotic outcomes particularly involving cytokines. The profibrotic cytokine transforming growth factor β (TGFβ) controls proliferation and cell differentiation, and in the kidney is a cause of fibrosis. Again, BS and GS exhibit a reduced gene expression of TGFβ (Calò *et al.*, 2003).

In addition, the downregulation of the RhoA/Rho kinase pathway alongside a short-term vasoconstriction is increasingly recognized as important in hypertension and cardiovascular remodeling (Calò and Pessina, 2007; Loirand *et al.*, 2006; Shimokawa and Takeshita, 2005; Budzyn *et al.*, 2006). Basal release of NO from the endothelium prevents the activation of RhoA/Rho kinase in smooth muscle through protein kinase G-dependent inhibitory phosphorylation of RhoA (Sauzeau *et al.*, 2003). Excessive production of reactive oxygen species inactivates NO with ensuing increased vascular tone, at least in part through elevated RhoA/Rho kinase activity (Takemoto *et al.*, 2002, Jin *et al.*, 2006). BS and GS patients were found to exhibit a parallel downregulation of RhoA/Rho kinase and upregulation of the nitric oxide system. In addition, the reduced p63RhoGEF and p115RhoGEF expression further delineates the major role of these proteins in the blunted activation of RhoA/Rho kinase pathway (Calò *et al.*, 2014a,b; Calò and Pessina, 2007; Pagnin *et al.*, 2004, 2005a). These features and the lack of endothelial dysfunction and cardiovascular remodeling (Calò *et al.*, 2008a, 2009) produce in BS and GS an opposite image of hypertension; this supports the involvement of RhoA/Rho kinase in the processes leading to cardiovascular remodeling and atherogenesis.

Insulin Sensitivity

BS and GS patients have a normal oral glucose tolerance test, a reduced baseline insulin level, and a markedly higher oral glucose insulin sensitivity compared with healthy normotensive individuals (Davis *et al.*, 2006).

In vitro, Ang II decreases the insulin receptor substrate-1 protein levels via Src, phosphoinositide-dependent kinase-1, and reactive'oxygen-species-mediated phosphorylation of Ser307 (Taniyama *et al.*, 2005), leading to impaired insulin signaling. Ang II is also thought to inhibit insulin signaling and reduce glucose transport via stimulation of RhoA/Rho kinase activity, inhibition of phosphoinositol 3 kinase, and its downstream Akt pathway (Sowers, 2004). Blocked ATR1 signaling in BS and GS results in Akt pathway activation, as shown by increased expression of HO-1 (Calò *et al.*, 2003, 2011), which is under Akt control (Martin *et al.*, 2004). In turn, Akt activates NOS (Wolfrum *et al.*, 2004; Mita *et al.*, 2005). These effects provide a mechanistic explanation for the higher insulin sensitivity found in these patients, as the parallel positive effects reported regarding insulin resistance in the presence

of Ang II AT1R blockers. In addition, in these patients there is an unaltered expression of p66shc, an adaptor protein with a major role in oxidative stress related responses, including those mediated by Ang II, which sensitizes the cells to apoptosis (Calò *et al.*, 1998, 2003, 2008c; Graiani *et al.*, 2005). All this is in marked contrast to type 2 diabetic patients who show the opposite (Avogaro *et al.*, 2003; Pagnin *et al.*, 2005b). These data confirm in humans the linkage of Ang II and glucose metabolism, and provide a molecular mechanism in a human model such as BS and GS for the positive effect of blocking the RAAS and RhoA/Rho kinase pathway on glucose tolerance, diabetes, and atherogenesis.

See also: Regulation of Potassium Homeostasis

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Angiogenesis☆

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Glossary

Angiogenesis In a strict sense, the formation of vessels from preexisting vessels. Generally, the development of new vessels.
Angiopoietins Factors implicated in the recruitment of accessory cells to the vasculature and vessel remodeling.
Arteriogenesis Formation of larger blood vessels including arterioles.
Endothelial cells Major cell type that is targeted by angiogenesis factors or inhibitors. Endothelial cells are in close contact with the blood and organize into tubular structures.
Fibroblast growth factors Pleiotropic growth factors that also induce angiogenesis.
Hypoxia-inducible transcription factors (HIFs) There are two HIFs, HIF-1α and HIF-2α, that regulate genes involved in angiogenesis.
Integrins Heterodimeric cell surface receptors for matrix molecules.
Lymphangiogenesis Formation of lymphatic vessels.
Mural cells Cells lining the vessel wall in close contact with the subendothelial matrix and endothelial basement membranes. Pericytes are among the principal mural cells and are found in capillaries.
Platelet-derived growth factors Growth factors implicated in the recruitment of accessory cells (mainly pericytes) to the vasculature.
Proteolytic enzymes Enzymes that degrade proteins such as extracellular matrix molecules. The major proteolytic systems for the degradation of matrix molecules are the plasmin/plasminogen activator–inhibitor system and matrix metalloproteinases.
Pruning Process that yields a mature remodeled vascular network.
RIP-Tag mouse model Transgenic mouse model with targeted expression of large T antigen in pancreatic β cells. A model for multistage carcinogenesis.
Vascular endothelial growth factors (VEGFs) The major regulators of angiogenesis.
Vasculogenesis Formation of blood vessels from progenitor cells

The formation of vascular channels, angiogenesis is a fundamental process that takes place during embryonic life and also plays a crucial role in the adult organism. This review covers an overview of mechanisms of angiogenesis and lymphangiogensis and the impact of angiogenesis research on the pathology and therapy of disease.

Overview of the Vascular System

The mammalian vascular system is made up of two circulatory networks, the blood and lymphatic vascular systems. The blood vascular system transports gases, nutrients, liquids, cells and chemical signals such as hormones to the tissues, and removes waste products. The lymphatic vascular system has complementary functions to those carried out by the blood vasculature. The lymphatic system collects fluid and macromolecules from within tissues (forming protein-rich lymph) and returns the fluid to the blood circulation via the thoracic duct.

Initial development of the blood vasculature occurs in the embryo via the process of *vasculogenesis*. Further remodeling and new vessel growth in the adult occur via *angiogenesis*, defined as the process of new blood vessel formation from the existing vasculature. Lymphatic vessel development occurs via the related process of *lymphangiogenesis*.

Basic Structure of Vessels

The vascular system is made up of large (macrovascular) blood vessels such as arteries and veins and small (microvascular) vessels such as capillaries, post-capillary venules and arterioles (see **Fig. 1**). Large vessels have a common basic structure and consist of three different layers. The tunica intima is made up of a central layer of endothelial cells (EC) forming the lumen of the vessel, surrounded by and attached to the vascular basement membrane (BM). Outside this is the tunica media, comprised of multiple layers of perivascular cells (smooth muscle cells or pericytes), and the outer tunica adventitia, which forms the outermost coat of the vessel and is formed from loose fibrous connective tissue, often continuous with the surrounding connective tissue. Microvessels are smaller and narrower than macrovessels. They are formed from an endothelial cell layer and basement membrane and

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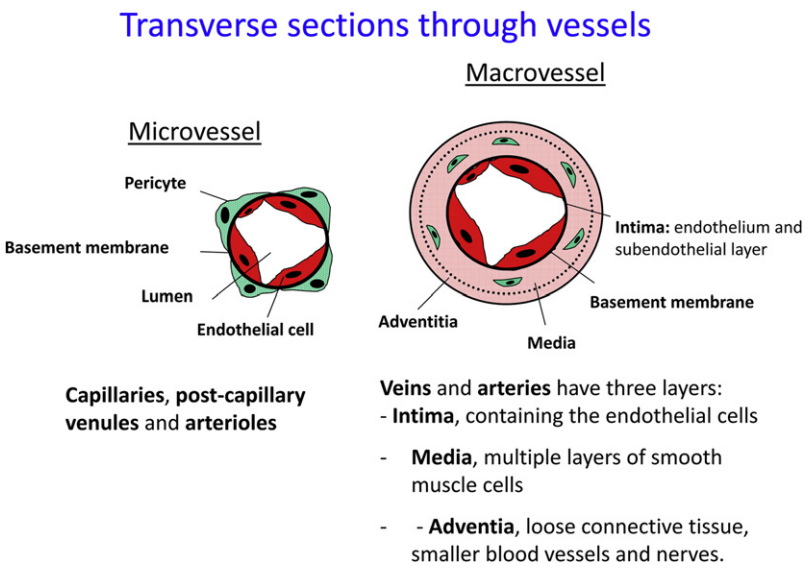

Fig. 1 Basic structure of blood vessels

are stabilized by pericytes. Within the vascular system, arteries carry afferent circulation away from the heart to the capillaries. They are thus exposed to the highest pressure and flow, and are surrounded by thick layers of smooth muscle cells (SMC). In contrast, veins carry efferent circulation back to the heart. Efferent circulation is less pressurized and veins typically have less surrounding smooth muscle and contain specialized valve structures to prevent backflow of blood. Capillaries form a branching and anastomosing network between small arterioles and venules, and are the sites for exchange of gases, nutrients and waste between the vasculature and organs. Capillaries show a remarkable heterogeneity and are uniquely adapted to suit the needs of a given tissue environment.

Structurally, lymphatic vessels show both similarities with and differences from blood vessels. Like blood vessels, the inner lining is made up of a single layer of endothelial cells (lymphatic endothelial cells, or LEC). Unlike blood vascular capillaries, lymphatic capillaries do not have a covering of perciytes, and very little basement membrane. Instead, LEC contact surrounding interstitial collagen directly via anchoring filaments composed of elastic fibers, which help to structurally support the vessel and prevent its collapse. Lymphatic vessels are generally larger in diameter than blood vessels, with loose junctions formed of layers of overlapping LEC. Larger vessels have a sparse covering of smooth muscle cells to aid unidirectional propulsion of lymph, as well as valves to prevent backflow.

Vasculature and Pathology

Angiogenesis plays roles in normal physiological processes such as wound healing, the female menstrual cycle and organ growth. It is a tightly regulated process in which new blood vessels form only as needed, with the vasculature remaining quiescent most of the time. Dysregulated angiogenesis leads to many pathological conditions. For example, vascular insufficiency is implicated in diseases including Alzheimer's, stroke, diabetes, atherosclerosis, Crohns disease, neonatal respiratory distress, nephropathy and osteoporosis. Conversely, excessive angiogenesis is involved in obesity, psoriasis, arthritis, diabetic retinopathy and many other diseases.

Arguably the most important aspect of excessive angiogenesis is that the growth and metastasis of solid tumors is angiogenesis-dependent. Like all cells, tumor cells require access to the blood vasculature in order to receive sufficient oxygen and nutrients to survive, and as a means of removing waste. A pre-vascular tumor grows until a critical size of 2–3 mm^2 is reached. At this point, insufficient oxygen and nutrients restrict the tumor growth, and it remains dormant, with cell proliferation balanced by apoptosis. In order for the tumor to grow any further, it must become vascularized via induction of blood vessel growth, a process described as the 'angiogenic switch' (see Fig. 2). Once this critical step occurs, the tumor is able to grow exponentially in size, and is also provided with a route to metastasize around the body. The vital importance of this critical step in the progression of cancer has led to major interest in the mechanisms and therapeutic control of blood vessel formation.

In recent years it has become clear that the lymphatic system plays a vital role in the spread of cancer around the body. The lymphatic vasculature is a primary route for metastatic tumor cells, presumably because the large diameter, lack of basement membrane and loose intercellular junctions of lymphatic vessels allow easier access than blood vessels. Tumor cells carried in the lymph gain access also gain access to the blood vascular system via the thoracic duct providing another route to distant tissues. Lymphatic vessel density of the primary tumor correlates with metastatic spread for a variety of tumors. However, some controversy remains as to whether tumor lymphatics are functional due to the high interstitial pressures within solid tumors.

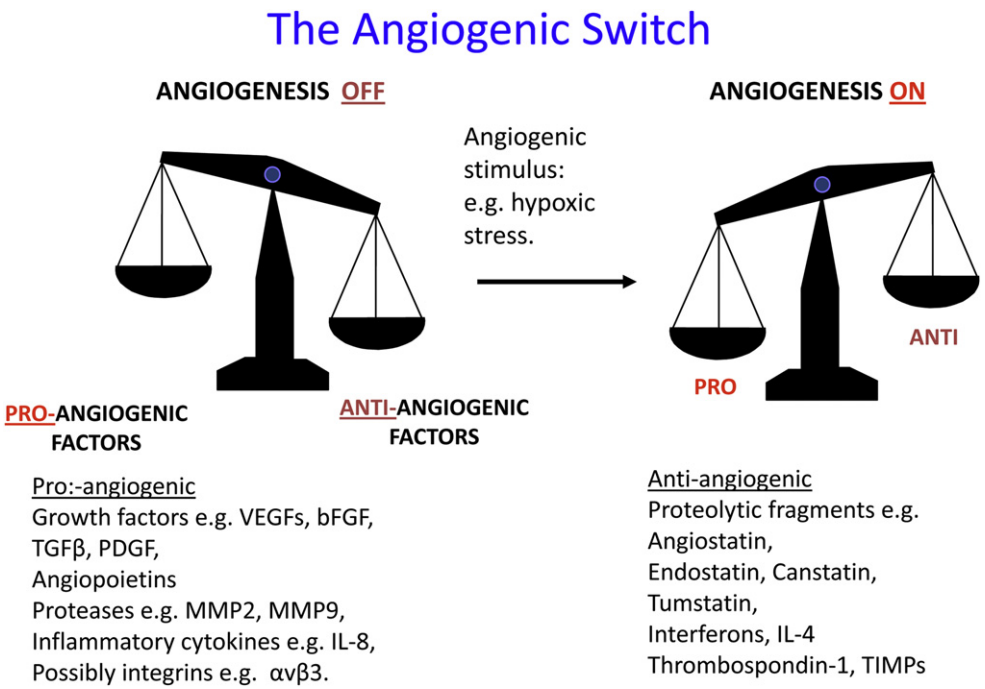

Fig. 2 A shift in the balance of pro- and anti-angiogenic factors leads to the 'angiogenic switch'. Following a known angiogenic stimulus such as hypoxic stress, pro-angiogenic factors begin to accumulate, and their effects eventually outweigh those of negative regulatory factors. The angiogenic switch is now 'on' and new vessel formation is induced.

Diabetic retinopathy remains the most common cause of acquired blindness among persons of working age in the industrialized world. The typical microangioathy changes include loss of pericytes and endothelial cells and thickening of the basilar membrane. Microaneurysms, that is, sites of outward ballooning of the capillary wall, are a common early feature of the disease. Occlusive microangioathy induces retinal ischemia and subsequent vitreoretinal neovascularization, whilst loss of hemato retinal barriers induces macular edema. Both complications threaten the patient's vision. Another diabetic condition where insufficient angiogenesis occurs is diabetic polyneuropathy seen in advanced-stage diabetes.

Another condition, where excessive angiogenesis occurs, is age-related macular degeneration. It was not until 1980 that macular degeneration was reported to be a significant cause of blindness in the United States. Since then, many studies have expanded upon the risks associated with new blood vessel growth in the aging retina, and have shown just how serious and widespread a public health issue AMD represents. Recently developed angiogenesis-therapies in this condition have yield to very significant results.

Basic Mechanisms of Vasoformation

Vasculogenesis

The blood vascular network is formed via two main processes, vasculogenesis and angiogenesis.

Vasculogenesis refers to the de novo formation of blood vessels, and is the mechanism of formation of a primitive blood vascular network in the embryo. Endothelial and hemtopoietic cells share a common progenitor, termed the hemangioblast. Aggregates of hemangioblasts (known as blood islands) form in the yolk sac, composed of inner cells which subsequently differentiate into hematopoietic cells, and outer cells which give rise to angioblasts (endothelial precursor cells). Angioblasts migrate and differentiate into mature endothelial cells, coalescing to form a vascular labyrinth, which is subsequently remodeled and extended by the related process of angiogenesis. For many years, it was thought that de novo blood vessel formation occurs only during embryonic development, and that all post-natal vascularization occurs via angiogenesis. However, in recent years it has become clear that endothelial precursor cells (EPC), which are defined as cells existing in differentiation stages between hemangioblasts and mature EC, are present in the adult bone marrow, at low levels in the blood circulation, and possibly in tissues. EPC are mobilized and recruited from their resident sites, and differentiate to mature EC in response to various factors such as VEGF, bFGF and insulin-like growth factor-1 (IGF-1). Such cells have been shown to contribute to neovascularization in the adult in a variety of settings, such as ischemic conditions, tumor growth and wound healing. The magnitude of participation of EPC in neoangiogenesis is still matter of debate. It has been argued that EPC contribute to new vessel formation by differentiating into mature EC, but a more likely explanation is by contributing to a pro-angiogenic environment via the release of cytokines such

as VEGF. The discovery of EPC in adult tissue has led to great interest in the potential therapeutic implications of using progenitor cells derived from bone marrow to aid re-vascularization in clinical settings, such as in repair of damaged heart and ischemic limb disease.

Angiogenesis

Angiogenesis is the process of new blood vessel formation from the existing vasculature. It is thus distinct from vasculogenesis in that new blood vessels arise from and extend a pre-existing vascular network. A second key difference is that angiogenesis occurs by division of mature endothelial cells rather than by differentiation of precursor cells. During sprouting angiogenesis, new vessels arise by extension of or branching from their vessel of origin. This process is now well characterized and can be divided into a series of non-discrete, overlapping stages, described below, and depicted in **Fig. 3**.

Initiation

In the adult, the vasculature is normally in a quiescent state. New vessels form only as required, in response to an angiogenic stimulus. In quiescent vasculature, inter-endothelial cell contacts are tight, and the vessel is stabilized by pericytes, which help to maintain EC in a quiescent state. The vessel must be destabilized in order for endothelial cells to migrate away from the parent vessel to form a new sprout. Current thinking suggests that a shift in the balance of pro- and anti-angiogenic factors regulates the 'decision' to induce vessel formation. Following a known angiogenic stimulus, pro-angiogenic factors begin to accumulate and their effects eventually outweigh those of negative regulatory factors, and new vessel formation is induced. Hypoxic stress, when tissues grow beyond the limit of oxygen diffusion is one such angiogenic stimulus. Low oxygen tension leads to upregulation of hypoxia-inducible transcription factors (HIFs) which induce expression of genes involved in angiogenesis, including nitric oxide synthase.

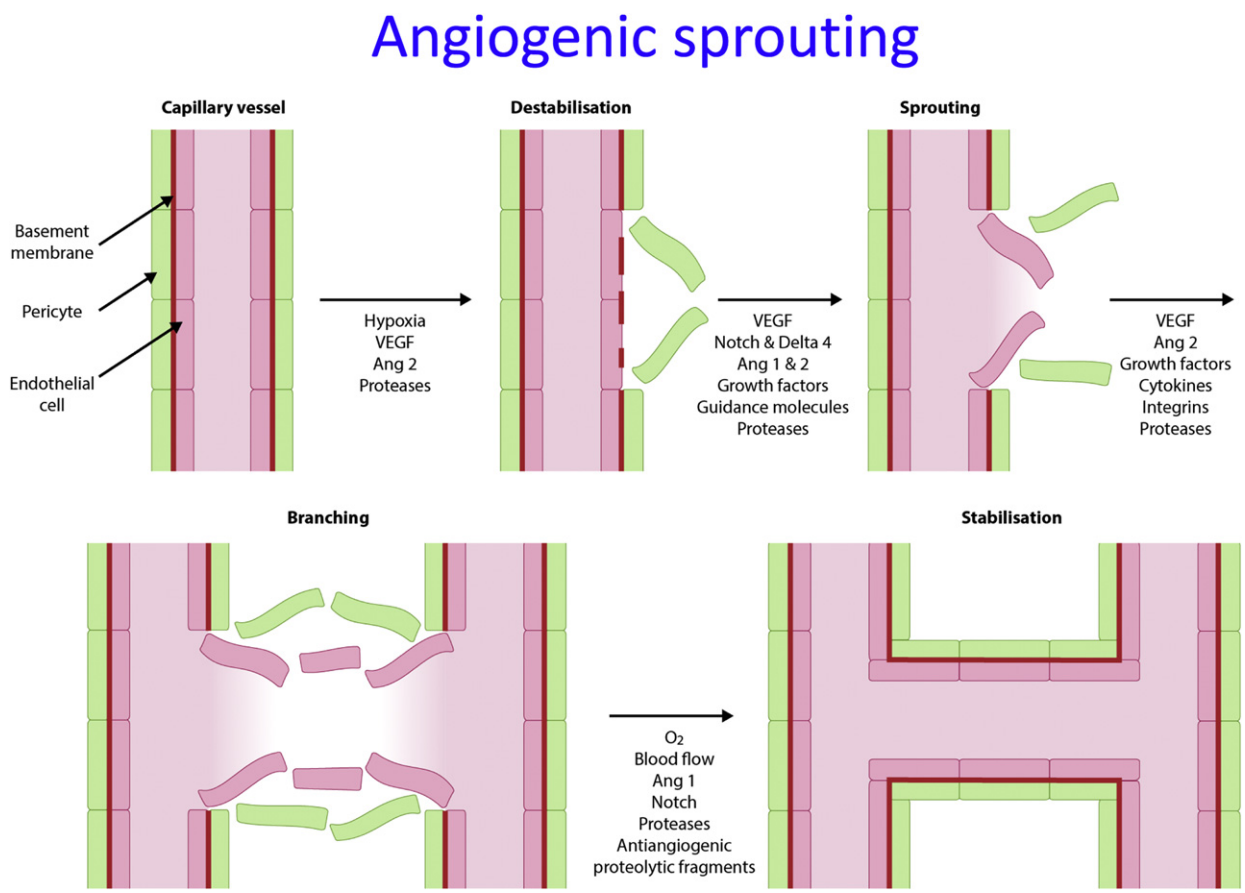

Fig. 3 Angiogenic sprouting. Resting vasculature is quiescent. In response to an angiogenic stimulus, endothelial cells are activated and the parent vessel is destabilised. Pericytes detach, and EC-EC contacts are loosened. Plasma proteins are extravasated and form a provisional scaffold. The basement membrane is degraded by the activity of proteases, and activated ECs proliferate and migrate towards the angiogenic stimulus, invading the ECM and forming a sprout. A new cord stucture is assembled, which is subsequently stabilised by the formation of new basement membrane and the attachment of pericytes.

Dilation and Destabilization of Parent Vessel

Induction of HIFs lead to expression of nitric oxide synthase and production of nitric oxide, which in concert with vascular endothelial growth factor (VEGF) leads to vasodilation. Inter-endothelial cell contacts are loosened via redistribution of inter-cellular adhesion molecules such as CD31 and vascular endothelial cadherin (VE-Cadherin) and pericytes detach from the basement membrane. Plasma proteins are extravasated from the dilated vessel, and form a provisional scaffold for migrating EC. Vessel destabilization must be tightly regulated to allow EC to leave the parent vessel, but without causing its collapse and regression. The process is regulated in a large part by the competing actions of angiopoietins in concert with the permeabalizing effect of VEGF.

Proliferation and Migration of Endothelial Cells

Endothelial cells in quiescent vasculature have a very low turnover. However, during new vessel formation, EC show a greatly increased rate of proliferation In order to migrate towards an angiogenic stimulus, EC produce proteases which are required for degradation of the vascular basement membrane, and to digest a path through the interstitial extracellular matrix. Endothelial cells migrate from the provisional scaffold, through the interstitial extracellular matrix (ECM) via the coordinated actions of proteases, integrins and endothelial mitogens such as VEGF. Each newly forming sprout has a single 'tip' cell which senses and responds to gradients of VEGF, guiding the nascent vessel. All other EC in the developing vessel, known as 'stalk' cells, proliferate and migrate after the tip cell without forming new sprouts. This process is regulated by Notch signaling.

Lumen Formation

Migrating EC assemble as a solid cord structure, which subsequently acquires a lumen. Studies have suggested that lumen formation occurs via an integrin-dependent pinocytotic mechanism. Vesicles form within individual EC, and fuse together to form vacuoles. Vacuoles form adjacent migrating chains of EC then merge to form the lumen. The diameter of the forming lumen is tightly regulated by VEGF isoforms and angiopoietins, as well as endogenous inhibitors of lumen formation such as thrombospondin-1 (TSP-1). An alternative explanation is that lumen formation occurs by cord hollowing. In this mechanism, two endothelial cells are brought in close contact by VE-cadherin. This contact is then partially broken by a de-adhesive process involving CD34-sialomucins, such as CD34 and podocalyxin. This is followed by recruitment of actin at the interface between two EC, which is under the control of ERM proteins, which are phosphorylated.

Vessel Stabilization

Maturation of newly formed vessels involves stabilization of the vessel structure by generation of a vascular basement membrane, and by the attachment of pericytes. Contact with the basement membrane induces EC quiescence, and promotes survival. The interaction of perivascular cells with nascent capillaries is vital for vessel stabilization, since vessels without a pericyte covering eventually regress. Endothelial-pericyte interactions are mediated in large part by platelet derived growth-factor –BB (PDFB-BB) signaling through the PDGF receptor β (PDGFRβ). Perivascular cells expressing PDGFβ are stimulated to differentiate and migrate towards PDGF-BB expressed by endothelial cells. Pericyte-endothelial cell contact requires EC proliferation, survival, migration and differentiation. Lack of pericyte coverage is believed to be responsible for the capillary fragility and impaired perfusion in diabetic retinopathy. Transforming growth factor-β (TGFβ) signaling contributes to vessel maturation by regulating differentiation of mural cells, and stimulating ECM production, and the action of angiopoietins is also required.

Remodeling and Maturation of the Vascular Network

After an initial burst of new vessel formation in response to an angiogenic signal such as hypoxia, formation of an ordered functional network occurs via vascular remodeling and 'pruning' of unnecessary vessels. This is a normal physiological mechanism to match perfusion with metabolic demand. Regression of immature vessels is induced on removal of angiogenic stimuli, but once vessels are stabilized by pericyte attachment, they become resistant to oxygen-induced regression. Thus, when the angiogenic switch is turned 'off' by accumulation of anti-angiogenic factors, or removal of pro-angiogenic stimuli, vessels must be actively maintained to avoid regression. Hemodynamic forces are also essential for vessel maintenance, via mechanisms which are still unclear, since vessels which are poorly perfused tend to regress. With maturation of the vascular network, EC also acquire the specialized characteristics required for vessel identity, including arterial, venous or lymphatic identity.

Detailed study of the lymphatic vasculature has only become possible in recent years with the discovery of lymphatic marker genes allowing discrimination of the (morphologically similar) lymphatic vessels from the blood vasculature. Lymphangiogenic sprouting appears essentially similar to angiogenic sprouting and is therefore thought to involve similar processes to those identified in angiogenesis. However, detailed knowledge of the precise mechanisms and molecules regulating the formation of new lymphatics is still lacking at this stage.

Key Factors Regulating Vascular Development/Therapeutic Targets

VEGF Family

The Vascular Endothelial Growth Factor (VEGF) family of secreted glycoproteins have long been recognized as prime regulators of angiogenesis. The VEGF family consists of five main members: VEGF-A (commonly known as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (Plgf). The VEGFs interact with a family of receptor tyrosine kinases known as vascular endothelial growth factor receptors (VEGFR)-1, −2 and −3. Neuropilins (NP)-1 and −2 are also able to functionally interact with the VEGFRs (see Fig. 4). As a general rule, the VEGF family proteins are expressed in a wide range of cell types, with the receptors having a much more restricted cell-type expression. VEGFR1 and VEGFR2 are expressed predominantly by endothelial cells, while VEGFR3 is expressed mainly by lymphatic EC in the adult.

VEGF-A (also known as VEGF) was first identified as a vascular permeability factor secreted by tumor cells and is the best characterized of the VEGF proteins. It is the most potent pro-angiogenic protein described to date, and is the major hypoxia-inducible pro-angiogenic factor. It is a key regulator of angiogenesis, vasculogenesis and hematopoiesis. VEGF-null mice die early in development (E8-9) due to a block of hematopoietic and vascular development, and there is a dose-dependent requirement for VEGF protein since deletion of a single allele is also embryonic lethal. Endogenous expression of VEGF by EC is also required to maintain vascular function and stability. VEGF exists as at least 7 isoforms formed through alternative splicing, known as VEGF 121, 145, 148, 165, 183, 189 and 206 with VEGF 165 being the effector of VEGF function. VEGF binds both VEGFR1 and VEGFR2, and signals primarily through VEGFR2 to promote EC proliferation and survival, sprouting angiogenesis and increase migration and invasion.

VEGFR2 deletion is embryonic lethal in mice as a result of defects in hematopoietic and endothelial precursors. VEGF binds VEGFR1 with a higher affinity than VEGFR2, however VEGFR1 exerts less activation of intracellular signaling and has therefore been proposed to act as a negative regulator of VEGFR2 function. On the other hand, VEGFR1 has been reported to heterodimerise with VEGFR2 forming a complex with stronger signaling properties than either VEGFR1 or VEGFR2 homodimers. VEGFR2 has recently been proposed to play a role in lymphatic development.

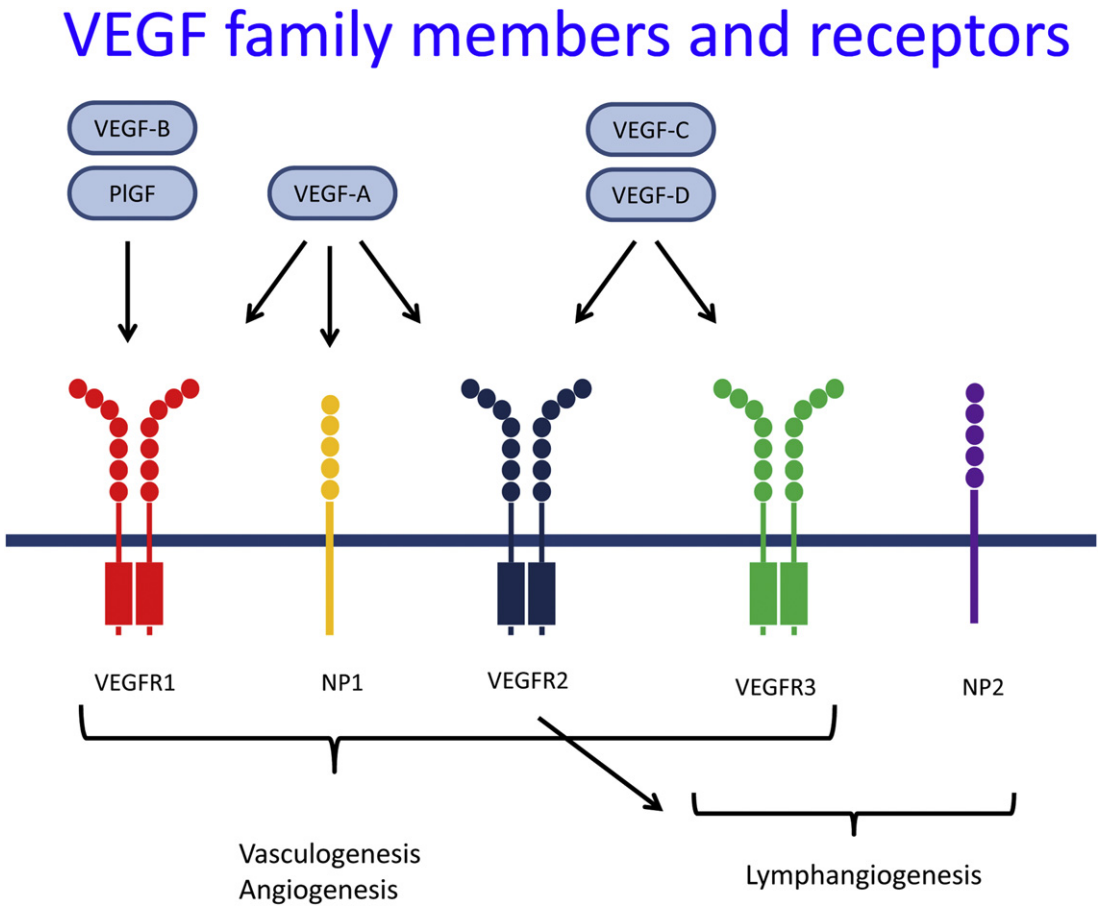

Fig. 4 VEGF family members and their receptors.

VEGFR3 plays a crucial role in embryonic blood vascular formation, however in later development its expression is restricted largely to the lymphatic endothelium. However, it has been shown to be re-expressed in the blood vasculature in certain pathological conditions such as on tumor vasculature, and blocking its function inhibits blood vascular endothelial cell tubulogenesis, suggesting that it can act in a pro-angiogenic role. VEGF-C is the critical growth factor regulating lymphangiogenesis, since the lymphatic vasculature fails to form in VEGF-C null mice. VEGF-C signals mainly through VEGFR3, although the proteolytically processed mature form can activate VEGFR2 in the blood vascular endothelium. Similarly, VEGF-D binds and activates VEGFR2 and VEGFR3.

Anti-angiogenic therapies currently in the clinic or in clinical trials focus largely on blocking VEGF signaling, either by sequestering VEGF or by inhibiting its receptors. The first FDA-approved anti-angiogenic therapy, Bevacizumab is a humanized monoclonal anti-VEGF antibody. It was first licensed in 2004 for the treatment of metastatic colon cancer in combination with standard chemotherapy, and is now licensed for used in a number of cancers including renal, ovarian, lung and glioblastoma multiforme. A derivative of Bevacizumab, Ranbizumab, has also been approved for the treatment of age related macular degeneration (AMD). Other approaches include the production of soluble decoy receptors, such as VEGF-Trap, a chimeric protein contains VEGF-binding domains of both VEGFR1 and VEGFR2. VEGF-Trap has also been licensed for the treatment of AMD, and is currently under investigation for therapeutic benefit in cancer treatment. Pegaptanib, an aptamer that selectively binds and neutrlises VEGF-165 has also been approved for AMD treatment.

A second approach to blocking VEGF signaling involves targeting the VEGFRs. Low molecular weight tyrosine kinase inhibitors such as Sorafenib, Sunitinib, Pazopanib and Everolimus are able to target multiple receptors including VEGFRs, PDGFRs and in some cases FGFRs. These drugs thus have the advantage of targeting multiple therapeutically relevant signaling pathways at once, and are used in the treatment of multiple cancers including renal cancer, liver cancer, and soft tissue sarcoma. A series of alternative receptor tyrosine kinase inhibitors are currently also in phase III clinical trials with the aim of treating a variety of cancers.

While anti-VEGF signaling therapy has a sound baiss, the clinical results of anti-angiogenic treatments to date have been perhaps less impressive than hoped for, largely due to the development of resistance in tumors treated with anti-angiogenic therapies. The reasons for resistance are manifold, but include the following:

- Upregulation of alternative pro-angiogenic signaling molecules, such as FGFs, Plgf, angiopoietins and ephrins in response to blockade of VEGF signaling.
- Upregulation of granulocyte colony stimulating factor (G-CSF) and stromal derived factor-1 (SDF-1), leading to recruitment of vascular progenitor cells from the bone marrow which can then participate in neovessel formation as described above. Existing blood vessels can also split (intusception) to form new vessels in the absence of sprouting angiogenesis. Co-option of existing blood vessels and vascular mimicry by tumor cells are also mechanisms to provide a tumor with a blood supply in the absence of sprouting angiogenesis.
- Increased perivascular cell coverage of tumor blood vessels. As discussed above, perivascular cell coverage stabilizes vessels and protects them from regression after withdrawal of VEGF stimulation.
- Selection of tumor cells harboring mutations rendering them relatively resistant to hypoxia, or lacking VEGF or VEGFRs.
- These have led to focus on the development of alternative therapies blocking other signaling pathways relevant to angiogenesis.

Angiopoietins

The Tie receptors Tie1 and Tie2 and their corresponding angiopoietin (Ang) ligands were identified as the second EC-specific tyrosine kinase receptor signaling system. The angiopoietin family consists of Ang-1, Ang-2, and Ang-4 (the mouse homologue of Ang-4 is known as Ang-3) as well as a set of angiopoietin-like proteins (Angptl1-7). Most studies have focused on Ang-1 and Ang-2 which bind the Tie2 receptor and have the best characterized roles in angiogenesis. The roles of Ang-1 and Ang-2 signaling are complex and context dependent. Ang-1 binds and directly activates the Tie2 receptor, and this can have pro-angiogenic roles, stimulating EC sprouting and vessel growth as well as being correlated with vessel density in a variety of tumors. However, contradictory anti-angiogenic roles have been proposed in pathological situations. Ang-1/Tie2 signaling can be broadly considered as promoting vascular stability. Ang-1 expressed by mural cells interacts with Tie2 expressed by EC, and it is involved in the induction of EC quiescence and stabilization of the vasculature via mediation of EC-perivascular cell interactions, antagonism of the permeability-inducing effects of VEGF, and promotion of EC survival via activation of the Akt signaling pathway.

Ang-2 deficient mice show defects in remodeling and maturation, with normal physiological vessel regression inhibited. Ang-2 binding to Tie2 does not result in receptor phosphorylation, and overexpression of Ang2 inhibits Tie2 signaling. This suggests that Ang-2 is a direct antagonist of Ang-1 and counteracts its effects on vessel stability. This has led to the concept of the balance between Ang-1 and Ang-2 acting as a switch between EC activation and vessel stability (see **Fig. 5**).Thus, constitutive Ang-1/Tie2 signaling would be expected to maintain the vasculature in a quiescent state, with Ang-2 signaling in response to pro-angiogenic signals allowing endothelial activation and sprouting. The removal of pro-angiogenic stimuli or accumulation of anti-angiogenic factors would reverse the balance, resulting in endothelial quiescence and stabilization of the newly-formed vasculature. Ang-2 is also required for lymphatic development. The role of Tie1 is unclear as it has no identified ligands but it is known to interact with Tie2, and its depletion from the endothelium of adult mice has been recently shown to inhibit tumor angiogenesis and growth by decreasing EC survival in tumor vessels without affecting normal vasculature.

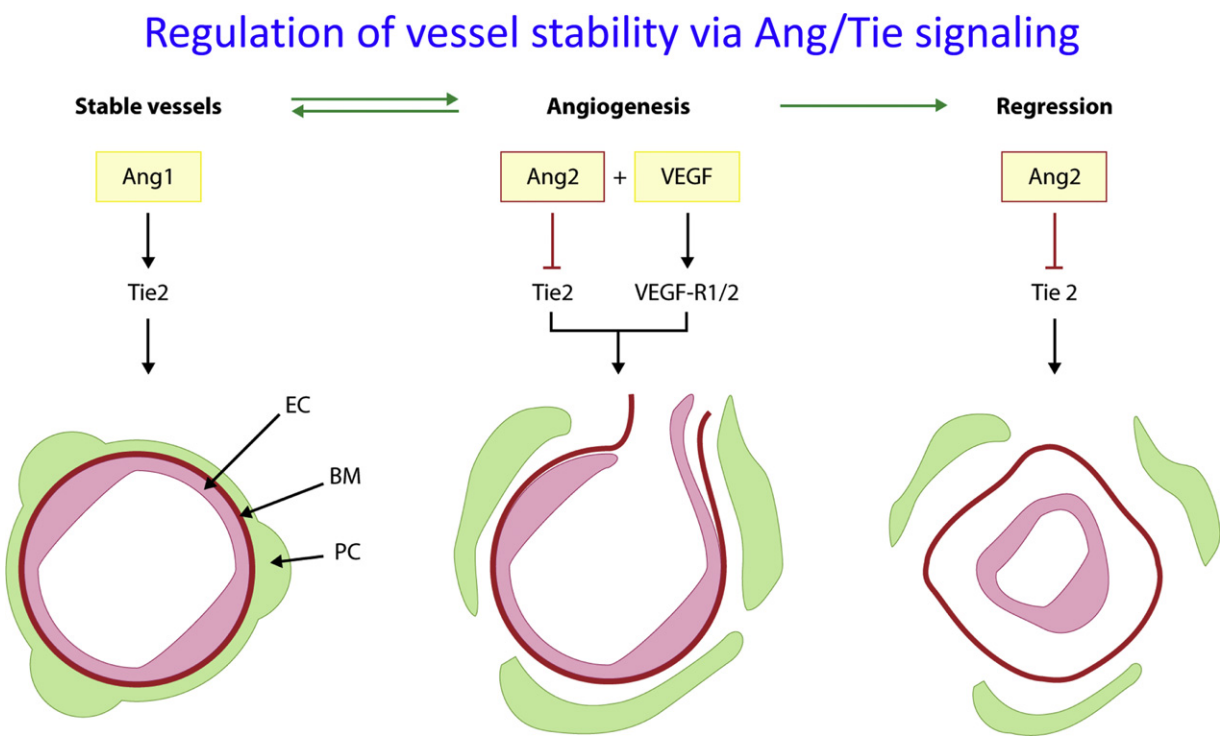

Fig. 5 Regulation of vessel stability by Ang/Tie signaling. In mature vessels Ang1 binds to Tie2 promoting recruitment and maintaining association of peri-endothelial support cells, thus stabilizing newly formed vessels. During initial stages of angiogenesis: Ang-2 binds Tie2 and blocks its activation, vessel structures become loosened, reducing EC contacts with matrix and peri-endothelial support cells, making the ECs more accessible and responsive to VEGF. VEGF binding to VEGF-R2 induces EC proliferation. Loosening of cell-matrix interactions via Ang-2/Tie2 binding in the absence of a growth or survival signals elicits endothelial cell death, likely by apoptosis, and therefore vessel regression.

The modulation of Ang/Tie signaling is of interest for therapeutic benefit. A number of drugs targeting Ang/Tie signaling, including monoclonal antibodies, peptibodies and aptamers against Ang1/Ang2 are currently in development or clinical trials, as well as tyrosine kinase inhibitors targeting Tie2.

Notch

The Delta/JaggedNotch signaling pathway is a conserved pathway regulating cell fate and differentiation, and includes four receptors (Notch 1–4), and five transmembrane ligands (Jagged1, Jagged2, Delta-like ligands (DLL) 1–4. The Receptor and ligands are tethered to the cell surface. Notch signaling enables cell-cell communication in multicellular organisms, and acts to influence cell proliferation, differentiation and apoptosis. In recent years, interest has focused particularly on the role of Dll4 interaction with Notch1 in angiogenesis. Dll4 expression is restricted to ECS and, similarly to VEGF, there is a dose-dependent requirement for Dll4 expression for vascular development. Expression of Dll4 is upregulated by VEGF, and strong in tumor vasculature compared to normal vessels. Surprisingly, despite its apparently clear pro-angiogenic role, a reduction in Dll4 signaling results in increased vessel density but shrinkage in tumor volume. Dll4-Notch1 signaling plays a role in specifying the phenotype of cells within growing sprouts, and therefore the number of branching sprouts formed. Dll4 is expressed on non-proliferative, highly motile 'tip' cells, and interacts with Notch1 on adjacent EC. This interaction results in repression of the 'tip' phenotype in these cells which take on a highly proliferative 'stalk' phenotype. Inhibition of Dll3 signaling therefore results in an expansion in the number of cells exhibiting the 'tip' phenotype, leading to increased sprout formation and the production of non-functional vasculature. The concept of 'abnormalization' of tumor vasculature is hoped to lead to the development of novel therapeutic agents to control pathological angiogenesis.

Pre-clinical data suggests that combining agents blocking VEGF and Dll4 is more effective than either drug used alone. Importantly, recent data also suggests that tumors which have become resistant to VEGF blockade remain sensitive to anti-DLL4 agents. A variety of antibodies targeting DLL4 are currently in clinical development.

Ephrins

The Ephs are a large family of receptor tyrosine kinases divided into classes A and B. They interact with their ligands, the ephrins, also divided into class A and B. Like Notch signaling, both Eph receptors and ephrin ligands are anchored to the cell surface,

requiring close proximity of cells for signaling to occur. A unique feature of ephrin signaling is that it is bi-directional, with both receptor and ligand activitiy signaling in the cells that bear them (known as 'forward' and 'reverse' signaling, respectively. The Eph/ephrin signaling mediates 'repulsive' roles, where receptor and ligand interaction results in repulsion of the receptor-bearing cell.

In the vasculature, Eph/ephrin signaling plays a variety of roles, including modulation of cell proliferation, migration, and cell-cell and cell-matrix interactions. Interest has focused on ephrin A2, which is overexpressed in multiple cancers, mediates VEGF-induced EC migration, and has been linked with vasculogenic mimicry. This is a process by which tumor cells form channels which 'mimic' blood vessels and is one means by which tumors become resistant to anti-angiogenic therapies. The monoclonal antibody EA5 targeting ephrin2A is in pre-clinical evaluation.

The interaction of EphB4 with its ligand ephrinB2 is also of interest, as both genes are essential for angiogenic remodeling and embryonic survival. EphB4/ephrinB2 interactions are important for the development of the vascular wall, since ephrinB2-defiicient pericytes fail to attach correctly to the nascent vessel. Furthermore, soluble ephrinB2 facilitates adhesion and migration of endothelial cells in culture, and blockade of EphB4 causes inhibition of angiogenesis in several pre-clinical models. EphB4/ephrinB3 are also linked to the development and patterning of the lymphatic vasculature. EphrinB2 is strongly expressed in the ECs of lymphatic collecting vessels, and mice engineered to express a mutant version of ephrinB2 showed disturbed maturation and patterning of the lymphatic vasculature. JI-101, a triple inhibitor of VEGFR2, PDGFRβ and EphB4 has entered early clinical evaluation.

Proteases

Several major classes of proteases have been identified as playing important roles in the processes that regulate angiogenesis. These include the Matrix Metalloproteases (MMPs), ADAMs (A Distintegrin And Metalloprotease domain), the related ADAMTSs (A Disintegrin And Metalloprotease with ThromboSpondin motifs), serine proteases, and some members of the cysteine cathepsins.

The primary role of proteases in angiogenesis is in remodeling of the extracellular matrix. For EC to exit the parent vessel, the vascular basement membrane must be degraded. Migration of activated EC into the interstitial ECM to form the new sprout also relies on the coordinated activity of proteases to digest the ECM, providing space for migration, as well as regulating adhesion and de-adhesion in the process of migration itself. While matrix remodeling is thus necessary for sprout formation to occur, this process must be tightly regulated. Insufficient proteolysis inhibits invasion of EC, however excessive matrix degradation results in collapse of the matrix scaffold, and prevents adhesion of EC, leading to EC death and vessel regression. The MMP family have distinct but overlapping substrate specificities, and collectively they can cleave virtually all components of the ECM. Furthermore, inhibition of MMP activity using broad spectrum inhibitors is able to completely block endothelial tube formation in *in vitro* and *ex vivo* angiogenesis models. This process is particularly dependent on a subset of cell-surface membrane-type (MT)-MMPs, particularly MT1-MMP. In vivo, proteolytic activity of MMPs, ADAMs and ADAMTSs is regulated by the Tissue Inhibitor of Metalloproteases (TIMPs).

Proteolysis contributes to angiogenesis via numerous mechanisms beyond simply providing spave for the new vessel to form. Partial proteolysis of native collagen during remodeling of the ECM results in exposure of cryptic sites, regulating processes such as adhesion and migration. Proteolytic activity also liberates growth factors such as VEGF, bFGF, tumor necrosis factor-α (TNFα), and insulin-like growth factor-1 (IGF-1) previously bound to the cell membrane or sequestered within the ECM. For example, MMP-9 was shown to activate the angiogenic switch in the Rip1Tag1 model of mouse carcinogenesis via the release of membrane-bound VEGF stores, in the absence of increased expression of either VEGF or its receptors.

Proteases and their inhibitors can also have anti-angiogenic activity. For example there is evidence that Plasminogen Activator Inhibitor-1 (PAI-1) is anti-angiogenic dependent on its local concentration. Certain ADAMTS including ADAMTS-1 and -8 also have anti-angiogenic roles, potentially by binding VEGF and also via the release of anti-angiogenic cleavage products of thrombospondins-1 and -2.

Integrins

Given their crucial role in communication between cells and their environment, it is unsurprising that several integrins are known to play roles in several aspects of vascular biology. A large body of evidence from both in vitro and in vivo studies has revealed roles for various integrins in angiogenesis including EC activation, survival and migration, ECM remodeling, vascular lumen formation, network remodeling via induction of cell death, vessel maturation and mediation of interactions between endothelial and perivascular cells.

Much attention has been focused on the role of $\alpha v\beta 3$ and $\alpha v\beta 5$, due to convincing evidence implicating both integrins in angiogenesis. Their inhibition blocks angiogenesis in a range of in vitro and in vivo angiogenesis models. However, generation of mice genetically engineered to lack either or both of these integrins show extensive developmental angiogenesis, suggesting that neither is required for vascular development. Furthermore, mice lacking $\alpha v\beta 3$ show enhanced tumor angiogenesis. These conflicting data suggest that $\alpha v\beta 3$ and $\alpha v\beta 5$ may have roles in negative regulation of angiogenesis. These may include binding of anti-angiogenic factors such as thrombospondin and angiostatin, mediating integrin-mediated cell death (IMD), in which presence of unligated $\alpha v\beta 3$ induces apoptosis in EC (even during an adherent state), or by negatively regulating activity of pro-angiogenic integrins or signaling cascades.

The role of integrins in the process of lymphangiogenesis is still a relatively unstudied area. Some integrins involved in angiogenesis are known to be expressed on lymphatic vasculature and some (e.g. $\alpha1\beta1$, $\alpha2\beta1$, $\alpha5\beta1$ and $\alpha4\beta1$) are known to be upregulated during pathological lymphangiogenesis. Possibly the most interesting data concerns $\alpha9\beta1$, which is expressed on lymphatic vasculature endothelium and absent from the blood vasculature. Mice deficient in $\alpha9$ survive until birth but die due to lymphatic defects 6–12 days later, suggesting an important role for this integrin in lymphangiogenesis.

Several integrin inhibitors are either in clinical use or are currently in clinical trials. These take the form of therapeutic antibodies, ligand mimetic peptides, or small molecule antagonists. Integrin inhibitors in the clinic target the integrin $\alpha4$ and platelet integrin $\alpha IIb\beta3$, with inhibitors in clinical trials targeting the RGD-binding integrins $\alpha v\beta1$, $\alpha v\beta3$, $\alpha v\beta5$ and $\alpha IIb\beta3$. Of these, the $\alpha v\beta3$/ $\alpha v\beta5$ inhibitor cilengitide, currently being tested in phase III clinical trials for glioblastoma, is the closest to approval.

Conclusion

Angiogenesis and lymphangiogenesis are fundamental mechanisms in embryonic and postnatal development which also play major roles in pathologies such as cancer, ocular neovascular disease, ischemic disease, and chronic inflammatory disease. Many molecules, receptors and intracellular signaling molecules have been implicated in vascular morphogenesis. These discoveries have offered novel opportunities for therapeutic intervention. There is an increasing repertoire of drugs with which to manipulate angiogenesis and new endothelial-specific genes with which to target the vasculature. Thus, angiogenesis research is at an exciting stage.

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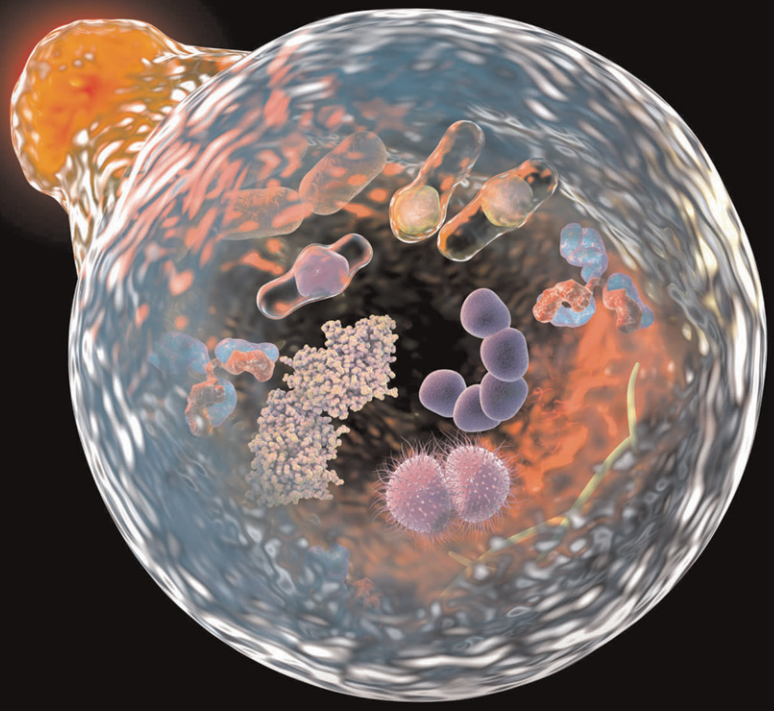

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DEDICATION

Professor Luciano Martini, 1927–2017

The other Editor in Chief of the Encyclopedia, Professor Luciano Martini, passed away on July 13th, 2017. He was an internationally acclaimed authority in the field of endocrinology, in particular neuroendocrinology, a brilliant and imaginative scientist, and an impressive and erudite scholar.

Luciano achieved the venerable age of 90, and his long career was full of outstanding scientific achievements, leadership positions in academia and in scientific societies, academies, and committees.

Luciano received his MD degree from the University of Milan in 1950. He then rapidly progressed through junior academic ranks up to the position of Professor and Chairman of the Department of Pharmacology at the University of Perugia in 1968, and subsequently, in 1972, he returned to his *alma mater*, the University of Milan, as full Professor and Chairman of the Department of Endocrinology, a post he held until 2001. He served in Milan as director of the training and research program entitled Physiology of Reproduction for nearly 20 years and attracted to his team top-class Italian and foreign scientists to address his main research interests of neuroendocrine regulation of reproductive functions.

Scientific severity, ethical integrity, fine perception, and deep farsightedness describe best Luciano's character as a scientist. He created in his institute a scientific research group devoted to experimental endocrinology, which grew over the years in size and visibility and became widely recognized internationally. Luciano published more than 400 peer-reviewed and highly cited papers mainly in the fields of neuroendocrinology, endocrine oncology, physiology of reproduction, and steroid and energy metabolisms.

Luciano was a prolific editor of scientific books and journals, which include the two volumes of *Neuroendocrinology* and the nine biennial volumes of *Frontiers in Neuroendocrinology*. He was Editor in Chief of *Comprehensive Endocrinology* published in 12 volumes and the first Edition of *Encyclopedia of Endocrine Diseases*. He served as President in many national and international scientific societies including the International Society of Neuroendocrinology, the Italian Society of Endocrinology, the International Society of Endocrinology, and the European Federation of Endocrine Societies. For his scientific achievements Luciano received honorary doctorates in the universities of Liège, Santiago de Compostela, Pécs, and Milan, and he was the recipient of numerous scientific awards and invited academy memberships.

Luciano's portrait could not be complete if one forgets to mention his life-time passion for music. He was a well-trained and accomplished pianist, a passionate music listener, and an enthusiastic connoisseur of all types of music. He also was an amateur in visual arts and deeply interested in history.

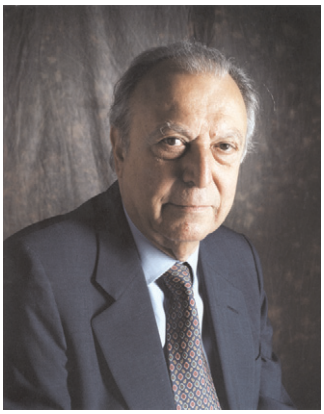
All of us who knew Professor Luciano Martini deeply mourn the loss of a great scientist and friend, the real "Il Maestro", teacher, colleague, and pioneer of modern neuroendocrinology. I trust Luciano would have been proud of this new edition of the Encyclopedia of Endocrine Diseases, and all of us having worked on its production would like to dedicate it to his memory.

Ilpo Huhtaniemi

Editor in Chief
Encyclopedia of Endocrine Diseases, 2nd edition

EDITORS IN CHIEF

Ilpo Huhtaniemi received his MD and PhD at University of Helsinki, Finland, did postdoctoral training in United States (UC San Francisco and NIH, Bethesda), and has been on sabbatical leave in Germany, United States and Scotland. In 1986–2002 he held the post of Professor and Chairman of Physiology at University of Turku, Finland. He moved in 2002 to UK to a Chair in Reproductive Endocrinology at Imperial College London, from which position he retired in 2015. He has received several national and international honors, amongst them a fellowship of The Academy of Medical Sciences, United Kingdom, and a Doctor Honoris Causa at the Medical University Łódź, Poland, and University of Szeged, Hungary. He was the Chief Managing Editor of *Molecular and Cellular Endocrinology* 1999-2017, has served in the Editorial Board of *Endocrinology and Endocrine Reviews* and is/has been the Editor or Editorial Board Member of several other scientific journals (e.g., *European Journal of Endocrinology*, *Clinical Endocrinology*, *Human Reproduction Update*, *Journal of Endocrinology*, *Molecular Human Reproduction*, *Reproduction*, *Asian Journal of Andrology*). He has extensive experience as Official of international scientific organizations (e.g., Past President of International Society of Andrology). His research interests include clinical and basic reproductive endocrinology, in particular the function of gonadotrophins and male reproductive endocrinology. He also has long-term interests in development of male contraception, hormone-dependent cancer, and the endocrinology of aging. He has authored about 700 peer-reviewed research articles and reviews, and his H-factor is 78.



Luciano Martini was born on May 14, 1927, in Milan, Italy. He obtained the degree of Medical Doctor "summa cum laude" on November 24, 1950, from the Faculty of Medicine of the University of Milan, Italy. He was Emeritus Professor of Pharmacology of the University of Perugia, Italy, and Emeritus Professor of Endocrinology of the University of Milan, Italy. He was Doctor Honoris Causa in Medicine of the Universities of Liège, Belgium, Santiago de Compostela, Spain, and Pécs, Hungary, and Doctor Honoris Causa in Biotechnological Sciences of the University of Milan, Italy. He was an author of more than 400 peer-reviewed scientific publications in the fields of endocrinology, neuroendocrinology, pharmacology, physiology of reproduction, steroid biochemistry, and basic oncology. He was elected member of the Accademia Nazionale dei Lincei (Italian National Academy) and of the American Academy of Arts and Sciences (Honorary Foreign Member).

Luciano Martini acted as Editor in Chief of the journal *Frontiers in Neuroendocrinology* from 1990 to 2001, and was a Member of the Editorial Board of *Endocrinology* (Foreign Consulting Editor, 1961–65), as well as of several other speciality journals, such as *Experimental and Clinical Endocrinology*, *Biochemistry*, and *Steroids*. He has acted as Editor of several textbooks (e.g., *Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1966–67, and *Clinical Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1977–82) as well of a series of books under the name *Comprehensive Endocrinology* (13 volumes), Raven Press, New York, 1979–84. He acted as Editor in Chief for the first edition of *Encyclopedia of Endocrine Diseases* (4 volumes), Academic Press-Elsevier, San Diego, 2004.

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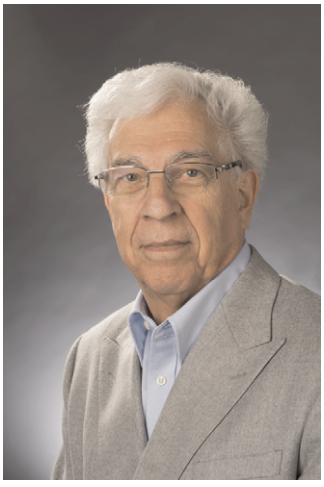
Professor **Jean-Jacques Body** has been trained as an endocrinologist and a medical oncologist. He was Head of the Department of Medicine at University Hospital Brugmann in Brussels and Full Professor of Medicine (Internal Medicine) at the Free University of Brussels, (ULB), Brussels, Belgium. He was previously Head of the Internal Medicine Clinic at Institute J. Bordet (Cancer Center of ULB). He has also developed the "Supportive Care Dept" at the same Institute. His particular research interests are osteoporosis and bone metastases. He has a long-standing interest for bone metabolism and turnover in osteoporosis and tumor bone diseases. He has authored or co-authored more than 250 international peer-reviewed papers and he counts more than 200 invited lectures for international meetings.

Felipe F. Casanueva is Professor of Medicine at University of Santiago de Compostela and Head of Department of Endocrinology and Nutrition at University Hospital Santiago. He has been President of the scientific societes, such as: European Federation of Endocrine Societies (EFES), The Pituitary Society, International Society of Endocrinology (ISE) and, Sociedad Española para el Estudio de la Obesidad (SEEDO). Has written more than 50 chapters in international books and published more than 700 papers in international journals. He has received several awards for research at national and international level, such as: Rey Jaime I to the Medical Research, Geoffrey Harris Prize in Neuroendocrinology, Fundación Lilly of Bio-medical Research Clinic, Fundacion Danone – Professional Career – Dr Carlos Martí Hennberg, European Hormone Medal by the European Society for Endocrinology (ESE); he has been named Honorary Doctorate in Medicine of the University of Łódź, Erciyes, and Belgrade, and Honorary Member of the European Society of Endocrinology.



Dr. **Jean-Louis Chiasson** is currently Full Professor of Medicine at the University of Montreal. He is Head of the Research Group on Diabetes and Metabolic Regulation at the Research Center of the Centre hospitalier de l'Université de Montréal (CRCHUM).

Dr. Chiasson obtained his MD at Laval University in Quebec City in 1967. He did his specialty training in Internal Medicine at Laval Univesity and in Endocrinology at McGill University. He then did a research Fellowship in Diabetes at Vanderbilt University in Nashville, Tennessee. In 1974–76 and 1978–80, he was appointed Assistant Professor in the Department of Medicine and Physiology respectively at Vanderbilt University. In 1980, he returned to Montreal as Assistant Professor in the Department of Medicine at the University of Montreal and as Endocrinologist at Hotel-Dieu Hospital, now merged into the Centre hospitalier de l'Université de Montréal.

Dr. Chiasson's research interests include the regulation of carbohydrate metabolism in health and diabetes, as well as the development and evaluation of new strategies for the treatment and prevention of diabetes and its vascular complications. He has contributed over 250 scientific publications and lectures nationally and internationally on various topics on diabetes mellitus, its pathogenesis, its treatment, and its prevention. His scientific contribution puts him in the prestigious club of the 100 most cited publications in the world in the field of diabetes.

Sophie Christin-Maitre received her MD at University of Paris XI and her PhD at University Paris VI, Pierre and Marie Curie, France. She did a postdoctoral training in United States (Massachusetts General Hospital, Harvard University, Boston); she specialized in reproductive medicine. She holds the post of Professor of Endocrinology at University of Sorbonne, Paris, France. She has been the head of the Adult Endocrine Unit, in Hôpital Saint-Antoine, Assistance-Publique Hôpitaux de Paris, since 2011. She is a member of the INSERM research unit UMR S_933, specialized in identifying new genes in reproductive disorders. Her interests include clinical and basic reproductive endocrinology, in particular the management of patients with Turner syndrome, patients with primary ovarian insufficiency, patients with hypogonadisms, and patients with abnormalities of sex development. She has authored approximately 150 peer-reviewed research articles and reviews.

Ulla Feldt-Rasmussen is Professor at Copenhagen University and Chief of Medical Endocrinology, National University Hospital. Her research interests involve the thyroid gland and autoimmunity, as well as pituitary and adrenal dysfunction.

She has published more than 410 papers in peer-reviewed journals on e.g., thyroid hormones and body composition, thyroid autoimmunity and cancer, cytokines as regulators of endocrine cells, influence of thyroid disrupting chemicals on thyroid cells, growth hormone deficiency related to body composition, bone metabolism and other pituitary axes, and transition from adolescent to adult care, as well as several aspects of Fabry disease. In recent years her group has embarked on studies on pituitary function after traumatic brain injury in a nationwide setting, and focusing on diagnostic accuracy of pituitary testing procedures. She has further authored numerous proceedings, textbook chapters, and other publications; as well as organized numerous international meetings and postgraduate courses, and has led several European projects and other collaborations within many areas of endocrinology.

Professor Feldt-Rasmussen reviews for international journals, and is an editorial board member of several endocrine journals. She belongs to many international professional organizations, including the Endocrine Society, ETA, ATA, ENEA, and GRS; she has served as Secretary-Treasurer of ETA and as President of the ETA Cancer Research Network.

Professor Feldt-Rasmussen serves on the advisory boards of several ad hoc endocrine committees, and has received many prestigious prizes including the Mayo Clinic's Haynes Lecturer's Award and ETA's Pinchera Research Prize.

Wouter W. de Herder M.D. Ph.D. (1960) is Professor of Endocrine Oncology at the Erasmus MC in Rotterdam, the Netherlands. In this University Hospital he is chairman of a multidisciplinary group for endocrine oncology (tumorwerkgroep endocriene tumoren) and he is head of the ENETS centre of excellence for neuroendocrine tumors. His major research interests are neuroendocrine and endocrine tumors.

Professor de Herder received his M.D. in 1985 and his Ph.D. in 1990 from the Erasmus University in Rotterdam, the Netherlands.

He is a member of several international and Dutch national societies, such as the Dutch Society for Endocrinology (NVE), the Endocrine Society (USA), the European Society of Endocrinology (ESE), European Neuroendocrine Tumor Society (ENETS) and the North American Neuroendocrine Tumor Society (NANETS). He served as a board member of the Dutch Society for Endocrinology (NVE) (2009–14). He served as chairman (2006–08) and vice-chairman of ENETS (2008–10) (European Neuroendocrine Tumour Society). He is member of the advisory boards of ENETS and NANETS.

Professor de Herder has (co-)published over 400 peer-reviewed papers and book chapters and is a reviewer for many international journals.

He is a member of the editorial boards of *Neuroendocrinology; Endocrinology, Diabetes & Metabolism Case Reports; Clinical Endocrinology*, and *Endocrine-Related Cancer*.

Professor de Herder has given over 200 invited presentations at Dutch national and international meetings.

Ieuan Hughes is currently Emeritus Professor of Pediatrics at the University of Cambridge and Honorary Consultant Pediatrician at Cambridge University Hospitals NHS Foundation Trust and Cambridge Biomedical Campus. He is the author of more than 300 papers and chapters across the whole range of paediatric endocrinology. His particular expertise is in disorders of sex development for which he coordinated the International Consensus on the approach to the investigation and management of this broad topic. Research interests focus on steroid enzyme deficiencies and molecular mechanisms of androgen action.
Professor Hughes has served on the editorial boards of several journals, including *Clinical Endocrinology*, *Journal of Clinical Endocrinology*, and *Metabolism* and *Archives of Disease in Childhood* where he was also the Associate Editor. He is Past-Secretary and President of the European Society for Pediatric Endocrinology and a recipient of the highest award of the Society, the Andrea Prader Prize. Professor Hughes is a James Spence Medallist of the Royal College of Pediatrics and Child Health for outstanding contributions to paediatric knowledge. He is a Fellow of the Academy of Medical Sciences, a Council Member of the Learned Society of Wales and a Trustee of two charities. The chapter on Disorders of Sex Development in *Williams Textbook of Endocrinology* (now in its 14e) by Hughes and co-authors is considered to be a definitive and up to date regular review of this topic, specific and key to pediatric endocrinology.

Dr. **Gregory Kaltsas** MD FRCP (Lon) is Professor in General Medicine and Endocrinology at the National and Kapodistrian University of Athens, Greece. He was trained in General Medicine in Athens, Greece and London, UK, and in Endocrinology at the Middlesex and St Bartholomew's Hospital, London, UK. He developed a particular interest in neuroendocrinology (pituitary and neuroendocrine tumors) and adrenal physiology and diseases. Upon returning to Greece he established a neuroendocrine network and he is currently running the European Neuroendocrine Tumor Society (ENETS) Center of Excellence at Laiko Hospital in Athens, Greece. He has served as a member of the advisory board of ENETS and of the Executive Committee of the European Neuroendocrine Association (ENEA) and he has been elected in the Executive Committee of the International Society of Endocrinology. He has recently been elected as a representative of the European Society of Endocrinology in the ExCo of the International Society of Endocrinology. He has published more than 300 original papers, reviews, and chapters and serves on editorial boards and as associate editor in several endocrine journals.

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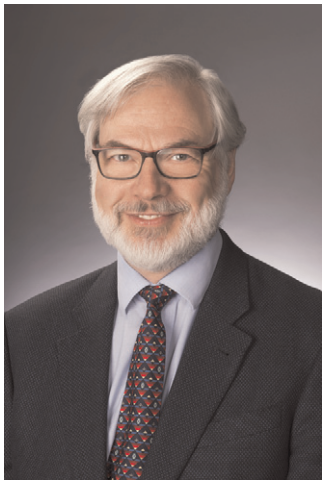

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She has authored over 300 peer-reviewed publications, 67 chapters and reviews mainly on male reproductive biology including characterization of the pharmacokinetics and efficacy of androgens in men, trials of hormonal male contraceptive, regulation of germ cell apoptosis, and reproductive aging. Dr. Wang served on the Executive Council, several committees and was the President of the American Society of Andrology (2006–07). She also served the International Society of Andrology as Secretary (2001–05) and Chair of the Program Organizing Committee (2005–09). She was President of the International Society of Andrology (2009–13). She is a member of the Research Group on Methods for the Regulation of Male Fertility of the World Health Organization since 1984 and Chairperson (1991–2002).

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PREFACE

The first Edition of the *Encyclopedia of Endocrine Diseases* was published in 2004. Because of the enormous development in the field it was found important to produce a completely revised and updated Second Edition of the Encyclopedia. The new Edition is a must-have one-stop reference covering every aspect of the physiological background, pathogenesis, clinical diagnostics, and therapeutic aspects of the wide array of endocrine and related metabolic diseases.

The functional balance of the body (homeostasis) is maintained by two regulatory circuits, i.e., the nervous and the endocrine systems. Among the most complex constructs in the body, the endocrine system comprises a group of glands that secrete hormones directly into the bloodstream, where they reach their specific receptors in other parts of the body, evoking specific intracellular signaling pathways leading to their biological effect. Many classically non-endocrine organs (e.g., the heart) have also turned out to have endocrine functions. The endocrine system maintains and regulates the body's homeostasis by using hormones to control metabolism, temperature, biological cycles, internal fluid volume, reproduction, growth, body composition, and development. The system is a marvel when functioning optimally, i.e., maintaining the body homeostasis. Unfortunately, there is a myriad of ways these processes, actions, and functions can go awry, resulting in various endocrine and metabolic diseases, which form the over-arching theme of the Encyclopedia.

The Encyclopedia is not meant as a primer on the subject of endocrinology, but instead intended to provide a comprehensive reference work on the extensive spectrum of diseases and disorders that can occur within the endocrine and metabolic system. The updated version of this groundbreaking encyclopedia is especially timely, as it covers the dramatic discoveries in the field of endocrinology and metabolism over the past 10 years, particularly with respect to novel diagnostic techniques and treatment approaches. In particular, there have been tremendous advancements in our understanding of the molecular basis of endocrine and metabolic diseases (mutations, epigenetics, signaling), as well as pathogenesis and therapy of the common forms of these diseases (e.g., diabetes, obesity, and endocrine malignancies).

The Encyclopedia offers a unique source of up-to-date information for the physicians and basic scientists working in the field. It is an essential resource for every clinician diagnosing and treating endocrine patients. The Encyclopedia also offers the prime source of information for students of medicine and science around the world, as well as basic research workers in academia, the pharma industry, and in other areas in need of information on endocrinology and metabolism. It also offers useful information for the lay public about normal and abnormal functions of hormones.

The Encyclopedia is intended to serve as a useful and comprehensive source of information spanning the many and varied aspects of the endocrine and metabolic system. The chapters have been written to be accessible to both clinical and nonclinical readers. The articles have been formatted in similar fashion and each is intended as a stand-alone presentation. Each article begins with a glossary list defining key terms that may be unfamiliar to the reader and are important for understanding the article. The body of the article begins with a brief introduction to the subject under discussion, bold headings lead the reader through the text, and figures and tables explain and illuminate most articles. The main text is followed by referenced citations to provide the reader with access to additional information on the topic, and cross-references lead the reader to related entries in the encyclopedia. The relatively short stand-alone articles have allowed us to recruit the best experts available for each topic.

Unlike the first Edition, where the articles were arranged in alphabetical order, the 2nd Edition is arranged in organ-based thematic order, where each organ-based group of diseases is presented as cluster of articles in the first four volumes. The fifth volume is a stand-alone compilation of all articles on pediatric endocrinology. The thematic organization gives the reader a better general view of the coverage of articles on a specific endocrine organ or disease type.

The Second Edition of the Encyclopedia builds of the first edition. Nevertheless, to bring a major reference work with such a broad scope from initial conception to final publication involved a great deal of planning and organization, together with the efforts of innumerable individuals. The authors of the first edition were invited to update their earlier texts. If this was not possible, the Section Editors invited another expert in the topic either to update the previous text or to write a de novo text; the latter happened in most of these cases. Hence, the Second Edition contains to a large extent totally new information, or at least the fluency of all texts has been scrutinized. Furthermore, all manuscripts have undergone peer-review arranged by the Section Editors.

Assembling a large volume of articles with the purpose to cover all essential topics of endocrine diseases posed multiple challenges. Coverage was a significant problem: on one hand some redundancy of the topics was almost impossible to avoid in places while, on the other, there were inevitable gaps. Some of these arose from late cancellations; others from oversights on our part. We can only promise to fill these gaps in future editions. We also note that as can be expected for a large multi-author compilation the individual articles do differ in detail and approach. We considered it more important to allow our experts substantial latitude in deciding how to present their topics than to apply rigid guidelines.

Most of the editing work of the Encyclopedia has been carried out by a highly competent board of 16 Section Editors, each of them internationally renowned experts in their respective field within clinical endocrinology. First, the broadest possible list of topics was compiled, aiming at the best possible coverage. Throughout the editorial process, the Section Editors supervised their subject area of expertise, recommended and corresponded with fellow editors and article contributors, reviewed the manuscripts, and continuously helped to refine the final list of topics. This has made the task of the Editor in Chief easy, mainly entailing the supervision of smooth progress of the project.

The Section Editors and their fields deserve being listed here: *Jean-Jacques Body* (Belgium, bone endocrinology), *Felipe F. Casanueva* (Spain, metabolism and obesity), *Richard N. Clayton* (United Kingdom, pituitary gland), *Jean-Louis Chiasson* (Canada, diabetes), *Sophie Christin-Maitre* (France, female reproduction), *Wouter W. de Herder* (The Netherlands, neuroendocrinology), *Ulla Feldt-Rasmussen* (Denmark, thyroid gland), *Ieuan Hughes* (United Kingdom, pediatric endocrinology), *Gregory Kaltsas*, Greece, and *Martin O. Weickert*, United Kingdom, (gastrointestinal hormones), *Jean-Marc Kaufman* (Belgium, endocrinology of aging), *André Lacroix* (Canada, adrenal cortex), *Franco Mantero* (Italy, adrenal medulla and endocrine hypertension), *Jorma Toppari* (Finland, endocrine disruptors), *Jacquetta Trasler* (Canada, endocrine epigenetics) and *Christina Wang* (United Kingdom, male reproduction).

The Elsevier editorial staff, *Will Smaldon*, *Laura Escalante Santos*, and *Kate Miklaszewska-Gorczyca*, have been of enormous help to the editors at every step during this long project. I admire the professionalism of everyone and am deeply indebted to all for their dedication and hard work to make the Encyclopedia the leading reference book of clinical endocrinology.

The authors of the individual chapters, more than 450 in total, were specifically selected by the Section Editors to represent the best available knowledge on the topic available. They all should be thanked for their dedication and the excellent quality of their contributions.

Ilpo T. Huhtaniemi
Editor in Chief

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Bone Cells: Osteoblast/Osteoclast/Osteocyte

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Glossary

Bone marrow mesenchymal stem cells (BMMSCs) Pluripotent cells isolated from the bone marrow, which are able to proliferate and self-renew, as well as to give rise to progeny of the osteogenic, chondrogenic, and adipogenic lineages.

Bone remodeling Physiological process by which bone maintains a dynamic steady state through sequential resorption and formation of bone at the same site by osteoclasts and osteoblasts.

Cleidocranial dysplasia (CCD) (*Alias* cleidocranial dysostosis) rare autosomal dominant disease characterized by hypoplastic or absent clavicles, dental anomalies, and delayed skeletal development. It is caused by a mutation of the 6p21gene coding for runt-related transcription factor (Runx)2.

Endosteal niche (*Alias* osteoblast niche) niche present in the endosteal part of the bone marrow. It includes osteoblast-like cells, called spindle-shaped N-cadherin$^+$/CD45$^-$ osteoblasts (SNOs) through which it regulates quiescence of long-term (LT)-hematopoietic stem cells (HSCs).

Hematopoietic stem cells (HSCs) Multipotent, self-renewing progenitor cells which give rise to all differentiated blood cells. HSCs can be found in bone marrow, peripheral blood, and umbilical cord blood.

High bone mass disease Rare genetic disease characterized by mandible enlargement and increased bone mass. It is caused by dominant missense mutations of the gene coding for the low density lipoprotein (LDL) receptor-related protein 5 (LRP5).

Matrix vesicles Membrane-enclosed vesicles secreted by odontoblasts, osteoblasts, and chondrocytes which serve as nucleation centers for the mineralization of calcified cartilage, bone, and dentin.

Osteoporosis pseudoglioma syndrome Autosomal recessive disease characterized by severe thinning of the bones (osteoporosis) and eye abnormalities that lead to vision loss. It is caused by an inactivating mutation of the gene coding for the low density lipoprotein (LDL) receptor-related protein 5 (LRP5).

Sclerosteosis Autosomal recessive disease characterized by bone overgrowth, sometimes associated with syndactyly. It is caused by inactivating mutation in the sclerostin (Sost) gene.

van Buchem disease Autosomal recessive bone dysplasia characterized by otosclerosis and thickening of skull, face, and trunk bones, also associated to facial paralysis and sight or hearing loss. It is caused by inactivating mutation in the sclerostin (Sost) gene.

Osteoblasts: Not Just Bone Building Cells

Osteoblastogenesis

Bone marrow mesenchymal stem cells are spindle-shaped nonhematopoietic stem cells accounting for 0.001%–0.01% of the nucleated fraction in the bone marrow (Pittenger *et al.*, 1999) and representing a reservoir of many progenitors of connective tissue cells, among them the osteoblasts.

One of the earliest steps in osteoblast differentiation is the commitment of MSCs toward an osteo/chondroprogenitor, which is expected to express the following markers: Runt-related transcription factor 2 (Runx2) and its downstream regulated genes Osterix (Osx) and Distal-less homeobox 5 (Dlx5). The role of Runx2 (*alias* core binding factor alpha 1, cbfa1, and osteoblast specific factor 2, Osf2) in osteogenesis is undisputed, since this transcription factor drives the expression of the principal osteoblast-specific genes (Ducy *et al.*, 1997). Consistently, missense mutations in the Runx2 gene cause cleidocranial dysplasia, an autosomal dominant disease characterized by hypoplastic or absent clavicles, dental anomalies, and delayed skeletal development (Lee *et al.*, 1997).

Runx2 is a direct target of Wnt/β-catenin pathway and, in turn, promotes the transcriptional expression of the osteoblast marker ALP and collagen I (Col1A1), bone sialoprotein (BSP) and BGLAP, that is the gene coding for osteocalcin (OCN). Another gene whose transcription is driven by Runx2 is Osx, also known as Sp7, which further pushes the osteo/chondroprogenitor toward the osteoblast lineage by promoting the expression of SATB2 (Tang *et al.*, 2011). This is a transcription factor belonging to the family of special AT-rich binding (Satb), whose haploinsufficiency causes craniofacial defects in humans (Britanova *et al.*, 2006). SATB2 interacts with both Runx2 and activating transcription factor 4, another crucial transcription factor as demonstrated by the fact that its knock down significantly impairs bone formation (Yang *et al.*, 2004). All these three factors cooperate to drive osteoblastogenesis.

Once formed, osteoprogenitors undergo a phase of proliferation; then they become preosteoblasts, expressing high amounts of ALP, which eventually differentiate into mature osteoblasts. These are very active cells producing bone matrix proteins, among which OCN is considered a late marker of osteoblast differentiation, while at this stage ALP expression progressively decreases (**Fig. 1**). In histological

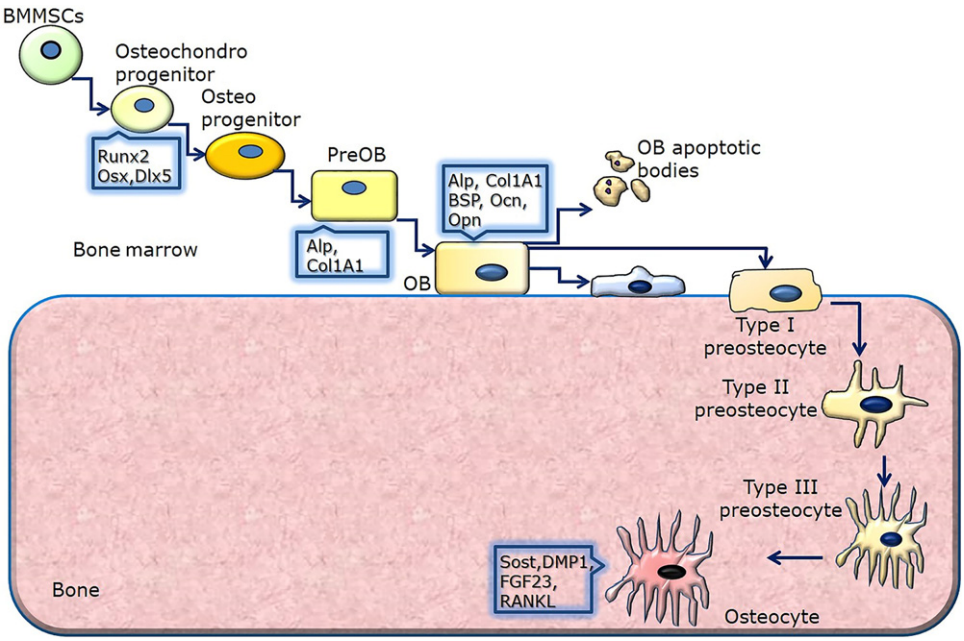

Fig. 1 Schematic representation of the multistep process of osteoblast (OB) and osteocyte differentiation. *BMMSCs*, bone marrow mesenchymal stem cells.

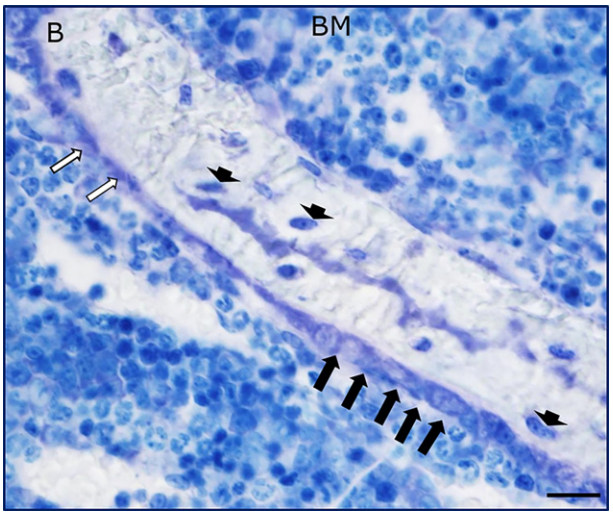

Fig. 2 Histological section of a mouse tibia, stained with toluidine blue. *Black arrows* indicate a row of osteoblasts on a bone trabecula, *white arrows* indicate bone-lining cells, *arrowheads* show the osteocytes. *B*, bone; *BM*, bone marrow; bar = 15 μm.

sections, active osteoblasts usually appear as large cuboidal cells arranged in single layer along the bone surface (**Fig. 2**, black arrows). They also present with cytoplasmic processes toward the bone matrix, which allow the contact with osteocytes.

We now have quite a complete picture of the main pathways driving osteoblastogenesis, which include Wnt and bone morphogenetic proteins (BMPs). The former is a family of at least 19 glycoproteins, which accounts for a broad range of functions in development, cell growth, and differentiation, while an aberrant activation of the Wnt pathway is causative of many cancers. In bone, the importance of the Wnt pathway came to light by studying two bone diseases, opposite in their phenotype, but both caused by a dysregulation of Wnt signaling: the osteoporosis pseudoglioma syndrome, due to an inactivating mutation of the Wnt co-receptor Lrp5, and the high bone mass syndrome, characterized by a gain-of-function mutation of the same gene (Gong *et al.*, 2001; Boyden *et al.*, 2002).

To transduce the signal, Wnt proteins interact with seven different G-coupled receptors belonging to the frizzled (Fzd) family and the Lrp5/Lrp6 coreceptors. Interaction of Wnt ligands with the tyrosine kinases Ryk and Ror as coreceptors has also been observed (Lu *et al.*, 2004). Two main intracellular pathways are triggered by Wnt, known as canonical or noncanonical. The former

relies on the stabilization of the β-catenin, which in turn translocates into the nucleus and activates the lymphoid-enhancing factor/T cell factor (Lef/Tcf) transcription factor. In particular, when Lrp5/6 and Fzd are not engaged by Wnt, the glycogen synthase kinase 3 (GSK3)-β and Ck1 serine kinases form the β-catenin destruction complex along with axin and the adenomatous polyposis of colon (APC) oncosuppressor protein. In this way, GSK3-β phosphorylates the β-catenin creating a docking site for the F-box-containing protein E3 ubiquitin ligase b-TrCP, eventually leading to its ubiquitination and subsequent proteasomal degradation (Aberle *et al.*, 1997). The interaction of Wnt with its receptor Fzd induces phosphorylation of the LRP5/6 receptor, which allows the destruction complex to be sequestered into multivesicular endosomes, thus allowing stabilization and accumulation of the β-catenin (Taelman *et al.*, 2010).

The alternative signaling pathway of Wnt, not involving β-catenin, depends on the G-protein-coupled function of the Fzd receptor, which in turn activates Rho and Rac GTPase and the downstream effectors JNK and p38 (Tu *et al.*, 2007). In osteoblasts, this pathway promotes their differentiation and is triggered by Wnt3a (Tu *et al.*, 2007). A Wnt/Ca^{2+}-dependent pathway has also been described, which requires phospholipase C (PLC) engagement, eventually leading to increased intracellular Ca^{2+} and activation of protein kinase C and calcium calmodulin-dependent kinase 2 (Sheldahl *et al.*, 1999).

Commitment of MSCs toward an osteoblast progenitor is driven by Wnt3a and Wnt10b which at the same time also inhibit adipogenic differentiation (Bennett *et al.*, 2007). The role of Wnt10b in osteogenesis has also been confirmed in transgenic mouse models overexpressing or knocked down for this protein (Bennett *et al.*, 2007; Stevens *et al.*, 2010).

Several Wnt antagonists affecting bone homeostasis have been identified, such as sclerostin (Sost), a molecule mainly produced by osteocytes, which inhibits Wnt/β-catenin pathway by binding to Lrp5 (van Bezooijen *et al.*, 2004). Two pathologies characterized by bone overgrowth, van Buchem syndrome and sclerosteosis, are due to inactivating mutations in the SOST gene (Brunkow *et al.*, 2001; Balemans *et al.*, 2002). Moreover, intermittent treatment with PTH inhibits Mef2c activity, eventually leading to a reduced expression of Sost (Koeller and Kneissel, 2005).

Other molecules counteracting the Wnt action are the Groucho/TLE family members, which are transcriptional corepressors that inhibit Wnt target gene transcription by competing with β-catenin for TCF/LEF binding (Daniels and Weis, 2005).

The second pivotal pathway for osteoblast differentiation belongs to the transforming growth factor beta-bone morphogenetic proteins (TGFβ-BMPs) family, which includes at least 30 members involved in several functions, such as embryonic development, cell proliferation, and differentiation. BMPs are mainly involved in the earliest phases of osteoblast differentiation, favoring the commitment of bone marrow MSCs toward an osteo/chondroprogenitor (Chen *et al.*, 2012). Osteoblast conditional knockout for BMP-2 and BMP-4 significantly affects osteogenesis (Bandyopadhyay *et al.*, 2006). Another BMP involved in osteoblast differentiation is the -7 isoform, which increases ALP activity in MSCs and nodule formation (Shen *et al.*, 2010).

Osteoblast differentiation is also regulated by fibroblast growth factors (FGFs), a family of 23 members, which are also involved in adipogenic differentiation. FGF-2, -4 and -8 increase Runx2 expression in MSCs. FGF-2, -9, and -18 also promote ALP activity and bone mineralization (Woei *et al.*, 2007).

Recent findings point out a role for microRNAs (miRNAs) in osteoblast homeostasis (van der Eerden, 2014). Indeed, a proadipogenic effect at the expense of osteoblastogenesis has been observed for miR-204 and miR-637, by targeting Runx2 and osterix, respectively (Huang *et al.*, 2010; Zhang *et al.*, 2011). With regard to miRNAs stimulating osteoblast differentiation, worth mentioning are miR194, miR216a, targeting c-Cbl, and miR26-a, targeting GSK3-β (Jeong *et al.*, 2014; Huang *et al.*, 2017; Li *et al.*, 2013). However, findings in this field are increasing more and more, broadening the picture of the regulation of osteoblast differentiation.

Osteoblast Functions

Bone matrix deposition and mineralization are the well-known functions of osteoblasts, which secrete collagen type I and noncollagenous proteins. The latter include osteopontin (OPN), osteonectin, BSP, dentin sialoprotein, and dentin matrix protein 1 (DMP1), all belonging to the small integrin binding ligand N-linked glycoproteins family (Fisher and Fedarko, 2003) and OCN.

The organic matrix, called osteoid, is destined to undergo mineralization, a process starting when local Ca^{2+} and phosphate (PO_4^{3-}) ions reach levels high enough to precipitate, leading to the formation of hydroxyapatite crystals. The process of bone mineralization occurs by means of the matrix vesicles. These are extracellular membrane-layered vesicles enriched in alkaline phosphatases and acidic phospholipids, which are secreted by chondrocytes and osteoblasts to shuttle hydroxyapatite crystals (Bonucci and Dearden, 1976; Anderson, 2003). The latter are then released into the extracellular space where they grow in clusters and fill the gaps within and between the collagen fibrils.

An intense crosstalk exists between osteoblasts and osteoclasts, with a reciprocal regulation, eventually leading to a coupling of their functions. From the side of osteoblasts, they regulate osteoclast life by producing macrophage-colony stimulating factor (M-CSF), receptor activator of NFkappaB ligand (RANKL), and osteoprotegerin (OPG) (Felix *et al.*, 1990; Lacey *et al.*, 1998; Simonet *et al.*, 1997), as will be described in more detail in the next paragraph. RANKL is mainly produced as a membrane-bound factor, thus requiring a cell–cell contact, and at low level as a soluble molecule. Mechanisms of cleavage of membrane bound RANKL by metalloproteinases (MMPs), such as MMP-14, allow the release of the soluble form (sRANKL). Other paracrine factors released by osteoblasts that regulate osteoclastogenesis are tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6.

Quite interesting is the recently emerged "endocrine" role of the osteoblasts, through the release of OCN, now considered a real hormone (Karsenty and Ferron, 2012). In its undercarboxylated form, this glycoprotein is able to regulate glycaemia by

stimulating pancreatic islets to produce more insulin, which in turn also affects osteoblast function by reducing OPG production (Lee *et al.*, 2007). Osteoblast-derived OCN also influences adipose tissue and male gonadal functions, by stimulating adiponectin and testosterone production, respectively (Ferron *et al.*, 2008; Oury *et al.*, 2011). Finally, a role for OCN in the regulation of learning and memory skills and in the prevention of anxiety and depression in mouse models has been recently ascertained (Oury *et al.*, 2013).

Bone and bone marrow are two tissues linked not only anatomically but also functionally. It is therefore conceivable that these organs can affect each other. Interestingly, some years ago, Zhang and colleagues identified a subpopulation of osteoblasts named, as suggested by their morphology, spindle-shaped N-cadherin^{+}/CD45^{-} osteoblasts (SNOs), which regulate hematopoietic stem cell (HSC) life (Zhang *et al.*, 2003). Consistent with their function, another peculiarity of the SNOs is their location close to the endosteal surface. This finding led to the identification in the bone marrow of the endosteal (i.e., osteoblast) niche, where SNOs are the nurse cells for the long-term (LT)-HSCs, guaranteeing their quiescent *status*. The SNOs-LT-HSCs interaction is likely determined by the Notch signaling, where Notch1 is expressed by HSCs and binds to its ligand Jagged1 expressed by SNOs, thus inhibiting HSC mobilization and differentiation (Varnum-Finney *et al.*, 2000). Another putative signaling pathway regulating HSC quiescence by SNOs is the angiopoietin-1 (Ang-1)/Tie2 (Arai *et al.*, 2004). Coherently with the role of osteoblasts in HSCs regulation, conditional ablation of osteoblasts results in depletion of LT-HSCs (Visnjic *et al.*, 2004), while PTH treatment increases the number of HSCs (Calvi *et al.*, 2003). Among the pathways involved in the regulation of this function, a regulatory role has been established for the SNOs, which also secrete the stromal cell-derived factor (SDF)-1, *alias* CXCL12, whose receptor, CXCR4, is expressed by HSCs. It has been demonstrated that high levels of SDF-1 on the surface of osteoblasts favor HSCs to return to the osteoblast niche (Varnum-Finney *et al.*, 2000).

In line with these findings, a recent paper from Kousteni's group points out a role for osteoblasts in the pathophysiology of the hematopoietic system, where they demonstrated that constitutive activation of beta catenin in osteoblasts induces acute myeloid leukemia, together with genome instability and chromosome mutations in the LT-HSCs (Kode *et al.*, 2014).

Osteocytes: Not Just Buried Alive Cells

Osteocyte Formation

Among the pool of mature osteoblasts, a small fraction (i.e., 15%) remains embedded in the bone matrix, giving rise to the osteocyte population, the most abundant and long lived cell type in the bone, while the remaining osteoblasts will become inactive flat osteoblasts, named lining cells, or will undergo apoptosis (**Fig. 1**). The peculiar spider-like morphology of osteocytes is due to several cytoplasmic processes located in canaliculi arranged in a complex network, which participate in an intracellular and an extracellular communication system. The intracellular communication system occurs between osteocytes or osteocytes and osteoblasts by means of gap junctions, mainly formed by connexin 43, through which they exchange signals, while the extracellular communication system occurs through the pericanalicular space and provides nutrients and oxygen to the osteocytes (Komori, 2016).

Osteocyte formation follows different phases, during which the osteoblast goes through the phases of type I preosteocyte stage, a type II preosteocyte, which is encased in the osteoid, type III preosteocyte, young osteocyte and old osteocyte, the latter embedded in a fully mineralized matrix (Franz-Odendaal *et al.*, 2006). This gradual evolution toward a mature osteocyte reflects molecular and morphological changes, which finally culminate in the formation of a stellate-shaped cell, encased in lacunae and expressing specific markers, such as Sost and DMP1 (**Fig. 1**).

Osteocyte Functions

Thanks to the tri-dimensional network of their cytoplasmic processes, osteocytes are classically considered mechanosensing cells, with the job of converting a mechanical stimulus into a biological signal. This requires the involvement of a protein complex, including the cilia-associated proteins PolyCystin (PC) 1 and PC2 (Xiao *et al.*, 2006), and of the cytoskeleton, by means of the focal adhesion protein complex. The latter includes multiple actin-associated proteins like vinculin, talin, and paxillin, and is activated by fluid flow, eventually leading to the stabilization of β-catenin (Santos *et al.*, 2010).

Besides their mechanosensing function, osteocytes are crucial regulators of bone remodeling. Indeed, it has been demonstrated that osteocyte apoptosis, occurring after menopause or by microfracture, primes bone resorption. This is accomplished by the secretion of factors stimulating osteoclast formation, including IL-1, IL-6, and, last but not least, RANKL (Kogianni *et al.*, 2008; Al-Dujaili *et al.*, 2011). Indeed, one recent finding identified osteocytes as the main source of RANKL in the bone, and the specific knockout of RANKL in osteocytes leads to an increased bone mass in adult mice (Nakashima *et al.*, 2011). Consistently, RANKL produced by osteocytes is required to induce bone loss caused by glucocorticoids (Piemontese *et al.*, 2016). Osteocytes also release OPG under the control of the Wnt/β-catenin pathway (Kramer *et al.*, 2010).

Other factors produced by osteocytes are interferon (INF)-β and TGF-β, which inhibit osteoclastogenesis (Hayashida *et al.*, 2014; Heino *et al.*, 2002), insulin-like growth factor (IGF)-1, whose deletion in osteocytes abolishes the loading-induced activation of the Wnt signaling (Lau *et al.*, 2013) and vascular endothelial growth factor, which is produced by apoptotic osteocytes thus promoting angiogenesis (Cheung *et al.*, 2011).

Going to the side of osteoblast regulation, the production by osteocytes of Sost and Dickkopf-related protein 1 (DKK-1) is well ascertained. These two inhibitors of the Wnt pathway are regulated by mechanical stimuli in osteocytes, with their expression significantly reduced in loading and increased in unloading conditions, respectively (Robling *et al.*, 2008). Taken together, all these findings point to the osteocytes as important determinants of bone remodeling.

Other unexpected functions for osteocytes have been identified over the years, such as the removal of the perilacunar matrix. Indeed, electron microscopy studies of tibiae from rats immobilized for 10 days showed periosteocytic osteolysis, characterized by fragmentation of collagen fibers and loss of mineral crystals (Krempien *et al.*, 1976). This perilacunar reabsorption seems to be exacerbated during lactation, as recently demonstrated by Qing *et al.* (2012). Moreover, a gene array of osteocytes from lactating animals showed an increase of tartrate-resistant acid phosphatase (TRAcP) and cathepsin K mRNA expression, which returned to basal levels after weaning (Qing *et al.*, 2012). Concurrent with the ability to regulate calcium homeostasis, osteocytic osteoplastic activity has also been described in lying hens previously fed with a low calcium diet and then subjected to calcium replacement (Zambonin-Zallone *et al.*, 1983).

Finally, osteocytes participate in the regulation of phosphate homeostasis in the kidney by producing FGF-23, a hormone that induces renal phosphate wasting, thus impairing mineralization (Liu *et al.*, 2006).

Osteoclasts: Not Just Any Bone Destroying Cells

Osteoclast Differentiation

Osteoclasts are multinucleated giant cells (from 4 to 20 nuclei) arising from the monocyte–macrophage lineage. The identification of the origin of these cells came after a key experiment by Walker, who showed the ability of osteopetrotic mice to recover bone resorption after bone marrow transplant from healthy mice (Walker, 1975). Ontogenesis of osteoclasts follows different steps and is triggered by two transcription factors: PU-1 and microphthalmia transcription factor, both promoting the commitment of HSCs toward a common macrophage/osteoclast progenitor expressing c-fms, the receptor for M-CSF (Tondravi *et al.*, 1997; Kawaguchi and Noda, 2000). The latter cytokine positively acts on the macrophage/osteoclast progenitor pool by stimulating their proliferation and survival through the activation of ERK and Akt (Biskobing *et al.*, 1995). Moreover, it increases the expression of RANK in osteoclast precursors, which is a key event that allows commitment toward an osteoclast (Arai *et al.*, 1999). At this point, the RANKL/RANK/OPG signaling takes place as the "king of the game." In particular, RANKL, which in bone is mainly produced by osteocytes, osteoblasts, and stromal cells, binds to its receptor RANK that in turn interacts with the TNF-receptor-associated factor (TRAF)-6 adaptor protein, thus triggering osteoclast differentiation by means of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). This is a dimeric transcription factor, whose knockout induces an osteopetrotic phenotype, thus demonstrating its importance in osteoclast differentiation (Franzoso *et al.*, 1997). NF-κB, along with c-Fos, stimulates the expression of another important transcription factor, the nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent (NFATc1) (Asagiri *et al.*, 2005). In parallel, RANKL interaction with TRAF6 also leads to Src engagement, which in turn influences osteoclastogenesis through the activation of the PI3K/Akt pathway (Wong *et al.*, 1999).

The RANKL/RANK pathway is essential for osteoclastogenesis; however it has to rely on a parallel signal triggered by two immune coreceptors, expressed on the surface of preosteoclasts, displaying the immunoreceptor tyrosine-based activation motif: Fc receptor common γ signaling chain (Fc$R\gamma$) and DNAX activating protein of 12 kDa (DAP12). It is known that in osteoclasts Fc$R\gamma$ interacts with osteoclast associated receptor (OSCAR) and paired immunoglobulin-like receptor-A, while DAP12 interacts with triggering receptor expressed in myeloid cells 2 and signal regulator protein β1. Engagement of these immunoreceptors results in the activation of PLCγ, which increases intracellular Ca^{2+}, thus promoting the calcineurin-dependent auto-activation of NFATc1 (Nakashima *et al.*, 2012).

The final event downstream of these RANKL pathways is the promotion of transcriptional expression of osteoclast-specific genes, such as TRAcP, calcitonin receptor, cathepsin K, matrix metalloproteinase (MMP)-9, OSCAR, and genes involved in osteoclast fusion, like CD44, macrophage fusion receptor, and dendritic cell-specific transmembrane protein (Cappariello *et al.*, 2014).

Coherently with the osteogenic role of Wnts, recent reports demonstrated that these molecules also participate in the regulation of osteoclast formation. Wnt3a inhibits 1,25-dihydroxyvitamin D_3-induced osteoclastogenesis in cocultures of bone marrow cells and osteoblasts (Yamane *et al.*, 2001), while osteoblast-derived Wnt16 inhibits RANKL-induced pathway and Nfatc1 expression by the noncanonical signaling (Movérare-Skrtic *et al.*, 2014). Similar effects were also observed for Wnt4 (Yu *et al.*, 2014). In contrast, a pro-osteoclastogenic effect has been demonstrated for Wnt5a, which increases osteoclastogenesis by a Ror2-mediated mechanism (Maeda *et al.*, 2012).

Osteoclast Functions

A mature osteoclast can be easily visualized in bone sections by the histochemical detection of TRAcP activity (**Fig. 3**A). To fulfill its principal function, that is bone resorption, the osteoclast must be polarized. Its plasma membrane is organized in different domains with distinct functions. The portion of the plasma membrane facing the vascular compartment is called the "basolateral membrane." Here, ion channels, sodium/potassium pumps, and the HCO_3^-/Cl^- anion exchanger 2 are localized (Lindsey

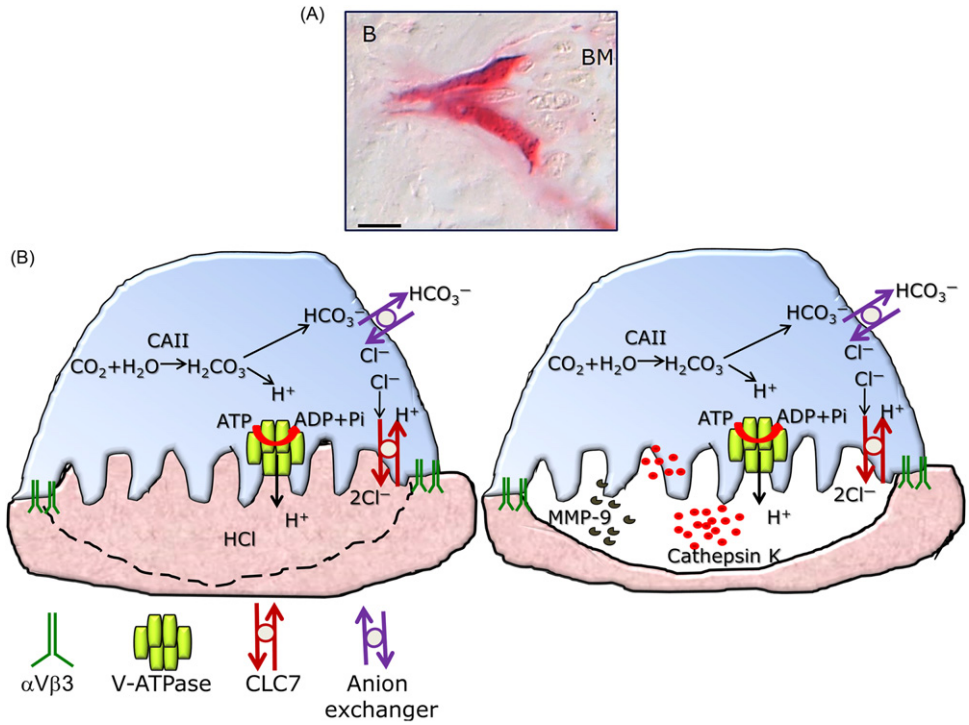

Fig. 3 (A) Histological section of a mouse tibia stained for the tartrate resistant acid phosphatase (TRAcP) activity to visualize the osteoclasts (purple spots, bar = 25 μm). (B) Cartoon showing the acidification process (left), which allows dissolution of the hydroxyapatite crystals, followed by the enzymatic digestion of the organic matrix (right).

et al., 1990). On the opposite side, the plasma membrane of the osteoclast facing the bone matrix is organized in several deep folds and constitutes the so called "ruffled border." Nearby it is possible to identify the "sealing zone" which allows the osteoclast to circumscribe the surface of the bone that will be reabsorbed. This is a circular outer domain containing characteristic adhesion structures, called podosomes (Marchisio *et al.*, 1984). They are very dynamic dot-like membrane protrusions with a core of actin filaments associated with a number of actin binding, adhesion, signaling, and regulatory proteins, including fimbrin, α-actinin, vinculin, talin, paxillin, gelsolin, tyrosine kinases, Wiskott–Aldrich syndrome protein, and Arp2/3. Before starting bone resorption, osteoclasts arrange podosomes in hook-like structures called actin rings, whose correct organization is pivotal to seal the resorption lacuna and allow the process of bone matrix degradation.

The dynamic nature of podosomes is guaranteed by a relatively fast polymerization/depolymerization of F-actins, resulting in a half-life turnover every 2–12 min. Integrin receptors, mainly $\alpha V\beta 3$ and to a lesser part $\alpha 2\beta 1$, contribute to osteoclast adhesion by forming a transmembrane bridge between the microfilaments and the adhesion proteins of the extracellular matrix. The $\alpha V\beta 3$-mediated signaling pathway involves PYK2 (belonging to the focal adhesion kinase family), Src, and Cbl, which are necessary for osteoclast polarization and bone resorption (Sanjay *et al.*, 2001). Interestingly, while $\alpha V\beta 3$ dominates in differentiated osteoclasts, $\alpha V\beta 5$ is the major integrin expressed by osteoclast precursors and the switch from $\alpha V\beta 5$ to $\alpha V\beta 3$ is a step required for osteoclast maturation (Inoue *et al.*, 1998).

Bone resorption can now start, with a phase of acidification that dissolves the hydroxyapatite crystals, followed by the enzymatic digestion of the organic components of the matrix (**Fig. 3**B). Briefly, carbonic anhydrase II catalyzes the chemical reaction of hydration of carbon dioxide (CO_2) to form carbonic acid (H_2CO_3) which gives rise to bicarbonate (HCO_3^-) and hydrogen (H^+) ions. The latter are pumped outside the osteoclasts into the resorption lacuna by means of a V-H^+-ATPase located in the ruffled border. To avoid the alkalinization of the cytosol, the HCO_3^-/Cl^- anion exchanger 2 allows the efflux of HCO_3^- and the influx of chloride ions (Cl^-) (Teti *et al.*, 1989), which is then released into the resorption lacuna by the $2Cl^-/1H^+$ antiporter (CLC7, chloride channel type 7) (Leisle *et al.*, 2011). In the resorption lacuna there is now HCl, which dissolves the hydroxyapatite crystals (**Fig. 3**B, left). This exposes the bone matrix that can be digested by cathepsin K and MMP-9 (**Fig. 3**B, right), while the debris is mainly removed by transcytosis and released at the basolateral domain (Salo *et al.*, 1997; Stenbeck and Horton, 2004).

Bone digestion releases growth factors stored in the matrix, such as BMPs, IGF-1, and TGF-β, which fuel the bone remodeling process because they attract the osteoblasts, thus inducing new bone formation. Interestingly, osteoclasts can also directly influence osteoblast behavior by producing the so-called clastokines (Drissi and Sanjay, 2016). Sphingosine-1-phosphatase is one of them along with TRAcP and complement factor C3a, the latter isolated from the conditioned medium of osteoclasts. Most clastokines have pro-osteoblastogenic effects (Pederson *et al.*, 2008; Hayman and Cox 2003; Matsuoka *et al.*, 2014; Teti, 2013), although others are antiosteoblastic, revealing the need of a balance between these two types of stimuli for physiological osteoblast activity.

As osteoblasts do, osteoclasts also contribute to hematopoiesis. This is supported by the evidence that treatment with anti-resorptive agents, which affect osteoclast behavior, such as bisphosphonates, increased the number of peripheral HSCs after mobilization induced by G-CSF (Takamatsu *et al.*, 1998). A similar effect was observed in osteopetrotic mice characterized by the lack of osteoclasts, while in OPG knock out mice, which instead present with a higher number of osteoclast, an opposite effect on HSC mobilization was noticed (Miyamoto, 2013).

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Bone Remodeling☆

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Glossary

Activation frequency The frequency at which a particular point on the bone surface will be reached by a basic multicellular unit.

Basic multicellular unit (BMU) A group of osteoblasts and osteoclasts that resorb a volume of bone and replace it with new bone.

Bone mineral density The weight of mineral per volume of bone tissue.

Bone tissue The total material found within a bone, including the bone marrow, fat, mineralized bone, and unmineralized osteoid.

Bone turnover An ambiguous term that is either the bone formation rate or the bone resorption rate and is meaningful only when these rates are equal.

Bone volume The bone material, including mineralized bone and unmineralized osteoid.

Mineral apposition rate The rate at which mineral is deposited at a discrete point on the bone surface; expressed in micrometers per day.

Mineralization density The weight of mineral per bone volume.

Osteoid Newly formed bone, composed primarily of lamellar collagen with small amounts of other bone proteins and glycoproteins, that forms a matrix that can accept deposition of hydroxyapatite crystals and that will become mineralized bone.

RANK A receptor on preosteoclasts and osteoclasts, which increases maturation or activity of the cells.

Runx2 A transcription factor that promotes osteoblast activity and is necessary for maturation and proliferation of osteoblasts.

Sclerostin A protein secreted by osteocytes that inhibits osteoblasts by blocking the wnt-signaling pathway.

Wall thickness The thickness (in three-dimensions) of a completed volume of new bone, measured perpendicularly from the interface with the bone marrow.

Introduction

Bone is such a solid substance that an orthopedic operating room resembles a carpenter's workshop, with its saws, drills, screws, and hammers used to repair broken and damaged furniture. This reinforces the concept that bones are mechanical structures; indeed, one of the major functions of the skeleton is to provide support to the rest of the body. The dynamic properties of the bone, however, are seldom considered, except to acknowledge that living bone, unlike the leg of a table, can repair a fracture. Actually, the skeleton is continually remodeling, responding to external forces and internal signals.

During childhood bones are formed at the growth plates and shaped by resorbing the outer surfaces. Once the growth plates have fused, the shape of a bone changes very slowly during life by formation at the periosteal surfaces. The diameters of the long bones gradually expand. The process of forming bone and changing the shape is termed modeling. There may also be some modeling on the trabecular surfaces of bone, where new bone is formed over a quiescent surface of older bone.

The newly formed bone is not permanent. Throughout life, quantities of bone are dissolved and then refilled with new bone. This process is termed remodeling, and it keeps the bone strong by removing old or damaged regions and replacing them with new bone. Overall, the cancellous bone in the entire skeleton is refreshed about every 5 years and the cortical bone every 12 years (Parfitt, 2002).

Bone remodeling always follows the same sequence. First there are signals to start remodeling a volume of bone. Then a cavity of bone is resorbed, and finally filled in with new bone.

Basic Multicellular Unit

The bone remodeling process involves a group of different kinds of cells, termed the basic multicellular unit (BMU) (Frost, 1969). The major bone cells are the osteoclasts and the osteoblasts.

☆*Change History*: January 2018. SM Ott made edits through-out the chapter to bring topics up to date. Larger sections were added about signaling pathways, canopies, and osteoporosis treatments.

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Osteoclasts

The osteoclasts are derived from the same hematopoietic stem cells that produce macrophages. These stem cells differentiate into preosteoclasts which circulate in the bloodstream. They express RANK (receptor receptor activator of NF-kappaB), a membrane receptor. When these cells encounter the RANK-ligand, they become active, fuse and form multinucleated cells which can resorb the bone.

Osteoblasts

The osteoblasts are derived from stromal stem cells that reside in the bone marrow. These cells can produce either adipocytes or preosteoblasts, depending on the condition. Situations that increase PPARγ (peroxisome proliferator-activated receptor gamma) will direct the stem cells to produce adipocytes. VEGF (vascular endothelial growth factor A) instead promotes preosteoblast formation.

The transcription factor Runx2 is a master switch for osteoblast differentiation. The second important transcription factor is Osterix. These transcription factors regulate expression of most of the osteoblast-specific genes. Several signaling pathways will increase expression of Runx2 and promote osteoblast maturation and proliferation. One of the most important is the wnt-signaling pathway. Other signaling pathways include BMP (bone morphogenic protein), TGF-β (transforming growth factor beta), PTH (parathyroid hormone), IGF-1 (insulin-like growth factor), FGF (fibroblastic growth factor), Notch, and Hedgehog. Not only are there multiple pathways, they also can interact with each other in a complex system of cell regulation (de Gorter and ten Dijky, 2013).

Some of the ligands for these pathways are in the general circulation, others from cells in the bone marrow. Some represent cell–cell communication between the osteoblasts and the osteoclasts. For example, the osteoblasts express RANK-ligand which signals maturation of osteoclasts, and also secrete osteoprotegerin, a decoy receptor that will prevent RANK signaling. Another example is even more complex: the mature osteoblasts secrete TGFß into the bone matrix, where it remains until the bone is resorbed in a subsequent bone remodeling cycle. Then, during bone resorption, the TGFß is released from the bone and activates receptors on the osteoclasts, which leads to secretion of the wnt ligand that will increase the wnt-signaling pathway in the osteoblasts (Weivoda *et al.*, 2016).

The mature osteoblasts form the bone matrix. They then either undergo apoptosis or further differentiate into osteocytes or lining cells. The osteocytes remain in the bone matrix and the lining cells form a boundary between the bone and the marrow.

Each BMU has a blood vessel. Other cells, including bone marrow stromal cells, bone marrow adipocytes, nerve cells, T-lymphocytes, and vascular endothelial cells, can influence the BMU but are not actively resorbing or forming bone.

Sequence of Bone Remodeling

A fundamental property of bone remodeling is that it occurs in discrete locations. In cancellous bone, the BMUs travel along the bone surface, spreading over an area. In the cortical bone, they tunnel through the bone, with the osteoclasts at the cutting edge and newly formed bone in the wake.

Each BMU performs its functions in the same sequence: origination and organization of the BMU, activation of osteoclasts and beginning of recruitment of osteoblasts, resorption of old bone, then a reversal phase, then formation of new bone matrix, then a quiescent period where there are no new cells but during this time mineralization continues (**Fig. 1**). Many of the details of this sequence have been elucidated by Parfitt (2001), and measurements of the timing of each phase were done by Eriksen *et al.* (1984). At one spot along the surface, the cycle takes about 170 days. The lifespan of a BMU is limited, it spreads along a surface and then ends, after most cells have died from apoptosis. Cortical BMUs can wander for months, usually in a straight line. Parfitt (1994) estimates the duration is 2–8 months. This is harder to measure in cancellous bone because the two-dimensional sections do not capture the entire serpentine course of the BMU (**Fig. 2**).

Origination

The osteocytes form a network within the bone, and these cells direct the bone remodeling (Dallas *et al.*, 2013). They are able to sense mechanical loading as well as hormonal signals. Small cracks caused by microdamage will initiate a BMU. Signals from osteocytes or lining cells that have detected mechanical stress, local cytokines from marrow cells, or systemic hormones that regulate mineral metabolism may also be involved in BMU origination. Possibly, some BMUs commence at random.

Mori and Burr demonstrated an association between fatigue damage and intracortical remodeling. They anesthetized mature dogs and applied a cyclic load to the radius, which caused asymptomatic microscopic cracks. Eight days later, an identical load was applied to the opposite radius. Histologic sections showed an equal number of cracks on each side. However, at the site with the earlier load, there was a significant increase in resorption cavities that were adjacent to the cracks. The temporal design of the study demonstrated that the resorption occurred after the fatigue damage (Mori and Burr, 1993).

Origination occurs only once for each BMU at a quiescent surface of the cancellous bone or on a surface nearest a crack in the cortical bone. Lining cells change their shape from that of flat, epithelial-like cells to rounded cells, thereby exposing some of the

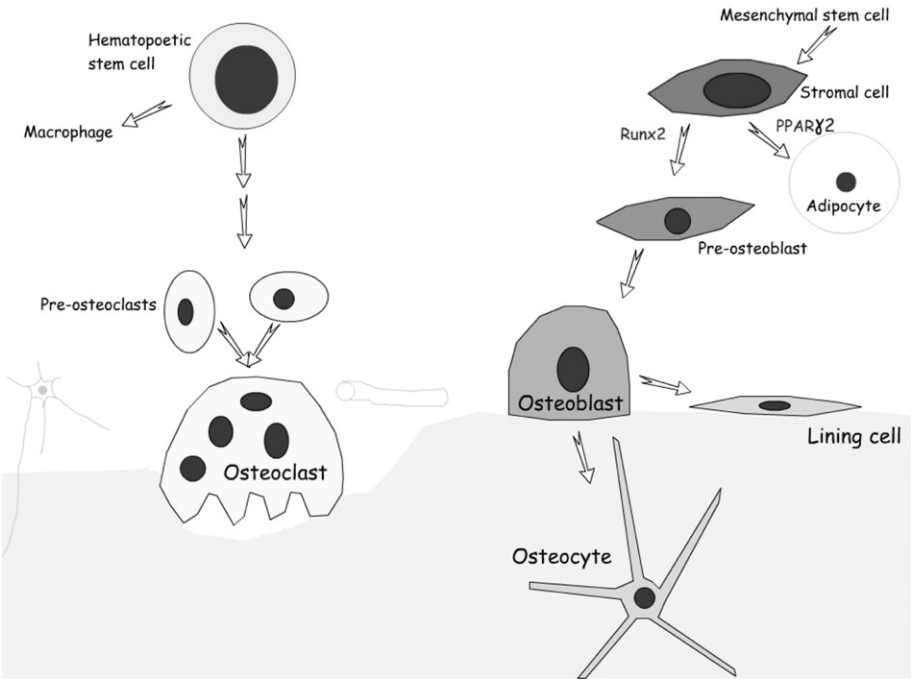

Fig. 1 Cells involved in bone remodeling. The osteoclasts resorb the bone and the osteoblasts form new bone. Osteocytes and lining cells remain after the BMU has finished remodeling a volume of bone.

collagen matrix. They also secrete collagenase to expose the bone mineral. The lining cells then fuse with endothelial cells to form a canopy above the bone surface that is connected with the vasculature (Jensen *et al.*, 2015). Within this canopy, osteocytes, and osteoclasts expressing RANK-ligand on their surface will attract the preosteoclasts which express the RANK receptor. The pre-osteoclasts fuse and become mature multinucleated osteoclasts.

Activation

Activation is a process that occurs at the cutting edge of the BMU, and as the BMU spreads, new surfaces undergo activation. The BMU "front" travels at a rate of approximately 10 μm/day and is followed by osteoblasts and new blood vessels. The original osteoclasts undergo apoptosis while the BMU is still progressing; thus, new cells must replenish the dying ones. Systemic hormones, growth factors, and interleukins may enlarge the precursor pool, but systemic factors cannot localize the preosteoclasts to the cutting edge.

Resorption

Preosteoclasts fuse when the RANK receptor encounters RANK-ligand on the surface of osteocytes or osteoblasts. At a given spot on the bone surface, resorption is rapid for the first 10 days, and continues for about a month. Once activated, the osteoclast becomes polarized and undergoes cytoskeletal changes that allow it to attach to bone (Teitelbaum, 2007). Small GTP-ases are necessary for these changes. The side of the osteoclast near the bone surface forms a "ruffled border" that will attach to integrins that were imbedded within mineralized bone matrix. The osteoclast seals off a circular surface of bone, creating a space between the bone and the ruffled border which is separated from the bone marrow (**Fig. 3**).

The integrins convey signals from the bone matrix to the osteoclast interior. The osteoclast then pumps hydrogen ions into the resorbing space by a hydrogen ATP-ase (the same pump is used by renal tubular cells to secrete acid into the urine). These ions are generated from water and carbon dioxide by carbonic anhydrase, and the bicarbonate is secreted into the bone marrow. Chloride flows through a chloride channel to maintain electrical neutrality. The acid environment will then dissolve the mineral from the bone. Also, the osteoclast secretes cathepsin K into the resorbing space; this degrades the collagen. The osteoclasts can also engulf debris through endocytosis, similar to macrophages, which are closely related cells.

During resorption, bone-derived growth factors are released, including TGF-β, insulin-like growth factor (IGF), and fibroblast growth factor (FGF), which were deposited into the matrix by the previous generation of osteoblasts. TGF-β is activated by the acid environment caused by osteoclastic proton secretion. These growth factors (delayed autocrine factors) account for the coupling between resorption and formation that is seen in normal situations (Weivoda *et al.*, 2016).

The osteoclasts eventually undergo apoptosis; the lifespan and activity of the osteoclasts determine the depth of the resorption cavity. The apoptosis may be promoted by TGFß and estrogen (Janssens *et al.*, 2005).

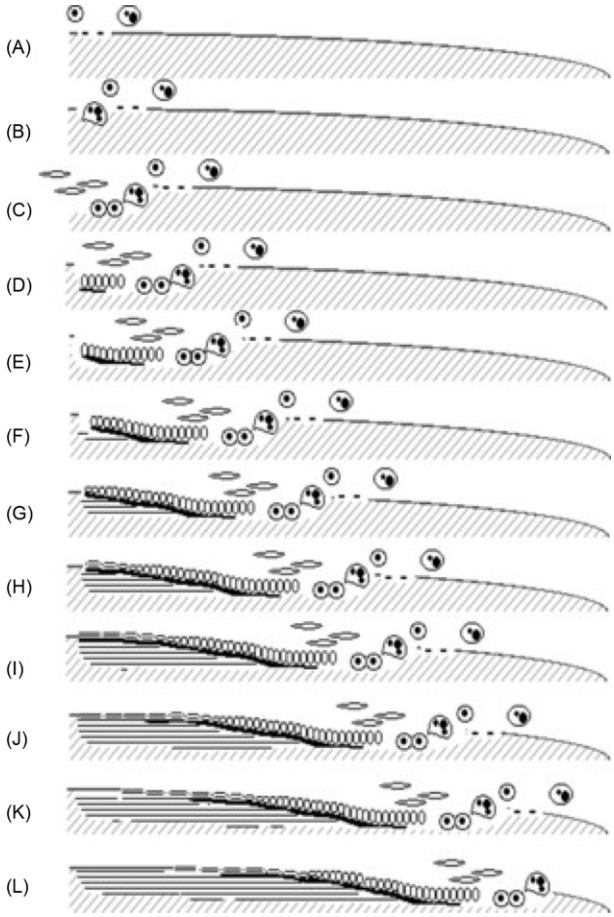

Fig. 2 The basic multicellular unit (BMU), moving along a cancellous surface. Each step represents approximately 10 days, and the BMU moves approximately 10 μm each day. (A) Origination of BMU: lining cells contract to expose collagen and fuse with endothelial cells to form a canopy over the bone. Osteocytes secrete factors that control osteoblast and osteoclast differentiation. (B) Preosteoclasts respond to RANK, fuse into multinucleated cells, and resorb a cavity. The resorbed bone contains growth factors which stimulate osteoblast formation, and the osteoclasts also secrete signals to the new osteoblasts to proliferate. (C) Mononuclear cells continue resorption. (D) Osteoblast team forms at the bottom of the cavity and starts forming osteoid. (E) Osteoblasts continue forming osteoid (shown as *black line*) and previous osteoid starts to mineralize (*horizontal lines*). (F–H) Osteoblasts continue formation and mineralization. (I and J) Osteoblasts begin to flatten; many undergo apoptosis, some differentiate into osteocytes and remain in the bone matrix. (K and L) Osteoblasts turn into lining cells; bone remodeling at initial surface (left of drawing) is now complete and that surface will be quiescent for years, but BMU is still advancing (to the *right*).

Formation

After the maximum eroded depth has been achieved, there is a reversal phase that lasts ~9 days. Reversal cells express Runx2 and other gene markers that are in the osteoblast family (Abdelgawad *et al.*, 2016). These cells, which are especially located between the osteoclastic bone resorption surface and the osteoblast formation surface, may play an important role in coupling between the osteoclastic resorption and osteoblastic formation (Delaisse, 2014). During this phase, the osteoblasts converge at the bottom of the cavity. The osteoblasts derive from precursors in the marrow stromal cells, which can differentiate into either osteoblasts or adipocytes, depending on the transcription factor runx2 (previously known asCbfa1) as well as PPARγ. The team of osteoblasts then begins to form osteoid, which is mainly composed of collagen but includes other bone proteins, growth factors, and proteoglycans. After about 15 days, the osteoid begins to mineralize. The osteoblasts continue to form and to mineralize osteoid until the cavity is filled or nearly filled, which takes about 120 days at any point on the surface.

At the bottom of the cavity, the new osteoblasts are plump and vigorous, have tall nuclei, and make a thick layer of osteoid. The cells then gradually flatten as they slow production, and finally they undergo apoptosis or mature into quiescent lining cells. Some of the osteoblasts differentiate into osteocytes and remain in the matrix. The osteocytes may secrete inhibitory factors, such as sclerostin, that slow the rate of bone formation as the resorbed cavity is nearly filled. As the BMU progresses, new osteoblasts are added, but only at the edge behind the osteoclasts.

The density of the osteoblasts at the formation site may vary. When the cells are more crowded, they are taller and narrower, and they collectively can make more osteoid than when there are fewer cells. Osteoporotic patients have the same rate of osteoid production per cell, but overall the wall thickness is decreased and the amount of newly formed bone is inadequate to fill the

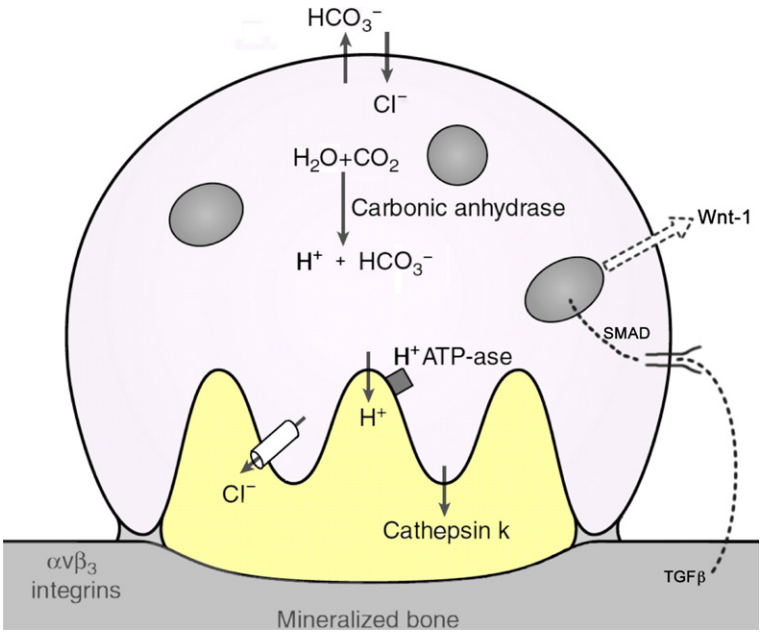

Fig. 3 Mature osteoclast resorbing bone. Integrins bind to the bone collagen, creating a space beneath the ruffled border. Carbonic anhydrase converts water and carbon dioxide to hydrogen and bicarbonate. The bicarbonate is exchanged for chloride, and the hydrogen is secreted into the resorbing space, dissolving bone. The chloride exits through a channel. Cathepsin K is secreted to break down collagen. Then TGFß from the bone is released and activated by the acidic environment, it activates receptors on the cell surface, signaling through SMADs to target expression of wnt-1, which is secreted and activates the osteoblast wnt-signaling pathway.

resorbed cavity. Some investigators think that bone formation is discontinuous (on and off), but studies with tetracycline labeling suggest that the osteoblast team does not take vacations (Ott, 1993).

Mineralization

Mineralization begins ~ 15 days after osteoid has been formed. In most situations (except in the case of osteomalacia), the average rate of osteoid formation and the rate of mineralization are the same and are measured by tetracycline labels.

After the BMU has completely restored the bone volume, the mineralization density continues to increase as the crystals become more densely packed. It is not known how long it takes to reach the maximum mineralization density, but indirect evidence suggests this takes approximately 3 years (**Fig. 4**).

The mineralization density is related to the bone formation rate. When the bone formation rate is high, mineralization density is low. When the bone formation rate is low, more of the bone is older and more highly mineralized (Boivin *et al.*, 2009).

Other Tissues That Are Within Bone

Vasculature

Each BMU is associated with a capillary. In cortical bone, the capillary grows along the excavated tunnel. On trabecular surfaces, small capillary-like structures are frequently seen adjacent to the osteoblasts. The cells that form these structures have characteristics that are unlike endothelial cells and resemble lining cells. In addition to supplying nutrients and a source of precursor cells, the vasculature may help direct the localization of the BMU.

Nerves

Anatomic studies have documented a dense and intimate innervation of bone tissue. The fibers contain markers for neural tissue, both sensory fibers and sympathetic fibers. Nerve endings are seen in contact with bone cells. Glutamate is expressed in the fibers that are in proximity to bone cells, suggesting a potential role of glutamatergic innervation in the bone remodeling process.

Adipocytes

Adipocytes and osteoblasts derive from the same precursors, and factors such as lipid levels can favor differentiation into adipocytes, which will reduce the number of available osteoblasts. The adipocytes also secrete leptin, but the ability of leptin to either stimulate or inhibit bone formation is still debated (Gimble, 2011).

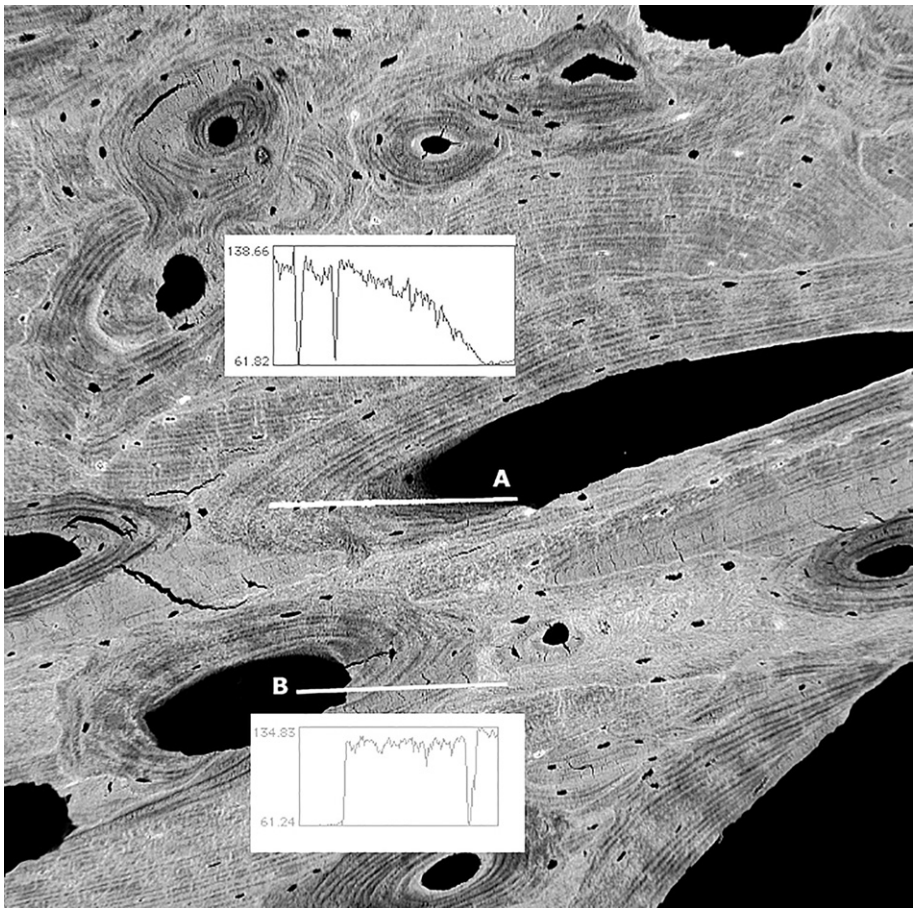

Fig. 4 Electron backscattered image of iliac cortical bone showing two new osteons, with plot profiles taken along the *white lines*. The line A profile shows that density is low in the newly formed osteoid (*dark gray*) and gradually increases as the bone becomes older (toward the left). The line B profile shows an abrupt change from the Haversian canal space to the bone; this line crosses a surface of bone resorption. The downward spikes in the plot profiles represent the osteocyte lacunae. Near the end of line B (right), there is higher mineralization after line B crosses the cement line into an area of older bone, which appears whiter on the image.

Control of Bone Remodeling

Mechanical Stress

The osteocytes, once thought to be trapped in the bone mineral, are actually active within their lacunae and canaliculae (Atkins and Findlay, 2012). They have multiple long cell processes that extend within the bone and form junctions with other osteocytes. The resulting network resembles a neuronal network, and the osteocytes are considered the brains of the bones A special cell process, like a cilia, can be found on these extensions, which is firmly attached to the mineral of the canalicular wall (McNamara *et al.*, 2009). When mechanical loads are applied to the bone, the cilia can detect fluid movement (Santos *et al.*, 2009), and thus the osteocytes can sense mechanical strains. They then stop secreting sclerostin (Robling *et al.*, 2008), which releases inhibition of the osteoblasts. The osteocytes also secrete stimulatory factors into the marrow, including prostaglandins, nitric oxide, and IGF. Where there is microdamage, the osteocytes undergo apoptosis, and as they lay dying they express RANK-ligand to signal osteoclasts to come and start the repair process.

Hormones That Regulate Mineral Metabolism

Parathyroid Hormone

Parathyroid hormone (PTH) and PTH-related protein can activate lining cells to secrete RANK ligand and originate a BMU. PTH may also affect osteoblast proliferation. When given as intermittent injections, it can bypass the bone remodeling system and stimulate bone formation directly on quiescent surfaces. PTH-related protein causes increased bone resorption and loss of bone during lactation.

Vitamin D

Vitamin D can also activate lining cells to secrete RANK ligand. It increases the proliferation of preosteoclasts and may play a role in osteoblast recruitment. Indirectly, it is important in the maintenance of serum mineral levels.

Calcitonin

Calcitonin receptors are present on osteoclasts, and when activated, the cells stop resorbing bone. The calcitonin gene-related protein appears to increase bone formation, but the mechanism is not clear.

Estrogen

Estrogen exerts a major effect on bone remodeling, possibly through different mechanisms. Several mechanisms are involved: (1) estrogen increases osteoclast apoptosis; (2) by suppressing interleukins and proinflammatory cytokine expression in bone marrow cells, estrogen decreases the number of osteoclasts; (3) by inhibiting the production of RANK-ligand, estrogen reduces the number and activity of osteoclasts; and (4) by increasing stromal cell/osteoblast cell expression of TGFß, estrogen inhibits osteoclast activity. Estrogen also has some positive effects on bone formation by acting as a mitogen to cells early in the osteoblast line, reducing apoptosis of osteoblasts, and increasing expression of TGFβ, bone morphogenetic proteins, and IGF-I (Modder *et al.*, 2011).

Bone Remodeling in Osteoporosis

When the bone resorption rate is higher than the bone formation rate, the bone volume will decrease, resulting in osteoporosis. The most common is postmenopausal osteoporosis with an increased bone resorption rate and a bone formation rate that is increased but not enough to match the increased resorption. Bone loss also occurs with normal resorption but low bone formation, as in some cases of renal osteodystrophy with adynamic bone. The most rapid bone loss is seen in diseases such as multiple myeloma where there is both increased bone resorption and decreased bone formation.

Aging Bone Loss

With aging, the osteoblasts lose their ability to fill the resorbed spaces. This is shown by age-related decreases in the wall thickness. When bone porosity increases, the remaining bone accumulates microdamage at an exponential rate. A vicious cycle is begun: bone mass decreases, so the remaining bone is subject to more fatigue damage that increases bone resorption, which may further weaken the bone and disrupt the osteocyte network.

Mechanisms of Action of Osteoporosis Medications

Understanding bone remodeling is important for predicting the response to therapeutic agents used for osteoporosis. Antiresorptive agents (estrogen, estrogen-receptor modulators, bisphosphonates, calcitonin, denosumab, and strontium ranelate) all decrease bone resorption, but they also decrease bone formation.

Bisphosphonates

The more potent nitrogen-containing bisphosphonates decrease the tetracycline-labeled bone formation rate by $\sim$95%. Small increases in bone volume occur while the BMUs that were forming bone continue to fill in the eroded cavities. Eventually, a new steady-state bone volume is reached, after which there is no further gain in bone volume because bone remodeling has been inhibited. Bone density will increase even after bone volume has reached a steady state because the mineralization density continues to increase. Women taking bisphosphonates have increasing bone density at the hip for $\sim$3 years; thereafter, the bone density does not increase further. The long-term ($>$7 years) consequences of prolonged suppression of bone formation rates are not known, but potentially fatigue damage and microcracks could accumulate. A bone biopsy study from patients who had taken bisphosphonates longer than 7 years showed increasing prevalence of microdamage (Ward *et al.*, 2016).

PTH and PTHrp

Intermittent parathyroid hormone and parathyroid hormone-related protein are anabolic agents that increases both bone formation and resorption, but the balance is positive, with large increases of bone density. Mineralization density decreases because a greater proportion of the bone is newly formed and incompletely mineralized. Therefore, anabolic agents have completely different mechanism of action from that of antiresorbing agents.

Sclerosin inhibitors

The newest category of medications studied in osteoporosis is sclerostin-inhibitors. By inhibiting sclerostin, wnt-signaling is increased and more osteoblasts are formed. The bone formation rate increases dramatically. The wnt-signaling also results in secretion of osteoprotegerin, which inhibits formation of mature osteoclasts, so that bone resorption is inhibited. The combination of decreased resorption and increased formation results in greater increases in the bone mass than the other medications (Cosman *et al.*, 2016).

Fluoride and the importance of bone quality

It is important to recognize that increasing the bone volume is not sufficient to improve the bone strength. Fluoride was a popular drug used to treat osteoporosis in the 1980s, and in 1990 a 4-year clinical trial found a 32% median increase of bone density in the spine (greater than any of the drugs mentioned above). The fracture rate, however, was not different than placebo in the spine and there were significantly more nonvertebral fractures (Riggs *et al.*, 1990). Bone biopsies from fluoride-treated patients show disorganized bone, high mineralization, abnormal crystals, and the bone strength was weaker (Kleerekoper and Balena, 1991). In growing children, bisphosphonates increase the bone volume and density, causing osteopetrosis, but these bones are more likely to fracture (Whyte *et al.*, 2003).

Other Mechanisms of Altering Bone Structure

Microcallus Formation

When trabeculae fracture, a microcallus can form that resembles the familiar "macrocallus" of a fractured bone. These microcallus formations in the spine increase after age 50, mostly in the lower thoracic and lumbar spine near the endplates. There are more in females than in males and even more in osteoporotic persons. These formations can account for up to 10% of the trabecular bone volume. Microcallus formations can allow creation of new trabeculae by forming bridges between existing trabeculae. They undergo resorption and modeling, eventually becoming mineralized and indistinguishable from trabecular bone. This mechanism of altering trabecular structure could be relatively important in patients with osteoporosis (Hahn *et al.*, 1995).

Direct Activation

In some situations, bone formation can also occur along surfaces in the absence of previous resorption. With fluoride therapy, bone formation surfaces increase markedly, but the bone is woven and not normal lamellar bone. Beagles treated with aluminum show new bone formation in some, but not all, experimental conditions, but this phenomenon is not seen in humans, who develop osteomalacia when exposed to parenteral aluminum. Intermittent PTH causes increases in the bone formation rate that occur too soon to be explained by the bone remodeling cycle. Measurements of the distance from new bone edges to cement lines also suggest that previously quiescent surfaces start to form bone. This is also seen with sclerostin antibody administration (Kim *et al.*, 2017).

Bone Arising From the Marrow Space

Patients with metastatic prostate cancer can develop osteoblastic lesions within the marrow spaces. First, spindle-shaped cells with characteristics of osteoblasts appear in the marrow spaces adjacent to the cancer cells, and then these calcify and become woven bone. The bone eventually becomes osteosclerotic (Roudier *et al.*, 2008).

Periosteal Bone Formation

The periosteal surfaces of cortical bone gradually expand throughout adult life. This compensates for loss of bone tissue because bone with a larger diameter has greater bending strength. Periosteal expansion occurs without prior bone resorption. In some cases, when there have been repetitive stresses on the bone, woven bone will form at the periosteal surface. This has been noted, for example, in young military recruits during basic training. The woven bone can be formed more rapidly than the lamellar bone formed during usual bone remodeling.

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Bone Structure and Biomechanics☆

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Introduction

The skeletal system is made up of specialized connective tissues, namely bone and cartilage. Bone is the main part of the system and the structure of bone can be usefully considered in several different ways that reflect its variety of functions and constituents. Its precise form and composition vary with location, nature having selected optimal designs for different roles. The purpose of bones are (1) to allow for locomotion by providing rigid support and sites for muscle action; (2) to provide a shield for vital internal organs and bone marrow; (3) to serve as a reservoir of ions, particularly calcium and phosphate, for maintenance of serum mineral homeostasis; and (4) to serve as an endocrine organ, modulating metabolism. In addition, the bone marrow serves as a source of precursor cells of the hematopoietic and mesenchymal lineage. Bone is a composite material with tough, resilient protein fibers embedded in a hard and rigid mineral matrix (Fig. 1). Its great advantage over sophisticated human-designed composite materials is its ability to repair itself and adapt by biological remodeling and turnover, by altering its mass, shape and properties, to meet the mechanical loads placed on it. Within a single skeletal element there are dramatically different forms of bone, some designed for load bearing, such as the cortical shaft, and some, such as trabecular bone in the metaphysis, providing both a supporting network and a large surface area for metabolic responses to the requirements of mineral homeostasis. Both of these types of bone are evident in the longitudinal section of the human tibia in Fig. 2.

Given its obvious structural role, bone is not always appreciated as one of the more complex and dynamic, living tissues in the body. Its cellular constituents include not only the bone-forming osteoblasts and the bone-resorbing osteoclasts, but also the matrix-embedded osteocytes which maintain communication with one another and with the bone surface from deep within the solid bone matrix through a dense, interconnected network of submicrometer-sized canaliculi. Interactions between bone cells and those of cartilage, blood vessels, teeth, and cells that degrade and/or remodel the extracellular matrix during growth are regulated in part locally, in part systemically, and in part by mechanisms still under intensive investigation.

Bone also contains pools of hematopoietic and mesenchymal stem cells. The osteoblast/osteocyte lineage originates from mesenchymal stem cells, which can also differentiate into other connective tissue cell types, including cartilage or fat. Osteoclasts differentiate from hematopoietic stem cells, primarily under the influence of the growth factor, receptor activator of NF-κB ligand (RANKL). Besides the obvious protection bones provide for brain, spinal cord, and thoracic organs, they also enclose the soft and vulnerable hematopoietic marrow, providing homing signals for colonization by hematopoietic and stromal stem cells. In addition, bone plays host to nerves, arteries, veins, and venous sinuses.

Macroscopic Structural Features

Types of Bone

There are two major types of bones in the skeleton: flat bones located in the axial skeleton (i.e., skull, scapula, mandible, and ilium) and long bones found in the appendicular skeleton (i.e., radius, tibia, femur, humerus, etc.). These two types of bone are distinguished not only by their overall anatomy, but also by their primary mechanism of development. In general, flat bones are formed by a developmental process in which bone-forming cells differentiate directly from the surrounding mesenchyme, a process called intramembranous ossification. Whereas long bones undergo endochondral ossification, in which a cartilage model, or anlagen, is replaced by bone during development and growth, in a sense recapitulating the evolutionary path from a non-mineralized to a mineralized endoskeleton.

Anatomy of Bone and Bone Compartments

The overall structure of a typical long bone is shown in Fig. 2, a longitudinal section of an adult human tibia. The shaft of the bone, called the diaphysis, consists of a hollow cylinder of dense, or compact, bone (C) surrounding a marrow space (Ma).

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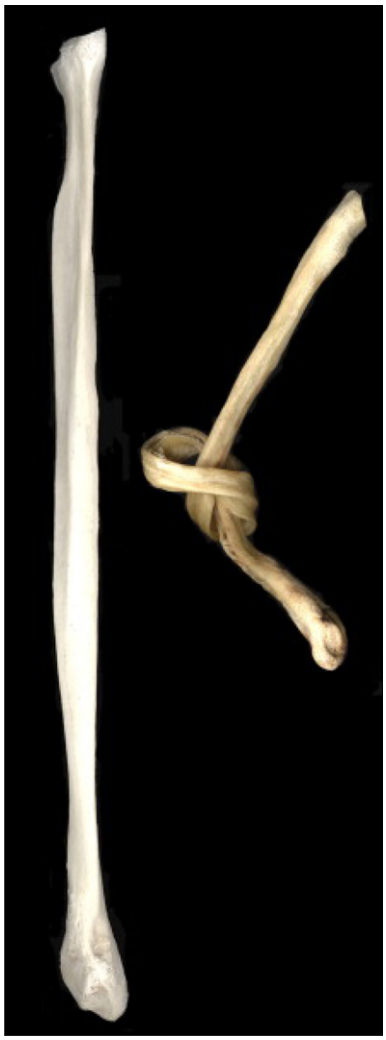

Fig. 1 Composition of bone. Human fibula before (left) and after (right) extraction with ethylenediaminetetraacetic acid (EDTA). EDTA removes the mineral by chelation, and what remains is the tough, fibrous protein matrix, 90% type I collagen. The demineralized bone matrix has very high tensile strength, greater than steel on a weight basis. It has, however, little resistance to compressive or bending forces, enabling it to be tied in a knot. The calcium phosphate crystalline form (hydroxyapatite) that comprises the mineral component has high compressive strength, but alone is brittle and has little resistance to tensile forces. Together they make the hard, somewhat flexible, composite material known as bone.

The bone at this location is also called cortical bone. The external surface of bone is covered by the periosteum and the internal surface (i.e., facing the marrow space) by the endosteum. These are fibro-cellular layers not present on the dried bone specimen in **Fig. 2**. At the end of the bone one sees the widened region called the epiphysis (E) which supports the articular cartilage that forms the cushioned interface of the joint. The region where the bone narrows, or flares, from epiphysis to diaphysis is called the metaphysis (Me). The metaphysis is a key structural and metabolic region that plays a crucial role in endochondral ossification and in mineral homeostasis. The metaphysis is occupied by cancellous, or trabecular, bone, organized in an open network of interconnecting rods and plates of bone (trabeculae) that transmits forces between the joint and the shaft of the bone. The trabeculae are oriented along stress lines and the surface covered with endosteum. As each delicate trabecular arch joins the bone shaft, the cortical bone thickens to bear the additional load being transferred (see **Fig. 2** inset). The crossing arches of bone trabeculae are reminiscent of human architectural solutions to the problem of transmitting the weight of a heavy roof to building's walls while leaving open interior spaces.

Due to its structure, trabecular bone has a much larger surface area exposed to marrow than cortical bone, and because bone resorption and formation occur on bone surfaces, trabecular bone is more metabolically active than cortical bone. As a result, changes in bone due to age, diet, disease or pharmacologic intervention occur sooner and are more pronounced in trabecular compared to cortical bone. Functionally, cortical bone fulfills more of a structural function (mechanical and protective) whereas trabecular bone serves to maintain serum mineral homeostasis, although trabecular bone is essential for transmitting loads from the joint surface to the cortex. In older individuals, cortical bone with high porosity (and therefore high surface area) may also have very high remodeling rates.

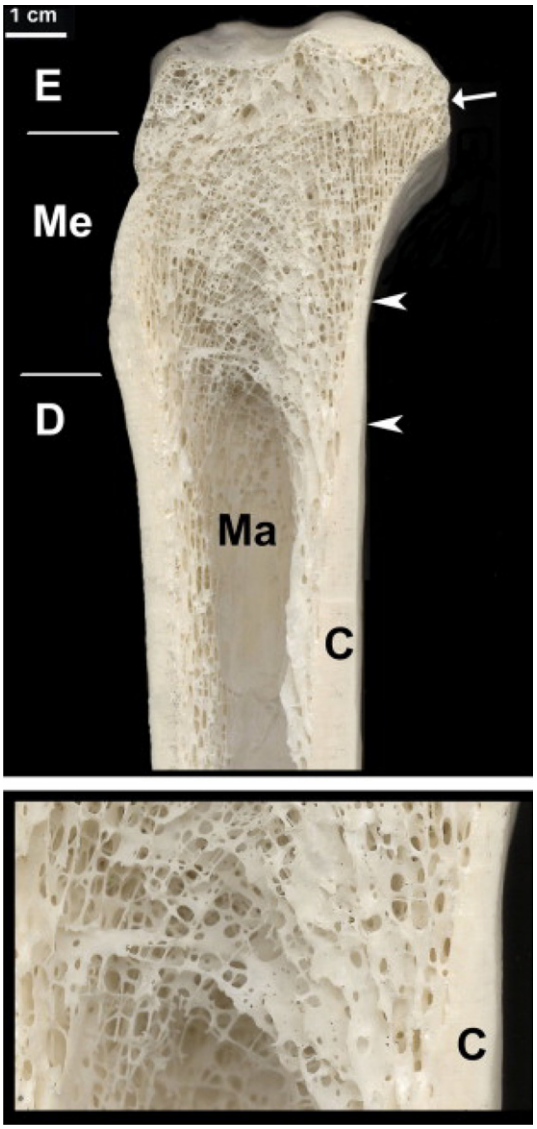

Fig. 2 Structure of a typical long bone. Longitudinal section of adult human tibia with all cellular material removed. Typically, a long bone can be divided into three regions. The epiphysis (E) of the tibia is the widened "knob" that articulates at the knee joint (the joint cartilage is removed in this specimen). The central shaft, or diaphysis (D), is a hollow cylinder of dense, high-strength cortical bone (C) which surrounds a marrow cavity (Ma). Between these two regions, the metaphysis (Me) is filled with a highly anastomosing network of bony trabeculae, that both distributes mechanical load between the joint and the bone shaft and provides an extraordinarily high surface area for metabolic activity. Between the epiphysis and the metaphysis, one can see the epiphyseal line (*arrow*), which is the mineralized remnant of the physis, the cartilage growth plate. In this adult, the growth plate has all turned to bone. The *arrowheads* indicate the area shown at higher magnification in the lower panel, which gives greater detail of the meshwork of metaphyseal trabeculae. Note that as more trabeculae join the cortical bone shaft, thereby transferring higher mechanical load from the joint, there is a corresponding thickening of the cortex (C) to bear the extra weight.

Of equal importance, the metaphysis provides signals and cell types required for the replacement of cartilage by bone during endochondral ossification in growing bones. When bones reach their adult size in humans and other large mammals, the layer of growth cartilage, called the physis or the epiphyseal growth plate, ceases its growth activity and becomes fully mineralized. The ossified remnant of the growth plate is visible as a boundary, called the epiphyseal line, between the epiphysis and metaphysis in Fig. 2 (*arrow*).

At higher magnification in Fig. 2, the inset shows in detail the enormous surface area achieved by the metaphyseal bone spicules. Bone being the body's chief reservoir of calcium and phosphorous, the metaphysis is a site of constant metabolic activity. Upon microscopic examination, during the process of bone remodeling it is common to find both bone formation (by osteoblasts) and bone resorption (by osteoclasts) occurring side-by-side on the same trabecular surface. This demonstrates that bone building and resorption are controlled not only by systemic signals such as hormones or circulating levels of minerals, but also by

local signals reflecting mechanical loading, genetic patterning, and/or microdamage. This dynamic state achieves an active balance that is poised to respond to environmental demands, maintaining mineral homeostasis over the long-term without compromising mechanical needs.

Bone Composition and Microscopic Structural Features

The basic composition of bone consists of cells and an extracellular matrix. The extracellular matrix itself is comprised of water, collagen, other noncollagenous proteins, and mineral. The specific composition of bone varies depending on skeletal sites (i.e., its function), age, diet, and disease. In general, however, the mineral (or inorganic phase) accounts for 60%–70% of the tissue, water accounts for 5%–10% and the organic components comprise the remainder. The calcified bone matrix is not inert, as cells (osteocytes) are embedded in the matrix, and are thought to play a critical role in local activation of bone remodeling.

Bone Cells

There are four principal bone cell types. Bone lining cells are a pool of quiescent, fibroblast-like cells that cover all bone surfaces (**Figs. 4**A and **5**). At least some bone lining cells are derived from previously active osteoblasts that have entered a quiescent phase. When called upon, they may differentiate into osteoblasts (**Fig. 3**B) that secrete the proteinaceous bone matrix called osteoid and subsequently matrix vesicles that deliver the mineral component and initiate the precipitation of hydroxyapatite crystals in the matrix. Note that unlike the nomenclature used for some other cell systems, osteoblasts are fully differentiated and functioning cells. As the layer of bone matrix deposited by osteoblasts thickens, a subset of the osteoblasts becomes fully surrounded by the matrix. Once encased in bone, the cell is called an osteocyte (**Fig. 3**C and **4**D). Each osteocyte inhabits a lacuna within the matrix and maintains contact with other cells, with circulating blood, and with the bone surface via a dense network of cytoplasmic processes that thoroughly permeate the bone matrix (see also section "The Haversian System—Metabolic Lifeline"). Osteocytes are

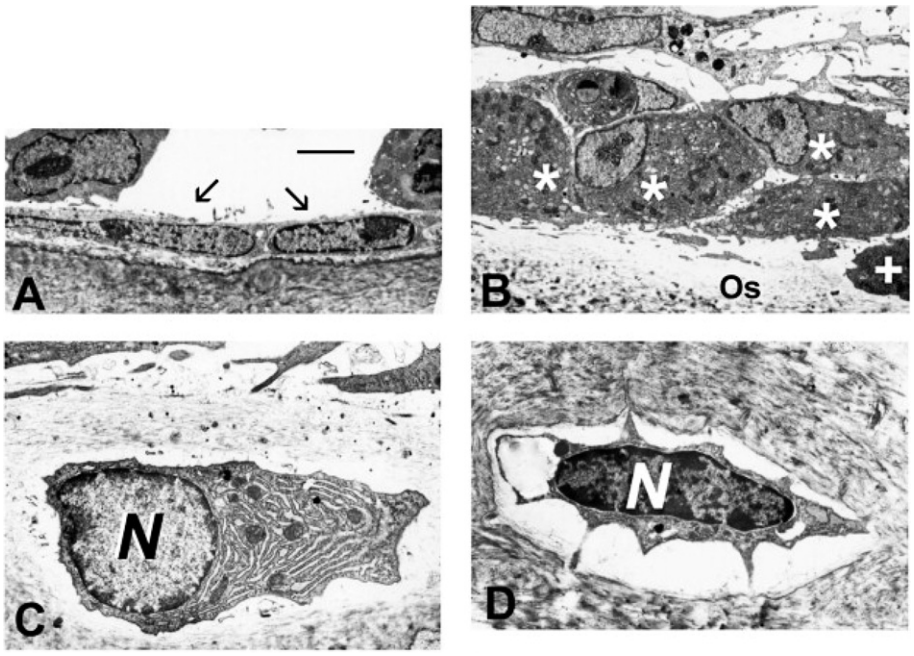

Fig. 3 Life cycle of bone-forming cells. (A) Quiescent, flat bone lining cells (*arrows*) cover the surface of bone. Above is marrow space with marrow cells at the right and left edges. Bone lining cells can differentiate into active osteoblasts when stimulated by parathyroid hormone or other osteogenic signals. (B) Osteoblasts (*asterisks*, *) actively secreting bone matrix. They are roughly cuboidal and contain well-developed organelles needed to synthesize and secrete bone matrix. They lie upon the layer of osteoid (Os) they have just secreted. Part of one osteoblast (+) at the right hand edge of the image can be seen in the process of being entrapped within the bone matrix, so becoming an osteocyte. (C) An osteogenic osteocyte is newly entrapped in bone and is still actively synthesizing matrix components: note the well-developed rough endoplasmic reticulum that fills the cytoplasm. Young osteocytes completely fill the lacuna that they inhabit. The lower margins of osteoblasts on the bone surface can be seen at the top of the image. (D) An osteolytic osteocyte. Late in their life cycle, osteocytes contract and also begin to degrade the surrounding bone matrix, creating unoccupied space within the lacuna. Some of the cytoplasmic projections that permeate the bone matrix can be seen around the cell's margin. Transmission electron micrographs of rat tibia; bar = 3.2 μm in A, 4.1 μm in B, 1.5 μm in C, and 1.7 μm in D. N in C and D = nucleus.

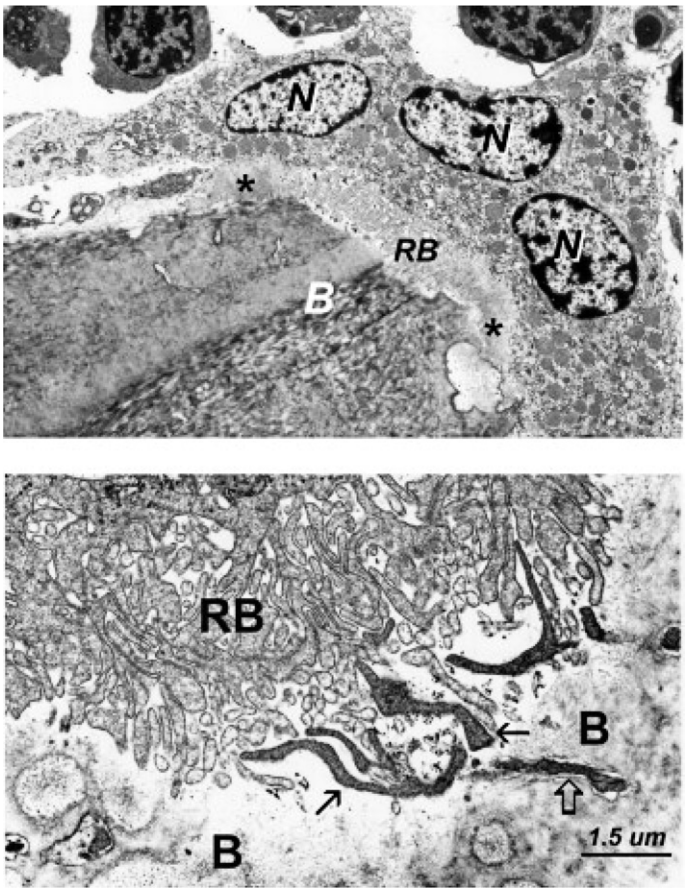

Fig. 4 The osteoclast. Upper panel: Three nuclei (N) are visible in this bone-resorbing osteoclast. It is firmly attached to the bone (B) by the so-called clear zone (*asterisks*), a dense ring of actin filaments that seals off the compartment under the cell where bone resorption occurs, the resorption lacuna. The ruffled border (RB) is a specialized region of highly invaginated plasma membrane where active secretion and resorption take place. Proton pumps in the ruffled border membrane acidify the extracellular compartment to dissolve the mineral, and proteolytic enzymes degrade the proteinaceous matrix. The dissolved bone is engulfed and transported through the osteoclast and released from another specialized domain on the free surface, the facultative secretory domain. Transit time through the cell is on the order of 20 min. Several marrow cells are visible along the top of the image. Lower panel: Higher magnification of a ruffled border reveals details of the elaborate ruffling that occurs over the area of bone (B) being resorbed. Several cytoplasmic processes of osteocytes can be seen in the process of being broken down (*arrows*), with one (*open arrow*) still inside its surrounding canaliculus. Transmission electron micrographs of rat tibia.

by far the most numerous bone cells, comprising 90%–95% of the total number of cells, with osteoblasts second at about 4%–6%, and osteoclasts third at about 1%–2% of cells.

The fourth cell type is the osteoclast (Fig. 4), which differentiates via fusion of mononuclear hematopoietic cells of the monocyte/macrophage lineage. This process occurs on the bone surface and results in very large, highly polarized, multinucleated cells. Osteoclasts form a tight seal against the bone surface, acidify the subcellular compartment (the resorption lacuna) to dissolve the mineral, and secrete proteolytic enzymes, especially cathepsin K and matrix metalloproteinase 9, to dissolve the proteinaceous bone matrix. The solubilized bone is endocytosed by the osteoclast, broken down further, transported through the cell and released from the free surface. The calcium and phosphate thus liberated are important in overall cell and tissue physiology.

Bone Matrix

The extracellular matrix of bone consists of crystalline precipitates of mineral (the hydroxyapatite form of calcium phosphate; see below) that permeate a dense protein substrate. Type I collagen fibrils comprise 90%–95% of the proteinaceous material in bone and provide very high tensile strength, which gram for gram is greater than that of steel. Also present are dozens of other glycoproteins, proteoglycans, integrin-binding proteins, other low-abundance collagens, growth factors, and water. Bone is first laid down as immature, "woven" bone, in which collagen fibrils are oriented randomly (Fig. 5). This bone is later resorbed by osteoclasts and replaced by osteoblasts as mature, lamellar bone, in which the collagen fibrils are ordered. Lamellar bone contains highly oriented type I collagen fibrils that are layered in alternating directions, much as the grain in individual layers of plywood, supplying maximal strength with minimal mass (Fig. 6).

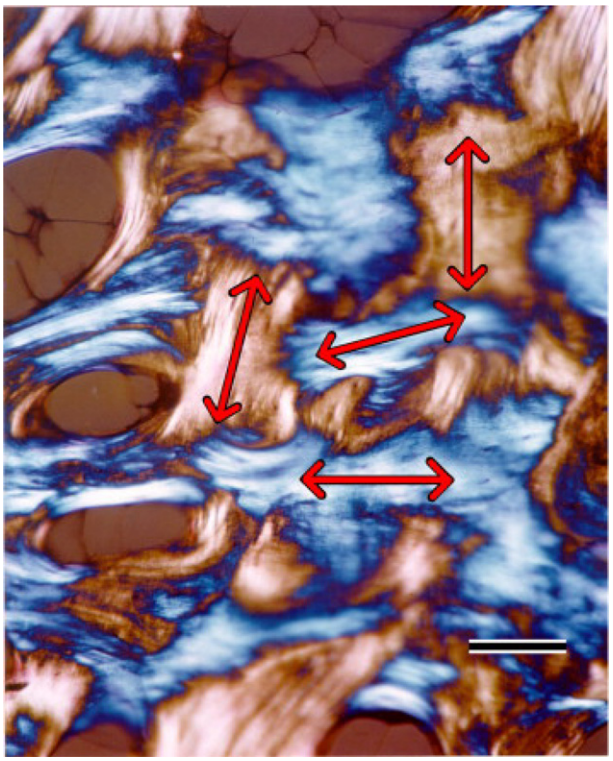

Fig. 5 Woven bone in polarized light (dog mandible). Collagen fibril orientation is visible in light micrographs with polarized illumination. *Double-headed arrows* indicate the orientations of several domains. This "first-generation" bone matrix is brittle and weak, and needs to be resorbed by osteoclasts and replaced by osteoblasts as mature, lamellar bone to attain full strength and resilience. Bar = 75 μm.

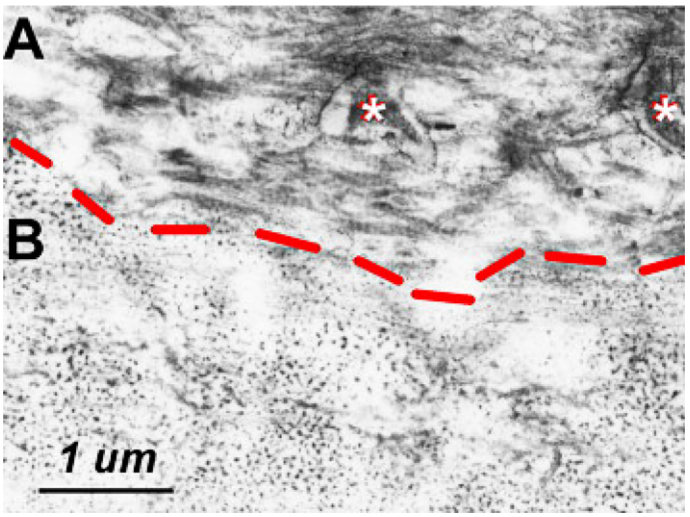

Fig. 6 Collagen fibrils in lamellar bone. Mature lamellar bone is so-called because of the layered, highly oriented collagen fibrils that comprise it, as seen in this electron micrograph. In the upper layer of matrix (A, above the *dashed line*), the cartilage fibrils travel in the plane of the image. In the lower layer (B), they project straight into the plane of the image and are seen end-on as small dots. As a result of this alternating orientation of the collagen fibrils, lamellar bone has much greater strength and resilience than woven bone. Small portions of osteocyte cytoplasmic projections are marked with *asterisks* (*). Transmission electron micrograph, rat tibia.

The processing of collagen needed to assemble this ordered extracellular matrix is complex, beginning within the osteoblast and continuing after secretion. Collagen molecules are assembled in the endoplasmic reticulum (ER) of osteoblasts into right-handed triple helices of highly posttranslationally modified monomers, designated α chains. Type I collagen consists of two α1[I]

chains and one α2[I] chain, each 1050 amino acids long and encoded by different genes, with a middle region rich in glycine, lysine, and proline. At both the amino (N-)- and carboxy (C-)-termini there are registration peptides that are not a part of the triple helix, but are required to align the monomers in proper registration. Within the ER, signal peptides are cleaved off and hydroxyl groups are added to the side chains of proline and lysine residues in hydroxyltransferase-mediated reactions that are vitamin C-dependent. The failure of this pathway in the absence of vitamin C is a major contributor to the pathogenesis of scurvy. Disulfide bonds form between the carboxy-terminal registration peptides of adjacent chains, aligning the monomers. Thus modified and registered, three monomers readily form right-handed, triple helical bundles 300 nm long and 1.5 nm in diameter wound in a C-to-N direction. O-linked sugars are added to some hydroxylysines and N-linked sugars to the terminal regions. The assembled trimers, or "procollagen" molecules, move to the Golgi, where they are packaged into secretory vesicles and secreted.

Once outside the cell, the terminal peptides are cleaved off and the resulting triple-helical molecules are called "tropocollagen." Tropocollagen then polymerizes in aligned bundles, with covalent bonds forming between C- and N-termini of adjacent trimers to form highly ordered collagen fibrils. The periodicity of the overlapping collagen molecules within the fibrils is seen at very high magnification as a banding pattern 67 nm in length (**Fig. 7**). The alternating orientation of the fibrils in successive layers of bone collagen fibrils that is crucial to the mechanical resilience and strength of bone can be seen in electron micrographs (**Fig. 6**). The surface of bone is generally covered by a "capsule" of collagen fibers arranged parallel to the surface, called the circumferential lamella. The exception to this occurs at the points of attachment of tendons and ligaments, whose collagen fibers penetrate the bone surface at an angle and become continuous with the bone collagen fibers. These are called Sharpey's fibers.

In addition to its high collagen content, the proteinaceous bone matrix secreted by osteoblasts (called "osteoid") contains many noncollagenous proteins, about 5%–10% of protein weight. Osteoid provides the microenvironment where mineral precipitates in a controlled way, and it is thought to mediate a wide range of other biological processes (Boskey and Robey, 2013). Among the bone matrix proteins are many growth factors, including the bone morphogenetic proteins (BMPs), members of the TGF-β gene family, some of which are used clinically to induce bone growth and repair. Other growth factors include platelet-derived growth factor, fibroblast growth factor a and b, and connective tissue growth factor. There is vitronectin, critical for integrin binding during osteoclast bone resorption, and a host of proteins having roles in regulating hydroxyapatite crystallization and facilitating remodeling by osteoclasts, such as osteopontin, osteonectin, osteocalcin, and matrix Gla proteins. There is also an assortment of highly glycosylated proteoglycans and glycosaminoglycans, among them aggrecan, perlecan, and hyaluronan, all of which assist in microstructure and water retention, and some of which may "mark" tissue destined to be bone rather than cartilage. Although many of these proteins are synthesized by osteoblasts, some may be absorbed from other sources, bone being a highly absorbent tissue. With such an array of cell regulatory molecules embedded within the bone matrix, it is clear that bone provides an information-rich environment that helps direct and integrate diverse cellular activities such as cell attachment, bone synthesis, collagen assembly, mineral deposition, and bone resorption, as well as responses to environmental cues such as mechanical loading or dietary status. Presumably some of these noncollagenous bone matrix proteins are also essential for the remarkable ability of osteocytes to survive while inhabiting lacunae deep within the matrix, interconnected via cytoplasmic processes that inhabit the profuse networks of cannaliculi, and for the formation and maintenance of the capillary network that thoroughly permeates the entire Haversian system (see below). Identifying specific biological functions for the many noncollagenous bone matrix proteins is an important area of ongoing research.

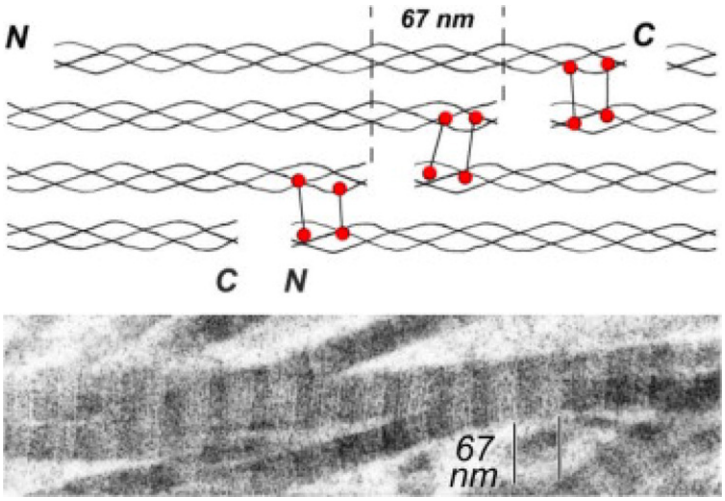

Fig. 7 Type I collagen fibrils in bone. The ends of adjacent tropocollagen trimers are staggered by 67 nm, as diagramed in the upper illustration. The N-terminal region of one trimer forms covalent disulfide bonds (represented as *ball-and-sticks*) with the C-terminal region of adjoining trimers. The spacing between the overlapped ends is seen as a banding pattern in high magnification electron micrographs, as in the lower panel, a transmission electron micrograph of rat tibia. The crosslinked trimers form collagen fibrils that constitute 90%–95% of the proteinaceous component of bone matrix.

Bone mineral is composed mainly of calcium and phosphate in the form of hydroxyapatite, $Ca_4(PO_4)_6(OH)_3$ (**Fig. 8**), which is secreted in matrix vesicles by osteoblasts. Thus, even though mineral deposition takes place outside the cells, it is a cell-regulated process. Matrix vesicles are seen near osteoblasts that supply mineral to the matrix and are roughly 100 nm in diameter. They contain alkaline phosphatase and other enzymes needed to produce high concentrations of calcium and phosphorous. The vesicles rupture, releasing their contents and permitting the precipitation of hydroxyapatite crystals—under the local control of components of the osteoid—that eventually permeate the osteoid completely to form the composite material we call bone. The mineral adds great rigidity and resistance to compressive and bending forces to the skeletal elements (**Fig. 1**). Bone mineralization is not completed immediately after the osteoid is secreted. Mineral accumulates over time, and can take 60 days or longer to be completed.

The crystal structure of bone mineral resembles pyrophosphate (Combes and Rey, 2010, p. 7), a fact which enables an important class of drugs called bisphosphonates—which mimic pyrophosphate in structure and charge—to bind to bone. These drugs are used clinically to block osteoclast bone resorption in osteoporosis and other conditions involving bone loss.

The Haversian System—Metabolic Lifeline

In large mammals, including humans, compact bone such as that found in the shafts of the limb bones, is too thick to permit adequate nutrient, signal, and waste exchange from the surface to sustain the embedded osteocytes. The evolutionary solution to this problem is the Haversian system, in which concentric cylinders of bone surround a nutrient artery and vein located within a central Haversian canal oriented parallel to the long axis of the bone (**Figs. 9** and **10**). The canals are typically about 50 μm in diameter and contain capillaries, and sometimes nerve fibers. The concentric rings of bone surrounding the canals are arranged much like growth rings in trees. One Haversian canal and its accompanying concentric lamellae is called an "osteon." Adjacent Haversian systems are interconnected at frequent intervals by blood vessels that travel in transverse passages called Volkmann's canals. Together, the Haversian and Volkmann's canals penetrate the entire bone substance and maintain communication among all the osteocytes embedded within the cortex. Note that small mammals such as rats and mice do not possess Haversian systems, their bones being small enough to permit survival of osteocytes without intraosseous circulatory elements.

Osteons, like other bony structures, are subject to remodeling. Successive waves of resorption by osteoclasts and formation by osteoblasts leave telltale structural evidence. The osteons most recently formed are generally the least densely mineralized and, more noticeably, have the most complete sets of rings. As new osteons form, they construct new bone in the territory cleared of older osteons by osteoclasts, sometimes overlapping more than one osteon's territory. This phenomenon is shown diagrammatically in **Fig. 9**B. This remodeling is essential to maintain the bones and to repair microdamage that accumulates over time. Metabolic studies have revealed that in the adult human, roughly 10% of the skeleton is remodeled per year. This implies that a complete skeletal replacement occurs about once a decade.

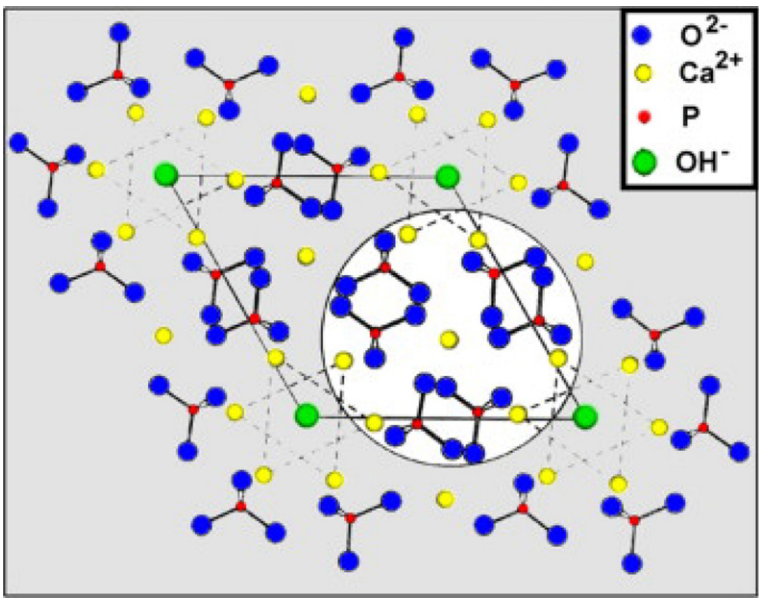

Fig. 8 Hydroxyapatite ($Ca_4(PO_4)_6(OH)_3$). The mineral crystals in bone are complex, with the so-called Posner cluster, having sixfold symmetry, highlighted near the center, and coordinated with the larger crystal structure around four hydroxyl groups marked by the *rhombus*. After Combes, C. and Rey, C. (2010). Amorphous calcium phosphates: Synthesis, properties and uses in biomaterials. *Acta Biomaterialia* **6**, 3362–3378.

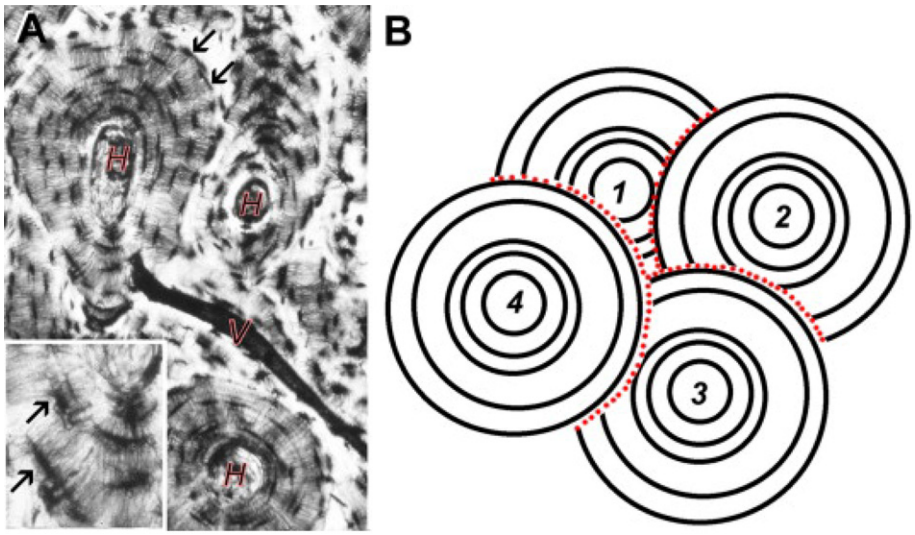

Fig. 9 Haversian systems and osteon remodeling sequence. Panel (A) is a transverse section of human cortical bone, polished and stained with India ink, without any cellular material. Three Haversian canals (H) and their associated structures are visible in the micrograph. A transverse Volkmann's canal (V) is also seen. These canals contain blood vessels that nourish the bone, and some also carry nerves. The concentric ring-shaped patterns, similar to tree rings, are produced by the osteocytes arranged around the central canals. Osteocytes occupy lacunae within the bone, several of which are indicated by *arrows* in the main panel and the inset. The collagen fibrils in successive rings are in alternating orientations. Each Haversian canal and its accompanying concentric rings (Haversian system) is called an osteon. The inset shows the dense meshwork of canaliculi that interconnect the osteocytes to permit transport of nutrients and signaling molecules from the blood vessels that occupy the canals throughout the entire bone volume. The diagram in (B) shows the succession of osteons due to remodeling over time. Osteons are not replaced on a one-to-one basis, but are resorbed and remodeled as controlled by local signals of microdamage, mechanical loading, and other factors. The oldest osteons (in this diagram, osteon #1) has been invaded by osteons 2, 3, and 4. Osteon #4 is the most recently formed, its territory impinging on the others. The intersecting boundaries are indicated by the *red dotted* lines.

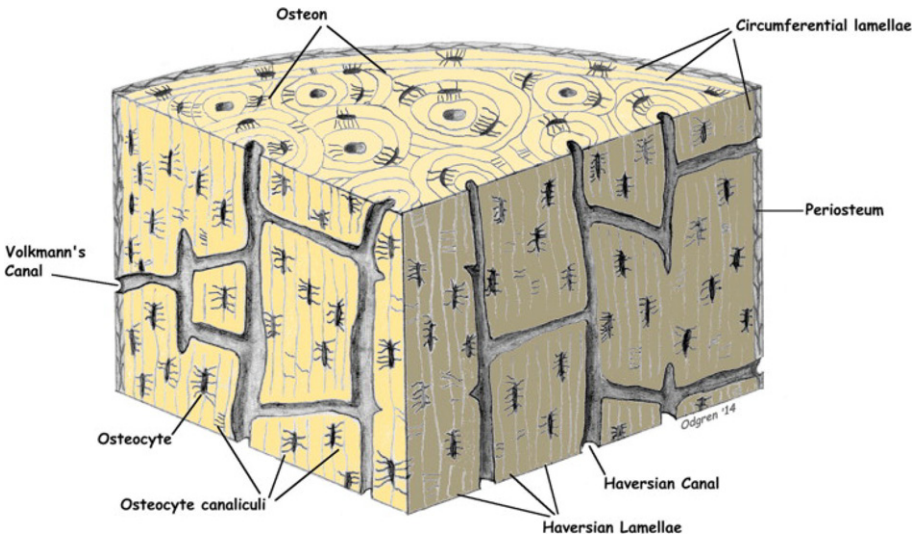

Fig. 10 Illustration showing Haversian bone (also called compact, or cortical, bone). Haversian and Volkmann's canals penetrate the bone, providing contact with the osteocytes that inhabit the bone. Cytoplasmic processes of the osteocytes occupy the canaliculi and permit responses to environmental stimuli such as mechanical loading and circulating levels of hormones, minerals, and other factors. At the inner (not shown) and outer surfaces, bone forms circumferential lamellae, in which the cells are sufficiently near the surface not to require Haversian organization to retain metabolic contact. In each layer of an osteon, the collagen fibrils are in different orientations to maximize tensile strength and flexibility.

Bone Biomechanics and Determinants Bone Strength

Bone is a unique material that has the ability to repair itself and adapt through modeling and remodeling which mediates changes in the traits that influence bone strength. Thus, diseases and drugs that impact bone remodeling will influence bone's resistance to fracture. Bone requires mechanical stress in order to grow and strengthen therefore physical activity is important to develop and

maintain bone strength. A fracture results from a structural failure of the bone, that is likely initiated at the material level, when the loads applied to the bone exceed its load-bearing capacity. Bone is a complex and heterogeneous material that is a consequence of its varied composition and microstructure that differ across anatomic sites and are dependent of age. The ability of a bone to resist fracture (or "whole bone strength") depends on the amount of bone (i.e., mass), the spatial distribution of the bone mass (i.e., shape and microarchitecture), and the intrinsic properties of the materials that comprise the bone (**Fig. 11**). Thus, properties at the cellular, matrix, microarchitectural, and macroarchitectural levels may all contribute to bone strength. Effects on the microscopic level are clinically relevant if they affect mechanical performance at more macroscopic levels.

In any discussion of bone strength, it is important to distinguish between the mechanical behavior of a whole bone as a structure (structural behavior) and the mechanical behavior of the bone tissue (material behavior). During any activity, a complex distribution of forces is applied to the skeleton. With the imposition of these forces, bones undergo deformations. This relationship between the forces applied to the bone and the resulting deformations characterize the structural behavior, or structural properties, of the whole bone. Thus, the size and shape of the bone, as well as the properties of the bone tissue influence structural properties. In contrast to the structural behavior, the material behavior, or material properties, of bone tissue is independent of the specimen geometry. Thus, the material properties reflect the intrinsic biomechanical characteristics of cortical and trabecular bone. Although the biomechanical properties of the whole bone are functionally the most important outcome, assessing bone material properties is critical for understanding the mechanisms that underlie changes in whole bone properties. The factors that are most likely to influence the structural and material behavior of bone will be presented in the sections that follow.

Role of Bone Geometry

The size of bone and its shape (i.e., distribution of mass) have an effect on overall fragility with larger bones being less susceptible to fracture (Bouxsein and Karasik, 2006; Bouxsein and Seeman, 2009; Crabtree *et al.*, 2001; Silva and Gibson, 1997). For example, decreased cross-sectional area of the radius is a risk factor for wrist fracture among both young girls (Skaggs *et al.*, 2001) and postmenopausal women (Ahlborg *et al.*, 2003), and individuals with smaller vertebral bodies have an increased risk of vertebral fracture (Gilsanz *et al.*, 1994, 1995; Duan *et al.*, 2001).

The loads applied to the skeleton generally are a combination of compression or tension forces with bending or torsional moments. The resistance to bending and torsional loading is particularly important because the highest stresses in the appendicular skeletal are due to these loading modes (Martin, 1993). The most efficient design for resisting bending and torsional loads involves distributing the bone mass far from the neutral axis of bending or torsion. The diameter and thickness of cortical bone varies in the skeleton, depending on the types of stresses that are applied to it in each location. For example, the mid-femoral neck adjacent to the shaft is elliptical, with the longer diameter in the superior–inferior direction and with greater cortical thickness inferiorly to minimize bending. On the other hand near the femoral head, stresses are mainly compressive and the geometry reflects this with the femoral neck being more circular and largely trabecular bone, with cortical bone of uniform thickness around its perimeter.

Role of Microarchitecture

The microarchitecture of bone is an important structural property and that is supported by experimental and clinical studies showing altered trabecular and cortical microarchitecture in subjects with fragility fractures compared to controls without fractures (Aaron *et al.*, 2000; Bell *et al.*, 2000; Ciarelli *et al.*, 2000; Crabtree *et al.*, 2001; Fields *et al.*, 2009; Legrand *et al.*, 2000; Thomas *et al.*, 2009).

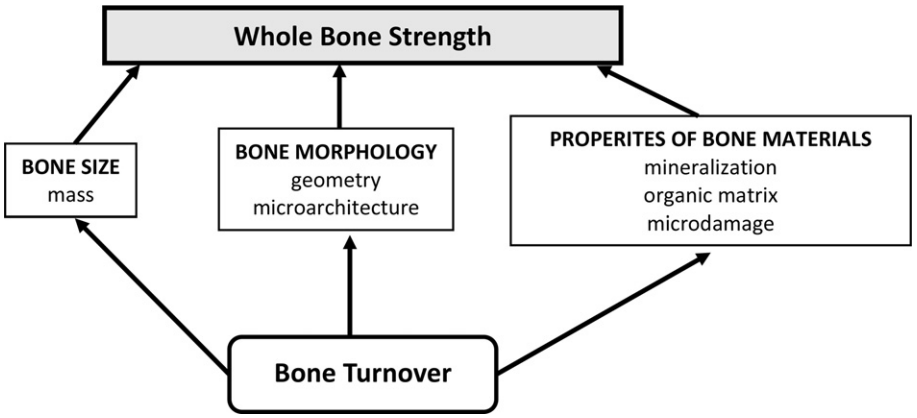

Fig. 11 Determinants of whole bone strength. Bone size, morphology, and bone tissue material properties contribute to whole bone strength. Bone turnover, or remodeling, is a crucial factor in controlling the quality and the quantity of bone.

Although bone density is among the strongest predictors of the mechanical behavior of trabecular bone, both empirical observations and theoretical analyses show that aspects of the trabecular microarchitecture influence trabecular bone strength as well (Gibson, 1985; Keaveny *et al.*, 2001; Rice *et al.*, 1988; Ulrich *et al.*, 1997, 1999). Trabecular bone microarchitecture can be described in terms of the number of trabeculae in a given volume, their average thickness, the average distance between adjacent trabeculae, the degree to which trabeculae are connected to each other, and their orientation relative to a given loading direction. The trabeculae are arranged in vertical and horizontal direction, the latter elements being critical to the bone's strength. Trabecular tissue can be modeled as a network of interconnected beams (horizontal trabeculae) and columns (vertical trabeculae) whereas the horizontal elements have the function of connecting and supporting the structure. A decrease in number of trabeculae reduces strength whereas loss of horizontal trabecular elements leads to more reduction in strength because it will markedly decrease the structure's buckling strength. Reduced strength due to the thinning of the trabeculae can be reversed with the appropriate treatment. Whereas, if the disconnectivity between the trabeculae (horizontal trabeculae disappear) increases the loss of resistance becomes irreversible and reduces the strength of the structure to a greater degree than accounted for by the loss of bone mass alone (**Fig. 12**). There is an association between the risk of fracture and the anisotropy (that is its mechanical properties are dependent upon direction of loading) of trabecular bone tissue. For example, after controlling for bone volume, trabecular bone architecture of specimens from the femoral head of individuals who had suffered a hip fracture was more oriented in a single direction than bone from unfractured individuals (Ciarelli *et al.*, 2000).

Cortical bone microarchitecture, such as cortical thickness and porosity, also play important roles in bone strength and skeletal fragility. Several studies, both in vitro (Bell *et al.*, 2000; Bousson *et al.*, 2001, 2004; Norman *et al.*, 2008; Zebaze *et al.*, 2010), and more recently with high resolution in vivo imaging (Burghardt *et al.*, 2010a,b; Macdonald *et al.*, 2011; Nishiyama *et al.*, 2010; Zebaze *et al.*, 2010), found marked increases in cortical porosity with aging due to accelerated remodeling (Zebaze *et al.*, 2010).

Role of Bone Matrix Properties

The features of the bone matrix itself influence bone mechanical properties. Thus, characteristic that affect bone mechanical properties include (but are not limited to) the composition of the matrix, the relative ratio of inorganic (i.e., mineral) to organic (i.e., water, collagen, and noncollagenous proteins); the degree of matrix mineralization; mineral crystal size, and maturation; the extent and nature of collagen cross-links; and the amount and nature of matrix microdamage (Burr, 2003; Follet *et al.*, 2004; Ottani *et al.*, 2001). The stiffness and strength of bone are positively related to the degree of matrix mineralization (Follet *et al.*, 2004), while collagen provides bone ductility and toughness.

Role of Bone Turnover

Bone is a complex living tissue that undergoes constant renewal to repair damage and adapt to mechanical loading in a healthy individual. The function of bone modeling and remodeling then influences the factors that determine whole bone strength, that is, bone size and shape, microarchitecture, and material properties. Bone turnover, or remodeling, is a crucial factor in controlling the quality and the quantity of bone. An imbalance between bone resorption and bone formation will either results in a net loss or gain of bone tissue. The onset of menopause is associated with increased bone turnover rate which leads to bone loss and an abnormal bone microarchitecture because of the cessation of estrogen production. Estrogen deficiency increases the lifespan of osteoclasts so that more bone is resorbed than is formed. This leads to increased porosity, bone thinning, and disconnection of trabeculae, ultimately resulting in a decrease in bone strength.

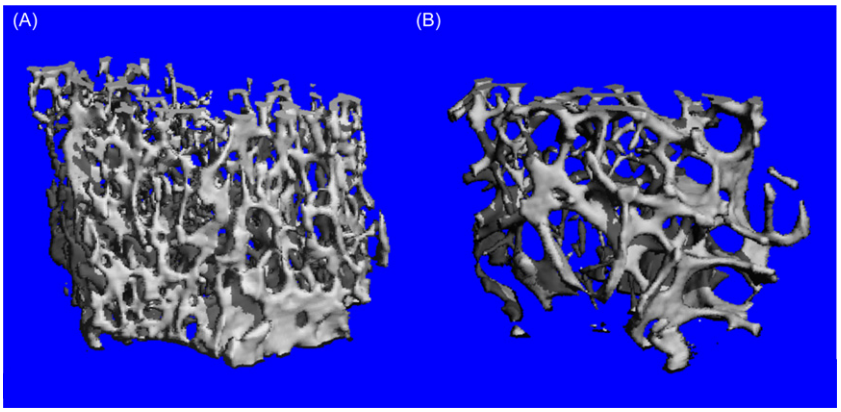

Fig. 12 Age-related changes in tibial trabecular architecture. 3D models of mouse tibial trabecular bone made from high resolution micro-CT. (A) 8 weeks old mouse (B) 52 weeks old mouse. Note the progressive loss of trabeculae, leading to a wider separation of trabecular elements, and unsupported vertical trabeculae.

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Bone and Bone Marrow; Interactions

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Abbreviations

BM	Bone marrow
C/EBPα	CCAAT/enhancer binding protein α
CEBPδ	CCAAT/enhancer binding protein δ
CSF	Colony-stimulating factor 3
CXCL12	C–X–C motif chemokine 12
Dex	Dexamethasone
DHA	Docosahexaenoïc acid
ECM	Extracellular matrix
EPO	Erythropoietin
FA	Fatty acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSC	Hematopoietic stem cells
IBMX	Isobutylmethylxanthine
IGF-1	Insulin growth factor-1
IL	Interleukin
LIF	Leukemia inhibitory factor
M-CSF	Macrophage-colony stimulating factor
MUFA	Mono-unsaturated fatty acid
NF-κB	Nuclear factor-kappa B
Osx	Osterix
PPARγ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acid
RANKL	Receptor activator of NFκB ligand
Runx2	Runt-related transcription factor 2
SFA	Saturated fatty acids
sFRP-1	Frizzled-related protein 1
SSC	Skeletal stem cells
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α

Introduction

Bone and bone marrow (BM) function closely together as a single entity, exhibiting functional, and structural interdependences. They form a unique system implicating cooperation and interactions of different cell types that are critical for the equilibrium of hematopoiesis and the maintenance of skeletal health.

Bone and Bone Remodeling

Bone is a mineralized connective tissue that constitutes the skeleton, together with the cartilage. In adult humans, the skeleton is composed of 80% cortical bone and 20% trabecular bone and, despite its inert appearance, it is completely renewed every 10 years (Bartl and Frisch, 2009; Clarke, 2008).

Cortical bone is a 90% calcified dense and compact tissue, with an annual remodeling rate of approximately 2.5%. By contrast, trabecular bone presents a spongy, honeycomb-like network that provides the structural support for BM, filling the intertrabecular spaces. Its renewal rate is elevated, reaching 25% per year.

The ratio of cortical to trabecular bone is not constant throughout the skeleton and any decrease in bone mass is therefore firstly manifest in bones containing a large fraction of trabeculae, as the vertebral column and the intertrochanteric region of the proximal femur which present a cortical to trabecular bone ratio of 25:75 and 50:50, respectively (Bonnick, 2010; Hunter and Sambrook, 2000).

Bone exhibits four programmed cell types (i.e., osteoblasts, bone lining cells, osteocytes, and osteoclasts) which cumulate their activities to assure a well-balanced system. Bone formation and bone resorption are indeed highly regulated processes involving complex interactions between osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells. These intercellular communications are crucial for the maintenance of skeletal integrity as their disturbance has been shown to result in abnormal bone mass and/or density and to lead to well-known bone diseases such as osteonecrosis, osteoporosis, and osteopetrosis.

Osteoclasts are highly specialized cells originated from a mononuclear hematopoietic precursor of the monocyte-macrophage lineage. Once activated, the osteoclastic precursors fuse to form a multinucleated osteoclast, which attaches to the endosteal bone surface and starts the bone resorption process (Baron, 2008).

Osteoblasts originate from a particular subset of BM stromal cells, the skeletal stem cells (SSCs), pluripotent cells that are both skeletal precursor cells and paracrine regulators of the BM microenvironment (Sacchetti *et al.*, 2016; Sworder *et al.*, 2015; van der Eerden and van Wijnen, 2017). SSCs are capable to differentiate into various cells of the mesenchymal lineages such as osteoblasts, adipocytes, and chondrocytes, depending on the presence of multiple environmental factors (Owen and Friedenstein, 1988; Prockop, 1997; Pittenger, 1999). Of note, although SSCs are a limited cell population, representing 0.001%–0.01% of the BM cell types (Pittenger, 1999), they remain one of the most promising types of stem cells used for cell-based therapies, already serving as therapeutic agents in a variety of diseases (Prockop, 2007; Wei *et al.*, 2013).

The principal function of osteoblasts is to synthesize the extracellular bone matrix and to further promote its mineralization. To fulfill their physiological role, osteoblasts do not function individually but are in contact with each other through gap junctions assuring intercellular communications.

Once the process of bone formation is achieved, most osteoblasts become gradually enclosed in the bone matrix and differentiate into osteocytes, the bone cells acting as mechanosensors and controlling the activity of bone forming and resorbing cells (Bonewald, 2011). Concomitantly, a small population of osteoblasts become flattened and die by apoptosis or remain as quiescent lining cells sited at the endosteal surface of bone (Florencio-Silva *et al.*, 2015). These bone lining cells are located on inactive bone surfaces, where neither formation nor resorption of bone occur (Miller *et al.*, 1989). Their physiological function is not completely understood, but they seem to play an important role in the process of bone remodeling since their activity was described as an essential prerequisite for subsequent bone formation. Indeed, by cleaning bone surface not adequately resorbed by osteoclasts and by depositing a thin collagenous matrix, bone lining cells prepare the surface where osteoblasts could start bone matrix production and mineralization (Everts *et al.*, 2002). Of note, during adulthood, quiescent bone lining cells may be activated by parathyroid hormone or nitric oxide and constitute a major source of osteoblasts (Matic *et al.*, 2016).

As osteoblasts and osteocytes, bone lining cells communicate via gap junctions (Prideaux *et al.*, 2016) allowing them to propagate a triggering signal that will initiate bone remodeling via recruitment of bone resorbing and forming cells.

Besides their principal role, osteoblasts regulate osteoclast maturation by the release of soluble factors (e.g., M-CSF, RANKL, IL-6, IL-1, TNF-α) that will create a microenvironment supporting osteoclastogenesis (Feng, 2009; Suda *et al.*, 1999). During the process of bone resorption, osteoclasts liberate factors (e.g., TGF-β, IGF-1) inhibiting their own activity and activating osteoblast function (Mohamed, 2008; Phan *et al.*, 2004).

The remodeling process is thus a coordinated series of events where osteoblasts and osteoclasts are coordinately regulated to assure an adequate coupling between bone formation and resorption.

Chondrocytes, originating also from SSCs, have a dual function as they regulate cartilage synthesis and degradation. The process of chondrogenesis requires aggregation and condensation of SSCs expressing specific extracellular matrix (ECM) and cell adhesion molecules. After proliferation, those particular SSCs differentiate into chondrocytes and start the production of cartilage-specific ECM molecules (e.g., aggrecan and type 2 collagen) that enlarge the cartilaginous shape (Goldring, 2012; Zuscik *et al.*, 2008). Chondrocytes are responsible for the turnover of the extracellular cartilaginous matrix in their surrounding area by regulating the synthesis of its major constituents and by producing cytokines and enzymes that will degrade it (e.g., IL-1β, TNF-α, IL-6, matrix metalloproteinases) (Goldring *et al.*, 2011; Tetlow *et al.*, 2001).

In case of joint disease, the equilibrium between cartilage synthesis and degradation is disrupted, supporting loss of ECM molecules and leading therefore to irreversible alteration of the cartilage, due to the limited replication potential of chondrocytes (Fox *et al.*, 2009). In such circumstances, cartilage turns into bone via the process of endochondral ossification. Chondrocytes proceed to terminal differentiation, increasing in size and leading to their hypertrophy. Subsequently, hypertrophic chondrocytes undergo apoptosis, generating cavities used by blood vessels to invade the remaining cartilaginous structure. Using the blood stream, SSCs enter the vascularized cartilage and invade the free spaces, where they differentiate into osteoblasts. The cartilage matrix serves then as a scaffold for endochondral bone formation (Goldring, 2012; Zuscik *et al.*, 2008).

Bone remodeling is therefore a complex process involving interconnected signaling between the four different types of bone cells, cooperating closely together to maintain a balanced bone mass (Sims and Martin, 2014). Moreover, since bone turnover occurs mainly at the endosteal surface where bone and bone marrow interact, commitment, proliferation, and differentiation of bone cells are also regulated by local signals and factors released by other BM surrounding cells (**Fig. 1**).

Bone Marrow and the Bone Marrow Niches

BM is one of the most dynamic and large tissues of the mammalian organism. It is a soft tissue located in the cavities of trabecular bones, presenting highly vascularized areas due to presence of a rich sinusoidal network of capillaries (Wang, 2012). BM is a complex organ, divided in arrays characterized by different cell populations that are interdependent (Heideveld and Van Den Akker, 2017). It contains two distinct lineages, the hematopoietic and the nonhematopoietic lineage, from which osteoclasts and osteoblasts originate, respectively. Therefore, BM exists in two appearances, varying in composition and function, the so-called red (hematopoietic) and yellow (nonhematopoietic) BM.

Red BM contains hematopoietic stem cells (HSC), the blood progenitor cells, and adipocytes; its red color is due to the presence of hemoglobin in erythroid cells. It is mainly devoted to hematopoiesis, contrary to yellow marrow, though to be made of 80% fat with few hematopoietic components (Małkiewicz and Dziedzic, 2012).

In mammalian fetus, BM is almost entirely red, since it is primarily composed of hematopoietic cells and does not contain fat. At birth, the conversion of red to yellow marrow starts, red marrow being subsequently replaced by yellow marrow following a well-defined pattern that evolves from the peripheral to the central skeleton (Gurevitch *et al.*, 2007). At the third decade of life, BM cavities are filled by 50%–70% yellow marrow (Moerman *et al.*, 2004; Rosen *et al.*, 2009).

In vertebrates, BM serves as the principal active site for hematopoiesis, allowing maintenance of homeostatic levels of circulating blood cells (Calvi and Link, 2014). HSC possess the ability to differentiate into mature blood cells and the capacity of self-renewal, a property essential to assure a permanent pool of stem cells, generating a continuous production of mature blood cells (Calvi and Link, 2014).

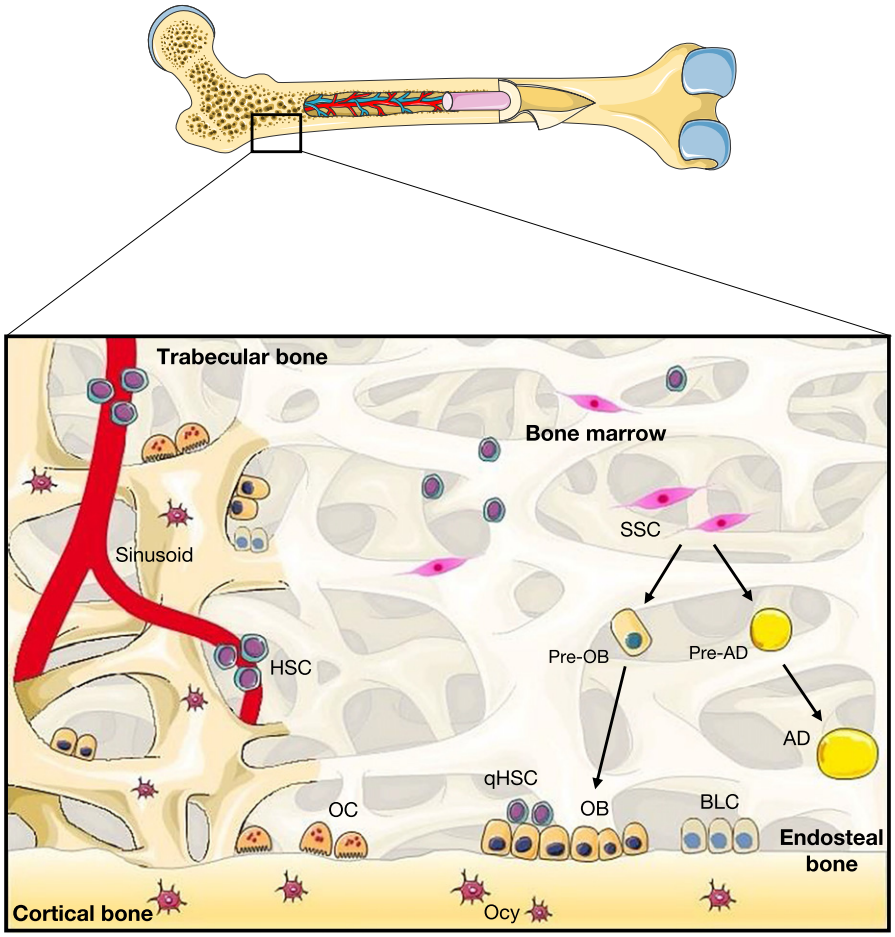

Fig. 1 Interactions in the bone marrow microenvironment. Bone marrow is the main place for hematopoiesis and for bone formation/resorption. Bone and bone marrow function closely together, as a single entity, exhibiting functional, and anatomical interdependences. Cells originated from different lineages communicate closely together to maintain a well-balanced microenvironment essential for normal hematopoiesis and osteogenesis. Bone marrow contains skeletal stem cells (SSC) that can differentiate into pre-osteoblasts (pre-OB) or pre-adipocytes (pre-AD), which mature to become fully functional (OB and AD). Endosteal bone surface principally includes bone-forming cells (OB) and bone-resorbing cells (OC), as well as osteocytes (Ocy), and bone lining cells (BLC). Quiescent hematopoietic stem cells (qHSC) are retained next the endosteum, in close contact with osteoblasts, whereas committed progenitors and differentiated hematopoietic cells (HSC) are localized near the perisinusoidal regions. Illustration realized thanks to Servier Medical Art (https://smart.servier.com/).

Mature HSC are located in a highly vascularized area of the BM due to the presence of a dense network of sinusoids and arterioles (Boulais and Frenette, 2015). Quiescent HSC are retained next to the endosteal bone surface, in close contact with osteoblasts that produce factors maintaining them in an undifferentiated state (**Fig. 1**). Physiological stress induces rapid mobilization of quiescent HSC and their migration into sinusoids. Once released into the vascular system, they home to particular organs and either restore their function or regenerate the stem cell pool (Heissig *et al.*, 2002). Therefore, the existence of two distinct HSC specialized microenvironments (also called "niche") generated by cell-dependent interactions has been proposed: the osteoblastic and the vascular niche (Del Fattore *et al.*, 2010; Yin and Li, 2006). The osteoblastic niche provides a quiescent microenvironment for HSC maintenance, while the vascular niche allows proliferation and differentiation of the blood cell precursors and their migration into blood vessels.

Bone and BM Cellular Interactions

Cellular Interactions Between Bone and Hematopoietic Cells

Cell populations sited in the BM are closely associated and communicate with each other. These reciprocal relationships are essential for maturation of both SSCs and HSC and, *in fine*, for maintenance of hemopoiesis and skeletal homeostasis. The cellular mechanisms involved in these interactions are not yet completely elucidated, but a large number of studies have already documented their close and intricate association.

Homeostasis of the hematopoietic system is maintained by highly regulated interactions among HSC and their microenvironment (Pontikoglou *et al.*, 2011). Numerous studies have already shown that SSCs as well as cells of the osteoblastic lineage, besides maintaining bone homeostasis, are fundamental regulators of hematopoiesis. They participate to the development of the HSC niche by releasing factors that support HSC migration, homing, self-renewal, proliferation and differentiation (Aqmasheh *et al.*, 2017). Clinical studies have shown that co-administration of SSCs and HSC promote hematological engraftment and prevent engraftment failure as well as poor graft function, making SSCs attractive candidates for cellular therapy in immune-based disorders, especially when HSC transplantation is needed (Koç *et al.*, 2000; Pontikoglou *et al.*, 2011; Zhao and Liu, 2016).

Likewise, a subset of specialized immature osteoblasts located in the endosteum is likely to play an important role in hematopoiesis by regulating function and maintenance of the hematopoietic progenitor cells. These particular osteoblasts support expansion of immature HSC and secrete factors (e.g., IL-6, IL-7, EPO or GM-CSF) regulating their activity (Calvi, 2006; Fazzi *et al.*, 2004; Zhang *et al.*, 2003; Rankin *et al.*, 2012; Taichman, 1994; Visnjic *et al.*, 2004). As well, HSC direct SSCs differentiation toward the osteoblastic lineage (Jung *et al.*, 2008). A cross-talk between HSC and osteoblastic cells seems thus to be essential for the optimal development of both populations.

Osteoclasts are also involved in the mobilization of hematopoietic progenitors. In a murine model, it was shown that inhibition of osteoclast differentiation severely affects the HSC pool and that functional recovery of the osteoclasts reestablishes the maintenance of the HSC niche, probably through an indirect effect on osteoblast turnover (Lymperi *et al.*, 2011).

The influence of BM adipocytes (BM-adipocytes) originating from SSCs, on the hematopoietic niches is still debated. On one hand, they produce factors (e.g., leptin and adiponectin) impairing expansion of the hematopoietic progenitors (Laharrague *et al.*, 2000; Yokota *et al.*, 2000). BM-adipocytes also negatively influence hematopoiesis by releasing growth factors inhibiting HSC proliferation such as neuropillin-1 and lipocalin 2 (Ambrosi *et al.*, 2017; Belaid-Choucair *et al.*, 2008; Miharada *et al.*, 2008; Naveiras *et al.*, 2009). On the other hand, the recent study of Mattiucci and coworkers proposes that BM-adipocytes support HSC survival and could play a role in their steady state maintenance by producing critical molecules such as C–X–C motif chemokine 12 (CXCL12), IL-8, colony-stimulating factor 3 (CSF3), and leukemia inhibitory factor (LIF) (Mattiucci *et al.*, 2017).

In physiological conditions, the various factors secreted by the different hematopoietic and nonhematopoietic cell types create a well-balanced BM microenvironment that is essential for optimal hematopoiesis and osteogenesis. An inadequate production of some of those factors, such as factors released by BM-adipocytes, could destabilize the system and disturb bone and red marrow interactions, leading to alteration of hemopoiesis and/or osteogenesis.

Cellular Interactions Between Bone and Nonhematopoietic Cells

In mammalians, BM is the only place where bone and fat (i.e., BM-adipocytes) are in close contact. Initially described as passive occupants and space fillers, BM-adipocytes are now recognized as an active organ that modulates the function and survival of neighboring cells by producing and secreting factors acting in paracrine and autocrine fashions.

In humans, adipocytes constitute the most abundant stromal cell population found in the BM at adult age. Like osteoblasts, BM-adipocytes originate from the pluripotent SSCs and a well-balanced differentiation of SSCs into the osteoblastic or adipocytic lineage is crucial for the maintenance of bone homeostasis (Muruganandan *et al.*, 2009).

In bone diseases such as osteonecrosis and osteoporosis, a shift toward a preferential differentiation of SSCs into adipocytes at the expense of the osteoblastic lineage is described, leading to excessive accumulation of BM-adipocytes in BM and decreased number of functional osteoblasts. The impact of abnormal BM-adipocytes accretion on skeletal integrity is currently under active investigation (Hardouin *et al.*, 2016; van der Eerden and van Wijnen, 2017) and it is proposed that adipocytes accumulated in the BM modulate bone remodeling through a paracrine action, influencing the physiology of osteoprogenitor and mature bone cells (Abdallah, 2017; Zhu *et al.*, 2015) as well as the activity of bone resorbing cells (Drosatos-Tampakaki *et al.*, 2014).

The impact of adipokines and cytokines secreted by BM-adipocytes on their surrounding cells is well documented (Kawai *et al.*, 2012; Lecka-Czernik, 2012; Rosen *et al.*, 2009).

Leptin, an adipokine secreted by BM-adipocytes, exerts complex effects on the skeleton (Ducy *et al.*, 2000; Hamrick *et al.*, 2005; Hamrick and Ferrari, 2008). Osteoblasts and osteoclasts express the leptin receptor, allowing a direct action of the adipokine on both cell types (Lecka-Czernik, 2012; Upadhyay *et al.*, 2015), in vitro studies having demonstrated that it promotes osteoblastic proliferation and differentiation and inhibits osteoclastogenesis (Cornish *et al.*, 2002). In vivo studies examining the impact of leptin administration or using leptin-deficient mice have highlighted its indirect inhibitory action on bone formation, that is, through the central nervous system via a hypothalamic relay (Ducy *et al.*, 2000; Takeda *et al.*, 2002).

As for leptin, the influence of adiponectin on bone cells is controversial. Most in vitro studies suggest that adiponectin has a positive action on bone formation, enhancing osteoblast proliferation and differentiation while inhibiting osteoclastogenesis. As such, mice treated with adiponectin show increased trabecular bone mass and mineralized osteoblasts, accompanied by a decreased number of functional osteoclasts (Oshima *et al.*, 2005). However, human clinical studies indicate an inverse relationship between circulating adiponectin levels and bone mass density (Chen *et al.*, 2017; Kajimura *et al.*, 2014; Kanazawa *et al.*, 2007, 2009; Luo *et al.*, 2005; Naot *et al.*, 2017).

Chemerin, a more recently identified adipokine, was shown to promote adipogenic differentiation while inhibiting osteoblastic differentiation (Muruganandan *et al.*, 2017).

BM-adipocytes also produce pro-inflammatory cytokines such as IL-1β, IL-6 or TNF-α which have been shown to increase osteoclast activity (Cao *et al.*, 2010; Suda *et al.*, 1999).

Moreover, they inhibit osteoblastic differentiation by blocking BMP2-induced osteoblastogenesis and by activating the proinflammatory NF-κB signaling pathway (Abdallah, 2017). BM-adipocytes secrete frizzled-related protein 1 (sFRP-1), an inhibitor of the Wnt signaling pathway, and co-culture experiments demonstrated that depletion of sFRP-1 abolishes this anti-osteoblastic effect (Taipaleenmäki *et al.*, 2011). Interestingly, in humans, BM-adipocytes display the particularity to express RANKL and OPG, and the RANKL/OPG ratio increases following dexamethasone treatment (Goto *et al.*, 2011; Hozumi *et al.*, 2009), a situation resembling that observed in mature osteoblasts. In mice, RANKL expression rises while OPG is reduced throughout adipogenesis and aging, an observation further supporting the link between adipogenesis and osteoclastogenesis (Takeshita *et al.*, 2014).

If the influence of cytokines and adipokines on skeletal health is documented by numerous studies, the impact of free fatty acids released by BM-adipocytes on bone cells was only deeply investigated during the last decade.

Maurin *et al.* were the first to demonstrate that co-culture of human osteoblasts and adipocytes isolated from mammary gland inhibits osteoblast proliferation via the release of arachidonic acid (C22:4) and docosahexaenoic acid (DHA; C22:6), two members of the omega-6 polyunsaturated fatty acid (PUFA) family (Maurin *et al.*, 2000, 2002). Later in vitro studies documented that human BM-adipocytes release large amounts of, palmitate (C16:0) and stearate (C18:0), two saturated fatty acids (SFA) disturbing the differentiation process and survival of SSCs and osteoblasts (Elbaz *et al.*, 2010; Lu *et al.*, 2012; Wang *et al.*, 2013).

Fatty acids serve as key sources of metabolic fuel in physiological conditions but it is recognized that a chronic exposure to high concentration of SFA favors their abnormal cellular accumulation and triggers cell dysfunction and organ injury through a process called lipotoxicity (Unger, 2002). As such, long-chain SFA are well known cytotoxic agents for several cell types including insulin secreting pancreatic β cells (Unger and Zhou, 2001), hepatocytes (Hetherington *et al.*, 2016), and cardiomyocytes (Kong and Rabkin, 2000).

We and others further characterized the molecular mechanisms responsible for the toxicity of palmitate in human SSCs and osteoblasts; furthermore, we demonstrated that the mono-unsaturated fatty acid (MUFA) oleate (C18:1) protects these bone cells from lipotoxicity (Fillmore *et al.*, 2015; Gillet *et al.*, 2015). Interestingly, unlike its well-known deleterious action in numerous cell types, palmitate is not cytotoxic for osteoclasts and enhances osteoclastogenesis while oleate counteracts palmitate-induced osteoclastogenesis (Drosatos-Tampakaki *et al.*, 2014).

SFA and MUFA have thus opposite actions on osteoblasts and osteoclasts and BM-adipocytes expansion could therefore create an imbalance between bone formation and bone resorption that may alter skeletal integrity, leading to bone loss, through the release of SFA.

In line with this proposal, an elevated SFA dietary intake has been reported to be associated with higher risk of bone loss in murine models (Chen *et al.*, 2016) as well as in humans (Corwin *et al.*, 2006) while investigations performed on Mediterranean population have shown a positive correlation between MUFA dietary intake and bone mass density (Pérez and Velasco, 2014; Savanelli *et al.*, 2017; Trichopoulou *et al.*, 1997). Human and animal studies have also highlighted the beneficial effect of an appropriate consumption of omega-3 PUFA on bone metabolism (Farina *et al.*, 2011; Li *et al.*, 2010; Sun *et al.*, 2003) while omega-6 PUFA appear to negatively impact bone remodeling (Casado-Díaz *et al.*, 2012; Kruger *et al.*, 2010; Orchard *et al.*, 2013). The clinical trial VITAL is currently investigating the effects of omega-3 fatty acid supplements on bone health and fracture risk (LeBoff *et al.*, 2015).

Disturbance of Bone and BM Interactions

Due to their particular secretory activity, disproportionate accumulation of BM-adipocytes could impair the concerted network signaling required to establish optimal bone remodeling or regeneration and, therefore, be implicated in the evolution of bone diseases characterized by BM fat accumulation such as osteoporosis and osteonecrosis.

BM-adipocytes and osteoblasts originate from SSCs. Accumulated evidence indicates that commitment of SSCs to the different lineages is directed by multiple cellular and molecular signals implicating critical transcriptional factors and that biological, chemical and physical factors regulate the balance between adipogenic and osteoblastic differentiation.

In vitro, the stemness of freshly isolated SSCs is therefore determined using well-established culture medium containing specific chemicals.

The couples PPARγ-C/EBPα and Runx2-osterix (Osx) are key transcriptional regulators of adipocyte and osteoblast differentiation, respectively (Zhang *et al.*, 2012).

To induce adipogenic differentiation, SSCs are usually cultured in medium supplemented with isobutylmethylxanthine (IBMX), indomethacin, dexamethasone (Dex), and insulin, factors that will, inter alia, stimulate genetic expression and activity of the transcriptional factors PPARγ and C/EBPα (Chen *et al.*, 2016; Pittenger, 1999).

Osteogenic medium contains Dex, L-ascorbic acid, and β-glycerophosphate (Pittenger, 1999), allowing cells to synthetize an extracellular matrix and further leading to upregulation of Runx2, Osx, alkaline phosphatase and osteocalcin expression, the major markers of osteoblast differentiation (Chen *et al.*, 2016; Huang *et al.*, 2007).

In vivo, SSCs physically interact with components of the BM microenvironment, for example, growth factors, adipokines, fatty acids and cytokines, released by surrounding cells as, notably, BM-adipocytes, that influence their differentiation (see previous

sections). More recently, it was demonstrated that human BM-adipocytes may also affect BM microenvironment through secretion of extracellular vesicles containing adipogenic mRNAs (i.e., PPARγ, CEBPα, and CEBPδ mRNA) (Martin *et al.*, 2015) that could be incorporated and translated by osteoblasts, further supporting the idea that BM-adipocytes modulate the osteoblastic phenotype (Morris and Edwards, 2016).

Moreover, SSCs cell–cell contact, physicochemical factors such as oxygen tension, ionic strength and pH, impact the fate of SSCs (McAdams *et al.*, 1997; Pattappa *et al.*, 2011; Zhang *et al.*, 2012). Pharmacological stress as glucocorticoid treatment or ethanol abuse also disturb the BM microenvironment and deregulate the maturation of bone precursor cells by favoring adipogenesis at the expense of osteoblastogenesis (Georgiou *et al.*, 2012).

The process of aging is a biological factor affecting bone mass and quality that may evolve to osteoporosis. It is characterized by decreased bone formation due to a decline of osteoblastogenesis associated with enhanced adipogenesis, leading to fat accumulation in the BM. SSCs isolated from aged subjects display a higher adipogenic potential concomitant to altered osteoblastic activity (Moerman *et al.*, 2004). Moreover, excessive degradation of the trabecular bone augments the trabecular space, which will be progressively infiltrated by marrow fat, generating a negative correlation between the amount of marrow fat and bone mass and density in old people (Griffith *et al.*, 2006; O'Neal *et al.*, 2011).

The loss of bone quality observed in aging and in pathological situations such as osteoporosis, diabetes, obesity and, puzzlingly, anorexia nervosa is currently definitely associated with an excessive expansion of BM-adipocytes (Devlin and Rosen, 2015). Likewise, clinical studies have highlighted accumulation of BM-adipocytes in the proximal femur of patients suffering from nontraumatic osteonecrosis of the femoral head (Mitchell *et al.*, 1986; Vande Berg *et al.*, 2006), a disease associated with low bone mineral density (Gangji *et al.*, 2018). Moreover, SSCs isolated from osteonecrotic patients are more susceptible to lipotoxicity and the fatty acid and cytokine composition of their BM-microenvironment is disturbed (Gillet *et al.*, 2017).

Having highlighted the impact of excessive BM-adipocyte accretion on skeletal health, the research is now focusing on the characterization of the qualitative aspects of BM adiposity.

Advanced imaging techniques allow for indirect evaluation of the BM lipid content and bone marrow aspiration permits the direct assessment of the BM environment lipid profile. Current studies reveal the dynamic lipid composition of BM, varying according to physiological and pathological situations. BM fat composition is enriched in SFA in patients with chronic diseases such as osteoporosis (Miranda *et al.*, 2016; Yeung *et al.*, 2005), osteonecrosis (Gillet *et al.*, 2017) or type-2 diabetes mellitus (Baum *et al.*, 2012; Patsch *et al.*, 2013).

Elucidation of the relationship between bone cells and BM-adipocytes could therefore help to get new insights on the pathophysiology of bone diseases characterized by marrow fat accumulation (Devlin and Rosen, 2015). In line with this proposal, studies could also be performed to clarify the influence of fatty acids on the survival and function of HSC and endothelial cells of the BM sinusoidal network.

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Bone Muscle Interactions and Exercise

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Abbreviations

1,25D	1,25dihydroxyvitamin D
25D	25hydroxyvitamin D
IGF	insulin-like growth factor
IL	interleukin
VDR	vitamin D receptor

Introduction

Bone and skeletal muscle are closely physically linked, with bone providing the scaffold for muscles to exert their force. With population aging, diseases of the musculoskeletal system are becoming more common. The increasing population obesity also has important effects on the skeleton and on muscle function. Sarcopenia, or loss of muscle mass and function and osteopenia or osteoporosis with associated fractures cause much of the disability in older life, with enormous societal and economic costs. The costs of these are estimated at $850 billion dollars a year in the USA (Connelly *et al.*, 2006).

Throughout life in people, muscle mass and bone mass are linked. This linkage requires direct mechanical interactions, and tissue cross-talk. Bones weaken with decreased muscle use in people and in animals. Congenital myotonic dystrophy and with other hereditary muscle disorders are examples of this effect. These patients are born with already thin long-bones, due to impaired and weaker fetal movements (Rodríguez *et al.*, 1988a,b). With weightlessness, astronauts lose bone and muscle mass rapidly. Bone mass and muscle strength is also rapidly lost with neurological injuries, for example, spinal cord lesions.

Bones release a number of osteokines, reviewed below. Growth hormone/insulin-like growth factor (IGF)-1, sex steroids and Wnt-signaling may help to coordinate the bone-muscle unit during fetal life and with adaptation to mechanical stimuli (Christoforidis *et al.*, 2005).

Development and Regulation of Bone and Muscle

Skeletal muscles and bones develop in close proximity to each other during early fetal life from the somatic mesoderm, and their development has been reviewed (Schweitzer *et al.*, 2010; DiGirolamo *et al.*, 2013).

Bone forms with a series of ordered steps. The first is condensation of mesenchymal precursors at future skeletal sites, followed by differentiation into chondrocytes to form the cartilage anlage (i.e., endochondral bone) or direct differentiation into osteoblasts (i.e., intramembranous bone) (DiGirolamo *et al.*, 2013) and mechanical cues are important during the process.

During life, bone continues to be remodeled. This occurs in response to loading, with an important role for sclerostin, and in order to repair microdamage. The cycle in the bone unit has been the subject of many reviews (Clarke, 2008). Bone is resorbed by osteoclasts, matrix is laid down, and then it is mineralized by osteoblasts. Mature bone cells (osteocytes) sense mechanical forces and may initiate both bone resorption and formation.

In fetal life, muscle development occurs next to skeletal development, at the same time. Pax3 is the master regulator in fetal life, and in adulthood this role is taken over by Pax7. Mesodermal precursors regulated by Pax3 commit to the myogenic lineage, and with external signals including Myf5 and MyoD fuse to multinucleated syncytia (Buckingham *et al.*, 2009). These syncytia ultimately form myotubes and then multinucleated muscle fibers. Some of the cells do not join with myotubes and instead remain on the edge of myofibres as a pool of Myf5/MyoD positive muscle precursors called satellite cells (Zammit *et al.*, 2002; Wang *et al.*, 2014; Hawke *et al.*, 2001; Sambasivan *et al.*, 2011; Seale *et al.*, 2000).

These developmental pathways for muscle and bone suggests the possibility that common signaling pathways could regulate their function and mass. To support this, mice with genetic defects in muscle development (MyoD knockout) display profound impairments in bone development and mineralization (Nowlan *et al.*, 2010). Signals thought to influence coordinated muscle/bone development include FGFs, transforming growth factors (TGFs), IGF-1 and other morphogens (Hamrick, 2012).

Exercise increases both muscle and bone mass, and disuse decreases both muscle and bone mass. There are obviously direct mechanical interactions, but evidence suggests that muscles may indirectly regulate bone repair via paracrine effects. After a fracture, covering the break with a muscle flap improves healing, even though the area is immobilized (Johnson *et al.*, 1996; Yoshizawa *et al.*, 1997). Conversely, injury to overlying muscle impairs fracture healing (Li *et al.*, 1997). Some of the secreted factor are shown in **Fig. 1** along with the effects of exercise and their effects on muscle and bone.

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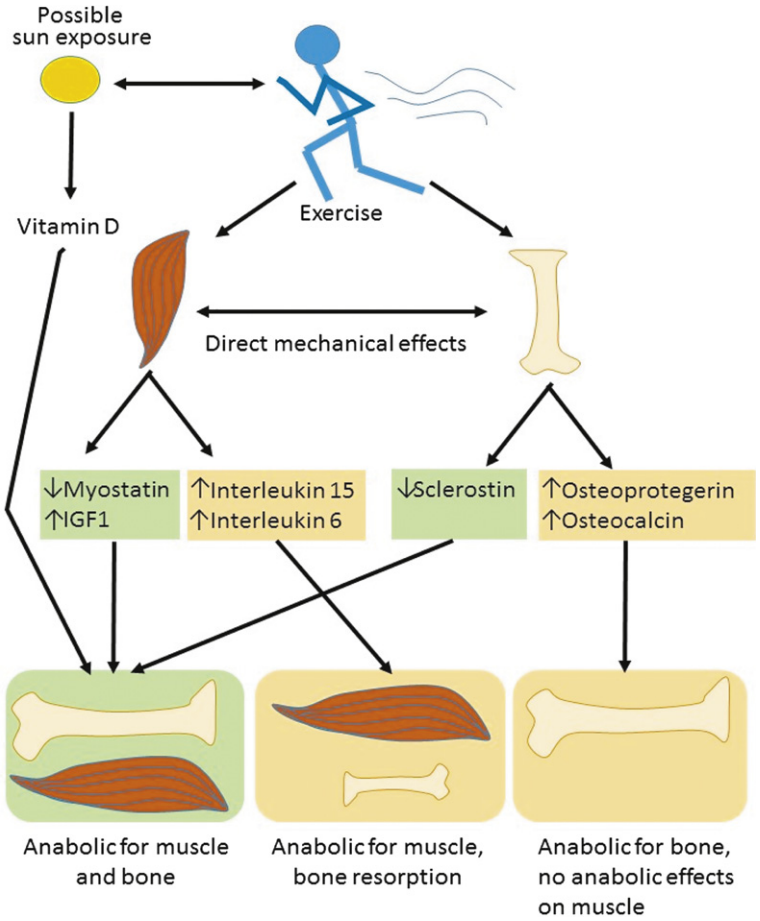

Fig. 1 Potential mechanisms underlying effects of exercise on bone and muscle. Exercise is often associated with sun exposure, generating increased vitamin D. Muscle decreases myostatin release and increases release of IGF1 (insulin like growth factor 1) and interleukins 6 and 16. Bone in response to exercise decreases sclerostin production, and increases osteoprotegerin (OPG) and osteocalcin. Osteocalcin increases muscle insulin sensitivity.

Vitamin D

Many kinds of exercise associate with sun exposure. Vitamin D is synthesized by conversion of 7-dehydrocholesterol in the skin with ultraviolet (UV) light exposure. Thus, vitamin D is not actually a true vitamin because humans can actually synthesize it, but it has retained the name for historical reasons.

Vitamin D is hydroxylated in the liver to form 25-hydroxyvitamin D (25D). This is the main circulating form and levels are used to assess vitamin D status. However, the active hormone is 1,25-dihydroxyvitaminD (1,25D). This is formed in kidney and a range of other cell types by 1α-hydroxylase. 1,25D binds to VDR, which is a member of the nuclear steroid hormone receptor superfamily. VDR usually heterodimerizes with RXR (the retinoid X receptor). It can, alternately, homodimerize with a second VDR. The VDR-dimer, when translocates to the nucleus, binds to vitamin D response elements (VDRE) in the DNA and regulates transcription (Lips, 2006).

There are hundreds of well-characterized gene regulation targets for activated VDR. Recent use of RNA-sequencing combined with chromatin immunoprecipitation (ChIP) has given new power to examine genes regulated by vitamin D in nonclassic target tissues such as liver (Ding *et al.*, 2013) and bone cells (Pike *et al.*, 2014) although this technology has not yet been applied to muscle. Vitamin D receptor (VDR) is a transcription factor, so mechanisms may include regulation of myokines, osteokines or perhaps regulation of mechano-stimulatory feedback.

People with deficiency, and particularly severe deficiency associated with rickets have some defects in muscle function (Girgis *et al.*, 2013a). Thus vitamin D represents another pathway by which muscle and bone interact, reviewed in (Gunton *et al.*, 2015).

Vitamin D in Bone

Bone is a classic vitamin D target, but it is also recognized to have important actions in muscle (Boland, 1986; Curry *et al.*, 1974; ADVANCE_Collaborative, 2008). Vitamin D deficiency causes rickets in children, and osteomalacia in adults due to impaired bone

mineralization. Vitamin D also stimulates calcium absorption from the intestine and decreases calcium loss in the urine. In the intestine VDR stimulates calbindin, and regulates some calcium channels. As well as active calcium absorption, passive intestinal absorption is possible if there is an appropriate calcium gradient.

Animal studies confirm these concepts. Mice with genetic deletion of VDR can still reverse rickets/osteomalacia when supplied with a diet very high in calcium and lactose; this has a "push" effect for calcium absorption. Even in people with genetic mutations in VDR, rickets can be healed with a similar diet. However, not all defects are corrected by this calcium "push." VDRKO mice on that diet still have atrophic muscle fibers and changes in osteoblast number, mineral apposition rate and bone volume (Endo *et al.*, 2003; Panda *et al.*, 2004).

Mouse models have enabled the delineation of direct effects of vitamin D in bone cells. Overexpression of VDR in mature osteoblasts increases bone formation, reduces bone resorption and inhibits bone loss with vitamin D deficiency (Gardiner *et al.*, 2000; Lam *et al.*, 2014). Recent data suggests that these mice respond more actively to mechanical loading by increasing bone formation and mineralization (Anderson *et al.*, 2013), a finding consistent with greater cortical bone mass and size in mice with elevated osteoblastic VDR levels, under normal or vitamin D-deficient conditions (Gardiner *et al.*, 2000; Lam *et al.*, 2014). The muscle phenotype of these mice has not been reported.

However, VDR activation in immature osteoblasts may have opposing effects; stimulating bone resorption and reducing bone mass (Yamamoto *et al.*, 2013). Treatment of early osteoblastic cells is also inhibitory, reducing cell activity and number (Shi *et al.*, 2007). The results in the osteoblast-specific VDR-null mouse suggests that VDR plays a systemic role to maintain serum calcium by inhibiting bone mineralization (Lieben *et al.*, 2012).

The osteoblast-specific VDR-null mouse suggests that VDR plays a more systemic role in inhibiting mineralization in order to maintain serum calcium levels (Lieben *et al.*, 2012). The direct role of VDR in osteocytes appears to be regulation of their development by effects on osteoprotegerin and maintaining calcium levels during deficiency (Takeda *et al.*, 1999). Therefore, local effects of vitamin D in bone may complement systemic vitamin D effects and may be anabolic or catabolic depending on the calcemic status and stage of bone cell differentiation.

Vitamin-D deficiency is associated with increased fracture risk and it appears that a significant proportion of this relates to muscle weakness and falls. The greatest effects are evident in those with lowest vitamin D levels (Girgis *et al.*, 2013a; Girgis *et al.*, 2014a). Low vitamin predicts future decline in physical performance (Wicherts *et al.*, 2007; Sohl *et al.*, 2013). Randomized clinical trials have been subjected to metaanalysis which confirms that vitamin D supplementation can decrease falls (Bischoff-Ferrari *et al.*, 2009). Coregulation of bone and muscle by vitamin D is suggested by coexistence of osteoporosis and sarcopenia in older, vitamin D deficient populations (Girgis *et al.*, 2013a; Onder *et al.*, 2008; Roth *et al.*, 2004; Visser *et al.*, 2003).

Vitamin D-related signals have also been implicated in muscle bone interactions (Tanaka *et al.*, 2014). Osteoglycin, produced by muscle cells under the control of vitamin D, regulates osteoblastic activity (Tanaka *et al.*, 2014). Decreased myostatin is associated with greater bone mass (Elkasrawy *et al.*, 2010), and this may be one means of the relative preservation of bone mass in VDR null mice, as vitamin D inhibits myostatin production from muscle cells (Garcia *et al.*, 2011; Girgis *et al.*, 2014b).

Vitamin D in Muscle

We and others have reviewed the roles of vitamin D in muscle (Girgis *et al.*, 2013a, 2014a; Boland, 1986; Ceglia, 2008). Whether vitamin D receptor is expressed in muscle was controversial. However, by RNA, Western immunoblot and by immunohistochemistry, VDR is present at low levels in cultured C2C12 myotubes (Girgis *et al.*, 2014b) and normal murine muscle (Girgis *et al.*, 2014c). VDR expression is often much higher in cell lines and isolated myocytes, and it is also higher in neonatal muscle. This pattern supports the concept of important developmental roles for VDR in muscle.

It has been long-recognized that vitamin D deficiency is associated with muscle weakness, especially proximal muscle weakness. There are electromyographic (EMG) features which resolve with correction of deficiency (reviewed in Girgis *et al.*, 2013a), but unfortunately the EMG findings are nonspecific. Human biopsy studies suggest that there may be preferential loss of type 2 muscle fibers which improves with supplementation (Sato *et al.*, 2005; Sorensen *et al.*, 1979). Children with rickets and adults with osteomalacia can have severe weakness (Girgis *et al.*, 2013a).

In people, it is challenging to control for sunlight and diet exposure; "healthier" individuals may have better diets and undertake more outdoor activities, including exercise, and have correspondingly better muscle and bone function. For this reason, it is useful to turn to animal models to examine the question of bone-muscle interactions and vitamin D.

Mice with mutations in vitamin D receptor are weak and can have low bone mass and less longitudinal growth. Studies of VDR-null mice where phosphate and calcium are aggressively replaced with a "rescue diet" may result in near-normal bone phenotype (Yeap *et al.*, 2014; Girgis *et al.*, 2013b). However, the muscle phenotype remains severe with a 40% decrease in muscle bulk (Girgis *et al.*, 2015). The size of muscle fibers is much smaller as well (Endo *et al.*, 2003).

Vitamin D is known to stimulate local vascular endothelial growth factor (VEGF) and IGF-1 production in muscle. Both factors are well known to have potential beneficial effects in bone. How large a contribution muscle vitamin D action makes to circulating levels of either hormone is not clear.

Another potential muscle factor which is regulated by vitamin D is IL-6 (interleukin-6). It is produced following exercise or contraction. It stimulates bone resorption, and may alter bone strength. It is decreased by vitamin D (Gannage-Yared *et al.*, 2003).

Potential Bone to Muscle Vitamin D Cross Talk

The osteocyte cell line MLO-Y4 expresses muscle anabolic factors IGF-1, MGF and VEGF after mechanical loading (Juffer *et al.*, 2012). Osteocalcin which is produced by osteoblasts has recently come to a new light for its role in regulating beta-cell function (Lalwani *et al.*, 2014). Its classic use is as a marker of bone formation. It is regulated by vitamin D and its gene contains a vitamin D response element (VDRE) indicating direct regulation by vitamin D (Girgis *et al.*, 2014d). It has potential effects in muscle, in which it alters mitochondrial function, insulin sensitivity (Lalwani *et al.*, 2014), and possibly strength in women.

FGF23 is another vitamin D responsive hormone produced by bone which has positive effects on cardiac and smooth muscle (Sherman *et al.*, 2014). The effects on skeletal muscle are the subject of current investigation.

Myokines

Myostatin

Myostatin is a hormone secreted from muscle, or a myokine. As implied by its name, it inhibits increases in muscle mass. There are several animal models of myostatin deficiency, including mice, dogs and cattle. One person with a mutation in myostatin has been found—he has a significant increase in muscle mass (Schuelke *et al.*, 2004). His bone density has not been reported. Muscle mass and bone density are both significantly increased in the myostatin mutant mouse (Hamrick *et al.*, 2006). Myostatin deficiency in this setting results in exercise-induced gains in bone mass and importantly, resistance to fracture (Hamrick *et al.*, 2006).

Myostatin expression is decreased in human muscle from 1 h after exercise with a persisting substantial decrease at 24 h (Louis *et al.*, 2007). The nadir in expression was 8 h after exercise. In that study the protocol was for 45 min of bike-riding. In another study of insulin resistant men, aerobic exercise at ~50% of VO_2 max for 6 months maintained a 21% decrease in circulating myostatin (Hittel *et al.*, 2010). Exercise at 50% or 80% of maximum lifting weight also significantly decreased myostatin, with a persisting decrease at 48 h (Schwarz *et al.*, 2016). In contrast, in older women, elastic band exercises did not alter myostatin although the exercise was enough to improve physical performance (Hofmann *et al.*, 2016). The "threshold" dose of exercise for duration or intensity of exercise is not known for myostatin, but half an hour at moderate intensity appears sufficient from the above studies.

Antibody inhibitors of myostatin in people have not produced consistent clinical results. One study in Duchenne's patients, where only two doses were received due to epistaxis and telangiectasias showed trends to improved muscle function (Campbell *et al.*, 2017). In androgen-deprived men, 28 days of myostatin inhibitor ACE-031 caused a small but significant increase in lean mass (2.2%) (Padhi *et al.*, 2014). The trials of inhibitors have mostly been of short duration, and or small in size, and there are no reports in people regarding whether there are or are not bone effects.

Interleukins

Interleukin 6 (IL6) was the first muscle-secreted cytokines to be identified (Pedersen *et al.*, 2008). It increases markedly in response to exercise, and it induces beneficial effects on metabolism and insulin sensitivity as well as myogenesis (Pedersen *et al.*, 2012). Similarly, interleukin 15 is released by exercising muscle. It has anabolic effects on muscle (Nielsen *et al.*, 2007). IL6 induces bone resorption.

Insulin-Like Growth Factor 1 (IGF1)

As well as liver and other tissues, muscle is able to synthesize and release IGF1. Its release is increased in response to exercise, and it has well described anabolic effects (Gregory *et al.*, 2013). Its use is banned in athletes due to its classification as a performance enhancing agent (Holt *et al.*, 2008). IGF1 increases bone formation (Yakar *et al.*, 2002) and bone mass (Tahimic *et al.*, 2013). However, there is a surprising lack of clinical trial evidence for beneficial effects on muscle function (i.e., strength or endurance) (Friedlander *et al.*, 2001; Mauras *et al.*, 2000; Vlachopapadopoulou *et al.*, 1995).

Osteokines

Sclerostin

Sclerostin is secreted by mature osteocytes during completion of osteon formation. It inhibits bone formation. Mutations in the SOST gene, coding for sclerostin, cause sclerosteosis with undetectable or low sclerostin levels, increased bone formation, very high bone mass and long-term neurological impairments due to entrapment of nerves (van Lierop *et al.*, 2012). Sclerostin antibodies have exciting potential in the treatment of osteoporosis. Sclerostin secretion by osteocytes increases in response to bedrest and decreases with muscle loading (Spatz *et al.*, 2012).

Vitamin D levels are inversely related to serum sclerostin. Sclerostin is a marker of osteocyte mechano-sensing, and it suppresses stimulation of bone formation by terminally differentiated osteocytes (van Lierop *et al.*, 2012). Thus, decreased sclerostin, seen with increased vitamin D, should increase bone mass.

Osteocalcin

Osteocalcin is released from osteoblasts, and is increased by exercise (Jurimae *et al.*, 2011). Its role in bone mineralization appears to be inhibitory; knockout mice have improved bone density and quality (Ducy *et al.*, 1996). It is not thought to have a major effect on muscle power, but improves muscle insulin sensitivity (Levinger *et al.*, 2016). That would improve responsiveness to insulin, but would also be expected to increase sensitivity to IGF1.

Osteoprotegerin

Osteoprotegerin (OPG) is part of the OPG/RANK/RANKL signaling system which is therapeutically targeted by denosumab. The effect of exercise on OPG is more difficult to interpret—some studies showing no effect, and some studies show an increase with exercise (Scott *et al.*, 2011). Increased OPG, by its function as a decoy receptor for RANKL would improve bone mass. Muscle does not express significant amounts of RANKL, so it is unlikely that OPG/RANK directly affects muscle function.

Conclusions

In summary, exercise induces direct mechanical effects on both bone and muscle. In addition, it regulates release of many myokines and at least some "osteokines" with effects on muscle and bone which are mostly positive. For people with sarcopenia or osteopenia, moderate exercise at 50% of maximal capacity for half an hour is sufficient to induce important changes in myostatin and sclerostin. Below that threshold, there is relatively little data. Since sclerostin decreases with load on bone, less intensive exercise which associates with some bone force may also be sufficient for benefit. This is an exciting and relatively fast-moving field. From muscle, myostatin and from bone, sclerostin are the targets of a range of therapeutic agents in current trials which have promise for preventing or treating these important aspects of frailty with disease or old age.

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Bone as an Endocrine Organ

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Introduction

The reasons that led to the hypothesis that bone might be an endocrine organ and that in this capacity, it should regulate energy metabolism and reproduction are probably as, if not more, important to understand as the mode of action of osteocalcin, the first hormone found to mediate endocrine functions of bone.

This hypothesis arose from cell biological and clinical observations that were placed anew in their original evolutionary context. From a cell biology perspective, bone is the only tissue in the body of vertebrates that contains a cell type, the osteoclast, the main function of which is to actively destroy the host tissue (Teitelbaum, 2000). It is as if bone had invented auto-immunity except that in that case it is a much-needed physiological process. This destruction, or bone resorption, occurs daily from birth to death in multiple locations in an organ that covers the largest surface in the body of vertebrates. Thus, because this process requires active lysis, bone resorption is likely to be energetically expensive. Bone resorption is only one aspect of a biphasic physiological process called bone modeling during childhood and bone remodeling during adulthood (Ducy *et al.*, 2000b). Bone formation is induced following bone resorption and is another energy demanding event since it entails the constant synthesis and secretion of collagenous protein. Importantly, the notion that there is a close link between bone physiology and energy metabolism is fully supported by clinical observations. Indeed, longitudinal bone growth stops in children and bone mass decreases in adults when access to food, that is, to energy, is severely limited (Legroux-Gerot *et al.*, 2005; Misra and Klibanski, 2011). Besides this link between bone (re)-modeling and energy metabolism, another well stablished and apparently unrelated clinical observation is that bone mass invariably declines in both sexes when gonadal functions end (Riggs *et al.*, 1998; Riggs and Melton, 1986). Considering these observations as a whole we formulated the hypothesis that there should be a coordinated regulation, endocrine in nature, of bone growth/mass, energy metabolism and reproduction.

This hypothesis, although seemingly vague at is birth, is in fact quite far reaching. A first justification of its application is the evolutional development of bone and the "peculiar" mechanism whereby it constantly renews itself. It simply implies that if hormones coordinating these physiological functions exist they most likely appear during evolution with bone, since it is the physiology of this tissue that requires their existence. In agreement with this prerequisite of our working hypothesis, leptin, a powerful endocrine regulator of bone mass that also controls fertility and energy metabolism, appears during evolution with bone. More to the point, the study of a mouse model of a partial gain of function of the leptin receptor showed that the amount of leptin signaling needed to influence bone mass is lower than the one needed to affect appetite, energy expenditure or fertility (Bjornholm *et al.*, 2007; Shi *et al.*, 2008). But the most original and far reaching implication of this working hypothesis was not to identify a new hormone regulating bone mass, but rather that bone should be an endocrine organ regulating energy metabolism and reproduction if not other physiological functions. It is the testing of this particular aspect of the working hypothesis that eventually led to a significant broadening of the field of bone endocrinology.

Osteocalcin Is a Bone Derived Hormone

As it is often the case in biology, the identification of a hormone synthesized by bone cells, regardless of its possible functions, required a degree of luck. At the beginning of the 90's when mouse genetics allowed physiologists to address fundamentally important questions of whole-organism physiology that could not possibly be addressed in vitro, osteocalcin was then and for several reasons the most mysterious protein in the field of bone biology. It was at the time the only protein that was solely synthesized by osteoblasts. Osteocalcin is subsequently secreted in the bone extracellular matrix (ECM) and is the most abundant non-collagenous protein of this ECM (Hauschka *et al.*, 1989; Lian *et al.*, 1978). Osteocalcin was also known to be abundant in the general circulation and its circulating levels correlate well with bone formation (Delmas *et al.*, 2000; Merle and Delmas, 1990). However why osteocalcin would be so abundant in the general circulation, was generally overlooked; yet, it is the tenth most abundant protein in bony vertebrates. Osteocalcin was known to undergo a post-translational modification whereby three glutamate residues are carboxylated to form GLA residues that traditionally confer to proteins harboring them a high affinity for mineral ions and mineral crystals (Hauschka *et al.*, 1989). Because of this post-translational modification, osteocalcin was thought to be implicated in the mineralization of the bone ECM. However, no firm evidence of such a function was available, this is why *Osteocalcin-null* mice were generated. To make a long story short, analysis of these mutants did not provide any evidence that osteocalcin was involved in any meaningful way, in bone ECM mineralization (Ducy *et al.*, 1996). Hence, taken at force value and in the most conservative interpretation what this experiment showed first is that the function of the 10th most abundant protein of our body does not affect the tissue that synthesizes it.

Even though their bones were normally mineralized, *Osteocalcin −/−* mice displayed some phenotypes that given the site of synthesis of osteocalcin and its abundance in the general circulation, could only be explained if this molecule was acting as a hormone. Indeed osteocalcin-null mice of either sex had larger fat pads without being obese and mutant mice bred poorly. The systematic study of these two phenotypes established that osteocalcin is a hormone regulating aspects of energy metabolism and male fertility (Lee *et al.*, 2007; Oury *et al.*, 2011).

Osteocalcin Regulates Energy Metabolism at Rest

The field of osteocalcin biology took from the onset the stand that it is unlikely for a hormone to have a single major function. As a result, at the time this article is being written, our knowledge about the functions of this hormone and our understanding of its mechanisms of action in its known target organs, remains a work in progress. For instance, if the increased adiposity of the *Osteocalcin −/−* mice could be ascribed to a decrease in energy expenditure and not to an increase in appetite another function regulated by bone (see below), the molecular bases of this function has not been elucidated yet.

On the other hand, what has begun to receive a molecular explanation are other ways osteocalcin affects energy metabolism. We should emphasize that throughout our investigation, the work was greatly helped by the availability of a loss of function (*Osteocalcin −/−*) and a gain of function mouse model for osteocalcin (*Esp −/−*), each of them serving as an internal control for the other, yet each model addressed a different question. The loss of function models allowed to answer the question of whether osteocalcin is necessary to up-regulate a variety of physiological processes. The gain of function models served as evidence that osteocalcin is also sufficient to up-regulate these functions. From a translational point of view this is of course of greater importance. Investigations conducted in mice fed a normal diet showed that osteocalcin is necessary and sufficient to promote *β*-cell proliferation in pancreatic islets, to enhance *Insulin* expression and secretion, to favor glucose uptake in peripheral tissues and as a result glucose homeostasis (Lee *et al.*, 2007; Wei *et al.*, 2014). The receptor mediating osteocalcin signal in pancreatic *β*-cells and other peripheral tissues is a GPCR called Gprc6a (Oury *et al.*, 2011; Wei *et al.*, 2014). Cell-specific gene deletion and genetic epistasis experiments has established that in vivo osteocalcin is the ligand of Gprc6a that explains the regulation of glucose homeostasis determined by their GPCR. Beyond the gain of function model of osteocalcin that the mice represent, the biological importance of osteocalcin regulation of glucose homeostasis was verified in wild type mice fed a cell high fat diet. Exogenous osteocalcin almost completely rescue the glucose intolerance of these animals (Ferron *et al.*, 2012).

Osteocalcin Regulates Energy Metabolism During Exercise

A question raised by the regulation of glucose homeostasis by osteocalcin is whether there was any difference between osteocalcin biology and insulin biology. An answer to this question was provided by studying the functions of osteocalcin during exercise, a physiological situation during which circulating levels of insulin decrease. An incentive to study this question came from the observation that during an endurance exercise, the circulating levels of osteocalcin nearly triple (Mera *et al.*, 2016a). Another reason to ask this question is that the mechanisms whereby muscle function increases during exercise remain poorly understood.

To be able to distinguish the role if any, of osteocalcin in muscle from its functions in other organs, this project was pursued after generating mice lacking the osteocalcin receptor only in myofibers. This investigation found that osteocalcin signaling in myofibers is necessary to increase exercise capacity because it favors uptake in myofibers of their two main nutrients: glucose and fatty acids, osteocalcin also enhances catabolism of these two nutrients to generate ATP (Mera *et al.*, 2016a). Hence, osteocalcin does have common function with insulin such as for instance glucose uptake in myofibers but unlike insulin, osteocalcin favors the catabolism of these nutrients in order to increase muscle function. Osteocalcin favors muscle function during exercise through another mechanism, by favoring the expression and release of interleukin-6 (IL-6) one of the first myokine ever described whose circulating were known to increase during exercise through unknown mechanism (Pedersen and Febbraio, 2012). In turn IL-6 signals in bone to favor bone resorption which is the mechanism whereby osteocalcin is decarboxylated and becomes bioactive (Ferron *et al.*, 2010). In other words there is a feed forward loop that takes place during exercise between bone and muscle. At the onset of exercise bone secretes osteocalcin which increases on the one hand nutrients uptake and catabolism in myofibers in order to produce ATP. On the other hand, osteocalcin signaling in myofibers enhances the production of IL-6 during exercise, IL-6 then signals into bone cells to promote the production of bioactive osteocalcin. Of note, at the same time it increases muscle function, osteocalcin signaling in myofibers also favors a gain in muscle mass (Mera *et al.*, 2016b).

As it is the case for the regulation of glucose homeostasis at rest, the regulation of muscle function and exercise capacity by osteocalcin signaling in myofibers is also sufficient to increase exercise capacity in WT mice. This was shown by delivering acutely just before an endurance exercise or chronically for 1 month bioactive osteocalcin to WT mice either young or old. Either mode of delivery resulted in a surprisingly high gain in exercise capacity in these mice (Mera *et al.*, 2016a), thus illustrating the therapeutic potential of osteocalcin in conditions such as age-related decrease in muscle function and mass.

Osteocalcin Regulates Male Fertility

The regulation of energy metabolism by osteocalcin fulfilled only one aspect of our original hypothesis. A second aspect was to determine whether osteocalcin also regulates reproductive functions. Since osteoporosis develops mostly in post-

menopausal women, it was natural to ask if osteocalcin influences fertility in females. To begin addressing this question we relied on a co-culture assay in which supernatants of mouse osteoblasts were added to ovary slices to determine if this would result in an increase in the secretion of sex steroid hormones. One negative control in this experiment was to treat testicular Leydig cells with supernatant of mouse osteoblasts. What was observed is that when cultured in the presence of supernatant of either WT or osteocalcin-null osteoblasts, ovary slices secreted the same amount of sex steroid hormones. In contrast Leydig cells of the testes did not secrete nearly as much testosterone when they were cultured in the presence of the supernatant of *osteocalcin −/−* osteoblasts, than when cultured in the presence of WT osteoblasts (Oury *et al.*, 2011). This effect was specific of osteoblasts since applying supernatant of other mesenchymal cells in culture, did not affect testosterone release by Leydig cells. As a corollary of this set of experiments, it was shown that osteocalcin favors in a dose dependent manner the release of testosterone by Leydig cells of the testes, because it favors the expression of all the genes encoding the enzyme necessary of testosterone biosynthesis (Oury *et al.*, 2011). Of note, osteocalcin does not increase the expression of the genes necessary to allow the conversion of testosterone to estrogens. These molecular and cell biological observations explain why circulating levels of testosterone are markedly lower in *osteocalcin −/−* and higher in *Esp −/−* than in WT mice. As a result male *osteocalcin −/−* mice have low testosterone impregnation and breed poorly (Oury *et al.*, 2011).

In a sense this investigation closed the first circle, after demonstrating that hormones such as leptin that are thought of exclusively belonging to the energy metabolism field are potent regulators of bone mass (Ducy *et al.*, 2000a), we had shown that indeed bone is an endocrine organ and that in this capacity it promotes several aspects of energy metabolism and male reproduction. Although these lines of research do not stop here, osteocalcin has more functions that those.

The Brain as a Target Organ of Bone; Part I: Through Osteocalcin

Greatly helped by our own work on leptin we suspected from the beginning that the functions of osteocalcin could not be restricted to the ones implied by the initial working hypothesis. Going beyond the working hypothesis was also prompted by the fact that *osteocalcin −/−* mice, were overtly passive. This phenotype could not be ascribed to their hypogonadism since it was observed in both male and female mice.

Analysis of this rather vague phenotype uncovered the importance of bone via osteocalcin for brain development and multiple brain functions. What was shown was, that when it is undercarboxylated osteocalcin crosses the blood brain barrier, binds to neurons of the dorsal raphe in the brainstem, the ventral tegmental area in the midbrain and the CA3 region of the hippocampus (Oury *et al.*, 2013). Osteocalcin also favors the synthesis of all monoamine neurotransmitters and decreases the synthesis of GABA, an inhibitory neurotransmitter. This occurs because osteocalcin signaling in neurons regulates the program of gene expression responsible for the synthesis of these neurotransmitters. Furthermore, it prevents neuronal apoptosis in the hippocampus. The consequences of these molecular events at the level of the entire animal are that *Osteocalcin −/−* mice displayed increased anxiety and depression and very poor learning skills and memory. Remarkably these phenotypes were dominant and also observed in *Osteocalcin +/−* mice (Oury *et al.*, 2013). Several lines of evidence demonstrated that this reflected the influence of bone on the brain and that this influence was independent of any other functions of osteocalcin. For instance, mice lacking *Osteocalcin* in an osteoblasts specific manner experienced the same molecular and phenotypic abnormalities, mice lacking *Gprc6a* that share with *Osteocalcin −/−* their metabolic and reproductive phenotypes did not have any behavioral abnormalities or other types of brain-related phenotypes that are seen in *Osteocalcin −/−* mice. The reason for that is that Gprc6a is not expressed in the brain. Finally delivering osteocalcin through intracerebroventricular infusion in *Osteocalcin −/−* mice at a dose that does not cross the blood brain barrier, rescued all molecular behavioral phenotypes, in *Osteocalcin −/−* mice (Oury *et al.*, 2013). This latter result indicated that it is osteocalcin itself that by signaling in the brain is responsible of all the central abnormalities noted in *Osteocalcin −/−* mice.

Osteocalcin is not expressed before E17.5 in the mouse thus it was surprising to note that the hippocampi of *Osteocalcin −/−* mice was significantly hypomorphic compared to the areas of WT mice (Oury *et al.*, 2013). That this latter phenotype was markedly more severe in *Osteocalcin −/−* mice born from *Osteocalcin −/−* mothers than in those born from *Osteocalcin +/−* mothers, allowed us to show that maternally derived osteocalcin crossed the placenta and is necessary for brain development of the embryo (Oury *et al.*, 2013). This observation identified osteocalcin as the first molecule that could explain the long noticed influence of health of the mother on the psychological health of the offspring. In agreement with this notion, delivering osteocalcin to *Osteocalcin −/−* mothers throughout their pregnancies corrected the brain development defects otherwise noticed in *Osteocalcin −/−* newborn mice.

Identification of Another Hormone Mediating Endocrine Functions of Bone: Lipocalin 2

The finding that bone is an endocrine organ formed the basis for one of the most intriguing questions in this area of research: *Does bone utilize more than one hormone to affect energy metabolism? And, in doing so, does it target additional, energy-regulating organs?* To address these questions, we generated mice lacking 50% of their osteoblasts and characterized their metabolic phenotype. These analyses showed that osteoblasts are primary cellular mediators of insulin signaling in all insulin target cells (Yoshikawa *et al.*,

2011). Remarkably, these studies also indicated that bone affects energy metabolism (a) in an additional osteocalcin-independent mechanism; and (b) by regulating an additional metabolic process, appetite. This latter study called for the identification of the anorexigenic signal(s) originating from osteoblasts. The route to identifying the new anorexigenic protein came from experiments showing that mice lacking the transcription factor *FoxO1* in osteoblasts ($FoxO1_{osb}-/-$ mice) have improved energy metabolism, partly due to an increase in osteocalcin bioactivity (Rached *et al.*, 2010). In addition, a microarray analysis identified Lipocalin 2 (LCN2) as a secreted protein upregulated in *FoxO1*-deficient osteoblasts. A comprehensive study of its pattern of expression revealed that *LCN2* is an osteoblast-enriched secreted protein that is expressed at least 10-fold higher in osteoblasts than in adipocytes or other cells in basal, non-stimulated conditions.

Regulation of Appetite by Bone

Determination of the putative metabolic functions of LCN2 and its cell of origin were obtained by generating mice lacking LCN2 in osteoblasts ($Lcn2_{osb}-/-$ mice) or in adipocytes ($Lcn2_{fat}-/-$ mice), because LCN2 had been considered an adipokine associated with obesity (Lin *et al.*, 2001; Mosialou *et al.*, 2017; Soukas *et al.*, 2000; Yan *et al.*, 2007). $Lcn2_{osb}-/-$ mice showed a 16.4% increase in food intake that correlated with increased gonadal fat weight (16.5%), total fat mass (19.6%) and a significant increase in body weight. In contrast, $Lcn2_{fat}-/-$ mice did not show any changes in food intake, fat or body weight. During growth, the increase in food intake was detected as early as 3 weeks of age, the earliest time point possible to measure appetite in weaned animals, and preceded the increase in blood glucose levels and body weight both of which manifested at 5 weeks of age. Corroborating the results obtained in $Lcn2_{osb}-/-$ mice, administration of recombinant LCN2 to lean wild type and to obese mice with a spontaneous inactivating mutation in the leptin receptor ($Lepr^{db/db}$ mice) suppressed appetite and body weight and improved glucose metabolism and insulin sensitivity throughout the entire duration of treatment.

In addition to the chronic effects of LCN2 on appetite suppression, its anorexigenic properties extend to acute post-prandial regulation of feeding. Indeed, expression in osteoblast and serum levels of LCN2 increase threefold in 1–3 h after refeeding mice. The increase in serum levels correlates with a suppression of food intake in wild type mice. This effect was specific to LCN2 and not due to other appetite regulating hormones or peptides because its inactivation in $Lcn2_{osb}-/-$ mice led to higher cumulative food intake and a twofold higher rate of food intake after refeeding. These results indicated that upregulation of *Lcn2* expression by osteoblasts following feeding may be a negative feedback mechanism to limit appetite after a meal in the mouse.

The Brain as a Target Organ of Bone; Part II: Through Lipocalin 2

How does LCN2 suppress food intake following its signaling in the hypothalamus? Understanding the mechanism of this action, revealed for a second time the ability of bone to signal to the brain. The observation that LCN2 does not regulate the expression of any peripheral hormones known to affect appetite and is not expressed in the hypothalamus (Mosialou *et al.*, 2017), raised the possibility that it may cross the blood brain barrier and act on areas that control appetite. Intraperitoneal injections of recombinant of LCN2 in $Lcn2-/-$ mice proved this hypothesis as they showed that LCN2 accumulated in the brain, mainly in the hypothalamus. Subsequent examination of hypothalamic pathways that affect appetite indicated that expression of only downstream effectors of the MC4R signaling pathway was modulated in a loss and gain of function mouse models for LCN2. It was subsequently shown that LCN2 binds to the MC4R in the paravetricular neurons of the hypothalamus with a K_d of 51.39 $\pm$ 4.78 nM and competed for binding with α-MSH at a K_i of 46.34 $\pm$ 1.11 nM. Moreover, ICV administration of LCN2 induced, *Fos* expression in PVH neurons of wild type but not $Mc4r^{-/-}$ and stimulated cellular activity of MC4R-expressing hypothalamic neurons, assessed by electrophysiological recordings. Lastly, administration of recombinant LCN2 suppressed appetite and decreased body weight in WT but not in $Mc4r^{-/-}$ mice indicating that MC4R mediates the anorexigenic function of LCN2 in vivo.

Lipocalin 2 Regulates Insulin Secretion and Production

An examination of the extent to which the anorexigenic function of LCN2 explains the metabolic phenotype of $Lcn2_{osb}-/-$ mice, revealed an additional function and direct target organ of this hormone. Pair-feeding experiments of $Lcn2_{osb}^{-/-}$ mice and their wild type littermates showed that whereas body weight, fat mass and insulin sensitivity were normalized, serum insulin levels and insulin secretion following glucose load remained compromised in $Lcn2_{osb}^{-/-}$ mice and as a result their glucose intolerance persisted. These results suggested that LCN2 may signal directly in β-cells to affect their functions. Indeed, LCN2 directly stimulated insulin secretion and β-cell proliferation in primary pancreatic islets. Therefore, whereas increased appetite accounts for the increase in fat mass, body weight and insulin resistance in $Lcn2_{osb}-/-$ mice, glucose intolerance likely reflects its direct action on pancreatic islets.

See also: Glucose Metabolism and Hormonal Regulation. Leptin

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Calcium Homeostasis in Humans

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Calcium is a major ion in humans with a total mass of more than 1000 g in adults and is present is different compartments. *Intracellular calcium* plays an essential role in a variety of cellular functions from the very start of evolution of life on earth onwards. The *cytosolic ionized calcium* concentration is very low (about 10–100 nM) but can change rapidly and transiently due to exchanges with *intracellular calcium pools* or with the much higher concentration of extracellular calcium. Intracellular ionized calcium plays a vital role as second messenger (translating a variety of extracellular signals), in neurotransmitter release, contraction of all types of muscle cells or in fertilization (Berridge *et al.*, 2003). Many enzymes require calcium ions as co-factors and the blood-clotting cascade is a crucial example of this role. Chelation of calcium is therefore a straightforward strategy to avoid blood clotting after blood sampling. *Extra-cellular calcium* in vertebrates can also be subdivided in different compartments: blood, interstitial fluid, and soft tissue matrix. Serum calcium, in all vertebrates and certainly in mammals, is tightly controlled by a variety of hormones as to maintain ionized calcium concentration within narrow limits (about 1.05–1.25 mmol/L). The total body pool of calcium is maintained by strict coordination by calcium transport in three major target tissues: gut, kidney and to a much lower extend also the skin. Transport of calcium via the placenta and mammary glands can also be very important but of course only during reproduction. In this short overview, I will discuss calcium homeostasis in its different compartments and the handling of calcium in its major target tissues as well as the (hormonal) regulation of serum calcium and body calcium content.

Total Body Calcium

The mature human fetus is born with about 30 g of calcium and this mass rapidly increases during the first years of life (about doubling from 30 to 60 g during the first 6 months of life). Thereafter, there is a gradual increase in total body calcium along with linear growth and thus with a more rapid increase during pubertal growth spurt. Peak bone mass, and thus also peak total body mass of calcium, is reached in the 3rd and 4th decade of life and is about 1000 g in adult females and about 1200 g of calcium in adult males. Later in life, there is a gradual decrease in total body calcium due to progressive loss of bone mass, with an accelerated loss of bone during a decade after menopause in women.

Bone as Mineralized Tissue

Most of the body store of calcium is found in the mineralized matrix of bone (and to a minor extend in teeth). This represents about 99% of total body calcium and is mainly stored as hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] embedded in collagen fibrils. The mineralization of bone matrix is an active process, mainly by osteoblasts and osteocytes (and dentinoblasts for teeth), with a complex regulation by local and systemic factors. These bone cells first synthesize and excrete the bone matrix proteins (osteoid) which later on, at a short distance from the bone forming cells, mineralize until about 70% of the bone is composed of minerals, with the remaining 30% as matrix proteins. Removal of bone minerals is only possible by combined removal of mineral and bone matrix and is mainly due to the activity of highly specialized cells, osteoclasts. More recently, it became clear that osteocytes, representing more than 90% of all bone cells, can also resorb bone and mineralize the perilacunal space. Bone is of course not a dead tissue. During growth, bone size regularly adapts in length and cross-sectional diameter by bone modeling. After the end of puberty, only bone remodeling is operational whereby old bone is removed and replaced by new bone formation. This process may take a few weeks for resorption and several months for new bone formation and mineralization. Ultimately, bone size, mass and structure remain identical during most of adult life. Later in life, bone resorption slightly exceeds formation with gradual loss of bone mass and bone strength. The bone left-over, however, is usually of normal structure and mineral density ("too little bone, but the bone there is, is normal"). The daily amounts of calcium removed and replaced by bone remodeling is about 300–500 mg of calcium, depending of total body size, age and other hormonal factors (see below). Apart from this bone remodeling, bone calcium can be released and replaced without cellular involvement.

Intracellular Calcium Homeostasis

About 1% ($\approx$1 g) of calcium is found in the intracellular compartment, blood and interstitial fluid. The intracellular calcium homeostasis is totally different from the regulation of extracellular calcium concentration. The cytosolic ionized calcium concentration is in the micromolar (10^{-6} M) range and thus about a thousand fold lower than the ionized calcium concentration in serum. This gradient is important for maintaining the electrochemical gradient (potential difference, whereby the cell interior is

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about 20 mV lower than the extracellular potential) across excitable cell membranes. Loss of this calcium gradient may jeopardize cell viability and this is prevented by a variety of active calcium pumps (requiring energy) at the cell membrane or at the membrane of cell organelles (such as mitochondria and endoplasmatic reticulum). These calcium permeable channels include transient receptor potential (TRP) and voltage-gated calcium channels, sodium-calcium exchangers (NCX) and ATP-dependent plasma membrane calcium pumps. Inside the cell, calcium is either in a free, ionized form or bound to cytosolic calcium buffers such as calbindins or parvalbumin, or stored in intracellular compartments such as mitochondria or endoplasmatic reticulum. These organelles also have specific calcium entry or exit pumps such as SERCA or mitochondria calcium uniporter pumps. All these pumps allow rapid, dynamic and transient fluctuations of intracellular free calcium concentrations needed for a variety of cellular functions such as muscle contractions, neural function or cellular functions in general.

Extracellular Calcium Homeostasis

The serum calcium concentration is tightly regulated by a diversity of hormones and maintained between 8.6 and 10.2 mg/dL (total calcium) or between 4.2 and 5.0 mg/dL for ionized calcium. The normal range may vary depending on the method used for measurement of total calcium. Indeed, total serum calcium is for about 50% bound to several proteins (which are negatively charged) and about 10% is bound to several organic acids (such as citrate) or other ions (such as PO_4 ions). The remaining free or ionized calcium is feed-back regulated (see below). Total calcium can fluctuate according to serum protein (especially albumin) concentrations. Ionized calcium can be directly measured or calculated from total calcium and protein/albumin concentrations. For each gram of calcium above or below 4 g of calcium per dL, 0.8 mg of total calcium has to be subtracted/added to calculate protein-corrected calcium. Acidosis or alkalosis such as in renal, respiratory or metabolic diseases, can disturb the equilibrium between bound and ionized calcium and can thus influence the real ionized calcium concentration and this usually requires direct measurements to estimate the true free calcium concentration. A number of diseases can increase or decrease ionized calcium concentration (as discussed in other chapters).

Calcium Transporting Tissues

Calcium homeostasis depends on the regulation of calcium transport in several major target tissues (**Fig. 1**).

Intestine

The intestine is the major calcium transporting tissue responsible for the transport of dietary calcium and calcium added to food by secretion of fluids by salivary glands, gastric, biliary, pancreatic and intestinal fluid secretion, adding a total of about 400 mg or more of calcium per day. Dietary calcium intake is dependent of dietary habits as the calcium content of food is highly variable. The highest concentration is found in cheese (700–1000 mg/100 g), and other dairy products (yoghurt and cow milk at 120 mg/100 mL) whereas, strangely, human milk has a much lower calcium content (30 mg/100 mL). Most fish and meat have a low calcium content (less than 20 mg/100 g). Therefore, dietary calcium intake is highly dependent on the regular use of dairy products. Adult populations with little or no use of dairy products such as in many African or Asian countries have a low calcium intake, whereas countries with high dairy consumption are at the upper range of calcium intake (e.g., The Netherlands). Breastfed infants receive sufficient calcium intake but after weaning their calcium intake depend on the region-specific nutritional habits. Lack of access to dairy products for many African (young) children may result in the risk of calcium deficiency rickets, especially when total calcium intake falls below 500 mg/d (Munns *et al.*, 2016). The mean calcium intake of many subjects around the world is frequently lower than the requirement for dietary calcium as defined by the Institute of Medicine or the major scientific societies for children and adults (about 1000 mg or 25 mmol per day). This implies that, in many countries, even more than half of the population may have lower than optimal calcium intake. Dietary calcium, as found in vegetable matter, is often complexed with phytates, oxalate, citrate or other organic acids and may therefore not easily be accessible for intestinal absorption. In the absence of sufficient dietary calcium, a calcium supplement may be required especially in subjects not willing or not able to consume dairy products or elderly subjects at risk for osteoporosis.

The intestinal absorption of calcium is dependent on many factors including the integrity of the intestinal cells, the composition of food (especially the presence or not of "chelators" of calcium) and vitamin D status. The intestinal absorption of calcium is partly an active, energy dependent transport through the cell and partly passive and paracellular. The active uptake is highly dependent on the presence of $1,25(OH)_2D$ and its genomic actions in the enterocytes. This active hormone stimulates the synthesis of TPRV6 channels which, after expression at the luminal site, allows the influx of calcium molecules. Thereafter, one or more intracellular calcium binding proteins such as calbindins (also largely dependent on the genomic action of $1,25(OH)_2D$) bind calcium ions and allow their transport to the serosal site of the enterocyte. Finally, an ATP-dependent calcium transport mechanism is needed to pump intracellular calcium against a concentration and electric gradient into the blood stream. These three set of calcium transporters are all vitamin D dependent but additional transport mechanisms, not yet fully identified, are probably also operational (Christakos *et al.*, 2016). The active calcium uptake is mainly located in the proximal part of the gut

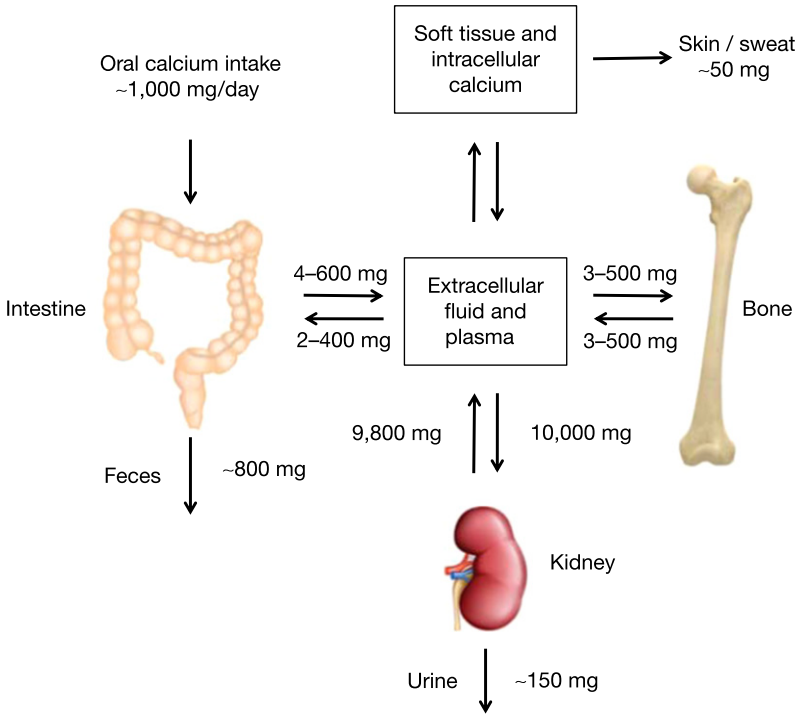

Fig. 1 The regulation of calcium transport in the major target tissues. Simplified overview of calcium homeostasis. The daily calcium intake is highly variable between persons and mainly depend on dairy intake. Dietary calcium is mixed with calcium present in all fluids reaching the intestine (from salivary, gastric, pancreatic, biliary, and intestinal origin). The large majority of dietary/exogenous and endogenous is not absorbed and excreted in the feces. The net calcium absorption is the difference between the total amount of calcium absorbed minus the sum of calcium in different intestinal secretions. The daily flux of calcium from (resorption) and into (mineralization) the bone compartment is in balance in normal healthy adults but can be positive (growth) or negative (old age/osteoporosis). The calcium loss by the skin is usually small. The kidney is responsible for the fine-tuning of the calcium balance. Serum ionized calcium is maintained by several calciotropic hormones. The flux of calcium into the fetus or in breast milk is not shown in this slide.

(duodenum and jejunum) whereas the more passive absorption is taking place in the more distal parts of the gut. The efficacy of intestinal calcium absorption can be highly variable and depend on age, $1,25(OH)_2D$ and other factors. It can exceed 60% in growing children and decrease to below 20% in case of lack of vitamin D action. The absorption efficacy is also higher in case of low calcium intake (probably highly $1,25(OH)_2D$-dependent). As shown in **Fig. 1**, the calcium intake is mixed with endogenous calcium secretion. The fractional calcium uptake then defines the net calcium absorption and fecal calcium excretion. In other words, true absorbed calcium is the total calcium absorbed from the calcium pool present in the intestines containing both dietary and digestive juice components. Net absorbed calcium is the difference between dietary calcium and fecal calcium and is the same as true absorbed calcium minus endogenous fecal calcium. At zero calcium intake, all the fecal calcium is endogenous and represents the digestive juice calcium, which has not been reabsorbed; net absorbed calcium at this intake is therefore negative to the extent of about 200 mg (5 mmol). In normal adults, fecal calcium excretion is about 0.8 g out of 1 g ingested with a net calcium absorption of 0.2 g/d. Although this seems to be only a 20% absorption, the fractional uptake of calcium (from exogenous and endogenous calcium together) is about 40% (**Fig. 1**). Correct measurements of calcium uptake is difficult and optimally requires either (1) a long term (more than 1 week) true calcium balance in specialized units (measurement of true calcium content of all food and fluids, measurement of urinary and fecal calcium content with color coding of start and end of observation period), or (2) a dual calcium isotope (preferably non-radioactive) study, whereby one isotope is given orally and one intravenously. These are very expensive and time consuming studies so that frequently a simplified method is used by measuring the absorption of a single orally given calcium isotope (Christakos *et al.*, 2016). **Table 1** summarizes the most frequent causes (physiologic or disease states) associated with increased or decreased efficacy of intestinal calcium absorption.

Kidney

Renal calcium handling is complex (Moor and Bonny, 2016). The urinary calcium excretion is dependent on the glomerular (ultra)filtration of calcium and reabsorption of this ion by renal tubular cells at different segments of the nephron. There is a massive amount (about 10 g) of calcium filtered per day (**Fig. 1**) and therefore about 99% needs to be reabsorbed. The proximal tubular cells are responsible for the large majority (65%–70%) of the reabsorbed calcium and is parallel with the massive reabsorption of water and sodium, and this absorption is mostly through a paracellular route and by passive diffusion and

Table 1 Altered efficacy of intestinal calcium absorption and its most frequent causes

Increased	*Decreased*
Growth	Vitamin D deficiency or low $1,25(OH)_2D$
Pregnancy	Chronic renal failure
Lactation	Hypoparathyroidism
Low dietary calcium intake	High dietary calcium intake
High $1,25(OH)_2D$ concentration (renal or extra-renal)	Aging
Primary hyperparathyroidism	Glucocorticoid excess (endogenous or exogenous)
Idiopathic hypercalciuria	Short bowel syndrome or "intestinal failure"

"solvent drag." The fine-tuning of calcium reabsorption takes place in the more distal parts of the tubuli (thick ascending limb of Henle, distal convoluted tubuli and collecting ducts). In the thick ascending loop, calcium reabsorption uses the paracellular pathway and is driven by the ion pumps, responsible for sodium and potassium transport, that create a transepithelial potential difference favoring calcium reabsorption. The calcium sensing receptor (CaSR) and several claudins play an important role in this ion transport. Overall, parathyroid hormone and calcitonin stimulate the active calcium uptake in the thick ascending loop. The distal tubular cells are responsible for the transcellular transport of calcium, first from the luminal site into the cytoplasma and later on by uphill (against a chemical and electrical gradient) transport to the serosal site (blood stream). The TRPV5 channel plays a very important role in this transport of calcium, in contrast to its related protein, TRPV6, that is responsible for the luminal transport of calcium in the intestine. Parathyroid hormone and calcitonin are the two major drivers of this reabsorption/excretion.

Overall, the urinary calcium excretion is kept well below 300 and 400 mg/d in adult women and men, respectively, but the median excretion varies between 100 and 200 mg/d. This minimal or "obligatory" urinary loss of calcium is already a good indicator of the minimal net intestinal calcium absorption needed to maintain a normal calcium homeostasis.

Hypercalciuria has many genetic or non-genetic etiologies and is closely linked to nephrocalcinosis and kidney stone formation (Coe *et al.*, 2016; Moor and Bonny, 2016).

Epidermal Loss of Calcium

Apart from loss of calcium in the urine and feces, some calcium is lost through the skin. This cannot be easily measured, but combined balance and isotope procedure has yielded estimates of daily insensible calcium losses in the range of 40–80 mg (1–2 mmol), and is unrelated to calcium intake. The loss of calcium through the skin is largely due to its presence in sweat and thus highly dependent on the total volume of sweat. The ion concentration of sweat (especially sodium but also calcium) is subject to acclimatization so that the total loss of ions can be minimalized in case of high transpiration. Overall, the normal loss of calcium through the skin is small (well below 100 mg/d). Calcium is an important regulator of skin homeostasis. Indeed, the epidermis of the skin is a stratified squamous epithelium composed of proliferating basal and differentiated keratinocytes. The skin is essential as physical and chemical barrier against infection and environmental insults, as well as preventing loss of water and ions from the body. Extra- and intracellular calcium is a key regulator of the proliferation and differentiation of keratinocytes.

Regulation of Extracellular Calcium Concentration

The pool of calcium in plasma is small in comparison with total body calcium but also in relation to the daily fluxes of calcium. The concentration of ionized calcium is plasma is tightly regulated within narrow limits by several mechanism and is vital for a variety of vital body functions, as mentioned in the introduction. The pool of ionized calcium in plasma is well below 500 mg and thus small in comparison with the amount of calcium filtered per day in the kidney (about 10 g) and amount of calcium released from bone (300–500 mg/d) or the dietary intake of calcium (about 1 g/d). Such a tight feed-back control of ionized calcium requires a calcium-sensing mechanism, appropriate signaling (hormonal) systems and effector target tissues. The sensor is the widely expressed, G-protein coupled receptor, *CaSR or calcium-sensing receptor*. This receptor is vital for the correct functioning of the parathyroid gland and its secretion of parathyroid hormone (**Fig. 2**). Mutations of this receptor results in an abnormal threshold for serum calcium, either too low as in case of an activating mutation or as hypercalcemia in case of a silencing mutation. This calcium sensing receptor is also highly important in the kidney to control calcium reabsorption. The *target tissues* responsible for short and long-term calcium homeostasis mainly include the intestine and the kidney as described above. *Several hormones* regulate the fluxes of calcium. Parathyroid hormone is a main stimulator of bone resorption and decreases the fractional excretion of calcium in the urine. Both mechanisms operate on a short term basis whereas PTH also stimulates the renal production of $1,25(OH)_2D$ and thus indirectly the intestinal absorption of calcium. The rapid effects of PTH on calcium handling in the kidney and on bone resorption are probably the main mechanisms allowing minute-to-minute maintenance of stable ionized calcium(Potts and Gardella, 2007). Vitamin D is metabolized into $1,25(OH)_2D$ in the kidney and then becomes a major calcium-regulating hormone. The vitamin D endocrine system started early in the evolution of vertebrates and is probable

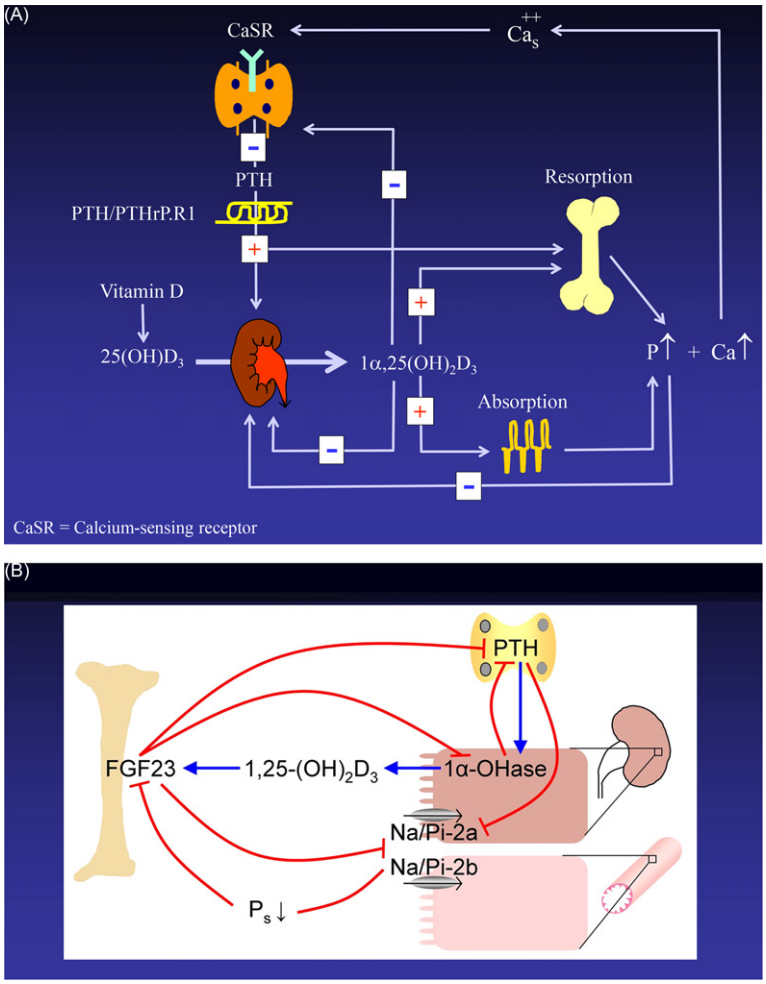

Fig. 2 (A) Control of PTH, FGF23, and vitamin D metabolism by calcium and phosphate. (B) Scheme of interaction between 1,25-$(OH)_2$D-VDR system and phosphate homeostasis.

the major hormone able to assure adequate supply of calcium even in situations of relatively low calcium intake (Bouillon and Suda, 2014). Vitamin D also plays a vital role in the normal mineralization of bone matrix but most of these effects are indirect by its effect on the intestinal absorption of calcium (Christakos *et al.*, 2016). Indeed, 1,25$(OH)_2$D stimulates the active transcellular uptake of calcium in the intestine by regulating all major genes/proteins necessary for calcium transport. Even the more passive paracellular uptake of calcium is probably partially regulated by the vitamin D hormone (Christakos *et al.*, 2016). The renal calcium handling is also partially under the control of the vitamin D endocrine system as VDR null mice loose more calcium in the urine than expected when compared with wild-type mice or mice with selective loss of VDR in the intestine. 1,25$(OH)_2$D thus stimulates the resorption of calcium in the renal tubuli and this is in line with the direct effect of 1,25$(OH)_2$D on several calcium-transporting genes in the kidney. 1,25$(OH)_2$D is able to prevent rickets by facilitating mineral deposition in bone matrix but also able to potently stimulate bone resorption and, in high concentration, also actively inhibits mineral deposition. This strongly indicate that the most important physiological role of this hormone is to maintain serum calcium homeostasis, and if needed to do so at the expense of bone calcium content.

Whereas PTH and 1,25$(OH)_2$D both increase the serum concentration of calcium, calcitonin is the only hormone able to decrease serum calcium by a combination of rapid and effective inhibition of bone resorption and increasing urinary calcium excretion. Its effects on osteoclasts is quite spectacular as it is able to immediately change the morphology of the osteoclasts whereby these large multinuclear cells loose contact with the bone and therefore stops bone resorption. The overall contribution of calcitonin to serum calcium homeostasis in mammals is, however, disputed (Davey and Findlay, 2013) as total loss of calcitonin after total thyroidectomy or large excess of calcitonin is case of medullary thyroid carcinoma hardly affects serum calcium homeostasis. Most experts therefore consider calcitonin to be a major calcium-regulating hormone for fish living in oceans with high calcium concentrations, except in situations of high bone turnover such as during lactation. PTH and especially 1,25$(OH)_2$D became more prominent hormones for animals living in an environment with lower dietary calcium access and in need for stronger bones to cope with higher gravity than in an aquatic milieu (Bouillon and Suda, 2014).

Fibroblast growth factor 23, FGF23, is a bone-derived hormone regulating phosphate homeostasis. Its secretion is stimulated by $1,25(OH)_2D$ and probably inhibited by PTH. Its receptor forms a complex with Klotho and then inhibits the membrane expression of the sodium-phosphate transporter in the renal tubular cells so that it inhibits renal phosphate reabsorption and thereby decreases serum phosphate. It therefore is also known as phosphatonin. In addition, it inhibits the renal 1α-hydroxylation (CYP27B1) of 25OHD and stimulates the degradation of 25OHD and $1,25(OH)_2D$ by enhancing 24R-hydroxylase (CYP24A1) activity. Therefore, despite being a major phosphate regulating hormone, FGF23 also has major indirect effects on calcium homeostasis, although excess or deficiency of FGF23 does not usually results in abnormal serum calcium concentrations due to compensatory activity of the other calcium regulating hormones.

Other hormones, such as sex steroid hormones, thyroid hormones, glucocorticoids, growth hormone, and IGF family members all have some effect on calcium and bone homeostasis by their effects in different target tissues. However, their effects are usually long-term effects and do not markedly influence short-term feed-back control of ionized serum calcium.

A large variety of diseases can result in hypo- or hypercalcemia, as discussed in other chapters of this handbook. These diseases either involve the calcium-sensing mechanism (CaSR mutations), the production, metabolism or action of calcium-regulating hormones (and their tissues of origin), or the target/effector tissues for calcium transport (especially kidney and intestine).

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Calcium-Sensing Receptor

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Abbreviations

ADH	Autosomal dominant hypocalcemia
BMD	Bone mineral density
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium-sensing receptor
FHH	Familial hypocalciuric hypercalcemia
GPCR	G-protein-coupled receptor
GWAS	Genome wide association studies
IGF-1	Insulin growth factor 1
NFAT	Nuclear factor of activated T cell
PTH	Parathyroid hormone
RANK-L	Ligand of receptor activator of NF-κB
TRAP	Tartrate-resistant acid phosphatase.

Introduction

CaSR is a 1078 amino acid protein that belongs to the third class of G-protein-coupled receptor (GPCR) family. It was cloned from bovine parathyroid cells in 1992 (Brown *et al.*, 1992) and two years later in human parathyroid cells and renal tubular cells (Aida *et al.*, 1995; Garrett *et al.*, 1995). It is located in caveolin-rich regions of the plasma membrane as a disulfide-linked homodimer, but may also be detected as a heterodimer with other members of the GPCR family (Kifor *et al.*, 1998). The CaSR molecule includes three domains: a large bi-lobed Venus-flytrap-like extracellular domain of 612 amino acids, a seven membrane-spanning domain of 250 amino acids and a C-terminal intracellular tail of 216 amino acids (Riccardi and Kemp, 2012). CaSR may bind cationic substances to its extracellular domain and may be considered as a sensor of environmental cations, which modulates cellular activities. Calcium ions are the main CaSR agonists, but it is also sensitive to divalent (Ni, Cd, Co, Mg, Zn) and trivalent (Gd, La, Tb, Eu) cations and to polycationic compounds like polyamines, aminoglycosides (neomycin, gentamycin) and poly-peptides (poly-L-arginine, β-amyloid) (Riccardi and Kemp, 2012). Calcium and the other agonists bind to the negatively charged residues in the pocket of the CaSR bilobed extracellular domain and induce a conformational change of the CaSR molecule that leads to the activation of an intracellular signaling cascade by the transmembrane and intracellular domains (**Fig. 1**). The intracellular signaling pathway induced by CaSR is tissue-specific and mediated by G-proteins (Magno *et al.*, 2011). Thus, CaSR may elicit the paracrine or autocrine adaptive responses of human cells to changes in extracellular calcium concentrations. CaSR is considered as ubiquitously expressed in human cells, but reaches its highest expression in parathyroid and renal distal tubular cells. In line with this tissue distribution, CaSR has acquired its main physiological role in parathyroid glands and renal distal tubules, because it enables their cells to regulate parathyroid hormone (PTH) secretion and calcium reabsorption in response to serum calcium changes (**Fig. 1**). The increase of serum calcium stimulates CaSR to inhibit calcium reabsorption in the renal distal tubules and PTH secretion in parathyroid glands; these responses restore normal serum calcium levels, whereas the opposite occurs when serum calcium decreases (Riccardi and Kemp, 2012). CaSR is also crucial for the activities of bone cells in bone remodeling and growth according to calcium levels in bone interstitium (Dvorak *et al.*, 2004). Finally, CaSR may enhance intestinal calcium absorption by stimulating 1-alpha-hydroxylation of vitamin D in the proximal tubule and acid secretion by parietal cells in the stomach (Riccardi and Kemp, 2012). All these effects indicate that CaSR is a key factor in calcium homeostasis, crucial to maintain serum calcium within the normal range and normal activities of parathyroid, bony and renal tubular cells (Riccardi and Kemp, 2012). In addition to its role in calcium metabolism, CaSR has been implicated in insulin secretion, adipocyte metabolism, smooth muscle cell activity and gastro-intestinal function (**Table 1**), but its effects in these tissues are less relevant than that in calcium-handling organs (Riccardi and Kemp, 2012).

CaSR and Parathyroid Glands

PTH has a crucial role in calcium homeostasis and the presence of CaSR on the plasma membrane of main cells of the parathyroid glands allows them to regulate PTH secretion according to the serum calcium concentration. In parathyroid cells, CaSR stimulation by serum calcium leads to adenylate cyclase inhibition, protein kinase C activation, diacylglycerol and inositol 1,4,5-triphosphate release and cellular calcium mobilization from intracellular stores, which decreases *PTH* gene transcription and PTH secretion (Kifor *et al.*, 1997). The opposite occurs in case of hypocalcemia, which may stimulate parathyroid cell activity. Hypocalcemia may also stimulate parathyroid cell proliferation to maintain high levels of PTH synthesis and secretion (Corbetta *et al.*, 2002). Prolonged hypocalcemia and low production of 1,25-dihydroxyvitamin D may result in nodular hyperplasia of parathyroid glands in patients with chronic kidney disease or hypophosphatemic rickets (Yano *et al.*, 2000). A reduced CaSR expression was found in parathyroid adenoma from patients with primary hyperparathyroidism who are less sensitive to the inhibitory effect of serum

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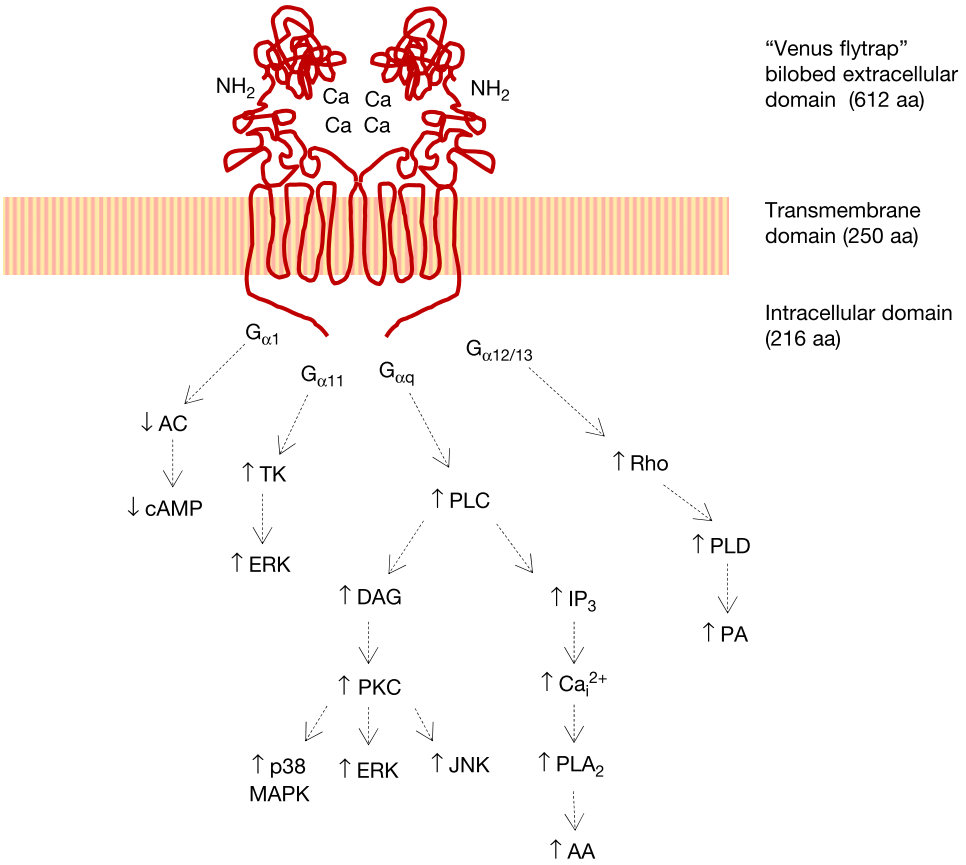

Fig. 1 Calcium ion binding to CaSR activates a complex network of intracellular signaling pathways through activation of different G-proteins. Stimulation of $G_{\alpha i}$ results in inhibition of adenylate cyclase (AC) and cAMP production. Stimulation of $G_{\alpha q}$ results in activation of phospholipase C (PLC) causing an accumulation of inositol 1,4,5-trisphosphate (IP3), rapid release of calcium from intracellular stores (Ca_i) and production of arachidonic acid (AA), as well as an increase of diacylglycerol (DAG) concentration, stimulation of proten kinase C (PKC) and activation of MAPK signal cascade: extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAP kinase. Stimulation of $G_{\alpha 11}$ activates tyrosine kinases (TK) resulting in ERK activation. Stimulation of $G_{\alpha 12/13}$ activates low molecular weight G protein Rho and phospholipase D leading to phosphatidic acid (PA) production (Riccardi and Kemp, 2012; Magno et al., 2011).

calcium on the PTH secretion. Higher concentrations of serum calcium are requested to inhibit PTH secretion in these patients and this is due to increased parathyroid gland set-point, which is the serum concentration of ionized calcium associated with the PTH serum concentration equal to 50% of that at the maximal parathyroid secretion. The defect in CaSR expression may contribute to determine hypercalcemia in primary hyperparathyroidism and may be caused by a decreased transcriptional activity of promoter 1 (Chikatsu *et al.*, 2000). A decrease in CaSR expression has been also observed in hyperplastic glands from patients with chronic kidney disease and secondary hyperparathyroidism and may contribute to their hyperplasia (Yano *et al.*, 2000).

CaSR and Kidney

CaSR can be detected in glomerular podocytes, mesangial cells and in cells of all renal tubular segments from humans and lab animals (Riccardi and Brown, 2010). Studies in lab animals suggested that CaSR might regulate glomerular filtration. These studies showed that activation of CaSR with an agonist drug (cinacalcet) may stabilize podocyte cytoskeleton and prevent disruption of the cytoskeletal structure caused by puromycin (Oh *et al.*, 2011); it may also decrease proteinuria and glomerular damage after subtotal nephrectomy in rats (Piecha *et al.*, 2008). Despite the relevance of these findings, no data concerning CaSR and glomerular function are available in humans.

The greatest tubular expression of CaSR may be found in the thick ascending limb of Henle's loop where CaSR is located in the basolateral membrane of the tubular cells and is activated by the increase in serum calcium. In this tubular segment, approximately 25% of filtered calcium is reabsorbed, mainly through a paracellular passive pathway. Microperfusion studies showed that CaSR inhibits paracellular calcium reabsorption in the ascending limb (Riccardi and Brown, 2010); this effect is mediated by the decrease of claudin-16 activity and the increase of claudin-14 expression. CaSR may upregulate claudin-14 expression by inhibiting

Table 1 CaSR expression and activity in different organs in humans (Riccardi and Kemp, 2012)

Organ	*CaSR effect*
Parathyroid glands	Inhibition of PTH secretion
Kidney	
Glomeruli	Prevention of proteinuria and glomerular sclerosis
Macula densa	Inhibition of renin secretion
Proximal tubule	Increased phosphate reabsorption
	Vitamin D hydroxylation
Ascending limb	Inhibition of paracellular calcium reabsorption
	Inhibition of sodium reabsorption
Convoluted distal tubule	Inhibition of active calcium reabsorption
Collecting duct	Proton excretion
	Urine dilution, antagonism of ADH activity
Bones	Osteoblast activation
	Osteoclast apoptosis
	Chondrocyte activation
Ovary	Oocyte maturation
Testis	Spermatozoa motility
Colon	Epithelial cell differentiation
	Fluid secretion
Duodenum	Cholecystokinin secretion
Intestine	Secretion of antidiabetic peptides
	Fluid reabsorption
Stomach	Gastrin secretion
	Acid secretion
Breast	Milk production
Pancreas	Insulin secretion
Central nervous system	Ischemia-induced injury
Glia cells	Brain ion homeostasis
Peripheral nervous system	Synaptic transmission
Adipose tissue	Lipolysis inhibition
Tongue	Kokumi taste
Arteries smooth muscle cells	Prevention of vascular calcification
Endothelial cells	Nitric oxide production
Prostate	
Heart	Cardiomyocyte hypertrophy
Blood	B lymphocyte activation
Skin	Epidermal differentiation, Cell adhesion
Thyroid gland	Calcitonin production

the production of two microRNAs, miR-9 and miR-374, suppressing claudin-14 transcription (**Fig. 2**). Claudin-16 and claudin-19 form channels in tight junctions, which drive paracellular reabsorption of calcium and magnesium, whereas claudin-14 inhibits permeability of these channels (Gong and Hou, 2014; Toka *et al.*, 2012). Claudin-16 may contribute to form tight-junction channels after phosphorylation by phosphorylase kinase A. CaSR may inhibit this phosphorilation and hamper claudin-16 location in tight-junctions and calcium reabsorption in this way (Ikari *et al.*, 2008). To decrease calcium transport in the ascending limb, CaSR may also reduce the transepithelial electric gradient driving paracellular calcium reabsorption (**Fig. 2**). This effect results from the CaSR-mediated production of arachidonic acid and 20-hydroxyeicosatetraenoic acid leading to the inhibition of the activity of sodium-potassium-chloride cotransport (NKCC2) and potassium recycling channels (Kir1.1, Kir4.1) (Gamba and Friedman, 2009; Cha *et al.*, 2011). As in the ascending limb CaSR is located in the basolateral cell membrane of distal convoluted tubules (Gamba and Friedman, 2009; Blankenship *et al.*, 2001); experiments in a canine cell model of distal convoluted tubules showed that CaSR inhibits active calcium reabsorption in this tubular segment.

Principal and intercalated cells of the collecting duct mainly express CaSR on their luminal membrane. Therefore, they are sensitive to calcium concentration in the tubular fluid that, to some extent, results from CaSR-modulated calcium reabsorption in the ascending limb and distal convoluted tubule. Its activation by luminal calcium may decrease cAMP production and aquaporin 2 trafficking and downregulate tubular cell response to vasopressin and water reabsorption (Bustamante *et al.*, 2008). In this tubular segment, CaSR activation may also stimulate urine acidification by enhancing proton pump function in the luminal membrane (Casare *et al.*, 2014).

CaSR is also expressed on the luminal membrane of proximal tubular cells and is sensitive to calcium in the glomerular filtrate. Cell culture experiments showed that CaSR antagonizes the PTH effect and promotes phosphate reabsorption via the NPT2 sodium-phosphate cotransporter by inhibiting cAMP production (Ba *et al.*, 2004). Here, it also promotes proton secretion and

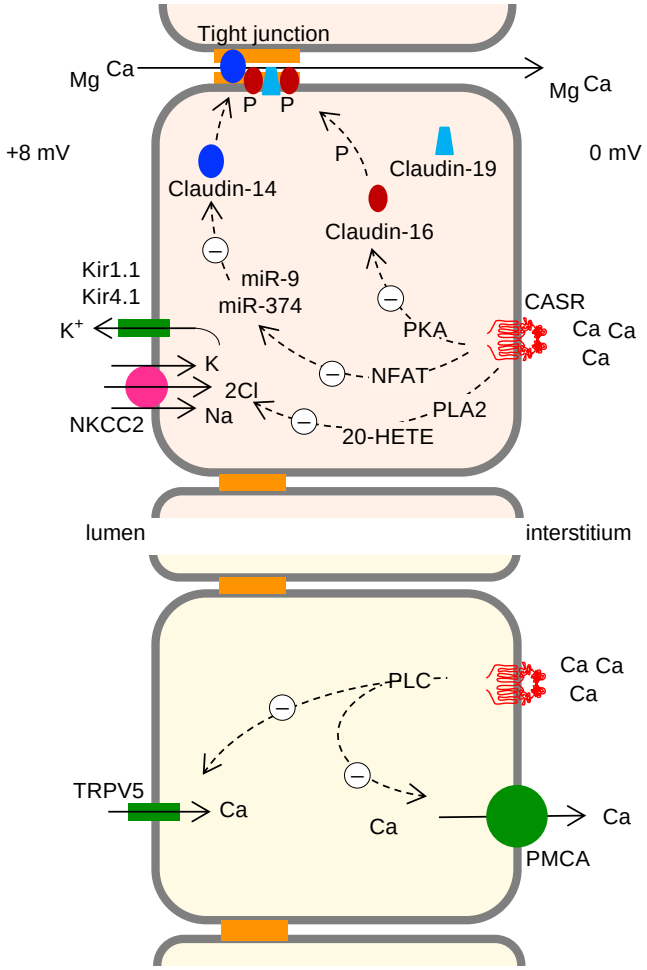

Fig. 2 Activities of CaSR in cells of the ascending limb of the Henle's loop (upper panel) and the cortical convoluted tubule (lower panel). In the ascending limb cells CaSR activation leads to (1) the inhibition of claudin-16 phosphorilation via proten kinase A (PKA) that hampers claudin-16 location in tight-junctions; (2) stimulation of claudin-14 expression through the activity of nuclear factor of activated T cell (NFAT) that inhibits the expression of two miRNA (miR9 and miR374) suppressing claudin-14 transcription; (3) reduction of sodium reabsorption through sodium-chloride-potassium cotransport carrier (NKCC2) and potassium recycling through specific channels (Kir1.1 and Kir4.1) mediated by arachidonic acid and 20-hydroxyeicosatetraenoic acid (20-HETE). These effects decrease tight-junction permeability to calcium and dissipate the electric gradient driving paracellular calcium reabsorption. In distal convoluted tubules, CaSR inhibits active calcium reabsorption by inhibiting basolateral calcium-pump (PMCA) activity and apical calcium channels by means of a pathway induced by phospholipase C (Gong and Hou, 2014; Toka et al., 2012; Ikari et al., 2008; Gamba and Friedman, 2009; Cha et al., 2011; Blankenship et al., 2001).

bicarbonate reabsorption through the NHE3 sodium–hydrogen exchanger (Capasso *et al.*, 2013), whereas it dampens 1-alpha-hydroxylation of 25-hydroxyvitamin D, thus, decreasing the production of the active metabolite of vitamin D (Egbuna *et al.*, 2009).

These findings demonstrate the integrated effects of CaSR in the kidney (**Fig. 3**). The main role of CaSR in the kidney tubule may be considered the inhibition of calcium reabsorption in the ascending limb and distal convoluted tubule that increases the risk of calcium-phosphate precipitation in the distal tubular lumen. However, this risk may be counterbalanced by the effect of CaSR in the collecting duct and the proximal tubule, where it is activated by tubular fluid calcium. CaSR in the collecting duct leads to urine acidification and dilution, while in the proximal tubule it decreases phosphate load to the distal tubule. Therefore, the effects of CaSR in the proximal tubule and collecting duct may promote calcium-phosphate solubility in urine and protect against calcium-phosphate crystalline precipitation (Renkema *et al.*, 2009).

CaSR has been also documented in the juxtaglomerular apparatus and specifically in juxtaglomerular cells and basolateral membrane of tubular cells at the macula densa. Juxtaglomerular cells secrete renin and are located in the lamina media of the afferent arteriole. CaSR activation in juxtaglomerular cells may inhibit renin release by activation of G_q protein, phospholipase C and inositol 1,4,5-triphosphate and reduction of adenylate cyclase activity and cAMP synthesis (Atchison and Beierwaltes, 2013; Ortiz-Capisano *et al.*, 2013). As a consequence, CaSR may reduce the stimulatory effect of renin on sodium reabsorption and arteriolar tone.

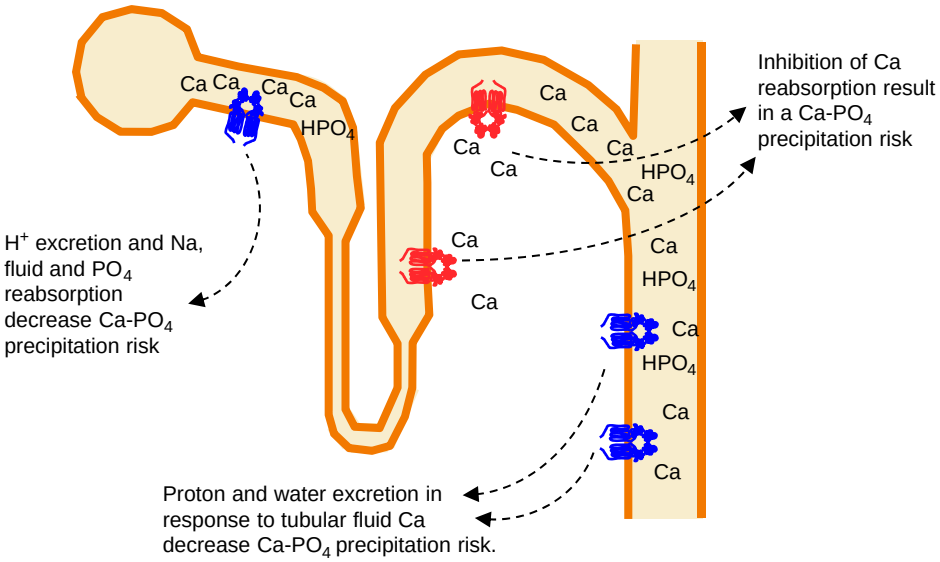

Fig. 3 CaSR decreased calcium reabsorption in the ascending limb and distal convoluted tubule. This results in calcium-phosphate precipitation risk in the tubular fluid, but this effect is counterbalanced by CaSR activities in proximal tubules and collecting ducts (Renkema *et al.*, 2009).

CaSR and Bones

There is ample evidence gathered over the last few years suggesting a strict relationship between CaSR and bone, involving regulation of the developing skeletal tissue and bone cell metabolism. Skeletal development begins at the early stages of embryogenesis and ends when peak bone mass is achieved in adulthood. During this time period the skeleton undergoes processes of elongation and modeling. The former is achieved by the coordinated differentiation, proliferation and maturation of chondrocytes in the growth plate. Extracellular calcium is important for chondrocyte differentiation and normal development of the growth plate. Calcium deficiency may cause rickets as a result of delayed chondrocyte differentiation and reduced matrix synthesis and mineralization (Thacher, 2003). Several studies showed that the extracellular calcium–sensing of chondrocytes is mediated by CaSR (Chang *et al.*, 2002, 2008). CaSR has been localized to maturing chondrocytes in the growth plate and its expression was found to increase as the cells hypertrophy, suggesting a role of the receptor in mediating terminal differentiation (Chang *et al.*, 2002). Furthermore, knocking down *CaSR* gene impaired differentiation and matrix mineralization of cultured chondrocytes induced by high extracellular calcium (Chang *et al.*, 2002). In vivo studies showed that mice with chondrocyte-specific ablation of the *CaSR* gene developed a shorter and undermineralized skeleton due to delayed differentiation of hypertrophic chondrocytes (Chang *et al.*, 2008). Hypertrophic chondrocytes from homozygous knockout mice showed a markedly reduced expression of IGF1 and IGF1 receptor, suggesting that CaSR promotes chondrocyte differentiation by enhancing IGF1 production or signaling (Chang *et al.*, 2002).

Mesenchymal progenitors are recruited at the end of chondrogenesis and they differentiate into cells of the osteoblastic lineage. The osteoblast differentiation is modulated by various local and systemic factors, including extracellular calcium. Studies performed on cells of the osteoblastic lineage demonstrated that changes in extracellular calcium affect cell proliferation, differentiation, and mineralization via the CaSR (Dvorak *et al.*, 2004; Thacher, 2003). Despite the evidence that CaSR mediates extracellular calcium-sensing in osteoblasts, its role in bone development in vivo has been controversial. The generation of a conditional CaSR knockout model, in which the receptor was broadly deleted across the osteoblast lineage (including pre-osteoblasts, proliferating, differentiating and mature osteoblasts and osteocytes) showed early lethality and skeletal abnormalities (retarded skeletal development and growth) (Chang *et al.*, 2008; Dvorak-Ewell *et al.*, 2011). From the second week of life, conditional knockout mice suffered long bone fractures along with deficient skeletal mineralization. Microcomputed tomography studies showed markedly reduced mineral content in both trabecular and cortical bone, decreased bone volume and altered trabecular architecture (Dvorak-Ewell *et al.*, 2011). Fluorescently labeled mineralizing surfaces exhibited a markedly delayed and disorganized mineralization of trabecular and cortical bone. Conversely, osteoclast numbers and activity were increased in the knockout mice and RANK-L mRNA expression was increased in femoral cortices. Cultured osteoblasts isolated from the knockouts showed an elevated osteoclastogenesis potential, stimulating the development of up to fivefold more TRAP-positive osteoclasts compared with cells from control mice (Dvorak-Ewell *et al.*, 2011). Therefore, CaSR may stimulate bone anabolism in the presence of calcium by promoting commitment, survival and differentiation of early osteoblasts and by suppressing local RANK-L-dependent osteoclastogenesis.

During bone resorption, the local calcium concentration in bone interstitium may reach 8–40 mM, and the surrounding cells are exposed to these high calcium levels. This stimulus is sensed by CaSR of cells populating the bone marrow. Mesenchymal stromal cells derived from rat bone marrow migrate and proliferate in a concentration-dependent manner and overexpress

osteogenic markers when stimulated with calcium (Gonzales-Vazquez *et al.*, 2014). Moreover, CaSR blockage inhibits the cellular response to stimulation with high concentration of calcium, indicating that CaSR is a key modulator of these cellular responses. During mice fetal life, the unusual calcium level in bone interstitial fluid also triggers hematopoietic stem cells to home to the endosteal niche within the bone marrow after migration from liver or spleen. Hematopoietic stem cells that express CaSR were able to home to bone marrow, whereas those from CaSR knockout mice could not (Adams *et al.*, 2006). This mechanism leads to the preferential localization of adult mammalian hematopoiesis in bones.

Disorders Caused by CaSR Gene Mutations and Antibodies Against CaSR

The human *CaSR* gene (3q13.3-21) spans 103 kb including eight exons and two promoters, P1 and P2 (**Fig. 4**) (Canaff and Hendy, 2002). Loss-of-function mutations of the *CaSR* gene cause familial hypocalciuric hypercalcemia (FHH, OMIM #145980) in heterozygous patients and severe neonatal hyperparathyroidism (NSHPT, OMIM #239200) in homozygous patients (Hofer and Brown, 2003; Pearce *et al.*, 1995). These patients develop hypercalcemia and low calcium excretion because mutated CaSR cannot inhibit PTH production and renal tubular calcium reabsorption appropriately.

FHH patients are usually asymptomatic; their serum PTH is normal-high and serum calcium moderately high; their calcium excretion is low and may be evaluated with the calcium-to-creatinine clearance ratio. The conventional formula to calculate calcium-to-creatinine clearance ratio is the following:

$$(\text{urinary calcium}/\text{total serum calcium}) \times (\text{serum creatinine}/\text{urine creatinine})$$

All variables in this formula are expressed as mmol/L; urine variables may be measured in 24 h urine or in 2 h urine collected in the morning during fast. Values of calcium-to-creatinine clearance ratio below 0.01 are typical of FHH patients, values between 0.01 and 0.02 may be indicative of FHH, but have uncertain meaning, and values above 0.02 are fully normal (Marx, 1996). This formula is "conventional" because, in a rigorous approach, the correct formula to calculate calcium-to-creatinine clearance ratio should include serum ionized calcium in the place of total serum calcium.

Serum PTH and calcium are markedly high in patients with severe neonatal hyperparathyroidism who also develop bone demineralization, chest deformity, fractures and failure to thrive from the first months of life. Histological analysis shows enlarged parathyroid glands, with evidence of hyperplasia. The disorder may be lethal in a few days unless patients undergo parathyroidectomy (Marx *et al.*, 1986).

Gain-of-function mutations of *CaSR* gene cause autosomal dominant hypocalcemia (ADH, OMIM #601198). These patients are characterized by high urinary calcium excretion and very low concentrations of serum PTH and calcium resulting in tetany and failure to thrive. A common finding in these patients is the extremely difficult outcome of treatment. A steady state of calcium and phosphate serum concentration is rarely reached, and nephrocalcinosis is a very frequent complication (Hu *et al.*, 2004; Mora *et al.*, 2006; Maruca *et al.*, 2017). Patients with the most severe activating mutations may also develop Bartter Syndrome type 5, a disorder caused by the inhibition of tubular sodium reabsorption by CaSR and leading to urinary potassium wastage and hypokalemia (Vezzoli *et al.*, 2006).

FHH and ADH are rare disorders and in addition to CaSR mutations, they may be caused by mutations of two other genes, *GNA11* (19p13.3) and *AP2S1* (19q13.3) causing FHH and ADH type 2 and 3, respectively. *GNA11* codes for the subunit α_{11} of G proteins used by CaSR for intracellular signaling. *AP2S1* codes for adaptor protein-2 σ subunit (AP2S1) involved in membrane protein internalization, including CaSR (Hannan *et al.*, 2016).

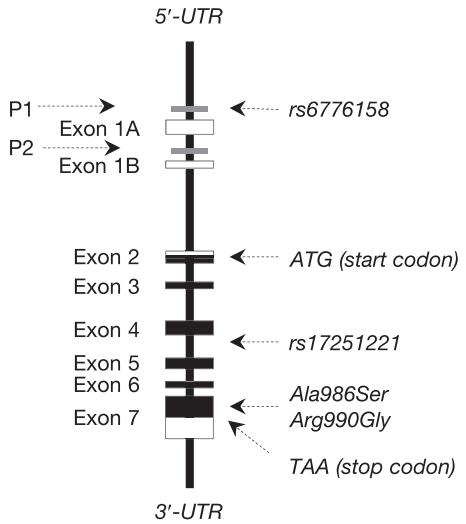

Fig. 4 The most significant polymorphisms in humans *CaSR* gene.

In addition to these genetic disorders, autoantibodies against CaSR may reduce or increase CaSR activity and cause acquired forms of hypoparathyroidism and hyperparathyroidism (Li *et al.*, 1996; Pallais *et al.*, 2004). Anti-CaSR antibodies have been found in patients with autoimmune polyendocrine syndrome, in one third of the patients with isolated acquired idiopathic hypoparathyroidism (Gavalas *et al.*, 2007) and in sporadic patients with primary hyperparathyroidism (Pallais *et al.*, 2004; Charrié *et al.*, 2009). The role of these antibodies in parathyroid disorders is under investigation, but they may be causal in a portion of cases (Makita *et al.*, 2007).

CaSR Gene Polymorphisms and Disorders of Calcium-Phosphate Metabolism

The involvement of CaSR in disorders of divalent ion metabolism has been studied by testing their association with genetic polymorphisms of the *CaSR* gene (**Table 2**). Genome wide association studies (GWAS) found that CaSR variants were associated with calcium and phosphate serum concentrations suggesting that it could be implicated in their regulation through its effects on PTH secretion and tubular reabsorption of calcium and phosphate (Kestenbaum *et al.*, 2010; O'Seaghdha *et al.*, 2010). GWAS findings indicated that rs1801725 (Ala986Ser, located in *CaSR* gene exon 7) and polymorphisms in linkage with it, like rs17251221 (located in exon 4), were associated with serum phosphate and calcium (O'Seaghdha *et al.*, 2010).

Two nonsynonymous polymorphisms of the human *CaSR* gene, rs1801725 (Ala986Ser) and rs1042636 (Arg990Gly), have been most extensively studied in humans because of their significant frequency (**Fig. 4**) (Chattopadhyay *et al.*, 2004). Both polymorphisms are located in exon 7, coding for the CaSR transmembrane and intracellular domains. In 1999, a Canadian study showed that subjects carrying the rarer allele at the polymorphism rs1801725 had higher values of serum calcium that increased with the number of copies of the minor allele (Chattopadhyay *et al.*, 2004). These findings were substantially confirmed in other studies (Hofer and Brown, 2003) and suggested that the minor allele at rs1801725 may decrease CaSR activity; however, no alteration of CaSR activity was observed when the minor allele of rs1801725 was tested in an in vitro assay using embryonic renal cells transfected with the *CaSR* gene (Pearce *et al.*, 1995). The minor allele of polymorphism rs1042636 was associated with higher urinary calcium excretion in stone formers, osteoporotic women and patients with primary hyperparathyroidism and could be responsible for idiopathic hypercalciuria (Pearce *et al.*, 1995; Marx, 1996). Experiments in embryonic renal cells transfected with the *CaSR* gene showed that the minor allele at rs1042636 caused a gain of CaSR function. This allele was also observed to increase CaSR sensitivity to calcimimetic and cell calcium oscillations; it also stimulated the activity of the endoplasmic reticulum calcium-pump that accumulates calcium within the endoplasmic reticulum (Terranegra *et al.*, 2010; Ranieri *et al.*, 2013). Therefore, the minor allele at rs1042636 could result in more efficient inhibition of calcium reabsorption in the ascending limb and increased calcium excretion (Riccardi and Brown, 2010). Hypercalciuria may predispose individuals to urinary stones and the polymorphism rs1042636 was associated with stones in some populations (O'Seaghdha *et al.*, 2010).

Another polymorphism, rs6776158, not changing amino acid sequence and located in promoter 1 was associated with calcium nephrolithiasis (**Fig. 4**) (Vezzoli *et al.*, 2013) because its minor allele was more frequent in calcium stone formers. Homozygous subjects for its minor allele had lower serum phosphate and tubular reabsorption of phosphate, consistent with a decreased CaSR influence in the proximal tubule. Accordingly, transcriptional activity of promoter 1 was decreased in two models of renal cells transfected with the minor allele at rs6776158 and CaSR expression was decreased in kidney medulla samples from patients carrying the minor allele at rs6776158 (Vezzoli *et al.*, 2013). This polymorphism was not associated with calcium excretion and could promote stones by decreasing the CaSR-mediated protective effects of urine acidification and dilution against calcium-phosphate precipitation within the tubular lumen. Contrary to what expected in the presence of a decreased CaSR expression, polymorphism rs6776158 was not related with serum calcium concentration in idiopathic stone formers; however, rs6776158 was in linkage with other polymorphisms of the 3′ untranslated region and intron 1, that were associated with stones and higher serum calcium values in patients with primary hyperparathyroidism (Vezzoli *et al.*, 2011). Findings of these studies are consistent with a decreased CaSR expression in calcium stone formers.

Table 2 *CaSR* gene polymorphisms (SNP) involved in human disorders. A list of *CaSR* gene polymorphisms is reported online in the NCBI database (http://www.ncbi.nlm.nih.gov/snp) and Calcium sensing receptor database (http://www.casrdb.mcgill.ca/)

SNP	*Domain*	*Alleles*	*Minor allele frequency*	*Amino acid change*	*Disorder associated with the minor allele*
rs1801725	exon 7	G > T	20%	986 A/S	Higher serum calcium Lower serum phosphate
rs1042636	exon 7	A > G	5%/50%[a]	990 R/G	Hypercalciuria Low serum calcium Calcium kidney stones
rs6776158	promoter 1	A > G	26%	None	Calcium kidney stones Lower serum phosphate
rs17251221	intron 4	A > G	19%	None	Higher serum calcium Higher serum magnesium

[a]5% in Caucasian population, 50% in Asian population.

Several studies investigated the association between *CaSR* polymorphisms and BMD with controversial findings. A genome-wide association study did not show any association between BMD and the *CaSR* gene locus in 2211 women and 1633 men (Gupta *et al.*, 2011). Other studies genotyped participants for rs1801725 polymorphism: a study performed in Swedish young women found a negative association of the minor allele T with BMD measured at the lumbar spine (Lorentzon *et al.*, 2001), whereas other studies did not find any association between the rs1801725 polymorphism and BMD measured at the lumbar spine, the femur or the distal forearm (Takacs *et al.*, 2002; Laaksonen *et al.*, 2009; Harding *et al.*, 2006). Conversely, two studies in patients with primary hyperparathyroidism showed that the minor allele T at the rs1801725 polymorphism was more frequent in patients who suffered from vertebral fractures; furthermore, carriers of the minor allele at rs1801725 plus two out of the following three factors, age, serum calcium concentration, and spinal BMD, showed a risk of fracture that was 4.7-fold higher than homozygotes for the more common allele (Eller-Vainicher *et al.*, 2014).

CaSR as a Drug Target

CaSR has become target for new categories of drugs, agonists or antagonists of CaSR (Nemeth and Goodman, 2016). Agonists are termed calcimimetics and may be distinguished in two groups according to their functional pattern. The first group may directly stimulate CaSR activity by binding to its extracellular domain pocket and reproducing the effect of calcium and other ligands. Calcimimetics of the other group may increase CaSR reactivity to calcium and other ligands acting as allosteric agonists interacting with the CaSR transmembrane domain (Nemeth and Goodman, 2016). Because of their stimulatory effect on CaSR, calcimimetics are used to inhibit parathyroid secretion in patients with primary hyperparathyroidism and hemodialysis patients with secondary hyperparathyroidism (Peacock *et al.*, 2009; Bover *et al.*, 2016). Two calcimimetic molecules are today available for the clinical practice: cinacalcet is a CaSR allosteric agonist used to treat both mentioned forms of hyperparathyroidism; etelcalcetide is a direct activator of CaSR, approved to treat hyperparathyroidism in hemodialysis patients. Additionally, the use of cinacalcet has been proposed in patients with FHH (Alon and VanDeVoorde, 2010). Treatment with calcimimetics also results in urinary calcium excretion and, therefore, hypocalcemia is the main, and potentially severe, complication of these drugs.

Antagonists of CaSR are called calcilytics and stimulate parathyroid gland secretion and calcium reabsorption in kidney tubules. Their clinical use in osteoporosis has been hypothesized because they could induce PTH pulsating secretion having an osteoanabolic effect in lab animals (Nemeth and Goodman, 2016). However, trials testing the calcilytic drug ronacaleret in osteoporotic patients failed to demonstrate a satisfying efficacy in this disorder, probably because it stimulated a chronic condition of "mild hyperparathyroidism" (Fitzpatrick *et al.*, 2012). Therefore, no calcilytic drug has till now been approved for the clinical use.

Conclusions

CaSR is a cation receptor through which extracellular calcium may influence cell activity, thus connecting the extracellular environment with the intracellular compartment. CaSR presence in parathyroid glands and kidney tubules has a specific relevance for calcium metabolism, because it ensures the strict control of the extracellular calcium concentration. Numerous findings have showed the relevance of CaSR for correct growth and healthy life, whereas genetic or acquired alterations of CaSR function may result in hypocalcemic or hypercalcemic disorders. *CaSR* polymorphic variants may contribute to individual variability of serum concentrations and urine excretion of calcium and phosphate; they may also predispose to calcium nephrolithiasis, whereas their effect on BMD and fracture risk remains controversial. As suggested by these functional and pathophysiological findings, CaSR has become an important target for the treatment of primary and secondary hyperparathyroidism by using calcimimetic drugs. On the contrary, calcilytic drugs have not yet found their clinical indications. However, in this field we are at the beginning of the story, because pharmacological modulation of CaSR activity could provide new instruments improving our strategy to treat disorders of calcium and phosphate metabolism or having in calcium a pathophysiological mediator.

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Further Reading

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Relevant Websites

An exhaustive list of CasR gene mutations and polymorphisms can be found in the NCBI database (http://www.ncbi.nlm.nih.gov/snp) and the Calcium sensing receptor database (http://www.casrdb.mcgill.ca/).

Phosphate Metabolism, Hyperphosphatemia, and Hypophosphatemia

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Nomenclature

DMP1	Dentin matrix protein 1.
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1.
GAlNAc-T3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3.
GALNT3	Polypeptide N-acetylgalactosaminyltransferase 3.
HFTC	Hyperphosphatemic familial tumoral calcinosis.
HHM	Humoral hypercalcemia of malignancy.
NPT	Sodium-phosphate cotransporter.
PHEX	Phosphate-regulating gene with homologies to endopeptidases on the X chromosome.
TIO	Tumor-induced osteomalacia.

Phosphate Metabolism

Phosphate is an essential anion in our body. Phosphate is one of components of bone and teeth. In addition, phosphate is a constituent of cell membrane, nucleic acids, high energy molecules like adenosine triphosphate (ATP), intracellular molecules such as 2,3-diphosphoglycerate, glucose-6-phosphate and many phosphorylated proteins. While it is not yet clear how intracellular phosphate concentration is regulated, extracellular phosphate level is maintained within a narrow range. Serum phosphate level is regulated by intestinal phosphate absorption, renal phosphate handling and shift between extracellular phosphate and phosphate in bone or intracellular space (**Fig. 1**). Renal phosphate handling is believed to be the most important determinant of serum phosphate level at least in a chronic state. Most phosphate filtered through glomeruli is reabsorbed in proximal tubules by type 2a and 2c sodium-phosphate cotransporters (NPT2a and NPT2b) (Segawa *et al.*, 2015). There are both saturable, transcellular route and nonsaturable, paracellular mechanisms in intestinal phosphate absorption. The saturable active transport is medicated by type 2b sodium-phosphate cotransporter (NPT2b) (Segawa *et al.*, 2015).

Regulatory Mechanisms of Serum Phosphate Level

Intestinal phosphate absorption and renal phosphate handling need to be regulated in a coordinated fashion in order to maintain serum phosphate level in a narrow range. Three hormones, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] and fibroblast growth factor 23 (FGF23), mainly regulate these intestinal phosphate absorption and renal phosphate handling.

PTH suppresses proximal tubular phosphate reabsorption by reducing the expression of NPT2a and NPT2b (Miyamoto *et al.*, 2004). PTH works by binding to PTH1 receptor or PTH/PTH-related protein (PTHrP) receptor (Juppner *et al.*, 1991). While PTHrP is not a hormone in a physiological state, it can induce hypophosphatemia by binding to and activating PTH1 receptor in patients with humoral hypercalcemia of malignancy (HHM) as discussed below. PTH also enhances 1,25$(OH)_2$D production in renal proximal tubules by stimulating *CYP27B1* expression which encodes 25-hydorxyivmitan D [25(OH)D]-1α-hydroxylase, an enzyme that convert 25(OH)D to 1,25$(OH)_2$D. PTH has been also reported to enhance FGF23 production (Fan *et al.*, 2016) (**Fig. 2**).

In addition to lowering serum phosphate level, PTH increases serum calcium (Ca) by enhancing osteoclastic bone resorption and Ca reabsorption in distal tubules. On the other hand, PTH secretion from parathyroid glands is mainly regulated by extracellular ionized Ca level through Ca-sensing receptor (Brown *et al.*, 1993). Activation of Ca-sensing receptor by extracellular Ca suppresses PTH secretion. Contrary, decreased serum Ca enhances PTH secretion. Thus, there is a negative feedback system between PTH secretion and serum Ca

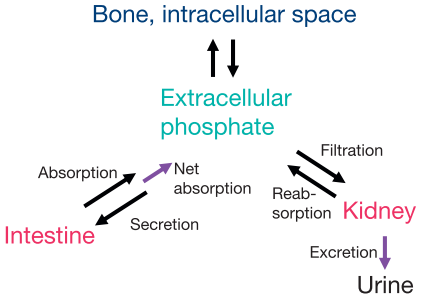

Fig. 1 Maintenance of extracellular phosphate level. Serum phosphate level is maintained by intestinal phosphate absorption, renal phosphate handling and shift between extracellular phosphate and phosphate in bone or intracellular space.

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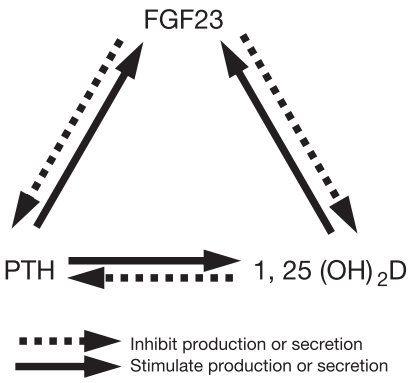

Fig. 2 Relationship among three major hormones affecting serum phosphate level.

level. It is not known whether there is a similar feedback system between PTH secretion and serum phosphate level. However, phosphate was shown to modulate PTH synthesis in animals (Naveh-Many and Nechama, 2007) (**Fig. 2**).

$1,25(OH)_2D$ stimulates intestinal phosphate absorption by enhancing the expression of NPT2b and works to increase serum phosphate by binding to vitamin D receptor (VDR) (Sabbagh *et al.*, 2011). In addition, $1,25(OH)_2D$ also increases serum Ca by stimulating intestinal Ca absorption and renal Ca reabsorption in distal tubules. $1,25(OH)_2D$ suppresses the synthesis of PTH and stimulates the production of FGF23 (Saito *et al.*, 2005).

FGF23 is produced by osteocytes/osteoblasts. FGF23 suppresses proximal tubular phosphate reabsorption by reducing the expression of NPT2a and NPT2b (Shimada *et al.*, 2004). FGF23 also decreases serum $1,25(OH)_2D$ level by suppressing the expression of *CYP27B1* and also stimulating *CYP24A1* expression (Shimada *et al.*, 2004). *CYP24A1* encodes an enzyme that works to reduce $1,25(OH)_2D$ level. From these effects, FGF23 reduces serum phosphate by inhibiting both intestinal phosphate absorption via reduced $1,25(OH)_2D$ and proximal tubular phosphate reabsorption. FGF23 is shown to work by binding to FGF receptor (FGFR)-Klotho complex (Urakawa *et al.*, 2006). While the expression of FGFR is not tissue-specific, Klotho is present in only limited tissues such as kidney, parathyroid gland and pituitary (Kuro-o *et al.*, 1997). In addition to reducing $1,25(OH)_2D$ level, FGF23 is reported to suppress PTH synthesis and secretion in animals (Ben-Dov *et al.*, 2007) (**Fig. 2**).

As shown above, PTH, $1,25(OH)_2D$ and FGF23 affect serum phosphate level. In addition, these hormones modulate the synthesis and/or secretion of other ones. Serum phosphate level is mainly maintained by the interplay of these hormone actions. In addition, several other hormones can affect serum phosphate level. For example, glucocorticoid can reduce serum phosphate by inhibiting proximal tubular phosphate reabsorption. Growth hormone enhances proximal tubular phosphate reabsorption through insulin-like growth factor I. Furthermore, serum phosphate can be changed by mechanisms independent of these hormone actions as discussed below.

Hyperphosphatemic Diseases

Causes

As discussed above, PTH, $1,25(OH)_2D$ and FGF23 are physiological hormones affecting serum phosphate levels. PTH and FGF23 reduces, and $1,25(OH)_2D$ works to increase serum phosphate level. Therefore, deficient actions of PTH or FGF23 result in hyperphosphatemia mainly by increased renal tubular phosphate reabsorption (**Table 1**). Enhanced signals from VDR also stimulate intestinal phosphate absorption. However, it is not common that frank hyperphosphatemia is induced by $1,25(OH)_2D$ actions if renal function is not impaired. Increased intestinal phosphate absorption can be compensated by enhanced urinary phosphate excretion in subjects with normal renal function.

Hypoparathyroidism is a disease characterized by impaired actions of PTH. This disease can be caused by impaired PTH secretion or resistance to PTH. In addition, severe hypomagnesemia also can cause hypoparathyroidism by both impaired PTH secretion and resistance to PTH. There are many causes for PTH-deficient hypoparathyroidism including gene mutations,

Table 1 Causes of hyperphosphatemia

1.	Impaired actions of PTH	Hypoparathyroidism
2.	Enhanced signals from vitamin D receptor	Granulomatous diseases, malignant lymphoma, vitamin D intoxication, overdose of active vitamin D…
3.	Impaired actions of FGF23	Hyperphosphatemic familial tumoral calcinosis (Mutations in *GALNT3*, *FGF23*, *Klotho*)
4.	Enhanced phosphate absorption	Phosphate enema, Phosphate containing laxatives…
5.	Impaired renal phosphate excretion	CKD, Acromegaly
6.	Shift from bone or intracellular pool	Bone metastases, hemolysis, diabetic ketoacidosis, tumor lysis syndrome, rhabdomyolysis…

autoimmunity, surgical removal, radiation, granulomatous diseases and so on. Resistance to PTH is observed in patients with pseudohypoparathyroidism.

Deficient actions of FGF23 cause hyperphosphatemic familial tumoral calcinosis (HFTC). This disease is characterized by hyperphosphatemia with enhanced proximal tubular phosphate reabsorption and ectopic calcification especially around large joints. Mutations in three genes have been shown to cause HFTC, *GALNT3*, *FGF23* and *Klotho* (Araya *et al.*, 2005; Benet-Pages *et al.*, 2005; Ichikawa *et al.*, 2007; Topaz *et al.*, 2004). FGF23 gene produces a peptide with 251 amino acids. A part of FGF23 protein is proteolytically cleaved between ^{179}Arg and ^{180}Ser before or during the process of secretion (Shimada *et al.*, 2001). Processed N-terminal and C-terminal fragments of FGF23 do not have an activity to reduce serum phosphate level (Shimada *et al.*, 2002). *GALNT3* gene encodes an enzyme called UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3). This enzyme attaches *N*-acetylgalactosamine to Ser or Thr residue as an initial step of mucin-type O-linked glycosylation. It has been shown that GalNAc-T3 initiates glycosylation of ^{178}Thr of FGF23 protein and works to prevent the processing of FGF23 between ^{179}Arg and ^{180}Ser (Frishberg *et al.*, 2007). Inactivating mutations in *GALNT3* are considered to make FGF23 protein susceptible for the proteolytic processing and cause deficient actions of FGF23. This is supported by FGF23 levels in patients with mutations in *GALNT3*. There are two kinds of assays for FGF23. Intact assay uses two antibodies that recognize N-terminal and C-terminal portion of the processing site of FGF23, respectively, and measures only biologically active full-length FGF23 (Yamazaki *et al.*, 2002). In contrast, C-terminal assay employs antibodies against C-terminal portion of FGF23 protein (Jonsson *et al.*, 2003). This C-terminal assay measures both full-length and cleaved C-terminal fragment of FGF23. FGF23 levels in patients with mutations in *GALNT3* were shown to be extremely high by C-terminal assay, but rather low by intact assay (Araya *et al.*, 2005). These results indicate that there is only a little full-length FGF23 protein while quite a large amount of processed C-terminal fragment is present in these patients. Patients with HFTC by mutations in *FGF23* also show high FGF23 levels by C-terminal assay and rather low FGF23 by intact assay (Frishberg *et al.*, 2007). A mutant FGF23 protein was shown to be trapped within Golgi while processed C-terminal fragment can be secreted (Benet-Pages *et al.*, 2005). However, other mechanisms are also proposed to explain impaired actions of FGF23 in these patients (Shawar *et al.*, 2016). FGF23 levels are high by both intact and C-terminal assay in a patient with HFTC by *Klotho* mutation (Ichikawa *et al.*, 2007). This condition is considered to be caused by resistance to FGF23 because Klotho works as a co-receptor for FGF23.

In addition to aberrant functions of these hormones, hyperphosphatemia can be induced by enhanced intestinal phosphate absorption, impaired renal phosphate excretion or shift from bone or intracellular pool (**Table 1**). Clinically, chronic kidney disease (CKD) is by far the most prevalent cause of hyperphosphatemia. During the progression of CKD, it is FGF23 that first increases followed by PTH and phosphate (Isakova *et al.*, 2011). FGF23 starts to increase in patients with CKD stage 2. It is not knows what triggers this increase of FGF23 in patients with early CKD. However, FGF23 and PTH are considered to enhance urinary phosphate excretion and prevent the development of hyperphosphatemia in these patients (Hasegawa *et al.*, 2010).

Clinical Manifestations

Acute hyperphosphatemia can cause hypocalcemia by physicochemical complex formation. Chronic hyperphosphatemia is a risk factor for ectopic calcification including vascular tissues. Hyperphosphatemia was shown to induce vascular smooth muscle cells to obtain osteoblastic characteristics (Nishizawa *et al.*, 2005). In patients with advanced CKD, hyperphosphatemia contributes to the development and progression of secondary hyperparathyroidism by enhancing PTH synthesis and parathyroid cell proliferation (Naveh-Many *et al.*, 1995; Naveh-Many and Nechama, 2007).

Treatment

Treatment of hyperphosphatemia is different depending on the causes. Hyperphosphatemia by drugs such as phosphate containing laxatives may improve spontaneously if the drug administration is stopped and renal function is maintained. Hyperphosphatemia by hypoparathyroidism usually improves by treating hypocalcemia. Hyperphosphatemia in patients with CKD is managed by dietary phosphate restriction and phosphate binders. Dialysis is the final method for patients with severe hyperphosphatemia especially when renal function is compromised. In patients with HFTC, acetazolamide has been tested in a couple patients together with phosphate restriction and phosphate binders (Lammoglia and Mericq, 2009; Yamaguchi *et al.*, 1995). Surgical treatment for calcified mass may be necessary in some patients with HFTC.

Hypophosphatemic Diseases

Causes

Hypophosphatemia also can be caused by aberrant signals from hormone receptos. As PTH binds to PTH1 receptor, enhanced signals from this receptor can cause hypophosphatemia. This is typically seen in patients with primary hyperparathyroidism. PTHrP is a cytokine regulating proliferation and function of many tissues including cartilage, bone, pancreas, keratinocyte, mammary gland and vasculature (Martin, 2016). However, in patients with HHM, PTHrP produced by tumor cells works as a systemic humoral factor and cause hypercalcemia and hypophosphatemia by binding to PTH1 receptor. Hypophosphatemia by

enhanced signaling from PTH1 receptor can also be found in patients with familial hypocalciuric hypercalcemia and Jansen's metaphyseal chondrodysplasia (Schipani *et al.*, 1995).

Vitamin D deficiency is defined by low circulatory 25(OH)D levels. Serum level of $1,25(OH)_2D$ in patients with vitamin D deficiency can be variable. It is not entirely clear why low 25(OH)D level causes impaired actions of vitamin D metabolites while $1,25(OH)_2D$ is considered to be an active hormone. Recent studies revealed that *CYP27B1* is expressed in several other tissues than kidney such as parathyroid gland, osteoblasts, keratinocytes and so on (Adams *et al.*, 2014). It is possible that vitamin D deficiency causes impaired synthesis of local $1,25(OH)_2D$ and results in impaired actions of local $1,25(OH)_2D$. Patients with vitamin D-dependent rickets show frank hypocalcemia and hypophosphatemia because of impaired systemic actions of $1,25(OH)_2D$. Some drugs such as diphenylhydantoin and rifampicin can reduce 25(OH)D level (Grober and Kisters, 2012).

Since the identification of FGF23, several kinds of genetic and acquired hypophosphatemic diseases have been shown to be caused by excessive actions of FGF23 (**Table 3**). Of these, X-linked hypophosphatemic rickets (XLH) caused by inactivating mutations in *phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX)* is by far the most prevalent cause of genetic hypophosphatemic rickets. Several terms such as vitamin D-resistant rickets and hypophosphatemic rickets have been used as a synonym for this disease. *PHEX* was identified in 1995 by positional cloning (The HYP Consortium, 1995). PHEX protein is a single membrane spanning protein which shows some homology to endopeptidases such as endothelin-converting enzyme-1 and Kell antigen (Turner and Tanzawa, 1997). It was shown that *Fgf23* is overexpressed in bone in model mice of XLH (Liu *et al.*, 2003). However, it is not clear how mutations in *PHEX* result in overproduction of FGF23 in bone. Mutations in *FGF23* in patients with autosomal dominant hypophosphatemic rickets (ADHR) changes ^{176}Arg or ^{179}Arg to other amino acids (ADHR Consortium, 2000). The mutant protein was shown to be resistant for the proteolytic processing of FGF23 protein between ^{179}Arg and ^{180}Ser (Shimada *et al.*, 2002; White *et al.*, 2001). It is known that symptoms and biochemical abnormalities can wax and wane with time in patients with ADHR (Imel *et al.*, 2007). It has been suggested that iron deficiency enhances FGF23 production and causes hypophosphatemia in patients with ADHR (Imel *et al.*, 2011). There are several other genes such as *dentin matrix protein 1 (DMP1)* (Feng *et al.*, 2006; Lorenz-Depiereux *et al.*, 2006) and *ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)* (Levy-Litan *et al.*, 2010; Lorenz-Depiereux *et al.*, 2010) whose mutations cause genetic FGF23-related hypophosphatemic rickets (**Table 3**). In these diseases, FGF23 is considered to be overexpressed in bone. However, it is largely unknown how mutations in these genes cause overproduction of FGF23.

FGF23 can also induce hypophosphatemia in patients with tumor-induced osteomalacia (TIO). TIO is a paraneoplastic syndrome usually caused by benign mesenchymal tumors (Minisola *et al.*, 2017). However, there are several cases of TIO caused by malignant tumors. These tumors are considered to be ectopically producing FGF23. FGF23 is also high in hypophosphatemic patients with intravenous administration of iron preparations (Schouten *et al.*, 2009; Shimizu *et al.*, 2009). It was reported that FGF23 is expressed in liver and can produce hypophosphatemia in patients with biliary atresia (Wasserman *et al.*, 2016).

Hypophosphatemia can also be caused by impaired intestinal phosphate absorption, renal phosphate wasting and shift into bone or intracellular pool independent of signals from these hormone receptors mentioned above (**Table 2**).

Differential Diagnosis

Renal tubular phosphate reabsorption can be evaluated by tubular maximum transport of phosphate per glomerular filtration rate (TmP/GFR) (Walton and Bijvoet, 1975). Low TmP/GFR in hypophosphatemic patients indicates impaired renal phosphate reabsorption. In addition, measurement of several hormones is useful for differential diagnosis of various causative diseases in hypophosphatemic patients (**Fig. 3**). Clinical information usually indicates the presence of conditions that cause phosphate shift into bone or intracellular pool. Hypophosphatemic patients by enhanced signaling from PTH1 receptor typically show

Table 2 Causes for hypophosphatemia

1. Enhanced signals from PTH1 receptor	Primary hyperparathyroidism Humoral hypercalcemia of malignancy (PTHrP-secreting tumor) Ectopic PTH-secreting tumor Familial hypocalciuric hypercalcemia Jansen metaphyseal chondrodysplasia (Activating mutations in *PTH1 receptor*)
2. Impaired signals from vitamin D receptor	Vitamin D deficiency Vitamin D-dependent rickets (VDDR) type 1 (Inactivating mutations in *CYP27B1*) Vitamin D-dependent rickets (VDDR) type 2 (Inactivating mutations in *VDR*) Drugs (diphenylhydantoin, rifampicin…)
3. Excess actions of FGF23 (**Table 3**)	
4. Impaired phosphate absorption	Malnutrition, Short bowel syndrome, Alcoholism, Drugs (antacids with aluminum or magnesium)…
5. Renal phosphate wasting	Fanconi syndrome, Hereditary hypophosphatemic rickets with hypercalciuria, Dent disease, Cushing syndrome, Drugs (adefovir dipivoxil, iphosphamide…)…
6. Shift into bone or intracellular pool	Hungry bone syndrome, Refeeding, Leukemia blast crisis, Treatment of diabetic ketoacidosis…

Table 3 FGF23-related hypophosphatemic diseases

	Responsible gene
X-linked hypophosphatemic rickets: XLH	*PHEX*
Autosomal dominant hypophosphatemic rickets: ADHR	*FGF23*
Autosomal recessive hypophosphatemic rickets 1: ARHR1	*DMP1*
Autosomal recessive hypophosphatemic rickets 2: ARHR2	*ENPP1*
McCune–Albright syndrome/Fibrous dysplasia of bone	*GNAS1*
Jansen-type metaphyseal chondrodysplasia	*PTH1R*
Hypophosphatemia, dental anomalies and ectopic calcification	*FAM20C*
Hypophosphatemia, skin and bone lesions	*HRAS, KRAS, NRAS*

Tumor-induced osteomalacia: TIO
Hypophosphatemia by iron polymaltose, saccharated ferric oxide or ferric carboxymaltose
Biliary atresia

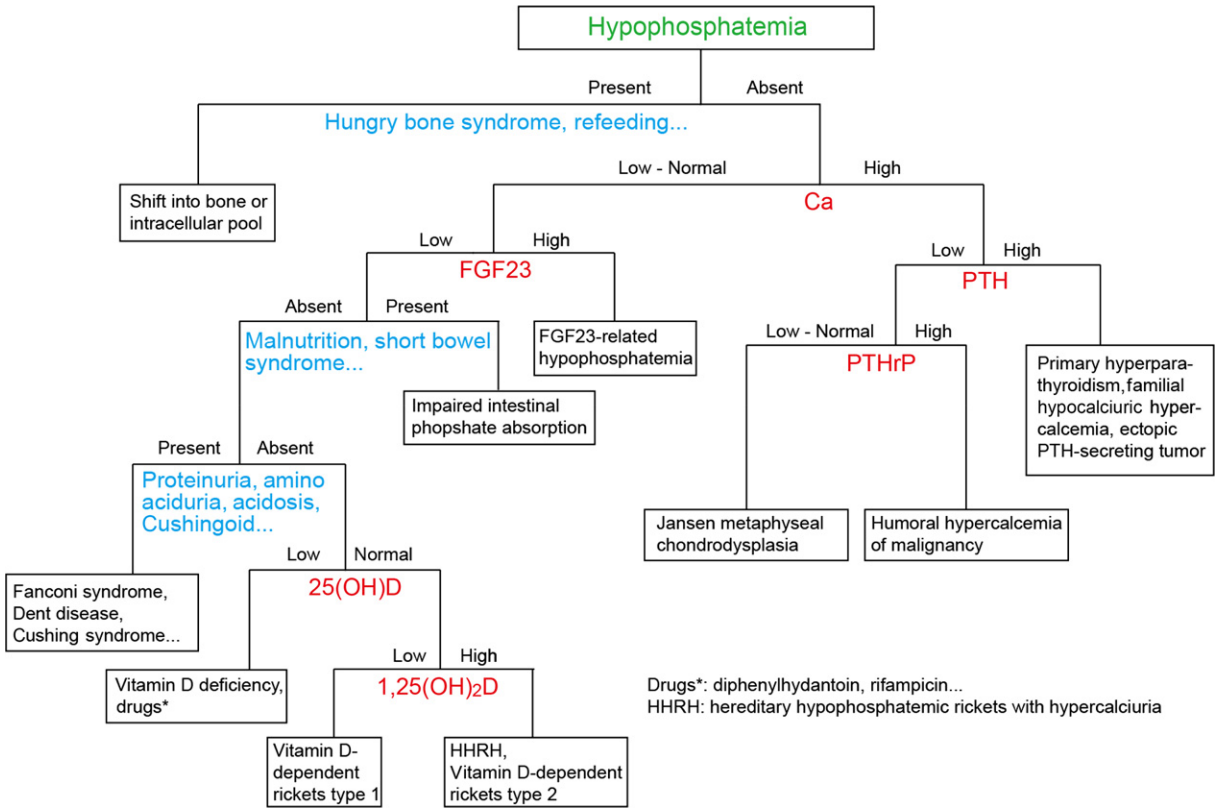

Fig. 3 Flowchart for differential diagnosis of hypophosphatemic diseases. PTH, $1,25(OH)_2D$ and FGF23 modulate the production or secretion of other hormones each other. PTH stimulates $1,25(OH)_2D$ and FG23 production. $1,25(OH)_2D$ enhances FGF23 production and suppresses PTH synthesis. FGF23 inhibits $1,25(OH)_2D$ production and suppresses PTH production and secretion.

hypercalcemia. High FGF23 level in hypophosphatemic patients indicates the presence of FGF23-related hypophosphatemic diseases. On the other hand, FGF23 is rather low in patients with chronic hypophosphatemia by other causes (Endo *et al.*, 2008). This suggests that endogenous FGF23 production is suppressed in these hypophosphatemic patients. Furthermore, other clinical data can indicate impaired intestinal phosphate absorption and renal phosphate wasting. Finally, measurement of vitamin D metabolites is useful for differential diagnosis of several hypophosphatemic diseases.

Symptoms

Hypophosphatemia can cause several symptoms. Because of decreased 2,3-diphosphoglycerate in red blood cells, severe hypophosphatemia causes tissue hypoxia. In addition, hypophosphatemia can reduce cellular ATP content and impair cellular function. Hypophosphatemia can result in heart failure, rhabdomyolysis, hemolysis, muscle weakness, arrhythmia and abnormalities in central nervous system such as delirium and coma.

Chronic hypophosphatemia causes rickets and osteomalacia characterized by impaired mineralization of bone matrix. Rickets develops in children before the closure of growth plate. Growth retardation and bone deformities such as genu valgus and valgum are typical problems of rickets. On the other hand, osteomalacia occurs in adult. Bone pain and muscle weakness are usual symptoms of patients with hypophosphatemic osteomalacia. While there are many causes for rickets and osteomalacia, chronic hypophosphatemia is present in most cases of rickets/osteomalacia.

Treatment

If the cause for hypophosphatemia can be corrected, that should be done. Acute symptomatic hypophosphatemia can be treated by parenteral phosphate administration. Treatment for hypercalcemia is usually more important for hypophosphatemic patients by enhanced signals from PTH1 receptor. Vitamin D deficiency is basically treated by native vitamin D and vitamin D-dependent rickets type 1 can be managed by a physiological amount of active vitamin D. Large amount of active vitamin D and calcium are used for patients with vitamin D-dependent rickets type 2 while the response is variable depending on the mutations in *VDR* gene.

Patients with TIO can be cured by complete surgical removal of responsible tumors. In addition, hypophosphatemia improves after cessation of intravenous iron preparations. Patients with other FGF23-related hypophosphatemic diseases including those with TIO whose tumors cannot be resected are currently treated with oral phosphate and active vitamin D. While these drugs are effective, they also have several limitations (Carpenter *et al.*, 2011). New treatment methods for patients with FGF23-related hypophosphatemic diseases are now being considered. Other chronic hypophosphatemia is usually treated by oral phosphate supplementation.

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Vitamin D☆

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Glossary

Alfacalcidol 1α-Hydroxyvitamin D.
Calcifediol 25-Hydroxyvitamin D.
Calcitriol 1,25-Dihydroxyvitamin D.
International unit Doses of the calciferols are often expressed in international units (IU), there being 40 IU/μg of cholecalciferol and 38.8 IU/μg of ergocalciferol.

Vitamin D is a term encompassing the two chemical entities cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). The vitamin Ds are secosteroids, which act as the substrate for the synthesis of a family of compounds involved in the regulation of calcium metabolism.

Introduction

Vitamin D is misnamed in that it is not a vitamin and this problem leads to fundamental confusion regarding its role in health and disease. Vitamins are usually defined as essential dietary constituents. Although vitamin D can occur in the diet, endogenous production is quantitatively much more important in most individuals. Therefore, vitamin D should be regarded as a substrate that is necessary for the synthesis of a family of hormones primarily recognized for their regulation of calcium metabolism. As a result, vitamin D status is not really a nutritional issue, but is determined by sunlight exposure and supplement use. The parent compounds are metabolized to a number of other secosteroids of varying, and sometimes uncertain, biological activity. Some of these compounds are important regulators of calcium metabolism, although they are recognized as having other important activities as well. Some are available as pharmaceuticals and play important roles in the management of conditions such as vitamin D deficiency, chronic renal failure, hypophosphatemia, and hypocalcemia. Novel pharmaceuticals, which may find applications in other conditions, are being developed within this class.

Structure and Synthesis of Vitamin D and Its Metabolites

Vitamin D_3 is a 27-carbon secosteroid (**Fig. 1**) that is derived from 7-dehydrocholesterol as a result of the action of ultraviolet (UV) light in the skin of animals (Bikle *et al.*, 2013). The UVB spectrum (wavelength 290–320 nm) results in the rupture of the bond between C-9 and C-10 in 7-dehydrocholesterol, leading ultimately to the production of vitamin D_3. A similar process takes place in some plants, leading to the conversion of ergosterol to vitamin D_2, which has a methyl group attached to C-24 and is widely used as a vitamin D supplement. With sustained exposure to sunlight, there is increased production of inactive vitamin D metabolites, such as lumisterol, providing a mechanism for preventing vitamin D intoxication. Cutaneous vitamin D production is related to the intensity of UVB irradiation and thus diminishes with increasing latitude. It is also diminished by skin pigmentation (particularly in individuals of African and Indian origin) and by advancing age. Covering the skin, whether with clothing or sunscreens, also provides an effective barrier to vitamin D production. Vitamin D_2 and vitamin D_3 have usually been regarded as having comparable biological effects, though some studies have suggested that metabolites in the vitamin D_3 series may have greater biological potency. The calciferols are fat-soluble and are absorbed primarily in the jejunum and ileum. This process involves bile acids and any disease state associated with malabsorption of fat is likely to be associated with malabsorption of vitamin D. Vitamin D is relatively uncommon in an unsupplemented diet, occurring in fish oils, egg yolk, butter, and liver. In the United States, supplementation of food (particularly dairy products) with vitamin D is common, though it has been demonstrated that actual levels of vitamin D in foods are sometimes widely different from those claimed on the packaging.

Transport

Following its synthesis in the skin or absorption from the gastrointestinal tract, vitamin D circulates bound to vitamin D-binding protein. Vitamin D-binding protein is encoded by a gene located on chromosome 4 and is structurally related to α-fetoprotein and

☆*Change History*: November 2017. Ian R Reid updated the text to this entire article. Figures and "Further Reading" replaced.

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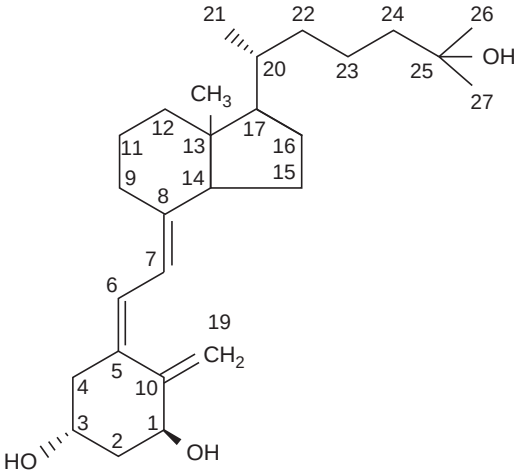


1,25(OH)$_2$D$_3$

Fig. 1 Structure of 1,25(OH)$_2$D$_3$.

albumin. It circulates at a concentration of 4–8 μM and there is one binding site for a vitamin D metabolite on each molecule. Thus, only approximately 5% of the binding sites are usually occupied. The affinities of the vitamin D metabolites for vitamin D-binding protein are in the following order: 25(OH)D$_3$ > 24,25(OH)$_2$D$_3$ > vitamin D$_3$ ≈ vitamin D$_2$ > 1,25(OH)$_2$D$_3$ > 1,24,25(OH)$_3$D$_3$.

Most circulating vitamin D-binding protein is synthesized in the liver. Its circulating concentrations are increased by pregnancy and estrogen therapy and are decreased in conditions associated with hypoproteinemia (e.g., liver disease, malnutrition, nephrotic syndrome) and inflammation. The role of vitamin D-binding protein is probably to prevent rapid fluctuations in the levels of the vitamin D metabolites. Vitamin D-binding protein deficiency in humans has not been identified but a knockout mouse does exist. This animal is more susceptible to hypocalcemia when fed a vitamin D-deficient diet than is a wild-type animal. Despite its size (58 kDa), vitamin D-binding protein appears to be filtered at the glomerulus and is then reabsorbed in the proximal tubule via the receptor protein, megalin, a member of the low-density lipoprotein receptor family. Mice deficient in megalin who survive long enough to develop a bone phenotype have severe rickets. Approximately 10% of vitamin D metabolites circulate bound to albumin. Once vitamin D metabolites have entered their target cell, their transport to the appropriate intracellular organelle may be facilitated by a different group of intracellular vitamin D-binding proteins, related to the heat shock proteins.

Activation

The first step in the activation of vitamin D is its 25-hydroxylation, to form 25-hydroxyvitamin D [25(OH)D]. This takes place in the parenchymal cells of the liver and is mediated by the enzyme from the cytochrome P450 family, coded for by the gene *Cyp2r1*. There appears to be very little regulation of the 25-hydroxylase and only in very advanced liver failure does its activity become limiting to normal vitamin D metabolism.

25(OH)D formed in the liver returns to the circulation bound to vitamin D-binding protein. If it is to be further hydroxylated, this takes place in the proximal tubular cells of the kidney. The entry of 25(OH)D into these cells is dependent on the action of megalin. Further activation of 25(OH)D is accomplished by 25-hydroxyvitamin D 1α-hydroxylase, which is also a mitochondrial P450 enzyme encoded by the *CYP27b1* gene. It shows significant homology to the 25-hydroxylase. The proximal tubular cells that contain the 1α-hydroxylase have parathyroid hormone (PTH) receptors, which stimulate enzyme activity. This accounts for the increased levels of 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] in states of hyperparathyroidism. The 1α-hydroxylase is also expressed in distal tubular cells and, again, megalin is present in these cells. In contrast to the proximal tubule, the PTH receptor is not present, but the receptor for calcitonin is and appears to have a stimulatory effect on 1α-hydroxylase activity. 1,25(OH)$_2$D directly inhibits the expression of the *Cyp27b1* gene at both sites in the renal tubule. The structure if 1,25(OH)$_2$D is shown in **Fig. 1**.

The kidney is clearly the most important site of 1,25(OH)$_2$D synthesis since anephric individuals have dramatically reduced concentrations of the hormone. However, 1,25(OH)$_2$D is still measurable in such patients, suggesting that there is extrarenal synthesis of the hormone. This 1α-hydroxylase gene is expressed in skin but even patients who are deficient in the gene still have measurable levels of 1,25(OH)$_2$D, suggesting that other enzymes might mediate 1-hydroxylation of 25(OH)D. Extrarenal synthesis of 1,25(OH)$_2$D clearly occurs in granulomatous diseases and in some lymphomas, where unregulated production of the hormone results in hypercalcemia. During pregnancy, the placenta produces 1,25(OH)$_2$D.

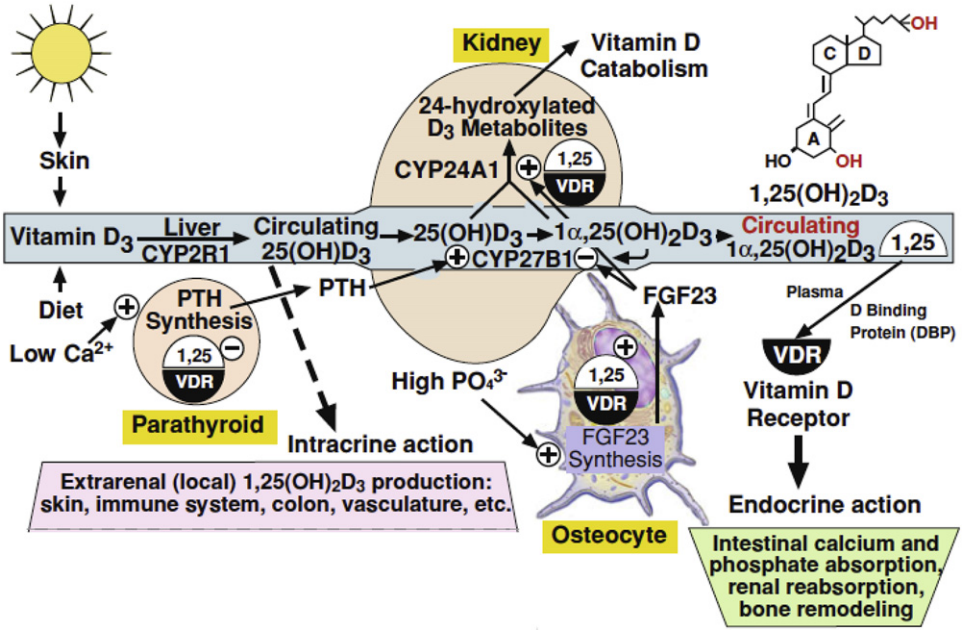


Fig. 2 Regulation of vitamin D metabolism, and principal site of its actions. Modified from Haussler et al., "Molecular Mechanisms of Vitamin D Action." Calcified Tissue International 92(2): 77–98, used with permission.

An alternative fate for 25(OH)D is to be hydroxylated on C-24, to produce 24,25-dihydroxyvitamin D. This conversion is mediated by vitamin D 24-hydroxylase, encoded by the gene *Cyp24a1*. This enzyme can also 24-hydroxylate $1,25(OH)_2D$ and is regarded as the principal pathway for deactivating vitamin D metabolites. As a consequence, it is widely expressed throughout the body, particularly in vitamin D target tissues, including the proximal and distal tubules of the kidney. Vitamin D metabolites then undergo further hydroxylations and side chain cleavage, resulting in the production of water-soluble calcitroic acid, which has no biological activity.

Regulation of Vitamin D Metabolism

The key points of regulation of vitamin D metabolism are the 1α-hydroxylase and the 24-hydroxylase (**Fig. 2**) (Haussler *et al.*, 2013). As noted above, the 1α-hydroxylase gene is directly regulated by PTH, calcitonin, and $1,25(OH)_2D$, the latter two hormones having the predominant effect under normocalcemic conditions, whereas the effect of PTH predominates over that of calcitonin under hypocalcemic conditions. Phosphate concentration is also an important regulator of 1α-hydroxylase activity, which is suppressed by high concentrations of phosphate and stimulated by low concentrations. Calcium concentration impacts on 1α-hydroxylase activity, mainly through its effect on circulating levels of PTH, though there is some evidence that it can directly influence enzyme activity as well. There is also evidence for effects of other growth-related hormones, such as estrogen, prolactin, and growth hormone, but these appear to be relatively minor.

$1,25(OH)_2D$ is the principal regulator of 24-hydroxylase activity. This is a genomic effect mediated by vitamin D-response elements in the promoter for the 24-hydroxylase gene. In the renal proximal tubule, PTH inhibits 24-hydroxylase activity, though it may have an opposite effect in the distal tubule. The induction of 24-hydroxylase by $1,25(OH)_2D$ also leads to accelerated catabolism of 25(OH)D. This explains the low levels of this metabolite seen in patients treated with calcitriol and in those suffering from primary hyperparathyroidism.

Actions of Vitamin D and Its Metabolites

Mechanisms of Action

The classic mechanism of action of vitamin D metabolites on their target tissues is similar to that of other steroid hormones and is mediated by the vitamin D receptor (VDR), a member of the steroid hormone receptor superfamily. Both $1,25(OH)_2D$ and 25(OH)D bind to this receptor, though the affinity of $1,25(OH)_2D$ for the receptor is 1000-fold higher. However, it should be remembered that 25(OH)D circulates in concentrations 1000-fold higher than those of $1,25(OH)_2D$, so both may contribute to receptor activation. The vitamin D metabolites bind to the VDR, which then forms a heterodimeric complex with the retinoic acid X receptor. This complex then interacts with vitamin D-response elements associated with a variety of genes. The VDR gene contains nine exons and encodes a protein of 427 amino acids. In humans, the gene is located on chromosome 12q. The DNA-binding domain is located in the amino-terminal

region and consists of a double zinc-finger structure. The ligand-binding domain is in the C-terminal region of the molecule. Mutations of the VDR have been identified in humans and result in vitamin D resistance, producing the clinical picture of rickets. Polymorphisms of the VDR gene have also been observed. These variations in nucleic acid sequence do not result in differences in the amino acid sequence of the expressed protein, but may impact on the transcription or stability of the mRNA for the VDR. Despite extensive clinical studies, it remains unclear whether these polymorphisms are consistently associated with changes in calcium metabolism or bone mass.

Calcium Metabolism

$1,25(OH)_2D$ is one of the classic calcitropic hormones. In humans and animals lacking the vitamin D receptor, the only abnormality observed at birth is in hair follicle development (Bouillon *et al.*, 2008). Subsequently, however, hypocalcemia and the development of rickets are observed. The abnormalities in bone development can be prevented by restoration of normal serum calcium concentration, suggesting that maintenance of normocalcemia and maintenance of normophosphatemia are the principal biological roles of the vitamin D metabolites. It is likely that this is achieved primarily by increasing the absorption of calcium in the small intestine (Carmeliet *et al.*, 2015). Vitamin D receptors are found throughout the small intestine but are most abundant in the duodenum. Administration of calcitriol to animals or humans leads to increases in intestinal calcium absorption, though the precise mechanism by which this occurs is not fully understood. $1,25(OH)_2D$ regulates the production of the cell membrane protein TRPV6 (a calcium-binding protein within the intestinal epithelial cell), calbindin D8k, and the activity of an ATP-dependent calcium pump at the basolateral membrane. It was thought that these proteins together facilitated the transport of calcium into, through and out of the intestinal epithelial cells, but there appears to be redundancy in the system since deletion of either *Trpv6* or *CaBP-9k* alone permits normal basal calcium absorption, though the double knockout does not. Calcium is also absorbed by a paracellular pathway present throughout the length of the intestine, in which transmembrane proteins known as claudins facilitate calcium transport across the tight junctions. Claudin expression in the distal intestine is regulated by $1,25(OH)_2D$. 1,25(OH)2D also stimulates intestinal phosphate absorption, through induction of Na^+-dependent phosphate co-transporters in enterocytes.

The direct effects of $1,25(OH)_2D$ on bone act in concert with those already described in the intestine, to maintain or increase serum calcium concentrations (Reid and Bolland, 2014). $1,25(OH)_2D$ acts on osteoblasts and their precursors, causing the production of RANK-L, which binds to pre-osteoclasts to stimulate their development into osteoclasts. This leads to an increase in osteoclastic bone resorption. However, other studies in vitro suggest an anabolic effect of $1,25(OH)_2D$ in osteoblasts with increased alkaline phosphatase activity, increased production of osteopontin and osteocalcin, and increased LRP5 expression, but the significance of this in human physiology is unclear. Although it has been suggested that $1,25(OH)_2D$ may directly influence skeletal mineralization, the majority of evidence suggests that this occurs indirectly, as a result of vitamin D effects on serum calcium and phosphate concentrations. A further important action in bone is on the osteocyte, where $1,25(OH)_2D$ stimulates production of fibroblast growth factor-23, which acts in the kidney to reduce phosphate reabsorption and to reduce 1α-hydroxylase activity. $1,25(OH)_2D$ also acts directly on the kidney, regulating calcium reabsorption in the distal nephron via a transcellular pathway similar to that in the proximal intestine. In addition, $1,25(OH)_2D$ induces its own catabolism by the 24-hydroxylase in the kidney.

The parathyroid glands are regulated by $1,25(OH)_2D$, where its binding to the vitamin D receptor directly decreases expression of the PTH gene. This genomic effect is reinforced by the action of $1,25(OH)_2D$ to increase serum calcium and thus diminish PTH secretion. The effects of vitamin D metabolites on PTH secretion are particularly important in the management of the secondary hyperparathyroidism of chronic renal failure. They may also be important in the prevention and treatment of primary hyperparathyroidism, since this condition may be exacerbated by the low levels of 25(OH)D with which it is frequently associated.

Other Tissues

$1,25(OH)_2D$ receptors are expressed in a wide variety of tissues other than those classically involved in mineral and bone homeostasis. In keratinocytes and in some white blood cells or their precursors, $1,25(OH)_2D$ has anti-proliferative and pro-differentiation effects. Thus, in psoriasis, it appears that the hyperproliferative state of skin cells can be controlled with the use of vitamin D analogues. There is also experimental evidence that some leukemic cell lines show similar responses to vitamin D metabolites. Despite the existence of promising preliminary results in these areas for a number of years, the vitamin D metabolites do not have an established therapeutic role in any of these malignant conditions. Vitamin D metabolites may also have direct actions on muscle cells, on adipocytes, in immune regulation, and on endocrine tissues, such as the pancreatic beta cell but, again, clear evidence of clinical benefit is lacking.

Assessment of Vitamin D Status

25(OH)D is the principal circulating vitamin D metabolite and it is the entity that should be assessed when determining an individual's vitamin D status. Because ingested or endogenously produced calciferols are converted to 25(OH)D with very little regulation, serum levels of this metabolite accurately reflect both excess and deficiency states. As noted above, 25(OH)D circulates bound to vitamin D-binding protein, so any condition associated with hypoproteinemia or inflammation may produce falsely

depressed levels of 25(OH)D. Caution is required in interpreting measurements of vitamin D metabolites in these situations, because it is uncommon to measure vitamin D-binding protein in routine clinical practice.

When assessing vitamin D status, it is important to consider whether the "normal" range—which varies with latitude—can be regarded as optimal. This has been addressed by Malabanan et al. (1998), who demonstrated that vitamin D supplementation suppressed parathyroid hormone levels only in subjects whose baseline serum 25(OH)D was <50 nmol/L. This suggests that 50 nmol/L is an appropriate target concentration for serum 25(OH)D.

Measurement of serum $1,25(OH)_2D$ is also available, though its value in clinical medicine is small. It is sometimes helpful in elucidating the cause of hypercalcemia, being high when hypercalcemia is a direct consequence of overproduction of $1,25(OH)_2D$, as in some granulomatous conditions and lymphomas. The widespread impression that $1,25(OH)_2D$ is the only biologically active vitamin D metabolite frequently leads to the inappropriate use of vitamin D assays and supplements. This belief results in the expectation that levels of $1,25(OH)_2D$ are all that will influence calcium metabolism, whereas this is clearly not the case. As vitamin D deficiency develops, serum 25(OH)D declines. In response to this decline, secondary hyperparathyroidism develops and with it there are increases in serum $1,25(OH)_2D$. Thus, the paradoxical situation can develop, in which a patient with clinical and histological evidence of osteomalacia has a serum 1,25(OH)D level that is either normal or supranormal.

In summary, measurement of serum 25(OH)D suffices for the detection of vitamin D deficiency or vitamin D intoxication, and measurement of $1,25(OH)_2D$ is usually indicated only in the diagnosis of difficult cases of hypercalcemia. Because vitamin D assays are expensive and of variable reliability, many clinicians provide low-dose supplements based on clinical risk factors for deficiency rather than assay results.

Therapeutic Use of Vitamin D and Its Metabolites

Vitamin D Deficiency and Osteoporosis

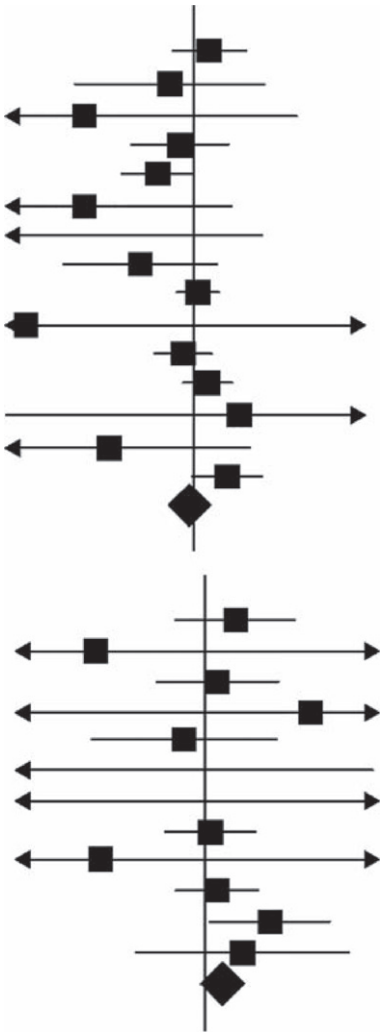
Vitamin D deficiency is common in the elderly, particularly those who are no longer fully independent and therefore less exposed to sunlight. The problem is often greater at higher latitudes, though it can also occur in very hot climates where the sun is often avoided because of the heat. Vitamin D deficiency leads to secondary hyperparathyroidism and a resulting increase in bone loss (Reid and Bolland, 2014).

Physiological supplements of calciferol (e.g., 400–800 IU/day) reduce parathyroid hormone concentrations (Bacon *et al.*, 2009) and lead to increases in bone density in those with 25(OH)D levels below 30–50 nmol/L (Reid *et al.*, 2017). Similar changes in biochemical end-points can be achieved with regular sunlight exposure for 15–30 min daily (Reid *et al.*, 1986). However, meta-analyses of untargeted use of vitamin D alone show no benefit on either bone density (Reid *et al.*, 2014) or fractures (**Fig. 3**) (Bolland *et al.*, 2014).

In a study which co-administered calcium with calciferol to frail elderly subjects, Chapuy et al. demonstrated a reduction of more than 25% in non-vertebral and hip fracture rates in a cohort of 3000 elderly women studied over a period of 3 years (Chapuy *et al.*, 1992). It should be noted that serum 25(OH)D levels in the placebo group during the study were about 14 nmol/L (after correction for errors in assay calibration) so many trial participants probably had osteomalacia. Setting this study aside, trials of calcium plus vitamin D are comparable to those of calcium alone in their effects on bone density and fracture (Tang *et al.*, 2007). Thus, supplementation is important for those at risk of severe deficiency and osteomalacia, but seems to produce no benefit in those who are already vitamin D replete, as defined above.

The dose–response relationship between calciferol intake and serum 25(OH)D levels is relatively flat up to intakes of several thousand international units per day (Gallagher *et al.*, 2012). Thus, this intervention is safe, and doses of 400–1200 IU/day are routinely used. Calciferol is stored in adipose tissue and has a half-life of many weeks. Therefore, it can be administered in larger doses less frequently. Single doses of up to 500,000 IU have been used to correct severe deficiency, and monthly oral supplements of 50,000 IU maintain 25(OH)D levels of $\sim$70 nmol/L (Bacon *et al.*, 2009). There are anecdotal reports of wide variability in the bioavailability of different preparations of calciferol, and dietary vitamin D intakes also vary widely from country to country. Therefore, each region needs to determine what regimen of replacement is safe and effective in that environment.

The use of vitamin D as a physiological supplement is fundamentally different from the use of high doses of calciferol or 1α-hydroxylated vitamin D metabolites (e.g., alfacalcidol, calcitriol) to pharmacologically manipulate intestinal calcium absorption. Both of these strategies bypass the normal homeostatic controls of vitamin D metabolism and therefore carry a significant risk of hypercalcemia and hypercalciuria. The use of pharmacological doses of calciferol has not been demonstrated to confer any beneficial effects on bone density. Trials of the use of alfacalcidol or calcitriol in the prevention and treatment of osteoporosis have shown mixed results, including both significant increases and significant decreases in bone density and fracture rates. This variability in results may be attributable to a different balance of the potentially beneficial effects on intestinal calcium absorption versus the potentially damaging effects on osteoclast recruitment in the different populations studied. As a result, alfacalcidol and calcitriol are not generally regarded as appropriate therapies for osteoporosis. A newer more potent analogue (Matsumoto *et al.*, 2011) has shown greater anti-fracture efficacy than alfacalcidol in a Japanese study, and is used for osteoporosis in that country.



Total fracture		
Lips et al. (1996)[26]	135/1291	122/1287
Komulainen et al. (1998)[29,30]	18/232	21/232
Pfeifer et al. (2000)[32]	3/74	6/74
Meyer et al. (2002)[34]	69/569	76/575
Trivedi et al. (2003)[38]	119/1345	149/1341
Avenell et al. (2004)[39]	6/70	11/64
Harwood et al. (2004)[40]	0/38	5/37
Flicker et al. (2005)[44]	25/313	35/312
Grant et al. (2005)[46]	387/2649	377/2643
Burleigh et al. (2007)[52]	1/100	3/103
Lyons et al. (2007)[54]	205/1725	218/1715
Smith et al. (2007)[55]	306/4727	279/4713
Prince et al. (2008)[58]	4/151	3/151
Pfeifer et al. (2009)[60]	7/121	12/121
Sanders et al. (2010)[63]	155/1131	125/1125
Vitamin D	1440/14536	1442/14493
Test for heterogeneity: $I^2 = 32\%$, $P = 0.11$		
Hip fracture		
Lips et al. (1996)[26]	58/1291	48/1287
Komulainen et al. (1998)[29,30]	1/232	2/232
Meyer et al. (2002)[34]	50/569	47/575
Bischoff et al. (2003)[35]	2/62	1/60
Trivedi et al. (2003)[38]	21/1345	24/1341
Avenell et al. (2004)[39]	1/70	3/64
Harwood et al. (2004)[40]	0/38	1/37
Grant et al. (2005)[46]	93/2649	90/2643
Burleigh et al. (2007)[52]	1/100	2/103
Lyons et al. (2007)[54]	112/1725	104/1715
Smith et al. (2007)[55]	66/4727	44/4713
Sanders et al. (2010)[63]	19/1131	15/1125
Vitamin D	424/13939	381/13895
Test for heterogeneity: $I^2 = 0\%$, $P = 0.8$		

Fig. 3 Random effects meta-analyses of the effect of vitamin D on total fracture and hip fracture. The meta-analytic relative risks were 0.97 (0.88–1.08) for any fracture and 1.11 (0.97–1.27) for hip fracture. From Bolland, M. J., Grey, A., Gamble, G.D., and Reid, I. R. (2014). The effect of vitamin D supplementation on skeletal, vascular, or cancer outcomes: a trial sequential meta-analysis. *Lancet Diabetes & Endocrinology* 2(4): 307–320. Used with permission.

In conclusion, suboptimal vitamin D status is very common in the elderly, mainly because of reduced sunlight exposure. The provision of a daily intake of 400–800 IU is a straightforward, safe, and inexpensive means of prevention and appears to produce substantial reductions in fracture rates in deficient populations.

Other Conditions

Failure of the 1α-hydroxylation of 25(OH)D is probably the single most important contributor to the hypocalcemia of renal failure and the resulting development of renal bone disease. Thus, the availability of $1,25(OH)_2D$ (calcitriol) and 1α-hydroxyvitamin D (alfacalcidol) as pharmaceuticals has revolutionized the management of calcium metabolism in renal failure. The development of hypercalcemia can be dose-limiting when using vitamin D metabolites to reverse secondary hyperparathyroidism in renal failure. This has led to the development of synthetic vitamin D analogues that suppress parathyroid hormone secretion but have less effect on serum calcium.

The 1α-hydroxyvitamin D metabolites have also revolutionized the management of other conditions associated with either hypocalcemia (e.g., hypoparathyroidism) or hypophosphatemia (e.g., X-linked-hypophosphatemia, oncogenic osteomalacia). Previously, these conditions were managed with very large doses of calciferol, which can result in sustained hypercalcemia because of calciferol's long half-life.

As noted above, the anti-proliferative actions of the vitamin D metabolites are being used in the management of psoriasis. There is also epidemiological evidence suggesting that high vitamin D levels are associated with a lower incidence of cardiovascular disease, infection, obesity and diabetes (Reid and Bolland, 2014). This work is potentially subject to a number of biases (e.g., people who are more physically active and therefore at lower risk of these conditions, spend more time outdoors and therefore

have better vitamin D status). Intervention studies are being carried out to assess the value of vitamin D supplementation in a number of conditions, but results to-date have not been encouraging.

Safety of Vitamin D and Its Metabolites

The use of replacement doses of calciferol is a very safe intervention, as would be expected since the intention is to restore circulating levels of 25(OH)D to the levels that are present in the healthy population. Thus, in the study by Chapuy in which more than 1600 women were treated with calciferol, the only individual who developed hypercalcemia was subsequently found to have primary hyperparathyroidism. A similar zero-incidence of significant hypercalcemia has been reported by other investigators using low-dose regimens of calciferol administration.

In contrast, with the use of pharmacological doses of calciferol, there are case reports of severe hypercalcemia, often of long duration and sometimes associated with renal failure. In trials, bolus dosing with 500,000 IU annually has been associated with increases in rates of fractures and falls (Sanders *et al.*, 2010), and daily dosing of $\geq$4000 IU has been associated with more frequent falls (Smith *et al.*, 2017). Calcitriol and alfacalcidol frequently cause hypercalcemia and hypercalciuria, but their short half-lives result in this being readily corrected by dose adjustment. However, close monitoring is necessary when these drugs are being introduced or their doses increased. Changes in calcium intake can also affect serum calcium concentrations in patients using these compounds.

The hypercalcemia of vitamin D intoxication was formerly attributed to increased intestinal calcium absorption, but bone resorption is also increased and bisphosphonates are effective in its treatment. The long duration of hypercalcemia associated with calciferol (Rizzoli *et al.*, 1994) means that this compound is substantially less safe at high doses than its more active and shorter half-life metabolites, such as alfacalcidol and calcitriol.

See also: Rickets and Osteomalacia

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Further Reading

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Nutrition and Bone

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Glossary

BMD and BMC Bone mineral density and bone mineral content internationally used parameters of bone mass and/or bone density.

Effect of Nutrition on Bone Health: General Considerations

The influence of nutrition on bone health is relatively weak, in addition to the negative effect of severe deficiencies. However, it is still significant, and it has the advantage that it can be modified, and actively used as an adjunctive prophylactic or therapeutic measure, for prolonged periods and even lifelong. For this reason, it deserves major attention. Stronger factors such as genetics or concomitant diseases and their pharmacological treatments cannot be changed in order to develop stronger bones.

The knowledge about the influence of nutrition on bone health stems from research. It is difficult to capture, because research is applied to all ages (embryos, babies, children, adolescents, adults, the elderly), to several nutritional constituents (calcium, proteins, vitamins), and to many nutrients (dairy products, meat, vegetable, fruits, and in particular milk, yogurt, onions, kale, blueberries, soya, mineral water, etc.). Research is performed with cross-sectional studies, and with longitudinal studies, where BMD could be used as an outcome (and in very large surveys, also fracture incidence), or with intervention trials, usually short-term, with markers of bone metabolism as an outcome. Some intervention trials produced results in BMD, but usually only when applied to states of nutritional deficiency at the baseline. In this context it has to be remembered that research results from animals cannot be applied directly to humans.

From all this heterogeneous data, the major conclusions are presented here. Unfortunately, single items of data of doubtful quality are often brought from this large puzzle of knowledge to the lay press and to dieticians, and directly applied to the public and to patients. However, the fact-based physicians and scientists base their knowledge and recommendations exclusively on research data, even in such a popular domain as nutrition.

Even when there is a correlation between bone health and a given nutrient, intakes beyond the physiological needs do not produce a benefit. The nutrient can even have a negative impact by displacing other useful nutrients within the frame of the required caloric intake. On the other hand, any nutritional deficiency results in low bone mass or low bone density, in growth delay during childhood, and in enhanced bone loss in adulthood. Nutritional interventions in deficiency states usually provide positive bone effects, which are difficult to reproduce in states of sufficiency.

There are ethnic differences in dietary needs for optimal bone metabolism and bone acquisition; these are not discussed in detail here

Dairy Products

General Considerations

Dairy products, such as milk, yogurt, curd, and cheese, are the best and the most important (but not the only) sources of dietary calcium, at least for Western nutrition. Among these, milk is the most studied dairy food. About half of nutritional calcium stems from dairy food. Higher figures were found in more superficial investigations, where minor nutrients, which all contain some calcium, were not included in the analysis. In Asia, dairy products are consumed less and contribute only 20%–23% of the total calcium intake. Some diets with no dairy products, which are meant to lower cholesterol, increase the risk of osteoporosis. A vegan diet is generally poor in calcium, despite the relatively high amount of calcium in certain vegetables and nuts.

The recommended calcium intake is 800–1000 mg calcium per day. A Western diet with no dairy products can deliver 250–300 mg calcium or more per day, when special attention is given to mineral water, calcium-rich vegetables, nuts, and seeds in sufficient amounts. On average, humans absorb about 30% of the calcium present in food.

As noted, the minimal requirement is set at 800 mg per day, and 1000 mg would not be excessive. In one large survey, milk intake was identified as being associated with mortality. This survey showed that ≥ 3 glasses of milk per day (≥ 600 mL) was associated with higher mortality in adults and with higher fracture incidence in women (Michaëlsson *et al.*, 2014). However, a meta-analysis of 29 prospective cohort studies on milk and mortality did not find such an association (Guo *et al.*, 2017), and several published comments contradict this survey.

Calcium deficiency disturbs growth and the maintenance of bone mass during adult life, and increases the risk of osteoporosis. It was recognized by 1985 that milk stimulates BMD by inhibiting bone turnover and resorption markers in postmenopausal women and in elderly men; this was reconfirmed later. Milk also increases IGF-1 (insulin-like growth factor 1) in elderly men.

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Dairy products provide more than calcium supplements, for example, protein, phosphorus, magnesium, etc. In postmenopausal women, the supplementation of a diet with milk improved the nutritional quality of the diet to a greater extent than calcium alone. But compliance to supplements might be better than adherence to milk, and therefore lead to a higher calcium intake.

The fear of increasing the cholesterol intake with dairy products concerns only high-fat products, including soft cheeses, butter, and cream. The cholesterol content of a menu of milk, hard cheese, and yogurt providing 1000 mg of calcium has <90 mg cholesterol. This is several times below the recommended intake. Eventual negative cardiovascular effects are due to the content of saturated fatty acids.

Bioavailability of Dairy Calcium

Calcium from dairy products is highly bioavailable, and well absorbed (20%–30%), but shows individual differences. Its absorption is facilitated by lactose and certain caseino-phospho-peptides, formed during the digestion of milk caseins. Like calcium supplements, calcium from dairy products exerts its biological effects immediately after intake, for example, inhibition of parathyroid hormone (PTH) secretion. For lactase deficiency, see the next article.

Effect of Nutritional Calcium on BMD

The assessment of the bone effect of dietary calcium is difficult. The assessment of food intake cannot definitively confirm that it reflects precisely the intake of the tested days, months, or years, and certainly not of the total adult life. In addition, the measurement of the effect on bone is problematic because of confounding factors, including first of all physical activity (known of for many years), but also lower alcohol intake and higher energy intake, which go along with the consumption of milk, or milk consumption during teenage years, which influences the results observed later in life. In addition, women who consume dairy products are less likely to smoke. For these reasons, contradictory results can be found in the literature.

Calcium is especially important in children and adolescents. There are four times more favorable results than negative ones in studies of subjects younger than 30 years, while in older subjects there is no overall effect (Weinsier and Krumdieck, 2000). This means that dairy calcium intake is mostly effective during growth. There are undoubtedly also positive associations between the calcium intake and BMD in adults according to large cross-sectional and longitudinal surveys or follow-up studies over long periods. If one might question the positive bone effects of nutritional calcium, there is no doubt that low calcium intake has a negative effect.

Low intake of dairy products is very often attributed to lactase deficiency. Lactase deficiency is found in 80% of US blacks, and in 80%–100% of Asians. It is rare in US Caucasians (15%) and in northern Europe (10%). In any case, it is of minor importance. First, self-perceived milk intolerance leads to self-imposed reductions in milk consumption, increased bone turnover, and increased risk of fracture, independent of the presence or absence of lactase deficiency. Second, fermented dairy products, such as mature hard and medium hard cheeses, as well as yogurts, do not contain lactose, and can be consumed by those with lactase deficiency. For this reason, lactose genotype and phenotype did not influence BMD in a population with high milk consumption. Intolerance to milk is often misinterpreted as lactase deficiency. Milk allergy, on the other hand, leads to a low calcium intake and to lower BMD values.

In children

In the first 6 months of life, human milk provides adequate amounts of calcium and phosphorus. Later, supplemental minerals are consumed in food.

A low calcium intake has a negative effect on growth and bone development, and a low milk intake in childhood is associated with lower BMD and smaller bones. The intake of dietary calcium, with and without vitamin D, correlates significantly with spine and total body BMC (bone mineral content; this replaces BMD during growth), but only in children with low baseline intakes, according to a meta-analysis of 21 randomized controlled trials (Huncharek *et al.*, 2008). The effect on BMD was still measurable in adult life, but no effect on hip fracture incidence could be found. In another study, women with a high intake during childhood and adolescence had not only a higher bone mass in adulthood, but also a smaller risk of fracture. A review in 2011 revealed that increasing calcium and vitamin D intake in infants and young children above the basic physiological needs had no long-term benefit. This shows again that nutrition has to meet the requirements and to avoid deficiencies, but intake beyond these needs does not offer additional benefits.

The age of accelerated growth seems to be especially sensitive to adequate intakes. In adolescent girls, consumption of milk, but of no other calcium source, was tightly associated with BMC and BMD, mainly at the hip, as well as with serum IGF-1 and negatively with serum PTH. Regular milk intake during growth was also associated with higher BMD in adulthood and postmenopause, either because of a higher peak bone mass, or as a consequence of a lifelong adherence to dairy products. Milk intake during teenage years was also associated with greater BMD of the total body in young adulthood.

The main knowledge stems from cross-sectional and follow-up studies. There are some interventional trials with milk, milk powder, and dairy products in children and adolescents showing a positive effect on BMD. This is not evident in adults and the elderly (Weinsier and Krumdieck, 2000), probably because the concomitant intake of protein, phosphorus, and IGF is particularly

essential during growth; milk improves the nutritional quality of the diet more than a calcium supplement. Milk undoubtedly stimulates growth. One glass of milk added to the diet of adolescent girls increased the bone accrual by 3 g per year, and a milk supplement of 300 mg calcium increased BMD. The bone effect is particularly evident in populations with a low calcium intake such as Asians, where supplementing the diet of children with milk powder or with milk equivalent to 1300 mg calcium enhanced bone accretion.

These positive results were opposed by a review that concluded that there is no reason for promoting milk or dairy consumption in children and adolescents (Lanou *et al.*, 2005). However, on the other hand, a more recent analysis of the studies with dairy food demonstrated that most of the observational studies and all interventional trials—this is the crucial point—showed a positive association with BMD or BMC (Heaney, 2009). Nevertheless, the effect of 2 years of school milk intake disappeared after withdrawal, probably because the calcium intake dropped again to low values.

In adults

The studies searching for a bone effect of nutritional calcium provided contradictory results in adults, except in high-risk populations and in populations with a low calcium intake. Positive results should be mentioned first in young adult women on oral contraceptives, where nutritional calcium showed a dose-dependent effect against the decline of BMD and BMC, especially with 1000–1100 mg calcium. Then, in adolescent mothers, dietary calcium was positively associated with the total body calcium of the infants. Finally, in elderly individuals, several studies showed that dairy intake was linked to higher bone density. However, in early postmenopausal women, where the prevailing influence on bone is estrogen deficiency, calcium intake does not correlate with BMD.

In general, in the elderly population as well as in children, nutritional calcium is more effective than calcium supplements. Only a cross-sectional survey of elderly subjects in the USA (National Health and Nutrition Examination Survey, or NHANES; Anderson *et al.*, 2012) concluded that a high calcium intake is not beneficial to bone. However, the sensitivity of this survey was questioned, because it could not reproduce the negative effect of a low intake.

Although short intervention trials, using bone markers as outcome, showed positive effects on bone metabolism, many intervention trials with milk, milk powder, and dairy products in adults showed no effect. However, the literature also reports several positive results. In an intervention trial, dairy products decreased bone loss in premenopausal women. Calcium supplementation with dairy products was associated with a higher hip BMD in men, and a smaller bone loss in men and women. Positive effects could also be found in postmenopausal Caucasian women and in the elderly. Positive bone effects were especially evident in countries with a low baseline intake of calcium, such as China or Malaysia.

Effect of Nutritional Calcium on Fracture Risk

In 1988, the protective effect of dietary calcium on the hip fracture risk was suggested by a follow-up study in elderly subjects. No other nutrient showed this effect. Not only the actual intake but also the intake of milk in childhood decreased the risk of fracture in adult women. However, one of the largest follow-up studies in postmenopausal women, the Nurses' Health Study (Feskanich *et al.*, 2003), showed no such association.

Only in 2011 was the question systematically examined. A meta-analysis of follow-up studies showed the absence of a significant association between milk intake and hip fracture incidence in women. In men, each glass of milk per day was associated with a marginally significant lower risk (Bischoff-Ferrari *et al.*, 2011). Although no association between milk and yogurt consumption and hip fracture risk could be found in the Framingham cohort, a year later the same authors found that the hip fracture risk was decreased by 42%, although not significantly, in elderly men and women who consumed regularly milk (Sahni *et al.*, 2014).

The opposite was found in two large Swedish cohorts. Surprisingly, women had a significant 9% greater risk of hip fracture for every glass of milk consumed per day, whereas no association was observed in men (Michaëlsson *et al.*, 2014). This increase of the fracture risk was thought to be due to galactose, because fermented milk and yogurt (all low in galactose) were linked to a significant decrease of the hip fracture risk by 11% per daily serving in women, as could be expected. In addition, cheese, which contains almost no galactose, was also associated with a decrease of the hip fracture risk by 14%. This discrepancy with milk remains mainly unexplained, and shows how difficult it is to assess dietary intakes over a long period. In a recent major study in Caucasian men aged 50 and older, and women past menopause, each additional serving of milk per day was associated with a significant 8% lower risk of hip fracture, as could be expected again. Here milk intake was assessed as a long-term cumulative average, and the intake was updated every 4 years, while in other studies it was assessed only once or twice, which cannot be guaranteed to reflect long-term habits.

While the antifracture effect of dairy products is debated, there is no doubt that low calcium intake increases the hip fracture risk (Warensjö *et al.*, 2011) and that a calcium intake above the maximal needs (over ca. 1500 mg/day) is not particularly protective, and is eventually even associated with higher cardiovascular risk (Michaëlsson *et al.*, 2013). One large European survey associated milk consumption with higher fracture risk and higher mortality (Michaëlsson *et al.*, 2014). But this could not be confirmed, and was contradicted by a meta-analysis of numerous studies (Soedamah-Muthu *et al.*, 2011) and by many published comments. There are no intervention trials with dairy products and fracture incidence as an outcome. This would need very large populations and long observation periods. Intervention trials with calcium supplements provided contradictory results. Only when combined with vitamin D could a small reduction in fracture risk be obtained.

Fortified Milk

Milk is an excellent carrier of supplemental calcium. Fortified with calcium and given to postmenopausal women over the course of 2 years, it delayed bone loss and decreased resorption markers. Calcium fortification of milk can optimize the calcium intake in infants, when human milk does not meet the requirements. In premature infants, fortification of mother's milk improved bone mineralization. Calcium-fortified soy milk is a valuable source of calcium, but is less effective than cow's milk. Calcium-enriched orange juice had less effect on infants than dairy food. Ice cream fortified with calcium is a convenient vector of alimentary calcium, since it lowered bone resorption.

The additional fortification of food with vitamin D, tested in several studies, provided essential results due to vitamin D. This vitamin is not discussed here, since it is not a nutrient.

Cheese and Yogurt

Cheese and yogurt have been tested specifically. The most relevant study is the 12-year follow-up Framingham Offspring study, which revealed that the intake of fluid dairy and milk was positively associated with BMD of the hip, but not of the spine, and that yogurt intake was positively associated with trochanter-BMD, while cheese intake was not associated with BMD. Fracture data were only suggestive for milk and yogurt intakes.

Some cheeses that are rich in sodium or in fat, as well as cream or ice cream, may even have a negative effect on bone (Weinsier and Krumdieck, 2000).

Yogurt: A small intervention study in postmenopausal women with a low dietary calcium intake of <600 mg per day showed that three servings of yogurt lowered N-telopeptides, a marker of bone resorption, to 22% lower values than the control snack did (Heaney *et al.*, 2002). This is mainly a calcium effect. Calcium from yogurt has a high bioavailability. In addition, yogurt contains proteins and phosphorus, which are also important for bone. Yogurt can be consumed by those with lactose intolerance.

In the large observational Framingham study mentioned above, participants consuming >4 servings of yogurt per week had a higher trochanteric BMD, while the association with femoral neck BMD was weak (P: 0.09) (Sahni *et al.*, 2013).

The additional effect of the probiotics in yogurt, as the promotion of calcium absorption, has only been documented in animal studies.

Cheese: Calcium in cheese is as well absorbed as calcium in yogurt, despite the absence of lactose which promotes the absorption of milk calcium. This concerns low-fat hard cheeses. They are rich in calcium, but usually also rich in sodium. When consumed regularly, these cheeses increase the sodium intake by several grams, which has to be taken into account. In prepubertal girls, cheese had a stronger effect on bone than calcium tablets with vitamin D, and appeared to be more beneficial for cortical bone mass accrual than supplements of calcium with or without vitamin D.

Fruits and Vegetables

When dairy intake is low, as for example, in Asian countries, calcium content in plants becomes crucial. It is difficult to match the requirements for calcium intake without dairy products in the Western-type diet. By including dairy products, lacto-vegetarians, but not vegans, are able to meet the recommended amounts of calcium. Vegetarianism is not a risk factor for osteoporotic fracture (Lanham-New, 2009). This is different for vegans. The vegan diet is, on average, low in protein and calcium, and increases the risk of osteoporosis. This negative effect on bone is not more important because of the alkalizing effect of fruits and vegetables, which protects bone (Burckhardt, 2016; Weaver, 2009). In several studies, a low acid load of the diet went with a higher BMD, and a low intake of fruits and vegetables was associated with lower BMD and vice versa (New *et al.*, 2004). The positive effect of fruits and vegetables is also due to the high potassium intake, which lowers calcium excretion. In general, a healthy diet which contains a lot of fruits and vegetables was associated with higher BMD (Hardcastle *et al.*, 2011). Alkalizing food promotes bone formation and inhibits bone resorption. The strongest inhibition of bone resorption and stimulation of BMD has been found to be caused by tomatoes, berries, salad, and green vegetables. Blueberries and plums contain anthocyanins, which inhibit bone resorption.

The calcium content of a vegetable does not indicate its nutritional value as a source of calcium. Some substances in vegetables decrease calcium absorption, such as oxalic acid (spinach, collard greens, sweet potatoes, rhubarb, beans) or phytic acid (fiber-containing whole-grain products and wheat bran, beans, seeds, nuts, and soy isolates). This explains, for example, the high availability of calcium in broccoli and kale (40.9%, higher than milk at 32.1%) which is low in oxalate, and the low availability of calcium in spinach (only 5.1%), which is rich in oxalate. Other substances enhance calcium absorption, such as lactose and certain caseino-phospho-peptides formed during the digestion of caseins from milk. Bioavailability is crucial, not the calcium content.

Nuts and Grain Products

Nuts are a natural source of dietary calcium, but they are usually consumed in small amounts. Sesame can be recommended for its exceptionally high calcium content (about 800 mg/100 g). One spoonful of sesame kernels provides 90–135 g of calcium. Some oriental food and sweets are rich in sesame.

Wholegrain products, such as ryebread, contain significant amounts of calcium. When consumed regularly, they can contribute significantly to the total calcium intake. However, bakery products made with wheat flour have little calcium. Bran interferes with calcium absorption; leavening improves it.

Tortillas are the second most important source of calcium among Mexican Americans.

Soy and Soy Products

Soy and some soy products are significant sources of protein ($\pm$ 38 g 100 g^{-1}) and calcium (almost 300 mg calcium 100 g^{-1}), although they contain less calcium than dairy products. Their content of oxalate and phytate is variable, and accordingly so is the bioavailability of their calcium. Similar to meat, soy protein contains the essential amino acids, which are lacking in other vegetal protein sources. Soy milk is often preferred to cow's milk because of its low-fat content. But its calcium content is also low. When fortified with calcium, soy milk becomes equivalent to cow's milk in terms of bioavailability of calcium. The intake of fortified soy milk is associated with less osteoporosis, and is comparable to the effect of dairy products. Tofu, a popular soy product, contains about 185 mg calcium 100^{-1} mg or several times more, depending on the use of calcium sulfate for its coagulation. The great variety of soy or tofu products, all different in their content of calcium and oxalate, excludes general statements.

Soy and soy products have a "bone sparing" effect because soy protein is not acidifying, contrary to meat. The pretended positive bone effect of their phytoestrogens, resp. isoflavones, could not be confirmed in several meta-analyses (Lagari and Levis, 2013).

Meat and Proteins

Bone health requires a sufficient protein intake, which is often lacking in populations that are advanced in age and/or malnourished. Protein directly promotes bone anabolism, stimulates IGF-1, and increases calcium absorption. The latter compensates for the increased urinary excretion of calcium.

Meat is the most important (but not the only) source of protein (ca. 27.5 g 100 g^{-1}) after dairy products (hard cheese ca. 28 g 100 g^{-1}). It contains the essential amino acids, such as soy, which are partially missing in vegetable proteins. The frequently formulated recommendation of 0.8 g protein kg^{-1} body weight (BW) per day is too low for an anabolic bone effect. With age, the caloric needs decrease, but the needs of protein do not. Therefore, protein intake should increase percentage-wise with age. However, low protein intake is frequent in the elderly. This increases hip fracture incidence.

Protein intake is correlated with BMD, as shown by several studies (Langsetmo *et al.*, 2015). There is some relationship with hip fracture. A meta-analysis of 2009 was not conclusive concerning the antifracture risk (Darling *et al.*, 2009). However, more recent studies were more positive (Fung *et al.*, 2017). A protein intake above the usual recommendations decreased the hip fracture risk, especially when the caloric intake was also increased. In older men, protein intake was associated with fewer hip fractures, but not with fewer clinical spine fractures.

The bone effect of protein is lacking when calcium intake is low, and is increased by calcium supplementation (Dawson-Hughes and Harris, 2002).

Meat is acidifying, which has a negative influence on bone (New *et al.*, 2004), but its positive bone effect is maintained if the overall nutrition contains enough alkalizing nutrients, such as fruits and vegetables. Vegetal proteins seem to have a stronger bone effect than meat, perhaps because of their low acid load, but they can miss essential amino acids. But in general, the effect of plant protein is questioned.

In children, a high protein intake increased bone modeling in a longitudinal study. In children with an intake of 1.5 g protein kg BW^{-1} per day, BMC and cortical bone surfaces were higher than in children consuming 1.2 g protein kg BW^{-1} per day. Protein supplements given to elderly patients with hip fractures diminished their bone loss.

Mineral Water

Calcium-rich mineral water can contain up to 500 mg calcium l^{-1}, especially some European types. Water's calcium is as well absorbed as calcium from milk. It lowers PTH and bone resorption markers within 1 h. The effects of calcium-rich mineral water on bone, including the improvement of BMD or the lowering of its decrease, were also observed in long-term studies, especially in women with a low calcium intake.Mineral waters can provide an alkali load, which is beneficial for bone. The rare mineral waters that are rich in both bicarbonate and calcium inhibit bone resorption even in calcium sufficiency, while calcium-rich waters with low bicarbonate show no effect.

Regular consumption of these rare waters with a very high fluoride content resulted in an elevated BMD, but this was mainly a Fluor effect and less a consequence of their alkalinity. Sulfate probably has no effect on urinary calcium excretion in humans.

Vitamins

Vitamin D is not a nutrient. Nutrition is a negligible source of vitamin D, since the skin delivers sufficient amounts if it receives enough ultraviolet irradiation. In northern Europe, nutrition delivers about 100 IU, far below the daily need of 800 IU. But fatty fishes are rich in vitamin D.

Vitamin A, resp. retinol accelerates bone catabolism and increases fracture risk, when taken in high amounts (Michaëlsson *et al.*, 2003). Vitamin A deficiency can also have a negative influence on bone, but few instances of this are known.

Vitamin K is a cofactor of carboxylase and as a result stimulates the solidification of the bone matrix (carboxylation of osteocalcin). For this reason, vitamin K deficiency is associated with decreased bone density and increased hip fracture risk. Vitamin K is mostly taken in with salads and green vegetables. Small amounts are present in cheeses and fermented soy beans. The daily need for an optimal bone effect is 109 µg vitamin K (Feskanich *et al.*, 1999). This exceeds the amount needed for optimal blood coagulation. The need for vitamin K is covered by the regular intake of salad and vegetables. Pharmacological studies with high doses of vitamin K had a positive effect on bone and decreased the fracture risk. However, these amounts cannot be obtained with nutrition.

Vitamin B12 and folic acid: Deficiency in vitamin B12 is associated with low BMD (Clemens, 2014), eventually because it decreases the synthesis of IGF. The need for vitamin B12 is usually covered by the intake of dairy products, meat, and fish. Folic acid is consumed with legumes, green vegetables, and wholegrain products. Deficiency in folic acid increases the level of homocysteinic acid, which is associated with lower bone density and higher fracture risk, since it leads to a stimulation of the formation of osteoclasts and disturbs bone formation.

Vitamin C: Vitamin C, which is found in many fruits and vegetables, has a positive influence on bone. However, in the published studies, this effect was not well separated from that of confounding factors. Nevertheless, in a longitudinal study over 4 years, vitamin C intake could be associated with a decrease of bone loss (Sahni *et al.*, 2008).

Food Patterns

Nutritional research succeeded in identifying many food items with a significant bone effect. But human nutrition is a mixture of a great number of nutrients with various bone effects. In general, nutrition rich in calcium has a higher health value than a nutrition poor in calcium, because of its general composition. To capture the effect of nutrition overall, recent studies linked nutritional patterns or habits to bone health, essentially in large cross-sectional surveys with hip fractures as an outcome. These surveys showed that adherence to the Mediterranean diet is associated with a decreased risk of hip fracture (Benetou *et al.*, 2013; Byberg *et al.*, 2016).

A generally healthy diet, as evaluated by various scores, is associated not only with fewer cardiovascular diseases, but also with fewer hip fractures, as well as a higher BMD (Hardcastle *et al.*, 2011).

Fortified Food

Many nutrients are fortified with vitamins and microelements. In terms of bone effects, fortification is usually achieved with calcium or vitamin D, and often with both. In order to strengthen the bone without negative side effects, the fortified food has to be consumed regularly, and the calcium content must be harmless in the case of high intake. The calcium used for fortification is the same as that used for supplements. It is served in the form of cow's milk, soy milk, orange juice, soft drinks, flour bread, cereals, snacks, rice and rice products, etc. Bioavailability varies with the calcium salt used and with the food itself, but only slightly. An especially high bioavailability was found for calcium sulfate in white bread. Calcium in orange juice is as well absorbed as it is from milk or supplements, and calcium carbonate in fortified bread is absorbed even more efficiently than calcium from milk. Fortification of nutrients that children like does not guarantee long-term intake.

See also: Regulation of Food Intake

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Bone Development During Childhood and Adolescence: Peak Bone Mass

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Abbreviations

Dxa	Dual X-ray absorptiometry
HR-pqct	High resolution peripheral quantitative computed tomography
pQCT	Peripheral quantitative computed tomography
QCT	Quantitative computed tomography

Introduction

Peak bone mass is the maximal amount of bone accrued within bone during the childhood and especially the during adolescence growth phase plus the consolidation that continues beyond the attainment of final height (**Fig. 1**). Almost half of the adult bone mineral mass is accrued by the skeleton in the 3–4 years following the onset of puberty, making adolescence the most critical period of skeletal bone mass development (Theintz *et al.*, 1992). At peak height velocity, adolescents have reached 90% of their adult stature, but only 57% of their adult bone mass (Bailey *et al.*, 1999), whereas by the end of the pubertal growth, more than 90% of the adult bone mass is formed (McCormack *et al.*, 2017; Henry *et al.*, 2004). The age of attainment of peak bone mass is influenced by the tempo of pubertal maturation, as reflected by a later peak in males compared to females and a later obtainment in late maturing males in comparison with early maturers (Chevalley *et al.*, 2009b; Darelid *et al.*, 2012).

It is generally assumed that optimizing bone mass accrual in childhood and adolescence is beneficial in the prevention of osteoporosis later in life, since it may compensate for aging-associated bone loss, reducing the risk bone fragility associated with adult onset osteoporosis (Bradney *et al.*, 2000). Furthermore, maximizing bone mass during the growth period might also prevent fractures during the building of the skeleton: children and adolescents with distal forearm fractures have deficits in cortical bone and microarchitecture at the distal radius, but also in bone mass at other bone sites (Chevalley *et al.*, 2011; Määttä *et al.*, 2015).

Mechanisms

Bone mass accrual during childhood and adolescence is mainly the result of an increasing bone size, although also significant changes in bone macro- and microstructure occur, especially during adolescence (Henry *et al.*, 2004). Bone mass as well as bone structure contribute to an increasing bone strength during childhood and adolescence in order to adapt to changing mechanical loads and increased muscle development.

Growth in bone length occurs via endochondral ossification at the growth plate, whereas changes in bone width result from periosteal expansion. The superior and inferior endplate contribute equally to the vertebral bone growth, whereas the growth plate

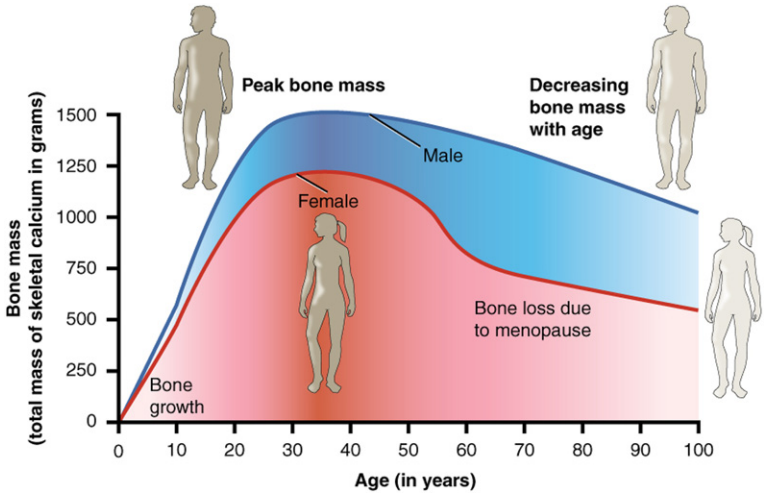

Fig. 1 Evolution of bone mass during life. From Wikimedia Commons, the free media repository.

at the distal radius contributes to 75% of the longitudinal growth of the radius, while about 45% of the longitudinal growth of the tibia is provided by the distal physe. Longitudinal and radial bone expansion occur in synchrony to maintain the bone strength in the growing skeleton (Xu *et al.*, 2009).

The greatest changes in bone mass occur at the cortical bone compartment, whereas the contribution of the trabecular compartment, particular in girls, in bone mass accrual is more static. The increase in the amount of trabecular bone during puberty is underpinned by thickening of trabeculae, with little to no change in number or separation of the trabecula (Gabel *et al.*, 2017). The increase in cortical bone is to a great extend reflected by an increase in cortical bone area during puberty (**Fig. 2**). Cortical bone mass accumulation at the long bones is not only driven by periosteal bone apposition, but also by endosteal bone resorption and formation. In the early stages of puberty both the periosteal and endocortical diameters of the bone increase significantly, whereas in late puberty there is a contraction of the endocortical diameter, increasing the cortical thickness. Periosteal apposition assembles most of the cortical mass in males, while endocortical apposition assembles about 25% of the cortical thickness in non-weight bearing bones (Seeman, 2001). While periosteal apposition slows down in later puberty, endocortical bone formation ensues continuing to increase the cortical thickness (Wang *et al.*, 2006; Ohlsson *et al.*, 2011).

Bone modeling, the process of adapting bone structure to loading by changing bone size and shape, as well as remodeling, the process of replacing old bone by a coupled action of osteoclasts and osteoblasts, are responsible for the accrual of bone mass and the changes in bone structure during childhood and adolescence (Slemenda *et al.*, 1997). Bone remodeling during childhood is characterized by a positive coupling between bone formation and bone resorption, while once final adult height is reached, the neutral coupling results in a stationary state of bone mass. In children about 3% more new bone is deposited than removed in the bone forming units. Remodeling is influenced by mechanical (muscle activity, weight bearing), systemic (calcium delivery, hormonal activities of IGF1 and sex steroids) as well as local (bone cell to bone cell and muscle and adipose tissue to bone tissue) interactions.

A high sensitivity of bone to loading, an increased intestinal calcium absorption, as well as an increased renal absorption of phosphate and an increased calcitriol production, probably related to the increased IGF-1 production during puberty, are some of the factors explaining the enormous capacity of adolescents to increase their bone mineral content in a short period of several years (Abrams *et al.*, 1995).

Changes in Bone Mass

The current understanding of bone mass acquisition during childhood and adolescence is largely based on the results of large longitudinal DXA studies (Bailey *et al.*, 1999; Zemel *et al.*, 2011).

Bone mass accumulation occurs heterogeneously in the skeleton during the skeletal growth period (Henry *et al.*, 2004). The peak bone mass gain is acquired earlier at the level of the femur neck than at the lumbar spine. The peak gain occurs last at the distal radius. The peak in bone mass accumulation during puberty lags by 0.7–1 year behind the peak acquisition of standing height, explaining in part the increased bone fracture rate around this period (McKay *et al.*, 1998).

Until puberty, no important differences in bone accrual exists between boys and girls, as observed by their similar increases in whole body and lumbar spine BMC (De Schepper *et al.*, 1991). From the onset of puberty, gender dysmorphisms in skeletal development occur, to a great extend related to the different actions of oestradiol and testosterone on bone mineral accrual and more specifically on periosteal bone apposition (Riis *et al.*, 1985). Girls have an earlier increase in bone mass accretion (12.5 vs. 14.2 years), but boys accumulate finally more bone than girls at all skeletal sites (Mølgaard *et al.*, 1999).

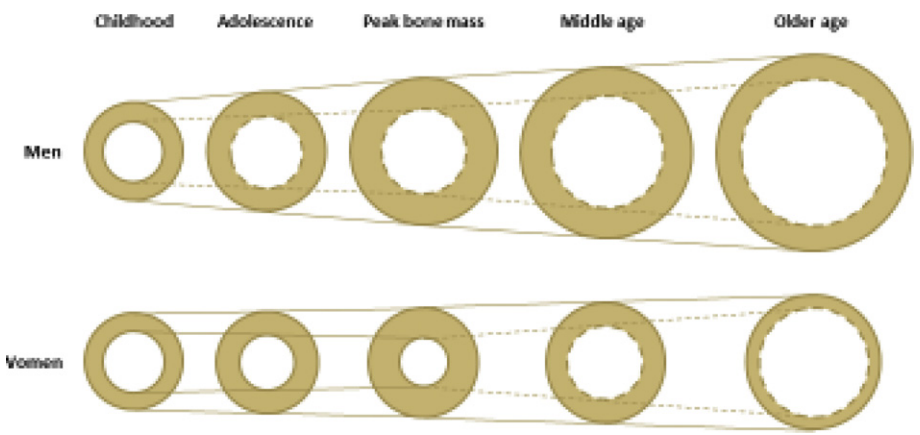

Fig. 2 Changes in cross sectional bone diameters during life.

Changes in Bone Mineral Density, Bone Structure, Bone Robustness, and Bone Strength

By the use of pQCT at the radius or tibial shaft, bone macrostructure, such as total bone cross sectional area, cortical cross sectional bone area, and the cortical thickness, as the net effect of periosteal expansion and endosteal resorption, can be measured easily in children from the age of 5 to 6 years (Neu *et al.*, 2001). Up to now, the growth related changes in bone mineral density and microstructure have been documented by only a limited number (and mainly transversal), QCT, pQCT, and HR-pQCT studies.

Since pQCT measurements in longitudinal studies are repeated in a volume of interest located relative to limb length and not at a fixed rate and bone elongation proceed symmetrically with varying contribution of distal or proximal growth plate depending on age and pubertal stage, observed differences in bone parameters might in part be related to positioning defects. Furthermore, changes in bone geometry and microstructure are site specific: cortical thickness increment at the radius remains relatively stable until late puberty, whereas at the tibial shaft, thickening of the cortex is more evident throughout the growth phase (Schoenau *et al.*, 2002; Roggen *et al.*, 2015). These differences can be explained by the greater forces at the weight bearing tibial site, contributing to greater differences in growth related adaptations. On the other hand, at the end of pubertal development trabecular bone mineral density and trabecular separation are lower at the radius compared to the tibia (Liu *et al.*, 2010).

Most bone parameters as studied by pQCT and HR-pQCT increase from childhood to early adulthood (Gabel *et al.*, 2017), although gains in trabecular BMD and cortical BMD are limited and even a transient decrease in cortical bone mineral density can be observed during midpuberty at the distal radius and tibia (Gabel *et al.*, 2017; Kirmani *et al.*, 2009; Wang *et al.*, 2006). Also at these sites, the porosity index decreases during pubertal development, although more rapidly in the females than the males (Nishiyama *et al.*, 2012). On the other hand, trabecular bone density at the lumbar spine increases linearly at the latest stages of pubertal development (stages 3–5) (Gilsanz *et al.*, 1988). The increase in trabecular BMD in the appendicular and axial skeleton is mainly explained by an increase in trabecular thickness rather than in trabecular number (Kirmani *et al.*, 2009). Using the individual trabecula segmentation technique, a greater increase in plate-like trabecula number and density than in rodlike structures has been evidenced during adolescence, at least in girls (Mitchell *et al.*, 2018).

Whereas cortical bone mineral density increases more in girls than boys at the tibia and the radius during puberty, trabecular bone volume fraction and cortical area are higher in boys, especially post puberty (Kontulainen *et al.*, 2006; Moyer-Mileur *et al.*, 2008; Wang *et al.*, 2010). On the other hand, cortical porosity declines more in females than in males during pubertal growth (Nishiyama *et al.*, 2012). The more porous cortices of boys reflect their higher rates of intracortical remodeling associated with their greater bone size, electing an increased demand for calcium. The peak of cortical porosity at the forearm aligns with the peak time of forearm fractures incidence in boys and girls (Kirmani *et al.*, 2009).

The robustness of the long bones, reflected by the size of the bone cross-section relative to the bone length decreases around the age of peak height, especially at the metaphysis (Rantalainen *et al.*, 2016). The persistent increase in bone diameter, although at a slower speed, into early adulthood in contrast to a stopped increasing bone length at the end of puberty, coincides with the wane in fracture risk in late adolescence (Xu *et al.*, 2009).

An increase of bone strength between 100% and 400% is observed during childhood and adolescence, depending on the skeletal site and the parameter measured. The most common pQCT derived indices of bone strength are the bone strength index at the distal ends of the long bones and the polar strength-strain index at the shaft. By the end of puberty, bone strength in males is 10%–38% higher in males than females, despite a slightly lower cortical bone mineral density and a greater cortical porosity (Nishiyama *et al.*, 2012). This is mainly due to their large bone diameters and their greater trabecular bone volume.

General Characteristics in Bone Mass and Bone Structure Development

Changes in bone mass and bone cortical parameters during growth are race dependent, even after correction for anthropometric differences: Black children and adolescents have a greater lumbar spine and whole body bone mineral content, as well as a greater cortical bone mineral density and size at the tibia (Kalkwarf *et al.*, 2007; Warden *et al.*, 2013).

The variability in most bone parameters increase with age, necessitating the use of a large reference sample to characterize the normal range of age related changes, especially during adolescence (Kalkwarf *et al.*, 2007; Zemel *et al.*, 2011).

Another important phenomenon in bone mineral accrual during growth is tracking. Tracking of bone mass has been documented especially during childhood but less during adolescence, even after correction for body height and weight changes (Loro *et al.*, 2000; Kalkwarf *et al.*, 2010; Foley *et al.*, 2009).

The timing of puberty appears of importance in bone mass accrual, as late menarche is associated with thinner cortices and lower trabecular BMD at the distal tibia and a lower areal bone mineral density at several sites (Chevalley *et al.*, 2009a).

Determinants of Bone Mass Accrual

Major factors influencing the accumulation of bone mass during childhood and adolescence, are heredity, life style (such as diet, physical activity, and cigarette smoking) and hormonal status.

Genetic factors play the greatest role in peak bone mass determination. About 40%–70% of the bone phenotype variance is derived from heritable variables, as suggested by family and twin studies (Havill *et al.*, 2007). Inheritance is polygenic and several

genomic loci have been identified that predict skeletal health in children (Duren *et al.*, 2011). Vitamin D receptor and estrogen receptor polymorphisms have been found to be related to bone mass in male and female adolescents.

On the other hand, a healthy life style including regular milk intake and regular sport activities, can increase bone mass accrual. Calcium supplementation during childhood or adolescence can modestly (2%–5%) increase bone mass, in general more at cortical sites and when habitual calcium intake is low (Bonjour *et al.*, 1997; Johnston *et al.*, 1992). Replacement of milk by soft drinks in female adolescents might compromise their optimal bone accrual, whereas the combination of vitamin D and calcium may enhance trabecular bone mineral density (Whiting *et al.*, 2004).

Physical activity is important for bone mass at all periods of life, but especially during the growth phase. This positive effect is mostly observed at weight bearing sites (lumbar spine and femoral neck), before pubertal onset and in males (Bielemann *et al.*, 2013; Bailey *et al.*, 1999; Behringer *et al.*, 2014). More physically active children accrue not only more bone mass, but develop also a more robust long bone geometry (greater cross sectional diameter, smaller endosteal bone diameter) than their less physically peers (Rantalainen *et al.*, 2015; Duckham *et al.*, 2014).

On the other hand, early initiation of smoking is inversely associated with late adolescence forearm and femur bone mineral density in girls, whereas bone acquisition at the lumbar spine might be reduced during adolescence by the use of contraceptive oral estrogens, especially those containing very low dose of EE (Cibula *et al.*, 2012; Dorn *et al.*, 2011).

During puberty, the dramatic changes in sex steroids and IGF-1, have been related to the rapid changes in bone structures (Vanderschueren *et al.*, 2006). Estradiol is required for the attainment of maximal peak bone mass in both sexes, whereas action of testosterone on stimulating periosteal apposition accounts for the larger size and thicker cortices of the adult male skeleton (Chevalley *et al.*, 2011; Gabel *et al.*, 2015).

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Bone Mass Measurement☆

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Glossary

Biochemical markers of bone turn-over (BTM's) Analytes (mostly different circulating collagen or procollagen fragments commonly called CTX and P1NP) measured in urine or serum to assess bone turn-over.

Bone mineral density (BMD) The amount of mineral present in a defined volume of bone. A "true density" (g cm^{-3}) can only be measured by CT scan. DXA measures an areal bone mineral density (BMD_a, g cm^{-2}), an apparent bone density which is the bone mineral content in g (BMC) divided by the area of bone scanned.

Dual X-ray absorptiometry (DXA) The most used technique to assess bone mass, using the absorption of two X-rays of different energies to separate that caused by mineral and that caused by soft tissues.

Finite element analysis (FEA) A mathematical technique using differential and integral calculus to simulate the resistance of bone to a defined force applied to its surface, using the structural parameters derived from QCT or HRpQCT.

High resolution peripheral computed tomography (HRpQCT) A CT scanner designed to measure peripheral bone density (radius, tibia) with a high resolution (80 μm). The apparatus allows to measure separately cortical and trabecular true density (g cm^{-3})and to measure structural parameters like cortical thickness and porosity, trabecular thickness or trabecular separation.

Least significant change (LSC) The minimal change necessary between two measurements to be significant rather than a measurement error as defined by the precision error of the measurement. In bone densitometry, the LSC is defined by the in vivo precision error of repeated measurements multiplied by the confidence level desired to be certain that a change in bone mineral density (BMD) or of bone markers (BCM) is real rather than due to a measurement error.

NHANES III—National Health and Nutrition Examination Survey III The study from which the reference values for BMD were established.

Prevalence The percentage of the population being studied that is affected by a particular disease at a given time. In bone densitometry, prevalence is usually applied to the percentage of the population above or below a certain standard deviation cut-point from the mean BMD of the reference population.

Quantitative ultrasound measurements (QUS) Ultrasounds are used to estimate bone strength (limited to the peripheral skeleton). Two main parameters are determined, the speed of sound (SOS) and broadband attenuation (BUA), which depend of bone density and probably microarchitecture.

Quantitative computed tomography (QCT) A radiologic tomographic technique allowing to compute a three dimensional view of the organs and to derive true volumic density (BMD_v, g cm^{-3}).

Risk The possibility of loss, injury, disadvantage, or destruction. In bone densitometry, risk is used to define either the relative or absolute risk of fracture at any given BMD value at a particular age.

Trabecular bone score (TBS) A score derived from image analysis of the DXA anteroposterior view of the spine to estimate trabecular architectural modifications susceptible to fragilize bone.

Vertebral fracture assessment (VFA) Vertebral heights measurement on a quick lateral view of the spine using DXA device to diagnose vertebral fractures at the time of BMD measurement.

Introduction

Bone mass measurements were developed by radiologists before World War II, but begun to be currently used in the clinic about 50 years ago, when Cameron *et al.* (1968) first described single photon absorptiometry (SPA) to measure radius bone mineral content (BMC) and apparent density (BMD). It was the introduction of dual X-ray absorptiometry (DXA) allowing quantification of the central skeleton (mainly spine and hip) that allowed the tremendous development of studies in osteoporosis. The clinical application of bone densitometry is one of the advances in the field of osteoporosis that has led to increased patient awareness of this increasingly prevalent disease. Bone densitometry has made it possible for clinicians to diagnose osteoporosis before the first fracture has occurred and to predict risk for fracture in postmenopausal women, men, and patients receiving glucocorticoids. It can

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be used as a surrogate marker to follow the efficacy of therapies and to examine those patients that might be osteoporosis-specific therapeutic nonresponders. Hence, there are three reasons clinicians perform bone mineral density (BMD) measurements: diagnosis using the World Health Organization criteria for osteoporosis, fracture risk prediction, and monitoring the natural progression of diseases that affect BMD or monitoring the therapeutic response to osteoporosis-specific treatments. This article examines each of these applications of bone mass measurements and provides principles of clinical application.

Diagnosis

In 1994, the World Health Organization (WHO) selected a bone mineral density (BMD) cut-point for defining the prevalence of osteoporosis in the Caucasian postmenopausal female population. The main intent of the WHO committee was to assess the prevalence of low bone mass in the population and relate bone mass to the anticipated lifetime fracture risk in order to advise health policymakers of the potential medical and economic burden of osteoporotic fractures in this population. The use of standard deviation (SD) scores (T-score) rather than absolute BMD in grams per centimeter squared was decided upon because of the known different absolute BMD calibrations that exist between the BMD measuring devices. A SD score mitigates some of the variance in absolute BMD measured by different manufacturer devices. In the densitometry nomenclature, T-score is the difference, expressed in SD units, between a BMD value observed in a patient and the mean BMD in the reference population during young adulthood (peak bone mass).

The WHO cut-point of $T = -2.5$ or lower used for the diagnosis of osteoporosis is based on a close association between prevalence at this cut-point and lifetime fracture risk of hip fractures or all fractures (hip, vertebrae, forearm, humerus, and pelvis). At the femoral neck, as assessed by the young, normal reference population database, 16% of postmenopausal women 50 years of age or older have a score of -2.5 SD or less, and the lifetime hip fracture risk after age 50 in postmenopausal Caucasian women is 16%. In addition, approximately 30% of this population has a score of -2.5 SD or less as measured at the hip, spine, and wrist, which approximates the lifetime fracture risk of all or global fractures after age 50. However, the relationship between low BMD and increased fracture risk is a gradient and not a threshold. Thus, the WHO created a separate classification, osteopenia, to recognize this gradient of risk and to help clinicians assess additional risk factors besides the level of BMD that may lead to increased fracture risk. In this regard, in the National Osteoporosis and Risk Assessment (NORA) dataset, although the 1-year fracture rates and relative risk for fracture/SD reduction in BMD were highest at T-scores of -2.5 SD or lower, the largest number of fractures, partially related to the larger sample size of the population with T-scores between -1.0 and -2.5 SD, was seen in this osteopenic category (Siris *et al.*, 2004). This conclusion derived from peripheral measurements was confirmed when considering central DXA (Pasco *et al.*, 2006).

The T-score based interpretation of BMD has been debated: though it resolves the problem of machine calibration, it only partially resolves that of a unifying definition of osteoporosis diagnosis. T-scores are indeed dependent on the mean and SD of the reference population, being lower if the mean is higher and/or SD lower (Chen *et al.*, 1998). So, the choice of the reference database has an impact on the diagnosis, independently of absolute BMD values, and using T-scores implies an agreement on which reference data base should be used for calculation, a question still controversial as far as gender and ethnicity are concerned. Abandonment of the T-score has been suggested for all BMD devices and skeletal sites with the exception of the hip, for which there is a uniformly accepted Caucasian database with NHANCEIII (Looker *et al.*, 1998). However, replacing T-scores by absolute BMD values would be impractical because of the lack of uniformity of the results between the different DXA devices. For example, the average BMD of the axial skeleton (L2–L4) in a healthy 20-year-old Caucasian women is 1.25 g/cm^2 by Lunar-GE and 1.00 g/cm^2 using a different device. Large comparison trials have been conducted to derive equations to convert values obtained from different devices. Though these equations can be useful for follow up when a patient was measured previously with another device, the residual error is too important for an exact standardized BMD to be calculated (Bonnick, 2010).

Regarding gender, two positions concurred: using the female NHANCE III database for both genders, or using gender specific reference data (Melton *et al.*, 2001; Binkley *et al.*, 2002). The tenants of the first position argue that males fracture at the same absolute BMD as females, those of the second that using female reference data for T-score calculation in males will result in underdiagnosing osteoporosis in men (Vallarta-Ast *et al.*, 2002). The percentage of men with T-scores <-2.5 SD at the femoral neck when the T-scores are calculated from a male database is 6% and that from the female database is 4% (Orwoll, 2000). Thus, when considering only the hip, the small differences in prevalence between the two genders using NHANES III databases will not miss many men at risk. On the other hand, when the prevalence of osteoporosis by WHO criteria is determined in men from a male or a female database by using the spine, wrist, and hip measurements, it is 19% when the T-score is calculated from a male database and 6% when calculated from a female database. Thus, when T-scores are calculated from a female database, men are underdiagnosed if the clinician examines multiple skeletal sites. Therefore, the number of men determined to be at risk for fracture is underestimated when all three central DXA-measured skeletal sites are combined if a female reference population database is used. In a recent longitudinal study however (Leslie *et al.*, 2014), it was shown that men were at slightly lower risk for incident major osteoporotic fractures than women for an equivalent lumbar spine BMD, supporting the use of a female Caucasian database to diagnose osteoporosis in men.

What about ethnicity? The NHANES III reference database provides the only head-to-head ethnic comparison in which both T- and Z-scores are calculated from both gender- and ethnic-specific databases for prevalence comparisons. Ethnic-specific databases were calculated from Caucasian, Hispanic, and African American male and female populations ranging in age from 20 to 80 years. There are no Asians populations in the NHANES III database. In this robust study, there are clear prevalence differences when T- or

Z-scores from different ethnic groups are calculated from a non-ethnic-specific database. However, no fracture data correlate with the T- or Z-scores from the NHANES III database. Hence, there are no data regarding whether there are differences in fracture rates as a function of the ethnicity-derived T-scores from this valuable dataset. Concerning Asians, there are recent studies showing important misclassification when Caucasian or Asiatic database were used for T-score calculation (Namwongprom *et al.*, 2012), but there are no fracture data either.

The only multiethnic head-to-head prevalence and fracture data derive from NORA, in which all SD scores were calculated from the Caucasian female reference population database. The relative risk (RR) per SDs decrease for global fractures are similar between Caucasians, American Hispanics, and Native Americans and lower for the American Asian and African American populations. On the other hand, the fracture rates across all ethnicities at T-scores <-2.5 SD are very similar. In the WHO-defined osteopenic category in NORA, American Asians had an unexpectedly low rate of fracture events. The reasons for this are unclear. However, because all of the risk calculations in NORA were done using a Caucasian female reference population database and fracture rates were not too dissimilar across ethnicities, at least for the U.S. multiethnic female postmenopausal population, it might be acceptable to calculate risk from a Caucasian female database, regardless of ethnicity. Native Chinese have a lower hip fracture risk (but similar vertebral fracture risk) despite having a much lower absolute BMD, even after adjusting for body mass index. It is possible that in the United States, lifestyle, nutrition, or even some elements of gene pool mix may result in non-Caucasian ethnic groups having the same fracture risk as Caucasians. The last ISCD recommendations suggest using uniformly white Caucasian female database for T-score calculation across genders and ethnicities (ISCD, 2015). The debate over reference data bases could be solved if the definition of osteoporosis changes towards one based on fracture risk, instead of epidemiology. But this will require a consensus over the fracture risk calculators, their validation, and the definition of a risk threshold.

A second important debate concerns the skeletal sites to measure (Faulkner *et al.*, 1999). Absolute BMD's measured at different skeletal sites are quite well correlated in the statistical sense, but do not predict each other tightly because residual variance is high (Mazess and Barden, 1990). Also, they change less or more rapidly with age and menopause according to the site measured, i.a., because of the different proportion of cortical and trabecular bone and because mechanical stress influences the bone physiology in supporting bones like femurs and tibias as compared with upper limb skeleton (radius). Hence, the evolution of T-scores with age or menopause may differ greatly between sites and if methods other than DXA are used for measurement (for instance, volumetric BMD of a central vertebra measured by QCT decreases much more rapidly than areal BMD of the same vertebra measured by DXA; conversely, the evolution of QUS parameters measured on peripheral bones (usually calcaneus) is much slower. Thus, if a low BMD in the postmenopausal population assessed by any BMD device and by any database is predictive of an increased fracture risk, a relationship also seen in the multiethnic U.S. population, for various reasons, T-scores obtained by peripheral devices may not always be as low as T-scores determined from central DXA devices and peripheral devices underestimate osteoporosis prevalence. The prevalence of WHO osteoporosis (≤ -2.5 SD) in the NORA dataset of postmenopausal women 50 years of age or older averaged 7% for all four peripheral devices combined, half of the prevalence that was determined by measuring only the femoral neck.

For these reasons, recommendations for the diagnosis of osteoporosis have been progressively adapted since 1994, limiting the technologies accepted to DXA and restricting the skeletal sites included for osteoporosis diagnosis to the lumbar spine, total hip and femoral neck (Hans *et al.*, 2006; ISCD, 2015), except in some special cases like primary hyperparathyroidism or extreme obesity where measurement of the 1/3 proximal radius remains advised. Thus, a central BMD should be performed in any patient with risk factors that would lead the clinician to suspect osteoporosis. Though a less expensive and more universally accessible peripheral BMD can be a first screening test, it must be followed by central BMD when the measured value is low, for instance if the calcaneal DXA T-score is ≤ 0 (Sweeney *et al.*, 2002) or if the heel measurement by ultrasound is <-1.0. However, a first screening using a peripheral device is not currently recommended (ISCD, 2015).

On the other hand, vertebral quantitative computerized tomography, which is more sensitive than DXA to document vertebral bone loss, underestimates T-scores and overestimate the prevalence of osteoporosis in the population. It is not accepted to diagnose osteoporosis according to the WHO definition.

A third important point in osteoporosis diagnosis is accuracy of the DXA measurement. Accuracy is the capacity of a method to give a result as close as possible to the "real" value of the object, here bone density, as a surrogate of bone strength. Accuracy as determined in cadaver studies is typically 3%–7%; it is influenced by soft tissue composition, mainly adipose tissue content, which differs from patient to patient: fat indeed absorbs less the X-rays than water, and changes the soft tissue correction (Blake *et al.*, 2013). Accuracy can also be affected by artefacts like spondylarthritis, which provokes a positive bias on the spine BMD. A (less important) effect of arthrosis has been observed at the hip site. Aortic calcifications can also increase artefactually spine BMD, so as the presence of radiological contrast product or metallic pieces in the measurement field. Eventually, as areal BMD is the ratio of BMC by estimated area, different positioning of the bone can give rise to substantial accuracy errors, particularly for the hip: a different rotation will modify the projected surface but is without effect on BMC. Hence the importance of formation and experience of the personal committed to bone density measurement.

A problem with the WHO osteoporosis T-score definition of osteoporosis is that, if it predicts adequately a higher risk of fracture, more than 50% of the fractures occur in persons in the osteopenic range (Pasco *et al.*, 2006). This is due in part to limitations of the definition of osteoporosis using the WHO criteria, not the least being that bone mass is only one component of bone strength, although an important one. Other factors designed globally as "bone quality" are also important. They include bone architecture (trabecular orientation and density, cortical thickness, geometry) and elastic properties, or brittleness, of the bone "fundamental material," this tight association of proteins, mainly collagen, and mineral, mainly hydroxyapatite. At the end of the next section, we shall briefly describe new techniques which can capture some of these properties and could add to the

accuracy of fracture prediction. This will never be perfect since fractures will most often result from random events (falls), themselves dependent upon non skeletal functions: muscular strength, sight, equilibrium… and from the force of impact, which depends of patient's weight. Some of these "non BMD factors" may be captured by clinical risk factors now included in several "fracture risk predictors," the most popular being the FRAX©.

It must be underlined that these considerations on osteoporosis diagnosis are valid only for post-menopausal women and men older than 50 years old. For younger people, and particularly for children, the use of T-scores is inappropriate and Z-scores, that is the difference between the BMD value measured in a patient and the mean of a sex and age matched reference population, must be used to diagnose a low bone mass (Z-score <– 2.0 SD).

Prediction

From a clinical point of view, fracture risk prediction is much more important than osteoporosis diagnosis, even if it is clear that both are linked, and that these two points of view should be reconciled in a more comprehensive definition of osteoporosis than the WHO T-score criteria. Work is in progress to try that reconciliation (see position paper by Siris *et al.* in 2014). It is fracture risk prediction which will allow to select groups of patients at high risk who will receive the highest benefit of osteoporosis treatment in terms of fracture prevention and thus to be most cost/benefit efficient.

Both central and peripheral BMD devices can predict an increased risk for fracture. Risk approximately doubles for each standard deviation reduction in BMD in the postmenopausal population. However, the risk associated with low BMD is very dependent on the age of the patient. Risk is far greater as age increases. In fact, the risk for hip fracture is approximately 4.5-fold greater at age 80 than at age 50, even at the same BMD or T-score (Hui *et al.*, 1988). Although some of the increased risk seen with advancing age may be related to an increase in falls, there are bone quality changes with aging that render a bone more susceptible to fracture at equivalent BMDs. Clinicians cannot capture these qualitative features with BMD devices.The best documented application of peripheral technologies is fracture risk prediction. However, estimated fracture risk varies widely when BMD is measured at different peripheral sites (Blake *et al.*, 2002). The first prospective observation documenting the ability of BMD technology to predict fracture risk was made in 1988 when forearm BMD was shown to predict an increased risk for nonspinal fractures in postmenopausal women (Hui *et al.*, 1988). This study was followed by a large metaanalysis that documented the ability of multiple technologies, both peripheral and central, to predict an increased risk for vertebral, nonvertebral, and hip fracture risk (Marshall *et al.*, 1996). It has been suggested that the femoral neck is a more robust skeletal site for predicting the risk of hip fracture (RR/SD = 2.4). Although this suggestion is based on the Study of ostoporotic fractures (SOF) head-to-head hip/heel ultrasound device study, the hip fracture risk that was predicted in NORA using wrist or heel DXA was also 2.4, and the receiver operating characteristic (ROC) curves of the two peripheral devices used in NORA match the ROC curves seen with femoral neck DXA obtained from SOF (Miller *et al.*, 2002). NORA was not a head-to-head comparison with hip DXA. Nevertheless, it appears that low peripheral device values in the postmenopausal population are powerful predictors of hip fracture risk in untreated postmenopausal women.

Whatever the measurement site and device, measurement of BMD alone predicts fracture risk with an insufficient sensitivity. There are several reasons therefore, inherent to the factors governing bone resistance and to factors unrelated to bone. Bone mass is indeed only one of the components of bone strength: geometry, architecture and the intrinsic quality of bone material (all grouped in what one call "bone quality"). Factors unrelated to bone are falls (a main determinant of hip fractures) and death, which is a competing risk with fractures. To increase the predictive value of bone mass measurements, models were constructed which take into account clinical risk factors such as age, BMI, prevalent fragility fractures, parental hip fracture, smoking, alcohol consumption or glucocorticoid excess (Kanis *et al.*, 2007). The clinical factors are supposed to take at least in part into account "bone quality." The most popular fracture risk calculator, developed under the auspices of the WHO, is FRAX®, which allows to calculate the absolute fracture risk for 10 years in men and women between 40 and 90 years old (Kanis *et al.*, 2011). There are other tools, like the Garvan nomogram (Australia) (Bolland *et al.*, 2011) or Q-fracture (England) (Hippisley-Cox *et al.*, 2014), differing by the duration of fracture prediction and by a number of risk factors taken into account. FRAX® has been constructed from a huge international database from which the absolute fragility fracture risk was estimated from clinical factors and BMD, and it is the only one which takes into account the competing risk of death. Prediction varies according to nationality and ethnicity, due to the distribution of risk factors and different risk gradients, and thus the model has to be adapted and validated for each population (Kanis *et al.*, 2012). FRAX® has been validated prospectively in different countries (a.o. Leslie *et al.*, 2010).

Taking into account the limitations of the WHO definition of osteoporosis, based on T-scores, in terms of recognition of the patients most at risk for fracture and thus for whom the cost/benefit ratio of treatment would be optimized, some experts plea for an extension of this diagnosis to persons with a history of hip fracture, independently of bone mass measurement, to osteopenic persons with a history of major osteoporotic fracture or to those with a 10 year fracture risk estimated by FRAX® higher than a predefined level (3% for a hip fracture or 20% for a MOF in the USA) (Siris *et al.*, 2014). This extended definition, for which measurement of bone mass remains essential, would have the advantage to reconcile diagnosis and fracture prediction. It would also reflect more accurately the clinical definition of osteoporosis as "a systemic skeletal disease characterized by low bone mass and microarchitecture deterioration of bone tissue, with a counterpart increase in bone fragility and susceptibility to fracture" (Consensus, 1993).

Among the clinical risk factors, one of the most important is the presence of a prevalent fragility fracture, among which vertebral fractures, which are frequently asymptomatic. A history of vertebral fracture increases greatly indeed the risk of further lesions. Hence the importance of vertebral fracture assessment (VFA) which has been added to modern DXA machines, based on a quick lateral view of the

spine (Rea *et al.*, 2000). Use of VFA allows to detect, at the time of bone mass measurement, if there are prevalent vertebral fractures, an observation which will change risk assessment and therapeutic decisions. The interpretation of VFA is not always that simple, particularly in patients with spine deformities, and fractures should be confirmed by a lateral spine X-ray when suspected.

Modern DXA machines also include a "Trabecular bone score (TBS)" tool, which allows to derive from image analysis an index reflecting the quality of trabecular organization. TBS could add to fracture prediction in some groups of patients with osteopenia or with diseases with an increased fracture risk associated with a normal or even supra-normal BMD, such as type 2 diabetes (Silva *et al.*, 2014). The introduction of the TBS value in the fracture risk calculator is now optional and has still to be validated in large cohorts of osteopenic patients (McCloskey *et al.*, 2015).

Bone geometry is also important for bone strength. With the Hip Structural Assessment (HSA), available on DXA and CT devices, it is possible to calculate the length of the femoral neck, the mineralized cross sectional area and the mass distribution around the longitudinal axis, and the ratio between the cortical thickness of the neck and the width of the femoral neck (called buckling ratio, BR), all parameters linked to mechanical resistance (Kaptoge *et al.*, 2008). Until now, there was no formal proof for these parameters to improve fracture risk prediction above BMD measurement. HSA has nevertheless allowed some interesting observations like that of a benefit for bone development of a high level physical activity (Maïmouna *et al.*, 2013). Femoral mapping by CT, as described by Poole *et al.* (2017), is perhaps more promising, showing that patients with hip fractures are characterized by regional bone loss limited to some areas of the superior femur. Vertebral and hip QCT also allow interesting structural studies, and include the possibility to simulate bone strength, an option which can be interesting in understanding the mechanism of action of drugs during osteoporosis treatment (Graeff *et al.*, 2015).

Eventually, high resolution peripheral quantitative computed tomography (HRpQCT) (and other high resolution radiographic techniques) allows to investigate volumetric cortical and trabecular BMD and even trabecular architecture; it is also possible, using finite element analysis (FEA), to calculate the resistance of bone to a simulated applied force. Some of the derived parameters predict fracture risk independently of BMD alone (Sornay-Rendu *et al.*, 2007; Vico *et al.*, 2008; Chevalley *et al.*, 2013; Edwards *et al.*, 2016; Ohlsson *et al.*, 2017). HR pQCT can also be applied to the investigation of the mechanism of action of anti-osteoporosis drugs (Tsai *et al.*, 2016).

The only technique allowing to test directly the resistance of bone fundamental material is reference point microidentation. Different instruments allow to measure in vivo the resistance of cortical bone to penetration by a calibrated weight. These are promising but invasive techniques which are used actually in a research context only (Jenkins *et al.*, 2016).

Monitoring

BMD, and also biochemical markers of bone turn over (BTM's), for which there is increasing evidence for clinical application (Cavalier *et al.*, 2016), allow to confirm that antiosteoporotic drugs have the expected effect (increase in BMD and decrease of BTM's for antiresorptive drugs, or increase of BTM's for anabolic drugs).

These surrogate markers are largely used in clinical trials to validate antiosteoporotic treatments, even if the primary end-point is the reduction of fracture risk. They are particularly useful in first phases trials, before undertaking longer and more costly trials powered on fractures (Leder *et al.*, 2015).

Although the FDA requires proof of a significant 3-year vertebral fracture reduction for registration of PMO osteoporosis-specific therapies, surrogate measurements can be accepted for bridging studies comparing analogous drugs belonging to the same class, or two administration schedules. For instance, the FDA has approved both the once-a-week alendronate and the once-a-week risedronate formulations not based on any prospective-derived fracture data but on evidence that these weekly formulations increase BMD and reduce BCM to equivalent degrees as daily dosing. Hence, the FDA acknowledges that these surrogate markers for bisphosphonates demonstrate improvement in bone strength and a reduction in fracture incidence. However, to the extent that the bone half-life may not reflect the bone biological, functional, bisphosphonate half-life, and because the bone binding and alteration in activation frequency of bisphosphonates differ between amino bisphosphonates, these assumptions may or may not be entirely valid. More recently, surrogate markers were also used as primary end-point in a study comparing 6–9 years of zoledronate treatment in osteoporosis (Black *et al.*, 2012).

The use of these surrogate markers is only justified if their evolution correlates with the reduction of fracture rate. This was shown in several studies examining the relationship between the changes in BMD and/or BTM's and the magnitude of reduction in either vertebral or non-vertebral incident fractures by robust metaanalysis of randomized, controlled clinical trials (Wasnich and Miller, 2000; Hochberg *et al.*, 2002). In general, the metaanalyses are in agreement that a portion (24%–54%) of the risk reduction of antiresorptive agents can be attributed to the increase in BMD. Also, these metaanalyses are in agreement that there is an approximately 24% reduction in vertebral fracture risk at the intercept (no change in BMD), indicating that other non-BMD mechanisms play a role in the improvement in bone strength. The metaanalysis of the relationships between the changes in BMD and BTM due to antiresorptive agents and the reduction in nonvertebral fractures shows that all of the effects of the antiresorptive agents that reduce the incident of nonvertebral fractures can be attributed to either the magnitude of increase in BMD or the magnitude of reduction in BCM of bone turnover. In this metaanalysis, risk reduction related to either the increase in BMD or the reduction in BTM could not be distinguished because the adjusted variances that the components contribute to risk reduction are so similar. In studies of newer antiresorptive agents of the same class (zoledronate) (Jacques *et al.*, 2012) or with a different mode of action (denosumab) (Austin *et al.*, 2012), the relation between BMD change and fracture rate reduction was comparable, as in

studies using an anabolic agent like teriparatide (Tanaka *et al.*, 2014). A predictive value of BMD changes on fracture rate reduction was also observed in daily practice (Leslie *et al.*, 2016).

Usually, not all the antifracture effect can be explained by BMD. By reducing bone turnover, there may be microarchitectural changes in bone not necessarily reflected in BMD measurements that lead to increased bone strength. Moreover, the areal BMD measurements that are routinely used in bone densitometry may underestimate the real magnitude of BMD increases because the areal BMD measured by DXA is a quotient of BMD by calculated area. The bone area may increase without any change or a small change in BMC; thus, the calculated BMD would decline, not necessarily due to any reduction in BMC but due to an increase in bone area. Evidence for this effect of bone area on calculated BMD derives from studies measuring changes in areal BMD by DXA versus quantitative computerized tomography (QCT), which measures the true bone mass and volumetric changes in bone mineral content. QCT changes were larger than DXA changes in this intermittent PTH study, even though BMC increased in both, suggesting that areal BMD measurements may underestimate the change in BMD (Hodsman *et al.*, 2003; Rehman *et al.*, 2003). Nevertheless, areal BMD at both baseline and longitudinal over time reflects both the basal bone strength as it relates to fracture prediction and the improvement in bone strength as it relates to changes in BMD that are induced by antiresorptives.

The use of sequential BMD measurements in the individual patient management was more subject to discussion, because they are neither a perfect indicator of pharmacological response or nonresponse to therapeutic interventions to antiresorptive agents and there is no formal proof that adding these measurements to the clinical follow up protocol improves the treatment efficacy. The prevention or reduction of fractures is the ultimate goal of treatment. However, for drugs which have been shown to increase BMD, and with a validated antifracture effect in clinical trials, changes in BMD can be a surrogate endpoint marker for monitoring the efficacy of osteoporosis-specific agents in real life. The rationale for follow up in individual patients is that: 1° since no treatment completely eliminates fracture risk, the occurrence of another (i.e., vertebral) fracture while on antiresorptive agents does not necessarily mean that the patient has not had a pharmacological effect of antiresorptive treatment; 2° clinicians are uncomfortable about waiting for another fracture event in order to define treatment effectiveness; thus, the use of surrogate markers to assist therapeutic strategies is analogous to lowering blood pressure or cholesterol to measure treatment effect of antihypertensive or cholesterol-lowering agents. As for all laboratory determinations, it is crucial to interpret changes in DXA values in the light of the intra-individual variability, which must be assessed in vivo. Thus, for DXA to be useful in follow up, you need an excellent precision, or reproducibility (Bonnick *et al.*, 2001). Precision depends on the quality of the DXA device but mostly of the experience of the DXA laboratory, which has to be alert to measurement drifts (use phantoms) and to be most meticulous in the patient preparation (no interfering objects in the field), positioning and in the image treatment (selected areas for calculation). The lowest precision is, as compared with the expected change during natural evolution or therapy, the sooner a significant evolution will be detected. For a clinician to know with certainty that a change in BMD between two measurements in an individual patient is real as opposed to an inherent measurement error of the DXA device or an error of the researcher performing the test, the in vivo precision error, which is the coefficient of variation % (CV%) between multiple measurements with repositioning, must be known for the DXA site. It is 1.0%–1.5% in normal subjects, thus much higher than the 0.3%–0.4% measured on phantoms. Performing daily phantom (in vitro) scanning is important in order to detect a "drift" in serial BMD values that may indicate the need to obtain manufacturer assistance to search for problems that affect machine performance, such as X-ray tube viability and software deterioration. However, phantoms do not move, whereas patients do. The densitometry community has set a standard requiring a 95% confidence limit for determining whether a difference between two BMD measurements in an individual patient is significant. Using this standard, the least significant change (LSC) in BMD is 2.8 times the CV % (precision error), thus 3%–5% for the spine or hip. If the independent precision error is not known for a DXA site, changes in BMD may be assumed to be significant (or nonsignificant) when in fact they may not be. Unfortunately, when the BMD change is within the LSC but misinterpreted to be significant, many patients are often taken off therapy or their therapy is changed. Unfortunately, none of the peripheral devices have shown BMD changes in any antiresorptive clinical trials, except recently for denosumab (Simon *et al.*, 2013).

The pertinence of DXA follow up and the frequency of sequential measurements remain debated. Because of the magnitude of BMD changes expected, and of in vivo precision of the DXA machines, a first control is generally proposed at 2 years, in order to surpass the LSC. Ideally, the treatment goal should be that BMD under treatment surpasses the critical value of a T-score above -2.5 (Kuroda *et al.*, 2012), but it is rarely the case in patients with very low basal values (Cummings *et al.*, 2017). Also, it is not proven that the threshold of -2.5 chosen for untreated patients is also valid for patients receiving treatment, since architecture is not or insufficiently restored and bone quality can be changed by treatment (Kanis *et al.*, 2014). As it is now recommended that antiresorptive treatment be suspended after 5–7 years, to avoid oversuppression of bone turn-over (treatment holiday), a DXA control should be done before treatment arrest, and after two years to see if bone gain is maintained (McNabb *et al.*, 2014). Biochemichal markers of bone turn-over (BTM's) can also be used to monitor the evolution of bone turn-over during treatment arrest.

The use of BTM's of bone turnover is now largely recommended indeed to assess early the biological response to the antiosteoporotic agent or to its withdrawal. Data suggest that if there is at least a 30% decline (LSC) in serum collagen cross-link markers 1–3 months after initiating a bisphosphonate, there is a greater likelihood that the 2-year follow-up repeat DXA will increase or at least not decline. In addition, data on the relationship between the magnitude of increase in BMD and the magnitude of decrease in BTM with the various antiresorptive agents suggest that the greater the decrease in BTM, the greater the risk reduction in vertebral as well as non-vertebral fractures, and prospective longitudinal studies have shown a relationship between BTM decrease and fracture risk reduction. No professional scientific organization recommends substitution of BTM for DXA as a means of monitoring therapy. The two are

complementary. The advantage of the 1- to 3-month BTM assessment is that it provides earlier feedback to the patient and the clinician: if it does decline below the LSC for BTM, then it is fair to assume that the patient will benefit from the treatment in the long run.

Conclusion and Perspectives

BMD testing has allowed clinical application in diagnosis, risk prediction, and monitoring of disease or therapy in osteoporosis. However, like all biological measurements, BMD testing at baseline and/or longitudinally is imperfect. First, it must be measured in laboratories with sufficient experience to get the best accuracy and precision, and to avoid pitfalls in its interpretation. Second, it is only useful if integrated in the clinical context. The definition of osteoporosis based on the calculation of T-scores proposed in 1994 by the WHO is evolutive. The acceptable methods for evaluating bone mass to diagnose osteoporosis were restricted to DXA and the skeletal sites to the spine and hip, limiting the use of peripheral measurements by any other methods to fracture prediction. The reference data bases acceptable to calculate T-score remains debated, though the trend is to use uniformly the female Caucasian values. The efforts to integrate prevalent fractures and absolute fracture risk calculation in the osteoporosis definition should eventually reconcile osteoporosis diagnosis and fracture prediction. A definition based on risk calculation only would be the most rational for clinical applications, but should await risk calculators to be uniformly validated and accepted, which is not yet the case, and a consensus on the risk level defining the disease and the intervention threshold. Perhaps that in the future, BTM's and new parameters describing bone architecture and quality, like TBS or HRpQCT, will allow to predict fractures with better positive and negative predictive value.

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X-Ray Based Imaging Methods to Assess Bone Quality

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Nomenclature

BMC	Bone mineral content. BMD = BMC/ volume
BMD	Bone mineral density measured in g/cm^3. BMD is measured by QCT and pQCT
aBMD	Areal bone mineral density measured in g/cm^2. aBMD is measured by DXA
CT	Computed tomography
DXA	Dual X-Ray Absorptiometry
FDA	US Food and Drug Administration
FEA	Finite element analysis
HR-pQCT	High resolution peripheral quantitative computed tomography
HSA	Hip structure (or strength) analysis; special analysis applied to DXA images to estimate bone strength
MrOS	A large epidemiological study funded by the National Institutes of Health. MrOs was initially designed to describe the epidemiology of osteoporosis and fractures in older men, including the identification of risk factors for fracture and bone loss. MrOS has described diagnostic methods for fracture assessment including identification of vertebral fracture
pQCT	Peripheral quantitative computed tomography
QCT	Quantitative computed tomography.
SOF	Study of osteoporotic fracture—a large epidemiological study in women. SOF, a nationwide research study funded by the National Institutes of Health, began in 1986. The study was originally focused on risk factors for fractures and falls and has grown to look at various determinants of successful aging
WHO	World Health Organization
TBS	Trabecular bone score

Glossary

3D-DXA Technique to estimate CT like images from one or multiple DXA images using atlas based procedures.

Attenuation The decrease in the intensity of X-rays when passing through matter; the extent of attenuation is a property of the material which is exposed to radiation and is quantitatively described by the linear attenuation coefficient.

CT X-ray based technique to produce cross-sectional images of a human subject, for example. Standard method in diagnostic radiology.

CT number The final result of the CT measurement; the CT number is calculated from the linear attenuation coefficient μ.

CT value Synonymous with CT number.

DEXA Dual energy X-ray absorptiometry (see also DXA).

DPA Dual photon absorptiometry using radioisotopic sources.

DXA Dual energy X-ray absorptiometry (manufacturer specific acronyms: DER, DEPR, QDR, DPX). Radiological technique for measuring areal bone density. Two different X-ray energy spectra are used.

FEA Finite element analysis (also termed FEM: finite element modeling); special analysis applied to CT images to estimate bone strength.

Helical CT Synonymous with spiral CT.

Hounsfield unit (HU) Unit of the CT number scale; the Hounsfield unit expresses the relative deviation of the measured linear attenuation coefficient from that of pure water, multiplied by 1000.

LSC Least significant change; the change between two measurements that can be measured with 95% confidence.

Motion artifact Artifact caused by movements of the object during data acquisition; in QCT the inconsistencies of the measured projections not only result in unsharpness, as in absorptiometry, but can also cause long-range artifacts in the CT image.

Pixel Abbreviation of "picture element."

QCT Quantitative computed tomography: CT with added calibration to quantify bone mineral density.

Partial volume artifact Artifact caused by severe inhomogeneities of the materials within the beam on the corresponding attenuation measurement (e.g., bone and air). The averaging of the incident intensity within the detector element is not equivalent to an averaging of the attenuation coefficient itself; this is a source of nonlinear errors and thereby inconsistencies for attenuation measurements along different directions.

ROI Region of interest; subset of pixels which lie within an arbitrary (circular, rectangular etc.) geometrical shape at a freely selectable position within a 2D image.

Spiral CT Method of CT scanning with continuous gantry rotation and simultaneous continuous object translation in z-direction; in contrast to the sequential scan technique, with spiral CT the volume to be examined is sampled continuously along the z-axis; during data acquisition the focus of the X-ray tube follows a spiral trajectory relative to the object; the z-interpolation is introduced as an additional step during data preprocessing and allows a retrospective and arbitrary selection of the positions at which images are reconstructed.

TBS Trabecular bone score; special analysis applied to DXA images to measure bone texture.

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VOI Volume of interest; subset of voxels which lie within an arbitrary (cubical, spherical etc.) geometrical shape at a freely selectable position within a 3D image cube.

X-ray absorption Basic physical ability of a material to absorb x-rays and to transform their energy into other forms of energy, such as visible light, heat or fluorescence; in diagnostic imaging this process is dominated by Compton scatter and photoelectric absorption.

X-ray attenuation The physical law, which quantitatively describes the attenuation of the incident X-ray intensity I_0 when passing through a homogeneous object of thickness d and total linear attenuation coefficient μ.; μ depends on the energy of the X-ray quanta; the intensity of the radiation I measured behind the object can be calculated from $I = I_0 e^{-\mu d}$ (Lambert–Beer law); it should be mentioned here that this formula only holds for ideal conditions, that is, in the absence of X-ray scatter and for monochromatic radiation, that is, when all incident quanta have the same energy level.

Introduction

Dual X-ray absorptiometry (DXA) is one of the best-researched quantitative imaging modalities in medicine. Its physical basis, technical features and limitations have been addressed in hundreds of papers and summarized in a report on bone densitometry of the International Commission on Radiation Units (ICRU) that also covers quantitative computed tomography (QCT) and describes performance measures used in densitometry (Kalender *et al.*, 2009). The primary output of DXA is areal bone mineral density (aBMD). Its clinical relevance for the diagnosis of osteoporosis, fracture prediction, and monitoring of treatment and age related changes has been investigated in multiple very large epidemiological and pharmaceutical studies of more than 2,00,000 subjects worldwide. aBMD is the basis of the WHO working definition of osteoporosis (Kanis and WHO Study Group, 1994). It is a strong predictor of fracture risk, but even in combination with other risk factors it cannot predict whether an individual subject eventually will fracture or not. Today, about 50–100 patients with osteoporosis must be treated to prevent one fracture (Albert and Reddy, 2017); vice versa, about 50% subjects who fracture do not have osteoporosis (Schuit *et al.*, 2004; Siris *et al.*, 2004).

Nevertheless, DXA remains the workhorse in clinical practice and aBMD serves as a powerful surrogate of bone quality. In patients with idiopathic and most types of secondary osteoporosis, tissue mineral density is normal, that is, bone loss is primarily caused by a loss/thinning of trabeculae and an increasing porosity/thinning of the cortex. Apparently, aBMD is such a strong surrogate of bone strength because the remaining bone is optimally distributed to support maximum strength for the given amount of bone.

In contrast to BMD, bone quality is not a well-defined physical parameter. Bone quality is often used as a concept to address deficiencies of BMD to fully explain bone strength and fracture risk (Donnelly *et al.*, 2014). A number of advanced imaging techniques have been developed to quantitatively asses aspects of bone quality. This article will introduce these techniques and will discuss their clinical value or to be more precise, their added clinical value to DXA, which is a well-standardized, widely available and affordable technique.

Concepts of Bone Quality

It is hypothesized that the determination of bone strength and fracture resistance requires a comprehensive assessment of "… bone quantity and bone quality, defined broadly as all geometric, micro architectural, and material factors (e.g., trabecular architecture, collagen crosslinking, mineralization, microcracks)…" (Donnelly *et al.*, 2014). These factors are controlled and regulated by a large number of cellular activities and by external forces that impact on bone (Turner, 2006). The concept of bone quality has been criticized as too vague (Sievanen *et al.*, 2007) but over the years a number of physical parameters that describe material and structural bone properties as well as bone geometry have been identified as determinants of ultimate force (or load) to fracture a bone and of energy to failure (**Fig. 1**). Material and structural properties can be experimentally determined from biomechanical testing of bone samples or whole bone specimen (Jepsen *et al.*, 2015; Turner and Burr, 1993).

Material properties that characterize bone tissue can be determined from the stress–strain curve obtained from mechanical testing of cortical or trabecular bone samples. Material properties subsume microscopic parameters such as collagen crosslinking, mineralization, microcracks, trabecular structure, and cortical porosity. Specific parameters are the elastic or Young's modulus E and the ultimate stress σ^{ult}, required for material failure. Toughness, the shaded area under the curve is the energy of mechanical deformation per unit volume prior to fracture. The Young's modulus is the slope of the linear portion of the stress–strain curve. For fatigue strength, multiple loading cycles are required.

Structural properties are determined from the force-displacement curve obtained from mechanical testing of whole bones. Whole bone structural properties depend on the material properties of the trabecular and cortical bone and on the specific bone geometry. As shown in **Fig. 1**, stiffness is the slope of the linear section of the force-displacement curve. It characterizes how much the entire bone deforms under load. F^{ult} is the ultimate force (sometimes also called ultimate load) that a bone can withstand before it breaks. Work or energy to failure, the area under the force displacement curve, is a measure of a structure's overall resistance to failure.

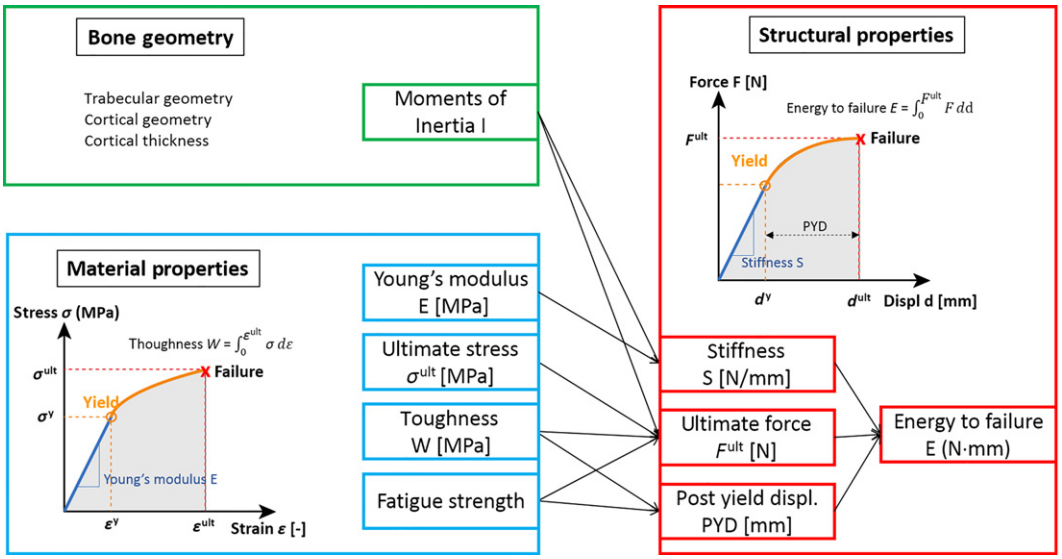

Fig. 1 Concept of bone quality.

An important contributor to energy to failure is post yield displacement PYD $= d^{ult} - d^{y}$. When loaded beyond the yield point, the deformation is no longer reversible, that is, elastic but plastic. The mechanical behavior of bone is altered and stays permanently deformed after unloading. If PYD is low the structure is called brittle, if it is high the structure is called ductile. A ductile bone can better deform plastically without fracturing than a brittle bone (Krichner, 2006; Peterlik *et al.*, 2006).

Whole-bone structural parameters depend on bone size, whereas tissue-level material parameters are size-independent. Typically, mechanical parameters are expressed in terms of load (N) and displacement (mm), whereas tissue-level material parameters are expressed in terms of stress (N/mm^2) and strain (mm/mm or %), that is, they are normalized to area. Whole bone strength is often used synonymously with ultimate load.

It is interesting that apparently, stress and force instead of strain and displacement are the independent parameters. However, an external force creates a strain/displacement that causes an internal reactive stress/force. Thus, stress and force are indeed the resulting parameters. In tension tests, typically, the displacement is controlled and stress is measured as dependent variable. Of course, in equilibrium the external force/force per area is equal to the internal force/stress.

In Vivo Imaging Techniques to Assess Bone Quality

The most widely applied quantitative imaging technique in osteoporosis is dual X-ray absorptiometry (DXA) to measure aBMD in g/cm^2. aBMD is a macroscopic surrogate of bone quality but it does not capture the underlying mechanisms that result in low or high BMD. Thus, a number of advanced imaging techniques have been developed to quantify bone quality beyond BMD (**Table 1**).

QCT

Computed tomography (CT) is an X-ray based technique resulting in an image of the total linear X-ray absorption coefficient μ. For clinical CT applications, the values of μ are calibrated to the X-ray attenuation of water (w) resulting in a CT number measured in Hounsfield units (HU):

$$\text{CT number} = \frac{\mu - \mu_w}{\mu_w} \times 1000[\text{HU}]$$

μ_w is the attenuation coefficient of distilled water at room temperature. In a calibrated scanner, the CT numbers of water and air are 0 and -1000, respectively. For quantitative computed tomography (QCT), an additional calibration is required to obtain BMD from the HU values (Kalender *et al.*, 2009). In contrast to aBMD from DXA, QCT measures a physical density and BMD values are given in g/cm^3. Typically, the subject is scanned on top of a calibration phantom with inserts of known BMD values (**Fig. 2**). There is a linear relation between measured CT numbers and known BMD values of the inserts. The resulting calibration equation is then applied to all bone voxels in the image for which BMD should be measured. This procedure is called simultaneous calibration. Recently a number of alternate approaches, for example, measuring a calibration phantom separately from the subject (Brown *et al.*, 2016) or using the absorption of internal organs and air (internal calibration) (Lee *et al.*, 2017) have been suggested. Thus, BMD measurements could be obtained from CT scans without calibration phantom but further validation of these techniques is required (Engelke *et al.*, 2015).

Table 1 Overview of advanced in vivo imaging techniques to assess bone strength and quality

Technique	*Advantages*	*Limitations*
DXA	● Standard method to quantify aBMD in the spine, hip, and forearm ● Used in almost all epidemiological and treatment studies during the last 20 years ● Low aBMD predicts vertebral and proximal femur fractures	● Projectional 2D technique ● No separation of trabecular and cortical bone ● aBMD depends on bone size ● Under treatment the increase in aBMD does not reflect the observed rate of fracture reduction
HSA	● Can be easily applied to DXA images ● Estimates bone strength parameters	● Requires many assumptions on hip geometry and BMD distribution ● No evidence that HSA parameters add value to standard DXA parameters with respect to monitoring BMD changes and for fracture risk assessment
TBS	● Can be easily applied to DXA images ● Has shown fracture prediction independent of aBMD	● Not fully clear what bone property is really quantified ● Not recommended to monitor BMD changes under bisphosphonate treatment
QCT	● Fast 3D imaging method ● Trabecular and cortical bone can be separated ● Cortical thickness, geometrical, and bone strength related parameters can be quantified ● QCT of the spine better predicts vertebral fractures than DXA ● Provides a more comprehensive insight into bone pathophysiology than DXA ● More sensitive than DXA to monitor changes of trabecular BMD	● Less standardized than DXA ● Analysis software not integrated in CT scanner and most of the software is not commercially available yet ● Spatial resolution not adequate to quantify trabecular architecture ● Generates a wealth of parameters but it is not fully clear yet, which ones should be used for which purpose
CTXA	● Estimation of DXA images of the hip from QCT scans ● T-scores of CTXA and DXA of the hip are equivalent –> CTXA can be used for diagnosis of osteoporosis	● See DXA
pQCT	● Same as for QCT with the exception of speed	● The forearm and tibia are less sensitive to pharmacological treatment ● Only one or two single slices are measured
HR-pQCT	● Trabecular architecture and cortical porosity can be quantified	● Scans length limited (1 cm in standard mode), long scans times, frequent motion artifacts
QCT-based FEA	● Integrates BMD and geometry to estimate bone strength ● FEA of the spine may better predict vertebral fractures than DXA	● Results depends on loading conditions assumed during the simulations ● Results depend on material properties assigned to the individual elements ● Expert analysis required
3D-DXA	● Estimation of CT-like 3D dataset from DXA scan including FEA	● Clinical validation pending

QCT was first developed to measure BMD of the spine (Genant *et al.*, 1982; Kalender *et al.*, 1987). After introduction of spiral CT (Kalender *et al.*, 1990), which allowed for rapid 3D scanning, QCT of the hip was introduced (Lang *et al.*, 1997, 2002). The analysis of QCT images starts either with a slice by slice or with a more advanced 3D segmentation process of the bone to be analyzed (Kang *et al.*, 2003). Then dedicated volumes of interest (VOIs) are defined. In contrast to DXA, QCT is not a projectional technique. Thus, cortical and trabecular bone compartments can be analyzed separately. Recommended VOIs for the spine are L1 + L2 (Engelke *et al.*, 2008) and for the hip, femoral neck, trochanter, intertrochanter, and of lesser importance the femoral head and shaft. For each VOI, integral, cortical, trabecular and sometimes, subcortical BMD, BMC and volume can be determined (**Fig. 3**). Cortical thickness and other geometrical parameters related to bone strength such as buckling ratio, section moduli, axial, and polar moments of inertia in the neck or cross-sectional area in the spine have also been analyzed (Borggrefe *et al.*, 2016; Khoo *et al.*, 2012; Museyko *et al.*, 2016). However, material or structural properties (**Fig. 1**) cannot directly be assessed by QCT.

For QCT of the spine and hip, clinical whole body CT scanners as manufactured by most large medical device companies are used. These are general purpose devices for radiological diagnosis. They provide a spatial resolution of about 0.5 mm. Consequently, in the reconstructed CT image thin structures such as cortical bone of the vertebral body ($\lesssim$0.5 mm) and the femoral neck ($\lesssim$1 mm) are blurred, a consequence of so-called partial volume artifacts. Thin structures appear to be thicker and have lower contrast than in reality. This results in accuracy errors of cortical BMD and thickness measurements, which increase with decreasing cortical thickness. Several cortical segmentation techniques have been proposed (Prevrhal *et al.*, 1999; Treece and Gee, 2015; Museyko *et al.*, 2017) and it is important to understand their respective impact on cortical measurements (Engelke, 2017). In order to reduce the impact of segmentation, the use of cortical BMC has been suggested (Newman *et al.*, 1998; Dougherty and Newman, 1999), which to a certain degree counterbalances the inaccurate decrease in cortical BMD and increase in cortical thickness.

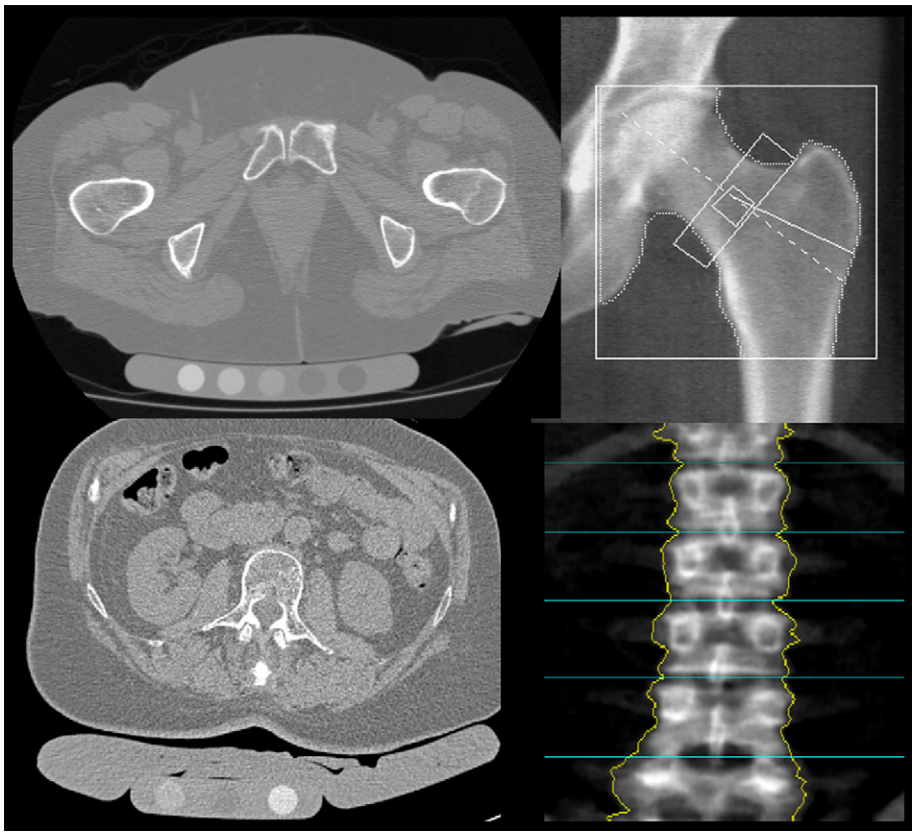

Fig. 2 QCT (left) and DXA (right) images of the hip (top) and spine (bottom). For QCT analysis the patient is scanned on top of a calibration phantom (hip scan on top of Mindways calibration phantom; Mindways Software, Inc., Austin, TX; spine scan on top of QRM Bone density calibration phantom (BDC); QRM Möhrendorf, Germany).

Peripheral and High-Resolution Peripheral QCT

Clinical whole body CT scanners can also be used for QCT of peripheral sites such as arms or legs (Engelke *et al.*, 2009a) but this is the domain of dedicated peripheral QCT (pQCT) scanners. They are widely used in pediatric research (Adams *et al.*, 2014), where CT scans of the spine or hip are avoided because of radiation exposure. Dedicated high-resolution pQCT (HR-pQCT) scanners with a spatial resolution of 120 μm (Burghardt *et al.*, 2013) have been developed to image trabecular bone structure at peripheral sites such as the distal radius and tibia (Laib *et al.*, 1998) (**Fig. 4**).

Cortical and trabecular regions of HR-pQCT scans can be segmented using semiautomatic (Laib *et al.*, 1998) or automatic methods (Nishiyama *et al.*, 2010; Buie *et al.*, 2007; Burghardt *et al.*, 2010a). In addition, a histomorphometric analysis can be used to assess the microstructure of the trabecular network. Average trabecular thickness (Tb.Th) and separation (Tb.Sp) are derived from bone volume fraction (BV/TV) and average number of trabeculae (Tb.N) (Laib *et al.*, 1998). BV/TV is determined from BMD of the trabecular compartment by assuming a density of 1200 mg/cm^3 for fully mineralized bone. Tb.N is directly measured using ridge extraction methods (Hildebrand and Rüegsegger, 1997). A newer HR-pQCT scanner model with improved spatial resolution allows for direct quantification of trabecular bone indices (Manske *et al.*, 2015) that do not depend on trabecular BMD.

Due to the high spatial resolution, larger cortical pores can be detected. Cortical porosity (Ct.Po) is calculated as percentage of void voxels in the cortex (Nishiyama *et al.*, 2010) after thresholding the image. Another method (Zebaze *et al.*, 2013) uses cortical density to estimate pores by assuming that BMD of fully mineralized bone is 1200 mg/cm^3. Both techniques showed strong correlations with synchrotron CT (Jorgenson *et al.*, 2015), but absolute values of porosity differ significantly.

Finite Element Analysis

FEA is widely used in mechanical engineering to calculate the strength and other properties of complex structures under the action of external forces. A given structure is divided into a large number of small finite elements of a simple geometry, for which deformations, stresses or even failure can be calculated easily if material properties are known for each element. In the musculoskeletal field, FEA is typically applied to CT datasets.

For the analysis of bone strength, two types of FEA are used (Pahr and Zysset, 2009). If the spatial resolution of the CT images is high enough, each trabecula can be segmented and divided into small finite elements. This approach is called μFE. Typical

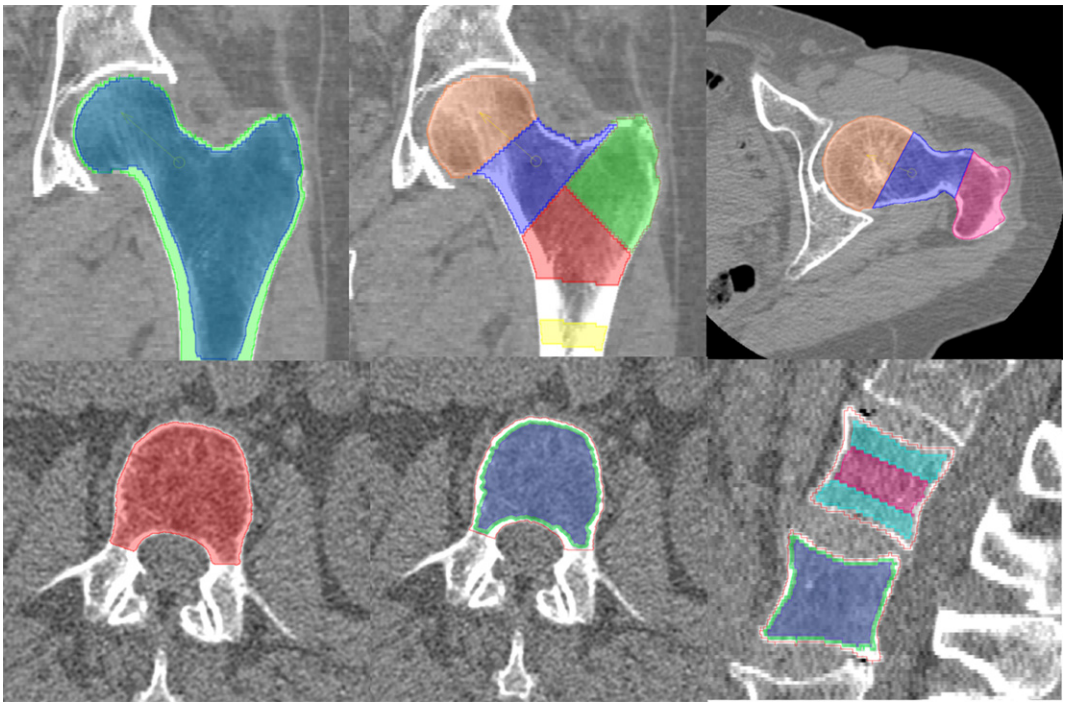

Fig. 3 Advanced QCT analysis of the hip (top) and spine (bottom). Top left: coronal MPR showing cortical and trabecular bone compartments; Top center: integral bone compartments of head (*brown*), neck (*blue*), trochanter (*green*), intertrochanter (*red*), and shaft (*yellow*) VOIs in coronal and top right in axial MPR. Bottom left and center: integral (*red*), subcortical (*green*), and trabecular (*blue*) bone compartments in axial view, integral bone is also shown as red contour; bottom right: sagittal MPR; In the upper vertebra the trabecular compartment is further subdivided in superior, mid and inferior compartments. 3D segmentation results are shown as 2D contours or overlays (Medical Image Analysis Framework (MIAF), University of Erlangen, Germany).

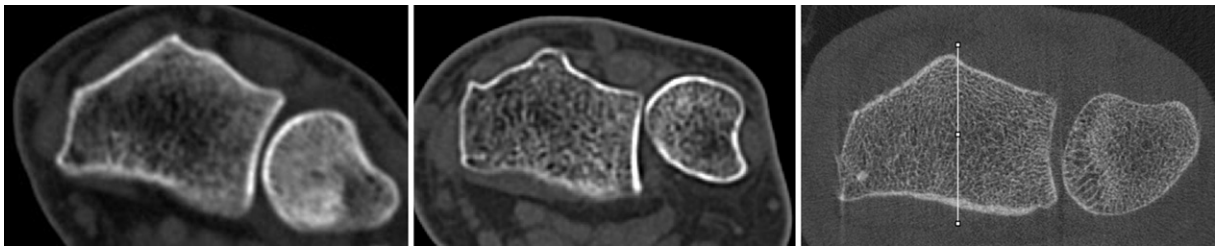

Fig. 4 Comparison of spatial resolution of distal forearm. Left and center: CT of the forearm using whole body CT scanner, left: standard body reconstruction kernel; center: bone reconstruction kernel; right: HR-pQCT scan (images from different patients).

applications are the analysis of trabecular bone samples imaged with μCT or the analysis of trabecular architecture in vivo using HR-pQCT scans of the distal radius or tibia. In CT scans of the spine and hip, the spatial resolution is much lower and a segmentation of individual trabeculae is not possible. Instead, a mesh is applied to the entire vertebral body or proximal femur resulting in individual elements with a size in the millimeter range (Eswaran *et al.*, 2009). Material properties are then assigned to each finite element based on its calibrated BMD value. These are the so-called homogenized FE models. The term homogenization denotes the averaging process used in determining the apparent material properties of the bone—marrow mixture (**Fig. 5**). The material properties are essential input parameters for FE analysis (Bayraktar *et al.*, 2004; Zysset, 2003). Another input is the so-called loading scenario describing the direction, magnitude, and insertion point(s) of the external force(s) (Bessho *et al.*, 2009; Buckley *et al.*, 2009). The FEA outcome also depends on the specific algorithm used for FEA simulation (Keyak and Rossi, 2000) and on criteria when the bone actually fails.

Vertebral bodies are typically loaded in compression. For the femur, usually a fall to the side is simulated (Keaveny *et al.*, 2014, 2018; Kopperdahl *et al.*, 2014). The outcome of FEA is a simulated load-displacement curve. The linear part of the curve, that is, stiffness, can be obtained with linear FEA. The simulation of the yielding of the curve, the maximal force and the energy to failure requires nonlinear FEA (Keyak, 2001; Nawathe *et al.*, 2015). For in vivo measurements, estimated failure load, reported in kN, is the most widely used parameter and is often reported as bone strength not to be confused with ultimate stress, which is a material variable.

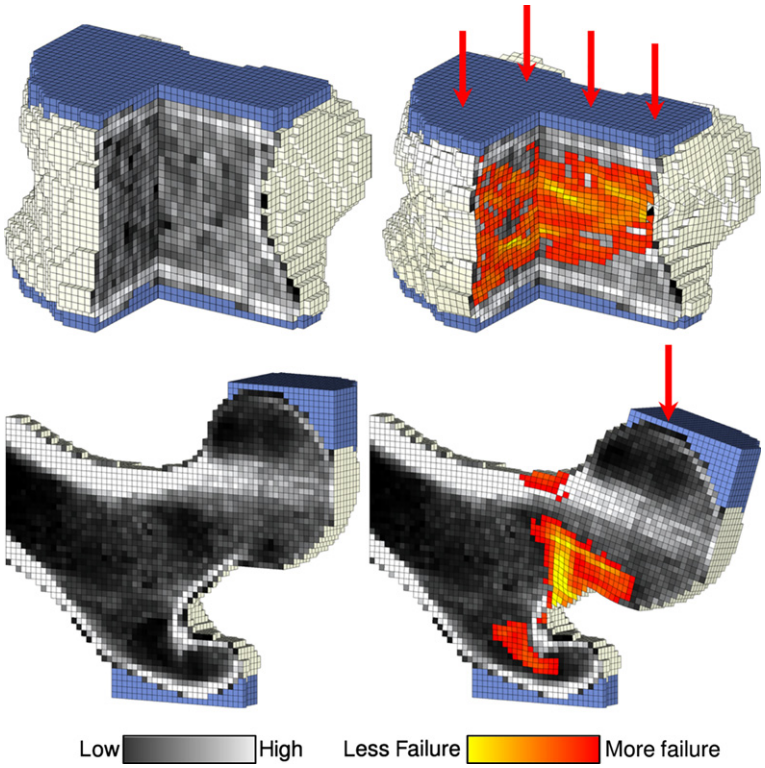

Fig. 5 Left: Cut-out views of finite element models depicting the distribution of volumetric BMD and derived from CT attenuation data from an L1 vertebral body and left proximal femur. Right: Nonlinear stress analysis results for compressive loading of the spine and a sideways fall of the hip with colors depicting bone tissue failure. Deformations are magnified. Courtesy: David Lee, O.N. Diagnostics, LLC, Berkeley, CA.

Advanced DXA Methods

Advanced DXA methods can be divided into two categories: 2D-based measurements such as hip strength analysis (HSA) or trabecular bone score (TBS) that are applied directly to the DXA image. In contrast, 3D measurements are derived from CT-like images estimated from one or multiple DXA images using atlas-based procedures.

The calculation of geometrical parameters related to bone strength such as cross-sectional area (CSA), cross-sectional moments of inertia (CSMI), section moduli or buckling ratio are based on the analysis of the BMC distribution along one-dimensional profiles in the DXA image. This technique was already applied to single-photon absorptiometry of the forearm (Martin and Burr, 1984). Hip Structural and a variant called hip strength analysis, both abbreviated as HSA, denote extensions to DXA of the hip (Beck *et al.*, 1990; Yoshikawa *et al.*, 1994). In simple objects such as homogeneous beams or pipes, the geometrical parameters determined by HSA are indices of resistance to bending (CSMI, section modulus, buckling ratio) and compression (CSA) (Beck, 2003). HSA for DXA of the hip is based on assumptions that the bone cross-section is circular at the femoral neck and shaft, and elliptical at the intertrochanteric region. It is further assumed that tissue mineral density is constant and that there is a constant proportion of cortical and trabecular bone in the cross-section (Beck, 2003; Uusi-Rasi *et al.*, 2006). Further, the one-dimensional profiles required for the analysis should be perpendicular the neutral axis of the structure. In a tube, which may be used to approximate a radius, this is the central axis. For the proximal femur, the neutral axis was determined using a curved beam model (Mourtada *et al.*, 1996). Despite all the limitations and assumptions, correlations between HSA parameters determined from DXA and the corresponding parameters directly obtained from QCT scans are high ($r = 0.89–0.95$) (Ramamurthi *et al.*, 2012).

Based on the curved beam model, 2D DXA images of the hip have been used to derive internal stresses directly (Mourtada *et al.*, 1996). A further refinement called composite curved beam analysis included the BMC distribution (Yang *et al.*, 2009). A stress distribution of the hip has also been calculated by 2D-FEA applied to DXA scans (Testi *et al.*, 1999). In a recent study, correlations of maximum load between DXA based 2D-FEA and QCT based 3D-FEA were $r^2 = 0.76$ (Dall'Ara *et al.*, 2016).

A further step toward a volumetric assessment of BMD and 3D geometry is 3D-DXA initially termed volumetric DXA (VXA) (Ahmad *et al.*, 2010). Statistical atlases generated from site-matched CT scans are used to determine shape and BMD variations across a population. An atlas is characterized by so-called Eigenvectors. About 10–20 eigenvectors suffice to describe the shape of an individual femur of this population with about 95% accuracy (Whitmarsh *et al.*, 2011). Originally, several (<5) DXA projections were taken from the proximal femur using different angles by rotating the C-arm of the DXA scanner. Newer techniques just use the standard DXA image (Whitmarsh *et al.*, 2011; Grassi *et al.*, 2017; Humbert *et al.*, 2012; Humbert *et al.*, 2017). The projection(s) are fitted to the statistical 3D atlas. The result is an estimation of the corresponding CT 3D dataset, which can be

segmented and analyzed using algorithms applied in QCT. For example, 3D-FEA results can be estimated from such a dataset. The opposite of 3D-DXA is CTXA, which generates 2D projectional images from a CT acquisition (Cann *et al.*, 2014). The purpose is to obtain DXA equivalent data that can be used for the diagnosis of osteoporosis according to the WHO definition.

Another potential application of 3D-DXA is the separation of cortical and trabecular bone compartments. In a validation study (Humbert *et al.*, 2012) of 157 subjects correlations between QCT and 3D-DXA were 0.95 for total hip cortical density and 0.91 for total hip cortical thickness with mean absolute differences of 72 mg/cm^3 or 7.6% and 0.33 mm or 18.3% for density and thickness, respectively. Results of three subjects are shown in **Fig. 6**. Graphs of correlations or Bland–Altman plots were not presented. Compared to QCT, 3D-DXA images are blurred, which is understandable as shape and density information is described by a limited number of Eigenvectors that is extremely small in comparison to about 3–4 million voxels of a femur in a CT dataset.

A different approach not related to strength is TBS (Harvey *et al.*, 2015), which performs a texture analysis of the BMD distribution of a DXA image of the spine. First attempts to use texture parameters to quantify trabecular architecture were developed using high resolution X-ray films of the calcaneus, where cortical bone is rather thin (Benhamou *et al.*, 1994, 2001). Obviously, the spatial resolution of DXA images is much lower than in X-rays. In addition, a DXA image not only includes the trabecular network but also mostly cortical structures such as the pedicles and spinal process. Therefore, the claimed association of TBS with trabecular structure has been criticized (Maquer *et al.*, 2016). However, TBS adds information to aBMD.

Clinical Relevance of Assessing Bone Quality

Several recent reviews have addressed the performance of imaging techniques assessing bone quality (Hunt and Donnelly, 2016; Link and Heilmeier, 2016; Griffith *et al.*, 2010; Bouxsein and Seeman, 2009). The International Society of Clinical Densitometry (ISCD) has instituted an evidence based consensus process to develop positions on how to use these techniques clinically (Engelke *et al.*, 2008, 2015; Zysset *et al.*, 2015; Broy *et al.*, 2015; Silva *et al.*, 2015). The official positions for DXA, QCT, FEA, HSA, and TBS are summarized in **Table 2**. Empty fields indicate that ISCD positions are not available for this technique. So far, no positions have been developed for DXA-based FE methods or HR-pQCT, although a number of publications have investigated performance characteristics of these techniques (Kawalilak *et al.*, 2016; Zhou *et al.*, 2016; van Rietbergen and Ito, 2015). By definition, techniques other than DXA cannot be used for the diagnosis of osteoporosis because the WHO definition is based on DXA

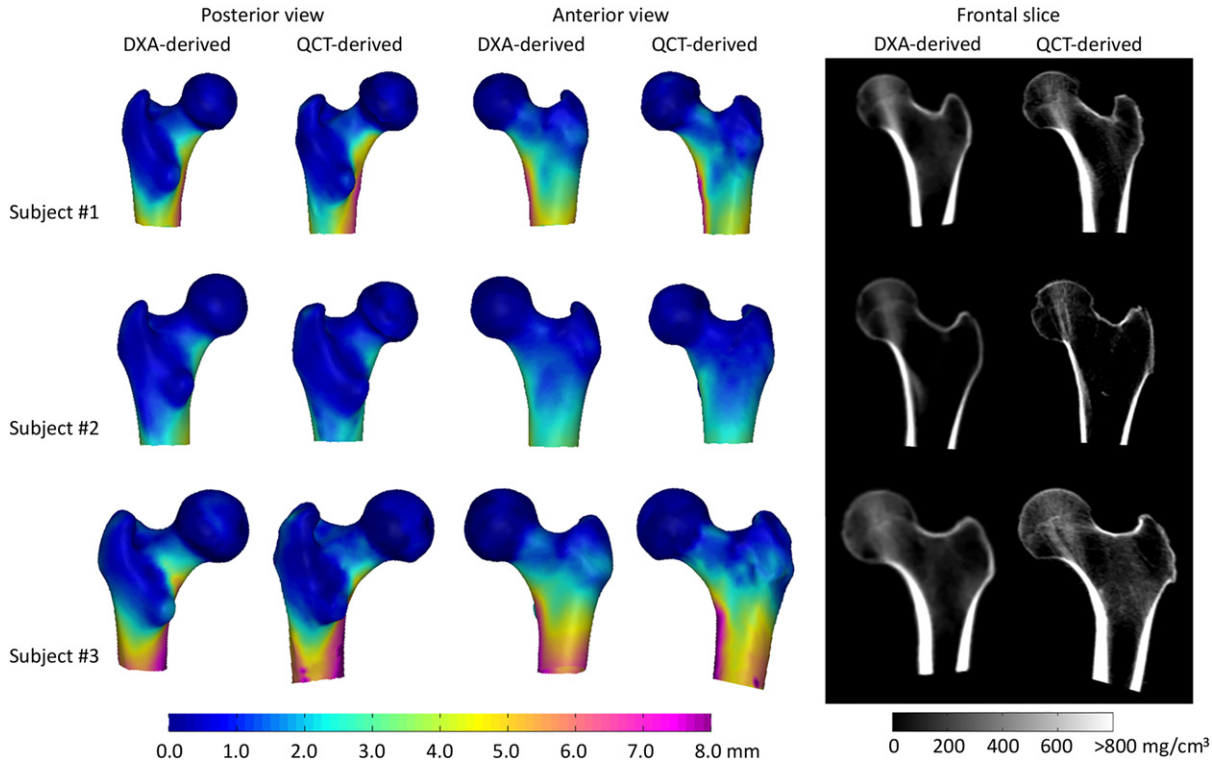

Fig. 6 Comparison between DXA- and QCT-derived femoral shapes, cortical thickness distributions (left), and frontal slices showing bone density values (right). Courtesy: Ludovic Humbert, Galgo medical, Barcelona, Spain; IEEE copyright line © 2017 IEEE; reproduced from Humbert, L., Martelli, Y., Fonolla, R., Steghofer, M., Di Gregorio, S., Malouf, J., Romera, J. and Barquero, L. M. (2017). 3D-DXA: Assessing the femoral shape, the trabecular macrostructure and the cortex in 3D from DXA images. *IEEE Transactions on Medical Imaging* **36**(1), 27–39.

Table 2 Official positions for women of the International Society of Clinical Densitometry (ISCD) on clinical utility of advanced imaging methods

	Location	*Diagnosis of osteoporosis*	*Site matched fracture prediction*	*Monitoring age related changes*	*Monitoring treatment effects*	*Initialization of treatment*
DXA based parameters						
Hip axis length	Hip	n	0[a]	n	n	n
HSA parameters		n	n	n	n	n
TBS	Spine	0[b]	0[b,c]	n[d]		0[c]
CT based parameters						
int BMD	Spine	n		0[f]	0[f]	y
	Hip	n		y	y	
trab BMD	Spine	n	y	y	y	y
	Hip	n	y	y	y	
CTXA	Hip	y				
int BMD	pQCT UD	n	y[e]	y		y
trab BMD	radius	n	y[e]	y		y
FEA strength	Spine	n	y	y	y	y
	Hip	n	y	y	y	y

[a]Association with hip fracture.
[b]Association with hip and spine fracture.
[c]Only in combination with other parameters.
[d]In patients under bisphosphonate treatment.
[e]Predicts hip fracture, no position for radius fracture.
[f]There was insufficient evidence at the time (2007) when ISCD positions for QCT of the spine were issued, however, there is now plenty of evidence that integral BMD of the spine can be used as for monitoring.
trab, trabecular; *int*, integral. For a complete list of positions, see www.ISCD.org. *y*, technique can be used; *n*, technique should not be used; field empty, no recommendation available.
Note: In men, there are fewer studies and several of the positions that are supported for women are not supported for men because of missing evidence.

T-scores. T-scores can be calculated for all techniques listed in **Table 1** but these are not equivalent to DXA T-scores (Engelke *et al.*, 2008). The only exception is CTXA of the hip for which T-scores can be directly calibrated to DXA hip T-scores (Khoo *et al.*, 2009).

CT Based Techniques

It is important to compare imaging techniques with DXA because aBMD is a strong surrogate of bone quality and it is unlikely that new techniques will replace DXA unless they show superior performance or add information. This is in particular important for CT based applications, because CT adds complexity, cost and radiation exposure. For typical QCT acquisition protocols, radiation exposure is about 1.5 and 2.5 mSv in the spine and hip, respectively (Engelke *et al.*, 2008). This is comparable to the natural background radiation of about 2.5 mSv but higher than for DXA (<0.01 mSv).

In vitro, aBMD correlates highly ($r = 0.6$–0.9) with bone strength of the femur (Keyak *et al.*, 1998; Cody *et al.*, 1999; Bousson *et al.*, 2006) and spine (Ebbesen *et al.*, 1999; Dall'Ara *et al.*, 2012; Mosekilde *et al.*, 1989; Hayes *et al.*, 1991). In studies, in which correlations with or predictions of bone strength have been assessed with multiple imaging modalities, typically QCT was a slightly better predictor than DXA (Bousson *et al.*, 2006; Lochmuller *et al.*, 2002) and FEA was superior to the other two (Cody *et al.*, 1999; Dall'Ara *et al.*, 2012; Danielson *et al.*, 2012; Johannesdottir *et al.*, 2017).

However, in vivo, FEA, and QCT improve hip fracture prediction or discrimination only marginally compared to DXA (Bousson *et al.*, 2011; Cheng *et al.*, 2007; Orwoll *et al.*, 2009; Johannesdottir *et al.*, 2011). Technical improvements are possible but probably non-bone related factors such as the risk of falling are important and independent contributors to assess hip fracture risk. FE models should not only focus on accurate prediction of bone strength, but also consider applied loads (Falcinelli *et al.*, 2014; Qasim *et al.*, 2016). As described above, the primary input to homogenized FE models is BMD, but anisotropy of trabecular bone is neglected. Integrating fabric, a parameter to assess anisotropy, into the FE model (Maquer *et al.*, 2015; Chevalier *et al.*, 2009) may also improve fracture risk prediction. For QCT, cortical thickness of the neck adds independent information to BMD for fracture prediction (Museyko *et al.*, 2016; Treece and Gee, 2015; Poole *et al.*, 2011). AUC values have been further increased by adding parameters describing the amount of adipose tissue and the distribution of fat within muscle. (Mühlberg *et al.*, 2018; Lang *et al.*, 2008, 2010).

In the spine, vertebral fracture risk assessment by QCT and FEA seem to be superior to DXA. Wang showed significantly higher hazard ratios and AUC values for ultimate load and integral BMD (AUC both 0.83) of the total vertebral body than for aBMD (0.76) (Wang *et al.*, 2012). Similar results in the spine were reported for Japanese women (Imai *et al.*, 2009). Evidence is still poor, based only on one longitudinal prospective and some cross-sectional studies but the findings are not surprising, as aBMD cannot

be measured separately for the vertebral body. aBMD includes cortical contributions from the spinal process and the vertebral arch and is falsely increased by degenerative changes and aortic calcifications.

With QCT but not DXA, differential BMD, and BMC effects in cortical, subcortical and trabecular BMD can be determined. Together with measurements of cortical thickness and of bone volume to potentially identify periosteal apposition, a detailed understanding of treatment effects has been achieved. In future, these differential effects combined with measurements of strength and trabecular architecture may be important for the characterization of different phenotypes of osteoporosis in order to develop individual treatment (Jepsen *et al.*, 2015; Litwic *et al.*, 2017; Vranken *et al.*, 2017; Edwards *et al.*, 2016).

Precision errors of QCT using advanced segmentation are comparable to DXA (Lang *et al.*, 1997; Museyko *et al.*, 2016; Engelke *et al.*, 2009b). However, QCT is more sensitive to monitor trabecular BMD changes than DXA because age or treatment related changes of trabecular BMD are higher than for integral BMD. Treatment related increases in bone strength in osteoporotic patients are usually higher than integral BMD increases (Zysset *et al.*, 2015) but precision errors for strength have not be published for strength so far.

QCT and FEA techniques are highly relevant for opportunistic screening, the use of existing clinical CT images for the diagnosis of osteoporosis and fracture prediction (Engelke, 2017). If widely implemented, opportunistic screening could significantly improve the identification of patients at high risk for fracture. A secondary analysis of CT data obtained for tumor and other routine clinical diagnoses could be used to categorize fracture risk as low, medium or high. It has been suggested that subjects with high fracture risk could be followed up with DXA or could be directly referred to counseling or appropriate intervention even without an additional DXA scan. Both scenarios are convenient and cost effective because DXA scans and associated logistics are not required. An additional benefit of the dual use of CT scans is the possibility to use a lateral scout view or a lateral projection of the spinal column to assess fractures.

The relevance of trabecular architecture for fracture prediction is not fully clear yet (Boutroy *et al.*, 2005; Folkesson *et al.*, 2011; Burghardt *et al.*, 2010b; Khosla *et al.*, 2006). In two recent studies, quantification of trabecular structure contributed moderately to fracture risk independent of DXA aBMD of the forearm (Sornay-Rendu *et al.*, 2007; Vilayphiou *et al.*, 2010) but results were not adjusted for integral, trabecular or cortical forearm BMD, which are also available from an HR-pQCT scan. Differences in trabecular architecture may be more relevant for phenotyping the association of bone with fracture (Litwic *et al.*, 2017; Edwards *et al.*, 2016). Trabecular architecture has also been estimated for the vertebral body using optimized acquisition and reconstruction protocols on clinical whole body CT scanners (Krebs *et al.*, 2009; Graeff *et al.*, 2007, 2015) but the technique is still in an experimental status.

Cortical BMD and thickness of the forearm are associated with severity of vertebral fractures (Sornay-Rendu *et al.*, 2009; Szulc *et al.*, 2011). The measurement of cortical porosity is relevant in patients with diabetes type 2, which despite normal aBMD and increased trabecular bone density have higher fracture risk than controls. As shown by HR-pQCT, these patients have reduced cortical vBMD (Burghardt *et al.*, 2010c) and in case of fragility fractures increased cortical porosity (Patsch *et al.*, 2013). For other applications, it is still debated whether at the spatial resolutions achievable with current HR-pQCT equipment, cortical porosity is just a surrogate of cortical BMD. Differential treatment effects on cortical and trabecular structure induced by a variety of antiosteoporotic drugs have been summarized recently (Lespessailles *et al.*, 2016): "Responses to therapies were treatment-specific and divergent effects in cortical and trabecular bone with antiresorptive or anabolic agents were observed." Often, effects were stronger in the tibia than in the radius. Results for cortical thickness and porosity depended on segmentation and revealed challenges to monitor changes, particularly in instances of endocortical or periosteal resorption or apposition.

DXA Based Techniques

A big advantage of 3D-DXA imaging techniques is the possibility to apply them directly to an existing DXA scan without additional imaging of the subject. Just special software is required. As standard DXA results are available, only information independent of aBMD will justify an additional analysis. For example the ISCD positions on HSA that were based on more than 50 studies concluded that this technique and other geometrical parameters such as the neck-shaft angle should not be used for fracture prediction because of lack of evidence that HSA parameters improved fracture prediction independent of aBMD. HSA should also not be used for monitoring of osteoporosis, because of insufficient precision (Broy *et al.*, 2015). The only exception with regard to fracture prediction was the neck axis length.

In contrast, TBS adds independent information for hip and spine fracture prediction and has been integrated as risk factor into the FRAX algorithm for calculation of fracture risk. Recently, a large metaanalysis of 14 prospective studies with 17,800 patients confirmed TBS as moderate aBMD independent risk factor of incident hip fractures (McCloskey *et al.*, 2016). Although the FDA has approved TBS for monitoring treatment effects in osteoporosis, the ISCD positions currently discourage monitoring in patients under bisphosphonate therapy due to small changes of TBS (Silva *et al.*, 2015).

It is too early to give clinical recommendations for 3D-DXA methods as most of the published studies have been dedicated to technical issues. In 65 cadavers the in vitro correlation between 2D-FEA derived and experimentally determined strength was $r^2 = 0.6$ (Naylor *et al.*, 2013). For 3D-FEA only a small pilot study in three cadavers has been published (Grassi *et al.*, 2017). In postmenopausal women of the large epidemiological study of osteoporotic fracture (SOF), estimated femoral strength from 2D-FE analysis of DXA scans was an independent predictor and performed at least as well as FN BMD in predicting hip fracture (Yang *et al.*, 2014). In a subgroup of men of the MrOS study similar results were reported. In a third study, again in women, AUC values

for femoral neck aBMD combined with 2D-FEA strength estimates (0.69) were slightly higher than for neck aBMD alone (0.66) (Naylor *et al.*, 2013).

Conclusion

Bone quality is no longer a cloudy concept but an ensemble of well-defined biomechanical parameters. Currently, FEA is the only in vivo imaging method to assess some of these parameters. Bone geometry can be measured by QCT, which is also the basis for FEA. DXA aBMD is a good surrogate of bone quality and currently remains the most important measurement in clinical routine. Hip fracture prediction remains challenging and more efforts should be directed to the integration of fall risk and muscle properties. QCT and FEA may eventually replace DXA for prediction of vertebral fractures but further validation is required. QCT and FEA will also play a dominant role in opportunistic screening.

Contrary to earlier expectations, differences in trabecular structure cannot be exploited to improve fracture prediction to a level necessary for an individual diagnosis. Homogenized FE models are surprisingly accurate. The correlation with strength is only slightly improved by μFEA (Pahr and Zysset, 2009; Zysset *et al.*, 2013), which requires the segmentation of the trabecular network. However, difference in the trabecular architecture may be important to identify phenotypes of osteoporosis for targeted treatment.

3D-DXA is an interesting development because advanced analysis methods such as assessment of cortical parameters or even FEA can be applied to DXA directly. This could revolutionize fracture prediction and monitoring of osteoporosis because DXA equippment is widely available. On the other hand, the achievable quality of 3D-DXA images may not be adequate to determine parameters of bone quality with sufficient accuracy.

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Bone Turnover Markers☆

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Introduction

Osteoporosis is the most prevalent metabolic bone disease and its impact is expected to rise throughout the world with the aging of the population (Harvey *et al.*, 2010). It is defined as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and consequent increase in fracture risk (Anon, 1993). Low bone mass, measured as bone mineral density (BMD), is asymptomatic and its important outcome is fracture, a cause of morbidity and mortality. This is especially true for hip fracture (Kanis and Johnell, 2005). Therefore, the clinical management focus in osteoporosis is to prevent or reduce the risk of fracture and follow the response to therapy. The identification of the patients to be treated relies on bone mineral density (BMD) measurement, most commonly using dual-energy X-ray absorptiometry (DXA) (Kanis *et al.*, 2008). Also, algorithms to estimate fracture risk based on BMD and other clinical features (age, gender, prior fractures, parental hip fracture history, body mass index, ethnicity, smoking, alcohol use, glucocorticoid use, rheumatoid arthritis, and secondary osteoporosis) such as FRAX are commonly used in clinical practice to guide the treatment of individual patients (Kanis *et al.*, 2011).

However, important gaps remain with interpretation of results particularly with regard to identification of individuals who would best benefit from intervention and, for those patients on treatment, the optimal manner in which response to treatment should be monitored. In this regard, there has been interest in the clinical potential of bone turnover markers (BTM) as tools to assess fracture risk, to monitor treatment, to predict response to treatments and to take the best intervention strategies (Vasikaran *et al.*, 2011). One of the great advantages of BTM is that they are measured in samples (blood or urine) that are easily collected. Also, their determination can be performed quite easily and levels provide information that is complementary to BMD. However, there are some weaknesses in the use of BTM in clinical practice. Among these, one can cite the biological, the preanalytical and the analytical variabilities. Indeed, BTM need to be measured in the morning(their concentrations are highest in the second half of the night and at time of waking whereas they are lowest in afternoon and evening), and in a fasting state (feeding leads to a significant decrease particularly for bone resorption markers) (Vasikaran *et al.*, 2011). Furthermore, no standardization of BTM assays exist, there are very few external quality control programs which include BTM, there are important method-to-method variations (Jørgensen *et al.*, 2017; Chubb *et al.*, 2015a) and many different BTM exist in the market, that have been used in various studies in the past, which makes it difficult to compare results. Therefore, in 2010, the International Osteoporosis Foundation (IOF)—International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Joint Working Group on Bone Marker Standards (WG-BMS) published an extensive review supporting the role of BTM in the management of patients with osteoporosis (Vasikaran *et al.*, 2011). The Working Group acknowledged that there was no perfect (or gold standard) BTM but recommended one bone formation marker (serum procollagen type I N-propeptide (PINP)) and one bone resorption marker (serum C-terminal telopeptide of type I collagen, (CTX)) be used as reference markers, to be measured by standardized assays in observational and intervention studies in order to assess their clinical performance as well as to provide data by which alternatives could be assessed thus enlarging the international experience of the application of these markers to clinical medicine. In 2012 the National Bone Health Alliance extended the literature review on this subject arriving at similar recommendations (Bauer *et al.*, 2012). The IFCC-IOF Working Group for the Standardization of Bone Marker Assays was established in 2012 to standardize or harmonize serum/plasma CTX and PINP assays depending on feasibility.

Bone Turnover Markers: Definition

BTM are biochemical products preferably measured in blood, but also in urine that reflect the metabolic activity of bone but without any function in controlling skeletal metabolism. Traditionally, they are categorized as markers of bone formation or bone resorption (**Table 1**).

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Table 1 Bone turnover markers: nomenclature, abbreviations and description

Marker	*Full name*	*Origin*	*Assay*	*Comments*
Resorption				
u-NTX	Urinary amino-terminal cross-linking telopeptide of type I collagen	Osteoclastic hydrolysis of type I collagen, generated by cathepsin K	Automated manual	Must be adjusted to levels of urinary creatinine Specificity: collagen type I, with highest contribution probably from bone Sources of variability: influenced by circadian rhythm
s-NTX	Serum amino-terminal cross-linking telopeptide of type I collagen			Specificity: collagen type I, with highest contribution probably from bone; smaller response to therapy may indicate some lack of bone specificity Sources of variability: influenced by renal function and circadian rhythm
u-CTX	Urinary carboxy-terminal cross-linking telopeptide of type I collagen			Must be adjusted to levels of urinary creatinine Specificity: collagen type I, with highest contribution probably from bone u-CTX is isomerized (β) or nonisomerized (α). Isomerized if not otherwise specified Sources of variability: influenced by circadian rhythm
s-CTX	Serum carboxy-terminal cross-linking telopeptide of type I collagen			s-CTX is always isomerized (β) Specificity: collagen type I, with highest contribution probably from bone Sources of variability: very dependent on time of day and food (must be collected after an overnight fast); influenced by renal function, liver function and circadian rhythm
s-ICTP or CTX-MMP	Carboxy-terminal cross-linking telopeptide of type I collagen	Osteoclastic hydrolysis of collagen generated by matrix metalloproteinases	Manual	Specificity: collagen type I, with highest contribution probably from bone. Results from MMP digestion of collagen and not responsive to usual treatments for osteoporosis Sources of variability: influenced by renal and liver function and circadian rhythm
u-DPD	Urinary deoxypyridinoline	Proteolytic hydrolysis of collagen, found in bone	Automated manual	Must be adjusted to levels of urinary creatinine Total or free (nonpeptide-bound) Specificity: highest contribution from bone, present in mature collagen only Sources of variability: independent of dietary sources, influenced by UV radiation and circadian rhythm
u-PYD	Urinary pyridinoline	Found in bone, cartilage, tendon, blood vessels	Automated manual	Must be adjusted to levels of urinary creatinine Total or free (nonpeptide-bound) Specificity: highest contribution from bone and cartilage, present in mature collagen only Sources of variability: independent of dietary sources; influenced by liver function, active arthritis and UV radiation, and circadian rhythm

Table 1 Continued

Marker	*Full name*	*Origin*	*Assay*	*Comments*
Formation				
s-OC	Serum osteocalcin	Hydroxyapatite-binding protein exclusively synthesized by osteoblasts and odontoblasts	Automated manual	Specificity: specific marker of osteoblast function but also liberated from bone matrix during resorption process Subject to rapid degradation in serum leads to heterogeneity of OC fragments: usually measured as intact [1–49] or N-mid [1–43] fragment, or can be undercarboxylated (ucOC) Sources of variability: influenced by renal function and circadian rhythms; large inter-laboratory variation
u-OC	Urinary osteocalcin		Manual	Adjusted to levels of urinary creatinine Specificity: specific marker of osteoblast function but also liberated from bone matrix during resorption process Mid (predominant fragments) or long (only longest fragment) in urine Sources of variability: influenced by renal function and circadian rhythm
s-ALP	Serum alkaline phosphatase (total)	Ubiquitous, membrane bound tetrameric enzyme located on the outer cell surface of various tissues: liver, bone, intestine, spleen, kidney and placenta	Automated manual	Specificity: nonspecific for bone (about 50% is liver isoform in healthy individuals Multiple assay methodologies Source of variability: very small circadian rhythm
s-BALP	Serum bone-specific alkaline phosphatase	Ubiquitous, membrane bound tetrameric enzyme located on the outer cell surface of osteoblasts	Automated manual	Specificity: specific for bone, but with a certain cross-reactivity with liver isoform (up to 20%) Source of variability: very small circadian rhythm Semantic confusion and may be expressed as “mass” (Ostase) or enzymatic units (ratio about 50% between both)
s-PICP	Procollagen type I C propeptide	Precursor molecules of collagen type I synthesized by osteoblasts	Manual	Specificity: mostly derived from bone collagen type I (around 90%). Short serum half-life. Regulated by hormones (thyroid, IGF-1) Source of variability: small circadian rhythm
s-PINP	Procollagen type I N propeptide		Automated manual	Specificity: mostly derived from bone collagen type I Assay: may recognise trimer alone (intact) or trimer and monomer (total PINP). Monomers accumulate in renal failure Source of variability: small circadian rhythm
Osteoclastic enzymes				
s-TRACP	Serum tartrate-resistant acid phosphatase	Includes two isoforms: type 5a (platelets, erythrocytes and other sources) and type 5b (osteoclasts)	Automated manual	Reflects the number of osteoclasts Only resorption marker that does not accumulate in renal failure
Cat K	Cathepsin K	Catalytic enzyme expressed by osteoclasts	Manual	

(*Continued*)

Table 1 Continued

Marker	*Full name*	*Origin*	*Assay*	*Comments*
				Could be a specific marker of osteoclast activity. Potential therapeutic target
Regulators of bone turnover				
OPG	Osteoprotegerin	Osteoblasts. OPG is a glycoprotein that circulates as a monomer or homodimer and may be bound to RANKL	Manual	Difficult to measure. We don't know which of the three molecular forms is more clinically relevant to measure. Circulating levels may be not reflecting local bone marrow production. Serum levels may not reflect accurately the risk of fracture or bone loss and serial changes may not reflect response to treatment
RANKL	Receptor activator of nuclear factor-kappaB ligand	Osteoblasts	Manual	Difficult to measure since serum levels are very low and because of analyte instability. Circulating levels may be not reflecting local bone marrow production. Serum levels may not reflect accurately the risk of fracture or bone loss and serial changes may not reflect response to treatment
SCL	sclerostin	Osteocytes	Manual	There is a very bad concordance between the assays that measure sclerostin. This has lead to confusion in clinical studies Also, according to the assays sclersotin seems to accumulate or not in CKD. The structure of active sclerostin is a triple loop, but many inactive fragments that corss-react at various extents with the antibodies used in the assays also seem to circulate. Sclerostin is a major pharmaceutical target.
DKK-1	Dickkopf-1 related protein	Osteocytes	Manual	Regulates bone metabolism through the Wnt signalling pathway by inhibiting osteoblast differentiation and FGF-23. Some data on the utility of assessment of DKK-1 and FGF-23 in osteoporosis have been published, but the importance of these mediators in the regulation of bone metabolism has not been established.
FGF-23	Fibroblast growth factor 23	Biologically active intact FGF23 has an estimated half-life of 20 to 60 minutes and is cleaved enzymatically into inactive C-terminal and N-terminal fragments	Automated manual	There are 2 types of assays: one that only recognizes intact-FGF23, and one that detects both intact-FGF23 and C-terminal fragments. In most CKD studies there appears to be a good correlation between intact and C-terminal FGF23 but in some diseases (iron deficiency, GALNT3 mutation) iFGF23 is lower whereas inactive C-terminal fragments are high. Correlate with left ventricular volume

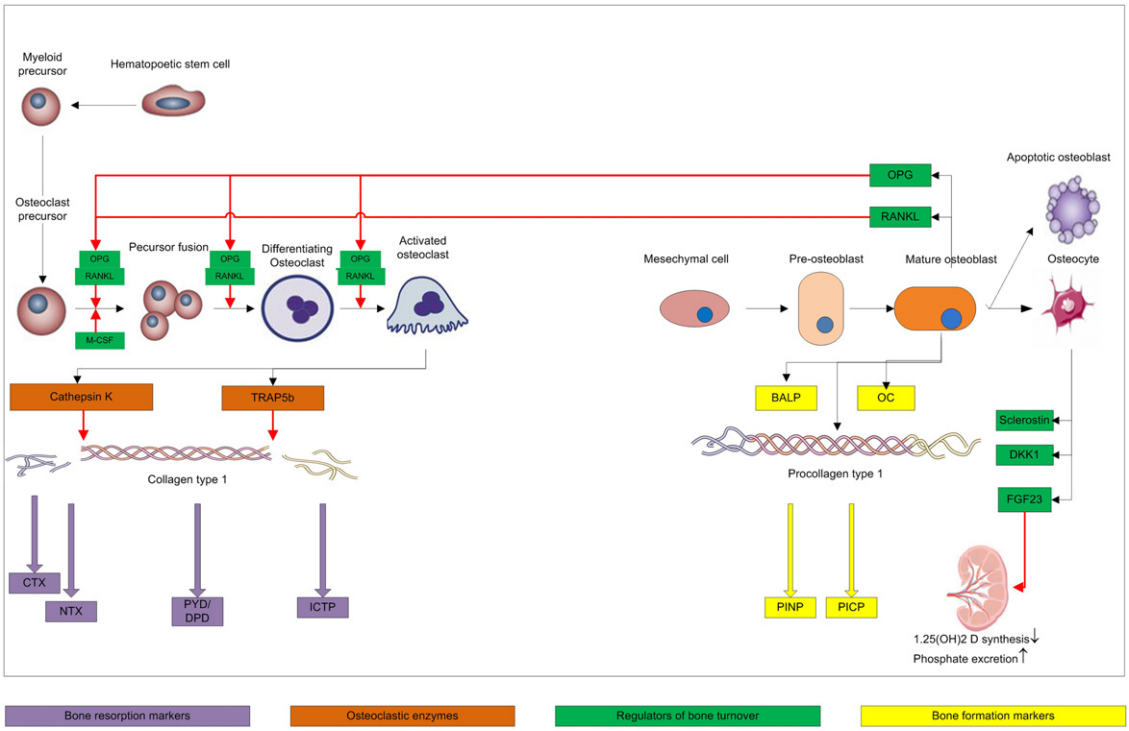

Fig. 1 Biochemical markers of bone turnover. Currently available markers include both enzymes and nonenzymatic peptides derived from cellular and noncellular compartments of bone. They are usually classified according to the metabolic process they are considered to reflect. Most biochemical markers of bone resorption are related to collagen breakdown products. Other markers of bone resorption include noncollagenous matrix proteins or osteoclast-specific enzymes. In contrast, markers of bone formation are either by-products of collagen neosynthesis, or osteoblast-related proteins. This distinction, however, is not absolute, since some markers reflect, at least in part, both bone formation and bone resorption (e.g., certain OC fragments). Furthermore, most of these molecules are also present in tissues other than bone, and nonskeletal processes may therefore influence their circulating or urinary levels. Finally, changes in markers of bone turnover are not disease specific but reflect, as an integral measure, alterations in the metabolism of the entire skeletal envelope independently of the underlying cause. Results of bone marker measurements should always be interpreted taking into account patient's clinical picture. The bone remodeling cycle lasts 150–200 days and is primarily mediated by osteoblastic signals which promote the differentiation and maturation of osteoclast precursors. RANKL (a product of osteoblast precursors and/or mature osteoblasts) is the key osteoblastogenic cytokine which in the presence of a permissive concentration of M-CSF is capable of inducing osteoclast formation and promoting osteoclast resorptive activity in the absence of any other cytokine. OPG (also a product of mature osteoblast and their precusrors but also a product of B cells) functions as a decoy receptor, preventing the association of RANKL with RANK receptor and thus moderating the osteoclastogenesis and bone resorption. The binding of RANKL to RANK initiates the differentiation of early osteoclast precursor into preosteoclasts. Preosteoclasts fuse with each other to produce multinucleated bone resorbing osteoclasts recognized by the expression of key osteoclast markers including TRAP5b, cathepsin K, and MMP9. Activated osteoclasts create resorption pits with low pH to dissolve the inorganic matrix and lysomal enzymes, such as TRAP and cathepsin K, effectively digest the exposed type-1 collagen releasing specific degradation products Osteoblasts are attracted to eroded bone surface and begin to form new osteoid. Type-1 collagen, abundant in osteoblasts, is secreted as a procollagen precursor molecule into the extracellular space where it is cleaved, by specific propeptidases, at the amino-and carboxy-terminals releasing pro-peptides into the blood. Initially hydroxyapatite crystals are deposited in the osteoid then a slower mineralization process continues over several months, followed by a period of quiescence. *Red arrow* indicate action, *black arrow* production. Modified (and enriched) from Krane, S. M. (2005). Identifying genes that regulate bone remodeling as potential therapeutic targets. *Journal of Experimental Medicine* **21** 201(6), 841–843; Wheater, G., Elshahaly, M., Tuck, S. P., Datta, H. K. and van Laar, J. M. (2013). The clinical utility of bone marker measurements in osteoporosis. *Journal of Translational Medicine* **11**, 201.

Biomarkers of Bone Formation

Markers of bone formation are direct or indirect products of active osteoblasts expressed during various phases of their development and reflect different aspects of osteoblast function (**Fig. 1**). The bone matrix is a complex structure composed of mineralized type I collagen. This protein is synthesized by the osteoblasts as a precursor molecule (procollagen) whose extension peptides at each end (procollagen type I N propeptide (PINP) and procollagen type I C propeptide (PICP)), are cleaved by enzymes during bone matrix formation and released into the circulation. The most abundant noncollagenous bone protein is osteocalcin (OC). It is also produced by osteoblasts during bone formation, and some proportion finds its way into the extracellular compartment where it can be measured. Of note, OC is also liberated from the bone matrix during bone resorption, which makes that OC cannot be considered as a "true" bone formation marker. Interestingly, OC has been shown to be involved in the energy metabolism by its interplay with insulin (Karsenty and Ferron, 2012), but this is out of the scope of this article. OC is

excreted by the kidneys and its fragments may also be measured in urine. Newly formed osteoid undergoes maturation followed by mineralization, and during this phase, alkaline phosphatase (ALP) is secreted by osteoblasts into the extracellular fluid and can be measured in serum. However, only about half of the total ALP activity in blood in healthy adults derives from bone, the other half being predominately of hepatic origin. Hence, total ALP measurement has a poor specificity and assays that detect more specifically the bone derived isoform (BAP) are available. Unfortunately, a certain intermethod variability also exists for this biomarker (Cavalier *et al.*, 2014).

Regulation of bone formation

The canonical Wnt-β-catenin signaling pathway controls the fate of mesenchymal stem cells (MSCs) and their differentiation into various cell lineages. This pathway enhances osteoblastic differentiation from MSCs, promotes osteoblast maturation and osteoblast and osteocyte survival and inhibits osteoclastogenesis (Baron and Kneissel, 2013; Plotkin and Bellido, 2016). This is achieved indirectly via the increase of the expression of osteoprotegerin (OPG) in osteoblasts and osteocytes. Dikkopf related protein (DKK-1) is a Wnt antagonist expressed in osteoblasts and in osteocytes at much higher levels (Paic *et al.*, 2009). Experimental studies in mice have shown that overexpression of DKK-1 in osteoblastic cells has as result decreased bone formation and low bone mass (Li *et al.*, 2006). Another antagonist of Wnt signaling is sclerostin, which is primarily expressed by mature osteocytes but not by early osteocytes or osteoblasts. Sclerostin binds to Wnt coreceptors and antagonizes downstream signaling (Poole *et al.*, 2005; Moester *et al.*, 2010; Sapir-Koren and Livshits, 2014). Absence of SOST (the gene that encodes for sclerostin) expression or secretion of sclerostin in humans has as result sclerosteosis (van Buchem disease) or craniodiaphyseal dysplasia, which are inherited diseases of high bone mass characterized by excessive bone mass formation. On the other hand overexpression of the SOST gene results in overproduction of sclerostin which in turn results in decreases in bone mass (Baron and Kneissel, 2013; Moester *et al.*, 2010; Sapir-Koren and Livshits, 2014).

Biomarkers of Bone Resorption

The commonly used bone resorption markers are degradation products of type I collagen, but noncollagenous proteins such as the enzyme of osteoclast origin tartrate resistant acid phosphatase 5B (TRAP-5B) have also been investigated as resorption markers. The pyridinium cross-links, pyridinoline (PYD), and deoxypyridinoline (DPD) are formed during the maturation of bone collagen, present in significant amounts in bone and dentine, released during resorption of bone and excreted in urine in the free and peptide-bound forms without being metabolized. The peptide-bound forms of PYD and DPD include the C-terminal and N-terminal cross-linking telopeptides (CTX, NTX) of the type I collagen molecule, and these are also released into the circulation and subsequently excreted in urine.

Osteoclastic Enzymes

Tartarate resistant acid phospatase (TRAP-5b)

TRAP-5b is produced by osteoclasts and is considered a good marker of bone resorption (Halleen *et al.*, 2000). During bone resorption osteoclasts secrete TRAP-5b which produces reactive oxygen species to digest bone degradation products in the microenvironment of bone matrix. If the action of TRAP-5b in the bone is still not fully understood it reflects both the osteoclast activity and number and has been shown to be inversely correlated with bone mineral density (BMD) in postmenopausal women and to predict an increased risk of hip or vertebral fracture (Nenonen *et al.*, 2005).

Cathepsin K

The enyme cathepsin K is a member of the cysteine protease family, and unlike other cathepsins has the unique ability to cleave both the helical and the telopeptide regions of collagen type I. It is a lysosomal enzyme and this active form is produced in vivo in the lysosomes is believed to occur in the low acid pH environment of the lysosomes and subsequently is released into bone resorption lacunae. Some of this cathepsin K is released into circulation and could be a specific biological marker of osteoclast activity. Increased levels of cathepsin K have been reported in individuals with postmenopausal osteoporosis, Paget disease, and rheumatic diseases (Chapurlat and Confavreux, 2016; Harvey *et al.*, 2010).

Regulation of bone resorption

Osteocytes produce cytokines that regulate osteoblast formation and survival (Plotkin and Bellido, 2016). Osteoclastogenesis involves the activation of the receptor activator of nuclear factor κB (RANK) in osteoclast precursors induced by RANK ligand (RANKL), which is produced mainly by osteoblastic cells (and in a lesser extent by osteocytes) (Kearns *et al.*, 2008). The antiosteoclastogenic cytokine OPG is expressed in both osteoblasts and osteocytes and it acts as a decoy receptor for RANKL, binds RANKL and competes RANK and therefore inhibits osteoclast differentiation (Harvey *et al.*, 2010; Anon, 1993; Kanis and Johnell, 2005). Moreover OPG is a target of the canonical Wnt signaling and experimental studies have shown that mice that lack β-canetin in osteoblasts exhibit low levels of OPG, increased numbers of osteoclasts and as a results low bone mass. These finding show that regulation of OPG by the Wnt-β-canting signaling has an important role in the control of bone resorption (Plotkin and Bellido, 2016; Weitzmann, 2013).

Regulators of osteoclastic–osteoblastic activity: Osteocytic signaling pathways

Osteocytes are the most abundant cells in bone and are essential the functioning of the human skeleton. They constitute >90% of bone cells. Osteocytes are differentiated osteoblasts, that live within the bone matrix and are highly connected not only among themselves but also with cells on the bone surface and the bone marrow. These "quiescent" cells are literally buried in the mineralized bone tissue and are very active. They coordinate bone acquisition during growth and the maintenance of healthy skeletal frame for movement support and the protection of essential organs. Osteocytes orchestrate the function of osteoblasts and osteoclasts by the production and secretion of factors that enable the skeleton to adapt to mechanical and hormonal changes. Moreover they secrete hormones that affect other tissues and regulate mineral homeostasis and hematopoiesis. Osteocytes produce and secrete factors that affect other bone cells by paracrine and/or autocrine mechanisms (RANKL, OPG and sclerostin) and secrete hormones (FGF23) that affect other tissues by endocrine actions. These pathways are now considered as potential therapeutic targets in an attempt to improve human skeletal health (Plotkin and Bellido, 2016; Dallas *et al.*, 2013; Bellido, 2014).

Phosphate metabolism and matrix mineralization

Fibroblast growth factor 23 (FGF23) is produced by osteocytes (and possibly by osteoblasts) and has a key role in the "bone-parathyroid-kidney" axis and the regulation of phosphate/calcium metabolism. FGF23 binds to one or several FGF receptors and the Klotho coreceptor in the renal proximal tubules and inhibits the expression of the types IIa and IIc sodium-phosphate cotransporters on the apical membrane of the renal proximal tubular cells. This leads to an increase in renal phosphate excretion. In contrast to PTH, which also induces phosphaturia and increases renal 1α-hydroxylase, FGF23 suppresses 1α-hydroxylase and stimulates renal 24-hydroxylase activity. This action leads to a decrease of 1,25-dihydroxy vitamin D ($1,25(OH)_2D$) production.

While FGF23 regulates phosphate and $1,25(OH)_2D$ levels, FGF23 is itself regulated by both phosphorus and $1,25(OH)_2D$ and probably by PTH. Indeed enhanced FGF23 production was observed after dietary phosphate loading and administration of $1,25(OH)_2D$ and PTH, while dietary phosphorus restriction reverses this trend. However the most significant increases of FGF23 levels have been observed in patients with chronic kidney disease (CKD). In patients with CKD, circulating levels of FGF23 rise progressively as renal function declines well before the onset of a critical reduction in the nephron number. These, early increased, FGF23 levels during CKD, are observed well before phosphate/PTH levels increase or $1,25(OH_2)D$ levels decrease, and the exact mechanisms and the triggers of this increase remain unknown. Several studies have shown that elevated levels of FGF23 are independently associated with greater risks of death, cardiovascular events progression to end-stage renal disease and premature allograft loss after kidney transplantation (Plotkin and Bellido, 2016; Shimada *et al.*, 2004; Hu *et al.*, 2013; Kuro-o, 2013; Urakawa *et al.*, 2006).

BTM Concentrations for Predicting Fracture Risk

Estrogen deficiency links to an osteoblastic overexpression of the Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) which cannot be adequately controlled by osteoprotegerin, its decoy receptor. This excess of RANKL activates RANK receptors, located on preosteoclasts and mature osteoclasts, leading to accelerated bone resorption (Boyle *et al.*, 2003). The overall imbalance between formation and resorption at the level of bone tissue is maintained for several decades after menopause, leading to increased risk of fracture. It may thus be logical to associate the high levels of BTM, and especially bone resorption markers to increased fracture risk.

The IOF-IFCC WG-BMS review by Vasikaran *et al.* described 22 studies, in which the relationship between bone turnover markers and incident fractures was examined (Vasikaran *et al.*, 2011). Eighteen of them showed that one or more markers were associated with risk of subsequent fracture with the concentration of bone resorption markers more consistently associated with fracture risk than bone formation markers. This was the case for studies in both men and women and seven of these studies even reported that in women with a low BMD, the presence of increased BTM had an additive effect on fracture risk. Since that time three more studies (reviewed in Morris *et al.*, 2016) have been published, including a metaanalysis. The metaanalysis examined the performance characteristics of PINP and CTX for fracture risk prediction in untreated individuals and found a modest, but significant association between both PINP and CTX concentrations at baseline and fracture risk (Johansson *et al.*, 2014). The results of this metaanalysis have to be examined with caution as it combined results for CTX generated by the two clinical laboratory automated assay methods currently available (IDS iSYS and Roche Cobas) and these assays do not appear to provide comparable values for CTX (Chubb *et al.*, 2015a). Similarly the PINP data were generated by different assays. In the Australian Health In Men Study the association of bone turnover markers with hip fracture incidence in older men was examined. Total osteocalcin (tOC), undercarboxylated osteocalcin (ucOC) and CTX were associated with hip fractures in univariate analyses, but only tOC remained significantly associated with incident hip fractures in multivariate analyses adjusting for age and glucocorticoid use (Chubb *et al.*, 2015b). In contrast to the above, a Japanese study of the Taiji cohort of both men and women failed to demonstrate a significant association between a broad range of BTM and incident fracture risk. However, the study was insufficiently powered for a fracture endpoint as this cohort included relatively young subjects (mean age approximately 60 years) resulting in a low number of osteoporotic fractures (32) during the 10-year follow-up period (Yoshimura *et al.*, 2011).

These more recent findings support the previous interpretation in the Vasikaran review (Vasikaran *et al.*, 2011).There are significant associations between bone turnover markers and incident fracture risk, though the association is modest. Most studies demonstrate a relation between bone turnover markers and fracture, yet there are limitations to the studies. These include the variable use of markers of bone formation (BAP, PINP, PICP, total osteocalcin, intact osteocalcin) and of bone resorption (ICTP, CTX, NTX-I, PYR, DPD, beta-CTX), differences in analytical assays and platforms, inconsistencies in expression of risk, as well as inconsistent predictive value for a specific marker in the individual studies reported (see **Table 1** for abbreviations of BTM).

BTM Concentrations for Monitoring Treatment

One of the great advantages of BTM over BMD is that they may rapidly show responses to the treatments used for osteoporosis. Hence, this rapid response (or the absence of response) may help the clinician decide on the dosage of the drug and the frequency of the administration -or even to change the treatment. Also, the percentage of variation of the BTM after treatment is quite large (e.g., bisphosphonates lead to a decrease of 50% of bone resorption marker, or even more) whereas the increment in BMD is quite modest (e.g., 5%–10%).

The use of BTMs for the monitoring of treatment requires a baseline assessment with a repeat measurement at some defined point during treatment. To interpret the variation observed between two results, one has to take into consideration two variations, namely the analytical variation (CV_a), expressed as the coefficient of variation (in percent) of the analytical method used to measure the BTM, and the intraindividual variation of the BTM (CV_i), also expressed in percent, which is the natural biological variation of the biomarker around its homeostatic set-point. In order to be confident ($P < 0.05$) that a change in a BTM value has occurred, then (assuming a normal distribution) the change in measured value must exceed $\sqrt{2} \times 1.96 \times \sqrt{(CV_i^2 + CV_a^2)}$ which is termed the "Least Significant Change (LSC) or "Reference Change Value" (RCV). Taking into consideration the fact that CV_a should at least be 1/4 of CV_i, we can come to the conclusion that LSC $\approx 3 \times CV_i$. For example, the LSC for CTX might be $3 \times 9.6 \approx 29\%$. In a woman with a baseline value of 500 ng/L for CTX, the LSC would be ± 145 ng/L, and so a significant decrease would be a value of 355 ng/L or below and a significant increase would be a value of 645 ng/L or above. Watch out that this is only true when the same preanalytical (time of sampling, sample type, fasting statutes, …) and analytical (same method) conditions are utilized. When monitoring treatment in clinical practice, it is also possible to note whether patients are below the geometric mean for a healthy young woman (35 μg/L for PINP, 280 ng/L for CTX). Indeed, before treatment, women with osteoporosis are above these thresholds in 90% of cases whereas on oral bisphosphonate therapy, 90% are below the threshold (Naylor *et al.*, 2016; Diez-Perez *et al.*, 2017).

The IOF-IFCC WG-BMS review (Vasikaran *et al.*, 2011) also reported seven studies concerning the relationship between change in BTM and fracture risk reduction with different drugs given for postmenopausal osteoporosis. These studies showed in general that the larger the decrease in BTM, the larger the reduction in fracture risk. One of the outcomes from such studies is to assess the extent to which a biological marker is a surrogate end-point for a clinical event, which is known as the "treatment effect explained." In the case of clinical trials for osteoporosis treatment, the clinical endpoint is fracture and the surrogate biological markers are BTM. The treatment effect explained was calculated for two of these seven studies, namely the VERT (Eastell *et al.*, 2003) and the MORE (Eastell *et al.*, 2003) trials). In the VERT trial, the change in u-CTX and u-NTX at 3–6 months explained between 54% and 77% of the fracture risk reduction with risedronate and in the MORE study, the change in PINP and OC explained 28% and 34%, respectively, of the vertebral fracture risk reduction with raloxifene. So, about half of the fracture risk reduction with these drugs, which work through the inhibition of bone turnover, could be associated with the measured change in BTM during the first year of treatment. However the review highlights many limitations to these studies: only a subset of patients generally had the BTM measured, so that the number of fractures considered was small, the size of the study was also generally quite small and BTM were not always collected in the correct way. Also, the IOF-IFCC WG-BMS also highlight some pitfalls in the statistical approach. There have now been two further studies that examine this question, one a follow-up analysis of zoledronic acid (HORIZON trial (Jacques *et al.*, 2012)) and the other a new analysis with bazedoxifene (Bruyère *et al.*, 2012), a selective estrogen receptor modulator, similar to raloxifene. In the HORIZON trial, the change in PINP at 1 year explained 58% of the treatment effect on new vertebral fracture (statistically significant), and there was a significant association with nonvertebral fracture. This figure was similar to the 54% treatment effect explained change in total hip BMD over 3 years and vertebral fracture. The effect explained by PINP was independent of that explained by total hip BMD, so the results of these two tests are complementary. Regarding the trial with bazedoxifene, the change in CTX at 1 year explained 16% and change in OC 6% of the treatment effect on new vertebral fracture (statistically significant). There was no overall reduction of nonvertebral fractures in this study so any relationship with marker change could not be tested. Once again the conclusions made in the original report (Vasikaran *et al.*, 2011) are at least partially supported by these new analyses. The treatment effect explained by BTM is at least as great as BMD. The finding of significant positive associations between the reduction in BTM and the reduction in fracture risk support the use of BTM in monitoring treatment. The limitation noted in the IOF-IFCC WG-BMS report that studies were often small subsets of the main trial was true for the zoledronic acid study but not for the bazedoxifene study, which is the largest study to date. The studies were also criticized for not obtaining samples under optimal conditions. This again was not true of these two studies as the patients from the bazedoxifene study were in the fasting state for the blood draw, a critical requirement for serum CTX.

Paget's Disease of Bone

Paget's disease of bone (osteitis deformans) is a chronic benign disorder of bone that generally affects one or several bones. It is characterized by a marked increase in bone turnover, leading to overproduction of bone of poor quality, responsible for hypertrophy, osteosclerosis, and bone fragility. BTM are routinely used for assessing the disease activity and for monitoring the efficacy of bisphosphonate therapy. Because of a marked increase in bone turnover, serum total ALP activity is the most commonly used bone marker for Paget's disease for both applications. Although serum total ALP is adequate to monitor most patients with active disease, this marker may lack sensitivity in patients with monostotic disease affecting a small bone and in patients with purely osteolytic lesions. Among more specific BTM, BALP, and PINP appear to be the most sensitive markers for assessing disease activity. These markers may be particularly interesting for patients with monostotic disease (Garnero and Delmas, 2004). On the contrary, osteocalcin lacks sensitivity probably because the fraction of newly synthesized osteocalcin that is incorporated into bone matrix may be increased because of the high mineral content of the woven bone. For bone resorption, urinary NTX and urinary nonisomerized α-CTX (and not β-CTX because pagetic bone matrix is characterized by an impaired degree of β -isomerization of type I collagen molecules) appear to be the most sensitive bone resorption markers both for assessing disease activity and for monitoring efficacy of bisphosphonate therapy. In 2014, the Endocrine Society published a clinical practice Guideline on Paget's disease of bone (Singer *et al.*, 2014). Regarding the diagnosis of the disease, the authors recommend with high quality evidence that after radiological diagnosis of Paget's disease, the initial biochemical evaluation of a patient should be done using serum total ALP or with the use of a more specific marker of bone formation when appropriate. They consider that the low cost and the universal availability of the total ALP is a great advantage over more specific formation biomarkers, but recommend that in patients with abnormal liver or biliary tract function, a more specific biomarker of bone formation should be used to assess response to treatment or follow evolution of the disease in untreated patients. In this situation, PINP is the best option and has great advantages over osteocalcin and BALP because of the lack of sensitivity of the first, and cross-reactivity with liver isoenzyme for the second marker. If PINP cannot be used because of cost or unavailability, plasma β-CTX and urinary α-CTX or NTx can be used. In untreated Paget's disease, α-CTX (measured by ELISA only) which contains an aspartyl-glycine motif derived from newly formed collagen, is raised proportionately more than β-CTX, which is formed from spontaneous isoaspartyl formation as the bone ages in response to treatment (albeit β-CTX slightly underestimates the response to treatment of very active disease due to the isomerization phenomenon). The advantage of telopeptide assays over bone formation assays is faster demonstration of a maximal decrease in bone resorption than in bone formation after treatment. Regarding the monostotic Paget's disease, the Working Group suggests (with a low quality evidence) that PINP or BALP and β-CTX or NTx should be used for assessing the activity of the disease, although these may be normal when evidence of disease activity is still clearly demonstrated on scintigraphy.

Effect of Renal Impairment on BTM Concentrations

Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD)

Bone health is very frequently altered in Chronic Kidney Disease (CKD) and these patients are at increased risk of fractures whether they are dialyzed (Jadoul *et al.*, 2006) or not (Nickolas *et al.*, 2008). Indeed, these patients are often characterized by either increased or decreased bone turnover, linked to over- or under-secretion of parathyroid hormone (PTH). The gold standard to evaluate bone turnover is bone biopsy. Unfortunately, use of bone biopsies to determine bone turnover is hampered by the invasive nature of the procedure and the difficulty for correct interpretation of the results, limiting its use to a few specialized centers (Torres *et al.*, 2014). In clinical practice repeated bone biopsies are problematic for the follow-up of the patients or to assess effect of a treatment. Hence, BTM are essential in clinical practice to evaluate bone turnover. In 2009 the international recommendations in nephrology, Kidney Disease: Improving Global Outcomes (KDIGO) guidelines (Moe *et al.*, 2009) recommended the measurement of PTH and BALP in the assessment of metabolic bone disease of CKD (CKD-MBD). BALP was selected because serum concentrations are unaffected by renal function since it is cleared by the liver and with a molecular weight above 50,000 D it is unlikely to be filtered at the kidney but as already mentioned, it does suffer from some analytical and clinical issues (Cavalier *et al.*, 2014).

Is There any Place for Other BTM in CKD-MBD?

PINP

PINP consists of three subunit chains of type 1 procollagen (2 pro-α1 chains and 1pro-α2 chain) that are noncovalently linked and is produced in equimolar amounts with collagen deposited in bone tissue (Koivula *et al.*, 2012). Once in the circulation, PINP is rapidly bound and internalized by liver endothelial cells through their scavenger receptors (Melkko *et al.*, 1994). In human serum, PINP is present in two major forms, an intact trimeric form and a monomeric form. This latter form tends to be elevated in CKD patients. PINP determination can be performed either with automated (Roche Elecsys/Cobas and IDS iSYS) or manual (Orion Diagnostica) methods but the "Total" PINP assay (Roche Elecsys/Cobas) recognizes both the trimeric form and the monomers whereas the "Intact" PINP assays (IDS iSYS and Orion Diagnostica) recognize the trimeric form only. In CKD patients, it has been shown that patients with a glomerular filtration rate (GFR) below 30 mL/min/1.73 m2 have PINP concentrations that are overestimated by the "Total" assay due to the cross-reactivity with the monomeric form (Cavalier *et al.*, 2013). Assays specific for "Intact" PINP are recommended for use with CKD patients. While IOF and IFCC recommend serum CTX as the bone resorption biomarker for clinical research studies in osteoporosis it is not

recommended in CKD-MBD by the KDIGO guidelines since serum PTH or BALP are more effective at predicting clinical outcomes or bone histology (KDIGO Working Group, 2013; Delanaye *et al.*, 2014).

CTX

Serum CTX concentrations in patients undergoing hemodialysis are some five times higher than those of the normal population due to its accumulation with decreased renal function and frequent secondary hyperparathyroidism (Delanaye *et al.*, 2014).

TRAP-5B

Tartrate resistant acid phosphatase 5B (TRAP-5B) may be a suitable alternative for the monitoring of the bone resorption in CKD patients as it presents very interesting features: its serum concentrations are not influenced by kidney function and it is a noncollagen bone resorption marker with serum concentrations significantly correlating with histological indices of osteoclast number, bone formation rate, and mineral apposition rate in uremic patients (Hu *et al.*, 2013). TRAP-5B has recently become available on the automated IDS iSYS platform which may increase its potential as a routine marker for clinical laboratories increasing the data on this marker since such information is scarce (Shimada *et al.*, 2004).

Interpretation and Reference Intervals of Bone Turnover Markers Concentrations

BTM reference intervals are useful for interpreting the results from osteoporosis patients but by themselves they are of limited value for fracture prediction in untreated, individual patients. The need to establish reference intervals from healthy premenopausal women aged 30–45 years when concentrations are at a nadir has been emphasized (Vasikaran *et al.*, 2011; Szulc *et al.*, 2017). Ideally the subjects used for these studies should have normal BMD at the spine (Vasikaran *et al.*, 2011), but also should not suffer from secondary hyperparathyroidism linked, for instance, to a vitamin D deficiency. Expert opinion also suggests that the mean of the premenopausal reference interval can be used as a treatment target for antiresorptive therapy (Vasikaran *et al.*, 2011; Delmas *et al.*, 2000). It is considered necessary to establish reference intervals for different geographic areas and ethnicities (Vasikaran *et al.*, 2011). Furthermore due to differences that currently exist between results from the different commercial clinical assays, current reference intervals need to be method specific and reference intervals from different methods cannot be used interchangeably. The different reference ranges of CTX and PINP obtained up to now in the literature have been exhaustively reviewed in (Morris *et al.*, 2016). The results of this review show that the largest variation between the reference intervals appear to be between the Roche and IDS-iSYS assays for CTX although data for the IDS-iSYS assay are limited. Most of the studies have been performed in Caucasian individuals and data are clearly lacking for other ethnicities, and especially for subjects from Asian origin, which are increasingly concerned by osteoporosis. The variation across geographic regions appears to be minor except for those from Saudi Arabia.

BTM and Bone Metastases

Various neoplastic diseases and their treatments have significant effect on bone health. Metastatic bone disease is mostly seen with specific cancer types (most commonly from breast, prostate, lung, kidney and multiple myeloma). Bone metastases (BM) are a major complication in the clinical management of neoplastic diseases because of its prevalence and because of the proven relationship between cancer cells and stromal cells in the bone. Moreover biomarkers can reflect multiple interactions of cancer cells with osteoclasts and osteoblasts and they can have a role in the pathways involved in cancer progression in the bone (Ferreira *et al.*, 2015). These interactions between the tumor and the bone results in increased rates of osteolysis and osteogenesis and release of high levels of several biochemical markers that their measurement can be performed in blood or urine. The measurement of CTX, NTX, PINP, PICP or bone specific ALP can provide information not only on the rates of bone resorption or formation but also on the effect of tumor growth on bone turnover (Ferreira *et al.*, 2015; Coleman *et al.*, 2011). Although several studies showed a value in the measurement of bone turnover markers in order to evaluate BM status and to evaluate the response to therapy (Brown *et al.*, 2005; Ulrich *et al.*, 2001; Fizazi *et al.*, 2009; Stopeck *et al.*, 2010), the most recent practice guidelines have not recognized a definitive role of bone turnover biomarkers in the interpretation of clinical outcomes and treatment response in patients with BM (Coleman *et al.*, 2014; Van Poznak *et al.*, 2011).

Bone markers have been used in clinical studies for the evaluation risk of bone metastases development after cancer treatment, for the diagnosis of bone metastases and for the prediction of patient's outcome. Detailed discussion can be found elsewhere (Ferreira *et al.*, 2015; Jung and Lein, 2014; Joerger and Huober, 2012).

We must note here that bone markers reflect the ongoing bone resorption and formation of the body as whole and do not provide information to a specific site of the skeleton (where the metastases are, and they are not disease specific. They reflect alterations in skeletal metabolism independent of the underlying cause (Seibel, 2005).

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Primary Hyperparathyroidism

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Introduction

Primary hyperparathyroidism (PHPT) is due to an increased secretion of parathyroid hormone (PTH) by one or more parathyroid glands. It represents the third most common endocrine disease and the most frequent cause of hypercalcemia in the general population. The classical presentation of PTHT was characterized by skeletal, renal, and neuromuscular manifestations. Nowadays the disease is generally diagnosed when asymptomatic, even though some patients may present bone loss and kidney stones. A normocalcemic variant of PHPT has recently been identified.

Epidemiology

Until the early 1970s PHPT was considered rare. In the 1970s the estimated prevalence varied from 1:1000 persons in the United States to 4.3:1000 persons in Sweden, where an even higher prevalence (21:1000 persons) was reported in 1990 in women aged 55–75 years. Recent data indicate that the estimated prevalence of PHPT in the general population in United States is 0.86%.

The incidence of PHPT has markedly increased following the introduction of serum calcium in the multichannel autoanalyzer. The incidence varies according to geographic areas and assessment measures. Data derived from the Rochester Epidemiological project firstly identified the asymptomatic variant and reported an incidence of about 30 per 100,000 person-years, with a decline in the late 1990s to 22 per 100,000 person-year. Recent data in the United States derived from the Kaiser Permanente Health Care database in California indicate an incidence of 79.6 per 100,000 person-years in women and 35.6 per 100,000 person-years in men of all races, with the highest rate among blacks. In Europe, a survey performed in Denmark has shown an increasing incidence over the period 1977–2010, up to an annual rate of 16 per 100,000. The increase was particularly evident in women after the age of 50 years. Epidemiological data are not available in other countries. Data from Latin America and Asia indicate that the symptomatic form still is the most common, even though a trend to an increased rate of asymptomatic cases has been reported over the last 10 years.

The incidence peaks in the sixth decade of life. Women are more affected than men, with female to male ratio of 3:1, whereas there is no sex difference before the age of 45 years. PHPT is rare in children and adolescent. In the latter cases it is important to consider a genetic cause.

Etiology

PHPT mostly presents as a sporadic disease (90%), but may be part of hereditary syndromes [e.g., multiple endocrine neoplasia (MEN) type 1, 2A, and 4, hyperparathyroidism-jaw tumor syndrome (HPT-JT), and familial isolated primary hyperparathyroidism (FIPH)]. A solitary, benign adenoma is found in 80%–85% of cases. The histology is characterized by an encapsulated collection of chief cells (less frequently oxyphil cells), surrounded by a rim of normal parathyroid cells. The remaining parathyroid glands are normal.

Multigland involvement (either multiple adenoma or hyperplasia of all parathyroid gland) is found in 15%–20% of cases and is more common in hereditary cases, particularly MEN1. It may present in an asynchronous manner and can be confused with a sporadic single gland disease when only one affected gland if detected at initial surgery. Rarely (less than 1%), PHPT is caused by a parathyroid carcinoma, in which mitoses, fibrous trabeculae, vascular or capsular invasion, and infiltration of surrounding tissue are present. In the absence of local infiltration or metastases, the diagnosis of parathyroid carcinoma may be difficult at histology. Genetic studies (*CDC73* gene mutations) and immunohistochemical studies [loss of parafibromin (the protein encoded by the *CDC73* gene) expression] may help in equivocal cases. Very rarely hyperparathyroidism may be caused by ectopic PTH production by a nonparathyroid neoplasia. Predisposing factors are external neck irradiation in childhood and long-term lithium therapy.

Pathophysiology

Control of PTH Secretion

Extracellular ionized calcium (Ca_o^{2+}), by interacting with the Ca^{2+} sensing receptor (CaSR) present on the surface of parathyroid cells, is the main regulator of PTH synthesis and secretion. There is an inverse sigmoidal relationship between Ca_o^{2+} concentration and PTH release: in adenoma, parathyroid cells are less sensitive to Ca_o^{2+}, the "set point" (i.e., the concentration of Ca_o^{2+} causing a

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50% inhibition of the maximal PTH secretion) is shifted to the right and each cell secretes more PTH. On the other hand, in hyperplasia the set-point for Ca_o^{2+} is unchanged and each cells secretes a normal amount of PTH.

1,25-Dihydroxyvitamin D ($1,25(OH)_2D$) suppresses the transcription of PTH gene and parathyroid cell proliferation. This effect is due to either circulating $1,25(OH)_2D$ or its local production by 1α-hydroxylation of circulating 25-dihydroxyvitamin D (25(OH)D).

Serum phosphate, by binding to Ca^{2+}, lowers serum calcium concentration and indirectly stimulates PTH secretion. In addition, a direct stimulatory effect seems to be mediated through an increased stability of PTH mRNA.

Finally, experiments performed in rats and parathyroid cell in culture indicates that fibroblast-growth factor 23 (FGF23) inhibits PTH synthesis and secretion and, perhaps, parathyroid cell proliferation. The role of FGF23 in the pathogenesis of PHPT in humans remains unclear. As a matter of fact, serum levels of FGF23 are increased in patients with PHPT likely reflecting an adaptive response to offset the increased levels of $1,25(OH)_2D$ secondary to the high serum concentration of PTH.

The pathophysiology of PHPT is characterized by the loss of the homeostatic control of PTH synthesis and secretion from one or more parathyroid glands, leading to excessive secretion of PTH by individual cells (adenoma) or increased parathyroid cell proliferation (hyperplasia), where each cell synthesizes and secrete a normal amount of PTH.

Target Organs of PTH

Bone and kidney are the major targets organ of PTH. In bone, PTH acts an osteoblast, osteoclasts, and osteocytes. The net effect depends on the dose and timing of PTH signaling. In healthy individuals PTH is secreted in a pulsatile manner, with a circadian rhythm, and the two opposite effects, namely stimulation of bone resorption and bone formation, are well balanced. In patients with PHPT the sustained increased concentration of PTH causes bone loss particularly at cortical sites, osteoporosis, and an increased risk of fragility fractures.

Molecular Pathogenesis

The molecular basis of PHPT remains unclear in the majority of cases, despite major progresses on the mechanisms responsible for parathyroid tumorigenesis has been performed in the last decades. Most parathyroid tumors are monoclonal, that is, the tumor is caused by an acquired genetic defect in key genes conferring to a single parathyroid cell a selective growth advantage. Conversely, hyperplastic parathyroid cells have a polyclonal origin, even though monoclonality has been shown in some cases. Two categories of genes controlling the growth of parathyroid cells and PTH synthesis/secretion may be involved: oncogenes and tumor-suppressor genes. Proto-oncogenes have a role in the control of cell proliferation, cell differentiation, and cell death. Several mechanisms are responsible for the conversion of a proto-oncogene to an oncogene, resulting in either an increased expression of its encoded protein or the synthesis of a new functioning protein. Most oncogenes bare dominant mutations and therefore abnormality of a single copy of the gene is sufficient for expression of the growth trait. Conversely, tumor suppressor genes encode for proteins that normally inhibit cell proliferation and one normal copy of the gene suffices to control cell proliferation. Therefore, inactivation of both copies of the genes is required for uncontrolled cell growth.

Germline mutations are commonly detected in hereditary forms, whereas somatic mutations may be found in sporadic cases, even though germline mutations have also been described. The studies of clonal DNA defects and inherited tumor predisposition syndromes have given a great contribution in the identification of genes involved in parathyroid tumorigenesis. The genetic abnormalities and the clinical manifestations are reported in **Table 1**.

Cyclin D1 gene

The cyclin D1 gene (*CCND1*) (initially named *PRAD1*) encoding cyclin D1 is an oncogene that is activated by a pericentromeric inversions of chromosome 11 that place the *CCND1* gene near the regulatory sequences upstream of the PTH gene. Thus, since PTH is highly expressed in parathyroid cells, the rearrangement leads to overexpression of cyclin D1 mRNA and protein, which cause parathyroid cell proliferation. This rearrangement has been found in up to 8% of sporadic parathyroid adenomas whereas cyclin D1 overexpression in 20%–40% of these tumors.

Multiple endocrine neoplasia type 1 (MEN1) gene

The multiple endocrine neoplasia type 1 (*MEN1*) tumor suppressor gene, at 11q13 encoding menin, is a "classic" tumor suppressor gene contributing to a cell's advantage through its biallelic inactivation. *MEN1* mutations (including large deletions) are found in up to 90% of familial MEN1 syndrome and are scattered in the coding regions of the gene. Up to 10% of patients may have de novo germline mutations. MEN1 mutations are also detected in up to 27% of familial isolated primary hyperparathyroidism (FIHP). In sporadic parathyroid adenomas, the loss of one *MEN1* allele has been detected in 20%–40% of cases together with a somatic inactivating mutation of the other allele in about 50% of these tumors.

Cyclin-dependent kinase inhibitor 1B (CDKN1B)/cyclin dependent kinase inhibitor (CDKI) gene

Germline mutations of the *CDKN1B* gene at 12p13.1, encoding the $p27^{kip1}$ cyclin dependent kinase inhibitor (CDKI), are responsible for the multiple endocrine neoplasia type 4 (MEN4). The most common phenotype of the 19 MEN4 syndrome described to date is represented by PHPT and pituitary adenomas. Somatic *CDKN1B* mutations have been identified in seven

Table 1 Hereditary forms of PHPT

Disorder (OMIM)	*Chromosomal locus*	*Gene (protein)*	*Pattern of inheritance*	*Clinical manifestations*
MEN 1 (131100)	11q13.1	*MEN 1* (menin)	Autosomal dominant	PHPT (95%), pancreatic neuroendocrine tumors (40%), anterior pituitary tumors (30%); less frequently adrenal adenoma, angiofibromas, carcinoid, collagenomas, lipomas
MEN 2A (171400)	10q11.21	*RET*	Autosomal dominant	Medullary thyroid carcinoma (90%), pheochromocytoma (50%), PHPT (20%)
MEN 4 (610755)	12p12.1	*CDKN1B* (p27)	Autosomal dominant	MEN-1 associated tumors in association with MEN 1-associated or nonassociated tumors (adrenal, thyroid, gonadal, renal)
HPT-JT (145001)	1q31.2	*CDC73* (parafibromin)	Autosomal dominant	PHPT (80%) due to parathyroid carcinoma (15%), ossifying fibromas of mandible and maxilla (30%), renal lesions and uterine, testicular, and thyroid tumors
FIHP (145000)	11q13.1, 1q31.2, 3q13.3–q21.1 and 6p24.2	*MEN1, CDC73, CaSR, GCM2*	Autosomal dominant	PHPT alone, without other manifestations typical of a syndromic form

Abbreviations: *OMIM*, online Mendelian inheritance in man; *MEN*, multiple endocrine neoplasia; *HPT-JT*, hereditary hyperparathyroidism-jaw tumor; *RET*, rearranged during transfection; *CDKN1B*, cyclin dependent kinase inhibitor 1B; *CaSR*, calcium sensing receptor; *GCM2*, glial cells missing homolog 2.

apparently sporadic parathyroid adenomas; one of them also harbored loss of heterozygosity (LOH) at 12p13.1. In five of seven cases, the mutation was germline. Biallelic loss of the *CDKN1B* is very rare in parathyroid tumors suggesting that this gene is a haploinsufficient tumor suppressor, but loss of p27 protein expression is a frequent finding.

No mutations of *CDK1C*, *CDKN2A*, or *CDKN2D* have been detected in sporadic parathyroid adenomas. Point mutations in *CDKN1A*, *CDKN2B*, or *CDKN2C* were detected in five parathyroid adenomas; all but one was germline or of undetermined germline/somatic status. Hypermethylation of *CDKN2A* and *CDKN2C* has also been found in parathyroid adenomas.

Cell division cycle 73 (CDC73) gene

Inactivating germline mutations of the tumor suppressor *CDC73* gene, located at 1q31 encoding parafibromin, are responsible for the hyperparathyroidism-jaw tumor (HPT-JT) syndrome. The mutations are scattered along the entire coding region of the gene although most are located in exons 1, 2, and 7. The majority of the mutations are nonsense leading to a truncate parafibromin protein. Germline *CDC73* mutations are also found in up to 15% of FIHP. Somatic mutations of this gene have also been reported in up to 70% of patients with sporadic parathyroid carcinoma, and in about one-third of them the mutation is germline. *CDC73* mutations are very rare in sporadic parathyroid adenoma.

PRUNE2 *gene*

Germline and somatic mutations of the prune homolog 2 (Drosophila) (*PRUNE2*) gene have been detected in 18% of patients with parathyroid carcinoma.

*Calcium-sensing receptor (*CaSR*) gene*

Loss-of-function mutations of the *CaSR* gene are responsible for familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) while activating mutations are detected in patients with autosomal dominant hypocalcemia. Loss-of-function mutations lead to a reduction of the sensitivity to extracellular calcium with alterations of the calcium-set-point in parathyroid cells. Patients with FHH and NSHPT develop little or severe parathyroid hyperplasia, respectively suggesting that *CaSR* may functions as a tumor suppressor gene. Mutations of *CaSR* gene have been detected in up to 7% of FIHP. However, no somatic mutations of *CaSR* gene have been found in sporadic parathyroid adenomas. Of note, a reduced expression of CaSR mRNA and protein has been detected in these tumors.

*Catenin B-1 (*CTNNB1*) gene*

Contradictory results have been reported on the role of mutations in the *CTNNB1*, the gene encoding for β-catenin. Almost 600 parathyroid adenomas have been studied for the presence of *CTNNB1* mutations with negative results. Two studies have identified a somatic homozygous stabilizing mutation in exon 3 of *CTNNB1* gene leading to a change of a serine to alanine at amino acid 37 (S37A) in up to 7% parathyroid adenomas. Discrepant results have also been reported on the Beta-catenin staining.

Clinical Presentation

In Western countries the "classical" symptomatic form of PHPT, characterized by hypercalcemia, renal (nephrolithiasis and nephrocalcinosis) and skeletal (osteitis fibrosa cystica, fragility fractures) manifestations, is rarely seen (Fig. 1). Nowadays, most patients diagnosed with PHPT lack these manifestations and present with subtle and nonspecific clinical features, like weakness, easy fatigability, anxiety, and mood alteration (asymptomatic PHPT). Nephrolithiasis is still the most common of the classic clinical manifestations (15%–20%), and silent stones are frequently detected at ultrasound. *Osteite fibrosa cistica* is very rarely observed and when present is associate with severe disease. Skeletal involvement can be documented by measurement of bone mineral density (BMD), which is typically reduced at sites rich in cortical bone (one third distal radius). Studies using peripheral high resolution quantitative CT has recently shown that both cortical and trabecular sites are targets of excess PTH. Trabecular bone involvement has recently been confirmed by the trabecular bone score (TBS), which shows reduced values at the lumbar spine. Fracture rate at vertebral and nonvertebral is increased in patients with PHPT. A recent prospective study has shown an increased rate of morphometric vertebral fractures in postmenopausal women with asymptomatic PHPT (Fig. 2), supporting the recommendation to perform vertebral imaging in patient's workout.

The clinical manifestation of the hereditary forms of PHPT are summarized in Table 1.

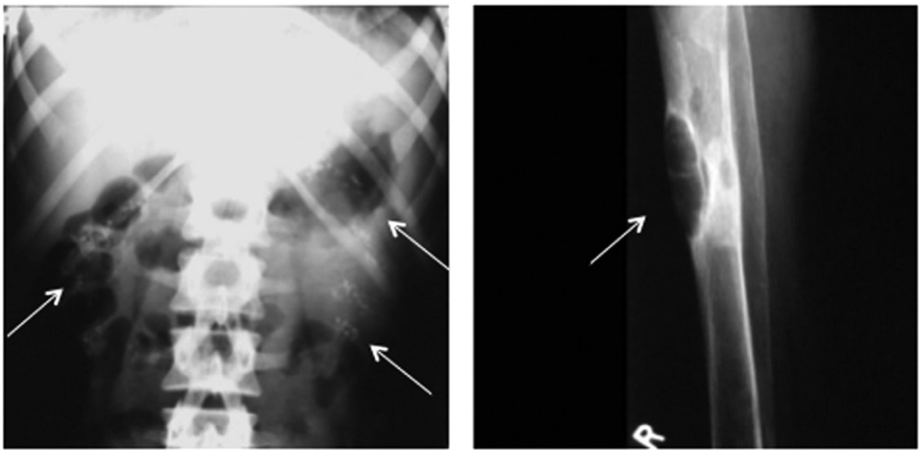

Fig. 1 *Left panel*: Bilateral nephrocalcinosis (*arrows*); *right panel*: *Osteite fibrosa cystica*.

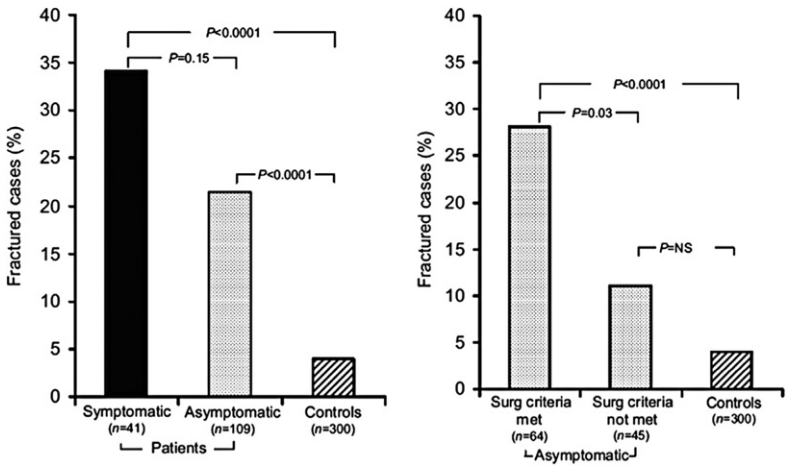

Fig. 2 Rate of morphometric vertebral fractures in postmenopausal women with PHPT. *Left panel*: Patients with symptomatic and asymptomatic PHPT and controls. *Right panel*: patients with asymptomatic PHPT, grouped according to whether they met or did not meet the criteria for parathyroidectomy and controls. *P* values refer to Odds ratios between different groups. Reproduced from Vignali, E., Viccica, G., Diacinti, D., Cetani, F., Cianferotti, L., Ambrogini, E., Banti, C., Del Fiacco, R., Bilezikian, J.P., Pinchera, A., Marcocci, C. (2009). Morphometric vertebral fractures in postmenopausal women with primary hyperparathyroidism. *The Journal of Clinical Endocrinology and Metabolism* 94, 2306–2312, with permission.

Nonclassical Manifestations

Cardiovascular abnormalities observed in "classical" PHPT, namely valvular and vascular calcification and left ventricular hypertrophy, are rarely seen. Hypertension is still rather common and subtle cardiovascular involvement (i.e., arrhythmias, increased vascular stiffness, and endothelial dysfunction) may be present in the patients with the modern form of PHPT. Increased cardiovascular mortality is present in patients with moderate to severe PHPT and persists after successful parathyroidectomy (PTx). Conversely, most, but not all studies indicate that patients with asymptomatic PHPT do not share this risk.

Psychological and cognitive complaints, like weakness, easy fatigability, depression, anxiety, and related symptomatology are common but not specific and can be present in many chronic diseases. Observational studies on cognitive function have been inconsistent and data on quality of life have recorded in three randomized trials shown either no difference or some degree of deterioration in psychological domain and mental component summary of the SF-36 between patients with asymptomatic PHPT and controls. PTx was associated with improvement in some SF-36 domains but the results varied among the three studies. Other studies have also shown that successful parathyroid surgery is associated with improvement in neurocognitive function, depressive and anxiety symptoms, and quality of life.

Normocalcemic PHPT

A new variant of PHPT has recently been identified, namely subject with persistently elevated serum PTH associated with repeatedly normal albumin-adjusted and ionized serum calcium (normocalcemic PHPT). This entity can only be detected if PTH is measured in individuals with normal serum calcium and can only be diagnosed when all other causes of secondary hyperparathyroidism (Table 2), particularly low vitamin D deficiency/insufficiency, have been excluded in in the diagnostic workout of patients with initial biochemical features of normocalcemic PHPT. In this regard a cut-off value of serum 25(OH)D >30 ng/mL should be taken. In borderline cases retesting after vitamin D and calcium supplementation may be advisable and may lead in some cases to the classical hypercalcemic PHPT. Other causes of secondary hyperparathyroidism, such renal failure, gastrointestinal diseases associated with malabsorption, hypercalciuria, and other metabolic bone diseases that could affect PTH levels (e.g., Paget's disease) should be considered and ruled out. Finally, the use of medications, which might affect PTH levels or calcium metabolism (estrogens, thiazide diuretics, lithium, bisphosphonates, denosumab, and anticonvulsants) should also be excluded. The epidemiology of normocalcemic PHPT has been investigated in various populations, often in selected gender and age groups. Data collected in nonselected populations indicate a prevalence of 0.4%–16.7%, using different biochemical cut off values for its diagnosis. This new phenotype may represent an early phase of mild hypercalcemic PHPT, but in some cases it may progress to a more severe form with nephrolithiasis, rapid loss of bone mass and factures. Finally, in other cases the biochemical and clinical profile of normocalcemic PHPT has remained stable over a few years of follow up. Successful PTx followed by decrease of serum calcium and normalization of PTH has been reported.

Evaluation and Diagnosis

The finding of hypercalcemia on routine blood testing or in postmenopausal women investigated for osteoporosis is typically the first clue to the diagnosis of PHPT. Hypercalcemia, preferably albumin-corrected total calcium (measured total calcium in

Table 2 Causes of secondary hyperparathyroidism[a]

Decreased intestinal calcium absorption
Vitamin D deficiency (serum 25OHD, <30 ng/mL)
Low calcium intake
Malabsorption
Bariatric surgery
Chronic pancreatitis
Small bowel diseases
Renal failure (eGFR, <60 mL/min)
Hypercalciuria
Drugs
Loop diuretics
Thiazide diuretics
Lithium (short term use)
Anticonvulsant
Antiresorptive agents
Bisphosphonates
Denosumab

[a]Condition characterized by an increased secretion of PTH in response to a physiologic stimulus (hypocalcemia) by the parathyroid glands.
eGFR, estimated glomerular filtration rate.

mg/dL + [0.8 × (4.0 − serum albumin in g/dL)] should be confirmed in repeated testing. It is important to remind that in mild cases of PHPT serum calcium levels may be in the (upper) normal range in repeated testing. Measurement of ionized calcium may be useful in selected patients either to discover those who have PHPT but normal serum calcium as well as to recognize those with artifactual hypercalcemia (hyperalbuminemia, thrombocytosis, Waldstrom's macroglobulinemia, and myeloma) in whom ionized calcium is normal. Serum PTH should then be measured and the finding of an elevated (or unexpectedly normal) concentration associated with hypercalcemia are virtually diagnostic of PHPT. It should be kept in mind that a similar biochemical profile could be observed in patients treated with lithium or thiazide diuretics, or affected by tertiary hyperparathyroidism and FHH. A careful medical history could help to rule out these possibilities. A low/undetectable serum PTH in a hypercalcemic patient excluded the diagnosis of PHPT and suggests a form of PTH-independent hypercalcemia (**Table 3**). The most common cause is hypercalcemia of malignancy, in which hypercalcemia is due to lytic bone metastases or by the secretion by the tumor of PTH-related peptide. This peptide shares with PTH the amino-terminal active part, but does not cross react with the PTH assay. The finding of increased PTH levels and consistently normal albumin-adjusted ionized serum calcium and no other causes of secondary hyperparathyroidism raise the possible diagnosis of normocalcemic PHPT (see before). The extremely rare possibility of parathyroid cancer should be suspected in patients, especially males, with markedly increased levels of serum calcium (>14 mg/dL) and PTH (3–10 times the upper normal limit).

Renal function should be evaluated and either creatinine clearance or eGFR calculated. Serum phosphate is normal or in the low-normal range in mild PHPT and low in severe cases. Twenty-four hours urinary calcium should also be measured and if calcium excretion is greater than 400 mg a more extended evaluation of the stone risk profile should be performed. Measurement of 24-h urinary calcium and creatinine levels is also of help in the differential diagnosis between PHPT and FHH, where the calcium to creatinine clearance ratio is usually <0.01, a finding never observed in PHPT. Bone turnover markers may be increased, particularly in patients with severe bone involvement. Vitamin D status should be assessed by measurement of serum 25(OD)D, which is frequently below the normal limit. Low 25(OH)D may further stimulate PTH secretion and account for further elevation of serum PTH.

Once the diagnosis of PHPT has been established, a hereditary form should be considered, particularly in patients aged less than 30 years and/or, a family history of hypercalcemia and neuroendocrine tumors (**Table 1**). In this setting serum calcium should be measured in first-degree relatives and genetic tests eventually performed.

Renal imaging by ultrasound or CT should be carried out in all patients, even in those with no history of nephrolithiasis, since silent kidney stones are common in patients with asymptomatic PHPT and occasionally detected also in patients with more severe form of PHPT. More rare is nephrocalcinosis (**Fig. 2**). Bone mineral density (BMD) by dual-energy X-ray absorptiometry (DXA) at

Table 3 Differential diagnosis of hypercalcemia

PTH-dependent
Sporadic PHPT
Hereditary PHPT
MEN 1
MEN 2
MEN 4
HPT-JT
FIHP
Tertiary hyperparathyroidism
Lithium
Thiazide diuretics
Paraneoplastic (ectopic PTH secretion)
PTH-independent
Malignancy
PTHrP
Osteolytic metastases
Multiple myeloma
Ectopic production of calcitriol
Granulomatous diseases
Lymphomas
Endocrine diseases
Hyperthyroidism
Adrenal insufficiency
Drugs
Vitamin D (active)
Sarcoidosis
Familial hypocalciuric hypercalcemia (FHH)

Abbreviations: *MEN*, multiple endocrine neoplasia; *HPT-JT*, hereditary hyperparathyroidism-jaw tumor; *FIHP*, familial isolated hyperparathyroidism; *PTHrP*, PTH-related peptide.

the lumbar spine, hip, and one-third distal radius should also be measured. Most patients show low values at the latter site, which is rich in cortical bone, with a relative preservation of bone mass the lumbar spine and to less extent at the hip, where the trabecular bone is more represented. However, high-resolution peripheral QCT and the trabecular bone score (TBS), which can be derived from the DXA data obtained at the lumbar spine, have clearly shown ultrastructure abnormalities at the cortical level. Vertebral imaging by X-ray or DXA [vertebral fracture assessment, VFA)] should also be routinely performed to detect silent vertebral deformities since vertebral fracture risk is increased also in asymptomatic patients. A more extensive X-ray study of the skeleton might be considered in patients with severe PHPT to detect *osteite fibrosa cystica*.

Parathyroid imaging studies (ultrasound and sestamibi scanning) has no values in the diagnosis of PHPT (**Fig. 3**, upper and central panels). Both are useful tools in patients selected for PTx to localize the abnormal parathyroid gland and particularly when the minimally invasive approach is used. Sestamibi scanning has the advantage to localize ectopic parathyroid gland outside the neck. In selected cases, particularly in remedial PTx, second-line imaging studies (CT and RM) should be considered (**Fig. 3**, lower panel).

Natural History

The natural history of PHPT varies according to its severity. Patients with symptomatic PHPT who either do not undergo surgery or are not cured by PTx usually have a progression of the disease, particularly recurrent kidney stones.

Three prospective clinical studies in which patients with asymptomatic PHPT were randomized to surgery of surveillance have shown improvement in the former, even if they did not meet the criteria for surgery, and stability in the latter over up to 2 years follow-up. Conversely, an increase, but not statistically significant, an increased rate of vertebral fractures was observed over 5 years in patients followed without surgery than in those cured by PTx. Long-term observational studies indicate that the disease remains stable in the majority for up to 8–9 years, but progression of the disease occurred in about one third of them, particularly in those with the longest follow up and younger age at the study entry.

Calcium intake should not be restricted and be similar to that recommended in the general population (preferably with food). Patients with a low vitamin D status should be supplemented with daily doses of 800–1000 IU of vitamin D or, alternatively, with weekly or monthly doses calculated on this daily doses. A serum level of 25(OH)D $>$ 20 ng/mL should be reached, but a higher target ($>$30 ng/mL) is suggested by some experts.

General principles for monitoring are reported in **Table 4**. Serum calcium and creatinine (eGFR) should be measured annually and BMD at lumbar spine, total hip and distal forearm measured every 1–2 years. Vertebral morphometry (by X-ray or VFA) should be performed in patients with back pain or height loss. If nephrolithiasis is suspected, 24-h urinary collection for biochemical stone risk profile and renal imaging (X-ray, ultrasound or CT) should be performed.

Parathyroid surgery should be recommended in the following instances: (i) increase of serum ($>$1 mg/dL above the upper normal limit); (ii) decrease of creatinine clearance $<$60 cm^3/min; (iii) discovery of nephrocalcinosis or kidney stones; (iv) decrease of BMD *T* score at any site $<$2.5 or greater than the least significant change (as defined by the International Society for Clinical Densitometry, even if the *T* score is not in the osteoporotic range); (v) occurrence of clinical fractures or morphometric vertebral fractures, even if asymptomatic.

Stability of serum calcium, creatinine, PTH, urinary calcium up to 10 years and BMD up to 8 years has been shown in patients with asymptomatic PHPT. Progression of the disease (worsening of hypercalcemia and decrease of BMD at the one-third distal radius and hip) has been reported in about one third of cases and is more likely to occur in patients with the longest follow up and younger age at the study entry.

Treatment

The aim of treatment is to normalize biochemical abnormalities and improve, and eventually completely revert the clinical manifestations of the disease.

Surgical Treatment

PTx represents the only definitive cure of PHPT. It is appropriate to consider for any patient with a confirmed diagnosis of PHPT and should be recommended in those with symptomatic disease. When successful PTx is followed by normalization of serum calcium and PTH, improvement of BMD and reduction of the risk of nephrolithiasis and fragility fractures. In experienced hands the rate of cure reaches 95%–98% with a negligible rate of surgical complications. It is therefore important that patients selected for surgery should be referred to an experienced parathyroid surgeon. A focused minimally invasive approach, guided by imaging studies associated with intraoperative monitoring of serum PTH, is the most commonly used surgical procedure in sporadic PHPT. In hereditary cases bilateral neck exploration by classical cervicotomy is still the recommended procedure since multigland involvement is common in these cases. In the rare cases where there is the suspicion of parathyroid carcinoma the "en-block" resection of the parathyroid lesion together with the ipsilateral thyroid lobe and the adjacent structures is recommended. During surgery, particular attention should be paid to avoid seeding of neoplastic parathyroid cells.

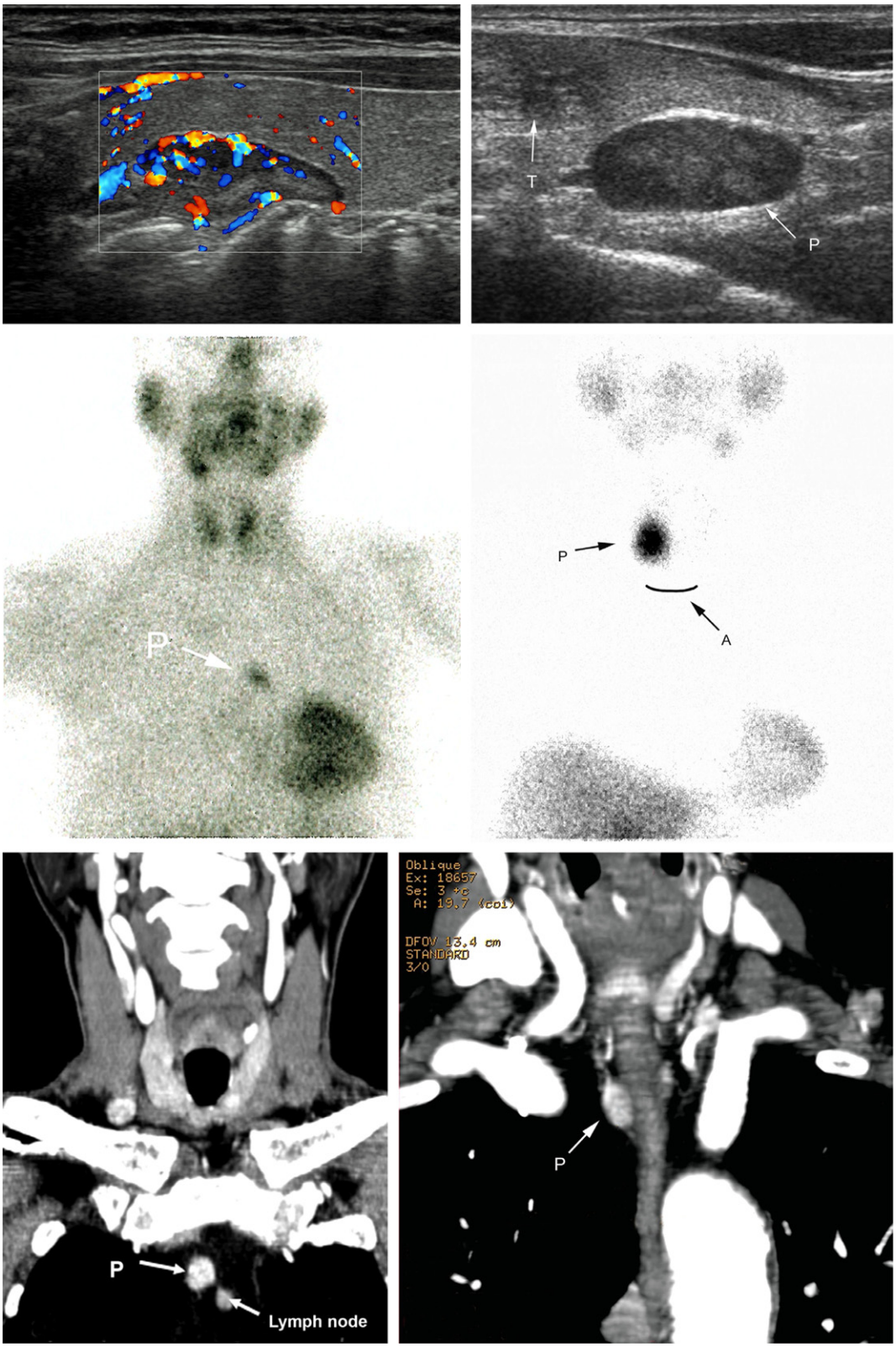

Fig. 3 Parathyroid imaging. *Upper panels*: Ultrasound. *Left:* Typical hypoechoic pattern and shape of a lower parathyroid adenoma (P) adjacent to the thyroid lobe (T). *Right*: Color power Doppler ultrasound pattern of a parathyroid adenoma, showing a diffuse intraparenchymal color flow signals. *Central panels*: ^{99}m-technetium-sestamibi scanning. *Left*: parathyroid adenoma (P) in the right lower neck; (A) indicates jugular dimple. *Right*: Parathyroid adenoma (P) located in the upper mediastinum (P). *Lower panels*. *CT scan*. *Left*: Coronal section showing a paraesophageal parathyroid adenoma (P). *Right*: Coronal section showing an ectopic parathyroid adenoma in the upper mediastinum (P) and an adjacent lymph node (L).

Patients with mild hypercalcemia [serum calcium <12 mg/dL (3 mmol/L) can proceed directly to surgery, following standard procedures. Conversely, when serum calcium is higher patients should receive preoperative treatment with iv saline eventually followed by loop diuretics, intravenous bisphosphonates, denosumab or cinacalcet to reduce serum calcium levels and, therefore decrease the risk of complications, particularly cardiac arrhythmias.

Surgery is also a reasonable option for patients with asymptomatic PHPT. Over the last 25 years four International workshops have provided guidance to select patients who should be referred for surgery or followed without surgery. The guidelines for surgery or monitoring according to the most recent guidelines are reported in **Table 5**. These guidelines mostly reflect the previous ones. New indications for surgery include: (i) silent vertebral fractures detected by X-ray, CT, MRI or DXA (vertebral fracture assessment, VFA), (ii) 24-h urinary calcium excretion >400 mg/dL and increased stone risk (evaluated by urinary stone risk profile) and subclinical kidney stone or nephrolcalcinosis detected by X-ray, ultrasound, and CT. PTx should be recommended to patients who meet one or more surgical criteria and it is estimated that about 60% of patients with asymptomatic PHPT meet the criteria for surgery. As mentioned before, some patients with mild PHPT may have subtle cardiovascular abnormalities and complain of psychological and cognitive symptoms. It is unclear whether and to what extent these abnormalities will be reversed after successful PTx; for the time being they are not considered as an indication for surgery. Nonetheless, in some cases the presence of one or more of these nonclassical manifestations associated with increased cardiovascular risk (metabolic syndrome, hypertension, ischemic heart disease) may contribute to advise surgery, if surgical risk is low. Randomized clinical trials have shown that also patients with sporadic asymptomatic PHPT and no surgical criteria may benefit from surgery, not only because it normalizes serum calcium and PTH, but also improves BMD and quality of life (QoL).

Particular attention should be paid in patients with hereditary forms of PHPT. In these patients multigland disease is common, occasionally with asynchronous involvement of the various parathyroid glands. Bilateral neck exploration, the classical surgical approach, carries an increased surgical risk and an increased recurrence/persistence of PHPT. Because of that in patients with mild hypercalcemia surgery can be temporarily postponed.

It is important to discuss with each individual patient the benefits and risks of PTx and the preference of the patients should be taken into account. Parathyroid surgery in experienced hands is safe, cost-effective, and associated with low perioperative morbidity. On the other hand, when performed by inexperienced surgeons, it may be associated with complications that could not be acceptable for a subject with a mild disease and a good QoL. In this regard, an initial advice of surgery may be reconsidered when preoperative imaging studies are negative in a patient with mild asymptomatic PHPT. As a matter of fact, under these circumstances the patient may feel uncomfortable to undergo a "blind neck exploration," which is a more extensive and risky surgical procedure.

Table 4 2013 guidelines for monitoring in patients with asymptomatic PHPT

- Serum calcium annually
- BMD (by DXA) at lumbar spine, hip or distal one-third distal radius every 1–2 years
- Vertebral fractures evaluation by X-ray, CT, MRI or VFA if clinically indicated (i.e., back pain, high loss)
- Serum creatinine (eGFR) annually
- 24-h urine biochemical stone risk profile, renal imaging by X-ray, ultrasound or CT if renal stones are suspected

Abbreviations: *BMD*, bone mineral density; *DXA*, dual X-ray absorptiometry; *CT*, computerized tomography; *MRI*, magnetic resonance imaging; *VFA*, vertebral fracture assessment (by DXA); *eFGR*, estimated glomerular filtration rate.

Bilezikian, J.P., Brandi, M.L., Eastell, R., Silverberg, S.J., Udelsman, R., Marcocci, C., Potts, J.T. Jr. (2014). Guidelines for the Management of Asymptomatic Primary Hyperparathyroidism: Summary statement from the fourth international workshop. The Journal of Clinical Endocrinology & Metabolism 99, 3561–3569.

Table 5 Guidelines for parathyroidectomy in patients with asymptomatic PHPT

- Age <50 years
- Serum calcium concentration >1 mg/dL above the upper normal limit
- BMD *T*-score (by DXA) ≤ -2.5 at lumbar spine, hip or one-third distal radius
- Vertebral fractures by X-ray, CT, MRI, or VFA
- Creatinine clearance <60 mL/min
- 24-h urine calcium >400 mg (10 mmol) and increased biochemical stone risk profile
- Nephrolithiasis or nephrocalcinosis by X-ray, ultrasound, or CT

Abbreviations: *BMD*, bone mineral density; *DXA*, dual X-ray absorptiometry; *CT*, computerized tomography; *MRI*, magnetic resonance imaging; *VFA*, vertebral fracture assessment (by DXA).

Bilezikian, J.P., Brandi, M.L., Eastell, R., Silverberg, S.J., Udelsman, R., Marcocci, C., Potts, J.T. Jr. (2014). Guidelines for the Management of Asymptomatic Primary Hyperparathyroidism: Summary statement from the fourth international workshop. The Journal of Clinical Endocrinology & Metabolism 99, 3561–3569.

Nonsurgical Management

Most patients with asymptomatic PHPT do not meet the criteria for PTx. These patients as well as those who decline surgery or have contraindications to surgery need to be monitored and eventually treated medically.

Medical treatment

Currently there is no effective medical treatment of PHPT, but there are options to improve some manifestations of the disease. Medical treatment may be considered in patients with contraindications to surgery or are unwilling to undergo PTx, as well as in those in whom surgery was unsuccessful. The choice of treatment should be guided by the aim of therapy.

Calcimimetics

The calcimimetic cinacalcet can be used to decrease serum calcium. Cinacalcet has been approved by the Food and Drug administration (FDA) for the "treatment of severe hypercalcemia in patients with PHPT who are unable to undergo PTx," and by the European Medical Agency (EMA) for the "reduction of hypercalcemia in patients with PHPT, for whom PTx would be indicated on the basis of serum calcium levels (as defined by relevant guidelines), but in whom surgery is not clinically appropriate or contraindicated." Serum calcium typically declines over a few weeks and may normalizes in most patients with moderate hypercalcemia. In addition, cinacalcet increases serum phosphate, slightly decreases PTH, but has no effect on BMD. Adverse events are dose-related: modest and transient when low doses of cinacalcet are used (up to 30–60 mg twice daily), but rather frequent, particularly gastrointestinal symptoms, and occasionally severe to require treatment withdrawal, when higher doses are employed, such as in patients with parathyroid carcinoma. The benefits of cinacalcet are sustained over time as long as the drug is used. Long-term safety of cinacalcet has not been established. Its rather high cost is another important limit for its long-term use.

Bisphosphonates

If the aim is to increase BMD antiresorptive therapy can be considered. Alendronate is the bisphosphonate that has been more extensively used and shown to be effective in reducing bone turnover markers and increase BMD. In a 2-year placebo-controlled study patients treated with alendronate (10 mg/daily) showed an increase of BMD at the lumbar spine and hip (6.8% and 4.1%, respectively), associated with a sustained decline of urinary NTX and bone-specific alkaline phosphatase. Serum total and ionized calcium and PTH and urinary calcium excretion did not change. Similar results have been observed in men. Over a short period of time the increase of BMD is comparable to that observed after successful PTx.

Combined therapy with cinacalcet and bisphosphonates

If a patient has a low BMD and serum calcium concentration appropriate for the use of cinacalcet, is reasonable to use combined therapy with bisphosphonates and cinacalcet. Indeed, limited data demonstrate that the combined therapy is associated with both the calcium lowering effect of cinacalcet and the skeletal advantage of bisphosphonates.

Pregnancy

PHPT can occasionally be diagnosed in pregnancy by the finding of hypercalcemia on routine blood testing. It is important to keep in mind that there are some distinctive features in the diagnosis and management of PHP during pregnancy (i) serum ionized calcium should preferably be measured because pregnancy is associated with hemodilution and occasionally hypoalbuminemia; (ii) the possibility of a hereditary form of PHPT should be sought because of the young age of pregnant women; (iii) a conservative approach, mostly hydration, should be considered in women with mild elevation of calcium; in the case of more severe hypercalcemia PTx in the second trimester and, in selected cases, cinacalcet (nonapproved in pregnancy) may be considered.

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Parathyroid Cancer☆

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Glossary

Bisphosphonates A group of drugs that inhibit osteoclast-mediated bone resorption.

Calcimimetics Allosteric stimulators of the calcium receptor that have been shown to lower serum parathyroid hormone and calcium concentrations in patients with primary hyperparathyroidism.

Hungry bone syndrome A period of hypocalcemia that ensues after successful parathyroidectomy, during which calcium is rapidly deposited into bone and usual intake may not be sufficient to maintain serum calcium within the normal range.

hypercalcemia Elevation of serum calcium to a point above the upper limit of normal for the particular laboratory performing the measurement.

Positron emission tomography A clinical technique that is reported to successfully localize parathyroid cancer deposits.

Preoperative localization Tests to localize the abnormal parathyroid gland prior to embarking on parathyroid exploration. The most common approaches are sestamibi scanning and ultrasonography.

Parathyroid carcinoma is an unusual neoplasm is characterized by a distinctive clinical profile that differs in several respects from that of hyperparathyroidism due to a benign parathyroid adenoma, which commonly presents with mild asymptomatic hypercalcemia (Shane and Bilezikian, 1982, Cohn *et al.*, 1985, Heath *et al.*, 1980, Silverberg *et al.*, 1989, Silverberg *et al.*, 1990, Parisien *et al.*, 1990). In this article, the clinical features, natural history, pathological features, diagnosis, and prognosis of parathyroid cancer are reviewed. Surgical approaches to parathyroid cancer are outlined as well as medical therapies of the hypercalcemia that accompanies recurrent or metastatic disease.

Incidence

In most series, parathyroid cancer accounts for less than 1% of patients with primary hyperparathyroidism (Brown *et al.*, 2011; Cohn *et al.*, 1985; Cryns *et al.*, 1994a; Hakaim and Esselstyn, 1993; Holmes *et al.*, 1969; Sandelin *et al.*, 1991; Schantz and Castleman, 1973; Shane and Bilezikian, 1982; Wang and Gaz, 1985; Wynne *et al.*, 1992) and 0.05% of all cancers (Hundahl *et al.*, 1999). It is possible that parathyroid cancer is somewhat more common in Japan and perhaps also in Italy, accounting for 5% of patients operated on for primary hyperparathyroidism in series reported from these countries (Fujimoto and Obara, 1987; Obara and Fujimoto, 1991; Obara *et al.*, 1993, 1997; Yoshimoto *et al.*, 1998). Approximately 400 cases of parathyroid carcinoma were reported in the English literature between 1930 and 1999. In 1999, the National Cancer Data Base reported 286 cases of parathyroid carcinoma (Hundahl *et al.*, 1999). In a recent study based upon the Surveillance, Epidemiology, and End Results (SEER) cancer registry, the incidence of parathyroid cancer increased between 1988 and 2003 (Lee *et al.*, 2007); 224 cases of parathyroid cancer were reported over the 16 years, with an incidence (per 10 million population) of 3.58 between 1988 and 1991 compared to 5.73 between 2000 and 2003. In addition, an Australian retrospective case series reported that 3 parathyroid carcinoma cases were reported between 1958 and 1990, while 11 were reported between 2001 and 2010 (Brown *et al.*, 2011). Possible reasons for this apparent rise in the incidence of parathyroid cancer include increased serum calcium screening, changing indications and increased surgical referrals for parathyroidectomy for asymptomatic hyperparathyroidism, and improvements in histopathological diagnosis.

Etiology

The etiology of parathyroid cancer is unknown. No clear pattern of predisposing factors has emerged in the cases that have been described. However, several clinical situations may predispose to the development of parathyroid cancer. Several cases of parathyroid carcinoma have been reported in patients with a history of neck irradiation (Christmas *et al.*, 1988; Ireland *et al.*, 1985; Mashburn *et al.*, 1987). Parathyroid cancer has also been reported within an adenoma or a hyperplastic parathyroid gland (Aldinger *et al.*, 1982;

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Berland *et al.*, 1982; Desch *et al.*, 1984; Guazzi *et al.*, 1982; Haghighi *et al.*, 1983; Kramer, 1970; Murayama *et al.*, 1977; Parham and Orr, 1987), in the setting of end-stage renal disease (see below), and in association with prolonged secondary hyperparathyroidism due to celiac disease (Boyle *et al.*, 1999). Despite these associations, Shantz and Castleman, in an extensive review of 70 cases, found no evidence for malignant transformation of previously pathologic tissue (Schantz and Castleman, 1973).

Parathyroid cancer has been reported in patients with familial hyperparathyroidism (Dinnen *et al.*, 1977; Kakinuma *et al.*, 1994; Leborgne *et al.*, 1975; Mallette *et al.*, 1974; Streeten *et al.*, 1992; Yoshimoto *et al.*, 1998), particularly those with autosomal dominant isolated hyperparathyroidism that is not part of the multiple endocrine neoplasia type 1 (MEN1) syndrome (Wassif *et al.*, 1993). In one such family, there was no evidence of antecedent hyperplasia in unaffected glands and chromosomal abnormalities commonly observed in other solid tumors were identified (a reciprocal translocation between chromosomes 3 and 4, trisomy 7, and a pericentric inversion in chromosome 9; Streeten *et al.*, 1992). Analyses of tumor DNA from one family member with parathyroid carcinoma showed no evidence of *ras* gene mutations, PTH gene arrangement, or allelic loss from chromosome 11q13, the locus of the gene for MEN1. MEN1 is most commonly associated with benign parathyroid disease, but there have been several reports of parathyroid cancer in patients with MEN1 (Agha *et al.*, 2007; del Pozo *et al.*, 2011; Dionisi *et al.*, 2002; Sato *et al.*, 2000; Shih *et al.*, 2009). The risk of parathyroid carcinoma is also greatly increased (15% prevalence) in patients with the hyperparathyroidism–jaw tumor syndrome (HPT-JT) (Dinnen *et al.*, 1977; Kakinuma *et al.*, 1994; Wassif *et al.*, 1993), which results from inactivating germline mutations in the *Hyperparathyroidism 2* gene (HRPT2) gene (Carpten *et al.*, 2002) (**Table 2**).

In 1996, Miki *et al.* reported a case of parathyroid cancer in a patient with end-stage renal disease and also reviewed 12 additional cases published between 1982 and 1996 (Miki *et al.*, 1996). Hyperplasia of the other parathyroid glands was present in all (Berland *et al.*, 1982; Greenberg *et al.*, 1989; Ireland *et al.*, 1985; Iwamoto *et al.*, 1990; Kodama *et al.*, 1989; Krishna *et al.*, 1989; Miki *et al.*, 1996; Sherlock *et al.*, 1985) and one had a history of prior neck irradiation (Ireland *et al.*, 1985). The diagnosis was made an average of 6 years after the start of hemodialysis. In all cases, parathyroid carcinoma was diagnosed during or after parathyroidectomy on the basis of local invasion ($n=5$), tumor pathology ($n=4$), or distant metastases ($n=2$). Hypercalcemia was less severe than usually observed in patients with parathyroid carcinoma and parathyroid hormone (PTH) levels were not unusually high for patients on maintenance hemodialysis. Only one patient died of hypercalcemia. The authors concluded that no preoperative features distinguished hemodialysis patients with parathyroid carcinoma from those with parathyroid hyperplasia and that the clinical course may be more benign because of the tendency for renal insufficiency to lower serum calcium levels.

Molecular Pathogenesis

Oncogenes and tumor suppressor genes have been implicated in the development of parathyroid tumors. In the 21st century, there have been significant advances in the understanding of the molecular pathogenesis of parathyroid carcinoma. In 2002, Carpten *et al.* reported a germline mutation in the tumor suppressor gene, HRPT2, which is located at 1q25-q31 in hyperparathyroidism–jaw tumor syndrome (HPT-JT) (Carpten *et al.*, 2002). HRPT2 encodes the protein, parafibromin (referring to the parathyroid tumors and jaw fibromas) and acts as a gene transcription regulator. In 2003, Howell *et al.* were the first to describe the role of HRPT2 in sporadic parathyroid carcinoma (Howell *et al.*, 2003). Shattuck *et al.* demonstrated HRPT2 mutations in 10 of 15 patients with sporadic parathyroid carcinoma; three patients were identified as having germline HRPT2 mutations, suggesting that patients with sporadic parathyroid cancer may instead have underlying HPT-JT syndrome (Shattuck *et al.*, 2003). HPT-JT syndrome and sporadic parathyroid cancer are now associated with inactivating germline and or somatic mutations in HRPT2 (Howell *et al.*, 2003; Shattuck *et al.*, 2003). Additionally, Krebs *et al.* reported that HRPT2 mutations were not present in 60 patients with sporadic parathyroid adenomas (Krebs *et al.*, 2005). Despite compelling evidence for HRPT2 mutations in the pathogenesis of parathyroid carcinoma, they are not universally detected and therefore are not an ideal screening test. However, assessing for HRPT2 mutations may prove helpful in the preoperative assessment of patients with hyperparathyroidism who have clinical features suggestive of parathyroid cancer (see below), in order to guide the initial surgical approach. Additionally, there is increasing evidence that mutations in MEN1 may also be involved in sporadic parathyroid cancer (Haven *et al.*, 2004, 2007).

Cyclin D1 or PRAD1 (parathyroid adenoma 1) is an oncogene located on chromosome 11q13; its protein product is a cell cycle regulator. Rearrangement of the cyclin D1 gene with the regulatory region of the PTH gene has been reported in 5% of parathyroid adenomas and the cyclin D1 oncoprotein is overexpressed in 18–40% of parathyroid adenomas (Hsi *et al.*, 1996; Tominaga *et al.*, 1999). In one study, overexpression of cyclin D1 protein was found in 90% of parathyroid cancers (Vasef *et al.*, 1999). However, it is uncertain whether overexpression of cyclin D1 protein is a causative or an associative phenomenon.

There is also strong evidence that inactivation of the tumor suppressor gene RB (retinoblastoma) on chromosome 13 may contribute to the development of parathyroid carcinoma. Together with cyclin D1, RB is important in cell cycle control. In one study, 11 parathyroid cancers lacked a RB allele and most had a complete absence of nuclear staining for the RB protein (Cryns *et al.*, 1994b). In contrast, only one parathyroid adenoma lacked the allele and none had abnormal staining for the RB protein. Several other investigators have also reported that allelic deletions on chromosome 13 are more common in parathyroid cancer than in benign primary hyperparathyroidism. These data strongly support the presence of a tumor suppressor gene on the long arm of chromosome 13, critical for the development of parathyroid carcinoma. However, as the deleted portion of chromosome 13 is large, it remains to be determined whether RB or a different gene on 13q will prove to be the primary causative tumor suppressor. Another important cell cycle regulator, the p53 tumor suppressor gene, does not appear to be a major contributor to the pathogenesis of parathyroid carcinoma (Cryns *et al.*, 1994a; Hakim and Levine, 1994).

Table 1 Parathyroid carcinoma and benign primary hyperparathyroidism: typical features

	Parathyroid carcinoma	*Primary hyperparathyroidism*
Female:Male ratio	1:1	3.5:1
Average age (years)	48	55
Asymptomatic	$<5\%$	$>80\%$
Serum calcium	>14 mg dl^{-1}	≤ 1 mg dl^{-1} above upper limit of normal
Parathyroid hormone	Markedly elevated	Mildly elevated
Palpable neck mass	Common	Rare
Renal involvement[a]	32–80%	4–18%
Skeletal involvement[b]	34–91%	$<5\%$
Concomitant renal and skeletal disease	Common	Rare

[a]Includes nephrolithiasis, nephrocalcinosis, and impaired renal function in the absence of any other etiology.
[b]Includes osteitis fibrosa, subperiosteal resorption, 'salt and pepper' skull, and diffuse osteopenia on plain radiographs.

Several locations have been identified for potentially important oncogenes or tumor suppressor genes that seem to be preferentially or exclusively found in parathyroid carcinomas as compared with adenomas. Tumor-specific gains or losses of chromosomal material suggest that oncogenes on chromosomes 1q, 5q, 9q, 16p, 19p, and Xq and tumor suppressor genes on chromosomes 1p, 3q, 4q, 13q, and 21q may be involved in the pathogenesis of parathyroid carcinoma.

General Considerations

The clinical features of parathyroid cancer are due primarily to the effects of excessive secretion of PTH rather than to infiltration of vital organs by the tumor (Shane and Bilezikian, 1982). Thus, signs and symptoms of hypercalcemia dominate the clinical picture in addition to symptoms of hyperparathyroid bone disease and renal involvement (renal insufficiency, nephrolithiasis, or nephrocalcinosis). It is important to consider parathyroid cancer in the differential diagnosis of parathyroid hormone-dependent hypercalcemia, particularly in symptomatic patients, as often the diagnosis of parathyroid carcinoma is made in retrospect when hypercalcemia recurs due to local spread or distant metastasis of the tumor. The best outcomes for patients with parathyroid cancer occur when there has been complete resection of the tumor at the time of the first surgery (Shane and Bilezikian, 1982). Several features of the patient with primary hyperparathyroidism should suggest a malignant rather than a benign disease (**Table 1**).

Demographic Features

Benign primary hyperparathyroidism is considerably more common in women than in men (3–4:1). In contrast, there is no association of gender with parathyroid carcinoma and the ratio of affected women to men is 1:1 in most reported series. Patients with primary hyperparathyroidism typically present in their fifties or sixties, whereas the average patient with parathyroid carcinoma is in his or her forties, approximately 10 years younger. However, two reviews (the Mayo Clinic experience (Wynne *et al.*, 1992) and the National Cancer Data Base (Hundahl *et al.*, 1999)) found that the average age of their patients was in the middle fifties. Thus, although it is reasonable to consider the possibility of parathyroid cancer when primary hyperparathyroidism is diagnosed in a man and or a younger individual, gender and age are of limited assistance in the setting of the individual patient.

Symptoms and Biochemical Characteristics

Benign primary hyperparathyroidism usually presents with mild hypercalcemia (within 1 mg dl^{-1} above the upper limit of normal) that is often asymptomatic and usually discovered incidentally (Heath *et al.*, 1980; Silverberg *et al.*, 1989). In contrast, the serum calcium level in parathyroid cancer is much higher, generally above 14 mg dl^{-1} or 3–4 mg dl^{-1} above the upper limit of normal (Shane and Bilezikian, 1982). Signs and symptoms of hypercalcemia, including fatigue, weakness, weight loss, anorexia, nausea, vomiting, polyuria, and polydipsia are almost always present. Bone pain, fractures, and renal colic are much more common when primary hyperparathyroidism is due to a parathyroid cancer than an adenoma. Extremely high levels of parathyroid hormone are unusual in benign primary hyperparathyroidism, where circulating concentrations are commonly less than twice the upper limit of normal. In contrast, parathyroid hormone levels in patients with parathyroid cancer are generally much higher, ranging from 3 to 10 times above the upper limit of normal. Oxyphil parathyroid adenomas may manifest some clinical features of parathyroid carcinoma, with markedly elevated serum calcium and PTH levels as well as large tumor sizes (Fleischer *et al.*, 2004). On the contrary, non-functional parathyroid carcinomas do not present with hypercalcemia or hyperparathyroidism, and often present with a neck mass (Wilkins and Lewis, 2009). Serum alkaline phosphatase activity is often frankly elevated in patients with parathyroid carcinoma, whereas levels are usually in the normal or upper-normal range in patients with benign primary hyperparathyroidism (Silverberg *et al.*, 1990). In patients with parathyroid cancer, there may be increased production of the N-terminal molecular form of PTH, distinct from hPTH(1-84); measurement of PTH by both third and second generation assays may be helpful in diagnosis, as the Third/Second Generation PTH Assay Ratio may be inverted (>1) (Cavalier *et al.*, 2010,

Table 2 Hyperparathyroidism–Jaw tumor syndrome (HPT-JT)

– Parathyroid tumors 1st presenting sign
– Ossifying fibromas of the mandible and maxilla
– Renal lesions → renal hamartomas and cystic kidney disease
– Uterine tumors

2014; Rubin *et al.*, 2007). Finally, patients with parathyroid carcinoma may have elevated levels of α- and β-subunits of human chorionic gonadotropin, whereas patients with primary hyperparathyroidism do not (Rubin *et al.*, 2008; Stock *et al.*, 1982).

Physical Findings

A palpable neck mass has been reported in 30–76% of patients with parathyroid carcinoma, but is distinctly unusual in benign primary hyperparathyroidism (Levin *et al.*, 1988). This important clinical finding constitutes another striking difference between benign and malignant parathyroid disease. Recurrent laryngeal nerve palsy in a patient with primary hyperparathyroidism who has not had previous neck surgery suggests local spread of the tumor and is also very suggestive of parathyroid cancer. In a patient with characteristics suggestive of malignancy in whom HPT-JT is considered in the differential diagnosis, other components of the syndrome must be addressed (**Table 2**). For such patients, transvaginal ultrasound in women to evaluate for uterine tumors, jaw radiography to evaluate for ossifying fibromas, and abdominal ultrasound to evaluate for renal tumors should be included in the preoperative evaluation (Schulte and Talat, 2012).

Target Organ Involvement

The classical target organs of parathyroid hormone – namely, the kidney and the skeleton – are affected with greater frequency and severity in parathyroid carcinoma than is typically observed in benign primary hyperparathyroidism (Shane and Bilezikian, 1982). The prevalence of renal involvement in benign primary hyperparathyroidism, including nephrolithiasis, nephrocalcinosis, and impaired glomerular filtration, is less than 20% (Heath *et al.*, 1980; Silverberg *et al.*, 1990). In contrast, renal colic is a frequent presenting complaint of parathyroid carcinoma. The prevalence of nephrolithiasis was 56% and the prevalence of renal insufficiency was 84% in the Mayo Clinic series (Wynne *et al.*, 1992), somewhat higher than in previous reports in which the prevalence of renal involvement has ranged from 32% to 60%.

Bone pain and pathologic fractures are also common features of parathyroid cancer. Patients with benign primary hyperparathyroidism rarely have skeletal complaints and specific radiologic signs are found in less than 5% of patients (Heath *et al.*, 1980; Silverberg *et al.*, 1989, 1990). In contrast, specific radiologic signs of hyperparathyroid skeletal disease are commonly seen in parathyroid carcinoma, with osteitis fibrosa cystica, subperiosteal bone resorption, 'salt and pepper' skull, absent lamina dura, and diffuse osteopenia reported in 44% to 91% of cases. Another distinguishing feature is the development of concomitant bone and stone disease in parathyroid cancer, whereas simultaneous and symptomatic involvement of the kidneys and skeleton is distinctly unusual in primary hyperparathyroidism.

Recurrent severe pancreatitis, peptic ulcer disease, and anemia occur with greater frequency in patients with malignant disease than in those with benign primary hyperparathyroidism.

Acute Primary Hyperparathyroidism

Primary hyperparathyroidism occasionally presents as acute parathyroid crisis, with marked elevations in serum calcium and parathyroid hormone levels and prominent symptoms of hypercalcemia. In such cases, it can be difficult to distinguish clinically from parathyroid cancer (Rock *et al.*, 2010). However, whenever patients present with marked elevations of serum calcium and parathyroid hormone, the diagnosis of parathyroid cancer should be considered in the differential, since the surgical approaches differ.

Summary

Features that should lead a physician to suspect that a patient with hypercalcemia and elevated parathyroid hormone levels has parathyroid cancer are shown in **Table 1**. However, patients with parathyroid cancer may occasionally present with signs and symptoms that are quite mild. Whether this is because the disease is diagnosed at an earlier stage is unclear. Conversely, some patients with benign primary hyperparathyroidism may present with more severe disease than is commonly seen. In such patients, distinguishing between benign and malignant disease on clinical grounds may be difficult or impossible, since profound hypercalcemia, renal disease, and osteitis fibrosa or diffuse osteoporosis may occur and even concomitant kidney and bone disease may be present (Levin *et al.*, 1988). In either situation, it is preferable to have a high index of suspicion for parathyroid carcinoma than to miss the opportunity for surgical cure by failing to consider it in the differential diagnosis.

Pathology

Gross Pathology

Certain operative findings are helpful in distinguishing between benign parathyroid adenomas and parathyroid carcinoma. Parathyroid adenomas are usually soft, round or oval in shape, and reddish-brown in color. In contrast, parathyroid carcinomas are usually lobulated and firm to stony-hard in consistency. Approximately half are surrounded by a dense, fibrous, grayish-white capsule that adheres tenaciously to adjacent tissues and makes the tumor difficult to separate from contiguous structures. If there is gross infiltration of adjacent thyroid, nerve, muscle, or esophagus, or if there are cervical node metastases, the diagnosis of carcinoma is a simple matter. Unfortunately, any one or all of these operative findings may be absent. Additionally, it is also important to note that a normal parathyroid gland weighs 25–35 mg and an average parathyroid adenoma weighs 0.5 g. Parathyroid carcinomas are usually larger than adenomas (DeLellis, 2005; Schantz and Castleman, 1973).

Histology

As is the case with many endocrine neoplasms, the histopathologic distinction between benign and malignant parathyroid tumors is difficult to make. In 1973, Schantz and Castleman, based on an analysis of 70 cases of parathyroid carcinoma, established a set of criteria for the pathologic diagnosis of this malignancy (Schantz and Castleman, 1973). These histologic features are as follows: (1) uniform sheets of (usually chief but occasionally oxyphil) cells arranged in a lobular pattern and separated by dense fibrous trabeculae; (2) mitotic figures within tumor parenchymal cells that must be distinguished from endothelial cell mitoses; and (3) capsular or vascular invasion. Unfortunately, neither of the first two features is pathognomonic of parathyroid carcinoma, both having been reported in parathyroid adenomas (Levin *et al.*, 1988). However, capsular invasion and vascular invasion correlate well with subsequent tumor recurrence and their presence greatly increases the likelihood that the tumor is malignant. Finally, the overall histologic pattern is probably more useful than any single feature in differentiating parathyroid carcinoma from adenoma (Fujimoto and Obara, 1987; Obara *et al.*, 1997). Cellular atypia, including nuclear pleomorphism and enlargement and macronucleoli, has been associated with a greater likelihood of malignancy (Bondeson *et al.*, 1993). The presence of more than one of the above-mentioned characteristics in a lesion should alert the pathologist.

Other Histologic Markers

Although several histologic techniques have been used to improve the diagnosis of parathyroid carcinoma, none have proved consistently helpful. Electron microscopy of parathyroid cancer reveals nuclear and mitochondrial alterations and evidence of increased secretory activity but is not useful in distinguishing benign from malignant tumors (de la Garza *et al.*, 1985; Holck and Pedersen, 1981; Smith and Coombs, 1984). Nuclear diameter is larger in parathyroid carcinomas than in adenomas, but is not very useful in the individual case (de la Garza *et al.*, 1985; Holck and Pedersen, 1981; Jacobi *et al.*, 1986; Lloyd *et al.*, 1979; Schantz and Castleman, 1973; Smith and Coombs, 1984). Measurement of nuclear DNA content by flow cytometry is of limited value both in establishing the diagnosis of parathyroid carcinoma and in predicting the invasive potential of the tumor. Although mean nuclear DNA content is greater and an aneuploid DNA pattern is more common in parathyroid carcinoma than in adenomas, aneuploidy occurs too frequently in parathyroid adenomas to be useful in differentiating benign from malignant parathyroid tumors.

Loss of parafibromin nuclear immunoreactivity has high sensitivity and specificity for parathyroid cancer and was first reported as a molecular marker for parathyroid cancer in 2004 (Tan *et al.*, 2004). However, follow-up studies have noted both false positive and false negative results limiting universal application parafibromin immunochemistry (DeLellis, 2008; DeLellis *et al.*, 2008). Immunohistochemical staining of RB protein with polyclonal antibodies is usually absent in parathyroid carcinomas and almost always present in parathyroid adenomas (Cryns *et al.*, 1994b; Subramaniam *et al.*, 1995). However, staining for RB protein with monoclonal antibodies was not useful in distinguishing between benign and malignant parathyroid tumors in one study (Farnebo *et al.*, 1999a). Similarly, immunostaining for the cell cycle-associated antigen Ki-67, a marker of proliferative activity, is not consistently useful for distinguishing carcinomas from adenomas (Abbona *et al.*, 1995; Farnebo *et al.*, 1999a).

Invasive growth of various neoplasms may be facilitated by tumoral secretion of proteolytic enzymes, such as gelatinase A. Farnebo *et al.* have reported that gelatinase A mRNA was detected in 14 of 18 unequivocal and 4 of 13 equivocal parathyroid cancers (Farnebo *et al.*, 1999b). The strongest signal was detected in the fibroblasts and macrophages at the tumor border, rather than in the tumor cells.

Natural History

Parathyroid carcinoma is an indolent, albeit tenacious, tumor with rather low malignant potential. It tends to recur locally at the operative site and spread to contiguous structures in the neck. Metastases occur late in the course of the disease with spread via both lymphatic and hematogenous routes. Cervical nodes (30%) and lung (40%) are involved most commonly, followed by liver (10%). Occasional involvement of bone, pleura, pericardium, and pancreas has been reported.

Management

Initial Surgery

The most effective therapy for parathyroid carcinoma is complete resection of the primary lesion at the time of the initial operation when extensive local invasion and distant metastases are less likely (Shane and Bilezikian, 1982). Therefore, both preoperative suspicion and intraoperative recognition are of paramount importance in the management of this cancer. This is particularly important in the era of minimally invasive parathyroidectomy, when full neck exploration with identification of all four parathyroid glands is less common than it used to be. To assess preoperative risk of parathyroid cancer, Schulte *et al.* recently proposed the '$<3+<3$ rule,' which refers to the size of parathyroid lesion (<3 cm) and the serum albumin-corrected serum calcium level (<3 mmol l^{-1}) to risk stratify patients. They suggest that patients with parathyroid tumors less than 3 cm in diameter and serum calcium levels less than 3 mmol l^{-1} (12 mg dl^{-1}) are at low risk for parathyroid carcinoma and can be managed surgically with local resection (Schulte and Talat, 2012). However, we feel that caution is warranted in applying this rule. The <3 cm criterion was based on data from the National Cancer Data Base report of 286 cases of parathyroid cancer; only 37.4% of patients had lesions >3 cm, while 23.4% were <3 cm and in 39.2% the size of the lesion was not reported. Similarly, in more than 300 patients with parathyroid cancer reported by Talat *et al.*, only 17% had lesions >3 cm, while 17% had lesions <3 cm and in 66% the size was unknown (Talat and Schulte, 2010). Thus, while parathyroid lesions >3 cm suggest higher risk of carcinoma, we believe that there are insufficient data to reliably categorize lesions <3 cm as low risk. Moreover, recent data demonstrate a decrease in the proportion of patients with tumor sizes less than 4 cm, suggesting that earlier referral and diagnosis may result in patients with parathyroid cancers that are not as large as those seen previously (Lee *et al.*, 2007). Therefore, an algorithm which uses a <3 cm size rule as a cutoff may result in missed opportunities for preoperative consideration of parathyroid carcinoma. This is important because those patients in whom the clinical presentation is suggestive of parathyroid carcinoma should have a thorough exploration of all four parathyroid glands, since parathyroid carcinoma has been reported to coexist with benign adenomas or hyperplasia (Anderson *et al.*, 1983; Boyle *et al.*, 1999).

When the gross pathologic findings suggest malignancy, the following steps should be taken:

- The lesion should be removed *en bloc* together with the ipsilateral thyroid lobe and isthmus.
- The trachea should be skeletonized.
- Any contiguous tissues to which the tumor adheres should be resected.
- Great care should be taken to avoid rupture of the capsule of the gland, which increases the likelihood of local seeding of the tumor.
- If the recurrent laryngeal nerve is involved in the tumor, it must be resected.
- Tracheoesophageal, paratracheal, and upper mediastinal lymph nodes should be excised.
- Extensive lateral neck dissection is indicated only when there is spread to the anterior cervical nodes.

The situation is more complex when the diagnosis is made in the early postoperative period on the basis of pathology, particularly in view of the controversy surrounding the histopathology of parathyroid carcinoma. However, a second neck exploration is indicated under the following conditions:

- The gross characteristics of the lesion are typical of a parathyroid cancer.
- The histology appears to be aggressive with extensive vascular or capsular invasion.
- The patient remains hypercalcemic.

If any of these conditions apply, the structures adjacent to the tumor site should be resected in the manner described above. If none of these features are present and the diagnosis was made solely on the basis of the microscopic characteristics, immediate reoperation may not be necessary since a simple complete resection of the tumor may be curative. However, careful observation of the patient is essential and frequent measurement of parathyroid hormone and serum calcium levels is necessary.

Prognostic Classification

While parathyroid carcinoma has no predilection for gender, there is some evidence that male patients have a higher relative risk of recurrence (Schulte and Talat, 2012) and death (Lee *et al.*, 2007; Schulte and Talat, 2012). Talat *et al.* developed two prognostic classification systems for disease outcome in 2010, which were validated in 2012 (Schulte *et al.*, 2012; Talat and Schulte, 2010). The first system divided patients into high and low risk categories. Low risk was based on the presence of capsular and soft tissue invasion whereas high risk was based on the presence of vascular invasion, lymph node metastases, invasion of vital organs, or distant metastases. The second system classified high risk patients into classes I–IV. Class 1 includes low risk patients (capsular and soft tissue invasion). Classes II–IV are all high risk; class II includes patients with vascular invasion only, class III includes those with lymph node metastases or organ invasion, and class IV includes those with distant metastases (Schulte *et al.*, 2012). These classification systems were validated based on 82 patients with parathyroid cancer with follow-up ranging from 2 to 347 months. Mortality was seen only in the high risk groups.

Medical Management of the Postoperative Patient with Parathyroid Cancer

The postoperative management of a patient with parathyroid cancer must include careful attention to the serum calcium level. Prolonged severe elevation of parathyroid hormone is usually associated with increased bone resorption and formation. The

amount of unmineralized bone matrix (osteoid) may be greatly increased. Sudden withdrawal of excess parathyroid hormone will permit rapid deposition of calcium and phosphorus into the excess unmineralized osteoid. This process may be associated with severe and symptomatic hypocalcemia ('hungry bone syndrome'). Although hungry bone syndrome should be regarded as a sign that the surgery has been at least temporarily successful, it can be dangerous and must be managed aggressively. The hypocalcemia may be severe and protracted, requiring large doses of intravenous calcium. If hyperparathyroidism was severe and there is biochemical or radiographic evidence of skeletal involvement (elevated alkaline phosphatase, radiographic lesions of hyperparathyroidism), the patient should remain in the hospital until the hypocalcemia can be controlled with oral calcium supplements. Sufficient supplemental calcium and calcitriol should be prescribed to maintain the serum calcium at the low end of the normal range. As the bones heal and the remaining parathyroid glands recover, the requirement for calcium will decrease, permitting gradual reduction of the doses of calcium and withdrawal of calcitriol. After this point, serum calcium and parathyroid hormone levels should be monitored every 3 months.

Surgical Management of Recurrent or Metastatic Disease

The management of recurrent or metastatic parathyroid carcinoma reflects the rather indolent biology of this cancer and, in contrast to many other tumors, is primarily surgical (Shane and Bilezikian, 1982). Recurrent carcinoma in the neck should be treated with wide excision of the involved area, including the regional lymph nodes and other involved structures. Since even very small tumor deposits may produce sufficient parathyroid hormone to cause severe hypercalcemia, resection of accessible distant metastases in lymph nodes, lungs, or liver should be performed (Obara *et al.*, 1993). Resection, even if incomplete, may provide significant palliation, resulting in periods of normocalcemia that range from months to years. In addition, surgical debulking of tumor deposits may make it easier to control the hypercalcemia medically.

Preoperative Localization of Recurrent Parathyroid Cancer

In patients with recurrent hypercalcemia, preoperative localization studies should be performed. However, careful palpation of the neck should not be neglected, since recurrence occurs earliest and most often at the original site and such tumors are frequently palpable. Thallium-201–technetium-99m scanning is useful in locating tumors in the neck and upper mediastinum (Fujimoto *et al.*, 1986; Johnston *et al.*, 1996; Obara *et al.*, 1990). Technetium-99m–sestamibi used concurrently with a handheld, gamma-detecting probe may also be useful for the intraoperative localization of abnormal parathyroid tissue (Martinez *et al.*, 1995). Thallium-201 is also helpful for situations in which the thyroid has been partially or completely resected or when pulmonary metastases are suspected. Computerized tomography and magnetic resonance imaging are useful adjuncts to ultrasonography in evaluation of the neck and are superior for detection of distant metastases in the chest or abdomen. Positron emission tomography with a positron-emitting analogue of 2-deoxyglucose ([^{18}F]fluorodeoxyglucose) has also been reported to successfully localize parathyroid cancer deposits. If noninvasive testing does not yield results, arteriography or selective venous catheterization may be useful. Fine-needle aspiration biopsy should be avoided in order to avoid seeding deposits of malignant tissue.

Radiation Therapy

In the past, the use of radiation therapy to control tumor growth and decrease hormone production was ineffective in the majority of cases. However, radiation to the neck after surgery for recurrence may be helpful in preventing tumor regrowth (Chow *et al.*, 1998; Levin *et al.*, 1988; Munson *et al.*, 2003). In a series from Princess Margaret Hospital in Canada, 6 patients with microscopic residual disease who received adjuvant radiation therapy were followed for 12 to 56 months without recurrence (Chow *et al.*, 1998). Adjuvant radiotherapy has been shown to improve risk of recurrence (Busaidy *et al.*, 2004; Clayman *et al.*, 2004; Munson *et al.*, 2003). Additionally, parathyroid cancer lung metastases may respond to radiofrequency ablation (Tochio *et al.*, 2010).

Chemotherapy

Attempts to control tumor burden with chemotherapy have been disappointing. Because of the rarity of parathyroid carcinoma, experience with various chemotherapeutic regimens is limited to scattered case reports. Several regimens (nitrogen mustard; vincristine, cyclophosphamide, and actinomycin D; adriamycin, cyclophosphamide, and 5-fluorouracil; and adriamycin alone) were not effective (Anderson *et al.*, 1983; Golden *et al.*, 1965; Grammes and Eyerly, 1980). Two patients have been treated with synthetic estrogens with some success (Goepfert *et al.*, 1966; Sigurdss *et al.*, 1973). A single patient with pulmonary metastases responded to treatment with dacarbazine, 5-fluorouracil, and cyclophosphamide with a decrease in PTH and normalization of serum calcium for 13 months (Bukowski *et al.*, 1984). Another patient responded to dacarbazine alone with a brief but significant decline in her serum calcium level (Calandra *et al.*, 1984). An 18-month remission after therapy with a regimenof methotrexate, doxorubicin, cyclophosphamide, and lomustine, associated with regression of a mediastinal mass and pleural effusion was reported in a patient with a nonfunctioning parathyroid carcinoma by (Chahinian *et al.*, 1981). Such approaches are probably worth trying if surgical measures fail.

Management of Hypercalcemia

When parathyroid carcinoma has become widely disseminated and surgical resection is no longer effective, cure is impossible. However, even at this juncture, relatively prolonged survival can be achieved with adequate control of hypercalcemia, although the extremely elevated PTH levels and the intensity of the associated bone resorption often make this goal difficult to realize.

Acute hypercalcemia associated with parathyroid carcinoma is treated in the same way as hypercalcemia due to any other cause (Bilezikian, 1992). Management includes infusion of saline to restore fluid volume and enhance urinary calcium excretion. After this has been accomplished, loop diuretics may be considered to further increase calciuresis. Agents that inhibit osteoclast-mediated bone resorption are virtually always necessary.

Antiresorptive Agents

Bisphosphonates

The bisphosphonates are a group of drugs that inhibit osteoclast-mediated bone resorption. Several of these drugs have shown some promise in the therapy of parathyroid carcinoma.

Clodronate (Cl_2MDP) lowers serum calcium in parathyroid carcinoma when administered intravenously (Jacobs *et al.*, 1981; Shane *et al.*, 1982). It is widely available in Europe and the United Kingdom, but it is not available in the United States. Pamidronate, when infused for periods ranging from 2 to 24 h and at doses ranging from 45 to 90 mg per day, has been at least transiently effective in lowering serum calcium levels in several patients with parathyroid cancer (Depapp *et al.*, 1994; Mann, 1985; Obara *et al.*, 1993; Sandelin *et al.*, 1991; Vainas *et al.*, 1997; Weinstein, 1991). A more potent bisphosphonate, zoledronate, has been approved in the United States for the treatment of hypercalcemia. Zoledronate, at doses of 4 and 8 mg, has been shown to be superior to pamidronate (90 mg) in the treatment of hypercalcemia of malignancy, in terms of both the response rate and the duration of response (Major *et al.*, 2001). Though zoledronate has not been specifically evaluated in parathyroid cancer, it is a reasonable approach to controlling hypercalcemia in these patients.

Denosumab

Denosumab is a monoclonal antibody that inhibits receptor activator of nuclear factor kB ligand (RANKL) and potently inhibits osteoclast activity. Vellanki *et al.* reported the first patient with parathyroid carcinoma and refractory hypercalcemia who responded to denosumab, 120 mg subcutaneously every month, with a reduction in serum calcium (Vellanki *et al.*, 2014). Initially, the patient was receiving dacarbazine, pamidronate and cinacalcet but had recalcitrant hypercalcemia. After the denosumab was started, he was eventually able to stop the dacarbazine and pamidronate. His serum calcium was maintained in the normal range on denosumab and cinacalcet.

Other Treatments

Calcimimetics

Under normal circumstances, parathyroid hormone secretion is mediated by a cell surface calcium-sensing receptor and this regulatory response is generally retained in benign parathyroid tumors. An allosteric modulator of the calcium receptor with calcimimetic properties, R-568, was first shown to lower serum parathyroid hormone and calcium concentrations in patients with primary hyperparathyroidism (Silverberg *et al.*, 1997). This same first generation calcimimetic was used to treat a patient with parathyroid carcinoma (Collins *et al.*, 1998), with control of serum calcium for 2 years without adverse effects. This was followed by a report by Silverberg *et al.* of 29 patients with inoperable parathyroid carcinoma treated with cinacalcet HCl, a second generation agent with a longer half-life, with doses starting at 30 mg twice daily to 90 mg four times a day (Silverberg *et al.*, 2007). Approximately two thirds of patients responded with a decrease in serum calcium levels.

Calcitonin

Although calcitonin inhibits osteoclast-mediated bone resorption, increases urinary calcium excretion, and has been reported to lower serum calcium in two patients with parathyroid cancer, it is a weak antiresorptive agent and is not effective in parathyroid carcinoma (Shane and Bilezikian, 1982).

Plicamycin

Plicamycin (mithramycin), another specific inhibitor of bone resorption, lowers serum calcium levels in parathyroid carcinoma (Singer *et al.*, 1970). It is administered intravenously at a dose of 25 $\mu g\ kg^{-1}$ over 4–8 h and may be repeated at daily intervals for up to 7 days until the serum calcium falls into an acceptable range (Bilezikian, 1992). Unfortunately, plicamycin has toxic effects on the liver, kidneys, and bone marrow that increase with the number of exposures. Intravenous bisphosphonates have largely supplanted plicamycin in the therapy of hypercalcemia.

Immunization

In 1999, Bradwell *et al.* reported a patient with parathyroid carcinoma metastatic to lungs and pleura who had severe hypercalcemia that was resistant to oral clodronate, intravenous pamidronate, octreotide, 5-fluorouracil, and streptozotocin. The patient

was immunized with human and bovine PTH peptides, followed with booster doses at 4 and 11 weeks (Bradwell and Harvey, 1999). Antibodies against PTH were detected at 4 weeks. Before therapy, serum calcium varied between 3.5 and 4.2 mmol l^{-1}. Serum calcium levels remained significantly lower (2.5–3.0 mmol l^{-1}) throughout the 6 months of observation. There was rapid improvement in her clinical condition and no significant adverse effects were observed. In 2004, there was another report of immunotherapy in a patient with metastatic parathyroid carcinoma (Betea *et al.*, 2004). In addition to improvement in clinical, hormonal, and biochemical measures, they reported, for the first time, an antitumor effect with a decrease in size of the pulmonary metastases.

Prognosis

The prognosis of parathyroid carcinoma is quite variable. No single characteristic correlates predictably with outcome. Lee *et al.* noted that increased survival rate was associated with younger age, more recent year of diagnosis, female gender, and lack of distant metastasis (Lee *et al.*, 2007). Talat *et al.* also noted that increased survival and lower risk of recurrence was associated with female gender and the absence of vascular invasion or lymph node metastasis (Hundahl *et al.*, 1999; Talat and Schulte, 2010). Early recognition and complete resection at the time of the initial surgery offer the best prognosis. The average time between surgery and the first recurrence is approximately 3 years, although intervals of up to 20 years have been reported. Once the tumor has recurred, complete cure is unlikely. However, prolonged survival is still common in these circumstances with palliative surgery and control of hypercalcemia. Five-year survival rates vary from 40% to 86%. The National Cancer Database survey reported 10-year survival to be approximately 49% (Hundahl *et al.*, 1999). To improve diagnosis and survival rates, endocrinologists and surgeons must be aware of the clinical characteristics of parathyroid cancer, include parathyroid cancer in the differential diagnosis of markedly elevated serum calcium and parathyroid hormone levels, and communicate prior to surgery regarding high risk patients, so that the initial operation is more likely to result in a definitive cure.

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Parathyroid Surgery☆

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Glossary

Autotransplantation Transfer of parathyroid tissue from its normal location, usually to a muscle bed in the neck or forearm in order to preserve parathyroid function following total parathyroidectomy.

Four-dimensional computed tomography (4D-CT) Preoperative imaging technique that adds the timing of contrast perfusion to traditional computed tomography to assist in localizing parathyroid adenomas.

Gamma probe Handheld counter used intraoperatiely to measure radioactivity following intravenous technetium-99m administration prior to the operation and provide real-time localization during minimally invasive radioguided parathyroidectomy.

Minimally invasive parathyroidectomy (MIP) Procedure that involves preoperative localization and intraoperative parathyroid hormone assay to identify and remove the single abnormal gland with limited exploration and without identifying the remaining parathyroid glands.

Primary hyperparathyroidism Disease caused by the hypersecretion of parathyroid hormone from one or more of the parathyroid glands, resulting in hypercalcemia. The diagnosis is made based on elevated calcium levels, elevated parathyroid hormone levels, and elevated or normal urinary calcium concentration.

Secondary hyperparathyroidism Disease associated with increased production of parathyroid hormone in response to external factors, such as chronic renal failure, leading to hyperplasia of all of the parathyroid glands.

Sestamibi scintigraphy [+/− single photon emission computed tomography (SPECT)] Preoperative nuclear medicine study that is used to localize hyperfunctioning parathyroid tissue. Sestamibi is combined with SPECT for three-dimensional perspective and improved localization.

Tertiary hyperparathyroidism Disease that develops in patients with prolonged secondary hyperparathyroidism who develop autonomous parathyroid function. It also may occur following renal transplant. It is treated with subtotal parathyroidectomy or total parathyroidectomy with autotransplantation.

History

Parathyroid surgery has advanced over the years through the slow process of trial and error. The original description of the parathyroid gland came in 1850 when Sir Richard Owen, a conservator of the Hunterian Museum, identified "a small compact yellow glandular body attached to the thyroid" while dissecting the remains of an Indian rhinoceros. Three decades later, a Swedish medical student, Ivar Victor Sandström, more completely described the "glandulae parathyroideae" in a manuscript entitled "On a New Gland in Man and Several Mammals." Around the same time, Anton Wolfer reported a case of post-operative tetany in a patient that had undergone a total thyroidectomy (and incidental total parathyroidectomy) by Theodor Billroth. It was Eugene Gley in Paris who ultimately made the connection between the parathyroid glands and tetany in 1891. In the early 1900s, it was postulated that removal of a solitary enlarged parathyroid may be used to treat the underlying bone disease of patients. Felix Mandl is credited with performing the first successful parathyroidectomy in 1920s on Albert Gahne, a 34-year-old tram car conductor who likely had a parathyroid carcinoma. Another famous case involves Charles Martell, a merchant marine captain who underwent seven operations before a parathyroid adenoma was ultimately found in the superior mediastinum.

Several technological advances over the past half century have refined parathyroid surgery. The routine measurement of serum calcium levels became possible with the advent of the serum channel autoanalyzer in the 1960s. This invention allowed for the early detection of hypercalcemia and led to a sudden increase in the incidence of hyperparathyroidism and a new population of patients with asymptomatic disease. Rosalyn Yalow and Solomon Berson won the Nobel prize in 1977 for developing the radioimmunoassay (RIA) that allowed the measurement of parathyroid hormone (PTH). Other important technical advances, including improved preoperative localization [high resolution ultrasound, sestamibi ± single photon emission computed tomography (SPECT), and four-dimensional computed tomography (4D-CT)] and rapid intraoperative PTH (IOPTH) assay, have allowed for the current era of the minimally invasive parathyroidectomy (MIP).

☆*Change History:* April 2016. EF Garner and H Chen has made changes throughout the text.

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Parathyoid Glands

Anatomy and Embryology

An understanding of the anatomy and embryologic variants of the parathyroid glands is critical for appropriate surgical management. During development the inferior and superior parathyroid glands arise from the third and fourth pharyngeal pouches, respectively. These glands migrate inferiorly and usually settle posterior to the thyroid. The inferior glands are typically located behind the inferior pole of the thyroid, while the superior glands are usually found behind the lateral lobes of the thyroid. However, the parathyroid glands may be found in an ectopic site in up to 16% cases (Phitayakorn and McHenry, 2006). The inferior glands are more likely to vary in location. It is important for the surgeon to be familiar with the common ectopic locations, including the mediastinum, intrathymic/intrathyroidal tissue, retroesophageal or paraesophageal space, within the carotid sheath, or high in the neck from failure of descent. One study found ectopic glands were most commonly found in the thymus (38%) followed by the retro/paraesophageal space (31%) (Roy *et al.*, 2013). Another study demonstrated that the retroesophageal space is the most common site of a missed parathyroid by low-volume surgeons (Chen *et al.*, 2010). Despite the variation in anatomy, the inferior parathyroid glands are generally located anterior and medial to the ipsilateral recurrent laryngeal nerve, while the posterior glands tend to be found posterior and laternal to this nerve.

Although the appearance of the parathyroid glands may vary, they are most commonly oval or bean-shaped with a yellow-brown color. The average weight of the normal adult parathyroid gland is 30-50 mg. The superior and inferior parathyroid glands typically receive their blood supply from branches of the inferior thyroid arteries. The venous drainage involves the superior, middle, and inferior parathyroid veins. The gland itself is composed of chief cells and oxyphil cells as well as adipose tissue and fibrovascular stroma.

Primary Hyperparathyroidism

Primary hyperparathyroidism is caused by the hypersecretion of parathyroid hormone (PTH) from one or more of the parathyroid glands. It can be caused by three different pathologic lesions: parathyroid adenomas, parathyroid hyperplasia, and parathyroid carcinoma. Parathyroid adenomas are benign neoplasms that are the source of most cases of primary hyperparathyroidism (80-90%). Adenomas usually involve a solitary gland, but occasionally involve more than one gland. The incidence of patients with primary hyperparathyroidism that have adenomas in two glands ("double adenoma") is between 2% and 15% (Abboud *et al.*, 2005; Alhefdhi *et al.*, 2014). Parathyroid hyperplasia, or the proliferation of parenchymal cells in all the parathyroid glands, is the second most common cause of primary hyperparathyroidism. Hyperplasia accounts for about 10-15% of cases. Parathyroid hyperplasia can be associated with multiple endocrine neoplasia (MEN) 1 (primary hyperparathyroidism, pancreatic tumors, and pituitary tumors) and MEN 2A (primary hyperparathyroidism, pheochromocytoma, and medullary thyroid cancer) syndromes. Parathyroid carcinoma is an invasive neoplasm that arises from the parenchymal cells of the gland and is an uncommon source of primary hyperparathyroidism ($<1\%$).

Clinical Presentation and Diagnosis

Historically, patients with primary hyperparathyroidism presented with the classic signs and symptoms of hypercalcemia, including the pentad of "painful bones, kidney stones, abdominal groans, psychic moans, and fatigue overtones." In recent years, due to the increased availability of laboratory testing, a biochemical diagnosis is often made prior to the development of symptoms during a routine health screening or workup for an unrelated medical condition. The development of the classic skeletal and renal complications is less commonly seen in modern times. The diagnosis is made based on an elevated serum calcium level and an elevated or inappropriately normal PTH level. A 24-h urine collection may help clarify the diagnosis and rule out familial hypocalciuric hypercalcemia (FHH). Patients with primary hyperparathyroidism will have normal or increased urinary calcium concentrations, while patients with FHH have low urinary calcium in the setting of high serum calcium levels.

Medical Alternatives

Whereas there is little question that the appropriate management of symptomatic primary hyperparathyroidism involves surgical excision of the involved gland, the management of the asymptomatic patient can be more challenging and the guidelines continue to evolve. There is still some debate regarding the role of parathyroidectomy for the asymptomatic patient with mild to moderate hypercalcemia. The primary question is whether these patients should undergo early parathyroidectomy or whether surveillance and medical therapy can be safely employed until the development of clinical sequelae. The most recent guidelines from the Fourth International Workshop on the Management of Asymptomatic Primary Hyperparathyroidism published in 2014 have been included (Table 1). New indications for surgery continue to be added to the guidelines. For instance, sleep disturbance and insomnia are common problems associated with primary hyperparathyroidism that are improved with surgery, but these are not part of the current operative criteria (Murray *et al.*, 2014).

Although surgery remains the only curative treatment, calcimimetics (e.g. cinacalcet) are a medical alternative for lowering serum calcium levels. A recent phase 3 clinical trial demonstrated that cinacalcet is well-tolerated and normalizes serum calcium levels in patients with primary hyperparathyroidism who had contraindications to surgery (Khan *et al.*, 2015). Although cinacalcet

Table 1 2013 Guidelines for surgery in asymptomatic primary hyperparathyroidism*

Measurement	*Indication for surgery*
Serum calcium	>1 mg dL^{-1}(0.25 mmol L^{-1}) over the upper limit of normal
Skeletal	a. BMD by DXA: T-score ≤ 2.5 at lumbar, spine, total hip, femoral neck or distal 1/3 radius
	b. Vertebral fracture by x-ray, CT, MRI, or VFA
Renal	a. Creatinine clearance <60 cc min^{-1}
	b. 24-h urine for calcium >400 mg day^{-1} (>10 mmol day^{-1}) and increased risk by biochemical stone risk analysis
	c. Presence of nephrolithiasis or nephrocalcinosis by x-ray, US, or CT
Age	<50

*Adopted from Bilezikian et al. Guidelines for the Management of Asymptomatic Primary Hyperparathyroidism: Summary Statement from the Fourth International Workshop. 2014.

lower serum calcium levels, it does not have a significant effect on serum PTH levels and bone mineral density does not change. Bisphosphonates, particularly alendronate, may improve the bone mineral density without effecting serum calcium levels. The role for combination therapy with both cinacalcet and bisphosphonates to improve both serum calcium levels and bone mineral density has not been sufficiently evaluated (Marcocci *et al.*, 2014). There is no role for restricting dietary calcium intake in patients with primary hyperparathyroidism who do not undergo surgery. Vitamin D sufficiency should also be maintained.

Localization Procedures

Pre-Operative Localization

Localization with imaging studies has become routine prior to parathyroid surgery. In the case of primary hyperparathyroidism, preoperative localization of a solitary adenoma allows for a minimally invasive approach. In addition, the location of the parathyroid glands can vary and preoperative identification is useful for operative planning. Localization is absolutely essential prior to reoperation. Many techniques have been used to identify abnormal parathyroid glands preoperatively, including nuclear imaging, ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). As the ability to perform a minimally invasive parathyroidectomy is dependent on accurate preoperative localization, careful selection of a localization technique is necessary and depends on several factors including surgeon preference, institutional factors, and patient characteristics (e.g. body habitus, contrast allergies).

Non-invasive techniques

The sestamibi scan remains the most widely used preoperative localization technique. Technetium-99m (^{99m}Tc) sestamibi accumulates in parathyroid adenomas as a product of the increased concentration of oxyphil cells which have high mitochondrial content. Sestamibi accumulates in both the parathyroid and thyroid glands, but washes out of the thyroid tissue more rapidly due to the lower mitochondrial activity. Parathyroid adenomas will have persistent sestamibi on delayed images (Fig. 1). Single-photon emission computed tomography (SPECT) has been added to the traditional planar images to provide a three-dimensional view and improved accuracy of localization. Sestamibi-SPECT is non-invasive, accurate and reliable. The sensitivity of sestamibi-SPECT can vary from 62% to 91% and the positive predictive value ranges from 84% to 96% (Cheung *et al.*, 2012). Surgeon interpretation and radiologist volume are factors that improve the success of sestamibi parathyroid localization (Zia *et al.*, 2012). One limitation of sestamibi scans includes low sensitivity with multigland hyperplasia and double adenomas. Another limitation is related to the presence of other metabolic active tissue (e.g., lymph nodes or thyroid pathology) that may cause a false positive with sestamibi scintigraphy.

Ultrasound may be used alone or in conjunction with sestamibi-SPECT. Parathyroid adenomas are often seen as hypoechoic masses on ultrasound. Localization with ultrasound has several advantages; it is easily performed, inexpensive, involves no radiation or contrast, and can also identify concomitant thyroid pathology. Ultrasound has a pooled sensitivity and positive predictive value of 76 and 93%, respectively (Cheung *et al.*, 2012). Ultrasound is limited by being operator dependent and its inability to evaluate mediastinal parathyroid tissue. There are several factors that may reduce the accuracy of ultrasound, including parathyroid hyperplasia, multiple gland adenomas, and thyroid nodules.

Computed tomography (CT) is another useful noninvasive technique used in preoperative localization. CT scanning can be helpful in identifying ectopic parathyroid glands. The sensitivity of this study for localizing parathyroid adenomas ranges from 46% to 87%. CT scans are readily available and allow for the possibility of needle-guided biopsy. The disadvantages of the technique include the use of intravenous contrast, radiation exposure, and cost. A newer technique known as four-dimensional computed tomography (4D-CT) involves the addition of timing of contrast perfusion to assist in localizing adenomas. In a meta-analysis of preoperative localization techniques, 4D-CT was demonstrated to have an improved sensitivity (89.4%) when compared with ultrasound (76.1%) and sestamibi-SPECT (78.9%) (Cheung *et al.*, 2012). 4D-CT may be useful when adenomas have not been localized on ultrasound or sestamibi-SPECT. A recent cost-analysis study revealed that the combination of sestamibi-SPECT and ultrasound with the addition of 4D-CT if necessary was the most cost-effective imaging method (Wang *et al.*, 2011). The availability and accuracy of these methods vary by institution, and thus, institutional factors often drive the preoperative imaging algorithm.

Magnetic resonance imaging (MRI) is typically used when other studies have been inconclusive. Unlike CT scans, MRI does not require ionizing radiation. However, MRI is expensive and interpretation can be difficult due to motion artifact. For this reason,

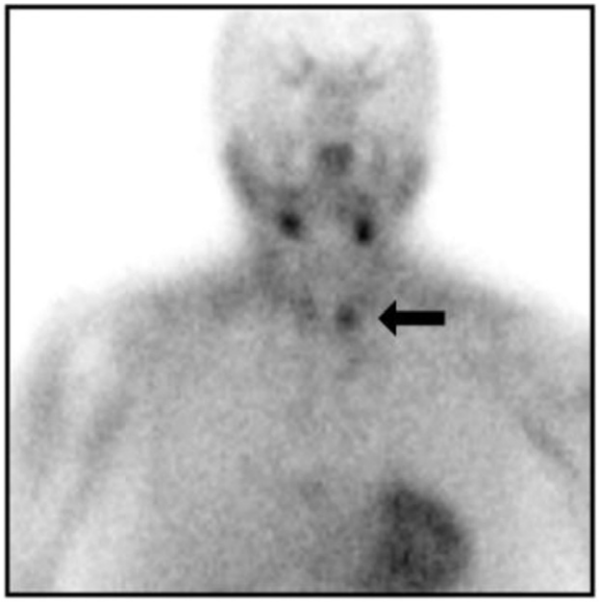

Fig. 1 Sestamibi scan localizing a left-sided, superior parathyroid adenoma (arrow) in the neck of a patient who subsequently underwent a MIP.

MRI should not be used as the first or only imaging study to evaluate parathyroid disease, but can be useful when added to the other studies mentioned.

Invasive techniques

Invasive localization techniques include parathyroid arteriography, selective venous sampling, and fine needle aspiration (FNA). These procedures are rarely used due to the capabilities of noninvasive imaging techniques and the potential complications associated with invasive procedures. Selective angiography of the inferior thyroid arteries can identify parathyroid adenomas as highly vascularized oval blushes and has a sensitivity of 60%. Selective venous sampling for PTH can help lateralize tumors. Bilateral internal jugular venous sampling for primary hyperparathyroidism has a sensitivity and positive predictive value of 80 and 71%, respectively (Ito *et al.*, 2007). This procedure has been shown to be safe and may be useful in the setting of negative sestamibi scanning and complex multigland disease (Ito *et al.*, 2007). Ultrasound-guided FNA can confirm parathyroid tissue through PTH sampling of a nodule, which is more sensitive than cytology. In addition, FNA may be useful in the evaluation of concomitant thyroid nodules identified on ultrasound.

Intraoperative Adjuncts

The rapid intraoperative PTH immunoassay has become an important tool to confirm all of the hyperfunctioning parathyroid tissue has been removed during the procedure. This assay was first used in the late 1980s, but has been refined over the years. The advantages of using intraoperative PTH (IOPTH) monitoring have been well described and include shorter operative times, less extensive dissection, decreased cost, and decreased failure rates for initial and reoperative parathyroidectomy. IOPTH testing is considered the most reliable perioperative adjunct. One study found that IOPTH monitoring has the highest sensitivity (99%), positive predictive value (99.6%), and accuracy (98%) when compared to other perioperative adjuncts (Chen *et al.*, 2005).

This laboratory test takes advantage of the short half-life of PTH (3-5 min) which allows for intraoperative monitoring. First, a PTH level is drawn prior to surgical incision to serve as a baseline level. The blood samples may be obtained through a peripheral intravenous catheter or arterial line. The equipment and the technician to run the assay are located within or close to the operating room to prevent delay due to transport of the sample. After gland excision, repeat PTH levels are measured at 5, 10, and 15 min post-excision. The surgery is concluded when the PTH drops by at least 50% of the baseline level. Several other algorithms for IOPTH monitoring have also been described. Propofol has traditionally been avoided because it was thought to alter intraoperative PTH levels. However, a randomized, prospective study demonstrated that propofol does not significantly alter PTH levels (Sippel *et al.*, 2004).

Radioguided minimally invasive parathyroidectomy requires the preoperative intravenous injection of 10 mCI technetium-99m sestamibi to be administered 1–2 h prior to surgery. Sestamibi remains in the hyperfunctioning parathyroid tissue longer and a handheld gamma counter is used to identify the abnormal parathyroid gland intraoperatively. Prior to incision, background counts are obtained over the thyroid isthmus to be used as a reference. Once the gland has been identified, in vivo counts are measured with the gamma probe. A parathyroid adenoma will generally produce an in vivo to background ratio of $>150\%$. The gland is then removed and an ex vivo count of the excised tissue is obtained by placing on the tip of the probe and directing the

probe away from the patient. The ex vivo count should be >20% of background levels. Any excised tissue containing radioactivity >20% of the background radioactivity is either a parathyroid adenoma or hyperplastic gland (">20% rule") (Murphy and Norman, 1999; Chen *et al.*, 2003). Most surgeons recommend confirming adequate excision with IOPTH.

Radio-guidance allows a more focused surgery and reduced operative time. While initially used for only well-localized parathyroid adenomas, the applications for radioguided parathyroid surgery continues to expand. This technique has now been applied to patients with multi-gland hyperplasia, secondary and tertiary hyperparathyroidism, and mediastinal adenomas with good results. In addition, radioguided techniques are even effective in patients with nonlocalizing setamibi scans (Chen *et al.*, 2009). Radioguided parathyroidectomy is safe for both the patient and operating room staff. Radiation exposure to the surgeon and staff has been shown to be minimal (Oltmann *et al.*, 2014).

Parathyroid Surgery

Surgery remains the only definitive treatment of primary hyperparathyroidism. Abnormal parathyroid glands are removed either through a bilateral neck exploration or a more focused parathyroidectomy if the abnormal gland is identified preoperatively. Cure rates with surgery are greater than 95%.

Bilateral Neck Exploration

The classic operation for primary hyperparathyroidism is a bilateral neck exploration with identification of all parathyroid tissue followed by the removal of any abnormal glands. This procedure is usually performed under general anesthesia, but may be performed under a regional cervical block. The patient is positioned supine with the neck extended. A 3-5 cm incision is made within a skin crease just below the thyroid isthmus centered on the midline. The strap muscles are dissected along the midline and retracted laterally. The surgeon then identifies all the parathyroid glands and removes any pathologically enlarged glands. IOPTH levels may be used to confirm adequate resection. The gamma probe can also be utilized. Failure to identify all four glands intraoperatively should prompt exploration of the common ectopic sites previously mentioned.

Bilateral neck exploration with parathyroidectomy provides a cure (defined as normocalcemia and normal PTH 6 months postoperatively) in greater than 95% of cases when performed by an experienced surgeon. This procedure is not as successful in less experienced hands as low-volume surgeons tend to have higher operative failure rates. Bilateral neck exploration provides the advantages of visualizing all of the glands and the potential to identify occult multiglandular disease. Complications include recurrent laryngeal nerve injury, hypoparathyroidism, hematoma and wound infection. When compared to a more focused parathyroidectomy, bilateral neck exploration is associated with longer operative times and higher rates of post-operative hypocalcemia (Bergenfelz *et al.*, 2002).

Minimally Invasive Parathyroidectomy

Primary hyperparathyroidism is the result of a solitary adenoma in about 85% of cases, and these cases are cured with the excision of the abnormal gland. Therefore, with the improvement in pre-operative localization studies, minimally invasive parathyroidectomy (MIP) has arisen as the standard of care for solitary adenomas with the goals of a more limited dissection, shorter operative times, and improved cosmesis. There is no difference in persistent or recurrent hyperparathyroidism in those undergoing MIP versus bilateral exploration. MIP can be performed under locoregional anesthesia in the ambulatory setting and is associated with shorter hospital stay and lower costs (Chen *et al.*, 1999). The complications of this procedure are similar to those seen with a bilateral exploration. In one series of 1065 patients that compared MIP to conventional exploration, MIP was associated with improvements in cure rates (99.4% vs. 97.1%) and complicate rate (1.45% vs. 3.10%) (Udelsman *et al.*, 2011).

While this procedure does not require the exposure of all the glands, it does rely on two important adjuncts: accurate preoperative localization and intraoperative PTH monitoring. Preoperative localization (i.e., sestamibi scanning or high resolution ultrasound) is critical for avoiding failure due to multiglandular disease. These studies should be reviewed by the operating surgeon prior to the operation. In addition, IOPTH monitoring is used to confirm the adequacy of the resection. Several criteria have been recommended for interpreting IOPTH values. If preoperative localization is negative or multiglandular disease is identified intraoperatively, the surgeon generally proceeds with a bilateral exploration as previously described. A radioguided technique may allow for a more focused surgery.

Prior to the operation, a large-bore peripheral intravenous catheter is placed for administration of medications and IOPTH monitoring. The procedure may be performed under general or locoregional anesthesia. The patient is positioned supine with the arms tucked. An inflated IV bag is placed to provide slight extension of the neck. A 1-4 cm transverse, slightly curved incision is made approximately one to two fingerbreadths below the cricoid cartilage in a skin fold centered along the midline. This incision may vary slightly based on localization. The dissection continues downward through the subcutaneous tissue and platysma. The strap muscles are then separated vertically along the midline and retracted laterally. Focused exploration is then performed based on preoperative localization studies and/or radioguidance. IOPTH values are monitored intraoperatively and results should be confirmed prior to completion of the procedure. If the IOPTH values fail to drop appropriately, further exploration for a second

adenoma or hyperplastic tissue is warranted. After confirmation the culprit gland has been excised, the strap muscles and platysma are reapproximated using absorbable suture.

Post-operative care includes calcium supplementation as well as counseling on the signs and management of hypocalcemia. Patients are instructed to take additional doses of calcium if they develop symptoms of hypocalcemia (e.g. numbness/tingling, muscle cramps). Postoperative pain can usually be managed with nonnarcotic medications. Most patients are discharge home the day of the procedure. Patients return to the clinic in 1-2 weeks after surgery where the serum calcium and PTH are checked. Vitamin D may be added for hypocalcemia refractory to calcium supplementation alone. The serum calcium and PTH values are rechecked at 6 months to confirm cure.

Variations on Minimally Invasive Parathyroidectomy

Endoscopic, video-assisted, and robotic approaches are variations on the minimally invasive parathyroidectomy. Similar to MIP, these techniques also rely on preoperative localization and IOPTH monitoring. These operations are typically performed under general anesthesia, but may provide the possible advantages of improved visualization of neck structures and improved cosmesis.

The first endoscopic parathyroidectomy was described by Michel Gagner in 1996 and has subsequently been modified by other surgeons. Access is gained at the manubrium where a 5-mm endoscope is introduced. Two or three additional trochars are added laterally in the neck for instruments. The procedure is performed under steady low pressure insufflation (<8 mmHg) to create an operative space. Disadvantages to this approach include the loss of operative space during suctioning, risk of hypercarbia from CO_2 absorption, longer operative times, and the development of subcutaneous emphysema. Due to some of these limitations, this approach has not gained widespread practice.

Minimally invasive video-assisted parathyroidectomy (MIVAP) is a procedure pioneered by Paolo Miccoli. A small 15 mm incision is made approximately 2 cm above the sternal notch. After dissecting the strap muscles off of the thyroid lobe, retractors are inserted to maintain the operative space. Traditionally, this procedure required a short period of insufflation to dissect out this space. Modifications have omitted this step such that it is now a gasless procedure. A 30°, 5-mm endoscope is then inserted into the incision followed by the introduction of small surgical instruments approximately 2 mm in size. The surgeon then proceeds by dissecting the thyroid gland from the strap muscles to gain exposure. A focused exploration is then performed based on pre-operative localization. The parathyroid gland is bluntly dissected under endoscopic vision and the vascular pedicle is ligated with either titanium clips or a ligature device. MIVAP has the advantages of improved cosmesis, decreased post-operative pain and improved patient satisfaction (Lombardi *et al.*, 2009). Robotic parathyroidectomy is a newer technique and has been successfully described at several centers. Although this approach provides adequate cure rates and improved cosmesis, it is limited by cost and longer operative times (Tolley *et al.*, 2014).

Re-Exploration

Persistent hyperparathyroidism is defined as the failure to achieve or maintain normocalcemia within 6 months following surgery. Recurrent hyperparathyroidism refers to hypercalcemia that reappears after 6 months of normalized serum calcium levels. The two most common reasons for failure of the initial parathyroid operation are failure of the surgeon to find the abnormal gland and missed multiglandular disease. Multi-gland disease accounts for up to 37% of patients undergoing reexploration. Double adenomas are also associated with a higher incidence of recurrent and persistent hyperparathyroidism (Alhefdhi *et al.*, 2014). Less commonly, a parathyroid carcinoma has not been recognized or adequately treated. The success of the initial operation is in part related to the experience of the operating surgeon (Chen *et al.*, 2010). An experienced surgeon is more likely to know the normal and ectopic site of parathyroid glands.

Reoperative parathyroid surgery is a more challenging operation and should be performed by a high-volume endocrine surgeon. Due to the increased complexity, patients should be selected for surgery based on an analysis of the risks and benefits. As the risk of complication increases with a second operation, surgery is typically reserved for symptomatic patients. Some surgeons recommend that patients who have undergone a prior neck operation or those with preoperative hoarseness should undergo fiberoptic laryngoscopy to document recurrent laryngeal nerve function.

Once the patient has been deemed to be a surgical candidate, the surgeon should review previous imaging studies, operative notes, and pathology reports. Additional imaging studies may be helpful in localizing the adenoma. Four dimensional-CT may be a useful tool for locating the hyperfunctioning tissue in the setting of the reoperative neck. One study evaluated 45 patients undergoing reoperative parathyroidectomy and found the sensitivity of 4D-CT localization was 88% compared to 54% with sestamibi localization (Mortenson *et al.*, 2008). Intraoperative adjuncts, including ultrasound, gamma probe and IOPTH monitoring may also be helpful in identifying the missing adenoma. A radioguided parathyroidectomy may be useful in the reoperative neck as it allows for a more focused approach and less dissection (Pitt *et al.*, 2009).

Secondary and Tertiary Hyperparathyroidism

Pathology and Clinical Presentation

Secondary hyperparathyroidism is the compensatory oversecretion of PTH in the setting of abnormalities in calcium metabolism. The majority of cases occur in patients with chronic kidney disease. Other causes of secondary hyperparathyroidism include

vitamin D deficiency, inadequate calcium intake, decreased intestinal absorption, and idiopathic hypercalciuria. Chronic kidney disease leads to hyperphosphatemia and decreased renal conversion of 24-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol, leading to a decrease in the intestinal absorption of calcium. As a result, hypocalcemia develops which leads to the stimulation of PTH secretion and parathyroid gland hyperplasia. Secondary hyperparathyroidism develops in approximately 90% of patients with chronic kidney disease. The clinical manifestations can be severe and include: skeletal symptoms (including classic osteitis fibrosa cystica), pruritis, extraskeletal calcifications, and calciphylaxis. Secondary hyperparathyroidism is typically managed with medical therapy directed at correcting serum calcium and phosphate levels into the physiologic range. Medical therapy consists of a low phosphate diet, administration of phosphate binders, calcium supplementation, vitamin D supplementation and dialysis. The recently developed calcimimetics, such as cinacalcet, function by increasing the sensitivity of the calcium-sensing receptor (CaSR) and thus lowering parathyroid hormone levels. These agents are typically added when patients on dialysis fail phosphate binders. Calcimimetics have been shown to effectively reduce PTH levels and decrease parathyroidectomy rates in this population (Ballinger *et al.*, 2014). Renal transplantation remains the definitive treatment for secondary hyperparathyroidism. However, emerging data suggest that a significant number of patients after renal transplantation still have hyperparathyroidism (Lou *et al.*, 2015).

Tertiary hyperparathyroidism develops as a result of the persistent stimulation of the parathyroid glands in patients with long-standing secondary hyperparathyroidism. These patients develop autonomous PTH production that is not suppressed even after correction of the abnormalities in calcium homeostasis. This scenario is most frequently encountered when a patient with renal failure and secondary hyperparathyroidism undergoes renal transplantation. Surgery remains the primary treatment for these patients.

Subtotal Versus Total Parathyroidectomy

Surgery is indicated for the management of secondary hyperparathyroidism when medical therapy fails (i.e., persistent hyperphosphatemia, hypercalcemia, or elevated PTH levels). Additional indications for parathyroid surgery include refractory symptoms of hypercalcemia (e.g., pruritus), intractable renal osteodystrophy (e.g., intractable bone pain, fractures), and calciphylaxis. Surgical therapy is the primary management of tertiary hyperparathyroidism after renal transplantation. The two accepted operations for the management of secondary and tertiary hyperparathyroidism include subtotal parathyroidectomy and total parathyroidectomy with parathyroid autotransplantation. Despite ongoing debate, neither procedure has been clearly distinguished as superior. The selection of an operative technique remains largely dictated by surgeon preference.

Subtotal parathyroidectomy involves identification of all four glands followed by the removal of "three and a half glands." A 40-60 mg remnant of normal-appearing parathyroid tissue is left in place and marked with nonabsorbable suture and/or a surgical clip in case reoperation is necessary. This procedure has the theoretical advantage of less postoperative hypocalcemia because the parathyroid remnant remains *in situ* and continues to function. One of the disadvantages of a subtotal parathyroidectomy is the need to perform a second cervical exploration for recurrent or persistent hyperparathyroidism, which can be challenging and carries the increased risk of recurrent laryngeal nerve injury. This is the most common operation for tertiary hyperparathyroidism.

Total parathyroidectomy with autotransplantation involves removal of all four glands followed by selection of the most suitable gland as an autograft. The most normal-appearing gland is chosen and a 40-60 mg portion of this gland is minced into small fragments for autotransplantation. This tissue may be confirmed as parathyroid gland on frozen section. Finally, approximately 10 gland fragments are autotransplanted into an acceptable site, most commonly the brachioradialis muscle of the nondominant forearm. This area should be marked with nonabsorbable or surgical clip in case future surgery is indicated for recurrence. The disadvantages of total parathyroidectomy include temporary post-operative hypocalcemia requiring aggressive medical therapy, risk of autograft failure, and difficulty in removing graft fragments at reoperation. Cryopreservation of the parathyroid tissue is an important adjunct and allows for future autotransplantation in the case of parathyroid transplant failure and persistent hypoparathyroidism. IOPTH monitoring and radioguided surgery may also be helpful to avoid missing a supernumerary gland during subtotal or total parathyroidectomy.

Parathyroid Cancer

Parathyroid carcinoma is a very rare malignancy with an incidence of <1 million population per year (Lee *et al.*, 2007). The majority of these tumors hypersecrete PTH and lead to symptoms similar to other causes of primary hyperparathyroidism. Parathyroid carcinoma accounts for less than 1% of cases of primary hyperparathyroidism. Preoperative diagnosis of this malignancy is often challenging as the clinical presentation can mimic that of benign disease. A high index of suspicion should accompany concerning features, including a very high serum calcium (>14 mg dL^{-1}) and a palpable neck mass. When these features are present, one should consider a metastatic workup prior to exploration. Intraoperatively, the parathyroid gland may appear grayish-white and is often adherent to the surrounding tissue. Fine-needle aspiration (FNA) is not recommended due to poor sensitivity as well as the risk of capsular rupture and possible seeding of the neck.

Surgery is the primary treatment for parathyroid cancer. If parathyroid carcinoma is suspected during the initial procedure, the surgeon should proceed with complete en bloc resection of the tumor with negative margins, including any of the locally invaded tissue such as the contiguous ipsilateral thyroid lobe. The most common operation involves a thyroid lobectomy. In the past, a

central lymph node dissection was recommended, but recent data suggest that this is not necessary for most patients with parathyroid cancer (Hsu *et al.*, 2014). Despite aggressive surgical management, tumor recurrence is common. Recurrence is best managed with reoperation as surgery is the most effective treatment in palliating the accompanying hypercalcemia. The multiple reoperations often result in higher rates of surgical complications. Mortality is usually secondary to the complications of uncontrollable hypercalcemia and not tumor burden.

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Hypercalcemia: Other Causes than Primary Hyperparathyroidism

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Introduction

The regulation of calcium homeostasis is aimed at maintaining extracellular calcium concentration and calcium balance as constant as possible, to protect the body against calcium overload and mineral deposition into soft tissues. Extracellular calcium concentration is thus maintained extremely stable, because of the high sensitivity of a variety of cell systems or organs, including the central nervous system, muscle, and exo-/endocrine glands, to small variations of extracellular calcium concentration (Rizzoli and Bonjour, 2006). Calcium homeostasis is controlled by a series of hormones and factors tightly interrelated in complex regulatory systems. The production of some of these agents is regulated by the concentration of the solute they are controlling, through negative feedback mechanisms.

Regulation of Extracellular Calcium Concentration

Extracellular calcium concentration is maintained in a dynamic equilibrium through fluxes occurring at the level of the intestine, bone and kidney (Rizzoli and Bonjour, 2006). At steady state, as in nongrowing individuals, the amounts of solutes entering the extracellular space are matched by the amounts leaving it. Approximately 50% of total calcium is bound to proteins, mostly albumin. To take into accounts variations in protein or albumin concentrations, when measuring total plasma calcium concentration, various corrections have been proposed. A simple one is Albumin-corrected calcemia = measured calcemia + 0.02 mmol/L per gram of albumin lower than 40 g/L. Alternatively, total protein can be used, provided there is no paraprotein, with the formula: Protein-corrected calcemia = measured calcemia/[(protein in g/L/160) + 0.55]. All the albumin or protein based corrections are not recommended for extreme values of albumin or protein. Furthermore they do not take into account variations in blood pH. Indeed, alkalosis is associated with a higher binding of calcium to albumin, hence a lower free calcium concentration. Acidosis is causing the reverse, that is, a higher unbound fraction.

To avoid the problem of the influence of albumin or protein concentration, the golden standard is the determination of the calcium bioactive form, that is, ionized calcium. However, the latter requires strict preanalytical conditions, without any contact of the blood sample with air, and measurement must be completed within 1 h after sampling. From an analytical point of view, ionized calcium determination is performed on blood gas analyzers with a specific electrode, which may complicate its utilization in large series of samples. A second sample (heparinated syringe or capillary sample) is generally required. Such a large volume to be collected in a child may preclude a routine ionized calcium determination in children. Because of the influence of pH on ionized calcium concentration, the latter must be interpreted at native pH, and not after an adjustment for pH 7.40.

Intestinal Fluxes

Net intestinal absorption of calcium represents the difference between the amounts of solutes absorbed and secreted into the gut lumen. In human, under normal conditions, fractional calcium intestinal absorption represents approximately 20% of ingested calcium. This percentage is higher during growth. Net intestinal absorption of calcium depends on dietary intakes, on the capacity of the intestinal wall to transport calcium, on the bioavailability of calcium present in the intestinal lumen, and on the secretory flux. The intestinal calcium active absorptive capacity is mainly controlled by calcitriol, which stimulates the transport through both genomic and nongenomic mechanisms (Rizzoli et al., 1977; Haussler et al., 2013). Parathyroid hormone is not exerting any direct effect on the intestinal cells for calcium absorption, but indirectly through calcitriol. The importance of bioavailability of calcium at absorptive sites is illustrated by the impairment of calcium absorption induced by the formation of complexes with anions, such as phosphate, sulfate, phytate or oxalate. For instance, the colonic mucosa is equipped with a powerful vitamin D-sensitive mechanism of calcium transport. However, the absorption is quantitatively little, since calcium in the large intestine lumen is under a form not available for absorption (Ammann et al., 1986). Modification of gut microbiota and lower intestinal content pH can increase distal tract calcium absorption (Ammann et al., 1988; Whisner and Castillo, 2018). At steady state, a 24 h urinary excretion of calcium is mainly the reflection of daily net intestinal calcium absorption.

Bone Fluxes

In average, about 1% of total bone calcium exchanges every month, through a mechanism involving bidirectional fluxes. The main regulators of these fluxes are parathyroid hormone and calcitriol, as well as numerous factors locally produced (Rizzoli and Bonjour, 2006; Manolagas and Parfitt, 2013; Bonewald, 2017). A large variety of substances either circulating or locally produced,

or present in the bone matrix, such as prostaglandins, thyroid hormones, glucocorticoids, sex hormones, growth factors, interleukins, lymphokines or myokines, components of the RANK-ligand/osteoprotegerin system, produced by the immune or hematopoietic systems, stromal or bone cells, are capable of influencing bone remodeling, and thereby the bidirectional calcium fluxes (Martin and Seeman, 2008; Martin and Sims, 2015). In fasting urine, calcium excretion related to creatinine is a direct reflection of net bone calcium exchange (Nordin and Peacock, 1969; Peacock et al., 1969). Indeed, after an overnight fast, calcium appearing in the urine mostly originates from bone. Multiplying the calcium to creatinine ratio by serum creatinine provides urinary calcium excretion per glomerular filtration rate unit.

Renal Fluxes

By controlling calcium excretion, the kidney plays a central role in calcium homeostasis (Rizzoli and Bonjour, 2006). At steady state, the amount of calcium appearing in the urine is the reflection of net fluxes into the extracellular fluid originating from intestine and bone. Approximately 75% of plasma calcium is ultrafiltrable. After filtration, more than 95% of calcium is reabsorbed. In proximal tubule, calcium reabsorption is tightly connected to that of sodium (Friedman, 2000). Then, 20% to 30% of filtered calcium are reabsorbed along the loop ascending limb, and 10% at the level of the distal tubule. Parathyroid hormone influences calcium reabsorption in the loop ascending limb and in the distal tubule (Friedman, 2000). Any change in renal tubular capacity to reabsorb calcium is able to induce variations of plasma calcium from 1.5 to 3.8 mmol/L (Nordin and Peacock, 1969; Peacock et al., 1969). This concept has been established by the study of the relationship between urinary calcium excretion and plasma calcium in patients suffering from a lack or an excess of parathyroid hormone.

Various situations or pharmacological agents can modulate the renal tubular reabsorption of calcium. Alkalosis stimulates renal tubular reabsorption of calcium, whilst acidosis decreases it. Thiazides and lithium salts increase the reabsorption of calcium, through mechanisms which are independent of parathyroid hormone (Rizzoli et al., 1981). Phosphate deficiency, pharmacological doses of calcitonin and loop diuretics are associated with an increase in calcium clearance (Friedman, 2000). Even large variations of the glomerular filtration rate do not cause major changes in calcemia, since the renal tubule can easily maintain the calcium excretion by modulating its reabsorptive capacity. A right shift in the relationship between urinary calcium excretion and calcemia indicates an increase in the renal tubular reabsorption of calcium, for instance through increased PTH levels. The latter can be quantified using an index calculated from a nomogram with fasting urinary and serum, calcium and creatinine values (Bonjour et al., 1988).

Calcium Homeostasis

A central role in the regulation of calcium homeostasis is played by parathyroid hormone produced by the parathyroid glands, which recognize small alterations in plasma calcium (Goltzman and Hendy, 2015; Hannan et al., 2016). Any change in plasma calcium is detected by a cell membrane-associated calcium-sensor/receptor, which can also be activated by other divalent cations. This 1078 aminoacid polypeptide belongs to the family of seven transmembrane domains cell membrane associated and guanine nucleotide binding protein-coupled receptors (Hannan et al., 2016).

Parathyroid hormone as well as a decrease in calcium and/or phosphate concentrations directly stimulate the synthesis of calcitriol at the renal level. The latter hormone contributes to an increase of plasma calcium through a mobilization of calcium from bone and a stimulation of intestinal calcium absorption (Haussler et al., 2013; Hefti et al., 1983).

Table 1 Mechanisms of hypercalcemia

	Increased bone resorption	*Increased renal tubular reabsorption of calcium*
Endocrine disorders		
Primary hyperparathyroidism	+	+
Hyperthyroidism	+ +	−
Malignancy (with or without bone metastases)	+ or + +	+ or −
Granulomatous disorders (calcitriol overproduction)	+ +	−
Immobilization	+ +	−
Drug-induced		
Vitamin D poisoning	+ +	−
Vitamin A intoxication	+	−
Milk-alkali syndrome	−	+
Thiazide diuretics	−	+
Lithium salts	−	+
Benign familial hypocalciuric hypercalcemia	−	+

Disorders of Extracellular Calcium Homeostasis (Table 1)

Any disturbance of the above described fluxes can result in an alteration of extracellular calcium homeostasis. An increase in intestinal absorption of calcium and in bone resorption can lead to hypercalcemia when the renal excretion capacity is overwhelmed (Buchs et al., 1991; Rizzoli et al., 1994b). When renal tubular reabsorption of calcium is stimulated, plasma calcium levels can rise, despite very minute changes of calcium influx into the extracellular fluid compartment (Nordin and Peacock, 1969; Peacock et al., 1969). Despite increased renal tubular reabsorption of calcium, urinary excretion is elevated as a consequence of a higher filtered load.

The predominance of stimulated renal tubular reabsorption of calcium or of increased bone resorption in determining an altered extracellular calcium homeostasis can be demonstrated in a variety of clinical disorders associated with hypercalcemia (Buchs et al., 1991) (**Fig. 1**).

Symptoms and Signs of Hypercalcemia

Hypercalcemia is associated with a variety of symptoms, for which there is no specific threshold of calcium concentration accompanied by clinical symptoms. Their severity is influenced by the rapidity of onset of hypercalcemia, age, comorbidities and

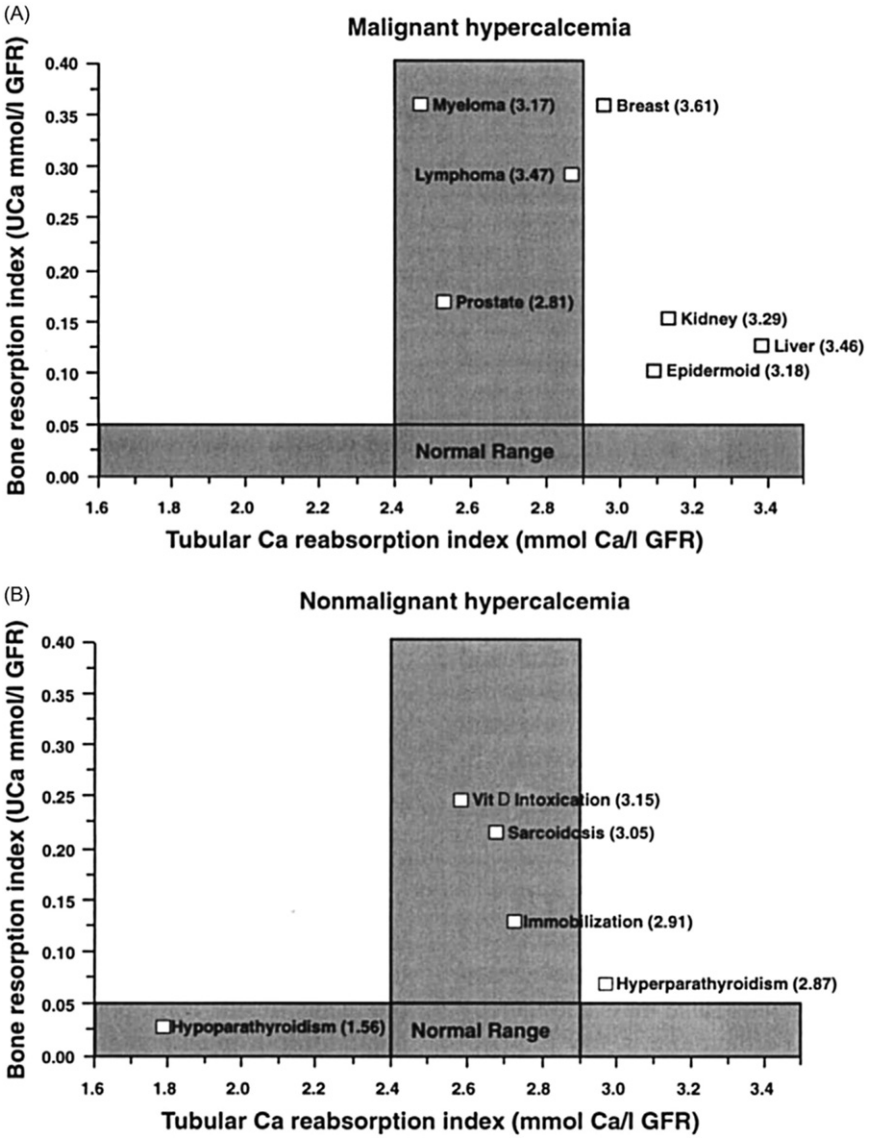

Fig. 1 Relationship between bone resorption, as evaluated by the bone resorption index (BRI) and tubular reabsorption of calcium index (TRCaI) in rehydrated patients with malignant (A) or nonmalignant (B) hypercalcemia. In parentheses is mentioned the mean plasma calcium concentration. This figure is taken from Buchs, B., Rizzoli, R. and Bonjour, J. P. (1991). Evaluation of bone resorption and renal tubular reabsorption of calcium and phosphate in malignant and nonmalignant hypercalcemia. *Bone,* **12**, 47–56.

concomitant medications. The symptoms can be divided into the four "O" categories. *Groan* (gastrointestinal tract): anorexia, dyspepsia, nausea, constipation, abdominal pain. *Moan* (neurological): fatigue, muscle weakness, poor concentration, confusion, irritability, even lethargy and coma. *Stone* (renal): dehydration, polydipsia, oliguria, renal function impairment, acute renal injury, nephrocalcinosis and vascular calcifications. *Bone*: this series of symptoms concerns more primary hyperparathyroidism, and is less prominent in other causes of hypercalcemia. Cardiac symptoms associated with elevated extracellular calcium concentrations comprises shortening of the QT interval in the electrocardiogram and increased risk of arrhythmia, particularly with concomitant hypokalemia.

Disorders Other Than Primary Hyperparathyroidism

In outpatients, the most frequent cause of hypercalcemia is primary hyperparathyroidism. Among other causes, malignancy accounts for the vast majority of hypercalcemia in hospitalized patients.

Endocrine Dysfunction: Hyperthyroidism

Mild elevation of serum calcium is frequently encountered in active hyperthyroidism. The mechanism is increased bone resorption (Baxter and Bondy, 1966). A feature of hyperthyroidism is a rather high phosphatemia, since thyroid hormone can directly stimulate renal tubular reabsorption of phosphate (Sorribas et al., 1995). Hypercalcemia resolves with correction of thyroid function.

Malignancy

Approximately one-half of people who die of cancer have bone involvement (Coleman, 2006). Different tumor types may have preferential sites of metastases; however, the vast majority of tumors metastases to bone. The term metastatic bone disease reflects the spread of a tumor to the bone. This term may be applied to solid tumors, as well as to multiple myeloma, where the tumor is intrinsic to the bone marrow (Rizzoli et al., 2013). In advanced breast or prostate cancer, metastatic bone disease is present in a large proportion of patients. Bone metastases may also be seen in 15%–30% of cancers of the lung, gastrointestinal tract (colon and stomach), and the genitourinary (bladder, kidney and uterus) (Roodman, 2004), as well as in advanced thyroid cancer and melanoma (Coleman, 2006). Skeletal related events include pathologic fractures (21%), spinal cord compression (1%), surgery (1%) and radiotherapy (8%) to bone and may or may not include hypercalcemia of malignancy. Hypercalcemia is a sign of advanced cancer disease. Only in neuroendocrine tumors or possibly in renal carcinoma, hypercalcemia may be detected before the tumor diagnosis.

Malignant cells release a number of molecules that favor osteoclastogenesis via the RANK/RANKL/OPG system like parathyroid hormone-related protein (PTHrP) detected by immunohistochemistry in about 90% of bone metastases from breast cancer (Powell et al., 1991; McCauley and Martin, 2012). RANKL has also been shown to trigger the migration to bone of melanoma and some epithelial cells that express the RANK receptor, such as breast cancer cells. Lytic bone lesions are observed on X-ray in about 95% of patients with advanced myeloma, in contrast to what is observed in lymphomas, although both entities are B-cells malignancies. Bone involvement is related to an excessive bone resorption through increased osteoclast number encountered in the close vicinity of myeloma cells (Bataille, 2015) together with a decreased osteoblast activity. In myeloma and lymphomas, there is a marked reduction in osteoblast activity. The plasma cells can release several factors such as DKK1, sFRP2 which act on the Wnt pathway and reduce the osteoblast number and activity (Tian et al., 2003). So the lesions observed in myeloma are predominantly osteolytic. In advanced metastatic bone disease, hypercalcemia reflects the release of large amounts of calcium mobilized due to breakdown of the calcified matrix. In some cases of multiple myeloma, the monoclonal immunoglobulin binds the calcium ion (Merlini et al., 1984). In these patients, the high total serum calcium is associated with normal ionized calcium and urinary calcium excretion, without symptoms or signs of hypercalcemia.

Hypercalcemia may occur even in the absence of bone metastases and is attributed to a humoral mechanism (Stewart et al., 1980) (**Table 2**). In humoral hypercalcemia of malignancy, increased renal tubular reabsorption of calcium represents the main pathogenetic mechanism, in which an effect of tumor-produced PTHrP is implicated (Stewart, 2005). Ectopic production of authentic PTH is a very rare phenomenon (Rizzoli et al., 1994a). The relative and quantitative contribution of calcium mobilization from bone, and of renal tubular reabsorption of calcium, to hypercalcemia induced by parathyroid hormone-related protein can be estimated in studying the model of thyroparathyroidectomized rats chronically infused with parathyroid hormone-related protein (Rizzoli et al., 1989). The elevation of plasma calcium is determined by both increased bone resorption and enhanced renal tubular reabsorption of calcium. However, the complete inhibition of bone resorption by a bisphosphonate, at a dose which fully normalizes fasting urinary calcium excretion, taken as a reflection of net bone resorption, is associated with an approximately 30% decrease, but not a correction of plasma calcium (Rizzoli et al., 1989). Thus, the residual hypercalcemia can be attributed to a renal tubular reabsorption effect, which accounts for more than two-thirds of the elevated plasma calcium in this experimental model. Indeed, it is well established that bisphosphonates are devoid of any direct effect on the renal handling of

Table 2 Bone invasion in hypercalcemia of malignancy.

Tumor type	*No. of subjects*	*% with bone invasion*	*Calcemia (mmol/L)*
Breast	35	100	3.61 ± 0.66
Epidermoid	21	48	3.18 ± 0.32
Myeloma	13	100	3.17 ± 0.24
Lymphoma	6	14	3.41 ± 0.45
Kidney	7	0	3.29 ± 0.20
Liver	3	33	3.47 ± 0.34
Prostate	3	100	2.81 ± 0.09
Miscellaneous	13	58	3.19 ± 0.35

From Buchs, B., Rizzoli, R. and Bonjour, J. P. (1991). Evaluation of bone resorption and renal tubular reabsorption of calcium and phosphate in malignant and nonmalignant hypercalcemia. *Bone*, **12**, 47–56.

Table 3 Hypercalcemic disorders with elevated calcitriol production

Eutopic secretion	Tumors: seminoma, PTHrP producing tumors
Ectopic production	Tumors: B- or T-cell lymphomas, Hodgkin disease, T-cell acute leukemia, eosinophilic granuloma
	Infection: tuberculosis, histoplasmosis, candidiasis, leprosy
	Other granulomatous diseases: sarcoidosis, silicosis, berylliosis, lipoid pneumonia, foreign body granuloma (e.g., silicon implant)

calcium (Rizzoli et al., 1992). Elevated PTHrP levels can lead to an earlier relapse after treatment of hypercalcemia by a bone resorption inhibitor (Rizzoli et al., 1999).

Granulomatous Disorders

Ectopic production of calcitriol is a cause of hypercalcemia occurring in some malignancies of the hematopoietic system as well as in various ganulomatous diseases (Kallas et al., 2010) (**Table 3**). In active sarcoidosis, up to 10% of the patients may become hypercalcemic, and even more hypercalciuric. Granuloma macrophages develop an increased activity of 1-alpha-hydroxylase enzyme leading to higher production and circulating levels of calcitriol, hence to a markedly elevated intestinal absorption of calcium.

Immobilization

Very rapidly after immobilization, there are an increase in bone resorption and an inhibition of bone formation, leading to calcium release into the extracellular fluid (Stewart et al., 1982). Hypercalcemia occurs when the urinary excretion capacity is overwhelmed. There is a clear age-dependency since disuse-induced hypercalcemia is very rare in the oldest old whilst it is more frequent in children and young adults.

Drug-Induced

Vitamin D excess due to iatrogenic administration of pharmacological doses of vitamin D is a rare cause of hypercalcemia. Large doses used to be given in the treatment of hypoparathyroidism before the availability of active vitamin D metabolites. Vitamin D excess is associated with increased calcium intestinal absorption and bone resorption (Rizzoli et al., 1994b). The latter is responsive to bone resorption inhibitors. Because of the prolonged half-life of the metabolite 25-hydroxyvitamin D, hypercalcemic–hypercalciuric syndrome can persist for several weeks to months, with an important morbidity and even permanent soft tissues damages.

Vitamin A intoxication-mediated hypercalcemia is very rare and can result from vitamin A supplementation or retinoic acid treatment for various disorders (Valentic et al., 1983). The pathogenic mechanism mainly involves increased bone resorption.

Milk-alkali syndrome encompasses hypercalcemia, metabolic acidosis and renal failure (Orwoll, 1982). It was described in patients with peptic ulcer treated with large amounts of milk and antacids, such as calcium carbonate. Whilst hypercalcemia is usually corrected with hydration and reduction of calcium intakes, renal function alterations may persist.

Thiazide diuretics stimulate calcium reabsorption in the distal tubule through a PTH-independent mechanism, particularly in situations with high bone turnover (Rizzoli et al., 1981; Wermers et al., 2007).

Lithium increases both PTH secretion, through parathyroid cells hyperplasia, and renal tubular reabsorption of calcium, which appears to be reversible upon cessation of lithium treatment (Khandwala and Van Uum, 2006; Bendz et al., 1996). Mild hypercalcemia has been reported in as many as 5% of lithium-treated patients.

Benign Familial Hypocalciuric Hypercalcemia

Various mutations in the calcium sensing receptor have been reported, which account for hyper- or hyposecretion of parathyroid hormone in relation with variations of extracellular calcium concentration (Hu and Spiegel, 2003). In familial hypocalciuric benign hypercalcemia, circulating levels of parathyroid hormone are insufficiently suppressed for the degree of calcemia, and the renal tubular reabsorption of calcium is increased through a parathyroid hormone-independent mechanism (Marx et al., 1982; Hannan et al., 2016). This disorder appears to be due to mutations associated with hypofunction of the cell membrane calcium sensing mechanism (Hannan et al., 2016). Thus, higher plasma calcium levels are necessary to inhibit PTH secretion. Interestingly, the same biochemical pattern can be encountered in patients treated with lithium salts (Bendz et al., 1996). Under these conditions, parathyroid hormone production is not suppressed despite higher circulating calcium levels. Calcium sensing receptor activity can be modulated by calcimimetics, which enhance its sensitivity to extracellular calcium (Marx, 2017).

Management of Hypercalcemic Disorders

Severe symptomatic hypercalcemia requires rapid intervention since the condition may be life-threatening. The first aim of therapy is to correct dehydration (Rizzoli and Bonjour, 1992). Fluid therapy comprises the administration of 2–3 L of isotonic saline over the first 24–48 h, with repeated measurements of plasma electrolytes, particularly potassium, because of the risk of severe hypokalemia. Hypovolemia may be present and further worsened during diuresis. Plasma calcium decreases not only by restoring intravascular volume and correcting thereby the hemoconcentration but also by altering proximal tubule calcium hyperreabsorption, which is the consequence of dehydration and volume contraction (Hosking et al., 1981). Thus, it is mandatory to replenish extracellular fluid volume, possibly under the continuous control of central venous pressure, particularly in older subjects with compromized cardiac function, and urine output, to maintain sodium diuresis and promote calcium excretion. To avoid possible fluid overload, loop diuretics could be administered. Loop diuretics impair calcium reabsorption in the ascending limb of Henle's loop and may help to enhance urinary calcium excretion (Suki et al., 1970). But their effect is rather weak, since the calcium reabsorbed in the ascending limb represents a small fraction of the filtered load. By causing acute volume loss, diuretics could further aggravate the volume contraction dependent hypercalcemia. In the management of hypercalcemia states, the combination of massive saline infusion with high doses of furosemide is of dubious efficacy to markedly decrease renal tubular reabsorption and may be associated with life-threatening imbalances in electrolytes concentration and fluid volumes. This historical therapeutic approach should be avoided nowadays. In case of hypernatremia, 5% dextrose should be given instead of saline. Attention should be paid to prevent immobilization to avoid additional disuse-induced stimulation of bone resorption and impaired bone formation.

Clinical improvement usually occurs after rehydration. The next step of therapy requires the correction of the disturbed calcium flux(es): the decrease in net bone resorption and/or in renal tubular calcium reabsorption. To modify the latter, pharmacological doses of salmon calcitonin, 400–600 IU given intravenously in perfusion over 12 to 24 h can be administered (the effect is however transient), or the oral calcimimetic cinacalcet. Both are reducing the renal tubular reabsorption of calcium. The use of the former should be restricted to very severe hypercalcemia with neurological symptoms, requiring a rapid decrease in calcemia.

The most efficacious and used agents in the management of nonhyperparathyroid hypercalcemia are certainly the bone resorption inhibitors bisphosphonates (Rizzoli and Bonjour, 1992; Berenson, 2002; Body et al., 2007). Pamidronate is given at the dose of 90 mg intravenously in 250–500 mL of saline or 5% dextrose over 2 h. Zoledronate is given at a dose of 4 mg intravenously in 250 mL saline or 5% dextrose over 30 min. Ibandronate is given at the dose of 4 mg in 100 mL saline or 5% dextrose over 30 min. In response to intravenous bisphosphonates, calcemia begins to drop by 48 h and reach a nadir by 4 to 5 days.

Conclusions

The physiology of calcium homeostasis is regulated by coordinated bidirectional calcium fluxes, occurring at the levels of intestine, bone and kidney. These fluxes are influenced by calciotropic peptides or steroid hormones, and by a variety of locally produced factors. In the control of extracellular calcium concentration, the kidney tubule reabsorptive capacity plays a central role. Management of hypercalcemic disorders other than primary hyperparathyroidism, includes the treatment of the hypercalcemia causing disease and normalization of the abnormal calcium fluxes. Bisphosphonates are an efficacious option in case of increased bone resorption.

See also: Hypercalcemia

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Parathyroid Hormone Related Protein (PTHrP)

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Abbreviations

GPCR G-Protein coupled receptor
PC Proprotein convertases
PTH Parathyroid hormone
PTHrP Parathyroid hormone related protein
PTHR1 Parathyroid hormone receptor 1
PTHR2 Parathyroid hormone receptor 2
cAMP Cyclic-adenosine monophosphate

Glossary

Osteoclasts Bone resorbing cells.
Osteoblasts Bone forming cells.
Paracrine Hormonal effects on local cells.

Introduction

The existence of a parathyroid hormone related (or like) protein was first postulated by Albright (1941), while discussing a patient with renal carcinoma, a solitary metastasis and severe hypercalcemia. Albright suggested that some of the tumors might be secreting PTH or something like it that induced the hypercalcemia (Albright, 1941). As with many of Albright's predictions, he was eventually proven correct, but not before the idea that the hypercalcemia was due to ectopic PTH production had become well-established (Omenn *et al.*, 1969). It was eventually the development of improved immunoassays in the 1970's, which revealed that the causative agent of the humoral hypercalcemia of malignancy (HHM) was distinct from *bona fide* PTH, with PTH even undetectable in numerous hypercalcemic patients (Benson *et al.*, 1974; Powell *et al.*, 1991; Roof *et al.*, 1971). In the late 1980s, parathyroid hormone related protein (PTHrP) was purified from a human lung cancer cell line, then cloned, sequenced and identified as the agent responsible for HHM (Moseley *et al.*, 1987; Suva *et al.*, 1987). Other confirmatory studies soon followed (Mangin *et al.*, 1988; Strewler *et al.*, 1987) that collectively demonstrated that this factor bound to the PTH receptor to induce cyclic-adenosine monophosphate (cAMP) production by activating adenylate cyclase downstream of the receptor (Horiuchi *et al.*, 1987; Kemp *et al.*, 1987), but was immunologically and genomically distinct from PTH. Thereafter, the gene encoding PTHrP, *PTHLH*, was isolated from multiple species, including human, rat, mouse (Mangin et al., 1989, 1990b; Suva *et al.*, 1989; Yasuda *et al.*, 1989) and more than 30 years of subsequent research have revealed a vast amount of information regarding the synthesis, metabolism, regulation and varied biological actions of PTHrP. Soon after its discovery, it became apparent that PTHrP was far more than an evolutionary accident that phenocopied the ability of PTH to regulate postnatal mammalian calcium and phosphate metabolism. If gene conservation across species and phyla is evidence of evolutionary and gene importance, then *PTHLH* clearly represents the ancestral gene and the basis for the subsequent gene duplication event yielding the *PTH* gene. Indeed, PTHrP has functions far beyond the limited physiologic role of PTH. This article will briefly consider PTHrP in the context of physiology and pathophysiology, and outline the gene structure, biosynthesis, metabolism, regulation and breadth of PTHrP's widespread biological functions.

PTHLH Gene

The PTHrP gene (*PTHLH*) has a more complex genomic organization than the *PTH* gene, but the two genes share similar intron-exon boundaries (**Fig. 1**). The amino acid sequence of PTHrP is highly conserved (up to amino acid 111) between human, mouse, rat, chicken and dog, suggesting important functions are likely residing in these regions. Studies over the past few decades, have demonstrated that PTHrP is the product of a single *PTHLH* gene localized on the p-arm of the human chromosome 12 (Suva *et al.*, 1989; Vasavada *et al.*, 1993; Yasuda *et al.*, 1989). Unlike the related human *PTH* gene, which is located on chromosome 11, the *PTHLH* gene is a complex transcriptional unit consisting of 9 exons and 3 unique promoters upstream of exons I, III and IV (**Fig. 1**) (Suva *et al.*, 1989; Vasavada *et al.*, 1993; Yasuda *et al.*, 1989). Transcription of the PTHLH gene can result in three mature human PTHrP peptides of 139, 141 and 173 amino acids. Multiple RNA transcripts of each of these peptides have been observed, validating the three peptides as the transcribed and translated products of the single human *PTHLH* gene. Gene splicing events result in the three variants of PTHrP: PTHrP(1–139), PTHrP(1–173), PTHrP(1–141) (Mangin *et al.*, 1988; Southby *et al.*, 1995).

Both PTHrP and PTH, as well as their shared common receptor, PTH receptor 1 (PTHR1), have been identified in teleost fish and in the cartilaginous elasmobranches (Devlin *et al.*, 1996; Trivett *et al.*, 1999; Danks et al., 1993, 1998). These data support the idea that *PTHLH* is indeed the original ancestral gene, with PTH and its subsequent control of postnatal calcium metabolism

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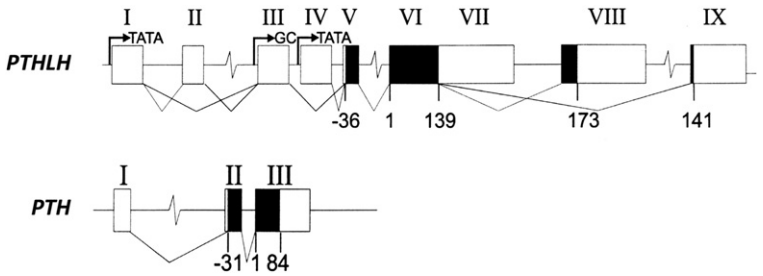

Fig. 1 *PTHLH* and *PTH* gene splicing. The nine exons of the human *PTHLH* gene are shown and three unique promoters upstream of exons I, III and IV. Transcription initiation position is at +1. During splicing three isoforms of PTHrP can be formed: PTHrP(1–139), PTHrP(1–173), PTHrP (1–141). The *lines* indicate different splicing events. Adapted from (Mangin, M., Ikeda, K., Dreyer, B. E. and Broadus, A. E. (1989). Isolation and characterization of the human parathyroid hormone-like peptide gene. *Proceedings of the National Academy of Sciences of the United States of America*, 86, 2408–2412; Suva, L. J., Mather, K. A., Gillespie, M. T., Webb, G. C., Ng, K. W., Winslow, G. A., Wood, W. I., Martin, T. J. and Hudson, P. J. (1989). Structure of the 5' flanking region of the gene encoding human parathyroid-hormone-related protein (PTHrP). *Gene*, 77, 95–105). The three exons of the human *PTH* gene are shown for comparison. Adapted from (Vasicek, T. J., Mcdevitt, B. E., Freeman, M. W., Fennick, B. J., Hendy, G. N., Potts, J. T., Jr., Rich, A. and Kronenberg, H. M. (1983). Nucleotide sequence of the human parathyroid hormone gene. *Proceedings of the National Academy of Sciences of the United States of America*, 80, 2127-2131). Transcription initiation position is at +1. During splicing 1 isoform of PTH, PTH(1–84) can be formed.

evolving by a gene duplication event. Interestingly, the structural organization of the chicken, mouse and rat PTHLH genes share substantial homology with the human *PTHLH* gene (Karaplis *et al.*, 1990; Mangin et al., 1990b; Moseley and Gillespie, 1995; Yasuda *et al.*, 1989; Thiede and Rutledge, 1990) although the rodent genes are considerably simpler than the human gene. The *PTHLH* gene in rodents appears to employ only a single TATA promoter and contains only four exons, suggesting that the greater complexity of the human PTHLH gene is a late evolutionary event (Karaplis *et al.*, 1990; Mangin et al., 1990a), given the relative simplicity of the mouse and rat genes.

In the human *PTHLH* gene, the presence of multiple promoters (**Fig. 1**) suggests that alternate promoter use maybe driving the tissue-specific and/or developmentally regulated expression of PTHrP. Since the *PTHLH* genes from all species described to-date have a functional proximal TATA promoter region, but may not have upstream promoters (**Fig. 1**), it is likely that the majority of the transcriptional regulation of PTHrP occurs through the proximal TATA promoter (5′ to exon IV).

The genomic organization and complexity of the human *PTHLH* gene that produces three splice variants of PTHrP with complex transcriptional regulation (**Fig. 1**) remains a scientific quandary. Indeed, there are currently only four reported situations in which PTHrP peptides are present in the circulation; (i) HHM where PTHrP expression by tumors stimulates bone resorption, (ii) lactation where PTHrP is made in the breast and enters the circulation (Grill *et al.*, 1992), (iii) fetal life where PTHrP regulates maternal-fetal calcium transport (Kovacs *et al.*, 1996; Abbas *et al.*, 1989; Rodda *et al.*, 1988), and (iv) breast cancer bone metastasis where PTHrP(12–48) has been measured in patient serum by mass spectrometry and likely regulates hematopoietic cell fate (Kamalakar *et al.*, 2017; Washam *et al.*, 2013). Since there is no evidence of PTHrP peptides in the circulation of normal humans, it appears that PTHrP actions, unlike PTH, are largely paracrine. It is these observations, along with the susceptibility of the PTHrP sequence to proteolysis, glycosylation and other posttranslational modifications that highlight the complexity of the molecule and its myriad functions, many of which we summarize below.

PTHrP Peptide(S) Biosynthesis, Metabolism and Nuclear Localization Motifs

In the context of diseases, the details and identity of circulating PTHrP is even more important. Less than 0.2 pmol/L of PTHrP peptides are detectable in the circulation in normal adult humans, although elevated levels (pg/mL) have been measured by mass spectrometry in the serum breast cancer bone metastasis patients (Washam *et al.*, 2013). PTHrP(12–48) has not been proposed or identified previously and thus may not have been detected by current PTHrP antisera. In fact, detection by existing PTHrP antisera is minimal (Washam *et al.*, 2013). Observations such as these, along with the finding that PTHrP is widely expressed in a variety of normal tissues drives the idea that PTHrP acts in a paracrine fashion. Sites of PTHrP expression represent both the regulated and constitutive pathways of protein secretion and suggest multiple modes of local release. Comparison of the human PTH and PTHrP peptide sequences reveals significant homology in the N-terminal region, with 8 of the first 13 amino acids identical and very little PTH homology observed through the rest of the sequence (Suva *et al.*, 1987). The N-terminal region (amino acids 1–14) comprises the PTH receptor (PTHR1) binding domain (**Fig. 2**). The extensive homology between PTH and PTHrP in this region accounts for the ability of PTHrP to bind to and activate PTHR1 in classic PTH target tissues (bone and kidney) and consequently to generate the paraneoplastic effects on calcium and phosphate metabolism characteristic of HHM. The amino acid sequence is conserved in various species from chicken, mouse and rat to dogs and humans indicating that PTHrP has an important evolutionary function.

In addition to PTHR1, a second human PTH2 receptor (PTHR2) has been identified (Behar *et al.*, 1996; Usdin, 2000; Usdin *et al.*, 1995) and which binds and is activated at high potency by PTH and by tuberoinfundibular peptide of 39 residues (TIP39)

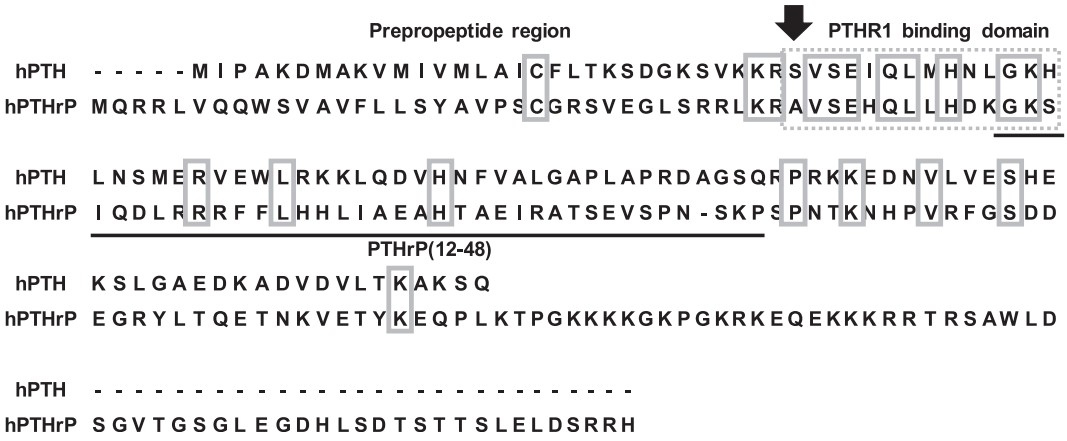

Fig. 2 *Comparison of human PTH and human PTHrP amino acid sequences.* The amino acid sequences of hPTH and hPTHrP are shown. Prepropeptide region is shown to the left of the *arrow*. *Arrow* identifies N-terminal amino acid of the secreted forms of both hPTH and hPTHrP. Amino acid sequence identity overlap between PTH and PTHrP enclosed in *gray*. Significant homology in the N-terminal, PTHR1 binding domain, enclosed in *dotted box*. Amino acid sequence of PTHrP(12–48) is underlined.

but not PTHrP (Usdin et al., 1995, 2002). Interestingly, despite the high homology at the N-terminus between PTH and PTHrP, the selective PTHR2 interaction with PTH and not with PTHrP is encoded by position 5 (Ile in PTH; His in PTHrP) (Behar *et al.*, 1996).

Interestingly, although the transcriptional control of *PTHLH* gene expression is complex, the regulation of individual PTHrP isoforms are also subject to a multitude of posttranslational modifications and enzymatic processing. Each PTHrP isoform; PTHrP (1–139), PTHrP(1–173) and PTHrP(1–141), can undergo numerous posttranslational modifications which are proteolytic and/or enzymatic in nature. These reactions result in various peptide fragments of PTHrP, and many fragments have been ascribed a known biological function (**Table 1**). In the amino acid sequence of PTHrP, the first 36 amino acids (− 36 to − 1) comprise of the prepropeptide region, (**Fig. 2**). The prepropeptide region is necessary for protein trafficking and secretion (Suva *et al.*, 1987). Proprotein convertases, which are a family of enzymes known to cleave peptides following basic amino acids or following an Arginine (R) (Constam and Robertson, 1999). Thus, proprotein or prohormone enzymes, PC1/3, Furin, PC2, PC4, PC5 and PACE4 were shown to cleave the prepropeptide region after the Arginine at position − 1 (**Fig. 3**). A proprotein enzyme-dependent endoproteolytic cleavage of Arginine at position 37 can give rise to the N-terminal fragment and other mid-molecule fragments like, 35–84, which have been associated with the transport of Ca2 + across the placenta to allow skeletal development of a fetus (Wu *et al.*, 1996; Hilliker *et al.*, 1996). The C-terminus 107–139 region (Valin *et al.*, 1999; Cornish *et al.*, 1997), formed by prohormone enzymatic cleavage of Arginine at 106, has been shown to inhibit osteoclast function and to increase osteoblast proliferation. An absence of any of these regions; N-terminal (Hilliker *et al.*, 1996), mid-molecule (Wu *et al.*, 1996) or the C-terminal, results in drastic bone abnormalities in vivo and decreased numbers and function of both osteoclasts and osteoblasts (Soifer *et al.*, 1992; Burtis *et al.*, 1992).

Potential enzymatic processing sites are scattered throughout the PTHrP primary amino acid sequence (**Fig. 3**), and it seems likely that processing of PTHrP takes place in an isoform- and tissue-specific fashion (Habener, 1981; Rholam and Fahy, 2009). The physiological implications of posttranslational processing of PTHrP are still poorly understood, however, it is clear that secreted forms of N-terminal, mid-regional, and C-terminal regions of PTHrP exist (**Table 1**). These peptides have a number of bioactivities that are presumably mediated via distinct receptors and signaling mechanisms to facilitate the broad spectrum of PTHrP effects (Rholam and Fahy, 2009; Deftos *et al.*, 2001). Despite this knowledge, the complex mechanisms driving the extensive endoproteolytic processing and/or metabolism of PTHrP are poorly defined, with even its native secretory forms having eluded precise identification (Burtis, 1992; Kamalakar *et al.*, 2017; Martin *et al.*, 1991; Orloff *et al.*, 1994).

However, although these specific peptide cleavage products have been predicted and/or demonstrated in transgenic animals and in vitro, no specific PTHrP peptide fragments, other than PTHrP(12–48), have been identified or sequenced from the circulation of patients (Kamalakar *et al.*, 2017; Washam *et al.*, 2013). PTHrP(12–48) is not a transcribed product, but is the result of proteolytic processing of the three known human PTHrP isoforms. Indeed, given the lack of direct information, in silico cleavage site analyses identifies the potential endoproteases involved in PTHrP(12–48) processing.

Lysyl endopeptidase (LysC) (Jekel *et al.*, 1983) and Peptidyl-Lys metalloendopeptidase (LysN) (Hori *et al.*, 2001) have cleavage specificities conducive to the N-terminal processing site of PTHrP(12–48) (**Fig. 4**). Although LysC and LysN are not expressed in humans, the chemical properties of these enzymes suggests that Lys11 may be a monobasic cleavage site. Indeed, the N-terminal processing at Lys11 follows the consensus rules and sequence motifs that typically characterize prohormone processing at monobasic sites (Devi, 1991; Schwartz, 1986). Thus, monobasic endoproteases with lysine substrate specificities likely participate in N-terminal processing of PTHrP at Lys11.

The C-terminus of PTHrP(12–48) ends in a proline residue at position 48 (**Fig. 4**). Proline contains a rigid pryrrolidine ring that imposes strong conformational restrictions on the peptide chain's structural architecture (Vanhoof *et al.*, 1995). Proline residues

Table 1 Reported PTHrP peptide fragments and their proposed functions

Peptide fragments	*Known functions*
Prepropeptide	
−36 to −1	Intracellular trafficking and polypeptide hormone secretion (Suva *et al.*, 1987)
N-terminal peptides	
1–14	Receptor binding and activation (Stewart *et al.*, 1987; Burtis *et al.*, 1992; Hilliker *et al.*, 1996)
14–36	Receptor binding and activation (Burtis *et al.*, 1992; Stewart *et al.*, 1987; Hilliker *et al.*, 1996)
12–48	Measured in patient serum by mass spectrometry and likely regulates hematopoietic cell fate (Washam *et al.*, 2013; Kamalakar *et al.*, 2017)
Mid-region peptides	
1–86	Ca^{2+} transport across placenta (Clemens *et al.*, 2001)
35–84	Ca^{2+} transport across placenta (Wu *et al.*, 1996; Hilliker *et al.*, 1996)
67–93	Nuclear localization sequence (NLS), brain development and function (Lam et al., 1999, 2002)
38–94	NLS +, Increases myeloma cell proliferation and survival (Caffario *et al.*, 2014)
C-terminal peptides	
1–108	Bicarbonate excretion from kidneys (Ellis *et al.*, 1990)
1–141	Bicarbonate excretion from kidneys (Ellis *et al.*, 1990)
107–139	Inhibits osteoclast activity, promotes osteoblast proliferation (Cornish *et al.*, 1997; Valin *et al.*, 1999)
142–173	Function unknown

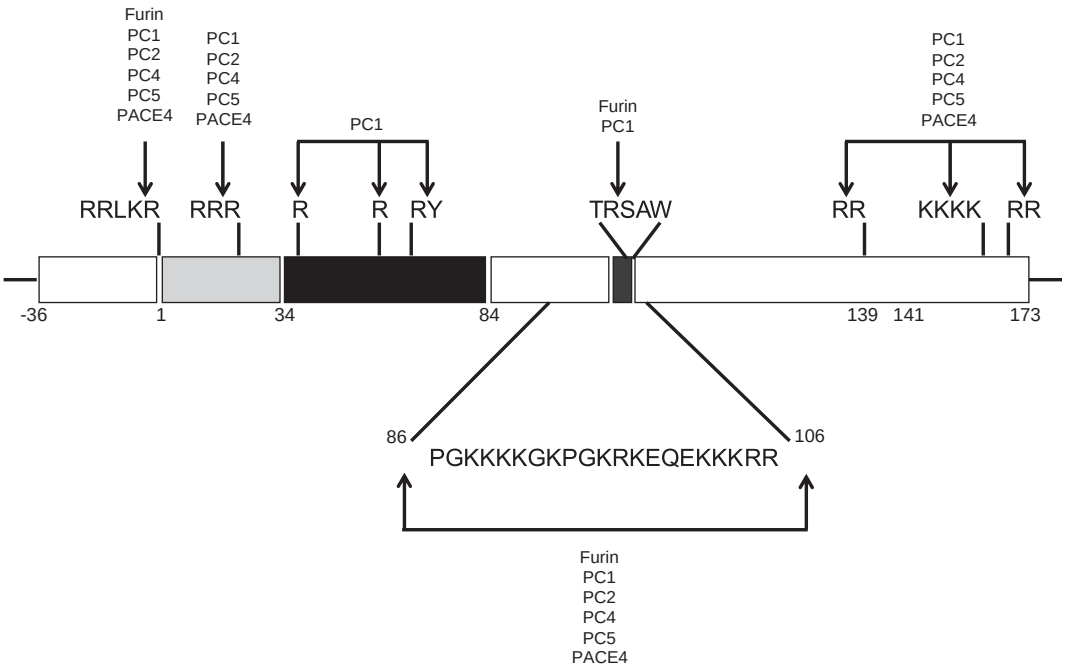

Fig. 3 Putative PTHrP processing sites. The PTHrP peptide from amino acid −36 to 173, including the prepropeptide region of PTHrP is shown. Furins, proprotein convertases (PC1,2,4, 5) and paired basic amino acid cleaving enzyme (PACE4) cleave at basic amino acid residues, Arginine (R), Lysine (K), Serine (S) and Prolines (P) to result in various *predicted* proteolytically and enzymatically cleaved active fragments of PTHrP.

are highly evolutionarily conserved, particularly in cytokines, neuropeptides, and growth factors, and are proposed to serve as beacons that mark biologically important protein regions associated with an array of regulatory, protective, destructive, and other biological activities (Kay *et al.*, 2000; Vanhoof *et al.*, 1995; Yaron and Naider, 1993). In this light, PTHrP proteolysis at position 48 is particularly intriguing. Prolyl endopeptidase (PEP) (Koida and Walter, 1976) or a related member of the serine type postprolyl dipeptidyl family of endoproteases, such as fibroblast activation protein-alpha (FAP-α) (Gass and Khosla, 2007), are strong candidates for the C-terminal processing of PTHrP(12–48) at Pro48 (**Fig. 4**). In sum, the in silico analysis suggests that lysine-specific monobasic and postprolyl endoproteases are likely involved in the N-terminal and C-terminal processing of PTHrP at positions Lys11 and Pro48. Since PTHrP(12–48) remains the only verified circulating PTHrP peptide, much interest still exists in determining the details of its processing and biologic function.

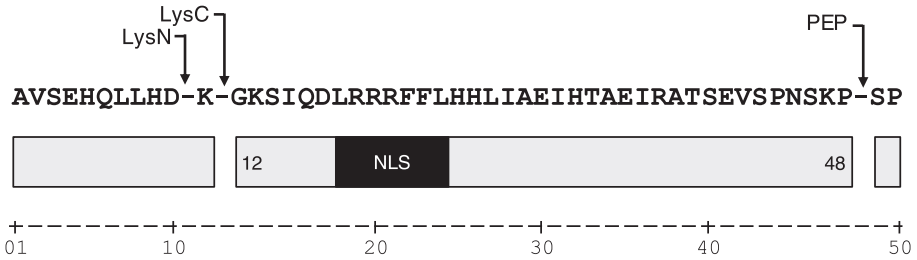

Fig. 4 *In silico PTHrP*(12–48) *Posttranslational Processing and Modification.* The PTHrP(1–50) amino acid sequence is shown. Lysine specific monobasic endoproteases (LysC/LysN) and postprolyl endoprotease family members (PEP) predicted to hydrolyze the Lys^{11} and Pro^{48} peptide bonds respectively. A noncanonical NLS between residues 19–21 suggests an intracrine function for PTHrP(12–48). *LysC* = Lysyl endopeptidase; *LysN* = Peptidyl-Lys metalloendopeptidase; *PEP* = proline endoprotease; *NLS* = nuclear localization signal.

In addition to the classical autocrine/paracrine actions mediated by the activation of PTHR1, mid-regional species of PTHrP have been shown to influence cell behavior through a nuclear pathway, involving endocytosis of PTHrP into neighboring cells and translocation to the nuclear compartment, where it functions to regulate cell death decisions (Luparello *et al.*, 2008) and proliferation in a variety of cell types (Lam *et al.*, 2002) Several noncanonical nuclear localization signals (NLS) have been proposed to exist in the various PTHrP isoforms, the most well-known being a bipartite nuclear/nucleolar localization sequence between residues 88 and 107 that mediates nuclear import of mid-regional species of PTHrP via importin-β1 (Fiaschi-Taesch and Stewart, 2003; Lam et al., 2001, 1999). A second tetrabasic (KKKK) nuclear localization motif spanning amino acid residues 147–150 has also been shown to have intracrine regulatory effects for PTHrP(1–173) in chondrocytes (Clemens *et al.*, 2001; Goomer *et al.*, 2000). In vitro studies have identified a CRM1-dependent leucine-rich nuclear export signal (NES) critical for nuclear export of PTHrP between residues 126 and 139 (Pache *et al.*, 2006). In addition to these motifs, the location of a nonconsensus NLS within PTHrP(12–48) (**Fig. 4**) raises some interesting possibilities that has been shown to target PTHrP(12–48) to osteoclasts (Kamalakar *et al.*, 2017). In sum, the precise actions of PTHrP in the nuclear compartment remain unclear, but are thought to include DNA binding transcription factor activity associated with regulation of gene transcription and/or nuclear organization (Luparello *et al.*, 2008), activation/suppression of the cell cycle via interaction with other nuclear proteins (Connell-Crowley *et al.*, 1997; Lam *et al.*, 2000; Swarthout *et al.*, 2002; Tovar Sepulveda *et al.*, 2002) as well as RNA/chromatin binding and nuclear trafficking (Aarts et al., 1999a, b; Sirchia *et al.*, 2007).

PTHrP Regulation and Function

Since its discovery, PTHrP has been shown to play major roles in a variety of fundamental biologic functions. Indeed, the observation that PTHrP is normally produced in a wide variety of tissues where it acts in a paracrine fashion has driven an intensive research effort. The following sections will summarize and highlight some of the most important advances.

Skeletal Development

The importance of PTHrP in the skeleton became evident when deletion of the *PTHLH* gene in mice resulted in early death due to respiratory failure, the result of defective rib formation (Karaplis *et al.*, 1994). In addition, this discovery also separated PTHrP from PTH, since the later deletion of the *PTH* gene revealed a much milder phenotype, (Miao *et al.*, 2002). Indeed, further interrogation of the role of PTHrP in skeletal development confirmed the important role of PTHrP in fetal bone development. *PTHLH* $-/-$ mice are early postnatal lethal, but haploinsufficient *PTHLH* $+/-$ mice are normal at birth and develop low bone mass at the age of 3 months. These mice demonstrated decreased recruitment of osteoblast precursors and increased osteoblast apoptosis compared to wild-type mice and as osteoblasts are crucial in osteoclastogenesis, an expected decrease in osteoclast formation was also observed in *PTHLH* $+/-$ mice (Amizuka et al., 1996, 1994; Karaplis *et al.*, 1994).

In the growth plate, differentiated chondrocytes produce Indian hedgehog (IHH) which signals undifferentiated chondrocytes to secrete PTHrP, which in turn signals to the differentiating hypertrophic chondrocytes to slow down the rate of chondrocyte differentiation, thereby, regulating developmental bone growth. This paracrine PTHrP/IHH signaling loop is fundamental to our understanding of endochondral bone formation and helped confirm the important role of PTHrP. In fact, PTHrP secretion is also regulated by TGF-β and IGF-1 (Alvarez *et al.*, 2002; Dexheimer *et al.*, 2016; Hilton *et al.*, 2005; Kronenberg, 2006), which are also active in the fetal growth plate. These findings have also been extended to articular joints, where PTHrP is produced in response to a variety of stimuli, including mechanical loading, to support the maintenance of articular cartilage in a similar paracrine PTHrP/IHH signaling loop (Broadus *et al.*, 1988; Ohta *et al.*, 2015; Pelosi *et al.*, 2013).

Tooth Eruption

PTHrP is produced by the enamel organ and loss of PTHrP leads to a lack of postnatal tooth eruption, a classic result of aberrant osteoclastogenesis (Philbrick *et al.*, 1998). Replacement of PTHrP in the enamel epithelial cells allows tooth eruption. Further studies have revealed that PTHrP drives osteoclasts to resorb bone to form crypts as a site for tooth eruption, in a paracrine manner. Interestingly osteoclasts do not express the PTH receptor, which warrants further investigation to uncover other receptors through which PTHrP presumably acts (Boabaid *et al.*, 2004). However, since PTHrP is a potent inducer of receptor activator of nf kappa b ligand (RANKL) is likely the regulatory mechanism. Currently, the periodontal ligament (PDL) is recognized to play an important role in root resorption of human deciduous teeth by odontoclasts (osteoclast-like cells) (Fukushima *et al.*, 2005), mediated by increased RANKL expression by the PDL. In addition, PTHrP induces Jagged1 expression in PDL cells, leading to both osteo- and odontoclastogenesis (Nakao *et al.*, 2009). The combined effects of PTHrP promotes tooth and alveolar bone resorption although the precise signaling mechanism for PTHrP in this setting remains unknown.

Smooth Muscle Relaxation

Next to a causal role in HHM, perhaps the most informative effect of PTHrP is its potent role relaxing effects in vascular smooth muscle. It has been known for almost 100 years that the injection of parathyroid extract in a variety of species induces dose-dependent increases in blood flow, resulting in decreased blood pressure (Charbon *et al.*, 1968; Collip and Clark, 1925; Collip *et al.*, 1925; Schleiffer *et al.*, 1979). Once PTHrP was identified, it became clear that the many reports of PTH and PTH peptides impact on smooth muscle beds (Mok *et al.*, 1987; Pang *et al.*, 1984; Qian *et al.*, 1999) was not the normal function of PTH, but the result of a local physiological role for PTHrP (Maeda *et al.*, 1999; Sutliff *et al.*, 1999). PTHrP is produced in the smooth muscle of the uterus, bladder, stomach and arteries where it acts a potent muscle relaxant (Francis *et al.*, 2003; Nishikawa *et al.*, 2013). Thus, following vasoconstriction, increased PTHrP synthesis and secretion provides a mechanism to limit or reverse the effect and relax smooth muscle. This mechanism is demonstrated in studies where vasoconstriction induced by angiotensin II elicits a rapid and dramatic increase in PTHrP production (Pirola *et al.*, 1993), a mechanism that is preserved in angiotensin II-induced kidney injury (Lorenzo *et al.*, 2002).

Mammary Gland Development and Calcium Transport

Much of our understanding of the role of PTHrP in mammary gland development comes from seminal studies rescuing the neonatal lethality of PTHrP $-/-$ mice (Wysolmerski *et al.*, 1995). The genetic rescue attained by targeting PTHrP expression to cartilage using the collagen II promoter, permitted study of PTHrP function in other tissues of the null phenotype (Wysolmerski *et al.*, 1995). In the absence of PTHrP expression, there is failure in the development of breast ducts. PTHrP stimulates calcium transfer from bone to breast milk during lactation as well as mother-to-fetus placental calcium transport (Ardeshirpour *et al.*, 2006; Kovacs *et al.*, 2002). Although the role of PTHrP in driving placental calcium transport is unquestioned, some controversy still remains regarding the identity of the primary driver(s) of postnatal breast development, with both PTHrP (Boras-Granic *et al.*, 2011) and RANKL (Gonzalez-Suarez *et al.*, 2010) potential candidates. These particular actions of PTHrP helped provide the original rationale for the misnamed "vicious cycle" of bone metastasis (Guise *et al.*, 1996), driven by PTHrP alone which has beleaguered the field for more than 20 years (Johnson and Suva, 2017).

PTHrP in Cancer

Although originally identified as the product of lung cancer cells, the importance of PTHrP expression in malignancy is not confined solely to the scenario of HHM. In many cancers, including lung, prostate and breast, patients with metastatic bone disease display an increase in serum calcium that is largely driven by PTHrP (Powell *et al.*, 1991; Soki *et al.*, 2012). Indeed, PTHrP expression and the activation of osteoclastogenesis and bone resorption is important, but there is significant evidence supporting the idea that tumor expression of a plethora of factors, in addition to PTHrP, is able to activate bone resorption (Johnson and Suva, 2017). These tumor-derived molecules exert a variety of effects on other cells residing in the local bone marrow micro-environment that favor tumor establishment and progression in bone (Johnson and Suva, 2017). In recent years it has become apparent that the bone and bone marrow compartment provides not only a receptive growth factor-enriched environment for arriving tumor cells but also favorable niches in which circulating/disseminated tumor cells can survive (Makhoul *et al.*, 2016). It is clear that the numerous interactions between invading tumor cells, host bone cells and the immune system that facilitate tumor progression in bone (and elsewhere) are the result of processes above and beyond the action of any single molecule. With the ongoing and intensifying focus on the mechanisms driving cancer development and bone metastasis, the future of effective therapy may well reside in the increased understanding of the common traits of bone metastatic breast cancer cells (Johnson and Suva, 2017) and not a simple reliance on any single agent, even if it is PTHrP.

Conclusion

Since the discovery of PTHrP in 1987, there has been significant efforts focused on gaining a deeper understanding of the physiological roles of both PTHrP and PTH. The data clearly indicate that endocrine PTH and paracrine PTHrP regulate bone and calcium metabolism in a cooperative fashion, with their interactions at their most complex during early development. It is also apparent that duplication of the *PTHLH* gene eventually gave rise to the *PTH* gene. This information provides an evolutionary argument to explain the many duplicated genes on chromosomes 11 and 12 as well as the many common regulators of both molecules. It goes without saying that PTHrP is a fundamental and essential regulatory hormone, significantly conserved across evolution. It plays a crucial role in physiology and pathophysiology yet, the identity of the specific PTHrP peptides in the circulation of patients has been elusive. As such, what specific PTHrP peptides are presented to the human PTHR1, the primary signaling receptor for PTH and PTHrP, in vivo remain largely unknown. Many important questions regarding the role of PTHrP in disease remain unanswered. Indeed, the questions perhaps most critical are *What is the importance of PTHrP entry into cells either receptor-mediated or not? What is the contribution of PTHrP* versus *other tumor-derived factors in the progression of cancer*? These and other unanswered questions will only be answered by continued efforts to uncover the underlying biology of this important molecule. Thus, the search to identify the PTHrP peptides and their biological characterization in both normal physiology and disease is an expanding and ongoing scientific endeavor, from which the results are eagerly anticipated.

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Physiology of Calcitonin and Its Therapeutic Uses☆

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Glossary

Bone mineral density (BMD) An indirect estimate of bone mineral content that is inversely correlated with the risk of future fractures. BMD is measured using dual energy X-ray absorptiometry (DXA) and expressed as the amount of mineralized tissue in the area scanned (g/cm^2). The BMD in the hip is considered the best predictor of hip fracture. The spine is a good location for assessing BMD changes early in menopause and monitoring the response to therapy; however, the location is subject to interference with artifacts such as osteophytes and extraskeletal calcification.

Calcitonin (CT) A 32-amino acid-polypeptide hormone produced in C cells of the thyroid gland. It is a potent inhibitor of osteoclastic bone resorption. It plays an important role in maintaining calcium homeostasis during the periods of calcium stress. Worldwide, synthetic forms of CTs, such as human, eel, and salmon, are used in the treatment of several bone-related diseases, including osteoporosis, Paget's disease, and hypercalcemia. Because of its analgesic effect, CT can be used to alleviate pain associated with acute, vertebral crush fracture syndrome.

Calcitonin gene-related peptide (CGRP) Calcitonin gene-related peptide, a 37-amino acid polypeptide that has neuromodulatory, nociception, and vasodilator functions.

Medullary thyroid carcinoma (MTC) Malignancy arising from C cells of the thyroid gland.

Osteoporosis A chronic, progressive skeletal disease, characterized by low bone mass and fragility and deterioration of bone microarchitecture; it increases fracture risks.

Introduction

C cells derive from the neural crest and migrate rostrally to become the parafollicular cells in humans and the ultimobranchial bodies in lower vertebrates. Calcitonin (CT) is synthesized by C cells located in the thyroid in mammals and in the ultimobranchial glands in lower vertebrate animals, secrete to the circulation, and act as a hormone throughout the body (Wimalawansa, 1989; Foster *et al.*, 1964). During this forward migration, C cells are expected to concentrate in the thyroid and ultimobranchial body (Wimalawansa, 1993a). However, some C cells may not progress to the thyroid and could be manifest in extrathyroidal tissues in the path way of its migration upwards (Wimalawansa and MacIntyre, 1991).

Calcitonin is an endogenous regulator of calcium homoeostasis, especially during "calcium stresses" protecting the skeleton, acting principally on bone. It also has a direct action on the kidneys and gastrointestinal secretory activity as well as direct and indirect effects on the central nervous system in modulating pain. Investigations into its central nervous system role suggest that, in addition to having an intrinsic analgesic effect, it may exert a modulator effect on other neuronal activities, directly at the sites at which it is known to be present, and indirectly, by a mechanism yet to be elucidated at other locations.

Currently, the principal indications for the therapeutic use of calcitonin are disorders involving hypercalcemia, Paget's disease (osteitis deformans), acute pancreatitis, high-bone turnover osteoporosis, pain associated with osteoporosis or bone metastases, and Sudeck's atrophy. Various types are in use—natural porcine calcitonin (pCT), synthetic human calcitonin (hCT), synthetic salmon calcitonin (sCT) (Salcatonin), and a synthetic eel calcitonin (eCT) analog (Elcatonin). One difficulty with treatment is that, until few years ago, injection was the only possible mode of administration. However, dosage forms using other routes are being developed, and a calcitonin nasal spray is now commercially available.

Structure–Activity of CT

The CT gene has been relatively conserved during evolution. Over the past five decades, 11 different sequences of CT, from 9 different species have been identified (**Table 1**). Six of the invariant amino acid residues in the CT molecules are clustered in the amino terminal, and two are at the carboxy terminal end of the molecule (Wimalawansa, 1989). Furthermore, as with several other bio-active hormones, all CT molecules, irrespective of the species, have a disulfide bridge in the N-terminus of the molecule, between the first and seventh amino acids making a ring structure, and have a proline-amide at the C-terminus of the molecule (Wimalawansa and MacIntyre, 1991). Intact disulfide bridge is essential for its receptor docking and thus, biological activity.

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Table 1 Amino acid sequences of the nine fully characterized and two "predicted" calcitonins*: The invariant residues are clustered at the two ends of the molecule

AA No.	*Man-1*	*Rat*	*S-1*	*S-2*	*S-3*	*Eel*	*Chick**	*Bov*	*Porc*	*Ovi*	*Man-2**
1	Cys	–	–	–	–	–	–	–	–	–	Tyr
2	Gly	–	Ser	Ser	Ser	Ser	Ala	Ser	Ser	Ser	Ser
3	Asn	–	–	–	–	–	Ser	–	–	–	–
4	Leu	–	–	–	–	–	–	–	–	–	–
5	Ser	–	–	–	–	–	–	–	–	–	–
6	Thr	–	–	–	–	–	–	–	–	–	–
7	Cys	–	–	–	–	–	–	–	–	–	
8	Met	–	Val	–	Val	Val	Val	Val	Val	Val	Leu
9	Leu	–	–	–	–	–	–	–	–	–	Gln
10	Gly	–	–	–	–	–	–	Ser	Ser	Ser	–
11	Thr	–	Lys	Lys	Lys	Lys	Lys	Ala	Ala	Ala	–
12	Tyr	–	Leu	Leu	Leu	Leu	Leu	–	–	–	–
13	Thr	–	Ser	Ser	Ser	Ser	Ser	Trp	Trp	Trp	Leu
14	Gln	–	–	–	–	–	–	Lys	Arg	Lys	–
15	Asp	–	Glu	–	–	Glu	Glu	–	Asn	–	Tyr
16	Phe	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
17	Asn	–	His	His	His	His	His	–	–	–	Lys
18	Lys	–	–	–	–	–	–	Asn	Asn	Asn	Asn
19	Phe	–	Leu	Leu	Leu	Leu	Leu	Tyr	–	Tyr	–
20	His	–	Gln	Gln	Gln	Gln	Gln	–	–	–	–
21	Thr	–	–	–	–	–	–	Arg	Arg	Arg	Met
22	Phe	–	Tyr	–	–	Tyr	Tyr	–	–	Tyr	–
23	Pro	–	–	–	–	–	–	Ser	Ser	Ser	–
24	Gln	–	Arg	Arg	Arg	Arg	Arg	Gly	Gly	Gly	Gly
25	Thr	–	–	–	–	–	–	Met	Met	Met	Ile
26	Ala	Ser	Asn	Asn	Asn	Asp	Asp	Gly	Gly	Gly	Asn
27	Ile	–	Thr	Thr	Thr	Val	Val	Phe	Phe	Phe	Phe
28	Gly	–	–	–	–	–	–	–	–	–	–
29	Val	–	Ser	Ala	Ala	Ala	Ala	Pro	Pro	Pro	Pro
30	Gly	–	–	–	–	–	Glu	Glu	Glu	Glu	Glu
31	Ala	–	Thr	Val	Val	Thr	Thr	Thr	Thr	Thr	Ile
32	Pro NH_2	–	–	–	–	–	–	–	–	–	–

Note: Amino acid sequences from human CT are illustrated in the column Man-1. Two predicted calcitonin sequences are highlighted with *. Variations of amino acid/sequences in other forms of CT molecules are indicated using standard, three-letter amino acid symbols. The invariant residues (illustrated as —) are clustered at the two ends of the molecule.

Abbreviations: *AA No.*, amino acid sequence number; *S*, salmon; *Bov*, bovine; *Porc*, porcine; *Ovi*, ovine; Man, predicted*.

For its key biological activities, hypocalcemic effects, and inhibition of osteoclasts, the intact molecule—all 32 amino acids—is required (Wimalawansa and MacIntyre, 1991). Nevertheless, substitution of some of these amino acids [e.g., some structurally (chemically) modified sCT and eCT molecules] is known to enhance their biological potency by delaying degradation (i.e., increases its circulatory and biological half-lives) but may enhance allergenicity (Wimalawansa and MacIntyre, 1991).

Calcitonin is produced within the C cells as a precursor molecule. Before secretion into the circulation, however, it undergoes a number of posttranslational modifications, including cleavage, disulfide bridge formation, and amidation of C-terminal amino acid, via converting proline into proline-amide. These highly conserved structural modifications are essential for the full biological activity of CT (Wimalawansa, 1989). The mature form of biologically active $CT_{(1-32)}$ that is secreted into the circulation has a short half-life.

Release of CT From C Cells and Its Measurement in Blood

The release of CT from C cells is stimulated by divalent cations, such as Ca^{2+} and Mg^{2+}, as well as by glucagon, dibutyryl cyclic AMP (cAMP), theophyllin, gastrin, cholecystokinin, and alcohol. Following the interaction of CT with its cell-surface receptors, second messenger cAMP dose-dependently increases, leading to its biological activity. Suppression of bone-resorbing activity occurs secondary to the cell surface CT receptor mediated increase of intracellular cAMP (Wimalawansa and MacIntyre, 1991). This function can be mimicked in organ and cell cultures by exogenous administration of its analogs, such as dibutyryl cAMP.

Immunoreactive calcitonin (i-CT) levels in plasma (or serum) can be measured accurately and precisely using various immunoassays, such as antibody-derived immunoassays or chemiluminescense. Immunoreactive CT levels in the blood have been

Table 2 Conditions associated with calcitonin underproduction and overproduction

Conditions associated with calcitonin underproduction	*Conditions associated with calcitonin overproduction*
Osteoporosis	Calcitonin-secreting tumors
• Postmenopausal	• Medullary carcinoma of the thyroid
• Pregnancy-induced osteoporosis	• C cell hyperplasia
• Senility	• Other (ectopic) hormone-secreting tumors
	• Multiple endocrine neoplasia syndromes
Secondary to other endocrine disorders	Hypercalcemia
Thyroidectomy	Neonatal hypocalcemia
Thyroid carcinoma (other than MTC)	Pseudohypoparathyroidism
Thyroiditis	Renal disorders including renal failure
	Pancreatitis
	Heroin and other drug addictions
	Graves' disease
	Atrophic gastritis
	Acute gastritis
	Pernicious anemia
	Peptic ulcer
	Gastrointestinal bleeding
	Stress, trauma
	Thyroid surgery
	Posttraumatic stress disorder
	Hepatic surgery
	Toxic shock syndromes
	Acute myocardial infarctions and strokes
	Lithium intoxication

used as a screening and diagnostic test in conditions with hypersecretion of CT. Premalignant and malignant disorders of C cells (i.e., medullary thyroid carcinoma or MTC) are such conditions (Wimalawansa and MacIntyre, 1991).

Table 2 illustrates causes of elevated and suppressed serum i-CT levels in humans. For diagnosis of latent disease (in those with familial diseases) and patients with MTC before clinical manifestation (as in the cases with some multiple endocrine neoplasia syndromes) or postoperative thyroidectomy to remove MTC tumors, a number of in vivo stimulation tests have been used, including injection of pentagastrin and infusion of calcium (Wimalawansa, 1989).

The first intraoperative immunoreactive (i-CT and i-PTH) assays were developed by the author in 1982, and data were presented at the U.K. Bone and Tooth Society meeting in London. Immunoreactive CT measurement was used as a mode to demonstrate the feasibility of measuring interoperative hormonal levels for the first time. The practical utility of these assays later was demonstrated in several series of patients undergoing thyroidectomy for MTC and removal of adenoma in those with primary hyperparathyroidism (Wimalawansa and Bailey, 1995; Wimalawansa, 1991). A number of conditions are associated with low- and high-serum i-CT levels (**Table 2**).

Efficacy of Commercially Available CT Formulations

Calcitonin can be administered parenteral or intranasal routes. Miacalcin nasal spray (Novartis Co.) is a synthetic salmon CT that is more potent than human CT, in part because of its relatively longer half-life in circulation (Wimalawansa, 1989). Its primary action is mediated through inhibition of bone resorption by osteoclasts. Each osteoclast is estimated to have >6 million CT receptors on its cell surface, so it is a prime target for CT actions; the direct action of CT on osteoclast cell suppression is bone resorbing activity.

Miacalcin nasal spray, 200 IU administered once daily, is an approved treatment for postmenopausal osteoporosis. Nevertheless, CT is the weakest among all the FDA-approved antiosteoporosis pharmaceutical agent in the market (Wimalawansa and Cooper, 1997). Thus, it usually is reserved for patients who refuse or cannot tolerate other antiosteoporosis agents, with the exception of patients experiencing acute bone pain (see later for more details). In general, CT nasal spray is recommended in conjunction with an oral calcium intake of 1200 mg (diet + supplements; or 500 mg of oral calcium supplement taken after supper) and 1000 IU of vitamin D to retard the rate of bone mass (Arantes *et al.*, 2016; Weaver *et al.*, 2016a,b).

In some countries, the only available form of CT is an injectable preparation. Injectable CT formulations, a dose of 40–60 IU administered three or four times per week, have been used for the prevention or treatment of osteoporosis; however, this regimen is less effective than all other antiosteoporosis agents. A smaller dose of injectable sCT, such as 10–40 IU three times per week, may have the same beneficial effect on bone and also decrease the chances of the onset of antibody-mediated resistance to CT; such a dose is also economical. Salmon CT has fewer side effects than does human CT with reference to nausea, but the main drawbacks with sCT are its high cost and loss of efficacy caused by the formation of neutralizing antibodies (Wimalawansa, 1993a).

Side effects are greatest with intravenous administration, less with intramuscular and subcutaneous injections, and least with intranasal administration (Wimalawansa, 1993a; Wimalawansa and Cooper, 1997). The recommended dosage of intranasal CT is 200 IU (one puff) per day, and its biological effects are similar to those seen with subcutaneous injections of 50 IU a day (Wimalawansa and Cooper, 1997).

Being a peptide, calcitonin preparations should be refrigerated and protected from direct sunlight to prevent degradation. Shelf lives of CT formulations are approximately 2 years. Nonhuman CTs can be allergenic, with roughly 50% of patients developing antibodies over 3–5 years (Wimalawansa, 1993a). However, this leads to actual clinical resistance (e.g., generation of neutralizing antibodies) in less than one-third of such patients (Wimalawansa, 1989; Wimalawansa and MacIntyre, 1991).

Adverse Reactions

Potentially Life-Threatening Effects

Calcitonin is a safe drug with few side effects (Wimalawansa, 1993a; Wimalawansa and Cooper, 1997). There are no potentially life-threatening adverse effects related to CT. There are no reports of deaths or long-term side effects attributable to CT use over 48 years of clinical experience (Wimalawansa, 1993a, 2004). The main drawback of CT therapy is its cost, local irritation when administered intranasally, and pain, flushing and nausea after parenteral administration (Wimalawansa and MacIntyre, 1991). Compared with other FDA-approved therapies, CT therapy has little effect on bone mineral density (BMD) and fracture reduction (Wimalawansa and Cooper, 1997).

The absorption of nasally administered CT is variable and poor, and a third of those who use nonhuman CT develop neutralizing antibodies in the long term; consequently CT further loses its efficacy (Wimalawansa, 1993a). In such patients, the dose may need to be increased by two- to threefold or more than the recommended doses to achieve the desired effects. Alternative routes of administration have been investigated, such as pulmonary, rectal, buccal [liposomes], depot preparations, and oral forms, but no real breakthrough has been reported yet.

Acute overdose

No overdoses have been reported with CT. Flushing and transient nausea and vomiting are the only key notable short-term symptoms.

Severe or irreversible adverse effects

Anaphylactic reactions have been reported rarely in association with nonhuman forms of CT therapy. As with other medications, such reactions are thought to be caused by allergenicity to excipients rather than the peptide itself.

Symptomatic adverse effects

CT generally is well tolerated. With injectable CT, the most commonly encountered adverse effects are pain at the injection site and cutaneous flushing that affects the upper body, particularly the face. This is noticed by at least a third of patients and probably occurs to a certain extent in most who take this medication. Depending on the route of administration, onset may be within seconds to minutes after administration and may last as long as 1 h.

Although nausea is common, it is mild. If it is troublesome, the problem may be alleviated by finding a suitable time for the administration of CT injections (e.g., avoiding immediately before or after a meal; taking it just before going to bed) or, for some patients, administering an antiemetic for the first few days. Increased urinary frequency (diuretic effect) occurs in as many as 10% of patients, but diarrhea is rare.

As many as one in four patients discontinue therapy because of adverse effects. Nevertheless, no major side effects have been observed with long-term treatment with CT (Wimalawansa, 1993a). With reference to the commonly used preparation of intranasal CT, the most common adverse effects are nasal irritation and runny nose. Rarely use of intranasal CT may be associated with nasal bleeding and exacerbation of asthma (Wimalawansa, 1993a).

Interference with clinical pathology tests

Calcitonin has no known technical or chemical interferences or interactions with laboratory tests.

Drug interaction

There are no known specific drug interactions between CT and other drugs (Wimalawansa, 1993a).

Physiological Roles of CT

Physiological concentrations of CT are likely to have a tonic effect to restrict osteoclastic bone resorption. The physiological role of CT is to maintain skeletal mass, particularly during periods of calcium stress, such as during the period of growth (infancy and

childhood), pregnancy, and lactation (Wimalawansa and Cooper, 1997). These physiological actions are geared to preserving the skeleton (Wimalawansa, 1989).

Maintaining Calcium Homeostasis During Calcium Stress

During pregnancy, calcium is retained by the fetus in increasing amounts irrespective of the calcium status of the mother (Wimalawansa and Cooper, 1997). During the period of lactation, calcium is secreted into milk against a concentration gradient (i.e., active calcium secretion into milk); this occurs at the expense of the maternal skeleton. Thus, if the maternal calcium and vitamin D intake are suboptimal, mothers who breast-feed for prolonged periods may experience significant bone loss during lactation. While testosterone and estrogen are stimulants for the synthesis of CT by C cells, i-CT levels in the blood are significantly lower in postmenopausal women and in older men.

This increased mobilization of calcium from the maternal skeleton occurs via the enhanced gene expression and secretion of parathyroid hormone-related protein [PTHrP; simulating fetal parathyroid hormone (with equivalent functions)] during these periods. In these situations, parallel increases in secretion of CT seem to exert a protective effect in preserving skeletal mineral content by preventing excessive osteoclast-mediated bone resorption that is stimulated by PTHrP. For example, the somewhat rare condition, pregnancy-associated osteoporosis is caused by a relative CT deficiency (suboptimal or lack, or secretion of biologically inactive form of CT from C cells) or severe maternal calcium deficiency, usually secondary to or malnutrition.

Antiinflammatory Properties

The antiinflammatory effect of CT is dose dependent. In acute experiments, CT seems to be involved in reducing the first-phase reactions of the inflammatory process as well as an antipyretic effects. In long-term experiments (e.g., Freund's adjuvant arthritis), sCT reduced edema and the response to pain stimuli, reduced the number and severity of intraarticular lesions, and consequently improved the range of movement in the joints affected by the arthritic process (Wimalawansa, 2009). In carrageenan edema, the potency of sCT is three times greater than that of hCT or pCT at equivalent doses of biological activity.

Calcitonin exhibits antiinflammatory properties in several models of acute and chronic inflammation in rats and mice arthritic models: (A) dextran, carrageenan, nystatin edema; yeast hyperthermia; and Freund's adjuvant arthritis in rats, and (B) histamine edema; acetic-acid-induced abdominal constriction; and pain secondary to inflammation in mice (Wimalawansa, 2009).

The antiinflammatory mechanisms of action probably involve a reduction in vascular permeability, reducing the synthesis and secretion of inflammatory cytokines such as interleukin-1 (e.g., IL-1), and stimulation of lysine decarboxylase activity, as with many nonsteroidal antiinflammatory agents (Wimalawansa, 2009). This effect seems to be independent of changes in tissue/cellular calcium levels.

In addition, a dose-dependent inhibition of prostaglandin and thromboxane biosynthesis is reported to occur as a result of partial inhibition of the activity of cyclooxygenase, the enzyme responsible for the first stage of arachidonic acid metabolism. However, such has not been established in humans. In patients with hypercalcemia of malignancy and those with increased prostaglandin levels, CT exerted antihypercalcemic and analgesic effects but had no effect on prostaglandin levels.

Cardiovascular Effects

Although CT is thought to have little or no effect on the cardiovascular system, a number of observations are of interest. Facial flushing is one of the most common side effects of CT (Wimalawansa, 2004, 2009). This side effect is usually associated with the peak plasma level of CT, especially in the early stages of treatment; it also frequently occurs after intravenous and intramuscular administration of CT, but it occurs less often after subcutaneous administration.

This phenomenon occurs after stress and emotions (e.g., when confronted with an embarrassment) and in postmenopausal women, as well as in pathological conditions, such as those with multiple paraneoplastic diseases (**Table 2**). The reaction is thought to result from a preferential release of various vasodilatory cytokine factors, including vasoactive intestinal polypeptide. In a rabbit model of immunoarteriosclerosis, long-term administration of pCT reduced the arteriosclerotic process; this was thought to be induced by inhibition of the formation of calcium deposits in arteries, which prevented calcium entry into vascular smooth muscle cells.

In rats, high doses of calcitonin (e.g., sCT, 100 IU/kg) have been reported to inhibit the arrhythmic response to aconitine and ouabain but not to adrenaline. Intravenous sCT produced a pressor response in rats after induction of hypotension by bleeding but was without effect in normotensive rats or those made hypotensive by other means. This pressor effect seems to be attenuated by chemical sympathectomy, which seems to indicate that CT may be potentiating the sympathetic outflow.

Calcitonin is a weak, vasoactive hormone. Its beneficial effect is evident in those with bone pain, sometimes called "migraine of the bone"; this is predominantly due to restoration of normal bone blood flow (i.e., reduction of blood flow to the affected bone, as in the case with Paget's disease of bone). In addition, CT's diuretic and natriuretic effects might be the result of an increase in renal blood flow, supporting the hypothesis that CT is a renal, vasoactive peptide (Wimalawansa, 2004, 2009).

Miscellaneous Effects

Less well-known effects of CT include certain interactions with other hormones, which seem to occur with high doses and thus appear to have no practical or therapeutic implications. Such effects include antistress, antiulcerogenic, and perhaps antiinflammatory properties. Other rare effects of CT include reduction in the synthesis of prostaglandin, inhibition of breast cancer cells in vitro, stimulation of plasminogen activator, and amylase secretion by salivary glands. Adverse effects, such as flushing and diarrhea in those with multiple endocrine neoplasia, metastatic bone syndrome, and after parenteral demonstration of CT, may be the result of excess episodic release of CT into the circulation.

Clinical Pharmacology

Considering the millions of cell surface CT receptors on each mature osteoclast, it is considered the main target for this hormone. Calcitonin directly suppresses the bone resorption activity of osteoclasts and thereby decreases bone resorption. When serum-ionized calcium increases, CT is secreted from the C cells into circulation (Wimalawansa, 1989). Osteoclast cells are highly sensitive to pico-gram (nmol) quantities of circulatory CT. This feedback mechanism will lead to rapid decreases in bone resorption, and thus the amount of calcium leaching from the skeletal surfaces into the circulation will decrease. This will bring back the tightly controlled ionized calcium levels toward the normal range (Wimalawansa and Cooper, 1997).

Osteoclasts are multinucleated giant cells (**Fig. 1**) and constantly create resorption cavities on bone surfaces (**Fig. 2**). In addition, when administered over several months, CT also reduces the number of osteoclasts by decreasing the rate of recruiting

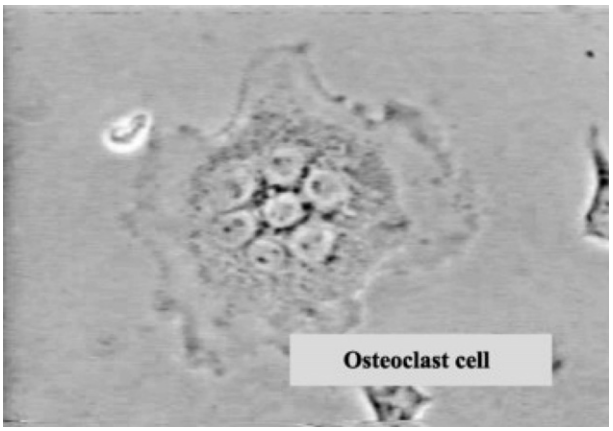

Fig. 1 An osteoclast cell: One of the largest cells in the body. Using their pseudopodia, osteoclasts move over the surface of bone, making resorption pits en route.

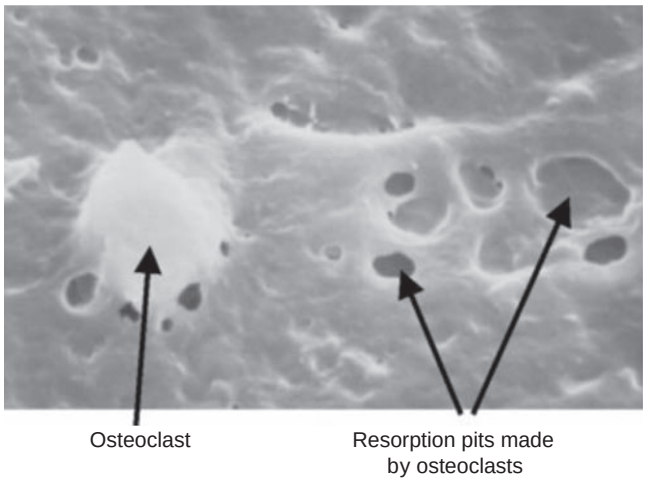

Fig. 2 Scanning electron micrograph of a bone slice illustrating an osteoclast and its movement path on the bone surface, showing a number of excavation pits made by the osteoclasts. These cells make a tight seal over the bone surface using its cell membrane pseudopodia, secreting acid and proteases to digest the underneath collagen matrix. Once a certain depth in a pit is formed, the osteoclast moves to a different site. Endogenous or exogenous CT will control this bone resorbing activity.

osteoclast progenitor cells into osteoclastic lineage (Wimalawansa, 1989). Considering its direct and potent actions in controlling osteoclasts, CT is a useful agent in patients with conditions associated with high bone turnover.

However, it is not clear whether the decrease in the number of osteoclasts is a consequence of an acute effect of CT on osteoclasts, an independent effect on its precursor cells, or both (Wimalawansa, 1991). Because of the rapid actions of CT on suppressing osteoclastic bone resorption, during the growth phase (also in growing rat models), administration of CT could lead to lowering of serum calcium levels and perhaps cause symptomatic hypocalcemia (Wimalawansa, 1989).

However, due to the relatively slow rate of bone turnover, a plasma calcium-lowering effect of CT is not seen in healthy adults. Therefore, the administration of CT has little or no effect on plasma calcium in ambulatory adults. Nevertheless, when bone turnover is high, as in children or in disease states (e.g., hypocalcemia of malignancy, Paget's disease, etc.), and during the first few days in those who are bedridden, the administration of CT may be followed by decreases in plasma calcium.

In the gastrointestinal system, several pharmacological actions of CT have been documented. CT enhances the intestinal secretion of sodium, potassium, chloride, and water, inhibits gastric acid secretion, and delays emptying (Wimalawansa, 1989). It also inhibits the secretion of several gastrointestinal regulatory peptides and hormones, including gastrin, insulin, pancreatic glucagon, motilin, pancreatic polypeptide, and gastric inhibitory peptide. Inhibitory effects on the secretion of pituitary hormones, including growth hormone, thyroid-stimulating hormone, and luteinizing hormone, also have been reported.

Pharmacokinetics

The most convenient and conventional analytical methods for i-CT are radioimmunoassay, chemiluminisum assays, and enzyme-linked immunosorbent assays. The usefulness of the radioimmunoassay and single site immunoassays are questionable because the testing may also detect inactive fragments and precursors. Pico-gram quantities of CT can be detected by such assays, and the biological activity of similar amounts can be detected using isolated osteoclast bioassay system (Wimalawansa, 1989, 1991).

Calcitonin is rapidly degraded by gastric contents and thus needs to be given parenterally. After CT is subcutaneously injected, the peak plasma concentrations are seen between 15 and 30 min. The plasma half-life of CT is short; approximately 4 min for porcine and hCT, but 15 min for sCT and eCT (Wimalawansa, 1989). Animal studies have shown that tissue uptake of labeled CT is greatest in the bone, liver, and kidney, but the volume of distribution varies widely. However, little information on plasma protein binding has been reported.

Loss of Efficacy of CT

The loss of efficacy following prolonged administration of CT is well known (Wimalawansa, 1989, 2009). This phenomenon is caused by a combination of downregulation of cell-surface CT receptors, development of neutralizing anti-CT antibodies (with nonhuman CTs), and enhanced catabolism of the synthetic peptide. This can be minimized with smaller doses of CT administered less frequently and switching nonhuman forms of CT to synthetic or genetically engineered human CT.

Primary Nonresponse

Some patients exhibit primary resistance to calcitonin, with little or no response to standard therapeutic doses. They may not have response or adverse effects even with high dosages of exogenously administered CT (e.g., 200–500 IU/day sCT or 2–5 mg/day hCT). Few people are in this category, but they may represent true nonresponders.

Secondary Resistance

Secondary resistance is having a good response at first but later failing to have a response or being unable to maintain the benefits of CT. Such secondary resistance occurs with all types of CTs, even in the absence of generating neutralizing antibodies. Those with Paget's disease treated for >3–4 years with CTs begin to experience a failure to respond to the therapy that is exhibited in serum alkaline phosphatase and urinary markers of bone turnover.

Another classic example is in those with malignancy-related hypercalcemia. Parenteral administration of 100–200 IU CT induced a decrease in blood calcium within the first 6–12 h. However, from day 3 or 4 onward, CT has little effect in maintaining serum calcium levels in those with hypercalcemia (i.e., lose its efficacy). The hypocalcemic response can be sustained in those patients only when CT is coadministered with a glucocorticoid or a potent, parenterally administered bisphosphonate or denosumab.

The Plateau Phenomenon

With prolonged administration of CT, some patients experience a "plateau" phenomenon. In many, this is attributable to downregulation of CT receptors and thus partial resistance to CT that requires higher dosages to obtain the same responses (e.g., reduction of serum alkaline phosphatase in those with active Paget's disease). However, it could also be caused by other complex

mechanisms, such as secondary hyperparathyroidism, development of anti-CT antibodies, and the development of a new lineage of calcitonin–nonresponsive osteoclasts (Wimalawansa, 1994a, 1997). Despite achieving a plateau phenomenon, for example, the inability to reduce serum bone-specific alkaline phosphates, some patients may continue to have symptomatic pain relief and histologic improvements.

Therapeutic Uses

Osteoporosis

Osteoporosis is a result of an imbalance between bone formation and resorption, resulting in a gradual, net loss of bone (decreased bone mass) and an associated loss of trabecular integrity and bone strength, which leads to fractures. CT has been used for more than four decades to treat various metabolic bone disorders that are characterized by accelerated bone resorption (Wimalawansa and Cooper, 1997; Chestnut, 1993). As in persons with postmenopausal osteoporosis, many persons with osteoporosis have increased bone turnover. While CT is a logical therapy for the prevention and treatment of osteoporosis (Wimalawansa, 1989, 1991), its effect in improving bone mass, bone strength, and fracture prevention is not striking (MacIntyre *et al.*, 1988; Toth *et al.*, 2005; Szucs *et al.*, 1992; Pontiroli *et al.*, 1991).

One of the first clinical studies suggesting the antifracture efficacy of nasally administered sCT was published by Overgaard and colleagues (Overgaard, 1994; Overgaard *et al.*, 1993; Overgaard and Christiansen, 1991); another study reported the results of analysis of rectally administered sCT (Overgaard *et al.*, 1992). The investigators studied the effects of intranasal sCT in a 2-year, double-blind, placebo-controlled trial of women 68–72 years randomized to receive 50, 100, or 200 IU CT or placebo daily. Among 162 women completing the study, spinal BMD increased by 1% (95% CI, 0.1–1.5) in the placebo group and 3% (95% CI, 1.8–4.2) in the group receiving 200 IU CT.

As with bisphosphonates, CT seems effective in patients with high bone turnover with little or no effects in those with normal bone turnover (Chestnut, 1993). Like other antiresorptive therapies, the administration of CT eventually decreases the bone formation phase, thereby producing a new, lower rate of bone turnover. Consequently, increases in bone mass are seen only during the first 2–3 years of treatment. Thereafter, a plateauing effect of BMD is observed. However, it may be possible to minimize this by administration of CT in a cyclical fashion (e.g., nasal CT, 200 IU/day, in cycles of 6 months). Although CT prevents bone loss associated with corticosteroid treatment, it is unclear how CT and other similar antiresorptive agents reduce bone loss because corticosteroids are thought to depress bone formation rather than stimulate bone resorption.

Although CT has been used for preventing bone loss and decreasing osteoporosis-associated fractures, its efficacy is much less than that of hormone replacement therapy, selective estrogen receptor modulator (SERMs) bisphosphonates, denosumab, and teriparatide. A reduction in the rate of bone loss, rather than a sustained increase in bone mass, is expected as the therapeutic goal with CTs. Nevertheless, for those who cannot tolerate or fail to have response to standard doses of Food and Drug Administration (FDA)-approved therapies, CT could be considered as an alternative therapy for the treatment of osteoporosis, especially when the potent agents are not appropriate, too costly, or cannot be tolerated because of adverse effects. CT is also an alternative therapy for women who cannot tolerate estrogen therapy for social, religious, or medical reasons (Pontiroli *et al.*, 1991).

Since the efficacy of CT is $<30\%$ that of other FDA-approved antiosteoporosis agents. Therefore, other FDA-approved drugs are recommended for the treatment of osteoporosis as the first line of treatment. CT also has been used for the treatment of osteoporosis in men (Wimalawansa and Cooper, 1997; Toth *et al.*, 2005; Szucs *et al.*, 1992).

Although its efficacy is questionable, its prolonged administration causes downregulation of receptors and/or the development of neutralizing antiCT antibodies (with nonhuman CT) and increased catabolism of CT (Wimalawansa, 1989). Administering small doses of CT less frequently and switching salmon or eel CT to a synthetic or genetically engineered human CT can minimize these problems.

Doses of injectable CT as small as 60 IU per week have been shown to have an effect in postmenopausal bone loss (Wimalawansa and Cooper, 1997; MacIntyre *et al.*, 1988). Calcitonin is more widely used in Southern Europe (e.g., Italy) and Japan than anywhere else in the world. In a small, 2-year dose-finding study using intranasal CT in postmenopausal women with osteoporosis, spinal BMD increased by 2% per year (MacIntyre *et al.*, 1988). There was no demonstrable effect on other skeletal sites, but compared with placebo a decreased vertebral fracture rate was observed.

Like other antiresorptive therapies, CT decreases the remodeling phase, thereby achieving a new lower rate of bone turnover (Wimalawansa and Cooper, 1997). Consequently, increases in bone densities generally are seen only during the first 2–3 years of treatment with CTs (Pontiroli *et al.*, 1985, 1989). At least in theory, it may be possible to overcome this by administering CT in a cyclical fashion.

Clinical studies

The Prevent Recurrence of Osteoporotic Fractures (PROOF) study is the only multicenter, randomized, controlled clinical trial (RCT) that demonstrates a fracture reduction with intranasal administered CT (Toth *et al.*, 2005; Szucs *et al.*, 1992; Chesnut *et al.*, 2000; Cummings and Chapurlat, 2000). It was a 5-year, multicenter, double-blind randomized study designed to test the efficacy of Miacalcin nasal spray. The objective was to test whether CT reduces the risk of new vertebral fractures in postmenopausal women with established osteoporosis (Chesnut *et al.*, 2008).

A total of 1255 women with at least one but not more than five vertebral compression fractures were randomized to receive Miacalcin nasal spray (doses of 100, 200, or 400 IU), or a placebo. All subjects received daily supplements of 1000 mg of calcium plus 400 IU of vitamin D (Chesnut *et al.*, 2000, 2008; Chestnut, 1996). **Fig. 3** illustrates the efficacy of sCT nasal spray on vertebral fracture reduction.

The results demonstrated that Miacalcin nasal spray, at the FDA-recommended dose of 200 IU daily, reduced the risk of new vertebral fractures as compared with placebo, but the doses of 100 and 400 IU did not. However, the study failed to demonstrate reduction of hip or nonvertebral fractures. The study also showed marginal reduction of a biochemical marker of bone resorption in the serum type I collagen cross-linked telopeptide (Silverman, 2003). Except for an increased incidence of rhinitis, the frequency of adverse events in all treatment groups was comparable to placebo.

Other studies that used CT as the interventional agent had small increments in spinal BMD and marginal antifracture efficacy (Chestnut, 1993; MacIntyre *et al.*, 1988). These studies using CT reported fewer fractures and consistently were underpowered to detect a statistical difference of fracture reduction. Synthetic salmon, human, and eel CTs have also been tested as a therapy for osteoporosis, but no conclusive human data exist on fracture reduction (Stevenson *et al.*, 1981). However, except for the PROOF study, no RCT data demonstrate a reduction of any type of fractures with CT.

There is some evidence to suggest that CT may prevent glucocorticoid-induced bone loss, but the efficacy is considerably less than that of bisphosphonates (Ezzat, 2010; Nakamuta *et al.*, 1996; Nishioka *et al.*, 1991; Roux and Dougados, 2002). However, in glucocorticoid-induced osteoporosis, it is unclear how CT and other antiresorptive agents reduce bone loss given that glucocorticoids predominantly suppress bone formation rather than stimulate bone resorption (Ezzat, 2010; Fudman, 1997). It is plausible that this is attributable to the effect of enhanced osteoclast apoptosis with CT, as demonstrated with amino-containing bisphosphonates.

In patients with established osteoporosis, CT may stabilize or modestly increase indexes of cortical and trabecular bone and total body calcium (MacIntyre *et al.*, 1988; Wimalawansa *et al.*, 1988). The increments in bone density seen with CT are small, appear to be transient, and likely are to be attributable to reduction in bone resorption with bone formation remaining unaffected until the remodeling spaces are filled. However, the reason for the reduction of fracture rate in light of the small increase in BMD is not yet understood.

In addition to their well-known effects on decreasing bone turnover, antiresorptive therapies such as estrogen, bisphosphonates, denosumab, and CT may have a positive effect on osteoblasts and osteocytes, enhancement of osteoclast apoptosis, and preservation of the microarchitecture of bone. This may result in a disproportionate reduction in fracture rates in comparison with a no or a small increase in true BMD.

New approach to reduce fracture rates

To improve the bone mass and strength and reduce fracture risks, it is logical to use an anabolic agent such as teriparatide first (once FDA approved, other bone-anabolic or biologics agent can also be used) and stabilize or maintain the gains achieved using a sequential use of an antiresorptive agent for a suitable duration (MacIntyre *et al.*, 1988). Such an approach could maximize the potential increase of the bone mass, strength, and microarchitecture, and the maintenance of such improvement with an antiresorptive agent for a few years should markedly reduce fracture rate. Nevertheless, this logical approach is not yet practiced in clinical setups.

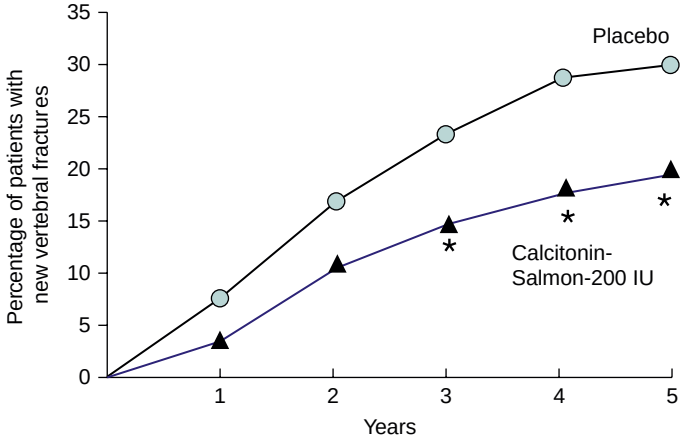

Fig. 3 Effects of intranasally administered sCT on new occurrence of vertebral fracture reduction. The cumulative percentages of subjects with vertebral fractures are indicated ($*P < 0.05$) Adapted from Chestnut III, C.H., Silverman, S., Andriano, K., *et al.*, (2000). A randomized trial of nasal spray salmon calcitonin in postmenopausal women with established osteoporosis: The prevent recurrence of osteoporotic fractures study. *The American Journal of Medicine* **109**, 267–276; Chestnut, C., 3rd, (1996). Postmenopausal osteoporosis: an overview. *American Journal of Therapeutics* **3**(10), p. 715–719.

Paget's Disease of Bone

Over the past few decades, CT has been used successfully as a treatment for Paget's disease of bone (Wimalawansa and Cooper, 1997). Several types of CT have been used for this purpose, but salmon CT is the most commonly used (Greenberg *et al.*, 1974; Woodhouse *et al.*, 1971). Paget's disease is a localized bone disease that is characterized by high bone turnover, so it is not surprising that CT is effective in decreasing the bone turnover in those with Paget's disease (Wimalawansa and MacIntyre, 1991). Calcitonin is particularly useful in this disease because it inhibits osteoclast activity and decreases the new osteoclast recruitment (Wimalawansa, 1989).

Inhibition of osteoclastic activity explains the short-term (or acute) effects of CT in reducing bone pain and deceasing markers of bone resorption, whereas long-term responses presumably are related to the decreased osteoclast numbers. In addition, CT alleviates Paget's-associated bone pain and improves clinical, biochemical, radiological, and histologic features of the disease. The major indication for CT therapy in Paget's disease is to control bone pain (Wimalawansa, 1991; Wimalawansa and Cooper, 1997).

One of the mechanisms by which CT relieves bone pain rapidly is via reducing the associated, excessive blood flow in Pagetic bones (Wimalawansa, 1989, 2009). It also alleviates some of the neurological signs and symptoms, such as the early spinal cord compression that is associated with Paget's disease (Wimalawansa and Cooper, 1997). Whether this is because of decreased local swelling or reduced inflammation is unknown. In general, treatment with CT decreases the biochemical markers of bone turnover, such as serum (bone-specific) alkaline phosphatase and N-telopeptide levels, by approximately 40%.

However, unlike potent agents such as bisphosphonates and denosumab, CT does not reduce the bone turnover markers (e.g., serum bone-specific alkaline phosphatase levels) into the normal range. Moreover, CT therapy is stopped, the levels go up again within a short period (Wimalawansa, 1989). Thus, the effectiveness of CT depends on its continued daily use; discontinuation of the drug rapidly reactivates the disease process, including the symptomatology. There are no major serious adverse effects associated with this treatment wit CT in patients with Paget's disease or any other disease.

Hypercalcemia

In patients with hypercalcemia caused by malignancy and hypercalcemic emergencies, CT produces a rapid reduction in blood calcium. However, its efficacy in controlling serum calcium levels lasts only 3–4 days (Cooper *et al.*, 1997; Wimalawansa, 1993b). A decrease in plasma calcium of 0.5–1.0 mmol/L is expected within 6–24 h of commencing parenteral CT therapy. However, in the absence of additional therapy, such as the concomitant administration of a bisphosphonate such as pamidronate or zoledronic acid, hypercalcemia recurs (Wimalawansa, 1997; Cooper *et al.*, 1997; Wimalawansa, 1995a).

Calcitonin is most likely to be effective in cases of hypercalcemia in which a generalized increase in bone resorption is a prominent feature. The calciuric effect of CT may also play a small role in reducing the increased plasma calcium (Cooper *et al.*, 1997). In patients with hypercalcemia, CT can be used to obtain an immediate calcium-lowering effect (Wimalawansa, 1994b,c, 1995a,b). However, for longer-term maintenance, the concomitant administration of intravenous bisphosphonate (e.g., pamidronate, zolendronate, etc.) is essential (Wimalawansa, 1993b, 1995a, 1997). In addition to controlling hypercalcemia, the latter agents also have an effect on controlling metastatic osteolysis.

Analgesic Action of CT

Use of CT to Control Bone Pain

Although the efficacy of CT in fracture reduction is a matter of controversy, there is convincing evidence for the efficacy of CT as an analgesic (Chiarini *et al.*, 1978) in patients with acute vertebral osteoporotic fractures (e.g., crush fracture syndrome) (Wimalawansa and Cooper, 1997). Because of its specific analgesic effect, CT can be useful as an adjunct therapy in the care of patients with acute osteoporotic fractures (Wimalawansa, 2009; Silverman and Azria, 2002).

Painful episodes in patients with osteoporosis are not caused by the osteoporotic process itself but by fractures. The presentation of such pain is acute, continuous, exacerbated by movement and ameliorated with rest. The pain can be severe and will ease with bed rest. Nevertheless, it is necessary to mobilize such patients as early as possible to avoid immobilization-associated complications, such as deep venous thrombosis (Wimalawansa, 2009). The short-term use of high-dose CT could assist pain relief in those with acute crush fracture syndrome and aid the early mobilization of such patients without resorting to opioid therapy (Wimalawansa, 2009).

In one double-blind placebo-controlled RCT, $>50\%$ of patients receiving daily nasal sprays of 200 IU CT had a significant response in mobility and functional capacity, whereas patients receiving placebo nasal spray experienced only a moderate response with respect to the same parameters (Wimalawansa, 2009). In addition, the analgesic actions of intranasal CT are rapid and lead to a meaningful reduction of pain in those with crush fracture syndrome (Wimalawansa, 2009).

A double-blind, double-placebo RCT examined for a period of 4 weeks the analgesic effect of daily administration of 200 IU intranasal CT, 100 IU CT administered intramuscularly, and a placebo ($n = 28$/group) in women with painful postmenopausal osteoporotic fractures. In the 24 patients receiving intranasal CT who completed the trial, a statistically significant reduction in pain score was measured by a visual analog scale by week two (Pontiroli *et al.*, 1985, 1989). For patients who received intramuscular CT and those who received a placebo, a significant decrease of pain scores was observed by 4 weeks (Wimalawansa, 2009).

The mechanism of the pain associated with acute fractures is different from that of the pain associated with fractures that happened a while ago. The most efficacious time to administer intranasal CT is immediately after a fracture (Wimalawansa, 2009). A significant pain reduction is observed within the first few hours and lasts for 4–5 days (Wimalawansa, 2009) (**Fig. 4**). The analgesic effect of CT diminishes over time as a consequence of tolerance or the spontaneous improvement that usually occurs over 4 weeks with most vertebral compression and wedge fractures.

Metastatic Bone Pain

Calcitonin has been used successfully for the treatment of bone pain in patients with metastatic bone disease. Analgesic effect has been observed in three-quarters of such patients. CT has also been used in the treatment of intractable pain from advanced malignancy, especially when injected into the subarachnoid space. In a series of 12 cancer patients, intrathecal injection of CT, together with chemotherapeutic agents, enabled reduction of opioid analgesic requirement by 80% (personal observation). It is likely that the main site of this analgesic action is in the hypothalamic "pain center," the central nervous system (Wimalawansa and Cooper, 1997; Wimalawansa, 1995a).

Although the mechanism of CT's analgesic action is not fully understood, it has been postulated to occur through direct action at the hypothalamic level and indirect action through interference with neurotransmitters such as serotonin and prostaglandin, independent of endorphin, and a peripheral action mediated via alteration of blood flow to the affected regions and inhibition of the factors such as inflammatory cytokines (Wimalawansa, 2009).

However, there have been no placebo-controlled studies conducted using CT in patients with metastatic bone pain. Jelic and colleagues used CT to treat 16 patients with pain caused by diffuse osteolytic or osteoblastic–osteolytic metastases who had not obtained satisfactory pain relief with opiate-type analgesics (Jelic *et al.*, 1995). Most of the patients on CT experienced a decrease in pain with an accompanying decreased intake of opiate-type analgesics (Jelic *et al.*, 1995; Kovcin *et al.*, 1994). Of the 16 patients, 2 were able to discontinue opiate intake completely, whereas 3 had no pain relief at all (Wimalawansa, 2009). Authors concluded that CT may be useful in relieving metastatic bone pain in some patients who experience resistance to opiate-type analgesics.

Pain Relief

Because of its effective pain-relief properties (analgesic effects), CT can be used as a treatment particularly for those with acute onset of back pain caused by vertebral crush fractures (Silverman and Azria, 2002). However, the use of the recommended,

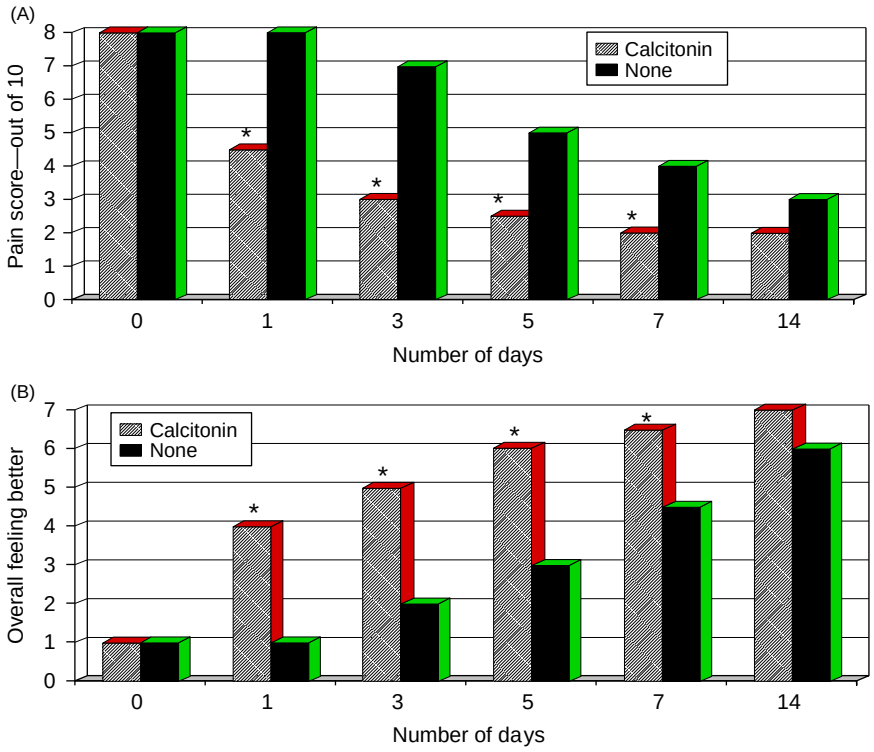

Fig. 4 (A) Effects of nasally administered sCT in reduction of pain after acute vertebral crush fractures ($n = 12$ per group; $^*P < 0.05$). (B) Overall feeling/well-being of patients after the use of nasally administered sCT after acute vertebral crush fractures ($n = 12$; $^*P < 0.05$). The data presented are inversely related to relief of pain after the administration of salmon calcitonin (Wimalawansa, 2009).

standard dosage of 200 IU CT administered intranasally daily does not relieve bone pain or pain associated with any other disorder. For this purpose, higher doses of intranasal CT, such as 800–1000 IU (i.e., 4–5 puffs), administered into alternative nostrils over a 10-min period is required (Wimalawansa, 2009).

As with its calcium-lowering effects in persons with hypercalcemia, the pain relief wears off after 3–4 days of use of high-dose intranasal CT. The goal is to reduce the pain quickly so that the patient can be mobilized out of bed, which prevents other complications, such as deep venous thrombosis. Therefore, such a condition requires a prescription of only one vial of Miacalcin (Wimalawansa and Cooper, 1997) (there is no reason to provide repeat prescriptions for this purpose) (Wimalawansa, 2009).

Continuation of CT beyond a few days is not beneficial because the agent loses its pain-relieving efficacy. The treatment regimen should change, and other reasons for continuation of pain when pain fails to subside should be sought. The principle of this approach is to mobilize patients to prevent inactivity-associated complications without resorting to using potent opioid analgesics that have additional adverse effects.

Miacalcin (CT) nasal spray is not approved for use as an "analgesic" by the FDA. Nevertheless, its analgesic actions of CT have been documented with the injectable forms and the nasal form (Wimalawansa and Cooper, 1997; Wimalawansa, 2009). The reduction in pain occurs significantly earlier than any demonstrated improvement in skeletal dynamics, and in some instances no objective improvement in the underlying skeletal condition can be observed.

Migraine

Migraine also reportedly responds to CT, but results are variable and the mechanism of action is unclear. However, CT has effects on vasoconstriction of certain atrial beds, which may indirectly help those experiencing migraine attacks. Daily intramuscular doses of 100 IU were significantly more effective than placebo in reducing the frequency, intensity, and duration of migraine (Brady *et al.*, 1991; Gennari *et al.*, 1986; Micieli *et al.*, 1988). Because of relatively less adverse effects, salmon CT has been investigated as a therapeutic agent for the treatment of migraine (Gennari *et al.*, 1986; Micieli *et al.*, 1988; Ustdal *et al.*, 1989).

However, administration of CT via injections is associated with a variety of gastrointestinal adverse effects, such as nausea, which in fact may worsen the situation. Thus, CT could aggravate certain migraine-associated problems, such as nausea. Salmon CT has also been administered prophylactically via the nasal route and shown to be effective in preventing attacks of migraine, and its actions are thought to be attributable to changes in local cytokines and hormones, such as ACTH, glucocorticoids, and endorphin (Ustdal *et al.*, 1989). However, disease-specific agents such as triptans have superseded therapies with CT.

Arthritis

There have been no placebo-controlled studies conducted using CT in patients with rheumatoid or osteoarthritis (Chiarini *et al.*, 1978; Manicourt *et al.*, 2005). However, anecdotal and corroborative evidence exists suggesting that pain and inflammation associated with various arthopathies may respond favorably to CT, at least partially (Manicourt *et al.*, 2005). An open-label study of 70 patients with osteoarthritis reported a decrease in spontaneous pain in those who were treated with 100 IU CT for a 40-day period (Dainotto *et al.*, 1985).

Eel- and salmon-CT have been shown to reduce inflammation and pain in persons with rheumatoid arthritis (Aida, 1991; Aida *et al.*, 1993, 1994). The mechanisms of action of these beneficial effects are thought to be mediated via regulation of immune responses through mononuclear cells/monocyte functions, and reduction of synthesis and release of immunoglobulins, and inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1 (IL-1) (Aida *et al.*, 1993, 1994; Ide and Suzuki, 2001).

A small controlled clinical studies have demonstrated an increase of axial BMD on those with rheumatoid arthritis after treatment with calcitonin (Kotaniemi *et al.*, 1996). Others have shown a possible synergy between calcitonin and active forms and analogs of vitamin D (Dottori *et al.*, 1982), and improved bone histomorphometric findings, such as reduction of resorption surface of trabecular bone (Kroger *et al.*, 1992). In addition, a randomized control clinical study (Sileghem *et al.*, 1992) and an observation study (Siamopoulou *et al.*, 2001) reported possible beneficial effects of intranasal sCT on reducing bone resorption and pain relief in adults with rheumatoid and children with juvenile rheumatoid arthritis. However, not all studies agree with such benefits in persons with arthritis (Stuart *et al.*, 1982).

Other Clinical Use of CT

Calcitonin has also been used successfully in the treatment of Sudeck's atrophy (Wimalawansa and Cooper, 1997; Wimalawansa, 2009; Dainotto *et al.*, 1985). Clinical experience suggests that the administration of CT shortens the duration of pain and healing of fractures, but this has not been confirmed. CT can be administered intrathecally, allowing a decrease in the (e.g., postoperative) requirements of narcotics in patients undergoing surgical procedures, such as hip replacement, under spinal anesthesia, and in patients with metastatic bone disease.

Conclusion

The 32-amino acid polypeptide hormone CT is a potent inhibitor of the osteoclast cells: a key mechanism that has been exploited with drugs for the treatment of bone disease with increased bone turnover. Therapy with CT is particularly effective in controlling osteoclastic bone resorption in disorders characterized by high bone turnover, including Paget's disease of bone, osteoporosis, Sudeck's atrophy, and statuses of hypercalcemia.

However, the efficacy of CT in the treatment of osteoporosis is significantly less than all other FDA-approved antiosteoporosis therapies. However, CT can be used effectively to control bone pain (e.g., crush fracture syndrome) that requires the use of higher doses (e.g., daily use of 800–1000 IU intranasal for 2–5 days). This allows for early mobilization of patients and thus prevents immobilization-associated complications; it also enables decreases (or elimination) in the use of potent analgesia. The efficacy on pain relief and the reduction of raised serum calcium levels do not last >3–4 days of continuous use.

See also: Premenstrual Syndrome (PMS)

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Hypoparathyroidism and Other Causes of Hypocalcemia

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Hypoparathyroidism

Grant *et al.* (2010) reported that postsurgical hypoparathyroidism is the cause of hypoparathyroidism in approximately 75% of adults with this disorder. Anterior neck surgery for thyroid or parathyroid disease is the usual explanation for postsurgical hypoparathyroidism. During anterior neck surgery, parathyroid glands may be damaged by compromised blood supply resulting from exploration of the neck structures, or their unexpected excision.

Patients usually develop postsurgical hypoparathyroidism manifest by hypocalcemia within a day or two after surgery. Postsurgical hypoparathyroidism is most often transient. Various hospitals have reported widely ranging rates of symptomatic hypoparathyroidism from 1% to 46% after thyroid cancer surgery (e.g., Lee *et al.*, 2010). Permanent postsurgical hypoparathyroidism only occurs in 1%–2% of cases on average, with a strong correlation with expertise of the surgeon.

Studies have shown that risk of postthyroidectomy hypoparathyroidism varies with staging of the thyroid cancer, and is dependent on extent of surgery. Roughly 50% of cases of stage IV thyroid cancer develop postsurgical hypoparathyroidism. Edafe *et al.* (2014) showed that patients undergoing thyroidectomy for autoimmune hyperthyroidism (Graves' disease) commonly experience both short- and long-term hypoparathyroidism. Most surgeries that lead to postsurgical hypoparathyroidism are done for thyroid or head or neck cancers, but the condition can also occur after surgery for benign causes such as goiter or primary hyperparathyroidism. Surgeons may use day 1 postoperative calcium and parathyroid hormone levels to assess patients for the likelihood of developing permanent hypocalcemia (e.g., Noordzij *et al.*, 2007).

Hypo- or hypermagnesemia may cause the appearance of biochemical hypoparathyroidism in patients who have not had neck surgery (Rude *et al.*, 1976; Cholst *et al.*, 1984). Typically low serum magnesium does not cause hypoparathyroidism unless it is reduced to 1.0 mg/dL or less. Wong *et al.* (1983) showed that as many as 11% of hospitalized patients may have hypomagnesemia, while as many as 9% may have hypermagnesemia. Hypomagnesemia commonly develops with gastrointestinal losses associated with vomiting due to increased alcohol intake, chronic diarrhea, steatorrhea, malabsorption, or intestinal resection. Rude (1997) summarized that hypomagnesemia also commonly develops due to renal tubular losses due to medications such as furosemide, aminoglycosides, cisplatin, cyclosporin, amphotericin B, pentamidine, tacrolimus, or proton pump inhibitors. Some patients have rare genetic disorders such as Gitelman syndrome causing renal tubular loss of magnesium and other analytes. Intensive care unit patients with serious illness often develop hypocalcemia due to multifactorial hypomagnesemia. Rude (2009) also described hypermagnesemia as not being unusual in the setting of late-stage chronic kidney disease in association with treatment with magnesium antacids, enemas, or infusions, or tocolytic therapy during labor, or acute renal failure due to rhabdomyolysis or tumor lysis syndrome.

Primary intestinal hypomagnesemia is a rare genetic cause of magnesium malabsorption leading to hypomagnesemia shortly after birth. This condition leads to deficient intestinal magnesium absorption, but some patients may also have defects in renal magnesium reabsorption. Affected individuals may develop tetany, muscle spasms, or seizures due to both hypomagnesemia and hypocalcemia, with biochemical evidence of hypoparathyroidism. Lifelong high oral magnesium supplementation is able to decrease symptoms and normalize serum calcium. This genetic disorder has been found to be due to mutations in the *TRMP6* gene on chromosome 9 (Schlingmann *et al.*, 2005; Voets *et al.*, 2004). The TRMP6 protein is a member of the transient receptor membrane potential channel family that associates with TRPM7, a calcium- and magnesium-permeable cation channel.

Other nonsurgical causes of hypoparathyroidism are less common. Toumba *et al.* (2007) showed that thalassemia major requiring multiple blood transfusions may lead to iron deposition in and destruction of the parathyroid glands, as may hemochromatosis on occasion. Carpenter *et al.* (1983) showed that Wilson's disease causing copper deposition in the parathyroid glands may sometimes result in hypoparathyroidism.

Goddard (1990) reported that metastases to the parathyroid glands may cause gland destruction leading to hypoparathyroidism in rare circumstances. External beam radiation therapy used to treat malignant disease in the neck may rarely cause hypoparathyroidism due to destruction of the parathyroid glands. Pauwels *et al.* (2000) demonstrated that high-dose radioactive iodine therapy for Graves' disease may also rarely cause hypoparathyroidism.

Inherited causes of hypoparathyroidism include autosomal dominant hypocalcemia (ADH), in which the CaSR is affected by an activating mutation, as shown by Egbuna and Brown (2008). This gain-of-function mutation alters the threshold for parathyroid hormone secretion by parathyroid cells in response to ambient ionized calcium, resulting in decreased or inappropriately normal PTH secretion despite hypocalcemia. Most of the activating mutations reported so far occur in the extracellular amino-terminal or transmembrane domains of the receptor. Mutant receptors evaluated so far show both increased sensitivity to extracellular calcium and increased signal-transduction capacity.

Since these activating mutations are also expressed in the CaSR on proximal renal tubular cells in the thick ascending limb of Henle, absolute or relatively increased 24-h urinary calcium is characteristic of the disorder. Most patients with ADH are

asymptomatic and have mild hypocalcemia with significant hypercalciuria. Occasionally patients may develop moderate or severe hypocalcemia. This form of CaSR-mediated hypoparathyroidism appears to cause increased risk of kidney stones compared to other forms of hypoparathyroidism. In one series by Lienhardt *et al.* (2001), almost half of the patients with ADH had kidney stones associated with their hypercalciuria. Because of the severity of hypercalciuria in this disorder, calcium supplements must be carefully monitored to prevent exacerbation of kidney stone risk.

Vargas-Poussou *et al.* (2002) demonstrated that occasional patients have CaSR activating mutations associated with a Bartter-like syndrome, suggesting that the CaSR also plays a role in sodium chloride regulation. These patients usually develop hypocalcemia, hypercalciuria, and kidney stones, associated with hypokalemic alkalosis, renal tubular sodium loss that may cause low blood pressure, hyperreninemic hyperaldosteronism, and increased urinary prostaglandin excretion.

Extensive burns occasionally lead to upregulation of the CaSR in affected tissues, resulting in lower than usual serum calcium and suppression of PTH secretion, resulting in hypocalcemia and hypoparathyroidism.

The second most common form of hypoparathyroidism after postsurgical hypoparathyroidism is thought to be autoimmune hypoparathyroidism. Michels and Gottlieb (2010) described that isolated antibody-mediated destruction of the parathyroid glands may occur, resulting in idiopathic hypoparathyroidism, or autoimmune destruction may occur in association with other autoimmune conditions as part of the autosomal recessive autoimmune polyglandular endocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome. Shikama *et al.* (2009) noted that mutations in the autoimmune regulator gene *AIRE* lead to this syndrome, causing abnormal thymic expression of tissue self-antigens, generation of auto-reactive T cells, and ultimate loss of central tolerance to specific self-antigens, and the consequent development of multiple autoimmune disorders. CaSR antibodies have been identified in some individuals with idiopathic hypoparathyroidism or the APECED syndrome (Blizzard *et al.*, 1966; Li *et al.*, 1996), but Brown (2009) indicated that it is not yet clear whether these autoantibodies are causative or markers of disease. Idiopathic autoimmune hypoparathyroidism most often occurs in teen-aged youth or young adulthood, but it may occur at any age. APECED usually presents in childhood, and is characterized by hypoparathyroidism, chronic mucocutaneous candidiasis, and Addison's disease, in addition to variable expression of endocrine and other autoimmune diseases. Variation in the clinical phenotype of individuals with identical mutations in the *AIRE* gene is incompletely understood, but this observation suggests that other genetic loci or environmental factors are important in the expression of the disorder.

Hypoparathyroidism may be diagnosed at birth or during childhood due to a variety of genetic mutations causing congenital syndromes, the most widely known being the DiGeorge (velocardiofacial) syndrome, as described by Goldmuntz (2005). This disorder is caused by abnormal development of neural crest cells in the third and fourth branchial pouches. In 90% of cases the syndrome is caused by heterozygous chromosomal deletion of the *TBX1* gene in the region of chromosome 22q11. Thirty-five genes have been identified in this region, so deletion of other genes, alone or in combination, could also cause this syndrome, but the *TBX1* gene is a major determinant of cardiac, thymus, and parathyroid cell phenotypes. A region on chromosome 10p (DiGeorge critical region II) has also been linked to this syndrome. DiGeorge syndrome is associated with distinctive facial abnormalities, cleft lip and/or palate, conotruncal cardiac anomalies, and mild-to-moderate immune deficiency. McDonald-McGinn and Sullivan (2011) indicated that hypocalcemia due to hypoparathyroidism has been reported in 17%–60% of affected children. DiGeorge syndrome is estimated to occur in as many as 1:2000–1:3000 births, with the incidence rate of new mutations estimated at 1:4000–1:6000. Because the clinical phenotype varies, findings may be subtle and therefore be overlooked, and mild hypocalcemia may be easily missed. Bassett *et al.* (2005) showed that in adults with chromosome 22q11.2 deletion, about half were hypocalcemic, with a median age at presentation of 25 years, and a maximum age of diagnosis of up to 48 years. This disorder may rarely be diagnosed for the first time as late as the mid-60s, with late-onset mild hypocalcemia, and is not infrequently diagnosed in affected parents in their 20s or 30s after birth of an affected child.

Finally, a number of other rare genetic mutations may cause hypocalcemia most often detected in infancy or childhood. Familial isolated hypoparathyroidism due to autosomal recessive or dominant mutations in the *pre–proPTH* gene on chromosome 11p15 (Parkinson and Thakker, 1992; Arnold *et al.*, 1990), or isolated parathyroid gland dysgenesis due to mutations in a number of critical transcription factors regulating parathyroid gland development such as *GCMB* (glial cells missing B) (Thomée *et al.*, 2005) or *GCM2* (glial cells missing 2) (Baumber *et al.*, 2005), *GATA3* (Van Esch *et al.*, 2000; Ali *et al.*, 2007), or Sry-box 3 (*SOX3*) (Bowl *et al.*, 2005), are thought to be very rare. Syndromic forms of genetic hypoparathyroidism include autosomal dominant hypoparathyroidism associated with deafness and renal anomalies, associated with mutations in the *GATA3* gene on chromosome 10p14-10-pter (Parvari *et al.*, 2002; Cassandrini *et al.*, 2006). Hypoparathyroidism has been very rarely associated with X-linked recessive mutations on Xq26-27, leading to disruption of SOX3 transcription (Labarthe *et al.*, 2006). The syndrome of autosomal recessive hypoparathyroidism, growth and mental retardation, and dysmorphism due to mutations in the *TBCE* gene on chromosome 1q42-q43 is another very rare cause of syndromic hypoparathyroidism (Rubin and Levine, 2009). Hypoparathyroidism with metabolic disturbances and congenital anomalies has been associated with rare maternal mitochondrial gene defects (Levine, 1999; Lui *et al.*, 2000).

Hypocalcemia

Hypocalcemia occurs for a variety of reasons other than hypoparathyroidism in both inpatients and outpatients. Hypocalcemia has multiple causes, and may occasionally occur in asymptomatic patients, but more commonly causes symptomatic tingling paresthesias, muscle cramps, or tetany in those with moderate hypocalcemia. More severe hypocalcemia is associated with

seizures, bronchospasm, laryngospasm, cardiac rhythm disturbances, loss of consciousness, or sudden death. These varying symptoms are due to the fact that ionized calcium plays a critical role in many tissues, with roles as varied as regulation of cellular secretion, muscle contraction, nerve function, and blood clotting.

About 50% of serum calcium is found circulating in the ionized form, not bound to proteins or anions. The majority of the remaining 45%–50% is bound by proteins, largely to albumin, with the remaining 5% bound to anions. Although the ionized form of calcium is the biologically active form of calcium, measurement of ionized calcium is more difficult and often not routinely available. Most laboratories report serum total calcium as the only form of serum calcium available to clinicians trying to assess calcium physiology in patients with complex disorders.

Several factors alter the results obtained for serum total and ionized calcium. Decreases or increases in serum albumin increase or decrease serum total calcium without changing serum ionized calcium. Decreases in serum albumin below 4.0 g/dL decrease serum total calcium by 0.8 mg/dL for each 1.0 g/dL decrease in serum albumin, with albumin-corrected serum calcium felt to be a more accurate measure of serum calcium than total calcium. Correspondingly, increases in serum albumin above 4.0 g/dL increase total calcium by 0.8 mg/dL for each 1.0 g/dL increase. Dehydration increases serum total calcium due to increased concentration of the blood. Serum pH below 7.4 increases serum ionized calcium, and serum pH above 7.4 decreases ionized calcium, without affecting serum total calcium levels. Circulating citrate or phosphate in the blood decreases serum total calcium, whereas monoclonal proteins may increase serum total calcium by binding to the calcium. Because variations in intravascular volume and calcium-binding proteins affect serum total calcium, ionized calcium should preferentially be measured in complex clinical situations associated with changes in volume status, albumin concentration, and/or blood pH. Under less complex clinical circumstances, serum total calcium corrected for serum albumin should be adequate.

Hypocalcemia is present when serum total calcium, albumin-corrected total calcium, and/or ionized calcium are below the lower limit of the normal range. Serum total calcium below 8.5 mg/dL (2.13 mmol/L), or ionized calcium below 4.80 mg/dL (1.20 mmol/L), is considered below normal in most clinical assays. Before launching into an exhaustive investigation of hypocalcemia, calculation of albumin-corrected serum calcium should be performed, and serum ionized calcium should be checked, if possible, to verify that it is decreased. Situations in which serum total or albumin-corrected calcium are decreased, but ionized calcium remains normal, may be due to binding protein abnormalities.

Biochemical Features of Hypocalcemia

Shoback (2008) summarized that serum calcium concentration represents the balance between calcium influx into extracellular fluid coming from intestinal absorption, skeletal resorption, and renal reabsorption, and calcium efflux from extracellular fluid coming from intestinal secretion, skeletal uptake, and renal excretion. Shafer and Shoback (2013) emphasized that hypocalcemia most commonly results from decreased skeletal resorption or intestinal absorption, associated with normal or increased renal excretion, but it may occur with normal calcium influx associated with increased renal excretion or skeletal mineralization. Mundy and Guise (1999) summarized that decreased skeletal resorption usually results from decreased osteoclast recruitment and activation, most often because of decreased PTH secretion, parathyroid hormone-related protein (PTHrP) release, or 1,25-dihydroxyvitamin D levels. Deficiencies of other cytokines that stimulate osteoclast recruitment or function, including interleukin (IL)-1α, IL-1β, IL-6, tumor necrosis factor-α, lymphotoxin, or transforming growth factor-β, usually lead to decreased skeletal resorption, but by themselves may not cause hypocalcemia. Decreased intestinal absorption of calcium is not uncommon, either due to decreased 1,25-dihydroxyvitamin D or malabsorption from other causes. Regardless of the cause of decreased calcium influx into extracellular fluid, serum calcium levels do not typically decrease unless the kidneys fail to compensate with appropriately increased urinary calcium reabsorption.

Other factors indirectly affect serum calcium. Decreased PTH or PTHrP lead to decreased renal tubular reabsorption of filtered calcium, resulting in increased urinary calcium loss. Increased fluid intake may result in hemodilution, and volume overload may result in polyuria resulting in increased renal calcium clearance. Physical activity may directly decrease bone resorption and thereby reduce serum calcium.

Kantham *et al.* (2009) demonstrated that the seven-transmembrane segment G protein-coupled extracellular calcium-sensing receptor (CaSR) plays a major role in regulation of extracellular calcium. This receptor is found on parathyroid, renal tubular, osteoblast, intestinal mucosal, and adipocyte cells, as well as other cells in other tissues. The CaSR regulates secretion of PTH by parathyroid cells and renal tubular reabsorption of calcium, as it regulates bone turnover and intestinal absorption of calcium. The CaSR has a large extracellular portion that binds ionized calcium, and a shorter intracellular portion that interacts with a variety of G proteins and signal-transduction pathways.

Symptoms and Signs

Patients with mild hypocalcemia may be completely asymptomatic, whereas those with severe hypocalcemia may lose consciousness due to profound metabolic derangements. The magnitude of symptoms and signs present depends largely on the severity and chronicity of the hypocalcemia (**Table 1**). Patients with chronically low levels of serum calcium may be asymptomatic except for a positive Chvostek's sign. Chvostek's sign may be present in up to 15% of healthy subjects without hypocalcemia,

Table 1 Signs and symptoms of hypocalcemia

Signs
Chvostek's sign
Trousseau's sign
Prolonged QTc interval
Posterior subcapsular cataracts
Basal ganglia and intracerebral calcifications
Papilledema or pseudotumor cerebri
Symptoms
Tingling paresthesias affecting fingertips, toe tips, or lips/tongue
Muscle cramps, twitching, or tetany
Abdominal cramps
Bronchospasm
Laryngospasm
Seizures
Depression
Generalized weakness
Congestive heart failure
Coma

however, so it is not pathognomic of hypocalcemia. Neuromuscular irritability is the most common cause of symptoms, ranging from tingling paresthesias around the fingertips, toes, lips, or tongue tip to tetany, carpopedal spasm, extremity muscle twitching or cramping, or abdominal cramps. McGreal *et al.* (1995) indicated that neuromuscular irritability is often most clearly demonstrated by eliciting facial muscle twitching after tapping over the facial nerve just anterior to the ear (Chvostek's sign), and Rehman and Wunder (2011) showed that carpal spasm may be caused by inflation of an upper arm blood pressure cuff to 20 mmHg above systolic blood pressure for 3 min (Trousseau's sign). Trousseau's sign may be present in up to 1%–2% of healthy subjects without hypocalcemia, so it is not pathognomonic of hypocalcemia either.

More severe hypocalcemia may cause a broader range of more severe symptomatology including bronchospasm, laryngospasm, seizures, cardiac dysrhythmias associated with QT interval prolongation, coma, or sudden death. Patients often report feeling weak, fatigued, or depressed until hypocalcemia is corrected.

Chronic hypocalcemia may result in calcification of the basal ganglia and other intracerebral structures on head CT, MRI, or X-rays. Patients may develop posterior subcapsular cataracts related to long-standing treatment-related increases in the calcium $\times$ phosphate product, or pseudotumor cerebri. Prolonged severe hypocalcemia may cause congestive heart failure due to cardiomyopathy, which may be reversible with appropriate management of hypocalcemia.

Differential Diagnosis

Causes of hypocalcemia may be broadly divided into PTH-mediated and non-PTH-mediated causes (**Table 2**). PTH-related hypocalcemia is most often due to PTH deficiency or resistance. A large portion of non-PTH-related causes are due to vitamin D deficiency or resistance, with a wide variety of other less common causes. This section briefly reviews the multiple causes of hypocalcemia due to conditions other than PTH deficiency.

PTH Resistance

Brandi (2011) described pseudohypoparathyroidism (PHP) as a complex disorder with several recognized subtypes, characterized biochemically by hypocalcemia, hyperphosphatemia, and hyperparathyroidism due to tissue unresponsiveness to PTH (**Table 3**). Most often, hypocalcemia is not detected at birth, but rather develops gradually during childhood. The historical gold standard for diagnosis of PHP was the Ellsworth–Howard test, in which bovine PTH was infused to evaluate whether urinary phosphorus and cyclic AMP increased as they should. In most forms of PHP, urinary cyclic AMP does not increase as seen in healthy controls. The Ellsworth–Howard test is no longer performed today due to the unavailability of bovine PTH for diagnostic testing, and the diagnosis is largely based on the constellation of biochemical findings, family history when present, and genetic analysis. Recombinant human PTH 1–34 (teriparatide) may be used in place of bovine PTH in modified Ellsworth–Howard protocols at some centers.

PHP is further classified as types 1a, pseudo-PHP, 1b, 1c, or 2. PHP type 1a is the most common subtype, and due to loss-of-function mutations in the coding region of the maternally inherited *GNAS* gene encoding the Gsα subunit of G proteins. Mantovani (2011) noted that in this disorder, Gsα protein expression is reduced by half. PHP type 1a patients have isolated PTH resistance at the renal tubule, resulting in blunted phosphaturic and cAMP responses to infused PTH. The blunted response is due to lack of normal signaling by the PTH receptor 1 due to the reduced stimulatory G protein expression.

Table 2 Differential diagnosis of hypocalcemia

Hypoparathyroidism
Acquired
Postsurgical
Autoimmunity
Hemochromatosis or transfusion-dependent thalassemia
Wilson disease
Parathyroid metastases
Neck irradiation
Hypo- or hypermagnesemia
Inherited
APECED syndrome
Isolated hypoparathyroidism: familial or X-linked
Autosomal dominant hypocalcemia
Parathyroid agenesis
Parathyroid hormone gene mutations
Syndromic forms of genetic hypoparathyroidism
PTH resistance
Types 1a, 1b, 1c, and 2
Hypomagnesemia
Vitamin D deficiency
Nutritional deficiency
Malabsorption
Inadequate sunlight exposure
Hyperpigmentation
Anticonvulsant therapy
Pseudovitamin D deficiency rickets (vitamin D-dependent rickets type 1)
Chronic kidney disease
Severe liver disease
Vitamin D resistance
Hereditary vitamin D-resistant rickets (vitamin D-dependent rickets type 2)
Others
Hyperphosphatemia
Chronic kidney disease
Tumor lysis syndrome
Rhabdomyolysis
Acute pancreatitis
Burns
Hungry bone syndrome
Osteoblastic metastases
Transfusion with citrated blood
Critical illness
Pseudohypocalcemia due to MRI gadolinium contrast agents
Electroconvulsive therapy
Medications
Hypocalcemia with decreased PTH levels
Medication-induced hypomagnesemia: cisplatin, diuretics, aminoglycosides, amphotericin
Medication-induced hypermagnesemia: magnesium-containing antacids or laxatives, tocolytic therapy
Cinacalcet
Alcohol abuse
Hypocalcemia with increased PTH levels
Calcium-chelating agents: EDTA, citrate, foscarnet, hydrofluoric acid
Vitamin D deficiency or resistance: phenytoin, phenobarbital, carbamazepine, valproic acid, isoniazid, theophylline, glutethimide, rifampicin
Antiresorptive agents: bisphosphonates, denosumab, estrogens, raloxifene, calcitonin, plicamycin, colchicine overdose
Loop diuretics
Proton pump inhibitors and H2-blockers
Glucocorticoid therapy
Others: propylthiouracil, dobutamine, calcium channel blockers, strontium-89, deferasirox, bicarbonate therapy

PHP type 1a is associated with Albright's hereditary osteodystrophy (AHO), which is characterized by obesity, round facies, mild mental retardation, and a skeletal phenotype involving short stature, brachydactyly of hands and/or feet, and heterotopic ossifications in subcutaneous tissues. This disorder is frequently associated with multiple hormone resistance involving thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), calcitonin, and/or growth hormone-releasing hormone (GHRH).

Table 3 Classification of pseudohypoparathyroidism

Type	*Gsα activity*	*Albright's HO*	*PTH resistance*	*Urinary cAMP response*	*Multiple hormone resistance*	*Molecular defect*
1a	↓	Yes	Yes	↓	Yes	Heterozygous *GNAS* mutations
1b	N	No	Kidney	↓	No	Imprinted *GNAS* defect
1c	N	No	Yes	↓	Yes	Unknown
Pseudo-PHP	↓	Yes	No	Normal	No	Heterozygous *GNAS* mutation
2	N	No	Kidney	Normal	No	Unknown

PseuoPHP, pseudopseudohypoparathyroidism; N, normal; HO, hereditary osteodystrophy; PTH, parathyroid hormone; cAMP, cyclic adenosine monophosphate.

Patients with PHP type 1a have renal tubule PTH resistance because they inherit a mutated maternally imprinted *GNAS* allele. *GNAS* alleles undergo differential imprinting in mothers and fathers, with tissue-specific expression of alleles in offspring. Only the maternal allele is expressed in the kidney and in the relevant endocrine organs associated with hormone resistance in this form of the disorder, but the rest of the body expresses both maternal and paternal alleles. An affected allele in other body tissues leads to haploinsufficiency and AHO expression, but PTH resistance occurs only in the tissues expressing the maternal allele.

Patients with paternally inherited *GNAS* mutations who have AHO without renal or endocrine gland resistance are designated as having pseudopseudohypoparathyroidism (pseudo-PHP). Patients with pseudo-PHP have a normal urinary phosphorus and cAMP response to PTH, unlike PHP type 1a patients. Patients with both PHP type 1a or pseudo-PHP may occur in the same kindred. In some families both forms are found in the same generation, but more often the two forms are found in different generations.

Brandi (2011) indicated that PHP type 1b patients lack typical features of AHO, but may have mild brachydactyly. Levels of Gsα in accessible tissues are normal, but patients have renal tubular PTH resistance without resistance to other hormones, although some may have mildly increased TSH levels. Skeletal manifestations are similar to those seen in patients with hyperparathyroidism, with bone loss or changes of osteitis fibrosa cystica. Mantovani (2011) reported that most cases are due to switching of the maternal *GNAS* allele to a paternal pattern of methylation, caused by microdeletions in the *STX16* gene located 220 kB centromeric from *GNAS* exon 1A, or deletions removing the differentially methylated region involving exon NESP55 or exons 3 and 4 of the antisense transcript. In both situations, inheritance of a mutation from a female, or spontaneous mutation of a maternally derived allele, removes the maternal *GNAS* epigenotype, leading to transcriptional silencing of the Gsα promoter in imprinted tissues, with little or no expression of either *GNAS* allele in these tissues.

Patients with PHP type 1c have normal Gsα activity and lack identifiable mutations in *GNAS*, and are thought to represent a variant of PHP type 1a.

PHP type 2 patients have normal cAMP production in response to administered PTH, but lack a phosphaturic effect. Little is known about the mutations involved, but because the biochemical picture is similar to that of severe vitamin D deficiency, it may be that most, if not all, cases of PHP type 2 are due to unsuspected vitamin D deficiency.

PTH causes the distal renal tubule to decrease phosphate reabsorption and increase calcium reabsorption. In patients with PHP type 1a, PTH has less distal tubule phosphaturic effect and no hypercalciuric effect, so these patients have reduced risk of kidney stones. Mantovani (2011) reported that in this situation, calcium supplements may be taken with less risk of calcium nephrolithiasis than in other causes of hypoparathyroidism.

Low serum magnesium may also increase PTH resistance. Rude (2009) indicated that magnesium repletion results in normalization or an increase in PTH levels, leading to normalization of serum calcium after a delay.

Vitamin D-Related Hypocalcemia

Hypocalcemia may be due to vitamin D deficiency or resistance. Thacher and Clarke (2011) implied that lack of adequate intake of dietary vitamin D or sunlight exposure may be the most common cause of hypocalcemia worldwide. Even if dietary intake is sufficient, malabsorption of vitamin D by the intestine can result from many causes and lead to hypocalcemia. Rare forms of vitamin D resistance due to renal 1α-hydroxylase deficiency or tissue vitamin D receptor mutations often lead to hypocalcemia. Leboff *et al.* (1999) indicated that hypocalcemia due to vitamin D deficiency or resistance may be moderate to severe, and lead to increased risk of hip fracture.

Vitamin D Deficiency

Ergocalciferol (vitamin D2) is obtained from plant sources or dietary supplements derived from irradiated fungi, and cholecalciferol (vitamin D3) is produced by synthesis in the skin with ultraviolet B light exposure, from animal sources, or dietary supplements. Both ergocalciferol and cholecalciferol are transported by vitamin D-binding protein in the blood to the liver, where they are converted by liver 25-hydroxylase to 25-hydroxyvitamin D2 and D3, and then to the kidneys, where small amounts are

renally 1α-hydroxylated to 1,25-dihydroxyvitamin D2 and D3. Serum total 1,25-dihydroxyvitamin D is the biologically active form of vitamin D in the body because of its 1000-fold higher affinity for the vitamin D receptor than 25-hydroxyvitamin D. Serum 1,25-dihydroxyvitamin D2 and 1,25-dihydroxyvitamin D3 are thought to have equal affinity for the vitamin D receptor. Lieben *et al.* (2011) summarized that serum total 1,25-dihydroxyvitamin D is able to stimulate active transport of calcium and phosphorus from the intestinal lumen into the blood by upregulating a number of intestinal transport proteins, especially when dietary intake of calcium or phosphorus is low. Serum 1,25-dihydroxyvitamin D also acts directly via vitamin D-binding sites on the PTH gene to suppress PTH transcription in parathyroid cells, resulting in reduced secretion of PTH. PTH also directly stimulates osteoclast and osteoblast cell recruitment and activation on bone surfaces. Total 1,25-dihydroxyvitamin D is able to suppress its own synthesis by renal 1α-hydroxylase, and to increase renal 24-hydroxylase to cause its own breakdown. Because decreased serum PTH, increased serum calcium, or decreased serum phosphorus each independently downregulate renal 1α-hydroxylase, hypoparathyroidism leads to reduced renal 1α-hydroxylase activity and decreased 1,25-dihydroxyvitamin D synthesis if serum calcium and phosphorus do not change in response to the decreased PTH level. Because vitamin D deficiency may result in both hypocalcemia and hypophosphatemia, this normally triggers upregulation of PTH synthesis that stimulates renal 1α-hydroxylase activity. Persistently increased serum PTH secretion from any cause often stimulates serum total and bone alkaline phosphatase activity, resulting in increased bone turnover, which may lead to bone loss over time. Severe and prolonged vitamin D deficiency may ultimately lead to increased unmineralized collagenous and noncollagenous matrix production in the skeleton, resulting in osteomalacia in adults, or rickets in children.

Van Schoor and Lips (2011) reported that vitamin D insufficiency, defined as serum 25-hydroxyvitamin D levels less than 20 ng/mL, and vitamin D deficiency, defined as serum 25-hydroxyvitamin D levels less than 10 ng/mL, are common in adults and children in most countries. Thomas *et al.* (1998) showed these are common as well as in hospitalized patients. Priemel *et al.* (2010) reported that serum 25-hydroxyvitamin D is the best marker of nutritional vitamin D intake, with levels below 10 ng/mL (25 nmol/L) considered deficient because of the higher prevalence of osteomalacia or rickets at this level. Optimal levels due to nutritional adequacy of intake continue to be debated, with most specialists recommending 30 ng/mL (75 nmol/L) for treatment of osteoporosis or metabolic bone disease. The 2011 U.S. Institute of Medicine report (2011) concluded that 20 ng/mL (50 nmol/L) was adequate to maintain skeletal health in healthy adults, and emphasized that optimal vitamin D levels have not yet been established for most human diseases. In light of the ongoing controversy regarding vitamin D optimal levels, vitamin D insufficiency is most often defined as levels between 10 and 20 ng/mL (25–50 nmol/L).

Vitamin D overdosing by patients is increasingly common, frequently leading to increased urinary calcium levels leading to nephrocalcinosis, but frank vitamin D toxicity resulting in hypercalcemia remains relatively uncommon. Most bone specialists regard serum 25-hydroxyvitamin D levels over 80 ng/mL (200 nmol/L) with concern, but it is uncommon to develop hypercalcemia unless serum 25-hydroxyvitamin D increases above 150 ng/mL (375 nmol/L).

Shoback (2008) indicated that any disorder that interferes with vitamin D absorption, production, transport, binding to vitamin D receptors in target tissues, or degradation may reduce vitamin D activity in tissues. Vitamin D deficiency may result from low sunlight exposure, especially in the elderly, those with hyperpigmented skin, or those using high-grade sun block to reduce sun exposure. Malnutrition, intestinal malabsorption, chronic liver disease, or later stage chronic kidney disease may interfere with adequate vitamin D absorption or production. Liamis *et al.* (2009) summarized that medications upregulating cytochrome P450 enzymes that normally break down vitamin D, such as anticonvulsants, including phenytoin, phenobarbital, carbamazepine, or valproate, may result in vitamin D insufficiency or deficiency if sunlight exposure or dietary or supplemental intake does not make up for more rapid metabolism.

Mutations in the CYP27B1 gene responsible for synthesis of renal 1α-hydroxylase give rise to pseudovitamin D deficiency rickets, previously known as vitamin D-dependent rickets type 1 (VDDR1) (St Arnaud *et al.*, 1997; Kitanaka *et al.*, 1998). These mutations result in partial or complete absence or reduced activity of the 1α-hydroxylase enzyme, causing synthesis of very low levels of 1,25-dihydroxyvitamin D, with resultant significant hypocalcemia and hypophosphatemia. This disorder is typically diagnosed shortly after birth or in infancy due to tetany, seizures, rickets, and failure to thrive. Vitamin D deficiency due to inadequate nutritional intake results in decreased serum calcium, phosphorus, and 25-hydroxyvitamin D levels, but serum 1,25-dihydroxyvitamin D levels usually remain normal until serum 25-hydroxyvitamin D levels decrease to less than 4 ng/mL (10 nmol/L). At this level, lack of adequate 25-hydroxyvitamin D availability leads to decreased production of serum 1,25-dihydroxyvitamin D. Pseudovitamin D deficiency rickets results in low serum calcium and phosphorus, increased PTH causing increased total and bone alkaline phosphatase, normal 25-hydroxyvitamin D, and very low or undetectable 1,25-dihydroxyvitamin D levels due to the inadequacy of renal 1α-hydroxylase activity.

Vitamin D-Resistant Rickets

Brooks *et al.* (1978) first reported that hereditary vitamin D resistance rickets, previously known as VDDR type 2, is a rare autosomal recessive disorder caused by vitamin D receptor mutations leading to resistance to the action of 1,25-dihydroxyvitamin D. This condition has been reported in association with mutations in the ligand-binding domain, DNA-binding domain, and other domains of the receptor. Resistance to 1,25-dihydroxyvitamin D action may be partial or complete. Children with this condition are usually identified before their 2nd year, but Malloy *et al.* (1990) reported that some children may not be diagnosed until their teenage years, similar to children with pseudovitamin D deficiency rickets. Presenting features include tetany, seizures,

rickets, and failure to thrive. Laboratory data confirms low serum calcium and phosphorus, increased PTH, increased total and bone alkaline phosphatase, normal 25-hydroxyvitamin D, and increased 1,25-dihydroxyvitamin D levels. Increased PTH caused by the hypocalcemia stimulates renal 1α-hydroxylase production of 1,25-dihydroxyvitamin D. The main biochemical feature of this disorder is increased serum 1,25-dihydroxyvitamin D, distinguishing it from pseudovitamin D deficiency rickets. Patients have significant scalp alopecia, with partial or total scalp alopecia in two-thirds of the kindreds reported. Hypocalcemia varies between different kindreds also. Patients do not respond to usual replacement doses of vitamin D or calcium, but may respond to high doses of activated vitamin D in some cases, depending on remaining vitamin D receptor activity. Those with no residual vitamin D receptor activity are unresponsive to vitamin D therapy, and usually require treatment with intravenous calcium and/or high-dose oral calcium.

Other Causes of Hypocalcemia

Hypocalcemia may occur due to a variety of other causes beyond those due to hypoparathyroidism or vitamin D deficiency or resistance.

Hyperphosphatemia may occur in later stage chronic kidney disease, leading to reduction in serum calcium because serum phosphorus complexes to calcium in extracellular fluid and leads to deposition of calcium × phosphate complexes in soft tissues. Stage V chronic kidney disease results in hyperphosphatemia causing decreased 1α-hydroxylase activity, leading to decreased 1,25-dihydroxyvitamin D production. Medications may cause hyperphosphatemia when taken in excessive amounts, including phosphate-containing laxatives or enemas. While not used very often any more, intravenous phosphorus given to lower serum calcium may cause hyperphosphatemia. Tumor lysis syndrome or rhabdomyolysis may result in acute hyperphosphatemia, thereby decreasing serum calcium acutely.

Large quantities of transfused citrated blood products may complex calcium to citrate in the circulation, resulting in acute hypocalcemia. Pancreatic lipase released into surrounding tissue fluids during acute pancreatitis may increase free fatty acids that can saponify calcium in the blood as it flows through the pancreas, resulting in significant lowering of serum calcium. Rapid new bone formation may cause such significant rapid uptake of calcium from the blood that normal homeostatic mechanisms are not able to compensate, and hypocalcemia develops, as is often seen with "hungry bone" syndrome after surgical cure of severe, long-standing hyperparathyroidism. Brasier and Nussbaum (1988) reported that higher risk of "hungry bone" syndrome is seen in older patients with higher preoperative PTH and serum alkaline phosphatase levels, and larger weight of the resected adenoma. Rapid new bone formation may also occur in patients with extensive osteoblastic metastases, leading to significant hypocalcemia. Vivien *et al.* (2005) reported that critical illness is often associated with multifactorial hypocalcemia.

Medications may directly cause hypocalcemia by a variety of mechanisms (**Table 3**). Prince *et al.* (2003) reported that certain gadolinium contrast agents for magnetic resonance imaging studies may cause pseudohypocalcemia within several hours of administration. Several of these agents, but not all, may cause falsely low serum calcium levels at less than 6.0 mg/dL, as these may interfere with colorimetric serum calcium assays. If spuriously low serum calcium is suspected, serum calcium should be rechecked using a different assay method to clarify the diagnosis. Asymptomatic patients with critical hypocalcemia reported after an MRI scan should be suspected of having an inaccuracy in their reported serum calcium level.

Rosen and Brown (2003) reported that many patients being treated for osteoporosis who are given potent antiresorptive agents, including oral and intravenous bisphosphonates, denosumab, or raloxifene, may significantly decrease serum calcium on occasion. Patients beginning these agents should be treated with adequate vitamin D and calcium supplementation before taking their first dose to minimize their risk of hypocalcemia.

Laboratory Assessment

Diagnosis and management of hypocalcemia is critically dependent on the laboratory studies available to the clinician. Initial evaluation of patients suspected of having hypocalcemia should include measurement of serum total calcium, albumin for calculating albumin-corrected serum calcium, ionized calcium if available, phosphorus, creatinine, PTH, 25-hydroxyvitamin D, and magnesium levels. Interpretation of serum calcium values is enhanced considerably by knowledge of serum phosphorus levels. Serum 1,25-dihydroxyvitamin D does not need to be routinely measured, but knowledge of this analyte is very important in certain cases.

Tandem mass spectroscopy is used to reliably measure serum 25-hydroxyvitamin D, but because this is expensive, this technique is frequently not available in most laboratories. Many laboratories still use antibody-based assays, but these are quite variable, and on average tend to overestimate serum 25-hydroxyvitamin D levels by about 30%. Priemel *et al.* (2010) reported that serum 25-hydroxyvitamin D levels less than 10 ng/mL (25 nmol/L) are regarded as deficient because of the significantly increased likelihood of osteomalacia. The Institute of Medicine (2011) considers a level of 20 ng/mL (50 nmol/L) to be sufficient for skeletal purposes in healthy adults. Serum 25-hydroxyvitamin D between 10 and 20 ng/mL (25–50 nmol/L) is interpreted as insufficient by most bone specialists. The normal upper limit for serum 25-hydroxyvitamin D is considered to be 50 ng/mL (75 nmol/L) by many bone specialists because hypercalciuria may occur above this level. Vitamin D toxicity is associated with hypercalcemia, but

this most commonly does not occur unless serum 25-hydroxyvitamin D is above 150 ng/mL (375 nmol/L). Occasionally this may occur when serum 25-hydroxyvitamin D is above 80 ng/mL (200 nmol/L).

Measurement of intact PTH should be done by reliable second- or third-generation assays. Most laboratories now use these types of PTH assay. These assays can reliably detect PTH levels at the low end of the normal range in patients with hypoparathyroidism. PTH assays tend to be less variable than 25-hydroxyvitamin D assays.

Serum magnesium deficiency or excess may both limit secretion of PTH (Cholst *et al.*, 1984; Wong *et al.*, 1983), so patients with apparent hypoparathyroidism with the biochemical phenotype of low serum calcium, increased serum phosphorus, normal creatinine, normal 25-hydroxyvitamin D, and low PTH should always have serum magnesium checked as part of their baseline evaluation. Increased 24-h urine magnesium in the setting of hypomagnesemia strongly suggests renal tubular magnesium wasting, rather than gastrointestinal loss of magnesium.

The biochemical phenotype of pseudohypoparathyroidism is usually low serum calcium, increased serum phosphorus, normal creatinine, normal 25-hydroxyvitamin D, and moderately increased PTH levels. Vitamin D deficiency is characterized by low serum calcium, low phosphorus, normal creatinine, and increased PTH levels.

Measurement of 24-h urine calcium and creatinine are important in determining the cause of hypocalcemia. 24-h urine calcium is often moderately increased in idiopathic hypercalciuria in the untreated patient, but is likely due to high-dose calcium or vitamin D supplementation in treated hypocalcemic patients. Markedly increased 24-h urine calcium and usually asymptomatic mildly decreased serum calcium may be due to autosomal dominant hypocalcemia. Untreated hypocalcemic patients may have decreased or low–normal 24-h urine calcium levels.

Management

The purpose of hypocalcemia treatment is to improve or eliminate symptoms, reverse skeletal demineralization if it is present, heal osteomalacia if present, maintain acceptable serum total and ionized calcium, and avoid hypercalciuria. Hypercalciuria is often defined as 24-h urine calcium above 300 mg. Bilezikian *et al.* (2011) reported that hypercalciuria may lead to chronic kidney disease, nephrolithiasis, or nephrocalcinosis. Patients with a need for urgent treatment due to tetany, seizures, laryngospasm, bronchospasm, cardiac rhythm disturbances, altered mental status, or severe hypocalcemia, typically require bolus intravenous calcium, usually given as calcium gluconate, followed by a slower intravenous infusion of calcium, and then oral calcium when able to tolerate this. One approach is to add ten 10-mL ampules of calcium gluconate, with 93 mg elemental calcium per ampule, to 900 mL of D5W, with 10 mL of this solution infused slowly over 10 min to improve symptoms, with repeat infusions given once or twice more as needed. A maintenance infusion with this solution is then begun at 10–100 mL/h to control symptoms and improve serum calcium toward the lower end of the normal range at around 8.5 mg/dL (2.12 mmol/L), with ionized calcium of around 4 mg/dL (1.0 mmol/L). The infusion rate is usually titrated to give 0.3–1.0 mg elemental calcium/kg/h.

Once the patient becomes stabilized on the intravenous infusion over several hours, and is able to take oral intake, an oral calcium supplement regimen is begun, giving the patient at least 500 mg elemental calcium three to four times a day. The calcium gluconate infusion is gradually slowed and then stopped as serum calcium approaches the target level of 8.0–8.5 mg/dL (2.00–2.13 mmol/L) and symptoms improve, providing the oral calcium supplements are tolerated.

Conventional management of chronic hypocalcemia involves mainly oral calcium and vitamin D supplementation which is occasionally supplemented with thiazide-type diuretics or magnesium. Once serum magnesium is below the normal range, total body magnesium deficiency is usually very large. Serum magnesium is a poor reflector of total body magnesium level because the vast majority of total body magnesium is located inside cells. Supplementation with oral magnesium tablets usually takes several months to fully replete body stores. As total body magnesium is gradually repleted, serum calcium and PTH levels gradually return toward normal.

Oral calcium supplements of any type will help restore serum calcium toward normal. In general, oral calcium carbonate or calcium citrate is used most commonly for this purpose because they are easily obtained and relatively inexpensive. Calcium carbonate is 40% calcium by weight, and calcium citrate 21% calcium by weight, so that more calcium citrate tablets need to be taken to achieve the same intake or elemental calcium. Calcium supplements are usually given in at least two divided doses each day, but sometimes up to four to six times a day, with dosing usually recommended at mealtimes to enhance absorption. The usual starting doses recommended are usually 500–1000 mg elemental calcium given two to three times each day, and these are then increased further as needed based on tolerability, compliance, and the clinical target. It should be emphasized that the calcium doses uses in Europe are lower than those used in the United States.

Because calcium tablets alone may be insufficient to achieve serum calcium of 8.0–8.5 mg/dL (2.0–2.13 mmol/L), vitamin D supplementation is usually started simultaneously. If renal function is normal, ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3) may be started at 1000–4000 International Units each day. With vitamin D deficiency, vitamin D 50,000 International Units once weekly to several times a week as needed, depending on intestinal absorption. Severe hypoparathyroidism or PHP typically require higher doses of vitamin D supplementation. Care must be taken with these longer-acting forms of vitamin D, however, as their half-life is prolonged due to storage in body fat, and toxic serum levels of 25-hydroxyvitamin D may take 6–9 months to come down after supplementation is stopped.

More often, because of concerns regarding toxicity, calcitriol (1,25-dihydroxyvitamin D) 0.25 mcg once or twice a day is started in place of vitamin D2 or D3 in the United States, whereas calcitriol or alfacalcidol (1α-hydroxyvitamin D) is used in Europe. The

half-life of these active forms of vitamin D is much shorter than vitamin D2 or D3, on the order of 1–3 days, so that improvement in absorption or offset of action occurs more rapidly. Patients with hypoparathyroidism in Europe are often treated with higher doses of active vitamin D than calcium. Commercial parenteral vitamin D is no longer available in the United States, but some hospital-compounding pharmacies produce intravenous vitamin D3 based on clinical need.

Patients who develop increased urinary calcium while on calcium and vitamin D supplementation, or who are unable to achieve or maintain serum calcium at or near their target range, may benefit from a thiazide-type diuretic to reduce their urinary calcium loss. Doses of hydrochlorothiazide or chlorthalidone of 12.5–25 mg a day may be useful, but some patients may require higher doses of 50 or 100 mg to reduce their 24-h urine calcium to the desired range of less than 300 mg.

Once- or twice-daily injections of PTH 1–34 (teriparatide; Forteo; Forsteo) have been used off-label in clinical trials lasting 1–3 years to more effectively control serum or urine calcium and phosphorus in patients with hypoparathyroidism (Bilezikian *et al.*, 2011; Winer *et al.*, 1996, 1998). This therapy is not approved by the FDA or European Medicines Agency (EMA) for treatment of hypoparathyroidism. Mannstadt *et al.* (2013) published the pivotal 6-month phase III clinical trial with recombinant human PTH 1–84 (Natpara; Natpar) in 2013, with FDA approval as an adjunct for treatment of hypoparathyroidism in January 2015, and similar approval by the EMA in April 2017. Several guidelines for use of recombinant human PTH 1–84 in hypoparathyroidism have been published (Brandi *et al.*, 2016; Bollerslev *et al.*, 2015).

Hasse *et al.* (1997) and Tolloczko *et al.* (1996) have reported a few patients who have received previous or simultaneous renal transplants have received parathyroid allograft transplants. Advances in stem cell technology may eventually permit induced pluripotent cells or stem cells to be used to create new parathyroid tissue in patients where it did not develop or has been removed.

Patients with pseudovitamin D deficiency rickets (VDDR1) require lifelong physiologic doses of 1,25-dihydroxyvitamin D3 and calcium therapy. Patients with hereditary vitamin D-resistant rickets (vitamin D-dependent rickets type 2) usually require pharmacologic doses of 1,25-dihydroxyvitamin D3 and calcium to overcome their resistance. Calcium supplements of up to 3000 mg elemental calcium each day may be required. High-dose therapy is continued until their undermineralized bones have mineralized, typically within 2–6 months. Lifelong close monitoring is required to monitor serum calcium and mineral metabolism measurements and clinical symptoms. Some patients do not respond to high-dose therapy despite serum 1,25-dihydroxyvitamin D3 levels more than 100 times the upper limit of normal. Long-term intravenous calcium infusions in combination with high-dose oral calcium supplements are usually used successfully in this case.

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Calcitonin Gene-Related Peptide (CGRP)☆

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Glossary

Calcitonin receptor-like receptor (CRLR) G protein-coupled receptor (GPCR); pharmacology of this receptor is defined by its interaction with a group of accessory proteins called receptor activity-modifying proteins (RAMPs). CRLR receptor functions as a CGRP receptor when dimerized with RAMP1 and as an adrenomedullin (AM) receptor when dimerized with RAMP2 or RAMP3.

CGRP receptor component protein (RCP) Intracellular protein that facilitates signal transduction through the CGRP receptor.

Migraine Complex neurological disorder characterized by severe head pain, nausea, and exacerbated sensitivity to external stimuli such as light and sound. It presents in one of two forms, migraine without aura or migraine with aura, with the latter characterized by visual early disturbances such as zigzag lines and blind spots.

Receptor activity-modifying proteins (RAMPs) A family of three single type I transmembrane proteins, RAMP1, RAMP2, and RAMP3, required for the transport, glycosylation, and pharmacological classification of the calcitonin receptor-like receptor (CRLR) as CGRP1 or adrenomedullin (AM) receptor.

Introduction

Calcitonin gene-related peptide (CGRP) is a product of alternative splicing of the calcitonin (CT) gene. CGRP is primarily located within unmyelinated, small diameter sensory C-, and thinly myelinated Aδ-nerve fibers. Its dual occurrence in nerves and non-neuronal locations suggests potential functions as a neuromodulator and effector mediator (for a recent review, see Russell *et al.*, 2014). Its biological activity is mediated by type II G protein-coupled receptors (GPCRs), known as the calcitonin receptor-like receptor (CRLR or CLR), which forms a receptor complex with the accessory proteins: receptor activity-modifying proteins (RAMPs) and receptor component protein (RCP), to modulate both its ligand-binding selectivity and its signal transduction. Structural and functional analysis of these multiple associations is providing new insights into molecular determinants of multiple CGRP functions and allow the design of new drugs to regulate CGRP activity under pathological conditions.

CT/CGRP Gene, Alternative Splicing, and Transcriptional Regulation

CGRP is a 37-amino acid neuropeptide discovered by Amara *et al.* (1982) in thyroid tissue of aging rats, where alternative splicing of RNA transcripts from the CT gene resulted in distinct mRNAs encoding CGRP. CT and α-CGRP are coded for by the same gene (CALCA) found on chromosome 11p. Depending on the tissue localization, the CALCA gene is processed by different combinations of the six exons contained within the gene. In thyroid "C" cells/parafollicular cells, 95% of the CALCA gene is processed to include exons 1–3 (forming the common region, translated into N-terminal peptide), exon 4 (calcitonin-coding gene), followed by poly-adenylation (forming the extra 16 amino acid-containing C-terminal peptide). In contrast, neuronal CALCA pre-RNA is processed to include exons 1–3 (as previously), exon 5 (CGRP-coding region), and exon 6 (CGRP 3′ non-coding exon) followed by 3′ polyadenylation, which is translated into α-CGRP precursor containing 121 amino acids. Both CT and CGRP precursors undergo post-translational processing. The process that allowed tissue-specific inclusion of the alternative 3′-terminal exon 4 is modulated by factors that bind to an intronic enhancer element that contains both 3′ and 5′ splice site consensus sequence elements. Although factors involved in the tissue-specific alternative splicing of the CALCA gene have not been identified, members of the serine- and arginine-rich RNA-binding protein family may participate in multiple interactions that coordinate individual recognition events during the early steps of RNA processing (Fig. 1).

A second CGRP gene (CALCB coding for β-CGRP) was predicted from cDNA analysis in rat and human. The β-CGRP gene encodes a molecule closely related to α-CGRP. β-CGRP differs from α-CGRP in one amino acid in rat and in three amino acids in human and mouse, resulting in >90% homology. Unlike CALCA, CALCB gene lacked poly A signal within exon 4, preventing

Adapted from the original reference module by: Mara J. Moreno and Danica B. Stanimirovic, National Research Council of Canada, Ottawa, Ontario, Canada. Edith Hamel, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada.

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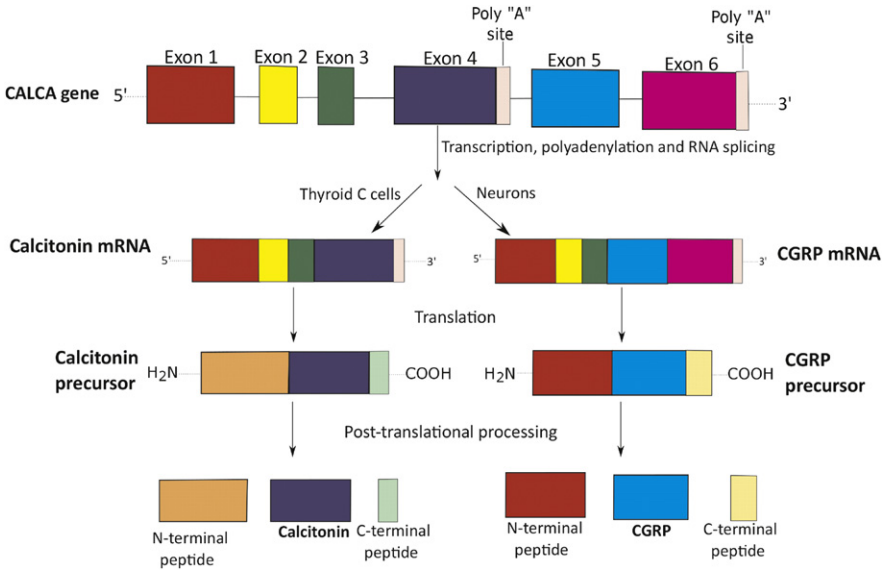

Fig. 1 CALCA gene processing yielding both calcitonin and α-calcitonin gene-related peptide (CGRP) in a tissue-specific manner. Boxes are exons; lines in between are introns. Adapted from Russell et al. (2014).

alternative splicing, hence β-CGRP is the only product of CALCB gene. Post-translational processing of α-CGRP and β-CGRP precursors resulted in the release of the bioactive peptides as well as non-functional N-terminal and C-terminal peptides. The structures of human α-CGRP and β-CGRP contain four clear domains: 1) The first 7 amino acids on the N-terminus, containing ring-like structure held by disulphide bridge between Cys2 and Cys7; 2) residues 8–18, consisting of an α helix; 3) residues 19–27 forming either a β- or γ- twist; 4) the last domain, made up of residues 28–37 including the C-terminus, forms the binding epitope. Traditionally, β-CGRP has been suggested to be localized to the CNS and gut, although a wider localization is now realized. However, as less is known about the specific function of β-CGRP, this chapter will only focus on the role of α-CGRP.

CGRP synthesis is upregulated in nerve damage and under inflammatory conditions. This is partly associated with nerve growth factor (NGF) release from keratinocytes and inflammatory cells, such as macrophages, playing an important role in nerve growth. The endogenous stimuli that drive CGRP release remain uncertain, although recent studies have focused on the role of various transient receptor potential (TRP) channels, such as TRP Vanilloid 1 (TRPV1) and TRP Ankyrin 1 (TRPA1), which are activated by a range of environmental (e.g. chemical, temperature) stimuli in addition to various endogenous stimuli (including reactive oxygen species and anandamide). The activation of these channels leads to an influx of cations, especially Ca^{2+} and Na^{+} resulting in Ca^{2+}-dependent exocytosis of CGRP from the nerve terminals.

Once released, the bioactivity of CGRP is partially dependent on its breakdown and removal system. Although the exact mechanism of CGRP metabolism remains unclear, studies in the skin has shown that CGRP is hydrolyzed by tryptase, which is released following mast cell activation or by neutral endopeptidases, which were identified in neurodegeneration field. Other enzymes involved in CGRP metabolism includes insulin-degrading enzymes (IDE) and endothelin-converting enzyme-1 (ECE-1). Independent of its breakdown, CGRP re-uptake into the nerve terminals has been proposed, especially in the mouse vas deferens, in guinea pig basilar artery and in rat dura mater encephali. Additionally, CGRP'S interaction with its receptor can lead to internalization and recycling. Hence, various removal mechanisms are important in regulating CGRP functions and may highlight future pharmacological targets in various disease conditions.

CGRP Receptors

Pharmacology of CGRP Receptors

CGRP is a member of a peptide family that also includes CT, amylin (AMY), adrenomedullin (AM) and intermedin. Although these peptides have only limited sequence identity, they share a number of structural features, including a six- or seven-amino acid ring formed by a disulphide bond at the N termini (implicated in triggering signal transduction), an amphipathic α-helix, and a C-terminal amide (responsible for the interaction of the molecule with the receptor). The structural convergence among these peptides results in a significant degree of cross-reactivity between receptors.

CGRP acts via type II GPCRs that primarily stimulate adenylate cyclase through the G_s subunit although full signaling pathway requires accessory proteins. CRLR was first isolated from rat pulmonary blood vessels. Despite initial transfection of human or rat CRLR into mammalian cells failing to elicit CGRP- or AM-induced responses, a stable expression of this gene in human embryonic kidney 293 cells resulted in increased density of high-affinity ^{125}I-CGRP binding sites and stimulation of intracellular cAMP levels

by CGRP. McLatchie *et al.* and Evans *et al.* showed the importance of two further accessory proteins on plasma membrane as well as cytoplasm to achieve full signaling function of CRLR.

Receptor Activity-Modifying Proteins

RAMPs are a family of single transmembrane domain proteins that are required for the transport, glycosylation and ligand specificity of CRLR (Fig. 2). They have a relatively long extracellular amino terminus, a single membrane-spanning domain and a short intracellular C terminus. Three members (RAMP1, RAMP2, and RAMP3) have been identified in this family by homology searches. They are 148, 175, and 148 amino acids long, respectively, with about 30% of sequence identity. RAMP1, although unable to bind CGRP on its own, can combine with CRLR to form a fully glycosylated receptor complex of 66 kDa. The crystal structure of human RAMP1 has been crystallized and residues on the extracellular domain important for cell-surface expression of CRLR.RAMP1 heterodimer have been identified (Kusano *et al.*, 2008). Interestingly, the combination of CRLR with RAMP2 or RAMP3 seemingly yields an AM receptor of 58 kDa (core glycosylated) that can cross-react with CGRP only at high concentrations.

RAMPs act as a chaperon for CRLR trafficking to the cell surface. When expressed individually, RAMPs and CRLR are retained intracellularly with CRLR sequestered in the endoplasmic reticulum (ER) and RAMP1 in both the ER and the Golgi system, predominantly as a disulfide-linked homodimer. When co-expressed, both proteins are detected on the cell surface, suggesting that the interaction between CRLR and RAMP1 facilitates their trafficking to the plasma membrane.

RAMP1 is also required for the terminal glycosylation of CRLR. Differential glycosylation of CRLR in the presence of RAMP1 or RAMP2 does not define receptor specificity for CGRP or AM. Moreover, core glycosylation is not sufficient for plasma membrane incorporation of CRLR in the absence of RAMPs. Human CRLR presents three asparagine (Asn) consensus glycosylation sites at positions 60, 112, and 117 of the amino acid sequence. Selective mutations of these sites have demonstrated that Asn117 is important for direct interaction of CGRP or AM with CRLR and RAMP1 or RAMP2 and, therefore, for the functionality of the receptor complex. However, Asn117 substitution does not affect N-glycosylation, association with RAMP1, and/or cell surface expression of receptor/RAMP1 heterodimers.

The extracellular N terminus of RAMP1 plays an important role in determining ligand specificity through association with the amino terminus of CRLR that results in formation of a CGRP-binding pocket. The observation that the first non-peptide CGRP receptor antagonist BIBN4096BS exhibits up to 200-fold higher affinity for human than rat CRLR, despite more than 90% sequence homology of CRLR between the two species, suggested that this selectivity is likely conferred by the interaction of BIBN4096BS with RAMP1 (homology ~71%). In support of this idea, tryptophan 74 in RAMP1 was shown to be responsible for the selective high-affinity binding of BIBN4096BS to the human CGRP receptor. Future structural analyses of CRLR/RAMP complexes are needed to map residues important for ligand recognition and provide a basis for designing therapeutics with high affinity and selectivity for these receptors.

RAMPs can also compete in regulating the function of CGRP/AM receptor function. For instance, co-expression of RAMP3 and RAMP1 results in reduced RAMP1-dependent CGRP receptor activity in a rabbit aortic endothelial cell line. Conversely, the expression of RAMPs is selectively regulated by different factors such as hypoxia and corticosterone. Therefore, balance of expression of various RAMPs, as well as their modulation by physiological or pathological conditions may affect the affinity, function, and "molecular switch" between CGRP and AM receptors. Many effects of AM are blocked by α-CGRP$_{8-37}$ and not by AM$_{22-52}$, a more selective AM receptor antagonist. In some cases, both α-CGRP$_{8-37}$ and AM$_{22-52}$ can partially inhibit CGRP- and AM-induced cAMP production. The first report documenting the interaction between CRLR and RAMP1 in neuroblastoma SK–N–MC cells showed that this complex selectively responds to CGRP but not to AM, suggesting that CRLR/RAMP1 heterodimers define a very selective CGRP1 receptor. Although α-CGRP has far lower affinity than does AM for CRLR/RAMP2 and CRLR/RAMP3

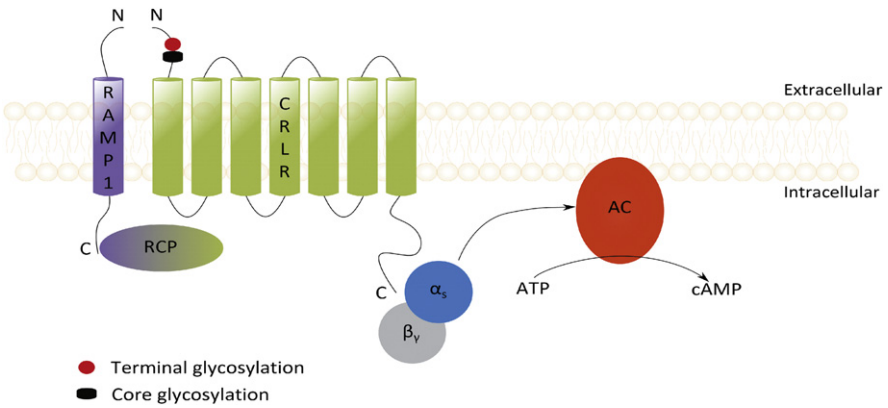

Fig. 2 A representative figure of Calcitonin Receptor Like Receptor (CRLR) and its interaction with associated accessory proteins Receptor Activity-Modifying Protein 1 (RAMP1) and Receptor Component Protein (RCP) which are vital for α-CGRP signaling. Activation of this receptor complex leads to a $G_{\alpha s}$ signaling pathway, activating adenylyl cyclase (AC) inducing cyclic adenosine monophosphate (cAMP) production.

complexes, the affinity of AM for CRLR/RAMP1 is comparable to that of αCGRP in some biological systems. Furthermore, in cells expressing higher levels of CRLR/RAMP1 than of CRLR/RAMP2 or-RAMP3, the effects of AM were blocked by $\alpha CGRP_{8-37}$ but not by AM_{22-52}. Hence, the CRLR/RAMP1 heterodimer defines a functional AM-sensitive receptor that is potently antagonized by $\alpha CGRP_{8-37}$ and distinct from CRLR/RAMP2, which is more sensitive to AM_{22-52}. This dual interaction of CRLR/RAMP1 complex with both αCGRP and AM may explain why many actions of AM are potently antagonized by $\alpha CGRP_{8-37}$. More recently, the possibility that CGRP may also act via CRLR and RAMP3 in human tissue has been investigated.

In summary, RAMPs contribute to various aspects of CGRP receptor biology: (1) trafficking of CRLR to the cell surface, (2) glycosylation of CRLR, (3) ligand specificity, and (4) receptor activity (see Hay and Pioszak, 2016 for more details). Furthermore, the importance of RAMPs in receptor pharmacology also extends to other members of the CT/CGRP family. As multiple AMY receptors arise from RAMP interaction with the CT receptor gene product, this indicates that the cellular profile of the CT/CGRP family of receptors, and potentially other class II GPCRs, can be dynamically regulated by the level and combination of these sets of proteins.

CGRP Receptor Component Protein (RCP)

In addition to RAMPs, the CGRP receptor complex seems to require another protein RCP for optimal function (Fig. 2). RCP is a 146-amino acid protein which is a peripheral membrane protein attached to the membrane by ionic interactions. RCP is expressed in CGRP-responsive tissues. CGRP binding and receptor density are not affected in RCP antisense-treated NIH3T3 cells, despite reduction in CGRP-mediated signal transduction, suggesting that RCP is not a chaperon protein for CRLR. The nature of the interaction between RCP and CRLR is unclear. It has been suggested that RCP may facilitate CRLR activation, couple the receptor to G proteins or other effector molecules, or coordinate the receptor–effector complex in the plasma membrane. The requirement for the expression of at least three proteins (CRLR, RCP, RAMPs) to form a functional CGRP receptor may explain the difficulty in determining the molecular identity of CGRP receptors. Structural analysis and antisense studies will allow determination of sites of interaction and assignment of functions to the emerging subset of accessory proteins that bind to and regulate the activity of GPCRs.

Signal Transduction

CGRP activates CGRP receptors, which stimulates adenylyl cyclase via $G_{\alpha s}$ increasing intracellular cAMP in a concentration-dependent manner. An increase in cAMP activates protein kinase-A (PKA), which then triggers multiple changes in the downstream signaling pathways. In some cases, the CGRP-induced increase in cAMP is followed by a membrane hyperpolarization associated with the activation of an adenosine triphosphate (ATP)-sensitive potassium channel. In blood vessels, CGRP increases nitric oxide (NO) production via two different pathways (Fig. 3): (1) stimulation of adenylyl cyclase followed by activation of PKA and subsequent phosphorylation of eNOS by PKB through a phosphatidylinositol 3-kinase (PI3K)-mediated mechanism or (2) induction of Ca^{2+} influx through voltage-gated calcium channels, an effect mediated by a cAMP-dependent PKA. Increased intracellular Ca^{2+} may stimulate eNOS and nNOS, leading to NO generation.

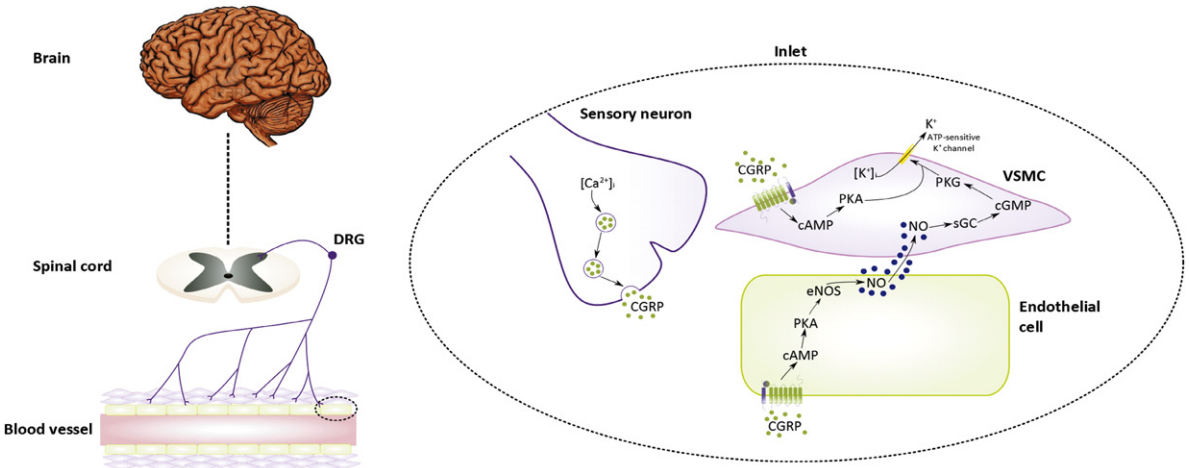

Fig. 3 A representative image of the role of CGRP in neurogenic vasodilation in peripheral and central nervous systems, which may play potential roles in migraine and other diseases, including inflammation and cardiovascular disease. Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CGRP, Calcitonin gene-related peptide; DRG, dorsal root ganglion; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial NOS; PKA, protein kinase A; PKG, protein kinase G; sGC, soluble guanylyl cyclase; VSMC, vascular smooth muscle cell.

Sometimes CGRP receptor activation does not stimulate adenylyl cyclase but instead activates PLC-β1 via pertussis toxin (PTX)-insensitive $G_{\alpha q/11}$ protein. This induces the formation of diacylglycerol, a direct activator of PKC, and inositol 1,4,5-triphosphate (IP_3), which binds to receptors on the ER and causes a transient release of Ca^{2+}. There is also solid evidence that CGRP, at high concentrations, can signal through G_i coupled to either calcium or potassium channels in various tissues. In summary, it appears that CGRP activates various signal transduction pathways via its receptor complex and the relevance of the accessory proteins, RAMPs and RCP in determining signal cascades triggered by CGRP receptor activation remains to be clarified.

CGRP Antagonists and Antibodies

Structure–activity characterization of various human α-CGRP (hα-CGRP) fragments demonstrated the potent antagonist properties of C-terminal fragments such as $CGRP_{8-37}$. Shorter C-terminal fragments, including $CGRP_{19-37}$ and $CGRP_{23-37}$, also displayed competitive antagonist properties although with much lower potency when compared with $CGRP_{8-37}$. The use of peptide-based receptor antagonists resulted in limited use *in vivo* and bioassays due to its short bioavailability, until the discovery of the first small molecule CGRP receptor antagonist, developed by SmithKline Beecham (SB-273779), with both binding K_i and cAMP bioassay IC_{50} of 0.31 μM. This finding was followed by the development of BIBN4096BS or olcegepant with $\sim$20 times higher potency compared to $CGRP_{8-37}$, exhibiting $>$200-fold higher affinity at human CGRP receptors (SK-N-MC cell line $K_i = 14.4 \pm 6.3$ pM) than at rat CGRP receptors (spleen $K_i = 3.4 \pm 0.5$ nM). However, as this antagonist is not orally active, subsequent compounds were identified. This included Merck's "Compound 2" and its optimization into telcagepant resulted in an antagonist with good oral bioavailability and K_i of 1.1 nM in CGRP receptors-expressing HEK293 cells, with similar pattern of higher potency at primates and human CGRP receptors compared to rodent tissues. Other compounds include Merck's MK-3207, with improved K_i at 0.024 nM, Boehringer Ingleheim's "Compound 1" with pK_i of 7.8, a weak antagonist for CGRP responses in humans cerebral and guinea pig basilar arteries, and Bristol-Myers Squibb (BMS) BMS-694153 that can be administered intranasally which may be suitable for treating acute migraine attacks.

Despite promising results during Phases I and II clinical trials of olcegepant and telcagepant for migraine, showing no cardiovascular side effects, long-term study in Phase III showed that the administration of telcagepant administered for 3 months as a prophylactic treatment resulted in elevated symptomatic transaminase level in 2 patients. In contrast, a longer-term Phase III study at 300 mg for intermittent, but repeated administration only reported 3 patients with transient and temporal increase in enzyme levels. Regardless, Merck discontinued the development of telcagepant and another compound MK-3207 in July 2011 due to liver toxicity. Currently, there are other novel CGRP receptor antagonists undergoing clinical trials, with little evidence of liver toxicity in the early phases, highlighting an exciting time for small molecule CGRP antagonist.

An alternative approach to inhibiting CGRP is the development of antibodies against CGRP or CRLR, shown to inhibit neurogenic vasodilation, lasting for 7 days without cardiovascular effects, suggesting long duration of treatment. Despite initial concerns regarding its inability to cross the blood–brain barrier, clinical trials have shown promising results in migraine patients. Currently, there are four companies (Amgen, Teva Pharmaceuticals, Eli Lilly & Co, Alder Pharmaceuticals) investigating monoclonal antibodies against CGRP, each in the process of or recently having completed their Phase II clinical trials, showing positive improvement in migraine symptoms of, at least, a third of individuals tested. Little or no adverse effects have been currently reported. The longer-term use of the CGRP antibodies on both episodic and chronic migraine and pain conditions is awaited.

CGRP Functions in Physiological and Pathological Conditions

CGRP in Migraine and Headache

Migraine is a complex neurovascular disorder, affecting 8% of men and 18% women, with 13% of sufferers experiencing more than one attack per week. While the mechanisms triggering this condition are debated, the role of CGRP in migraine headache is widely supported by both clinical and experimental evidence. It is thought that the activation of the trigeminovascular system, releasing neuropeptides, including CGRP resulted in the pain experienced by sufferers. The trigeminal ganglion consists of bipolar neurons that send fibers to the meninges, to the meningeal (dural) and cerebral (pial) blood vessels involved in the manifestation of head pain and (centrally) to the caudal brainstem and upper cervical spinal cord. Activation of the trigeminovascular system causes peripheral release of neuropeptides that induce meningeal blood vessel dilation, blood flow increase and plasma protein extravasation, in part by central reflex activation of the perivascular parasympathetic system via the superior salivatory nucleus. This inflammatory process is also thought to cause sensitization of nerve fibers and, consequently lowering of the nociceptive threshold. Additionally, intravenous administration of CGRP can induce migraine-like attacks in sufferers while plasma CGRP levels in patients suffering an attack was elevated and normalized with anti-migraine agents, such as triptans. However, not all patients show such prominent response in CGRP levels, which may suggest that CGRP can be used as a biomarker to identify migraine patients who will benefit from inhibiting CGRP (as described in the "antagonist and antibodies" section).

CGRP in the Central Nervous System

CGRP immunoreactivity is widely distributed in the central nervous system (CNS), suggesting its involvement in various brain functions. Anatomical and functional studies in animal models showed that CGRP is involved in sensory, cognitive and motor

activities. A comprehensive review of biological roles of CGRP in the CNS was published by Van Rossum *et al*. CGRP participates in sensory functions such as olfaction, vision, hearing, and taste. CGRP-immunoreactive fibers are localized in the glomerular, mitral, and plexiform cell layers of the olfactory bulb as well as in other areas of the olfactory system, including the accessory and anterior olfactory nuclei. Although no functional evidence is available for a role of CGRP in audition and vision, the presence of immunoreactive CGRP cells in auditory structures or in visual areas, such as superior colliculus and lateral geniculate nucleus, strongly suggests that CGRP may be involved in these functions. CGRP is also expressed in sensory nerve endings in taste buds and in their central projections terminating in the rostral part of the solitary tract nucleus. CGRP neurons have been found in the amygdala and in circuits that project to the amygdala. Behavioral studies indicate that these projections may play an important role in learning by regulating gustatory, nociceptive, and acoustic information during aversive conditioning. CGRP plays an essential role in synaptic plasticity, erasing the fear memory and facilitating long-term potentiation in the central nucleus of the amygdala in rats.

A potential role for CGRP in neurological disorders such as dementia and depression has been supported by anatomical, biochemical, and clinical data. CGRP receptors are often localized in dopamine (DA)-containing neurons. Direct administration of CGRP into the brain markedly affects DA release and metabolism in selected brain regions as well as learning and memory. On the other hand, both psychomimetics and antipsychotic drugs influence regional brain concentrations and release of CGRP *in vivo*. Altogether, these findings support the involvement of CGRP in DA-related disorders. In dementia patients, cerebrospinal fluid (CSF) levels of both CGRP-LI and CT-LI are lower, but their concentration ratio is similar to that of age-matched healthy controls. This may be explained by a general neuronal loss or, alternatively, by a down-regulation of the CT/α-CGRP gene expression. In depressed patients, only CT-LI appears to be diminished, resulting in an increase of the α-CGRP/CT ratio. This most likely reflects an altered splicing mechanism favoring the formation of α-CGRP mRNA. Although more studies are needed to draw definitive conclusions, combined measurements of CGRP-LI and CT-LI in CSF may be of diagnostic and perhaps prognostic value in dementia and affective CNS disorders, including depression.

CGRP in the Cardiovascular System

CGRP and its receptors are found throughout the cardiovascular system, specifically in the media, intima and endothelial layer of blood vessels. CGRP-containing nerves form perivascular networks around blood vessels and the heart. CGRP participates in various functions, including modulation of vascular tone, microvascular permeability, cell proliferation, apoptosis and inflammation. In healthy subjects, CGRP plasma levels can vary between 2 and 35 pmol L^{-1} in a gender-independent manner and it possessed a circadian rhythm, with higher amounts released during nocturnal periods. CGRP is established as a potent microvascular vasodilator, 10-fold higher than prostaglandins and 10–100 times greater than acetylcholine and substance P. Brain *et al.* (1985) showed that local administration of CGRP caused reddening, due to increased blood flow in the cutaneous vasculature. Picomole amounts of CGRP results in an erythema lasting for 5-6 h. Positive chronotropic and inotropic responses in the heart are observed after intravenous CGRP administration. However, the physiological significance of circulating CGRP remains unknown. Various studies have supported the role of CGRP in regulating peripheral vascular tone and regional organ blood flow in physiological conditions through either endothelium-dependent or endothelium-independent mechanisms. Of note, plasma levels of CGRP are raised in pregnancy. Despite these responses, CGRP is not involved in regulating baseline blood pressure in normal individuals. Antagonism of CGRP receptor or inhibition of CGRP using antibodies had no effect on baseline cardiovascular hemodynamics in various animal species and humans. Additionally, the α-CGRP specific KO mice showed no changes in their baseline blood pressure and blood flow. Nevertheless, the commonly used CT/αCGRP KO mice displayed a significantly higher mean blood pressure at baseline, compared to WT counterparts.

Changes in CGRP levels have been reported with various cardiovascular pathologies including hypertension, obesity and cardiogenic shock associated with sepsis. The role of CGRP in obesity has been studied, with evidence that α-CGRP KO mice are protected from diet-induced obesity. CGRP levels are slightly raised in obese female humans and pre-obese Zucker rats. In some cases, increases in CGRP levels may be a compensatory mechanism to counteract pathological vasoconstriction or increased plasma volume such as that observed in myocardial ischemia or pregnancy, respectively. Nevertheless, other studies reported reduced or unchanged levels of CGRP in the plasma of patients with essential hypertension compared to normotensive controls. This discrepancy may be due to variations in treatment, sampling methods or stage/types of hypertension. There is increasing evidence that CGRP can play a protective role in the cardiovascular system but its precise protective mechanisms remain unclear (see Russell *et al.*, 2014).

Recently, a study demonstrated that α-CGRP KO mice have enhanced hypertension following continuous infusion of AngII for up to 28 days in mice, associated with worsened vascular dysfunction, inflammation and oxidative stress. Moreover, deletion of α-CGRP caused enhanced cardiac and renal damage in DOCA-salt induced hypertension, and exacerbated cardiac dysfunction in pressure overload-induced heart failure. Indeed, acute systemic administration of CGRP is beneficial in experimental models of hypertension and patients with congestive heart failure. Further research into the therapeutic potential of CGRP has been limited due to its peptide nature and short half-life ($\sim$10 min in plasma). Other ways of modulating endogenous CGRP release to influence blood pressure regulation have relied on the activation of TRP channels such as TRPV1 and TRPA1 expressed on sensory neurons. Whilst deletion of TRPV1 and TRPA1 has no effects on baseline cardiovascular hemodynamics and limited role in AngII-induced hypertension, activation of TRPV1 using its agonist rutaecarpine protects against phenol-induced hypertension in rats,

associated with increased vascular and neuronal CGRP. Other ways include the development of long lasting CGRP agonist. Notably, there is presently one stabilized α-CGRP peptide analogue from Novo Nordisk, with a half-life of ~10 h (patent number WO 2011/051312). Nilsson *et al.* (2016) recently demonstrated that 2-week treatment with this analogue had potential anti-diabetic effects and, reduced both food intake and body weight in diet-induced obese rats and the Leptin deficient mouse model (ob/ob mice). This suggests that long acting CGRP analogues may have a therapeutic potential in treating type 2 diabetes through positive metabolic effects. The effects of such analogues in cardiovascular diseases remains to be elucidated.

Role of CGRP in Vascular Disorders Associated With Aging and Raynaud's Phenomenon

Wimalawansa *et al.* demonstrated age- and circadian rhythm-related changes of CGRP tissue content. With aging, CGRP levels decrease in neural cells and increase in both the thyroid gland and circulation. The relevance of these changes in deteriorating hemodynamics in the elderly is unclear. CGRP content also decreases in major arteries such as aorta, carotid, cerebral and coronary arteries, a phenomenon likely linked to vasomotor dysfunction and higher risk of ischemic cerebrovascular and cardiovascular events in the elderly. An age-dependent reduction in CGRP nerve function, including decreases in neuronal CGRP mRNA levels, neurogenic CGRP release and CGRPergic nerve-mediated vasodilation were observed in spontaneously hypertensive rats, without any change in expression in normotensive rats. Chronic blockade of the angiotensin system using temocapril and losartan protects against the reduction in perivascular sensory nerve innervation in a blood pressure-independent manner. Hence, hypertension can modulate CGRP-containing nerves and availability of endogenous CGRP as one ages.

Local cold exposure causes a transient vasoconstriction followed by a gradual increase of blood flow back to baseline level, often referred to as vasodilation. This is important to protect against local cold-induced vascular injury. This phenomenon was first described by Thomas Lewis in 1930 and involved both the sensory and sympathetic nerves. There is recent evidence from Aubdool *et al.* (2014) showing that cold activates TRPA1 in the vasculature and sensory nerve denervation or pharmacological blockade of the CGRP receptor using BIBN4096 can impair the cold-induced vasodilator response. This highlights the major participation of CGRP which is released downstream of TRPA1 activation by cold from the nerves. A lack of reflex vasodilation in response to local cold exposure is normally observed in patients with Raynaud's phenomenon. Small unmyelinated nerves containing CGRP are extensively present in digital skin, where they are involved in nociception and modulating vascular tone. There is a deficiency in digital perivascular CGRP-containing nerves in Raynaud's phenomenon. Intradermal or intravenous administration of CGRP increase peripheral cutaneous perfusion as measured by laser Doppler flowmetry and cutaneous temperature. However, the numbers of patients studied have been small and further studies are required to understand the therapeutic potentials of CGRP in the Raynaud's phenomenon.

CGRP in Cutaneous-Neuroimmune Interactions

Interest in the role of CGRP in the skin arose from initial studies showing that it is a highly potent microvascular vasodilator. Endogenous CGRP is released from the sensory afferent nerve terminals and causes neurogenic vasodilation. However, emerging evidence has shown that CGRP can be released by other cells, including keratinocytes and immune cells, highlighting their potential role in mediating acute and chronic cutaneous immunity. Acute application of nanomolar concentration of CGRP in response to topical application of various inflammatory mediators, such as histamine and leukotriene B_4 onto hamsters' cheek pouch resulted in reduced edema responses to these substances, suggesting an anti-inflammatory effect of CGRP. The anti-inflammatory potential of CGRP was further highlighted in models of delayed-type and contact hapten-induced hypersensitivity, where epicutaneous pre-treatment of CGRP resulted in dose-dependent reduction in hypersensitivity, which was locally mediated. In contrast, pro-inflammatory effects of CGRP have been reported, especially in the role of CGRP in priming dendritic cells and T-cells in driving a Th2 response, increasing cytokines production, hence proposing an important role in atopic dermatitis and other skin inflammatory conditions.

In addition to immunomodulatory responses in the skin, CGRP can enhance keratinocytes proliferation and epidermal thickness *in vitro*. In support for this, sensory nerve denervation in a spontaneous psoriasis model (K5-Tie2 model) improve the skin symptoms which were later blunted by systemic CGRP administration. This suggests that CGRP is an important neuropeptide in driving psoriatic skin phenotype. However, it remains unknown whether CGRP acts directly by driving keratinocyte proliferation or through pro-inflammatory response. Indeed, CGRP plasma levels are elevated in both psoriatic and atopic dermatitis patients, although this trend does not necessarily indicate causality. While most research has mainly focused on the role of α-CGRP in the skin-sensory nerve interactions, recent study has shown the importance of *β*-CGRP, the main isoform expressed in keratinocytes, in skin samples from chronic pain patients as well as animal models of pain. Hence, our knowledge on the role of CGRP in skin has improved from its role in neurogenic vasodilation, to more complex interactions between the sensory nerve terminal-keratinocyte-immune responses, with multiple involvement of CGRP at each step of the interaction. Understanding these pathways may lead us to a novel indication of targeting CGRP in various skin conditions.

CGRP in Arthritis

Arthritic patients are known to have increased levels of CGRP in their plasma and synovial fluid, with increased sensory innervation throughout the arthritic joints. Modulation of CGRP activity can affect important disease components in both rheumatoid

arthritis (RA) and osteoarthritis (OA). CGRP receptors are expressed in the joint afferents and upregulated following arthritis induction in animal models of arthritis. When CGRP are released in the periphery, it activates the synovial vascular cells causing acute vasodilation, followed by endothelial cell proliferation and angiogenesis leading to chronic inflammation. Local administration of the TRPV1 agonist capsaicin and surgical denervation on arthritic ankle joints in rats reduced both peripheral and DRG levels of CGRP, and reduced the development of joint inflammation. Treatment with the CGRP receptor antagonist $CGRP_{8-37}$ inhibits the proliferation of synovial cells, cytokines production and MMPs expression in RA, in addition to reduced peripheral sensitization and decreased CRLR mRNA levels in both the DRG and large joint afferent neurons in both RA and OA animals. A high affinity neutralizing antibody to CGRP, LY2951742 has recently been shown to be efficacious in pre-clinical *in vivo* models of OA pain and remains to be studied in OA patients.

Concluding Remarks

There is sufficient evidence to highlight the potential therapeutic benefit of drugs modulating abnormal CGRP functions in pathological conditions. In the past few years, considerable progress has been made in understanding the complexity of CGRP receptor structure and pharmacology. The development of synthetic, non-peptide CGRP receptor antagonists and antibodies, and their apparent benefit in migraine, implies a major role of CGRP in migraine. This is an exciting stage where we are able to understand the complexity of CGRP in a range of physiological and pathological pathways, which may highlight the potential for targeting CGRP therapeutically. As the use of the antagonists and antibodies increases, it will continue to be important to monitor adverse effects, considering the multiple biological activities of this peptides. Separately, there is a potential possibility that CGRP agonists may also have a protective role in condition involving the cardiovascular system, but this is presently still at a research stage.

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Epidemiology of Fractures

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Introduction

Bones are rigid structures that provide architectural support for the body to allow mobility and locomotion. At a tissue level, bones are an intricate combination of mineralized connective tissue, bone marrow, endosteum, periosteum, blood vessels, and nerves. The connective tissue is composed of an organic matrix (composed of 95% type 1 collagen and 5% noncollagenous protein), inorganic mineral content (calcium phosphate and hydroxyapatite), and cells. There are two types of bone, cortical and trabecular bone of which contribute to bone structure and strength. Cortical bone is tightly compacted, forms the outer rim of long bones, and accounts for 80% of the adult bone mass (Augat and Schorlemmer, 2006). Trabecular bone is porous and less regular, being composed of plates and rods, and has a high turnover due to a massive surface area. It accounts for the remaining 20% of adult bone mass. The strength of a material is determined by the spatial distribution and the material composition. The latter is a fine balance between the material's ability to resist excessive strain and adequate elasticity to allow it to absorb energy. Generally, bone is 60% mineralized, but if this mineralization increases, it can lead to increased stiffness and thus brittleness, which increases the risk of fracture.

Fractures, defined as a crack or break in the cortex of a bone, occur due to excessive force being applied to the bone. They are a major cause of morbidity and mortality throughout the life course, and the resultant financial burden placed on global health economies is huge. In 27 European countries, fractures are estimated to account for €32 billion of direct and associated costs. In youth and early adulthood, they are more commonly caused by significant trauma; however, as aging occurs, the proportion of fractures occurring due to bone fragility increases, until predomination.

This chapter will review the epidemiology of fractures as a whole before focusing on hip, vertebral, and distal forearm fracture. We will then examine the morbidity, mortality, and wider financial implications of fragility fractures and peruse secular trends and geographic variation.

Fracture Epidemiology

Fracture incidence has a bimodal distribution with peaks in youth and older adulthood (Curtis *et al.*, 2016; Moon *et al.*, 2016). The young tends to experience long-bone fractures, around puberty, due to significant trauma that are more common in males than females. However, after the age of 50, the fracture incidence increases rapidly in the female population leading to a 1:2 (male-to-female) ratio. This growth in fracture incidence is due to an increase in hip, distal radial, and vertebral fracture. Risk factors for fracture in adult life include obesity, physical inactivity, vitamin D insufficiency, and possibly dietary calcium intake, tobacco smoking, alcohol consumption, and use of exogenous estrogens.

The prevalence of fractures, divided according to anatomical location, was recorded in the European POSSIBLE study (Freemantle *et al.*, 2010). Nearly 2000 fractures were sampled with 30% wrist, 23% vertebral, 11% ankle, 9% forearm, 8% foot, 7% hip, 7% rib, and 7% upper arm fractures. The remainder is depicted in **Fig. 1**. Hip, wrist, and vertebral fractures account for nearly two-thirds of prevalent fractures and are common sites for fragility fractures.

In the year 2000, the incidence of fragility fractures was approximately 9 million, and it was therefore extrapolated that a fragility fracture occurs once every 3 s (Johnell and Kanis, 2006). Of these, 1.6 million were hip, 1.4 million were clinical vertebral, and 1.7 million were distal forearm fractures (Johnell and Kanis, 2006). Other sites included humerus, rib, tibia, pelvis, and nonhip femoral fractures.

Global variation in fracture incidence is thought to be due to factors including the effect of ethnicity on BMD, bone geometry and microarchitecture, diet, and behavior (Curtis *et al.*, 2016). Incidence in fracture increases with increasingly northerly latitude (Oden *et al.*, 2014; Wahl *et al.*, 2012) that suggests a possible role for vitamin D.

In the United Kingdom, about half of women and a fifth of men, over the age of 50, will experience a fragility fracture during their lifetime (van Staa *et al.*, 2001). These fractures therefore represent an enormous issue for health-care systems and governments.

Hip Fracture

Examining hip fractures as a whole, 80% occur in females, and the incidence of hip fractures increases exponentially with age. Over the age of 50 years, fractures are considerably more common in females with a 2:1 (female-to-male) incidence ratio (Curtis *et al.*, 2016). This higher rate of hip fracture in women is due to an increased incidence and longer life span (Gallagher *et al.*, 1980; Cooper *et al.*, 1992b). There is a preponderance for middle and older age, with 80% occurring in those >35 years. Of the 1.6 million hip fractures in the United Kingdom in 1990, 1.19 million occurred in females and 463,000 in males (Cooper *et al.*, 1992b).

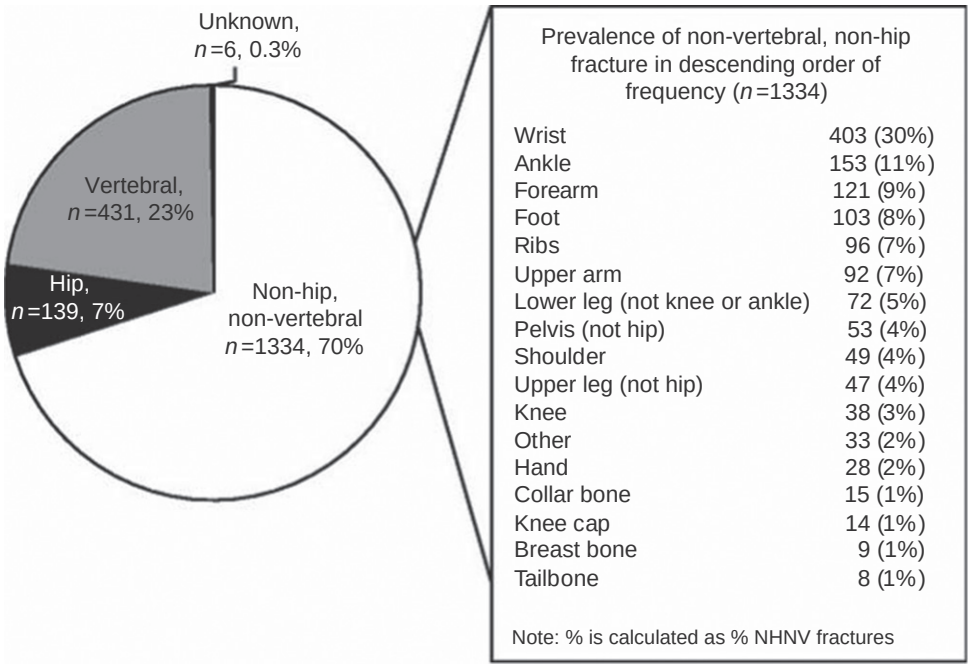

Fig. 1 Fracture frequency in clinical practice: result from the POSSIBLE study (Freemantle et al., 2010).

In terms of traumatic mechanisms, although, in youth, hip fractures are largely due to substantial trauma, the majority of hip fractures are generally the result of a fall from standing height (Blain *et al.*, 2016). The direction of fall is also important, with falls forward less commonly associated with hip fracture than falls sideways.

Seasonality also plays a significant role with a marked increase in winter months (in those countries with temperate climates). Many of these occur indoors and so may not be due to environmental hazards of increased precipitation or icy conditions but may be due to reduced efficacy of neuromuscular reflexes and a reduction in natural light during this season.

An American study investigating a wide range of ecological correlates of hip fracture found a significant relationship with latitude (hip fracture more common in the south), poverty level, rural land use, and fluoridated water and a negative relationship with water hardness and sunlight hours in January and nonsignificant relationships with activity level, cigarette smoking, alcohol consumption, Scandinavian heritage, and obesity (Jacobsen *et al.*, 1990).

Indeed, variation in the incidence of hip fracture is observed between populations of differing ethnicity and race both within nations (Curtis *et al.*, 2016) and internationally (Oden *et al.*, 2015). Across Europe, there is an 11-fold variation in the prevalence of hip fracture (depicted in **Fig. 2**) that is not explained by smoking, obesity, alcohol consumption, migration, and physical activity levels (Elffors *et al.*, 1994). Global variation in hip fracture is seen in **Fig. 3**.

Hip-fracture mortality is considerable, and the vast majority requires admission to hospital for surgical intervention. The potential, acute complications they may experience include surgical complications, anesthetic risks, postoperative infections, and pressure sores, leading to 8% of men and 3% of women dying during hospital admission for the condition (CotUS, 1993). In the United States, there are 31,000 excess deaths (of 300,000 fractures in the sample population) within a 6-month period post fracture. At 12 months, the postfracture survival is significantly less at 63.3% (vs. 90.0% expected) for females and 74.9% (vs. 91.1% predicted) for males (van Staa *et al.*, 2001). Hip-fracture patients continue to have an increased mortality for up to 10 years post fracture (Bliuc *et al.*, 2009). From these four time points, it can be observed that the risk of death from fracture peaks initially and then gradually reduces with time. Mortality is increased in those with poor prefracture functional status and comorbidities. Encouragingly, however, a US medicare database suggests that hip-fracture mortality rates are decreasing (Brauer *et al.*, 2009). This may be due to improved preoperative, operative, and postoperative care and better rehabilitation.

Hip fractures have a huge effect on independence, with 50% of patients who were walking prior to the fracture rendered nonambulatory afterward. This and other comorbid factors lead to 14% of 50–55 year olds and 55% of >90 year olds requiring nursing home placement after the fracture.

Vertebral Fracture

Vertebral fractures are the most common osteoporotic fractures globally affecting 30%–50% of people over the age of 50 years (Bouxsein and Genant, 2010). Establishing the precise epidemiology of vertebral fractures is complicated by two main factors. Firstly, between 65% and 75% are clinically silent (Kanis *et al.*, 2012; Fink *et al.*, 2005) and are picked up incidentally on

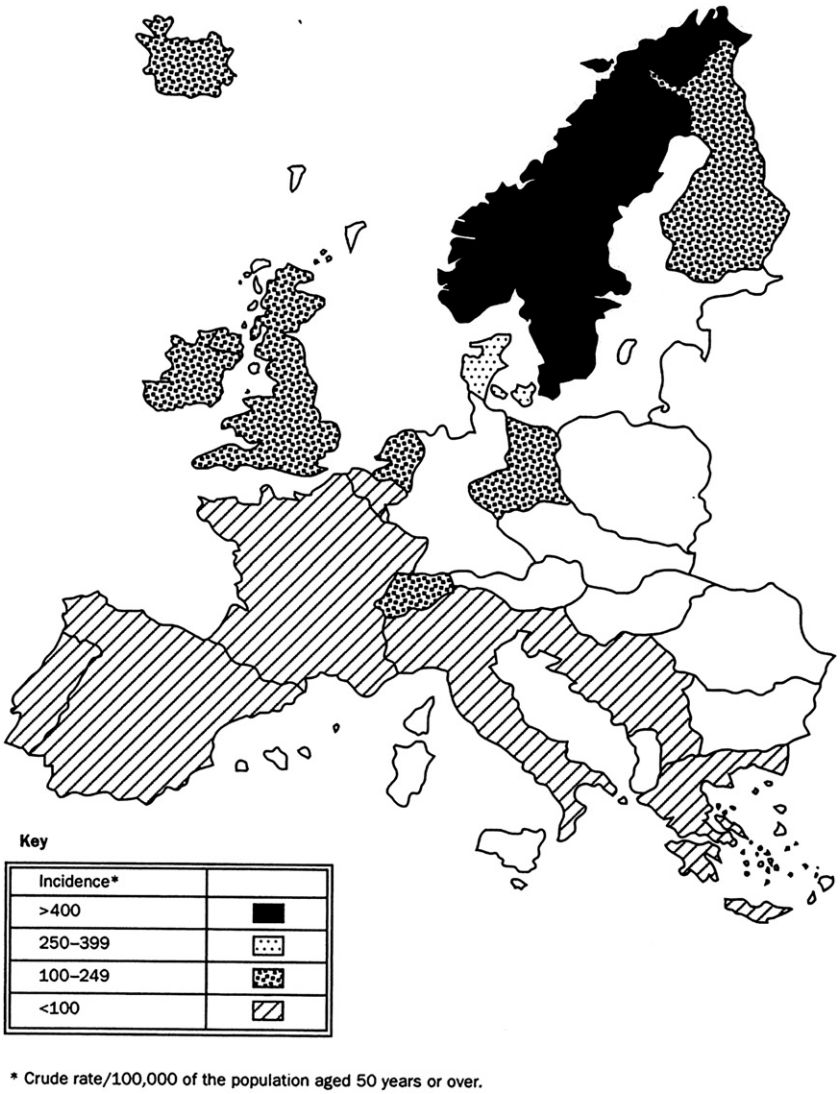

Fig. 2 The geographic variation in hip-fracture prevalence across Europe.

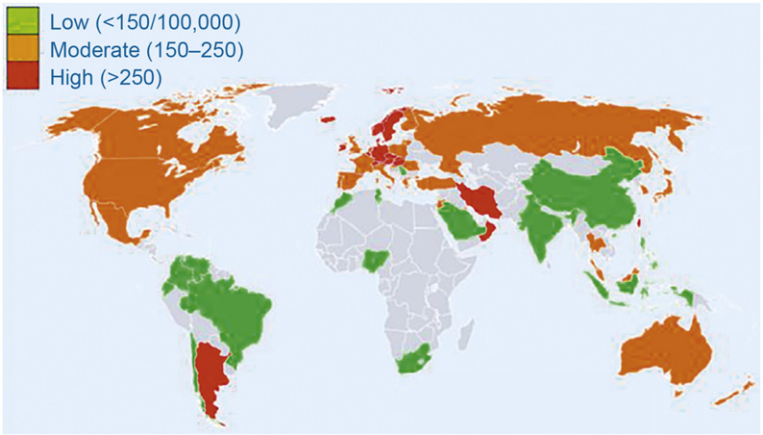

Fig. 3 The global variation in hip-fracture prevalence (Kanis et al., 2012).

radiography. Indeed, research shows that even on radiography, the presence of vertebral fractures is often not documented or even missed (Eastell *et al.*, 1991). Secondly, vertebral fractures can be defined clinically or morphometrically, with differing classifications of the latter being available. Morphometric analysis quantifies or semiquantifies the fracture according to measures of vertebral height, but there is variation concerning decision thresholds (O'Neill *et al.*, 1996).

The frequency of vertebral fractures divided according to vertebra affected is depicted in **Fig. 4**. As can be seen, the most commonly affected vertebrae are at T6–T8, the site of the most pronounced dorsal kyphosis (Cooper *et al.*, 1992a), and L1, at the thoracolumbar junction, the location of synthesis of the more rigid thoracic spine and the more mobile lumbar spine.

In terms of the etiology of vertebral fracture, males were found to have a higher prevalence than women aged 50–64, and it is postulated that this may be due to a higher incidence of trauma. Fractures in elderly women are commonly the result of normal activities, though they may be asymptomatic. Indeed, only 30%–40% of vertebral fractures come to medical attention (Cooper *et al.*, 1992a) as seen in **Fig. 5**.

The EVOS study, which is the largest study of vertebral fractures to date, included 15,000 individuals from 19 European countries. In this study, the age-standardized population prevalence was 12.2% in women and 12.0% in men (aged 50–79 years) (O'Neill *et al.*, 1996). There was marked variation across the continent with a threefold difference in the prevalence of vertebral fracture in different countries, the highest being in Scandinavia. This variation was partially explained by physical activity levels and measures of adiposity (O'Neill *et al.*, 1996). The CaMoS study, a five-year follow-up of over 9000 participants based in Canada, also found a similar prevalence of vertebral fractures between the sexes with a male-to-female prevalence ratio of 1:1 (Jackson *et al.*, 2000). The study of fractures (Kado *et al.*, 1999) and Rochester (Melton *et al.*, 1993) studies, based in the United States, were studies of females alone and found a prevalence rate of 20%–23% for vertebral fracture.

The age-standardized rates do vary by geography, with the highest (in studies combining hospitalized and ambulatory fractures of the vertebrae) seen in South Korea, the United States, and Hong Kong and the lowest rates in the United Kingdom (Ballane *et al.*, 2017). This variation can also be observed in the LAVOS study that found an overall prevalence of 14.8% across Latin America but a significant difference between the highest country rate in Mexico and the lowest in Puerto Rico (Clark *et al.*, 2009). Indeed, vertebral fracture prevalence is low in Latin America, with a study of osteoporotic women demonstrating a prevalence that was twice as low as that observed in EVOS (O'Neill *et al.*, 1996) and CaMoS (Jackson *et al.*, 2000).

In Asian studies, there is generally greater variation in prevalence rates across the region, with a 1.42 difference between the highest prevalence rates in Vietnam and the lowest rates in China (Ballane *et al.*, 2017). Heterogeneity was observed on an intranational and international basis. Interestingly, a pan-Asian study observed higher rates of vertebral fracture in men than women in Japan, Thailand, Indonesia, and Hong Kong at a female-to-male ratio of 0:6 (Kwok *et al.*, 2012). However, studies performed in individual countries have shown a preponderance for fractures in females with prevalence ratios of 1:24 in Korea (Shin *et al.*, 2012), 1:15 in Vietnam (Ho-Pham *et al.*, 2012), and 1:65 in Taiwan (Tsai *et al.*, 1996).

A recent study summarized the worldwide prevalence of osteoporotic vertebral fractures (Ballane *et al.*, 2017). The findings are summarized in **Table 1**.

The incidence of vertebral fractures in those aged 75–79 is 13.6 and 29.3 fractures per 100 persons per year for females and males, respectively (Felsenberg *et al.*, 2002). This does vary between studies, as demonstrated by the Rotterdam study of 3000 participants, followed up over 4–7 years, which found incidences of 1470 and 590 per 100,000 persons per year in women and men, respectively (Van der Klift *et al.*, 2002). The female-to-male ratio was 2:5, and interestingly, the presence of a fracture at baseline increased the incidence of vertebral fracture in women (26% vs. 5%) and men (9% vs. 3%).

Vertebral fractures are associated with an increased mortality that persists for >1 year post fracture (Bliuc *et al.*, 2009; Cooper *et al.*, 1993). The UK CPRD study observed a 12-month survival rate in women of 86.5% (vs. 93.6% expected) and 5-year survival of 56.5% (vs. 69.9% expected). Survival is reduced by the presence of comorbidities.

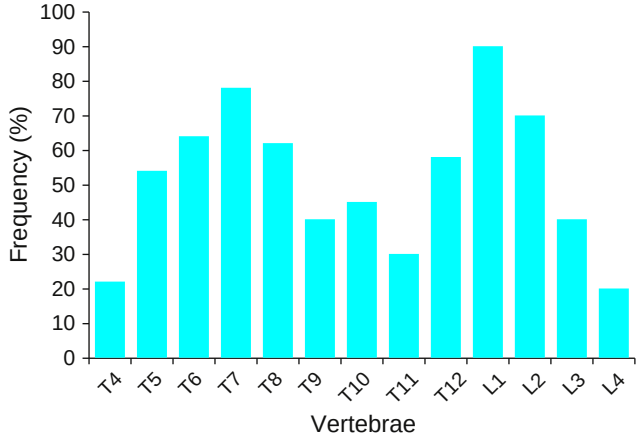

Fig. 4 Frequency of vertebral fractures according to vertebral level (Ismail et al., 1999).

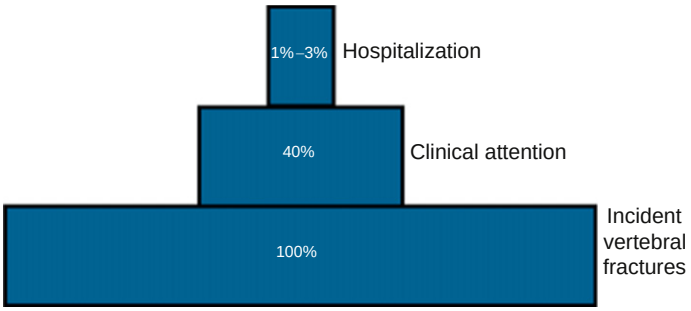

Fig. 5 The outcome of vertebral fractures (Cooper *et al.*, 1992a).

Table 1 Global prevalence rates of vertebral osteoporotic fracture (Ballane et al., 2017)

Geographic location	*Population demography*	*Prevalence rate (%)*
The Americas		
North America	White women ≥50 years	20–24
Latin America	Women ≥50 years	11–19
Asia		
Japan	≥65 years	24
Indonesia	≥65 years	9
Middle East		
Lebanon		20

Vertebral fractures lead to debilitating symptoms including back pain, kyphosis, and height loss that have significant effects on quality of life. Indeed, measures of quality of life reduce with increasing number of fractures.

Distal Forearm Fractures

Fragility fractures of the distal forearm are often the sequelae of a fall onto an outstretched arm. The incidence in men is relatively low and stable between the ages of 20 and 80 years. There is a marked preponderance for the female sex, with a larger sex prevalence ratio, of 1:4 (male to female) observed for distal radial fractures than for hip or vertebral fractures (van Staa *et al.*, 2001). Rates for those aged 50 and over are 39.7 and 8.9 per 10,000 persons per year for women and men, respectively (Curtis *et al.*, 2016).

Conversely to hip or vertebral fracture, there is no associated rise in mortality with wrist fractures, though increased morbidity does occur with only "fair to poor" function reported at 6 months after fracture (Chrischilles *et al.*, 1991).

Interfracture Relationships

Fragility fractures are thought to cluster with an increased risk of further fractures observed after the index fracture (Johnell *et al.*, 2004). A meta-analysis of 11 population-based cohort studies including 15,259 males and 44,902 females found that a history of prior fracture led to an 86% increase in the risk of a new fracture (Kanis *et al.*, 2004). Indeed, findings from the EVOS study of vertebral fracture found that vertebral fragility fracture was strongly predictive of incident hip fracture with a rate ratio of 2.8–4.5 (Ismail *et al.*, 2001). This risk increased with each new fracture.

Early Life

Early-life programming may affect an individual's predisposition to fracture. A low birthweight, which is taken as a surrogate measure for a poor quality of intrauterine environment, is associated with lower bone mass as an adult and subsequent increase in the risk of hip fracture (Harvey *et al.*, 2014; Cooper *et al.*, 2001; Javaid *et al.*, 2011; Baird *et al.*, 2011). There are also data to support a role for maternal vitamin D as the MAVIDOS trial demonstrated a benefit, in terms of childhood bone mass, if vitamin D supplementation was taken by the mothers of infants born in winter months (Cooper *et al.*, 2016). Certainly, peak bone mass is a significant contributor to the adult risk of osteoporosis (Hernandez *et al.*, 2003) and is likely determined by a genetic–environmental interaction that could be mediated by epigenetic factors.

Children

The epidemiology of fractures in children has been investigated in two studies, one based in Sweden and the other in the United Kingdom.

The Swedish study in Malmo investigated fracture in a pediatric population and found that the incidence of facture was 212 and 257 per 10,000 for females and males, respectively. Indeed, 27% of girls and 42% of boys had experienced a fracture by the age of 16 (Landin, 1983). Again, a dichotomy between the sexes is seen in this age group and is hypothesized to be due to a higher prevalence of trauma in males in this age group. The most common fractures were of the distal radius, followed by phalangeal fractures (Landin, 1983). Interestingly, a 10-year follow-up study found reduction in the incidence of fracture by 10% (Tiderius *et al.*, 1999).

The UK CPRD study of the incidence of pediatric fracture found 137 per 10,000 persons per year in total. In boys, fractures were more common occurring at 169 per 10,000 persons per year compared with girls at 103 per 10,000 persons per year. This extrapolates to 30% of UK boys having a fracture before their 18th birthday compared with 19% of UK girls. The most common fracture site, similar to the Malmo study, was the radius/ulna with 29.7 per 10,000 persons per year. There was also an ethnic discrepancy in the frequency of fractures, with a ratio of 1:2 black to white and South Asians at an intermediate risk as seen in **Fig. 6**.

Geographic variation in the rate of fracture is observed (**Fig. 7**) and is suspected to be due to concurrent variation in ethnicity and socioeconomic status (Moon *et al.*, 2016) The greatest fracture incidence was observed to be at the age of pubertal commencement, aged 14.5 years in males and 11.5 years in females (Kanis *et al.*, 2012). This may be due to this being the point at which there is the greatest discrepancy between vertical growth and gain in volumetric bone density (Walsh *et al.*, 2012; Holroyd *et al.*, 2016).

In terms of comorbid associations with fracture risk, childhood obesity increases the risk (Goulding *et al.*, 2000). Those children who fracture are more likely to have a lower bone mineral density than those who do not fracture, emphasizing the importance of this physiological variable in childhood and in old age. Physical activity, despite being associated with increased bone mass, is also associated with an increased risk of trauma and thus fracture (Clark *et al.*, 2008).

The Cost of Fragility Fractures

The cost of fragility fractures increases with increasing population size and thus increases with time.

In 1997, a conservative estimate for the costs of hip fracture alone was $131.5 billion worldwide by the year 2050 (Johnell, 1997).

In 2010, the cost of fragility fractures across 27 European Union countries was calculated to be €39 billion. Of this total, €26 billion were for the direct costs of treating the incident fracture, €11 billion for the cost of long-term care following fracture, and €2 billion for the cost of preventative strategies.

The cost of fracture varies by site with hip fracture accounting for 55% of costs, vertebral fractures 5%, wrist fractures 1%, and all other fractures 38%.

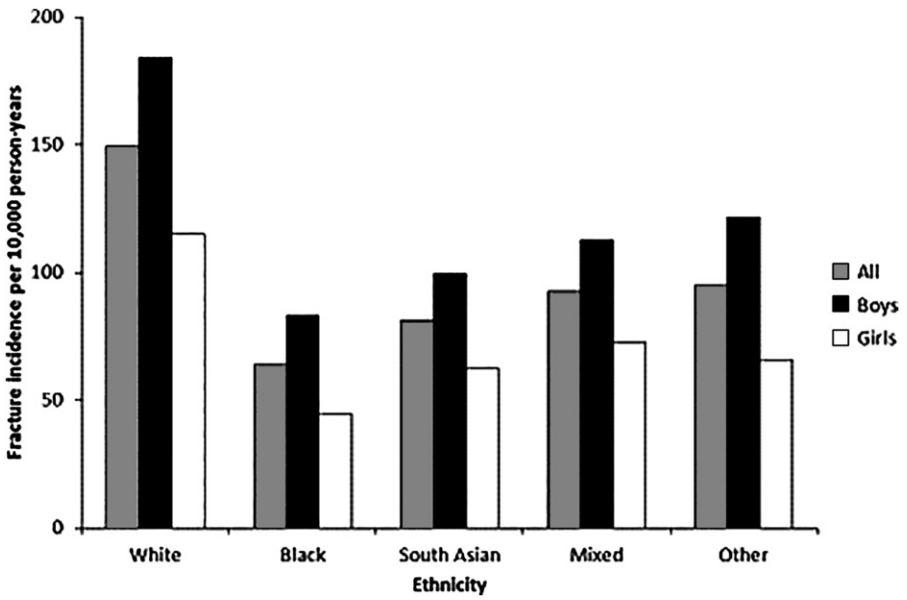

Fig. 6 Fracture incidence per 10,000 persons per year among UK children, 1988–2012, by ethnicity (Moon *et al.*, 2016).

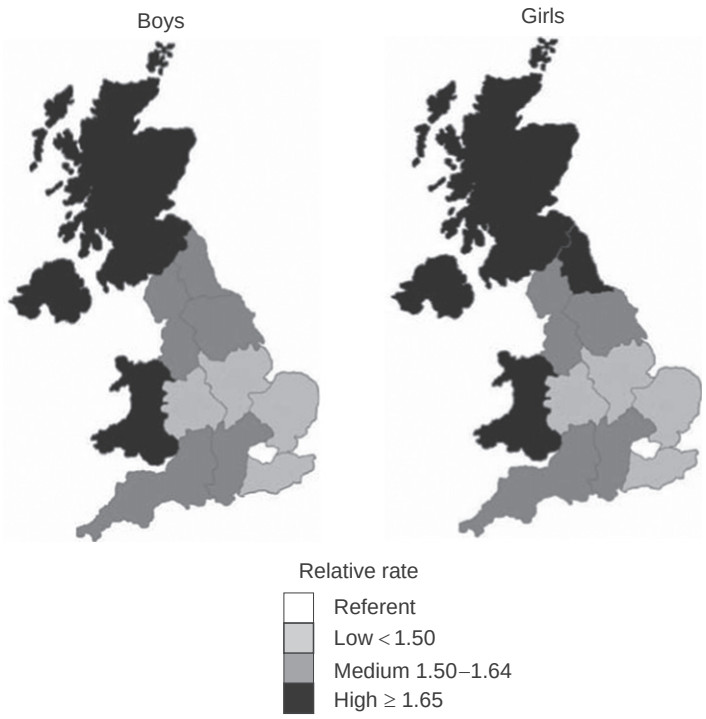

Fig. 7 The regional variation in pediatric fracture incidence across the United Kingdom (compared with Greater London) (Moon *et al.*, 2016).

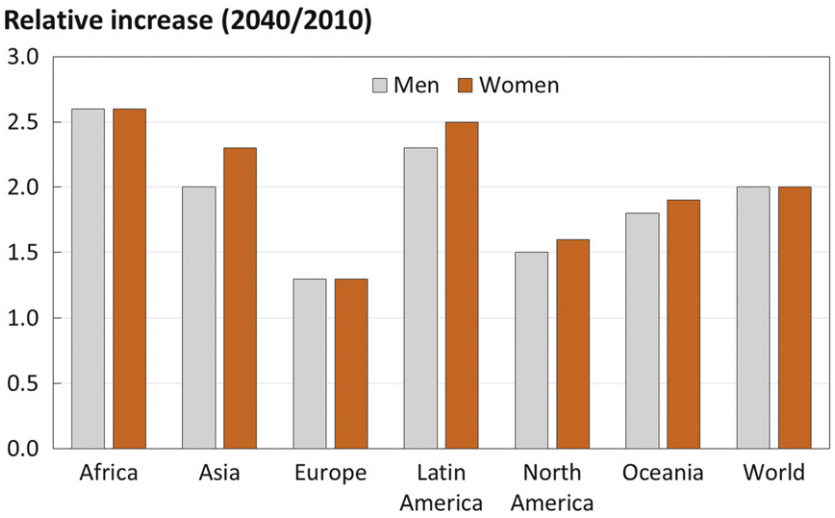

Fig. 8 Number of men and women at high fracture risk in 2040 relative to 2010, by continent (Oden *et al.*, 2015).

The Future

The number of fractures rose by 25% from the year 1990 to 2000 (Johnell and Kanis, 2006). Numbers are suspected to continue to rise, and the proportional increase in men is predicted to be higher than in women.

The total number of individuals currently aged 60 or greater is thought to be 901 million (equal to 12% of the global population). Europe has a higher proportion in this higher age bracket, likely due to increased life expectancy. However, by 2050, it is estimated that the majority of areas (except Africa) will have at least a quarter of their populations aged 60 or greater with a concurrent rise in the prevalence of hip fracture increasing form 1.66 million in 1990 to 6.26 million in 2050 (Gullberg *et al.*, 1997; Cooper *et al.*, 1992a).

Fracture risk will also increase with an estimated doubling of those at high risk of fracture aged greater or equal to 50 years by 2040 (see **Fig. 8**).

Interestingly, changes have been observed over time with regard to fracture site. From 1990 to 2012, there was no change seen in the rate of fracture; however, significant variation in the site of fracture occurred (van der Velde *et al.*, 2016).

Conclusion

As highlighted in this article, fragility fractures are common, often associated with increased mortality and manifold morbidity. They have an ever-rising financial cost, and therefore, the continued study and understanding of the epidemiology of fragility fractures are vital for coherent, rigorous, and effective health-care strategy.

See also: Sexual Function in Aging Men

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Pathogenesis of Osteoporosis

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Introduction

The strength of the skeleton and its ability to withstand trauma is determined by a multitude of diverse factors. Skeletal development and homeostasis is a complex and tightly regulated process, and any condition that disrupts normal bone physiology has the potential to cause osteoporosis and increase the risk of fragility fracture. This article reviews the numerous determinants of skeletal strength and highlights the various scenarios in which osteoporosis can develop.

Fractures occur when the skeleton is subjected to a force that exceeds its resilience. Such force often results from trauma, such as a fall. Fragility fractures are fractures that result from minimal trauma (i.e., a fall from ground level). Many individuals with osteoporosis are at increased risk of falling, as the result of contributors such as frailty, impaired balance, sensory dysfunction (including poor vision and neuropathy), polypharmacy, and hazards in the home environment. This article will address factors that affect the intrinsic ability of the skeleton to resist fracture. However, extrinsic factors such as the force resulting from a fall should not be overlooked as important causes of fracture.

Definitions

Osteoporosis is a condition characterized by low bone mass and decreased bone strength, resulting in an increased propensity for fracture. Bone mass refers to the "quantity" of the bone mineral and is dependent on both the highest bone density that an individual achieves in their lifetime (i.e., peak bone mass), as well as the rate and duration of bone loss after peak bone mass has been attained, which is greatly dependent on bone turnover. Bone quantity can be assessed using dual-energy X-ray absorptiometry (DXA). DXA is a widely available, relatively inexpensive, and noninvasive technique that provides a two-dimensional estimate of an individual's bone mineral density (BMD) in a specific region of interest, usually the spine or the hip. Low BMD is predictive of fracture risk and has accordingly become recognized as a surrogate for the presence of osteoporosis (World Health Organization Study Group, 1994).

However, BMD by DXA does not take into account bone "quality," which is thought to be another important determinant of skeletal strength (Kanis, 2002). Bone quality encompasses the material properties of the bone matrix and bone mineral, as well as bone micro- and macro-architecture. These parameters are more challenging to assess clinically than bone quantity. The widespread use of techniques to assess the quality of the bone, such as high resolution peripheral quantitative computed tomography (HR-pQCT) and bone histomorphometry, is limited by several factors, including poor availability, high cost, and, in the case of histomorphometry, invasiveness. Although bone quality is not routinely assessed in the clinical setting, it is important to recognize that BMD alone does not capture the full picture of skeletal strength, and that the condition of osteoporosis encompasses abnormalities of both bone quantity and bone quality. Determinants of bone strength are outlined in **Table 1**.

The terms "primary osteoporosis" or "age-related osteoporosis" can be used to describe the development of skeletal fragility in the context of aging, which is largely mediated by a decline in circulating sex hormone concentrations. A more specific term, "postmenopausal osteoporosis," refers to the presence of primary osteoporosis in older women, resulting from the estrogen deficiency that accompanies menopause. The term "secondary osteoporosis" denotes osteoporosis that is not explained by age and/or age-related sex hormone deficiency alone and is often the consequence of underlying illness or medication use. Conditions that are associated with reduced bone mass and increased skeletal fragility are set out in **Table 2**.

Table 1 Components of skeletal strength

Component	*Contributors*
Bone mass	Peak bone mass
	Rate and magnitude of bone loss (bone turnover)
Material properties	Organic phase (collagen volume, cross-linking, advanced glycation end products)
	Mineral phase (hydroxyapatite, mineralization)
	Microcracks
Micro-architecture	Trabecular thickness, connectivity
	Cortical porosity
Macro-architecture	Hip axis length
	Diameter

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Table 2 Causes of secondary osteoporosis

Category	*Risk factor*
Lifestyle factors	Smoking
	Excess alcohol consumption
	Low body weight, poor nutrition
	Immobilization
	Falls
Underlying disease states	
Endocrine	Hyperparathyroidism
	Untreated hyperthyroidism
	Hypogonadism
	Type 1 and type 2 diabetes mellitus
	Cushing syndrome
	Acromegaly
Malabsorptive	Inflammatory bowel disease
	Short gut syndrome
	Celiac disease
Hematologic	Multiple myeloma
	Mastocytosis
	Thalassemia
Rheumatologic	Rheumatoid arthritis
	Systemic lupus erythematosus
	Marfan syndrome
	Ehlers–Danlos syndrome
Other	Hyponatremia
	Chronic kidney disease
	Chronic liver disease
	Spinal cord injury
	Organ transplantation
	Human immunodeficiency virus
	Idiopathic hypercalciuria
	Ankylosing spondylitis
	Anorexia nervosa
Drugs	Aromatase inhibitors
	Gonadotropin-releasing hormone agonists
	Depot medroxyprogesterone
	Glucocorticoids
	Thiazolidinediones
	Antiepileptic drugs
	Selective serotonin reuptake inhibitors
	Proton pump inhibitors
	Heparin

Determinants of Skeletal Strength

Peak Bone Mass

Peak bone mass is generally reached by the mid-20s and influences bone mass, and thus an individual's propensity to fracture, later in life (Heaney *et al.*, 2000). Gains in bone mass throughout childhood and adolescence are achieved largely via the process of bone modeling. Modeling involves formation of new bone on the periosteal (outer) surface, along with removal of old bone on the endosteal (inner) surface (Seeman, 2003), resulting in large changes in bone size and shape. Genetic factors have a large influence on peak bone mass, and studies of twins and families have repeatedly demonstrated that $>50\%$ of inter-individual variance in peak bone mass is genetic (Ralston and Uitterlinden, 2010; Krall and Dawson-Hughes, 1993; Pocock *et al.*, 1987; Hernandez-De Sosa *et al.*, 2014). Emerging evidence suggests a role for epigenetics in skeletal development (Marini *et al.*, 2016; Earl *et al.*, 2010), and the degree of DNA methylation in the perinatal period has recently been shown to be predictive of bone

mass in early childhood (Curtis *et al.*, 2017). In addition to genetic factors, bone development throughout adolescence is responsive to circulating sex hormones. Animal studies suggest that periosteal apposition is inhibited by estrogen and stimulated by testosterone (Turner *et al.*, 1989). Accordingly, at the time of puberty, periosteal apposition is reduced in girls, and increased in boys (Seeman, 2003).

The developing skeleton is also influenced by mechanical loading (Ruff, 2003), and both body weight and physical activity have been highlighted as modifiable environmental factors that have a strong impact on peak bone mass (Rubin *et al.*, 1999; McGuigan *et al.*, 2002; McKay *et al.*, 2005; Sundberg *et al.*, 2002). Dietary calcium intake has mostly been found not to play a significant role in determining peak bone mass (Rubin *et al.*, 1999; McGuigan *et al.*, 2002; Lanou *et al.*, 2005). Chronic systemic illness in childhood or adolescence can lead to arrested skeletal development (Soyka *et al.*, 2000), and the presence of growth hormone deficiency (Giustina *et al.*, 2008), type 1 diabetes (Pan *et al.*, 2014), and amenorrhea (Rubin *et al.*, 1999) have all been associated with failure to achieve expected peak bone mass, as has the use of glucocorticoid medications (Avioli, 1993).

Bone Turnover

The skeleton remains active following achievement of peak bone mass, undergoing constant change to repair damage, preserve structure, and maintain adequate circulating concentrations of bone minerals (Parfitt, 1982; Calvo *et al.*, 1996). After skeletal maturity is reached, such changes are primarily achieved through the process of bone remodeling, rather than modeling. Remodeling involves the removal of old bone from an area of the bone surface and replacement with new bone at the same site. At any given time, approximately 20% of the trabecular bone surface is undergoing remodeling (Hill, 1998), with the majority of the skeleton being replaced over the period of a decade in older adults (Manolagas, 2000).

Remodeling results from the concerted actions of the cells that resorb old bone (osteoclasts) and those that form new bone (osteoblasts). Together, these two cell types are known as a "basic multicellular unit," and their remodeling activities are largely orchestrated by osteocyte cells, which reside within the bone matrix and send signals to the osteoclasts and osteoblasts in response to mechanical loading and humoral stimuli (Neve *et al.*, 2011; Martin and Sims, 2005). The processes of bone resorption and formation are usually tightly coupled, although resorption generally starts to exceed formation beginning in the early 30s, leading to net losses in bone mass from that time onwards (Drake *et al.*, 2015). Factors that worsen this imbalance, such as the rapid decrease in estrogen that occurs in women at the time of menopause, can result in profound bone loss as well as reductions in bone quality. Many of the causes of secondary osteoporosis presented in **Table 2** also contribute to uncoupling of bone resorption and formation (Calvo *et al.*, 1996).

Resorption

Bone resorption is carried out by osteoclasts. Osteoclasts and osteoclast precursor cells express the receptor activator of nuclear factor-kappa B (RANK). Simulation of RANK by its ligand (RANK ligand [RANKL]), which is secreted by both osteoblasts and osteocytes (Nakashima *et al.*, 2011), results in osteoclast differentiation and recruitment to the region of the bone surface where resorption is to take place (Eghbali-Fatourechi *et al.*, 2003). Osteoclast recruitment is also facilitated by macrophage colony stimulating factor (M-CSF) (Tanaka *et al.*, 1993). Mature osteoclasts adhere to the bone surface and secrete hydrochloric acid and acidic proteases such as cathepsin K, resulting in degradation of the bone matrix and liberation of calcium, phosphate, and fragments of type I collagen (Ross, 2013).

Formation

Following resorption of bone by osteoclasts, deposition of new bone is carried out by osteoblasts. These cells arise from the mesenchymal cell lineage, and a number of signal transduction cascades are involved in their differentiation (reviewed in De Gorter and Ten Dijke, 2013). Activation of the transcription factor Runx2 and induction of the canonical wingless (Wnt) pathway are two particularly critical steps in the osteoblast differentiation process. Runx2 determines the fate of osteoblast precursor cells, promoting differentiation of mesenchymal stem cells into osteoblasts, rather than cartilage or adipose cells (De Gorter and Ten Dijke, 2013). Conditions that impair Runx2 activity can predispose to osteoporosis by promoting the differentiation of mesenchymal stem cells into adipose cells rather than osteoblasts (Grey, 2008; Benvenuti *et al.*, 2007; Rzonca *et al.*, 2004; Akune *et al.*, 2004). Activation of the canonical Wnt pathway is also required for osteoblast differentiation (De Gorter and Ten Dijke, 2013). The proteins sclerostin and dickkopf-related protein 1 (DKK-1) are antagonists of the Wnt pathway, and conditions that increase their presence result in suppressed bone formation (Cosman *et al.*, 2016; Pinzone *et al.*, 2009).

Osteoblasts initiate the formation of new bone by secreting type I collagen and noncollagenous proteins, including osteocalcin, osteopontin, and bone sialoprotein, onto the bone surface (Neve *et al.*, 2011), with type I collagen creating a scaffold onto which calcium and phosphate can deposit. After osteoblasts complete their part in the bone formation process, some become "buried" in the bone and undergo further differentiation into osteocytes (Dallas *et al.*, 2013).

In addition to depositing new bone, mature osteoblasts are involved in the regulation of osteoclast activity. Osteoblasts secrete RANKL, as well as another important regulatory protein, osteoprotegrin (OPG). While RANKL stimulates osteoclast differentiation and recruitment and promotes bone resorption (Eghbali-Fatourechi *et al.*, 2003), OPG does the opposite. OPG acts as a "decoy receptor" for RANKL, preventing binding of RANKL to RANK and thereby inhibiting osteoclast activity (Boyle *et al.*, 2003). Factors that alter the ratio of circulating RANKL and OPG can therefore lead to the uncoupling of bone resorption and formation.

Expression of RANKL is upregulated by interlukin-1 (IL-1), tumor necrosis factor α (TNF- α), glucocorticoids and vitamin D (Hadjidakis and Androulakis, 2006; Ross, 2013), and downregulated by estrogen (Eghbali-Fatourechi *et al.*, 2003). OPG expression is upregulated by estrogen and transforming growth factor β (Hadjidakis and Androulakis, 2006). Parathyroid hormone (PTH) also has an important influence on the RANKL to OPG ratio. Sustained elevations in circulating PTH concentrations lead to increased production of RANKL and M-CSF, promoting bone resorption (Ross, 2013), while intermittent cyclical exposure to PTH results in the stimulation of bone formation at a greater rate than resorption and leading to net gains in bone density.

Regulation of remodeling

Osteocytes are integral to the regulation of bone remodeling (Dallas *et al.*, 2013). Osteocyte cell bodies sit within the bone matrix, each extending several dendritic processes to form a network that connects these cells to the vasculature, the bone surface, and one another (Dallas *et al.*, 2013). These cells constantly assess for changes in the condition of the skeleton and signal to osteoclasts and osteoblasts to ensure that bone remodeling is adjusted accordingly. Like osteoblasts, osteocytes express surface receptors for PTH and Wnt, and have the ability to secrete RANKL and OPG (Xiong *et al.*, 2011). Mechanical loading of osteocytes, such as by weight-bearing exercise, results in the inhibition of sclerostin and activation of the Wnt pathway, as well as downregulation of RANKL. This leads to osteoblast differentiation and osteoclast inhibition (Tatsumi *et al.*, 2007; Dallas *et al.*, 2013). Mechanical unloading results in the reverse: increased expression of sclerostin and RANKL (Dallas *et al.*, 2013; Xiong *et al.*, 2011). Osteocyte apoptosis also serves a regulatory purpose, promoting bone resorption via recruitment of osteoclasts (Gu *et al.*, 2005). Several stimuli can trigger osteocyte apoptosis, including local bone microdamage, oxygen deprivation due to immobilization, withdrawal of estrogen, and glucocorticoid exposure (Gu *et al.*, 2005).

Material Properties

All bone is made up of both cellular and extracellular components. The cellular constituent is comprised of the osteoclasts, osteoblasts, and osteocytes, as described above. The extracellular component of bone ensures that the skeleton is both strong and flexible, and can be categorized into organic and mineral phases. The organic phase is mostly comprised of type I collagen (90%) (Young, 2003), and also contains many noncollagenous proteins ($\sim$5%), lipids ($\sim$2%), and water. Type I collagen provides a structural blueprint for mineral deposition (Young, 2003). Aberrations in collagen production and cross-linking are associated with reduced skeletal strength and increased risk of fracture (Saito *et al.*, 2006b). Collagen can undergo post-translational modifications via the process of non-enzymatic glycation. States of low bone turnover can lead to the accumulation of advanced glycation end products through non-enzymatic glycation, and this has been shown to contribute to skeletal fragility in animal models (Tang *et al.*, 2009). Although the specific roles of noncollagenous proteins, lipids, and water within the organic phase are incompletely understood, noncollagenous proteins appear to be important for maintaining the structural integrity of type I collagen, and are also involved in cell signaling and mineralization (Boskey, 2013).

The mineral phase of the extracellular matrix makes up 60%–70% of the dry weight of the human skeleton and lends hardness and rigidity to bone (Boskey, 2007). This phase is comprised of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], which consists primarily of the minerals calcium and phosphorus (Boskey, 2007). Mineralization of bone is dependent on the presence of adequate amounts of calcium, phosphorus, and the enzyme alkaline phosphatase which breaks down the mineralization inhibitor, pyrophosphate (Neve *et al.*, 2011; Sapir-Koren and Livshits, 2014). Some osteoblast-secreted noncollagenous proteins, such as bone sialoprotein, have also been found to promote mineralization (Malaval *et al.*, 2008). In states of deficiency of one or more of these factors, under-mineralization of bone, known as osteomalacia, results. Prolonged exposure to drugs such as fluoride and high-dose etidronate can also interfere with the mineralization process (Jones and Sambrook, 1994).

Microdamage to the bone has been postulated as a potential mechanism by which skeletal fragility is increased. This term refers to small "microcracks" within the bone matrix, resulting from repeated loading of the skeleton (Seref-Ferlengez *et al.*, 2015). If not repaired via remodeling, these cracks have the potential to extend, leading to a reduced ability of the bone to withstand force (Seref-Ferlengez *et al.*, 2015). In animals, suppression of bone remodeling with alendronate has been shown to promote the development of microdamage (Allen *et al.*, 2006). In humans, the presence of microdamage appears to have little impact on the mechanical properties of the skeleton when compared to other parameters, such as trabecular volume (Follet *et al.*, 2011).

Micro-Architecture

Each bone in the body is composed of both a cortical and a trabecular compartment, and bone micro-architecture concerns the characteristics of each of these compartments. The cortical component forms the bone's hard outer layer. Cortical bone lends strength and stability to the skeleton, being relatively resistant to bending and torsion (Felsenberg and Boonen, 2005). This bone is laid down in a lamellar manner and has a smooth appearance. Inside the cortical shell sits a delicate network of trabecular bone, which has a spongy, cancellous appearance. The number, spacing, and thickness of the trabeculae are important determinants of skeletal strength (Felsenberg and Boonen, 2005), and trabecular bone is also the primary location for bone remodeling and mineral metabolism. The majority of bone turnover—around 70%—occurs at trabecular bone surfaces, although this type of bone only comprises 20%–30% of the skeleton. Cortical bone, on the other hand, comprises 70%–80% of the skeleton and accounts for only 30% of bone turnover (Felsenberg and Boonen, 2005). Different bones have differing trabecular and cortical compositions.

For instance, the vertebrae are >75% trabecular bone, whereas the neck of the femur is approximately 65% trabecular, and the proximal radius is >95% cortical (Riggs *et al.*, 2004).

Alterations in trabecular and cortical micro-architecture can be associated with increased skeletal fragility (Nishiyama *et al.*, 2013; McCloskey *et al.*, 2016). Age-related bone loss is associated with reductions in trabecular number, and loss of trabecular continuity, which are more frequently observed in patients with osteoporotic fractures than in controls (Parfitt *et al.*, 1983). States of high bone turnover contribute to thinning of the trabeculae, as activation of a greater number of basic multicellular units increases the likelihood that resorption lacunae will be in close proximity to one another or even directly opposite one another on the same trabeculae. This trabecular thinning represents an additional mechanism, beyond loss of overall bone mass, by which increased bone turnover might increase fracture risk (Garnero *et al.*, 1996; Rogers *et al.*, 2000). In conditions where bone resorption is increased, such as aging, loss of cortical bone is also observed, and eventually this lamellar bone develops a porous appearance (Zebaze *et al.*, 2010; Bala *et al.*, 2014). In the perimenopausal period, trabecular bone loss exceeds cortical bone loss. However, by 15 years after menopause, cortical remodeling exceeds trabecular remodeling, as a result of the development of cortical porosity or "trabecularization," which provides increased surface area for remodeling (Zebaze *et al.*, 2010). The development of cortical porosity is more profound in women than men (Macdonald *et al.*, 2011), and this parameter can be used to distinguish between postmenopausal women who have fractured and those who have not, independent of BMD by DXA (Bala *et al.*, 2014; Patsch *et al.*, 2013). Both diabetes and hyperparathyroidism have also been shown to promote the development of cortical porosity (Patsch *et al.*, 2013; Vu *et al.*, 2013).

Characteristics of cortical and trabecular bone can be assessed in detail with HR-pQCT, although this technique is not presently available for routine clinical use. While DXA does not permit detailed assessment of cortical and trabecular micro-architecture, it is possible to estimate trabecular bone quantity using DXA images from the lumbar spine, which is done via calculation of a "trabecular bone score." Trabecular bone score has been shown to independently predict fracture risk and was recently incorporated into the WHO FRAX fracture risk calculator (McCloskey *et al.*, 2016).

Macro-Architecture

Skeletal strength is also influenced by the macro-architecture, or geometry, of the bone. In particular, an evaluation of the Study of Osteoporotic Fractures cohort demonstrated that shorter hip axis length is associated with a lower risk of fracture (Faulkner *et al.*, 1993). Correspondingly, an increase in average hip axis length observed in New Zealand women between the 1950s and 1990s was found to correlate with increases in age-adjusted hip fracture rates throughout this timeframe (Reid *et al.*, 1994). Other macro-architectural parameters, such as outer diameter of the proximal femur and neck-shaft angle have been associated with fracture risk in some populations but not others (Broy *et al.*, 2015). Ahlborg and colleagues have demonstrated that periosteal apposition increases at the time of menopause, resulting in increases in the medullary and periosteal diameter of the radius (Ahlborg *et al.*, 2003). These geometric changes may help to offset the effects of rapid loss of bone mass in the early postmenopausal period.

Causes of Skeletal Fragility

Postmenopausal Osteoporosis

In most individuals, loss of bone mass begins in the early 30s, when the rate of resorption starts to exceed formation (Drake *et al.*, 2015). In women, bone loss accelerates around the time of menopause when ovarian estrogen production ceases, often exceeding 2%–5% per year in the perimenopausal period (Hannan *et al.*, 2000). This translates into losses of 20%–30% of bone density at trabecular sites and 5%–10% at cortical sites in the decade following menopause (Riggs *et al.*, 2004; Khosla and Riggs, 2005). Within 10 years of menopause, bone loss begins to slow again, continuing at a rate of 0.5%–1.0% throughout the remainder of the lifespan (Khosla and Riggs, 2005; Cummings, 1993). In addition to the losses in bone mass observed on DXA following menopause, postmenopausal osteoporosis is also characterized by alterations in bone micro-architecture, including reductions in trabecular number and thickness, and increasing cortical porosity (Zebaze *et al.*, 2010).

Postmenopausal osteoporosis primarily results from the estrogen deficiency that occurs at the time of menopause, which leads to the uncoupling of bone resorption and bone formation. Estrogen receptors are expressed by osteoclasts, osteoblasts, and osteocytes, and estrogen has been shown to exert effects on each of these cell types, primarily through the estrogen receptor α (Manolagas *et al.*, 2013). Estrogen suppresses the production of RANKL by osteoblasts (Eghbali-Fatourechi *et al.*, 2003) and upregulates OPG expression, in addition to suppressing the pro-osteoclast factors M-CSF, IL-1, IL-6, TNF-α, and prostaglandins (Drake *et al.*, 2015), and stimulating apoptosis of mature osteoclasts and osteoclast precursors (Drake *et al.*, 2015). The presence of estrogen also results in upregulation of growth factors such as insulin-like growth factor-1 and TGF-β in osteoblasts (Khosla and Riggs, 2005), differentiation of mesenchymal stem cells into osteoblasts rather than adipocytes, and suppression of osteoblast and osteocyte apoptosis (Khosla *et al.*, 2011). Estrogen may also play a role in osteocyte mechano-sensing. In a mouse model, knockout of the estrogen receptor α results in failure to evoke an anabolic bone response with mechanical loading (Lee *et al.*, 2003). Thus, the net effect of the estrogen deficiency that develops at the time of menopause is a rapid uncoupling of bone resorption and formation. This effect can be attenuated by the administration of exogenous estrogen (Cauley *et al.*, 2003, Eghbali-Fatourechi *et al.*, 2003).

Male Osteoporosis

Men ultimately lose less bone mass than women, as they do not undergo an accelerated period of loss as women do following menopause. However, older men experience gradual reductions in bone mass, at an average rate of 0.5%–1.0% per year (Drake *et al.*, 2015; Cummings, 1993). Although osteoporosis is related to a secondary cause in roughly half of men, the likelihood of straightforward age-related osteoporosis increases in men older than 65–70 years of age (Gennari and Bilezikian, 2013). Age-associated reductions in circulating sex hormone levels appear to impact upon the loss of bone mass and development of skeletal fragility in aging men. Over the course of the male lifespan, sex hormone binding globulin levels increase by >2-fold. This corresponds to decreases in the amounts of bioavailable testosterone and estrogen by approximately half (Khosla *et al.*, 1998). This reduction in estrogen concentrations is particularly important, as estrogen status appears to be more reflective of BMD than testosterone in men (Drake *et al.*, 2015), and correspondingly, estrogen replacement has been shown to have a more marked effect on reducing bone turnover than testosterone replacement in men who are deficient in both hormones (Falahati-Nini *et al.*, 2000).

Additional Causes of Age-Related Osteoporosis

The observation that both women and men begin to lose bone mass as early as the mid-30s (Drake *et al.*, 2015), in the absence of significant changes in endogenous estrogen and testosterone production, has led to the postulation that the development of age-associated osteoporosis cannot be attributed to sex hormones alone (Manolagas, 2010). Indeed, additional age-related factors have been shown to promote bone loss via mechanisms that appear to be at least partially independent of circulating sex hormones. Cellular senescence develops with aging as the result of multiple processes, including accumulation of oxidative stress, telomere shortening, and impairment of DNA repair mechanisms (Marie, 2014). Bone cell senescence is associated with reduced osteoblast and osteocyte differentiation, as well as alterations in the stiffness and quality of the bone matrix (Marie, 2014). One mechanism by which cellular senescence has been shown to affect the skeleton is through the increased production of mitochondrial reactive oxygen species, resulting in oxidative stress (Marie, 2014). The presence of oxidative stress attenuates Wnt signaling and promotes osteoblast and osteocyte apoptosis (Manolagas, 2010). A potential role for oxidative stress in the development of skeletal fragility is corroborated by the finding that genetic knockdown of NADPH oxidase 4, an enzyme involved in the production of reactive oxygen species, protected against bone loss in ovariectomized mice (Goettsch *et al.*, 2013). The effects of sex hormone deficiency and cellular senescence on the bone seem to be at least partially independent of one another, but existing evidence suggests that they are not mutually exclusive. For instance, the effects of oxidative stress on the skeleton appear to be augmented by estrogen deficiency (Almeida *et al.*, 2007), and can be at least partially attenuated via treatment with estrogen (Manolagas *et al.*, 2013; Almeida *et al.*, 2010).

Secondary Osteoporosis

Declines in bone quantity and alterations in bone quality are anticipated consequences of aging. However, changes that are more profound than expected for an individual's age and sex suggest the presence of a secondary cause. Although the majority of postmenopausal women with osteoporosis have primary disease, a secondary contributor may be identified in up to 50% of men and premenopausal women (Klibanski, 2001). Secondary causes of osteoporosis include lifestyle factors, underlying disease states, and the use of medications. As skeletal homeostasis is tightly intertwined with the regulation of other organ systems, it is not surprising that a diverse multitude of secondary causes exist. Here, the pathogenesis of some of the more common and well-characterized causes of secondary osteoporosis is reviewed.

Monogenic syndromes

While much of the hereditary contribution to bone mass and bone strength appears be polygenic (Xie *et al.*, 2015) or epigenetic (Marini *et al.*, 2016; Earl *et al.*, 2010), a few monogenic syndromes result in low bone mass and/or increased propensity to fracture. These include osteogenesis imperfecta, which most often results from mutations in the genes encoding one of the two alpha chains that form type 1 collagen (COL1A1 and COL1A2) (Marini *et al.*, 2007), although mutations in genes encoding proteins responsible for post-translational modifications of type 1 collagen can also lead to this disorder. While the severity of osteogenesis imperfecta is mutation-dependent, many individuals present with low bone mass and fractures in childhood (Ralston and Uitterlinden, 2010). Osteoporosis pseudoganglioma syndrome results from a loss-of-function mutation in LDL-receptor related protein 5 (LRP5), a receptor that is required for activation of the Wnt pathway (Gong *et al.*, 1996, 2001). Affected individuals have low bone mass and frequent fractures in childhood. Mutations in the gene encoding aromatase (Morishima *et al.*, 1995) (which is responsible for the conversion of androgens into estrogen in peripheral tissues) and of the estrogen receptor (Smith *et al.*, 1994), also result in the development of osteoporosis. These single gene disorders are rare, being present in a very small proportion of individuals with osteoporosis.

Lifestyle factors

Large observational studies have demonstrated that several lifestyle factors are associated with low bone mass and fracture risk, including smoking, alcohol use, low body weight, poor nutritional status, and immobility. Smoking and excess alcohol consumption (≥ 3 units per day) are two well-established fracture risk factors (Kanis *et al.*, 2007). Low body weight is also associated

with reduced BMD and fracture (Reid, 2010). In addition, nutrition has been highlighted as a determinant of fracture risk in observational studies, with both low protein intake (Langsetmo *et al.*, 2015) and low fruit and vegetable intake (Benetou *et al.*, 2016) being associated with increased risk (Bischoff-Ferrari *et al.*, 2011). Dietary calcium intake is not consistently related to bone density (Bischoff-Ferrari *et al.*, 2009; Tai *et al.*, 2015), bone loss (Reid *et al.*, 2015) or fracture risk (Bolland *et al.*, 2015). The mechanical loading of bone that results from weight-bearing physical activity has a stimulatory effect on osteocytes and promotes bone formation (Tatsumi *et al.*, 2007; Dallas *et al.*, 2013). Correspondingly, exercise has been associated with higher BMD and reduced likelihood of fracture (Farmer *et al.*, 1989; Gregg *et al.*, 1998), with immobility being associated with low bone mass and increased fracture risk (Holm and Hedricks, 1989).

Underlying diseases

Many diseases have been associated with the presence of osteoporosis and predisposition to fracture. Several of these conditions affect the skeleton via more than one mechanism, and in many cases the mechanism(s) remain incompletely elucidated. Some of these diseases are discussed in further detail below, grouped according to their principal effect on the bone. This topic has also recently been comprehensively reviewed elsewhere (Emkey and Epstein, 2014).

Diseases that result in excess bone resorption include those that lead to sex hormone deficiencies, as well as hyperparathyroidism, hyperthyroidism, inflammatory disorders, and some hematologic conditions. In keeping with the established effects of sex steroids on the skeleton, conditions that result in hypogonadism are associated with high bone turnover and net loss of bone mass. These include hypothalamic amenorrhea, hemochromatosis, Turner syndrome, and Klinefelter syndrome (Emkey and Epstein, 2014). In persons with hyperparathyroidism, sustained elevations in circulating PTH concentrations lead to increased production of RANKL and M-CSF, promoting bone resorption (Ross, 2013). Hyperparathyroidism preferentially affects areas rich in cortical bone, such as the distal radius (Khan and Bilezikian, 2000). In hyperthyroidism, activation of the thyroid hormone receptor by excess thyroid hormone (T3) results in high bone turnover; the increased incidence of osteoporosis in individuals with subclinical hyperthyroidism also suggests a direct effect of TSH on the bone (Emkey and Epstein, 2014). Pro-inflammatory conditions such as chronic obstructive pulmonary disease, rheumatoid arthritis and inflammatory bowel disease lead to upregulation of cytokines such as IL-6 and TNF-α, both of which promote bone resorption (Emkey and Epstein, 2014). Diseases that involve infiltration of the bone marrow, such as systemic mastocytosis and multiple myeloma, are also associated with increases in inflammatory mediators such as RANKL, M-CSF, and IL-6 at sites of bone marrow infiltration (Emkey and Epstein, 2014; Rossini *et al.*, 2014). In addition, thalassemia major has been associated with increased RANKL production and high bone turnover (Morabito *et al.*, 2007).

Conditions that result in impaired absorption of calcium and vitamin D from the digestive tract, such as inflammatory bowel disease and celiac disease have been associated with the development of secondary hyperparathyroidism (Lamb *et al.*, 2002; Keaveny *et al.*, 1996). These conditions also appear to contribute to bone loss and impaired skeletal strength via promotion of systemic inflammation and RANKL production (Fornari *et al.*, 1998; Moschen *et al.*, 2005). Malabsorptive bariatric surgery, particularly Roux-en-Y gastric bypass, has also been associated with the development of secondary hyperparathyroidism and a high bone turnover state (Switzer *et al.*, 2017).

Several conditions have been shown to reduce bone formation and lead to low bone turnover, including growth hormone deficiency, Cushing syndrome, and diabetes. Growth hormone has an anabolic effect on osteoblasts, and adult-onset growth hormone deficiency is characterized by low bone mass, primarily at cortical sites, and increased fracture risk (Emkey and Epstein, 2014). Excess endogenous glucocorticoid production, as in Cushing syndrome, is also associated with impaired osteoblast function (Emkey and Epstein, 2014). Individuals with diabetes are at increased risk of fracture (Sellmeyer *et al.*, 2016). Persons with type 1 diabetes have lower BMD than their disease-free counterparts, which may reflect failure to achieve peak bone mass (Pan *et al.*, 2014). HR-pQCT imaging of individuals with type 2 diabetes has demonstrated increased cortical porosity (Burghardt *et al.*, 2010; Farr *et al.*, 2014). Mounting evidence suggests that the skeletal abnormalities and increased risk of fracture observed in this population are the result of low turnover disease (Bouillon *et al.*, 1995). Animal studies have demonstrated that abnormal glucose uptake by osteoblasts results in reduced activity of the transcription factor Runx2 (Wei *et al.*, 2015), which is required to direct the differentiation of mesenchymal stem cells into osteoblasts rather than adipose and cartilage cells. In a rat model with insulin-dependent diabetes, expression of Runx2 was reduced, while expression of the Wnt pathway inhibitors DKK-1 and sclerostin was increased (Hie *et al.*, 2011), highlighting another mechanism by which this condition may impede bone formation. Beyond its effects on bone turnover, diabetes also appears to adversely affect the bone matrix. Prolonged hyperglycemia promotes the development of advanced glycation endproducts and alters collagen cross-linking in rats (Saito *et al.*, 2006a), which may explain why fracture risk in persons with diabetes is increased independently of BMD (Schwartz *et al.*, 2011).

Altered bone matrix has also been identified in other disease states, such as the connective tissue disorders Marfan syndrome and Ehlers–Danlos syndrome (Emkey and Epstein, 2014), and Paget's disease, which results in deposition of woven rather than lamellar bone at sites of disease activity (Ingram *et al.*, 1996).

Drugs

Several medications are associated with increased fracture risk. The mechanism(s) by which some of these agents, such as glucocorticoids, contribute to the development of skeletal fragility is well established. In other cases, the pathophysiology remains unclear. Some of the more widely used medications that impact upon bone density and fracture risk are briefly reviewed here. Drugs with adverse skeletal effects have also been extensively reviewed elsewhere (O'Sullivan and Grey, 2015).

Not surprisingly, medications that reduce circulating sex hormone levels also reduce bone density and increase fracture risk (Emkey and Epstein, 2014). These include aromatase inhibitors, which are used in the adjuvant treatment of hormone-receptor positive breast cancer, gonadotropin-releasing hormone agonists, most frequently used in the treatment of prostate cancer, and the injectable contraceptive agent depot medroxyprogesterone acetate (Emkey and Epstein, 2014).

Prolonged treatment with glucocorticoids is the most common iatrogenic cause of osteoporosis, and the principal effect of these agents on the skeleton is to impair bone formation (Reid, 2000). The use of these medications is associated with dose-dependent skeletal effects, including rapid declines in bone mass and an increased risk of fracture (Reid, 2000; Weinstein, 2011). Histomorphometric assessment has demonstrated reductions in trabecular bone mass of $>25\%$ over the first 6 months of glucocorticoid therapy (LoCascio *et al.*, 1990). An increased risk of fracture also becomes apparent within the first 3 months of therapy (Steinbuch *et al.*, 2004), and fracture incidence is higher than expected for the changes in BMD observed in individuals taking these medications (Weinstein, 2011). Glucocorticoids suppress osteoblast proliferation and stimulate osteoblast and osteocyte apoptosis (Reid, 2000). While glucocorticoid exposure also inhibits osteoclastogenesis, these agents extend the osteoclast lifespan and increase RANKL production while exerting a suppressive effect on OPG (Emkey and Epstein, 2014; Humphrey *et al.*, 2006), the net result being uncoupling of bone formation and resorption (Weinstein, 2011).

Treatment with thiazolidinedione (TZD) medications, used primarily for the treatment of type 2 diabetes, has been associated with a 1.5 to 2-fold increased risk of fracture (Dormandy *et al.*, 2009; Home *et al.*, 2009; Kahn *et al.*, 2008). The use of TZDs is associated with modest reductions in bone density that do not appear to be reversed with cessation of therapy (Billington *et al.*, 2015). These medications have inconsistent effects on bone turnover markers (Billington *et al.*, 2015). The mechanism(s) by which treatment with TZDs increase fracture risk are unclear at present, although preclinical data indicates that they might reduce osteoblast activity by promoting the differentiation of mesenchymal stem cells into adipose cells rather than osteoblasts (Grey, 2008; Benvenuti *et al.*, 2007; Rzonca *et al.*, 2004; Akune *et al.*, 2004).

The use of antiepileptic drugs has been associated with low bone density and increased fracture risk (Meier and Kraenzlin, 2011). In the case of anticonvulsants that induce the cytochrome P450 enzyme system in the liver (such as phenytoin, carbamazepine, primidone, and topiramate), the increased fracture risk has been attributed to increased conversion of 25-hydroxyvitamin D into inactive metabolites (Meier and Kraenzlin, 2011). This has the potential to lead to 1,25-hydroxyvitamin D deficiency and promote the development of secondary hyperparathyroidism (Meier and Kraenzlin, 2011). Accordingly, supplementation with 2000 or 4000 IU of vitamin D has been shown to stabilize BMD in adults taking longterm antiepileptics (Mikati *et al.*, 2006). The mechanism by which antiepileptic agents that do not induce cytochrome P450 impact the bone is unclear, although recent data suggest that some of the newer anticonvulsants (such as levetiracetam) may not have significant effects on the skeleton (Lee *et al.*, 2010).

Other commonly prescribed medications that have been associated with increased fracture risk include proton pump inhibitors and the selective serotonin reuptake inhibitor class of antidepressants (Bedimo *et al.*, 2012; Emkey and Epstein, 2014). The mechanisms by which these medications increase skeletal fragility remain poorly understood.

Summary

Skeletal strength and resilience to fracture is dependent upon both bone quantity and bone quality. Bone quantity is determined by an individual's peak bone mass and the subsequent rate and degree of bone loss, whereas bone quality reflects the micro- and macro-architecture of the bone, as well as the condition of the bone matrix. Conditions that influence these determinants of bone strength have the potential to promote the development of osteoporosis and increase the risk of fragility fracture. The most common cause of skeletal fragility is postmenopausal osteoporosis, which results primarily from rapid declines in endogenous estrogen production at the time of menopause. While men do not undergo a similar phase of accelerated bone loss, they do experience gradual age-related reductions in estrogen and testosterone concentrations, contributing to steady declines in bone mass with aging. Cellular senescence also contributes to the development of osteoporosis with advancing age. Beyond age-related contributors, several secondary causes of osteoporosis have been identified, although the pathogenic mechanisms remain unclear in many cases. Disease states and medications that result in hypogonadism or glucocorticoid excess are established causes of secondary osteoporosis, and their underlying pathophysiology is well understood. On the other hand, the presence of diabetes and the use of medications such as TZDs, proton pump inhibitors, and selective serotonin reuptake inhibitors represent emerging risk factors for osteoporosis and fracture for which a better understanding of pathogenesis is required.

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Role of Estrogens and Androgens in Osteoporosis

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Introduction

Osteoporosis is a skeletal disease characterized by compromised bone strength, which predisposes a person to an increased risk of fracture (NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy, 2001). Bone strength is determined not only by bone mineral density (BMD) but also by bone size and macroarchitecture (particularly cortical bone area, but also cortical thickness and trabecular bone volume), microarchitectural features such as cortical porosity, trabecular thickness and perforations, as well as bone material properties (Seeman and Delmas, 2006).

As early as the 1940s, estrogen deficiency was blamed for post-menopausal osteoporosis and androgen deficiency for male osteoporosis (Albright *et al.*, 1941). However, by the late 1990s it was realized that estrogens also played an important role in the pathophysiology of male osteoporosis, and estrogens were even implicated in bone loss in old age as well as age-related secondary hyperparathyroidism (Riggs *et al.*, 1998). More recently however, there is growing awareness that aging has an independent and overriding influence on bone loss and fractures in both genders. Indeed, there is an exponential increase in fracture risk in elderly of both genders (see section "Fractures") resulting not only from an increasing risk of falls (Riggs *et al.*, 2006) but also from distinctive mechanisms of age-related bone loss such as oxidative stress, cellular senescence, hypercortisolism etc. (Farr *et al.*, 2017; Khosla *et al.*, 2011a; Khosla *et al.*, 2011b; Manolagas, 2010; Ucer *et al.*, 2017).

The aim of this chapter is to provide an overview of how estrogens, androgens and their receptors influence skeletal development and maintenance. However, their effects are gender-specific (estrogens play a key role in both genders whereas androgens may provide some additional benefit in young men), time-specific (i.e. due to enhanced development of peak bone mass, prevention of bone loss later in life, or both), compartment-specific (i.e. different in cortical vs. trabecular bone; see **Table 1**) and dose-dependent (i.e. estrogen levels below certain thresholds are associated with bone loss, whereas supraphysiological sex steroid levels generally confer no added skeletal benefits). Thus, to understand the role of sex steroids, we will first outline the effects of age and gender on cortical and trabecular bone as well as fracture risk.

Gender Differences in Bone Development, Bone Loss and Fracture Risk

Fractures

Gender is a key clinical risk factor for fractures, second only to age. The lifetime risk of osteoporotic fractures after the age of 50 years is as high as 45%–55% in women, compared to 20%–25% in men in high-risk Caucasian populations (Ahmed *et al.*, 2009; Lippuner *et al.*, 2009; Nguyen *et al.*, 2007). Also for hip fractures, the male: female incidence ratio is about 1:2 and remarkably constant worldwide despite >10-fold geographic differences in fracture incidence (Kanis *et al.*, 2012). Still, this means that men account for about one-third of the 9 million osteoporotic fractures worldwide, with some gender differences according to the fracture site (men accounting for 30% of hip and 39% of spine fractures, only 25% of humerus and 20% of forearm fractures, but 54% at other sites e.g. ribs) (Barrett-Connor *et al.*, 2010; Johnell and Kanis, 2006).

Fracture incidence starts to increase around the age of menopause in women, and then further accelerates in parallel in both genders after the age of 70 years (**Fig. 1**) (Bergström *et al.*, 2008; Curtis *et al.*, 2016; Laurent *et al.*, 2013). The gender difference in fracture risk can be explained by musculoskeletal sexual dimorphism i.e. the fact that men develop greater bone and muscle mass during puberty and adulthood, and do not experience accelerated bone loss after midlife as post-menopausal women do.

Table 1 Summary of the time-, gender- and compartment-specific (cortical vs. trabecular bone) effects of estrogens and androgens

	Estrogens				*Androgens*			
	Cortical bone		Trabecular bone		Cortical bone		Trabecular bone	
	Women	Men	Women	Men	Women	Men	Women	Men
PBM	++	++	++	+	−	+	?	+
Midlife	−	−	−	−	−	−	−	−
Old age	++	++	++	++	−	−	−	−

++ = very important role, + = important role, ? = uncertain role, − = no established role.

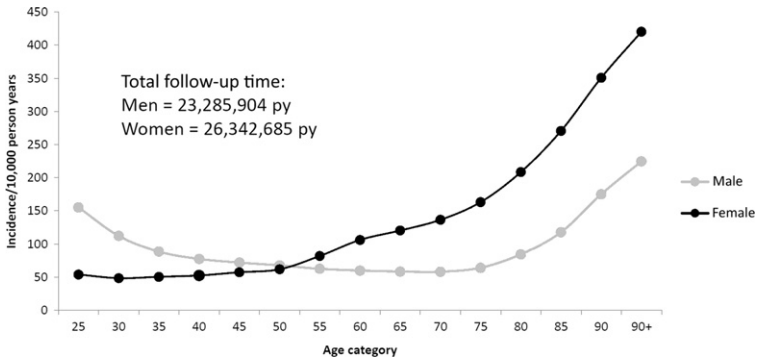

Fig. 1 Age- and sex-specific incidence rates of any fractures among adults in the United Kingdom, between 1988 and 2012, based on data from the Clinical Practice Research Datalink. Reproduced, with permission, from Curtis *et al.* (2016).

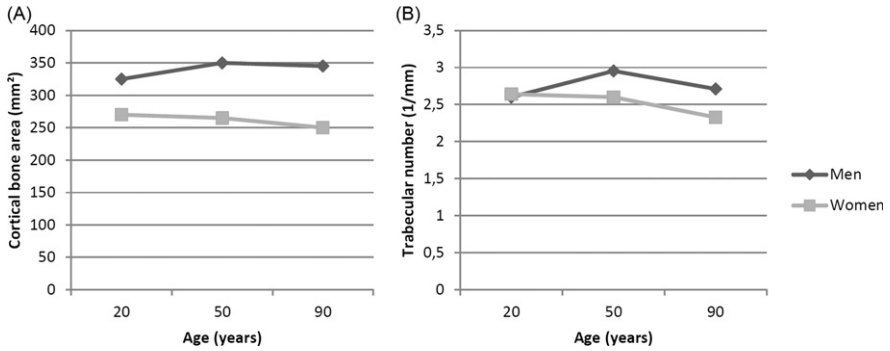

Fig. 2 (A) Cortical bone area at the tibia and (B) trabecular number at the distal radius, at the approximate ages of peak bone mass, middle age and in old age (20, 50 and 90 years, respectively), in men (dark gray) and women (light gray). Adapted and reproduced, with permission, from Khosla *et al.* (2006b) and Lauretani *et al.* (2008).

Cortical and Trabecular Bone Development and Maintenance

The structural development and maintenance of the skeleton can be divided into three phases: peak bone mass (PBM) acquisition, midlife, and old age. Men between 20 and 30 years have about 25% greater bone mass (whole body bone mineral content, BMC) (Boot *et al.*, 2010). However, this is not surprising given the fact that men are on average 8% taller than women (because dual-energy X-ray absorptiometry is a projectional technique and in 3D, $1.08^3 \approx 1.25$) (Almeida *et al.*, 2017). Gender differences in PBM are however not uniform, but time- and site-specific (e.g. PBM is reached earlier at the hip than at the spine). More importantly, these differences are explained almost entirely by the fact that young men develop *wider* cortical bone diameters, even after adjustment for height (Baxter-Jones *et al.*, 2011; Berger *et al.*, 2010; Henry *et al.*, 2004; Lorentzon *et al.*, 2005; Walsh *et al.*, 2009). Other determinants of bone strength also show sexual dimorphism (Almeida *et al.*, 2017) but the key determinant is bone width. The latter is determined by periosteal expansion and offers important biomechanical advantages, because bone strength scales to the fourth power with bone diameter. In fact, young men have similar cortical thickness as women, slightly lower cortical volumetric BMD, greater cortical porosity and lower trabecular bone volume in the spine, but these elements are outweighed entirely by greater cortical bone size (Berthold *et al.*, 2006; Burt *et al.*, 2016; Hansen *et al.*, 2014; Khosla *et al.*, 2006b; Kirmani *et al.*, 2009; Macdonald *et al.*, 2011; Nishiyama *et al.*, 2012; Riggs *et al.*, 2004).

Midlife may be a period during which men continue to consolidate their cortical bone assets. Trabecular bone volume declines early after PBM in both genders, but in men, some studies suggest that this is mainly due to loss of trabecular thickness (which is biomechanically less deleterious). Thus, both cortical bone cross-sectional area and trabecular number increase towards midlife in men, whereas they slightly decline in women (**Fig. 2**) (Khosla *et al.*, 2006b; Lauretani *et al.*, 2008; Riggs *et al.*, 2008). What drives these changes remains incompletely understood, but they occur independently of changes in sex steroids.

After menopause and in aging however, two pathophysiological alterations disturb the remodeling process (i.e. the resorption of bone by osteoclasts and bone formation at previously resorbed sites by osteoblasts). Collectively, the activities of these cells are coupled in basic multicellular units (BMUs). The first disturbance is an increased bone turnover with an increased number of BMUs. As a result, there are more osteoclasts resorbing pits at any given moment throughout the skeleton, even though osteoclast activity within each BMU is not more vigorous (Szulc and Seeman, 2009). Secondly, the osteoblasts in these BMUs fail to completely refill the resorption cavity. This leads to a small deficit in bone formation compared to resorption, which is amplified by the increased number of remodeling cycles. At the structural level, these two pathophysiological processes results in trabecular thinning and eventually

perforations and loss of trabecular number, widening of the medullary cavity with trabecularization of the cortex and increased cortical porosity. All of these structural features of skeletal decay are exaggerated in women compared to men (**Fig. 2**), in association with their greater bone turnover and lower estrogen levels after menopause (Khosla *et al.*, 2006a; Khosla *et al.*, 1998; Riggs *et al.*, 2006; Riggs *et al.*, 2004; Riggs *et al.*, 2008). Indeed, there is no "andropause" equivalent, and older men generally have higher serum estrogen concentrations than post-menopausal women. Periosteal expansion continues into old age and appears to represent a compensatory mechanism for bone loss from endosteal resorption (Pye *et al.*, 2017). This explains why periosteal apposition in old age may be even higher in women, nevertheless the net balance between endosteal resorption and periosteal expansion is more negative in women, leading to greater cortical thinning (Ahlborg *et al.*, 2013; Seeman, 2002; Zebaze *et al.*, 2010).

In conclusion, male gender is a protective factor that approximately halves the lifetime risk of osteoporotic fractures by both increasing PBM and decreasing subsequent bone loss. In the next section, we will discuss how sex steroids contribute to these gender differences.

Effects of Sex Steroids or Cortical and Trabecular Bone

Endogenous androgens and estrogens are C19 and C18 steroid hormone ligands of the androgen receptor (AR) and estrogen receptors (ERα and ERβ), respectively. Testosterone (T), the principal circulating androgen, can be converted by 5α-reductase enzymes to the more potent androgen dihydrotestosterone (DHT), and by the aromatase enzyme (CYP19A1) to 17β-estradiol (E2), the principal ligand of ERα. Thus, T has a dual mode of action, both androgenic as well as estrogenic.

Role of the Estrogen Receptors (ERα and ERβ)

Clinical evidence

Before bisphosphonates became available, estrogen replacement therapy together with calcium- and vitamin D-supplementation constituted the only therapeutic option for osteoporosis in post-menopausal women. Similarly, young men with hypogonadotropic hypogonadism are at increased risk for osteoporosis, which is reversible by T replacement therapy (Finkelstein *et al.*, 1987). Furthermore, surgical or pharmacological castration e.g. for breast or prostate cancer increases the risk of osteoporosis and fractures (Greenspan *et al.*, 2013; Hamilton *et al.*, 2010; Shahinian *et al.*, 2005). However, since hormone deficiency or deprivation results in both androgen as well as estrogen deficiency, the respective contributions of androgen- or estrogen-receptor signaling cannot be distinguished. Nevertheless, selective estrogen-receptor modulators (SERMs) are antiresorptive in men with low E2 levels (Uebelhart *et al.*, 2004), and prevent fractures in men receiving androgen deprivation therapy for prostate cancer, attesting to the importance of estrogen signaling even in men (Smith *et al.*, 2013). Also in male-to-female transsexuals, pharmacological E2 prevents bone loss, even despite androgen suppression (Lapauw *et al.*, 2008; Mueller *et al.*, 2011; Ruetsche *et al.*, 2005).

A more direct way of examining this issue is by animal or human models with genetic receptor or enzyme dysfunctions, discussed in the next paragraph. However, it is important to note that all of the effects in these knock-out animal models may be developmental, even though some phenotypes manifest at older age. Nevertheless, also after skeletal maturity, aromatase inhibition in adult animals or humans results in bone loss not only in women but also in men which cannot be compensated by AR actions alone (Falahati-Nini *et al.*, 2000; Finkelstein *et al.*, 2016; Leder and Finkelstein, 2005; Vanderschueren *et al.*, 2000). Finally, a large number of epidemiological studies (reviewed in Vanderschueren *et al.*, 2014) show that free or bioavailable E2 levels, are associated with bone loss and fracture risk in older men.

Genetic studies

Case reports of severe osteoporosis in men or women with aromatase or ERα deficiency attest to the importance of estrogens for bone health (Bilezikian *et al.*, 1998; Quaynor *et al.*, 2013; Smith *et al.*, 1994; Taes *et al.*, 2009). Notably, periosteal circumference was normal in an ERαKO man, supporting a role for the AR in periosteal bone formation (Smith *et al.*, 2008). Also in population studies, polymorphisms in the *ESR1* (ERα), *CYP19A1* (aromatase) or *COMT* (an estrogen-metabolizing enzyme) gene are associated with bone mass or fracture risk (Eriksson *et al.*, 2008; Gennari *et al.*, 2004; Limer *et al.*, 2009; Paternoster *et al.*, 2013; Van Pottelbergh *et al.*, 2003). In young rodent models, aromatase knock-out results in severely disturbed cortical and trabecular bone acquisition in both genders, despite increased T and IGF-1 levels (Matsumoto *et al.*, 2006; Öz *et al.*, 2001). In ERαKO mice, cortical bone development is impaired in both genders. Trabecular bone volume is also decreased by ERαKO in females but normal or even paradoxically increased in males, because of hypothalamic-pituitary feedback and high androgen levels signaling through the AR (Lindberg *et al.*, 2002; Sims *et al.*, 2003; Vidal *et al.*, 2000).

In female rodent models, ERβ seems to have a weaker opposite, inhibitory role on the stimulatory effects of ERα (Nicks *et al.*, 2016; Windahl *et al.*, 2001; Windahl *et al.*, 1999). However, any role for ERβ in males or in humans remains unconfirmed (Almeida *et al.*, 2017). G protein-coupled receptor 30 (GPR30) has also been proposed as an ER (and therefore renamed G protein-coupled ER 1 or GPER1), although this remains controversial. In any case, GPR30 appears redundant for the effects of estrogens on cortical and trabecular bone, although it may play a role at the growth plate (Vanderschueren *et al.*, 2014).

In summary, estrogens are indispensable for normal cortical and trabecular development as well as maintenance, not only in women but also in men (**Table 1**).

Target cells and genes

To identify whether the target cells for these effects of estrogens are osteoclasts, osteoblasts, osteocytes or other cell types, conditional KO models using Cre/LoxP technology have been used, in which ERs or AR can be deleted in specific cell types (Davey *et al.*, 2004). Based on such models, estrogens appear to stimulate cortical thickness through ERα in the osteoblast cell lineage in female mice, although only transiently in male mice (Almeida *et al.*, 2013; Määttä *et al.*, 2013a; Melville *et al.*, 2014). In female mice, it remains debated whether trabecular bone volume is maintained through actions of ERα in either osteoclasts (Almeida *et al.*, 2013; Martin-Millan *et al.*, 2010; Nakamura *et al.*, 2007; Seitz *et al.*, 2012) or osteoblasts and osteocytes (Kondoh *et al.*, 2014; Määttä *et al.*, 2013a; Melville *et al.*, 2014; Windahl *et al.*, 2013a). In male mice, the effect of estrogens on trabecular bone is not mediated via osteoclasts, but possibly via late osteoblasts or osteocytes (although not osteoblast precursor cells) (Määttä *et al.*, 2013a; Ucer *et al.*, 2015; Windahl *et al.*, 2013a).

The downstream actions of ERα can be mediated in four different ways. Firstly, translocation to the nucleus and regulation of target genes by direct binding to estrogen response elements (ERE), which are specific DNA sequences recognized by the ERα. This classical mode of action requires pioneer factors which modulate chromatin availability (such as GATA4; Miranda-Carboni *et al.*, 2011) and co-activators, of which SRC-1 is the best-known example. As a consequence, trabecular bone loss in SRC-1 knock-out mice is due to estrogen resistance in females and androgen resistance in males (Mödder *et al.*, 2004; Yamada *et al.*, 2004). Secondly, sex steroid receptors (both ERα and AR) may bind and influence (positively or negatively) the signaling of other transcription factors, e.g. NF-κB, AP-1 or RUNX2 (Baniwal *et al.*, 2009; Martin *et al.*, 2015; McCarthy *et al.*, 2003). Thirdly, both of the previous two (direct and indirect) genomic signaling mechanisms may be mediated by the unliganded ERα (but not the AR), and this ligand-independent effect can stimulate or repress gene expression (Almeida *et al.*, 2013; Rudnik *et al.*, 2008). Fourthly, both liganded ERα and AR may stimulate kinase-mediated extranuclear signaling at the plasma membrane, which is called non-genomic (or non-genotropic) signaling (Kousteni *et al.*, 2001; Kousteni *et al.*, 2002; Kousteni *et al.*, 2003). Knock-in mouse models in which the AR or ER are replaced by a mutant incapable of DNA binding demonstrate the physiological consequence of this mode of action, although these effects are modest and usually opposite to the classical genomic effects (Almeida *et al.*, 2010; Pang *et al.*, 2012; Syed *et al.*, 2011; Syed *et al.*, 2007; Syed *et al.*, 2005). The latter mode of action is involved in the ERα regulation of cortical bone mass and its regulation of oxidative stress in bone cells and their mechanoresponsiveness (see below) (Manolagas, 2010). An E2 dendrimer conjugate which selectively stimulates extranuclear estrogen signaling maintains protective effects on cortical bone without stimulating the uterus (Bartell *et al.*, 2013; Farman *et al.*, 2017), which supports further drug development selectively exploiting this pathway.

The direct target genes of ERα in osteoblasts, and even more so in osteoclasts or the target genes of AR in any target cell, remain poorly understood (Krum, 2011). Many target genes have been proposed, but few have been extensively validated (Almeida *et al.*, 2017; Vanderschueren *et al.*, 2014). Recent studies suggest that estrogens stimulate osteoblast expression and cleavage of Fas ligand to induce anti-resorptive effects via pre-osteoclast apoptosis, while other studies suggest involvement of SDF-1 (also known as Cxcl12) (Garcia *et al.*, 2013; Krum *et al.*, 2008; Ucer *et al.*, 2017; Wang *et al.*, 2015).

Rodent studies have suggested that female ERαKO mice also have an altered bone formation response to mechanical loading (Lee *et al.*, 2003). However, this seems to be a ligand-independent effect of ERα, while ERαKO in male mice, gonadectomy or ARKO has the opposite effect i.e. enhanced skeletal mechanoresponsiveness (Callewaert *et al.*, 2010a; Saxon *et al.*, 2012; Sinnesael *et al.*, 2015b; Windahl *et al.*, 2013b). Whether the altered mechanoresponsiveness in these models is explained by altered bone turnover (Vanderschueren *et al.*, 2014), altered background physical activity or whether these findings are generalizable to humans, remains unknown.

Collectively, these studies suggest that ERα actions are important for cortical and trabecular bone development in both genders, although more work is needed to fully elucidate the target cell(s) and molecular mechanisms of these effects.

Role of the Androgen Receptor (AR)

Rodent studies have demonstrated that androgens stimulate periosteal bone expansion in growing animals and have antiresorptive effects on trabecular bone through a direct effect of AR and independent of aromatization (Beck *et al.*, 2014; Movérare *et al.*, 2003; Venken *et al.*, 2006). In contrast to the strikingly osteopenic phenotypes resulting from selective estrogen deficiency, studies in androgen-insensitive or AR knock-out (ARKO) rodents reveal a milder cortical and trabecular bone phenotype (Callewaert *et al.*, 2009; Kawano *et al.*, 2003; MacLean *et al.*, 2008; Venken *et al.*, 2006; Yeh *et al.*, 2002), which is quite normal compared to female reference values. The same probably applies to XY women with androgen insensitivity syndrome, although less studies are available (Han *et al.*, 2008). Also in the general population, polymorphisms which negatively influence AR signaling such as long CAG repeats, have been not been negatively associated with male bone mass—in fact in some studies, a positive association due to hypothalamic-pituitary feedback and increased estrogen actions has been observed (Huhtaniemi *et al.*, 2009). In other words, androgens are responsible for the greater cortical bone expansion and trabecular PBM seen in young adult males compared to females, but they are not indispensable for normal cortical and trabecular bone development as estrogens are.

More recent cell-specific ARKO models have suggested that the effect of androgens on trabecular bone development is, at least partly, mediated through effects of the AR in osteoblasts and osteocytes. There is some evidence that the AR in osteoblasts enhances trabecular bone mass in female mice (Määttä *et al.*, 2013b), but whether or to what extent androgens promote bone mass in women (e.g. with hyperandrogenism due to polycystic ovary syndrome) remains unknown. Most likely, the effect of

androgens on trabecular bone involves reduced bone resorption, rather than an anabolic or increased bone formation effect (Chiang *et al.*, 2009; Notini *et al.*, 2007; Sinnesael *et al.*, 2013; Ucer *et al.*, 2015). The expression of the AR in osteoclasts as well as any direct contribution thereof to the antiresorptive effect of androgens remains questionable (Sinnesael *et al.*, 2015a; Ucer *et al.*, 2015). Remarkably, the key effect of androgens on periosteal bone expansion does not appear to be mediated by the AR in osteoblast-lineage cells either (Ucer *et al.*, 2015).

Hence, the quest to identify the—extraskeletal—target cell(s) responsible for the effect of androgens on cortical bone remains open. Although the AR exerts well-known anabolic actions on muscle, which may in turn positively influence bone mass, the effects of androgens on bone are certainly not entirely explained by muscle-bone interactions (Laurent *et al.*, 2016a; Laurent *et al.*, 2016c).

Similarly, it is known that rising serum T during early puberty in males indirectly regulate growth hormone and insulin-like growth factor 1 (IGF1) levels, which may partially (but not fully) explain the periosteal bone expansion induced by androgens in early puberty (Callewaert *et al.*, 2010b). The perinatal T surge on the other hand is a major regulator of pubertal growth hormone secretion and ultimate height, but it appears redundant for the inhibition of trabecular bone resorption and periosteal expansion induced by androgens (Sims *et al.*, 2006). Finally, there is some evidence that low IGF1 may contribute to fracture risk as well as frailty in older men (Boonen *et al.*, 2002; Ohlsson *et al.*, 2011). Still, the role of the somatotropic axis, by itself as well as its contribution to the effects of sex steroids in male or female osteoporosis, remain poorly understood.

Whether inducible AR deletion at later age has any direct detrimental skeletal effects in skeletally mature mice, remains yet unanswered. This is an important topic for future research because a direct role of androgens in the development of osteoporosis in older men has not been confirmed (Finkelstein *et al.*, 2016; Idan *et al.*, 2010; Laurent *et al.*, 2015). However, some epidemiological studies suggest that the combination of low serum E2 and T may be associated with higher fracture risk than low E2 alone (Amin *et al.*, 2006; Woo *et al.*, 2012). Although androgens may have a minor independent effect on bone turnover markers (Khosla, 2015), on risk of falls and fractures independent of BMD (Khosla, 2010; Meier *et al.*, 2008; Orwoll *et al.*, 2006), and on trabecular bone in the spine of mice treated with strong AR antagonists (Wu *et al.*, 2016), clearly more research is needed.

In summary, the beneficial skeletal effects of androgens mainly arise during puberty, by establishing greater trabecular PBM and greater radial bone expansion in young men. On the other hand, an independent effect of androgens in older men remains hitherto unconfirmed (**Table 1**). The development of osteoporosis in older men is mainly associated with estrogen deficiency, especially when serum T concentrations fall below 200 ng/dL and consequently, E2 serum levels below 10 pg/mL (Finkelstein *et al.*, 2016).

Other Players in the Gonadotropic Axis: SHBG and FSH

T, DHT and E2 are bound with high affinity to sex hormone-binding globulin (SHBG) and non-specifically to bulk carrier proteins such as albumin. Consequently, only about 1%–5% of these active sex steroids circulate freely i.e. not bound to proteins. According to the free hormone hypothesis however, this small percentage constitutes the active sex steroid because only non-protein-bound hormones are proposed to diffuse across cell membranes to active their nuclear receptors. Calculated or measured free T or E2 concentrations are often used in clinical practice as well as in studies related to osteoporosis, although many fundamental and practical concerns surround this approach (Laurent and Vanderschueren, 2014). Nevertheless, numerous studies show that higher SHBG levels are associated with greater BMD and cortical bone size in young men, but higher bone loss and fracture risk in older men and post-menopausal women (Cawthon *et al.*, 2016; Eriksson *et al.*, 2006; Hsu *et al.*, 2015; Hsu *et al.*, 2016; Lee *et al.*, 2008; Mellström *et al.*, 2008; Nielson *et al.*, 2017; Vanbillemont *et al.*, 2010; Vandenput *et al.*, 2016; Vanderschueren *et al.*, 2014). This may be explained by the fact that SHBG is sensitive metabolic marker rather than by its effect on free sex steroids, given the lack of a bone phenotype in SHBG-transgenic mice (Laurent *et al.*, 2016b).

Finally, there are experimental animal studies suggesting that increased follicle-stimulating hormone (FSH) levels per se contribute to (peri-)menopausal bone loss, although this remains controversial and evidence in humans remains limited (Allan *et al.*, 2010; Sun *et al.*, 2006; Zhu *et al.*, 2012).

Role of Estrogens and Androgens in the Diagnosis and Treatment of Osteoporosis

Role of Sex Steroids in Osteoporosis Work-Up

The role of sex steroids in the diagnosis of osteoporosis is generally limited. Osteoporosis is most commonly a disorder of post-menopausal women. Although all post-menopausal women have low E2 concentrations, those with the lowest serum E2 levels have the lowest cortical and trabecular volumetric BMD and the highest fracture risk (Khosla *et al.*, 2005; Lee *et al.*, 2008). However, there is no clinical utility of E2 or SHBG measurements in post-menopausal women, neither for fracture risk assessment nor to predict benefits of osteoporosis therapies, even in hormone-replacement therapy (Crandall *et al.*, 2015).

In contrast to post-menopausal women, most aging men retain relatively normal serum T concentrations, although free or bioavailable T concentrations decline more substantially because SHBG increases in older men (Bhasin *et al.*, 2011; Jardi *et al.*, 2018). Although low E2 and high SHBG (particularly at a threshold <16 pg/mL total E2 or <12 pg/mL of bioavailable E2 (Amin *et al.*, 2000; LeBlanc *et al.*, 2009; Mellström *et al.*, 2008; Woo *et al.*, 2012)) are associated with greater fracture risk in older men, the

clinical utility of measuring these sex steroids in older men is limited (Orwoll *et al.*, 2017). Still, current guidelines recommend measuring serum T concentrations in the work-up for male osteoporosis (Watts *et al.*, 2012). This may be useful in younger men with unexplained "idiopathic" osteoporosis, while it may have little usefulness in a geriatric population.

Sex Steroid Receptors as Treatment Targets in Osteoporosis

Estrogen replacement therapy

Since the Women's Health Initiative study, estrogen-replacement therapy (colloquially known as hormone replacement therapy, HRT) is no longer broadly recommended for the prevention of chronic diseases including osteoporosis in post-menopausal women, due to an increased risk of breast cancer, dementia, gallbladder disease, stroke, deep vein thrombosis (DVT), pulmonary embolism and urinary incontinence (Manson *et al.*, 2013). However, HRT is still a first-line treatment option for vasomotor symptoms. Excess adverse events were mainly observed with combination HRT with conjugated equine estrogen and medroxyprogesterone acetate (CEE + MPA). CEE alone still increased the risk of stroke, DVT, gallbladder disease and urinary incontinence relative to placebo, although mainly in older women. In women aged 50–59 years however, the risk/benefit ratio was favorable (Manson *et al.*, 2013).

Both CEE and CEE + MPA significantly prevented hip fractures (− 33% for both groups), vertebral fractures (− 36% and − 32% respectively) and total fractures (− 28% and − 24% respectively) (Manson *et al.*, 2013). In general however, HRT is not recommended for fracture prevention per se, given the availability of more effective treatments with less side-effects, such as bisphosphonates (Recker *et al.*, 2007). Nevertheless, in women with low or moderate fracture risk who use HRT for vasomotor symptoms, another antiresorptive therapy may not be required as long as HRT is continued. However, post-menopausal women at high risk for fractures should be treated with osteoporosis drugs such as bisphosphonates (Almeida *et al.*, 2017).

Selective estrogen receptor modulators (SERMs)

SERMs such as tamoxifen, raloxifene, bazedoxifene and lasofoxifene bind to the ERα ligand binding pocket and are thus estrogen antagonists in the presence of E2 and partial agonists in the absence of estrogens. Thus, they induce bone loss in premenopausal women but prevent bone loss in post-menopausal women. Notably, SERMs have tissue-specific effects: they have beneficial agonistic effects on bone and desirable antagonistic effects on breast tissue, but unwanted antagonistic vasomotor effects and adverse agonistic effects on the liver (promoting venous thromboembolism) and (in case of tamoxifen only) on the uterus. This tissue-specificity is explained by tissue-specific differences in recruitment of ERα co-activators and co-repressors (Shang and Brown, 2002). Full agonism of ERα requires two co-activator interaction surfaces, called activation function (AF)-1 and 2. The bulky side-chain of SERMs hinders approximation of helix 12 and recruitment of co-activators to AF-2, which is situated in the ligand-binding domain (Almeida *et al.*, 2017). Tissue-specificity is further determined by AF-1, which is situated on the ERα aminoterminal domain. Mice lacking ER AF-1 display a normal response to E2 in the liver and almost full responsiveness in cortical bone, but no response in trabecular bone and only weak effects on the uterus (Börjesson *et al.*, 2011). All available SERMs, as well as non-nuclear ER agonists such as estrogen dendrimer conjugate require a functional AF-1 (Börjesson *et al.*, 2013; Börjesson *et al.*, 2016; Farman *et al.*, 2017). Interestingly, ICI 182,780 (a potent ERα antagonist) works as an agonist on trabecular bone and on the uterus of ovariectomized ERα AF-2 null mice (Moverare-Skrtic *et al.*, 2014). Further work is needed to develop compounds that selectively exploit beneficial ERα effects on bone without unwanted effects on other organs.

Randomized trials have demonstrated that SERMs prevent vertebral fractures (Cummings *et al.*, 2010; Ettinger *et al.*, 1999; Silverman *et al.*, 2008). Lasofoxifene additionally reduces non-vertebral fractures as well as coronary heart disease and stroke (Cummings *et al.*, 2010), while for raloxifene and bazodoxifene only post-hoc analyses suggest prevention of non-vertebral fractures in high-risk subgroups (Delmas *et al.*, 2003; Silverman *et al.*, 2012). Hip fracture prevention has not been demonstrated however, and for bazedoxifene evidence of breast cancer chemoprophylaxis is lacking. In clinical practice, raloxifene or lasofoxifene may be useful in relatively young post-menopausal osteoporotic women with low risk of hip fractures or venous thromboembolism, or in women with contraindications to other drugs (Almeida *et al.*, 2017).

As mentioned above, SERMs appear to exert similar effects in men with severe hypogonadism and compromised E2 levels (Doran *et al.*, 2001; Uebelhart *et al.*, 2004), however they are not approved for men. In randomized trials, raloxifene and toremifene also reduced bone turnover and increased BMD in men receiving androgen-deprivation therapy for prostate cancer, while toremifene reduced incident vertebral fractures (Smith *et al.*, 2004; Smith *et al.*, 2013). However, due to an increased risk of venous thromboembolism, SERMs are not currently approved for this indication either (Alibhai *et al.*, 2017).

Testosterone

Several randomized trials have examined the effects of T therapy in post-menopausal women. Although a significant improvement in sexual function has been suggested by a recent meta-analysis, a deleterious effect on lipid profiles, increased risk of acne and hirsutism, and no effect on BMD was found, while long-term safety data remain lacking (Elraiyah *et al.*, 2014).

In recent years, there has been a tremendous increase in the prescription of T for adult or older men in some countries, particularly in the United States. While T replacement therapy has established benefits in men with congenital or acquired hypogonadism (Basaria, 2014; Finkelstein *et al.*, 1989), its use in so-called "late-onset hypogonadism" is much more controversial. Notably, the definition of a clinical syndrome associated with "low T" levels in older men remains controversial. According to the

European Male Aging Study criteria of total $T < 11$ nmol/L, free $T < 220$ pmol/L and at least three sexual symptoms, only 3.2% of men 60–69 years and 5.1% of those 70–79 years would classify as late-onset hypogonadism (Wu *et al.*, 2010). Secondly, not only adverse effects on the prostate but particularly adverse cardiovascular events have drawn much attention (Basaria *et al.*, 2010; Budoff *et al.*, 2017). The stimulatory effects on the prostate may be blunted by concomitant 5α-reductase inhibitors, without affecting the musculoskeletal benefits (Amory *et al.*, 2004; Bhasin *et al.*, 2012; Borst *et al.*, 2014; Matsumoto *et al.*, 2002).

T therapy has produced significant but modest BMD increases in some randomized trials (Amory *et al.*, 2004; Kenny *et al.*, 2010; Nair *et al.*, 2006) but not others (Christmas *et al.*, 2002; Emmelot-Vonk *et al.*, 2008; Snyder *et al.*, 1999). The benefits of T appeared limited to men with baseline low T levels and treated for longer duration with T at relatively high doses, with possibly greater effectiveness of i.m. injections (Borst and Yarrow, 2015; Giannoulis *et al.*, 2012; Snyder *et al.*, 1999; Tracz *et al.*, 2006).

More recently, the Testosterone Trials investigated the efficacy and safety of T therapy in men with late-onset hypogonadism. After 1 year, transdermal T increased the primary outcome of spine trabecular volumetric BMD by QCT by 6.8%. Additional significant increases were seen on femoral and spine cortical and trabecular bone and estimated strength; the effect was greater on trabecular than on cortical bone, and greater on the spine than on the hip (Snyder *et al.*, 2017). T gel increased lumbar spine areal BMD by only 1.2%, while femoral BMD was not significantly affected. Importantly however, evidence that T reduces fractures remains lacking. Therefore, current guidelines in male osteoporosis recommend that hypogonadal men with low T and low fracture risk may not need additional anti-osteoporotic medications, while men at increased fracture risk should be treated with approved fracture prevention drugs (Watts *et al.*, 2012).

Selective androgen receptor modulators (SARMs)

In contrast to the well-established tissue-specific agonistic/antagonistic effects of SERMs, the development of SARMs has been largely disappointing. Notably, SARMs maintain anabolic effects on bone and muscle with less agonistic effects on reproductive organs, but true tissue-specificity has hitherto not been demonstrated (Dubois *et al.*, 2012; Dubois *et al.*, 2015). Most SARMs cannot be converted to DHT, which may well explain why their deleterious effect on the prostate is diminished (but often not completely absent); they can be regarded more as "marginators" (greater dose-dependent margin between wanted and unwanted effects) than as true modulators (Almeida *et al.*, 2017). Unfortunately, SARMs are not aromatizable either, which is problematic given the key role of estrogens for osteoporosis in older men. In a randomized trial, DHT treatment in older men reduced BMD, probably because gonadotropins and E2 were concomitantly suppressed (Idan *et al.*, 2010). These results are in line with the Hypogonadism and Estrogen Removal (HER) trial, which has shown that the effects of T on cortical and trabecular bone and bone turnover in older men depends largely if not exclusively on estrogens (Finkelstein *et al.*, 2016). Despite beneficial effects in hypogonadal animal studies, few clinical trials with SARMs are available, and none have demonstrated positive effects on BMD (Dalton *et al.*, 2011; Dobs *et al.*, 2013; Papanicolaou *et al.*, 2013). Furthermore, the potential cardiovascular side-effects of T raise concern for further SARM development. Nevertheless, other therapies which could reverse muscle and bone loss (i.e. concomitant osteoporosis and sarcopenia) in elderly of both genders are eagerly awaited (Laurent *et al.*, 2016a).

Summary

At PBM, men develop wider bones due to greater periosteal bone expansion, as well as greater trabecular bone mass. Estrogens are essential for cortical and trabecular bone development in both genders; however, key elements of musculoskeletal sexual dimorphism are determined by androgen effects of periosteal bone formation and trabecular bone resorption. During midlife, men continue to consolidate their cortical and trabecular bone assets, likely independent of sex steroids. Cortical and trabecular bone loss with aging however is dependent on estrogen deficiency in post-menopausal women, as well as in some men (particularly those with serum $T < 200$ ng/dL and $E2 < 10$ pg/mL) and high SHBG. Although the clinical role of sex steroids in the diagnosis and treatment of osteoporosis in general is limited, there is growing interest and attention for bone loss and fracture risk in patients treated with hormone deprivation therapies for breast and prostate cancer. Further research is needed to investigate the cellular and molecular mechanisms by which sex steroid regulate bone metabolism, which could potentially lead to the development of new drugs to exploit their musculoskeletal benefits without adverse reproductive or cardiovascular effects.

See also: Estrogen and the Male. Human Aromatase Deficiency. Hypogonadism and Testosterone Therapy in Elderly Men. Reproductive and Nonreproductive Actions of Testosterone

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Osteoporosis in Premenopausal Women☆

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Glossary

Aromatase inhibitors Compounds that inhibit Aromatase in order to reduce production of estrogenic steroid hormones. These drugs are used for the treatment of breast cancer.

Anorexia nervosa (AN) An eating disorder that is characterized by the lack or loss of appetite, known as anorexia. Other features include excess fear of becoming overweight; body image disturbance; significant weight loss; refusal to maintain minimal normal weight; and amenorrhea. This disorder occurs most frequently in adolescent females.

Amenorrhea Absence of menstruation for at least 6 months (primary: it never occurred,) Secondary. The absence of vaginal bleeding for at least 6 months in women who previously have had menstruations.

Bone mineral density (BMD) A measurement of the density of bone as determined by dual-energy X-ray absorptiometry (DEXA). It is generally measured at the hip and lower spine.

Bulimia A condition characterized by gorging and purging behaviors, where the purging is induced by vomiting, laxatives, or diuretic use.

DEXA Dual X-ray absorptiometry. A noninvasive method, based on the differential absorption of X-rays. It is primarily used for quantitating bone mineral content (BMC) and density (BMD), especially for the diagnosis of osteoporosis, and also in measuring body composition.

Depo Provera (DMPA; depot medroxyprogesterone acetate) An injectable progesterone contraceptive that works in part by inhibiting the pituitary gonadotropins, which in turn suppress ovulation and ovarian steroidogenesis.

Gonadotropin-releasing hormone (GnRH) agonist A synthetic version of the naturally occurring decapeptide: GnRH: A decapeptide that stimulates the synthesis and secretion of both pituitary gonadotropins, luteinizing hormone and follicle stimulating hormone. GnRH is produced by neurons in the septum preoptic area of the hypothalamus and released into the pituitary portal blood, leading to stimulation of gonadotrophs in the anterior pituitary gland.

Hypothalamic amenorrhea (HA) Lack of menses caused by impairment of normal hypothalamic function leading to absent or decreased secretion of GnRH. It is often related to anorexia nervosa, to intense exercise or stress.

Hyperprolactinemia Increased levels of prolactin in the blood, which may be associated with amenorrhea and galactorrhea. Relatively common etiologies include prolactinoma, medication effect.

Laurence–Moon Syndrome An autosomal recessive condition characterized by hypogonadism; spinocerebellar degeneration; mental retardation; retinitis pigmentosa; and obesity. This syndrome was previously referred to as Laurence–Moon–Biedl syndrome until Bardet–Biedl syndrome was identified as a distinct entity. (From *The New England Journal of Medicine* **1989**, *12*; *321*(15): 1002–1009).

Osteoporosis A systemic disease characterized by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk. A condition characterized by a reduced amount of bone leading to an increased susceptibility to fractures (*T* score lower than 2.5 SD from the mean peak value on DEXA scan).

Osteopenia Decreased bone density (*T* score between 1 and 2.5 SD below the mean peak value on DEXA scan) (Figure).

Oligomenorrhea Abnormally infrequent menstruation.

Premature ovarian failure (POF) also called premature ovarian insufficiency (POI) menopause, premature The premature cessation of menses (Menstruation) when the last menstrual period occurs in a woman under the age of 40. It is due to the depletion of ovarian follicles. Premature menopause can be caused by diseases; ovariectomy; radiation; chemicals; and chromosomal abnormalities.

Prader–Willi Syndrome An autosomal dominant disorder caused by deletion of the proximal long arm of the paternal chromosome 15 (15q11-q13) or by inheritance of both of the pair of chromosomes 15 from the mother (Uniparental Disomy) which are imprinted (Genetic imprinting) and hence silenced. Clinical manifestations include mental retardation; muscular hypotonia; hyperphagia; obesity; short stature; hypogonadism; strabismus; and hypersomnolence. (Menkes, Textbook of Child Neurology, 5th edn., p. 229).

Turner syndrome A syndrome of defective gonadal development in phenotypic females associated with the karyotype 45,X (or 45,XO). Patients generally are of short stature with undifferentiated gonads (streak gonads), sexual infantilism, hypogonadism, webbing of the neck, cubitus valgus, elevated gonadotropins, decreased estradiol level in blood, and congenital heart defects. Noonan syndrome (also called Pseudo-Turner syndrome and Male Turner syndrome) resembles this disorder; however, it occurs in males and females with a normal karyotype and is inherited as an autosomal dominant.

☆*Change History*: February 2018. Rozenberg updated an article (from Kimberly Elford Paul Claman University of Ottawa and Ottawa Hospital, Ottawa, Ontario, Canada). The figures were not from that article but were referenced.

This article is an update of Kimberly Elford and Paul Claman, Osteoporosis in Reproductive Age Women: Impact of Sex Steroid Replacement, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 441–446.

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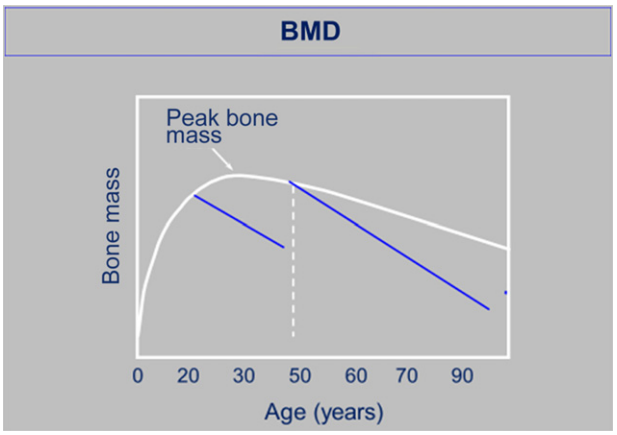

Fig. 1 It is essential to try to identify whether low bone mass in premenopausal women is related to failure to attain peak bone mass (which may for instance be of genetic origin, or have occurred during child development), to previous bone loss that has stopped, or ongoing bone loss, as the therapeutic attitude will differ.The white curb represents schematically the mean BMD evolution with age. The first *blue* line represents the BMD evolution of a patient suffering from BMD loss starting at a young age before the onset of menopause. The second blue line represents the BMD evolution of a patient suffering from BMD loss starting at the onset of menopause.

Introduction

Although osteoporosis is generally considered to be a disease mostly affecting older women after menopause, it can occur at any age, occasionally affecting premenopausal women. According to Callegari *et al.* (2017), about 2%–7% of young Australian women have a bone mass below 2 standard deviations for their age group (Z Score of less than -2).

Cauley *et al.* (2012) evaluated the value of bone turnover markers for predicting fracture risk in women across menopausal transition in 2305 women.

They observed that a higher urinary NTX excretion measured before menopause and across menopause is associated with a higher risk of fracture.

Premenopausal osteoporosis may result from insufficient bone accrual, premature bone loss or both. It is essential to try to identify whether low bone mass in premenopausal women is related to failure to attain peak bone mass (which may for instance be of genetic origin or have occurred during childhood development), to early bone loss that has been contained or ongoing bone loss because the therapeutic attitude will differ (**Fig. 1**). All of the etiological and risk factors that induce bone loss after menopause may also lead to premenopausal osteoporosis. One should strive to correct etiological and risk factors associated with osteoporosis (such as smoking, alcohol consumption, sedentary lifestyle). In the following article we will focus on reproductive factors associated with osteoporosis and occurring at a young age, that is, before the usual onset of menopause. We will not, however, discuss, steroid induced osteoporosis, cancer induced osteoporosis, hyperparathyroidism, Cushing syndrome, celiac disease and other forms of malabsorption, idiopathic hypercalciuria and connective tissue disorders, even though these conditions also need to be excluded when confronted with premenopausal women who have osteoporosis.

Assessment of Premenopausal Osteoporosis

The clinical investigation involves an osteoporosis assessment consisting of BMD measurements and determination of other risk factors for osteoporosis such as a personal history of low-trauma fractures, a family history of osteoporotic fractures, lifestyle factors, medications and serum level of 25OHD. In addition, since many women with premenopausal osteoporosis present reproductive dysfunction (often amenorrhea), clinical management will include the evaluation of its origin. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, prolactin, thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG) will be measured to evaluate the cause of amenorrhea.

Etiologies

The major causes of hypo-estrogenemia induced osteoporosis in premenopausal women are documented in **Table 1**.

Hypothalamic Amenorrhea

Hypothalamic amenorrhea (HA) is a prevalent condition and the most common cause of young-adult-onset amenorrhea, with the exception of pregnancy. It can be precipitated by eating disorders, excessive exercise, anorexia nervosa (AN), hyperprolactinemia,

Table 1 Amenorrhea which may induce osteoporosis in the young premenopausal woman. The aetiologies in bold are the principal causes

Hypothalamic amenorrhea (HA)
Functional hypothalamic amenorrhea
Exercise
Stress induced
Eating disorders
Anorexia nervosa
Severe illness
Medically induced
GnRH agonists (used previously for endometriosis, previously for myomata or breast cancer treatment), eventually combined with aromatase inhibitors
Inflammatory or infiltrative diseases
Brain tumors—e.g., craniopharyngioma
Cranial irradiation
Traumatic brain injury
Other syndromes—Prader–Willi, Laurence–Moon–Biedl, leptin mutations
Pituitary dysfunction
Hyperprolactinemia
Other pituitary tumors—acromegaly, corticotroph adenomas (Cushing disease)
Other tumors—meningioma, germinoma, glioma
Genetic causes of hypopituitarism
Empty sella syndrome
Pituitary infarct or apoplexy
Ovarian dysfunction
Gonadal dysgenesis (Turner syndrome, 46,XY*)
Other causes of primary ovarian insufficiency (POI)
Post-surgery, inflammation endometriosis
Post chemotherapy, irradiation

Depo Provera use, gonadotropin-releasing hormone (GnRH) agonist use and functional hypothalamic amenorrhea. AN is the major concern for osteoporosis.

Osteoporosis in Patients With Anorexia Nervosa (AN)

Conservative estimates of the lifetime prevalence of anorexia nervosa (AN) range from 0.6% to 4.2% (Gielen *et al.*, 2017). AN is 10 times more common in women than in men and mainly affects young women (median age of onset 18 years old) (Grinspoon *et al.*, 2000; Gielen *et al.*, 2017). About half of these patients suffer from osteoporosis and almost all have osteopenia (Grinspoon *et al.*, 2000; Gielen *et al.*, 2017). Amenorrhea is common and the typical triad consists of low energy intake (generally related to eating disorders), osteoporosis and amenorrhea (**Fig. 2**).

Concerns should be raised if a patient's history reveals the absence of three or more cycles. Amenorrhea seems to be very common in athletes, affecting between 3% and 66% of them and about 15%–62% of young, female athletes are concerned by eating disorders. The incidence of osteoporosis in adults who presented the triad during adolescence and young adulthood is unknown (Grinspoon *et al.*, 2000; Gielen *et al.*, 2017).

AN is associated with marked endocrine changes that also impair healthy bone turnover. As mentioned before, amenorrhea is common. In adolescents with AN, menarche is often delayed, contributing to low BMD. However, the severity of bone loss in women with AN is greater than in those with normal-weight hypothalamic amenorrhea, indicating that, in addition to estradiol deficiency there are other factors including nutritional deficiencies and hormonal abnormalities that contribute to bone loss. GH resistance, low levels of IGF-1, hypercortisolemia and low levels of testosterone have all been implicated in AN-associated osteopenia and osteoporosis. Abnormalities in hormones regulating appetite (oxytocin, leptin and peptide tyrosine tyrosine (PYY)) may play an additional role. Therefore, the etiology of bone loss in patients with AN is multifactorial (Gordon, 2010; Gielen *et al.*, 2017).

Biological Markers of Bone Turnover, Bone Loss and Bone Fragility, Fracture Risk in AN Patients

Klibanski's team observed an uncoupling of bone turnover in adults with AN, with a decrease in markers of bone formation and an increase in markers of bone resorption. However, in adolescents with AN, there is a low bone turnover state with a decrease in levels of bone formation and resorption, as opposed to an increased bone turnover during normal adolescence (Misra and Klibanski, 2006). Veronese *et al.* conducted a meta-analysis of vitamin D status in AN patients and observed that AN patients who

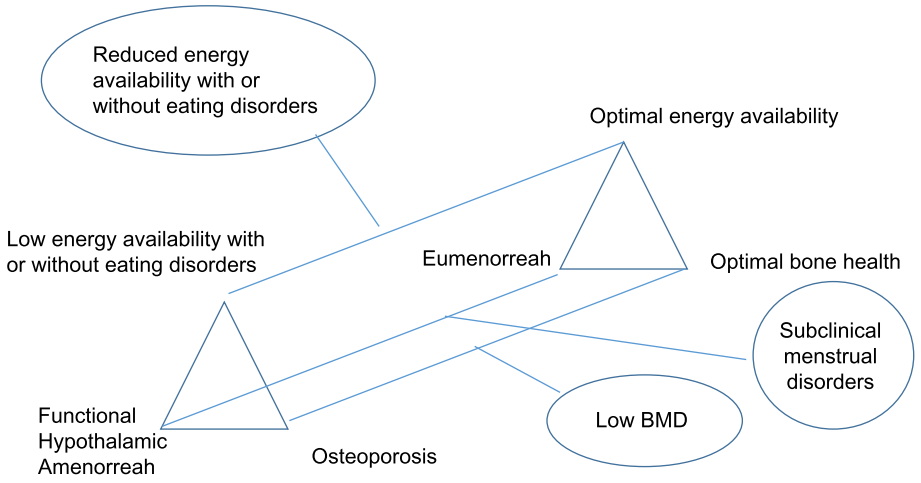

Fig. 2 The female athlete triad refers to the inter-relatedness of eating disorders, amenorrhea, and osteoporosis. Adapted from Catherine Gordon Meet the Professor course at the ASBMR Annual Conference 2012.

did not take vitamin D supplements had significantly lower levels of 25OHD and 1,25(OH)2D (Veronese *et al.*, 2015). Both trabecular and cortical bone sites are affected in AN, but since there is a marked estrogen deficiency component in these women, trabecular osteoporosis (such as at the lumbar spine) is more marked and common (Misra and Klibanski, 2014). Moreover, HR-pQCT studies reported that their bone microarchitecture, marrow adiposity and bone strength are affected (Misra and Klibanski, 2014). AN patients often have a reduced peak bone mass, increasing their risk of fracture (Theintz *et al.*, 1992).

Not surprisingly, therefore, a two- to sevenfold increase in fractures has been reported in patients with AN (Rigotti *et al.*, 1991; Vestergaard *et al.*, 2002; Strokosch *et al.*, 2006). Rigotti *et al.* followed a series of 27 women with AN for a median of 25 months and concluded that anorexic women may have an increased risk of fracture (RR 7.1; 95% CI 3.2–18.5) and that low BMD is not rapidly reversed after recovery from AN (Rigotti *et al.*, 1991). Vestergaard *et al.*, using a case control Danish Nationwide register study, also observed an increased fracture risk in AN subjects, which persisted more than 10 years after the diagnosis (Vestergaard *et al.*, 2002).

Assessment and Management of Osteoporosis in AN

AN patients need to be encouraged to gain weight. A restored, acceptable BMI (between 18 and 25 kg/m^2) will generally help to recover a normal reproductive function, which can be determined by a regular menstrual cycle (without hormone therapy). Restauration of body weight will also improve BMD although a complete catch-up does not always occur (Misra and Klibanski, 2006). In addition, AN patients should take adequate amounts of calcium (e.g., 1200 mg) and vitamin D (e.g., 800 IU) daily, from diet and supplements.

High-dose estrogen contraception is not an effective treatment option for AN-associated bone loss in adolescents or adults. Indeed, prospective trials have failed to show a benefit of combined estrogen–progestin oral contraceptives in treating AN-associated bone loss even though one may be tempted to restore menstruation in these patients using oral contraceptives. Unfortunately, several studies have failed to observe a protective effect of the "pill" containing 50 µg ethynilestradiol (Strokosch *et al.*, 2006). On the other hand, lower-dose physiological estrogen replacement, using MHT such as 100 µg transdermal 17-beta-estradiol with cyclic micronized progesterone, resulted in BMD gains at the spine and hip compared with placebos. The therapy did not, however, restore BMD to normal (Misra *et al.*, 2011). Although AN patients are also androgen-deficient, its replacement is currently not recommended.

Limited data suggest that bisphosphonates may benefit iwomen with AN-associated bone loss. For instance, 1 year of risedronate treatment resulted in a modest gain in BMD compared with placebo (Miller *et al.*, 2011). Another study, however, failed to report any improvement between alendronate and placebo (Golden *et al.*, 2005). Furthermore, there are safety concerns with bisphosphonates, especially in younger patients who may want to become pregnant. Bisphosphonates should only be prescribed to non-pregnant women of reproductive age and those who will not become pregnant during the treatment period. Bisphosphonates are usually not prescribed in such cases but can be used by older AN patients. Denosumab and teriparatide should also not be used as first line therapies in young patients suffering from AN-induced osteoporosis.

Conclusion

AN predominantly affects young women and results in the disturbance of most endocrine axes. This will lead to osteoporosis in about half of the patients and to osteopenia in almost all of them with added suffering due to fractures. Multidisciplinary management is needed, involving a gynecologist, an endocrinologist, a pediatrician (depending of the patient's age), a psychologist, a nutritionist and a bone specialist. BMD and an endocrine evaluation is mandatory in these patients. Other pathologies

associated with weight loss, amenorrhea and bone loss should be excluded. Optimal therapy aims to restore body weight and the recovery of a regular cycle. A healthy lifestyle is therefore mandatory. Calcium and vitamin D supplementation is recommended in all patients with AN. MHT is indicated in adolescent girls with sustained low body weight, low BMD and amenorrhea when other causes of amenorrhea have been excluded. The bisphosphonates, denosumab and teriparatide may be useful in adult women with severe bone loss.

Hyperprolactinemia

Hyperprolactinemia is a relatively common clinical problem. Its prevalence ranges from 0.4% in an unselected adult population to as high as 9%–17% in women with reproductive diseases. It was also found in 5% of women at a family planning clinic, 9% of those with young-adult onset amenorrhea and 17% among women with polycystic ovary syndrome. (Biller *et al.*, 1999). It may result from a micro (or less often macro) prolactinoma or be drug induced. The clinical spectrum involves anovulation, oligomenorrhea, amenorrhea, infertility, galactorrhea, and osteoporosis. Hyperprolactinemia leads to the inhibition of pulsatile secretion of hypothalamic GnRH with resultant hypothalamic amenorrhea or menstrual irregularity. Estrogen-deficient hyperprolactinemic women are at high risk for the development of osteoporosis. Klibanski and Greenspan (1986) and Schlechte *et al.* (1992), have shown that women with hyperprolactinemia have reduced bone densities compared to controls. Although loss of bone density worsens with lower estrogen levels, there is no correlation between prolactin levels and bone density. Prolactin receptors have been found in human bone, but it is unlikely that hyperprolactinemia exerts a significant, direct effect on calcium metabolism or bone mineral content. However, it is clear that the estrogen deficiency found in hyperprolactinemic women is directly responsible for osteopenia. Park and Song, 1995 showed that the decline in bone mass observed in hyperprolactinemic women follows a rapid course between ages 20 and 25 and then starts to slow down. Fortunately, bone loss partially recovers after the restoration of gonadal function with dopamine agonist therapy. Menstrual dysfunction and bone loss do not occur in all hyperprolactinemia women. Only 30% of the subjects in Klibanski and Greenspan's study in 1986, were found to suffer from bone loss. It is likely that only those women with increased prolactin and amenorrhea or menstrual irregularities (indicating a hypoestrogenic state) are at risk for osteoporosis.

Treatment

When hyperprolactinemia is found, a careful history must be taken to rule out the use of dopamine-inhibiting medications or hypothyroidism because these can cause hyperprolactinemia. Imaging of the pituitary gland using computed tomography or magnetic resonance imaging is indicated to rule out a pituitary adenoma. Normally, menstruating hyperprolactinemic women who do not have pituitary tumors and those in whom fertility is not desired may not require therapy. Indeed, some women suffer from hyperprolactinemia associated with the "big big molecule of prolactin" which is biologically inactive (called macroprolactinemia), and is detected by the same radioimmunoassay as the biologically active prolactin (Biller *et al.*, 1999). This may explain many cases of the very high prolactin levels sometimes found in normally ovulating women which require no treatment. Dopamine agonist is the mainstay of treatment. In women with menstrual irregularities, Bromocriptine and Cabergoline are the most commonly used dopamine agonists and are effective in normalizing serum prolactin, resulting in the resumption of menstrual regularity. Dopamine agonist therapy not only regulates menstruation but also helps to prevent bone loss and reduces long-term fracture risk in hypoestrogenic hyperprolactinemic women. However, the presence of a pituitary macroadenoma may require surgical or radiological management (Biller *et al.*, 1999).

Drug Induced

Depo Provera Use

Depot medroxyprogesterone acetate (DMPA) is a safe injectable contraceptive that works by inhibiting pituitary gonadotropin secretion with resultant suppression of ovarian steroidogenesis. Most DMPA users become amenorrheic within the first year of use. Circulating estrogen concentrations in women using DMPA are maintained at levels similar to those found in the early follicular phase of normally menstruating women. Controversy exists regarding whether a constant low estradiol level leads to bone loss in DMPA users.

Kaunitz *et al.* (2008) conducted a systematic review of studies published in PubMed from 1996 to 2006, evaluating changes in BMD after discontinuation of DMPA. Ten primary clinical or observational studies addressing this issue were identified. BMD consistently went back to baseline or near base-line values following DMPA discontinuation in women of all ages. This recovery in BMD was seen as early as 24 weeks after therapy cessation and persisted for as long as women were followed up. BMD in past DMPA users was similar to that in non-users. The authors concluded that bone loss occurring with DMPA use is reversible and is not likely to be an important risk factor for low bone density and fractures in older women, although data on fracture risk in DMPA users are lacking (Kaunitz *et al.*, 2008).

Some investigators have found significantly lower BMD in DMPA users than in controls. Others, however, have been unable to show significant deterioration in BMD after years of use. A long-term study by Tang *et al.* (1999) found significantly lower BMD of the lumbar spine and hip in women who used DMPA for more than 5 years compared to nonusers of the same age, body mass

index, smoking status and who had the same calcium intake. Longitudinal research needs to be undertaken to determine whether DMPA use causes significant loss in BMD and whether any deficit in bone density is carried forward into postmenopausal life thereby increasing fracture risk. Substantial recovery of bone mass was reported, in 1994, by Cundy *et al.* more than 1 year after discontinuation of DMPA. Candidates for injectable contraception should be informed that the use of DMPA is associated with a slight decrease in bone mineral density (BMD), which is largely, if not completely, reversible. There should be no specific limit to the length of time that the DMPA contraceptive is prescribed, regardless of the woman's age. Monitoring BMD is not recommended in users of DMPA for contraceptive purposes. Finally, the consensus statement declared that, although calcium and vitamin D supplements are beneficial in skeletal health for women in general, it should not be recommended solely based on a woman's use of DMPA (Guilbert *et al.*, 2009).

On the other hand, DMPA may not be indicated in women with suspected osteoporosis or with a high prevalence of osteoporosis risk factors. Similarly, DMPA may not be the first choice of contraception in adolescents who have not achieved peak bone mass.

Conclusion

Scientific evidence shows that DMPA is a valid contraceptive option for women. Its potential impact on BMD must be balanced against the significant individual, family and social consequences of unintended pregnancy.

Gonadotropin-Releasing Hormone Agonist (GnRHa or LHRHa) Use

Gonadotropin-releasing hormone agonists suppress ovarian function through the inhibition of the hypothalamic–pituitary axis and are used in the management of several conditions in women's health. They may be indicated for severe endometriosis, before surgery or before IVF. They also used to be indicated for the preoperative treatment of uterine fibroids but since the development of ulipristal acetate, they have largely been abandoned for such cases (Rozenberg *et al.*, 2017). They may be used to induce chemical castration in breast cancer patients (this subject will be covered in another chapter). Using GnRHa for more than 6 months may result in extreme bone loss. Data suggest that after 6 months of use, discontinuation of treatment is associated with a virtually complete recovery of any bone lost during the time of therapy. For long-term use, low-level estrogen and/or progesterone add-back therapy has been suggested. The concept of add-back therapy is that there may be a threshold level of estrogen that is high enough to prevent bone loss but low enough to attenuate the development of endometriosis.

Premature Ovarian Failure

Premature ovarian failure (also known as premature menopause) is defined as menopause before the age of 40. It can be "natural" or "iatrogenic" such as after bilateral oophorectomy. It may be either primary or secondary. In the majority of cases of primary POF, the cause is unknown. Chromosome abnormalities (especially X chromosome), follicle-stimulating hormone receptor gene polymorphisms, inhibin B mutations, enzyme deficiencies and autoimmune disease may be involved. Secondary POF is becoming more prevalent since survival rates following treatment of malignancies using surgery, radiotherapy and chemotherapy continue to improve. Diagnosis should be confirmed by an elevated FSH level, over 40 IU/L and an estradiol level lower than 50 pmol/L, in the absence of bilateral oophorectomy. Further assessments should include thyroid function tests, autoimmune screening for polyendocrinopathy, karyotype (below the age of 30) and bone mineral density. Untreated early ovarian failure increases the risk of osteoporosis, cardiovascular disease, dementia, cognitive decline and Parkinsonism. The mainstay of treatment is hormone therapy which must be continued until the average age of the natural menopause. With regards to fertility, while spontaneous ovulation may occur, the best chance of achieving pregnancy is through donor oocyte in vitro fertilization. It is essential that women receive adequate information as they may find it a difficult diagnosis to accept. It is recommended that women with POF be seen in a specialized unit capable of dealing with their multiple needs (Vujovic *et al.*, 2010).

Treatment

One should aim at treating the underlining disorder. Risk factors need to be corrected (alcohol, smoking). Calcium, vitamin D, and weight bearing exercise are recommended for most women with premenopausal osteoporosis. Exercise is not recommended in AN patients, however. Pharmacotherapy is not indicated in premenopausal women with low bone mineral density without fractures or without evidence of accelerated bone loss. There are very few data available to guide clinicians in the use of classical pharmacologic therapies used in postmenopausal women (bisphosphonates, selective estrogen receptor modulators (SERMs), teriparatide (parathyroid hormone [PTH] 1–34), and denosumab). These treatments should be reserved for rare patients with fractures or ongoing secondary causes of osteoporosis and bone loss. Women with fractures or accelerated bone loss in the setting of estrogen deficiency or amenorrhea may benefit from estrogen replacement. Replacement of estrogen in premenopausal women who are estrogen deficient may have beneficial effects on bone mass (women with POI and to a lesser extend women with AN) (Gielen *et al.*, 2017).

Conclusion

Bone density measurement is recommended for premenopausal women with known secondary causes of osteoporosis or history of a fragility fracture. During the evaluation of premenopausal women with osteoporosis, secondary causes of osteoporosis need to be excluded. One should determine whether there is ongoing excessive bone loss. Adequate calcium, vitamin D, and life style should be recommended. In women with a secondary cause of osteoporosis etiological treatment should be targeted. In the absence of evidence of accelerated bone loss or fractures, conservative management without specific other medical therapy and only follow up should occur. In rare cases of premenopausal osteoporosis, for instance those associated with amenorrhea and osteoporosis induced by chemotherapy, will other medication such as bisphosphonates or denosumab be used.

See also: Delayed Puberty and Hypogonadism; Female. Menopausal Treatment

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Secondary Osteoporosis

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Introduction

Osteoporosis is a skeletal disease characterized by decreased bone mass and microarchitectural changes in bone tissue that increase the susceptibility to fracture. It is estimated that 10 million Americans over the age of 50 have osteoporosis, and tens of millions more are at risk. Bone mineral density testing has been the accepted standard for screening for this disease, and is routinely targeted at postmenopausal females by general clinicians.

Secondary osteoporosis is loosely defined as low bone mineral density or increased risk of fragility fracture caused by any factor other than aging or postmenopausal status. A wide array of diseases, treatments, and medications can affect bone quality in men and women of all ages, which is often not reflected in bone density testing. In light of the significant public health burden caused by osteoporosis, a recognition and understanding of these factors is of utmost importance. The following is an organ-system based review of the more common causes of secondary osteoporosis, with a focus on pathogenesis.

Endocrinology

Glucocorticoid Excess

Endogenous overproduction or systemic administration of glucocorticoids is a well-known cause of bone loss, and is the most common iatrogenic cause of osteoporosis seen in clinical practice (Weinstein, 2011). The pathogenesis includes diverse effects on three main cell types in bone biology, changes in calcium handling, as well as increased falls related to steroid-induced myopathy (Natsui *et al.*, 2006; Canalis *et al.*, 2007). As many as 30%–50% of patients receiving chronic glucocorticoid therapy will experience a fracture, making this a major contributor to the morbidity and mortality associated with steroid-responsive chronic diseases such as lupus, rheumatoid arthritis, giant cell arteritis, transplant recipients, inflammatory bowel disease, and COPD (Steinbuch *et al.*, 2004; Angeli *et al.*, 2006).

Glucocorticoids cause decreased production of osteoblast precursors and increased apoptosis of mature osteoblasts (Weinstein *et al.*, 1998; O'Brien *et al.*, 2004). Histomorphometric studies have confirmed decreased numbers of osteoblasts on cancellous bone in patients with glucocorticoid-induced osteoporosis (GIO) with associated thinning wall width. Decreased osteoblast differentiation is in part mediated through inhibition of wingless (Wnt)/B-catenin signaling, as the expression of Dickkopf-1 and sclerostin, both antagonists of Wnt signaling, can be enhanced by glucocorticoids (Ohnaka *et al.*, 2005; Gifre *et al.*, 2013). Enhanced apoptosis of osteocytes is also seen, which are the main mechanosensing cells of bone that contribute to the ability to withstand significant compressive forces.

While glucocorticoid therapy can decrease osteoclast production, the lifespan and activity of osteoclasts are increased, thereby maintaining their effect relative to the decreased osteoblastic number and activity. Studies have shown increased production of receptor activator of NF-kB ligand (RANKL) which promotes osteoclastic activity and survival, and suppression of osteoprotegerin (OPG), a natural decoy receptor for RANKL (Hofbauer *et al.*, 1999; Humphrey *et al.*, 2006). Additionally, glucocorticoids have been shown to confer a prosurvival effect directly to osteoclasts, independent of alterations in RANKL and OPG, in the early stages of steroid exposure in mice (Jia *et al.*, 2006). The bone abnormalities may also be enhanced by the decreased sex hormone secretion because of suppressed hypothalamic-pituitary function.

Bone loss occurs quickly with glucocorticoid therapy and appears to be biphasic, with as much as a 6%–12% loss in BMD within the first year, followed by a continued annual loss with ongoing therapy (LoCascio *et al.*, 1990). Biochemical markers of bone turnover are affected as early as 1–2 months following initiation of therapy, and the bulk of bone loss is seen within the first 6 months (Saag *et al.*, 1998). Addressing the deleterious effects of steroid therapy on bone is imperative early on in the course, particularly if long term use (i.e., 90 days or longer) or moderate to high dose (>7.5 mg/day) is planned. Risk factors associated with GIO include advanced age, low BMI, dose and duration of glucocorticoid, as well as other traditional factors (Weinstein, 2012). Bone density testing is recommended at the onset of therapy as a general screening, though changes in the mechanical qualities of bone may occur prior to any changes reflected in BMD testing. Treatment recommendations are based on dose, additional risk factors, prior fractures, and an updated guideline was recently published, and both anabolic and antiresorptive therapies may be used in addition to calcium and vitamin D supplementation (Buckley *et al.*, 2017; Saag *et al.*, 2007).

Primary Hyperparathyroidism

Parathyroid hormone (PTH) is critically involved in calcium and phosphorus homeostasis, and continuous exposure to elevated levels in primary hyperparathyroidism (PHPT) are known to be catabolic to the skeleton. The contribution to osteoporosis is so

well established that in patients with PHPT significant metabolic bone disease is generally considered an indication for parathyroidectomy (Bilezikian *et al.*, 2009). The majority of patients with PHPT are postmenopausal females, with an incidence as high as 1 in 500 reported in this population (Bilezikian *et al.*, 2009). PHPT preferentially affects areas rich in cortical bone more so than cancellous bone, therefore evaluating distal forearm and femoral neck BMD is recommended (Khan and Bilezikian, 2000). Vertebral fracture prevalence is increased, both in symptomatic and asymptomatic patients (Cipriani *et al.*, 2015).

The precise mechanism of bone loss in PHPT is not completely understood. Histomorphometrically, persistently elevated levels of PTH induce a state of high bone turnover and have been associated not only with a low BMD but also with alterations in the mineralization density distribution and collagen crosslinks affecting bone stiffness and quality (Roschger *et al.*, 2007; Zoehrer *et al.*, 2008). At the cellular level, compared with healthy controls, patients with PHPT had a low OPG/RANKL ratio favoring a state of bone resorption, which improves following parathyroid surgery (Szymczak and Bohdanowicz-Pawlak, 2013). The effects of PHPT on Wnt pathway signaling is still being elucidated. Taken together, the finding of increased bone turnover, low BMD, and reduced bone quality appear to confer an increased risk of fracture in PHPT, highlighting the need for proactive screening of bone disease.

Hyperthyroidism

Thyrotoxicosis is an established cause of high-turnover osteoporosis, and many studies have shown a consistent decrease in BMD and increase in fracture risk in patients with untreated hyperthyroidism (Vestergaard and Mosekilde, 2003; Ross, 1994). While bone loss has been documented at all skeletal sites, there is preferential involvement of cortical bone, suggesting the need to screen distal forearm BMD (Greenspan and Greenspan, 1999; Svare *et al.*, 2009). While the cause of the hyperthyroidism may not matter, the severity is an important factor, as a TSH of <0.1 mU/L was associated with a threefold increase risk of hip fracture and a fourfold increase risk of vertebral fracture in a cohort of postmenopausal females, even in subclinical thyroid disease (Blum *et al.*, 2015; Bauer *et al.*, 2001).

The effect of thyrotoxicosis on bone was felt to be mediated by the effects of T3 through its interaction with nuclear receptors TR-α and TR-β, and excessive stimulation of these receptors leads to accelerated bone remodeling and ultimately osteoporosis. Additionally, recent evidence has shown that thyroid stimulating hormone (TSH) may be a negative regulator of bone turnover, as decreased expression of TSH-receptor leads to a high turnover state and low BMD (Abe *et al.*, 2003). Bone-protective effects of TSH include increased osteoblastic differentiation, and inhibited osteoclast formation (Baliram *et al.*, 2017). This has important implications for other situations with low TSH such as subclinical hyperthyroidism and use of thyroid replacement medications in cancer, etc. In hypothyroidism the use of highly sensitive TSH measurements has allowed a more accurate control of patients on replacement therapy and it is recommended that values be kept in mid normal range to avoid bone sequelae (Garber *et al.*, 2012).

Growth Hormone Deficiency

Growth hormone (GH) is an important determinant of longitudinal bone growth and development of skeletal maturity, and children with GH deficiency exhibit short stature and reduced peak bone mass (Giustina *et al.*, 2008). The anabolic effect of GH on osteoblasts appears to be dependent upon insulin-like growth factor 1 (IGF-1), whose production by the liver and osteoblasts is stimulated by GH (Andreassen and Oxlund, 2001). The local interaction of IGF-1 and its binding proteins regulate GH receptor (GHR) on osteoblasts, mediating some effects of GH on bone (De Jesus *et al.*, 2009). A marked reduction in bone turnover is seen histomorphometrically in adult-onset GH deficiency, primarily affecting cortical bone (Bravenboer *et al.*, 1996). This is associated with a lower BMD, and a fracture risk two to three times that of osteoporotic patients without GH deficiency (Wüster *et al.*, 2001). Treatment with GH replacement has been associated with a decrease in the fracture rate (Mazziotti *et al.*, 2006).

Acromegaly

Acromegaly is associated with significant increase in markers of bone turnover due to the effect of excess GH and IGF-1 on osteoblasts and osteoclasts (Ueland *et al.*, 2006). Studies on the effect of acromegaly on BMD are nuanced due to the structural changes in bone influencing the accuracy of testing, as well as the common finding of concurrent hypogonadism. Overall, the BMD in acromegalics is generally preserved, with some showing a relative decrease in trabecular bone density (Kayath and Vieira, 1997; Scillitani *et al.*, 2003). Despite a preserved BMD, several studies document increased bone turnover and an increased incidence of vertebral fractures compared with a control population, influenced by factors such as the duration of active acromegaly, serum IGF-1 levels, and postmenopausal status (Mazziotti *et al.*, 2013, 2015; Bonadonna *et al.*, 2005).

Male Hypogonadism

Androgens are important for developing and maintaining skeletal health in men (Clarke and Khosla, 2009). Serum testosterone levels decline with aging starting around the 5th decade of life in men, a time after which approximately 85% of cortical bone loss occurs (Mohr *et al.*, 2005; Ebeling, 1998). In addition to aging, medications such as androgen deprivation therapy (ADT) cause an abrupt decline in androgen levels. In vitro studies have shown that androgens, via the androgen receptor, can stimulate

osteoblastic cell proliferation, upregulate TFG-b and IGF-1 (involved in bone formation), and downregulate IL-6 (involved in bone resorption) (Kasperk *et al.*, 1989, 1997). Testosterone also contributes to bone health indirectly in men via its conversion to estrogen by aromatase in peripheral tissues (including osteoblasts and osteocytes), and men with low estrogen, or decreased aromatase activity, can present with low BMD (Riggs *et al.*, 2002; Leder *et al.*, 2003; Miedlich *et al.*, 2016). Estrogens play a role in both bone resorption and bone formation in men. Several large studies have shown a relationship between low serum estradiol levels in men and low BMD (Amin *et al.*, 2000; Travison *et al.*, 2009). In addition to these direct effects on bone, low testosterone may also be associated with increased falls, a clear risk factor for fractures (Vandenput *et al.*, 2017).

Measurement of sex hormone levels can be valuable in the workup and prognostication of bone health in men with hypogonadism. While low testosterone levels are associated with decreased bone mass and muscle strength, a much stronger correlation with fracture risk is seen with low bioavailable estradiol levels or high sex hormone binding globulin (SHBG) levels (LeBlanc *et al.*, 2009; Barrett-Connor *et al.*, 2000; Vandenput *et al.*, 2016). The highest correlation with fracture was when both the bioavailable estradiol level was low *and* the SHBG level was high. Hypogonadism, whether from aging, surgery, or a medication, is a major cause of osteoporosis in men, and the relative contributions of alterations in estradiol, testosterone, and SHBG need to be better defined. In symptomatic patients, testosterone replacement therapy can also serve to improve the bone density at the spine and later the hip, though concern of side effects and lack of antifracture efficacy affect its primary use to treat hypogonadal bone disease (Seftel *et al.*, 2015).

Diabetes Mellitus

Both type 1 (T1DM) and 2 diabetes mellitus (T2DM) are increasingly associated with deleterious effects on the skeleton (Inzerillo and Epstein, 2004; Epstein and Leroith, 2008). Compared with healthy controls, there was a 12-fold increased risk of hip fracture in T1DM and a 1.7-fold increase in T2DM seen in the Iowa Women's Health Study (Nicodemus *et al.*, 2001). A similar increase in fracture risk was seen in the Women's Health Initiative in those with T2DM, even after adjusting for frequent falls and BMD changes (Bonds *et al.*, 2006). T1DM is associated with decreased BMD and failure to achieve peak bone mass (Pan *et al.*, 2014). In T2DM, however, there is often a normal or elevated BMD, implicating a decline in the quality of the bone as a contributor to fracture risk (Ma *et al.*, 2012). In T2DM, structural changes such as increased intracortical porosity, as well as decreased material strength seen in micro-indentation testing, may explain fracture risk despite lack of BMD changes (Burghardt *et al.*, 2010; Farr *et al.*, 2014).

The osteoporosis associated with DM is one of low bone turnover, with decreased markers of osteoblastic activity (Bouillon *et al.*, 1995). Insulin and amylin have anabolic effects on bone, and their decrease in T1DM may lead to impaired bone formation likely through decreased IGF-1 (Kemink *et al.*, 2000; Fowlkes *et al.*, 2011). Additionally, mouse models of insulin-dependent diabetic osteopathy have shown an increased expression of Dkk1 and SOST, both antagonists of Wnt signaling and osteoblastogenesis (Hie *et al.*, 2011). The accumulation of advanced glycation endproducts and lower enzymatic collagen crosslinks contribute to altered biomechanical features of diabetic bone showing the importance of glycemic control over time (Khosravi *et al.*, 2014).

In diabetes there is increased bone marrow adiposity which has been linked with osteoporosis (Rosen and Bouxsein, 2006). Peroxisome proliferator activator receptors (PPARs), especially PPAR-gamma, are transcription factors and nuclear receptors involved in glucose metabolism, and have been shown to be potent regulators of adipogenesis. The mesenchymal stem cells in the bone marrow microenvironment can experience an adipogenic or osteogenic fate, and PPARs and their ligands rosiglitazone and pioglitazone appear to drive the differentiation toward adipocytes and away from osteoblasts (Kawai and Rosen, 2010). This has important implications as these agonists are commonly used in the treatment of T2DM (see below under drugs). In addition to the above cellular mechanisms of bone compromise in DM, other factors such as retinopathy-induced visual impairment, neuropathy-induced balance problems, renal osteodystrophy from dialysis, and renal transplantation all add to the fracture burden. In addition, sarcopenia contributes to an increased risk for falls and fractures in this population (de Liefde *et al.*, 2005). Close attention should be given to bone health in diabetics of all ages, as fractures are an increasingly recognized complication.

Vitamin D Deficiency

Deficiency of vitamin D is leads to reduced intestinal absorption of calcium and phosphorus, resulting in secondary hyperparathyroidism and demineralization of bone (Sai *et al.*, 2011). The main sources of vitamin D are diet and sunlight exposure; lack of fortification of foods and increased appreciation of the harmful skin effects of excessive sunlight exposure have contributed to the amount of vitamin D deficiency seen in clinical practice. Serum 25(OH)D is best indicator for vitamin D status in clinical practice, and while 20 ng/mL is generally considered the threshold for defining insufficiency with regards to bone health, optimal calcium absorption and control of secondary hyperparathyroidism have been seen closer to 30 ng/mL (Sai *et al.*, 2011; Heaney, 2004). Extreme examples of the skeletal effects of vitamin D deficiency are seen in cases of osteomalacia, or rickets in children, where there is a softening of the bones caused by an increase in the unmineralized osteoid component of the bone matrix.

Due to the difficulty involved in isolating the effect of vitamin D on clinical outcomes, and the heterogeneity of the investigations, the magnitude of its effect on bone loss is debated, but most studies agree that levels of 25(OH)D below 20 ng/mL are associated lower BMD (Bischoff-Ferrari *et al.*, 2004a; Lips *et al.*, 2001). For similar reasons, the effect of vitamin D deficiency on fracture risk is difficult to quantify, but large population studies of both men and women agree that hip fracture risk is higher in those with a 25(OH)D level below 20–25 ng/mL (Cauley *et al.*, 2008, 2010; Looker and Mussolino, 2008). The contribution of vitamin D deficiency to muscle weakness and falls is debated, though recent review suggests supplementation can reduce falls in

adults (Bischoff-Ferrari *et al.*, 2004b; Kalyani *et al.*, 2010). While there is debate about the definition of optimal vitamin D level for bone health, there is agreement that levels below 20 ng/mL are associated with lower BMD, increased fracture risk, and decreased efficacy of pharmacologic and nonpharmacologic interventions for osteoporosis.

Rheumatologic and Inflammatory

Systemic Lupus Erythematosus

Low BMD is described in up to 50% of female patients with systemic lupus erythematosus (SLE) (Yee *et al.*, 2005). In addition to traditional risk factors, bones of SLE patients are affected by use of corticosteroids and other immunosuppressive drugs, physical inactivity, and vitamin D deficiency on the basis of sun avoidance (Yee *et al.*, 2005; Bultink, 2012; Lane, 2010). While HR-pQCT studies have documented decreased bone strength in SLE patients on glucocorticoids, a recent study saw similar changes in SLE patients without steroid exposure implicating effects of the disease rather than medications (Tang *et al.*, 2013). Osteoclast-inducing inflammatory cytokines are known to be elevated in SLE and contribute to bone loss (Walsh *et al.*, 2005). An elevated risk of fracture is seen, some citing as high as 12.5% of patient experiencing fractures, with age at diagnosis, duration of disease, and glucocorticoid use increasing that risk (Ramsey-Goldman *et al.*, 1999; Lee *et al.*, 2007). As SLE commonly affects premenopausal females, heightened awareness of the deleterious skeletal effects and fracture risk is important to prompt early screening.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis associated with several forms of skeletal remodeling including marginal joint erosions, periarticular osteopenia, and systemic osteoporosis. Low BMD and an increased risk for fracture have been seen in RA patients compared with control populations, though confounding variables make the underlying mechanism of bone loss somewhat difficult to elucidate. These include age, female sex, physical inactivity, disease severity, and use of medications such as glucocorticoids. An association between disease severity and bone loss has been observed, though there has been no consistent correlation between joint erosions and low BMD (Lodder *et al.*, 2004; Solomon *et al.*, 2009). Many of the cytokines and growth factors known to be involved in RA-associated inflammation, such as IL-1, IL-6, TNFa, can promote osteoclastic activity themselves or modulate the expression of the osteoclastogenic factor RANKL and its inhibitor OPG (Goldring and Gravallese, 2000).

Further insights into the control of bone turnover in RA have come from studies of RANKL and OPG. In early, active, untreated RA patients, an increased RANKL:OPG ratio, as well as increased CTX levels, were predictors of rapid and persistent bone damage over an 11 year follow up (Geusens *et al.*, 2006; van Tuyl *et al.*, 2010). Consistent with these findings, TNFa blockade, an effective treatment of the inflammatory process, has been associated with decreased systemic bone loss, and these effects correlate with a fall in serum RANKL levels (Vis *et al.*, 2006). In addition to an increased RANKL:OPG ratio, increased levels of Dkk-1 and sclerostin, inhibitors of bone formation, have been found in the serum of RA patients (Terpos *et al.*, 2011). Treatment with inhibitors of IL-6, was associated with a favorable decrease in the RANKL:OPG ratio as well as a drop in the Dkk-1 and sclerostin levels and less bone mass loss (Terpos *et al.*, 2011; Tanaka *et al.*, 2014). Clearly, monitoring for bone loss and instituting timely therapy is of utmost importance in RA.

Ankylosing Spondylitis

Ankylosing spondylitis (AS) is a chronic arthritis characterized by inflammation in synovial joints as well as the entheses. In contrast to RA, the inflammation can be accompanied by increased bone formation, particularly at areas of entheseal inflammation such as at ligament insertion sites in the spine. Osteoporosis is a known complication of AS, which can often go undiagnosed because of the young, mostly male population affected. Osteoporosis is seen in up to 25% of patients with AS, with osteopenia seen up to 50% of the time, and is usually associated with disease activity and duration (Ghozlani *et al.*, 2009). The fact that bone loss can be seen early in the disease process argue that immobility is not the primary cause (van der Weijden *et al.*, 2012). Vertebral and nonvertebral fractures are seen with increased frequency in patients with AS contributing to the morbidity of the disease, and are associated with advanced age, longer disease duration, and degree of structural changes (Ghozlani *et al.*, 2009; Mitra *et al.*, 2000; Muñoz-Ortego *et al.*, 2014). Chronic systemic inflammation is apparent, as is progressive immobility, which both are thought to contribute to the bone loss. Recent data indicate that there are negative changes in the bone microarchitecture, bone strength, and vBMD in AS patients, which will prompt further investigations into the pathogenesis of bone loss in this group, and treatment directions (Nigil Haroon *et al.*, 2015). Bone health, activity levels, and fracture prevention are a significant aspect of caring for this disease, and need to be addressed early in disease course.

Neurologic

Spinal Cord Injury/Immobilization

Spinal cord injury (SCI), or any situation that results in prolonged immobilization and skeletal unloading, is associated with bone loss and an increased risk of fracture (Szollar *et al.*, 1998; Zehnder *et al.*, 2004). While DEXA is an important tool for screening for

low BMD, the most affected areas of the skeleton in SCI patients appear to be the distal femur and proximal tibia, areas not routinely accessible, limiting the value of the test. Initially following SCI there is massive mobilization of calcium from the mineral phase of bone, which can lead to complications such as hypercalcemia and hypercalciuria. Animal studies have shown an increase in osteoclasts and bone resorption surfaces after only 72 h of rat leg immobilization, and decreased trabecular bone volume at 10 days (Weinreb *et al.*, 1989). In human SCI patients, there is a minor initial increase in markers of bone formation with a profound and sustained increase in bone resorption markers that peaks at 10–16 weeks after immobilization (Chantraine *et al.*, 1986; Roberts *et al.*, 1998). Following a stroke and immobilization, sclerostin levels are elevated, implicating a role for antagonizing the Wnt pathway in the skeletal response to mechanical unloading (Gaudio *et al.*, 2010). Any situation where there will be prolonged offloading of the skeleton, such as with injuries or as extreme as with space travel, can cause profound bone density loss and needs to be addressed proactively (Vico *et al.*, 2000).

Parkinson's Disease

Parkinson's disease (PD) is a progressive neurologic disorder associated with reduced mobility and an increased risk of falling (Pickering *et al.*, 2007). Compared to age and sex-matched controls, there is a decrease in femoral neck and lumbar spine BMD, 25-OH vitamin D levels, and an increase in bone turnover markers in patients with PD (Abou-Raya *et al.*, 2009; Ozturk *et al.*, 2016). There is a significantly increased risk of bone fracture in PD, particularly at the proximal femur, which is likely affected by disease duration and severity and propensity for falls (Johnell *et al.*, 1992; Invernizzi *et al.*, 2009). In addition to traditional risk factors affecting this population, such as advanced age and female gender, contribution to bone loss by reduced mobility, low BMI, poor nutrition, and vitamin D deficiency appear to increase the risk of osteoporosis (Vaserman, 2005). Screening BMD and vitamin D levels are recommended at the onset of disease as appropriate therapy may prevent progression of bone loss and risk of fracture (Cummings and Eastell, 2016).

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic demyelinating neurologic condition often leading to profound disability and functional limitation. Low BMD, particularly at the hip, has been consistently shown in MS compared to a control population in both male and female patients (Ozgocmen *et al.*, 2005; Weinstock-Guttman *et al.*, 2004). While factors such as vitamin D deficiency, frequent courses of glucocorticoid therapy, and female gender are considered risk factors, the most important determinants of bone loss are degree of functional impairment, advanced age, and duration of disease (Ayatollahi *et al.*, 2013). In a large multinational longitudinal cohort of women (GLOW), MS was one of the comorbidities that appeared to contribute significantly to fracture risk, though quantifying the specific contribution of MS to fracture risk has yet to be done (Dennison *et al.*, 2012). Vitamin D deficiency is very prevalent in MS, though the extent of its role in the pathogenesis of bone loss in MS is unclear (Nieves *et al.*, 1994).

Hematologic/Oncologic

Multiple Myeloma/Monoclonal Gammopathy of Undetermined Significance

Hematologic malignancies can directly and indirectly affect bone contributing to generalized osteoporosis and pathologic fractures, bone pain, and hypercalcemia. Multiple myeloma (MM) has the highest incidence of bone involvement of all malignant diseases. It is estimated that 70% of patients present with bone pain in MM, and up to 60% of patients will develop a pathologic fracture during their course (Kyle *et al.*, 2003). The skeletal involvement of MM is a major contributor to the morbidity and mortality associated with MM, and up to 90% of patients will develop generalized osteoporosis or lytic bone lesions (Roodman, 2004).

The bone loss in MM is driven by the uncoupling of bone turnover, with marked increase in osteoclastic bone resorption and a decrease in the rate of bone formation, leading to generalized osteolysis. In the bone marrow microenvironment, MM cells produce multiple factors that increase osteoclast production, activity, and survival. By binding to bone marrow stromal cells, MM cells stimulate production of osteoclastogenic cytokines such as RANKL, M-CSF, and IL-6 by the stromal cells and production of osteoclastogenic factors IL-3 and macrophage inflammatory protein 1a (MIP-1a) by the MM cells (Gunn *et al.*, 2006; Giuliani *et al.*, 2004; Choi *et al.*, 2000). The RANKL:OPG axis is altered in MM, with an increase in the ratio observed favoring production and survival of osteoclasts (Giuliani *et al.*, 2001). Osteoblasts are involved in MM-related bone disease with normal or low levels of bone formation markers seen despite increased resorption, as well as decreased osteoid formation and osteoblastic activity observed histologically (Zangari *et al.*, 2011). Recent work has focused on over-expression of DKK-1, a Wnt signaling inhibitor, by plasma cells in MM patients with bone lesions, as well as increased levels of DKK-1 in MM cells and circulating in the plasma (Tian *et al.*, 2003). Consistent with this finding, anti DKK-1 treatment in a murine model of MM resulted in prevention of the suppression of osteoblast number and surface seen in MM, and an increase in the bone formation rate (Heath *et al.*, 2009).

Monoclonal gammopathy of undetermined significance (MGUS) is the most common plasma cell disorder with a prevalence of nearly 7% in the population age 80 or older (Bida *et al.*, 2009). MGUS is a premalignant condition, with roughly a 1% risk per year of progression to MM. Despite the lack of osteolytic lesions seen in MGUS, there is an increased risk of fracture particularly at

axial sites, and the level of the paraprotein in the blood does not seem to correlate with this increased risk (Melton *et al.*, 2004). RANKL levels are elevated in MGUS, and recent studies suggest that there could be bone architectural changes in MGUS, as well as a rise in serum DKK-1 and MIP-1a before progression to MM (Ng *et al.*, 2011; Politou *et al.*, 2004). Measurement of serum protein electrophoresis and immunofixation are important in the evaluation of elderly patients with osteoporosis, particularly those with unexplained fragility fractures, as that may be the first clue to an underlying plasma cell disorder that requires monitoring and potential treatment.

Thalassemia Major

Metabolic bone disease is a known complication of thalassemia major (TM), with the incidence of osteoporosis reported as high as 50%, and an additional 45% of patients have osteopenia (De Sanctis *et al.*, 2013). The precise pathogenesis of bone disease in TM is incompletely understood. Contributing factors include delay in sexual maturation, hypogonadism, decreased growth hormone and IGF-1 levels, diabetes, hypothyroidism, and vitamin D deficiency (De Sanctis *et al.*, 2013). There is a decrease in the activity of osteoblasts and an increase in bone resorption, leading to a decrease in BMD (Perisano *et al.*, 2012). Bone histomorphometry studies in adolescents with TM showed increased osteoid thickness, osteoid maturation time, and mineralization lag time, all of which indicate impaired bone maturation and mineralization (Mahachoklertwattana *et al.*, 2003). Voskaridou *et al.* showed elevated serum levels of Dkk-1 and sclerostin in thalassemic patients compared with healthy controls, as well as increased bone turnover markers, all of which correlated with low BMD (Voskaridou *et al.*, 2012). This may give clues to the pathogenesis of thalassemic bone disease, and sclerostin may make a reasonable therapeutic target. In terms of osteoclastic activity in TM, recent studies have shown elevated circulating levels of RANKL, a relatively preserved levels of OPG, yielding a shift in the RANKL:OPG ratio favoring bone resorption (Morabito *et al.*, 2007). Bone marrow expansion, another feature of TM, can have effects on cortical thickness of bone and increased fragility (Perisano *et al.*, 2012). Delays in recognition of TM-associated bone disease can lead to serious consequences of fractures and skeletal deformities as survival improves, making screening BMD and controlling associated risk factors very important early in the disease. Bisphosphonates are a reasonable starting place for treatment when warranted (Giusti *et al.*, 2016).

Systemic Mastocytosis

In systemic mastocytosis, there is diffuse infiltration of mast cells and their products such as histamine, prostaglandins, leukotrienes, and cytokines (IL-1, IL-3, IL-6) into various tissues of the body. Diagnosis is based on mast cells infiltration on a bone marrow biopsy as well as the finding of a c-kit point mutation or elevated serum tryptase levels. Osteoporosis at the spine, as well as vertebral compression fractures, is a common finding in both men and women with mastocytosis (Rossini *et al.*, 2011). Given the often mild or nonspecific symptoms in indolent mastocytosis, the true incidence of this disease and the magnitude of its contribution to idiopathic osteoporosis is unknown. One series of asymptomatic men with idiopathic osteoporosis showed bone marrow infiltration with mast cells in 9% of cases (Brumsen *et al.*, 2002). The role of mast cells in bone turnover is still being elucidated, but a deficiency appears to induce a low remodeling state, whereas an abundance is associated with accelerated bone loss (Chiappetta and Gruber, 2006). Older age, lower DKK-1 levels, and alcohol use have also been shown to be associated with increased fracture risk (Greene *et al.*, 2016). Serum IL-6 levels are elevated in patients with aggressive mastocytosis, as well as in those with bone pain and osteoporosis, implicating a role for this osteoclastogenic cytokine in the pathogenesis of the bone loss (Theoharides *et al.*, 2002). All patients with systemic mastocytosis warrant BMD testing and evaluation for vertebral fractures, and clinicians need to consider this disease in working up patients with idiopathic osteoporosis, and consider treatment when appropriate with bisphosphonates.

Infectious Disease

Human Immunodeficiency Virus (HIV)

Osteoporosis is common in HIV infection, and has become a more important issue as treatment advances have allowed the HIV-infected population to enjoy longer life expectancy. Up to 70% of patients with HIV have evidence of low bone density, and the risk appears to increase with exposure to antiretroviral therapy (Brown and Qaqish, 2006). Multiple factors contribute to the pathogenesis of bone loss in HIV infection, including smoking and alcohol use, low BMI, chronic inflammatory response, hypogonadism, decreased physical activity, and growth hormone deficiency. There is a significantly increased risk of fracture, with a more than fivefold higher risk of hip fracture compared with a healthy population (Güerri-Fernandez *et al.*, 2013). While bone loss is described independent of medications, use of highly active antiretroviral therapy (HAART) has been associated with higher incidence of osteopenia possibly driven by increased osteoclastic activity and bone resorption and vitamin D deficiency (Marques de Menezes *et al.*, 2016; Nylén *et al.*, 2016; Mastaglia *et al.*, 2017). Within as little as 2 years after initiating HAART, a 2%–6% loss in BMD is seen, as well as an increased risk of fracture, and this effect may stabilize over time (Yin *et al.*, 2012).

Recent attention has focused on the effect of the viral infection itself on pathogenesis of bone loss. Analysis of expression levels of miRNAs in HIV patients showed changes in genes involved in the TGFb and Wnt signaling pathways, implicating potentially

altered osteoblastogenesis and osteoclastogenesis, which requires further study (Del Carpio-Cano *et al.*, 2013). With regards to the effect of the infection, HIV viral protein R and HIV glycoprotein, gp120 can both upregulate RANKL (Fakruddin and Laurence, 2003, 2005). The proinflammatory cytokines induced by HIV infection include TNFa, and OPG, both of which can affect osteoclast development and function (Amorosa and Tebas, 2006). The infection itself can also increase TNF-related apoptosis-inducing ligand (TRAIL) which binds OPG, limiting the capacity of OPG to regulate RANKL-associated osteoclastogenesis (Herbeuval *et al.*, 2005). Combining this with experience of decreased BMD and increased fracture incidence, attention to bone health is of paramount importance in those infected with HIV.

Nephrology

Idiopathic Hypercalciuria

Idiopathic hypercalciuria (IH) is defined as urinary excretion of calcium >4 mg/kg/day in women and >4.5 mg/kg/day in men without any underlying metabolic cause. There is an association between hypercalciuria and low BMD, and the prevalence is increased among Ca-containing stone-formers (Sakhaee *et al.*, 2011; García-Nieto *et al.*, 2003). This is consistent with studies that report a fourfold increased risk of vertebral fracture observed among urolithiasis patients compared with healthy controls (Melton *et al.*, 1998). The deleterious skeletal effects of hypercalciuria in the absence of stone formation is not as well established as in stone formers, and consideration should be given to a radiographic evaluation for asymptomatic stones in osteopenic patients, as this could alter management decisions (Miller, 2012). Clearly bone loss needs to be aggressively addressed in stone forming IH, and the significance of increased urinary Ca in the absence of stone formation needs to be determined by the clinician on a case-by-case basis. Decreased BMD is even seen in children with IH, and is associated with decreased 25-OHD3 levels (Artemiuk *et al.*, 2015).

The precise mechanism of bone loss in IH remains incompletely understood despite recent advances. Bone histomorphometry studies have consistently documented decreased osteoblastic activity, mineralization rates, and osteoid surfaces (Sakhaee *et al.*, 2011; Malluche *et al.*, 1980). IH is characterized by increased intestinal calcium absorption, increased bone resorption, and decreased renal tubular calcium reabsorption (Heilberg and Weisinger, 2006; Pak *et al.*, 2005). In 40%–60% of hypercalciuric stone formers elevated circulating 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) levels are found, as well as increased monocyte expression of vitamin D receptor (VDR) (Coe *et al.*, 1982; Favus *et al.*, 2004). Animal studies have confirmed role of 1,25(OH)2D3 in urinary calcium concentration and decreased BMD (Frick *et al.*, 2013; Ng *et al.*, 2014). The significance of these findings needs to be determined in humans, but they begin to provide insights into potential pathogenic mechanisms of IH-related bone disease.

Psychiatry

Anorexia Nervosa

Anorexia nervosa (AN) is an eating disorder characterized by an obsessive fear of gaining weight and refusal to maintain an adequate body weight, 10 times more common in females than in males. Significant caloric restriction and periods of amenorrhea are the norm. Low BMD is a near universal finding in AN, often occurring in young adolescent females during a time of critical bone mass accrual, with as many as 50% of young females with a z-score of less than -1 (Misra *et al.*, 2004). Studies have documented the detrimental effects of interruption of normal menstruation during adolescence on adult BMD (Wiksten-Almströmer *et al.*, 2009). The risk of fracture during childhood and early adult years is nearly 60% higher for AN females compared with healthy-weight controls (Faje *et al.*, 2014). Contributors to osteoporosis in this population include the low peak bone mass accrual, decreased muscle mass, hypogonadism, decreased IGF-1 levels, and increased cortisol (Misra and Klibanski, 2011). Increased bone marrow adiposity is seen in AN, despite decreased visceral adiposity, and this correlates inversely with BMD as it does in other disease states (Bredella *et al.*, 2009). During treatment of AN, bone density gains were more associated with increased fat mass, rather than increased total body weight (Achamrah *et al.*, 2017). Even in successfully treated AN, adults will have an increased risk of fracture due to the low peak bone mass achieved, and screening for osteoporosis should be considered at a younger age.

Gastrointestinal

Celiac Disease

Celiac disease affects around 1% of the world's population, and is characterized by inflammatory changes in the duodenum with loss of normal small intestinal villi affecting dietary absorption. While classically thought to begin in childhood, it is now more common to be diagnosed as an adult. The effect of celiac disease on bones has been established for a long time, with osteomalacia historically complicating childhood disease, and low BMD affecting all ages. There is as much as a 17-fold higher prevalence of

celiac disease among patients with osteoporosis as compared to controls, suggesting the need for more intensive screening in this population (Stenson *et al.*, 2005). It is estimated that 40% of adults diagnosed with celiac disease will have osteoporosis, affecting both women and men (Meyer *et al.*, 2001). Low bone mass complicates all ends of the celiac spectrum, from severe to asymptomatic disease (Mazure *et al.*, 1994). A recent systematic review reported an increased risk of hip and nonhip fractures in celiac patients compared with controls (Heikkilä *et al.*, 2015).

The pathophysiology of bone loss in celiac disease is multifactorial, centered around inflammation, malabsorption, and decreased intestinal absorption. Decreased absorption of calcium and vitamin D, as well as frequent avoidance of dietary calcium, contribute to a net negative calcium balance and compensatory elevation in PTH (Keaveny *et al.*, 1996; Chakravarthi *et al.*, 2012). The secondary hyperparathyroidism has been correlated with increased bone turnover and decreased BMD (Valdimarsson *et al.*, 2000). There has been a lot of attention recently on intestinal and systemic inflammation in celiac disease, with elevated levels of inflammatory cytokines detected and correlate with bone loss (Fornari *et al.*, 1998). The RANKL:OPG axis has been extensively studied in celiac, and an increased ratio is seen favoring bone resorption (Taranta *et al.*, 2004; Fiore *et al.*, 2006). In addition to the above factors, low BMI, hypogonadism, decreased physical activity often contribute to the bone loss associated with celiac disease. Gluten free diet can improve the status of the bone disease, though the magnitude of the improvement is less in adult patients than in children.

Inflammatory Bowel Disease

Crohn's disease and ulcerative colitis are two forms of chronic inflammatory bowel disease (IBD) and are associated with bone loss, with as many as 40% having evidence of osteopenia (Frei *et al.*, 2006). Risk of fracture appears to be elevated, though the magnitude varies in different epidemiological studies (Vestergaard and Mosekilde, 2002; Loftus *et al.*, 2002; Bernstein *et al.*, 2000). The pathophysiology is multifactorial, including the effect of osteoclastogenic inflammatory cytokines, intestinal malabsorption, low BMI, malnutrition, decreased physical activity, and use of glucocorticoids (Rodriguez-Bores *et al.*, 2007). Low bone mass has been described in new-onset disease prior to any steroid therapy (Lamb *et al.*, 2002). Lower BMD is associated with increased cumulative dose of prednisolone and lower weight in young adults with IBD (Laakso *et al.*, 2014).

Calcium intake and absorption is often poor in this population, and as many as 50% of patients with IBD are vitamin D deficient (Ulitsky *et al.*, 2011; Vernia *et al.*, 2014). In Crohn's disease, the primary tissue involved is at the terminal ileum, where the majority of absorption of fat-soluble vitamins occurs, and resection of the terminal ileum has been described as one of the most important determinants of developing osteoporosis (van Hogezand *et al.*, 2006). The RANKL/OPG system has been implicated in the metabolic bone effects of IBD, as elevated plasma levels of OPG are seen, as well as increased release of OPG from inflamed colonic mucosa (Moschen *et al.*, 2005). The elevated OPG levels correlate with lower BMD, and are felt to be a response to osteopenia and RANKL driven osteoclastogenesis. There is little debate about the development of bone loss in IBD, though the magnitude of the effect is still in question. Routine BMD testing, dietary questioning on calcium intake, and measuring of 25-OH vitamin D levels are recommended for all patients at the onset of disease.

Bariatric Surgery

Obesity now affects nearly 30% of the US population, and using bariatric surgery to treat morbid obesity has become increasingly popular (Ogden *et al.*, 2014; Buchwald and Oien, 2009). Malabsorptive and restrictive bariatric procedures are successful for weight loss and improvement in comorbid conditions such as diabetes and hypertension, but there has been growing recognition of the negative skeletal effects that occur following the surgeries. Decrease in BMD has been consistently demonstrated in the 1 year following bariatric surgery, as high as a 10% loss at the femoral neck and 3% loss at the lumbar spine (Vilarrasa *et al.*, 2009). The decrease in BMD is similar following Roux-en-Y gastric bypass and sleeve gastrectomy procedures (Vilarrasa *et al.*, 2013). Decreased calcium intake and absorption, low vitamin D, secondary hyperparathyroidism are common, though advanced age, menopausal status, and greater lean mass loss seem to correlate more with degree of bone loss. Most studies to date use areal BMD (aBMD) measurements to quantify bone density following surgery, a technique susceptible to artifactual changes in the setting of obesity and profound weight loss, but a recent systematic review showed a significant decline in hip BMD and a trend toward decrease at the spine at 1 year (Kalani *et al.*, 2017).

Bone turnover markers are elevated as quickly as 3 months following bariatric surgery and persist through an 18-month follow-up study (Sinha *et al.*, 2011). This indicates a high turnover state consistent with the effect of elevated PTH. The same study also showed that, even after a transient improvement in vitamin D and PTH levels following surgery (likely from aggressive repletion), they both return to preoperative levels by 1 year. Another intriguing observation is that following gastric bypass surgery plasma ghrelin levels fall, and the degree of this fall correlate with the degree of bone loss over 1 year (Carrasco *et al.*, 2014). Ghrelin is a gut derived peptide involved in energy homeostasis and growth hormone secretion, as well as a direct mitogenic and antiapoptotic effect on osteoblasts (Fukushima *et al.*, 2005). At this point, there are no consistent long-term data on fracture risk following bariatric surgery. In addition to maintaining adequate calcium and vitamin D intake, all patients should have screening BMD done at or before surgery, and have it closely monitored along with markers of bone turnover.

Pulmonary

Chronic Obstructive Lung Disease (COPD)

COPD and osteoporosis share many common risk factors such as smoking use, advanced age, and reduced physical activity (Xiaomei *et al.*, 2014). The addition of chronic steroid use, systemic inflammation, reduced muscle mass, hypogonadism, and vitamin D deficiency augment the risk of bone loss in COPD patients. In a large study of 2699 newly diagnosed COPD patients, the prevalence of osteoporosis was elevated compared to a non-COPD population (RR 3.1) (Soriano *et al.*, 2005). Radiographic emphysema, regardless of other factors or airway obstruction, is a risk factor for bone loss, indicating a possible common pathway between lung parenchymal damage and bone loss (Bon *et al.*, 2011). There is significant body composition change in COPD patients, and the loss of fat-free mass is associated with osteopenia (Bolton *et al.*, 2004). The increased risk of osteoporosis seems to be present in both steroid-users and nonusers, though the rate of fractures is likely higher among those that frequently use oral or inhaled corticosteroids, have more severe COPD, and low muscle mass (de Vries *et al.*, 2005; Loke *et al.*, 2011; Gonçalves *et al.*, 2017). Systemic inflammation in a feature COPD, including elevated levels of osteoclastogenic cytokines such as TNFa and IL-6 (Gan *et al.*, 2004). Vitamin D deficiency is reported in nearly half of patients with COPD, and the level is associated with both BMD and severity of the lung disease (Romme *et al.*, 2013). Adding to the importance of addressing bone health in COPD patients, is the fact that vertebral fractures negatively affect pulmonary function in general, thereby increasing the morbidity of both disease processes (Schlaich *et al.*, 1998).

Transplantation Medicine

Transplantation Osteoporosis

Transplantation is an increasingly viable option for the treatment of end-stage diseases of the kidney, heart, endocrine pancreas, liver, and lung as well as for many hematological disorders. Immunosuppressive medications have improved patient and graft survival, while at the same time allowing for better recognition of long-term complications of transplantation, including osteoporosis and fractures (Ebeling, 2009). Posttransplant bone loss is multifactorial. The primary contributors are the immunosuppressive medications, namely glucocorticoids and calcineurin inhibitors (CIs), though factors such as decreased mobility, poor nutrition, hypogonadism, and the underlying disease all play a role (Epstein *et al.*, 2003). High doses of glucocorticoids are often used, especially in the immediate posttransplant time period, causing significant deleterious effects to bone by mechanisms reviewed above. Cyclosporine (CsA) and tacrolimus (FK506) are the principle CIs used following a transplant, and both have been shown to cause acute, rapid, and severe bone loss (Movsowitz *et al.*, 1988; Kirino *et al.*, 2004). Histomorphometrically, a high-turnover state of bone remodeling was seen, with the resorption far exceeding the bone formation (Movsowitz *et al.*, 1989).

Clinically, the bulk of the bone loss is in the first 6–12 months following transplantation, a time when close monitoring of BMD and other risk factors is of utmost importance. BMD at the spine tends to improve especially at 1 year but cortical bone loss at the femur continues to decline. Fragility fracture rates are generally elevated, and vary depending upon the organ transplanted, osteoporotic BMD, underlying condition, dosage and duration of immunosuppression, and preexisting bone disease. Fractures add significantly to the morbidity associated with transplantation, and every effort should be made to address bone loss in a timely manner in this population.

Drugs Associated With Bone Loss

Depot Medroxyprogesterone Acetate (DMPA)

The injectable contraceptive DMPA (Depo-Provera) has been associated with decreased BMD which has received tremendous attention because of its popularity as an effective form of contraception. In 2004, the US Food and Drug Administration attached a black-box warning to its label, suggesting it should only be used as a long-term form of contraception only of other methods are inadequate despite lack of long term serious skeletal events (Cromer *et al.*, 2006). As a progestin-only contraceptive, it induces a state of estrogen deficiency, which is the primary mechanism of its effect on bones (Cundy *et al.*, 1991). A decrease in BMD is often seen in the first 2 years of therapy, though the loss has been shown to be mild and dose-dependent, worse with prolonged use, and reversible upon discontinuation (Curtis and Martins, 2006; Clark *et al.*, 2006; Kaunitz *et al.*, 2008; Lange *et al.*, 2017). While anything that can negatively affect bone mass, particularly during development and adolescence warrants caution and discussion with patients, the use of DMPA as an effective form of contraception should not be avoided solely on the basis of its mild skeletal effects.

Aromatase Inhibitors

Adjuvant treatment of hormone receptor positive breast cancer with an aromatase inhibitor (AI) is highly effective at decreasing the reoccurrence of the disease in postmenopausal women. By blocking the peripheral conversion of androgens to estrogen, they

reduce endogenous production of estrogen by nearly 90%. This reduction in circulating estrogen has a deleterious effect on the skeleton, and induces a state of increased bone turnover (Eastell *et al.*, 2006). For example, after 5 years of therapy in the Arimidex, Tamoxifen, Alone, or in Combination (ATAC), anastrazole was associated with a decline in BMD at the spine and total hip of 6.08% and 7.24%, respectively (Eastell *et al.*, 2008). A retrospective study of nonosteoporotic AI users over 3 years, not on bisphosphonate therapy, showed a steady decline in BMD as well as trabecular bone score, and altered hip geometry, indicating bone structural changes occur early on in therapy (Hong *et al.*, 2017). There is an increase in the fracture occurrence during AI therapy, that seems to decrease upon cessation of therapy (Forbes *et al.*, 2008). The presence of low BMD prior to treatment seems to affect the fracture incidence, as 9.8% of women with baseline osteoporosis had a fracture during 3 years of AI therapy, compared with 5.6% of those without baseline osteoporosis (Bouvard *et al.*, 2014). Denosumab is proving to be an effective alternative to bisphosphonate therapy for these patients (Nakatsukasa *et al.*, 2017).

Thiazolidinediones

Thiazolidinediones (TZDs) are ligands for PPARγ, and are commonly used in DM2 for improved glycemic control and insulin sensitization. Evidence for the effects of TZDs on bone come from several mouse models, where PPARγ deficiency is associated with increased bone mass, and activation of PPARγ with rosiglitazone resulted in increased marrow adipocytes, reduced bone formation, and decreased expression of osteoblastogenic transcription factors (Ali *et al.*, 2005). In the bone environment, PPARγ controls differentiation of cells of mesenchymal and hematopoietic lineages, and its activation can shift away from bone formation and osteoblastogenesis and increase adipogenesis (Beck *et al.*, 2013). The increase in bone marrow fat is similar to that which is seen with the aging process. Additionally, PPARγ activation can inhibit the Wnt/B-catenin pathway and decrease IGF-1 production, both of which have negative effects on bone formation (Moldes *et al.*, 2003; Lecka-Czernik *et al.*, 2007).

There is accumulating evidence that TZD use is associated with bone loss in humans, particularly in women. In the ADOPT study (A Diabetes Outcome Progression Trial), a randomized controlled trial of 4360 patients with DM2 comparing treatment with rosiglitazone, metformin, and glibenclamide, there was an increased incidence of fractures among women taking rosiglitazone (9.3%) compared with metformin and glibenclamide (5.1% and 3.5%, respectively) (Kahn *et al.*, 2008). In a subset of this study, a significant increase in a serum marker of bone resorption (C-telopeptide) was seen in women taking rosiglitazone, not with the other agents; there was no increase in markers of bone formation (Zinman *et al.*, 2010). A meta-analysis confirmed the increased risk of fracture among female long-term users of TZDs, along with decline of BMD at the total hip and spine of 1%–2% (Loke *et al.*, 2009). Evaluation of bone health should happen before prescribing TZDs for DM, and if fracture risk is elevated, other hypoglycemic medications are preferable.

Acid-Suppressive Medications

Acid-suppressive medications, proton-pump inhibitors (PPIs) and H2 receptor antagonists (H2RAs), are some of the most commonly prescribed medications worldwide. Studies have recently been reporting increased adverse consequences of chronic acid suppression, one of which has been an increased risk of fracture. In a 1997 study of male hip fractures, Grisso *et al.* found an increased risk in those that used cimetidine, an H2RA (Grisso *et al.*, 1997). Several case–control studies have been done since that time and have shown an increased risk of osteoporotic fractures among PPI users (Yang *et al.*, 2006; Vestergaard *et al.*, 2006; Gray *et al.*, 2010). The fracture data on H2RAs have been a bit more inconsistent, with some showing a slight increased risk, and others actually showing a slightly decreased risk. The length of time and dose used appear to increase the adverse skeletal events (Grisso *et al.*, 1997). The effect of chronic acid suppression on BMD is unknown, with stable or mild decreases seen over short-term follow up (Gray *et al.*, 2010; Yu *et al.*, 2008). In that study, females had a higher risk of nonspine fractures, and the increased risk in men was only seen in those with poor calcium intake.

The leading hypothesis for the mechanism of adverse skeletal events in the setting of acid suppression was that it induces a state of hypochlorhydria and affects calcium absorption leading to a negative calcium balance (Graziani *et al.*, 1995). Recent studies have refuted that finding that calcium absorption is the cause (Hansen *et al.*, 2010). The difficulty in controlling for multiple confounding factors has limited our understanding of the full scope of the effect of acid suppressive medications on bone health, and while the overall fracture risk may be small, the large number of people potentially affected make this a serious issue. There is a possible increased risk of fracture in patients cotreated with bisphosphonates and PPIs, and careful consideration of the need for PPIs should be given to these patients (Yang *et al.*, 2015). At this point, it is advisable to recommend optimal calcium intake in people that need PPIs, address other contributors to fracture risk, and limit the use to the lowest dose and shortest time possible. The calcium supplement which may be the most suitable is calcium citrate because of its better absorption profile (Heller *et al.*, 2000).

Antiepileptic Drugs

The association between antiepileptic drugs (AEDs) and metabolic bone abnormalities has been observed since the 1960s, when anticonvulsants and osteomalacia were linked together. Since that time there have been several cohort and prospective studies that have shown an association of AEDs with low BMD and increased fracture risk (Farhat *et al.*, 2002; Vestergaard *et al.*, 2004; Lee *et al.*,

2010). The pathophysiology was felt to be primarily through induction of the cytochrome P450 enzyme system in the liver, which would convert 25(OH)D to an inactive metabolite, leading to less active vitamin D, less calcium absorption, and increase in PTH levels (Meier and Kraenzlin, 2011). In fact, in a randomized trial, Mikati *et al.* showed that using high dose vitamin D supplementation in patients on AEDs could help stabilize the BMD at the spine and total hip compared with low dose vitamin D supplementation (Mikati *et al.*, 2006). Some studies have found an association of lower BMD even with AEDs that do not induce the cytochrome P450 system, suggesting an alternative mechanism. A recent retrospective study of some of the newer anticonvulsants (gabapentin, topiramate, levetiracetam) did not show any adverse effects on BMD (Lee *et al.*, 2012). A meta-analysis of 22 studies showed a significant increase in both enzyme-inducing and nonenzyme-inducing AEDs, particularly with phenytoin, phenobarbital, and topiramate (Shen *et al.*, 2014). Clearly more studies need to be done to definitively link AEDs with bone loss and fractures, and it is recommended to screen for vitamin D deficiency, particularly in those on enzyme-inducing AEDs, and address other aspects of bone health on an individual basis.

Selective Serotonin Reuptake Inhibitors

Selective serotonin reuptake inhibitors (SSRIs) are widely prescribed antidepressant medications that have recently received a lot of attention for negative effects on bone and fracture risk. They act by potently blocking the serotonin transporters (5-HTT) which are located in the CNS as well as in the periphery, such as bone (Bliziotes *et al.*, 2001). While depression itself has been linked with bone loss, cross-sectional studies have supported an association between decreased BMD and SSRI use in both men and women (Diem *et al.*, 2007; Haney *et al.*, 2007; Richards *et al.*, 2007). Longitudinal studies have documented a 1.6-fold greater decline in BMD in those using SSRIs compared with nonuser (Diem *et al.*, 2013). These studies, however, have been contrasted by investigations of several large databases (The National Health and Nutrition Examination Survey III, Women's Health Initiative, and Study of Women's Health Across the Nation) that did not reveal an association of BMD decrease with SSRI use (Spangler *et al.*, 2008; Kinjo *et al.*, 2005). Even though there is discrepancy on the effect of SSRI on BMD, most agree that there is an increased risk of fragility fractures among depressed individuals on SSRIs (Richards *et al.*, 2007; Spangler *et al.*, 2008; Moura *et al.*, 2014).

The exact mechanism of bone loss among users of SSRI is unknown, and recent focus has been on the effect of serotonin on osteoblasts. Circulating serotonin of gut origin was shown to reduce osteoblast proliferation, an effect modulated by LDL-receptor related protein 5 (LRP5) (Yadav *et al.*, 2008). The interaction of LRP5, serotonin, and the Wnt pathway is being elucidated, and will guide our understanding of SSRI use and bone effects (Warden *et al.*, 2010). While not always possible, use of SSRIs should be avoided in those at high risk of fracture.

Heparin

The long-term use of unfractionated heparin has been associated with reduced BMD, increased rates of bone loss, and an increased risk of fracture (de Swiet *et al.*, 1983; Barbour *et al.*, 1994). Using rat histomorphometry and bone turnover markers, it has been shown that extended us of heparin reduces bone formation and increases bone resorption; low molecular weight heparin (LMWH) only seems to reduce bone formation markers, possibly contributing to its lessened effects on bone (Muir *et al.*, 1997). However there has been a recent report of postpartum osteoporosis with fractures in women treated with LMWH during pregnancy (Ozdemir *et al.*, 2015). Recently, long-term use of heparin is mostly limited to treatment of thromboembolism during pregnancy, and as such bone loss should be a consideration in these women.

Antihypertensives

The frequency of use of antihypertensives increases with advanced age, as does the incidence of falling, which has led to numerous studies of the effects of these medications on BMD and fracture risk (Wiens *et al.*, 2006). There is an increased risk of falls when initiating any antihypertensive medication in an elderly patient, particularly within the first few weeks, which has obvious implications on fracture risk (Butt *et al.*, 2013). Diuretics appear to have differential effects on bone based on their mechanism of action, as loop diuretics increase urinary calcium excretion and are associated with elevated markers of bone turnover and lower BMD, while thiazide diuretics decrease urinary loss of calcium and may have protective effects on bone and the risk of fracture (Wiens *et al.*, 2006; Rejnmark *et al.*, 2006). Despite this, a recent prospective study identified increased fracture risk with both thiazide and loop diuretics (Paik *et al.*, 2016). Baseline osteoporosis status and fracture risk should be considered when placing a patient on an antihypertensive medication, especially with the elderly.

Summary

As outlined in this article, there are many disease states and medications that can affect the metabolic activity of bone and its relative strength and risk of fracture. It is important for the clinician to consider the scope of these conditions when evaluating a patient with osteoporosis, as well as keeping bone health in mind when treating patients with these diseases and medications,

even if they fall out of the normal scope of the elderly and postmenopausal women. This approach can help to limit the added morbidity and mortality an osteoporotic fracture to their other comorbid conditions.

See also: A Review of Skeletal Dysplasias for the Pediatric Endocrinologist. Cushing Syndrome; Screening and Differential Diagnosis. Cushing Syndrome—Unilateral Adrenal Adenoma. Long-Term Complications of Hypercortisolism. Medical Therapy of Hypercortisolism. Osteogenesis Imperfecta. Overview of Glucocortcoids. Pediatric Cushing Disease. Thyroid Disease and Bone

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Osteoporosis; Prevention and Ca—Vitamin D Treatment

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Physiology and Development

As the principal repository for calcium in the body, the skeleton is tasked not only with the role of protecting our inner organs and being a scaffold for locomotion but also serves as a necessary source of calcium to maintain normal serum calcium levels during fasting or other times of inadequate supplies of dietary calcium, including compromised active calcium absorption in vitamin D deficiency. Because of the safety implications it is useful to briefly reconsider how calcium homeostasis is regulated in normal physiology.

Keeping serum calcium concentrations within a narrow range is absolutely necessary for normal neuromuscular and cardiac function whereas the body is tolerant to relatively large decreases in bone mineral content, at least in the short term. Accordingly, the normal parathyroid'calcium axis is finely tuned through the sigmoid calcium-PTH curve to respond to small decreases in serum calcium by large and rapid increases in PTH which will increase mobilization of calcium from the skeleton and increase renal reabsorption of calcium. From a practical point of view this means that skeletal calcium content is the junior partner in the relationship between bone and the extracellular fluid, so that the calcium need of the skeleton is sacrificed to maintain adequate serum levels in circumstances of inadequate supply. It is possible to override part of this regulation by treating patients with potent anti-resorptive drugs, in which case the skeletal stores of calcium cannot be mobilized by an increase in PTH and in such circumstances patients may develop hypocalcaemia if not calcium and vitamin D replete.

As shall be briefly addressed below, incorporation of calcium and phosphate into osteoid is an active, vitamin D dependent process so increasing serum calcium will not in itself promote bone mineralization, let alone bone formation. The PTH axis is not so well tuned for dealing with increased serum calcium levels as PTH levels are fully suppressed at very modestly elevated serum calcium levels with no additional decrease possible. However, renal autoregulation responds to hypercalcemia with hypercalciuria and polyuria. The latter reduces the risk of nephrocalcinosis but comes with a risk of dehydration, which may ultimately worsen hypercalcemia. Apart from into the urine and into the skeleton, calcium can be deposited into plaques in atherosclerotic vessel walls or in extreme circumstances—such as renal failure with a high calcium phosphate product—into other soft tissues. It has become clear that vascular calcification, too, is not driven by passive diffusion but results from an active, highly regulated and incompletely understood process as reviewed in detail elsewhere (Evrard *et al.*, 2014).

As for vitamin D, 1,25-OH_2D is of course required both for the mineralization process in newly formed bone and for active calcium absorption from the gut. There are some safeguards in place, however. Firstly, conversion of 25-OHD to 1,25-OH_2D is increased by increasing PTH levels and thus by any declines in serum calcium. Second, given a large enough intake of calcium, the passive, non-vitamin D mediated, absorption of dietary calcium can be considerable though this can only be deposited in the skeleton if 1α-hydroxylated vitamin D and phosphate is present in the bone microenvironment in sufficient amounts. Interestingly, the micro-environment of bone contains a full local apparatus with regulation of hydroxylases in marrow stromal cells by substrate induction and feedback suppression (Zhou *et al.*, 2010), which does not only affect local availability of 1,25-OH_2D but also feeds forward into the FGF23 axis for global phosphate regulation (Nguyen-Yamamoto *et al.*, 2017). From a practical point of view, the buffering of 1,25-OH_2D makes this hormone non-informative as regards whether a person is vitamin D deficient or not. So while 1,25-OH_2D is arguably the most potent entity, serum 25-OH_2D is the clinical indicator of vitamin D status and vitamin D stores.

Mineralization of Bone and Osteomalacia

Osteomalacia denotes a delayed mineralization of newly formed bone tissue. This may have many causes but the most common one and the only one relevant to this article is vitamin D deficiency. By bone biopsy with tetracycline double labelling the diagnosis is simple and straightforward, with the delay in mineralization evident through increased thickness of the osteoid (above 12.5 μm) and a mineralization lag time of over 100 days (Parfitt *et al.*, 2004; Dempster *et al.*, 2013). Clinical clues generally included raised alkaline phosphatase though this can be missing (Bisballe *et al.*, 1991). Patients with nutritional osteomalacia are generally identified not by bone biopsy but by very low serum 25OHD, usually accompanied by raised alkaline phosphatase and PTH. Though a risk factor for development of osteoporosis as the secondary hyperparathyroidism accelerate thinning of trabeculae and cortices with time, osteomalacia is a distinct disease entity and one that is eminently treatable and where the response in terms of BMD increase far outstrips what we see in patients with osteoporosis proper.

Prevention of Development of Osteoporosis

Low calcium diets are unusual in Western Europe and the United States, except in persons with lactose intolerance, but such diets are prevalents in large parts of the world (Balk *et al.*, 2017). In theory, effects of calcium supplements on BMD in the vitamin D

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replete, skeletally mature person should be limited to reversal of deficiency and consist of no more than a filling of resorption space brought about by a reduced PTH drive on bone resorption. A meta-analysis of 51 studies of BMD and calcium supplements, of which 13 studies used calcium in combination with vitamin D (Tai *et al.*, 2015) demonstrated an increase in BMD of 1%–2% with calcium supplementation, chiefly in the first study year. There was no appreciable difference between calcium alone trials and combined (CaD) trials. A similar analysis of vitamin D trials found marginal BMD effects only, with studies showing marked heterogeneity and a potential publication bias favoring positive studies (Reid *et al.*, 2014). In large population based cohorts (Swanson *et al.*, 2015; Cauley *et al.*, 2011), there were found inverse associations between serum vitamin D fracture risk, though interactions were reported with ethnicity in the latter study. Among Danish women, low vitamin D levels appear to be predictive of increases risk of fractures only if secondary hyperparathyroidism is present (Rejnmark *et al.*, 2011).

Interestingly, meaningful preservation of BMD in older persons after vitamin D treatment appear to be confined to those with 25OHD levels below 30 nmol/L at the start of treatment (Reid *et al.*, 2017). In children (Händel *et al.*, 2015), studies of the relationship between calcium intake and fracture risk have produced conflicting results with a possible interaction with race, though milk avoidance was linked to a higher occurrence of childhood fractures. However, it is far from clear that fractures in childhood are indicative of an increased likelihood of osteoporotic fractures in adulthood. Effects of calcium and vitamin D supplementation on BMD in healthy children appear to be small and transitory (Winzenberg et al., 2006, 2010). In adults, studies relating dietary calcium intake to the risk of osteoporotic fractures are dominated by observational studies and these have generally failed to show a clinically meaningful relationship (Bolland *et al.*, 2015).

Treatment of Osteoporosis and Prevention of Osteoporotic Fractures

As a sole treatment, calcium and vitamin D given in combination appears to marginally reduce the risk of osteoporotic fractures in older persons (Avenell *et al.*, 2014). Studies where subjects were recruited based on a prior osteoporotic fracture generally failed to demonstrate reduction in hip fracture risk with this intervention (Avenell *et al.*, 2004; Harwood *et al.*, 2004; Porthouse *et al.*, 2005; Grant *et al.*, 2005) while metaanalysis found a risk reduction averaging 18% (Avenell *et al.*, 2014) collectively for studies in persons not selected based on a prior osteoporotic fracture (Chapuy et al., 1992, 2002; Dawson-Hughes *et al.*, 1997; Salovaara *et al.*, 2010; Jackson *et al.*, 2006), though many of these studies were short of statistical significance for hip fracture outcomes when viewed separately. The risk reduction for fractures in general was much smaller (HR 0.95; 95% CI 0.90–0.99) but in contrast with hip fractures there was no appreciable difference according to prior osteoporotic fracture status (Avenell *et al.*, 2014). Vitamin D given without calcium did not significantly reduce the risk of fractures. We have recently learned that some of the inter-individual differences in anti-fracture efficacy may have a genetic basis. In the Women's Health Initiative (WHI) study (Wang *et al.*, 2017) calcium and vitamin D supplementation only resulted in significant benefits in a genetically defined subpopulation. This difference was seen despite limitations in study design in terms of parallel intake of personal supplements. While interesting, this does not provide useful guidance to physicians at present and of course requires replication in other studies or populations.

Table 1 shows the use of calcium and vitamin D supplements in pivotal trials of key osteoporosis medications in use today. Because calcium and vitamin D supplements were used as a mandatory adjunct to both active treatment and placebo in the great majority of trials there is a lack of high quality evidence that the treatments work equally well if given without supplementation. At the same time, the mandatory co-administration of supplements means that the fracture risk reduction seen with the osteoporosis

Table 1 Calcium and vitamin D used as adjuncts to osteoporosis medications in pivotal RCTs

		Calcium		*Vitamin D*		
Drug	*Trial*	*Mandatory*	*If low*	*Mandatory*	*If low*	*Percent supplemented*
No mandatory supplement						
Alendronate	FIT (Black, Lancet 1996)		500 mg		250 IU	82%
Calcium supplement mandatory						
Risedronate	VERT (Harris, JAMA 1999)	500 mg			500 IU	100%
Calcium and vitamin D supplement mandatory						
Raloxifene	MORE (Ettinger, JAMA 1999)	500 mg		400 + IU		100%
Teriparatide	Neer (NEJM 2001)	1000 mg		400 + IU		100%
Ibandronate	BONE (Delmas, OI 2004)	500 mg		400 IU		100%
Zoledronic acid	HORIZON (Black, NEJM 2007)	1000 + mg		400 + IU		100%
Bazedoxifen	Silverman (JBMR 2008)	<1200 mg		400 + IU		100%
Lasofoxifene	PERL (Cummings, NEJM 2011)	1000 mg		400 + IU		100%
Denosumab	FREEDOM (Cummings, NEJM 2009)	1000 mg +		600–800 IU		100%
Abaloparatide	ACTIVE (Miller, JAMA 2016)	1000 mg +		400–800 IU		100%[a]
Romosozumab	FRAME (Cosman NEJM 2016)	500–1000 mg		600–800 IU		100%

[a]Protocol allowed stopping supplements within certain limits.

medications in the trials represents what can be achieved over and above the effect of calcium and vitamin D supplementation. Finally, these trials of course provide no information on the safety of supplements as they were had to be given in both the active arm and the placebo arm of the studies. Biologically, it is hard to support the notion that dietary sources of calcium and vitamin D would not be equally effective and permit similar efficacy of specific anti-osteoporosis pharmaceuticals but this should be considered lower degree evidence that is, expert opinion rather than experimental evidence.

Safety Concerns

Aside from actual vitamin D intoxication, which remains a rarity, the safety concerns—justified or not—associated with calcium and vitamin D supplements at representative doses of 10–20 μg vitamin D and 500–1000 mg calcium daily are generally cardiovascular or renal (Abrahamsen, 2017). Much of the data available on the safety of calcium and vitamin D supplementation and the comparative safety of dietary intake comes from observational studies (Sluijs *et al.*, 2014; Li *et al.*, 2012; Adebamowo *et al.*, 2015; Yang *et al.*, 2016; Langsetmo *et al.*, 2013; Khan *et al.*, 2015; Paik *et al.*, 2014; Michaelsson *et al.*, 2013) that should not be dismissed but which are vulnerable to unmeasured confounding. We may use supplements because we are particularly health conscious and active, in above average health and with a low rate of disease events compared with that of the background population, or users may be those of us who rightly perceive themselves to be in poor health and nutrition and then try to ward off health consequences including osteoporotic fractures by taking the same supplements. Similar concerns apply to some extent also to dietary intake. Metaanalysis of RCTs suggests excess cardiovascular events in persons randomized to calcium and vitamin D supplementation (Bolland *et al.*, 2011), though the Womens Health Initiative CaD trial, which was the most influential trial in the analysis, was further restricted by exclusion of users who took own supplements. This removed the balancing created by the randomization process and essentially reduces the level of evidence to that of an observational study. There is an ongoing debate and controversy about how best to interpret the imperfect data on calcium and vitamin D safety collected in the clinical trials (Abrahamsen, 2017; Prince and Zhu, 2011; Abrahamsen and Sahota, 2011).

Fortification and the Public Health Perspective

It is important to be aware that vitamin D fortification is far from universal in the Western world and when in place it is generally there to prevent only severe rickets, not to achieve "normal" serum vitamin D levels of 50 or 75 nmol/L. Even so, many European countries have opted not to put in place mandatory food fortification programmes (Spiro and Buttriss, 2014). Hence, only infant milk formula and follow-on formula formally require fortification with vitamin D in the European Union. Sweden, Finland and Iceland fortify some food groups. Low-fat milks are fortified while in Sweden and Iceland while Finland has vitamin D fortification of fat spreads, milk, and milk alternatives. In the United Kingdom, most margarines and fat spreads are fortified with vitamin D though there is no mandatory fortification. Food fortification seeks to provide a small amount of vitamin D—about 5 μg. This is just enough to avoid rickets but will not lead to any risk of toxicity even in persons on highly unusual diets. Recognizing that it is very difficult to tailor fortification programmes to meet the vitamin D needs of housebound elderly people with low overall food intake, vitamin D supplements of 10 or 20μg daily are often recommended by health authorities in groups at particularly high risk of vitamin D deficiency.

Directions for Research

We are fortunate that some large vitamin D trials with multiple health outcomes are in progress and should be reporting in the next years. Hence, first reports from the New Zealand VIDA study—in which 5100 healthy volunteers aged 50–84 years were randomized to 100,000 IU cholecalciferol each month for up to 4 years—have been released in the past months demonstrating a threshold for BMD effects as discussed above (Reid *et al.*, 2017). The supplementation did not prevent falls or fractures (Khaw *et al.*, 2017). In the ongoing VITAL trial (Bassuk *et al.*, 2016; LeBoff *et al.*, 2015), 25,875 trial men and women were randomized in a 2 × 2 factorial design to a double-blind, placebo-controlled intervention consisting of cholecalciferol 2000 IU/d and Omacor fish oil (1 g/d) with the primary outcomes being prevention of cancer and CVD. Finally, the D-Health study in Australia has recruited 21,315 participants for a 5 years intervention study of 60,000 IU monthly cholecalciferol or placebo with 5 years additional register-based follow-up, aimed at capturing mortality and cancer outcomes (Neale *et al.*, 2016). These studies are likely to shed new light on the pros and cons of vitamin D supplementation on large disease areas but no studies are in place to better understand the role of calcium supplementation (Abrahamsen, 2017). This should be a major research priority given the considerable scientific uncertainty, which leaves clinicians at a loss of how best to advise their patients and the topic is also of importance in the light of low calcium diets being the order of the day in large parts of Asia and South America (Balk *et al.*, 2017).

How to Act in Daily Clinical Practice

There is ample evidence that very low vitamin D levels should be avoided as they translate to a higher risk of low BMD and fractures. Osteomalacia is imminently treatable and results in improvements in BMD that surpasses what can be achieved in osteoporosis. There is no consistent evidence that vitamin D supplementation given without calcium supplementation reduces the risk of osteoporotic fractures in the general population but some evidence that small reductions in fracture risk can be achieved in older people if supplemented by modest amounts of calcium and vitamin D, likely due to a large effect in a small number of people and no effect in the majority. Given the relatively low quality RCT evidence it is difficult to be dogmatic about the need for calcium and vitamin D supplements, at least in healthy people in the Western hemisphere with a varied diet. There seems to be a tendency for prescribers to use somewhat small calcium and vitamin D supplement amount as adjuncts to osteoporosis medications compared with what was used in the pivotal RCTs; this pragmatic approach is not unreasonable though it is nominally a deviation from the principles of evidence based medicine. If we have learned one thing from the past two decades of vitamin D and calcium research then it is that one size does not fit all and that supplements should be given according to individual requirements.

See also: Physical Activity and Exercise

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Osteoporosis Treatment: Bone-Forming Agents

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Glossary

Antiresorptive osteoporosis treatments Treatments that improve bone mass and reduce fracture risk by inhibition of bone resorption. This group includes bisphosphonates, RANKL antibody, estrogen, testosterone, and selective estrogen receptor modulators.

Bone mineral density (BMD) The mineral content in bone measured by DXA (dual energy Xray absorptiometry) or quantitative computer tomography (qCT).

Bone remodeling A coupled process of bone resorption followed by bone formation, which maintains skeletal integrity throughout adulthood. In osteoporosis, bone resorption usually exceeds bone formation, leading to bone loss.

Osteoporosis Osteoporosis is a systemic skeletal disease, characterized by reduced bone mass and deteriorated bone architecture, resulting in an increased risk of fractures. Osteoporosis is defined by WHO as BMD more than 2.5 standard deviations below peak bone mass (T-score < -2.5).

Parathyroid hormone (PTH) An 84 peptide hormone produced by the parathyroid glands.

Sclerostin A peptide produced almost exclusively by the osteocytes. Sclerostin binds LRP5 and other agonists of the wnt-pathway, and thereby inhibits osteoblast activity and bone formation.

Introduction

Antiresorptive treatments are well established for the treatment of osteoporosis. They increase BMD and prevent fractures—vertebral fractures more so than hip and nonvertebral fractures. Some patients with severe osteoporosis, many fractures, and/or very low BMD or patients diagnosed at a young age with osteoporosis are difficult to treat optimally with antiresorptives, as fractures often reoccur in these patients. There is thus a need for new treatments that can increase bone mass and perhaps more importantly restore bone architecture (**Table 1**).

There are currently two approved treatments in this category: teriparatide and abaloparatide. At the time of writing, abaloparatide is only approved in the United States. A new treatment, romosozumab, has just recently finished the phase III clinical trial program and is undergoing evaluation by the authorities for approval.

Teriparatide

Both parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP) exert their effects on bone by activating the PTH type 1 receptor (PTH1R) (Juppner *et al.*, 1991). It has been recognized that the effect of activation of the receptor differs whether the activation is sustained or intermittent. Sustained activation as seen in primary hyperparathyroidism activates both osteoblasts and osteoclasts, and increases bone formation as well as bone resorption. Intermittent activation, on the other hand, activates primarily osteoblasts and therefore primarily increases bone formation (Tam *et al.*, 1982). The first approved PTH-based treatment for osteoporosis is teriparatide (Dempster *et al.*, 1993). Teriparatide is the first 34 amino acids of the natural occurring PTH. Teriparatide is a peptide and therefore has to be administered parentally to avoid destruction in the stomach and proximal intestine if administered orally. The half-life of teriparatide is short; 60 min, and teriparatide is therefore administered as a daily subcutaneous injection, usually by the patients themselves (Satterwhite *et al.*, 2010). Teriparatide primarily stimulates recruitment and activity of the osteoblasts, and thereby increases bone formation. But since bone formation and bone resorption are physiologically coupled processes, bone resorption is subsequently stimulated, causing the net anabolic response to level off over time (McClung *et al.*, 2005).

The effects of teriparatide were first examined in a placebo-controlled study (Neer *et al.*, 2001) comprising women with severe osteoporosis, defined as having two or more vertebral fractures, or one vertebral fracture in combination with reduced bone mass (T-score < -1). The women were randomized to teriparatide in two different doses or placebo. All women received calcium and vitamin D substitution. The study was planned for 3 years but was stopped prematurely after a median study period of 21 months, because preclinical studies showing an increased risk for osteosarcoma in rats treated early in life or lifelong with teriparatide (Vahle *et al.*, 2004). The clinical study demonstrated robust increases in BMD: 9.7% at the lumbar spine and 2.8% at the femoral neck in women treated with teriparatide 20 μg daily. The risk of morphometric vertebral fractures and nonvertebral fractures was reduced by 65% and 53%, respectively. Adverse effects were hypercalcemia, dizziness, and leg cramps. Posthoc analyses revealed that the effect on nonvertebral fractures was positively correlated with treatment duration (Lindsay *et al.*, 2009). Since then, head-

Table 1 Summary of the main effects seen in clinical trials on bone mineral density (BMD) and fracture risk reduction of anabolic treatments of osteoporosis

Treatment	*Patients*	*Pharmacodynamics*	*Comparator*	*Increase in BMD*	*Fracture risk reduction*
Teriparatide	Postmenopausal women with prior vertebral fractures	Intermittent activation of the PTH type 1 receptor and thereby activation of osteoblasts	Placebo (21 months)	lsBMD 9.7%[a] fnBMD 2.8%[a]	Vertebral 65%[1] Nonvertebral 53%[a]
Teriparatide	Patients treated with glucocorticoids	Intermittent activation of the PTH type 1 receptor and thereby activation of osteoblasts	Alendronate (18 months)	lsBMD 7.2%[b] thBMD 3.8%[b]	NA
Abaloparatide	Postmenopausal women with osteoporosis	Intermittent activation of the PTH type 1 receptor and thereby activation of osteoblasts	Placebo (18 months) Teriparatide All had alendronate for additional 6 months	lsBMD 11.2%[c] thBMD 4.2%[c] lsBMD 12.8%[d] thBMD 5.5%[d]	Vertebral 84%[c] Nonvertebral 43%[c] Vertebral 87%[d] Nonvertebral 52%[d]
Romosozumab	Postmenopausal women with osteoporosis	Inhibition of sclerostin that inhibits the osteoblast and stimulates osteoclast recruitment	Placebo (12 months) All had denosumab during year 2	lsBMD 13.3%[e] thBMD 6.8%[e] lsBMD 13.3%[e] thBMD 6.8%[e]	Vertebral 73%[5] Nonvertebral ns[e] Vertebral 75%[5] Nonvertebral ns[e]
Romosozumab	Postmenopausal women with severe osteoporosis	Inhibition of sclerostin that inhibits the osteoblast and stimulates osteoclast recruitment	Alendronate (12 months) All had alendronate during year 2	lsBMD 13.7%[f] thBMD 6.2%[f] lsBMD 15.2%[f] thBMD 7.1%[f]	Vertebral 37%[6] Nonvertebral ns[f] Vertebral 48%[6] Nonvertebral 48%[f]
Combination therapy	Anabolic and antiresorptive	Inhibition of RANKL and thereby recruitment of osteoclasts (Denosumab) and intermittant activation of the PTH type 1 receptor and thereby activation of osteoblasts (Teriparatide)	Teriparatide (24 months) Denosumab (24 months)	lsBMD 12.9%[g] thBMD 6.3%[g]	NA

[a]Teriparatide 20 μg daily for 21 months (Neer *et al.*, 2001).
[b]Teriparatide 20 μg daily in comparison with Alendronate 10 mg daily for 18 months (Saag *et al.*, 2007).
[c]Abaloparatide 80 μg daily for 18 month (Miller *et al.*, 2016).
[d]Abaloparatide 80 μg daily for 18 month, all participants were treated with Alendronate for an additional 6 months (Cosman *et al.*, 2017).
[e]Romosozumab 210 mg monthly for 12 months, all participants received denosumab during the second year (Cosman *et al.*, 2016).
[f]Romosozumab 210 mg monthly in comparison with alendronate 70 mg weekly for 12 months, all patients were treated with Alendronate during the second year (Saag *et al.*, 2017).
[g]Denosumab 60 mg every 6 months in combination with teriparatide 20 μg daily for 24 months (Tsai *et al.*, 2013).
lsBMD: lumbar spine BMD, *fnBMD*: femoral neck BMD, *thBMD*: total hip BMD, *NA*: data not available.

to-head comparisons with antiresorptive treatments have been performed. Hadji et al. carried out a study investigating the effect of teriparatide in comparison with risedronate on back pain in postmenopausal women with prevalent vertebral fractures. The study demonstrated more robust increases in bone mass and a significant reduction in new vertebral fractures with teriparatide in comparison with risedronate (Hadji *et al.*, 2012). The VERO study compared the antifracture efficacy of teriparatide and risedronate in postmenopausal women with severe osteoporosis, and demonstrated that teriparatide is superior to risedronate for the prevention of vertebral, multiple vertebral, and clinical fractures (Kendler Dl *et al.*, 2017).

Histomorphometric and microCT-based investigations of bone biopsies from patients treated with teriparatide have demonstrated improvements in bone architecture (Jiang *et al.*, 2003). This has been confirmed by CT-based analyses of vertebrae and hips in patients. These analyses have revealed an increase in estimated bone strength by finite element analyses (Graeff *et al.*, 2009; Borggrefe *et al.*, 2010).

Teriparatide has also been investigated in men with severe osteoporosis (Orwoll *et al.*, 2003). This study, just like the study in postmenopausal women, was stopped prematurely and the median treatment duration was 12 months. No data on the effect on fracture are available; however, the changes seen in spine and hip BMD were similar to the increases seen in postmenopausal women, and teriparatide is therefore also approved for the treatment of male osteoporosis. Finally, the effect of teriparatide has been investigated in patients treated with glucocorticoids. Glucocorticoid-induced osteoporosis is pathophysiologically characterized by two phases. The first phase is characterized by stimulation of osteoclasts and bone resorption, whereas the second and permanent phase is characterized by inhibition of osteoblasts and osteocytes (Compston, 2010). In patients who had received glucocorticoids for more than 3 months and therefore are likely to be characterized by reduced bone formation, the effect of teriparatide in comparison with alendronate was investigated (Saag *et al.*, 2007). Bone mass at the spine and hip increased significantly more with teriparatide than with alendronate. Fractures were not an endpoint in this study; however, 10 vertebral fractures were seen in 165 patients (6.1%) treated with alendronate compared to 1 vertebral fracture in 171 patients (0.6%) treated with teriparatide. The difference was thus statistically significant.

In summary, teriparatide is a bone-forming treatment that increases bone mass at both trabecular and cortical sites, and prevents vertebral and nonvertebral fractures. The treatment duration is limited to 24 months and should be followed by an antiresorptive treatment. Teriparatide has been investigated in postmenopausal women, men, and patients treated with glucocorticoids.

Abaloparatide

The PTH1R has two different high-affinity conformations termed R^0 and RG, and responses of prolonged duration are observed with ligands that bind efficiently to the R^0 state, whereas short-duration responses are seen with ligands that bind more selectively to the RG state (Hoare *et al.*, 1999; Dean *et al.*, 2008). Abaloparatide is PTHrP (1–34) with some modifications (Hattersley *et al.*, 2016). Early studies showed that it induces bone formation without stimulating resorption and causing hypercalcemia (Culler *et al.*, 2001); it has also demonstrated that this effect may be due to a lower affinity for the RG conformation and thus a short activation period. The affinity to the RG conformation was shown to be lower than that of teriparatide (Hattersley *et al.*, 2016). In a phase III trial 2463 postmenopausal women were randomized to abaloparatide 80 μg daily, teriparatide 20 μg daily, or a placebo for 18 months (Miller *et al.*, 2016). Abaloparatide and teriparatide both increased BMD at the lumbar spine and hip sites compared to the placebo. Abaloparatide increased BMD more at the hip sites throughout the study period and initially also at the lumbar spine. In addition, the effect on biochemical markers of bone formation and resorption differed between the two treatments. Teriparatide stimulated bone formation and resorption throughout the study period, whereas the effect of abaloparatide on bone formation was less prominent after the first 6 months. Also, resorption was less increased with abaloparatide compared with teriparatide. Abaloparatide and teriparatide reduced the risk of new vertebral fractures by 84% and 80%, respectively. Abaloparatide reduced the risk of nonvertebral fractures by 43%, which was significant; however, the reduction was not different from the nonsignificant reduction seen with teriparatide. Abaloparatide and teriparatide reduced the risk of major osteoporotic fractures (upper arm, forearm including wrist, hip, shoulder, spine) by 67% and 30%, respectively. The reduction seen with abaloparatide was significantly different from the placebo and teriparatide, whereas the effect of teriparatide was not significantly different from the placebo. More women discontinued abaloparatide (9.9%) compared with the placebo (6.1%) and teriparatide (6.8%) due to adverse events. Hypercalcemia was less frequent with abaloparatide (3.4%) than with teriparatide (6.4%), but more common than with the placebo (0.4%).

The study was extended for an additional 6 months for the patients who had been treated with abaloparatide and placebo during the first 18 months. During the additional 6 months, all patients were treated with alendronate 70 mg weekly. BMD at the spine and hip sites increased in both groups (Cosman *et al.*, 2017). The reductions in vertebral, nonvertebral, and major osteoporotic fractures in women treated with abaloparatide/alendronate in comparison with women treated with placebo/alendronate were 87%, 52%, and 58%, respectively.

In summary, abaloparatide is a bone-forming treatment that increases BMD at both cortical and trabecular sites, and may be superior to teriparatide in preventing some types of fractures. Abaloparatide has not been investigated in men with osteoporosis or patients with glucocorticoid-induced osteoporosis.

Romosozumab

Bone formation by the osteoblasts can be stimulated via the canonical wnt-pathway in which lipoprotein-related peptide (LRP) 5 and −6 binds to the frizzled receptor and promotes bone formation (Poole *et al.*, 2005). The osteocytes are terminally differentiated osteoblasts that are imbedded within the bone matrix and control bone formation by producing sclerostin that prevents the binding of LRP5 and −6 to the frizzled receptor and thereby *inhibits* bone formation (Van Bezooijen *et al.*, 2004). Sclerostin also stimulates the release of RANKL by osteocytes and thereby the recruitment and activation of osteoclasts. Sclerostin was identified as the result of genetic analysis of families with sclerosteosis (Beighton *et al.*, 1976). Sclerosteosis is a recessive disease characterized by skeletal overgrowth, syndactyly, and nerve compression syndromes. Linkage analysis of families with sclerosteosis

and the related syndrome of Van Buchem disease localized both disease genes to chromosome 17q12–q21 (Balemans *et al.*, 1999) and subsequent positional cloning identified loss of function mutations in the SOST gene, which encodes sclerostin as the cause of sclerosteosis (Balemans *et al.*, 2001; Brunkow *et al.*, 2001). Given that individuals with loss of function mutations affecting SOST have a phenotype restricted to bone, sclerostin emerged as a highly attractive therapeutic target for the treatment of osteoporosis. Accordingly, neutralizing antibodies that act as inhibitors of sclerostin (McColm *et al.*, 2014; Padhi *et al.*, 2011) have emerged as highly promising agents for the treatment of osteoporosis.

Romosozumab is an antibody against sclerostin that has demonstrated bone-forming potential (Padhi *et al.*, 2011). The effect of romosozumab on bone turnover and BMD has been investigated in women in a phase II trial (McClung *et al.*, 2014). The women were randomized to treatment for 12 months with one of five different doses of romosozumab either monthly or 3-monthly, alendronate 70 mg weekly, teriparatide 20 mg daily, or a placebo. Treatment with romosozumab 210 mg monthly increased the bone formation marker, serum PINP by 91% after 1 month, but the increase leveled off over the following months, and at the end of the treatment period, serum PINP was 20% below baseline level. In addition, the bone resorption marker serum CTX decreased by 41% 1 week after administration of the first dose of romosozumab and then slightly increased to a level 26% below baseline at 12 months. Thus, romosozumab appears not only to stimulate formation but also to inhibit resorption. The fact that formation is decreased after 12 months is somewhat surprising, but it has been suggested to be caused by depletion of osteoblast progenitors or a compensatory increase in other inhibitors of bone formation such as dickkopf (Ferrari, 2014). The suppression of bone resorption is most likely caused by a reduction in the osteocyte production of RANKL due to the inhibition of sclerostin (Atkins and Findlay, 2012). Romosozumab 210 mg monthly increased lumbar spine BMD 11.3% which was significantly more than teriparatide, alendronate, and the placebo, which increased lumbar spine BMD by 7.1%, 4.1%, and 0.1%, respectively. A similar pattern was seen at the total hip where BMD changed by 4.1%, 1.3%, 1.9%, and -0.7% in women treated with romosozumab, teriparatide, alendronate, or a placebo, respectively. The effect of romosozumab and teriparatide on trabecular and cortical bone was further investigated in substudies of this phase II study using qCT of the spine and hip. These analyses demonstrated that romosozumab improves estimated bone strength both at the spine and hip more than teriparatide (Keaveny *et al.*, 2017; Genant *et al.*, 2017).

The effects of romosozumab have been further investigated in two phase III trials. The FRAME study investigated 7180 women with osteoporosis and found that treatment with romosozumab for 12 months increased BMD at the spine and total hip by 13.3% and 6.8%, respectively, and reduced the risk of vertebral and clinical fractures by 73% and 36%, respectively, in comparison with a placebo (Cosman *et al.*, 2016). The study was extended for a second year, where all patients received denosumab 60 mg every 6 months. This led to further increases in BMD, and the difference in fracture rates seen between the two treatment groups in the first year was maintained. The ARCH study included 4093 women with severe osteoporosis and investigated the effect of romosozumab in comparison with alendronate for 12 months. In the second year, all women received alendronate (Saag *et al.*, 2017). Romosozumab followed by alendronate increased BMD at the lumbar spine and total hip by 15.2% and 7.1%, respectively, and reduced the risk of vertebral, hip, and nonvertebral fractures by 48%, 38%, and 19%, respectively, in comparison with alendronate. A numeric imbalance in serious adverse cardiovascular events was observed during the first 12 months in the ARCH study, with more events seen in the romosozumab-treated women.

In the clinical setting, bone-forming agents are often not used first line, but only after insufficient response or treatment failure with antiresorptive treatments. The STRUCTURE study compared the effect of teriparatide and romosozumab for 12 months in women with osteoporosis previously treated with bisphosphonates (Langdahl *et al.*, 2017). The study demonstrated that both treatments substantially stimulated bone formation at the spine. At the hip sites, the responses to the two treatments were different. Romosozumab stimulated BMD and bone strength, estimated by finite element analysis of CT scans at the hip, whereas BMD and bone strength did not improve with teriparatide. This difference can probably be explained by the different mechanism of action of the two treatments: teriparatide stimulates bone formation and subsequently bone resorption, whereas romosozumab stimulates bone formation and at the same time inhibits bone resorption. The study duration was only 12 months and usually teriparatide is used for 24 months.

In summary, romosozumab represents a new treatment concept in osteoporosis: dual-acting as it appears to stimulate formation as well as inhibit resorption. It increases BMD substantially at both trabecular and cortical sites, and demonstrates strong antifracture efficacy that in one study was superior to the effect seen with alendronate.

Combination Treatment

Throughout the years, several treatments have been available for osteoporosis, and therefore an even larger number of combinations of therapies have been possible. Attempts have been made to demonstrate additive or synergistic effects of different combinations, but initially with disappointing results. Thus, in the PaTH trial, BMD in patients treated with a combination of teriparatide and alendronate did not increase more than with either drug alone. In fact, alendronate even appeared to impair the bone-forming effect of teriparatide (Black *et al.*, 2003; Finkelstein *et al.*, 2003). Similar results were found in a study combining risedronate and teriparatide (Walker *et al.*, 2013). In accordance with these findings, a rodent study showed that chronic exposure to a bisphosphonate blunted the response to teriparatide (Gasser *et al.*, 2000). It was suggested that this was caused by the osteoblasts being exposed to bisphosphonates while in the circulation. In accordance with this hypothesis, it was shown that a single infusion of zoledronic acid (which is rapidly cleared from the circulation) in combination with daily teriparatide for 1 year

increased lumbar spine and total hip BMD by 7.5% and 2.3%, respectively, whereas zoledronic acid alone resulted in increases of 4.4% and 2.2%, respectively, and teriparatide alone provided increases of 7.0% and 1.1%, respectively (Cosman *et al.*, 2011).

Recently, in the DATA trial the combination of teriparatide 20 μg daily and denosumab 60 mg every 6 months was compared with either treatment alone (Tsai *et al.*, 2013). After 24 months, BMD at the lumbar spine had increased by 12.9%, 9.5%, and 8.3%, and total hip BMD increased by 6.3%, 2.0%, and 3.2% in the combination, teriparatide, and denosumab groups, respectively. Taken together, the studies suggest that a combination of teriparatide and zoledronic acid gives "the best of both worlds," the significant increase in hip BMD seen with zoledronic acid combined with the significant increase in spine BMD seen with teriparatide, whereas the combination of denosumab and teriparatide appears even to have an additive or synergistic effect. None of the studies, however, was powered to allow for conclusions regarding antifracture efficacy.

Conclusion

A number of exciting drugs for the treatment of osteoporosis are emerging. In particular, the new anabolic options (romosozumab and abaloparatide) are promising, as they efficiently increase both hip and spine BMD and moreover may have greater antifracture efficacy than teriparatide.

The DATA trial demonstrated for the first time an additive effect of anabolic and antiresorptive therapy on BMD; however, antifracture efficacy with combination therapy remains to be demonstrated.

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Osteoporosis Treatment: Sequential and Combination Therapy

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Glossary

Abaloparatide An anabolic drug that is a parathyroid hormone-related protein (PTHrP) analog.

Bisphosphonate A pyrophosphate analog compound with high affinity to hydroxyapatite that causes osteoclast inhibition.

Denosumab A monoclonal antibody that inhibits the receptor activator of nuclear factor kappa-B ligand (RANKL) and thereby inhibits the maturation of osteoclasts.

Romosozumab An anabolic monoclonal antibody that targets sclerostin, a Wnt antagonist that blocks osteoblast proliferation and function.

Teriparatide An anabolic drug that contains the first N-terminal 34 amino acids of human parathyroid hormone (PTH).

Introduction

Osteoporosis drugs may be categorized as either antiresorptive or anabolic, though these labels are not precise as all currently available antiresorptive agents also inhibit bone formation and all currently available anabolic agents also stimulate bone resorption. In contrast to many other chronic conditions, such as hypertension, that often warrant multidrug regimens, the standard of care for osteoporosis is monotherapy. It remains the case, however, that no single agent is curative for osteoporosis and thus potential combination treatment strategies continue to be actively investigated. Early trials combining two antiresorptives drugs (generally oral bisphosphonate with hormonal agents) were not found to be more effective than monotherapy and thus the more recent focus of study has been in combining antiresorptive and anabolic drugs, an approach that was hypothesized to benefit from their contrasting mechanisms of action (Bone *et al.*, 2000; Greenspan *et al.*, 2003; Harris *et al.*, 2001; Lindsay *et al.*, 1999; Palomba *et al.*, 2002; Sanad *et al.*, 2011; Tiras *et al.*, 2000; Tseng *et al.*, 2006; Wimalawansa, 1995). Notably, the past decade has also seen advances in our understanding of the importance of the order of antiresorptive and anabolic treatment and in how drug sequence may influence long-term efficacy. This article reviews the published clinical trials that have assessed the efficacy of various antiresorptive and anabolic combinations and sequences in the treatment of postmenopausal osteoporosis.

Combination Antiresorptive and Anabolic Treatment

Parathyroid hormone (PTH) analogs, such as teriparatide (PTH 1–34), increase bone mineral density (BMD), improve trabecular microarchitecture, and reduce fracture risk (Neer *et al.*, 2001; Black *et al.*, 2003; Tsai *et al.*, 2016; Hansen *et al.*, 2013). As discussed above, however, it has been hypothesized that limiting the bone-resorbing effects of PTH by the co-administration of an antiresorptive drug may improve its therapeutic effect, particularly in cortical bone (Uihlein and Leder, 2012). The combinations that have been investigated include combining PTH analogs (PTH 1–84 and teriparatide) with (1) estrogen and selective estrogen receptor modulators, (2) oral and intravenous bisphosphonates, and (3) denosumab, a monoclonal antibody that inhibits the receptor activator of nuclear factor kappa-B ligand (RANKL) (Deal *et al.*, 2005; Cosman *et al.*, 2001, 2011; Lindsay *et al.*, 1997; Ste-Marie *et al.*, 2006; Black *et al.*, 2007; Finkelstein *et al.*, 2010; Schafer *et al.*, 2012; Tsai *et al.*, 2013; Leder *et al.*, 2014).

Combination of Estrogen or Selective Estrogen Receptor Modulators and PTH Analogs

Combinations of PTH analogs with estrogens or raloxifene, a selective estrogen receptor modulator, have been studied in multiple small clinical trials, most of which examined the effect of adding a PTH analog to ongoing hormone replacement therapy (Deal *et al.*, 2005; Cosman *et al.*, 2001; Lindsay *et al.*, 1997; Ste-Marie *et al.*, 2006). Conclusions about the efficacy of this combination, however, are limited as these studies generally lacked monotherapy comparison groups. Similarly, in a double-blind, placebo-controlled trial study of 137 postmenopausal women randomized to receive teriparatide 20-mcg daily with either raloxifene 60-mg daily or placebo for 6 months, combination treatment increased total hip BMD more than teriparatide monotherapy but a raloxifene monotherapy control group was not included (Deal *et al.*, 2005). The short duration of this latter study also limits clinically relevant conclusions.

Combination of Bisphosphonates and PTH Analogs

The majority of combination therapy trials have focused on combinations of PTH analogs and bisphosphonates. In the parathyroid hormone and alendronate (PaTH) study, 238 postmenopausal women with osteoporosis were randomized to receive

Table 1 Comparison of 12-month BMD changes in three randomized controlled trials of combination therapy with concurrent initiation of antiresorptive and anabolic drugs

Study	*Sample size*	*Regimen*	*12-month % change at posterior-anterior spine*	*12-month % change at total hip*
Black et al. (2003)	119	PTH 1–84	6.3	0.3
	60	Alendronate	4.6	~3
	59	Both	6.1	1.9
Cosman et al. (2011)	138	Teriparatide (with placebo IV)	7.0	1.1
	137	Zoledronic acid	4.4	2.2
	137	Both	7.5	2.3
Tsai et al. (2013)	31	Teriparatide	6.2	0.7
	33	Denosumab	5.5	2.5
	30	Both	9.1	4.9

1 year of PTH 1–84100-mcg daily, alendronate 10-mg daily, or both (Black *et al.*, 2003). After 12 months, spine BMD increased similarly in all three groups (**Table 1**), whereas at the total hip, combination therapy increased BMD more than the PTH 1–84 alone but similarly to alendronate (**Table 1**). Importantly, PTH monotherapy increased spine trabecular volumetric BMD (vBMD) nearly twofold more than combination therapy (as assessed by QCT). At the hip, trabecular vBMD increased similarly among the three groups whereas cortical vBMD decreased with PTH 1–84, remained unchanged with combination treatment, and increased with alendronate. While early increases in bone formation marker type I collagen propeptide (P1NP) were observed with combination therapy, these increases were not sustained over 12 months. Moreover, combination treatment suppressed bone resorption marker serum c-telopeptide (CTX) significantly less than alendronate monotherapy. Qualitatively similar findings were also reported in separate studies assessing the combination of alendronate and teriparatide (at 40 mcg daily, double the FDA approved dose) and the combination of ibandronate and teriparatide (Finkelstein *et al.*, 2010; Schafer *et al.*, 2012). The combination of teriparatide and an intravenous bisphosphonate was assessed in a clinical trial of 412 postmenopausal women randomized to receive 12-months of teriparatide 20-mcg daily, zoledronic acid 5-mg, or both (Cosman *et al.*, 2011). While spine BMD increased more in the combination group at early time points, gains at 12-months were similar in the combination therapy and teriparatide monotherapy groups (**Table 1**). Combination therapy also demonstrated larger early BMD increases at the hip, though by 12-months gains were equivalent in the combination therapy and zoledronic acid monotherapy groups. Of note, in patients treated with combination therapy, serum CTX was suppressed initially but increased above baseline values at month-12.

Taken together, these trials demonstrate that combinations of PTH analogs and bisphosphonates do not provide significant additive skeletal effects in postmenopausal women. Based on the bone turnover marker results described above, it was hypothesized that the lack of benefit was due to the key role bone resorption plays in mediating the anabolic effects of PTH analogs, though the inability of bisphosphonates to fully inhibit PTH analog-induced bone resorption provides an alternate explanation.

Combination of Denosumab and Teriparatide

In contrast to combinations of PTH analogs and bisphosphonates, the combination of denosumab and teriparatide has clear additive effects on BMD. In the Denosumab and Teriparatide Administration (DATA) trial, 94 osteoporotic postmenopausal women were randomized to receive teriparatide 20-mcg daily, denosumab 60-mg every 6 months or both for 2 years (Leder *et al.*, 2014). Combination treatment increased spine, total hip, and femoral neck BMD more than either group after 1 year and this advantage was maintained during the second year (**Table 1** and **Fig. 1**) (Tsai *et al.*, 2013; Leder *et al.*, 2014). The 2-year net gains in spine and hip BMD realized with combined denosumab/teriparatide were larger than can currently be achieved with 2-year courses of any approved single drug (Neer *et al.*, 2001; Bolognese *et al.*, 2012; Cosman *et al.*, 2017; Black *et al.*, 1996; Harris *et al.*, 1999; Ettinger *et al.*, 1999). Similar additive effects of combined teriparatide/denosumab were observed in volumetric BMD, skeletal microarchitecture, and estimated bone strength of the distal tibia and radius as assessed by high-resolution peripheral QCT (HR-pQCT) (Tsai *et al.*, 2016). Specifically, combined teriparatide/denosumab increased total vBMD and cortical thickness more than both monotherapy groups whereas cortical porosity increased in the teriparatide monotherapy group but remained unchanged in the combination group. Together, these microarchitectural changes resulted in greater estimated bone strength (as assessed by micro-finite element analysis) in the combination therapy group as compared to the teriparatide monotherapy group at both peripheral sites.

The mechanisms underlying the unique efficacy of the specific teriparatide/denosumab combination remain unclear but the pattern of changes in bone turnover markers provides a possible explanation. Specifically, serum CTX was identically suppressed in women treated with combination therapy and denosumab monotherapy whereas bone formation markers in these two groups deviated. Bone formation remained stable in the combination therapy group for the initial 3-months of treatment and then declined modestly (though not to the level observed in the denosumab monotherapy group) (**Fig. 2**) (Leder *et al.*, 2014). The divergent pattern in bone resorption and formation marker changes in the combined teriparatide/denosumab group suggests that when given together, denosumab fully inhibits teriparatide-induced bone resorption while not interfering with teriparatide-induced "modeling-based" bone formation.

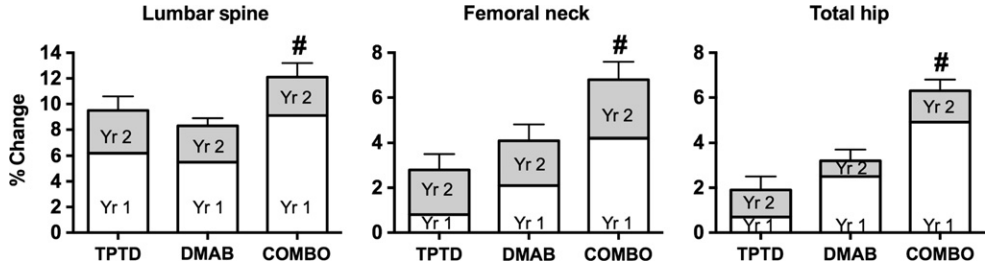

Fig. 1 Mean percent change (±SEM) in BMD from months 0–12 (in *white*) and 12–24 (in *gray*) in women treated with teriparatide (TPTD), denosumab (DMAB), or combination therapy (COMBO). #$P < .05$ versus both other groups at 24 months. Adapted from Leder, B.Z., Tsai, J.N., Uihlein, A.V., Burnett-Bowie, S.A., Zhu, Y., Foley, K., Lee, H. and Neer, R.M. (2014). Two years of denosumab and teriparatide administration in postmenopausal women with osteoporosis (the DATA extension study): A randomized controlled trial. *The Journal of Clinical Endocrinology and Metabolism* **99**, 1694–1700.

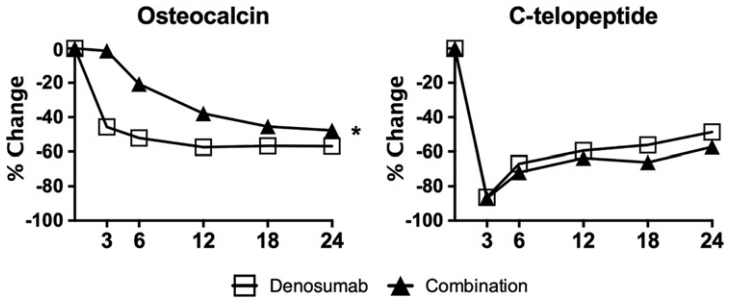

Fig. 2 Mean percent change (±SEM) in serum osteocalcin and c-telopeptide from month 0–24 in subjects treated with denosumab monotherapy or combination teriparatide/denosumab. *$P < .05$ versus the other group at all time points for osteocalcin. Adapted from Leder, B.Z., Tsai, J.N., Uihlein, A.V., Burnett-Bowie, S.A., Zhu, Y., Foley, K., Lee, H. and Neer, R.M. (2014). Two years of denosumab and teriparatide administration in postmenopausal women with osteoporosis (the DATA extension study): A randomized controlled trial. *The Journal of Clinical Endocrinology and Metabolism* **99**, 1694–1700.

In summary, while bisphosphonate-containing combinations with PTH analogs do not lead to additive effects, the combination of denosumab and teriparatide appears to be is a promising approach that, even in the absence of fracture-efficacy studies, could be considered in patients with severe osteoporosis.

Sequential Treatment

Switching from Anabolic to Antiresorptive Therapy

Due to the observed increased risk of osteosarcoma in animal studies with PTH and parathyroid hormone-related protein (PTHrP) analogs, the use of teriparatide and abaloparatide (a PTHrP analog recently approved in the United States) is limited to 2 years (Langdahl *et al.*, 2017). And while the fracture risk reduction of teriparatide appears to be maintained for up to 18 months after discontinuation, BMD decreases almost immediately when the drug is stopped (Black *et al.*, 2005; Kaufman *et al.*, 2005; Lindsay *et al.*, 2004). Thus, the use of these agents should generally be followed by an alternate osteoporosis drug. In the extension of the PATH study discussed above, postmenopausal women who had received 1 year of PTH 1–84 were then randomized to receive either placebo or alendronate for an additional year (Black *et al.*, 2005). While alendronate increased BMD at the spine, total hip, and femoral neck, those who received placebo experienced no gain in femoral neck and total hip BMD and a −1.7% decline in spine BMD. In the separate EUROFORS (The EUROpean Study of FORSteo) study, postmenopausal women with severe osteoporosis who had received 1 year of teriparatide were randomized to continue teriparatide, switch to raloxifene, or stop treatment for an additional year (Eastell *et al.*, 2009). Spine BMD increased in those who received teriparatide, remained stable in those who received raloxifene, and decreased in those who did not receive treatment. Femoral neck BMD increased in all three groups (with greater gains in the teriparatide group compared to the no-treatment group) and total hip BMD increased only in those who received active treatment.

In an extension of the large double-blind placebo controlled abaloparatide registration trial, the 1139 postmenopausal women who had received either placebo or abaloparatide in the initial 18-month phase were all transitioned to open-label alendronate and spine, total hip, and femoral neck BMD increased in both groups (Cosman *et al.*, 2017). Additionally, the antifracture efficacy observed during the initial 18-month placebo-controlled portion of the trial was maintained during the open-label alendronate phase.

The transition from the as yet unapproved anabolic sclerostin inhibitor, romosozumab, to alendronate was also investigated in the recently published placebo-controlled ARCH trial (Active-Controlled Fracture Study in Postmenopausal Women with Osteoporosis at High Risk) (Saag *et al.*, 2017). In this study, postmenopausal women with osteoporosis who had received either romosozumab 120 mg monthly or alendronate 70 mg weekly for 12 months then received open-label weekly alendronate for 2 years. In the group who originally received romosozumab, alendronate maintained the comparative increases in spine and total hip BMD as well as the beneficial effect on vertebral, clinical, nonvertebral, and hip fracture incidence.

In the extension of the DATA study (DATA-SWITCH), women who originally received teriparatide or combined therapy were switched to 2-years of denosumab whereas those who originally received denosumab were switched to 2-years of teriparatide (Leder *et al.*, 2015). In the group who received 2 years of teriparatide followed by 2 years of denosumab, BMD continued to increase at all measured sites resulting in net 4-year gains of 14.0% at the spine and 6.6% at the total hip (**Fig. 3**). Women who received combination therapy followed by denosumab experienced mean 4-year gains of 16.0% at the spine and 8.6% at the total hip (**Fig. 3**). The transition from teriparatide-to-denosumab also significantly increased total vBMD, trabecular vBMD, cortical vBMD, cortical thickness, and estimated stiffness at both the distal tibia and distal radius (Tsai *et al.*, 2017).

Switching from Antiresorptive to Anabolic Therapy

Most patients who are treated with anabolic drugs have already been treated with antiresorptive agents for extended periods. Nonetheless, it has become increasingly clear that switching from a bisphosphonate to an anabolic drug may result in transient cortical BMD loss and diminished BMD gains at sites of predominately trabecular bone, such as the lumbar spine (Finkelstein *et al.*, 2003, 2010; Boonen *et al.*, 2008; Ettinger *et al.*, 2004; Cosman *et al.*, 2009; Miller *et al.*, 2008). An example of this phenomenon is illustrated in a clinical trial in which 59 women who previously received either alendronate or raloxifene for 18–36 months were then administered 18-months of teriparatide (Ettinger *et al.*, 2004). In these women, spine BMD increased more in those who had previously received raloxifene than those who had received alendronate and total hip BMD decreased by −1.8% in the initial 6-months of teriparatide therapy in those previously treated with alendronate and then reverted to baseline with continued therapy. In a separate substudy of the EUROFORS trial, 24-months of teriparatide was administered to postmenopausal women who were either treatment-naïve or had prior antiresorptive use and spine BMD increased more in the treatment-naïve group than those with prior antiresorptive exposure (87% of the prior antiresorptive drugs were bisphosphonates) (Obermayer-Pietsch *et al.*, 2008). This pattern of blunted increases in spine BMD and either stable BMD or transient mild hip BMD loss when an oral bisphosphonate is followed by teriparatide has also been observed in several additional studies (Boonen *et al.*, 2008; Ettinger *et al.*, 2004; Cosman *et al.*, 2009; Miller *et al.*, 2008).

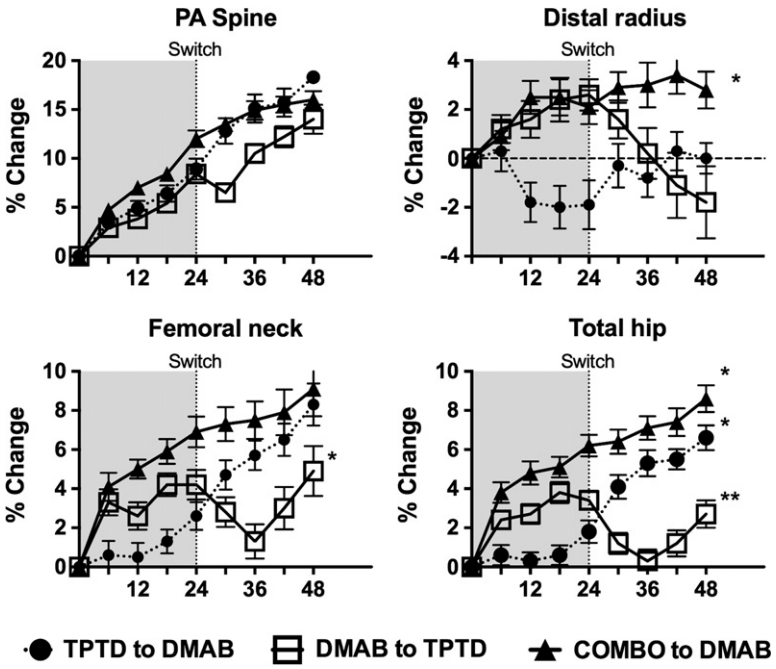

Fig. 3 Mean percent change (±SEM) in BMD from months 0–48 in subjects treated with teriparatide followed by denosumab (TPTD to DMAB), denosumab followed by teriparatide (DMAB to TPTD), and combination therapy followed by denosumab (COMBO to DMAB). $^{*}P < .05$ versus both other groups at month-48. $^{**}P < .0005$ versus both other groups at month-48. Adapted from Leder, B.Z., Tsai, J.N., Uihlein, A.V., Wallace, P.M., Lee, H., Neer, R.M. and Burnett-Bowie, S.A. (2015). Denosumab and teriparatide transitions in postmenopausal osteoporosis (the DATA-switch study): Extension of a randomised controlled trial. *Lancet* **386**, 1147–1155.

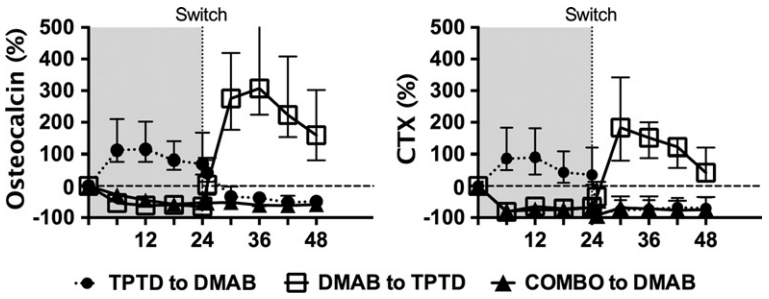

Fig. 4 Mean percent change (±SEM) in serum osteocalcin and c-telopeptide (CTX) from month 0–48 in subjects treated with teriparatide followed by denosumab (TPTD to DMAB), denosumab followed by teriparatide (DMAB to TPTD), and combination therapy followed by denosumab (COMBO to DMAB). Adapted from Leder, B.Z., Tsai, J.N., Uihlein, A.V., Wallace, P.M., Lee, H., Neer, R.M. and Burnett-Bowie, S.A. (2015). Denosumab and teriparatide transitions in postmenopausal osteoporosis (the DATA-switch study): Extension of a randomised controlled trial. *Lancet* **386**, 1147–1155.

In a recently published comparative efficacy clinical trial in which 436 postmenopausal women who had been previously treated with at least 3-years of bisphosphonate therapy were randomized to receive either 1-year of teriparatide or romosozumab, BMD at the spine, total hip, and femoral neck increased significantly more in patients treated with romosozumab than with teriparatide (Langdahl *et al.*, 2017). Despite this apparent comparative advantage, however, it should be noted that the romosozumab-induced increases in BMD appear to be lower than those observed in separate studies performed in treatment-naïve subjects, suggesting that the effects of romosozumab may also be blunted in the setting of prior bisphosphonate exposure (McClung *et al.*, 2014; Cosman *et al.*, 2016).

The transition from denosumab-to-teriparatide appears to result in a unique maladaptive pattern of high-turnover bone loss. In the DATA-Switch study previously described, women switched to teriparatide after 2-years of denosumab experienced 6-months of declining BMD at the spine, 12-months of declining BMD at the total hip and femoral neck, and progressive bone loss through all 24-months of treatment at the distal radius (**Fig. 3**) (Leder *et al.*, 2015). Notably, the decline in hip BMD after the switch resulted in the complete reversal of the denosumab-induced BMD increases achieved in the initial 2-years of denosumab monotherapy. At the distal tibia and distal radius, the transition from denosumab-to-teriparatide resulted in progressive decreases in total vBMD and cortical vBMD, increases in cortical porosity, and reduced bone strength as assessed by micro-finite element analysis (Tsai *et al.*, 2017). Moreover, the bone loss associated with this specific drug transition was accompanied by a dramatic stimulation of both bone formation and resorption. Specifically, levels of bone turnover markers increased 200%–300% 6–12 months after the drug transition and remained elevated even after 24-months (**Fig. 4**) (Leder *et al.*, 2015). This unprecedented stimulation of bone remodeling is concerning given that the more modest stimulation of bone turnover that occurs when denosumab is discontinued without a transition to teriparatide is associated with an increased risk of multiple vertebral compression fractures (Bone *et al.*, 2011; Cummings *et al.*, 2017).

Adding One Class of Drug to Another

When deciding to transition from one class of drug to another, an overlapping strategy may be preferable in some instances. In a trial of 198 postmenopausal women who received at least 18-months of alendronate or raloxifene, strategies of adding or switching to 18-months of teriparatide were compared (Cosman *et al.*, 2009). In the group who initially received raloxifene, spine and total hip BMD increased similarly in the add and switch groups whereas in the group who initially received alendronate, adding teriparatide increased spine BMD more than switching to teriparatide (hip BMD did not differ). Additionally, no early decreases in hip BMD were observed when teriparatide was added to either ongoing raloxifene or alendronate.

In a trial of 125 postmenopausal women with osteoporosis who had received 9-months of teriparatide, the effects of adding raloxifene, adding alendronate, or continuing teriparatide for 9 months were compared (Muschitz *et al.*, 2013). Spine BMD increased more in the raloxifene-add group than the group who continued teriparatide monotherapy whereas total hip BMD increased more in the alendronate-add group than both other groups during months 9–18. Additionally, in a 12-month extension to this study in which teriparatide was discontinued in all three groups, the greatest BMD gains were observed in women who consecutively received 9-months teriparatide, 9-months of combined teriparatide/alendronate and 12-months of alendronate monotherapy (Muschitz *et al.*, 2014).

Summary

In summary, when given sequentially, anabolic therapy should be given prior to bisphosphonates in order to achieve maximal gains in spine and hip BMD. The sequence of denosumab followed by teriparatide should be avoided due to high bone turnover BMD loss, especially given the known increased risk of vertebral fractures after denosumab alone is discontinued. While the

combination of bisphosphonates and PTH analogs do not result in additive effects, the combination of denosumab and teriparatide is uniquely effective. Lastly, approaches that overlap anabolic and antiresorptive therapy in either order deserve additional study.

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Osteoporosis: Treatment Gaps and Health Economics

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Introduction

Despite many advances in the diagnosis of osteoporosis, the assessment of fracture risk, the development of therapies to reduce the risk of fractures, and the production of best practice guidelines, many studies indicate that a minority of men and women at high fracture risk actually receive treatment (Harvey *et al.*, 2017a). Even in patients who sustain a fragility fracture, fewer than 20% actually receive therapies to reduce the risk of fracture in the year following the fracture (Giangregorio *et al.*, 2006; Kanis *et al.*, 2014a), with particularly poor rates of treatment for older women and those who live in long term care. Disparities in use of fracture risk assessment tools such as FRAX® vary one thousand-fold worldwide, with a far greater variability than the 30-fold range of crude, or 10-fold range of age-standardized hip fracture worldwide, indicating a large gap in service provision (Kanis *et al.*, 2012, 2014b). Limitations in access to the internet, lack of national assessment guidelines for osteoporosis in many countries, and the availability of alternative assessment algorithms may partially explain these differences (Kanis *et al.*, 2014b). Not only is lack of assessment and lack of treatment of those at very high risk of further fracture such as hip fracture a concern, most worrying is the downward trend in people being treated after hip fracture, demonstrated both in the USA and UK populations (Solomon *et al.*, 2014; Hawley *et al.*, 2016). The precise causes for this trend are likely to be several, including the recent reimbursement changes in the US, and the massive inflation of concerns regarding potential rare side effects of long-term bisphosphonate treatment such as osteonecrosis of the jaw and atypical femoral shaft fractures. In this review, we give an overview of the treatment gaps at all levels, potential underlying reasons, and possible approaches to help reverse the situation.

The Osteoporosis Treatment Gap

There are now data, from both Europe and the United States, demonstrating substantial disparities between the number of individuals at high fracture risk, or who have experienced a low trauma fracture, and the number who receive appropriate assessment and treatment for osteoporosis (Harvey *et al.* 2017a). Thus in the UK, analysis of the Clinical Practice Research Datalink (CPRD) has demonstrated substantial gaps in both primary and secondary prevention. The probability of being prescribed any antiosteoporosis drug after hip fracture in the UK increased from only 7% in 2000 to 46% in 2010 (Klop *et al.*, 2014). This trend was more marked in patients $\geq$75 years. The increase in prescribing of antiosteoporosis drugs was complemented by a similar increase in vitamin D/calcium provision. The cumulative incidence of antiosteoporosis therapy was greater at any given point in time in women (8% in 2000, 51% in 2010) than in men (4% in 2000, 34% in 2010). Despite $<$50% of hip fracture patients receiving treatment, more recent data suggest a plateau and a possible decrease in prescriptions from around 2011 (Hawley *et al.*, 2016). Furthermore, there appears to be substantial geographic heterogeneity in the UK amongst treatment rates following hip fracture. For example, in a CPRD analysis based on the UK healthcare regions, the odds ratio for antiosteoporosis medication following a hip fracture was 1.29 (95% CI: 0.89, 1.87) for the North East and 0.56 (95% CI, 0.43, 0.73) in South Central regions, the North West being the referent. These geographic differences in prescribing persisted over the 5-years of follow-up (Shah *et al.*, 2017). Additionally, data from the GLOW study, a prospective observational study of over 60,000 older women recruited from primary care practices in 10 countries across US, Europe, and Australia, showed that $>$80% of women with a fragility fracture did not receive osteoporosis treatment (Greenspan *et al.*, 2012).

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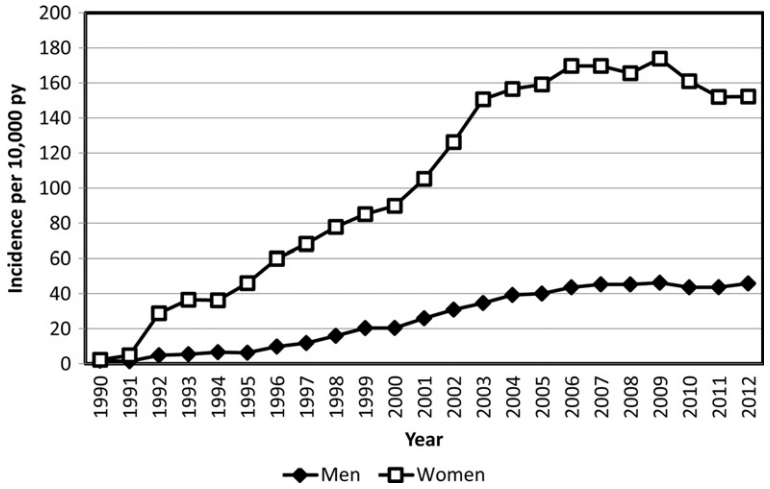

Fig. 1 Incidence of antiosteoporosis medication prescription from 1990 to 2012 in the UK population aged 50 years or over. Reproduced with permission from van der Velde, R.Y., Wyers, C.E., Teesselink, E., Geusens, P.P., van den Bergh, J.P., de Vries, F., Cooper, C., Harvey, N.C., van Staa, T.P. (2017). Trends in oral anti-osteoporosis drug prescription in the United Kingdom between 1990 and 2012: Variation by age, sex, geographic location and ethnicity. *Bone* **94**, 50–55.

These differences by geography, ethnicity and time for secondary fracture prevention are consistent with findings relating to primary use of antiosteoporosis medications. Between 1990 and 2012, rates of antiosteoporosis medication prescription in the UK rose from 2.3 to 169.7 prescriptions per 10,000 person-years amongst women from 1990 to 2006, but this was followed by a plateau and then a 12% decrease over 2009 to 2012 (**Fig. 1**). Prescription rates rose less steeply in men from 1990 to 2007 and plateaued from 2008 onwards. There were marked differences in prescription of antiosteoporosis medications according to ethnicity and geographic location (van der Velde *et al.*, 2017).

In Europe, treatment uptake for osteoporosis increased progressively up to 2008, thereafter plateaued, and has subsequently fallen in more recent years (**Fig. 2**). The phenomenon is most marked in the case of the bisphosphonates and is evident on a country by country basis (Svedbom *et al.*, 2013). The number of patients treated in each country was computed from IMS Health sales data for 2010, adjusted for suboptimal adherence, and expressed as treatment years (Hernlund *et al.*, 2013). The use of hormone replacement therapy was excluded since the majority of women take this treatment for menopausal symptoms rather than for osteoporosis. The proportion of patients eligible for treatment depended on defining an intervention threshold that is, the risk of fracture above which treatment can be recommended. In this report, the intervention threshold set was at the FRAX-based 10-year fracture probability equivalent to women with a prior fragility fracture without knowledge of BMD as adopted in several European guidelines (Kanis *et al.*, 2013a; Lekamwasam *et al.*, 2012a; Compston *et al.*, 2017). Thus, the intervention threshold can be likened to a "fracture threshold" expressed in terms of fracture probability. The study showed a very wide inter-country variation in the treatment penetration of individuals at high risk for osteoporotic fractures. The treatment gap varied from 25% in Spain to 95% in Bulgaria. Large treatment gaps were identified in countries with populations at both high and low risk of fracture. In total in the EU, it was estimated that, out of the 21.3 million men and women who exceeded the risk level, 12.3 million were untreated in 2010 (Hernlund *et al.*, 2013). These figures are conservative since an undetermined proportion of low risk women will have received treatment (Diez-Perez *et al.*, 2011). In an international prospective study, low uptakes of pharmacological intervention after hip fracture was also observed. Amongst 1795 patients who sustained a low-energy hip fractures in ten countries (Australia, Austria, Estonia, France, Italy, Lithuania, Mexico, Russia, Spain, and the United Kingdom), only 27% were prescribed pharmacological fracture prevention after the hip fracture (Svedbom *et al.*, 2013).

Data from the US suggest a similar pattern. In a large retrospective analysis of nearly 100,000 men and women aged 50 years or more who were hospitalized for hip fracture over a period of 1 year, based on U.S. administrative insurance claims data, the uptake of osteoporosis medication within 12 months after discharge from hospital was examined (Solomon *et al.*, 2014). The estimated probability of receiving osteoporosis medication within 12 months after discharge from hospital was 28.5% over this time period but varied by year. Indeed, the rates declined significantly over a 10-year interval, from 40.2% in 2002 to 20.5% in 2011 (Solomon *et al.*, 2014). Congruent findings come from analysis of the US Medical Expenditure Panel Survey, demonstrating a marked reduction in the prevalence of bisphosphonate use amongst women from 2007 onwards. Rates in men appeared to decline, albeit from a much lower baseline, over the same period (Jha *et al.*, 2015).

Reasons Underlying the Treatment Gap

There appear to be many factors in the poor rates of treatment for osteoporosis, including the insufficient implementation of strategies to effect primary and secondary prevention. Fundamentally, primary prevention is always made difficult by the concept

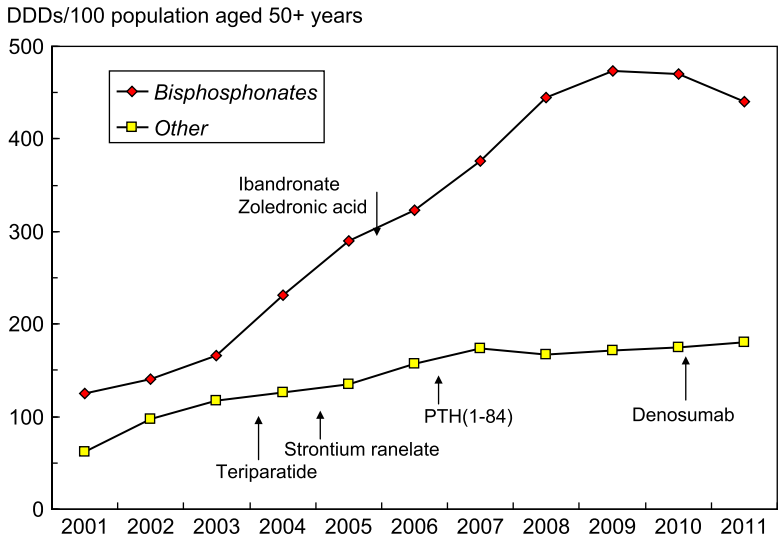

Fig. 2 Estimated sales (defined daily doses (DDDs)/100 population aged 50+ years) from 2001 to 2011 in the European Union. Reproduced with permission from Hernlund, E., Svedbom, A., Ivergard, M., Compston, J., Cooper, C., Stenmark, J., McCloskey, E.V., Jonsson, B., Kanis, J.A. (2013). Osteoporosis in the European Union: Medical management, epidemiology and economic burden: A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Archives of Osteoporosis* **8**, 136.

of managing a future "risk", rather than treating a disease event which has already happened. However, a hip fracture, for example, is a devastating life event, with a 20% associated reduced survival compared with non-fracture peers (Harvey *et al.*, 2010); in the analogous situation of an acute myocardial infarction, it is difficult to imagine a situation in the developed world in which it would be acceptable for <50% of such cardiac patients receiving preventative treatments such as aspirin, statins, and anti-hypertensives (Austin *et al.*, 2008). It is apparent that musculoskeletal disease may be viewed both by patients and policymakers as a lower priority than outcomes such as myocardial infarction and cancer (Harvey *et al.*, 2017a). Conversely, the Global Burden of Disease initiative has demonstrated musculoskeletal disease to be a leading cause of disability worldwide (Harvey *et al.*, 2014). These observations suggest a mismatch between the severity of the condition, and associated perceptions; this is well documented in the large international GLOW cohort, in which many women underestimated their fracture risk compared with their peers (Siris *et al.*, 2011). Against this backdrop, studies cited above have clearly demonstrated a successful increase in antiresorptive treatment rates both in primary and secondary prevention over the last 15 years. Advances in risk assessment and policy, for example through the use of risk calculators such as FRAX (Kanis *et al.*, 2011, 2016), guidance on intervention (Kanis *et al.*, 2013a), together with the availability of generic bisphosphonates, have helped to improve the clinical situation. It is therefore tragic in the context of this maturing field that treatment rates, both before and after a fracture, have declined in recent years, despite inexorable expansion of the population at risk (Oden *et al.*, 2015). Two studies have suggested possible reasons for this. Jha *et al.* examined relationships between medication use (data from the Medical Expenditure Panel Survey and National Inpatient Sample in the US), internet search activity for alendronate between 2006 and 2010, and media reports of safety concerns (Jha *et al.*, 2015). Against the backdrop of a decline in bisphosphonate use by >50% between 2008 and 2012, there were marked spikes of internet search activity corresponding to events such as a 2006 lawsuit filed against Merck for Fosamax allegedly causing osteonecrosis of the jaw, a major ABC World News feature on Fosamax and atypical femoral fractures in 2010, and several other media reports of such rare, but serious side effects. Similar findings have come from the Australian Longitudinal Study on Women's Health (Peeters *et al.*, 2014). Consistent with the US data, total use of antiosteoporosis medications increased over the period 2000 to 2007 but then decreased from 2007 to 2010. Despite relaxing indications for bone density testing and a subsidy for antiosteoporosis medications, the decline coincided with adverse media stories such as a major report on osteonecrosis of the jaw in 2007 (Peeters *et al.*, 2014).

Whilst serious long-term adverse side-effects of bisphosphonates are very rare in absolute terms (with incidences in the range of 1/100,000 to 1/10,000 per year) (Adler *et al.*, 2016), the approach to risk/benefit communication has largely amongst the media (as demonstrated above) and unfortunately amongst physicians and policymakers, been very much on the side of declaring risk. Recognition that the underlying disease is associated with substantial morbidity and increased mortality, with fracture risk markedly reduced by antiosteoporosis medications, seems largely under-articulated in these discussions. Indeed, the recent UK National Institute for Health and Care Excellence (NICE) guidance on multi-morbidity (Farmer *et al.*, 2016) specifically targeted bisphosphonates for review after 3 years treatment despite evidence for longer term efficacy and safety being more reliable than for other treatments considered. Such concerns do very little to improve osteoporosis care in the setting of declining treatment rates overall. Reassuringly in terms of the risk-benefit ratio, a recent study in the Danish population has demonstrated that users of alendronate still have a reduced risk of fracture compared with matched controls even after 10 years use, and that the number of

Table 1 Number of central DXA units available in the EU27 countries per million of the general population

Country	*DXA units/million*	*Country*	*DXA units/million*	*Country*	*DXA units/million*
Austria	28.7	Germany	21.1	Netherlands	10.7
Belgium	53.0	Greece	37.5	Poland	4.3
Bulgaria	1.2	Hungary	6.0	Portugal	26.9
Cyprus	23.9	Ireland	10.0	Romania	2.4
Czech Republic	5.2	Italy	18.6	Slovakia	10.7
Denmark	14.6	Latvia	4.9	Slovenia	27.1
Estonia	8.9	Lithuania	3.4	Spain	8.4
Finland	16.8	Luxemburg	2.0	Sweden	10.0
France	29.1	Malta	9.7	UK	8.2

hip fractures prevented is still substantially greater than the number of subtrochanteric fractures occurring even by the end of a decade of bisphosphonate treatment (Abrahamsen *et al.*, 2016). It is patently clear that the field needs to dramatically improve its approach to communicating the risks and benefits of treatments, and to robustly counter ill-informed adverse media stories in a timely fashion.

Healthcare Policies and Osteoporosis Assessment

Osteoporosis, in comparison with comparable non-communicable diseases, has rarely attracted proportionate levels of attention from healthcare providers and governments, and an individual nation's policy on access to bone densitometry with dual-energy X-ray absorptiometry (DXA) and its reimbursement will greatly influence the assessment and treatment of this disease. The International Osteoporosis Foundation (IOF) has published various regional audits (https://www.iofbonehealth.org/regional-audits) covering the European Union, Eastern Europe and Central Asia, Latin America, North America, the Middle East and Africa, Asia Pacific in terms of epidemiology, burden and costs of osteoporosis. Taking Asia Pacific as an example, whilst Australia, Hong Kong, Japan, New Zealand, Republic of Korea and Singapore had 12–24 DXA machines per million of population, China, India, Indonesia, Pakistan, Philippines, Sri Lanka and Vietnam were greatly under-resourced with less than 1 DXA machine per million of population. In addition, BMD testing and osteoporosis treatment were not fully reimbursed by insurance or healthcare policies in many countries, which served as a barrier to accessing treatment.

In Europe, it was assumed that 11 DXA machines per million of population were needed to provide adequate osteoporosis care. 16 European countries fell into this category of adequate provision, and 9 countries were considered to have very inadequate provision with <8.4 DXA units per million (Bulgaria, Czech Republic, Hungary, Latvia, Lithuania, Luxembourg, Poland, Romania, and the United Kingdom). **Table 1** shows the number of DXA units per million of population in the EU27 countries as estimated in 2010 (Kanis *et al.*, 2013b). Reimbursement for DXA scans was extremely variable between EU member states in terms of the criteria required and level of imbursement awarded—interestingly in some countries reimbursement for DXA was only offered if the BMD measurement demonstrated osteoporosis (Bulgaria and Switzerland), only if after fracture (Germany), or only if seen by a specialist (Poland).

Though no official IOF audit is available for North America, reimbursement for treatment also varies greatly, depending on each individual patient's health insurance plan. However, healthcare reform is evolving in the USA from fee for service to supporting improved quality, prevention and care coordination with financial incentives to encourage healthcare professionals or systems to report on or improve patient outcomes. However, performance measures on osteoporosis assessment remain low compared to other major chronic diseases, and a major drop in reimbursement for DXA scans in the office setting has led to a fall in the number of DXA providers and more than 1 million fewer DXA scans performed per annum (Overman *et al.*, 2015). Recent evidence suggests that this coincides with a plateau in the secular decline in age- and sex-adjusted hip fracture rates which had been apparent up until 2012 (Lewiecki *et al.*, 2016).

Approaches to Closing the Gap

Identification of Patients at High Risk of Fracture

It is apparent from the evidence described above, that osteoporotic fractures place a huge burden on societies across the world. It is well known that osteoporosis is a silent disease until a fracture occurs. Patient perception of fracture risk is often underestimated (Grover *et al.*, 2014; Gregson *et al.*, 2014), so initiation of primary prevention is usually reliant on health care practitioners. It is unsurprising therefore that secondary prevention (identifying individuals for treatment on the basis of a low trauma fragility fracture occurring) is the approach most often taken as the starting point for fracture prevention. However, whatever approach is taken to the reduction of fracture risk, it is critically important to place this within the context of local factors, such as the

background population fracture risk, prevalent patterns and risk factors, funding constraints and willingness of healthcare providers to pay for treatment.

Secondary Fracture Prevention

Following attendance to a healthcare practitioner with a new fracture, it is important to assess fracture risk in a straightforward way, and to treat if appropriate. Several methods have been explored—some staff based, some IT-based and others a combination of the two. The most successful systems usually focus on a multi-disciplinary Fracture Liaison Service (Eisman *et al.*, 2012; Mitchell, 2013), incorporating orthogeriatricians, rheumatologists, other physicians and clinical nurse specialists. They work in a multi-disciplinary team to ensure that medical management of patients admitted with fracture is optimized, both whilst in hospital and for future fracture prevention, ideally with a lead clinician responsible for coordinating the team (Drew *et al.*, 2016). The International Osteoporosis Foundation has recently instituted "a global campaign to facilitate the implementation of coordinated, multi-disciplinary models of care for secondary fracture prevention." The "Capture the Fracture®" (http://www.capturethefracture.org/) initiative has provided guidance on secondary fracture prevention, and also a global map, with a quality grading scheme, on which, subject to application, secondary fracture prevention services can be documented (Akesson *et al.*, 2013). There is currently huge variation, not only between, but also within countries, and in the availability, scope and quality of secondary prevention facilities. The Capture the Fracture initiative, aimed at raising the quality and coverage of fracture liaison services providing secondary prevention for osteoporosis, should provide a clinically valuable and cost-effective contribution to service improvement (Mitchell *et al.*, 2016).

Further important initiatives around case finding of fragility fractures centre around vertebral fractures—around 12% of postmenopausal women with osteoporosis have at least one vertebral deformity, with less than a third of these individuals coming to clinical attention (Cooper *et al.*, 1992). Primary care based screening strategies (Clark *et al.*, 2012), and history-taking strategies distinguishing back pain likely to relate to vertebral fracture from other types of back pain may facilitate detection of these fractures (Clark *et al.*, 2016). In addition, consistent reporting of radiographs, CT scans and the incorporation of vertebral fracture assessment in DXA scans will help with secondary fracture prevention in individuals with prevalent osteoporotic vertebral fracture.

Primary Fracture Prevention

In osteoporosis, as in any non-communicable chronic disease, there is clearly a balance between the benefits of a systematic screening approach leading to widespread treatment, with associated increased cost and risk of side-effects, and a case-finding strategy focused on those at greatest individual risk, with associated problems of under-treatment. Although, DXA screening is standard in the US (at the age of 65 years in women, and age 70 in men, and in individuals over the age of 50 years who have suffered an adult fracture) (Cosman *et al.*, 2014), in the majority of countries population screening is not judged to be cost-effective and primary prevention is focused more on opportunistic case-finding, triggered by the presence of clinical risk factors (Kanis *et al.*, 2013a; Lekamwasam *et al.*, 2012a,b; Compston *et al.*, 2017). A seven-centre randomized controlled trial of the effectiveness and cost-effectiveness of screening older women in primary care for the prevention of fractures (the UK SCOOP study), in which approximately 12,500 older women were randomized to either normal care or screening and subsequent treatment (based upon the FRAX risk assessment tool), has recently demonstrated that this intervention leads to a reduction in hip fracture risk (Shepstone *et al.*, 2012, 2017).

Health Economic Considerations

There are two major approaches to the health economic assessment in a particular condition. Firstly, one can assess the cost-effectiveness of the intervention, and set the threshold for intervention, for example FRAX probability, accordingly. Alternatively, one can derive a clinically informed and appropriate intervention threshold, and use cost-effectiveness analysis to validate a threshold. The 2017 National Institute for Health and Care Excellence (NICE) updated Multiple Technology Appraisal (MTA) on bisphosphonate use in osteoporosis (NICE, 2017) illustrates how, for a common disorder, the former approach, with strict application of cost-effectiveness thresholds for relatively inexpensive drugs, may lead to counter-intuitive and potentially harmful guidance (Sims, 2017; Harvey *et al.*, 2017b). The MTA incorporates the development of fracture risk calculators based on individualized clinical risk factors, such as FRAX and QFracture, (both recommended by NICE for the assessment of fracture risk in certain sections of the population (NICE, 2012)), and also the widespread availability of low-cost generic forms of the main oral and intravenous bisphosphonates. This latter development has led, in the NICE analysis, to cost-effectiveness at very low risk thresholds, resulting in an appraisal which recommends that, amongst individuals who qualify for osteoporosis assessment on the basis of the NICE Clinical Guideline CG146 on fracture risk assessment (NICE, 2012), treatment with oral bisphosphonates may be instituted above a 1% probability of major osteoporotic fracture (hip, spine, wrist or humerus) over 10 years, or above 10% for intravenous bisphosphonates. These health-economic-derived thresholds create a real danger of excessive bisphosphonate prescription in the general population (Sims, 2017), with treatment of substantial numbers of people who are at very low individual fracture risk; for example, every person eligible for assessment under CG146, including all women aged ≥ 65 and men ≥ 75 years,

would be recommended treatment if the MTA recommendations were interpreted as intervention thresholds (Kanis *et al.*, 2008). Very rare, but serious, side-effects of bisphosphonate treatment, such as atypical femur fracture and osteonecrosis of the jaw, would be observed far more commonly in the population than at present. Furthermore, the risk/benefit balance for individuals at low risk would be adversely affected, in contrast to the very clearly positive benefit/risk ratio associated with intervention at more clinically appropriate treatment thresholds (Adler *et al.*, 2016; Compston *et al.*, 2017; Rizzoli *et al.* 2011). In contrast, whilst the derivation of treatment thresholds is necessarily arbitrary, the UK National Osteoporosis Guideline Group (NOGG) used the second approach, developing its guidance on the basis of clinical appropriateness, setting the threshold at the age-specific 10-year FRAX probability of fracture equivalent to women having already sustained a fracture. Thus, economic thresholds were not used to set intervention thresholds but, more appropriately, to validate the use of clinically driven intervention thresholds. This approach, which avoids inappropriate over-treatment of older individuals and under-treatment of younger individuals, has been shown to be cost-effective (Kanis *et al.*, 2008), and has been adopted in many countries (Kanis *et al.*, 2016).

The cost effectiveness of individual therapies for osteoporosis have recently been comprehensively reviewed (Hiligsmann *et al.*, 2015). Of the 1794 articles identified across a range of databases, 39 studies fulfilled the inclusion criteria. These covered 14 different countries and within them 9 active interventions were assessed. When the interventions were compared with no treatment, active antiosteoporosis drugs were generally cost-effective in postmenopausal women aged over 60–65 years with low bone mass, especially amongst those with prior vertebral fractures. Factors which increased cost-effectiveness included higher individual fracture risk and medication adherence.

Summary

Whilst assessment for fracture risk, and use of antiosteoporosis medications, have increased markedly over the last 20 years, there is evidence from the United Kingdom, United States, and continental Europe that treatment rates have declined substantially in the last 5 years. Concerns amongst patients and clinicians around rare side effects of anti-resorptives, compounded by dramatic and widespread media reports, have been complemented by adverse changes in reimbursement in the US, and reflected in new guidance. Indeed, many doctors, dentists, and patients are now more frightened of the rare but serious side effects than they are of the disease and the fractures that arise. Notwithstanding, the lay press is simply the messenger bringing news and opinion from the scientific community, some or much of which may be ill-judged. The paradox arises that we seek to treat individual patients to the highest standards but at the same time bring disservice and disadvantage to the wider osteoporosis community. It is now time for us all to accept a long overdue collective responsibility for our failures and to work cohesively to improve the management of our patients.

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Cancer Treatment-Induced Bone Loss (CTIBL)

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Introduction

Bone loss is associated with an increased risk of fractures and comorbidities. Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fractures. Furthermore, recent evidence suggests that the process of bone loss may play an important role in implanting tumor cell aggregates in bone (metastatic niche) (Ibrahim *et al.*, 2013; D'Oronzo *et al.*, 2015).

Drugs used in cancer treatment (like endocrine therapy, androgen deprivation therapy, and chemotherapy) may induce bone resorption, bone loss and a consequently high risk of bone fractures.

Therefore, assessing which patients are at risk, performing an early detection of bone loss, and implementing preventive measures and treatment is critical. Measures to secure bone health should be implemented early in the course of disease (Ibrahim *et al.*, 2013).

Risk Factors

Identification of patients at risk for cancer treatment -induced bone loss (CTIBL) is key to prevent further bone loss. In this setting, the following risk factors should be considered (D'Oronzo *et al.*, 2015; Doo and Shapiro, 2013; Hadji, 2015).

- Endocrine factors, such as hyperparathyroidism, vitamin D deficiency and menopause.
- Immobility, which prevents osteocyte-regulated bone turnover.
- Genetic factors, since certain gene polymorphisms (of IL-6, IL-1 receptor, and IL-1R antagonist) have been associated with a lower bone mass density (BMaD) in post-menopausal women.
- Family history of osteoporosis.
- Smoking history.
- Alcoholism.
- Chronic corticosteroid treatment (>6 months).
- Older age (>65 years).
- Body mass index <24.
- Low calcium and vitamin D dietary intake.
- Family and/or personal history of previous vertebral/hip fractures.

Diagnosis

Several diagnostic exams can be performed to better characterize bone loss and the risk of fractures.

Initially, a detailed medical history, physical exam, and medication review is required to look for risk factors.

A comprehensive laboratory assessment is necessary and should include serum levels of calcium, phosphate, 25-hydroxyvitamin D, parathyroid hormone, hemoglobin, C-reactive protein, alkaline phosphatase, thyroid-stimulating hormone, creatinine clearance and protein electrophoresis (serum and/or urine).

A dual energy X-ray absorptiometry (DEXA) scan of the hip, lumbar spine and femoral neck is the best way to determine BMaD and a predictor of fracture risk. It is highly precise, with a moderate cost and low radiation exposure. Periodic BMaD evaluations are recommended in women with iatrogenic ovarian failure (those that have treatment-related amenorrhea for >1 year should have BMaD assessed), in postmenopausal women treated with aromatase inhibitors (AIs), and in all breast cancer patients with fracture risk factors. BMaD detection is also mandatory in men on ADT. (D'Oronzo *et al.*, 2015) Z-scores ≤ -2.0 are considered below the expected age range, whereas Z-scores > -2 are within the expected age range (Doo and Shapiro, 2013).

The World Health Organization (WHO) provides a clinical tool—the Fracture Risk Assessment tool (FRAX)—to evaluate the 10-year probability of major osteoporotic fractures based on several risk factors. However, anticancer treatments are not included as a specific risk factor (D'Oronzo *et al.*, 2015; Doo and Shapiro, 2013). Also, current fracture risk assessment tools are based on data from healthy postmenopausal women and do not properly address treatment risks in younger premenopausal women (Coleman *et al.*, 2014).

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BMaD-based osteoporosis treatment guidelines in postmenopausal women do not generally apply to premenopausal women, as the relationship between bone mass and fractures is not the same in both groups. Nevertheless, it is an indication for further evaluation in premenopausal women (Becker and Cohen, 2017).

Cancer Treatments Leading to Bone Loss

Endocrine Therapy (Figs. 1 and 2)

The early days of endocrine therapy (ET) for breast cancer date back to 1896, when Beatson performed the first oophorectomy in premenopausal women. Today, after >100 years of investigation, ET plays a major role in breast cancer treatment, both in early disease and metastatic setting. Indeed, most breast cancer diagnosis belong to the ET-responsive subtype, accounting for 70% of all cases.

According to the European and American Guidelines, ET is indicated in patients with detectable estrogen receptor (ER) expression (defined as ≥1% of invasive cancer cells) (Senkus *et al.*, 2015). The choice of agent is primarily determined by patient's menopausal status. It is acknowledged that in premenopausal women estrogen production is dependent on ovarian tissue, whereas in postmenopausal women aromatase enzyme converts androgens to estrogens in the adrenal gland, inhibiting CYP-19 cytochrome P450 enzyme.

Although bone loss induction differs according to ET regimen, 78% of women with breast cancer have at least one secondary cause of bone loss besides cancer or cancer-related therapies. Among secondary causes, the most common is vitamin insufficiency, with 38% of women presenting with vitamin D below 30 ng/mL [74.9 nmol/L]. Other causes of bone loss include idiopathic hypercalciuria and normocalcemic hyperparathyroidism (Camacho *et al.*, 2008).

The mechanisms by which estrogen regulates bone remodeling are not fully understood. Estrogen is known to affect osteoclastogenesis and osteoclast function through its effects on local cytokines and growth factors (produced by either bone cells or adjacent marrow cells estrogen). Furthermore, it regulates the life span of mature osteoclasts via induction of the Fas/Fas-ligand (FasL) system, thereby providing a rational for the osteoprotective function of estrogen, as well as of selective estrogen receptor modulators (SERMs). Both estrogen and SERMs primarily act by regulation of gene transcription via estrogen receptors (estrogen receptor alpha [ERα] and estrogen receptor beta [ERβ]) (Couse and Korach, 1999; Shang and Brown, 2002).

Several ETs are currently available, including aromatase inhibitors (AIs)—which include steroidal (exemestane) and non-steroidal inhibitors (letrozole, anastrozole)–, SERMs—like tamoxifen and raloxifene–, and estrogen receptor downregulators—like fulvestrant.

Anastrozole was the first selective non-steroidal AI to be used in clinical practice. It works by inhibition of conversion of androstenedione or testosterone to estrone, causing a decrease in bone mineral density (BMiD). Exemestane and letrozole are more recent, third-generation, agents of this class.

In vivo animal studies suggest that, due to its androgenic structure, exemestane may be more bone-sparing than letrozole (Lønning *et al.*, 2005).

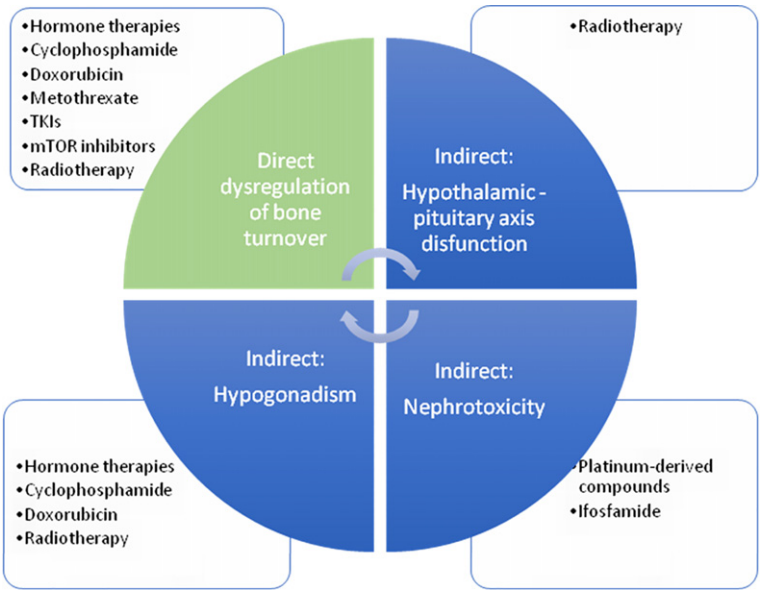

Fig. 1 Mechanisms of cancer treatment-induced bone loss. Adapted from D'Oronzo, S., et al. (2015). Cancer treatment-induced bone loss (CTIBL): Pathogenesis and clinical implications. *Cancer Treatment Reviews* **41**(9), 798–808.

Drug	Mechanism of boneloss
Hormone therapies	Hypogonadism, decrease OB proliferation, increase OB apoptosis
Platinum-derived compounds	Nephrotoxicity (hypomagnesemia)
Ifosfamide	Nephrotoxicity (oxidative stress) tubular damage with hypophosphatemia
Cyclophosphamide	Hypogonadism, decrease bone formation and resorption
Doxorubicin	Hypogonadism, decrease OB formation, increase OC formation and activation
Methrotexate	Decrease OB proliferation, osteocyte apoptosis
TKIs	Bone-damaging effect: hypocalcemia and secondary hyperparathyroidism Bone sparing effect: decrease OC differentiation and activity, increase OB activation
Radiotherapy	Hypogonadism, imbalanced activities of OBs and OCs
OB: osteoblast; OC: osteoclast; TKIs: tyrosine kinase inhibitors	

Fig. 2 Type of treatment and respective mechanisms of cancer treatment-induced bone loss. Adapted from D'Oronzo, S., et al. (2015). Cancer treatment-induced bone loss (CTIBL): Pathogenesis and clinical implications. *Cancer Treatment Reviews* **41**(9), 798–808.

Tamoxifen, a type II competitive inhibitor of estradiol at its receptor, acts as an inhibiting agent (anti-estrogen) in the mammary tissue, but as a stimulating agent in cholesterol metabolism, bone density, and cell proliferation in the endometrium (Shang and Brown, 2002).

Osteoprotection observed with SERMs is, at least partially, mediated by osteoclastic ERα in trabecular bone, and life span of mature osteoclasts is regulated through activation of the FasL signaling.

SERMs ERα-activated seem to truncate the already short lifespan (approximately 2 weeks) of differentiated osteoclasts, by inducing apoptosis through activation of the Fas/FasL system (Takashi Nakamura, 2007).

As an increasing number of women is exposed to AIs, these agents' adverse effects on the skeleton are important to understand. AIs lead to a marked increase in bone resorption, with a two to fourfold increase in bone loss compared to the postmenopausal physiologic BMiD loss (Hadji, 2009). Randomized controlled trials (RCTs) including an AI for 5 years showed an increase in the absolute fracture risk of around 10%, suggesting that one in every 10 women will eventually experience a bone fracture. Real world data indicate 18%–20% fracture risk after 5 years of follow-up. After conclusion of AI treatment, bone turnover normalizes and BMD and fracture risk can partially recover.

For those advocating an AI treatment extension for up to 10 years, a further increase in fracture risk, corresponding to 2%–3% per year, has been registered and should be taken into account.

For most pre-menopausal women eligible for ET, a 5-year tamoxifen 20 mg/day treatment, with or without ovarian suppression, is the option. The exception is for premenopausal women with HR-positive breast cancer at higher risk of recurrence 5 years, for whom the indication is for AIs plus ovarian suppression. The more sudden and severe estrogen deprivation, the greater the magnitude of bone loss. Bone loss is more rapid in premenopausal women receiving both ovarian suppression therapy (GnRH agonist) and an AI.

Patients undergoing ovarian suppression and those taking AIs are at an increased risk of bone loss and should be advised to have an adequate calcium and vitamin D3 intake. Additionally, a periodic assessment of BMD (by dual energy X-ray absorption [DEXA] scan) should be undertaken by these patients.

Some clinical studies investigated the impact of osteoporosis in clinical fractures. The MA-27 trial compared exemestane and anastrozole as adjuvant treatment in postmenopausal women and did not find a difference in the frequency of new clinical fractures between both treatments (4% for each) (Goss *et al.*, 2013). In the ATAC (Arimidex [anastrozole], Tamoxifen, Alone or in Combination) trial, 9366 postmenopausal women with invasive operable breast cancer who completed primary therapy were randomly assigned to receive anastrozole, tamoxifen, or both. More fractures were observed in patients on anastrozole compared with patients on tamoxifen. Also when looking at BMD in trials with AIs, BMD of the lumbar spine (LS) and total hip (TH) was significantly reduced in postmenopausal women receiving AIs versus tamoxifen or placebo. In the ATAC substudy, there was a 6% and 7.2% BMD loss with AI, and a 2.8% and 0.74% increase with tamoxifen, respectively. The annual fracture incidence was higher in women receiving anastrozole (11% vs. 7.7%) throughout the 5-year treatment period; at the sixth year, fracture rate decreased in women previously assigned to anastrozole; and in years 7–9, fracture rates were similar with both treatments, suggesting that AI-related fracture rates will not be a major event when treatment cessation occurs (Howell *et al.*, 2005).

The Intergroup Exemestane Study (IES) also demonstrated that postmenopausal women receiving tamoxifen for 2–3 years who continued on tamoxifen or switched to exemestane experienced a greater decline in BMD at the lumbar spine (2.7%) after 6 months compared with those who remained on tamoxifen (no change at either site) (Coleman *et al.*, 2007).

In the MA-17 trial, 5149 postmenopausal women were randomized to letrozole versus placebo after completion of 5 years of tamoxifen treatment. A total of 256 women registered a clinical fracture (5.3% of patients assigned to letrozole compared with 4.6% assigned to placebo) (Perez *et al.*, 2006).

Overall, analysis of data on the use of AIs, both continuously or with switch, shows that ET is associated with a significant bone mass density and that it correlates with treatment extent. Discussion about the trade -off between quality of life (Qol) and treatment duration, considering risk of fracture, osteoporosis management, and cancer-associated risk, is a matter of concern and debate in daily clinical practice.

Androgen Deprivation Therapy (ADT) (Figs. 1 and 2)

Androgen deprivation therapy (ADT) is a standard prostate cancer treatment. ADT was initially accomplished by orchiectomy, but it is currently increasingly performed by medical castration with gonadotrophin releasing hormone (GnRH) agonists (e.g., Leuprolide, Goserelin) and antagonists (e.g., Degarelix), given alone or in combination with androgen receptor antagonists (e.g., Flutamide, Bicalutamide, Nilutamide) (Saad *et al.*, 2008).

Hormone therapies are not equivalent concerning bone loss. Non-steroidal anti-androgens monotherapy, although not standard, provides some degree of bone protection. To evaluate the effects of anti-androgens on bone health, Smith and colleagues randomized 52 prostate cancer patients without bone metastases to receive Leuprolide or Bicalutamide, a nonsteroidal anti-androgen that competitively binds to androgen receptors. Results showed that bone loss is more relevant with Leuprolide and suggested a potential protective effect of Bicalutamide in bone fracture onset (Smith *et al.*, 2004; D'Oronzo *et al.*, 2015; Sieber *et al.*, 2004).

Patients under castration for a prolonged period of time can have long survival, and the impact of treatment becomes particularly important at this stage. BMaD declines within months of initiation of ADT, reflecting the rapid decrease in sex steroid levels, which reach nadir within 2–4 weeks. Rates of annual bone loss reported in prospective studies range from 2% to 8% at the lumbar spine and from 1.8% to 6.5% at the femoral neck, compared with 0.5%–1.0% in the general population of aging men, and with 2.0% in women undergoing early menopause (Grossman *et al.*, 2011).

Testosterone reduction to castrate levels is associated with a decline in serum estrogens (which result from testosterone conversion by peripheral aromatization). Estrogens are essential in bone formation and resorption in men, and low levels are associated with BMaD loss, increased fracture risk, increased body fat and decreased muscle mass (Saad *et al.*, 2008; D'Oronzo *et al.*, 2015). Estrogens also stimulate osteoclast apoptosis and suppress osteoblast apoptosis. As a result, in an estrogen deficiency setting, as the one elicited by ADT, osteoclast numbers increase and osteoblast numbers decrease, resulting in bone resorption. Estrogen deficiency is also associated with increased levels of cytokine -promoting bone resorption, including TNF-α and IL-1α. These cytokines increase RANKL expression, further stimulating bone resorption (Lipton *et al.*, 2012).

Overall, long-term ADT is associated with significant and progressive BMaD decline, which correlates with treatment extent, and the risk of frailty fractures increases as BMaD decreases. Fracture events significantly correlate with shorter survival in men with prostate cancer (Brufsky, 2008).

Chemotherapy (Figs. 1 and 2)

Chemotherapy may induce bone loss due to premature ovarian failure and to direct effects of cytotoxic treatment. Brufsky, 2008).

By inducing ovarian failure, chemotherapy reduces endogenous estrogens and leads to an increased bone turnover, bone loss, and fracture risk. These effects are associated with deregulation of osteoblast (OB) and/or osteoclast (OC) differentiation and activity.

Indirect effects of chemotherapy include the development of chronic renal disorders and electrolyte abnormalities, as well as a pro-apoptotic effect on both osteoblasts and osteocytes, and accelerated differentiation of bone marrow stromal cells into adipocytes (D'Oronzo *et al.*, 2015). Small observational trials have confirmed that, irrespective of ovarian function, postmenopausal women also experience chemotherapy-associated bone loss (Robinson *et al.*, 2005; Saad *et al.*, 2008).

Chemotherapy-associated bone loss has been reported in breast cancer within 1 year of initiation of adjuvant chemotherapy (Brufsky, 2008). Among premenopausal women receiving chemotherapy, the rate of bone mineral density (BMiD) loss may approach 3%–8% at the lumbar spine within 12 months of initiating chemotherapy. In postmenopausal women, data suggests that patients receiving adjuvant chemotherapy can lose 1%–10% bone mass within 1 year of chemotherapy (Van Poznak, 2017).

Risk factors for chemotherapy-induced ovarian failure include age—older premenopausal women are at higher risk–, chemotherapy type—risk associated with alkylating drugs, such as cyclophosphamide, is higher than with taxanes–, genetic factors, and higher doses and longer extent of chemotherapy (Shapiro, 2017; Doo and Shapiro, 2013).

Several bone loss mechanisms can be considered regarding the type of chemotherapy (D'Oronzo *et al.*, 2015):

– Platinum compounds, especially cisplatin, may induce kidney failure. Kidney impairment impacts bone health as a result of electrolyte disorders, which include acute and chronic hypomagnesaemia. Low magnesium serum levels affect bone turnover

by impairing the H + /K + ATPase pathway, involved in regulation of bone extracellular pH and the VitD synthesis enzyme 1,a-hydroxylase;
- Ifosfamide is a nephrotoxic drug, potentially affecting bone health;
- Cyclophosphamide exerts direct effects on bone turnover by suppressing bone formation and resorption and also induces ovarian failure;
- Doxorrubicin may compromise bone health by increasing osteoclast differentiation and reducing fibroblast and osteoblast-forming units. Doxorubicin may further impair bone health through dose-dependent ovarian failure.
- Metotrexate affects osteoblast proliferation and differentiation.

Other Treatments (Figs. 1 and 2)

Glucocorticoids are used in Oncology as an integral part of antiemetic chemotherapy regimens, as infusion reaction preventive medications, and as analgesics. Long term treatments can be responsible of iatrogenic osteoporosis. Furthermore, in patients with brain primary and secondary tumors, glucocorticoids are used to manage neurological symptoms associated with peritumoral edema. They mainly affect the trabecular bone, inducing an early and rapid decline of BMaD due to bone resorption, followed by a slower demineralization phase due to the impaired osteoblast activity (D'Oronzo *et al.*, 2015).

Radiotherapy can induce bone loss through multiple mechanisms, including indirect ones, such as iatrogenic hypogonadism, hyperparathyroidism and electrolyte disorders, and direct toxicity based on the imbalance between osteoblasts and osteoclasts (D'Oronzo *et al.*, 2015).

Prevention

Lifestyle measures, such as weight exercises, avoiding alcohol and smoking, limiting caffeine consumption, and achieving and/or maintaining a normal body weight, are beneficial to prevent CTIBL (Finkelstein and Wu, 2017; Ibrahim *et al.*, 2013; D'Oronzo *et al.*, 2015; Hadji *et al.*, 2008; Becker and Cohen, 2017).

Supplementation with calcium (1200 mg/day) and vitamin D (800 IU) to reach serum levels of at least 30 ng/mL should also be ensured to maintain bone turnover. Furthermore, maintaining these levels is known to reduce the risk for hip fractures in elderly women (Brufsky, 2008; Ibrahim *et al.*, 2013; D'Oronzo *et al.*, 2015; Hadji *et al.*, 2008).

Treatment

There are currently no FDA-approved agents for CTIBL prevention or treatment. However, conventional therapies for osteoporosis management have been successfully used to date.

An expert consensus recommends antiresorptive therapy for patients receiving AIs and having a T-score < -2.0 or two or more clinical risk factors for fracture (**Fig. 3**) (Coleman *et al.*, 2014).

A number of clinical trials have shown efficacy of bisphosphonates in preventing chemotherapy- and AIs-induced BMaD reduction in premenopausal women (**Table 1**) (Vehmanen *et al.*, 2004; Delmas *et al.*, 1997; Gnant *et al.*, 2008; Ellis *et al.*, 2008). Dosing and administration route is usually different for preventive and treatment purposes. Patients should be warned about contraindications for invasive dental procedures during bisphosphonate treatment, as this can be a risk factor for osteonecrosis of the jaw (ONJ). This treatment should not be offered to patients with esophageal disorders (if taken po), chronic kidney disease (eGFR <30–35 mL/min) or who have undergone certain types of bariatric surgery (if taken po). Calcium and vitamin D serum levels should be corrected, and supplementation should be used even with normal serum levels, as bisphosphonates can cause hypocalcemia.

Types of Bisphosphonates (Table 2)

- Oral (Clodronate, Alendronate, Risedronate, Ibandronate): Patients should be instructed to take these medications in an empty stomach, with plenty of water and in the upright position. Afterwards, they should stay upright for an hour time due to the limited oral bioavailability of these medications (0.5%–1%), which can be severely impaired by food. Oral bisphosphonates can be associated with (sometimes severe) esophagitis, constipation, or stomach discomfort. Alendronate improves bone mass density and reduces the risk of fractures in men with osteoporosis, although conflicting results have been reported for the oral bisphosphonates Alendronate and Risedronate (Ringe *et al.*, 2004; Brufsky, 2008; Saad *et al.*, 2008). Clodronate showed efficacy in bone loss prevention in premenopausal breast cancer patients with chemotherapy-induced ovarian failure (Saarto *et al.*, 1997).
- Intravenous: Intravenous (IV) bisphosphonates include Pamidronate and Zoledronic acid. Like oral bisphosphonates, although not formerly approved in this indication, they are sometimes used to treat CTIBL. IV bisphosphonates can also be used in men (Boonen *et al.*, 2012; Brufsky, 2008; Saad *et al.*, 2008). A 2007 phase III trial conducted by Gnant *et al.* revealed that Zoledronic

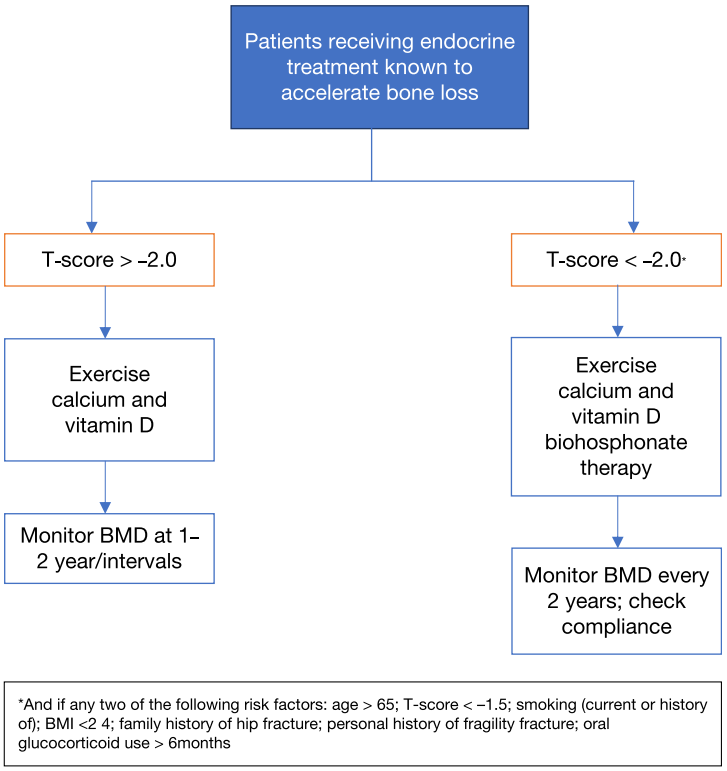

Fig. 3 Recommended bone health management algorithm in women receiving aromatase inhibitor (AI) therapy for breast cancer. Adapted from Coleman, R., *et al.* (2014). Bone health in cancer patients: ESMO Clinical Practice Guidelines, *Annals of Oncology* **25** (Supplement 3), iii124–iii,137.

Table 1 Trials of antiresorptive agents for preventing CTIBL in postmenopausal women with breast cancer

Antiresorptive agent (trial)	*N*	*BMaD study, n°*	*Dosing*	*Treatment duration, years*
Zoledronic acid (ZO-FAST)	1065	1065	4 mg iv q6mo	5
Zoledronic acid (*Z*-FAST)	602	602	4 mg iv q6mo	5
Zoledronic acid (*E*-ZO-FAST)	527	527	4 mg iv q6mo	5
Zoledronic acid (N03CC)	558	395	4 mg iv q6mo	5
Denosumab (HALT-BC)	252	252	60 mg sc q6mo	2
Risedronate (SABER)	154	111	35 mg po/week	2
Risedronate	87	87	35 mg po/week	5
Clodronate	61	61	1600 mg po/day	3
Risedronate (ARBI)	213	70	35 mg po/week	2
Risedronate (IBIS-II)	613	59	35 mg po/week	5
Ibandronate (ARIBON)	131	50	150 mg po/month	2
Risedronate	118	11	35 mg po/week	1

BMaD: Bone mass density; iv: intravenous; sc: subcutaneous; po: per os; q6month: every 6 months.

Adapted from Hadji, P., et al. (2011). Management of aromatase inhibitor-associated bone loss in postmenopausal women with breast cancer: practical guidance for prevention and treatment. Annals of Oncology 22, 2546–2555.

Acid (4 mg every 6 months) can prevent adjuvant endocrine therapy-associated bone loss in premenopausal patients. Results showed that endocrine treatment without Zoledronic acid led to significant ($P = 0.001$) overall bone loss after 3 years of treatment (BMaD, 14.4% after 36 months; mean T score reduction, 1.4). In contrast, BMaD remained stable in Zoledronic acid-treated patients compared with endocrine therapy alone ($P = 0.0001$). No interactions with age or other risk factors were observed (Gnant *et al.*, 2007). Zoledronic acid should be infused for a period of 15 min. The dosage of 5 mg yearly has also been shown to prevent osteoporosis fractures (D'Oronzo *et al.*, 2015; Zhang *et al.*, 2012; Gnant *et al.*, 2007).

– Denosumab, marketed under the brand name *Prolia* (60 mg every 6 months), is approved to increase bone mass in patients with increased risk of fractures in several indications, including (i) postmenopausal women with osteoporosis; (ii) men receiving ADT for non-metastatic prostate cancer; and (iii) women receiving adjuvant AI therapy for breast cancer (Lipton *et al.*,

Table 2 Bisphosphonates dosing

Agent	*Indication/dose (prevention CTIBL)*
Alendronate	70 mg oral weekly
Ibandronate	150 mg oral monthly
Risedronate	35 mg oral weekly
Zoledronic acid	5 mg IV yearly 4 mg IV q6month
Clodronate	1600 mg PO id
Pamidronate	60 mg IV every 12 weeks
Denosumab	60 mg every 6 months sc

CTIBL: cancer treatment induced bone loss; iv: intravenous; sc: subcutaneous; po: per os; q6month: every 6 months.

2012; Ellis *et al.*, 2008). In one trial, Denosumab (60 mg every 6 months) was also associated with an increase in BMaD and a reduction in new vertebral fractures incidence among men receiving ADT for non-metastatic prostate cancer (Smith *et al.*, 2009).

Regarding safety concerns, both bisphosphonates and denosumab are well tolerated, although Zoledronic acid is associated with more acute phase reactions and renal dysfunction, and less hypocalcemia episodes. Frequency of ONJ is equivalent with both treatments. Nevertheless, a less intensive schedule of bisphosphonates and Denosumab for bone mass preservation is associated with a lower rate of side effects (D'Oronzo *et al.*, 2015).

Bone Turnover Biomarkers

Although fracture risk rises exponentially as the BMaD declines, many frailty fractures occur in individuals who are not at risk according to the conventional densitometric criteria for osteoporosis, suggesting the need for a complementary assessment with biochemical markers of bone turnover. Markers of bone formation include bone alkaline phosphatase (ALP) and procollagen type I N-terminal propeptide (PINP), whereas serum C-terminal cross-linked telopeptide of type I collagen (S-CTX), urinary C-terminal telopeptide of type I collagen (U-CTX), and urinary NTX (U-NTX) are bone resorption markers. Concurrent dosage of plasmatic VitD, PTH, and calcium increases the accuracy of bone health assessment. Several studies have shown that levels of S-CTX and bone ALP could be predictive of vertebral and hip fractures, although their high individual variability due to age, gender, ethnicity, comorbidities and diet prevents routine application in clinical practice (D'Oronzo *et al.*, 2015; Lipton *et al.*, 2012).

Bone turnover markers are not yet established as routine clinical markers in any population, and their value for management of individual patients is not determined to date (Doo and Shapiro, 2013).

See also: Hormonal Treatment of Breast Cancer

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Bone Metastases; Basic Aspects

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Nomenclature

BMP-7	Bone morphogenetic protein 7
CaSR	Calcium-sensing receptor
CDH11	Cadherin 11
CTGF	Connective tissue growth factor
CTSK	Cathepsin K
CX43	Connexin 43
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	Couple chemokine (C–X–C) receptor type 4
DKK1	Dickkopf-1
DTC	Disseminated tumor cell
ET-1	Endothelin-1
ERK 1/2	Extracellular signal-regulated kinase ½.
FGF9	Fibroblast growth factor 9
GAS6	Growth arrest-specific 6
HIF	Hypoxia-inducible factor
HSC	Hematopoietic stem cell
IHH	Indian Hedgehog
LIF	Leukemia inhibitory factor
LOX	Lysyl oxidase
MAPK	Mitogen-activated protein kinase
MSK1	Mitogen- and stress-activated kinase 1
OPG	Osteoprotegerin
PDGF	Platelet-derived growth factor
PTH-rP	Parathyroid hormone-related peptide
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt-related transcription factor 2
SIBLINGs	Small integrin-binding ligand N-linked glycoproteins
SNO	Spindle-shaped N-cadherin + osteoblast
SOST	Sclerostin
TGFβ-2	Transforming growth factor-β2
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TSP-1	Thrombospondin-1
VCAM-1	Vascular cell adhesion protein 1
Wnt	Wingless/int

Introduction

Bone metastases, the spread of cancer to the bones, occur in more than 1.5 million patients with cancer world-wide and are most commonly associated with cancers of the prostate, lung, and breast, with incidence rates as high as 75% of patients with metastatic disease (Weilbaecher *et al.*, 2011). Metastatic cancer cells residing in the bone marrow alter the functions of bone-resorbing (osteoclasts) and bone-forming (osteoblasts) cells (Weilbaecher *et al.*, 2011). By disrupting the physiological balance between bone resorption and bone formation, metastatic cells promote skeletal destruction (Weilbaecher *et al.*, 2011). Weakened bones due to skeletal metastases can then lead to occurrence of skeletal-related events, such as fractures and compression of the spinal cord, bone pain and disability, contributing substantially to morbidity and mortality in patients with advanced cancer (Weilbaecher *et al.*, 2011). Here we provide an overview of the mechanisms of bone metastasis formation.

Bone metastasis is a stepwise sequence of events that include tumor cell colonization of the bone marrow, construction of a cancer niche, tumor cell interactions with bone cells (osteoclasts, osteoblasts) and release of signals from the resorbed bone matrix that promote skeletal tumor growth. Molecular mechanisms that mediate each of these events are described below.

Bone Colonization by Tumor Cells

Pre-metastatic Niche

Experimental studies suggest that, even before becoming clinically detectable, primary tumors release soluble factors into the bloodstream that induce the formation of a tumor growth-favoring microenvironment (called "pre-metastatic niche") in distant organs in order to attract tumor cells to these sites (Peinado *et al.*, 2017). Among these soluble factors, exosomes may play a prominent role. Exosomes are small vesicles (30–120 nm) containing DNA, RNA [messenger RNA (mRNA), microRNA (miRNA) and other noncoding RNAs], lipids and proteins that are secreted by all types of cells in culture, including tumor cells. Exosomes are also found in body fluids (blood, urine, saliva, and breast milk) (Vlassov *et al.*, 2012). In cancer, tumor-derived exosomes express distinct cell surface receptors (integrins) that are addressing them to specific organs (Hoshino *et al.*, 2015). Specifically, tumor-derived exosomal integrins $\alpha6\beta4$ and $\alpha6\beta1$ are associated with lung metastasis, while exosomal integrin $\alpha v\beta5$ is linked to liver metastasis in animals (Hoshino *et al.*, 2015). These tumor-derived exosomes are up-taken by organ-specific cells (S100A4-positive fibroblasts and Kupffer cells in lungs and liver, respectively), which then produce promigratory and proinflammatory

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molecules within pulmonary and hepatic tissues, thereby preparing the soil to facilitate the arrival of tumor cells at these sites (Hoshino *et al.*, 2015). We, and others, showed that tumor cell integrins ITGBL1, $\alpha 2\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 6$ are associated with breast or prostate cancer bone metastasis (Zhao *et al.*, 2007; Sottnik *et al.*, 2013; Dutta *et al.*, 2014; Li *et al.*, 2015), suggesting that they could address exosomes to bone. Integrin-independent exosomal mechanisms are also involved. In melanoma, the transfer of the MET oncoprotein from tumor-derived exosomes to bone marrow progenitor cells promotes the metastatic process in lungs and bone (Peinado *et al.*, 2012). Tumor-derived exosomal miRNAs can also reprogram or educate target cells that take up exosomes toward a pro-metastatic and proinflammatory phenotype, thereby creating a pre-metastatic niche (Peinado *et al.*, 2017). The main action of miRNAs is the negative regulation of gene expression by base-pairing to the 3′-UTR of target mRNAs, resulting in translational repression or mRNA degradation (Beermann *et al.*, 2016). In cancer, miRNAs function either as tumor suppressors or as oncogenes, and their dysregulation contributes to metastasis in specific organs, including the skeleton (Beermann *et al.*, 2016). With regards to bone metastasis, there are evidence that exosomal miR-141 and miR-940 produced by prostate cancer cells promote the osteogenic differentiation of mesenchymal cells in the bone marrow microenvironment, which then facilitates the formation of bone metastases with an osteoblastic phenotype (Hashimoto *et al.*, 2018; Ye *et al.*, 2017).

Another important class of molecules involved in the formation of the pre-metastatic niche is the lysyl oxidase (LOX) family whose primary function is to drive collagen crosslinking and extracellular matrix stiffness (Peinado *et al.*, 2017). Hypoxia-inducible factor-1 (HIF-1) induces the expression of LOX in breast tumors and secreted LOX catalyzes collagen crosslinking in the lungs, facilitating the recruitment of bone marrow-derived cells and the subsequent colonization of the pulmonary tissue by breast cancer cells (Erler *et al.*, 2009). In bone, we and others have shown that tumor-derived LOX from breast and colon cancer cells generates pre-metastatic osteolytic lesions in animals by impairing bone homeostasis through the stimulation of osteoclast formation induced by RANKL and the inhibition of osteoblast formation (Cox *et al.*, 2015; Reynaud *et al.*, 2017). Thus, by acting systemically, LOX contributes to the formation of a pre-metastatic niche in the bone marrow.

Factors Promoting the Homing of Tumor Cells to the Bone Marrow

Chemokines, which are small proteins (8–14 kDa) belonging to the cytokine family, play a crucial role in promoting the migration of tumor cells to the bone marrow and, more specifically, to the pre-metastatic niche (Weilbaecher *et al.*, 2011). They are produced by stromal cells, which are associated with the pre-metastatic niche, and released in the bloodstream. For instance, the chemokine C-X-C motif chemokine ligand 12 (CXCL-12) is produced in large amounts in the bone marrow, lungs, and liver. Metastatic cells from breast or prostate cancers that express CXCR-4, the CXCL-12 receptor, migrate preferentially to these three organs due to the chemotactic properties of CXCL-12 (Weilbaecher *et al.*, 2011). Several other chemokines (CXCL-10, CXCL-13, CXCL-16, CX3CL-1, CCL22) are also produced by the bone microenvironment and have been implicated in the homing of tumor cells to bone (Weilbaecher *et al.*, 2011; Lee *et al.*, 2012; Lee *et al.*, 2012).

Construction of a Cancer Niche in the Bone Marrow

The fate of tumor cells after they enter the bone marrow remains the most elusive aspect of metastasis formation. Disseminated tumor cells (DTCs) in the bone marrow may be nonproliferative and remain dormant for several years, therefore never develop into overt bone metastases (Weilbaecher *et al.*, 2011). What enables DTCs in the bone marrow to remain dormant and how do they acquire the competence to form overt metastases? These questions come under intense scrutiny. Current hypotheses gaining ground are that DTCs must build a cancer niche by producing their own niche components to anchor and survive in the bone microenvironment, or bind to extracellular matrix components of the pre-metastatic niche that are produced by recruited stromal cells and/or occupy native bone marrow niches in which hematopoietic stem cells reside (Oskarsson *et al.*, 2014). By building this cancer niche, DTCs can enter dormancy and thus survive treatment with chemotherapeutics, which only target proliferating tumor cells.

Extracellular Matrix Niche and Tumor Survival

The extracellular matrix components tenascin C and periostin have been shown to play important roles in the construction of cancer niche in mouse models of cancer. Tenascin C is a hexameric protein produced by tumor cells that acts in an autocrine manner to support tumor cell survival. Periostin is a protein produced by stromal cells from the pre-metastatic niche that supports in a paracrine manner tumor cell survival. Tenascin C and periostin bind tightly to each other and to tumor cell surface integrins $\alpha 9\beta 1$ and $\alpha v\beta 3$, respectively, which leads to the activation of Wnt- and Notch-dependent intracellular signaling pathways (Oskarsson *et al.*, 2014; San Martin *et al.*, 2017). Other extracellular matrix components, including SIBLINGS (osteopontin, bone sialoprotein, dentin matrix protein 1), osteonectin, connective tissue growth factor (CTGF, CCN2), and nephroblastoma overexpressed (Nov, CCN3), have also been associated with bone metastasis formation (Trotter and Yang, 2016). Osteopontin has been involved in early bone colonization through binding to tumor cell surface integrin $\alpha v\beta 3$ (Trotter and Yang, 2016). Other SIBLINGs, osteonectin, CTGF and Nov are involved at a late stage in the bone metastatic disease, promoting tumor cell invasion and growth (Trotter and Yang, 2016; Croucher *et al.*, 2016).

Bone Niches and Tumor Dormancy

Osteogenic and vascular niches are crucial regulators of hematopoietic stem cell (HSC) behavior in the bone marrow. During homeostasis, signals coming from the osteogenic niche keep HSCs dormant, which preserves their long-term self-renewal potential, whereas the vascular niche is oxygenated and stimulates proliferation and differentiation of HSCs to maintain hematopoiesis (Winkler *et al.*, 2012). A current hypothesis gaining ground is that common mechanisms are likely to be involved in maintaining HSC and DTC dormancy in the osteogenic niche. "Dormancy is a stage in cancer progression where the cells cease dividing but survive in a quiescent state while waiting for appropriate environmental conditions to begin proliferation again. Quiescence is the state where cells are not dividing but at arrest in the cell cycle in G0-G1" (https://en.wikipedia.org/wiki/Cancer_dormancy). Quiescent DTCs are therefore protected against chemotherapy-induced apoptosis.

The osteogenic niche is home to a particular spindle-shaped N-cadherin + osteoblast (SNO) cell population. SNO cells regulate dormancy of HSCs through interaction with N-cadherin. It has been shown that E-cadherin-expressing breast cancer cells home to SNO cells through interactions with N-cadherin (Sosa *et al.*, 2014; Kan *et al.*, 2016). Another molecule that regulates HSC dormancy is growth arrest-specific 6 (GAS6), which is an osteoblast-derived ligand of the MER, TYRO3 and AXL (also known as UFO) tyrosine kinase receptors. When prostate cancer cells bind to osteoblasts in the niche they increase their expression level of AXL and GAS6 signaling inhibits tumor cell proliferation (Sosa *et al.*, 2014; Kan *et al.*, 2016). Osteoblasts also express the chemokine CXCL-12, which mediates adhesive interactions with HSCs by binding to CXCR4, the receptor for CXCL-12. It has been observed that prostate cancer cells, which express CXCR4, can directly compete with HSCs for osteogenic niche support (Shiozawa *et al.*, 2011).

The vascular niche is composed of endothelial cells that express specific cell adhesion molecules (*E*- and P-selectins), E-selectin being a crucial component that mediates HSC attachment and proliferation (Winkler *et al.*, 2012). Using real-time in vivo microscopy of breast tumor xenografts, it has been shown that dormant tumor cells were preferentially located in E-selectin-rich vascular regions in which they were anchored through SDF-1/CXCR4 interactions (Price *et al.*, 2016). These results (Price *et al.*, 2016) are in contrast with previous findings showing that dormant prostate cancer cells, which express CXCR4, compete with HSCs for osteogenic niche support (Shiozawa *et al.*, 2011). Ghajar *et al.* (2013) also reported that the bone marrow microvasculature might constitute a dormant niche for breast cancer cells. Specifically, they showed that the endothelium-derived extracellular matrix protein thrombospondin-1 (TSP-1) induces sustained dormancy of breast cancer cells in vivo. Overall, these experimental data suggest the concept of niche support for DTCs is still in the early stages of investigation, and warrants further investigation.

Other factors produced by osteoblasts or bone marrow mesenchymal stem cells, including bone morphogenetic protein 7 (BMP-7), transforming growth factor-β2 (TGFβ-2), leukemia inhibitory factor (LIF), and exosomal miR-23b, can also induce tumor dormancy in different types of cancer (Ono *et al.*, 2014; Sosa *et al.*, 2014; Johnson *et al.*, 2016).

Osteomimicry

During the time DTCs are in the bone marrow, they are able to adapt, exiting and re-entering a dormant state, and undergoing further selection to acquire a full complement of metastasis-colonization functions that they did not express before. In this context, tumor cells in the bone marrow express bone-associated genes (*RUNX2*, *CTSK*, *OPG*, *SPARC*, *CDH11*, *CX43*, *DKK1*), which are normally expressed by osteoblasts or osteoclasts (Bellahcène *et al.*, 2007; Le Gall *et al.*, 2007; Fradet *et al.*, 2011; Weilbaecher *et al.*, 2011). This process is called osteomimicry. For instance, the disruption of *RUNX2* activity in breast cancer cells abolishes their ability to form osteolytic lesions in vivo (Pratap *et al.*, 2011). Similarly, the incidence of prostate cancer bone metastasis is reduced greatly when cadherin-11 (CDH11) expression by tumor cells is silenced (Weilbaecher *et al.*, 2011). Moreover, paired immunochemistry on primary breast tumor samples and associated liver, lung or bone metastases showed that only bone metastatic cancer cells express bone proteins such as cathepsin K, osteonectin, cadherin-11, connexin-43 and RUNX2, demonstrating the clinical relevance of this process (Bellahcène *et al.*, 2007; Le Gall *et al.*, 2007; Fradet *et al.*, 2011).

Dormant Cell Reactivation

Two mitogen-activated kinases (MAPKs), namely p38 and extracellular signal-regulated kinase (ERK), control the switch of tumor cells between dormancy and active growth, respectively (Sosa *et al.*, 2014). Some of the molecular mechanisms that regulate this switch in the bone marrow have been identified. For example, mitogen- and stress-activated kinase 1 (MSK1), a downstream effector of the p38 and ERK1/2 signaling pathways, has been involved in the regulation of tumor dormancy in estrogen receptor (ER)-positive breast cancer cells that are metastatic to bone (Gawrzak *et al.*, 2018). Specifically, p38 depletion in tumor cells decreases MSK1 expression, and MSK1 depletion increases the capacity of poorly metastatic ER-positive breast cancer cells to form overt metastasis in animals (Gawrzak *et al.*, 2018). Thus, MSK1 is a dormancy enforcer and a negative regulator of metastasis initiation.

Bone might also provide an environment that enables dormant cell reactivation. Within a mouse bone metastasis model, indolent breast cancer micrometastases were found to overexpress adhesion protein vascular cell adhesion protein 1 (VCAM-1), which promotes the recruitment of osteoclast precursors by binding to osteoclast integrin $\alpha4\beta1$, leading to osteoclast formation and osteoclastic bone resorption (Sosa *et al.*, 2014). The mechanisms underlying VCAM-1 silencing and re-expression in these indolent bone marrow micrometastases are unknown. However, bone resorption likely creates an environment that promotes

tumor cell reactivation (Croucher *et al.*, 2016). In this respect, TGFβ1 and TGFβ2 exhibit competing functions on behavior of tumor cells in the bone marrow (Sosa *et al.*, 2014). TGFβ2 promotes tumor cell dormancy through Smad1/5 and p38 signaling, whereas TGFβ1 switches off dormancy, leading to rapid tumor growth in vivo (Sosa *et al.*, 2014). Thus, TGFβ1 released from resorbed bone could contribute to dormant cell reactivation (Weilbaecher *et al.*, 2011).

As exemplified with VCAM-1 and TGFβ1, there is a growing body of evidence suggesting that osteoclasts may be responsible for the reactivation of dormant DTCs (Weilbaecher *et al.*, 2011; Sosa *et al.*, 2014; Croucher *et al.*, 2016). It is well known that manipulating the bone microenvironment in mice by stimulating osteoclast activity through vitamin D deficiency, or through estrogen or androgen deprivation enhances skeletal tumor burden and bone destruction (Weilbaecher *et al.*, 2011; Croucher *et al.*, 2016). In this context, TGFβ1 that is released from resorbed bone not only switches off DTC dormancy, but also plays a central role in most of the events leading to tumor expansion in bone and bone destruction (Weilbaecher *et al.*, 2011). For instance, bone-derived TGFβ1 stimulates the expression of the Notch ligand Jagged-1 in bone metastatic tumor cells, which in turn promotes bone metastasis by activating the Notch pathway in supporting bone cells. Specifically, Jagged-Notch signaling directly promotes osteoclast differentiation and bone resorption, and it stimulates the release of IL-6 from osteoblasts to promote tumor cell proliferation (Sethi *et al.*, 2011). In addition, the therapeutic targeting of Jagged-1 with a monoclonal antibody inhibits bone metastasis formation in animals (Zheng *et al.*, 2017). Thus, osteoclastic bone resorption is likely to play an important role at an early stage in the establishment of bone metastasis.

Tumor-Derived Factors Mediating Osteolytic or Osteoblastic Bone Metastases

At a late stage in the progression of skeletal lesions, the patterns of bone metastases ranged from mostly destructive or osteolytic (in breast cancer, lung cancer, or myeloma), to mostly bone-forming or osteoblastic (in prostate cancer). There is however always an imbalance between bone formation and bone resorption. Bone metastasis is therefore a spectrum between these two extremes where, at one end, predominantly osteolytic lesions are associated with high osteoclast activity and reduced osteoblast activity and, at the other end, bone metastases that are predominantly osteoblastic have a high osteoblast activity and a reduced osteoclast activity (Weilbaecher *et al.*, 2011). Different molecular mechanisms associated with these different patterns of bone metastasis have been identified and are described below.

Osteolytic Lesions

Several factors secreted by tumor cells during the course of development of malignant osteolytic lesions stimulate osteoclast activity and bone resorption. Among them, parathyroid hormone-related peptide (PTHrP) was the first to be recognized as involved in malignant osteolysis. PTHrP stimulates in osteoblasts the expression of the cytokine receptor activator of nuclear factor-κB ligand (RANKL), which binds to its receptor RANK on osteoclast precursors, leading to the formation of new osteoclasts and therefore to osteoclastic bone resorption (Weilbaecher *et al.*, 2011). The transcription factor RUNX2 contributes to the ability of tumor cells to activate osteoclasts by upregulating Indian Hedgehog (IHH) expression, which in turn stimulates PTHrP-induced RANKL in osteoblasts and subsequently promotes RANK/RANKL-dependent activation of osteoclasts (Pratap *et al.*, 2011). Likewise, the transcription factor MAF mediates breast cancer bone metastasis through the control of PTHrP (Pavlovic *et al.*, 2015). Subsequent studies, described below, showed that TGFβ released from resorbed bone induces PTHrP production from tumor cells that results in enhanced bone resorption (Weilbaecher *et al.*, 2011). Overall, these studies (Pratap *et al.*, 2011; Weilbaecher *et al.*, 2011; Pavlovic *et al.*, 2015) demonstrate the importance of PTHrP in the pathogenesis of osteolytic lesions. Several other molecules produced by tumor cells, including interleukins (IL-6, IL-8, and IL-11) and granulocyte macrophage-colony stimulating factor (GM-CSF), stimulate osteoclast activity through the activation of the RANK/RANKL pathway (Weilbaecher *et al.*, 2011). Platelet-derived lysophosphatidic acid (LPA) also supports progression of osteolytic bone metastases in breast cancer. We showed that LPA, by binding to its receptor LPA1 at the tumor cell surface, promotes tumor cell proliferation and the LPA1-dependent secretion of IL-6 and IL-8, which in turn promotes osteoclast-mediated bone resorption (Boucharaba *et al.*, 2004). Moreover, the functional blockade of LPA action on its receptor, using a LPA1 antagonist, substantially reduces progression of osteolytic bone metastases in animals (Boucharaba *et al.*, 2006).

Tumor cells not only stimulate osteoclast activity, but also inhibit osteoblast activity, thereby worsening the imbalance between bone formation and bone resorption, and promoting bone destruction (Weilbaecher *et al.*, 2011). Specifically, tumor cells secrete activin A (a member of the TGF-β superfamily of growth factors), noggin (a BMP antagonist), and dickkopf-1 (DKK-1) and sclerostin (SOST-1) [two Wingless/int (Wnt) protein antagonists], all of them suppressing osteoblast differentiation (Weilbaecher *et al.*, 2011; Zhu *et al.*, 2017). For instance, MDA-MB-231 and MCF-7 breast cancer cells express SOST-1 and an anti-SOST antibody reduces tumor cell migration and invasion in vitro, and prevents formation of osteolytic lesions in animals (Zhu *et al.*, 2017).

Osteoblastic Lesions

One of the most well studied mediators is endothelin-1 (ET-1), which stimulates osteoblast proliferation and inhibits osteoclast activity and motility. Among patients with prostate cancer, those with bone metastases have far higher circulating levels of ET-1,

compared to those with localized cancer (Weilbaecher *et al.*, 2011). Gene expression analysis in mouse primary osteoblasts revealed that treatment with ET1 upregulates multiple osteoblast-stimulatory factors (e.g., IL-6 and RANKL) and downregulates DKK-1 (Rosano *et al.*, 2013). Stimulatory effects of ET-1 on osteoblasts are mediated by two receptors, ETAR and ETBR, which activate similar signaling pathways. Osteoblast proliferation and bone metastasis are both inhibited by ETAR antagonists atrasentan and zibotentan, as well as by the dual ETAR and ETBR antagonist bosentan, highlighting the prominent role played by ET-1 in the formation of osteoblastic lesions (Rosano *et al.*, 2013). Other osteoblast-stimulatory factors (VEGF, BMP-6) have also been shown to participate to the formation of osteoblastic lesions (Dai *et al.*, 2005; Kitagawa *et al.*, 2005). Osteoblasts express VEGF membrane receptors Flt1 and KDR and the treatment of animals with the Flt1 receptor inhibitor PTK787 blocks the formation of prostate cancer osteoblastic lesions in animals (Kitagawa *et al.*, 2005). Likewise, the treatment of animals with an antibody to BMP-6 decreases the formation of osteoblastic bone metastases induced by prostate cancer cells (Dai *et al.*, 2005). The osteoblast-stimulatory factors BMP-2, adrenomedullin, Wnt, and fibroblast growth factor 9 (FGF9) have been also involved in the formation of osteoblastic lesions (Logothetis and Lin, 2005). In addition, prostate-specific antigen (PSA), as a serine protease that cleaves PTHrP in the PTH-like domain, could also indirectly contribute to the formation of osteoblastic lesions by neutralizing osteoclast-stimulatory effect of PTHrP (Cramer *et al.*, 1996).

Osteoprotegerin (OPG) is a soluble RANKL inhibitor produced by osteoblasts that inhibits RANKL/RANK interaction. OPG levels are increased in patients with prostate cancer bone metastases. In addition, OPG can be expressed in breast and prostate cancer cells. OPG expressed by prostate cancer cells inhibits bone resorption, thereby contributing to the osteoblastic trait of most prostate cancer bone lesions (Corey *et al.*, 2005). In addition, OPG can promote tumor cell survival by binding to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Croucher *et al.*, 2016).

Contribution of Bone to Metastasis Progression

Bone tissue is an abundant reservoir for growth factors, including TGFβ, insulin-like growth factors (IGFs) and platelet-derived growth factors (PDGFs) (Weilbaecher *et al.*, 2011). When released from the resorbed bone matrix, TGFβ acts on tumor cells, via SMAD- and COX2-dependent signaling pathways, which stimulates the expression of factors such as PTHrP, Jagged-1, IL-11, and prostaglandin E2 (Weilbaecher *et al.*, 2011; Sethi *et al.*, 2011; Croucher *et al.*, 2016). Bone-derived IGFs and PDGFs stimulate tumor growth (Weilbaecher *et al.*, 2011). Calcium is another factor released from bone during osteoclastic resorption. Calcium binds on tumor cells (breast, prostate) via a calcium-sensing receptor (CaSR) and promotes tumor cell proliferation and migration and secretion of PTHrP and epiregulin (Weilbaecher *et al.*, 2011; Boudot *et al.*, 2017). Epiregulin is an EGF-like ligand whose expression is increased in osteolytic lesions in vivo. It decreases *OPG* expression in osteoblasts, thereby contributing to the progression of osteolytic lesions (Boudot *et al.*, 2017).

Summary and Conclusion

In this review, we described early events that allow tumor cells to disseminate and colonize in the bone marrow and we highlighted the prominence of pre-metastatic and metastatic niches in mediating homing and dormancy of tumor cells, respectively. We also discussed the importance of the osteoclast activity for reactivation of dormant tumor cells. Finally, we explained how, at a later stage, tumor cells induce osteolytic or osteoblastic lesions. To date, no effective treatments for bone metastasis exist, only therapies that aim to limit the bone degradation such as denosumab (anti-RANKL monoclonal antibody) and bisphosphonates. The future challenge will lie in our ability to develop therapeutics targeting dormancy in order to address residual disease in patients.

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Bone Metastases; Clinical Aspects

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Incidence and Clinical Features of Bone Metastasis

Metastasis to the skeleton represents a serious and, currently irreversible, step in the progression of solid and hematological malignancies. Both the incidence of bone metastases and median survival after their diagnosis vary according to the primary tumor (**Table 1**). Due to the high incidence of breast and prostate tumors and the relatively long survival following bone metastasis development (typically 2–3 years for breast cancer, but potentially improving with immunotherapy, and 2–4 years for prostate cancer), these tumors account for up to 70% of the prevalence of cancer-related skeletal lesions (Coleman, 2006). Hernandez *et al.* (2015) estimated the 2012 prevalence of patients with bone metastases in the United States as approximately 300,000. The primary malignancies were represented by breast cancer in approximately one third of cases and prostate tumors in more than a quarter. In approximately 40% of breast cancer patients who develop distant metastasis, bone is the first site of relapse and, ultimately, more than 70% of patients who die from breast cancer have evidence of skeletal metastasis (Coleman, 2006). In advanced prostate cancer, the frequency of bone metastasis is even higher, probably greater than 80%, whereas in advanced lung cancer, the frequency of bone metastasis is much less at around 30%. Since survival after bone metastasis development in lung cancer is relatively short (typically 12–18 months) (Chen *et al.*, 2016), skeletal complications have represented less of a long-term health issue than for patients with breast or prostate cancer, though again survival is potentially improving with immunotherapy.

Regardless of the primary tumor, the axial skeleton (i.e., spine, ribs, pelvis and skull) is more frequently involved than the appendicular one. Among long bones, femora and humeri are commonly affected by bone metastases.

How Do the Clinical Features of Bone Metastases Arise?

Normal bone homeostasis is maintained by the continuous action of specialized bone resorbing cells (osteoclasts) and bone forming cells (osteoblasts) which, together, act to maintain and continuously renew the skeleton. In healthy individuals, bone resorption and bone formation are coupled and perfectly balanced in location, time, and amount. Osteoblasts are activated by numerous diverse cytokines and growth factors including Wnt-family members (Takada *et al.*, 2009), endothelin-1 (Clines *et al.*, 2007), bone morphogenetic proteins (Zhang *et al.*, 2009), fibroblast growth factor (Bosetti *et al.*, 2010) and transforming growth factor-β, TGFβ (De Gorter *et al.*, 2011) resulting in the increased deposition of new bone matrix. Bone resorption results from the action of osteoclasts which differentiate from osteoclast precursors to form active osteoclasts and this occurs in response to the action of Nuclear Factor-κB ligand (RANKL) binding to the receptor activator of nuclear factor-κB (RANK) upon osteoclast precursor cells (Suda *et al.*, 1999). This balance between bone-formation and bone-resorption, which depends on a range of diverse cytokines and growth factors is perturbed in differing ways by bone-metastatic cancer cells.

The mechanisms which lead to colonization of bone by metastatic cancer cells are the subject of active research. Recent data has provided a molecular basis for the "seed and soil" hypothesis, originally proposed by Stephen Paget in 1889 (Paget, 1989). The progress of tumor cells from initial colonization to tumor growth appears to require distinct niches within the bone, including the bone remodeling compartment (BRC)—a site of active bone resorption (Parfitt, 2001).

Active tumor cells in bone cause disruption of the fine-tuned balance between osteoblast and osteoclast activity, either causing excess bone resorption and bone destruction (as associated with lytic bone lesions), for example, in most breast cancers, renal cell carcinoma, multiple myeloma, thyroid cancer, melanoma (Macedo *et al.*, 2017; Selvaggi and Scagliotti, 2005; Taube *et al.*, 1994), or increased levels of bone formation (as in osteosclerotic bone lesions, e.g., in prostate cancer and lung cancers), though the new

Table 1 Incidence of bone metastases from different primary tumors and relative median survival (Macedo *et al.*, 2017; Coleman, 2006)

Primary tumor site	*Incidence of bone metastases*	*Median survival after bone metastasis diagnosis*
Breast	65%–75%	19–25 months
Prostate	65%–75%	12–53 months
Thyroid	60%	48 months
Lung	30%–40%	6–7 months
Bladder	40%	6–9 months
Kidney	20%–25%	12 months
Skin (Melanoma)	14%–45%	6 months

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bone is formed with an irregular structure (Macedo *et al.*, 2017). In many cases (e.g., in breast cancer), mixed lesions are present having both osteolytic and osteosclerotic character. In all cases of bone metastases, the rates of bone resorption are increased and bone formation is often increased in response, even in lytic disease.

Several mechanisms promote osteolytic lesions including the release by cancer-cells of PTHrP, which in turn promotes the release of RANKL by osteoblasts and the RANKL-RANK mediated activation of osteoclasts. Osteoclast activation in turn releases growth factors and cytokines trapped within the bone matrix which then act upon the cancer cells to promote their further proliferation in a "vicious cycle" of bone destruction (Guise, 2010; Guise, 2013). The secretion of PTHrP by cancer cells which metastasize to bone may be intrinsic to the cancer cells themselves, as gene copy-number analysis recently identified amplification of the gene encoding Musculoaponeurotic Fibrosarcoma (MAF) transcription factor within bone-metastatic breast cancer cells, and an identified target of gene activation by MAF was PTHrP (Pavlovic *et al.*, 2015).

The mechanisms involved in osteoblastic lesions are still poorly understood. Prostate-specific antigen (PSA) has the ability to cleave PTHrP thus inhibiting osteoclast activation and switching the balance over towards bone formation (Keller *et al.*, 2001). More recently evidence has been obtained that prostate cancer secreted bone-morphogenetic protein-4 (BMP4) can convert tumor-associated endothelial cells within the bone marrow into osteoblasts, and this phenotypic conversion may account for the abnormal bone formation observed within prostate cancer (Lin *et al.*, 2017).

Skeletal Related Events and Bone Turnover Markers

Once bone metastases are established, patients may experience serious skeletal complications during the subsequent course of the disease, with consequent progressive disability. These complications of bone metastases are collectively termed skeletal related events (SREs) and have been widely used in clinical trials for assessing bone metastasis-induced skeletal complications and their response to bone-targeted therapies. SREs include pathological fractures (such as in long bone and vertebral fracture), the need for radiation to bone for pain relief, the need for surgery to bone and development of spinal cord compression. Some studies have included hypercalcemia of malignancy within the SRE definition. In most cases, hypercalcemia is associated with osteolytic bone metastases being, thus, more frequent in breast and lung malignancies than in prostate tumors (Coleman, 2006).

The frequency of SREs is relatively independent of the tumor of origin, with the most common being the need for radiation in patients with bone metastasis from breast, lung or prostate malignancies (Kuchuck *et al.*, 2013; Ulas *et al.*, 2016; Klaassen *et al.*, 2017). Pathological fractures are the second most frequent SRE, while spinal cord compression is relatively uncommon; however, this represents a medical emergency requiring urgent attention.

The socio-economic impact of SREs is very high. A multicenter observational study involving four European countries (UK, Germany, Italy and Spain) investigated the use of healthcare resources by patients with bone metastases from solid or hematologic malignancies who developed SREs. Approximately one-third of patients needed hospitalization, whose median duration reached 41.1 days in case of spinal cord compression (Hoefeler *et al.*, 2014). Another observational study investigated the need for palliative radiotherapy in both breast and prostate cancer patients, showing a total cost for each patient of $7,457US and $7,553US respectively; spine and hip were the most frequently irradiated sites (Hess *et al.*, 2012).

Normal bone metabolism (bone resorption and equivalent bone formation) results in release of a variety of intermediates into the circulation or urine. Many of these are derived from the breakdown or formation of type I collagen and have been evaluated as markers which can report in a "real time" manner on the current rates of bone formation and bone resorption and how these differ from normal. Originally these markers were developed for use in assessment of benign bone diseases such as osteoporosis. However, they are being used increasingly in oncology, either to give information on the skeletal effects of cancer treatments (e.g., in the bone loss induced by aromatase inhibitors in breast cancer or androgen deprivation therapy, ADT, in prostate cancer) or to reflect the effects of cancer cells on bone function in metastatic bone disease. A list of the most commonly used bone turnover markers is included in **Table 2**.

Table 2 Commonly used bone turnover markers

Turnover marker	*Formation/resorption*	*Urine/serum*
Bone-specific alkaline phosphatase	Formation	Serum
P1CP	Formation	Serum
P1NP	Formation	Serum
Osteocalcin	Formation	Serum
NTX	Resorption	Urine
α/β CTX	Resorption	Urine
Calcium	Resorption	Serum/Urine
1-CTP	Resorption	Serum
CTX	Resorption	Serum

1-CTP, type 1 collagen C telopeptide; NTX, N-telopeptide of type 1 collagen; P1NP, N-terminal propeptide of type 1 procollagen; P1CP, C-terminal propeptide of type 1 procollagen; CTX, C-telopeptide of type 1 collagen.

Whilst bone turnover markers have proved extremely valuable as surrogate efficacy endpoints, especially in early trials of bone-targeted agents, and are widely accepted as evidence of efficacy in this context (see below), it should be noted that they are not approved by regulators as endpoints for registration in Phase III studies.

Several studies have analyzed correlations between bone turnover markers and the risk of SREs. In 121 unselected, consecutive patients with histologically proven malignancy and bone metastases (mainly from breast and prostate cancer), NTX levels were correlated with the number of skeletal-related events and/or death ($r = 0.62$, $P < .001$ for 0–3 months and $r = 0.46$, $P < .001$ for 4–6 months, respectively) (Brown *et al.*, 2003). This study was the first to indicate a strong correlation between the rate of bone resorption and the frequency of skeletal complications in metastatic bone disease, demonstrating the utility of NTX in the prediction of patients most likely to experience skeletal complications and thus benefit from bisphosphonate treatment. In a further study (Brown *et al.*, 2005), baseline and recent bone marker levels were found to be predictive of negative clinical outcomes in patients with bone metastases secondary to prostate cancer and to nonsmall cell lung cancer and other solid tumors (breast cancer not included in this study). NTX levels were more consistent prognostic indicators than bone-specific alkaline phosphatase for all tumor types, reflecting the key role of osteolysis in the development of skeletal complications.

In a study involving 1824 patients, a significant correlation was described between high sBALP levels and the risk of SREs, especially in prostate cancer, while moderate-high levels of uNTX were associated with doubled risk of skeletal complications ($P < .001$ in both instances) (Coleman *et al.*, 2005). By exploring data deriving from placebo-controlled phase III trials of zoledronate in advanced tumors, Brown *et al.* (2010) observed a significant correlation between high baseline levels of the two markers and the risk of SREs. On the other hand, normal baseline NTX correlated with lower risk of pathological fractures ($P < .0001$) in patients with bone metastases receiving zoledronate. Furthermore, patients whose NTX remained permanently within the normal range, exhibited a significant reduction of skeletal complications ($P \leq .0005$), as compared to those with raising NTX (Lipton *et al.*, 2013). Interestingly, normalization of NTX during the first 3 months of bisphosphonate therapy, in patients with high baseline levels of this marker, was associated with reduced risk of skeletal complications, as well as improved survival (Lipton *et al.*, 2008).

A number of clinical variables have been also investigated for their correlation with SRE occurrence. In particular, Wang *et al.* analyzed 1143 patients with bone metastases and developed three different prediction models, identifying the visual analog scale (VAS) for pain as the strongest predictor of SREs. Other clinical variables, such as Mirels score, Frankel classification of spinal cord injuries and serum calcium levels, contributed to SRE prediction (Wang *et al.*, 2016).

More recently, a related terminology termed symptomatic skeletal events (SSE) has been used in clinical studies. The SSE definition is almost identical to SRE but excludes asymptomatic pathologic fractures (Parker *et al.*, 2013). Although clinically more relevant than SREs, SSEs have not been widely investigated so far. Two recent studies analyzed the incidence and economic impact of SSEs in patients with bone metastases from breast and prostate cancer, respectively. In both cases, the need for radiotherapy was the most common event, occurring in 59.5% of breast cancer patients and 84.1% of men with prostate cancer (Yanae *et al.*, 2017; McKay *et al.*, 2017).

Cancer-induced bone pain is one of the most significant causes of morbidity and quality of life (QoL) deterioration, that should be periodically monitored through specific scales (e.g., VAS). Pain features and presentation may vary from vague and intermittent discomfort to severe, sharp and persistent aches, progressively worsening and exacerbated by physical activity (Milgrom *et al.*, 2017). Sometimes, patients describe bouts of intermittent, severe pain (i.e., breakthrough pain) whose management requires specific expertise in palliative care. Bone pain pathogenesis is multifactorial and includes periosteum stretching by space-occupying masses, nerve root compression, endosteal nerve ending stimulation by chemical agents (e.g., cytokines, bradykinin, prostaglandins, histamine, etc.) secreted by both tumor and stromal cells, which stimulate ion channels and sensitize afferent neurons. Pathological fractures further contribute to bone pain, whose features vary according to the skeletal site. Moreover, back pain represents the first symptom of spinal cord compression, generally localized in the overlying area and worsened by activities that increase intradural pressure, such as sneezing, coughing and straining. Local pain may precede the onset of other neurological signs (i.e., weakness and paralysis) by weeks or even months (Milgrom *et al.*, 2017; Coleman, 2006).

Patients' QoL should be routinely assessed, especially when bone metastases have been detected. Tharmalingam *et al.* (2008) reviewed data from 47 studies which applied 24 different questionnaires, aimed at QoL evaluation, to 10,844 patients with bone metastases. Most studies employed the European Organization for Research and Treatment of Cancer QoL Questionnaire version 3 (EORTC QLQ-C30), consisting of 30 questions related to global health, systemic symptoms, limitation in daily functions and potential financial difficulties. More recently, a supplement of the abovementioned questionnaire has been developed, which focuses more specifically on bone metastasis-related symptoms (EORTC QLQ-BM22) (Chow and Bottomley, 2009; Lin and Pakpour, 2016).

Diagnosis of Bone Metastases

Although bone metastasis is usually identified during follow up in patients with a known cancer history, patients may present with bone metastases with no previous cancer diagnosis. Also, diagnosis of bone metastasis can be difficult in elderly patients, in whom osteoporosis and degenerative skeletal diseases can mimic cancer-related conditions. Thus, a careful clinical evaluation of the patient is always necessary, together with a record of the past medical history.

The radiographic features of bone metastases vary according to the osteoblastic, osteolytic or mixed pattern primary tumor from which they arise. Among imaging techniques, plain radiograph is usually the first to be performed following symptoms of bone pain. It is cheap, fast and specific, although its sensitivity is low (44%–50%) and lesions smaller than 1 cm might be undetected, especially when bone mineral content is almost unaffected. Medullary lesions are even more difficult to be identified because of poor contrast (Vinholes *et al.*, 1996).

Following plain radiograph, bone scintigraphy with technetium-99m is widely used to diagnose bone metastasis and to assess progression. It is much more sensitive than plain radiograph (except for multiple myeloma bone lesions), but it is poorly specific (Macedo *et al.*, 2017). The bone-seeking technetium-99m is absorbed onto the hydroxyapatite in bone, to an extent reflective of osteoblastic activity, with preferential uptake of tracer at sites of active bone formation. The bone scan therefore reflects osteoblast activity (as well as bone vascularity), thus highlighting active osteogenesis areas, regardless of their neoplastic, inflammatory or traumatic nature. False-negative scans might occur in the presence of pure lytic metastases, such as those associated with multiple myeloma, as well as in rapidly evolving osteolytic lesions. On the other hand, osteoblastic bone metastases are clearly visualized, unless they are very slow growing (Algra *et al.*, 1991).

Both computed tomography (CT) and magnetic resonance imaging (MRI) have higher sensitivity than plain radiograph (70%–100% and 80%–100%, respectively) and are useful to characterize spinal lesions. The former clearly shows both osteolytic and osteoblastic lesions, while describing any extensions to surrounding tissues. Notably, CT scan is very useful to localize lesions for biopsy (Rosenthal, 1997). MRI enables the entire spine evaluation, as well as the demonstration of spinal cord compression/infiltration (Shah and Salzman, 2011).

18F-Fluoro-D-glucose positron emission tomography (18F-FDG-PET) is another functional technique showing all those metastatic sites, including bone metastases, with high glycolytic rate. It is superior to bone scintigraphy for the detection of skeletal lesions from multiple myeloma, breast cancer (Jambor *et al.*, 2016) and lung cancer (Marom *et al.*, 1999). However, it is less reliable in detecting renal and prostate cancer bone metastases, possibly due to their low glycolytic rate (Cook *et al.*, 2016).

Measurement of bone turnover markers (BTM) has been widely attempted to detect the presence of early-stage skeletal lesions, before they become radiologically evident (Lumachi *et al.*, 2016; Elfar *et al.*, 2017; Klepzig *et al.*, 2009; Kong *et al.*, 2007; Du *et al.*, 2014; Zhang *et al.*, 2016). Although some diagnostic utility has been reported in detecting bone metastasis in lung cancer (Coleman *et al.*, 2011a; Lumachi *et al.*, 2013), this has not been supported in other cancers and their use in detecting the presence of bone metastases is not approved in clinical practice (Van Poznak *et al.*, 2011).

Bone Targeted Agents and Treatment of Bone Metastases

A more detailed account of treatment of bone metastases may be found in Chapter XX.

The discovery and development of pharmaceutical agents which specifically target bone has transformed the treatment of bone metastases and continues to do so. Bone-targeted agents have become standard of care for the systemic treatment and prevention of skeletal complications in patients with bone metastases from a range of solid tumors as well as multiple myeloma. Their current use is based on the results of large randomized controlled clinical trials over the last 20 years. They include the bisphosphonates, the fully humanized synthetic antibody denosumab and the radiopharmaceutical, radium-223. Whilst none of these currently has curable implications for established bone metastasis, they have greatly improved quality of life in terms of reduction in skeletal complications and extended survival in the case of radium-223 in prostate cancer. A summary of the key trials which supported the development of bone-targeted agents is included in **Table 3**.

Bisphosphonates are pyrophosphate analogs which are relatively safe, have few interactions and are usually well tolerated. Having homed to bone, bisphosphonates are internalized by osteoclasts resulting in disruption of osteoclast function and increased osteoclast apoptosis with the overall effect of reducing bone resorption. The bisphosphonate family contains variable side chains which determine their relative potency and precise mechanism of action (Roelofs *et al.*, 2010). Nitrogen-containing bisphosphonates (e.g., ibandronate and zoledronic acid) are especially potent, as they inhibit enzymes of the mevalonate pathway, further slowing down bone turnover. Nonnitrogen containing bisphosphonates, such as clodronate and alendronate, act via the formation of adenosine triphosphate analogs, which cause osteoclast apoptosis.

In oncology, in addition to their standard use in hypercalcemia of malignancy, bisphosphonates are being increasingly employed in the treatment of bone metastases and, more recently, in the adjuvant setting in prevention of metastasis in breast cancer. Studies in both benign disease (Eastell *et al.*, 2010) and in patients with cancer (Brown *et al.*, 2007), have shown that the effects of even a single dose of zoledronic acid on suppression of bone resorption, is extremely long-lasting, potentially out to several years.

Like bisphosphonates, denosumab acts to decrease osteoclast activity and thus reduce bone resorption. Denosumab inhibits receptor activator of nuclear factor κ ligand (RANKL), which is expressed on osteoblasts and released into the bone microenvironment where it activates its receptor [RANK] on osteoclast precursors. Since denosumab directly blocks osteoclast activity, it thereby reduces bone loss. Radium-223(as radium chloride), is an α particle-emitting radiopharmaceutical, chemically in the same periodic group as calcium. It binds strongly to bone, where it interacts with the hydroxyapatite in metabolically active areas such as bone metastases. It emits high linear energy transfer radiation, but with a relatively small range, which results in highly localized effects, acting directly at the site of bone metastases with minimal damage in adjacent tissues. It is associated with minimal toxicity

Table 3 Key trials for the development of bone targeted agents in metastatic bone disease

Agent/trial	*Accrual*	*Population*	*Intervention*	*Comparator*	*Primary outcome*
Clodronate					
Paterson *et al.* (1993)	173	Breast cancer with bone metastases	Oral Clodronate 1600 mg daily	Placebo	Skeletal morbidity rate 218.6 vs. 304.8 per 100 patient-years ($P < .001$)
Tubiana-Hulin *et al.* (2001)	144	Breast cancer with bone metastases	Oral Clodronate 1600 mg daily	Placebo	Time to new SRE 244 vs. 180 days ($P = .05$)
Ibandronate					
Body *et al.* (2004)	564	Breast cancer with bone metastases	Oral Ibandronate 50 mg daily	Placebo	Skeletal morbidity period rate 0.95 vs. 1.18 ($P = .004$)
Body *et al.* (2003)	466	Breast cancer with bone metastases	IV Ibandronate 2 or 6 mg daily	Placebo	Skeletal morbidity period rate 1.19 vs. 1.48 ($P = .004$)
Pamidronate					
Hortobagyi *et al.* (1996)	380	Breast cancer with ≥ 1 bone metastasis	IV Pamidronate 90 mg every 4 weeks	Placebo	Proportion of patients with SRE 65% vs. 46% ($P < .001$)
Theriault *et al.* (1999)	372	Breast cancer with ≥ 1 bone metastasis	IV Pamidronate 90 mg every 4 weeks	Placebo	Skeletal morbidity rate 56% vs. 67% ($P = .027$)
Berenson *et al.* (1996)	392	Multiple myeloma Stage III with ≥ 1 bone metastasis	IV Pamidronate 90 mg every 4 weeks	Placebo	Proportion of patients with SRE 24% vs. 41% ($P < .04$)
Zoledronate					
Barrett-Lee *et al.* (2014)	1404	Breast cancer with ≥ 1 bone metastasis	IV Zoledronate 4 mg every 3–4 weeks	Oral ibandronate 50 mg daily	Rate ratio for SRE 1.148 (95%CI 0.967–1.362)
Rosen *et al.* (2003)	1648	Breast cancer or multiple myeloma with bone metastasis	IV Zoledronate 4 mg (or 8 mg) Every 3–4 weeks	IV Pamidronate 90 mg every 3–4 weeks	Proportion of patients with ≥ 1 SRE 47% vs. 51% Multiple SRE risk HR 0.84 (0.72–0.98) ($P = .003$)
Morgan *et al.* (2010)	1970	Multiple myeloma newly diagnosed	IV Zoledronate 4 mg every 3–4 weeks	Oral clodronate 1600 mg daily	Overall survival HR 0.84 (0.74–0.96) $P = .0118$
Saad *et al.* (2004)	643	Metastatic castrate-resistant prostate cancer	IV Zoledronate 4 mg (or 8 mg) every 3–4 weeks	Placebo	Proportion of patients with ≥ 1 SRE 38% vs. 49% ($P = .028$)
Rosen *et al.* (2004)	773	Lung cancer and other solid tumors (excludes breast and prostate cancer)	IV Zoledronate 4 mg (or 8 mg) every 3–4 weeks	Placebo	Proportion of patients with ≥ 1 SRE 39% vs. 48% ($P = .039$)
Denosumab					
Fizazi *et al.* (2011)	1904	Castrate-resistant prostate cancer	SC Denosumab 120 mg every 4 weeks	IV Zoledronate 4 mg every 4 weeks	Time to first SRE 20.7 vs 17.1 months HR 0.82 (0.71–0.95) (superiority $P = .008$)
Stopeck *et al.* (2010)	2046	Breast cancer with ≥ 1 bone metastasis	SC Denosumab 120 mg every 4 weeks	IV Zoledronate 4 mg every 4 weeks	Time to first SRE HR 0.82 (0.71–0.95) (superiority $P = .01$)
Henry *et al.* (2011)	1776	Solid tumors with bone metastases (excluding breast or prostate primary)	SC Denosumab 120 mg every 4 weeks	IV Zoledronate 4 mg every 4 weeks	Time to first and subsequent SRE HR 0.84 (0.71–0.98) (noninferiority $P = .0007$)
Raje *et al.* (2017)	1718	Multiple Myeloma Newly diagnosed, symptomatic	SC Denosumab 120 mg every 4 weeks	IV Zoledronate 4 mg every 4 weeks	Time to first SRE 22.82 vs 23.98 months (noninferiority $P = .01$) Overall survival HR 0.90 (0.70–1.16) ($P = .41$)
Radium 223					
Parker *et al.* (2013)	921	mCRPC with symptomatic bone metastases and no visceral metastases	Radium-223 50KBq/kg every 4 weeks	Placebo	Time to first SSE 15.6 vs 9.8 months HR: 0.66; $P < .001$

and is well-tolerated. Currently approved for treatment of bone metastatic prostate cancer, extensive current trials are exploring its use in combination with chemotherapy and endocrine treatments in breast and prostate cancer.

The definitive criterion for clinical assessment of response of bone accepted by regulators is the reduction in SREs, as used in the large randomized trials of bone targeted agents discussed above. However, for development of new therapies, including combination of agents, bone turnover markers have been extensively used as surrogates in early trials and in selection of patients for clinical studies. Since increased bone resorption is the hallmark of bone metastasis, reductions in bone resorption markers such as NTX and CTX (and often consequent reduction also in formation markers such as P1NP and BSAP) have been widely interpreted as success criteria in early studies (Coleman *et al.*, 2011a). A major advantage for such studies is that the response is very fast (usually within 6 weeks), much more rapid than assessment via SREs or imaging.

This approach has proven to be reliable when later assessed in larger studies using SRE endpoints. For example, in the SRE breast cancer studies which demonstrated superiority or noninferiority of denosumab to zoledronic acid (see **Table 3**), the early change in bone markers induced by the two bone targeted agents reflected these differences (Stopeck *et al.*, 2010).

Adverse Effects of Bone Targeted Agents

The most serious adverse effect of bisphosphonates and denosumab is the occurrence of osteonecrosis of the jaw (ONJ), which involves painful bone destruction and potential infection. Treatment of ONJ can be challenging with only slow healing despite measures such as local debridement and antibiotic therapy. At the therapeutic doses of potent bisphosphonates (e.g., zoledronic acid) and denosumab used in metastatic bone disease, the incidence risk is about 1% per annum during therapy (Van Poznak *et al.*, 2011). The risk of developing ONJ can be mitigated by improved awareness of ONJ among oncologists, dental practitioner and oral surgeons, good dental hygiene and avoidance of dental procedures during therapy. Essential dental work should be completed before commencing bisphosphonates or denosumab. Based on our now much improved understanding of ONJ, comprehensive guidelines have been produced for its prevention, diagnosis, and management (Ruggiero *et al.*, 2014).

With the exception of ONJ, the adverse effects of bone-targeted agents are relatively mild and include a transient acute phase response and the possibility of renal impairment with intravenous bisphosphonates and gastrointestinal effects or diarrhea with oral agents. In the metastatic setting, there is consensus that the benefits of treatment with bisphosphonates or denosumab almost always outweigh the risks (Coleman *et al.*, 2014).

Bone Targeted Agents and Prevention of Bone Metastases

Preclinical and early clinical studies of bone targeted agents such as bisphosphonates in bone metastatic cancers showed that, in addition to modulating the bone microenvironment, these agents have direct antitumor, antiangiogenic and immunomodulatory effects (Aviles *et al.*, 2007; Daubine *et al.*, 2007; Mystakidou *et al.*, 2005; Santini *et al.*, 2007). There is therefore a rationale for the use of bisphosphonates in early disease in a strategy to prevent or reduce development of bone metastasis.

In one trial involving early breast cancer patients with proven micro-metastases (on bone marrow evaluation), adjuvant use of oral clodronate appeared to delay and prevented the development of bone metastases (Diel *et al.*, 2008). Similarly, Powles *et al.* (2006) showed that daily treatment with clodronate 1600 mg for two years significantly reduced the 5-year incidence of bone metastases (HR 0.59, 95%CI 0.398–0.887; $P = .009$) and overall survival (HR 0.77, 95%CI 0.591–0.999; $P = .048$).

Large studies of zoledronic acid have shed new light on the utility of bisphosphonates in the adjuvant setting in preventing bone metastasis. The ABCSG12 trial included 1803 premenopausal women with endocrine-receptor positive early breast cancer and showed that the addition of 6-monthly intravenous zoledronate for 3 years to ovarian suppression and tamoxifen/anastrozole prevented around a third of disease recurrences (HR 0.64, 95%CI 0.46–0.91; $P = .01$) after 7 years follow-up (Gnant *et al.*, 2009). A subsequent trial (the AZURE study), was performed in a wider subpopulation of early breast cancer patients, in which pre- and postmenopausal patients with Stage II and III breast cancer received standard adjuvant therapy $\pm$ an intensive schedule of zoledronic acid. Whilst this study failed to show a significant benefit in disease recurrence in the overall population studied (HR 0.98; $P = .73$), preplanned analysis of the postmenopausal subgroup showed a significant reduction in disease-free survival at 5 years (HR 0.75, 95%CI 0.59–0.96; $P = .02$) (Coleman *et al.*, 2011b). These results were mirrored by those of the NSABP-34 trial that showed that addition of oral clodronate led to disease-free interval improvement in women >50 years (HR 0.75, 95%CI 0.57–0.99; $P = .045$) (Paterson *et al.*, 2012).

This benefit from adjuvant bisphosphonates among older, postmenopausal women was confirmed in a meta-analysis of data from all randomized clinical trials evaluating adjuvant bisphosphonate use in the early breast cancer. Among a total of 18,766 patients (pre- and postmenopausal), there was a significant reduction in the rate of recurrence in bone (RR 0.83, 95%CI 073–0.94; $2P = .004$) (Coleman, 2016). This effect was found to be greater among the 11,767 postmenopausal women (RR 0.72, 95%CI 0.60–0.86; $2P = .0002$), who were also found to derive a breast cancer-specific overall survival benefit (RR 0.82, 95%CI 0;73–0.93; $2P = .002$). Neither benefit was demonstrated among premenopausal women (HR 0.92; $2P = .42$ and HR 1.00; $2P = .35$, respectively).

Mounting evidence of their benefit in the postmenopausal setting has now led to the recent consensus recommendation that adjuvant bisphosphonates be considered in the adjuvant treatment of early breast cancer in postmenopausal women, as well as

premenopausal women for whom ovarian suppression is a planned component of their adjuvant endocrine therapy (Hadji *et al.*, 2016).

The RANKL-inhibitor, denosumab was shown in the ABCSG18 trial to reduce the incidence of fractures in postmenopausal women receiving adjuvant aromatase inhibitors (Gnant *et al.*, 2015) and preliminary analysis of data from 3425 women in this trial suggests a disease-free survival benefit (HR 0.816, $P = .051$) that is comparable to the use of adjuvant bisphosphonates (Gnant *et al.*, 2015). The ongoing Phase 3 double-blind placebo-controlled D-CARE trial that is evaluating adjuvant denosumab in locally advanced breast cancer (Goss *et al.*, 2011) is expected to provide more-definitive evidence. This is expected to report in 2018.

Preventing prostate cancer bone metastasis using bone targeted agents has also been evaluated in a number of studies, though with mixed success. In the STAMPEDE trial for example, addition of zoledronic acid to androgen deprivation ± docetaxel did not show survival benefit (James *et al.*, 2016). A randomized, placebo-controlled trial involving 2406 castrate-resistant nonmetastatic prostate cancer patients, showed that administration of subcutaneous denosumab 120 mg every 4 weeks led to a significant increase in bone-metastasis free survival (HR 0.85 95%CI 0.73–0.98 $P = .028$) (Smith *et al.*, 2012). There was however no overall survival benefit seen (HR 1.01 $P = .91$) and a notable increased risk of osteonecrosis of the jaw (cumulative incidence of 4.6% at 3 years) in the denosumab arm resulted in its failure to obtain a license for routine adjuvant treatment in prostate cancer.

Prognostic and Predictive Biomarkers

As outlined above, biomarkers of bone turnover have proved valuable in assessing the current bone resorption and bone formation status of patients with established bone metastases and in monitoring response to bone targeted therapy. Several studies have also demonstrated their value in predicting risk of impending SREs in patients with established bone metastases. For example, (Brown *et al.*, 2005) showed that, patients with prostate cancer and high NTX levels, had a higher risk of skeletal complications.

(RR = 3.25, 95% CI = 2.26 to 4.68, $P < .001$) compared with patients with normal NTX levels. For nonsmall cell lung cancer and other solid tumors, excluding breast cancer (combined analysis), the corresponding figures were RR = 1.79, 95% CI = 1.15 to 2.79, $P = .010$). The association of bone marker levels with SRE risk persists was also observed in patients treated with bisphosphonates (Coleman *et al.*, 2005).

Could these bone turnover markers also have a role in early cancer, in assessing the future risk of developing bone metastasis and the response to bone-targeted treatment? Early studies suggested this may be possible in lung cancer (Papotti *et al.*, 2006), but it is only more recently that this possibility has been addressed in large, breast cancer studies, which open up exciting possibilities for biomarker development.

Lipton *et al.* (2011) investigated β-CTX in 621 postmenopausal early breast cancer patients in a 5-year phase III trial of tamoxifen ± octreotide. Over 7.9 years median follow-up, higher pretreatment β-CTX was associated with shorter bone-only recurrence-free survival (HR 2.8, 95%CI 1.05–7.48, $P = .03$). However, there was no significant association with first recurrence at other distant sites.

Very recently, Brown *et al.* (2018) have carried out a preplanned analysis of data from the AZURE trial to determine whether serum bone turnover markers in early disease have clinical utility in identifying patients at high risk of developing bone metastasis. When considered as continuous variables, pretrial values of P1NP, CTX and 1-CTP were each prognostic for future bone recurrence at any time ($P = .006$; $P = .009$; $P = .008$ respectively). In categorical analyses based on the normal range, high baseline P1NP (>70 ng/mL) and CTX (>0.299 ng/mL), but not 1-CTP (>4.2 ng/mL) were also prognostic for future bone recurrence ($P = .025$; $P = .033$; $P = .099$ respectively). None of the markers were prognostic for overall distant recurrence that is, they were bone metastasis-specific and none of the markers were predictive of treatment benefit from zoledronic acid.

In a proteomics-based study (Westbrook *et al.*, 2016) identified the proteins macrophage-capping protein (CAPG) and PDZ domain–containing protein (GIPC1) as being associated with the development of bone metastasis and this was subsequently tested clinically in an analysis of pretrial tissue samples from patients in the AZURE study. When analyzed as a novel composite biomarker (CAPG/GIPC1) the results showed that high expression of both CAPG and GIPC1 was associated with a higher risk of development of bone metastasis (HR 4.5, 95%CI = 2.1 to 9.8, $P < .001$) and death (HR 1.8, 95%CI 1.01 to 3.24, $P = .045$). The composite biomarker was bone metastasis-specific, that is, it was not associated with nonskeletal relapse. Moreover, the composite biomarker was predictive for treatment benefit since, in patients with high CAPG and high GIPC1, adjuvant zoledronate reduced the risk of first disease recurrence in bone by a 10-fold factor.

In another study using the AZURE trial tissue resource, absence of 16q23 (MAF) amplification was associated with improved disease outcomes in patients treated with zoledronic acid (Pavlovic *et al.*, 2015). In patients with MAF-negative tumors, but not in patients with MAF positive tumors, zoledronic acid was associated with higher invasive-disease-free survival than was control treatment (HR 0.74, 95%CI 0.56–0.98), but in patients with MAF-positive tumors who were not postmenopausal at randomization, zoledronic acid was associated with lower invasive-disease-free survival (HR 2.47, 95%CI 1.23–4.97) and overall survival (2.27, 95%CI 1.04–4.93) than control treatment (Coleman *et al.*, 2017).

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Bone Metastases; Medical Treatment

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Introduction

The aim of treatment for bone metastases is typically palliative with cure only rarely a realistic aim in highly chemo-sensitive tumors such as lymphoma and germ cell malignancies. The choice and sequence of therapies varies, depending on the underlying disease and the extent of disease beyond the skeleton. External beam radiotherapy, endocrine and cytotoxic treatments, targeted therapies, radioisotopes and, in some tumors, immunotherapy are all relevant. In addition, orthopedic intervention may be necessary for the structural complications of bone destruction. Optimal management requires a multidisciplinary team that includes not only medical and radiation oncologists but also orthopedic surgeons, radiologists, nuclear medicine physicians and specialists in palliative medicine. Local therapy is frequently relevant throughout the clinical course of the disease while multiple lines of systemic therapy are likely to be necessary to regain control of the disease as resistance to each treatment develops.

Localized external beam radiotherapy is effective in relieving bone pain and may promote bone healing and improved bone structure. Overall, response rates of around 85% are reported, with complete relief of pain achieved in half of patients (Agarawal *et al.*, 2006). However, systemic treatments to reduce tumor burden and/or improve bone structure are the mainstay of management.

Systemic Treatment of Bone Metastases

Therapy for bone metastases should be directed against the tumor cell to reduce tumor cell proliferation and at normal osteoclasts to inhibit the tumor induced osteoclastic bone resorption. Chemotherapy, biologically targeted agents, and endocrine treatments have direct antitumor effects, whereas the bisphosphonates and denosumab target osteoclasts in order to improve the structural integrity of affected bones and reduce skeletal morbidity. In general, direct systemic anticancer treatment for metastatic bone disease is similar to that indicated for other metastatic manifestations of the malignancy and will be dictated by the underlying tumor type. Chemotherapy can be more hazardous for patients with extensive bone disease, because of both poor bone marrow tolerance after replacement of functioning marrow by tumor and the effects of previous irradiation. Primary prophylaxis with hematopoietic growth factors may be required to enable chemotherapy to be administered safely.

Bone metastases are most prevalent in patients with breast, prostate and lung cancers and most patients will experience bone pain and skeletal complications including pathologic fracture. The developments in systemic therapy for solid tumors including the emergence of a range of targeted treatments for patients with specific mutations (e.g., b-raf in melanoma, epidermal growth factor receptor and ALK in lung cancer) as well as immunotherapy with immune checkpoint inhibitors across multiple tumor types appear to be as relevant for patients with bone metastases as they are for those with metastatic disease at other sites.

Skeletal morbidity is also a major problem in patients with multiple myeloma, and either widespread lytic metastases or diffuse osteopenia can occur. Most patients with myeloma respond rapidly to treatment and can now expect a median survival of around 5 years. However, despite these dramatic tumor responses, bone healing is rare, with lytic lesions persisting despite control of the disease due to persistent inhibition of osteoblast function.

Targeted Radionuclide Therapy

The therapeutic use of radioactive-labeled tracer molecules is an area of considerable interest and research. Because the radiation dose can be delivered more specifically to the tumor across multiple sites and normal tissues partially spared unnecessary irradiation, radionuclide therapy has a number of advantages over external beam radiotherapy.

Radionuclide therapy began with the treatment of bone metastases from thyroid cancer with radioiodine (^{131}I) and is now well established for the treatment of patients with significant uptake of ^{131}I into the metastases. Long-term palliation may be possible (Qiu *et al.*, 2011) and other than a radiation reaction within the salivary glands and a low but not insignificant 1%–2% incidence of radiation-induced leukemias, ^{131}I treatment is generally well tolerated.

Currently, radionuclide therapy is most widely used for bone metastases from prostate cancer. Strontium-89, a β emitter that imitates calcium and is taken up preferentially at sites of new bone formation, has been shown to localize at the sites of prostatic bone metastases and deliver between 10 and nearly 100Gy to an individual bony metastatic site (Jansen *et al.*, 2010). The dose of radiation to the bone marrow is only about one tenth of that to the bone metastases; however, significant bone marrow suppression may occur and limit subsequent use of chemotherapy. Pain relief occurs in up to 80% of patients, with 10%–20% becoming pain free and a median duration of symptomatic response of around 6 months (Jong *et al.*, 2016). Samarium-153 is

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both a β- and a γ-ray emitter and, through linkage of the isotope to ethylene diamine tetramethylene phosphonate (EDTMP), concentrates preferentially in skeletal metastases. ^{153}Sm-EDTMP can provide excellent palliation of pain in both breast and prostate cancer and, because of its short half-life and more limited adverse effects on bone marrow function, is easier to administer on a repeated basis (Sartor *et al.*, 2004).

Most recently the bone-seeking, α particle–emitting radiopharmaceutical ^{223}radium chloride (Ra-223) has been developed primarily for the treatment of metastatic prostate cancer. Ra-223 mimics calcium and preferentially forms complexes with the bone mineral hydroxyapatite in areas such as bone metastases where there is usually increased osteoblast activity. The radiation emitted from Ra-223 is of high linear energy and has ultra-short penetration (<100 μm; 2–10 cell diameters) resulting in a highly localized antitumor effect in tumor cells adjacent to the bone surface with little adverse effects to the surrounding normal tissue (Nilsson *et al.*, 2005). Following a placebo controlled randomized phase 3 trial in castration resistant prostate cancer (CRPC) which produced a 3.6 month improvement in median survival over best supportive care, Ra-223 has been approved for the treatment of bone metastases from CRPC. Skeletal morbidity was also reduced by Ra-223 over and above that achieved by a bisphosphonate (Parker *et al.*, 2013). Treatment with Ra-223 was well tolerated and improved quality of life with no significant long-term toxicities identified (Nilsson *et al.*, 2016). Ra-223 is now being studied earlier in the course of the disease, as well as in other diseases including metastatic breast cancer, in combination with both endocrine and cytotoxic agents.

Bone Targeted Agents

In the past three decades bone targeted agents have become established as a valuable additional approach to the range of current anticancer treatments. Most experience has been with the bisphosphonates but, in the past decade, antibody treatment directed at inhibiting RANK ligand has become the treatment of choice in a number of disease settings.

The bisphosphonates are pyrophosphate analogs, characterized by a P-C-P–containing central structure that promotes their binding to the mineralized bone matrix and a variable R′ chain that determines the relative potency, mechanism of action and adverse effect profile (Roelofs *et al.*, 2010). After administration, bisphosphonates bind rapidly to exposed bone mineral around resorbing osteoclasts. Then, during bone resorption, bisphosphonates are internalized by the osteoclast, disrupt several biochemical processes involved in osteoclast function and ultimately result in apoptotic cell death.

The molecular mechanisms of action of the bisphosphonates are well established, with nitrogen-containing bisphosphonates shown to inhibit enzymes of the mevalonate pathway that catalyze the posttranslational modification of several proteins including the small guanosine triphosphatases such as Ras and Rho (Roelofs *et al.*, 2010). Bisphosphonates that do not have a nitrogen atom in the R′ chain, such as clodronate, induce osteoclast apoptosis through the generation of cytotoxic adenosine triphosphate analogs (Xu *et al.*, 2013).

After intravenous administration of a bisphosphonate, the kidney excretes approximately one third of the injected dose, and the remainder is taken up by bone (Daley-Yates *et al.*, 1991). Oral bisphosphonates are poorly and variably absorbed from the gut and must be taken on an empty stomach and food and drink avoided for an hour after taking.

Bisphosphonates have been used successfully in the treatment of a wide range of bone conditions characterized by increased osteoclastic bone resorption. In oncology they have become the standard treatment for tumor-induced hypercalcemia and the prevention of skeletal morbidity and, as shown below, they now also have a role in prevention of metastasis in the adjuvant setting of postmenopausal women with early breast cancer (Coleman *et al.*, 2014).

Bisphosphonates to Prevent Skeletal Morbidity and Relief of Bone Pain

Osteoclast activation is the key step in the establishment and growth of bone metastases. Biochemical data indicate that bone resorption is of importance not only in classic “lytic” diseases such as myeloma and breast cancer but also in prostate cancer with its osteoblastic looking metastases on radiographs. Despite the osteoblastic appearance, values of resorption markers are at least as high as those seen in breast cancer and other solid tumors reflecting the importance of osteoclastic bone resorption in this disease setting as well (Brown *et al.*, 2005). As a result, the osteoclast is a key therapeutic target for skeletal metastases irrespective of the tissue of origin.

Although radiotherapy has been the treatment of choice for localized bone pain for decades, the bisphosphonates provide an additional treatment approach for the relief of bone pain across a range of tumor types. The effects seems to be independent of the nature of the underlying tumor or radiographic appearance of the metastases, with sclerotic lesions responding similarly to lytic metastases (Wong and Wiffen, 2002). In a randomized trial, the effect of a single intravenous dose of the bisphosphonate, ibandronate, was similar to that achieved by an 8 Gy dose of external beam radiotherapy (Hoskin *et al.*, 2015).

During the 1990s and early 2000s, the bisphosphonates became the standard of care for the treatment and prevention of skeletal complications associated with bone metastases in patients with solid tumors or multiple myeloma (Coleman *et al.*, 2014). Randomized, placebo controlled trials used skeletal related events (SREs) as the primary end point for these studies; either the number of patients experiencing one or more SREs, or the time to the first SRE or the rate of SREs as determined by either a simple annual rate or more complex multiple event analysis techniques. SREs included the occurrence of pathologic long bone and vertebral fractures, the need for radiation for pain relief or to treat or prevent structural bone damage, development of spinal cord

compression or a requirement for surgery to bone. Episodes of hypercalcemia of malignancy were also included in some definitions.

Breast Cancer

The greatest experience with bisphosphonates is in the management of bone metastases from breast cancer, where the value of the agents is undisputed (Van Poznak *et al.*, 2011). Both the oral agents, clodronate and ibandronate, as well as the intravenous formulations of pamidronate, ibandronate and zoledronic acid have useful clinical efficacy (Coleman *et al.*, 2014). Oral ibandronate has obvious practical attractions over intravenous treatments to both patients and health care providers (Body *et al.*, 2004). However, in a randomized comparison with intravenous zoledronic acid, ibandronate did not meet the strict statistical criteria for noninferiority defined in the study protocol (Barrett-Lee *et al.*, 2014). Pamidronate was the first intravenous bisphosphonate to be systematically evaluated and has clinically important efficacy on skeletal morbidity, quality of life, pain and analgesic use in metastatic breast cancer (Hortobagyi *et al.*, 1996; Theriault *et al.*, 1999) and multiple myeloma (Berenson *et al.*, 1996).

Zoledronic acid is the most potent bisphosphonate available and is the bisphosphonate of choice in most clinical settings and health care systems. A placebo-controlled trial of zoledronic acid showed that the percentage of patients with at least one SRE after 1 year on treatment was reduced from 50% in the placebo group to 30% with zoledronic acid ($P = 0.003$). Zoledronic acid also significantly delayed the time to first SRE ($P = 0.007$) and reduced the overall risk of SREs by 41% ($P = 0.019$) (Kohno *et al.*, 2005). When compared to pamidronate, zoledronic acid reduced the overall risk of experiencing an SRE by a further 20% ($P = 0.025$) (Rosen *et al.*, 2003). Additionally, control of bone resorption was greater at all time points with the more potent zoledronic acid. The increased potency, however, did not influence either time to progression or overall survival.

Multiple Myeloma

Myeloma is exemplified by a marked increase in osteoclast activity and suppression of osteoblastic activity. Early studies of bisphosphonates for myeloma evaluated oral clodronate and showed beneficial effects on disease progression and a reduction in skeletal morbidity. However, intravenous bisphosphonates are the treatment of choice for most patients with multiple myeloma. 3–4 weekly intravenous pamidronate significantly reduced the proportion of patients developing SRE(s), time to first SRE and skeletal morbidity rate in comparison to placebo (Berenson *et al.*, 1996). Additionally, QOL scores, performance status and pain scores were all favorably influenced by pamidronate therapy. Zoledronic acid has similar activity to pamidronate in terms of control of skeletal morbidity (Rosen *et al.*, 2003). However, zoledronic acid also appears to modulate the course of the underlying disease. In a large randomized trial conducted in the United Kingdom by the Medical Research Council in newly diagnosed patients with myeloma who were receiving chemotherapy (including stem cell transplantation where appropriate) and either intravenous zoledronic acid or daily oral clodronate, patients treated with zoledronic acid had a better chance of survival, with an improvement in median OS of 5.5 months ($P = 0.04$) (Morgan *et al.*, 2010).

Prostate Cancer

Despite early evidence of pain relief and inhibition of biochemical markers of bone resorption in patients with osteoblastic bone lesions associated with advanced prostate cancer, the role of bisphosphonates in prevention of skeletal morbidity was slow to evolve. After, unimpressive results with either oral clodronate (Dearnaley *et al.*, 2005) or intravenous pamidronate (Small *et al.*, 2003), zoledronic acid was investigated in patients with CRPC and bone metastases; the increased potency of this compound translated into improved clinical benefit with a 36% reduction in the overall risk of skeletal complications. Bone pain was also reduced at all time points (Saad *et al.*, 2004).

Other Tumors

The pathophysiology of bone metastases has many similarities across tumor types and bisphosphonates likely to be of value in preventing skeletal morbidity across the range of tumors involving the skeleton. A randomized trial in each individual tumor type with bone metastases was not feasible and so a placebo controlled "basket trial" of all solid tumor types with bone metastases other than breast or prostate cancer with zoledronic acid was performed. This showed that zoledronic acid reduced the proportion of patients with at least one SRE (39% vs. 48%, $P = 0.039$), almost doubled the time to the first SRE (314 days vs. 168 days, $P = 0.021$) and reduced the risk for SRE(s) by about 30% ($P = 0.003$) compared with placebo (Rosen *et al.*, 2004).

RANKL Inhibition to Prevent Skeletal Morbidity and Relief of Bone Pain

RANKL is a member of the TNF superfamily and is the ligand for OPG. RANKL is expressed on the surface of osteoblasts and released into the bone microenvironment, where it binds to and activates its receptor, RANK, on immature osteoclasts to increase

the number and activity of functional osteoclasts. Denosumab is a fully human, synthetic antibody that specifically binds to RANKL preventing its interaction with RANK in a way similar to that of OPG (Brown and Coleman, 2012).

A single subcutaneous dose of denosumab causes rapid suppression of bone turnover; the maximal amount of urinary NTX suppression (about 70%–80%) was reached after 7 days and was sustained over the observation period of 84 days of the study (Body *et al.*, 2006). In a dose-finding study in patients with breast cancer and bone metastases, doses between 60 mg and 180 mg and dosing intervals of 4–12 weeks were evaluated (Lipton *et al.*, 2007). Differences between treatment groups in bone resorption responses were small, but following modeling of the pharmacodynamic profiles, treatment with 120 mg of denosumab every 4 weeks was selected to provide the optimal balance of efficacy and tolerability. Additionally, a randomized, phase 2 study in patients with bone metastases and urinary NTX levels >50 nmol/mmol creatinine despite treatment with an intravenous bisphosphonate, showed that switching to denosumab was more likely to normalize NTX levels than continuing with bisphosphonate treatment (Fizazi *et al.*, 2009). Moreover, considerably fewer patients treated with denosumab experienced a first SRE during time on study.

Subsequently, three identically-designed double-blind, phase 3, registration studies of denosumab were performed (Stopeck *et al.*, 2010; Fizazi *et al.*, 2011; Henry *et al.*, 2011). These studies included patients with bone metastases resulting from breast cancer ($n = 2046$) (Stopeck *et al.*, 2010), CRPC ($n = 1901$) (Fizazi *et al.*, 2011), and other advanced solid tumors or multiple myeloma ($n = 1776$) (Henry *et al.*, 2011). Patients were randomly assigned to receive four weekly subcutaneous injections of denosumab (120 mg) or intravenous zoledronic acid (4 mg), with supplements of calcium and vitamin D. The primary end point was the time to first SRE.

In patients with bone metastases resulting from breast and prostate cancers, denosumab was statistically superior to zoledronic acid in delaying the first SRE (Stopeck *et al.*, 2010; Fizazi *et al.*, 2011). In patients with bone metastases resulting from other advanced solid tumors and myeloma, denosumab was not inferior to zoledronic acid but failed to demonstrate statistical superiority over bisphosphonates (Henry *et al.*, 2011). Overall, denosumab treatment delayed the occurrence of all types of SREs.

Recently, a formal comparison of denosumab and zoledronic acid in multiple myeloma was completed. This study met its primary endpoint, demonstrating noninferiority to zoledronic acid in delaying the time to first on-study SRE ($P = 0.01$) and benefits in a number of secondary endpoints including an increase in time to progression with denosumab (Raje *et al.*, 2017). This study is likely to lead to regulatory approval and more widespread use of denosumab in myeloma.

Disease Modifying Effects of Bone Targeted Agents in Metastatic Disease

Other than the beneficial effect of zoledronic acid on survival in multiple myeloma described earlier, no significant effects on disease-related end points such as time to progression and survival have been demonstrated in individual trials within the metastatic disease setting, despite the favorable effects on skeletal morbidity. Similarly, in the trials comparing denosumab to zoledronic acid no differences in either disease progression or survival were seen, despite the improved efficacy of denosumab in prevention of skeletal morbidity. Interestingly however, exploratory analyses performed using data from three randomized trials of zoledronic acid versus placebo suggested a beneficial effect of zoledronic acid on survival in patients with highly aggressive or advanced metastatic bone disease (Coleman *et al.*, 2013). It was thought most likely that these possible survival benefits result from prevention of SREs such as fractures and hypercalcaemia that are likely to preclude or delay introduction of effective anticancer treatments rather than any direct effects on the underlying cancer.

Adverse Events

The severity of adverse events related to bisphosphonates is generally mild and the benefits of treatment almost always outweigh the risks (Coleman *et al.*, 2014). With the exception of the acute phase response, causing transient fever, myalgia, and bone pain, adverse effects after administration of intravenous bisphosphonates are infrequent, provided the drug is infused at the recommended dose and duration. Intravenous bisphosphonates are associated with renal damage, notably when administered by rapid infusion. With use of the recommended infusion times, renal monitoring with dose reductions or delays as appropriate and adequate hydration of the patient, renal adverse events are uncommon. With oral agents, upper gastrointestinal toxicity (esophagitis and dyspepsia) or diarrhea may occur and may compromise adherence to treatment.

Denosumab does not impact on kidney function, which obviates the need for renal monitoring. Acute-phase reactions are also less common but hypocalcemia is more frequent and can be severe. All patients should be encouraged to take calcium and vitamin D supplements, and monitoring of serum calcium levels is advised, particularly in patients with impaired renal function.

The more serious potential adverse event from long-term treatment with bone-targeted agents is the development of osteonecrosis of the jaw (ONJ), particularly when administered parenterally on a monthly schedule. ONJ is characterized by painful bone destruction, secondary infection, and delayed healing in the mandible and/or maxilla (Ruggiero *et al.*, 2006). The risk of ONJ appears to be related to duration of treatment, occurring at around 1% per year on therapy (Migliorati *et al.*, 2011). As with intravenous bisphosphonates, the most important adverse event associated with the intensive dose schedule of denosumab used in oncology settings is ONJ. In the phase III registration trials ($n = 5677$), a similar proportion of patients treated with zoledronic acid and denosumab experienced ONJ (1.3% vs. 1.8%, respectively) (Saad *et al.*, 2012).

Treatment of ONJ is difficult and the management focus should be on prevention by increasing awareness amongst oncologists, dentists and maxillo-facial surgeons. Good dental hygiene and avoidance of dental procedures during therapy significantly reduce the risk of ONJ. Comprehensive guidelines now exist for prevention, diagnosis, and management of ONJ (Ruggiero *et al.*, 2014).

Optimum Use of Bone-Targeted Agents in Persons With Metastatic Bone Disease

Consensus guidance recommendations indicate that all patients with radiologically confirmed bone metastases from breast cancer should start taking a bisphosphonate or denosumab at the time of diagnosis of bone metastasis and continue with this treatment indefinitely (Coleman *et al.*, 2014; Van Poznak *et al.*, 2011). The development of an SRE is not necessarily a sign of treatment failure or a signal to stop treatment; evidence is now available to confirm that bone-targeted agents delay second and subsequent complications, not just the first event.

Bisphosphonate treatment—specifically zoledronic acid—or denosumab is also appropriate for patients with CRPC. In hormone sensitive prostate cancer, bisphosphonates may be indicated to prevent treatment induced bone loss but, because initial treatment of prostate cancer is so effective at controlling the underlying disease, the risk of SREs from metastatic involvement is low and does not usually justify routine use of monthly denosumab or zoledronic acid. Patients with other tumors and metastasis to bone should be considered for bone-targeted treatment if bone is the dominant site of metastasis or the patient has bony symptoms or a raised level of a biochemical marker of bone metabolism.

Despite the clinical benefits of bone-targeted treatments, only a proportion of events is prevented, and not all patients will experience a skeletal event despite the presence of metastatic bone disease. Accurate prediction in an individual patient of the need for and likely benefit from bone-targeted treatments is lacking; elevated bone marker levels, a previous SRE, increasing numbers of lesions, and lytic rather than sclerotic appearance of lesions on radiograms are associated with a higher risk for SREs and may be taken into consideration (Coleman *et al.*, 2014; Lipton *et al.*, 2008).

Several trials have investigated whether the interval between bisphosphonate infusions can be increased to take advantage of the prolonged pharmacologic effect in bone, improve convenience, reduced treatment costs and potentially minimize the risk of ONJ. Several small trials have suggested that reducing the frequency of administration did not significantly diminish the efficacy of zoledronic acid. However, these trials were underpowered to show noninferiority of the less intensive schedules with any reliability (Hutton *et al.*, 2013; Hortobagyi *et al.*, 2017). More recently, the noninferiority of less frequent zoledronic acid administration was shown in the much larger CALGB 70604 trial ($n = 1822$) (Himelstein *et al.*, 2017). No statistically significant differences in efficacy were seen between zoledronic acid monthly or every 3 months for 2 years. 29% of patients in both treatment groups experienced ≥ 1 SRE and there were no differences in time to first SRE or pain scores. These data support the use of the three monthly schedule, potentially from the outset as tested in the trial and certainly after loading of the skeleton with a few months of treatment with zoledronic acid. It is not known if the frequency of administration of denosumab can also be reduced. The mechanism of action is completely different, with no accumulation of the antibody in the skeleton, and there are data to show that both bone resorption biomarker levels and vertebral fracture rates increase within a few months of stopping treatment (Tsourdi *et al.*, 2017).

Prevention of Bone Metastases

Breast Cancer

The potential for bone targeted agents to modify the process of metastasis and have important effects on disease outcomes has been the focus of extensive laboratory and clinical research for several decades (Wilson and Coleman, 2012). The early metastasis prevention trials with oral clodronate in early breast cancer produced apparently inconsistent results in terms of disease recurrence. Then, the disease modifying impact of adding zoledronic acid every 6 months for 3 years to ovarian suppression therapy (plus tamoxifen or anastrazole) was shown to reduce disease recurrence by about one third ($P = 0.01$) (Gnant *et al.*, 2009), benefits that were confirmed with longer follow up at a median of 84 months (Gnant *et al.*, 2015a).

Subsequently the AZURE trial, performed in a broader patient population showed no overall benefit for the addition of an intensive schedule of zoledronic acid to standard adjuvant therapy and limited the clinical application of the ABCSG-12 results (Coleman *et al.*, 2011). Nevertheless, in a prespecified subgroup analysis, postmenopausal women receiving zoledronic acid showed improved disease-free and overall survival and these data, alongside the results of ABCSG 12, formulated the hypothesis that the benefits of adjuvant bisphosphonates may be restricted to women with low levels of reproductive hormones, either through the occurrence of menopause or induced by ovarian suppression therapy. This hypothesis was strengthened by both preclinical data (Ottewell *et al.*, 2014) and the results of the NSABP-B34 trial evaluating the addition of daily oral clodronate to standard adjuvant therapy; improved disease-free survival was seen in an exploratory subset analysis of women >50 years at trial entry (Paterson *et al.*, 2012).

To address this hypothesis systematically, the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) collected individual patient data from all randomized trials to evaluate in a meta-analysis the effects of adjuvant bisphosphonates on disease outcomes

and, specifically, whether menopausal status influenced treatment efficacy (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) *et al.*, 2015). Data from 18,766 randomized women were collected and included 3453 and 2106 breast cancer recurrences and deaths respectively. Bisphosphonates reduced first distant recurrence in bone by about one sixth ($2P = 0.004$) and confirmed a significant interaction between treatment efficacy and menopausal status. There were no demonstrable benefits in premenopausal women but in 11,767 postmenopausal women highly significant reductions, not only in bone recurrence (RR = 0.72; 95%CI 0.60–0.86, $2P = 0.0002$) but also, most importantly, in breast cancer mortality (0.82; 95%CI 0.73–0.93, $2P = 0.002$) were seen. Treatment effects appeared to be similar irrespective of the estrogen receptor status or grade of primary tumor, and consistent across the different bisphosphonate agents tested or the dosing schedule employed (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) *et al.*, 2015). The recently reported SWOG trial that compared adjuvant treatment with oral clodronate, oral ibandronate or intravenous zoledronic acid supports the meta-analysis suggestion of a probable class effect with no differences in disease recurrence between the three treatments (Gralow *et al.*, 2015).

The meta-analysis findings are changing clinical practice and both European (Hadji *et al.*, 2016) and North American (Dhesy-Thind *et al.*, 2017) consensus panels of breast cancer experts have recommended use of adjuvant bisphosphonates in post-menopausal women with early breast cancer, especially those at intermediate or high risk of relapse.

Denosumab is clearly an effective intervention to prevent fracture (Gnant *et al.*, 2015b) and preliminary data suggest it may also reduce disease recurrence (Gnant *et al.*, 2015c). However we do not know if this agent also reduces breast cancer mortality and await the results of the placebo controlled D-CARE trial (NCT01077154) for an answer.

Prostate Cancer

Prostate cancer spreads almost exclusively to bone and provides the ideal clinical setting for the evaluation of bone-targeted treatment to modify the course of the disease. In a placebo controlled trial of denosumab in men with nonmetastatic CRPC who were at high risk for bone metastasis by virtue of either a high PSA ($\geq$8.0 ng/mL) and/or short PSA doubling time ($\leq$10.0 months), denosumab significantly increased bone metastasis-free survival by a median of 4.2 months compared with placebo ($P = 0.028$) and delayed time to symptomatic first bone metastases (Smith *et al.*, 2012). However, this benefit was offset by the relatively high cumulative incidence of ONJ (4% at 3 years) and has not led to regulatory approval of denosumab in this clinical setting or changed clinical practice.

In the STAMPEDE trial in men starting ADT for prostate cancer, the impact of zoledronic acid alone or in combination with chemotherapy on disease recurrence was assessed. Docetaxel had a major impact on relapse and overall survival but zoledronic acid, despite extending the time to first skeletal complication, had no impact on survival (James *et al.*, 2016). Other studies of bisphosphonates to prevent bone metastases in prostate cancer have also failed to show benefit (Wirth *et al.*, 2015; Denham *et al.*, 2014); the biological rationale for lack of efficacy in the context of men with prostate cancer on androgen deprivation therapy when clear benefit has been seen in postmenopausal breast cancer requires further investigation.

Lung Cancer

Exploratory studies testing the potential use of bone targeted agents to prevent bone metastases in lung cancer have not identified any impact on the subsequent course of the disease (Scagliotti *et al.*, 2012). An ongoing trial with denosumab (SPLENDOR) has completed accrual and will report in the near future.

Treatment of the Skeletal Complications of Bone Metastases

Bone metastases cause considerable morbidity including pain, impaired mobility, hypercalcemia, pathological fracture, spinal cord or nerve root compression, and bone marrow infiltration. In two randomized trials that included patients with breast cancer and multiple myeloma receiving chemotherapy, the mean skeletal morbidity rates (number of skeletal events per year) in the absence of bisphosphonates were 3.5 and 2.0, respectively (Hortobagyi *et al.*, 1996; Theriault *et al.*, 1999; Berenson *et al.*, 1996). However, despite the clinical importance of metastatic bone disease and the huge expenditure on medical care for skeletal complications, relatively little thought has been given until recently as to how best to coordinate clinical management and deliver optimum care for patients with bone metastases.

Bone Pain

Bone pain is the most common type of pain from cancer. It is usually the manifesting symptom and is caused by a variety of factors, including periosteal stretching, compression or infiltration of nerve roots, reflex muscle spasm, and the local effects of cytokines. Appropriate analgesics and coanalgesics are essential to promptly relieve pain in addition to radiotherapy, systemic anticancer therapy and bone targeted treatments. A low threshold for seeking expert advise from a palliative care specialist is recommended.

Hypercalcemia of Malignancy

Hypercalcemia is an oncologic emergency that is typically associated with metastatic bone disease although, in tumors producing significant amounts of parathyroid hormone related peptide, it can occur in the absence of bone involvement. Clinical features include nausea, vomiting, dehydration, and confusion. The signs and symptoms vary considerably from patient to patient and are often nonspecific, affecting many systems in the body; they may easily be mistaken for symptoms of the underlying cancer or associated treatment if there is not an astute awareness of the possibility of hypercalcemia. If untreated, a progressive rise in serum calcium leads to deterioration in renal function and level of consciousness. Death ultimately ensues as a result of cardiac arrhythmias and renal failure.

Intravenous bisphosphonates, in conjunction with rehydration, are well established as the treatment of choice for hypercalcemia. Approximately 70%–90% of patients will achieve normocalcemia, resulting in relief of symptoms and improved QOL. Zoledronic acid is the most effective bisphosphonate for the acute treatment of this metabolic emergency (Major *et al.*, 2001). Denosumab is an alternative therapeutic option having been shown to be effective in around two thirds of patients with persistent hypercalcemia after recent bisphosphonate treatment (Hu *et al.*, 2014).

Summary

The management of bone metastases requires an experienced multidisciplinary team to ensure timely diagnosis and the appropriate integration of local and systemic treatments. The effects of tumor cells on bone cell function (especially on osteoclast activity) underpin the rationale for the use of bone-targeted treatment to reduce skeletal morbidity (Weilbaecher *et al.*, 2011). These bone-specific treatments are now an accepted part of routine clinical management. Additionally, the disruption of bone remodeling results in release of collagen fragments, which seems to have value in predicting skeletal events, prognosis, and monitoring of response.

Further developments in our understanding of the pathophysiology of bone metastases can be expected to provide new therapeutic strategies. Already, improved knowledge of the signaling molecules involved in regulating osteoclast function—notably OPG and RANKL—has led to the development of highly active targeted therapies for cancer-induced bone disease, and other novel therapeutic approaches are in clinical development. During the next 5 years, several of these compounds can be expected to gain regulatory approval, and ultimately combinations of bone-targeted therapies may be recommended to further reduce the clinical burden of metastatic bone disease.

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Multiple Myeloma Bone Disease

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Introduction

Multiple myeloma is a plasma cell dyscrasia characterized by bone marrow infiltration by monoclonal plasma cells and the presence of a monoclonal immunoglobulin in serum. It is the second most common hematological malignancy (Kyle and Rajkumar, 2004; Palumbo and Anderson, 2011). Symptomatic disease is characterized by one or more myeloma defining events including anemia, renal impairment, hypercalcemia, and bone disease (Greipp *et al.*, 2005). Approximately 85% of the patients present with osteopenia at diagnosis, while the degree of bone disease is correlated both with the extent of the tumor burden and prognosis (Palumbo and Anderson, 2011; Kyle *et al.*, 2003; Terpos *et al.*, 2003a,b). Bone metabolism is maintained by the balance between bone resorption and bone formation, however in multiple myeloma this balance which is mainly regulated by osteoclasts and osteoblasts is impaired. Myeloma cells secrete several factors that inhibit osteoblast bone formation and enhance osteoclast activity, while the microenvironment supports tumor expansion (Quail and Joyce, 2013; Podar *et al.*, 2009). Animal studies have demonstrated that increase of bone formation or inhibition of osteoclasts not only reduce myeloma burden but reduce the risk for myeloma bone disease as well (Choi *et al.*, 2001; Edwards *et al.*, 2008). Therefore, since disease progression depends on the interactions of myeloma cells with the microenvironment, treatment agents are aiming to restore bone homeostasis by targeting either osteoclasts or osteoblasts, or both.

Bone Marrow Mesenchymal Stromal Cells

Bone marrow stromal cells (BMSCs) is compiled by several hematopoietic (red and white blood cells) and mesenchymal (osteoprogenitors, osteoblasts, chondrocytes, adipocytes, fibroblasts, and endothelial cells) cells (Mendelson and Frenette, 2014) and participate indirectly in hematopoiesis by secreting chemokines, cytokines, and growth factors such as IL-6, G-CSF, GM-CSF, CXCL12, IL7, and LIF. These factors are involved in proliferation and differentiation of hematopoietic stem cells and progenitor cells (Mendelson and Frenette, 2014; Morrison and Scadden, 2014; Panaroni *et al.*, 2014; Panaroni and Wu, 2013). Mesenchymal cells originate from a mesenchymal stem cell (MSC), while B lymphocytes are differentiated in the bone marrow (Fridenshtein *et al.*, 1968). This explains that if, deficiency of osteoprogenitors and preosteoblasts is induced in vivo, the common lymphoid precursor cells are depleted (Visnjic *et al.*, 2004), while deletion of the α subunit in the G can lead to B cell differentiation at the pro-B cell stage arrest (Wu *et al.*, 2008a,b). In contrary, deletion of the parathyroid hormone (PTH) receptor, arrests the differentiation of a pro-B cell into a mature one. Following that, mature B cells are accumulated in the bone marrow and this occurs along the bone surface. This phenomenon is mainly due to overexpression of the vascular cell adhesion molecule 1 (VCAM1) by bone and stromal cells (Panaroni *et al.*, 2015). Myeloma cells interact with BMSCs either directly with cell–cell contact or via secretion of paracrine factors. These interactions stimulate secretion of BMSC-derived factors that upregulate the expression of antiapoptotic and cell cycle regulating proteins (Hideshima *et al.*, 2004; Neri *et al.*, 2007; Tai *et al.*, 2006; Asano *et al.*, 2011). More specifically, cell–cell interactions are mediated via adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and VCAM-1and very late antigen 4 (VLA-4). These interactions enhance MM cells retention and lead to increased osteolytic bone lesions (Michigami *et al.*, 2000; Markovina *et al.*, 2010; Hao *et al.*, 2011). Other factors such as interleukin 6 (IL-6) also promote of myeloma progression (Hideshima *et al.*, 2007). IL-6 is secreted by both MM and BMSCs as a result of different signaling pathways (such as NF-kB and Notch) stimuli (Chauhan *et al.*, 1996; Urashima *et al.*, 1995; Radtke and Raj, 2003; Nefedova *et al.*, 2004). Furthermore, other secreted cytokines like TNF-α and IL-1β can lead to bone disease by osteoblast inhibition, IL-6 upregulation, and osteoclast activation (Galson *et al.*, 2012; Lee *et al.*, 2013). Other factors secreted include vascular endothelial growth factor (VEGF), IL-1, insulin-like growth factor (IGF-1), TGF-β, angiopoietin-1 (Ang-1), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and basic-fibroblast growth factor (bFGF) (Hideshima *et al.*, 2005; Gupta *et al.*, 2001). These factors are associated with osteoclastogenesis, increased angiogenesis, and tumor growth (Giuliani *et al.*, 2011). Levels of serum Activin A have been shown to be increased in patients with advanced bone disease (Terpos *et al.*, 2012; Vallet *et al.*, 2010). Activin A is a factor activating osteoclasts and inhibiting osteoblast differentiation. However, it is not secreted by MM cells (Vallet *et al.*, 2010). MSCs from myeloma patients were found to secrete activin A, therefore suggesting a possible genetic defect in BMSCs of these patients (Vallet *et al.*, 2010). In preclinical models it has been demonstrated that targeting activin A can reverse osteoblast inhibition and improve this way myeloma bone disease. Furthermore, lenalidomide which is an immunomodulatory agent increases the levels of activin A secreted from the BMSCs, leading to osteoblastogenesis inhibition. Therefore, the combination an antiactivin A antibody with lenalidomide based regimens could represent a potential new therapeutic strategy for the management of myeloma bone disease (Scullen *et al.*, 2013). BMSCs in myeloma patients secrete growth differentiation factor 15 (GDF15), which supports stem cell survival and regeneration (Corre *et al.*, 2007, 2012; Tanno *et al.*, 2014; Reagan *et al.*, 2014). All the above-

mentioned findings highlight the important role of the microenvironment in the myeloma disease progression (Corre *et al.*, 2012; Tanno *et al.*, 2014; Feng *et al.*, 2010; Eda *et al.*, 2016). Proteasome inhibitors target the interactions between MM cells and BMSCs, inhibit cell growth and reduce the secretion of cytokines necessary to enhance myeloma cells survival (Hideshima *et al.*, 2001). Furthermore, bortezomib increases osteoblast differentiation by acting directly on MSCs (Mukherjee *et al.*, 2008). A different way to decrease myeloma cell adhesion to stromal cells involves the inhibition of CXCL-12/CXCR-4 axis, which is upregulated by VLA-4/VCAM-1 interaction, by using a CXCR4 inhibitor named AMD3100. Sensitivity to chemotherapy can be achieved by mobilization mechanisms (Azab *et al.*, 2009).

Cells of the Osteoblast Lineage

Osteoblasts are mononuclear cells which originate from mesenchymal stem cells. They contain alkaline phosphatase, which is a marker of osteoblastic activity (George *et al.*, 2006). Osteoblasts normally locate near the bone surface and their main function is bone formation (McSheehy and Chambers, 1986; Owen *et al.*, 1991). The osteoblasts that become part of mineralized matrix are called osteocytes (Giuliani *et al.*, 2006; Holtrop, 1990). Osteolineage cells affect primarily myeloma bone disease via the activation of two transcription factors: the runt-related transcription factor 2 (RUNX2) and the osterix (OSX) (Zaidi, 2007). These factors are essential for osteoblasts maturation and depend on the canonical Wnt signaling pathway.

Transcription Factor Runx2/Cbfa

Runx2/Cbfa1 is a transcription factor involved in formation and differentiation of osteoblasts from BMSCs and mesenchymal stem cells. The activation of Runx2/CBFA1 increases the expression of osteoblastic markers. In preclinical models, mice Runx2/CBFA1-negative do not have osteoblastic activity (Giuliani *et al.*, 2005). The expression of Runx2/CBFA1 in myeloma patients with bone disease is significantly lower than that in normal subjects or patients without bone disease. Myeloma cells inhibit osteoblast formation via cell-to-cell interactions and by suppressing Runx2/Cbfa1 activity.

Wingless-Related Integration Site (Wnt) Signaling Pathway

Wnt pathway is critically involved in bone remodeling (Giuliani *et al.*, 2005) via activation, gene expression, cell proliferation, migration, and differentiation (Albers *et al.*, 2013; van Amerongen *et al.*, 2012). Furthermore, Wnts control embryogenesis, organ formation and development, and regeneration of human tissues (Wan *et al.*, 2013). Wnts are classified as canonical when they have the capacity to inhibit phosphorylation of β-catenin and its degradation and as noncanonical when β-catenin levels remain the same (Bartis *et al.*, 2013). Wnt proteins bind to Wnt receptor and its co-receptors LRP5/LRP6 (low-density lipoprotein receptor-related protein) leading to β-catenin stabilization. Consequently, β-catenin accumulates in cytoplasm, translocates into the nucleus, and stimulates the expression of osteoblastic related genes (Westendorf *et al.*, 2004). When the Wnt signal is absent, the proteasome phosphorylates and degrades β-catenin. β-Catenin is the main factor regulating OPG expression from osteoblasts. Actually, it is demonstrated that inactivation of gene for LRP5 leads to an osteoporosis-pseudoglioma syndrome in humans while gain of function mutations in LRP5 leads to a syndrome of hereditary high bone density (Boyden *et al.*, 2002). Wnt pathway is regulated by extracellular soluble antagonists (Kawano and Kypta, 2003). Members belonging to the Dickkopf (DKK) family bind to LRP5/LRP6 while secreted frizzled-related proteins (sFRP), such as sFRP-2 and sFRP-3 bind to Wnt proteins. This results in suppressed Wnt signaling and reduced osteoblast function.

Dickkopf-1 (DKK-1) Protein

DKK1 is mainly expressed by osteoblasts and BMSCs, and antagonizes the Wnt pathway. This results in inhibition of osteoblasts maturation and increased bone formation. DKK-1 is also produced by myeloma cells and inhibits differentiation of osteoblast precursor. In patients with lytic lesions, DKK-1 was shown to be overexpressed in bone marrow biopsies. Furthermore, DKK-1 was also increased in the serum of MM patients (Politou *et al.*, 2006), while gene expression levels and serum levels of DKK-1 have been correlated with the extent of bone disease (Kaiser *et al.*, 2008; Tian *et al.*, 2003). The high expression of DKK1 has as a result that mesenchymal stem cells do not differentiate to osteoblasts (Tian *et al.*, 2003). Antibodies against DKK-1 (BHQ880) have shown encouraging results in patients with myeloma (Iyer *et al.*, 2014), however their development seems to be currently discontinued.

Secreted Frizzled-Related Protein-2 (sFRP-2)

sFRP-2 is produced from myeloma cells and inhibits osteoblast differentiation induced by bone morphogenetic protein 2 (BMP-2) (Oshima *et al.*, 2005). It is considered as a decoy receptor interfering with Wnt by binding to its receptor, frizzled. It has been demonstrated that patients with advanced bone disease have elevated levels of sFRP-2 in myeloma cells (Oshima *et al.*, 2005).

Sclerostin

Sclerostin is a Wnt inhibitor, produced by osteocytes which inhibits osteoblast-induced bone formation (Moester *et al.*, 2010). The production of sclerostin is upregulated by glucocorticoids, while intermittent parathyroid hormone (PTH) inhibits the production by osteocytes. Furthermore, sclerostin inhibits the canonical Wnt pathway by antagonizing Wnts for binding to LRP-5/6 receptor, leading to beta-catenin degradation. Patients with myeloma have elevated levels of circulating sclerostin and this correlates with adverse disease characteristics features and extensive bone disease. Patients with pathological fractures at diagnosis have extremely high levels of sclerostin compared with all the others, whereas sclerostin seems to correlate with bone-specific alkaline phosphatase and with C-telopeptide of collagen type 1 (Terpos *et al.* 2012a,b). Sclerostin seems to be the most appropriate target in myeloma bone disease. Romosozumab, is a monoclonal antibody against sclerostin, that increased the bone mass in patients with post-menopausal osteoporosis (McClung *et al.*, 2014). Clinical trials in myeloma setting are highly anticipated.

Interleukin-7

IL-7 increases osteoclastic activity and at the same time inhibits osteoblast maturation. IL-7 levels are increased in samples from bone marrow plasma in myeloma patients compared to healthy controls. IL-7 inhibits osteoblast differentiation by decreasing the promoter activity of Runx2/Cbfa1 in osteoblasts (Giuliani *et al.*, 2005).

Transforming Growth Factor-Beta (TGF-β)

TGF-β is a growth factor that is released from bone matrix during bone resorption and inhibits osteoblastic differentiation via Runx2 and remote deletion homoeobox 5 (DLX-5) pathways (Takeuchi *et al.*, 2010). It has been demonstrated that myeloma cells growth might be suppressed by TGF-b inhibition leading to promotion of the differentiation and maturation of osteoblasts (Lu *et al.*, 2016).

Growth Factor Independence-1 (Gfi1)

Gfi1 is a multifunctional transcription factor that binds DNA via specific Zn-finger domains mediating this way chromatin remodeling. This may result to long-term changes regarding gene expression. Elevated levels of Gfi1 block Runx2 induction by osteogenic signaling. MM cells increase Gfi1 expression, inhibit osteoblastogenesis, and reduce bone formation in MM bone disease (D'Souza *et al.*, 2011).

Adipocytes

Adipocytes are not directly implicated in bone disease. Wnt signaling suppresses adipocyte lineage which is regulated by PPARγ signaling pathway (Kang *et al.*, 2007; Fairfield *et al.*, 2018). Furthermore, adipocytes accumulation in the marrow increase with age from 30% to 70% in elderly people (Scheller *et al.*, 2015). Respectively, bone formation decreases with aging. Obesity has been shown to be correlated with increased risk of myeloma and is also associated with increased levels of adipocytes (Landgren *et al.*, 2010; Islam *et al.*, 2005; Wallin and Larsson, 2011). Patients with MM have higher abdominal fat and increased fat metabolic activity by FDG-PET/computed tomography (CT) when compared to MGUS (Veld *et al.*, 2016). Recent preclinical data suggested that an unhealthy diet may be involved in tumor progression (Lwin *et al.*, 2015). Adipocytes seem to support myeloma growth by secreting adipocyte-specific cytokines (adipokines) (such as leptin, adipsin, insulin, and resistin) and growth factors (IL-1β, IL-6, IL-10, IL-12, TNFα, monocyte chemoattractant protein 1 (MCP-1), IGF-1, VEGF, SCF, and bFGF) (Caers *et al.*, 2007; Sprynski *et al.*, 2009, 2010; Greco *et al.*, 2015; Sakurai *et al.*, 2013). Furthermore, adipocytes have a protective effect on myeloma cells against apoptosis induced by chemotherapy through autophagy mechanisms (Liu *et al.*, 2015). Bone marrow adipocytes secrete lipids that can be used as energy bank for cell proliferation (Zub *et al.*, 2015). Increased serum levels of free fatty acids were found in myeloma patients when compared to healthy controls (Jurczyszyn *et al.*, 2015). Some fatty acids had antimyeloma effect (Nagata *et al.*, 2015; Abdi *et al.*, 2014), however a recent study involved lysolipids in the origin of monoclonal gammopathies (Nair *et al.*, 2016). This probably is due to an extensive underlying immune activation that could represent an initiation mechanism in sporadic monoclonal gammopathies. Furthermore, increased levels of free fatty acids are correlated with upregulation of PPARγ signaling, which enhances adipogenesis and therefore myeloma survival and osteoblast inhibition. A recent in vitro study showed direct lipotoxic effect on human osteoblasts (Wang *et al.*, 2013; Gunaratnam *et al.*, 2014). Moreover, adipogenesis during aging and obesity affects bone renewal (Ambrosi *et al.*, 2017). To conclude, marrow fat seems to represent an important emerging regulator of tumor growth and bone disease development and, therefore, in the future it might play crucial role in the upcoming therapeutic strategies.

Osteoclasts

The underlying pathophysiology of myeloma bone disease consists of upregulation of osteoclast activity resulting in increasing bone resorption and therefore to bone lesions (Bataille *et al.*, 1989). The amount of osteoclast has been associated with disease progression (Terpos *et al.*, 2003a,b Valentin-Opran *et al.*, 1982 Taube *et al.*, 1992). Myeloma cells secrete osteoclastogenic cytokines, including IL-1, IL-3, IL-6, TNFα, MIP-1α, MIP-1β, and decoy receptor 3 (DcR3) (Roodman, 2009 Lee *et al.*, 2004a,b Nanes, 2003 Kitaura *et al.*, 2004 Abe *et al.*, 2002a,b Colucci *et al.*, 2009). Furthermore, bone cells and MSCs and bone cells also produce osteoclastogenic cytokines. BAFF, activin A, CXCL12, IL-6, and VEGF play critical role in osteoclast differentiation through the receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) (Giuliani *et al.*, 2001; Pearse *et al.*, 2001; Roux *et al.*, 2002).

RANK

RANK is a transmembrane signaling receptor, which belongs to the tumor necrosis receptor superfamily. RANKL is mainly expressed by the surface of osteoclast precursors, while it is produced by BMSCs, osteoblasts and activated T-lymphocytes (Hsu *et al.*, 1999; Nakagawa *et al.*, 1998). MM cells adhesion to BMSCs acts as stimuli for RANKL expression by osteoblasts. Osteoblasts promote osteoclast differentiation through activation of NF-κB and JunN-terminal kinase pathways (Ehrlich *et al.*, 2005). Moreover, RANKL inhibits osteoclast apoptosis. RANKL has direct effects on mature osteoclasts resulting to actin ring formation and cytoskeletal rearrangements. The TNF receptor associated factor 6 (TRAF6) is a regulatory molecule which promotes osteoclasts formation when RANK binds to RANKL (Häcker *et al.*, 2011). TRAF6 binds to RANK and leads to the activation of the downstream NF-Kb (Jules *et al.*, 2010) and activation of MAPK pathways (JNK, p38). The activation of NF-Kb pathway increases c-Fos expression, which consequently binds and interacts with the nuclear factor of activated T cells (NFAT-c1) initiating osteoclast gene transcription. Denosumab is a humanized monoclonal antibody that binds to RANKL resulting in inhibition of B136 bone resorption (Body *et al.*, 2006). Denosumab currently is approved for the treatment of bone metastases due to solid tumors. In a phase 3 clinical trial which enrolled >1700 patients with newly diagnosed MM, denosumab was compared to zoledronic acid. The first available data of this study revealed that denosumab is not inferior to zoledronic acid while it seems to have a safer renal profile while it possibly increases progression-free survival (Terpos *et al.*, 2017).

OPG

OPG is a soluble decoy receptor for RANKL and member of the tumor necrosis factor receptor superfamily (Lacey *et al.*, 1998). It is produced by osteoblasts and BMSCs and reduces osteoclastogenesis by blocking the interactions of RANKL with RANK. Its expression IL-1b, TNF-a, TGF-b, estradiol, and 17b-oestriol regulate OPG expression. In preclinical studies OPG-deficient mice developed severe osteopenia and osteoporosis (Bucay *et al.*, 1998; Mizuno *et al.*, 1998; Simonet *et al.*, 1997). In healthy subjects, the RANKL/OPG ratio is very low. In contrary in MM patients osteoprotegerin (OPG) is decreased and correlates significantly with advanced bone disease (Seidel *et al.*, 2001).

RANKL/OPG Ratio

Several studies demonstrate that myeloma cells induce the RANKL expression by stromal cells in the bone microenvironment through direct cell-to-cell interactions (Giuliani *et al.*, 2001) and also express directly RANKL (Farrugia *et al.*, 2003; Heider *et al.*, 2003). In contrary, myeloma cells decrease OPG levels via reduced OPG secretion by stromal cells and osteoblasts (Qiang *et al.*, 2008). Furthermore, they produce syndecan-1 (CD138) which is a transmembrane proteoglycan binding to the heparin-binding domain of OPG mediating its lysosomal degradation by myeloma cells (Standal *et al.*, 2002). All these result in an increased RANKL/OPG ratio in the bone marrow microenvironment that increases osteoclast activity. The increased ratio of RANKL/OPG ratio has been associated with poor prognosis and severity of bone disease (Terpos *et al.*, 2003a,b). Agents targeting the normalization of the RANKL/OPG by reducing RANKL and increasing OPG managed to delay bone resorption and tumor growth in preclinical models (Croucher *et al.*, 2001a,b; Terpos *et al.*, 2004, 2005; Heath *et al.*, 2007).

Chemokine (C–C Motif) Ligand 3 (CCL3) (Also Known as Macrophage Inflammatory Protein 1-Alpha—MIP-1α)

CCL3 belongs to the CC chemokine family and is involved in the recruitment and activation of monocytes and monocyte-like cells, including osteoclast precursors (Wolpe *et al.*, 1988). It induces differentiation of osteoclast progenitors and increases osteoclast formation (Abe *et al.*, 2002a,b; Choi *et al.*, 2000; Oyajobi *et al.*, 2003). This is performed dose-dependently via the receptors CCR1 and CCR5, which are expressed by osteoclasts. CCL3 also enhances the effect of IL-6 and RANKL on osteoclast formation (Han *et al.*, 2001). CCL3 levels were elevated in the both in bone marrow and plasma of myeloma patients and correlated with disease features and activity. Furthermore, CCL3 was elevated in the blood of myeloma patients with extensive bone disease, but not in MGUS patients (Politou *et al.*, 2004; Terpos *et al.*, 2003a,b). Moreover, CCL3 gene was shown to be the

gene most highly correlated with advanced bone disease in multiple myeloma (Magrangeas *et al.*, 2003) CCL3 also acts directly on myeloma cells, promoting their growth, survival, and migration (Lentzsch *et al.*, 2003).

Interleukin-3

IL-3 is secreted from activated lymphocytes. IL-3 levels were increased in myeloma cells and in bone marrow plasma from patients with myeloma. IL-3 along with CCL3 and RANKL increase osteoclast activity and therefore bone resorption. Furthermore, IL-3 enhances the growth of myeloma cells independently of IL-6 (Ehrlich *et al.*, 2005; Lee *et al.*, 2004a,b).

Interleukin-6

IL-6 is produced by BMSCs and is involved in inflammatory and immune procedures and in bone metabolism through the Ras/mitogen-activated protein (MAP), extracellular signal-regulated kinase (ERK) cascade, signal transducer and activator of transcription3 (STAT 3) and phosphoinositide 3-kinase/Akt cascade pathways. IL-6 promotes survival and growth for both myeloma cells and osteoclasts. IL-6 mainly increases the early osteoclast precursors that differentiate into mature osteoclasts. Serum levels of IL-6 are increased in myeloma and correlate with progression free survival, disease stage and activity (Kyrstsonis *et al.*, 1996). IL-6 levels were elevated in patients with osteolytic bone lesions when compared with those without bone disease or with patients with MGUS (de la Mata *et al.*, 1995). Preclinical studies have shown reduction in RANKL expression with anti-IL-7 and anti-IL-6 (mAb-1339) antibodies (Fulciniti *et al.*, 2009). However, no osteoclastogenic inhibition and antimyeloma effect was demonstrated.

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a glycoprotein that plays a critical role in MM migration and survival (Taylor *et al.*, 2012). Recently it has also been implicated in osteoclastogenesis. VEGF activates the osteoclasts by binding to VEGFR-1 receptor which is predominantly expressed on osteoclasts. Myeloma cells, on the other hand secrete VEGF stimulated by IL-6 and therefore activates osteoclasts (Tanaka *et al.*, 2007). Furthermore, VEGF enhances bone resorption and increases survival of mature osteoclasts (Tanaka *et al.*, 2007). Moreover, VEGF induces IL-6 production from BMSCs, while IL-6 stimulates VEGF secretion by myeloma cells. This mechanism suggests the existence of paracrine interactions between BMSCs and myeloma cells which is triggered by VEGF and IL-6 (Dankbar *et al.*, 2000).

Osteopontin

Osteopontin is a protein that presents normally in the human bone, kidney, teeth, and epithelial tissues. It is involved in adhesion, apoptosis, angiogenesis, and tumor growth (Standal *et al.*, 2004). Increased levels of osteopontin were identified in patients with advanced myeloma disease when compared with asymptomatic MM or MGUS subjects. Furthermore, increased levels of OPN were correlated with increased bone destruction.

Stromal-Derived Factor-1a (SDF-1α)

SDF-1a is produced by BMSCs and mediates its activity through CXCR4 receptor which is expressed on hematopoietic stem cells, lymphocytes, osteoclast precursors, and malignant cells. SDF-1a upregulates the expression of the matrix degrading enzyme, matrix metalloprotease 9 (MMP-9), which promotes recruitment, activation, and migration of the osteoclasts and is crucially involved in hematopoietic stem cells homing, migration, and tumor proliferation (Alsayed *et al.*, 2007). Plasma levels of SDF-1a are elevated in myeloma patients when compared with healthy subjects and are correlated with the presence of bone lesions. A CXCR4 inhibitor, BKT140, has shown that induces myeloma cells apoptosis and reduces osteoclast activity (Beider *et al.*, 2011).

Activin-A

Activin-A is a dimeric glycoprotein, which belongs to the transforming growth factor-β (TGF-β) family. It regulates several biological functions, including hormonal homoeostasis, gonadal functions, muscle growth, immunity, inflammation, and bone remodeling (Hedger *et al.*, 2011). Activin-A which is produced by osteoblasts and its antagonist lead to autocrine regulation of extracellular matrix mineralization and formation (Eijken *et al.*, 2007). Activin-A stimulates osteoclasts and probably inhibits osteoblasts (Fuller *et al.*, 2000; Kawabata *et al.*, 2007). It is elevated in myeloma patients with advanced disease and correlates with adverse disease features, extensive bone disease, and poor prognosis (Terpos *et al.*, 2012). Sotatercept, is an activin-A inhibitor, which was administered in patients with MM in a phase 2 study and showed that it could probably increase bone mineral density (Abdulkadyrov *et al.*, 2014).

Similarly, increased expression of Bruton's tyrosine kinase (BTK) in osteoclasts and tumor cells has been correlated with tumor growth and bone destruction (de Weers *et al.*, 1994; Shinohara *et al.*, 2008). Therefore, a BTK inhibitor, ibrutinib, has demonstrated satisfying results in reduction of osteolytic bone disease by inhibiting NF-κB pathway (Rushworth *et al.*, 2013; Tai *et al.*,

2012). The in vivo combination of another BTK inhibitor, CC-292, with carfilzomib demonstrated synergistic activity and increased bone formation (Eda *et al.*, 2014). Osteoclasts originate from the monocyte/macrophage lineage and share similar immune receptors and functions (Charles and Aliprantis, 2014; Wu *et al.*, 2008a,b) and interact with T lymphocytes in a direct way (Takayanagi, 2009). T cells secrete osteoclastogenic cytokines (such as RANKL and IL-1β), however osteoclasts suppress T lymphocyte proliferation maintaining this way bone remodeling balance (Grassi *et al.*, 2011). Other cells of the myeloid lineage such as macrophages, dendritic cells, and myeloid-derived suppressor cells (MDSCs), are considered to create a protective environment for tumor cells by preventing apoptosis and by T cells suppression (Gorgun *et al.*, 2013; Leone *et al.*, 2015; Zheng *et al.*, 2009). Recently, it was demonstrated that osteoclasts enhance myeloma cell survival via direct inhibition of T cells proliferation and therefore T-cell-mediated cytotoxicity (An *et al.*, 2016). This results to tumor escape from immunological surveillance (Topalian *et al.*, 2015), and highlights the critical role of osteoclasts in osteolytic bone disease. Therefore, osteoclasts should be considered as potential therapeutic targets for multiple myeloma.

Osteocytes

Osteoblasts and osteoclasts are the major compounds of bone remodeling, however osteocytes are considered to be the main regulators of bone homoeostasis (Bellido, 2013). Osteocytes are approximately 90%–95% of all bone cells, while osteoclasts and osteoblasts fewer than 10%. The osteocyte is defined as a cell surrounded by mineralized matrix. Osteocyte apoptosis is linked to bone remodeling as a result of what is called the osteocyte cell death (Plotkin, 2014). The regions that contain apoptotic osteocytes are targets for bone resorption, resulting in increased bone formation (Noble, 2005). The available data suggest that osteocytes are the major source of RANKL driven osteoclast formation (Xiong and O'Brien, 2012). Myeloma patients with bone disease seem to have fewer viable osteocytes and increased osteoclasts compared to than healthy controls or patients without bone lesions, and this is mainly the result of increased osteocyte apoptosis. Moreover, the expression of OPG is downregulated in the osteocytes by myeloma cells, and this leads to a more pro-osteoclastogenic RANKL/OPG ratio. Furthermore, the osteocytes seem to be the exclusive source of sclerostin production (Westendorf *et al.*, 2004). In murine models the inhibition of the osteocyte-produced sclerostin led to increased bone mass and prevented bone destruction (McDonald *et al.*, 2017). Bortezomib, which is a proteasome inhibitor, has demonstrated bone formation effect (Mohty *et al.*, 2014), by maintaining the viability of osteocytes by reducing their apoptosis rate (Toscani *et al.*, 2016).

Bone Disease Management

Bone disease which is mainly due to increased osteoclastic activity accompanied by decreased osteoblastic function leads to skeletal-related events (SREs). Bisphosphonates, radiotherapy, balloon kyphoplasty and orthopedic surgery, and very recently denosumab are the main therapies used for the management of myeloma bone disease.

Bisphosphonates

Based on the results of phase 3 studies, pamidronate and zoledronic acid (ZA) have demonstrated reduction in SREs compared to placebo (Berenson *et al.*, 1996, 2001; Rosen *et al.*, 2001a). In the first study, ZA was as effective as pamidronate in SREs reduction for patients treated with conventional chemotherapy (Rosen *et al.*, 2001a, 2003). In the second study, 30 mg versus 90 mg intravenous pamidronate monthly demonstrated similar results regarding time to SRE and SRE-free survival time (Gimsing *et al.*, 2010). The third study, compared intravenous ZA with oral clodronate, and it was found that ZA reduced the SRE risk compared to clodronate in all patients included, irrespectively of the presence of lytic bone lesions at diagnosis. Furthermore, ZA improved overall survival (OS) by 10 months in patients with lytic bone lesions at diagnosis (Morgan *et al.*, 2010, 2011). A more recent meta-analysis revealed survival advantage of ZA versus placebo (Mhaskar *et al.*, 2012). Therefore, the European Myeloma Network (Terpos *et al.*, 2013) recommends that all myeloma patients with adequate renal function (creatinine clearance >30 mL/min) and osteolytic bone disease at diagnosis should be treated with ZA (4 mg, over an at least 15-min infusion, every 3–4 weeks) or pamidronate (90 mg, in a 2–4-h infusion, every 3–4 weeks), intravenously. However, symptomatic patients, without bone disease as assessed by conventional radiography, can be treated with ZA as well.

Management of Bisphosphonates Side-Effects

Intravenous BPs can cause acute phase reactions, inflammatory reactions at the injection site, hypocalcemia, hypophosphatemia, renal impairment (RI), and osteonecrosis of the jaw (ONJ) (Terpos *et al.*, 2013; Berenson *et al.*, 1997; Dimopoulos *et al.*, 2006). For hypocalcemia prevention, all patients should receive calcium with vitamin D3 supplementation (at least 600 mg calcium per day and 400 IU vitamin D3 per day).

Renal impairment is due to acute tubular damage and can be observed with both pamidronate and ZA, but the true incidence of this adverse event remains unknown, considering that renal dysfunction is also a common myeloma complication (Morgan

et al., 2010; Terpos *et al.*, 2013; Dimopoulos *et al.*, 2006). Thus, patients with mild or moderate renal impairment need dose adjustments of ZA, according to the summary of the product characteristics.

Osteonecrosis of the jaw is an uncommon complication (ONJ) of BP. Retrospective studies suggest that ONJ is usually observed with ZA, after dental procedures, and seems to be associated with the prolonged administration of the BP (Dimopoulos *et al.*, 2006). Preventive dental measures may reduce ONJ incidence (Dimopoulos *et al.*, 2009). In cases of ONJ, BP should be discontinued and can later be re-administered whenever ONJ has healed.

Denosumab

The most promising agent targeting RANK/RANKL/OPG signaling pathway is the humanized monoclonal antibody denosumab. Due to its high affinity and specificity for RANKL, it can prevent RANK activation, osteoclastogenesis, and osteoclast activation. Recently, denosumab met the primary endpoint of a randomized, double blind, phase 3 study by demonstrating no inferiority compared with zolendronic acid in delaying time to first on-study SRE in patients with newly diagnosed MM. Furthermore, it was proven superior to zolendronic acid regarding time to first on-study SRE in a 15-month landmark analysis. Interestingly, a PFS advantage was seen in denosumab arm; something that needs further investigation as this was an exploratory endpoint in the study. Denosumab is not cleared though the kidneys, providing patients with renal impairment a novel therapeutic modality. Compared with zoledronic acid, patients treated with denosumab had lower rates of renal adverse events (17.1% vs. 10.0%, respectively; $P < 0.001$) (Terpos *et al.*, 2017). Based on these results FDA approved on 5th January 2018 denosumab for the prevention of skeletal-related events in patients with multiple myeloma.

Radiotherapy

Radiotherapy is mainly used for solitary plasmacytomas, symptomatic spinal cord compression, extremely painful lytic lesions, and to prevent pathological fractures. For painful osteolytic lesions, a dose of 3000 cGy in 10–15 fractions seems to be adequate.

Balloon Kyphoplasty and Vertebroplasty

These techniques are used for the management of painful vertebral compression fractures, and approximately 80% of patients with pain which does not respond to pain killers, experience pain relief A (Berenson *et al.*, 2011). All recent data, including a phase III study and a meta-analysis, suggest that balloon kyphoplasty is the treatment of choice for the reduction of pain due to cancer-related vertebral fractures and may be associated with reduced rates of cement leakage (Berenson *et al.*, 2011; Bhargava *et al.*, 2009).

Surgery

The administration novel antimyeloma treatment regimens has reduced the need for surgery during the past decade. Currently, surgery should be used only to fix pathological fractures of the long bones, to prevent and restore axial skeleton in cases of unstable spinal fractures and for spinal cord compression with bone fragments within the spinal route.

Conclusions

Osteolytic bone disease is one of the most common feature of multiple myeloma and is caused by factors produced by myeloma cells that consequently stimulate osteoclasts and inhibit osteoblasts. The cross talk between myeloma cells and the bone marrow stromal microenvironment is extremely complex. Although both overall survival and quality of life of patients with MM has dramatically improved the past decade after the incorporation of novel therapeutic agents, myeloma still remains an incurable disease. Progressive disease depends on the interactions within the stromal compound, therefore agents targeting these interactions are essential for disease treatment and bone disease. Bisphosphonates are the cornerstone of treating and preventing bone disease in MM. Other novel agents, such as denosumab represent promising potential therapeutic tools in the treatment of MM-induced bone disease. These novel agents alone or in combination with bisphosphonates and antimyeloma treatment are expected to extend our therapeutic options for myeloma bone disease in the near future. Further understanding of the underlying pathophysiology is needed.

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Diabetes and Bone

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Abbreviations

BMD Bone mineral density
HRpQCT High resolution peripheral quantitative computerized tomography
PTH Parathyroid hormone
T1D Type 1 diabetes
T2D Type 2 diabetes

Introduction

Diabetes is a condition with increased glucose levels in the blood (hyperglycemia). Usually glucose is transported into the cells via the glucose transporters (GLUT) mediated for some of the GLUT (GLUT4) by insulin. Diabetes may occur when insulin is absent (type 1 diabetes—T1D) or when transportation of glucose is diminished due to insulin resistance type 2 diabetes (T2D), that is, in T2D insulin is present, but not in sufficient quantities. The glucose levels are roughly speaking determined by intake of glucose, glucose transport into and from the tissues (i.e., storage as glycogen in the liver or muscles mediated by insulin and degradation of glycogen by glycolysis mediated by say glucagon). Glucose may also be lost in the urine either through increased filtration brought about by high glucose levels in the blood or alterations in the GLUT in the kidney—these transporters differ from the GLUT by also transporting sodium (SGLT2—or sodium glucose transporters type 2).

In T1D insulin is mandatory for treatment, otherwise ketoacidosis may occur. In T2D a number of subcutaneous (s.c.) and oral treatment alternatives are available. Metformin may decrease glucose absorption, improve insulin resistance, and affect glycogen storage in the liver. Sulphonylureas and glinides increase insulin secretion from pancreas, and are thus dependent on functioning beta-cells.

Insulin may be used in insulin secretion is decreased.

Glucagon-like peptide increases feeding-related insulin release. GLP1 may either be given as injection as an analogue or the amounts in plasma may be increased by inhibiting the degradation of GLP by inhibiting the enzyme that degrades GLP (dipeptidyl peptidase 4 inhibitors).

The thiazolidinediones may improve insulin sensitivity by affecting the peroxisome proliferator-activated receptor gamma (PPAR-gamma). SGLT2 inhibitors may increase glucose loss in the urine and thus decrease blood glucose levels.

Diet is essential in order to control glucose delivery, for example, is starch a complex carbohydrate, which is slowly turned into glucose by amylase in the intestine, whereas pure glucose demands more insulin in order to process it quickly. Exercise is encouraged in order to metabolize glucose (usage in the muscles) as this improves insulin sensitivity. Fat deposition especially in the liver may increase insulin resistance by increasing degradation of the insulin produced in pancreas and transported to the blood through the liver via the portal vein. Exercise further diminished fatty depositions in the liver, gut omentum and peripherally thus improving insulin sensitivity.

Diabetes may be accompanied by a number of complications such as impaired eye sight (diabetic retinopathy), decreased kidney function (diabetic nephropathy), and atherosclerosis. Impairment of bone function may be a further complication of diabetes, and the impairment of collagen structure and function may share some features in the vessel walls and in the collagen in bone. Atherosclerosis is brought about by deposition of cholesterol and fatty acids in the vessel walls, which may then calcify, either directly or by vascular smooth muscle cells transforming into bone forming cells.

Bone consists of a mineralized matrix (mainly hydroxyapatite, which is a calcium–phosphorous compound), an unmineralized matrix (mainly consisting of collagen, osteocalcin—which in turn mediates signaling with the pancreas and insulin, and a number of other proteins), and cells that mediate bone turnover (formation, i.e., creation of new bone and resorption, i.e., degradation of old bone).

The density of the mineralized matrix (bone mineral density—BMD) may be assessed by sending two weak X-ray beams through the body (DXA or dual X-ray absorptiometry—dual for two, X-ray for radiation, and A for absorptiometry, as the fraction of the X-ray absorbed is measured).

Phosphate is absorbed passively from the intestine in abundant quantities and filtrated into the urine. Parathyroid hormone (PTH) may increase loss of phosphate in the urine. Vitamin D may increase phosphate absorption in the intestine.

Calcium is absorbed from the intestine either passively or actively mediated by vitamin D. Both vitamin D and PTH may increase reabsorption of calcium from the urine.

Calcium and phosphate are then built into the mineralized matrix as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Collagen is formed as a triple helix from protein chains. First it is formed as pro-collagen, from which N- and C-terminal fragments may be measured in blood (P1NP is the N-terminal fragment of pro-collagen of type 1 collagen). The chains are hydrolyzed, glycosylated, and bonded by disulfate bridges. Each triple helix chain is then bound to other chains by cross-linking. Degradation of cross-linked collagen may thus release fragments such as CTX (C-terminal fragment of cross linked collagen).

Collagen is rich in glycine and proline. The hydroxylation forms hydroxylysine, which is likely to undergo glycation by nonenzymatic glycation (the Maillard reaction), **Fig. 1**. This is one form of advanced glycation end products (AGE). These AGE in turn have their own receptor (RAGE).

Diabetes has been associated with an increased risk of fractures (Vestergaard, 2007; Janghorbani *et al.*, 2007) disproportionate to the changes in bone mineral density (BMD) (Vestergaard, 2007). Especially in patients with T2D where an increased BMD was accompanied by an increased risk of fractures. In fact a decreased risk should be expected (Vestergaard, 2007). In T1D a much higher increase in hip fracture risk was seen than predicted from the observed decrease in BMD (Vestergaard, 2007). A special bone phenotype may thus be proposed in patients with diabetes.

In order to understand changes in bone, bone biomechanical competence, turnover, and fracture risk in diabetes, it is necessary to comprehend bone turnover and bone biomechanical competence.

Normal Bone Turnover and Competence

In order to understand normal bone biomechanical competence (Burr and Turner, 2003), one may contemplate the hypothetical example why it is relatively easy to break a piece of chalk for writing on a blackboard, while the phalanx of the fifth finger—which holds similar dimensions—may not be broken so easily despite containing less calcium and thus in theory on a DXA scans would seem less strong.

Several reasons for this exist; the bone contains both a mineral matrix and collagen. The mineral matrix (hydroxyapatite in bone and calcium carbonate in the chalk) adds strength but at the same time fragility. Like an atypical femur fracture the chalk will usually break in a relatively right angle while the fracture of a phalanx more likely will be a spiral fracture.

Unlike the chalk, bone contains collagen fibers which—like rebar in concrete—adds bending and compressive strength (**Fig. 2**). Cross-linking of collagen may further add to bone strength. Like re-bar the addition of collagen (a soft material) to a rigid material like the calcified material (like concrete in a building) created a composite material, which is much stronger and lightweight than the individual components would suggest.

Besides collagen, the unmineralized matrix contains osteocalcin and a number of other proteins.

Bone (Fig. 2)

In addition to collagen, the macro-architecture (cortex and marrow cavity) and micro-architecture (trabeculi, Haversian system) adds biomechanical competence.

Further to this, crystal structure and cross-binding adds to the biomechanical competence (**Fig. 3**). A similar cross-binding is not seen in the calcium carbonate crystals of chalk which may be less perfect hexagons than hydroxyapatite, and thus be less densely packed with less cohesion between the individual crystals and is thus easier to crush.

The architecture with a solid shell—the cortex—and a hollow marrow filled with trabeculi, which criss-cross the marrow cavity, further adds to bone biomechanical competence. The criss-crossing of trabeculi is a much stronger structure than a solid beam, especially the cross-linking and triangular structures created add strength (**Fig. 4**).

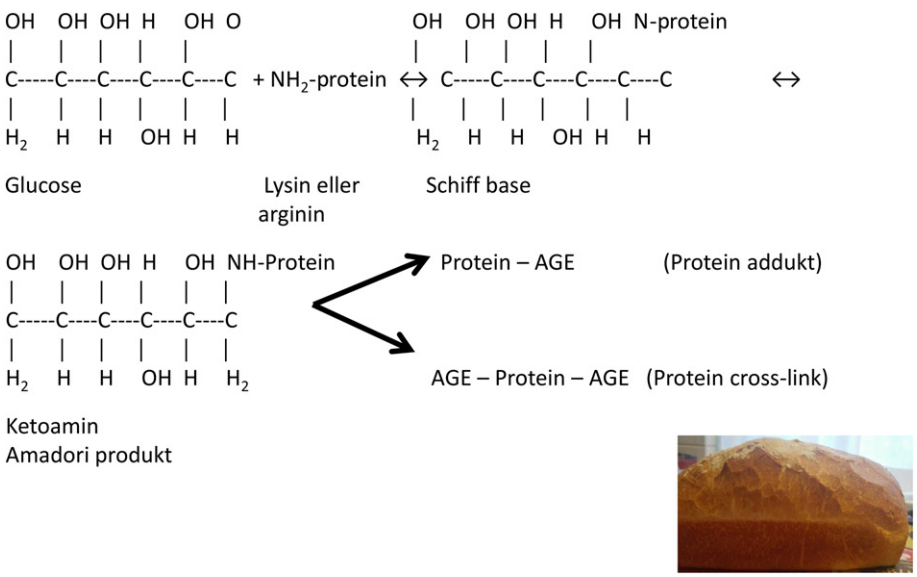

Fig. 1 The Maillard reaction and formation of advanced glycation end-products. This is the same reaction that turns bread yellow during baking.

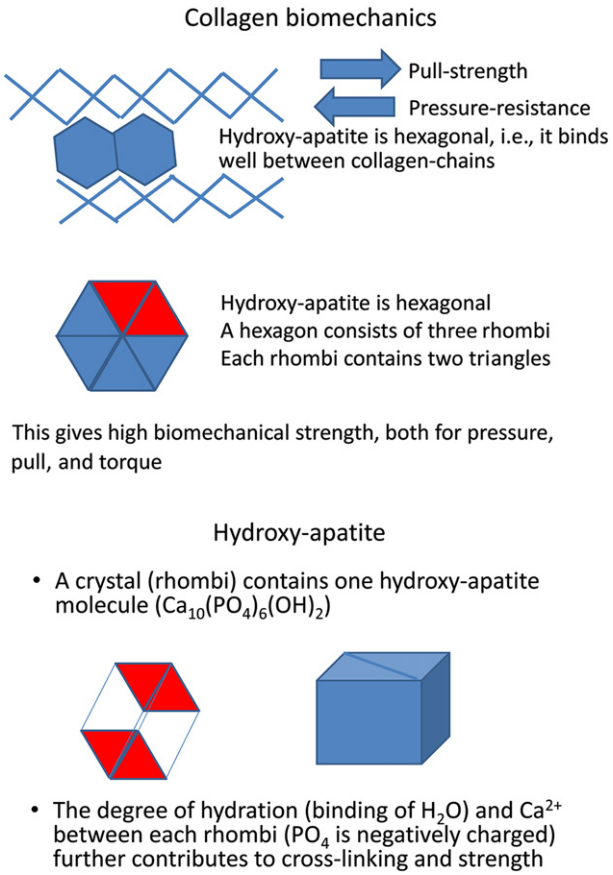

Fig. 2 The Maillard reaction and formation of advanced glycation end-products. This is the same reaction, which makes bread golden during baking.

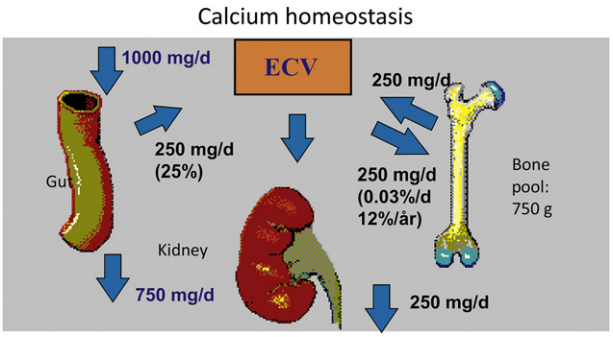

Fig. 3 Shows the normal calcium balance in adults.

This micro-architecture with trabeculi and fenestrations of the cortex (cortical porosity) may be visualized with modern high-resolution CT scanners (High-resolution peripheral quantitative computerized tomography—HrpQCT).

Bone is a living tissue, which contains osteoclasts, osteoblasts, and osteocytes. These contribute to turnover of bone and the way crystals are formed from hydroxyl-apatite, collagen is produced, and the mineralized and unmineralized matrixes are laid down. Diabetes may affect all of these processes.

Effects of Diabetes on Bone Biomechanical Competence

Diabetes may modify several of the processes mentioned above, and other factors associated with diabetes (obesity in T2D) and insulinopenia in T1D may also contribute,

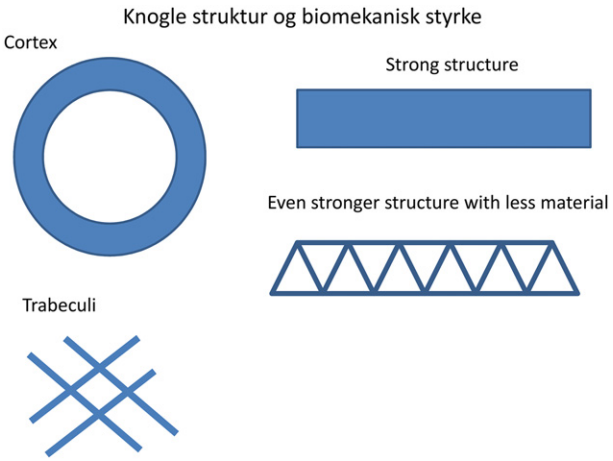

Fig. 4 Bone structure and biomechanical competence.

Turnover

In general dynamic histomorphometric studies on patients with diabetes are few in humans, especially in patients with T1D.

Animal studies (Follak *et al.*, 2004; Abbassy *et al.*, 2010; Fujii *et al.*, 2008; Glajchen *et al.*, 1988; Hamada *et al.*, 2007; Picke *et al.*, 2016) and studies in T2D in humans have suggested a low-turnover state in patients with diabetes (Manavalan *et al.*, 2012). Manavalan *et al.* (2012) in five T2D patients found reduced mineralizing surface, bone formation rate, osteoid surface, and osteoblast surface. Mineral apposition rate decreased with increasing blood glucose. Krakauer *et al.* in a mixed population of T1D and T2D patients among eight osteopenic patients reported markedly reduced bone formation rate and other histological indexes of osteoblast recruitment and function compared with those in nondiabetic control subjects (Krakauer *et al.*, 1995). On the other hand did Armas *et al.* not find any differences in dynamic or other histomorphometric parameters in patients with T1D (Armas *et al.*, 2012).

The disturbances in dynamic histomorphometry seem reversible with good metabolic control (Follak *et al.*, 2004; Fujii *et al.*, 2008; Picke *et al.*, 2016). In humans, markers of bone turnover in general have pointed at a reduced turnover expressed as osteocalcin (Starup-Linde *et al.*, 2015; Hygum *et al.*, 2017), CTX (Hygum *et al.*, 2017), and P1NP (Hygum *et al.*, 2017).

Bone Density and Structure (Micro and Macro-Architecture)

The findings using HRpQCT have differed. One study using HRpQCT in T2D showed similar trabecular and cortical architecture compared to normal controls, and the same increase in cortical porosity with fractures in T2D and controls (Patsch *et al.*, 2013; Heilmeier *et al.*, 2016).

One study reported increased cortical porosity and thus decreased density in the radius in T2D after adjustment for body weight (Yu *et al.*, 2015). Similarly a study in T2D patients with microvascular complications showed a decreased cortical porosity and cortical bone density, while T2D patients without microvascular complications had HRpQCT parameters comparable to controls (Shanbhogue *et al.*, 2016).

One further study showed an increased pore volume, but not statistically significantly increased porosity (Paccou *et al.*, 2016).

Increased trabecular number and cortical thickness have been reported in T2D without adjustment for weight (Patsch *et al.*, 2017), which was confirmed in a further study in T2D which showed a better microarchitecture in T2D than in controls (Nilsson *et al.*, 2017).

In T1D patients without microvascular complications had similar HRpQCT parameters to controls, while patients with microvascular complications had larger total and trabecular bone areas, lower total, trabecular, and cortical volumetric BMD, and thinner cortex at the radius, and lower total and trabecular vBMD, at the tibia in comparison to controls with microvascular complications after adjustment for weight (Shanbhogue *et al.*, 2015).

From these results it seems less likely that architecture as such lend a major contribution to the increased frailty at least in T2D and T1D, although a contribution may be seen with the presence of microvascular complications.

As an alternative to traditional DXA and HRpQCT, trabecular bone score (TBS) has been shown to be decreased in T1D (Neumann *et al.*, 2016) as well as in unspecified diabetes (Kim *et al.*, 2015; Leslie *et al.*, 2013) and TBS is related to fracture risk in nonspecified diabetes (Leslie *et al.*, 2013) and T2D (Choi *et al.*, 2016; Bonaccorsi *et al.*, 2017). One feature that deserves attention is that TBS—in contrast to areal BMD—may decrease with increasing BMI (Langsetmo *et al.*, 2016), which may interact with the high body-mass phenotype of T2D. This may be due to bone marrow adiposity as also shown by MRI to be altered in T1D (Abdalrahaman *et al.*, 2015).

Bone Quality

Studies using microindentation have shown a reduced biomechanical competence both in T2D (Farr *et al.*, 2014) and in adipose subjects (Sundh *et al.*, 2016). As mentioned patients with T2D have an increased areal BMD, but also an increased risk of fractures, and patients with T1D have an elevated risk of hip fractures not mirroring the decrease in BMD seen in these patients (Vestergaard, 2007). Both of these findings indicate a decrease in bone biomechanical competence as also found with microindentation.

The reasons for this decrease in biomechanical competence may be several. Einhorn *et al.* (1988) in an animal experimental study found a decrease in the perfection of crystal formation in diabetic rats, decreased calcium/phosphorus ratio of the ash, and decreased ash content in the tibial metaphyses with increased ash content in the tibial diaphyses compared to control animals. Bone osteocalcin content was increased in the metaphyses of the diabetic rats. Absolute measures of stiffness, torsional strength, and energy absorption were decreased in the bones of the diabetic rates. The latter is in accordance with the findings from the studies using micro-indentation.

In humans studies by Starup-Linde have suggested that mineralization may be disturbed with an increased formation of mineralized matrix to unmineralized matrix (Starup-Linde, 2015), which—like the chalk mentioned above—may make the bone look dense on DXA but frail on biomechanical testing. High sclerostin levels indicate that the bone tries to counter the hypermineralization (Hygum *et al.*, 2017; Heilmeier *et al.*, 2015), and with very high levels a decreased risk of fractures is actually seen in T1D (Starup-Linde *et al.*, 2016a) possibly indicating a successful balancing of mineralized versus nonmineralized matrix. However, similar findings were not seen in T2D (Heilmeier *et al.*, 2015; Starup-Linde *et al.*, 2016a), possibly indicating that the balance was not obtained in these patients.

A further important contributor to decreased biomechanical strength is glycation of collagen and formation of advanced glycation end products such as pentosidine.

In animal experimental diabetes, pentosidine and other advanced glycation end products are abundant in bone and the biomechanical competence of bone is reduced (Saito *et al.*, 2006a; Saito and Marumo, 2010). Similar findings have been done in human femur specimens (Saito *et al.*, 2006b).

In humans, serum markers of advanced glycation end products such as pentosidine (Yamamoto *et al.*, 2008) have associated with increased risk of fractures, whereas the opposite was the case for their receptor (esRAGE) (Neumann *et al.*, 2014; Yamamoto *et al.*, 2009; Yamamoto and Sugimoto, 2016).

The glycation of collagen and formation of AGE may alter the way bone cells interact with it (Hein *et al.*, 2006) so that increased AGE formation decreased the number of osteoblasts thus leading to a decreased formation of new bone.

Hyperglycemia may alter the function of bone cells. In osteoclasts, high glucose levels inhibited RANKL-induced osteoclastogenesis (Xu *et al.*, 2015) and decreased Tartrate resistant acid phosphatase (TRAP) activity and the pit resorption area for osteoclasts (Xu *et al.*, 2013). For the osteoblasts, high glucose levels increase mineralization at 12 mM levels and especially at 24 mM levels (Garcia-Hernandez *et al.*, 2012). PPAR-gamma expression may also be upregulated by hyperglycemia (Botolin and McCabe, 2006), which may induce an osteopetrotic phenotype. Hyperglycemia and high osmotic pressure for 24 h increased the production of collagen type I in osteoblasts and decreased the expression of BAP; (Cunha *et al.*, 2014) thus the effects of glucose on mineralization are unclear. Osteocyte like cells exposed to high glucose levels decrease expression of sclerostin protein, but not RANKL (Tanaka *et al.*, 2015) in contrast to what is seen in humans.

However, these are isolated cells and due to the complex interaction between osteocytes, osteoclasts, and osteoblasts, differences may be seen in vivo.

Body Weight and Feeding

T2D patients are often overweight, whereas T1D patients usually are normal weight or even underweight. The obesity in T2D may be brought about by increased energy intake (more meals and/or higher amounts of food per meal) and/or decreased physical activity with decreased energy consumption.

Higher BMI is associated with higher BMD (De Laet *et al.*, 2005), and the higher BMI in T2D may thus to some degree explain the higher BMD in these patients. The increased sclerostin levels may this partially explain the body's attempt to halt the increased mineralization induced by the higher body weight.

Ingestion of food is followed by a decrease in both resorptive and formative markers of bone formation (Henriksen *et al.*, 2003). In general the reduction in bone resorption is larger (around a factor of two to four depending on food item) than the reduction in bone formation following oral ingestion of any food item including oral glucose (Henriksen *et al.*, 2003). This response is brought about by GLP-2 (Henriksen *et al.*, 2003) and can be abolished using somatostatin, which inhibits GLP-2 (Clowes *et al.*, 2003). Intravenous (i.v.) Administration of glucose does not elicit a similar response despite release of insulin (Westberg-Rasmussen *et al.*, 2017), indicating that the response with decreased resorption and formation is strictly limited to oral feeding.

If T2D patients feed excessively and frequently this may induce a prolonged decrease in bone resorption favoring bone formation, which is then sought countered by sclerostin. Also as old bone may not be resorbed properly, the ratio between collagen and mineralized matrix may change.

The fact that similar changes are seen in HRpQCT in T2D and obesity may support the effects of obesity (Farr *et al.*, 2014; Sundh *et al.*, 2016).

Obesity may thus be a double-edged sword, increasing BMD, but decreasing bone quality. Inflammation from the adipose tissue and fat-rich diet may contribute to this (Cao, 2011).

Calcitropic Hormones and Other Biochemical Markers

As mentioned, sclerostin levels are markedly higher in both T1D and T2D (Heilmeier *et al.*, 2015; Starup-Linde *et al.*, 2016a). However, in animal models the role of sclerostin has been debated (Pereira *et al.*, 2017).

With hyperglycemia, hyperglycosuria usually leads to an increased loss of calcium in the urine. This can—in the absence of compensatory increased intake of calcium and vitamin D—lead to a negative calcium balance and thus bone loss (McNair *et al.*, 1979). Poorly controlled diabetes thus may lead to bone loss from increased calcium loss (McNair *et al.*, 1981). Usually this should be accompanied by secondary hyperparathyroidism, which is not the case in diabetes (McNair *et al.*, 1981). Especially the PTH response to hypocalcemia may be blunted (Schwarz *et al.*, 1992). However, as BMC and BMD are not decreased in T2D, this mechanism probably plays a minor role.

Vitamin D levels tend to be lower in T2D (Starup-Linde and Vestergaard, 2016), while PTH levels are not elevated (Starup-Linde and Vestergaard, 2016). Some of the decrease in vitamin D in T2D may perhaps be related to obesity—vitamin D being fat soluble and thus potentially deposited in the fatty tissue and not being available for normal feedback on PTH.

Proportionally lower than expected PTH may affect the bone anabolic properties of this hormone (Campbell *et al.*, 2016).

Usually low PTH should lead to decreased calcium absorption in the gut and reabsorption in the kidneys. However, as BMC and BMD are not decreased in T2D, this mechanism probably plays a minor role.

Effects of Treatments

Diet and exercise

Usually weight loss is encouraged in obese patients with T2D. A randomized controlled trial in overweight patients with T2D showed that weight loss was associated with a decrease in BMD (Daly *et al.*, 2005), while exercise training seemed to prevent the loss of BMD (Daly *et al.*, 2005). In patients treated by gastric by-pass, which is used for weight reduction in morbidly obese patients with T2D, a decrease is seen in BMD (Giusti *et al.*, 2005).

Hypoglycemia, falls, and fractures

An additional explanation for more fractures with normal to high BMD could be more traumas related to, for example, more falls due to hypoglycemia. However, the increased risk of fractures in diabetes is present even after adjustment for this, and although a relationship between hypoglycemia and fracture exists, this is weak (Vestergaard *et al.*, 2005).

Among the drugs used to treat diabetes, insulin and sulphonylureas may induce hypoglycemia and this fractures (Starup-Linde *et al.*, 2017), whereas most other oral and s.c. treatment principles usually do not induce major hypoglycemia.

Other causes for falls in diabetes may be impaired eyesight, neuropathy with impaired postural control, and sequelae to say cardiovascular problems and stroke. However, these seem to be of minor importance (Vestergaard *et al.*, 2009).

Effect of drug for glucose control

In animal studies insulin may revert the negative effects on bone following diabetes. However, in humans the picture is somewhat more unclear. Insulin (Vestergaard *et al.*, 2005), metformin (Vestergaard *et al.*, 2005; Josse *et al.*, 2017), and sulphonylureas (Vestergaard *et al.*, 2005) may along with dipeptidyl peptidase four (DPP-4) inhibitors (Monami *et al.*, 2011; Dombrowski *et al.*, 2017) show a trend toward lowering fracture risk. For GLP-1 agonists, the effect on fracture risk may depend on the type, with a decreased risk following use of liraglutide and an increased risk following use of exenatide (Su *et al.*, 2015). SGLT2 antagonists do not seem to affect fracture risk (Ruanpeng *et al.*, 2017; Zinman *et al.*, 2015).

Thiazolidinediones (glitazones) are associated with an increased risk of fractures (Loke *et al.*, 2009), although differences exist between the various types of glitazones (Josse *et al.*, 2017).

TZDs act by improving insulin sensitivity through activation of the nuclear receptor, peroxisome proliferator-activated receptor γ (PPAR-γ) (Yki-Jarvinen, 2004). The TZDs affect the differentiation of mesenchymal stem cells (Tornvig *et al.*, 2001). Normally the common mesenchymal progenitor stem cell can differentiate into among others osteoblasts and bone marrow adipocytes (Nuttall *et al.*, 1998). TZDs increase adipogenesis at the expense of osteoblasts, leading to bone loss (Lecka-Czernik *et al.*, 2002; Ali *et al.*, 2005). Other effects by PPARγ agonists include decreased circulating IGF-I concentrations (Lecka-Czernik *et al.*, 2007), and a decrease in estrogen levels, as treatment has been shown to affect the synthesis of sex steroids by inhibiting the aromatase pathway which is the main source for estrogen in postmenopausal women (Rubin *et al.*, 2005).

However, as mentioned the picture is not uniform (Palermo *et al.*, 2015), as insulin may be associated with an increased risk of fractures possibly linked to hypoglycemia, especially seen with short acting insulins (Josse *et al.*, 2017). Also some studies fail to find a decreased fracture risk with the use of DPP-4 inhibitors and GLP-1 agonists, including a recent meta-analysis (Driessen *et al.*, 2017).

For other types of drugs against diabetes, no change in fracture risk was seen (Vestergaard *et al.*, 2005).

Other effects of drugs used to treat patients with diabetes

Many patients with diabetes receive antihypertensive drugs. In general these do not increase, but may rather decrease the risk of fractures (Rejnmark *et al.*, 2006a).

Also statin use is frequent among patients with diabetes, and statins have been associated with a decreased risk of fractures (Starup-Linde *et al.*, 2016b). However, newer research have pointed at an increased fracture risk with very low LDL levels (Starup-Linde *et al.*, 2016b; Peña *et al.*, 2015) possibly from interference with the LRP5 and Wnt signaling system.

Effects of Drugs Against Osteoporosis in Diabetes

Bisphosphonates

Diabetes is a low-turnover condition and antiresorptive drugs reduce turnover. Concern has thus been raised about the safety of such drugs in diabetes. However, the fracture reducing potential seems similar in patients with and without diabetes for antiresorptive drugs (Vestergaard *et al.*, 2011).

Osteonecrosis of the jaw and other inflammatory jaw events—although rare—are associated with bisphosphonate use (Vestergaard *et al.*, 2012), and diabetes is per se a risk factor for osteonecrosis of the jaw and other inflammatory jaw events (Vestergaard *et al.*, 2012). However, these events seem rare (Vestergaard *et al.*, 2012).

The relationship between atypical femur fractures and diabetes is not known in detail (Saita *et al.*, 2015) although diabetes and long-term bisphosphonates share low bone turnover as a common risk factor.

PTH

Diabetes is a low turnover condition in terms of bone and interest has thus been shown on the bone anabolic properties of PTH or analogues. One study indicated that teriparatide may increase insulin resistance (Anastasilakis *et al.*, 2007). Like for bisphosphonates, the relative fracture risk reduction and increase in BMD were similar between patients with or without T2D (Schwartz *et al.*, 2016).

Conclusions

Diabetes seems associated with an increased risk of fractures, which may prove yet a complication of this disease. Changes in micro-architecture may neither explain the increase except in patients with microvascular disease, nor may increase risk of falls especially those related to hypoglycemia. Changes in calcitropic hormones may also play a minor role. This leaves changes in bone biomechanical competence as the presumed main cause of the increased risk of fractures. Some antidiabetic drugs may to some degree modify the risk of fractures.

See also: Lifestyle Diabetes Prevention. Type 2 Diabetes

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Further Reading

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Paget's Disease of Bone

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Glossary

Etiology The study or theory of the factors that cause disease and the method of their introduction to the host; the cause of origin of a disease or disorder.

Giant cell tumor A rare but benign bone tumor comprised of mesenchymal stromal cells and multinucleated giant cells with characteristics of osteoclasts.

Osteoblasts The cells responsible for bone formation, derived from differentiation of mesenchymal stem cells.

Osteoclasts Multinucleated bone resorbing cells formed by differentiation and fusion of mononuclear precursor cells of hematopoetic origin.

Osteosarcoma A malignant primary neoplasm of bone derived from cells of the osteoblast lineage. Almost all osteosarcomas in adults are secondary to Paget's disease of bone.

Epidemiology

Three major determinants of susceptibility to PDB have been identified; increasing age, ethnicity and male gender (van Staa *et al.*, 2002). Age is by far the strongest risk factor. Paget's disease is uncommon below the age of 50 but the prevalence doubles with each decade thereafter to affect about 5% of women and 8% of men aged 80 in the United Kingdom. The prevalence of PDB also varies widely in different countries (Corral-Gudino *et al.*, 2013). The disease occurs most commonly in the United Kingdom where it is estimated to affect about 2% of people over the age of 55 as assessed radiographically. The disease is also common in other European countries such as France, Spain and Italy and in people of European descent who have emigrated to other regions of the world, such as Australia, New Zealand, the United States of America, and Canada. In contrast Paget's disease is rare in Scandinavian countries, the Indian subcontinent, and Asian countries. These ethnic differences in prevalence of PDB persist after migration highlighting the importance of genetic factors in etiology. Archeological studies of skeletal remains in the UK have been performed and are consistent with a model whereby susceptibility to PDB arose as the result of genetic mutations that predispose to the disease in North West Europe many centuries ago, with spread to other regions of the world through emigration (Mays, 2010).

Pathophysiology

At a tissue level, active PDB is characterized by a marked increase in bone remodeling at affected sites (**Fig. 1**). Osteoclasts are increased in number and size and contain many more nuclei than normal. Some contain nuclear inclusion bodies which were

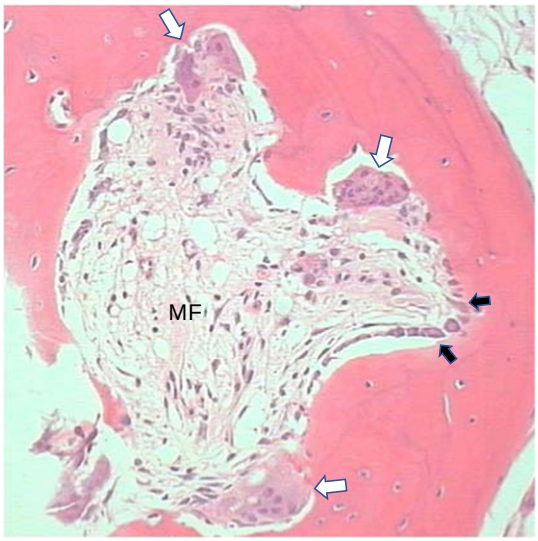

Fig. 1 Bone histology in active Paget's disease. Hematoxylin and eosin stained section of bone from a patient with active Paget's disease. Several large osteoclasts are visible (*white arrows*) as well as osteoblasts forming new bone (*black arrows*). There is extensive marrow fibrosis (MF).

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originally considered to represent viral nucleocapsids (Rebel *et al.*, 1974). However more recent evidence suggests that these structures could be aggregates of undegraded proteins due to defects in the autophagy pathway in PDB (Daroszewska *et al.*, 2011; Helfrich and Hocking, 2014). Osteoblast numbers and new bone formation is also markedly increased in PDB but the bone is laid down in a disorganized fashion (woven bone) which is mechanically weak. Other histologic abnormalities characteristic of active PDB include increased vascularity of bone and bone marrow fibrosis.

Genetic factors play a key role in the pathogenesis of PDB (Ralston and Albagha, 2014; Cundy, 2018; Singer, 2015). Familial clustering is common and there is a sevenfold increases risk of developing the disease in first degree relatives of patients as compared with controls (Siris *et al.*, 1991). In a proportion of families, the disease is inherited in an autosomal dominant trait with an age-dependent increase in penetrance (Morissette *et al.*, 2006; Hocking *et al.*, 2000; Morales-Piga *et al.*, 1995). There are marked ethnic differences in susceptibility to PDB. These disease is common in people from North West Europe, Italy, and Spain. The ethnic differences in susceptibility persist in European migrants to countries such as Australia, New Zealand, and Canada where PDB occurs rarely in the indigenous population (Ralston and Albagha, 2014; Gardner *et al.*, 1978). This is consistent with a founder effect due to carriage of genetic variants that predispose to PDB across the world (Lucas *et al.*, 2005). At the present time 12 genes or loci with robust evidence for association with PDB have been identified as summarized in **Table 1**. However, for some of these variants, PDB is only one component of a multisystem proteinopathy which is characterized by myopathy and neurodegeneration as well as PDB.

Research into the genetic basis of PDB has provided several insights into the molecular pathways that regulate bone remodeling (reviewed by Ralston and Albagha (2014)). For example, discovery of mutations in *SQSTM1* as a cause of PDB uncovered a previously unsuspected role for this gene in regulating osteoclast activity through effects on RANK induced NFκB signaling and its interaction with the signaling protein CYLD. Similarly, studies of the 10p13 locus identified *OPTN* as an important gene in the regulation of bone metabolism through effects on the NFκB pathway and interferon signaling (Obaid *et al.*, 2015).

Other loci associated with PDB lie close to genes that play a role in osteoclast differentiation or activity. For example, the susceptibility locus on the 1p13 locus is 87Kb upstream of *CSF1* which encodes macrophage colony stimulating factor, a protein that is essential for the early stages of osteoclast differentiation. The 8q22 locus lies close to the *DCSTAMP* gene which is essential for the formation of multinucleated osteoclasts. At present, it seems likely that predisposition to PDB at these loci is mediated by variation in expression of these genes, or by linkage disequilibrium with rare variants within the genes themselves but further research is needed to clarify this. For other loci such as 7q33 (*NUP205*), 14q32 (*RIN3*), and 15q34 (*PML*), but the mechanism by which PDB occurs is unclear since these genes have not previously been implicated in bone metabolism. Mutations of the *HNRNPA1/HNRNPA1B1* and *VCP* genes result in a multisystem disease characterized by accumulation of abnormal protein aggregates in muscle and neurones. This supports the hypothesis that abnormalities of protein degradation play a key role in PDB the mechanisms by which osteoclast activation occurs in these diseases remains unclear.

Although susceptibility to PDB is strongly influenced by genetics, environmental factors also play a role. Evidence of this comes from epidemiological studies which have shown significant decreases in disease prevalence and severity in most countries over the past 25 years (Corral-Gudino *et al.*, 2013). It is of interest, however, that in the Campania region of Italy, the severity has increased and in some regions of Spain, no major changes in prevalence or severity of PDB have been observed. Various factors have been suggested as possible triggers for PDB including dietary calcium and vitamin D deficiency; exposure to environmental toxins; repetitive biomechanical loading of affected bones; and local trauma. The only environmental factor that has been studied experimentally is chronic paramyxovirus infection. This was suggested as a possible disease trigger based on the identification of nuclear inclusions in osteoclasts that were thought to resemble measles virus (Rebel *et al.*, 1974). However, more recent work has shown that these structures are morphologically distinct from measles virus particles on ultrastructural analysis (Helfrich *et al.*, 2000). Furthermore, attempts to detect evidence of paramyxovirus nucleic acids and proteins in patient material have yielded

Table 1 Genes and loci for PDB

Locus	*Syndrome*	*Discovery*	*Effect size*	*Allele frequency*[a]	*Likely gene(s)*	*Mechanisms of PDB*	*Author*
1p13	PDB	GWAS	Moderate	Common	*CSF1*	Unknown	Albagha *et al.* (2011)
1q42	PDB/GCT	Linkage	Large	Very rare	*ZNF678*	Unknown	Divisato *et al.* (2016)
5q35	PDB	Linkage	Large	Rare	*SQSTM1*	NFκB activation	Laurin *et al.* (2002) Hocking *et al.* (2002)
7p15	PDB/MSP	NGS	Large	Very rare	*HNRNPA2B1*	Unknown	Kim *et al.* (2013)
7q33	PDB	GWAS	Moderate	Common	*NUP205*	Unknown	Albagha *et al.* (2011)
8q22	PDB	GWAS	Moderate	Common	*DCSTAMP*	Unknown	Albagha *et al.* (2011)
9p13	IBM-PFD	Linkage	Large	Very rare	*VCP*	Unknown	Watts *et al.* (2004))
10p13	PDB	GWAS & linkage	Moderate	Common	*OPTN*	NFκB activation	Albagha *et al.* (2011)
12q13	PDB/MSP	NGS	Large	Very rare	*HNRNPA1*	Unknown	Kim *et al.* (2013)
14q32	PDB	GWAS	Moderate	Common	*RIN3*	Unknown	Albagha *et al.* (2011)
15q24	PDB	GWAS	Moderate	Common	*PML*	Unknown	Albagha *et al.* (2011)
18q21	PDB	GWAS	Moderate	Common	*TNFRSF11A*	Unknown	Albagha *et al.* (2011)

[a]In the general population.

GWAS, Genome wide association study; NGS, next generation sequencing; MSP, Multisystem proteinopathy; IBM-PFD, inclusion body myopathy, Paget's disease and frontotemporal dementia.

inconclusive results and a recent large scale study showed no evidence of an enhanced immune response to paramyxoviruses in PDB as one would expect if a slow virus infection was the cause (Visconti *et al.*, 2017). Although experimental studies have shown that targeted over expression of measles virus nucleocapsids protein to osteoclasts can result in abnormalities of osteoclast function and bone turnover (Kurihara *et al.*, 2011) over-expression of other viral proteins can do the same (Ruddle *et al.*, 1993). Currently therefore the role of viral infection as a trigger factor or perpetuating factor in PDB remains controversial.

Clinical Features

Many individuals with PDB do not suffer symptoms related to the disease and it has been estimated from radiological survey data that between 7% and 14% of patients come to medical attention (van Staa *et al.*, 2002; Tan and Ralston, 2014). In those that do, musculoskeletal pain is the single most common presenting complaint affecting 38% of individuals, followed by bone deformity (20.3%), pathological fractures (10.6%), and deafness (5.9%) (Tan and Ralston, 2014). In one case series about 20% of patients who had presented clinically were truly asymptomatic. In these patients, the disease had been picked up on the basis of a raised alkaline phosphatase (ALP) on blood testing or an X-ray performed for other reasons. It is important to emphasize that the causes of musculoskeletal pain in PDB are often multifactorial; they included pain associated with increased metabolic activity of the disease; and pain secondary to osteoarthritis which is more common in PDB that age-matched controls (van Staa *et al.*, 2002) and pain associated with nerve compression syndromes. It was previously assumed that deafness in patients with PDB of the skull is due to auditory nerve compression but evaluation of the mechanisms of deafness in case series of patients with PDB has revealed that in most cases, the auditory nerve is intact and the deafness is of a conductive type due to involvement of the cochlea (Monsell *et al.*, 1995).

Most patients with PDB do not have clinical signs. Deformity of the facial bones, skull and femurs can occur but this is only detectable clinically when the disease is quite advanced. Paget's disease of the tibia can often be detected clinically and in patients with active disease there may be warmth in the overlying skin. Clinical signs related to nerve compression syndromes of spinal stenosis may also be evident in some patients. Osteosarcoma is a rare complication of PDB which is estimated to affect about 0.01% of patients. The presentation is usually with a sudden increase in pain and swelling of an affected site. The prognosis of osteosarcoma in PDB is poor even with aggressive treatment unless the lesion can be surgically excised (Ruggieri *et al.*, 2010). Giant cell tumors may also be observed in PDB and these are associated with mutations in the *ZNF687* gene (Divisato *et al.*, 2016). Rare complications of PDB that are seldom seem nowadays include high output cardiac failure and hypercalcemia in patients that have been immobilized. Vascular calcification is more common in patients with PDB as compared with controls and there is evidence that the risk of cardiovascular disease is increased (van Staa *et al.*, 2002).

Investigations

The diagnosis of PDB can usually be made on plain radiographs which show coarsening of the trabecular pattern in cancellous bone, cortical thickening, areas of osteolysis alternating with osteosclerosis, pseudofractures, and bone expansion (**Fig. 2**). Predominantly

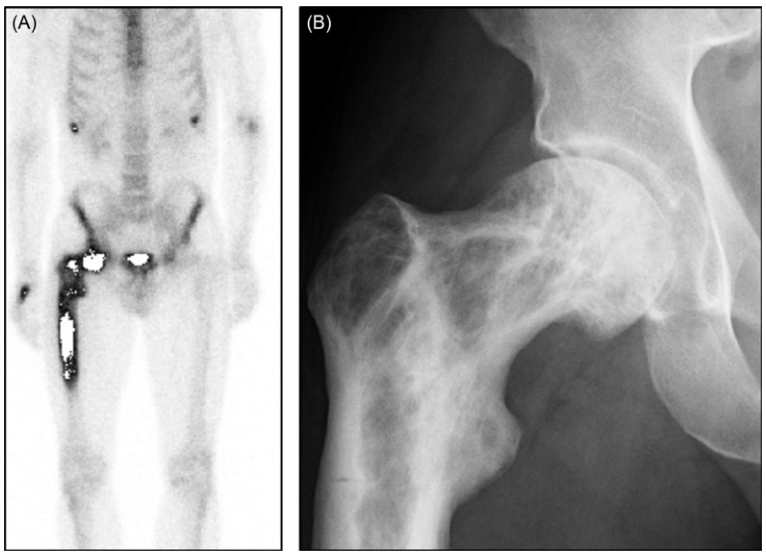

Fig. 2 Radiologic and scintigraphic features of Paget's disease. Panel A: Radionuclide bone scan of a patients with Paget's disease affecting the upper right femur. There is intense tracer uptake affecting the upper two-thirds of the femur. Panel B: X-ray of the upper femur from then same patient showing coarsening of trabeculae throughout the upper femur with areas of osteosclerosis alternating with areas of osteolysis. The right femur is enlarged and a pseudofracture is just visible on the lateral aspect of the femoral shaft at the level of the lesser trochanter.

osteolytic lesions may be observed in the early phase of the disease. Radionuclide bone scans are more sensitive that plain X-rays at detecting early lesions and are the preferred means of documenting the extent of PDB. The typical picture is one of a homogenous increase in tracer uptake affecting a whole bone or part or an affected bone (**Fig. 2**). In the long bones, tracer uptake typically starts at the metaphysis and extends proximally or distally to affect the diaphysis. Bone scans can be negative in PDB which is metabolically inactive however, and it's important to consider performing both forms of imaging in the assessment of patients suspected to have the disease. Routine biochemical screening in patients with PDB typically shows an isolated elevation in serum total alkaline phosphatase (ALP) with otherwise normal liver function tests and calcium biochemistry. Vitamin D deficiency is common but this simply reflects the fact that PDB tends to affect older people where vitamin D deficiency is common. Specialized biochemical markers of bone turnover are typically raised in patients with active PDB. Recently, Al-Nofal and colleagues (2015) conducted a systematic review of the clinical value of several biochemical markers in PDB including serum total alkaline phosphatase (ALP), serum bone specific alkaline phosphatase (BSALP), urinary collagen type 1 cross-linked N-telopeptide (uNTX), urinary collagen type 1 cross-linked C-telopeptide (uCTX); serum CTX and procollagen type I N-terminal propeptide (P1NP). This showed that in untreated patients, levels of all markers were associated with extent of PDB as determined by bone scans with no significant difference in performance between individual markers. Following bisphosphonate treatment, the bone formation markers BSAP, ALP, and P1NP showed the greatest changes and the best correlation with indices of disease activity on bone scan.

Management

Optimal management of PDB requires a multidisciplinary approach. All patients should be carefully assessed for the presence of symptoms that might be due to increased bone turnover as well as for complications such as spinal stenosis, secondary osteoarthritis and fracture that might need surgical treatment. It's also important to consider the possibility that pain in a patient with PDB might be due to an unrelated musculoskeletal condition.

Bisphosphonates

Bisphosphonates are the treatment of first choice for bone pain that is thought to be due to increased metabolic activity in PDB. Bisphosphonates are potent inhibitors of osteoclastic bone resorption which have been shown to be significantly more effective than placebo in relieving bone pain associated with PDB (Corral-Gudino *et al.*, 2017). Nitrogen containing bisphosphonates (aminobisphosphonates) are now used in virtually all patients since they are more effective at reducing bone turnover than simple bisphosphonates such as etidronate and tiludronate and have a longer duration of action (Corral-Gudino *et al.*, 2017). Comparative studies have suggested that zoledronic acid is superior to pamidronate and risedronate in improving bone pain secondary to PDB. Vitamin D deficiency should always be corrected prior to administration of bisphosphonate therapy to reduce the risk of hypocalcaemia. Bisphosphonates are contraindicated in patients with renal impairment; zoledronic acid and alendronic acid are licensed for use in patients with a GFR of >35 mL/min whereas pamidronate and risedronate are licensed to a GFR of >30 mL/min. Treatment regimens and common adverse effects of the bisphosphonates that are used most frequently in PDB are shown in **Table 2**. In addition to the common adverse effects shown in **Table 2**, bisphosphonate therapy has been associated with atypical femoral fractures, uveitis, and osteonecrosis of the jaw. Symptomatic atrial fibrillation has also been reported as an adverse effect of zoledronic acid.

There is no evidence to support the use of prophylactic bisphosphonate therapy to prevent complications of PDB although a trial is in progress to address this issue in carriers of *SQSTM1* mutations (http://www.isrctn.com/ISRCTN11616770).

Table 2 Bisphosphonates in Paget's disease

Drug	*Posology*	*Most common adverse effects*
Oral		
Risedronate	30 mg/day orally for 2 months	Dyspepsia, esophagitis
Alendronic acid[a]	40 mg/day orally for 6 months	Dyspepsia, esophagitis
Intravenous		
Pamidronate	30–60 mg/day i.v. for 3 days	Acute phase response, hypocalcaemia
Zoledronic acid	5 mg i.v.	Acute phase response, hypocalcaemia

[a]Not licensed in the UK or Europe for Paget's disease.

Attempts to normalize bone turnover by repeated courses of bisphosphonate therapy confers no advantage over symptom-directed treatment in PDB and there is evidence that with long term therapy, this approach may be harmful by increasing the risk of fracture and requirement for orthopedic surgery (Tan *et al.*, 2017; Langston *et al.*, 2010).

Analgesics

Many patients with PDB require analgesic therapy with paracetamol, non-steroidal antiinflammatory drugs and antineuropathic agents, either as an adjunct to bisphosphonate therapy in the treatment of pain due to increased metabolic activity or to control symptoms associated with secondary osteoarthritis or other conditions.

Calcitonin

Calcitonin can be effective at improving bone pain and reducing biochemical markers of bone turnover in PDB. It is now seldom used due to the fact that bisphosphonates are more convenient to administer, cheaper and have fewer side effects.

Denosumab

Denosumab is an osteoclast inhibitor which is a monoclonal antibody that neutralize RANKL. It is a powerful inhibitor of bone resorption. Although Denosumab is not licensed for PDB it has been found to be effective in isolated cases and represents a potential treatment option for patients where other drugs are contraindicated (Reid *et al.*, 2016).

Surgery

Orthopedic surgery is frequently required in PDB patients for fracture fixation, correction of deformity, treatment of spinal stenosis and for the treatment of co-existing osteoarthritis. Clinical experience indicates that the outcome of orthopedic surgery is good in PDB, although the presence of bone deformity increases complexity of the surgery and blood loss is increased, even with preoperative bisphosphonate therapy (Wegrzyn *et al.*, 2010).

Monitoring and Retreatment

It is usual to keep patients with PDB under periodic review every 6–12 months at which point the patient can be assessed clinically and biochemical markers of bone turnover checked. Total ALP concentrations are the most widely used biochemical marker of disease activity in routine clinical practice, although P1NP and BSALP are slightly more sensitive. Some patients may require repeated courses of treatment for PDB. The most common reason for retreatment is recurrence of bone pain thought to be due to recurrence of metabolic activity in PDB. This should be suspected clinically if the patient presents with pain localized to an affected site and there is biochemical evidence of increased bone turnover (such as an elevated ALP value). It should be noted however that if only a single bone is affected (monostotic PDB) there may be increased metabolic activity in the presence of normal biochemistry. In this circumstance, a therapeutic trial of bisphosphonate can be considered. It should be noted that the availability of potent bisphosphonates such as zoledronic acid with a prolonged duration of action has meant that biochemical recurrences of disease activity are now relatively rare especially in elderly patients with PDB (Cundy *et al.*, 2017).

Summary

Paget's disease of bone is a skeletal disorder characterized by focal increases in bone remodeling affecting one or more bones. Genetic factors play a key role in pathogenesis of PDB and many cases are caused by mutations in the *SQSTM1* gene. Bone pain is the most common symptom, affecting up to 38% of those who present clinically. Bisphosphonates are indicated for the treatment of bone pain thought to be due to increased metabolic activity of PDB.

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Heterotopic Ossification and Calcification

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Glossary

Abnormal injury/immune response (AIR) The aberrant activation and suppression of the immune system, often follows a bimodal pattern, i.e., the activation of the innate immune responses is often followed by an anti-inflammatory adaptive immune response. The AIR and the subsequent abnormal release of endogenous mediators can lead to secondary insults and/or other dysfunctions.

Acquired HO A common clinical complication following traumatic events, such as spinal cord injury (SCI), traumatic brain injury (TBI) and joint arthroplasty.

Bone morphogenetic protein (BMP) A member of transforming growth factors-β (TGF-β) superfamily.

Endochondral ossification An indirect ossification that requires cartilage as an intermediator.

Hereditary HO Hereditary HO such as fibrodysplasia ossificans progressiva (FOP) and progressive osseous heteroplasia (POH) is rare but debilitating genetic disorders. FOP is characterized by progressive heterotopic endochondral ossification in connective tissues, while *POH* is characterized by intramembranous ossification inside skin and subcutaneous tissues.

Heterotopic calcification (HC) The deposit of calcium-based salts and crystals within tissues other than normal bone and enamel. HC can be considered as an atypical HO.

Heterotopic ossification (HO) The process by which bone tissue forms outside of the skeleton.

Intramembranous ossification A direct ossification, i.e., bone formation in the primitive connective tissue by direct differentiation of mesenchymal stem cells (MSCs).

Mesenchymal stem cells (MSC or marrow stromal cell) Adult multipotent stem cells that derived from the mesoderm, and have the capacity to differentiate into osteocytes, chondrocytes and adipocytes.

Introduction

Heterotopic ossification (HO) is the pathologic transformation of soft tissue into structurally normal bone through either endochondral or intramembranous ossification. Acquired HO is often a serious and costly complication of traumatic tissue damage whereas hereditary HO, such as fibrodysplasia ossificans progressiva (FOP) and progressive osseous heteroplasia (POH), is fatal genetic disorder characterized by progressive ossification in soft tissues.

Heterotopic calcification (HC, also called heterotopic mineralization), on the other hand, is abnormal deposition of calcium salts in tissues other than bone and enamel. HC can be considered as an atypical HO. Vascular calcification, a specific HC, is a frequent complication of atherosclerosis and diabetic vasculopathy.

All forms of HO are serious heterogeneous medical complications that could be costly or even fatal, but these disorders have not received due attentions. As a result, the current understanding of the mechanism of HO formation is very limited, and there is no effective treatment for these disorders either.

The purpose of this article is to raise the attention by updating our current understanding and identifying key knowledge gaps; however, since it is almost impossible to cover all aspects of HO at reasonable depth, this article will focus only on highly conserved or highly impact aspects of HO. Hopefully, our discussion will not only raise the attention of general audience but also identify potential knowledge gaps for professionals in this area.

Classification and Incidence

HO can be divided into different categories. Based on etiology, HO can be divided into acquired HO (aHO) and hereditary HO. aHO is the most common form of HO, which was first described in World War I and was frequently reported following the traumatic events (Shehab *et al.*, 2002; Garlan, 1991; Potter *et al.*, 2006, 2010), including (1) the spinal cord injury (SCI) induced HO, which affects about 22%–29% patients, often forming HO around hips, knees and shoulder joint (Ohlmeier *et al.*, 2017; Potter *et al.*, 2006; Brady *et al.*, 2017); (2) the traumatic brain injury (TBI) induced HO, which affects about 5%–20% patients, often forming HO around shoulders, elbows and knees (McAuliffe *et al.*, 1997; Ranganathan *et al.*, 2015; Brady *et al.*, 2017); (3) the severe burn injury induced HO, which affects up to 60% patients (Thefenne *et al.*, 2017; Schneider *et al.*, 2017; Foster *et al.*, 2017); (4) the hip arthroscopy induced HO, which affects 4.7%–60% patients (Bedi *et al.*, 2012; Beckmann *et al.*, 2014); and (5) the major elbow trauma induced HO, which affects 14%–35% patients (Cai *et al.*, 2015; Park *et al.*, 2010).

Hereditary HO, including fibrodysplasia ossificans progressiva (FOP) and progressive osseous heteroplasia (POH), is rare but life-threatening disease. The incidence of FOP is about one in two millions (Kaplan *et al.*, 2008; Pignolo *et al.*, 2011, 2016; Zhang

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et al., 2013), and POH is even rarer (only about 60 confirmed patients worldwide) (Job-Deslandre, 2004; Jüppner, 2002; Kaplan *et al.*, 2004; Elli *et al.*, 2016).

HC can be considered as an atypical HO. vascular calcification, a specific type of HC, affects about 30% patients with peripheral vascular disease (Kusumbe *et al.*, 2014; Mizobuchi *et al.*, 2009; Fuery *et al.*, 2017).

Pathophysiology of HO

HO is an injury induced pathological process, and typical HO includes the sequential stages of inflammation, fibro-proliferation/angiogenesis, condensation, and final endochondral ossification (Davies *et al.*, 2017; Thilak *et al.*, 2015; Culbert *et al.*, 2014; Pignolo *et al.*, 2015).

Inflammation

Inflammatory response, triggered by injury, is responsible for the initial tissue degeneration and the subsequent events. Various cytokines, such as TNF-a, IL-1 and IL-6, secreted by the innate and adaptive immune cells, such as macrophages, mast cells and T cells, are pro-inflammatory cytokines that are able to facilitate the immune response and accelerate the death of damaged cells. On the other hand, IL-4, IL-10 and TGF-β1 were reported to be anti-inflammatory cytokines and contribute mainly to tissue repairing (Serra *et al.*, 2017; Netea *et al.*, 2015; Shintani *et al.*, 2017; van Dyken and Locksley, 2013; Mauri and Bosma, 2012; Josefowicz *et al.*, 2012). This normal inflammatory/injury response generally plays beneficial role and is essential for normal wound healing; however, this process could be dysregulated, especially in the context of traumatic injuries.

Abnormal inflammation/injury response (AIR) is commonly considered as a key common mechanism of both acquired and genetic HO (Kraft *et al.*, 2016). For example, Thilak *et al.* (2015) reported that the increased level of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), two clinical indicators of inflammation, were closely associated with HO following total hip arthroplasty. Citak *et al.* (2016) also suggested that elevated level of serum creatine kinase (CK) was recommended as a novel biomarker of HO. Forsberg *et al.* (2014) tested the expression of 24 inflammatory cytokines in the patients with high-energy trauma and found interleukin (IL)-3 promoted HO formation while serum IL-12 and IL-13 showed negative correlation with HO. Evans *et al.* (2012) found that pro-inflammatory cytokine, IL-6 was rapidly up-regulated within 6 days of high-energy penetrating war injuries, but after this acute stage, anti-inflammatory cytokine IL-10 exhibited significant trend of upregulation. Consistently, numerous trauma-induced animal models confirmed the crucial role of AIR in HO (Genêt *et al.*, 2015; Huang *et al.*, 2014; Kan *et al.*, 2009, 2011, 2014).

In FOP, a hereditary HO, AIR is thought to be responsible for flare-up, which is a pre-condition and crucial for subsequent HO (Pignolo *et al.*, 2016). Consistently, several FOP mice models, either gain-of-function of BMP receptor (ACVR1) or overexpression of ligands, demonstrated that AIR dramatically facilitates HO process (Kan *et al.*, 2004; Kan and Kessler, 2011; Yu *et al.*, 2008; van Dinther *et al.*, 2010). For example, inflammation-associated cytokines, such as IL-1, monocyte chemoattractant protein-1 (MCP-1) and TNF-a, were all elevated either in saliva or serum of FOP mouse model (Nfatc1-Cre/caAcvr1$^{fl/wt}$) (Sung Hsieh *et al.*, 2017), and activin A, a member of TGF-β superfamily which is strongly associated with AIR, was reportedly up-regulated in FOP model (Alessi Wolken *et al.*, 2017; Hino *et al.*, 2017; Upadhyay *et al.*, 2017; Hino *et al.*, 2015; Hatsell *et al.*, 2015). However, strangely, Hildebrand *et al.* (2017a) showed no significant difference of pro-inflammatory cytokines in FOP patients serum compared to unaffected healthy children. This could be due to inappropriate sample collection time (since AIR usually occurred immediately after injury); alternatively, inconspicuous change in systemic level might not reflect the local inflammation.

It is worthy to mention that even though HC only partially recapitulates the complex process of HO, the degree of inflammation is also closely associated with HC. However, the role of inflammation in HC is probably a bit different from that in the typical HO process. For example, in vascular calcification, the initial pro-inflammatory promotes microcalcification, which, in turn, induces further inflammation, and this vicious cycle can be broken by adaptive immune response, which, instead, promotes fibrosis and osteogenic transdifferentiation, and ultimately leads to macrocalcification (Hildebrand *et al.*, 2017a).

Fibrosis and Angiogenesis

One feature of normal wound healing is fibro-proliferation of local stem/progenitor cells and the ingrowth of new capillaries or angiogenesis; however, this normal process may also exceed what is needed for optimal repairing. In fact, both HO and HC suffered from pathologic fibrosis and angiogenesis. Although it is still unclear how fibrosis and angiogenesis are regulated, it is believed that abnormal cell–cell interaction and tissue microenvironment directly lead to activation of tissue-specific pluripotent stem/progenitor cells which eventually leads to fibrosis (Kan *et al.*, 2009; Dey *et al.*, 2017; Ranganathan *et al.*, 2016; Kaplan *et al.*, 2016). High level of local trophic factors, such as EGF and FGF, might not only facilitate the fibro-proliferation but also up-regulated neovascularization. Consistently, Agarwal *et al.* (2017a) showed the closely association of vasculature with HO by in vivo imaging through combining reflectance and Raman spectroscopy and indicated HO formation depended on angiogenesis. Interestingly, Cocks *et al.* (2017) also demonstrated that vascularity of HO was closely associated with maturity of bony lesion, i.e., less vasculature was observed in early stages of HO, but in mature HO, blood vessel numbers (BVN) are much higher.

Endochondral Ossification

In typical HO process, following AIR and fibrosis/angiogenesis, progenitor cells initiate the chondrogenic differentiation, under the influence of microenvironment factors, including factors of biophysical (mesenchyme condensation) and biochemical (osteogenic factors, such as BMPs), into terminal differentiated cells and form mature ectopic bone through endochondral ossification (Kan *et al.*, 2013, 2017). Alternatively, mesenchymal stem/progenitor cells directly differentiate into osteoblasts and osteocytes through intramembranous ossification (Cairns *et al.*, 2013; Pignolo *et al.*, 2015), such as in POH.

Cellular Origin of HO

One key knowledge gap is that the exact cellular origin of HO is still unclear (Kan and Kessler, 2014). Few investigators proposed that endothelia-derived progenitors contribute to HO via endothelial-mesenchymal transition (EndMT) mainly in the context of FOP (Medici *et al.*, 2010; Barruet *et al.*, 2016; Pang *et al.*, 2016). Others also indicated that EndMT participated in acquired HO formation (Sun *et al.*, 2016; Zhang *et al.*, 2016). However, Wosczyna *et al.* (2012) demonstrated that Tie2-cre labeled multipotent stem cells (originally thought to be endothelial progenitors) were mesenchymal origin. Consistently, study of another putative endothelial marker, VE-cadherin, did not support the idea that endothelial cells are involved in HO formation.

Hematopoietic cells were also reported to contribute to HO (Kaplan *et al.*, 2007). For example, Suda *et al.* (2009) first demonstrated that CD45 + circulating osteogenic progenitor cells expressed both hematopoietic and osteoblastic marker in vitro, and contribute to fibro-proliferative region in vivo, and even directly differentiation into osteoblasts. However, recent study found that CD45 + cells did not give rise to mature osteoblasts either in normal skeletogenesis or HO in triple transgenic mouse model (CD45-cre;Z/RED;Col2.3GFP) (Otsuru *et al.*, 2017).

Overall, the current consensus is that mesenchymal stem cells (MSCs) (Friedenstein *et al.*, 1966; Frenette *et al.*, 2013) are probably the main cellular origin of HO. For example, Kan *et al.* demonstrated that both Glast-creERT and Gli1-creERT labeled mesenchymal progenitors contribute to HO (Kan *et al.*, 2013, 2017). Dey *et al.* (2016) also suggested that tissue-specific mesenchymal progenitors contributed to distinct aspects of HO, i.e., SCX + tendon-derived progenitors participate in endochondral HO of tendon and MX1 + cells contribute to HO in muscle.

Molecular Mechanisms

Another key knowledge gap is that the exact molecular mechanism of HO is still unclear, even though various conserved signaling pathways have been implicated. The emerging picture seems to support the idea that dysregulation of BMP and activin A signaling might play a central role in HO, and that other conserved signaling pathways, such as Hedgehog (HH), Wnt/β-catenin and fibroblast growth factors (FGF), are also involved, either through cross-talk with BMP signaling or through other independent mechanisms (**Fig. 1**).

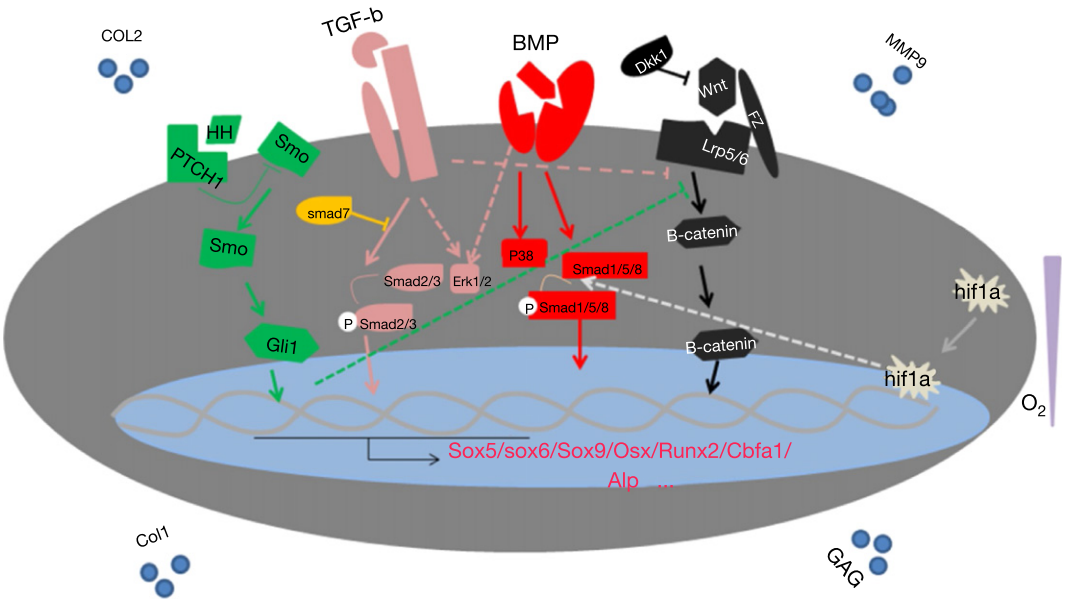

Fig. 1 Current working model of HO.

BMP/TGF-β Signaling

Even though BMP signaling is the most extensively studied pathway in HO, the exact roles of BMP in HO, either in acquired HO or hereditary, is still unclear; nevertheless, current data seem to suggest that the roles of BMP signaling could be very complicate and context-dependent.

For example, in FOP, at least three mechanisms have been proposed: (1) activates BMP signaling without exogenous BMP ligands (Kaplan *et al.*, 2009; Agarwal *et al.*, 2015). (2) Gain-of-function of BMPRI (Bagarova *et al.*, 2013), and hyperactivity of BMP signaling after ligand stimulation (Katagiri *et al.*, 2017; Bragdon *et al.*, 2011). (3) Mutant ACVR1 abnormally transduces BMP signaling in response to activin-A, which in turn, causes abnormal phosphorylation of smad1/5/8, and eventually leads to cartilage and bone formation in vivo (Hildebrand *et al.*, 2017b). This specific mechanism is proposed based on Hatsell *et al.*'s surprising finding that activin A could triggers ectopic ossification in an animal model of FOP (Hatsell *et al.*, 2015).

In aHO, BMPs were also thought to be critical for HO, but the detailed mechanism of BMP signaling in aHO is still largely unknown; however, Jackson *et al.* found that the expression of BMP ligands and BMPR1A are all elevated in a traumatic HO model. Kang *et al.* also showed that BMPR1A synergistically with BMPR2A, responding to high level of BMP ligands, phosphorylated SMADs and promoted osteogenesis. (Kang *et al.*, 2014; Peterson *et al.*, 2014, 2015; Liu *et al.*, 2014; Jackson *et al.*, 2011). Recently, Agarwal *et al.* (2017b) suggested that BMP ligands expression is essential for HO formation, however, loss of single type I BMP receptor, i.e., ACVR1, ALK2, or ALK3 are not sufficient to block HO; nevertheless, loss of both ACVR1 and ALK3 significantly reduces HO. Consistently, TGF-βs were also thought important for traumatic HO. For example, TGF-β2 is highly expressed in the HO lesion following total hip arthroplasty (THA) (Jackson *et al.*, 2011; Toom *et al.*, 2007; Suutre *et al.*, 2009; Sato *et al.*, 2007).

HH Signaling

HH signaling was also an important regulator of HO. For example, in POH, loss of GNAS encoding Gαs, were reported to enhance HH signaling, since Gαs is an activator of PKA which is an inhibitor of HH signaling (Cairns *et al.*, 2013; Regard *et al.*, 2013; Zhang *et al.*, 2012). In aHO, up-regulation of BMPs will promote IHH expression and lead to activation of ptch1 and eventually cause endochondral bone formation (Stoeger *et al.*, 2002; Sugita *et al.*, 2013; Yasuda *et al.*, 2010). Collectively, it seems safe to assume that HH signaling plays crucial roles in HO, but the exact roles of HH signaling or the crosstalking between HH and BMP or other signaling pathways are still largely unknown.

Wnt Signaling

Wnt pathway is also important for HO, especially for chondrocytes maturation and osteoblast differentiation (Gu *et al.*, 2015; Guerrero *et al.*, 2014; Su *et al.*, 2015). In the context of POH, Wnt signaling was demonstrated to be a downstream of HH signaling, and further studies found that Wnt/β-catenin target gene expression inversely correlates with HH signaling (Ptch1, Gli1 and Hip1). Thus disruption of the balance between Wnt/β-catenin and HH signaling may contribute to POH. In vitro experiment also demonstrated that BMP and Wnt signaling also coordinately regulate osteogenic differentiation of MSCs, i.e., both Smad1/5/8 and LRP5 (a ligand of wnt pathway) were up-regulated during osteogenic differentiation of MSC.

Diagnosis

The early diagnosis of HO is still challenging. Soft tissue swelling, severe pain, and joint restriction, could be pre-conditions, therefore suggestive of HO, but the definite diagnosis is dependent on cutting edge imaging technology and the development of specific early metabolic biomarkers of HO.

For imaging-based diagnosis, radiography (X-ray), ultrasonography, three-phase bone scanning and computed tomography (CT), magnetic resonance imaging (MRI) and Raman spectroscopy were all reported to be useful as tools (Onat *et al.*, 2017; Mercuri and Saltzman, 2017). Each technique has its own advantages and disadvantages. X-ray is often used to provide rough evaluation of mature HO. CT and MRI are commonly used in HO quantification other than early detection. Three-phase bone scanning is sensitive for early HO detection but lack of specificity. Ultrasonography appears to be the most suitable first-line imaging modality for the diagnosis of HO. Of note, a novel imaging technology, Raman spectroscopy has potential to define earlier mineralized collagen within HO lesion (Agarwal *et al.*, 2017a; Peterson *et al.*, 2013).

Additionally, the studies of earlier HO markers at metabolic level attract more attention recently. Serum alkaline phosphatase were shown to increase after 6 weeks in patients with HO (Evans *et al.*, 2014; Lee *et al.*, 2016). Matrix metalloproteinase-9 (MMP-9) are elevated in HO and suggested to be a in vivo HO marker (Evans *et al.*, 2014; Davis *et al.*, 2011; Shi *et al.*, 2017). Levels of inflammatory factors, chemokines, calcium, phosphorous, ESR, CRP and CPK were also demonstrated to be closely associated with HO (Qin *et al.*, 2014; Yang *et al.*, 2017; Edsberg *et al.*, 2017).

Table 1 Characterization of the therapeutic strategies to prevent HO

Therapeutic strategies	*Advantages*	*Disadvantages*	*Clinical application?*
NSAIDs	Simple, cheap, extensive effects on various HO and higher efficiency in HO reduction	Uncertainty adverse effects, such as chronic pain and impaired physical function	Yes
Radiation	Simple, noninvasive and higher efficiency	High expense, undetermined radiotherapeutic dose and higher adverse effects	Yes
Biphosphonates	Simple, wide use in HO. broader effects in each HO stage	Limited efficiency, occur after treatment cessation, higher adverse effects	Yes
Surgical resection	Clearly resection of HO lesions	Restricted surgical location, HO recurrence, invasive and high expense	Yes
Small molecular drugs	More specific, artificially synthesized. Easily absorbable and degradation	A certain adverse effects	Part of drugs
Specific neutralized reagents	More specific, artificially synthesized, easily absorbable and degradation, lower adverse effects	Limited effective range	No
Gene modification	Simple, cheap and most specific	Limited effective target, unstable and uncertain adverse effects	No

Treatment

There are no efficient treatments for either acquired or genetic HO (**Table 1**). However, nonsteroidal anti-inflammatory drugs (NSAIDs), radiation, bisphosphonates and surgical resection were commonly used to relieve or prevent HO. NSAIDs is first line treatment, because of its anti-inflammatory role in early stage of HO (Rath *et al.*, 2016; Beckmann *et al.*, 2014). Radiation therapy was also helpful for reducing local inflammatory response after injury and preventing MSCs osteogenic differentiation (Hoff *et al.*, 2013; Popovic *et al.*, 2014). Bisphosphonates are anti-absorptive agents, and several studies demonstrated it could induce osteoclast apoptosis and inhibit calcification (McClure, 1983; Taravati *et al.*, 2017). Operative intervention is limited to acquired mature HO resection but not for FOP. Although clinical improvement after operation is appreciated, there is still significant risk of recurrence (Agarwal *et al.*, 2017c).

Recently, preclinical studies showed that gene modification, potential small molecular drugs/inhibitors and several neutralizing reagents were also beneficial to HO. For example, specific and efficient inhibition of osteogenic genes, such as sox9, runx2, osterix and ALP were reported to be potential HO therapy. microRNAs, 18–25 nucleotide RNA molecules that target messenger RNA (mRNA) for cleavage or translational repression, showed enormous potential in HO prevention (Guérit *et al.*, 2013; Zhang *et al.*, 2017). It was reported that miR-203, miR-132-3p, miR-499, miR-563 and miR-574-3p alleviate ectopic bone formation either by targeting runx2 or sox9 or other related osteogenic genes (Tu *et al.*, 2016; Sun *et al.*, 2016; Xu *et al.*, 2016; Qu *et al.*, 2016; Lim *et al.*, 2016). Moreover, short interfering RNAs (siRNAs) which facilitate posttranscriptional silencing of target genes, also prevent HO via decreasing expression of runx2 and osterix (Shrivats *et al.*, 2015a; Shrivats *et al.*, 2015b). Overexpression of smad7 in injured tendon will downregulate EndMT and inhibited HO formation.

Small molecular drugs or inhibitors repress several conserved signaling pathways of HO could also be potential HO therapy. Dorsomorphin and its derivatives are BMPR inhibitors, such as LDN193189 and LDN212854, significantly decreased HO formation (Yu *et al.*, 2008; Peterson *et al.*, 2014). But the use of LDN212854 in HO prevention is also at great risk of adverse effects (Agarwal *et al.*, 2017b). PX478 and rapamycin, were reported to inhibit hif1a signaling to efficiently alleviate both genetic and acquired HO (Agarwal *et al.*, 2016). Imatinib, an inhibitor of platelet-derived growth factors (PDGF) signaling pathway, recently are also considered as a desirable inhibitor of hif1a, c-kit and MAPK, to effectively ameliorate HO symptoms (Werner *et al.*, 2013; Kaplan *et al.*, 2017). Chakkalakal *et al.* (2016) showed palovarotene, an inhibitor of retinoic acid receptor γ (RARγ), significantly prevented HO as well corrected skeletal defect. Interestingly, Cromolyn, an inhibitor or stabilizer of mast cells, recently were demonstrated to be a great help to HO reduction through specifically diminishing the number of de-granulating mast cells in pre-osseous lesions (Brennan *et al.*, 2017).

Other, more specific drugs, such as neutralizing antibodies, showed favorable effects in HO treatment via targeting abnormally activated signals. For example, Hino *et al.* and hatsell *et al.* proposed that activin a-specific-neutralizing antibody or broad-acting BMP and activin blockers like ACVR2A-Fc and ACVR2B-Fc could be a new strategic regime for FOP (Hino *et al.*, 2017). Agarwal *et al.* (2017b) also indicated ALK3-Fc is a translational drug to HO treatment with reduced risk of adverse effects. However, none of above-mentioned approaches have been commonly accepted in clinics, and further studies are needed to clarify the potential issues.

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Kidney Stones☆

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Glossary

Lithotripsy The crushing of a calculus.
Nephrolithiasis The presence of calculi in the kidney.
Oxalate A salt of oxalic acid; an end product of metabolism.
Struvite Crystals of magnesium ammonium phosphate.
Supersaturation The presence of more substance in a solution than can be dissolved permanently.

Stones of the urinary tract have plagued humankind since antiquity, with the oldest recorded stone having been found in an Egyptian mummy. Hippocrates himself warned against the unskilled management of bladder calculi. In industrialized societies, upper renal tract stones predominate, with bladder stones being relatively rare. The passage of a kidney stone is associated with significant morbidity and expense. Indeed, the current estimated annual cost to the health care system in the United States is $5 billion. For the most part, the major burden of the disease is borne by young and otherwise healthy individuals. Knowledge of the composition of the stone, coupled with an understanding of the physicochemical and physiological principles underlying stone formation, can guide timely and appropriate investigation and intervention.

Epidemiology of Kidney Stones

The majority of urinary tract stones are kidney stones. The change from bladder to kidney as the site of lithiasis has paralleled national wealth. The frequency of kidney stones is also linked to the prosperity of the nation, as indicated by the significant reduction in stone formation in Europe temporarily associated with World War I and World War II. The common factor may be the increase in dietary protein intake (particularly meat) resulting in an increased acid load for the kidney to handle. Bladder stones remain a problem in Developing World children and in older men with urinary stasis. In North America, a proportion of the increase in rates of kidney stones has been linked to obesity, with a higher prevalence seen in people with a BMI exceeding 30 kg m^{-2}.

There is wide geographic variation in the rates of nephrolithiasis. The lifetime risk of developing a kidney stone is ~3% in Asia, 10% in Europe and North America, and 20% in Saudi Arabia. In addition to dietary variations in the countries, there is a potential role for climate effects. Stone formation appears to be more common in warmer climes; indeed, there is a 'Stone Belt' in the southern United States. Occupations associated with increased sweating, and reduced fluid intake also appear to predispose to renal tract stones. The presumptive mechanism relates to the development of more concentrated urine with an increased likelihood of crystal formation.

Stones are more common in men than in women, with a prevalence of 10.6% and 7.1%, respectively, however the incidence of stone formation in females is on the rise. The peak age for stone formation is the third decade of life for both sexes; there is a second, smaller peak during the sixth decade of life for women. It has been postulated that this may be associated with the increased renal calcium throughput associated with menopause. The recurrence rate for stone formation is high but relatively unpredictable. Nonetheless, ~75% of patients who have formed a kidney stone can be expected to have a recurrent episode within 20 years. Table 1 shows the relative frequency of kidney stones based on their chemical composition.

Stones are not always chemically pure; for example, many calcium stones contain small amounts of hydroxyapatite and uric acid.

Etiology of Kidney Stones

The majority of cases of renal stone disease are idiopathic in origin. Despite this, a family history of kidney stones can be found in 30–45% of patients. Although this may be the result of shared environmental factors (e.g., climate, diet), there has been substantial interest and progress in the understanding of genetic causes of stone disease. Furthermore, it is well established that several medical conditions predispose to stone formation. Table 2 shows those conditions associated with an increase risk of renal stone disease.

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Table 1 Chemical composition of kidney stones (percentages)

Composition	*Approximate frequency (%)*
Calcium oxalate (pure) or mixed with calcium phosphate	60
Calcium monohydrogen phosphate (brushite)	10
Struvite	5–10
Uric acid	15–20
Cystine	1
Other	1

Table 2 Factors associated with stone formation

Genetic condition
Metabolic disorders
0. Cystinuria
0. Primary hyperoxalurias (1 and 2)
0. Xanthinuria
0. 2,8-dihydroxyadeninuria
0. Orotic aciduria
Disorders of uric acid metabolism
0. Lesch–Nyhan syndrome
0. Phosphoribosyl pyrophosphate synthase hyperactivity
0. Glucose-6-phosphatase deficiency
0. Adenine phosphoribosyl transferase deficiency
Renal chloride channel defects
0. Dent's disease
0. X-linked hypophosphatemic rickets
0. X-linked recessive nephrolithiasis
Renal tubular acidosis
Disease process
Medical conditions
0. Primary hyperparathyroidism
0. Renal tubular acidosis
0. Autoimmune
0. Malabsorption syndromes
0. Inflammatory bowel disease
0. Hyperthyroidism
0. Sarcoidosis
0. Hypercalcemia of malignancy
Surgical conditions
0. Intestinal resection
0. Jejunoileal bypass
0. Gastric bypass surgery (Roux-en-Y)
Anatomic abnormalities
0. Medullary sponge kidney
0. Horseshoe kidney
0. Solitary kidney
0. Ureteric obstruction
0. Caliceal diverticulum
Medication
Calcium supplements
Vitamin D
Vitamin C
Sulphonamides
Triamterene
Indinavir
Ephedrine
Ciprofloxacin

Calcium-Containing Stones

Calcium-containing stones account for more than 70% of all renal tract stones. From a physicochemical point of view, the fact that they form is hardly surprising given that urine is almost always in a state of supersaturation for calcium salts. Factors that

predispose to the development of idiopathic calcium stones include increased urinary concentrations of calcium and oxalate, alterations in inhibitors of stone formation, and nidus for stone formation.

The concentration of urinary calcium is increased by low urine volume and hypercalciuria. Low urine volume promotes stone formation by increasing the concentration of urinary calcium and by decreasing the urine flow rate. Hypercalciuria is commonly transmitted in an autosomal dominant fashion. Absorptive hypercalciuria is the most common cause of hypercalciuria and is postulated to be due to hyperresponsiveness to vitamin D in the jejunal mucosa. Resorptive hypercalciuria due to primary hyperparathyroidism occurs in ~5% of individuals with recurrent stone formation. Primary renal hypercalciuria due to a defect in renal tubular calcium reabsorption occurs in 5% of individuals with recurrent stone formation. A diet high in sodium is associated with increased urinary calcium excretion.

The sources of oxalate are the metabolism of amino acids (e.g., serine, glycine, hydroxyproline), metabolism of ascorbate, and dietary intake (10–20%). Oxalate is excreted by the kidney. The urinary concentration of oxalate is much lower than that of calcium. Increases in absolute excretion of oxalate result in larger proportional changes in urinary oxalate concentration when compared with calcium. Increased absorption of oxalate is seen with a low-calcium diet and enteric hyperoxaluria. The latter occurs with malabsorption syndromes associated with small bowel resection, chronic diarrhea or inflammatory bowel diseases. Calcium is bound by free fatty acids allowing increased colonic absorption of unbound oxalate. Inherited hepatic enzyme defects, primarily hyperoxaluria types I and II, are associated with severe hyperoxaluria that commonly presents during childhood with multiple kidney stones and may lead to renal failure.

Citrate is an important inhibitor of stone formation. Urinary citrate forms a soluble complex with calcium and decreases the concentration of free calcium available for crystal formation. Citrate also inhibits crystal propagation by unknown mechanisms. Hypocitraturia can occur as an isolated finding or may be due to chronic systemic metabolic acidosis, renal tubular acidosis, hypokalemia, a high-protein diet, and possibly a high-sodium diet.

Urinary glycoproteins inhibit stone formation at various stages. These proteins include Tamm–Horsfall protein, bikunin, nephrocalcin, and a urinary form of prothrombin fragment 1. The possible role of abnormal glycoproteins in recurrent stone formation is under investigation.

Uric acid is the product of purine metabolism. A diet high in purines can lead to hyperuricosuria and uric acid crystals that act as nidus for calcium oxalate precipitation and promote calcium stone formation. One third of patients with isolated hyperuricosuria have endogenous uric acid overproduction. In these cases, dietary restriction of protein does not affect uric acid concentrations in the urine.

Alterations in urinary pH, including both acidic ($pH < 5.5$) and alkaline ($pH > 6.7$), can predispose to calcium stones. Acidic pH increases the amount of undissociated uric acid available for calcium oxide stone formation. Conversely, alkaline urine increases monohydrogen phosphate available for formation of calcium monohydrogen phosphate (brushite) stones.

Uric Acid Stones

Pure uric acid stones are uncommon. They are radiolucent. The factors that predispose to the development of uric acid stones are the urine concentration of uric acid and low urine ($pH < 5.5$). Low urine volume increases the risk for uric acid stones by the same mechanisms discussed previously for calcium stones. Chronic hyperuricosuria is associated with gout, a high-purine diet, and inborn errors of metabolism. Acute hyperuricosuria may occur during treatment of myeloproliferative disorders.

Over the last ten years, the increasing prevalence of metabolic syndrome has been highlighted as the leading cause of uric acid stone formation. The proposed mechanism suggests that renal steatosis and lipotoxicity leads to decreased ammonia (NH_4^+) excretion, thereby dropping urinary pH.

Struvite Stones

Struvite stones are composed of magnesium ammonium phosphate. These stones develop in the context of urinary tract infection with organisms that produce urease (e.g., Proteus, Klebsiella, Serratia, Mycoplasma). The urinary pH and concentration of ammonia are increased by the breakdown of urea. Struvite stones grow rapidly, particularly in areas of the pelvicalyceal system that do not drain adequately, and may form stag-horn calculi. These stones usually present with symptoms of urinary tract infection. Struvite stones are difficult to treat because they are themselves infected.

Cystine Stones

Cystine stones develop as a result of cystinuria, an autosomal recessive trait or autosomal dominant with incomplete penetrance, associated with decreased proximal tubular reabsorption of cystine and other amino acids. Cystine stones develop due to the low solubility of cystine in the urine. Low urine volume and acidic urine promote stone formation.

Investigation of Patients with Kidney Stones

There is some controversy as to how extensively to investigate patients who have passed a single kidney stone. The controversy is centered around the low mortality and relatively low morbidity of the condition as well as the lack of specific therapy for most

forms of renal stone disease. Many clinicians favor the institution of general therapeutic maneuvers without investigation. The European Association of Urology has suggested a classification for stone formers based on frequency of stone passage and relative risk of recurrence. This classification can be adapted to direct investigation.

Analysis of the calculus, where possible, is the cornerstone of the investigation and rational therapy of nephrolithiasis. Patients with infection-related (struvite) stones and cystine stones can be readily identified and treatment can be planned. Patients who make uric acid stones are at high risk for recurrence and require detailed investigations as to the cause of their hyperuricosuria. For calcium stone formers, the European Association of Urology classification divides patients according to whether they are first-time or recurrent stone formers, whether or not there are residual stone fragments, and (for those recurrent stone formers) whether their disease results in rare (mild) or frequent (severe) passage of stones. Also highlighted for aggressive management is a group of patients who are at a predetermined specific risk for recurrence (e.g., primary hyperparathyroidism).

Table 3 suggests a rational approach to investigation in patients who have passed a single stone. This will detect patients with hyperuricemia and hypercalcemia as well as those with infected urine. The presence of a low bicarbonate level with a urine pH that is not maximally acidified would suggest the presence of renal tubular acidosis.

For patients who have frequent stone passage or a significant number of residual stones visible on radiographs, an attempt to establish the lithogenicity of the urine is a reasonable strategy. Commercially available software can calculate the relative supersaturation for calcium oxalate and calcium phosphate if required. **Table 4** outlines those additional 24-h urinary investigations that may be useful for recurrent stone formers. Optimal values for these parameters for minimal lithogenicity are shown in parentheses.

Rational Therapy for Kidney Stone Disease

The treatment of kidney stones can be divided into general and specific measures. The power of general advice in terms of fluid intake and diet should not be underestimated. It has been suggested that the 'stone clinic effect' can result in a 5-year reduction in new stone passage of 60%.

Dietary Intervention

Given that stone formation is the end result of the process of crystal formation, aggregation, and growth from a supersaturated solution, the most rational therapy would be to modify the solution such that the potential energy for crystal formation within the solution is lowered. The logical way in which to do this is to increase fluid intake. Consumption of enough fluid such that the daily urine volume is >2.5 l has been associated with significantly reduced rates of stone formation in large observational studies over prolonged periods of time. Furthermore, a randomized prospective study performed by Borghi and colleagues at the University of Parma, Italy, demonstrated that kidney stone patients randomized to drink enough fluid to generate a urine volume >2.5 lday^{-1} had a significantly lower rate of recurrence of their stones than did the group of patients randomized to receive no strict fluid prescription. In addition, the time to recurrence was significantly longer in the fluid-treated group.

From large observational studies, it appears that many different beverages provide some protection against stone formation. Data exist to support the beneficial effects of coffee (both caffeinated and decaffeinated), tea, beer, and wine in this regard. In contrast, the ingestion of grapefruit juice and apple juice was shown to increase the risk of stone formation in men followed in the Health Professionals Follow-up Study. Grapefruit juice also increased the risk of stone formation in women observed as part of the Nurses' Health Study; however, the mechanism of this effect is unclear.

Although it would seem to be intuitive that a reduction of calcium in the diet would reduce the frequency of kidney stones, that is not the case. The most compelling evidence of the failure of a low-calcium diet to ameliorate renal stone disease came from a prospective randomized study in men performed by Borghi and colleagues. This landmark study compared a diet containing 100 mmol day^{-1} of calcium with a diet containing 30 mmol day^{-1} of calcium. Members of the latter group were also required to lower their sodium intake to $\sim$50 mmol day^{-1} and their animal protein intake to 52 g day^{-1}. The group on the normal-calcium,

Table 3 Basic investigations following passage of a single stone

Serum analysis
0. Complete blood count
0. Biochemical profile
0. Electrolytes, bicarbonate, creatinine, urea
0. Ionized calcium, or calcium corrected for albumin concentration, phosphate
0. Parathyroid hormone
0. Urate
Urine analysis
0. Urine culture
0. Urine pH

Table 4 Suggested investigations for frequent stone formers

24-h urine collection:	
Acidified urine (6 mol l^{-1} HCl)	
Calcium	(<7.5 mmol male) (<6 mmol female)
Phosphate	(<35 mmol)
Oxalate	(<350 μmol)
Standard urine	
Volume	(>2.5 l)
Sodium[a]	(50–100 mmol)
Creatinine[b]	(7–16 mmol male) (5–14 mmol female)
Urate	(<4.5 mmol)
Citrate	(>2.5 mmol)
Magnesium	(>3.0 mmol)

[a]To assess compliance with dietary recommendations.
[b]To establish accuracy of 24-h collection.
Note. Number in parentheses indicate desirable values for low urine lithogenicity.

low-sodium, low-protein diet had a significantly reduced risk of recurrent stone formation, although the effect took more than 3 years to manifest. The American Urological Association (AUA), recommends limiting sodium and non-dairy protein intake while maintaining a calcium intake of 1000–1200 mg day^{-1} in patients with calcium stones.

Few well-designed studies have addressed other dietary components. The combination of a low-protein, high-fiber diet was not shown to be preventive of recurrent calcium oxalate stones in a prospective study performed under the auspices of the Kaiser Permanente Medical Care Program.

Pharmacological Intervention

Calcium stones

The optimal initial treatment for idiopathic hypercalciuria is the administration of a thiazide diuretic such as hydrochlorothiazide or chlorthalidone (12.5–50.0 mg day^{-1}). Thiazide diuretics indirectly and directly increase calcium reabsorption in the proximal and distal tubules. Randomized trials have shown a 20% risk reduction for the development of new calcium oxalate stones. Routine monitoring for serum electrolyte abnormalities is necessary. Hypokalemia can lead to hypocitraturia and so must be avoided. If hypercalciuria persists, amiloride (5 mg day^{-1}) may be added to further reduce calcium excretion and correct hypokalemia. Potassium citrate (30–80 mEq day^{-1}) is indicated for patients with hypocitraturia or those with recurrent calcium stones in whom other metabolic abnormalities have been addressed. Urinary citrate is increased by decreased reabsorption induced by alkalinization. Patients with hyperuricosuria are at risk for the development of uric acid crystals that serve as a nidus for calcium oxalate crystal formation and have been shown to benefit from administration of allopurinol. Oral calcium and cholestyramine have been considered to bind oxalate and diminish urinary oxalate; however, there is no strong evidence for their use in idiopathic calcium oxalate stones. Oral calcium to bind oxalate and potassium citrate to correct acidosis may be beneficial in enteric hyperoxaluria.

Uric acid stones

The most important aspect of the pharmacological management of uric acid stones is alkalinization of the urine to a range of 6.0–6.5. Potassium citrate (30–80 mEq day^{-1}) is preferable to sodium bicarbonate because it avoids the sodium load. During potassium citrate therapy, the urine pH must be monitored and not allowed to rise above 7.0 because this will promote the formation of calcium phosphate stones.

Struvite stones

Struvite stones are chronically infected. Treatment of struvite stones with antibiotics may help to reduce the rate of growth of the stones; however, definitive therapy for struvite stones usually involves surgical excision of the stones. In patients whom surgery is not feasible or has failed, a trial of acetohydroxamic acid (AHA), a urease inhibitor, can provide benefit in patients where residual or recurrent struvite stones are present.

Cystine stones

Cystine stones are treated with the usual general measures of increased fluid intake and restriction of sodium intake. Alkalinization of the urine to a pH above 7.0 using potassium citrate increases the solubility of cystine. If these measures are not effective in reducing the rate of stone formation, α-mercaptoproprionylglycine (tiopronin), penicillamine, and captopril may be effective. These medications cleave the disulfide bond and increase the solubility of cystine.

Table 5 Situations where alternatives to ESWL may be required

Situation	*Explanation*	*Alternative*
Cystine stones	Resistant to fragmentation	Medical management
Struvite stones	Infected, hardcore	Combined strategy
Ectopic kidney (including renal transplant)	Targeting problem	PCNL
Pregnancy	Safety concerns	Conservative management until after delivery
Large stones (>2.5 cm)	Risk of steinstrasse or multiple residual fragments	PCNL
Bleeding diathesis	Risk of hemorrhage	Correction of problem, medical management, or direct surgical approach

Surgical Intervention

The surgical management of renal stone disease has been revolutionized by the advent of extracorporeal shock wave lithotripsy (ESWL) and the growth of endourological procedures. Invasive surgery is rarely required except in cases of complicated infected stones. The success rate of ESWL is close to 95% for individual cases. Concerns about the procedure relate to possible long-term complications (although the procedure has been in use for more than 25 years) and to the fact that failure to clear all of the stone fragments after lithotripsy will inevitably lead to an increased risk of recurrence. For ESWL to be effective, the patient must not be too large or too small for the machine. The stone must be targetable and amenable to fragmentation by the shock wave. Once the stone has been broken, there must be a clear drainage pathway given that the presence of ureteric obstruction is a contraindication to ESWL. Not infrequently, ancillary procedures, such as ureteric stenting and endourological fragment removal, are required after ESWL. **Table 5** details several other situations where ESWL may be less suitable.

Although the shock wave can be delivered externally (as in ESWL), direct application of the shock to the stone is possible using minimally invasive techniques such as percutaneous nephrolithotripsy (PCNL). Under these circumstances, a scope is passed through a percutaneous nephrostomy and fragmenting energy is applied directly to the stone. For ureteric stones, endourological procedures using rigid or flexible ureteroscopes are more likely to be successful than is ESWL alone. Larger kidney stones, >2 cm, could be managed with flexible ureteroscopy and laser lithotripsy if PCNL is contraindicated (in cases of morbid obesity, anticoagulation or bleeding disorders).

Acute complications of ESWL are infrequent. Loin pain and ureteric obstruction from stone fragment passage (steinstrasse) occur in ~6% and 2.5% of cases, respectively. Rarer complications include renal hematomata and direct shock wave damage to other viscera. Long-term development of hypertension has been reported by several groups and is the subject of ongoing investigations. Similarly, reduction in renal function following bilateral ESWL has led to recommendations that a period of months be allowed to pass before a second kidney is treated by this method.

The removal of stones by ESWL or other surgical methods should not be considered a cure of the renal stone disease. Close attention to medical management is required to prevent recurrence, either from growth of residual stone particles or from de novo stone formation in a susceptible individual.

Management of Acute Renal Colic Due to Stone Passage

The passage of a kidney stone is usually associated with severe pain, hematuria, and nausea and vomiting. Frequently, the person is unable to find a comfortable position and can be seen to be writhing on the bed. This is in contradistinction to persons with severe peritonitis who usually lie very still and resist movement. Despite being called 'colic,' the pain of stone passage is usually constant and may be in the flank or radiate to the groin. Radiation to the groin area suggests that the stone is in the middle or lower part of the ureter.

If the stone is passed, it should be sent for analysis. Other important investigations in the acute setting should be designed to rule out urosepsis and obstruction as well as to exclude other diagnoses. Helical computed tomography scanning offers high sensitivity and specificity for the diagnosis of ureteric stones and can provide significant information if nephrolithiasis is not the correct diagnosis. Investigations aimed at determining the cause of the kidney stones can be delayed until the acute episode is over.

Patients with acute renal colic can be managed supportively. Kidney stones smaller than 4 mm can be expected to pass spontaneously over the course of 48 h with symptomatic relief. It should be remembered that disappearance of symptoms does not always imply stone passage, and a confirmatory test is required if the stone is not voided. Indications for surgical intervention during the acute phase include nonpassage of an obstructing stone, urosepsis with obstruction, and ongoing pain. Acute interventions may include percutaneous nephrostomy followed by ESWL, percutaneous lithotripsy, and cystoscopy with retrieval of the stone. Invasive surgery is rarely required.

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Pseudohypoparathyroid States

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Introduction

Pseudohypoparathyroid (PHP) states are a heterogeneous group of rare metabolic disorders that are characterized by end-organ resistance to parathyroid hormone (PTH) action. In PHP disorders, plasma PTH levels are elevated in the face of hypocalcemia and hyperphosphatemia, whereas these are low or absent in true hypoparathyroidism (PTH deficiency) (Mantovani and Spada, 2006; Kelsey, 2010; Weinstein *et al.*, 2001).

The syndrome was described for the first time in 1942 by Albright *et al.*, who reported three patients with hypocalcemia and hyperphosphatemia, along with skeletal abnormalities, including short stature, brachydactyly, and obesity. The diagnostic basis of these cases was their reduced calcemic and phosphaturic response to the administration of parathyroid extract. This was the first recognized hormone resistance syndrome, and was named "pseudohypoparathyroidism," as opposed to primary hypoparathyroidism (Albright *et al.*, 1942).

The constellation of developmental and skeletal abnormalities was further expanded to include mental retardation, round facies, dental hypoplasia, subcutaneous calcifications, shortening of metacarpal (particularly the third, fourth, and fifth) and metatarsal bones, and were collectively called Albright's hereditary osteodystrophy (AHO) (Thakker, 2011). Further reports described the presence of AHO without hypocalcaemia, and this variant was called pseudopseudohypoparathyroidism (PPHP).

Further developments in the understanding of PTH resistance syndromes came from the identification of the PTH receptor and its coupling to the α-subunit of the stimulatory G protein (Gsα), thereby activating cAMP levels (Chase *et al.*, 1969; Aurbach *et al.*, 1992).

Measurement of urinary cAMP, in response to synthetic PTH administration, permitted the differentiation between PHP type 1 (PHP1) and type 2 (PHP2). In PHP1 patients a blunted cAMP response to PTH was observed whereas in PHP2 the cAMP response was preserved. However, in both types the phosphaturic response was deficient, indicating a defect distal to cAMP generation in the PTH-signaling transduction pathway (Drezner *et al.*, 1973).

Recent applications of molecular biology techniques have allowed the distinction of PHP1 into PHP1a, which results from heterozygous inactivating mutations of the *GNAS*, and PHP1b, which is due to methylation defects in *GNAS* locus, known to be an imprinted gene (Turan *et al.*, 2015). Finally, PHP1c appears to be a variant of PHP1a, in which a specific mutation disrupts receptor-mediated activation of adenyl cyclase but does not affect receptor-independent activation of the enzyme (Turan *et al.*, 2015) (Table 1).

The aim of this article is to provide an overview of the current understanding of the pathophysiology, the genetic biology and phenotypes of the PTH resistant states.

Physiology of PTH Action

PTH is an 84-amino acid peptide that is secreted from the parathyroid chief cells and plays an important role in the maintenance of calcium homeostasis. The secretion of PTH is regulated by specific calcium-sensing receptors (CaSR) on the surface of the chief cells that monitor serum calcium levels (Tfelt-Hansen and Brown, 2005).

Table 1 Classification and characteristics of the various pseudohypoparathyroid disorders

Disease	*AHO*	*PTH resistance*	*Additional hormone resistance*	*Erythrocyte Gsa activity*	*PTH infusion*	*Parental origin*	GNAS *defect*
PHP1a	Yes	Yes	Yes	Reduced	Blunted urinary cAMP and phosphate excretion	Maternal	Inactivating mutations
PHP1c	Yes	Yes	Yes	Normal	Blunted urinary cAMP and phosphate excretion	Maternal	Inactivating mutations
PPHP	Yes	No	No	Reduced	Normal response	Paternal	Inactivating mutations
POH	No	No	No	Normal or reduced	Normal response	Paternal	Inactivating mutations
PHP1b	No	Yes	No	Normal	Blunted urinary cAMP and phosphate excretion	Maternal	Methylation defects
PHP2	No	Yes	No	Normal	Normal cAMP Blunted phosphate excretion	NA	None

PHP, pseudohypoparathyroidism; *PPHP*, pseudopseudohypoparathyroidism; *POH*, progressive osseous heteroplasia; *AHO*, Albright's hereditary osteodystrophy; *PTH*, parathyroid hormone; *Gsα*, G protein stimulating α-subunit; *cAMP*, cyclic adenosine monophosphate.

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The hormone binds to a seven-transmembrane G-protein-linked receptor that is usually referred as the PTH/PTHrP receptor, because it also binds PTH-related peptide (PTHrP). This receptor is coupled to a Gs signaling pathway that leads to increased intracellular cAMP production and phospholipase C, which mediate the systemic actions of PTH on its target tissues (Juppner *et al.*, 1991; Gensure *et al.*, 2005).

The main targets of PTH action are the kidney and bone. In the kidney, PTH rapidly increases fractional reabsorption of calcium in the distal renal tubules. The renal effects of PTH are reinforced by low calcium levels, as sensed by the CaSR. This receptor is also present within the renal tubule, where it increases calcium reabsorption when serum calcium levels are reduced.

Additionally, PTH and low calcium concentrations directly stimulate 1α-hydroxylase expression in the proximal renal tubules and increase the production of 1,25-dihydroxyvitamin D ($1.25(OH)_2D$). The latter, acting in the small intestine, stimulates calcium absorption.

PTH is also an important regulator of phosphate metabolism. It acts on the proximal renal tubule and inhibits the activity of the sodium-dependent phosphate transporter (NPT-2), thereby increasing phosphate excretion (Lee and Partridge, 2009).

At the bone, PTH stimulates osteoblasts to secrete receptor activator of nuclear factor kappa-B ligand, which increases osteoclast activity, leading to increased bone resorption and the release of calcium and phosphate into the blood (Datta and Abou-Samra, 2009).

Thus, calcium homeostasis is regulated in a coordinated way by PTH, $1.25(OH)_2D$, and calcium itself in order to ensure the maintenance of its levels within the physiologic range.

Pathophysiology of PTH Resistance

The hallmark of all forms of PHP is the defect in renal response to PTH action. Specifically, PTH resistance appears to occur only in the proximal renal tubule, whereas the thick ascending tubule remains unaffected (Stone *et al.*, 1993). Therefore, the renal phosphaturic effect of PTH is reduced, leading to hyperphosphatemia, and the production of $1.25(OH)_2D$ is also decreased, contributing to hypocalcemia (Chase *et al.*, 1969).

Since the anticalciuric action of PTH is exerted in the distal tubule, this effect appears to remain intact in all PHP patients. This explains the hypocalciuria, the preservation of renal function and the absence of renal stones that are seen in PHP patients. The cell-specific defect in PHP is consistent with the cell-specific imprinting of *GNAS*. In accordance with this, the response to vasopressin that also acts in the distal tubule appears to be preserved in patients with PHP (Moses *et al.*, 1986).

The bone remodeling response to PTH appears to be intact in patients with PHP (Ish-Shalom *et al.*, 1996). However, the clinical effects on bone density are variable ranging from reduced bone mineral density to osteitis fibrosa cystica and to osteosclerosis (Burnstein *et al.*, 1985; Jacobson, 1985; Balkissoon and Hayes, 1999; Sbrocchi *et al.*, 2011). Whether this variability is due to differences in the skeletal response to PTH or to differences in the circulating levels of PTH and/or $1.25(OH)_2D$, that may have a predominant resorptive or anabolic effect in PHP, remains unknown (Long *et al.*, 2010).

In addition to changes in the regulation of calcium and phosphate homeostasis, PTH resistance appears to cause also perturbations in uric acid excretion. In particular, hypouricemia due to inappropriate increase in urinary excretion of uric acid has been reported in a Greek family with PHP1b (Laspa *et al.*, 2004). Uric acid is secreted and reabsorbed along the proximal renal tubules by the action of a urate-anion exchanger, which is expressed in tubular epithelial cells and involved in the regulation of uric acid excretion. It appears, therefore, that PTH is likely involved in the renal handling of uric acid excretion and this effect is disturbed in PHP patients.

Molecular and Clinical Characteristics of PHP States

Molecular Structure and Expression of *GNAS*

The *GNAS*, located on the long arm of chromosome 20, is a complex transcriptional locus that, in addition to Gsα, gives rise to multiple gene products through the use of alternative promoters and alternative splicing of downstream exons (Blatt *et al.*, 1988; Turan and Bastepe, 2013).

Gsα is encoded by exons 1–13 (Kozasa *et al.*, 1988). Exons 2–13 can also be used with three other alternative first exons, located upstream of exon 1, in order to produce additional novel transcripts. These include extra-large Gsα (XLas), which are expressed only from the paternal allele, the neuroendocrine secretory protein 55 (NESP55) and the noncoding A/B (Swaroop *et al.*, 1991; Kehlenbach *et al.*, 1994; Ischia *et al.*, 1997; Ishikawa *et al.*, 1990). There are also shortened neural transcripts of Gsα and XLas, termed GsαN1 and XLN1, respectively, which terminate prematurely before exon 4 (Crawford *et al.*, 1993; Pasolli *et al.*, 2000). In addition to these transcripts the *GNAS* locus also encodes a noncoding transcript in the opposite direction, termed *GNAS* antisense transcript (GNAS-AS1), which consists of five distinct exons and is involved in the imprinting of the *GNAS* locus (Hayward and Bonthron, 2000; Wroe *et al.*, 2000) (Fig. 1).

Genomic imprinting results in the expression of a gene according to its parental origin. Genes subjected to parental imprinting contain a number of differentially methylated regions (DMRs). Is seems that the methylated promoter is associated with non-expressed transcripts, whereas the non-methylated promoter drives the expression (Ideraabdullah *et al.*, 2008; Barlow and Bartolomei, 2014).

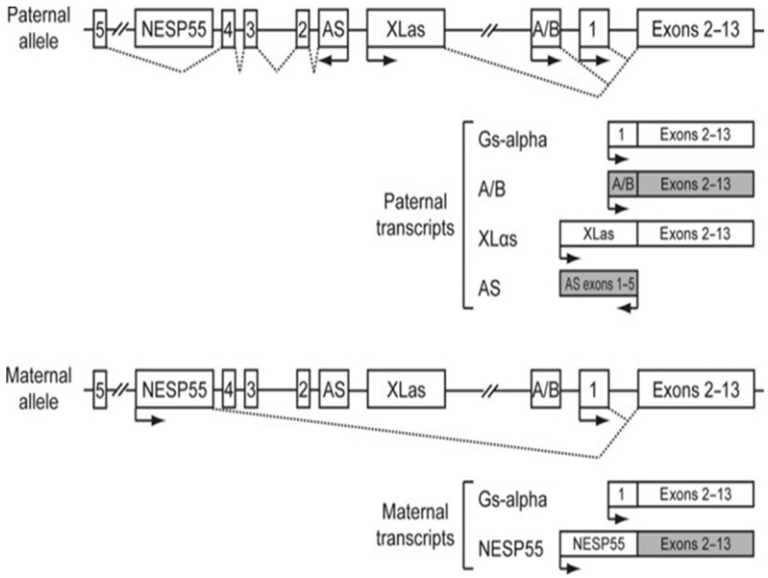


Fig. 1 *GNAS* genomic structure and encoded transcripts. Gsα is encoded by exons 1–13. Other transcripts produced by using alternative first exons that splice on to exons 2–13 are A/B, XLαs, and NESP55. An AS noncoding transcript is also produced in the opposite direction using distinct exons. Gsα is transcribed from both the paternal and maternal allele, except in selected tissues (proximal renal tubules, thyroid, gonads, and pituitary), in which expression occurs only from the maternal allele. A/B, XLαs, and AS transcripts are paternally expressed, and NESP55 transcripts are maternally expressed. Exons are represented by boxes. Arrows indicate the direction of transcription of the different paternal and maternal transcripts. Dashed lines join exons that are spliced to produce the different transcripts. Shaded boxes represent noncoding transcripts. Reproduced from Lemos, M. C., Thakker, R. V. (2015). GNAS mutations in pseudohypo-parathyroidism type 1a and related disorders. *Human Mutation* **36**, 11–19.

The *GNAS* is an imprinted locus and contains a number of DMRs that enclose the promoters of the different transcripts. Due to differential methylation of their promoter, most transcripts originate from one parental allele only. The XLαs, A/B *και GNAS-AS1* promoters are methylated on the maternal allele and are exclusively paternally expressed. Conversely, the NESP55 transcript that is methylated on the paternal allele shows exclusively maternal expression (Plagge *et al.*, 2008; Hayward *et al.*, 1998a,b).

Gsα promoter lacks methylation and is biallelically expressed in most cells. However, in some cells (pituitary somatotrophs, proximal renal tubular cells, thyroid epithelial cells, gonadal cells, neonatal brown adipose cells) paternal Gsα expression is silenced. The mechanisms of this silencing effect are still undetermined. Cis-acting elements that control tissue-specific paternal imprinting of Gsα appear to be located within the exon A/B which is imprinted (Yu *et al.*, 1998; Williamson *et al.*, 2004; Mantovani *et al.*, 2002; Germain-Lee *et al.*, 2002; Liu *et al.*, 2003; Chen *et al.*, 2009; Hayward *et al.*, 2001).

In humans, two imprinting control regions (ICRs) of *GNAS* have been identified within or close to the *GNAS* locus. One is located within the syntaxin 16 gene (*STX16*) that controls the imprinting of the A/B DMR only (Bastepe *et al.*, 2003). The other, encompassing exons 3 and 4 of the *GNAS-AS1*, controls the imprinting over the entire *GNAS* locus (Bastepe *et al.*, 2005; Richard *et al.*, 2012; Chillambhi *et al.*, 2008).

Genetic Disorders Resulting from *GNAS* Mutations

Pseudohypoparathyroidism type 1α (PHP1a)

PHP1a is an autosomal dominant disease caused by heterozygous inactivating mutations on the maternal allele of *GNAS* that involve exons 1–13 encoding Gsα (Patten *et al.*, 1990; Weinstein, 1998) (for a list of mutations, please see OMIM entry 139320 at http://www.ncbi.nlm.nih.gov). Homozygous mutations that disrupt completely Gsα activity are not compatible with life (Yu *et al.*, 1998, 2000).

Patients with PHP1a have renal PTH resistance, manifested as hypocalcemia, hyperphosphatemia, elevated serum PTH levels and a blunted cAMP and phosphate excretion following administration of PTH, together with the features of AHO (Chase *et al.*, 1969). The same individuals may also have resistance to other hormones which act via G-protein-coupled receptors and whose target tissues show predominant expression of Gsα from maternal allele, such as thyrotropin stimulating hormone (TSH), gonadotrophins and growth hormone releasing hormone (GHRH). Primary hypothyroidism without goiter, hypogonadism and growth hormone deficiency are commonly associated endocrinopathies (Weinstein *et al.*, 2001; Mantovani *et al.*, 2002; Germain-Lee *et al.*, 2002; Liu *et al.*, 2003). By contrast, responsiveness to other hormones (eg, adrenocorticotropic hormone and vasopressin) is normal in tissues in which both parental alleles are expressed (Moses *et al.*, 1986). Clinical features depend on the mono- or biallelic transcription of Gsα in tissues.

Individuals with PHP1a demonstrate a 50% reduction in Gsα activity in erythrocyte membranes, although this Gsα deficiency appears to have no adverse effect on red cell function (Levine *et al.*, 1983a,b). The 50% decrease in Gsα activity in patients with inactivating mutations in *GNAS* is utilized as a diagnostic test for making the distinction of the PHP subtypes (Turan *et al.*, 2015; Brix *et al.*, 2014).

The reported *GNAS* mutations are scattered in the entire coding region, with one hot spot in exon 7 accounting for 17.8% of cases (Lemos and Thakker, 2015). In general, there is no correlation between type or location of the Gsα mutation and onset of the disease or severity of the endocrine resistance in patients with inactivating *GNAS* mutations. However, a temperature sensitive Gsα mutant (A366S) that causes testotoxicosis due to constitutive activity at the lower temperature of the testes, but instability at body temperature, has been described in two boys (Nakamoto *et al.*, 1996).

PTH resistance usually develops during the first year of life, with hyperphosphatemia and elevated PTH preceding hypocalcemia. The severity of PTH resistance varies significantly between patients, even within the same family, and some of them remain normocalcemic throughout life (Gelfand *et al.*, 2006; Maupetit-Méhouas *et al.*, 2008). In addition, PHP patients are not prone to hypercalciuria, unlike patients with primary hypoparathyroidism, and maintain normal renal function. The delayed onset of PTH resistance may be explained by recent studies in knocked out mice, which demonstrated that the manifestation of PTH resistance caused by the maternal loss of Gsα occurs after early postnatal life, due to gradual development of paternal Gsα silencing in renal proximal tubules (Germain-Lee *et al.*, 2005; Turan *et al.*, 2014). However, nutritional factors and the involvement of Gsα-independent signaling pathways, may also contribute to the latency of PTH resistance, and this awaits further elucidation.

The associated TSH resistance also becomes clinically manifest over childhood and adolescence, but may occasionally present as hypothyroidism at neonatal screening. Hypogonadism, particularly in females, usually manifests as delayed or incomplete sexual maturation, amenorrhea or oligomenorrhea, and/or infertility. Growth hormone deficiency due to resistance to GHRH has also been reported, and appears to be more frequent in adults than in children.

Pseudohypoparathyroidism type 1c (PHP1c)

PHP1c individuals develop the same clinical and laboratory abnormalities as patients with PHP1a, including AHO and peptide hormone resistance. However, in contrast to PHP1a, *in vitro* assessment of Gsα activity reveals no abnormality (Mantovani and Spada, 2006; Weinstein, 1998; Linglart *et al.*, 2002). In a subset of designated PHP1c patients, there have been documented inactivating mutations on the maternal allele of the *GNAS* gene. These mutations are located in the α5-helix concerning the extreme carboxyl-terminus of Gsα, whereas usually PHP1a-associated mutations are distributed throughout the gene. This region contains the major sites for interaction between Gsα and G-protein-coupled receptors. It appears that mutations located at the carboxy-terminal end of Gsα (encoded by exon 13) can disrupt or strongly impair the ability for receptor-coupling, yet preserve cAMP generation through the activation of the adenyl cyclase (Thiele *et al.*, 2011).

Up to date there have been reports about two PHP1c patients in whom the disease is caused by epigenetic changes involving the *GNAS* locus. However, several patients with the diagnosis of PHP1c neither show epigenetic nor molecular genetic changes in *GNAS*, demonstrating that PHP1c may be caused by a variety of pathogenetic mechanism (de Nanclares *et al.*, 2007).

Pseudopseudohypoparathyroidism (PPHP)

Patients with PPHP exhibit the somatic features of AHO in the absence of PTH resistance. Both PHP1a and PPHP results from the same inactivating mutations of the *GNAS* and coexist in the same kindred, but never in the same sibship. The development of one or the other type of the disorder depends on the gender of the parent transmitting the molecular defect. Thus, paternal transmission leads to PPHP, whereas maternal transmission of the same mutation causes PHP1a (Wilson *et al.*, 1994; Davies and Hughes, 1993).

This parent-of-origin mode of inheritance for hormone resistance could be explained by the tissue-specific paternal imprinting of Gsα. In some, but not all tissues, the paternal allele is silenced and Gsα expression is predominantly maternal. This has been documented for the renal proximal tubule, thyroid gland, gonads and pituitary. On the other hand, expression of Gsα in the adrenal gland is biallelic. In the case of a maternal mutation, a significant loss of Gsα activity exists in those tissues in which paternal Gsα is silenced, thus leading to hormone resistance. When inherited paternally, the same mutation, does not severely affect the Gsα activity in tissues where the allele is already silenced.

Heterozygous inactivating mutations of Gsα lead to 50% loss of protein activity in non imprinted tissues where Gsα expression is biallelic, such as skin, fibroblasts, erythrocytes, white adipose tissue, bone and growth plate chrondrocytes (Yu *et al.*, 1998; Linglart, 2007; Long *et al.*, 2007; Wang *et al.*, 1992; Mantovani *et al.*, 2004; Mantovani *et al.*, 2003). Clinical findings related to those tissues are due presumably to Gsα haploinsufficiency and are present independently of the parental origin of the mutation. Thus, it is thought that AHO features result primarily from Gsα haploinsufficiency in tissues where Gsα expression is biallelic. Growth plate chondrocytes, that lack either the paternal or the maternal Gsα allele, differentiate into hypertrophic chondrocytes earlier than wild-type chondrocytes in a chimeric mouse model (Bastepe *et al.*, 2004). However, obesity and cognitive impairment occur predominantly in patients with PHP1a rather than PPHP, indicating involvement of Gsα imprinting in these AHO features (Mouallem *et al.*, 2008; Long *et al.*, 2007).

A recent study has provided evidence of hormone resistance in a PPHP patient with a novel paternal mutation in *GNAS*. This finding suggest that PTH and other hormone resistance may not be an exclusive feature of PHP1a and could also be observed in patients with PPHP (Turan *et al.*, 2014).

Progressive osseous heteroplasia (POH)

POH is a rare manifestation of AHO, characterized by severe heterotopic ossifications, which is progressive and affects skeletal muscle and deep connective tissues (tendons, ligaments, fascia), leading to ankylosis of affected joints and growth retardation of affected limbs later in life (Sanchez *et al.*, 2011).

Patients with POH have germline *GNAS* mutations, in the absence of hormone resistance and features of AHO. In the majority of cases, paternally inherited mutations have been detected, suggesting that the deficiency of a paternally expressed *GNAS* product in the development of POH (Shore *et al.*, 2002; Ahmed *et al.*, 2002; Adegbite *et al.*, 2008; Lebrun *et al.*, 2010).

Recent studies suggest that POH frequently follows dermomyotomes and shows bias of lesions towards one side or the other, similar to the lesions observed in McCune-Albright syndrome, which is due to mosaic mutations of *GNAS* that cause constitutive GSα activity. These observations suggest that the underlying cause of POH may be second-hit postzygotic mutations affecting tissues in patients with inherited heterozygous inactivating Gsα mutations (Cairns *et al.*, 2013; Pignolo *et al.*, 2015). In this context, it was recently shown that Gsα ablation causes heterotopic ossification through activation of Hedghoc signaling (Regard *et al.*, 2013). Further studies in human samples are awaited in order to better understand the mechanisms of the disease.

Hormone Resistance due to Epigenetic Alterations of *GNAS*

Pseudohypoparathyroidism type 1b (PHP1b)

Individuals with PHP1b lack typical features of AHO, and hormone resistance is confined to the actions of PTH in renal proximal tubules. Recent studies, however, have shown that some AHO features, as mild brachydactyly, may also be present in such patients. In addition, some PHP1b patients may also have mildly elevated TSH levels, indicating TSH resistance, whereas GH secretion appears to be conserved (Liu *et al.*, 2003; Mantovani *et al.*, 2007).

Most PHP1b cases are sporadic (80–85%) but some cases are familiar and show an autosomal dominant mode of inheritance (AD-PHP1b). PTH resistance in PHP1b is inherited only from female obligate carriers in a manner similar to that of type PHP1a (Linglart *et al.*, 2007; Juppner *et al.*, 1998; Bastepe *et al.*, 2001).

PTH infusion in patients with this syndrome results in deficient stimulation of nephrogenous cAMP or urinary phosphate excretion, whereas Gsα bioactivity in erythrocyte membranes is normal (Levine *et al.*, 1983a,b). Furthermore, *GNAS* mutations are not found in PHP1b. However, the AD-PHP1b gene maps to a telomeric region on chromosome 20q containing the *GNAS* locus (Juppner *et al.*, 1998; Levine *et al.*, 1983a,b). Subsequently, *GNAS* imprinting defects have been identified in patients with familiar and sporadic forms of PHP1b resulting in the absence of expression of the maternal Gsα. As the paternal allele is normally silenced in proximal renal tubules, the maternal allele silencing results in marked reduction of Gsα levels leading to PTH resistance.

The most consistent finding in these patients is the loss of methylation at the exon A/B DMR in the maternal allele, leading to silencing of the downstream maternal Gsα promoter (Izzi *et al.*, 2012).

In familiar cases of PHP1b, microdeletions of the *STX16* have been identified and are associated with loss of methylation at the A/B DMR. These include a recurrent 3 kb (Bastepe *et al.*, 2003) and a 4.4 kb (Linglart et al, 2005) and more recently a 24.6 kb deletion all within the *STX16* (Elli *et al.*, 2014). It seems that *STX16* harbor a cis-acting control element crucial for the establishment of the methylation imprint at exon A/B. Recently, deletions within the *NESP55* and/or the *GNAS-AS1* regions have been identified in some AD-PHP1b kindreds, in whom affected individuals show loss of methylation of all the maternal *GNAS* imprints, in addition to the A/B DMR (Bastepe *et al.*, 2005; Chillambhi *et al.*, 2010; Richard *et al.*, 2012).

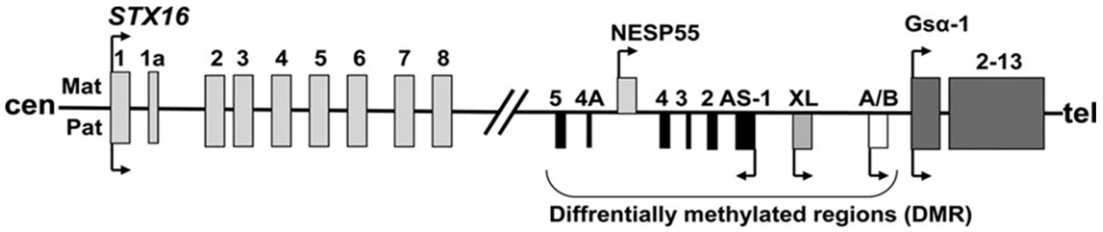
Sporadic forms do not present deletions within the *GNAS* or *STX16* loci but show broad loss of imprinting at the *GNAS* locus including loss of methylation of the A/B DMR and loss of imprinting affecting at least one additional DMR (gain of methylation at NESP55, loss of methylation at GNAS-AS1 and/or XLas) (Izzi *et al.*, 2012). A few cases have been shown to be due to paternal uniparental disomy involving whole or part of chromosome 20 encompassing the *GNAS* locus (Dixit *et al.*, 2013; Bastepe *et al.*, 2011; Fernandez-Rebollo *et al.*, 2010). In this situation, two normal copies of *GNAS a*re both inherited from the father causing absence of maternal Gsα expression, hence PHP1b. However, the genetic causes of most cases of sporadic PHP1b remain unknown (Fig. 2).

In conclusion, there are no differences between the phenotypes of patients affected with familiar or sporadic forms of PHP1b. Therefore, diagnosis of PHP1b relies on the identification of loss of methylation at the A/B DMR of *GNAS*, the characterization of the methylation pattern of the entire *GNAS* locus, and the existence of deletions within the *STX16* or *GNAS* or paternal uniparental disomy (Linglart *et al.*, 2013).

Recently imprinting defects in *GNAS* were also reported in a large proportion of patients with PHP1a phenotype in which no mutations were found in Gsα coding exons, suggesting that the clinical and genetic features of PHP1a and PHP1b may overlap (Elli *et al.*, 2014; Fernandez-Rebollo *et al.*, 2013; Mantovani *et al.*, 2010a).

Diagnostic Aspects and Therapy

The presence of PTH resistance (hypocalcemia, hyperphosphatemia, and elevated PTH), in association with the features of AHO, are the basis for the clinical diagnosis of PHP1a. The demonstration of a blunted response of cAMP and phosphate excretion after PTH administration may contribute to diagnosis, although it is rarely necessary in clinical practice. Patients with PHP1a should



		NESP55	AS	XL	A/B
Normal	Mat	-	+	+	+
	Pat	+	-	-	-
pat UPD Chr. 20	Pat	+	-	-	-
	Pat	+	-	-	-
STX16 Del (3 & 4.4 kb)	Mat	-	+	+	-
	Pat	+	-	-	-
GNAS NESP55 Del (18.9 kb)	Mat		+	+	-
	Pat	+	-	-	-
GNAS NESP55/AS Del (4.0/4.7 kb)	Mat		-	-	-
	Pat	+	-	-	-
GNAS AS Del (4.2 kb)	Mat	-/+	-	-	-
	Pat	+	-	-	-
Sporadic PHP-Ib	Mat	-/+	-/+	-/+	-
	Pat	+	-	-	-

Fig. 2 Genetic and epigenetic defects causing PHP1b: Maternal *STX16* deletions cause isolated *A/B* loss of methylation, maternal deletion of *NESP55* leads to isolated *A/B* loss of methylation with hemizygosity in NESP55. However, maternal deletions affecting *AS* exons 3 and 4 result in a loss of methylation at all maternal *GNAS* imprints. Sporadic PHP-Ib cases show loss of methylation at exon *XL*, the promoter of *AS*, and exon *A/B*, and a gain of methylation at *NESP55*. Note that the methylation changes at the NESP55, AS promoter, and XL in sporadic PHP-Ib cases are often, but not always, partial. Please note that the figure is not drawn to scale. Reproduced with permission by Turan, S., Bastepe, M. (2013). The GNAS complex locus and human diseases associated with loss-of-function mutations or epimutations within this imprinted gene. *Hormone Research in Paediatrics* **80**, 229–241 (http://dx.doi.org/10.1159/000355384), S. Karger AG, Basel.

also be screened for any associated endocrinopathy, in particular hypothyroidism and hypogonadism. Patients with PPHP exhibit most of the somatic features of AHO, in the absence of PTH resistance, and their diagnosis may be facilitated in the context of a family history of PHP1a (Mantovani, 2011).

In general, PHP1a patients should be monitored annually with blood testing (PTH, calcium, phosphate, TSH) and urinary calcium excretion. Particular attention should be given to height, growth velocity and pubertal development in children as well as screening with provocative tests for GH deficiency. Careful physical examination should be performed annually to detect the presence and follow up the evolution of specific AHO features, including radiological evaluation of brachydactyly.

Several tests have been developed to assess Gsα *in vitro*, but are not used for routine analysis. These include semi-quantitative immunoblot analysis as well as functional assays of Gsα. The application of these assays to patients with PHP1 showed that patients with AHO and multihormone resistance had about 50% reduction in Gsα activity (PHP1a), whereas patients without clinical features of AHO and hormone resistance that was limited to PTH (PHP1b) had normal levels of Gsα activity. On the other hand, patients with PPHP or POH, who carry *GNAS* mutations on their paternal allele were also found to have a similar 50% reduction in Gsα activity (Levine, 2012).

These findings reflect the effect of *GNAS* haploinsufficiency in erythrocytes, which normally express both copies of *GNAS*, leading to a 50% reduction in Gsα activity regardless of the parental origin of the *GNAS* defect. The normal Gsα activity in PHP1b indicates the absence of paternal imprinting of Gsα in erythrocytes, so both alleles are expressed regardless of an epigenetic methylation defect.

However, the recent description of PHP1b patients with *GNAS* methylation defects who have mild features of AHO and partial resistance to TSH supports the notion that PHP1a and PHP1b may share overlapping phenotypes. Indeed, a recent study found that Gsa activity was partially reduced in patients with mild AHO but normal in those without these features. Taken together, these findings indicate that features of AHO are manifestations of Gsα deficiency in relevant tissues and argue against the association of AHO with a specific *GNAS* genotype. Thus, a threshold level of Gsa activity is necessary to assure a normal phenotype (Mantovani, 2011; Lemos and Thakker, 2015).

Genetic testing, through DNA sequencing of the *GNAS* will identify a mutation in most cases of PHP1a, PPHP, and POH, and rarely in PHP1c. However, it cannot distinguish between these disorders or determine the paternal origin of the mutation.

In patients with PHP1b, epigenetic defects can be identified by methylation analysis of DMRs in the *GNAS* locus. Familiar and sporadic forms of PHP1b have different patterns of methylation of the three DMRs, but loss of methylation at the maternal exon A/B DMR is a consistent finding in all patients (Turan and Bastepe, 2015).

Treatment of PTH resistance is similar to that of hypoparathyroidism and consists of administration of active vitamin D metabolites (eg, calcitriol) and oral calcium supplements to maintain normocalcemia. Additional endocrine disorders, in patients with PHP1a or PHP1c, in particular hypothyroidism and hypogonadism, should be screened for and corrected if necessary. There are no specific treatments for the various AHO features (Mantovani, 2011).

Regarding GH deficiency in patients with PHP1a, to date there is no conclusive evidence indicating that GH replacement therapy is beneficial. The observation that individuals with PPHP have short stature as their relatives with PHP1a, without having any endocrine abnormalities appears to challenge the primary role of GH deficiency in determining growth in PHP1a. However, a pilot study in a group of prepubertal short PHP1a children, treated with GH, reported a significant increase in height velocity, providing the first evidence that treatment of GH deficiency, if started early, would be a potentially effective therapy (Mantovani *et al.*, 2010b). Further studies are needed to clarify this matter.

Conclusions and Future Perspectives

Recent advances in the molecular genetic characterization of patients with PTH resistance have revealed novel pathogenic mechanisms that explain the phenotypes of the various forms of PHP disorders and provided insights into the understanding of *GNAS* function and epigenetic regulation. These advances have also led to the recognition of the complexity of the *GNAS* locus and the understanding that the various forms of PHP share overlapping characteristics.

However, further work in this interesting field remains to be done. This must address the allelic imbalance in *GNAS* expression, and in particularly, clarify the mechanism of tissue-specific expression of maternal or paternal *GNAS* alleles. The genetic modifiers that account for the development of POH in some patients and PPHP in others require further investigation. Research is also needed in order to clarify the genetic basis for the imprinting defects in patients with PHP1b.

Finally, the possible involvement of the imprinted *GNAS* locus in the development of obesity and the regulation of energy balance opens up new research avenues for the investigation of the role of imprinted genes in energy metabolism.

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Thyroid Gland: Anatomy and Physiology☆

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Glossary

Follicle A hollow sphere lined by a single layer of epithelial cells called thyrocytes, and filled with colloid.

Iodotyrosines Precursors of thyroid hormones resulting from iodination of tyrosyl residues of thyroglobulin. They include monoiodotyrosine and diiodotyrosine.

Iodothyronine Thyroid hormones resulting from coupling of two iodotyrosines.

Thyrocyte (follicular cell) The cell type that takes up iodide from the bloodstream and incorporates it in the thyroglobulin, the backbone of the colloid, thus producing iodotyrosines and iodothyronines.

Thyronamines Compounds with a chemical structure similar to thyroid hormones, in which the carboxylate group is replaced with the alanine side chain. Compared with thyroid hormones, their biological actions are either in the same or in the opposite direction.

Embryology

It is beyond the scope of this article to provide a detailed review of the embryological development of the hypothalamic thyrotropin-releasing hormone (TRH)-secreting neurons and the pituitary thyrotrophs. Concerning the hypothalamus, suffice it to say that, similarly to the releasing factors for other pituitary hormones, TRH is synthesized in parvocellular, not magnocellular, neurons of the paraventricular nucleus. On a historical note, the identification and characterization of TRH in 1970 and other releasing hormones by Roger Guillemin and Andrew Schally permitted these two scientists to share the Nobel Prize in Medicine 7 years later.

Concerning pituitary organogenesis, suffice it to say that it is dictated by the orderly expression of cell-specific transcription factors, including Titf1/Nkx2.1, Rpx/Hesx-1, Pax-6, Sox-3, Lhx-3, Prop-1, Pit-1, and TEF. Some of these genes are involved in the formation of other specific populations in the adenohypophysis. Accordingly, depending on the mutated gene, congenital secondary hypothyroidism may or may not be accompanied by other pituitary hormone deficiencies. Adenohypophysis anlage is recognizable at 4–5 weeks of gestation, but the hypothalamic-pituitary unit becomes mature only by 20 weeks. Within the anterior pituitary, the thyrotrophs are placed anteromedially and anterolaterally, and account for less than 10% of all the cell types (Aaron *et al.*, 2007).

The thyroid gland is the first endocrine gland to develop in humans. The thyroid gland originates from a diverticulum located in the median ventral wall of the pharynx (called the thyroid diverticulum). During the fourth week of embryonal development, an endodermal thickening (thyroid placode) appears in the midline floor of the primitive pharynx between the first and second pharyngeal pouches, dorsal to the aortic sac (Fancy *et al.*, 2010). This primitive thyroid tissue is hollow at first, but soon becomes solid (thyroid bud) and penetrates the underlying mesenchymal tissue, descending anteriorly through the thyroglossal duct to the hyoid bone and laryngeal cartilages in order to reach the lower neck. As the thyroid tissue migrates downward, it passes just anteriorly to the hyoid bone and laryngeal cartilages. The thyroid gland is initially spherical and then assumes a more bi-lobed configuration as it enlarges; a major increase regards its lateral portions (lobes) in comparison to the median connecting portion (isthmus) (Fancy *et al.*, 2010). Whereas in mice thyroid organogenesis takes about 1 week, in humans it takes a much longer time, and thyroid hormones synthesis is not evident before the 11th week of gestation (Szinnai *et al.*, 2007).

Whereas the epithelial cells, the most abundant cell type in thyroid, derive from endostyle, an endodermal area containing iodine-concentrating cells, C-cell precursors derive from the neural crest bilaterally to the fourth pharyngeal pouches and are located in the ultimobranchial bodies. Epithelial cells' differentiation is assumed to be the consequence of signals from the heart primordium, which is close to the ventral pharyngeal endoderm during the early embryogenesis. This hypothesis is supported by the frequent association of congenital cardiac malformations with congenital hypothyroidism. In addition, the close association of the thyroid and heart partly accounts for thyroid migration, which ends at day 45–50 (Santisteban, 2013). Differentiated follicular cells (thyrocytes) are polarized cells with a basolateral and an apical surface; the first faces the extrafollicular space, while the second faces the follicular lumen. This polarity is functionally paramount, as iodine uptake occurs at the basolateral side, whereas thyroid hormone secretion occurs at the apical side (Nilsson and Fagman, 2017).

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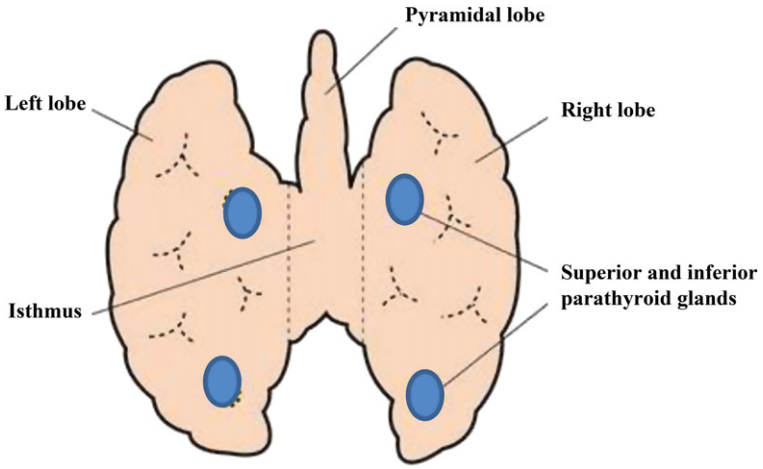


Fig. 1 Macroscopic posterior view of the thyroid.

During embryogenesis, thyroid development depends on the expression of a number of transcription factors, the most important being TTF-1 (thyroid transcription factor-1), PAX8 (paired box gene 8), FOXE-1 (forkhead box E1), and HHEX (hematopietically expressed homeobox). TTF-1 (also called NKX2–1) is a single polypeptide in humans and regulates the transcription of thyroglobulin, thyroid peroxidase (TPO), and thyrotropin (TSH) receptor genes in the follicular cells (Kratzsch and Pulzer, 2008). Moreover, TTF-1 promotes the expression of HHEX, FOXE1, and (weakly) PAX8. In turn, HHEX, PAX8, and FOXE1 regulate each other (Nilsson and Fagman, 2017). PAX8 plays a fundamental role in cell differentiation, in maintenance of the differentiated state, and in proliferation. FOXE-1 is essential in promoting migration of the follicular cells and seems to be involved in their survival and/or differentiation. HHEX is an early marker of thyroid cells with a putative effect in maintaining the expression of TTF-1 and PAX8 during thyroid organogenesis (Kratzsch and Pulzer, 2008). Hence, deletion of any one of the genes encoding HHEX, TTF-1, PAX8, or FOXE1 inevitably confers athyreosis or severe thyroid hypoplasia (Nilsson and Fagman, 2017). Currently available data indicate that gene expression undergoes significant changes during thyroid organogenesis and confirm the existence of unknown factors at least as critical as TTF-1, PAX8, FOXE-1, and HHEX (Kratzsch and Pulzer, 2008).

Anatomy

The thyroid gland is a highly vascularized organ located anteriorly in the neck between the C_5 and T_1 vertebrae, deep in the platysma, sternothyroid, and sternohyoid muscles. The thyroid weighs 15–20 g and weighs more in men than in women; the thyroid weighs approximately 1 g in a newborn and increases by about 1 g/year until age 15. It is an H-shaped, soft and reddish parenchymal organ, consisting of two lobes (left and right) and one isthmus that binds them together (**Fig. 1**). Each lobe is approximately 4 cm in length, 2 cm in width, and 2–3 cm in thickness. The isthmus measures about 2 cm in width, 2 cm in height, and 2–6 mm in thickness.

The superior extremity (called the superior horn) lies lateral to the inferior constrictor muscle and posterior to the sternothyroid muscle, while the inferior part (inferior horn) extends to the levels of the fifth or sixth tracheal ring. In the posterolateral section, the gland overlaps the carotid sheath and its components. About 50% of individuals present a pyramidal lobe (Morgagni's or Lalouette's pyramid), arising from either lobe or the superior portion of the isthmus and directed upward, usually to the left (**Fig. 1**) (Braun *et al.*, 2007).

The thyroid is enveloped by the layers of the deep cervical fascia and covered by the strap muscles anteriorly and the sternocleidomastoid muscle more laterally. The true thyroid capsule is firmly adherent to the gland, developing projections into the thyroid, forming septae and dividing it into lobes and lobules. The posterior layer of the thyroid capsule is thick. Posteriorly, the middle layer of the deep cervical fascia condenses to form the posterior suspensory ligament of Berry, connecting the thyroid lobes to the cricoid cartilage and the first two tracheal rings. In the posterior surface of the lateral lobes are located the parathyroid glands; normally there are four (two superior and two inferior), and these are roundish, and about the size of a grain of rice (**Fig. 1**).

Histology

Microscopically, thyroid is divided into lobules; each lobule consists of 20–40 round follicles that vary considerably in size, with a diameter ranging from 45 to 250 μm. In the newborn, follicles are small and grow slowly (**Fig. 2**).

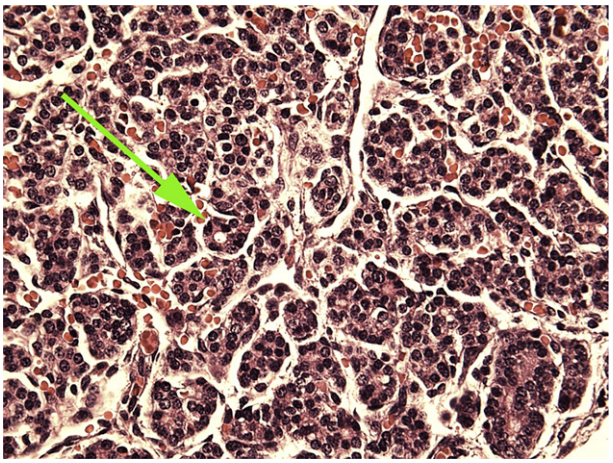


Fig. 2 Thyroid follicle of a newborn (*green arrow*), greatly different in size from that observed in adult ones (see **Fig. 3**).

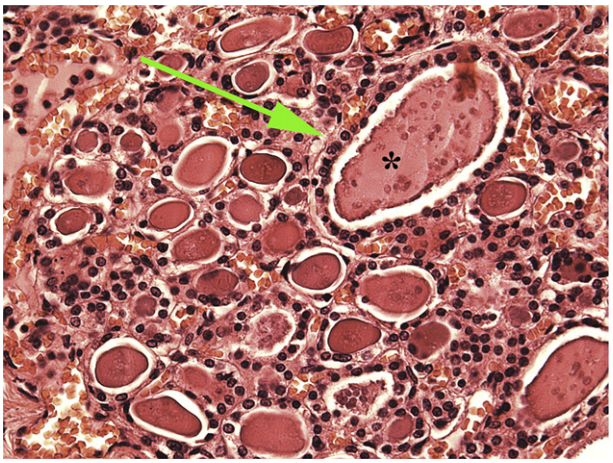


Fig. 3 Evident thyroid follicles in the adult (*green arrow*) lined by a single epithelium filled with colloid (*).

Each follicle is lined by a single cuboidal layer of epithelium (9–13 μm) with a thin basement membrane filled with acidophilic colloid-core. Thyrocytes have a definite polarity, with their apices directed toward the lumen of the follicles and their basis toward the basement membrane. The apical surface of the epithelial cells has numerous microvilli extending to the colloid, while the spheroid nuclei are located at the same level in all cells, mainly near to their basis (**Fig. 3**). Thyroid is the only human gland in which the hormonal product is stored extracellularly (viz. in the colloid).

Mitoses are infrequent, being evident only in young people. Thyrocytes are characterized by a pale acidophilic or amphophilic cytoplasm in which lysosomal bodies, granules, and secretory vacuoles are evident. Immunohistochemistry shows that normal follicular epithelium contains thyroglobulin, low-molecular weight keratin, epithelial membrane antigen, and vimentin. Follicles are embedded in a small amount of a loose connective tissue that forms the gland stroma, in which blood vessels, nerves, and lymphatics are present.

C-cells are dispersed between follicles, mainly in the posterolateral portion of the lobes, or are located beyond the basement membrane within the follicles, close to thyrocytes (Nilsson and Fagman, 2017). As noted above, C-cell precursors derive from the neural crest. They constitute about 0.1% of thyroid cells, and their identification is possible only using immunohistochemical methods for calcitonin. Moreover, the numerous dense-core granules of C-cells show immunoreactivity for neuron-specific enolase (NSE), chromogranins A and B, synaptophysin, and carcinoembryonic antigen (CEA). The stromal compartment surrounding follicles consists of fibroblasts derived from the neural crest (Kameda *et al.*, 2009), and includes also macrophages and mast cells, which recently were reported to have a role in thyroid cancer development (Visciano *et al.*, 2015).

Vascular supply of the thyroid gland is conspicuous, bilaterally represented by the superior thyroidal artery (from the external carotid) and inferior thyroid artery (from the succlavia). Exceptionally, another artery, the thyroid IMA artery (also known as Neubauer's artery), originating from either the common carotid or the anonymous troncus, may be present (Mohebati and Shaha, 2012). The thyroid contains a rich network of capillaries surrounding follicles. Venous blood drains through two sets of vessels:

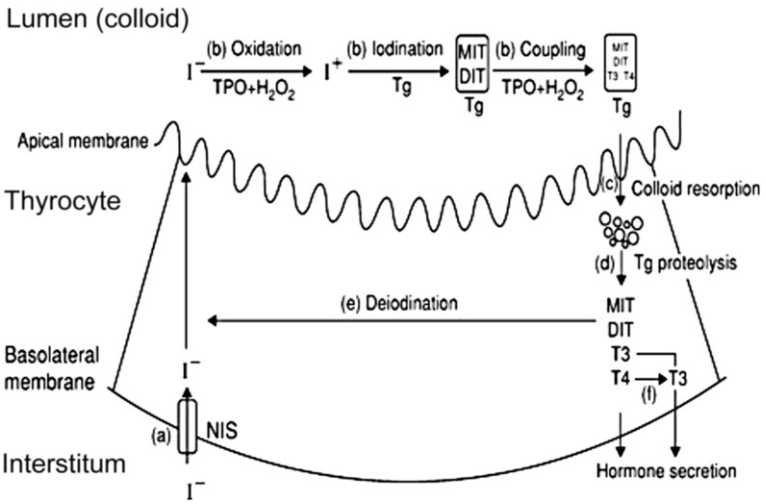


Fig. 4 Schematic diagram of thyroid hormone biosynthesis in and release from the thyrocyte. Subsequent metabolic steps are: (a) iodide transport via the Na^+/I^- symporter (NIS) inhibited by ClO_4^- and SCN^-; (b) oxidation of I^- to I^+ and iodination of tyrosine residues in thyroglobulin (Tg), and coupling of monoiodotyrosine (MIT) and diiodotyrosine (DIT) to thyroxine (T_4) or triiodothyronine (T_3), catalyzed by thyroid peroxidase (TPO) and inhibited by propylthiouracil and methimazole; (c) colloid resorption, inhibited by lithium and I^-; (d) proteolysis of Tg, inhibited by I^-; (e) deiodination of MIT and DIT; and (f) deiodination of T_4, inhibited by propylthiouracil.

superior and medial thyroidal veins realize a plexus, which drains into the external jugular vein, whereas inferior thyroidal veins realize a plexus in front of the trachea joining the brachiocephalic vein (Mohebati and Shaha, 2012).

A rich lymphatic network is present in the thyroid. Intraglandular and subcapsular lymphatics drain into the internal jugular lymph nodes. In particular, the superior lymph node group drains the upper gland and medial isthmus, while the inferior group drains the lower gland.

The thyroid nerves originate from the superior and middle cervical sympathetic ganglia. These fibers are vasomotor, indirectly influencing thyroid secretion (Mohebati and Shaha, 2012). Moreover, adrenergic fibers realize a network, which ends near the follicular basement membrane; adrenergic receptors are also present in follicular cells.

Physiology

Hormonogenesis in the thyrocyte can be subdivided into three main steps: iodide uptake; iodide oxidation and organification; and secretion of thyroid hormones. These steps are summarized in **Fig. 4**.

First Step: Iodide Uptake

All living beings are capable of taking up iodine and incorporating it into proteins. Iodinated compounds are of the utmost importance in regulating diverse functions in invertebrates devoid of the thyroid gland (Nilsson and Fagman, 2017). In humans and most vertebrates, the thyroid gland has evolved to save and store iodine. The thyroid produces iodinated molecules, iodotyrosines, and iodothyronines, the latter including thyroid hormones (T4 and T3) (Nilsson and Fagman, 2017).

Iodine is ingested with a number of food including dairy products, grains, and meat. Upon ingestion, organic iodine is reduced to inorganic iodide (I^-), the chemical form needed for the biosynthesis of thyroid hormones. Approximately 150 μg iodide are required by the thyroid gland for its daily activity, but in certain conditions, such as pregnancy and breastfeeding, iodide requirements are greater (Pennington and Young, 1991).

The thyroid and kidney are the most iodine-hungry organs. Indeed, the thyroid actively takes up iodine from the bloodstream, where its concentration is approximately 30 times lower than in the thyroid (Eskandari *et al.*, 1997). Particularly, the sodium/iodide symporter (NIS), located in the basolateral membrane of the follicular cell, entraps iodide from the circulation into cytoplasm against its electrochemical gradient, together with sodium ions. Unlike iodide, sodium entry into the cell is down its gradient, and results in energy production, which is required for the inward translocation of iodide. In turn, the sodium/potassium pump maintains the sodium gradient (Ferreira *et al.*, 2005). The next step is iodide efflux, namely its passive (down its electrochemical gradient) translocation from the cytoplasm to the apical side of the polarized thyrocyte, and the subsequent transport through the apical membrane. Crossing of the apical membrane was previously assumed to depend on pendrin and a putative apical iodide transporter, but the latter was recently ruled out. Nevertheless, a chloride channel was shown to mediate iodide efflux together with pendrin (Twyffels *et al.*, 2014; Rodriguez *et al.*, 2002).

Second Step: Iodide Oxidation and Organification

Upon its entry into the cytoplasm of the polarized thyrocyte, iodide moves apically, where it is oxidized and covalently bound to thyroglobulin (Tg). This step requires TPO and H_2O_2.

TPO is a 100 kDa heme-containing protein that belongs to the same family of human peroxidase, together with lactoperoxidase, myeloperoxidase, and eosinophil peroxidase (Godlewska *et al.*, 2017). Posttranslational modifications including glycosylation, heme fixation, proteolytic trimming, and dimerization are essential to obtain the mature protein (Godlewska *et al.*, 2017).

TPO acts as an H_2O_2 donor and oxidizes iodide. The resulting compound may be I^+ or OI^- (hypoiodite); both are capable of interacting with Tg (Kopp, 2013). H_2O_2 is generated by a NADPH oxidase system including DuOX (or thyroid H_2O_2-generating enzyme, THOX).

Tg, the most abundant protein of the thyroid, is a large glycosylated protein with more than 2700 amino acids and molecular mass of 660 kDa, representing the largest 1% of proteins in the vertebrate proteome (Lee *et al.*, 2008; Di Jeso and Arvan, 2016). Tg contains at least 66 tyrosyl residues, with slight differences between species. The number of tyrosines that are iodinated varies with iodine intake. Particularly, there is a hierarchy in iodination of tyrosines, so that tyrosine at position 5 is one of the most favored (see below) (Di Jeso and Arvan, 2016). There is evidence that Tg antigenicity depends on post-translational modifications, including iodination and glycosylation (Targovnik, 2013; Benvenga *et al.*, 1997).

Glycosylation of 10% of the total Tg weight occurs in the rough endoplasmic reticulum and in the Golgi apparatus, where N-linked oligosaccharides are acquired. Glycosylation is essential for the tertiary structure and the normal folding of Tg, which occurs also by interaction of Tg with endoplasmic reticulum oxidoreductase and molecular chaperones, such as calnexin and calreticulin (Di Jeso and Arvan, 2016). Within the endoplasmic reticulum, but before intracellular transport to the Golgi complex, two 12S (330 kDa) monomers are dimerized into a stable 19S (660 kDa) molecule. Tg represents the scaffold of the colloid in the follicular lumen, and acts as a depot of thyroid hormones and iodine (Targovnik, 2013; Di Jeso and Arvan, 2016). From this point of view, thyrocytes are more similar to exocrine cells than to the other major endocrine glands (Nilsson and Fagman, 2017).

Iodination of Tg results in monoiodotyrosine (MIT) and diiodotyrosine (DIT), depending on the number of iodine ions incorporated in Tg. Subsequently, when a MIT (donor) is coupled to a neighboring DIT (acceptor), 3,5,3′-triiodothyronine (T3) is generated, whereas when a DIT (donor) is coupled to another neighboring DIT (acceptor), 3,5,3′,5′-tetraiodothyronine or thyroxine (T4) is generated. Coupling of noniodinated tyrosine donor to a DIT acceptor forms 3,5-diiodothyronine (T2), whose effects on adiposity and body weight are still a matter of debate (Lanni *et al.*, 2005; Vatner *et al.*, 2015). Finally, 3,3′,5′-triiodothyronine (reverse T3 or rT3) accounts for only 0.9% of thyroid hormones released in the circulation. This results from either unfavorable coupling of a donor DIT to an acceptor MIT, or deiodination of T4 by type 1 or type 3 deiodinases (Bianco *et al.*, 2002). Structures of iodotyrosines and iodothyronines are shown in **Fig. 5**.

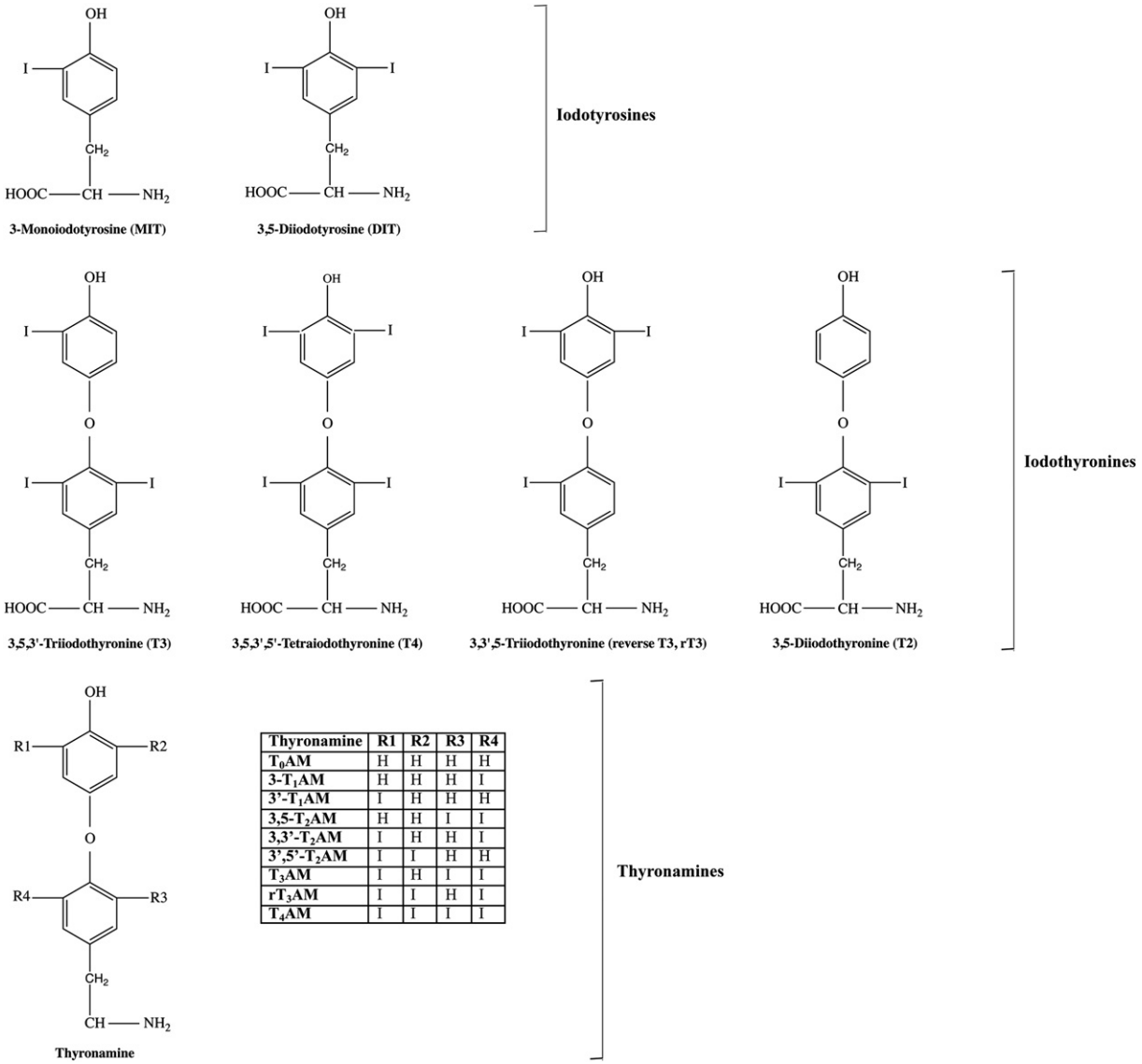
The major thyroid hormones forming sites are at the extreme N-terminus (T4) and C– terminus (T3 and T4) (Di Jeso and Arvan, 2016). Indeed, four main hormonogenic DIT-acceptor tyrosines were identified at position 5, 2554, 2747, and 1291, the first being the most efficient in T4 formation, while the third was the most efficient in T3 formation (Di Jeso and Arvan, 2016; Lamas *et al.*, 1989). Furthermore, formation of DIT and T4 are favored over MIT and T3, respectively. In iodine-sufficient areas the ratio of DIT:MIT:T4:T3 per molecule of Tg is 5:5:2.5:0.7, whereas in iodine-deficient areas, DIT:MIT and T4:T3 ratios are increased. Even if three or four thyroid hormones are synthetized per molecule of Tg, this process is warranted at extremely low levels of iodination (even 4 mol I^-/mol Tg) (Di Jeso and Arvan, 2016). The thyroid produced T3 accounts for only 20% of total T3; the remainder was obtained peripherally by T4 deiodination.

The iodide pool of the follicular unit includes also that resulting from deiodination of MIT and DIT. This part of the pool is recycled or further organified, or alternatively moved to the bloodstream (Rosenberg *et al.*, 1961). The daily turnover rate of the iodide pool is about 1% (Delange, 1998).

Although thyronamines were discovered in the 1950s, only in 2004 were they identified as ligands of a class of G protein-coupled receptors called trace-amine associated receptors. Thyronamines are structurally related to thyroid hormones as they have an identical carbon skeleton, but differ from the thyroid hormones in terms of the absence of the carboxylate group of the alanine side chain, which is replaced by an ethylamine chain (**Fig. 5**) (Chiellini *et al.*, 2017). Thyronamines include nine compounds differing for either the number or the position of the iodine atoms, the most abundant being 3-T_1AM (**Fig. 5**) (Chiellini *et al.*, 2017). These compounds were initially considered catabolites of thyroid hormones. However, recent observations suggest that thyronamines result from decarboxylation of thyroid hormones by ornithine decarboxylase, not by the aromatic amino acid decarboxylase as first reported (Hoefig *et al.*, 2015). Biosynthesis of 3-T_1AM occurs also in the intestine via the intermediate metabolite 3,5-T2, as demonstrated in thyroid cancer patients after thyroidectomy or radioiodide treatment (Hoefig *et al.*, 2011). So far, only two thyronamines (3-T_1AM and T_0AM) have been detected in vivo, particularly in the blood, heart, liver, adipose tissue, thyroid, and brain of rats and other animals. The other thyronamines are synthetically derived (Piehl *et al.*, 2011).

Third Step: Secretion of Iodothyronines

Tg is internalized in the thyrocytes through the apical membrane via micropinocytosis, namely vesicle-mediated endocytosis. Thus, invaginations of the apical membrane by pseudopods formation form colloid droplets (Bernier-Valentin *et al.*, 1991). These droplets release their content into endosomes, where Tg is sorted based on iodine content: whereas the highly iodinated molecules are fused with prelysosomes and then to lysosomes, those that are poorly iodinated are recycled and return back to the apical



Thyronamine	R1	R2	R3	R4
T_0AM	H	H	H	H
3-T_1AM	H	H	H	I
3'-T_1AM	I	H	H	H
3,5-T_2AM	H	H	I	I
3,3'-T_2AM	I	H	H	I
3',5'-T_2AM	I	I	H	H
T_3AM	I	H	I	I
rT_3AM	I	I	H	I
T_4AM	I	I	I	I

Fig. 5 Structures of the main iodotyrosines, iodothyronines, and thyronamines. Concerning thyronamines, only two (3-T_1AM and T_0AM) have been detected in vivo so far.

membrane, where they are secreted into the follicle lumen (Kostrouch *et al.*, 1993). Lysosomal endopeptidases, such as cathepsins B, D, and L, cleave Tg, thus releasing T3 and T4 (Dunn *et al.*, 1991). Direct cleavage within the follicle lumen has been also proposed (Tepel *et al.*, 2000). Proteolytic cleavage of Tg occurs at four major cluster sites, called A, B, C, and D, which fall at around residue 500, 990, 1800, and 2515, respectively (Dunn *et al.*, 1991). Three additional cleavage sites have been also found at residue 240, between residues 1142 and 1184, and at residue 597 (Gentile and Salvatore, 1993).

Upon their release into the cytoplasm, thyroid hormones reach the basolateral membrane with unknown mechanisms, and finally enter the circulation (Vickers *et al.*, 2012).

Regulation of Thyroid Hormones Biosynthesis

Thyroid hormones biosynthesis and metabolism is regulated by at least three factors: TSH-induced stimulation, iodine availability, and deiodinases activity.

TSH stimulates most if not all steps of thyroid hormones biosynthesis, from the uptake of iodine (by enhancing NIS expression) to internalization of Tg from the follicular lumen and consequent secretion of thyroid hormones into the bloodstream. TSH secretion is stimulated by TRH, which is in turn produced by neurons of the paraventricular nucleus of the hypothalamus, and prevents thyroid undersupply (Hoermann *et al.*, 2015). In order to prevent hyperstimulation by TSH, and to restore the individual set point of the hypothalamus–pituitary–thyroid axis, there are multiple negative feedback loops. Indeed, the

thyroid hormone inhibits both TRH and TSH secretion (Hoermann *et al.*, 2015). Concerning the inhibition of TRH release, it involves TRH-secreting neurons and tanycytes (Hoermann *et al.*, 2015). Also, the homeostatic relationship between TSH and FT4 is defined by a kite-shaped curve (Dietrich *et al.*, 2012). In addition, there is an ultrashort feedback loop by TSH on its own secretion by the thyreotrophs (Prummel *et al.*, 2004).

Iodine availability regulates thyroid hormones biosynthesis and secretion (Song *et al.*, 2010). When iodine availability is insufficient, T3 and T4 are inadequately synthetized, TSH increases, and goitrogenesis occurs. In addition, conversion of T4 to T3 is enhanced. In contrast, excessive iodine exposure leads to inhibition of thyroid hormones' biosynthesis by blocking H_2O_2 production and Tg iodination (the Wolff-Chaikoff effect) (Wolff and Chaikoff, 1948).

Thyroid hormone activation and inactivation are regulated by the deiodinases. Type 2 deiodinase (D2) acts on the outer ring of T4, converting it into T3; by contrast, type 3 deiodinase (D3) inactivates T4 and T3, deiodinating their inner ring and converting them into rT3 and T2, respectively. In addition, type 1 deiodinase (D1) acts both on the outer and inner ring. Thyroid contains especially D1 and D2 (Bianco, 2013).

Thyroid Hormones Circulation in the Bloodstream and Biological Actions

Similarly to steroid hormones, thyroid hormones are hydrophobic molecules, and therefore have to be carried in plasma by transporter proteins. Indeed, the free fraction of thyroid hormones is very low (0.03% of T4 and 0.3% of T3). The three major carriers are thyroxine binding globulin (TBG), transthyretin, and albumin. In addition, there are a number of minor carriers, such as lipoproteins, immunoglobulins, and serine protease inhibitors (serpins) (Benvenga, 2013).

TBG is the most important carrier of the thyroid hormone in blood in most mammals. It is a four-carbohydrate-chain glycoprotein that belongs to the serpin family, and peaks between α_1 and α_2 at zone electrophoresis (Benvenga, 2013). Other minor serpins that bind to thyroid hormones are α_1-antitripsin, α_1-chymotripsin, antithrombin III, and cortisol binding globulin. All the serpins have one thyroid hormone binding site with a relative higher affinity for T4 compared with T3 (Benvenga *et al.*, 2002). α_1-acid glycoprotein and sex hormone binding globulin are nonserpin proteins demonstrated to be minor T4 carriers (Benvenga, 2013).

Transthyretin is a homotetramer forming a cylindrical channel, which carries thyroid hormones and vitamin A in distinct sites. There are two sites for thyroid hormones, but only one is available, due to the much lower K_a of the second site (Neumann *et al.*, 2001).

From a phylogenetical point of view, serum albumin is the most ancient carrier. It has five binding sites for the thyroid hormones in its two subdomains (A and B). Albumin also binds sterol-derived hormones. Interestingly, other two homologues of albumin, vitamin D binding protein and α-fetoprotein, are capable of binding thyroid hormones (Benvenga, 2013).

All classes of lipoproteins can bind T4, T3, and rT3. Particularly, thyroid hormones interact with apoA, apoB100, apoC, and apoE, and this interaction is inhibited by lipids (Benvenga and Robbins, 1996).

Transport of thyroid hormones into cells relies on monocarboxylate transporters (MCT) 8 and 10, which are responsible for both the influx and the efflux of the thyroid hormones, and are ubiquitous. Another transporter of the thyroid hormones is the organic anion transporting polypeptide 1C1 (OATP1C1), which is particularly expressed in the astrocytes, where it is involved in T4 uptake (Mayerl *et al.*, 2014).

Upon its entry into the cell, T3, not T4, binds the thyroid hormone receptor (TR), which is a member of the nuclear receptor superfamily. There are two isoforms of TR (α and β), encoded by different genes located in different chromosomes (chromosomes 17 and 3, respectively) (Cheng *et al.*, 2010). Each isoform has three variants (α1, α2, α3 and β1, β2, β3). Of note, TRα2 and TRα3 are splicing variants of TRα1 that do not retain T3-binding activity. TR expression is spatially and temporally specific, as TRα is expressed mainly in the brain from the early stages of embryonic development, while TRβ is expressed mainly in the brain, liver, kidney, thyroid, heart, and retina (TRβ2) at a later stage of development (Cheng, 2000).

TR is a single polypeptide with a carboxyl-terminal ligand-binding domain (LBD), which interacts with coregulators (either activators or repressors) and participates in homodimerization (dimerization between two TR) and heterodimerization (dimerization between TR and retinoid X receptor). The binding of T3 to TR induces structural changes that lead to displacement of corepressors, recruitment of coactivators, and transcription activation, which is also regulated by other molecules, such as p53 and β-catenin (Cheng *et al.*, 2010). TR contains also a central, highly conserved domain, which interacts with the thyroid hormone response elements (TRE) (Wagner *et al.*, 1995).

For the purpose of this article, suffice it to say that mutations may occur in genes encoding either TRα or TRβ. Mutations of the TRβ gene lead to resistance to the thyroid hormone, which is a syndrome characterized by signs of various degree, including goiter, tachycardia, short stature, decreased IQ, and elevated thyroid hormone concentrations in serum together with nonsuppressible TSH (Weiss and Refetoff, 2000). Only a few cases of homozygosity for TRβ mutations have been reported so far (Ferrara *et al.*, 2012). Mutations of the TRβ gene have been also found in thyroid cancer and TSH-secreting pituitary adenoma (Cheng *et al.*, 2010). Concerning TRα, in mice, mutations in both alleles lead to death shortly after birth, while mutation of one allele results in dwarfism and abnormal lipid metabolism, and a phenotype different from that found in mutations of TRβ (Kaneshige *et al.*, 2001). Indeed, no mutation of TRα1 has been found in patients with resistance to thyroid hormones, indicating that TRα1 and TRβ have distinct functions. The phenotype resulting from TRα1 mutations is variable according to the location and type of mutation. These differences in the resulting phenotype might stem from the different interaction of TRα1 with various corepressors, with variable repression of different target genes (Cheng *et al.*, 2010).

In addition, thyroid hormones act directly in mitochondria stimulating cellular respiration. T3 or T2 binds a specific site in the mitochondrial inner membrane. Even if T2 is as potent as T3, it has a more rapid action (Horst *et al.*, 1989), and therefore its therapeutic use has been recently proposed (Lanni *et al.*, 2005). Thyroid hormones also induce mitochondrial heat generation, which depends on both basal proton leak and inducible proton leak; the latter is regulated by the uncoupling proteins, whose synthesis is stimulated by the thyroid hormone (Brand and Curtis, 2002).

The effect of the thyroid hormone in inducing thermogenesis had been used to treat obesity until 1978, when the US Food and Drug Administration issued a warning against it, due to severe heart and bone side effects. Subsequently, analogs of the thyroid hormone maintaining its thermogenic action, called thyromimetics, were synthetized (Yehuda-Shnaidman *et al.*, 2014). The main strategies to obtain stable thyromimetic molecules are the introduction of a bulky group at 5′ position for antagonism for TR, the replacement of iodine atoms to achieve resistance to metabolic deactivation, the change of bridging oxygen, and the replacement of the polar amino acid group at position 1 to change binding to TR (Hirano and Kagechika, 2010). Thyromimetics are TRβ-selective compounds that do not bind to TRα, which mediates the cardiac activity of the thyroid hormones. Some of these thyromimetics were proven efficient in treating obesity and dyslipidemia (Yehuda-Shnaidman *et al.*, 2014). However, despite TRβ selectivity, they can still interact with TRα, giving rise to heart and bone side effects (Unnikrishnan *et al.*, 2012). Also, because TRβ mediates the hepatic effects of the thyroid hormone as well as the negative feedback of the thyroid hormone in the hypothalamus, thyromimetics may induce both hepatic hyperthyroidism and systemic hypothyroidism due to hypothalamus–pituitary–thyroid axis suppression (Yehuda-Shnaidman *et al.*, 2014).

Except for direct action of the thyroid hormone in the mitochondrion, its effects have been long ascribed to genomic mechanisms. Only in the past decade the existence of a number of nongenomic effects of thyroid hormone has been demonstrated. These effects are, by definition, not mediated by the interaction of T3 with its nuclear receptor and protein synthesis, and therefore they have a much more rapid onset (minutes or hours) (Hammes and Davis, 2015). Furthermore, nongenomic actions are initiated by T3, T4, or rT3 binding to nontruncated TR, or truncated TR, or integrin $\alpha v\beta$ at the level of cell membrane, cytoplasm, and cytoskeleton. Activation of certain kinases (protein kinase C, mitogen activated protein kinases) ensues, with gene transcription or activation of the Ca-ATPase (Davis *et al.*, 2016). Nongenomic actions of the thyroid hormone might mimic the effects of estrogens in certain tumors by supporting cell proliferation and angiogenesis (Hammes and Davis, 2015).

Finally, recent investigations have highlighted a neural route of action of the thyroid hormone, originating in the hypothalamus at the level of T3-responsive nuclei, such as the paraventricular, ventromedial, and arcuate nucleus, and the preoptic and anterior areas. The activation of these areas, via the sympathetic and parasympathetic branch of the autonomic nervous system, regulates metabolism in liver and brown adipose tissue (Zhang *et al.*, 2017).

Thyronamines in the blood bind with high affinity to apoB100, with consequent very low free concentrations in serum, and interact with a class of G protein-coupled receptors called trace-amine associated receptors, but also with adrenergic receptors (Chiellini *et al.*, 2017). Biological effects of thyronamines are partly in the opposite direction of T3, since they reduce heart rate, cardiac output, metabolic rate, and body temperature. However, thyronamines also have actions that are synergic to T3, as they stimulate lipid metabolism over the carbohydrates one and neurological responses (Chiellini *et al.*, 2017). Like monoamine neurotransmitters, thyronamines have an ethylamine chain, and may also act as neuromodulators (Ianculescu and Scanlan, 2010).

See also: Thyroglobulin

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Thyroid Gland Development, Molecular Biology☆

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Glossary

Congenital hypothyroidism Alteration of thyroid functions in newborns; when this condition is associated with a primary thyroid disease, it is characterized by an elevated thyroid-stimulating hormone (TSH) level and decreased thyroxine (T4) in the bloodstream.

DNA-binding domain Protein domain capable of recognizing and binding to specific DNA sequences.

Epigenetics The study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence.

Gene targeting A complex of methods that allows a defined gene to be modified; in cultured embryonic stem (ES) cells, it provides a tool for the generation of genetically modified mice.

G protein-coupled receptor A transmembrane receptor that interacts with a G protein associated with the cytosolic surface of the cell membrane; G proteins are so named for their ability to bind guanine nucleotides.

Knockout (null) mouse Genetically modified mouse in which both alleles of an endogenous gene have been inactivated.

Penetrance The frequency with which a genotype manifests itself in a given phenotype.

Transcription factor DNA-binding protein required for a promoter to function at full level; it recognizes and binds to a specific sequence located in regulatory elements of a target gene and exercises its function by interacting with other components of the transcription apparatus.

Introduction

The thyroid gland displays a peculiar architecture characterized by the presence of spheroidal structures, called follicles, composed of a single layer of epithelial cells (thyroid follicular cells, TFCs) surrounding a closed cavity (follicular lumen). TFCs are highly specialized cells responsible for the production of thyroid hormones and express a specific set of genes, whose protein products perform functions essential for hormonogenesis such as thyroglobulin (Tg), thyroperoxidase (TPO), sodium–iodide symporter (NIS), thyrotropin receptor (Tshr), pendrin, and thyroid oxidase (THOX). In adults, TFCs are found in the thyroid gland, located in front of the trachea at the base of the neck. However, they are originally derived from a group of endodermal cells, located in the posterior portion of the mouth cavity, in the primitive pharynx. These cells migrate caudally, reach their definitive position, and finally accomplish their terminal differentiation. In humans, this process requires approximately 7 weeks; a thyroid primordium is visible at the beginning of the 3rd week, whereas follicular organization appears by the 10th week.

The thyroid also contains a second population of hormone producing cells named parafollicular cells or C cells, which are neuroendocrine in nature and primarily synthesize calcitonin, a natural antagonist to parathyroid hormone. C cells are also of endoderm origin and arise from the ultimobranchial bodies (De Felice and Di Lauro, 2004).

The organogenesis of the thyroid follows the same pattern in all mammals. Hence, it was possible to use animal models to define the various steps involved in thyroid morphogenesis in detail and to understand the molecular mechanisms underlying the development of the thyroid gland.

This article focuses on the results obtained from both mouse models and patients affected by congenital hypothyroidism, results that have demonstrated how a complex network of factors is required for a correct morphogenesis of the thyroid.

Morphological Aspects of Thyroid Gland Development

During mouse development, the thyroid primordium is visible by embryonic day (E) 8.0 to 8.5. At this stage, the thyroid primordium, called thyroid anlage, appears as a thickening of the endodermal epithelium in the midline of the floor of the primitive pharynx, in the presumptive sublingual region. At E 9.5, the thyroid primordium buds from the primitive pharynx and begins to migrate caudally, forming an endodermal-lined diverticulum that descends in the tissue in front of the neck. At this stage,

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the migrating thyroid primordium is still connected to the pharyngeal floor by a narrow channel, the thyroglossal duct. At E 11.5, the thyroglossal duct disappears and the thyroid primordium loses all connections with the floor of the pharynx and reaches its final destination, in front of the trachea, by E 13.5. Then, the thyroid expands and follicular organization appears. By E 14.0, the differentiation process of TFCs begins and thyroid-specific genes are expressed. Finally, 2 days later, thyroxine is detected in the fetal thyroid (De Felice and Di Lauro, 2004).

Molecular Aspects of Thyroid Gland Development

During the past years, it has become possible to identify a number of genes expressed in various stages of thyroid development and their role(s), as deduced by the phenotypes obtained in mouse models where such genes have been mutated (inactivated) by gene targeting.

The discovery that the transcription factors TTF-1 (thyroid transcription factor-1), Foxe1 (forkhead box e1, formerly called TTF-2), Pax8 (paired box gene 8), and Hhex (hematopoietically expressed homeobox) are expressed not only in mature thyroid cells but also in their precursors offered a useful tool for the exploration of the genetic basis of the developmental process of the thyroid gland (Lazzaro *et al.*, 1991; Plachov *et al.*, 1990; Thomas *et al.*, 1998; Zannini *et al.*, 1997).

At E 8.5, in the primitive pharynx, the endodermal cells fated to become TFCs are univocally specified by the expression of TTF-1, Foxe1, Pax8, and Hhex. These factors are also present in other embryonic tissues, but all four are coexpressed only in the presumptive thyroid bud from the moment a thickening composed by proliferating cells appears in the midline of the floor of the primitive pharynx. When the thyroid diverticulum forms and begins its migration, the thyroid primordium still expresses TTF-1, Foxe1, Pax8, and Hhex, and the simultaneous presence of these four factors will remain for the rest of the life as a hallmark of differentiated TFCs.

The expression of TTF-1, Foxe1, Pax8, and Hhex is necessary but not sufficient to guarantee the morphogenesis of the thyroid. Other genes also are required for the correct development of the gland. Among these, Tshr (thyroid-stimulating hormone receptor) seems to be involved in the final steps of differentiation of TFCs (**Table 1**).

TTF-1

TTF-1, also known as Nkx2.1, was first identified as a nuclear factor able to bind to specific sequences that are present in both *Tg* and *TPO* genes. TTF-1 is a transcription factor that recognizes and binds to specific DNA sequences via a 60-amino acid DNA-binding domain called homeodomain. The protein is encoded by a single gene, *Titf1* in mice and *TITF1* in humans, located on chromosomes 12 and 14q13, respectively.

During embryonic life, TTF-1 is expressed in the developing thyroid, lungs, diencephalon, and posterior pituitary. Studies in *Titf1* knockout mice have demonstrated that this factor is absolutely necessary for the correct development of the thyroid. *Titf1* null mice display a perinatal lethal phenotype characterized by severe defects in both lung and forebrain development as well as a lack of thyroid and posterior pituitary. At an early stage of development, in the absence of TTF-1, the thyroid primordium forms in its correct position, but already by E 10.0 the thyroid primordium appears to be smaller than that in wild-type embryos and soon undergoes degeneration, probably due to an apoptotic process. Hence, TTF-1 is dispensable for the initial commitment of thyroid cells but is required for the survival and subsequent differentiation of these cells. Because in the absence of TTF-1 thyroid cells disappear before the gland accomplishes its final differentiation, it is not possible to assess the role of TTF-1 in vivo in the adult thyroid. However, experiments performed in cultured differentiated thyroid cells have demonstrated that TTF-1 is relevant for the expression of thyroid-specific genes. The role of TTF-1 in embryonic life is being investigated. TTF-1 is present in organs derived from the ventral wall of the anterior foregut (thyroid and lungs) but is absent in the esophagus, which derives from the dorsal wall of the gut. Furthermore, in the absence of TTF-1, there is no septation between the trachea and esophagus. Hence, a possible role of TTF-1 could be to contribute to the specification of the anterior part of the gut. However, we do not know which genes TTF-1

Table 1 Timing and Molecular Phenotype of Thyroid Development in Humans and Mice

Embryonic stage		*Histological feature*	*Gene expression*[a]			*Hormonogenesis*
Mice(day)	*Humans (week)*		*TTF-1 Foxe1 Pax8 Hhex*	*Tg TPO Tshr*	*NIS*	
8.5	3.0	Thyroid anlage	+	−	−	−
9.5	3.5	Thyroid primordium migration initiates	+	−	−	−
11.5	4.0–5.5	Thyroglossal duct disappears	+	−	−	−
13.5	6.5–7.0	Thyroid reaches final destination				
14.5	8.5	Expansion and functional differentiation	+	+	−	−
16.5	10.0	Onset folliculogenesis	+	+	+	+

[a]The expression of the indicated genes has only been studied in the mouse embryo.

controls during thyroid morphogenesis. TTF-1 appears to act in both early and late stages of morphogenesis through phosphorylation on multiple serine residues. Studies on the phenotype of *Titf1* null embryos or in the absence of phosphorylated TTF-1 indicate that this transcription factor regulates the expression of some signaling molecules. TTF-1, presumably in its phosphorylated form, is also required for the maintenance of an ordered follicular architecture and function in the differentiated thyroid. Finally, it should be noted that TTF-1 is also expressed in the ultimobranchial bodies and embryonic C cells.

Pax8

Pax8 is a transcription factor characterized by the presence of a 128-amino acid DNA-binding domain (paired domain) that recognizes and binds to a single site present in *Tg* and *TPO* promoters. The gene encoding Pax8 (called *Pax8* in mice and *PAX8* in humans) is located on chromosome 2 in both species. During embryonic life, Pax8 is expressed since E 8.5 in the kidneys, brain, and thyroid primordium. In *Pax8* knockout mice, the thyroid is severely affected, follicular cells cannot be detected, and the mice die within a few weeks. In the absence of Pax8, at an early stage of embryogenesis, the thyroid primordium forms, buds from the gut, and begins its migration. However, the thyroid primordium is nearly undetectable by E 12.0. Hence, like TTF-1, Pax8 is required for the survival of thyroid precursor cells.

Experiments in cultured cells have demonstrated that Pax8 can activate transcription of thyroid-specific genes at their chromosomal locus. The coexpression of Pax8 and TTF-1 in thyroid cells only has suggested that these factors can cooperate in the stimulation of thyroid genes. Indeed, studies have provided us with the demonstration that Pax8 and TTF-1 directly interact in vivo in differentiated thyroid cells. Our knowledge of the function of Pax8 in the developing thyroid is still scarce. Based on the reported anti-apoptotic function of *PAX* genes, it has been proposed that the loss of TFCs through apoptosis might be the underlying mechanism of the observed thyroid dysgenesis in Pax8 null embryos. It has been demonstrated that in the absence of Pax8, the expression of both Foxe1 and Hhex is strongly reduced in the precursors of follicular cells. Pax8 could then have a specific upper role in the regulatory pathway controlling the development of the thyroid.

Foxe1

Foxe1 is a transcription factor characterized by the presence of a forkhead domain as a DNA-binding domain. It is encoded by the *Foxe1* gene in mice and the *FOXE1* gene in humans, located on chromosomes 4 and 9q22, respectively. Early during development, at E 8.5, Foxe1 is expressed along the whole foregut, at variance with TTF-1 and Pax8, whose presence in the gut is restricted to the thyroid anlage. *Foxe1* knockout mice display a perinatal lethal phenotype characterized by a severe cleft palate and athyreosis or ectopia of the thyroid. Analysis of thyroid development shows that in the absence of Foxe1 at E 8.5, the thyroid anlage is specified, but at E 9.5, thyroid precursor cells are still on the floor of the pharynx, showing a clear defect of migration. At E 15.0, the *Foxe1* knockout embryos exhibit either a small sublingual thyroid remnant or no thyroid gland at all. Hence, Foxe1 is absolutely necessary to promote migration of TFC precursors. Furthermore, Foxe1 also could be implicated in the control of the survival of thyroid cells, as shown by the absence of thyroid in many *Foxe1* knockout mice.

Hhex

Hhex is a homeodomain containing the transcription factor encoded by the *Hhex* gene in mice and the *HHEX* gene in humans, located on chromosomes 19 and 10q23, respectively. During embryonic life, Hhex is present in the developing thyroid and in several organs derived from the foregut endoderm. In *Hhex* null embryos at E 8.5, the thyroid anlage is visible, but already at E 9.5 the thyroid primordium is absent or hypoplastic and the expression of TTF-1, Foxe1, and Pax8 is down-regulated.

It is possible that Hhex is implicated in the regulation of the expression of TTF-1, Foxe1, and Pax8 in the thyroid primordium. Hhex could then be required to maintain the expression of these transcription factors in the thyroid.

Tshr

Tshr is a protein that belongs to the superfamily of G protein-coupled receptors encoded by the *Tshr* gene in mice and the *TSHR* gene in humans, located on chromosomes 12 and 14q31, respectively. Tshr is detected in the developing thyroid after the completion of the migration of the primordium, during the same stage at which Tg appears and before the first evidence of follicular organization in the gland.

In adults, thyroid-stimulating hormone (TSH) is the main physiological agent implicated in the regulation of the main functions of the thyroid and exerts its cellular effects by binding to Tshr. Inactivating mutations in this receptor cause severe hypothyroidism during postnatal life. During the early stages of thyroid development, a functional Tshr is not required but is necessary for the final step of follicular cell differentiation. Indeed, in the absence of a correct TSH/Tshr signaling, the expression of both TPO and NIS in TFCs is strongly down-regulated.

Table 2 Mouse and human phenotypes following inactivation of some genes normally expressed during thyroid development

Protein	*Gene*		*Tissue expression*	*Phenotype*		*Inheritance in humans*
	Mouse	*Human*		*Null mice*	*Patients carrying inactivating mutations*	
TTF-1	*Titf1*	*TITF1*	Thyroid Lung Brain Posterior pituitary	Thyroid and pituitary absent; severe defects in lung and diencephalon	Mild thyroid hypoplasia; respiratory distress; atassia	Dominant
Pax8	*Pax8*	*PAX8*	Thyroid Kidney	Thyroid absent	Thyroid hypoplasia	Dominant
Foxe1	*Foxe1*	*FOXE1*	Thyroid Esophagus Palate Hair follicles Anterior pituitary	Thyroid absent or ectopic; cleft palate	Athyreosis; cleft palate; spike hair; choanal atresia	Recessive
Tshr	*Tshr*	*TSHR*	Thyroid	Thyroid hypoplasia	Thyroid hypoplasia	Recessive

Thyroid Specification

Studies in mouse, Xenopus and zebrafish have identified key roles for fibroblast growth factors (Fgfs) and bone morphogenetic proteins (Bmps), presumably derived from the cardiogenic mesoderm, in inducing thyroid fate in competent but yet undifferentiated anterior endoderm cells. These studies indicated that Fgf2 and Bmp4 are key players of an evolutionarily conserved mechanism for thyroid specification (Nilsson and Fagman, 2017). Moreover, when conditioned in 3D culture and stimulated with TSH, cells derived from mouse embryonic stem cells overexpressing Nkx2-1 and Pax8 are able to generate fully functional thyroid follicular, capable of rescuing thyroid hormone levels in athyreotic mice (Antonica *et al.*, 2012). This exciting discovery opens the door to regenerative therapy for patients with CH, though the field needs further advances to make this a realistic possibility.

Molecular Genetics of Thyroid Dysgenesis

In 85% of cases, permanent congenital hypothyroidism (CH) detected in newborns is due to impaired development of the thyroid. Defects in the mechanisms that allow the growth, survival, migration, or differentiation of the thyroid primordium can result in thyroid dysgenesis (TD). This term indicates an ectopic or hypoplastic thyroid (or both) as well as the absence of the gland (athyreosis). It has been demonstrated that in some patients, TD is due to mutations in genes involved in thyroid development and already identified in mouse models such as *TITF1*, *PAX8*, *FOXE1*, and *TSHR*. These results confirm that the products of these genes are required for a correct thyroid morphogenesis (**Table 2**). Moreover, newly discovered predisposing genetic factors are described in familial CH cases associated mainly with a normal or small thyroid in situ.

TITF1

Some patients affected by a syndrome characterized by severe neurological disturbances, respiratory distress, and thyroid alterations have been found to carry heterozygous mutations within the *TITF1* gene. Nevertheless, the signs and symptoms of the three disorders are not always observed in combination and moreover, their severity varies greatly (Abu-Khudir *et al.*, 2017). When tested in vitro, the various mutations described encode for proteins that do not display either functional activity or a dominant negative effect on the wild-type form. These data indicate that a reduced expression of TTF-1 is not compatible with the normal development of lungs, brain, and thyroid in humans. On the contrary, mice carrying only a functional allele of *Titf1* show a very mild, possible strain-dependent phenotype.

PAX8

Mutations in *PAX8* have been described in sporadic and familial cases of congenital hypothyroidism with TD. The phenotype of the patients ranges from mild hypothyroidism to severe hypoplasia of the thyroid. As in the case of TTF-1, all affected individuals are heterozygous from the mutated allele, and in the familial cases transmission is autosomal dominant with incomplete penetrance and variable expressivity (Ramos *et al.*, 2014). This dominant effect of *PAX8* mutations could be due to a gene dose

requirement (haploinsufficiency). In these patients, the single functioning allele is not able to produce a sufficient amount of Pax8 to support the normal development of the gland.

FOXE1

Homozygous missense mutations in the *FOXE1* gene have been described in cases affected with Bamfort syndrome. This syndrome is characterized by cleft palate, bilateral choanal atresia, spiky hair, and congenital hypothyroidism with TD. All patients show athyreosis, whereas in mice the absence of Foxe1 causes either athyreosis or defects in thyroid migration. All the reported FOXE1 mutations occurred within the forkhead domain (FHD) and led to either partial or complete loss of the DNA-binding ability and of the transcriptional activity of the mutated protein.

TSHR

Mutations in the *TSHR* gene have been identified in patients affected by congenital hypothyroidism with thyroid hypoplasia and compensatory increased TSH secretion. The disease is inherited as an autosomal recessive trait, and the phenotype ranges from asymptomatic hyperthyrotropinemia to congenital hypothyroidism with severe hypoplasia of the thyroid. The different penetrance of the phenotype is likely due to the residual mutant receptor activity (Grasberger and Refetoff, 2017). Nonfunctional mutations in the two alleles produce severe hypothyroidism (uncompensated TSH resistance), while mutations in at least one allele with residual receptor function produce either mild hypothyroidism (partial compensation) or isolated hyperthyrotropinemia (full compensation).

Nkx2.5/Csx

Nkx2.5/Csx, located at 5q34, is a member of the NKx2 family. During early embryonic development of the mouse, Nkx2.5 is expressed in the progenitors of the myocardial cells, in the pharyngeal endoderm and in the developing thyroid (from E8.5 to E11.5). A smaller sized thyroid bud was detected in Nkx2.5 null embryos, suggesting that Nkx2.5 is required during organogenesis of the thyroid. Mutations in NKX2.5 gene have been detected in patients with congenital heart disease (CHD) and patients with either thyroid ectopy or athyreosis. However, pedigree and functional data suggested that NKX2.5 mutations were not a major contributor in the pathogenesis of thyroid dysgenesis, though their role as a genetic modifier cannot be excluded (van Engelen *et al.*, 2012).

GLIS3

GLIS3 biallelic mutations have been reported in consanguineous familial cases with variable manifestations of neonatal diabetes, CH, polycystic kidneys, glaucoma, hepatic fibrosis and exocrine pancreatic deficiency (Senée *et al.*, 2006). Most cases have a thyroid gland in situ; athyreosis is described only in one case (Dimitri *et al.*, 2011).

JAG1

Jagged ligands, expressed on the cell surface, interact with Notch receptors on adjacent cells, resulting in receptor cleavage and translocation to the cell nucleus. The disruption of the Notch signal leads to thyroid dysgenesis in zebrafish, suggesting an essential role of Notch pathway in thyroid development (Porazzi *et al.*, 2012). Heterozygous jagged 1 protein (JAG1) mutations have been reported in Alagille syndrome type 1 (ALGS1) which is characterized by variable involvement of liver, heart, skeleton, eye and facial defects. de Filippis *et al.* (2016) described 2 ALGS1 patients with thyroid hypoplasia and four CHTD patients (one with thyroid ectopy, one with a normal gland in situ and two with thyroid hypoplasia) with heterozygous JAG1 mutations. jag1a/b morphant zebrafish showed variable defects of thyroid development, indicating that JAG1 can contribute to the pathogenesis of thyroid dysgenesis.

BOREALIN

Homozygous mutations of CDCA8/BOREALIN were found in 2 consanguineous cases and monoallelic mutations in 2 sporadic cases of CHTD (Carre *et al.*, 2017). Borealin is expressed in thyrocytes and is a major component of the Chromosomal Passenger Complex (CPC), implicated in chromosome segregation and cytokinesis.

NTN1

Deletion in Netrin-1 gene (NTN1) was reported in one patient with congenital heart (VSD) and thyroid ectopy (Opitz *et al.*, 2015) and in 1 case of sporadic thyroid ectopy (Dr. Michel Polak, personal communication). Functional studies in zebrafish revealed that thyroid migration is disturbed in ntn1a morphant due to lack of proper guidance exerted by dysplastic pharyngeal vessels.

Conclusion

Although early in development TTF-1, Foxe1, Pax8, and Hhex are expressed in the thyroid primordium, none of them is essential for the initial steps of thyroid morphogenesis. Recent studies have identified fibroblast growth factors (Fgfs) and bone morphogenetic proteins (Bmps) as key players of an evolutionarily conserved mechanism for thyroid specification. However, additional factors required for thyroid anlage specification are still to be identified. It is possible that the identification of the genes controlling TTF-1, Foxe1, Pax8, and Hhex expression can give us information on how thyroid precursor cells begin their differentiation. Mutations in genes involved during the initial steps of thyroid morphogenesis could be responsible for thyroid agenesis (i.e., absence of the formation of a thyroid anlage). TTF-1, Foxe1, Pax8, and Hhex are transcription factors whose role is to control the expression of target genes that ultimately actuate a specific developmental program. The identification of these genes is still a matter of study. A number of cases of congenital hypothyroidism with TD could be due to mutations in genes that are targets of the transcription factors TTF-1, Foxe1, Pax8, and Hhex. Nevertheless, causal mutations have been found in a minority of CH cases, mainly to orthotopic hypoplasia, whereas the commonest developmental anomaly, thyroid ectopy, remains unexplained, indicating the involvement of still unidentified genes or alternative mechanisms. In this context, more recent studies describe new genes or pathway (e.g., GLIS3, JAG1) involved in thyroid development that may provide new clues for understanding the CH pathogenesis. The existence of alternative mechanisms for CH pathogenesis is also supported by the elevated frequency of discordance for CH phenotype between monozygotic twins. This latter finding suggests the involvement of non-Mendelian mechanisms, including post zygotic somatic mutations, environmental or epigenetic factors, that might predispose to or protect from the full expression of a CH genetic background. Interestingly a recent systematic NGS analysis performed in a large CH population reveals a frequent oligogenic origin of CH (de Filippis *et al.*, 2017). This data, consistent with a previously proposed animal model of a multifactorial pathogenesis of CH (Amendola *et al.*, 2005), may constitute a suitable explanation for the variable expressivity and penetrance of genetic defects previously reported in several CH familial settings.

See also: Thyroglobulin

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Hypothalamus–Pituitary–Thyroid Axis

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Glossary

Chaperones Proteins that assist the noncovalent folding or unfolding and the assembly or disassembly of other macromolecular structures.

Circadian rhythm Physiological activity that occurs approximately every 24 h, or the rhythm of such activity.

Deiodinase Enzymes containing selenium, in the form of the amino acid selenocysteine, and important in the activation and deactivation of thyroid hormones.

Glycoprotein hormones Family of glycosylated, cysteine-rich proteins consisting of an alpha and beta subunit.

G-proteins Family of proteins involved in transmitting signals from a variety of different stimuli outside a cell into the inside of the cell.

Hypothalamus Region of the diencephalon at the base of the brain in which specialized nerve cells originate and produce neuropeptides that regulate the secretion of hormones from the pituitary gland.

N-glycosylation The addition of sugar chains at the amide nitrogen on the side chain of asparagine.

O-glycosylation The addition of sugar chains on the hydroxyl oxygen on the side chain of hydroxylysine, hydroxyproline, serine, or threonine.

Pituitary Small gland, the hypophysis cerebri, connected to the base of the brain by a stalk and located in a depression in the sphenoid bone (the sella turcica).

Thyroglobulin Large glycoprotein that is synthesized in the thyroid gland and is the substrate for thyroid hormone formation.

Thyroxine (T4) 3,5,30,50-Tetraiodothyronine, the major hormonal product of the thyroid gland, formed by the coupling of two diiodotyrosine residues within the thyroglobulin molecule.

Triiodothyronine (T3) Active form of thyroid hormone produced in the thyroid gland by the coupling of a monoiodotyrosine and a diiodotyrosine residue in thyroglobulin, and in other organs by the deiodination of a 30'iodine atom from thyroxine.

Thyroid gland activity is the result of a dynamic regulating network that include the hypothalamic thyrotropin-releasing hormone (TRH), the pituitary glycoprotein hormone thyroid-stimulating hormone (TSH), and the circulating thyroid hormones with their feedback effects at the hypothalamic and pituitary levels. The first findings of a possible relationship between the pituitary and the thyroid was described more than 150 years ago from Niepce but only in the middle of the last century it was possible to isolate the first thyrotropin-releasing factor from porcine hypothalamus (Schally *et al.*, 1966) and to establish the primary structure of the TSH (McKenzie, 1960; Liao and Pierce, 1971; Fiddes and Goodman, 1979).

TRH

Structure

TRH is the most important endogenous stimulator of the HPT axis (**Fig. 1**). It is a small pyroGlu-His-Pro-NH2 tripeptide derived from a larger precursor which is posttranslationally cleaved by prohormone convertases (PC1/3, PC2), carboxypeptidase E, pyroglutamyl cyclase, and peptidylglycine α-hydroxylating monooxygenase.

Synthesis and Secretion

The human preprohormone *TRH* gene mapped on chromosome 3. TRH is synthesized and released from specific hypothalamic neurons within the paraventricular nucleus and then transported into the median evidence via axons of specialized nerve. It is then released, by membrane depolarization through Ca^{2+}-dependent exocytosis, into the hypophyseal portal blood system thus reaching the anterior pituitary gland where it stimulates the thyrotropes through the interaction with its specific receptor, the TRH receptor. The TRH has a very short half-life that ranges from 2 to 6 min, since it is rapidly inactivated by proline endopeptidase, pyroglutamyl peptidase I (PPI), and the membrane-bound PPII, which hydrolyze active TRH in the hypothalamus and pituitary (Iversen, 1991).

Regulation

The regulation of the TRH neuron activity is under the control of multiple factors (e.g., leptin, NPY, environment), that act either directly or indirectly, although the thyroid hormones are surely its most potent negative regulators. Indeed, thyroid hormones

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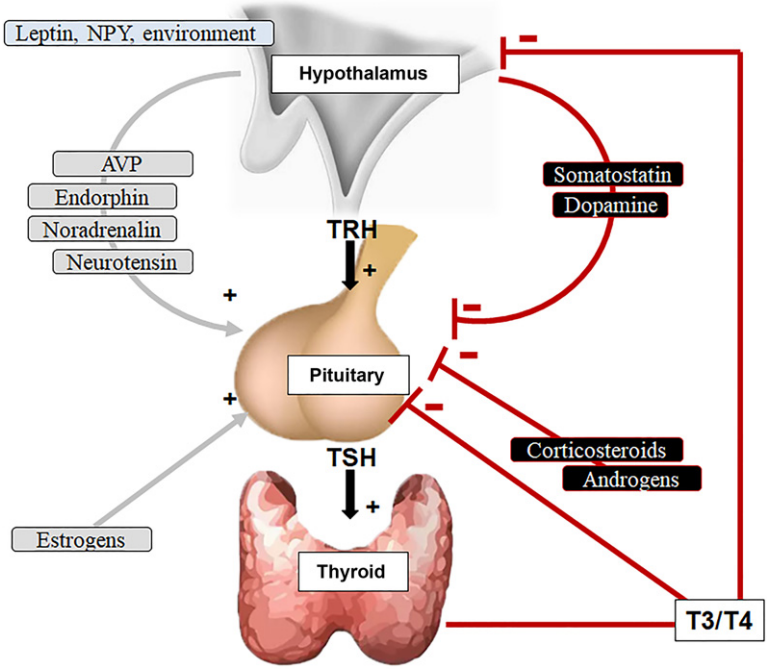

Fig. 1 Schematic representation of the hypothalamus–pituitary–thyroid axis.

interact with their specific nuclear receptors on TRH neurons to reduce TRH synthesis and secretion. Moreover, other factors, such as somatostatin, dopamine, glucocorticoids, adrenergic agents regulate the pituitary TSH response to TRH, but it is difficult to distinguish between hypothalamic and pituitary effects.

TRH Receptor

TRH receptor belongs to the family of the seven-transmembrane domain G protein coupled receptors. It is mainly expressed in the anterior pituitary, in neuroendocrine brain regions, in the autonomic nervous system, and in the brainstem. Upon TRH binding it mediates its signaling by the recruitment of the Gq protein thus leading to the stimulation of the phospholipase C. This will induce the intracellular accumulation of inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG) and thus the increase of intracellular Ca^{2+} concentrations and phosphokinase C activation (Engel and Gershengorn, 2007). TRH receptor expression on the thyrotropes is regulated by thyroid hormones but not by TRH (Chiamolera and Wondisford, 2009).

TRH Action on the Pituitary Gland

TRH induces secretion and synthesis of pituitary TSH. TRH injection in humans causes up to a 22-fold increase in TSH within half an hour. Continuous intravenous TRH infusion over 4 h results in a biphasic TSH surge, being the first peak the early release of preformed TSH, and the second peak the release of newly synthesized TSH. TRH has also been proven to affect the bioactivity of TSH by acting on its glycosylation pattern. Moreover, TRH is also known to stimulate pituitary prolactin secretion and may induce hyperprolactinemia in some hypothyroid patients. PRL levels are reduced by 40% in TRH-null mice, such as in the patients with a complete resistance to TRH (Collu *et al.*, 1997; Bonomi *et al.*, 2009). Although TRH is dispensable for pregnancy and lactation (Bonomi *et al.*, 2009), it is needed for lactotroph function, principally during lactation in mice.

TSH

Structure

TSH is a member of the glycoprotein hormones family together with the luteinizing hormone (LH) and follicle stimulating hormone (FSH), originated from the pituitary, and CG which has a placental origin. As demonstrated by the available crystal structure of the CG and FSH (Lapthorn *et al.*, 1994; Fan and Hendrickson, 2005), glycoprotein hormones are heterodimers characterized by a common alpha subunit (92 amino acids) and a specific beta subunit (TSH: 118 aa, FSH: 111 aa, LH: 121 aa, and

CG: 145 aa). Each subunit belongs to the "cysteine-knot growth factors" superfamily since it is characterized by six cysteines linked by disulfide bridges (Grossmann *et al.*, 1997). These latter stabilize the structure of each subunit that can be displayed in a "Y" shape, formed by three loops: loop 1 and 3 are parallel to one end of the subunit, and loop 2 extends at the other end. The heterodimer is than formed by the two subunits that are hinged head to tail and substantially back to back forming a protein slightly curved with loops alpha 1, alpha 3, and beta 2 at one end and loops beta 1, beta 3, and alpha 2 at the other end. The beta subunit of glycoprotein hormones in then characterized by a long C-terminal end after loop 3, that will make the so called "determinant loop." This is a short loop in the center of the dimer, between cysteines 10 and 12, that encloses the alpha subunit as a safety belt and it was indeed called "seat-belt" (Shupnik *et al.*, 1989; Magner, 1990; Lapthorn *et al.*, 1994). The glycoprotein hormones are characterized by various N-glycosylation sites and indeed the carbohydrate residues constitute around 20% and 10% respectively of the alpha and beta subunit molecular weights. A supplementary O-glycosylation site is also placed on the free alpha subunit (Shupnik *et al.*, 1989, 1994; Magner, 1990, 1994).

Synthesis and Secretion

TSH is synthesized in the adenohypophysis by the thyrotropes, which represent approximately 5% of the pituitary endocrine cells and are sited in the anterior median part of the gland. TSH biosynthesis results from several stages inside the rough endoplasmic reticulum and the proximal and distal Golgi (Magner, 1990, 1994; Cohen and Wondisford, 2013) where specific enzymes act modifying the posttranslational subunit precursors and adding the carbohydrate chains. The signal peptide allows the translocation of both alpha and beta precursors across the membrane of the rough endoplasmic reticulum where the cleavage of the signal peptide and the glycosylation of some asparagine residues take place. These precursors, which are rich in mannose, are preassembled in the rough endoplasmic reticulum in conjunction with a dolichol phosphate carrier and then moved to the glycosylation sites. The glycosylation process plays a key role in allowing the correct folding of the two subunits in forming the heterodimer. Glycoprotein are maintained in the rough endoplasmic reticulum until the end of the process by chaperone molecules, and then, heterodimers move to the following compartment for the posttranslational modifications leading to the formation of complex oligosaccharide chains. O-glycosylation of alpha subunit excess takes place in the proximal Golgi compartment, while sialylation and sulfation processes of the oligosaccharide complex in the Golgi distal compartment. TSH heterodimers, thus, enter the secretion pathway and are transported into small secretory granules. The pituitary content of immunoreactive TSH is about 80–200 mIU. TSH and the free subunits are released by exocytosis in a pulsatile manner with an average of 13 pulses/24 h. The daily production of TSH is 75–150 mIU with a circadian rhythm characterized by a nocturnal rise with a peak after numbness and a nadir in midafternoon. TSH is water-soluble and thus circulates as a free molecule in the circulatory torrent. It acts interacting with a specific receptor, the TSH receptor expressed on the membrane of the thyroid cells, and it is subjected to renal and hepatic metabolism with a clearance of about 40–60 mL/min. TSH half-life ($t/2$) is about 50–80 min, although its $t/2$ is modified according to the presence of hypo- or hyperthyroidism, being respectively augmented or reduced, or to the degree of glycosylation.

Regulation

TSH synthesis and secretion is regulated by several factors. TRH represents the most important stimulating factor, while the circulating thyroid hormones act as negative regulators through the feedback mechanism at the hypothalamic–pituitary level (**Fig. 1**) (Hollenberg, 2013). TRH binds to the TRH receptor expressed on the membrane of the pituitary thyrotropes inducing the intracellular pathways of the IP3 and DAG. This stimulates the glycosylation of the alpha and beta subunits and finally the secretion by exocytosis of TSH by increasing the cytosolic free Ca^{2+}. Nevertheless, a prolonged stimulation of the TRH receptor by its ligand is demonstrated to cause a "downregulation" phenomenon with the internalization of the receptors. On the contrary, thyroid hormones act both on the hypothalamus and pituitary with a negative feedback mechanism. Triiodothyronine (T3), decreases the TRH synthesis from the hypothalamus both inhibiting the gene-expression of the TRH precursor (prepro-TRH) and increasing the TRH degradation by specific enzymatic activities. Moreover, T3 is able to inhibit the TSH secretion and then the synthesis at the pituitary level through the interaction with the T3 nuclear receptors at this level.

Other factors are then involved in the complex regulation of the TSH synthesis and release, and thus, in turn, of the hypothalamus–pituitary–thyroid axis. This is the case of somatostatin and dopamine, two hypothalamic factors able to inhibit TSH secretion. Somatostatin was demonstrated to decrease TSH basal level and its response to TRH stimulation, and its long acting analogs are proved to be effective in treating the TSH-secreting pituitary adenomas (Beck-Peccoz *et al.*, 1996). Dopamine effect on TSH secretion is instead more complex and it is exercised both at the median eminence and pituitary level. TSH inhibition due to dopamine is more evident in females and in hypothyroidism. Moreover, the use of dopamine agonist in the treatment of pituitary adenomas secreting PRL and/or GH has not been proven to induce a simultaneous condition of central hypothyroidism with low TSH, since in this case the thyroid hormones negative feedback mechanism is prevailing. On the contrary, the infusion of high doses of dopamine for a prolonged period may cause central hypothyroidism. Contrary to dopamine, adrenergic substances seem to be able to induce the release of TSH from the human pituitary, although their role remains uncertain. Finally, high doses of corticosteroids, such as in the Cushing syndrome, stress conditions, anorexia nervosa, psychiatric disorders, and non-thyroidal illnesses, has been proven to decrease basal and TRH-stimulated TSH secretion. Sex hormones seems also to influence TSH response to TRH stimulation, being the estrogens able to increase and the androgens able to inhibit this stimulated secretion.

TSH Receptor

TSH receptor, which belongs to the superfamily of the G protein coupled receptors (Parmentier *et al.*, 1989), is placed on the basolateral membrane of thyrocytes (about 1000 receptors per cell) and mediates all TSH actions on the thyroid gland besides being the target for different autoimmune thyroid diseases (Parmentier *et al.*, 1989; Grossmann *et al.*, 1997). It is encoded by a specific 10 exons gene that maps on chromosome 14. TSH receptor extracellular domain is encoded by the first nine exons, while the last exon codes for the other two domains, the transmembrane and the intracellular ones. It is a 744 amino acids glycoprotein characterized by an N-terminal ECD with leucine-rich repeats and 6 N-glycosylation sites, and a C-terminal portion comprising a 7 spanning transmembrane domain and an intracellular tail. There is a 70% homology to the C-terminal portion and 40% to the N-terminal part between the three glycoprotein hormone receptors, the TSH and LH–FSH receptors. A crystal structure of the TSH receptor in complex with a human monoclonal stimulant antibody was published in 2007 (Sanders *et al.*, 2007), as well as a bioinformatic model of TSH receptor complexed with its natural ligand (Krause *et al.*, 2012). The interaction of the TSH with the extracellular domain of the TSH receptor and the consequent modifications of the transmembrane domain, leads to the recruitment of the G-proteins and the following activation of the downstream intracellular pathways, the cyclic adenosine monophosphate (cAMP) and the Ca-IP3 pathways (Sanders *et al.*, 2007; Krause *et al.*, 2012). The intracellular cAMP elevation activates cAMP-dependent protein kinase, which promotes the phosphorylation of cytosolic proteins, leading to some specific response of the thyroid cells. The elevation of IP3 induces and increase in the cytosolic Ca^{2+} which, in turns, is responsible for the cellular effects, while diacylglycerol activates a protein kinase that is responsible for specific biological effects that are still poorly understood.

TSH Action on the Thyroid Gland

TSH acts almost exclusively on thyrocytes, where it controls, through different mechanism and kinetics, cells activity (thyroid hormones synthesis and secretion) and their general metabolism (mitotic activity, carbohydrate, lipid, and protein) (Sanders *et al.*, 2007; Krause *et al.*, 2012). Indeed, TSH, via the cAMP pathways, enhances the thyroglobulin gene-expression thus stimulating the synthesis of this protein which is essential for the biosynthesis of the thyroid hormones. Furthermore, another cAMP mediated effect of TSH is represented by the stimulation also of the expression of the Na^{+}–I^{-} symporter which allow the active transport of the iodide inside the thyrocytes. Moreover, TSH stimulates the iodination of thyroglobulin and the synthesis of thyroid hormones by increasing the generation of H_2O_2, substrate of the thyroid peroxidase, by the action of specific enzymes, the so called "dual oxidase." This latter effect is mediated via the IP3/DAG intracellular pathways. In addition, TSH, via cAMP, fosters the thyroglobulin endocytosis and thyroid hormones secretion by thyroglobulin hydrolysis. In addition, TSH exerts other effect on the thyrocytes including the activation of the pentose pathway by increasing the production of H_2O_2 and the stimulation of the cell growth and proliferation (Kimura *et al.*, 2001).

See also: Central Hypothyroidism. Thyroid-Stimulating Hormone (TSH; Thyroptopin). TSH Function and Secretion

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Thyroid-Stimulating Hormone (TSH; Thyroptopin)☆

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Historical Aspects

In 1916, Bennett M. Allen at Kansas University and Philip E. Smith at Cornell and Berkeley, California, working independently, observed that thyroid follicles from thyroidectomized tadpoles contained less colloid and that the tadpoles consequently grew far more slowly (Magner, 2014; Allen, 1916; Smith, 1916). These findings substantially validated the theory of a pituitary factor that stimulates the thyroid gland. In the late 1950s, the introduction of improved ion exchange chromatography resulted in the elucidation of the primary structure of thyroid-stimulating hormone (TSH), or thyrotropin, as a glycoprotein, composed of two peptide chains, which is secreted from the thyrotrophic cells that are basophilic and constitute approximately 5% of all anterior pituitary cells (Pelletier *et al.*, 1978).

Structure and Metabolism

The α (alpha) subunit with a 92-amino acid sequence, which is virtually identical to that of human chorionic gonadotropin (hCG), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), forms the effector region that stimulates adenylate cyclase (involved in the generation of cAMP). In contrast, the β (beta) subunit (TSHB) containing a 118-amino acid sequence is unique to TSH and therefore determines its receptor specificity (Porcellini *et al.*, 2003).

TSH is released in a pulsatile manner, resulting in both circadian and ultradian rhythms of its serum concentrations: the latter should be borne in mind when interpreting clinical laboratory findings (Brabant *et al.*, 1990).

TSH, which is excreted via the kidneys, has a half-life of about an hour in healthy individuals and is likely to be dependent on the degree of glycosylation, since de-glycosylated TSH is more rapidly degraded. This mechanism is regulated by the hypothalamic thyrotropin-releasing hormone (TRH), which regulates the glycosylation process of TSH, a fact that explains the observed accelerated metabolism of TSH in hyperthyroidism and its retarded metabolism in hypothyroidism (Ridgway *et al.*, 1974).

Regulation and Physiology

TRH is the main regulator of TSH. The stimulated effect on pituitary TSH is mediated via the nucleus paraventricularis and nucleus dorsomedialis, both of which contain a high number of TRH positive cells: these, together with the very high number of TRH content in the projection areas of the eminentia mediana, release TRH into the thyrotrophic area of the pituitary as well as into the neurohypophysis and the extrahypothalamic central nervous system. The effect of TRH on TSH is mediated via a calcium-dependent process, in vitro studies having shown that interaction of TRH with its specific pituitary receptors rapidly results in activation of phosphatoinositol metabolism, the latter acting as a "second messenger" system (Weintraub *et al.*, 1985). This leads to release of calcium from the intracellular storage space, accompanied by a quick inflow from the extracellular space and an increase in intracellular calcium activity, which mediates the release of TSH (Brenner-Gati and Gershengorn, 1986).

Somatostatin, another peptide generated and secreted by the hypothalamus, has an opposite effect by decreasing or inhibiting the release of TSH. There are also other as yet undetermined factors that may have a stimulating action on TSH, such as catecholamines through their interaction with the TRH, presumably histamine, via stimulation of TRH, and vitamin D (1,25 (OH) D3), via its interaction with intracellular calcium (D'Emden and Wark, 1987).

TSH stimulates the thyroid gland to synthesize and secrete thyroxine (T4) and triiodothyronine (T3) which, depending on their concentration in the blood, regulate the pituitary release of TSH. Feedback loops, from which most control mechanisms originate, are especially important in the endocrine system, with negative feedback being the more frequent. One example of a negative feedback circuit occurs in the case that T3 and T4 concentrations are low, whereby the secretion of TSH is increased; in contrast, when T3 and T4 concentrations are high TSH secretion is decreased (Estrada *et al.*, 2014). TH additionally exerts a negative effect at hypothalamic levels (**Fig. 1**). The negative effects on TSH synthesis are mediated via attenuation of hypothalamic stimulation by reducing the number of TRH receptors in the pituitary as well as by a direct action mediated via nuclear receptors suppressing the generation of the TSH subunits.

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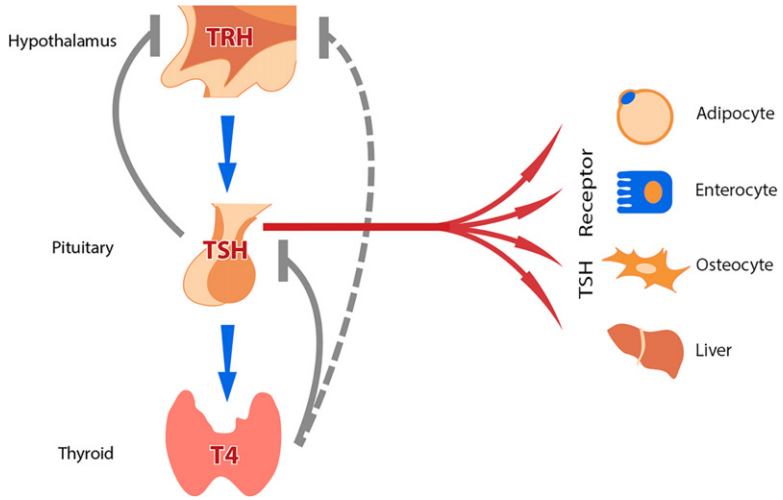

Fig. 1 TRH is released from the hypothalamus and activates the pituitary synthesis and secretion of TSH, which in turn stimulates the thyroid to secrete thyroid hormones (TH). In a paradigm of a negative feedback mechanism, TSH inhibits TRH secretion, while TH inhibits TSH and potentiates the inhibitory action of TSH to TRH release. TSH exerts various effects on a variety of organs in the periphery via stimulation of its receptor.

Stimulation of the G protein-coupled TSH receptor, which is found mainly in thyroid follicular cells, increases T3 and T4 production and secretion (Parmentier *et al.*, 1989). The TSH receptor consists of a seven-transmembrane (serpentine) domain with a large 400 amino acid extracellular NH2 domain, which is thought to exert an inhibitory action (Dumont *et al.*, 2000). This occurs via a cascade leading to thyroid hormone synthesis and secretion in the following manner: (1) upregulation of the sodium-iodide symporter (NIS) activity at the basolateral membrane of follicular cells, this elevating intracellular concentrations of iodine (iodine trapping); (2) iodination of the precursor protein thyroglobulin (Tg) in the follicular lumen, iodination of the tyrosyl residues of Tg precede TH biosynthesis, the latter depending on the interaction of iodide, TG, hydrogen peroxide (H_2O_2), and thyroid peroxidase (TPO) at the apical plasma membrane of thyrocytes: this results in thyroid hormone biosynthesis coming under the tonic control of TSH; (3) stimulation of the conjugation of iodinated tyrosine residues, this resulting in the formation of T4 and T3 which remain attached to the thyroglobulin protein; (4) increased endocytosis of the iodinated thyroglobulin protein across the apical membrane back into the follicular cell; (5) stimulation of proteolysis of iodinated thyroglobulin to form free T4 and T3; (6) secretion of T4 and T3 across the basolateral membrane of follicular cells to enter the circulation (Carvalho and Dupuy, 2017).

Pars Tuberalis TSH

It was recently shown in animals that breed seasonally that a long-day stimulation of the pituitary gland may induce TSH secretion from the pars tuberalis (PT), which locally activates thyroid hormone within the hypothalamus: this subsequently stimulates gonadotropin-releasing hormone followed by gonadotropin secretion, leading to gonadal growth (Nakayama and Yoshimura, 2018).

Diagnosis and Reference Levels

Measurement of TSH concentrations is an important component of the thyroid function test when an excess (hyperthyroidism) or deficiency (hypothyroidism) of thyroid hormones is suspected. The sensitivity of the TSH assay has been considerably improved over the last two decades, rendering it today's most reliable screening test for thyroid dysfunction. However, the interpretation of the results should additionally take into consideration both TSH and FT4 concentrations, while T3 measurements may be necessary in only a minority of cases. For the monitoring of hypothyroid patients on thyroxine, measurement of TSH alone is usually sufficient. An increase in TSH above the reference levels indicates under-replacement or poor compliance, whereas a significant reduction in TSH suggests over-treatment. A low or low-normal TSH value in the differential diagnosis may also be an indicator of pituitary disease.

In 2002, the National Academy of Clinical Biochemistry (NACB) recommended age-related reference limits for children, these usually being higher than in adults, starting from about 1.3 to 19 mIU/L for normal-term infants at birth, dropping to 0.6–10 mIU/L at 10 weeks old, to 0.4–7.0 mIU/L at 14 months and gradually declining during childhood and puberty till reaching adult levels, 0.4–4.0 mIU/L (Baloch *et al.*, 2002). Importantly, as TSH levels increase, FT3/FT4 ratios also rise until age 40, then

continuously decline with increasing age, thus reflecting a diminishment in T4 to T3 conversion rate which most likely forms part of the aging process (Strich *et al.*, 2016).

The reference interval for TSH varies significantly by age, sex, hour of day, and ethnicity, though it is unaffected by time of year. While females have significantly higher TSH than males, the magnitude of these differences did not exceed 0.1 mIU/L or 0.1 ng/dL, respectively. Although the 2.5% TSH reference interval remains constant through the day and age ranges, the upper limit (97.5%) of the TSH reference interval increases from 6.45 to 7.55 mIU/L with age, mainly due to a progressive increase in the amplitude of the nocturnal TSH surge (Ehrenkranz *et al.*, 2015).

The TSH threshold for initiating treatment is still not well defined due to the lack of large randomized controlled trials. However, it has been widely accepted that treatment of subclinical hypothyroidism should be started at TSH $\geq$ 10 mIU/L and subclinical hyperthyroidism at TSH $<$ 0.1 mIU/L. Recommendations on screening are controversial, though most guidelines advocate that thyroid function should be checked in patients at risk for hypothyroidism, those over 60 and those with known cardiovascular disease and heart failure (Floriani *et al.*, 2018).

Challenges in the Interpretation of TSH Results

Heterophilic antibodies (HA—including human anti-mouse antibodies (HAMA) and rheumatoid factor (RF)) may interfere with TSH measurement, causing a higher or, rarely, lower TSH result as compared to the actual true TSH level (Mongolu *et al.*, 2016). HA may occur in up to 4.4% of samples tested, generating a discrepancy between TSH and free T4 values and, most importantly, between laboratory values and patients' conditions. Macro-TSH-endogenous antibodies bind to TSH reducing its activity, while TSH-isomers-natural variations of the TSH molecule, with lower activity, exert a lesser effect, the pituitary gland being thus forced to produce more TSH to overcome this deficiency (Hattori *et al.*, 2016).

These factors must be kept in mind so as to avoid skewed results.

Extrathyroidal Effects of TSH

TSH receptors are found in various cells, while experimental data have revealed an important role of TSH in a range of extra-thyroidal organs and systems (Fröhlich and Wahl, 2016). Two outstanding examples are the observations that TSH supported T-cell development in the thymus and improved natural killer cell activity (Provinciali *et al.*, 1992) and that thymocytes responded in a time-dependent manner upon stimulation with TSH, with application at 0900 h inducing proliferation but application at 1800 h having no effect. The latter underlines the importance of circadian changes in TSH levels and TSH receptor expression (Winczyk and Pawlikowski, 2000). Below an update on the physiology of TSH receptor in enterocytes, osteocytes, and adipocytes.

TSH and Enterocytes

Exogenous application of TSH can induce intraepithelial lymphocyte development in the intestinal epithelium, though not in athymic mice. Furthermore, TSH regulates the activity of the intraepithelial immune system, while the pattern of TSH-immunoreactive cells in the mucosa is changed from the upper and lower parts of the intestinal crypts to the stained cells in the apical region of the villi on infection with rotavirus (Wang and Klein, 1995; Fröhlich and Wahl, 2016). It is of note that TSH in the small intestine is specifically localized to regions below the crypt-villus units. Upon investigation, these disclosed, on the one hand, high-level TSH staining in the lower crypts in the absence of IL-7 staining and, on the other hand, TSH and IL-7 co-staining in the upper crypt regions. In rotavirus-infected mice, a notable difference was observed between TSH staining pattern in infected and in noninfected animals. Interestingly, 2 and 3 days postinfection TSH expression was high in and close to the apical villi where virus infection was most severe (Scofield *et al.*, 2005). It is thus probable that TSH exerts immunomodulatory activity on inflammation of the intestine.

TSH and Osteocytes

Both hyperthyroidism and hypothyroidism interfere with inhibitors of Wnt signaling, dickkopf-1 (DKK1), and sclerostin and have been associated with bone loss and osteoporosis (Tsourdi *et al.*, 2015). Osteoclasts, originating from multipotent progenitors in bone marrow, differentiate to osteoclast precursors, fusing and becoming mature (multinucleated) osteoclasts, a process primarily mediated by the receptor activator of nuclear factor kappa B (RANK) binding to its cellular receptor. The sensitivity of osteoclasts to TSH is regulated by estrogen, a lack of which results in the bone loss seen in postmenopausal women—and particularly those under TSH suppression with L-T4 (Martini *et al.*, 2008; Fröhlich and Wahl, 2016). Both estrogen binding to estrogen receptor alpha and TSH binding to TSH receptors inhibit RANKL signaling, leading to bone degradation. Estrogen acts by increasing osteoprotegerin, which binds RANKL and prevents it from binding to RANK, while TSH receptor activation, by antagonizing RANKL signaling and inhibiting RANKL-induced osteoclast formation and stimulation of osteoblast differentiation, increases bone mass.

TSH and Adipocytes

While the effect of subclinical hypothyroidism on lipid profiles is not as yet clarified, overt hypothyroidism is known to be associated with increased lipid profiles, this relationship being strongly influenced by gender and age as well as by degree of thyroid failure (Alamdari *et al.*, 2015). The TSH receptor (TSHR) is present in both white adipose tissue and brown adipose tissue (BAT) and is increased during brown adipocyte differentiation. Moreover, TSH increases basal and T3-stimulated uncoupling protein-1 (UCP1) and DIO2 expression as well as DIO2 activity and lipolysis (Martinez-deMena *et al.*, 2015).

TSH interacts with insulin on differentiated human adipocytes modulating their respective intracellular signaling. TSHR is upregulated by insulin and low-TSH, whereas it is downregulated by high TSH and T3. Given that TSH inhibits insulin-stimulated Akt phosphorylation in adipocytes, while insulin reduces the ability of TSH to activate PKC and stimulate lipolysis (Felske *et al.*, 2015), it is evident that TSH is involved in lipolysis and maintenance of thermogenesis. Moreover, TSH may stimulate cholesterol biosynthesis, this hypothesis recently being supported by the observation of a bidirectional relationship between TSH-β (TSHB) and total- and LDL-cholesterol (LDL-c) in adipose tissue (Moreno-Navarrete *et al.*, 2017). Of particular note, interventional reduction of cholesterol leads to decreased TSHB mRNA, while, conversely, excess cholesterol upregulates TSHB mRNA in human adipocytes. This underscores the significance of TSH as a paracrine factor and modulator in cholesterol metabolism. In this line of evidence, TSH was recently positively associated with hepatic PCSK9 expression (Gong *et al.*, 2017). In subclinical hypothyroid patients with higher serum TSH levels, significantly higher serum PCSK9 levels, along with increased LDL-c concentrations, were reported as compared with those of matched euthyroid persons. The above results point to a regulating role of TSH in hepatic PCSK9 expression, which further aggravates the state of dyslipidemia.

See also: Thyrotoxicosis; Diagnosis. Thyroid and Infertility. Thyroglobulin. Radioactive Iodine. Thyroid and Irradiation. Thyroid Disease and Bone. Iodine Deficiency. Thyroid Carcinoma. Lithium. Postpartum Thyroid Dysfunction. Thyroid Imaging. Amiodarone and Thyroid. Hypothyroidism Subclinical. Hypothalamus–Pituitary–Thyroid Axis. Central Hypothyroidism. Graves' Orbitopathy. Thyroid Function and Depression. Drug Effects and Thyroid Function. Hashimoto's Thyroiditis. Diagnosis of Hypothyroidism. Thyroid Autoimmunity. Thyroiditis, Infectious and Subacute. Myxedema Coma. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Thyroid Disease and Pregnancy. Thyroid Disorders in the Elderly. Thyroid Function Tests

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TSH Function and Secretion☆

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Introduction

Thyrotropin (thyroid-stimulating hormone (TSH)), produced in the anterior pituitary gland, is released into the systemic circulation, and acts on the thyroid gland to produce secretion of thyroxine (T4) and triiodothyronine (T3). T4 and T3 are carried in the circulation in association with binding proteins. Their free hormones (free T4 (FT4) and free T3 (FT3)) enter peripheral cells and interact with genes in the cell nucleus. FT4 and FT3 regulate protein synthesis that affects metabolic processes of the body (e.g., growth, energy, intestinal motility, cardiac output and pulse rate, hair growth, body temperature). TSH synthesis is regulated by feedback of FT4 and FT3 on both pituitary TSH-producing cells and hypothalamic cells producing TSH-releasing hormone (TRH). This negative feedback system is the major regulator of hypothalamic-pituitary-thyroid (HPT) axis especially in conditions where primary thyroid dysfunction is present. However, it is becoming increasingly evident that control of the HPT axis is much more complex than previously thought. For example, changes in circadian rhythm, food intake, and environmental temperature result in predictable changes of thyroid hormone that seem to override classic negative feedback regulation (Costa-e-Sousa and Hollenberg, 2012). Much work is still needed to elucidate these relevant neuroendocrine pathways.

Genetics and Embryology of Pituitary Development

The anterior pituitary originates from an invagination of oral ectoderm by the 4th or 5th week of gestation (Rathke's pouch), which migrates to meet the posterior pituitary (an extension of neural ectoderm of the floor of the forebrain near the hypothalamus). The vascular connection between the hypothalamus and pituitary (portal circulation) begins to develop by 7 weeks of gestation and is established by 20 weeks (Kronenberg and Williams, 2008). Functional development of the anterior pituitary is determined by a cascade of transcription factors under complex temporal regulation, resulting in commitment of differentiated cell types including thyrotrophs or TSH secreting cells between 12 and 24 weeks gestation (Scully and Rosenfeld, 2002). Mutations affecting transcription factors involved early in pituitary development (HESX1, SOX2, SOX3, OTX2, LHX3, LHX4) may present as syndromic hypopituitarism with craniofacial abnormalities such as septo-optic dysplasia and holoprosencephaly (Parks *et al.*, 1999). Defects in transcription factors expressed later in pituitary development will manifest with varying combinations of pituitary hormone deficiencies. Defects in PROP1 and POU1F1 (PIT1) can all result in TSH deficiency (Mehta and Dattani, 2008).

Thyroid Function in the Fetus and Infant

Fetal Thyroid Physiology

The fetal HPT axis functions for the most part independently from that of the mother. The maternal delivery of TSH to the fetus via transplacental passage is negligible. In contrast there is transfer of maternal T4 and T3 to fetal blood, and this is essential for normal early fetal neurogenesis (Haddow *et al.*, 1999; Patel *et al.*, 2011). During the first 8–16 weeks of gestation, the human fetal thyroid synthesizes only minute amounts of T4 and T3. TRH has been found in fetal whole-brain extracts by 30 days and in the hypothalamus by 9 weeks of gestation. TSH can be detected in fetal blood by 10–12 weeks. As a result of maturation of the HPT axis, fetal hypothalamic expression of TRH and production of TSH and T4 progressively rise from mid gestation to peak during the month prior to term. TSH then remains higher than maternal levels possibly reflecting a higher set point for feedback (Kratzsch and Pulzer, 2008). T3 levels in fetal blood remain low until 26–30 weeks of gestation, a consequence of high levels of type III deiodinase activity (which converts T4 to inactive reverse T3) in peripheral fetal and placental tissues (Patel *et al.*, 2011).

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Neonatal Thyroid Physiology

At delivery, the sudden cool temperature stimulates a dramatic rise in plasma TSH during the first hour of life. The levels of TSH rise to a peak of more than 50 mU L^{-1} at 30 min of age, stimulating a rise in serum total and free T4 (FT4) and T3 (FT3). In full-term infants with normal thyroid function, TSH levels are generally below 20 mU L^{-1} at 24 h of age and are 1.3–16.0 mU L^{-1} by 4 days of age. By 6 weeks of age, TSH concentrations approximate those of adults. In full-term infants, serum T4 and FT4 concentrations fall gradually during the subsequent 4–6 weeks, and serum T3 levels rise gradually after the neonatal period to achieve mature levels by 2–12 weeks of age. By 6 months of age, the levels of T4 and FT4 remain slightly higher than those of older children and adults, and the circadian pattern of TSH secretion begins to appear. These observations suggest that hypothalamic–pituitary regulation of TSH secretion matures gradually from 6 weeks to 6 months of postnatal age.

Thyroid Function After Premature Birth

In preterm infants, the neonatal rise of TSH and the rise in T4, FT4, and FT3 are blunted. By 4 days of age, TSH levels have declined to 0.8–6.9 mU L^{-1}. T4 and FT4 levels in preterm infants exceeding 30–32 weeks gestation increase progressively over the ensuing 4–8 weeks to values comparable to those of term infants. In contrast, levels of T4 and FT4 in very low-birthweight infants (less than 30 weeks or less than 1200–1500 g) decline progressively after day 1, reaching a nadir at 1–2 weeks of age. This drop appears to be the result of many factors, including nutritional problems, alterations in thyroid binding globulin, immaturity of HPT axis, immaturity of the thyroid gland itself, and increased tissue utilization of T4 (LaFranchi, 1999). This relative hypothyroxinemia is most profound in those infants born most prematurely.

TSH Structure and Glycosylation

TRH has been shown to be necessary for TSH synthesis, posttranslational glycosylation, and secretion of a fully bioactive TSH molecule from the pituitary. TSH is composed of an α-subunit (identical to that of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG)) and a unique β-subunit. For TSH to act, both the α- and β-subunits interact with the G protein-coupled TSH receptor. Both subunits undergo posttranslational glycosylation in response to TRH. The glycosylation patterns influence the bioactivity of the TSH molecule (Persani, 1998). There are two oligosaccharide chains on the α-subunit and one on the β-subunit. Highly sialylated chains (as in recombinant TSH) have decreased bioactivity, decreased hepatic clearance, and longer half-life. Conditions with altered TSH glycosylation resulting in abnormal bioactivity are listed in the following table (Rose, 2000):

Conditions of decreased TSH bioactivity	Mixed hypothyroidism (central hypothyroidism with elevated TSH)
	Primary hypothyroidism
	Nonthyroidal illness
Conditions of increased TSH bioactivity	Resistance to thyroid hormone
	TSH-secreting pituitary adenomas

The TSH β-subunit gene was cloned in 1988, allowing research leading to production of recombinant TSH. Key domains of the β-subunit allowing ligand receptor interaction were identified, potentially permitting engineering of TSH molecules with increased bioactivity (Weintraub and Szkudlinski, 1999).

Regulation of TSH Synthesis and Release

TSH-Releasing Hormone (TRH)

Like GH, TSH is synthesized in anterior pituitary cells in response to a releasing hormone. TRH is secreted from the hypothalamus and transported to the pituitary via the venous portal system. Dopamine and somatostatin (both of which inhibit TSH release) are also transported from the hypothalamus (Rose, 2000). The TRH (G protein-coupled) receptor has been identified on pituitary cells and also in the central and peripheral nervous systems.

Circadian Variation in TSH Concentration

In persons of all ages after infancy (6 months), TSH secretion normally occurs in a circadian pattern with lower concentrations in the afternoon, a nocturnal surge beginning after 1900 h, and higher concentrations between 2200 and 0400 h. Peak TSH levels between 2200 and 0400 h are followed by a gradual decline between 0400 and 1000 h to nadir TSH values. Small frequent pulses occur as an overlay on this overall "sin wave"-shaped circadian pattern. Thus, at least one-third of the trophic influence of TSH on

the thyroid gland occurs during the hours of sleep. Central hypothyroidism (FT4 in the lowest third of the normal range) can be confirmed in children older than 1 year by the am to pm TSH ratio or the TSH surge test (Rose, 2010).

Despite the clear description of the daily pattern of TSH variation in the literature, the neuroendocrine influences that result in this circadian pattern have not yet been fully characterized. Factors regulating the timing and amplitude of the TSH surge may include an endogenous surge in hypothalamic TRH secretion at night, timing of onset of sleep, psychiatric disturbance, and/or altered pituitary sensitivity to endogenous TRH. Substances that appear to influence pituitary sensitivity to TRH include α-adrenergic stimuli, somatostatin, endogenous opiates, dopamine, cortisol, leptin, and GH concentrations.

TSH Function at the Receptor

TSH Receptor Binding

TSH is secreted from the pituitary into the blood, stimulating synthesis and release of T4 and T3 from the thyroid gland as well as influencing uptake of iodine. Both subunits of TSH interact with the G protein-coupled cell membrane receptor to generate the second messenger, cyclic AMP (cAMP), within the cell. The TSH receptor has an extracellular glycosylated portion and an intracellular portion with a cytoplasmic "tail" involved in G protein coupling and signal transduction. Both portions undergo posttranslational processing, influencing receptor-binding affinity of TSH. Mature complex glycosylation of the extracellular portion is required for TSH binding; if there are only mannose-type sugars, TSH binds with only low affinity (Persani *et al.*, 1998; Rose, 2000).

Results of TSH Receptor Binding

TSH receptor binding leads to increased synthesis of iodothyronines and to their storage in thyroid colloid in association with thyroglobulin. TSH receptor binding also leads to increased release of fully formed T4 and small amounts of T3 from the colloid. Plasma T4 and T3 circulate in the serum bound to thyroid-binding globulin and albumin, with only small amounts that are "free" or unbound. FT4 undergoes intracellular deiodination to FT3, which interacts with DNA to influence cellular mRNA and protein synthesis. Both T4 and T3 provide negative feedback at the hypothalamus and pituitary to modulate TRH and TSH secretion.

TSH Receptor Mutations

A TSH receptor mutation database has been established to allow easy access to the many identified TSH receptor mutations and their clinical consequences (Trulzsch *et al.*, 1999; Kronenberg and Williams, 2008; Paschke, 2013). Inactivating mutation can present as TSH resistance or in rare cases congenital hypothyroidism with eutopic thyroid. To date, over 50 loss-of-function mutations have been identified in humans. Nearly 100 gain-of-function mutations have been documented. Activating mutations of the TSH receptor in focal tissue may result in hyperfunctioning thyroid nodules/carcinoma or multinodular goiter. Expression of these mutations in all thyroid cells can result in thyrotoxicosis presenting during the neonatal period (and persisting beyond 6 months of age), late infancy, childhood, or adulthood (Trulzsch *et al.*, 1999; Kronenberg and Williams, 2008; Paschke, 2013).

Elevated TSH Secretion

Primary Hypothyroidism

Congenital hypothyroidism

Primary hypothyroidism (reduced function of the thyroid gland itself with low FT4 and high TSH) is the most common form of hypothyroidism both in the general population and in cancer survivors. There is now universal screening for congenital primary hypothyroidism in the United States. American newborn screening programs measure either TSH (with secondary T4 assay) or total T4 by radioimmunoassay (with secondary TSH) in blood spot samples obtained after 24 h of age. Hypothyroxinemia is defined as a total T4 value below 90% of the samples screened on that day. Measurement of TSH in hypothyroxinemic infants identifies those with primary hypothyroidism. TSH values often exceed 50–100 mU L^{-1} in infants with thyroid agenesis and other forms of permanent primary hypothyroidism. TSH levels exceeding 16–20 mU L^{-1} between 24 and 96 h of age, or 5.0–7.7 mU L^{-1} between 1 and 12 months of age, may reflect primary thyroid dysfunction (LaFranchi, 2011; American Academy *et al.*, 2006). Rarely, congenital primary hypothyroidism results from a genetic defect (Knobel and Medeiros-Neto, 2003; Nicholas *et al.*, 2016; Lof *et al.*, 2016).

Hypothyroidism in preterm infants

It should be recognized that very low-birthweight infants are at an eightfold increased risk for the development of transient primary hypothyroidism with low T4 levels and marked elevations in TSH (LaFranchi, 1999; Rapaport *et al.*, 2001). Transient primary hypothyroidism in preterm infants may, in some cases, be induced by transdermal absorption of iodine antiseptics. On the other hand, in iodine-deficient geographic regions, transient hypothyroidism is more likely related to insufficient iodine intake. Transient primary hypothyroidism may be accompanied by a delayed rise in TSH that is not detected until several weeks after birth (American Academy *et al.*, 2006). The prevalence of permanent congenital primary hypothyroidism in preterm infants is comparable to that in term infants. There is concern that permanent congenital primary hypothyroidism may be masked in premature infants due to drug administration that suppresses TSH, hypothalamic–pituitary immaturity, and other effects of neonatal illness. Many centers now rescreen at-risk infants as they approach discharge but it is unclear if the rescreening tests are just detecting a transient problem versus true permanent hypothyroidism (Leger *et al.*, 2014).

Acquired primary hypothyroidism

Primary hypothyroidism may be acquired after infancy and may represent a late presentation of thyroid dysgenesis or dyshormonogenesis. Most commonly, acquired primary hypothyroidism is due to thyroid autoimmunity associated with thyroid peroxidase antibodies (as in Hashimoto's thyroiditis). In acquired primary hypothyroidism, TSH may be as elevated as 1800 mU L^{-1}. The frequency of primary hypothyroidism is increased in chromosome abnormality syndromes such as Turner, Klinefelter, and Down syndromes. Most patients with mildly elevated TSH concentrations have been considered to have mild or "compensated" hypothyroidism. However, mild primary hypothyroidism has potential clinical importance, given that children with mild TSH elevation and T4 levels within normal limits typically grow less well than do other children. In addition to slow growth, mild hypothyroidism causes fatigue, dry skin, constipation, increased sleep requirement, and cold intolerance. Thyroid replacement therapy in mild hypothyroidism improves growth velocity, lipid profile, and energy level (Rose, 1995).

Primary hypothyroidism after cancer

Primary hypothyroidism (with TSH elevation) is most likely to occur in patients who have received mantle irradiation for Hodgkin's disease, craniospinal radiation for medulloblastoma, or total body irradiation prior to bone marrow transplantation. Primary hypothyroidism is rarely isolated in cancer survivors, who may also have GH deficiency, adrenocorticotropic hormone (ACTH) deficiency, and/or pubertal disorders (Sklar, 1997; Yeung *et al.*, 1998).

Thyroid Hormone Resistance and TSH-Secreting Adenoma

In thyroid hormone resistance and TSH-secreting adenoma, TSH is measurable (inappropriately) in the presence of elevated T4 and elevated FT4. In resistance, this biochemical picture is associated with few or no symptoms of hyperthyroidism. Patients may have goiter but may be euthyroid or even hypothyroid. In contrast, in TSH-secreting adenomas, patients are clinically hyperthyroid. Pituitary TSH-secreting adenomas are uncommon in general and are particularly rare in the pediatric age group.

Depressed TSH Secretion

Hyperthyroidism

Low serum TSH concentration is an appropriate response to excessive thyroid hormone levels resulting from either endogenous or exogenous source. Graves' hyperthyroidism is a result of antibodies to the TSH receptor, binding in a manner that activates the intracellular G protein signaling pathway as if TSH were binding at the receptor. A hypersecreting thyroid nodule (making thyroid hormone independent of TSH stimulation) will also suppress the TSH concentration. Hyperthyroidism in McCune–Albright syndrome is an example of a chronically activated G protein pathway.

Central Hypothyroidism

Reduced TSH surge

A blunted or absent nocturnal TSH surge is a characteristic of central hypothyroidism, suggesting a loss of the normal circadian variation in TRH (and thus a reduction in total TSH release) and loss of one-third to one-half of the daily trophic stimulus to the thyroid gland. In central hypothyroidism, FT4 is low or in the lowest third of the normal range, with a normal daytime TSH. Measurement of the nocturnal TSH surge has improved sensitivity for detection of central hypothyroidism compared with the TRH test. Central hypothyroidism (FT4 in the lowest third of the normal range) can be confirmed by the am to pm TSH ratio or the TSH surge test (Rose, 2010).

Low-normal T4 and FT4

Many patients with central hypothyroidism maintain normal daytime TSH concentrations and iodothyronine concentrations that are just below the normal range or in the lowest third of the normal range. These patients remain clinically puzzling and, therefore, are often not treated with thyroid replacement medication. Failure to recognize and treat central hypothyroidism can result in poor growth in children and in a less than optimal state of health in children and adults. Subtle hypothyroidism may be associated with depression and mild hyperlipidemia.

A cause of short stature

In children referred to endocrinology for evaluation of apparent idiopathic short stature, approximately 13% had isolated central hypothyroidism (a blunted TSH surge associated with low or low-normal FT4 in the absence of any other pituitary hormone disturbance). The incidence was 33% of children with height shorter than -2 SD who had an FT4 in the lowest third of the normal range. Growth velocity significantly improved in children during treatment with levothyroxine compared with that in children who had a normal TSH surge and who were otherwise clinically similar (Rose, 1995). Children with known hypopituitarism also show blunting of the nocturnal TSH surge. Familial central hypothyroidism has been recognized to be caused by an IGSF1 gene mutation or altered TSH beta subunits (Garcia *et al.*, 2017; Tenenbaum-Rakover *et al.*, 2016; Nicholas *et al.*, 2017).

Thyroid function after head injury

Disturbances in thyroid function are commonly observed in head-injured patients, including low T3 and low T4, usually with a normal level of TSH (Reifschneider *et al.*, 2015; Giuliano *et al.*, 2016). Reverse T3 is often elevated. Thyroid axis injury may still be quite difficult to identify in newly injured patients because of the prolonged half-life of T4 (7 days).

Central hypothyroidism after cancer

Central hypothyroidism is recognized in as many as 65% of patients after brain tumor or nasopharyngeal tumor, in more than 35% after bone marrow transplant, and in as many as 15% after leukemia, suggesting that central hypothyroidism (hypothalamic–pituitary–thyroid dysregulation) may be quite common in cancer survivors (Rose, 2001; Rose *et al.*, 2016). In contrast, primary hypothyroidism occurs most commonly after total body radiation or radiation to the nasopharynx, neck, or spine (Oudin *et al.*, 2016). Central and mixed hypothyroidism both occur in patients after radiation to the head and include brain tumors, nasopharyngeal tumors, and total body radiation (Rose, 2001). Chemotherapy alone can cause primary or central hypothyroidism, but the frequency of this occurrence is much less than that after radiotherapy.

Central hypothyroidism in adults

Central hypothyroidism can be quite difficult to recognize in adults where slowed growth rate is not available as a sign. Symptoms of hypothyroidism (e.g., asthenia, edema, drowsiness, adynamia, skin dryness) may be of gradual onset and be unrecognized until therapy is begun and the patient feels better (Razvi *et al.*, 2016).

Mixed Hypothyroidism

Mixed hypothyroidism consists of central hypothyroidism with TSH elevation. It has been described in survivors of childhood cancer and in women with Sheehan syndrome (postpartum pituitary necrosis). With reduced TRH from the hypothalamus, TSH may be abnormally glycosylated and of lower biological activity (Persani, 1998; Persani *et al.*, 1998). Thus, mild elevation of serum TSH concentrations (5–14 mU L^{-1}) may be seen in central hypothyroidism; the differentiation from primary hypothyroidism can be made by documentation of a blunted or absent TSH surge. In Sheehan syndrome patients, elevated 24-h TSH was observed with blunting of the TSH surge, suggesting dysregulated release of biologically inactive TSH.

Diagnosis of Hypothyroidism

Individual Set Point for TSH and FT4

FT4 is currently the best measure of thyroid status along with serum TSH. FT4 remains fairly stable in an individual over the years at an optimal "set point" for thyroid function (Andersen *et al.*, 2003). If FT4 production from an injured thyroid gland declines (as in mild primary hypothyroidism), the intact pituitary secretes more TSH. Thus, in primary hypothyroidism, a serum sample for TSH at 0800 h is the best test (Rose, 2010; Fitzgerald and Bean, 2016).

Monitoring

Yearly measurements of TSH and FT4 and growth surveillance are recommended in children at risk for hypothyroidism, such as in girls with Turner syndrome, children with Down syndrome, and childhood cancer survivors. Earlier diagnosis of mild hypothyroidism will allow earlier intervention to improve growth velocity and quality of life. In a healthy person with slow

growth or other symptoms of hypothyroidism, criteria for starting thyroid hormone therapy without further diagnostic testing include (1) TSH above 4 mU L^{-1} at 0800 or 0900 h or above 3 mU L^{-1} between 1000 and 2000 h (regardless of the FT4 value) and (2) FT4 at or below the lower limits of the normal range for the assay (regardless of the TSH value). If FT4 is in the upper two-thirds of the normal range and TSH does not meet the preceding criteria, no thyroid therapy is needed, and thyroid status and growth (in children) should be reviewed in 1 year in patients at risk. If FT4 is in the lowest one-third of the normal range without TSH elevation, the AM-PM TSH ratio should be measured (Rose, 2010). The combination of history of head injury or cranial or total body radiation, slow growth rate, normal weight gain, no intercurrent illness, delayed bone maturation, and declining FT4 or FT4 below the lower limits of normal or TSH above the upper limits of normal should be diagnostic of hypothyroidism.

Therapy for Hypothyroidism

Starting Thyroid Hormone Therapy

Thyroid hormone doses at initiation of therapy are much higher in infants (10–12 μg kg^{-1} BW day^{-1}) and toddlers (5–8 μg kg^{-1} BW day^{-1}) than in children (3 μg kg^{-1} BW day^{-1}) and adolescents or adults (1.25–1.50 μg kg^{-1} BW day^{-1}). These doses approximate 100 μg per meter squared per day.

Typical initial thyroid replacement dose in healthy young people with TSH of less than 30 mU L^{-1} and without risk of cardiac decompensation is 3 μg levothyroxine per kilogram body weight given daily in the morning (Khandelwal and Tandon, 2012). If TSH is more elevated or if there are concerns about medical stability, starting with one-quarter of this dose and increasing by a quarter dose each month can permit more gradual physiological and psychological adjustments to the new metabolic state. In general, because levothyroxine has a long half-life of about 6 days, it is useful to measure thyroid levels only after 4 weeks.

Thyroid Dose Adjustment

Thyroid dose should then be adjusted as follows: In primary hypothyroidism, TSH is the most useful test to monitor during therapy. The target should be a TSH of 0.5–1.5 mU L^{-1}, without symptoms of overreplacement. In central or mixed hypothyroidism, therapy with levothyroxine rapidly suppresses TSH before resolution of clinical symptoms, so TSH is not useful to measure. The recommendation is to monitor FT4 during therapy, adjusting the dose to achieve FT4 just above the middle of the normal range and without symptoms of hypo- or hyperthyroidism (Khandelwal and Tandon, 2012).

Thyroid Replacement and Adrenal Function

Because thyroid therapy can result in improved metabolic clearance of many substances such as cortisol, thyroid hormone replacement can result in clinical decompensation of patients with unrecognized adrenal insufficiency. In patients at risk, it is necessary to evaluate for primary adrenal insufficiency and/or hypothalamic ACTH deficiency and to provide such patients with hydrocortisone prior to initiating thyroid therapy.

See also: Hypothalamus–Pituitary–Thyroid Axis. Thyroid-Stimulating Hormone (TSH; Thyroptopin). TSH (Thyrotropin) Receptor. TSH-Producing Adenomas and Resistance to Thyroid Hormones.

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TSH (Thyrotropin) Receptor☆

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Glossary

G protein-coupled receptor The largest superfamily of cell surface receptors that couple to heterotrimeric G (GTP-binding) proteins and mediate cellular responses to a diverse array of signaling molecules, including peptides, glycoprotein hormones, neurotransmitters, phospholipids, and odorants, as well as photons. A common structural feature is the presence of seven transmembrane-spanning segments connected by alternating intra- and extracellular loops, with the N terminus located on the extracellular side and the C terminus located on the intracellular side of the membrane.

Graves' disease An organ-specific autoimmune thyroid disease, characterized by the presence of stimulating antithyrotropin receptor autoantibodies in patients' sera that mimic the action of thyrotropin, thereby overstimulating the thyroid gland and causing goiter and hyperthyroidism.

Leucine-rich repeats (LLRs) A motif comprising 20 to 30 aa tandemly repeated sequences found in numerous proteins of diverse origins and with varied functions, forming amphipathic β-sheet structures and likely mediating specific protein–protein interactions. The consensus sequence proposed for the TSH receptor is xLxxTxxLTxLPxxAFxxLxxLxxL, where x is any amino acid. Ribonuclease A inhibitor was the first protein with LLRs to have its three-dimensional structure solved.

Introduction

TSH receptor (TSHR) is the primary molecule regulating both differentiated function and growth of thyroid follicular cells. On binding of TSH, TSHR transduces signals through G_s–cyclic AMP (cAMP) and G_q–phospholipase C cascades. The former regulates iodide uptake and expression of thyroid-specific proteins, such as thyroid peroxidase, thyroglobulin, and the sodium–iodide symporter, and the latter regulates iodide efflux and H_2O_2 production. TSHR involves three thyroid pathologies: autoimmune thyroid disease, oncogenesis, and congenital thyroid dysfunction. TSHR is one of the main autoantigens in autoimmune thyroid disease; autoantibodies against TSHR stimulate thyroid cells in Graves' disease patients [thyroid stimulating antibodies (TSAb)] or block TSH action in some patients with hypothyroidism (TSH-blocking antibodies (TBAb)). Ectopic expression of TSHR may play a role in the pathogenesis of extrathyroidal manifestations of Graves' disease (ophthalmopathy and pretibial myxoedema). Gain- and loss-of function mutations of the receptor cause hyperfunctioning adenoma/non-autoimmune congenital hyperthyroidism and congenital hypothyroidism, respectively.

Structure and Function of TSH Receptor

The TSHR protein consists of 764 amino acids (aa) (including a 21 aa signal peptide) and is structurally and functionally divided into two domains: the large N-terminal extracellular domain (ECD) (aa 1-~415), a unique feature of GPHR subfamily and the transmembrane domain (TMD), a characteristic of the GPCR superfamily. From its N-terminus, the TSHR ECD comprises a cysteine-rich sequence called cysteine-box 1 (C-b1) (aa ~24-~41), leucine-rich domain (LRD) containing eleven leucine rich repeats (LRR) (aa ~35-~280) and the hinge region containing the C-b2, C-b3 and C-peptide (aa ~281-~415). The TMD contains seven transmembrane helixes connected with extracellular and intracellular loops, and ends with the C-terminal tail (see Fig. 1A).

TSHR undergoes a variety of posttranslational modifications. The nascent TSHR (~95 kDa) first transforms to the precursor protein attached to mannose-type oligosaccharides on six N-linked glycosylation sites (NxS/T) (~100 kDa) in the endoplasmic reticulum and then to the mature protein with complex oligosaccharides (~120 kDa) in the Golgi apparatus, which is thereafter expressed on the cell surface. Thus, TSHR is heavily glycosylated and contains an ~25 kDa glycan, which corresponds to 40% of the molecular mass of the A-subunit (see below).

TSHR undergoes intramolecular cleavage into two subunits: the TSH-binding A-subunit and the transmembrane B-subunit. This cleavage apparently occurs at multiple sites in the hinge region of the mature receptor on or near the cell surface, presumably by a membrane-associated matrix metalloproteinase, resulting in the removal of a peptide corresponding to an approximately 50 aa insertion (aa 317–366, called C peptide). Although these two subunits are still linked by disulfide bridges at this point, their reductions by a cell surface enzyme, possibly protein disulfide isomerase, releases the A-subunit, a phenomenon called "receptor shedding" (see Fig. 1B and C). The B subunit remains embedded in the plasma membrane. Cleavage does not affect the receptor

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This article is an update of Yuji Nagayama, TSH Receptor (Thyrotropin Receptor), In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 632–635.

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function in terms of high TSH-binding and TSH-mediated signal transduction. However, it is generally thought that the free A-subunit is more immunogenic than the single-chain receptor or two-subunit receptor, and may be the main autoantigen in Graves' disease.

There are 11 cysteines in the ECD, clustering in three distinct regions; C-b-1, 2 and 3. There are two disulfide bridges in the C-b1 between C24 and C29, and C31 and C41, and three in the C-b2/3 between C283 and C398, C284 and C408, and C301 and C390 (Fig. 1).

Other posttranslational modifications include (1) sulfation of Y385, which is required for high-affinity binding for TSH, but not TSAb; (2) palmitoylation of C699 in the C-terminal cytoplasmic tail of the receptor, which positively controls the rate of intracellular trafficking of the receptor; and (3) the formation of oligomers, which rapidly dissociate into monomers upon TSH binding.

The crystal structures of TSHR aa 1–260 complexed with TSAb monoclonal (M22) or TBAb monoclonal (K1-70) were resolved, from which, together with that of FSH-FSHR ECD complex, the detailed structure of TSHR ECD bound to TSH, TSAb and TBAb were determined. As shown in Fig. 2, the amino acid residues on the receptor involved in binding for all the ligands widely distributes over the concave of the LRD, and, in case of TSH and TSAb, further extend to C-b1 and the hinge region. Thus, the ligand binding site(s) on the receptor are conformational and consists of multiple, discontinuous amino acid sequences. As mentioned above, Y385 binds to TSH, but not antibodies. Finally the binding sites for TSH, TSAb and TBAb are substantially overlapping.

TSH Receptor Gene

The TSHR gene consists of 10 exons and 9 introns and spans > 60 kb on human chromosome 14q. Exons 1–9 encode most of the ECD, with each exon corresponding to a LRR, and exon 10 encodes part of the ECD and the TMD. These findings indicate that the TSHR gene is derived from the integration of multiple genes, each encoding a LRR into a prototypic intronless G protein-coupled receptor gene. The full-length TSHR cDNA is approximately 4 kb long and contains a single open reading frame of 2292 bp.

Multiple TSHR mRNAs are detected in the thyroid tissue; there are major transcripts of ~4 kb and several other minor transcripts. The former correspond to the full-length receptors with distinct 3′-untranslated regions and the latter correspond to alternatively spliced, truncated receptors lacking the TMD.

The promoter region of the TSHR has characteristics typical of those of housekeeping genes with multiple transcription start sites. TSH induces a transient increase and subsequent down-regulation of TSHR expression under in vitro culture conditions, whereas TSHR mRNA levels are relatively stable in vivo. The minimal sequence required for thyroid-specific expression and negative regulation by TSH is located at −195 to −39 bp of the 5′-flanking region.

TSHR is also expressed in the extrathyroidal tissues such as fat tissues and retro-orbital fibroblasts, which may be relevant for extrathyroidal manifestations of Graves' disease.

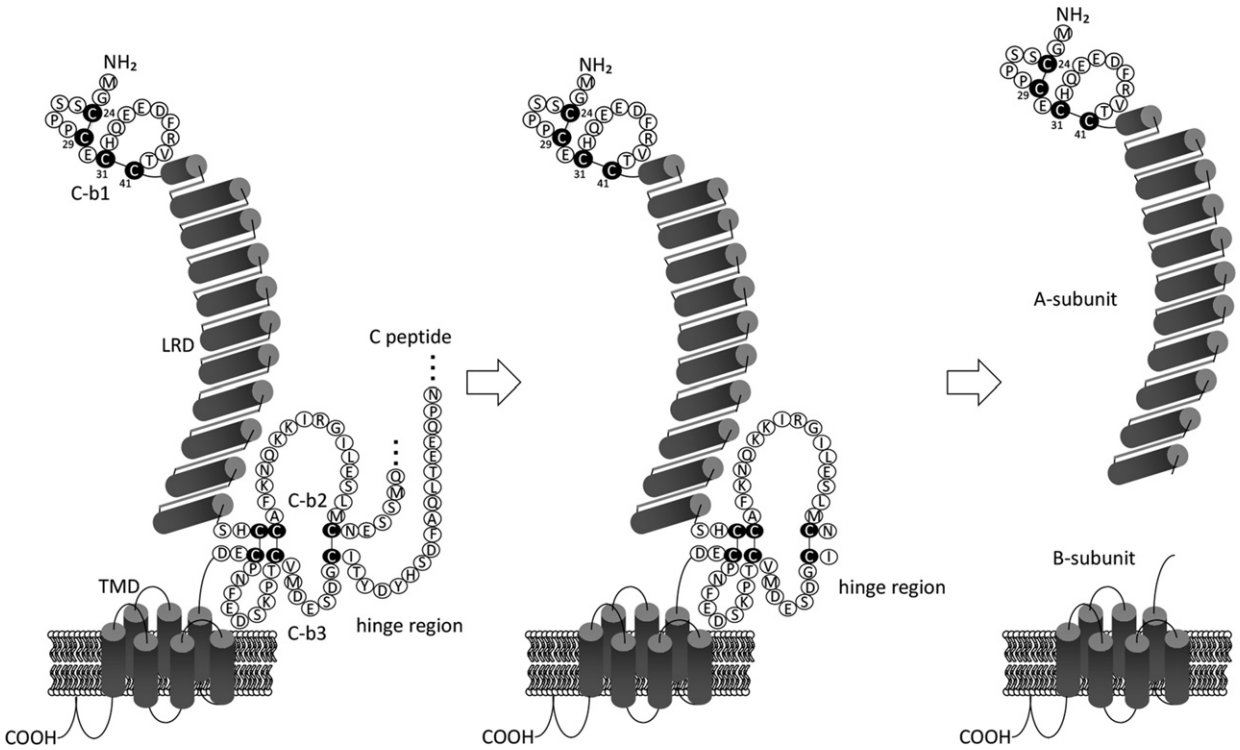

Fig. 1 Schematic representations of TSH holoreceptor (A), two-subunit receptor (B) and separated two-subunits (C).

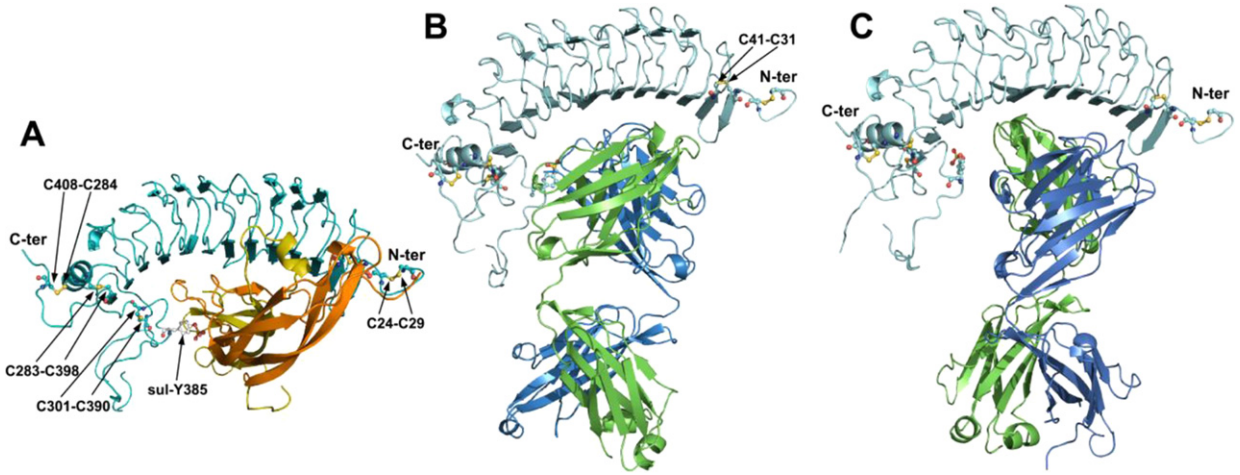

Figure 2 Comparative models of (A) the TSH-TSHR ECD complex (conformational arrangements show a final stage in the proposed activation mechanism when sulphated Tyr 385 is bound to the hormone; see Figure 10D), (B) the M22-TSHR ECD complex and (C) the K1-70-TSHR ECD complex. The TSHR ECD is shown in cyan, the hormone α subunit is shown in olive and β subunit in orange. Antibody light chains are shown in green and heavy chains in blue. The N- and C-termini of the TSHR ECD, disulphide bonded cysteines and the TSHR sulphated Tyr385 are marked. Reproduced, with permission of the copyright holder RSR Ltd, from Figure 9 of Furmaniak *et al.* (Horm Metab Res.47,735-752,2015).

Autoimmunity

As mentioned above, the TSHR autoantibodies act as agonists in patients with Graves' disease (TSAb) or as antagonists in some patients with autoimmune hypothyroidism (TBAb). To detect these antibodies, two assays have become available. One is a bioassay for TSAb that measures agonist-mediated cAMP production using thyroid cells of various species or mammalian cells stably expressing recombinant TSHR. The other is competition of antibodies for ^{125}I-, chemiluminescence- or biotin-labeled TSH (or M22) binding to TSHR [TSH-binding inhibiting antibody (TBIAb)]. Although the TBIAb assay has long used porcine thyroid membrane as an antigen, a highly sensitive TBIAb assay with recombinant human TSHR has become commercially available. However, this assay cannot discriminate between stimulating and blocking antibodies.

TSHR autoantibodies can be experimentally elicited by traditional immunization approaches using the soluble TSHR protein with classical adjuvants, which, however, are usually without noticeable TSAb activities. In contrast, immunization of mice with syngeneic cells co-expressing TSHR and major histocompatibility complex class II antigen or genetic immunization with plasmid- or adenovirus-encoded human TSHR can effectively induce TSAb and Graves'-like hyperthyroidism. Since Graves' disease is an antibody-mediated disease, it has long been believed that the T helper 2 cell (Th2)-based immune response is predominant in Graves' disease. However, studies with these animal models challenge this concept, suggesting that the Th1 immune response may be more critical than previously anticipated or that the Th1/Th2 paradigm may be too simplistic to explain the pathogenesis of Graves' disease. Th17, a newly identified immune response, unlikely participates in the pathogenesis in animal models.

Development of Graves' disease is controlled by both genetic and environmental factors. The TSHR gene is one of the candidates for polygenic, inherited predisposing gene of Graves' disease. The TSHR gene polymorphisms conferring susceptibility are located in the intron 1, regulating relative expression levels of alternatively spliced form of the receptor (similar to the A-subunit) in the thyroid and of the holoreceptor in the thymus.

Naturally Occurring Mutations

Numerous mutations in the TSHR gene causing loss- or gain-of-function have been identified. Constitutively activating mutations were found in hyperfunctioning thyroid adenomas and autosomal-dominant congenital non-autoimmune hyperthyroidism. Most of the mutations are point mutations, resulting in a single amino acid substitution, and are localized in the TMD. TSHR is "noisy," that is, it displays significant constitutive activity even in the absence of agonist. Unliganded ECD likely constrains the receptor activity. In contrast, loss-of-function mutations in congenital hypothyroidism associated with resistance to TSH can be found in any region of the receptor. Furthermore, a unique mutation (K183R), which increases the sensitivity to human CG and causes a type of familial gestational hyperthyroidism, has also been identified.

See also: Sodium/Iodide Symporter (NIS). Thyrotoxicosis; Diagnosis. TSH Function and Secretion

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Thyroid Hormone Metabolism☆

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Abbreviations

ADRA2	α2-Adrenergic receptor
DIO	Deiodinase
K_m	Michaelis–Menten constant
LAT	L-type amino acid transporter
MCT8	Monocarboxylate transporter 8
OATP	Organic anion transporter
Pi3K	PI3-kinase
RXR	Retinoic acid receptor
TAAR	Trace amine-associated receptor
TBG	Thyroxine-binding globulin
Tetrac	Tetraiodothyro-acetic acid
TH	Thyroid hormone (T4,T3)
THM	Thyroid hormone metabolites
T4	3,3′,5,5′-Tetraiodo-L-Thyronine (Thyroxine)
T3	3,3′,5-Triiodo-L-Thyronine
rT3	3,3′, 5′-Triiodo-L-Thyronine (reverse T3)
3,5-T2	3,5-Diiodo-L-Thyronine
3-T1AM	3-Iodo-thyronamine
TR	T3 Receptor
TRH	Thyrotropin releasing hormone
Triac	3,3′,5-Triiodothyro-acetic acid
TRPM8	Transient receptor potential cation channel subfamily M member 8
TSH	Thyrotropin, thyroid stimulating hormone
TTR	Transthyretin

Glossary

Conjugation The 4′-OH group of THM that carry iodine substituents at the phenolic ring is rather acidic and is conjugated by sulfotransferases or glucuronyltransferases. These reactions generate THM with higher solubility than those of the native highly hydrophobic iodothyronines. Conjugated THM enter the bile excretion pathway and may undergo enterohepatic circulation. Sulfation of THM occurs in the fetus and may contribute to the generation of a more soluble reservoir form of thyroid hormones. Sulfatases cleave the 4′-*O*-sulfate ester of THM and liberate the free thyroid hormone.

Deiodination The iodinated thyroid hormones thyroxine (T_4) and 3,3′,5-triiodothyronine (T_3) and their THM undergo several steps of enzymatic reductive deiodination either at the 5′ (phenolic ring or outer ring) or 5 position (tyrosyl ring or inner ring), leading to the release of iodide and the formation of hormonally active T_3 from T_4 or inactive metabolites such as reverse T_3 (3,3′,5′-triiodothyronine), 3,3′-T_2, or monoiodinated metabolites. Also, 4′-O-sulfated THM including thyronamines can be deiodinated. The action of deiodinases as "gatekeepers" to nuclear action of the active thyroid hormone T_3 is essential for the proper coordinated development of vertebrate organisms.

Euthyroid sick syndrome or low T_3 syndrome A clinically relevant constellation in the course of a critical illness, with rapid decrease in serum concentrations of T_3, increased serum reverse T_3, and unaltered or decreased production rate of T_4 while thyrotropin (TSH) remains normal or even decreases. Persistence of this syndrome and decreased T_4 concentrations are associated with an increased mortality. In critically ill patients, mean nocturnal TSH secretion and the number of TSH surges are markedly reduced. This syndrome develops by a combination of interference of proinflammatory cytokines in the negative feedback regulation at several levels, disturbance of hypothalamic stimulation of the thyroid and growth hormone axes, and poor tissue perfusion resulting in decreased expression of 5′-deiodinases (DIO1, DIO2) and increased expression of 5-deiodinase (DIO3) activity.

Iodothyronines Iodinated derivatives of the thyroid hormones T_4 and T_3 that are formed by sequential deiodination of T_4 and T_3 or that are sulfated or glucuronidated metabolites. Apart from T_3, the other iodothyronines, including T_4, do not bind to the nuclear T_3 receptor in vivo and therefore are not thyromimetically active. 3,5-T2 also exerts thyromimetic activity probably via mitochondrial mechanism of action and at high concentrations via T_3 receptors.

Thyroid hormone The thyroid produces and secretes T_4 and T_3, which are iodinated derivatives of the amino acid tyrosine. T_4 is considered as prohormone that acts primarily after 5′-deiodination to T_3, the active thyroid hormone. Some rapid T_4 effects may be exerted via binding and activation of $\alpha v\beta 3$ integrin receptors at the plasma membrane. Thyroid hormones regulate the development, differentiation, growth, energy, and structural metabolism of higher vertebrates, including humans. Thyroid hormones act in a permissive way in close cooperation with other hormonal, neuronal, and nutritive signals. Deficiency of iodine or thyroid hormones during pregnancy leads to severe impairment of the development of the central nervous

☆*Change History*: February 2018. Josef Köhrle has updated the chapter. New information especially on recent developments of pre-receptor control of T3 ligand availability for T3 receptors, which is controlled by thyroid hormone transmembrane transporters and deiodinase isoenzymes, was added and recent findings on the role of thyroid hormone metabolites such as thyronamines and thyroacetic acids and their receptors and mechanism of action was included.

This article is an update of Josef Köhrle, Thyroid Hormone Metabolism, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 480–489.

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system (cretinism) and impairment of many metabolic functions in the adult organism.

Thyroid hormone metabolites This term comprises the classical thyroid hormones T_4 and T_3, their deiodinated metabolites and their recently re-discovered decarboxylated metabolites, the thyronamines 3-iodo-thyronamine and the iodine-free thyronamine as well as the oxidized metabolites of the latter two compounds, i.e., 3-iodo-thyroacetic acid and thyroacetic acid, all of which show various pharmacological actions distinct from those of the classical thyroid hormones T_3 and T_4.

Introduction

The roles of the essential trace element iodine and of the thyroid gland for development and normal function of vertebrate organisms, especially in humans, have been known for a long time (Hoefig *et al.*, 2016), but it was not until 1915 that the iodine containing prohormone thyroxine (T_4) was isolated by Edward C. Kendall. Charles R. Harington and George Barger performed the correct structure analysis and chemical synthesis of T_4 in 1927. In 1952 Jack Gross and Rosalind Pitt-Rivers concomitantly with the French team Jean Roche, Serge Lissitzky, and Raymond Michel identified the bioactive TH 3,3′,5-triiodothyronine (T_3), the "real TH" (**Fig. 1**). Various enzymes metabolizing the amino acid-derived TH have been characterized during the past 65 years. These enzymes generate a complex pattern and network of T_4-derived iodinated metabolites (THM), some of which have specific biological functions that differ from those of the bioactive TH T_3. T_3 exerts its influence mainly by modulation of action of the ligand-dependent transcription factors, the nuclear T_3 receptor forms (TRα and TRβ) (Tata, 1999). In addition to this key mechanism of action, several other biological effects of T_4, T_3 and THM derived therefrom have been reported. However, these effects are less well characterized (e.g., activation of plasma membrane associated integrin receptor $\alpha v\beta 3$, several intracellular kinase cascades [e.g., Pi3K] modulation of the organization of the cytoskeleton, or direct influence of THM on mitochondrial function). Some of these rapid effects also involve ligand binding to TR, but do not require DNA binding and transcription modulation of the TR-THM complexes. Iodothyronines comprise iodinated metabolites of the prohormone T_4 that have an intact diphenylether structure which is generated by coupling of two iodinated tyrosines residues of thyroglobulin during biosynthesis of TH in the thyroid gland. The sole source of T_4 in humans and higher animals are the angiofollicular units of the thyroid gland, whereas the other THM are formed intracellularly in various organs and tissues (Bianco *et al.*, 2002; Köhrle, 2000). T_4 is a highly hydrophobic, lipid-soluble compound that circulates in the blood bound to three main distribution proteins: thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin. T_4 and THM are excreted mainly via the bile and the feces. Iodide liberated

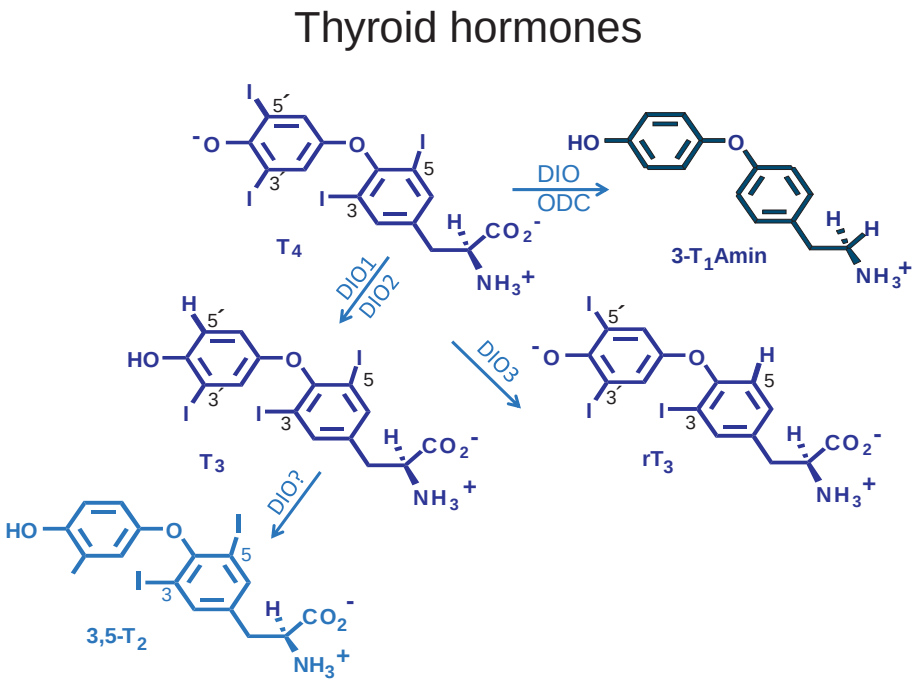

Fig. 1 Enzymatic deiodination of the prohormone T_4 to the thyromimetically active T_3 by the selenoenzymes type 1 or type 2 5′-deiodinase and inactivation of T_4 to rT_3 by type 3 5-deiodinase (DIO). 3,5-T_2 is a further thyromimetic TH metabolite, probably generated by DIO activity. 3-T_1AM represents a novel TH metabolite mainly antagonistic to T3 action. 5′-D, 5′-deiodination (phenolic or outer-ring deiodination); 5-D, 5-deiodination (tyrosyl- or inner-ring deiodination). Numbers indicate substituent positions (*n′*) at the phenolic (outer) ring or (*n*) at the tyrosyl (inner) ring.

from the THM by enzymatic deiodination, together with a fraction of nutritional iodide uptake, is excreted into the urine. Urinary iodide concentrations provide a valuable indicator of the iodine supply of the organism.

T_4 and THM are derivatives of the amino acid tyrosine and carry several ionic charges in solution. Therefore, efflux of T_4 and T_3 from the thyrocytes and uptake of THM into target cells require transport processes across cellular membranes such as facilitated, active or passive carrier-mediated transport, which is catalyzed by several specific proteins such as members of the monocarboxylate transporter (MCT8. MCT10), organic anion transporter (OATP1c1) or L-type amino acid transporter (LAT1, LAT2) families, which are not yet fully characterized with respect to their relevance and functional characteristics (Hennemannn *et al.*, 2001; Wirth *et al.*, 2014). Mutations of the *MCT8* gene cause a severe X-linked neuromuscular developmental disorder, the Allan–Herndon–Dudley syndrome (Groeneweg *et al.*, 2017). Tissue- and development-specific expression of these TH transmembrane transporters contributes to the bioavailability of THM in cells and organs and provides a first level of control for metabolism of THM by intracellular enzymes that depend on substrate availability (Köhrle, 2007). No enzymes metabolizing THM in the blood or in the interstitial space have been identified. The free THM concentrations in serum and interstitial space are very low in the picomolar range due to the binding of T_4 and T_3 by the high-affinity distribution proteins TBG, TTR, and albumin. With exception of 3-T1AM that has high affinity to apolipoprotein B100, the other THM exhibit lower affinity for these binding proteins than T_4 and T_3 and thus might be more available to metabolic transformations.

Enzymatic Deiodination of Thyroid Hormone Metabolites

Sequential Reductive Monodeiodination Both Activates and Inactivates Thyroid Hormone Metabolites

Reductive deiodination of the prohormone T_4 generates iodothyronine metabolites with different biological function (**Fig. 1**) (Dentice *et al.*, 2013). Deiodination at the 5′-position of the phenolic (outer) ring of T_4 produces the thyromimetically active hormone T_3. Deiodination at the 5-position of the tyrosyl ring (inner ring) generates reverse-T_3 (rT_3), a metabolite devoid of thyromimetic action at the nuclear T_3 receptors but with the potential to act as a competitor to T_4 in the 5′-deiodination reaction. rT_3 circulates in the serum of normal persons in concentrations similar to those of T_3. DIO enzymes are expressed in a development-, tissue-, and cell-specific manner and are regulated by different factors (Köhrle, 2002). All three DIO enzymes belong to the class of selenocysteine-containing proteins and are encoded by three different genes. With few exceptions, selenium availability is not a limiting factor in humans for the expression of the three DIO selenoenzymes, which rank very high in tissue-specific selenium supply among the selenoproteins. However, in vitro and animal studies indicate selenium-dependent regulation of the three DIO enzymes. Only during severe selenium deficiency, under protein-free diets due to metabolic diseases (phenylketonuria), long-term total parenteral nutrition, or malabsorption syndromes selenium deficiency may impair expression of deiodinase enzymes and T_3 formation in children and adults. All three DIO isoenzymes are obligatory intracellular integral membrane proteins with their active sites oriented toward the cytosol. Evidence has been presented that inactivation of the DIO enzymes, especially DIO2, occurs by the ubiquitin-associated proteasome protein degradation system (Bianco *et al.*, 2002). Reductive deiodination of THM by DIO isoenzymes requires reduced cellular cosubstrates such as thioredoxins or glutathione, while reduced dithiols (DTT or DTE) are used as cosubstrates for in vitro activity measurements (Köhrle, 2002).

Two 5′-Deiodinases Are Involved in Activating Thyroid Hormone Metabolism

5′-Deiodination at the phenolic ring is catalyzed by two enzymes, the type 1 and the type 2 5′-deiodinase (DIO1 and DIO2, respectively), which differ in various aspects as indicated in **Table 1** (Köhrle, 2000; Bianco *et al.*, 2002). DIO1 is less selective in substrate specificity than DIO2, which preferentially deiodinates T_4 with a low-nanomolar Michaelis–Menten constant (K_m) for this substrate. DIO2 produces T_3 mainly for the local supply in tissues expressing this enzyme, which renders these tissues independent from circulating T_3. DIO2 does not contribute significantly to circulating plasma T_3, which is mainly provided by DIO1 action on T_4 in liver, kidney, and thyroid tissues expressing high DIO1 activity in normal healthy subjects. The extent to which thyroid DIO1 activity contributes to T_3 secreted by the thyroid gland, which also produces T_3 apart from T_4 during TH synthesis especially under conditions of iodine deficiency and in Graves' autoimmune thyroid disease, is unclear. In the latter constellation, DIO2 activity also adds to thyroid T_3 production from T_4 because DIO2 expression in Graves' disease is stimulated by TSH receptor-stimulating autoantibodies, similar to TSH stimulation of DIO2 in autonomous adenoma. Thyroid stimulation by TSH or TSH receptor-stimulating autoantibodies also enhances the ratio of T3 versus T4 formation in thyroglobulin. DIO1 also contributes to the inactivation of T_4 by deiodination at the tyrosyl ring, generating rT_3 and degrading T_3 to 3,3′-T_2. Especially, sulfated iodothyronines are good substrates of DIO1 (Visser *et al.*, 1998).

Type 3 5-Deiodinase Inactivates Thyroid Hormones

The third deiodinase, 5-deiodinase (DIO3), removes iodine from the 5 position of the tyrosyl ring (inner-ring deiodination) (**Fig. 1**). DIO3 is responsible for the majority of rT_3 formation and T_3 degradation and is considered the main TH-inactivating enzyme (Dentice *et al.*, 2013; Bianco *et al.*, 2002). Expression of DIO3 occurs in several tissues and cells that do not respond to TH action, especially in fetal tissues, undifferentiated progenitor and tumor cells. Thus, DIO3 can be considered as one of the

Table 1 Properties of the Deiodinase Enzymes

	Type 1 5′-deiodinase DIO1	*Type 2 5′-deiodinase DIO2*	*Type 3 5-deiodinase DIO3*
Function and substrate preference	Systemic > local T_3 production; degradation of rT_3 and sulfated T4, T3 and other THM; rT3 > T4S > T3S > T4, T3	Local > systemic T_3 production; T4 > T3	Inactivation of T_4 and T_3; T3 > T4
Expression	Liver, kidney, thyroid, pituitary; at low levels in heart and many other tissues; loss of expression in tumors; higher expression in hyperthyroidism	Pituitary, brain, brown adipose tissue, skin, placenta; thymus, pineal; glial cells, tanycytes; higher expression in hypothyroidism; overexpression in mesothelioma cell line	Placenta, uterus, brain, fetal tissues; many other tissues, except normal adult pituitary and adult healthy liver, thyroid, kidney; re-expression in pathological organs (liver, pituitary); overexpression in hemangioma, activated macrophages, neutrophils and several solid tumors
Subcellular location	Endoplasmatic reticulum in liver; inner plasma membrane in kidney and thyroid	Inner plasma membrane; p29 subunit associated with F-actin respectively perinuclear vesicles	Endoplasmatic reticulum
Human gene location	1p32–p33	14q24.3	14q32
Enzyme stimulation or induction	T_3, retinoids; TSH and cAMP in thyroid only; testosterone in liver; selenium; carbohydrate	cAMP; FGF; β-adrenergic agonists; nicotine; phorbolesters via PKC; ANP and CNP via cGMP in glial cells	T_3, FGF, EGF; selenium
Inhibition	PTU, iodoacetate, aurothioglucose, genistein, F21388, iopanoate, xanthohumol, dexamethasone	T_4, rT_3; iopanoate, xanthohumol, F21388	Iopanoate, xanthohumol

Abbreviations used: ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide; DTE, dithioerythreitol; DTT, dithiothreitol; EGF, epidermal growth factor; F21388, 5′-deiodinase inhibitor; FGF, fibroblast growth factor; PKC, protein kinase C; PTU, 6-*n*-Propyl-2-thiouracil; THM, thyroid hormone metabolites.

components of a relay of "gatekeepers" of TH action that prevent cellular T_3 action from occurring at an inappropriate time, location, or concentration (Köhrle, 2000). Because DIO3 can act on both T_4 (forming rT_3) and T_3 (forming 3,3′-T_2), it can perform the gatekeeping function in cell types that are exposed to T_4 and/or T_3 and thus might act independently of the expression pattern of cell type-specific TH transporters, restricting the uptake of these TH. On the other hand, the development-, tissue-, and cell-specific expression pattern of the two 5′-deiodinase enzymes provides a system of systemic or local production of the thyromimetically active nuclear receptor ligand T_3. Expression of the DIO isoenzymes in hypothalamic TRH neurons and the thyrotrope cells of the anterior pituitary controls the local production and concentrations of T_3, which suppresses gene expression and secretion of TRH and TSH, respectively (Gereben *et al.*, 2015). Therefore, DIO isoenzymes play a key role in negative feedback regulation of TRH and TSH by circulating TH concentrations. Recently, a delicate antidromic balance of coordinated expression of DIO2 and DIO3 isozymes has been reported during commitment, differentiation and proliferation of various progenitor cells and tumor stem cells (Dentice *et al.*, 2013). While proliferation seems to require high levels of DIO3 activity resulting in low local T3 concentration, cellular differentiation is characterized by high DIO2 activity and enhanced local T3 production delivering the thyromimetic ligand T3 for binding to T3 receptors, which may function as tumor suppressor genes.

Intracellular Targeting and Compartmentalization of Active and Inactive Iodothyronines

Because all three DIO isoenzymes are located inside the cell with active sites exposed toward the cytosol (**Fig. 2**), their action also depends on the availability of the main substrate T_4, which reaches the cytosol via cell-type-specific T_4 carriers such as MCT8. T_3 entering the cells via the same or distinct TH transmembrane transporters may therefore bypass the control exerted by the intracellular DIO network and gain direct access to the intracellular T_3 receptors. T_3 formed by the high activity of 5′-DI in liver, kidney, or thyroid might not be targeted to the nuclear T_3 receptor of the same cells but rather channeled toward efflux and export

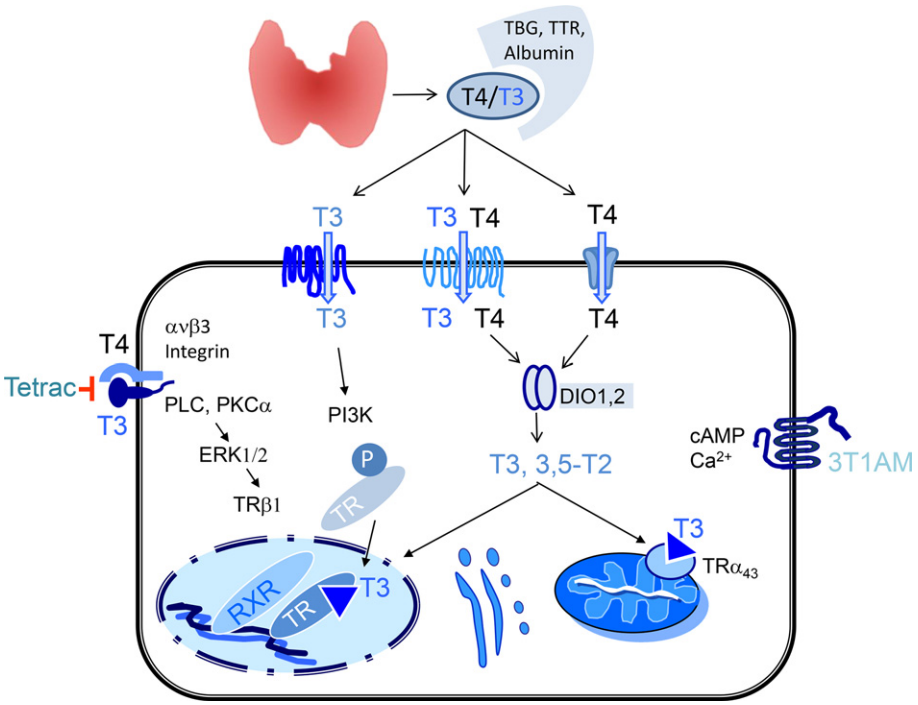

Fig. 2 Schematic representation of TH synthesis by the thyroid; transport and distribution by the serum-binding proteins, thyroxine-binding protein (TBG) and transthyretin (TTR); cellular uptake by thyroid hormone transporters such as MCT8, OATP1, LAT2; intracellular activation of the prohormone T_4 to the thyromimetically active hormone $T_3$by Dio; binding of T_3 to nuclear, cytosolic or mitochondrial T_3 receptors; and modulation of transcription of T_3-regulated genes. T_4 and T_3 activate and Tetrac inhibits the integrin receptor $\alpha v\beta 3$; TH also activate cytosolic kinase cascades; $3T_1AM$ activates plasma membrane signaling via TAAR, ADRA2 or TRPM8; RXR, retinoic acid receptor; TR, T_3 receptor.

into circulation via TH transmembrane transporters and thus reach (other) target cells only after its transient circulation in the blood pool of TH.

Iodothyronine Deiodinases Participate in the Control of Cell-Type-Specific Production and Action of Thyroid Hormone

It has long been known that TH action, mainly exerted by the thyromimetically active T_3, and to some extent also by 3,5-T_2, depends on the cellular expression pattern of T_3 receptors, which mediate TH action on gene expression (Tata, 1999). The cloning of several isoforms of (nuclear) TH receptors, encoded by two different genes (TR-α and TR-β), which occur in different splice forms that bind either both ligand and DNA (TR-α_1, TR-β_1, TR-β_2, and several variants) or only hormone-responsive elements of DNA (TR-α_2), added to the complexity of the regulation of TH action. This complexity has become even more complicated by the demonstration that in many instances the cellular location of the T_3 receptor differs from that of T_3 formation by the DIO1 or DIO2 enzymes. Two examples are the distinct location of Dio2-forming T_3 and the T_3 receptor-mediating T_3 action in the development of the inner ear hair cells and cochlea or the retina development of the eye. Whereas T_3 receptors are located in the sensory epithelium of the inner ear, the Dio2 enzyme producing the active ligand T_3 is located in the surrounding connective tissue cells in a development-dependent manner. This implies that T_3 produced from T_4 must be released from the connective tissue stromal cells and taken up by the sensory epithelial cells. Therefore, defects in both the time-dependent and the spatial organization of this network of ligand availability might lead to disturbed hearing function, known to be affected by fetal or congenital hypothyroidism. During retina development of the eye, a close correlation between the expression of T_3 receptors, Dio2 and Dio3 is reported. Local overexpression of Dio3, removing the active ligand T_3, disturbs the development of the eye. Coordinated temporal compartmentalization of the T_3 receptors, the ligand-activating Dio2, and the ligand-inactivating Dio2 also occurs at the implantation site of the embryo in the uteroplacental unit and the fetal epithelium. Again, the expression patterns of the three Dio isoenzymes appear strictly associated with different cell types in a time- and space-dependent manner, thus allowing for strictly controlled ligand availability to the developing fetus. In the latter case, especially the temporal expression pattern of the T_3- and T_4-inactivating enzyme Dio3, is essential for the control of TH transfer from the maternal to the fetal compartment because it is well-known that excessive exposure of the fetal tissues and the fetal brain to TH leads to irreversible brain damage such as occurs in uncontrolled maternal hyperthyroidism during pregnancy. Comparable antidromic developmental and cell-type specific patterns of expression of TH transmembrane transporters (e.g., MCT8, MCT10 and OATP1c1), DIO isozymes and TR forms have been

reported and studied during fetal and neonatal brain development (Wirth *et al.*, 2014). Recent evidence—especially established during molecular analysis of defects underlying the Allan–Herndon–Dudley syndrome caused by MCT8 mutations—indicates that prereceptor control of local availability of the thyromimetically active ligand T3, which is binding and modulating TR function, is a key mechanism for adequate brain development. While endothelial cells express TH transmembrane transporters and deliver TH to brain cells, astrocytes and tanycytes are the main cell types expressing Dio2, generating the active ligand T3 for neurons, which are devoid of Dio2 but express TR and Dio3 inactivating this hormonal signal. Further detailed analysis is required to map the temporal and spatial expression patterns of TH transmembrane transporters on brain cells during development and in the adult differentiated brain.

Amphibian Metamorphosis Is a Paradigm of Spatial and Temporal Control of Thyroid Hormone Expression

Similar patterns of spatial and temporal control of the concentration of active ligand T_3 have been demonstrated for the developmental program of expression of T_3 receptors, DIO enzymes and TH transmembrane transporters during amphibian metamorphosis that is under fundamental control by TH (Tata, 1999). Although the thyroid gland provides the prohormone T_4 and a small amount of circulating T_3 (which might be needed to prime the metamorphic events), the local production and removal of the active ligand T_3 are under the control of the TH transmembrane transporter and DIO network, which is expressed in a cell-type-specific manner. Disruption of these organized networks of control of ligand availability for T_3 receptors might occur during various diseases, affecting either the cellular organization of tissues and or the expression of the three DIO enzymes.

Aberrant and Inappropriate Expression of Deiodinases

The concept of the pre-receptor control of availability of the thyromimetically active ligand T_3 by appropriate expression of TH transmembrane transporters and DIO isoenzymes (Köhrle, 2000; Köhrle, 2007) has gained support by the identification of several pathological constellations in which aberrant or inappropriate expression of DIO isoenzymes has been observed. The first example is the overexpression of DIO3 in some infantile hemangiomas leading to extremely severe hypothyroidism that in some cases cannot be treated with excessive doses of administered T_4—a syndrome called "consumptive hypothyroidism." The opposite constellation is found for some mesothelioma tumors, in which overexpression of DIO2 leads to elevated T_3 production. For DIO1, less clear-cut alterations of expression have been described in humans. Remarkably low DIO1 expression has been reported for several solid tumors, in part associated with altered expression of TR forms or even TR mutants, while DIO1 re-expression in these tumor cells prevents their proliferation and migration in vitro.

However, several animal experimental models with altered expression or regulation of Dio1 activity have been described. Decreased (hepatic) expression of Dio1 leads to constellations characterized by normal to elevated serum T_4, decreased or normal serum T_3, and elevated rT_3. The latter parameter might be the leading sign of decreased expression of Dio1 because rT_3 is almost exclusively degraded by this Dio enzyme, whereas its production occurs via Dio3 and, to a minor extent, Dio1. Therefore, the ratio of serum T_3/rT_3 might be the most sensitive parameter of altered Dio1 activity. Recent studies in various animal models, mimicking the nonthyroidal illness, low T3 syndrome, also revealed evidence for de novo expression of Dio3 under such conditions in activated monocytes and neutrophils or parenchymal tissues such as liver (van der Spek *et al.*, 2017). Thus, both elevated Dio3 and/or impaired Dio1 activity might contribute to decreased T_3 and elevated rT_3 concentrations in blood.

Several polymorphisms have been reported in the *DIO* genes gene in association with altered plasma TH concentrations and THM ratios. In combination with a further polymorphism of the TSH receptor gene, clear-cut alterations of the setpoint of the pituitary feedback control of TH secretion and homeostasis are apparent (Peeters *et al.*, 2003a). A polymorphism in the *DIO2* gene leading to an amino acid exchange in the enzyme has been found to be associated with obesity in diabetic patients and linked to neurodegenerative disorders.

Low T_3 Syndrome or Euthyroid Sick Syndrome

A long-standing clinically relevant conundrum is the low T_3 syndrome or the euthyroid sick syndrome, characterized by low serum concentrations of T_3, elevated rT_3, and normal, elevated, or, in later stages, decreased serum T_4 without appropriate compensatory elevation of serum TSH concentrations (de Vries *et al.*, 2015). This peculiar hormone constellation has initially been explained by decreased hepatic T_3 formation by Dio1, normal or decreased TH synthesis, and lack of pituitary stimulation of TSH secretion (**Table 2**). Many facets of regulation of thyroid hormone homeostasis contribute to this syndrome, which is observed during carbohydrate starvation, acute and chronic illness, after surgical intervention, trauma, infection, sepsis, or after administration of certain drugs that inhibit (hepatic) DIO1 activity. In addition to decreased hepatic and, in some cases, impaired renal and thyroidal DIO1 activity, the remarkable interruption of the normal negative feedback of TH at the hypothalamic and pituitary level is a hallmark of this syndrome. Experiments using various animal models, including knockout mice models, suggest that elevated concentrations of proinflammatory cytokines (interleukins-1 and -6, tumor necrosis factor-α, and interferon-γ) under these conditions contribute to the inhibition of Dio1 activity while concomitant hypoxia and altered cellular redox state may elevate Dio3

Table 2 Pathogenesis of low serum concentrations of T_3 and T_4 in the euthyroid sick syndrome of nonthyroidal illness

Low T_3/T_4 state
Decreased hepatic Type I 5′-deiodinase activity
Increased Type III 5-deiodinase activity
Decreased concentrations of serum thyroid hormone-binding proteins
Increased circulating inhibitors of binding of T_3 to serum proteins
Decreased tissue uptake of T_3 by peripheral tissues
Decreased TRH, TSH and thyroid hormone secretion
Hypercortisolism
Elevated proinflammatory cytokines (IL-1, IL-6, and TNF-α)
Interference of drugs with serum binding, tissue uptake, and 5′-deiodination (dopamine, glucocorticoids, propranolol, uremic compounds)

Abbreviations used: IL, interleukin; TRH, thyrotropin releasing hormone; TSH, thyroid-stimulating hormone; TNF-α, tumor necrosis factor-α.

activity and thus contribute to the interruption of the negative feedback of TH at the central level (Papanicolaou, 2000). In addition, both TH binding in serum and cellular uptake appear to be changed under these circumstances. If recovery or causal treatment of the underlying disease are not possible, this condition may worsen because T_4 production decreases ("low T_4 syndrome"), a condition with a bad prognosis and high mortality rate. Attempted treatment or substitution with TH (T_4 or T_3) were unsuccessful and even worsened the situation by generating a negative nitrogen balance or an even more pronounced catabolic constellation.

Support for this concept was provided in a clinical study on intensive care patients. A systematic analysis of the activities of the three DIO isoenzymes in biopsies of postmortem tissues (liver and skeletal muscle) and the corresponding serum concentrations of TH, together with other routine clinical chemistry parameters, revealed a re-expression of hepatic DIO3 in critically ill patients, which is similar to the pattern in fetal human liver (Peeters *et al.*, 2003b). This finding may explain the rapid decrease in circulating T_3 and the increase in rT_3 in combination with decreased hepatic DIO1 activity. Lowest DIO1 and highest DIO3 activities were observed in patients who died from cardiovascular complications, suggesting that poor tissue perfusion alters the normal expression levels of these two DIO enzymes. No significant expression of DIO2 activity was found in both kidney and muscle. In addition, successful disruption of this vicious cycle of the euthyroid sick syndrome has been demonstrated in intensive care patients who received a combined stimulation by the hypothalamic releasing hormones TRH and growth hormone-releasing peptide, whereas administration of either component alone was not successful in restoring the growth hormone (GH)—TH axes in severe illness (Van den Berghe *et al.*, 1999). Pulsatile secretion of the pituitary hormones GH, TSH, and prolactin was reamplified by this combination of releasing factors, which also substantially increased circulating concentrations of insulin-like growth factor-1, T_4, and T_3 while avoiding an increase in rT_3. Apparently, such interference at the hypothalamic level of regulation enabled this metabolic improvement and the restoration of normal feedback loops. Whether this treatment regimen can be used for all variants of the euthyroid sick syndrome remains to be established. Independent support for this hypothesis derives from observations in children and adult patients treated with GH. This regimen led to decreased T_4 and rT_3 and increased serum T_3 concentrations independent of alterations in TSH and suggests stimulation of hepatic DIO1 activity by GH. This interpretation is supported by several studies of animal models indicating increased hepatic Dio1 expression and activity after GH treatment. No data are available on a GH-dependent decrease in hepatic DIO3, which is normally not expressed in rodent liver. Whether concomitant tissue-specific downregulation of DIO2 expression, as described for skeletal muscle, is additionally involved in adaptation mechanisms required for the cellular decrease in T3 concentration observed in the euthyroid sick syndrome remains to be studied.

Tissue-Specific Expression Patterns of Deiodinase Enzymes and Homeostasis of Net T_3 Formation

The observations of hepatic re-expression of Dio3 and decreased activity of Dio1 in severe illness suggest that the developmental pattern of expression of deiodinases can be reverted under certain conditions or that an alteration of the cellular composition of a given tissue results in marked alterations in the net balance of homeostasis of T_3 formation. Decreased expression of DIO1 without evidence of a concomitant increase in DIO3 expression is also found in tumors of the thyroid, kidney, prostate, and testis. In pituitary adenoma, decreased expression of DIO1 is associated with elevated DIO3 activity, whereas the normal pituitary shows only low levels of this enzyme. Depending on the type of pituitary tumor, DIO2 expression is increased or reduced. Treatment of human cell lines derived from tumors expressing deiodinase activity with agents modulating chromatin methylation or histone acetylation or administration of retinoic acid, an agent known to induce cell differentiation, leads to re-expression of DIO1 activity, even in cell lines that have lost DIO1 expression. These observations indicate that several regulatory factors involved in development and differentiation exert marked influence on the production and homeostasis of T_3, which is essential for maintenance of the differentiated state and the metabolic function of various cell types, especially epithelial cells. Therefore, adequate production of thyromimetically active T_3 seems to be a key parameter of the normal cell and functional tissue, which is under a

complex network of control by potent factors involved in development, proliferation, differentiation, and maintenance of function of a vertebrate organism.

Metabolism of Thyroid Hormones at the Amino Acid Side Chain

TH and iodothyronines derived therefrom have an alanine amino acid side chain that can undergo oxidative decarboxylation and deamination, as found for other amino acids. Only limited in vivo experimental evidence is available on enzymatic decarboxylation of iodothyronines by ornithine decarboxylase to yield metabolites with an amine side chain, such as 3-Iodothronamine (T1AM) (Hoefig *et al.*, 2016). 3T1AM circulates in human blood in nanomolar concentrations and shows high-affinity binding to apolipoprotein B100. T1AM exerts a number of pharmacological effects via cell membrane G-protein coupled receptors, members of the family of trace amine- associated receptors (TAAR), adrenergic receptors (ADRA2) and the transient receptor potential cation channel subfamily M member 8 (TRPM8). Among those T1AM actions are some opposed to those of T3 (e.g. on cardiac function). T1AM administration affects memory, learning, behavior and pain sensation, decreases insulin and increases glucagon secretion, and high doses were reported to reversibly decrease body temperature by several degrees. Thyronamines are inactivated by enzymatic oxidation via inhibitor-sensitive monoamine oxidases. The oxidized product of T1AM, i.e. 3-iodothyroacetic acid, also shows some central effects at high concentrations.

Oxidative decarboxylation of T_4 and T_3 generates relevant circulating concentrations of both tetraiodothyro-acetic acid (Tetrac) and the corresponding triiodothyro-acetic acid (Triac), both of which can be measured in human serum. The enzymes catalyzing these reactions have not been characterized in detail and it is not clear whether thyronamine formation is an obligatory intermediary step in their formation. However, the metabolites Tetrac and Triac are of clinical relevance because Tetrac is a good substrate for the 5′-deiodinases and a ligand for the $\alpha v\beta 3$ integrin receptor while Triac is a potent ligand for nuclear T_3 receptors. Whether endogenous Triac reaches the nuclear T_3 receptor target in vivo and modulates the expression of T_3-regulated genes in humans is unclear. Triac has a short biological half-life compared to that of T_3 and is used in pharmacological doses for treatment of TH resistance and amelioration of the Allan–Herndon–Dudley syndrome. Triac efficiently suppresses elevated TSH but does not lead to hyperthyroid conditions in heart, liver, and other organs due to its rapid degradation and lack of accumulation in tissues.

Conjugation of Thyroid Hormones by Glucuronidases and Sulfotransferases

Glucuronidation

Multiple UDP-glucuronyltransferases appear to be involved in the glucuronidation of TH. The 4′-OH group of iodothyronines is rather acidic, depending on the iodine substitution pattern of the phenolic ring, and Tetrac and Triac are better substrates for glucuronidation than their parent compounds T_4 and T_3. Glucuronidated iodothyronines, which do not bind TR, show increased solubility in water and are rapidly excreted via the bile, but they may also undergo enterohepatic circulation. Drugs and agents interfering with UDP-glucuronyltransferases might therefore affect TH metabolism and elimination as indicated by findings on the effects of several halogenated aromatic compounds (PCB and dioxins) acting as endocrine disruptors in animal models (Gutleb *et al.*, 2016). Compounds that increase T_4 glucuronidation decrease serum concentrations of T_4 (e.g., phenobarbital and several halogenated aromatic compounds).

Sulfation

The second important pathway of TH conjugation of the phenolic 4′-hydroxyl group is catalyzed by sulfotransferases, which belong to a large superfamily of enzymes with tissue- and development-specific expression patterns. Sulfation accelerates the deiodination of different iodothyronines by Dio1 and initiates irreversible hormone degradation (Visser *et al.*, 1998). T_3 sulfate found during pregnancy in fetal and maternal circulation might function as a reservoir from which active T_3 is recovered by tissue sulfatase activity. T_2 sulfate, also found in fetal circulation, might represent an elimination form of excess TH secreted toward the maternal circulation when fetal type I deiodinase activity is still low. Significant sulfotransferase activity has been found in fetal tissues. Relevant sulfatase activities that might liberate T_3 from circulating T_3-sulfate have been observed in (maternal) placenta and liver during pregnancy as well as in other tissues. Changes in sulfation metabolism of TH have also been observed in adaptation to selenium deficiency and during fasting. Furthermore, several endocrine disruptors and drugs alter sulfation-related metabolism of TH and interfere with enterohepatic recycling of THM.

Drugs Interfering With Thyroid Hormone Metabolism

Several drugs and pharmaceuticals interfere with TH metabolism, especially their deiodination and conjugation reactions. Many phenolic aromatic, mono- or polycyclic agents compete with THM binding and deiodination by DIO isoenzymes. With few exceptions, these agents specifically interfere with one or more of the DIO enzymes. Several drugs routinely used in clinics interfere

with DIO1. Most efficient inhibition is observed for some iodinated oral cholecystographic X-ray contrast agents (ipodate or iopanoic acid), the antiarrhythmic drug amiodarone and its metabolites, the synthetic glucocorticoid dexamethasone, some antiphlogistic and antiinflammatory agents, and the large group of plant secondary phenolic metabolites (flavonoids, isoflavonoids, aurones, and chalcones). The latter compounds are contained in significant amounts in daily consumed plant-derived food, but it is unclear whether their consumption contributes to goitrogenesis under conditions of inadequate iodide supply. Many of these DIO-inhibiting compounds lead to a constellation of serum TH reminiscent of the low T_3 syndrome: normal or elevated T_4, low T_3, elevated rT_3, and normal TSH. The main effect of these compounds is inhibition of DIO1. Some agents are potent inhibitors of all three deiodinases (e.g., iopanoic acid, xanthohumol). No selective or isoenzyme-specific inhibitors of 5′-DII and 5-DIII are known. The antithyroid drug 6-*n*-propyl-2-thiouracil (PTU) and the isoflavone genistein are selective inhibitors of DIO1 in most species. However, the clinical use of PTU as an antithyroid drug is based mainly on its inhibition of TH synthesis catalyzed by the thyroperoxidase and less so via inhibition of DIO1 activity. The compound aurothioglucose is a potent inhibitor of DIO1 but also inhibits the other deiodinases at higher concentrations due to the presence of a selenocysteine residue in the active sites of all three DIO isoenzymes. The synthetic flavonoid F21388, derived from natural compounds, is a potent inhibitor of DIO1 and DIO2 and has been shown to modify transplacental TH transfer and TH transport to the fetal brain. The development of isoenzyme-specific inhibitors may provide the possibility for tissue- and cell-specific interference of TH action in a manner similar to that documented for the steroid field, for which selective inhibitors of steroid activation (e.g., aromatase inhibitors) and inactivation are already in clinical use.

See also: Thyrotoxicosis; Systemic Manifestations

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Sodium/Iodide Symporter (NIS)☆

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The thyroid hormones (T_3 and T_4) play a key role in prenatal and early development, and have often been called master regulators of intermediary metabolism throughout life—for good reason, given the myriad effects they have in virtually all tissues. Their impact on human health cannot be overstated. Of all metabolic diseases, thyroid maladies are second in frequency only to diabetes. Iodine, an environmentally scarce element, is an essential constituent of the thyroid hormones, which are the only iodine-containing hormones in vertebrates. Humans' only source of iodide (I^-) is their diet, and the first step in the biosynthesis of the thyroid hormones is the active transport of I^- into the thyroid follicular cells (Carrasco, 1993; Ravera *et al.*, 2017). This step is mediated by the sodium/iodide symporter (NIS), a key plasma membrane transport protein located at the basolateral surface of these cells (Portulano *et al.*, 2014). Although NIS was only identified at the molecular level in 1996, when the cDNA encoding it was first isolated (Dai *et al.*, 1996), the ability of the thyroid to actively take up I^- has been a mainstay of the clinical management of thyroid disease since 1946 (Seidlin *et al.*, 1946). The treatment for thyroid cancer based on administering radioiodide post-thyroidectomy, the most effective internal radiation cancer therapy devised to date, depends on the ability of most thyroid cancer cells to express functional NIS, which ensures that the administered radioiodide is selectively accumulated via NIS in these cells only, destroying them but sparing non-NIS-expressing cells (Ho *et al.*, 2013).

NIS relies on the sodium (Na^+) electrochemical gradient, maintained by the sodium/potassium ATPase, to drive the active transport of I^- into the cell. By coupling the inward transport of Na^+ down its electrochemical gradient to the translocation of I^- against its electrochemical gradient across the basolateral plasma membrane, NIS avidly concentrates I^- in the thyroid at concentrations over 40 times those inside thyroid cells (Ravera *et al.*, 2017). The K_m of NIS for I^- transport is 10–30 μM, and its K_m for Na^+ is 40–60 mM (Portulano *et al.*, 2014). In rats, NIS is a 618 amino acid protein (Dai *et al.*, 1996), whereas in humans, it is slightly larger, with 643 amino acids (Smanik *et al.*, 1996, 1997). The experimentally tested secondary structure model for NIS shows a hydrophobic protein with 13 transmembrane segments (TMSs), an extracellular amino terminus, and an intracellular carboxy terminus (Levy *et al.*, 1997, 1998a,b). Although NIS is N-glycosylated at three positions, N-glycosylation is not essential for I^- transport or for trafficking NIS to the plasma membrane (Li *et al.*, 2013). NIS is a phosphoprotein, and most phosphorylation occurs on its carboxy terminus (Riedel *et al.*, 2001). The carboxy terminus is also important for NIS trafficking and for its localization at the basolateral plasma membrane (Dohan *et al.*, 2007) (**Fig. 1**).

NIS has turned out to be a fascinating molecule and has yielded many surprises. Among them are these: it transports substrates with rather different geometries [e.g., I^- (spherical), perchlorate (ClO_4^-) and perrhenate (ReO_4^-) (both pyramidal), and thiocyanate (SCN^-) (planar)] (Eskandari *et al.*, 1997); it transports different substrates with different stoichiometries (Dohan *et al.*, 2007; Paroder-Belenitsky *et al.*, 2011) [I^-, chlorate (ClO_3^-), and SCN^- electrogenically ($2Na^+$/anion), but ReO_4^- and the environmental pollutant ClO_4^- electroneutrally ($1Na^+$/anion)]; and it has a very long half-life (over 5 days) (Riedel *et al.*, 2001). Moreover, although NIS was previously thought to be a thyroid-specific protein, it is now known to be expressed in various extrathyroidal tissues, including salivary glands, stomach, choroid plexus, lactating (but not non-lactating) breast, placenta, small intestine, and kidney (Tazebay *et al.*, 2000; Mitchell *et al.*, 2001; Spitzweg *et al.*, 2001; Morgenstern *et al.*, 2005; Di Cosmo *et al.*, 2006; Portulano *et al.*, 2014; Riesco-Eizaguirre *et al.*, 2014; Marti-Climent *et al.*, 2015). That NIS is expressed in the lactating breast is particularly significant, because it supplies I^- to the nursing newborn through the milk (Dohan *et al.*, 2003). Strikingly, NIS is also endogenously expressed in some breast cancers, both in primary tumors (Tazebay *et al.*, 2000) and in metastases (Wapnir *et al.*, 2003, 2004), suggesting that radioiodide ($^{131}I^-$) could potentially be used to treat breast cancer similarly to how it is used to treat thyroid cancer. In addition, NIS has also been experimentally expressed exogenously by gene transfer in cancers that do not express it endogenously to render them susceptible to radioiodide treatment (Portulano *et al.*, 2014; Ravera *et al.*, 2017).

Considering that NIS plays a key role in clinical medicine, particularly in the treatment of thyroid disease, and that its use is being extended beyond thyroid disease by carrying out gene transfer studies, it is imperative to understand how NIS works at the molecular level. A longstanding question in the field has been how NIS can transport I^- so efficiently when serum I^- concentrations are submicromolar and the K_M of NIS for I^- is 10–30 μM. Using statistical thermodynamics, it has been shown that NIS has a low intrinsic affinity for I^- (K_d = 224 μM), which increases 10-fold (K_d = 22.4 μM) when the symporter has two Na^+ ions bound to it. Therefore, at physiological Na^+ concentrations, ~79% of NIS molecules are occupied by two Na^+ ions, enabling them to bind and translocate I^- extremely efficiently (Nicola *et al.*, 2014). Furthermore, the mechanism by which NIS binds and releases its substrates has begun to be elucidated. To that end, specific amino acid positions that are crucial for NIS function have been identified. Some of these positions were ascertained to be critical when mutations at these positions were found in patients with congenital I^- transport defect (ITD) (Levy *et al.*, 1998c; Dohan *et al.*, 2002; De La Vieja *et al.*, 2004; De la Vieja *et al.*, 2005, 2007; Li *et al.*, 2013; Paroder *et al.*, 2013; Nicola *et al.*, 2015). ITDs are characterized by extremely low or no I^-

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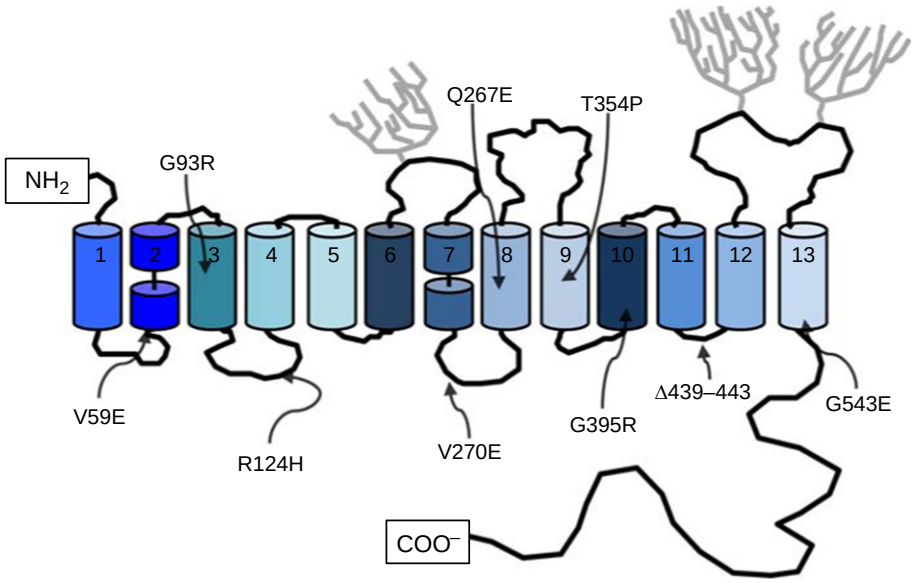

Fig. 1 Experimentally tested NIS secondary structure model. TMSs are shown as cylinders labeled 1 through 13; *black lines* connecting the cylinders represent extracellular and intracellular segments; and trees represent N-linked glycosylation at sites N225, 489, and 502. The single-letter amino acid code is used to label NIS amino acid substitutions identified in patients with ITD and experimentally investigated by determining the effects of several amino acid substitutions at the relevant positions. Δ indicates a deletion. From Ravera, S., Reyna-Neyra, A., Ferrandino, G., Amzel, L. M. and Carrasco, N. (2017). The sodium/iodide symporter (NIS): Molecular physiology and preclinical and clinical applications. *Annual Review of Physiology* **79**, 261–289.

accumulation in the thyroid and a low saliva-to-plasma I^- ratio. If not treated early in life, ITD can lead to hypothyroidism, goiter, and impaired physical and intellectual development. By determining the molecular requirements at these positions and combining these findings with data from molecular dynamics simulations using a NIS homology model (Paroder-Belenitsky *et al.*, 2011; Ferrandino *et al.*, 2016), it is becoming clearer that the two Na^+ ions bind cooperatively—that is, the binding of the first Na^+ increases the affinity of NIS for the second one and for I^-. In addition, the second Na^+ is coordinated by more residues than previously thought (Ferrandino *et al.*, 2016). The fully loaded complex (NIS, 2 Na^+ ions, and I^-) is translocated into the interior of the cell, where all three substrates are released, and the unloaded symporter returns to its original conformation facing the extracellular milieu to begin the cycle anew (Nicola *et al.*, 2014).

In the last two decades, NIS has become central in the use and optimization of gene transfer studies, because it can be used as both a reporter and a therapeutic gene (see Ravera *et al.*, 2017 and Portulano *et al.*, 2014 for detailed discussion). In addition, surprising data on the intricate metabolic crosstalk that takes place between several organs, including the thyroid, liver, pancreas, and adipose tissue, have been obtained in the recently generated NIS knockout (KO) mice (Ferrandino *et al.*, 2017a). Studies using this drug-free mouse model of hypothyroidism have also yielded compelling evidence that, at extremely high serum I^- concentrations, I^- can enter the thyroid via passive routes, as previously suspected, compensating for the absence of functional NIS molecules in patients with ITD. Indeed, when NIS KO mice were fed a standard chow diet, they exhibited reduced serum levels of T_4 but, surprisingly, their serum levels of T_3 and thyroid stimulating hormone (TSH) were similar to those of wild-type (WT) mice. Given that the chow diet supplies approximately 6 μg I^- per gram of food, which is 40 times the recommended daily amount of I^- for mice (National Research Council (U.S.). Subcommittee on Laboratory Animal Nutrition, 1995), these findings indicate that some I^- enters the thyroid passively via non-NIS routes (Ferrandino *et al.*, 2017a). In contrast, when NIS KO mice are fed a diet supplying the recommended minimum daily amount of dietary I^- for rodents [minimum I^- diet (MID); 0.15 μg per gram of food], they show markedly lower serum T_4 and T_3 and drastically greater serum TSH levels than WT mice, as expected.

It has been proposed that hypothyroidism-induced non-alcoholic fatty liver disease (NAFLD) is due to reduced thyroid hormone signaling in the liver, which reduces fatty acid utilization and results in triglyceride accumulation (Liu *et al.*, 2007; Mullur *et al.*, 2014). Several drug-free models of hypothyroidism were used to better define the mechanisms underlying hypothyroidism-induced NAFLD (Ferrandino *et al.*, 2017b). WT mice placed on a low I^- diet (LID) supplying one-tenth the daily recommended amount of I^- for rodents (0.01 μg per gram of food) become mildly hypothyroid, as shown by lower serum T_3 and T_4 and greater serum TSH than euthyroid mice (WT mice on a chow diet). These mildly hypothyroid mice had a higher fat mass and lower lean mass than, and overall similar body weight to euthyroid mice. Mildly hypothyroid mice readily developed NAFLD, even though hepatic genes known to be downregulated in hypothyroidism (Dozin *et al.*, 1986; Jump, 1989; Zavacki *et al.*, 2005; Visser *et al.*, 2016) were found to be expressed at similar or greater levels in these than in euthyroid mice. Expression of β-oxidation genes was similar between mildly hypothyroid and euthyroid mice, and mitochondria from LID mice oxidized ^{14}C-palmitate and generated CO_2 and acid-soluble metabolites at the same rate as mitochondria from euthyroid mice, suggesting that lipid utilization does not

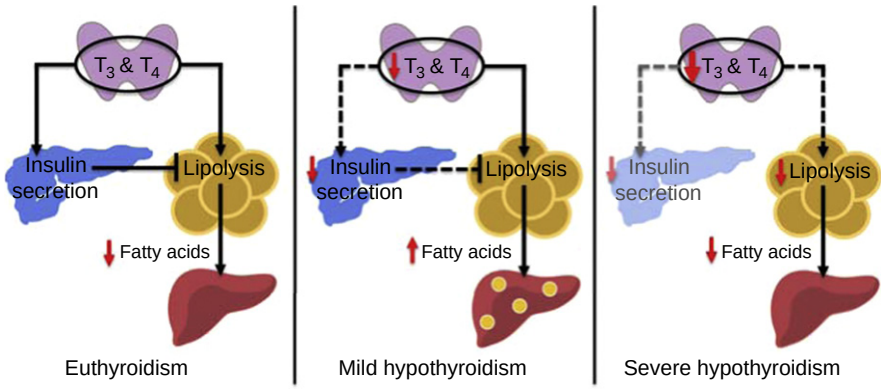

Fig. 2 Overview of the mechanisms that are involved in the development of hypothyroidism-induced NAFLD in mild hypothyroidism and those that protect against NAFLD in severe hypothyroidism. In euthyroidism (left panel), insulin is secreted in response to a meal, which inhibits lipolysis in the adipose tissue, and prevents NAFLD. In mild hypothyroidism (middle panel), insulin secretion is impaired; as a result, lipolysis is not suppressed, fatty acids are accumulated as triglycerides in the liver, and NAFLD develops. In severe hypothyroidism (right panel), adrenergic stimulation of lipolysis is constitutively suppressed, which protects against the development of NAFLD. Adapted from Ferrandino, G., Kaspari, R. R., Spadaro, O., Reyna-Neyra, A., Perry, R. J., Cardone, R., Kibbey, R. G., Shulman, G. I., Dixit, V. D. and Carrasco, N. (2017). Pathogenesis of hypothyroidism-induced NAFLD is driven by intra- and extrahepatic mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* **114**(43), E9172–E9180.

contribute to the development of hypothyroidism-induced NAFLD. Mildly hypothyroid islets had impaired insulin secretion mostly during the second phase of insulin release. The impaired insulin secretion in mildly hypothyroid mice prevents the suppression of lipolysis caused by insulin in fed conditions, leading to increased fatty acid delivery to the liver, where triglyceride accumulation thus occurs. Mildly hypothyroid mice also have increased levels of the key lipolysis enzyme hormone sensitive lipase phosphorylated on activating positions in their visceral adipose tissue under fed conditions. Furthermore, insulin infusion during a euglycemic–hyperinsulinemic clamp study suppressed glycerol and palmitate turnover in euthyroid mice, but failed to suppress turnover in mildly hypothyroid mice, suggesting that insulin action in the adipose tissue is impaired in these mice.

Adrenergic stimulation of lipolysis was not impaired in mildly hypothyroid conditions, but it was in conditions of severe hypothyroidism (NIS KO mice on a LID). Consistent with the impairment of lipolysis, severely hypothyroid mice showed significantly lower fasting serum glycerol and fatty acid levels than mildly hypothyroid mice, along with reduced activating phosphorylations of hormone sensitive lipase in their visceral adipose tissue. Strikingly, severely hypothyroid mice are protected against development of NAFLD, even though they exhibit reduced expression of hepatic genes previously shown to be down-regulated in hypothyroidism (Dozin *et al.*, 1986; Jump, 1989; Zavacki *et al.*, 2005; Visser *et al.*, 2016). These results suggest that hypothyroidism-induced NAFLD is not due to reduced thyroid hormone signaling in the liver, as previously surmised, but rather to unsuppressed lipolysis, which occurs in mildly hypothyroid but not in severely hypothyroid conditions (Ferrandino *et al.*, 2017b) (**Fig. 2**).

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Thyroid Peroxidase☆

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Glossary

Immune tolerance Unresponsiveness of the immune system to antigens that potentially can elicit an immune response. Tolerance to self-antigens is accomplished via central and peripheral mechanisms.

Gene mutation Alteration in DNA structure resulting from substitution, insertion or deletion of nucleotides.

Gene polymorphism Allele variant in a population with a frequency $\geq 1\%$.

Heme Iron containing prostethic group covalently bound to TPO that is essential for its enzymatic activity.

Genetics of Thyroid Peroxidase

Thyroid peroxidase (**Fig. 1**), also called thyroperoxidase (TPO) is a 100 kDa, glycosylated heme-protein bound to the apical membrane of the thyroid follicular cell.

TPO belongs to the same family of human peroxidases, together with lactoperoxidase, myeloperoxidase and eosinophil peroxidase, with which TPO shares 48%, 47% and 47% sequence homology, respectively (Godlewska *et al.*, 2017; Le *et al.*, 2015).

The *TPO gene* has been mapped at 2p13. It comprises 17 exons, spanning more than 150 kb of DNA. Two different TPO cDNAs, hTPO-1 (3048 bp) and hTPO-2 (2877 bp) were isolated in 1987 from a human thyroid cDNA library (Kimura *et al.*, 1987). Compared to hTPO-1, hTPO-2 has a 171 bp deletion that results in a 57 amino acid shorter protein, and is produced by alternative splicing of the primary *TPO gene* transcript (Kimura *et al.*, 1987). Particularly, a proximal histidine is linked to the iron center of the enzyme and is located in the 171 bp sequence that is deleted in hTPO-2. Since this sequence is critical for heme binding, and therefore for enzymatic activity, hTPO-2 was later proved not to have peroxidase activity. Compared to hTPO-1, hTPO-2 is also broken down more rapidly and is not capable to reach the cell surface. The *hTPO-1 gene* encodes for a 933 amino acid protein, which consists of a large extracellular region (called ectodomain, and containing a myeloperoxidase-like domain, a complement controlled protein-like domain, and an epidermal growth factor-like domain), a short transmembrane region, and a cytoplasmic tail (Godlewska et al., 2017; Libert *et al.*, 1987) (**Fig. 2**).

A fully active isoform devoid of the EGF-like domain, called isoform4, was also described (Ferrand *et al.*, 2003). Worthy of note, the 510–567 region contained in the extracellular domain, is homologous to the heme-binding region of the bovine cytochrome C oxidase polypeptide 1, which is encoded by the mitochondrial genome. Based on this observation, some authors have hypothesized that a mitochondrial gene module contributed to the evolution of the *TPO gene* (Libert *et al.*, 1987).

Thyroid Peroxidase Mutations and Variants

It is intuitive supposing that mutations of the *TPO gene* result in partially or totally deficient iodide organification and, consequently, in hypothyroidism. A number of studies have screened patients with either congenital or acquired hypothyroidism for TPO mutations in different countries (Bakker *et al.*, 2000; Nascimento *et al.*, 2003; Wu *et al.*, 2002). Thyroid dyshormonogenesis accounts for 10%–15% of all cases of congenital hypothyroidism, and results from disruption at any stage of iodide organification due to mutations of TPO, thyroglobulin (Tg), H_2O_2-producing system, or administration of TPO inhibitors. So far, more than 80 mutations in the *TPO gene* have been identified, resulting in variable impairment of TPO bioactivity (Belforte *et al.*, 2012). Defective TPO activity has a prevalence of 1 in 40,000 newborns. TPO gene mutations have mostly autosomal recessive inheritance, and are associated with qualitative or quantitative defects in TPO activity (Baş *et al.*, 2014; Ris-Stalpers and Bikker, 2010). In a recent study, Cangul *et al.* have identified 21 TPO mutations in 28 patients with thyroid dyshormonogenesis in a Turkish consanguineous community (Cangul *et al.*, 2013). Furthermore, two missense mutations in the *TPO gene*, Glu799Lys and Arg648Gln, have been reported in an inbred Amish population (Pannain *et al.*, 1999). Most TPO mutations, such as Arg412His substitution or the nonsense mutation at Glu596, are associated with intellectual disability and goiter due to compensatory hyperplasia (Mittal *et al.*, 2016). Moreover, an iodide organification defect that resulted from the expression of a mutant TPO

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Fig. 1 Predicted tertiary structure of the human thyroid peroxidase 1 (devoid of the heme group) retrieved by using the free web portal Phyre2 available at http://www.sbg.bio.ic.ac.uk/phyre2 (Kelley et al., 2015).

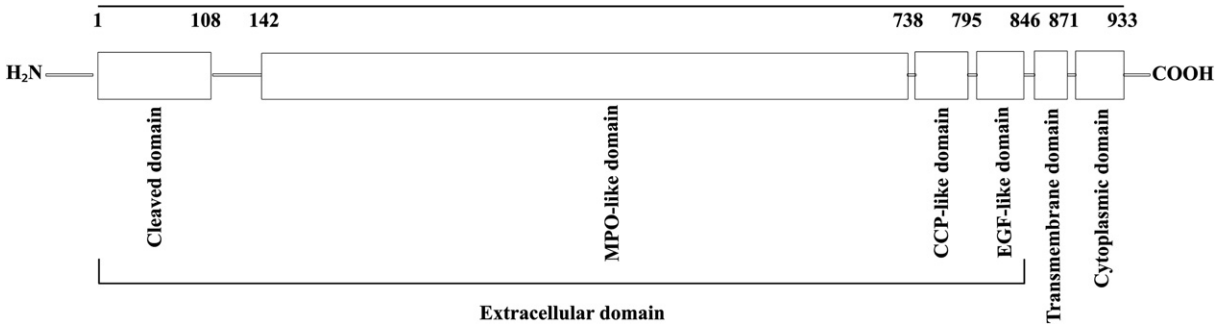

Fig. 2 Schematic presentation of TPO domains. *MPO*: myeloperoxidase; *CCP*: complement control protein-like domain; *EGF*: epithelial growth factor.

(Arg693Trp) paternal allele associated with the lack of maternal allele transcript was reported in three siblings of an Italian family (Fugazzola *et al.*, 2003).

Recent researches have revealed that single nucleotide polymorphisms (SNP) of the *TPO gene* associate with autoimmune hypothyroidism, as TPO variants are recognized as nonself antigens by the immune system. Indeed, the prevalence of TPOAb is as high as 80% in subjects with hypothyroidism (Fatourechi, 2009). Recently, the substitution of asparagine with threonine at position 217 and/or the substitution of threonine with proline at position 725, were reported to increase chances of developing subclinical hypothyroidism by 1.5–5.6-fold in Iranian and Indian populations (Khoshi *et al.*, 2017; Balmiki *et al.*, 2014). Conversely, the Asp666Asp polymorphism was found to be protective for hypothyroidism (Balmiki *et al.*, 2014).

In two large genome-wide association studies carried out in Caucasian and Asian populations, the authors reported different loci associated with TPO positivity. Interestingly, two of those (rs11675434 and rs2071403) are in linkage disequilibrium and are located near the *TPO gene* (Medici *et al.*, 2014; Kwak *et al.*, 2014). The polmorpishm rs11675434 has been associated with Graves' ophthalmopathy, especially in males and in patients whose onset occurs $\geq$45 years (Kuś *et al.*, 2017), but not with Graves' disease itself (Kuś *et al.*, 2017). The involvement of the TPO antigen in the pathogenesis of Graves' ophthalmopathy is confirmed by the expression of TPO and related mRNA in the orbital tissue of such patients (Kuś *et al.*, 2017). However, in contrast with anti-TSH

receptor antibodies, the role of TPOAb has to be understood, since some studies have concluded that higher serum levels of TPOAb are associated with decreased risk of Graves' ophthalmopathy (Lantz *et al.*, 2014).

Thyroid Peroxidase Activity

TPO is synthetized in polyribosomes and undergoes glycosylation in the Golgi apparatus. Upon packaging into vesicles, TPO reaches the apical membrane. TSH stimulates all these steps (Kuliawat *et al.*, 2005). Different glycosylation patterns of TPO account for its heterogeneity using immunoblotting (Gardas *et al.*, 1997). Other posttranslational modifications contributing to the formation of the mature protein are proteolytic trimming (cleavage of the N-terminal 1–108 residue), iron-protoporphyrin IX (heme) covalent binding, and dimerization at level of the extracellular domain (Le *et al.*, 2015). Despite previous crystallographic studies, the structural interpretation of these data remains obscure, so that an experimentally determined structure is still lacking. In this regard, the dimeric myeloperoxidase has been used as a reliable template for TPO (Baker *et al.*, 1994). Interestingly, both in TPO and in myeloperoxidase dimerization occurs at the same site (Cys296), where a disulfide bond links together the two monomers. Recently, Le *et al.* have proposed two models for spatial orientation of TPO: whereas in the *trans* model the cavity containing the heme group faces the follicular lumen, in the *cis* one the prosthetic group faces the apical membrane (Le *et al.*, 2015).

The heme group is essential for enzymatic activity of TPO, namely oxidation of inorganic iodide (I^-), and coupling of iodotyrosine residues on Tg. Particularly, TPO acts as a H_2O_2 donor and oxidizes I^- to I^+, an unstable ion that is capable to bind to Tg (Kopp, 2013). Controversy exists on whether or not iodination of Tg occurs via the production of a TPO-bound iodinated intermediate (Kessler *et al.*, 2008).

TSH stimulates all the steps of thyroid hormones biosynthesis, including TPO production and targeting to the apical membrane of the thyrocyte. In this regard, a number of transcription factors, such as the thyroid transcription factors-1 and 2, and the paired box gene 8 may have a key role in TPO expression (Gerard *et al.*, 1988).

Autoimmunity Against Thyroid Peroxidase

Experimentally, thyroiditis can be induced in mice by injecting purified thyroid antigens from other species, like porcine TPO or TPO peptides or recombinant TPO ectodomain (Ng and Kung, 2006). For instance, transgenic H2-k mice expressing T cell receptor for human TPO, when exposed to the TPO 536-547 peptide develop severe thyroiditis and hypothyroidism (Quaratino *et al.*, 2004). More recently, alternative approaches such as injections of fibroblasts coexpressing TPO and MHC class II, expression of TPO using plasmids or viral vectors or transgenic mice (TAZ10) expressing a human T-cell receptor specific for TPO but lacking other functional T cells and B cells, have been also used (Flynn *et al.*, 2004; Quaratino *et al.*, 2004; McLachlan and Rapoport, 2014).

TPO is a major thyroid antigen together with Tg and the TSH receptor. TPO has been isolated and identified only in the 80s as the "thyroid microsomal antigen," previously reported as a target of the autoimmune process in Hashimoto's thyroiditis (Czarnocka *et al.*, 1985; Libert *et al.*, 1987). However, similarly to TgAb, TPOAb are found also in patients with Graves' disease, underlining that both Hashimoto's thyroiditis and Graves' disease are characterized in their initial stage by a common breakdown of self-tolerance and TPOAb production even before overt thyroid disease (Marcocci *et al.*, 1982; McLachlan and Rapoport, 2014). In this regard, up to 10% of euthyroid subjects can be found positive for TPOAb, and are at risk of developing an autoimmune thyroid disease, as demonstrated by precociously altered ultrasound echogenicity (Acar *et al.*, 2013).

Tg has higher immunogenicity compared to TPO and the TSH receptor, as Tg is more abundant in thyroid and is larger. However, glycosylation of Tg is similar to TPO, 12% and 10%, respectively, but lower than TSH receptor A-subunit (40%) (Chazenbalk *et al.*, 2005). In addition, efficacy of the immune response after immunization with the membrane-bound TPO is greater compared to the soluble Tg or TSH receptor A-subunit (McLachlan and Rapoport, 2014). Since there are patients that produce both TgAb and TPOAb, bispecific "TgPO" antibodies have been postulated. These antibodies might discriminate patients with autoimmune hypothyroidism (with a prevalence as high as 35%–41%) from antibody-positive euthyroid individuals and from patients with nonautimmune hypothyroidism (Estienne *et al.*, 1999). Alternatively, certain patients produce concomitantly TPOAb and TgAb because TPO and Tg share cross-reactive epitopes, such as residue 119–126 of TPO and 2763–2770 of Tg (McLachlan and Rapoport, 2014).

A number of investigations have demonstrated two immunodominant regions within the TPO molecule, called immunodominant region A and B (Ruf and Carayon, 2006; Czarnocka *et al.*, 1997). Approximately 25% of TPOAb of patients with autoimmune thyroid disorders bind to the immunodominnat region A, while 50% of them bind to the immunodominant region B (Jastrzebska-Bohaterewicz and Gardas, 2004). In tridimensional models, the immunodominant region A consists in epitopes scattered across the TPO molecule, and including the complement controlled protein-like domain and the myeloperoxidase domain. In contrast, the immunodominant region B clusters close to the dimer interface at the myeloperoxidase-like domain (Le *et al.*, 2015). It is likely that the interaction of TPO with autoantibodies leads to conformational changes consisting in dissociation of dimers into monomers in order to access to immunodominant region B epitopes (Baker *et al.*, 1994; Le *et al.*, 2015). These data are confirmed by other studies according to which T cells and autoantibodies interact with TPO epitopes

downstream of amino acid 109, namely beyond the cleaved N-terminal region (McLachlan and Rapoport, 2007). Interestingly, even if the heme group is pivotal for the enzymatic activity, it is not involved in TPO autoantibody recognition (McLachlan and Rapoport, 2014). Even though in vitro TPOAb show complement-dependent and antibody-dependent cytolysis activity, in vivo the apical membrane-bound enzyme is not accessible to TPOAb, thus it has been proposed that TPO would interact with autoantibodies either after conformational changes upon micropinocytosis of colloid droplets, or after transcytosis from the basolateral membrane via the Fc receptor (Rebuffat *et al.*, 2010; Estienne *et al.*, 2002). Interestingly, TSH specifically stimulates the expression of the autoantibody binding domains of the TPO molecule in vitro (Rasmussen *et al.*, 1999).

The first step in the development of autoimmune diseases is the breakdown of immune tolerance, namely the initiation of the response against self-antigens. Disruption of immune tolerance has been hypothesized to occur both at central and peripheral levels (McLachlan and Rapoport, 2014). The autoimmune regulator (AIRE) gene greatly influences the thymic expression of thyroid antigens, which is pivotal in central tolerance to thyroid antigens, including TPO. This is confirmed by the high prevalence of TPOAb and autoimmune hypothyroidism in patients with autoimmune polyglandular syndrome type 1 who carry mutations in the AIRE gene (Perniola *et al.*, 2008). At a peripheral level, regulatory CD4+ CD25+ Foxp3+ T cells (Treg) are essential in maintaining immune tolerance. Indeed, mice depleted of Treg lymphocytes develop thyroiditis (McLachlan *et al.*, 2007).

Autoimmune thyroid diseases are supposed to develop when one or more environmental triggers hit genetically predisposed individuals (Benvenga and Guarneri, 2016; Burek and Talor, 2009). Concerning environmental factors, for the purpose of the present chapter we will herewith address only infectious microorganisms, disregarding the role of other factors, such as radiation and iodine, for which we refer to specific entries of this *Encyclopedia*.

According to the molecular mimicry hypothesis, antigens of certain microorganisms may share epitopes with thyroid autoantigens, eliciting an immune response directed against both these microorganisms and thyroid. For instance, antibodies directed to *Yersinia Enterocolitica* lipoprotein and its outer membrane proteins cross react with the TSH receptor, stimulating it, thus triggering Graves' disease in predisposed individuals. Also, the outer membrane proteins of *Y. Enterocolitica* contain cross-reactive T cell epitopes and have mitogenic effect on B cells (Wang *et al.*, 2010; Guarneri *et al.*, 2011). Finally, germline gene precursors of TSH receptor antibodies have been reported to contain binding sites for *Y. Enterocolitica* proteins (Hargreaves *et al.*, 2013). Other investigations demonstrated a similar trigger role also for Coxsackie virus B and other enteroviruses for Graves' disease and Hashimoto's thyroiditis, respectively. In this regard, hepatitis C virus has been recently reported in autoimmune and nonautoimmune hypothyroid patients (Kraemer *et al.*, 1998; Di Domenicantonio *et al.*, 2014). Overall, bacterial (*Y. Enterocolitica*, *Borrelia Burogdorferi*, *Helicobacter Pylori*, *Rickettsia Prowazeki*), viral (enteroviruses, cytomegalovirus), fungal (Candida), and protozoan (*Toxoplasma gondii*) triggers may break down immune tolerance either via molecular mimicry or T cell cross-reactive epitopes (Benvenga and Guarneri, 2016). Alternatively, recently published bioinformatics data have highlighted the role of peptides from microorganisms, such as *Y. Enterocolitica*, *B. Burgdorferi*, and *Clostridium Botulinum* that are capable, similarly to thyroid autoantigens, to bind certain HLA-DR motifs. Consistency between in silico prediction of cross-reactivity and experimental results confirms the above-mentioned data (Benvenga and Guarneri, 2016).

Thyroid Peroxidase in Cancer

A number of studies have highlighted the relation between thyroid autoimmunity and breast cancer. Indeed, women with hyperthyroidism are more prone to develop breast cancer, in contrast with women with hypothyroidism (Søgaard *et al.*, 2016). In early investigations carried out in the 90s, it stood out that the prevalence of TPOAb and other thyroid autoantibodies was higher in women with breast cancer (Rasmusson *et al.*, 1987; Giani *et al.*, 1996; Smyth *et al.*, 1998). Based on these findings, a protective role of TPOAb has been postulated, so that TPOAb positivity can favorably influence prognosis of these patients (Giustarini *et al.*, 2006). Other studies did not corroborate these data (Jiskra *et al.*, 2007). It has been hypothesized that one or more shared antigens between breast cancer and thyroid cells are capable to induce an immune response. Common candidate antigens are the sodium/iodide symporter (NIS), expressed both in thyroid and in breast, or cross-reactive epitopes in TPO and lactoperoxidase, the former being expressed in thyrocytes, whereas the latter in breast cells (Muller *et al.*, 2014). However, the role of thyroid autoimmunity in the development of breast cancer is not fully understood. In vivo experiments have shown that T3 can stimulate the estrogen receptor in breast-cancer derived cell lines, enhancing their proliferation (Hall *et al.*, 2008).

Only recently hTPO-1 isoform and TPO mRNA were found in breast carcinoma samples, and also in the peritumoral tissue (Muller *et al.*, 2014) In this regard, Godlewska *et al.* have demonstrated that TPO expression is four-fold higher in normal breast compared with breast cancer (Godlewska *et al.*, 2017). TPO expressed in breast cancer is immunogenically similar to thyroid TPO, since it is recognized by monoclonal antibodies directed to the immunodominant regions A and B. In addition, TPO mRNA expression inversely correlates with tumor progression, namely the less differentiated the cancer, the lower TPO mRNA expressed, so that compared to well-differentiated and moderately differentiated tumors, in poorly differentiated tumors TPO mRNA expression is 2.3 and 1.6-fold lower, respectively (Godlewska *et al.*, 2017).

TPO and its spliced forms are detected also in thyroid cancer regardless of histotype and stage, although their expression is lower compared with normal thyroid tissue (Di Cristofaro *et al.*, 2006). Studies on three-dimensional structure of TPO isoforms revealed that it might differ compared to the structure of full-length TPO, and this difference may impact on their immunogenicity (Godlewska *et al.*, 2017). Certain TPO variants have been associated with the risk of developing thyroid cancer. In a case control-study carried out in two European populations, two polymorphisms (rs2048722 and rs732609) showed a negative or positive association with differentiated thyroid cancer in the Italian population and in the Spanish one, respectively (Cipollini *et al.*, 2013).

See also: Smoking and the Thyroid. Thyroid Autoimmunity. Thyroid Gland: Anatomy and Physiology

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Thyroglobulin☆

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Glossary

DNA A nucleic acid that constitutes the genetic material of all cellular organisms and the DNA viruses.

Epithelial cells Cells that form the barrier between an organism and its external environment.

RNA A linear, usually single-stranded polymer of ribonucleotides found in all living cells.

Transcription The formation of a nucleic acid molecule using a template of another molecule, particularly the synthesis of an RNA molecule using a DNA template.

The follicular structures of the thyroid gland are composed of a single layer of epithelial cells encompassing a lumen that is filled with the most abundant thyroidal protein: thyroglobulin (Tg), Tg functions as a scaffold protein for thyroid hormone synthesis and as a storage protein for thyroid hormone and iodine, several other proteins, such as thyroid peroxidase and thyroid oxidase, are involved in thyroid hormone synthesis, On stimulation of the thyroid by thyrotropin, several processes, including Tg synthesis, are up-regulated in the thyrocytes, favoring thyroid hormone synthesis.

The Thyroglobulin Gene

The thyroglobulin (Tg) gene, mapped to human chromosome 8q24.2–q24.3, covers at least 300 kb of genomic DNA. The coding sequence is divided over 48 exons separated by intronic sequences varying in size up to 64 kb (Mendive *et al.*, 2001). The complete gene sequence is available on GenBank (accession number NT 008150).

Thyroglobulin Expression, Synthesis, and Maturation

Initial transcription of the Tg gene is regulated by the transcription factors TTF1 (NKX-2.1), TTF2 (FKHL15), and PAX8. The 8.7-kb Tg mRNA is the most highly expressed transcript in normal thyrocytes, with an expression level of 2.7%. The open reading frame of Tg consists of 8307 bp. Because of the use of alternative polyadenylation cleavage sites, there is variation in the size of the 3′ untranslated region. Furthermore, polymerase chain reaction (PCR) showed that 11 alternatively spliced Tg mRNA variants occur. The most abundant and largest Tg mRNA encodes a protein of 2768 amino acid residues, including the signal peptide. After translation of the mRNA, the posttranscriptional route of Tg starts with the signal peptide directing the uptake in the endoplasmic reticulum (ER) where the first carbohydrate molecules are attached and Tg obtains its tertiary and quaternary structure. At least seven molecular chaperones are known to guide this process to ensure correct folding. Tg that "passes" the ER quality control is directed to the Golgi apparatus where glycosylation proceeds and is subsequently transported to the follicular lumen. If the alternative spliced mRNAs are translated, it is possible that these Tg molecules are considered to be "misfolded" by the ER quality control and never reach the follicular lumen. On the other hand, these alternatively spliced transcripts may result in a population of Tg-like proteins that could down-regulate thyroid hormone production because the configuration of the protein is less optimal for thyroxine formation. Moreover, alternatively spliced Tg molecules might have a lower degree of iodination and could be involved in the recently proposed feedback function of Tg when poorly iodinated follicular Tg suppresses the transcription of thyroid-specific transcription factors TTF1, TTF2, and PAX8 (Van de Graaf *et al.*, 2001).

Functional Domains of Thyroglobulin

The normal Tg protein product is a homodimer containing 10% carbohydrates, with a molecular weight of 660,000. The protein shows several characteristic sequences, and specific functions are attributed to some of these (summarized in **Table 1**) (Targovnik *et al.*, 2017).Glycosylation is a key event in Tg maturation. Approximately three-quarters of the potential glycosylation sites in Tg are occupied. Human Tg contains different types of carbohydrate units. Common are the "polymannose" units consisting of mannose and *N*-acetylglucosamine and the "complex" unit that has a core of three mannose residues with several chains of *N*-

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Table 1 Summary of thyroglobulin characteristic sequences

Thyroglobulin (amino acid residue nr)	*Characteristics*
−19	5′ amino acid
−19 ↔ −1	Signal peptide
1	5′ amino acid after signal peptide cleavage
59, 93, 181, 467, 479, 512, 731, 799, 930, 1203, 1332, 1348, 1699, 1757, 1852, 1896, 2105, 2233, 2274, 2565	Glycosylation sites
5, 1291, 2554, 2747	Iodothyronine acceptor sites
130, 847, 1448	Iodothyronine donor sites
	Domains with cysteine-rich repeated sequences
12 ↔ 1191, 1492 ↔ 1546	Type 1
1437 ↔ 1484	Type 2
1584 ↔ 2168	Type 3
2192 ↔ 2716	Acetylcholine esterase homologous domain
1440 ↔ 1443, 1454 ↔ 1457, 1471 ↔ 1474	Thioredoxin boxes
789 ↔ 1173	Heparin-binding domain
2749	3′ amino acid

acetylglucosamine, galactose, and fucose or sialic acid extending from it. Both types of carbohydrate complexes are linked to peptide through an asparagine-*N*-acetylglucosamine bond. Moreover, human thyroglobulin (hTg) contains two additional carbohydrate units linked to the peptide chain through the hydroxyl group of serine or threonine.

Tg contains several tyrosine residues that are preferentially iodinated. By coupling of two iodotyrosine residues, one functioning as a donor residue and the other as acceptor residue, thyroxine is generated at the acceptor site. The selectivity of tyrosine residues that become iodinated appears to be strongly dependent on the stereo-specific structure of the molecule. The main product is thyroxine and in much lesser quantities, 3,5,3′-triiodothyronine (I3) and 3,3′5′ triiodothyronine (reverse I3) are formed.

The N-terminal and the central part of the monomer includes three types of repetitive motifs comprising cysteine-rich repeat domains covalently bound by disulfide bonds (11 type 1, 3 type 2, and 5 type 3).

The function of the 11 type 1 cysteine-rich repeats in the molecule, which is described in bovine and human Tg, is not clear. It has been shown that these repeats are protein modules that can act as pH-dependent binders and reversible inhibitors of the proteases implicated in Tg degradation and thyroxine release.

ACHE-like domain is required for protein dimerization and consequently plays a critical structural and functional role in the TG protein, that is essential for intracellular transport of TG to the site of its hormonogenesis.

Three conserved thioredoxin boxes have been identified in mammalian Tg, and studies on a bovine Tg fragment revealed a role for these boxes in self-assisted disulfide-bond formation leading to the intramolecular cross-linking of luminal Tg.

In rat Tg, the heparin-binding domain is proposed to bind to megalin that participates in the endocytosis of Tg–T4 complexes from the follicular lumen.

Thyroglobulin Synthesis Defects

The Tg synthesis defect has been studied extensively in three animal species. In Afrikander cattle, a homozygous nonsense mutation in exon 9 results in truncated Tg protein of 75 kDa due to the conversion of arginine 697 to a premature stop codon. In this case, an alternatively spliced mRNA lacking exon 9 sequence also is present, encoding a Tg protein of 250 kDa. In Dutch goats, a homozygous nonsense mutation in exon 8 changes tyrosine 296 to a stop and results in a truncated Tg protein of 40 kDa, causing hypothyroidism with goiter. Administration of extra iodine to homozygous mutant Dutch goats restores euthyroidism, but goiter persists. Furthermore, in a mouse model (cog/cog mouse), congenital goiter is linked to the Tg locus and is caused by the transition of leucine 2366 in the Tg protein to proline. This defect results in an ER storage disease.

Patients with this autosomal-recessive condition display a wide range of thyroid dysfunction, ranging from severe hypothyroidism to euthyroidism, congenital goiter or goiter appearing shortly after birth and elevated thyroidal radioiodine uptake. The plasma Tg concentration is usually low, especially in relation to the thyroid-stimulating hormone (TSH) concentration, and intravenous injection of TSH does not increase the plasma Tg concentration. Patients classified in the category "Tg synthesis defects" often have abnormal circulating iodoproteins, mainly iodinated albumin, and excrete iodopeptides of low molecular weight in the urine.

To date, one hundred seventeen deleterious mutations in the human TG gene have.

been identified: 19 splice site mutations, 23 nonsense mutations, 57 missense mutations,13 deletions and 4 single nucleotide insertions or duplication. Mutations of TG gene have been also associated with endemic and nonendemic simple goiter. The p.

C1058R and p.C1977S mutations are the most frequently identified TG mutations in Japanese population, whereas the frequent mutation p.R277* is found in Caucasian populations.

Nucleotide substitutions or deletion in acceptor or donor splice sites can cause exon skipping in the TG gene resulting in an altered ability to transfer an iodophenoxyl group from the donor site to the acceptor iodotyrosine.

The 23 nonsense mutations generate truncated proteins missing functional domain and could represent targets for nonsense mediated mRNA decay (NMD) pathway, a RNA surveillance mechanism that degrades mRNAs containing premature terminated codons.

Sixteen missense mutations involved Cys residue and may eliminate disulfide bonds and alter the normal conformational structure of the TG, possibly preventing the interaction of hormonogenic acceptor and donor sites. Finally, nine missense mutations were reported in the ACHE homology domain: functional analyses indicate that these mutations may result in retention of the TG protein inside the ER and degradation via the proteasome system, as already observed in the cog/cog congenital goiter mouse (Targovnik *et al.*, 2011).

See also: Genetic Factors in Thyroid Disease. Hashimoto's Thyroiditis. Nontoxic Goiter. Thyroid Autoimmunity. Thyroid Carcinoma. Thyroid Gland: Anatomy and Physiology. Thyroid Gland Development, Molecular Biology. Thyrotoxicosis; Diagnosis. Thyrotoxicosis Factitia

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Serum Thyroid Hormone-Binding Proteins

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Glossary

Free thyroid hormone The fraction of thyroxine (0.03%) and triiodothyronine (0.3%) not bound to thyroid hormone-binding proteins and metabolically active at the tissue level.

Thyroxine-binding globulin The major thyroid hormone-binding protein; it binds two-thirds to three-fourths of thyroid hormone in serum.

Thyroxine-binding globulin deficiency and excess Conditions characterized by a deficiency or excess, respectively, of the major thyroid hormone-binding protein; both thyroxine-binding globulin deficiency and thyroxine-binding globulin excess can be either acquired or inherited (X-linked).

Transthyretin A protein involved in the transport of both thyroid hormones and retinol-binding protein, thus being involved also in vitamin A transport.

Introduction

Thyroid hormones, thyroxine (T_4), and triiodothyronine (T_3), are reversibly bound in the bloodstream to a set of carrier proteins (thyroid hormone-binding proteins) mostly of liver origin (Bartalena, 1990). Major thyroid hormone-binding proteins include thyroxine-binding globulin, transthyretin, and albumin. These three proteins account for the binding of most thyroid hormone (95%) (Bartalena and Robbins, 1993). Minor thyroid hormone-binding proteins include lipoproteins (about 5% binding) and, under certain circumstances, immunoglobulins (both IgM and IgG) (**Table 1**). (Benvenga, 2013; Benvenga and Robbins, 1993). The interaction of thyroid hormone-binding proteins with thyroid hormones is such that most T_4 (99.97%) and T_3 (99.7%) are bound, and only a minute fraction of thyroid hormone is unbound or free. The latter, however, is metabolically active at the tissue level, thus accounting for thyroid status. Acute variations in thyroid hormone synthesis and secretion are followed by changes in the proportion of bound hormone, which allow maintenance of the normal free thyroid hormone concentration (Bartalena, 1990). Thus, thyroid hormone-binding proteins exert a buffering action that protects the body against acute variations in thyroid hormone production. Furthermore, thyroid hormone-binding proteins form an important extrathyroidal pool of thyroid hormone and may facilitate its uniform cellular distribution. Thyroid hormone-binding proteins may also help to avoid iodine urinary loss by conferring macromolecule properties to the small thyroid hormone molecules. Finally, thyroxine-binding globulin, like corticosteroid-binding globulin, may be involved in targeting the amount of delivered hormone by its site-specific, enzymatic cleavage (Benvenga, 2013). In this regard, release of thyroid hormone from thyroxine-binding globulinat the infected/inflamed tissue site might provide a relevant amount of iodide and iodine for a significant bactericidal/antiinflammatory action.

Molecular and Physicochemical Properties of Thyroid Hormone-Binding Proteins

Thyroxine Binding Globulin

Thyroxine-binding globulin is a 54-kDa single-polypeptide chain glycoprotein synthesized by the liver. The single-copy gene encoding for human thyroxine-binding globulin, located on the long arm of the X chromosome (Xq22.2), consists of five exons spanning 5.5 kilobase pairs. The mature protein is composed of 395 amino acids. The amino acid sequence of human thyroxine-binding globulinhas revealed a high degree of homology with other members of the serine protease inhibitors (SERPIN) family, including corticosteroid-binding globulin, α_1-antitrypsin, and α_1-antichymotrypsin.

Thyroxine-binding globulin contains approximately 20% of carbohydrates by weight organized in four asparagine-linked oligosaccharide chains with an average of 10 sialic acid residues. Carbohydrates affect the half-life of thyroxine-binding globulin in serum since deglycosylation is associated with a rapid clearance of the protein by the liver. In addition, deglycosylation slightly reduces thyroxine-binding globulin immunoreactivity, T_4-binding activity, and stability. Thyroxine-binding globulinhas only one binding site for thyroid hormones, and binds T_4 with a higher affinity than T_3. Because of its very high affinity for thyroid hormones, thyroxine-binding globulin, although present in serum at a much lower concentration than transthyretin or albumin, binds approximately 65% of T_4 and 75% of T_3 (**Table 1**).

Thyroxine-binding globulin concentration in normal adult human serum ranges from 12 to 20 mg/L (**Table 2**), with a maximal T_4-binding capacity of 0.14–0.25 mg T_4/L. Thyroxine-binding globulin is detectable in the 12-week-old fetus; concentrations in newborns are higher than in adults, decline until mid adulthood, and increase in the elderly.

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Table 1 Human serum thyroid hormone-binding proteins

Protein	*Bound Hormone (%)*	
	T4	*T3*
Thyroxine-binding globulin (TBG)	65	75
Transthyretin	11	10
Albumin	20	9
Lipoproteins	3	6
Immunoglobulin M or G	?	?

Table 2 Characteristics of the major thyroid hormone-binding proteins

Characteristics	*Protein*		
	TBG	*TTR*	*Albumin*
Molecular weight (kDa)	54	56	66
Structure	Monomer	Tetramer	Monomer
Carbohydrates (%)	20	0	0
Serum concentration (mg/L)	12–20	250	40,000
Gene location (chromosome)	Xq22.2	18	4 (humans)
Site of synthesis	Liver	Liver Choroid Plexus Retina Pancreas	Liver

TBG: Thyroxine-Binding Globulin; *TTR*: Transthyretin.

Transthyretin

Transthyretin is a 56-kDa nonglycosylated protein composed of four identical 127-amino acid subunits, (**Table 2**). Transthyretin has two identical thyroid hormone binding sites, but normally only one of them is occupied. The normal serum transthyretin concentration is 250 mg/L (**Table 2**), corresponding to maximal binding capacity of 2 mg T_4/L. Transthyretin binds approximately 10% of T_4 and 10% of T_3. In addition, transthyretin also binds retinol-binding protein, thereby being involved in vitamin A transport. Transthyretin is mostly of liver origin, but it is also synthesized in pancreatic islet cells, retina, and epithelial cells of choroid plexus in both rats and humans. Transthyretin synthesized in the choroid plexus may be important for brain development by maintaining the appropriate T_4 concentration in the central nervous system and favoring its uniform distribution in different areas of the central nervous system (Bartalena, 1990).

The single-copy transthyretin gene is located on chromosome 18 and composed of four exons spanning 7.3 kilobase pairs. The 5′-flanking region has a highly homologous DNA sequence across species, suggesting its crucial role in the regulation of transthyretin gene expression.

Albumin

Albumin is a 66-kDa nonglycosylated protein composed of 585 amino acids (**Table 2**). It has a relatively strong binding site for thyroid hormone and several additional sites with much lower affinity. Its serum concentration is very high (40 g/L), and the percentage of thyroid hormone bound to albumin is about 20% of T_4 and 10% of T_3.

The single-copy albumin gene is located on the long arm of chromosome 4, linked to vitamin D-binding α_2-globulin, whereas in mice the gene is located on chromosome 5, close to the α-fetoprotein gene. There is 90% homology between the human albumin gene and the corresponding gene in rodents. Most automated methods, however, are liable to unpredictable and variable artefacts, which clinicians should be aware of.

Lipoproteins

Lipoproteins are complex molecules composed of a protein moiety (apolipoprotein) and a lipid (both polar and nonpolar) moiety. They bind approximately 3% of T_4 and 6% of T_3. High-density lipoproteins are the major lipoprotein plasma carriers of thyroid hormones through a specific interaction with their apolipoproteins (A-I, A-II, A-IV, C-I, C-II, C-III, and E). These

apolipoproteins have a single thyroid hormone binding site encoded by exon 3 (exon 2 for apolipoprotein A-IV) of the respective gene. The thyroid hormone binding site on apolipoproteins is distinct from the apolipoprotein portion that binds to cell lipoprotein receptors. The physiological role of thyroid hormone binding to lipoproteins is unsettled, but lipoproteins may facilitate enterohepatic circulation, transplacental passage, and central nervous system distribution of thyroid hormones, and they may be involved in thyroid hormone delivery to target tissues with cell surface receptors for apolipoproteins (Benvenga, 2013).

Variations in Thyroid Hormone-Binding Proteins

Acquired Variations

Thyroxine binding globulin

Many drugs and pathophysiologic conditions (**Table 3**) cause changes in the serum TBG concentration, through variations in either thyroxine-binding globulin synthesis or metabolic clearance rate. Hyperthyroidism and hypothyroidism cause a slight decrease and increase, respectively, in serum thyroxine-binding globulin levels by affecting liver synthesis of the protein. Pregnancy and estrogen therapy cause an increase in serum thyroxine-binding globulin concentrations likely due to the longer half-life of thyroxine-binding globulin in the circulation because of estrogen-induced increased sialylation of the protein. Serum thyroxine-binding globulin values are also increased in patients with acute or chronic hepatitis and in a significant proportion of cases of hepatocarcinoma. Whereas in hepatitis the increase in thyroxine-binding globulin is probably the consequence of thyroxine-binding globulin release from damaged liver cells, in hepatocarcinoma the underlying mechanism may be increased liver synthesis of thyroxine-binding globulin. Patients with nephrotic syndrome have a reduced thyroxine-binding globulin concentration due to massive renal protein loss. Losses of TBG through peritoneal membrane are likely to account for the decrease in the thyroxine-binding globulin concentration observed in patients with chronic renal failure undergoing regular peritoneal dialysis. Serum thyroxine-binding globulin (but not corticosteroid-binding globulin) levels are increased in AIDS patients, possibly due to associated hepatitis or to a specific enhancement of thyroxine-binding globulin hepatic synthesis. Patients with diabetic ketoacidosis often have decreased serum thyroxine-binding globulin levels, which might be related to the lack of stimulation of liver protein synthesis by insulin. Starvation or extreme protein-calorie malnutrition cause a decrease in serum thyroxine-binding globulin concentration likely related to decreased hepatic synthesis of the protein. These effects, as well the decrease in thyroxine-binding globulin that occurs in severe terminal illness, may be mediated by inhibition of thyroxine-binding globulin synthesis caused by interleukin-6. Minor variations in serum thyroxine-binding globulin concentration have been reported in several other pathophysiologic conditions (**Table 3**).

Table 3 Acquired serum thyroxine-binding globulin (TBG) variations

Condition/drug	*Serum TBG concentration*
Hypothyroidism	Increased
Pregnancy	Increased
Acute and chronic hepatitis	Increased
Hepatocellular carcinoma	Increased
AIDS	Increased
Oat Cell Carcinoma	Increased
Estrogens	Increased
Perphenazine	Increased
5-Fluorouracil	Increased
Heroin, Methadone	Increased
Clofibrate	Increased
Mitotane	Increased
Hyperthyroidism	Decreased
Nephrotic syndrome	Decreased
Chronic renal failure	Decreased
Diabetic ketoacidosis	Decreased
Starvation	Decreased
Cushing syndrome	Decreased
Acromegaly	Decreased
Androgens	Decreased
Anabolic steroids	Decreased
Glucocorticoids	Decreased
L-Asparaginase	Decreased
Interleukin-6	Decreased

Table 4 Acquired serum transthyretin (TTR) variations

Condition/drug	*Serum TTR concentration*
Insulinoma	Increased
Glucagonoma	Increased
Gastrointestinal carcinoids	Increased
Depression	Increased
Androgens	Increased
Anabolic Steroids	Increased
Glucocorticoids	Increased
Perphenazine	Increased
Parkinson's disease	Increased
Protein-calorie malnutrition	Decreased
Liver diseases	Decreased
Nephrotic syndrome	Decreased
Cystic fibrosis	Decreased
Estrogens	Decreased

In addition to estrogens, other drugs cause an increase in serum thyroxine-binding globulin concentration, including 5-fluorouracil, clofibrate, heroin, and methadone (**Table 3**). Conversely, administration of androgens, anabolic steroids, glucocorticoids, and L-asparaginase decreases serum thyroxine-binding globulin levels (**Table 3**) (Bartalena, 1990).

Transthyretin

The serum transthyretin concentration is often decreased in patients with severe nonthyroidal illness, particularly during protein-calorie malnutrition, nephrotic syndrome, liver diseases, and cystic fibrosis (**Table 4**). In such circumstances, serum transthyretin levels decrease, whereas TBG and albumin concentrations may remain normal. Both decreased liver synthesis of transthyretin (possibly mediated by interleukin-6) and its accelerated degradation contribute to these changes. Transthyretin may be increased in patients with pancreatic endocrine tumors (insulinomas or glucagonomas) or gastrointestinal carcinoids, probably due to transthyretin synthesis by the neoplasm. Transthyretin levels are increased in the central nervous system but not in the serum of patients with endogenous depression or with Parkinson's disease (after adrenal medullary autotransplantation). These changes probably reflect an increased transthyretin synthesis by the choroid plexus. Many drugs affect serum transthyretin concentration, and the effect is often the converse of that on thyroxine-binding globulin. Thus, estrogens decrease serum transthyretin concentration and androgens, anabolic steroids, and glucocorticoids increase serum transthyretin concentrations (**Table 4**). Although the underlying mechanisms are not completely understood, variations in transthyretin synthesis likely contribute to these changes (Benvenga, 2013).

Albumin

The albumin concentration is decreased in many acute and chronic nonthyroidal illnesses. These variations occur concomitantly and are always associated with the previously mentioned similar changes in serum TBG and TTR concentrations.

Inherited Variations

Thyroxine binding globulin

Familial forms of thyroxine-binding globulin deficiency and thyroxine-binding globulin excess, both inherited as X-linked traits, exist (Ferrara *et al.*, 2015). These defects involve the thyroxine-binding globulin gene rather than the rate of thyroxine-binding globulin disposal. Complete thyroxine-binding globulin deficiency, partial thyroxine-binding globulin deficiency, and thyroxine-binding globulin excess are distinguished according to serum thyroxine-binding globulin levels in hemizygous subjects. When TBG deficiency is complete, affected males have no detectable thyroxine-binding globulin in serum, whereas carrier females have half the normal serum thyroxine-binding globulin levels. In partial thyroxine-binding globulin deficiency, serum thyroxine-binding globulin, concentration in heterozygous females is usually higher than half the normal value. In the presence of excess thyroxine-binding globulin, the serum concentration of the protein is usually two- to fourfold higher than normal.

Complete thyroxine-binding globulin deficiency occurs in about 1 in 15,000 newborn males (Refetoff, 1990). 27 thyroxine-binding globulin variants have been associated with complete thyroxine-binding globulin deficiency. In most cases, a single nucleotide substitution, a frameshift due to nucleotide deletion, or multiple nucleotide deletions are the mechanisms leading to early termination of translation and truncation of the thyroxine-binding globulin molecule. Mutations may also occur outside the coding region of the thyroxine-binding globulin gene.

Partial thyroxine-binding globulin deficiency is more common and occurs in 1 in 4000 newborns. 18 different thyroxine-binding globulin variants, characterized by missense variations, cause variable degrees of decreases in the serum thyroxine-binding

globulin concentration. Some of these variants are unstable, have a reduced binding affinity for T_4 and T_3, or show an abnormal migration pattern on isoelectric focusing. In 5% of families studied in Refetoff's laboratory in Chicago, the thyroxine-binding globulin deficiency was not due to mutations in the thyroxine-binding globulin gene, but rather in a liver-specific enhancer, whose activity was reduced.

Inherited thyroxine-binding globulin, excess is rare and occurs in about 1 in 25,000–30,000 newborns. The pathophysiological basis of thyroxine-binding globulin excess has been shown to be thyroxine-binding globulin gene amplification (duplication and triplication), whereas no mutations in the coding and promoting regions have been detected.

Transthyretin

Many transthyretin variants characterized by single amino acid substitutions have been described, mostly in patients with familial amyloidotic polyneuropathy, amyloidotic cardiomyopathy, or senile systemic amyloidosis (Pappa *et al.*, 2015). Some of these transthyretin variants have a reduced binding affinity for thyroid hormone. A different transthyretin variant characterized by an increased affinity for T_4 is responsible for a pattern of euthyroid hyperthyroxinemia (i.e., transthyretin-associated hyperthyroxinemia).

Albumin

A well-characterized inherited albumin variation transmitted as an autosomal dominant trait is familial dysalbuminemic hyperthyroxinemia, which is characterized by the presence in serum of an albumin variant with increased affinity for thyroid hormones. In many cases, the albumin variant has increased affinity for T_4 only; in other instances, an increased affinity for T_3 and/or reverse T_3 is also present. Three different single nucleotide substitutions have been identified as the molecular basis for the increased albumin affinity for thyroid hormone.

The inherited absence of albumin (analbuminemia) and the polymorphism called bisalbuminemia have negligible effects on thyroid hormone transport because the decrease in albumin levels is partially compensated for by a slight increase in TBG and TTR levels.

Effects of Variations in Thyroid Hormone-Binding Proteins on Thyroid Function Tests

Variations in thyroid hormone-binding protein concentrations or affinity profoundly affect serum total thyroid hormone concentrations. This is particularly true for thyroxine-binding globulin because it has a major role in thyroid hormone binding. Accordingly, a decrease or an increase in the serum thyroxine-binding globulin concentration lead to a decrease or an increase, respectively, in serum total thyroid hormone levels. Although the latter changes are similar to those found in hypothyroidism and hyperthyroidism, respectively, they do not reflect thyroid hypofunction or hyperfunction because they are not associated with variations in the metabolically active, free (unbound) thyroid hormone fraction. Similar considerations are tenable for Familial Dysalbuminemic hyperthyroxinemia and transthyretin-associated hyperthyroxinemia. Therefore, thyroxine-binding globulin excess, familial dysalbuminemic hyperthyroxinemia, and transthyretin-associated hyperthyroxinemia are among the most important causes of euthyroid hyperthyroxinemia. The latter may be independent of thyroid hormone-binding protein variations and caused by drugs (e.g., amiodarone, propranolol, iodinated contrast agents, and L-thyroxine), resistance to thyroid hormones, or the acute phase of some psychiatric disorders (**Table 5**).

Thus, should serum total thyroid hormone measurement provide results that are in contrast with the clinical picture, a thyroid hormone-binding proteins abnormality should be suspected and searched for. The correct definition of thyroid status requires measurement of serum free thyroid hormones and thyrotropin concentrations. This approach is particularly useful when thyroid hormone-binding proteins (e.g., thyroxine-binding globulin excess or familial dysalbuminemic hyperthyroxinemia) coexist with thyroid disorders, such as Graves' disease or Hashimoto's thyroiditis. In these circumstances, serum total thyroid hormone levels may be normal in hypothyroid patients, whereas the increased levels of hyperthyroid patients may not easily be distinguished from the increased concentrations due to thyroid hormone-binding protein abnormalities.

Table 5 Causes of euthyroid hyperthyroxinemia

Thyroxine-binding globulin excess
Transthyretin-associated hyperthyroxinemia
Familial dysalbuminemic hyperthyroxinemia
Amiodarone
Propranolol
Iodinated contrast agents
L-thyroxine therapy
Resistance to thyroid hormone
Acute phase of psychiatric disorders

Because serum free thyroid hormone determination is crucial for the assessment of thyroid status and to avoid inappropriate treatment for hyperthyroidism or hypothyroidism, it is essential to select methods for free thyroid hormone measurement that are not affected by the abnormal thyroid hormone-binding protein concentration or affinity. The two-step methods in which free hormone is first separated from protein-bound hormone by dialysis, ultrafiltration, column adsorption chromatography, or immunoadsorption provide the most reliable results. In fact, in the second step (immunoassay) the tracer is not in contact with thyroid hormone-binding proteins, thus preventing interaction between the two and consequent artifactual results.

Conclusions

Thyroid hormone-binding proteins exert functions that are important for thyroid physiology. They provide a buffering action, preventing abrupt changes in serum thyroid hormone levels; function as a storage system for thyroid hormones; they are involved in targeted delivery of thyroid hormone at the tissue level, thus facilitating thyroid hormone cellular distribution. TBG is the major thyroid hormone-binding protein in serum since it binds approximately two-thirds to three-fourths of T_4 and T_3. Both inherited and acquired variations of the major thyroid hormone-binding proteins (thyroxine-binding globulin, transthyretin, and albumin) have been demonstrated. These variations do not modify thyroid status but do affect the results of serum total thyroid hormone measurement and may lead to incorrect diagnosis and inappropriate treatment for hyperthyroidism or hypothyroidism. Thus, for a correct definition of thyroid status, determination of free T_4 and T_3 by assays that are not influenced by thyroid hormone-binding proteins is required.

See also: Genetic Factors in Thyroid Disease. Thyroid and Infertility. Thyroid Function Tests. Thyrotoxicosis; Diagnosis

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Thyroid Hormone Receptors☆

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Glossary

Cofactor (corepressor or coactivator) Ancillary molecule that binds to the nuclear receptor and dictates the negative or positive response to the receptor. Basically a corepressor binds to an unliganded receptor and is released upon binding of the ligand, which allows for the binding of the coactivator. The cofactors interact with the transcription machinery.

Dimerization Association of two molecules of the receptor. This can be an homodimerization (two molecules of the thyroid hormone receptor) or an heterodimerization (one molecule of thyroid hormone receptor and one molecule of retinoic acid receptor).

Transcription machinery All the molecular complex assembled on the regulatory region of the genes.

Dominant negative effect Disturbance of the functioning of the normal receptor (wild-type receptor) by a mutant receptor or an inactive isoform.

The thyroid hormones exert their biological effects essentially through a classical pathway grounded by nuclear receptors (Cheng *et al.*, 2010; Sap *et al.*, 1986), the so-called thyroid hormones receptors (TRs). There is also growing evidence of a nonclassical pathway involving membrane receptors (Davis *et al.*, 2016). The biological effects of the two pathways are also named genomic effects, a regulation of the expression of target genes on the one hand, and nongenomic effects, usually more rapid effects, on the other hand. The present article will concentrate on the classical pathway.

Mechanisms of Action of Thyroid Hormones on Their Nuclear Receptors

The nuclear thyroid hormone receptors (TRs) belong to the superfamily of nuclear receptors (Cheng *et al.*, 2010), as well as steroid receptors, vitamin D receptor, and retinoic acid receptors.

They bind to specific regions of the genes that they regulate, the hormone responsive elements (made of two half sites), here the TRE for thyroid hormone responsive elements (**Fig. 1**), and influence positively or negatively the transcription of the genes, that is the production of the messenger RNA. This regulation of the gene transcription is mediated by an interaction between the TR and either coactivators or corepressors, and then with histone acetylase/deacetylase system. The TR is thus a piece of the transcription machinery, similarly to other nuclear receptors (Cheng *et al.*, 2010; Vella and Hollenberg, 2017).

However, the TR behaves differently with other steroid receptors in that it is already localized in the nucleus of the cells and bound to the TREs in absence of the ligand. This unliganded TRE-bound state already participates to the regulation of transcription of the target gene: repression if the gene is positively regulated by thyroid hormones, or activation if it is a negatively regulated gene. Surprisingly, owing to the tremendous amount of studies on the field, the mechanism of negative regulation by thyroid hormones, that is, the regulation of TSH, is still poorly understood (Vella and Hollenberg, 2017).

In addition, the TR can bind as monomer, as homodimer and can heterodimerize with the retinoic acid receptor RXR (Cheng *et al.*, 2010; Williams *et al.*, 1991; Yen, 2001). The binding as monomer, homodimer or heterodimer is probably dictated by the spatial organization of the TREs, as well as the liganded/unliganded state.

The DNA binding of the TR, as well as the interaction with dimerization partners and cofactors should be viewed as a dynamic, permanently changing process, rather than a static combination, although the latter is a helpful model to describe the transcription machinery.

The Thyroid Hormone Receptor Protein Structure

Like the other nuclear receptors, the TRs are modular structures built of several domains (Cheng *et al.*, 2010; Yen, 2001), (**Fig. 2**).

The DNA binding domain, the C domain, includes two zing fingers responsible for the contact and the specificity of binding to TREs. Each zinc finger contains four cysteines coordinated by a zinc anion. Two regions, the P and D boxes, also participate in the specificity of recognition of the DNA sequence and of the spacing between the two half sites of the TRE.

☆*Change History:* March 2018. Claire Briet, Frederic Illouz and Patrice Rodien participated to the *de novo* writing of this chapter. Figure 1 is a new one. Figure 2 is the simplified version of previous figure 2.

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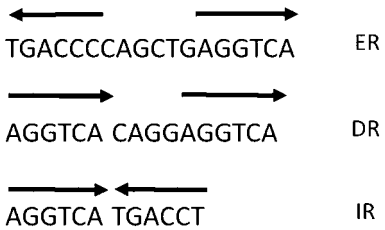

Fig. 1 Thyroid hormone responsive elements (TRE). The two half sites AGGTCA are shown in different orientations. *ER*: Everted repeat, *DR*: Direct repeat with four nucleotide space, *IR*: Inverted repeat or palindrome.

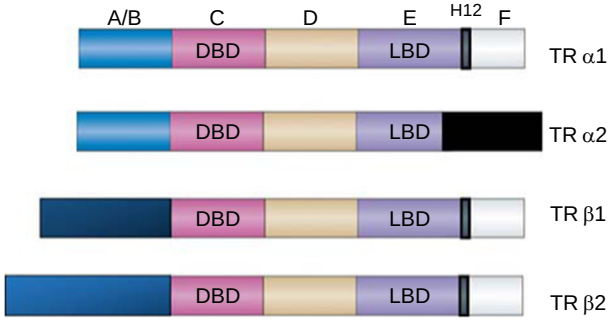

Fig. 2 Structure of the four main isoforms of thyroid hormone receptors with domains A–F indicated. *DBD*: DNA binding domain, *LBD*: ligand binding domain, *H12* Helix 12.

The ligand binding domain, also named the E/F domain, is made of several alpha helixes, delimitating the ligand pocket. The twelfths and last helix can adopt different positions depending on the presence or absence of the ligand. In brief, it moves from a mobile and remote position in absence of ligand to a closer and fixed position and closes the ligand pocket, as the lid of a mouse trap. In addition, by contacting the other parts of the ligand binding domain, it masks the anchoring sites for the corepressors which can no longer bind to the receptor, and contributes to the delineation of the anchoring surface for the coactivators. Again the opening/closing of the binding pocket as well as the blockade/liberation of the anchoring sites for cofactors have to be envied as dynamic processes with specific configurations favored rather than frozen by the binding to DNA, ligand and cofactors. The ligand binding domain also includes some regions involved in the homo or heterodimerization.

It is largely accepted that the active ligand of the TRs is triiodothyronine (T3) and that T4 is essentially a prohormone. More recently, it was shown that T4 can also activate the TRα in some conditions. This may depend on the cellular context (Schroeder *et al.*, 2014).

The D domain, also called the hinge region, between the DNA binding domain and the ligand binding domain, is involved in the biding of corepressors, whereas the A/C domain is involved in the binding of coactivators.

Much information has been obtained with the study of truncated or chimeric forms of the TRs which has allowed to identify different regions spread along the linear representation of the protein and preferentially interacting with DNA, ligand, cofactors, dimerization partners. This makes it hard to understand how the receptor functions. Only the three dimensional structure, in models and cristalls, can reconcile all the data by packing remote distant regions together, and building the functional domains.

Several Isoforms With Different Tissue Distributions

There are two genes in human, encoding the TRs. The α gene (THRA), c-erbA-α is located on chromosome 17 and the β gene (THRB) c-erbA-β on chromosome 3. From both genes are several isoforms produced: TRα1 and 2 and TRβ 1,2 and TRβ3 (at least in rodents) (Williams, 2000). The different isoforms are produced by alternate splicing and different transcription starting sites. Also an additional gene is present on the c-erbA-α locus on the reverse direction, coding for rev-erb α and β, involved in the circadian rythms and metabolic regulations (Kojetin and Burris, 2014).

The TRα1, β1, and β2 are *bona fida* receptors for thyroid hormones, whereas TRα2 has a truncated ligand binding domain and does not transmit the thyroid hormones signal. Truncated forms of TRα and TRβ, lacking the DNA binding domain, but able to bind the hormone have also been described (Chassande *et al.*, 1997).

The role of TRα2 and the truncated forms is still mysterious. TRα2 has been shown to be able to inhibit the function of the other TRs, in vitro, when dimerization with them. However, the physiological role of such a dominant negative effect in vivo, is not known so far. Similarly, the truncated forms, unable to bind DNA, may be able to regulate the activity of full length receptors (Chassande *et al.*, 1997).

The different TRs have preferential, not exclusive, organ and tissue distribution which leads to the concept of α or β regulation in different organs (Cheng *et al.*, 2010).

For example, the hypothalamus and pituitary gland functions are mainly regulated by the TRβ receptors especially the TRβ2. The liver mainly expresses the β1 receptor, whereas the heart can be seen as an α organ, as well as the brain. This is, however, an oversimplification, as TRα1 is also active in the liver, some genes in the heart are also regulated by the TRβ, though less importantly than TRα, and, the heart rhythm is largely dependent on TRα. The bone development appears mainly dependent on TRα, but TRβ is also involved. Finally it was shown that the high TSH observed in TRβ KO mice, as expected, was further increased by the double knock out TRα and TRβ (Gauthier *et al.*, 1999, 2001; Macchia *et al.*, 2001). This illustrates the redundancy, overlap and possible rescue between the different isoforms.

The preferential regulation by TRα or TRβ of different genes is thus the result of the relative expression of the isoforms in the organ, and as nicely shown in the liver, of the nonhomogenous expression of both the target genes and the TR isoforms in the same organ (Zandieh Doulabi *et al.*, 2002). In addition, the availability of the different coactivators and corepressors will modulate the effect of thyroid hormones differently in different cells.

Targeting Specific Isoforms for Specific Effects

Some biological effects of thyroid hormones, may be sought out in therapeutic. Among them, the capacity to lower blood cholesterol which is dependent on TRβ1 is a pharmaceutical goal. However, the α effects such as the heart and bone response to thyroid hormones, limit their potential use in metabolic disease. Targeting exclusively the TRβ, leaving the TRα unaffected, with specific agonist is thus an interesting approach for metabolic disorder. TRβ agonists have been largely investigated, with success in animal models. There are less data in human models and some concerns on potential liver and cartilage toxicity have emerged (Delitala *et al.*, 2017; Ladenson *et al.*, 2010; Sjouke *et al.*, 2014). However, it should be kept in mind that the isoform specificity of these agonists is far from perfect. Indeed some agonists do only display a preferential binding and activation of TRβ rather than an exclusive one, with a very small difference in affinity for both α and β receptors (Borngraeber *et al.*, 2003). Due to the high homology of TRα and TRβ ligand binding domains, it is indeed quite surprising that it was possible to design specific agonists (Wagner *et al.*, 2001). Also, as mentioned above, it appears that T4, can be a ligand for TRα, depending on the type of coactivator interacting with the receptor (Schroeder *et al.*, 2014). The organ specificity agonists also relays on tissue diffusion of the agonist (Takahashi and Izuchi, 2016; Trost *et al.*, 2000).

In addition, if targeting the TRβ to lower cholesterol in order to prevent or treat metabolic disorders appears efficient, it was shown that the fat deposition in arteries, leading to atheroma, is dependent on TRα (Billon *et al.*, 2014).

Along with the design of isoform specific agonists, the design of specific antagonists may be of value (Baxter *et al.*, 2002). For example, antagonizing the TRα, may be of help in treating heart rhythm disorders, or osteoporosis. In fact, desethylamiodarone, the active metabolite of amiodarone, has been shown to be an antagonist of TRα and TRβ (van Beeren *et al.*, 1996). This may participate in its antiarrhythmic action.

Human Rare Diseases of Thyroid Hormone Receptors

The resistance to thyroid hormone (RTH) syndrome has been of great value to understand the mechanisms of action of TRβ (Dumitrescu and Refetoff, 2013). Indeed the heterozygous state of loss of function mutations in patients affected by the syndrome, in agreement with the autosomal dominant transmission, leads to the concept of dominant negative effect, explained by the homo or heterodimerization of the TRs.

The more recent description of first cases of patients harboring TRα mutations will undoubtly lead to a better comprehension of the distinct roles of TRα and TRβ. Interestingly, in the majority of cases with mutations affecting both TRα1 and TRα2, the phenotype was not different from the one in patients harboring a mutation affecting exclusively TRα1 (Moran and Chatterjee, 2015).

See also: Resistance to Thyroid Hormone. Thyroid Hormone Action. Thyrotoxicosis; Systemic Manifestations. TSH (Thyrotropin) Receptor

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Thyroid Hormone Action☆

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Glossary

ATPase Any enzyme that converts energy-rich ATP to ADP to catalyze a process.

Coactivator Class of proteins that can bind to regulatory transcription factors. The intrinsic histone-modifying activity of coactivators leads to activation of transcription.

Corepressor Class of ubiquitous proteins that can form a physical link between regulatory transcription factors and enzymes that modify histone proteins so that transcription is repressed.

Deiodinase Group of three enzymes that catalyze the removal of an iodine atom from iodothyronines. Iodothyronines may contain up to four iodine atoms. The principal actions are conversion of T_4 to T_3 by deiodinase type I and type II and conversion of T_4 and T_3 to reverse T_3 and T_2 by deiodinase type III.

Histone protein Highly abundant nuclear protein. In sets of four, it forms scaffolds around which DNA is wound.

Hormone response element Short, specific nucleotide sequence in the promoter of a gene that is recognized by the DNA-binding part of a hormone-dependent transcription factor. Examples are thyroid hormone response elements and estrogen response elements, which confer thyroid hormone and estrogen responsiveness, respectively, to the promoter.

Promoter Sequence of DNA that precedes the transcribed part of a gene and to which RNA polymerase binds to begin transcription. A promoter may be several thousand nucleotides long and contains multiple binding sites for transcription factors.

RNA polymerase Enzyme that catalyzes the synthesis of an RNA molecule on a DNA template. The DNA sequence of a gene is thus transcribed into messenger RNA, which is subsequently translated into protein.

Transcription factor Any protein that is required to initiate or regulate transcription. There are general factors and gene-specific ones, such as DNA-binding hormone receptors.

Introduction

The two forms of thyroid hormone found in the circulation are T_4 (3,5,3′,5′-tetraiodothyronine or thyroxine) and T_3 (3,5,3′-triiodothyronine). Although T_3 is the biologically most active form, T_4 may elicit specific effects and in this article the singular form "thyroid hormone" is used to include both. Perhaps the best known examples indicating the wide range of effects of thyroid hormone are the induction and progression of metamorphosis in amphibians and the marked stimulation of the overall metabolic rate in mammals. Although critical fluctuations in circulating thyroid hormone levels occur during development and differentiation in most species, hormone levels are remarkably constant throughout life with a reproducible circadian fluctuation in most mammals and humans. Depending on the underlying mechanism, some effects of thyroid hormone are quite rapid, whereas most take hours, reflecting both the genomic and nongenomic pathways of thyroid hormone action.

Perhaps not surprising, the mechanisms by which thyroid hormone exerts its myriad effects are complex and certainly not completely understood. A large number of processes are directly affected by thyroid hormone, but the full physiological effect is often the result of additional, secondary effects. As appears to be the case for various steroid hormones, nonnuclear actions may contribute to the full spectrum of direct effects of thyroid hormone, but evidence for this is relatively sparse. In contrast, following the seminal work of Tata and Oppenheimer in the 1960s and 1970s it was generally accepted by the early 1980s that regulation of gene expression, mediated by nuclear thyroid hormone receptors (TRs), is the principal mechanism of action of the hormone. The independent discoveries of multiple T_3-binding nuclear receptors in 1986 opened the way to a detailed analysis of transcriptional regulation as the molecular basis of thyroid hormone action. This mode of action proves to be particularly complex, and together with an increasing amount of information on the role of local thyroid hormone metabolism in different tissues, it is becoming clear that thyroid hormone action can be modulated at the level of individual tissues throughout life.

☆*Change History*: January 2018. Y Watanabe and RE Weiss updated in sections "Introduction," "Growth and Development," "Other Iodothyronines," "Mechanisms of Thyroid Hormone Action," "Extranuclear Action," "Thyroid Hormone Response Elements," "Stimulation of Gene Transcription by T3," "Bone," "Liver," and "Further Reading" and added the keywords and the reference section.

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Evolution of Thyroid Hormone Action

Information on the origins and earliest effects of thyroid hormone has increased our understanding of the effects in higher organisms. A brief overview of the evolutionary development of thyroid hormone action and its major effects is therefore given, followed by a description of the mechanisms of action and the tissue-specific effects of thyroid hormone in mammals, including man.

Synthesis of Thyroid Hormone in Invertebrates

Spontaneous iodination of tyrosine residues in proteins occurs in the iodine-rich marine environment. Subsequent coupling of two iodinated tyrosines and digestion of proteins may release iodothyronines, including T_4 and T_3, and these thyroid hormones are indeed found in significant amounts in marine invertebrates. At least in some species of jellyfish, organification of iodine to form T_4 actually appears to play a role in the timing of the asexual reproductive cycle in a process called strobilation, which may be likened to metamorphosis in amphibians. T_4 production is therefore an ancient biochemical process with possibly a physiological role in the most primitive invertebrates, predating the evolution of specialized thyroid cells. Such cells are found in the immediate precursors of vertebrates, the protochordates (sea squirts). These cells actively form T_4-containing proteins but are not yet organized in the typical thyroid follicle structure. Furthermore, release and subsequent absorption of T_4 appear to be dependent on digestion of the secreted proteins in the gut.

Synthesis of Thyroid Hormone in Vertebrates

Follicular thyroid cells, in which thyroid cells enclose a follicular lumen containing iodinated proteins (thyroglobulin), are found in the most primitive living vertebrates, the sea lampreys. At this point in evolution, thyroid cells have acquired a protein-digesting enzyme that allows the release of T_4, and in adult lampreys the gland has become endocrine. Interestingly, during the larval stage of this organism, which lasts up to 5 years and constitutes the better part of its life, the thyroid cells are still nonfollicular and T_4 secretion is exocrine followed by absorption. Upon metamorphosis, these epithelial cells form thyroid follicles and secreted T_4 is then directly absorbed by the vascular system. Therefore, the development of the lamprey seems to recapitulate the evolutionary acquisition of the endocrine thyroid gland.

All vertebrates contain follicular thyroid cells, although the localization and organization into a discrete gland vary greatly, from scattered follicles in the subpharyngeal area in lampreys to follicles located mostly in the kidneys in fish, a disk-shaped gland next to the heart in turtles, two widely separated globules on either side of the trachea in birds, and a bilobed gland connected by an isthmus straddling the trachea in lizards, as is also the typical structure of the thyroid gland in all mammals. Despite these differences, the circulating levels of free T_3 and T_4 are within one order of magnitude throughout the vertebrate lineage, with the exception of some species of fish.

Modes of Action and Effects of Thyroid Hormone in Invertebrates

The presence of T_4 or T_3, particularly in marine organisms, is not proof of a physiological function given the spontaneous generation of iodothyronines under certain conditions. Nevertheless, iodothyronines appear to have effects in invertebrates as mentioned previously, although direct evidence for these effects is limited. A stronger case for a physiological role for thyroid hormone in primitive organisms is perhaps made by the presence of the specialized T_4-producing cells in lampreys. This has interesting implications for the primordial mode of action of thyroid hormone. Phylogenetic analysis of the large family of DNA-binding nuclear receptors, of which the TRs are members, shows that the common ancestral proteins were DNA-binding factors without ligand-binding properties. The capacity to selectively bind hormones and other regulatory compounds evolved relatively late. In the case of the thyroid receptors, it appears to have occurred during the development of the vertebrate lineage. The earliest effects of thyroid hormone must then be assumed to be extranuclear, and this mode of action may still be relevant in higher organisms.

Modes of Action and Effects of Thyroid Hormone in Vertebrates

Development of the large diversity of effects in vertebrates is linked to the adaptive progression of tissue distribution of the T_3 receptors (TRs) and mutations within the receptors allowing interactions with other DNA-binding proteins or accessory factors. Genes may have developed thyroid hormone responsiveness by acquiring the specific sequence in their promoter region that is required for binding of TRs. In contrast, the part of the receptor that recognizes this six-base pair sequence is highly conserved in this family of receptors and therefore not a factor in the diversification of T_3 action. Further development of (variable) tissue

responsiveness is probably also linked to the evolving expression patterns of the thyroid hormone-metabolizing deiodinases, which are present throughout the vertebrate lineage.

Growth and Development

The receptor-mediated effects of thyroid hormone on growth and development are evident in all vertebrates. The most striking of these is the transition of the larval amphibian from an aquatic vegetarian organism to a terrestrial carnivore. This experiment can be done with a salamander by simply adding T_4 to the aquarium water. The ensuing metamorphosis leads to radical changes in the appearance of the animal, including replacement of the fin-like tail and appendages with legs and claws and a round tail; replacement of a smooth skin with a thicker keratinized skin; the loss of gills and acquisition of lungs; development of eyelids; complete restructuring of the mouth, tongue, and intestinal tract to allow a mostly carnivorous diet; and numerous biochemical changes to accommodate a different respiratory and metabolic physiology. The increase in thyroid activity and thyroid hormone levels at metamorphic climax in amphibians triggers and sustains the process. Cells in all tissues undergoing metamorphic changes express TRs, and thyroid hormone induces or represses the expression of many genes. In addition, responsiveness of tissues is actually increased by T_3-dependent stimulation of the expression of TRs, as is the case in the restructuring of the tail. A surge in thyroid hormone levels is similarly critical in early growth and differentiation in fish, reptiles, and birds. All these effects have their counterparts in the fetal and perinatal development in mammals, particularly with respect to brain development. In humans, the critical involvement of thyroid hormone is illustrated by the striking and mostly irreversible effects of congenital hypothyroidism on growth, metabolism, reproduction, and mental development (cretinism). The surge in T_3 and T_4 levels reaches its peak in humans during the first 2 months after birth, coinciding with the phase of maximal cortical growth. This growth spurt starts during the third trimester when fetal thyroid activity increases and the dependence on maternal thyroid hormone wanes. However, thyroid hormone is required for central nervous system development beginning in the 10th week of fetal life, coinciding with the appearance of TRs in neuronal tissue. Relatively low levels of thyroid hormone are essential throughout intrauterine development to ensure proper timing and progression of the various phases of brain development. Studies in rats show that T_4 levels are critical because neurons preferentially take up this iodothyronine and then convert it to T_3. Monocarboxylate transporter 8 (MCT8) is a specific transporter of thyroid hormone and involved in thyroid hormone transport in the brain. Individuals with *MCT8* mutations have severe mental and neurological impairment. Studies of 3,5-diiodothyropropionic acid (DITPA), a thyroid hormone receptor agonist, have shown that it is dependent on MCT8 and is a potential therapy for the patients with *MCT8* mutations (Di Cosmo *et al.*, 2009).

Metabolism

The basal metabolic rate (BMR), which is the sum of all energy-consuming processes of an individual when completely at rest, is reduced in humans by 40% in hypothyroidism, whereas it is increased by 50% in hyperthyroidism. Thyroid hormone has essentially the same stimulatory effect on metabolism and concomitant O_2 consumption in other mammals and birds. It was previously thought that this effect played a crucial role in the evolutionary development of endothermy since metabolism in cold-blooded animals appeared unresponsive to thyroid hormone. However, this effect proved dependent on body temperature and the BMR of fish, amphibians, and reptiles is similarly responsive to T_3, albeit the extent of the effect is somewhat less than in mammals. Thyroid hormone does not appear to play a major role in thermoregulation in warm-blooded animals, and the effect on energy turnover, and hence heat production, is considered to be a second-order phenomenon.

The T_3-dependent stimulation of the BMR is the sum of increased energy consumption and substrate metabolism. The major energy-consuming processes in all tissues at rest are maintenance of the mitochondrial proton (H^+) gradient; maintenance of the gradients of calcium (Ca^{2+}), sodium (Na^+), and potassium (K^+) over various cellular membranes; and synthesis of DNA, RNA, and proteins. The effect on the BMR is not primarily the result of the stimulation of a single process, as has long been maintained for the effect of thyroid hormone on the sodium–potassium pump (Na^+–K^+-ATPase). Cellular ion homeostasis is nevertheless an important aspect of the effect of thyroid hormone on the BMR. This homeostasis involves the energy-dependent transport of Ca^{2+}, Na^+, and K^+ ions against gradients across the plasma membrane, as well as across intracellular membrane systems in the case of Ca^{2+}, to maintain their critical cytosolic concentrations. These processes account for a considerable part of the metabolic rate in mammals due to the passive and facilitated leak of ions through membranes. The expression and activity of the major ion-pumping ATPases are stimulated by thyroid hormone, as are the passive-leak characteristics of membranes. The proliferation of some intracellular membranes is also increased by thyroid hormone, and together these effects result in increased ion fluxes and ATP consumption. Proliferation of mitochondria as well as their capacity for oxidative phosphorylation are also stimulated by thyroid hormone, ensuring sufficient ATP production to meet the greater demand due to increased ion cycling and other processes.

To fuel the extra ATP turnover, thyroid hormone increases the rate of glucose production by the liver and increases the availability of the necessary substrates—amino acids, glycerol, and free fatty acids from proteins and fat. Thyroid hormone stimulates the breakdown of protein and fat, but at the same time it stimulates the synthesis of these compounds. This cycling of substrates, which consumes a considerable amount of energy, appears futile, but it allows for the regulated liberation of the substrates mentioned previously.

Which Iodothyronines Are Active in Mammals?

T_4 and T_3

The thyroid produces mainly T_4, whereas 80% of circulating T_3 results from peripheral enzymatic deiodination of T_4, predominantly in the liver. The protein-bound and free plasma levels of T_4 in all species that have been studied are substantially higher than those of T_3. In humans, the difference is approximately 40-fold for total hormone and 5-fold for free hormone. Nevertheless, as noted previously, T_3 is considered to be the active form of thyroid hormone in mammals. Although T_4 can affect the expression of thyroid hormone-responsive genes, the affinity of the thyroid hormone receptors for T_4 is considerably lower compared to that for T_3. In some thyroid hormone effects, such as the effect on neurons and the downregulation of pituitary thyroid-stimulating hormone (TSH or thyrotropin) production, T_3 cannot substitute for T_4 and T_4 appears to be the active form. In the latter case, as in neurons, the genes encoding the TSH-α and TSH-β subunits are indeed responsive to T_3, but the pituitary cellular T_3 content is determined by T_4 uptake and subsequent deiodination rather than by the plasma T_3 levels.

Other Iodothyronines

Progressive deiodination of T_4 and T_3 by the three known deiodinases gives rise to a number of different triiodothyronines and diiodothyronines. Of these, reverse T_3 (3,3′,5′-triiodothyronine) and 3,5-T_2 (3,5-diiodothyronine) have also been shown to elicit T_3-like effects. Because the affinity of the TRs for these compounds is negligible and the effects do not require de novo protein synthesis, the mechanism of action must be extranuclear. In the case of stimulation of mitochondrial activity by 3,5-T_2, it was indeed shown to involve a direct interaction of this iodothyronine with mitochondrial enzymes.

Enzymatic removal of the amino group (deamination) of T_4 and T_3 gives 3,5,3′,5′-tetraiodothyroacetic acid (Tetrac), 3,5,3′-triiodothyroacetic acid (Triac), and 3,5-diiodothyropropionic acid (DITPA), respectively. Tetrac can be deiodinated to Triac, and Triac has an even higher affinity for TRs than T_3. When administered at concentrations comparable to those of T_4 and T_3, these compounds show T_3-like effects. These thyroid hormone analogs have the potential to rescue thyroid hormone signaling (Groeneweg *et al.*, 2017a,b; Ferrara *et al.*, 2015).

Although it cannot be ruled out that in certain circumstances cellular concentrations of these metabolites may reach levels high enough to elicit significant effects, they are generally considered to be of little or no physiological relevance based on the known low plasma concentrations.

Mechanisms of Thyroid Hormone Action

In the following sections, the mechanisms of the primary actions of thyroid hormone are presented (i.e., those mediated by T_3 receptors and those that may involve direct interaction with a target). Additional actions may include effects on the stability of specific proteins or the messenger RNAs (mRNAs) encoding these proteins. However, there are few examples, and the mechanism is not understood. **Fig. 1** summarizes the modes of action that are discussed here. The first step in thyroid hormone action is actually transport of the thyroid hormone (T_3 or T_4) into the cell. We have learned much in the last several years about the classes of transporters responsible for intracellular thyroid hormone transport. Specifically the proton linked monocarboxylate transporter family, organic anion transporter family, L-type amino acid transporters, and Na^+-taurocholate co-transporting polypeptide have all been identified as thyroid hormone transporters across the blood–brain barrier and the intestinal wall (Groeneweg *et al.*, 2017c;

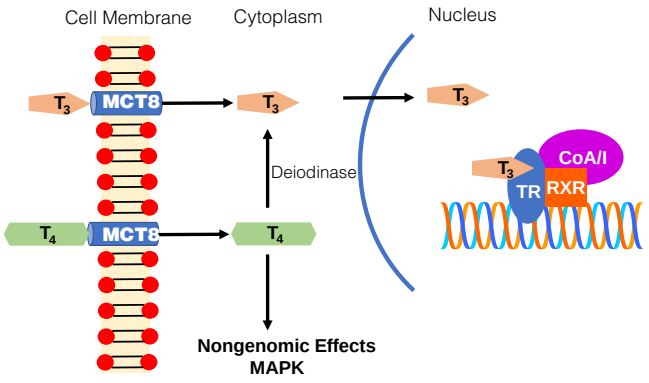

Fig. 1 Modes of action of thyroid hormone. T4 and T3 are actively taken up by the cell by specific transporters (MCT8). Deiodinases can convert T4 to T3 in the cytoplasm. T3 is translocated to the intranuclear milleu whereupon it binds to a complex of up to 40 proteins, one of which is a typical thyroid hormone receptor (TR). Once TH binds TR there is a release of the co-represors and retinoic acid x receptor (RXR) and recruitment of a coactivator. There is also a nongenomic pathway of TH action mediated by MAP-kinase (MAPK).

Brent, 2012). We do not have any information about the mechanism of transport of the thyroid hormone from the cytoplasm into the nucleus.

Extranuclear Action

Stimulation by T_4 and T_3 of the activity of the plasma membrane Ca^{2+}-pump (Ca^{2+}-ATPase) in red blood cells, which do not have a nucleus, is the best documented example of an extranuclear thyroid hormone effect. The in vitro effects correlate with increased activity of this enzyme in erythrocytes taken from hyperthyroid patients and decreased activity in those from hypothyroid patients. In vitro and in vivo studies also show that T_3 and T_4 stimulate glucose uptake in a variety of tissues, increase heart rate, and induce vasodilation, with effects appearing within minutes. Similarly, in various cultured cell types, the addition of physiological amounts of T_3 or T_4 almost instantly stimulates Ca^{2+} influx or the activity of proteins involved in cellular signaling processes. These effects, however, are transient and it is not clear whether they are relevant under normal conditions. The molecular mechanism underlying the mostly membrane-associated rapid effects is also unclear. There is evidence of a direct interaction of T_3 or T_4 with membrane-bound proteins, thereby altering their properties. Based on a large body of data, an alternative hypothesis proposes that T_3 and T_4 affect membrane properties in a less specific way by becoming part of it. The physicochemical properties of the molecules are such that the lipophilic, iodinated phenolic part inserts readily in the outer leaflet of the lipid bilayer, whereas the hydrophilic amino and carboxyl groups prevent the molecule from crossing the membrane (it is for this reason that specific thyroid hormone transporters are required for cellular uptake). This partitioning in the membrane at normal levels of T_3 and T_4 reduces its fluidity, and this will affect the activity of membrane-associated enzymes.

Such a mechanism may also account for the immediate effects of T_3 and T_4 on many mitochondrial membrane-bound enzymes, although this is a matter of debate. Irrespectively, the number of mitochondria, mitochondrial membrane density, and mitochondrial activity generally increase with increasing thyroid activity. This may in part be a secondary response to greater ATP demand. However, the presence of high-affinity thyroid hormone-binding proteins in the inner mitochondrial membrane in hormone-responsive tissues, but not in refractory tissues such as testis and spleen, suggests a unique pathway of thyroid hormone action in mitochondria. These proteins are known to be truncated forms of the TR-α protein, known as p43 and p28, and indeed act as T_3-dependent transcription factors of genes encoded in the mitochondrial genome. As with T_3, specific binding sites for T_2 were identified in the inner membrane of rat liver mitochondria, however, the mechanism of T_2 action on mitochondria is poorly understood (Davis *et al.*, 2016). Apart from this relatively rapid receptor-mediated action, several nuclear-encoded mitochondrial enzymes are regulated via nuclear TRs.

Changes in thyroid status also lead to alterations in the lipid composition and properties of membranes, most notably affecting the saturation of fatty-acyl chains of the phospholipids. Such changes have also been shown to affect membrane-associated enzymes. These lipid alterations are thought to be related to the effects of thyroid hormone on the expression of lipid desaturases. Any subsequent effects on membrane-associated processes are therefore secondary to a nuclear-mediated action of the hormone.

Receptor-Mediated Nuclear Action

There are two known genes for thyroid hormone receptors (i.e., TR-α and TR-β). Multiple receptor isoforms and related proteins are expressed through alternative splicing of mRNAs. Of these, TR-α_1, TR-β_1, TR-β_2, and TR-β_3 are bona fide TRs in that they bind T_3 and confer transcriptional regulation of target genes. Other forms, such as TR-α_2 (DNA binding but not hormone binding) and the ΔTR-α/ΔTR-β forms (hormone binding but not DNA binding), can act as dominant negative factors interfering with TR action. These factors may increase the capacity for tissue-specific fine-tuning of thyroid hormone effects. Some in vivo data support such a role, but the precise physiological relevance in different tissues is unknown.

Studies of receptor-knockout mice lacking either functional TR-α or TR-β receptors indicate a large degree of redundancy between both isoforms. The molecular mechanism of transcription regulation is considered identical for the TR-α and TR-β isoforms, which is supported by in vitro analyses. Some isoform-specific effects are observed in knockout mice as well as in cases of TR-β mutations in humans, including cardiac pacemaking (TR-α_1), development of hearing (TR-β_1), and thyroid hormone feedback regulation (TR-β_2). These dependencies reflect tissue-restricted expression of TRs rather than gene-specific regulatory mechanisms.

Regulation of Gene Transcription

Transcription of a gene's DNA into mRNA requires recruitment of the RNA polymerase complex to general transcription factors that are already bound to the promoter region of the DNA, immediately upstream of the coding sequence. The rate of expression of a gene is basically determined by the ease with which these complexes are formed. Promoter activity and transcription rate are therefore terms that describe the frequency with which transcription of a gene is initiated following assembly of the complex. Gene transcription is primarily modulated by factors that either facilitate and stabilize the assembly of the transcription machinery or disrupt it. The RNA polymerase complex that transcribes the gene is exceptionally large, containing more than 100 different proteins. In contrast, nuclear DNA in its compact form is not readily accessible for such large complexes. Every 200-base pair stretch of DNA is wrapped around a disk-shaped histone protein complex forming a string of so-called nucleosomes that compact the DNA further by combining with other proteins. The resulting chromatin structure, however, is dynamic and can be made accessible to large DNA-binding complexes by modification of the histone proteins. Addition of acetyl groups to histones relaxes

the chromatin structure, whereas deacetylation closes it. Two classes of enzymes are responsible for this reversible chemical modification: histone acetyl transferases (HATs) and histone deacetylases (HDACs). These proteins are key factors in the regulation of gene expression because they can be recruited by transcription factors, including nuclear hormone receptors.

Thyroid Hormone Response Elements

The TRs belong to the large family of ligand-dependent transcription factors, which includes receptors for steroid hormones, retinoic acid and related retinoids, and vitamin D. Specific DNA sequences (response elements) are required in the promoters of genes to allow binding of these receptors, making the gene hormone responsive. Receptors in this family recognize the six-base pair element AGGTCA and interaction of two receptors (dimerization) is typically required for functional activity. The DNA motif to which each receptor binds is consequently referred to as a half-site, and the spacing and orientation of the half-sites determine receptor specificity of the response element. In the case of TRs, the optimal arrangement of half-sites in a thyroid hormone response element (TRE) is either a direct repeat spaced by four base pairs, a palindrome, or an everted repeat with a six-base pair gap (**Fig. 2**).

Deviations from the optimal half-site sequence and, to a lesser extent, from the optimal spacing between them are common in naturally occurring TREs. This variation is responsible for large differences between TREs in TR-binding affinity and stimulation of promoter activity. The relaxed constraints placed on TRE structures are evident in **Fig. 3**, which shows several natural TREs. TREs often consist of three or four half-sites allowing for binding of additional TRs, which further increases promoter activation. In addition, a promoter may contain several TREs contributing to the overall T_3 responsiveness.

TRs readily form heterodimers on TREs with the retinoid X receptor (RXR) with greater binding affinity than that of TR homodimers. Transcription activation by T_3 is also higher, requiring no ligand for RXR. RXRs are also considered the natural partner for TRs in vivo because of the ubiquitous presence of these proteins.

Interactions between different hormone receptors within the family are possible given their similar structure and DNA-binding properties. Some studies support this, showing, for instance, that estrogen receptors can interfere with TR action on T_3-responsive promoters, whereas TRs can bind to and block estrogen response elements in vitro. Moreover, it was shown that T3 decreased expression of estrogen receptors in testis of gold fish (Marlatt *et al.*, 2012). This so-called crosstalk may be relevant in tissues in which both hormone signaling routes are active, such as the hypothalamus in this example, but in vivo data in the hypothalamus

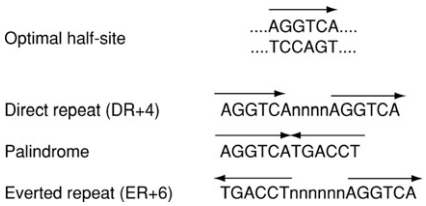

Fig. 2 The optimal half-site for TR binding is depicted as in double-stranded DNA. Only the top strand of DNA is depicted in the three TREs that confer high T_3 responsiveness to a promoter. The *arrows* indicate the orientation of half-sites in a TRE, and "n" can be G, A, T, or C. The hexamer sequence and optimal half-site arrangements are derived from studies of synthetic TREs.

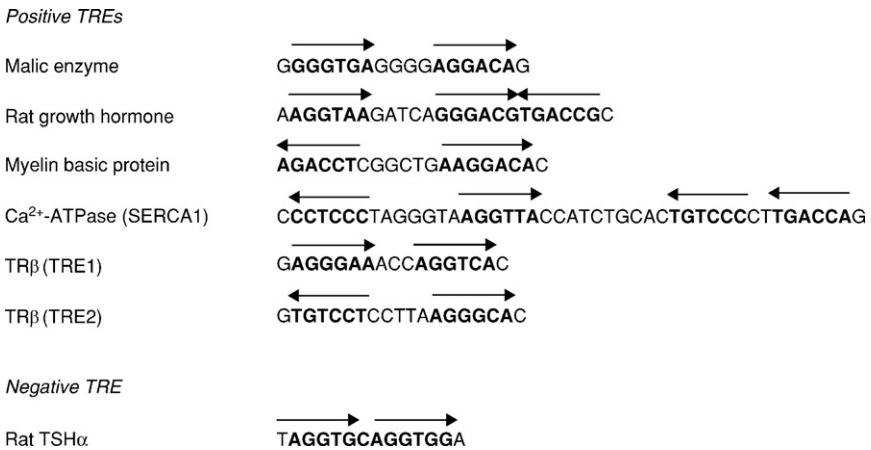

Fig. 3 A selection of natural TREs illustrating the variation in half-site sequence and spacing between half-sites. All indicated half-sites are functional in these TREs and the invariable central two Gs (Cs) are essential for TR binding. TREs from the rat growth hormone gene and the Ca^{2+}-ATPase gene from rat skeletal muscle are examples of composite response elements. Both TREs from the human TR-β promoter contribute to the T_3 responsiveness of this gene. The TRE from the rat TSH-α gene is an example of a half-site structure conferring a negative T_3 response.

are lacking. Also, crosstalk between TRs and peroxisome proliferator-activated receptor (PPAR) which plays a key role in lipid metabolism, carcinogenesis, and cardiovascular diseases was identified. In vitro and in vivo studies indicated that TR-β_1 repressed the transcriptional activity of PPARg via binding to PPAR response element (Araki *et al.*, 2005).

Stimulation of Gene Transcription by T_3

One aspect of TRs that is unique in the nuclear receptor family is the fact that TRs and TR–RXR heterodimers are bound to TREs in the unliganded state, whereas the other receptors require ligand for DNA binding. Furthermore, binding of unliganded TRs results in a substantial repression of basal promoter activity. Activation of transcription is induced by binding of T_3 to the TR. This not only relieves the repression but also further stimulates promoter activity. **Fig. 4** summarizes the current model of the in vivo molecular mechanism underlying this action of T_3. The essential classes of factors involved are depicted, but different proteins exist within each class, some of which show tissue-specific expression. Transcription is repressed in the absence of T_3 because the TR recruits a histone deacytelase to the promoter by means of a ubiquitous corepressor protein such as nuclear receptor corepressor (NCOR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) that serves as an adapter, having binding sites for both TR and the HDAC protein. Binding of T_3 induces a marked conformational change in the TR, which disrupts binding of the corepressor complex. Instead, binding of coactivator proteins such as steroid receptor coactivator 1 (SRC-1) is now possible. Several such proteins form a complex with histone acetylase activity. This in turn opens up the local chromatin structure, allowing the assembly of an active transcription complex. Additional direct interactions of the TRs with basal transcription factors appear to further facilitate this process. In vivo studies have shown that coactivator and corepressor are involved in TR sensitivity and transcriptional response to the hormone, for instance, lack of corepressor induces a higher percentage of the receptors which bind to T_3 and coactivators and results in increasing TR sensitivity (Astapova, 2016). This role of the corepressor has also been supported by a result that patients with TBL1X defect, which is part of the NCOR complex and plays a role in its interactions with chromatin, were considered to have increased TR sensitivity to thyroid hormone (Heinen *et al.*, 2016).

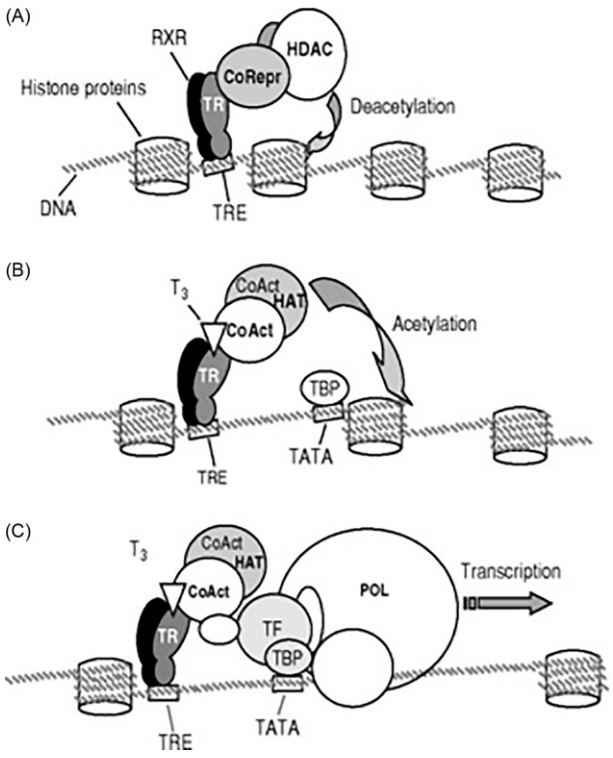

Fig. 4 Model of the T_3-dependent stimulation of transcription in a T_3-responsive gene. (A) Basal or repressed state in the absence of T_3. DNA is in its compacted form wound around histone proteins (nucleosomes) and bound to other ubiquitous proteins (not depicted). TR–RXR dimers are bound to the TRE. TR forms a complex with a protein with histone deacetylase activity (HDAC), which maintains the compact structure. The interaction is through a corepressor protein (CoRepr). (B) Activation. The corepressor complex is replaced by coactivator proteins (CoAct) upon binding of T_3 to its receptor. Histone acetylase activity (HAT) in the CoAct proteins opens up the nucleosome structure. The TATA box-binding protein (TBP) is a general transcription factor that binds to the DNA sequence TATA at the transcription initiation site. (C) Transcription. Following assembly of multiple general transcription factors (TF), which is stabilized by proteins that interact with the TR complex, the RNA polymerase complex (POL) is recruited to the promoter and transcription ensues.

Table 1 Genes known to be transcriptionally regulated by thyroid hormone[a]

	Gene function
Gene transcription stimulated by T_3	
BDNF	Brain-derived neurotropic factor. Stimulation of expression of this brain-specific regulatory factor may in part explain the critical role of thyroid hormone in brain development
Myelin basic protein	Major protein component of the insulating myelin sheaths surrounding axons
β_1-Adrenergic receptor	Stimulation of expression of this receptor accounts for the potentiating effect of thyroid hormone on the adrenergic responsiveness of many tissues, such as liver, fat, skeletal muscle, and heart
TR-β_1	Thyroid hormone receptor β_1. Stimulation of expression of this TR is particularly important for the timing of brain development
HCN2	Hyperpolarization-activated cyclic nucleotide-gated channel. Cardiac ion channel involved in pacemaker activity
MHC-α	Myosin heavy-chain α. One of the two ATPase proteins responsible for cardiac contraction. MHC-α imparts faster contraction at higher energy consumption than MHC-β. MHC-β is the predominant isoform in human heart
SERCA2a	Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase. Ion pump that regulates intracellular calcium, playing a key role in the contraction–relaxation cycle of the heart. SERCA2a is also expressed in skeletal muscle
SERCA1	Isoform of SERCA2a but exclusively expressed in skeletal muscle. It can be expressed at much higher levels than SERCA2a and its activity may account for up to 50% of the total energy consumption of contracting muscle
GLUT4	Insulin-responsive glucose transporter. Responsible for insulin-stimulated uptake of glucose, particularly in skeletal and cardiac muscle and adipose tissue
PEPCK	Phospho-enol-pyruvate carboxy kinase is a key enzyme in gluconeogenesis in liver and skeletal muscle
Malic enzyme	Malic enzyme, or malate dehydrogenase, catalyzes the last oxidation step in the citric acid cycle in mitochondria
Cholesterol hydroxylase	Cholesterol 7α-hydroxylase is a key enzyme in the hepatic degradation of cholesterol into bile acids
Na^+/K^+-ATPase	Ubiquitous ion pump in the plasma membrane of cells responsible for maintaining the gradients of sodium and potassium between the extracellular and intracellular space
MyoD and myogenin	Two different transcription factors that drive the expression of many muscle-specific genes during the differentiation of skeletal muscle (myogenesis)
rGH	Rat growth hormone. The TRE in this pituitary gene was the first to be analyzed in detail. A similar TRE has not been identified in the human GH gene
Deiodinase type 1	This enzyme is responsible for the peripheral production of T_3 through deiodination of T_4. It is primarily expressed in liver and kidney
UCP1	Stimulation of expression of uncoupling protein 1 is responsible for the T_3-induced thermogenic response in brown adipose tissue. It uncouples mitochondrial respiration, thereby converting oxidative energy directly to heat
Gene transcription inhibited by T_3	
MHC-β	Myosin heavy-chain β. Slower and more energy-efficient isoform of the two myosin ATPases responsible for cardiac contraction. The hypothyroid rodent heart expresses almost exclusively MHC-β, which is nearly completely replaced by MHC-α in hyperthyroidism. This shift occurs to some extent in the hyperthyroid human heart
TSH-α and-β	Both anterior pituitary genes encoding the α- and β-subunits of thyroid-stimulating hormone (TSH or thyrotropin) are negatively regulated by thyroid hormone. This is part of the negative feedback loop regulating plasma thyroid hormone levels
TRH	Thyrotropin-releasing hormone, synthesized in the hypothalamus, is the principal positive regulator of TSH synthesis. Repression of transcription of this gene is another aspect of the feedback inhibition of thyroid hormone production

[a]One or more TREs have been identified in the promoters of these genes.

Inhibition of Gene Transcription by T_3

The mechanism of T_3-induced repression of transcription, such as in the case of the TSH-α, TSH-β, and thyrotropin-releasing hormone (TRH) genes, is not understood. Corepressors and coactivators appear to be equally involved in this mode of regulation but with exactly opposite effects compared to the positively regulated genes (i.e., unliganded TRs stimulate transcription, which is abolished after T3 binding). Although TRE-like binding sites or even single half-sites have been implicated in some downregulated promoters, a consensus negative TRE has not been defined.

Many genes are expected to be directly regulated by T_3 based on the responsiveness of expression under various conditions. The list of promoters that are shown to contain functional TREs is continuously growing. A selection of such genes are presented in Table 1 together with a short description of the function of each gene.

Effects of Thyroid Hormone on Target Tissues

The large number of different processes, factors, and mechanisms summarized in the previous sections provide an explanation for the variety of tissue-specific actions of thyroid hormone. The responsiveness of tissues with respect to transcription of specific genes is dependent on the mix of TRs and their numerous cofactors, the details of which are poorly understood. Furthermore, the rate of cellular T_3 and/or T_4 uptake and local activity of hormone-metabolizing deiodinases will determine a tissue's T_3 concentration. As noted previously, many of the physiological effects of altered thyroid hormone levels are secondary to a limited number of direct

actions of T_3, and a large-scale analysis of gene expression indicates that the current list of genes that are directly regulated by T_3 is far from complete.

The effects of thyroid hormone on some of the major responsive tissues, based on both clinical and animal experimental data, are described in the following sections. The emphasis is on the effects for which the primary targets of thyroid hormone are known.

Brain

The development of the central nervous system is severely impaired in the absence of sufficient levels of thyroid hormone. The incomplete maturation of the brain is evidenced by neurological defects and mental retardation in humans. The timing of connections between axons, dendrites, and their targets in the developing brain is critical for the formation of the neuronal network. Diminished axonal growth and reduced dendritic arborization are evident in cerebral cortex, hippocampus, and cerebellum and in the visual and auditory cortex of hypothyroid rats. The expression of many genes is dependent on T_3, but only a few have been shown to be directly regulated, such as myelin basic protein and brain-derived neurotropic factor. The action of T_3 on the other genes may in part be permissive, which means that T_3 is essential for the action of another factor. For example, the stimulation of expression of ornithine decarboxylase by growth hormone in the brain is absolutely dependent on thyroid hormone. This enzyme is essential in nucleic acid and protein synthesis, and thyroid hormone alone has no effect on its expression. Interestingly, in hypothyroidism all enzymes that are dependent on thyroid hormone eventually reach normal levels of expression. It therefore appears that the timing of thyroid action is critical, and that there is a transient period of hormone responsiveness during the development of the brain.

Bone

Development and growth of bone are critically dependent on thyroid hormone, as evidenced by the typical short stature of adults when neonatal hypothyroidism remains untreated. The cell types that are involved in bone formation (osteoblasts) and bone resorption (osteoclasts) are both stimulated by T_3, with effects on several critical enzymes. In hyperthyroid patients, increased bone formation and resorption result in a net loss of bone thickness and increase in porosity, leading to greater risk of fractures. Some studies have shown that TR-α1 is the predominant TR expressed in bone and that T_3 actions are anabolic during growth but catabolic in the adult skeleton via increased osteoclastic bone resorption (Williams and Bassett, 2017). However, little is known about the primary targets and mechanism of action of T_3 in bone cells, mainly because these cells are difficult to study in culture. Thyroid hormone is required for the effects of growth hormone on bone growth and development, which involve the production of insulin-like growth factor 1. Some of the effects of T_3 may therefore result from this permissive action of thyroid hormone. However, studies using TR knockout mice indicate that many of the effects of T_3 on bone metabolism are nuclear mediated and independent of growth hormone.

Heart

Cardiovascular effects are among the most prominent clinical manifestations of thyroid disease. The expression of key cardiac proteins is dependent on thyroid hormone, as evidenced by the profound changes in cardiac performance in the transition from hypothyroidism to hyperthyroidism. Thyroid hormone directly regulates the expression of genes involved in membrane depolarization, muscle contraction, Ca^{2+} handling, and adrenergic signaling (**Table 1**). These actions include positive and negative regulation of transcription. In addition, extranuclear effects on membrane ion fluxes contribute to the increase in heart rate and a reduction in vascular resistance. All these effects combine to increase the performance of the heart, with a concomitant increase in energy turnover. A key factor is the stimulation of the Ca^{2+}-pump of the sarcoplasmic reticulum, whereas its inhibitory protein phospholamban is reduced. The sarcoplasmic reticulum membrane system is the intracellular store for Ca^{2+} required for each contraction. The higher Ca^{2+}-pump activity increases the amount of Ca^{2+} that can be released from the sarcoplasmic reticulum as well as the speed with which it can be taken up again during relaxation. The result is an increased contractile capacity of the heart. Together with the increase in heart rate, the hemodynamic load placed on the heart increases with higher T_3 levels. Such mechanical load is a major independent stimulus for growth of the heart and responsible for the progressive cardiac hypertrophy seen with increasing thyroid hormone levels. The combined effects of T_3 on cardiac function and vascular resistance result in an increase in cardiac output adapted to the higher overall metabolic demand made by the organism.

Skeletal Muscle

The overall effect of thyroid hormone on skeletal muscle is similar to that described previously for the heart: that is, an increase in performance at the expense of a higher energy turnover. Stimulation of the rates of contraction and relaxation by thyroid hormone is a classic observation in thyroidology. Changes in expression of genes similar or identical to those in cardiac tissue underlie this effect, but multiple isoforms and cell-specific and innervation-dependent factors make for a less straightforward regulation by T_3. Generally, T_3 induces a shift in the expression of myosin-ATPase proteins, which determine the rate of contraction, from the slow myosin heavy-chain form (MHC I) to faster MHC II forms. MHC I is identical to MHC-β in heart and this gene is also negatively regulated in skeletal

muscle by T_3 at the promoter level. Direct regulation is not established for the multiple MHC II forms that are similar, but not identical, to cardiac MHC-α. As in heart, the relaxation rate is determined by the level of expression of the Ca^{2+}-pump of the sarcoplasmic reticulum. Muscle also expresses the SERCA2 form present in the heart, but T_3 preferentially stimulates the expression of a second isoform (SERCA1). This protein is virtually identical to the SERCA2 form, but the promoter of the SERCA1 gene is completely different, with different TREs allowing much higher expression of the protein compared to the SERCA2 form. Proliferation of the sarcoplasmic reticulum membrane is also stimulated by T_3. The expression of the fast MHC isoforms and the high Ca^{2+}-pump levels increase the energy cost of contraction two- or threefold in the transition from hypothyroidism to hyperthyroidism. The economy of force production is consequently reduced by T_3 since absolute force production is not affected by these changes.

The timing of expression of a series of fetal and adult MHC isoforms during development is dependent on thyroid hormone. Similar to brain, the adult expression patterns are ultimately obtained irrespective of the thyroid status. This is not the case for the SERCA1 gene, which is absolutely dependent on thyroid hormone. Effects of neonatal hypothyroidism on muscle development in rats are fully reversible by treatment with thyroid hormone.

Liver

Plasma levels of cholesterol, particularly low-density lipoprotein (LDL) cholesterol, are increased in hypothyroid patients. This is the result of decreased LDL cholesterol uptake by the liver (due to reduced LDL receptor numbers) and reduced hepatic metabolism of cholesterol. These effects can be reversed by treatment with thyroid hormone. The enzyme cholesterol 7α-hydroxylase is the rate-limiting step in the degradation of cholesterol into bile acids, and its expression is stimulated by T_3 at the promoter level. Whether the expression of LDL receptors is also directly stimulated by T_3 is not known. In vivo studies have shown that thyroid hormone reduces cholesterol in LDL receptor-knockout mice (Goldberg *et al.*, 2012). TRβ-selective thyroid hormone analogs were expected a role as lipid-lowering drugs. However, clinical trials of the first generation TRβ-agonists were discontinued because of adverse effects of thyroid hormones (Mondal and Mugesh, 2017).

T_3 stimulates the expression of a number of enzymes involved in lipogenesis, including malic enzyme, glucose-6-phosphate dehydrogenase, and fatty acid synthase. Malic enzyme is particularly responsive and the promoter of this gene contains a strong TRE. Interestingly, the same enzyme is expressed in brain, but it is unresponsive to T_3. In liver, several signal routes involved in energy metabolism, such as insulin signaling, converge on the promoter of this gene and interact with the T_3 signal. For instance, the stimulatory effect of T_3 on malic enzyme expression is increased 10-fold when a normal diet is replaced by a high-carbohydrate/fat-free (lipogenic) diet.

Synthesis of glucose (gluconeogenesis) from precursors such as amino acids and glycerol is an important liver function. T_3 stimulates the expression of key enzymes in this process, including pyruvate carboxylase, glucose-6-phosphatase, and phospho-enol-pyruvate carboxy kinase. A TRE has been identified in the promoter of the phospho-enol-pyruvate carboxy kinase gene.

In addition, T_3 stimulates the expression of the deiodinase type I gene, which converts T_4 to T_3. This enzyme is responsible for most of the circulating T_3 in the body and its promoter contains several TREs.

Adipose Tissue

Differentiation of preadipocyte cells into mature white adipose tissue is dependent on thyroid hormone, and the same key enzymes involved in lipid metabolism in liver are influenced by T_3 in fat tissue. High levels of T_3 shift the balance between fat synthesis and breakdown to net lipolysis, resulting in generalized fat mobilization and loss of body fat stores. Although brown adipose tissue is virtually absent in adults, as it is in most larger mammals, newborns have appreciable amounts of this type of fat tissue. It gets its color from the cytochromes in mitochondria, which are particularly abundant in these fat cells. In rodents, brown adipose tissue is the site of the thyroid hormone-dependent extra heat production in response to prolonged cold exposure. Circulating thyroid hormone levels do increase, but this is not essential for cold adaptation and the action of the hormone appears to be permissive. Surprisingly, however, the adipocytes greatly increase their T_3 content by increasing the expression of deiodinase type II. Like type I, this enzyme converts T_4 to T_3 and its expression is stimulated by the higher levels of circulating adrenergic hormones triggered by the cold stress. The increase in T_3 in the brown adipocyte in turn induces the transcription and expression of a protein called thermogenin or uncoupling protein 1, which uncouples respiration in mitochondria. This means that the energy produced by the numerous mitochondria in these cells is not stored in the form of ATP but rather directly released as heat. This mechanism is equally important in maintaining body temperature in newborns and it is an example of how thyroid hormone action may be regulated at the level of individual tissues.

See also: Myxedema Coma. Resistance to Thyroid Hormone. Thyroid Hormone Receptors. Thyrotoxic Storm. Thyrotoxicosis; Systemic Manifestations

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Resistance to Thyroid Hormone☆

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Glossary

Dominant-negative effect (DNE) When mutant TRs interfere with functions of the wild-type TRs.

Resistance to thyroid hormone (RTH) A syndrome of reduced target tissue responsiveness to thyroid hormone, usually inherited in an autosomal dominant manner.

Retinoic acid receptor (RXR) Thyroid hormones form heterodimers with RXRs and bind to TRE.

Thyroid hormone receptor (TR) Nuclear receptor that mediates thyroid hormone transcription.

Thyroid response element (TRE) Specific DNA sequences located near the transcription start point of thyroid hormone-regulated genes.

Introduction

The syndromes of resistance to thyroid hormone (RTH) are generally characterized by reduced tissue responsiveness to thyroid hormone (TH) despite elevated circulating levels of free T_3 and/or T_4 (Refetoff et al., 1993). It is associated with an inappropriately normal or raised serum TSH characteristic of impaired thyrotroph sensitivity. The original clinical description of RTH was reported by Refetoff *et al.* (1967) in two subjects with deaf-mutism, delayed bone age, and stippled epiphyses. Despite these symptoms of hypothyroidism, serum levels of TH were high. Furthermore, administration of exogenous TH failed to produce the expected metabolic effects and failed to suppress thyrotropin-releasing hormone (TRH)-stimulated TSH. These observations lead to the diagnosis of RTH. Twenty years later, the molecular basis of the syndrome was found to be due to a mutation in the TH receptor β (THRB and *TRβ*) gene (Sakurai *et al.*, 1989; Usala *et al.*, 1990). To date, 186 different mutations in the *TRβ* gene, found in $>$ 3000 individuals in 500 families, have been described. As our knowledge of the different thyroid function phenotypes has evolved, it has become clear that RTH is part of a larger group of conditions known as impaired sensitivity to thyroid hormone (Refetoff *et al.*, 2014). Those with classic RTH due to TRβ mutations are known as RTH-beta. This is to distinguish them from a group of patients with mutations in the *TH receptor alpha* (*THRA* and *TRa*) gene RTH-alpha who have low serum T_4, borderline high T_3, and very low rT_3, with normal to elevated TSH concentrations (Espiard *et al.*, 2015; Moran *et al.*, 2013, 2014; Tinnikov *et al.*, 2002; Bochukova *et al.*, 2012; van Mullem *et al.*, 2012; Tylki-Szymanska *et al.*, 2015). A high ratio of free T3 to free T4 seems to be a common finding in the 14 cases of RTH-alpha described to date. Two other syndromes belonging to the group of impaired sensitivity to thyroid hormone include patients with gene mutations affecting TH metabolism, such as SBP2 (Di Cosmo *et al.*, 2009; Dumitrescu *et al.*, 2005) or transport via MCT8 (Dumitrescu *et al.*, 2004; Friesema *et al.*, 2004, 2010; Papadimitriou *et al.*, 2008). The latter is associated with severe psychomotor defects. Knowledge of the molecular mechanisms involved in mediation of TH action allows the recognition of the phenotypes caused by genetic defects in the involved pathways.

The inheritance pattern of RTH-beta is autosomal dominant in most cases. There is a small subgroup (about 10%) of patients with apparent RTH-beta phenotype (elevated T4, T3, and nonsupressed TSH) but without mutations in the *THRB* gene, and they are referred to as non-TR RTH. The molecular basis for their condition is unknown.

Although the initial description of RTH was associated with signs of hypothyroidism, the phenotype of RTH is variable, both between families and among family members with identical TH receptor (TR) mutations. The majority of the patients present with mild-to-moderate symptoms. This variability in clinical manifestation may be due in part to the severity of the hormonal resistance, the effectiveness of compensatory mechanisms, the presence of modulating genetic factors, and the effects of prior therapy.

Although the apparent insensitivity to TH may vary in severity, the common feature characteristics of the syndrome are goiter, growth delay, elevated serum levels of free T_3 and/or T_4, and normal or slightly increased TSH level that responds to TRH.

☆*Change History:* October 2017. GA de Carvalho, TM Fighera, and RE Weiss updated all sections and deleted Table 2, and further information has been included in the text.

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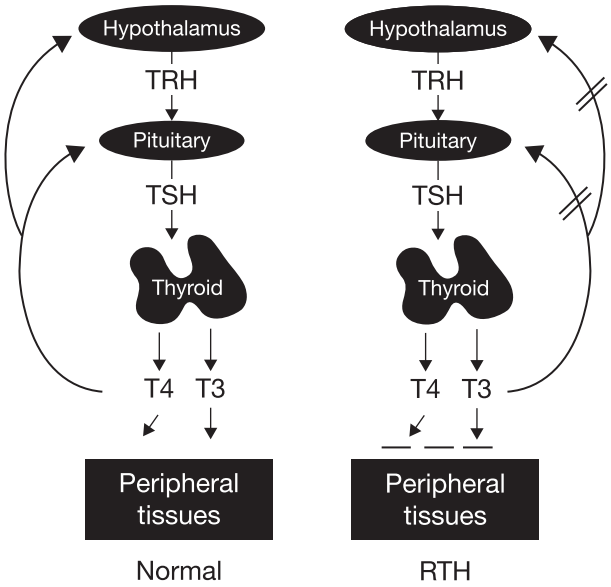

Fig. 1 Schematic representation of the hypothalamic–pituitary–thyroid axis. Under normal conditions, the hypothalamus secretes TRH that stimulates the pituitary to produce TSH that stimulates the thyroid to produce mainly T_4. T_4 is metabolized in the peripheral tissues to T_3 and, in turn, THs feedback in an inhibitory fashion on the hypothalamus and pituitary. Thyroid hormone action on pituitary and peripheral tissues occurs when T_3 binds to its receptor; in RTH-beta, the feedback is inhibited due to decreased sensitivity of the pituitary to TH, resulting in elevated TSH with increased T_4 secretion. The peripheral tissues also have decreased sensitivity to TH in RTH-beta.

Thyroid Physiology

Biological Effects of Thyroid Hormone

THs are known to induce metamorphosis in amphibians by mediating remodeling of specific tissues and organs. In humans, severe TH deprivation during intrauterine life and in the immediate postnatal period results in impaired neurogenesis and growth retardation. In adult life, hypothyroidism affects the function of many organs and results in alterations of thermogenesis and behavior.

Thyroid Hormone Regulation

Several mechanisms are responsible for modulating the supply of TH to tissues: (1) The hypothalamus–pituitary–thyroid axis controls TH synthesis and releases from the thyroid gland via stimulation by TSH, (2) the generation of T_3 from T_4 at the tissues via the action deiodinases may modulate tissue responsiveness to TH, and (3) autoregulation of expression of TR can also modulate the effect TH has on a tissue, for example. The more receptor, the greater the response; the less receptor, the less response for a given amount of hormone. TSH synthesis and secretion is stimulated by TRH, a tripeptide derived from the hypothalamus. TSH originating from pituitary gland in turn stimulates TH synthesis and secretion. Although T_4 is the predominant form of TH released from the gland and T_3 generated at the tissue level is the biologically active hormone, T_4 can also bind to the TR but with 100-fold less affinity. T_3, generated from the conversion of T_4 in the pituitary and hypothalamus by type II deiodinase, controls the synthesis and secretion of TSH and TRH through a negative feedback loop (**Fig. 1**). Finally, proper TH action requires an intact TH, its transport across cell membrane, hormone activation through intracellular metabolism, cytosolic processing, nuclear translocation, binding to the TRs, and interaction with coregulators or other postreceptor effects mediating the TH effect.

The signs, symptoms, and laboratory abnormalities observed in RTH are due to reduced peripheral tissue and pituitary responsiveness to TH, resulting in impaired negative feedback of T_3 on TSH/TRH and impaired peripheral tissue action of T_3.

Mechanisms of TH Action

TH was once thought to solely enter the cell by a process of concentration-dependent passive diffusion after dissociation from the serum hormone-binding proteins. However, several molecules have been identified as putative TH transmembrane transporters. TH action, for the most part, is mediated through specific TRs in the cell nucleus, although pathways of nongenomic action of TH have been described (Bassett *et al.*, 2003).

TRs are members of the nuclear hormone receptor superfamily together with retinoic acid receptors (RXRs), 9-*cis* retinoic acid receptors, vitamin D3 receptors, and peroxisome proliferator-activated receptors (Cheng *et al.*, 2010). TRs act as ligand-dependent transcription factors that increase or decrease the expression of target genes depending on whether the gene promoters contain, respectively, positively or negatively regulated TH-response elements.

There are two genes, *THRA* and *THRB*, located on chromosomes 17 and 3, respectively, which encode TRs. TRs are cellular homologues of the viral oncogene erb-A. Each gene generates at least two proteins by alternative splicing: TRα_1, TRα_2, TRβ_1, and TRβ_2.

The relative expression of two TR genes and the distribution of their products vary from tissue to tissue and during different stages of development. Furthermore, TH differentially regulates the expression of two TR genes and their isoforms.

The THRα and THRβ proteins are structurally alike, which suggests that they might have similar functions. However, the identification of patients with mutations in *THRA* and their significant different clinical presentation to that of patients with mutations in *THRβ* suggests that the THR isoforms have distinct physiological roles that extend beyond differences in tissue distribution (Refetoff and Dumitrescu, 2007). In patients with mutations in *THRA*, the HPT axis is not impaired as it is in patients with RTH-beta (Bochukova *et al.*, 2012). These clinical observations are supported by findings in which THRβ was identified as the receptor that modulates negative regulation of the HPT axis (Abel *et al.*, 2001; Gauthier *et al.*, 2001).

The α and β TR isoforms have a well-conserved DNA-binding domain (DBD or C domain) separate from the ligand (T_3)-binding domain (LBD or E domain) by a hinge domain (D domain). TRα_2, due to a carboxyl-terminal sequence difference, does not bind TH, and the molecule does not function as a TR (**Fig. 2**).

TRs homodimerize (TR/TR) or form heterodimers with RXRs (TR/RXR) and bind to specific DNA sequences termed TH-responsive elements (TREs), which are located near the transcription start point of TH-regulated genes.

On genes upregulated by TH, the unliganded TRs suppress basal gene transcription activity by interacting with a class of nuclear proteins known as corepressors such as the nuclear receptor corepressor (NcoR) and the silencing mediator for retinoid and TH receptor (SMRT). Conformational changes of TR, produced by T3 binding, enhance the occupation of TREs by TR/RXR heterodimers, release the corepressor complex, and recruit coactivators such as steroid receptor coactivator 1 (SRC-1), among others, all exhibiting histone acetyl transferase activity. The latter modifies the structure of the chromatin and, through loosening the nucleosome at the site of the promoter, activates gene transcription (**Fig. 3**). The mechanisms involved in the genes that are downregulated by TH, such as TSH, are less well understood but likely to involve the same classes of molecules on a different TREs that cause corepressors to become "activators" and coactivators to become "repressors."

Molecular Basis of RTH

Location of Mutations Associated with RTH

RTHβ Mutations

The precise incidence of RTH-beta is unknown. Because most neonatal screening programs are based on the determination of TSH only and RTH-beta is rarely identified by this means (Weiss *et al.*, 1990), a limited neonatal survey by measuring blood T_4

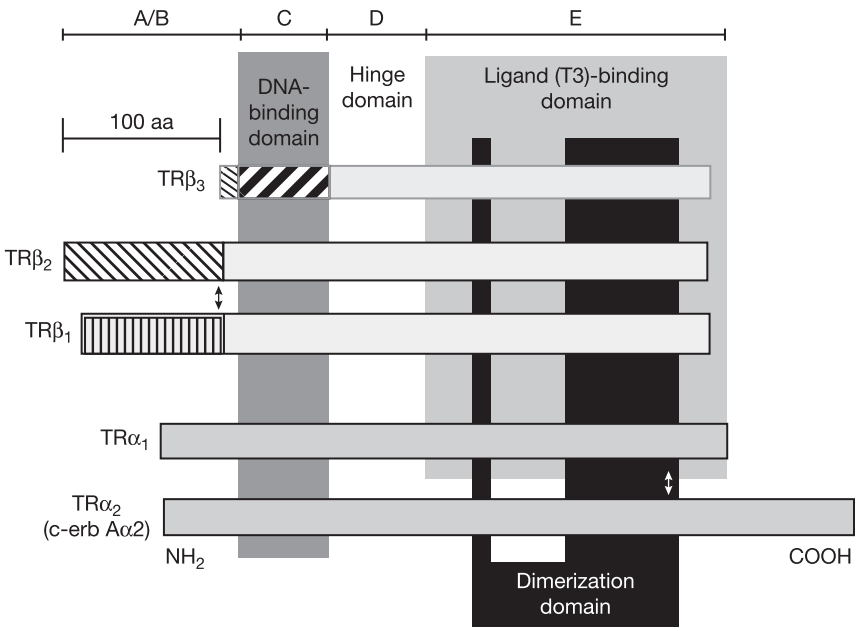

Fig. 2 Schematic representation of the principal TH receptor (TR) forms and their functional domains. Each of the two TR genes (THRA and THRB) expresses two major protein forms (1 and 2) by alternative splicing (*arrows*). The shading pattern of the bars corresponds to the degree of amino acid sequence similarity (identical, similar, or completely different). Differences are most marked in the amino termini (NH_2) of the TRs. The different carboxyl terminus (COOH) of the α2 completely abolishes T_3 binding, and this product is not considered to be a TR proper. The functional domains are also identified by a letter code and by the stippled backgrounds.

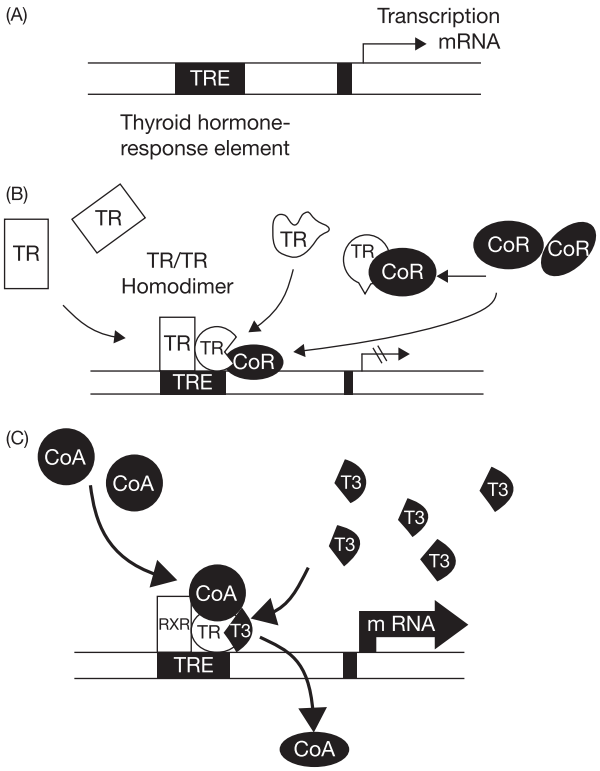

Fig. 3 Mechanism of TH action. Schematic representation of the elements involved in the mediation of TH action through the regulation of T_3-responsive genes expression (A). The unliganded TR homodimerizes with TR, associates with CoR (corepressor), and exerts the suppressive effect on genes positively regulated by TRE (B). Formation of a TR–T_3 complex resulting in disassociation of CoR, occupation of TRE by TR/RXR heterodimers, and recruitment of CoA (coactivator) to enhance transcription of genes positively regulated by TRE (C).

concentration suggested the occurrence of one case/40,000 live births (Lafranchi *et al.*, 2003; Tajima *et al.*, 2009) with equal frequency in both genders. Familial occurrence of RTH-beta has been documented in 75% of cases, while the incidence of sporadic cases is 21%. Inheritance is autosomal dominant. Transmission was clearly recessive in only one family (Refetoff *et al.*, 1967; Takeda *et al.*, 1992).

One hundred eighty-six different mutations in the *THRB* gene have been identified in subjects with RTH-beta belonging to 500 families. The majority (430 families) have a single nucleotide substitutions resulting in single amino acid replacements in 419 instances and stop codons in 11 others (**Table 1**). Deletions, insertions, and duplication were identified in 20 families. The majority of mutations have occurred in three "hot spots" located the LBD (exon 8–10) of TRβ (**Fig. 4**), and identical mutations have developed independently in different families (Weiss *et al.*, 1993).

The RTH index family had homozygous deletion of the *TR*β_1 allele, representing the only example of recessive inheritance of RTH. In all other cases, the inheritance pattern of RTH is autosomal dominant, involving a single nucleotide substitution or small deletions. All *THRB* gene mutations are located in the functionally relevant domain of T_3 binding and its adjacent hinge region. The mutant receptors cannot either bind TH or have reduced affinity. The result of this is that the corepressor is not released, and the gene is not activated. Other receptor mutants have been found that do bind T_3 but release the corepressor slower than normal or have a weaker interaction with the coactivator.

Ten to fifteen percent of families with RTH do not have mutations in the TRβ. These non-TR RTH subjects have a phenotype similar to those with TRβ mutations including baseline thyroid function tests and metabolic and TRH responsiveness to exogenously administered TH. Since non-TR RTH also has an autosomal dominant mode of inheritance, it is believed that other transcriptional cofactors involved TH action could be responsible for the hormone resistance. In support of this are studies of hypothalamic/pituitary/thyroid axis of knockout mice that lack the SRC-1 (coactivator) or the RXR and show that the absence of a cofactor can cause RTH. Further work is required to determine whether there are other forms of hormonal resistance in the non-TR RTH subjects in which the same cofactor may be responsible for mild but multiple hormone resistances.

Dominant Negative Effect

Mutant TRβs interfere with function of the wild-type TRs, a phenomenon termed dominant-negative effect (DNE). Mutant TRβ receptors are still able to bind to TRE and dimerize with normal TRs or RXRs, interfering with the function of the normal TRs. There is a strong correlation between the DNE measured *in vitro* and decreased release of transcriptional corepressor from TRs.

Table 1 Type of TRβ gene mutations

Type		*Number of occurrences at different sites*	*Number of families*	*Effect on TRβ*
Substitution	Single nucleotide	148	430	Single a.a. substitution; premature stop (C434X, K 443X, E445X, C446X, E449X)
	Dinucleotide	3	3	Single a.a. substitution (P453Y, P453 Y); premature stop (F451X)
Deletion	Single nucleotide	4	0	FrSh and stop (441X) of two a.a. extension
	Trinucleotide	5	6	Single a.a. deletion (T276D, T337D, M430D, G432D)
	Eight nucleotides FrSh normal stop at a.a. 461	1	1	
All coding sequences	1	1	Complete deletion	
Insertion	Single nucleotide	7	14	FrSh and two a.a. extension
	Trinucleotide	1	1	Single a.a. insertion (328S)
Duplication FrSh and two a.a. extension	Seven nucleotides	1	1	
Mutation at CpG dinucleotides		10	184[a]	42.8% of 430 families with single nucleotide substitutions and 46.1% of 191 similar families studied in the authors laboratory
De novo mutations b 43[b]	Total		[c]	20.6% of 209 families studied in the authors laboratory
	In CpGs	6	[c]	48.8% of the *de novo* mutations
No TRβ gene mutations		[d]	40[e]	14.0% of 243 families studied in the authors and in whom the TRHB gene was sequenced

[a]Not included are seven families in which the mutation did not follow the rule of G to A or C to T transition.
[b]Families with TRβ gene mutations excluding those with a single affected individual when both parents were not tested.
[c]Not counted as publications do not always include parental genotype.
[d]Nonapplicable.
[e]Total number of families underestimated because usually they are not reported.
a.a., amino acid; *FrSh*, frameshift

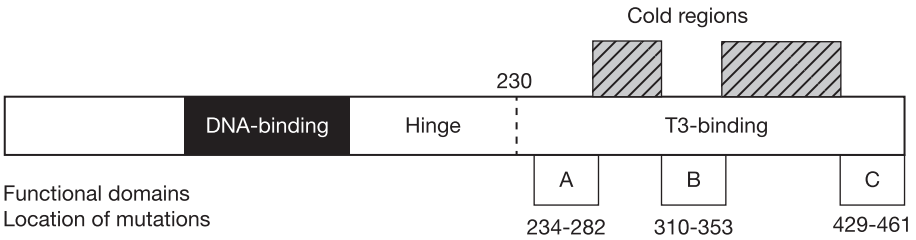

Fig. 4 Location of natural mutations in the TRβ gene associated with RTH-beta. Schematic representation of the TRβ and its functional domains for interaction with TREs (DNA binding) and with hormone (T_3 binding). The mutations that have been found in the TRβ cluster in three areas of the T_3-binding domain. The amino acid positions between which the mutations are found are indicated below the boxes (A, B, and C). Note the "cold regions" of 28 and 75 amino acids, devoid of mutations associated with RTH-beta.

For a DNE to occur, the TR mutant must bind to TRE, which may explain why no mutations have been identified in the DBD and why in the family with a deletion of all coding sequences of the TRβ gene the heterozygotes did not manifest the phenotype of RTH. Interestingly, one subject with homozygous deletion of TRβ Thr-337 (amino acid deletion) with dominantly inherited RTH pattern manifested the most severe form of RTH.

Subjects heterozygous for a *THRB* gene deletion are normal because the expression of a single TRβ allele is sufficient for normal function. RTH-beta manifests in homozygotes completely lacking the *THRB* gene and in heterozygotes that express a mutant THRB with DNE. The most severe form of RTH-beta, with extremely high TH levels and signs of both hypothyroidism and thyrotoxicosis, occurs in homozygous individuals expressing only mutant TRβs (Wu *et al.*, 2005; Yen *et al.*, 2006; Ferrara *et al.*, 2012). The more severe phenotypes in the subjects homozygous for mutant THRBs indicate a stronger DNE and support the notion that mutated THRB interferes with the function of THRA.

Pathophysiology

The lack of negative feedback of T_3 on TSH result in persistent TSH secretion and thyroid gland stimulation resulting in increased TH synthesis and secretion. The normal or slightly increased TSH usually responds by a further increase to the administration of TRH, distinguishing this from subjects with a TSH-secreting pituitary adenoma.

The serum TSH of RTH-beta patients is identical immunologically to the serum TSH of normal controls. It does not contain an excess of glycoprotein α-subunits (TSHα), typically found in the serum of patients with TSH-producing pituitary tumors. However, the TSH has an increased biological potency *in vitro*, perhaps explaining how normal TSH levels produce goiter and hypersecretion of TH by the thyroid gland. Another explanation but also unproved possibility to explain the goiter of RTH-beta subjects with normal serum TSH could be augmented thyrocyte sensitivity to TSH through increased density of TSH receptor units.

Various mechanisms can be postulated to show the tissue differences in TH resistance within the same subject and among individuals. The distribution of receptor isoforms varies from tissue to tissue (Lazar, 1993). This likely accounts for greater hormonal resistance in the liver as compared with the heart.

Several of the clinical features encountered in some patients with RTH-beta may be the manifestation of selective tissue deprivation of TH during early stages of development. The severity of symptoms is related to the relative expression of the mutant allele (and perhaps cofactors) in the particular tissue. For example, cardiac tissue predominantly expresses TRα. Most RTH-beta subjects present with tachycardia, because of their abnormal TRβ, they over produce TH, but since the heart has TRα, which is normal, tachycardia results.

Thyroid tissue obtained by biopsy or at surgery revealed various degrees of hyperplasia of the follicular epithelium, supporting the idea of structural heterogeneity of the follicles. Little can be said about the pathological findings in tissues other than the thyroid because of unavailability of autopsy data from patients with RTH-beta. Metachromasia in fibroblasts is present in patients with RTH-beta and in patients with myxedema due to TH deficiency, although treatment with the hormone failed to induce a disappearance of the metachromasia in fibroblasts from patients with RTH-beta.

Clinical Features

Clinical classification

The clinical presentation of RTH-beta is highly variable both between families and among members of the same family with identical TR mutations. The majority of individuals appear to be euthyroid, and therefore, they are asymptomatic. Some may manifest symptoms suggestive of TH deprivation such as growth retardation, impaired cognitive ability, and hypercholesterolemia, while others show signs of TH excess such as tachycardia, advanced bone age, or hyperactivity. Not uncommonly, individuals have symptoms of both TH deficiency and excess.

Subjects with RTH-beta that appear to be eumetabolic and maintain near-normal serum TSH concentrations have been classified as having generalized resistance to TH (GRTH). In such individuals, the defect seems to be compensated by the high endogenous levels of TH. In contrast, patients with equally high levels of TH appear to be hypermetabolic because they are restless and hyperactive or have a rapid heart rate. Such individuals have been classified as having selective pituitary resistance to TH (PRTH). Subdivision of RTH-beta into generalized RTH-beta (GRTH) and peripheral RTH-beta (PRTH) is an artifact arising from the subjective nature of symptoms and poor specificity of signs. Subjects harboring the same mutations, and even belonging to the same family, have been classified as having GRTH and PRTH. Furthermore, clinical studies have shown that the responses of peripheral tissues markers to TH action were equally attenuated. The classification of RTH-beta into GRTH and PRTH can therefore be viewed as opposite ends of the spectrum of a single disease.

Clinical findings

The phenotype of RTH-beta is variable, with most patients presenting with mild-to-moderate symptoms. In children, investigation leading to the diagnosis has been undertaken because of goiter, hyperactive behavior, learning disabilities, or developmental delay. Most adults seek medical attention because of goiter or rapid heart rate. RTH-beta has been diagnosed in infancy as a result of routine neonatal screening programs that measure both TSH and T_4 and to hormonal and DNA analyses performed in infants born to parents known to have RTH-beta. In adults, abnormalities found on routine thyroid testing, in the absence of clinical findings, have been responsible for the diagnosis of RTH-beta.

The majority of untreated subjects are eumetabolic at the expense of high levels of TH. The degree of this compensation for the tissue hyposensitivity to the TH is variable among individuals and in different tissues. As a consequence, clinical and laboratory evidence of TH deficiency and excess often coexists.

Many attempts have been made to demonstrate tissue hyposensitivity *in vitro*. The common finding of paradoxical responses to TH is difficult to explain. They are possibly due to (a) variable interaction of the mutant TR with the α and β isoforms of the normal TRs, (b) variable expression of the α and β TR genes in different tissues, and (c) differences in the regulation of expression of the TRα and TRβ genes by the hormone-activated TR, which in some tissues may be in opposite directions.

On physical examination, goiter is the most common abnormality, occurring in 66–95% of cases and almost always detected by ultrasonography. Gland enlargement is usually diffuse. Tachycardia occurs in up to 80–90% of subjects with RTH-beta and is caused by the excess of TH acting on the TRα gene and often brings the patient to the physician. Careful evaluation of subjects with RTH-beta has shown that one-half has learning disabilities, often associated with ADHD and, on the average, lower intellectual

quotients (IQ). However, frank mental retardation (IQ < 60) occurs in only 3% of the cases. RTH-beta can present with mild-to-moderate growth retardation and delayed bone maturation in 25% of the cases. Hearing defects have been detected in almost one-quarter of patients. Other features, such as frequent ear, nose, and throat infections; decreased bone mass; deafness; hypotonia; and seizures, have been recognized.

The course of the disease is as variable as its presentation. Some subjects have normal growth and development and lead a normal life. Others present variable degrees of mental and growth retardation. There is some evidence that the severity of resistance tends to improve as the subject gets older as determined by thyroid function tests. This has been shown in the TRβ knockout mouse model of RTH-beta (Weiss *et al.*, 2002) and is supported from some published studies in humans.

Diagnosis and Differential Diagnosis

Serum Levels of Thyroid Hormones and TSH

Elevation in the concentration of serum free T4 is a *sine qua non* requirement for the diagnosis of RTH-beta. It is often accompanied by high serum levels of T3 with nonsuppressed TSH.

In RTH-beta, serum T_3 and T_4 values range from just above to several folds the upper limit of normal. Reverse T_3 concentration is also high in these patients. The degree of T_3 and T_4 elevation is usually congruent, resulting in a normal T_3 to T_4 ratio. This contrasts with the disproportionate increase in serum T_3 concentration characteristic of autoimmune thyrotoxicosis. The concentration of serum thyroglobulin reflects the level of TSH-induced thyroid gland activity. The extrathyroidal conversion of T_4 to T_3 has been normal.

The fractional uptake of radioiodide by the thyroid gland is high. The iodide appears to be normally organified since no discharge of trapped iodide has been observed following the administration of perchlorate.

TRH Testing

A diagnostic feature of the syndrome is the presence of normal or slightly elevated serum TSH levels and preservation of its response to TRH despite elevated TH levels. The TSH response to TRH is either normal or exaggerated; the latter is more common in patients receiving antithyroid drug therapy or with history of prior surgery or radioiodide therapy. The bioactivity of secreted TSH is normal or increased, and the concentration of its free α-subunit is not disproportionately high. In contrast, the α-subunit to TSH ratio is elevated in TSH-secreting tumors.

Responses to the Administration of TH and Other Drugs

The administration of supraphysiological doses of TH suppresses TSH secretion resulting in the decrease and eventually the abolition of the TSH response to TRH. In subjects with RTH-beta, L-T4 given in doses of up to 1000 μg/day and L-T3 up to 400 μg/day may be necessary. Various responses of peripheral tissues to the administration of TH have been quantitated. Most notable are measurements of the BMR, pulse rate, reflex relaxation time, serum cholesterol, lipids, enzymes, SHBG, and urinary excretion of hydroxyproline, creatine, and carnitine. Either no significant changes were observed, or they were much reduced relative to the amount of TH given.

As expected, the administration of the TH analog, 3,5,3′-triiodo-L-thyroacetic acid (TRIAC), to patients with RTH-beta produced attenuated responses. A standardized diagnostic protocol, using short-term administration of incremental doses of L-T_3, is recommended. The three doses given in sequence to an adult are a replacement dose of 50 μg/day and two supraphysiological doses of 100 and 200 μg/day. The hormone is administered in a split dose every 12 h, and each incremental dose is given for 3 days. Doses are adjusted in children and in adults of unusual weight to achieve the same level of serum T_3.

Administration of glucocorticoids promptly reduced the TSH response to TRH and the serum T4 concentration (Refetoff *et al.*, 1993). The β-adrenergic blockers, propranolol and atenolol, produce a significant reduction in heart rate.

Family Studies

Once a diagnosis of RTH-beta is suspected, testing of thyroid function in siblings and parents can be helpful. Confirmation of the same phenotype in other related individuals make the diagnosis of RTH-beta more likely. TSH-secreting adenoma as such tumors has not been reported to be familial. Identification of an abnormal phenotype in an asymptomatic family member will allow for a correct diagnosis and may prevent unnecessary diagnostic testing and treatment. From a practical standpoint, a physician may find it difficult to arrange for thyroid testing on asymptomatic relatives; however, considering the cost of an MRI, documentation of an inherited disorder is more cost-effective.

Imaging Studies

Imaging studies are not necessary for the diagnosis of RTH-beta. While an MRI of the pituitary is oftentimes ordered to satisfy the physician that a TSH-secreting adenoma (TSHoma) is absent, it cannot be justified as routine in the workup for RTH-beta due to the fact that a small microadenoma may be missed by the MRI and 10% of normal individuals may have some abnormality that may not have any clinical significance.

Nevertheless, one condition that is most similar to RTH-beta in terms of thyroid function testing is a TSHoma and is often most confused with RTH-beta. In both conditions, the free TH levels can be elevated to the same degree, and the serum TSH will not be suppressed. While the TRH stimulation test and alpha subunit can be helpful, oftentimes, distinguishing between the two diagnoses can be challenging unless (1) there is a clear genetic diagnosis such as a mutation in the THRB or (2) there is familial evidence (see earlier) of the phenotype. However, it should be noted that there have been several case reports of patients with both RTH-beta and TSHomas occurring simultaneously (Ando *et al.*, 2001; Krysiak *et al.*, 2011; Teng *et al.*, 2015; Watanabe *et al.*, 1993).

Genetic Studies

The use of genetic testing in a family with RTH-beta for mutations in the *THRB* genes is no longer limited to research laboratories as Quest Diagnostics and others offer *THRB* gene sequencing for a definitive diagnosis. Once a mutation is identified, screening of other family members for the mutation can be more sensitive than screening for the phenotype due to the mild and variable nature of the RTH-beta phenotype. Confirmation of the mutation in a newborn may eliminate the need for treatment. Confirmation of the mutation in a fetus, at some point, may allow us to prescribe intrauterine treatment, if necessary.

Summary of Recommended Diagnostic Evaluation

1. Usual presentation—persistently high serum levels of TH with nonsuppressed TSH. Given the inherited nature of RTH-beta, normal thyroid tests are never seen in subjects who have not been treated.
2. Confirm the elevated serum levels of TH and exclude TH transport defect or antibody interference in the assays.
3. Measurement of thyroid function tests in other family members.
4. In sporadic cases, exclude the presence of a pituitary adenoma by measurement of α-subunit in serum.
5. Demonstrate a blunted TSH suppression and metabolic response to the administration of supraphysiological doses of TH.
6. Perform genetic studies when indicated.

Treatment

General Considerations

No specific treatment is available to fully correct the defect. Fortunately, in most cases of RTH-beta, the partial tissue resistance to TH appears to be adequately compensated by an increase in the endogenous supply of TH, and no treatment is necessary in these cases (Hassan and Koh, 2008).

In cases where previous erroneous diagnosis has occurred resulting in postsurgical or postradiation hypothyroidism, treatment with TH can be started. Serum TSH level can be used as a guideline for hormone dosage.

Some patients with RTH-beta present with peripheral tissues relatively more resistant than pituitary. Thus, compensation for the defect at the level of peripheral tissue is incomplete. In such instances, administration of supraphysiological doses of TH is indicated. Since the dose varies among affected subjects, it should be individually determined by assessing tissue responses.

Patients without prior antithyroid treatment but with more severe thyrotroph resistance and symptoms of thyrotoxicosis may also require therapy. Common symptoms are hyperactivity; tachycardia; and, less commonly, diarrhea. Usually, symptomatic treatment with a β-adrenergic-blocking agent would suffice. Local symptoms due to goiter size can be reduced by administration of a single high dose of L-thyroxine given every other day.

The exact criteria for treatment of RTH-beta in infancy have not been established. This is often an issue when the diagnosis is made at birth or in early infancy. In infants with elevated serum TSH levels, subclinical hypothyroidism may be more harmful than treatment with TH. Indications for treatment may include a TSH level above the upper limit of normal, retarded bone development, and failure to thrive. Treatment with L-T_3 may improve the symptoms of ADHD in a significant proportion of children that also have RTH-beta.

Although RTH-beta is a relatively rare condition, the physician should be aware that common diseases, such as autoimmune-mediated hypothyroidism and hyperthyroidism, can occur in subjects with RTH-beta. Concurrence of Graves' disease and RTH-beta would significantly alter the therapeutic and diagnostic approach to the care of these patients, but the overall goal would be to obtain clinical euthyroidism with a normal serum TSH.

Pharmacologic Therapy

TRIAC has been used successfully to decrease the serum TSH and TH levels, to reduce goiter size, and to alleviate some of the symptoms attributed to the effect of TH on peripheral tissues. However, the concomitant effects of TRIAC on markers that measure TH action on peripheral tissues, as well as on heart rate, are minimal probably because the decrease in TH levels is offset by the intrinsic specific thyromimetic effect of TRIAC (Radetti *et al.*, 1997). The ability of TRIAC to suppress TSH without an increase in the thyromimetic effect on peripheral tissues is due to properties of this TH analog: its higher affinity for the β, but not α TR as compared with T_3, and its more rapid degradation. The mechanism mediating a similar effect attributed to D-T_4 is less well understood.

Dopaminergic drugs and somatostatin analogs have had limited use because of side effects and low success rate in maintaining TSH suppression.

General guidelines for the treatment with TH, usually L-T_4, are (1) elevated serum TSH levels, (2) failure to thrive that cannot be explained on the basis of another illness or defect, (3) unexplained seizures, (4) developmental delay, and (5) history of growth or mental retardation in affected members of the family.

The future direction in the treatment of RTH-beta will be the use of "designer drugs" that have been designed to interact with a specific mutant TR. Such a TH analog would have a strong affinity for the mutant TR and could eliminate the DNE. The rarity of the syndrome combined with the fact that RTH-beta is not a life-threatening illness, the expense of the drug, and the necessary trials to obtain approval, even as an orphan drug, make the widespread availability of these drugs something for the future.

Management of Pregnancy and RTH-Beta

Little is known about the ideal management of a pregnant mother with RTH-beta. Different considerations may need to be made for whether the fetus also has RTH-beta. In such an instance, one may predict that an increase level of maternal TH is necessary for the early development of a similarly affected fetus but could be detrimental to an unaffected fetus. On the other hand, it is unknown whether a normal mother would be able to provide the appropriate amount of TH to an RTH-beta fetus (e.g., when the father is affected or a *de novo* mutation). A retrospective study of a large family the RTH-beta, due to THRB 243Q, demonstrated that pregnancy loss was increased by threefold in affected mothers, but not in couples with an affected father and unaffected mother (Anselmo *et al.*, 2004). Unaffected infants born to affected mothers had lower birth weights and suppressed serum TSH concentrations. Management of pregnancies in mothers with RTH who are carrying unaffected fetuses may warrant the use of antithyroid medication depending on the well-being of the fetus. In such mothers, free T4 should be maintained not higher than 20% above the upper limit of normal with the use of propylthiouracil. There is no basis for regular treatment of unaffected mothers carrying affected fetuses unless the fetus has a large goiter or is in distress. In such cases, treatment with intra-amniotic infusion of L-thyroxine could be considered. Further investigation is necessary for the evaluation of these important questions.

Management of Differentiated Thyroid Cancer and RTH-Beta

As more cases of RTH-beta are diagnosed, it is not unusual that there would be more cases of differentiated thyroid cancer found in RTH-beta patients. However, there is no direct evidence that RTH-beta is causative for thyroid cancer in humans. Mouse models with homozygous TRβ defects do have an increased incidence of differentiated thyroid cancer. The challenge in RTH-beta with thyroid cancer is the TH replacement following thyroidectomy. Such patients can require as much as 1 g of L-thyroxine daily to suppress the TSH.

See also: Thyroid Function Tests. Thyroid Hormone Action. Thyroid Hormone Receptors. TSH-Producing Adenomas and Resistance to Thyroid Hormones

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TSH-Producing Adenomas and Resistance to Thyroid Hormones☆

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Glossary

Hyperthyroidism Clinical condition characterized by excessive secretion of thyroid hormones in the presence of normal tissue response to thyroid hormone action.

Hypothalamic – pituitary – thyroid axis Axis composed of the hypothalamus (thyrotropin-releasing hormone [TRH] and other molecules produced in the brain), the pituitary (thyroid-stimulating hormone [TSH]), and the thyroid (thyroxine [T4] and triiodothyronine [T3]).

Pituitary tumors Neoplastic lesions of the pituitary; they are generally adenomas and rarely carcinomas, and they may be nonfunctioning or functioning with hypersecretion of prolactin (PRL), growth hormone (GH), adrenocorticotropic hormone (ACTH), gonadotropins, or thyroid-stimulating hormone (TSH).

Resistance to thyroid hormones An inherited disease characterized by a reduced responsiveness of target tissues to thyroid hormone action.

Introduction

Hyperthyroidism usually results from thyroid stimulation by anti-thyroid-stimulating hormone (TSH) receptor autoantibodies or toxic adenomas, most of them due to activating mutation of TSH receptor, and very rarely by TSH-producing adenomas (TSH-omas) or other causes of central hyperthyroidism such as pituitary resistance to thyroid hormone (RTH) action. However, during the past decades with the advent of ultrasensitive immunometric assays routinely performed as a firstline test of thyroid function, patients with central hyperthyroidism have been recognized with increasing frequency. Patients with central hyperthyroidism have normal or elevated circulating TSH levels in the presence of high thyroid hormone concentrations. The most frequent cause of central hyperthyroidism is a TSH-producing pituitary adenoma. However, a subset of patients with RTH may present with signs and symptoms of hyperthyroidism and may be confused with patients with TSH-omas. It is mandatory to distinguish RTH patients from those with TSH-omas because the treatment of the latter is completely different from that of RTH patients.

TSH-producing adenoma is a rare disorder, accounting for about 0.5–2% of all pituitary adenomas. The prevalence in the general population is 1–2 cases per million. However, this figure is probably underestimated because the number of reported cases of TSH-omas tripled during the past decade or so. The increased incidence of TSH-omas has been futher confirmed by recent data obtained from The Swedish Pituitary Registry, demonstrating an increased TSH-omas incidence over time (0.05 per 1 million per year in 1990–94 to 0.26 per 1 million per year in 2005–09), the national prevalence in 2010 being 2.8 per 1 million inhabitants. The increased number of reported cases of TSH-omas is due principally to the introduction of ultrasensitive immunometric assays for TSH as a firstline test for the evaluation of thyroid function. Indeed, many patients previously thought to be affected with Graves' disease were correctly diagnosed as patients with TSH-omas or, alternatively, with RTH based on the finding of measurable serum TSH levels in the presence of elevated thyroid hormone concentrations.

Pathology

All TSH-oma cases originate from pituitary thyrotropes, with the exception of two individuals who showed ectopic nasopharynx TSH-omas. Only one TSH-producing carcinoma has been reported in the literature. Most TSH-omas are macroadenomas, with microadenomas accounting for less than 15% of all reported cases. The occurrence of invasive macroadenomas is particularly high in patients with previous thyroid ablation by surgery or radioiodine. This aggressive transformation of the pituitary tumor resembles that occurring in Nelson syndrome after adrenalectomy for Cushing's disease.

By light microscopy, tumoral thyrotropes generally appear to be chromophobic, with atypical nuclei and mitoses, and so they are often mistakenly recognized as pituitary malignancy or metastasis from distant carcinomas. By electron microscopy, these tumors are monomorphous and characterized by a poorly developed Golgi apparatus and a low number of small secretory granules aligned mainly under the plasma membrane.

Approximately 75% of TSH-omas secrete TSH alone, which is often accompanied by an unbalanced hypersecretion of the α-subunit of glycoprotein hormones. Hypersecretion of growth hormone (GH) and/or prolactin (PRL), resulting in acromegaly and/or amenorrhea/galactorrhea syndrome, is present in approximately 25% of tumors, whereas the occurrence of mixed TSH/

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gonadotropin adenomas is rare and no association with adrenocorticotropic hormone (ACTH) hypersecretion has been documented. This may be due to the fact that GH and PRL share with TSH the common transcription factor Pit-1.

As for most pituitary tumors, the pathogenesis of TSH-omas remains largely unknown. Screening studies for genetic abnormalities that may be responsible for tumor formation were generally negative. Anecdotal reports showed overexpression of the Pit-1 gene, absence of thyroid hormone receptor isoforms, and (recently) mutations in the beta isoform of the receptor leading to an altered feedback mechanism that is possibly responsible for the tumor formation. However, available data concern only a low number of tumors and are too preliminary to draw definite conclusions on transcriptional and/or expression abnormalities in TSH-omas.

Clinical Manifestations

TSH-omas may occur at any age, although most patients are in the third to sixth decade of life. Unlike the female predominance seen with other common thyroid disorders, TSH-omas occur with equal frequency in males and females. Patients with TSH-omas present with signs and symptoms of hyperthyroidism that are sometimes milder than expected on the basis of circulating thyroid hormone levels. Signs and symptoms of hyperthyroidism are frequently associated with those related to the mass effects of the pituitary adenoma. Visual field defects are present in approximately 40%, headache in 20% and partial or total hypopituitarism in 25% of patients (**Table 1**).

Most patients have a long history of thyroid dysfunction, often misdiagnosed as Graves' disease, and approximately one-third had inappropriate thyroidectomy or radioiodine thyroid ablation. In some acromegalic patients, signs and symptoms of hyperthyroidism may be clinically missed because those of acromegaly overshadow them. The presence of goiter, frequently uni- or multinodular ($\sim$90% of reported cases), is the rule. Also, in patients with previous thyroidectomy, thyroid residue may regrow as a consequence of TSH hyperstimulation. Progression toward functional autonomy or differentiated carcinomas seems to be infrequent. Bilateral exophthalmos occurred in a few patients who subsequently developed autoimmune thyroid disorders, whereas unilateral exophthalmos due to orbital invasion by pituitary tumor was reported in three patients with TSH-omas.

Diagnosis

Serum Thyroid Hormone and TSH Levels

Serum total and free thyroid hormone levels are definitely high in patients with TSH-omas, whereas TSH levels may be elevated or in the normal range (**Table 2**). In some patients with TSH-omas, the findings of normal TSH in the presence of high levels of free thyroxine (FT4) and free triiodothyronine (FT3) were demonstrated to be due to an increased biological activity of secreted TSH molecules.

Particular clinical situations and possible laboratory artifacts causing a biochemical profile similar to that characterizing central hyperthyroidism should be considered. Measuring free, instead of total, thyroid hormones may recognize most of the confusing conditions, including genetic alterations or drugs that may case quantitative/qualitative alterations of T4-binding globulin, albumin, or transthyretin leading to increases in thyroid hormone levels, particularly T4. Laboratory artifacts, such as those caused by circulating anti-T4 and/or anti-T3 autoantibodies that interfere in most immunometric assays, may cause falsely high levels of free thyroid hormones, whereas heterophilic antibodies directed against or cross-reacting with mouse immunoglobulin G (IgG), as well as anti-TSH autoantibodies in patients previously treated with bovine TSH or contaminated pituitary extracts, may lead to incorrect evaluation of the actual TSH concentrations.

Pituitary Glycoprotein Hormone α-Subunit

A helpful diagnostic tool for the diagnosis of TSH-omas is the determination of serum concentrations of the α-subunit, the subunit common to all pituitary glycoprotein hormones. Secretion of the α-subunit in these tumors is in excess not only of the TSH-β

Table 1 Clinical features of patients with TSH-omas

Feature	*Patients with TSH-omas (%)*
Goiter	91
Thyroid nodules	69
Severe thyrotoxicosis	30
Previous thyroidectomy	31
Menstrual disorders	33
Macroadenomas	84
Visual filed defects	40
Headache	20

Source Updated from data published through September 2013.

subunit but also of the intact TSH molecule, resulting in an α-subunit/TSH molar ratio generally higher than that recorded in controls matched for age and sex and with similar circulating levels of TSH, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) (Table 2).

Parameters of Peripheral Thyroid Hormone Action

Because patients with central hyperthyroidism may present with mild signs and symptoms of thyroid hormone overproduction, the measurements of several parameters of peripheral thyroid hormone action have been proposed to quantify the degree of hyperthyroidism. Some of these parameters, and in particular sex hormone-binding globulin (SHBG) and cross-linked carboxyterminal telopeptide of type I collagen (ICTP), have been used to differentiate hyperthyroid patients with TSH-omas from those with RTH (Table 2); patients with TSH-omas have levels of these indexes into the hyperthyroid range, whereas they are into the normal range in RTH patients.

Dynamic Testing

Several stimulatory and inhibitory tests are useful for the diagnosis of TSH-omas. Classically, the T3 suppression test has been used to assess the presence of a TSH-oma because complete inhibition of TSH secretion after T3 (80–100 μg per day for 8–10 days) has never been recorded in patients with TSH-omas (Table 2). It is worth noting that in patients with previous thyroid ablation, T3 suppression seems to be the most sensitive and specific test in assessing the presence of a TSH-oma. In approximately 93% of patients, TSH levels do not increase after TRH injection. The lack of TSH response to TRH may also be useful in unusual situations where TSH-omas coexist with primary hypothyroidism.

The majority of patients with TSH-omas maintain the sensitivity to native somatostatin or its analogues, and these tests may be predictive of the efficacy of long-term treatment in most patients.

Imaging Studies

When considering the diagnosis of a TSH-oma, full imaging studies, particularly nuclear magnetic resonance imaging (MRI) or high-resolution computed tomography (CT), are mandatory. Various degrees of suprasellar extension or sphenoidal sinus invasion are present in two-thirds of cases.

Differential Diagnosis

In a patient with signs and symptoms of hyperthyroidism, the presence of elevated thyroid hormone and detectable TSH levels rules out primary hyperthyroidism. When the existence of central hyperthyroidism is confirmed and the presence of methodological interferences is excluded, several diagnostic steps have to be carried out to differentiate a TSH-oma from RTH (Table 2). Indeed, the possible presence of neurological signs and symptoms (e.g., visual defects, headache) or clinical features of concomitant hypersecretion of other pituitary hormones points to the presence of a TSH-oma. Furthermore, the presence of alterations of pituitary content at MRI or CT scan strongly supports the diagnosis of a TSH-oma. Nevertheless, the differential diagnosis may be difficult when the pituitary adenoma is undetectable by MRO or CT scan or in the case of confusing lesions such as empty sella or pituitary incidentalomas. In these cases, elevated α-subunit concentrations or high α-subunit/TSH molar ratios and TSH

Table 2 Differential diagnosis between TSH-omas and RTH

Parameter	*TSH-omas*	*RTH*	P
Female/Male ratio	1.29	1.41	NS
Familial cases (percentages)	0[a]	86	<0.01
TSH (mU l^{-1})	2.9 ± 0.5	2.4 ± 0.4	NS
FT4 (pmol l^{-1})	39.8 ± 4.1	31.2 ± 2.3	NS
FT3 (pmol l^{-1})	13.9 ± 1.2	12.3 ± 1.0	NS
SHBG (nmol l^{-1})	121 ± 19	60 ± 5	<0.02
Lesions at CT or MRI (percentages)	98	7	<0.01
High α-subunit levels (percentages)	71	2	<0.01
High α-subunit/TSH m.r. (percentages)	82	2	<0.01
Absent or impaired TSH response to TRH test (percentages)	93	3	<0.01
Abnormal TSH response to T3 suppression test[b] (percentages)	100	100	NS

[a]Excluding the three families with Men-1 reported in the literature.

[b]Werner's test (80–100 μg T3 for 8–10 days). Quantitatively normal responses to T3 (i.e., complete inhibition of both basal and TRH-stimulated TSH levels) have never been recorded in either group of patients. Although abnormal in quantitative terms, TSH response to T3 suppression test was qualitatively normal in RTH patients.

Note. Only patients with intact thyroid were taken into account. Data are obtained from patients followed at our institute and are expressed as means ± SE.

unresponsiveness to TRH stimulation and/or to T3 suppression tests favors the presence of a TSH-oma. Moreover, in contrast to RTH patients, familial cases of TSH-omas have never been documented. Finally, an apparent association between TSH-oma and RTH was recently reported in a young Japanese woman, although genetic investigations of possible mutations in T3 receptor $\beta 1$ were not carried out. Nonetheless, the occurrence of TSH-omas in RTH patients is theoretically possible and, therefore, should be carefully considered.

Treatment and Follow-Up

Surgical resection is the recommended therapy for TSH-producing pituitary tumors. However, a radical removal of large adenomas, which still represent the majority of TSH-omas, is particularly difficult due to the local invasion of the tumor. Particular attention has to be paid to presurgical preparation of the patient. Antithyroid drugs or octreotide along with propranolol should be used to restore euthyroidism. If surgery is contraindicated or declined, as well as in the case of surgical failure, pituitary radiotherapy may be undertaken with the recommended dose of no less than 45 Gy fractionated at 2 Gy per day or 10–25 Gy in a single dose if a stereotactic gamma unit is available. The criteria of cure of patients operated and/or irradiated for TSH-omas have not been clearly established due to the rarity of the disease and the great heterogeneity of parameters used. In particular, clinical remission of hyperthyroidism with normalization of thyroid hormones, TSH, and α-subunit or α-subunit/TSH molar ratio, and with the disappearance of neurological symptoms, has been considered for the evaluation of the efficacy of surgery or radiotherapy in patients with TSH-omas. In our experience, undetectable TSH levels 1 week after surgery are likely to indicate complete adenomectomy provided that presurgical treatments were stopped at least 10 days before surgery. The most sensitive and specific test to document the complete removal of the adenoma remains the T3 suppression test. In fact, only patients in whom T3 administration completely inhibits basal and TRH-stimulated TSH secretion appear to be truly cured. No data on the recurrence rates of TSH-omas in patients judged to be cured after surgery or radiotherapy have been reported. Although earlier diagnosis has improved the surgical cure rate of TSH-omas, several patients require medical therapy to control the hyperthyroidism. Dopamine agonists, and particularly bromocriptine, have been employed in some TSH-omas with variable results, with positive effects being observed mainly in some patients with mixed PRL/TSH adenomas. Today, the medical treatment of TSH-omas rests on long-acting somatostatin analogues such as octreotide LAR and lanreotide SR. Treatment with these analogues leads to a reduction of TSH and α-subunit secretion in nearly all cases, with restoration of the euthyroid state in the majority of them.

Somatostatin analogs treatment induce the normalization of circulating thyroid hormone levels in more than 90% of patients and a significant decrease in goiter size in about 30% of cases. Somatostatin analog treatment induces a significant tumor mass shrinkage in about 40% of patients and vision improvement in about 70% of them.

Resistance to octreotide treatment has been documented in only 4% of cases.

According to recently published guidelines, somatostatin analogs treatment is indicated in patients not cured by surgery or to prepare patients to surgery, surgery remaining the therapy of choice for TSH-omas.

See also: Sodium/Iodide Symporter (NIS). TSH Function and Secretion

Further Reading

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Thyroid Function Tests☆

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Thyroid function tests are used for the diagnosis of all thyroid disorders, but are particularly important for the diagnosis of thyroid hormone deficiency (hypothyroidism) or excess (hyperthyroidism or thyrotoxicosis). Most frequently used are *in vitro* blood tests, but *in vivo* tests with radioisotopes can also provide information on thyroid function.

Pathophysiology

The thyroid is well known for its important stores of hormones, and the circulating thyroxine (T4) represents a large reservoir that is bound mainly to an interalpha glycoprotein, thyroxine-binding globulin (TBG), whereas other T4-binding proteins are transthyretin (prealbumin) and albumin. TBG has the highest affinity of these three proteins for T4. TBG's Ka value is $1 \times 1010\ M^{-1}$ compared with 7×10^{-7} and $7 \times 105\ M^{-1}$ for transthyretin and albumin, respectively.

The maximal binding capacity of TBG is about 250 nmol l^{-1} serum (20 μg/100 ml); and under most pathophysiological conditions, it is the limiting buffering system that determines the ratio of protein bound to the free T4. This ratio is extremely in favor of protein binding because the free fraction represents only 0.02% of the total serum T4.

There are three conditions for altering effective TBG concentrations.

The first condition is the inherited trait of x-linked absence (1:5000 births) or autosomal-linked increased TBG (1:25000) concentrations. In addition, many point mutations have been described, resulting in genetic variants, with perhaps the most striking one being found in Australia, where close to 30% of the aborigines present with a genetic variant due to direct consequence of the amino acid substitution and/or altered glycosylation of the protein. There is also a genetic variant where TBG concentrations are increased. This is due to gene duplication.

Second, serum TBG concentrations may vary as a function of glycosylation. A highly glycosylated TBG has a longer half-life. Estrogens increase glycosylation. Consequently, during pregnancy, TBG concentrations are very high. Androgens and glucocorticoids have the opposite effect. Obviously, TBG concentrations can also increase due to increased synthesis stimulated, for instance, by thyroid hormones and estrogens.

The third condition is competition for the T4- binding site of TBG by substances such as phenytoin, high doses of salicylate, and free fatty acids (FFAs). Phenytoin, rifampicin, and carbamazepine also alter T4 metabolism by accelerating its hepatic metabolism, inducing the cytochrome P450 complex. Other drugs stimulating the cytochrome P450 may act likewise, yet few are sufficiently potent to be clinically relevant.

Of particular clinical importance is the displacing of T4 from TBG by high FFA levels. This may occur in vivo, particularly under heparin treatment, which activates serum lipase activity. More important, FFAs can also be generated in vitro either in the presence of heparin or by repeated freezing and thawing of serum. This can result in increases of FFA levels to 3 mmol l^{-1} or more, and such values result in an increase of the free T4 levels.

Normal thyroid function is dependent on an adequate intranuclear concentration of triiodothyronine (T3) that is again a function of the transfer of T3 and T4 from plasma to the cell. For both hormones, only the free fraction can enter the peripheral cell. If the free concentration falls, the pituitary will be stimulated to secrete thyroid-stimulating hormone (TSH) in order to restore adequate free T4 and T3 concentrations. This will occur when free T4 concentrations change due to altered TBG levels. The kinetics of the protein changes are slow (half-life = 6 h for transthyretin and 4 days for TBG).

For a long time, it was believed that the changes in serum concentrations of TBG would not affect thyroid hormone economy. Recently, it has been shown that in treated but severely hypothyroid patients, pregnancy or estrogen treatment increases the demands of T4 treatment as evidenced by an increase in serum TSH values. This is the first evidence that the increased total T4 pool under estrogen treatment cannot be achieved solely by decreasing the metabolic rate and metabolism of T4. Therefore, under physiological circumstances, there is a discrete adaptation of thyroidal secretion to ensure an unchanged thyroid function. Consequently, in hypothyroid patients treatment must be adjusted.

TBG binds T3 with a lower affinity than T4, the affinity constant for T3 being 5×10^{-8} M. The T3 serum levels are less affected by changes in serum TBG. Despite the fact that T3 is the active thyroid hormone, other factors markedly reduce the diagnostic value of T3:

1. T3 is mainly an intracellular hormone, and the circulating pool represents no more than 15–20% of its whole body pool.
2. T3′s production is mainly non-thyroidal and is markedly influenced by the metabolic state of the organism.
3. T3′s production is controlled by monodeiodination, and its degradation mainly by conjugation.

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Monodeiodination plays a crucial role in the control of serum T3 levels. Three enzymes – deiodinase types 1, 2, and 3 – have been characterized. They belong to the very rare selenoproteins. They differ markedly concerning their functional characteristics. Quantitatively, the most important enzyme is deiodinase type 1, which mainly is found in the liver and kidney but in small amounts it is ubiquitously present. It is localized at the inner plasma membrane. Under physiological conditions, approximately 30% of T4 is converted by this enzyme to T3. The expression and activity of this enzyme are stimulated by T3; for example, in hyperthyroidism the activity is very high, whereas in hypothyroidism it is very low. However, many other non-thyroidal factors affect its activity. In any catabolic state – malnutrition, starvation, severe infections, or metabolic or malignant diseases – its activity is decreased and this is reflected in decreased serum T3 levels. Another example is drug interferences such as glucocorticoids and amiodarone, which are potent inhibitors of deiodinases. Stimulation of deiodinase type 1 can be observed with overfeeding and/or a high-carbohydrate intake.

The activity profile of deiodinase type 2 is very different. It has a very low affinity constant (Km). The enzyme that is found in the pituitary and brain is most active when T4 is absent. It is inhibited with increasing T4 concentrations. It is directly implicated in the control of serum TSH. Both enzymes attack the outer ring of T4. The third enzyme, deiodinase type 3, deiodinates only at the inner ring, a function that it shares with deiodinase type 1 under particular conditions. Interestingly, deiodinase type 3 is localized at the outer cell membrane, and by degrading T4, it reduces its entry into the cell.

Thyroidal production of T3 is minimal compared with that of T4. Without iodine deficiency, T3 represents less then 5% of the secreted T4. There are at least two conditions where this percentage can increase: in iodine deficiency and in hyperthyroidism. In hyperthyroidism, the thyroidal deiodinase is strongly stimulated so that some of the T4 is converted to T3 during the process of secretion. In iodine deficiency, this mechanism is also operating. In addition the poorly iodinated thyroglobulin (Tg) has a high percentage of T3 that can be up to 50% of the amount of T4. This contributes to a higher proportion of T3 in serum, and the T3-to-T4 ratio can increase from the physiological ratio of approximately 2–4% in hyperthyroidism and to much higher values in severe iodine deficiency. If selenium deficiency is associated with iodine deficiency, the increased ratio of T3 to T4 is not seen because the lack of selenium affects the activity of the deiodinases. Clinically, the increased T3-to-T4 ratio was previously of diagnostic value for T3 toxicosis that is now diagnosed more adequately by a suppressed serum TSH in the presence of a still normal free T4. For practical purposes, one can conclude that the serum levels of T3 are influenced by many factors other than thyroidal secretion and are, therefore, of lower diagnostic value than free T4 values.

rT3 is the inactive counterpart of T3 and has been advocated as a diagnostic tool, but today none of them remains valid. Nevertheless, it is an interesting parameter that gives a good mirror image of T4-to-T3 conversion. If deiodinase type 1 is inhibited, serum rT3 levels increase. There are two reasons for this. First, rT3 needs deiodinase type 1 to be degraded and accumulates if the enzyme is low. Second, there may be some shift from T4-to-T3 conversion to rT3.

Methods of Serum Free T4 and T3 Measurements

As mentioned previously, free T4 represents an extremely small fraction of the total T4. Today, for routine purposes, no fully satisfactory free T4 measurement is available. The best, but cumbersome methods use ultrafiltration or equilibrium dialysis. The latter technique was the first reliable method; however, it can yield falsely elevated values if FFAs are artificially increased by the presence of in vitro heparin or by repeated freezing and thawing. Both methods cannot be used in routine practice.

Two different approaches for measuring the free T4 indirectly are available: the free T4 or T3 index test, which is based on the fact that the product of total serum T4 times the result of the T3 resin uptake gives an arbitrary index that roughly correlates with free T4 values. Artifactual results with T3 resin uptake methods are frequently used and the results are similar to the findings using commercial free T4 methods.

The so-called direct measures of free T4 and T3 are methods based on chemiluminescent assays. They cannot measure directly the free fraction since these concentrations are far too low to be measured. Therefore, commercial companies have developed T4 analogs that do not bind to TBG and are distributed only within the free hormone fraction. In addition, this fraction is artificially increased by competitors of T4 for TBG. These methods have the advantage over the free T4 index in that they yield absolute concentrations of free T4 and their interpretation is straightforward. However, because of technical limitations, they are also prone to artifactual values, where unknown competitors for T4 binding to TBG have been described. Consequently, some commercial methods will have a tendency of too low values in severe illnesses. Some of these problems can be overcome by more cumbersome techniques (two step methods), but even these methods are not free of artifactual results, since theyhe tend to give too high values in severe illness.

In ambulatory medical practice, these commercial methods are satisfactory and render total T4 and T3 methods unnecessary. In addition, the clinician should rely first on serum TSH, with serum free T4 and T3 levels being useful adjuncts for the refinement of diagnosis and monitor treatment and for excluding some extremely rare thyroidal conditions.

These are TSHomas (pituitary tumors secreting TSH) and thyroid hormone resistance. In TSHomas, serum TSH values are normal or slightly increased, yet both serum free T4 and T3 are increased and are associated -with clinical signs of hyperthyroidism. In thyroid hormone resistance, serum TSH can be normal, low, or slightly increased in the presence of increased free T4 and T3. However, the clinical picture is composite, with the heart often being the sole organ with signs of hyperthyroidism.

Serum TSH

Measurement of serum TSH values is the first line diagnostic test. Its sensitivity is very high, whereas its specificity is slightly lower (90%) because drugs and severe illness can decrease serum TSH levels in euthyroid patients. The predominant role of serum TSH as a diagnostic parameter is due to the fact that it is the most sensitive parameter of peripheral thyroid hormone action. It increases exponentially when serum free T4 decreases. It differs from the clinically more relevant effects of T3 on heart, liver, brain, and other tissues for the following reasons:

1. The intracellular regulation of thyroid hormone metabolism in the pituitary and brain does not exist in peripheral tissues.
2. The action of thyroid hormones in the pituitary is inhibitory (on dejodination?). In the periphery, T3 has multiple actions some stimulatory, others are inhibitory.
3. TSH secretion is controlled by the hypothalamus: thyrotropin-releasing hormone (TRH), somatostatin, dopamine and leptin to mention some of these factors.

Clinical Use of Serum TSH Measures

Serum TSH is measured with immunometric assays using chemiluminescent probes. Progress has been constantly made in this field, and the lower detection limit of the newest assays is 0.02 mU l^{-1} or less. Yet At 0.02 mU l^{-1}, most immunometric assays have a coefficient of variation of 20%, whereas at higher TSH values, this decreases to 5% or less. A coefficient of variation of 20% is the upper limit of what is clinically acceptable.

The clinician should be aware that these assays measure only the immunological, and not the biological properties of TSH. This is not relevant when hyperthyroidism is diagnosed, but there is good evidence of discordance between immunological and biological effect in hypothalamic hypothyroidism, in the fetus and (possibly) in severely ill patients.

It also appears that antibodies recognize variable degrees of TSH glycosylation that occur in several thyroid pathologies. Different results in the range of serum TSH from 3 to 7 mU l^{-1} have been reported and are possibly related to such differences in antibodies.

TSH is secreted in a pulsatile manner and has a circadian rhythm. TSH spikes do not exceed 0.5 mU ml^{-1}. Serum TSH levels are highest during the evening (at 23:00 h), that is, during the first hours of sleep. The peak is displaced in shift workers at night. In young individuals, the mean serum TSH levels are in the morning (0.9 ± 0.3 mU l^{-1}) and at 23:00 h (1.9 ± 0.6 mU l^{-1}). In the elderly, serum TSH levels tend to be slightly lower (0.7 ± 0.6 and 1.3 f 0.9 mU l^{-1}, respectively). These fluctuations are rarely critical for the clinical evaluation of thyroid function. The normal range extends from 0.4 to 4.0 mU l^{-1}. Recent studies tend to narrow the normal range to 0.4–2.5 mU l^{-1}, with a median of 1.0–1.5 mU l^{-1}. African American population have a slightly higher mean serum TSH than the Caucasian population (**Figure 1**). From the clinical point of view, a serum TSH of 0.4 mU l^{-1} or less needs investigation. One study suggested that serum TSH values of 2.5–4 mU l^{-1} already represent an increased risk of developing overt thyroid disease, with the annual incidence of clinically open disease being approximately 5%. The concomitant presence of thyroid autoantibodies probably increases this risk. The significance of low serum TSH depends on the degree of serum TSH suppression that can be classified into three different degrees:

1. Serum TSH of 0.1–0.4 mU l^{-1} (for some authors, to 0.6 mU l^{-1}). This is frequently seen in subclinical hyperthyroidism. Serum free T4 values are normal and serum free T3 values are only rarely increased. Such serum TSH values are not fully specific for subclinical hyperthyroidism because they can also be found in severe non-thyroidal illness with or without glucocorticoid or dopaminergic treatment. In these latter situations, serum free T4 and T3 levels tend to be decreased or in the lower normal range. The differential diagnosis can be established by clinical observation and repetitive measures. If repeatedly detected, a slight decrease of serum TSH is compatible with partial thyroid autonomy, caused by a hot nodule, a multinodular goiter, or euthyroid Graves' disease. For the differential diagnosis thyroid scintigraphy can be helpful in these situations. Recent iodine contamination may play an important role in this situation and can be the origin of transient subclinical hyperthyroidism. Rarely, such serum TSH Ievels can be measured in pituitary or hypothalamic insufficiency, but in these conditions the changes in thyroid function are mostly accompanied by other pituitary hormone insufficiencies.
2. Serum TSH 0.05–0.1 mU l^{-1} (for some authors, 0.02–0.1 or 0.2 mU l^{-1}). The differential diagnosis is similar to the previously mentioned one. In addition, serum free T4 and/or T3 levels tend to be at the upper limit or slightly increased. Some authors consider that even a small increase in free T3 stands for overt and not subclinical hyperthyroidism. Suppressed serum TSH levels are the goal of T4 treatment in some patients with differentiated thyroid cancer. These patients present with serum free T4 levels at the upper limit of normal, and in one-third of these patients free T4 levels will be slightly increased. Serum T3 levels are in the normal range because of absent thyroidal T3 secretion. Low serum TSH levels are frequent in panhypopituitarism but not in hypothalamic disease. In serious systemical disease it is rare to find such low serum TSH levels unless the patients are treated with large doses of dopamine and/or glucocorticoids.
3. Serum TSH less than 0.02 mU l^{-1}, such values are encountered only in endogenous hyperthyroidism. Some assays will allow measuring levels as low as 0.002 mU l^{-1}. There are no data correlating the very low serum TSH Ievels with the clinical severity of hyperthyroidism. It is now well established that reactivation of TSH secretion after successful therapy for hyperthyroidism lag

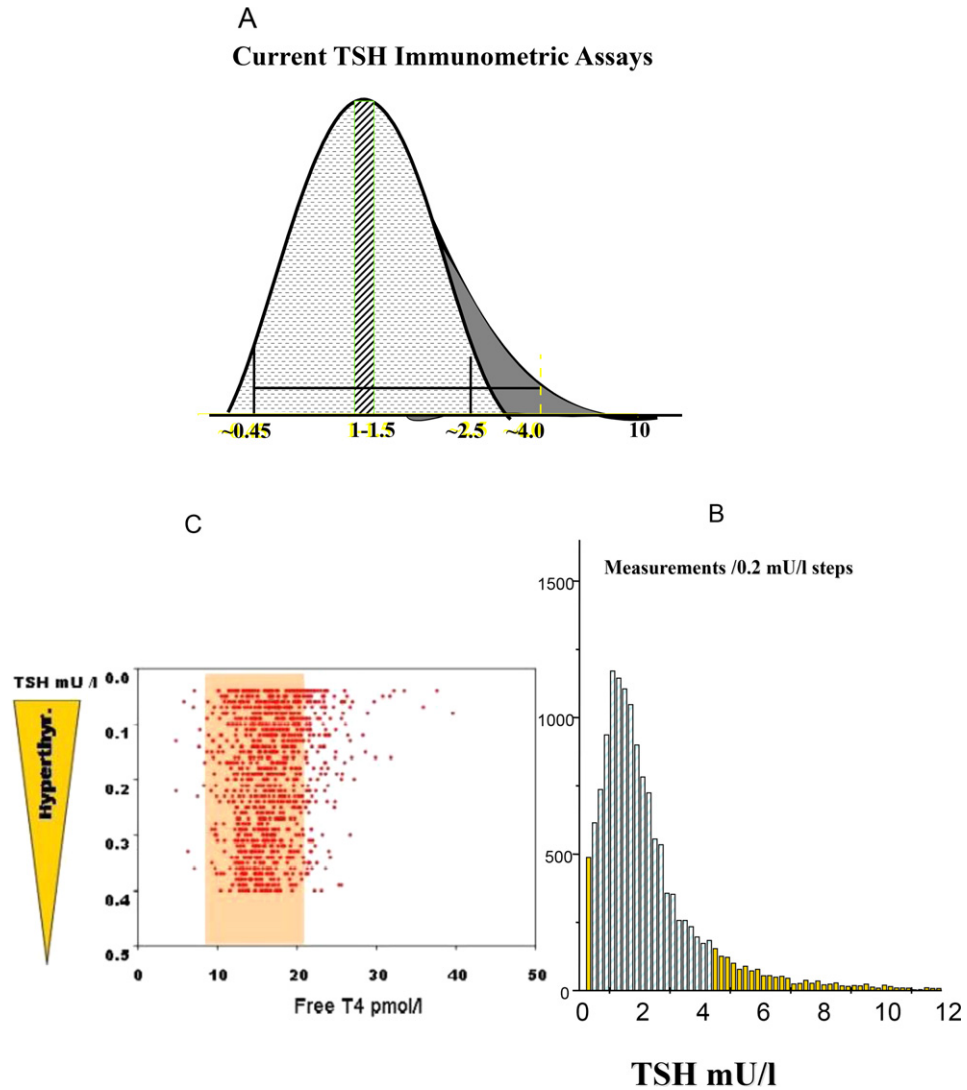

Fig. 1 Current TSH immunometric assays. (a) The area between 0.45 and 2.5 mU l^{-1} represents the values for a population (mean $\pm$ 2 SD) with documented absence of thyroid diseases. The bordering tails indicate that this population is not separated from the borderline pathological cases that present with TSH levels above 2.5 mU l^{-1} The majority of these cases have an euthyroid autoimmune thyroiditis (figure courtesy of Professor Carole Spencer). (b) Free T4 values in the subclinical hyperthyroid range are shown as a function of decreasing serum TSH values. Even though some free T4 values are above the normal range, the majority fall within the normal limits. (c) Consecutive serum TSH determinations (16000) over 10 months in a diagnostic laboratory are shown. It includes known and unknown pathologies, the limit being arbitrarily set at 12 and 0.2 mU l^{-1}. It documents what is shown in panel a: the groups of euthyroidism, subclinical hyper- or hypothyroidism do not present as individual peaks. The free T4 values shown in panel b are extracted from the lower end of TSH values in panel c. The data in b and c were obtained by courtesy of UNILABS, SA, Geneva.

behind the normalization of peripheral thyroid hormones and the improvement of the clinical condition by several weeks. There is no clear explanation for this finding, but it is important to realize that in this situation serum TSH only cannot be used as a valid parameter of euthyroidism.

Use of TSH Measurements in the Case of Artifactual Free T4 Values

Two diagnostic pitfalls can occur when serum TSH measurements are the only thyroid hormone parameter measured.

Serum TSH measurements are essential for excluding thyroid dysfunction in two situations with apparently increased free T4 levels. One abnormality involves T4 binding to albumin or transthyretin. These proteins have a high binding capacity but low affinity for T4. There exist however congenital variants with high affinity for T4. In addition, some neuroendocrine tumors secrete very large amounts of normal transthyretin (familial dysalbuminemic hyperthyroxinemia (FDH and familial or acquired

hyperthyroxinemia due to transthyretin excess). Today most methods for free T4 measurement yield normal free T4 values but former kits measured erroneously high free T4 values. Few laboratories are equipped to identify the modified albumin or transthyretin. If this methodology is not available, differential diagnosis with pituitary tumors or thyroid hormone resistance should include serum T3, serum TSH alpha-subunit, a TRH test (rarely necessary), and possibly magnetic resonance imaging.

Serum T3 and the alpha-subunit are increased in TSHomas and in thyroid hormone resistance. Basal serum TSH and the TRH test are normal in the case of abnormalities in serum transport proteins, whereas the response to TRH tends to be exaggerated in thyroid hormone resistance. In most but not all cases of TSHomas, there will be no response to TRH stimulation.

Another artifactual increase of free T4 values is encountered in the presence of autoantibodies to T4 or T3, even though most modern kits are no longer affected by such antibodies.

Artifacts of TSH Measures

There is only one condition where serum TSH values can be artifactually increased, namely in the presence of antibodies against mouse immunoglobulin (IgG) in the patient's serum. Monoclonal mouse IgGs are part of ail immunometric assays and, therefore, will be trapped by these antibodies. Recently, immunometric assays have overcome this problem. If necessary, the artifactual increase in serum TSH can be diagnosed by omitting anti-TSH antibodies in the assay and/or by a TRH test.

The well established overwhelming diagnostic power of serum TSH measurements can raise two questions. First, should one restrict the first line diagnostic parameters of thyroid dysfunction to TSH only, thereby omitting free T4 measures? This might be justified for systematic screening, yet free T4 measurements are inexpensive and will be ordered mostly during further workup of the patient's thyroid disease. Second, should serum TSH be used systematically as a screening test of the adult female population over 50 or even over 35 years of age and be repeated at regular intervals (e.g., every 5 years)?, At present the scientific societies do not recommend such procedures.

Special Techniques

The main indications for the TRH test (200 jug intravenously, blood sampling at 0, 15, 30, 60, and 120 min) are pituitary diseases. For primary thyroid dysfunction, it has lost all of its indications except those mentioned previously. In most countries, intravenous TRH is no longer available, with the nasal spray being used instead. This way of testing represents a stronger and more prolonged TSH stimulation. Blood should be sampled before and 30 and 60 min after the TRH for measuring TSH or 3 h later for measuring an increase in serum T3.

Measuring the alpha-subunit of TSH, the common subunit of all pituitary glycoproteins, is important in the case of TSHomas and can be used in the gonadotropinomas as well.

Thyroglobulin

Physiologically, Thyroglobulin (TG) is released in small amounts from the thyroid. It is estimated that 1 g of tissue corresponds to 1 $\mu g\,l^{-1}$ serum TG if serum TSH is in the normal range. Disruption of the normal thyroid structure results in leakage of TG, and high serum levels are found in goitrous patients. In multinodular goiter, TG levels can reach very high levels that overlap with those found in metastatic thyroid cancer patients. Marked and transient increases can be seen after ^{131}-iodine (^{131}I) treatment and after thyroid surgery. High levels are also found in Graves' disease and in functioning (and occasionally even nonfunctioning) thyroid adenomas.

The measurement of serum TG levels has benefited from the advances in immunometric assays. Today, an assay should have a functional sensitivity (with a coefficient of variance of 20%) of less than 0.5 $pg\,l^{-1}$, and interference of TG antibodies should be kept to a minimum. In practice, there are considerable differences among the commercial kits, and each laboratory should determine the sensitivity of its assay. Two additional controls are, therefore, recommended:

1. TG antibodies should be measured, and some authors recommend not using serum concentrations of TG if anti-Tg antibodies are present.
2. Another control consists in adding pure TG to a separate sample of the patient's serum. A recovery of 80–120% of the added TG excludes major interference. Adding small amounts of external TG (1 $\mu g\,l^{-1}$ rather than 50 $\mu g\,l^{-1}$) is recommended. Some authors consider that the recovery test does not guarantee absence of interference. It is hoped that technical improvements of immunometric assays will allow to minimize most of these problems.

Indications

TG measurements are useful in neonatology if a dysthyroid condition is suspected. Low levels are found in thyroid agenesis or dysgenesis whereas high levels are measured in thyroid hormone resistance and in some rare inborn errors of thyroid hormone synthesis. They are a valuable adjunct to scintigraphy and thyroid ultrasound.

Low TG values allow to differentiate between thyrotoxicosis facticia and endogenous hyperthyroidism. In the presence of a normal-sized or even moderately increased thyroid, the ingested L-thyroxine will suppress TG levels to values of less than 10 $\mu g\,l^{-1}$. Yet in the presence of very large goiters, TG levels will often not be suppressible.

In addition, in subacute thyroiditis, an increase TG levels has been documented. The measurement of TG values is, therefore, not recommended in these conditions.

Importantly, TG measures are not useful in the differential diagnosis between benign nodular goiter and thyroid carcinoma because TG levels tend to be increased and overlap in both conditions. In an occasional patient with an unidentified pulmonary or bone metastasis, a very high TG level may help to establish the origin of the tumor.

Besides the rare indications for TG measurements mentioned before, the main indication for TG measurements remains in the context of the surveillance of differentiated follicular or papillary thyroid cancer. It allows to easily detect recurrences (**Table 1**). TG levels are a function of the tumor mass and tumor differentiation. TG levels tend to be lower in tumors that are more aggressive and are absent in undifferentiated cancers.

The surveillance of well differentiated thyroid cancers using TG concentrations measurements is now established. In this context, it is critical not to rely only on basal TG levels but also interprete the values in the context of TSH stimulation as well. The use of recombinant TSH has greatly facilitated the surveillance because its use is equally valuable than inducing severe hypothyroidism.

If TG values are more than 2 $\mu g\,l^{-1}$, the presence of thyroid tissue is certain, indicating the presence of neoplastic tissue in the case of complete surgical or ^{131}I ablation. The changes in its concentration over time parallel the evolution of the tumor. TG levels are particularly important for diagnosing absence of disease. For this purpose, one cannot always rely on non-measurable TG levels ($<0.5\ pg\,l^{-1}$) under T4 treatment. If the patient is rendered hypothyroid, or if the patient receives recombinant TSH and serum TG values do not exceed the critical limit of 2 $\mu g\,l^{-1}$, the patient can be considered cured. In these situations, thyroid scintigraphy is not necessary.

Recently, a complementary method for determining the presence of tumor tissue has been advocated in cases of interference in serum measurements of TG by anti-Tg antibodies. The presence of circulating thyroid follicular tells is obtained by using real-time quantitative reverse transcription – polymerase chain reaction (RT-PCR) based on the amplification of TG mRNA. If normal thyroid tissue has been destroyed, the presence of TG mRNA strongly supports recurrence of disease. The method is sensitive and independent of circulating anti-Tg antibodies, but it is currently limited to specialized research laboratories.

Autoantibodies

For clinical use, three types of antibodies are of interest: the antithyroperoxidase (anti-TPO), the anti-thyroglobulin antibodies antibodies (anti-Tg), and the anti-TSH-receptor antibodies antibodies (anti-TRAb). Among the TRAb, one can distinguish blocking and stimulating antibodies. The recently discovered anti-sodium-iodide symporter antibodies do not seem to play an important diagnostic role.

The thyroid antibodies belong to the IgG class, and only for anti-TPO antibodies complement fixing and cytotoxic activity have been described. The antigenic sites of the TPO have been well characterized; there are six of them. For anti-Tg and anti-TRAb, this is less well known because the antigenic sites are mainly conformational and not linear. Recently, it has been possible to produce functioning anti-TRAb.

Indications to measure thyroid antibodies can serve diagnostic, follow-up, and screening purposes. TPO antibodies are the most sensitive antibodies for the diagnosis of any thyroid autoimmune process, and they are widely used in combination with the TSH for diagnosing the etiology of a thyroid disease. They are also useful in the follow-up of Graves' disease because their titer may reflect the activity of the disease. In Hashimoto's thyroiditis, the follow-up of these patients measuring antibodies may lead to confusion and is not generally recommended. There is no specific indication for TG antibodies except the clinical condition mentioned before (follow-up differentiated thyroid cancer) However, in rare occasions, the TPO antibodies may be negative, whereas TG antibodies will reveal thyroid disease.

The TRAb are not always useful for the diagnosis of Graves' disease. Similarly to TPO antibodies, their titer may follow the activity of the disease. They can also be present in primary hypothyroidism and in postpartum thyroiditis; therefore, they are not always a reliable tool for the diagnostic of Graves' disease.

Table 1 Diagnostic use of serum Thyroglobulin (TG) levels in well differentiated thyroid cancer

TSH< 0.1 mU l^{-1} *TG value*	*TSH> 30 mU l^{-1}*	*TG antibodies*	*^{131}I scintigraphy*	*Loco-regional tumor*	*Distant metastasis*	*'Cure'*
	$<2\ \mu g\,l^{-1}$	negative	negative	excluded	excluded	most likely
$<2\ \mu g\,l^{-1}$		negative	–	not excluded	Unlikely	not proven
2–10 $\mu g\,l^{-1}$		negative	positive or negative	certain	Unlikely but possible	No
	2–10 $\mu g\,l^{-1}$	negative	positive or negative	certain	Unlikely	No
$>10\ \mu g\,l^{-1}$		negative or positive	mostly positive	certain	not excluded	No

There is one rare but clear indication for measuring these antibodies, namely in pregnant patients with Graves' disease in whom the thyroid has been removed. In these cases, one has no clinical indication about the severity of the autoimmune process and, on rare occasions, placental transfer of a high titer of TRAb may cause neonatal hyperthyroidism.

Screening with anti-TPO antibodies is advocated during pregnancy because their presence indicates increased risk of post-partum thyroiditis.

In Vivo Isotope Imaging

Diagnostic in vivo isotopic imaging will yield information on thyroidal uptake of the isotope and functional morphology of the thyroid. Two isotopes, ^{123}-iodine and Tc^{99m} pertechnetate are used. There is still some indication for ^{131}I for whole body scintigraphy of patients with thyroid cancers. The place for other isotopes, such as thallium and gallium, has not been established. Thallium has been used for identifying thyroid metastases, and gallium 67 is taken up by inflammatory cells, particularly mast tells, and therefore can be used in some cases of destructive thyroiditis such as subacute thyroiditis (Table 2).

^{123}-Iodine

The physical characteristics of ^{123}I are given in Table 2. It is close to an ideal isotope in so far that the irradiation of the thyroid is minimal. Iodide, is taken up specifically by the gastric mucosa. Thyroidal uptake occurs very rapidly. In the case of concomitant food intake, the uptake is delayed and occurs by intestinal absorption.

The iodine uptake depends on the activity of the sodium – iodide symporter (NIS), formally called iodide trapping of the thyroid. Once transported into the cell, the free intrathyroidal iodide is immediately incorporated into TG. the free iodide pool of the thyroid is only increasedin pathological conditions This can be tested by the perchlorate discharge test. Once stored in the colloid as TG, the labeled T4 will remain in the thyroid for a long time, depending on the total amount of stored colloid. In most conditions, the reserve in TG is huge; therefore, the turnover of this iodide is slow, even though the most recently formed colloid is preferentially resorbed and secreted. As a rule of thumb, only 1–2% of the accumulated dose will be secreted per day. Therefore, the disappearance of the isotope will be mainly a function of its physical half-life.

Most of the iodine is secreted as T4 and to a lesser extent as T3, and only a minor part of iodide is lost.

Iodine contamination is one of the major technical problems resulting in invalid thyroid imaging. In individuals with a normal thyroid and a daily iodide intake of 200 μg or more, a single dose of 30 mg iodide together with the tracer will reduce the uptake to background levels. A similar effect can be obtained with half the dose of iodide given over several days. However, it should be noted that this applies only to a completely normal thyoird gland. In areas of moderate iodine deficiency where small multi-nodular goiters are frequent, the suppression of ^{123}I uptake by iodine needs higher and longer treatment with similar or higher doses. Iodine contamination is a frequent event because large amounts of iodine containing contrast media are injected during axial CT tomography. These substances are highly water soluble and are eliminated within 4 to 6 weeks. Iodide is rapidly eliminated by the renal route (clearance of 50 ml min^{-1} and more). Accordingly, elimination is decreased in renal insufficiency.

Today, the major culprit of long-term iodine contamination is amiodarone. This substance and its biologically active metabolite, desethylamiodarone, have an approximate half-life of 4–6 weeks. However, iodine contamination from its degradation products can last for months or up to 1–2 years. Historically, other organic iodine compounds can be responsible for even longer lasting contaminations. For instance, dyes used for myelography give rise to lifelong contamination and can even pass the placenta resulting in a low iodine uptake into the thyroid gland of children.

Among the non-iodinated substances interfering in thyroidal uptake, methimazole, propylthiouracil, and perchlorate should be mentioned, with the latter being a specific inhibitor of the iodide symporter. In severe non-thyroidal illness, thyroid function and thyroid uptake may be depressed. Drugs such as glucocorticoids (30–60 mg of prednisone) dopamine and its analogs as well as the sandostatin analogue octreotide have multiple impacts, but the most significant one is a reduction of TSH secretion with a

Table 2 Isotopes for thyroid imaging

	^{123}I	^{131}I	^{99m}Tc	F^{18} Thioglucose
Half-life	13 h	8 days	6 h	110 min
Emission	Gamma	Beta, Gamma	Gamma	Gamma
Radiation exposure (rad)mCi^{-1}	13	1300	0.13	
Diagnostic dose (μCi)	300	50 (or 5–10 000)	2000	
Route	Oral	Oral	iv	iv
Absorption	Gastric	Gastric		
Particularities				Uptake by metabolically active cells
Maximal uptake	4–8 h	4–8 h	20–40 min	

subsequent reduction in thyroid function. Older literature also mentions sulfonamides, but the more modern drugs of this class have not been reported to interfere.

$Tc^{99}m$ Pertechnetate

$Tc^{99}m$ is close to ideal for studying the trapping of iodide by the thyroid. It is easily available and cheap. Because it can only give information on anion trapping, the measurements are performed within 1 h after intravenous injection. The thyroidal uptake of$Tc^{99}m$ is approximately 10 times lower than that for ^{123}I (0.4–4%). The precise normal range is also dependent on iodine intake; therefore, it must be validated for each laboratory. As compared with ^{123}I, $Tc^{99}m$ may occasionally result in false positive imaging of nodules that may erroneously be taken for functioning nodules.

It should be noted that on many occasions thyroid imaging with isotopes has been replaced by ultrasonography. The indications for scintigraphy could be summarized as follows. In hyperthyroidism, $Tc^{99}m$ is as valid as ^{123}I for the differential diagnosis of nodular or diffuse goiter. If the thyroid is diffusely enlarged without concomitant exophthalmos or pretibial myxedema, a positive uptake will allow excluding acute and/or subacute thyroiditis and will help establishing a diagnosis of thyrotoxicosis factitia and/or iodine contamination. In the case of a multinodular goiter with a moderate decrease of serum TSH (<0.6 mU l^{-1}), the scintigraphy may confirm autonomous regions. In the case of very painful thyroid and inflammatory symptoms, an absent thyroid uptake may confirm the diagnosis of subacute thyroiditis. In the case of an isolated euthyroid thyroid nodule, the uptake will identify a cold nodule, even though $Tc^{99}m$ may give false positive results and the predominant position of this investigation has been superseded by cytology (fine needle aspiration).

In most cases, ultrasound evaluation of the thyroid gland is as useful as or gives even more information than scintigraphy. Nevertheless, the two examinations can be complementary. Scintigraphy will allow identifying an inactive nodule of the thyroid that the ultrasound may reveal to be cystic, mixed, or solid. In multinodular goiter, the scintigraphy will clearly give the patchy appearance of the functioning and (possibly) autonomous tissue. However, its value in describing inactive or cold nodules in this situation is limited.

^{131}-Iodine (^{131}I)

The disadvantage of the ^{131}I isotope is the rather large delivered dose of irradiation to the patient. In thyroid cancer patients, ^{131}I has kept its place for diagnostic procedures, even though the use of TG is reducing its indications. During the follow-up of these patients, whole body scintigraphies are obtained with ^{131}I. Ideally, TSH levels have to be increased (>30 mU l^{-1}). This classically obtained by stopping T4 or T3 substitution or, more recently, by injecting recombinant human TSH.

Hypothyroidism is obtained by switching 7 weeks before ^{131}I treatment to T3 substitution that has to be stopped 12–14 days before radioisotope therapy. Serum TSH levels should increase to more than 30 mU l^{-1}, and the efficiency of the whole body scan or treatment can be increased by concomitant regimen recommendations such as avoidance of iodized salt and iodine rich bread, eggs, and fish. A small dose of diuretics (thiazides) can also be recommended to decrease the circulating iodide pool. Large diagnostic doses of ^{131}I are used (5–10 mCi) because the uptake of the cancerous cell is much lower than normal and is mostly markedly below 0.5% of the administered dose. The whole body scintigraphy is performed 3–4 days after administration of the radioactive iodide. A ^{131}I scintigraphy or therapy should not be repeated before 8–12 weeks because it induces a stunting effect (i.e. transiently abolished uptake). This technique is very sensitive for detecting small metastases and in most cases correlates very well with the measured serum TG levels. However, it has been reported that there can be some dissociation and that serum TG levels can be increased even in the absence of residual ^{131}I iodide uptake. It is thought that these patients have metastasis and should benefit from a more complete workup with subsequent surgery and/or ^{131}I treatment.

Rare and/or Experimental Methods for Thyroid imaging

The perchlorate discharge test can be performed with ^{123}I or ^{131}I. It is a rarely used test that was described initially for detecting inherited defects of intrathyroidal iodine metabolism (absence of sodium – iodide symporter, absence of Tg, and dehalogenase defect). It allows detection of organification defects that can be seen in autoimmune thyroiditis and Graves' disease treated with ^{131}I. Today, this test is not performed in routine practice.

Positron emission tomography with fluorodeoxyglucose is a very promising technique for detecting metabolically active tissue. The active principle, Deoxyglucose, can be taken up by cells but cannot be metabolized. Malignant cancer cells have an increased glucose rnetabolism. Initially, it was thought that this test would yield the best results in poorly differentiated, and hence metabolically very active tumors. However, experience has shown that even differentiated thyroid cancers can occasionally be identified with this technique.

See also: Diagnosis of Hypothyroidism. Nontoxic Goiter. Postpartum Thyroid Dysfunction. Resistance to Thyroid Hormone. Serum Thyroid Hormone-Binding Proteins. Thyroid and Infertility. Thyroid Disease and Pregnancy. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyrotoxicosis; Diagnosis. TSH-Producing Adenomas and Resistance to Thyroid Hormones

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Epidemiology of Thyroid Disease☆

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Glossary

Epidemiology The study of the distribution and determinants of disease prevalence and incidence.

Hyperthyroidism The earliest measure of thyroid overactivity is a decline in serum thyrotropin (TSH) (subclinical hyperthyroidism) followed by an increase in serum triiodothyronine (T_3) and an increase in serum thyroxine (T_4).

Hypothyroidism The earliest biochemical abnormality in hypothyroidism is an increase in serum TSH associated with normal serum T_4 and T_3 concentrations (subclinical hypothyroidism or mild thyroid failure), followed by a decline in serum T_4, at which stage most patients have symptoms and benefit from treatment (overt hypothyroidism).

Screening The identification of unrecognized disease by a test or examination that can be applied rapidly to differentiate apparent well persons who probably have a disease from those who probably do not.

Iodine Deficiency Disorders

Iodine is an essential component of the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) produced by the thyroid gland. The ideal dietary allowance of iodine recommended by World Health Organization (WHO) in adults is 150 μg of iodine per day which increases to 250 μg per day in pregnancy and lactation. Iodine deficiency impairs thyroid hormone production and has adverse effects throughout life, particularly early in life as it impairs cognition and growth (WHO, 2017) (**Table 1**).

Iodine deficiency is recognized as a global problem with large populations at risk who are living in an environment where the soil has been deprived of iodine. This arises from past glaciation, compounded by the leaching effects of snow, water, and heavy rainfall, which removes iodine from the soil. The mountainous regions of Europe, the Northern Indian Subcontinent, the extensive mountain ranges of China, the Andean region in South America, and the lesser ranges of Africa are all iodine deficient. Iodine deficiency remains a significant problem despite major national and international efforts to increase iodine intake, primarily through the voluntary or mandatory iodization of salt. In 2007, the WHO estimated that 2 billion people, including 285 million school-age children, still had iodine deficiency, defined as a urinary iodine (UI) excretion of less than 100 μg/L (**Fig. 1**). Recent epidemiological data suggest that iodine deficiency is an emerging issue in industrialized countries, previously thought of as iodine-sufficient. International efforts to control iodine deficiency are slowing, and reaching the third of the worldwide population that remains deficient poses major challenges (Vanderpump, 2017).

In areas where the daily iodine intake is less than 50 μg, goiter is usually endemic, and when the intake falls below 25 μg/day, congenital hypothyroidism is seen. The prevalence of goiter in areas of severe iodine deficiency can be as high as 80%. Iodization programs are of proven value in reducing goiter size and in preventing goiter development and cretinism in children. Autonomy can develop in nodular goiters, occasionally leading to thyrotoxicosis, and iodization programs can also induce thyrotoxicosis, especially in those older than age 40 with nodular goiters (Zimmermann and Boelaert, 2015).

Severe iodine deficiency results in congenital hypothyroidism which is a condition associated with severe learning disabilities, deafness, and impaired motor development. Endemic congenital hypothyroidism had been long recognized to be associated with severe iodine deficiency when the daily iodine intake falls below 25 μg. Severe iodine deficiency may be associated with impairment in the psycho-neurological outcome in the progeny because both mother and offspring are exposed to iodine deficiency during gestation (and the postnatal period). Controlled studies performed in iodine-deficient regions have confirmed that iodine supplementation eliminated new cases of congenital hypothyroidism, reduced infant mortality, and improved cognitive function in the general population (Pharoah *et al.*, 1971). The effects of mild-to-moderate iodine deficiency on cognition are less well known than those of moderate-to-severe deficiency but it is assumed that there is a continuum of disability with more subtle impairments of the intelligence quotient (IQ) and motor ability associated with less severe deficiency (Zimmermann and Boelaert, 2015). A systematic review of available published studies from 1980 to 2011 examined the relationship between iodine and mental development of children 5 years old and under and found that regardless of study design, iodine deficiency had a substantial impact on mental development which translated into 6.9–10.2 IQ points lower in iodine deficient children compared with iodine replete children (Bougma *et al.*, 2013). Methodological concerns included weak study designs, the omission of

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Table 1 The spectrum of iodine deficiency disorders

Fetus	Abortions
	Stillbirths
	Congenital anomalies
	Increased perinatal mortality
	Endemic cretinism
Neonate	Neonatal goiter
	Neonatal hypothyroidism
	Endemic mental retardation
	Increased susceptibility of thyroid gland to nuclear radiation
Child/adolescent	Goiter
	(Subclinical) hypothyroidism
	Impaired mental function
	Retarded physical development
	Increased susceptibility of thyroid gland to nuclear radiation
Adult	Goiter with its complications
	Hypothyroidism
	Impaired mental function
	Spontaneous hyperthyroidism in the elderly
	Iodine-induced hyperthyroidism
	Increased susceptibility of thyroid gland to nuclear radiation

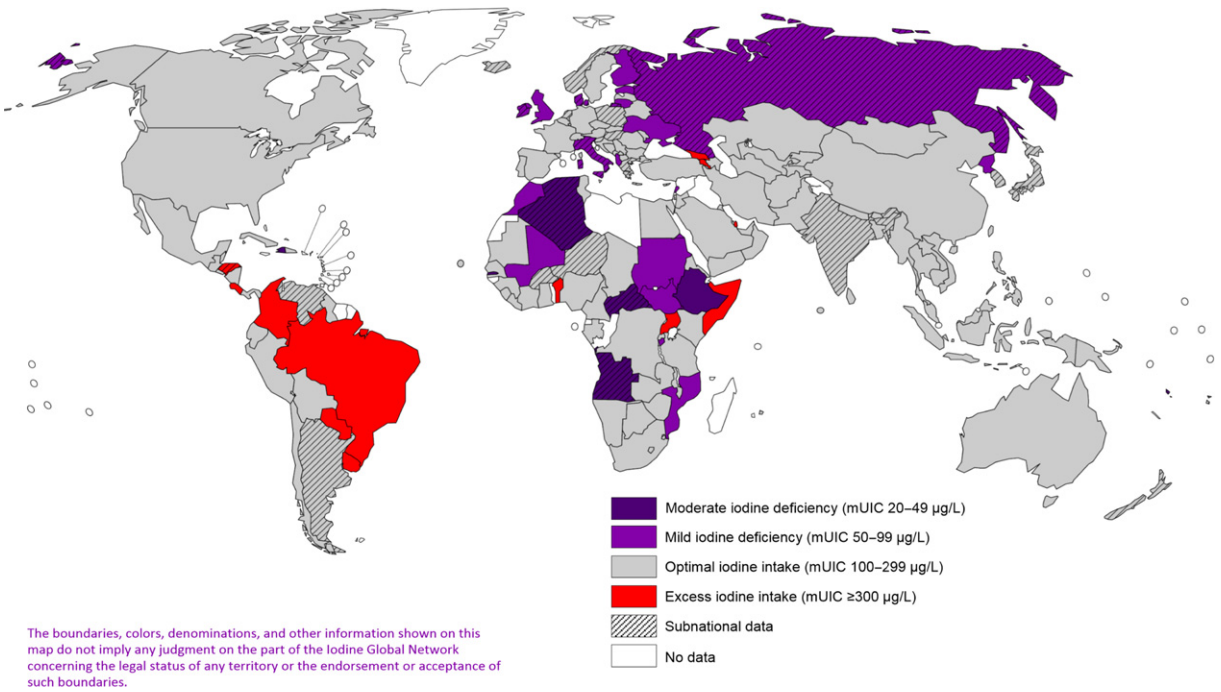

Fig. 1 Global scorecard of iodine nutrition 2014–15. Based on median UI concentration in school-age children (WHO, 2017).

important confounders, small sample sizes, the lack of cluster analyses, and the lack of separate analyses of verbal and nonverbal subtests.

Hypothyroidism

The earliest biochemical abnormality is an increase in serum thyrotropin (TSH) concentration associated with normal serum free T_4 and T_3 concentrations (subclinical hypothyroidism or mild thyroid failure), followed by a decrease in serum free T_4 concentration, at which stage, most patients have symptoms and benefit from treatment (overt hypothyroidism). Hypothyroidism is an insidious condition with a significant morbidity, and the subtle and nonspecific symptoms and signs may be mistakenly

attributed to other illnesses, particularly in postpartum women and the elderly (Chaker *et al.*, 2017). In iodine-replete communities, the cause is either chronic autoimmune disease [atrophic autoimmune thyroiditis or goitrous autoimmune thyroiditis (Hashimoto's thyroiditis)] or destructive treatment for hyperthyroidism, which may account for up to one-third of cases of hypothyroidism in the community (Vanderpump, 2011).

Congenital Hypothyroidism

Congenital hypothyroidism affects approximately 1 newborn in 3500–4000 births and is the most treatable cause of mental retardation (Ford and LaFranchi, 2014). There is an inverse relationship between age at diagnosis and IQ in later life. In iodine-replete areas, 85% of the cases are due to sporadic developmental defects of the thyroid gland (thyroid dysgenesis), such as the arrested migration of the embryonic thyroid (ectopic thyroid) or a complete absence of thyroid tissue (athyreosis). The remaining 15% have thyroid dyshormonogenesis defects transmitted by an autosomal recessive mode of inheritance. Iodine deficiency (<25 μg/day), particularly in preterm infants, accounts for many cases in Europe, Asia, and Africa. Clinical diagnosis occurs in $<5\%$ of newborns with hypothyroidism because symptoms and signs are often minimal. As a result, it is not possible to predict which infants are likely to be affected. Without prompt diagnosis and treatment, most affected children gradually develop growth failure, irreversible mental retardation, and a variety of neuropsychological deficits.

The apparent incidence of congenital hypothyroidism has more than doubled in recent years because of several factors, including more inclusive diagnostic criteria, shifting demographics, and increasing survival of preterm infants (Wassner and Brown, 2015). The greatest increase has occurred in mildly affected children. Congenital hypothyroidism may be transient or persistent, but the natural history cannot be predicted by severity at diagnosis. In premature infants, who are especially vulnerable to hypothyroidism, the rise in serum TSH may be delayed and therefore detected only by routine follow-up screening.

Spontaneous Hypothyroidism

In iodine-replete communities, the prevalence of spontaneous hypothyroidism is between 1% and 2%, and it is more common in older women and 10 times more common in women than in men (Vanderpump, 2011). Studies in Northern Europe, Japan, and the United States have found the prevalence to range between 0.6 and 12 per 1000 women and between 1.3 and 4.0 per 1000 in men investigated. The prevalence is higher in surveys of the elderly in the community. A lower prevalence is seen in areas of iodine deficiency. The testing of hospital inpatients, predominantly elderly women, might be expected to reveal a higher proportion of unsuspected hypothyroidism, but this is not supported by the available studies, which confirm a prevalence of 2%.

Subclinical Hypothyroidism

A substantial proportion of the population, particularly elderly women who live in iodine-replete areas, have circulating thyroid antibodies [antithyroid peroxidase (microsomal) and antithyroglobulin antibodies] and normal thyroid function. The presence of these antibodies correlates with the presence of focal thyroiditis in biopsy and in postmortem material of patients with no evidence of hypothyroidism during life. Patients with hypothyroidism caused by either atrophic or goitrous autoimmune thyroiditis usually have high serum titers of the same antibodies (Biondi and Cooper, 2012).

With respect to epidemiological studies, the definition of subclinical hypothyroidism varies from any increase in serum TSH to values >10 mU/L or, more stringently, a serum TSH value >10 mU/L and a positive test for circulating thyroid antibodies in serum. The term implies that patients should be asymptomatic, although symptoms are difficult to assess, especially in those in whom thyroid function tests have been checked because of nonspecific complaints such as tiredness.

There has been controversy about the upper limit of the reference range for serum TSH (Wartofsky and Dickey, 2005; Surks *et al.*, 2005). Reference ranges are derived from a reference population that comprises a large group of subjects who do not have thyroid disease and are otherwise well. By convention, a reference range usually only comprises 95% of a reference population. Thus, 2.5% of "normal" individuals will fall above the reference range and 2.5% will fall below the range. For serum TSH, the reference population shows a log normal distribution and has a diurnal variation with the reference range in thyroid disease-free individuals typically cited as between 0.4 and 4.0 mU/L. The serum TSH reference range varies in different ethnic communities, trimesters of pregnancy and progressively shifts toward higher concentration with age. Analysis of the National Health and Nutrition Examination Survey (NHANES) III data suggest that the reference range for serum TSH rises with age as the 97.5 centile for those subjects aged >80 years was 7.49 mU/L and 70% had a serum TSH $>$ the population defined upper limit of the reference range of 4.5 mU/L of whom only 40% were antithyroid antibody positive (Surks and Boucai, 2010).

Spontaneous recovery has also been described in subjects with subclinical hypothyroidism, although the frequency of this phenomenon is unclear. In 1 study, 37% of patients normalized their serum TSH levels over a mean follow-up time of 32 months (Meyerovitch *et al.*, 2007). Normalization of serum TSH concentrations is more likely to occur in patients with negative antithyroid antibodies and serum TSH levels <10 mU/L, and within the first 2 years after diagnosis (Biondi and Cooper, 2012).

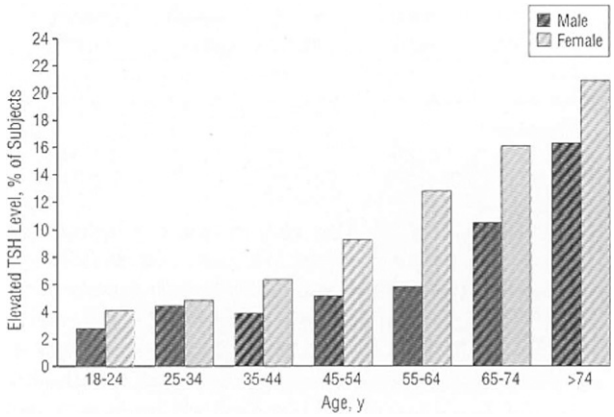

Fig. 2 The percentage of 25,682 subjects with a high serum TSH concentration, by sex and decade of age, in the Colorado Thyroid Disease Prevalence Study (Canaris et al., 2000).

Table 2 The effect of environmental iodine intake on the prevalence of subclinical thyroid disease

Iodine status	*Subclinical hypothyroidism*	*Subclinical hyperthyroidism*
Deficient	1%–4%	6%–10%
Replete	4%–9%	1%–2%
Excess	18%–14%	<1%

In the Whickham survey, 8% of women (10% of women older than age 55) and 3% of men had subclinical hypothyroidism (Tunbridge *et al.*, 1977). A cross-sectional screening survey of 25,682 subjects older than age 18 attending a health fair in Colorado found that 9% of the population, excluding 1525 on levothyroxine therapy, had an elevated serum TSH level; of these, <1% had overt hypothyroidism. Among those with a high serum TSH concentration, 74% had a value between 5.1 and 10 mU/L and 26% had a value greater than 10 mU/L. The percentage of subjects with a high serum TSH concentration was higher for women than men in each decade of age, and ranged from 4% to 21% in women and 3% to 16% in men (Canaris *et al.*, 2000) (**Fig. 2**). In the NHANES III survey serum TSH concentrations increased with age in both men and women and were higher in whites than blacks, independent of serum antithyroid antibody concentrations (Hollowell *et al.*, 2002). Approximately 2% of adolescents aged 12–19 years had a serum TSH greater than 4.5 mU/L. Subclinical hypothyroidism is found at higher frequency in areas where iodine intake is high, but most cases are not of autoimmune origin (Laurberg *et al.*, 2010) (**Table 2**).

Incidence of Hypothyroidism

In the 20-year follow-up of the Whickham cohort, the mean annual incidence of spontaneous hypothyroidism in surviving women was 3.5 per 1000, increasing to 4.1 per 1000 if all cases are included, including those who had received destructive treatment for hyperthyroidism (Vanderpump *et al.*, 1995). The probability of a woman developing hypothyroidism at a particular time (i.e., the hazard rate) increased with age to 14 per 1000 in women aged 75–80. The mean annual incidence in men (all spontaneous except for one case of lithium-induced hypothyroidism) was 0.6 per 1000. Either raised serum TSH or positive thyroid antibodies alone or in combination were associated with a significantly increased risk of hypothyroidism in women and men. The odds were greatly increased when both risk factors were present and each had a similar effect (**Table 3**). In the surviving women, the annual risk of developing spontaneous hypothyroidism was 4% in those who had both raised serum TSH levels and were thyroid-antibody positive, 3% if only serum TSH was raised, and 2% if only thyroid antibodies were positive. The cumulative incidence of hypothyroidism over 20 years was 55%, 33%, and 27%, respectively. The probability of developing hypothyroidism in women increases linearly when serum TSH is higher than 2 mU/L; this is further increased if women are antithyroid antibody positive or decreased if antithyroid antibody negative (**Fig. 3**). The development of hypothyroidism also correlated with the strength of titer of antithyroid microsomal antibodies at first survey. A positive family history of any form of thyroid disease, the presence of a goiter at either the first or the follow-up survey, or parity at first survey were not associated with an increased risk of hypothyroidism. The prognostic importance of positive thyroid antibody tests and increasing serum TSH levels has been confirmed in other cohort studies (McGrogan *et al.*, 2008).

Data from a large population study in Tayside, UK, have demonstrated that the standardized incidence of primary hypothyroidism varied between 3.90 and 4.89 per 1000 women per year between 1993 and 2001. The incidence of hypothyroidism in men significantly increased from 0.65 to 1.01 per 1000 per year ($P = 0.0017$). The mean age at diagnosis of primary hypothyroidism decreased in women from 1994 to 2001 (Flynn *et al.*, 2004; Leese *et al.*, 2008).

Table 3 Development of spontaneous hypothyroidism in surviving women and men at 20-year follow-up of Whickham survey: odds ratios (with 95% confidence interval) (Vanderpump et al., 1995)

Women	
Serum TSH raised, regardless of thyroid antibody status	14 (9–24)
Thyroid antibody positive, regardless of serum TSH status	13 (8–19)
If thyroid antibody negative, effect of raised serum TSH alone	8 (3 – 20)
If thyroid antibody positive, additional effect of raised serum TSH	5 (2 – 11)
If serum TSH normal, effect of thyroid antibody positive alone	8 (5–15)
If serum TSH raised, additional effect of thyroid antibody positive	5 (1–15)
Serum TSH raised and thyroid antibody positive combined	38 (22–65)
Men	
Serum TSH raised, regardless of thyroid antibody status	44 (19–104)
Thyroid antibody positive, regardless of serum TSH status	25 (10–63)
Serum TSH raised and thyroid antibody positive combined	173 (81–370)

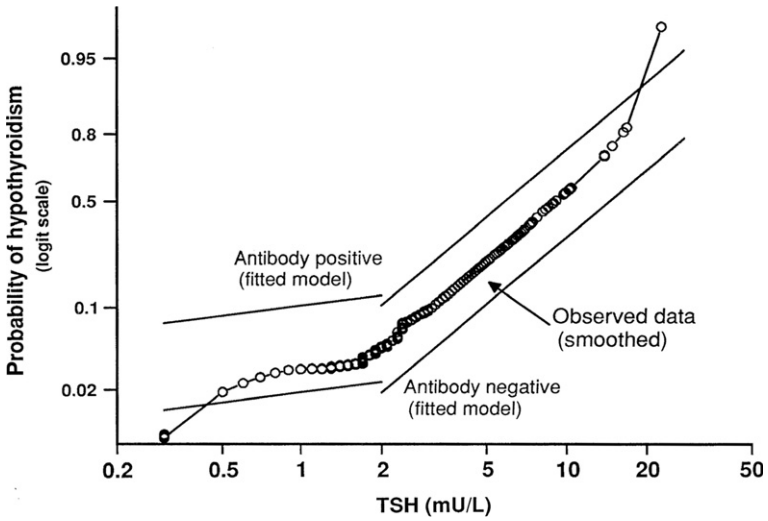

Fig. 3 Probability for development of hypothyroidism within 20 years with increasing values of serum TSH at first Whickham survey in 912 survivors (Vanderpump et al., 1995).

Iatrogenic Hypothyroidism

After destructive treatment for hyperthyroidism (either radioiodine therapy or surgery), the incidence of overt hypothyroidism is greatest in the first year (Vanderpump, 2011). Factors that influence outcome are age, gland size (small glands are more likely to lead to hypothyroidism postoperatively), remnant size, and iodine intake (high intake is associated with recurrence). Radioiodine therapy for Graves' hyperthyroidism, toxic or nontoxic nodular goiter, or autonomously functioning thyroid adenomas can cause hypothyroidism months or years later. A fixed high dose of radioiodine (550 MBq), administered with the intent of ablating the thyroid and inducing early hypothyroidism, results in permanent hypothyroidism in at least 50% of patients by 1 year. Subclinical hypothyroidism is commonly found either post-radioiodine therapy or post-surgery in up to 50% of apparently euthyroid patients. It may be evident for only a few months, but more often it represents a stage in the progression toward overt thyroid failure. If serum TSH remains raised, then the rate of progression toward overt hypothyroidism is 2%–6% per year after either treatment. Treatment of Graves' disease with antithyroid drugs alone is also associated with the eventual development of hypothyroidism from either autoimmune thyroiditis or the presence of TSH-blocking antibodies in 5%–20% of cases.

Other iatrogenic causes of hypothyroidism are surgery and external irradiation for head and neck cancer and drugs used to treat nonthyroid conditions, including amiodarone, lithium carbonate, tyrosine kinase inhibitors, interferon-α, interleukin-2, and immune checkpoint inhibitors. In addition, patients with hypothyroidism who are taking levothyroxine may become hypothyroid if given drugs that decrease absorption (e.g., cholestyramine and iron salts) or drugs that increase its clearance (e.g., phenytoin and carbamazepine or an estrogen-induced increase in the serum concentration of thyroxine-binding globulin). Poor compliance with levothyroxine therapy or suboptimal treatment may also result in hypothyroidism (Vanderpump, 2011).

Hyperthyroidism

In epidemiological studies, the clinical diagnosis of thyrotoxicosis should be supported by measurements of serum T_4 or T_3 and TSH concentrations. Biochemical tests of thyroid function may reveal the diagnosis before it is clinically apparent. A rise in serum T_3 and fall in serum TSH are the earliest measures of thyroid overactivity, followed by a rise in serum T_4. Hyperthyroidism has a significant short-term morbidity and long-term morbidity and mortality. The most common cause of hyperthyroidism in iodine-replete communities is Graves' disease, followed by toxic multinodular goiter, whereas rarer causes include an autonomously functioning thyroid adenoma, thyroiditis, excessive levothyroxine replacement or drugs including amiodarone, lithium carbonate, tyrosine kinase inhibitors, interferon-α, and immune checkpoint inhibitors (De Leo *et al.*, 2016). In epidemiological studies, however, the etiology is rarely ascertained.

The prevalence of hyperthyroidism in women is between 0.5% and 2%, and is 10 times more common in women than in men in iodine-replete communities (Vanderpump, 2011). In NHANES III, in those subjects who were neither taking thyroid medication nor reported a history of thyroid disease, 2 per 1000 had "clinically significant" hyperthyroidism, defined as a serum TSH concentration <0.1 mU/L and a serum total T4 concentration >170 nmol/L (Hollowell *et al.*, 2002). The prevalence data in elderly persons show a wide range between 0.4% and 2.0% and a higher prevalence is seen in iodine deficient areas (Laurberg *et al.*, 2010). The reported prevalence rates for previously undiagnosed hyperthyroidism in hospitalized patients, between 0.3% and 1%, are consistent with community surveys (Vanderpump, 2011).

Subclinical Hyperthyroidism

The introduction of assays for serum TSH that were sensitive enough to distinguish between normal and low concentrations allowed subjects with subclinical hyperthyroidism to be identified. Subclinical hyperthyroidism is defined as a low serum TSH concentration and normal serum T_4 and T_3 concentrations, in the absence of hypothalamic or pituitary disease, nonthyroidal illness, or ingestion of drugs that inhibit TSH secretion such as glucocorticoids or dopamine (Biondi and Cooper, 2012). Epidemiological studies differ in the definition of a low serum TSH concentration and whether the subjects included were receiving levothyroxine therapy.

In studies using sensitive serum TSH assays (detection limit, 0.01 mU/L), approximately 2% of subjects have subnormal serum TSH and 1% have an undetectable serum TSH (Vanderpump *et al.*, 1995). The Colorado study, which screened more than 25,000 healthy volunteers found 2% of the population to have a subnormal serum TSH, with more than half on levothyroxine therapy (Canaris *et al.*, 2000). In the NHANES III study the prevalence was highest in those subjects aged 20–39 years and those aged greater than 79 years (Hollowell *et al.*, 2002). In this study the percentage of subjects with serum TSH concentrations less than 0.4 mU/L was significantly higher in women than men, and black subjects had significantly lower mean serum TSH concentrations, and therefore a higher prevalence of subclinical hyperthyroidism (0.4%) than whites (0.1%) or Mexican Americans (0.3%). The prevalence of subnormal serum TSH concentrations is higher in iodine-deficient populations (6%–10%), due to functional autonomy from nodular goiters (Laurberg *et al.*, 2010) (**Table 2**).

Among subjects with subclinical hyperthyroidism, those with low but detectable serum TSH values may recover spontaneously when re-tested. Nonthyroidal illness is an important cause of false positive serum TSH test results. There are limited data on the risk of progression of subclinical hyperthyroidism to overt hyperthyroidism. In those subjects with an undetectable serum TSH and a confirmed etiology as determined by thyroid scintigraphy due to Graves' disease or nodular disease, it has been calculated that the annual incidence is approximately 5%–8% (Schouten *et al.*, 2011). A large population study in Tayside, Scotland, followed 2024 subjects with at least 2 serum TSH measurements below the reference range for at least 4 months for up to 7 years (Vadiveloo *et al.*, 2011). Few subjects developed hyperthyroidism (0.5%–0.7%) and the percentage of those reverting to normal increased with time and this was more common in those with a baseline serum TSH between 0.1 and 0.4 mU/L.

Incidence of Hyperthyroidism

In the 20-year follow-up of the Whickham cohort, the mean annual incidence of hyperthyroidism in women was 0.8 per 1000 (Vanderpump *et al.*, 1995). No new cases were detected in men. The estimated probability of developing hyperthyroidism in women at a particular time (the hazard rate) averaged 1.4 per 1000 between the ages of 35 and 60. The incidence data for overt hyperthyroidism in men and women from large population studies are comparable at 0.4 per 1000 women and 0.1 per 1000 men per year, although the age-specific incidence varies considerably (McGrogan *et al.*, 2008). The peak age-specific incidence of Graves' disease was between 20 and 49 years in two studies but increased with age in Iceland and peaked at 60–69 years in Malmö, Sweden. The only available data for a black population from Johannesburg suggest a 10-fold lower annual incidence of hyperthyroidism in black Africans than in European whites: 0.09 per 1000 women and 0.007 per 1000 men (Vanderpump, 2011). In a large population study in Tayside, Scotland, 620 incident cases of hyperthyroidism were identified with an incidence rate of 0.77 per 1000 per year (95% confidence interval (CI), 0.70–0.84) in women and 0.14 per 1000 per year (95% CI, 0.12–0.18) in men (Flynn *et al.*, 2004). The incidence increased with age, and women were affected two to eight times more than men across the age

range. Further analysis suggested that the incidence was increasing in women but not in men between 1997 and 2001 (Leese *et al.*, 2008).

Postpartum Thyroiditis

Postpartum thyroiditis (PPT) is a transient, destructive autoimmune thyroiditis that occurs between weeks 12 and 16 postpartum in 40%–50% of women and the presence of antithyroid microsomal antibodies early in pregnancy increases the risk of developing PPT (De Leo and Pearce, 2017). It presents as a temporary, usually painless, episode of hypothyroidism, occasionally preceded by a short episode of hyperthyroidism. Approximately 10% of women entering antenatal clinics at 16 weeks of gestation are antithyroid peroxidase antibody (antithyroid microsomal antibody) positive, which is comparable to the prevalence of thyroid antibodies in community surveys (Vanderpump, 2011). A proportion of these women will have subclinical hypothyroidism with a high serum TSH, but most will be euthyroid. However, after delivery a transient, destructive autoimmune thyroiditis that occurs between the 12th–16th week postpartum will develop in 50% of antithyroid peroxidase positive women, as ascertained in early gestation, clinically apparent as PPT. Up to about 25% of women progress to permanent hypothyroidism within approximately 5 years following an episode of PPT, particularly those with high antibody titers. It is not clear whether pregnancy alters the final incidence of autoimmune thyroid disease or merely quickens the development of thyroid disease (Alexander *et al.*, 2017).

Diffuse and Nodular Goiter

The most common thyroid disease is simple (diffuse) goiter. The clinical grading of thyroid size is subjective and imprecise. The WHO grading system recognizes that an enlarged thyroid gland may be palpably but not visibly enlarged. Examiner variation is greatest in deciding whether a thyroid that is palpable but not visible is normal (WHO stage O-A) or enlarged (WHO stage O-B). Interexaminer variation may also lead to differences in classifying goiter as diffuse or multinodular. There is also considerable overlap between the five WHO grades based on clinical criteria and thyroid volume estimated by ultrasonography. Ultrasonography has been used in epidemiological studies to assess thyroid size, resulting in much higher estimates of goiter prevalence than in studies in which goiter size was assessed by physical examination.

In cross-sectional surveys, the prevalence of diffuse goiter declines with age, the greatest prevalence is in premenopausal women, and the ratio of women to men is at least 4:1. In the Whickham survey, among the women 26% had a goiter; the frequency ranged from 31% in those aged less than 45 years (mostly diffuse) to 12% in those aged over 75 years (who had a higher proportion of nodular goiter) (Tunbridge *et al.*, 1977). Longitudinal studies confirm the decreasing frequency of diffuse goiter with age (Vanderpump *et al.*, 1995).

This decline in frequency of diffuse goiters with age is in contrast to the increase in frequency of thyroid nodules and antithyroid antibodies with age. In the Whickham survey, $<1\%$ of the men but 5% of the women had thyroid nodules detected clinically, and the frequency increased to 9% in women aged >75 (Tunbridge *et al.*, 1977). A higher prevalence of nodular goiter is found in areas of iodine deficiency in Europe, such as Italy, Germany, and Denmark. The only available longitudinal data suggest an annual incidence for nodules of 1 per 1000 and that, once formed, they tend to remain present and benign for a long period of time (Vanderpump, 2011).

With the increasing use of sensitive imaging techniques, an increasing proportion of thyroid nodules are detected incidentally. Many nodules are detected because of their size or anterior position in the neck, or the skill of the physician performing the examination but most thyroid nodules will not be clinically recognized. Up to 50% of nodules >1 cm detected by ultrasound are undetected by clinical examination. The prevalence of thyroid incidentaloma as an unexpected, asymptomatic thyroid nodule discovered during the investigation of an unrelated condition, is 67% with ultrasonography imaging, 15% with computed tomography or magnetic resonance imaging of the neck, and 1%–2% with fluorodeoxyglucose positron emission tomography (Russ *et al.*, 2014).

Thyroid Cancer

The clinical presentation of thyroid cancer is usually as a solitary thyroid nodule or increasing goiter size. Although thyroid nodules are common, thyroid cancers are rare. The four major histological types are papillary, follicular, medullary, and anaplastic, and each displays a different epidemiology. The annual incidence of all thyroid cancers ranges between 1 and 10 per 100,000 population in most countries and is two to four times more frequent in women than men (Perros *et al.*, 2014; Haugen *et al.*, 2016). Papillary and follicular tumors, which comprise 60%–90% of the total, are rare in children and adolescents, but their incidence increases with age in adults.

Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy and worldwide constitutes 50%–90% of differentiated follicular cell-derived thyroid cancers. Papillary thyroid microcarcinomas (diameter, ≤ 1 cm) are found in 4%–36% of adults postmortem in population-based studies. Most diagnoses of PTC occur in patients 30–50 years old (median age, 44 years),

and the majority (60%–80%) occur in women. The reported increase in the incidence of these carcinomas in recent years may be attributed to an improvement in pathological techniques. The increasing incidence may partially reflect earlier and increased detection of small (subclinical) papillary cancers secondary to more widespread use of neck ultrasonography and fine-needle aspiration of very small thyroid nodules. An analysis of the National Cancer Institute's Surveillance, Epidemiology, and End Results database found an increase in the rates of differentiated thyroid cancer of all sizes, including tumors >4 cm. In addition, incidence-based mortality increased between 1974 and 2013, from 0.40 to 0.46 per 100,000 person-years. This suggests a true increase in the incidence of papillary thyroid cancer (Lim *et al.*, 2017), Although the incidence of thyroid cancer is rising, death rates (0.5 per 100,000 men and women per year) have not changed significantly between 2003 and 2012. External radiation exposure, particularly in childhood, is a major risk factor for papillary cancer. Four years after the nuclear accident at Chernobyl, a dramatic increase in the incidence of childhood thyroid cancer (almost exclusively papillary tumors in which a translocation of the RET gene occurs) was recorded in the regions most exposed (Tuttle *et al.*, 2011). There is no documented association between radioiodine therapy for thyrotoxicosis and subsequent development of thyroid cancer in adults.

Follicular thyroid cancer occurs relatively infrequently compared to papillary cancer and accounts for approximately 15% of all thyroid cancers. There is an increased frequency of follicular to papillary carcinoma (5:1) in iodine-deficient endemic goiter areas. It tends to be a malignancy of older persons, with a peak incidence of between ages 40 and 60 years and is approximately three times more common in women than in men.

Medullary thyroid cancer (MTC) occurs in both sporadic and hereditary forms. The highest incidence of sporadic disease occurs in the fifth decade. Hereditary MTC can be inherited as an autosomal dominant trait with a high degree of penetrance associated with multiple endocrine neoplasia type 2 syndrome or as familial MTC without any other endocrinopathies. It can be diagnosed before clinical presentation by genetic and biochemical screening. Anaplastic thyroid cancer is very rare and is more frequent in populations with endemic goiter. Thyroid lymphoma is also uncommon, constituting approximately 2% of extranodal lymphomas and occurring predominantly in older women. Up to one-third of patients have a history of goiter, whereas some have established autoimmune thyroiditis and may be taking levothyroxine therapy.

Screening for Thyroid Disease

In the 1970s, screening programs for congenital hypothyroidism were developed in which TSH was measured in heel-prick blood specimens to detect this condition as early as possible. The value of screening for congenital hypothyroidism is unquestioned, but only done routinely in approximately 30% of the world's birth population (Ford and LaFranchi, 2014).

Certain groups within the adult population who should have an assessment of thyroid function at least once to detect thyroid dysfunction include those with a goiter or thyroid nodule, atrial fibrillation, dyslipidemia, subfertility, or osteoporosis. There is a high frequency of asymptomatic thyroid dysfunction in unselected patients with diabetes mellitus, and assessing thyroid function in the annual review of patients with diabetes appears cost-effective. The threefold increase in the prevalence of thyroid antibodies in patients with breast cancer suggests that it may be worth screening this group for thyroid dysfunction. There is no consensus on whether healthy pregnant women should be screened for thyroid disorders or PPT although it has been shown to be cost-effective in analytical models (Dosiou *et al.*, 2012). However, because women with type 1 diabetes are three times more likely to develop postpartum thyroid dysfunction, it is recommended that all such diabetic women should be tested in the first trimester for thyroid antibodies.

Any woman with a history of PPT should be offered annual surveillance of thyroid function due to the possible long-term risk of permanent hypothyroidism. Because of the high prevalence of hypothyroidism in people with Down syndrome and Turner syndrome, an annual check of thyroid function is recommended. Thyroid function tests are indicated every 6 months for those receiving amiodarone, lithium and immune checkpoint inhibitor therapy and every 12 months following head and neck irradiation. Following destructive treatment for thyrotoxicosis by either radioiodine or surgery, indefinite annual surveillance for the development of hypothyroidism is required.

Controversy exists as to whether healthy adults living in an area of iodine sufficiency benefit from screening for thyroid disease. The benefit from a screening program must outweigh the physical and psychological harm caused by the test, diagnostic procedures, and treatment (Tunbridge and Vanderpump, 2000; Rugge *et al.*, 2015). The prevalence of unsuspected overt thyroid disease is low, but a substantial proportion of subjects tested will have evidence of thyroid dysfunction, with approximately 10% with subclinical hypothyroidism and 1% with subclinical hyperthyroidism. No appropriately powered prospective, randomized, controlled, double-blinded interventional trial of either L-T4 therapy for subclinical hypothyroidism or antithyroid therapy for subclinical hyperthyroidism exists (Biondi and Cooper, 2012).

Although epidemiological studies have shown an association between subclinical hypothyroidism and coronary heart disease in younger people (<65 years) or those with high serum TSH (>10 mU/L) (Rodondi *et al.*, 2010), recent evidence suggests that in older people, higher serum TSH and lower free T_4 concentrations within the euthyroid range are associated with lower risk of multiple adverse events including mortality (Cappola *et al.*, 2015). Treatment in those who are symptomatic, pregnant, or preconception, aged <65 years appears justified (Pearce *et al.*, 2013).

A meta-analysis demonstrated that endogenous subclinical hyperthyroidism was associated with increased risk of total, coronary heart disease (CHD) mortality and incident atrial fibrillation (AF) (Collet *et al.*, 2012). The highest risk of CHD mortality and AF is noted when the serum TSH is <0.10 mU/L. Subclinical hyperthyroidism might be associated with an increased risk for

hip and non-spine fractures, but additional large, high-quality studies are needed (Wirth *et al.*, 2014). Treatment may be indicated in patients older than 65 years with serum TSH < 0.1 mU/L to potentially avoid these serious cardiovascular events, fractures, and the risk of progression to overt hyperthyroidism (Biondi *et al.*, 2015). Any potential benefits of therapy in subclinical hyperthyroidism must be weighed against the morbidity associated with the treatment of hyperthyroidism.

From the available evidence, the following recommendations may be justified for an iodine-replete community:

- Screening for thyroid dysfunction in women younger than age 50 and in men is not warranted in view of the relatively low point prevalence of unsuspected overt thyroid dysfunction.
- Case-finding in women during menopause or during visits to a primary care physician with nonspecific symptoms is justified due to the high prevalence of subclinical hypothyroidism.
- If increased serum TSH is found at screening, then measurement should be repeated 2 months later together with free T_4 measurement after excluding nonthyroidal illness, drugs, etc.
- Treatment with levothyroxine is recommended if the serum TSH is ≥ 10 mU/L, irrespective of whether free T_4 is low.
- Subjects with a serum TSH between 5 and 10 mU/L and normal free T_4 are at increased risk of developing hypothyroidism, and repeat measurement of serum TSH is warranted at least every 3 years if not annually.
- If a suppressed serum TSH is found at screening, it should be remeasured 2 months later, and if it is still suppressed, free T_3 should be measured.
- After levothyroxine replacement is initiated, for whatever indication, long-term follow-up with at least an annual measurement of serum TSH is required.

See also: Causes of Hypothyroidism. Graves' Disease. Graves' Orbitopathy. Hypothyroidism Subclinical. Postpartum Thyroid Dysfunction. Thyroid Carcinoma. Thyroiditis, Infectious and Subacute

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Genetic Factors in Thyroid Disease☆

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Glossary

Genetic association analysis The most frequently used method for detecting predisposing genes; it compares the frequency of a certain allele in the affected population with its frequency in a control population. Genetic association analysis is quite sensitive and detects genes with a small predisposing effect (risk factors).

Genetic heterogeneity The presence of multiple genes causing the same phenotype.

Linkage analysis A procedure that employs polymorphic genetic markers whose precise location in the genome is known. By following the cosegregation of alleles of these markers with the disease phenotype in families, linkage establishes the likelihood that the genetic locus examined contains the unknown gene responsible for the disease phenotype. Genetic linkage was originally designed for purely Mendelian disorders but theoretical work supports its use in polygenic diseases. Linkage detects necessary genes with substantial effects on the predisposition to disease. It is therefore relatively insensitive when compared with genetic association analysis. However, its results are robust when the data set is large and stringent statistical criteria are used for significance.

Linkage disequilibrium A phenomenon whereby two alleles at two loci in close spatial relationship in the genome are found associated more often than predicted by their individual population frequencies and by the distance between them on the genome. Linkage disequilibrium indicates one of two phenomena: selective advantage of the two alleles combined together or an admixture of two populations with different frequencies of the two alleles.

LOD score In its classical form, linkage yields an LOD (logarithm of differences) score, an absolute number that represents the logarithm of the likelihood of linkage of a given phenotype with a genetic location. An LOD score of 3 or more is considered a strong evidence for linkage in a complex disease and represents an absolute likelihood of 1000 to 1 in favor of linkage.

Microsatellite markers Short sequences of tandem repeats (multiple repeats of the same couple of bases) that are ubiquitous within the genome. Alleles of microsatellite markers are readily detected by polymerase chain reaction amplification. Given their high degree of polymorphism (8–20 known alleles on average at each locus), microsatellite markers are especially suitable for linkage analysis.

Online Mendelian Inheritance in Man (OMIM) The National Center for Biotechnology Information maintains an online, searchable catalogue of inherited human diseases publicly available on the World Wide Web at www.ncbi.nlm.nih.gov. All registered diseases and disease loci are given a unique identifying number.

Single nucleotide polymorphisms (SNPs) Single base substitutions found across the whole genome (in both coding and noncoding regions). SNPs are mostly detected by restriction fragment length polymorphism analysis. The degree of polymorphism of SNPs is much lower than in microsatellite markers and therefore SNPs are more suitable for use in association studies.

Whole genome screen A technique for scanning the whole genome for linkage with a known phenotype. Whole genome screening employs a set of multiple microsatellite markers evenly spaced across the genome and tests each location for linkage to the target phenotype in well-characterized families. Cosegregation of each single marker with the disease phenotype is then analyzed using computerized linkage algorithms. Eventually, a map of the likelihood of linkage across the genome is obtained.

Introduction

When ancient vertebrates migrated from the iodine-rich oceanic environment to the iodine-poor terrestrial environment, evolution provided the new organisms' thyroid with an extremely complex and specialized machinery, completely devoted to the avid collection and storage of iodine, for the sole purpose of continuously providing enough substrate for the synthesis of thyroid hormone. As a very specialized organ, the thyroid therefore expresses, along with common housekeeping genes, a unique subset of genes, whose products are mostly enzymes involved in iodine metabolism and/or thyroid hormone synthesis. On one hand, some of these enzymes serve as unique and possibly confined targets (antigens) for the immune aggression characteristic of autoimmune

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thyroid diseases (AITD), which are very common conditions in humans, whose etiology is still incompletely understood. The susceptibility to these diseases has long been recognized to be largely hereditary in nature. Because of the high prevalence in the general population and because of the large amount of available data on genetic factors, AITD are given special emphasis in this article.

On the other hand, a wide range of thyroid disorders have been described in which a single inherited point mutation in one thyroid-specific gene causes clinically significant derangements in thyroid physiology and function, with or without hereditary goiter. Given their strong negative effect on survival, the latter conditions are distinctly rare but are usually inherited in a Mendelian fashion and therefore represent an important part of the genetics of thyroid disease. Thyroid cancer and goiter are also quite prevalent. Although epidemiological studies indicate that environmental factors are by far predominant in causing these disorders, emerging evidence shows significant inherited susceptibility to these disorders as well.

The Monogenic Diseases of the Thyroid Gland

Inherited Diseases of the Thyrotropin Receptor

The human thyrotropin receptor (TSHr) was cloned in 1989. The TSHr is a protein receptor, one of the G protein-coupled, seven-transmembrane domain receptors. The TSHR gene is located on chromosome 14q31. The primary structure of the TSHr is closely related to that of the follicle-stimulating hormone and luteinizing hormone receptors. Activation of the TSHr is the key event leading to both increased thyroid hormone secretion and growth. Because of its central role in the regulation of thyroid physiology, the TSHr has been the focus of constant research in the history of endocrinology. In more recent studies, however, inherited defects of the TSHr have been described as a cause of clinically relevant thyroid disease.

Activating Mutations of the TSHR

Site-directed mutagenesis initially showed that single-point mutations in critical regions of the TSHR were capable of constitutively activating the TSHr, in the absence of its natural ligand, thyrotropin [or thyroid-stimulating hormone (TSH)]. Such mutations, if found in vivo, would be expected to cause thyroid growth and hyperthyroidism. Indeed, naturally occurring, activating mutations of the *TSHR* have been described in a significant fraction of human toxic adenomas and toxic multinodular goiters (Kleinau and Biebermann, 2014). Mutations of the *TSHR* causing distinct hyperfunctioning nodules are always somatic, that is, are found only within the nodular tissue. The nodules containing the mutation therefore represent monoclonal expansions of an initially mutated single thyroid cell and the condition is not hereditary. Since activation of the TSHr induces not only thyroid growth but also differentiation, these neoplasms are only very rarely malignant. More rarely, however, germ-line activating mutations of the *TSHR* have been described. The phenotype caused by these mutations is termed autosomal-dominant nonautoimmune hyperthyroidism [Online Mendelian Inheritance in Man (OMIM) 603372]. Although in many cases, this disease is inherited in a dominant fashion, at least half of the cases described thus far (>20) in the literature are sporadic; that is, the mutation is found only in the index case and not in the parents (de novo mutation) (Hébrant *et al.*, 2011). The classical phenotype is characterized by severe congenital or neonatal hyperthyroidism and goiter. TSHr antibodies are usually negative and this helps differentiate the disorder from the other relevant cause of neonatal hyperthyroidism, the transplacental passage of maternal TSHr-stimulating antibodies. Treatment with antithyroid drugs can usually control hyperthyroidism, but relapses are the rule. Thyroid growth is not contrasted by antithyroid drugs and the goiter evolves from initially diffuse to multinodular early during childhood, requiring surgery. Milder cases have been described, with later onset of hyperthyroidism and goiter. Several mutations of the *TSHR* have been identified as being responsible for the syndrome ("SSFA-GPHR" http://www.ssfa-gphr.de/). The relationship of genotype to phenotype has been found to be somewhat inconsistent in large families with multiple members carrying the same mutation. This indicates that other factors (genetic or environmental) play a role in determining the phenotype, at least with some of the known mutations.

More rarely, inherited mutations of the *TSHR* cause an inordinate responsiveness of the receptor to the placental hormone β human chorionic gonadotropin (β-HCG), which is closely related to TSH. In these cases, the effect of the mutation becomes evident only when high levels of β-HCG are present, that is, during pregnancy, hence the appellation of familial gestational hyperthyroidism for this peculiar disease (OMIM 603373). Other than in pregnancy, the carriers of this rare mutation have a completely normal thyroid function.

Inactivating Mutations of the *TSHR*

Inactivating mutations of the *TSHR* manifest themselves with the clinical phenotype of resistance to TSH (OMIM 275200). Patients have often been detected at birth with severe primary congenital hypothyroidism (elevated TSH and low thyroxine) and a normally located but hypoplastic thyroid. In other cases, only mild, subclinical hypothyroidism has been found. Clinical phenotypes of TSH resistance correlate with the residual mutant receptor activity (Grasberger and Refetoff, 2017). Nonfunctional mutations in the two alleles produce severe hypothyroidism (uncompensated TSH resistance), while mutations in at least one allele with residual receptor function produce either mild hypothyroidism (partial compensation) or isolated hyperthyrotropinemia (full compensation). More recently, a new subgroup of TSH resistance (nonclassic TSH resistance) that is characterized

by paradoxically high thyroidal iodine uptake has been reported. The inheritance of TSH resistance is typically recessive, though heterozygous *TSHR* mutations play a more prominent role in the pathogenesis of isolated nonautoimmune hyperthyrotropinemia diagnosed after the neonatal period.

Inherited Defects of Thyroid Hormone Biosynthesis and Processing

Defective synthesis of thyroid hormone from the thyroid results in overt or compensated primary hypothyroidism. Since thyroid growth is largely independent of the enzymatic pathways involved in the biosynthesis of thyroid hormone, an ongoing TSH elevation as a consequence of low circulating thyroid hormone results in goiter. Therefore, the clinical phenotype of this group of diseases is characterized by hereditary goiter, usually early in onset, and various degrees of impairment of thyroid function, ranging from euthyroidism to overt primary hypothyroidism. Additional clinical features may be observed when the defect has consequences on other organs, as in Pendred syndrome. The phenotype may be further characterized by the application of sophisticated thyroid function tests, such as the perchlorate challenge test or the measurement of thyroid hormone by-products. Because of their great impact on the overall health and survival of affected individuals, some of these diseases are quite rare and mostly recessive. However, their recognition has greatly enhanced the understanding of thyroid gland metabolism.

Pendred Syndrome

Pendred syndrome (OMIM 274600) is an autosomal-recessive disease characterized by congenital deafness, early-onset goiter, and euthyroidism, though subclinical hypothyroidism is frequently observed in iodine-deficient areas (Wémeau and Kopp, 2017). The prevalence of Pendred syndrome is estimated at 1/1000. The sensory deafness is often due to a malformation of the inner ear in which the cochlea is replaced by a single cavity (Mondini's defect). However, an enlarged vestibular aqueduct (EVA), endolymphatic sac, and endolymphatic duct on magnetic resonance imaging (MRI) of the ear have been shown to be more specific signs. Except for its unusually early onset, the goiter is clinically not distinguishable from the more common endemic multinodular goiter. A positive perchlorate discharge test with typical malformations on MRI imaging of the inner ear establishes the diagnosis. The disease has been mapped by linkage analysis to chromosome 7q31. The cloned gene (termed *SLC26A4*) encodes a multifunctional anion exchanger protein (pendrin) expressed in the adult human thyroid and in the human cochlea and kidney. In the thyroid gland, pendrin is expressed at the apical membrane and functions as an iodide/chloride exchanger involved in iodide efflux into the lumen. Close to 200 different mutations in the *SLC26A4* gene have been reported. Individuals with true Pendred syndrome are either homozygous or compound heterozygous for mutation in the *SLC26A4* gene. Biallelic mutations in the *SLC26A4* gene have no or only a mild thyroidal phenotype, indicating that other components are likely involved in iodide efflux. Intriguingly, some patients with nonsyndromic EVA harbor monoallelic mutations in the *SLC26A4* gene, suggesting an oligogenic mechanism with concomitant mutations in either the *KCNJ10* or the *FOXE1* genes (Wu *et al.*, 2010; Yang *et al.*, 2009), although this seems to be a rare event.

Sodium–Iodide Symporter Defect (OMIM 601843)

This extremely rare disease is characterized by an autosomal-recessive mode of inheritance, severe congenital hypothyroidism, and multinodular goiter. The diagnosis is clinically established on the basis of a very low thyroidal uptake of radioiodine in the presence of goiter. An additional feature is a partial response in terms of thyroid hormone production to high oral doses of potassium iodide. The disease is caused by homozygous or compound heterozygous inactivating mutations of the sodium–iodide symporter gene, located on chromosome 19p33.2–p12 (Targovnik *et al.*, 2017).

Thyroglobulin Defects (OMIM 274900)

Patients with this autosomal-recessive condition display a wide range of thyroid dysfunction, ranging from severe hypothyroidism to euthyroidism, low serum thyroglobulin concentration, congenital goiter, or goiter appearing shortly after birth and elevated thyroidal radioiodine uptake. Thyroglobulin is a very large protein whose gene is located on chromosome 8q24.2–q24.3. To date, more than one hundred deleterious mutations, including splice-site, nonsense, and missense, have been identified and characterized in patients with the disease (Targovnik *et al.*, 2017).

Thyroperoxidase Defects (OMIM 274500)

Patients with thyroperoxidase defects have severe congenital hypothyroidism and various degrees of goiter. The disease is autosomal-recessive and is diagnosed by a positive perchlorate discharge test, in the absence of clinical features of Pendred syndrome. The gene for thyroperoxidase has been mapped to chromosome 2p25 and about one hundred deleterious mutations that cause iodide organification defect, total in most cases, have been identified and characterized (Ris-Stalpers and Bikker, 2010).

Dual Oxidase 2 Defects (OMIM 606759)

In the thyroid gland, dual oxidase 2 (*DUOX2*) is responsible for H_2O_2 generation which is used by thyroid peroxidase for the iodine organification. The prevalence of *DUOX2* mutations in thyroid dyshormonogenesis is variable among different series but invariably high. To date, near one hundred mutations have been reported, with a high inter and intrafamilial phenotype variability. In particular, there is no correlation between the class of mutation (truncated, missense, monoallelic, biallelic) and the phenotype in terms either of TSH levels or of discharge rate or of CH duration (transient or permanent). Genetic, epigenetic, and environmental modulators may account for the lack of genotype–phenotype correlation (Muzza and Fugazzola, 2017).

Dual Oxidase Maturation Factors 2 (OMIM 612772)

Dual oxidase maturation factors (DUOXA) 2 form functional heterodimeric complexes with DUOX2, which enable the exit of the dimer from ER and the translocation to the plasma membrane. The prevalence of *DUOXA2* variants in cases with congenital hypothyroidism is very low; indeed, only seven different variants have been reported in eight unrelated cases showing the same phenotype variability observed for *DUOX2* mutations (Muzza and Fugazzola, 2017).

Dehalogenase Defects (OMIM 274800)

Deiodinases (or dehalogenases) are a group of enzymes capable of deiodinating metabolites of thyroid hormone degradation, such as diiodotyrosine (DIT) and monoiodotyrosine (MIT). As such, they greatly contribute to the intrathyroidal pool of iodine by deorganifying "used" organic iodine and making it available for new hormone synthesis. Patients with a dehalogenase defect are incapable of reusing MIT or DIT and develop hypothyroidism and goiter secondary to urinary loss of iodine. The defect is inherited in an autosomal-dominant fashion and is diagnosed by an elevated urinary excretion of administered labeled diiodotyrosine and monoiodotyrosine. Also characteristic is a prompt resolution of hypothyroidism when dietary supplementation with high doses of iodine is given (Targovnik *et al.*, 2017).

Developmental Defects of the Thyroid and Congenital Hypothyroidism

Congenital hypothyroidism (CH) has an incidence of approximately 1 in 2000–3000 newborns. Universal screening for the disease is available in most countries and allows early detection and treatment. Although CH can be observed in any severe form of the defects of thyroid synthesis described above, it is most often due to an abnormal in utero development of the thyroid gland, varying from its complete absence (thyroid agenesis) to various degrees of ectopy and hypoplasia (thyroid dysgenesis). Hereditary forms of the latter are very rare, most likely due to the severity of the consequences of CH, which include infertility. A number of genes involved in thyroid organogenesis have been identified and their role in the pathogenesis of CH has been partially clarified (Abu-Khudir *et al.*, 2017). The thyroid transcription factor 1 (*TTF-1*; OMIM 600635), mapped to chromosome 14q21, has been shown in animal models to be necessary for normal early thyroid organogenesis. Despite its central role, mutations in *TTF-1* have been identified in only a few patients with congenital hypothyroidism due to thyroid dysgenesis. TTF-2, also known as FOXE1 (on chromosome 9q22; OMIM 241850), is also highly expressed during thyroid ontogenesis. A familial case of thyroid agenesis associated with cleft palate and spiky hair has been shown to be due to homozygous missense mutations of *TTF-2* and was named Bamforth–Lazarus syndrome. Paired-box gene 8 (PAX-8; OMIM 167415) is another transcription factor involved in thyroid organogenesis and regulation of the transcription of thyroid-specific genes, such as thyroglobulin and thyroid peroxidase. Nonsense and missense mutations of *PAX-8* have been observed in both sporadic and familial cases of thyroid dysgenesis. In addition, newly discovered predisposing genetic factors (e.g., *GLIS3*, *JAG1*) are described in familial CH cases associated mainly with a normal or small thyroid in situ.

Despite these encouraging findings, the etiology of the large majority of cases of CH and thyroid dysgenesis remains unknown. It is likely that, in addition to other genetic factors, epigenetic or environmental factors play an important role.

Genetic Factors in Thyroid Autoimmune Diseases

The Clinical Phenotype

It is important to recognize that these diseases represent a group of diseases rather than a homogenous entity and that several clinical features may occur independently of one another, defining a variety of possible phenotypes. In general, the autoimmune thyroid diseases (AITD) are defined by the presence of a thyroid lymphocytic infiltrate, associated with serological evidence of thyroid autoimmunity in the form of circulating antibodies reactive to thyroid antigens and various degrees of thyroid dysfunction, ranging from profound hypothyroidism, as in the case of atrophic Hashimoto's thyroiditis (OMIM 140300), to severe hyperthyroidism, as in the typical Graves' disease (OMIM 275000). A number of other features may or may not be associated, such as the presence of Graves' ophthalmopathy, an autoimmune disease of the orbital tissues, typically observed in a relevant number of patients with hyperthyroid Graves' disease but occasionally also found in patients with Hashimoto's thyroiditis. Pretibial

myxedema is a puzzling inflammatory process of the dermis, localized to the pretibial regions, that is also associated (more rarely than ophthalmopathy) with Graves' disease. Finally, depending on various factors that are not completely understood, the thyroid gland may be enlarged as a consequence of either massive lymphocytic infiltration or ongoing stimulation; it may be normal in size or it may be strikingly reduced in size, as in the case of primary myxedema.

Evidence for a Role of Genetic Factors in the Pathogenesis of Autoimmune Thyroid Disease

A number of lines of evidence strongly indicate an important influence of genetic factors in the etiology of these diseases. Indeed, the prevalence of the disease in first-degree relatives of patients with the disease (probands) is significantly higher than in the general population. In early studies conducted in the late 1950s and 1960s, the prevalence of positive antithyroglobulin or antimicrosomal antibody tests in relatives ranged from 45% to 55%, compared to a general population prevalence of approximately 15%. More stringent classical segregation analysis in families with autoimmune thyroid disease confirmed these earlier results, indicating a Mendelian dominant mode of inheritance for thyroid autoantibodies, at least in some families. In a further refinement of these findings, some investigators have indicated that in some families not only the predisposition to form thyroid autoantibodies is strongly hereditary, but also that even the fine molecular specificity of such antibodies can be inherited. In summary, there seems to be a clear role for genetic factors in the formation of thyroid antibodies, at least in families in which the clinical disease exists in one member. The situation was different when normal subjects with thyroid autoantibodies but without clinical Hashimoto's thyroiditis were selected as probands. In one such study, only 30% of relatives had a positive result, compared to approximately 16% expected from population data, indicating at best a polygenic inheritance with low penetrance. Thus, the relatively common antithyroid autoantibody phenotype may represent the consequence of a strong genetic influence only in families with overt AITD. Less clear data are available when one looks at the inheritance of the full AITD phenotype. Approximately 33% of relatives of patients with AITD were found to be affected, resulting in a relative risk for the disease in sibs, also termed λ(s), as high as 16.9.

Another way of estimating the role of the genetic contribution to the etiology of a disease is studying twins. In both Graves' disease and Hashimoto's thyroiditis, studies in twins have shown concordance rates well below 100% in identical twins (approximately 30% in Graves' disease and 55% in Hashimoto's thyroiditis). However, much lower concordance rates (close to zero) have been observed in dizygotic twins. Thus, whereas a lower concordance rate in dizygotic twins indicates the presence of genetic factors, the less-than-100% concordance rate observed in identical twins indicates a role for environmental factors as well (incomplete penetrance). Interestingly, 80% of identical twins and 40% of dizygotic twins of Hashimoto's thyroiditis patients had circulating thyroid autoantibodies. Again, these data suggest a dominant mode of inheritance with high (80%) penetrance for the thyroid autoantibody trait, within a framework of a general predisposition to thyroid autoimmune disease.

Predisposing Genes in AITD

The evidences summarized above have encouraged in the past few decades studies aimed at the identification of the genes involved in the genetic predisposition to AITD. The majority of the susceptibility genes include genes of thyroid autoantigens and genes involved in the immune response. Aberrant activity of these immune-regulatory genes due to polymorphisms would potentially lead to a breakdown in immune tolerance and ultimately to autoimmunity. Through linkage and association analyses, genome screening, and genome wide association studies (GWAS), several single nucleotide polymorphisms (SNPs) in genes including *TSHR*, *FOXP3*, *CD25*, *CTLA-4*, *CD40*, and the *HLA* have been identified as possible candidates.

TSHR

GWAS studies allowed to establish a strong association of the *TSHR* gene with Graves' disease. Subsequent fine-mapping studies narrowed the disease-susceptibility region to a 40 kb sequence in intron 1, where at least five Graves' disease-associated SNPs in tight linkage disequilibrium were identified (rs179247; rs2284720; rs12101255; rs12101261; and rs2268458). Further functional analyses of *TSHR* intron 1 polymorphisms provided direct evidence of a link between central tolerance and *TSHR* intron 1 SNPs (Stefan *et al.*, 2014).

FOXP3

FOXP3 (forkhead box P3) is an X-linked gene that can act as both a transcriptional repressor and an activator for primarily immunological genes. The mouse mutant *Scurfy*, a line defective in FOXP3, is characterized by massive hyperproliferation and multiorgan infiltration of CD4 + T cells and is lethal in hemizygous males. In humans, mutations in *FOXP3* lead to an X-linked syndrome which includes immune dysregulation, polyendocrinopathy, and enteropathy. Various *FOXP3* single nucleotide polymorphisms and microsatellite markers have been reported to be associated with autoimmune thyroiditis (AITD), though the role of these variants in thyroid autoimmunity has not been clarified (Lee *et al.*, 2015).

CD25

CD25, also known as the α-subunit of the IL-2 receptor, is involved in the regulation of T cell function. Similar to mice with impaired FOXP3, IL-2Rα deficient mice exhibit lethal lymphoproliferative disorder and a severe autoimmunity. Case-control and GWAS studies from the UK reported that CD25 was significantly associated with Graves' disease with elevated serum concentrations of sIL-2Rα (Lee *et al.*, 2015).

CD40

CD40 is a tumor necrosis factor receptor (TNF-R) predominantly expressed on antigen presenting cells (APC) like B cells, but also on thyroid epithelial cells, and plays a fundamental role in B-cell activation and antibody secretion. Defective or absent CD40 activity results in a variety of profound immune system deficiencies. Moreover, several single nucleotide polymorphisms within the *CD40* gene have emerged as causative genetic variants that predispose to GD. The most well-studied polymorphism, a-1C/T polymorphism (rs1883832) in the 5′ UTR Kozak sequence, was found to have significant association with GD with a relative risk of 1.6 among individuals carrying the CC genotype. The C allele has been associated with higher persistent levels of thyroid peroxidase and thyroglobulin antibodies, while the T allele and TT genotype were found to carry a protective effect and even linked to later-age onset of GD. Functional studies indicated that the -1C/T *CD40* SNP increases the translation efficiency of CD40 synthesis of 13%–35%; this could potentiate the activation of APCs, the costimulation of the T cell, and the CD40 expression on thyroid cells leading to an overall proinflammatory and autoimmune response (Lee *et al.*, 2015).

CTLA-4

The human *CTLA-4* gene is located on chromosome 2q33 and produces a protein expressed on T cells only after antigen or mitogen stimulation. It subsequently interacts with activated T cells carrying a CTLA-4 receptor (B-7) causing their silencing or death. Therefore, CTLA-4 acts as a key downregulator of the immune response and represents an excellent candidate for an autoimmunity gene. Consistently, mice defective in CTLA-4 function (CTLA-4 −/−) develop rapidly fatal multiorgan lymphocytic infiltrates, a condition that resembles human autoimmune diseases. Several *CTLA-4* SNPs have demonstrated risk for thyroid autoimmunity. An A/G SNP at position 49 in exon 1, an (AT)n microsatellite polymorphism in the 3′ untranslated region of exon 4, and the CT60 polymorphism in the 3′UTR have consistently been associated with both Graves' disease and Hashimoto's thyroiditis. Functional studies have shown that these polymorphisms lead to a reduced CTLA-4 expression and/or stability that would predispose to autoimmunity by decreasing suppression of T cell activation and proliferation.

The *HLA* Genes

The *HLA* gene complex is located on the short arm of the sixth chromosome (6p21) and encodes a large number of genes mostly (but not only) involved in the regulation of the immune response. In general, genes within the HLA complex have been subdivided into three classes. Class I includes the HLA class A, B, and C antigens, which are widely expressed on several tissues. Class I genes are mostly involved in direct cytotoxic reactions leading to the killing by the immune system of epithelial cells carrying exogenous antigens, mostly viral in nature. Class II includes the HLA DR, DP, and DQ antigens, mostly expressed on immune cells. These antigens are mostly involved in the presentation of antigen within the immune system. This process is central to the development of the normal immune response and, depending on several factors, it will lead to either the selection and amplification of antigen-specific T and B cells or to the silencing of these cells (tolerance). Class III includes genes coding for complement factors, heat shock proteins, tumor necrosis factor α, and several other proteins not directly involved in the immune response. The *HLA* genes (especially class I and class II) are highly polymorphic and some alleles show striking linkage disequilibrium. Because of its function, the HLA complex has been widely studied in relation to autoimmune diseases, including AITD. Graves' disease was initially shown to be associated with the HLA-B8 allele in several studies involving Caucasian patients with relative risks from 1.5 to 3. However, the HLA-B8 antigen has been subsequently recognized to be in linkage disequilibrium with the class II allele HLA-DR3. Indeed, additional association studies have shown a slightly higher relative risk (2.5–4) when this allele was studied in Caucasian Graves' disease patients. It was concluded that the HLA-DR3 allele was more relevant than HLA-B8 in determining the inherited susceptibility to GD. GD has also been found to be associated with (relative risk 3.8) the allele HLA-DQA1*0501, also in linkage disequilibrium with DR3, as well as with the allele DRB1*0301, in linkage disequilibrium with DR3. Hashimoto's thyroiditis has been occasionally found to be associated with HLA-DR3, HLA-DR4, and HLA-DR5, with similarly low relative risks, ranging from 2 to 7. It is interesting that different haplotypes of the HLA have been involved, as Graves' disease and Hashimoto's thyroiditis are often found in different members of the same family, portending a common genetic background. In keeping with the low relative risks obtained in association studies, linkage studies, including a whole genome screen analysis, have shown no evidence in favor of linkage with the HLA. In summary, genes within the HLA complex have been consistently shown to be involved in the genetic predisposition to AITD. However, as exemplified by low relative risks and negative linkage, the HLA complex failed to explain the large hereditary predisposition to the diseases shown by family studies. This shows that mutations within the HLA system are not absolutely required or sufficient to cause AITD, representing risk factors with a small overall effect.

Genetic Factors in Simple and Multinodular Goiter

The main etiologic factor in goiter is indisputably iodine deficiency, a worldwide problem that affects almost 1 billion people around the world. In areas with iodine deficiency, the prevalence of goiter in the general population is >10%. Epidemiologists have traditionally distinguished "endemic" goiter, observed in geographic areas of iodine deficiency, from "sporadic" goiter, observed in areas of iodine sufficiency. The accuracy of the distinction is, however, questionable, as the clinical phenotype of the two forms of the disease is indistinguishable, except for areas of extreme iodine deficiency, where large goiters are associated with cretinism and hypothyroidism. In addition to iodine deficiency, other factors are likely involved in the pathogenesis of this common disease. This hypothesis stems from several circumstantial observations. In areas with iodine deficiency, not all exposed subjects develop goiter. Universal iodine supplementation strikingly reduces but does not abolish the disease and goiter is observed in the absence of iodine deficiency, as mentioned above. Additional environmental factors, such as tobacco smoking, naturally occurring goitrogens, and pharmacologic goitrogens, have been demonstrated. Sex hormones are also likely to be involved as goiter is more prevalent in women living in areas with mild iodine deficiency. The observation of familial clustering of the disease is a daily experience of all endocrinologists, especially in areas with mild or no iodine deficiency, and this has led to the hypothesis that inherited factors coincide in the predisposition to the development of goiter. The familial clustering of the disease has also been reported in several epidemiological surveys, mostly from areas of endemic iodine deficiency. As in the case of the AITD, a number of twin studies have been performed. These have shown that the environmental factors are largely predominant when cases are drawn from endemic regions, as shown by similar concordance rates between dizygotic and monozygotic twins. However, large, population-based studies in twins have shown significantly different and overall higher concordance rates between monozygotic and dizygotic twins (**Table 1**). Interestingly, the intrinsic population prevalence of goiter in these latest studies is much lower than in the two older studies, performed in the 1960s in areas of iodine deficiency. One possible explanation for these apparently discrepant data is that once the effect of the major environmental factor (iodine deficiency) is removed from the population, cases with a stronger genetic effect emerge. In this view, the distinction between endemic and sporadic goiter seems to be justified. Despite evidence in favor of relevant genetic influences, these have not been clarified yet. As in many common diseases, there is no identifiable mode of inheritance and multiple, relatively frequent gene variants are likely to play a role. Among thyroid-specific genes markers, polymorphisms in the genes for thyroid peroxidase, thyroglobulin, sodium iodine symporter, pendrin, and thyrotropin receptor have been associated with goiter. Genetically linked nonthyroid-specific loci in familial goiter have been reported in several studies. Studies in single large kindreds with highly prevalent and apparently dominant transmission of multinodular goiter have indicated linkage of the phenotype to a locus (termed *MNG-1*) located on chromosome 14q31. The same studies have ruled out the *TSHR* (which resides in the same chromosomal region) from the linked region. *MNG-1* has been confirmed as a susceptibility locus in other large families. Interestingly, *MNG-1* overlaps with *GD-2*, a susceptibility locus for Graves' disease. It is therefore possible that the region contains a novel thyroid-specific growth factor and/or antigen. *MNG-2* was mapped on chromosome X by linkage analysis in an Italian family with goiter across three generations. *MNG-3*, located on chromosome 3, has been identified in two independent Japanese pedigrees. Other loci on chromosome two, three, seven, and eight have been identified by linkage analysis in Danish, German, and Slovakian families (Knudsen and Brix, 2014). These findings are encouraging but were obtained in a small subset of families with a seemingly large genetic effect and their epidemiological relevance to goiter found in the general population is unknown. Monogenic disorders of thyroid hormone metabolism represent a rare cause of hereditary goiter, with or without hypothyroidism. These conditions, described in some detail above, are distinct from the commonly encountered sporadic or endemic goiter in that there is usually an earlier appearance of goiter, a more distinct familial pattern (usually autosomal-recessive), and several degrees of thyroid dysfunction. Although it is conceivable that more subtle defects in any of these genes might be involved in the pathogenesis of goiter, extensive screening of the general population for association and/or linkage to known genes is lacking.

In summary, genetic predisposition is probably important in the etiology of simple goiter, both sporadic and endemic. In endemic areas, though, the effect of environmental factors (iodine deficiency) is predominant and widespread, making goiter very prevalent. In contrast, in areas with normal iodine intake, genetic factors are more relevant, but with a smaller prevalence. As a consequence, the prevalence of goiter is strikingly reduced and the phenotype is more clearly clustered in families. It is likely that multiple different genes are involved in the predisposition to simple goiter, but thus far only a few of these have been identified.

Table 1 Available studies in twins in populations with different prevalence of goiter

Year of the study	*Geographic location*	*Concordance rate in monozygotic twins*	*Concordance rate in dizygotic twins*	*Population prevalence of goiter*
1967	United Kingdom	24%	12%	25.3%
1967	Greece	89%	73%	53.0%
1999	Denmark	42%	13%	1.5%
2005	Denmark	67%	17%	1.5%

Note. In populations with a lower prevalence, a higher concordance rate is observed in monozygotic twins when compared with dizygotic twins, suggesting a stronger genetic predisposition.

Genetic Factors in Nonmedullary Thyroid Cancer

Thyroid cancer of follicular origin (OMIM 188550) is the most frequent endocrine tumor and more than 90% cases are sporadic, due to somatic genetic alterations mostly affecting the mitogen-activated protein kinase pathway. Only 3%–9% of all thyroid cancers are familial nonmedullary thyroid cancer (FNMTC) cases, defined by the presence of thyroid cancer in 2 or more first-degree relatives, in the absence of predisposing environmental factors (Peiling Yang and Ngeow (2016)). Clinically, FNMTC has been reported to be more aggressive and more often multifocal than its sporadic counterpart. Only 5% of all FNMTCs in the syndromic form have well-defined driver germline mutations; the associated syndromes include Cowden syndrome, familial adenomatous polyposis, Gardner syndrome, Carney complex type 1, and Werner syndrome. Cowden disease (OMIM 158350) is an autosomal-dominant disease, characterized by multiple hamartomas of the skin, breast, thyroid, brain, and endometrium. Facial trichilemmomas are a distinctive feature of the syndrome. Other findings include craniomegaly, scrotal tongue, and cerebellar neoplasias. A significant increase in the incidence of breast cancer is seen in Cowden syndrome families. Up to 25% of patients have thyroid cancer, and 60% of cases have thyroid nodules benign thyroid nodular disease. Cowden syndrome has been mapped to chromosome 10q23.31 and the mutated gene, termed *PTEN* for phosphatase (OMIM 601728), has been isolated. The PTEN protein is expressed in several tissues and functions as a tyrosine phosphatase able to dephosphorylate activated regulator proteins, thus downregulating proliferation pathways. Several mutations of *PTEN* have been found in families with Cowden syndrome, without a clear-cut genotype–phenotype relationship. Several in vitro studies suggest that PTEN acts as a tumor suppressor gene. This observation is in keeping with an autosomal-dominant mode of inheritance and with the observation that most of the mutations identified thus far cause a loss of function. Moreover, in several families with many features of the syndrome, no mutation of the gene has been found. Germline mutations in *SDHB-D*, *PIK3CA*, or *AKT1* genes and *KILLIN* promoter methylation have been identified in *PTEN* mutation-negative patients. Familial adenomatous polyposis (FAP; OMIM 175100), also termed Gardner syndrome, is inherited in an autosomal-dominant fashion. The most prominent feature of the syndrome is early-onset colonic cancer and multiple widespread, preneoplastic polyps of the colon. Extracolonic manifestations include congenital hypertrophy of the retinal pigment epithelium in up to 90% of affected persons and have been used as a marker of the disease before genetic testing became available. Other features of the syndrome include benign bone tumors and hepatoblastomas. In women with the syndrome, the risk of papillary thyroid cancer has been estimated to be 160-fold higher than in the general population. Papillary thyroid cancer in the setting of FAP is often detected before age 30, is multifocal, and displays unusual pathological features. The gene responsible for FAP has been mapped to chromosome 5q21 and was designated adenomatous polyposis of the colon (APC). As expected by the autosomal mode of inheritance, APC functions as a tumor suppressor gene and detected mutations associated with the phenotype cause a loss of function of the gene product. The adenomatous polyposis of the colon (APC) gene product, expressed on the membrane of several epithelial cell types, is part of the regulatory β-catenin destruction complex in the Wnt/beta-catenin pathway. The inactivating APC mutation promotes gene transcription leading to cell proliferation. Carney complex type 1 is an autosomal-dominant disease characterized by the following features: myxomas of soft tissues; skin and mucosal pigmentation (blue nevi); schwannomas, tumors of the adrenal and pituitary glands and testicle. A patient is considered to have Carney complex if two major criteria are present or if one major criterion is present and a first-degree relative has Carney complex or an inactivating *PRKAR1A* mutation. Mutations in *PRKAR1A* gene were identified in 73% of cases. This gene encodes the type 1A regulatory subunit of protein kinase A (PKA) and its loss of function leads to enhanced signaling by PKA.

Werner syndrome is an autosomal-recessive disease, associated with mutations of the *WRN* gene on chromosome 8p11-21. It is characterized by premature aging, scleroderma-like skin changes, cataracts, premature graying and/or thinning of scalp hair, short stature, which onset over 10 years of age. Thyroid cancer was observed in 16% of patients and occurred at a younger age.

Nevertheless, 95% of FNMTC is nonsyndromic with less well-defined genetic susceptibility. To date, four susceptibility genes have been identified: *SRGAP1* (12q14), *TITF-1/NKX2.1* (14q13), *FOXE1* (9q22.33), and *HABP2* gene (10q25.3). In particular, the role of SRGAP1 and TITF-1/NKX2.1 remains to be validated in a larger FNMTC cohort, while data supporting the association between *FOXE1* gene variants and FNMTC are not entirely consistent. Finally, the G534E variant of the *HABP2* gene was reported as the underlying genetic defect in large kindred with FNMTC; nevertheless, this postulated role was not confirmed in additional cohorts. Chromosomal loci reported to be associated with nonsyndromic FNMTC include *TCO* (19q13.2), *fPTC/PRN* (1q21), *FTEN* (8p23.1-p22), *NMTC1* (2q21), *MNG1* (14q32), 6q22, 8q24, and 4q32. However, the candidate genes at these genetic loci remain unknown. In summary, though the vast majority of cases of NMTC are sporadic, a few cases present in the setting of recognized hereditary syndromes. Knowledge of these clinical conditions is important to the clinician, in order to perform appropriate screening for associated conditions and to provide genetic counseling to individuals at risk.

Familial Medullary Thyroid Cancer

Approximately 5% of thyroid cancers originate from the parafollicular cell lineage. These cancers are termed medullary thyroid cancer (MTC) and account for approximately 15% of all thyroid cancer-related deaths. In contrast to NMTC, MTC is quite often familial (in approximately one-fourth of the cases). Familial medullary thyroid cancer (FMTC) arises in the setting of multiple endocrine neoplasia (MEN) type II syndromes or as an isolated disease. In MEN IIA (OMIM 171400), FMTC is associated with pheochromocytomas and parathyroid adenomas, and in MEN IIB (OMIM 162300), patients present a marfanoid habitus,

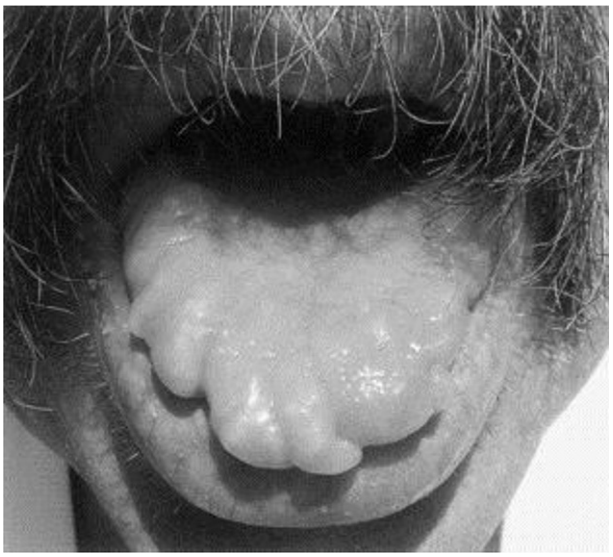

Fig. 1 Typical lingual neuromas in a patient with MEN IIB

mucosal neuromas, and ganglioneuromatosis, in addition to the above-described features (see **Fig. 1**). In isolated FMTC (OMIM 155240), there are no extrathyroidal manifestations. The inheritance of these closely related conditions is autosomal-dominant, with high penetrance. In MEN IIA and MEN IIB, MTC is found in almost 100% of carriers by the end of third decade of life if sought by biochemical screening, whereas pheochromocytomas occur in 50% of carriers and parathyroid tumors in 25% of carriers. Despite these clearly distinct phenotypes, all three conditions are due to germline mutations in the *RET* proto-oncogene sequence. The *RET* proto-oncogene maps to chromosome 10q11.2 and encodes a membrane-bound tyrosine kinase receptor. Specific mutations have been associated with the different subtypes of MEN II (Accardo *et al.*, 2017). Causative mutations detected thus far in MEN IIA are almost entirely limited to a few cysteine residues located in the extracellular domain. These mutations induce critical conformational changes in the gene product, causing constitutive activation of the receptor with ongoing production of intracellular proliferative signals. Interestingly, there is quite a strict genotype–phenotype relationship. Mutations involving codons 609, 611, 618, 620, and 634 are found exclusively in families with MEN IIA or with FMTC and mutations involving noncysteine codons 768, 804, and 891 have been found only in families with FMTC, although even in these families, occasional cases of pheochromocytoma and hyperparathyroidism are found. Since the molecular genetics of the two conditions partially overlap, other genes must be involved in determining the phenotype. In contrast, mutations at codon 918 (M918T) are found in almost all families with MEN IIB. This mutation causes a conformational change in the intracellular tyrosine kinase binding pocket and allows for constitutive kinase activation in the absence of dimerization. The *RET* gene is also mutated in many cases of Hirschsprung's disease, although most mutations detected in this disorder induce the loss of function of the mutant allele. Surprisingly, Hirschsprung's disease has been found in some families with MEN IIA.

Detailed knowledge of the molecular genetics of these aggressive diseases has greatly enhanced the ability of clinicians to detect persons at high risk for MENs. Genetic testing for RET mutations has become widely available for family members of patients and allows early, preventive treatment, which is expected to greatly increase the life expectancy of patients.

See also: Medullary Thyroid Carcinoma. Serum Thyroid Hormone-Binding Proteins. Thyroglobulin. Thyroid Carcinoma. Thyrotoxicosis; Diagnosis. Thyrotoxicosis; Overview of Causes

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Environmental Goitrogens☆

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Glossary

Goiter An abnormally enlarged thyroid gland, detected either by palpation or by ultrasound.

Goitrogen A substance that induces goiter formation.

Introduction

Goiter is a heterogeneous term that refers to diffuse or nodular enlargement of the thyroid gland. Goiters are commonly seen in populations with mild to moderate iodine deficiency. Although iodine deficiency is recognized as the primary driver of goitrogenesis, several other genetic and environmental factors are also known to contribute. Environmental goitrogens are naturally occurring or synthetic substances that interfere with normal thyroid physiology. Most do so by inhibiting the sodium-iodide symporter (NIS), thyroid peroxidase (TPO) activity, or peripheral conversion of thyroxine (T4) into triiodothyronine (T3) by type 1 5′-deiodinase (Gore *et al.*, 2015). Other potential mechanisms of environmental goitrogens include increased thyroid hormone clearance, binding to binding proteins, or transmembrane thyroid hormone transport (Gore *et al.*, 2015).

Types of Goitrogens

Perchlorate

Perchlorate is an inorganic anion used in the manufacturing of rocket propellant, fireworks, airbags, and other explosives. There is extensive evidence of contamination of surface and groundwater with perchlorate, and it has been detected in a variety of food types. Perchlorate has been a historical treatment for Graves disease and continues to be used in the treatment of amiodarone-induced thyrotoxicosis (Gharib *et al.*, 2016). As shown through animal studies, perchlorate competitively inhibits the NIS, potently reducing thyroidal iodide uptake. This leads to decreased thyroid hormone levels and increased thyroid cell hypertrophy, hyperplasia, and thyroid mass (Ting *et al.*, 2006). There appears to be a rebound increase in radioactive iodine uptake (RAIU) following discontinuation of perchlorate exposure, suggesting that the perchlorate-induced NIS inhibition is reversible (Braverman *et al.*, 2005).

Studies of human volunteers have shown that single doses of perchlorate as low as 2.2 mg or repeated administration of doses as low as 5.2 μg/kg/day for a 2-week period reduce iodide uptake in a dose-dependent fashion (Ting *et al.*, 2006; Greer *et al.*, 2002). However, human studies of volunteers given perchlorate and of workers occupationally exposed to perchlorate have not shown changes in thyroid hormone or thyroid-stimulating hormone (TSH) levels at similar levels of perchlorate exposure (Eisenbrand and Gelbke, 2016). Interestingly, Braverman *et al.* (2005) showed that workers in an ammonium perchlorate production plant, who had chronic intermittent exposure to perchlorate, had similar RAIU to non-exposed controls after several shifts at the plant, but increased RAIU and decreased iodine excretion compared to controls after several days off, suggesting that long-term adaptation is present. Furthermore, thyroid volume was not enlarged in exposed workers compared to controls.

Although clinical studies of perchlorate exposure have not consistently shown an impact on thyroid function tests, epidemiological evidence suggests an association between perchlorate exposure and thyroid dysfunction, with decreased T4 and increased TSH (Eisenbrand and Gelbke, 2016). For example, data from the 2001–2002 National Health and Nutrition Examination Survey (NHANES) showed a significant positive association between urinary perchlorate and serum TSH levels in women, and a significant negative association between urinary perchlorate and serum total T4 levels in women with urinary iodine <100 μg/L (Blount *et al.*, 2006). While these results suggest that perchlorate exposure in the population is associated with a rise in TSH and fall in total T4 levels, another NHANES analysis of a slightly different population did not confirm this relationship (Bruce *et al.*, 2013). Epidemiologic studies of pregnant women have had conflicting results about whether urinary perchlorate levels correlate with changes in serum T4 and TSH levels, as have studies comparing perchlorate levels in drinking water with neonatal

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thyroid function testing (Steinmaus, 2016). It has been theorized that NIS may be upregulated in response to exposure to substances inhibiting iodide uptake, which could explain the discrepancy between the clearly documented inhibition of iodide uptake and the lack of change in thyroid function tests in humans.

In 2011, the United States Environmental Protection Agency determined that perchlorate should be regulated as a contaminant, as outlined in the Safe Drinking Water Act (United States Environmental Protection Agency, 2011). However, considerable controversy exists regarding defining a safe level for perchlorate, and determining how much attention should be given to this substance compared to other NIS inhibitors such as thiocyanate and nitrate, as these substances exist at substantially higher environmental levels than perchlorate (Eisenbrand and Gelbke, 2016).

Thiocyanate

Thiocyanate is one of the breakdown products of glycosinolates and cyanide. Glycosinolates are thioglycosides found in cruciferous vegetables of the *Brassica* family, including cabbage, bamboo shoots, kale, sprout, broccoli, brussel sprouts, turnips, and mustard. Cyanide is a metabolite of cyanogenic glucosides found in plants such as cassava, corn, apricot, almond, cherries, lima beans, linseed, bamboo shoots, and sweet potatoes. Cyanide is also found in cigarette smoke, which is thus a major source of thiocyanate. Thiocyanate has also historically been used in the treatment of hypertension. Thiocyanate is frequently found in human urine samples, suggesting its significant absorption from the environment (Corey *et al.*, 2017).

Thiocyanate competitively inhibits NIS, leading to decreased iodide uptake. Thiocyanate also inhibits TPO-mediated organification and appears to increase iodide efflux. The effect on TPO-mediated coupling is less clear (Willemin and Lumen, 2017). Although thiocyanate is a less potent inhibitor of NIS than perchlorate, the substantially higher environmental levels and longer half-life of thiocyanate as compared to perchlorate results in its greater contribution to environmental goitrogenic risk (Corey *et al.*, 2017; Eisenbrand and Gelbke, 2016).

Both animal and human data support the role of thiocyanate in goitrogenesis. Animals fed diets high in glycosinolates can develop goiter in a dose-dependent fashion. This holds true across multiple different species (Eisenbrand and Gelbke, 2016). In humans, several studies have suggested higher rates of goiter in smokers compared to non-smokers (Brix *et al.*, 2000), which is believed to be a consequence of the higher burden of thiocyanate in this population. This effect seems to be reversible, as former smokers have goiter rates similar to that of lifelong non-smokers (Knudsen *et al.*, 2002). The finding of persistent goiter after correction of iodine deficiency has been shown to be positively correlated with higher urinary thiocyanate levels (Marwaha *et al.*, 2003). However, evaluation of NHANES data showed only a modest association between higher urinary thiocyanate levels and lower T4 levels (Steinmaus *et al.*, 2013), and administration of thiocyanate-fortified milk to human volunteers did not have any impact on thyroid function tests (Dahlberg *et al.*, 1984).

Cassava is amongst the most well-known dietary sources of thiocyanate, and is a staple food in many parts of the world, including the Democratic Republic of Congo, where it is thought to contribute to high rates of goiter (Gaitan and Dunn, 1992). In regions of endemic goiter, ingestion of cassava has been shown to reduce iodide uptake compared to ingestion of a control rice meal, while ingestion of cassava in non-endemic regions did not (Delange and Ermans, 1971). This suggests regional variation in the goitrogenic potential of these plants. Different processing methods alter the degree of thiocyanate formation from cassava-containing foods (Mlingi *et al.*, 1996; Gaitan and Dunn, 1992).

Other breakdown products of glycosinolates found in cruciferous vegetables include isothiocyanate and 5 vinyl oxazolidine-2-thione (also known as goitrin). Isothiocyanate can be metabolized in a number of ways leading to thiocyanate or other antithyroidal substances with thiourea-like effects (Gaitan, 1990). Goitrin has also been shown to reduce iodide uptake in the thyroid gland in humans and animals (Felker *et al.*, 2016).

Overall, it is likely that dietary thiocyanate exposure from a typical diet including *Brassica* vegetables is not sufficient to cause significant inhibition of iodide uptake. However, thiocyanate exposure may exacerbate the effect of iodine deficiency, and lead to hypothyroidism in a dose-dependent manner (Felker *et al.*, 2016). This has been illustrated in a case report of an 88-year-old women who presented with myxedema coma after ingesting up to 1.5 kg of raw bok choy per day for several months (Chu and Seltzer, 2010). This patient had no history of thyroid dysfunction, and the presumed cause of her profound hypothyroidism was the antithyroidal effect of prolonged high dose exposure to glycosinolates and their breakdown products in bok choy.

Nitrates

Nitrates are found throughout the environment, occurring in certain vegetables, preservatives, and as contaminant from fertilizers. Nitrates can also be synthesized by humans, animals, and by certain microorganisms. Varying levels of nitrates have been documented in food sources such as lettuce, as well as in drinking water. Epidemiological studies have shown an association between high nitrate levels (>50 mg/L) in drinking water and larger thyroid glands associated with subclinical thyroid disease in children (Eisenbrand and Gelbke, 2016; De Groef *et al.*, 2006). The association between high nitrate levels in drinking water and thyroid hypertrophy has also been found in adults. This relationship appears to hold true especially in the setting of iodine deficiency.

Similar to perchlorate, nitrates inhibit thyroidal iodide uptake by NIS. Although they are much less potent at NIS inhibition than both thiocyanate and perchlorate, their relatively higher environmental concentration conveys a potentially greater risk towards goitrogenesis. Furthermore, while urinary thiocyanate and perchlorate levels have been decreasing over time, urinary

nitrate levels have remained stable. Thus, the relative contribution of nitrates towards environmental goitrogenesis risk is increasing (Corey *et al.*, 2017).

Flavonoids

Flavonoids are phenolic compounds that exist in several forms, and are found in various foods. The most prevalent source of flavonoids in the Western diet is soy. Flavonoids inhibit the activity of TPO, preventing organification and iodotyrosine coupling, and thus decrease thyroid hormone synthesis. They have also been shown to decrease the activity of type 1 deiodinase, inhibiting peripheral metabolism of thyroxine to triiodothyronine. Soybeans contain isoflavones and coumestans, both types of phytoestrogens, which also inhibit the activity of TPO. They are associated with goiter development in animals (Matovinovic, 1983); however, this goiter development appears to be mitigated by increasing iodine intake.

The majority of evidence does not support an association between soy intake and overt thyroid dysfunction in human adults (Eisenbrand and Gelbke, 2016). However, soy does appear to interfere with the absorption of oral levothyroxine used in the treatment of hypothyroidism. Moreover, a retrospective study of hypothyroid infants showed a greater proportion of infants with persistently elevated TSH levels in those fed soy formula versus milk formula. Soy intake in infancy may also be associated with an increased risk of developing autoimmune disease in adolescence (Fort *et al.*, 1990). Infants in general appear more susceptible to the effects of soy, as evidenced by higher serum isoflavone concentrations in infants on soy formula than in adults consuming soy foods. Despite this, there is no evidence to support the development of goiter in soy-fed infants since the introduction of iodine fortification (Messina and Redmond, 2006).

C-glycosylflavones are a different type of flavonoid found in pennisetum millet (pearl millet), a common food item in areas of Asia and Africa, including rural western Sudan. Goiter prevalence has been found to be significantly higher in these rural areas than in nearby urban areas, where rates of borderline iodine deficiency are similar, but dietary millet ingestion is reduced (Gaitan *et al.*, 1995). However, assessment of the effect of pearl millet is complicated by the fact that this mill*et al*so contains thiocyanate, which, as discussed above, can cause NIS inhibition. Vitexin, a type of C-glycosylflavone in millet, has been shown to inhibit coupling and organification of iodine in rodents. This effect may be even more pronounced in humans due to the breakdown of vitexin to a more potent metabolite (Gaitan *et al.*, 1995). As with cassava, different methods of processing the millet may alter its goitrogenic potential. Dehulling the millet leaves behind a flour with a much lower amount of C-glycosylfavones and thiocyanate, but is not a particularly desirable processing technique as it also removes most of the millet's protein content (Gaitan and Dunn, 1992).

Other types of flavonoids found in different types of millet have also been shown to have antithyroidal properties (Sartelet *et al.*, 1996). As a final example of the potential impact of flavonoids on the thyroid, catechins, a type of flavonoid found in green tea, have been shown in animal studies to significantly decrease levels of T3 and T4 and increase levels of TSH. Histologic examination of rats injected intraperitoneally with catechins revealed thyroid follicular hypertrophy and hyperplasia (Chandra and De, 2013). Human data on catechins is not yet available.

Resorcinol

Resorcinol is a phenolic chemical found in dermatologic medications, used in several industrial applications (e.g. photography, tanning, tire manufacturing), and found in roasted barley, canned molasses, adhesives, hair dyes, and cosmetics, among other products. Coal and shale deposits lead to higher levels of resorcinol in drinking water.

Resorcinol is thought to inhibit TPO activity. As reviewed by Lynch *et al.* (2002), animal studies demonstrate conflicting impact of resorcinol on thyroid weight and function. Some studies support the goitrogenic effect of resorcinol, but only when exposed to persistently high levels, either through daily oral exposure, subcutaneous administration in oil (but not aqueous solutions), or very high-dose twice-daily dermal application.

Studies of humans with occupational exposure to resorcinol have not shown any impact on thyroid function. Epidemiologic support for the goitrogenic effect of resorcinol in humans comes from data showing higher resorcinol levels in drinking water in areas with a high prevalence of goiter as compared to nearby areas with lower prevalence of goiter but similar urinary iodine levels. However, this is confounded by several other differences in the study populations and their drinking water, precluding any firm conclusions.

There are several case reports of hypothyroidism and/or goiter resulting from extremely high-dose (>30 mg/kg/day) long-term topical application of resorcinol-containing medications to extensive areas of the body, especially when applied to areas with open sores or ulceration. Goiter and hypothyroidism resolved upon discontinuation of resorcinol in these cases. Reassuringly, standard dose application of resorcinol-containing medications to intact skin does not appear to have an impact on thyroid function (Lynch *et al.*, 2002).

Phthalates

Phthalates are chemicals used as plasticizers and in adhesives, solvents, cosmetics, and other products. The mechanism by which phthalates interfere with thyroid function is not entirely understood. Animal studies have shown enhancement of NIS expression and iodide uptake but antagonism at the thyroid receptor (Gore *et al.*, 2015).

Both animal and human epidemiologic data support the hypothesis that phthalates lead to reductions in thyroid hormone levels (Eisenbrand and Gelbke, 2016). Several studies have also shown a positive association between urinary phthalates and serum TSH, although the overall strength of this association is weaker than that between urinary phthalates and serum thyroid hormone levels (Gore *et al.*, 2015; Dirtu *et al.*, 2013; Wu *et al.*, 2013). As with resorcinol, phthalates are among the many chemicals found to be present at higher levels in drinking water consumed in areas with higher goiter prevalence (Lynch *et al.*, 2002).

Fluoride

Many countries have environmental sources of excess fluoride, such as from drinking water, or household and agricultural agents. Fluoride exposure is associated with dysfunction of several organ systems. The mechanism by which fluoride influences the thyroid gland is currently unclear. It may relate to reduction in thyroid hormone synthesis, altered hormone transport, or decreased peripheral conversion of T4 into T3 (Barberio *et al.*, 2017). Animal models suggest that the goitrogenic effect of fluoride may be mediated through increased levels of nitric oxide and vascular endothelial growth factor (VEGF), as increased deposition of VEGF in thyroid tissue has been demonstrated in response to fluoride intake (Liu *et al.*, 2012).

Animal models support the hypothesis that high doses of fluoride can impair thyroid function and are associated with reduced levels of T3, T4 and TPO. However, human data has been mixed. Some, but not all, epidemiologic studies show a positive association between higher fluoride concentration and serum TSH levels. Larger population studies from England and Canada have yielded conflicting results about a potential association between higher fluoride levels and prevalence of overt hypothyroidism (Barberio *et al.*, 2017; Peckham *et al.*, 2015). More studies are needed to better understand the impact of fluoride on thyroid function.

Calcium

Excess calcium, as can be found in hard water, has also been postulated to contribute to goitrogenesis. Animal studies show that high levels of administered calcium can lead to an increase in thyroid weight, hypertrophy and/or hyperplasia of thyroid follicular cells, and higher T4 and TSH levels but lower T3 levels. This is accompanied by assays demonstrating inhibition of TPO activity, increase of thyroidal Na^+/K^+-ATPase activity, and reduced type 1 deiodinase activity (Chandra *et al.*, 2012). In humans, several studies have suggested an association between the presence of hard water and an increased prevalence of goiter (Chandra *et al.*, 2016). Calcium is also known to interfere with the absorption of oral levothyroxine, and patients should be counseled to take their thyroid pills several hours apart from any calcium-containing products (Garber *et al.*, 2012).

Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are industrial chemicals previously used for a wide variety of reasons, such as the synthesis of plasticizers, carbonless copy paper, adhesives, paints and inks. Despite being banned since 1979 due to teratogenic and carcinogenic concerns, these chemicals remain measurable in the environment, including in fish, animals, and humans. PCBs have been suggested to interact with the thyroid hormone receptor, alter binding to transport proteins, and affect hepatic clearance of thyroid hormone (Miller *et al.*, 2009).

Several animal studies suggest that PCBs have a goitrogenic effect. For example, salmon exposed to PCBs in their environment were observed to have higher rates of goiter than those that were not exposed (Brucker-Davis, 1998), and rats experimentally exposed to PCBs also demonstrated goitrogenesis. However, there does not appear to be a link between PCBs and goitrogenesis or thyroid dysfunction in humans, even when exposed to potentially lethal levels (Matovinovic, 1983).

Cadmium

Cadmium is a heavy metal found in batteries, dye pigment, and used in manufacturing plastic. Human exposure is through tobacco and contaminated food and water, or occupational exposure during cadmium processing. Cadmium can accumulate within organs, including the thyroid gland. Animal studies have shown that cadmium can lead to goitrogenesis and follicular hyperplasia, inhibition of TSH secretion, and reduction in serum T4 levels; the effect of cadmium on T3 levels varies between studies. C-cell hyperplasia has also been reported. Human data regarding the impact of cadmium on thyroid function and goitrogenesis is extremely limited (Jancic and Stosic, 2014).

Bisphenol A

Bisphenol A (BPA) is a synthetic compound found in many products, including plastic toys, food packaging, and in the lining of canned foods and beverages (Gore *et al.*, 2015). It is a common contaminant in food and water, and nearly all Americans have detectable levels of BPA in their urine. Animal studies suggest that BPA may have both agonistic and antagonistic effects on the thyroid hormone receptor (Gore *et al.*, 2015). Human epidemiologic studies have shown conflicting associations between BPA and T4 levels (Gore *et al.*, 2015), but a neutral or inverse association between BPA and serum TSH levels (Gore *et al.*, 2015; Meeker and

Ferguson, 2011; Meeker *et al.*, 2010). BPA is among the list of endocrine-disrupting chemicals believed to be associated with obesity. One proposed mechanism of this link between BPA and obesity is through BPA-induced thyroid dysfunction (Gore *et al.*, 2015). Overall, there is no convincing data in humans to suggest that BPA has a goitrogenic effect.

Other

A number of other synthetic substances have been identified, which may interfere with various steps in thyroid hormone synthesis, transport, and metabolism (Howdeshell, 2002). Polybrominated diphenyl ethers (PBDEs) are one example. PBDEs are used as flame retardants, and have been shown in animal studies to alter binding of thyroid hormone to its receptor and to be associated with decreased T4 levels. However, the effect of PBDEs on TSH has been inconsistent and human data is lacking (Gore *et al.*, 2015). Triclosan and paraben have been inconsistently associated with higher thyroid hormone and/or lower TSH levels, but generally not with overt hypothyroidism or goitrogenesis (Eisenbrand and Gelbke, 2016). Overall, the interaction of these substances with the thyroid hormone receptor is thought to be complex, and the in vivo effects of these substances is not yet clear (Gore *et al.*, 2015).

Summary

Many natural and synthetic environmental factors have been found to be associated with disruption of normal thyroid physiology and thus potentially the development of goiter, often in the setting of iodine deficiency. Perchlorate, thiocyanate, nitrate, and flavonoids are the most well-studied, but much remains to be discovered regarding their clinical impact on thyroid disease. Infants and iodine-deficient individuals appear to be most sensitive to the exposure to goitrogens. However, direct causation is often difficult to establish between various substances and goiter formation, given the ethical limitation of performing randomized controlled trials using potentially toxic substances in humans. Thus, the bulk of available literature comes from animal studies, which must be interpreted with caution given important differences in thyroid physiology between animals and humans, and epidemiologic data, which is often subject to confounding and bias. Furthermore, attempts to look at the simultaneous effect of multiple environmental agents is complicated by their potential interactions with one another. For example, higher perchlorate intake may lead to increased iodine excretion, thus masking concomitant iodine deficiency. Safety predictions based on assessment of potential goitrogen levels in food and water often conflict with associations drawn from exposures estimated from urinary levels. Moreover, identifying a specific environmental exposure as the root cause of goiter development in an individual patient remains challenging, except perhaps in cases of exposure to extraordinarily high levels of a known environmental goitrogen. Given the growing number of chemicals we are exposed to in modern society, it is important to improve our understanding of the impact of various environmental exposures on our health, including their effects on goitrogenesis and thyroid functioning. Future studies should focus on elucidating the source, mechanism of action, and endocrine effects of environmental goitrogens; defining a safe level of exposure to these substances will be crucial.

See also: Smoking and the Thyroid. Thyroid Carcinoma. Nontoxic Goiter

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Iodine Deficiency☆

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Glossary

Endemic goiter Thyroid enlargement due to iodine deficiency in a community.

Iodized salt The addition of iodine to dietary salt to correct iodine deficiency.

Urinary iodine The concentration of iodine in urine, the recommended measure for monitoring iodine nutrition.

Optimal Iodine Nutrition

Need for Iodine

Iodine is an integral part of the thyroid hormones, thyroxine (T4) and 3,5,3′-triiodothyronine (T3) its importance in thyroidology has been established for over a century. Thyroid hormones are essential for health making optimal iodine nutrition a key determinant of health in the individual and the population. Iodine was first identified in the thyroid in 1895 (Baumann, 1896) and in 1917 it was demonstrated that goiters were caused by iodine deficiency and could be prevented by iodine supplementation (Marine and Kimball, 1917).

The effects of iodine deficiency are translated through inadequate thyroid hormone production. Most ingested iodine is broken down into iodide (I^-) in the stomach and duodenum and transferred across the gut mucosa into the bloodstream. In healthy individuals the absorption of iodide can be as high as 90%. Following absorption, it is either excreted via the kidney or concentrated by the thyroid, where a sodium/iodide symporter transports it as iodide into the thyroid gland (**Fig. 1**).

The concentration gradient varies with the availability of iodide and other factors. Under conditions of iodine sufficiency, approximately 10%–20% of a radioactive iodine dose is retained by the thyroid 24 h after administration.

Once in the thyroid, iodide migrates to the apical surface of the cell, where a complex series of reactions oxidizes and attaches it to tyrosyl residues of thyroglobulin, a large glycoprotein (molecular size 660 kDa) that is synthesized on the endoplasmic reticulum of the thyrocyte, glycosylated in the Golgi, and processed by molecular chaperones. Key factors in the iodination of thyroglobulin include thyroperoxidase, a 103 kDa protein synthesized by the thyroid, hydrogen peroxide, the production of which requires both calcium and NADPH, and NADPH oxidase. Further action of the thyroperoxidase results in a coupling of two iodinated tyrosyls to form thyroxine. At this point, the thyroglobulin molecule is mature and contains the inactive precursors diiodotyrosine and monoiodotyrosine and the thyroid hormones thyroxine and triiodothyronine. Approximately one-third of thyroglobulin's iodine is in one or two residues of thyroid hormone.

Thyroglobulin is stored in the lumen of the thyroid follicles, where it makes up the bulk of the colloid. For retrieval, it reenters the thyroid cell, where endosomal and lysosomal proteases break it down and release free T4 and T3 into the circulation. The remaining iodine in thyroglobulin, from diiodotyrosine and monoiodotyrosine, is removed by a deiodinase and returned to the thyroid pool for recycling. This is an important mechanism for iodine conservation.

Iodine insufficiency results in substantial adaptation by the thyroid in response to increased secretion of TSH. Higher TSH results in increased plasma iodide clearance through NIS expression. As a result, there is reduced renal iodide excretion. TSH also stimulates the breakdown of thyroglobulin and results into preferential secretion of T3. If iodine deficiency cannot be compensated for by these approaches, many individuals will then develop a goiter.

Amounts of Iodine Required

Experimental and clinical observations have produced recommendations for daily iodine intake. These include iodine turnover and thyroidal radioiodine uptake studies. They are also based on calculations of daily thyroid hormone production and metabolism in balance studies, the amount of thyroxine required for adequate replacement in subjects who are athyreotic, and metabolic disposal rates. The estimated average requirement (EAR) of iodine in men and nonpregnant or lactating women has been set at 95 μg/day. The corresponding recommended daily allowance (RDA) (defined as the EAR plus twice the coefficient of variation in the population, rounded to the nearest 50 μg) is 150 μg/day. **Table 1** shows recommendations by the Food and Nutrition Board of the U.S. National Academy of Sciences for daily iodine intake. The World Health Organization, the Iodine Global Network (IGN) and UNICEF have recommended almost identical values. Setting an upper limit for iodine ingestion has been more difficult. The Food and Nutrition Board recommended 1100 μg/day as a safe upper dose for adults.

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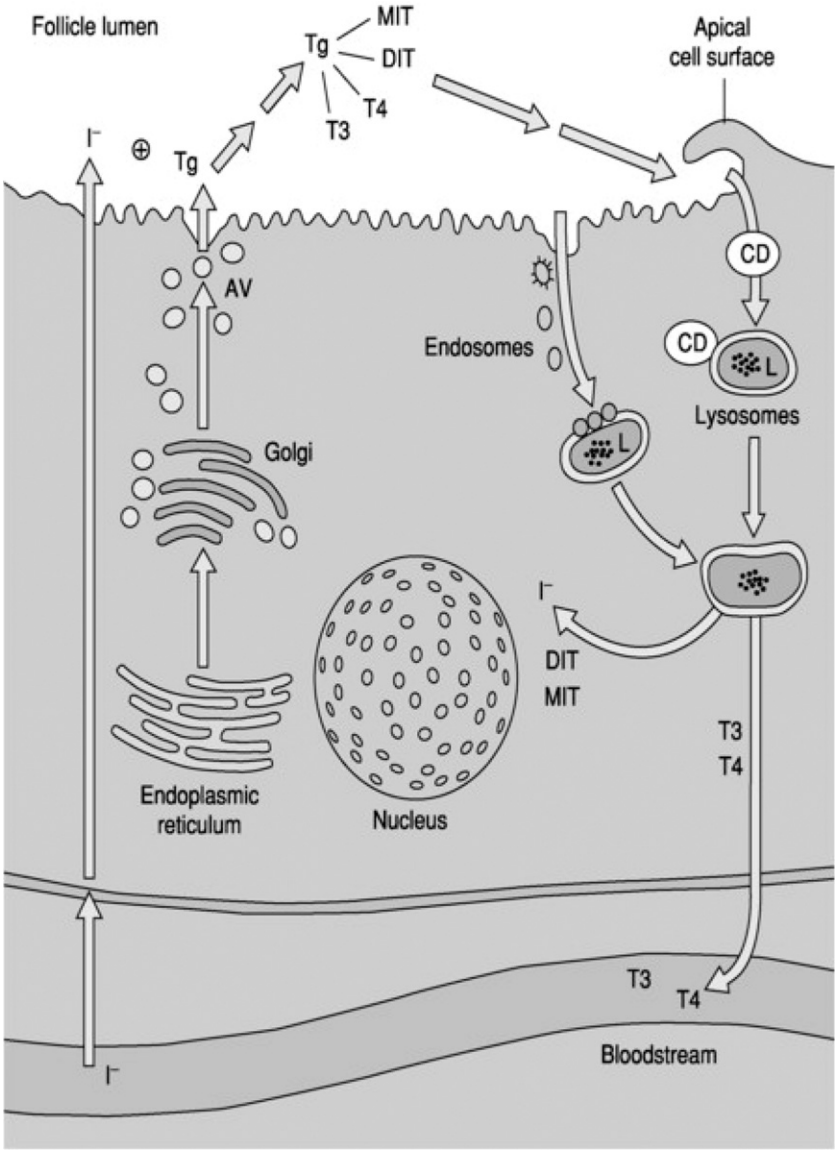

Fig. 1 Diagram of a thyroid cell to show thyroid hormone synthesis and release. *AV*, apical villi; *CD*, colloid droplets; *DIT*, diiodotyrosine; *L*, lysosomal proteases; *MIT*, iodotyrosine; T3, 3,5,3′-triiodothyronine; *T4*, thyroxine. Reprinted from Dunn, J. T. and Dunn, A. D. (2002). Thyroid physiology. In Besser, C. M. and Thorner, M. O. eds. *Comprehensive Clinical Endocrinology*, 3rd edn., p. 132, Copyright 2002, with permission from Elsevier.

Most people can tolerate much higher doses without apparent ill effects. Large amounts of iodine inhibit the thyroid's ability to make thyroid hormone, producing goiter and hypothyroidism, but many people can tolerate several milligrams per day. People with autoimmune thyroid diseases, such as Graves' disease and Hashimoto's disease, and many of their relatives are more sensitive to iodine excess.

Assessment of Iodine Nutrition

In practice there are four methods for assessing iodine nutrition in populations: urinary iodine concentration the goiter rate, serum TSH and serum Tg levels. To an extent they reflect different periods of iodine deficiency. Urinary iodine is a sensitive indicator of recent iodine intake (days) whereas Tg shows an intermediate response (weeks to months), while changes in the goiter rate reflects much longer term iodine nutrition (months to years).

Urinary Iodine Concentration

Over 90% of ingested iodine eventually appears in the urine. Thus, in the steady state, the urinary iodine excretion is a good marker of iodine nutrition. For epidemiological purposes, the concentration itself, in micrograms per liter, is usually satisfactory

Table 1 Recommendations on dietary intakes of iodine

	RDA (μg/day)	*AI (μg/day)*	*UL (μg/day)*
Adult men	150		1100
Adult women	150		1100
Pregnancy	220		1100
Lactation	250		1100
Children			
1–3 years	90		200
4–8 years	90		300
9–13 years	120		600
14–18 years			900
Infants			
0–6 months		110	
7–12 months		130	

Iodine requirements during pregnancy, lactation and the neonatal period and indicators of optimal iodine nutrition (Delange, 2007).

Note. RDA, Recommended daily allowance; *AI*, adequate intake (estimated when insufficient data for RDA; values set high to avoid possible insufficiency); *UL*, tolerable upper limit (infants not included; insufficient data).

for gauging the nutritional status of a community. Changes in hydration affect the urinary iodine concentration in individuals but these tend to smooth out in the population median. Occasionally, urinary iodine is expressed as micrograms of iodine per gram of creatinine, but other nutritional abnormalities, such as low protein intake, influence creatinine excretion and the concentration alone is satisfactory and simpler.

The urinary iodine concentration is the single most useful test in assessing populations. **Table 2** relates median urinary iodine concentration to different degrees of iodine nutrition; for deficiency it also presents an index of its severity and, therefore, the priority for corrective action. Urinary iodine concentration also detects excess as well as deficiency. However UIC does not represent an individual's iodine status and at least 10 spot samples are needed to reliably identify an individual's iodine status.

Thyroid Size

Iodine deficiency diminishes thyroid hormone production. The pituitary responds with increased TSH secretion, causing the thyroid to increase hormone production, and the gland enlarges as part of this adaptive response. The degree of enlargement, called "goiter," is proportional to the severity of the iodine deficiency and can be assessed by palpation, classifying it as "no enlargement," "palpable goiter," or "visible goiter," the latter two constituting the "total goiter rate." Thyroid enlargement is one of the earliest and most apparent features of iodine deficiency and palpation is a simple technique that requires some skill but no special instruments. Rapid surveys can be carried out in schools. When the iodine deficiency is severe, such surveys provide a quick and satisfactory basis for recognizing the presence of iodine deficiency and the need for its correction. This was the most common form of assessment for much of the 20th century. However, as iodine nutrition has improved, the degree of thyroid enlargement in such surveys has become more subtle and the results have become correspondingly less accurate.

Ultrasonography provides a quantitative and reproducible assessment of thyroid size. The technique is simple. Portable instruments can easily be used in the field and require only several minutes for each measurement. Normative data exist for iodine-sufficient individuals, related both to age and to body surface area, and can be used for comparison.

TSH

Serum TSH rises with iodine deficiency and the threat of hypothyroidism. In populations the median serum TSH increases, but frequently remains within the normal range of the TSH assay, unless the iodine deficiency is quite severe. For this reason, the TSH is not a very sensitive indicator of iodine deficiency in the general population, but its use in newborns is an exception. Most developed countries have neonatal screening for early detection and treatment of congenital hypothyroidism, which occurs in approximately 1 in 3000 births in iodine-sufficient populations. Iodine deficiency causes transient neonatal hypothyroidism and a resulting rise in TSH, so the incidence of transient hypothyroidism, as detected on neonatal screening, increases and correlates with the degree of deficiency in the community. Introducing neonatal screening only to detect iodine deficiency is not cost-effective, but if this program is already in place, valuable information about iodine nutrition can be obtained.

Table 2 The median urinary iodine concentration as an index of community iodine nutrition

Median urinary iodine concentration (μg/L)	*Corresponding approximate iodine intake (μg/day)*	*Iodine nutrition*
<20	<30	Severe deficiency
20–49	30–74	Moderate deficiency
50–99	75–149	Mild deficiency
100–199	150–299	Optimal
200–299	300–449	More than adequate
>299	>449	Possible excess

Serum Thyroglobulin

The normal thyroid secretes small amounts of thyroglobulin into the bloodstream, detectable by serum assays. Increased thyroid activity from any cause (including iodine deficiency) increases serum thyroglobulin and this correlates well with the degree of iodine deficiency. Urinary iodine is a more practical measure for iodine nutrition, but the serum thyroglobulin is worth considering if blood if being obtained for other purposes.

Serum Thyroid Hormone Levels

Iodine deficiency causes the median serum T4 to decrease and the serum T3 to increase. However, these changes may be subtle and the altered values may still be within the normal range. In practice, these have not been cost-effective measures to assess population iodine deficiency.

Radioactive Iodine Uptake

Iodine deficiency increases the thyroid's uptake of iodine and this can be demonstrated by administering a tracer dose of radioactive iodine (^{131}I or ^{123}I). The test is cumbersome, particularly in the field, and is not recommended for assessing the iodine nutrition of a population.

Survey Technique

School-age children are a convenient group to study, because they are easy to assemble and reflect recent rather than remote iodine nutrition in a community. The study sample must be representative, take into account the degree of homogeneity within the population being sampled, and address school attendance, rural location, and socioeconomic status. Failure to consider these factors may lead to an unrepresentative survey that does not sufficiently assess the poorer, remote rural areas that are likely to harbor the worst iodine deficiency. The most important sample is the urinary iodine. The most spartan surveys can simply collect urine samples and transfer them to a central laboratory. More extensive data may include an estimate of thyroid size, particularly if obtainable by ultrasonography. Many countries select a few sentinel sites and monitor them periodically to detect trends. Older female school children are particularly useful to study as they reflect likely iodine status of women who may potentially become pregnant and has revealed important iodine deficiency in the United Kingdom (Vanderpump *et al.*, 2011).

Overview of Iodine Deficiency

Consequences of Iodine Deficiency

Thyroid hormone, and therefore iodine, is essential for health. The thyroid hormones modulate many chemical reactions in the body and are necessary for development of the brain and proper growth. Failure to have adequate iodine causes goiter, hypothyroidism, cretinism, reproductive failure, decreased child survival, iodine-induced hyperthyroidism, and socioeconomic deprivation. Whilst it is well established that correction of severe iodine deficiency has clear benefit, there is also emerging evidence that correction of mild-moderate iodine deficiency has modest benefits (Taylor *et al.*, 2013) although a recent large randomized blinded controlled trial failed to establish clear benefits on obstetric and offspring cognitive outcomes (Gowachirapant *et al.*, 2017).

Goiter

Thyroid enlargement is an early and easily recognized feature of iodine deficiency. It results from increased TSH stimulation. It can be regarded as an adaptation, and if the iodine deficiency is mild, further damage does not necessarily follow. Goiter is not the worst result of iodine deficiency, but it is not inconsequential. The enlarged thyroid may compress adjacent structures, requiring eventual surgery or other treatment. Prolonged thyroid hyperplasia causes thyroid nodules (lumps), which may occasionally be malignant or become autonomous and overproduce thyroid hormone. This is a particular problem in individuals who have been exposed to chronic iodine insufficiency.

Damaged Reproduction

This is the most severe consequence of iodine deficiency. Its spectrum begins with infertility and includes complications of pregnancy and neonatal death. The need for iodine increases in pregnancy to supply the fetus and also because maternal renal losses are greater. The iodine-deficient pregnant woman may experience gestational hypertension, abnormal fetal presentation, stillbirths, and goiter.

Her offspring faces additional severe risks, with death being the worst. Several epidemiological studies have shown dramatic improvement in child survival with correction of iodine deficiency. For example, child mortality decreased by 50% after iodization of irrigation water in China. Other studies found that child survival increased when iodine was administered during pregnancy and during the first 6 months of life. An extensive literature shows similar results in animals, where stillbirths and abnormal progeny are a common consequence of iodine deficiency.

The developing brain needs thyroid hormone for proper maturation. It is particularly crucial in the first trimester. Iodine through thyroid hormone is necessary for the myelination, neuronal differentiation and migration as well as synaptogenesis.

Failure to have adequate thyroid hormone during this critical period, which extends from the first trimester until age 2 can result in permanent mental retardation. In fact, iodine deficiency is the most common cause of preventable mental impairment.

The extent of the brain damage varies over a wide spectrum. Occasionally, it is subtle, requiring specific testing to show impairment. At the other end is cretinism, characterized by severe mental retardation, various other neuromuscular abnormalities, short stature, and deaf–mutism. The incidence of cretinism in severely iodine-deficient areas is uncertain, because many die early or are secluded by defensive family members. Some severely deficient villages have reported incidences of $>10\%$ and in such communities the probability is great that most of the population has some degree of impairment.

Data from two large randomized controlled trials of correcting low thyroid function identified after 12 weeks during pregnancy (Lazarus *et al.*, 2012; Casey *et al.*, 2017) failed to show clear benefits on offspring IQ which further indicate that early or preconception iodine correction is essential.

Iodine-Induced Hyperthyroidism

Iodine-induced hyperthyroidism, (Jod-Basedow phenomenon), can occur in individuals receiving iodine supplementation or from sudden high exposure to iodine for example, radiographic contrast. It is most common in elderly individuals with long standing nodular goiter exposed to chronic iodine deficiency undergoing iodine supplementation. Iodization programs temporarily increase the risk of iodine induced hyperthyroidism; particular in the elderly.

Fortunately, this trend is usually short-lived and the individual can be adequately treated by the usual techniques available for hyperthyroidism from other causes. Although this complication is regrettable, its damage to the community is mild compared to that from continuing iodine deficiency and iodine-induced hyperthyroidism should not be used as an argument for preventing the correction of iodine deficiency. This complication occurs because of preceding iodine deficiency, so it is reasonable to regard it as another of the iodine deficiency disorders.

Interestingly effects may vary between countries when correcting mild-to-moderate iodine deficiency. In Denmark, they observed an increase in rates of hypo and hyperthyroidism. In China increases were observed in the incidence of subclinical hypothyroidism and autoimmune thyroiditis, but not in overt hypo- or hyperthyroidism.

Socioeconomic Damage

Iodine deficiency not only hurts the individual, but also the community in which he or she lives. Their domestic animals risk similar complications from iodine deficiency and they have more stillbirths and produce fewer eggs, less meat, and less wool. Correction of iodine deficiency in a community can result in dramatic improvement in work performance, per capita income, and school performance. For example, one metaanalysis concluded that an average of 13.5 IQ points were lost from moderately severe iodine-deficient communities when compared with iodine-sufficient peers.

Means for Correcting Deficiency

Vehicles available for delivering iodine to deficient communities include salt, vegetable oil, water, tablets, drops, and occasionally others. The following features are important in selecting a vehicle: (Baumann, 1896) provision of physiological amounts of iodine at regular, preferably daily, intervals; (Marine and Kimball, 1917) easy distribution; (Delange, 2007) affordable cost; (Vanderpump *et al.*, 2011) acceptability to target population; and (Taylor *et al.*, 2013) safety. In principle, the problem is straightforward: the population is deficient, so iodine must be provided; the critical issue is to see that the prescribed iodine reaches the target correctly.

Iodized Salt

Iodization of salt has is the most common strategy for supplementing a population's iodine intake and has several advantages over other fortification vehicles. Almost uniquely salt is consumed by almost everyone consistently throughout the year. Iodine fortification of salt is inexpensive, easy to monitor and does not affect the taste. An additional benefit is that salt manufacturing in a country is usually limited to a few countries allowing easy implementation at scale.

Salt should be fortified with an iodine concentration depending on a regions salt intake. A level of between 20–40 mg iodine/kg is usually recommended. Iodine can be added to the salt as either potassium iodide or as potassium iodate. Iodate is more resistant to humidity and salt impurities and is therefore the preferred choice in tropical countries and in those with salt of poorer quality. Effective storage is also essential as substantial losses (up to 90%) can occur if porous packing is used.

Iodization of salt can be utilized throughout the food chain (universal salt iodization). This is the ideal strategy for correcting iodine deficiency however certain challenges need overcoming. Key challenges include overcoming reluctance from industry and lack of ongoing political support.

Success in implementing the use of iodized salt varies tremendously. Countries that import all their salt can achieve effective iodization by enforcing strict quality control at the borders or at designated processing plants. This situation has made it possible for a number of countries, for example, Nigeria and Zimbabwe, to achieve universal use of iodized salt fairly quickly. Other countries may have numerous salt deposits scattered throughout their territories and introducing iodization into the local salt trade is a much more daunting task.

Constant vigilance and political will is necessary even in high income countries retail outlet surveys conducted in the United Kingdom demonstrated that most commercial salt brands lacked adequate iodine (Bath *et al.*, 2013; Lazarus and Smyth, 2008). Another issue is that successful public health campaigns have aimed at preventing cardiovascular disease through reduced salt consumption. To address this WHO forum has indicated that strategies to reduce salt intake and increase iodine fortification are not contradictory (George Institute for Global Health, 2013).

Iodized Oil

Iodinated vegetable oils have been used as X-ray contrast materials for over 70 years. The most common of these, Lipiodol, has 480 mg of iodine per milliliter. Its first use for prophylaxis in iodine deficiency, in the 1950s, proved dramatically effective. For the next 20 years, its administration was principally by intramuscular injection, with a single dose providing adequate coverage for several years. Subsequently, oral use has been more popular. Capsules containing 200 mg iodine, one or two capsules per administration, provide satisfactory coverage for approximately 1 year. Iodized oil has the advantage of bringing iodine supplementation instantly to the subject on administration and avoids the complexities of altering commercial patterns of salt. Its greatest use has been for women and young children with moderate to severe deficiency, to buy time while awaiting the implementation of effectively iodized salt. The disadvantages are that iodized oil requires direct contact with each person, is more expensive than iodized salt, and provides uneven amounts of iodine over the duration of its effects, with a large amount being lost in the first few days. Well over 50 million people have received iodized oil and it remains a useful alternative under specified conditions.

Iodized Water

Water, like salt, is necessary for all humans, regardless of geographic or socioeconomic situation. As in salt, the addition of iodine to water can provide a constant physiologic dose.

Several approaches have been used. One of the simplest is to add a concentrated iodine solution to vessels containing drinking water. A program using this approach has been used in schools and homes in northern Thailand for several decades. Addition of iodine to running drinking water has been used in several countries (e.g., Italy, Malaysia, Thailand) by diverting some water through a bed containing iodine as crystals or on a solid support and then reintroducing it to the main stream. Another approach uses diffusers, commercially available polypropylene baskets containing iodide that is slowly released into a water source, such as wells. Finally, an innovative system in western China periodically drips potassium iodate into irrigation water. Follow-up studies have shown impressive benefits to people and domestic animals, at a cost of approximately US $0.02 per person per year.

Molecular iodine (I_2) has powerful bactericidal properties and is frequently used for water purification. Many communities needing water purification are also iodine deficient, so a system delivering molecular iodine can solve two problems at the same time.

Iodization of Milk

This traditionally has been an adventitious source of iodine which occurs due to the use of iodophors in the dairy industry for cleaning milk cans and teats rather than through deliberate fortification. This is likely to be a declining source of iodine for two reasons; school children in particular are drinking less milk, and the increasingly widespread of organic milk which contains less iodine.

Iodine Tablets and Solutions

Tablets containing potassium iodide are occasionally used for iodine supplementation. They can be taken daily at 100 or 200 μg, weekly at 1 mg iodine, or at less frequent intervals up to several months, in correspondingly larger doses. The aim is to provide approximately 150 μg iodine/day. The smaller doses may be used as supplements in special circumstances, such as pregnancy, and are also frequently incorporated into antenatal vitamin and mineral preparations. However the iodine content of prenatal multivitamins is very variable (Bath *et al.*, 2014) and many do not contain less than 150 mcg.

Some countries, such as the United States, stockpile tablets containing 65 or 130 mg potassium iodide to block thyroidal uptake of radioactive iodine in case of a nuclear event, but these amounts are far too excessive for routine prophylaxis against iodine deficiency.

Even remote health posts in developing countries frequently have iodine solutions for topical antisepsis. These can be appropriately diluted and used for iodine supplementation, again aiming at approximately 150 μg iodine/day. Such programs can be quite effective, but require responsible oversight to see that the correct dose is actually taken.

Choice of Iodine Vehicle

Iodized salt is by far the favored vehicle for most situations. It does not require individual contact, is technologically simple, is low in cost, and provides a constant daily supplement. However, implementation of an effective iodized salt program is not always easy. In some countries, it requires a massive reorganization of the salt trade, so that an iodization step can be reasonably interpolated. For countries that import salt or have only a few large producers, introducing iodization is usually straightforward. Other countries may have many small salt farmers scattered over a large area who harvest the product simply and sell it locally. Some progress has been made by iodizing their product through cooperatives and other methods, but with only partial success.

When iodized salt cannot be effectively introduced within a reasonable time, other methods need to be recruited. Oral iodized oil can be distributed rapidly through community health systems and provides a stopgap while awaiting salt iodization. Iodized water should be considered, depending on water sources and appropriate supervision. Iodized tablets and solutions are effective for individuals, but require careful administration.

More recently bread has been used as iodine fortification, this is an attractive target as bread is widely consumed with similar intakes throughout the year. This approach has been introduced successfully in Australia, Denmark, and New Zealand (Seal *et al.*, 2007; Clifton *et al.*, 2013; Skeaff and Lonsdale-Cooper, 2013). However follow on studies in New Zealand indicate that whilst effective it is still not as good as universal salt iodization (Edmonds *et al.*, 2016).

Many other vehicles have been occasionally used and may have a niche in certain locales. Examples are brick tea, sugar, candy, fried bananas, and fish sauce. For each, one must ask whether its iodization will reach the right part of the population, especially the poor, women, and children, and in the right amounts. A possible danger is that several different forms of iodine supplementation may converge in an individual and produce iodine excess.

Iodine Supplementation Strategies for Pregnancy and Lactation

Women of child-bearing age are a key target for iodine supplementation programs. The correction of iodine deficiency in pregnancy results is in reduced infant mortality and reduced incidence of cretinism and is economically advantageous. Iodine requirement are increased by around 50% in pregnancy due increased renal losses and the need to maintain FT4 levels. As a result iodine deficiency can occur in pregnancy, despite iodine sufficiency in the general population. Universal salt iodization remains the best strategy for ensuring iodine sufficiency in pregnancy. Key guidelines are shown below in **Table 3**. It is also intriguing that both European and American Thyroid associations recommend 150 mcg daily for iodine supplements, even though the United States is more iodine sufficient than most of Europe. Patient education is key and planning prior to pregnancy may be essential as iodine supplementation may need to be undertaken well in advance of pregnancy for maximum benefit. Observational data suggests that consumption of iodized salt may need to be undertaken 2 years prior to pregnancy (Moleti *et al.*, 2008).

Iodine Deficiency Disorders Control Programs

Background

Programs for controlling iodine deficiency are organized by national governments or Health Ministries, as they relate to the public health of a country or region, and are best underpinned by government statute. This provides these programs with a legal

Table 3 Summary of key guidelines for iodine supplementation in pregnancy

Guideline	*Guidance*
American Thyroid Association Stagnaro-Green *et al.* (2011)	To achieve a total of 250 mcg iodine ingestion daily in North America all women who are planning to be pregnant or are pregnant or breastfeeding should supplement their diet with a daily oral supplement containing 150 mcg of iodine
Endocrine Society De Groot *et al.* (2012)	Preconception and during pregnancy and breastfeeding, women should increase their daily iodine intake to 250 mcg. Iodine intake during pregnancy and breastfeeding should not exceed 500 mcg/day
European Thyroid Association Lazarus *et al.* (2014)	Women who are pregnant, lactating, or planning a pregnancy should ingest daily supplements containing 150 mcg iodine
WHO Andersson *et al.* (2007)	Women who are pregnant or breastfeeding take a daily oral iodine supplement so that the total daily intake is 250 mcg

framework, a sense of direction and the organizational capability that is required for such a task. Furthermore, governments have the ability to enforce mandatory legislation, ensuring their sustainability in the long term. However, national or regional governments need to be convinced of the need for controlling iodine deficiency, by individual experts and local or international "expert" organizations (e.g., WHO, IGN—previously ICCIDD—and UNICEF). Although governments take a lead role, the task of organizing successful programs requires a coordinated effort between many governmental and nongovernmental agencies for example, food and salt industries, education and health authorities. The involvement of the private sector has been a feature in many countries with successful programs.

Components of An Iodine Control Program

The WHO in a joint declaration with UNICEF and the ICCIDD (de Benoist and Delange, 2007), recommended the following principles for devising a successful national iodine deficiency control program:

(a) Assessment—Assessment of the magnitude of the problem of iodine deficiency involves collection of regional and national data with adequately powered surveys giving an accurate picture
(b) Communication—The findings of these assessments need to be conveyed to political authorities, health professionals and the public. Political authorities understand the socio–economic consequences of iodine deficiency best, while health professionals appreciate its health consequences and their easy prevention. The public should be educated about the advantages of iodized salt and their use in preventing damage to their children, thereby creating a demand for iodized salt. The importance of establishing channels of communication with the government and public health education programs cannot be overestimated.
(c) Action Plan—Developing an action plan is largely the responsibility of the government in association with national and international stakeholders. A national multidisciplinary committee involving experts from medicine, nutrition, education, salt and food industries, media etc. needs to be established and should be chaired by a senior Ministry of Health appointed officer, who is responsible for devising the action plan. The active involvement of the food and salt manufacturing industries is vital at this stage.
(d) Implementation—The implementation of the action plan for controlling iodine deficiency should be primarily focused on maximizing household access to iodized salt. If quality control measures for salt iodination and other well defined criteria are met, this would help in the elimination of iodine deficiency from regions that implement such programs
(e) Monitoring—this phase of iodine deficiency control is often neglected, sometimes because of economic reasons. Monitoring requires logistical support and laboratory expertise, often not available in low income countries. The lack of monitoring has led to the failure of previously successful programs in the past. An important principle in monitoring is that it needs to be done both at the level of the salt manufacturer and the household and needs to be done reasonably frequently. The availability of iodine can change frequently and unpredictably with alterations in the physical, political, commercial, and cultural landscape. Natural disasters and civil unrest can disrupt salt plants and distribution patterns, a new government may overturn established patterns of health care and regulatory enforcement, changes in the management and structure of salt companies may profoundly affect the market for iodized salt, and understanding and concern about iodine nutrition and the importance of iodized salt may wax and wane with policy makers and consumers. Because at least some of these factors are likely to occur in any country, careful monitoring of iodine nutrition is essential to maintain effective optimal iodine intake in a population.

Sustaining Optimal Iodine Nutrition

The sustainability of a successful salt iodination program depends on many factors. Consistent and strong government support is of prime importance. Governments should be committed to enforcing penalties in the event of failure to follow legislation. The food and salt industries should continue to be totally committed to the task at hand. Regular monitoring of the adequacy of salt iodination and availability of iodized salt to households, and population surveys using modern technology (such as

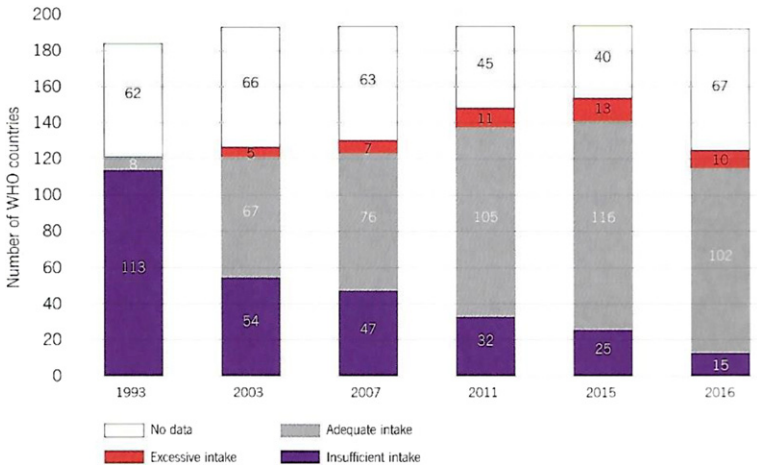

Fig. 2 Number of iodine deficient countries between 1993 and 2016.

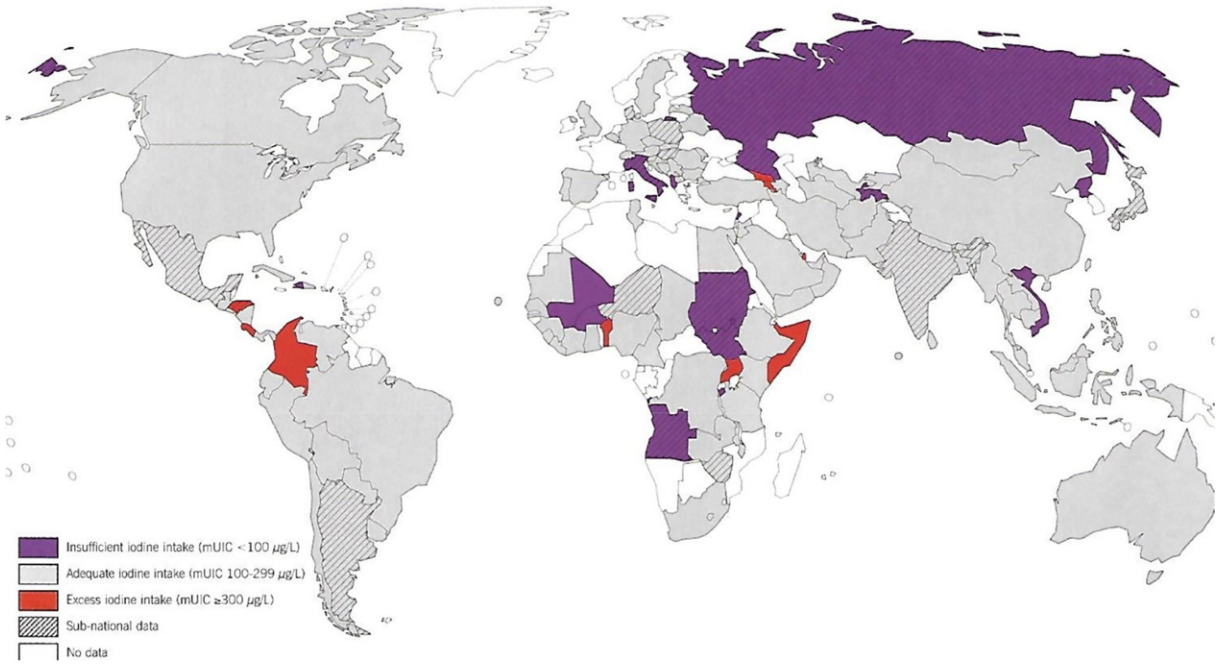

Fig. 3 An overview of current global iodine status.

ultrasound thyroid volume measurement in schoolchildren; urinary iodine measurement etc.) should be undertaken to identify failing areas. Remedial action would then need to be undertaken if necessary. Health education of the consequences of iodine deficiency, and the cheap and effective remedy of iodized salt, should be consistently and continuously conveyed to both health professionals and the general public. The public need also to be convinced that salt iodination and salt reduction are compatible health policies.

Global Status

Until recently, very few countries were iodine sufficient with key exceptions being the United States, Canada, Australia, and Switzerland. Severe iodine deficiency occurs in new mountains (e.g., the Andes, Alps, Himalayas), in zones with frequent flooding (e.g., India, Bangladesh), and in far inland areas (e.g., Central Asia, Central Africa). However, other places are not immune and even inhabitants of small islands (e.g., the Azores, Cape Verde) may have significant deficiency. Thus, inadequate iodine is the baseline condition for most parts of the world and poses a constant threat, unless corrected either by natural means, that is, availability of foods from iodine-sufficient regions, or by intention—that is, prophylactic programs, usually with

iodized salt. The largest concentrations are found in occasional natural deposits, especially in northern Chile, and in association with oil and gas deposits (e.g., in Japan).

More recently iodine deficiency has been declining (Fig. 2) as evidenced by reduced goiter rates and increased urinary iodine concentrations although areas of deficiency still remain (Fig. 3).

Obstacles to the Global Elimination of Iodine Deficiency

The past decade has seen remarkable progress in working toward the elimination of iodine deficiency. At the same time, approximately half the world's population still live in countries that harbor some deficiency. In 1990, the World Summit for Children, sponsored by the United Nations and attracting heads of state from most countries in the world, pledged the virtual elimination of iodine deficiency by 2000. That goal failed. A second conference, in May 2002, repeated the pledge, changing the target date to 2005. Reaching it is theoretically possible, but depends on accelerated action from countries and international agencies.

The correction of iodine deficiency is conceptually straightforward. People lack iodine, so they need to obtain it. A choice of good methods for providing iodine exists. Of these, iodized salt is preferred, but the alternatives are also acceptable. Iodized salt has been in use for 80 years. Some countries have adopted it with dramatic success. Why, then, does iodine deficiency continue in so much of the world?

Countries and their inhabitants are unique. Even though a country may share the same geography and ethnic mix as its neighbors, it may differ in many ways that influence the success of an iodization program. Some of the more common reasons that programs are not successful are as follows.

Difficulties in Implementing Iodized Salt

As already pointed out, some countries have numerous remote salt deposits that are easily mined and sold locally. Other countries have only a few large salt producers, but those do not comply with the law. Still others import their salt, but its iodization is not enforced, and much also enters as contraband.

The compliance and cooperation of the salt industry are crucial to successful iodization. Producers need education on its importance, assistance with technical issues, and constructive actions to make it economically attractive. They must be assured of a market for iodized salt, especially if it costs much more than the noniodized products. The consumer must learn to demand iodized salt because of its health benefits, even if the price is greater.

Lack of Awareness

Consumers, salt producers, and government officials must appreciate the damage from iodine deficiency and the importance of its correction. All levels need vigorous communication efforts.

Insufficient Data

Information about iodine nutrition is fragmentary, not only in the developing world but also in many socioeconomically advanced countries. Baseline surveys are necessary in order to plan action. Countries such as Australia and New Zealand have recognized their current iodine deficiency only recently. Other countries, such as Canada, though unlikely to be deficient, do not have recent national data. If a government is unwilling or unable to obtain such information, then other groups, such as endocrinologists, universities, or independent national coalitions, should.

Inadequate Government Support

The government is ultimately responsible for the public health of its citizens, including their iodine nutrition. Policy makers must be convinced of the importance of iodine nutrition and of the means for its correction and this effort may require extensive lobbying. The government should have specific laws and enabling regulations to support universal salt iodization and should designate responsibility to a specific unit with sufficient budget to carry out the program. Often government support is strong during periods of great publicity and activity by the unit, but this enthusiasm may taper with new personnel or budget constraints.

Poor Monitoring

Because iodine deficiency is a chronic condition, it needs regular long-term monitoring. The government must provide for follow-up surveys and respond promptly to any reports that the control program is not working. National coalitions can help to keep iodine nutrition in constant view and to prod the government when lapses in control occur.

Summary

People need iodine because it is an essential component of the thyroid hormones, which influence a variety of vital biochemical reactions in the body. Insufficient iodine, and therefore insufficient thyroid hormone production, causes pregnancy complications, neonatal and infant death, mental retardation, goiter, hypothyroidism, deaf–mutism, and low economic productivity. The recommended daily intake for an adult is 150 μg, with larger amounts recommended during pregnancy and lactation. The best measure of iodine nutrition is the urinary concentration of iodine.

Salt is the most effective vehicle for delivering iodine to a deficient population and can be iodized by simple inexpensive techniques. Other delivery systems are iodized vegetable oil, iodized water, and iodine tablets. Success in achieving iodine sufficiency depends on an effective program, usually the responsibility of the Ministry of Health and supported by many other sectors, both public and private, especially health providers and the salt industry. Key components in the program are communication, education, and monitoring of both iodine nutrition and salt iodine content.

Approximately half of the world's population lives in countries with iodine deficiency and risks its consequences. Most of these individuals are in the developing world, but many in Western Europe also continue to be deficient. Approximately 70% of households in the developing world use adequately iodized salt. The United Nations and heads of states have pledged virtual elimination of iodine deficiency by the year 2005, but achieving this goal requires an intensified effort.

See also: Smoking and the Thyroid. Thyroid and Infertility. Thyroid Carcinoma. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Nontoxic Goiter☆

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Glossary

Nontoxic goiter A diffuse or nodular enlargement of the thyroid gland that is not associated with hyperfunctioning of the thyroid and that does not result from an autoimmune or inflammatory process. Also known as "euthyroid goiter."

Radioiodine treatment Administration of radioactive iodine to humans with the aim of inhibiting the function of thyroid follicular cells or destroying them.

Recombinant TSH Recombinant thyroid stimulating hormone used to stimulate thyroid follicular cells prior to treatment with radioactive iodine.

Thyroidectomy Surgical removal of the thyroid gland.

Thyroxine treatment Synthetic thyroid hormone given to patients with thyroid hormone deficiency to normalize their thyroid function. Thyroid hormone replacement is typically administered in the form of an oral tablet containing thyroxine (T4).

Definition

Nontoxic goiter is defined as a diffuse or nodular enlargement of the thyroid gland that is not associated with hyperthyroidism and that does not result from an autoimmune or inflammatory process. Endemic goiters are those present in greater than 10% of the population due to chronic iodine deficiency. By contrast, sporadic goiters develop in areas of normal iodine intake, and typically occur in younger individuals. This article will focus on the evaluation and management of patients with sporadic, nontoxic goiters.

Introduction

Iodine deficiency is the most important risk factor for goitrogenesis, and even modest levels of iodine deficiency may contribute to goiter formation. Additional risk factors for goitrogenesis include female gender, increasing age, history of smoking, and various dietary and environmental factors (Hegedus *et al.*, 2016). Moreover, twin and family studies suggest a hereditary contribution to the development of nontoxic goiter. Although the responsible genetic mechanisms are incompletely understood, linkage studies have identified several candidate genes (Krohn *et al.*, 2005; Paschke, 2011). *MNG1* is a candidate locus on chromosome 14q31 that was first identified in a large Canadian family and later confirmed in a kindred from Germany (Paschke, 2011). An extended genome wide linkage analysis identified four novel candidate loci on chromosomes 2q, 3p, 7q, and 8p. The 3p locus was of particular relevance as it was identified in 20% of families with nontoxic goiter and appears to have an autosomal dominant pattern of inheritance, thus representing a plausible genetic mechanism for goitrogenesis (Bayer *et al.*, 2004). Weaker genetic defects, or combinations of genetic variations in different genomic regions may also contribute to goiter predisposition, which may not be detected by linkage studies. Thus, development of nontoxic goiter is likely driven by the interaction of environmental risk factors and individual genetic susceptibility (Paschke, 2011).

Nontoxic goiter is often a precursor of toxic multinodular goiter. The natural history of nontoxic goiter includes an average increase in thyroid volume by 4.5% per year accompanied by increasing nodularity and autonomy of the thyroid gland (Berghout *et al.*, 1990). If goiter volume increases significantly, nontoxic goiters may result in compression of surrounding structures leading to dyspnea, dysphagia, or globus sensation, necessitating treatment. However, most nontoxic goiters exhibit slow growth, and can be managed conservatively (Hegedus *et al.*, 2003).

Diagnosis

The clinical presentation of nontoxic goiter can vary depending on the size, nodularity, location, and functional status of the thyroid gland. Patients may be asymptomatic and present only due to the incidental finding of gland enlargement or nodularity on

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diagnostic imaging done for other reasons. Alternatively, they may present due to detection of an enlarging neck mass, or symptoms of compression of surrounding structures, as discussed below.

The general approach to a patient with goiter includes a detailed history and physical examination, serum thyroid-stimulating hormone (TSH) evaluation, and ultrasound (US) examination of the thyroid. Additional evaluation may include thyroid scintigraphy, primarily in the setting of low serum TSH, fine needle aspiration biopsy of suspicious nodules, or cross-sectional imaging of suspected substernal goiter.

History and Physical Examination

The history for a patient with nontoxic goiter should focus on assessment of thyroid gland enlargement, compressive symptoms, thyroid dysfunction, and risk factors for thyroid cancer. Clinicians should inquire about the discovery of the goiter, rate of change or growth, and associated pain or tenderness. Enlarged goiters may cause compression of surrounding structures such as the trachea, esophagus, recurrent laryngeal nerve, or thoracic cavity. Mild tracheal compression does not cause any symptoms; however, as the trachea becomes narrower, patients may complain of exertional or positional dyspnea, stridor, choking sensation, or cough. Dyspnea is typically exacerbated in the recumbent position. Occasionally, patients complain of acute worsening of symptoms of tracheal compression, which can be seen with bleeding into a thyroid nodule or cyst. Compression of the esophagus is rare due to its posterior location, but when present, leads to dysphagia. Compression of the recurrent laryngeal nerve may result in hoarseness of voice. A goiter that extends inferiorly into the mediastinum (i.e., substernal goiter) may manifest symptoms of thoracic outlet obstruction, such as facial plethora or dilated neck veins.

The history should include assessment of symptoms associated with hyperthyroidism, which are not present in patients with nontoxic goiters, and would therefore point to an alternative diagnosis. These symptoms are discussed elsewhere. Patients should also be assessed for risk factors for thyroid cancer, such as history of head and neck irradiation, and previous personal or family history of thyroid cancer.

General inspection of the patient includes listening for dyspnea, stridor, or hoarseness of voice. Palpation of the goiter should focus on assessment of its size, nodularity, position, texture, mobility, tenderness, and the presence or absence of cervical lymphadenopathy. If it is not possible to palpate the lower edge of the goiter, the goiter may extend into the thoracic cavity. Suspected substernal goiters should be evaluated by percussion over the sternum listening for dullness, and special maneuvers such as Pemberton's sign. This maneuver is conducted by having the patient elevate both arms above their head for one minute. A positive sign is the finding of facial plethora, facial swelling, or dilatation of neck veins, and is indicative of thoracic outlet obstruction. Rare complications of enlarged goiter include paralysis of the phrenic nerve, potentially resulting in elevated diaphragm or dyspnea, or paralysis of sympathetic nerve chains, resulting in Horner syndrome. Horner syndrome is characterized by unilateral miosis, ptosis, and anhidrosis.

Investigations

Assessment of Thyroid Function

The serum TSH level should be measured for all patients with diffuse or nodular goiter. If the TSH concentration is below the lower limit of normal, it is necessary to measure the level of free thyroxine (T4) to distinguish between subclinical or overt hyperthyroidism. As the name implies, "nontoxic" goiters are typically not associated with suppressed serum TSH levels. However, the progression of nontoxic goiter to toxic multinodular goiter represents a continuum of disease, and therefore, patients with nontoxic goiter may present with euthyroidism or subclinical hyperthyroidism. In patients with a subnormal TSH, thyroid scintigraphy (in the form of ^{99m}Tc pertechnetate or ^{123}I) should be performed to determine the functional status of the nodules. The scintigraphy images should be compared to thyroid ultrasound images to map the location of hyper- or hypo-functioning nodules. Thyroid scintigraphy can also help determine whether a substernal mass represents functioning thyroid tissue. Furthermore, if radioactive iodine (RAI) treatment is being considered to reduce thyroid volume (see below), measurement of the radioiodine uptake (%) helps to guide radioactive iodine dosing and the decision of whether or not to use recombinant TSH. If the serum TSH is above the upper limit of normal and the concentration of free T4 is low, chronic autoimmune thyroiditis should be considered as a possible cause of the thyroid enlargement. This diagnosis can be confirmed by measurement of serum thyroid peroxidase (TPO) antibodies.

Assessment of Thyroid Nodules

Thyroid ultrasonography is recommended for all patients with suspected thyroid nodules (Haugen *et al.*, 2016; Gharib *et al.*, 2016). Thyroid ultrasonography gives detailed information about thyroid gland size, presence of thyroid nodules, and characterization of malignancy risk within each nodule. Nodules should be evaluated in the manner outlined by clinical practice guidelines (Haugen *et al.*, 2016; Gharib *et al.*, 2016). Broadly speaking, this includes selective use of fine needle aspiration biopsy based on a nodule's sonographic features of malignancy, size, and rate of growth over time.

Assessment of Compression of Surrounding Structures

If local obstruction is suspected, contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) of the neck and chest should be performed to assess the exact size and anatomic location of the goiter and its relationship to surrounding structures in the neck and mediastinum. In addition, the smallest cross sectional area of the trachea should be measured (Bahn and Castro, 2011). Cross sectional imaging is also helpful to assess for substernal extension of the goiter and to rule out thoracic outlet obstruction. It should be noted that iodine-containing contrast agents may lead to hyperthyroidism in patients with autonomously functioning thyroid tissue, and therefore should be used with caution in patients at high risk of cardiac decompensation from thyrotoxicosis. Clinicians may consider pretreatment with antithyroidal medications (including perchlorate) or beta-blockers as needed.

If tracheal compression is suspected based on clinical features or imaging, pulmonary function tests should be obtained to assess the degree of upper airway obstruction. It should be noted that tracheal compression affects inspiratory flow more than expiratory flow. Measurement of forced expiratory volume in one second/peak expiratory flow ratio over 8 mL/L/min has a high sensitivity and specificity for upper airway obstruction, while measurement of forced expiratory flow at 50% of vital capacity ($FEF_{50\%}$)/forced inspiratory flow at 50% of vital capacity ($FIF_{50\%}$) has a high sensitivity but poorer specificity for upper airway obstruction (Sorenson *et al.*, 2014). The smallest cross sectional area of the trachea ("SCAT") has been shown to significantly positively correlate with the $FIF_{50\%}$ (Bonnema *et al.*, 2008).

Treatment of Nontoxic Goiter

The presence of a diffuse or multinodular goiter is not an indication for treatment on its own. The most important indications for treatment are compression of surrounding structures or presence of a suspicious nodule. There is no thyroid volume threshold beyond which treatment is deemed necessary, as there is poor correlation between goiter volume and presence of compressive symptoms. Treatment should also be considered in the case of progressive growth where development of compression is likely to occur, if even mild compression would be poorly tolerated, or if there is significant intrathoracic growth. Occasionally, treatment is considered for cosmetic reasons and patient preference.

Choice of Treatment

There are three traditional modalities for treatment of nontoxic goiter: thyroidectomy, radioiodine, and levothyroxine suppressive therapy. Clinicians should assess the most appropriate modality for their patients based on treatment risks and benefits, availability, and patient factors such age, comorbidity, and personal preference. Thyroidectomy is the standard treatment, especially for young and otherwise healthy patients, and when prompt decompression of vital structures is required. Radioiodine treatment with or without recombinant TSH is an alternative to surgery for older patients and for those who are poor surgical candidates, as well as in the case of recurrent goiters. Levothyroxine suppressive therapy is rarely efficacious for treatment of nontoxic goiter.

Thyroidectomy

Surgery is the first-line therapy for the treatment of nontoxic goiter, as it results in fast decompression of vital structures with alleviation of obstructive symptoms, and provides tissue material for pathological examination. The most common procedure is a subtotal or total thyroidectomy performed with an open approach (Dralle *et al.*, 2014). When performed by experienced surgeons, thyroidectomy is a safe and effective procedure. Potential complications of thyroid surgery include voice changes or vocal cord paralysis resulting from damage to the recurrent laryngeal nerve, or surgical hypoparathyroidism. Rarely, bilateral recurrent laryngeal nerve damage may result in tracheomalacia or tracheal obstruction requiring intubation. Surgical morbidity is greater in patients with very large goiters or those undergoing repeat neck surgery. The incidence of postsurgical hypothyroidism occurs in 100% of patients with total thyroidectomy necessitating lifelong levothyroxine supplementation.

Radioactive Iodine

RAI treatment has emerged as an attractive alternative to thyroidectomy in patients who are either poor surgical candidates or those who have declined surgery. RAI treatment of nontoxic goiter typically involves a single orally administered dose of 3–5 MBq/g thyroid mass of radioactive iodine (^{131}I) (Bonnema and Hegedus, 2012). Goiter volume reduction varies from 30% to 50% by five years after administration of therapy (Bonnema and Hegedus, 2012). In addition to the reduction in thyroid volume, the treatment also reduces compressive symptoms for a majority of patients. Recurrent goiter growth after 3–5 years occurs in approximately 10% of patients. A second RAI treatment can be considered in these cases.

The degree of thyroid volume reduction depends on three factors: the baseline thyroid volume, the amount of radioiodine uptake of the gland (RAIU), and the administered dose of ^{131}I. Most patients with nontoxic goiter have normal or low RAIU in the gland. Therefore, in larger goiters or those with low RAIU, higher doses of ^{131}I are needed to achieve adequate goiter volume

reduction. Most studies looking at RAI therapy for nontoxic goiter thus used a dosing algorithm that takes into account both thyroid weight and 24-h RAIU; an absorbed dose of 100 Gy is usually targeted, although the superiority of calculated versus fixed dosing is controversial (Gharib *et al.*, 2016). Another strategy to overcome the relatively low RAIU in nontoxic goiters is the use of recombinant TSH (rhTSH) prior to ^{131}I administration. Low dose rhTSH given 24 h prior to RAI therapy has been shown to increase the 24 and 96 h RAIU, thus allowing lower doses of applied radiation to result in equivalent thyroid volume reduction (Fast *et al.*, 2010a). Moreover, the use of rhTSH with RAI leads to an additional 15%–20% thyroid volume reduction, when compared to the same dose of applied radiation alone (Lee *et al.*, 2015). Several studies have tried to delineate the optimal dose and timing of rhTSH. Theoretically, the optimal dose of rhTSH would be the lowest dose that achieves a satisfactory increase in RAIU, timed such that the RAIU peaks at the same time as RAI administration. Such a dose would maximize the intended effects of RAI while minimizing the risk of thyroid swelling and thyrotoxicosis. Although there is no universally accepted dose, the optimal dose is likely between 0.03 and 0.1 mg, and the optimal timing is likely 24 h prior to RAI administration (Fast *et al.*, 2010b; Nielsen *et al.*, 2005).

Early side effects of RAI therapy include transient radiation thyroiditis (tenderness and swelling of the thyroid gland) and hyperthyroidism, both of which are usually self-limited. Although rhTSH may slightly increase the degree of radiation thyroiditis and hyperthyroidism, this is a dose-dependent effect, and can be minimized by using low dose rhTSH (0.03–0.1 mg) (Bonnema and Hegedus, 2012; Fast *et al.*, 2010b). Moreover, several studies have shown the safety of RAI with or without rhTSH, even in the setting of very large goiters (volume > 150 mL) (Lee *et al.*, 2015; Albino *et al.*, 2010; Bonnema *et al.*, 1999). Notably, there have been no reported cases of respiratory compromise induced by this therapy. Nevertheless, transient swelling caused by RAI therapy may in theory temporarily worsen tracheal compression, and thus caution should be used when treating high risk patients. High-risk patients include those with very large goiter, very small cross-sectional area of the trachea, or significant upper airway obstruction detected by flow-volume loops. Pretreatment of these patients with glucocorticoids can be considered, although the evidence for this practice is limited. A rare consequence of RAI therapy is the onset of Graves' disease induced by elevation of TSH receptor antibodies (Nygaard *et al.*, 1997). A late side effect of RAI therapy is the development of hypothyroidism necessitating levothyroxine supplementation, which occurs in 20%–50% of cases after 5 years (Le Moli *et al.*, 1999). Treatment with high doses of radioactive iodine is potentially carcinogenic, however, such high doses are rarely required in this setting.

All patients receiving RAI for the treatment of nontoxic goiter should be followed for alleviation of obstructive symptoms, thyroid function tests, and thyroid volume as assessed by neck ultrasonography. Although RAI is an appealing treatment for patients with nontoxic goiter unable or unwilling to undergo surgery, its universal adoption will require more data showing its safety and efficacy in various populations, where the ambient iodine levels may differ from those of previously studied European cohorts.

Levothyroxine Suppressive Therapy

TSH is the most important growth stimulator of normal thyroid tissue. Treatment of patients with levothyroxine is based on the hypothesis that the growth of a goiter is also TSH-dependent and that suppression of TSH secretion will result in the reduction of thyroid volume or at least prevent further growth of the goiter. However, this assumption has not been borne out in the literature. In a randomized clinical trial by Wesche *et al.* (2001) comparing levothyroxine to radioactive iodine therapy for sporadic nontoxic goiter, levothyroxine failed to shrink goiter volume after two years of therapy (median goiter size reduction − 1%). Other studies have shown a very modest reduction in thyroid volume in small goiters, but negligible thyroid volume reduction in large goiters (Shimaoka and Sokal, 1974). In addition, the subclinical hyperthyroidism induced by levothyroxine suppressive therapy has been associated with an increased risk of atrial fibrillation, increase in bone turnover markers, and significant decline of bone mineral density particularly in postmenopausal women (Wesche *et al.*, 2001; Knudsen *et al.*, 1998). Most patients are not suitable candidates for levothyroxine suppressive therapy for the treatment of nontoxic goiter (Fast *et al.*, 2008). Given the lack of efficacy and potential detrimental impact on cardiovascular and bone health, this past therapy should be abandoned.

See also: Environmental Goitrogens. Radioactive Iodine. Smoking and the Thyroid. Thyroglobulin. Thyroid Function Tests. Thyroid Imaging

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Thyroid Nodule☆

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Introduction

A thyroid nodule is characterized by excessive growth and structural and/or functional transformation of an area within the normal thyroid tissue (**Fig. 1**). In the absence of thyroid dysfunction, autoimmune thyroid disease, thyroiditis, and thyroid malignancy, it can be described as a simple nodule. The main concern of patients and physicians is to diagnose the few cancers (approximately 5%) as rapidly and cost-effectively as possible and reduce superfluous thyroid surgery (Hegedüs, 2004; Burman and Wartofsky, 2015).

Although important, the clinical evaluation of nodule size, morphology, and function is highly inaccurate. Thus, up to 50% of subjects with a solitary palpable thyroid nodule are found to have multiple nodules when investigated by ultrasound. Additionally, up to 50% of subjects with a normal gland by palpation have thyroid nodules when investigated by ultrasound. This is one of the reasons for the increasing use of imaging in such patients (Hegedüs, 2004).

Almost any thyroid disease can appear as a thyroid nodule. The spectrum ranges from the incidental finding in a normal gland to diffuse, uni- or multinodular and cystic enlargement of the thyroid gland. The manifestations are related to those of growth and functional autonomy, leading to cosmetic and pressure symptoms, and that of hypersecretion of thyroid hormones. A number of diagnostic and therapeutic aspects are unresolved and recent European and North American investigations and guidelines have disclosed some differences in the management (Paschke *et al.*, 2011).

Etiology

It is well accepted that nodular thyroid diseases belong to the group of diseases referred to as complex diseases. Such diseases are common, vary in severity, and are multifactorial. Thus, the clinical entity is the net effect of all the contributing genetic and environmental factors (Hegedüs *et al.*, 2003).

Although iodine deficiency is the major environmental factor, other factors are clearly important since nodular thyroid disease also exists when there is abundant iodine intake (Carlé *et al.*, 2014). Constitutional factors such as gender are implicated, as illustrated by a 5–10:1 ratio of affected females to males. Other risk factors include cigarette smoking, naturally occurring goitrogens, emotional stress, irradiation, and infection.

Evidence of a genetic effect is provided by twin studies demonstrating that concordance rates for nodular thyroid disease are much higher in monozygotic twins than in dizygotic twins. The heritability has been estimated to be 82% in a non'iodine deficient goiter area. The remaining 18% is the result of individual specific environmental factors not shared by the twins (e.g., cigarette smoking). Studies assessing the role of specific candidate genes in the etiology of nodular thyroid disease have not provided a clear picture, mostly because too small and too few families have been studied. Although single genes may play a role in certain families, it is thought that genetic heterogeneity (i.e., no single gene is either necessary or sufficient for disease development) is highly likely (Hegedüs *et al.*, 2003).

The following sequence of events appears to lead to the development of nodular thyroid disease: First, iodine deficiency or other goitrogenic factors induce thyroid hyperplasia. Second, due to increased proliferation during this stage, mutagenesis is increased. In the case of hot or toxic nodules, these mutations confer constitutive activation of the cyclic AMP cascade (e.g., thyroid-stimulating hormone receptor and $G_s\alpha$ protein mutations). This eventually leads to stimulation of iodine uptake and metabolism, thyroid hormone synthesis and release, and hyperthyroidism. In the case of cold thyroid nodules, a similar mechanism, but with mutations in genes that favor dedifferentiation (e.g., *ras* oncogene), is suggested. These latter mutations initiate growth but not function of the affected thyroid cells (Eszlinger *et al.*, 2017).

Epidemiology

Unfortunately, our knowledge is somewhat hampered by a lack of population-based longitudinal studies using sensitive diagnostic imaging (e.g., ultrasound) allowing distinction between uninodular and multinodular disease and morphologic as well as functional characterization. Despite these shortcomings, there is a clear pattern of increased thyroid nodularity with decreasing

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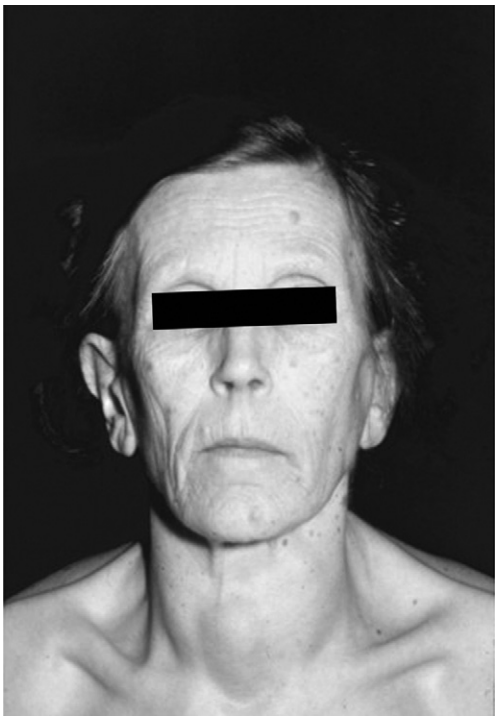

Fig. 1 Patient with a clinically solitary thyroid nodule located in the midline of the anterior neck.

iodine intake. The fact that nodules exist in the face of iodine sufficiency and even iodine excess emphasizes the importance of other environmental etiologic factors acting in concert with genetic factors (Carlé *et al.*, 2014).

In the Whickham survey (United Kingdom), a solitary thyroid nodule was present in 5.3% of women and 0.8% of men (6.6:1 ratio). Size and function of the nodules were not indicated. In the Framingham, Massachusetts, study, a solitary thyroid nodule was present in 6.4% of women and 1.6% of men. Both investigations used clinical evaluation (palpation) and were performed in an iodine-sufficient area. If ultrasound is used, the prevalence of thyroid nodules >10 mm is usually 20%–30%, increasing with age and in areas with insufficient iodine intake (Carlé *et al.*, 2014). In autopsy studies, 50% or more have either single or multiple thyroid nodules. If investigated using isotope scintigraphy, approximately 10%–15% of all nodules are autonomously functioning (taking up the isotope, hot or toxic), whereas 85%–90% are nonfunctioning (cold, no isotope uptake). The incidence of clinical disease is estimated to be 0.1% by palpation, corresponding to a lifetime risk of 5%–10%.

Natural History

The natural history with respect to growth and function varies and is difficult to predict in a given patient since no specific growth parameters exist. Therefore, it is difficult to decide whether a patient can be monitored without treatment or should be offered treatment before the nodule grows any more.

In the Framingham survey, new nodules appeared with an incidence of 1 per 1000 individuals per year, resulting in an estimated lifetime risk for developing a nodule of 5%–10%. After exclusion of the minority of patients who have rapid growth and symptoms and clinical suspicion of malignancy, and who are therefore offered treatment, nodules on average do not change significantly over time. Nodules that increase in size are predominantly solid and carry a higher risk of harboring thyroid malignancy than those predominantly cystic, which are more prone to decrease in size or even disappear. In many patients, ultrasound will identify additional nodules not evident at clinical investigation. Given time, many patients will be classified as having multinodular goiter.

In the subgroup of hot nodules, the rate of evolution into a toxic nodule is approximately 4% annually. The risk is closely related to nodule size. If the nodule is >3 cm, the risk is 20% within 6 years, whereas the risk is only 2%–5% if nodule size is <2.5 cm.

Diagnosis

Clinical Evaluation

There is no clear-cut relation between thyroid nodule size, morphology, and function, on the one hand, and the complaints, quality of life and treatment effects in the individual patient, on the other hand (Sørensen *et al.*, 2014; Cramon *et al.*, 2015). The

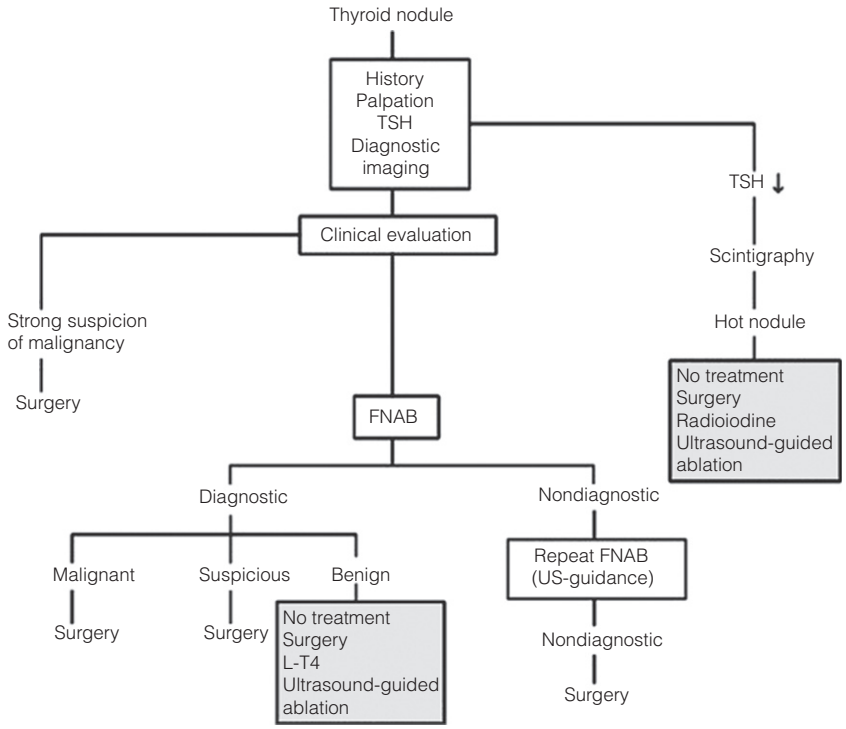

Fig. 2 Management algorithm outlining a cost-effective evaluation and treatment of the solitary thyroid nodule. In case of strong suspicion of malignancy, surgery is advised irrespective of fine needle aspiration biopsy (FNAB) results. In case of a nondiagnostic result, repeat FNAB yields a satisfactory aspirate in 50% of patients. FNAB guided by ultrasonography (US-guidance) allows sampling from the periphery of a solid nodule or solid part of a mixed solid–cystic nodule, increasing the sufficiency rate. The options in case of a diagnostic FNAB include those for both solid and cystic nodules. In case of recurrent cysts, the possibilities are reaspiration, surgery, or ethanol injection. The *shaded boxes* indicate treatment options. Ultrasound-guided ablation covers: Ethanol sclerotherapy as well as thermal ablation by laser, radiofrequency, high frequency ultrasound, or microwaves (Gharib et al., 2013).

majority of patients have few or no clinical symptoms. Therefore, given normal thyroid function and exclusion of malignancy, many need no treatment. A rough management algorithm for the majority of patients with a thyroid nodule is shown in **Fig. 2**.

Manifestations

When present, the most important symptoms and signs are caused by compression of structures in the neck or, rarely, in the upper thoracic cavity. In addition to various degrees of neck disfigurement, which by itself can merit treatment, the symptoms are related to compression of the trachea or esophagus. The symptoms of tracheal compression are dyspnea, stridor, cough, and choking sensation, but respiratory distress is rare unless the nodule extends into the thoracic cavity (Hegedüs *et al.*, 2003; Sørensen *et al.*, 2014). An acute exacerbation may be caused by hemorrhage into a nodule or by upper respiratory infections causing endotracheal swelling. Complaints due to esophageal compression are less common, as is vocal cord paralysis caused by stretching and/or compression of the recurrent laryngeal nerves.

A high proportion of functioning nodules cause slight or overt hyperthyroidism due to excessive secretion of thyroid hormones. This condition, with or without pressure symptoms or cosmetic complaints, may by itself merit treatment.

Clinical Examination

The evaluation of a patient with a thyroid nodule comprises a careful history and physical examination focusing on inspection of the neck, including regional lymph nodes, the upper thorax, and palpation of the thyroid. This clinical evaluation should preferably be done with the patient swallowing gulps of water and the head tilted slightly backward. Observer variation is very high, and the specificity and sensitivity of the diagnosis of a thyroid nodule are low. Detection of nodules depends on their size, morphology, and location within the thyroid parenchyma; the anatomy of the patients neck; and, most important, the training of the physician. The patient is usually unaware of the presence of a nodule smaller than 1.5–2 cm in diameter, and a nodule of 1.0 cm or less usually escapes detection by the physician (Hegedüs, 2004; Burman and Wartofsky, 2015).

Table 1 The most important clinical factors increasing the likelihood of thyroid malignancy in a patient with normal thyroid function and a solitary thyroid nodule

Family history of thyroid malignancy
Rapid nodule growth (especially during levothyroxine therapy)
Very firm or hard nodule
Fixation to adjacent structures
Vocal cord paralysis (evidenced by laryngoscopy)
Regional lymphadenopathy (enlarged regional lymphnodules)
Detection of distant metastases
Age <20 or >60 years
Male sex
History of head and neck irradiation
Diameter of nodule >4 cm and partially cystic
Compression symptoms (dysphagia, hoarseness, dyspnea)

Assessment of Risk of Malignancy

A family history of benign goiter suggests a benign disorder but is not proof thereof. Medullary thyroid carcinoma or even papillary or follicular thyroid carcinoma in the family should raise suspicion. The risk of harboring cancer is highest in the young and the old, and the risk is higher in men than in women. Head or neck irradiation during childhood for a number of benign conditions leads to clinically evident thyroid abnormality in 10%–40% of these individuals 5–40 years later. Thyroid carcinomas, mainly papillary carcinomas, are seen in 30% of those with thyroid abnormality. The importance of fallout radiation is tragically evidenced by the epidemic of childhood papillary thyroid cancer seen in Belarus and Ukraine after the Chernobyl nuclear accident. Rapid nodule growth (weeks to months) and symptoms of local invasion, such as pain, dysphagia, hoarseness, or dyspnea, suggest a carcinoma; however, only a minority of patients have these symptoms. Growth during thyroid hormone therapy is particularly worrisome. Sudden growth is most likely a thyroid cyst or hemorrhage into a previously undetected nodule. A hard and immobile nodule is suggestive of thyroid carcinoma, as is same-sided lymphadenopathy. Virtually all patients with thyroid carcinoma have normal thyroid function, as do most patients with benign thyroid nodules. On the other hand, thyroid hyperfunction normally rules out thyroid malignancy. In case of a high clinical suspicion of malignancy (**Table 1**), thyroid surgery should be recommended, irrespective of a benign needle biopsy, since the likelihood of malignancy is very high.

Laboratory Investigations

The only relevant biochemical test that is routinely needed is serum thyrotropin (TSH), which if normal indicates normal thyroid function. Subnormal serum TSH values should lead to determination of free thyroxine (T_4) and free triiodothyronine (T_3) in serum. If serum TSH is decreased on repeat examination, treatment of this hypermetabolic state should be offered independent of whether free T_4 and/or free T_3 are elevated, especially in the elderly, since this condition is associated with increased morbidity and mortality (Lillevang-Johansen *et al.*, 2017). Isotope scintigraphy is recommended and will most likely demonstrate a functioning nodule. Most patients have normal serum TSH, including those with thyroid malignancy. Elevated serum TSH with or without decreased serum free T_4 suggests that the patient has chronic autoimmune thyroiditis (Hashimoto's thyroiditis). This can be verified by demonstrating thyroid autoantibodies in serum. The latter condition may be an independent risk factor for thyroid malignancy.

Thyroid autoantibodies against thyroid peroxidase or thyroglobulin do not aid in the differentiation between malignant and benign nodules. However, they are markers of an increased risk of developing hypothyroidism (Hashimoto's thyroiditis) and hyperthyroidism (Graves' disease) spontaneously or secondary to surgery or treatment with radioactive iodine.

Calcitonin, a hormone produced by the parafollicular C cells of the thyroid gland, is the only clinically relevant biochemical marker of medullary thyroid carcinoma, which accounts for approximately 5% of all thyroid carcinomas. Basal or pentagastrin-stimulated serum calcitonin measurement is more sensitive than thyroid biopsy in detecting medullary thyroid carcinoma. However, there is no consensus on its routine use in patients with thyroid nodules (Paschke *et al.*, 2011).

Diagnostic Imaging

Neck palpation is very imprecise with regard to the determination of thyroid nodule morphology and size. For this reason, imaging methods are increasingly used, although no imaging method can accurately differentiate benign and malignant nodules.

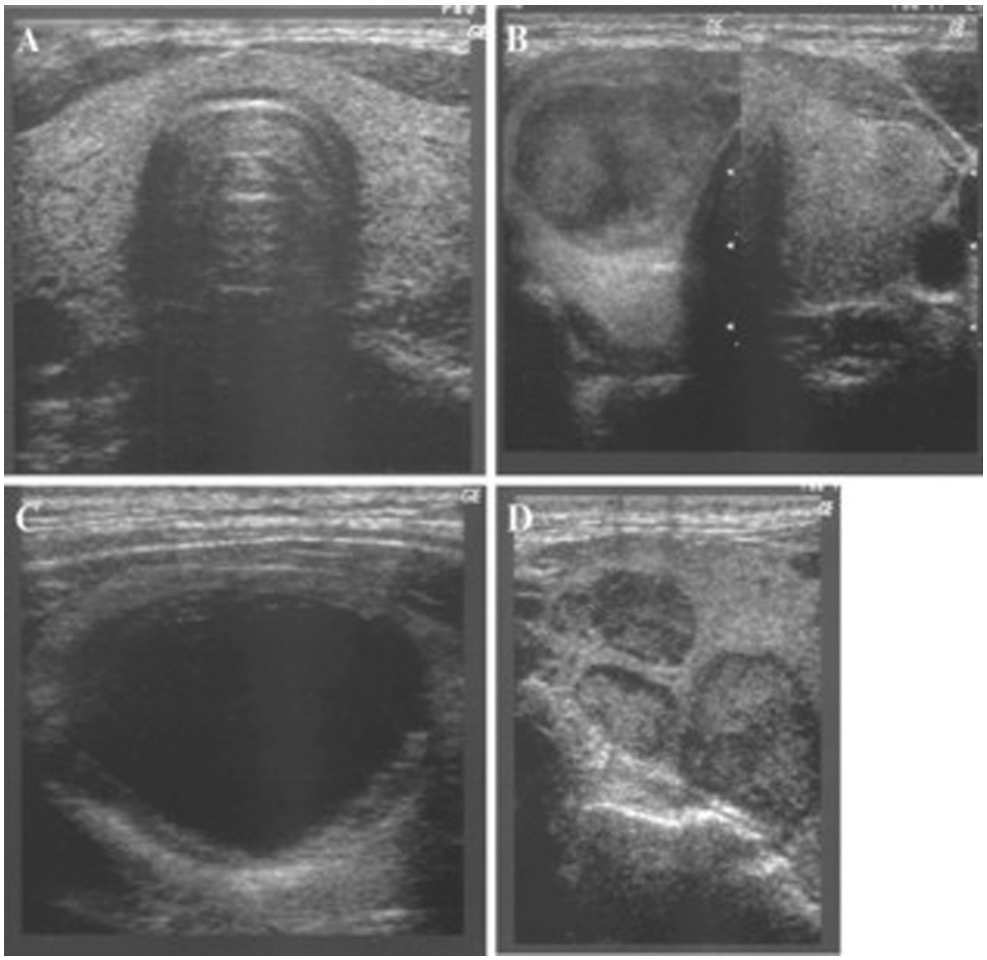

Fig. 3 Various appearances (morphological patterns) of a thyroid nodule using ultrasonography. (A) Normal thyroid tissue. (B) Solitary solid hypoecchoic (*dark*) thyroid nodule in the right thyroid lobe (left) surrounded by normal thyroid tissue (medium *gray*). (C) Solitary cyst (*black area*) surrounded by normal thyroid tissue. (D) Multiple nodules with varying ecchogenicity (multinodular gland).

Ultrasonography

This very sensitive technique with a high resolution has had a dramatic impact on clinical practice. When used in patients with a goiter, it has been shown to alter management in more than half of these patients. The increasing and widespread use, whether initially or during follow-up, is related to high availability, low cost, little discomfort to the patient, and its nonionizing nature. It allows determination of total thyroid volume, individual nodule size and echogenicity, and morphology of extranodular tissue and the evaluation of regional lymph nodes. Color-flow Doppler provides additional information regarding regional blood flow and nodule vascularity (**Fig. 3**). It distinguishes solid from cystic lesions and aids in the performance of accurate biopsies, punctures, and therapeutic procedures, such as percutaneous ethanol injection and thermal ablation procedures (Gharib *et al.*, 2013). Although there is no ultrasonographic pattern, alone or in combination with other techniques that may be considered specific for thyroid malignancy, characteristics such as hypoechogenicity, microcalcifications, increased nodular flow, and decreased elasticity are all predictive of malignancy to some extent. However, fine needle aspiration biopsy, preferably guided by ultrasonography, is far more accurate for this distinction (Gharib *et al.*, 2016; Haugen *et al.*, 2016).

Scintigraphy

Although the resolution of isotope imaging can be enhanced to 5–10 mm by tomography, this resolution is still far below that of ultrasonography. Therefore, scintigraphy is not so much used for evaluation of morphology as for evaluation of the regional uptake of the isotope and thereby the determination of functionality of the thyroid nodules (**Fig. 4**). Nodules with uptake by scintigraphy (hot or toxic) almost never harbor clinically important malignancy, although rare exceptions do exist. In an unselected population of patients with a thyroid nodule, 80%–90% of the nodules were nonfunctioning (cold). The a priori risk of malignancy is probably no higher than 5% for such a nodule. Scintigraphy is inaccurate in estimating thyroid and nodule size as well as in diagnosing malignancy.

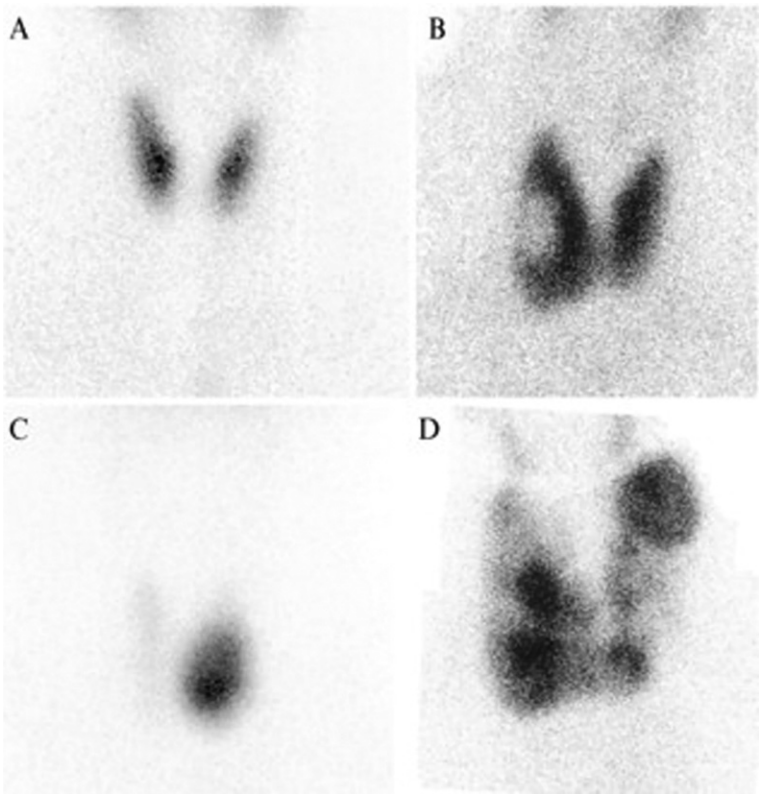

Fig. 4 Various appearances (morphological patterns) of a thyroid nodule using scintigraphy. (A) Normal uptake in two thyroid lobes. (B) Solitary nonfunctioning (cold) nodule in the right thyroid lobe (left). (C) Solitary functioning (hot or toxic) nodule in the left thyroid lobe (right). (D) Multiple nodules with varying degrees of isotope uptake (multinodular gland).

Computed Tomography and Magnetic Resonance Imaging

Computed tomography (CT) and magnetic resonance imaging (MRI) are expensive, time-consuming, and not readily available for imaging thyroid nodules. Their major strength is their ability to diagnose and assess the extent of substernal/intrathoracic thyroid tissue much more precisely than any other method. Both methods are well suited for visualizing the trachea and demonstrating narrowing of the tracheal area or a decrease in its volume. However, this measure correlates poorly with lung function.

Importantly, the increasing use of ^{18}F-fluorodeoxyglucose positron emission tomography (PET), for non-thyroid indications, has led to the incidental detection of focal uptake in the thyroid in around 2%–3% of cases. Approximately one in three of such patients harbor thyroid malignancy, predominantly papillary thyroid cancer (Soelberg *et al.*, 2012).

Fine Needle Aspiration Biopsy

Fine needle aspiration biopsy (FNAB) provides the most direct and specific information about a thyroid nodule and is used by virtually all thyroid specialists in the initial evaluation of a patient with a solitary thyroid nodule or a dominant nodule in a multinodular goiter (Gharib *et al.*, 2016; Haugen *et al.*, 2016). It is without complications, inexpensive, and easy to learn to perform. Use of FNAB has reduced the number of thyroid surgeries by approximately 50%, doubled the surgical yield of thyroid cancer, and reduced the overall cost of medical care for these patients by 25%.

The technique involves the use of a 5- to 20-mL plastic syringe with a 22- to 27-gauge needle. The skin is cleaned with alcohol, and sometimes skin infiltration with 1 or 2 mL of 1% lidocain is used. The needle attached to the syringe is inserted perpendicular to the anterior surface of the neck. Negative pressure is applied, and as soon as bloody fluid in the hub of the needle appears, pressure is released and the needle withdrawn. No fluid should enter the syringe. If the nodule is a cyst, or partly cystic, the aspiration should be followed by FNAB of any residual solid component. Investigation of the cyst sediment rarely aids in the diagnosis of malignancy. After withdrawal, the needle is detached and the specimen is evacuated onto a slide. The specimen should be smeared immediately. Often, air drying is used and a number of staining methods are available.

Diagnostically useful FNAB specimens are obtained in approximately 80% of the cases and rebiopsy typically reduces the number of insufficient samples by half. The number of sufficient samples increases with operator experience, use of ultrasound guidance, the number of aspirations, when the nodule is solid, and with increasing cytopathologist experience, but it is highly dependent on the criteria used for adequacy of a sample. The relative distribution of FNAB results is given in **Table 2**.

Table 2 Etiology of thyroid nodules and the relative distribution of fine needle aspiration biopsy results[a]

Etiology	*Distribution (%)*
Benign (no evidence of malignancy)	70
Colloid nodule	
Cyst	
Thyroiditis (acute, subacute, or chronic)	
Suspicious	10
Follicular neoplasia	
Malignant	4
Follicular carcinoma	
Papillary carcinoma	
Medullary carcinoma (C-cell carcinoma)	
Undifferentiated carcinoma (anaplastic)	
Lymphoma	
Metastasis	
Nondiagnostic (insufficient)	16[b]

[a]Data are representative of the author's institution.
[b]The number of nondiagnostic results can be halved by rebiopsy.

Diagnostic accuracy of FNAB at large depends on the classification of the 10%–15% of suspicious lesions, of which 15%–25% are malignant. If regarded negative, sensitivity will decrease and specificity will increase. If regarded positive, the converse is true. Patients with suspicious, malignant, and nondiagnostic FNAB results (after reaspiration) should be operated on (**Fig. 2**). If this strategy is followed, the risk of postponing the diagnosis of malignancy in the approximately 70% of cases in which nonsurgical therapy is an option can be reduced to 1%. Repeat FNAB during follow-up of nodules left untreated will virtually eliminate the risk of overlooking thyroid malignancy. Neither elaborate classification systems for suspicious FNAB findings nor the use of large-needle biopsy increase diagnostic accuracy considerably. Attempts to include biochemical analysis of thyroid cyst fluid or immunodetection of various candidate molecules, such as thyroid peroxidase or lectin-related molecules, in the evaluation of thyroid cytology are still in the experimental stage. Current focus on increasing diagnostic accuracy is very much on molecular tests. Thus a "rule in" approach employing the detection of BRAF, NRAS, HRAS, and KRAS and PAX8/PPARG- and RET/PTC rearrangements is increasingly used on cytology material for cytologically indeterminate samples. A "rule out" approach, using gene expression classifiers, has been adopted by many, especially in case of follicular tumors. However, dependent on the clinical setting, the risk of overlooking malignancy is still around 3%–5%, which precludes its clinical use in many organizations and lack of consensus on their routine implementation from the thyroid specialty societies (Burman and Wartofsky, 2015; Eszlinger *et al.*, 2017; Gharib *et al.*, 2016; Haugen *et al.*, 2016).

Treatment

There is no ideal treatment for the thyroid nodule. Furthermore, despite availability of thyroid specific quality of life instruments, e.g. the ThyPRO (Watt *et al.*, 2014), little information is available regarding influence on quality of life of any of these therapy options (Cramon *et al.*, 2015). Neither are there large-scale studies comparing, head-to-head, the available therapies. The optimal therapy varies depending on the size and morphology of the nodule and whether it is functioning (**Fig. 2**; **Table 3**). Although nodules 1–1.5 cm or larger should undergo FNAB, treatment is often not necessary once malignancy has been ruled out. In the subcentimetric nodule, FNAB need not routinely be performed and treatment is rarely indicated. However, there is a continuous debate on not overlooking clinically significant malignancy on the one hand, and avoiding superfluously diagnosing and treating microcarcinomas, most of which will never become clinically significant, on the other hand (Gharib *et al.*, 2016; Haugen *et al.*, 2016; Burman and Wartofsky, 2015).

Levothyroxine Therapy

Although on the decline, some still use thyroid suppression with levothyroxine (L-T4) in the management of solid thyroid nodules in the euthyroid patient. The aim is to shrink existing nodules, considered to be a favorable sign indicating that the nodule is benign. TSH suppression seems most beneficial in the subgroup of patients with small, solid nodules. Approximately 20% of solitary solid nodules actually regress as a result of L-T4 therapy, and cessation of therapy leads to rapid regrowth. On average, long-term therapy is without significant nodule-reducing effect. Growth can be suppressed or slowed, and the formation of new nodules may be prevented. However, this necessitates that serum TSH is suppressed to subnormal values, which may have adverse effects. This degree of TSH suppression, called mild or subclinical hyperthyroidism, is associated with an increased risk of atrial fibrillation, other cardiac side effects, and reduced bone density, potentially leading to osteoporosis. Even more grave is the recently shown TSH-suppression related time-dependent increase in mortality (Lillevang-Johansen *et al.*, 2017) L-T4 is without effect in the cystic nodule and in patients with spontaneously low serum TSH

Table 3 Advantages and disadvantages of the treatment options for the solitary thyroid nodule

Treatment	*Advantages*	*Disadvantages*
Levothyroxine	Outpatient Low cost May slow nodule growth Possibly prevents new nodule formation	Low efficacy Lifelong treatment Regrowth after cessation Adverse effects (bone and heart) Not feasible with TSH suppressed
Surgery[a]	Prompt relief of symptoms Nodule ablation Definite diagnosis	Inpatient High cost Anesthesiological risk Surgical risk Vocal cord paralysis Hypoparathyroidism Hypothyroidism Bleeding and infection Scar
Radioiodine[b]	Outpatient Low cost	40% size reduction Contraceptives needed in fertile women Side effects Radiation thyroiditis Graves' disease Hypothyroidism Long-term cancer risk unknown
Ethanol injection and ultrasound-guided thermal ablation techniques (laser, radiofrequency, high-frequency ultrasound and microwave therapy)	Outpatient Relatively low cost compared to surgery Thyroid function preserved	Repeat treatment may be needed Low efficacy in large nodules Operator dependency Side effects[c] Pain Transient dysphonia Thyroiditis Extranodular fibrosis Complicates subsequent cytological interpretation

[a]In this case, unilateral operation limiting the risk of side effects.
[b]It can only be used in the nodule with uptake, whether thyroid function is increased or not.
[c]Except for various degrees of pain, side effects are rare.

with or without elevated thyroid hormone levels. For these reasons, its use is questionable; at most, it can be used for a limited time period in younger patients with small nodules, in whom treatment is in fact least necessary.

Surgery

When there is malignant or suspicious cytologic features and/or symptoms due to the nodule, surgery is often recommended, especially for younger patients and in cases in which there are large nodules (Gharib *et al.*, 2016; Haugen *et al.*, 2016). The preferred operation is a unilateral removal of the affected lobe. The frequency of complications decreases with increasing experience and specialist training and is generally low. Complications include temporary and permanent unilateral vocal cord paralysis (1%–2% and 0.5%–1.0%, respectively), temporary and permanent hypocalcemia (1.0% and 0.5%, respectively), and wound hematomas and infections (0.5% and 0.3%, respectively). The risk of complications increases with the extent of operation. In the patient with normal thyroid function postoperatively, there is no indication for routine L-T4 treatment since this does not seem to hinder thyroid growth in the long term, at least in iodine-sufficient regions.

Although an option, surgery is rarely used in the hyperthyroid patient with a toxic nodule. In this situation, radioactive iodine treatment is the preferred treatment, although there are no comparative studies (Bonnema and Hegedüs, 2012).

Radioactive Iodine

If the patient has hyperthyroidism (toxic nodule), antithyroid drugs (methimazole or propylthiouracil) can normalize thyroid function but disease recurrence is the rule when medication is stopped. With the exception of a few patients who have a large nodule, in which case surgery may be indicated, radioactive iodine is the treatment of choice (Bonnema and Hegedüs, 2012). This is also the case for the clinically euthyroid patient with a functioning (hot) nodule without hyperthyroidism, in whom treatment

may be dictated by the nodule size, which may cause compression or cosmetic disturbances. In addition, radioactive iodine treatment is used to prevent hyperthyroidism (annual risk of approximately 4%).

A cure rate (i.e., normalization of thyroid function and the appearance on a thyroid isotope scintigram) of 75% is seen, and the nodule shrinks 30%–40% following a single dose of radioactive iodine. Side effects are few, with rare cases of radiation thyroiditis and transition to Graves' disease. The risk of hypothyroidism is approximately 10% after 5 years and poorly related to the dose of radioactivity. The long-term risk of malignancy is unknown but considered negligible.

Radioactive iodine has no effect in the nonfunctioning (cold) thyroid nodule, whether solid or cystic. In the future, the possibility of stimulation with recombinant human TSH before radioactive iodine treatment may lead to an increased iodine uptake and also an effect in the solid, cold nodule.

Ethanol Injection and Thermal Ablation Techniques

Ethanol (70%–100%) causes local small vessel thrombosis and coagulative necrosis, leading to fibrosis and permanent tissue ablation. It has been used in both autonomously functioning thyroid nodules and nonfunctioning thyroid nodules, whether solid or cystic. If multiple injections are used, complete cure (normal serum TSH and isotope scintigraphy) can be achieved in 60%–70% of patients with toxic nodules and 70%–80% with hot nodules. A single ethanol instillation (after aspiration) in thyroid cysts reduces recurrence to approximately 20% compared to approximately 50% after aspiration alone. In solitary solid, nonfunctioning thyroid nodules, approximately 50% of patients are relieved of their clinical symptoms based on a 50% nodule volume reduction. Additional injections have little effect. It is an option for patients who do not wish to undergo radioiodine treatment or surgery. However, it often necessitates repeat treatment to obtain complete cure. The long-term effects are unknown, and the treatment is not devoid of side effects. The procedure requires the special technical skill that can be obtained only at a center familiar with interventional ultrasound. While recommended for benign recurrent thyroid cysts, specialty societies do not recommend it for routine therapy of solid thyroid nodules due to the risk of side-effects. In such nodules, current studies focus on thermal ablation techniques, which are only available in a limited number of centers.

Ultrasound-guided thermal ablation, whether with laser, radiofrequency, high-frequency ultrasound or microwaves, have been introduced for solid and cystic solitary, benign, nonfunctioning thyroid nodules. Most experience is available for laser and radiofrequency therapy (Gharib *et al.*, 2013; Papini *et al.*, 2014; Ha *et al.*, 2015). One treatment session results in a nodule reduction of 50%–70% or more. It may be repeated and significantly reduces pressure symptoms and cosmetic complaints. These results are similar to those obtained using ethanol therapy. The fact that the spread of energy with thermal destruction can be better controlled, as opposed to chemical destruction by injection of ethanol, may favor these techniques in the long term. Controlled studies with surgery as the gold standard, and comparison of not only efficacy and side-effects but also quality of life and cost are awaited and may promote routine use.

See also: Thyroid Carcinoma. Thyroid Fine Needle Aspiration Cytology. Thyroid Imaging

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Thyroid and Irradiation☆

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Glossary

Chernobyl accident The explosion of the nuclear power plant in Chernobyl, Ukraine, on 26 April 26, 1986.

Differentiated thyroid carcinoma An endocrine malignant neoplasm arising from the follicular thyroid cell, which exhibits thyroid differentiation features.

External irradiation The delivery of radiation to the human body by any external source of radiation.

Effective radiation dose It is the tissue-weighted sum of the equivalent doses in all specified tissues and organs of the human body and represents the stochastic health risk (the probability of cancer induction and genetic effect) to the whole body. It is used to assess the potential for long-term effects that might be related to radiation exposure; it takes into account: (1) radiation dose, (2) the relative harm level of the radiation used (not all radiation is the same), (3) the sensitivities of each organ to radiation (e.g., bone marrow is more sensitive than bone); the SI unit for effective radiation dose is the sievert (Sv).

Fukushima accident The accident of the nuclear power plant in Fukushima, Japan, on March 11, 2011.

Internal irradiation The delivery of radiation to the body by ingested or inhaled radioactive isotopes.

Radiation dose Amount of energy deposited in tissue as a result of an exposure to ionizing radiation. It is used to calculate the dose uptake in living tissue in radiation protection and radiology. The unit of measurement for absorbed dose is the gray (Gy).

Introduction

Radiation exposure of the thyroid gland, like in many organs of the body, may result in DNA damage. Depending on the modality and severity of irradiation, the damage to the thyroid may cause cell death or may be less severe leading to specific genetic abnormalities. The thyroid is particularly vulnerable to the effect of ionizing radiation with the pediatric population being the most sensitive. Diagnostic and therapeutic medical radiation represents the largest source of man-made radiation exposure. However, the most striking increase in the incidence of thyroid cancer has occurred after Chernobyl nuclear plant accident, among children and adolescents who lived in the most contaminated areas of Belarus, the Russian Federation, and Ukraine.

This article reviews the sources of thyroid radiation, radiation risk to the thyroid gland and the most relevant health consequences on the thyroid after external and internal irradiation.

Source of Radiation Exposure

People are exposed to natural radiation sources (background radiation) as well as human-made sources on a daily basis. Natural radiation comes from naturally occurring radioactive materials found in soil, water, and air. The average background radiation is about 3.0 mSv/year, but is some regions can be as high as 6 mSv/year (e.g., Ramsar, Iran—the annual radiation ranges between 0.7 and 131 mSv). The average exposure to medical X-rays, estimated to be about 2.2 mSv, is related mostly to computer tomography imaging, nuclear medicine procedures or external beam radiation treatment. Beyond medical radiation nuclear accidents and nuclear bomb testing can be a source of high radiation dose in some regions.

Both external radiation (therapeutic or accidental) and internal radiation (caused by therapeutic or accidental irradiation due to radioactive isotopes of iodine) have been associated with thyroid diseases in vitro as well as in vivo (Sinnott *et al.*, 2010).

Factors Affecting Sensitivity to Develop Radiation-Induced Thyroid Cancer

Age and Sex

Young age at the time of radiation exposure is a major risk factor with a strong inverse relationship between age at exposure and the risk of thyroid cancer development. According to a pooled analysis of 12 studies including 970 thyroid cancer patients exposed

☆*Change History*: March 2018. Barbara Jarzab, Daria Handkiewicz-Junak and Jolanta Krajewska. Updated for Thyroid damage after radiation; age and sex; genetic predisposition; post-Chernobyl thyroid cancer, biology of post-Chernobyl thyroid cancer, glossary, and further reading and added the section for source of radiation exposure; Fukushima nuclear power plant accident; Prevention of thyroid cancer after internal contamination with radioactive iodine; Table 1.

This article is an update of Furio Pacini, Irradiation, Thyroid and, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 108–113.

to radiation in childhood (before age of 20), recently published, patients exposed to external radiation before the age of four demonstrated a fivefold greater risk per Gy of developing thyroid carcinoma comparing to those aged 10–14 years (Veiga *et al.*, 2016). The excess risks (ERs) of thyroid cancer among the Japanese atomic bomb survivors were 9.5, 3.0, 0.3, and 0.2 in the age categories of 0–9, 10–19, 20–39, and over 40 years of age at the time of the bombing, respectively (Pacini, 2004). However, above the age of 15 the risk was not longer statistically significant (Sinnott *et al.*, 2010).

Another question is related to in utero exposure of the thyroid. Accidental fetal exposure occurred after the Chernobyl accident. The data coming from a screening study of thyroid cancer prevalence among individuals exposed in utero to iodine isotopes suggested that such exposition may lead to an increased risk of thyroid cancer approximately 20 years later (Sinnott *et al.*, 2010). Among other effects of in utero radiation exposure, observed after Chernobyl, were dose dependent and significant reductions in head and chest circumference without any significant effect of birth weight (Hatch *et al.*, 2017). The effects of accidental medical exposure in pregnant women depend on gestational age and how much is taken up by mother's thyroid gland. The carcinogenic potential of such exposure is not known (Sinnott *et al.*, 2010).

The risk of thyroid cancer after exposure to external radiation was believed to be two- to threefold higher in females than in males, but this gender effect was not confirmed in the recent pooled analysis of 12 studies, mentioned above, and was not found in contaminated children after the Chernobyl accident (Veiga *et al.*, 2016; Iglesias *et al.*, 2017). Thus, gender does not seem to influence the risk of developing radiation-induced thyroid cancer.

Genetic Predisposition

There are some data suggesting that genetic predisposition, such as defects in the DNA repair mechanisms, may play a role in developing radiation-induced thyroid cancer. It was reported that in some families several irradiated individuals developed thyroid cancer more often, that could be expected by chance (Iglesias *et al.*, 2017). Patients who experience one radiation-related cancer are more likely to develop a second radiation-related cancer (Pacini, 2004). A higher risk was demonstrated in children irradiated for neuroblastoma and Hodgkin disease than in those, who underwent radiation treatment due to other reason (Rubino *et al.*, 2002). However, studies available so far have not identified genetic determinants that modify individual predisposition to radiation-induced childhood thyroid cancer. Genome-wide association studies using adult sporadic thyroid cancers and Belarusian cases aged 0–18 years at the time of the accident pointed out that a common single nucleotide polymorphism marker, rs965513, located in the FOXE1 vicinity at chromosome 9q22.33 showed a strong correlation with both sporadic and radiation-related thyroid cancer (Takahashi *et al.*, 2010).

Thyroid Damage After External Irradiation (Pacini, 2004)

Irradiation to the head and neck for the treatment of distinct benign conditions (e.g., enlargement of the thymus, tonsils, adenoids, or neck lymph nodes; skin angioma; acne; otitis; tinea capitis) used to be a common procedure applied in children since 1920. The relationship between irradiation and thyroid carcinoma for the first time was reported in 1950 (Duffy and Fitzgerald, 1950) and subsequently confirmed in numerous studies (Hanford *et al.*, 1962; Albright and Allday, 1967; DeGroot and Paloyan, 1973). In 1970 in the United States up to 76% of children diagnosed with thyroid carcinoma had a history of previous radiation exposure (Pacini, 2004). Therefore, since 1970 external radiation for benign diseases has been totally abandoned in nearly all countries (Pacini, 2004). In Europe the incidence of radiation-induced thyroid carcinoma in children and adolescent was substantially lower: 10% in France and 7% in Italy (Pacini *et al.*, 1997).

Based on the data of the National Council on Radiation Protection (NCRP), the risk estimate (EAR) for radiation-induced thyroid cancer is 2.5×10^{-4}/Gy/year for persons exposed under 18 years of age, whereas for adults the risk per year is assumed to be half of this value. Because of their smaller number of years at risk, the lifetime risk for adults is about one-fourth the risk for children. In a pooled analysis of five cohorts, the EAR is 4.4×10^{-4}/Gy/year for persons exposed before 15 years of age—the highest risk group. Little risk concerns persons after 20 years of age, and it is close to zero in persons after 40 years of age (Pacini, 2004).

In a recent study, which included 12 large cohorts with 927 thyroid cancers after radiation exposure, the risk of thyroid cancer significantly increased after a mean dose to the thyroid during childhood as low as 0.05–0.1 Gy (50–100 mGy). The relative risk increased supralinearly through 2–4 Gy, leveled off between 10 and 30 Gy and declined thereafter, probably because of cell killing, but the overall risk remained significantly elevated above 50 Gy (Veiga *et al.*, 2016).

In children exposed to a dose of 1 Gy to the thyroid, the relative risk of thyroid carcinoma ranges among series from 5.1 to 8.5. It was estimated that 88% of the thyroid cancers in this group of patients are attributable to radiation exposure (Sinnott *et al.*, 2010). Although radiation-induced differentiated thyroid cancer (DTC) used to be considered as more aggressive the recently published data discredited this opinion. A retrospective analysis, evaluating 257 DTC patients treated in the United States between 1951 and 1987 with median follow up of 27 years, showed significantly longer overall survival in patients with DTC developed after radiation exposure comparing to DTC without previous radiation exposure, 43 versus 38 years, respectively. However, regarding the percentage of recurrences and DTC-specific mortality the differences between the groups were not significant (White *et al.*, 2016).

In most studies, the minimal latency period between the time of radiation exposure and the development of thyroid cancer ranges between 5 and 10 years. A shorter interval was noticed after the Chernobyl accident probably due to the large number of contaminated children among whom thyroid cancer was diagnosed earlier (Iglesias *et al.*, 2017). The risk peaks at 20–35 years and decreases thereafter remaining still elevated even 45–60 years after exposure (Iglesias *et al.*, 2017; Lubin *et al.*, 2017).

The specific effect of diagnostic radiation exposure on thyroid cancer risk, previously believed to be controversial, has been unequivocally demonstrated by a meta-analysis of nine studies, just published. The overall diagnostic radiation exposure significantly increased the risk of thyroid cancer (the odds ratio [OR] = 1.52). Considering a particular radiological examination both computed tomography and dental X-ray were associated with increased odds of thyroid cancer, 1.46 and 1.69, respectively, whereas chest X-ray and mammography not. Both head/neck and chest diagnostic exposure increased the risk of thyroid cancer, OR 1.31 and 1.71, respectively. Conversely, the other body site exposure did not (Han and Kim, 2017).

High-dose radiation therapy to the neck (>20 Gy) results in a high rate of hypothyroidism but also in an increased risk of thyroid cancer with OR of 9.8 (Sigurdson *et al.*, 2005; Hancock *et al.*, 1991). The thyroid damage is probably related to the distance between the thyroid gland and the radiation field. If this is far from the thyroid, as in the case of thoracic or abdominal radiation fields in children, the thyroid gland may receive minimal radiation doses of some 100 mGy, not enough to cause hypothyroidism but sufficient to trigger thyroid cancer (Pacini, 2004). Interestingly, the meta-analysis of 13 studies evaluating the risk of thyroid cancer in individuals living near nuclear power plants did not demonstrate any significant increase in thyroid cancer incidence and mortality. The pooled estimates did not show different risk patterns by sex, exposure definition, or reference population (Kim *et al.*, 2016).

Thyroid Disorders After Exposure to Radioactive Iodine

Radioiodine (^{131}I) is commonly used in the treatment of benign thyroid conditions as well as in differentiated thyroid cancer. Nowadays, the use of RAI in the diagnostics of benign thyroid disease plays a minor role due to widely accessible thyroid sonography.

Radioiodine administration leads to direct and indirect radiobiological effects on affected tissues. The direct effect is related to ionization of the thyroid cell and results in DNA damage, whereas indirect effect leads to the production of free radicals that react with the critical macromolecules. Beta decay is responsible for causing DNA mutation and death in affected cells and other cells up to several millimeters away. For this reason, lower doses of ^{131}I may be more dangerous than higher doses, since they do not kill thyroid cells and may lead to carcinogenesis as a result of the irradiation. Therefore ^{131}I administration, particularly its lower activity in children is disputable.

Swedish analysis that involved 34,104 patients exposed to ^{131}I diagnostic activities with a mean estimated thyroid dose of 110 cGy demonstrated a small increase in the number of observed thyroid cancer in comparison to the expected number, 67 versus 50 cases respectively. It has to be emphasized that this increase was confined to subjects in whom thyroid scan was performed due to suspicion of thyroid cancer. These differences disappeared if the analysis was carried out in patients tested for other reasons. The authors concluded that the diagnostic ^{131}I administration showed no health effect on thyroid. However, one should be cautious as only a minority of patients in Swedish cohort were children (Hall and Holm, 1997).

Thyroid Effects of the Chernobyl Nuclear Accident (Pacini, 2004)

The accident at the nuclear power plant in Chernobyl, Ukraine, occurred on 26 April 1986 and dispersed large quantities of radioactive isotopes into the atmosphere, including ^{131}I, ^{133}I, ^{132}Te rapidly decaying to ^{132}I, ^{134}Ce, and ^{90}Sr. Among the most contaminated territories were southern Belarus, northern Ukraine, and adjacent oblasts of Russian Federation (Pacini, 2004).

Volatile radioactive isotopes could be first inhaled or later ingested. The time at which ingestion occurred varied considerably, but the milk chain, particularly in children, played the major role. Several factors contributed to the high radiation exposure of the population, in particular: the lack of or delay in protective procedures such as advising and evacuating the people at risk and distributing iodine prophylaxis, and moderate iodine deficiency of the most contaminated regions leading to increased iodine uptake. The thyroid is the only tissue in the body able both to take up and store iodine. Its contamination depends on amounts of uptaken radioiodine, thyroid mass itself and the magnitude of contamination. In addition, regardless of the level of contamination, the thyroid dose is always higher in children than in adults, and the uptake in children is similar to that observed in adults but the thyroid mass is smaller and the thyroid dose per gram of tissue is greater and extremely high in newborns and very young children (Pacini, 2004). These entire factors resulted in increased incidence rate of childhood thyroid cancer following the Chernobyl accident.

Post-Chernobyl Thyroid Cancer

The first information of any increase in malignancy rate among individuals exposed to fallout was reported in 1990, only 4 years after the accident, when growing incidence of thyroid cancer was noticed in Minsk and Kiev (Williams, 2008). The magnitude of

this increase and the geographical and temporal distribution of the cases strongly suggest that thyroid cancer was due to the reactor explosion and particularly to the huge amount of iodine radioisotopes released (Pacini, 2004). It is estimated that 7000 thyroid cancer cases occurred among the 2 million highly contaminated subjects, who were younger than 18 years at the time of the accident. In the most contaminated area of Belarus, the Gomel region, thyroid cancer incidence following the accident, between 1986 and 1996 was the highest—13 per 100,000 children per year, whereas a baseline incidence was lower than 1 per 100,000 children per year. However, the questions why only some people exposed to the same radiation dose develop cancer, whether it is just chance or depends on genetic or environmental factors remain open (Thomas *et al.*, 2011).

According to dosimetric data the mean estimated thyroid dose was nearly 700 mSv in Belarus. In Ukraine, 79.0% of children received a thyroid dose less than or equal to 300 mSv, 10.5% received between 300 mSv and 1 Sv, and 10.5% received more than 1 Sv. In most of the children, who developed thyroid cancer, the estimated thyroid dose has been less than or equal to 300 mGy. However, an excess thyroid cancer incidence has been observed even in areas where the mean thyroid dose in children was significantly lower; 50–100 mGy (Pacini, 2004).

Biology of Post-Chernobyl Thyroid Cancer

In earlier cases after Chernobyl accident, nearly virtually all thyroid cancers were papillary of the solid subtype, which was the unique characteristic observed after the Chernobyl accident. Subsequently, the proportion shifted to the classic subtype, which is less aggressive and metastatic, and is the common subtype in sporadic childhood PTC. Diffuse sclerosing variant, cribriform type and Warthin-like variant were rare, whereas tall cell and columnar cell variants were absent (LiVolsi *et al.*, 2011).

Rearrangements of the *RET* proto-oncogene (mostly *RET/PTC1*) are found in nearly 30%–70% of the childhood PTC. Molecular studies of the early Chernobyl-related tumors found that a very high proportion showed a RET rearrangement (Elisei *et al.*, 2001), and almost all were *RET/PTC3*. It was speculated that this rearrangement might be a marker for radiation-induced tumors. Over time, the proportion of tumors with RET rearrangement has declined. Radiation exposure is an efficient inducer of DNA double-strand breaks, therefore it is highly expected to cause gains or losses of DNA. A variety of copy number alterations (CNAs) have been identified in childhood thyroid cancers after the Chernobyl accident, mostly gains of DNA, and these were compared with CNAs in sporadic cases, in which losses were more frequent than gains. Ricarte-Filho and coworkers identified kinase fusion oncogenes aberrantly activated MAPK signaling pathway in post-Chernobyl radiation-induced thyroid cancers (Ricarte-Filho *et al.*, 2013). However, most studies have failed to demonstrate specific CNAs associated with radiation exposure except one study that described a unique gain of chromosome 7q11, which was absent in all unexposed cases (Hess *et al.*, 2011).

Several studies have been carried out and some of them reported gene expression changes unique to radiation-induced childhood PTCs, whereas others have failed to identify the signatures (Maenhaut *et al.*, 2011). Importantly, the identified genes substantially varied between the studies, with few recurrent genes. More recently, gene expression profiles in normal thyroid tissues and tumor tissues obtained from exposed and unexposed children after the Chernobyl accident were compared (Handkiewicz-Junak *et al.*, 2016). These studies that managed to eliminate most of confounding factors (e.g., environment) identified a significant, but subtle, differences in gene expression that are associated with low-dose radiation exposure.

Fukushima Nuclear Power Plant Accident

Twenty-five years after Chernobyl accident, on March 11, 2011, large amounts of radioactive isotopes, including 131-I, were released after Fukushima Daiichi (Japan) nuclear power plant accident. In terms of severity, the accident at the Fukushima was rated at level 7 on the International Nuclear and Radiological Event Scale, which matches the rating to the Chernobyl accident in 1986. However, the radiation dose to the thyroid gland was much lower because the authorities ordered shielding, evacuation from the most contaminated territories, and food restriction. Furthermore, the thyroid uptake of iodine was low in relation to the high iodine alimentary intake. Doses of less than 15 mSv were reported in 99% of children aged 0–14 years and the average thyroid dose was <1 mSv (maximal 33 mSv) (Nagataki *et al.*, 2013). Soon after the accident thyroid ultrasound screening began for those who were from the areas, where the radiation doses were the highest. In total, more than 300,000 subjects aged 18 years or younger at the time of the disaster were screened between 2011 and 2014. One hundred and one cases of thyroid cancer were found in the screened population (preliminary baseline screening), and a similar incidence was found in a Japanese control population of nonexposed children and adolescents (Suzuki, 2016; Yamashita *et al.*, 2017). Since these cases were discovered soon after the accident, there is no clear evidence related these cases to radiation exposure after the accident. The second round (the first full-scale survey), completed between 2014 and 2016, revealed another 71 cases with malignant or suspicious for malignancy fine needle aspiration biopsy results. Among 44 individuals, who underwent surgery, thyroid cancer was confirmed in all cases (Yamashita *et al.*, 2017).

Further follow-up of the exposed population is essential in obtaining the full picture of the long-term thyroid cancer risk at a very low radiation doses. Fukushima Health Management Survey is the largest health-monitoring project, currently ongoing. It involves the Basic Survey to estimate the external radiation dose received during first 4 months following the accident, when the airborne activity was at peak, and four detailed surveys: thyroid ultrasound examination, comprehensive health check-up, mental health and life-style survey, and survey of expectant and nursing mothers (Yamashita *et al.*, 2016).

Table 1 World Health Organization Guidelines for potassium iodide prophylaxis following a nuclear accident

Issue	*Details*
Potassium iodide dosage	Age dependent: Neonate (<1 month): 16 mg/day 1 months to 3 years: 32 mg/day 3–12 years: 65 mg/day >12 years of age and adults: 130 mg/day
Preparation form	Potassium iodine tablets Lugol solution (130 mg = 1 mL)
How long	A single dose of KI is usually sufficient for adequate protection for 24 h In the event of prolonged or repeated exposure, public health authorities may advise taking KI tablets more than once
Who should receive	The first group to get prophylaxis (most sensitive): Pregnant and breastfeeding women infants and children <18 years of age
Avertable radiation dose to thyroid to start prophylaxis	10 mGy (most sensitive group)

Other Thyroid Consequences of Radiation Exposure (Pacini, 2004)

Radiation exposure of the thyroid gland may also cause other thyroid abnormalities including hypothyroidism, acute thyroiditis, and the possibility of developing of autoimmune thyroid disorders. Thyroid cancer and benign thyroid nodules after radiation exposure represent the stochastic effects, whereas hypothyroidism and acute thyroiditis depend on the radiation dose.

Hypothyroidism was reported in 34% of 1677 patients treated with radiation therapy due to Hodgkin disease (Hancock *et al.*, 1991). Hypothyroidism was diagnosed in 43 among 2587 atomic bomb survivors in Nagasaki. Twenty-seven of these individuals were thyroid antibody positive and 16—thyroid antibody negative, without sex differences. There was an association noticed between thyroid dose and the prevalence of antibody positive but not antibody negative hypothyroidism, what might suggest an underlying autoimmune thyroid disorder (Nagataki *et al.*, 1994). Hypothyroidism is also a frequent consequence of internal radiation as observed in patients treated with radioiodine.

External irradiation to the head and neck may result in the development of thyroid autoimmunity. A significant increase in thyroid autoimmunity in exposed children comparing to nonexposed individuals was noticed 6–8 years after the Chernobyl accident, 19.5% versus 3.8% respectively (Pacini *et al.*, 1998). Similarly, an increased incidence of thyroid antibodies was observed in children who underwent radiation for benign disorders. More than two-thirds of irradiated patients demonstrated variable degrees of thyroid lymphocytic infiltration several years before thyroidectomy for nodular thyroid lesions. In patients who received radiation of the neck for Hodgkin's disease, 3% or more developed Graves' disease (a 7- to 20-fold excess risk) and 1% developed thyroiditis. The possible pathomechanism is related to the release of thyroid antigen from damaged thyroid cells, regardless of the modality of thyroid exposure (Pacini, 2004).

Prevention of Thyroid Cancer After Internal Contamination With Radioactive Iodine

Stable iodine, administered as potassium iodide (KI), inhibits the thyroid uptake of radioactive iodine by more than 98% if it is administered several hours before contamination, by 90% at the time of the contamination, and by 50% if it is given 6 h after the accident. Uptake will be low during 48–72 h and then will re-increase. Stable iodine does not protect against any other radioactive substances. The Guidelines proposed by World Health Organization for potassium iodide prophylaxis following a nuclear disaster are summarized in **Table 1**.

Polish experiences after the Chernobyl accident strongly support the necessity of stable iodine administration to prevent thyroid gland damage. KI administration, implemented in Poland within the first 4 days following the start of exposure, led to a reduction in committed thyroid dose between 40% and 60%. It has been estimated that about 90% of children under the age of 16 showed thyroid radiation dose below the predicted mean maximal burden (<50 mSv) (Nauman and Wolff, 1993).

See also: Thyroid Carcinoma. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Thyroid Imaging

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Introduction

The thyroid gland may be affected by different diseases with variable outcome and prognosis. A crucial step for the correct management of these disorders is the early diagnosis, the proper differential diagnosis, and, when appropriate, an accurate disease staging. Beyond clinical and laboratory data, different imaging modalities are available to evaluate thyroid disorders. These imaging modalities include both morphological and functional imaging methods, sometimes combined by using hybrid devices. The aim of this chapter is to provide a comprehensive overview on the thyroid imaging of thyroid moving from the basic characteristics of the different imaging methods and ancillary techniques used for evaluation and diagnosis to their applications in a wide range of thyroid disorders encompassing Graves' disease, toxic multinodular goiter, toxic adenoma, thyroiditis, nontoxic goiter, benign thyroid nodules, and the different forms of thyroid carcinoma.

Thyroid Ultrasonography

Since 1990s ultrasonography (US) has become the most diffused thyroid imaging tool. In fact, US allows to estimate thyroid size and evaluate its morphology (Haugen *et al.*, 2016; Gharib *et al.*, 2016). Also, a main power of US is the detection of thyroid lesions which may be not evident at palpation or other imaging techniques (Haugen *et al.*, 2016; Gharib *et al.*, 2016). **Table 1** details the main strengths of thyroid US. The procedure is safe, takes few time (i.e. 10–15 min), is relatively cheap, and does not require specific preparation of patient.

Assessment of Diffuse Thyroid Diseases

The thyroid gland is more echo-dense than the adjacent structures. Thyroid volume can be estimated by ellipsoid volume formula applied to each lobe, and a size between 7 and 20 mL can be considered as the normal reference in adult population (Rago *et al.*, 2006; Shapiro, 2003; Lyshchik *et al.*, 2004; Deveci *et al.*, 2007; Vejbjerg *et al.*, 2006; Trimboli *et al.*, 2008). As a general rule, the normal cells/colloid ratio gives homogeneous and normoechoic thyroid presentation at US, while higher cells/colloid ratio determines hypoechogenicity. Then, normal thyroid appearance at US (i.e. normoechoic and homogeneous echostructure combined with normal size) theoretically correlates with normal thyroid function and negative thyroid antibodies (Trimboli *et al.*, 2010, 2012); on the contrary, hypoechoic and inhomogeneous thyroid structure is associated with thyroid dysfunction and is highly suspicious for autoimmune diseases (i.e., Hashimoto or Graves' disease) (Marcocci *et al.*, 1991; Rago *et al.*, 2001). The presence of nodules within the gland does not influence the global thyroid function with the exception of autonomously functioning nodules which can be detected by scintigraphy. **Fig. 1** illustrates the normal thyroid US presentation, while **Figs. 2** and **3** show two cases of Hashimoto thyroiditis and Basedow disease.

Assessment of Thyroid Nodules

The role of US to identify thyroid nodules at risk has become the most relevant in clinical practice. In this context, it is well known that some US specific features can help to discriminate nodules at higher risk from those with no need to be further investigated. Overall, based on the US risk stratification, up to 80% of thyroid cancers are correctly identified (Haugen *et al.*, 2016; Gharib *et al.*, 2016). However, specificity of US in this context was reported as low. In fact, thyroid nodules are very common (their prevalence achieves 70% of European adult population) and US can detect a lot of clinically non-relevant nodules. As a consequence, performing US in patients with no specific risk factors (i.e. previous neck irradiation) or clinical indications is strongly discouraged. **Table 2** illustrates the reliability of the US characteristics useful to discriminate nodules suspicious for malignancy from those with low/very-low suspicious.

Remarkably, none of these features is accurate alone and the combination of all features achieves relevance to identify malignant nodules. As the most relevant novelty over the recent years in the field of thyroid US, several international societies have been published multi-points score systems to assess the risk of thyroid lesions. The major aim of these US systems was to cover the potential limitation of using each US parameter as a single predictor of malignancy. Firstly the so called TIRADS (Thyroid Imaging Reporting and Data System) was proposed by radiologists (Horvath *et al.*, 2009); there was a 6-points system. Following, the American Thyroid Association (ATA) proposed a 5-score scale (Haugen *et al.*, 2016) and, more recently the American Association Clinical Endocrinologists/American College of Endocrinologists and Associazione Medici Endocrinologi (AACE/ACE/AME)

Table 1 Main goals of thyroid US examination

What thyroid US can evaluate	*How*	*When is useful*
To verify the presence of gland in the correct position and its correct development	Localizing the entire gland and its shape	To exclude thyroid agenesia
To estimate thyroid size	Applying the ellipsoid volume formula to each thyroid lobe	In the initial evaluation of all patients and during their follow-up
To assess the presence of and risk for autoimmune thyroid disease	Evaluating the ecogenicity ed. echostructure of the gland	In presence of thyroid dysfunction
To assess the relationship between thyroid and other cervical organs	Examining if trachea and esophagus are damaged by increased thyroid size	In presence of cervical compressive symptoms (i.e. dysphagia and dyspnea)
To detect thyroid nodules	During thyroid evaluation	In all patients undergoing thyroid US
To discriminate nodules suspicious for malignancy from those with poor risk	Searching for the presence of suspicious US features in each one nodule	In each one patient with nodular disease
To follow-up nodules	Assessing change in size and echostructure of nodules over the time	In each one patient with nodular disease
To identify cervical lymph nodes suspected to be thyroid metastases	Examining neck levels (II to VI)	In each one patient with nodular disease or followed-up for thyroid cancer
To guide thyroid interventions	Performing the procedures under real time US-guide	During biopsy, percutaneous ethanol injection, or thermal therapy

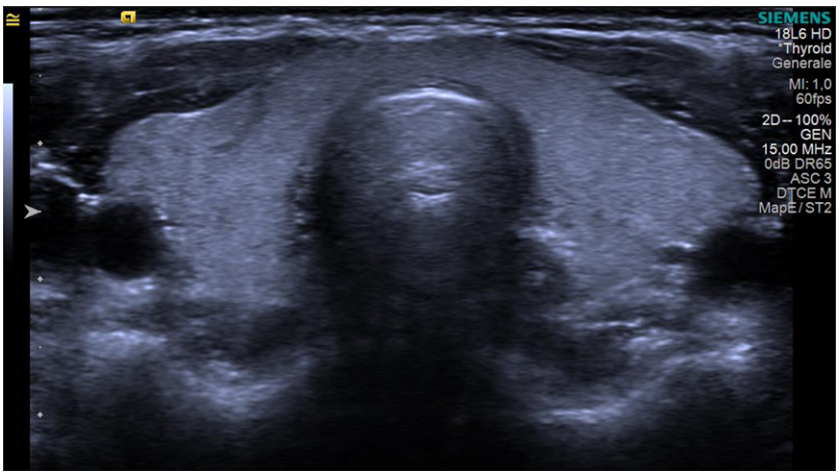

Fig. 1 Normal thyroid presentation at ultrasonography (bilobar transaxial section).

(Gharib *et al.*, 2016), and British Thyroid Association (BTA) (Perros *et al.*, 2014) systems were reported. Despite of different interpretation of the specific US parameters of the experts boards, these four US systems seem to furnish similar performance. **Table 3** summarizes and compares these four US systems. To date, a few papers have investigated the performance of these US systems in clinical cohorts of patients, and the results should suggest that ATA guidelines (Haugen *et al.*, 2016) have good sensitivity and in thyroid nodules (Yoon *et al.*, 2016; Xu *et al.*, 2017) and in the selected category of nodules with previous indeterminate cytology (Trimboli *et al.*, 2017; Grani *et al.*, 2016). However, these papers reported retrospective series, and further studies with prospective design are required. **Figs. 4** and **5**. Illustrate the presentation of one non-suspicious nodule and one nodule at risk for malignancy. In **Fig. 6** is shown an inelastic lesion at elastographic assessment.

Ultrasound-Guided Thyroid Interventions

US is pivotal in assisting fine-needle aspiration cytology (FNAC) of thyroid nodules and neck lymph nodes. Until the 1990s FNAC was performed without US guidance and its accuracy was suboptimal. In the last years US-guided FNAC has been worldwide diffused with improvement of cytologic diagnosis. Moreover, complications are very rare and FNAC can be performed in ambulatory office (Haugen *et al.*, 2016; Gharib *et al.*, 2016). Cytologic evaluation is essential to assess thyroid nodules with suspicious US presentation: the larger part of nodules are assessed at FNAC, while a rate of 15–25% of thyroid cytology is inconclusive due to inadequate material (Thy 1, Category I) or indeterminate diagnosis (Thy 3, Category III or IV). Nodules with Thy 1 or Category I need to repeat FNAC, and those lesions with repeated inadequate sample should be addressed to diagnostic

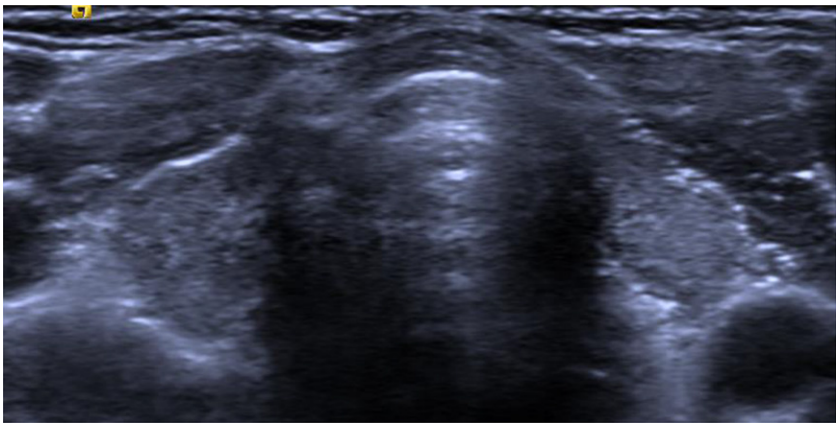

Fig. 2 Hashimoto's thyroiditis: thyroid size is markedly reduced, echogenicity is reduced with inhomogeneous echostructure (bilobar transaxial section).

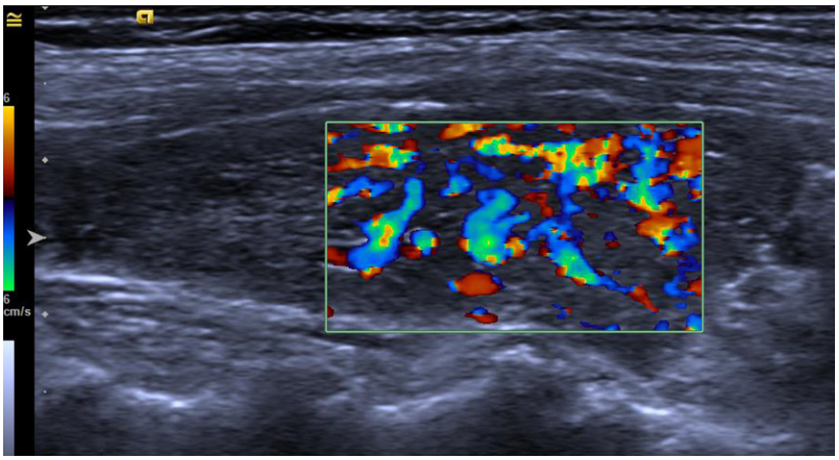

Fig. 3 Graves' disease: echogenicity is reduced with irregular echostructure. A diffuse increase of parenchymal vascularization is observed at color-Doppler evaluation (right lobe, sagittal section).

surgery or other biopsy (see below) (Haugen *et al.*, 2016; Gharib *et al.*, 2016). Generally, a 23–27 gauge needle attached to a syringe is used. Several fashions can be adopted for thyroid FNAC, being the free-hand mode the most popular. In a parallel approach the needle is inserted parallel to the probe or at an angle of that: the needle can be viewed as it traverses the nodule. A perpendicular approach is also largely used due to its simplicity.

As above mentioned, US is accurate in identifying suspected recurrence of thyroid cancer in enlarged lymph nodes. Also in these cases, US-guided FNAC has been reported as highly accurate.

Finally, US is highly useful also for guidance of core needle biopsy (CNB). In the last decade, several papers described the use of CNB as a second-line approach to assess those thyroid nodules with prior indeterminate or inadequate FNAC report (Trimboli and Crescenzi, 2015). This biopsy has been included in AACE/AME/ETA guidelines (Gharib *et al.*, 2016). In **Fig. 7** is shown a US scan during thyroid FNAC.

The "Problem" of Small Non-palpable Thyroid Nodules

Thyroid micro-nodules (i.e. nodules with larger diameter below 1 cm) may be detected at US in up to 70% of adult European population and the large majority of these are detected during US of other neck structures (i.e., carotid, jugular veins, etc.) (Horvath *et al.*, 2009) Rarely, one micro-nodule harbors a cancer, and this is generally a well-differentiated papillary micro-carcinoma with indolent behavior. It is recognized that US has poor accuracy in lesions with size <1 cm, and the above mentioned US risk factors are difficult to assess due to the small size of the nodule. Also, FNAC may be not practicable, and cytologic sample often is unsatisfactory to achieve a diagnosis. Remarkably, there is no consensus in this context between international US systems (Haugen *et al.*, 2016; Gharib *et al.*, 2016; Horvath *et al.*, 2009; Perros *et al.*, 2014).

Table 2 Performance of US features in detecting thyroid cancer

Feature	*Accuracy*	*To be considered*
Echogenicity	Has high sensitivity: thyroid cancers are often solid and hypoechoic because of their high cells/colloid ratio	Some cancers (i.e., medullary carcinoma) may manifest as mixed or spongiform. Rarely, papillary cancer has a cystic presentation
Microcalcifications	Is the most specific feature	They may be detected in up to 20% of thyroid cancers. Macrocalcifications do not raise the risk for cancer
Hypoechoic halo	Is the most accurate feature to identify a benign nodule	Rarely, papillary cancers have a halo.
Shape	Nodules with taller than wide shape should be viewed as at higher risk	There is no a fixed cut-off to define a nodule taller than wide
Vascularization	A few cancers (up to 20%) have intranodular vascularization. The color Doppler evaluation may be performed during conventional US assessment	The enthusiastic studies of 1990s have not been confirmed. This feature has low accuracy
Elastography	Theoretically, thyroid cancer is hard. The elastographic examination may be useful to increase the sensitivity of conventional US	Different color scales and elastographic methods have been reported and future studies have to be performed before to routinely introduce thyroid elastography
Margins	Up to 30% of thyroid cancers appear with irregular, spiculated, or blurred borders	The identification of these feature requires expert US examiners

Table 3 Nodule's ultrasound features included in current classification systems

	ATA	*AACE/ACE/AME*	*BTA*	*TIRADS*
Echostructure	Yes	Yes	Yes	Yes
Echogenicity	Yes	Yes	Yes	Yes
Margins/halo	Yes	Yes	Yes	Yes
Shape	Yes	Yes	Yes	Yes
Calcifications	Yes	Yes	Yes	Yes
Vascularization	No	Yes	Yes	Yes
Elastography	No	Yes	No	No
Size cut-off to select for FNA	Yes	Yes	No	No
>1.0 cm	High or intermediate suspicion	high risk		
>1.5 cm	Low suspicion			
>2.0 cm	Very low suspicion	intermediate or low risk		

Cervical Ultrasonography in the Follow-Up of Thyroid Cancer

After initial treatment, i.e. surgery and iodine-131 ablation, differentiated thyroid carcinomas (DTC) recur in about 20%. In the vast majority of these cases the relapse of disease occurs in the neck, being frequently discovered in cervical lymph nodes and rarely in the thyroid bed (Haugen *et al.*, 2016; Gharib *et al.*, 2016). Then, US is essential to be performed routinely during the follow-up of these patients. In 2013 the European Thyroid Association (ETA) task force on US in thyroid cancer follow-up has assessed a sort of guidelines on this topic. There, sensitivity and specificity of several US signs were reported. Of very high utility for clinical practice, the authors described the rate of non-metastatic lymph nodes with specific US signs; as the most relevant annotation, microcalcifications and cystic changes are never recordable in normal nodes, while round shape, peripheral vascularization, and hyperechogenicity are rarely present in these nodes. Thus, these have to be taken into account as major risk factors (Leenhardt *et al.*, 2013).

In addition to the in-office work, US should be useful in the operating room during surgery; this intraoperative US examination can significantly improve the localization of metastases to be excised reducing the risk of further operations. In **Fig. 8** is illustrated a metastatic cervical lymph node detected at US.

Thyroid Scintigraphy

Radioactive iodine was first employed for the diagnosis and therapy of thyroid diseases in the 1950s and thyroid scintigraphy with either iodine or iodine-analogue isotopes remains the only method able to characterize autonomously

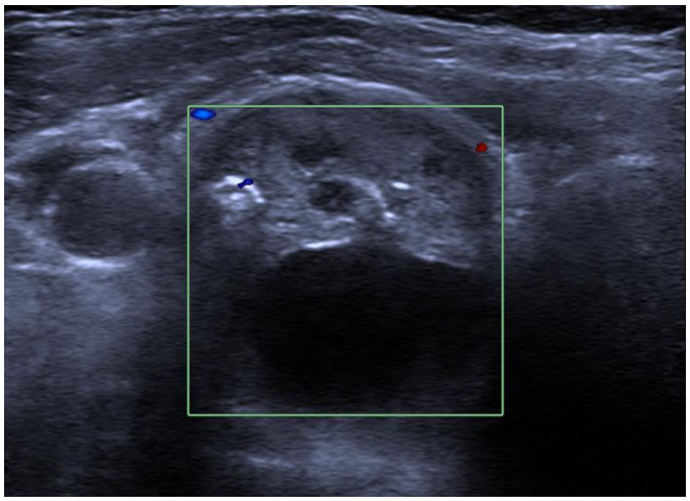

Fig. 4 A spongiform nodule with anechoic area of the right thyroid lobe: this lesion can be assessed as not suspicious for malignancy.

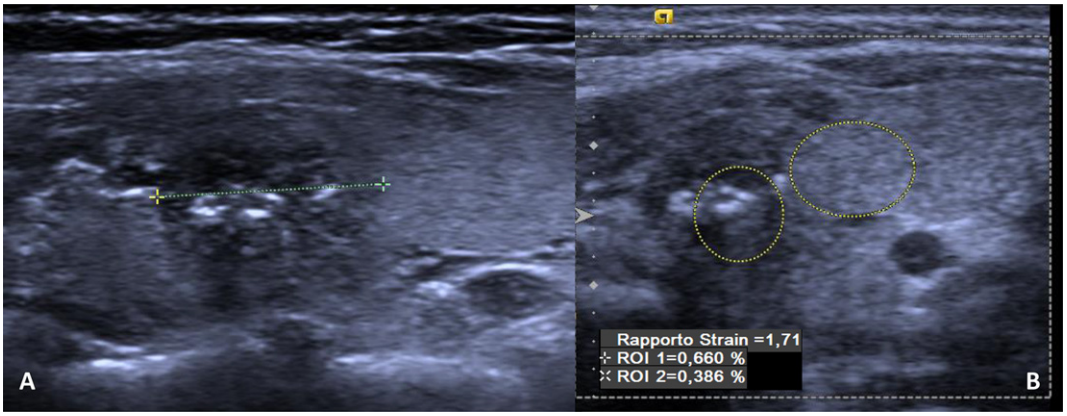

Fig. 5 Suspicious thyroid nodule at ultrasonography: the lesion is solid, hypoechoic, with microcalcifications and blurred margins (A); at elastography a high strain ratio between normal parenchyma and nodule is observed (B).

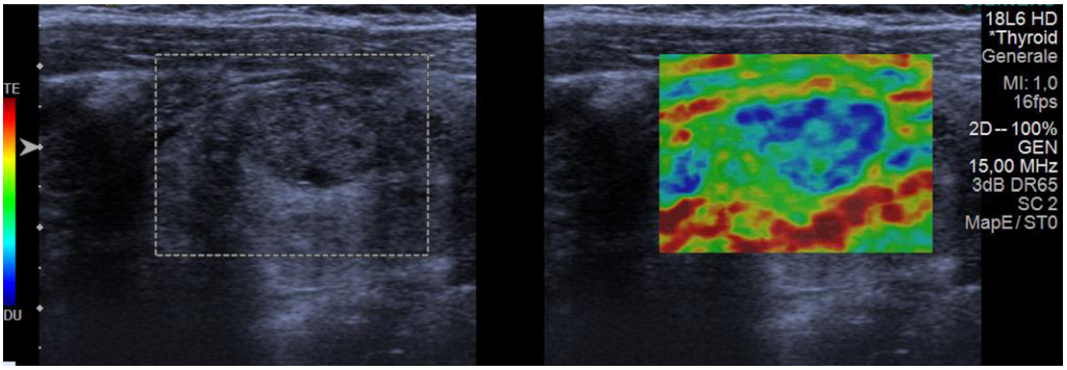

Fig. 6 Sonoelastography: the inelastic thyroid nodule appears as blue (hard) while the surrounding tissue is green (soft).

functioning nodules (Haugen *et al.*, 2016). Additionally, whole body scan (WBS) is pivotal in patients (to be) treated with radioiodine for DTC. Recently, tracers to evaluate the proliferation rate of the thyroid cells became also available. Finally, novel imaging technologies such as single-photon emission computed tomography (SPECT) and hybrid SPECT/computed tomography (SPECT/CT) consistently increased the quality of nuclear medicine images allowing sophisticated quantification procedures (Gharib *et al.*, 2016). Basically, thyroid scintigraphy may performed by using radiotracers describing the function of follicular cells and radiotracers mapping the proliferative activity of follicular cells, respectively.

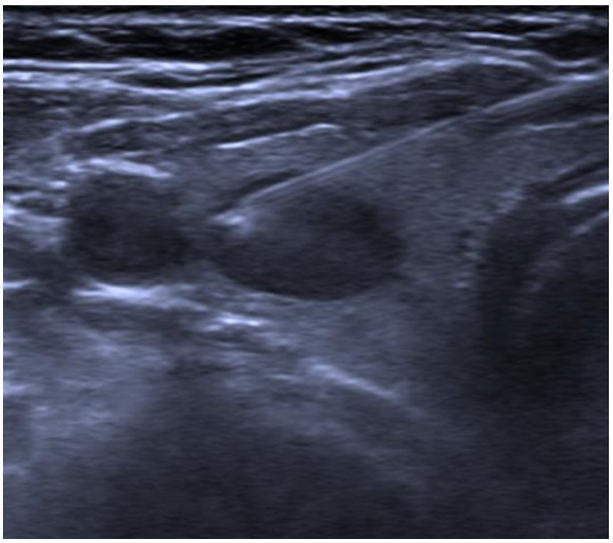

Fig. 7 Ultrasound-guided FNAC: the tip of the needle is placed inside the lesion assuring the optimal cytologic sampling.

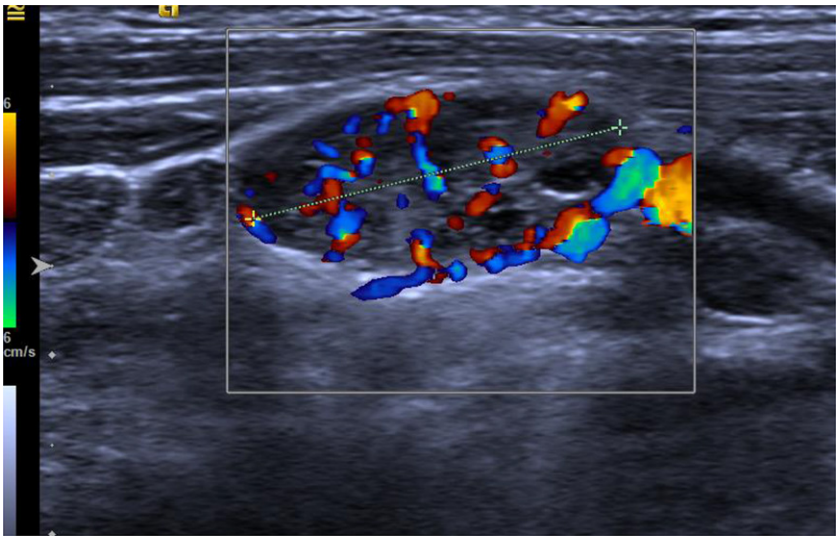

Fig. 8 Ultrasound presentation of a neck lymph node metastasis: the node is hypoechoic with cystic areas and increased and irregular vascular signal.

Tracers Mapping the Function of Follicular Thyroid Cells

Normal thyroid tissue is characterized by the unique capability of its follicular cells to trap and to process stable iodine (I) which is subsequently incorporated in thyroglobulin (Tg) in order to form thyroid hormones. The I uptake into the follicular cells is regulated by the sodium-iodide symporter (NIS), a transmembrane protein that carries sodium and iodine from the blood into the follicular cells (Dohán *et al.*, 2003). The NIS allows the thyroid trapping of different radioactive thyroid tracers. Iodine-123 (^{123}I) is an ideal thyroid radiopharmaceutical due to low radiation burden and optimal imaging quality, as opposed to the use of iodine-131 (^{131}I), which is strongly discouraged for routine diagnostic use (excepted thyroid cancer management) because of its much higher radiation burden to the thyroid. The thyroid uptake of a different tracer, ^{99m}Tc-pertechnetate, is also related to NIS expression. Importantly, it is not a substrate for any metabolic pathways and a complete washout from thyroid cells occurs in about 30 min. However, although the thyroid does not organify ^{99m}Tc-pertechnetate, in the majority of cases the uptake and imaging data provide all the information needed for accurate diagnosis (Giovanella *et al.*, 2014). ^{99m}Tc-pertechnetate has a shorter half-life and a preferred energy for scintigraphic imaging compared to ^{123}I. Additionally, it is cheaper and readily available in nuclear medicine departments. As a consequence, it has generally been adopted as the primarily used thyroid tracer in clinical practice (Meller and Becker, 2002).

Tracers Mapping the Proliferation of Follicular Thyroid Cells

The ^{99m}Tc-MIBI is a lipophilic cation that crosses the cell membrane and penetrates reversibly into the cytoplasm via thermodynamic driving forces and then irreversibly passes the mitochondrial membrane using a different electrical gradient regulated by a high negative inner membrane potential (Heston and Wahl, 2010). The cancer cells, with their greater metabolic turnover, are characterized by a higher electrical gradient of mitochondrial membrane, thus determining an increased accumulation of ^{99m}Tc-MIBI compared to normal cells. The characteristics of various nuclides used for the visualization of the thyroid gland are shown in **Table 4**.

Instrumentations and Methodology

Thyroid scans are obtained by a gamma-camera equipped with a parallel-hole collimator. Sometimes dedicated "pin-hole" collimators are employed to increase focal resolution but a significant geometric distortion should be taken into account in this case. Planar images, acquired in the anterior view for some minutes, provide a reliable map of thyroid function and metabolism. Whole-body scans are obtained by double-head gamma camera to obtain simultaneously anterior and posterior images covering the entire body. Additional SPECT and SPECT/CT images may be very useful in case of intra-thoracic goiter, ectopic thyroid tissue and thyroid cancer metastases. **Table 4**.

In addition to qualitative evaluation of thyroid maps, tracer uptake can be measured by semi-quantitative indexes (i.e. TcTU: ^{99m}Tc-pertechnetate thyroid uptake, RAIU: Radioactive Iodine Uptake). Remarkably, the uptake of these tracers is strictly related to the stable I in plasma and any overload of I [due to dietary intake (i.e. kelp), drugs (i.e. amiodarone), or iodinated radiological contrast media] causes a competitive interference with the radioactive tracer uptake by the NIS (Reinhardt *et al.*, 1998). In addition, medication such as thyroid hormones and anti-thyroid drugs affect the pituitary-thyroid axis and, thereby, the tracer uptake by the thyroid gland. Therefore, a thorough medical history should be obtained prior to administration of the radiopharmaceutical, and interfering drugs should be discontinued for an appropriate period of time (i.e. T3: 2 weeks, T4: 4 weeks, anti-thyroid drugs: 3–7 days) and, if necessary, the investigation should be delayed correspondingly.

Clinical Applications of Thyroid Scintigraphy

Thyroid Imaging with ^{99m}Tc-Pertechnetate and ^{123}I-Iodide

Thyroid scintigraphy, with both ^{99m}Tc-pertechnetate and ^{123}I, reflects the metabolic rate of thyroid cells and are mainly employed to distinguish different causes of hyperthyroidsm and to assess the functional activity of thyroid nodules. In addition it is also performed to detect and locate ectopic thyroid tissue (including the differential diagnosis of congenital hyperthyroidism).

Hyperthyroidism

The etiology of hyperthyroidism should be determined in order to correctly address the treatment. Thyroid uptake measurements are indicated when the diagnosis is in question (except during pregnancy and usually during lactation) and distinguishes causes of hyperthyroidism having elevated uptake over the thyroid gland (i.e. true hyperfunction) from those with near-absent uptake (i.e. thyrotoxicosis due to destructive thyroiditis or exogenous interferences) (**Fig. 9**). Radioactive iodine uptake (RAIU) test was traditionally indicated in such cases, especially by US thyroidologist; however it may be easily substituted by ^{99m}Tc-pertechnetate scan that is immediately available in any nuclear medicine laboratory, cheap and fast (i.e. a diagnosis is obtained in 15–20 min). Notably, a ^{99m}Tc-pertechnetate or ^{123}I-iodide scan, mapping the distribution of thyroid activity, should be always obtained when the clinical presentation suggests nodular Graves' disease, thyroid autonomy or multinodular toxic goiter.

Assessment of the functional activity of thyroid nodules

Thyroid autonomy appears as one (unifocal autonomy) or more (multifocal autonomy) hyperactive areas (i.e. hot nodules) while the tracer uptake is variably reduced in normal, TSH-dependent, thyroid tissue (**Fig. 10**). A timely diagnosis of thyroid autonomy

Table 4 Thyroid scintigraphy: tracers and procedures

	$^{99m}TcO_4$-	^{123}I	^{131}I	^{99m}Tc-MIBI
Administration	i.v.	o.a.	o.a.	i.v.
Activity (adults)	74–111 MBq	7.4–14.8 MBq	74–200 MBq	185–370 MBq
Technique	Planar (ev SPET or SPET/CT)	Planar (ev SPET or SPET/CT)	Planar whole body (ev SPET or SPET/CT)	Planar (ev SPET or SPET/CT)
Acquisition start	15 min p.i	4 and 24 h p.o.	4 and 24 h p.o.	15 and 60–120 min p.i.
Acquisition time	5 min	10 min	10 min	10 min
Effective dose (mSv/MBq)	0.013	0.20	0.20	0.009

Abbreviations: $^{99m}TcO_4$-, ^{99m}Tc-pertechnetate; i.v., intravenous; o.a., oral administration; mSV, milliSievert.

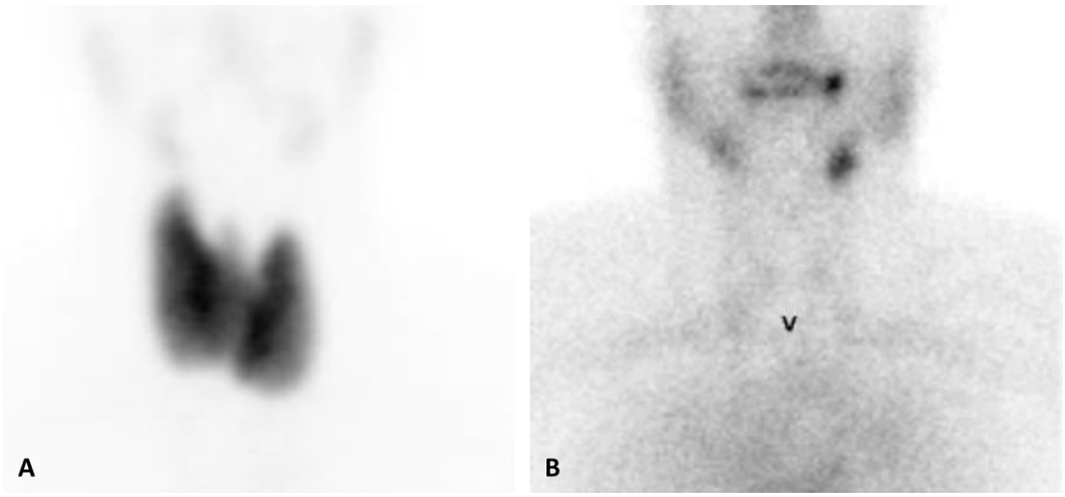

Fig. 9 ^{99m}Tc-pertechnetate scan: Graves' disease (A) and destructive thyroiditis (B).

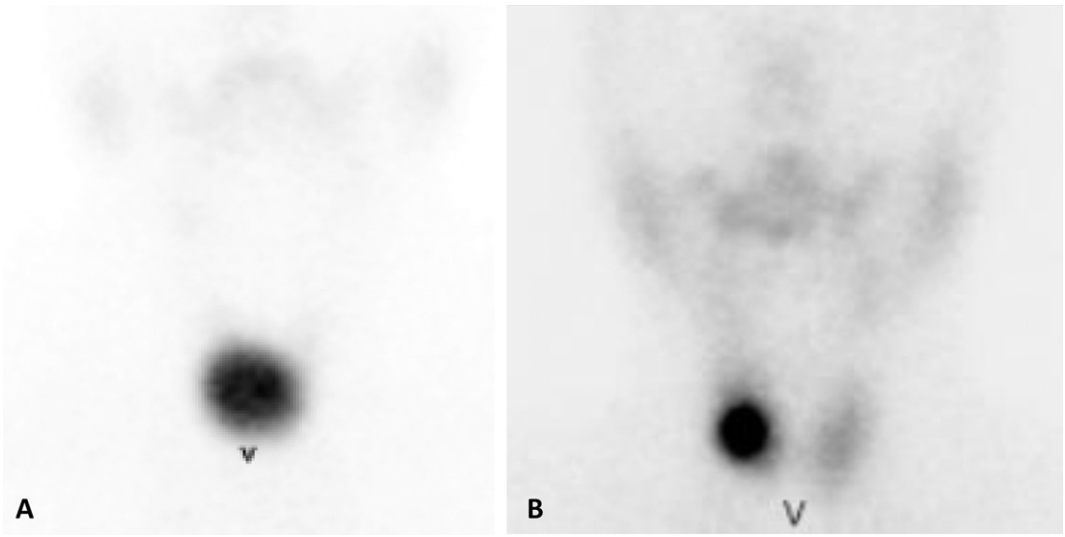

Fig. 10 ^{99m}Tc-pertechnetate scan presentation of an autonomously functioning thyroid nodules with complete (A) or partial (B) functional suppression of normal thyroid tissue.

allows early treatment avoiding progression toward manifest hyperthyroidism (Meller and Becker, 2002; Giovanella et al., 2016a, b). Of even higher importance, autonomous nodules extremely rarely harbor malignancy and current clinical guidelines suggest refraining from FNA of hyperactive nodules (Haugen *et al.*, 2016; Perros *et al.*, 2014). Importantly, although almost all thyroid cancers are nonfunctioning (i.e. cold nodules), the majority of these nodules are benign (i.e. 90–95%) which greatly reduces the specificity of a thyroid scan. Accordingly, a thyroid scan is generally performed when nodules occur in patients with low or low-normal TSH levels. However, the relationship between thyroid autonomy and TSH levels is affected by the degree of iodine sufficiency and varies widely regionally (Treglia and Giovanella, 2015; Treglia *et al.*, 2015). In summary, while autonomous nodules are almost invariably accompanied by decreased TSH levels (i.e. <0.1–0.4 mUI/L) when iodine supply is adequate, the bulk of autonomous tissue may be insufficient to suppress the TSH level in iodine-depleted thyroids, especially in the early phases of autonomy. As a consequence, different indications are given in current clinical guidelines (Haugen *et al.*, 2016; Gharib *et al.*, 2016; Paschke *et al.*, 2010; **Table 5**).

Independently from the iodine status and the TSH levels, a thyroid scan is also recommended in patients with a large multinodular goiter in order to select suspicious nodules for fine needle aspiration and/or to identify a benign compensated functioning adenoma. Thyroid scintigraphy may also help when evaluating nodules with indeterminate cytology readings (i.e. follicular proliferation) as diagnostic surgery can be safely omitted when hot nodules are demonstrated. As in very rare cases the appearance of a thyroid nodule may be discordant on radioiodine and pertechnetate scans due to iodide organification defects in the nodule that results in a rapid washout of radioiodine (i.e. so-called "trapping only nodule"), ^{123}I is recommended (Giovanella, 2009).

Table 5 Indications for ^{99m}Tc-pertechnetate and ^{123}I thyroid scintigraphy in different clinical guidelines

ATA (United States)	*AACE/AME/ETA (US and Europe)*	*DGN/DGE (Germany)*
Nodules >10–15 mm and subnormal TSH	Nodules and TSH level below the lower limit of reference range *Iodine-deficient regions*: consider performing scintigraphy to exclude autonomy for thyroid nodules even if TSH is normal.	Thyroid nodules >10 mm

Congenital hypothyroidism and ectopic thyroid tissue

^{99m}Tc-pertechnetate scan remains the most accurate test for the detection of ectopic thyroid tissue (Noussios *et al.*, 2011; **Fig. 11**). Despite new molecular genetic insight into congenital hypothyroidism the ^{123}I or, preferably, ^{99m}Tc-pertechnetate scan remains the most accurate test for the detection of ectopic thyroid tissue and the differential diagnosis between thyroid dysgenesis (60–70% of cases), athyreosis (10–30% of cases) and inherited disorders of thyroid metabolism (10–20% of cases) (Meller and Becker, 2002). In particular, thyroid scan is the highly accurate for the detection and location of thyroid dysgenesia while neck ultrasound miss the correct diagnosis in about 50% of cases (De Bruyn *et al.*, 1990; **Fig. 12**).

Thyroid Imaging with ^{99m}Tc-MIBI

^{99m}Tc-MIBI uptake within the nodule reflects its abundance of actively functioning mitochondria and therefore its oxidative burden. ^{99m}Tc-MIBI scan has been used by several authors to investigate thyroid nodules, in order to differentiate between benign and malignant nodules. This can be based on the intensity of uptake of ^{99m}Tc-MIBI within the nodule and/or by assessing an eventual increase in uptake within the nodule over time, in comparison with radiotracer wash-out from normal thyroid tissue (Theissen *et al.*, 2009). A meta-analysis (Treglia et al., 2013a, 2013b, 2013c), including twenty-one studies, demonstrated that ^{99m}Tc-MIBI scan is a sensitive diagnostic tool in predicting malignancy of thyroid nodules in which malignancy is suspected on the basis of conventional diagnostic techniques. Of note, only hypofunctioning thyroid nodules (i.e. "cold" nodules) should be evaluated by ^{99m}Tc-MIBI scan. In fact, an increased metabolic rate in hyperfunctioning thyroid nodules (i.e. "hot" nodules) is responsible for the higher ^{99m}Tc-MIBI uptake within autonomously functioning thyroid tissue. In this setting, as a second-line investigation, ^{99m}Tc-MIBI scan was found to be significantly more accurate than mutation analysis (i.e. mutations for *KRAS*, *HRAS* and *NRAS* and for *BRAF* and translocations of PAX8/PPARγ, RET/PTC1 and RET/PTC3) in patients with a cytological diagnosis of follicular neoplasm (Saggiorato *et al.*, 2009; Giovanella et al., 2016a, 2016b; Campennì *et al.*, 2016; **Figs. 13** and **14**). Therefore, combined FNA/^{99m}Tc-MIBI scan strategies are potentially cost-effective in the management of solitary or dominant thyroid nodules leading to a lower rate of unnecessary thyroidectomies (Verburg *et al.*, 2014; Wale *et al.*, 2014). Then, in patients with a thyroid nodule cytologically diagnosed as a follicular proliferation, semiquantitative analysis of ^{99m}Tc-MIBI scintigraphy should be the considered for differentiating benign from malignant nodules.

Whole Body Imaging with $^{131/123}$I-Iodide

Post-treatment whole body scan

For many years, postoperative management of thyroid cancer patients included radioiodine administration, followed by a post-therapy whole body scan (PT-WBS) obtained 2–10 days after radioiodine administration (1.1–7.4 GBq) (Luster *et al.*, 2008; Haugen *et al.*, 2016). The PT-WBS is highly accurate to show the degree of radioiodine uptake, determine the extension of the disease, identify situation at risk (requiring additional actions) and predict prognosis. Currently, SPECT/CT is added to conventional WBS: the synergistic combination of functional and anatomic information has been found to have many advantages over traditional planar imaging in different clinical settings (Avram, 2012). Advantages of SPECT/CT include accurate anatomic localization of radioiodine uptake foci and clear discrimination between normal thyroid remnant and neck lymph node metastases (**Fig. 15**). Due to CT-based attenuation correction, SPECT/CT can reveal more foci of pathologic activity as compared to planar studies, solve difficult diagnostic interpretations and reveal metastatic lesions to unexpected sites or tissues. The size and iodine avidity of metastatic lesions can be assessed on the CT and SPECT components, respectively, providing information about the likelihood of response to ^{131}I therapy properly addressing clinical decisions on alternative therapeutic options (i.e. surgical excision or external-beam radiation therapy for large lesions or targeted drugs for diffuse non–iodine-avid metastatic disease) (Chen *et al.*, 2008; **Fig. 16**). Finally, when a physiologic mimic of disease is suspected on planar images, SPECT/CT clarifies their interpretation, thereby avoiding false-positive diagnoses (Kohlfuerst *et al.*, 2009; Glazer *et al.*, 2013).Therefore, performing a PT-WBS is recommended in all patients receiving radioiodine ablation after surgery (Luster *et al.*, 2008; Haugen *et al.*, 2016). However, the current American Thyroid Association (ATA) Practice Guidelines for Thyroid Cancer Management, published in 2016 emphasize postoperative management according to stage and risk stratification based on clinical-pathologic criteria to assess the prognosis for an individual patient, decide on the use of ^{131}I therapy, decide the frequency and intensity of follow-up (Haugen *et al.*, 2016). Consequently, staging with PT-WBS cannot be performed when ^{131}I therapy is omitted with the potential to underestimate the recurrence risk in non-ablated patients.

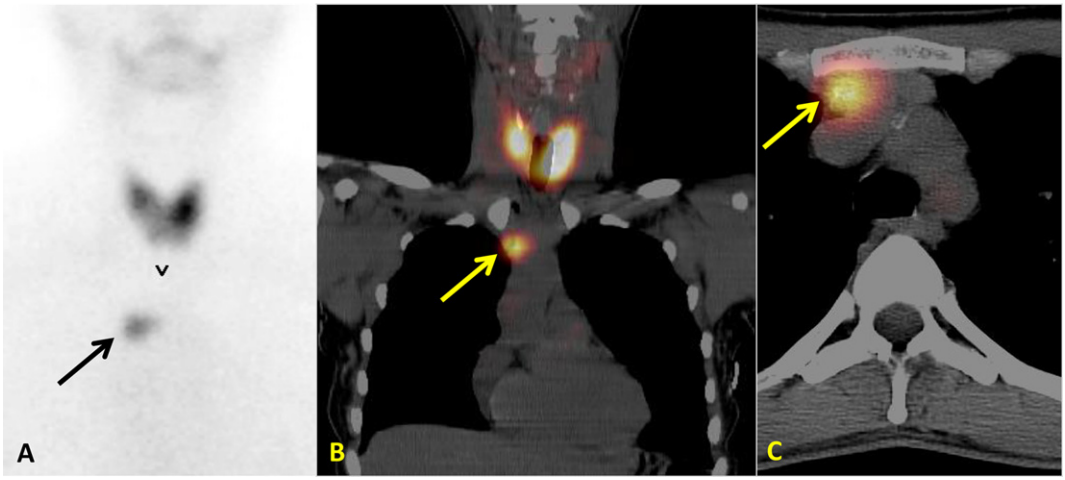

Fig. 11 ^{99m}Tc-pertechnetate planar (A) and SPECT/CT (B, coronal; C, axial) presentation of a substernal ectopic thyroid tissue (arrow).

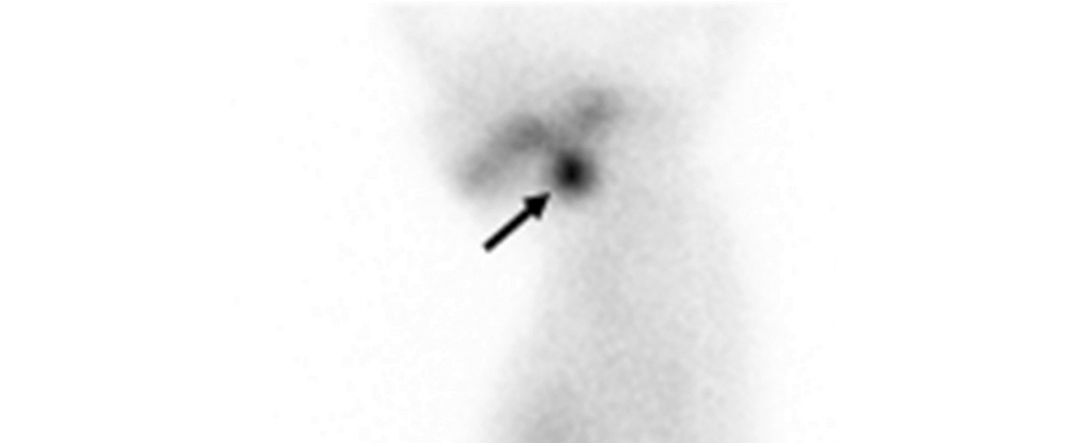

Fig. 12 ^{99m}Tc-pertechnetate scan presentation of ectopic (lingual) thyroid tissue in a newborn with congenital hypothyroidism.

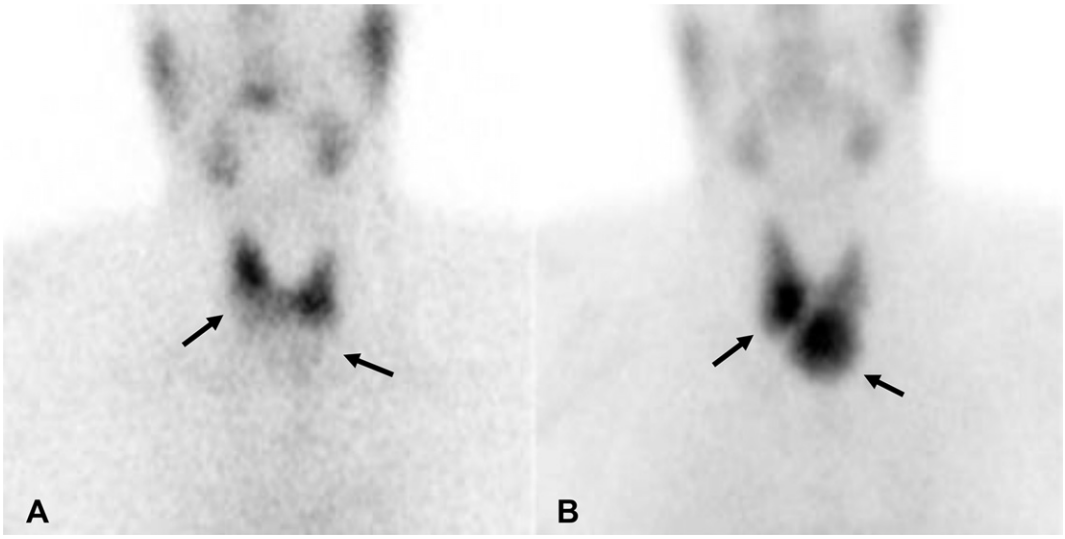

Fig. 13 ^{99m}Tc-pertechnetate (A) and ^{99m}Tc-MIBI (B) scans: cold and MIBI active nodules in both lobes (arrows). Histopathology: multifocal invasive follicular variant of papillary thyroid carcinoma.

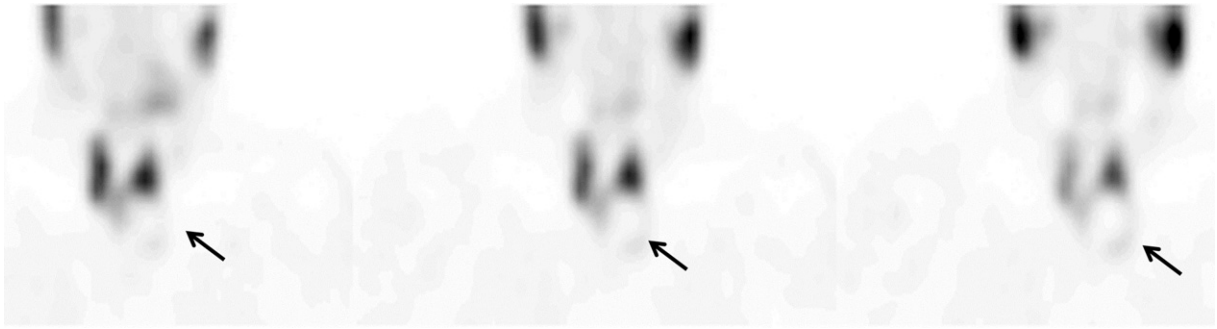

Fig. 14 ^{99m}Tc-MIBI scans of a cold nodules in the left thyroid lobe. No significant retention of the tracer is observed within the nodule (arrows). Histopathology: nodular hyperplasia.

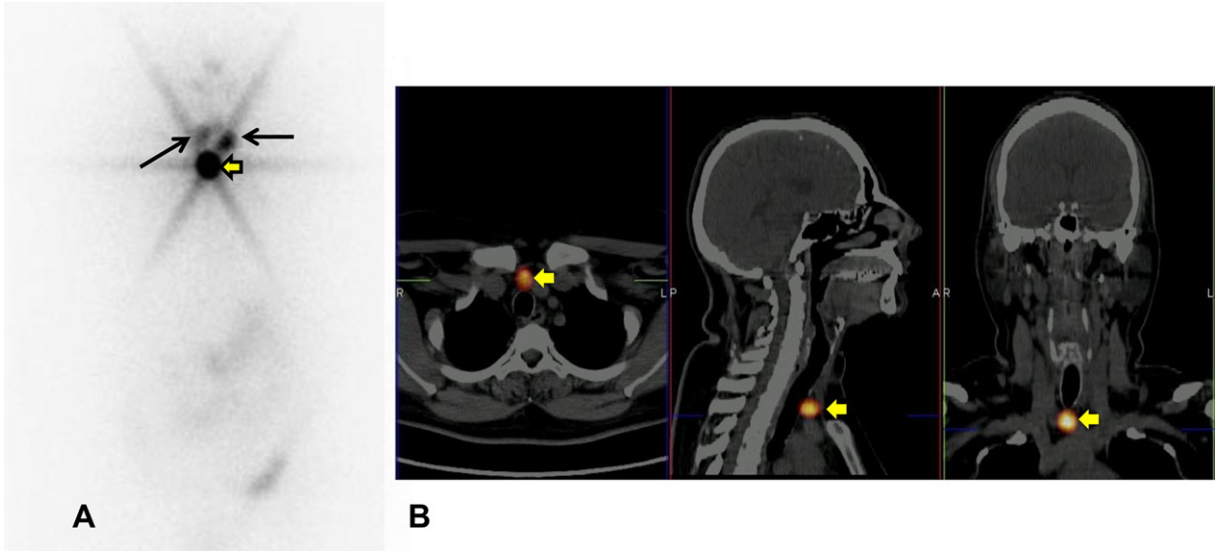

Fig. 15 Post-ablation ^{131}I WBS (A) and SPECT/CT (B) in a young female affected by papillary thyroid carcinoma (pT2 Nx) showing remnant tissue within the thyroid bed (arrows) and a central neck lymph-node metastases (yellow arrow head).

Diagnostic whole body scan (post-surgery)

When ^{131}I therapy is omitted (*see the previous paragraph*) valuable information on disease status, remnant uptake, and the presence of residual radioiodine-avid disease may be obtained by performing a diagnostic radioiodine WBS (DxWBS). This test could alter management and potentially benefit outcome as it provides the opportunity of identifying patients with unsuspected regional and distant metastases. Particularly, DxWBS can detect metastases in normal-sized cervical lymph nodes (frequently not visible on neck ultrasonography), pulmonary micrometastases (that may remain undetected on X-ray and CT scans), and bone metastases before cortical disruption is visible on bone radiographs (Avram et al., 2013, 2015). Questions regarding the potentially negative impact of DxWBS with ^{131}I on radioiodine therapeutic efficacy for successful remnant ablation (i.e. "stunning") may be mitigated or avoided by the use of either low-activity ^{131}I (74–111 MBq) or alternative isotopes such as ^{123}I (7.4–14 MBq) (Yap and Murby, 2014; Barwick *et al.*, 2010; **Fig. 17**).

The role of diagnostic whole body scan in follow-up of DTC patients

Measuring serum Tg and performing a diagnostic whole body scan (DxWBS) was for a long time the standard for follow-up of patients with DTC. More recently, however, the role of DxWBS was questioned as many studies demonstrated that PT-WBS with additional SPECT/CT is superior in terms of diagnostic accuracy to DxWBS. Accordingly, the DxWBS usually is no longer necessary in low and intermediate risk patients with negative PT-WBS scan, if complete response to therapy is achieved (Pirich and Schweighofer-Zwink, 2017). In contrast, the DxWBS may have a role in the follow-up of patients with high or intermediate risk of persistent disease especially in patients with abnormal uptake outside the thyroid bed on PT-WBS or poorly informative PT-WBS due to extensive thyroid remnants, and patients with circulating anti-Tg antibodies and associated risk of false-negative Tg measurements (Haugen *et al.*, 2016).

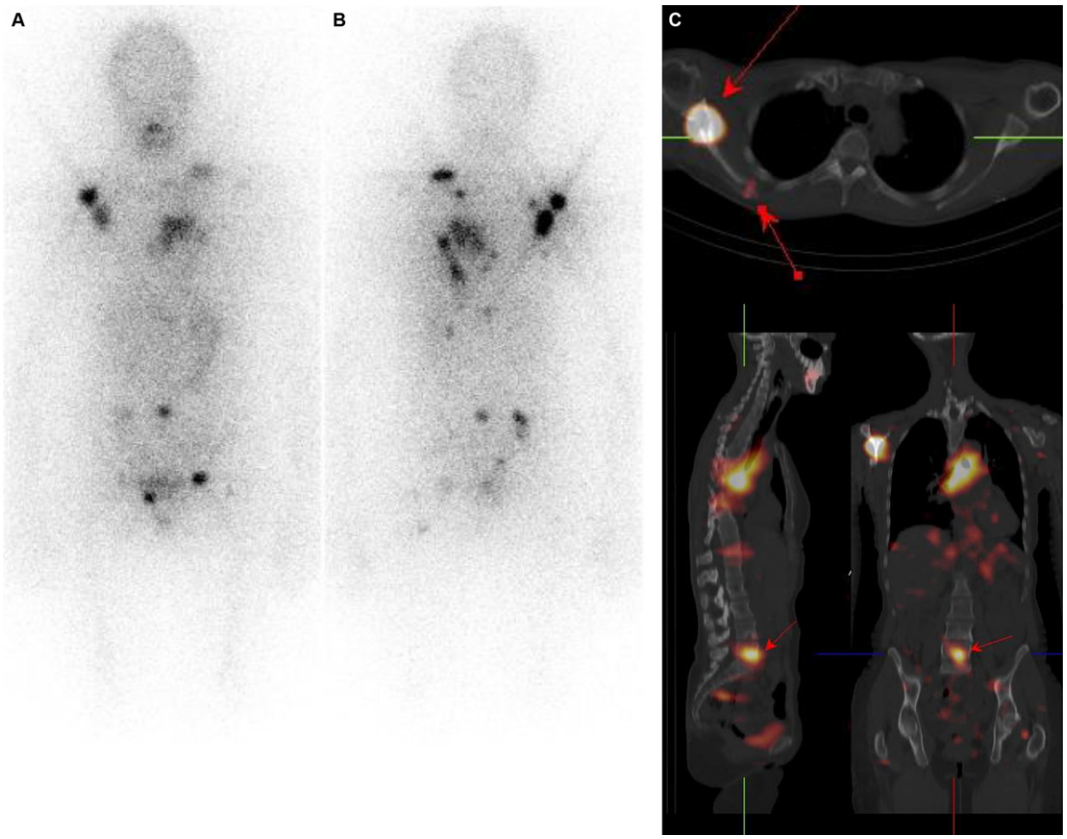

Fig. 16 Post-therapy ^{131}I WBS (A, anterior; B, posterior views) and SPECT/CT (C) in a 50 years old female affected by papillary thyroid carcinoma (pT2 N1b) showing multiple visceral and skeletal radioiodine-avid metastases.

Computed Tomography and Magnetic Resonance Imaging

Because of its high iodine content, the thyroid gland attenuates more than nearby soft tissues, appearing slightly hyperdense at CT imaging. If possible, CT should be performed without contrast media in patients with thyroid disorders and when a scintigraphy for thyroid disease is planned because the iodine load received may delay treatment with radioiodine and may also lead to thyrotoxicosis (i.e. Jod-Basedow). The normal thyroid gland has homogeneous MRI signal intensity slightly greater than neck musculature in T1-weighted images. Normal thyroid glands appear as hyperintense in T2-weighted images. Thyroid carcinomas are isointense or slightly hypointense lesions on T1-weighted images and hyperintense lesions on T2-weighted images compared with normal thyroid tissue (Hoanga *et al.*, 2013). Overall, computed tomography (CT) and magnetic resonance imaging (MRI) is not as sensitive as ultrasonography for the evaluation of intrathyroid lesions (i.e. nodules) (Loevner *et al.*, 2008). Consequently, the role of CT in thyroid disorders is mainly limited to presurgical evaluation to assess the extent of the disease, substernal components, or relationship with extrathyroidal structures, i.e., the trachea, esophagus, or vascular structures by both benign (i.e. substernal multinodular goiter) and malignant thyroid tumors (**Fig. 18**; Loevner *et al.*, 2008).

Furthermore CT/MRI scans are also be used to evaluate extrathyroidal manifestations of thyroid disorders (i.e. Graves' orbitopathy). Thyroid nodal metastases commonly occur in the central compartment (level VI) and the lateral nodal groups (levels II-IV). While US is highly sensitive in evaluating lateral neck compartments, its accuracy greatly decrease in central compartment and retropharyngeal and retroesophageal lymph node groups cannot be assessed. The presence of superior mediastinal nodes may preclude surgery for curative intent so CT or MRI may be indicated if there are predictors of mediastinal disease such as lateral nodes or large and/or invasive primary tumor. Indeed, cross-sectional imaging is pivotal if local invasion is suspected with a similar accuracy for MRI and CT in predicting local invasion of the esophagus, trachea/larynx and recurrent laryngeal nerve, the prevertebral space posteriorly and the mediastinum inferiorly (Hoanga *et al.*, 2013). The main sign for tracheal and esophageal invasion on both MRI and CT is a mass contacting the circumference of these organs. Other findings suggesting tracheal invasion are deformity of the lumen, focal mucosal irregularity or thickening, and intraluminal mass. The esophageal wall is more difficult to evaluate than the trachea because it is usually not distended with air (**Fig. 19**).

On MRI, the most suspicious finding for esophageal invasion is a focal T2 signal in the outer layer of the esophageal wall. On CT, the radiologist should look for loss of the normal esophageal wall and lumen. Invasion of the RLN can be predicted on MRI and CT by effaced fatty tissue in the tracheoesophageal groove where the nerve courses. Other imaging features of RLN invasion are signs of vocal cord dysfunction and 25% or more of the circumference of the primary tumor abutting the capsule at the posterior

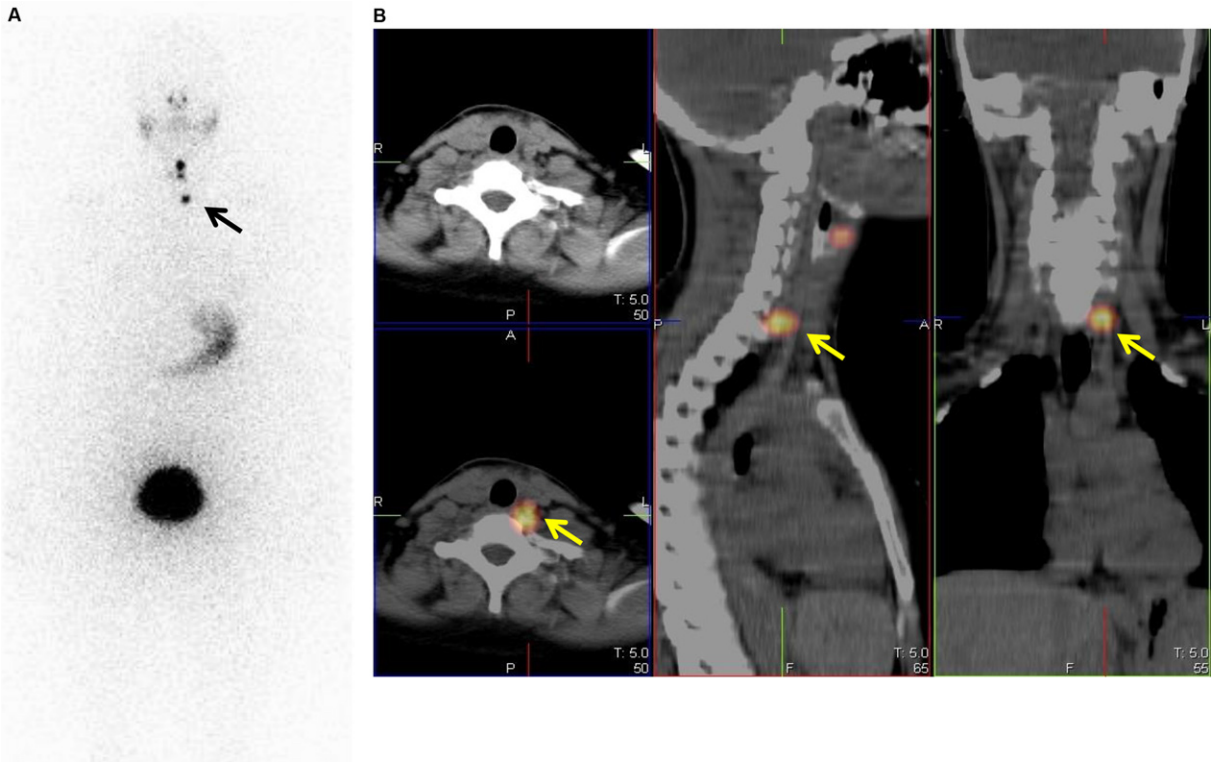

Fig. 17 Post-surgery ^{123}I WBS (A, anterior view) and SPECT/CT (B) in a 34 years old female affected by papillary thyroid carcinoma (p1b Nx) showing residual thyroglossal duct and a radioiodine-avid lymph node metastases (arrow).

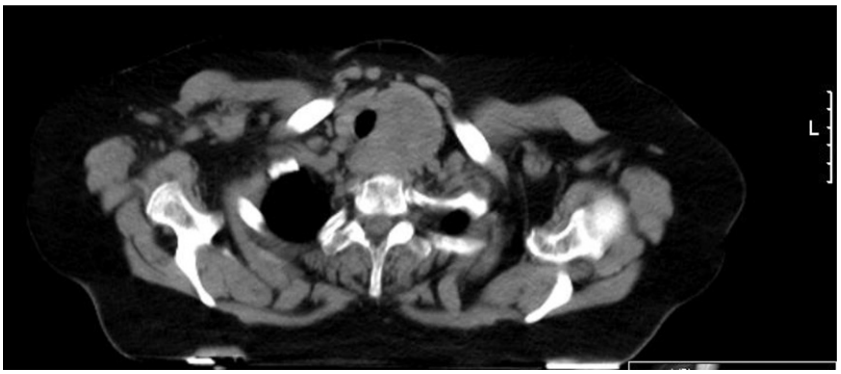

Fig. 18 Large goiter, mainly affecting the left thyroid lobe, with lateral deviation and compression of the trachea.

portion of the thyroid (sign of posterior extracapsular invasion) (Seo *et al.*, 2010). Finally, that more accurate findings for arterial involvement on CT/MRI were arterial compression/deformation or fat/fascial plane deletion. In general, these invasive findings preclude the patient from curative surgery and treatment regimen may be changed to a palliative approach (Hoanga *et al.*, 2013). After primary treatment CT/MRI can be readily performed for assessment of thyroid recurrence, even if it is not dedifferentiated or retropharyngeal nodal groups involvement is suspected (Kaplan *et al.*, 2009; **Fig. 20**).

Currently, however, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography/computed tomography (PET/CT) is generally performed in these case providing more accurate combined metabolic and morphologic data (*see the paragraph below*).

Positron Emission Tomography (PET)

PET is an established diagnostic imaging method in oncology. PET/CT is a hybrid technique which combines morphological information obtained by CT with functional data provided by PET. In the last years PET/MRI has emerged as another useful hybrid imaging technique in oncology (Treglia and Sadeghi, 2014).

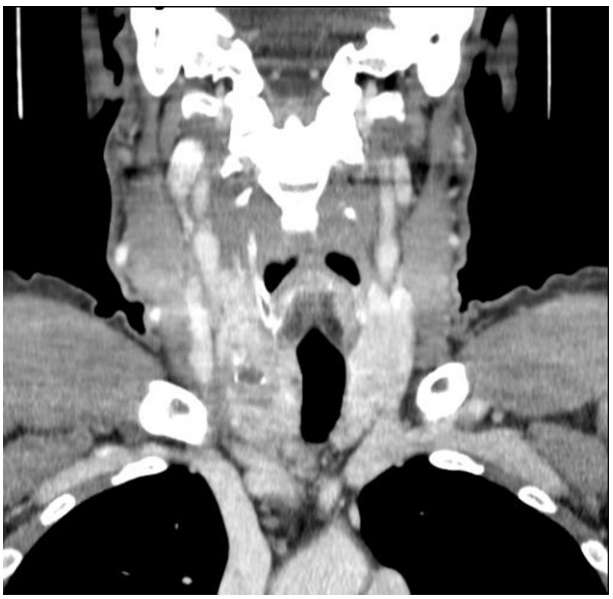

Fig. 19 Poorly differentiated thyroid carcinoma diffusely involving the thyroid gland: inhomogeneous contrast-enhancement, especially within the right thyroid lobe (also containing macrocalcifications). Mediastinal dislocation of the lower portions of the thyroid gland with mild tracheal deviation.

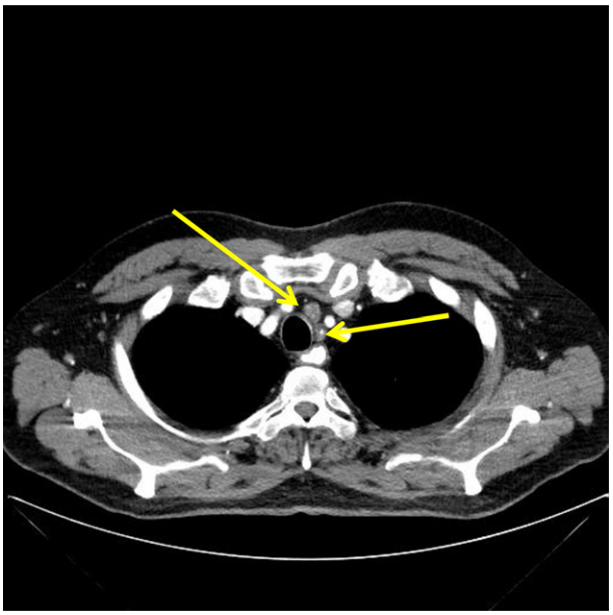

Fig. 20 Differentiated thyroid carcinoma: left paratracheal lymph node metastases (arrows) containing microcalcifications.

Table 6 The most used PET radiopharmaceuticals for thyroid tumors

Radiopharmaceutical	*Function evaluated*	*Thyroid tumors evaluated*
Fluorine-18-fluorodeoxyglucose (^{18}F-FDG)	Glucose metabolism	More aggressive thyroid tumors
Iodine-124 (^{124}I)	Iodine metabolism	Well differentiated thyroid tumors
Fluorine-18-dihydroxyphenylalanine (^{18}F-DOPA)	Amino acid uptake, decarboxylation and storage	Medullary thyroid carcinoma
Somatostatin analogues labeled with Gallium-68 (^{68}Ga-SSA)	Somatostatin receptor expression	Medullary thyroid carcinoma

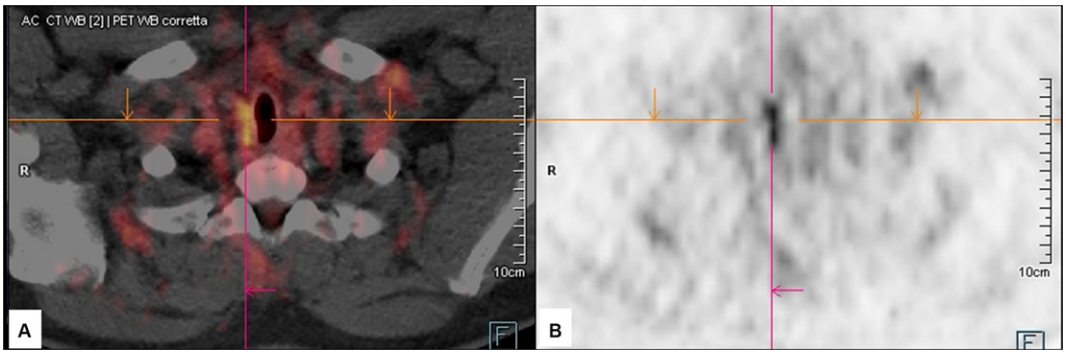

Fig. 21 Axial ^{18}F-FDG PET/CT (A) and PET (B) images in a differentiated thyroid carcinoma patient with increasing serum thyroglobulin levels after thyroidectomy and negative radioiodine whole-body scan. PET/CT showed an area of increased radiopharmaceutical uptake corresponding to a tissue located in the right thyroid bed and corresponding to DTC relapse.

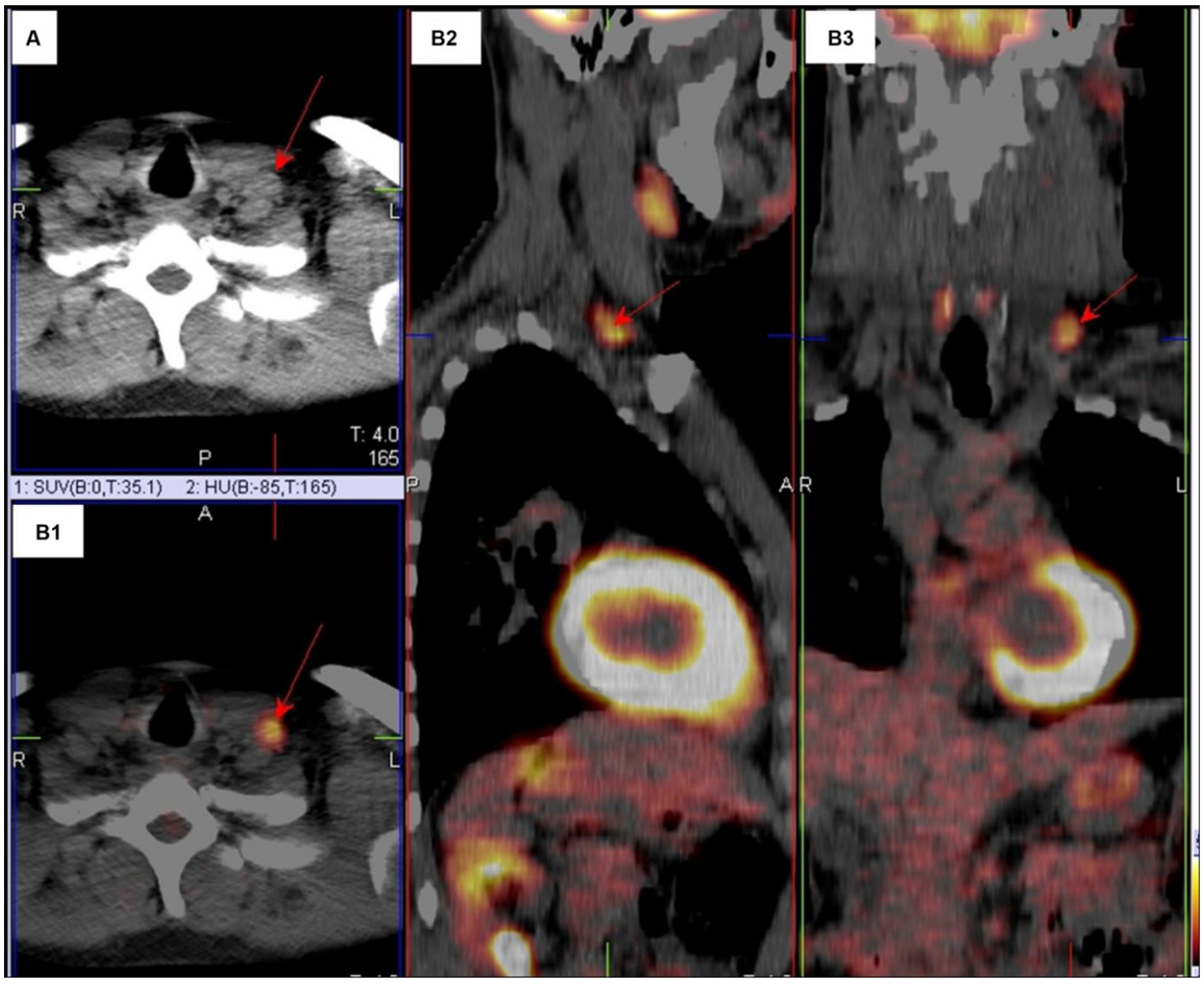

Fig. 22 Axial CT (A) and ^{18}F-FDG PET/CT images in axial (B1), sagittal (B2) and coronal (B3) projection in a differentiated thyroid carcinoma patient with increasing serum thyroglobulin levels after thyroidectomy and negative radioiodine whole-body scan. PET/CT showed an area of focal increased radiopharmaceutical uptake corresponding to a left laterocervical lymph node (arrow) suspicious for tumor relapse (diagnosis confirmed by surgery).

^{18}F-FDG is the most frequently used PET radiopharmaceutical for oncological diseases, including thyroid tumors. This glucose analogue is trapped by tumor cells via the glucose transporters (GLUTs) which are overexpressed in aggressive tumors; in addition, overexpression of hexokinase-1 promotes ^{18}F-FDG uptake in cancer cells (Giovanella *et al.*, 2013). Beyond ^{18}F-FDG, other PET radiopharmaceuticals evaluating different metabolic pathways are available to evaluate thyroid tumors (**Table 6**).

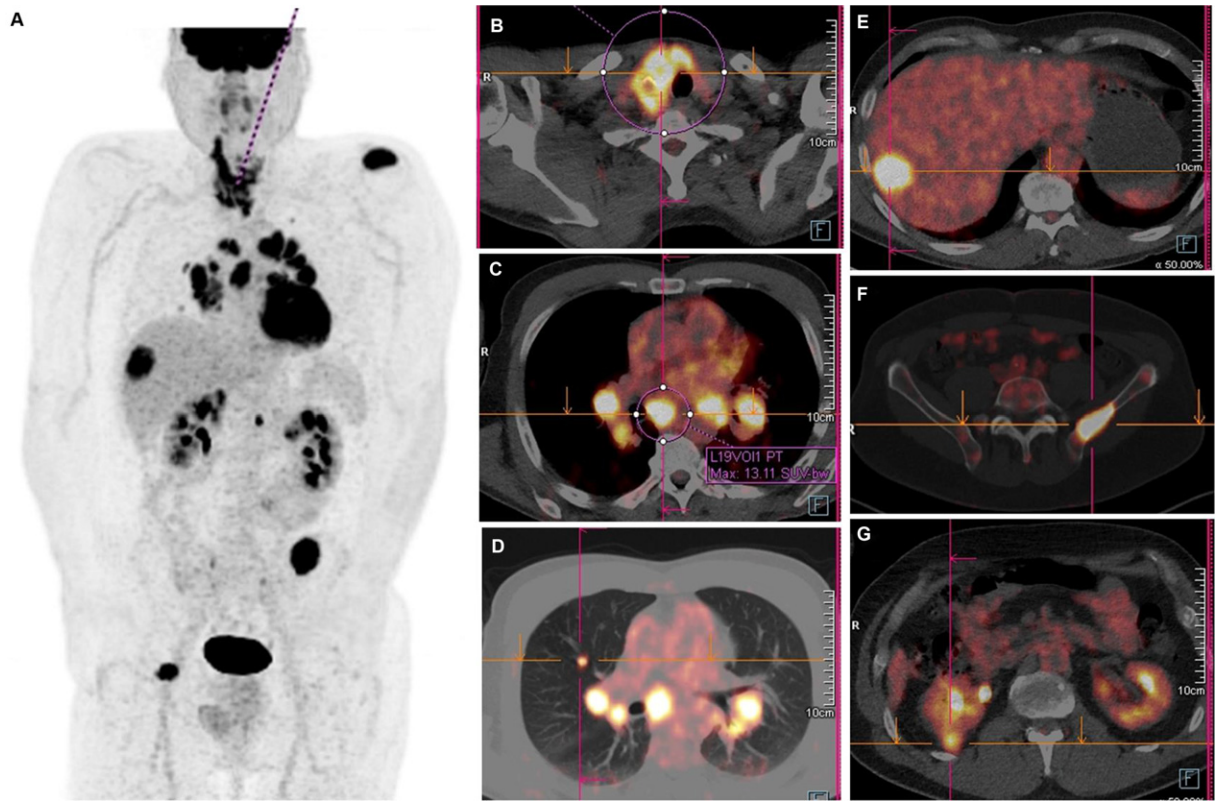

Fig. 23 ^{18}F-FDG PET maximum intensity projection image (A) and axial ^{18}F-FDG PET/CT images (B-G) in a patient with poorly differentiated thyroid carcinoma showing increasing radiopharmaceutical uptake in the thyroid (B) corresponding to the primary tumor site. Furthermore multiple lymph nodal (C), lung (D), liver (E), bone (F) and renal (G) metastases were detected by ^{18}F-FDG PET/CT.

Iodine-124 (^{124}I) has been used to evaluate the iodine metabolism in DTC. Fluorine-18-dihydroxyphenylalanine (^{18}F-DOPA), which assesses the amino acid uptake, decarboxylation and storage, and somatostatin analogues labeled with Gallium-68 (^{68}Ga-SSA), which evaluate the somatostatin receptor status, are PET radiopharmaceuticals useful for diagnosis of recurrent medullary thyroid carcinoma (MTC) (Treglia et al., 2013a, 2013b, 2013c).

During the last decades PET/CT and PET/MRI using different radiopharmaceuticals have been increasingly used in patients with thyroid tumors. Furthermore the usefulness of ^{18}F-FDG PET in assessing indeterminate thyroid nodules at fine needle aspiration biopsy (FNAB) and the clinical relevance of thyroid incidental ^{18}F-FDG uptake at PET imaging have been evaluated (Treglia et al., 2013a, 2013b, 2013c).

Role of PET in DTC

DTC cells expressing the sodium'iodine symporter take up radioiodine. In a small percentage of DTC the tumor cells are less differentiated and the radioiodine uptake capacity is reduced; these cells multiply more rapidly, their glucose metabolism is increased and consequently their ability to take up ^{18}F-FDG is higher (Giovanella *et al.*, 2013).

According to international guidelines routine preoperative ^{18}F-FDG PET is not recommended in the majority of DTC patients. In fact the sensitivity of ^{18}F-FDG PET for the detection of cervical lymph node metastases in the pre-operative setting is relatively low (30%–40%) in DTC. ^{18}F-FDG PET can also detect inflammatory lymph nodes, which reduces the specificity of this imaging method in many patients with DTC (Haugen *et al.*, 2016). Invasive DTC has been reported to occur in 10%–15% of patients at the time of diagnosis. In this group of DTC ^{18}F-FDG PET may be sensitive in some patients for neck or mediastinal involvement and may reveal distant metastases as well (Haugen *et al.*, 2016).

Currently, the most valuable role of ^{18}F-FDG PET in the work-up of DTC is in the post-operative setting, in particular in high-risk DTC patients with increasing serum Tg levels and a negative DxWBS (**Figs. 21** and **22**; Haugen *et al.*, 2016). If no disease sites are identified on conventional imaging or DxWBS or serum Tg levels are elevated out of proportion to minor disease found on conventional imaging, ^{18}F-FDG PET should be performed to detect recurrent or metastatic disease (Treglia et al., 2013a, 2013b, 2013c). In this setting ^{18}F-FDG PET/CT showed a good diagnostic accuracy with pooled sensitivity and specificity of 83% and 84%, respectively. Factors influencing ^{18}F-FDG PET/CT sensitivity included tumor dedifferentiation, larger tumor burden, and TSH stimulation (Haugen *et al.*, 2016). In patients with a TSH-stimulated serum Tg < 10 ng/mL, the sensitivity of ^{18}F-FDG PET is lower. Nevertheless it should be taken into account that in case of aggressive pathological variant of thyroid cancer low amounts of serum Tg

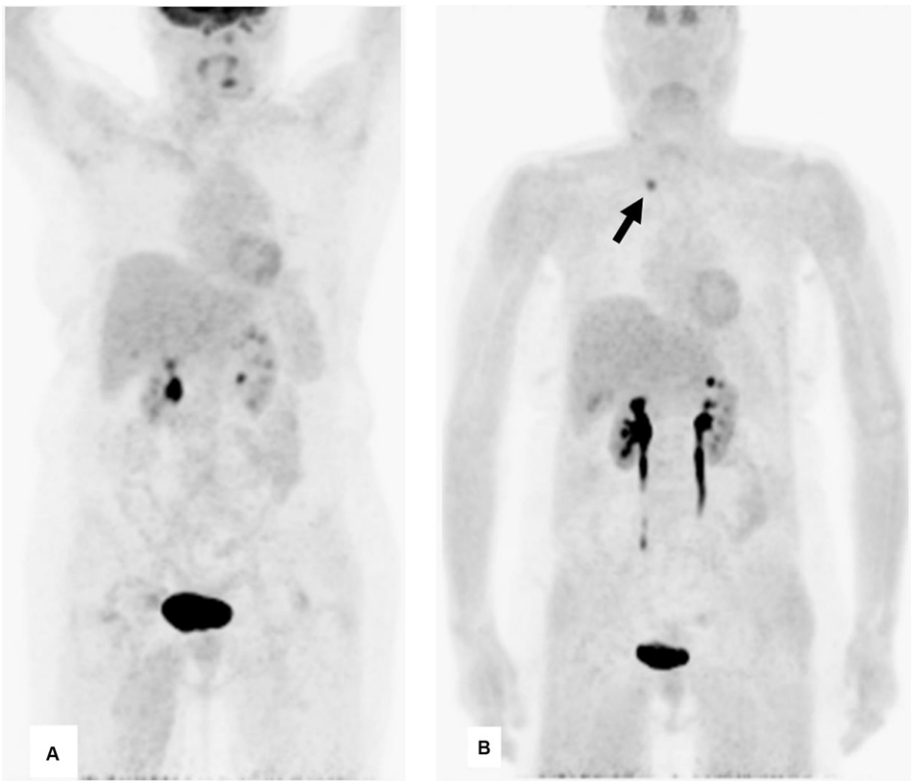

Fig. 24 ^{18}F-FDG PET (A) and ^{18}F-DOPA PET (B) maximum intensity projection images in a patient with recurrent medullary thyroid carcinoma based on increased serum calcitonin levels. ^{18}F-FDG PET was negative whereas ^{18}F-DOPA PET showed an area of increased radiopharmaceutical uptake corresponding to a right laterocervical lymph node (arrow) suspicious for tumor relapse (diagnosis confirmed by surgery).

can be produced. Furthermore, in patients with undetectable serum Tg levels but with persistent Tg antibodies the level of serum Tg cannot be reliably assessed and ^{18}F-FDG PET may localize disease in some of these patients (Haugen *et al.*, 2016). Lastly the accuracy of ^{18}F-FDG PET significantly improves when the Tg doubling time is less than 1 year, independently of the absolute serum Tg value (Giovanella *et al.*, 2013). When indicated ^{18}F-FDG PET findings might alter the indications for ^{131}I treatment or the decision for surgical removal of small tumor foci with ^{18}F-FDG uptake in some DTC patients (Haugen *et al.*, 2016).

^{18}F-FDG PET may be also useful in DTC as prognostic tool in patients with metastatic disease to identify lesions and patients at highest risk for rapid disease progression and disease-specific mortality (Treglia and Giovanella, 2015; Treglia *et al.*, 2015) and in the evaluation of post-treatment response following systemic or local therapy of metastatic or locally invasive disease (Haugen *et al.*, 2016).

About ^{124}I PET, it is used as a dosimetric and also as a diagnostic tool to localize recurrent disease in DTC (Haugen *et al.*, 2016). ^{124}I PET provides images of higher spatial resolution and lesion contrast than either planar or tomographic imaging with ^{131}I, although the impact of this improved lesion detection compared to ^{131}I imaging in DTC remains to be proven. The combination of ^{18}F-FDG and ^{124}I PET allows detection of non'iodine-avid lesions and discrimination from simultaneously occurring iodine-positive lesions, thus improving restaging in recurrent DTC. The pre-treatment dosimetry by using ^{124}I-PET may result in a significant alteration in the therapeutic procedure compared to standard therapy with fixed activities of ^{131}I (Treglia et al., 2013a, 2013b, 2013c).

Role of PET in Aggressive Histological Subtypes of Thyroid Cancer

Aggressive histological subtypes of thyroid cancer are rare and have a poor prognosis. The most important aggressive subtypes of thyroid cancer are Hürthle cell carcinoma, anaplastic and poorly differentiated thyroid carcinomas. Aggressive histological subtypes of thyroid cancer show aggressive clinical behavior with high glucose metabolism and intense ^{18}F-FDG uptake at PET. In these patients ^{18}F-FDG PET may be indicated for staging and for prognostic purposes and, in selected cases, for evaluating the efficacy of therapy (**Fig. 23**; Treglia et al., 2013a, 2013b, 2013c).

Role of PET in MTC

PET imaging with different radiopharmaceuticals is not recommended for routine initial screening of patients with a FNAB and/or serum calcitonin levels suggestive for MTC, but it may have a role in the post-operative setting in detecting recurrent MTC based on increased serum calcitonin levels. In fact, MTC recurrences are often difficult to detect using conventional morphological imaging

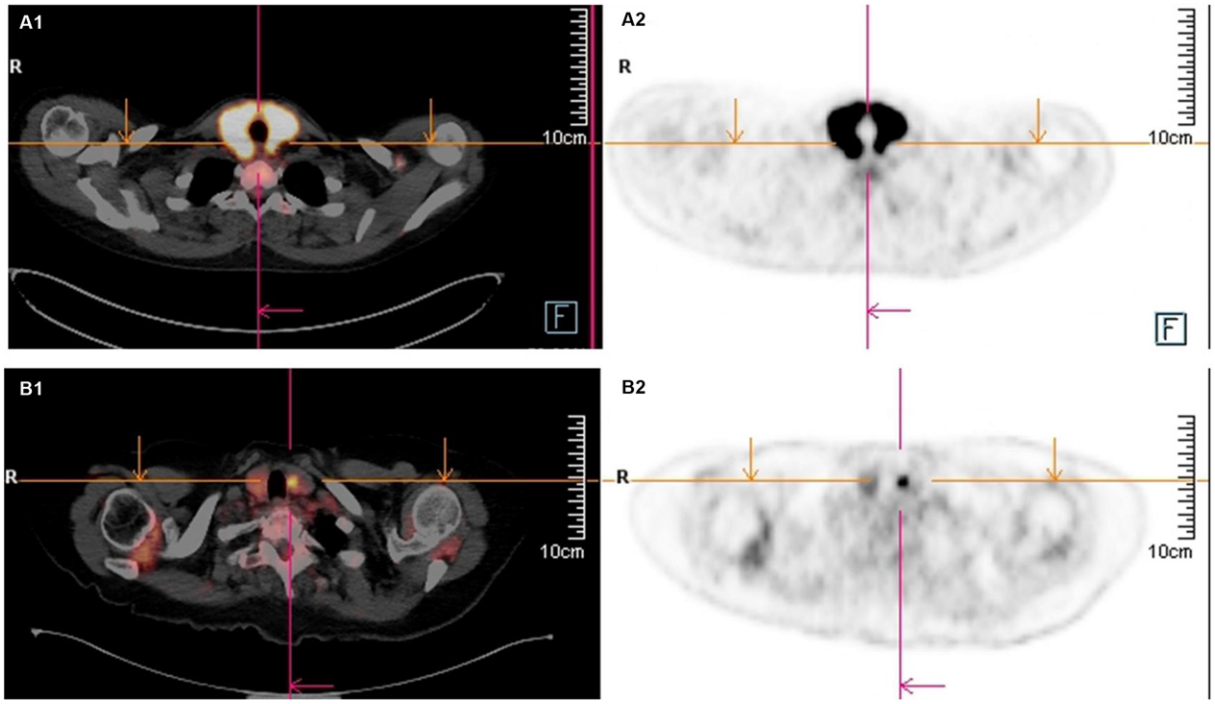

Fig. 25 Axial ^{18}F-FDG PET/CT (A1, B1) and PET (A2, B2) images showing diffuse (A1, A2) and focal (B1, B2) patterns of incidental ^{18}F-FDG uptake in the thyroid gland in two oncological patients evaluated by ^{18}F-FDG PET/CT for non-thyroid tumors.

and traditional scintigraphic methods (Treglia *et al.*, 2016). ^{18}F-FDG PET could be very helpful in detecting MTC recurrences in those patients in whom a more aggressive disease is suspected. To date, ^{18}F-DOPA seems to be the most useful PET radiopharmaceutical in detecting recurrent MTC based on rising levels of calcitonin (**Fig. 24**). ^{68}Ga-SSA PET may be useful to select patients who could benefit from therapy with radiolabeled somatostatin analogues. Anyway, the different PET radiopharmaceuticals reflecting different metabolic pathways seem to show a complementary role in detecting recurrent MTC (Slavikova *et al.*, 2013).

Role of ^{18}F-FDG PET in Thyroid Nodules with Indeterminate FNAB and Significance of Thyroid Incidental ^{18}F-FDG Uptake

According to international guidelines ^{18}F-FDG PET is not routinely recommended for the evaluation of thyroid nodules with indeterminate cytology. False-negative findings of ^{18}F-FDG PET in this setting is low; conversely, a positive ^{18}F-FDG-PET result does not identify cancer because approximately 50% of these patients had benign nodules (Haugen *et al.*, 2016).

Sometimes ^{18}F-FDG PET reveal thyroid incidental uptake (TIU) of the radiopharmaceutical which may have a focal or a diffuse pattern (**Fig. 25**). Diffuse TIU at ^{18}F-FDG PET can be considered at low risk of malignancy, being more likely associated with thyroiditis or diffuse thyroid autonomy. Conversely, focal TIU at ^{18}F-FDG PET may represent both benign and malignant lesions with a risk of malignancy of about 35%. Therefore, a complete work-up including laboratory examinations, ultrasonography and FNAB should usually be obtained to exclude malignant lesions in focal TIU detected by ^{18}F-FDG PET (Bertagna *et al.*, 2012).

See also: Amiodarone and Thyroid. Graves' Disease. Hyperthyroidism in Graves' Disease. Nontoxic Goiter. Radioactive Iodine. Thyroid Disorders in the Elderly. Thyroid Nodule. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyrotoxic Storm. Thyrotoxicosis; Diagnosis. Thyrotoxicosis Factitia. Toxic Adenoma. Toxic Multinodular Goiter

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Thyroid Fine Needle Aspiration Cytology☆

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Introduction

The fine needle aspiration (FNA) biopsy of the thyroid gland was developed at Karolinska Hospital in Stockholm, Sweden in the 1950s. A preliminary report by Söderström was published in Acta Medica Scandinavica in 1952. Thyroid nodule FNA cytology has since been recognized as the most accurate way of diagnosing thyroid cancer. However, thyroid nodules are a common clinical problem and FNA is not indicated for all thyroid nodules. The prevalence of palpable thyroid nodules in iodine-sufficient areas is estimated to 5% in women and 1% in men. When thyroid ultrasound is performed in randomly selected persons, nodules are found in as many as 19%–68%. The frequency is highest in women and the elderly. The aim should be to identify clinically relevant cancers in persons who would benefit from intervention, while avoiding excessive diagnostic procedures in persons who would not benefit from it. All patients must undergo careful clinical examination. Clinical signs and symptoms of hypo- or hyperthyroidism should be registered, as well as possible patient reports of rapid growth of the thyroid/nodule, as well as family history of thyroid disease. Palpation of the thyroid should include an estimate of the size of the thyroid, prevalence of multiple or a single nodules as well as their consistence. Serum TSH should be measured. Patients with nodules or goiter causing compression symptoms in the neck should be referred for surgery. Neck ultrasound in combination with FNA, when adequate, should be performed preoperatively. FNA should not be performed if the patient anyway is unlikely to be referred for surgery because of comorbidities and advanced age. In general, small, nonpalpable (<1 cm), incidentally found nodules can often be followed up conservatively while larger nodules (>1 cm) and those with suspicious sonographic features should undergo FNA. The Bethesda system for reporting thyroid cytopathology (TBSRTC) should be used for reporting the FNA findings.

FNA Techniques

A wide variety of needles of varying diameters and lengths are available for FNA use with 27- to 22-gauge needles used for thyroid FNA. The Zajdela technique using a bare needle without syringe or a variety of syringe holders for suction technique are used (Table 1). Cellular material is obtained by the cutting action of the trailing edge of the needle and is retained in the needle core by forward motion and capillary tension.

Most patients only experience complications similar to those of a blood draw as bleeding, bruising and local pain during the procedure. Occasionally hematoma appears after FNA of vascular lesions. Severe complications are rare.

According to the local practice, samples can be triaged for various stainings and ancillary techniques. Air dried direct smears are stained by May-Grunwald-Giemsa of Diff-Quick stains. Polychrome Papanicolaou stain requires wet fixed smears. The material can also be fixed by 95% ethyl alcohol and cytocentrifuged on slides. Liquid-based preparations concentrate cells into monolayer removing obscuring blood. Interpretation requires a learning curve.

Cell block refers to the processing of sediment, blood clots, or grossly visible pieces of tissue from cytologic specimens that are processed into paraffin block and stained by hematoxylin-eosin. Cell blocks can be prepared by various techniques, such as plasma-thrombin, agar or histogel techniques.

The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC)

TBSRTC was introduced to thyroid gland FNA diagnostics in 2007 and it has become the most widely used reporting system for thyroid cytopathology in the world. Use of the TBSRTC terminology has been endorsed by the revised 2015 ATA guidelines and 2016 AACE/ACE/AME guidelines. In addition, national endocrine and cytopathology societies all around the world recommend TBSRTC in daily practice. A second edition of TBSRTC is currently being prepared and will be available by 2018.

Introduction

TBSRTC diagnostic categories are shown in Table 2. Each category has an implied risk of malignancy and evidence-based clinical management guidelines. For some of the general categories, subcategorization is recommended.

☆Change History: February 2018. Camilla Schalin-Jantti updated the text in the chapter.

This article replaces the one by Armando Bartolazzi, Thyroid Fine Needle Aspiration Cytology, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 430-441.

Table 1 FNA techniques

Technique	*Zajdela/French technique*	*Swedish technique*
Description	Without suction, nonaspiration, capillary method	With suction
Pros	Less blood dilution	Quantitatively more material
	More concentrated specimen	Possible to drain cystic lesion
	Needle easy to conceal	
	Better fine motor control	
Cons	Quantitatively less material	More blood dilution
	Impossible to drain cystic lesion	Less concentrated specimen
		Syringe holder hard to conceal
		Worse fine motor control

Table 2 The Bethesda system for reporting thyroid cytopathology

Diagnostic category	*Risk of malignancy*	*Management*
Nondiagnostic/unsatisfactory	N.D.	Repeat FNA
Benign	0–3	Clinical follow-up
Atypia of undetermined Significance/Follicular Lesion of Undetermined Significance (AUS/FLUS)	5–15	Repeat FNA
Follicular neoplasm	15–30	Surgical lobectomy
Suspicious for malignancy	60–75	Total thyroidectomy or lobectomy
Malignant	97–99	Total thyroidectomy

N.D. not determined.

Nondiagnostic/Unsatisfactory

TBSRTC introduced criteria for adequacy as other terminology systems also did. Unsatisfactory smears contain less than six groups of follicular cells with fewer than 10 cells per group. Not preserved or poorly preserved and obscured follicular cells also belong to this category. Approximately 5%–20% of thyroid FNAs are nondiagnostic. Inflammation rich specimen are an exception to the adequacy rule: thyroid FNA with abundant inflammatory cells only can be placed in the benign category. The nondiagnostic rate can be reduced by ultrasound-guided FNA, rapid on site evaluation (ROSE) and use of liquid-based preparations. Thyroid cysts can lead to false positive and false negative results. In TBSRTC, cysts aspirates lacking follicular cells belong to the nondiagnostic category (**Fig. 1**).

Benign

A benign result is the most common thyroid FNA interpretation: 60%–70% of thyroid FNAs belong to the benign category. This category is associated with a very low risk of malignancy. Benign category results are further sub-classified as benign follicular nodules, thyroiditis, or other less common entities such as acute infection-related inflammation, amyloid goiter, and minocycline-related changes.

Benign follicular nodule

All follicular lesions are a mixture of micro- and macrofollicles. The predominant pattern should be focused. Benign follicular nodule specimen are composed of predominantly macrofollicles, honeycomb clusters and sheets and colloid. Degenerative background with macrophages and hemosiderin and oncocytic metaplasia may occur (**Figs. 2** and **3**). Benign FNAs originates from multinodular goiter, adenomatous nodule or macrofollicular follicular adenoma. Micro:macrofollicular lesions (50:50) are diagnostic pitfalls. The reason is mixed nature of the lesion or contamination by normal thyroid macrofollicles.

Thyroiditis

Chronic lymphocytic thyroiditis (Hashimoto thyroiditis) is the most common form of thyroiditis. The cytologic features include cytoplasm-rich oncocytic cells with possible atypia. On background lymphocytes/plasma cells are revealed. There may be germinal center fragments. Colloid is usually scant or absent (**Fig. 4**).

Subacute (de Quervain) thyroiditis cytologic features include granulomatous inflammation with multinucleated giant cells and lymphoid cells.

Riedel thyroiditis aspirates are hypocellular or acellular due to dense fibrosis. There may be mixed inflammation with myofibroblasts and fragments of fibrosis.

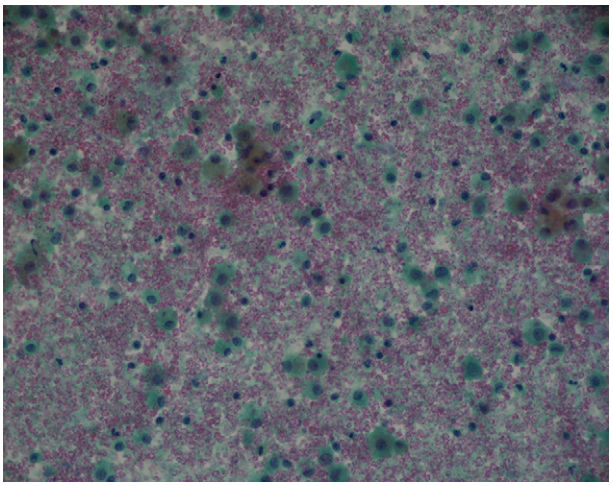

Fig. 1 Numerous macrophages and siderophages in bloody background. Cyst material only is diagnosed in TBSRTC as nondiagnostic sample. Papanicolaou stained cytospin, 400 × magnification.

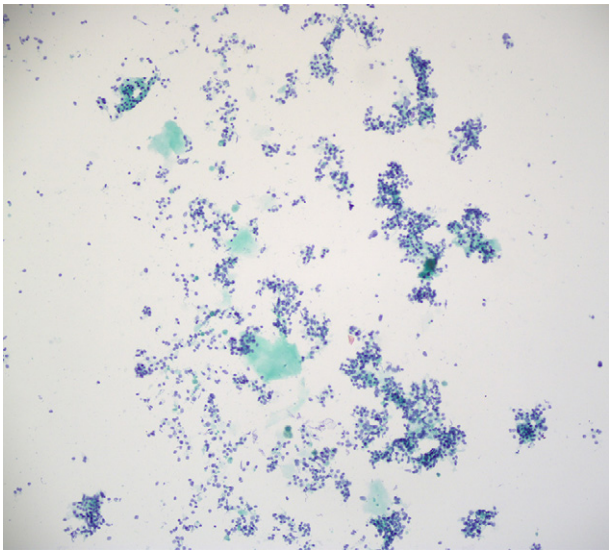

Fig. 2 Thyroid follicular cells with monotonous nuclei in variously sized groups and fragments in goiter. Notice focal oncocytic metaplasia. Colloid detected on background. TBSRTC benign category. Papanicolaou stained cytospin, 100 × magnification.

Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance (AUS/FLUS)

AUS/FLUS category is a heterogeneous and partially controversial group. Generally, samples contain architectural or nuclear atypia that is more than would be expected in benign lesions but that falls short of being suspicious for follicular neoplasm or malignancy. A specimen is often compromised by low cellularity or from artifacts such as blood contamination or air-drying.

Different scenarios for AUS/FLUS can be divided into three main categories: nuclear atypia, architectural atypia and compromised specimen. The most common different scenarios are listed in Table 3.

The risk of malignancy for AUS/FLUS in the original Bethesda system is estimated to be 5%–15%, however, the results in resected cases were reported to be on average 34%, with 6%–97% range. The reason behind this is the heterogeneity of the AUS/FLUS category. Unfortunately, not all AUS/FLUS cases are submitted to histopathological examination and a 100% cyto-histopathological correlation is rare. In the majority of the studies, less than half of AUS/FLUS cases are histopathologically verified.

Recommended management after AUS/FLUS is repeated FNA. Two AUS/FLUS result in lobectomy. Molecular markers are recommended in AUS/FLUS category in 2nd TBSRTC edition.

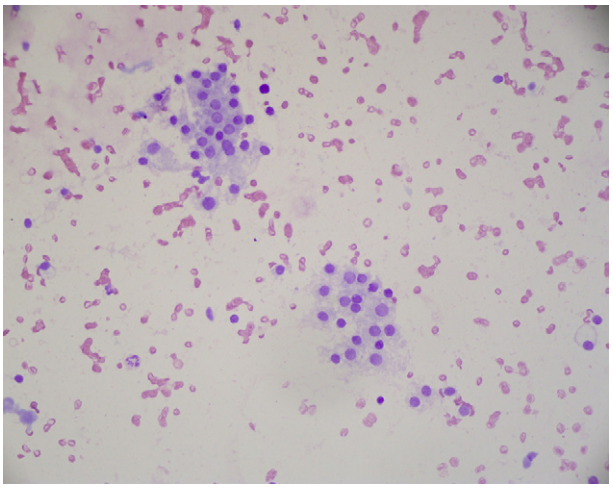

Fig. 3 Benign follicular cells in two groups in goiter. TBSRTC benign category. May-Grunwald-Giemsa stained imprint, 400 × magnification.

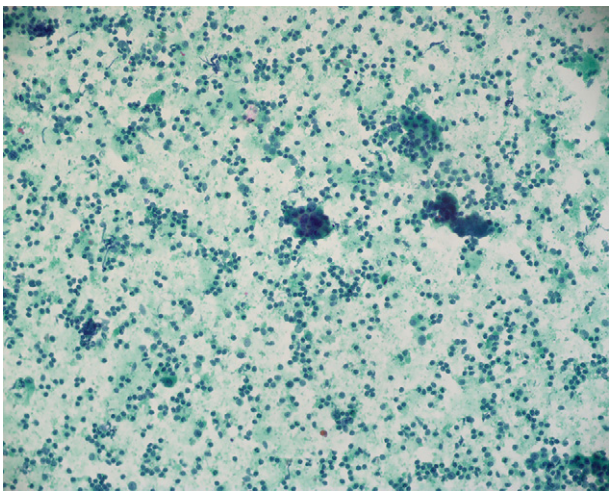

Fig. 4 Rich lymphocytes/plasma cells inflammatory background with sparse follicular oncocytic cells in lymphocytic thyroiditis. TBSRTC benign category. Papanicolaou stained cytospin, 200 × magnification.

Follicular Neoplasm/Suspicious for Follicular Neoplasm

It is not possible to distinguish between follicular adenoma and follicular carcinoma based upon cytologic material. Cytologically, cellular aspirate consists of follicular cells arranged in microfollicular or, rarely, trabecular pattern. Cells are uniform in size, significantly crowded. Nuclei are round and mildly hyperchromatic (Fig. 5). If the aspirate is composed almost entirely of oncocytes (Hürthle cells), the nodule represents oncocytic variant of follicular neoplasm and this fact should be reported.

The recommended management is surgical lobectomy. Cytology–histology correlation usually reveals cellular adenomatous nodule, follicular adenoma, follicular carcinoma, and follicular variant of papillary carcinoma.

Suspicious for Malignancy

The majority of primary thyroid malignancies have distinctive cytologic features and are easily diagnosed by FNA. The aim of separating the suspicious category from the malignant category is to preserve the very high positive predictive value of the malignant category without compromising the overall thyroid FNA sensitivity.

A suspicious for malignancy interpretation is rendered when some of the diagnostic features are either absent or equivocal. The diagnosis is an indication for surgery. Perioperative frozen section is recommended.

Table 3 Most common AUS/FLUS scenarios

Prominent microfollicular architecture
Predominance of oncocytes and oncocytes only
Nuclear features suggestive of papillary carcinoma
Focally enlarged nuclei
Atypical cyst-lining cells
Atypical lymphoid infiltrate
Clotting and air-drying artifacts

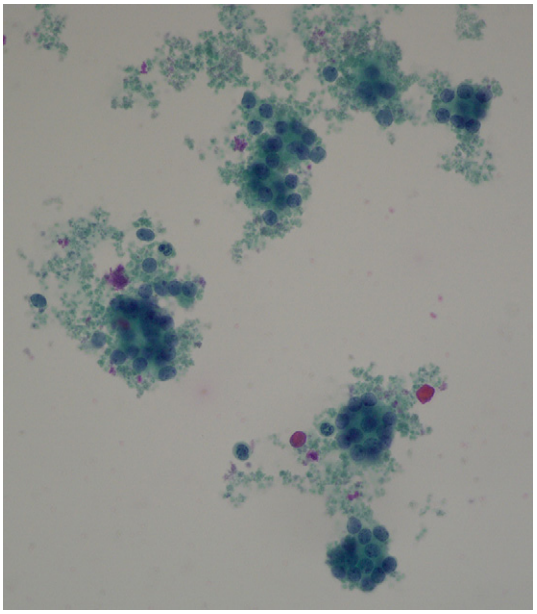

Fig. 5 Follicular cells arranged in microfollicle in follicular proliferation. TBSRTC follicular neoplasm category. Papanicolaou stained cytospin, 400 × magnification.

Malignant

A malignant thyroid FNA diagnosis accounts for 4%–8% of all thyroid FNAs and the majority of them represent papillary carcinoma. The recommended management is total thyroidectomy.

Papillary carcinoma

Papillary carcinoma is the most common endocrine malignancy with excellent prognosis for the majority of its variants. Smears are usually very cellular. Follicular cells are arranged in groups, syncytial sheets, papillary fragments and fronds. Follicular cells are cuboidal, columnar, polygonal, flattened or hobnailed. Cells are larger than normal follicular cells with increased nuclear: cytoplasmic ratio. Large usually oval or elongated nuclei have irregular membrane and are overlapping. Intranuclear pseudoinclusions, nuclear grooves and prominent nucleoli are present (Figs. 6 and 7).

Poorly differentiated carcinoma

Prognosis is intermediate between well- and undifferentiated thyroid carcinomas. Definite diagnosis requires histologic examination. Aspirates are highly cellular with monotonous, small to intermediate-sized cells containing bland nuclei. Cells in microfollicles are arranged in nests ("insulae"), trabecular, solid clusters, and as single discohesive cells. Colloid is scant, necrosis and mitoses are common.

Anaplastic (undifferentiated) carcinoma

Anaplastic carcinoma is an aggressive malignancy with rapidly progressive disease course. Isolated cells in highly cellular aspirates have marked nuclear polymorphism, prominent nucleoli and abundant cytoplasm. Dirty background contains necrotic debris, inflammatory cells and scant to absent colloid. Mitoses and apoptotic bodies are common (Fig. 8).

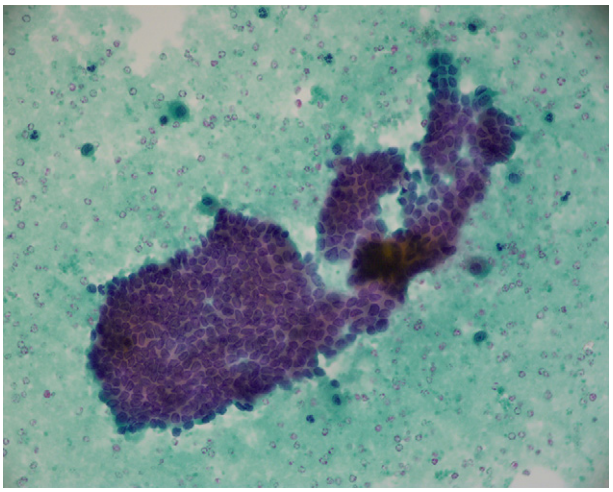

Fig. 6 Papillary fragment with enlarged cells with overlapped nuclei with irregular membrane. Notice cystic background in papillary carcinoma. TBSRTC malignant category. Papanicolaou stained cytospin, 400 × magnification.

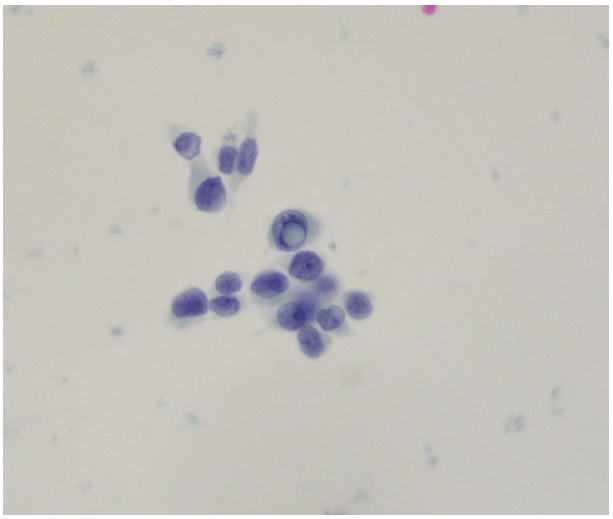

Fig. 7 Nuclear pseudoinclusion in papillary carcinoma. TBSRTC malignant category. Papanicolaou stained cytospin, 400 × magnification.

Medullary carcinoma

Medullary thyroid carcinoma arises from C-cells and comprises 5%–10% of all thyroid malignancies. Hypercellular aspirates contain loosely cohesive to noncohesive cells. In addition to single cells, small clusters are papillary-, trabecular-, microfollicular-, or syncytially patterned. Variably sized and shaped cells are spindle, polygonal, oncocytic, or bipolar with common binucleated and multinucleated cells. Amyloid can be detected on background, colloid is absent. Differential diagnosis regards other tumors, such as anaplastic carcinoma and oncocytic neoplasms may be difficult based on cytology only. However, positivity for calcitonin and other neuroendocrine markers is diagnostic. Increased serum calcitonin and CEA concentrations are hallmarks of medullary thyroid carcinoma.

Lymphoma

Malignant lymphomas can arise in the thyroid gland as primary malignancies or involvement can be part of systemic disease. The most common type is diffuse large B-cell lymphoma (60%–80%). Lymphoma is almost always associated with lymphocytic thyroiditis. Hypercellular aspirates contain monotypic lymphoid cells in noncohesive sheets of single cells. Nuclear chromatin is relatively fine. Background karyorrhexis is often extensive.

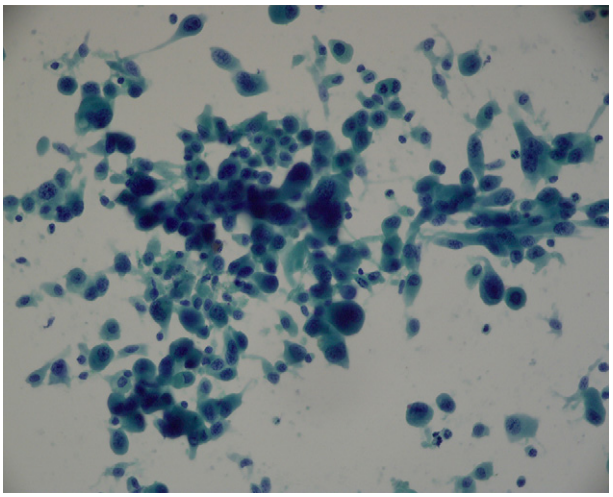

Fig. 8 Atypical cells with abundant cytoplasm with enlarged pleomorphic nuclei with prominent nucleoli in anaplastic carcinoma. TBSRTC malignant category. Papanicolaou stained cytospin, 400 × magnification.

Secondary tumors

Tumors secondarily involving the thyroid gland are metastases from distant organs or direct extension of tumors from adjacent organs. The thyroid gland is a vascular organ and therefore predisposed to metastases. The incidence varies in surgical versus autopsy study series. The incidence depends on the incidence of the underlying tumor. The involvement can form three different patterns: (1) solitary large nodules, (2) multiple small discrete nodules and, (3) diffuse involvement. The cytologic picture depends on the primary malignancy. Aspirates are cellular with two distinct cell populations. Background necrosis and mitotic figures are common. Immunocytochemistry and clinical history are of great importance.

Indeterminate Categories

The term is used for three categories: AUS/FLUS, Follicular Neoplasm and Suspicious for Malignancy. The indeterminate thyroid FNA comprises 15%–30% of all thyroid FNAs and continues to present a challenge for clinical management.

Non-Invasive Follicular Thyroid Neoplasm With Papillary-Like Nuclear Features (NIFTP)

The recently described new thyroid gland low-risk neoplasm redefines a group of follicular encapsulated or well demarcated tumors, with predominant follicular growth pattern without vascular or capsular invasion, tumor necrosis and high mitotic activity. This subset of tumors was before classified as follicular variant of papillary carcinoma. The new entity should be managed as follicular adenomas, that is, lobectomy is indicated. Cytologically, NIFTP is follicular-patterned. Nuclei are enlarged, pallor, grooved and overlapped. Pseudoinclusions are rare. Papillae and psammoma bodies are absent. As a consequence, risk of malignancy in several TBSRTC categories has changed. It has modified the cytologic criteria for classifying follicular-patterned FNAs. An optional note about possible NIFTP on selected cases is warranted.

Immunohistochemistry

Most of the thyroid lesions are relatively easy to interpret on FNA. Immunocytochemistry is the method to detect tissue antigens by utilizing specific antibodies and visualize antigen-antibody reaction by microscopically visible chromogen. Immunocytochemistry can be performed on cytological cell blocks or on cytological smears as well as on liquid-based specimens.

In thyroid FNA, immunocytochemistry can be used to differentiate follicular-derived cells from C-cells. Parathyroid lesions can be also distinguished from follicular lesions by immunocytochemistry. Poorly differentiated, anaplastic and secondary carcinoma can be distinguished from malignant lymphoma by specific antibodies. Furthermore, prognostic markers can be detected by immunocytochemistry (Table 4).

Molecular Cytology

Common genetic alterations detected in differentiated thyroid carcinomas are BRAF and RAS mutations and RET/PTC and PAX8/PPARγ rearrangements, respectively. A panel of the listed molecular alterations was successfully tested in indeterminate cytology

Table 4 Common immunohistochemical diagnostic markers used in immunocytochemistry

Follicular cells	Thyroglobulin, thyroid transcription factor-1 (TTF-1)
C-cells, medullary carcinoma	Calcitonin
Parathyroid gland	Parathyroid hormone
Papillary and follicular carcinoma	Cytokeratin-19, HBME-1, galectin-3, thyroid peroxidase (TPO)

specimens. Larger commercially available tests such as Afirma, ThyroSeq, ThyGenX-ThyraMIR and RosettaGX are available for FNA thyroid ancillary diagnostics. They are convenient in bringing objective results and in avoiding waiting for repeat FNA. Nevertheless, they increase the overall diagnostic costs.

Recently, an aberrant microRNA (miRNA) profile was described in thyroid carcinomas as compared to normal thyroid tissue. Both downregulation and upregulation of numerous miRNAs was found. miRNA-222, miRNA-221 and miRNA-146b are upregulated in papillary carcinoma. Currently, miRNA testing is not recommended as clinical routine in indeterminate cytology nodules due to overlapping profiles. Several studies demonstrated discrepancy between miRNA profiles in surgically resected tissues and FNA material.

Quality Control and Assurance

The specimen- should be monitored from the moment it arrives in the laboratory until the clinician receives the report. Turnaround time should be determined in the laboratory. Cytology–histology correlations should be systematically monitored. AUS: malignant ratio can be calculated for the individual pathologist and the whole laboratory. This can be used as a performance measure for thyroid FNA.

See also: Medullary Thyroid Carcinoma. Thyroid Carcinoma. Thyroid Nodule. Toxic Adenoma. Toxic Multinodular Goiter

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Thyroid Carcinoma☆

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Introduction

Thyroid carcinoma (TC) is the most frequent malignant neoplasm of the endocrine system, representing about 4% of all human malignant neoplasms (National-Cancer-Institute Surveillance, n.d.) and is considered a rare disease. Different histotypes are distinguished on the basis of the morphological features, degree of differentiation and cell origin (**Fig. 1**). Some of these histotypes are really very rare and require to be managed in referral center by expert endocrinologists.

Epidemiology

The TC incidence has been increased from 2.4/100.000 subjects in the 50s, up to 10.2/100,000 in the recent years (Burke *et al.*, 2005). This increase of incidence was likely due to the increase in the diagnosis of small papillary microcarcinoma (mPTC) (Davies and Welch, 2014; McLeod *et al.*, 2013) likely related to the progressive and widely use of the neck ultrasound (US) in the clinical practice. Nevertheless, recently an increase of bigger tumors and more advanced cases have also been observed, thus raising the question of whether we are facing a true increase of TC incidence (Morris and Myssiorek, 2010).

The male:female ratio is 1:3, resulting in a different incidence of 5.2/100.000 in males and 15.5/100.000 in females. The mean age at diagnosis is around 45 years but recently an increase of the mean age has been also observed with a plateau around 50 years (National-Cancer-Institute Surveillance, n.d.). Age at diagnosis is also an important prognostic factor, with a worse prognosis if the onset of the disease is >55 years (UICC, 2016). Children are very rarely affected and, although a more aggressive phenotype is observed at diagnosis, the prognosis is usually good with a very low rate of death (Sassolas *et al.*, 2013).

Cancer related death is relatively low, with a risk of death of 0.5/100,000 subjects per year in both men and women; during the last 20 years, mortality remained stable despite the increase in incidence (Davies and Welch, 2014).

Pathological Features and Classification

As shown in **Table 1**, the classification of TC is based on the degree of differentiation, on their cellular origin and morphology. Well differentiated thyroid cancer (DTC) are so defined because the tumor cells maintain the typical features of normal follicular cells such as the production and secretion of thyroglobulin (Tg), the ability to take up iodine and to respond the thyrotropin stimulus (TSH). Papillary (PTC) histotype is the most frequent representing the majority of all TC followed by follicular (FTC) and then medullary (MTC) thyroid cancer. Several variants of PTC are nowadays well recognized (**Fig. 2**) and demonstrated to have different biological behavior (Shi *et al.*, 2016). Poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC) are both rare representing 5%–8% and 2%–3% of all TC, respectively. Other tumors can be present in the thyroid such as lymphomas and sarcomas or metastases of other human malignancy (Wood *et al.*, 2004) (**Table 1**). They are all very rare but they must be taken into consideration for a differential diagnosis when an undifferentiated tumor is diagnosed.

Risk Factors

The main risk factor of DTC is represented by the exposure to ionizing radiations, especially during the period of infancy, childhood and adolescence (Veiga *et al.*, 2012). Several studies evaluated the consequences of the external radiation therapy on pediatric subjects suffering from benign diseases of the head and neck, such as tinea capitis, acne, chronic tonsillitis, skin angiomas, thymic hyperplasia. They showed a significantly higher incidence of thyroid nodules and then thyroid cancer compared to nonirradiated subjects (Brooks, 1973). Moreover, after the explosion of the Chernobyl nuclear power plant in 1986, a few years later the incidence of the DTC increased dramatically in children of Belarus and Ukraine and, to a lesser extent, in those of Russia, affected by the radioactive fallout (Kazakov *et al.*, 1992; Baverstock *et al.*, 1992).

Other risk factors are represented by the presence of a concomitant thyroid disease (e.g., chronic autoimmune thyroiditis) in the family; deficient food intake of iodine (Belfiore *et al.*, 1992); hormonal factors and pregnancy (ratio females/males close to 1 before puberty and after menopause, between 2 and 4 in the average age of life).

Living close to active volcanoes has been also reported as a risk factor to develop TC. In several volcanic island the incidence of TC is greater than in other regions and in particular in Sicily, the population living in the areas close to the ETNA volcano has a higher prevalence of TC than those living in the same region but far from the volcano (Pellegriti *et al.*, 2009).

☆*Change History:* March 2018. Rossella Elisei has updated the text of the article.

This article is an update of Martin Jean Schlumberger and Sophie Leboulleux, Thyroid Carcinoma, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 385–394.

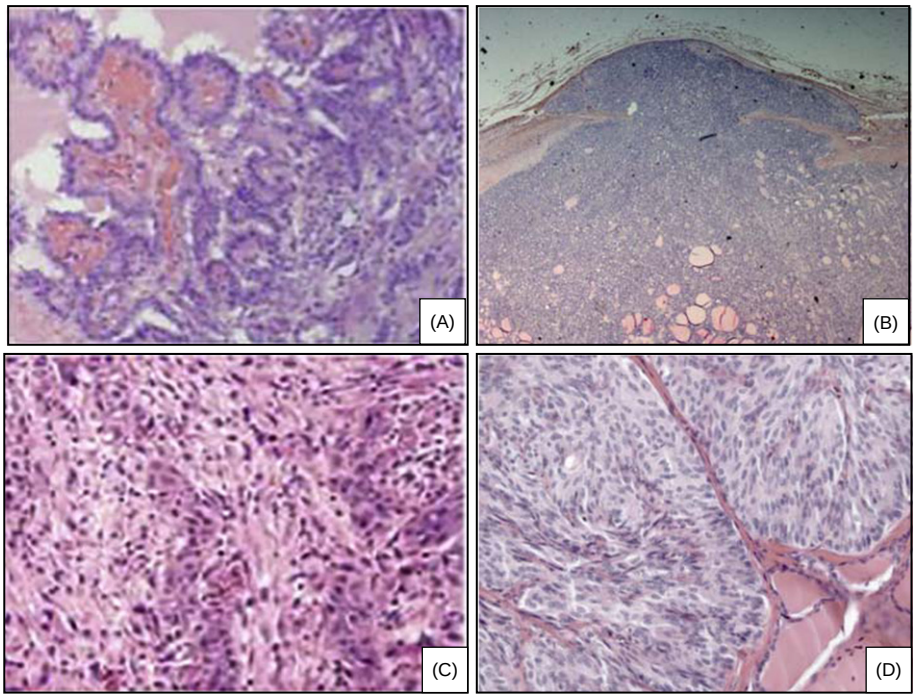

Fig. 1 Different histotypes of thyroid cancer. Panel (A) Papillary thyroid cancer; panel (B) Follicular thyroid cancer; panel (C) Anaplastic thyroid cancer; panel (D) Medullary thyroid cancer.

Table 1 Classification of thyroid cancers according with their origin, degree of differentiation and morphology

Cell origin	*Degree of differentiation*	*Histotypes, variants and prevalence (% of all TC)*
Thyroid cancers originating from follicular cells	Well differentiated thyroid cancers	- Papillary thyroid cancer (PTC), (80–85%): - Classic variant; - Follicular variant; - Diffuse sclerosing variant; - Hobnail variant; - Solid variant; - Tall cell variant; - Columnar cell variant - Hürthle cell variant (oncocytic)
		- Follicular thyroid cancer (FTC) (5%–10%): - Minimally invasive; - Widely invasive; - Hürthle cell (oncocytic) carcinoma;
	Poorly and undifferentiated thyroid cancer	- Poorly differentiated (PDTC) (5%) - Anaplastic thyroid cancer (ATC) (2%)
Thyroid cancers originating from parafollicular C -cells		- Medullary thyroid cancer (MTC), (5%): (a) Sporadic form (75% of all MTC) (b) Hereditary form (25% of all MTC): - Multiple endocrine neoplasia type 2A (MEN2A) - Multiple endocrine neoplasia type 2B (MEN2B) - Familial medullary thyroid cancer (FMTC)
Other cancer with different cellular origin but localized in the thyroid		Lymphomas Sarcomas Hemangiosarcomas Metastases from other human tumors (mainly kidney, lung, breast and colon malignancies)

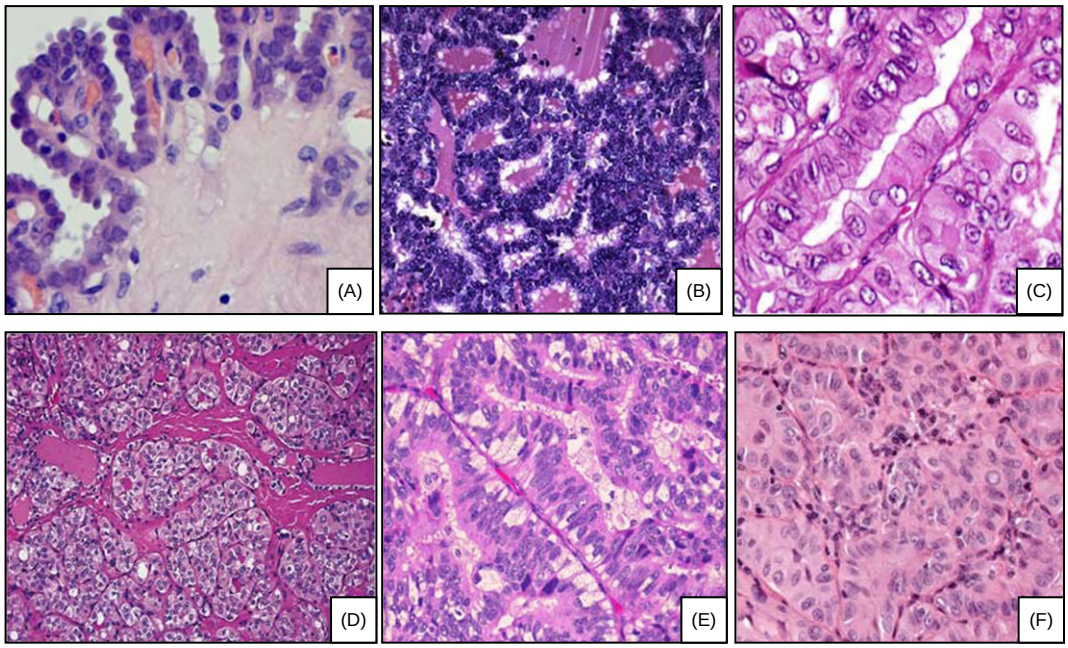

Fig. 2 Different histological variants of papillary thyroid cancer. Panel (A) Classical variant; panel (B) Follicular variant; panel (C) Tall cell variant; panel (D) Solid variant; panel (E) Columnar variant; panel (F) Hürthle (oncocytic) variant.

Obesity and several human cancers appear to be correlated (Strong *et al.*, 2017; Colditz and Peterson, 2018). As far as a relationship between TC and obesity is concerned, several controversial studies have been reported so far and this issue is still not well defined (Pappa and Alevizaki, 2014).

Recently, a few studies have shown a direct causal relationship between elevated serum TSH levels and a higher prevalence of DTC (Fiore and Vitti, 2012). On this regard, it is worth noting that elevated TSH is necessary to develop TC in mouse models transfected with oncogenes, thus suggesting a promoting role of TSH (Franco *et al.*, 2011). However, a true causative role of TSH in thyroid tumorigenesis has not been demonstrated yet.

No risk factors are known for MTC as well as for PDTC and ATC. For these latter a longstanding goiter can be considered a risk factor since in many cases they are discovered in patients with a medical history of goiter (Molinaro *et al.*, 2017). Nevertheless, there are cases of ATC rapidly developing from an apparent normal thyroid without any history and type of risk exposure.

Clinical Manifestations

The most frequent clinical manifestation of TC is a thyroid nodule, isolated or in the context of a multinodular goiter. Sometimes, a lump in the laterocervical region(s) of the neck can be the first appearance, especially in children. Only advanced cases or ATC can show up with a local symptom such as swallowing difficulties or change of the voice tone. Rarely, very advanced MTC can arrive at clinical observation for general symptoms such as diarrhea or flushing that are likely due to the elevated levels of serum calcitonin (Ct) and other peptides produced by this tumor.

PTC usually remains localized in the thyroid gland, has a rather slow growing rate and in the majority of cases the surgical treatment alone can cure the patient. In about 25% of cases, and in particular in young patients, it can metastasize through the lymphatic vessels to the lymph nodes, in particular to the cervical ones and the upper mediastinum. At variance, FTC has a more aggressive clinical behavior (Grani *et al.*, 2017). Unlike PTC, metastases occur mainly by blood vessels and are located in lungs and bones. Although more rarely, the brain and the liver parenchyma can also be sites of metastases. Frequently, bone metastases, particular in the vertebrae, can be the first clinical manifestation of FTC, especially in older patients (**Fig. 3**).

From a prognostic point of view an advanced stage and an advanced age at diagnosis are the most important poor prognostic factors for survival in all TC (Elisei and Pinchera, 2012). However, as previously said, with the exception of ATC that is almost invariably lethal, both PTC and FTC mortality is rather low and the major concern is the risk of recurrence that can happen in 15%–20% of PTC and/or FTC also several years after the initial treatment. For this reason, in 2009, the American thyroid association experts developed a new classification related to the risk of structural recurrence by defining three levels of risk: low, intermediate and high (Cooper *et al.*, 2009). This classification is very important to plan the therapeutic and follow up strategies (**Table 2**).

As far as MTC prognostic factors are concerned, also in this case an advanced stage at diagnosis and in particular the presence of distant metastases represent a high risk for death within 10 years, while the presence of lymph nodes in the absence of distant

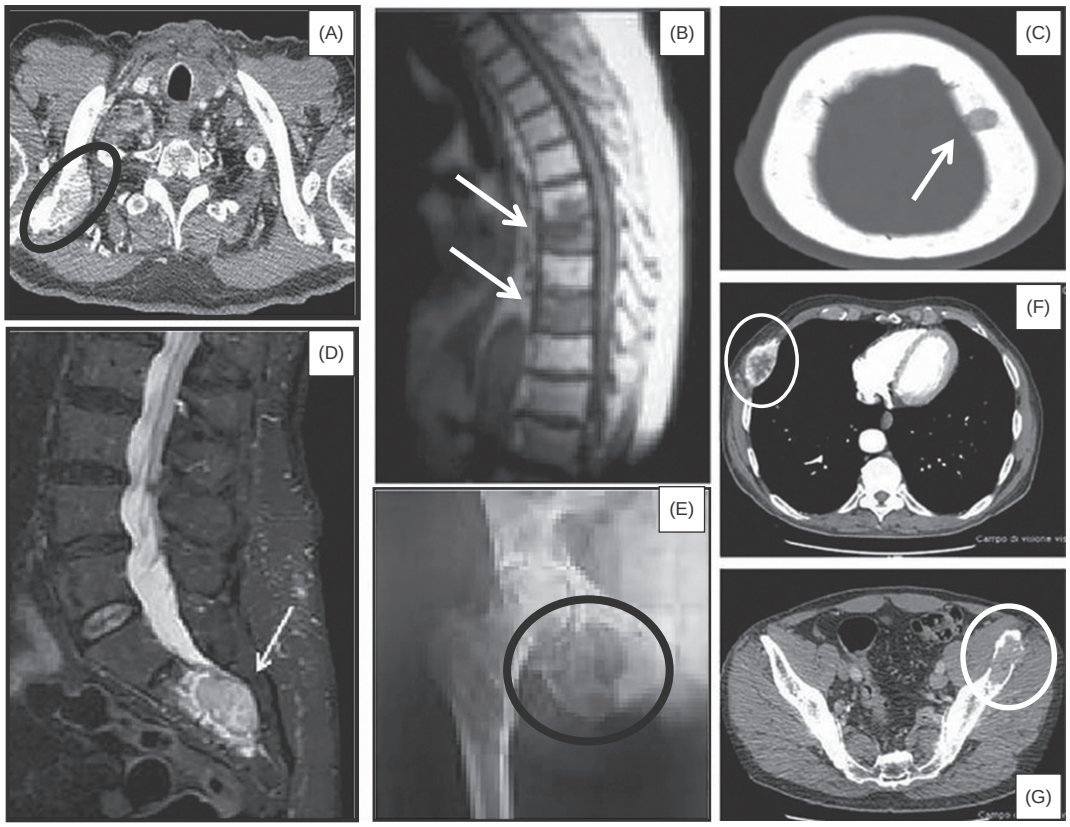

Fig. 3 Typical cases of bone metastases of follicular thyroid cancer indicated by *arrows* or *circles*. Panel (A) left clavicular lesion; panel (B) lytic vertebral lesions; panel (C) skull lesion; panel (D) sacral lesion; panel (E) right ischiatic lesion; panel (F) big lesion of the VI left rib; panel (G) left iliac crest lesion with extension in the gluteus.

metastases represents a high risk of disease persistence with a very low probability to obtain a definitive cure with the initial treatment.

Initial Treatment

Once the diagnosis of TC has been done, usually by cytology, the first therapeutic approach is surgery. A complete resection of the disease is the intent of this procedure but, since TC have different biological behaviors depending on their pathological features and particularly on the extension of the disease, a more tailored surgical approach is nowadays recommended. A similar concept has been developed for the radioiodine (131-I) treatment and, at variance with the past decades, nowadays a more personalized therapy is required thus confining the use of 131-I for the ablation of the postsurgical remnant to selected cases. This new therapeutic approach is described below.

Surgery

For many years, total thyroidectomy (TTx) and radioiodine remnant ablation (RRA) represented the initial treatment of all patients with PTC and FTC (Mazzaferri and Jhiang, 1994; Molinaro *et al.*, 2013). Although most of the DTC have a slow evolution and a favorable prognosis, they can be potentially lethal neoplasms, especially those cases, representing 20% of all DTC, that lose both the ability to produce Tg and to take up 131-I thus becoming de-differentiated and radioiodine refractory (RAR). Therefore, the initial treatment, which represents the most important step, should aim to reach a complete surgical removal of the tumor but avoiding iatrogenic complications which are more frequent when the surgery is more extended (Chen *et al.*, 2017).

Until a few years ago the surgical treatment of PTC and FTC, usually consisted in TTx in all cases. At variance, the most recent international consensus guidelines suggest that when tumors are smaller than 4 cm, unifocal, without a history of previous head and neck irradiation or familiarity for TC and the neck US can exclude the presence of cervical lymph node metastases, lobectomy alone can be sufficient and preferred for a lower risk of surgical complication (Haugen *et al.*, 2016).

When TTx is planned the question to perform also a central neck node dissection must be taken into consideration. A lot of controversial opinions and evidence have been reported about the need to add this procedure prophylactically. The higher risk to

Table 2 ATA 2009 risk stratification system with proposed modifications in ATA 2016 guidelines: indication for radioiodine postsurgical remnant ablation

Risk of structural disease recurrence	*Histological features*	*Radioiodine remnant ablation (RRA)*
Low risk (LR)	*Papillary thyroid cancer (with all of the following):* ● No local or distant metastasis; ● All macroscopic tumor has been resected ● No tumor invasion of loco-regional tissues or structures ● The tumor does not have aggressive histology (e.g., tall cell, hobnail variant, columnar cell carcinoma) ● If ^{131}I is given, there are no RAI-avid metastatic foci outside the thyroid bed on the first posttreatment whole-body RAI scan ● No vascular invasion ● Clinical N0 or $\leq$ 5 pathologic N1 micrometastasis ($<$0.2 cm in largest dimension) ● Intrathyroidal, encapsulated follicular variant of papillary thyroid cancer (NIFTP)* ● Intrathyroidal, well differentiated follicular thyroid cancer with capsular invasion and no or minimal ($<$4 foci) vascular invasion ● Intrathyroidal, papillary microcarcinoma, unifocal or multifocal, including BRAFV600E mutated (if known) *Follicular thyroid cancer minimally invasive $<$ 4 cm*	Not recommended
Intermediate risk (IR)	● Microscopic invasion of tumor into the perithyroidal soft tissues (mETE) ● RAI-avid metastatic foci in the neck on the first posttreatment whole-body RAI scan ● Aggressive histology (e.g., tall cell, hobnail variant, columnar cell carcinoma) ● Papillary thyroid cancer with vascular invasion ● Clinical N1 or $>$ 5 pathologic N1 with all involved lymph nodes $<$3 cm in largest dimension ● Multifocal papillary microcarcinoma with ETE and BRAFV600E mutated (if known)	Recommended in selected cases
High risk (HR)	● Macroscopic invasion of tumor into the perithyroidal soft tissues (gross ETE) ● Incomplete tumor resection ● Distant metastasis ● Postoperative serum thyroglobulin suggestive of distant metastasis ● Pathologic N1 with any metastatic lymph node $\geq$3 cm in largest dimension ● Follicular thyroid cancer with extensive vascular invasion ($>$4 foci of vascular invasion)	Usually recommended

have surgical complications, and in particular hypoparathyroidism, against a modest reduction of risk of recurrence makes this procedure very unpopular (Viola *et al.*, 2015). At variance, the central neck node dissection is at the present mandatory when an MTC is diagnosed. The only exception is when a prophylactic thyroidectomy is performed in children carrying a *RET* oncogene mutation without any evidence of disease and undetectable levels of serum Ct (Wells *et al.*, 2015).

The lymph node dissection of the latero-cervical compartment(s), on the other hand, is still a matter of discussion. Usually, in DTC it is performed only in case of laterocervical lymph node metastases already evident at neck US before the surgical treatment and this approach has been approved by all experts involved in the preparation of the DTC guidelines (Haugen *et al.*, 2016). At variance, no unanimous consensus was reached by the experts involved in the preparation of guidelines for MTC management (Wells *et al.*, 2015) since some of them suggested to perform laterocervical dissection based on the levels of preoperative serum Ct and independently from neck US evidences (Machens and Dralle, 2010). Sometimes echographic features of laterocervical lymph nodes are not clear enough to decide if a lymph node is metastatic or inflammatory. In these cases a fine needle aspiration cytology (FNAC) and the measurement of either Tg or Ct in the washout of the needle used for FNA will reveal the correct diagnosis (Pacini *et al.*, 1992; Boi *et al.*, 2007).

In advanced cases and in particular in ATC the TTX can be not feasible for the infiltration of other local structures such as trachea, esophagus, laryngeal nerves, and blood vessels. In these cases the surgeon should envisage if a good debulking (i.e., R1) can be obtained and in this case it is always convenient to proceed. However, if this would not be the case and just a biopsy of the tumor can be the best surgical option, it is better to decline the surgical treatment and start with other therapeutic strategies (Haddad *et al.*, 2015).

Radioiodine Therapy

Radioiodine (131-I) therapy can be used both for post surgical thyroid remnant ablation and for the treatment of distant metastases. While it is still the gold standard therapy for the treatment of distant metastases in well differentiate thyroid cancer, both PTC and FTC, its role for remnant ablation has been recently revised and limited to all high risk and some selected intermediate risk cases.

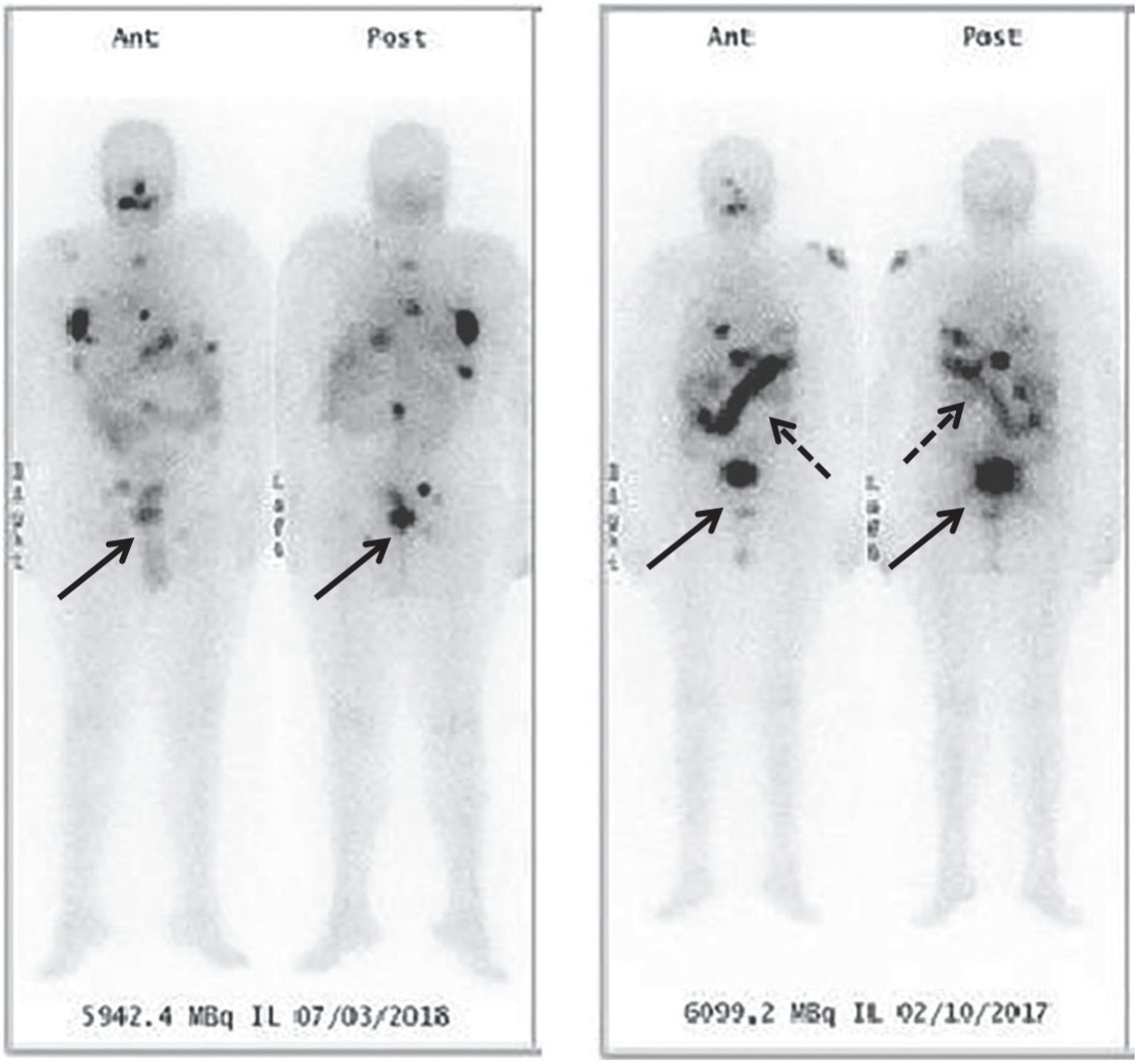

Fig. 4 Typical examples of post therapeutic whole body scans (WBS) showing several lesions still able to take up 131-I. The interpretation of the WBS must take into consideration also the physiological areas of uptake or iodine accumulation that should not be confused with metastatic lesions such as bladder (*continuous line arrows*) and colon (*dot line arrows*).

Radioiodine for remnant ablation (RRA)

The rationale of the RRA is represented by two major concerns:

1) Ablation of the normal thyroid remnant that can produce Tg which can reduce its specificity as tumor marker.
2) Adjuvant therapy of microscopic residual disease a/o RAI-avid metastatic foci, particularly in PTC which is multifocal in 40%–50% of cases.

It is well recognized that this approach facilitates the follow up of DTC, but there is currently much debate surrounding the real clinical impact of not only TTx, but especially RRA on both recurrence and survival-specific rates (Sacks *et al.*, 2010; Mazzaferri and Jhiang, 1994), particularly in low (LR) and intermediate (IR) risk DTC (Sawka *et al.*, 2008; Schvartz *et al.*, 2012; Cooper *et al.*, 2009; Haugen *et al.*, 2016). For this reason, the international referral guidelines (Haugen *et al.*, 2016; Cooper *et al.*, 2009), proposed an initial risk classification based on the histological features of DTC, to better identify the patients, mainly those with a high or intermediate-high risk tumor, who could really benefit from RRA (**Table 2**).

Usually the decision making to perform or not RRA is based on the presence of aggressive histopathological features (i.e., severe extrathyroidal extension, aggressive variants of PTC, vascular invasion, lymph node metastasis, distant metastasis). At the moment, there are no stratification analyses to determine the real efficacy of RRA on the recurrence rate during the time. Furthermore, cancer related death (CRD) is almost negligible in the LR group and very rare in the IR group thus the real clinical challenge nowadays in the management of DTC is to identify and treat the recurrences as soon as possible but avoiding the overtreatment of all the other non recurrent cases (Haugen *et al.*, 2016).

An important decision making role in the opportunity to perform RRA has been recently recognized for both serum Tg and neck US performed 3 months after the TTx (Rosario *et al.*, 2016). If neck US is negative and postsurgical Tg is already <0.2 μg/L the disease can already be considered in clinical remission and patients can avoid RRA, independently from the level of risk (Matrone *et al.*, 2017a).

Once the decision to perform RRA is taken, remnant ablation should be performed with the lowest but efficacy activity of 131-I and after stimulation with recombinant TSH (rhTSH) to avoid hypothyroidism that is very demanding for patients. Several uncontroversial studies demonstrated that low activities as 30 mCi of 131-I combined with the rhTSH stimulation will display the same results as higher amount of activities either after LT4 withdrawal or rhTSH stimulation (Mallick *et al.*, 2012; Schlumberger *et al.*, 2012). Higher activities of 131-I must be reserved to advanced cases with incomplete surgical treatment or cases with already known distant metastases.

RRA is not indicated in MTC since C cells are unable to take up 131-I although in the past some evidences of effectiveness was demonstrated for a "by-stander" effect due to 131-I entered in adjacent follicular cells (Nusynowitz *et al.*, 1982). Nowadays this practice is not recommended. Thus MTC can be either cured or not by the first surgery after which few other therapeutic options are available (Orlandi *et al.*, 2001). Even less effective is 131-I in ATC and should not be used. High activities can be used in PDTC with the hope that at least a minimal ability to take up iodine is persisting.

Radioiodine for metastatic lesions

Almost 2/3 of DTC metastatic lesions are able to take up iodine (**Fig. 4**) and 131-I can be used with subsequent administrations until the evidence of clinical benefit (Durante *et al.*, 2006; Pitoia *et al.*, 2014). No limits of cumulative dose have been identify and courses of high activities of $^{131\text{-}I}$ can be repeated several times if renal and/or liver and/or lung functions are not yet compromised. From a clinical point of view it is indicated not to repeat 131-I courses in less than 6 months especially if lung lesions are present where the risk of pulmonary fibrosis can become a life treating event (Hebestreit *et al.*, 2011). Although 131-I courses can be repeated many times without a real cumulative dose limitation, it is known that after a total of 600 mCi of 131-I it is very difficult to definitively cure the patient only with this type of treatment (Martins-Filho *et al.*, 2010).

The Follow Up of Thyroid Cancer Patients

About 80% of DTC and 30% of MTC can show an excellent response to initial treatment and patients can be considered in clinical remission with a risk of recurrence of 1%–3% in both cases (Tuttle *et al.*, 2010; Pellegriti *et al.*, 2003). About 50% of the recurrences usually show up within 5 years from the diagnosis while the others are commonly spread during the rest of the patients' life (Leung *et al.*, 2011). The other 20% of DTC and 70% of MTC usually have persistent disease that can be either biochemical, when only the specific biochemical marker is detectable without any evidence of metastases, or structural when imaging procedures show the presence of local or distant metastases (Tuttle *et al.*, 2010).

It is evident that the follow up of both the cured and not cured patients is fundamental for the benefit of the patients. The two specific and sensitive markers of DTC and MTC are serum Tg and Ct, respectively. Patients with an excellent response to initial therapy have undetectable levels of these markers and their de novo detected positivity is expression of recurrence as well as the persistence of detectable levels is expression of persistent disease (Tuttle *et al.*, 2010). Patients must be submitted to periodical controls whose schedule is very much dependent from the disease status. When the disease is apparently cured, patients can be monitored every 12 months with the measurement of serum Tg or Ct and neck US since, both in DTC and MTC, the most frequent site of recurrence is in the neck with lymph node metastases. Whenever the serum marker, either Tg or Ct in DTC and MTC respectively, turns out to be detectable other imaging procedures must be used to search for metastases especially if the neck US is negative. In cases with biochemical persistent disease the trend of increase of the serum marker becomes more relevant than the absolute value per se. In particular the doubling time (DT) of both the serum Tg and Ct can predict the outcome of the disease with a higher risk of death when the DT is <0.5–1 year (Miyauchi *et al.*, 2011; Meijer *et al.*, 2010). In these cases imaging procedures such as computerized tomography scan (CT), magnetic resonance (MRI), bone scintigraphy or positron emission tomography (PET) should be performed to find the sites of the metastatic lesions and evaluate their rate of growth. As far as the radiopharmaceutical to be used in PET imaging is concerned, 18 Fluoro deoxyglucose (18FDG) is useful for metastatic DTC while 18 Fuoro-di idrossi-fenil-alanina (18DOPA) is preferred in metastatic MTC for a higher specificity. Nevertheless, both in DTC and MTC imaging techniques, and particular PET scan, are usually positive only when the disease is rather advanced and serum markers are significantly elevated (i.e., >10 ng/mL in DTC and >150 pg/mL in MTC) (Agate *et al.*, 2014; Haugen *et al.*, 2016). A preferential use of some imaging techniques is usually recommended according to the anatomical district to be analyzed (Giraudet *et al.*, 2007).

It is well known that approximately 25%–30% of patients with DTC have serum Tg antibodies (TgAb) (Pacini *et al.*, 1988; Fiore *et al.*, 2011), which interfere with Tg measurement, causing either false negative or false positive results depending on the type of assay used (Latrofa *et al.*, 2016; Spencer *et al.*, 2014). TgAb are the expression of an associated lymphocytic thyroiditis (LT) or the expression of an immune reaction to DTC (Latrofa *et al.*, 2012). In these cases, serum Tg loses its function as tumor marker, but the change of the serum TgAb levels over the time can be used as a "Tg surrogate" since it has been demonstrated that in cured DTC patients, the TgAb levels decline to reach negativization (Verburg *et al.*, 2013; Chiovato *et al.*, 2003). At the same time, the persistence of stable levels of TgAb for a long period of time or the increase of TgAb levels after TTx plus/minus RRA represent an alert about the possibility of persistence or recurrence of DTC (Kim *et al.*, 2008; Tsushima *et al.*, 2013; Yamada *et al.*, 2014).

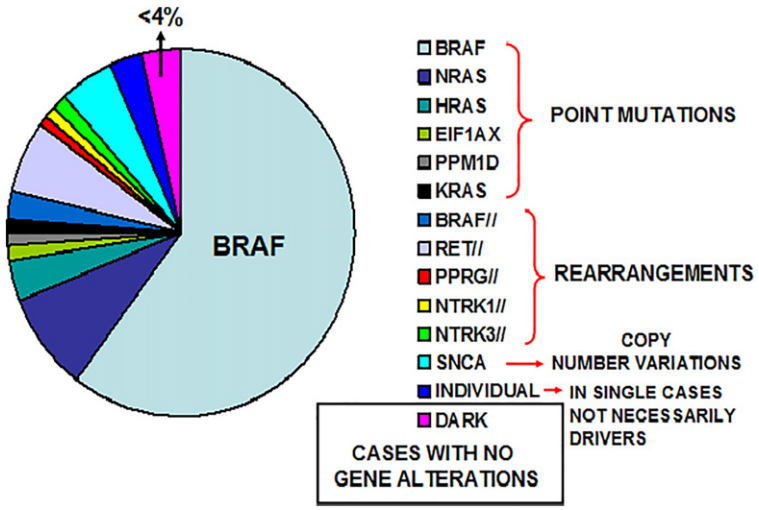

Fig. 5 Schematic representation of the prevalence and types of differentiated thyroid cancer oncogenes according to the data obtained by the TCGA (Cancer-Genome-Atlas-Research-Network, 2014): only a 4% of cases are still orphan of driver mutations.

Currently, this concept is so well established that the new international referral guidelines for the management of DTC include the TgAb evaluation after initial treatment as essential to assess the ongoing risk stratification (Haugen *et al.*, 2016).

Serum Tg becomes less significant as tumor marker in PDTC and even less in ATC. In fact because of the lost of differentiation features they produce less or null amount of Tg. In a few cases of dedifferentiated MTC also serum Ct production can be reduced and serum Ct levels be improperly low with respect to the tumor burden. In these latter cases serum carcinoembrionic antigen (CEA) levels can be useful to monitor the progression of the disease (Giovanella *et al.*, 2008).

Therapy of Advanced Cases

Radiorefractory metastatic DTC cannot be further treated with 131-I and other therapies must be considered when the disease becomes progressive. However, until recently, no effective therapeutic options were available for patients with any type of advanced thyroid cancer. In fact, classical cytotoxic chemotherapies have shown only a transient and poor response rate (10%–20%), with no prolongation of survival in response to the use of either a single therapeutic agent or in combination (Orlandi *et al.*, 1994; De Besi *et al.*, 1991). The significant improving of our knowledge of the molecular mechanisms underlying thyroid carcinogenesis and the description of drugs able to inhibit the catalytic activity of tyrosine kinases (TK) involved in the tumor process has opened up an era of targeted cancer therapies that represent new and important therapeutic options (Viola *et al.*, 2016).

The Rationale of Target Therapy

Since 1986, when the first activated oncogene (i.e., *RET/PTC*) was found in the DNA extracted from an irradiated PTC tumor and transfected into a cell line (Fusco *et al.*, 1995) up to 2014, when the genomic landscape of DTC has been fully revealed (Cancer-Genome-Atlas-Research-Network, 2014), a lot of interest has been growing for the possibility to use these oncogene alterations as targets of the new tyrosine kinase inhibitors (TKI) (Bible and Ryder, 2016).

The most common activated oncogenes in the different types of TC are reported in **Fig. 5**. Somatic activating point mutations of BRAF (i.e., V600E) and RET/PTC rearrangements are the most frequent altered oncogene in DTC. They are usually mutually exclusive but in advanced cases and especially in PDTC and ATC the presence of several alterations in the same tumor tissue has been observed and correlated to a greater aggressiveness and poor prognosis. A high prevalence of both *P53* and *TERT* promoter mutations are found in these cases (Landa *et al.*, 2016; Giordano, 2018; Yoo *et al.*, 2016).

RET oncogene is the most important driver in MTC tumorigenesis and somatic mutations are present in about 90% of sporadic advanced cases (Romei *et al.*, 2016). *RAS* oncogene is also altered but in a lower percentage of cases, usually in about 20% of *RET* negative cases (Ciampi *et al.*, 2013). Germline mutations are present in the hereditary forms of MTC that represent about 25% of all MTC. The genetic screening of *RET* mutations is nowadays recognized as the most important tool to early identification of gene carriers and plan the best therapeutic strategy (Elisei *et al.*, 2013a).

In the majority of cases, the activated oncogenes involved in thyroid tumorigenesis induce cell proliferation and tumor-transformation via the mitogen-activated protein kinase (MAPK) pathway, also known as the Ras-Raf-MEK-ERK pathway, and/or PI3K/AKT/mTOR pathway that is the other most important signaling mechanism in thyroid tumor-transformation. TKI can stop

Table 3 Major adverse events (AE) and their prevalence (%) in the four phase III studies

AE	*Sorafenib*	*Lenvatinib*	*Vandetanib*[a]	*Cabozantinib*
Hand–foot skin reaction	76	32	45	50
Diarrhea	69	60	33	63
Alopecia/change of color	67	nd	nd	34
Rash/desquamation	50	nd	45	nd
Fatigue	49	59	24	41
Weight loss	46	46	10	48
Hypertension	40	68	32	33
Metabolic—lab (other)	35	45	nd	42
Anorexia	32	50	21	46
Oral mucositis	23	36	nd	34
Pruritus	21	nd	15	nd
Nausea	21	46	77	43
Proteinuria	nd	31	nd	nd
Headache	nd	28	26	nd
Dysphonia	nd	24	nd	nd

[a]Vandetanib has some peculiar cutaneous AE such as eritrodermia and photosensitisation likely due to its anti epidermal growth factor receptor; nd = not determined.

Table 4 Local treatments to take into consideration before starting a systemic therapy

Different types of local treatments
a) Other surgeries
b) Thermoablation
c) Alcohol ablation
d) Laser ablation
e) Transarterial chemoembolization (TACE)
f) Transarterial radioembolization (TARE)
g) External radiotherapy (conventional or stereotaxic)
h) Endotracheal stent

the tumor cell growth by blocking these activated pathways and exerting a cytostatic action thus significantly slowing down the tumor progression (Bible and Ryder, 2016).

Tyrosine Kinase Inhibitors

Although several TKIs have been tested in advanced and progressive TC only four drugs, two for RAR-DTC and PDTC and two for MTC, have been approved by both FDA and EMA for the therapy of these patients (Viola *et al.*, 2016). Sorafenib and lenvatinib are the TKI for the treatment of advanced and progressive RAR-DTC and PDTC (Brose *et al.*, 2014; Schlumberger *et al.*, 2015). Vandetanib and cabozantinib are those for the treatment of advanced and progressive MTC (Wells *et al.*, 2012; Elisei *et al.*, 2013b) but vandetanib can also be used for symptomatic patients and in children with metastatic MTC (Fox *et al.*, 2013). They have been tested in phase III clinical trials and a significant increase of the progression free survival (PFS) has been demonstrated in all these studies. Similar adverse events (AE), but with different prevalence and degree of severity, have been shown for all the four drugs (**Table 3**). However, a good knowledge of these AE, their prevention whenever possible, the early report of their appearance from patients and the possibility to reduce the daily dose of the TKI without losing their cytostatic activity, allow to find a good compromise between continuing the therapy and maintaining a good quality life (QoL) (Matrone *et al.*, 2017b).

The major concern is "when" to start the TKI therapy that should be not too late but also not too early because of the AE of the drugs that can impair the QoL of TC patients, which is usually rather good despite the presence of a metastatic disease. Moreover, TC, particularly de-differentiated cases, are slowly growing and a long interval of time will be required to become clinically significant (Kwong *et al.*, 2014). For this reason there is a general consensus that TKI must be started when the metastases are affecting several organs, are at least 1.5–2 cm in their bigger diameter and are progressing according to radiological criteria (i.e., RECIST) (Schlumberger *et al.*, 2014). Moreover, to be considered relevant to start TKI, the progression should involve several lesions simultaneously and the possibility to use a local treatment must be considered anytime the growth is regarding a single lesion (**Table 4**).

No TKI have been approved for ATC so far. Several phase II studies have been performed with good results (Sosa *et al.*, 2014; Smallridge *et al.*, 2013; Savvides *et al.*, 2013; Bible *et al.*, 2012; Copland *et al.*, 2006) but not enough to start phase III studies and

TKI may be used only as "off label" drugs or "compassionate use." ATC still remains a lethal tumor with a median survival of 6–8 months and the therapy should be addressed to avoid suffocation whenever possible (Molinaro *et al.*, 2017).

Conclusions

Thyroid carcinoma is represented by a variety of histotypes characterized by different biological behaviors. The majority of them, particularly those well differentiated, are cancers that have a good prognosis and a lifelong survival. For this group of patients the risk of an overtreatment is the most important risk since they are frequently cured with the initial treatment represented by TTx and RRA, if indicated. However, a not negligible subset, particularly those that lose the ability to take up iodine and those that are undifferentiated from the beginning, require the application of therapeutic procedures varying from further surgeries, external radiotherapy, thermoablation, chemoembolization and/or systemic therapies. For these reasons and for the rarity of the disease TC should be managed and treated in referral centers and by multidisciplinary teams.

See also: Environmental Goitrogens. Genetic Factors in Thyroid Disease. Hashimoto's Thyroiditis. Iodine Deficiency. Medullary Thyroid Carcinoma. Thyroglobulin. Thyroid and Irradiation. Thyroid Autoimmunity. Thyroid Nodule. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Medullary Thyroid Carcinoma☆

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Introduction

MTC is derived from the parafollicular cells (C-cells) of the thyroid gland, which are of neural origin. Although they reside in the thyroid, C-cells do not play a role in thyroid function; they secrete amines, polypeptides and prostaglandins. Calcitonin is the main secretory product of C-cells and is widely used for the diagnosis of this tumor. Importantly, CT is also a useful marker after thyroidectomy for the follow-up of these patients and the identification of relapse or progression of disease (Wells *et al.*, 2015; Elisei *et al.*, 2004). Carcinoembryonic antigen (CEA) is another, non-specific, marker of the disease.

The clinical presentation includes enlargement of the thyroid and quite frequently palpable cervical lymph nodes. Approximately 10% of patients may have distant metastases already at the time of diagnosis (Kebebew *et al.*, 2000; Modigliani *et al.*, 1998). Postoperatively, the diagnosis of MTC raises some important questions involving disease staging, further therapeutic management in case of disease persistence as well as the identification of familial disease.

MTC is inherited in 25% of cases, frequently in the context of multiple endocrine neoplasia (MEN) syndromes which comprise familial MTC (FMTC), MEN 2A and MEN 2B variants. Mutations in the "rearranged during transfection" (*RET*) proto-oncogene are responsible for the transmission of familial disease. Genetic screening is performed in all patients diagnosed with MTC in order to identify familial disease within the group of apparently sporadic cases; early intervention may be performed in gene carriers. The time of prophylactic thyroidectomy is planned according to the risk level for aggressive disease associated with the identified affected *RET* codon (Wells *et al.*, 2015). Investigation for the coexistence of other tumors such as pheochromocytoma and primary hyperparathyroidism in MEN 2 cases is mandatory.

Thyroidectomy with or without lymph node dissection is the standard treatment for MTC in the majority of cases. The completeness of the initial surgery is of great importance for cure of the disease. Stage at diagnosis, tumor size, lymph node metastases and postoperative CT levels are important predictors of disease free survival and disease progression (Kebebew *et al.*, 2000; Verbeek *et al.*, 2015; Saltiki *et al.*, 2014; Roman *et al.*, 2006).

The majority of MTC cases with undetectable postoperative CT levels does not relapse and can achieve very high cure rates. In cases with elevated postoperative CT levels the clinical course varies. Estimation of CT and CEA doubling time helps predict progression and outcome. Locoregional therapies for metastatic lesions are of value as well as palliative or adjuvant therapy depending on the site. Two tyrosine kinase inhibitors (TKIs), vandetanib and cabozantinib, have been recently approved for the treatment of metastatic MTC; these agents can induce clinical response and disease stabilization (Wells Jr *et al.*, 2012; Elisei *et al.*, 2013b). An overview of the recent developments in the presentation, diagnosis, clinical course and management of both sporadic and inherited MTC are presented in this article.

Epidemiology

Sporadic MTC may present at any age but is most frequently encountered in the 4th–6th decade of life. Recent studies have shown that its incidence may be higher than previously believed as it has been found that 0.5%–1.3% of multinodular goiters may harbor unsuspected small MTCs. Small MTCs are being discovered more frequently due to the routine CT measurement in nodular disease, as well as to the increased use of high resolution ultrasound. However, the clinical significance of these may be different from the classical MTC (Alevizaki *et al.*, 2012; Elisei *et al.*, 2004; Valle and Kloos, 2011; Scheuba *et al.*, 2007). Moreover, incidental MTCs may be found in 0.2%–0.8% of autopsies; the majority of these are microcarcinomas (Baudin *et al.*, 1998; Beressi *et al.*, 1998; Valle and Kloos, 2011). Interestingly, in 10%–15% of MTCs the diagnosis is made only postoperatively (Ahmed and Ball, 2011; Valle and Kloos, 2011).

Histology

In 75% of MTCs there is amyloid deposition in the stroma which has a characteristic appearance. Immunohistochemistry is positive for CT and CEA staining. In the majority of cases with familial disease C-cell hyperplasia is also seen. This is considered a pre-malignant condition in cases of familial disease. It should be noted that C-cell hyperplasia can also occur in 20% of thyroid

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samples, in the absence of malignancy, frequently in the context of Hashimoto's thyroiditis (Albores-Saavedra and Krueger, 2001). In case of familial disease the tumors are multicentric more frequently compared to sporadic disease (Leboulleux *et al.*, 2004).

Clinical Picture: Diagnosis

MTC patients usually present with a single firm hard nodule in the upper middle region of thyroid lobes or less frequently with palpable lymph nodes. Thyroid ultrasound may show features of malignancy such as microcalcifications and hypoechogenicity with irregular margins and occasionally infiltrated lymph nodes. Fine needle aspiration (FNA) cytology is diagnostic in only 50% of the cases (Trimboli *et al.*, 2014). It may be false negative, may indicate papillary thyroid cancer or unspecified malignancy. Serum CT measurement along with FNA-calcitonin may improve the diagnostic accuracy in patients at risk for MTC and avoid false negative or inconclusive results from cytology (Wells *et al.*, 2015; Trimboli *et al.*, 2016).

The clinical picture of MTC may vary and patients are frequently asymptomatic. In 20% of the cases diarrhea may be the presenting symptom due to prostaglandin release by the tumor. Rarely, Cushing syndrome may be present due to ectopic ACTH secretion by MTC cells as well as flushing due to the release of biogenic amides from the tumor. In familial cases of MEN 2 syndrome the symptoms of pheochromocytoma may appear first (10%–50% of cases). Pheochromocytoma is bilateral in 60%–80% of cases and is usually benign although malignant pheochromocytomas have also been reported. Pheochromocytoma symptoms depend on the degree of catecholamine release; when diagnosed early, it may be asymptomatic. Hyperparathyroidism may appear in the 3rd decade of life in 10%–25% of MEN 2A patients and is usually mild and slow-progressing.

Cervical lymph node involvement may be detected at diagnosis and may be present in up to 20%–30% of MTC patients with tumors ≤ 1 cm. This percentage increases to 50% in cases with tumors >1–4 cm and up to 90% in larger tumors (De Groot *et al.*, 2006b; Scollo *et al.*, 2003). Lymph node metastases usually involve the central and lateral compartments. However, contralateral and upper mediastinal involvement may occur in locally invasive tumors, in which case the prognosis is worse (Machens *et al.*, 2005; Scollo *et al.*, 2003). Distant metastases may involve the lung, liver, bones and less frequently the brain and skin. Occasionally, in 10% of cases, distant metastases may be present already at diagnosis (Roman *et al.*, 2006).

Serum CT measurement is valuable in the preoperative evaluation (Machens and Dralle, 2010). Practically all patients have elevated CT levels. In recent years it has been suggested that CT measurements should be included in the laboratory assessment of multinodular goiter (Costante *et al.*, 2007; Alevizaki *et al.*, 2012; Elisei *et al.*, 2004). The European Thyroid Association (ETA) recommends one measurement of CT levels in all cases while the American Thyroid Association (ATA) guidelines are more reluctant to generalize this recommendation (Daniels, 2011; Wells *et al.*, 2015; Elisei *et al.*, 2013a). It should be noted that measurement of CT is a preventive action that leads to the earlier diagnosis, earlier intervention and higher cure disease rates (Alevizaki *et al.*, 2012, Elisei *et al.*, 2004). It should however be recognized that in a small percentage of cases CT may be slightly elevated in the absence of MTC; such cases are chronic renal failure, smoking, proton pump inhibitors intake, neuroendocrine tumors or Hashimoto's thyroiditis (Gibelin *et al.*, 2005). Another cause of falsely elevated CT may be the presence of heterophilic antibodies (Preissner *et al.*, 2005). It has been reported that up to 50% of patients who have been operated for multinodular goiter on the basis of elevated CT levels did not have MTC in the final histology (Hahm *et al.*, 2001).

In a recent study a cut-off value of 65 pg/mL for basal CT levels has been proposed as a threshold for detecting MTCs larger than 1 cm. However, microMTCs or even C-cell hyperplasia cannot always be discriminated (Kwon *et al.*, 2015). For investigation of marginally elevated CT levels stimulation tests either with intravenous calcium or pentagastrin administration may be useful (Mian *et al.*, 2014, Fugazzola, 2013, Machens *et al.*, 2009). In both familial and sporadic MTCs a threefold increase of CT levels or an absolute level of >100 pg/mL after stimulation is considered abnormal. Gender-specific CT thresholds may better differentiate between C-cell hyperplasia and MTC cases (Mian *et al.*, 2014; Machens *et al.*, 2009; Fugazzola, 2013). It is recommended that laboratories determine their own reference levels for stimulated CT.

Serum CT levels in MTC patients are usually associated with tumor size (Machens *et al.*, 2005; Cohen *et al.*, 2000). Rarely a "hook effect" may occur and give erroneously low levels due to very high serum CT levels interfering with the immunoassay. This should be examined when inappropriately low serum CT levels are found in a patient with large tumor burden. Furthermore, rarely calcitonin-negative or low-calcitonin tumors have been reported; in such cases MTC cells are poorly differentiated and may have aggressive biological behavior (Trimboli and Giovanella, 2015). In these cases other markers such as procalcitonin and CEA may be useful (Trimboli *et al.*, 2015; Karagiannis *et al.*, 2016; Giovanella *et al.*, 2008). CEA is a useful, although non-specific, marker which is produced from the cancerous cells (Barbet *et al.*, 2005). When CEA levels are >100 at diagnosis, lymph node involvement and also distant metastases are usually present.

In cases with familial disease, catecholamines and metanephrines should be measured in plasma or 24 h urine. In the presence of a positive result, adrenal imaging should be performed. In case a pheochromocytoma is diagnosed, this should be surgically removed before the operation for MTC. Furthermore, calcium measurements should be performed to exclude the presence of hyperparathyroidism. Serum chromogranin A may be elevated only in case of large tumor burden. Elevated chromogranin A levels in a patient with moderately elevated CT levels may indicate the presence of pheochromocytoma or other neuroendocrine tumor (De Groot *et al.*, 2006a). MTC is a neuroendocrine tumor and thus other hormones, bioactive amines and neuropeptides may be ectopically produced; these include ACTH, calcitonin gene-related peptide, histaminase, neuron specific enolase, prostaglandins, serotonin, somatostatin, vasoactive intestinal peptide, gastric inhibitory peptide, etc.

Familial Disease

In about 25%–30% of MTC the disease is inherited in the context of the multiple endocrine neoplasia (MEN) 2A or 2B syndromes or as familial MTC (FMTC). In MEN 2A, practically all patients develop MTC, which is accompanied by pheochromocytoma in 50% and primary hyperparathyroidism in 25%–30% of the cases. Rarely, cutaneous lichen amyloidosis and Hirschsprung disease may also be present in MEN 2A patients. In the rare MEN 2B syndrome, MTC may be associated with 50% lifetime risk of pheochromocytoma, mucosal neuromas, ganglioneuromatosis of the intestinal tract, medullated corneal nerves, musculoskeletal abnormalities and a characteristic phenotype with Marfanoid habitus. FMTC is a clinical variant of MEN 2A characterized by the isolated existence of MTC not accompanied by any other endocrine neoplasia. It is interesting that cases initially considered as FMTC have been subsequently re-classified as MEN 2 as pheochromocytomas developed at later generations (Moers *et al.*, 1996). Compared to sporadic MTC, hereditary MTC is usually, but not always, bilateral, multicentric and associated with C-cell hyperplasia. The distribution of familial cases is shown in **Fig. 1**.

The gene responsible for the transmission of the predisposition for hereditary MTC is the *RET* (Rearranged during Transfection) proto-oncogene. The characterization of the *ret.* mutation in a family allows the genetic screening of relatives allowing diagnosis of the disease at early stages; the disease outcome has thus significantly improved, because preventive rather than curative thyroidectomy can be performed (Spinelli *et al.*, 2010). The wide application of genetic testing has also resulted in the recognition of previously undiagnosed hereditary disease in cases among those initially considered as sporadic. The widespread application of molecular testing has resulted in an increase in the incidence of hereditary MTC (Machens *et al.*, 2013). Interestingly, in sporadic cases of MTC, somatic *RET* mutations are present in tumor cells in approximately 50% of cases; some studies have correlated these somatic mutations with higher proliferation rate and adverse prognosis (Elisei *et al.*, 2008; Mian *et al.*, 2011).

The *RET* Gene

The *RET* gene is located in chromosome 10q11.2, spans 21 exons and encodes a tyrosine kinase transmembrane receptor; it was first described by Takahashi *et al.* in 1985 (Takahashi *et al.*, 1985). It has three distinct domains: an extra-cellular ligand-binding segment with a cadherin-like region, a calcium-binding site and a juxtamembrane cysteine-rich region critical for receptor dimerization, a hydrophobic transmembrane domain and an intra-cellular part with two tyrosine kinase subdomains that mediate the downstream signaling pathways. The structure of the gene is shown in (**Fig. 2**).

In the physiologic state, *ret.* dimerization is required for autophosphorylation of the intracellular tyrosine residues and, subsequently, for receptor activation to ensue. Ligands binding *ret.* include the glial cell-line derived neurotrophic factor, neurturin, persepin, and artemin. The physiologic role of RET during development is to transmit signals in cells of neural origin. In hereditary MTC, germline gain-of-function mutations, present in 95%–98% of cases, lead to ligand-independent dimerization of mutant *ret.* proteins and constitutive activation of the tyrosine kinase domain and its downstream transduction pathways (Santoro *et al.*, 1995). These molecular abnormalities result in proliferation of tissues derived from neural crest cells, including C-cells and adrenal medulla cells.

Mutations in the cysteine-rich extra-cellular ligand-binding segment lead to ligand–independent receptor dimerization, whereas mutations in the transmembrane domain augment noncovalent interactions and closer proximity between the RET monomers. Regarding the intra-cellular part, mutations in the tyrosine kinase domain induce access of ATP to its binding site, whereas mutations in the intracellular catalytic core (such as M918T) result in preferential binding of the substrate and activation of the downstream signaling pathway (Santoro *et al.*, 1995).

The majority of recognized mutations are located in exons 5, 8, 10, 11, and 13–16; this information is important in the planning of the panel that has to be screened for hereditary MTC in all patients presenting with MTC (Elisei *et al.*, 2013a; Machens *et al.*, 2013). Novel *RET* mutations are continuously recognized. >150 germline *RET* mutations, accessible in electronic databases

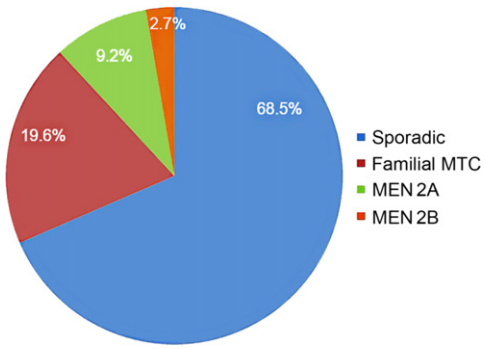

Fig. 1 Distribution of MTC category ($n = 184$). The percentage of familial disease may vary from 25% to 31% in various studies. Data derived from Sarika, H. L., Papathoma, A., Garofalaki, M., Saltiki, K., Pappa, T., Pazaitou-Panayiotou, K., Anastasiou, E. & Alevizaki, M. (2015). Genetic screening of patients with medullary thyroid cancer in a referral center in Greece during the past two decades. *European Journal of Endocrinology* **172**, 501–509.

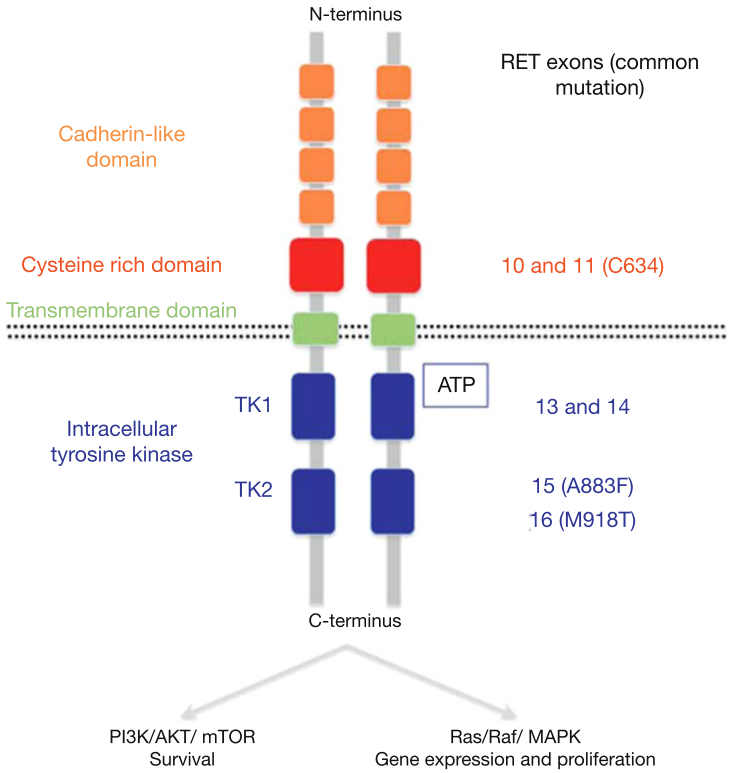

Fig. 2 Schematic representation of the RET tyrosine kinase receptor (TK: tyrosine kinase domain).

(e.g., www.arup.utah.edu/database/MEN2) have been identified to date. A list of confirmed pathogenic *RET* mutations associated with MEN 2 syndromes is presented in **Table 1**.

During *RET* genetic analysis polymorphisms may be found, the significance of which is unclear. The European Thyroid Association suggests validating the oncogenicity of novel *RET* mutations with in silico and in vitro testing (Elisei *et al.*, 2013a; Machens *et al.*, 2012). An appropriate population should serve as a control to exclude gene polymorphisms lacking any functional relevance (Elisei *et al.*, 2013a, Machens *et al.*, 2012).The term unclassified is used to describe mutations reported in families or individuals with suspected or known hereditary MEN 2 disease, who do not however meet the clinical criteria of a specific MEN 2 syndrome.

Genotype-Phenotype Correlation

In MEN 2 syndromes a genotype-phenotype correlation has been recognized (Elisei *et al.*, 2013a; Eng *et al.*, 1996). Most typical MEN 2A cases harbor mutations in the cysteine-rich region of the extra-cellular ligand-binding segment, specifically in exon 10 (codons 609, 611, 618, 620) and exon 11 (630 and 634) (Romei *et al.*, 2010). In a large multicenter Italian study, the most prevalent mutations in MEN 2A syndrome were Cys634Arg (35.3%) and Cys634Tyr (24.7%) (Romei *et al.*, 2010); in pedigrees with codon 634 mutations the prevalence of pheochromocytoma and primary hyperparathyroidism is significantly higher (Frank-Raue *et al.*, 1996; Machens *et al.*, 2001). A few European large-scale studies have investigated the distribution of *RET* mutations (Raue and Frank-Raue, 2009; Elisei *et al.*, 2007; Jindrichova *et al.*, 2004; Paszko *et al.*, 2007; Sarika *et al.*, 2015; Saltiki *et al.*, 2017) and have shown slightly different mutation spectra. Raue *et al.* reported that the distribution of the detected mutations has changed over the last decades. Earlier, mutations involving codon 634 in exon 11 were the most frequent, while recently mutations in exons 8, 10, 13, 14, and 15 are increasingly recognized (Raue and Frank-Raue, 2009). This change may be attributed to the different ethnic origin of the study populations, the wider testing including more than just the hot spot region, as well as to the extent of genetic screening now performed in apparently sporadic MTCs, which may potentially be re-classified as hereditary MTCs.

Regarding MEN 2B, in >95% of cases the mutation is localized in codon 918 of exon 16 (ATG to ACG) and rarely in codon 883 of exon 15 (Wells *et al.*, 2015). The course of MTC in MEN 2B syndrome caused by the M918T mutation is more aggressive, with cancer development as early as in the first year of life; this can be explained from the structure of the protein; Met918 is an important element of the substrate recognition pocket in the intracellular catalytic core of RET and is closely related to the conformational change of the kinase and constitutive RET activation in a monomeric form of RET.

FMTC has a more indolent course and the distribution of the mutations concerns a wider region of the *RET* gene; the most frequently affected sites include exons 10 (codons 609, 611, 618, and 620) and more rarely exon 11 (codon 634); mutations in the

Table 1 List of *RET* pathogenic mutations and their association with MEN 2 syndromes (arup database last accessed November 30, 2017)

Location in gene	*Mutation*	*Protein change*	*Classification*	*Phenotype*
Exon 5	c.874G > A	p.V292M	Pathogenic	MEN 2A
Exon 5	c.874G > A	p.V292M	Pathogenic	MEN 2A
Exon 5	c.961G > A	p.G321R	Uncertain	Unknown
Exon 5	c.1013C > T	p.T338I	Uncertain	Unknown
Exon 7	c.1512_1517delGGAGGG	p.505_506del	Pathogenic	MEN 2A
Exon 8	c.1529C > T	p.A510V	Uncertain	Unknown
Exon 8	c.1531G > A	p.E511K	Uncertain	Unknown
Exon 8	c.1544_1545delinsCT	p.C515S	Pathogenic	Unclassified
Exon 8	c.1545C > G	p.C515W	Pathogenic	Unclassified
Exon 8	c.1573C > T	p.R525W	Uncertain	Unknown
Exon 8	c.1585_1593dupGAGGAGTGT		Pathogenic	FMTC
Exon 8	c.1591T > C	p.C531R	Uncertain	Unknown
Exon 8	c.1597G > A	p.G533S	Uncertain	Unknown
Exon 8	c.1597G > T	p.G533C	Pathogenic	MEN 2A and FMTC
Exon 9	c.1649G > A	p.G550E	Uncertain	Unknown
Intron 9	c.1760-12G > A		Uncertain	Unknown
Exon 10	c.1799G > A	p.R600Q	Uncertain	Unknown
Exon 10	c.1807A > C	p.K603Q	Uncertain	Unknown
Exon 10	c.1817A > G	p.Y606C	Uncertain	Unknown
Exon 10	c.1825T > A	p.C609S	Uncertain	Unknown
Exon 10	c.1825T > C	p.C609R	Pathogenic	MEN 2A and FMTC
Exon 10	c.1825T > G	p.C609G	Pathogenic	MEN 2A
Exon 10	c.1826G > A	p.C609Y	Pathogenic	MEN 2A and FMTC
Exon 10	c.1826G > C	p.C609S	Pathogenic	MEN 2A
Exon 10	c.1826G > T	p.C609F	Pathogenic	MEN 2A
Exon 10	c.1827C > G	p.C609W	HSCR variant	No MEN 2 disease
Exon 10	c.1831T > A	p.C611S	Pathogenic	Unclassified
Exon 10	c.1831T > C	p.C611R	Pathogenic	Unclassified
Exon 10	c.1831T > G	p.C611G	Pathogenic	FMTC
Exon 10	c.1832G > A	p.C611Y	Pathogenic	MEN 2A and FMTC
Exon 10	c.1832_1833delinsAT	p.C611Y	Pathogenic	Unclassified
Exon 10	c.1832G > C	p.C611S	Uncertain	Unknown
Exon 10	c.1832_1833delinsCT	p.C611S	Pathogenic	Unclassified
Exon 10	c.1832G > T	p.C611F	Pathogenic	MEN 2A and FMTC
Exon 10	c.1832_1833delinsTT	p.C611F	Pathogenic	Unclassified
Exon 10	c.1833C > G	p.C611W	Pathogenic	MEN 2A and FMTC
Exon 10	c.1852T > A	p.C618S	Pathogenic	MEN 2A and FMTC
Exon 10	c.1852T > C	p.C618R	Pathogenic	MEN 2A and FMTC
Exon 10	c.1852T > G	p.C618G	Pathogenic	MEN 2A
Exon 10	c.1853G > A	p.C618Y	Pathogenic	MEN 2A and FMTC
Exon 10	c.1853G > C	p.C618S	Pathogenic	MEN 2A and FMTC
Exon 10	c.1853G > T	p.C618F	Pathogenic	MEN 2A and FMTC
Exon 10	c.1854C > G	p.C618W	Pathogenic	Unclassified
Exon 10	c.1857C > T	p.F619F	Uncertain	Unknown
Exon 10	c.1858T > A	p.C620S	Pathogenic	MEN 2A
Exon 10	c.1858T > C	p.C620R	Pathogenic	MEN 2A and FMTC
Exon 10	c.1858T > G	p.C620G	Pathogenic	MEN 2A
Exon 10	c.1859_1860delinsTG	p.C620L	Uncertain	Unknown
Exon 10	c.1859G > A	p.C620Y	Pathogenic	MEN 2A and FMTC
Exon 10	c.1859G > C	p.C620S	Pathogenic	MEN 2A and FMTC
Exon 10	c.1859G > T	p.C620F	Pathogenic	MEN 2A and FMTC
Exon 10	c.1859G > T	p.C620F	Pathogenic	MEN 2A and FMTC
Exon 10	c.1860C > G	p.C620W	Pathogenic	MEN 2A
Intron 10	c.1879 + 4A > G		Uncertain	Unknown
Exon 11	c.1888T > C	p.C630R	Pathogenic	MEN 2A and FMTC
Exon 11	c.1889G > A	p.C630Y	Pathogenic	MEN 2A
Exon 11	c.1889G > C	p.C630S	Uncertain	Unknown
Exon 11	c.1889G > T	p.C630F	Pathogenic	FMTC
Exon 11	c.1891_1893delGAC		Pathogenic	MEN 2A
Exon 11	c.1891G > A	p.D631N	Uncertain	Unknown
Exon 11	c.1891G > T	p.D631Y	Pathogenic	MEN 2A

(*Continued*)

Table 1 Continued

Location in gene	*Mutation*	*Protein change*	*Classification*	*Phenotype*
Exon 11	c.1891G>T	p.D631Y	Pathogenic	MEN 2A
Exon 11	c.1892A>C	p.D631A	Uncertain	Unknown
Exon 11	c.1892A>G	p.D631G	Uncertain	Unknown
Exon 11	c.1892A>T	p.D631V	Uncertain	Unknown
Exon 11	c.1892A>T	p.D631V	Uncertain	Unknown
Exon 11	c.1892_1903dupACGAGCTGTGCC		Pathogenic	MEN 2A
Exon 11	c.1893C>A	p.D631E	Uncertain	Unknown
Exon 11	c.1893C>T	p.D631D	Uncertain	Unknown
Exon 11	c.1893C>T	p.D631D	Uncertain	Unknown
Exon 11	c.1894G>A	p.E632K	Uncertain	Unknown
Exon 11	c.1895A>G	p.E632G	Uncertain	Unknown
Exon 11	c.1896_1900delinsCGTGC		Pathogenic	MEN 2A
Exon 11	c.1900T>A	p.C634S	Pathogenic	MEN 2A
Exon 11	c.1900T>C	p.C634R	Pathogenic	MEN 2A and FMTC
Exon 11	c.1900T>G	p.C634G	Pathogenic	MEN 2A
Exon 11	c.1900_1908dupTGCCGCACG		Pathogenic	MEN 2A
Exon 11	c.1901G>A	p.C634Y	Pathogenic	MEN 2A and FMTC
Exon 11	c.1901G>A	p.C634Y	Pathogenic	MEN 2A and FMTC
Exon 11	c.1901G>A	p.C634Y	Pathogenic	MEN 2A and FMTC
Exon 11	c.1901G>A	p.C634Y	Pathogenic	MEN 2A and FMTC
Exon 11	c.1901G>C	p.C634S	Pathogenic	MEN 2A and FMTC
Exon 11	c.1901G>T	p.C634F	Pathogenic	MEN 2A and FMTC
Exon 11	c.1901_1902delinsTG	p.C634L	Pathogenic	MEN 2A
Exon 11	c.1902C>G	p.C634W	Pathogenic	MEN 2A and FMTC
Exon 11	c.1903C>G	p.R635G	Uncertain	Unknown
Exon 11	c.1903C>T	p.R635C	Uncertain	Unknown
Exon 11	c.1906delinsGACCTGTGCCGCC		Pathogenic	Unclassified
Exon 11	c.1907C>T	p.T636M	Uncertain	Unknown
Exon 11	c.1919C>G	p.A640G	Uncertain	Unknown
Exon 11	c.1921G>T	p.A641S	Uncertain	Unknown
Exon 11	c.1942G>A	p.V648I	Uncertain	Unknown
Exon 11	c.1942G>A	p.V648I	Uncertain	Unknown
Exon 11	c.1942G>A	p.V648I	Uncertain	Unknown
Exon 11	c.1946C>T	p.S649L	Uncertain	Unknown
Exon 11	c.1946C>T	p.S649L	Uncertain	Unknown
Exon 11	c.1946C>T	p.S649L	Uncertain	Unknown
Exon 11	c.1946C>T	p.S649L	Uncertain	Unknown
Exon 11	c.1946C>T	p.S649L	Uncertain	Unknown
Exon 11	c.1947G>A	p.S649S	Uncertain	Unknown
Exon 11	c.1995C>G	p.H665Q	Uncertain	Unknown
Exon 11	c.1996A>G	p.K666E	Pathogenic	MEN 2A
Exon 11	c.1997A>G	p.K666R	Uncertain	Unknown
Exon 11	c.1997A>T	p.K666M	Uncertain	Unknown
Exon 11	c.1998G>T	p.K666N	Uncertain	Unknown
Exon 11	c.1998delinsTTCT		Pathogenic	MEN 2A
Exon 11	c.2057G>A	p.S686N	Uncertain	Unknown
Exon 11	c.2098A>T	p.M700L	Uncertain	Unknown
Exon 12	c.2248G>C	p.A750P	Uncertain	Unknown
Exon 13	c.2304G>C	p.E768D	Pathogenic	MEN 2A and FMTC
Exon 13	c.2304G>T	p.E768D	Pathogenic	Unclassified
Exon 13	c.2304G>T	p.E768D	Pathogenic	Unclassified
Exon 13	c.2309G>A	p.R770Q	Uncertain	Unknown
Exon 13	c.2311G>A	p.D771N	Uncertain	Unknown
Exon 13	c.2330A>G	p.N777S	Uncertain	Unknown
Exon 13	c.2332G>A homozygous	p.V778I	Uncertain	Unknown
Exon 13	c.2332G>A	p.V778I	Uncertain	Unknown
Exon 13	c.2342A>G	p.Q781R	Pathogenic	Unclassified
Exon 13	c.2342A>G	p.Q781R	Pathogenic	Unclassified
Exon 13	c.2370G>C	p.L790F	Pathogenic	MEN 2A and FMTC
Exon 13	c.2370G>T	p.L790F	Pathogenic	MEN 2A and FMTC
Exon 13	c.2371T>A	p.Y791N	Uncertain	Unknown

Table 1 Continued

Location in gene	*Mutation*	*Protein change*	*Classification*	*Phenotype*
Exon 14	c.2410G > A	p.V804M	Pathogenic	MEN 2A and FMTC
Exon 14	c.2410G > C	p.V804L	Pathogenic	Unclassified
Exon 14	c.2410G > C	p.V804L	Pathogenic	Unclassified
Exon 14	c.2410G > T	p.V804L	Pathogenic	MEN 2A and FMTC
Exon 14	c.2410G > T	p.V804L	Pathogenic	MEN 2A and FMTC
Exon 14	c.2413G > A	p.E805K	Uncertain	Unknown
Exon 14	c.2452G > A	p.E818K	Uncertain	Unknown
Exon 14	c.2456G > T	p.S819I	Uncertain	Unknown
Exon 14	c.2497C > T	p.R833C	Uncertain	Unknown
Exon 14	c.2522C > T	p.P841L	Uncertain	Unknown
Exon 14	c.2523G > A	p.P841P	Uncertain	Unknown
Exon 14	c.2529G > T	p.E843D	Uncertain	Unknown
Exon 14	c.2530C > T	p.R844W	Uncertain	Unknown
Exon 14	c.2531G > A	p.R844Q	Uncertain	Unknown
Exon 14	c.2531G > T	p.R844L	Uncertain	unknown
Exon 14	c.2531G > T	p.R844L	Uncertain	Unknown
Exon 14	c.2535C > T	p.A845A	Uncertain	Unknown
Exon 14	c.2543T > C	p.M848T	Uncertain	Unknown
Exon 14	c.2556C > G	p.I852M	Uncertain	Unknown
Exon 14	c.2556C > G	p.I852M	Uncertain	Unknown
Exon 15	c.2641C > G	p.L881V	Uncertain	Unknown
Exon 15	c.2647G > A homozygous	p.A883T	Pathogenic	Unclassified
Exon 15	c.2647_2648delinsTT	p.A883F	Pathogenic	MEN 2B
Exon 15	c.2656C > T	p.R886W	Uncertain	Unknown
Exon 15	c.2671T > G	p.S891A	Pathogenic	MEN 2A and FMTC
Exon 15	c.2673G > A	p.S891S	Uncertain	Unknown
Exon 15	c.2711C > G	p.S904C	Uncertain	Unknown
Exon 15	c.2711C > T	p.S904F	Pathogenic	Unclassified
Exon 15	c.2719A > G	p.K907E	Uncertain	Unknown
Exon 15	c.2720A > T	p.K907M	Uncertain	Unknown
Exon 16	c.2735G > A	p.R912Q	Uncertain	Unknown
Exon 16	c.2735G > C	p.R912P	Pathogenic	Unclassified
Exon 16	c.2753T > C	p.M918T	Pathogenic	MEN 2B
Exon 16	c.2753T > C	p.M918T	Pathogenic	MEN 2B
Exon 16	c.2752A > G	p.M918V	Uncertain	Unknown
Exon 18	c.3025A > G	p.M1009V	Uncertain	Unknown
Exon 19	c.3049G > A	p.D1017N	Uncertain	Unknown
Exon 19	c.3122T > G	p.V1041G	Uncertain	Unknown

non-cysteine regions of *RET* have also been described, such as exon 14 (codon 804), exon 15 (codon 891), exon 16 (codon 912), exon 8 (codons 532 and 533), and exon 13 (codons 768, 790 and 791) (Figlioli *et al.*, 2013).

Genetic Screening and Clinical Applications

All scientific societies now recommend genetic testing in patients with MTC. According to the European Thyroid Association (ETA) guidelines, analysis of the *RET* gene should be performed in all patients with apparently sporadic or familial MTC with the following order for testing *RET* gene exons (based on the frequency they appear): 10, 11, 14, 15, 13, 8, 5, and 16 (Elisei *et al.*, 2013a). The clinical features should also be taken into account; if there is suspicion of MEN 2B syndrome, exon 16 should be analyzed first (Elisei *et al.*, 2013a). In the large study of Romei *et al.* with 729 MTC patients, sequencing eight exons of the *RET* gene led to the re-classification of 6.5% of MTCs to hereditary and the identification of 41.1% of gene carriers within the MTC kindreds; 50% of them underwent total thyroidectomy and 90% remained disease-free after a 6-year follow up (Romei *et al.*, 2011). These cases would have remained undiagnosed using only conventional genetic testing. In the same line, one study from Greece recently reported an appreciable prevalence (7.75%) of the G533C exon 8 mutation in apparently sporadic MTCs and suggested that this site should be routinely screened in MTC patients presenting without any obvious family history (Sarika *et al.*, 2012; Saltiki *et al.*, 2017).

The widespread application of genetic screening has revolutionized the management of MTC patients and the counseling of family members carrying the mutated *RET* gene. Early identification of gene carriers allows timely prophylactic thyroidectomy at an early stage before lymph node involvement (Spinelli *et al.*, 2010). This approach significantly decreases the incidence of

persistent or recurrent disease (Machens *et al.*, 2003; Skinner *et al.*, 2005). Several studies have evaluated the beneficial role of prophylactic thyroidectomy in asymptomatic carriers; the site of the mutation, patient's age and basal CT levels are important parameters that have direct impact on the long-term outcome (Pappa and Alevizaki, 2016).

In the recent ATA guidelines *RET* mutations have been classified according to risk level and aggressiveness of MTC in three groups: moderate (ATA-MOD), high (ATA-H), highest (ATA-HST) risk level of aggressiveness (Wells *et al.*, 2015) (**Tables 1** and **2**). The ATA-HST category includes patients with MEN 2B (mutation in *RET* codon M918T), the ATA-H category includes MEN 2A patients with a C634 *RET* mutation and the category ATA-MOD includes the rest of familial MTC patients (Wells *et al.*, 2015). ATA-HST mutation harbors the highest risk for MTC developing very early in life with increased metastatic potential and prompts for total thyroidectomy within the first year of life. For ATA-H mutation it is advised that gene carriers undergo surgery within the first 5 years of life. Concerning the third class, ATA-MOD, the risk of MTC developing at a young age is lower, and although thyroidectomy before age 5 is advocated, it is suggested that it could be postponed if CT values (baseline and stimulated), and neck ultrasound are still normal (**Table 2**). Age-appropriate prophylactic thyroidectomy according to the risk classification of the mutations may improve disease-free survival (Shepet *et al.*, 2013). Recent studies demonstrated that stimulated CT values could represent a safe and reliable tool to personalize timing of thyroidectomy independent of the *RET* mutation and the gene carrier age (Elisei *et al.*, 2012).

Treatment

The primary treatment of MTC is surgical. Total thyroidectomy is recommended for both familial as well as for sporadic disease, as C-cells are distributed in both lobes and multifocality may be observed in sporadic cases as well. Preoperative levels of CT and CEA are also valuable to determine the extent of the initial surgery as these markers have been strongly correlated with the extent of lymph node metastases (Machens and Dralle, 2010; Machens *et al.*, 2007; Wells *et al.*, 2015).The decision for lymph node dissection is based on ultrasound findings, preoperative CT levels as well as the specific *RET* mutation in familial cases, according to the ATA guidelines risk stratification for each mutation (Wells *et al.*, 2015). Patients without lymph node involvement in the preoperative ultrasound and no evidence of distant metastases should undergo central compartment dissection at the initial surgery as lymph node invasion is frequently present at diagnosis (Wells *et al.*, 2015). In patients with preoperatively confirmed cervical lymph node involvement, lateral and central compartment dissection should be performed (Scollo *et al.*, 2003). In those with basal preoperative CT >200 pg/mL and positive ipsilateral lymph nodes a contralateral neck dissection should be considered; however, such a procedure is not curative in most cases (Wells *et al.*, 2015; Miyauchi *et al.*, 2002). In case of locally advanced disease debulking with removal of as much tissue as possible is recommended, however in many cases surgical resection is not feasible. Thoracic surgery may be needed for infiltrated upper-mediastinum lymph nodes (Wells *et al.*, 2015).

The prognosis is better in familial disease presumably because of earlier diagnosis and treatment. In young gene carriers with familial MTC and normal CT levels the necessity for prophylactic lymph node dissection is questionable. Pheochromocytoma should be excluded before thyroidectomy is performed. In case of a single pheochromocytoma, this should be removed. The probability of a second contra-lateral tumor within the next 10 years is 60%–80%. Adrenal cortex sparing surgery has been advocated in some centers (Castinetti *et al.*, 2014). In case of primary hyperparathyroidism the surgical procedure may vary according to the findings: either a single adenoma or 3.5 parathyroids may be removed, while in some centers total

Table 2 Overview of the American Thyroid Association (ATA) recommendations regarding prophylactic thyroidectomy for *RET* mutation carriers

ATA risk category	*Mutation*	*Thyroidectomy*	*Rationale for central neck dissection*	*Annual cervical ultrasound and serum basal CT*
Highest (ATA-HST)	M918T	– First year of life	– Suspicious lymph nodes present – Aim to preserve parathyroid glands	
High (ATA-H)	C634 A883F	– By5 years of age or sooner if CT high	– CT levels >40 pg/mL – Positive imaging – Suspicious lymph nodes during surgery	– Start at age 3 years
Moderate (ATA-MOD)	G533C C609,611,618,620,630 D631Y K666E E768D L790F V804 S891A R912P	– CT elevated or $\approx$ age 5 years if long term follow up difficult	– CT elevated	– Start at age 5 years

parathyroidectomy and a parathyroid implant in the forearm are performed (Alevizaki and Saltiki, 2015).The evaluation and management algorithm for MTC patients is shown in **Fig. 3**.

Disease Course and Prognosis

The detection of disease at earlier stages and the optimized surgical techniques are of paramount importance for the survival of MTC patients and for the possibility of cure (Cupisti *et al.*, 2007). The best outcome and highest 10 year survival rates are achieved with a successful first operation (Scollo *et al.*, 2003). Important prognostic factors are tumor size, lymph node invasion, disease stage, pre- and post-operative CT levels and age at diagnosis.

The overall 10-year survival rate is 65% but depends on the stage at diagnosis. In stage I it is 95%, stage II 93%, stage II 71%, and 20%–40% in stage IV patients. Of patients with no lymph nodes at diagnosis, biochemical and clinical cure may be achieved in 75%–90% of the cases while, of those with lymph node metastases at diagnosis only 20%–30% will have remission. The 10 year survival rate is 75% (Scollo *et al.*, 2003; Kebebew *et al.*, 2005; Modigliani *et al.*, 1998; Bergholm *et al.*, 1997). In cases with over 10 positive lymph nodes, this remission rate decreases to 4% (Scollo *et al.*, 2003; Pelizzo *et al.*, 2007). Compliance to ATA guidelines results in fewer local reoperations and more frequent biochemical cure of the disease (Verbeek *et al.*, 2015).

During follow-up, CT measurement appears to be the most sensitive marker for the evaluation of disease persistence and progression (Barbet *et al.*, 2005; De Groot *et al.*, 2006a; Kebebew *et al.*, 2005; Siironen *et al.*, 2016; Cho *et al.*, 2016). CT and CEA measurement are performed 3 months postoperatively (since both have long half-lives). When postoperative CT is undetectable the probability of relapse decreases to 0%–5% (Kebebew *et al.*, 2005; Modigliani *et al.*, 1998).

In a substantial proportion of patients postoperative CT may be detectable, indicating persistent disease. Many patients with lymph node metastases and/or elevated postoperative CT will have a long survival (Siironen *et al.*, 2016). In patients with persistent disease and postoperative CT > 100–200 pg/mL it is important to localize the disease and select the appropriate therapeutic approach. For disease localization various imaging methods are available depending on the organ that needs to be explored. Thus neck ultrasound, computed tomography of the thorax, magnetic resonance imaging (MRI) of the liver and bone MRI may be performed (Giraudet *et al.*, 2007). Positron emission tomography–computed tomography (FDG-PET/CT and F-DOPA-PET/CT) have poor sensitivity unless the tumor progresses rapidly (Wells *et al.*, 2015; Giraudet *et al.*, 2007; Skoura *et al.*, 2012).

CT and CEA levels are monitored every 6–12 months at follow-up. Short doubling time of CT and CEA has been proposed as the best indicator of disease progression (Meijer *et al.*, 2010; Laure Giraudet *et al.*, 2008). Specifically, CT doubling time shorter than 2 years correlates with structural disease progression (Barbet *et al.*, 2005; Laure Giraudet *et al.*, 2008). Fluctuations in

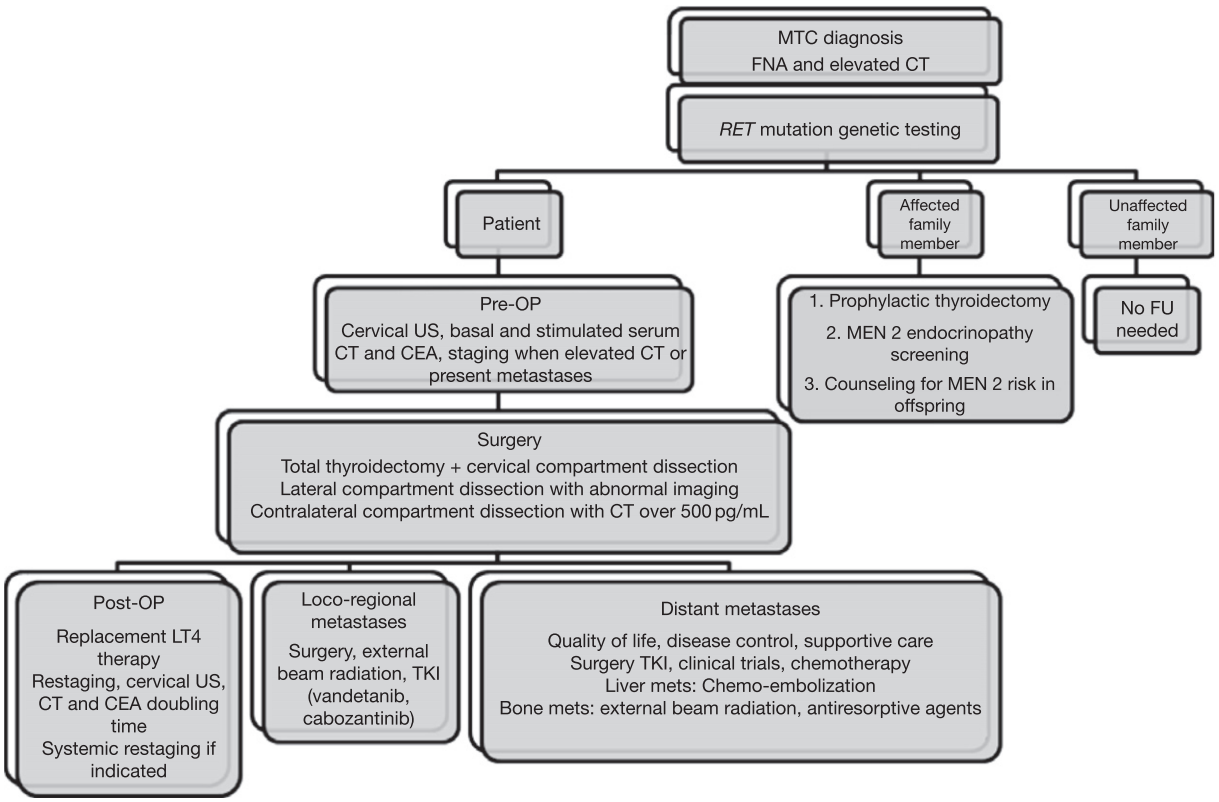

Fig. 3 Evaluation and management algorithm for MTC patients.

successive measurements of CT levels (20%–30%) do not indicate disease progression. A short CEA doubling time also indicates tumor progression. Increasing CEA levels in case of declining CT levels postoperatively may be a marker of dedifferentiation (Mendelsohn *et al.*, 1984). Tumor progression needs to be validated with imaging techniques, similar to those used preoperatively. The RECIST criteria (response evaluation criteria in solid tumors) are used to evaluate the structural disease progression before and during systemic treatment.

Patients with persistent disease with measurable CT but negative imaging need close follow-up as the location of the disease may eventually be identified in 40% within the following 10 years. In these patients the 10-year survival is 86% (Yip *et al.*, 2011). Patients with postoperative CT levels >400–500 pg/mL are likely to have distant metastases. When present, the 10-year survival is 20%–40%. However identifying the metastasis site is not always successful. MTC metastases are found in the liver, lungs, bones, and rarely in other organs. The lung metastases are usually small in size or micronodular. Liver metastases are usually small and mimic hemangiomas; therefore MRI is needed for the differential diagnosis. Bone metastases are either osteolytic or osteoblastic and will show-up in the bone MRI or the bone scan. It should be noted that MTC has a slow progression even in the case of distant metastases; occasionally, no disease progression is observed over a long period even without any systemic treatment.

Management of Metastatic Disease

In patients with local and regional recurrence of disease the main treatment is surgery, before which the extent of the tumor as well as the presence of distant metastases must be taken into consideration. In cases of locally invasive tumors endangering aero-digestive structures external radiation is proposed. In distant metastases palliative locoregional therapies may provide local control. For hepatic metastases chemoembolism may be used to decrease tumor mass and symptoms or to stabilize sizeable lesions. Chemoembolization is effective in lesions <3 cm and with limited liver involvement. External radiation in bone metastases may offer pain relief, protect from fractures and preserve motility. Surgery and/or stereotactic irradiation for brain metastases and radiofrequency ablation in lung, bone and liver metastases have been used (Baere De *et al.*, 2015; Wells *et al.*, 2015) (**Fig. 3**). Local interventions should preferably be performed before the initiation of systemic therapy. They may also stabilize the disease in patients with slow progression and thus delay the initiation of systemic therapy. For diarrhea control, loperamide is administered, whereas analgesic medication is administered for painful metastases. Somatostatin analogs may help in the control of diarrhea but have no other place in the management of metastatic MTC.

Classical chemotherapy is not currently used as it does not prolong survival. Radioiodine has no place in the management of MTC (Wells *et al.*, 2015).

The recent progress in the understanding of the oncogenic pathways in MTC and the identification of specific molecular alterations has played a crucial role in the development of molecular targeted therapies, mainly tyrosine kinase inhibitors (TKIs) (**Table 3**). Their main targets are the RET kinase, the vascular endothelial growth factor receptor (VEGFR), the epidermal growth factor receptor (EGFR), and other factors participating in signaling downstream pathways involved in tumorigenesis, such as the mesenchymal-epithelial transition (MET) pathway, mutations in MET gene with activation in c-MET/hepatocyte growth factor (HGF), as well as the activation of RAS and mTOR factors in RET-mutated tumors. Receptors for fibroblast growth factor (FGFR)

Table 3 Tyrosine kinase inhibitors used in patients with metastatic MTC

Tyrosine kinase inhibitors (references)	*Molecular targets*	*PFS (vs. placebo) in months (% increase)*	*ORR (%)*	*Major adverse events*
Vandetanib (Wells *et al.*, 2012)	RET, VEGFR2, VEGFR3, EGFR, PDGFR	30.5 (19.3) 58%	45	Fatigue, rash, dry skin, photosensitivity, folliculitis, diarrhea, anorexia, weight loss, nausea, asthenia, hypertension, QT prolongation
Cabozantinib (Elisei *et al.*, 2013b)	VEGFR2, RET, c-MET, KIT, AXL, FLT3, Tie2	11.2 (4) 180%	28	Diarrhea, abdominal discomfort, fatigue, hypertension, mucositis, hand-foot syndrome, anorexia, weight loss, GI perforation, hemorrhage, fistula formation
Sorafenib	RET, VEGFR, PDGFR, RAF, c-KIT, FLT3	17.9	21	Hand-foot syndrome, hypertension, diarrhea, infection, leukopenia, musculoskeletal pain, alopecia, rash, anorexia, fatigue
Sunitinib	RET, VEGFR1-3, PDGFR, c-KIT, FLT3, CSF1R		32	Leukopenia, fatigue, diarrhea, hand-foot syndrome, musculoskeletal pain
Pazopanib (Bible *et al.*, 2014)	VEGFR1-3, c-KIT, FGFR, PDGFR	9.4	14	Fatigue, anorexia, diarrhea, abnormal liver tests, hypertension
Lenvatinib (Schlumberger *et al.*, 2016)	VEGFR1-3 FGFR1-4 PDGFRα, c-KIT, RET	12.6	50	Weight loss, hypertension, fatigue, diarrhea, dehydration, anorexia, nausea, stomatitis, proteinuria

PFS, progression free survival; *ORR*, objective response rate; *AE*, adverse events; *GI*, gastrointestinal.

and for platelet-derived growth factor (PDGFR) may also participate in tumor angiogenesis (Papotti *et al.*, 2000; Plaza-Menacho *et al.*, 2014).

The rare cases with symptomatic or rapidly progressive metastatic disease are candidates for receiving molecular targeted therapy (Wells *et al.*, 2015; Links *et al.*, 2015; Hadoux *et al.*, 2016) which aims to stabilize disease and to potentially prolong survival. Such therapy should not be used in patients with only biochemical disease progression without structural disease or in asymptomatic patients with small metastatic lesions and no evidence of progression (Wells *et al.*, 2015; Hadoux *et al.*, 2016).

Two multikinase inhibitors, vandetanib and cabozantinib have been approved by FDA (Food and Drug Administration) and EMA (European Medicines Agency) for use in progressive metastatic MTC. Vandetanib inhibits VEGFR2, VEGFR3, RET, EGFR. In a large randomized controlled phase-3 trial vandetanib showed, compared to control group, increased progression-free survival (30.5 vs. 19.3 months) and objective imaging response (45% vs. 13%), irrespective of various factors such as *RET* mutation status, progression rate, disease location and disease extent at baseline (Wells Jr *et al.*, 2010). Cabozantinib inhibits RET kinases, VEGFR2 and c-met. Its efficacy was documented in a large randomized controlled phase-3 clinical trial where improved progression-free survival (11.2 vs. 4 months) and objective imaging response (28% vs. 0%) was found compared to placebo, irrespective of age, tumor burden and location, progression rate or prior TKI treatment (Elisei *et al.*, 2013b) (**Table 3**). Both drugs have been associated with disease stabilization in 30% and partial regression in 35% of cases (Elisei *et al.*, 2013b; Schlumberger *et al.*, 2012). However, the estimation of the effect of these agents on overall survival is difficult because the majority of patients have a relatively long life expectancy. The presence of either germline or somatic *RET* mutation may predict response to TKI treatment (Verbeek *et al.*, 2011; Fox *et al.*, 2013). In sporadic tumors the most common somatic mutation is *RET* M918T (85%) and its presence is associated with higher proliferation rate and more aggressive disease. RAS mutations, usually without coexisting *RET* mutations, are also present in 0%–43% of intermediate risk MTC tumors (Moura *et al.*, 2015).

Treatment with TKIs should be discontinued in cases with disease progression. A switch to another TKI may be helpful in maintaining disease control. TKIs have substantial adverse effects in 30%–60% of patients (**Table 3**). General not specific symptoms, cutaneous adverse events, gastrointestinal symptoms as well as hypertension, hypocalcemia and TSH increases are frequent but they can usually be treated symptomatically. Serious adverse events occur in 2% of patients. The most serious of these are the prolongation of QT interval in the electrocardiogram caused by vandetanib and the development of gastrointestinal fistulas and life threatening bleeding resulting from the use of cabozantinib. Dose reductions are required in 35% of patients treated with Vandetanib and 79% of those under cabozantinib. Discontinuation of treatment has been reported in 12% for Vandetanib and 16% for cabozantinib.

Other multikinase inhibitors (pazopanib, lenvatinib) are currently being studied and show promising response (Bible *et al.*, 2014; Schlumberger *et al.*, 2016) (**Table 3**). Novel drug molecules as well as combined targeted therapies may prove efficient. Finally, other treatments (labeled antiCEA-antibodies and DTPA, octreotide analogs labeled with Yttrium-90) have shown some response, however they present significant toxicity (Wells *et al.*, 2015).

Conclusions

MTC prognosis has significantly improved in recent years because of the timely diagnosis through routine CT screening in nodular disease, the availability of better quality ultrasound, the wide application of genetic screening and improved surgical procedures. Overall the most important advancement in the field has been the wide availability of genetic testing which allows screening and early intervention in familial disease. This should be performed in all MTC patients, those with positive family history as well as those with the apparently sporadic form. The course of disease may vary but is generally of slow progression. Tyrosine kinase inhibitors represent another important advancement in the management of patients with distant metastases and progressive disease.

See also: Thyroid Carcinoma. Thyroid Fine Needle Aspiration Cytology. Genetic Factors in Thyroid Disease. Thyroid Nodule

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Causes of Hypothyroidism☆

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Glossary

Congenital hypothyroidism Hypothyroidism present at birth.

Craniopharyngiomas Tumors arising from Rathke's pouch.

Dysgerminomas Germ cell tumors.

Hashimoto's thyroiditis Chronic goitrous autoimmune thyroiditis

Hemangiomas Tumors composed of blood vessels; the most common tumors of infancy, characterized by rapid growth during the first year of life, followed by involution and gradual regression by adolescence.

Pituitary adenomas Benign tumors arising in adenohypophyseal cells.

Rathke's pouch Invagination of part of the roof of the primitive nasopharynx; transforms later in the development into the adenohypophysis.

Thyroid agenesis Absent thyroid gland due to developmental disorder during embryogenesis.

Thyroid ectopia Thyroid gland located at aberrant position.

Wolff–Chaikoff effect Obstruction of the organic binding of iodine and its incorporation into hormone, caused by large doses of iodine; usually transient, but in large doses in susceptible people, the effect can be prolonged and cause iodine myxedema.

Introduction

Hypothyroidism, a syndrome characterized by the clinical and biochemical manifestations of thyroid hormone deficiency in the target tissues of thyroid hormone, may be caused by an abnormality in the thyroid gland itself (primary hypothyroidism) or by an abnormality in the pituitary or hypothalamus causing insufficient stimulation of the thyroid gland by thyroid-stimulating hormone (TSH) (central hypothyroidism). In rare cases, the abnormality is located in the target tissues of thyroid hormone ("peripheral" hypothyroidism) (Wiersinga, 2017). Within each of these, some of the disorders are genetic. Occurrence of central hypothyroidism is often related to hypothalamo-pituitary disease, and thus often associated with other pituitary hormone dysfunctions. The primary causes of hypothyroidism are very dependent on environmental factors, and the global distribution is therefore very variable. In areas with iodine deficiency, this is the main cause of hypothyroidism, while autoimmunity accounts for most cases in iodine sufficient areas (**Table 1**).

Central Hypothyroidism

The reduced secretion of thyroxine (T_4) due to deficient TSH secretion by the pituitary can be caused directly by lesions in the pituitary (secondary hypothyroidism) or indirectly by lesions in the hypothalamus via diminished secretion of TSH-releasing hormone (TRH) (tertiary hypothyroidism). The term central hypothyroidism is preferred because some lesions involve both sides, preventing a clear-cut distinction between secondary and tertiary hypothyroidism.

One would expect decreased serum TSH concentrations in central hypothyroidism, but normal or even slightly elevated TSH concentrations are not infrequently observed. This apparent paradox is explained by an aberrant glycosylation of TSH in these patients resulting in reduced biological activity of the TSH molecule while maintaining its immunoreactivity in conventional TSH immunometric assays (Beck-Peccoz and Persani, 1994; Persani *et al.*, 2000). Central hypothyroidism is also associated with a decrease in the nocturnal surge of TSH that may contribute to insufficient thyroid stimulation (Feldt-Rasmussen and Klose, 2016; Persani, 2012).

The incidence of central hypothyroidism in the general population is generally assumed to be very low: 1–2 cases per 100,000 persons per year (Feldt-Rasmussen and Klose, 2016). However, several studies have indicated that it might not be so rare after all (Beckett and Toft, 2003; Feldt-Rasmussen and Klose, 2016; Preiss *et al.*, 2008). The main reason for overlooking cases is the current screening strategy for hypothyroidism based on only TSH measurement (Beckett and Toft, 2003; Feldt-Rasmussen and Klose, 2016; Preiss *et al.*, 2008), while inclusion of free T4 in the programme might increase the diagnostic sensitivity (Beckett and Toft, 2003; Preiss *et al.*, 2008). This is not generally advocated for financial reasons and lack of good evidence (Dayan, 2001; Stockigt, 2011; Weetman, 1997). The gender distribution of central hypothyroidism is nearly equal. The occurrence of central

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Table 1 Causes of hypothyroidism

Central (hypothalamic/pituitary) hypothyroidism
Loss of functional tissue
Tumors (pituitary adenoma, craniopharyngioma, meningioma, germinoma, glioma, metastases)
Trauma (surgery, irradiation, head injury)
Vascular (ischemic necrosis, hemorrhage, stalk interruption, aneurysm of internal carotid artery)
Infections (abscess, tuberculosis, syphilis, toxoplasmosis)
Infiltrative (sarcoidosis, histiocytosis, hemochromatosis, lymphocytic, immunoglobulin G4 hypophysitis, hypophysitis after treatment with monoclonal antibodies and granulomatous hypophysitis)
Congential (pituitary hypoplasia, septo-optic dysplasia, basal encephalocele)
Functional defect in TSH biosynthesis and release
Mutations in genes encoding for TRH receptor, TSH-β, or Pit-1 (pituitary-specific positive transcription factor 1)
Drugs: dopamine, glucocorticoids, L-thyroxine withdrawal, somatostatin analogues, interferons, RXR-selective ligands
Recovery after hyperthyroidism with sustained or temporary suppressed TSH
Primary (thyroidal) hypothyroidism
Loss of functional thyroid tissue
Chronic autoimmune thyroiditis e.g. Hashimoto's thyroiditis
Reversible autoimmune hypothyroidism (silent and postpartum thyroiditis, cytokine-induced thyroiditis, immune therapy (antibodies against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) or programmed cell death protein (PD-1) and its ligand PD-L1))
Surgery and irradiation (^{131}I or external irradiation)
Infiltrative and infectious diseases, subacute thyroiditis
Thyroid dysgenesis/agenesis
Functional defects in thyroid hormone biosynthesis and release
Congenital defects in thyroid hormone biosynthesis
Iodine deficiency and iodine excess
Drugs: antithyroid agents, lithium, Amiodarone, natural and synthetic goitrogenic chemicals/agents, mitotane
"Peripheral" (extrathyroidal) hypothyroidism
Thyroid hormone resistance
Infantile hemangioma

hypothyroidism peaks during childhood and in adults 30 to 60 years of age. TSH deficiency caused by loss of functional tissue usually becomes manifest after the development of growth hormone and gonadotropin deficiency, and before loss of corticotropin and prolactin production (Feldt-Rasmussen and Klose, 2016).

Childhood

Congenital cases are due to pituitary hypoplasia, midline defects such as septo-optic dysplasia (TSH deficiency in 20% of patients), Rathke's pouch cyst, or rare "loss-of-function" mutations in genes encoding for the TRH receptor, the TSH-β-subunit, or Pit-1. Pit-1 is a pituitary-specific transcription factor confined to nuclei of somatotropes, lactotropes, and thyrotropes in the anterior pituitary, and Pit-1 deficiency results in growth hormone and prolactin deficiency and sometimes also in TSH deficiency. However, most childhood cases of central hypothyroidism are caused by craniopharyngiomas (TSH deficiency in 53% of patients) or cranial irradiation (e.g., for dysgerminoma or hematological malignancies) (Beck-Peccoz *et al.*, 2017; Feldt-Rasmussen and Klose, 2016; Lando *et al.*, 2001; Persani, 2012).

Adulthood

Adult cases of central hypothyroidism are most frequently due to pituitary macroadenomas (TSH deficiency in 10%–25% of patients), pituitary surgery, or irradiation (Feldt-Rasmussen and Klose, 2016; Persani, 2012; Schmiegelow *et al.*, 2003). TSH deficiency sometimes disappears after selective removal of a pituitary adenoma. Cranial radiotherapy for brain tumors causes hypothyroidism in 65% of patients, depending on the radiation dose, and its onset varies between 1 and 26 years after the irradiation. Radiotherapy for pituitary tumors is followed by hypothyroidism in at least 15% of patients (up to 55% when combined with surgery). Less common causes are severe head trauma, ischemic necrosis of the pituitary from postpartum hemorrhage (syndrome of Sheehan) or severe shock, pituitary apoplexy (hemorrhage in a pituitary adenoma), and immune therapy-induced hypophysitis, immunoglobulin G4 hypophysitis or lymphocytic hypophysitis. Lymphocytic hypophysitis is likely an autoimmune disease, presenting as a pituitary mass with hypopituitarism predominantly in women during pregnancy and the postpartum period.

Dopamine infusions in critically ill patients may cause central hypothyroidism by inhibition of pituitary TSH secretion. A huge excess of glucocorticoids (e.g., in Cushing syndrome) also dampens TSH secretion. Removal of the dopamine or steroid excess restores TSH release. A transient inhibition of TSH release is observed after withdrawal of long-term T_4 treatment in TSH-

suppressive doses, lasting for about 6 weeks, but can be longer in cases of very long-term treatment at very high dosages. Due to the same mechanism, TSH release can be inhibited for a very long time during recovery after hyperthyroidism.

Primary Hypothyroidism

The hallmark of primary hypothyroidism is an elevated serum TSH. The pituitary senses the lower plasma concentrations of T_4 and responds by increasing the release of TSH. Primary hypothyroidism is a very prevalent disease worldwide, especially in iodine-deficient regions. The incidence in the adult population is 4.1 cases per 1000 women per year and 0.6 cases per 1000 men per year. Thus, the most frequent causes of primary hypothyroidism in any given population depend very much on environmental factors, of which iodine intake is the most important (Zimmermann and Boelaert, 2015). Primary hypothyroidism is therefore mainly caused by iodine deficiency in areas of low iodine intake of the population (Zimmermann, 2009), but it is also very common in iodine-sufficient regions. In those areas, however, the cause is mainly due to chronic autoimmune thyroiditis such as Hashimoto's thyroiditis, followed by thyroidectomy and ^{131}I treatment. The incidence of congenital hypothyroidism is approximately 1 in 3500 newborns.

In a Danish epidemiological study from the DanThyr cohorts, Carle *et al.* (2006) described during a 4-year period from 1997 to 2000 (2,027,208 person-years) 685 new diagnosed cases of overt hypothyroidism; the incidence rate was 32.8 per 100,000 person-years (standardized to the Danish population). Nosological types of hypothyroidism were: spontaneous (presumably autoimmune) 84.4%, post-partum 4.7%, amiodarone-associated 4.0%, subacute thyroiditis 1.8%, previous radiation or surgery 1.8%, congenital 1.6% and lithium-associated 1.6%. Crude incidence rates were 29.0 around Aalborg and 40.6 in an area of Copenhagen. The higher incidence rate of hypothyroidism in the area with higher iodine intake was caused solely by more cases of spontaneous (presumably autoimmune) hypothyroidism, whereas the incidence of non-spontaneous hypothyroidism (all types combined) was significantly lower in the area with higher iodine intake.

Childhood

Congenital primary hypothyroidism can be caused by structural loss of thyroid tissue due to thyroid agenesis or an ectopic thyroid gland or by functional defects in thyroid hormone biosynthesis due to loss-of-function mutations in genes encoding the TSH receptor, the sodium iodide symporter, thyroglobulin, or thyroid peroxidase (Hannoush and Weiss, 2017; Persani and Bonomi, 2017). Neonatal mass screening programs will detect nearly all cases of congenital primary hypothyroidism. Chronic autoimmune thyroiditis is rare during childhood.

Adulthood

Hypothyroidism secondary to chronic autoimmune thyroiditis is caused mainly by destruction of thyrocytes. Its atropic variant is much more common than the goitrous variant, which is the original definition of Hashimoto's thyroiditis. Hashimoto's thyroiditis is, however, now often used synonymous with chronic autoimmune thyroiditis. The incidence is higher in women than in men and increases with advancing age. Still other variants are silent or painless thyroiditis and postpartum thyroiditis (occurring in 4%–6% of women during the first year after delivery and in up to 25% of women with type 1 diabetes mellitus), which are mostly self-limiting diseases with spontaneous recovery. Another type of reversible autoimmune hypothyroidism is induced by treatment with cytokines such as interleukin-2 and interferon-α, associated with the occurrence of thyroid peroxidase antibodies.

Hypothyroidism after subtotal thyroidectomy is less common after surgery for nontoxic or toxic nodular goiter (15% of patients) than after surgery for Graves' hyperthyroidism (40% of patients after 10 years). Most patients become hypothyroid during the first year after surgery. Likewise, hypothyroidism after ^{131}I therapy is less common in patients with toxic nodular goiter (6%–13%) than in patients with Graves' hyperthyroidism (up to 70% after 10 years). External radiotherapy of the neck for lymphomas or head and neck cancer causes hypothyroidism in 25%–50% of patients, depending on pretreatment with iodine-containing radiographic contrast agents, radiation dose, and duration of follow-up.

Hypothyroidism is very rarely caused by thyroid infections or thyroidal infiltration during the course of invasive fibrosis, cystinosis, or amyloidosis. In contrast, hypothyroidism during the recovery phase of subacute thyroiditis or postpartum thyroiditis is not uncommon. Iodine excess may induce hypothyroidism by failure to escape from the Wolff–Chaikoff effect. Sources of iodine excess include an iodine-rich diet (e.g., seaweed) and iodine-containing medications such as potassium iodide, kelp, topical antiseptics, radiographical contrast agents, and amiodarone.

Lithium inhibits thyroidal iodide transport and release of thyroid hormones. Long-term lithium treatment results in goiter (up to 50% of patients) and hypothyroidism (20% of patients), usually occurring during the first 2 years of treatment and particularly in patients with preexisting thyroid peroxidase antibodies. Furthermore the newly developed immune therapeutical drugs may induce thyroiditis as a side effect.

"Peripheral" Hypothyroidism

Hypothyroidism due to abnormalities outside the hypothalamus, pituitary, and thyroid is rare. Impaired sensitivity to thyroid describes a process that interferes with the effectiveness of thyroid hormone and includes defects in thyroid hormone action, transport, or metabolism. Several genomic causes have been described and the field has expanded immensely over the past years (Hannoush and Weiss, 2017; Persani and Bonomi, 2017). The most common cause of thyroid hormone resistance is a genomic mutation of the thyroid hormone receptor TRβ_1 fails to transmit the proper signal, causing symptoms and signs of thyroid hormone deficiency in certain target tissues. Serum TSH and serum T_4 and triiodothyronine (T_3) are increased - a remarkable combination of test results. The disease is frequently detected only during adulthood. Three cases of infants with massive hepatic hemangioma and hypothyroidism with markedly elevated serum TSH have been described (Huang *et al.*, 2000). The hypothyroidism was caused by high levels of type 3 iodothyronine deiodinase activity in the hemangioma tissue, which catalyzes the degradation of T_4 into the inactive reverse T_3 and the degradation of T_3 into the inactive 3,3′-T_2.

See also: Drug Effects and Thyroid Function. Epidemiology of Thyroid Disease. Hashimoto's Thyroiditis. Hypothyroidism Subclinical. Postpartum Thyroid Dysfunction. Thyroid Autoimmunity. Thyroid Disorders in the Elderly. Thyroiditis, Infectious and Subacute

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Diagnosis of Hypothyroidism☆

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Glossary

Accuracy The proportion of all test results, both positive and negative, that are correct.

Hypothyroidism Syndrome characterized by the clinical and biochemical manifestations of thyroid hormone deficiency in the target tissues of thyroid hormones.

Negative predictive value The probability of not having the disease when the test result is negative (normal).

Nosology The systematic classification of diseases.

Positive predictive value The probability of disease in a patient with a positive (abnormal) test result.

Sensitivity The proportion of people with the disease who have a positive test for the disease.

Specificity The proportion of people without the disease who have a negative test for the disease.

Syndrome A group of manifestations that together are characteristic of a specific disease.

Introduction

The diagnosis of hypothyroidism can be divided into two phases. During the first phase, it should be ascertained whether thyroid hormone deficiency exists (diagnosis of the hypothyroid syndrome). If so, the second phase is aimed to establish which disease is causing the lack of thyroid hormone (nosologic diagnosis) (Stockigt, 2016).

Diagnosis of the hypothyroid syndrome starts with the history and physical examination of the patient, followed by laboratory tests in case of sufficient and appropriate clinical suspicion. The rationale for history, including family history, and for clinical examination is to increase the pretest likelihood of hypothyroidism so that fewer patients need hormone tests, thereby increasing the diagnostic accuracy of the laboratory test results. In some cases, however, a diagnostic laboratory screening strategy may be used for example, in relation to investigations for infertility (Baloch *et al.*, 2003; Feldt-Rasmussen *et al.*, 2011; Stockigt, 2016). The rationale for a nosologic diagnosis is to detect possible cases of reversible hypothyroidism and to increase awareness of the possible existence of conditions associated with a specific cause. In all situations it is important to exclude confounders (e.g. drug and assay interference) that can distort conclusions of diagnoses (Baloch *et al.*, 2003; Feldt-Rasmussen *et al.*, 2011; Esfandiari and Papaleontiou, 2017).

Syndromal Diagnosis

Clinical Assessment

None of the symptoms and signs of hypothyroid patients is specific for the syndrome, but the simultaneous occurrence of a number of symptoms and signs adds to the specificity. Consequently, statistical methods have been applied based on the relative frequency of symptoms and signs in hypothyroid patients and in controls. A simple score is derived awarding 1 point each for the presence of 12 symptoms and signs; correction for age is done by adding 1 point for individuals below 55 years of age (**Table 1**). The positive predictive value for hypothyroidism is 96.9% with a score of 6 points or more; the negative predictive value for exclusion of hypothyroidism is 94.2% with a score of 2 points or less. Approximately 62% of overt hypothyroid patients and 24% of subclinical hypothyroid patients are classified as clinically hypothyroid by this score (Seshadri *et al.*, 1989; Zulewski *et al.*, 1997).

Biochemical Assessment

The ideal test would be one that accurately measures the consequence of thyroid hormone deficiency in target tissues. However, tissue function tests such as serum cholesterol lack sufficient sensitivity and specificity to be of much use. Without doubt, serum thyroid-stimulating hormone (TSH) is currently the best single assay for detection of hypothyroidism (Wiersinga, 1989; Baloch *et al.*, 2003; Esfandiari and Papaleontiou, 2017), although presence of TSH isoforms may compromise results (Estrada *et al.*, 2014). Test characteristics of the serum TSH assay for the diagnosis of abnormal thyroid function are 98.8% sensitivity, 94.3%

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Table 1 Accuracy of 12 symptoms and signs in the diagnosis of primary hypothyroidism (percentages)

Symptoms and signs	*Sensitivity*	*Specificity*	*Positive predictive value*	*Negative predictive value*
Symptoms				
Hearing impairment	22	98	90	53
Diminished sweating	54	86	80	65
Constipation	48	85	76	62
Paraesthesia	52	83	75	63
Hoarseness	34	88	73	57
Weight increase	54	78	71	63
Dry skin	76	64	68	73
Physical signs				
Slow movements	36	99	97	61
Periorbital puffiness	60	96	94	71
Delayed ankle reflex	77	94	92	80
Coarse skin	60	81	76	67
Cold skin	50	80	71	62

Note: Hypothyroid, $\geq$6 points; intermediate, 3–5 points; euthyroid, $\leq$2 points.

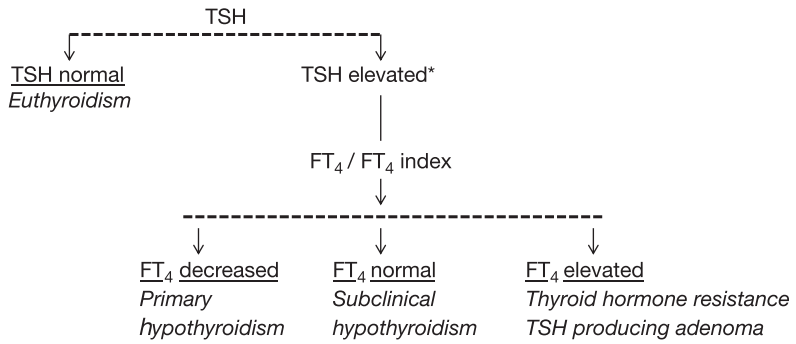

Fig. 1 Flow diagram for the biochemical diagnosis of hypothyroidism. *, if TSH is elevated it is important to exclude confounders for TSH elevation such as recovery after nonthyroidal illness, heterophilic antibodies or other interfering factors in the patient's serum, or transitory high TSH. FT4: Serum free thyroxine; FT4 index: measure of the total serum thyroxine concentration corrected for binding proteins = free thyroxine estimate.

specificity, 83.9% positive predictive value, and 99.7% negative predictive value. The high diagnostic accuracy of the TSH assay is caused by the exquisite sensitivity of the pituitary for small changes in serum thyroid hormone concentrations, displaying a log-linear relationship (Baloch *et al.*, 2003). Because of the negative feedback of thyroid hormone on the TSH release from the pituitary, a fall in serum thyroxine will result in elevated TSH levels in serum, and a rise in serum thyroid hormone concentrations will suppress serum TSH. This, however, requires an intact and stable hypothalamo–pituitary–thyroid axis (Baloch *et al.*, 2003), which is probably the only situation where the relationship between serum TSH and serum free thyroxine (fT4) has been reported to be log-linear (Baloch *et al.*, 2003; Esfandiari and Papaleontiou, 2017).

Fig. 1 depicts a flow diagram for the biochemical diagnosis of primary hypothyroidism. If TSH is normal, euthyroidism is nearly certain, and no further tests are necessary. However, central hypothyroidism (due to TSH deficiency) may be overlooked because serum TSH in this condition is usually normal or decreased. Fortunately, clinical examination often provides sufficient clues to suspect hypothalamic or pituitary disease such as symptoms arising from space-occupying lesions in the sella or from overproduction of pituitary hormones. As a rule, lack of gonadotropins occurs before the onset of TSH deficiency; therefore, the presence of regular menstrual periods in women or normal potency in men renders central hypothyroidism unlikely (Feldt-Rasmussen and Klose, 2016). The low incidence of central hypothyroidism (1–3 per 100,000 persons per year) does not warrant routine free thyroxine (FT_4) measurements after a normal TSH test result, although the possibility is being discussed (Waise and Belihetz, 2000; Beckett and Toft, 2003; Preiss *et al.*, 2008; Beck-Peccoz, 2017).

If TSH is elevated, a decreased serum FT_4 value indicates overt hypothyroidism and a normal FT_4 points to subclinical hypothyroidism. In both instances, the thyroid gland itself is at fault (primary hypothyroidism). The very rare combination of elevated TSH and elevated FT_4 allows the diagnosis of thyroid hormone resistance or TSH-producing adenoma (Feldt-Rasmussen and Klose, 2016; Beck-Peccoz, 2017).

It is important to recognize possible confounders in the assays for thyroid function tests or the pathophysiology of other situations that carry the risk for false interpretation. Estimates of FT_4 concentrations are globally widely measured on different platforms in Clinical Biochemical Laboratories, and they display a huge interlaboratory variability (Baloch *et al.*, 2003; Welsh and Soldin, 2016). Furthermore, changes in thyroid hormone binding proteins and use of many drugs may distort the results and their

interpretation. In such cases a total T4 measurement combined with an assessment of binding proteins (free T4 index) gives more information and more reliable results (Baloch *et al.*, 2003; Feldt-Rasmussen *et al.*, 2011; Welsh and Soldin, 2016; Esfandiari and Papaleontiou, 2017). If TSH is elevated it is important to exclude confounders for TSH elevation such as during recovery after nonthyroidal illnesss, heterophilic antibodies or other interfering factors in the patient's serum, or transitory high TSH (Baloch *et al.*, 2003; Stockigt, 2016; Esfandiari and Papaleontiou, 2017). Reversibility is verified by repeat serum TSH after 3–6 months, where serum TSH then will be normalized.

Nosologic Diagnosis

The cause of the hypothyroidism may reveal itself in many instances from the history (e.g., recent delivery, exposure to iodine excess, presence of other autoimmune diseases (Hollowell *et al.*, 2002; Feldt-Rasmussen, 2015; Bliddal *et al.*, 2017a,b; Kahaly and Frommer, 2017), family members with autoimmune thyroid disease or other autoimmune diseases, use of antithyroid drugs, thyroid surgery or ^{131}I therapy, intake of certain other drugs, hypothalamo–pituitary disease) and physical examination (although many patients will have no goiter), and clinical scoring (Seshadri *et al.*, 1989; Zulewski *et al.*, 1997). In iodine deficient areas, this deficiency as cause should be suspected.

The presence of thyroid peroxidase antibodies (and/or thyroglobulin antibodies) (Baloch *et al.*, 2003; Bliddal *et al.*, 2017a,b) in serum indicates chronic autoimmune thyroiditis, which is the most prevalent cause of hypothyroidism in the Western world (Carlé *et al.*, 2006). Serum TSH measurement is therefore paramount in the differential diagnosis, sometimes supplemented with measurement of serum thyroglobulin antibodies. Thyroid scans usually show low and inhomogenous uptake of the radioisotope in chronic autoimmune thyroiditis. Preserved thyroidal radioiodine uptake and homogenous distribution of the tracer increases the likelihood of reversible hypothyroidism (due to iodine excess).

Aberrant thyroid tissue (e.g., lingual thyroid, ovarian goiter) can be detected by a thyroid scintiscan, while hypothyroidism due to subtle enzyme and other abnormalities can be more challenging, as in for example, Pendred syndrome where genetic testing for a mutation in the pendrin gene may be required. This will be dealt with in other articles (Baloch *et al.*, 2003).

Spontaneous recovery of hypothyroidism occurs in the course of subacute and postpartum thyroiditis and sometimes during the first 6 months after subtotal thyroidectomy or ^{131}I therapy. It is unlikely in the final stages of chronic autoimmune thyroiditis, but may be present during the initial phases of autoimmune thyroiditis with fluctuating hyperthyroidism (autoimmune destructive release of thyroid hormone from the gland) alternating with hypothyroidism or stimulating thyroid hormone receptor antibodies alternating with blocking ones (Kraiem *et al.*, 1992; Diez *et al.*, 2005).

See also: Hypothyroidism Subclinical. Thyroid Function Tests. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Central Hypothyroidism☆

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Glossary

Thyrotopin (TSH) Pituitary heterodimeric molecule belonging to the glycoprotein hormone family, including also gonadotropins (FSH, LH, CG) and the recently identified thyrostimulin. TSH and gonadotropins are constituted by a common alpha subunit and a beta subunit which confers the specific hormone properties to the heterodimer. TSH synthesis and secretion occur in specific pituitary cells (thyrotropes), are negatively regulated by thyroid hormone feedback mechanism and positively influenced by hypothalamic Thyrotropin-Releasing Hormone. TSH is the major stimulator of thyroid function and postnatal growth. It binds to a specific G protein-coupled receptor, which consists of seven membrane-spanning domains and preferentially coupled to Gs. TSH binding is mainly associated with the activation of adenylate cyclase and cAMP pathway. However, recent evidences support a relevant functional role also to Gq coupling and consequent activation phospholipase C and the inositol-phosphate/diacylglycerol pathway.

Thyrotropin-Releasing Hormone (TRH) Tripeptide (L-pyroglutamyl-histidyl-prolineamide) secreted by hypothalamic neurons. Cell bodies are located in the medial parvocellular portion of the paraventricular nucleus, and project neuronal endings to the median eminence, where TRH is secreted. The neuro-hormone reaches pituitary target cells through the portal system and the pituitary stalk. TRH synthesis and secretion are mainly under the control of negative thyroid hormone feedback mechanism and are influenced by several other hypothalamic factors. TRH is the major positive regulating factor of thyrotropin synthesis and secretion and also stimulates prolactin secretion. TRH binds to a specific G-protein coupled receptor, which is preferentially coupled to a Gq. TRH binding is associated with the activation of phospholipase C and the inositol-phosphate/diacylglycerol pathway.

Definition

Central hypothyroidism (CeH) is a rare form of hypothyroidism, representing about 1 out of 1000 hypothyroid subjects (Nebesio *et al.*, 2010; Kempers *et al.*, 2006; Price and Weetman, 2001). It is the consequence of defects in thyrotropin (TSH) secretion, finally resulting in an insufficient stimulation of an otherwise normal thyroid gland. In central hypothyroid cases, a combined hypothalamic-pituitary impairment is usually found (Persani and Bonomi, 2017; Schoenmakers *et al.*, 2015; Persani, 2012).

Diagnosis

The missed diagnosis of CeH represents the main drawback of the reflex-TSH strategy for the screening of thyroid function (Persani, 2012; LaFranchi, 2010; Miyai *et al.*, 1971; Kempers *et al.*, 2006). The serum concentrations of immunoreactive TSH and free thyroxine (FT4) in a series of CeH patients clearly indicate that the diagnosis of these patients cannot be reached by using single TSH measurements. Indeed, FT4 is the parameter with the highest sensitivity and specificity for the diagnosis of hypothyroidism in a population of patients with hypothalamic-pituitary lesions (Persani, 2012; Koulouri *et al.*, 2011; Yamada and Mori, 2008; Alexopoulou *et al.*, 2004; Ferretti *et al.*, 1999). As in primary hypothyroidism, total T4 allows only the recognition of patients with severe impairment of thyroid function. The finding of low FT4, measured by reliable immunoassays, in the presence of low/normal/or even elevated immunoreactive TSH concentrations is consistent with the diagnosis of CeH (**Fig. 1**). Free thyroxine index is a valid alternative parameter for the diagnosis and management. Nevertheless, TSH concentrations in the range of mild primary hypothyroidism can be found also among patients with CeH with prevalent hypothalamic damage and could lead to misdiagnosis (Persani and Bonomi, 2017; Persani, 2012). The exclusion of primary thyroid failure and of interference in TSH measurement retains a fundamental role in confirming the central origin of the thyroid dysfunction (Persani, 2012). Exclusion of primary hypothyroidism should be documented by the absence of any sign of direct thyroid lesion (negative antithyroid antibodies, gland of small/normal size and normal structure at ultrasound). Interference possibly leading to false decrease of TSH concentrations in noncompetitive modern immunoassays includes anti-TSH autoantibodies: exclusion of interference in TSH measure may be obtained by dilution or recovery tests. Indeed, the existence of concomitant or preexisting hypothalamic-pituitary disease, such as combined pituitary hormone deficiencies or

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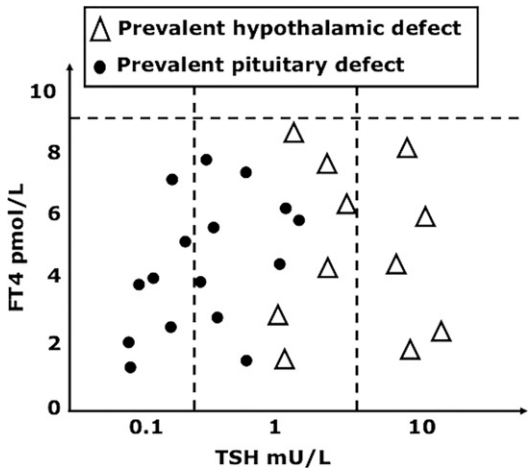

Fig. 1 Serum levels of FT4 and TSH in patients with central hypothyroidism. *Dotted lines* indicate the lower or upper limits of the normal ranges. The evident lack of correlation between TSH and FreeT4 demonstrates that quantitative impairment of TSH secretion does not completely explain the pathogenesis of CeH, and indicates the involvement of qualitative alterations of hypothalamic regulation in a large portion of CeH patients and in particular in those with prevalent hypothalamic defects.

alterations at imaging of sella region, strongly supports the diagnosis of CeH. The history of previous traumatic brain injury or vascular accidents can also support a CeH diagnosis.

Confirmation of CeH can be obtained by TRH testing, in the settings where this test is still currently available (Persani, 2012; van Tijn *et al.*, 2008; Darzy and Shalet, 2005; Mehta *et al.*, 2003; Rose *et al.*, 1999; Faglia, 1998). The finding of blunted or delayed/prolonged responses of immunoreactive TSH after intravenous TRH injections confirm a prevalent pituitary or hypothalamic defect, respectively. Others have not found the test more useful than measurement of basal serum TSH (Hartoft-Nielsen *et al.*, 2004; Yamakita *et al.*, 2001). Another characteristic of CeH is the blunting of nocturnal TSH surge (Darzy and Shalet, 2005; Yamakita *et al.*, 2001; Rose *et al.*, 1999). Both of these anomalies can become useful to support a hidden CeH diagnosis that may be suspected in the presence of FT4 levels in the lower part of the normal range, as in mild primary thyroid failure.

Transient or reversible forms of CeH can be associated with drugs able to inhibit TSH secretion or during recovery from thyrotoxicosis (Persani, 2012; Haugen, 2009).

Finally, before giving the diagnosis of CeH, one should exclude the presence of the so-called "nonthyroidal illnesses" (NTIs) leading to the biochemical picture of the "low T3 syndromes" (Persani, 2012). These conditions include acute (e.g., myocardial infarction, severe infections, extended burns) or chronic (e.g., liver or kidney insufficiency) diseases, or fasting (as in anorexia nervosa), or recovery from general surgery and occur also in patients with intact hypothalamic-pituitary-thyroid axis. NTIs are generally associated with low T3, but normal TSH and FT4, and fall of circulating TSH and FT4 can be seen only in prolonged and manifest disease states as the likely consequence of the suppression of hypothalamic TRH gene expression.

Clinical Presentation

Depending on the cause of CeH, some characteristics of the disease may vary greatly. Genetic CeH can be either isolated or combined with other pituitary hormone deficiencies (CPHD) and can be associated with growth retardation, delayed puberty and/or neurological defects (Persani, 2012; Castinetti *et al.*, 2016; Schoenmakers *et al.*, 2015; Raivio *et al.*, 2012; Pfäffle and Klammt, 2011). Neonatal forms of CeH are not recognized by the screening programs for congenital hypothyroidism based on primary TSH evaluation on dried blood spots (Persani, 2012; LaFranchi, 2010; Bonomi *et al.*, 2009; van Tijn *et al.*, 2008; Miyai, 2007; Kempers *et al.*, 2006), and neurological defects may be particularly severe in patients with TSHβ defects (Persani and Bonomi, 2017; Libri *et al.*, 2013; Ramos *et al.*, 2010; Bonomi *et al.*, 2001; Dacou-Voutetakis *et al.*, 1990; Hayashizaki *et al.*, 1989; Miyai *et al.*, 1971). Nevertheless, several genetic forms generate a mild and/or progressive onset of hypothyroidism beyond the vulnerable period of central nervous system development during infancy (Persani and Bonomi, 2017; Schoenmakers *et al.*, 2015; Bonomi *et al.*, 2009; Collu *et al.*, 1997). Some genetic forms should also be suspected in adolescents or adults with the biochemical features of CeH but lacking a plausible cause of acquired CeH. Some particular clinical features are indicative of specific gene defects (such as macrorchidism for *IGSF1*, or audiometric defects for *TBL1X* defects) (Joustra *et al.*, 2013, 2016; Heinen *et al.*, 2016; Sun *et al.*, 2012).

Nongenetic CeH is typically sporadic and acquired in most cases as the consequence of large sella tumors (Schoenmakers *et al.*, 2015; Persani, 2012). Among the lesions potentially leading to CeH, those that are more frequently associated with hypothalamic defects include large pituitary macroadenomas with suprasellar extension, craniopharyngiomas and suprasellar tumors, cranial irradiation, vascular accidents, and head traumas. In these situations, defects of posterior pituitary function with diabetes insipidus as well as visual field defects, due to compression of the optic nerves, may be concomitant. These lesions generally affect both pituitary and hypothalamic functions to various extent leading to hypothyroidism, always combined with defects of other anterior pituitary hormones and frequently associated with high prolactin levels due to stalk resection or compression. The association with signs and symptoms secondary to the altered secretion of other pituitary hormones (e.g., menstrual disorders, decreased libido, hair loss, galactorrhea, pallor, altered lipid metabolism, etc.) or to local compression (visual defects, headache, etc.) may cover the specific hypothyroid manifestations in several cases. Therefore, the evaluation of TSH and FT4 should always be included in the biochemical evaluation of patients with hypothalamic-pituitary diseases (Feldt-Rasmussen and Klose, 2016), and mild CeH should be suspected also in cases presenting with significant decrements of FT4 levels toward the lower limits of normal.

Pathogenesis

The pathogenesis of typical CeH is due to defects affecting the TRH/TSH function. These defects occur in various pathological conditions including large pituitary macroadenomas with suprasellar extension, craniopharyngiomas and suprasellar tumors (such as meningiomas or gliomas), cranial irradiation, vascular accidents (including Sheehan syndrome), head traumas (including traumatic delivery), infiltrative diseases (such as sarcoidosis) (Schoenmakers *et al.*, 2015; Persani, 2012; Barbesino *et al.*, 2012). As shown also in **Fig. 1**, quantitative impairment of pituitary TSH reserve does not completely explain the pathogenesis of CeH, and indicates the involvement of qualitative alterations in a large portion of CeH patients and in particular in those with prevalent hypothalamic defects. Several patients with CeH of hypothalamic origin have normal or even elevated circulating TSH levels. The lack of any signs of primary thyroid insufficiency (absent antithyroid autoantibodies and normal response to exogenous TSH injection) suggested that hypothyroidism in these patients could result from the secretion of biologically inactive TSH molecules (Persani, 2012). This hypothesis was indirectly supported by the blunted thyroid hormone response to endogenous TRH-stimulated TSH, despite exaggerated and prolonged responses of endogenous TSH to the tripeptide. The very low bioactivity of TSH molecules circulating in several patients with CeH of various origins (idiopathic, secondary to pituitary or hypothalamic tumors, or due to cranial irradiation or Sheehan syndrome) was obtained by means of different bioassays. The reduced bioactivity was due to an impaired binding of circulating TSH to its specific thyroid receptor. Chronic TRH treatment restored both TSH receptor binding and bioactivity, indicating that this hypothalamic factor is necessary for the secretion of TSH molecules with structural features essential for appropriate thyroid stimulation. Lectin affinity chromatography showed that the oligosaccharide structure of TSH molecules circulating in CeH is altered.

Genetic defects associated with CeH in humans and the relative clinical features are reported in **Table 1**.

Table 1 List of genes known to be involved in the pathogenesis of CeH and the corresponding phenotypes

Genes (OMIM #)	*Phenotypes (OMIM #)*	*Inheritance*
TSHβ (188540)	Severe isolated CeH of neonatal onset with high α-GSU, pituitary hyperplasia (275100)	Recessive
TRHR (188545)	Isolated CeH with blunted TSH/PRL response to TRH and apparently uneventful infantile development, and with childhood (growth retardation) to adulthood onset	Recessive
IGSF1 (300137)	CeH associated with low PRL, partial GH deficiency, and macro-orchidism	X-linked
TBL1X (300196)	Mild isolated CeH, normal TRH test, audiometric defects	X-linked
POU1F1 (173110)	Moderate/severe CeH of neonatal to childhood onset combined with GH and PRL defects, prominent forehead, mid-face hypoplasia, depressed nose (613038)	Dominant or recessive
PROP1 (601538)	Moderate/severe CeH of neonatal to childhood onset, combined with GH, PRL, LH/FSH defects, and ACTH deficiency, pituitary hypo – /hyperplasia (262600)	Recessive
HESX1 (601802)	Severe panhypopituitarism associated with septo-optical dysplasia (SOD), supernumerary/hypoplastic digits (182230)	Dominant or recessive
SOX3 (313430)	Hypopituitarism with pituitary hypoplasia, variable ectopic posterior pituitary, persistent cranio-pharyngeal canal and mental retardation (312000)	X-linked
OTX2 (600037)	Hypopituitarism with pituitary hypoplasia, ectopic posterior pituitary, ano/microphtalmia, retinal dystrophy (610125)	Dominant
LHX3 (600577)	Hypopituitarism with conserved ACTH function and associated with pituitary hypo- or hyperplasia, short/rigid cervical spine and variable deafness (221750)	Recessive
LHX4 (602146)	Combined anterior pituitary defects associated with abnormalities of cerebellum and small sella turcica (262700)	Dominant
LEPR (601007)	Severe obesity and hyperphagia combined with delayed puberty and mild thyrotropin defect	Recessive

Treatment

As in primary hypothyroidism, treatment of CeH is based on the administration of L-thyroxine (Persani, 2012; Wiersinga *et al.*, 2012; Beck-Peccoz, 2011). In this condition, particular attention should be paid to the recognition of possible concomitant corticotropin (ACTH) defects leading to partial adrenal insufficiency before starting L-thyroxine therapy. Failure to recognize this concomitant defect may lead to acute adrenal insufficiency during initial days of therapy. Therefore, if a combined corticotrope deficiency is suspected, the screening for adrenal insufficiency is recommended. Alternatively, cautionary treatment with corticosteroids may be advised before starting L-thyroxine.

L-thyroxine replacement therapy should be tailored for each patient, as in primary hypothyroidism (Persani, 2012; Koulouri *et al.*, 2011; Klose *et al.*, 2013; Feldt-Rasmussen and Klose, 2016). Nevertheless, this goal is not easily reached in CeH patients because TSH measurement loses its value as monitoring index. Therapy should be tailored in order to maintain FT4 (and FT3) concentrations in the middle of the reference range, provided that blood for hormone measurement is withdrawn before the morning administration of L-Thyroxine (Persani, 2012; Koulouri *et al.*, 2011). Evaluation of parameters of thyroid hormone action (such as cholesterol, bone markers or sex hormone-binding globulin) could be helpful in cases of doubt. Replacement doses range 1.1–1.7 $\mu g\,kg^{-1}$ per day in the large majority of adult patients, and are generally lower in those over 65 years. Inappropriate treatment should be suspected on the basis of the following conditions: (a) careful clinical evaluation uncovering manifestations of under- or overtreatment regimens; (b) circulating FT4 values in the lower or upper tertile of normal range suggesting under- or overtreatment, respectively; (c) circulating TSH values >0.5 mU L^{-1} generally suggesting undertreatment; (d) addition or removal of replacement therapies for multiple pituitary hormone deficiencies, such as growth hormone or gonadotropin deficiencies.

See also: Hypothalamus–Pituitary–Thyroid Axis. Hypothyroidism Subclinical. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Hashimoto's Thyroiditis☆

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Glossary

Autoimmunity The condition in which the immune system of an organism attacks its own healthy cells, tissues, or organs, resulting in their damage.

Iodine and the thyroid Iodine is an essential element for the production of thyroid hormone. Thyroid antibodies and autoimmune hypothyroidism are more common in iodine-sufficient areas than in iodine-deficient areas.

Euthyroid Relating to or characterized by normal thyroid function.

Goitrous Affected with goiter; that is, having an enlargement of the thyroid gland.

Hypothyroidism A pathological condition due to insufficient production of thyroid hormones; diminished activity of the thyroid gland.

Thyroglobulin antibodies Auto-antibodies against the protein thyroglobulin, a substrate for the synthesis of the thyroid hormones and a storage of the inactive forms of thyroid hormone and iodine.

Thyroid antibodies Antibodies targeting against components on the thyroid tissues. Clinically relevant thyroid autoantibodies are the antithyroid peroxidase antibodies (TPOAb), thyrotropin receptor antibodies (TRAbs) and thyroglobulin antibodies (TgAb).

Thyroperoxidase antibodies Auto-antibodies against the thyroperoxidase (TPO), an essential enzyme involved in thyroid hormone synthesis.

Hashimoto's thyroiditis is a common, lifelong, organ-specific autoimmune disease. Hashimoto's thyroiditis is associated with hypothyroidism and represents the one end of the clinical-pathological spectrum of the different phenotypes of autoimmune thyroid disease, with its counterpart being Graves' disease associated with hyperthyroidism.

Introduction

In his original article published in 1912 (Hashimoto, 1912), Hakaru Hashimoto described four goitrous patients with a typical histological pattern: diffuse lymphocytic infiltration and fibrosis of the thyroid gland, with a variable degree of atrophy and eosinophilic changes of the thyroid follicular cells. In 1956, Roitt *et al.* (1956) and Rose and Witebsky (1956) detected antibodies against thyroid microsomes (later identified as thyroid peroxidase antibodies Czarnocka *et al.*, 1985) in the serum of patients with Hashimoto's goiter.

Nowadays, the term Hashimoto's disease or Hashimoto's thyroiditis is widely used as a synonym for chronic lymphocytic thyroiditis, chronic autoimmune thyroiditis or autoimmune hypothyroidism, an organ-specific autoimmune disease characterized by lymphocytic infiltration of the thyroid gland and thyroid peroxidase antibodies in serum. Two main clinical types of chronic autoimmune thyroiditis exist marked by the presence or absence of a goiter but in any case, there are no serological markers that can distinguish between them. Purists might argue that the term Hashimoto's disease should be reserved for the less common goitrous variant of chronic autoimmune thyroiditis as originally described by Hashimoto.

Epidemiology

The presence of thyroid peroxidase antibodies (TPOAb) in serum correlates with the presence of focal thyroiditis in biopsy and postmortem material (Yoshida *et al.*, 1978) and is thus a reliable marker of chronic autoimmune thyroiditis. More than 90% of the patients with Hashimoto's thyroiditis are positive for TPOAb and it has been proposed that TPOAb have superior diagnostic value than the thyroglobulin antibodies (TgAb). Therefore, for many physicians TPOAb positivity suffices for the confirmation of Hashimoto's thyroiditis. Although this is true for the vast majority of Hashimoto's thyroiditis patients, there are patients where TgAb positivity is the only serological marker of thyroid autoimmunity (Pedersen *et al.*, 2011).

The prevalence of serum TPO-Ab in women rises from 15% at ages 18–24 years to 24% at ages 55–64 years, whereas the prevalence in men is much lower (~3%) with no age trend. The prevalence of subclinical hypothyroidism, is very high

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(75 per 1000 women and 28 per 1000 men) in the general adult population (Tunbridge *et al.*, 1977; Vanderpump *et al.*, 1995) and increases with advancing age. The prevalence of overt hypothyroidism in adults is 18 per 1000 women and 1 per 1000 men. It has been estimated that 90% of noniatrogenic hypothyroidism in iodine sufficient areas is due to thyroid autoimmunity.

Clinics

Most patients (~70%) with chronic autoimmune thyroiditis are asymptomatic and remain euthyroid. Many factors have been proposed as the determinants of the progression toward development of hypothyroidism. High iodine intake might be involved in light of a higher prevalence of autoimmune hypothyroidism in iodine-sufficient regions as compared with iodine-deficient regions (Bülow Pedersen *et al.*, 2002; Pedersen *et al.*, 2011). Overt hypothyroidism in patients on a very rich iodine diet (e.g., seaweed, kelp) can be reversible on avoiding the source of the iodine excess.

Current smoking is a known risk factor for Graves' disease but it has been showed that it is protective for Hashimoto's disease (Effraimidis and Wiersinga, 2014). Moderate alcohol consumption is associated with a reduction in the risk of Hashimoto's thyroiditis (Effraimidis *et al.*, 2012). Current studies don't allow definite conclusions regarding the use of selenium supplementation for Hashimoto's thyroiditis (van Zuuren *et al.*, 2014). Low vitamin D levels have been reported as a risk factor for various autoimmune diseases but the picture is less clear regarding Hashimoto's thyroiditis (Effraimidis and Wiersinga, 2014).

In the atrophic variant of chronic autoimmune thyroiditis (atrophic myxedema), fibrosis is the predominant feature along with lymphocytic infiltration. In the less common goitrous variant originally described by Hashimoto, the histology remains essentially unaltered after 20 years. The goiter is diffuse and has a firm "rubbery" consistence; it does not regress in 43% of patients despite T_4 treatment. Some patients have an initial transient hyperthyroid stage labeled as Hashitoxicosis. It disappears in a few months and can be caused either by autoimmune destruction with release of thyroid hormones into the circulation or to TSH receptor-stimulating antibodies, which may give way to TSH receptor-blocking antibodies (McLachlan and Rapoport, 2014).

The clinical features, the diagnosis and the treatment of the hypothyroidism of the Hashimoto's thyroiditis is discussed in the chapters Hypothyroidism, diagnosis of; Hypothyroidism, treatment of.

Overt autoimmune hypothyroidism demands in the vast majority of the cases lifelong treatment with L-thyroxine, but juvenile and adolescent Hashimoto's thyroiditis may be self limiting.

See also: Causes of Hypothyroidism. Drug Effects and Thyroid Function. Hypothyroidism Subclinical. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Postpartum Thyroid Dysfunction. Thyroglobulin. Thyroid and Infertility. Thyroid Carcinoma. Thyroid Function and Depression. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Relevant Website

http://www.thyroidmanager.org/chapter/hashimotos-thyroiditis1/

Systemic Manifestations of Hypothyroidism☆

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Glossary

Autoimmune thyroid disease A disease in which clinical manifestations are due to the reaction of the immune system against thyroid antigens.

Central hypothyroidism Hypothyroidism due to deficient stimulation of an intrinsically normal thyroid gland by thyrotropin; can be the consequence of an anatomical or functional disorder of the pituitary gland, the hypothalamus, or both.

Primary hypothyroidism Hypothyroidism due to a deficient function of the thyroid gland.

Introduction

Hypothyroidism may affect persons of both sexes and all ages. The clinical expression of thyroid hormone deficiency varies considerably between individuals. It is influenced mainly by the age of the patient and the rate at which hypothyroidism develops, whereas it is largely independent of its cause. Most adult patients complain of a slowing of physical and mental activity.

Hypothyroidism may be overt or subclinical, with the latter being defined as increased serum thyrotropin (TSH) and normal free thyroxine (T4) and triiodothyronine (T3) concentrations. Even among patients with overt hypothyroidism, the severity is variable. At one extreme are patients who have few symptoms and signs of hypothyroidism, and at the other extreme are those with myxedema coma.

Clinical Aspects of Hypothyroidism at various Ages

Infantile and Juvenile Hypothyroidism

Hypothyroidism in the newborn results in severe mental deficiency, neurological impairment, and physical retardation unless treatment is initiated within weeks after birth. Early diagnosis and treatment of congenital hypothyroidism, consequent to systematic screening for this condition during the neonatal period, results in the disappearance of the clinical picture of the disease, at least in developed countries. Hypothyroidism in children is characterized mainly by retarded growth and impaired mental performance. Infantile hypothyroidism leads to sexual immaturity, and juvenile hypothyroidism causes a delay in the onset of puberty followed by anovulatory cycles in females. Precocious puberty may occur rarely.

Thyroid hormone is essential for normal growth and maturation of the skeleton. Deficient thyroid hormone production *in utero* and in the neonate retards growth and delays skeletal maturation. Deficiency during early life also leads to an abnormal stippled appearance of the epiphyseal centers of ossification (epiphyseal dysgenesis). Before puberty, thyroid hormones also play a major role in the maturation of bone. Impairment of linear growth leads to dwarfism, where the limbs are disproportionately short in relation to the trunk. Bone age is retarded in hypothyroid children.

Hypothyroidism in the Adult

In the adult, the clinical manifestations of hypothyroidism may be profound, but they are reversible. The development of spontaneous hypothyroidism is usually slow, and many patients seek medical attention for variable and nonspecific symptoms. In contrast, patients who develop hypothyroidism rapidly (i.e., when replacement therapy is discontinued in patients with primary hypothyroidism or after the gland is surgically removed) have more symptoms. In such patients, manifestations of overt hypothyroidism are present by 6 weeks. In general, older patients tend to have fewer symptoms and signs of hypothyroidism than do young adults.

In adults, common features of hypothyroidism include easy fatigability, tiredness, coldness, mild weight gain, constipation, menstrual irregularities, and muscle cramps. Drowsiness and slowing of intellectual and motor activity are often reported. Sensitivity to cold is suggested by the use of more blankets on the bed. Women frequently complain of hair loss, brittle nails, and dry skin. Periorbital puffiness may be present. Stiffness and aching of muscles may be attributed to rheumatism. Constipation may occur. Numbness and tingling of the extremities are frequent. Physical findings include cool dry skin, a puffy face and hands, a hoarse husky voice, and slow reflexes.

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Clinical Features of Hypothyroidism in the Elderly

Hypothyroidism in the elderly is often atypical and elusive and often lacks the classic clinical features present in younger patients. This is due to a combination of several factors, including the insidious onset, the ambiguity of several signs and symptoms (e.g., fatigue, weakness, cold intolerance, dry skin, hair loss, constipation, poor appetite, depression and/or mental deterioration, hearing loss, cardiomegaly, congestive heart failure) that may be attributed to normal aging, and the frequent coexistence of several age-associated diseases.

The most relevant clinical findings that lead one to suspect hypothyroidism in the elderly are an unexplained increase in serum cholesterol, severe constipation, congestive heart failure (particularly when it presents as restrictive cardiomyopathy), and macrocytic anemia (as a consequence of folate deficiency or coexistent autoimmune gastritis and pernicious anemia).

Clinical Aspects of Hypothyroidism Due to Various Etiologies

Primary Hypothyroidism

Primary hypothyroidism in adults results mainly from autoimmune thyroiditis, is more common in women than in men, and occurs between 40 and 60 years of age. In these patients, clinical features of hypothyroidism may be accompanied by the typical goiter of Hashimoto's thyroiditis. When present, the goiter is usually firm in consistency, generally moderate in size, and often lobulated (although well-defined nodules are unusual).

Other organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus, Addison's disease, premature ovarian failure, celiac disease, hypoparathyroidism, and myasthenia gravis, may also coexist. Patients with primary hypothyroidism may also complain of vitiligo and alopecia. Primary autoimmune hypothyroidism may also occur as a component of either the type I or the type II polyglandular autoimmune syndrome. The specific association of primary hypothyroidism and primary adrenal cortical insufficiency is known as Schmidt syndrome. The rare type I syndrome consists of at least two of the triad of Addison's disease, hypoparathyroidism, and chronic mucocutaneous candidiadis; other autoimmune disorders, such as alopecia, chronic autoimmune thyroiditis, and malabsorption syndrome, may also be present. Autoimmune thyroid disease is reported in 10 to 12% of these patients. Type I polyglandular autoimmune syndrome presents more often during childhood. The type II syndrome is more common and usually presents during adulthood. Addison's disease, Hashimoto's thyroiditis, and type 1 diabetes are the most common endocrine deficiencies found in these patients, although gonadal failure, pernicious anemia, and vitiligo are observed in a significant percentage.

Central Hypothyroidism

The clinical picture of central hypothyroidism varies depending on the severity of thyroid failure, the extent of associated hormone deficiencies, the age of the patient, and the nature of the underlying lesion. Central hypothyroidism is due to TSH deficiency caused by either hypothalamic or pituitary disease. The differentiation of central hypothyroidism from primary hypothyroidism is important for the institution of the proper therapy. The clinical features of central hypothyroidism are similar to those of primary hypothyroidism, although the former are generally less pronounced. The skin is pale and cool, but it is not as coarse and dry as in primary hypothyroidism. Periorbital and peripheral edema are uncommon in patients with central hypothyroidism. Loss of axillary, pubic, and facial hair, as well as thinning of the lateral eyebrows, is more pronounced. The tongue is not enlarged, and hoarseness of the voice is not as prominent as it is in primary hypothyroidism. The heart tends to be small, and blood pressure is low. Atrophic breasts and amenorrhea are found in women. Body weight is more likely to be reduced than to be increased. Defects in growth hormone (GH) and gonadotropin secretion usually precede TSH insufficiency, and in most cases adrenocorticotropin hormone (ACTH) secretion is the last to be affected. Growth failure with delayed skeletal maturation results from GH deficiency in children. Hypoglycemia may occur. Gonadotropin insufficiency results in impotence, loss of libido, and diminished beard growth in men and results in amenhorrea, infertility, and atrophy of the breasts in women. ACTH deficiency leads to weakness, postural hypotension, and depigmentation of the areole and other normally pigmented areas of the skin. Symptoms and signs that arise directly from the hypothalamic or pituitary lesion may precede, accompany, or even obscure manifestations of pituitary failure. The manifestations of a sellar mass include headache and symptoms secondary to compression of adjacent structures with visual field disturbances and ophthalmoplegia.

Diagnostic Accuracy of the Clinical Features of Hypothyroidism

Several attempts have been made to develop a clinical score system that, based on the most frequent symptoms and signs of hypothyroidism, could accurately predict the diagnosis of thyroid failure in individual patients.

During the 1960s, Billewicz and colleagues described a diagnostic index that scored the presence or absence of various signs and symptoms of hypothyroidism. However, at that time, modern laboratory thyroid function tests were not available to validate the diagnostic accuracy of such a score. More recently, Zulewski and colleagues proposed a convenient clinical score that is both

Table 1 Sensitivity and specificity of the 14 symptoms and signs of hypothyroidism and analysis of their positive and negative predictive values (percentages)

Symptoms and signs	*Sensitivity*	*Specificity*	*Positive predictive value*	*Negative predictive value*
Ankle reflex	77	93	92	80
Dry skin	76	64	68	73
Cold intolerance	64	65	65	64
Coarse skin	60	81	76	67
Puffiness	60	96	94	71
Pulse rate	58	42	50	50
Sweating	54	86	79	65
Weight increase	54	77	70	63
Paresthesia	52	82	75	63
Cold skin	50	80	71	61
Constipation	48	85	76	62
Slow movements	36	99	96	61
Hoarseness	34	87	73	57
Hearing loss	22	97	90	53

Table 2 Scoring of symptoms and signs of hypothyroidism

	On the basis of	*Score*	
		Present	*Absent*
Symptoms			
Diminished sweating	Sweating in the warm room on a hot summer day	1	0
Hoarseness	Speaking voice, singing voice	1	0
Paresthesia	Subjective sensation	1	0
Dry skin	Dryness of skin, noticed spontaneously, requiring treatment	1	0
Constipation	Bowel habit, use of laxative	1	0
Impairment of hearing	Progressive impairment of hearing	1	0
Weight increase	Recorded weight increase, tightness of clothes	1	0
Physical signs			
Slow movements	Observe patient removing his/her clothes	1	0
Delayed ankle reflex	Observe the relaxation of the reflex	1	0
Coarse skin	Examine hands, forearms, and elbows for roughness and thickening of skin	1	0
Periorbital puffiness	This should obscure the curve of the malar bone	1	0
Cold skin	Compare temperature of hands with that of examiner	1	0

Note. For clinical judgment, add 1 point to the sum of symptoms and signs present in women under 55 years of age. Hypothyroid, more than 5 points; intermediate, 3 to 5 points; euthyroid, less than 3 points.

easy to determine and sensitive for individual assessment of the severity of thyroid failure. The frequencies of the 14 more common symptoms and signs of overt hypothyroidism are shown in **Table 1**. The most frequent features in hypothyroid patients were prolonged ankle reflex (77%) and complaints about dry skin (76%). A reduced pulse rate and cold intolerance were recorded with a high frequency in euthyroid controls and so were excluded from this score. The sensitivity and specificity of each symptom and sign of hypothyroidism, and the analysis of their positive and negative predictive values, are shown in **Table 1**. **Table 2** shows the scoring system of symptoms and signs of hypothyroidism. Because a correlation analysis revealed a significant correlation of these scores with age, a simple age-correcting factor was defined by adding 1 point to the sum of symptoms and signs in women under 55 years of age. According to this analysis, the following diagnostic ranges for the clinical judgment with the age-corrected score were defined: hypothyroid, more than 5 points; intermediate, 3 to 5 points; euthyroid, 0 to 2 points.

Organ System Manifestations of Hypothyroidism

Cutaneous Manifestations and Changes in the Connective Tissues

The cutaneous changes observed in hypothyroidism belong to the most classic and frequent findings of the disease (**Table 3**). In more than 80% of patients with primary hypothyroidism, the epidermis is dry, rough, cool, and covered with fine superficial scales. This is an expression of decreased cutaneous metabolism, reduced secretion of sweat and sebaceous glands, vasoconstriction, thinning of the epidermis, and hyperkeratosis of the stratum corneum. The face is puffy, pale, and expressionless at rest

Table 3 Cutaneous signs and symptoms of hypothyroidism (percentages)

Cutaneous manifestation	*Frequency*
Cold intolerance	50–95
Nail abnormality	90
Thickening and dryness of hair and skin	80–90
Edema of hands, face, and eyelids	70–85
Change in shape of face	70
Malar flush	50
Nonpitting edema	30
Alopecia	30–40
Pallor	25–60
Decreased sweat secretion	10–70

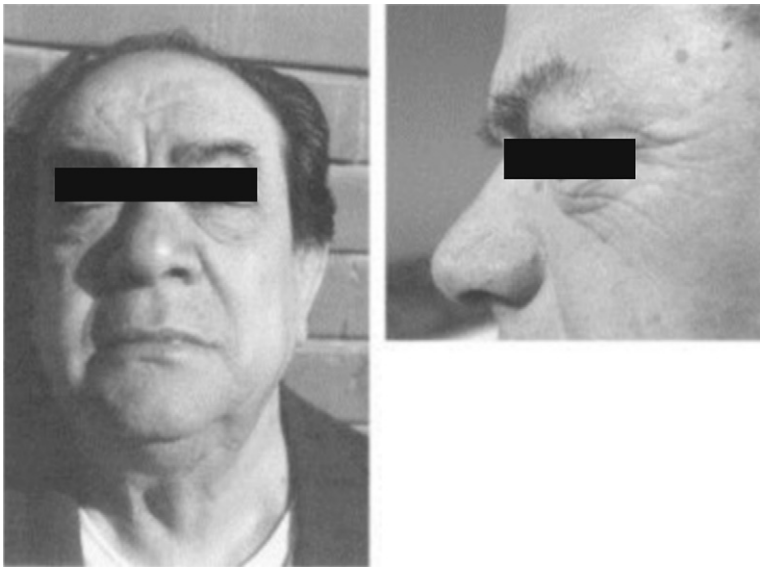

Fig. 1 Photographs of a hypothyroid patient.

(Fig. 1). The palpebral fissure may be narrowed because of blepharoptosis. The tongue is usually large, and some patients will complain of this problem. The tongue is smooth if pernicious anemia coexists. The voice is husky, low-pitched, and coarse due to the enlargement of the tongue and the thickening of the pharyngeal and laryngeal mucous membranes. The speech is deliberate and slow.

Hair Follicles and Nails

The hair is dry, dull, and coarse, and it grows slowly, becoming sparse and falling out readily. Loss of scalp, genital, and beard hair may also occur. Hair may be lost from the temporal aspects of the eyebrows (Queen Anne's sign). The nails, through retardation of growth, become thickened and brittle, striated in both transverse and longitudinal grooves, showing frequent deformities.

Cardiovascular Changes

Lack of thyroid hormones causes multiple alterations in the cardiovascular system (Table 4). Bradycardia, cardiomegaly, and low-voltage complexes on the electrocardiogram (ECG) are well-known features. The decrease in pulse rate approximately parallels the decrease in the body's metabolic rate. Myocardial contractility is reduced. The cardiac output at rest is decreased due to reduction in both stroke volume and heart rate, reflecting loss of the inotropic and chronotropic effects of thyroid hormones. Peripheral vascular resistance at rest is increased, and blood volume is reduced. These hemodynamic alterations cause narrowing of pulse pressure, prolongation of circulation time, and decreased blood flow to the tissues. Arterial blood pressure is often mildly increased. Hypertension is present in 10 to 20% of patients with hypothyroidism. Diastolic hypertension is usually restored to normal after treatment. Few symptoms associated with the cardiovascular system are referred from patients with hypothyroidism. Exertional dyspnea and exercise intolerance are probably due to skeletal muscle dysfunction.

Table 4 Cardiovascular signs and symptoms in hypothyroidism

Symptoms
Dyspnea
Decreased exercise tolerance
Angina
Signs
Low pulse rate
Increased systemic vascular resistance
Diastolic hypertension
Cardiomegaly
Pericardial effusion
Peripheral nonpitting edema
Low voltage ECG, nonspecific ST–T changes

On physical examination, certain findings can suggest hypothyroidism. The heart rate is lowered, the pulse pressure is narrowed, and the carotid upstroke and left ventricular apical impulse are diminished. The heart sounds are diminished in intensity. This finding is due largely to effusion into the pericardial sac of fluid rich in protein and glycosaminoglycans.

Electrocardiographic Changes

Electrocardiographic changes include sinus bradycardia, prolongation of the PR interval, low amplitude of the P wave and QRS complex, alterations of the ST segment, and flattened or inverted T waves. Although suggestive of myocardial ischemia, these waveform changes often disappear during T4 substitution therapy. Pericardial effusion is probably responsible for the low amplitude.

Respiratory Changes

Respiratory troubles are rarely a major complaint in hypothyroid patients. However, hypothyroidism may cause respiratory problems through (1) depression of the respiratory center in the brain, (2) disturbed neural conduction and/or neuromuscular transmission to the respiratory muscles (due to hypothyroid neuropathy), (3) diseased respiratory muscle function (due to hypothyroid myopathy), and/or (4) changes in the alveolar–capillary membranes and the surfactant lining the alveoli, leading to impaired gas exchange.

Fatigue and dyspnea on exertion are frequent symptoms. Dyspnea is a frequent complaint of myxedematous patients, but it is also a common symptom among well persons. Congestive heart failure of separate origin, pleural effusion, anemia, obesity, and/or pulmonary disease may be responsible for dyspnea.

Gastrointestinal Changes

The gastrointestinal manifestations of hypothyroidism are listed in **Table 5**. Poor appetite can be a leading symptom in hypothyroid patients. Anorexia can reasonably be interpreted as the reflection of a lowered food requirement. Although two-thirds of hypothyroid patients report weight gain, it is of modest degree and due largely to retention of fluid by the hydrophilic glycoprotein deposits in the tissues. True obesity is not a feature of hypothyroidism per se.

Constipation is frequently present and is the result of lowered food intake and decreased peristaltic activity. Atrophy of the gastric and intestinal mucosa and myxedematous infiltration of the bowel wall may be present at histological examination. Immune gastritis is often observed in hypothyroid patients with autoimmune thyroiditis. As many as 50% of patients with autoimmune hypothyroidism have achlorhydria, 25% have circulating antibodies directed against the gastric parietal cells or intrinsic factor, and 2 to 10% have pernicious anemia caused by impaired absorption of vitamin B12.

Symptoms or signs of disturbed liver or exocrine pancreatic function are usually not encountered, but biochemical tests may suggest disease. The association of liver disease and hypothyroidism is suggestive of a multisystem autoimmune disease affecting both the liver (e.g., chronic active hepatitis, primary biliary cirrhosis) and the thyroid. Structural liver damage is unusual in hypothyroidism per se. Serum glutamine–oxaloacetic transaminase (GOT), lactate dehydrogenase (LDH), and creatin–phosphokinase (CPK) levels are elevated in patients with hypothyroidism. These enzymes return to normal over 2 to 4 weeks during treatment. Urinary amylase levels may be increased.

Cerebral and Neurological Changes

Thyroid hormone is essential for the development of the central nervous system. Deficiency during fetal life or at birth causes hypoplasia of cortical neurons with poor development of cellular processes, retarded myelination, and reduced vascularity.

Table 5 Gastrointestinal manifestations of hypothyroidism

Symptoms
Anorexia
Gaseous distension
Constipation
Signs
Prolonged gastric emptying
Prolonged intestinal transit time
Slowed intestinal absorption
Ascites
Elevated liver enzymes
Gallbladder hypotonia

Table 6 Neurological and psychiatric manifestations in hypothyroidism

Neurological symptoms or signs
Somnolence, lethargy
Slow speech
Impaired cognitive functions
Headache
Paresthesias
Cerebellar ataxia
Deafness
Vertigo
Delayed relaxation of deep tendon reflexes
Psychiatric syndromes
Depression
Bipolar disorders
Affective psychosis

Deficiency of thyroid hormone beginning during adult life causes less severe manifestations that usually reverse after treatment with thyroid hormone.

Table 6 lists the numerous symptoms suggesting either neurological or psychiatric disorders in patients with moderate to severe hypothyroidism. In adult and elderly patients, mental changes may go unrecognized for a long time because of their slow development and because they may mimic cerebral atherosclerosis. However, an unusual complacency, fatigue, and pronounced somnolence or even lethargy, together with a prolonged reaction time, should suggest the possibility of hypothyroidism. All intellectual functions, including speech, are slowed. There is loss of initiative, and slow-wittedness and memory defects are common. Myxedematous dementia in the elderly patient may be mistaken for senile dementia. Memory is undoubtedly impaired, and attention and the desire to think are reduced. The emotional level seems definitely low, and irritability is decreased. Except during the terminal stage, reasoning power is preserved. Sensory phenomena are common. Numbness and tingling of the extremities are frequent. Mononeuropathies occur in hypothyroidism, as attested to by the high incidence of carpal tunnel syndrome (i.e., compression of the median nerve at the wrist). Nocturnal paresthesia and pain in the median nerve distribution in one hand or both hands is a common manifestation of this condition.

The tendon reflexes are slow, especially during the relaxation phase, producing the characteristic "hung-up reflexes." This phenomenon is due to a decrease in the rate of muscle contraction and relaxation rather than to a delay in nerve conduction.

Electroencephalographic changes include slow alpha wave activity and general loss of amplitude.

Muscle–Skeletal Changes

Muscles

In patients with hypothyroidism, disordered muscle function often is the predominating feature of the clinical syndrome. Generalized muscular hypertrophy, accompanied by easy fatigue and slowness of movements, occurs in some myxedematous children or adults.

Muscle symptoms such as myalgia, muscle weakness, stiffness, cramps, and easy fatigability are very prevalent in hypothyroid patients. The symptoms are aggravated by exposure to cold. They are also prominent during the rapid onset of hypothyroidism after surgery or 131-iodine therapy.

Skeletal system: calcium and phosphorus metabolism

In the adult skeleton, thyroid hormone deficiency decreases recruitment, maturation, and activity of bone cells, leading to decreased remodeling that is especially reflected in the impaired function of the osteoclasts. Despite this decrease in osteoclastic

Table 7 Changes in serum lipids in hypothyroidism

Total cholesterol	Increase
LDL cholesterol	Increase
HDL2 cholesterol	Modest increase
HDL3 cholesterol	No change
Triglycerides	No change or modest increase

activity, trabecular bone volume and bone mineral density appear to be comparable to those in age-matched normals, presumably because of the corresponding decrease in osteoblastic activity. The concentrations of calcium and phosphorus in serum are usually normal, but calcium may be slightly elevated. Serum alkaline phosphatase levels are often decreased, as are serum osteocalcine levels. Because the levels of parathyroid hormone are often slightly increased, some degree of resistance to its action may be present. Serum concentrations of 1,25-dihydroxycholecalciferol are also increased.

Hematological Changes

Anemia is present in as many as two-thirds of hypothyroid children and adolescents and in approximately one-third of adults with hypothyroidism. Anemia is usually mild. In two reports on a large series of patients with hypothyroidism from various causes, the incidence of anemia ranged from 32% to as high as 84%. Anemia in hypothyroidism may be a normochromic and normocytic anemia due to the diminished oxygen requirements and decreased production of erythropoietin, or it may result from a specific depression of the marrow that lacks thyroid hormone. The anemia may be macrocytic, sometimes from deficiency of vitamin B12. Folate deficiency from malabsorption or dietary inadequacy may also cause macrocytic anemia.

Granulocyte, lymphocyte, and platelet counts are usually normal in hypothyroidism. Hypothyroid patients may have bleeding symptoms such as easy bruising, menorrhagia, and prolonged bleeding after tooth extraction. The most frequent defects in hemostasis are prolonged bleeding time, decreased platelet adhesiveness, and low plasma concentrations of factor VIII and von Willebrand factor.

Changes in the Reproductive Tract

In both sexes, thyroid hormones influence sexual development and reproductive function. Infantile hypothyroidism leads to sexual immaturity, and juvenile hypothyroidism causes a delay in the onset of puberty followed by anovulatory cycles. Paradoxically, primary hypothyroidism may also cause precocious sexual development and galactorrhea.

In adult men, hypothyroidism may lead to impotence, lack of libido, and/or (rarely) testicular tubular involution. The testicles are histologically immature if hypothyroidism preceded puberty and show tubular involution if the onset of hypothyroidism was after puberty. In adult hypothyroid men, semen analysis is usually normal. In adult women, severe hypothyroidism may be associated with diminished libido and failure of ovulation. In general, hypothyroid women complain of menorrhagia as well as (occasionally) oligo and amenorrhea. Plasma gonadotropins are usually in the normal range in primary hypothyroidism, and the pulsatile gonadotropin release during the follicular phase is normal, but the ovulatory surge of luteinizing hormone (LH) might not happen. Secretion of progesterone is inadequate, and endometrial proliferation persists, resulting in excessive and irregular breakthrough menstrual bleeding. The anovulation is reflected in the frequent finding of a proliferative endometrium. These changes may be due to a deficient secretion of LH. Mild to moderate hyperprolactinemia is a frequent finding in hypothyroid women with or without galactorrhea. It is attributed to the stimulatory effect of increased thyrotropin-releasing hormone (TRH) on prolactin secretion. Fertility is reduced, and spontaneous abortion may result, although many pregnancies are successful.

The literature contains many reports of pregnancy in untreated hypothyroid women. Euthyroid neonates born to hypothyroid mothers during pregnancy have been reported to achieve lower IQs later in life. When treatment has been started during pregnancy, a normal child is usually produced, but abortion is frequent in myxedematous women. Pregnancy-induced hypertension is two to three times more common in hypothyroid women. Low birthweight may be secondary to premature delivery for gestational hypertension. The incidence of various congenital abnormalities may be increased, but recent studies do not report an increased risk of fetal death or congenital anomalies with proper treatment.

Other Endocrine Glands

Hypothyroidism decreases GH secretion, and hypothyroid children have a dramatic retardation of growth. Retarded growth caused by hypothyroidism appears to result from deficient secretion of GH as well as from impaired action of GH. Many hypothyroid children have subnormal serum GH response to insulin–induced hypoglycemia. GH secretion is decreased in hypothyroidism related to an increase in hypothalamic-somatostatinergic tone and results in low serum insulin-like growth factor-1 (IGF-1) concentrations. Serum IGF-2, IGF-binding protein-1 (IGFBP-1), and IGFBP-3 also fall, whereas IGFBP-2 rises. These changes are reversible with treatment.

Thyrotroph hyperplasia caused by primary hypothyroidism may result in sellar enlargement, particularly when the condition has remained untreated for a long period of time. Patients with severe hypothyroidism may have an increase in serum prolactin level that correlates with the level of serum TSH, and some patients develop galactorrhea.

Metabolic Changes

The decrease in energy metabolism and heat production is reflected in the low basal metabolic rate (BMR), decreased appetite, cold intolerance, and slightly lower basal body temperature. Measurement of the resting energy expenditure is rarely performed nowadays. In patients with complete athyreosis, it falls between 35 and 45% below normal. Both the synthesis and the degradation of proteins are decreased (the latter especially so), resulting in a nitrogene balance that is usually slightly positive.

In hypothyroidism, absorption of glucose from the gastrointestinal tract is slowed and peripheral glucose assimilation is retarded. At the same time, glycerol release from adipose tissue is slowed and the availability of amino acids and glycerol for gluconeogenesis is decreased. The oral glucose tolerance curve is characteristically flat, and the insulin response to glucose is delayed. Degradation of insulin is slow, so the sensitivity to exogenous insulin may be increased. Despite the easily demonstrable abnormalities in carbohydrate metabolism in hypothyroidism, clinical manifestations of these abnormalities are seldom conspicuous. A variety of abnormalities in plasma lipid concentrations occur in hypothyroidism (**Table 7**). Plasma free fatty acid concentrations are normal, whereas plasma concentrations of triglycerides, phospholipids, and low-density lipoprotein (LDL) cholesterol are well elevated. Biosynthesis of fatty acids and lipolysis is reduced. In general, the changes bear a reciprocal relationship to the level of thyroid activity.

The increased serum cholesterol may represent an alteration in the substrate steady-state level caused by a transient proportionally greater retardation in degradation than in synthesis. The increase of serum cholesterol is largely accounted for by an increase of LDL cholesterol. Interestingly, the LDL particles of hypothyroid patients are also susceptible to increased oxizability. The increase of high-density lipoprotein-2 (HDL2) cholesterol but not of HDL3 cholesterol is due to diminished activity of cholesteryl ester transfer protein and hepatic lipase (which is involved in the conversion of HDL2 to HDL3). The occasional modest increase of serum triglycerides has been related to decreased lipoprotein lipase activity in postheparin plasma.

See also: Hypothyroidism Subclinical. Myxedema Coma. TSH Function and Secretion. Treatment of Hypothyroidism

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Treatment of Hypothyroidism☆

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Definition of Hypothyroidism

Primary hypothyroidism is characterized by reduced secretion of thyroid hormones by the thyroid gland. It can be overt (increased serum TSH with low thyroid hormone) or subclinical (increased serum TSH with thyroid hormone at the lower limit of their respective reference range) (Biondi and Wartofsky, 2014). Secondary hypothyroidism is due to an insufficient stimulation of a normal thyroid gland due to a deficiency in TSH as a result of hypothalamic or pituitary disease and can be diagnosed when thyroid hormone levels are low and serum TSH is inappropriately low-normal or slightly elevated (Biondi and Wartofsky, 2014). $<1\%$ of cases of hypothyroidism are due to TSH deficiency.

Iodine deficiency is the most common cause of persistent hypothyroidism in the world. Hashimoto's thyroiditis, an autoimmune disease, represents the most common cause of acquired subclinical or overt hypothyroidism in adults in areas of iodine sufficiency. Treatment with radioiodine for hyperthyroidism may induce thyroid hormone deficiency in about 82% of patients with Graves' disease and about 60% of patients with toxic adenoma and toxic multinodular goiter (Biondi and Cooper, 2008). Moreover, acquired hypothyroidism can also develop after total and partial thyroidectomy and external radiotherapy of the head and neck.

Transient hypothyroidism is characterized by a period of reduced thyroid function with elevated TSH, which is followed by recovery to a euthyroid state. Transient causes of a TSH increase in adults are frequently related to viral, autoimmune, infiltrative or infectious disorders of the thyroid gland. Postpartum thyroiditis can be developed after delivery in women with individual or personal and/or family history of autoimmune thyroid disorders, autoimmune endocrine conditions or nonendocrine autoimmune disorders.

Some drugs can interfere with thyroid function, (iodine-containing compounds, lithium carbonate, cytokines, tyrosine kinase inhibitors (TKIs), amiodarone) (Biondi and Wartofsky, 2014; Biondi and Cooper, 2008). This drugs can induce transient hypothyroidism, although a persistent thyroid hormone deficiency can be developed specially in patients with preexisting thyroid autoimmunity.

Replacement Therapy With Levothyroxine

Replacement therapy with thyroid hormone is indicated as a lifelong treatment in patients with persistent hypothyroidism (Biondi and Wartofsky, 2014; Biondi and Cooper, 2008). Synthetic levothyroxine (L-T4) has largely replaced animal thyroid extract in the treatment of hypothyroidism since 1950. Today, L-T4 represents the first-line treatment in patients with hypothyroidism because of its long half-life (about 7 days) and safe biochemical profile after once daily administration. It is generally considered well tolerated, and associated with good patient compliance.

The aim of replacement therapy with L-T4 is to restore biochemical and clinical euthyroidism.

Treatment with L-T4 is able to prevent the progression of subclinical to overt hypothyroidism avoiding a severe hypothyroid state (myxedema). L-T4 improves the symptoms, quality of life, and high risk of cardiovascular complications associated with hypothyroidism.

Two important metaanalyses provided sufficient evidence to justify treatment of patients with subclinical hypothyroidism (SHypo) and a serum TSH level above 10 mIU/L, to reduce increased risk of coronary heart disease (CHD) and heart failure (HF) (Rodondi *et al.*, 2008; Gencer *et al.*, 2012).

On this basis, L-T4 monotherapy is recommended by international guidelines in patients with overt hypothyroidism and in presence of subclinical hypothyroidism when serum TSH is above 10 mIU/L (Cooper and Biondi, 2012; Garber *et al.*, 2012; Pearce *et al.*, 2013). Treatment of patients with mild SHypo (TSH levels 4.5–9.9 mU/L) remains controversial. Evidence suggests that treatment could be indicated in patients with positive antithyroid antibody tests and a progressively rising TSH levels, new onset of symptoms or depression, goiter, or cardiovascular risk factors (Cooper and Biondi, 2012; Garber *et al.*, 2012; Pearce *et al.*, 2013).

Starting Dose of L-T4

Usually body weight is considered a good indicator for calculating an appropriate starting dose of L-T4. Most patients are well treated with a narrow dose window that varies according to body weight from 1.6 to 1.8 μg/kg/day for replacement therapy in young and middle-aged patients (Biondi and Wartofsky, 2014).

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The etiology and severity of hypothyroidism, age, sex, body mass index, and any underlying physiological or pathological conditions (pregnancy and comorbidities) may affect the L-T4 dose. Some conditions may be associated with increasing (e.g., pregnancy, glomerular disease, malabsorption, drugs interfering with L-T4 absorption, increased metabolism or deiodinase activity) or decreasing the L-T4 dosage (weight loss, withdrawal of drugs interfering with L-T4, and atrial arrhythmias) (Biondi and Wartofsky, 2014). Elderly patients require lower doses of L-T4 than younger patients and serum TSH should be normalized to a higher target level (>6.0 mIU/L) (Biondi and Wartofsky, 2014; Biondi and Cooper, 2008).

An initial full replacement dose is safe and effective in reversing symptoms of hypothyroidism in young adults (Biondi and Wartofsky, 2014). On the contrary, replacement therapy should be started with low doses of L-T4 (25–50 μg/d) in patients older than 50–60 years without evidence of CHD (Biondi and Wartofsky, 2014). This dosage should be titrated gradually with a progressive increase every 2–3 weeks until euthyroidism is reached. In patients with severe ischemic heart disease, the starting dose should be even lower (12.5 μg/d) with a progressive increase of 12.5 μg/d every 4–6 weeks (Biondi and Wartofsky, 2014). Some patients may need coronary revascularization before achieving euthyroidism because the progressive L-T4 may trigger severe angina or arrhythmias.

Exogenous Subclinical Hyperthyroidism

The pituitary thyrotropes is very sensitive to small changes in serum thyroid hormone concentrations, therefore, an unintentional suppressed serum TSH concentration may be developed during L-T4 replacement therapy, leading to exogenous subclinical hyperthyroidism (Biondi and Cooper, 2008). This hypermetabolic state can impair the psychological, social, and physical quality of life and can be associated with the development of some important cardiovascular risk factors (increased heart rate and left ventricular mass and enhanced risk of atrial arrhythmias) (Biondi and Wartofsky, 2014; Cooper and Biondi, 2012). Elderly patients with exogenous subclinical hyperthyroidism may be asymptomatic and the onset of atrial fibrillation may be the first symptom of overtreatment with L-T4. Current evidence supports the association of suppressed serum TSH during L-T4 therapy with an increased risk of fractures in elderly, postmenopausal women, and people with risk factors for osteoporosis.

Treatment with L-T4 should be carefully monitored, especially in elderly patients, because over-treatment can increase mortality from cardiovascular disease (Biondi and Wartofsky, 2014; Biondi and Cooper, 2008; Cooper and Biondi, 2012).

Drugs Interfering With L-T4 Administration

It is good practice to annually assess serum TSH during L-T4 therapy to ensure compliance and determine whether a change in L-T4 dose is required.

Table 1 Drugs interfering with L-T4 administration and causes of L-T4 malabsorption

Amiodarone
Glucocorticoids
Blocking drugs
Sertraline
Phenytoin
Carbamazepine
Rifampicin
Aluminum hydroxide
Antacids
Cholestyramine
Sucralfate
Aluminum hydroxide
Ferrous sulfate
Calcium carbonate
Dietary fiber supplements

Table 2 Conditions inducing L-T4 malabsorption

Lactose intolerance
Atrophic gastritis
H pylori infection
Intestinal infections
Liver cirrhosis, obstructive liver disease
Pancreatic diseases
Pancreatic insufficiency
Previous gastrointestinal surgery

Some drugs may interfere with L-T4 requirement (**Table 1**). Malabsorption can be responsible of persistent hypothyroidism despite the administration of high doses of L-T4 (**Table 2**) (Biondi and Wartofsky, 2014).

Recently, liquid formulations of L-T4 and oral soft gelatin capsules (containing L-T4 dissolved in inactive ingredients, gelatin, and glycerin) have become available in Europe and the United States (Biondi and Wartofsky, 2014). This new formulations, which are therapeutically equivalent to the same preparation in tables by pharmacokinetic analysis, have a different dissolution and absorption profile. They allow a 100% absorption from the gastrointestinal (GI) tract and can be useful in patients with malabsorption (Biondi and Wartofsky, 2014).

Combined Treatment With L-Thyroxine and L-Triiodothyronine

Levothyroxine is a pro-hormone which is periferically converted to T3, the active hormone, by type 2 deiodinase. This conversion is usually efficient in the majority of thyroidectomized patients to ensure tissue euthyroidism. Polymorphisms in the D2 gene have been detected in about 20% of the population and could explain why some hypothyroid patients continue to experience symptoms consistent with thyroid hormone deficiency despite restoration of biochemical euthyroidism (Panicker *et al.*, 2009; Biondi and Wartofsky, 2012). This minority of thyroidectomized patients continues to complain of mood changes, cognitive dysfunction, lethargy, fatigue and weight gain despite adequate L-T4 replacement doses. They have poor quality of life during monotherapy with L-T4.

The increase in L-T4 dose is usually ineffective to improve symptoms suggesting that the restoration of normal thyroid function during L-T4 does not accurately reflect euthyroidism in all tissues in some patients. Metaanalyses of intervention trials have reported no benefit of combination treatment of L-T4 and L-T3 in symptomatic patients with biochemical euthyroidism. Further prospective randomized controlled studies are required to clarify this issue. In the meantime, available guidelines suggest to prescribe combination therapy with LT4 and L-T3 in hypothyroid patients with persistent symptoms during L-T4 (Wiersinga *et al.*, 2012).

This treatment should be managed only by skilled specialists and requires a careful follow-up to detect and prevent possible adverse effects. Serum concentrations of T3, T4, and TSH should remain within their respective reference ranges during this combination regime, in particular avoiding overtreatment with suppressed TSH concentrations. T3 should ideally be given in a slow-release form but this drug is not available today.

See also: Central Hypothyroidism. Diagnosis of Hypothyroidism. Hypothyroidism Subclinical. Systematic Manifestations of Hypothyroidism. Thyroid Function and Depression

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Myxedema Coma☆

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Glossary

Bradycardia Slow heart rate, generally below 60 bpm.
CNS Central nervous system, that is, brain and spinal cord.
Hypercalcemia Higher than normal blood calcium concentration.
Hypercarbia Higher than normal blood carbon dioxide content.
Hyperkalemia Higher than normal blood potassium concentration.
Hypoglycemia Low blood glucose level.
Hyponatremia Low blood sodium concentration.
Hypothermia Lower than normal body temperature.
Hypoxemia Lower than normal blood oxygen content.
Monodeiodination Refers to peripheral metabolism of thyroxine (T4) with removal of one iodine molecule to produce triiodothyronine (T3).
Myxedema Hypothyroidism, generally of a severe form.
Tachycardia Rapid heart rate, generally greater than 100 bpm.

Introduction

Myxedema coma is a state of severe life-threatening hypothyroidism that is classically associated with decreased mentation. It is a clinical diagnosis, and no one specific laboratory value can establish the diagnosis. The typical myxedema coma case presents as an elderly woman with all the features of long-standing hypothyroidism but with stupor or coma and hypothermia. This severe form of hypothyroidism is most commonly seen during the winter months, which might point to external temperature as a significant environmental factor influencing onset. Several other factors, such as hypoglycemia, hyponatremia, hypoxemia, and hypercapnia (**Table 1**), have been associated with myxedema coma. All are thought to contribute to the presentation of the disease, but in many cases it remains unclear whether these factors may reflect cause or effect of the myxedema coma. Most cases of myxedema coma are due to primary hypothyroidism, with less than 10% being related to central hypothyroidism.

Clinical Features

Usually present are all of the classic features of hypothyroidism such as dry, coarse, and scaly skin; sparse or coarse hair; nonpitting edema of the periorbital regions, hands, and feet; macroglossia; hoarseness; and delayed deep tendon reflexes (Popoveniuc *et al.*, 2014). Decreased respiratory drive can cause respiratory depression; together with decreased ventilatory response to hypercapnia, CO_2 narcosis settles in and worsens the central nervous system (CNS) depression (Wilson and Bedell, 1960; Zwillich *et al.*, 1975). Hypoventilation is also exacerbated by depressed function of the respiratory muscles. Rarely, pleural effusions or ascites can diminish respiratory function by reducing lung volumes as well as macroglossia by obstructing the upper airway.

Multiple other organ systems are typically involved in patients with myxedema coma. Hyponatremia and decreased glomerular filtration rate are seen frequently. The two most likely causes are a loss of the kidney's ability to excrete a water load because of decreased delivery of water to the distal nephron and an increased production of antidiuretic hormone. Urinary sodium is usually normal.

Decreased intestinal motility or even paralytic ileus and megacolon can be seen, with patients presenting with abdominal pain, constipation, and nausea. Cardiac enlargement, bradycardia, and decreased cardiac contractility are common features of cardiovascular involvement in myxedema coma, but congestive heart failure is rare. Nonspecific electrocardiogram changes are seen frequently. When cardiovascular collapse occurs, vasopressors and thyroid hormone replacement are required for improvement.

Routinely, these patients develop CNS depression with lethargy and then stupor or even coma. Approximately 25% of patients with myxedema coma have generalized seizures, and administration of any CNS depressants can exacerbate this.

Patients suffering from myxedema coma are susceptible to infection. What makes this aspect of their presentation more elusive is that some of classic symptoms and signs of myxedema coma, such as hypothermia (which can be seen in up to 75% of these

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Table 1 Precipitating and exacerbating factors for myxedema coma

Hypothermia
Infections
Cerebrovascular accidents
Drugs
Anesthetics
Tranquilizers, barbiturates, sedatives, narcotics
Amiodarone, beta blockers, lithium
Discontinuation of thyroxine therapy
Burns
Trauma
Gastrointestinal bleeding
Metabolic disturbances exacerbating myxedema coma
Hypoglycemia
Hyponatremia
Hypoxemia
Hypercapnia
Acidosis
Hypercalcemia

patients) and bradycardia, may mask the classic signs of infection (e.g., fever, tachycardia). Pulmonary infections may exacerbate any respiratory dysfunction.

Laboratory Diagnosis

As in uncomplicated hypothyroidism, a significantly elevated thyroid-stimulating hormone (TSH) with very low or undetectable thyroid hormone levels is seen. The degree of TSH elevation can vary a lot between patients; a TSH level seen in a patient with mild signs and symptoms of hypothyroidism could be comparable to that seen in another patient with myxedema coma. Similarly, it is not unheard of for patients to be functional with undetectable levels of thyroid hormone (at least for some period of time).

An exception to the rule of an elevated TSH level may be the patient with central hypothyroidism due to pituitary disease in whom both low thyroid hormone and low TSH levels would be seen. However, even in this case it is possible for the TSH to be in the normal range or even somewhat elevated, possibly reflecting bioinactive TSH.

Treatment

Given the high mortality associated with myxedema coma, treatment must be instituted as soon as diagnosis is strongly suspected. Thyroid hormone therapy alone might not be enough in the presence of the multiple system organ dysfunction associated with myxedema coma. Ventilatory support is often required and helps to prevent respiratory failure, a common cause of death in these cases. An intensive care unit is always the best place in which to care for these patients. Mechanical ventilation and empirical antibiotic therapy are often indicated together maintenance of oxygenation while monitoring blood gases. It is important to realize that prolonged ventilatory support may be required despite adequate therapy of the hypothyroidism (Wilson and Bedell, 1960; Zwillich *et al.*, 1975).

Correction of hyponatremia is of equal importance. This can easily contribute to the altered mental status of these patients, especially if the serum sodium concentration is less than 120 mEq/L. Intravenous saline and dextrose are used to correct any volume depletion and to provide minimal nutritional support. If serum sodium concentration is less than 120 mEq/L, use of small amounts of hypertonic saline may be indicated; however, this requires very close monitoring of sodium concentration changes to avoid central pontine myelinolysis (Skowsky and Kikuchi, 1978). The intravenous fluids may be warmed to help correct hypothermia. Thyroid hormone will ultimately restore body temperature, and external heat with warming blankets must be used with great caution because they may act to cause vasodilation and provide too precipitous a fall in peripheral vascular resistance. Rather than external warming, it would be more prudent to use ordinary blankets or increase the ambient room temperature.

If there is any suspicion of adrenal insufficiency, stress dose corticosteroids should be given after a baseline cortisol level is drawn. The presence of signs such as hypoglycemia, hyponatremia, hyperkalemia, and hypotension is highly suggestive. This can be of extreme importance because correction of the hypothyroidism without correction of adrenal insufficiency can precipitate an adrenal crisis. Rarely, these patients require vasopressor support for hemodynamic stability.

The most important part of therapy is the replacement of the thyroid hormone deficiency. Which regimen of thyroid hormone administration to use remains controversial. Administration of levothyroxine (T4) by itself may result in insufficient levels of

triiodothyronine (T3) because of inadequate monodeiodination of T4 to T3 in sick patients. On the other hand, T4 therapy provides a steady smooth rise of the hormone level and is less likely to be associated with any adverse effects. A commonly used dosing regimen involves administration of a high dose (300–600 μg) intravenously the first day to replete the body's stores and then about 50–100 μg daily intravenously or orally after that (Holvey *et al.*, 1964).

Administration of T3 has the advantage that its onset of action is much faster than that of T4, an advantage that can be very important for patients' survival. The drawback is that there may be potential for an increased incidence of complications, especially in those patients who have primary cardiac diseases or who are at risk for arrhythmias or ischemia. Intravenous preparations of T3 are available, and common doses for myxedema coma are 10–20 μg intravenously every 4 h for the first day and then 10 μg every 6 h for 1–2 days. Oral administration is usually possible after that (Pereira *et al.*, 1982).

Another approach is to administer both T4 and T3 initially. T4 is given at a dose of 4 μg/kg lean body weight (approximately 200–300 μg) followed by 100 μg 24 h later and then 50 μg daily either intravenously or orally as tolerated. The initial T3 dose is 10 μg intravenously every 8–12 h until the patient is able to tolerate oral intake. The response to therapy can be quite variable, and close monitoring of all such patients is extremely important.

See also: Systematic Manifestations of Hypothyroidism. Thyroid Hormone Action. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Treatment of Hypothyroidism

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Hypothyroidism, Subclinical☆

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Glossary

Hypothyroidism Absolute or relative deficiency of thyroid hormones at the peripheral target tissues.

Peripheral target tissues of thyroid hormone action The effects of thyroid hormones are ubiquitous and not restricted to one target cell. Peripheral target tissues of thyroid hormone action include the brain, liver, muscle, hearth and others. Consecutively, in addition to measuring thyroid hormone and thyrotropin levels, clinical and metabolic effects of hypothyroidism are to be evaluated at the peripheral target tissues. Assessments of these tissue effects include standardized clinical scores measurement of lipid profile, or assessment of cardiac and skeletal muscle function.

Thyrotropin Thyroid-stimulating hormone (TSH) is a glycoprotein hormone released by the anterior pituitary gland that stimulates the thyroid gland to release thyroxine and triiodothyronine. The release of thyrotropin is triggered by the action of thyrotropin-releasing hormone (TRH) (or factor; TRF), a substance found in the hypothalamus of the brain. Thyroxine (T4) and its active metabolite triiodothyronine (T3) inhibit the further release of thyrotropin by a negative feedback mechanism.

Definition

Subclinical hypothyroidism or mild thyroid failure is defined by an elevated serum TSH level accompanied by normal concentrations of circulating thyroid hormones (T4 and T3), in the presence or absence of clinical signs and symptoms. Recent research has identified significant changes of thyroid hormone action at the peripheral target tissues, which are of clinical relevance and need treatment in selected patients.

Introduction and Epidemiology

An increase in the secretion of thyroid-stimulating hormone (TSH) is the earliest biochemical sign of impending thyroid failure. In this early form of hypothyroidism free thyroxine (fT4) levels are still within the laboratory reference range. However, they are below a critical individual set point, which leads to stimulation of pituitary TSH by negative feedback mechanism.

Due to the use of TSH measurement for early detection of thyroid dysfunction, subclinical hypothyroidism has been detected with increasing frequency in recent years. It occurs in 3%–16% of the general population with a particularly high prevalence observed in areas of iodine sufficiency and in women over 60 years. Recently it has been suggested that the upper limit of normal for TSH shifts to higher levels with age (Surks and Hollowell, 2007) thus the prevalence in elderly people may not be as high as previously thought and it is important to consider the age-specific reference range for TSH when establishing the diagnosis.

Etiology

The causes of subclinical hypothyroidism are the same as for those of overt hypothyroidism. Most patients have chronic autoimmune (Hashimoto's) thyroiditis with elevated serum levels of antithyroid peroxidase antibodies. According to epidemiological studies autoimmune thyroiditis is found in 40%–67% of patients with subclinical hypothyroidism. The other major causes are prior treatment for hyperthyroidism (e.g., radioiodine or surgical treatment), thyroid surgery for epidemic goiter, and drug induced subclinical hypothyroidism (e.g., lithium, amiodarone). Other causes of elevated TSH levels and normal peripheral thyroid hormone concentrations that must be included in the differential diagnosis include noncompliance with thyroxine replacement therapy in overt hypothyroid patients (up to 40% of patients), recovery from severe nonthyroidal illness, and mutations causing inactivation of the thyrotropin receptor.

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Natural Course and Prognostic Factors

Hypothyroidism is a graded phenomenon and therefore some patients with subclinical hypothyroidism will progress to overt disease over time. Based on epidemiological studies progression to overt thyroid failure has been reported to occur in 3%–18% of affected patients per year. In an epidemiological study, the "Whickham survey," nearly 1900 survivors of a normal population evaluated in 1972 were re-examined 20 years later, in 1992. In this survey 97 females with subclinical or overt hypothyroidism have been detected with a prevalence of 9.3% and a calculated incidence of 0.41% per year. The risk factors for the development of hypothyroidism were a raised TSH level and/or positive antithyroid antibodies. For females with a raised serum TSH a mean annual risk for developing hypothyroidism of 26% over 10 years could be calculated.

In a prospective study of 82 female patients with known subclinical hypothyroidism we investigated the natural course of this syndrome with regular evaluations at yearly intervals. Over a mean period of 9.2 years 28% of the patients progressed to overt hypothyroidism, 68% remained in the subclinical state, and 4% reverted to a normal TSH. The incidence of overt hypothyroidism was correlated with the initial serum TSH concentrations. The calculated 10 years rate of overt hypothyroidism was 0% for TSH levels of 4–6 mU L^{-1}, but 43% for values of 6–12 mU L^{-1} and 77% for TSH levels above 12 mU L^{-1} (**Fig. 1**).

Clinical Manifestations and Benefits of Treatment

Symptoms

One of the most controversial aspects concerning subclinical hypothyroidism is whether affected patients are symptomatic and, therefore, may benefit from thyroid hormone replacement. Based on case–control studies, nearly 30% of patients with subclinical hypothyroidism have symptoms that are suggestive of thyroid hormone deficiency. Symptoms and signs of hypothyroidism may be very vague and non-specific, and hence are easily overlooked in an individual patient. This limitation can be overcome by a systematic assessment of clinical signs and symptoms of hypothyroidism with an easy scoring system (Zulewski *et al.*, 1997). Some but not all studies reported significant improvement in symptoms of hypothyroidism after thyroxine replacement assessed by different clinical scores. A meta-analysis of 12 clinical trials and a subsequent systematic review found no benefit of thyroxine therapy compared to placebo, especially if the TSH level was below 10 mIU L^{-1} (Villar *et al.*, 2007; Rugge *et al.*, 2015). This might particularly be true for the older population: a recent randomized controlled trial evaluating whether thyroxine treatment provides clinical benefits in 737 patients of at least 65 years of age and persisting TSH levels between 4.6 and 19.99 mIU L^{-1} showed negative results (Stott *et al.*, 2017).

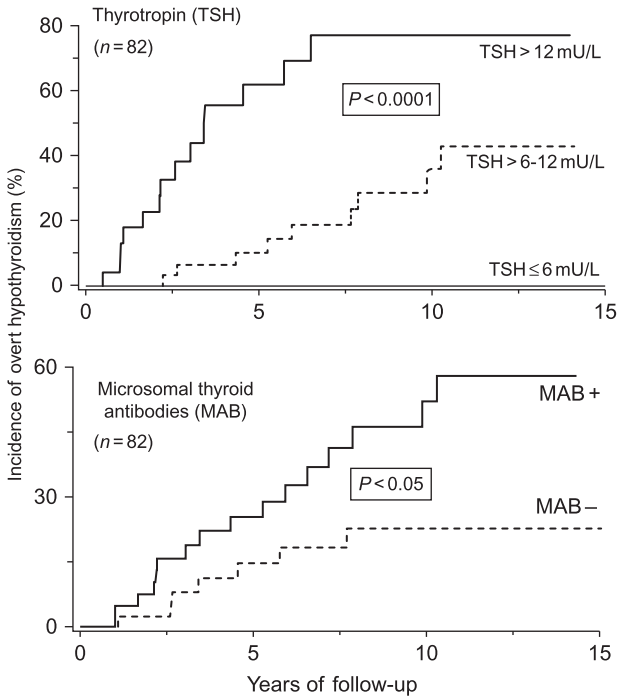

Fig. 1 Kaplan–Meier estimates of incidence of overt hypothyroidism according to TSH and microsomal thyroid antibody levels. Natural course and spontaneous evolution without treatment. From Huber, G., Staub, J. J., Meier, C., Mitrache, C., Guglielmetti, M., Huber, P., Braverman, L. E. (2002). Prospective study of the spontaneous course of subclinical hypothyroidism: prognostic value of thyrotropin, thyroid reserve, and thyroid antibodies. *Journal of Clinical Endocrinology and Metabolism* 87(7), 3221–3226, with permission.

Table 1 Indications to screen for hypothyroidism

Signs and symptoms
Symptoms suggestive of hypothyroidism
Goiter
Endocrine ophthalmopathy
Dyslipidemia
Infertility
Risk factors for hypothyroidism
Personal history of autoimmune diseases
Thyroidectomy
Neck surgery
Radioactive iodine therapy
External radiotherapy of the neck
Family history of autoimmune thyroid disease
Comorbidities
Pituitary or hypothalamic lesions
Infiltrative disorders (e.g., amyloidosis, sarcoidosis, hemochromatosis, AIDS)
Down syndrome
Turner syndrome
Drugs
Iodine-containing drugs (e.g., amiodarone, radiographic contrast agents)
Lithium carbonate
Cytokine (e.g., interferone a)
Tyrosine-kinase inhibitor

Cognitive Function and Neuropsychiatric Dysfunction

Several reports suggest that subclinical hypothyroidism is associated with neuropsychiatric disease with higher scores on scales of depression and anxiety in affected patients. Neuropsychiatric parameters (i.e., reaction time and figure identification) as well as memory scores were significantly improved in some intervention trials assessing the effect of thyroxine treatment in patients with subclinical hypothyroidism (mean TSH levels at baseline: 7–12 mU L^{-1}). However other studies including a recent meta-analysis do not support the association of subclinical hypothyroidism and neuropsychiatric diseases (Pasqualetti *et al.*, 2015).

Atherosclerosis and Cardiovascular Risk Factors

Overt hypothyroidism is associated with increased cardiovascular morbidity. The relationship between subclinical hypothyroidism and atherosclerotic risk factors has been widely investigated, with major interest on serum lipid abnormalities. Several cross-sectional studies found serum lipid concentrations, mainly total cholesterol levels, within the normal range, whereas others have detected significant elevations of total cholesterol and/or LDL-cholesterol concentrations, especially in smokers (Muller *et al.*, 1995). In addition, reduced HDL-cholesterol levels were reported in some studies. Data are conflicting whether thyroxine replacement shows a beneficial effect on serum cholesterol levels. Our "Basel Thyroid Study" was the first randomized trial demonstrating significant effect of thyroxine on total cholesterol and atherogenic LDL-cholesterol levels (Meier *et al.*, 2001). These results were confirmed by other randomized trials evaluating patients mainly with serum TSH concentrations >10 mIU L^{-1}. However a meta-analysis including seven trials analyzing lipid levels after T4 replacement revealed no lowering effect of treatment on total cholesterol, HDL, LDL, triglycerides, apolipoprotein A and B and lipoprotein (a). Besides lipid abnormalities several mechanisms are discussed to be involved in the possible association between subclinical hypothyroidism and atherosclerosis. These include elevated markers of inflammation, vascular reactivity, endothelial effects and hypercoagulable state.

Several observational and prospective cohort studies observed an increased risk for coronary heart disease in patients with subclinical hypothyroidism. A meta-analysis considering data from a total of 55,287 participants (3450 with subclinical hypothyroidism) showed that the risk for coronary events and mortality—but not for total mortality—increases with higher concentrations of TSH, especially if >10 mIU L^{-1} (Rodondi *et al.*, 2010). The risk persisted after adjustment for age, sex or preexisting cardiovascular disease. Conversely, minimal TSH elevations up to 6.9 mIU L^{-1} were not associated with an increased cardiovascular risk. In another study subclinical hypothyroidism had no influence on mortality in elderly individuals (median age 83 years) over a follow-up period of 5 years (Waring *et al.*, 2012).

Subclinical hypothyroidism may also have an impact on heart failure. Myocardial function has been shown to be slightly impaired in patients with subclinical hypothyroidism. The identified functional abnormalities include impaired myocardial contractility and diastolic function, at rest or with exercise. A pooled analysis of data from several prospective cohort studies showed a significant increase of heart failure events in patients with TSH >10 mIU L^{-1} compared to euthyroid controls

Table 2 Causes of transient TSH elevation and of altered thyroid function tests

Transient TSH elevation
Subacute, silent or postpartum thyroiditis
Recovery from nonthyroidal illness
Altered thyroid function tests without thyroidal illness
Assay variability
Autoantibodies to TSH
Heterophilic antibodies or rheumatoid factors interfering with immunometric assays
Drugs (e.g., amiodarone)
Others
Morbid obesity
Untreated adrenal insufficiency
TSH producing pituitary adenomas
Resistance to thyroid hormone
Mutation of the TSH receptor

(Gencer *et al.*, 2012). Minimal elevations were not associated with an increased risk of heart failure (levels <6.9 mIU L^{-1}) and in patients with moderate augmented TSH levels (7.0–9.9 mIU L^{-1}) the increased risk was not statistically significant.

In summary, data of cardiovascular mortality and all-cause mortality are conflicting. The former seems to be significantly elevated in patients with TSH levels above 10 mIU L^{-1}, but especially in elderly individuals and in those with lower TSH elevations the association is not clear.

So far it remains unclear, whether cardiac risk may be influenced by thyroxine replacement therapy. Although several cardiovascular risk factors and surrogate cardiovascular endpoints (e.g., dyslipidemia, vascular smooth muscle proliferation, ventricular function) may improve under treatment, current data cannot clearly conclude whether cardiovascular outcome can be optimized. In an analysis of the United Kingdom General Practitioner Research Database thyroxine replacement was associated with reduced ischemic heart events in patients aged 40–70 years, but was not beneficial in older individuals (Razvi *et al.*, 2012). In a recent retrospective register-based cohort study analyzing 1192 patients with established heart disease and subclinical hypothyroidism (mean age 74 years) thyroxine treatment was not associated with a significant benefit nor risk of all-cause mortality, major adverse cardial events or hospital admission (Andersen *et al.*, 2016). Thus, appropriately powered randomized clinical trials are needed to answer this fundamental question. Similarly, the risk of cardiovascular effects of a potential overtreatment with thyroxine has yet to be established.

Others

In patients with subclinical hypothyroidism, abnormalities in peripheral-nerve function and neuromuscular activity, skeletal muscle abnormalities, intraocular pressure and ovulatory dysfunction have been described in subclinical hypothyroidism. However, the role of subclinical hypothyroidism in these circumstances, especially in reproductive abnormalities, is not well defined. The prevalence of subclinical hypothyroidism seems to be elevated in women with infertility compared to women with confirmed fertility (Abalovich *et al.*, 2007); moreover, thyroid autoimmunity was linked to an increased risk of unexplained subfertility according to a meta-analysis of four studies (van den Boogaard *et al.*, 2011). Conversely, a recent prospective cohort study found no association between mild preconception subclinical hypothyroidism or the presence of antithyroid antibodies and fecundity (Plowden *et al.*, 2016). During pregnancy, subclinical hypothyroidism is associated with complications such as placental abruption, pregnancy loss and neonatal death, hence replacement therapy is mandatory (Maraka *et al.*, 2016).

Diagnostic and Therapeutic Recommendations

Screening and Diagnosis

Screening for thyroid disease is still a controversial issue (Surks *et al.*, 2004; Gharib *et al.*, 2005). TSH screening in women older than 35 years of age has been shown to be cost-effective. Some authors favor TSH screening for thyroid dysfunction in asymptomatic adults, others deny this policy because the effects of subsequent thyroxine therapy are not beneficial in all patients and put them at risk for potential overtreatment with adverse cardiovascular or skeletal effects. Subclinical hypothyroidism is a frequent finding in women older than 40 years (affecting about 10% of the female population of this age) and its clinical presentation may be subtle. Therefore, TSH screening should be advocated if risk factors are present (**Table 1**) and by a case-finding approach, focusing on patients visiting their physicians for an unrelated reason. Since smoking impairs both thyroid hormone secretion and thyroid hormone action, smoking status should be considered in the evaluation of patients in whom hypothyroidism is suspected.

If the initial screening test ist positive with an elevated TSH level, a second measurement (TSH and fT4) after 1–3 months ist needed to confirm the diagnosis and exclude a transient elevation. **Table 2** summarizes causes of transient TSH elevations and altered thyroid hormone tests, which have to be considered in the differential diagnosis. Further evaluation of patients includes a

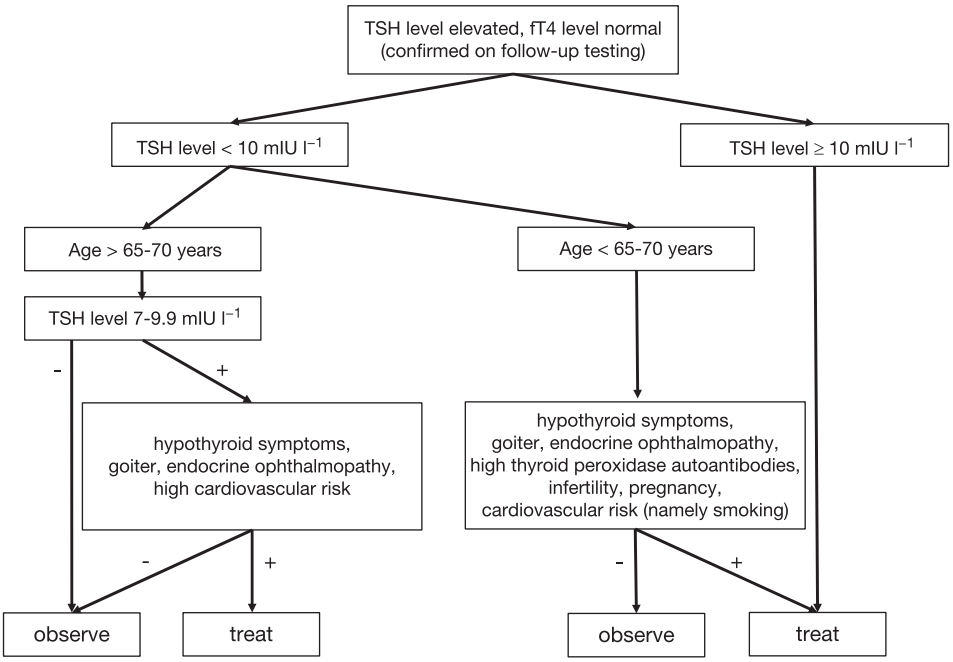

Fig. 2 Algorithm for the management of patients with subclinical hypothyroidism.

thorough history and a examination for a thyroid gland enlargement and a endocrine ophthalmopathy. Although most patients will have chronic autoimmune thyroiditis, we do not routinely measure antithyroid peroxidase antibodies. Similarly, an ultrasound of the thyroid is not required in the management of subclinical hypothyroidism unless there are additional clinical indications (Pearce *et al.*, 2013).

Replacement Therapy With Thyroxine

The goal of treating patients with mild thyroid failure is to reverse clinical and metabolic alterations by hormone supplementation and, in addition, to prevent progression of the subclinical form to the overt stage of hypothyroidism with its considerable morbidity and possible mortality. Thyroxine treatment is indicated in virtually all younger patients who have elevated TSH levels above 10 mU L^{-1}. Routine replacement of asymptomatic patients with TSH levels between 4.5 and 10 mU L^{-1} remains controversial (Cooper and Biondi, 2012). Patients at risk with special conditions like goiter, thyroidectomy, depression, infertility, pregnancy, high thyroid peroxidase autoantibodies, endocrine ophthalmopathy and hypercholesterolemia, in particular in the presence of other cardiovascular risk factors, such as smoking and hypertension may benefit from early treatment. In patients with minimal or moderate TSH elevations (<10 mU L^{-1}) with no clinical or metabolic changes treatment can be withhold, but annual follow-up is required (**Fig. 2**).

Overtreatment can produce overt or subclinical hyperthyroidism with its consequences on morbidity (e.g., atrial fibrillation and osteopenia) and mortality. Therefore, fine-tuning of thyroxine replacement therapy with the goal to restore serum TSH to physiological, euthyroid levels is mandatory.

See also: Causes of Hypothyroidism. Central Hypothyroidism. Diagnosis of Hypothyroidism. Epidemiology of Thyroid Disease. Hashimoto's Thyroiditis. Postpartum Thyroid Dysfunction. Systematic Manifestations of Hypothyroidism. Thyroid and Infertility. Thyroid Autoimmunity. Thyroid Disorders in the Elderly. Thyroid Function and Depression. Thyroiditis, Infectious and Subacute. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Thyroid Autoimmunity☆

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Nomenclature

AIRE gene The autoimmune regulator gene
CD Cluster of differentiation
CTLA-4 Cytotoxic T-lymphocyte–associated antigen 4
FasL Fas ligand
FOXP3 Forkhead box protein 3
GO Graves orbitopathy
HLA Human leukocyte antigen
IL Interleukin
LATS Long-acting thyroid stimulator
MHC Major histocompatibility complex
TBII TSH receptor binding inhibiting antibodies
Tg antibodies Thyroglobulin antibodies
TGF-β Tumor growth factor
Th cells T-helper cells
TPO Thyroid peroxidase
Tregs Regulatory T cells
TSH Thyroid stimulating hormone

Glossary

Autoimmune thyroiditis A predominantly lymphocytic inflammatory response in the thyroid gland caused by autoreactivity to thyroid autoantigens, especially thyroglobulin, thyroid peroxidase, or the TSH receptor.
Cytokines Soluble factors released by leukocytes and, in some cases, other cell types that have immunomodulatory properties; T cells can be categorized by their profile of cytokine expression.
Epitope The part of an antigen recognized during an immune response. T-cell epitopes are short linear peptide sequences of the antigen, whereas B-cell epitopes are not limited to amino acid sequences and may be composed of discontinuous sequences that constitute the three-dimensional part of the antigen to which an antibody binds.
HLA The human leukocyte antigen, a gene complex encoding the major histocompatibility complex (MHC) proteins in humans, which encodes specialized cell surface molecules able to present antigenic peptides to T-cell receptors and is thus an integral part of the adaptive immune system. Some HLA alleles have been associated with an increased risk of specific autoimmune diseases (i.e., HLA-DR3 and Graves' disease or Hashimoto's thyroiditis).
Thyroid autoantibodies Antibodies, usually of the IgG class, directed against thyroid autoantigens, such as thyroglobulin, thyroid peroxidase, or the TSH receptor.
Tolerance A state of nonresponsiveness to an antigen recognized by the immune system that depends on how the antigen is presented to the T cell or B cell; usually categorized as central (thymic) or peripheral, depending on the site at which tolerance is induced.

Introduction

Autoimmune disease affects the thyroid gland more often than any other organ (McLeod and Cooper, 2012). Autoimmune thyroid diseases comprise a spectrum of disorders, representing two clusters of pathogenic mechanisms. Hashimoto's thyroiditis (a term used synonymously with chronic autoimmune thyroiditis and autoimmune hypothyroidism) and postpartum thyroiditis/painless thyroiditis share a predominately T cell-mediated autoimmunity, while Graves' disease is characterized by a primarily humoral response and the presence of antithyroid stimulating hormone (TSH) receptor antibodies (McLachlan and Rapoport, 2013; Rapoport *et al.*, 1998).

The most common type of thyroid autoimmunity is focal thyroiditis, which is found in 40% of all Caucasian women and 20% of Caucasian men (**Table 1**) (Vanderpump, 2011). The condition is half as common in Japanese and black Americans, and in all races it increases in incidence with age. Focal thyroiditis is closely associated with the presence of antibodies against thyroid peroxidase (TPO) and, often, thyroglobulin, but thyroid function is either normal (also designated asymptomatic autoimmune thyroiditis or only mildly affected (subclinical hypothyroidism), as indicated by an elevated thyroid-stimulating hormone (TSH) concentration and normal free thyroid hormone levels. The incidence of focal thyroiditis is increased in thyroid cancer, and its presence is associated with a slightly better prognosis.

Postpartum thyroiditis is defined as the appearance of transient thyroid dysfunction during the year after delivery, occurring particularly in women who have preexisting subclinical thyroid autoimmunity (Pearce, 2015). Silent or painless thyroiditis is a

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Table 1 Types of thyroid autoimmunity

Type	*Thyroid antibodies*	*Thyroid function*	*Other features*
Focal thyroiditis	TgAbs or TPOAbs, in most cases at low titer	Normal or subclinical hypothyroidism	May progress to overt hypothyroidism
Silent thyroiditis	TPOAbs, in most cases, TgAbs variable	Transient thyrotoxicosis and/or hypothyroidism	Most commonly occurs one year after delivery as postpartum thyroiditis; may progress to overt hypothyroidism
Hashimoto's (goitrous) thyroiditis	TgAbs and/or TPOAbs, usually high titer; occasionally TBAbs	Euthyroid or hypothyroidism	Associated rarely with thyroid lymphoma
Atrophic thyroiditis (primary myxedema)	TgAbs and/or TPOAbs at variable titer; TBAbs in up to 20% of cases	Hypothyroidism	May evolve from Hashimoto's thyroiditis
Graves' disease	TSAbs in all cases; TPOAbs in 80% and lower frequency of TgAbs	Hyperthyroidism and rarely hypothyroidism	Strongly associated with GO

Abbreviations: *GO*, Graves' orbitopathy; *TBAb*, TSH receptor-blocking antibodies; *TgAbs*, thyroglobulin antibodies; *TPOAbs*, thyroid peroxidase antibodies; *TSAb*, TSH receptor-stimulating antibodies.

similar transient disturbance in thyroid function in nonpregnant patients. In both types of thyroiditis, there is classically a phase of thyrotoxicosis caused by release of stored thyroid hormone and then a phase of hypothyroidism as destruction continues, before resolution. However, in many individuals only one phase may be observed. In approximately half of cases, either type may progress over 5–10 years to overt hypothyroidism.

Autoimmune hypothyroidism is caused by destruction of the thyroid follicles leading to low circulating thyroid hormone concentrations and an elevated TSH. Two main types are identified histologically, marked by the presence or absence of a goiter, but some patients progress from goitrous to atrophic thyroiditis and no clear autoimmune features distinguish one type from the other (Caturegli *et al.*, 2014).

Graves' disease is characterized by the presence of antibodies that stimulate the TSH receptor, resulting in elevated circulating thyroid hormone levels and suppressed TSH levels (Smith and Hegedus, 2016). In some patients, there is progression to autoimmune hypothyroidism, and in others there is fluctuation between hyperthyroid and hypothyroid phases over weeks or months. The reason for this might sometimes be TSH receptor antibodies penduling between stimulating and blocking varieties (McLachlan and Rapoport, 2013), and it could indicate the close relationship between the various types of thyroid autoimmunity. Graves' orbitopathy is an orbital disorder in which there is swelling of the extraocular muscles due to glycosaminoglycan accumulation and water trapping; orbital fat content may also increase (Bahn, 2010). These events cause a variety of soft tissue changes, including periorbital edema, proptosis, diplopia, and, in severe cases, optic nerve compression. Approximately 90% of patients with Graves' orbitopathy have Graves' disease, 5% have autoimmune hypothyroidism, and almost all of the remaining have subtle evidence of thyroid autoimmunity.

Epidemiology

With the reservation that the prevalence and incidence of autoimmune thyroid diseases depend highly on the iodine intake in a given population, approximately 10%–16% of women of fertile age have TPO antibodies and/or thyroglobulin antibodies (Bliddal *et al.*, 2015; Pedersen *et al.*, 2011), and approximately 30%–50% of these develop postpartum thyroiditis following pregnancy (Feldt-Rasmussen *et al.*, 1990). Thus, approximately 5% of pregnancies are followed by postpartum thyroiditis, although clinically significant features occur in only a small percentage of these cases. However, a substantial number of these women has persistent hypothyroidism at 1 year postpartum (Stagnaro-Green *et al.*, 2011), with a high risk for permanent hypothyroidism in the longer term (Sarvghadi *et al.*, 2005). The general prevalence of autoimmune hypothyroidism in women is 1% or 2%, and in men it is 5–10 times less frequent (McLeod and Cooper, 2012). Approximately 2% of individuals with thyroid antibodies and presumed focal thyroiditis progress to overt autoimmune hypothyroidism annually; the rate is more than double if there are thyroid antibodies and subclinical hypothyroidism (Vanderpump *et al.*, 1995). The peak incidence of autoimmune hypothyroidism occurs in individuals between 50 and 60 years of age. It is important to be aware of the burden of autoimmune thyroid disease, its geographic differences, and trends over time (McLeod and Cooper, 2012). For example, an increased incidence of overt hypothyroidism as well as thyroid autoimmunity was seen after iodine fortification of salt in Denmark during prospective monitoring of the effect of iodine fortification on thyroid function and autoimmunity (Pedersen *et al.*, 2007; Pedersen *et al.*, 2011). During the same period there was a doubling of prescriptions for thyroid replacement therapy (Cerqueira *et al.*, 2011).

Graves' disease accounts for 60%–80% of all cases of thyrotoxicosis in iodine-replete areas, while it is lower in mildly iodine deficient populations; for example, 38% in a Danish population study (Carle *et al.*, 2006; Laurberg *et al.*, 1991). It has a prevalence of approximately 1% in women, peaking in those aged 30–60 years. In men, the frequency is 5- to 10-fold lower than in women. Approximately 50% of patients with Graves' disease have clinically obvious thyroid-associated orbitopathy (or Graves' orbitopathy), but subclinical evidence of this complication can be found in more than 90% of cases by using imaging techniques (Bahn, 2010).

Pathological Features

In Hashimoto's thyroiditis, the thyroid is enlarged, usually symmetrically, due to a diffuse infiltration by lymphocytes, plasma cells, and macrophages, together with the formation of lymphoid germinal centers. Thyroid cells undergo hyperplasia and oxyphil metaplasia, with the latter leading to the formation of Hürthle or Askanazy cells. Progressively, however, thyroid cell destruction occurs, together with variable amounts of fibrosis. In primary myxedema (atrophic thyroiditis), there is more extensive fibrosis and follicular destruction and minimal or moderate lymphoid infiltration (Caturegli *et al.*, 2014).

In Graves' disease, there is hypertrophy and hyperplasia of the thyroid follicles, which have columnar, folded epithelium and less colloid than usual. There is a variable degree of lymphoid infiltration, sometimes with germinal center formation. All these changes are largely reversed by antithyroid drug treatment (Smith and Hegedus, 2016). The changes seen in Graves' orbitopathy consist of variable lymphocytic infiltration of the extraocular muscles and orbital connective tissue, edema, and, in the later stages, fibrosis and possibly muscle atrophy. Fat content may increase, especially late in the disease process, and mast cells may be prominent (Shan and Douglas, 2014). The levator palpebrae muscle fibers are enlarged. Lymphocytic infiltration of the eyelids is uncommon, but can often be seen in the lacrimal glands. This might be due to a concomitant occurrence of Sjögren syndrome (Bliddal *et al.*, 2017) and not Graves' orbitopathy per se.

Determinants of Thyroid Autoimmunity

Like almost all autoimmune disorders, those associated with thyroid autoimmunity are the result of a complex interaction between a large number of genetic, environmental, and endogenous factors, with the same disease resulting from different combinations of factors in different patients. The contribution of genetic factors to disease susceptibility is unknown, but concordance rates in monozygotic twins are approximately 20%–30%, considerably higher than in dizygotic twins (Brix and Hegedus, 2012). Different autoimmune thyroid diseases may occur in the relatives of a proband in a single family, and there is a well-known set of associations with other autoimmune diseases (**Table 2**), suggesting that these share common susceptibility factors, most likely genetic (Bliddal *et al.*, 2017).

Genetic Factors

In common with the majority of autoimmune diseases, the highly polymorphic alleles of the major histocompatibility complex (MHC) molecules, called human leukocyte antigen (HLA) in man, have been the most intensively studied and are

Table 2 Diseases associated with thyroid autoimmunity

Endocrine disorders
Type 1 diabetes mellitus
Addison's disease
Premature ovarian failure
Lymphocytic hypophysitis
Autoimmune polyglandular syndrome types 1 and 2
Organ-specific autoimmunity
Vitiligo
Alopecia areata
Pernicious anemia
Celiac disease and dermatitis herpetiformis
Myasthenia gravis
Autoimmune serositis
Chronic active hepatitis
Primary biliary disorders
Crohn's disease
Ulcerative colitis
Autoimmune thrombocytopenia
Autoimmune hemolytic anemia
Nonorgan-specific autoimmunity
Rheumatoid arthritis
Systemic lupus erythematosus
Sjögren syndrome
Sarcoidosis
Relapsing polychondritis
Systemic sclerosis
Other conditions
Breast cancer
Depression or bipolar affective disorder
Recurrent miscarriage

the most frequently associated genetic factors in thyroid autoimmunity (**Table 3**). There are several reasons why HLA alleles might confer susceptibility to autoimmune thyroiditis (Weetman, 2003). The MHC class II genes are crucial for antigen presentation (**Fig. 1**), and it is possible that only certain class II molecules can bind particular autoantigenic epitopes and thus initiate an autoimmune response (determinant selection). Particular class II alleles may also be critical for the thymic selection of T cells, either permitting the evolution of autoreactive T cells (positive selection) or preventing their development (negative selection); in the latter case, the allele would have a protective effect. Alternatively, some MHC class II alleles may determine selection of an increasingly better defined subpopulation of regulatory T cells (Tregs), previously called suppressor cells (McLachlan and Rapoport, 2014). Finally, associations between MHC class II alleles and autoimmune disease may also be related to the effects of polymorphisms in other genes in linkage disequilibrium with MHC class II molecules, such as complement components or the cytokine tumor necrosis factor.

Table 3 Major HLA associations in thyroid autoimmunity

Condition	*Ethnic group*	*Association*
Hashimoto's thyroiditis/autoimmune hypothyroidism	Caucasian	HLA-DR3, DR4
	Chinese	HLA-B46, DR9
	Japanese	HLA-DR53, DR9
Postpartum thyroiditis	Caucasian	HLA-DR5, possibly DR3
Graves' disease	Caucasian	HLA-DR3 (DR17 and DR18 subtypes)
	Japanese, Korean	HLA-DR5, DR8, DPB1*0501 (DQB1*0501 protective)
	United States Black	None
	South African Black	HLA-DR1, DR3
Graves' orbitopathy	Caucasian and Japanese	As for Graves' disease; HLA-DPB1*0201 may be protective

Associations between specific major HLA alleles and thyroid autoimmune disease entities according to ethnic groups.
Abbreviations: *HLA*, human leukocyte antigen.

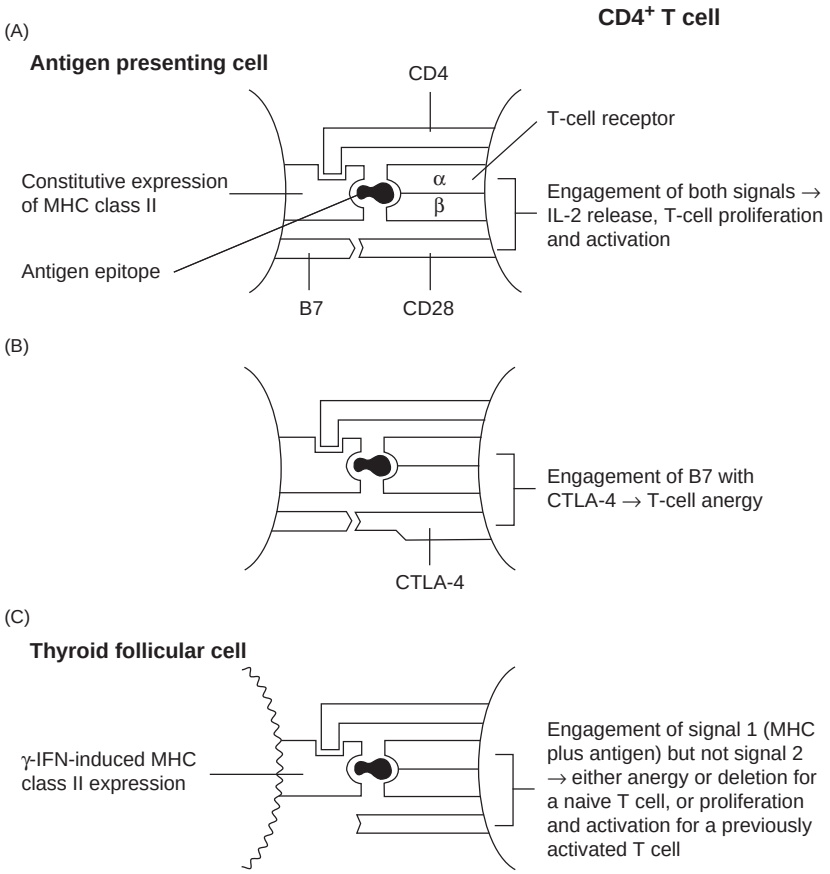

Fig. 1 Pathways of antigen presentation leading to $CD4^+$ T-cell stimulation. (A) Acute antigen presentation or anergy; (B) Chronic antigen presentation; (C) Antigen presentation by endocrine cell. Reproduced from first edition with kind permission from Weetman, A. P. (2003). Autoimmune thyroid disease: Propagation and progression. *European Journal of Endocrinology* ***148***, 1–9.

However, it is clear that many individuals have HLA-DR alleles that confer susceptibility but never develop autoimmune thyroid disease. Conversely, approximately half of individuals with autoimmune thyroid disease do not have any known HLA allele associated with susceptibility. The inevitable conclusion is that other genes must be involved, and these may act independently or interact with MHC-encoded susceptibility, making the determination of their effect more difficult. This genetic diversity is also demonstrated by the three to four times higher concordance rate for Graves' disease in monozygotic twins compared to dizygotic twins (Brix and Hegedus, 2012).

According to current evidence (Lee *et al.*, 2015; McLachlan and Rapoport, 2014), susceptibility genes to thyroid autoimmune disease can be categorized as either thyroid specific (thyroglobulin, TSH receptor) or immune-modulating (*FOXP3*, *CD25*, *CD40*, *CTLA-4*, *HLA*), with HLA-DR3 carrying the highest risk. Of these susceptibility genes, *FOXP3* and *CD25* play critical roles in the establishment of peripheral tolerance while the HLA genes are pivotal for antigen presentation and CD40 and CTLA-4 determine T-cell activation. Polymorphisms in these immune-modulating genes, in particular, significantly contribute to the predisposition for Graves' disease and Hashimoto's thyroiditis and, not surprisingly, many other autoimmune diseases. Emerging evidence suggests that single nucleotide polymorphisms in the immunoregulatory genes may induce functional hindrance to proper development of central and peripheral tolerance and alter the interaction of T cells with antigen presenting cells. *CTLA-4* is an attractive candidate autoimmunity gene because the CTLA-4 molecule is critical to the termination of immune responses (**Fig. 1**) and CTLA-4 knockout mice develop a lymphoproliferative syndrome associated with widespread lymphocytic infiltration of tissues. The susceptibility conferred by CTLA-4 polymorphisms is modest and similar in both Graves' disease and autoimmune hypothyroidism; despite initial observations, these polymorphisms do not confer any heightened risk of complications such as Graves' orbitopathy. Moreover, the same polymorphisms are shared with other disorders, such as type 1 diabetes mellitus, vitiligo, and Addison's disease, and therefore seem to confer a general propensity to develop organ-specific autoimmunity, which in turn may partially explain the frequent co-occurrence of these disorders.

Mutations in the autoimmune regulator (*AIRE*) gene give rise to more or less severe phenotypic forms of polyautoimmunity (Bliddal *et al.*, 2017; McLachlan and Rapoport, 2014; Weetman and DeGroot, 2016), while other genes may be involved in the thymic T-cell selection process such as those coding for apoptosis for example, Fas (CD95). As indicated above, polyautoimmunity is most often due to increased genetic susceptibility to autoimmune diseases in general and thyroid autoimmunity in particular, as recently reviewed in Bliddal *et al.* (2017) and further substantiated in **Table 4**.

Environmental Factors

The importance of environmental factors in causing autoimmune thyroiditis is illustrated by the incomplete concordance in monozygotic twins (Brix and Hegedus, 2012) and the lack of any family history of autoimmunity in many patients. Genetic factors are more likely to exert their effects in early onset cases, in cases with more than one autoimmune disease in an individual (Ottesen *et al.*, 1995), or in case of polyautoimmunity (Bliddal *et al.*, 2017; Kahaly and Frommer, 2018), while environmental factors, which require exposure of the individual, likely operate at later stages in life, but matters are even more complex because there are certainly interactions between genetic and environmental factors. Our inadequate knowledge stems largely from the difficulty of analyzing such heterogeneous interactions.

Infections are frequently suggested to play a role in producing autoimmunity by causing release of autoantigens from damaged tissue, by molecular mimicry in which epitope sequences of the organisms provoke an immune response against matching host protein sequences, or by altering target cell surface molecules that render the cell susceptible to autoimmune attack. However, there is no good evidence that thyroid autoimmunity is precipitated by infection and, indeed, subacute thyroiditis caused by viral infection of the thyroid is characterized by recovery of normal thyroid function. *Yersinia* infection has been examined as a potential trigger because there is evidence that the organism contains TSH-receptor-like sequences, but any role is likely to be small, at best, because few patients with Graves' disease have evidence of infection. There is even less reliable support for the involvement of retroviruses, but the subject of infectious relationship with thyroid autoimmunity is still being explored (Kohling *et al.*, 2017). Thus, the emerging research field of gut microbiota is also of interest in autoimmune thyroid disease research. Studies on this topic are, however, still sparse (Kohling *et al.*, 2017).

Iodide intake may determine the frequency of thyroid autoimmunity, because there is epidemiological evidence that increases in dietary iodide are associated with an increase in the incidence of thyroid antibodies, autoimmune hypothyroidism, and Graves' disease. Animal models of autoimmune thyroiditis and epidemiological studies (see earlier) have provided support for such an enhancing role; conversely, iodide depletion appears to be protective. Excess iodide may be toxic to thyroid cells or may have immunological effects, including enhanced immunogenicity of thyroid autoantigens (Zimmermann and Boelaert, 2015).

Major stress may precipitate Graves' disease, at least based on evidence from retrospective surveys, and such an effect could be produced by the neuroendocrine consequences of stress, especially on glucocorticoids (Morshed *et al.*, 2012; Mizokami *et al.*, 2004). Smoking is weakly associated with Graves' disease and is the strongest known determinant of Graves' orbitopathy. The reasons for these associations are not known, but it is possible that smoking may either modulate the immune response in some way (e.g., by affecting cytokine production) or enhance orbital fibroblast responses secondary to partial hypoxia.

At low doses, radiation may initiate thyroid autoimmunity because both Graves' disease and autoimmune hypothyroidism have been reported after mantle irradiation for lymphoma, and there is an increase in thyroid antibodies in the survivors of irradiation from nuclear weapon or reactor fallout (Agate *et al.*, 2008), though only transitory. Some drugs may also precipitate thyroid autoimmunity,

Table 4 Polyautoimmune endocrine syndromes including thyroid disease

Clusters	*Proposed pathogenic mechanism*
Autoimmune polyendocrine syndromes	
Type I (APECED or Whitaker syndrome): Mucosal and cutaneous candida infections, Addison's disease, hyposplenism, hypoparathyroidism, and multiple autoimmune presentations that is, hypothyroidism, hypogonadism, vitiligo, alopecia, pernicious anemia, chronic autoimmune hepatitis	*Mutation of autoimmune regulator (AIRE)* gene involved in central tolerance development. Phenotype possibly affected by HLA subtypes
Type II (Schmidt's syndrome): Addison's disease and hypothyroidism or type 1A diabetes as well as pernicious anemia, primary hypogonadism, vitiligo, coeliac disease, myasthenia gravis (by some further classified in types III and IV according to specific entities of the above)	Polygenetic with increased risk of disease linked to specific HLA-DR and HLA-DQ genotypes
Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome	
Immune-dysfunction, enteropathy, dermatitis, autoimmune endocrinopathies (often type 1 diabetes, autoimmune thyroid disease), autoimmune skin diseases (i.e., bullous pemphigoid), multiple organ involvement	Mutation of *FOXP3* gene on the X-chromosome
Multiple autoimmune syndromes (MAS)	
Type I: Myasthenia gravis, thymoma, polymyositis, and giant cell myocarditis	
Type II: Sjögren syndrome, rheumatoid arthritis, primary biliary cirrhosis, scleroderma, and autoimmune thyroid disorders	
Type III: Autoimmune thyroid disease, myasthenia and/or thymoma, Sjögren syndrome, pernicious anemia, idiopathic thrombocytopenic purpura, Addison disease, insulin-dependent diabetes, vitiligo, autoimmune hemolytic anemia, systemic lupus erythematosus	Genetic predisposition with phenotype HLA B8 and/or DR3 or DR5 seems to be an important factor
Thyrogastric cluster	
Autoimmune thyroiditis, chronic gastritis/pernicious anemia, autoimmune adrenalitis (Addison's)	Polygenetic
Lupus-associated cluster	
Autoimmune hemolytic anemia, immune thrombocytopenia, systemic lupus erythematosus, rheumatoid arthritis, autoimmune hepatitis, Sjögren syndrome	Polygenetic
Trisomy 21 and Turner syndrome	
Chronic thyroiditis, type 1A diabetes and others	Chromosomal abnormalities
Kearns–Sayre syndrome	
External ophthalmoplegia, retinal degeneration, diabetes, thyroiditis, and hypoparathyroidism	Mitochondrial myopathy

Abbreviations: *APECED*, Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; *FOXP3*, forkhead box P3; *HLA*, human leucocyte antigen.
Reproduced with kind permission from Bliddal, S., Nielsen, C. H. and Feldt-Rasmussen, U. (2017). Recent advances in understanding autoimmune thyroid disease: The tallest tree in the forest of polyautoimmunity. *F1000Research 6*, 1776.

particularly in those who are genetically predisposed; examples include lithium, α-interferon, and other cytokines. For example, Graves' disease occurs in one-third of multiple sclerosis patients treated with monoclonal antibodies to deplete T cells (Rotondi *et al.*, 2017).

Endogenous Factors

Pregnancy is associated with amelioration of autoimmune thyroiditis, and patients with Graves' disease can usually stop drug treatment during the last trimester. However, a rebound in the autoimmune process occurs during the year after delivery. This is when postpartum thyroiditis emerges and Graves' disease appears with an increased incidence. These changes are largely unexplained but presumably relate in part to massive fluctuations in various hormones during and after pregnancy, and may constitute a similarity to reconstitution of immunity after the general immunosuppression during pregnancy (Singh and Perfect, 2007). Sex hormones are likely the major determinants of the increased risk of thyroid autoimmunity in women, but thyroid epigenetics in the form of X chromosome inactivation seems a player in patients with autoimmune thyroid disease (Yin *et al.*, 2007). However, the causes of the increased risk in Turner and Down syndromes are unknown. Weetman (2013) has envisaged the multifactorial causes of the typical autoimmune thyroiditis as conforming to the Swiss-cheese model of catastrophe in which multiple small genetic (and nongenetic) events, none in themselves sufficient to precipitate autoimmune destruction, line up like the holes in slices of such cheese to allow the untoward event to occur (**Fig. 2**).

Thyroid Autoantigens

Understanding any autoimmune disease is contingent on a thorough knowledge of the nature of the autoantigens involved and their B- and T-cell epitopes. In addition to aiding in the determination of the etiology and pathogenesis of an autoimmune disease,

autoantigen identification is central to assays for antibodies that may be markers or predictors of disease or for the delineation of particular subsets of patients, and it may lead to novel treatments, such as that based on modified T-cell peptide epitopes. Indeed, our poor understanding of the pathogenesis of Graves' orbitopathy is due directly to an inadequate understanding of the autoantigens involved. A summary of the main thyroid autoantigens is shown in **Table 5**.

Thyroglobulin

This was the first tissue autoantigen to be identified, based on the pioneering work by Witebsky *et al.* (1955), who immunized rabbits with thyroid extract in adjuvant, eliciting Tg antibodies and thyroiditis, and work by Roitt *et al.* (1956), who first showed the presence of Tg antibodies in patients with Hashimoto's thyroiditis. Thyroglobulin is the thyroid hormone storage system; tyrosine molecules at hormonogenic sites are iodized to form T3 and T4. The protein is secreted into the follicular lumen, in which it is endocytosed and hydrolyzed to release T3 and T4. Iodination of the molecule is important for its immunogenicity in animal models of autoimmune thyroiditis: there is no clear evidence that this is important in humans.

Two major epitopes and one minor conformational epitope exist on each thyroglobulin subunit for human autoantibodies, but the B-cell immune response becomes even more diverse with chronicity and increasing antibody titer, and some autoantibodies in Hashimoto's thyroiditis bind to linear determinants of thyroglobulin. The majority of Tg antibodies belong to the IgG-1 and IgG-4 subclasses (McLachlan *et al.*, 1987), but do not fix complement because the spacing of the autoantigenic epitopes prevents cross-linking of C1q subunits (Forouhi *et al.*, 1987; McLachlan and Rapoport, 2014; Weetman and DeGroot, 2016). In

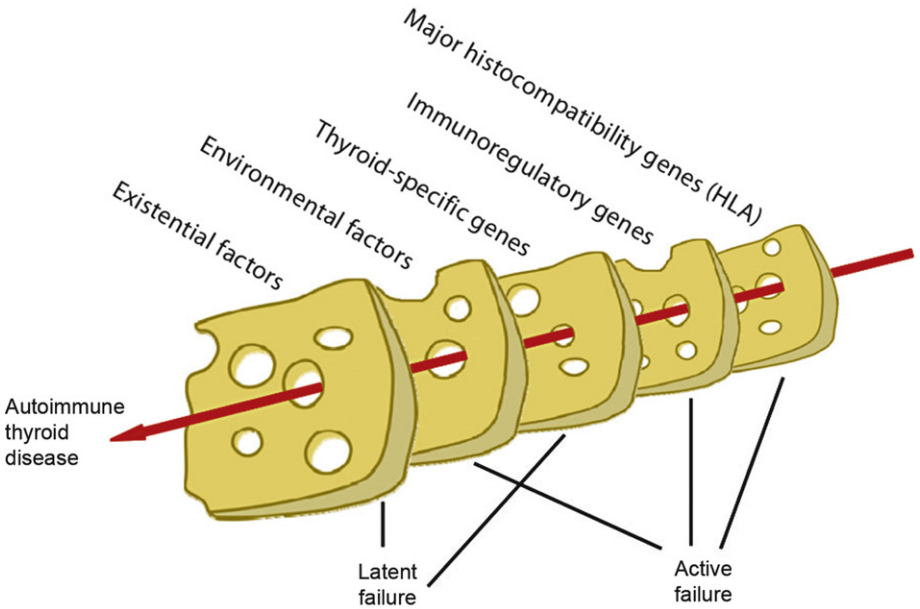

Fig. 2 Swiss cheese model for the causation of autoimmune thyroid disease. A Swiss cheese model for the causation of autoimmune thyroid disease, showing the effect of cumulative weaknesses lining up to allow a catastrophic event to occur, like the holes in slices of cheese. Each of the broad slices represented is composed of many individual components, which also have to line up. The Swiss cheese model for human accident causation incorporates active failures (e.g., pilot error) and latent failures (which lie dormant until the accident for example, aircraft maintenance deficiencies). Some factors contributing to the initiation of Hashimoto thyroiditis may be regarded as latent (e.g., aging, growing up in a hygienic environment) and others as active (e.g., possession of an HLA allele which permits presentation of a thyroid autoantigen). Reproduced with kind permission from Weetman, A. P. (2013). The immunopathogenesis of chronic autoimmune thyroiditis one century after hashimoto. *European Thyroid Journal* ***1***, 243–250.

Table 5 Key properties of thyroid autoantigens

	Chromosomal location	*Size (kDa)*	*Protein type*	*Functions*	*Homology*
Thyroglobulin	8	660	Iodinated glycoprotein	T3 and T4 prohormone and storage	Acetylcholinesterase
Thyroid peroxidase	2	105–110	Hemoprotein enzyme	Iodination and coupling of tyrosine	Myeloperoxidase
TSH receptor	14	85	G protein-coupled receptor	Thyroid stimulation by TSH	Related receptors (e.g., luteinizing hormone)
Na^+/I^- symporter	19	65–77	Transmembrane transporter	Iodide uptake	Na^+/glucose transporter

the past, Tg antibodies were detected by immunofluorescence or hemagglutination assays, but these have generally been superseded by enzyme-linked immunosorbent assays and radioimmunoassays, which express levels in units related to a standard antibody preparation (Verburg *et al.*, 2013). The most sensitive assays indicate that low levels of Tg antibodies can be detected in up to 20% of the healthy female population.

Thyroid Peroxidase

There are two forms of thyroid peroxidase, TPO-1 and -2, but only the first has enzymatic activity and is expressed on the cell surface, where it catalyzes the iodination and coupling of tyrosine molecules to form T3 and T4. Originally, antibodies against TPO were called microsomal antibodies because they bound to an antigen in this subcellular fraction, although they were also recognized from immunofluorescence to bind to the apical microvillar surface of the thyroid cells. It was subsequently shown that binding of these complement-fixing microsomal antibodies could be inhibited by TPO-specific monoclonal antibodies, and studies of recombinant TPO confirmed that essentially all the binding to thyroid microsomes is directed against TPO.

Further work localized two linear B-cell epitopes, C-2 (amino acids 590–622), and C-21 (amino acids 710–722), but these are only recognized by antibodies arising late in the disease process. In the early phase of any autoimmune response involving TPO, antibodies are directed against conformational epitopes in two overlapping domains, A and B, and relative antibody binding to each of these domains is fairly constant over time. This narrow response seems to be related to the marked restriction of the immunoglobulin variable (V) region gene usage in TPO antibody formation, with each domain being recognized by antibodies encoded by different V gene sequences, indicating a genetic component to the control of antibody production. In contrast, T-cell epitopes are heterogeneous, with several different epitopes typically recognized by T cells from patients and only partially shared between patients. Such diversity is typical of a chronic immune response in which only one dominant T-cell epitope may initiate the process, but this is followed by "spreading" of the response to involve other epitopes over time, including so-called cryptic epitopes that only become exposed during the phase of tissue destruction.

TPO antibodies are measured using the same techniques as those used to measure Tg antibodies and are also found in healthy subjects in the absence of overt thyroid disease (Mariotti *et al.*, 1987). They are predominantly of the IgG-1 and IgG-4 subclasses (McLachlan *et al.*, 1987). Approximately one-fourth of patients with thyroid autoimmunity have TPO antibodies that can also bind thyroglobulin, so-called TGPO antibodies (Estienne *et al.*, 1999; Ruf *et al.*, 1992). Their pathological significance is unknown, and more recent evidence has indicated that antibodies against both thyroglobulin and TPO in the same individual arise due to breaking self-tolerance to each individual autoantigen separately (McLachlan and Rapoport, 2014).

TSH-Receptor

This receptor is similar to other G-coupled receptors and comprises two subunits, corresponding to the extracellular and transmembrane domains, joined by disulfide bonds. The fundamental importance of the TSH-receptor to Graves' disease was first established by the identification of a long-acting thyroid stimulator (LATS) in the serum of Graves' patients that matched the thyroid stimulatory action of TSH but had a much longer time course and, hence, could not be TSH. LATS was soon identified as an IgG antibody and assays were developed for these thyroid-stimulating antibodies that relied on the ability of IgG to stimulate cyclic AMP production—that is, the second messenger produced from TSH-receptor interaction with its ligand. Other intracellular signaling pathways may be activated by certain TSH-receptor antibodies, which may lead to goiter formation.

Considerable effort has been directed at identifying the B-cell epitopes on TSH-receptor recognized by antibodies, but the results are not conclusive. However, multiple species of TSH-receptor antibodies exist and some actually prevent TSH from stimulating the receptor without causing any stimulation themselves (Davies and Latif, 2015; McLachlan and Rapoport, 2014). The effect of these blocking antibodies is hypothyroidism. Other "neutral" TSH-receptor antibodies bind but have no functional effects. Although the detection of TSH-receptor-stimulating and-blocking antibodies depends on bioassays, most commonly measuring the cyclic AMP responses of the FRTL-5 rat thyroid cell line or Chinese hamster ovary cells transfected with TSH-receptor; these are cumbersome and not suited to rapid diagnostic testing. Antibodies that bind to TSH-receptor can be measured simply in solid-phase assays that estimate competition between antibody and radiolabeled TSH for binding to the receptor. Results from assays of these so-called TSH-binding inhibiting immunoglobulins (TBIIs) correlate well with the results of thyroid-stimulating antibodies in thyrotoxic patients, but TBII is also positive when TSH receptor blocking antibodies are present. Therefore, a positive TBII cannot be equated with the presence of the TSH-receptor-stimulating antibodies that cause Graves' disease.

There are multiple B-cell epitopes in the TSH-receptor that are generally conformational, comprising discontinuous segments that overlap the TSH binding site. Antibodies that bind to the carboxyl terminus of the extracellular domain have blocking activity, and those against the amino terminus have stimulating activity. Further dissection of these epitopes may allow the development of solid-phase assays for TSH-receptor-stimulating and -blocking antibodies that would render bioassays obsolete. In some Graves' patients, the TSH-receptor-stimulating antibody response is restricted in terms of light and heavy-chain use, implying genetic control of these strongly pathogenic antibodies. Another noteworthy feature is the very low concentration of these antibodies in sera, which makes their study difficult.

T-cell epitopes are heterogeneous and no clear dominant epitope has been identified. Nonetheless, the production of TSH-receptor antibodies is T-cell dependent, and presumably there are such dominant epitopes in the earliest phase of disease.

Other Autoantigens

The Na^+/I^- symporter is recognized by antibodies in 5%–30% of patients with Graves' disease or autoimmune hypothyroidism. Some of these antibodies have weak blocking effects on symporter activity in vitro, but in vivo iodide uptake is not a rate-limiting step in thyroid hormone synthesis and it is unknown whether there is any pathogenic role for these antibodies. A poorly characterized second colloid antigen (i.e., in addition to thyroglobulin) has been recognized for decades based on immunofluorescence studies with patient sera, but its nature is obscure. It has been suggested that a set of thyroid growth-stimulating and growth-inhibiting antibodies, separate from TSH-receptor antibodies, cause goitrous and atrophic thyroiditis, but this hypothesis is controversial and these antibodies are poorly characterized.

The biggest enigma is the nature of the orbital autoantigen(s) in Graves' orbitopathy. To explain the close association between this condition and thyroid autoimmunity, especially Graves' disease, the existence of an orbital autoantigen that cross-reacts with one in the thyroid has been proposed. The most attractive candidate is the TSH-receptor, which is expressed by the preadipocyte subset of fibroblasts; however, whether this is sufficient to explain pathogenesis is debated (Davies and Latif, 2015; Wall, 2014). A wide variety of extraocular muscle and fibroblast antigens have been suggested during the past 20 years based on enzyme-linked immunosorbent assays and immunoblotting studies, and it seems likely that several autoantigens are targets of the autoimmune response, at least in the later stages of disease, akin to the situation in autoimmune thyroiditis and type 1 diabetes mellitus.

Breach of Self-Tolerance

Central self-tolerance develops in the thymus and is essential for self-/nonself discrimination. Expression of the *AIRE* gene allows medullary thymic epithelial cells to express thousands of self-proteins specific for other organs, for example, the thyroid gland, and present peptides derived from these proteins in the context of MHC molecules to T cells under maturation. As a consequence, T cells with strong reactivity against self-peptides are eliminated. The system is, however, not a perfect one; some self-reactive T cells are not deleted and must be regulated peripherally in order to avoid development of autoimmune disease (McLachlan and Rapoport, 2014; Weetman and DeGroot, 2016).

Peripheral self-tolerance is normally ensured by Tregs that suppress autoimmune responses partly via mechanisms that involve cell-to-cell contact, and partly via production of immunoregulatory cytokines such as interleukin (IL)-10 and tumor growth factor (TGF)-β. Some T cells leave the thymus as Tregs (natural Tregs), while other Tregs (inducible Tregs) are induced from naïve T cells in the periphery under the influence of TGF-β. A shift in this balance between Tregs and pro-inflammatory T-cell subsets may lead to autoimmune diseases (**Fig. 3**) (Bliddal *et al.*, 2017; McLachlan and Rapoport, 2014). While many investigators have failed to show reduced Treg proportions or functional capacity in human thyroid autoimmunity, there is some evidence of a skewed balance between thyroglobulin-specific Tregs and pro-inflammatory T-helper-17 (Th17) cells in Hashimoto's thyroiditis (Kristensen *et al.*, 2015). Interestingly, overexpression of a truncated form of FOXP3, the transcription factor governing Treg induction, has been observed in Hashimoto's thyroiditis (Kristensen *et al.*, 2015).

Recently, a subset of B cells known as regulatory B cells (Bregs) have been shown to contribute to maintenance of peripheral tolerance by virtue of IL-10, transforming growth factor (TGF)-β, Fas ligand (FasL), and TRAIL expression (Fillatreau *et al.*, 2002; Klinker and Lundy, 2012; Mauri *et al.*, 2003). Particularly transitional B cells are enriched with Bregs. Significantly lowered frequencies of IL-10 producing B cells have been observed in new-onset Graves' disease patients (Zha *et al.*, 2012) as well as in Hashimoto's thyroiditis where transitional B cells showed impaired capacity to suppress T-cell proliferation and production of pro-inflammatory cytokines (Yu *et al.*, 2017).

Pathogenesis of Autoimmune Hypothyroidism

Cell-Mediated Effects

T cells play a critical role in the pathogenesis of autoimmune thyroiditis in animals, and considerable evidence supports a similar role in humans. $CD4^+$ T-helper (Th) cell and $CD8^+$ cytotoxic T (Tc) cells accumulate in the thyroid and the majority of these cells express markers of activation. Although some studies have suggested that these T cells are clonally restricted, others have found diverse uses of T-cell receptor Vα and Vβ genes, and this type of polyclonal response is in agreement with the known multiplicity of autoantigens and their epitopes and the likely spreading of antigenic determinants over time. Circulating T cells have an increased percentage of $HLA\text{-}DR^+$ T cells, marking a state of activation, and the proportion of $CD8^+$ T cells is probably decreased, but these are features of many autoimmune disorders and may have no autoantigen-specific cause or effect. More importantly, circulating and intrathyroidal T cells from patients with autoimmune hypothyroidism can be activated by and proliferate in response to thyroglobulin or thyroid peroxidase.

Conventional antigen presentation in the initiation of an immune response is primarily a function of dendritic cells, which can process antigen and present it with appropriate costimulatory molecules (**Fig. 1**). B cells and macrophages can also present antigen under appropriate conditions, and for B cells there is the additional feature that specific antigens can be processed because these are taken up by binding to antigen-specific antibody, the B-cell receptor, on the B-cell surface. This allows cells to up-concentrate

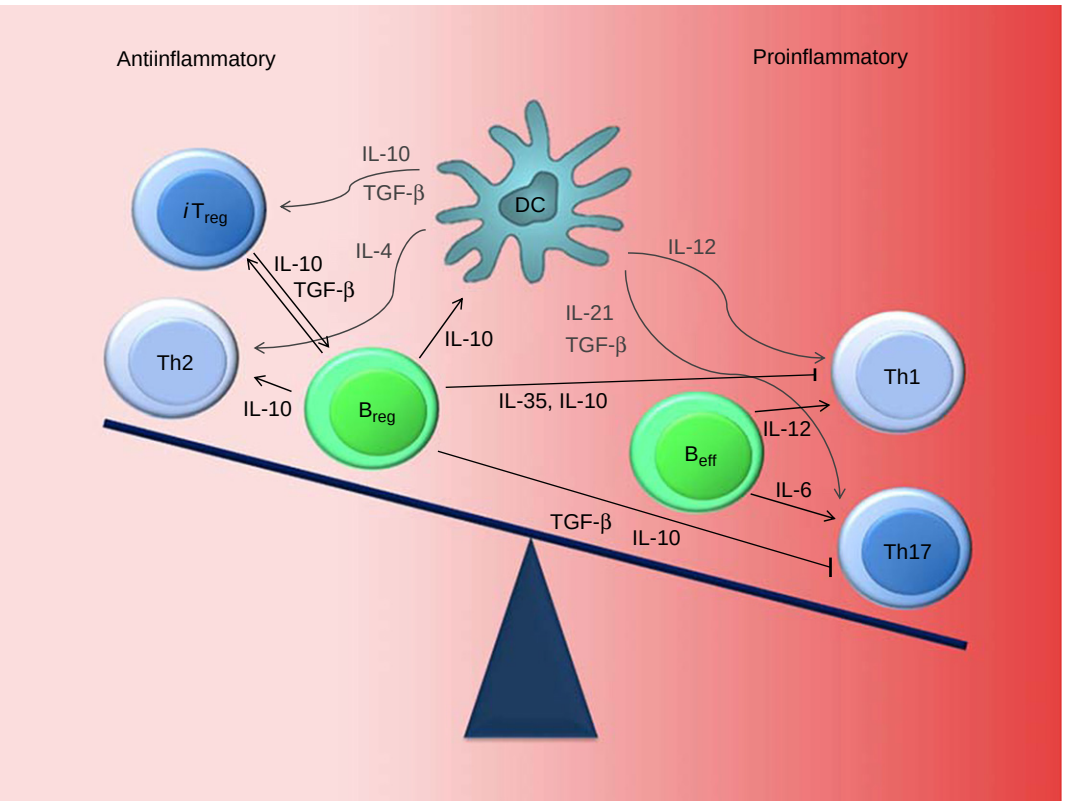

Fig. 3 B-cell and T-cell subsets involved in autoimmunity. Peripheral self-tolerance is ensured by regulatory T cells and regulatory B cells. Autoimmunity (loss of self-tolerance) is considered to be due to a shift in favor of pro-inflammatory cells. *Abbreviations*: iT_{reg}, induced regulatory T cells; $Th_{1,2,17}$, Effector CD4+ T-cell subsets with different cytokine profiles; B_{reg}, regulatory B cells; B_{eff}, effector B cells. Reproduced with kind permission from Bliddal, S., Nielsen, C. H., and Feldt-Rasmussen, U. (2017). Recent advances in understanding autoimmune thyroid disease: The tallest tree in the forest of polyautoimmunity. *F1000Research 6*, 1776.

antigens that are present in small quantities in the circulation which favors antigen-presentation by B cells over that of dendritic cells that simply sample a variety of antigens from the surroundings for presentation. Both types of antigen-presenting cells accumulate in the thyroid gland at the early stages of autoimmune thyroid disease. Like T cells and B cells, dendritic cells may be immunogenic or tolerogenic—and may thereby drive T cells into the appropriate directions—but their role in the pathogenesis of autoimmune thyroid disease is poorly understood.

When it was discovered that thyroid follicular cells express MHC class II molecules in autoimmune hypothyroidism and Graves' disease, there was considerable interest in the idea that this could be the precipitant of the autoimmune process because the thyroid cell would be capable of presenting thyroid autoantigens to any specific T cells in the vicinity. It is now clear that such MHC class II expression is a secondary rather than primary event, depending on the production of interferon-γ by locally infiltrating T cells, which clearly must have been activated by other antigen presenting cells. Moreover, the initiation of an immune response depends on the delivery of a second costimulatory signal to T cells at the same time as the first signal, namely the MHC class II molecule complexed with the antigenic epitope (**Fig. 1**). Failure to provide this second signal leads to anergy or even deletion—that is, the opposite outcome to activation. In general, thyroid cells do not appear to express B7 (CD80 or CD86), the most important costimulatory molecule, although there is a single report suggesting its expression in Hashimoto's thyroiditis. However, it seems that MHC class II expression by thyroid cells, from a teleological standpoint, is a protective mechanism to provide peripheral tolerance, for instance, in the event of viral thyroiditis.

Once activated, T cells can be restimulated by cells presenting antigen in the absence of B7 and thus, in the setting of autoimmune thyroiditis, MHC class II expression of thyroid cells may have an important role in expanding the intrathyroidal pool of T cells and thus exacerbating the autoimmune process (**Fig. 1**). It is also possible that differences in the regulation of T cells at this critical stage of disease (e.g., through genetically determined variability in class II expression or cytokine availability) may determine whether a focal thyroiditis progresses to full-blown disease.

Following these observations, it has become clear that thyroid follicular cells express a vast array of immunologically active molecules, including adhesion molecules and cytokines (**Table 6**). Many of these are induced by the cytokines known to be present in the intrathyroidal milieu in autoimmune thyroid disease, especially IL-1, tumor necrosis factor-α, and γ-interferon. Their expression tends to exacerbate the autoimmune response, although in some cases, such as the upregulation of complement regulatory proteins, the cell is protected from immune attack.

Since the original observation that Th cells may be divided into Th1 and Th2 cells on the basis of their cytokine production (interferon-γ and IL-4, respectively), investigators have suggested that Th cells producing IL-17, IL-9, and IL-22 be designated Th17, Th9, and Th22 cells, respectively. Of these subsets, Th1, Th2, and Th17 cells have been well-described in relation to autoimmune thyroid disease. The nature of the cell presenting antigen, and particularly the cytokines it secretes, plays a key role in determining the direction of the Th-cell response. Traditionally, an increased Th1 cell response has been viewed as a main cause of Hashimoto's thyroiditis by means of triggering a strong lymphocyte inflammatory infiltrate of the thyroid (thyroiditis) and, consequently, thyroid gland destruction. However, cytokines related to Th17 responses have also been shown to be excessively present in Hashimoto's thyroiditis and Graves' disease (Ramos-Levi and Marazuela, 2016; Shao *et al.*, 2018). Notably, a high degree of plasticity exist between Th17 cells and inducible Tregs, allowing these subsets to be converted to each other, and a skewed balance between them is likely to contribute to the pathogenesis of Hashimoto's thyroiditis (**Fig. 3**) (Kristensen *et al.*, 2015; Yang *et al.*, 2008). Of notice, cytokine profiles have been characterized by varying methods, that is, from isolated peripheral blood mononuclear cells, cells from thyroid gland tissue, or by ex vivo stimulation of such cells.

How thyroid cells are destroyed is unresolved. Data from animal models indicate that $CD8^+$ T cells kill thyroid follicular cells, and perforin-containing cells exist in the thyroid infiltrate in human autoimmune thyroid disease. Recently, attention has focused on apoptosis as a major mechanism for thyroid cell loss. Thyroid follicular cells in Hashimoto's thyroiditis overexpress Fas (CD95), likely the result of locally released cytokines, especially IL-1β. This could lead to an increased risk of thyroid cell destruction through interaction with $CD8^+$ T cells that bind to Fas and thereby trigger apoptosis. Several groups have described expression of FasL on thyroid follicular cells in autoimmune thyroid disease, although normally this molecule is restricted to $CD8^+$ T cells and sites of immunological privilege (e.g., trophoblast and testis), where it prevents T-cell recognition of antigen by deleting these T cells through Fas engagement. In some studies, IL-1β upregulated FasL expression by Hashimoto's thyroid cells in vitro, leading to the suggestion that exposure to this cytokine in vivo may cause suicide or fratricide within the Fas-positive thyroid cell population. However, questions have been raised over technical aspects of these experiments and, even if correct, the proportion of $FasL^+$ thyroid cells is often low. Upregulation of Bcl-2 and other antiapoptotic intracellular proteins, especially in Graves' disease, may limit thyroid cell death. Finally, the opposite view of FasL expression could be taken; namely, that such expression may protect thyroid cells from immune attack by creating the conditions of immunological privilege. Much work remains to be done before the roles of Fas/FasL and other cytotoxic pathways in the thyroid can be determined.

Humoral Effects

Overwhelming evidence indicates that thyroid antibodies alone cannot cause thyroid cell destruction. Tg antibodies do not fix complement. These antibodies and those against TPO and the Na^+/I^- symporter can cross the placenta and yet neonates born to mothers with high levels of such antibodies have normal thyroid function, as do many adults who have detectable thyroid antibodies. Experimental data indicate that these antibodies cannot access their antigens within an intact thyroid follicle. Nonetheless, there may be a secondary role for TPO antibodies: they cause tissue injury once the follicular architecture is disrupted by cytokines or T-cell-mediated cytotoxicity. Also, there is in vitro evidence that Tg antibodies and TPO antibodies can affect antibody-dependent, cell-mediated cytotoxicity by engaging Fc receptors on natural killer cells. There is certainly widespread complement activation within the thyroid in both Hashimoto's thyroiditis and Graves' disease. Although heightened expression of complement regulatory proteins (**Table 6**) may protect the thyroid cells from overt lysis, sublethal complement attack impairs the metabolic response of thyroid cells to TSH and induces cytokine production, which may exacerbate the autoimmune process.

Table 6 Immunological active molecules expressed by thyroid cells in autoimmune thyroiditis

Molecule	*Functions*	*Upregulatory factors*
MHC class I	Recognition by $CD8^+$ cytotoxic T cells	γ-IFN, TNF increase basal expression
MHC class II	Recognition by $CD4^+$ helper T cells	γ-IFN induces expression; enhanced by TNF
Adhesion and homing molecules (ICAM-1, LFA-3, Hermes-1, CD44, NCAM)	Binding of T and NK cells	γ-IFN, IL-1, TNF
Cytokines (IL-1, IL-6, IL-8, IL-13, IL-15)	Multiple effects on bystander lymphocytes	γ-IFN, IL-1, TNF
Complement regulatory proteins (CD46, CD55, CD59)	Prevention of lethal complement attack	γ-IFN, IL-1, TNF
CD40	Binds CD40 ligand on T cells, acting as a costimulatory	γ-IFN, IL-1
Nitric oxide	Tissue injury	γ-IFN, IL-1, TNF (decreased by IL-4, α-IFN, TGF-β)
Fas/Fas ligand	Apoptosis	IL-1

Abbreviations: *CD*, cluster of differentiation; *ICAM-1*, intercellular adhesion molecule-1; *IFN*, interferon; *IL*, interleukin; *LFA-3*, lymphocyte function-associated antigen 3; *NCAM*, neural cell adhesion molecule; *NK cells*, natural killer cells; *TGF*, transforming growth factor; *TNF*, tumor necrosis factor.

Thyroid autoantibodies may further promote presentation of thyroglobulin and TPO and induce proinflammatory cytokine responses by enhancing their uptake by antigen-presenting and cytokine-producing cells (Nielsen *et al.*, 2009; Nielsen *et al.*, 2004).

TSH-receptor-blocking antibodies, in contrast, have pathogenic effects, causing hypothyroidism. This is most clearly demonstrated in the transient neonatal hypothyroidism that follows placental transfer. This effect lasts only as long as the antibodies persist and is therefore a functional rather than cytotoxic effect. As many as 20% of patients with hypothyroidism may have TSH-receptor-blocking antibodies, and these appear particularly common in patients with atrophic rather than goitrous thyroiditis in Asia, whereas in other regions TSH-receptor-blocking antibodies show no such dichotomy. Unexplained fluctuations in these antibodies (and sometimes in coincident TSH-receptor-stimulating antibodies) account for most, if not all, cases of hypothyroidism that show spontaneous recovery. The role of other potentially cytotoxic molecules, such as lymphotoxin-α, nitrous oxide, and reactive oxygen metabolites, in causing thyroid injury is unclear (**Fig. 4**).

Pathogenesis of Graves' Disease

Cell-Mediated Effects

Similar findings as those for autoimmune thyroiditis are found for Graves' disease, the latter also being caused by a T-cell abnormality, but the clinical presentation of hyperthyroidism being caused by production of the pathognomonic TSH-receptor-stimulating antibodies as described in the above. Thus, *Graves' hyperthyroidism is antibody-mediated but is predominantly a Th1-type cytokine disease* (Rapoport and McLachlan, 2014). Circulating T cells show an increase in HLA-DR$^+$ and a decrease in CD8$^+$ subsets, and the thyroid infiltrate comprises CD4$^+$ and CD8$^+$ cells with little evidence of clonal restriction. Thyroglobulin-, TPO-, and TSH-receptor-specific T cells can be demonstrated in the circulation and thyroid infiltrate, but there is no evidence of a specific defect in their regulation. The same issues have been raised regarding antigen presentation as those for autoimmune hypothyroidism, and there is a mixed intrathyroidal cytokine profile with evidence of various Th-cell responses. These observations help explain the transition of Graves' disease to autoimmune hypothyroidism seen in some patients spontaneously and in others years after treatment with antithyroid drugs. There is also indirect evidence that hypothyroidism is more likely to occur after radioiodine or surgery in Graves' patients with the most marked autoimmune responses against thyroglobulin and TPO.

Humoral Effects

Graves' disease is the prime exemplar of a disease caused by antibodies with a stimulatory action (previously termed type V hypersensitivity). The role of TSH-receptor-stimulating antibodies was discussed previously. Virtually all patients have detectable TSH-receptor-stimulating antibodies, provided the most sensitive assays are used. The few who do not, most likely have antibodies, but in an amount that is below the current limit of detection. The level of TSH-receptor-stimulating antibodies correlates loosely with the severity of hyperthyroidism due to the ongoing intrathyroidal T-cell-mediated events, the secondary effects of TPO antibody causing thyroid injury, and the simultaneous presence of TSH-receptor-blocking antibodies in some patients. There is a much clearer relation between

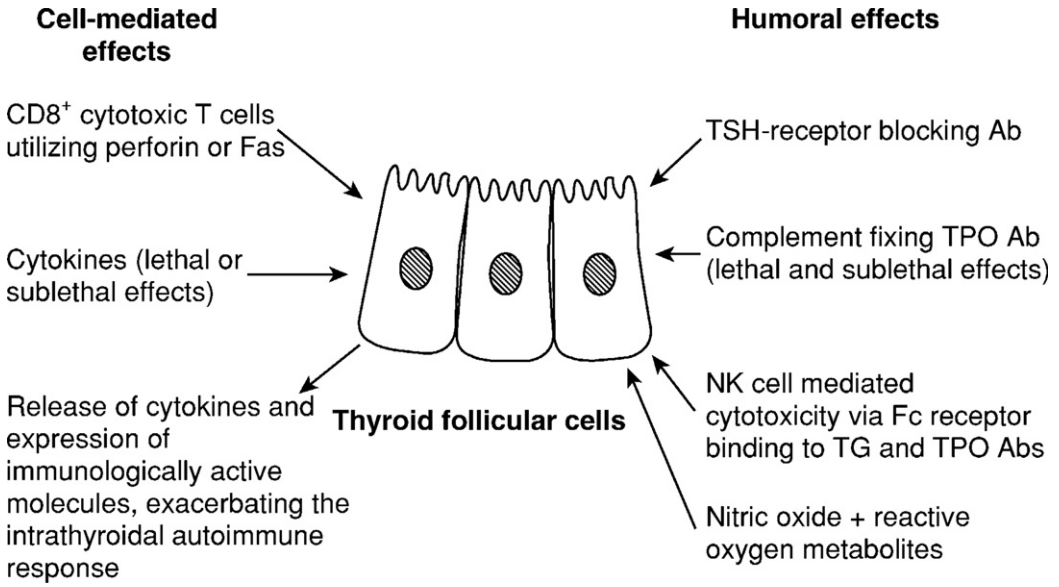

Fig. 4 Pathogenetic mechanisms in autoimmune hypothyroidism. *Abbreviations*: *Ab*, antibody; *NK cell*, natural killer cell; *TPO*, thyroid peroxidase; *TG*, thyroglobulin.

the maternal level of TSH-receptor antibodies in women with Graves' disease in the last trimester of pregnancy and the occurrence of neonatal thyrotoxicosis, suggesting that the first two processes, which do not affect fetal or neonatal thyroid, are most important.

Effects of Treatment

Spontaneous remission of Graves' disease appears to be unusual, unless autoimmune hypothyroidism intervenes. Accurate data are not available, but clinical remission was seen in only 10%–20% of patients treated decades ago with beta-blockers, and these patients had the mildest degree of thyrotoxicosis (Codaccioni *et al.*, 1988). Remission of other receptor antibody-mediated diseases, such as myasthenia gravis, is also rare. Therefore, the 40%–50% remission rate of Graves' disease after treatment with the antithyroid drugs carbimazole, methimazole, or propylthiouracil indicates an immunomodulatory effect of these agents. TSH-receptor antibody levels decline during treatment and remain low in those who do not relapse, although there is not a clear correlation between levels of TSH-receptor antibodies and relapse/remission rate after medical treatment (Feldt-Rasmussen *et al.*, 1994). It may, however, depend on the method used for measurement since a recent study found higher predictive value by a method assessing thyroid stimulating antibodies by a bioactivity stimulation assay, than when using TBII (Kwon *et al.*, 2016). Furthermore, levels of nonthyroid autoantibodies do not decline during medical antithyroid therapy. This apparent paradox is most likely explained by the actions of antithyroid drugs on the thyroid cells, including suppression of cytokine, reactive oxygen metabolite, and prostaglandin production. As a result, the thyroid lymphocytic infiltrate declines and the production of thyroid antibodies are curtailed. Remission is not expected in patients in whom the autoimmune process has spread beyond the thyroid gland because antithyroid drugs do not easily influence any extrathyroidal autoreactivity; this is in accord with the observation that patients with the most severe disease do poorly on antithyroid drugs.

Subtotal thyroidectomy usually leads to cessation of thyroid antibody production, although this may continue in some patients due to the persistence of specific B cells in the bone marrow and lymph nodes. Thyroid antibodies show a characteristic rise and then fall usually during the year after ^{131}I therapy: this may be due to release of autoantigen from the damaged thyroid, followed by death of the intrathyroidal lymphocyte population. TSH-receptor antibodies have, however, been demonstrated to remain in the circulation years after radioiodine therapy, unlike after thyroidectomy or only medical treatment (Laurberg *et al.*, 2008).

Activated T cells increase in the circulation in the first few months after ^{131}I and these cells, by homing to the orbit, may be responsible for the exacerbation of Graves' orbitopathy seen in up to 15% of patients after such treatment. This worsening can be prevented by glucocorticoids (Shiber *et al.*, 2014).

Graves' Orbitopathy

The pathogenesis of Graves' orbitopathy is complex and unclear. The extraocular muscles are the main focus of the autoimmune process, but occasional patients appear to have a primary expansion of the retro-ocular connective tissue, including fat. A separate process is likely involved in the clinical sign of lid retraction (which is more extreme than in any type of thyrotoxicosis), at least based on the histological findings. There is no evidence that tissue destruction plays a role in initiating these changes, although extraocular muscle cell damage may occur late in severe disease; thus, attempts to find cytotoxic autoantibodies or lymphocytes seem ill founded. Apart from lid retraction, which could be mediated by the sympathetic overactivity secondary to thyrotoxicosis, no convincing signs of Graves' orbitopathy have been reported in neonates born to women with Graves' orbitopathy, arguing against a primary role for orbital autoantibodies (Weetman and DeGroot, 2016). However, antibodies that activate insulin-like growth factor I (IGF-I) receptor have been detected in patients with Graves' orbitopathy and have been shown to synergistically enhance TSH action and stimulate fibroblast action in the orbit (Smith *et al.*, 2017). Thus, the most likely explanation for Graves' orbitopathy is that fibroblasts in the extraocular muscles and orbital connective tissue are activated by cytokines from the infiltrating T cells and macrophages. IL-1β, tumor necrosis factor, and γ-interferon all affect fibroblast function and may cause the increase in expression of HLA-DR and other immunologically active molecules found in Graves' orbitopathy. More important, they enhance glycosaminoglycan production, leading to water trapping and edema. An identical or related pathway of cytokine-induced activation may lead to fibrosis later in disease, the so-called burnt-out stage of Graves' orbitopathy. Smoking may exacerbate this pathogenetic process by affecting cytokine secretion or action or by inducing partial hypoxia, which increases glycosaminoglycan production. Indeed, as the orbital contents increase, the pressure effects within the confines of the bony orbit may lead to partially hypoxic and congested conditions with impaired venous drainage, which in turn may cause further edema in addition to being a proinflammatory environment. In such a scenario, relatively modest initial increases in orbital tissue volume may quickly increase as in congestive orbitopathy. Anatomical differences between the capacity of the two orbits in an individual may accommodate different degrees of swelling, leading to the asymmetric form of Graves' orbitopathy seen in 5%–10% of cases. Another factor accounting for Graves' orbitopathy is the likely heightened sensitivity of the orbital fibroblast population to cytokine activation compared to that of fibroblasts from other sites. Together with the presumed intraorbital location of a thyroid cross-reactive autoantigen, this susceptibility may explain the localization of disease.

Approximately 1% of patients with Graves' disease develop thyroid dermopathy, often situated over the shins, where it is called pretibial myxedema. These patients almost always have marked Graves' orbitopathy, and dermopathy seems to be a more flagrant

form of the same process, in which immunologically mediated fibroblast activation occurs within the dermis, presumably via the action of cytokines. Dermopathy is usually localized to sites where fluid retention and injury occur, suggesting that a cascade of events similar to those of Graves' orbitopathy may cause worsening of the disease.

The expanding knowledge of the immunologic processes involved in autoimmune thyroid disease has great potential in the understanding and of treatment of otherwise treatment-resistant Graves' orbitopathy. The B-cell-depleting agent rituximab improves Graves' orbitopathy (as well as numerous other autoimmune diseases) (El *et al.*, 2006; Salvi *et al.*, 2015). Also, targeting and thus inhibiting the IGF-1-receptor by use of teprotumumab significantly improved outcome in 88 patients with Graves orbitopathy (Smith *et al.*, 2017). More recently, a randomized trial of the effect of adding the B-cell and T-cell-suppressant mycophenolate to prednisolone treatment in patients with active moderate-to-severe Graves' orbitopathy failed to show improvement of response rates, although results in subgroup analyses were improved (Kahaly *et al.*, 2018). Thus, immunomodulatory drugs may become key players in understanding and treating severe Graves' orbitopathy in years to come.

Conclusion

Thyroid autoimmunity is the most common autoimmune phenomenon in humans and is caused by T-cell and B-cell self-reactivity against the thyroid gland. Several clinically overt, common, and distinct thyroid diseases result from thyroid autoimmunity, although there are also many individuals who have subclinical evidence of thyroid autoimmunity. The pathogenesis is most often multifactorial including genetic susceptibility (i.e., HLA-DR3 alleles), environmental factors (iodine consumption, smoking) and endogenous factors (female sex, previous pregnancies, increasing age). Future studies (i.e., studies of next-generation sequencing, immune regulation, and microbiota) will further evolve our understanding of this heterogenous group of autoimmune diseases and help identify potential beneficial targets of treatment in those with treatment-resistant disease.

See also: Causes of Hypothyroidism. Graves' Disease. Graves' Orbitopathy. Hyperthyroidism in Graves' Disease. Hypothyroidism Subclinical. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Postpartum Thyroid Dysfunction. Pretibial Myxedema. Thyroglobulin. Thyroid and Infertility. Thyroid Carcinoma. Thyroid Function and Depression. Thyroid Peroxidase. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Relevant Website

http://www.thyroidmanager.org/about/ —Thyroid Disease Manager non-profit up-to-date analyses of the thyroid by thyroid experts.

Thyrotoxicosis; Overview of Causes☆

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Glossary

Graves' disease Autoimmune thyroid disease characterized by goiter, hyperthyroidism, and ophthalmopathy (in its classic form, also known as "Merseburg triad," from the name of the small village in Germany where Karl von Basedow first observed a patient).

Hyperthyroidism Active overproduction of thyroid hormone by the thyroid follicular epithelial cells that can cause thyrotoxicosis.

Thyrotoxicosis The clinical syndrome that results from the exposition of tissues to an excess of circulating thyroid hormone.

Thyrotoxicosis factitia Thyrotoxicosis due to a voluntary surreptitious ingestion of excess thyroid hormone preparations with various purposes.

Toxic adenoma Benign thyroid tumor characterized by autonomous overproduction of thyroid hormone (in most cases due to a somatic activating mutation of the thyrotropin receptor gene) functioning autonomously.

Classification

The various causes of thyrotoxicosis can be classified according to their pathogenic mechanism. In **Table 1**, the most common forms of thyrotoxicosis are categorized into two broad groups: thyrotoxic syndromes of thyroidal origin and thyrotoxic syndromes of nonthyroidal origin. The first group, the most frequent one, can be further divided into forms associated with thyroid hormone hypersecretion (hyperthyroidism) and into forms characterized by the release of preformed hormones, secondary to destructive processes (destructive thyrotoxicosis). The second group includes a heterogeneous group of rare disorders in which the thyroid gland is not primarily involved as a source of thyroid hormone. Thyroid radioiodine uptake (RAIU) and thyroid 99m-pertechnetate scintigraphy although not always performed nowadays, have for many decades represented the primary tests in broadly defining the cause of thyrotoxicosis (De Leo *et al.*, 2016; Ross *et al.*, 2016).

Causes of Hyperthyroidism

Graves' Disease

The most frequent cause (40%–70% according to the iodine intake in the population) of thyrotoxicosis is Graves' disease (Carle *et al.*, 2011). The prevalence of Graves' disease may be as high as 1%, with a predominance of the female sex. Graves' disease is an autoimmune disorder characterized, in its classic form, by goiter, hyperthyroidism, ophthalmopathy (Merseburg triad), and (less frequently) dermopathylocalized in the pretibial region (pretibial myxedema). Hyperthyroidism is caused by an autoimmune reaction to the thyroid, leading to the production of autoantibodies to the thyrotropin (TSH) receptor (TSH receptor-stimulating antibodies (TSABs)) (Chiovato *et al.*, 1994). These antibodies stimulate the TSH receptor on thyroid follicular cells. Because there is no feedback of thyroid hormone on the production of TSABs, uncontrolled stimulation of the receptor causes a diffuse enlargement of the thyroid gland and overproduction and release of thyroid hormone that lead to hyperthyroidism. In some cases, thyroid nodularities can be felt as a consequence of either long-standing disease or preexisting nodular goiter. Physical examination reveals signs of Graves' ophthalmopathy in 30%–45% of patients, and when studied with imaging techniques, suggestive findings can be observed in up to 70% of cases. Local myxedema is a peculiar and rare skin manifestation of Graves' disease characterized by edema, inflammation, and lymphocytic infiltration localized mostly to the pretibial dermis. It occurs almost exclusively in patients who also have Graves' ophthalmopathy (Bartalena *et al.*, 2016; Ross *et al.*, 2016; Smith and Hegedüs, 2016).

Toxic Adenoma

Toxic adenoma is a quite frequent cause of hyperthyroidism, especially in iodine-deficient countries, where its prevalence has been reported to be as high as 4.5% (10% of all cases of thyrotoxicosis) (Carle *et al.*, 2011). A lower prevalence (2.7%) has been reported in iodine-sufficient areas. Toxic adenomas are benign isolated thyroid tumors that function autonomously. The nodular tissue acquires the capability of producing thyroid hormones independently of TSH stimulation. The increased thyroid hormone secretion first suppresses pituitary TSH secretion and eventually leads to overt hyperthyroidism. Because of TSH suppression, the

☆*Change History:* March 2018. C Marcocci and R Rocchino updated Table 1 and References list.

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Table 1 Classification of known causes of thyrotoxicosis, epidemiology, and distinctive diagnostic features

Group	*Disease*	*Relative frequency (%)*	*Dstinctive features*	*Neck RAIU*	*Serum TSH*	*Serum thyroglobulin*
Thyrotoxicosis of thyroidal origin associated with hyperthyroidism	Graves' disease	40–70*	Diffuse goiter, ophtalmopathy, positive TRABs	High	Undetectable	High
	Toxic adenoma	5	Single "hot" nodule at thyroid scan	High	Undetectable	High
	Toxic multinodular goiter	20–40*	Multiple "hot" nodules at thyroid scan	High	Undetectable	High
	Iodine induced thyrotoxicosis	<1	High urinary iodine	Low to high	Undetectable	High
	TSH-secreting adenomas	<1	Inappropriately high TSH level	High	Normal to high	High
	Familial gestational hyperthyroidism	Extremely rare	Pregnancy-associated DNA analysis	Presumably high	Undetectable	High
	Trophoblastic tumors	<1	High chorionic gonadotropin	High	Undetectable	High
	Neonatal thyrotoxicosis	<1	Positive TRABs	High	Undetectable	High
	Familial hypersensitivity to hCG	<1	TSH receptor mutation with inc. sensitivity to hCG	High	Undetectable	High
	Non-autoimmune congenitaland familial hyperthyroidism	Extremely rare	TSH receptor gene mutations by DNA analysis	High	Undetectable	High
Associated with thyroid destruction	Subacute thyroiditis	3	Neck pain, high erythrocte sedimentation rate	Low	Undetectable	High
	Silent thyroiditis	3	Positive thyroid autoantibodies	Low	Undetectable	High
	Type II amiodarone-induced thyrotoxicosis	<1	High urinary idine, highserum interleukin-6	Low	Undetectable	High
Thyrotoxicosis of non-thyroidal origin	Factitious thyrotoxicosis	<1	Low serum thyroglobulin	Low	Undetectable	Low
	Thyroid hormone intoxication	<1	Low serum thyroglobulin	Low	Undetectable	Low
	Dermoid tumors (struma ovarii)	Very rare	Abdominal RAIU	Low	Undetectable	High
	Metastatic differentiated thyroid cancer	Very rare	Bone RAIU (whole body scan)	Low	Undetectable	High

*According to iodine intake in the population and endemic goiter areas (Carle et al., 2011).
TRAbs: Antibodies against TSH receptor; *RAIU*: Radioiodine uptake; *hCG*: human chorionic gonadotropin.

extranodular thyroid tissue becomes functionally quiescent and may undergo some degree of atrophy. Toxic adenomas are more frequent in the aged population and in females. The natural history of toxic adenoma is characterized by slow growth over many years with a progression of its functional properties through various stages. During the early stages, the excess of secretion of thyroid hormones is not sufficient to completely suppress TSH secretion (partial autonomy) and the extranodular tissue. With further growth of the nodule, TSH suppression becomes complete, whereas circulating thyroid hormones are in the upper range of normality (complete autonomy). Eventually, overt thyrotoxicosis ensues with frankly elevated thyroid hormone levels. Recent studies have shown that somatic mutations of the TSH receptor gene are the cause of 20%–80% of toxic adenomas. In other cases, the Gs-α-subunit of the TSH receptor coupled adenyl cyclase is mutated. Both kinds of mutations cause permanent activation of the TSH receptor intracellular signaling pathway in the absence of the natural ligand (TSH); therefore, they fully explain the autonomous functioning of the nodules (Tonacchera *et al.*, 1998; Ross *et al.*, 2016; Siegel and Lee, 1998).

Toxic Multinodular Goiter

Toxic multinodular goiter is also more frequent in iodine-deficient countries and primarily affects women (Carle *et al.*, 2011). Epidemiological studies have clearly shown that toxic multinodular goiter represents the long-term outcome of many long-standing nontoxic goiters; therefore, it is most often found in aged persons. Somatic activating mutations of the TSH receptor have

been observed occasionally in toxic multinodular goiter as well. The natural history of toxic multinodular goiter is similar to that of toxic adenoma, with a slow formation of multiple autonomously functioning nodular areas in the setting of an overall nodular goiter. Progression of autonomous function may lead to subclinical hyperthyroidism and overt thyrotoxicosis. The clinical manifestations are scanty due to the advanced age of patients and the slow progression of thyrotoxicosis (Hegedus 2004; Siegel and Lee, 1998).

TSH-Secreting Adenoma

Hyperthyroidism caused by excessive TSH secretion by a pituitary adenoma (central hyperthyroidism) is very rare. TSH-secreting adenomas completely or partially lose feedback regulation by thyroid hormone and, therefore, cause sustained stimulation of the thyroid gland, leading to the development of goiter and hyperthyroidism. The severity of thyrotoxicosis is highly variable, ranging from modest elevations to very high levels of thyroid hormones; therefore, the patient may report few or no symptoms or may present with overt thyrotoxic symptoms. TSH-secreting adenomas may cosecrete growth hormone (GH) and prolactin, with clinical presentation of acromegaly and galactorrhea. Most TSH-secreting adenomas are macroadenomas and may lead to hypopituitarism and visual field defects (Beck-Peccoz *et al.*, 1996; Tjörnstrand and Nyström, 2017).

Human Chorionic Gonadotropin-Dependent Hyperthyroidism

Human chorionic gonadotropin (hCG) is secreted in large amounts by placental tissue in normal pregnancy and also by trophoblastic tumors such as hydatidiform mola. hCG has a partial homology with TSH and can act as a weak TSH agonist. Therefore, large amounts of hCG in the bloodstream can over stimulate the thyroid gland and cause hyperthyroidism (Ross *et al.*, 2016; Reinwein *et al.*, 1988; Ross, 1998).

Trophoblastic Tumors

In hydatidiform mola, hyperthyroidism is caused by tumor secretion of large quantities of hCG. However, overt thyrotoxicosis is observed in only a minority (10%) of patients with extraordinarily high levels of hCG (>3 million IU/L) (Ross *et al.*, 2016).

Hyperemesis Gravidarum

Hyperemesis gravidarum is characterized by prominent nausea and vomiting, weight loss ketosis, and electrolyte abnormalities. It is a poorly understood early complication of pregnancy associated with inappropriately high levels of circulating hCG that causes clinical or subclinical hyperthyroidism in about one-third of cases (Pearce, 2015; Ross *et al.*, 2016).

Familial Gestational Hyperthyroidism

This inherited pregnancy-associated form of hyperthyroidism has been described in one family. Hyperthyroidism was due to a mutation in the TSH receptor gene, causing increased sensitivity of the receptor to the effect of hCG. For this reason, hyperthyroidism manifests only during pregnancy and recurs every time an affected woman becomes pregnant (Pearce, 2015; Ross *et al.*, 2016).

Fetal and Neonatal Thyrotoxicosis

TSABs in the serum of mothers with Graves' disease can cross the placenta and cause fetal and neonatal hyperthyroidism through direct stimulation of the fetal thyroid. The disease can be very severe and is characterized by tachycardia, jaundice, heart failure, and failure to thrive. A goiter is usually present. The thyrotoxicosis is transient and resolves within 3 months after birth because there is no source of TSABs in the neonate (Zimmerman, 1999).

Nonautoimmune Congenital and Familial Hyperthyroidism

Recently, a new form of congenital hyperthyroidism has been reported. The disease is caused by a germ line de novo mutation of the TSH receptor gene, causing constitutive permanent activation of the receptor and, therefore, diffuse goiter and overproduction of the thyroid hormone, in turn causing severe hyperthyroidism in the neonate. The clinical presentation is similar to the presentation of neonatal thyrotoxicosis, and the diagnosis should be suspected when no history of Graves' disease is present in the mother. Familial nonautoimmune hyperthyroidism has been described in two kindreds. In this case, hyperthyroidism is due to dominant inherited activating mutations of the TSH receptor, but because the effect of the mutation is mild, hyperthyroidism and goiter develop only during adult age. The clinical manifestations are mild and variable in patients bearing the same mutation (Ferraz and Paschke, 2017; Ross *et al.*, 2016).

Causes of Destructive (Low-RAIU) Thyrotoxicosis

Subacute Thyroiditis

Transient thyrotoxicosis occurs in approximately 50% of patients with subacute thyroiditis. Subacute thyroiditis is an inflammatory disorder of the thyroid, probably due to a viral agent. The viral origin is suggested by the frequent association with a history of recent upper respiratory tract infection.

Clinically, patients with subacute thyroiditis present with pain in anterior neck, variable degrees of thyroid swelling, and systemic symptoms such as malaise and fever. From a histopathological point of view, the thyroid gland indicates a leukocytic and granulomatous infiltrate with follicles disruption. Thyrotoxicosis results from the destruction of thyroid follicles by the inflammatory process with release of preformed thyroid hormones. When present, thyrotoxicosis lasts for 3–8 weeks and is sometimes followed by a phase, also transient, of mild hypothyroidism. Complete recovery of thyroid function eventually occurs, but permanent hypothyroidism has also been reported (Akamizu *et al.*, 2013; Weetman and McGregor, 1994).

Painless Thyroiditis

Painless thyroiditis is probably due to an autoimmune thyroid disorder that can generate an inflammation of the gland with chronic lymphocytic infiltration closely resembling that of Hashimoto's thyroiditis. Similar to subacute thyroiditis, the disease is characterized by a transient phase of thyrotoxicosis, but neck pain and general symptoms are usually absent. Circulating thyroid autoantibodies are found in the majority of cases, and the progression to spontaneous permanent hypothyroidism is observed in as many as 20% of cases in long-term follow-up. In some cases, painless thyroiditis is precipitated by radiotherapy in the region of the neck for a variety of neoplasms that induce thyroid damage with discharge of preformed thyroid hormone. Cytokine treatments, particularly with interferon-1 alpha and interleukin-2, have also been shown to cause a clinical syndrome resembling classical painless thyroiditis. These cytokines may also cause classical Graves' disease by acting as generic triggers of thyroid autoimmunity in predisposed individuals. The most likely mechanism in these cases is activation of preexisting thyroid autoreactive T-cell clones. Findings suggestive of painless thyroiditis are the presence of a goiter, low radioiodine uptake with transient thyrotoxicosis, and occasional progression toward hypothyroidism. These cases may be classified as a variant of Graves' disease with predominant cytotoxic aspects, quickly leading to a clinical picture of Hashimoto's thyroiditis. Painless thyroiditis may be seldom associated with Graves' ophthalmopathy (Akamizu *et al.*, 2013).

Postpartum Thyroiditis

Postpartum thyroiditis is a subacute thyroid inflammation that occurs during the early postpartum period in susceptible woman. It represents a variant of painless thyroiditis. Postpartum thyroiditis is a rather common disorder, occurring in 5%–10% of pregnancies. Most women with postpartum thyroiditis have circulating antithyroglobulin and antithyroperoxidase antibodies before or during the onset of the disease. Some human leukocyte-associated antigen(HLA) haplotypes, such as B35, confer a clear-cut predisposition to the disorder, making it distinct from classical Hashimoto's thyroiditis. From a clinical point of view, the disease is similar to painless thyroiditis with absence of nonspecific or local symptoms and with transient thyrotoxicosis that occurs in approximately 50% of cases. Approximately one-third of cases present with a second phase of transient hypothyroidism that may develop up to 10 years later. The risk of recurrent postpartum thyroiditis in subsequent pregnancies is as high as 70% in women who have already had an episode (Masiukiewicz and Burrow, 1999).

Other Forms of Destructive Thyrotoxicosis

Rarely, destructive thyrotoxicosis can be precipitated by anterior neck injuries. Thyrotoxic crises following thyroid surgery were frequent at the beginning of thyroid surgery era. It has become extremely rare considering the optimal preparation of patients with antithyroid drugs and the refinement of surgical procedures. Finally, thyrotoxicosis may transiently worsen or recur in patients who are treated with radioiodine for Graves' disease, toxic adenomas, and multinodular toxic goiter. This phenomenon can be explained with two mechanisms: ongoing thyroid hyperfunction before radioiodine fully takes effect and radiation-induced thyroid destruction (De Leo *et al.*, 2016; Ross *et al.*, 2016).

Iodine- and Amiodarone-Induced Thyrotoxicosis

Iodine-induced thyrotoxicosis, secondary to the consumption of large amounts of iodine through the diet or other routes (some medications and diagnostics (e.g., contrast media, disinfectants, drugs) and some foods), has been recently reported with increased frequency. Thyrotoxicosis can precipitate through several mechanisms. When high doses of iodine are administered, the normal thyroid responds with inhibition of organification. This is an autoregulatory defense mechanism known as the Wolff–Chaikoff effect. Eventually, an escape phenomenon ensues and thyroid hormone synthesis resumes. The protection mechanism appears to be defective in autonomous thyroid tissue, and excess iodine or even simple dietary supplementation may precipitate hyperthyroidism in patients with a preexisting thyroid disorder through the "jodbasedow" phenomenon. Most of these disorders

Table 2 Thyroid disorders predisposing to iodine-induced thyrotoxicosis

- Autonomous or pretoxic thyroid adenoma
- Nontoxic, autonomous, or pretoxic multinodular goiter
- Euthyroid or "latent" Graves' disease
- Graves' disease in remission after or during antithyroid drug treatment

(Table 2) are characterized by thyroid autonomy; therefore, iodine-induced thyrotoxicosis is by far more prevalent in areas of iodine deficiency and in aged patients (Stanbury *et al.*, 1998).

Among drugs, the antiarrhythmic amiodarone deserves a special mention due to the dual mechanism by which it can cause thyrotoxicosis. One tablet of 200 mg of amiodarone contains approximately 75 mg of organic iodide and will release approximately 8 mg of free iodine, a tremendous amount when compared with the daily recommended dose of 200 μg. This amount of iodine can precipitate hyperthyroidism in predisposed patients simply through the jodbasedow phenomenon in a manner similar to that of iodine of other sources (type I amiodarone-induced thyrotoxicosis). However, amiodarone has been shown to be directly cytotoxic to thyroid follicular cell in vitro and can precipitate a form of thyrotoxicosis similar to that observed in subacute thyroiditis and due to the release of preformed hormones (type II amiodarone-induced thyrotoxicosis). The distinction between the two forms is crucial because the treatments are quite different. A mixed form of amiodarone-induced thyrotoxicosis may occur (Martino *et al.*, 1984; Ross, 1998).

Thyrotoxicosis of Extrathyroidal Origin

Thyrotoxicosis Factitia

Thyrotoxicosis factitia is due to the voluntary surreptitious ingestion of excess thyroid hormone preparations with the purpose of mimicking thyrotoxicosis. It should be distinguished from iatrogenic thyrotoxicosis, which is caused by excessive doses of thyroid hormones administered by the physician or inadvertently taken by the patient. However, the term has been widely applied to all forms of thyrotoxicosis due to the ingestion of thyroid hormone (Mariotti *et al.*, 1982). True thyrotoxicosis factitia is most often observed in women with psychiatric disturbances. Very often, thyroid hormone is taken to reduce weight or to receive medical attention. Denial of thyroid hormone consumption may be extreme in these patients, and the diagnosis is rarely obtained at history taking. Sometimes, thyroid hormone is inadvertently taken as a component of "herbal" or "alternative" medications, usually for weight-reducing purposes. Finally, accidental grinding of cattle thyroids in hamburger meat has been reported to be the cause of an outbreak of thyrotoxicosis among hamburger consumers in the United States.

Struma Ovarii

Struma ovarii is a rare teratoma of the ovary that may contain functional thyroid follicular tissue, among others. Struma ovarii causes overt thyrotoxicosis only rarely, depending on the amount of follicular tissue present in the neoplasia. Because complete TSH suppression occurs in struma ovarii, the neoplastic thyroid tissue is assumed to be functionally autonomous to cause thyrotoxicosis. A suspicion of the struma ovarii can be confirmed at the time of RAIU by scanning the pelvic area with the probe. The presence of functional thyroid tissue can be demonstrated by the finding of significantly increased uptake of iodine in the ovarian region. Computed tomography or ultrasound scan will confirm the presence of an ovarian mass (De Leo *et al.*, 2016; Ross *et al.*, 2016).

Functional Metastatic Thyroid Cancer

Differentiated thyroid cancer, even when metastatic and with large tumor burdens, rarely produces physiologically relevant amounts of thyroid hormone. On the other hand, thyroid follicular cancers with extensive bone metastases may cause thyrotoxicosis (Ross *et al.*, 2016).

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Thyrotoxicosis; Diagnosis☆

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Glossary

Thyroglobulin A large thyroid glycoprotein molecule that functions in the storage of iodine and from which the thyroid hormones thyroxine and triiodothyronine are formed.

Thyroid radioiodine uptake (RAIU) A functional thyroid test, based on the use of radioactive isotopes of iodide, that is useful in the determination of the etiological diagnosis of thyrotoxicosis.

Thyroid 99m-pertechnetate scintigraphy A functional thyroid test, based on the use of radioactive isotopes of iodide, that is useful in the determination of the etiological diagnosis of thyrotoxicosis.

Thyroid ultrasound A harmless and useful technique for examining the thyroid gland. It uses high-frequency sound waves that are emitted and received by a transducer (a handheld instrument) and penetrate the body. By electronic conversion, the sound waves are arranged into the picture image seen on a screen. It allows the presence of thyroid nodules to be determined and is helpful in the diagnosis of autoimmune diseases of the thyroid gland.

Thyroxine (T4) and triiodothyronine (T3) Hormones secreted by the thyroid gland. They are essential for the function and metabolism of every cell in the body.

Diagnosis of Thyrotoxicosis

Thyrotropin Measurements

Measurement of serum thyrotropin (TSH) by immunometric assay is probably the single most useful test for confirming the presence of thyrotoxicosis. The assays of the past generation are very sensitive (detection limit 0.001 mU/L) and can measure TSH concentrations well below the normal range. Because pituitary TSH secretion is tightly downregulated by thyroid hormone level, TSH is undetectable in most cases of thyrotoxicosis. The only remarkable exception is TSH-secreting adenomas, in which high or inappropriately normal TSH levels are found in spite of overt thyrotoxicosis. A subclinical thyrotoxic status can be found when TSH levels are detectable but low (<0.4 mU/L). Low or undetectable TSH levels can also be observed in a number of conditions not associated with thyrotoxicosis (**Table 1**).Very rarely, the presence in the serum of anti-mouse immunoglobulin antibodies may interfere with the TSH assay, causing falsely elevated TSH levels (Braverman, 2000; Ross *et al.*, 2016).

Thyroid Hormone Measurements

Low serum TSH concentrations alone are not sufficient to obtain a quantitative measure of the severity of thyrotoxicosis. The estimate measurement of circulating thyroid hormone levels is mandatory in all patients being evaluated for the disorder. Only the very small amount of nonprotein-bound or free thyroxine (FT4) and free triiodothyronine (FT3) in the blood can enter the cell and interact with its specific receptor. Estimation of total T4 (tT4) and total T3 (tT3) can be easily measured in blood, but because of the large variations of the binding protein concentrations in healthy individuals, this might not parallel the free thyroid hormone concentrations. This makes the estimate value of tT4 and tT3 measurements somewhat less useful in the evaluation of thyrotoxicosis. Free thyroid hormone estimate level measurements, although not completely exempt from flaws, are more satisfactory. In most iodine-sufficient countries, a single FT4 estimate measurement is sufficient to confirm or reject the suspicion of thyrotoxicosis. In contrast, in iodine-deficient countries, a significant proportion of hyperthyroid patients (up to 12%) may have normal estimate FT4 levels with elevated FT3 levels (T3toxicosis). Conversely, FT4 estimate can be falsely elevated in conditions causing reduced peripheral conversion of T4 to T3 such as amiodarone or high-dose propranolol treatment. We prefer to assess both FT4 andFT3 levels together with TSH to obtain a complete baseline panel of the thyroid function status in every patient where the diagnosis of thyrotoxicosis is suspected. A condition characterized by low or undetectable TSH level and normal free thyroid hormone levels, detected occasionally at routine thyroid function testing or in patients complaining of mild thyrotoxic symptoms, is termed "subclinical thyrotoxicosis" (Bartalena *et al.* 1996, Ross *et al.* 2016).

☆*Change History*: March 2018. Marcocci and Rocchi updated Differential Diagnosis section, Table 2, as well as References.

This article is an update of Claudio Marcocci and Roberto Rocchi, Thyrotoxicosis: Diagnosis, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 543-550.

Table 1 Causes of low serum TSH levels in the absence of thyrotoxicosis

Nonthyroidal chronic or acute illness
Starvation and malnutrition, anorexia nervosa
Hypopituitarism
Hypercortisolism
Major depression
Anorexia nervosa
Early pregnancy
Drugs
Dopamine agonists
Somatostatin
Glucocorticoids
Triiodoacetic acid

Differential Diagnosis of Thyrotoxicosis: Laboratory and Instrumental Investigations

History and physical examination are usually sufficient to identify the cause of thyrotoxicosis. However, in some cases, a careful differential diagnosis is needed to establish an etiological diagnosis. This is necessary to plan the correct treatment. For many years, radioiodine uptake (RAIU) has representing a mainstay of the differential diagnoses of thyrotoxicosis. RAIU performed by administering a minimal (tracer) dose of radioactive iodine and then measuring the percentage of administered radioactivity accumulated in the thyroid. Thyroid 99m-pertechnetate scintigraphy is another in vivo imaging technique that gives informations on the etiology of the thyrotoxicosis and in most cases it can replace RAIU reducing the radiation hazard. Actually 99mTc scintigraphy is more environmentally appropriate. Thyroid 99m-pertechnetate scintigraphy represents an indirect method of estimating the thyroidal intracellular mechanisms for iodine trapping. Whenever excessive active formation of thyroid hormone takes place in the thyroid gland, a brighter scan of the thyroid gland is obtained. Therefore, a bright 99mTc scintigraphy identifies true hyperthyroidism (e.g., with thyroid hyperfunction). In contrast, thyrotoxicosis with a feeble or absent scan of the thyroid gland indicates either thyroidal destruction, with release of preformed hormone, or an extrathyroidal source of thyroid hormone. In thyroid destruction, the damaged follicular cell loses its capability of iodine trapping (Mariotti *et al.*, 1982). Conversely, when exogenous hormones are administered in excess, the suppression of the pituitary secretion of TSH causes a block of the trapping capacity of follicular cells. (De Leo *et al.*, 2016; Bartalena *et al.*, 2016).

Graves' Disease

Anti-TSH Receptor Antibodies

A mainstay in the diagnosis of Graves' disease resides in serum detection of TSH receptor antibodies(TRABs). TRABs can be measured by different methods. TRABs were originally detected with in vivo bioassays, but this method has been replaced by in vitro systems. In the clinical practice, the detection of TRABs has, nowadays, become a routine test in presence of a thyrotoxicosis of unknown origin. The most commonly used assay is based on the displacement of radiolabeled TSH from its receptor by the patients' sera. Antibodies detected with this method have been termed "TSH binding-inhibiting immunoglobulins" (TBIIs). This test does not provide any information on the biological activity of the detected antibodies and, therefore, cannot distinguish between antibodies with stimulating activity and those with blocking activity that can also be detected in thyroid autoimmune disorders. The stimulating activity of TRAB scan be tested by employing cellular systems carrying a functional TSH receptor (e.g., Chinese hamster ovary cells transfected with cloned human TSH receptor) and detecting the release of cyclic AMP (cAMP)in the culture medium on challenge with serum or purified immunoglobulins (Chiovato *et al.*, 1994). In a modification of the assay, antibodies with blocking activity can be detected as well. Antibodies stimulating the thyroid activity are the cause of hyperthyroidism in Graves' disease (Burch, 1993). The assay is quite expensive and requires cell culture capabilities, making it available only to research centers. For clinical purposes, the TBII assay is most frequently used. By past-generation assays, positive TBII tests are found in 75%–95% of patients, with a high specificity (99%). Therefore, the TBII test represents an excellent tool in the diagnosis of Graves' disease. In spite of its efficiency, a TBII test is needed only in the minority of cases where the clinical picture is unclear, for example, in the differential diagnosis of hyperemesis gravidarum, in the nodular variants of Graves' disease that must be differentiated from toxic nodular goiter, and in patients with exophthalmos without thyrotoxicosis (euthyroid Graves' disease) (Ross *et al.* 2016). Therefore, the TBII test should be considered a second-line test in the diagnosis of Graves' disease. The finding of TBIIs after long-term treatment with antithyroid drugs may be useful to identify patients prone to recurrence of hyperthyroidism. Recently, an in vitro antibodies stimulating the thyroid activity bioassay based on a Chinese hamster ovary cell-line that express chimeric TSHr has been developed by Kahaly and coworkers (Li *et al.*, 2013). The bioassay

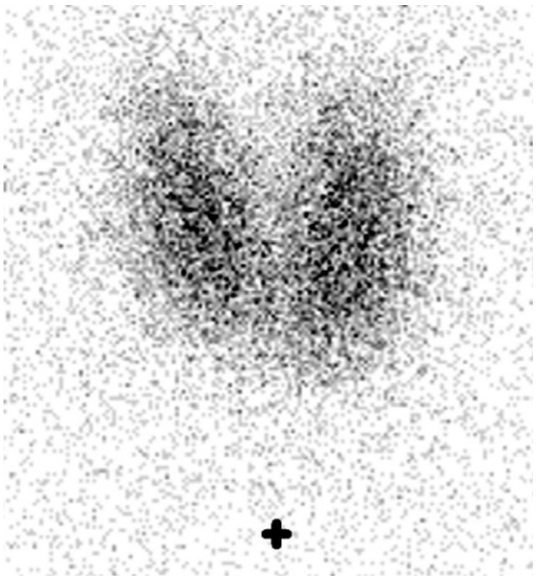

Fig. 1 Thyroid scanning (performed with radioiodine) in Graves' disease. It should be underlined that both radioiodine and 99m-pertechnetate can be taken up by the thyroid cell, but the only radioiodine can be organificated, scan images of thyroid nodules can be different.

detects both antibodies with stimulating activity and those with blocking activity and measures the net activity of a mixture of both types of antibodies. The antibodies stimulating the thyroid activity bioassay is approximately 20-fold more sensitive than commercially available TBII assay.

Antithyroid Peroxidase and Antithyroglobulin Antibodies

Antithyroid peroxidase (AbTPO) antibodies can be found by commercial radioimmunoassays in up to 90% of patients with untreated Graves' disease, whereas antithyroglobulin (AbTG) antibodies are less frequently positive (in about 50%–80% of cases). However, both antibodies are also present in other forms of thyroid autoimmune disorders, some of which may cause thyrotoxicosis, such as postpartum thyroiditis and silent thyroiditis. A relatively high percentage (up to 25%) of positive tests is also found in normal individuals, especially women. Thus, anti-TPO and anti-TG tests do not establish the diagnosis of Graves' disease as the cause of thyrotoxicosis but may be useful as complementary tests in confirming the presence of thyroid autoimmunity (Mariotti *et al.* 1990).

Thyroid Scan and RAIU

99m-pertechnetate is a radioactive tracer with 4–6 h of half-life, that can be administered i.v. and electively trapped by the thyroid gland and therefore used to obtain a true thyroid scan. Thyroid scintigraphy based on 99m-pertechnetate administration is an easy and safe exam, that is helpful in identifying the etiology of the hyperthyroidism In particular cases a RAIU can be still performed. A high value of 24th hour RAIU is always found as a distinctive feature in untreated hyperthyroid Graves' disease patients. In some cases, the 3rd- or 6th-hour value can be even higher than the 24th-hour value, as an expression of an extremely high iodine turnover. The test is useful for ruling out transient thyrotoxicosis due to hashitoxicosis or painless or subacute thyroiditis, factitious thyrotoxicosis, and type II amiodarone-induced thyrotoxicosis. Thyroid scanning in Graves' disease is useful when coexisting nodules are detected by palpation and their functional status needs to be evaluated. Thyroid imaging can be performed with radioiodine (131-I or 123-I) at the time of RAIU or by using 99m-pertechnetate (**Fig. 1**). 131-I has a higher half-life (8 days) if compared with 123-I (12 hours), but it is cheaper than 123-I. Due to its shorter half-life, the use of 123-I is indicated in young people (Martino *et al.*, 1988; Hegedus, 2004; Smith and Hegedüs, 2016; Siegel, 1998).

Thyroid Ultrasound

The thyroid gland in Graves' disease hyperthyroidism has a typical ultrasound pattern. Because of both the reduction in the colloid content and the lymphocytic infiltrate, the gland becomes diffusely hypoechoic. A similar pattern is also observed in goitrous autoimmune thyroiditis. Therefore, thyroid ultrasound can be useful for confirming the suspicion of thyroid autoimmunity during the evaluation of thyrotoxicosis. Moreover, thyroid ultrasound scanning allows an accurate measurement of the goiter size. This information is important in choosing the most appropriate treatment. Finally, thyroid ultrasound accurately distinguishes true thyroid nodules from the lobulations that can be felt occasionally at palpation in Graves' disease glands. Therefore, the

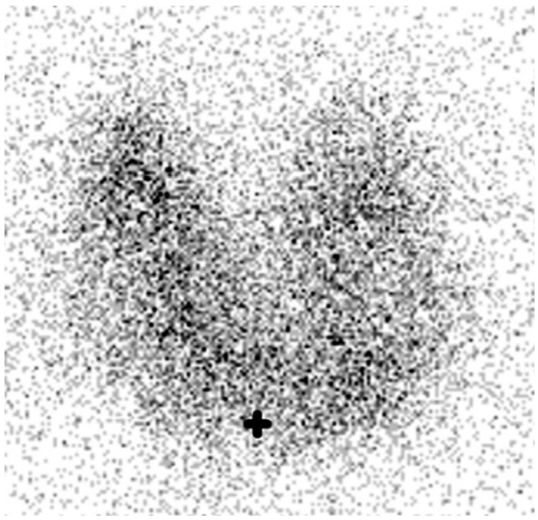

Fig. 2 Thyroid scanning (performed with 99m-pertechnetate) in toxic multinodular goiter. Thyroid ultrasound scan is also useful in measuring the size of the goiter. Moreover, relative to radionuclide scanning images, it is helpful in identifying cold nodules. Fine needle biopsy should be performed in any palpable dominant nodule that is cold at scan.

information provided by thyroid ultrasound is quite useful in the initial evaluation of Graves' disease patients, although it is not strictly needed from a diagnostic standpoint (Marcocci *et al.* 1991, Smith and Hegedüs 2016).

The measurement of blood flow to the thyroid gland by color flow Doppler ultrasound has also been used experimentally in Graves' disease patients. In untreated Graves' disease, the color Doppler pattern is characterized by markedly increased signals with a patchy distribution. In the setting of a hypoechoic pattern at ultrasound, the detection of an increased blood flow allows the distinction from Hashimoto's thyroiditis. Therefore, color Doppler studies of the thyroid gland can be useful, as is 99mTc scan, indistinguishing Graves' disease from other forms of thyrotoxicosis such as amiodarone-induced destructive thyrotoxicosis, subacute thyroiditis, and painless thyroiditis (in which the blood flow is reduced or absent) (Ross *et al.*, 2016).

Toxic Adenoma

The presence of a toxic adenoma must always be suspected in a thyrotoxic patient with a single thyroid nodule revealed at neck palpation. In confirming the diagnosis of thyrotoxicosis, it is important to measure both FT4 and FT3 levels because T3 toxicosis is distinctly frequent in toxic adenomas. At 99 m'technetium or radioiodine thyroid scanning, the nodule will appear "warm" when only partial autonomy is present, with the extranodular thyroid tissue clearly visible. In this case, parallel thyroid function tests will show a low but detectable TSH and thyroid hormone levels that are normal or at the upper limit of the normal range. When TSH is completely suppressed (e.g., complete autonomy, overt thyrotoxicosis), the scan will show only the autonomous nodule with complete suppression of the extranodular tissue. Ultrasound scanning of the neck provides no direct diagnostic information on the functional property of the nodule, but it is useful in detecting coexisting nodules (which can eventually be cold at scan) and accurately defines the size of the nodule. Preliminary reports have shown a distinctive color Doppler pattern in autonomously functioning thyroid nodules, characterized by an increased blood flow in the nodular tissue, in good correlation with radionuclide scans. This technique is not able to distinguish benign nodules from malignant ones; therefore, it is of limited value. Fine needle aspiration biopsy is not useful in the evaluation of a thyroid hot nodule and provides inconclusive findings that often show undetermined follicular neoplasm. The risk of malignancy in hot nodules is extremely low, although they are occasionally reported. Therefore, in the presence of a low TSH, fine needle aspiration is needed only when coexisting nodules detected by palpation or ultrasound are cold at radionuclide scanning. Further imaging, such as neck X rays, barium swallow, and computed tomography scans, may be needed in selected patients with large nodules to evaluate the presence of significant tracheal and/or esophageal compression. It is important to remember that computer tomography scan, when done with this purpose, should always be performed without the administration of iodinated contrast media because these may worsen thyrotoxicosis or precipitate it in partially autonomous nodules (Siegel, 1998; Ross *et al.*, 2016).

Toxic Multinodular Goiter

The same alterations of thyroid function tests described in toxic adenomas can be observed in toxic multinodular goiter. Physical findings and history are often sufficient to suspect the presence of a toxic multinodular goiter. Thyroid radionuclide scanning is

Table 2 Laboratory investigations in the differential diagnosis of the syndrome of inappropriate Secretion of TSH

Test	*TSH-secreting adenomas*	*Resistance to thyroid hormone*	*Comment*
Peripheral markers of thyroid hormone action	High	Normal to high	Non specific
Alpha-subunit/TSH molar ration	>1	1	High during menopause
TSH after T3 suppression test	Unchanged or slightly reduced	Frankly reduced or suppressed	Hazardous in elderly and cardiac patients
TSH after TRH	Unchanged	Increased	
Thyroid hormones after 3 months of somatostatin analogs treatment	Reduced	Unchanged	
Pituitary imaging	Positive	Negative	Confirmatory

quite useful for identifying and mapping autonomous nodules and cold nodules that are often coexistent (**Fig. 2**). Scanning is also useful as an adjunct to TRAB measurement in distinguishing true toxic multinodular goiter from Graves' disease hyperthyroidism over imposed on a preexisting nontoxic multinodular goiter. RAIU is always elevated, unless iodine overload is present, but is not always necessary to establish the diagnosis. Thyroid 99m-pertechnetate scan can show an irregular distribution of the tracer with one or more "warm" nodules with suppression of the extranodular thyroid tissue (Siegel 1998, Tonacchera *et al.* 1998).

TSH-Secreting Adenomas

Patients with TSH-secreting adenomas present a detectable TSH in the presence of clearly elevated circulating thyroid hormone levels (inappropriate secretion of TSH). · The first step in the evaluation of inappropriate secretion of TSH is making sure that interferences in the measurement of TSH (by heterophilic antibodies) or thyroid hormone levels (by thyroid hormone antibodies) are not the cause of the laboratory findings. Once these interferences are ruled out, further investigations are required to differentiate patients with TSH-secreting pituitary adenomas from those with resistance to thyroid hormone. TSH-secreting adenomas abnormally secrete the α-subunit of TSH in molar excess with respect to TSH. A serum α-subunit/TSH ratio greater than one is observed in approximately 90% of patients with TSH secreting adenomas. High ratios can also be observed in postmenopausal women and even in normal individuals, making this test alone unable to establish the diagnosis (Faglia *et al.* 1987, Ferraz 2017). Growth hormone (GH), insulin-like growth factor-1 (IGF-1), and prolactin serum measurements are useful because approximately 30% of TSH-secreting adenomas cosecrete these hormones. Dynamic tests may be useful for demonstrating the unresponsiveness of TSH in patients with TSH secreting adenomas. In most TSH-secreting tumors (92%), the TSH level fails to increase in response to a standard TRH stimulation test, whereas a normal or increased response is observed in resistance to thyroid hormone. It can be useful to investigate the response of pituitary TSH to exogenous T3. T3 is administered orally, and the dose is increased every 3 days (up to 200 μg daily). Before every increase, basal and TRH-stimulated TSH levels are measured together with peripheral markers of thyroid hormone action. Only partial or no suppression of TSH secretion is observed in TSH-secreting adenomas, whereas complete suppression is observed in resistance to thyroid hormone. The test is contraindicated in elderly patients and in patients with arrhythmias and/or coronary artery disease. Available tests in the differential diagnosis of the syndrome of inappropriate secretion of TSH are described in **Table 2** (McDermott and Ridgway 1998).

Pituitary imaging is very important in confirming the diagnosis. Fully 90% of TSH-secreting adenomas are larger than 1 cm at diagnosis and, therefore, are easily detected at pituitary magnetic resonance imaging (MRI) scanning. As a complement, radiolabeled octreotide pituitary scintigraphy can be used and may be particularly useful for detecting small tumors (Tjörnstrand and Nyström, 2017).

Human Chorionic Gonadotropin-Dependent Thyrotoxicosis

The diagnosis of thyrotoxicosis during hyperemesis gravidarum can be particularly difficult. Because of weight loss and malnutrition, FT3 levels may be disproportionately low or even normal in comparison with FT4 levels due to a reduced peripheral conversion of T4 to T3. The TSH level is often low during early normal pregnancy, but it is seldom undetectable as it is in true thyrotoxicosis. The only distinctive laboratory feature is an inappropriately high human chorionic gonadotropin (hCG) level, but a large overlap with normal pregnancies exists. Therefore, the diagnosis of thyrotoxicosis in hyperemesis gravidarum relies mainly on the clinical picture and on appropriate exclusion of other more common forms of hyperthyroidism by specific testing. It is important to remember that RAIU, as well as any other in vivo radioisotopic procedure, is absolutely contraindicated during pregnancy. The presence of a trophoblastic tumor should be suspected when thyrotoxicosis is diagnosed in an amenorrheic woman, especially when a palpable abdominal mass is present. The diagnosis is readily confirmed by the finding of extremely high circulating hCG levels and a pelvic mass at ultrasonography. Given the extreme rarity of the disorder, no diagnostic guidelines are

Table 3 Common sources of iodine contamination

Foods
Seaweed and seaweed containing foods (Japanese cuisine)
Food supplements
Kelp and other seaweed derivatives
Vitamin supplements
Radiological contrast agents
Intravenous and oral (e.g., Gastrografin, Renografin, iopanoic acid, sodium ipodate)
Antiseptics
Betadine
Iodoform gauze
Drugs
Amiodarone
Expectorants
Iodine solutions
Lugol's solution, SSKI (saturated solution of potassium iodide), KI (potassium iodide)

available for familial gestational hyperthyroidism. The diagnosis can be suspected only on a history of recurrent pregnancy-associated hyperthyroidism and can be confirmed only by the demonstration of a mutation of the TSH receptor gene, available in very few research laboratories worldwide (Ross *et al.* 2016, Masiukiewicz and Burrow 1999).

Fetal and Neonatal Hyperthyroidism

Mothers with a past or current history of Graves' disease should be carefully monitored throughout pregnancy. The persistence of high TRAB levels in the maternal serum by the end of pregnancy, when the transplacental passage is maximal, is a predictor of hyperthyroidism in the neonate. Fetuses of mothers with Graves' disease who have been previously treated with radioiodine or surgery may be at higher risk because they lack the protective effect of antithyroid drugs administered to the mothers. The presence of a fetal heart rate >160 beats per minute, in the absence of other fetal abnormalities, is suggestive of fetal hyperthyroidism. It is very useful to test neonatal cord blood at the time of delivery for thyroid function tests and TRABs. When the mother has been treated with high-dose antithyroid drugs, the neonate should be retested 10 days after birth given that transplacental passage of methimazole or propylthiouracil may initially mask hyperthyroidism. A TRAB-negative neonatal hyperthyroidism, in the absence of a maternal history of Graves' disease, should direct diagnosis to the suspicion of nonautoimmune congenital hyperthyroidism. Nowadays, the diagnosis can be confirmed only by sequencing of the TSH receptor gene (Pearce, 2015; Zimmerman, 1999).

Iodine-Induced Thyrotoxicosis

Iodine urinary excretion in patients with thyrotoxicosis due to excessive iodine consumption is always high, and excessive iodine consumption should always be suspected when hyperthyroidism appears abruptly in patients with a history of nodular thyroid disease. A careful history often identifies the source of iodine, and all patients should be asked about recent exposure to any of the compounds listed in **Table 3**. With the exception of type II amiodarone-induced thyrotoxicosis, RAIU is usually low in thyrotoxic patients with heavy iodine contamination, but it is almost never $<1\%$, a feature that allows distinction from subacute and painless thyroiditis. Thyroid 99m-pertechnetate scintigraphy usually does not give image of the gland (Ross, 1998; Martino *et al.*, 1984).

Amiodarone-Induced Thyrotoxicosis

In the presence of amiodarone-induced thyrotoxicosis, further testing is required to distinguish between type I (nondestructive) and type II (destructive) forms given that treatments may be radically different. Because of the concomitant underlying presence of thyroid disease, such as Graves' disease or nodular thyroid disease, type I amiodarone-induced thyrotoxicosis differs somewhat from other forms of iodine-induced thyrotoxicosis. It usually can be diagnosed with appropriate tools. Accordingly, RAIU is usually low but by definition never $<1\%$. In contrast, in type II amiodarone-induced thyrotoxicosis, RAIU is always $<1\%$ and often no clear underlying thyroid disorder can be identified. In both cases, thyroid 99m-pertechnetate scintigraphy, generally, does not give image of the gland. High circulating interleukin-6 levels have been proposed as a useful marker of thyroid tissue destruction, and color flow Doppler ultrasound imaging shows a distinctive absence of vascularization in the gland (Martino *et al.*, 1984, 1988).

Subacute, Painless, and Postpartum Thyroiditis

Subacute, painless, and postpartum thyroiditis are classically characterized by a feeble 99mTc scan during the thyrotoxic phase. This test alone, in the presence of a suggestive clinical presentation, allows the diagnosis in nearly all cases. High-titer anti-TG and anti-TPO antibodies are usually found in postpartum and painless thyroiditis as a marker of prominent thyroid autoimmunity, whereas only weakly and transiently positive tests are occasionally found in subacute thyroiditis. A very high ($>$ 50 mm/h) erythrocyte sedimentation rate is an additional distinctive diagnostic feature in subacute thyroiditis. Other inflammation indexes may be high in subacute thyroiditis, and a mild leukocytosis is often observed. As in other thyroidal destructive processes, interleukin-6 levels are elevated in all three disorders and, therefore, have no differential values. Ultrasound findings are generally characterized by patchy areas of hypoechogenicity in subacute thyroiditis, whereas a more diffuse hypoechoic pattern, closely resembling Hashimoto's thyroiditis, is found in postpartum and painless thyroiditis. The color flow Doppler pattern shows reduced vascularity in all three disorders. Occasionally, and especially when patients are first seen during the recovery or hypothyroid phase, a more subtle picture can emerge from testing, with a low but not nil RAIU (as well as with thyroid 99m-pertechnetate tracer) and with only mild elevations of the erythrocyte sedimentation rate, making the differential diagnosis more difficult (Woolf 1980, Masiukiewicz and Burrow 1999).

Thyrotoxicosis of Extrathyroidal Origin

An extrathyroidal source of thyroid hormone should always be suspected when more frequent causes of low RAIU thyrotoxicosis have been ruled out. When thyrotoxicosis factitia is suspected, a serum thyroglobulin measurement can be extremely useful in confirming the diagnosis because this disorder represents the only condition (TgAb in which thyrotoxicosis is associated with an undetectable thyroglobulin level. Indeed, the presence of TgAb may cause falsely low thyroglobulin levels. Given the high prevalence of thyroid nodules in the general population, especially in iodine deficient areas, it is also useful to perform ultrasound scanning of the neck because, in the presence of thyroid nodules, thyroglobulin may be elevated in spite of the assumption of exogenous thyroid hormone (Ross *et al.* 2016).

The suspicion of struma ovarii can be confirmed at the time of RAIU by simply scanning the pelvic area with the probe. The presence of functional thyroid tissue is demonstrated by the presence of significantly increased uptake of iodine in the ovarian region. Further imaging (computed tomography or ultrasound scan)will confirm the presence of an ovarian mass. When the source of thyroid hormone is metastatic thyroid follicular cancer, the presence of the latter is usually evident from the history. Because all patients with differentiated thyroid cancer after thyroidectomy take L-T4 in TSH-suppressive doses, thyroid function tests should be repeated after tapering the medication to rule out iatrogenic thyrotoxicosis. Confirmation is obtained with whole body radioiodine scanning that will show multiple foci of uptake in several skeletal regions (Ross *et al.*, 2016).

See also: Thyroid Function Tests. Thyroid Imaging. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyrotoxicosis; Overview of Causes

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Thyrotoxicosis; Systemic Manifestations☆

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Glossary

Arrhythmia Irregularity of the heartbeat.
Dyspnea A subjective difficulty in breathing, usually caused by heart or lung disease.
Goiter An enlargement of the thyroid gland.
Hormone A chemical compound synthesized in one organ and carried in the blood to another organ.
Hyperphagia Condition of overeating.
Polydipsia Excessive and prolonged thirst.
Polyuria Excessive excretion of urine.
Tachycardia Rapid beating of the heart, with a rate of >100 beats per minute.
Vitiligo The appearance of nonpigmented white patches bordered by hyperpigmented areas on otherwise normal skin; the skin lesions are of variable size and often symmetrical.

Introduction

The clinical features of thyrotoxicosis depend on the severity and duration of the disease, the age of the patient, the presence or absence of extrathyroidal manifestations, and the specific disorder producing the thyrotoxicosis. Older patients tend to show fewer findings, and the rare patient with "apathetic" hyperthyroidism will lack nearly all of the usual clinical manifestations of thyrotoxicosis.

Peripheral Manifestations

Nearly all organ systems in the body are affected by thyroid hormone excess (Trzepacz *et al.*, 1989). The high levels of circulating thyroid hormones are responsible for most of the systemic effects observed in these patients (**Tables 1** and **2**).

Skin and Appendages

Cutaneous manifestations are nearly always present when hypermetabolism is significant (Jabbour, 2003). The patient feels hot and prefers a cold environment. Active sweating occurs under circumstances that would provoke no response in normal persons. The hand of the thyrotoxic person is erythematous, hot, and moist in a state of hot hyperhidrosis. Occasionally, diffuse pruritus or urticaria occurs. Pigmentation may be increased and is often diffuse, or localized in areas such as the knuckles, and skin creases. Vitiligo of variable extent may also occur (Jabbour, 2003).

The hair is fine and friable, and hair loss can be excessive. Alopecia areata and loss of axillary, pubic, body, and eyebrow hair have been noted since the initial description by von Basedow but are uncommon.

Localizing nonpitting edema occurs along the shins (so-called pretibial myxedema); it can also occur elsewhere, generally on extensor surfaces (**Fig. 1**). The lesion reflects the deposition of increased amounts of glycosaminoglycans in the subcutaneous connective tissue (Herskovitz *et al.*, 2017).

Thyroid acropachy is the rarest manifestation of Graves' disease (**Fig. 2**). Nearly all reported patients have had localized myxedema as well as hyperthyroidism and ophthalmopathy.

The nails become shiny and may be soft and friable. In many patients, the nails are separated prematurely from the nail beds (onycholysis). Onycholysis is not specific to thyrotoxicosis, but when it occurs in this setting, it usually begins under the distal central portion of the fourth fingernail.

Eyes

Retraction of the upper eyelid, evident as the presence of a rim of sclera between the lid and the limbus, is frequent in all forms of thyrotoxicosis and is responsible for the bright-eyed "stare" or "fish eyes" of the patient with thyrotoxicosis (**Fig. 3**). Lid lag is caused by the

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Table 1 Systemic effects of thyrotoxicosis

System	*Effects*
General	Weight loss, fatigue, heat intolerance, insomnia, nervousness, tremulousness
Skin	Fine, warm, and moist; hyperpigmentation; hyperhidrosis; onycholysis; fine and friable hair; urticaria
Eye	Exophthalmos, lid edema, lid lag, globe lag, chemosis, ophthalmoplegia, optic nerve involvement
Cardiovascular	Tachycardia, overactive heart, widened pulse pressure, bounding pulse; occasionally cardiomegaly, signs of congestive heart failure, paroxysmal tachycardia, atrial fibrillation
Gastrointestinal	Hyperphagia, increased thirst, increased frequency of stools or diarrhea, elevated liver function tests, hepatomegaly
Muscular	Tremulousness, weakness of proximal muscles, quickened and hypermetric reflexes, myopathy, muscle atrophy, periodic paralysis
Nervous	Anxiety, inability to concentrate, irritability, restlessness, lability, depression, psychiatric reactions
Respiratory	Dyspnea
Renal	Mild polyuria, polydipsia
Skeletal	Osteopenia or osteoporosis
Hematopoietic	Anemia (usually normochromic or normocytic), lymphocytosis, splenomegaly, lymphadenopathy, enlarged thymus
Endocrine	Irregular menses or amenorrhea, gynecomastia, decreased fertility
Metabolic	Elevated serum calcium, decreased serum magnesium, increased bone alkaline phosphatase, hypercalciuria

Table 2 Clinical findings in patients with Graves' hyperthyroidism and controls (percentages)

	Hyperthyroid	*Controls*
Cases	880	880
Symptoms		
Palpitations	65	13
Increased perspiration	45	7
Heat intolerance	55	8
Weight loss	61	13
Weight gain	12	21
Increased appetite	42	5
Decreased appetite	11	6
Increased number of bowel movements	22	2
Increased appetite with weight loss	24	0
Tiredness	69	41
Irritability	45	18
Nervousness	69	15
Signs		
Fine finger tremor	69	6
Pulse rate $\geq$ 90 beats/min	19	78
Atrial fibrillation[a]	3	–
Thyroid size ($\times$ normal)	1.9 $\pm$ 0.6	1.3 $\pm$ 0.4

[a]The presence of atrial fibrillation was not assessed in controls.

fact that the upper lid lags behind the globe when the patient is asked to gaze downward; globe lag occurs when the globe lags behind the upper lid when the patient gazes slowly upward. These ocular manifestations appear to be the result of increased adrenergic activity. It is important to differentiate these ocular manifestations from those of infiltrative ophthalmopathy characteristic of Graves' disease.

Cardiovascular System

The vascular manifestations of thyrotoxicosis constitute some of the most profound and characteristic symptoms and signs of the disorder (Klein and Danzi, 2007). Tissue blood flow is increased in response to accelerated metabolism and increased oxygen consumption. Hemodynamic changes in thyrotoxic patients are characterized by an elevated cardiac output and a decreased peripheral vascular resistance. The mechanism responsible for the reduced vascular resistance is unclear. Thyroid hormone itself may be involved directly through its action on smooth muscles of blood vessels. Moreover, the finding in thyrotoxic patients of elevated levels of plasma adrenomedullin and proadrenomedullin-N-terminal 20-peptide, which have a potent vasodilatory activity, raises the possibility that these substances might also be involved in the decrease of vascular resistance in these patients.

Tachycardia is nearly always present, even at rest. The heart rate is also elevated during sleep; this helps to distinguish tachycardia of thyrotoxic origin from that of psychogenic origin. The pulse pressure is widened as a result of both an increase in cardiac output and a decrease in peripheral vascular resistance. Other common cardiovascular symptoms include exercise

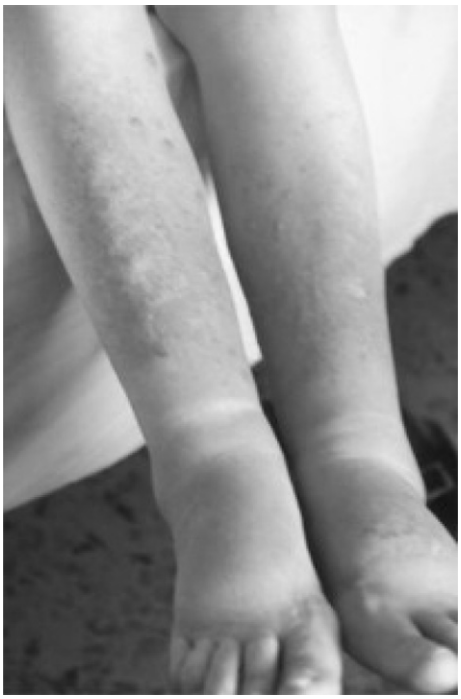

Fig. 1 Dermopathy of Graves' disease. Marked thickening of the skin is noted, usually over the pretibial area. Thickening will occasionally extend downward over the ankle and the dorsal aspect of the foot but almost never above the knee.

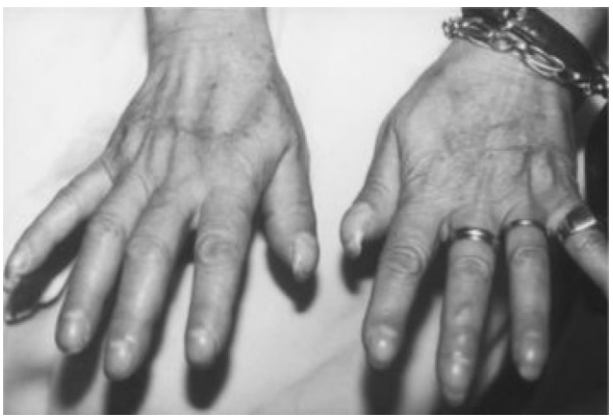

Fig. 2 Thyroid acropachy. Photograph of the hands in a patient with acropachy shows clubbing and swelling of fingertips.

intolerance and dyspnea on exertion. The latter is usually present with sustained activity but may also arise with an activity as limited as climbing a flight of stairs. Because of the diffuse and forceful nature of the apex beat, the heart may appear to be enlarged on physical examination, but echocardiography is usually normal.

In aged thyrotoxic patients, the cardiovascular manifestations may be limited to resting tachycardia. Other classic thyrotoxic symptoms may be absent, possibly due to the relative paucity of adrenergic activity.

Thyrotoxic patients may have chest pain similar to angina pectoris in nearly all respects, probably caused by either relative myocardial ischemia or coronary artery spasm. However, in older patients, the increased myocardial oxygen demand due to thyrotoxicosis may unmask coronary artery disease. The plasma level of homocysteine, an independent risk factor for cardiovascular disease, in thyrotoxic patients did not differ significantly from that in controls.

Heart sounds are loud and ringing, and a systolic murmur—or even a late diastolic or presystolic murmur—may be present at apex. Auscultation may reveal a systolic ejection murmur and a gallop rhythm caused by rapid flow of blood through the aortic outflow tract. Systolic murmurs may arise from valve prolapse, left ventricular dilatation, or dysfunction of the mitral valve apparatus (Anakwue *et al.*, 2015). Mild edema sometimes occurs in the absence of heart failure. Heart rarely occurs in thyrotoxic patients unless an underlying cardiac disease is also present.

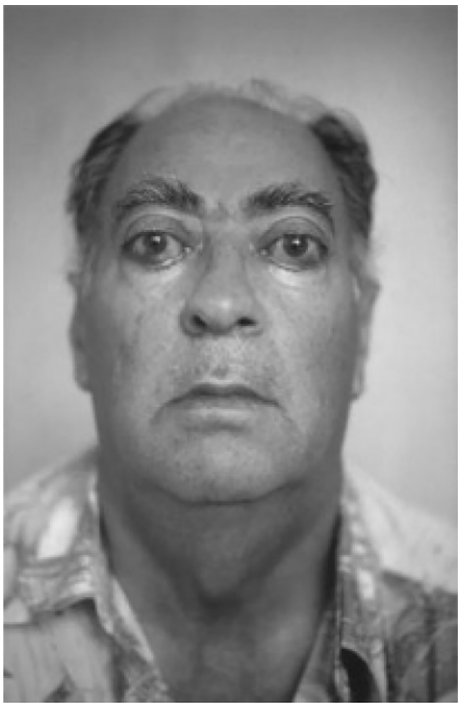

Fig. 3 Clinical presentation of Graves' ophthalmopathy. Photograph shows proptosis, marked conjunctival injection and chemosis, and retraction of lower eyelids.

Cardiac arrhythmias are common with thyrotoxicosis and are almost invariably supraventricular. Approximately 10% of patients with thyrotoxicosis have atrial fibrillation, and a similar percentage of patients with otherwise unexplained atrial fibrillation are thyrotoxic. This manifestation may be the presenting symptom of thyrotoxicosis, particularly in the elderly, and the risk of developing persistent atrial fibrillation is approximately three times that in normal individuals. Paroxysmal supraventricular tachycardia may be demonstrable or may be suggested by the history. Ventricular premature contractions are rare. Angina pectoris and myocardial infarction may occur rarely in the absence of coronary artery disease.

Nonspecific electrocardiographic changes may occur in thyrotoxicosis. A shortening of the PR interval is common, secondary to the increased rate of conduction through the atrioventricular node.

Thyrotoxicosis alone may determine heart failure in both old patients and (much less often) young patients (Burch and Wartofsky, 1993). In large clinical studies, thyrotoxic patients with heart failure were generally old and, therefore, at risk for underlying heart disease and had chronic thyrotoxicosis. Elderly patients with rhythm disturbances, including atrial fibrillation, are at the greatest risk for heart failure (Chiovato *et al.*, 1997; Boelaert *et al.*, 2010). In the absence of atrial fibrillation, heart failure is rare. In the absence of underlying heart disease or in young patients, the heart failure is thought to be "high output." High-output heart failure might not be a true heart failure; instead, it might be a circulatory congestion caused by fluid retention. In thyrotoxicosis, cardiac output is potentially near to maximal at rest and cannot increase in response to exercise, stress, surgery, or pregnancy. As a consequence, atrial filling pressures rise, leading to pulmonary and peripheral edema. This situation may be worse if atrial fibrillation is present. Left ventricular function is impaired because the persistent tachyarrhythmia alters this function. Sustained tachycardia causes abnormal ventricular systolic and diastolic function that resolves when arrhythmia is treated. β-Adrenergic receptor blockade-mediated slowing of the heart rate can rapidly reverse even severe degrees of left ventricular dysfunction in thyrotoxic patients.

Gastrointestinal System

The classical gastrointestinal manifestations of thyrotoxicosis are rapid intestinal transit, increased frequency of semiformed stools, and weight loss from increased caloric requirement or malabsorption (Kyriacou *et al.*, 2015). These changes are not necessarily frequent. An increase in appetite, both during and between meals, is a common symptom but is usually not seen in patients with mild disease. In severe disease, the increased intake of food is usually inadequate to meet the increased caloric requirements, and weight loss occurs. Anorexia, rather than hyperphagia, sometimes accompanies severe thyrotoxicosis. It occurs in approximately one-third of elderly patients and contributes to the picture of "apathetic" thyrotoxicosis.

Frequent bowel movements are significantly more common in thyrotoxic patients than in normal controls. Diarrhea is rare. When constipation is present before the development of thyrotoxicosis, bowel function may become normal. More often, stools are less well formed and the frequency of bowel movements is increased. Gastric emptying and intestinal motility are increased,

and these changes appear to be responsible for slight malabsorption of fat. Gluten enteropathy and Graves' disease may coexist more frequently than can be accounted for by chance due to their common autoimmune origin.

Hepatic dysfunction occurs in thyrotoxicosis, particularly when the disease is severe; hypoproteinemia and increased serum alkaline phosphatase and transaminase levels may be present. In severe cases, hepatomegalia and jaundice may be found.

Nervous System

Hyperactivity, emotional lability, distractibility, and anxiety observed in thyrotoxicosis may reflect changes in the nervous system, but the pathogenetic mechanisms remain obscure (Trzepacz *et al.*, 1988). The reaction to all sorts of stimuli is distinctly excessive. Examination reveals a fine rhythmic tremor of the hands, tongue, or slightly closed eyelids. Emotional lability causes patients to lose their tempers easily and to have episodes of crying without any apparent reason. Crying may be evoked by merely questioning patients about the symptom. In rare cases, mental disturbance may be severe. Rarely, patients develop visual or auditory hallucinations or a frank psychosis. It is probable that thyrotoxicosis makes manifest an abnormality already present rather than inducing a psychosis de novo. Anxiety is characterized by restlessness, shortness of attention span, and a compulsion to be moving around despite a feeling of fatigue (Swee du *et al.*, 2015).

Fine hand tremor is a frequent finding and may sometimes mimic that of Parkinsonism, and a preexisting Parkinsonian tremor can be accentuated. Chorea seldom appears as a manifestation of thyrotoxicosis. The neurological manifestation of thyrotoxic crisis rarely includes coma and status epilepticus. Patients with a convulsive disorder may become more difficult to control with the usual medications, and seizures may appear in patients who never manifested such symptoms previously.

The electroencephalogram of most thyrotoxic patients reveals an increased fast wave activity. The basal metabolic rate tends to correlate with the frequency of brain waves, but the correlation is usually poor at the extremes of thyroid abnormality.

Muscle

Muscle weakness and fatigue are frequent (Kung, 2007). In most instances, they are not accompanied by objective evidence of local disease of muscle except for the generalized wasting associated with weight loss. Weakness is often most prominent in the proximal muscles of the limbs, causing difficulties in climbing stairs or in maintaining the leg in an extended position. In severe untreated cases, muscle wasting occasionally occurs as a predominant symptom (thyrotoxic myopathy). In extreme forms, the patient may be unable to rise from a sitting or lying position and may be virtually unable to walk.

Muscle manifestations affect men with thyrotoxicosis more commonly than they do women and may overshadow other manifestations of the syndrome. In severe forms, the myopathy involves mainly distal muscles of extremities and the muscles of the trunk and face. The involvement of ocular muscles may mimic myasthenia gravis. Graves' disease occurs in approximately 3%–5% of patients with myasthenia gravis, and approximately 1% of patients with Graves' disease develop myasthenia gravis (Kung, 2007). Myasthenia gravis associated with Graves' disease has a mild expression characterized by preferential involvement of the eye muscles. Another myopathy sometimes observed in association with thyrotoxicosis is hypokalemic periodic paralysis. It is characterized by sporadic attacks (which may last from minutes to many hours), most commonly involving flaccidity and paralysis of legs, arms, and/or trunk, although any muscle can be involved. Episodes can occur spontaneously, after carbohydrate ingestion, or after exercise. Hypokalemic periodic paralysis is most frequent in Asians.

Respiratory System

Dyspnea is present in the large majority of severe thyrotoxic patients, and several factors may contribute to this condition, including reduction of vital capacity, decreased pulmonary compliance, weakness of the respiratory muscles, and increase in respiratory dead space ventilation. In some cases, it is difficult to separate patients with pure respiratory muscle weakness from patients who have only decreased lung compliance. Manifestations of respiratory muscle dysfunction include rapid shallow respirations, respiratory dyskinesis, hypoventilation, respiratory acidosis, and easy fatigability. Most patients with overt thyrotoxicosis have diminished proximal muscle strength. Pulmonary function returns to normal when the eumetabolic state is restored (Siafakas *et al.*, 1992).

Renal System

Most of the renal effects in thyrotoxic patients produce no symptoms except mild polyuria. Renal plasma flow and glomerular filtration rate are increased in thyrotoxicosis, probably because of the increase in cardiac output and decrease in peripheral resistance (Iglesias *et al.*, 2017). Intrarenal vasodilation also occurs. The mean 24-h urine creatinine excretion is significantly lower in thyrotoxic patients than in normal individuals. The latter finding has been attributed to loss of muscle mass and occurs despite an increase in urea clearance. These changes are normalized when a normal metabolic state is restored.

Thyrotoxicosis is generally not associated with abnormalities in water metabolism. Serum electrolytes are usually normal. Some thyrotoxic patients have polydipsia, with 24-h urine volumes up to 3–4 L. Polyuria in these patients is due to increased thirst, as in primary polydipsia, and could be secondary to an increase of plasma angiotensin II concentration.

Skeletal System: Calcium and Phosphorus Metabolism

Thyrotoxicosis is associated with an increase of bone turnover and eventually bone loss, especially in postmenopausal women (Williams and Basset, 2018). Patients with a long-standing history of thyrotoxicosis may have overt osteoporosis and an increased risk of fractures (Cummings *et al.*, 1995). Bone turnover is increased, but the increase in bone resorption is relatively greater than that of bone formation, so the urinary excretion of calcium, phosphorus, and hydroxyproline is increased. As a consequence of this acceleration in bone resorption, hypercalcemia may occur in a significant proportion of patients with thyrotoxicosis. Total serum calcium may be slightly increased in up to 27% of these patients, and the ionized serum calcium level may be increased in up to 47%. The concentration of alkaline phosphatase and osteocalcin is also frequently increased. Parathyroid hormone and 1,25-dihydroxy-vitamin D3 levels tend to be low as a result of the increased calcium released from bone. Excretion of calcium in the feces is also increased in thyrotoxic patients. The secretions of the gastrointestinal tract are altered in thyrotoxicosis, and the transit time of calcium in the intestine is shortened.

Hematopoietic System

The red blood cells are usually normal, but the red blood cell mass is increased. The increase in erythropoiesis appears to be due both to a direct effect of thyroid hormones on the erythroid marrow and to an increased production of erythropoietin. A parallel increase in plasma volume also occurs; therefore, hematocrit value is normal.

The most common red blood cell morphological abnormality is microcytosis, which is found in at least 37% of patients. The cause of this change is unclear. Iron deficiency is occasionally reported in thyrotoxic states. Microcytosis usually resolves with the restoration of euthyroidism. Some patients with severe thyrotoxicosis may develop a normocytic anemia. Defective iron use has been shown to occur in thyrotoxic patients and may be responsible for the development of anemia. Approximately 3% of patients with Graves' disease have pernicious anemia, and a further 3% have antibodies to intrinsic factor but normal absorption of vitamin B12. Autoantibodies against gastric parietal cells are present in about one-third of patients with Graves' disease, and the requirements for vitamin B12 and folic acid appear to be increased.

The total white blood cell count is often low because of a decrease in the number of neutrophils. The absolute lymphocyte count is normal or increased, leading to a relative lymphocytosis. The numbers of monocytes and eosinophils may also be increased.

Blood platelets and the intrinsic clotting mechanism are normal. However, the concentration of factor VIII is often increased and returns to normal when thyrotoxicosis is treated. Furthermore, there is an enhanced sensitivity to coumarin anticoagulants because of an accelerated clearance of vitamin K-dependent clotting factors.

Endocrine System

Thyrotoxicosis affects the secretion of most pituitary hormones, in particular the secretion of growth hormone (GH), prolactin (PRL), adrenocorticotropin (ACTH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH).

Children with thyrotoxicosis grow more rapidly than do normal children. Growth acceleration in thyrotoxicosis suggests that GH secretion might be greater than normal. However, serum GH concentrations are lower in thyrotoxic patients than in normal individuals. This decrease is probably due to the increased metabolic clearance rate. Serum insulin-like growth factor-1 (IGF-1) concentration is higher in thyrotoxic patients and returns to normal values after restoration of the euthyroid state.

Thyrotoxicosis has several effects on adrenocortical function and adrenocortical hormone metabolism, with an increased clearance of the latter. The half-life of cortisol is shortened, but both the number of bursts of ACTH and the resulting burst of cortisol secretion are increased and maintain serum cortisol levels. The plasma concentration of corticosteroid-binding globulin is normal. The urinary excretion of the free cortisol and 17-hydroxycorticosteroids is normal or slightly increased, whereas the urinary excretion of 17-ketosteroids may be reduced. The rate of turnover of aldosterone is increased, but its plasma concentration is normal. Plasma renin activity is increased, and the sensitivity to angiotensin II is reduced.

β-Adrenergic receptor blockade ameliorates most of the cardiovascular manifestations of thyrotoxicosis. This suggests that catecholamines play a role in their genesis, but the secretion rate and plasma levels of epinephrine and norepinephrine are normal in thyrotoxic patients. Indeed, the apparent sympathetic hyperactivity appears to be the consequence of a direct effect of thyroid hormones on peripheral tissues.

Thyrotoxicosis during early life may cause delayed sexual maturation, although physical development is normal and skeletal growth may be accelerated.

Reproductive System

Thyrotoxicosis, after puberty, influences the reproductive function, especially in women. An increase in libido occurs in both genders. The intermenstrual interval may be prolonged or shortened, and menstrual flow initially diminishes and ultimately ceases. Fertility may be reduced. The incidence of miscarriage, premature delivery and pre-eclampsia may be increased by maternal thyrotoxicosis. High maternal thyroid hormone levels may lead to the suppression of fetal TSH, lower fetal weight and fetal death. In some women, menstrual cycles are predominantly anovulatory with oligomenorrhea; however, in most women, ovulation occurs. With treatment, menstrual cycles return to their regular pattern. Thyrotoxicosis in prepubertal girls may result in slightly delayed menarche. In premenopausal women with thyrotoxicosis, basal plasma concentrations of LH and FSH are normal but may display an enhanced responsiveness to LH-releasing hormone.

An increase in sex hormone-binding globulin is a prominent feature of thyrotoxicosis and is responsible for many of the alterations in steroid metabolism. Because of the increase in sex hormone-binding globulin, the metabolic clearance rates of testosterone and (to some extent) estradiol are decreased (Rosner, 1990; La Vignera *et al.*, 2017). Testosterone levels are elevated because of the increased concentration of sex hormone-binding globulin. Free testosterone levels tend to be normal. The metabolic clearance rate of estradiol is normal, suggesting that tissue metabolism of the hormone is increased. Conversion rates of androstenedione to testosterone, estrone, and estradiol, as well as conversion rates of testosterone to dihydrotestosterone, are increased. Extragonadal conversion of androgens to estrogens is increased, and this could be the mechanism responsible for gynecomastia observed in a consistent minority of thyrotoxic men. A reduction of sperm motility (astheno-zoospermia) has been reported in about 60% of adult thyrotoxic patients; it normalize after treatment with antithyroid drugs. Other seminal fluid alterations namely low sperm count and an increased number of spermatozoa with altered morphology has been found in about 42% and 40%, respectively. Premature ejaculation, erectile dysfunction, hypoactive sexual desire has also been reported. However, the major limitations of the studies performed so far is that semen parameters have been performed in patients from infertile couples in whom fertility of the partner has not always been studied.

Energy Metabolism: Protein, Carbohydrate, and Lipid Metabolism

One of the most prominent symptoms in the hyperthyroid patient is heat intolerance. The symptom reflects an increase in the basal metabolism of many substrates. The increase in metabolic activity results in increased consumption of adenosine triphosphate and oxygen. Despite the increased food intake, a state of chronic caloric inadequacy often ensues, depending on the degree of increased metabolism, and becomes more pronounced with age. In addition to losing fat stores, there is often a loss of muscle mass, making weakness a common complaint. Both synthesis and degradation of proteins are increased, with the latter increased to a greater extent than the former, so that there is a net decrease in tissue protein content.

The oral glucose tolerance test is often abnormal. The most common abnormality is a faster rise in plasma glucose after glucose ingestion, but some patients have a delayed peak plasma glucose or a peak value that is higher than that in normal individuals. These abnormalities may reflect changes in glucose absorption rather than metabolism given that many patients who have abnormal oral glucose tolerance have normal responses to intravenous glucose administration. Preexisting diabetes mellitus is aggravated by thyrotoxicosis, with one cause being increased degradation of insulin.

Both synthesis and clearance of cholesterol and triglycerides are increased, but the latter effect predominates, so that serum levels are generally low. Plasma phospholipid and low-density lipoprotein cholesterol concentrations fall, whereas high-density lipoprotein cholesterol levels increase. Finally, thyroid hormones may influence cholesterol metabolism by increasing its conversion to bile acid and its clearance through the membrane surface low-density lipoprotein receptors. Although fatty acid synthesis is increased in both adipose tissue and liver, degradation of most lipids appears to be stimulated out of proportion to synthesis; consequently, body lipid deposits become depleted and plasma concentrations of various lipid components fall.

Several studies have investigated the relationship between leptin level and thyroid status. With the exception of a couple of reports suggesting a relative hypoleptinemia, most clinical studies have found no effect of thyrotoxicosis on leptin levels.

See also: Graves' Orbitopathy. Hyperthyroidism in Graves' Disease. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Thyroid Disease and Bone. Thyroid Disorders in the Elderly. Thyroid Hormone Action. Thyroid Hormone Metabolism. Thyrotoxic Storm

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Thyrotoxicosis; Treatment☆

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Introduction

The treatment of thyrotoxicosis is directed to reduce overproduction of thyroid hormones by inhibiting their synthesis or release or by ablating thyroid tissue with surgery or radioiodine. Amelioration of the impact of thyroid hormones on peripheral tissues is an additional objective of treatment. There are several means of accomplishing these goals, and their efficacy depends, to some extent, on the cause of thyroid hormone hypersecretion. Because of this, the pathogenesis of hyperthyroidism should be determined by means of history, physical examination, and selected laboratory tests before making a decision on the plan of therapy. The patient should participate in the choice of treatment and must be informed of the therapeutic alternatives. This article discusses the various therapeutic options and the treatment of the three most frequent causes of hyperthyroidism: toxic diffuse goiter (Graves' disease), toxic adenoma, and toxic multinodular goiter. In addition, it briefly discusses other rare etiologies of hyperthyroidism and exogenous thyrotoxicosis.

Medical Treatment

Antithyroid Drugs (Thionamides)

The main action of thionamides (e.g., methimazole, MMI; carbimazole, propylthiouracil, PTU) is to inhibit the organification of iodide and the coupling of iodotyrosines, thereby blocking the synthesis of thyroid hormones. Because carbimazole is almost completely converted to MMI in the body, the effects of these two drugs are comparable. PTU has the additional effect of inhibiting the conversion of thyroxine (T_4) to triiodothyronine (T_3) in peripheral tissues (He *et al.*, 2004). Despite this property of PTU, in most patients, MMI therapy results in a more rapid normalization of serum T_4 and T_3. This may be due to the greater intrinsic potency of MMI and to its longer duration of action. The main pharmacological features of antithyroid drugs are summarized in **Table 1**. The half-life in plasma of MMI is approximately 6 h, whereas that of PTU is approximately 1–2 h.

Both MMI and PTU are extremely effective in controlling hyperthyroidism; therefore, in most instances, the choice between the two drugs is largely a matter of personal preference and local availability. Because these compounds do not inhibit iodide transport or block the release of stored thyroid hormones, control of hyperthyroidism is not immediate and in most cases will not occur for 2–6 weeks.

MMI should be used in virtually every patient who chooses antithyroid drugs, except during the first trimester of pregnancy when PTU is preferred, in the treatment of thyroid storm, and in patients with minor reactions to MMI who refuse RAI therapy or surgery (De Groot *et al.*, 2012; Hegedüs *et al.*, 2011; Laurberg and Andersen, 2015).

The starting dose should be targeted to the degree of thyroid dysfunction, because a too low dose will not restore an euthyroid state in patients with severe disease and an high dose can cause iatrogenic hypothyroidism if used in patients with mild disease. Most physician would start treatment with a dose of 10 mg three times daily for MMI and 200 mg three times daily for PTU (Elbers *et al.*, 2011).

The latest guidelines of the American Thyroid Association recommend a more personalized approach, suggesting that the staring dose of MMI should chosen according to the severity of hyperthyroidism: 5–10 mg if free T_4 is 1–1.5 times the upper limit of normal; 10–20 mg if free T_4 is 1.5–2 times the upper limit of normal; and 30–40 mg if free is T_4 2–3 times the upper limit of normal (Ross *et al.*, 2016). These rough guidelines should be tailored to the individual patient, incorporating additional information on symptoms, gland size, and total T_3 levels where relevant. Further treatment can be continued according to two different strategies: (1) titration mode: the daily dose of MMI and PTU should progressively be reduced according to the levels of thyroid hormones and TSH and treatment maintained for 12–18 months; (2) block and replace mode: the starting daily dose of MMI and PTU is maintained for the entire treatment period, combined with L-thyroxine to maintain euthyroid levels. However, the latest guidelines of the American Thyroid Association indicate that this therapeutic approach is not generally recommended because of the higher rate of side effect compared with the titration mode (Ross *et al.*, 2016).

Serious side effects (**Table 2**) are not common with antithyroid drugs, being observed in approximately 3 of every 1000 patients. Patients should be informed of their side effects and the necessity of informing the physician promptly if they should

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Table 1 Pharmacological features of thionamides

	Methimazole	*Propylthiouracil*
Serum half-life (hours)	4–6	1–2
Serum protein binding (percentage)	–	80–90
Metabolism of drug during illness		
Severe liver disease	Decreased	Normal
Severe kidney disease	Normal	Normal
Levels in breast milk	Higher	Low

Table 2 Side effects of thionamides

Major (<1%)
Rare
Agranulocytosis
Very rare
Severe vasculitis and Lupus-like syndrome
Cholestatic jaundice
Toxic hepatitis
Aplastic anemia
Thrombocytopenia
Minor (1–5%)
Common
Cutaneous manifestations (e.g., rash, pruritus, urticaria)
Transient granulocytopenia
Arthralgia
Less common
Fever
Gastrointestinal manifestations
Loss of taste sensation

develop pruritic rash, jaundice, acholic stools or dark urine, arthralgias, abdominal pain, nausea, fatigue, fever, or pharyngitis. Before starting the therapy and at each subsequent visit, the patient should be alerted to stop the medication immediately and call their physician if there are symptoms suggestive of agranulocytosis or hepatic injury (Kim *et al.*, 2001).

Agranulocytosis (granulocyte count <500 per cubic milliliter), the most feared problem, may be observed with both MMI and PTU, but a low dose of MMI is safer than a high dose of either drug. Agranulocytosis may develop suddenly and usually occurs during the first months of therapy. It typically presents with fever and evidence of infection. In addition to prompt discontinuation of the antithyroid drug, treatment of agranulocytosis involves the administration of broad-spectrum antibiotics and growth factors to stimulate recovery of bone marrow. Patients usually recover within 2–3 weeks, but a few deaths from this complication have been reported. Hepatitis, vasculitis, and lupus-like syndromes are rare complications but make the discontinuation of antithyroid drugs mandatory. Minor side effects are much more frequent, occurring in 1%–5% of patients. Among them, pruritus, rash, and (less commonly) urticaria are the most prominent manifestations. These side effects may resolve spontaneously despite continued therapy, or using concurrent antihistamine therapy but they generally call for substitution of one thionamide for the other, although cross-sensitivity to these drugs may occur **Table 2**.

According to the latest guidelines of the American Thyroid Association, prior to start antithyroid drugs, patients should have a baseline complete blood count, including white blood cell (WBC) count with differential, and a liver profile including bilirubin and transaminases (Ross *et al.*, 2016). There is insufficient information to recommend for or against routine monitoring of liver function tests or white blood cell counts in patients taking antithyroid drugs. Clinical judgment is required. Liver function and hepatocellular integrity should be assessed in patients taking MMI or PTU who experience pruritic rash, jaundice, light-colored stool or dark urine, joint pain, abdominal pain or bloating, anorexia, nausea, or fatigue.

β-Adrenergic Antagonist Drugs

These drugs are an integral part of the management of hyperthyroidism. Blockade of β-adrenergic receptors rapidly ameliorates some manifestations of thyrotoxicosis such as tremor, palpitation, and anxiety (Ventrella and Klein, 1994). β-Adrenergic antagonists do not affect thyroid hormone synthesis and release; therefore, they should not be used alone except for short periods before and/or after radioiodine therapy in selected patients preparing for thyroid surgery or in patients with self-limited forms of thyrotoxicosis (Tagami *et al.*, 2012). Propranolol, a nonselective beta1 and beta2-blocker, is frequently used to treat this condition.

It is highly lipid soluble, allowing it to become sufficiently concentrated in tissues to inhibit monodeiodinase activity and has a short half-life.

Since the introduction of propranolol, a number of new agents with a longer duration (e.g., atenolol, metoprolol, nadolol), or with greater cardioselectivity (e.g., atenolol, metoprolol, bisoprolol), have become available. Calcium channel blockers, such as verapamil or diltiazem can be used to reduce heart rate in patients who cannot tolerate beta blockers.

The usual contraindications of β-adrenergic antagonists, such as asthma, should be considered. Despite the initial concern, propranolol or other β-adrenergic antagonists are widely used in thyrotoxic heart disease in view of the notion that tachycardia and tachyarrhythmia are the most prominent factors involved in this condition.

Iodide and Iodine-Containing Agents

Inorganic iodide given in pharmacological doses (as Lugol's solution or as saturated solution of potassium iodide, SSKI) decreases its own transport into the thyroid, inhibits iodide organification (the Wolff–Chaikoff effect), and rapidly blocks the release of T_4 and T_3 from the gland. However, after a few days or weeks, its antithyroid action is lost and thyrotoxicosis recurs or may worsen. The usual dose of Lugol's solution is three to five drops three times daily and that of SSKI is one drop three times daily. Short-term iodide therapy is used to prepare patients for surgery, usually in combination with a thionamide drug. Iodide is also used in the management of severe thyrotoxicosis (thyroid storm) because of its ability to inhibit thyroid hormone release acutely.

Iopanoic acid and sodium ipodate, widely used in the past as oral cholecystographic agents, may be useful in the management of thyrotoxicosis (Tyer *et al.*, 2014). These compounds have a dual action: they produce a fall in the serum concentration of thyroid hormones (resulting from the block of thyroid hormone secretion due to the inorganic iodide released from the drug) and inhibit the peripheral conversion of T_4 to T_3. Some reports have confirmed the efficacy of these compounds as a primary therapy of hyperthyroidism. These agents may be particularly useful when a rapid decrease in T_3 levels is desired. Although an early escape from the therapeutic effect has been suggested, other studies have not found this to be the case for up to nearly 2 years of therapy.

Perchlorate

Perchlorate interferes with accumulation of iodide by the thyroid. In conjunction with thionamides, it has been used successfully in the treatment of amiodarone-induced hyperthyroidism. Gastric irritation and toxic reactions (aplastic anemia) limit the long-term use of perchlorate in the management of hyperthyroidism.

Glucocorticoids

Glucocorticoids in high doses inhibit the peripheral conversion of T_4 to T_3. In Graves' hyperthyroidism, glucocorticoids appear to decrease T_4 secretion by the thyroid, but the efficiency and duration of this effect are unknown. In severe hyperthyroidism, short-term glucocorticoid administration may be used as a general supportive treatment. The immunosuppressive effect of glucocorticoids in high doses is commonly exploited in the treatment of ophthalmopathy and dermopathy of Graves' disease but not in the management of uncomplicated Graves' hyperthyroidism (Marcocci and Marinò, 2012; Bartalena *et al.*, 2015, 2016).

Rituximab

Rituximab (RTX) is a human/murine chimeric monoclonal antibody which targets CD20, a transmembrane protein present on immature and mature B cells, but absent on most pro-B cell or plasma cells. It was originally used in the treatment of B cell lymphomas and subsequently RTX proved useful in the treatment of a variety of autoimmune diseases. So far, three studies have been conducted examining the effect of RTX on thyroid function in Graves' hyperthyroidism and ophthalmopathy Stan and Salvi (2017). The results of these studies suggest that RTX may prolong remission for hyperthyroidism over that seen with antithyroid drugs, at least in mild Graves' disease.

Radioiodine

Among different radioactive isotopes of iodine, ^{131}I is the agent of choice in the treatment of thyroid hyperfunction. After oral administration, radioiodine is completely absorbed, rapidly concentrated, oxidized, and organified by thyroid follicular cells. The destruction of thyroid cells produced by radioiodine results from the ionizing effects of β-particles that have a path length of 1–2 mm. Specifically, 1 μCi of ^{131}I retained per gram of thyroid tissue delivers approximately 70–90 rads. Biological effects of radioiodine include necrosis of follicular cells, shorter survival and impaired replication of nondestroyed cells, and vascular

occlusion. In the long run, there is atrophy and fibrosis, as well as a chronic inflammatory response that ultimately may result in thyroid failure.

The goal of radioiodine therapy of hyperthyroidism is to destroy sufficient thyroid tissue to cure hyperthyroidism with one dose of ^{131}I. The administered dose is calculated on the basis of thyroid size and uptake of ^{131}I using the following formula:

$$\text{Dose (mCi)} = \text{Estimated thyroid weight (g)} \times \text{Planned dose } (\mu\text{Ci/g})/\text{fractional 24 h radioiodine uptake.}$$

In other centers, standard fixed doses are given. Because of radiation safety restrictions, in some centers, especially in Germany, repeated 2- to 3-mCi doses of radioiodine are administered. It is a common experience that small glands appear to be destroyed more readily by radioiodine than do larger ones and that toxic adenomas or toxic multinodular goiters are usually more radioresistant than Graves' glands (Ma *et al.*, 2016).

The therapeutic effect of radioiodine is delayed, and in some patients up to several months may be required for the complete control of hyperthyroidism (Villagelin *et al.*, 2015). Thus, a course of antithyroid drugs before the administration of radioiodine is frequently used, particularly in cases of severe thyrotoxicosis, to avoid the inconveniences of persistent hyperthyroidism. In selected patients with significant comorbidity, antithyroid drugs may also be administered following radioiodine, but doing so may decrease the efficacy of treatment.

Radioiodine therapy may lead to worsening of Graves' ophthalmopathy when present. This worsening can be prevented by concomitant glucocorticoid therapy, beginning after radioiodine administration (Bartalena *et al.*, 2015, 2016).

In the past, the major concerns with radioiodine therapy derived from the possible carcinogenic effects of ionizing radiation and from the risks of congenital malformations in offspring of women treated during their childbearing years. No association was found between radioiodine administration and thyroid cancer in large epidemiological studies. Similarly, there is no evidence that radioiodine therapy for hyperthyroidism increases the risk of leukemia or solid tumors. No association between radioiodine treatment for hyperthyroidism and congenital abnormalities in subsequent offspring has been observed.

Thyroidectomy

Subtotal or near-total thyroidectomy is performed in Graves' disease and toxic multinodular goiter, whereas lobectomy is the procedure of choice in toxic adenoma. Restoration of euthyroidism before surgery is mandatory (Fortuny *et al.*, 2015). The classical approach combines a course of thionamide treatment to restore and maintain euthyroidism and the preoperative administration of iodide for approximately 10 days to decrease blood flow of the gland. Care must be taken not to discontinue or decrease the dose of antithyroid drugs when iodide is added. We do not favor a preoperative program based only on the use of a β-adrenergic antagonist associated with iodide. Euthyroidism is not restored in these patients because iodide alone normalizes the concentration of thyroid hormones in serum only a few days before the operation but does not produce "tissue" euthyroidism. Furthermore, the risk of thyroid storm is not completely prevented even when high-dose propranolol is administered for several days after surgery.

Possible complications of thyroid surgery include thyroid storm (which is extremely rare nowadays), bleeding, injury to the recurrent laryngeal nerve, and hypoparathyroidism. In particular, the risk of laryngeal nerve injury and hypoparathyroidism cannot be disregarded. Although an incidence of these complications of $<2\%$ is reported from clinics with wide experience in thyroid surgery, much higher figures of up to 10%–15% are encountered in some series. These complications are, of course, less frequent when lobectomy for toxic adenoma is performed.

Choice of Therapy

The choice of treatment for Graves' hyperthyroidism involves both physician's prejudice, advantage and disadvantage of available options and, last but not least the patient's preference.

Graves' Disease

All patients should receive treatment with MMI for restoring euthyroid function. Afterwards two strategies can be considered: (1) maintaining MMI therapy for up to 12–18 months, hoping that meanwhile the mechanism responsible of thyroid hyperfunction goes into permanent remission (in up to 30% of cases); (2) definitive treatment with radioiodine of surgery (near total thyroidectomy). Long-term MMI therapy is usually employed in children and adolescent ad also appropriate to use for patients who are not willing to consider as neither radioiodine or surgery as first option.

Radioiodine could be the first option for middle-aged and elderly people, but can also be considered for young adults. The reasons for choosing radioiodine could be the relative high recurrence rate of hyperthyroidism after antithyroid drugs an the notion that radioiodine is effective, inexpensive, and safe. When radioiodine is considered in patients with mild and active Graves' ophthalmopathy, particularly if smokers and with recent onset of hyperthyroidism, a course of prednisone (0.2–0.3 mg/kg body weight for 8 weeks) should be advise to avoid the possible worsening of eye disease which has been reported to occur in about

15% of cases (Marcocci and Marinò, 2012; Bartalena *et al.*, 2015). Acute complications of radioiodine are extremely rare if patients are rendered euthyroid with antithyroid drugs for a period of time sufficient to deplete intrathyroidal stores of hormones.

Surgery should be advised to patients with large goiters, in whom the likelihood that MMI therapy may induce long-term remission of hyperthyroidism is low and in young patients with releasing hyperthyroidism after MMI therapy who are unwilling to receive neither a second course of MMI therapy or radioiodine. Patients should be informed of adverse events of surgery even though in experienced hand thyroidectomy is a rather safe procedure (Fortuny *et al.*, 2015).

Hypothyroidism should be the aim, and not an adverse event, of either radioiodine of surgery in patients with Graves' disease since hypothyroidism make very unlikely the relapse of hyperthyroidism and is easily and stably corrected by levothyroxine.

It is still debate which treatment modality for Graves' hyperthyroidism and ophthalmopathy, especially in those with moderate-to-severe eye disease (Bartalena *et al.*, 2016). In these patients it is important to promptly restore euthyroidism with antithyroid drugs. Two strategies have been proposed over the last 20–30 years, namely consider definitive treatment with radioiodine or surgery, as appropriate and at the same time star the specific treatment for the ophthalmopathy (Bartalena *et al.*, 2015, 2016). This approach is aimed to a permanent control of thyroid dysfunction and likely to short the active phase of the disease. The other option is to maintain the patients under antithyroid drug therapy until the treatment of ophthalmopathy is completed and the disease is inactive and to consider permanent treatment of hyperthyroidism thereafter. To date there is no evidence supporting either of the two options. A recent study has evaluated the outcome of long-term antithyroid drugs (as the block-and-replace [B-R] regimen) [median of 41 months (range: 24–132)] for Graves' hyperthyroidism in patients with ophthalmopathy by assessment, after discontinuation of B-R therapy of the recurrence rate of hyperthyroidism and the course of Graves' ophthalmopathy and its association with recurrent hyperthyroidism and/or 131-I therapy. The study suggests that a long term antithyroid drug treatment is associated with a lower recurrence rate of hyperthyroidism ($\sim$37%). With the regimen employed, recurrence of hyperthyroidism and recurrence of hyperthyroidism followed by treatment with 131-I appears not to be a likely cause of relapse of ophthalmopathy.

Thionamides are the first-choice treatment in pregnant women with hyperthyroidism. Radioiodine therapy is contraindicated during pregnancy, and surgery is restricted to exceptional cases. Both PTU and MMI cross the placenta and, in excessive doses, may cause hypothyroidism and goiter in the fetus and the neonate. PTU was until recently the preferred choice particularly in the United States because, compared to MMI, is less lipid soluble and more highly protein bound, and therefore its placental transfer appears to be lower. However, there is no difference between PTU and MMI in suppressing fetal thyroid function (Laurberg and Andersen, 2015). The drug chosen should be given at the lowest possible dose (but not higher than 10 mg MMI or 100 mg PTU daily as maintenance dose), aiming to maintain maternal-free T_4 and free T_3 in the high–normal range. A recent large international looking to antithyroid drug treatment-related birth defects confirmed an association between maternal use of MMI and omphalocele and choanal atresia in offspring. The study identified three malformations (situs inversus, unilateral renal agenesis/dysgenesis, and cardiac outflow tract lesions) associated with prenatal PTU exposure. The results were, however, of marginal statistical significance and the authors point out that a firm conclusion that PTU is teratogenic will require further studies. For the time being, PTU, if available, is recommended by the Endocrine Society of Endocrinology as the first-line drug for treatment of hyperthyroidism during the first trimester of pregnancy because of the possible association between MMI use and specific congenital abnormalities that may occur during early organogenesis. In this regard, it would be appropriate to shift from MMI to PTU when the pregnancy is planned. Recent analyses reported by the US Food and Drug Administration indicate that PTU may rarely be associated with severe liver toxicity. For this reason a change treatment from PTU to MMI is recommended after the completion of the first trimester. It reasonable to monitor liver function in pregnant women on PTU every 3–4 weeks. MMI may also be prescribed if PTU is not available or if a patient cannot tolerate or has an adverse response to PTU.

Graves' disease is the main cause of hyperthyroidism in children and adolescents. AT this age, the peak incidence of the disease is between 11 and 15 years of age, but it may occur in children under 5 years of age. Antithyroid drugs, radioiodine, and surgery have been successfully used in children, but thionamides is usually considered the first-choice treatment.

Toxic Adenoma and Multinodular Goiter

Hyperthyroidism due to toxic adenoma responds well to radioiodine, and the nodule is partially reduced by this form of therapy. In severe hyperthyroidism, pretreatment with thionamides is indicated, but radioiodine should be given before restoration of extranodular thyroid function to limit the rate of postradioiodine hypothyroidism.

Surgery is a more appropriate choice in large ($>$5 cm) nodules, particularly if associated with compressive symptoms (**Table 3**). Young age also favors surgery with respect to radioiodine. Lobectomy is indicated in classic toxic adenoma in otherwise normal thyroid glands. When single hyperfunctioning thyroid nodules occur in a multinodular goiter, subtotal or near-total thyroidectomy is indicated.

Toxic multinodular goiter is more frequent in patients older than 50 years, who might have an increased risk of surgery because of concomitant comorbidities (**Table 3**). Therefore, many patients with toxic multinodular goiters receive radioiodine. Hyperthyroidism is cured in virtually all cases, although more than one dose of ^{131}I may be required. However, the reduction in thyroid size is only partial because of the presence of intrathyroidal calcifications, fibrosis, and large areas of nonfunctioning tissue. Nevertheless, in patients with large mediastinal goiters and contraindications to surgery, radioiodine may be used with beneficial effects on thyroid hyperfunction and partial relief of compressive symptoms.

Table 3 Factors favoring specific forms of treatment of toxic adenoma and uninodular and toxic multinodular goiter

Surgery
Young-adult age
Large goiter or adenomas >5 cm
Low radioiodine uptake and large areas of poorly functioning tissue within the goiter
Airway obstruction or compression of other structures
Refusal to take radioiodine
Radioiodine
Small adenomas (<5 cm)
Old age
Contraindications to surgery

Surgery is generally regarded as a better option in patients with large goiters, especially if there is evidence of substernal extension and compression of airways and blood vessels (Fortuny *et al.*, 2015). Surgery is also indicated in those instances when radioiodine uptake by the gland is relatively low, as frequently occurs in large multinodular goiters. In the latter cases, when there is a true contraindication to surgery, the application of recombinant human TSH before radioiodine therapy should be considered. The use of recombinant human TSH in this setting is off-label and not generally recommended.

Treatment of Other Etiologies of Hyperthyroidism and Thyrotoxicosis

It is of importance to recognize other etiologies in order to choose the most appropriate therapeutic option and long-term surveillance.

Administration of moderate or high doses of iodine may induce thyrotoxicosis in patients with or without apparent preexisting thyroid disease. There are numerous sources of iodine, for example drugs, contrast agents, disinfectants, and food components. A notorious iodine-containing agent is the antiarrhythmic drug amiodarone, which may induce thyrotoxicosis (AIT) because of its high iodine content and/or a drug-induced thyroiditis (Bogazzi *et al.*, 2012). If possible, amiodarone should be discontinued. Patients with type 1 AIT, that is., caused by increased hormone synthesis because of exposure to high amounts of iodine, are preferably treated with methimazole (initially 40–60 mg/day, followed by gradual adjustment of the dose), but the response to thionamides is modest. In selected patients, treatment with potassium perchlorate (1 g/day for 4–6 weeks) can be considered. Potassium perchlorate is a drug that can cause aplastic anemia and its use should be limited to patients who cannot be controlled by methimazole, or who are allergic to thionamides. For patients with AIT Type II, that is., cytotoxic destruction of thyrocytes, prednisone (0.5–0.7 mg/kg body weight per day) is recommended, with tapering of the starting dose of prednisone once euthyroidism has been restored. Because the distinction between AIT Type I and II is difficult and not always clear, and because some patients have mixed forms of AIT, these therapies are occasionally combined.

Any form of thyroiditis, including subacute thyroiditis, silent thyroiditis and, occasionally, Hashimoto's thyroiditis may be accompanied by mild symptoms of thyrotoxicosis, particularly in the early phases of the disease, because the disruption of thyroid follicles can result in an increased release of stored iodothyronines. In these diseases treatment with beta-blocking agents is often sufficient. The thyrotoxic phase may be followed by transient or permanent hypothyroidism. Symptomatic treatment with nonsteroidal antiinflammatory drugs or aspirin is often sufficient. A subset of patients needs therapy with prednisone for variable amounts of time. Therapy with levothyroxine may be necessary during the hypothyroid phase of the illness.

See also: Subclinical Hyperthyroidism. Antithyroid Drugs. Radioactive Iodine. Thyrotoxicosis Factitia. Toxic Adenoma. Toxic Multinodular Goiter. Hyperthyroidism in Graves' Disease. Graves' Orbitopathy. Thyrotoxic Storm. TSH-Producing Adenomas and Resistance to Thyroid Hormones. Thyroid Disease and Pregnancy. Thyroid Disorders in the Elderly

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Subclinical Hyperthyroidism☆

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Introduction

Subclinical hyperthyroidism (SHyper) is a disorder characterized by subnormal or undetectable serum thyrotropin concentration (TSH) with serum free triiodothyronine and serum free thyroxine levels in the normal range for the general population, more often in the upper half (Biondi and Cooper, 2008; Cooper and Biondi, 2012).

The advent of the immunometric measurement of serum TSH concentration resulted in a progressive increase in the diagnostic rate of SHyper. Subsequently, a number of clinical investigations have established that this condition is not a simple biochemical abnormality but rather a mild tissue thyrotoxicosis "sensu strictu."

Etiology

SHyper can be transient or persistent and related to exogenous or endogenous causes (Biondi and Cooper, 2008, 2010; Cooper and Biondi, 2012). The exogenous variant of SHyper is related to TSH-suppressive treatment with levothyroxine (L-T4) in patients with differentiated thyroid carcinoma or to an unintentional excessive replacement L-T4 therapy in hypothyroid patients (Biondi and Cooper, 2010). About 200 million people worldwide take L-T4 and might be at risk for exogenous SHyper.

The endogenous variant is related to Graves' disease (GD), autonomously functioning thyroid adenoma (TA), and multinodular goiter (MNG) (Biondi and Cooper, 2008; Cooper and Biondi, 2012).

While GD is the most common cause of SHyper in younger patients (<65 years) in iodine-replete areas, TA and toxic MNG are relatively more frequent in iodine-deficient areas and in older persons (≥ 65 years).

Epidemiology and Natural History

Considering geographical aspects (iodine intake) and methodological differences (sensitivity of TSH assays), the prevalence of endogenous SHyper ranges between 0.6% and 16% and is, in general, more frequent in female and elderly patients (Biondi and Cooper, 2008; Cooper and Biondi, 2012). As for the spontaneous evolution of endogenous SHyper, prospective studies have demonstrated that overt hyperthyroidism develops frequently in patients with undetectable serum TSH compared to patients with low but detectable serum TSH (Vadiveloo *et al.*, 2011). Overt hyperthyroidism is often precipitated by iodine supplementation or by iodide contrast agents.

Clinical Implications

SHyper can be associated with signs and symptoms of mild thyroid hormone excess resembling adrenergic over activity (Biondi *et al.*, 1993, 1994). In general, SHyper negatively affects the quality of life of adult patients and induces supraventricular arrhythmias (atrial premature beats in middle-aged people and atrial fibrillation in the elderly) (Biondi *et al.*, 1993, 1994, 1996, 2000; Sawin *et al.*, 1994). Various authors have reported SHyper patients scoring higher on a symptom rating scale (specifically designed for clinically overt hyperthyroid patients) than euthyroid controls (Biondi *et al.*, 1993, 1994; Mercuro *et al.*, 2000). These findings clearly indicate that the term "subclinical hyperthyroidism" is a misnomer. Patients with persistent SHyper can have important alterations involving the morphology and function of the cardiovascular system and/or bone and mineral metabolism (**Table 1**) (Biondi *et al.*, 1993, 1994, 1996, 2000; Mercuro *et al.*, 2000; Fazio *et al.*, 1995). Therefore, SHyper is not merely a laboratory condition, but rather a mild tissue hyperthyroidism that may be particularly dangerous in the elderly.

Effects on the Cardiovascular System

Cardiovascular morbidity and mortality are significantly increased in patients with endogenous SHyper (Collet *et al.*, 2012; Gencer *et al.*, 2012; Parle *et al.*, 2001).

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Table 1 Clinical implications of subclinical hyperthyroidism

- Impaired quality of life
- Higher heart rate
- Increased prevalence of supraventricular arrhythmias and increased risk of AF
- Increased left ventricular mass
- Impaired diastolic function
- Impaired systolic function on effort
- Increased cardiovascular morbidity and mortality
- Reduced bone mineral density
- Increased risk of fractures

An increased risk of atrial fibrillation (HR 1.68, 95% CI 1.16–2.43) has been reported in a recent meta-analysis on individual participant data from five prospective cohort studies in patients with SHyper. The risk was higher for TSH levels <0.1 mU/L compared to 0.1–0.44 mU/L (HRs 2.54 vs. 1.63) (Collet *et al.*, 2012).

SHyper has also been associated with relevant changes in left ventricular properties. Increased left ventricular mass, in some cases above the hypertrophic threshold, is a consistent finding in adult SHyper patients, variably associated with impaired diastolic function and reduced exercise tolerance (Biondi *et al.*, 1993, 1994, 2000; Mercuro *et al.*, 2000).

A meta-analysis on 718 patients with endogenous SHyper has recently reported that the risk of coronary heart disease events is higher in patients with serum TSH <0.1 mU/L (HR 1.21, 95% CI 0.99–1.46) (Collet *et al.*, 2012). Moreover, a pooled analysis of individual participant data from six prospective cohort studies has demonstrated that patients with TSH levels <0.10 mU/L may have a higher risk of heart failure than euthyroid controls (HR 1.94, 95% CI 1.01–3.72) (Gencer *et al.*, 2012). Cardiovascular mortality is increased in patients with endogenous SHyper (HR 1.29, 95% CI 1.02–1.62) and this risk is higher for TSH levels <0.1 mU/L compared to levels between 0.1 and 0.44 mU/L (HRs 1.84 vs. 1.24) (Collet *et al.*, 2012).

Effects on Bone and Mineral Metabolism

Although clinically overt hyperthyroidism is a well-recognized risk factor for osteoporosis and fractures, it is still debatable whether this is also the case for persistent SHyper. Serum concentration of osteocalcin and urinary excretion of markers of collagen bone degradation and reabsorption (pyridinoline cross-links, hydroxyproline, and telopeptide type I) have been found to be increased in patients with SHyper. Congruent with these findings, various studies have showed a significant reduction in bone mineral density and an increased risk of fractures in women with SHyper; both features become more apparent during the postmenopausal period and in elderly patients (Greenspan and Greenspan, 1999; Bauer *et al.*, 2001).

Management Strategies

In general, the treatment of SHyper should be modulated according to the underlying etiology, the patient's age, and the presence of comorbidities (Biondi *et al.*, 2015; Ross *et al.*, 2016). In patients with exogenous SHyper, in whom the L-T4 dose cannot be reduced (e.g., in patients with high-risk thyroid cancer), beta-blocking drugs can attenuate the effect of adrenergic overactivity on the cardiovascular system, thereby mitigating cardiac abnormalities and improving the quality of life. Conversely, in all cases where L-T4 may be tailored (i.e., in benign thyroid diseases and in some cases of low-risk thyroid cancers), this is the mandatory treatment, eventually corroborated by beta blockade in case of persistent symptoms of increased adrenergic overactivity (Biondi and Cooper, 2010). The treatment of endogenous SHyper, similarly to that of overt hyperthyroidism, is aimed at redressing euthyroidism by means of antithyroid drugs, radioiodine, or surgery. Bisphosphonates and/or estrogens may be used in postmenopausal women and in individuals who have a high risk of fractures (Biondi *et al.*, 2015; Ross *et al.*, 2016).

See also: Antithyroid Drugs. Radioactive Iodine. Thyrotoxicosis; Diagnosis. Thyrotoxicosis; Overview of Causes. Thyrotoxicosis; Treatment

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Antithyroid Drugs☆

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Glossary

Graves' disease Graves' disease is the most common cause of hyperthyroidism, accounting for approximately 75% of cases. An autoimmune disease, it results from the production of autoantibodies directed to the thyrotropin (TSH) receptor which stimulate the thyroid gland to secrete excessive quantities of thyroxine (T4) and triiodothyronine (T3).

Hyperthyroidism This is the clinical condition arising from sustained raised concentrations of T4 and/or T3 in the circulation resulting from the excessive and deregulated synthesis and secretion of thyroid hormones, T4 and T3 by the thyroid gland. In this situation, TSH secretion is suppressed and serum TSH concentrations are undetectable or low. Thyrotoxicosis is defined as an excess of T4 and T3 concentration in serum resulting from the release of intrathyroid thyroid hormones through inflammatory cytolysis of thyroid tissue as in subacute thyroiditis or certain forms of autoimmune thyroiditis.

Thionamides A group of antithyroid drugs comprising carbimazole and its active metabolite, methimazole, and propylthiouracil. Their main action is to inhibit the iodination of tyrosyl residues in thyroglobulin, one of the early stages of thyroid hormone synthesis.

Antithyroid drugs are used in the treatment of hyperthyroidism of Graves' disease. The antithyroid drugs carbimazole (CBZ) and methimazole (MMI), as well as propylthiouracil (PTU), are heterocyclic compounds known as thionamides that contain a thiourylene group. Antithyroid drugs inhibit thyroid hormone synthesis hence opposing the overproduction of the hormones due to Graves' disease.

Introduction

Antithyroid drugs are rapidly and almost completely absorbed from the gastrointestinal tract, with peak serum concentrations at 1 or 2 hours. CBZ and MMI are interchangeable since CBZ is immediately metabolized in serum to MMI, 10 mg of CBZ yielding 6 mg of MMI. In the blood, MMI is virtually non-protein-bound while PTU is about 80% protein-bound. The half-life of MMI is 3 to 5 hours, that of PTU 1 to 2 hours. MMI and PTU cross the placenta and are transferred into breast milk, with a somewhat greater rate for the former. Both MMI and PTU are actively transported and concentrated into the thyroid, where they inhibit the synthesis of triiodothyronine (T3) and thyroxine (T4), principally by interfering with the iodination of tyrosine. They serve as preferential substrate for the iodinating intermediate of thyroid peroxidase so that oxidized iodine is diverted from the tyrosyl iodination sites in thyroglobulin. This intrathyroidal inhibitory effect of MMI and PTU on thyroid hormone synthesis outlasts by several hours, and longer for MMI than PTU, the presence of the drugs in blood. Consequently, MMI may be given as one daily dose, PTU two or three. The iodinated antithyroid drugs are desulfurated and further oxidized to inactive metabolites (Kampmann and Hansen, 1981; Cooper, 2005).

Two other effects of antithyroid drugs have to be discussed:

- PTU, but not CBZ/MMI, at high dose, inhibits T4 to T3 conversion by blocking type 1 iodothyronine deiodinase, not only in peripheral tissues, but also within the hyperactive thyroid gland (Laurberg *et al.*, 2007). This effect would be of clinical significance in the management of thyrotoxic crisis, when it is important to lower the raised serum T3 concentration as quickly as possible.
- Antithyroid drug therapy of hyperthyroid Graves' disease may be associated with a remission of the disease far more often than would be the case spontaneously. Moreover, it is common observation that, during antithyroid drug treatment, the serum level of circulating autoantibodies against the TSH receptor (TSH receptor antibodies, TRAb) declines and may even become undetectable. Hence, the concept of the immunosuppressive effect of the antithyroid drugs. However, the crucial question is whether this effect results from a direct action of the antithyroid drugs or would be associated with the restoration of the euthyroid state (Laurberg, 2006). While there are experimental in vitro data in favor of the former, the fact that remissions are independent from the dose of antithyroid drugs and that remissions as well as decrease of serum TRAb level are also observed

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with non-thionamide drug treatment (perchlorate, for instance) or after partial thyroidectomy is in favor of the latter, that is, the restoration of the euthyroid state. Obviously, more is to be learned in this domain.

Depending on availability in given countries, CBZ or MMI is the drug of choice in all areas of the world. PTU is no longer the first-line antithyroid drug in the Americas because of the risk of liver side-effects, uncommon but severe (see below). Currently, PTU is restricted to use during the first trimester of pregnancy. Methimazole is available as 5- and 10-mg tablets and CBZ as 5- and 20-mg tablets. PTU is available as 50-mg tablets.

The Treatment of Graves' Disease: Overview

The perfect single treatment of Graves' disease—an autoimmune condition with a relapsing and remitting long-lasting course—which would cure every patient, is not available, yet. In Graves' disease, the hyperfunction of the thyroid gland, responsible for the overproduction of thyroid hormone, results from the presence of TRAb that activate the TSH receptor. Currently available treatments are not targeted to the causal immune system derangement but to the resultant hyperthyroidism. There are two types of treatment strategies:

(a) *Antithyroid drug administration*: Antithyroid treatment restores euthyroidism in all patients in a few weeks but, in addition, after a 12–18 month-long continuous treatment course it may lead to a long-lasting (2 years or more) remission of the disease in approximately 40% of the patients
(b) *Eradication of thyroid tissue*: the radical/ablative strategy using thyroid irradiation with radioactive iodine or surgical thyroid ablation. Obviously, these two types of intervention are followed by a definitive hypothyroid state requiring substitutive levothyroxine treatment.

Antithyroid drug treatment is used not only in the first option strategy, but also in the second, to restore the euthyroid state before the radical treatment.

Initial identification of the patients more likely to go into remission after the long-term cure with antithyroid drug treatment remains uncertain at the individual level. However, prognostic criteria have been identified. Clinical characteristics such as younger age, male gender, and large thyroid gland volume are associated with a higher risk of relapse. Biologically, the severity of hyperthyroidism as assessed by a marked elevation of the serum concentration of T4 and/or of T3 (current assays measure the free forms of T4 called free T4-FT4, rather than the total T4-TT4; assays for FT3 or TT3 are both in use) and a high initial level of TRAb is also associated with a high relapse risk. Recently, it has also been observed that *PTPN22* C/T polymorphism and HLA subtypes *DQB1*02, DQA1*05, and DRB1*03* are independent predictors for recurrence (Vos *et al.*, 2016). Although these markers are highly significantly predictive only at group level, they are helpful to orient the discussion of the treatment selection with a given patient. This is why the treatment strategy remains somewhat empirical, individually tailored, also influenced by cultural/educational regional biases, by the prejudices of the physician and the patient, and by the local expertise availability, for example, trained team in thyroid surgery, or nuclear medicine center. In the past, there were major differences in the management of Graves' disease from center to center and between countries. For instance, radioiodine treatment was the first-line therapeutic option in the United States, antithyroid drugs in Europe and in Japan. Thanks to the work of several thyroid associations, guidelines and recommendations have been published so that therapeutic option differences between specialists tend to level off.

Antithyroid drugs are not normally indicated in the treatment of toxic nodular goiter, a condition which requires a radical/ablative therapy since no remission is to be expected, unless, in fragile or elderly patients, prior restoration of euthyroidism is necessary. There is no role for antithyroid drugs in subacute or postpartum thyroiditis, in which the thyrotoxicosis does not result from the overproduction of thyroid hormone but is due to the release of preformed thyroid hormones from the damaged gland (Ross *et al.*, 2016).

The Treatment of Graves' Disease: Management of the Therapy With the Antithyroid Drugs

In practical terms, the treatment of Graves' disease must be considered as a two consecutive step process. The initial one, following the diagnosis and the evaluation of the severity of the case, aims at restoring the euthyroid state using antithyroid drugs, the second, at implementing the more appropriate treatment strategy to cure the disease.

Initiation of Antithyroid Drug Treatment: Treatment Strategy Selection

The objective is to reach normal or near-normal serum T4 and T3 concentrations by 6–8 weeks. The initial dose of CBZ or MMI is 30–40 or 15–30 mg daily, respectively, depending on the severity of the hyperthyroidism assessed clinically and on the degree of serum T4 and T3 elevation. For instance, 30 mg/day of MMI is considered as advisable for severe cases (FT4 $\geq$ 90 pmol/L), 15 mg/day of MMI for mild and moderate cases. Clinical evaluation and the control of serum T4 and T3 levels are mandatory after 3–4 weeks of treatment. As the serum concentrations of T4 and T3 decrease, the daily dose of MMI can then be reduced to 10–20 mg and that of CBZ to 20–30 mg. Further adjustment of the dose is mandatory on the basis of measurements of serum

concentrations of T4 and T3 in order to prevent iatrogenic hypothyroidism due to inappropriately high doses of antithyroid drug. Adjunct treatments are not discussed here.

In newly diagnosed patients, the end of this first phase is the point in time when to select or confirm, and/or implement, the subsequent treatment strategy, either the "medical" one with the prolongation of the antithyroid drugs for a total of 12–18 months, or the "radical/ablative" with radioactive iodine administration or thyroidectomy.

Antithyroid Drug for the "Medical" Option

This is the more frequent option selected in the newly diagnosed patients. It requires patient's adhesion to treatment and regular follow-up. The antithyroid drug may be prescribed alone—the so-called "titration method", or in combination with the thyroid hormone levothyroxine (T4)—the "block and replace" method.

Duration of treatment

Approximately 40% of the patients in whom this modality has been elected remain in remission for the 2 years after the end of the antithyroid drug treatment. However, this percentage is significantly decreased if treatment duration is shortened to less than 12–18 months. On the contrary, the prevalence of remission is not increased by prolonging therapy for up to 3 years. There are a few reports of very long, or ultra-long (a few years up to 10 years) antithyroid drug administration (Azizi *et al.*, 2005). This approach may be considered in some patients provided the dose of antithyroid drug required of is very low ($<$5–10 mg/day), the drug is well tolerated, the patient is compliant to treatment and follow-up and, accordingly, there is no indication of radical/ablative treatment.

Management of the dose of antithyroid drug

With the titration method, the dose of the antithyroid drug must be closely adapted to the evolution of the thyroid hormone levels and, after 10–12 weeks of treatment, of the levels of serum TSH levels no longer suppressed as the result of hyperthyroidism. Usually, a maintenance dose of 5 or 5–15 mg of MMI or CBZ, respectively, may be achieved. The decrease in antithyroid drug dose requirement parallels that of the degree of thyroid overstimulation. Adaptation of antithyroid dose prevents iatrogenic hypothyroidism. After a further 6–12 month treatment, possibly at even lower dosage, antithyroid drugs may be stopped, with the patients being closely followed up.

The combination of both antithyroid drug and levothyroxine in the block and replace allows maintaining the high initial dose of antithyroid drug after the patient is euthyroid without the risk of iatrogenic hypothyroidism thanks to the substitutive daily dose of 100–150 μg of levothyroxine. In this method, it is the dose of levothyroxine which is adjusted to maintain the serum TSH level at the lower reference range.

While the two methods have been shown to be equivalent in term of remission rate, the block-replace regimen might be simpler to monitor since iatrogenic hypothyroidism is prevented and controls may be less frequent. As explained below, the block-replace regimen should not be used during pregnancy.

There is no single good marker to determine when to stop the antithyroid drug treatment after the 12–18 month course. However, the absence of reduction of the goiter volume and/or the persistence of thyroid hypervascularity, and, in the case of the titration method, persistently suppressed level of TSH and the requirement for a full dose of antithyroid drugs are indicative of persistent disease activity and, consequently, of recurrence at drug withdrawal. Similar information is provided by persistently elevated serum levels of TRAb.

Antithyroid Drug Treatment and the Radical/Ablative Strategy

Treatment with radioactive iodine

Although not mandatory, except in severe forms of hyperthyroidism and in fragile patients, many centers elect to moderate the degree of hyperthyroidism with antithyroid drugs before radioiodine administration. Also, since radioactive iodine requires approximately 6–8 weeks to be effective, antithyroid drug may be continued for 6 weeks after irradiation. Antithyroid drugs confer some degree of radioresistance to the thyroid tissue so that the drug must be discontinued and resumed 5–7 days before and after radioactive iodine administration. Alternatively, the dose of radioactivity may be increased by 15%–25%, a recommendation currently of less importance taken into account the general rule of using larger ablative doses of radioactive iodine.

Surgical thyroid ablation

As a rule, as much as possible, euthyroid state must be restored before thyroidectomy is considered in order to prevent post-operative thyroid crisis. Usually, an 8–16 week antithyroid drug treatment is appropriate. Care must be taken to prevent iatrogenic hypothyroidism due to an excessive dose of antithyroid drug that could favor goitrogenesis and thyroid gland hypervascularisation.

Side Effects of Antithyroid Drugs

Patients should be informed of the potential adverse effects of antithyroid drugs. They usually occur within the first 3–6 weeks of treatment. They appear to be dose-related for CBZ/MMI, more than for PTU. The adverse effects may be minor, the more frequent, or severe, much more uncommon (Cooper, 2005).

The more frequent are minor, occurring in approximately 5% of the patients, as cutaneous rashes of the urticaria or macular type, arthralgia or myalgia. Minor forms of rashes do not require stopping CBZ/MMI treatment and may resolve with antihistamine. There is a 50% cross-sensitivity between CBZ or MMI and PTU so that it was usual to switch to the alternative antithyroid drug in the event of a minor adverse reaction, a possibility to adapt to the current restriction of the use of PTU. A potentially severe adverse effect, migratory polyarthritis, may occur alone or in association with the rash and resolves within 4 weeks of stopping treatment.

Severe, potentially life-threatening or even lethal, adverse reactions may occur. These are uncommon. Agranulocytosis is the more dreadful. It may occur in 0.2%–0.7% of the patients, with a greater risk in older patients, with high doses of antithyroid drugs and during the first three months of treatment, but it may occur later, at reinitiating of the drug. Agranulocytosis is defined as a granulocyte count $<500/mm^3$. It should not be mistaken for the mild granulocytopenia (granulocyte count $<1500/mm^3$) sometimes observed in patients with Graves' disease before the treatment is started, so that it is useful to have a baseline white cell count available. If baseline neutrophil count is less than $1000/mm^3$, it is recommended that treatment with antithyroid drug be reconsidered. Agranulocytosis is of sudden onset, therefore routine monitoring of the white blood cell count is not recommended. Agranulocytosis is characterized by fever, throat infection and malaise and patients should be instructed to contact their medical practitioner as soon as they present with these signs and symptoms. Antithyroid drug must be stopped immediately. Along with proper management (isolation, broad-spectrum antibiotics therapy), the white blood cell count returns to normal within 1–3 weeks, usually. The benefit of the treatment with granulocyte-colony-stimulating factor is unsettled. Occurrence of agranulocytosis is an absolute contraindication to further antithyroid drug therapy. Hepatotoxicity of the antithyroid drugs is a severe side-effect, occurring in 0.1%–0.2% of the patients, either as cholestatic hepatitis or toxic hepatitis (Lin *et al.*, 2017). The prevalence is about the same with CBZ/MMI and PTU. Onset of hepatotoxicity of PTU may be acute and, if not recognized lead to hepatic failure in adults and in children and adolescents, requiring liver transplantation, or even to death (Glinoer and Cooper, 2012). Accordingly, the use of PTU is no longer recommended as the first-line treatment for Graves' hyperthyroidism, except in special conditions (vide infra). Since untreated hyperthyroidism is associated with a 30%–40% incidence of a biochemical liver test abnormality, it might be useful to obtain a baseline liver profile and to reconsider the treatment with antithyroid drug if transaminases levels are more than five times the upper limit of normal. Antinuclear-cytoplasmic-antibodies (ANCA)-associated small-vessel vasculitis related to antithyroid drugs has been recently reviewed (Balavoine *et al.*, 2015). Association with PTU is more frequent than with CBZ/MMI (75% vs 25%). Occurrence of ANCA is more frequent in young patients and on antithyroid drug therapy of long duration. Only 15% of ANCA-positive patients treated with antithyroid drugs exhibited clinical evidence of vasculitis, corresponding to 3% of all patients so treated. Clinical presentations (systemic symptoms, renal, lung, skin manifestations) of ANCA-associated vasculitis related to antithyroid drugs are extremely heterogeneous. Drug withdrawal is usually followed by rapid clinical improvement of vasculitis and a favorable prognosis. Because the decreasing use of PTU, ANCA screening is not systematically recommended on antithyroid drug therapy.

Antithyroid Drugs in Pregnancy

The incidence of thyrotoxicosis during pregnancy, in majority due to Graves' disease, approximates 0.1% (Mestman, 1998). During pregnancy, untreated hyperthyroid Graves' disease may cause maternal congestive heart failure and increases the risk of spontaneous abortion, premature labor, low birth weight, fetal death, and also of malformation in the infants. As other organ-specific autoimmune diseases, Graves' disease, after a transient exacerbation of clinical symptoms likely related to the first-trimester peak of human chorionic gonadotropin, usually tends to spontaneously improve or even remit in the second half of pregnancy. Maternal circulating antibodies cross the placenta, therefore high level of TRAb in the mother may cause fetal hyperthyroidism. Antithyroid drugs also cross the placenta. Consequently, restoration of euthyroidism in the mother requires that the dose of the antithyroid drug be maintained at the minimum to avoid fetal hypothyroidism. Since the transfer of thyroxine from mother to fetus is limited, the "block-replace" method is inappropriate during pregnancy, except in the more severe cases of maternal hyperthyroidism requiring full dose antithyroid drug treatment. In usual cases, the classical rule of thumb is to adjust the antithyroid drug dose so that the maternal serum FT4 level is maintained at the upper normal limit (Alexander *et al.*, 2017). In a good proportion of the cases, however, antithyroid treatment can be withdrawn after midpregnancy. Close clinical and biological surveillance of the mother, as well as multidisciplinary endocrinological, obstetrical and when appropriate, neonatal management, including expert fetal thyroid ultrasonography, are mandatory especially if the maternal level of TRAb is high, in order not to overlook fetal dysthyroidism. Measurement of the TRAb concentration in maternal serum before delivery is helpful since a high level is predictive of neonatal thyrotoxicosis. Hyperthyroidism occurs in 2%–10% of babies born to women with active Graves' disease.

Antithyroid drugs may be teratogenic and birth defects of all types are observed in 2%–3% of exposed children. The high risk period encompasses gestational weeks 6–10, the major period of organogenesis. Birth defects are more severe in newborns of CBZ/

MMI (the "CBZ/MMI embryopathy" representing approximately less than half of all defects: choanal atresia, esophageal atresia, omphalocele, omphalomesenteric duct abnormalities, aplasia cutis) than PTU (urinary system, face and neck region malformations) treated mothers. Consequently, PTU should be preferred as early as possible in the first trimester of pregnancy, or even prior to conception and patients on CBZ/MMI should be switched to PTU (Andersen *et al.*, 2013). Subsequently, patients may be switched back to CBZ/MMI.

Antithyroid Drugs and Breast-Feeding

In hyperthyroid patients in whom lactation is not impaired antithyroid drug treatment is not a contraindication to breast-feeding. Only minute amounts of the antithyroid drugs are detected in breast milk ranging from 0.01% to 0.1% for PTU and CBZ/MMI, respectively, providing the baby with a dose well below a therapeutically active dose. Nevertheless, it is recommended that the dose of antithyroid drug to the mother should not exceed 20 mg/day dot CBZ/MMI or 450 mg/d for PTU. A classical well-controlled dose–effect study of 88 lactating mothers treated with doses of MMI up to 20 mg daily showed no deleterious effects in thyroid function and physical and intellectual development of breast-fed infants (Azizi *et al.*, 2000).

See also: Graves' Disease. Hyperthyroidism in Graves' Disease. Subclinical Hyperthyroidism. Thyroid Disease and Pregnancy. Thyrotoxic Storm. Thyrotoxicosis; Treatment. Toxic Multinodular Goiter

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Radioactive Iodine☆

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Glossary

Differentiated thyroid cancer The most common endocrine malignant neoplasm arising from the follicular thyroid cell, which exhibits thyroid differentiation features.

Hyperthyroidism An excessive production of thyroid hormone by the thyroid gland in the course of Graves' disease, multinodular goiter with autonomous nodule, and/or autonomously functioning thyroid nodule.

Radioactive iodine (RAI) Radioisotopes of iodine used in the diagnostics and treatment of benign thyroid diseases and thyroid cancer, mainly I-131 (therapy) and/or I-123 (diagnostics).

Introduction

Iodine plays an essential role for normal functioning of the thyroid gland, its homeostasis, and thyroxine (T4) and triiodothyronine (T3) production. Iodine is actively transported into thyroid cells via the sodium–iodide symporter (NIS) and accumulates in the thyroid follicles being a substrate for thyroid hormone production. Normal thyroid takes up to 20%–30% of orally administered iodine, whereas in patients with hyperthyroidism this rate may increase even up to 80%.

The ability to uptake radioiodine (RAI) by thyroid cells in the same way as normal nonradioactive iodine was reported in the late 1930s. The first therapeutic RAI activity was administered to a patient with Graves' disease by Dr. Saul Herz in Massachusetts General Hospital in early 1941. In 1942 Keston and coworkers demonstrated RAI avidity in metastatic lesions of differentiated thyroid carcinoma (DTC). A year later, in 1943 in New York, United States, Dr. Samuel Seidlin applied RAI for the first time in a clinical practice to treat metastatic DTC. During the subsequent years RAI has been widely implemented in clinical thyroidology. Currently RAI is used in diagnostics and treatment of both benign thyroid diseases and DTC.

Radiation Physics

Only two among more than 20 known iodine radioactive isotopes are used in a daily clinical practice: I-123 for diagnostics and I-131 for both diagnostics and treatment. Iodine-131 is a beta- and gamma-emitter (principle $\beta^{-} = 0.807$ MeV; gamma rays ranging from 80 to 637, mainly 364 keV), showing a physical half-life of 8.02 days. Gamma radiation is related to the necessity of patients' isolation and the use of a strict radiation protection in treated subjects, whereas beta radiation serves for therapeutic purposes. The average range of the 0.807 MeV beta particles in soft tissues is about 1–2 mm, what results in high intrathyroidal/intratumor doses. Beta decay is responsible for causing DNA mutation and death in affected cells and other cells up to several millimeters away. For this reason, lower doses of I-131 may be more dangerous than higher doses, since they do not kill thyroid cells and may lead to cancerogenesis as a result of the irradiation. Therefore I-131 administration, particularly its lower activity in children is disputable.

Iodine-123 is characterized by a shorter half-life of 13.13 h and lower gamma radiation emission energy of 159 keV. It does not emit beta radiation. Such properties make I-123 an excellent isotope for thyroid imaging. It delivers far less radiation (1%) to the thyroid that of I-131. In addition the use of I-123 in the DTC diagnostics is not related to so called "stunning" of the tissue and loss of RAI avidity due to its low radiation burden. However, a high cost limits a clinical use of I-123.

Technetium-99m (Tc-99m) is another radioisotope used in the diagnostics of thyroid diseases, also transported into thyroid cells via NIS. It emits gamma rays with photon energy of 140 keV and half-life of nearly 6 h.

Biological Effects of RAI

Radioiodine administration leads to direct and indirect radiobiological effects on affected tissues. The direct effect is related to ionization of the thyroid cell and results in DNA damage, whereas indirect effect leads to the production of free radicals that react with the critical macromolecules.

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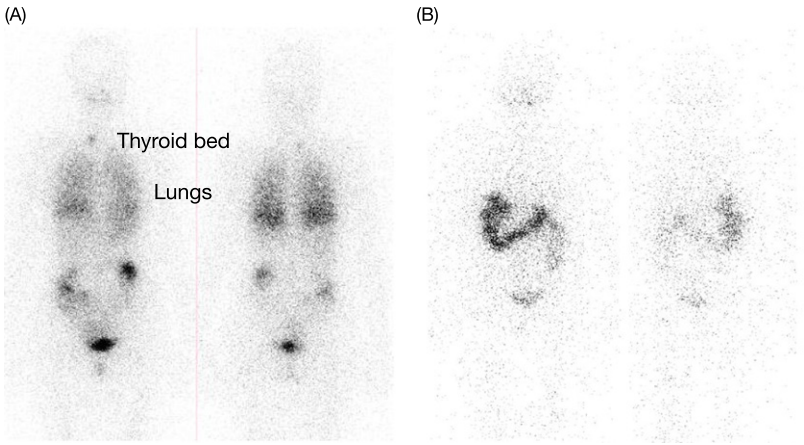

Fig. 1 Whole body scintigraphy in a DTC patient: (A) performed after the first RAI therapy—RAI uptake in thyroid bed and in both lungs; (B) performed after three courses of RAI treatment (complete remission). Physiological RAI uptake in digestive system and urinary tract is also present.

Among basic factors that determine the effectiveness of RAI therapy are the effective half-life of I-131, discrete energy of its β decay, the effective range of β radiation, used therapeutic activity, the ability to take up radioiodine, and the absorbed total radiation dose. The biological factors influencing the efficacy of RAI treatment in DTC are defined as "five R" and include radiosensitivity, repair, redistribution, reoxygenation, and repopulation (Suwiński and Gawkowska-Suwińska, 2001).

Radioactive Iodine in the Diagnostics of Thyroid Diseases

The use of RAI in the diagnostics of benign thyroid disease plays a minor role due to widely accessible thyroid sonography. Thyroid scanning allows visualization of the thyroid gland and functioning thyroid tissue elsewhere in the body, identifying the reason of hyperthyroidism, and evaluating thyroid enlargement and functionality of thyroid nodules. Currently, the indications for RAI thyroid scintigraphy in benign thyroid conditions are limited to patients, in whom RAI therapy is considered and in case of retrosternal goiter (technetium should not be used for the diagnostic of retrosternal goiter due to bone attenuation).

RAI uptake measurement is recommended in patients before RAI therapy to provide the information regarding the feasibility of RAI treatment and to calculate RAI activity.

Whole body RAI scintigraphy is still widely used in DTC patients. It allows visualization of the thyroid remnants and functioning, RAI-avid metastases (**Fig. 1**).

Radioactive Iodine in the Treatment of Thyroid Disease

Benign Thyroid Disease (Allahabadia and Franklyn, 2004; Stokkel *et al.*, 2010; Dietlein and Schmidt, 2014; Wong and Goh, 2014; Ross *et al.*, 2016)

The purpose of RAI therapy in benign thyroid disease is to resolve hyperthyroidism and/or to reduce the goiter volume. Indications for RAI administration involve the presence of a single toxic nodule, toxic nodular goiter, and Graves' disease. RAI treatment may be considered in selected cases of nontoxic nodular goiter, particularly in patients in whom surgery is contraindicated.

Pretreatment diagnostics should involve laboratory examinations (fT3, fT4, TSH, TRAb), thyroid scintigraphy (the assessment of the presence autonomous thyroid tissue or hypofunctioning lesions) with 24 h RAI uptake (at least 20% of administered RAI activity should be taken up by the thyroid gland to achieve a sufficient treatment effect), thyroid sonography (to assess thyroid volume and the presence of thyroid nodules), fine needle aspiration biopsy (to exclude malignancy), and pregnancy test (in women of childbearing potential).

Hyperthyroid patients are usually pretreated with antithyroid drugs to achieve clinical euthyreosis before RAI administration. One should remember that antithyroid drug has to be withdrawn at least 3–10 before RAI therapy because these drugs attenuate radiation effect in thyroid. Because RAI treatment of Graves' disease may lead to a transient exacerbation of hyperthyroidism, according to the American Guidelines β-adrenergic blockade should be considered even in asymptomatic patients. If it is clinically indicated antithyroid drug may be reintroduced 3–10 days following RAI administration. Patients with Graves' orbitopathy if not already on steroids therapy should be given prednisolone to avoid eye disease worsening after RAI administration.

Regarding autonomously functioning thyroid nodule TSH level before RAI treatment in patients with an autonomous thyroid nodule should be <0.1 uIU/mL to protect healthy thyroid tissue against radiation.

Recombinant human TSH (rhTSH) administration before RAI therapy may be considered to increase the absorbed radiation dose in selected cases of nontoxic nodular goiter. However, so far it is not a routine clinical practice.

The aim of RAI therapy in hyperthyroid patients is to restore euthyreosis, what is possible mainly in nonimmunological autonomous thyroid nodules. The only exception is Graves' disease in which RAI therapy has to result in hypothyroidism to effectively prevent recurrent hyperthyroidism. To calculate an optimal RAI activity to cure hyperthyroidism on one hand and to avoid hypothyroidism on the other hand is still challenging. The choice between estimation with administration of "fixed" RAI activities versus calculation, based on RAI uptake measurements, depends on physicians or site preference. Radioiodine doses used in the treatment of toxic or nontoxic nodular goiter have been already empirically established. The recommended absorbed radiation dose used in the treatment of toxic and nontoxic nodular goiter ranges between 100 and 150 Gy, requiring about 3.7–5.5 MBq per gram of thyroid tissue, corrected for the 24 h I-131 uptake. In patients with autonomous nodules the recommended absorbed radiation dose is 300–400 Gy. Considering patients with Graves' disease the lower absorbed RAI dose of ~150 Gy seems to be sufficient to restore euthyreosis because the radiosensitivity is higher than in other thyroid diseases. While higher absorbed doses, ranged between 200 and 300 Gy, are required for complete ablation of thyroid tissue.

Indications for RAI therapy in children, mainly due to Graves' disease are disputable due to a potential risk of development of thyroid carcinoma after irradiation. Children, who received RAI below the age of 5 years has twofold higher risk of thyroid neoplasm than children treated between 5 and 9 years of age and fivefold higher than children treated between 20 and 14 years of age. Therefore RAI therapy should be avoided in children below 5 years of age, whereas it may be considered in older children.

Differentiated Thyroid Cancer (Haugen *et al.*, 2016)

The ability to take up radioiodine is preserved in DTC cells therefore RAI has become a basic treatment modality in DTC. However, its routine use following thyroid surgery has been a matter of debate during the recent years, particularly in low-risk DTC due to the lack of unequivocal proofs, fulfilling evidence-based medicine criteria, confirming its efficacy. The primary goal of postoperative RAI administration after total thyroidectomy includes

(a) remnant ablation to facilitate further DTC monitoring and recurrence detection by thyroglobulin measurement or whole body scanning,
(b) adjuvant therapy to improve disease-free and overall survival by destroying potential undetected micrometastases, and
(c) RAI therapy to improve disease-specific and disease-free survival in high-risk patients.

Current 2016 ATA Guidelines (Haugen *et al.*, 2016) do not routinely recommend remnant ablation in ATA low-risk DTC. RAI adjuvant therapy should be considered in ATA intermediate-risk patients and it is definitely recommended for ATA high-risk patients. These guidelines met with EANM criticism (Verburg *et al.*, 2017). In case of RAI-avid inoperable, locally advanced or disseminated DTC RAI still constitutes the most effective first-line treatment.

ATA 2016 Guidelines (Haugen *et al.*, 2016) recommend a low RAI activity of 30 mCi for remnant ablation in patients after total thyroidectomy, whereas higher activities may be considered for patients after less than total or near-total thyroidectomy with a larger volume of thyroid remnants or for adjuvant treatment. When postoperative RAI therapy is given to treat suspected microscopic residual disease, in absence of macroscopic distant metastases, high RAI activities up to 150 mCi are recommended. Considering RAI therapy for loco-regional or metastatic DTC ATA 2016 Guidelines do not precise which method has to be used: empiric high RAI activity versus blood/or body dosimetry versus lesional dosimetry because there is a lack of evidences supporting of any approach.

DTC cells show expression of the genes responsible for RAI avidity (NIS, pendrin, and thyroid peroxidase genes), however this expression is significantly lower comparing to the normal thyroid cells. Moreover, there are some differences between primary tumor cells and metastases. Therefore a healthy thyroid tissue has to be surgically removed before RAI treatment. In addition, to increase RAI uptake in DTC lesions, TSH stimulation either by L-thyroxine withdrawal or rhTSH administration is necessary.

Considering pediatric DTC, the current ATA Guidelines for Children (Francis *et al.*, 2015) do not routinely recommend postoperative RAI treatment in low-risk DTC children, whereas intermediate and high-risk patients should first be evaluated for evidence of persistent disease. RAI administration is recommended only if persistent DTC is confirmed. Thyroxine withdrawal is preferred to achieve TSH stimulation in children. The routine administration of rhTSH is not recommended in children due to a limited data concerning its use in this age group. This recommendation also raised controversies in Europe. However, in selected cases, when endogenous stimulation has to be avoided due to comorbidities or is impossible, ATA Guidelines accept the use of rhTSH.

RAI Treatment-Related Complications

Acute Side-Effects

Benign thyroid disease: Acute radiation thyroiditis and transient goiter swelling may be observed in patients in whom RAI was given due to hyperthyroidism in the course of Graves' disease, toxic nodular goiter, or due nontoxic nodular goiter. Transient elevation of free T3 and T4, related to the release of thyroid hormone from disrupted thyroid cells, occurs usually approximately 7 days

following RAI administration. In some cases analgesics, β-blockers, or even steroids might be required. Some patients, particularly those with uncontrolled hyperthyroidism and large goiter, may develop thyroid storm. Autoimmune thyroiditis may be rarely noticed in patients with autonomous nodule or toxic goiter (Stokkel *et al.*, 2010).

Differentiated thyroid cancer: Acute radiation sialadenitis or gastrointestinal disturbances are common complication observed in DTC patients treated with higher RAI activities. Acute radiation thyroiditis may be observed in DTC patients with large thyroid remnants. Among other acute complications, noticed in DTC patients, are low platelets and leucocyte number due to transient myelosuppression, tumor swelling, or tumor bleeding. In rare cases, particularly when DTC metastases are localized in the central nervous system and respiratory tract, tumor swelling may be life threatening. In such cases RAI has to be administered under steroids cover (Suwiński and Gawkowska-Suwińska, 2001).

Late Side-Effects

Benign thyroid disease: Shorter cell survival, impaired replication of surviving thyroid cells, atrophy and fibrosis, and a chronic inflammatory response resembling Hashimoto thyroiditis are among long-term effects responsible for the development of hypothyroidism following RAI treatment in hyperthyroidism and nontoxic nodular goiter. The risk of hypothyroidism increases over time therefore lifelong TSH monitoring is necessary. However, it has to be emphasized that in Graves' disease hypothyroidism is rather an unavoidable consequence than side effect (Stokkel *et al.*, 2010).

Differentiated thyroid cancer: Late RAI treatment-related side-effects, noticed in DTC patients, involve salivary glands fibrosis, lung fibrosis (in patients treated due to highly RAI-avid lung metastases), hypogonadism, and bone marrow aplasia. The estimated bone marrow absorbed dose after the administrated RAI activity of 100 mCi is 0.3–1.0 Gy. It should not exceed 2 Gy (Suwiński and Gawkowska-Suwińska, 2001).

Another important issue is the risk of radiation-induced neoplasms, among them leukemia, breast, urinary bladder, and colon cancer (Brown *et al.*, 2008; de Vathaire *et al.*, 1997; Rubino *et al.*, 2003). The risk of secondary malignancies in DTC patients treated with RAI may increase with higher RAI cumulated activity. A relative risk of the development of radiation-induced leukemia is approximately 2.3. Considering solid cancer a relative risk ranges between 1.2 and 2.5 and it may be even higher regarding breast cancer—3.4. However, one should remember that any paper analyzing the risk of secondary malignancies did not consider a possible genetic predisposition common for thyroid cancer and other neoplasms. However, the supporters of RAI therapy in DTC believe that benefits resulting from RAI administration significantly exceed the risk of potential secondary malignancies.

Contraindications for RAI Administration

Pregnancy constitutes of an absolute contraindication for RAI treatment. Also according to 2016 ATA Guidelines (Haugen *et al.*, 2016) women of childbearing potential should avoid pregnancy for 6–12 months following RAI therapy. Some specialists also recommend 3-month waiting period for men to avoid any potential transient chromosomal abnormalities.

Radioactive iodine should not be given to nursing women. 2016 ATA Guidelines (Haugen *et al.*, 2016) recommend to stop breastfeeding or pumping for at least 3 months before RAI administration. The use of dopaminergic agents may be useful to decrease breast exposure in recently nursing women, however the risk of serious side-effects related to their administration to suppress lactation should be considered.

Uncontrolled hyperthyroidism and tracheal stenosis constitute relative contraindications for RAI therapy in benign thyroid disease. RAI therapy may lead to transient elevation in free T3 and T4 levels approximately 7 days after RAI administration therefore patients should be pretreated with antithyroid drugs. It is worthy to be considered that antithyroid drugs protect thyroid against ionizing radiation. In rare cases, when antithyroid drugs or surgery are contraindicated I-131 may be given under the cover of steroids and β-blockers. A large goiter caused tracheal stenosis, with tracheal diameter <1 cm, may be treated with RAI under steroids cover. When tracheal diameter is <5–6 mm surgery rather than RAI is recommended due to a high risk of serious dyspnea after therapy (*Stokkel et al.*, 2010).

The association between RAI administration and the worsening of eye disease has been shown therefore EUGOGO and EANM Guidelines recommend the use of steroids cover in patients demonstrating eye symptoms, treated with RAI. According to EANM Guidelines severe Graves' orbitopathy is a contraindication for RAI therapy (Stokkel *et al.*, 2010), whereas EUGOGO statement is not so categorical (Bartalena *et al.*, 2016).

Hypersensitivity reaction is very unlikely due to very low stable iodine content of RAI preparations (0.05–0.18 μg).

See also: Graves' Disease. Hyperthyroidism in Graves' Disease. Nontoxic Goiter. Subclinical Hyperthyroidism. Thyroid Imaging. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyrotoxic Storm. Thyrotoxicosis; Treatment. Toxic Adenoma. Toxic Multinodular Goiter

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Thyrotoxicosis Factitia☆

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The term "thyrotoxicosis factitia" describes thyrotoxicosis due to the voluntary and surreptitious ingestion of excess thyroid hormone(s). The ingested thyroid hormone is most frequently represented by thyroxine (T4), but triiodothyronine (T3) or a mixture of these two hormones (T4 and T3) may be the product responsible for the occurrence of this syndrome.

Thyrotoxicosis factitia should be distinguished from iatrogenic thyrotoxicosis, which is related to excessive doses of thyroid hormones erroneously given by the physician or inadvertently taken by the patient (**Table 1**).

Thyrotoxicosis factitia is usually observed in psychiatrically disturbed patients. They are almost invariably women who have psychoneurotic disturbances such as obsessive preoccupation with body weight, conflict with gender identity, hysterical personality, and emotional instability. By voluntarily inducing thyrotoxicosis, they often seek attention of their partners to their "sickness." Alternatively, these women can be driven by the erroneous idea that thyroid hormones can help them to look younger or to lose weight. The main psychopathological disturbances range from anorexia to depression, anxiety panic disorder, or phobic disorders. Acute schizophrenia like psychosis has been reported as main clinical manifestation of thyrotoxicosis factitia in few cases.

Patients with thyrotoxicosis factitia usually deny the deliberate assumption of excess thyroid hormones. The age of these patients appears to have been increasing during recent decades (Bogazzi *et al.*, 1999).

Clinical Features

The clinical picture of thyrotoxicosis factitia does not differ from that of classical spontaneous hyperthyroidism (Bartalena *et al.*, 1996; Roti *et al.*, 1993). Patients may complain of tachycardia, tremors, loss of weight, increased perspiration, heat intolerance, extreme anxiety and nervousness, increased bowel activity and/or insomnia. Goiter and ophthalmopathy, as seen in Graves' disease, are absent. However, the presence of goiter does not exclude the diagnosis of thyrotoxicosis factitia. Thyroid pain and tenderness, commonly observed in subacute thyroiditis, are absent (**Table 1**). Cardiovascular complications (e.g., tachyarrhythmias, heart failure, myocardial infarction) as well bone loss (osteopenia) may occur as a consequence of prolonged excess thyroid hormone ingestion. These complications are more likely to take place in the older patients.

Poisoning with thyroid hormone may be particularly severe, in selected patients: the burst of enhanced, excessive sympathetic activity arising from thyrotoxicosis factitia has been proposed as a cause of Takotsubo cardiomyopathy (Tsao *et al.*, 2010); clinical presentation may consist in thyroid storm, febrile confusion and fatal malignant hyperthermia (Artl *et al.*, 2012).

Diagnosis

Diagnosis of thyrotoxicosis factitia requires a high grade of suspicion (Rose *et al.*, 1969; Cohen *et al.*, 1989).

Laboratory evaluation reveals the typical increase in free T4 (FT4) and free T3 (FT3) concentrations associated with undetectable serum thyrotropin (TSH) levels. An isolated increase in serum FT3 concentration associated with low/absent FT4 levels and suppressed TSH levels indicates that the ingested thyroid hormone preparation contained only T3. Circulating autoantibodies to thyroglobulin or thyroperoxidase, as well TSH receptor autoantibodies, responsible for Graves' disease, are usually absent. Thyroidal radioactive iodine uptake (RAIU) is characteristically very low or suppressed (**Table 2**). Scan should be extended to the abdomen, to exclude rare cases of struma ovarii. Serum thyroglobulin concentration is typically markedly reduced or undetectable in thyrotoxicosis factitia (Mariotti *et al.*, 1982; Hamolsky, 1982 and **Table 2**). Accordingly, its measurement is a useful tool for differentiating thyrotoxicosis factitia from other thyrotoxic conditions associated with low RAIU values (**Table 2**). However, the presence of thyroglobulin antibodies may generate either a false-positive or a

Table 1 Sources of exogenous thyroid hormone excess

1	Iatrogenic thyrotoxicosis
2	Accidental (or suicidal) thyrotoxicosis
3	Surreptitious ingestion of thyroid hormones (thyrotoxicosis factitia)
4	Inadvertent ingestion of thyroid hormone (hamburger thyrotoxicosis)
5	Dietary pills

☆*Change History:* March 2018. Bogazzi and Martino updated Clinical Features, Diagnosis, References and Table 2.

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Table 2 Differential diagnosis of main forms of thyrotoxicosis with low-suppressed thyroidal radioiodine uptake

	Subacute thyroiditis	*AIT2*	*Thyrotoxicosis factitia*	*Struma ovarii*
Serum free thyroid hormones	Increased	Increased	Increased	Increased
Serum TSH	Undetectable	Undetectable	Undetectable	Undetectable
Serum thyroglobulin	Increased	Increased/normal	Low/undetectable	Increased/normal
Urinary iodine excretion	Normal	Increased	Normal	Normal
Thyroid echogenicity	Markedly reduced and not homogeneous	Slightly reduced, homogeneous	Normal	Normal
Thyroidal increased vascularity	No	No	No	No
Thyroid pain/tenderness	Yes	No	No	No
Radioiodine abdomen uptake	No	No	No	Yes
Pathogenesis	Destructive thyroiditis	Destructive thyroiditis	Surreptitious ingestion of thyroid hormone	Ectopic functional thyroidal tissue
Duration	Weeks	Months	Unpredictable	Weeks to months

AIT, Amiodarone-induced thyrotoxicosis type 2 (i.e., destructive thyroiditis induced by amiodarone).

false-negative thyroglobulin result depending on the used immunoassays (Jahagirdar *et al.*, 2008). Measurement of thyroid hormones in stool may be useful for identifying the abnormally high fecal excretion of ingested thyroid hormones, particularly, in these patients (Bouillon *et al.*, 1993). Urinary iodine excretion is normal (**Table 2**). Color flow Doppler sonography of the thyroid shows an absent increased vascularity and normal-low peak systolic velocity in spite of the thyrotoxic state (Bogazzi *et al.*, 1999) (**Table 2**).

The patient usually denies the surreptitious thyroid hormone intake; in those situations, it may be necessary to hospitalize him or her to be sure that the deliberate ingestion does not continue. Under strict medical controls, a rapid improvement in the clinical and laboratory features of thyrotoxicosis is frequently observed after hospital release unless reasons for surreptitious thyroid hormone intake are identified.

Treatment

Treatment of thyrotoxicosis factitia obviously requires withdrawal of thyroid hormones. It may be useful to associate a short-term treatment with beta- adrenergic blocking drugs to control tachycardia and tremors promptly. However, for a full recovery of the patient, psychiatric aid or counseling is mandatory in all cases (Da Silva *et al.*, 2009).

See also: Drug Effects and Thyroid Function. Thyroglobulin. Thyroid Imaging. Thyrotoxicosis; Diagnosis

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Thyrotoxic Storm☆

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Glossary

Euthyroid sick syndrome In patients with non-thyroidal systemic illness, laboratory tests that include a low total T4, a normal or high free T4, a normal TSH, and a low serum T3, that is a "low T3 syndrome".

Hemoperfusion Experimental technique of filtration of blood through extracorporeal special filters or adsorbents to remove excess thyroid hormone.

Hyperglycemia Elevated blood concentration of glucose.

Ophthalmopathy Pathologic condition of the orbital content; in Graves' disease marked by swollen eye muscles, increased fat content, conjunctival injection, proptosis, pressure sensation, and in most severe form, optic nerve compression and risk of blindness.

Proptosis Forward protrusion of the eye globes; exophthalmos.

Sick euthyroid syndrome A state of altered thyroid function tests in response to systemic illness without primary thyroid pathology. Laboratory tests typically include a low total T4, a normal or high free T4, a normal TSH, and a low serum T3, that is, a "low T3 syndrome".

Tachycardia A rapid heart rate, generally greater than 100 bpm.

Thyrotoxic storm Severe hyperthyroidism with systemic decompensation.

Clinical Features

Usually, the presentation of thyrotoxic storm follows some specific precipitating event such as surgery, burns, emotional stress, or even vigorous palpation of the thyroid gland. Most of these patients will have some or all of the classic signs and symptoms of thyrotoxicosis such as goiter and Graves' ophthalmopathy. However, in older patients, particularly those who may have an underlying toxic multinodular goiter rather than Graves' disease, the thyrotoxic storm may present as so-called masked or "apathetic" thyrotoxicosis. The clinical presentation includes tachyarrhythmias other than sinus tachycardia, and signs and symptoms of congestive heart failure may be present. Systolic hypertension with widened pulse pressure is a common finding at least initially. Cardiac compromise may be seen in relatively young and middle-aged patients without known antecedent cardiac disease. Postural hypotension in the common setting of volume depletion secondary to vomiting or diarrhea may be demonstrable. The extreme expression of the latter is with shock and vascular collapse, which has a very high risk of mortality. Gastrointestinal manifestations include presentations such as an acute abdomen, intestinal obstruction, diffuse abdominal pain, hepatomegaly, splenomegaly, and various abnormalities in liver function tests. Congestive failure or hepatic necrosis may cause the liver to be tender to palpation. The presence of jaundice is another serious prognostic sign.

Distinguishing between true storm and uncomplicated infection in a thyrotoxic patient may be quite difficult because of the likely presence of signs of tachycardia and fever in both. In such cases, fever that is seemingly out of proportion to an apparent infection along with profound diaphoresis strongly suggests thyrotoxic storm. It is imperative at this point to initiate an intensive treatment plan because no other clear-cut signal of the presence of thyrotoxic crisis may present itself prior to the inexorable decline of vital functions in the patient and an attendant high risk of mortality. As the thyroid crisis progresses, symptoms of central nervous system dysfunction may appear, including increasing agitation and emotional liability, confusion, paranoia, psychosis, and finally even frank coma.

Laboratory Findings

Diagnosing thyrotoxic storm using laboratory findings is extremely difficult because of relatively similar thyroid test results in storm as in uncomplicated thyrotoxicosis. Indeed, serum total triiodothyronine (T3) levels actually may be within normal limits, probably due to the fact that these patients have some underlying illness that precipitated the storm and that is responsible for altering the thyroid function tests in the direction of these patients, that is, congruent with the "low T3" or "euthyroid sick syndrome." This may initially obscure a diagnosis of coexistent thyrotoxicosis. Perhaps the most rapid confirmation of the diagnosis in a patient with previously undiagnosed thyrotoxicosis may be obtained by performance of a 2-h radioiodine uptake, although it should be feasible to obtain the result of serum T4 determination within a few hours on an emergency basis in most

☆*Change History*: February 2018. Leonard Wartofsky did miscellaneous edit of text, expanded glossary, and updated and added new reference citations.

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hospitals today. However, initiation of therapy should not be postponed when there is a high index of suspicion merely because one is awaiting laboratory confirmation of the diagnosis.

Other laboratory abnormalities often include modest hyperglycemia in the absence of diabetes mellitus. Moderate leukocytosis with a mild shift to the left is common even in the absence of infection. Increased serum calcium levels may be seen, perhaps due to both hemoconcentration and the known effects of thyroid hormone on bone resorption, but other serum electrolytes are usually normal. Hepatic dysfunction in thyrotoxic storm will result in elevated levels of serum lactate dehydrogenase, aspartate aminotransferase, and bilirubin.

Because serum cortisol levels are usually slightly elevated in thyrotoxic individuals, a normal value may be interpreted as being inappropriately low. In view of the known coincidence of adrenal insufficiency with Graves' disease, one should maintain a reasonably high index of suspicion for this disorder, particularly if there is hypotension and suggestive electrolyte abnormalities. It would be prudent to obtain a serum sample for cortisol determination prior to the administration of corticosteroid. Even in the absence of adrenal insufficiency, adrenal reserve may be exceeded in thyrotoxic crisis due to the inability of the adrenal gland to meet the demand placed on it as a result of the accelerated turnover and disposal of glucocorticoids that occur in thyrotoxicosis.

Pathogenesis

The precise pathogenesis underlying the progression of uncomplicated hyperthyroidism to the precipitation of thyrotoxic storm is likely not to be the same in all cases and remains incompletely understood, although the magnitude of serum hormone levels per se does not appear to be a critical factor. However, acute discharge of hormone from the thyroid gland resulting in rather sudden changes of its concentration, in the appropriate clinical setting, certainly can trigger crisis. This may be seen after 131-I therapy, withdrawal of propylthiouracil (PTU) therapy, vigorous palpation of the thyroid, or administration of lithium, stable iodine, or iodinated contrast dyes.

A possible interaction between the effects of excessive levels of circulating thyroid hormone and the catecholamines has been proposed. This is evidenced by the dramatic clinical improvement that follows the use of agents that either deplete their tissue levels, such as reserpine, or block β-adrenergic receptors, such as propranolol. Although these agents are useful adjuncts to therapy, they should not be used by themselves because they might not prevent the occurrence of storm.

Treatment

We believe that to avoid a fatal outcome, it is important to implement a four-pronged approach to the management of thyrotoxic storm. The relative importance for survival of each arm of therapy will vary in a given patient. First, specific antithyroid drugs must be used to decrease the increased thyroid production and release of thyroxine (T4) and T3. The second part of management consists of treatment intended to block the effects of the remaining, but excessive, circulating concentrations of free T4 and T3. The third component addresses any underlying precipitating illness such as infection or ketoacidosis. The final arm of therapy is composed of those several specific treatments that are directed against the underlying systemic decompensation that may be characterized by fever, congestive failure, shock, and the like. In view of the poor prognosis associated with incompletely treated thyrotoxic storm, no one component of this four-pronged therapeutic approach should be neglected.

Therapy Directed Against the Thyroid Gland

Inhibition of new synthesis of the thyroid hormones is achieved by administration of thionamide antithyroid drugs, such as PTU and methimazole (Tapazole), given orally. There are no available parenteral preparations of these compounds in the United States although intravenous PTU or methimazole may be available in parts of Europe. Either methimazole or PTU may also be administered via the rectum if necessary. In view of the gravity of thyrotoxic storm, thionamide doses are much higher than those for otherwise uncomplicated thyrotoxicosis. Some experienced clinicians believe that PTU will provide more rapid clinical improvement because it has the additional advantage of inhibiting conversion of T4 to T3, a property not shared by methimazole. Separate treatment must be administered to inhibit the continuing release of T4 and T3 into the blood because thionamides act to reduce new hormone synthesis but have no effect on thyroidal secretion of preformed stores of hormone. Either inorganic iodine or lithium carbonate may be used for this purpose. Iodides may be given either orally as Lugol's solution or as a saturated solution of potassium iodide (eight drops every 6 h). When iodine is administered together with full doses of antithyroid drugs, dramatic decreases in serum T4 can be seen. The sequence of administration of these agents is extremely important. Use of iodine without prior thionamide dose is contraindicated because the iodine will provide extra substrate to enrich hormone stores within the gland, thereby generating the potential for further exaggeration of thyrotoxicosis.

In patients who may be allergic to iodine, lithium carbonate may be used as an alternative agent to inhibit hormonal release, although some caution has been raised in regard to its use in the setting of storm. This drug also may be used in thyrotoxic patients who are known to have serious toxic reactions to the thionamides.

Therapy Directed Against Ongoing Effects of Thyroid Hormone in the Periphery

For the purpose of acutely reducing the circulating hormones, peritoneal dialysis and plasmapheresis have been employed, as has experimental hemoperfusion through a resin bed or charcoal columns. Such aggressive management should be considered in severe cases.

Beta blockers are also commonly used. Propranolol is the agent most commonly used in the United States today. Large doses, such as 60 to 120 mg every 6 h, are used in crisis or impending crisis. Indeed, because of the more rapid metabolism of the drug in severe thyrotoxicosis, even larger oral doses, or preferably intravenous doses, should be given. Initial intravenous doses should be given cautiously, whereas the patient's cardiac rhythm is monitored continuously. Added benefits of β-adrenergic blockade in these patients include improvement in agitation, convulsions, psychotic behavior, tremor, diarrhea, fever, and diaphoresis. Very short-acting β-adrenergic antagonists, such as esmolol or landiolol, can be given intravenously instead of propranolol. For esmolol, an initial intravenous dose is 0.25 to 0.5 mg/kg given in 5 to 10 min, followed by a continuous infusion of 0.05 to 0.1 mg/kg/min. For patients with asthma or who present other concerns if treated with beta blockers, short half-life calcium antagonists such as verapamil have been employed.

Therapy Directed Against the Precipitating Illness

In most cases of thyrotoxic storm, therapy is not complete unless a diagnosis of the possible precipitating event is made and early treatment as indicated for that underlying illness is implemented.

It is important to be alert to the fact that conditions such as ketoacidosis, pulmonary thromboembolism, and stroke may underlie thyrotoxic crisis, particularly in the obtunded or psychotic patient, and require the same vigorous management ordinarily indicated. In the patient with thyrotoxic crisis in whom none of the latter precipitating factors is apparent, a diligent search for some focus of infection must be carried out. Empirical, broad-spectrum antibiotic coverage may be warranted while awaiting results of cultures. In most patients who survive thyrotoxic crisis, clinical improvement is dramatic and demonstrable within 12–24 h.

Therapy Directed Against Systemic Decompensation

Fluid depletion caused by the hyperpyrexia, diaphoresis, vomiting, or diarrhea must be vigorously replaced to avoid vascular collapse. Fluid management must be individualized. Shock may be refractory to cautious fluid resuscitation in younger patients, whereas judicious replacement of fluids is necessary in elderly patients with congestive heart failure or other cardiac compromise. Intravenous fluids containing 10% dextrose in addition to electrolytes will allow repletion of the depleted hepatic glycogen. For fever, acetaminophen, rather than salicylates, is the preferred antipyretic because salicylates inhibit thyroid hormone binding and could increase free hormone, thereby transiently worsening the thyrotoxic crisis.

Vasopressor therapy may become necessary on a temporary basis to provide adequate hemodynamic support if hydration with intravenous fluid replacement is not effective. Stress dose glucocorticoids have been given on empirical grounds on the basis of postulated relative adrenal insufficiency. This approach has the added benefit of further blockade of peripheral conversion of T4 to T3, and this is an additional justification for their use.

See also: Antithyroid Drugs. Radioactive Iodine. Thyroid Hormone Action. Thyroid Imaging. Thyrotoxicosis; Systemic Manifestations

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Graves' Disease☆

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Glossary

Antigen presenting cells (APCs) APCs are immune cells mediating immune responses by processing and presenting antigens to lymphocytes. APCs include dendritic cells, macrophages, Langerhans cells and B cells.

Autoantibody An antibody directed against one or more of the patient's own (self) proteins.

Autoimmunity Dysregulation of the immune system leading to recognition of "self" components (autoantigens), which are attacked by autoantibodies and/or autoreactive T lymphocytes.

Dyscrasia The term derived from the ancient Greek medicine meaning the imbalance of the four humors which were believed to be present in the body: blood, black bile, yellow bile, and phlegm. This imbalance was considered the direct cause of all disease.

Glycosaminoglycans (GAGs) GAGs are long unbranched polysaccharides consisting of a repeating disaccharide unit. Hyaluronic acid is the GAG most commonly found in thyroid dermopathy.

Goiter An enlarged thyroid gland.

Gut microbiota (formerly called gut flora) Gut microbiota is the microbe population living in the intestine, Its role in human health and disease is becoming clearer when high throughput sequencing technologies have become available.

Hyperthyroidism A condition caused by increased secretion of thyroid hormones by the thyroid gland, leading to a hypermetabolic state and overactivity of the sympathetic nerve system.

Next generation sequence Next generation sequence is a high-throughput sequencing allowing to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing.

Radioiodine or radioactive iodine Radioactive isotopes of iodine: 123I is used for diagnostic (thyroid scan) purposes, while 131I is used to achieve thyroid ablation.

Single nucleotide polymorphisms (SNPs) SNPs are the most common type of genetic variation among people. Although most SNPs have no effect on health, some of these may help to predict an individual's response to certain drugs, susceptibility to environmental factors such as toxins, and risk of developing diseases.

Susceptibility locus An allele that increases the risk of disease occurrence, but is neither necessary nor sufficient for disease expression.

TSH receptor (TSHR) Receptor for thyroid-stimulating hormone (TSH) secreted by the pituitary; through binding to its receptor, TSH stimulates thyroid hormone synthesis and secretion.

Introduction

The first descriptions of autoimmune hyperthyroidism were reported from 1802 to 1840 by four physicians (Parry, Flajani, Graves, and Basedow). However the real etiology was discovered only in the 1950s and 1960s when thyroid stimulating class G immunoglobulins were identified. Interestingly, Basedow in his original publication hypothesized that the combination of tachycardia, goiter and exophthalmos was due by a dyscrasia of the blood, suggesting that a circulating factor was responsible for syndrome.

Graves' disease (GD) is the most common cause of hyperthyroidism in iodine sufficient areas: the annual incidence rate has been estimated of 14–50 cases per 100,000 persons.

Although, the disease may affect every age, the peak incidence is between 30 and 50 years. The lifetime risk of developing GD is higher in women compared to men, with a 6:1 ratio.

Etiology

GD is an autoimmune disorder but its etiology is not completely understood and is likely multifactorial, as a result of a combination of genetic and environmental factors.

☆*Change History:* March 2018. Irene Campi and Mario Salvi have added Keywords and Etiology and Pathogenesis paragraphs have been updated and expanded. Diagnosis and Therapy paragraphs have been added and Table 1 has been included. The list of references has been updated.

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Genetic Factors

The familial clustering and the studies in twins suggest genetic factors in the pathogenesis of the disease. Wiersinga et al. found that 33% of the families included in a study had cooccurrence of Hashimoto's thyroiditis (HT) and GD indicating a common genetic basis for autoimmune thyroid disorders (AITD). HLA loci have been associated with an increased risk of GD development in different population, in particular HLA-B8 and HLA-DR3 in Caucasians, HLA-A2 in Japanese, and of HLA-B46 in Chinese.

With next generation sequence (NGS) techniques, several other susceptibility loci for GD have been identified. In particular, two SNP locates in intron 1 the TSHR gene have been found in two large independent Caucasian datasets of GD patients; another locus associated with GD predisposition is CD40, which is a well-known gene regulating B-cell responses. Thyroglobulin, CTLA-4 and *PTPN22* have been associated with both GD and HT. The R620W SNP of *PTPN22* (*gene coding for* lymphoid tyrosine phosphatase) is a gain of function variant that enhance the inhibitory properties of the protein on T cells. This may confer a sort of resistance of self-reactive T cells from thymic deletion, thus inducing autoimmune reactions. Genetic factors may also influence the severity of GD: CTLA-4 polymorphism are associated with GO development.

Environmental Factors

Iodine intake may have a role in the development of GD, since the prevalence of hyperthyroidism is higher in iodine-deficient than in iodine-sufficient areas. In 1991, Laurberg et al. conducted a survey in two European areas with a divergent iodine intake (East-Jutland and Iceland with low and high iodine intake, respectively) and found that hyperthyroidism from toxic nodular goiter was more common in East-Jutland, while the incidence of GD was significantly higher in Iceland, especially in younger individuals. In a relatively isolate rural population of Italy, an increased incidence of HT and hypothyroidism was found 15 years following a salt iodization program. The risk of development of Graves' orbitopathy does not seem not influenced by salt iodization.

Infectious diseases have been often evoked as a potential trigger for autoimmunity. It has been suggested that viral infections, by increasing serum IFN-α levels, may increase the risk of GD. Yersinia enterocolitica (YE) might cause GD by a mechanism of molecular mimicry of the outer membrane porin F protein of the bacterium and a leucine-rich domain of TSHR. Healthy female relatives of AITD patients have been found to have an increased prevalence of anti-YE antibodies. In contrast, a prospective study did not find any correlation between YE infection and the development of thyroid dysfunctions or de-novo occurrence of thyroid antibodies. Recently, the role of gut microbiota has been investigated; Zhou L et al. found a decrease of Bifidobacterium and Lactobacillus and an increase of Enterococcus in hyperthyroid patients compared with controls. Further studies are needed to better understand these issues; data coming from a EU funded project, which is currently exploring the role of gut microbiota and antibody responses of food derived antigens in GD, will probably increase our knowledge on the pathogenesis of GD (Covelli & Ludgate). Smoking, which is an established risk factor for GO, also increases the risk of developing GD.

Pathogenesis

Hyperthyroidism is caused by autoantibodies against the TSH receptor (TRAbs) which mimic the action of its natural ligand (TSH). The biochemical feature of GD is characterized by high circulating free thyroid hormones (TH) causing TSH suppression, due to negative feed-back of TH on the pituitary gland. The elevated serum FT3 concentrations found in GD are mainly due to the increased intrathyroidal conversion of T4 to T3 by deiodinase type 2 (DIO2). Whether TRAbs are also responsible for the extrathyroidal manifestations of GD (GO, pretibial myxedema and acropachy) is still controversial. Although the TSHR is expressed on fibroblasts derived from orbital retrobulbar tissues and the pretibial skin, the coexpression of other antigens such as the IGF-1 receptor has been evoked as a potential trigger for GO. Autoantibodies against thyroid peroxidase (TPO-Ab) are also detected in approximately 70% of GD patients and their titers are related to the degree of lymphocytic infiltration of the thyroid gland in Hashimoto's thyroiditis (HT). The importance of these antibodies in GD is unclear, but they may explain why some patients with long standing GD become hypothyroid. In addition, the occurrence of primary hypothyroidism in GD patients may also be due to the presence of TRAbs with a TSHR-antagonist action.

Diagnosis

GD diagnosis is usually straightforward especially when extrathyroid manifestations are present. The clinical manifestations of an overt hyperthyroidism are generally easily recognizable (**Table 1**).

TSH and FT4 assay are often used as a first line test to confirm the diagnosis of GD.

Thyroid ultrasonography (US) is the most cost-effective way to image the thyroid gland; in GD US scan is useful to estimate the thyroid volume and to study palpable nodules; the estimation of thyroid volume is particularly useful in order to plan future therapies such as radioiodine or total thyroidectomy.

Although not essential, nowadays TRAb are routinely measured to support the diagnosis of GD and to predict response to therapy, since this assay has become easily available. TRAb are also important to predict the risk of neonatal hyperthyroidism in newborn of woman affected with GD, even in those previously submitted to radioiodine (RAI) or total thyroidectomy.

Table 1 Signs and symptoms of hyperthyroidism

Hyperthyroidism
Heat *intolerance* and excessive sweating
Weight loss with increased appetite
Goiter, thyroid thrill and bruit
Irritability, emotional lability, insomnia, hyperkinetic behavior
Fatigue, muscle wasting, and weakness
Tachycardia, rarely paroxysmal tachycardia, arrhythmias (atrial fibrillation), dyspnea, and angina
Tremors
Rarely hypokalemic periodic paralysis (more common in Asians)
Menstrual irregularity or amenorrhea, infertility
Increased frequency of stools
Polyuria
Ankle edema (without cardiac disease)
Thinning of hair, alopecia, change in texture of skin and nails (onycholysis)
Hypermetric reflexes
Graves' Orbitopathy and other systemic manifestation
Exophthalmos
lids edema
Lid retraction
Pain or irritation of eyes
Blurred vision/diplopia
Lid lag
Chemosis
Decreased visual acuity and decreased color discrimination in case of optic neuropathy
Pretibial myxedema
Acropachy
Hyperpigmentation or vitiligo

Therapy

The therapy of Graves' disease (GD), aims at achieving restoration of euthyroidism as rapidly as possible, especially when associated to GO. In fact, rapid control of hyperthyroidism may induce spontaneous improvement of milder GO and may increase responsiveness to immunosuppressive treatments for moderate to severe GO. Available treatments for Graves' hyperthyroidism include antithyroid drugs as the initial approach, and subsequent definitive ablation of thyroid tissue by surgery or radioiodine therapy.

The choice among the different therapeutic approaches for GD is based on several considerations such as the patient's age, thyroid volume, the presence and the degree of GO. Thioamides are the most common anti thyroid drugs which act by inhibiting the enzyme thyroid peroxidase, thus reducing the synthesis of TH in the thyroid; propylthiouracil (PTU) also has an inhibitory effect on DIO2. Methimazole (MMI) and carbimazole (CMI) (converted in vivo to MMI) are the first choice, because of their higher efficiency and low toxicity, while PTU is preferred during the first trimester of pregnancy or in case of intolerance to MMI/CMI. Thioamides are usually well tolerated; the most common side effect of MMI, found in about 10% of the patients, is a skin reaction (urticaria and dermatitis) which is generally responsive to antihistamines and reversible with the titration of the drug. PTU has been associated with hepatic failure. Agranulocytosis is an uncommon but severe side effect of thioamides and requires discontinuation of the drug.

Restoration of permanent euthyroidism is generally obtained only after radioiodine (RAI) or surgery, as spontaneous remission of hyperthyroidism after MMI discontinuation is infrequent. In patients with or without preexisting GO, low dose steroid prophylaxis should be prescribed after RAI, in order to prevent worsening or de novo occurrence of GO. No conclusive data are available so far in the literature on the possible risk of GO reactivation after thyroidectomy.

Extrathyroid Manifestations of Graves' Disease

Orbitopathy, pretibial myxedema (PTM) and thyroid *acropachy* are extrathyroid manifestations that may occur in patients with GD. Hyperthyroidism is the most common manifestation, and some ophthalmopathy occurs in 25%–50% of these patients, although severe eye disease is rather rare and develops in only approximately 5% of patients. Pretibial myxedema or dermopathy is present in 1% of patients, usually in those with GO. Acropachy is even less frequent and it is associated with both GO and PTM. It is characterized by digital clubbing, swelling of digits and toes, and periosteal reaction of extremity bones. Skin biopsy demonstrates typical findings of pretibial myxedema (fibroblast activation and glycosaminoglycan deposition). Although isolated case of

GO (the so-called euthyroid GO or EGO), PTM and *acropachy* have been described, these signs are usually correlated with the degree of severity of the autoimmune process.

The natural course of GD suggests that the thyroid disorder and GO are pathogenically linked. In fact, it supports the theory of GD as a multiple-organ disease: autoantibodies against a thyroidal antigen may cross-react with a similar antigen in the skin and the retrobulbar tissues. Radioactive iodine treatment leads to a slow destruction of the thyroid, and thyroidal proteins (including the TSH receptor) leak into the circulation and are presented to the immune system. A minority of patients subsequently mount an autoimmune response to the TSH receptor, leading to hyperthyroidism, and a small number also develop GO. The nature of this antigen is unknown, but the TSH receptor is a good candidate because TSH receptor autoantibodies are the cause of the hyperthyroidism and the TSH receptor is expressed on retrobulbar fibroblasts and possibly also on skin fibroblasts in patients affected by PTM.

See also: Antithyroid Drugs. Epidemiology of Thyroid Disease. Graves' Orbitopathy. Hyperthyroidism in Graves' Disease. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Postpartum Thyroid Dysfunction. Pretibial Myxedema. Radioactive Iodine. Thyroid and Infertility. Thyroid Autoimmunity. Thyroid Imaging. Thyrotoxicosis; Systemic Manifestations

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Relevant Website

http://www.thyroidmanager.org/

Hyperthyroidism in Graves' Disease☆

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Glossary

Hyperthyroidism Overproduction of thyroxine and triiodothyronine by an overactive thyroid gland.

Orbitopathy Inflammatory condition of the orbital tissues causing protrusion of the eye globe and diplopia. Most frequent extrathyroidal manifestation of Graves' disease.

Pretibial myxedema and acropachy Other more rare extrathyroidal manifestations of Graves' disease characterized by remodeling of the dermis and of the bone-connective tissue of the extremities.

T_3 receptor Receptor for triiodothyronine (T_3) present in the cytoplasm and nucleus of most human cells; after T_3 binding, this T_3 receptor complex influences gene transcription.

Thyrotoxicosis Elevated thyroxine (T_4) and triiodothyronine (T_3) levels, regardless of their cause; may result from an overactive thyroid gland, but also may result from exogenous T_4 and/or T_3 use or from destruction of the thyroid gland.

Thyroxine (T_4) Inactive prohormone produced by the thyroid gland and containing four iodine atoms.

Triiodothyronine (T_3) The active thyroid hormone mainly produced locally in the tissues by deiodination of thyroxine; approximately 20% of circulating T_3 is produced by the thyroid gland itself.

TSH—receptor The target of TSH in a physiological state and of autoreactive immunoglobulins in Graves' hyperthyroidism.

Thionamides Antithyroid drugs such as methimazole, carbimazole and propylthiouracil.

Introduction

Hyperthyroidism is the main clinical feature of Graves' disease (GD) and is due to the excess of thyroid hormones produced by follicular cells, as a result of stimulation by autoantibodies against the thyroid-stimulating hormone (TSH) receptor.

Manifestations of hyperthyroidism in Graves' patients were first described by Caleb Perry in the first half of 19th century but the link with thyroid hyperfunction was recognized only after the description of Robert Graves and Karl A. von Basedow.

As in all autoimmune diseases, when self-tolerance is broken, T cells recognize self-antigens and B cells produce antibodies targeting host cells. In this case, autoantibodies are directed against the TSH receptor. The binding of these antibodies causes an overfunction of the follicular cell, and thus thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), thyroglobulin and small amounts of an iodinated albumin-like protein and iodotyrosines are released into the blood stream at increased rates.

The overproduction of hormones by the thyroid itself is defined as hyperthyroidism and is the main cause of thyrotoxicosis, the clinical pattern due to the exposure of peripheral tissues to excess thyroid hormones.

Clinical Manifestations

The presenting symptoms are mainly attributable to the effect of the excessive serum concentrations of thyroid hormones; ocular changes and abnormalities of the skin and connective tissue are instead more related to the multi-organ autoimmune involvement.

The classical clinical picture includes weight loss, despite increased appetite, weakness, dyspnea, palpitations, increased bowel movement, irritability, profuse sweating, sensitivity to heat or increased tolerance to cold and tremor. The most common clinical manifestations are outlined in **Table 1**.

The involvement of the entire body in the hyperthyroid syndrome is due to the widespread distribution of T_3 receptors in tissues, although the cardiovascular system and the muscles are the main targets for thyroid hormones. Tachycardia is the most common clinical sign of hyperthyroidism and it may eventually lead to atrial fibrillation, especially in elderly patients. A recent study has shown that older age, elevated serum free T4 concentrations at diagnosis and male gender were independently associated with the risk of atrial fibrillation (Boelaert *et al.*, 2010).

In hyperthyroid patients a fine, rapid tremor of the outstretched fingers and generalized tremor, involving also the tongue, may be evident. Muscular weakness occurs in nearly all patients and this may lead to myalgia and fatigue of the larger proximal limb muscles. The weakness of respiratory muscles can induce dyspnea on exercise. 20%–40% of patients, mainly women, can experience friable nails and diffuse loss of scalp hair.

☆*Change History:* February 2018. Danila Covelli and Mario Salvi updated all sections of the previous manuscript. Tables and Figures are unchanged.

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Table 1 Common clinical manifestations of thyrotoxicosis

Symptoms	Nervousness, hyperactivity
	Fatigue, weakness, diminished sleep
	Increased perspiration, heat intolerance
	Palpitations
	Tremor
	Weight loss despite increased appetite
	Menstrual disturbances
Signs	Hyperactivity
	Tachycardia, atrial fibrillation
	Systolic hypertension
	Warm moist skin
	Fine skin and hair
	Tremor
	Hyperreflexia
	Stare and eyelid retraction
Only in Graves' disease	Diffusely enlarged thyroid (goiter)
	Orbitopathy
	Pretibial myxedema and acropachy

The increased production of thyroid hormones initially may induce a condition of hyperactivity, which is perceived favorably by some patients, who may then delay the time of reporting to a doctor and therefore the time of diagnosis. Later on, nervousness, anxiety, irritability, attention deficits, and some memory loss may also be misinterpreted as a syndrome of anxiety or depression. The change in the body weight is variable: some patients are wasting, but on the average the weight loss in 2–10 kg. The weight loss is the most frequent complaint of hyperthyroid patients seeking medical advice.

Hyperdefecation is not uncommon and is associated with increased motility of the gastrointestinal tract. Interestingly, some studies have evidenced an imbalance of gut microbiota in hyperthyroid patients (Zhou *et al.*, 2014). Since the gut microbiota has an essential role in the function of the immune system, it would not be surprising that its alteration may be involved in the pathogenesis of Graves' disease. What is not clear at the moment is if the microbiota imbalance is the cause or the consequence of the bowel discomfort.

In women, oligomenorrhea is common, but fertility is not severely impaired. In men, there is no evidence of an effect of hyperthyroidism on spermatogenesis, but the biological activity of estrogens is increased and mild gynecomastia may be found.

The action of high thyroid hormones on the palpebral Muller's muscles generally induce lid retraction causing a sort of a frightened expression in the patient. This sign will disappear when the euthyroid state is restored. On the other hand, proptosis of the eyes, lid swelling, redness or edema of the conjunctiva and ocular pain or diplopia suggest associated orbitopathy (GO) and this needs to be accurately diagnosed and classified. Approximately one third of patients with GD have some evident clinical signs and/or symptoms of GO.

Most of the systemic effects of hyperthyroidism disappear when the euthyroid state is achieved. Interestingly, increasing age was associated with reduced adjusted odds ratio for the presence of most classical symptoms, except for weight loss and shortness of breath, and smokers had increased odds ratios for most symptoms (Boelaert *et al.*, 2010).

Mortality is increased in both treated and untreated hyperthyroidism and mainly in males (Schwensen *et al.*, 2017) and is related to the cumulative periods in which serum TSH concentrations are low. This implies that it may not be caused by the fact that the disease has not been treated, but rather to the inability to keep patients euthyroid during the course of therapy (Lillevang-Johansen *et al.*, 2017).

There is no clear evidence for direct toxic effects of elevated thyroid hormones on the liver, although a mild elevation of transaminases is common during the initial phase of the disease. Thus, liver function should be assessed at least once before starting thyrostatic drugs in order to be able to properly monitor the safety of the treatment. Serum alkaline phosphatase concentrations may correlate with serum T3, with a pattern showing an equal distribution of the bone and the liver isoenzyme. After therapy, serum alkaline phosphatase tends to increase, and the bone isoenzyme prevails, probably due to the skeletal repair (Cooper *et al.*, 1979). Patients with even mild increases in thyroid hormone lose bone mass, especially if postmenopausal and not receiving estrogen therapy. Severe and premature osteoporosis might be seen in disease with a longer duration or not properly treated. Even if the skeletal mass is recovered with therapy, bone density in not usually restored in the elderly (Cooper *et al.*, 1979). Hypercalcemia and hypercalciuria are common in hyperthyroid patients, but usually only moderate. While serum magnesium concentrations may decrease, serum phosphate concentrations are generally higher. Red blood cells may be microcytic, but anemia is usually not seen. Mild thrombocytopenia, due to shortened platelet survival may occur: if persistent after restoration of euthyroidism, autoimmune thrombocytopenia should be excluded.

Diagnosis

When only some of the signs and symptoms described above are present it may take long to consider a thyroid disease as the cause of the clinical manifestations. It is the combination of several signs and symptoms and their persistence that will lead the physician

to suspect hyperthyroidism. All patients with suspected hyperthyroidism should undergo a comprehensive history (including iodine intake or exposure, drugs, family history for thyroid and autoimmune diseases) and physical examination, including measurement of pulse rate, blood pressure, respiratory rate, and body weight. In addition, thyroid size, presence or absence of thyroid tenderness, symmetry and nodularity; pulmonary, cardiac, and neuromuscular function and presence of eye signs or pretibial myxedema should be assessed (Bahn *et al.*, 2011).

When thyrotoxicosis is suspected, the biochemical diagnosis is straightforward and characterized by elevated serum concentrations of T4 and T3 in the presence of low or undetectable serum TSH concentrations. Usually, both serum free T4 and T3 concentrations are elevated and in GD serum T3 is relatively higher than serum T4.

In milder hyperthyroidism, as in the earliest stages of GD, only serum T3 concentrations may be elevated (T3 toxicosis).

The presence of prominent eyes and lid retraction, the anxious behavior, the increased volume of the neck, due to the presence of a goiter, signs of ocular involvement and a family history of autoimmune disease help distinguish GD from other forms of hyperthyroidism (e.g., toxic multinodular goiter, toxic adenoma) or thyrotoxicosis (e.g., silent or subacute thyroiditis, exogenous thyroid hormone use). The measurement of serum TSH-receptor autoantibodies (TRAb) will help confirm this diagnosis.

A recent study has shown that the measurement of TRAb for the diagnosis of GD, when compared to radioactive iodine uptake (RAIU) determination, reduced costs by 47% and resulted in a 46% quicker diagnosis (McKee and Peyerl, 2012). TRAb bioassays have a sensitivity of 96%–97% and a specificity of 99% for GD (Diana *et al.*, 2017).There are two standardized methods for measuring serum TRAb. Third generation TSH Binding Inhibiting Immunoglobulin (TBII) competition assays are very precise but unable to distinguish the TSH-R antibody subtypes (stimulating or inhibitor). Newer bioassays for the Thyroid Stimulating Immunoglobulin (TSI) specifically detect simulating antibodies by measuring the ability of TSI to increase the intracellular level of cAMP.

RAIU should only be performed when the diagnosis is not clear, despite clinical examination, thyroid function assessment and TRAb detection. The iodine uptake pattern in GD is diffuse, unless there are coexistent nodules or fibrosis. Technetium 99 may also be used. The total body radiation exposure with technetium is lower than that with 123 iodine scintiscans.

When RAIU is contraindicated, such as during pregnancy or breastfeeding, thyroid ultrasound is performed. Increased color Doppler flow may be helpful in confirming the diagnosis of thyroid hyperactivity. About 70% of patients with GD have been reported to exhibit a low thyroid echogenicity pattern at ultrasonography (Vitti *et al.*, 1992). A thyroid scan should be performed also in the presence of thyroid nodularity at the neck examination or at a thyroid scintigram.

Management

The first goal of the treatment is to promptly alleviate the signs and symptoms of thyrotoxicosis. Physicians should recommend to temporarily avoid pregnancy to all thyrotoxic fertile women and to quit smoking, especially in patients who have some degree of ocular involvement.

Beta-adrenergic blocking drugs are recommended in all patients with symptomatic thyrotoxicosis, especially in elderly patients and those with resting heart rates in excess of 90 beats per minute or coexistent cardiovascular disease. In the latter, reduction of intense activity is also suggested.

Anti-thyroid drugs such as methimazole (MMI), carbimazole (CBZ; rapidly converted in methimazole in the serum) and propylthiouracil (PTU) reduce the synthesis and the secretion of thyroid hormones.

Initial daily therapy with 10–30 mg of MMI or CBZ or with 300–400 mg of PTU will render most patients euthyroid in 4–6 weeks. PTU has a shorter duration of action and is usually administered two or three times daily. When euthyroidism has been achieved, the physicians can choose between progressively decreasing the dose ("titration method") or maintaining the same dose and adding L-thyroxine ("block and replace treatment").

MMI or CBZ should not be used during the first trimester of pregnancy, when PTU is preferred, in the treatment of thyroid storm, and in patients with minor reactions to MMI who refuse RAI therapy or surgery. The most common side effects during thyrostatic treatment are a skin rash and mild abnormalities of liver function tests. Interestingly, thyrotoxicosis itself can induce cutaneous irritation and mildly abnormal liver function tests in up to 30% of patients and this may be misdiagnosed as an adverse effect of antithyroid drugs. Drug-induced cutaneous reactions are more common with PTU or higher dose MMI (30 mg/day) compared with lower dose MMI (15 mg/day) and usually appear after a median of 18–22 days of treatment, significantly earlier than transaminase elevations (median 28 days). They usually spontaneously regress in a few days, thus can be managed by using anti-histaminic drugs and not discontinuing the thyrostatic treatment. PTU may cause transient elevations of serum aminotransferases in approximately one-third of patients. Significant elevations to threefold above the upper limit of normal are seen in up to 4% of patients taking PTU, more than observed with MMI. Hepatotoxicity is usually seen in the first 120 days of treatment.

Agranulocytosis is a very rare, but life-threatening, event induced by thyrostatic drugs.

It occurs usually in the first 90 days of therapy and it manifests with acute pharyngitis and fever. Prompt discontinuation of the medical treatment is mandatory: this will generally allow the leukocyte count to normalize within a week.

No consensus exists on the usefulness of periodic monitoring of WBC counts and liver function tests for predicting drug-induced adverse reaction. Thus, patients have to be warned of discontinuing medications and measuring WBC in case of fever and sore-throat during therapy and to assess liver function in case of pruritic rash, jaundice, light-colored stool or dark urine, joint pain, abdominal pain or bloating, anorexia, nausea, or fatigue. A recent study provided evidence that switching from one thyrostatic

drug to another is safe as far as the occurrence of minor side effects, but not in the case of agranulocytosis or other serious side effects, because of the risk of cross-reactivity between anti-thyroid medications.

Serum TSH, free T4 and T3 should be tested about 4–6 weeks after initiation of therapy, depending on the severity of the thyrotoxicosis, and the dose of medication should be adjusted accordingly. TSH may remain suppressed for several weeks after starting therapy, therefore serum T3 and T4 concentrations are better variables for monitoring therapy. Anti-thyroid treatment is usually continued for 12–24 months, after which time up to 50% of patients may achieve remission of hyperthyroidism.

Measurement of serum TRAb levels before withdrawing thyrostatic drugs is suggested in order to assess the chances of remission. GD is considered in a remission phase when serum TSH, free T4, and T3 concentrations remain normal for 1 year after the discontinuation of anti-thyroid therapy. The remission rate varies considerably between geographical areas. A lower remission rate has been described in men, smokers (especially men), and those with large goiters (>80 g). Higher initial doses of MMI (60–80 mg/day) do not improve remission rates, while they increase the risk of side effects: thus they are not recommended.

During disease remission, thyroid function should be tested at 2- to 3-month intervals for the first 6 months, then at 4- to 6-month intervals for the following 6 months, and then every 6–12 months in order to eventually detect relapse as early as possible. The patient should be counseled to contact the treating physician if symptoms of hyperthyroidism are recognized or in case of an upcoming pregnancy. In general, factors that may predict remission are: a decrease in goiter size, normalization of serum TSH levels during treatment, decline or negativity of serum TSH receptor antibodies titers, and the patient not smoking.

If hyperthyroidism persists after 18–24 months of medical treatment or relapses soon after a period of remission, a definitive treatment by surgical thyroidectomy or radioactive iodine ablation (RAI) is suggested. In elderly patients, or in people with contraindication to RAI and surgery, a long term medical treatment may be continued as the only treatment option. Definitive treatment is also suggested in women planning a pregnancy: near-total or total thyroidectomy may be indicated if the woman desires pregnancy within 6 months, RAI ablation can otherwise be programmed at least 12 months before in particular situations.

The long-term quality of life (QoL) following treatment for GD was found to be the same in patients randomly allocated to either of the available treatment options. The goal of RAI therapy in GD is to control hyperthyroidism by rendering the patient hypothyroid; this treatment is very effective, provided that a sufficient radiation dose is taken up by the thyroid. This can be accomplished equally well by either administering a fixed 131-iodine activity or by calculating the dose based on the size of the thyroid and its ability to trap RAI. Low activities are not recommended in order to avoid more than one cycle of treatment. Thyrostatics must be withdrawn 2–3 days before RAI in order not to reduce the success rate of the treatment. A special diet is not required before RAI therapy, but nutritional supplements that may contain excess iodine and seaweeds should be avoided for at least 7 days.

RAI can induce a transient exacerbation of hyperthyroidism due both to follicular damage and increase of TRAb levels. Moreover persistent hyperthyroidism has been found to be associated with a long-term increase in cardiovascular and cerebrovascular deaths (Boelaert *et al.*, 2013). Thus, using beta-blockers or resuming anti-thyroid drugs 3–7 days after RAI might be considered in the presence of severe hyperthyroidism, in elderly patients and when severe comorbidity is an issue.

A recent meta-analysis found no increase in overall cancer risk after RAI treatment for hyperthyroidism, thus this treatment is a safe option. However, a trend towards increased risk of thyroid, stomach, and kidney cancer was also recently reported (Hieu *et al.*, 2012). Conception after RAI ablation should be delayed for at least 6–12 months and, anyway, until stable euthyroidism is achieved. In men, a delay of 3–4 months is suggested, to allow for turnover of sperm production.

Thyroid function must be assessed within the first 30–45 days after RAI and then monitored at 4- to 6-week intervals for 6 months, or until the patient becomes hypothyroid and is stable on thyroid hormone replacement. Hypothyroidism may occur from 4 weeks on, with 40% of patients being hypothyroid by 8 weeks and $>80\%$ by 16 weeks (Stan *et al.*, 2013). Since TSH may take longer to normalize or increase, FT4 should be the variable to be assessed also during the patients' follow-up and when serum FT4 concentrations fall below normal range, L-thyroxine replacement treatment should be commenced. Overt hypothyroidism should be avoided, especially in patients with GO, since it may trigger disease reactivation. If hyperthyroidism persists after 6–12 months following one cycle of RAI therapy, retreatment may be advised.

Surgical ablation is generally preferred for patients with larger goiters, in the presence of multinodular goiter or when the patients require a quick restoration of euthyroidism.

Patients should be rendered euthyroid prior to the surgical procedure with thyrostatic treatment, with or without beta-adrenergic block. Total thyroidectomy has a nearly 0% risk of recurrence, whereas near total thyroidectomy may result in 8% risk of persistence or recurrence of hyperthyroidism at 5 years (Guo *et al.*, 2013). The most common complications of total thyroidectomy are hypocalcemia due to hypopara-thyroidism, recurrent or superior laryngeal nerve injury, postoperative bleeding, and complications related to general anesthesia.

After thyroidectomy, L-thyroxine is started at a daily dose appropriate for the patient's weight and age, and serum TSH concentrations are first measured 6–8 weeks postoperatively. PTH and calcium levels need to be monitored in all patients immediately after surgery. The detection of low intact PTH concentrations (<10–15 pg/mL) in the immediate postoperative setting appears to predict symptomatic hypocalcemia and need for calcium and calcitriol (1,25 vitamin D) supplementation. Thus, postoperative routine supplementation with oral calcium and calcitriol is continued until serum calcium concentrations remain normal over a 24-h period, upon withdrawal.

In the pediatric population anti-thyroid drugs are the first-line treatment and MMI is preferred, since PTU is associated with an increased risk of hepatotoxicity, particularly in children. In case of disease relapse or persistent hyperthyroidism, RAI or surgical ablation are suggested following the same indications adopted for deciding therapy in the adult patients.

See also: Antithyroid Drugs. Graves' Disease. Graves' Orbitopathy. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Postpartum Thyroid Dysfunction. Pretibial Myxedema. Radioactive Iodine. Thyroid Autoimmunity. Thyroid Imaging. Thyrotoxicosis; Systemic Manifestations

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Pretibial Myxedema☆

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Glossary

Autoimmunity Dysregulation of the immune system leading to recognition of "self" components (autoantigens), which are attacked by autoantibodies and/or autoreactive T lymphocytes.

Glycosaminoglycans (GAGs) Long unbranched polysaccharides consisting of a repeating disaccharide unit. Hyaluronic acid is the GAG most commonly found in thyroid dermopathy.

Graves' disease Condition caused by anti-TSH receptor antibodies leading to increased secretion of thyroid hormones by the thyroid gland.

Graves' orbitopathy The most common extrathyroidal manifestation of Graves' disease characterized by inflammation of the peri- and retro-orbital tissues with forward displacement of the eye globe and altered ocular motility.

TSH receptor (TSHR) Receptor for thyroid-stimulating hormone (TSH) secreted by the pituitary; through binding to its receptor, TSH stimulates thyroid hormone synthesis and secretion.

Ultrasonography (US) Is a noninvasive procedure that can measure skin thickness, identifying two skin layers, the epidermis, dermis and subcutaneous tissue.

Introduction

Pretibial myxedema is an infiltrative lesion of the derma and the subcutaneous tissue, associated with autoimmune thyroid diseases (Smith *et al.*, 1989; DeGroot, 2000–2015), typically localized within the skin of the pretibial region, but occasionally described also in other areas of the limbs, such as the toes or the radial skin (Forgie *et al.*, 1994; Rice *et al.*, 2010; Singh *et al.*, 1985; Hasani-Ranjbar and Mohajeri-Tehrani, 2008). It was firstly described by Von Basedow in 1840 as a nonedematous fattening of the lower parts of the legs in a patient with thyrotoxicosis. The prevalence of pretibial myxedema has been estimated to be 0.5%–4% in patients with Graves' disease, and as high as 15% in those who have severe Graves' orbitopathy (Fatourechi, 2005) and markedly elevated serum TSH receptor antibodies (TRAb).

Etiology

Although the pathogenesis of pretibial myxedema is not known, it is thought that both mechanical and immunological factors may contribute to the pathophysiology of this disorder (Bahn, 2010). Prolonged standing and trauma to soft tissues may act as a trigger for the induction of pretibial myxedema, and once the immune reaction has been activated, the infiltrating T lymphocytes may lead to the release of cytokines, which then stimulate cell proliferation, the synthesis of glycosaminoglycans and the expression of immunomodulatory molecules (Bahn, 2010). Pretibial myxedema arises from the deposition of glycosaminoglycans, namely hyaluronic acid and chondroitin sulfate, either in the affected skin derma or in the surrounding tissues. Accordingly, the accumulation of these highly hydrophilic compounds produces edema and consequently the disruption of the dermal collagen and elastic fibers. This results in malfunctioning of the subcutaneous lymphatic network (Bull *et al.*, 1993).

Clinical Description

Pretibial myxedema has been classified into three clinical forms:

1. Diffuse pretibial myxedema: presented as diffusely cutaneous, but not definitely elevated over the surface of skin, of one-third to three fourths of the lower leg or more. The swelling is hard and is characterized by hyperpigmentation and/or nonpitting edema of the skin associated with dilated follicular openings;
2. Nodular- or plaque-like myxedema: the nodule is a circumscribed, solid lesion with a diameter of <2 cm. It can be elevated over the skin but is definitely palpable. A plaque is a superficial and flat-topped protrusion over the surface of skin, may be the only lesion or a major lesion of plaque variant. Its diameter is generally >1 cm. and the color may be reddish or pigmented.
3. Elephantiasic myxedema: this is characterized by a dramatic diffuse enlargement of the lower extremities with plaques, nodules, or masses scattered on the smooth or polypoid or papillary surface (Lan *et al.*, 2014; Chung-Leddon, 2001). The lesions of pretibial

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This article is an update of Mark F. Prummel, Graves' Dermopathy, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 349-350.

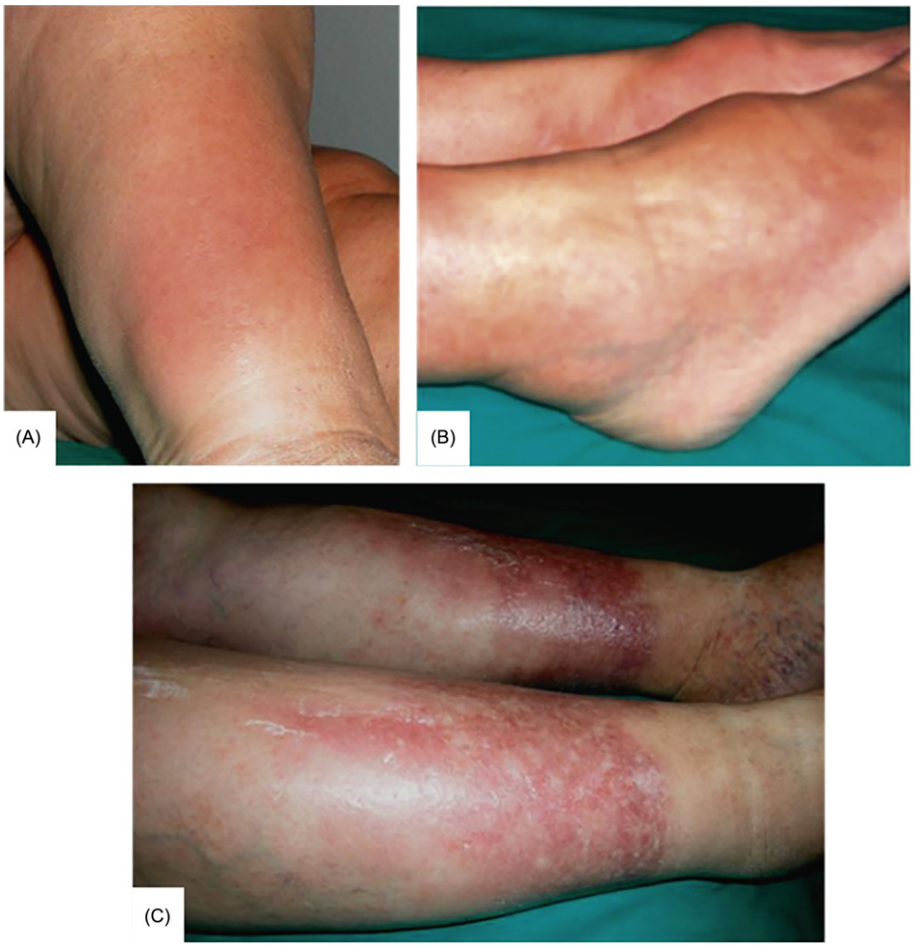

Fig. 1 Clinical appearance of pretibial myxedema (PTM). (A) Nodular-like PTM. The hyperemic nodule is localized on the right pretibial region. (B) Plaque-like PTM: bilateral myxedematous lesions located on the tarsal area. (C) Elephantiasic PTM.

myxedema are also characterized by morphological diversity, inflammation, infiltration, and hyperplasia. In most cases, pretibial myxedema is mild and subclinical (Fatourechi *et al.*, 1994) with only aesthetic implications (Ramos *et al.*, 2015). If lesions are extended, they may cause local discomfort and when the feet are also involved, difficulties with wearing shoes and walking may occur.

In **Fig. 1** the clinical appearance of pretibial myxedema.

Diagnosis

The diagnosis of pretibial myxedema is clinical and is based on the characteristic pretibial lesions, the presence of Graves' orbitopathy and history of thyrotoxicosis. Recently, ultrasonography (US) has become a noninvasive and simple procedure that can be used to accurately measure and study skin thickness. US is performed with a 13–16 MHz probe and two skin layers, one that includes the epidermis, dermis and of subcutaneous tissue and a second one that includes the subcutaneous tissue up to the muscular fasciae, are measured. The accuracy of this technique in patients has been validated by Salvi et al. (1994). A schematic representation of the skin layers measured and a US image of a patient's myxedema identified as D1 and D2 are shown in **Fig. 2**. If the clinical diagnosis of pretibial myxedema is uncertain and US imaging is not helpful, a confirmatory biopsy may be performed (Schwartz *et al.*, 2002). Histologically, the dermal tissue is characterized by wide spaces between collagen bundles, under hematoxilin and eosin staining, and accumulation of abundant mucopolysaccharides acid, through Alcian blue, coloidal iron or toluidine blue staining. Normal or slightly elevated amounts of fibroblasts may also be observed (Fatourechi, 2005).

Treatment

Treatment is often not required because the lesions are usually asymptomatic and up to 50% of patients have been reported to achieve a complete spontaneous remission, after several years (Fatourechi, 2005).

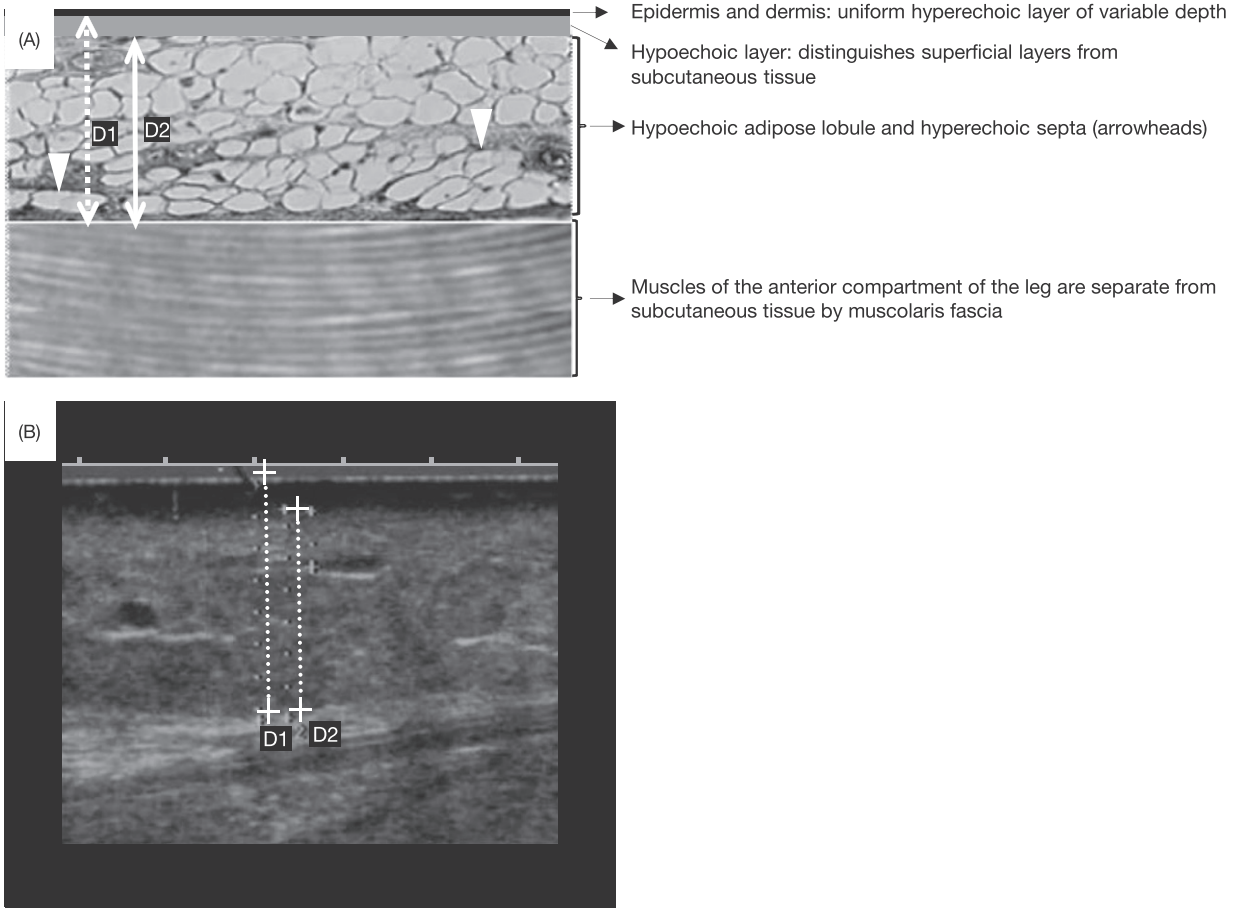

Fig. 2 (A) Schematic representation of skin layers. (B) US study by 13 MHz probe of the pretibial skin of a patient.

Only when there is local discomfort or the lesions become unsightly some treatment should be considered. In more severe cases, in which there is significant skin discomfort as well as a cosmetic issue, a number of therapeutic approaches have been proposed including compressive dressings with topical corticosteroids (Kriss *et al.*, 1967; Takasu *et al.*, 2010), intradermal injections of glucocorticoid (GC) (Fatourechi, 2005; Lang *et al.*, 1975) and hyaluronidase (Deng and Song, 2011; Rosman, 1950). Glucocorticoids have potent antiinflammatory properties, inhibiting hyaluronan synthesis and inducing skin atrophy (Stuhlmeier and Pollaschek, 2004; Gebhardt *et al.*, 2010). Hypodermal injections of triamcinolone have not been extensively used and evidence of a long-term efficacy of this therapy is lacking (Fatourechi, 2005). The occurrence of important adverse reactions, such as hypopigmentation, skin atrophy (Rosman, 1950; Friedman *et al.*, 1988) and lumpy skin (Takasu *et al.*, 2010) has been reported and may have hampered a more wide spread use of this treatment modality. An alternative route of steroid administration that may avoid the skin degeneration associated with hypodermal injection into the subcutaneous tissue is mesotherapy, also known as intradermotherapy. This treatment is carried out with needles 4–6 mm long and has been shown to induce significant and permanent reduction of pretibial dermal infiltration and improvement of the patient discomfort (Vannucchi *et al.*, 2013). By Cox's regression analysis models of the existing literature on pretibial myxedema treatment, Lan et al. have recently reported that pretibial myxedema variants influence the efficacy of intralesional steroid injection (Lan *et al.*, 2014). Nonsevere forms of pretibial myxedema generally respond well to therapy, while severe variants, such as giant plaque myxedema, are often resistant to treatment with intralesional steroids. In those cases, combined therapy with subcutaneous steroid injection could attain a therapeutic response satisfactory for patients (Chen *et al.*, 2016; Fatourechi *et al.*, 1994). More severe variants can even make patients disabled.

Conclusions

Pretibial myxedema is an uncommon dermopathy associated with autoimmune thyroid diseases. To date, it has been widely accepted that most cases of pretibial myxedema are asymptomatic and tend to resolve spontaneously. However, it is important to always examine the pretibial region of patients with Graves' disease to promptly diagnose the more severe forms of pretibial myxedema and eventually start the adequate treatment that can slow down the progression of this extrathyroidal manifestation.

See also: Graves' Disease. Graves' Orbitopathy. Hyperthyroidism in Graves' Disease. Thyroid Autoimmunity

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Graves' Orbitopathy☆

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Glossary

Apical crowding Enlarged eye muscles in the posterior part of the orbit, the apex, where the optic nerve leaves the orbital cavity; local pressure of the swollen eye muscles may compress the optic nerve, resulting in sight loss.

Conjunctiva Transparent mucous membrane covering the inner surface of the eyelids and the outer surfaces of the cornea and sclera.

Extraocular eye muscles Seven muscles behind each eyeball; four rectus muscles and two oblique muscles move the eyeball, and one (levator palpebrales) lifts the upper eyelid.

Lagophthalmos Incomplete closure of the eyelids, leading to exposure of the cornea during sleep.

Ocular torticollis Abnormal head posture to correct for double vision; typically, the head is tilted backward to correct for impaired elevation of the eyeballs.

Orbit A recess in the skull containing the eyeball and behind the extraocular muscles, vessels, nerves, and connective tissues; apart from the eyeball, the orbital cavity is completely surrounded by bones.

Proptosis Forward displacement of the eyeball; it is also referred to as exophthalmos.

Sclera White membrane surrounding the entire eyeball except for the anterior transparent part that is covered by the cornea.

Epidemiology

The incidence of GO, also known as thyroid-associated ophthalmopathy or endocrine ophthalmopathy is 42.2 per million per year (20.1% of the incidence of Graves' hyperthyroidism). The incidence of moderate-to-severe and very severe GO has been reported as 16.1 per million per year in the general population, with a female to male ratio of 5:1 and peak incidence between 40 and 60 years, whereas the prevalence of moderate-to-severe and very severe GO in patients with Graves' hyperthyroidism varies between 4.9% in Denmark and 6.1% observed in Italy (Perros *et al.*, 2017). A recent study comparing characteristics of referrals to EUGOGO centers, showed a trend toward less active and less severe GO in patients referred in 2012 as compared to 2000.

Pathology

GO is characterized by swelling of the extraocular muscles and of the retrobulbar fat and connective tissues. The normally ribbon-like eye muscles, with a total volume of 3.5 mL, can become grossly (up to fourfold) enlarged (**Fig. 1**). This increase in volume occurs in the bone-confined space of the orbital cavity and therefore the increase of the inflamed orbital tissue causes forward displacement of the eyeball. The volume of the orbit is 26 mL, and it has been shown that an increase in muscle volume of only 4 mL will lead to a proptosis of 6 mm. Although all eye muscles can become enlarged, the inferior and superior rectus muscles are preferentially affected, followed by the medial and lateral eye muscles. The reason for this difference is unknown. In addition to the swelling of the eye muscles, an increase in the volume of retrobulbar fat and connective tissues is frequently seen. The disease is usually bilateral, but in 15% one eye seems preferentially involved. However, on computer-assisted tomography (CT) scanning, bilateral involvement of the eyes is frequently observed in these patients. True unilateral eye disease is rare and occurs in only 5% of patients. These patients often have no evidence of Graves' thyroid disease (Euthyroid GO) (Perros *et al.*, 2017), making it difficult to establish the diagnosis. In many instances, bilateral eye disease and hyperthyroidism will develop later during follow-up.

Etiology

Like Graves' hyperthyroidism, GO is an autoimmune disorder with a multifactorial background. The majority of patients have family members afflicted by Graves' hyperthyroidism, but a family history of orbitopathy itself is uncommon.

☆*Change History*: March 2018. The m/s has been prepared by Mario Salvi; new paragraphs: abstract end epidemiology; paragraphs expanded: pathology, relation with hyperthyroidism; the classification of GO according to the EUGOGO Consensus and GUIDELINES has been introduced in the clinical diagnosis and management paragraphs; the reference list has been updated.

This article is an update of Mark F. Prummel, Graves' Ophthalmopathy, In Encyclopedia of Endocrine Diseases, edited by L Martini, Elsevier, New York, 2004, Pages 357–363.

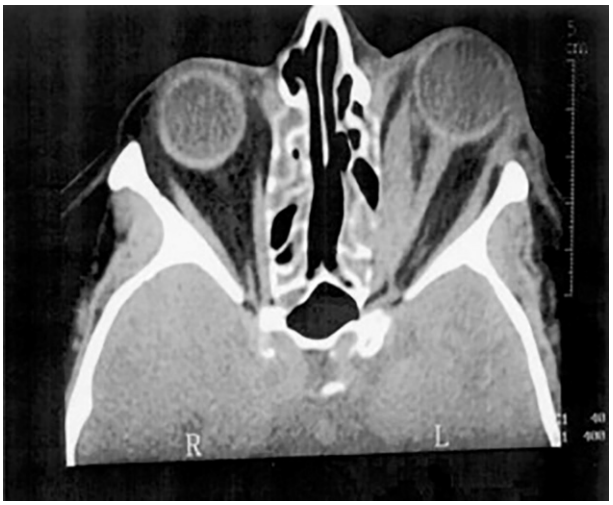

Fig. 1 CT scanning of the orbits of a patient with unilateral GO. The eye muscles in the right orbit are small and ribbon-like, whereas one eye muscle (the medial rectus) on the left is obviously swollen.

Certain exogenous and environmental factors are necessary to bring about the eye disease in patients with (a genetic predisposition for) Graves' hyperthyroidism. No genetic loci have been found to be associated with an increased risk for orbitopathy, with the possible exception of a CTLA-4 polymorphism (Smith and Hegedüs, 2016).

Pathogenesis

The main players involved in the pathogenesis of GO, in the so called "active phase," in which disease progresses to cause increasing orbital involvement with deterioration of visual function and of the patient's quality of life, are basically three: (1) the antigens expressed on the target organ of inflammation, namely the thyrotropin receptor (TSH-R) and the insulin-like growth factor I receptor (IGF-IR) on the fibroblasts; (2) the cytokines and other humoral factors involved in the various stages of disease progression; and (3) the immune effector cells, B, and T cells (Salvi, 2014).

Early inflammatory changes are characterized by sparse mononuclear cell infiltrates within the muscle endomysium and the connective tissue. The majority of cells are T lymphocytes, CD4+ or CD8+, and B lymphocytes are also observed. HLA-DR expression by interstitial cells—including fibroblasts, but not muscle fibers—are observed in both early and late stages. As lymphocytes, plasma cells and macrophages increase in number, fibroblasts proliferate producing collagen and mucopolysaccharides. Muscles become enlarged and edematous and fibrous with an increase in fat content, mucin, and water. Proliferation of fibroblasts subsequently leads to scarring and muscle fibrosis and sclerosis. Generally muscle fibers are spared in GO.

The TSH-R is uniquely targeted in Graves' disease by pathogenic autoantibodies known as thyroid-stimulating immunoglobulins (Smith and Hegedüs, 2016). These autoantibodies are detected in the serum of patients with Graves' disease with or without orbitopathy. The TSH-R has been found expressed in orbital tissues and this is suggestive of its potential contribution to the pathogenesis of GO. Antibodies to the TSH-R are not always detectable in patients with GO and therefore it is plausible that other autoantigens are also involved. IGF-IR has been shown to be overexpressed by orbital fibroblasts and by T cells and B cells in GD. Immunoglobulins that activate the IGF-IR signaling have been detected in patients with GD, and may synergistically enhance the effect of TSH-R antibodies on the orbital target antigens.

A subpopulation of fibroblasts (preadipocytes and/or mesenchymal stem/stromal cells) may differentiate into adipocytes. Orbit connective/adipose tissue and extraocular muscles are particularly rich in glycosaminoglycans (GAGs) mainly chondroitin sulphate and hyaluronan (HA), an especially hydrophilic molecule. HA synthases are strongly expressed by orbital fibroblasts and upregulated in the presence of various cytokines. Enlargement of intraorbital tissues is largely accounted for by the accumulation of GAG and edema within the connective tissue, both within and outside the muscles.

Relationship With Graves' Hyperthyroidism

GO is usually seen in patients with Graves' hyperthyroidism. In the majority of patients, the eye disease develops concomitantly with or some months after the onset of the thyroid disease. However, in 25% of patients, the eye disease precedes a diagnosis of hyperthyroidism by a number of months. At times, there may be considerable delay between the onset of the two manifestations, even of several years. In a small minority (5%), patients with orbitopathy present with primary hypothyroidism instead of hyperthyroidism. The pathogenesis of this thyroid failure is unclear but may be due to so-called TSH-blocking autoantibodies

against the TSH receptor. These antibodies render the thyroid gland devoid of the stimulating effect of TSH, resulting in atrophy and hypothyroidism.

Three situations/factors appear to precipitate the occurrence of GO: (1) Treatment of hyperthyroidism with radioiodine can potentially worsen GO especially in smokers and in the presence of markedly elevated T3 levels and in patients with shorter disease duration. It is likely that GO deterioration results from exacerbation of thyroid immune reactions, as evidenced by the rise in blood of circulating anti-TSH receptor antibodies (TRAb) which occurs 3–5 months after radioiodine. (2) Iatrogenic hypothyroidism has been recognized as a risk factor for GO. (3) Smoking has been identified as a strong risk factor for the development of this eye disease. Smoking increases the risk of orbitopathy 7.7-fold and is especially associated with more severe eye disease and resistance to therapeutic interventions. The reason behind the influence of smoking on the eye disease is unknown.

Clinical Manifestations

GO manifests with clinical heterogeneity and the signs and symptoms may be summarized in the so-called NO SPECS classification (**Table 1**). This classification system was first developed in 1969, was subsequently revised on a number of occasions, and now serves as a good memory aid. According to EUGOGO (Bartalena *et al.*, 2016), the following criteria of disease severity have recently been proposed:

Mild GO: Patients whose features of GO have only a minor impact on daily life insufficient to justify immunosuppressive or surgical treatment. They usually have one or more of the following: minor lid retraction (<2 mm), mild soft tissue involvement, exophthalmos <3 mm above normal for race and gender, no or intermittent diplopia, and corneal exposure responsive to lubricants.

Moderate-to severe GO: Patients without sight-threatening GO, whose eye disease has sufficient impact on daily life to justify the risks of immunosuppression (if active) or surgical intervention (if inactive). They usually have two or more of the following: lid retraction >2 mm, moderate or severe soft tissue involvement, exophthalmos >3 mm above normal for race and gender, inconstant or constant diplopia.

Sight-threatening GO (very severe GO): Patients with dysthyroid optic neuropathy (DON) and/or corneal breakdown.

Class 1: Only Signs, No Symptoms

This refers to the upper eyelid retraction frequently observed in patients with Graves' hyperthyroidism. The lid retraction causes stare and lid lag on downward gaze (Von Graefe's sign) and can be due to swelling of the superior levator muscle. However, thyrotoxicosis per se can also induce this sign by increasing the sympathetic tone, so Von Graefe's sign is sometimes also present in hyperthyroidism not caused by Graves' disease. Sympathetic overactivity is not the only cause of lid retraction given that upper eyelid retraction frequently remains present when orbitopathy patients are rendered euthyroid. If severe, lid retraction may lead to lagophthalmos, that is, incomplete closure of the eyelids at night.

Class 2: Soft Tissue Involvement

This entails chemosis (edema of the conjunctiva), conjunctival injection and redness, swelling of the caruncle, and swelling of the upper and lower eyelids (periorbital swelling). These findings are partly explained by impaired venous drainage as a result of the increase in volume of the retrobulbar tissues. Periorbital swelling is also due to herniation of retrobulbar fatty tissues through openings in the orbital septum covering the retrobulbar cavity.

Table 1 NO SPECS classification of eye changes in GO

Class	*Description*	*Signs and symptoms*	*Cause*
0	No signs or symptoms	—	—
1	Only signs, no symptoms	Lid retraction, stare, lid lag	Increased sympathetic tone
2	Soft tissue involvement	Swelling of eyelids, chemosis, photophobia, grittiness	Impaired venous drainage, herniated orbital fat
3	Proptosis	Exophthalmos	Increased retrobulbar pressure pushing globe forward
4	Extraocular muscle involvement	Restricted eyeball motility (often with diplopia)	Swollen eye muscles
5	Corneal involvement	Keratitis, corneal ulcer	Overexposure of cornea
6	Sight loss	Decreased visual acuity due to optic nerve involvement, impaired color vision, visual field defects	Pressure on optic nerve, apical crowding

Class 3: Proptosis

Because of the confining bony orbital walls, the swollen retrobulbar tissues have no other outlet than pushing the globe forward. Hence, exophthalmos may be seen as "nature's own decompression."

Class 4: Extraocular Muscle Involvement

One can easily imagine that swelling of the normally very thin extraocular eye muscles leads to impaired mobility. If the impairment is asymmetrical, the patient will have double vision. However, if the impairment is symmetrical, no diplopia will occur. Sometimes, the patient will keep the neck in a certain position (usually bent backward) to correct for impaired motility. This so-called ocular torticollis may lead to painful neck muscles.

Class 5: Corneal Involvement

Exophthalmos, lid retraction, lagophthalmos, and less frequent blinking all contribute to an excessive exposure of the cornea to air that can lead to inflammation of the cornea (keratitis). Early signs are photophobia, a gritty sensation, intolerance to contact lenses, and blurred vision. This phenomenon is different from diplopia in that the abnormal images disappear after blinking.

Class 6: Sight Loss

Sight loss can occur if the enlarged eye muscles compress the optic nerve. This can occur in the apex of the orbital cavity where the optic nerve leaves the orbit. On CT scanning, no room is seen between the optic nerve and the swollen muscles; this is called "apical crowding." Early signs of optic nerve involvement are impaired color vision and visual field defects. This severe complication is more often seen in males and in patients without significant proptosis. In those patients, a tight orbital septum precludes forward displacement of the globe, causing a rise in retrobulbar pressure that is damaging to the optic nerve.

Quality of Life

In view of these many and different clinical manifestations, it is not surprising that patients suffer from a diminished quality of life. The changes in appearance as a consequence of proptosis and periorbital swelling can be profound. Diplopia will hamper many activities in daily life such as reading and driving. In fact, studies have found that even patients with mild to moderately severe eye disease already have a markedly decreased sense of well-being. They rate their degree of social and role functioning lower than do patients with other chronic diseases such as diabetes mellitus. Use of a disease-specific quality of life questionnaire developed with the aid of patient self-support groups has shown unequivocally that GO is a seriously disabling disorder. The disease leads to feelings of social isolation in as many as 40% of these patients. Half of the patients notice unpleasant reactions from others, and many do not want to appear in photographs. As a consequence, as many as 70% of patients with mild to moderately severe orbitopathy report a marked decrease in self-confidence.

Diagnosis

The diagnosis of GO is almost always clinical, at least in the bilateral forms, but it may be less obvious in patients with unilateral disease or in patients without a thyroid disorder. There are no diagnostic procedures that may unequivocally confirm a diagnosis of GO, but several imaging procedures may be helpful. CT scanning of the orbits typically will reveal swelling of the extraocular eye muscles, of the retrobulbar tissues, or of both (**Fig. 2**). Magnetic resonance imaging (MRI) will also help suggest edematous swelling, by recording a prolonged T2 relaxation time. Other diagnostic imaging procedures, including ultrasound or octreotide scintigraphy, have not proven to be accurate or are very expensive. However, enlargement of the eye muscles or connective tissues is not definitive proof of the existence of orbitopathy. Other diagnoses that should be ruled out are lymphomas or metastases of carcinomas to the orbit and the rare orbital pseudotumor. The combination of various signs and symptoms of GO, together with evidence of an underlying autoimmune thyroid disease, will help the physician make the correct diagnosis.

General Management

The first step in the management of a patient with GO is to achieve adequate control of the underlying thyroid disorder (Bartalena *et al.*, 2016). Restoration of the euthyroid state frequently brings about some degree of amelioration of inflammation and even of

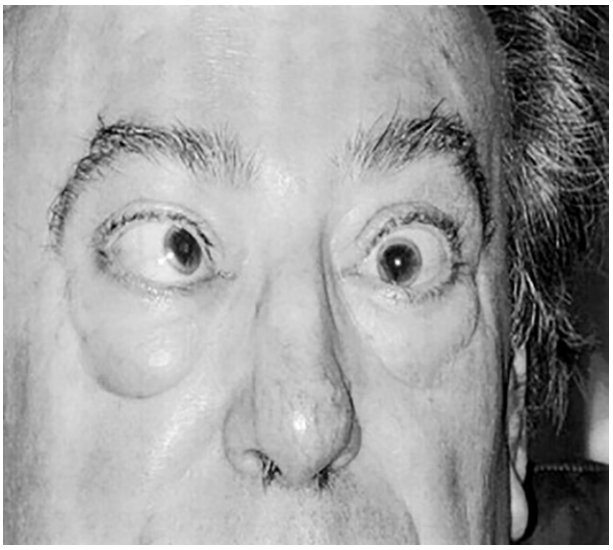

Fig. 2 A patient with severe GO. Note the abnormal eyeball position, resulting in double vision, swelling of the eyelids, increased lid aperture, and periorbital swelling.

diplopia in a few weeks. Generous application of lubricants (eye drops should be applied at least six times daily) prevents corneal damage and the use of a lacrimal gel at night protects the exposed cornea during sleep. Further therapeutic measures depend on the severity and stage of the eye disease.

Disease Activity

From observations by the Australian physician, F. F. Rundle, it has become clear that the disease has a tendency toward spontaneous improvement (**Fig. 3**). After reaching a peak in severity, the signs and symptoms gradually ameliorate over a highly variable period of time, from several months to a number of years. However, in most patients, a complete restoration to the premorbid state is hardly reached (Campi *et al.*, 2016). Immunosuppressive therapies are to be effective during the active disease stage only, whereas rehabilitative surgery is the treatment of choice for residual disease in the inactive stage. When symptoms and signs of inflammation are progressing and the eye condition is rapidly deteriorating, GO is in its active phase. When the eye signs have become stable for at least 6 months, GO can be considered inactive. However, in most patients with moderately severe disease, the assessment of disease progression relies on continuous monitoring of the eye changes on consecutive clinical examinations. A clinical activity score (CAS) has been developed by attributing a score to the clinical signs characteristic of the active phase of GO, upon clinical examination (**Table 2**).

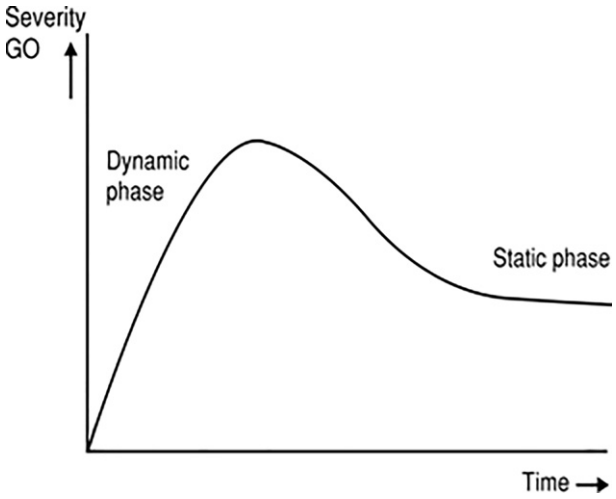

Fig. 3 Rundle's curve showing the natural history of GO and its tendency toward spontaneous improvement over a variable period of time. Note that the *x* axis (time) is not defined.

Table 2 The clinical activity score for assessing disease activity in GO

Parameter	*Points*
Pain or oppressed feeling on globe	1
Pain on attempted up, side, and/or down gaze	1
Redness of eyelids	1
Redness of conjunctiva	1
Swelling of eyelids	1
Swelling of caruncle	1
Chemosis	1
Active disease	$>2/7$

A CAS of three or more points will suggest active disease, if confirmed in two consecutive visits at 2–4 weeks-interval. Patients with active and progressive GO will be more likely to respond to immunosuppressive treatment, aimed at modifying the natural course of disease as shown by the Rundle's curve.

Management During the Active Stage

Mild GO

One option during the initial phase of the disease is to observe the patient over the course of several months. When GO is mild, the patient can simply be observed as the disease natural course is toward spontaneous improvement. Adequate antithyroid treatment and lubrication of the cornea will alleviate some of the symptoms until the disease has become inactive. In more severe cases, nonsurgical (immunosuppressive) treatment is indicated.

Moderate-Severe GO

To date, glucocorticoids (GC) represent the first line treatment for active moderate-severe GO, since they exert an antiinflammatory and, at high doses, also an immunosuppressive effect (Bartalena *et al.*, 2016). Response to treatment much relies on interindividual variability that leads to either treatment failure or drug induced toxicity. A significant response to therapy has been demonstrated for oral GC (prednisone). High doses of oral GC (daily administration of 50–100 mg of prednisone tapered down in 4–5 months) are effective on inflammatory signs of active GO in about 50%–60% of cases but the relapse of symptoms, after discontinuation or dose tapering, is not uncommon. Oral therapy is however more often associated to long term side effects, including hepatotoxicity, Cushing syndrome, osteoporosis, glaucoma, and diabetes mellitus. More recent studies have shown that pulsed intravenous methylprednisolone (ivMP) is more effective (70%–80% responders) and has a better safety profile compared with oral prednisone. A recent randomized study from EUGOGO has shown that a cumulative dose of 7.5 g of methylprednisolone is more effective in patients with moderate-severe GO than lower doses (5 g or 2.25 g, respectively), although it is associated with more frequent adverse events. Therefore high doses should be used in more severe cases of GO while intermediate doses (5 g) may be effective in most patients with moderate disease. A full cycle of treatment with ivMP should never exceed a total cumulative dose of 8 g. The limitation of ivMP treatment is that 20%–30% of patients are poorly responsive or unresponsive at all and that approximately 10%–20% of patients have disease relapse following drug withdrawal. The morbidity and mortality of GC therapy in GO patients have been estimated to 6.5% and 0.6%, respectively (Zhang *et al.*, 2011). Acute liver failure and cardiovascular events associated with GC administration are potentially fatal and careful monitoring during steroid therapy is mandatory. Marked increase of liver enzymes is the most common adverse event associated with ivMP, while acute liver failure seems caused by a direct toxic effect of GC on hepatocytes and appears to be dose-related. The onset of this damage is often not predictable, but preexistent viral hepatitis is probably associated with an increased risk. In patients with elevation of liver enzymes during ivGC therapy, the possibility of an autoimmune hepatitis should be ruled out.

Second line treatment is considered when patients do not respond to steroids or present with disease relapse after discontinuation of therapy. According to the recent EUGOGO Guidelines (Bartalena *et al.*, 2016), the decision on which therapeutic alternative is to be pursued in this situation is to be shared with the patient. This is because, among alternatives, the re-proposal of steroids, alone or in combination with other modalities of treatment, remains a possibility.

Retrobulbar irradiation has been used in active GO for many decades. Radiotherapy probably exerts its effect through the killing of infiltrating lymphocytes and activated (GAG-secreting) fibroblasts. The overall response rate is of approximately 65% and is effective mostly on periorbital swelling and inflamed extraocular muscle with a reduction of the degree of diplopia, but with only very little, and clinically insignificant, effect on proptosis. Radiotherapy is generally administered in 10 daily fractions of 200 cGy. Improvement of eye signs occurs earlier when corticosteroids are associated (usually some effect is seen within 2–4 weeks), whereas the beneficial effect of irradiation may take 3–6 months. The association with oral prednisone improves efficacy and the therapeutic response rate, although is almost always associated with side

effects. Combination therapy with ivMP has been reported more efficacious than radiotherapy alone, but it has not been challenged in randomized controlled trials. Radiotherapy causes no significant untoward effects and is safe in the long term, the only contraindication being diabetes mellitus, because irradiation can aggravate diabetic retinopathy.

Cyclosporine given as monotherapy is ineffective, but can be combined with oral prednisone, in which case a lower dose of prednisone can be used to achieve a similar response rate.

Many studies of B-cell function carried out in experimental animal models have provided evidence for their contribution to human autoimmune disease, recently reconsidered due to the therapeutic benefit of B-cell depleting therapies (Salvi *et al.*, 2015). Rituximab (RTX) is a humanized chimeric anti-CD20 monoclonal antibody whose variable (antigen-binding) region is derived from a mouse antibody that binds the CD20 antigen. It is approved for clinical use in non-Hodgkin's lymphoma and rheumatoid arthritis, but has recently been used off label in several other autoimmune diseases. The rationale of its use in GD is the blockade of the pathogenic TSH-R autoantibody by specifically targeting TSAb, the stimulating subpopulation of immunoglobulins targeting the TSH-R. The results of two randomized clinical trials employing RTX in GO have recently been published. One study showed GO inactivation after RTX in 100% of patients compared to 69% after IVMP and no disease relapse. The treatment was not effective on proptosis, but RTX proved to be more effective than steroids on motility and quality of life. Another study did not find RTX effective in treating active GO, when compared to placebo. Reasons for the conflicting results of these two studies are unknown, but differences in the duration of GO in the patients' population included may have had an important impact on the treatment outcomes (Stan and Salvi, 2017). The results of this work seem to suggest that RTX may act as a disease modifying therapy, compared to steroids. Further and larger randomized controlled trials are needed for definitive data on the potential disease modifying role of RTX and its superiority over standard treatment with steroids.

In vitro studies of orbital fibroblasts have shown that IGF-IR–targeting antibodies may inhibit the effect of IGF-I, thyrotropin, TSAb, and immunoglobulins isolated from patients with Graves' disease. Based on these data, a trial of teprotumumab, a fully human IGF-IR–inhibitory monoclonal antibody has recently been conducted and has shown promising results, especially in reducing the patients' proptosis. Patients on teprotumumab also showed reduction of the CAS, improvement of the quality of life (both the visual-functioning subscale and combined scales when compared to placebo). The marked reduction in proptosis was similar to that reported after orbital decompressive surgery. The findings suggest that therapy with teprotumumab in patients with active GO may modify the natural course of disease (Smith *et al.*, 2017).

Sight-Threatening GO

In patients with optic nerve involvement, characterized by apical crowding on CT scanning, visual field defects, and/or a decrease in visual acuity, a more aggressive approach is warranted. In those patients, methylprednisolone pulses can be administered (1000 mg intravenously on three consecutive days, repeated once 1 week later, followed by oral prednisone for 3–5 months), and this may even be combined with orbital irradiation. In case of an unsuccessful outcome, generally in about 60% of patients, acute surgical decompression of the orbits is the only option left.

Surgical Treatment

Once the disease has become inactive, most patients still experience disabling and often disfiguring symptoms, even after otherwise successful immunosuppressive therapy. These remaining manifestations can be treated successfully with various targeted surgical procedures by an experienced orbital surgeon (Bartalena *et al.*, 2016). The surgical procedures should be done in a strict order. First, an orbital decompression is performed if there is significant proptosis. During this procedure, several of the orbital walls are partly removed to increase the retrobulbar space. The degree of proptosis reduction varies with the number of walls removed. A three-wall decompression (coronal, transantral, and swinging eyelid approaches) typically results in a 6- to 8-mm reduction in proptosis. Complications include numbness of parts of the skin and worsening or even induction of diplopia. This latter complication occurs in 10%–20% of patients and is the reason why a decompression should always be done before squint (strabismus) surgery.

Squint surgery consists of a recession or resection of restricted extraocular eye muscles. The tendon of the muscle is severed from the eyeball and reinserted in a new position determined preoperatively during extensive orthoptic evaluations. Binocular single vision in all directions of gaze is seldom achieved, but single vision in the primary and reading positions is reached in 50%–80% of patients after one operation. Thus, frequently two or even more procedures are required to obtain an acceptable situation.

The final step in the rehabilitation of these patients consists of eyelid surgery. Upper eyelid retraction can be treated by recessing the levator muscle with or without a sclera interpositioning. Upper eyelid surgery is difficult, and in most patients various procedures (under local anesthesia) are needed. Lower eyelid surgery is often successful after just one attempt. Finally, redundant connective and fatty tissues can be removed by a blepharoplasty of the upper and/or lower eyelids.

See also: Epidemiology of Thyroid Disease. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Thyroid Autoimmunity. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Smoking and the Thyroid☆

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Glossary

Chronic autoimmune thyroiditis (Hashimoto' thyroiditis) Autoimmune disease characterized in many instances by goiter in the presence of euthyroidism or hypothyroidism, and circulating thyroid autoantibodies to thyroglobulin and/or thyroid peroxidase.

Graves' disease Autoimmune disease typically characterized by diffuse goiter, eye involvement (Graves' orbitopathy), and, less frequently, dermopathy (pretibial myxedema). It is caused by thyroid-stimulating hormone receptor antibodies.

Thyroid hormones Hormones secreted by the thyroid gland, represented mostly by thyroxine (T4) and triiodothyronine (T3).

Effects of Smoking on Thyroid Function

Several studies have been carried out to ascertain whether smoking is associated with variations in thyroid homeostasis. Two large surveys (the NHANES III survey in the USA and the 5th Tromso study in Norway) showed that smokers have lower serum thyrotropin (TSH) concentrations than nonsmokers (Belin *et al.*, 2004; Jorde and Sundsfjord, 2006). In the Tromso study higher serum free thyroxine (FT4) and free triiodothyronine (FT3) in smokers were also reported (Jorde and Sundsfjord, 2006). In another study from Norway (HUNT study) active smokers had lower serum TSH levels than former smokers (Asvold *et al.*, 2007). In addition, there might be an inverse relationship between the number of daily cigarettes and TSH concentration, because heavy smokers tend to have lower serum TSH levels compared to mild-to-moderate smokers (Asvold *et al.*, 2007). Whether passive smoking may be associated with similar changes is unsettled. Conversely, smoking withdrawal is associated with a small and slow decrease in serum FT4 and a small and slow increase in serum TSH concentrations. This might suggest that cigarette smoking stimulates thyroid function, which would then be readjusted after smoking cessation (Wiersinga, 2013). One possible explanation for the above changes might reside with stimulation of the sympathetic nervous system, leading to stimulation of thyroid hormone secretion, in turn decreasing TSH release (Bartalena *et al.*, 1995). It should be mentioned that results in the literature are not, however, unequivocal, and several factors may influence the results, such as the inclusion of heavy versus moderate smokers, the evaluation of short-term versus long-term effects, or differences in age and body mass. In addition, the presence of substances in cigarette smoke that stimulate drug metabolism might also account for a decrease in thyroid hormone levels, since induction of liver microsomal enzymes involved in the metabolism of thyroid hormones might result in an enhanced hormonal catabolism (Bartalena *et al.*, 1995; Wiersinga, 2013). In summary, cigarette smoking seems to be associated with overall minor and controversial changes in thyroid function tests, whose pathophysiological relevance seems to be marginal. The most frequently observed change is a decrease in serum TSH concentration, reversible after smoking cessation (**Table 1**).

Smoking and Goiter

Several reports have documented that smoking is associated with an increased prevalence of goiter (Hegedus *et al.*, 1985; Berthelsen and Hegedus, 1994; Knudsen *et al.*, 2002; Vestergaard, 2002) (**Table 1**). In addition, it was shown that heavy smokers have an increased frequency of nodular goiter. These findings may be related to iodine intake, because many of these studies were performed in iodine-deficient areas, whereas no significant differences in the prevalence of goiter among smokers and nonsmokers were found in iodine-sufficient areas. It is likely that cigarette smoking represents only a cofactor and its goitrogenic effect becomes more apparent when other goitrogenic factors, particularly iodine deficiency, are also present.

It is not completely clear how cigarette smoking can contribute to the development of goiter, but a candidate goitrogen in smoke is thiocyanate, produced by detoxification of hydrogen cyanide. The role of thiocyanate in the pathogenesis of endemic goiter has been clearly shown in the presence of iodine deficiency (Wiersinga, 2013). Body fluids of smokers contain increased thiocyanate concentrations. The increase in the thyroid volume/birth weight ratio in newborns parallels the increase in cord serum thiocyanate levels, taken as an index of maternal smoking habits, suggesting that smoking during pregnancy may be a relevant cause of thyroid gland enlargement in the newborn. The effects of thiocyanate and other cigarette smoking products (nicotine,

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Table 1 Smoking and the thyroid

	Effect	*Relevance*
Thyroid function	• Increase in serum free thyroid hormone and decrease in serum thyrotropin concentrations	Marginal
Goiter	• Goitrogenic effect, increase in multinodularity	Relevant cofactor in iodine-deficient areas
Thyroid cancer	• Reduced risk of thyroid cancer in current smokers	Possible protective effect (related to decreased TSH?)
Graves' hyperthyroidism	• Increased risk of occurrence of Graves' hyperthyroidism in smokers • Increased risk of recurrence of hyperthyroidism after antithyroid drug withdrawal	Relevant
Graves' orbitopathy	• Increased prevalence of smokers among patients with orbitopathy • Increased risk of developing severe forms • Decreased and slower response to immunosuppressive treatment	Relevant
Chronic autoimmune thyroiditis	• Lower prevalence of thyroid autoantibodies in smokers • Protective role against progression to hypothyroidism	Doubtful

cotinine) have also been studied in vitro using porcine thyroid follicles in culture; while nicotine and cotinine did not inhibit iodide transport or thyroid hormone synthesis, thiocyanate, at concentrations equivalent to those reached in the serum of smokers, inhibited iodide transport and iodine organification while increasing iodide efflux (Bartalena *et al.*, 1995). The possible role of thiocyanate is supported also by studies showing that serum thyroglobulin is increased concomitantly with a slight decrease in serum TSH concentration among smokers in a population living in a mild iodine deficiency area (Hegedus *et al.*, 1985). In addition to underscoring the role of thiocyanate, these findings may explain the interaction of cigarette smoking and iodine deficiency in the development of goiter.

In summary, it appears that smoking plays a role in the development of goiter and most reports agree on a higher prevalence of goiter, particularly multinodular goiter, in smokers (**Table 1**). Although it is conceivable that many others (and yet unidentified) smoke products may contribute, thiocyanate appears, for the time being, to be the most likely culprit, in view of the high circulating levels found in smokers and in view of its complex effects on thyroid function. Smoking-related goitrogenesis appears to be frequent, particularly in iodine-deficient areas, where smoke may also represent a relevant cause of neonatal thyroid enlargement.

Smoking and Thyroid Cancer

Cigarette smoking seems to be associated with a decreased risk of thyroid cancer, particularly the papillary histotype. In a study of 90,713 Radiologic Technologists followed for 24 years, smokers had a low hazard ratio of 0.54 (95% CI, 0.35, 082) of developing compared to nonsmokers, while the risk in former smokers (0.91, 95% CI, 0.67, 1.22) did not differ from that of nonsmokers (Meinhold *et al.*, 2009). Likewise, in a pooled analysis of 14 case-control studies, thyroid cancer risk was reduced in current smokers (odd ratio, 0.6; 95% CI, 0.6, 0.7), but not in former smokers (Mack *et al.*, 2003). Finally, in a large a pooled analysis of five prospective USA studies involving 413,979 women and 434,953 men, the risk of thyroid cancer increased with body mass index (BMI), and this effect was even stronger in never smokers (Kitahara *et al.*, 2011) Because an increase in BMI is associated with an increase in serum TSH, and the latter is associated with an increased risk of thyroid cancer, it might be conceived that the protective effect of smoking be related to the TSH-lowering effect of smoking (see previous paragraph).

Smoking and Thyroid Autoimmune Disorders

Cigarette smoking has a number of immunological effects involving both humoral and cellular components of the immune system. These include a depression of natural killer cell activity, an increase in the total number of T lymphocytes, with a relative decrease in $CD4^+$ (helper) and increase in $CD8^+$ (suppressor) subpopulations in heavy smokers (Bartalena *et al.*, 1995). Serum immunoglobulin (Ig) G, IgM, and IgA levels are decreased by 10%–20% in the serum of smokers, whereas IgE levels are increased in light-to-moderate smokers and decreased in heavy smokers (Bartalena *et al.*, 1995). A lower level of immunosuppression has been observed in mice as an effect of smoking. The numerous immunological effects of smoking may have some relevance in human pathophysiology. For example, it has been shown that, although smoking is associated with Crohn's disease, ulcerative colitis is strongly associated with not smoking. The underlying mechanism might not necessarily be related to immunological effects of smoking, but to substances contained in smoke that might be somehow beneficial to ulcerative colitis patients. An association between rheumatoid arthritis and cigarette smoking, as well as an increased prevalence of antinuclear antibodies in smokers, has been reported.

Graves' Hyperthyroidism and Orbitopathy

Graves' disease is an autoimmune disease contributed by both genetic and environmental factors (Bartalena, 2013). The latter may include stressful life events and infections. Smoking represents an additional and important environmental factor (Prummel and Wiersinga, 1993). An increased prevalence of smokers has been observed in patients with Graves' disease and smoking increases the risk with an odd ratio of 3.30 (2.09–5.22) (Prummel and Wiersinga, 1993; Bartalena *et al.*, 2000). Smoking cessation seems to reduce the risk of occurrence of Graves' hyperthyroidism. In addition, smoking increases the risk of recurrence of hyperthyroidism following antithyroid drug treatment withdrawal (Glinoer *et al.*, 2001). No association between smoking habits and toxic nodular goiter has been found.

Why should cigarette smoking be associated with an increased risk of developing Graves' disease? Smoking might simply be a coincidental, unrelated factor and Graves' patients might smoke more because they are nervous and stressed. Stress is indeed associated with an increased desire to smoke. It should be mentioned that, in most cases, Graves' patients smoked prior to the development of hyperthyroidism. Alternatively, smoking might enhance other activities or factors that bear the true responsibility for the occurrence of the disease. However, the possibility cannot be excluded that smoking may be effectively involved in the pathogenesis of the disease. This might occur through different smoke-related immunological mechanisms, such as an alteration of the TSH receptor structure, an enhancement of responsiveness to other factors responsible for the initiation of the disease, or an impairment of restoration of tolerance to thyroid autoantigens. Evidence that these hypothetical mechanisms participate in the development of Graves' disease is lacking.

Graves' orbitopathy is the most frequent extrathyroidal manifestation of Graves' disease and represents a complex therapeutic problem (Bartalena and Fatourechi, 2014). A possible relationship between cigarette smoking and Graves' orbitopathy was initially suggested in a small series of patients and subsequently confirmed in a large number of patients in different series (Bartalena *et al.*, 2000). Furthermore, there seems to be a relationship between smoking and disease severity (Prummel and Wiersinga, 1993). Interestingly, the current number of daily cigarettes smoked, but not lifetime tobacco consumption, appears to be an independent risk factor, and ex-smokers have a lower risk of developing severe Graves' orbitopathy than current smokers with a comparable lifetime tobacco consumption (Bartalena *et al.*, 2000). Interestingly, the prevalence of Graves' orbitopathy in newly diagnosed Graves' patients seems to be declining in recent years: a decrease in the proportion of smokers in the population might be a relevant factor for this trend (Tanda *et al.*, 2013). This association between cigarette smoking and Graves' orbitopathy is unexplained. In addition to direct irritative effects and smoke-related increase in oxidative stress, cigarette smoking might influence ongoing immune reactions in the orbit. It is well accepted that cytokines play an important role in the pathogenesis of Graves' orbitopathy. Cigarette smoking may affect the process, because smoking-induced hypoxia in the retrobulbar tissue increases the release of cytokines from orbital fibroblasts in vitro. Furthermore, it has been reported, but not unequivocally confirmed, that smokers, compared to nonsmokers, have lower circulating levels of soluble interleukin-1 (IL-1) receptor antagonist levels, an anti-cytokine antagonizing the effects of IL-1.

Cigarette smoking may also affect treatment outcomes of Graves' orbitopathy (**Table 1**). A more favorable response to immunosuppressive treatment for moderate-to-severe Graves' orbitopathy is more frequent in nonsmokers than in smokers. In addition, smokers may have a delayed response to treatment. In patients with nonsevere Graves' orbitopathy, radioiodine treatment may cause a progression of eye disease in approximately 15% of cases; postradioiodine progression is more likely to occur in smokers, whereas improvement of preradioiodine orbitopathy with the concomitant glucocorticoid treatment is more frequent in nonsmokers (Bartalena *et al.*, 1998).

In summary, although the mechanisms whereby cigarette smoking operates remain to be elucidated, the relationship between smoking, Graves' hyperthyroidism, and Graves' orbitopathy is well established, in terms of the epidemiology of the disease and its influence on the severity of the disease and the efficacy of its management. Accordingly, Graves' orbitopathy patients should be vigorously encouraged to refrain from smoking, even though it remains to be proven that smoking withdrawal beneficially affects the course of the disease.

Chronic Autoimmune Thyroiditis

Chronic autoimmune thyroiditis is the most common cause of spontaneous hypothyroidism in the adults. Several studies have shown that, at variance with Graves' disease and orbitopathy, smoking exerts a protective effect against hypothyroidism of autoimmune origin (**Table 1**). In the large HUNT study from Norway, the odds ratio for the occurrence overt and subclinical hypothyroidism in women were 0.60 (0.38–0.95) and 0.54 (0.46–0.66), respectively; figures were even lower in men (Asvold *et al.*, 2007). Three studies from the Netherlands, Denmark, and Iran showed that smokers have a lower prevalence of thyroid autoantibodies (anti-thyroid peroxidase and/or anti-thyroglobulin) than nonsmokers (Wiersinga, 2013). Smoking cessation has been associated with an increased risk of occurrence of positive tests for thyroid autoantibodies and development of autoimmune hypothyroidism. However, the risk of hypothyroidism after smoking withdrawal seems to be transient and disappears after 3 years or more after smoking cessation. Although results are not unequivocal, in most studies smoking apparently does not play a relevant role in the occurrence of postpartum thyroid disorders.

Mechanisms whereby smoking may have a protective effect on chronic autoimmune thyroiditis and related hypothyroidism remain obscure. They might, however, be related to receptor-mediated effects of nicotine or other tobacco alkaloids, causing a shift from Th1 and Th17 responses to Th2 responses.

Concluding Remarks

The relationship between cigarette smoking and the thyroid is complex and not yet completely understood. Marginal changes in thyroid function have been described in smokers, but they are unlikely to have a relevant pathophysiological significance. More clearly established is the goitrogenic effect of smoking, probably related to the action of thiocyanate (and possibly of other compounds liberated in smoke). This effect might be particularly important in iodine-deficient areas. An interesting observation is the high prevalence of smokers among patients with Graves' disease, but it is unclear whether this has a pathogenic importance or merely represents an epiphenomenon of behavioral changes related to thyroid hyperfunction. Even more striking is the association between smoking and Graves' ophthalmopathy. Although the underlying mechanisms need to be elucidated, smoking might play a role in the pathogenesis of Graves' ophthalmopathy and unfavorably affect its course. Conversely, cigarette smoking seems to play a protective effect on chronic autoimmune thyroiditis (and related hypothyroidism) and thyroid cancer.

See also: Environmental Goitrogens. Nontoxic Goiter. Thyroid Peroxidase

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Toxic Adenoma☆

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Glossary

Autonomy The ability of thyrocytes to function and produce thyroid hormones (thyroxine and triiodothyronine) without stimulation from thyrotropin.

Constitutive thyrotropin (TSH) receptor activation State of activation of the TSH receptor in the absence of TSH caused by a mutation in the TSH receptor gene.

^{131}Iodine (^{131}I) A radioisotope of iodine.

Scintiscan Radionuclide scanning of the thyroid after administration of technetium (as isotope ^{99}Tc in the form of pertechnetate), or iodine (as isotope ^{123}I or ^{131}I), used for a functional assessment of the thyroid's ability to take up iodine.

Sodium-iodine symporter (NIS) A transporter of the basolateral membrane of thyroid follicular cells that facilitates uptake of iodine by active transport of two sodium cations and one iodide anion.

Thyrocyte Epithelial cells within the thyroid gland that are responsible for thyroid hormone synthesis. Also known as follicular cells.

Thyrotoxicosis Also known as hyperthyroidism. Systemic alteration of metabolism in which increased levels of thyroid hormone in the serum lead to biochemical and/or clinical signs of excess thyroid hormone at the tissue level.

Definition

The term solitary toxic adenoma refers to a benign monoclonal thyroid tumor with autonomous thyroid hormone production. This term is interchangeable with "toxic thyroid nodule" and "hot nodule." Increased thyroid hormone synthesis from these nodules leads to suppression of thyroid-stimulating hormone (TSH) and thyrotoxicosis.

Introduction

Epidemiology

Individuals with solitary toxic adenoma may present with a wide spectrum of disease, ranging from subclinical hyperthyroidism (suppressed TSH with normal levels of triiodothyronine and/or thyroxine) to overt thyrotoxicosis. Solitary toxic adenoma may occur as a solitary nodule within an otherwise normal thyroid gland (solitary toxic adenoma) or as multiple nodules in the setting of a multinodular goiter (also known as toxic multinodular goiter or abbreviated as TMG). For the purposes of this discussion, the term "solitary toxic adenoma" can be assumed to refer to a solitary toxic nodule.

The overall prevalence of both solitary toxic adenoma and TMG is inversely correlated with the iodine consumption of the population. Recent data suggests that solitary TA accounts for 5%–10% of cases of hyperthyroidism while toxic MNG represents 6%–47%; both conditions are relatively more common in regions with mild or moderate iodine deficiency (Carlé *et al.*, 2011; Laurberg *et al.* 2010). In populations that are iodine sufficient, solitary toxic adenoma has a similar prevalence to TMG, and represents the second most common cause of hyperthyroidism after Graves' disease (Laurberg *et al.*, 2010). Solitary toxic adenoma predominantly affects older individuals; increased incidence is observed after the sixth decade (Carlé *et al.*, 2011). While it is a common cause of hyperthyroidism in adults, this condition is exceedingly rare in children with only a few cases described in the literature (Grob *et al.*, 2014). Similar to other thyroid diseases, there is a female preponderance with a female to male ratio of 1.4:1 (Carlé *et al.*, 2011).

Etiology

Solitary toxic adenoma is understood to arise from acquired genetic mutations in the TSH receptor gene or the α-subunit of the G_s protein ($G_{s\alpha}$). TSH stimulates thyroid function by binding to the TSH receptor, which, through coupling to $G_{s\alpha}$, causes intracellular

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generation of the second-messenger cyclic AMP (cAMP) (**Fig. 1**). As demonstrated by in vitro cell culture experiments, TSH receptor mutations in solitary toxic adenoma cause constitutive activation (in the absence of the ligand TSH) of the cAMP signaling cascade. Stimulation of the cAMP cascade leads to increased iodide uptake by upregulation of the sodium iodine symporter (NIS), increased thyroid hormone synthesis, and growth of thyrocytes. Therefore, mutations in the TSH receptor gene or G_s protein ($G_{s\alpha}$) confer a growth and functional advantage to the affected thyroid cell leading to the development of an autonomously functioning thyroid nodule (**Fig. 2**) (Krohn and Paschke, 2001). Gradually, the increasing cell mass in the autonomously functioning thyroid nodule reaches a level of thyroid hormone production that exceeds the normal demand. Initially, this manifests as subclinical hyperthyroidism, but continued growth eventually results in biochemical hyperthyroidism and clinically apparent thyrotoxicosis (**Fig. 2**). Accordingly, the risk of hyperthyroidism is increased with larger nodule size (Sandrock *et al.*, 1993).

Activating mutations of the TSH receptor have also been implicated in familial non-immune hyperthyroidism and sporadic congenital non-autoimmune hyperthyroidism. In solitary toxic adenoma, the somatic mutation of the TSH receptor initially affects only a single cell, which undergoes clonal proliferation to ultimately form a hot nodule, whereas in the aforementioned disorders, a germline mutation in the TSH receptor affects all thyroid cells. **Fig. 3** shows a compilation of known somatic activating TSH receptor mutations in solitary toxic adenoma, which can be identified in up to 70% of cases (Trülzsch *et al.*, 2001; Gozu *et al.*, 2006). Mutations in $G_{s\alpha}$ have been detected in 1%–3% of cases. The cause of the remaining 20%–30% is currently unknown. Given the clonal origin of solitary toxic adenomas, there are presumably additional genetic mechanisms that have yet to be elucidated. Solitary toxic adenomas with known somatic TSH receptor or $G_{s\alpha}$ mutations lack a clear genotype/phenotype correlation, suggesting that their effects may be modulated by other mechanisms such as downstream signaling events, cross-talk with other signaling cascades, and negative feedback (Krohn *et al.*, 2005; Frenzel *et al.*, 2005; Kursawe and Paschke, 2007). Recently, mutations in the *EZH1* gene were found in 27% of solitary toxic adenomas and were strongly associated with those harboring TSH receptor mutations, suggesting a possible "two hit" model whereby *EZH1* mutations promote additional growth in the setting of underlying constitutive activation of the cAMP pathway (Calebiro *et al.*, 2016).

Diagnosis

Patients with toxic adenoma may present with signs and symptoms of hyperthyroidism or mass effect from a large nodule or goiter. Typically, the patient will present with features suggestive of hyperthyroidism, including anxiety, hyperactivity, weight loss despite increased appetite, palpitations, tremor, and heat intolerance (**Table 1**) (Ross *et al.*, 2016). Of note, these classical symptoms of hyperthyroidism are frequently absent in elderly patients with solitary toxic adenoma or TMG (Thomas *et al.*, 1970). Conversely, individuals may notice a palpable or visible lump in the neck, or experience mechanical symptoms such as difficulty swallowing or shortness of breath, due to local compression of the esophagus or trachea, respectively (Haugen *et al.*, 2016).

All patients presenting with a thyroid nodule should undergo a complete history and physical examination of the thyroid gland and cervical lymph nodes. The focus of the history and physical should be to assess the patient for signs and symptoms of hyperthyroidism, look for clues as to the etiology of hyperthyroidism, assess for obstructive symptoms, and evaluate the patient for their risk of thyroid malignancy.

As per clinical practice guidelines, upon confirmation of the presence of a thyroid nodule by neck ultrasonography, all patients with a nodule should have an evaluation of serum TSH (Haugen *et al.*, 2016). If the TSH value is subnormal or low normal, the

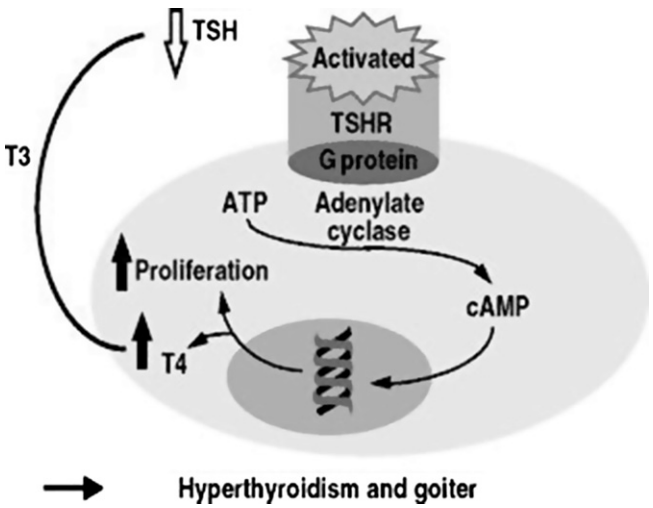

Fig. 1 Signal transduction in an autonomously functioning thyroid cell. The cAMP cascade is stimulated by the constitutively activated TSH receptor (or less frequently by the $G_{s\alpha}$ protein). cAMP stimulates thyroid function and growth, ultimately resulting in a phenotype of hyperthyroidism and goiter.

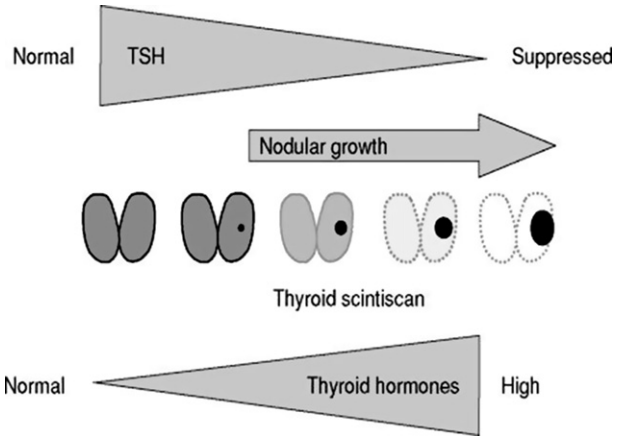

Fig. 2 Development of thyroid autonomy. A thyroid cell with a somatic gain-of-function TSH receptor mutation has a growth and functional advantage over unaffected thyrocytes. The autonomous nodule causes increased thyroid hormone synthesis independent of the TSH level. If the nodule reaches a critical mass, TSH Is suppressed and thyrotoxicosis becomes clinically apparent. On scintiscan, the toxic adenoma appears as a "hot" nodule due to increased NIS expression within the nodule with suppression of surrounding thyroid tissue.

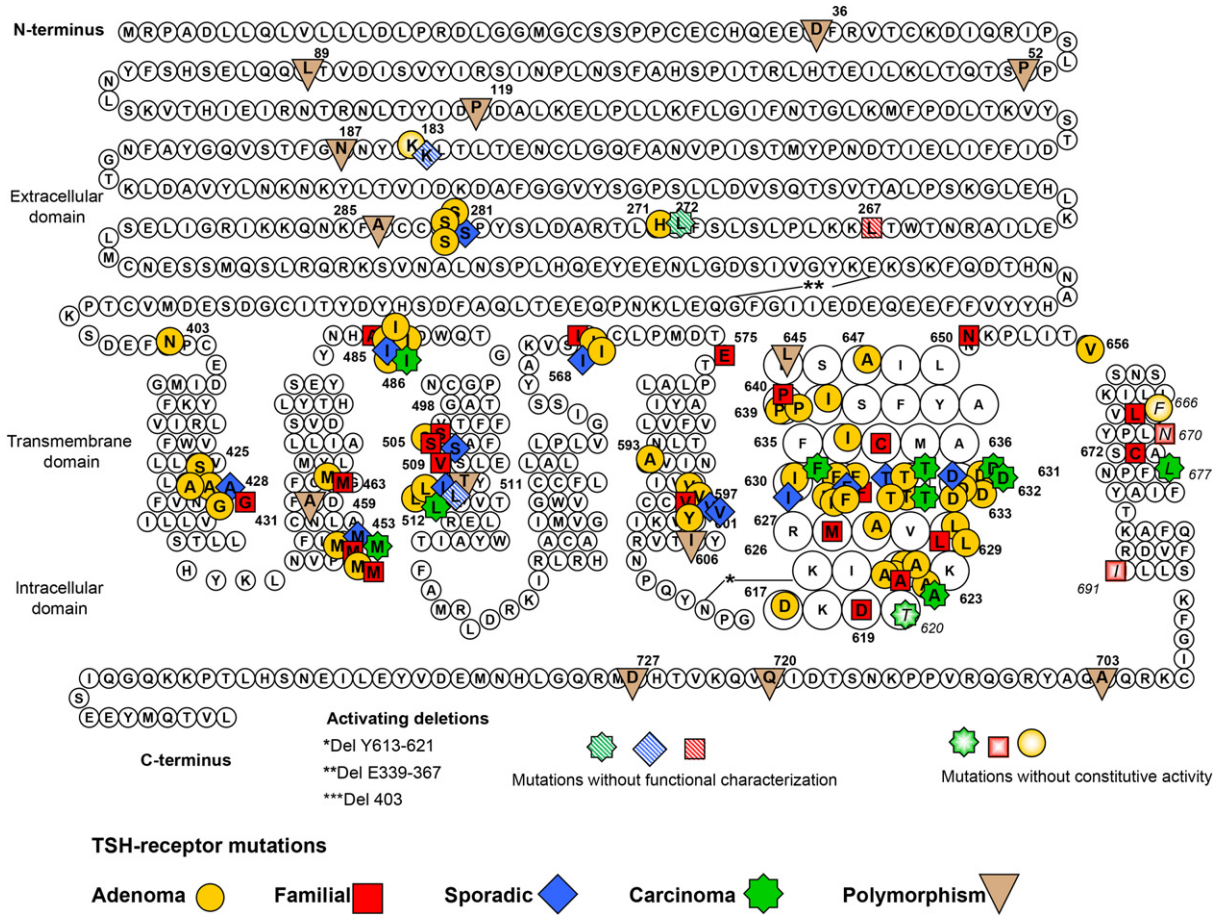

Fig. 3 All constitutively activating TSHR mutations and polymorphisms (nonfunctional variants) according to TSHR database (http://tshreceptor-mutation-database.org/) and all constitutively activating TSHR mutation publications since the 2012 update of the TSHR mutation database published as of 12/2017according Google Scholar and PubMed using the search terms "TSHR mutation" and "thyrotropin receptor mutation." Mutations are characterized with at minimum basal and TSH stimulated cAMP but ideally with linear regression analysis (LRA) of constitutive activity as a function of TSHR expression determined by 125I-bTSH binding or FACS analysis compared to the wild-type. Those without functional characterization are included based on their association with a clinical phenotype only.

Table 1 Clinical manifestations of thyrotoxicosis

History	*Physical exam*
Anxiety	Hyperactivity
Fatigue	Tachycardia/arrhythmia
Increased perspiration	Systolic hypertension
Heat intolerance	Warm, moist skin
Tremor	Tremor
Hyperactivity	Hyperreflexia
Palpitations	Proximal muscle weakness
Appetite change (usually increased)	
Weight loss	
Menstrual disturbances	

Note: Classic features of hyperthyroidism are often absent in the elderly.

next step is radionuclide scanning to determine whether the nodule is "hot" (i.e., displaying increased tracer uptake relative to surrounding tissue). Hot nodules very seldom harbor malignancy (<1% of patients), therefore, cytologic evaluation is not required in these cases, however, this risk can be as high as 29% in children (Haugen *et al.*, 2016; Pazaitou-Panayiotou *et al.*, 2012; Eszlinger *et al.*, 2014).

Diagnosis of a toxic adenoma is based on the presence of three characteristics: (1) thyroid function tests in keeping with overt hyperthyroidism (suppressed TSH, elevated thyroid hormones [triiodothyronine (T3) and (T4)]), or subclinical hyperthyroidism (suppressed TSH, T3, and T4 normal), (2) presence of a palpable and/or sonographically localized thyroid nodule (3) increased radioiodine or ^{99m}Tc-pertechnetate uptake in the nodule concomitant with decreased uptake in the surrounding extranodular thyroid tissue. (**Fig. 4**). If these three features are met, then one can conclude that an autonomous functioning nodule is the cause of hyperthyroidism, and the diagnosis of solitary toxic adenoma is confirmed. However, it should be noted that in some situations, patients with solitary toxic adenoma may have normal thyroid function tests; thyroid scintigraphy has been shown to be more sensitive than serum TSH for detection of solitary toxic adenoma (Ianni *et al.*, 2013).

Natural History

Longitudinal studies on patients with solitary toxic adenoma have demonstrated stability or insidious progression of nodule growth and development of hyperthyroidism. A previous study that followed 287 patients for up to 15 years demonstrated that only 9% of patients had interval nodule growth of >1 cm in size and 86% of patients had no detectable change in size (Sandrock *et al.*, 1993). Patients presenting with euthyroid solitary toxic adenoma show progression to thyrotoxicosis at a rate of approximately 4% per year. Risk of progression to hyperthyroidism is increased with older age, adenomas larger than 3 cm, and in individuals living in iodine deficient regions (Corvilain, 2003). However, as the driver of solitary toxic adenoma is an acquired mutation causing autonomy, spontaneous remission will not occur except perhaps in the rare scenario of infarction of the nodule. Therefore, once a solitary toxic adenoma causes hyperthyroidism, it is unlikely that the hyperthyroidism will spontaneously resolve and definitive treatment will be required.

Particularly in regions with iodine deficiency, exposure to excess iodine is a known precipitant of thyrotoxicosis in patients with solitary toxic adenoma or other conditions with thyroid autonomy. This clinical scenario can be seen following administration of iodinated contrast media used for computed tomography scanning or angiography. Thyrotoxicosis can occur weeks to months after the exposure to an iodine load and typically has a self-limited course; thyroid storm is rare (Lee *et al.*, 2015). If possible, administration of iodinated radiocontrast should be avoided in patients with overt hyperthyroidism or those at risk of serious decompensation should acute worsening of thyrotoxicosis occur. In these cases, prophylaxis using sodium perchlorate and a thionamide prior to radiocontrast administration should be considered (Ross *et al.*, 2016; Nolte *et al.* 1996; Lawrence *et al.*,1999; Vagenakis *et al.*, 1972). However, current guidelines recommend against routine prophylaxis for all patients after radiocontrast administration (Ross *et al.*, 2016).

Treatment

It is imperative that patients with solitary toxic adenoma be counseled about the advantages, disadvantages and potential risks of the available treatment modalities, discussed below. When deciding on a treatment, the clinician should take an individualized management approach with consideration of disease factors, comorbidities, and patient preference.

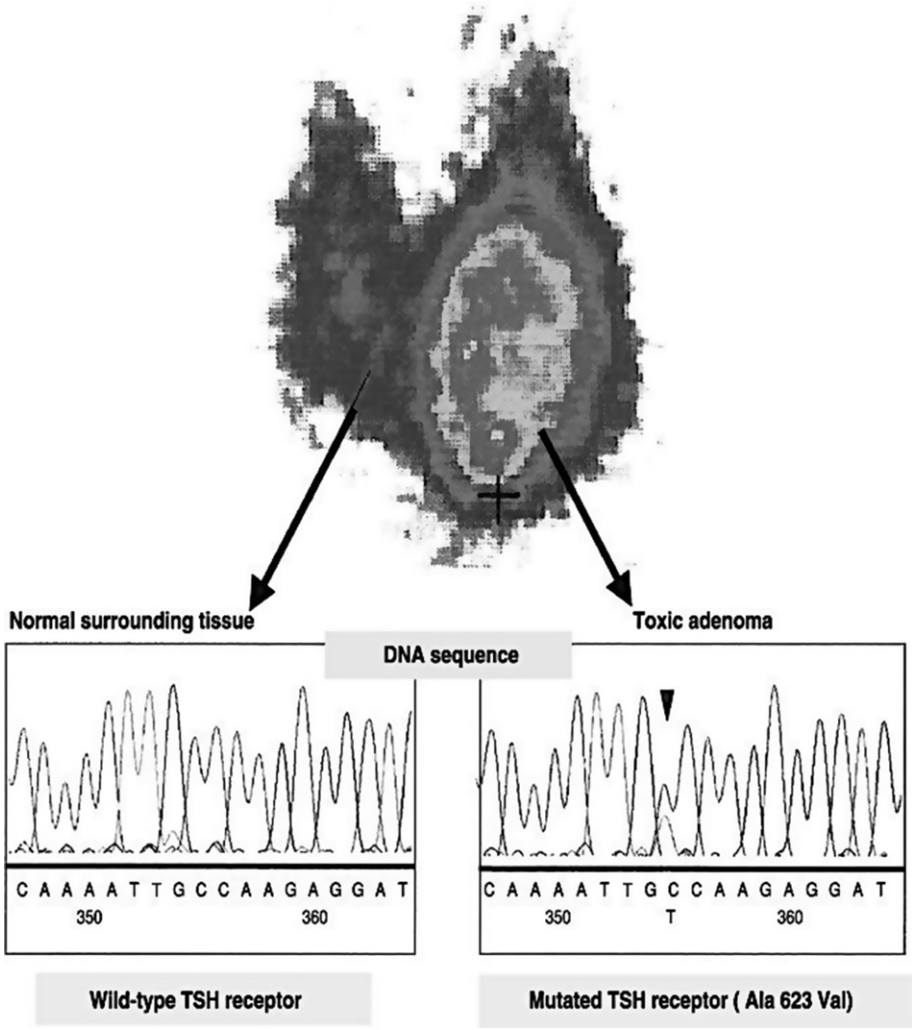

Fig. 4 Typical scintiscan finding of a TA that led to the notion of a "hot" nodule. A somatic gain-of-function TSH receptor mutation is the cause of a toxic adenoma. The mutation is present only in the hot nodule, whereas the wild-type TSH receptor sequence can be found in the normal thyroid tissue (hence the term somatic mutation).

Medical Therapy

In certain cases such as severe thyrotoxicosis or in patients at significant risk of complications due to worsening of hyperthyroidism, pre-treatment with thionamides and/or beta blockers should be considered. Thionamides such as carbimazole or methimazole should be used as first line. Beta blockers can be used as adjunctive therapy to control adrenergic symptoms such as anxiety, tremor, and palpitations. Achieving biochemical control of hyperthyroidism with a thionamide may take several weeks due to the 7 day long half-life of T4 and intra-thyroidal stores of thyroid hormone. Prior to initiation of therapy, patients should be counseled about common side effects including skin rashes (1%–5%) arthritis and rare but serious side effects including hepatotoxicity and agranulocytosis ($<0.5\%$) (Ross *et al.*, 2016).

Definitive Management

^{131}I therapy and surgery are the mainstays of definitive therapy. Alternative minimally invasive procedures are also now available, and these are discussed below. Therapy with ^{131}I therapy exploits the increased iodine uptake that is characteristic of solitary toxic adenoma, resulting in preferential accumulation of ^{131}I in the nodule and progressive destruction of the solitary toxic adenoma by ^{131}I beta radiation, with sparing of the surrounding normal thyroid tissue. ^{131}I can either be administered as an empiric fixed dose (10–20 mCi) or a calculated activity based on the size of nodule (150–200 μCi per gram) corrected for 24-h radioactive iodine uptake. The goal of treatment should be to administer a single dose of ^{131}I that will alleviate hyperthyroidism. ^{131}I therapy may lead to transient worsening of hyperthyroidism and swelling of the thyroid gland. Therefore, it should be used with caution in patients who are at risk of decompensation from cardiac disease, and in those with large goiters with compressive symptoms.

Following treatment with ^{131}I and depending on the treatment modalities, the cumulative incidence of hypothyroidism is 7.6% at 1 year, 46% at 10 years, and 60% at 20 years (Ceccarelli *et al.*, 2005). Patients who have received ^{131}I ablation should have follow-up thyroid function tests to monitor for the development of hypothyroidism. Radioiodine therapy results in the gradual resolution of hyperthyroidism, which occurs over weeks to months. Therefore, ^{131}I ablation is not the preferred treatment choice when immediate alleviation of hyperthyroidism is desired. Furthermore, ^{131}I is contraindicated in several clinical situations including pregnancy, lactation, women planning to conceive within the next 6 months, or in patients with known or suspected thyroid cancer (Ross *et al.*, 2016).

Surgery can provide rapid cure of thyrotoxicosis for patients with solitary toxic adenoma. The recommended surgical approach in solitary toxic adenoma is ipsilateral lobectomy, allowing the majority of patients to maintain euthyroidism post-operatively without thyroid hormone replacement. However, thyroid surgery is an invasive procedure that carries risks including permanent hypoparathyroidism (in case of total thyroidectomy) and vocal cord paralysis due to injury of the recurrent laryngeal nerve. Although these complications are rare in the setting of experienced, high volume surgeons, they should be discussed with patients prior to embarking on this treatment option. Relative contraindications to hemithyroidectomy include significant medical comorbidities precluding surgical candidacy or limited life expectancy. In patients that are poor surgical candidates, long-term treatment with low dose thionamides can be considered as an alternative to surgery or ^{131}I ablation (Ross *et al.*, 2016).

Alternative Treatment Approaches

Percutaneous ethanol injection involves several ethanol injections into the solitary toxic adenoma under ultrasound guidance by experienced clinicians. This procedure has good efficacy with an overall cure rate of $>90\%$ and a low complication rate (Tarantino *et al.*, 2008). A recent study evaluated radiofrequency ablation in a small multicenter cohort of 44 patients (Sung *et al.*, 2015). In this procedure, an electrode is inserted into the thyroid nodule under sonographic guidance, and delivery of thermal energy induces tissue necrosis. Normalization of thyroid function occurred in around 70% of patients and there were no major complications. Similarly, laser ablation can be used to treat solitary toxic adenoma by delivery of laser photons leading to rapid heating and necrosis, however, the efficacy of this approach has been inconsistent (Papini *et al.*, 2016). Cumulative experience with these novel alternative approaches is limited and as such, they should only be employed at specialized centers in circumstances where surgery and I^{131} are contraindicated.

See also: Radioactive Iodine. Thyroid Fine Needle Aspiration Cytology. Thyroid Imaging

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Toxic Multinodular Goiter☆

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Glossary

Autonomy The ability of thyrocytes to function and produce thyroid hormones without stimulation from thyrotropin.
Constitutive thyrotropin (TSH) receptor activation State of activation of the TSH receptor in the absence of TSH caused by a mutation in the TSH receptor gene.
Euthyroid goiters The most common form of thyroid enlargement without clinical or laboratory evidence of abnormal thyroid hormone production.
Goitrogens Substances such as food or drugs that disrupt normal thyroid hormone production, leading to increased stimulation and growth of the thyroid gland, that is, formation of a goiter.
Scintiscan Radionuclide scanning of the thyroid after administration of technetium (as isotope ^{99}Tc in the form of pertechnetate), or iodine (as isotope ^{123}I or ^{131}I), used for a functional assessment of the thyroid's ability to take up iodine.
Sodium-iodine symporter (NIS) A transporter found in the basolateral membrane of thyroid follicular cells that facilitates uptake of iodine by active transport via exchange of two sodium cations and one iodide anion.
T4 Thyroxine
T3 Triiodothyronine
Thyrocyte Epithelial cells within the thyroid gland that are responsible for thyroid hormone synthesis. Also known as thyroid follicular cells.
Thyrotoxicosis Also known as hyperthyroidism. A systemic alteration of metabolism in which increased levels of thyroid hormone production result in biochemical and/or clinical signs of excess thyroid hormone.
Toxic thyroid adenoma Benign monoclonal thyroid tumor with autonomous thyroid hormone production. This term is interchangeable with "toxic thyroid nodule" and "hot nodule."
TSH Thyroid-stimulating hormone; a pituitary hormone that regulates the functioning and growth of thyroid follicular cells.

Definition

Toxic multinodular goiter is a heterogeneous disorder, which is characterized by a mixture of thyroid nodules that are hyper-, iso-, and hypo-functioning by scintigraphic evaluation. The overall degree of thyroid autonomy in the gland determines the clinical status of the patient, which can range from euthyroidism to subclinical or overt hyperthyroidism.

Introduction

Goiter is a heterogeneous term that refers to diffuse or nodular enlargement of the thyroid gland, and can be associated with normal, increased, or decreased thyroid hormone production. Goiters are commonly seen in populations with mild to moderate iodine deficiency. The term toxic multinodular goiter more specifically refers to multiple autonomously functioning thyroid nodules on a background of iso- or hypo-functioning nodules. Toxic multinodular goiter arise from longstanding non-toxic multinodular goiters. In toxic multinodular goiter, the cumulative functional status from all nodules results in a net increase in thyroid hormone production, manifesting as either subclinical or overt hyperthyroidism (Krohn *et al.*, 2005).

Epidemiology

Recent data suggests that toxic multinodular goiter accounts for approximately 6% of all cases of hyperthyroidism in iodine sufficient populations (Laurberg *et al.*, 2010). However, the overall prevalence of toxic multinodular goiter is inversely correlated with the iodine status of the population, and in regions of iodine deficiency toxic multinodular goiter is the most common cause of hyperthyroidism, accounting for almost 50% of cases (Laurberg *et al.*, 2010; Carlé *et al.*, 2011). The introduction of iodized salt in previously iodine

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deficient regions has successfully led to a decline in the incidence of toxic multinodular goiter (Laurberg *et al.*, 2010). Toxic multinodular goiter is predominantly seen in older individuals; the median age of affected individuals is 75 years. A steep rise in incidence of toxic multinodular goiter is observed after the fifth decade, and continues to increase steadily with age thereafter. Similar to other thyroid diseases, toxic multinodular goiter has a female preponderance with a F:M ratio of 6:1 (Carlé *et al.*, 2011). Pazaitou-Panayiotou *et al.* (2012) reviewed the risk of malignancy in toxic multinodular goiter, and found a range in the literature from 1.8% to 8.8%. However, the common finding of incidental papillary microcarcinomas of the thyroid gland, along with the fact that several studies failed to accurately discriminate between malignancies occurring inside and outside of the hyperfunctioning nodule, has likely led to an overestimation of malignancy risk in toxic multinodular goiter. Given the overall low risk of malignancy in autonomous nodules, several guideline societies recommend against routine cytologic evaluation of these lesions (Haugen *et al.*, 2016; Gharib *et al.*, 2016).

Etiology

The development of toxic multinodular goiter is thought to occur in the following key steps: (1) iodine deficiency or exposure to other goitrogens leads to increased functional activity of the thyroid and thyrocyte hyperplasia; (2) nodular transformation occurs when increased proliferation and oxidative damage of thyrocytes induces somatic mutations of the TSH receptor or other genes that stimulate thyrocyte growth and/or function; (3) increased cell division, clonal formation and proliferation leads to subsequent formation of thyroid nodules (Krohn *et al.*, 2005). These steps are illustrated in **Fig. 1**, and will be discussed in detail below.

Previously multinodular goiter was regarded solely a consequence of iodine deficiency and persistent TSH elevation. Though iodine deficiency is recognized as the primary driver of goitrogenesis in most cases, TSH is not elevated in patients with multinodular goiter and several other contributing factors have been identified. Female gender has been associated with the development of multinodular goiter; this is thought to be due to the growth promoting effects of female sex steroid hormones on thyrocytes. Smoking has been implicated in multinodular goiter formation, presumably due to the competitive inhibition of the sodium iodine symporter (NIS) by the thiocyanate present in tobacco. Statins, lithium, and dietary goitrogens also appear to predispose individuals to developing multinodular goiter (Knobel, 2016). Numerous environmental chemicals are now recognized to disrupt thyroid function and subsequently contribute to multinodular goiter development; these are discussed in detail elsewhere.

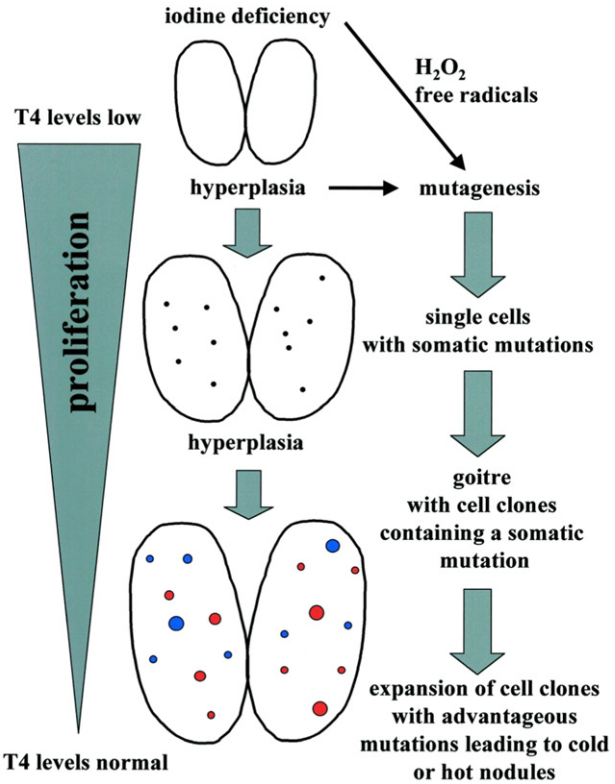

Fig. 1 Hypothesis for the evolution of clonal thyroid nodules in multinodular goiters. Hyperplasia due to iodine shortage increases the likelihood for somatic mutations that may confer a growth and functional advantage. Further expansion of cell clones ultimately results in "hot" and "cold" nodules that characteristically prevail together in multinodular goiters. Reproduced with permission from Krohn, K. and Paschke, R. (2001). Clinical review 133: Progress in understanding the etiology of thyroid autonomy. *Journal of Clinical Endocrinology & Metabolism* **87**(7), 3336–3345.

Individual genetic susceptibility is thought to play a crucial role in the development of multinodular goiter, as evidenced by familial clustering of goiters and higher concordance rates observed in monozygotic twins. Genes involved in thyroid hormone synthesis including thyroglobulin (TG), thyroid peroxidase (TPO), pendrin (PDS), NIS, and the TSH receptor have all been implicated as candidate genes. Genome wide linkage studies have identified several loci in families with multinodular goiter showing a dominant inheritance pattern (Neumann *et al.*, 1999; Bayer *et al.*, 2004; Krohn *et al.*, 2005). In summary, the development of multinodular goiter is likely a complex interplay of genetic and environmental factors, although the specific molecular mechanisms are not yet completely understood.

Exposure to iodine deficiency and the other aforementioned goitrogens leads to an increase in thyrocyte number and functional activity. Increased thyroid function induces activation of H_20_2, which is an important substrate in thyroid hormone synthesis. This causes increased generation of free radicals that may damage genomic DNA and subsequently cause spontaneous mutations and inhibit mutational repair (Krohn *et al.*, 2005). Some of these acquired mutations occur in areas such as the TSH receptor gene or in the α-subunit of the G_s protein ($G_{s\alpha}$) and confer a functional and/or proliferative advantage to the thyrocyte by causing a constitutive activation of the cAMP signaling cascade. Stimulation of the cAMP cascade leads to increased iodide uptake by upregulation of NIS, increased thyroid hormone synthesis, and growth of thyrocytes. Autonomous nodules are therefore likely to develop from small clones that contain these advantageous mutations within a multinodular goiter (Hébrant *et al.*, 2011). Over time, the autonomous function of these nodules may reach a level where net thyroid hormone production is increased causing subclinical or overt hyperthyroidism, leading to transformation of multinodular goiter to toxic multinodular goiter.

Natural History

Many patients with toxic multinodular goiter have a long-standing history of multinodular goiter, with evolution of thyroid autonomy over a number of years (Krohn *et al.*, 2005). The development of hyperthyroidism is insidious in onset, manifesting initially with subclinical hyperthyroidism and eventually progressing to overt hyperthyroidism. Longitudinal studies have demonstrated that around 10% of individuals with euthyroid goiter will progress to toxic multinodular goiter over a mean 12 year follow up period (Elte *et al.*, 1990; Wiener, 1987). The degree of thyroid autonomy is correlated with thyroid volume, which increases over time. Thus, advanced age is a risk factor for progression to toxic multinodular goiter (Carlé *et al.*, 2011; Krohn *et al.*, 2005). In rare cases, transient thyrotoxicosis due to hemorrhagic infarction can occur, followed by regression in size and autonomy (Hamburger and Taylor, 1979).

Autonomous function in patients with toxic multinodular goiter can be exacerbated by exposure to excess iodine, such as with iodinated contrast media in the setting of computed tomography scanning or angiography (Lee *et al.*, 2015). Thyrotoxicosis can occur weeks to months after the exposure to an iodine load and typically has a self-limited course; thyroid storm is exceedingly rare (Lee *et al.*, 2015). If possible, administration of iodinated radiocontrast should be avoided in patients with overt hyperthyroidism or those at risk of serious decompensation due to thyrotoxicosis. Alternatively, prophylaxis using sodium perchlorate and/or a thionamides prior to radiocontrast administration should be considered (Ross *et al.*, 2016).

Diagnosis

Patients with toxic multinodular goiter may come to the attention of physicians in several different ways. Firstly, they may present due to signs and symptoms of hyperthyroidism such as anxiety, tremor, palpitations, weight loss, heat intolerance, or increased frequency of bowel movements (summarized in **Table 1**). It is important to note that these classic symptoms of hyperthyroidism may be absent in the elderly (Thomas *et al.*, 1970). Secondly, subclinical or biochemical hyperthyroidism may be detected due to

Table 1 Clinical manifestations of thyrotoxicosis

History	*Physical exam*
Anxiety	Hyperactivity
Fatigue	Tachycardia/arrhythmia
Increased perspiration	Systolic hypertension
Heat intolerance	Warm, moist skin
Tremor	Tremor
Hyperactivity	Hyperreflexia
Palpitations	Proximal muscle weakness
Appetite change (usually increased)	
Weight loss	
Menstrual disturbances	

Note: Classic features of hyperthyroidism are often absent in the elderly.

routine screening of serum TSH. Alternatively, patients may present due to detection of a thyroid nodule or goiter by neck palpation, ultrasonography, or due to symptoms suggestive of obstruction, such as dysphagia, dyspnea, or cough. Therefore, the clinical pathway for the diagnostic workup of patients with toxic multinodular goiter will depend upon the presenting complaint.

In all cases, clinicians should begin their assessment with a detailed history and physical examination focused on delineating signs and symptoms of hyperthyroidism, obstruction, and risk factors for thyroid cancer. Physical examination of the thyroid may reveal diffuse, painless enlargement of the gland with multiple palpable nodules. Examination should also include palpation of the lower edge of the thyroid gland and percussion over the manubrium listening for dullness, to assess for retrosternal extension of the goiter.

Following this, patients should undergo measurement of serum TSH $+/-$ free T4 and free T3. Laboratory testing may reveal euthyroidism (normal TSH/T4/T3), subclinical hyperthyroidism (low TSH, normal T3/T4), or overt hyperthyroidism (low TSH, high T3/T4). Neck ultrasonography should be obtained to confirm the location, size, and number of thyroid nodules, the size of the gland, and presence of lymphadenopathy. Ultrasound of the thyroid is also a crucial step in risk stratification of thyroid nodules and determination of the need for fine needle aspiration biopsy. This is discussed in detail elsewhere.

If subclinical or overt hyperthyroidism exists in the context of solitary or multiple thyroid nodules, the next step is thyroid scintigraphy to determine the functional status of the gland and associated nodules. Scintigraphy also provides information about possible substernal extension of the goiter (Bahn and Castro, 2011; Becker *et al.*, 1996). Hyper-functioning nodules have a very low risk of malignancy, and fine needle aspiration biopsy of these lesions is not recommended (Pazaitou-Panayiotou *et al.*, 2012; Haugen *et al.*, 2016; Gharib *et al.*, 2016). As previously mentioned, toxic multinodular goiter is a heterogeneous condition and nodules can exhibit variable scintigraphic patterns including normal or decreased uptake, circumscribed areas of increased uptake with suppression of surrounding thyroid tissue, or areas of increased uptake without suppression of the surrounding parenchyma. In patients without complete scintigraphic suppression of the surrounding parenchyma, thyroid hormones may be administered prior to suppression scintigraphy in order to differentiate autonomous from normal thyroid tissue.

The clinical diagnosis of toxic multinodular goiter is based on the presence of three characteristics: (1) palpable and or sonographically localized thyroid nodules; (2) increased radioiodine or ^{99m}Tc-pertechnetate uptake in nodular structures typically concomitant with a decreased uptake in surrounding extranodular thyroid tissue; (3) excess thyroid hormone production causing thyrotoxicosis, concurrently with the suppression of TSH secretion from the pituitary gland (see **Fig. 2**).

It is important to differentiate toxic multinodular goiter from other causes of hyperthyroidism including Graves' Disease, solitary toxic adenoma, and painless or subacute thyroiditis. In iodine-deficient areas, the distinction between autoimmune and non-autoimmune forms of hyperthyroidism (i.e., Graves' Disease and toxic multinodular goiter) may be challenging due to a mixed clinical presentation. For example, patients may have Graves' Disease superimposed on pre-existing nodular thyroid disease. In this case, the scintiscan may be suggestive of toxic multinodular goiter but the presence of thyroid autoantibodies is suggestive of Graves' Disease. Another diagnostic challenge may occur in sporadic or familial autosomal dominant non-autoimmune hyperthyroidism when a germline TSH receptor mutation is found in the setting of undetectable TSH receptor antibodies (Ferraz and Paschke, 2017).

Treatment

Management of patients with toxic multinodular goiter should take into account several clinical and demographic factors, in addition to patient values and preferences. The goal of therapy is rapid and lasting elimination of the hyperthyroid state and alleviation of obstructive symptoms. Therefore, the main indications for treatment include a large goiter causing compressive symptoms or thyrotoxicosis. Subclinical hyperthyroidism has been associated with higher risk of atrial fibrillation, osteoporosis, and an increase in 10-year cardiovascular mortality (Krohn *et al.*, 2005). Current guidelines suggest that treatment for subclinical hyperthyroidism should be initiated for the following groups of patients: those >65 years old with cardiac risk factors, heart disease, or osteoporosis; post-menopausal women who are not taking medications for bone protection; and symptomatic patients (Ross *et al.*, 2016).

Medical Therapy

In patients with severe thyrotoxicosis or those at significant risk of complications due to worsening of hyperthyroidism, pre-treatment with thionamides and/or beta blockers should be considered. Low dose thionamides such as carbimazole or methimazole should be used as first line. Beta blockers can be used as adjunctive therapy to control adrenergic symptoms such as anxiety, tremor, and palpitations. Achieving biochemical control of hyperthyroidism typically takes several weeks as thionamides block new thyroid hormone synthesis, but intra-thyroidal stores continue to be released until they are depleted. Prior to initiation of therapy, patients should be counseled about common side effects of thionamides including skin rashes (1%–5%), arthritis and rare but serious complications such as hepatotoxicity and agranulocytosis ($<0.5\%$). Anti-thyroidal drugs are not routinely recommended for long-term use because they cannot induce remission in nodular thyroid disease. However, long term anti-thyroidal drug use can be considered when definitive therapy is contraindicated, such as in the elderly or those with limited life expectancy.

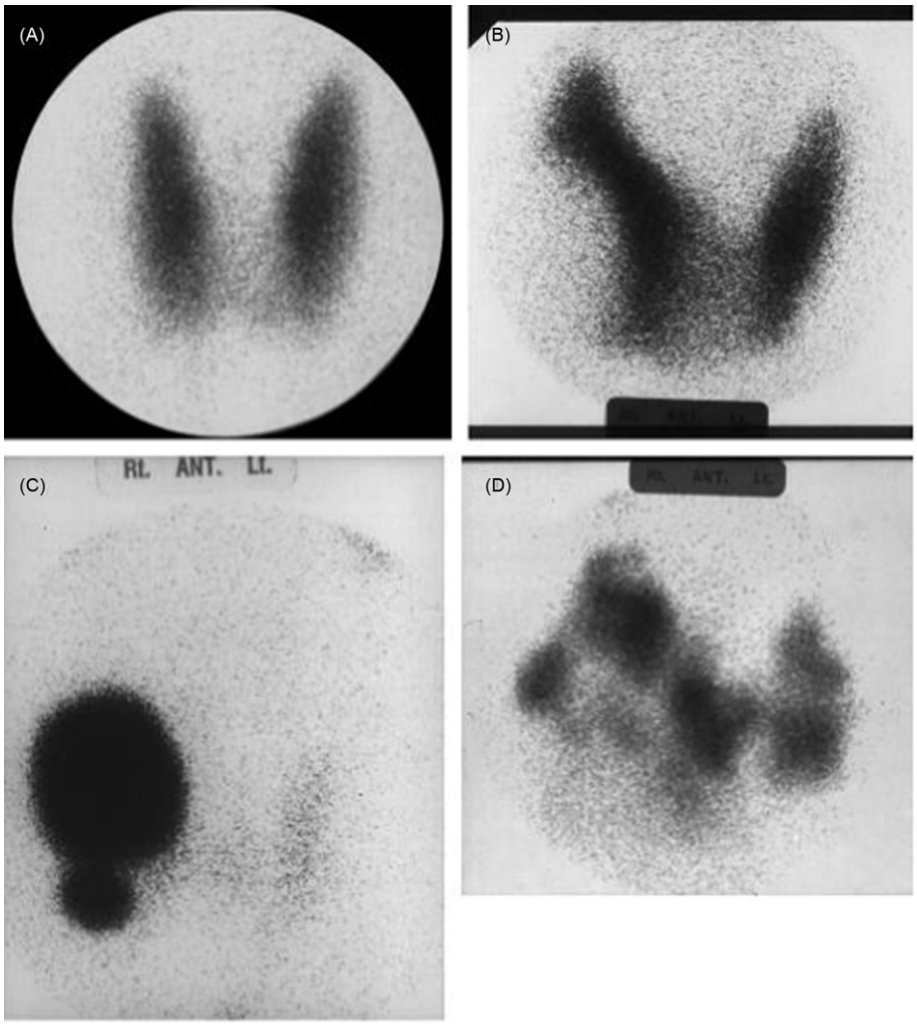

Fig. 2 Four different patterns of thyroid scintigraphy illustrating (A) Normal thyroid gland. (B) Nonfunctioning "cold" nodule in the right thyroid lobe. (C) Hyperfunctioning "hot" nodule in the right thyroid lobe with suppression of the remaining thyroid parenchyma. (D) Multinodular goiter with heterogeneous uptake of an enlarged thyroid gland, with patchy areas of normal, decreased, and increased ^{123}I uptake. Reproduced with permission from Gharib, H. and Papini, E. (2007). Thyroid nodules: Clinical importance, assessment, and treatment. *Endocrinology and Metabolism Clinics of North America* **36**, 707–735.

Definitive Management

Definitive treatment of toxic multinodular goiter includes surgical resection of the thyroid gland or radioactive iodine. The risks, benefits, and alternatives of each treatment should be discussed with the patient prior to choosing a therapy. Factors that influence the choice of treatment modality are outlined in **Table 2**.

Surgery

Surgery is the preferred treatment option if immediate eradication of hyperthyroidism is desired, if thyroid malignancy is suspected or confirmed, if obstructive symptoms are present due to large goiter with mass effect, or if coexisting hyperparathyroidism exists that requires simultaneous treatment. In contrast, patients with previously operated or irradiated necks, or those with increased surgical risk are not ideal surgical candidates. Relative contraindications to surgery for toxic multinodular goiter include significant comorbidities, limited life expectancy, and pregnancy.

If surgery is chosen as the treatment for a patient with toxic multinodular goiter, pre-treatment with a thionamide $+/-$ beta blockade is recommended to achieve euthyroidism, thus minimizing the risk of intraoperative and postoperative complications from hyperthyroidism. Preoperative iodine should not be administered in this setting due to the risk of exacerbating hyperthyroidism. The preferred surgical procedure for treatment of toxic multinodular goiter is a near-total or total thyroidectomy. Thionamides should be stopped after surgery, while ß-adrenergic blockade should be slowly tapered following the operation. The

Table 2 Factors influencing the choice of treatment modality for toxic multinodular goiter

Factors that favor radioiodine treatment	*Factors that favor surgical treatment*
Small to medium sized goiter	Large goiter or compressive symptoms
Previously operated or irradiated neck	Desire for rapid termination of hyperthyroidism
Increased surgical risk of patient	Suspicion of thyroid malignancy
	Low radioiodine uptake
	Coexisting hyperparathyroidism requiring treatment

risk of treatment failure or need for repeat treatment is <1% following near-total or total thyroidectomy, however the risk of permanent hypothyroidism requiring thyroid hormone replacement is 100%. Potential surgical complications include risk of permanent hypoparathyroidism (<1.0%) or vocal cord paralysis due to recurrent laryngeal nerve injury (<1.0%). Although these complications are rare in the setting of experienced, high volume surgeons, they should be discussed with patients prior to embarking with this treatment option.

Radioactive ^{131}Iodine (RAI)

In patients with contraindications to or a preference for avoiding surgery, RAI therapy can be used to eradicate hyperthyroidism and reduce the thyroid gland volume. Contraindications to RAI use include pregnancy, lactation, coexisting thyroid cancer, or cases where individuals are unable to comply with radiation safety guidelines. In addition, RAI treatment may lead to transient worsening of hyperthyroidism and swelling of the thyroid gland, and should be used with caution in patients at high risk of decompensation from thyrotoxicosis or those at risk of tracheal obstruction.

RAI therapy may exacerbate hyperthyroidism leading to tachyarrhythmias, and in extremely rare cases, thyroid storm (Koornstra *et al.*, 1999). Therefore, in preparation for RAI, pretreatment with beta blockade should be considered in patients with a pre-existing cardiac history or in the elderly. Thionamides may also be used to achieve euthyroidism in patients at high risk of decompensation from worsening hyperthyroidism. However, thionamides may reduce the efficacy of RAI treatment when used in the week before or after RAI administration, therefore, they are routinely held during this interval (Walter *et al.*, 2007; Ross *et al.*, 2016). The recommended RAI activity for treatment of toxic multinodular goiter is based on goiter size, and aims to deliver 150–200 μCi (5.55–7.4 MBq) per gram of tissue corrected for 24-h RAI uptake (Ross *et al.*, 2016).

It should be noted that an elevated or normal TSH at the time of RAI administration will lead to radiation exposure of perinodular normal thyroid tissue. If goiter volume reduction is a goal of treatment, this property may be leveraged to achieve a greater reduction in goiter size at the expense of permanent hypothyroidism. Patients with large goiters should be monitored after administration of RAI for symptoms of compression such as hoarseness, dysphagia, or dyspnea. Significant symptoms such as these can be treated with corticosteroids. The failure rate of RAI in treatment of toxic multinodular goiter is around 15% (Nygaard *et al.*, 1999). The response rate, characterized by alleviation of hyperthyroidism and goiter volume reduction, increases with time and is around 80% by 6 months post-administration of RAI (Nygaard *et al.*, 1999; Kang *et al.*, 2002). The risk of hypothyroidism is 6% at 1 year, and 72% within 26 years following RAI administration for hyperthyroidism (Holm *et al.*, 1982).

Patients who have received ^{131}I ablation should have their thyroid function tests monitored at 1–2 months post treatment, including measurement of serum TSH, free T4, and total T3. Biochemical monitoring should occur every 4–6 weeks for the first 6 months, and annually thereafter (Ross *et al.*, 2016). The long-term management of patients with toxic multinodular goiter is directed at the detection of thyroid dysfunction (hypothyroidism after RAI treatment or relapse of hyperthyroid state), relapse of goiter, and treatment of surgical complications (e.g., calcium and/or vitamin D administration in the case of hypoparathyroidism).

See also: Antithyroid Drugs. Radioactive Iodine. Thyroid Fine Needle Aspiration Cytology. Thyroid Imaging

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Thyroiditis, Infectious and Subacute☆

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Glossary

Genetic susceptibility Heritable trait based on histocompatibility genes.
Fine needle aspiration biopsy Aspiration of the contents of a thyroid nodule for diagnostic evaluation.
Hypothyroidism Clinical state of hypometabolism characterized by low circulating concentrations of thyroid hormones.
Pyriform sinus fistula Communication between the pyriform sinus and the thyroid gland, most commonly on the left side. It is the most common cause of bacterial thyroiditis.
Radioactive iodine uptake Ability of the thyroid gland to trap iodine.
Thyroiditis Inflammation of the thyroid gland, most often autoimmune in nature (chronic) but may also be caused by infection (subacute and acute).
Thyrotoxicosis Clinical state of hypermetabolism characterized by elevated circulating concentrations of thyroid hormones.

The term thyroiditis is defined as inflammation of the thyroid gland. It is a heterogeneous group of disorders which can be classified according to their symptoms development as acute, subacute and chronic. However, it should be underlined that there is a considerable overlap in their clinical presentation. The acute infectious thyroiditis (caused by pathogenic microorganisms) and subacute thyroiditis (precipitated by viral infection in genetically predisposed individuals) are illustrated in this article.

Infectious Thyroiditis

Infectious thyroiditis, also known as acute suppurative thyroiditis, is a potentially life-threatening disorder caused by invasion of the thyroid by infectious organisms, usually bacterial, fungal, parasitic and mycobacterial infections.

Introduction

Infections of the thyroid are rare, with only about 300 and 100 cases reported in the literature in adults and children, respectively (Yamada *et al.*, 2009). This is due, in large part, to the resistance of the thyroid gland to infection through protective mechanisms, including the rich blood supply to and lymphatic drainage from the thyroid gland; the high glandular content of iodine and peroxidase, which may be bactericidal; and the separation of the thyroid from other structures of the neck by fascial planes and the complete, protective fibrous encapsulation of the gland. The most common predisposing factor to infections of the thyroid appears to be preexisting thyroid disease. Simple goiter, nodular goiter, Hashimoto's thyroiditis, or thyroid carcinoma have been observed in up to two-thirds of women and one-half of men with infectious thyroiditis. The most frequent predisposing factor in children and adolescents is a fistula from the pyriform sinus that extends to the thyroid capsule (Rich and Mendelman, 1987; Sai Prasad *et al.*, 2007). Immunocompromised patients, such as those infected with human immunodeficiency virus or those on immunosuppressive drugs, are particularly at risk for bacterial thyroiditis (Pearce and Farwell, 2003). Thyroidal infections are potentially life threatening, especially if the diagnosis is delayed and appropriate antimicrobial therapy is not instituted, with an overall mortality of up to 12% (Berger *et al.*, 1983; Paes *et al.*, 2010).

Bacterial Infections

Bacterial infections are the most common cause of infectious thyroiditis, comprising about the 75% of cases (Paes *et al.*, 2010; Yu *et al.*, 1998); thus, the presentation, diagnosis, and management of bacterial thyroiditis are discussed in detail. The seminal observation regarding the pathogenesis of bacterial thyroiditis was made in 1979 when Takai *et al.* reported seven cases of infectious thyroiditis due to a fistula originating from the left pyriform sinus (Takai *et al.*, 1979). Subsequently, it has been reported that a pyriform sinus fistula is the most common route of infection in acute suppurative thyroiditis, especially in children and in patients with recurrent episodes. Pyriform sinus fistulae is primarily located in up to 90% on the left side (Miyauchi *et al.*,

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1990). Additional reports have identified the following routes of thyroidal infection: infected embryonic cysts from the third and fourth brachial pouches, patent thyroglossal duct fistulae, infections of the retropharyngeal space (pharyngitis and tonsillitis) or the lateral pharyngeal spaces (pharyngitis, tonsillitis, parotitis, otitis, and mastoiditis), and perforations in the esophagus. Case reports of hematogenous spread from infections of the skin, lower respiratory tract, urinary tract, and gastrointestinal tract have been described. Hematogenous inoculation occurs more often in adults than in children, usually in the presence of other underlying illnesses. Infectious thyroiditis has also been reported as a complication of thyroid surgery or after fine needle aspiration biopsy (Ünlütürk *et al.*, 2013).

Clinical Presentation and Diagnosis

Bacterial thyroiditis is often preceded by an upper respiratory infection, which may induce inflammation of the fistula and promote the transmission of pathogens to the thyroid (**Table 1**). More than 90% of patients present with thyroidal pain, tenderness, fever, and local compression resulting in dysphagia and dysphonia. Signs and symptoms of systemic toxicity may be present. The thyroid is tender to palpation, with unilateral or bilateral lobar enlargement, firm swelling that moves on swallowing and it is associated with erythema and warmth of the skin. Cervical lymphadenopathy is not a prominent feature unless there is a predisposing pharyngitis.

The differential diagnosis of bacterial thyroiditis can be divided into nonthyroidal and thyroidal causes (**Table 2**). Essentially all of the nonthyroidal causes are infectious in origin and present as discrete painful masses. Subacute thyroiditis is the most common cause of a painful thyroid and often results in both local and systemic symptoms similar to those seen in bacterial thyroiditis. The pain in acute suppurative thyroiditis does not relocate during the clinical course of the disease which can be a key point in the differential diagnosis with subacute thyroiditis.

Thyroid function tests are usually in the normal range in patients with bacterial thyroiditis (**Table 1**), but both thyrotoxicosis and hypothyroidism have been reported (Goldani *et al.*, 2006; Luiz *et al.*, 2013). Thyrotoxicosis is transient due to destruction of the thyroid tissue. Fine needle aspiration biopsy is the best laboratory test in the evaluation of infectious thyroiditis and is diagnostic in most cases, especially when tenderness is limited to a solitary nodule or a localized area and subacute thyroiditis has been ruled out. Gram stain and culture of the fine needle aspirate will reveal the causative organism in the overwhelming majority of the cases. Most imaging studies are adjunctive and are best reserved for patients for whom the diagnosis is unclear.

In adults, *Staphylococcus aureus* and *Streptococcus pyogenes* are the offending pathogens in the majority of cases (Paes *et al.*, 2010; Yu *et al.*, 1998). In children, *S. aureus*, *S. pyogenes*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae* and *Haemophilus influenza* account for most cases. Anaerobic bacteria are also isolated from the infected thyroid and they are usually members of the oropharyngeal flora (Chi *et al.*, 2002).

Management and Prognosis

Once acute suppurative thyroiditis is suspected, the initiation of empiric antibiotic treatment is essential before the culture results are known, taking into consideration the potential mortality of the acute suppurative thyroiditis. In adults antibiotic therapy should cover gram-positive cocci. An antistaphylococcal β-lactam such as nafcillin or cefazolin combined with an aminoglycoside (gentamicin) or monotherapy with a third-generation cephalosporin are appropriate initial regimens (Chow, 2009). In penicillin-allergic patients or if methicillin resistant *S. aureus* is suspected, vancomycin can be chosen. Anaerobic coverage (e.g., clindamycin or β-lactam/β-lactamase inhibitor) should also be included as oral anaerobes may be involved in acute suppurative thyroiditis, especially in pediatric or recurrent cases. For children empiric antibiotic therapy should provide adequate coverage for *S. aureus* and *S. pyogenes* (Schlossberg, 2015). A broader spectrum of antimicrobial therapy should be considered in patients with human immunodefiency virus infection. Empirical antibiotic therapy should be adjusted according to the microbiological susceptibility and modified after the culture results are available.

In patients with compromised airway, an urgent transcutaneous or open-surgical drainage should be considered. In stable patients, therapeutic ultrasound guided fine needle aspiration biopsy may be appropriate.

Since a pyriform sinus fistula is the most common route of infection in bacterial thyroiditis, a barium swallow, computed tomography, or magnetic resonance imaging of the neck should be performed to search for communicating fistulae in most patients, especially in children with the first episode and in all patients with recurrent episodes. Such fistulae must be surgically excised or endoscopically chemo- or electrical-cauterized for definitive cure and prevention of recurrent infection.

With prompt and appropriate treatment, the prognosis of the acute suppurative thyroiditis is excellent unless the diagnosis is delayed and antimicrobial therapy is not initiated during the acute phase.

In survivors, complete recovery is the norm, although there have been reports of transient hypothyroidism, vocal cord paralysis (which may also be transient), and recurrence of infection as sequelae of acute bacterial thyroiditis.

Fungal Infections

Although rare, fungal infections of the thyroid are the next most common cause of infectious thyroiditis, comprising 15% of reported cases (Paes *et al.*, 2010; Yu *et al.*, 1998). The overall fatality rate associated with fungal thyroiditis is high as most

Table 1 Clinical features and the diagnostic tests in the evaluation of infectious thyroiditis and subacute thyroiditis and their comparison

	Characteristic	*Acute thyroiditis*	*Subacute thyroiditis*
History	Fever	Always	Half of the patients
	Thyrotoxicosis	Uncommon	Half of the patients
	Sore throat/preceding upper respiratory infection	Very common	About half of the patients
Physical examination	Left side affected	In about 90% of the cases	Not specific
	Erythema of overlying skin	Very common	Not common
Lab tests	White blood cell count	Usually elevated; nonspecific test	Less than 50% of the patients has leukocytosis; nonspecific test
	Erythrocyte sedimentation rate	Almost always elevated; nonspecific test	Almost always highly elevated >50 mm/h; normal ESR places the diagnosis of subacute thyroiditis in question
	Thyroid function tests	Usually normal; hypothyroidism and thyrotoxicosis can be found	More than 50% of the patients has thyroid function tests indicative of thyrotoxicosis
	Liver enzymes	Usually normal	Common abnormal
	Screening for human immunodeficiency virus	Prudent	Not necessary
Imaging examinations	Fine needle aspiration biopsy	Diagnostic in the vast majority of cases; test of choice; special stains considered necessary	Not necessary
	Radionucleide imaging	Adjunctive test; radioiodine or 99mTc best; provides information regarding overall gland function	Radioactive iodine or 99mTc uptake is always low, usually less than 2% after 24 h; if the radioactive iodine uptake is more than 5% the diagnosis is unlikely
	Ultrasonography	Adjunctive test; might help in the differential diagnosis; unifocal hypoechoic lesions; helpful in identifying pyriform sinus fistulae and/or spread of the infection	Adjunctive test; might help in the differential diagnosis; usually multiple and bilateral lesions in subacute thyroiditis. Not specific
	Neck radiography	Adjunctive test; tracheal deviation; presence of gas indicates abscess with anerobic organisms	Not indicated
	Barium swallow	Adjunctive test; helpful in identifying pyriform sinus fistulae	Not indicated
	Computed tomography	Adjunctive test; helpful in identifying pyriform sinus fistulae and/or spread of the infection	Not indicated
	Magnetic resonance imaging	Adjunctive test; helpful in identifying pyriform sinus fistulae and/or spread of the infection	Not indicated
Clinical Course	Response to glucocorticoid treatment	Transient	Significant clinical improvement within 24 h after the initiation of steroids; absence of rapid improvement would call the diagnosis into question
	Incision and drainage required	Often, especially in patients with compromised airway	Never
	Piriform sinus fistula	The most common route of infection, especially in children and in patients with recurrent episodes	Never

patients with fungal thyroiditis have disseminated fungal infection. The predominant offending organism is Aspergillus species, with the majority of the cases found at autopsy. Virtually all of the affected patients were immunocompromised and had disseminated disease. Asymptomatic infection of the thyroid with *Pneumocystis jiroveci* (former carinii) is found in more cases as the number of immunocompromised patients increase. Case reports of fungal infections of the thyroid have included *Coccidioides immitis, Cryptococcus neoformans, Candida albicans, Histoplasma capsulatum, Pseudallescheria boydii,* and *Nocardia asteroids* (Goldani *et al.*, 2006). There is a tendency of patients with fungal thyroiditis to be hypothyroid (62.5% of the cases) (Yu *et al.*, 1998).

Table 2 Differential diagnosis of the painful neck mass

Nonthyroidal
Infected thyroglossal duct cyst
Infected branchial cleft cyst
Infected cystic hygroma
Cervical adenitis
Cellulitis of the anterior neck
Parathyroid hemorrhage
Thyroidal
Subacute thyroiditis
Infectious thyroiditis
Acute hemorrhage into a cyst or into a benign or malignant nodule
Rapidly enlarging thyroid carcinoma (differentiated, medullary, anaplastic, lymphoma)
Painful Hashimoto's thyroiditis
Radiation thyroiditis
Globus hystericus

Mycobacterial Infections

The true incidence of infection of the thyroid with Mycobacterium tuberculosis has been reported around 10% (Paes *et al.*, 2010; Yu *et al.*, 1998). Infections with atypical mycobacteria, including *M. cheloni* and *M. avium-intracellulare*, have also been described. Mycobacterial thyroiditis had a tendency to cause hypothyroidism (50% of the cases) (Luiz *et al.*, 2013; Yu *et al.*, 1998).

Parasitic Infections

Parasitic agents such as *Echinococcus granulosus* and Cysticercus species have involved the thyroid on rare occasions. If echinococcal infection is suspected, biopsy of the lesion is contraindicated due to spillage and rupture of the cyst contents, and specific serologic testing should be performed. Surgical removal is the preferred mode of treatment, with antiparasitic agents useful as adjunctive therapy and for inoperable cases. Involvement of the thyroid with *Strongyloides stercoralis* has been described only in the setting of disseminated disease in immunocompromised patients.

Viral Infections

The most common infectious organism found in the thyroid at postmortem examination in patients with acquired immune deficiency syndrome is cytomegalovirus, occurring in the setting of disseminated cytomegalovirus infection. However, symptomatic thyroidal infection with cytomegalovirus has not been reported. Thyroiditis has been associated with mumps parotitis, although this is rare.

Subacute Thyroiditis

Subacute thyroiditis is a self-limited inflammatory disorder of the thyroid characterized by neck pain or discomfort, a predictable course of thyroid dysfunction, and disruption of the thyroid follicular cell architecture.

Introduction

Subacute thyroiditis was first described in 1895 by Mygind (1895) in 18 patients with "Thyroiditis akuta simplex." The Swiss surgeon De Quervain described the pathology of the disorder in 1904 and he differentiated it from other forms of thyroiditis (Engkakul *et al.*, 2011). Therefore, the disorder often bears De Quervain's name. It is also referred as subacute granulomatous thyroiditis, subacute thyroiditis, subacute painful thyroiditis, and pseudogranulomatous thyroiditis.

Epidemiology

Subacute thyroiditis is the most common form of painful thyroiditis (Engkakul *et al.*, 2011) and it has been estimated to account for up to 5% of all clinical thyroid abnormalities (Farwell, 2006) and to occur at the rate of 1 case per 5 cases of Graves' disease and in 1 case per 15–20 cases of Hashimoto's thyroiditis (Guimaraes, 2016). Subacute thyroiditis is more common in women (female to male ratio is 3 to 7:1), between the ages of 40 and 50 years and it is rare in children (Carlè *et al.*, 2011; Fatourechi *et al.*, 2003; Nishihara *et al.*, 2008).

Pathogenesis and Etiology

Subacute thyroiditis is presumed to be caused by a viral infection (Fraser and Harrison, 1952) or a postviral inflammatory course in genetically predisposed subjects. The initial hypothesis of a viral or postviral inflammation was supported by epidemiological and clinical data such as a seasonal distribution of the disease (higher in summer), its association with outbreaks of other viral illnesses, the antecedent respiratory infection, the association with viral-type prodrome such as myalgia, malaise and fatigue, and the fact that it is a self-limiting disorder.

The disease was thought to have a higher incidence in summer (Kitchner and Chapman, 1989; Martino *et al.*, 1987; Nishihara *et al.*, 2008) but not all studies confirmed a seasonal variation (Benbassat *et al.*, 2007; Bennedbaek and Hegedus, 1997; Fatourechi *et al.*, 2003). Subacute thyroiditis has been linked to outbreaks of other viral illnesses, including Coxsackievirus, mumps, measles, adenovirus, Ebstein–Barr virus, influenza viruses (H1N1 included), adenovirus and others (Engkakul *et al.*, 2011). There are reports of subacute thyroiditis following influenza vaccination (Altay *et al.*, 2016; Hernán Martinez *et al.*, 2011; Hsiao *et al.*, 2006). Patients with subacute thyroiditis have at least fourfold increase in viral antibodies during the disease course (Desailloud and Hober, 2009; Volpe *et al.*, 1967) or positive viral cultures of the thyroid tissue. However, virological evidence is not present in all subacute thyroiditis patients series (Luotola *et al.*, 1998; Mori *et al.*, 1998).

In addition to the possible viral precipitation of the development of subacute thyroiditis, a genetic predisposition in class I major histocompability antigen status has been suggested. An association between subacute thyroiditis and HLA-Bw35 haplotype has been reported in 72% of the patients with subacute thyroiditis and in different ethnic groups (Nyulassy *et al.*, 1977; Ohsako *et al.*, 1995; Yeo *et al.*, 1981). Genetic susceptibility is suggested in addition by the familial clustering (Kramer *et al.*, 2004; Zein *et al.*, 2007) and the simultaneous development of the disorder in identical twins with HLA-Bw35 (Hamaguchi *et al.*, 2005). Another haplotype, the HLABw67 haplotype, has been reported in Japanese patients, although the association is not as strong as that reported for HLA-Bw35, the disease has a mild course and has been correlated with a seasonal appearance (Ohsako *et al.*, 1995).

It has been proposed that an antigen provided either directly from a virus or from virus-induced host tissue damage binds to HLA molecules on macrophages. The resulting antigen-HLA complex is recognized and activates cytoxic T-lymphocytes that then damage thyroid follicular cells because of molecular mimic. The immune reaction is not self-preserved, like in the autoimmune thyroid disease, and therefore the disease is self-limited.

Although thyroid autoimmunity is not believed to play a central role in subacute thyroiditis, autoimmune abnormalities associated with the disorder have been described. Thyroid antibodies (thyroglobulin and thyroid peroxidase antibodies) have been found in 42%–64% of patients with subacute thyroiditis (Volpe *et al.*, 1967). Those antibodies gradually decrease to low or even undetectable levels after recovery. Antibodies directed against the thyroid stimulating hormone receptor have been reported (Strakosch *et al.*, 1978; Tamai *et al.*, 1991, Wall *et al.*, 1982). It is likely that the thyroid antibodies develop secondary to the tissue damage caused by the viral infection.

Clinical Features

As mentioned previously, a viral prodrome is common, and half of the patients have a history of upper respiratory infection with symptoms such as sore throat, weakness, low-grade fever, myalgias and dysphagia. The clinical manifestation of subacute thyroiditis develops gradually over few days or weeks. Anterior neck pain in the region of the thyroid is predominant in various degrees and is present in up to 96% of the patients (Fatourechi *et al.*, 2003). Pain involves part of a lobe, one lobe or the whole thyroid. It frequently radiates to the upper neck, jaw, throat, ear or even the upper chest. Commonly, the patient initially consults an ear, nose, and throat physician or a dentist because of pain in the area of the throat or mandible. Frequently, the pain will migrate from one side to the opposite thyroid lobe after a few weeks. Exacerbation of the pain occurs by moving the head, swallowing or coughing. Symptoms may be limited in the neck and the head but most patients have systemic symptoms like malaise, myalgia, arthralgia, anorexia and fever that can reach 40°C.

Symptoms of thyrotoxicosis are frequent since thyrotoxicosis occurs in approximately 50% of affected individuals including the typical symptoms of thyrotoxicosis like diaphoresis, nervousness, tremor, heat intolerance, palpitations, tachycardia, and weight loss. Usually the symptoms of thyrotoxicosis are mild-to-moderate and the pain in the neck dominates the clinical picture. Rarely, the clinical presentation of subacute thyroiditis may be so dramatic in onset and so pronounced in severity that obstructive symptoms may develop.

Physical examination may disclose signs of hypermetabolism, with tachycardia, diaphoresis, and tremor. Palpation of the neck generally reveals a slight to moderate enlargement of the thyroid with the one lobe larger than the other. The thyroid gland is very firm to hard, exquisitely tender. Tenderness may be so pronounced that patients may try to prevent palpation by the examiner. The overlying skin is occasionally erythematous.

The inflammation of the thyroid is transient and the illness is self-limited even if the patient is not treated. Transient hyperthyroidism may be followed by a transient hypothyroidism (**Fig. 1**). Recovery is almost always complete but it has been reported that up to 15% of the patients eventually develop permanent hypothyroidism requiring treatment with thyroxine (Fatourechi *et al.*, 2003). Recurrence may occur in 1.3%–4.0% of the patients 6 to 21 years after the first episode (Fatourechi *et al.*, 2003; Iitaka *et al.*, 1996).

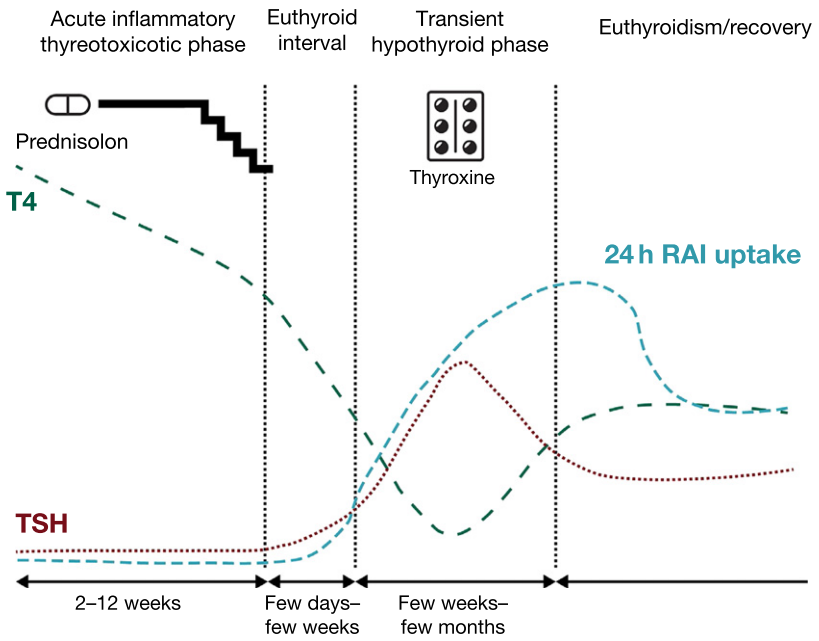

Fig. 1 The clinical course of subacute thyroiditis. The clinical course of subacute thyroiditis generally composed of four phases. The initial, acute inflammatory thyrotoxic phase which is characterized by pain, tenderness, and thyrotoxicosis due to leakage of thyroid hormones from the destruction of the thyroid folicles. The acute phase may last from 2 to 12 weeks. In this phase treatment with glucocorticoids is necessary. Following a euthyroid interval occurs which lasts up to several weeks. A transient hypothyroid phase then follows, which may last from a few weeks to a few months. Rarely, during the hypothyroid phase, levo-thyroxine therapy may be necessary. An asymptomatic recovery phase then follows. Virtually all patients with subacute thyroiditis achieve restoration of normal thyroid function, although permanent hypothyroidism has been reported in up to 15% of patients. In most cases, the entire episode of subacute thyroiditis rarely lasts more than 6 months. Not all patients with subacute thyroiditis progress through all four phases of the disorder since only approximately 50% of patients develop transient hypothyroidism. *RAI*: radionuclide uptake including 99mTc. *TSH*: thyroid stimulating hormone. *T4*: thyroxine.

Laboratory and Imaging Findings

Biochemical thyrotoxicosis with suppressed TSH and elevated levels of free/total T4 and free/total T3 occurs in approximately half of patients with subacute thyroiditis. T3 is not disproportionately elevated as it is in some Graves' patients reflecting the intra-thyroidal T3 and T4 content and the ratio of T4 to T3 in serum is <20. In general, the thyrotoxicosis associated with subacute thyroiditis is mild or at most modest in its severity. The thyrotoxicosis is transient and it is followed by a usually asymptomatic, transient period of overt or subclinical hypothyroidism.

The erythrocyte sedimentation rate is almost always >50 mm/h and may be as high as more than 100 mm/h. A normal sedimentation rate in a patient with a painful anterior neck mass places the diagnosis of subacute thyroiditis in question. C reactive protein may also be elevated, but is less diagnostic than the sedimentation rate. A slightly elevated total white blood cell count is present in half of the patients (Greene, 1971; Niklaus-Müller *et al.*, 1994). Serum thyroglobulin concentration is elevated during the acute phase of subacute thyroiditis, reflecting the destruction of the thyroid follicular architecture and release of thyroglobulin into the circulation. Thyroglobulin measurement should not be part of the routine evaluation of patients with subacute thyroiditis. Other laboratory findings, not routinely needed in order to confirm the diagnosis, are a mild normochromic, normocytic anemia and elevated liver enzymes.

Radioactive iodine uptake is always low, usually less than 2% after 24 h, in contrast to Graves' disease. Indeed, if the radioactive iodine uptake is more than 5% after 24 h, the diagnosis of subacute thyroiditis is unlikely. The absent uptake of iodine by the thyroid is due to destruction of the iodine-trapping mechanism from the inflammatory process as well as from inhibition of TSH secretion by excess circulating thyroid hormone. It is important to perform a radionucleide uptake test in patients with suspected subacute thyroiditis in order to rule out other causes of anterior neck pain as well as other types of thyrotoxicosis. In routine clinical practice a 99mTc-scintigraphy provides similar low uptake and is at the same time associated with lower irradiation to the patient and the environment.

Ultrasonography is not required as part of the evaluation of patients with subacute thyroiditis. Findings show diffuse areas of hypoechogenicity. In the Color Doppler ultrasonography, the thyroid has low-to-normal vascularity in contrast with the hyper vascularity shown in patients with Graves' disease (Bennedbaek and Hegedus, 1997). Fine needle aspiration is not routinely recommended in the evaluation of suspected subacute thyroiditis but may be useful to distinguish patients with unilateral involvement of subacute thyroiditis, acute pyogenic thyroiditis, hemorrhage into a cyst or thyroid cancer. Fine needle aspiration in subacute thyroiditis shows giant cells and pseudogranulomas.

Table 1 summarizes typical clinical and laboratory manifestations of subacute thyroiditis.

Differential Diagnosis

The differential diagnosis of subacute thyroiditis is shown in **Table 2** and includes both thyroid and nonthyroid disorders. Subacute thyroiditis is the most common thyroid etiology of anterior neck pain. Hemorrhage into a cyst or benign nodule is the second most common thyroidal cause of neck pain. Hemorrhage is neither presented by symptoms of infection (such as fever, weakness, myalgias) nor of thyrotoxicosis. In addition, the thyroid pain is predominantly unilateral and hemorrhage is sudden in onset, while subacute thyroiditis develops in days or weeks. The radioactive iodine or 99mTc-uptake in patients with hemorrhage is normal, and a radionuclide scan would reveal a filling defect. Both acute infectious thyroiditis and subacute thyroiditis are characterized by fever, thyroid pain and tenderness. However, in the case of acute infectious thyroiditis the fever is higher and the pain is predominantly unilateral. Very rare infectious thyroiditis may be presented with hyperthyroidism and usually the thyroid function is normal. The white blood cell count in acute infectious thyroiditis is significantly higher than the leucocytosis in subacute thyroiditis and the diagnosis can be confirmed by ultrasonography and fine needle aspiration biopsy. Patients with cellulitis of the anterior neck are not thyrotoxic and they do not have discrete masses on palpation. The patient with an infected thyroglossal duct cyst or branchial cleft cyst are euthyroid and the painful lesion is fluctuant and in more superior (thyroglossal duct cyst) or lateral (branchial cleft cyst) locations.

Rapidly growing thyroid cancer or primary thyroid lymphoma is an unusual cause of thyroid discomfort and anterior neck pain (called also "malignant pseudothyroiditis") (Yang *et al.*, 2006).Those patients do not have clinical features of inflammation and the neck mass is nontender and usually rock hard. In addition, an ultrasound can be helpful in differentiating these conditions vs. subacute thyroiditis.

Radioactive iodine therapy for hyperthyroidism or thyroid cancer or incidental irradiation of the thyroid during external beam radiotherapy to the neck can cause clinical thyroiditis with anterior neck pain and/or swelling. In those cases, the etiology of the inflammation is always obvious.

Treatment and Clinical Course

Treatment of subacute thyroiditis directs toward relief of the thyroid pain and tenderness and the control of thyrotoxic symptoms when present. Patients with mild symptoms may need no treatment.

For the patients who need treatment, anti-inflammatory therapy in form of a nonsteroidal anti-inflammatory drug (NSAID) or prednisolone is indicated. In mild cases, initiating acetylsalicyclic acid (2600 mg daily) or an NSAID like naproxen (500–1000 mg daily divided in two doses) or ibuprofen (1200–3200 mg daily in three to four divided doses) is reasonable. If the NSAID treatment fails and there is no clinical improvement after 2–3 days or in severe cases, prednisolone in a divided daily dose of 30–60 mg (usually 40 mg) should be initiated. Glucocorticoids provide pain relief within hours after their administration. Lack of significant improvement within 24 h after the initiation of steroids is uncommon and the absence of rapid improvement would call the original diagnosis into question. The prednisolone can be tapered after approximately 1 week and once the pain is relieved with a reduction by 5–10 mg every 5–7 days. If the pain and swelling recurs, prednisolone should be resumed. Recurrence of the pain during the prednisolone therapy occurs often in the contralateral lobe, in approximately 20% of patients (Mizukoshi *et al.*, 2001).

Thyrotoxic symptoms may be controlled by the use of beta-blocking agents, with the dose depending on the severity of symptoms. Propranolol in a dose of 40–120 mg or atenolol in a dose of 25–50 mg daily is usually sufficient to control symptoms of thyrotoxicosis, but higher doses may sometimes be needed.

Thionamides are not recommended since the thyrotoxicosis results from release of preformed hormone into the circulation rather than from increased synthesis. Therefore, drugs that inhibit thyroid hormone synthesis, such as thyroid-blocking agents, have no beneficial effect. Sodium iopodate has also been used in the management of the hyperthyroidism of subacute thyroiditis. Thyroidectomy can be employed in cases with prolonged, disabling pain.

It has been reported that patients who have a history of subacute thyroiditis may develop subtle, permanent thyroid abnormalities. It has also been reported that those patients may be sensitive to the inhibitory effects of exogenously administered iodides by exhibiting elevations in serum TSH concentrations, even years after having had subacute thyroiditis (Roti *et al.*, 1990).

See also: Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyroid Imaging. Hypothyroidism Subclinical. Causes of Hypothyroidism. Epidemiology of Thyroid Disease

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Postpartum Thyroid Dysfunction☆

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Glossary

Antibodies Molecules produced by the immune system, often directed at tissues in the body.
Depression Clinical state of low mood.
Immune Biological process of recognizing self from nonself.
Postpartum Time period of up to 1 year following delivery of a baby.
Screening Performance of a test or procedure in order to detect a specific disease or increased chance of development of disease in a particular group.
Thyroid Butterfly-shaped endocrine gland at the front of the neck.

Historical Background

H. Roberton, a General Practitioner in New Zealand, is credited with the first description of postpartum hypothyroidism as a distinct clinical entity. Thyroid dysfunction was described in the postpartum period more than a century ago, but a possible cause-and-effect relationship to pregnancy and delivery was not clarified until much later. Walfish and Ginsberg first described the thyrotoxic phase, which preceded the hypothyroid phase in the biphasic form of postpartum thyroiditis (PPT). Evidence for changes in thyroid autoimmune markers during pregnancy and the postpartum period (i.e., a decrease in thyroid autoantibodies during pregnancy and an increase after delivery) was obtained later. Amino's work produced a resurgence of interest in the subject, and since then numerous studies from different parts of the world have described the syndrome and its incidence, pathophysiology, and long-term outcome.

Immune Changes During Pregnancy and the Postpartum Period

Normal Women

The immunological changes that occur during pregnancy are directed toward inducing tolerance to the growing fetus. Failure of such tolerance results in rejection and miscarriage. The fetal trophoblast, maternal T cells, and maternal antibody production are all modulated in ways that enhance tolerance and reduce rejection.

The fetal trophoblast enhances tolerance by means of four main mechanisms. First, the failure to express MHC class I/II molecules prevents maternal T cells from mounting a response to paternal antigens. Second, the expression of the HLA-G gene reduces natural killer cell function and activates CD8 T cells, enhancing suppressor function. Third, complement activation is inhibited by the expression of proteins CD46, CD55, and CD59. Finally, Fas ligand is produced, enhancing tolerance.

Maternal T helper cell (Th) cytokine responses shift from a Th1 (γ-interferon and interleukin-2 (IL-2)) to a Th2 (IL-4, IL-5, and IL-10) secretion pattern. Potentially damaging cytotoxic and cytolytic reactions give way to relative immune suppression and tolerance. T-cell subsets that serve a suppressor function increase and natural killer cells decrease during early pregnancy, with postpartum rebound. There has been great interest recently in the role of regulatory T cells (Treg), previously called T suppressor cells, in preventing rejection of the fetal "allograft" in pregnancy. These cells are either thymic or peripheral in origin, and are thought to suppress the activity of immune competent cells (natural killer cells, dendritic cells, B cells, and CD4+ and CD8+ T cells) by various mechanisms. Treg numbers increase during the first and second trimester in both maternal blood and in the uterus, and may be partly estrogen driven. They rapidly decrease during the postpartum period. Although their exact role is currently being clarified, it does not seem to be limited to tolerance induction alone and a more central role is postulated.

The significant hormonal changes that occur during pregnancy have a profound effect on the production of immune tolerance. Placental progesterone is largely responsible for the shift to a Th2 response and may enhance other immune regulators as well. Placental estrogen and growth hormone also make a significant contribution to these changes. Furthermore, increased placental corticotropin-releasing hormone production stimulates the maternal adrenal glands to produce a state of hypercortisolism. There is also an increased production of catecholamines. A moderate increase in 25-hydroxy vitamin D_3 and more significant increases in

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1,25-dihydroxy vitamin D_3 also occur. Through intermediate mechanisms, all these changes suppress proinflammatory cytokine formation and promote a shift from a Th1 to Th2 response.

Maternal immune mechanisms return to a normal nonpregnant state in the first few months to 1 year after delivery. However, there may be a rebound increase in some elements of the autoimmune reaction which may aggravate existing autoimmune disease or precipitate it for the first time in predisposed women.

Women With PPT

The immune changes that occur in the postpartum period are reminiscent of the recently described phenomenon of "immune reconstitution" that follows treatment with drugs such as alemtuzumab (CAMPATH1) used in multiple sclerosis, and highly active antiretroviral therapy used in HIV disease. It is plausible that pregnancy induced Treg cells, decline in numbers soon after delivery, and their relative insufficiency in relation to preexisting thyroid reactive T cells will set off a "destructive" thyroiditis resembling a Th1 type of reaction, resulting in PPT. The behavior of antibodies to thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) during pregnancy and the postpartum period have been well documented. This "heightened" immune response in the first few months of the postpartum period may be caused by several factors.

Microchimerism

There is a considerable influx of fetal cells into the maternal circulation at the time of delivery. These cells may persist for short or long periods of time in the maternal host. Evidence suggests that these "chimeric" cells may induce an immune reaction in the host by causing a breakdown in immune tolerance. One short-term effect may be the rebound immune enhancement seen in the postpartum period, which may cause an exacerbation or new onset of some autoimmune diseases.

T-Cell Changes

In addition to changes to Treg described earlier, evidence suggests that activation of both circulating and intrathyroidal T cells occurs in PPT. Both Walfish and Stagnaro-Green demonstrated the expression of MHC class II molecules and a higher percentage of increased CD4:CD8 ratios in subjects who developed PPT. In a prospective study of TPOAb-positive women, Kuijpens showed a higher percentage of MHC II-expressing T cells in subjects who subsequently developed PPT compared with those who did not. However, Jansson's group did not demonstrate a difference in circulating subsets of T lymphocytes in thyrotoxic and hypothyroid PPT subjects compared with normal controls. This group demonstrated a relative increase in intrathyroidal B cells and a relative decrease in CD8 cells (resulting in an increased CD4:CD8 ratio) in subjects with PPT.

PPT as an Immune-Mediated Disease

Several features of PPT point to the central importance of immune mechanisms in its pathogenesis (**Table 1**). The majority of women who develop PPT are positive for markers of thyroid autoimmunity (i.e., TPOAb and TgAb). In our experience, all such women have TPOAb during early pregnancy. These TPOAb positive subjects have a 33%–50% chance of developing PPT. Evidence suggests that antibodies with a dual specificity for Tg and TPO may also be found in PPT at a higher prevalence than in normal control subjects. However, several investigators have reported PPT in TPOAb-negative women. In such women, the etiology of PPT is unclear. The histological changes occurring within the thyroid gland, with immune cell infiltration typical of autoimmune thyroid disease, give further credence to the immune pathogenesis of the disease. Fine needle biopsy of the thyroid gland in women with PPT shows lymphocytic infiltration and follicle formation reminiscent of Hashimoto's thyroiditis. Several studies have shown HLA haplotype restrictions in PPT, which are commonly seen in autoimmune thyroiditis such as Hashimoto's and Graves' disease.

The subclasses of TPOAb that are able to activate the complement cascade (IgG_1–IgG_3) increase during the postpartum period (**Fig. 1**) and are associated with both phases of PPT. Jansson reported an increase in IgG_1 in hypothyroid PPT. Hall demonstrated an increase in IgG_2 and IgG_3 in biphasic PPT, with the increase in IgG_3 coinciding with the thyrotoxic phase. Briones-Urbina confirmed the IgG_1 and IgG_2 changes but found low IgG_3 levels. However, these investigators also confirmed that IgG_4, which is incapable of influencing the complement cascade, remains relatively unchanged. This raises the interesting possibility of a

Table 1 Immunohistological features of PPT suggesting an autoimmune etiology

Presence of thyroid antibodies	TPOAb (majority) Thyroglobulin antibodies and antibodies with dual specificity (minority)
Postpartum increase in TPOAb subclasses capable of activating complement	IgG_1–IgG_3
Histological changes	Lymphocyte infiltration and follicle formation within the thyroid gland
HLA haplotype restriction	Similar to autoimmune thyroiditis

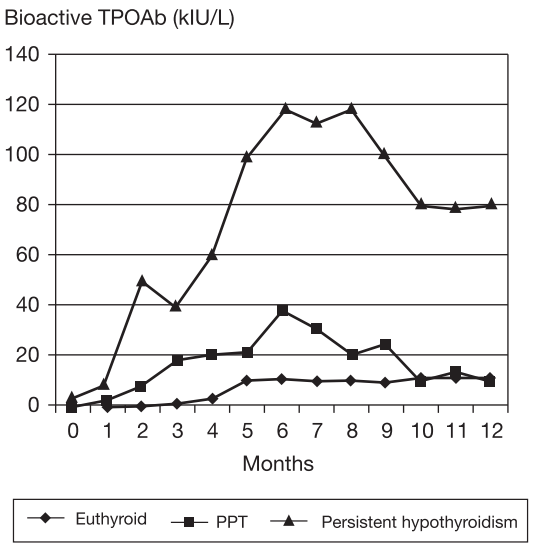

Fig. 1 Biologically active TPOAb levels in antibody-positive women who were euthyroid or had transient or persistent postpartum hypothyroidism. Antibody concentrations returned to normal in those with PPT but remained elevated in subjects with persistent hypothyroidism.

pathogenetic role for these antibodies in PPT, perhaps through complement activation. A sublethal antibody-directed, complement-mediated attack on thyroid cells may result in increased secretion of thyroid hormones, producing the thyrotoxic phase of the disease. However, a more severe complement-mediated attack may produce damage to the follicular architecture of the gland and produce hypothyroidism. Conclusive evidence for such complement activation in PPT is lacking; studies have been unable to demonstrate terminal complement complexes (markers of complement activation) in TPOAb-positive women who developed PPT.

Clinical Features and Management of PPT

Incidence of PPT

There is a wide variation in the reported worldwide incidence of PPT. This variation may be explained by true geographic differences in incidence (reflecting genetic heterogeneity and other factors) or by methodological discrepancies in studies reported from different locations. Variability of factors such as diagnostic criteria, length of follow-up after delivery, frequency of postpartum blood sampling, and differences in hormone assay methodology may have contributed to this variation. An average incidence of 5%–7% is acceptable for unselected pregnant women from most iodine-replete populations.

Predisposition to PPT

Women with TPOAb (and TgAb alone in $<5\%$), type 1 diabetes mellitus, other autoimmune disorders, and previous PPT are at increased risk of developing PPT. Studies from Cardiff show that approximately 50% of women with TPOAb during the early stages of pregnancy develop PPT. Other studies have shown this proportion to vary between 30% and 52%. Therefore, TPOAb is a marker of risk for the development of PPT but remains a weak predictor. There is a higher prevalence of PPT in subjects with type 1 diabetes mellitus. Gerstein followed 40 of 51 pregnant subjects with type 1 diabetes, of whom 10 developed thyroid dysfunction (1 due to Graves' disease), and Alvarez-Marfany followed 28 of 41 similar women, of whom seven developed thyroiditis. Therefore, the incidence is approximately 25% in women with type 1 diabetes mellitus. Recent but limited evidence suggests that gestational diabetes mellitus may also be a risk factor for PPT. The link between other autoimmune disorders and PPT is also significant. Studies have shown that SLE (14%) and chronic viral hepatitis (25%) were associated with a higher incidence of PPT. Similarly, there was a higher incidence of clinical and laboratory features of Sjögren syndrome in subjects with a history of PPT. However, the highest incidence of PPT is found in women who have had a previous episode of the disease. Sixty-nine percent of women who have TPOAb and had PPT following a previous pregnancy will develop PPT during the next pregnancy. However, only 25% who are TPOAb positive and remain euthyroid will develop thyroid dysfunction during the next pregnancy. There is limited evidence that previous autoimmune thyroid disease is also a risk factor for PPT.

There is evidence of a role for environmental factors, such as the presence of a goiter and smoking and a family history of thyroid disease, in the causation of PPT. However, additional studies are needed to confirm this finding.

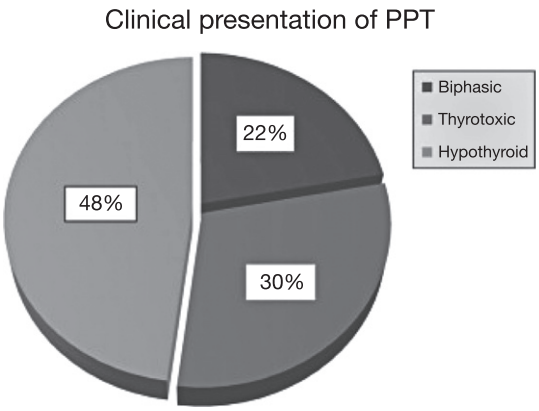

Fig. 2 Clinical types of PPT. The thyrotoxic phase usually precedes the hypothyroid phase.

Types of PPT and Clinical Features

Classically, PPT occurs after a full-term delivery. PPT occurring after early loss of pregnancy between 5 and 20 weeks of gestation has been reported.

PPT is a biphasic disease in about one-fifth of subjects (Fig. 2). A transient thyrotoxic phase is followed by a period of recovery and then a hypothyroid phase. The thyrotoxic phase occurs at a median of 13 weeks postpartum and lasts 1 or 2 months; it is probably due to the release of preformed thyroid hormone resulting from destruction of thyroid follicles. The hypothyroid phase that follows occurs at a median of 19 weeks postpartum, lasts longer (approximately 4–6 months), is accompanied by significant symptoms, and results from autoimmune follicular destruction and reduced hormone synthesis. Some women may require thyroxine replacement therapy during this phase. Rarely, the hypothyroid phase may precede the thyrotoxic phase. In some women, the two phases of PPT may occur independently of each other and either clinical or biochemical evidence of thyrotoxicosis or hypothyroidism alone develops during approximately the same postpartum periods as described previously. Significantly, as many as 30% of women who have TPOAb and PPT may develop permanent hypothyroidism requiring thyroxine replacement therapy by the end of the first postpartum year.

The symptoms of the thyrotoxic phase are mild and self-limiting. Fatigue, palpitations, weight loss, irritability, and heat intolerance are more commonly found in subjects with thyrotoxic PPT than in euthyroid postpartum women. They may also have tremor, nervousness, and psychological symptoms. The mild and nonspecific nature of these symptoms may cause diagnostic confusion, and they may be missed if a high index of suspicion is not maintained.

The hypothyroid phase lasts longer and may cause considerable morbidity. Fatigue, loss of concentration, constipation, muscle and joint pains, and stiffness are common complaints. Some of these symptoms may occur before the abnormalities in biochemical thyroid function become evident and also persist after euthyroidism is achieved. Three out of seven studies reported in the literature demonstrated a relationship between postpartum depression and PPT, that is, only 43%. But the rest did not find a significant relationship. The mechanism of depression in hypothyroid PPT is speculative but may be related to the reduced 5-hydroxytryptamine drive seen in this condition or to known cytokine release associated with this phase, affecting neurotransmission.

Management of PPT

The timing of symptoms in relation to pregnancy and delivery, the presence of thyroid antibodies in the majority of women who develop symptoms, and the pattern and timing of biochemical thyroid dysfunction should alert clinicians to PPT in women at risk. Symptoms and the presence of a goiter are unhelpful in differentiating PPT from other causes of thyroid dysfunction in the postpartum period. The presence of eye signs in the thyrotoxic phase, however, favors a diagnosis of Graves' disease.

The thyrotoxic phase is distinguished from an exacerbation of quiescent or a new onset of Graves' disease relatively easily by radioiodine uptake scanning observing appropriate precautions if breastfeeding. Uptake is consistently low in PPT but high in Graves' disease. When postpartum thyrotoxicosis occurs in women with previously known Graves' disease, a low uptake confirms PPT (on the background of quiescent Graves' disease). However, care needs to be taken in the use of radioiodine as a diagnostic tool in nursing mothers. Technetium scans may be preferable in them. The presence of thyrotrophin receptor antibodies and increased thyroid blood flow on color flow Doppler thyroid ultrasonography favors the diagnosis of Graves' disease. Standard thyroid ultrasonography, thyroglobulin estimation, and IL-6 measurement are of little practical value. Specific antithyroid drug therapy is not required in the thyrotoxic phase because symptoms and biochemical thyroid function return to normal in a few weeks. Occasionally, a beta-blocker may be indicated for symptom relief.

The hypothyroid phase usually follows the thyrotoxic phase in the biphasic form of PPT, and should be anticipated with periodic follow-up. But in a considerable minority (48%), hypothyroidism occurs alone and is the only manifestation of PPT in these women. An elevated TSH level at the appropriate time postpartum in women who are most likely TPOAb positive should alert clinicians to the diagnosis. This phase lasts longer and is associated with considerable morbidity. In a minority, it may result

Table 2 Long-term follow-up of PPT

Author	*Follow-up (years)*	*No. of subjects*	*Thyroid dysfunction (includes subclinical hypothyroidism or abnormal TRH test)*
Nikolai	3	25	6
Vargas	1	42	8
Tachi	5–16	44	10
Lervang	2	23	2
Jansson	5	50	13
Othman	2–4	43	10
Solomon	0.9–3.7	55	20
Kuijpens	2.5–3	14	6
Premawardhana	5–11.5	98	24
Lucas	3.3	42	5
Barca	2	49	30

in early permanent hypothyroidism, as described previously. Thyroxine therapy is indicated with a trial of withdrawal at 9–12 months. It is possible to withdraw thyroxine therapy in the majority of women at the end of this period, but a recurrence of symptoms associated with increased TSH levels on follow-up indicates the need for permanent replacement therapy.

Long-Term Outcome Following PPT

The long-term outcome of PPT has been examined in several studies (Table 2). In our series, permanent hypothyroidism occurred as early as 9 months postpartum in approximately 30% of subjects who were TPOAb positive and had PPT. These women required thyroxine replacement therapy to maintain normal clinical and biochemical thyroid function. A review of long-term follow-up studies of PPT from geographically different locations reported a 12%–61% prevalence of permanent hypothyroidism. The variability may in part be due to differences in the definition of PPT and long-term thyroid dysfunction, length of follow-up, and ascertainment. We followed 98 TPOAb-positive women (of whom 48 developed PPT) and 70 TPOAb-negative controls for 66–140 months. Forty-six percent of women who developed PPT were hypothyroid (some subclinically) at the end of the follow-up period compared with only 4% of women who were TPOAb positive but did not develop PPT and 1.4% of women who were TPOAb negative. The rate of conversion to hypothyroidism in women who were TPOAb positive and developed PPT was 7.1% per year, higher than that reported for women in community-based follow-up studies. Investigators from different areas of the world have confirmed a high prevalence of hypothyroidism at the end of variable follow-up periods in women who developed PPT. In a study of Japanese women with a similar length of follow-up after PPT (mean, 8.7 years), Tachi found a 29% prevalence of permanent hypothyroidism. In a Swedish study, Jansson found a 30% prevalence of hypothyroidism at 5 years. In Brazil, Barca found a 61% prevalence of hypothyroidism at the end of a 2-year period of follow-up after PPT. The reason for this high prevalence of relatively early hypothyroidism is unclear.

Women with a high risk for long-term permanent hypothyroidism in these studies (a) had higher TPOAb concentrations and higher TSH levels during the hypothyroid phase of PPT, (b) were older, (c) were multiparous, and (d) had greater hypoechogenicity on thyroid ultrasound scanning during the initial illness.

The nature and mechanism of persistent thyroid damage following the initial episode of PPT remains speculative. Several investigators have indicated the distinct possibility of a persistent but subtle abnormality (probably autoimmune in nature) of thyroid function and morphology in these women. Iodine perchlorate discharge tests were abnormal in 41% of Italian and 64% of Welsh women studied 3 and 7 years, respectively, following PPT, suggestive of a persistent organification defect. Furthermore, we found a significantly higher prevalence of thyroid ultrasound hypoechogenicity (due to autoimmune destruction) at 4–8 weeks postpartum in women who were TPOAb positive and developed PPT (45%) compared with antibody-positive women who did not develop PPT (17%) and to antibody-negative women (1.5%). There was a significantly higher prevalence of persistent abnormalities in the first group after 66–144 months of follow-up (although mildly reduced from the postpartum period), indicative of persistent autoimmune destruction following the initial episode of PPT. It seems likely that a persistent but low-grade immune destructive process severe enough to produce echogenic changes in the thyroid gland continues to occur in these women, although maximal damage occurs at the time of PPT.

These findings indicate the need for long-term follow-up of women who are TPOAb positive and who develop PPT. A relatively high incidence of early permanent hypothyroidism in these women and a higher than normal annual conversion rate to hypothyroidism (compared with that of TPOAb-positive women who do not develop PPT and women from community surveys) indicate the need for long-term follow-up. An increased TSH level with or without symptoms of hypothyroidism on annual (or more frequent) thyroid function testing is an indication for thyroxine replacement therapy.

Screening for PPT and Its Prevention

There is no consensus about screening for PPT. This relates as much to the absence of a highly sensitive and specific marker for prediction as to the lack of appreciation of the clinical problem and the long-term effects of PPT among endocrinologists in

general. The significant morbidity of PPT in the first postpartum year, the likelihood that approximately three-fourths of women who have PPT will have an episode in a future pregnancy, the high prevalence of long-term thyroid dysfunction following PPT, and the availability of effective treatment should make a screening strategy useful. Some authorities recommend a selective screening strategy aimed at only those women who have a high risk of developing PPT (i.e., women with type 1 diabetes mellitus and those who have had PPT in a previous pregnancy). However, a screening strategy should take into account the fact that PPT may also occur in TPOAb-negative women. It is salutary to remember that the prevalence of diseases for which antenatal screening is currently recommended is considerably lower than that of PPT in women of childbearing age.

TPOAb has been proposed as a marker for the prediction of PPT, although the timing of its measurement remains a point of contention. As mentioned previously, when measured in early pregnancy, TPOAb is present in approximately 10% of women. However, only approximately half of these develop PPT, raising questions about the sensitivity of TPOAb as a predictor. Ten studies have examined TPOAb as a predictor of PPT. No firm conclusions can be drawn from these studies for several reasons. Most investigators measured microsomal antibodies, whereas some measured antibodies to TPO (the specific microsomal antigen). The assay methods used and the timing of antibody measurement were variable. Whereas some investigators measured antibodies in the antepartum period, others measured them at delivery and in the postpartum period. These studies, however, showed that thyroid antibodies have a sensitivity of 0.45–0.89 and specificity of 0.91–0.98. Positive predictive value and relative risk were 0.31–0.78 and 20–50.7, respectively. A report confirmed the cost-effectiveness of a screening program. It is not known whether screening will be improved by thyroglobulin estimation, measuring ultrasound thyroid volume or assessment of complement activation.

Although the benefits of preventing PPT seem fairly clear (avoiding symptoms of thyroid dysfunction and preventing permanent hypothyroidism), no single strategy is currently recommended. An early study offering levothyroxine or iodine to TPOAb positive women showed no difference in the incidence of hypothyroidism either in the treatment or control groups. Another study evaluated giving iodine to TPOAb positive women during pregnancy compared with giving iodine during both pregnancy and the postpartum periods. There was no difference in the incidence or severity of PPT between the groups given iodine compared with the group given placebo. The administration of selenium to TPOAb positive women decreased the incidence of PPT and permanent hypothyroidism, and also caused a significant decrease in TPOAb titer in the postpartum period. The effect of radioactive iodine treatment of Graves' disease on the incidence of subsequent PPT was retrospectively studied by Japanese investigators. They found a reduced incidence of PPT in those who received radioactive iodine for Graves' (2.1%) compared with those who had subtotal thyroidectomy (23.6%) and thionamides (55.1%). But both these sets of data are preliminary and need to be replicated in further studies. Therefore, although the benefits of preventing PPT are self-evident, the jury is still out about a specific strategy in doing so.

Conclusion

PPT is a common endocrine disorder affecting young women. The exact mechanisms of cellular damage have not been determined, although the autoimmune destructive nature of the disease has long been recognized. The immune perturbations of pregnancy and the postpartum period account for the modulation of thyroid autoimmunity, which is the hallmark of the disease, and the timing and nature of clinical and biochemical changes. Although the majority of patients have a short and self-limited illness, some have a prolonged and symptomatic disorder that requires specific therapy. The recognition of short- and long-term morbidity, and the need for permanent thyroxine supplementation in a significant minority of subjects following PPT, has raised the important but unsettled issue of screening for PPT, targeted perhaps at those at highest risk. We await the discovery of a sensitive and specific screening tool.

See also: Thyroid-Stimulating Hormone (TSH; Thyroptopin). Graves' Disease. Hypothyroidism Subclinical. Hyperthyroidism in Graves' Disease. Hashimoto's Thyroiditis. Causes of Hypothyroidism. Thyroid Autoimmunity. Epidemiology of Thyroid Disease. Thyroid Function Tests

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Thyroid and Infertility

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Abbreviations

ART Assisted reproductive technology
FSH Follicle stimulating hormone
ICSI Intracytoplasmic sperm injection
IVF In vitro fertilization
LH Luteinizing hormone
TPOAbs Thyroid peroxidase antibodies

Glossary

Assisted reproductive technology Fertility treatments involving the handling of the woman's eggs and thus does not include isolated stimulation to achieve ovulation or insemination. The most common ART procedure is in vitro fertilization (IVF).
Cretins A person suffering from cretinism, a condition of severely stunted physical and mental growth due to untreated congenital hypothyroidism. Often due to iodine insufficiency during pregnancy and childhood.
FSH Follicle stimulating hormone. Involved in female ovulation and male spermatogenesis.
Gonads Reproductive organs producing sex hormones and cells. In women the ovaries (estrogen, progesterone, and egg cells) and in men the testes (testosterone and sperm cells).
Infertility The inability for a man and a woman of reproductive age to conceive by regular intercourse without use of contraception over a time period of 1 year.
IVF In vitro fertilization. Procedure in which eggs are extracted to be fertilized in the laboratory and after developing for 2–5 days are then transferred to the uterus.
LH Luteinizing hormone. Involved in female ovulation and male testosterone production.
Overt hypothyroidism TSH above reference range and T4 below reference range, or TSH above 10 mU/L.
Subclinical hypothyroidism TSH above upper reference range, but below 10 mU/L, with normal T4 levels.
T4 treatment Replacement of thyroid hormones with synthetic thyroxine.

Introduction

Infertility (the inability for a man and a woman of reproductive age to conceive by regular intercourse without use of contraceptives over a time period of 1 year) affects 10%–20% of couples. Causes are divided into male factor (30%), female factors (35%), both male and female factors (20%) and no known cause (15%). Female factors include endometriosis, tubal factors (often due to previous infection, that is, with chlamydia trachomatis), or ovulatory disorders (hypothalamic/pituitary/ovarian cause). The latter can be due to thyroid disease.

Thyroid hormones are essential to reproduction. Although, many nonthyroidal endocrine abnormalities can lead to infertility (hyperprolactinemia, hypopituitarism, growth hormone deficiency, congenital adrenal hyperplasia to name a few), thyroid disease is common in women of reproductive age. Most often such thyroid disease in young women is concurrent with thyroid autoimmunity representing additional risks to the chance of obtaining and sustaining a pregnancy. Knowledge of the reproductive challenges associated with thyroid disorders and how to diagnose, monitor and treat those, is therefore important to any clinician encountering women of reproductive age.

Key points
- Infertility is defined as the inability for a man and a woman of reproductive age to obtain pregnancy by regular intercourse over a time period of 1 year.
- 10%–20% of all couples in Western Countries experience infertility.
- Thyroid disease increases the risk of infertility.
- Hypothyroidism induces a physiological suppression of the hypothalamic–pituitary–gonadal axis.
- Ovarian hyperstimulation increases the demands for thyroid hormone production, which is difficult to honor in women with low iodine intake or thyroid autoimmunity.
- Thyroid function should be fully restored before initiation of assisted reproductive technology to secure sufficient maternal thyroid hormone supply for fetal development, especially fetal brain development.
- All women attending Fertility Clinics should be screened for TSH level and consulted with an Endocrinologist in case of aberrations.

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Mechanisms of Thyroid Function in Fertility

The mechanisms governing the interplay between thyroid dysfunction and infertility are not well understood. A vast range of animal studies have demonstrated an effect of abnormal thyroid hormone levels on gonad maturation, morphology and function. However, results vary between the numerous investigated species and depending on the developmental stage at which thyroid dysfunction occurs.

In rats, it has been shown that thyroid hormones are involved in regulating testicular development by altering expression of enzymes involved in masculinization (increasing activity and expression of type 1 steroid 5-alpha-reductase and androgen receptor and downregulating aromatase (P450arom/cyp19a1) activity and expression) (Castaneda Cortes *et al.*, 2014; Ulisse *et al.*, 1994). During male rat and mice development, levothyroxine (T4) administration led to an early gonadal development with decreased testicular size at maturation (Krassas *et al.*, 2010). Spermatogenic function was unaltered in thyroidectomized rams, while sperm maturation was compromised. This was restored upon administration of T4 replacement in moderate doses (Chandrasekhar *et al.*, 1986). In both male and female rats, thyroidectomy followed by either a hypothyroid or a iatrogenic hyperthyroid state led to a decrease in luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels (Bruni *et al.*, 1975). Normalization of gonadotropin levels were achieved with euthyroidism. In another study of female rats, inducing hypo- or hyperthyroidism led to a significant rise and fall, respectively, of progesterone levels. Both conditions led to lower LH and estradiol levels, and morphological changes in the ovaries, ovarian duct and uterus (Ajayi *et al.*, 2013).

The association between thyroid function and infertility may in some cases be mediated by thyroid disrupting chemicals. In a study of mice exposed to polybrominated diphenyl ethers, a class of brominated flame retardants, reduced thyroid hormone levels were associated with suppressed spermatogenesis (Sarkar *et al.*, 2016). Finally, thyroid autoantibody-presence was demonstrated in human follicular fluid (Monteleone *et al.*, 2011) and was associated with an increased rate of fetal resorption in female rats and mice (Lee *et al.*, 2009; Matalon *et al.*, 2003). While this is suggestive of immune processes being involved in the reproductive challenges posed by autoimmune thyroid disease, thyroperoxidase antibodies (TPOAbs) are generally not considered pathogenic, but rather an epiphenomenon.

In humans, thyroid hormones have been associated with altered peripheral estrogen and androgen metabolism, hyperprolactinemia, serum hormone-binding globulin levels, and abnormal pulsatile release of LH (Krassas *et al.*, 2010). In a study by Jacobs et al., thyrotropin releasing hormone (TRH) was shown to increase prolactin levels (Jacobs *et al.*, 1971). Snyder et al. demonstrated that compared to euthyroid controls, the prolactin response to TRH stimulation was increased in hypothyroid patients and blunted in hyperthyroid patients (Snyder *et al.*, 1973). Treatment with T4 and antithyroid drugs, respectively, normalized prolactin responses in both patient groups (Snyder *et al.*, 1973). As such a significant positive correlation exist between thyroid stimulating hormones (TSH) and prolactin levels explaining at least some of the ovulatory changes occurring in patients with thyroid disease (Fourman and Fazeli, 2015; Honbo *et al.*, 1978). Furthermore, sex-hormone-binding globulin levels are increased in hyperthyroidism being the predominant reason for a reduced metabolic clearance rate of estrogen and thus higher serum levels (Ridgway *et al.*, 1975). Other studies have found normal gonadotropin levels in both hypo- and hyperthyroidism (despite changes in metabolic clearance rate and sex-hormone-binding globulin levels) (Krassas *et al.*, 2010). Finally, altered responses of LH to gonadotropin releasing hormone stimulation indicate an effect of thyroid hormones on the hypothalamic–pituitary–gonadal axis (Gordon and Southren, 1977; Krassas *et al.*, 2010; Tanaka *et al.*, 1981).

Also, local effects of thyroid hormones on reproductive organs are well-substantiated by the findings of thyroid hormones and expression of thyroid hormone receptors, deiodinases and transporters in reproductive organs as illustrated in **Fig. 1** (Vissenberg *et al.*, 2015). These effects are likely governed by an interplay with hormones of the hypothalamic–pituitary–gonadal axis (Catalano *et al.*, 2007).

As such, alterations in thyroid hormone economy directly impact the hypothalamic–pituitary–gonadal axis and function of reproductive organs in both men and women. The following sections will focus on clinical studies, diagnosis, and treatment of thyroid dysfunction in women with infertility before briefly touching upon male infertility.

Thyroid Function and Infertility

Although knowledge of the biologic mechanisms is still expanding it has long been known that thyroid disease is associated with altered fertility. In 1840, Carl Adolph von Basedow described how menstruations ceased in women with protruding eyeballs and swelling of the thyroid gland (Huth and Murray, 2006; von Basedow, 1840). In 1892, a case report was published on ovarian atrophy and amenorrhea in a thyroidectomized woman (Hofmeister, 1892). In the following decades, the understanding of menstrual irregularities alongside with thyroid disorders was broadened. In a case report of irregular menstruation in a 5-year-old girl with overt hypothyroidism (ceasing upon thyroxine replacement), the author commented on the otherwise "well-known fact that puberty is invariably delayed in cretins" (Kendle, 1905; Lederer, 1954).

Overt Hypothyroidism

Overt hypothyroidism is defined as a thyrotropin (TSH) level above accompanied by free T4 (FT4) levels below reference ranges, or a TSH level above 10 mU/L irrespective of FT4 levels. The prevalence is approximately 0.5%–2% in background populations depending on iodine nutritional status (Hollowell *et al.*, 2002; Vanderpump, 2011). Women with overt hypothyroidism are likely to have reduced reproductive capability. In a case series by Scott et al., 56% of 50 patients with clinical hypothyroidism reported of

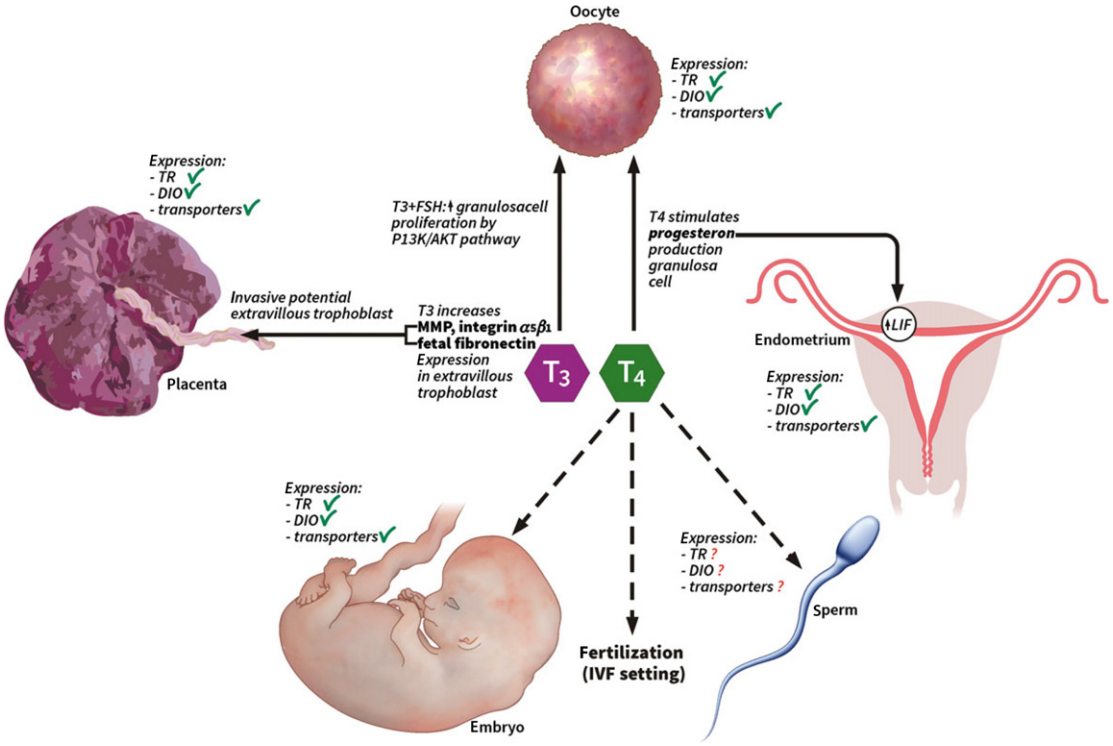

Fig. 1 Thyroid hormone action in reproduction. Multiple local mechanisms of thyroid hormone action have been associated with the reproductive system. *Solid lines* indicate an effect of T4 treatment; dotted lines indicate associations without evidence for causality. Abbreviations: *Akt*, protein kinase B; *DIO*, deiodinases; *IVF*, in vitro fertilization; *LIF*, leukemia inhibiting factor; *MMP*, metalloproteinases; *P13K*, phosphatidylinositol 3′-kinase; *TR*, thyroid hormone receptor. Figure adapted with permission from Vissenberg, R. *et al.* (2015). Pathophysiological aspects of thyroid hormone disorders/thyroid peroxidase autoantibodies and reproduction. *Human Reproductive Update* **21**, 378–387.

menstrual irregularities most of whom had menorrhagia (Scott and Mussey, 1964). In a controlled study of 178 women referred to a thyroid clinic, normal menstrual patterns were reported by 31.8% of hypothyroid and 35.3% hyperthyroid women as compared to 87.8% of healthy controls ($P < 0.001$) (Joshi *et al.*, 1993). Self-reported reproductive challenges (including infertility) was only significantly more prevalent in hyperthyroid patients, but this was mainly driven by an increased pregnancy loss rate. Interestingly, in many of the patients, menstrual irregularities had preceded other symptoms of thyroid dysfunction (Joshi *et al.*, 1993). The study suffered from a small number of patients and unknown thyroid autoimmunity status. In a larger study by Krassas et al. (1999), significantly more hypothyroid women experienced menstrual disturbances than age- and weight-matched controls (23.4% vs. 8.4%, $P < 0.001$). The severity of menstrual irregularity was associated with higher TSH levels. Upon T4 treatment, there was no difference between the two groups (Krassas *et al.*, 1999). More recently, Quintino-Moro et al. conducted interviews about reproductive history with 193 women with Graves' disease (18–50 years old) and 66 women with Hashimoto's thyroiditis (18–60 years old) (Quintino-Moro *et al.*, 2014). None of the included women had sought fertility treatment. Self-reported infertility rates were 52.3% in patients with Graves' disease and 47.0% in patients with Hashimoto's thyroiditis. However, there were no significant differences in number of achieved pregnancies, pregnancy losses or live births between women reporting of infertility and those who did not (Quintino-Moro *et al.*, 2014).

Among infertile patients, overt hypothyroidism is uncommon and will often have been diagnosed in the primary sector inflicting a bias in studies of thyroid function among patients seeking fertility treatment (Poppe et al., 2008b). In 438 women referred for various reasons of infertility, Poppe et al. only found two women with overt hypothyroidism (both antibody-positive) (Poppe *et al.*, 2002). Although median TSH levels were higher in patients with female infertility compared to controls, the prevalence of TSH above reference range was not different (Poppe *et al.*, 2002). In a Finnish cohort of 299 women seeking fertility treatment for the first time, 3.3% had been diagnosed with overt hypothyroidism prior to referral, and at time of screening a total of 12 women (4%) had elevated TSH levels (Arojoki *et al.*, 2000).

Subclinical Hypothyroidism

Subclinical hypothyroidism is defined as a TSH level above reference ranges (but below 10 mU/L) and FT4 within the reference range. The prevalence in background populations ranges approximately 5%–10% with substantial variations depending on national iodine

status, sex, ethnicity and age (Hollowell *et al.*, 2002; Vanderpump, 2011). Many cases are likely transient as described in follow-up studies illustrating normalization upon repeated measurements in up to 50% of cases (Meyerovitch *et al.*, 2007).

The prevalence of subclinical hypothyroidism in infertile women has ranged from 0.7% to 43% in different studies reflecting variations in study designs, analytical methods and definitions of subclinical hypothyroidism (Poppe et al., 2008b). In a retrospective study of 244 women attending a fertility clinic, Abalovich et al. found subclinical hypothyroidism in 13.9% as compared to 3.9% in fertile controls ($P < 0.002$). Among the infertile women, those with precocious ovarian failure, anovulation, and tubal disturbances had the highest prevalences of subclinical hypothyroidism (40%, 22.2%, and 18.2%, respectively). These results were subsequently recalculated to an increased risk of unexplained subfertility in subclinical hypothyroidism with an odds ratio of 4.0 (95% CI 1.7–9.8) (van den Boogaard *et al.*, 2011). Recently, Orouji et al. (2018) compared TSH levels in 187 women referred for unexplained infertility to 52 women with normal fertility (referred for severe male infertility). The women with unexplained fertility had higher TSH levels in both a priori testing (1.95 vs. 1.66 mU/L, $P = 0.003$) and adjusted analyses taking into account age, BMI and smoking status ($P < 0.01$). Furthermore, the prevalence of TSH above 2.5 mU/L was almost doubled in women with unexplained infertility (26.9% vs. 13.5%, $P < 0.05$).

Several studies of patients in fertility treatment have investigated the association between varying degrees of thyroid hypofunction, that is, increased TSH levels, and treatment outcomes. Thus pregnancy rates were lower in infertile women with an elevated TRH-stimulated TSH response (used as indicator of thyroid hypofunction prior to more sensitive TSH assays) (Gerhard *et al.*, 1991). Cramer et al. showed that higher TSH levels significantly predicted a reduced fertilization rate of oocytes (Cramer *et al.*, 2003). However, in a retrospective study of 627 women, Chai et al. did not find ART outcome (clinical pregnancy, live birth and pregnancy loss rate) to be associated with subclinical hypothyroidism (nor thyroid autoantibody-presence) (Chai *et al.*, 2014). Thus, evidence differs with regards to an impact of smaller aberrations of TSH on fertility treatment outcome.

Hyperthyroidism

In women of reproductive age the most common cause of hyperthyroidism is Graves' disease, an autoimmune disease characterized by low TSH and high T4 levels and pathognomonic TSH receptor stimulating or blocking receptor antibodies.

Krassas et al. investigated 214 premenopausal thyrotoxic women and 214 healthy controls and found menstrual irregularities in 21.5% and 8.4%, respectively (Krassas *et al.*, 1994). This was fewer than in previous studies of thyrotoxicosis where hypomenorrhea, oligomenorrhea or, especially, amenorrhea was found in the majority of patient populations (Goldsmith *et al.*, 1952; Krassas *et al.*, 1994)—possibly reflecting more severe thyroid disease due to less sensitive diagnostic testing. As mentioned above, in a more recent study 52.3% of 193 women with Graves' disease reported of having experienced infertility (Quintino-Moro *et al.*, 2014). Although prevalences vary, little doubt remains that hyperthyroidism affect menstrual frequency and fertility. However, subclinical hyperthyroidism has not been associated with a poor treatment outcome.

Thyroid Autoimmunity

Increasing attention is paid to the role of thyroid autoimmunity in reproduction, especially TPOAbs. Thyroid autoimmunity is common in women of reproductive age and many studies have failed to show an increased prevalence among infertile women (Poppe et al., 2008b). Poppe et al. did find a relative risk of 2.3 (CI: 1.02–5.12, $P = 0.045$) of having thyroid antibodies in women who had a female cause for infertility compared to healthy controls (Poppe *et al.*, 2002). Especially, women with endometriosis were more often thyroid antibody-positive in line with hypotheses of endometriosis being an immunological disorder (Eisenberg *et al.*, 2012). The first controlled study to illustrate increased polyautoimmunity in infertile patients was conducted by Roussev et al. who performed multiple immunological testing of 108 women with reproductive failure (45 with unexplained infertility) (Roussev *et al.*, 1996). Since, thyroid antibodies as a marker of an underlying immunological breach causing reproductive failure has been extensively investigated (Bliddal et al., 2017a; Bliddal and Feldt-Rasmussen, 2014).

A meta-analysis of four studies found subfertility to occur more often in thyroid antibody-positive women (OR 1.5, 95% CI 1.1–2.0) (van den Boogaard *et al.*, 2011). Although there may be an increased prevalence of thyroid autoantibodies among infertile women this did not affect the chance of achieving clinical pregnancy (van den Boogaard *et al.*, 2011). In women undergoing ART, Unuane et al. found the crude cumulative live birth rate after six cycles to be 47% in both 333 TPOAb-positive women and 2019 TPOAb-negative women (Unuane *et al.*, 2016). In a more recent meta-analysis of women in IVF/ICSI treatment (Busnelli *et al.*, 2016), women with thyroid autoantibodies did not differ compared to antibody-negative women in fertilization rate (odds ratio 1.11 (95% CI: 0.97–1.27), $P = 0.13$) nor to clinical pregnancy rate (odds ratio 0.9 (95% CI: 0.77–1.06), $P = 0.22$). However, thyroid antibody-positivity was significantly associated with a higher miscarriage rate (odds ratio 1.44 (95% CI: 1.06–1.95), $P = 0.02$) and reduced live birth rate (odds ratio 0.73 (95% CI: 0.54–0.99), $P = 0.04$) (Busnelli *et al.*, 2016). In 2017, these findings were confirmed by one of the largest to date study of 1468 infertile women (Seungdamrong *et al.*, 2017). Thus, presence of TPOAbs without thyroid dysfunction does not seem to affect the chance of obtaining pregnancy by ART, but rather increase the risk of pregnancy loss.

Thyroid Function Testing in Infertile Patients

Based on the findings described in the above, thyroid function testing of all women seeking fertility treatment is endorsed by multiple international guidelines with the aim of correcting thyroid disease before starting fertility treatment (Alexander *et al.*, 2017; De Groot *et al.*, 2012; Practice Committee of the American Society for Reproductive Medicine, 2015). However, several caveats exist in the interpretation of results.

Pitfalls in Thyroid Function Testing in General

Thyroid function tests are influenced by the presence of concurrent or recent illness, assay interference, biologic variation of TSH secretion and sensitivity, and interindividual differences (Baloch *et al.*, 2003). Acute illness (often severe) can lead to nonthyroidal illness syndrome (transient central hypothyroidism with low TSH in the presence of low triiodothyronine (T3)) giving rise to misinterpretation of an apparently normal TSH level. TSH assays can be obscured by heterophilic antibodies or other interfering antibodies. Thus, repeated measurements by an alternative assay with a minimum of 2 to 3 weeks apart is mandatory for a diagnosis of, especially subclinical, thyroid disease. Noteworthy, TSH reference ranges often span a factor of 10 reflecting the large interindividual differences in the background population. The intraindividual thyroid function fluctuations are much smaller and are to be considered based on an individual biological set-point (Andersen *et al.*, 2002; Feldt-Rasmussen *et al.*, 1980). Comparison with the patient's own previous TSH measurements is a good guidance for correct interpretation.

Affection of Thyroid Function Test During Fertility Treatment

Up to 99% of circulating thyroid hormones are bound to thyroid binding globulin. As estrogen increases the liver's production of globulin, any ART procedure stimulating the ovaries and thus increasing estrogen levels, will affect thyroid function variables. These changes even occur remarkably more abrupt than those seen in normal pregnancy (Feldt-Rasmussen and Mathiesen, 2011). The increase in thyroid binding globulin will result in an increased binding capacity and therefore a transient reduction in the circulating FT4 levels (Muller *et al.*, 2000). By negative feedback, TSH will rise to compensate for this reduction (Gracia *et al.*, 2012; Muller *et al.*, 2000). Poppe et al. demonstrated that in previously euthyroid women, FT4 levels were lower and TSH levels higher after ovarian stimulation, and this was significantly more so in women with thyroid autoantibodies (Poppe *et al.*, 2004). Thus, in women with a latent low thyroid reserve (i.e., low iodine intake, thyroid autoimmunity (Feldt-Rasmussen *et al.*, 2011; Glinoer *et al.*, 1994)), the extra demand for thyroid hormone production during controlled ovarian stimulation can trigger (sub)clinical hypothyroidism (see **Fig. 2**).

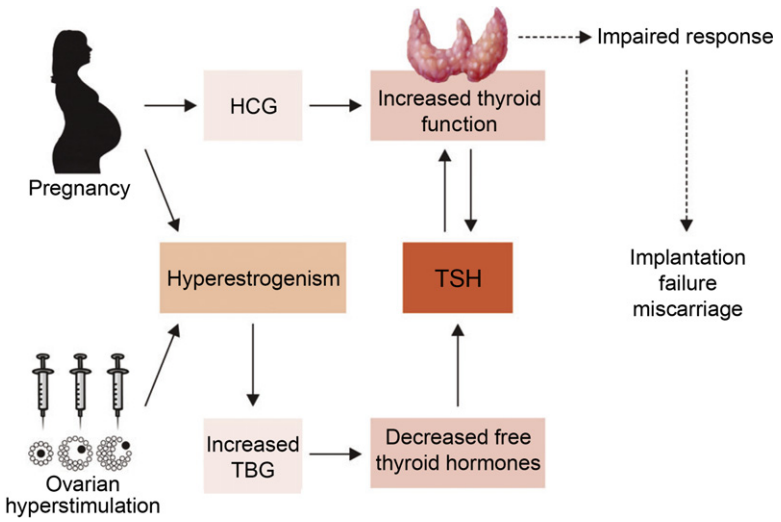

Fig. 2 Thyroid hormone production during pregnancy in ART. In healthy pregnancies, a steep increase in human chorionic gonadotropin levels in the first trimester result in an increased thyroid hormone production by cross-reaction with the TSH-receptor. Ovarian hyperstimulation during ART results in high estrogen levels that stimulate the liver's production of thyroid binding globulin. As a result, more free thyroid hormone is bound and by negative feedback increase pituitary TSH secretion. The combination of physiological pregnancy-adaptation and ovarian hyperstimulation pose high demands on the thyroid's capability of thyroid hormone production. This may be impaired in women with a low iodine reserve or thyroid autoimmunity possibly resulting in an increased risk of implantation failure and miscarriage. Abbreviations: *ART*, assisted reproductive technology; *hCG*, human chorionic gonadotropin; *TSH*, thyroid stimulating hormone. Figure adapted with permission from Colicchia, M. *et al.* (2014). Molecular basis of thyrotropin and thyroid hormone action during implantation and early development. *Human Reproductive Update* **20**, 884–904.

A potentially life-threatening complication of controlled ovarian stimulation is the ovarian hyperstimulation syndrome characterized by cystic enlargement of the ovaries and a risk of shock due to increased capillary permeability and ovarian neoangiogenesis. In two cases, women with known hypothyroidism developed ovarian hyperstimulation syndrome after ART (Poppe et al., 2008a; Skweres *et al.*, 2014). TSH levels steeply increased from normal prepregnancy levels to overt hypothyroidism at the time the women presented with symptoms of ovarian hyperstimulation syndrome. TSH levels normalized after a substantial increase in T4 treatment dose. However, cases of spontaneous ovarian hyperstimulation syndrome in hypothyroidism have also been reported (Borna and Nasery, 2007; Kanza *et al.*, 2013; Sridev and Barathan, 2013).

Screening for TSH and FT4 levels (Baloch *et al.*, 2003) before initiating fertility treatments is thus very important. Should pregnancy be achieved without recognizing low maternal free thyroxine levels, fetal development is at cost.

Special Considerations Related to Pregnancy

In pregnancy, the fertilized egg and (upon implantation) the placenta produces the glycoprotein hormone human chorionic gonadotropin (hCG). The beta-subunit of hCG is capable of binding to the TSH receptor thus stimulating thyroid hormone production. During the first trimester hCG levels steeply increase and thus also thyroid hormone levels, thereby reducing TSH levels by negative feedback. Furthermore, the increased estrogen levels in pregnancy will increase thyroid binding globulin levels and thus total T4 as described in the above (Glinoer, 1999). It is therefore physiological for TSH to decrease to a lower level than prepregnancy and total T4 to increase up to approximately 150% (Alexander *et al.*, 2017). Placental deiodinases involved in transport of maternal thyroid hormones to the fetus, and altered renal clearance of iodine, also affect thyroid hormone status during pregnancy (Glinoer, 1999). In, ART the high level estrogen treatment and subsequent elevation in TSH levels has been shown to exceed pregnancy appropriate targets for TSH, especially in women with preexisting hypothyroidism. The combined stress on the thyroid by ART and pregnancy may thus have important clinical implications for screening and thyroid hormone supplementation (Gracia *et al.*, 2012).

Treatment of Thyroid Diseases During Fertility Treatment

Thyroid hormones play an integral part of fetal development. During pregnancy the fetus rely on a maternal supply of thyroid hormones. This is especially the case in the first and early second trimesters where fetal brain development depends on thyroid hormones, but the fetus has no independent thyroid hormone production (Bernal, 2007). Although unwanted to the woman, the inability to obtain and sustain pregnancy in thyroid disease can therefore be physiologically relevant. Furthermore, it has been hypothesized that even smaller and transient thyroid function impairments may cause implantation failure or miscarriages (Colicchia *et al.*, 2014). Restoring thyroid function before initiating ART is therefore essential to fetal development and will often result in improved fertility. Thus, there is little disagreement on the benefits of treating overt thyroid disease. However, subclinical thyroid disease and isolated thyroid autoimmunity constitute particular challenges, not least due to lack of evidence of benefit.

T4 Treatment in Infertility

Women with known hypothyroidism should increase their T4 dosage before start of ART due to the marked and abrupt changes—in case of spontaneous pregnancy, when becoming pregnant—in order to be able to meet the increased thyroid hormone requirements during pregnancy. Alexander et al. (2004) prospectively followed women with hypothyroidism and concluded that levothyroxine dosage needed to be increased by 50% in the first trimester. A rapid increase in levothyroxine requirements between gestational week 6 and 16 prompted the authors to suggest increments of 30% as soon as pregnancy was confirmed (Alexander *et al.*, 2004). In a small study of 18 infertile women with well-controlled hypothyroidism, Davis et al. compared pregnancies achieved by nongonadotropin and gonadotropin stimulation and found levothyroxine dosage increments to be comparable between groups (30.6% and 32.4%, respectively) (Davis *et al.*, 2007). However, TSH levels were higher in women after controlled ovarian stimulation (3.8 vs. 2.2 mU/L) and lack of significance was likely due to the small number of investigated women. Thus, it is recommended to increase T4 treatment dosage prior to ART and to monitor TSH levels closely in early pregnancy.

T4 Treatment and Outcome of ART

T4 treatment may improve fertility in hypothyroid and subclinically hypothyroid infertile women. Thus Yoshioka found T4 treatment of subclinically hypothyroid women (TSH $>$ 3 mU/L) to improve pregnancy rates and shorten the period of infertility (Yoshioka *et al.*, 2015). In addition to improved fertility, T4 treatment could also improve obstetric outcome of the pregnancies achieved. Abalovich et al. followed 114 hypothyroid women. Women who were not sufficiently treated with T4 during pregnancy experienced pregnancy loss in 60% of cases and preterm delivery in 20% of cases. In comparison none of the euthyroid women had pregnancy loss or premature deliveries (Abalovich *et al.*, 2002). In accordance with this, Busnelli et al. found no difference in pregnancy or delivery rate after ART when comparing 137 hypothyroid women with 274 controls as long as TSH levels were kept below 2.5 mU/L (Busnelli *et al.*, 2013). One study did show a poorer outcome of fertility treatment in women with

hypothyroidism despite sufficient treatment (Scoccia *et al.*, 2012). However, the treatment group more often had competing male factor infertility and a poorer embryo fertilization rate, which could impact results. A randomized controlled trial of the effect of T4 treatment in subclinically hypothyroid women undergoing ART, showed no difference in pregnancy rates, but a reduced pregnancy loss rate (no miscarriages vs. 33.3% in controls, $P = 0.021$) and increased live birth rate ($P = 0.039$) in the treatment group (Kim *et al.*, 2011). Accordingly, a meta-analysis of prospective randomized control trials of T4 treatment in a total of 222 subclinically hypothyroid women undergoing ART found that achieving euthyroidism prior to ovulation induction increased live birth rates (RR 2.76, 95% CI: 1.20–6.44) (Velkeniers *et al.*, 2013).

As part of the ongoing debate on slight thyroid dysfunction in pregnancy is the impact on child neurodevelopment. Observational studies in fertile women found an association between slight maternal thyroid dysfunction and poorer child brain functioning (Haddow *et al.*, 1999; Pop *et al.*, 1999). However, in a large randomized controlled trial on maternal subclinical hypothyroidism or hypothyroxinemia no difference in IQ between the children of treated versus untreated mothers was found in the children at the age of 5 (Casey *et al.*, 2017).

Recommendations for Screening and T4 Treatment in ART

Potentially harmful effects of maternal overtreatment with levothyroxine are not yet clarified, but T4 treatment is generally considered safe. Thus, further studies are needed to define the selection of women who require levothyroxine replacement and to determine the benefits of a predictive dose adjustment strategy. This give rise to discrepancies in guidelines from various scientific societies (Alexander *et al.*, 2017; Lazarus *et al.*, 2014; Practice Committee of the American Society for Reproductive Medicine, 2015). However, due to the possible impact on fertility, obstetric outcome and fetal development, guidelines recommend to screen all patients attending fertility clinics for TSH. The potential downside of this screening strategy is the delay of fertility treatment and overtreatment without clear evidence of benefit (Chan and Boelaert, 2015; Taylor *et al.*, 2014; Velasco and Taylor, 2018). Despite limited evidence showing a benefit of T4 treatment of subclinical hypothyroidism, considering potential benefits, limited side effects, and ART-related thyroid hormone requirements, guidelines recommend T4 treatment for all subclinically hypothyroid women (TSH above upper limit of reference range) in ART. Furthermore, in women with TSH levels between 2.5 and 4.5 mU/L, to perform supplemental TPOAb-measurements and, in case of TPOAb-positivity, T4 treatment (Alexander *et al.*, 2017; Practice Committee of the American Society for Reproductive Medicine, 2015). The treatment goal is a TSH level below 2.5 mU/L in the first trimester of pregnancy (Alexander *et al.*, 2017), although this threshold is somewhat arbitrary (Brabant *et al.*, 2015).

Thyroid Autoimmunity in Euthyroidism

The association between TPOAb-positivity and the risk of pregnancy loss is well substantiated (Alexander *et al.*, 2017; Bliddal and Feldt-Rasmussen, 2014). An increasing amount of evidence also suggests an increased risk of preterm delivery although this finding is less evident (Bliddal et al., 2016, 2017b; Bliddal and Feldt-Rasmussen, 2014; Korevaar *et al.*, 2018). In 2005, Negro et al. conducted a randomized placebo-controlled trial investigating the effect of T4 treatment in TPOAb-positive euthyroid women undergoing ART (Negro *et al.*, 2005). Pregnancy rates were comparable between groups. Euthyroid TPOAb-positive women ($n = 72$) had higher pregnancy loss rates than euthyroid TPOAb-negative women ($n = 412$) (RR 2.01, 95% CI: 1.13–3.56, $P = 0.028$), but there was no difference between the treatment and the placebo group (Negro *et al.*, 2005). However, in a similar trial from the same research group, T4 treatment of euthyroid TPOAb-positive women attending prenatal care had no effect on pregnancy loss rate, but was associated with significantly lower preterm delivery rates (7% vs. 22.4%, $P < 0.05$) (Negro *et al.*, 2006).

In a recently published blinded randomized controlled trial, euthyroid thyroid antibody-positive women undergoing ART were randomized to receive either T4 treatment ($n = 300$) titrated according to continuously monitored TSH levels or placebo ($n = 300$) (Wang *et al.*, 2017). The women underwent the same ART protocol and cases of spontaneous pregnancy were excluded. Approximately one third of women in each group achieved pregnancy and among those, pregnancy loss rates were 10.3% (11 of 107) in the intervention group and 10.6% (12 of 113) in the control group ($P = 0.94$). There were no differences among intervention and control group in terms of live-birth (RR 0.98, 95%CI 0.78–1.24, $P = 0.86$) and risk of preterm delivery (RR 1.13, 95% CI 0.65–1.96, $P = 0.67$) (Wang *et al.*, 2017).

Results are currently awaited from two randomized controlled trials of T4 administration in TPOAb-positive women with infertility or recurrent pregnancy loss; the UK based multicenter TABLET study and the multinational T4life study with preterm delivery and live birth as the respective primary outcomes (T4life Trial, 2017; TABLET Trial, 2017). Meanwhile, the recent ATA guidelines (not taking into account the study by Wang et al.) recommend that "administration of levothyroxine to TPOAb positive, euthyroid women undergoing ART may be considered given its potential benefits in comparison to its minimal risk" (Alexander *et al.*, 2017).

Although, Litwicka et al. found that prednisolone treatment of thyroid antibody-positive women may improve outcome of IVF (Litwicka *et al.*, 2015) this is not recommended by guidelines and neither is treatment with intravenous immunoglobulin therapy (Alexander *et al.*, 2017).

Hyperthyroidism

As with hypothyroidism, treatment of hyperthyroid women normalizes menstrual patterns. Hyperthyroidism should be well controlled before and during pregnancy. In women with Graves' disease, thyroid stimulating or blocking autoantibodies can cross the placenta and affect the fetal thyroid hormone receptors as well. Antithyroid drugs given to the mother also cross the placenta to a higher extent than the maternal thyroid hormones needed for fetal development. Thus, to avoid complications such as iatrogenic fetal hypothyroidism and teratogenic effects of medication this calls for outmost attention from and collaboration between fertility specialists, endocrinologists, obstetricians and pediatricians (Andersen et al., 2017, 2013; Bliddal *et al.*, 2011). Due to the substantial comorbidities related to antithyroid treatment, treatment of subclinical hyperthyroidism is not recommended.

Male Infertility and the Thyroid

Although thyroid dysfunction is far less common in men than in women, both thyroid hypo- and hyperfunction affect male fertility. Clinical studies investigating male thyroid function and fertility suggest an association of both hyper- and hypothyroidism on spermatogenesis, ejaculate volume and erectile function (Krassas *et al.*, 2010). In hypothyroid men with hypogonadotropic hypogonadism, testosterone levels rose and prolactin levels fell when restoring euthyroidism (Donnelly and White, 2000). In hyperthyroid men, erectile dysfunction (prevalent in 78.9%) was ameliorated by restoring euthyroidism (Krassas *et al.*, 2008).

However, thyroid disorders are far less prevalent in men than in women and so far no clinical value has been shown of screening males seeking fertility treatment (Lotti *et al.*, 2016). Instead a targeted case-finding approach in case of suspected hypogonadotropic hypogonadism is advisable.

Conclusion

Thyroid hormones affect the reproductive capability by altering responses of the hypothalamic–pituitary–gonadal axis, changing metabolic clearance of sex hormones and affecting function of local reproductive tissues. Both hypo- and hyperthyroidism in men and women have been associated with infertility. With proper treatment, fertility is restored. Although thyroid autoantibodies constitute an additional risk for infertility they do not seem to reduce chances of obtaining pregnancy by ART, but rather increase the risk of pregnancy loss. Fertility treatment with controlled ovarian stimulation put extra stress on the thyroid gland with a risk of unmasking subclinical cases of thyroid hypofunction. Screening for TSH levels should be performed in all women seeking fertility treatment. However, diagnostic challenges and evolving treatment strategies call for at close collaboration between fertility specialists and endocrinologists. Securing sufficient thyroid hormone economy is detrimental to the chance of achieving and sustaining a healthy pregnancy with an optimal thyroid hormone supply to the fetus.

See also: Graves' Disease. Hashimoto's Thyroiditis. Hypothyroidism Subclinical. Iodine Deficiency. Serum Thyroid Hormone-Binding Proteins. Thyroid Autoimmunity. Thyroid Disease and Pregnancy. Thyroid Function Tests. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Thyroid Disease and Pregnancy☆

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Glossary

Hashimoto's thyroiditis Autoimmune inflammation of the thyroid gland.
Human chorionic gonadotropin (hCG) A hormone secreted by the placenta.
Hyperemesis gravidarum Excessive vomiting during pregnancy.
Hyperthyroidism The condition resulting from the effects of excessive production of thyroid hormone.
Hypothyroidism The condition resulting from the effects of deficient production of thyroid hormone.

Thyroid Size and Function During Pregnancy

An increase in the size of the thyroid gland during pregnancy has been shown in some studies but not others. In regions of adequate iodine intake, the thyroid gland does not typically enlarge during pregnancy, whereas it often enlarges in areas of relative iodine insufficiency.

In regions of Europe where there is reduced iodine intake (average iodine intake of 50–75 μg day^{-1}), such as Belgium and southern France, thyroid volume (as measured by ultrasound) increased approximately 25% in pregnant women. Reevaluation of the thyroid size 1 year postpartum showed that thyroid size was still increased in approximately half of the women. However, pregnant women in southwest France treated with 100 μg iodine per day did not develop thyroid enlargement. Therefore, adequate iodine intake is the most significant factor influencing the size of the thyroid gland.

A number of physiological changes during pregnancy influence thyroid hormone production, transport, and disposal (Table 1). An increase in iodide clearance by the kidney starts in the latter part of the first trimester and continues throughout pregnancy. Serum human chorionic gonadotropin (hCG), which peaks in the first trimester, stimulates thyroid function and size by acting as a thyroid-stimulating hormone (TSH) receptor agonist. Estrogen increases thyroxine-binding globulin (TBG), and thyroid hormone production increases to fill the expanded TBG pool and maintain a normal free thyroxine concentration. Thyroxine disposal is accelerated by type 3 5-deiodinase (D_3) expressed at high levels in the placenta and uterus. There is also an increased volume of thyroid hormone distribution in the plasma. All these factors contribute to an increase in thyroid hormone production during pregnancy that is required to maintain the euthyroid state.

Total serum thyroxine (T_4) and triiodothyronine (T_3) concentrations increase during normal pregnancy due to the estrogen-induced increase of serum TBG, the principal serum-binding protein for both T_4 and T_3. The serum TBG concentration increases early in pregnancy and tends to peak in midpregnancy, approximately 2.5-fold higher than the prepregnancy level. The increase in serum TBG concentration is such a consistent change in early pregnancy that a "normal" serum TBG level in the first trimester is considered an indication of impending miscarriage. The increase in serum TBG concentration is primarily due to an estrogen-induced increase in TBG sialylation that reduces its clearance. The extrathyroidal pool of T_4 must increase more than twofold to maintain normal free T_4 concentration. Free T_4 and T_3 concentrations generally remain within the normal range, although modestly increased or reduced values have been reported. These differences in the results of free hormone measurements may be related to thyroid hormone changes that are a consequence of relative iodine deficiency, the stage of pregnancy, and methodological variables of the free hormone assays used. The regulation of thyroid function by the hypothalamic–pituitary axis is not

Table 1 Physiological changes in pregnancy and their influence on thyroid function tests

Physiologic change	*Serum thyroid test change*
Increased thyroid gland size (women in areas of insufficient iodine intake)	Increased thyroglobulin
Estrogen-mediated increase in serum levels of TBG	Elevated total T_4 and T_3 concentrations
Increased plasma volume	Increased T_4 and T_3 pool
First-trimester elevation in serum hCG	Transient reduction in TSH and probable increase in free T_4 concentrations
Increased expression of type 3 deiodinase (D_3) in placenta and uterus	Accelerated degradation of T_4 and T_3

Abbreviations used: T_4, thyroxine; T_3, triiodothyronine; hCG, human chorionic gonadotropin; TBG, thyroxine-binding globulin.

☆*Change History:* December 2015. AM Leung and JM Hershman has updated affiliation details and few changes in text.
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affected by pregnancy. Alterations of serum TSH in pregnancy reflect changes in thyroid function. The most uniform change during pregnancy is a reduction in serum TSH that corresponds to the peak of serum hCG in the first trimester. Generally, serum TSH remains in the normal range, although subnormal values were reported in approximately 15% of women from an area of relative iodine insufficiency. The reduction of serum TSH is likely the result of an increase in circulating free thyroid hormones at this time. The increase in free T_4 level is most likely mediated by direct stimulation of the thyroidal TSH receptor by hCG.

Hypothyroidism During Pregnancy

The previously described physiological factors that influence thyroid function during pregnancy — increased iodine clearance, increased serum TBG concentration, increased D_3 activity in the placenta and uterus, and increased volume of thyroid hormone distribution in the plasma — are a "stress" on the thyroid gland. Women with normal thyroid glands in iodine-sufficient areas can compensate for these changes, but women in iodine-insufficient areas or those with underlying hypothyroidism may not be able to maintain euthyroxinemia. Although the fetal thyroid gland begins to function at 10–12 weeks of development, maternal thyroid status influences fetal development throughout pregnancy. Up to 40% of the cord blood thyroid hormone concentrations at delivery arise from the mother.

Iodine Deficiency

Iodine deficiency remains an important cause of maternal hypothyroidism in a number of areas throughout the world, including parts of Africa, Asia, and Europe. It is estimated that as many as 500 million people, including up to 30% of school-age children, live in areas of iodine deficiency, despite a worldwide program to eradicate iodine deficiency through universal salt iodization. Approximately 70% of the world's population has access to iodized salt. The World Health Organization recommends that pregnant women receive 250 μg of iodine per day. Since iodine clearance increases during pregnancy, pregnant women in areas of relative iodine insufficiency are at an increased risk of iodine deficiency. In the United States, a survey conducted from 1988 to 1994 indicated that, compared to a similar study conducted from 1971 to 1974, the percentage of women of childbearing age with low urinary iodine (<5 μg dL^{-1}) increased from 4% to 15% and that of pregnant women increased from 1% to 7%. The American Thyroid Association recommends the supplementation of iodine sources from the diet with an oral supplement containing 150 μg iodine during preconception, pregnancy, and lactation.

Hashimoto's Disease

The most common cause of hypothyroidism during pregnancy is chronic lymphocytic thyroiditis, also referred to as Hashimoto's thyroiditis. There is a marked female predominance of Hashimoto's thyroiditis, with a female to male ratio of approximately 7:1. Hypothyroidism can also be seen as a consequence of radioiodine or surgical treatment of Graves' disease, thyroid cancer, or goiter. Transient hypothyroidism in the postpartum period is most often due to postpartum lymphocytic thyroiditis. Hypothyroidism can also occur after external radiation for cervical neoplasms and may also be caused by the medications lithium, amiodarone, or interferon.

Women may first be diagnosed with hypothyroidism during pregnancy. A survey of thyroid function studies of a sample of healthy individuals without known thyroid disease showed a high incidence of undiagnosed hypothyroidism. Thyroid tests were performed from 1988 to 1994 and showed that 4.3% of the group had undiagnosed subclinical hypothyroidism and 0.3% had undiagnosed overt hypothyroidism. Positive anti-thyroid peroxidase antibodies were found in 11.3% of the population without known thyroid disease. The incidence of abnormalities was significantly higher in women than in men. Approximately one-fourth of hypothyroid women have menstrual irregularity, with oligomenorrhea more common than hypomenorrhea and menorrhagia.

Complications

A variety of maternal and fetal complications of pregnancy have been associated with hypothyroidism. Although severe hypothyroidism can interfere with conception, women have become pregnant even when profoundly hypothyroid. Correction of subclinical hypothyroidism with thyroxine replacement has been associated with conception in women with ovulatory defects. Although complications of pregnancy are not uniformly seen in hypothyroidism, a number of complications are observed with a higher frequency in women who are hypothyroid during pregnancy. Among women who experience a miscarriage, a high percentage have positive anti-thyroid antibodies, and some have mild or overt hypothyroidism. Although it is possible that reduced thyroid hormone levels and thyroid autoantibodies play a pathogenic role in miscarriage, it is more likely that thyroid autoantibodies are a marker for antibodies to other fetal or placental components that result in miscarriage. Other complications of pregnancy associated with hypothyroidism include placental abruption, pregnancy-induced hypertension, and preterm delivery. In a study of 150 pregnancies complicated by overt or subclinical hypothyroidism, the incidence of miscarriage and preterm delivery was much higher in patients with incompletely treated hypothyroidism than in those receiving adequate thyroxine

replacement. Term deliveries were seen in approximately 20% of women with inadequately treated hypothyroidism and approximately 95% of women receiving adequate thyroxine replacement at the time of delivery. Table 2 summarizes the effects of hypothyroidism on pregnancy and fertility.

Increased Thyroxine Dose

In women known to be hypothyroid, the thyroxine dose should be titrated to a low-normal TSH prior to conception, if possible. A number of studies suggest that maternal euthyroxinemia is important in early gestation, prior to fetal thyroid gland functioning at 10–12 weeks. Approximately 50–70% of hypothyroid women require an increase in thyroxine dose during pregnancy. Women who are completely athyreotic (as a consequence of radioiodine therapy or surgical thyroidectomy) have the least thyroid reserve and are likely to need the greatest increase in thyroxine dose. Women with some thyroid reserve will need only a small increment or no change in thyroxine dose. The average required increase in thyroxine dose is approximately 50 μg. Women should generally be advised to increase their thyroxine dose by 30% immediately upon confirmation of pregnancy, perhaps by taking two extra tablets of thyroxine each week.

It is also important to caution women about taking calcium and iron supplements simultaneously with thyroxine since this may interfere with thyroxine absorption. Many women may take such supplements for the first time during pregnancy. Thyroid function should be evaluated in the first trimester with subsequent monitoring every 4–6 weeks, with dose adjustments as necessary to keep the serum TSH within the pregnancy-specific ranges (first trimester, 0.1–2.5 mIU L^{-1}; second trimester, 0.2–3.0 mIU L^{-1}; third trimester, 0.3–3.0 mIU L^{-1}). The dose can be returned to the prepregnancy dose immediately after delivery.

Fetal Development

The influence of hypothyroidism on fetal development is controversial. When identified and started on thyroxine replacement soon after birth, infants with congenital hypothyroidism have generally been found to have normal intellectual and neurological development. Some investigators have identified subtle defects of cognitive function in treated hypothyroid children. The placenta does not usually allow thyroxine to cross between mother and fetus. In congenital hypothyroidism, however, there is evidence that in the presence of a large maternal-to-fetal gradient, thyroxine will cross. This permits sufficient thyroxine for the fetus for development. The intellectual development of the offspring of hypothyroid mothers is thought by some to be modestly reduced. One study showed a small but significant reduction in the IQ of 8-year-old children born to mothers with untreated hypothyroidism.

The potential benefits and cost-effectiveness of antenatal thyroid function screening during pregnancy has been controversial. One study of nearly 22,000 women did not demonstrate a difference in the IQ of 3-year-old children born to women who were screened and treated for hypothyroidism (as determined by either TSH, FT_4, or both) if found, compared to children of hypothyroid women whose hypothyroidism was not treated. In contrast, another study based on a large population-based cohort in the Netherlands reported that hypothyroxinemia during pregnancy was associated with increased risks of expressive language and cognitive delays when evaluated in infants age 18–30 months.

Hyperthyroidism During Pregnancy

Graves' disease is the most common clinically recognized cause of hyperthyroidism in pregnant women. The majority of patients have previously identified Graves' disease, although it can also develop for the first time during pregnancy. Most women with hyperthyroidism have oligomenorrhea or amenorrhea.

Table 2 Hypothyroidism and fertility/pregnancy

Maternal complications	*Fetal complications*
Ovulatory dysfunction	Low birth weight
Miscarriage (highly associated with positive anti-TPO antibodies regardless of thyroid status)	Perinatal mortality (some studies show a small increase; others show no difference)
Pregnancy-induced hypertension	Increase in congenital anomalies (studies vary, with some showing a small increase and others no difference)
Placental abruption	Intellectual development (some studies show a small reduction in IQ at 8 years of age)
Preterm delivery	

TPO, thyroid peroxidase.

Table 3 Hyperthyroidism and pregnancy

Maternal complications	*Fetal complications*
Preeclampsia	Low birth weight
Preterm labor	Increased perinatal mortality
	Neonatal hypothyroidism (due to excessive treatment of mother with antithyroid drugs)
	Neonatal hyperthyroidism (due to transplacental passage of maternal thyroid-stimulating immunoglobulin)

Complications

Graves' disease is associated with a variety of complications during pregnancy (Table 3). There can also be significant fetal complications as a consequence of antithyroid drug treatment. Preterm labor and infants small for gestational age are associated with Graves' disease. Approximately 1 in 100 infants from mothers with Graves' disease will have neonatal Graves' disease. Since this results from maternal thyroid-stimulating immunoglobulin (TSI) crossing the placenta, the fetus is at risk even if the mother has been definitively treated with radioiodine or surgery. Some advocate measuring maternal TSI levels in the serum at 26–28 weeks gestation to determine the risk of neonatal Graves' disease, although the predictive value of this measurement is uncertain.

Treatment

There is controversy regarding the appropriate treatment for Graves' disease in anticipation of pregnancy, and the recommendations vary with the severity of disease and the region of the world. Some advocate definitive treatment of Graves' disease with radioiodine prior to pregnancy. This eliminates the possibility of Graves' disease complicating pregnancy, although the thyroxine dose during pregnancy must be carefully monitored and adjusted. Some endocrinologists recommend against radioiodine treatment in women of reproductive age because of fear of radiation dose to the ovaries. Although limited data are available, in follow-up of a small group of women treated as children with high-dose radioiodine for thyroid cancer, there was no increase in the incidence of congenital anomalies. In another study, radioiodine use for thyroid cancer was associated with a delay in childbearing in women across most of the reproductive lifespan.

In most cases of mild to moderate Graves' disease in women of reproductive age, treatment with antithyroid drugs is used. Most authorities recommend a treatment goal of keeping the free T_4 level at the upper normal to slightly elevated level, rather than normalization of the serum TSH level. Studies that have correlated fetal thyroid hormone levels with maternal levels indicate that when the mother is treated to euthyroidism, there is a much greater risk of fetal hypothyroxinemia. The total dose of antithyroid drug should be limited to restrict its transplacental passage. The choice of drug is controversial. A limited number of studies suggest that propylthiouracil (PTU) has less transplacental passage and is found in the breast milk to a lesser extent than methimazole, although PTU has also been associated with rare acute livery injury. However, methimazole therapy has been associated with aplasia cutis, a rare congenital defect manifest as loss of cutaneous structures in the crown of the head, and other more severe embryopathies. In Europe, the most popular drug is carbimazole, which is metabolized to methimazole and has not been associated with aplasia cutis. The American Thyroid Association guidelines for the management of thyroid dysfunction in pregnancy recommend the use of PTU during the first trimester to minimize the risk of embryopathies, and this should then be switched to methimazole during the second and third trimesters to minimize the risk of PTU-induced liver injury.

In general, Graves' disease becomes less active during pregnancy as general immunosuppression occurs in the later stages of gestation. The dose of medication required for treatment is often considerably less in the second and third trimesters. During the postpartum period, however, there can be a flare of Graves' disease, as is seen with many other autoimmune diseases. Some women may have their first manifestation of Graves' disease in the postpartum period. This must be differentiated from postpartum thyroiditis. Although a radioiodine uptake study can distinguish between these conditions, this cannot be easily performed in women during lactation, due to the risk of transmitting radioiodine to the nursing infant. Serum TSH receptor antibodies can be tested to support a diagnosis of Graves' disease. Sequential thyroid function tests show spontaneous improvement or transition to a hypothyroid state in those with thyroiditis, whereas those with Graves' disease have persistent hyperthyroidism.

Human Chorionic Gonadotropin (hCG)-Associated Hyperthyroidism

There is an exponential increase in serum hCG concentrations during the first trimester of pregnancy, with peak levels occurring at 10–12 weeks of pregnancy. *In vitro* studies using cells transfected with the human TSH receptor demonstrate that hCG binds the TSH receptor and activates adenylate cyclase. This is likely responsible for the reduction in serum TSH seen in most women in the first trimester of pregnancy. There are also a number of clinical manifestations of excess hCG (Table 4).

Hyperemesis Gravidarum

Hyperemesis gravidarum is characterized by prolonged and severe nausea and vomiting in early pregnancy and is associated with a loss of 5% body weight, dehydration, and ketosis. It occurs in approximately 1–1.5% of pregnancies, is more prevalent in Asian

Table 4 Spectrum of hCG action in pregnancy

Nature of hCG elevation	*Clinical consequences and changes in thyroid function*
Physiologic elevation in first trimester	Mild elevation of serum free T_4, mild reduction in serum TSH concentration
Exaggerated first-trimester hCG elevation (commonly seen with multiple gestations)	Greater increase in serum free T_4 and greater reduction in serum TSH concentration; associated with hyperemesis gravidarum
Pathologic elevation from hydatidiform mole or choriocarcinoma	Marked elevation in serum free T_4 and free T_3 concentrations; suppression in serum TSH; clinical thyrotoxicosis observed in some patients
Increased "sensitivity" to hCG due to a TSH receptor mutation	Gestational thyrotoxicosis

Abbreviations used: hCG, human chorionic gonadotropin; TSH, thyroid-stimulating hormone.

women than in Caucasians, and is more common with multiple gestations. Abnormalities of serum chemistries include hyponatremia, hypokalemia, hypochloremic alkalosis, and abnormalities of liver function. There is a positive correlation between the severity of vomiting and serum hCG level.

The majority of hyperemesis patients have a suppressed serum TSH level and increased free thyroxine concentration. Free thyroxine and TSH levels normalize when the hyperemesis resolves in the second trimester. In general, these patients do not have clinical features of hyperthyroidism or goiter. The hyperthyroidism is most likely due to a less sialylated hCG, which stimulates the TSH receptor to a greater extent, although it has a reduced serum half-life.

In most patients, the increased thyroid function of hyperemesis gravidarum is self-limited and subsides with the disappearance of vomiting. In a small percentage of patients, however, there is clear clinical evidence of hyperthyroidism; this has been termed transient hyperthyroidism of hyperemesis gravidarum or gestational thyrotoxicosis. In these situations, the diagnosis of Graves' disease should be excluded. Treatment with antithyroid drugs does not influence the course of hyperemesis gravidarum, even when associated with elevated thyroid function tests. If they are used, these drugs should be discontinued as soon as thyroid function tests return to normal and the vomiting subsides.

Trophoblastic Tumors

Clinical thyrotoxicosis has been associated with excessive hCG produced by trophoblastic tumors, hydatidiform mole, and choriocarcinoma. The hCG produced by these tumors is used as a marker for the diagnosis and management of these patients. The serum concentration of hCG in affected patients may be several-fold higher than the peak levels of normal pregnancy. Molar pregnancies are estimated to occur in approximately 1 in 1500 pregnancies in the United States and 1 in 1000 pregnancies in the United Kingdom, and they are significantly more common in Asian and Latin America populations.

Molar pregnancies are usually diagnosed before 20 weeks of gestation because of abnormal vaginal bleeding. Toxemia and hyperemesis gravidarum may also occur in molar pregnancy with greater frequency than in normal pregnancy. Thyroid function abnormality ranges from subclinical hyperthyroidism to increased free thyroxine levels with minimal clinical features of hyperthyroidism and even to severe thyrotoxicosis causing atrial fibrillation and congestive heart failure. In general, the manifestations of hyperthyroidism probably depend on the severity and duration of the increased thyroid hormone levels, which are in turn proportional to the hCG level, and the hCG level is proportional to the mass of the tumor.

TSH Receptor Mutation Sensitive to hCG

A very rare but informative cause of gestational thyrotoxicosis has been reported. In this case, a women and her mother both had a similar history of hyperemesis and hyperthyroidism during pregnancy. The TSH receptor had an A-to-G heterozygous mutation at codon 183 in exon 7, resulting in substitution of arginine for lysine in the middle portion of the extracellular domain of the TSH receptor. *In vitro* studies indicated that cells transfected with the mutant TSH receptor were much more sensitive to hCG than those expressing the wild-type TSH receptor. The hyperthyroidism during pregnancy in this family is most likely due to activation of this mutant TSH receptor by hCG.

See also: Antithyroid Drugs. Serum Thyroid Hormone-Binding Proteins. Thyroid and Infertility. Thyroid Function Tests. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Thyroid Disorders in the Elderly☆

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Epidemiology

Thyroid disorders are common in the general population, and their prevalence increases with age. Based on data from NHANES III it is estimated that 10.4 million people in the United States have thyroid disease, goiter, or take thyroid medication, and an additional 8.7 million have biochemical evidence of thyroid dysfunction (Hollowell *et al.*, 2002). In NHANES III, 4.6% of participants had hypothyroidism, and 1.3% had hyperthyroidism. Measuring serum thyroid stimulating hormone (TSH), Bagchi *et al.* screened more than 900 community dwelling older adults in Detroit, MI and found increased prevalence of hypothyroidism (defined in this study as TSH >6 mU/L) and hyperthyroidism (TSH <0.1 mU/L) compared with the general population: 6.9% and 2%, respectively. The prevalence was higher in subjects older than 75 years as compared to the 55- to 64-year age group (Bagchi *et al.*, 1990). Increased incidence of hypothyroidism or raised TSH with age has also been reported from Great Britain (Vanderpump *et al.*, 1995), in more than 25,000 participants in state health fairs in Colorado(Canaris *et al.*, 2000) and in a population wide study including $>300{,}000$ residents of Tayside, Scotland (Flynn *et al.*, 2004).

Diagnosis of Thyroid Disease in the Elderly

In the presence of an intact hypothalamic-pituitary axis, serum TSH remains the most reliable means of assessing thyroid function. As for most other laboratory parameters, clinicians rely on a reference range, derived from population studies, to determine whether an individual's thyroid function is within the reference range and thus can be considered "normal." In recent years, published reports have shown that the TSH reference range might be different in the elderly population, therefore adding to the complexity of diagnosis of thyroid disease in this age group. Using data from two NHANES surveys, Surks and Hollowell showed that the TSH distribution curve shifts toward higher concentrations with age, suggesting that an increase in the TSH reference limits, especially its upper limit, might be appropriate in the elderly (Surks and Hollowell, 2007). This finding was later confirmed in population studies from Australia (Bremner *et al.*, 2012; Yeap *et al.*, 2017) and Scotland (Vadiveloo *et al.*, 2013). Investigators of the Cardiovascular Health Study also showed that in more than 800 adults with mean age of 85 years who were followed for 13 years, TSH increased over time and it was not associated with increased or decreased risk of mortality (Waring *et al.*, 2012). Other investigators have shown increased serum TSH levels in healthy centenarians (Atzmon *et al.*, 2009) and decreased risk of all-cause mortality in elderly individuals with TSH values of 5–10 mIU/L (Selmer *et al.*, 2014). These findings raise concern about assuming that small increases in TSH in the elderly, up to 8 mIU/L, represent hypothyroidism as well as routinely treating this group with levothyroxine. These findings together imply that subclinical hypothyroidism may be significantly over diagnosed in the elderly when the conventional upper TSH limit is employed. If so, treating mild elevations of TSH with levothyroxine in advanced age may be inappropriate unless an age-specific reference limit was employed. This is of particular concern since 20–40% of elderly people treated with levothyroxine have decreased serum TSH, indicating over-treatment, especially when the risks of adverse events due to iatrogenic hyperthyroidism are taken into account (Mammen *et al.*, 2015).

Another important consideration in diagnosis of thyroid disease in this age group is the higher prevalence of concurrent illness and use of medications which can lead to thyroid dysfunction, for example, amiodarone and iodinated contrast agents, or affect interpretation of thyroid tests, for example, steroid hormones and antiseizure medications. Careful consideration of recent illnesses and exposures is essential for accurate interpretation of test results and before treatment, which is often life long, is started.

Hypothyroidism

In Iodine sufficient areas, Hashimoto's thyroiditis is the most common cause of hypothyroidism. Prevalence of hypothyroidism due to Hashimoto's disease is higher in women than men and increases with age. Overt hypothyroidism is diagnosed when TSH is higher than 10 mIU/mL and concentration of free thyroxine is lower than the lower reference limit (Garber *et al.*, 2012). The classic symptoms and signs of hypothyroidism, including constipation, cold intolerance, dry skin, fatigue, and muscle cramps can be subtle or attributed to aging, side effect of medication, or concurrent illness. Hypothyroidism may thereby not be diagnosed unless free T4 and TSH are determined.

☆*Change History:* September 2017. V Tabatabaie and MI Surks updated the text in the chapter.

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Elderly patients with hypothyroidism should be treated with levothyroxine (Garber *et al.*, 2012). Based on expert opinion, for patients without evidence of coronary artery disease, a starting dose of 50 mcg daily is recommended. If coronary artery disease is possible, reducing the starting dose to 12.5–25 mcg daily is appropriate. After either starting treatment or a change in levothyroxine dosage, serum TSH should be measured after 6–8 weeks and the dosage adjusted appropriately.

Hyperthyroidism

In contrast to young hyperthyroid patients who often present with classic sign and symptoms such as heat intolerance, tremor, palpitations, and anxiety, manifestations of hyperthyroidism in the elderly are often subtle and can go unnoticed for prolonged periods of time. Apathy, depression, weight loss, and fatigue often predominate in the elderly and can be attributed to the normal process of aging or intercurrent illness. Cardiovascular manifestations are also more common in this age group, with many patients first being diagnosed with hyperthyroidism when they present with cardiac arrhythmias, particularly atrial fibrillation (Boelaert *et al.*, 2010).

Effect of hyperthyroidism on cardiovascular and bone health in the elderly deserves special attention. Atrial fibrillation, a known complication of untreated hyperthyroidism, is much more common in the elderly hyperthyroid population than in young people (Osman *et al.*, 2007). Furthermore, elderly hyperthyroid patients are at increased risk for mortality due to cardiovascular causes. Franklyn *et al.* showed that after ablative doses of radioactive iodine and starting thyroid hormone replacement, this increased mortality risk returned to that of the background population (Franklyn *et al.*, 2005). The effect of hyperthyroidism in increasing bone turnover is also well recognized, especially in elderly postmenopausal women. A history of hyperthyroidism has been shown to be associated with increased risk of hip fracture in women (Cummings *et al.*, 1995).

Subclinical Thyroid Disorders

Effect of subtle changes of thyroid function in the elderly has been long debated. In recent years a number of population-based and retrospective studies have contributed to our understanding of the importance of subclinical hypothyroidism and subclinical hyperthyroidism in the elderly.

Subclinical Hypothyroidism

Subclinical hypothyroidism is defined as a serum TSH that is higher than the upper limit of the reference range, but lower than 10 mIU/mL, in the presence of normal free T4. Most experts agree that individuals with serum TSH higher than 10 mIU/mL are more likely to progress to overt hypothyroidism than if TSH is raised but <10 mIU/L. If TSH >10 mIU/L is a stable finding over time, or progressively increases, treatment with levothyroxine is generally recommended, even when free T4 remains within the reference range. Milder subclinical hypothyroidism, that is, when TSH is higher than upper reference limit but <10 mIU/L, has not been consistently shown to affect metabolic or cognitive health or mortality risk in elderly patients.

As part of Cardiovascular Health Study, Cappola *et al.* studied 679 patients with subclinical hypothyroidism at least 65 years old and showed no association between persistent subclinical hypothyroidism and coronary heart disease, heart failure, and cardiovascular death (Hyland *et al.*, 2013). In a pooled analysis of close to 50,000 individual participants with subclinical hypothyroidism, investigators in Thyroid Studies Collaboration showed an increased risk of stroke and fatal stroke in participants 18–64 years of age, but not in those older than 65 (Chaker *et al.*, 2015). These investigators also showed no association between subclinical hypothyroidism with TSH up to 9.9 mIU/L and risk of heart failure, although this risk increased when TSH exceeded 10 mIU/L (Gencer *et al.*, 2012). In contrast, Tseng *et al.* studies Taiwanese adults with subclinical hypothyroidism, defined in their study as TSH 5–19.96 mIU/L and showed increased mortality as compared to euthyroidism regardless of age; surprisingly this association was seen only in those with smaller elevations of TSH (5–9.99 mIU/L), and not in those with higher TSH values (10–19.96 mIU/L) (Tseng *et al.*, 2012).

Similarly, no association between subclinical hypothyroidism and frailty (Virgini *et al.*, 2015), risk of MI in older women (LeGrys *et al.*, 2013), impairment of cognitive function or increased incidence of depression (Yeap *et al.*, 2012) has been demonstrated.

Subclinical Hyperthyroidism

Subclinical hyperthyroidism is defined as a serum TSH that is lower than the reference range, with free thyroxine and T3 concentrations that are within the reference range. Several studies have shown an association between subclinical hyperthyroidism and adverse cardiovascular and skeletal effects in the elderly; this association seems to be more robust when TSH concentration is <0.1 mIU/L).

In a recent pooled study of individual participant data from 13 prospective cohorts, investigators in Thyroid Studies Collaboration demonstrated an increased risk of hip and other fractures, particularly when TSH levels was <0.1 mIU/L (Blum *et al.*,

2015). The same group also demonstrated an increased risk of heart failure in older individuals with TSH values of <0.1 mIU/L (Gencer *et al.* 2012). Subclinical hyperthyroidism has also been show to be associated with increased risk of atrial fibrillation, with a higher attributable risk seen in individuals older than 65 years of age as compared to younger people (Selmer *et al.*, 2012). Other groups have shown that subclinical hyperthyroidism in the elderly is associated with higher incidence of heart failure (Nanchen *et al.*, 2012).

Conclusion

Disorders of the thyroid are common and their diagnosis and treatment pose specific challenges in the elderly. Before making therapeutic decisions, which are often life long, healthcare providers should familiarize themselves with considerations specific to this vulnerable age group.

See also: Causes of Hypothyroidism. Hypothyroidism Subclinical. Thyroid Imaging. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyrotoxicosis; Systemic Manifestations

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Drug Effects and Thyroid Function☆

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Glossary

Amiodarone Medication used to prevent disturbances of heart rhythm. It is high in iodine content and has multiple side effects, including several affecting the thyroid.

Check point inhibitors Anti-PD-1/PD-L1 are a novel class of inhibitors that function as a tumor suppressing factor via modulation of immune cell–tumor cell interaction. These checkpoint blockers are rapidly becoming a highly promising cancer therapeutic approach that yields remarkable antitumor responses with limited side effects.

Deiodinase Family of tissue enzymes that catalyze the removal of an iodine atom from thyroid hormones, as in the conversion of thyroxine to triiodothyronine (T3), an activating step, or the further deiodination of T3, an inactivating step.

Furosemide Diuretic that is a potent inhibitor of thyroxine and triiodothyronine binding to thyroxine-binding globulin.

Heparin Anticoagulant that releases tissue lipases into the circulation. After blood sampling, lipases can lead in vitro to an increased concentration of nonesterified fatty acids, which can displace thyroxine and triiodothyronine from thyroxine-binding globulin.

Lithium Medication that is used for bipolar or manic–depressive illness and that can impair thyroid hormone release, leading to goiter and/or hypothyroidism.

Nonesterified fatty acids (NEFA) Family of hydrophobic products of triglyceride hydrolysis that circulate at a concentration of 0.5–2.0 mmol/L, of which only a minute fraction is unbound, the rest being carried predominantly on albumin. At concentrations >3 mmol/L (see heparin), NEFA can displace thyroxine and triiodothyronine from thyroxine-binding globulin.

Phenytoin (diphenylhydantoin, Dilantin) Anti-epileptic medication that competes for thyroxine and triiodothyronine binding to thyroxine-binding globulin, accelerates the clearance of thyroid hormones, and influences the secretion of thyroid-stimulating hormone.

Thyroid-stimulating hormone (TSH) Two-chain glycoprotein from the anterior pituitary that increases iodide trapping, increases hormone synthesis and release, and stimulates thyroid growth. It is regulated by negative feedback from the circulating concentration of free thyroxine.

Thyroxine (T4) Major secretory product from the thyroid gland, which normally produces approximately 0.1 mg/day. T4 circulates at a total concentration of 50–150 nmol/L, of which 99.97% is protein bound. It is the standard medication used for thyroid hormone replacement.

Thyroxine-binding globulin (TBG) α2-Globulin of hepatic origin that carries approximately 70% of circulating thyroxine (T4) and triiodothyronine (T3). Its concentration is normally approximately 300 nmol/L, being about one-third occupied. Its concentration and capacity is increased by estrogen and several other medications. A wide range of substances can displace T4 and T3 from TBG.

Triiodothyronine (T3) Major metabolically active thyroid hormone that circulates in a total concentration of 1–3 nmol/L, of which 99.7% is protein bound. It is secreted directly from the thyroid gland, but formed mainly from peripheral monodeiodination of thyroxine, by deiodinases in liver, kidney, brain, pituitary.

Introduction

The effects of drugs can be complex, because some agents, for example, amiodarone, glucocorticoids, and diphenylhydantoin, can influence the pituitary–thyroid axis in several ways. There have been significant advances in the understanding of the thyroid-related effects of cytokines, monoclonal antibodies, heparin, phenytoin, amiodarone, and inhibitors of hormone binding to plasma proteins. Importantly and often neglected, abnormal thyroid function, either thyrotoxicosis or hypothyroidism, can influence the effect and potential toxicity of a wide range of therapeutic agents, predominantly by altering their metabolism or clearance.

Altered Release of Thyroid Hormone From the Gland

Iodide at a high dosage acutely decreases the release of thyroid hormone from the gland, apparently by inhibition of proteolysis of thyroglobulin (Bürgi, 2010). This effect is occasionally used in the management of complicated thyrotoxicosis, when standard

☆*Change History*: November 2017. Ulla Feldt-Rasmussen and Åse K Rasmussen updated extensively all sections of the chapter, introduced an in-text reference list and updated the tables and figures.

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Table 1 Principal medications that influence thyroid hormone or TSH levels

Agents inhibiting thyroid hormone production
Antithyroid drugs, amiodarone, lithium, iodide (large doses), iodine containing contrast media
Agents altering the extrathyroidal metabolism of thyroid hormone

– Inhibit conversion of T4 to T3
Propylthiouracil, glucocorticoids, propranolol, amiodarone, iodine-containing contrast media
– Stimulate T4 and T3 degradation
Carbamazepine, barbiturates, rifampicin, phenytoin, sertralin

Agents altering binding of T4 and T3 to plasma proteins

– Increase concentration of TBG
Estrogen, heroin, methadone, clofibrate, 5-fluorouracil, perphenazine
– Decrease concentration of TBG
Glucocorticoids, androgens, L-asparaginase, nicotinic acid
– Displace T4 and T3 from binding proteins
Furosemide, (**Fig. 1**), salicylates, phenytoin, fenclofenac, heparin (**Fig. 2**)

Agents inducing thyroiditis
Amiodarone, interleukin-2, interferon-α, interferon-β, γ-interferon, sunitinib, monoclonal antibody therapy (the check point inhibitors: nivulomab, pembrolizumab, ipilimimab)
Agents affecting TSH secretion

– Increase serum TSH concentration
Lithium, dopamine receptor blockers, L-dopa inhibitors, cimetidine, clomiphene, amiodarone, amphetamine
– Decrease serum TSH concentration
Thyroid hormones, dopamine, L-dopa, glucocorticoids, growth hormone, somatostatin, octreotide, interferon-α

Agents impairing absorption of ingested T4
Aluminum hydroxide, ferrous sulfate, cholestyramine, calcium carbonate, calcium citrate, calcium acetate, iron sulfate, colestipol, sucralfate, soya preparations, kayexalate, ciprofloxacin, sevelamer, or proton pump inhibitors

TSH, thyrotropin; *T4*, thyroxine; *T3*, triiodothyronine; *TBG*, thyroid hormone binding globulin.

anti-thyroid drugs cannot be used or are insufficient. Iodine containing contrast agents e.g. those used in coronary angiography can have similar effect due to iodine load (Bonelli *et al.*, 2017), and lithium also inhibits thyroid hormone release as a result of decreased proteolysis of thyroglobulin (Lazarus, 2009).

Inhibition of 5′-(Outer Ring) Deiodination of T4

The outer ring deiodinase, or 5′-deiodinase, present predominantly in liver, kidney, thyroid, and heart, designated type 1, is a selenoprotein that catalyzes the peripheral conversion of T4 to triiodothyronine (T3) and reverse T3 (rT3) to 3,3′T2. This activity is markedly diminished by caloric deprivation and in catabolic states. Activity of this enzyme is also inhibited by numerous drugs (**Table 1**), resulting in decreased T3 and increased rT3 serum concentrations. Several iodinated compounds, including amiodarone (Rosene *et al.*, 2010), also inhibit the pituitary 5′-deiodinase type 2, an effect that results in decreased formation of T3 in the pituitary, leading to a slight increase in serum TSH, generally within the normal reference range (Harjai and Licata, 1997).

Beta-blockers differ from one another in their effect on 5′-deiodination. Propranolol in high doses diminishes the production of T3, an effect due to a quinidine-like membrane stabilizing effect, rather than specific beta-blockade (Wiersinga, 1991). This T3-lowering effect is not seen with other beta-blockers, such as atenolol, metoprolol, or labetalol. The symptomatic benefit of beta-blockers in thyrotoxicosis is independent of their influence on serum T3. A subnormal serum T3 concentration is a normal response to illness or caloric deprivation that is potentially beneficial; this acute response should not be interpreted as hormone deficiency (Van den Berghe, 2014), although measurements of free T4 may be indicative of such condition in a number of commercially available free T4 assays (Sapin *et al.*, 2000). An experimental model of this non-thyroidal illness condition using infusion of salsalate in healthy volunteers found that an acute release of T4 and T3 from circulating transport proteins, induced by this inhibitor of binding, can result in large and rapid redistribution of T4 and T3 into tissue compartments associated with transiently reduced peripheral tissue 5′-monodeiodination and deranged TSH regulation (Wang *et al.*, 1998). Thus, during non-thyroidal illness, or with the use of medications that can lower serum T3 (Van den Berghe *et al.*, 1994), the possibility of thyrotoxicosis should not be dismissed on the grounds of normal serum T3, if this diagnosis is supported by T4 excess and suppression of TSH.

Enhanced Thyroid Hormone Metabolism and External Loss

There is usually little effect from medications that enhance thyroid hormone clearance if the pituitary–thyroid axis is normal, but effects may be profound when an individual is dependent on exogenous T4. Hepatic T4 and T3 metabolism is enhanced by numerous agents that stimulate the cytochrome P450 system, among others rifampicin, phenytoin, carbamazepine, and barbiturates (Curran and DeGroot, 1991). External loss of protein as in severe proteinuria in nephrotic syndrome or severe protein loosing enteritis includes loss of thyroid hormones bound to TBG and thus the major circulating reservoir mainly of T4. These patients also require higher dosages of T4 if on replacement therapy, and they may even require commencement of T4 if decompensating to hypothyroidism with prior euthyroidism before the protein loss (Chandurkar *et al.*, 2008).

Altered Concentration of Plasma-Binding Proteins

Estrogens, whether endogenous or exogenous, commonly affect tests of thyroid function by increasing total serum T4 and T3 due to an increase in the serum concentration of thyroxine-binding globulin (TBG), but free T4 and T3 remain normal. Estrogens increase the glycosylation of TBG, which slows its clearance, leading to a higher serum concentration, or binding capacity, with normal binding affinity. Transdermal estrogens do not show this effect to the same extent, because they have less influence on hepatic proteins. In pregnancy, the mean total serum T4 concentration is increased by approximately 30%–50%, with over 40% of values above the normal reference range in the second and third trimesters (Feldt-Rasmussen and Mathiesen, 2011). This estrogen induced increase in TBG can result in a higher requirement of T4 replacement dose in women treated for hypothyroidism (Arafah, 2001). Conversely, antiandrogen therapy for breast cancer can decrease this requirement (Arafah, 1994). It is not certain whether other drugs that alter the serum concentration of TBG (**Table 1**) increase the synthesis or retard the clearance of TBG.

Competition for Thyroid Hormone Binding to Plasma Proteins

Thyroid hormone-binding proteins show wide cross-reaction with various drugs (**Table 1**; Lim *et al.*, 1988). In contrast, the binding proteins for corticosteroids, vitamin D, and the sex hormones are more specific for a single family of ligands. Substances that interfere with T4 and T3 binding are themselves bound to albumin. Because of differences in relevant total therapeutic concentration and albumin binding, the hierarchy of drug competitor potency in vivo in undiluted serum differs markedly from the rank order of drug affinity for isolated TBG (Stockigt, 2001; Surks and Sievert, 1995).

Serum dilution and albumin concentration need to be clearly defined in the interpretation of in vitro drug competition studies, because the concentrations of free ligand and competitor(s) and the number of unoccupied binding sites do not maintain the relationship that exists prior to serum dilution.

Therapeutic concentrations of phenytoin and carbamazepine induce an artifact with an increased free fraction of T4 by 40%–50% in undiluted serum, but no increase in free T4 when assayed in diluted serum (Surks and Defesi, 1996). Similarly, the T4-displacing effect of furosemide is most obvious (**Fig. 1**) in methods with the lowest sample dilution (Hawkins, 1998).

Any substance that shares albumin-binding sites with a competitor can influence the free concentration of that competitor. Indoles and furans that accumulate in renal failure can displace drugs from albumin and may thus indirectly accentuate drug

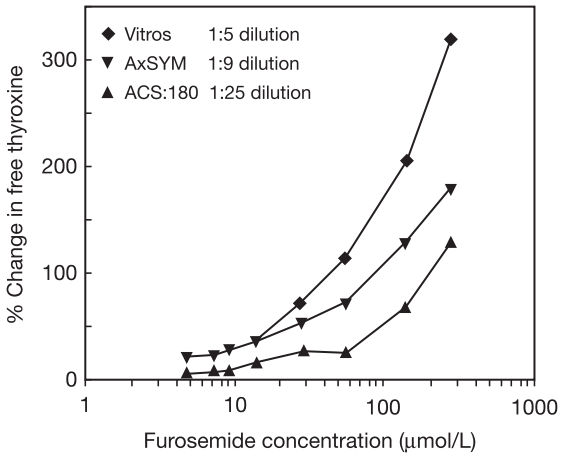

Fig. 1 Effect of furosemide on estimates of serum free T4 by three methods, showing that T4 displacement is progressively lost with increasing sample dilution. The least diluted serum sample most closely reflects the in vivo effect. Redrawn from Hawkins, R. C. (1998). Furosemide interference in newer free thyroxine assays. *Clinical Chemistry* **44**, 2550–2551.

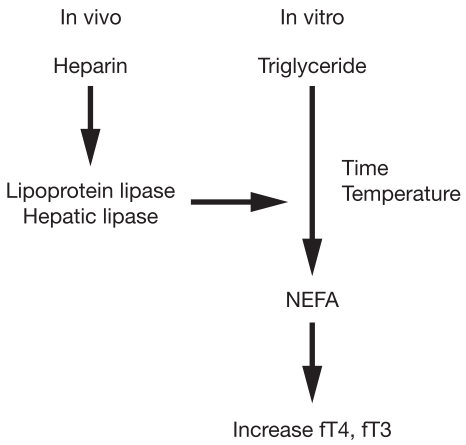

Fig. 2 Heparin-induced changes can markedly increase the apparent concentration of serum free T4. Heparin in vivo (left) liberates lipoprotein lipase from vascular endothelium. Lipase acts in vitro (right) to increase the concentration of free fatty acids to >3 mmol/L, which displaces T4 and T3 from TBG. Generation of non-esterified free fatty acids (NEFA) is increased by sample storage or incubation and by high serum triglyceride. The T4-displacing effect is accentuated at low albumin concentrations. Concept from Mendel, C. M., Frost, P. H., Kunitake, S. T. and Cavalieri, R. R. (1987). Mechanism of the heparin-induced increase in the concentration of free thyroxine in plasma. *Journal of Clinical Endocrinology and Metabolism* **65**, 1259–1264.

effects on hormone binding; i.e., the free hormone concentration can be influenced by substances that have little direct effect on hormone binding (Lim *et al.*, 1993).

The effect of heparin on increasing the apparent free T4 concentration in vitro is an example of spurious competition. Heparin treatment causes an in vivo release of lipases, followed by generation of non-esterified free fatty acid during sample storage or incubation in vitro, leading to much higher non-esterified free fatty acid concentrations in the assay tube than were present in vivo (see Fig. 2). Heparin doses as low as 10 units can cause this effect after prolonged sample incubation, especially if serum triglyceride is increased. Low-molecular-weight heparin preparations have a similar effect. This artifact may account for reports of method-dependent increases in apparent free T4 concentrations in hospitalized subjects who are often exposed to heparin (Mendel *et al.*, 1987).

Agents That Modify Immune Function and Induce Thyroiditis

Treatment of hepatitis C with interferon-α is frequently associated with thyrotoxicosis or hypothyroidism, sometimes in a sequence that suggests thyroiditis; the abnormalities are often transient, with resolution occurring over several months after treatment is ended. The prevalence of this effect has been estimated at 15%–30%, with greater frequency in females and those with positive thyroid peroxidase antibodies (Hsieh *et al.*, 2000). In contrast, thyroid dysfunction is less common during interferon-β treatment of neurological disorders, such as multiple sclerosis (Durelli *et al.*, 2001; Coles *et al.*, 1999).

Monoclonal antibody treatment for multiple sclerosis may lead to persistent autoimmune thyroid dysfunction. Six to thirty months after treatment, one-third of previously euthyroid patients who received a 5-day course of the humanized anti-CD52 monoclonal antibody developed antibodies against the TSH receptor (Shang *et al.*, 2017). Check point inhibitors act by promotion of the effector T cell response to tumors. The drugs enhance effector T cells by blockade of programmed cell death protein (PD-1) (Shang *et al.*, 2017) and its ligand PD-L1 (pembrolizumab, nivulomab) or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (ipilimumab). The drugs have endocrinological side-effects because of exacerbation of autoimmunity, and thyroiditis is reported in up to 43% of patients treated with nivulomab, but much rarer after treatment with pembrolizumab and ipilimumab (Barroso-Sousa *et al.*, 2017; Abdel-Rahman *et al.*, 2016). The check point inhibitors can also induce autoimmune conditioned hypothyroidism or Graves' hyperthyroidism (Shang *et al.*, 2017).

Altered Secretion of Thyroid-Stimulating Hormone

Glucocorticoids, either endogenous or exogenous, are potent inhibitors of thyroid-stimulating hormone (TSH) secretion (Haugen, 2009). Dopaminergic drugs, even at doses that do not influence blood pressure, cause profound inhibition of TSH secretion; after cessation of dopamine infusion, there is a rapid reversal of TSH suppression within a few hours (Haugen, 2009; Van den Berghe *et al.*, 1994).

Cytokines directly affect TSH secretion (Pang *et al.*, 1993). For example, infusion of interferon-α in normal volunteers results in a 60%–70% decrease in serum TSH within 8–12 h, prior to any significant change in serum thyroid hormone concentrations

Table 2 Medications with multiple effects on the pituitary–thyroid axis

Glucocorticoids
Inhibit TSH secretion
Impair thyroid hormone release from the gland
Inhibit 5′-deiodination of T4 and rT3
Decrease concentration of TBG
Amiodarone
Delivers iodine load
Iodine-induced thyrotoxicosis (Jod-Basedow)
Iodine-induced hypothyroidism
Causes unique form of thyrotoxicosis due to thyroiditis
Retards T4 clearance
Inhibits 5′-deiodination (type 1 and type 2) of T4 and rT3
Augments TSH secretion
Phenytoin
Inhibits T4 and T3 binding to TBG
Accelerates T4 clearance and metabolism

TSH, thyrotropin; *T4*, thyroxine; *T3*, triiodothyronine; *rT3*, reverse triiodothyronine; *TBG*, thyroid hormone binding globulin.

(Corssmit *et al.*, 1995). In addition, cytokines can induce or aggravate autoimmune thyroid dysfunction (McLachlan and Rapoport, 2014).

During long-term amiodarone therapy, TSH tends to be high in relation to serum free thyroxine (T4), because this drug, or its active metabolite, desethylamiodarone, acts as an antagonist at thyroid hormone receptors (Rosene *et al.*, 2010). Heavy amphetamine abuse can result in higher than normal levels of serum TSH and may cause TSH-induced hyperthyroxinemia.

Impaired Thyroxine Absorption

Numerous agents (**Table 1**) can impair the absorption of ingested T4, some probably by binding T4 in the bowel lumen by e.g. complex formation with the drug (Mersebach *et al.*, 1999). Such agents have little effect if the pituitary–thyroid axis is intact, but they may make fixed oral T4 dosage inadequate in hypothyroid patients who have no capacity to increase T4 production. This effect can generally be minimized by avoiding concurrent ingestion of T4 and potential inhibitors of absorption or use liquid formulations of levothyroxine preparations (Vita *et al.*, 2017).

Clinical Issues With Particular Drugs

Amiodarone

Amiodarone affects thyroid status in a complex manner (**Table 2**). Benign euthyroid hyperthyroxinemia, with high total and free T4, normal or subnormal serum T3, and increased serum rT3, occurs in up to one-third of amiodarone-treated subjects. This abnormality requires no treatment—the diagnosis of amiodarone-induced thyrotoxicosis should not be based on T4 excess alone.

In iodine-replete regions, the predominant amiodarone-induced thyroid problem is hypothyroidism, which is especially likely to occur on a background of thyroid autoimmunity. Routine replacement with T4 is effective, but therapy may need to be modified because of associated heart disease.

The most difficult abnormalities that result from amiodarone therapy are two unpredictable forms of thyrotoxicosis, one due to iodine excess and the other attributed to a unique form of persistent thyroiditis with specific intracellular inclusion bodies. Severe life-threatening thyrotoxicosis can occur rapidly, without premonitory abnormalities of thyroid function. Weight loss, deterioration of cardiac function, and severe myopathy are the important clues to this diagnosis. There can be poor correlation between circulating thyroid hormone levels and the clinical features of amiodarone-induced thyrotoxicosis, perhaps because of interaction of this drug, or its active metabolite desethylamiodarone, with thyroid hormone receptors.

A distinction between these two forms of amiodarone-induced thyrotoxicosis by color Doppler flow studies has been reported. If blood flow is increased (Easton *et al.*, 2002) the thyrotoxicosis may be directly due to iodine excess; standard anti-thyroid drugs, with the possible addition of potassium perchlorate, may be appropriate first-line therapy. In contrast, glucocorticoids are generally preferred in the thyroiditis variant that shows markedly decreased thyroid blood flow. Emergency thyroidectomy may be necessary in some cases of severe amiodarone-induced thyrotoxicosis that cannot be controlled medically.

At the other end of the spectrum, short-term amiodarone for atrial fibrillation after catheder ablation gave only a very transient change of thyroid function (Diederichsen *et al.*, 2016).

Iodine-Based Contrast Agents

Most used contrast agents in radiology for CT scans and angiographies are iodine-based, and may thus put the thyroid function at risk. In current practice, nonionic low or isoosmolar preparations are used almost exclusively for intravascular injections (Beckett *et al.*, 2015; Thomsen, 2011). Iodine based contrast agents for an average examination contain free iodine equal to around five times the daily recommended dose for intake of iodine (Thomsen, 2011). Yet, for some CT scans approximately 500–600 mg of iodine per kilogram of bodyweight is recommended to obtain adequate imaging of hepatic parenchymal enhancement (Spampinato *et al.*, 2017).

It is generally considered, that patients with normal thyroid function are not at risk (Thomsen, 2011), even though urinary iodine can be increased (Lee *et al.*, 2015a,b). However, thyroid dysfunction after exposure to iodine-based contrast media has been described in persons with completely normal thyroid function and no apparent abnormality in their thyroid glands (Lee *et al.*, 2015a,b). The effect on the thyroid gland can be both one of hypo- and hyperthyroidism as for other iodine compounds (Thomsen, 2011; Lee *et al.*, 2015a,b). In a group of 810 consecutive patients with ischemic heart disease undergoing cardiac angiography, 58 (7.2%) had hyperthyroidism before the procedure, but more patients developed hyperthyroidism after the iodine load (10%) (Bonelli *et al.*, 2017).

In another study (Vassaux *et al.*, 2017) the thyroid perturbing effect from iodine-based contrast agents seemed to be unrelated to the free iodine content, but rather an iodine unrelated direct effect on the sodium iodide receptor (NIS), causing a dramatic drop in their numbers.

Independent on the amount of contrast media used, patients with untreated Graves' disease and/or multinodular goiter and thyroid autonomy, the elderly, and those living in areas where dietary iodine deficiency is common should be identified, and the risk of inducing thyrotoxicosis through excess iodine absorption should be reduced (Beckett *et al.*, 2015). Use of iodinated contrast agents should be avoided immediately before planned radioactive iodine imaging or therapy, because the iodine may reduce radioactive iodine uptake. It is also advised that consultation with an endocrinologist may be beneficial before administration of an intravenous contrast agent (Thomsen, 2011; Beckett *et al.*, 2015).

Lithium

This agent, widely used in the management of manio–depressive illness, has several effects on the pituitary–thyroid axis, the most important being the effect of inhibiting thyroglobulin hydrolysis and hormone release. Lithium exacerbates, or may possibly cause, autoimmune thyroid disease of the Hashimoto type, leading to eventual primary hypothyroidism, often with goiter. Women with positive anti-peroxidase antibodies are especially likely to be affected. Among 690 lithium-treated Scottish patients, 14% of women and 4.5% of men developed various grades of thyroid failure (Lazarus, 1998, 2009). There are also reports of lithium-induced thyrotoxicosis of probable autoimmune origin.

It is generally recommended that TSH, T4, and anti-peroxidase antibodies be assessed before commencement of lithium therapy, with serial evaluation of thyroid status every 6–12 months during treatment or if a goiter develops. Thyroxine replacement is recommended if there is TSH excess.

Phenytoin

This anti-epileptic drug commonly results in subnormal serum total T4, with an apparent lowering of free T4, not accompanied by the anticipated increase in TSH. Such findings are not easily distinguishable from central hypothyroidism due to pituitary deficiency. The effect on free T4 may be spurious, because the T4-displacing effect of phenytoin is poorly reflected by assays that use diluted serum, leading to an underestimate of the free T4 concentration.

It remains difficult to make an accurate assessment of thyroid status in hypopituitary patients who are taking T4 together with phenytoin or carbamazepine; serum TSH is not useful and free T4 estimates can be misleading. Phenytoin accelerates T4 clearance by induction of cytochrome P450 enzymes (Curran and DeGroot, 1991), so that the replacement dose may need to be increased. Treatment with phenytoin or carbamazepine can make previously optimal treatment of primary hypothyroidism inadequate or may unmask diminished thyroid reserve.

Effects of Thyroid Status on Drug Effects

In general, thyrotoxicosis increases drug clearance, whereas hypothyroidism may markedly retard drug disposal. During thyrotoxicosis, standard drug dosage may be ineffective, as, for example, in the reputed "insensitivity" of thyrotoxic patients to digitalis preparations. A higher than normal dosage may be required, but as the thyrotoxicosis comes under control, digitalis toxicity can occur unless dosage is adjusted.

Severely hypothyroid subjects are abnormally sensitive to narcotics, sedatives, and analgesics, due to diminished clearance of these substances. This may be recognized as prolonged respiratory depression after anesthesia, when hypothyroidism has not been previously recognized (O'Connor and Feely, 1987).

Anticoagulant therapy is an exception to this rule (Kellett *et al.*, 1986). Consumption of coagulation factors tends to be more rapid in active thyrotoxicosis, with a tendency toward increased responsiveness and lower coumarin dose requirements. An increase in dosage may be required as control of thyrotoxicosis is achieved.

See also: Causes of Hypothyroidism. Hashimoto's Thyroiditis. Sodium/Iodide Symporter (NIS). Thyroid Autoimmunity. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyrotoxicosis Factitia

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Lithium☆

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Introduction

In the first half of the 20th century, lithium was used as a salt substitute in patients with cardiac failure, but severe toxicity prevented its acceptance. In 1949, during an investigation into the role of uric acid in manic patients, Australian psychiatrist J. F. J. Cade noticed that guinea pigs that had been given lithium urate became less startled. This led to the trial administration of lithium in manic depressive patients and then to the widely acclaimed studies of Schou and colleagues from Denmark defining the clinical effectiveness of this ion in psychiatry. It is the most effective mood stabilizer for the treatment of bipolar disorder, but it is toxic at only twice the therapeutic dosage and has many side effects. The endocrine effects of lithium may be regarded as comprising effects of lithium on cell function as well as on the influence of the ion on specific endocrine glands or systems.

Cell Function

The transport of lithium has been studied extensively in red cells. There are four separate pathways of lithium transport: the sodium–lithium countertransport system, the bicarbonate pathway, the sodium–potassium ouabain-sensitive pump, and the so-called passive leak pathway. These systems contribute variably to total lithium transport. The intra- to extracellular concentration ratio, which is a steady-state distribution ratio, is most dependent on the countertransport pathway, although both passive leak and the bicarbonate pathways make a contribution, at least in humans. Lithium inhibits the formation and action of the adenylate cyclase cAMP system in many tissues, but the precise mode of action is unclear. This may have adverse effects in many endocrine and other systems. For example, the immunological modulatory effects of lithium are thought to be mediated at least in part by its inhibitory action on lymphocyte cAMP. Lithium also affects the signal transduction system involving inositol phospholipids. The effect on phosphatidyl inositol may influence the calcium supply to a cell, thereby affecting function. Attention has also been directed to the action on regulator network GSK3 (glycogen synthase kinase 3). The action through inositol monophosphatase is intriguing but to date no small molecule antagonists are available. It has been reported that ebselen (an antioxidant) inhibits the enzyme and exhibits lithium-like actions at many levels, including enzymatic, inositol recycling, and animal behavior. This drug lowers inositol concentrations in human brain suggesting that this may be an important action of lithium. Lithium may also affect cellular endocrine function by separate actions on membrane transport and intracellular enzyme function.

At the neuronal level, lithium reduces excitatory (dopamine and glutamate) but increases inhibitory (GABA) neurotransmission. Lithium also appears to reduce the oxidative stress that occurs with multiple episodes of mania and depression. These actions of lithium are complex and interrelated in defining the therapeutic action of the drug.

Lithium and the Thyroid

Administration of lithium to humans causes inhibition of thyroidal hormone release and, to a lesser extent, inhibition of intrathyroidal biosynthesis of thyroxine. In patients already possessing thyroid antibodies (thyroid peroxidase or antithyroglobulin), lithium exposure leads to an increase in titer of these antibodies and early onset of hypothyroidism. The clinical effects of lithium are characterized by goiter without antibodies and euthyroidism, antibody-negative goiter with accompanying hypothyroidism, euthyroid goiter with positive antibodies, and the development of autoimmune thyroiditis with hypothyroidism. Screening thyroid function and thyroid antibodies is recommended before beginning lithium therapy.

Lithium increases iodine retention and inhibits thyroid hormone release. It has therefore been evaluated as an adjunct in the radioiodine treatment of Graves' and toxic nodular hyperthyroidism. The likelihood of cure was significantly greater in those patients receiving adjuvant lithium and this supports its use in improving the efficacy of radioiodine in the therapy of Graves' disease and the management of toxic nodular goiter. It has beneficial effects in the management of some patients with thyroid cancer.

Some patients receiving lithium have developed thyrotoxicosis with or without positive thyroid-stimulating hormone receptor-stimulating antibodies. There is no consensus that lithium causes this hyperthyroidism immunologically or in any other way.

☆*Change History:* September 2017. Abstract, body part of the text, and the reference list have been updated and some older references have been removed. J.H. Lazarus updated text and references.

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Lithium and Calcium

Lithium alters the calciostat and renders the parathyroid cell less sensitive to calcium. It can stimulate the release of human parathyroid hormone in vitro which may account for the increase in set point in response to calcium. Some patients develop parathyroid adenomas accompanied by an increase in serum calcium and immunoreactive parathyroid hormone. Parathyroid hyperplasia has also been described more often than expected but of patients who do develop hyperparathyroidism more than half have single adenomas.

Other Endocrine Effects

Diabetes insipidus (DI) occurs in up to 40% of patients within a few days of beginning lithium therapy. DI is usually nephrogenic, with high circulating concentrations of arginine vasopressin and an impairment of the renal response to this hormone. Aquaporins are water channels inside of proteins expressed in renal tubules and collecting ducts. The greater the activation of aquaporins, the greater water reabsorption in renal collecting ducts, reducing the volume of urine. When the expression of aquaporins is inhibited, polyuria ensues. The binding of antidiuretic hormone (ADH) to the V2 receptor stimulates the expression of aquaporins in the kidney. Lithium inhibits the expression of these aquaporins in the renal collecting duct, mainly aquaporin 2 (AQP2), by mechanisms still not fully understood. Lithium may also inhibit antidiuretic hormone release from the posterior pituitary, leading to central DI but this only occurs in a few cases.

The clinical effect of lithium on adrenal function is minimal, although urinary aldosterone concentration increases soon after starting the drug. An increase in body weight is commonly seen in patients receiving lithium. The effects of the drug on glucose metabolism are not thought to be of clinical significance.

See also: Thyroid-Stimulating Hormone (TSH; Thyroptopin). Thyroid Function and Depression

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Amiodarone and Thyroid☆

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Glossary

Action potential Changes in the plasma membrane during depolarization and the subsequent repolarization of cardiomyocytes. The duration of the action potential is reflected by the QT time of the electrocardiogram (interval between the start of the QRS-complex and the end of T-top). Class III anti-arrhythmic drugs (such as amiodarone) act via prolongation of the action potential.

Colloid Proteinaceous substance in thyroid follicles, containing sizable quantities of iodine, thyroglobulin, and thyroid hormone.

Deiodination Enzymatic removal of iodine atoms from organic compounds.

Euthyroidism Normal thyroid function.

Lysosome A membranous bag of hydrolytic enzymes; this organelle is used for the intracellular digestion of macromolecules.

Organification The incorporation of iodine atoms in organic compounds.

Thionamides Class of anti-thyroid drugs (such as carbimazole, methimazole, and propylthiouracil) that inhibit thyroid hormone synthesis by interfering with organification.

Thyroid hormone receptor Protein that, after binding of triiodothyronine (T3), binds to specific DNA sequences in the promoter region of T3-dependent genes, thereby modulating the transcription of these genes.

Wolff-Chaikoff effect Decreasing yield of organic iodine from increasing doses of inorganic iodide.

Amiodarone is a potent antiarrhythmic drug, widely used in the treatment of atrial fibrillation as well as of life-threatening ventricular or supra-ventricular arrhythmias and to prevent sudden death in selected patients. However, the drug has side effects on several tissues, including the thyroid gland.

Pharmacology

Amiodarone is highly lipophilic; this explains its tissue distribution, with the highest concentrations in the adipose tissue, liver and lung, and to a lesser content the thyroid gland (**Table 1**). A daily dose of amiodarone results in urinary iodine of approximately 14,000 μg in 24 h, largely exceeding the recommended daily dose of 200 μg. Amiodarone metabolism occurs mainly through N-dealkylation, leading to formation of the main active metabolite, desethylamiodarone (DEA). Other metabolic pathways of amiodarone include deiodination and glucuroconjugation; excretion is mainly through biliary excretion; the average half-life of amiodarone and DEA is 40 days and 57 days, respectively, accounting for their long-lasting effects (Wiersinga *et al.*, 1997).

Antiarrhythmic Properties

Amiodarone is a class III antiarrhythmic drug, mostly acting through the inhibition of myocardial Na-K ATPase activity, which eventually increases the refractory period. However, amiodarone also has class I (decrease in conduction velocity through blockade of Na channel), class II (antiadrenergic effect reducing β-adrenergic receptor) and class IV (suppression of Ca-mediated action potentials) actions. Owing to its multiple antiarrhythmic effects, amiodarone is used in patients with supraventricular and ventricular tachyarrhythmias, atrial fibrillation (when other therapies are ineffective) and to prevent sudden cardiac death in selected patients.

Effect of Amiodarone on Thyroid Function Tests

Euthyroid patients under amiodarone therapy undergo to changes of thyroid function tests (**Table 2**). Within 2 months after starting amiodarone therapy, a transient increase in serum TSH concentrations occurs, whereas serum thyroid hormone concentrations remain unaffected. During chronic ($\geq$3 months) amiodarone therapy, TSH usually normalizes, serum free T_4 (FT_4)

☆*Change History:* February 2018. Fausto Bogazzi and Enio Martino updated: sinopsis, amiodarone and thyroid dysfunction, amiodarone-induced thyrotoxicosis, references, Table 3.

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Table 1 Tissue distribution of amiodarone (A) and desethylamiodarone (DEA) in human autopsies

Tissue	*A (μg/g)*	*DEA (μg/g)*	*A/DEA*
Adipose tissue	316	76	4.2
Liver	391	2354	0.12
Lung	198	952	0.21
Kidneys	57	262	0.22
Heart	40	169	0.24
Muscle	22	51	0.43
Thyroid	14	64	0.22

Table 2 Effect of amiodarone on thyroid function tests in euthyroid patients

	Short-term	*Long-term*
Thyroxine (T4)	Increased	Normal to Increased
Triiodothyronine (T3)	Reduced	Normal to Reduced
Reverse T3 (rT3)	Increased	Increased
Thyrotropin (TSH)	Increased	Usually normal

and reverse T_3 (T_3) concentrations increase and serum free T_3 (FT_3) levels decrease. These changes are due to an inhibitory effect of amiodarone on intracellular T_4 transport and pituitary D2 activity, with a consequent reduction in intracellular T_3 generation, and thyroid hormone binding to its cognate pituitary receptor, whereas in the chronic therapy an inhibitory effect of amiodarone on hepatic D1 activity prevails (Martino *et al.*, 2001). These changes, however, do not indicate a thyroid disease.

Effects of Amiodarone at the Molecular Levels

Amiodarone is a benzofuranic iodine-rich drug with a molecular structure resembling that of thyroid hormones (**Fig. 1**); amiodarone DEA, exert thyroid hormone receptor (TR)-mediated thyromimetic effects. These participate in the developing some hypothyroid-like effects occurring in euthyroid subjects under amiodarone therapy. Amiodarone causes down-regulation of myocardial genes, including sarcoendoplasmic reticulum calcium ATPase-2 (SERCA2a) and α-myosin heavy chain (αMHC), and increased expression of β-myosin heavy chain (βMHC), all features observed in hypothyroidism. In addition, downregulation of the hepatic LDL receptor, leading to an increase in serum cholesterol concentrations, has been attributed to a direct effect of amiodarone on the LDL receptor gene expression CA. These effects are the consequence of the competitive binding of amiodarone and DEA with thyroid hormone to TRβ (Martino *et al.*, 2001).

Amiodarone and Thyroid Dysfunction

Amiodarone-induced thyroid dysfunctions are due to the excessive iodine load or to the drug itself. The iodine load is responsible for the occurrence of amiodarone-induced-hypothyroidism (AIH) and for amiodarone-induced thyrotoxicosis type 1 (AIT-1), whereas the intrinsic property of the drug is linked to the amiodarone-induced thyrotoxicosis type 2 (AIT-2) (Newman *et al.*, 1998).

Effects of Iodine Load

A normal thyroid gland responds to iodine load through an intrinsic autoregulatory mechanism, which acutely blocks thyroid hormone synthesis (Wolff-Chaikoff effect) and causes an increase in serum TSH concentration. The normal thyroid gland then escapes this block by reducing iodine transport and intrathyroidal concentrations to levels insufficient to maintain the Wolff-Chaikoff effect. Amiodarone can inhibit iodide transport into the thyroid by either an iodine-independent mechanism or a decrease in the sodium-iodide symporter mRNA expression (Bogazzi *et al.*, 2012).

Failure to escape from the Wolff-Chaikoff effect may occur in patients with an apparently normal thyroid gland thus causing amiodarone-induced hypothyroidism (AIH). In these patients AIH may be a transient phenomenon, and the drug withdrawal,

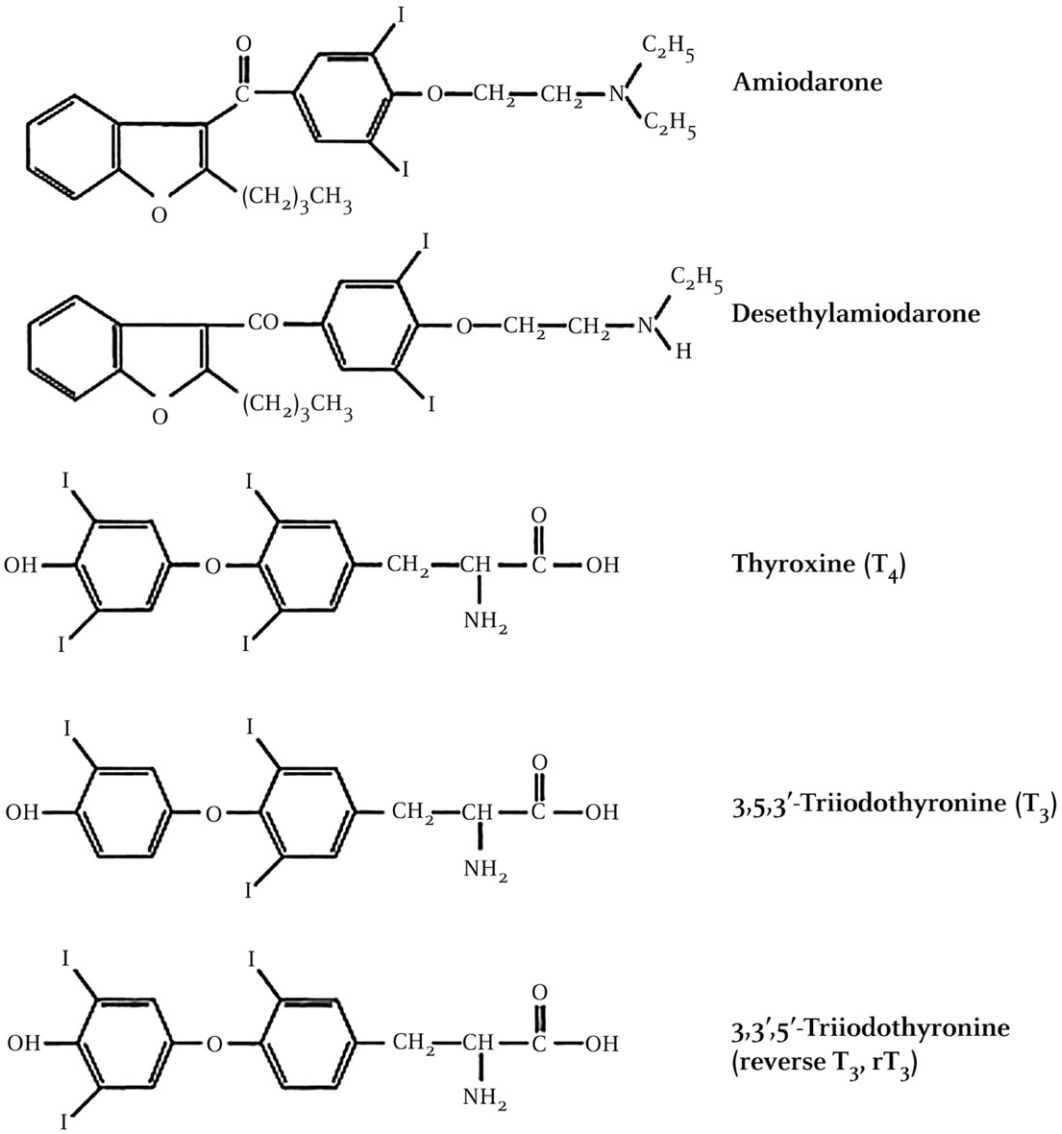

Fig. 1 Molecular structure of amiodarone, desethylamiodarone, and thyroid hormone.

when feasible, may restore euthyroidism. On the other hand, the huge iodine load caused by amiodarone may accelerate the progression towards hypothyroidism in euthyroid patients with preexisting chronic autoimmune thyroiditis. In these patients, hypothyroidism is usually permanent (Bogazzi *et al.*, 2012).

Excessive iodine is the cause of type 1 amiodarone-induced thyrotoxicosis (AIT), a form of iodine-induced hyperthyroidism, in which iodine load unveils underlying thyroid autonomy or latent Graves' disease and triggers the occurrence of hyperthyroidism (Jod-Basedow phenomenon).

Effects Intrinsic to the Molecular Structure

Amiodarone and DEA have proapoptotic and direct cytotoxic effects on thyroid follicular cells. Histopathological studies, both in animal models and in patients with AIT submitted to total thyroidectomy, showed disruption of thyroid structure, including follicular damage, a reduced number of mitochondria, an increased number of lysosomes and dilation of endoplasmic reticulum. The above findings are in keeping with drug-induced damage of the thyroid gland, resembling those occurring in other thyroid-destructive processes, such as subacute thyroiditis.

While, as mentioned above, type 1 AIT is a true form of iodine-induced hyperthyroidism, type 2 AIT is a destructive thyroiditis usually occurring in a substantially normal thyroid gland. Further evidence lending support to this notion is its self-limiting outcome, the usually prompt response to glucocorticoids, and the absence of thyroid hyperfunction.

Epidemiology of Amiodarone-Associated Thyroid Dysfunction

Prevalence of amiodarone-induced thyroid dysfunction is influenced by environmental iodine supply: hypothyroidism is relatively more frequent in iodine-sufficient areas, thyrotoxicosis in iodine-deficient areas. A proportion of 2% and 12% of patients under chronic amiodarone therapy may develop thyrotoxicosis in areas with high or low iodine intake, respectively. Conversely, hypothyroidism ranges from 13% (high iodine intake) to 6% (low iodine intake) (Trip *et al.*, 1991).

Amiodarone-Induced Hypothyroidism

Underlying chronic autoimmune thyroiditis and female gender are predisposing risk factors for developing AIH; each factor carries on average a relative risk of 7. Because chronic autoimmune thyroiditis has a much higher prevalence in women, the relative risk of AIH in women with chronic autoimmune thyroiditis is 13. In these patients, hypothyroidism is permanent despite amiodarone withdrawal, and requires levothyroxine (L-T_4) replacement.

Clinical features of AIH do not differ from those of patients with hypothyroidism of different causes, although goiter is rare. Diagnosis is confirmed by low serum FT_4 and elevated serum TSH concentrations.

AIH is easily managed by L-T_4 replacement. Serum TSH should be maintained in the upper third of the normal range. Amiodarone therapy can be continued, if needed, irrespective of the presence or absence of an underlying thyroid disorder. Hypothyroidism does not remit after drug discontinuation in patients with chronic autoimmune thyroiditis. Conversely, amiodarone withdrawal results in the restoration of euthyroidism in most patients without underlying thyroid autoimmunity.

Amiodarone-Induced Thyrotoxicosis

Over the years, proportion of patients with type 1 and type 2 AIT has changed, at least in Italy: while the number of patients affected by type 1 AIT did not change, the number and proportion of type 2 AIT patients increased. The practical consequence is that, for the time being, endocrinologists will face mainly with amiodarone-induced destructive thyroiditis; consensus exists on the differentiation of the two main forms of AIT, although mixed forms may exist.

Differentiation of the two main forms of AIT is crucial, although challenging, because therapeutic options and outcome greatly differ. The available different diagnostic procedures are aimed to reveal a thyroidal increased hormonal synthesis or an increased released of preformed hormones (Type I and Type II, respectively), often, performed in a rapid sequence in the same patient (**Table 3**) (Bogazzi *et al.*, 2010).

Clinical features of AIT are variable and indistinguishable from those of patients with spontaneous hyperthyroidism or other forms of thyrotoxicosis. However, some peculiar manifestation may herald AIT: apathetical hyperthyroidism, unexplained worsening of the underlying cardiac disease, or an unexplained increased sensitivity to warfarin.

Management of AIT patients is challenging. Based on the different pathogenic mechanisms, type 1 and type 2 AIT should be managed using different medical therapies. In the therapeutic management of AIT, first of all, the underlying heart disease and the cardiovascular compensation should be considered. If cardiovascular conditions are not critical, a medical therapy can be considered.

In type 1 AIT treatment should control the increased thyroid hormone synthesis, using thionamides. Since an iodine replete thyroid gland is less responsive to thionamides, higher doses than those usually prescribed in spontaneous hyperthyroidism are necessary (40–60 mg methimazole); perchlorate, when available, can be added during the first weeks of treatment (no >1 g/day for 4–6 weeks) to reduce the intrathyroidal iodine load. However, type 1 AIT patients usually have an underlying thyroid disorder,

Table 3 Characteristics of amiodarone-induced thyrotoxicosis Types I and II

	Type I	*Type II*
Underlying thyroidal functional autonomy	Yes	No
Pathogenetic mechanism	Excessive thyroid hormone synthesis due to iodine excess	Excessive thyroid hormone release due to destructive thyroiditis
Goiter	Usually diffuse or nodular goiter	Occasionally small diffuse goiter
Thyroid radioiodine uptake	Low, normal, or high	Low
Anti-thyroid antibody	Sometimes present	Sometimes present
FT4/FT3 ratio	<4	>4
Onset after Amio initiation	Short	Dealayed
Spontaneous remission	Less likely	More likely
Preferred drug treatment	Thionamides	Glucocorticoids
Subsequent hypothyroidism	Unlikely	Possible

which may require a definitive therapy. This may be relevant, because thyroid ablation may make continuation of amiodarone therapy (if needed from the cardiac standpoint) perfectly feasible. Radioiodine therapy is not feasible in the short run due to the persistently high intrathyroidal iodine content, whereas, total thyroidectomy may be programmed, once euthyroidism has been restored (Martino *et al.*, 2001; Bogazzi *et al.*, 2010, 2012, 2018; Trip *et al.*, 1994).

In type 2 AIT, the primary goal of medical therapy is to abate the destructive and inflammatory process damaging the thyroid gland. This is usually achieved by the use of oral glucocorticoids (starting dose, prednisone 0.5 mg/kg/day, or equivalent doses of other synthetic glucocorticoids). In type 2 AIT time to restore euthyroidism may occasionally be exceptionally long. Overall, the time required to restore euthyroidism during glucocorticoid therapy is a function of thyroid volume (estimated with standard echography) and serum concentrations of free thyroxine at the diagnosis of AIT; those two parameters may be considered surrogate of the grade of thyroid damage (Bogazzi *et al.*, 2012). Glucocorticoid therapy may be continued for several weeks to months if cardiac conditions are stable; however, deterioration of the cardiovascular function should dictate a re-evaluation of the therapeutic options. Under these circumstances, thyroid ablation by total thyroidectomy represents a valid option also in type 2 AIT.

Amiodarone-induced thyrotoxicosis (AIT) occurs in patients with preexisting cardiac disease and may represent an emergency condition because of the detrimental effects of excess thyroid hormone on underlying heart abnormalities. When AIT represents an imminent risk for cardiac function, it should be managed without delay, because the late resolution of thyrotoxicosis is associated with a high mortality rate. Under these circumstances, emergent thyroidectomy may represent the most effective and rapid way for resolving thyrotoxicosis (Bogazzi *et al.*, 2018).

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Thyroid Disease and Bone

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Glossary

Bone mineral density (BMD) The mineral content in bone measured by DXA (dual energy X-ray absorptiometry) or quantitative computer tomography (qCT).

Bone remodeling A coupled process of bone resorption followed by bone formation, which maintains skeletal integrity throughout adulthood. After peak bone mass is reached at age 20–30 years, bone resorption usually exceeds bone formation and this imbalance leads to age-related bone loss.

Osteoblast The bone forming cell.

Osteoclast The bone resorbing cell.

Osteocyte The mature osteoblast embedded on bone matrix. The osteocyte is the major controller of bone remodeling and responds to hormones, strain, and microdamage.

Osteoporosis Osteoporosis is a systemic skeletal disease, characterized by reduced bone mass and deteriorated bone architecture resulting in an increased risk of fractures. Osteoporosis is defined by WHO as BMD more than 2.5 standard deviations below peak bone mass (T-score < -2.5).

TSH Thyroid stimulating hormone.

T3 and fT3 Triiodothyronine and free triiodothyronine. The biologically active thyroid hormone.

T4 and fT4 Thyroxine and free thyroxine. The thyroid prohormone, which is secreted by the thyroid gland and subsequently converted to T3.

Effects of Thyroid Hormones on Bone

The thyroid hormone transporter MCT8 and the thyroid receptor alpha are present in osteoblasts (Bassett and Williams, 2016). In vitro studies have shown that T3 stimulates proliferation of osteoblasts, the bone forming cell (Kassem *et al.*, 1993). The same studies also showed that T3 stimulates the early phases of osteoblast differentiation. Bone resorption is also influenced by thyroid hormone; however, it is less clear if there is a direct effect of thyroid hormone on osteoclasts, the bone resorbing cell or if the effect is indirect through the osteoblast or the osteocyte (Bassett and Williams, 2016). Osteocytes are difficult to investigate in vitro and therefore the effect of thyroid hormones on osteocyte function is still unknown.

If T3 is administered to healthy women in high doses for a week, rapid and pronounced increases are seen in bone turnover (Langdahl *et al.*, 1996d, 1997). The most pronounced effects were seen on markers of osteoclast activity, but also markers of bone formation were affected.

In healthy postmenopausal women with thyroid hormones and thyroid stimulating hormone (TSH) within the normal ranges included in an observational study of the prevalence of osteoporotic fractures (Murphy *et al.*, 2010), it was demonstrated that the level of free thyroxine (T4) in the circulation was negatively associated with Bone Mineral Density (BMD) at the hip and with changes in BMD at the hip over time. Regression analyses demonstrated that higher levels of free triiodothyronine (T3) and T4, although still within the normal ranges were associated with an increased risk of non-vertebral fractures. Similarly, higher levels of TSH were associated with decreased risk of non-vertebral fractures. These findings have subsequently been confirmed by a recent meta-analysis that found that higher free T4 and lower TSH within the normal reference ranges were associated with 22%–25% increased risk of hip fractures (Aubert *et al.*, 2017).

In recent years, there have been speculations whether TSH had an effect on bone independent of T3 or T4. Animal and in vitro studies have suggested that TSH inhibits bone remodeling directly. To investigate the importance of TSH in humans, patients with Graves's disease where thyroid hormone as well as TSH signaling is increased because TSH receptor antibodies stimulates the TSH receptor and increase T4 and T3 production have been examined. The studies have showed that patients with Graves's disease lose bone to the same extent as patients with nonautoimmune causes of hyperthyroidism, suggesting that stimulation of the TSH receptor does not protect against T4/T3 induced bone loss and therefore probably plays a minor, if any role in humans (Bassett and Williams, 2016).

Childhood Thyroid Disease Affects Bone Development

Hypothyroidism in childhood is associated with growth retardation, delayed skeletal development and subsequently short stature due to defective osteochondral ossification (Bassett and Williams, 2016). The impaired osteochondral ossification results in delayed closure of the fontanelles, skull sutures and in severe cases epiphyseal dysgenesis. Initiation of substitution therapy leads to rapid bone growth and skeletal maturation and normal adult height is achieved in most cases (Salerno *et al.*, 2001).

Hyperthyroidism in children stimulates bone growth and accelerates ossification of both intramembranous and endochondral bone. This may in severe cases lead to premature fusion of growth plates and thereby early cessation of growth and permanent short stature. Closure of the cranial sutures may result in craniosynostosis. Craniosynostosis may also be seen as a consequence of untreated hyperthyroidism during pregnancy (Bassett and Williams, 2016).

Hypothyroidism

Hypothyroidism in adults leads to a low bone turnover state in the skeleton. Remodeling of the skeleton is less frequent than in euthyroid individuals and the balance between bone resorption and the subsequent bone formation is in favor of bone formation (Eriksen *et al.*, 1986). These changes would tend to increase BMD to a level above age-matched normal, however, since turnover is reduced it would take years for the changes to become measurable. Most studies therefore find that patients with hypothyroidism have normal BMD (Vestergaard and Mosekilde, 2002). There is no documented increased fracture risk in patients with hypothyroidism.

Substitution with T4 and/or T3 restores bone turnover. No long-term studies of the effect of substitution therapy in hypothyroidism has been conducted. Short term studies have not shown significant changes in bone mass (Ross, 1993; Boeving *et al.*, 2011; Cole *et al.*, 1997; Celi *et al.*, 2011). Cross-sectional studies of patients who have been on long-term substitution therapy have shown that these patients have BMD indistinguishable from healthy controls (Langdahl *et al.*, 1996a; Salerno *et al.*, 2004).

Cross-sectional population based studies have demonstrated an increased risk for fracture in hypothyroid patients substituted with thyroid hormones (Vestergaard and Mosekilde, 2002; Flynn *et al.*, 2010) and this increased risk has been suggested to be associated with overtreatment (Ko *et al.*, 2014).

The effect of subclinical hypothyroidism on fracture risk has been investigated and a recent meta-analysis did not find an increased risk for fracture in patients with subclinical hypothyroidism (Blum *et al.*, 2015).

Goiter

If goiter is not associated with hyperthyroidism or suppressed TSH, goiter is not associated with changes in bone metabolism or bone mass. Treatment of goiter may, however, affect the skeleton. Treatment of goiter with radioactive iodine does not affect the skeleton, whereas treatment with T4 may affect the skeleton negatively (Wesche *et al.*, 2001; Knudsen *et al.*, 1998). Goiter associated with subclinical hyperthyroidism; normal T3 and T4, but suppressed TSH, is associated with bone loss that can be prevented by treatment with radioiodine (Faber *et al.*, 1998).

Hyperthyroidism

Hyperthyroidism is associated with increased bone turnover (Pantazi and Papapetrou, 2000) and a negative balance at each remodeling site (Eriksen *et al.*, 1985). The increased bone turnover is restored to normal when hyperthyroidism is treated, independently of whether the treatment is radioiodine, surgical or medical (Pantazi and Papapetrou, 2000; Langdahl *et al.*, 1996b,c).

The increased bone turnover and the negative remodeling balance lead to bone loss (Vestergaard and Mosekilde, 2003). Bone mass increases when hyperthyroidism is treated (Vestergaard and Mosekilde, 2003) and patients who have had normal thyroid function for more than 4 years have similar bone mineral density as healthy controls (Langdahl *et al.*, 1996b,c).

Hyperthyroidism is associated with increased risk of fractures (Vestergaard and Mosekilde, 2003). This risk seems to decrease after treatment, however remains above the risk found in the background population (Vestergaard *et al.*, 2000). The studies have not been able to show if it is the loss of BMD, the high bone turnover, or the thyroid disease itself which cause the increased risk of fracture. The BMD losses are usually less than 10%, but the increases in bone turnover may be significantly higher and it is well-known that high bone turnover leads to thinning of trabeculae and loss of trabeculae and to cortical porosity and thinning of the cortex. These changes will destabilize bone and compromise bone strength. If the patient is young the majority of these changes may be reversible, but in postmenopausal women loss of trabecular connectivity and cortical thinning are predominantly irreversible. It is therefore likely that the high bone turnover is more important than the loss of BMD for the increased fracture risk.

Population based investigations have demonstrated that history of hyperthyroidism is an important risk factor for future fractures (Cummings *et al.*, 1995) (**Table 1**).

Subclinical Hyperthyroidism

Subclinical hyperthyroidism is defined as circulating concentrations of T3 and T4 within the normal reference ranges and circulating TSH below the reference range.

Table 1 Bone turnover, bone mineral density, fracture risk and management of bone health in patients with thyroid disease

Treatment	*Bone turnover*	*Bone mineral density*	*Fracture risk*	*Management of bone health*
Hypothyroidism	Normal/ reduced	Normal/ increased	Normal/low	As in euthyroid patients
Goiter	Normal	Normal	Normal	As in euthyroid patients If long-term T4 therapy, consider DXA
Subclinical hyperthyroidism (suppressed TSH)	Increased/ normal	Reduced/ normal	Increased	If long-term lasting, consider DXA
Hyperthyroidism	Increased	Reduced	Increased	Treat hyperthyroidism and advise on bone friendly lifestyle Consider DXA when euthyroid for 6 months
TSH suppression therapy	Normal/ increased	Normal/ decreased	Normal/increased (post-menopausal women)	Advise on bone friendly lifestyle Reduce TSH suppression to minimum Consider DXA

Subclinical hyperthyroidism is associated with bone loss and increased risk of fractures. A recent meta-analysis found that TSH below 0.01 mU/L is associated with a two-fold increase in hip fractures and a 3.5-fold increase in vertebral fractures (Blum *et al.*, 2015). In a large Danish register based study excluding patients with a history of thyroid or pituitary diseases or use of any kind of thyroid medication, it was demonstrated that a single measurement of TSH below the normal reference range was associated with increased risk of hip fractures (Abrahamsen *et al.*, 2014). The duration and severity of the suppression of TSH was associated with increased risk of both hip and major osteoporotic fractures.

TSH Suppressive Therapy

TSH suppression therapy is used in patients thyroidectomised due to thyroid cancer. This is a situation similar to mild hyperthyroidism or subclinical hyperthyroidism and therefore associated with increased bone turnover. There is some controversy if TSH suppressive therapy leads to bone loss as some studies did not demonstrate this (Reverter *et al.*, 2005) whereas others did (Sugitani, 2005). The differences may be related to the women included in the studies as Sugitani *et al.* demonstrated that bone loss was only seen in postmenopausal women. This was later confirmed in a meta-analysis that also included a few studies in men and found no bone loss in men receiving TSH suppressive therapy (Quan *et al.*, 2002).

Management of Patients With Thyroid Diseases

Hypothyroidism

Patients with untreated hypothyroidism have low risk of osteoporosis. Patients receiving replacement therapy have low risk of osteoporosis, however, may have an increased risk of fractures during the first years of treatment or if oversubstituted. Therefore, general screening for osteoporosis in this group of patients is not recommended, however, DXA may be considered in postmenopausal women and elderly men if other risk factors for osteoporosis are present.

Hyperthyroidism

Patients with hyperthyroidism have high bone turnover and lose bone mass. The risk of developing osteoporosis and fractures depends on age, gender and menopausal status.

The most important intervention is treatment of hyperthyroidism. In addition, the patient should be advised about bone healthy lifestyle measures; avoid smoking, physical activity and sufficient intake of calcium and vitamin D. In postmenopausal women and elderly, it is advised to consider DXA when the patient has had normal thyroid function for 6 months.

Subclinical Hyperthyroidism or Suppressed TSH Found on Routine Biochemical Screening

Individuals with suppressed TSH may have increased risk of fractures, however, the risk depends on many other risk factors. DXA may be considered as part of the work-up that should be done in these patients.

TSH Suppressive Therapy

Patients receiving suppressive therapy probably have an increased risk of developing osteoporosis and fractures, however the risk depends on the level of suppression of TSH, age, gender, menopausal status, and other risk factors. The suppression of TSH should therefore be the least acceptable in the context of the cancer disease and performing DXA should be considered in postmenopausal women.

Treatment of Osteoporosis in Patients With Thyroid Diseases

Treatment of osteoporosis comprises general measures; secure sufficient calcium and vitamin D intake, avoid smoking, and be physical active and engage in exercise and specific treatment. Medical treatment of osteoporosis can be divided into antiresorptive and anabolic treatment and choice of initial treatment and long-term management follows the general recommendations for treatment of osteoporosis as there are no clinical trials specifically investigating treatment of osteoporosis in patients with thyroid diseases.

Conclusion

Thyroid status affects bone status. Individuals with thyroid function in the upper end of the normal reference range have lower BMD and increased risk of fragility fractures compared with individuals with thyroid function in the middle or lower end of the reference range.

Hypothyroidism generally does not pose a risk to bone health, only long-term over substitution with thyroid hormones may lead to increased risk of fractures.

Overt hyperthyroidism is accompanied by increased bone turnover, bone loss, and increased risk of fractures. The risk of fractures may not be completely reversible in postmenopausal women and elderly men and therefore it is recommended to perform DXA when the patient has been euthyroid for 6 months.

Subclinical hyperthyroidism is also associated with bone loss and increased risk of fractures and should therefore be treated. Similarly, TSH suppression therapy in patients with thyroid cancer is associated with bone loss, especially in postmenopausal women and bone friendly lifestyle should be advised and DXA considered. Osteoporosis in patients with thyroid disease should be managed in accordance with general guidelines.

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Thyroid Function and Depression☆

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Glossary

Apathetic thyrotoxicosis Hyperactivity of the thyroid gland in which the classic signs and symptoms are subtle; occurs chiefly in middle-aged and elderly persons.

Bipolar disorder Psychiatric syndrome with both episodes of mania and depression. Formerly called manic-depressive illness.

Depression Psychiatric syndrome with decreased mood, loss of interest and lack of energy.

Dysphoria Decreased mood, bad mood.

Hyperthyroidism A condition of excessive functional activity of the thyroid gland and excess secretion of thyroid hormones marked by goiter, tachycardia or atrial fibrillation, widened pulse pressure, palpitations, fatigability, nervousness and tremor, heat intolerance and excessive sweating, ocular signs, and emotional lability.

Hypothyroidism Deficiency of thyroid activity that, in adults, is most common in women and is characterized by a decrease in basal metabolic rate, tiredness and lethargy, depression, sensitivity to cold, and menstrual disturbances.

Levothyroxine Monosodium salt of the levoisomer of the thyroid hormone thyroxine; used for replacement therapy in reduced or absent thyroid function; administered orally.

Liothyronine Synthetic levoisomer of the thyroid hormone triiodothyronine, which is more potent and has a more rapid action than thyroxine; used for thyroid replacement or supplementation in hypothyroidism and nontoxic goiter; administered orally.

SSRI Selective serotonine reuptake inhibitor.

Tca Tricyclic antidepressant.

Thyroid gland Endocrine gland normally situated in the lower part of the front of the neck and consisting of two lobes, one on either side of the trachea and joined in front by a narrow isthmus; secretes, stores, and liberates (as necessary) the thyroid hormones, which require iodine for their elaboration and which play the major endocrine role in regulating the metabolic rate.

Thyrotropin A glycoprotein hormone of the anterior pituitary that has affinity for and specifically promotes the growth of, sustains, and stimulates the hormonal secretion of the thyroid gland.

Thyrotropin-releasing hormone A tripeptide elaborated by the paraventricular nucleus of the hypothalamus or obtained by synthesis that stimulates release of thyrotropin from the anterior pituitary gland.

Thyroxine A crystalline iodine-containing hormone, considered the major hormone elaborated by the thyroid gland, that is formed from thyroglobulin and transported mainly in the blood serum by thyroxine-binding globulin; chief function is to increase the rate of cell metabolism.

Triiodothyronine An organic iodine-containing compound liberated from thyroglobulin by hydrolysis and thought to be formed by the conjugation of one molecule each of monoiodotyrosine and diiodotyrosine and by the partial deiodination of thyroxine intrathyroidally and in the periphery; has several times the biological activity of thyroxine; thought to be the "tissue-active" form of thyroid hormone.

Introduction

In 1888, a committee appointed by the Clinical Society of London established that thyroid hormone is important for central nervous system (CNS) development and function. Although early investigations suggested that adult brain tissue was not responsive to thyroid hormone due to its failure to increase the consumption of oxygen, thyroid hormone receptors have been demonstrated in the neonatal and adult brain (Dezonne *et al.*, 2015). Furthermore, it has been shown that thyroid hormone regulates normal neuronal growth and synaptogenesis (Stenzel and Huttner, 2013). The fact that hypothyroidism could lead to depression and be reversed by thyroid hormone administration was established by Asher in 1949 (Asher, 1949). Since then, attention has shifted to the potential role of thyroid hormone, particularly liothyronine (T_3), as adjuvant therapy for euthyroid patients who have depression. T_3 has been reported to influence the effect of antidepressants either by hastening the onset of response or by converting a nonresponder into a responder (Joffe, 2011). Although it has been given as adjuvant therapy for refractory depression for more than four decades, efficacy is moderate, and it is paramount to find biomarkers to identify preferential responders (Joffe, 2011).

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Clinical Manifestations of Depression and Thyroid Disease

Major depression affects on average 5% of the population across all countries (Kessler and Bromet, 2013). The diagnosis is made in those who present with a depressed or irritable mood, and have a lack of interest or pleasure and reduced energy for at least 2 weeks in combination with accompanying symptoms (**Table 1**). The initial episode of depression most frequently occurs during the fourth or fifth decade of life but may occur at any age. If left untreated, the duration of an episode can vary greatly, from a few months to 1 or more years. Occasionally, it will persist as chronic depression. A family history of mood disorders is common (McIntyre, 2016).

As in thyroid disease, depression occurs more often in women than in men, with peak incidence varying between countries and across ages (Bromet *et al.*, 2011). The prevalence of autoimmune thyroid disease is highest among women more than 40 years of age (Ajjan and Weetman, 2015). Most individuals presenting with depression do not have biochemical evidence of thyroid dysfunction; despite this, depression and thyroid dysfunction share many clinical features (see **Table 2**). In those cases where patients present with depression and findings that suggest thyroid dysfunction, it may be difficult to distinguish clinically which entity is the cause of the symptoms.

Regulation of the Hypothalamic–Pituitary–Thyroid Axis

The fundamental actions of the hypothalamus–pituitary–thyroid axis are based on stimulation of the pituitary thyrotrophs balanced by a negative feedback inhibition. The hypothalamus regulates the synthesis and release of thyrotropin (TSH) through the secretion of thyrotropin-releasing hormone (TRH). TRH is produced in the paraventricular nucleus of the hypothalamus and transported to the specialized nerve terminals in the median eminence before being liberated into the hypophyseal portal blood. The basophilic cells of the anterior pituitary produce TSH in response to TRH. TSH is liberated in the circulation and stimulates the thyrocytes to produce thyroid hormones via the thyrotropin receptor. Once thyroxine (T_4) and T_3 are liberated, they exert a negative feedback inhibitory effect on TSH and TRH secretion (see **Fig. 1**). Apart from thyroid hormones, there are other inhibitors of TSH secretion, including somatostatin, dopamine, glucocorticoids, and certain cytokines (see **Table 3**), while α-adrenergic agonists stimulate the thyrotrophs (Persani, 2012).

Table 1 Associated symptoms of major depression

- Reduced self-esteem and self confidence
- Feelings of worthlessness or guilt
- Recurrent thoughts of death or suicide
- Decreased concentration and attention
- Motor agitation or retardation
- Insomnia or hypersomnia
- Appetite or weight change

Table 2 Clinical findings common to depression and hypothyroidism (Bathla *et al.*, 2016)

- Anorexia
- Apathy
- Anhedonia
- Constipation
- Lethargy
- Anergia
- Decreased libido
- Dysphoria
- Fatigue
- Somnolence
- Melancholia
- Weight gain or weight loss

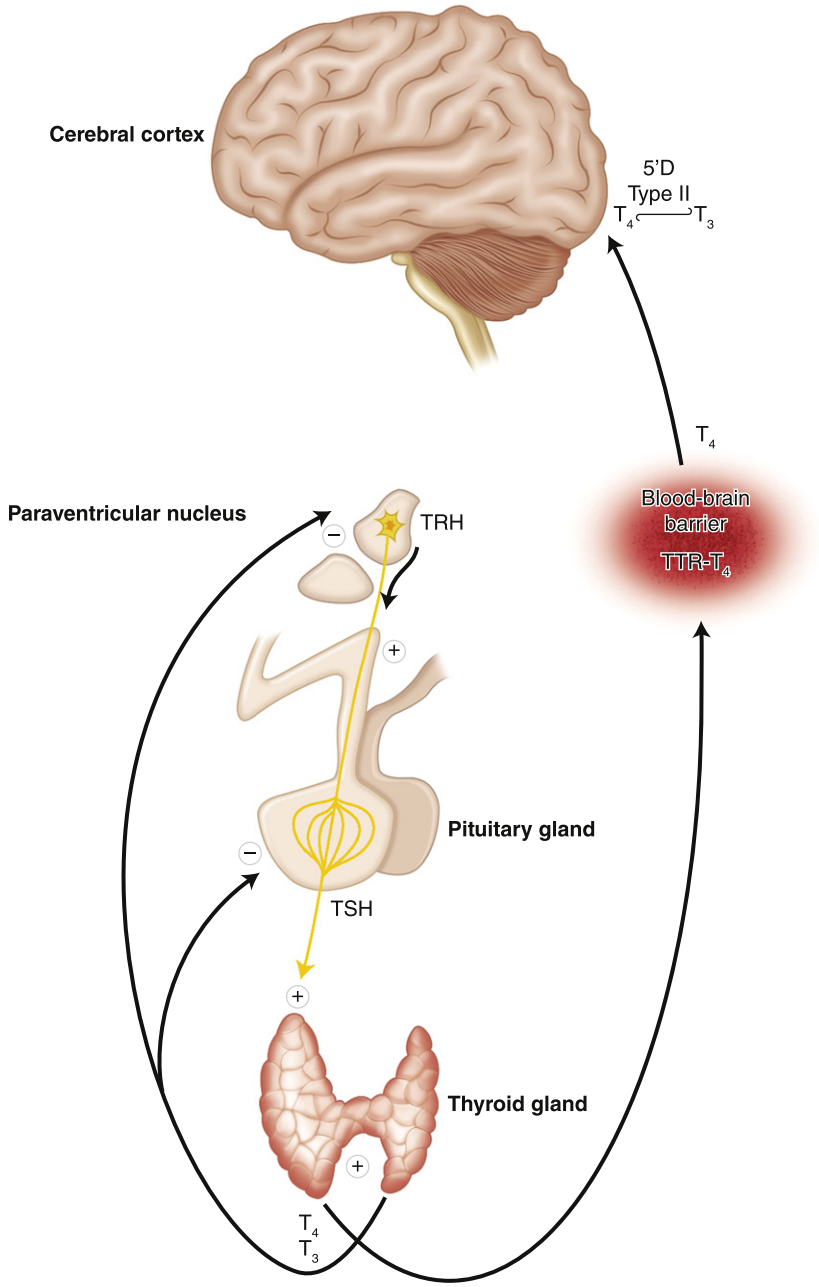

Fig. 1 The hypothalamic–pituitary–thyroid axis. Once T_4 is in the circulation, it crosses the blood–brain barrier with the aid of transthyretin. In the cerebral cortex, T_4 is converted into T_3 by 5′-deiodinase type II. Finally, T_3 is inactivated by 5-deiodinase (5D) into diiodothyronine (T_2). T_3 is the active thyroid hormone at the cellular level.

The most abundant thyroid hormone produced by the thyroid gland is T_4. The monodeiodination of T_4 in extrathyroidal tissues gives rise to 80% of the T_3 in the peripheral circulation, with the remainder being derived from direct thyroid gland secretion. Within the CNS, local deiodination activity in the brain is of great importance because it enables the CNS to maintain an optimal T_3 content. Type II 5′-deiodinase and 5-deiodinase are the major mediators of this process. The former, which is responsible for the conversion of T_4 to T_3, is found in the cerebral cortex, pituitary, and hypothalamus. The latter is also found in the cerebral cortex but not in the anterior pituitary. It results in degradation of T_3 and inactivates T_4, yielding reverse triiodothyronine (rT_3). In addition, 5-deiodinase activity predominates in the euthyroid state, and the rT_3 produced plays a role in the regulation of local deiodination (see Fig. 1). The cortex has the highest level of activity of these enzymes, followed by the midbrain, pons, hypothalamus, and brainstem (in that order).

Table 3 Conditions with a blunted TSH response to exogenous TRH (Persani, 2012)

- Depression
- Elderly males
- Acromegaly
- Hyperthyroidism
- Cushing syndrome
- Alcoholism
- Medications
- Glucocorticoids
- Dopamine
- Phenytoin
- Somatostatin
- Fasting
- Nonthyroidal illness

Alterations of the Hypothalamic–Pituitary–Thyroid Axis in Depression

Patients with depression can present many abnormalities of the hypothalamic-pituitary-thyroid axis (see **Fig. 2**). The most widely recognized disturbance is blunting of the TSH response to TRH stimulation (Arem and Cusi, 1997;Duval *et al.*, 2015). Defined as a TSH rise of less than 5 mU/L, this phenomenon occurs in 25%–30% of depressed individuals. Normalization of this response is noted once clinical recovery ensues. It differs from thyrotoxicosis in which the TSH response is flat and the circulating T_3 levels are elevated, and may rather be caused by central hypothalamic suppression, as seen during fasting and in nonthyroidal illness. A blunted TSH response is therefore not unique to depression but occurs in other clinical settings (Persani, 2012) (see **Table 3**).

Another common abnormality of the hypothalamic–pituitary–thyroid axis is held to be an increase in total and/or free thyroxine (FT_4) levels, although still within the conventional normal range, that regress after successful treatment of depression (Jackson, 1998). It has been reported that some patients admitted with acute psychosis, including depression, manifest a transient elevation above normal levels of T_4 and/or FT_4 and occasionally TSH. These findings usually resolve spontaneously within 2 weeks (Roca *et al.*, 1990).

In a fetal rat hypothalamic culture system, Luo and Jackson demonstrated an increase in TRH gene expression by glucocorticoid exposure (Luo and Jackson, 1999). This effect is notable because human depression is characterized by hypercortisolemia, which we believe leads to activation of the TRH neuron and, consequently, thyroid function. The hypercortisolemia of depression probably results from impaired function of the hippocampus, a locus for glucocorticoid negative feedback of the hypothalamic–pituitary–adrenocortical axis (MacQueen and Frodl, 2011). Although glucocorticoids generally inhibit the thyroid axis in humans and rats in vivo, a "functional" disconnection of the hypothalamus from the rest of the brain (as is postulated to occur in depression) would remove this inhibitory influence from the hypothalamus. Indeed, a fornical lesion that severs the hypothalamus from hippocampal regulation will increase thyroid function (Shi *et al.*, 1993). In many ways, the hypothalamus in culture is analogous to a deafferentated hypothalamus in vivo. Luo and Jackson hypothesized that the direct stimulation of the TRH neuron by glucocorticoids seen in vitro is overridden in vivo by an inhibitory influence emanating from the hippocampus in the normal human or rat but not in some persons with clinical depression. In these individuals, activation of the TRH neuron could lead to increased hypothalamic TRH secretion with down-regulation of the TRH receptors. This could result in a blunted TSH response to exogenous TRH and spillover of TRH into the cerebrospinal fluid (CSF), increased levels of which have been reported. In addition, Jackson and Lou have explored a direct effect of antidepressants on the TRH neuron to explain the reversal of hyperthyroxinemia with successful treatment of clinical depression. The results showed that the selective serotonin reuptake inhibitors (SSRIs) and the tricyclic antidepressants (TCAs) inhibit TRH secretion (Jackson and Luo, 1998). These studies suggest that the fall in circulating T_4 levels seen with antidepressant medication might reflect a direct effect on the TRH neuron and consequent reduced activation of the thyroid axis. Antidepressants may also be clinically efficacious by enhancing T_4-to-T_3 conversion in the CNS (Bauer *et al.*, 2008).

As mentioned previously, T_3 is the active thyroid hormone in the brain. It has been hypothesized that depression leads to inhibition of type II deiodinase, possibly due to the elevated cortisol levels in this disorder (Bauer *et al.*, 2008). Therefore, T_4 is converted to rT_3 by 5-deiodinase. This would explain the elevated levels of rT_3 in the CSF of individuals with unipolar depression.

Transthyretin, a T_4 transport protein, is synthesized by the choroid plexus and accounts for up to 25% of the protein in the CSF. It has a relative binding affinity of 39.3% for T_4 and of only 1.4% for T_3; thus, it is unlikely that transthyretin plays a significant role in the transport of T_3 across the blood–brain barrier. Interestingly, in a study of eight patients with refractory depression, CSF levels of transthyretin were much lower when compared with nine patients with neurological disease but without depression. The authors of this study proposed that low levels of TTR could give rise to "brain hypothyroidism" with normal peripheral thyroid hormone concentrations in depression (Sullivan *et al.*, 2006).

Autoimmune thyroiditis is found in 7% of depressed patients (Gold *et al.*, 1981; Haggerty, *et al.* 1997) and may be associated with an exaggerated response to TRH stimulation, indicating a subtle subclinical hypothyroidism. In bipolar patients the

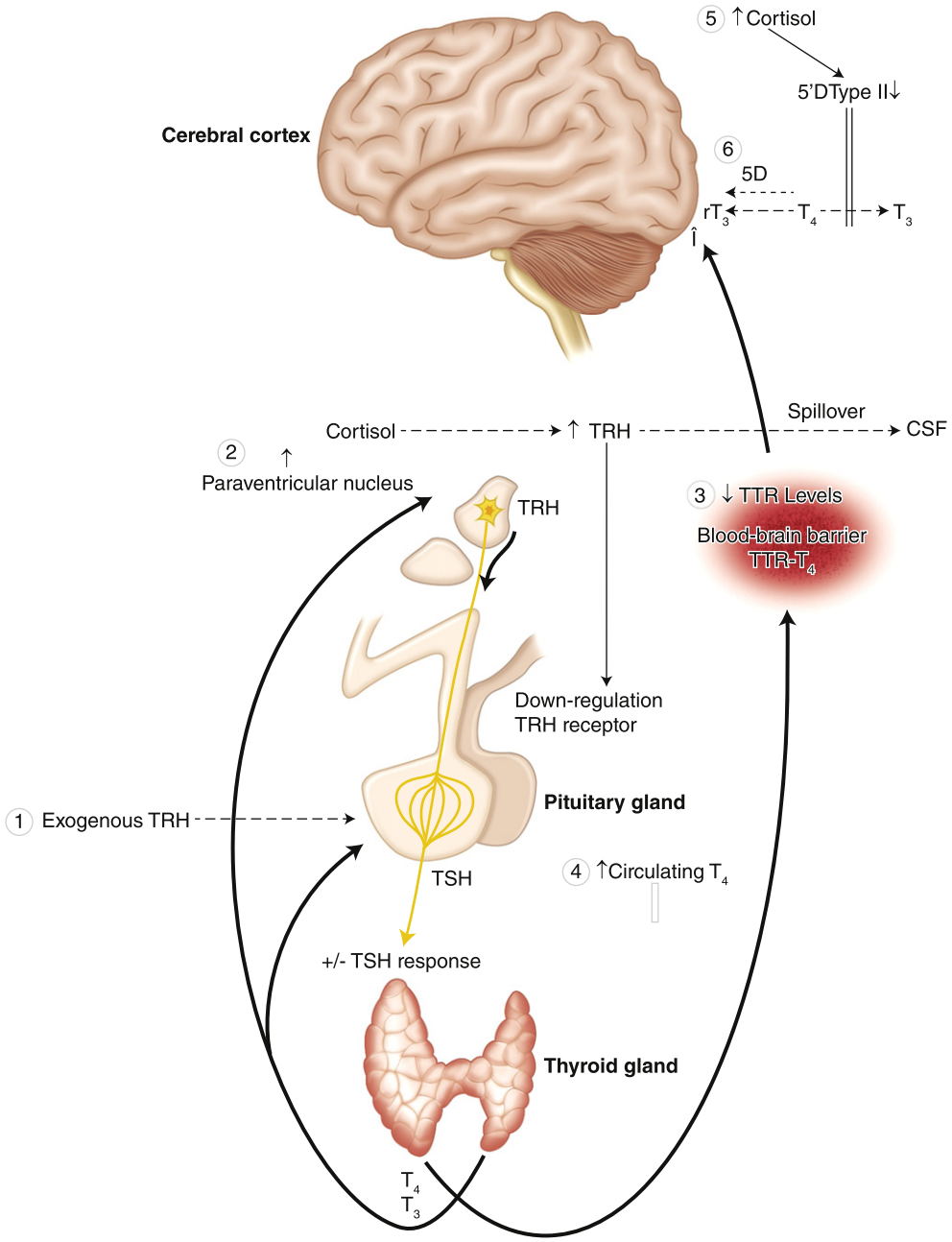

Fig. 2 Alterations of the hypothalamic–pituitary–thyroid axis in depression: (1) Blunted (+/−) thyrotropin (TSH) response to exogenous thyrotropin-releasing hormone (TRH) administration (Haggerty, *et al.* 1993). (2) Elevated cortisol levels activate the TRH neuron, which can lead to down-regulation of the TRH receptor (Jackson, 1998; Tsigos and Chrousos, 2002). (3) Decreased levels of transthyretin (TTR), a thyroxine (T_4) transport protein, have been found (Hatterer *et al.*, 1993; Sullivan *et al.*, 2006). (4) Circulating T_4 levels have been found to be elevated (Jackson, 1998). (5) Inhibition of 5′-deiodinase type II (5DType II) occurs, possibly due to elevation of cortisol, resulting in decreased triiodothyronine (T_3) (Bauer *et al.*, 2008; Brouwer *et al.*, 2006). (6) T_4 is converted into reverse T_3 (rT_3), resulting in higher rT_3 levels in the CSF (Bauer *et al.*, 2008).

prevalence of autoimmune thyroiditis is higher with 28% carrying anti-TPO antibodies (Kupka *et al.*, 2002) and up to 50% in one series of patients with i rapid cycling bipolar disease (four or more episodes of manic depression per year) (Bauer *et al.*, 1990). Furthermore, women with postpartum thyroiditis often suffer from depression. In a study of 145 women with presence of antithyroid antibodies, 47% had significant depressive symptoms. When these women were compared with a control group, with negative titers to antithyroid antibodies, only 32% presented with depressive symptoms regardless of the thyroid function abnormalities. Among the antibody-positive individuals, 62 had episodes of thyroid dysfunction; a total of 27 had signs of hypothyroidism, 11 had signs of hyperthyroidism, and 24 showed biochemical evidence of hyperthyroidism followed by hypothyroidism (Harris *et al.*, 1992). A recent systematic review (Barbuti *et al.*, 2017) found evidence of increased circulating

thyroid autoantibodies in depressed and mixed episode bipolar patients and pointed at a study on bipolar twins that suggests that autoimmune thyroiditis is related to the genetic vulnerability to develop bipolar disease rather than to the disease process itself.

Alterations in the circadian rhythm of TSH have been described, including absence of the normal nocturnal TSH surge that may result in an overall diminution of thyroid hormone secretion (Bartalena *et al.*, 1990). This suggests that there may be a degree of central hypothyroidism in some patients with depression. Furthermore, sleep deprivation, which has an antidepressant effect, leads to restoration of TSH and an elevation in the levels of T_4 and T_3 (Parekh *et al.*, 1998).

Depression in Thyroid Disease

Hypothyroidism

Hypothyroidism indicates any degree of thyroid hormone deficiency. The epidemiology and causes of this are described in the chapter on Hypothyroidism. Studies on adult patients with hypothyroidism have demonstrated decreased cerebral blood flow and consumption of oxygen and glucose (27% below normal) (Bauer *et al.*, 2009; Kinuya *et al.*, 1999). Results from positron emission tomography (PET) studies indicate that TSH levels correlate inversely with global and regional blood flow as well as cerebral glucose metabolism. In addition, when PET studies were performed in individuals with severe hypothyroidism of short duration, the brain activity was globally reduced without the regional modifications observed in primary depression. As noted previously, T_3 in the brain is derived from deiodination of T_4 and intracerebral generation of T_3 increases as serum concentrations of T_4 decline, to maintain the intracellular T_3 concentrations until serum T_4 has been depleted. Depression has been described in all grades of hypothyroidism (Thomsen *et al.*, 2005). The occurrence of depression is believed to be higher in those who have a history of a first-degree relative with depression, but it has not been studied if this also relates to first degree relatives more often having thyroid autoimmunity. One study showed that the presence of thyroid autoimmunity may be more important for depression than hypothyroidism per se (Watt *et al.*, 2012), which may be related to an occurrence of Hashimoto's encephalopathy in some patients (Montagna *et al.*, 2016). Up to 10% of patients admitted for treatment of depression are found to have subclinical or frank hypothyroidism. However, in a large study of patients with subclinical hypothyroidism depression was not associated with increased depressive symptoms among older adults at high cardiovascular risk (Blum *et al.*, 2016). Clinical manifestations are indistinguishable from non-thyroid-related depression. Response is usually refractory to antidepressants alone; on certain occasions, it has also been resistant to thyroid hormone replacement.

Hyperthyroidism

Hyperthyroidism can be seen in up to 3% of individuals over 60 years of age. Typically, Graves' disease is still the most common cause of thyrotoxicosis in the elderly in some populations, but toxic multinodular goiter or adenomas are more frequent than they are in young people, and very prevalent in populations with current or recent low iodine intake (Laurberg *et al.*, 2006). The classic findings of hyperthyroidism are not always seen in the elderly. The term "apathetic thyrotoxicosis" has been used to address such a condition, which may be associated with depressive symptoms (see **Table 4**), that are relieved by antithyroid treatment. The laboratory data show a suppressed TSH with or without frank elevation of T_4 and T_3. In studies of patients with thyrotoxicosis, depression has been documented in up to 30%–60% of the cases, although such a high prevalence is controversial. Other studies showed much lower prevalence (Bove *et al.*, 2014), and subclinical hyperthyroidism with suppressed TSH and normal circulating thyroid hormones did not seem to have a higher depression tendency in one study (Almeida *et al.*, 2011), but probably in another (Blum *et al.*, 2016).

Thyroid Hormone Supplementation in Depression

In the evaluation of thyroid hormone as adjuvant therapy for depression, there is a need to exclude patients with borderline TSH elevation as well as those with detectable antithyroid antibodies. This population may respond favorably to thyroid hormone

Table 4 Manifestations of apathetic thyrotoxicosis

- Anorexia
- Apathy
- Depression
- Confusion/slow mentation
- Weight loss
- Constipation
- Atrial fibrillation
- Angina exacerbation
- Muscle atrophy

supplementation simply because of underlying hypothyroidism. In addition, when variables of thyroid function are evaluated, the antithyroid peroxidase (anti-TPO) antibody should be included. It is conceivable that patients with evidence of autoimmune thyroid disease might benefit from treatment with levothyroxine because the treatment corrects a cryptic underlying decrease in thyroid function. Patients with rapid cycling bipolar disease may benefit from pharmacological doses of levothyroxine. Such cases may reflect an aberrant expression of hypothyroidism. In studies involving thyroid hormone as adjuvant therapy in depression, T_3 has been used more broadly than T_4 (Joffe, 2011). Cooke and colleagues reported that T_3 augmented the response to antidepressant therapy in T_4-replaced hypothyroid patients in a randomized controlled trial during a 3-week period (Cooke *et al.*, 1992). Joffe found T_3 to be significantly more effective than T_4 in patients with depression who did not respond to TCAs. However, because T_4 equilibrates in tissues more slowly than does T_3, 6–8 weeks of T_4 therapy may be required for adequate comparison of its efficacy with the more rapidly acting T_3 (Joffe and Singer, 1990).

Thyroid Hormone Use to Hasten or Enhance Antidepressant Response

When the hormone is added to the antidepressant at the outset of the study (and the patients therefore are not selected as nonremitters) it is conceptualized as an enhancement study (Joffe, 2011).The therapeutic response of antidepressants is delayed up to 1 month. Therefore there is a tradition for attempts to hasten this effect. All such studies on thyroid hormones and TCAs are more than 40 years old and employed T_3 as accelerator of a response. The studies included small number of patients, poorly defined patient groups and suboptimal dosing but showed promising results (Joffe, 2011). The four published studies on SSRIs point in different directions with one showing a hastened effect (Posternak *et al.*, 2008), two showing an enhanced effect (Cooper-Kazaz *et al.*, 2007; Posternak *et al.*, 2008) and two studies showing no effect (Appelhof *et al.*, 2004; Garlow *et al.*, 2012).

Thyroid Hormone Use to Augment the Effect of Antidepressants

After 1st line treatment for depression slightly less than one third remit and less than half of the patients respond (Trivedi *et al.*, 2006b). With a second line strategy of either shift or augmentation only further 25% receive remission or response (Rush *et al.*, 2006; Trivedi *et al.*, 2006a). As a consequence, there is much interest in the possibility that augmentation therapy. As mentioned T_3 has been the most widely used in doses from 20 to 50 μg daily. In 8 of 11 open label studies more than 50% of the patients showed response (Joffe, 2011). In only 3 of the studies fewer patients responded—one on inpatients were all 14 patients showed no response (Birkenhager *et al.*, 1997), one with only 5 of 20 patients resistant to imipramine and interpersonal therapy responded (Thase *et al.*, 1989) and one with 7 of 20 responding (Iosifescu *et al.*, 2005). In one blinded add-on of T_3 study on patients nonresponsive to amitriptyline or imipramine 8 of 12 patients showed marked response (Goodwin *et al.*, 1982). One of the largest randomized studies published to date, took 51 nonresponders to TCAs and added either T_3, lithium, or placebo to the antidepressant and found that 10 of 17 subjects responded to T_3, nine of 17 responded to lithium and three of 16 responded to placebo (Joffe *et al.*, 1993). The largest study was part of the STAR*D trial, on 142 patients who had not achieved remission or who were intolerant to an initial prospective treatment with citalopram and a second switch or augmentation trial (Nierenberg *et al.*, 2006). They were randomly assigned to augmentation with lithium ($N = 69$) or with T_3 ($N = 73$) for up to 14 weeks. It was found that remission rates with lithium and T(3) augmentation were modest and did not differ significantly (16% and 25% respectively). But the lower side effect burden and ease of use of T_3 augmentation suggest that it has slight advantages over lithium in this patient group. Another double blind cross over study failed to find difference between T_3 and placebo (Gitlin *et al.*, 1987). However, it only included 16 patients who showed no responsiveness to just 4 weeks of imipramine whereafter T_3 was added for only 2 weeks before cross over, which can make it difficult to interprete the response.

Studies on T_4 as augmenting agent are few, they are carried out on few patients of different subtypes of depression with doses of T_4 of 100–500 μg daily. They all showed some antidepressant effect (Joffe, 2011). In a study by Stamm *et al.* (2014) 62 bipolar depressed patients were randomized to levothyroxine or placebo adjunctive to continuing treatment with mood stabilizer and/or antidepressant medication. The T_4 group did numerically but not statistically better than the placebo group.

Moreover, studies have focused on whether a relationship can be detected between the swiftness in reaching a clinical remission or the risk of recurrence and the baseline thyroid hormone levels. Cole *et al.* (2002) observed that both lower values of free T_4 and higher values of TSH were significantly associated with longer times to remission, that is, slower response to treatment in bipolar depressed patient. Correspondingly, Amann *et al.* (2017) in a study on comorbidity found that particularly hypothyroidism was associated with an increased risk of manic relapse in bipolar disorder. The authors questioned whether adjuvant therapy with thyroid hormone may optimize the management of patients presenting in the depressive phase of bipolar disorders. Joffe and colleagues (Joffe and Marriott, 2000) looked at the relationship between basal thyroid hormone levels and the life course of depressive illness in 75 outpatients with unipolar major depressive disorder. The significant positive predictors of recurrence were comorbid anxiety, number of previous episodes of depression, prolonged course of episodes, and level of T_3. An increase in T_3 was associated with a 22% decrease in the risk of recurrence.

Although adjuvant therapy may help nonremitters, observers must be cautious about this conclusion for many reasons. First and most important, we do not know with certainty which clinical and/or biochemical variables determine the subset of depressed patients who will benefit from adjuvant treatment. Second, the clarification of the thyroid status was not universally reported in

the studies that are available, and unrecognized "subclinical" hypothyroidism or autoimmune thyroid disease could have led researchers to overestimate the therapeutic response to T_3. Third, the relationship between T_3 dose and clinical response is unclear. Fourth, because the longest duration of T_3 administration in studies has been only a few weeks, the extent of treatment remains imprecise, although it has been recommended by some investigators that thyroid augmentation be discontinued 8–12 weeks after a response and then reinstituted if symptoms recur. Fifth, studies regarding long-term side effects from use of thyroid hormones in depressed patients are not available, and when instituting this form of therapy, the potential effect of thyroid hormone over-replacement on risk of heart arrhythmias, osteoporosis and muscle loss/myopathy has to be strongly taken into account, as indicated in recent recommendations on safety monitoring (Rosenthal *et al.*, 2011; Touma *et al.*, 2017). Finally, longer and larger randomized, double-blind, placebo-controlled trials are needed.

Use of Thyrotropin-Releasing Hormone in Refractory Depression

The therapeutic use of TRH in mood disorders is based on the fact that this hormone has direct effects on the CNS independent of pituitary and thyroid stimulation. High-affinity receptors to TRH are found throughout the brain especially, in the amygdala and the hippocampus, and modulate the effects of serotonin and dopamine. In addition, in some studies the concentrations of TRH in the CSF of untreated individuals with mood disorders were elevated. It remains unclear whether this corresponds to a natural compensatory mechanism or to a pathological occurrence. Marangell *et al.* (1997) administered 500 μg of TRH intrathecally in a double blinded fashion to eight drug-free patients with refractory depression compared to sham spinal punctures a week apart. Five patients had a clinically significant, rapid, and robust but short-lived improvement in mood after TRH infusion, but not after the sham puncture. Systemic thyroid function was unaltered. Intrathecal TRH might thus be a positive modulator of mood, but this has not been pursued in further studies.

Conclusions

Hypothyroidism predisposes patients to depression that may be wholly reversed by thyroxine replacement. When first seen for depression, most patients have normal circulating TSH, T_3, and T_4. However, 15% may have subclinical hypothyroidism and have a TSH hyper-response to TRH stimulation. Another 10%–15% may show evidence of autoimmune disease. In this setting, thyroxine may alleviate depressive symptoms alone or in combination with antidepressants. The clinical features of depression often resemble those of hypothyroidism and may reflect brain hypothyroidism despite systemic euthyroidism. The mechanism may be related to impaired conversion of T_4 to T_3 in the brain due to inhibition of type II 5′-deiodinase, possibly by increased levels of circulating cortisol and/or reduced transport of T_4 across the blood–brain barrier. Another possibility is a functional polymorphisms in type 1 deiodinase, DIO1-C785T, which was associated with efficacy of T3 but not placebo supplementation, and at baseline a lower serum T3 was found (Cooper-Kazaz *et al.*, 2009). This would provide a possibility for predicting a positive response of adjuvant T3 therapy in individual depressed patients. Adjuvant therapy with T_3 for patients receiving antidepressant medication has been postulated to augment responsiveness to therapy in about a quarter of cases of refractory depression, possibly by correcting brain hypothyroidism. TCAs and SSRIs have been reported to inhibit TRH gene expression in vitro, a fact that might explain the normalization of serum T_4 levels following treatment; TCAs also enhance the conversion of T_4 to T_3 in the brain, providing another potential explanation for their therapeutic efficacy in depression. A possible role of intrathecal TRH administration as modulator of mood requires further studies.

See also: Hashimoto's Thyroiditis. Hypothyroidism Subclinical. Lithium. Measuring Impact of Benign Thyroid Diseases on Quality of Life. Thyroid Autoimmunity. Thyroid-Stimulating Hormone (TSH; Thyroptopin)

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Measuring Impact of Benign Thyroid Diseases on Quality of Life

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Glossary*

GO-QLS Graves' ophthalmopathy quality of life scale
GO-QOL Graves' orbitopathy quality of life instrument
Item A question (including any introductory legends) and its associated response options, within a PRO
Multi-item scale A condensation of responses to a number of items. Most often a simple summation of arbitrary numerical weights associated with each response option
Patient-reported outcome (PRO) and Patient-reported outcome measure (PROM) Any report of the status of a patient's health condition that comes directly from the patient, without interpretation of the patient's response by a clinician or anyone else (FDA 2009)
QOL Thyroid eye disease quality of life scale
Quality of life (QoL) The subjective assessment of the impact of disease and its treatment across the physical, psychological, social, and somatic domains of functioning and well-being (Revicki *et al.* 2000)
Short Form (36) Health Survey (SF-36) The most widely used instrument for measuring generic quality of life (or rather: health status), that is, independent of any specific disease or condition
ThyDQoL Underactive thyroid-dependent quality of life questionnaire
ThyPRO Thyroid-related patient reported outcome
ThyPRO-39 Thyroid-related patient reported outcome, 39-item version
ThySRQ Thyroid symptom rating questionnaire
ThyTSQ Thyroid treatment satisfaction questionnaire

Introduction

In recent years, a paradigm shift has occurred in medical care. Patient-centered care has become the goal in most clinical settings and the value of an intervention is increasingly judged by its ability to maximize health, with an emphasis on the degree to which longevity and health-related quality of life (QoL) are impacted. QoL assesses the patient's point of view concerning how they feel and what they are able to do in everyday life. It has been defined as the subjective assessment of the impact of disease and its treatment across the physical, psychological, social, and somatic domains of functioning and well-being (Revicki *et al.*, 2000, Barofsky, 2012). QoL is measured by standardized questionnaires (patient-reported outcomes, PRO), where responses to questions are converted to and summarized in numeric values that quantify the attributes being measured. Typically, multi-item scales are used, where several items measuring the same construct are summarized into one scale, to reduce random measurement error and to simplify reporting. Findings are generally ordered within three main domains: physical, mental and social/participation, in accordance with the definition of health-related QoL (Ware, 2003).

For QoL assessments to be valid, it is essential to apply tools with appropriate measurement properties for the population and research question under study. The International Society of Quality of Life Research recently recommended minimum requirements for PROs used in patient-centered outcomes and comparative effectiveness research (Reeve *et al.*, 2013). The definitions and recommendations are presented in **Table 1**.

There are two broad categories of QoL measures, disease specific and generic measures. Disease specific QoL measures typically emphasize evaluation of symptoms, functioning and patient perceptions that relate to a narrowly defined disease or condition. Generic measures assess broad categories of functioning and well-being that can be affected by a multitude of conditions, and enable comparisons between the relative burdens of diseases and their treatment benefits. Both types of measures have been widely used to quantify QoL. However, because generic QoL measures favor broad functional health and well-being concepts over symptoms and problems that are more specific, they are sometimes found to be less sensitive to differences among clinically relevant groups and to be less responsive to small changes in health (Guyatt *et al.*, 1999, Patrick and Deyo, 1989).

Thyroid diseases affect QoL (Bianchi *et al.*, 2004, Watt *et al.*, 2006, Elberling *et al.*, 2004, Mishra *et al.*, 2013, Cramon *et al.*, 2015, Bove *et al.*, 2014), work role function (Nexo *et al.*, 2014, Nexo *et al.*, 2015), as well as morbidity and mortality (Brandt *et al.*, 2012, Thvilum *et al.*, 2013). A model of the theoretical relationships between thyroid pathophysiology and QoL impact is illustrated in **Fig. 1**. Pathophysiological mechanisms lead to physical and mental symptoms and impair functioning and well-being, which leads to impaired participation in life and overall QoL.

Generic measures can be applied in patients with thyroid diseases; for example, the Short Form (36) Health Survey (SF-36) has been applied in a number of studies. This may be advantageous, when comparison with other patient groups is

Please also see Table 1 for descriptions of concepts concerning measurement properties.

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Table 1 Measurement properties: patient-reported outcomes measurement properties—definitions and recommended minimum requirements

Measurement property	*Definition*	*Recommendation*
Conceptual and measurement model	The conceptual model provides a description and framework for the targeted construct(s) to be included in a PRO measure. The measurement model maps the individual items in the PRO measure to the construct	A PRO measure should have documentation defining and describing the concept(s) included and the intended population(s) for use. In addition, there should be documentation of how the concept(s) are organized into a measurement model, including evidence for the dimensionality of the measure, how items relate to each measured concept, and the relationship among concepts included in the PRO measure
Reliability	The degree to which a PRO measure is free from measurement error	The reliability of a PRO measure should preferably be at or above 0.70 for group-level comparisons, but may be lower if appropriately justified. Reliability can be estimated using a variety of methods including internal consistency reliability, test–retest reliability, or item response theory. Each method should be justified
Internal consistency reliability	The degree of the interrelatedness among the items in a multi-item PRO measure	
Test–retest reliability	A measure of the reproducibility of the scale, that is, the ability to provide consistent scores over time in a stable population [2]	
Validity	The degree to which a PRO instrument measures the PRO concept it purports to measure	
Content validity	The extent to which the PRO measure includes the most relevant and important aspects of a concept in the context of a given measurement application	A PRO measure should have evidence supporting its content validity, including evidence that patients and experts consider the content of the PRO measure relevant and comprehensive for the concept, population, and aim of the measurement application.
Construct validity	The degree to which scores on the PRO measure relate to other measures (e.g., patient-reported or clinical indicators) in a manner that is consistent with theoretically derived a priori hypotheses concerning the concepts that are being measured	A PRO measure should have evidence supporting its construct validity, including documentation of empirical findings that support predefined hypotheses on the expected associations among measures similar or dissimilar to the measured PRO
Criterion validity	The degree to which the scores of a PRO measure are an adequate reflection of a "gold standard."	
Responsiveness	The extent to which a PRO measure can detect changes in the construct being measured over time	A PRO measure for use in longitudinal research study should have evidence of responsiveness, including empirical evidence of changes in scores consistent with predefined hypotheses regarding changes in the measured PRO in the target population for the research application
Interpretability of scores	The degree to which one can assign easily understood meaning to a PRO measure's scores	A PRO measure should have documentation to support interpretation of scores, including what low and high scores represent for the measured concept
Minimal important difference (MID)	The smallest difference in score in the outcome of interest that informed patients or informed proxies perceive as important, either beneficial or harmful, and that would lead the patient or clinician to consider a change in the management	
Burden	The time, effort, and other demands placed on those to whom the instrument is administered (respondent burden) or on those who administer the instrument (investigator or administrative burden)	A PRO measure must not be overly burdensome for patients or investigators. The length of the PRO measure should be considered in the context of other PRO measures included in the assessment, the frequency of PRO data collection, and the characteristics of the study population.
Translation of the PRO measure		A PRO measure translated to one or more languages should have documentation of the methods used to translate and evaluate the PRO measure in each language. Studies should at least include evidence from qualitative methods (e.g., cognitive testing) to evaluate the translations

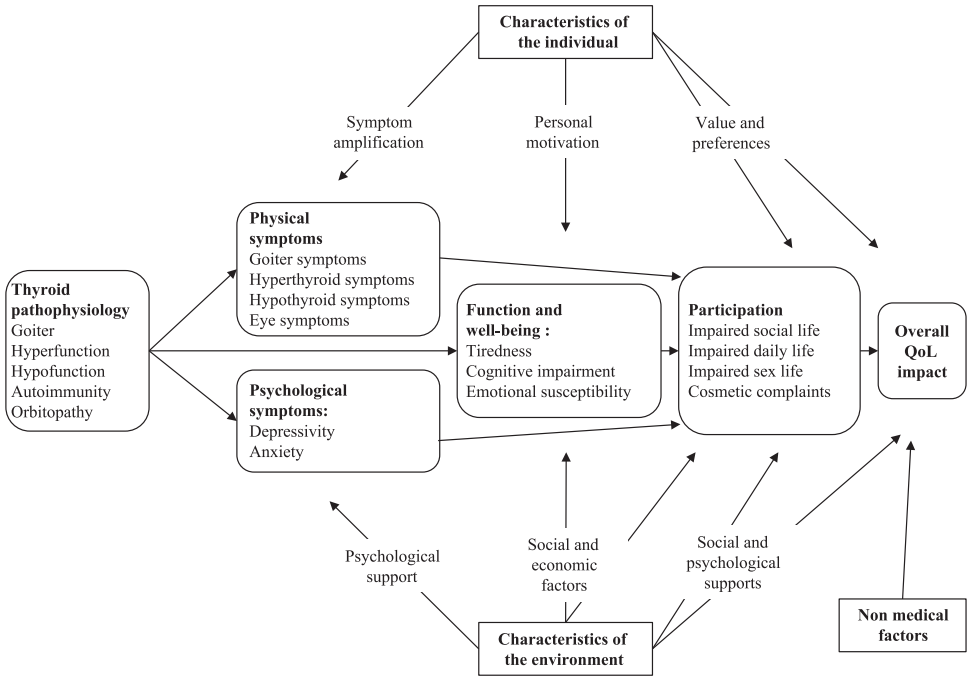

Fig. 1 Conceptual Model linking clinical pathophysiological variables and thyroid-relevant aspects of QoL. Based on Wilson, I. B. and Cleary, P. D. (1995). Linking clinical variables with health-related quality of life. A conceptual model of patient outcomes. *The Journal of the American Medical Association* **273**, 59–65.

important, for example, in descriptive studies quantifying impact on QoL of thyroid diseases overall. However, a thyroid-specific measurement has been found more sensitive to change than a generic measure (Watt *et al.*, 2014a), and such measurements thus are more relevant in studies evaluating effect of treatment and course of disease as well as when comparing clinically different groups of thyroid patients.

Focus in this article is thus on the properties of existing thyroid-specific PRO measures.

Thyroid-Specific PRO Measures

Instruments for Patients with Hyperthyroidism

The Hyperthyroidism Complaint Questionnaire (HCQ) measures "somatic and mental discomfort … typical for patients suffering from hyperthyroidism" (Fahrenfort *et al.*, 2000) and consists of 31 dichotomous items requiring a dual response: currently present and formerly present; two overall summary measures are generated: Past and Present Complaints, respectively. Eleven items concern physical symptoms, 6 are about emotional distress, 6 evaluate fatigue, 3 concern cognitive functions whereas existential problems, sleeping problems, anxiety, sexual function and social function were covered by one item each. The development was based on interviews with a small sample of patients (not documented). In a study including 303 patients previously treated for hyperthyroidism, internal consistency reliability (**Table 1**), was 0.93 for both scales. Item-total correlations were generally low, some were 0.21, questioning the justification of the overall summary score. A relationship between overall score and degree of self-reported thyroid dysfunction was found, but no further description of the validity of the instrument has been provided. The HCQ has not been used in any subsequent study.

Instruments for Patients with Graves' Orbitopathy

The Graves' Orbitopathy Quality of Life Questionnaire (GO-QOL) is a disease specific QOL-instrument for patients with Graves' orbitopathy (Terwee *et al.*, 1998, 1999, 2001). The development was based on a review of existing eye QOL-measures, as well as open-ended questionnaires from 24 patients and was pretested in 8 patients. Detailed description of these content validity studies have not been published. The GO-QOL consists of 16 items summarized in two scales: "Visual Functioning" and "Cosmetic Complaints"; that is, two distal QoL-concepts, albeit not as distal as "overall QoL". No proximal concepts such as physical symptoms are measured. Subsequent studies comprising 70–164 well-described patients have shown excellent test-retest and internal consistency reliability (Terwee *et al.*, 1998, 1999) and have supported its construct validity (Terwee *et al.*, 1998, 1999, 2001) in terms of exploratory factor analyses and examinations of con- and divergent validity. Further, good responsiveness was found (Terwee *et al.*, 2001). Modern psychometric methods have not been applied. According to the European Group of Graves' Orbitopathy (EUGOGO) website, the GO-QOL is available in 15 languages (www.

eugogo.eu). The Korean version has been clinically (not cross-culturally) validated (Choi *et al.*, 2012). GO-QOL has been applied in numerous subsequent clinical studies (Terwee *et al.*, 2002, Marcocci *et al.*, 2011, Kashkouli *et al.*, 2011, Park *et al.*, 2004, Wickwar *et al.*, 2015, Prummel *et al.*, 2004, European Group on Graves' Orbitopathy (EUGOGO) *et al.*, 2009, Ponto *et al.*, 2011, 2015, Fichter *et al.*, 2013, Jellema *et al.*, 2014, Lin *et al.*, 2013, Bartalena *et al.*, 2012, Dickinson *et al.*, 2004, Wemeau *et al.*, 2005). Cross-cultural validity does not seem to have been quantitatively assessed, but a forthcoming validation of the English version is described in a published trial protocol (Rajendram *et al.*, 2008). A non-validated modified version has also been applied in one clinical study (Finamor *et al.*, 2004).

In an attempt to develop a measure with high correlation with clinical variables, Yeatts developed the nine-item *GO-QLS* questionnaire (Yeatts, 2005). The development was based on analyses of responses from 256 patients to 105 existing generic and eye-specific items. The best discriminating items fitting a one-factor analysis were selected. The resulting nine-item measure does not seem to have been implemented in subsequent clinical studies yet.

Recently, a three-item Graves' orbitopathy-specific PRO has been developed, the TED-QOL (Fayers and Dolman, 2011). It consists of three visual analogue scale-like single items measuring overall QoL impact, interference with daily activities and satisfaction with appearance, respectively. The development was very briefly described, test-retest reliability was found to be good, convergent and discriminant validity was confirmed, compared with similar or dissimilar aspects from GO-QOL and GO-QLS and meaningful correlation with clinical variables were found. It has been translated and validated (not cross-culturally though) in Korean (Son *et al.*, 2014), but clinical studies applying the measure or studies reporting other important aspects of validity such as responsiveness, have not yet been published.

Instruments for Patients with Hypothyroidism

For the purpose of detecting patients developing hypothyroidism in a follow-up system after radioiodine-treatment, Barker and colleagues used a patient-completed questionnaire about nine classical symptoms of hypothyroidism (Barker and Bishop, 1969, Gardner and Barker, 1975). The evaluation of the instrument focused on its screening abilities, that is, ability to detect biochemical hypothyroidism. As with the Zulewski index, a simple summation of the number of symptoms were as effective as more complex weighting and scoring systems and if patients with five or more symptoms was referred to further diagnostic procedures, this would include 70% of the hypothyroid and 40% of the euthyroid patients.

For the analysis of the relationship between symptoms and biochemical thyroid status, Canaris and colleagues developed a 16–17 item hypothyroid symptom index (Canaris *et al.*, 1997), sometimes referred to as the *Colorado Thyroid Symptom Survey*. The questionnaire was developed on the basis of a combination of a literature study and expert evaluation. It was tested for readability and with regards to its ability to discriminate between hypothyroid and euthyroid patients. A modest correlation with TSH was found.

A modified version of the questionnaire was used in the large Colorado Thyroid Disease Prevalence Study (Canaris *et al.*, 1997) and in a subsequent screening study (Canaris *et al.*, 2013). Generally, low discriminating properties of individual symptoms were found. The proportion of hypothyroid patients increased with increasing number of symptoms present and with increasing weighted score; the total score had moderate discriminative power.

The *Chronic Thyroid Questionnaire (CTQ)* is a hypothyroidism *and* patient-specific QOL-questionnaire. It consists of 104 items, each representing a specific complaint, covering four domains: "Physical Complaints," "Mood and Emotions," "Energy and General Well-Being," and "Cognitive Complaints" (Jaeschke *et al.*, 1994, 1996). The development of the CTQ was quite thorough. Based on a literature review, a list of symptoms or problems related to hypothyroidism, potentially responsive to treatment and likely to influence the QoL of the patients, was generated (Jaeschke *et al.*, 1994). This list was expanded through interviews with endocrinologists and patients. The scoring of the CTQ is unusual: Of the 104 complaints, each patient identifies applicant items and rates the degree of discomfort represented by these items. Thus, for a patient with two of the 104 complaints, the instrument consists of two items, whereas a patient with 22 complaints rates 22 items. This approach increases the potential sensitivity of the measure to improvements in the individual patient, but it makes between-patient comparisons and interpretations of what is actually measured difficult and new complaints arising from intervention are ignored in longitudinal studies. The CTQ has not been validated in any subsequent studies, but applied in one subsequent study (Jorde *et al.*, 2006). A modified version was applied in two other studies ((Clyde *et al.*, 2003, Hoang *et al.*, 2013); however, the modifications were not described.

The *Thyroid Symptom Questionnaire (TSQ)* consists of 12 items: 6 items on cognitive complaints, 5 items on physical symptoms and 1 item on fatigue, summarized in one overall score (Saravanan *et al.*, 2002). The items were selected on the basis of patient-responses to a notice in the British Thyroid Foundation newsletter, inviting patients to tell about persisting complaints despite replacement therapy with L-thyroxine. Moderate correlations with the generic QOL-questionnaire General Health Questionnaire (GHQ-12) were found, but no other evidence of validity has been presented. The measure does not seem to have been adopted in subsequent clinical studies.

The *Underactive Thyroid-Dependent Quality of Life Questionnaire (ThyDQoL)* (McMillan *et al.*, 2004). ThyDQoL is a 20-item questionnaire measuring impact of hypothyroidism on various domains of QOL: overall QoL (2 items), limitations in usual activities (6 items), social function (4 items), fatigue (2 items), emotional well-being (2 items), sexual function, cosmetic complaints, weight problems, and bodily discomfort (1 item each). Items are scored individually in a two-step procedure: both impact and importance of the items are rated, and the item score is derived by multiplication of these two ratings. An average weighted impact score is obtained by summing all applicable weighted domain scores (i.e., not the two overall QoL items), divided by number of domains applicable to the individual. Content validity was ensured through interviews with 38 hypothyroid

patients, as part of the development process (McMillan *et al.*, 2004). In a subsequent validation study, appropriate internal consistency reliability was found. Construct validity in terms of scale validity as evaluated by exploratory factor analyses was also examined. Although the authors conclude that an identified two-factor solution was un-interpretable, it could be argued, that a picture of one set of items clustering around an aspect of participation and another around symptoms and well-being within the patient, was indeed drawn (Table 3 in McMillan *et al.* (2008)); but a one-factor solution also seems well justified. Criterion validity was not explicitly addressed, but a tendency towards worse perceived negative impact on QoL was observed among patients with overt hypothyroidism, compared to patients with subclinical disease. One problem with the two-step importance rating approach is the reduced inter-individual comparability of the measure and the susceptibility to a confounding effect of coping. These results regarding validity have been reproduced for the German version (Quinque *et al.*, 2013). In that study, also criterion validity was evaluated (correlation with clinical variables), and only partial support for criterion validity was found; of note, however, only 25 patients and 27 healthy controls were included in the criterion validation study. ThyDQoL has been applied in a clinical study evaluating effect of L-thyroxine on subclinical hypothyroidism (Razvi *et al.*, 2007) and in a study of impact of transient hypothyroidism among patients with thyroid cancer (Smith *et al.*, 2015). In the latter, scores worsened during hypothyroidism, supporting the clinical validity of the measure. Also, it is specified as an outcome in a published trial protocol (Wilkes *et al.*, 2013).

During the development of the ThyDQoL, symptoms related to hypothyroidism were extracted into a separate instrument, the *Thyroid Symptom Rating Questionnaire, ThySRQ* (McMillan *et al.*, 2004). The motivation was the observation, that the symptoms were "too specific in nature to be important for many aspects of life, for example, voice problems, or because some patients were unsure whether they were attributable to hypothyroidism." ThySRQ consists of 15 items, covering both physical, psychological, and functional (tiredness, cognition) symptoms. Items are reported separately, since factor analyses could not support summation (McMillan *et al.*, 2008). Similarly to ThyDQoL, items are completed in a two-step procedure: first, whether it is experienced, then, the extent to which it causes distress. To the authors' surprise, high internal consistency reliability was found. Along with ThyDQoL, it was applied in patients with subclinical hypothyroidism (Razvi *et al.*, 2007) and is included as outcome in a published trial protocol (Wilkes *et al.*, 2013). A simplified version was applied in a study in Brazil; no information regarding method of translation or validation thereof was presented (Vigario Pdos *et al.*, 2013). In a third study applying the measure, an average sum-score was analyzed and reported, despite the evidence against the validity of such an approach (Smith *et al.*, 2015).

In parallel with ThyDQoL and ThySRQ, a *Thyroid Treatment Satisfaction Questionnaire*, the *ThyTSQ*, was developed (McMillan *et al.*, 2004). The questionnaire has two parts: seven items measuring satisfaction with present treatment and four items with past treatment, each rated from 6 (very satisfied) to 0 (very dissatisfied). In a validation study, excellent internal consistency reliability, good acceptance and evidence of unidimensionality, justifying reporting as a single summated scale, for the two multi-item scales (Past and Present Satisfaction) (McMillan *et al.*, 2006). It has been applied in the abovementioned L-thyroxine study (Razvi *et al.*, 2007) and in a published study protocol (Wilkes *et al.*, 2013).

Instrument for all Benign Thyroid Diseases

The *Thyroid-related quality of life patient-reported outcome (ThyPRO)* is 85-item instrument covering all benign thyroid diseases summarized in 13 multi-item and one single-item scale(s): 4 scales measuring physical symptoms (goiter symptoms (11 items), hyperthyroid symptoms (8 items), hypothyroid symptoms (4 items) and eye symptoms (8 items)), 2 scales measuring mental symptoms (anxiety (6 items) and depressivity (7 items)), 3 scales measuring well-being and function (emotional susceptibility (9 items), cognitive complaints (6 items) and tiredness (7 items)), 4 scales measuring aspects of impaired participation (impaired social life (4 items), impaired daily life (6 items), impaired sex life (2 items) and cosmetic complaints (6 items)) and overall QOL impact (1 item). It takes 14 min to complete, on average, and each items is rated on a 5-point Likert scale from no symptoms/problems $=0$ to severe symptoms/problems $=4$. The average score of items in a scale is divided by four and multiplied by 100 to yield thirteen 0–100 scales, with higher scores indicating worse health status. The development was based on a literature review (Watt *et al.*, 2006), interviews with 15 thyroid experts and 80 thyroid patients (Watt *et al.*, 2007) and qualitatively validated and refined in 6 rounds (4–10 patients each round, 31 in total) of cognitive interviewing (Watt *et al.*, 2008). Subsequently it was substantially validated using classical (Watt *et al.*, 2009, Rasmussen, 2016) and modern (Watt *et al.*, 2014c, Watt *et al.*, 2014b) psychometric methods as well as clinimetrics (Watt *et al.*, 2010, Watt *et al.*, 2014a) which found good internal consistency and test-retest reliability, appropriate construct validity and measurement invariance, as well as good discriminant validity and ability to detect relevant clinical change. As the only thyroid-related PRO, cross-cultural validity has been approved (Watt *et al.*, 2015a) and by October 2017 linguistically validated versions are available in 19 languages and undergoing adaptation in another 11 languages. ThyPRO (or its short-form ThyPRO-39, see below) is or has been in use in more than 60 clinical trials world-wide (personal communication). Published studies includes cross-sectional, longitudinal and interventional studies in patients with autoimmune hypothyroidism (Winther *et al.*, 2016, Watt *et al.*, 2012, Stott *et al.*, 2017), cross-sectional and longitudinal studies of hyperthyroidism (Cramon *et al.*, 2016, Bove *et al.*, 2014, Bukvic *et al.*, 2015, Taieb *et al.*, 2016), cross sectional, longitudinal as well as interventional studies on non-toxic goiter (Cramon *et al.*, 2015, Bukvic *et al.*, 2014, Mishra *et al.*, 2013, Zivaljevic *et al.*, 2015, Fast *et al.*, 2014, Graf *et al.*, 2011) and two published randomized clinical trial (RCT) protocols (Winther *et al.*, 2014, Watt *et al.*, 2013). Due to the coverage across all benign thyroid diseases, the instrument is rather lengthy.

The *Short form thyroid-related quality of life patient-reported outcome (ThyPRO-39)* was extracted from the 85-item ThyPRO, based on previous patient interviews, validation studies and item response modeling (Watt *et al.*, 2015b). It consists of 39 items, summarized in the same scales as the 85-item version, except for impaired sex life, which was omitted. In addition, mental symptoms, well-being and function and participation scales were further summarized in one overall composite score. High level of agreement between the two versions was found and good measurement properties were preserved, when repeating the psychometric and clinimetric analyses described above. Although the extracted version had appropriate measurement properties, it has not yet been validated as a stand-alone measure.

Comparison of Instruments

Of the identified instruments, only few have been developed in accordance with current standards, as shown in **Table 2**: Three instruments for patients with Graves' orbitopathy: GO-QOL, GO-QLS, and TED-QOL and one triplet of instruments for patients with hypothyroidism, ThyDQoL, ThySRQ and ThyTSQ and ThyPRO/ThyPRO-39, for patients with any benign thyroid disease, and thus the only PRO validated for use among patients with non-toxic goiter and hyperthyroidism.

Table 2 Existing thyroid-related PRO instruments

Measurement property documented	*ThyPRO*	*ThyPRO39*	*HCQ*	*GO-QOL*	*GO-QLS*	*TED-QOL*	*Barker*	*Canaris*	*CTQ*	*TSQ*	*ThyDQoL*	*ThySRQ*	*ThyTSQ*
Conceptual model	+	+	−	+	+	−	−	−	−	−	+	+	+
Reliability:													
Intern. consist. reliability	+	+	+	+	+	−	−	−	−	−	+	+	+
Test–retest reliability	+	+	−	+	−	+	−	−	−	−	−	−	−
Validity:													
Content validity	+	+	+	+	(+)[a]	+	−	+	+	−	+	+	+
Construct validity	+	+	−	+	(+)	+	−	−	−	−	(+)	(+)[b]	+
Criterion validity	+	+	−	+	+	+	+	+	−	−	(−)[c]	(−)[c]	(+)[d]
IRT models applied	+	+	−	−	−	−	−	−	−	−	−	−	−
Responsiveness	+	+	−	+	−	−		−	−	−	−	−	−
Interpretability of scores	(+)[e]	(+)[e]	−	(+)	−	−	−	−	−	−	−	−	−
MID	(+)[e]	(+)[e]	−	+	−	−	−	−	−	−	−	−	−
Burden (*n* of items)	85	39	31	16	9	3	9	16	104	12	20	15	11
Language versions	16	19	1	15[f]	1	2	1	1	1	1	3	2	3
Target population:													
Non-toxic goiter	+	+	−	−	−	−	−	−	−	−	−	−	−
Toxic nodular goiter	+	+	+	−	−	−	−	−	−	−	−	−	−
Graves' hyperthyroidism	+	+	+	−	−	−	−	−	−	−	−	−	−
Graves' orbitopathy	+	+	−	+	+	+	−	−	−	−	−	−	−
Hypothyroidism	+	+	−	−	−	−	+	+	+	+	+	+	+[g]
Domains covered:													
Physical symptoms	+	+	+	−	−	−	+	+	+	+	−	+	−
Psychological symptoms	+	+	+	−	−	−	−	−	+	+	+	+	−
Well-being	+	+	+	+	−	−	−	−	+	+	+	+	−
Function	+	+	+	+	+	+	−	−	+	−	+	−	−
Sex life	+	−	+	−	−	−	−	−	−	−	+	−	−
Participation	+	+	+	+	+	+	−	−	+	−	+	−	−
Overall QoL	+	+	−	−	−	+	−	−	−	−	+	−	−
Treatment satisfaction	−	−	−	−	−	−	−	−	−	−	−	−	+
Methodological publications	15	15[h]	1	4	1	2	1	1	1	0	3	3	3
Application publications	11	11	(1)	15	0	0	0	2	1 (+2)	1	2	2 (+1)	2

[a]Not based on patient-interviews.
[b]A total score was not fully justified by factor analyses.
[c]No significant relationship with clinical variables, but study design not specifically aimed at criterion validity.
[d]PRO scores correlated in a meaningful direction with some clinical variables. Criterion validity only partially supported.
[e]MID has been published as a conference paper and responsiveness data as well as general population norms offer interpretability-information.
[f]Cross-cultural validity not yet quantitatively assessed.
[g]Only applicable to patients currently receiving treatment.
[h]High level of agreement, repetition and nestedness allows extrapolation from the full version validation studies.
Description of development and validation, including number of associated studies published, in accordance with the terminology presented in Table 1.

Spectrum of Concepts Measured by Thyroid-Specific PROs

As evident from Table 2, some instruments focus on very specific manifestations of disease impact, for example, physical symptoms (Barker and Canaris indices, for example), whereas other focus on broader aspects of QoL, for example, impact on social functioning (TED-QLS, for example). These broader aspects may also be termed "distal" concepts (corresponding to the right, "downstream" part of the theoretical QOL-model (Fig. 1)), whereas aspects more closely associated with a particular condition may be referred to as" proximal" aspects (corresponding to the left part of the QOL-model). Disease- or condition-specificity of an instrument may in fact be obtained with both categories. Proximal outcomes may do so by selecting symptoms specific for, or very relevant to, a specific condition. Distal outcomes may acquire specificity by attributing the concept in focus to the relevant condition. As an example of the former approach, the Canaris index measures symptoms characteristic of hypothyroidism. In contrast, the TED-QLS asks about impact of eye-disease on overall QoL, daily function and well-being, using only one item for each of these. This latter approach is tempting, due to the ease of administration, and because it can easily be applied to a wide variety of diseases, using the same format. However, with this approach, precision and reliability is a concern. Moreover, the standard approach for ensuring content validity may be less suited in this situation: In a study comparing TED-QLS with GO-QOL and GO-QLS, patients were asked if the items covered their situation. Since the questions are so broad, it is difficult to bring up an issue, which cannot be categorized under one of these aspects. In such a situation, a more elaborate approach is needed, to evaluate content validity. For example, in-depth cognitive interviewing, exploring whether patients truly consider all relevant subaspects, when they formulate their replies to such broadly formulated questions. Proximal items offer the advantage of providing cues for comprehension and retrieval to the respondent. ThyPRO includes both proximal and distal aspects, reported separately. Proximal aspects are administered first, to stimulate retrieval, and distal aspects are administered in the end of the questionnaire. Aspects measured by other instruments not covered by ThyPRO are "treatment satisfaction" and "positive effects of the disease", measured in ThyDQoL and ThyTSQ, respectively. Thus, studies focusing on treatment satisfaction among patients with hypothyroidism could use the ThyTSQ.

Future Directions

Availability of a validated PRO for patients with thyroid diseases enables studies evaluating currently unresolved clinical questions. For example, studies evaluating if treatment of mild or subclinical thyroid disease improves QoL, including the question of whether certain subgroups (e.g., various strata of TSH-elevation) benefit more than others.

It could also be argued, that the role of thyroxine/thyronine combination therapy for patients not satisfactorily treated with standard thyroxine supplementation alone is not clarified yet. A pragmatic RCT, testing the treatment approach suggested by the European Thyroid Association (Wiersinga *et al.*, 2012), or a new slow-release combination tablet, could be set up, including clinical and genetic biomarkers (e.g., deiodinase gene polymorphisms), preferably in a multi-cultural setting.

A study evaluating if various TSH-targets lead to differences in QoL would also provide important clinical insights into a previously debated and studied issue (Jonklaas *et al.*, 2014, Walsh *et al.*, 2006, Wartofsky and Dickey, 2005, Surks *et al.*, 2005).

Ideally, QoL-outcome of the various treatment modalities available for Graves' disease (including titration vs. block-replacement (Abraham *et al.*, 2005)) should also be evaluated in RCTs in analogy to a previous unique Swedish study (Torring *et al.*, 1996).

No studies on routine clinical use of PRO assessments among patients with thyroid diseases have been published.

Several features of thyroid diseases could make routine PRO assessments particularly well suited for these patients: (1) Thyroid hormones affect many organ systems and have profound effect on the central nervous system and thereby mental health. (2) QoL-issues have major influence on choice of intervention (e.g., physical symptoms and cosmetic concern in non-toxic goiter). (3) Some patients experience reduced QoL despite adequate thyroid function on treatment and may experience lack of congruence between the focus of the endocrinologist and themselves (Nexo *et al.*, 2015). (4) The diseases are often chronic and occur in all ages, including during working life. (5) Thyroid diseases are rarely life-threatening, and thus focus on and relevant addressment of QoL-issues may have relatively large impact. In contrast, most research on routine use of PRO have been among patients with cancer, who face a life-threatening disease, serious adverse effects, potential disease progression and treatment failure.

From a scientific point of view, thyroid diseases may also be an advantageous group for studies of the effect of PRO-implementation in clinical practice. For example, independent markers of management (e.g., TSH) exist and can be used as independent effect markers. Also, the existence of different, yet linked, thyroid diseases allows for comparison of effects among the different diagnostic groups.

As regards ThyPRO, several features position it as a good candidate for a PRO-implementation trial. First, it is relevant to patients and clinicians (Watt *et al.*, 2007), easy to understand and complete (Watt *et al.*, 2008), framed within a clinical and theoretical understanding (Watt, 2008; Watt *et al.*, 2014b) and with good measurement properties, including responsiveness to treatment. Second, it encompasses both proximal (symptoms) and distal (participation, overall QoL) aspects, enabling analyses of differing effects and uses of such. Third, despite being disease-specific, general population norms are available for interpretation-optimization.

PROs have become a natural, integral part of clinical research. Although the optimal use in routine clinical practice still has to be identified, clinical implementation for descriptive purposes seems well justified at present. Thereby data regarding usual course of disease and treatment can be described and possibly predictors for reduced QoL after treatment can be identified. Such data

could improve other patient-engaging activities, such as shared decision-making, for which a tool has recently been developed (Brito *et al.*, 2015). More detailed information about impact on QoL can improve such tools.

Further patient-engagement, also in design of clinical trials, is recommended.

See also: Thyrotoxicosis; Systemic Manifestations. Thyroid-Stimulating Hormone (TSH; Thyroptopin). Hashimoto's Thyroiditis. Thyroid Autoimmunity. Graves' Orbitopathy. Graves' Disease. Hyperthyroidism in Graves' Disease

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Further Reading

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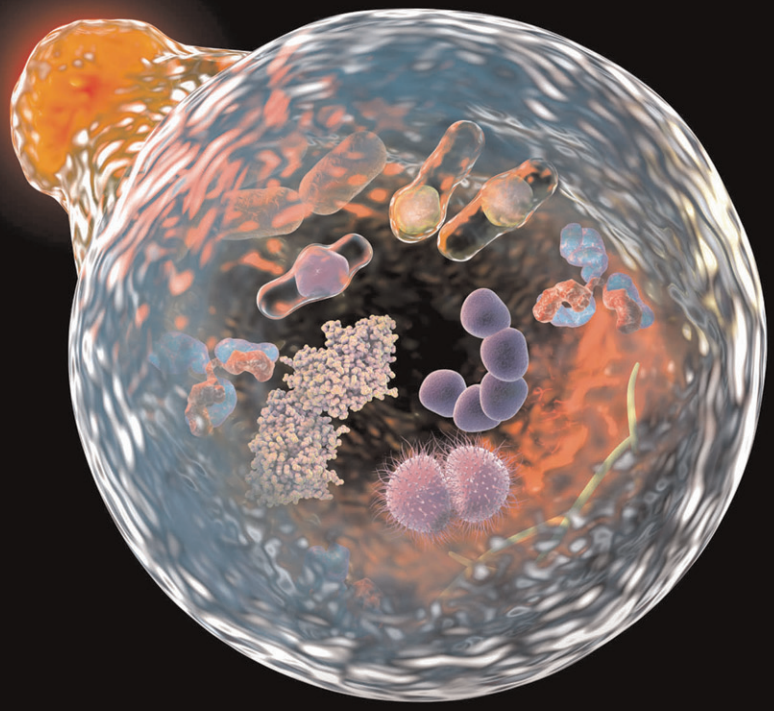

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DEDICATION

Professor Luciano Martini, 1927–2017

The other Editor in Chief of the Encyclopedia, Professor Luciano Martini, passed away on July 13th, 2017. He was an internationally acclaimed authority in the field of endocrinology, in particular neuroendocrinology, a brilliant and imaginative scientist, and an impressive and erudite scholar.

Luciano achieved the venerable age of 90, and his long career was full of outstanding scientific achievements, leadership positions in academia and in scientific societies, academies, and committees.

Luciano received his MD degree from the University of Milan in 1950. He then rapidly progressed through junior academic ranks up to the position of Professor and Chairman of the Department of Pharmacology at the University of Perugia in 1968, and subsequently, in 1972, he returned to his *alma mater*, the University of Milan, as full Professor and Chairman of the Department of Endocrinology, a post he held until 2001. He served in Milan as director of the training and research program entitled Physiology of Reproduction for nearly 20 years and attracted to his team top-class Italian and foreign scientists to address his main research interests of neuroendocrine regulation of reproductive functions.

Scientific severity, ethical integrity, fine perception, and deep farsightedness describe best Luciano's character as a scientist. He created in his institute a scientific research group devoted to experimental endocrinology, which grew over the years in size and visibility and became widely recognized internationally. Luciano published more than 400 peer-reviewed and highly cited papers mainly in the fields of neuroendocrinology, endocrine oncology, physiology of reproduction, and steroid and energy metabolisms.

Luciano was a prolific editor of scientific books and journals, which include the two volumes of *Neuroendocrinology* and the nine biennial volumes of *Frontiers in Neuroendocrinology*. He was Editor in Chief of *Comprehensive Endocrinology* published in 12 volumes and the first Edition of *Encyclopedia of Endocrine Diseases*. He served as President in many national and international scientific societies including the International Society of Neuroendocrinology, the Italian Society of Endocrinology, the International Society of Endocrinology, and the European Federation of Endocrine Societies. For his scientific achievements Luciano received honorary doctorates in the universities of Liège, Santiago de Compostela, Pécs, and Milan, and he was the recipient of numerous scientific awards and invited academy memberships.

Luciano's portrait could not be complete if one forgets to mention his life-time passion for music. He was a well-trained and accomplished pianist, a passionate music listener, and an enthusiastic connoisseur of all types of music. He also was an amateur in visual arts and deeply interested in history.

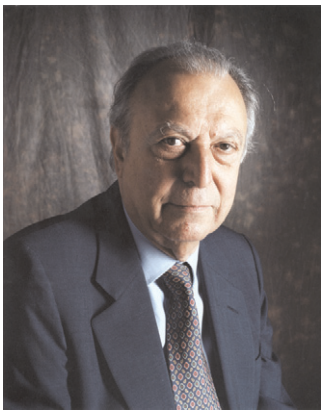
All of us who knew Professor Luciano Martini deeply mourn the loss of a great scientist and friend, the real "Il Maestro", teacher, colleague, and pioneer of modern neuroendocrinology. I trust Luciano would have been proud of this new edition of the Encyclopedia of Endocrine Diseases, and all of us having worked on its production would like to dedicate it to his memory.

Ilpo Huhtaniemi

Editor in Chief
Encyclopedia of Endocrine Diseases, 2nd edition

EDITORS IN CHIEF

Ilpo Huhtaniemi received his MD and PhD at University of Helsinki, Finland, did postdoctoral training in United States (UC San Francisco and NIH, Bethesda), and has been on sabbatical leave in Germany, United States and Scotland. In 1986–2002 he held the post of Professor and Chairman of Physiology at University of Turku, Finland. He moved in 2002 to UK to a Chair in Reproductive Endocrinology at Imperial College London, from which position he retired in 2015. He has received several national and international honors, amongst them a fellowship of The Academy of Medical Sciences, United Kingdom, and a Doctor Honoris Causa at the Medical University Łódź, Poland, and University of Szeged, Hungary. He was the Chief Managing Editor of *Molecular and Cellular Endocrinology* 1999-2017, has served in the Editorial Board of *Endocrinology and Endocrine Reviews* and is/has been the Editor or Editorial Board Member of several other scientific journals (e.g., *European Journal of Endocrinology*, *Clinical Endocrinology*, *Human Reproduction Update*, *Journal of Endocrinology*, *Molecular Human Reproduction*, *Reproduction*, *Asian Journal of Andrology*). He has extensive experience as Official of international scientific organizations (e.g., Past President of International Society of Andrology). His research interests include clinical and basic reproductive endocrinology, in particular the function of gonadotrophins and male reproductive endocrinology. He also has long-term interests in development of male contraception, hormone-dependent cancer, and the endocrinology of aging. He has authored about 700 peer-reviewed research articles and reviews, and his H-factor is 78.



Luciano Martini was born on May 14, 1927, in Milan, Italy. He obtained the degree of Medical Doctor "summa cum laude" on November 24, 1950, from the Faculty of Medicine of the University of Milan, Italy. He was Emeritus Professor of Pharmacology of the University of Perugia, Italy, and Emeritus Professor of Endocrinology of the University of Milan, Italy. He was Doctor Honoris Causa in Medicine of the Universities of Liège, Belgium, Santiago de Compostela, Spain, and Pécs, Hungary, and Doctor Honoris Causa in Biotechnological Sciences of the University of Milan, Italy. He was an author of more than 400 peer-reviewed scientific publications in the fields of endocrinology, neuroendocrinology, pharmacology, physiology of reproduction, steroid biochemistry, and basic oncology. He was elected member of the Accademia Nazionale dei Lincei (Italian National Academy) and of the American Academy of Arts and Sciences (Honorary Foreign Member).

Luciano Martini acted as Editor in Chief of the journal *Frontiers in Neuroendocrinology* from 1990 to 2001, and was a Member of the Editorial Board of *Endocrinology* (Foreign Consulting Editor, 1961–65), as well as of several other speciality journals, such as *Experimental and Clinical Endocrinology*, *Biochemistry*, and *Steroids*. He has acted as Editor of several textbooks (e.g., *Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1966–67, and *Clinical Neuroendocrinology*, a textbook in 2 volumes, Academic Press, New York, 1977–82) as well of a series of books under the name *Comprehensive Endocrinology* (13 volumes), Raven Press, New York, 1979–84. He acted as Editor in Chief for the first edition of *Encyclopedia of Endocrine Diseases* (4 volumes), Academic Press-Elsevier, San Diego, 2004.

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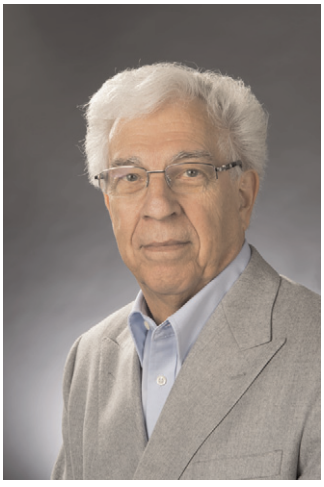


Dr. **Jean-Louis Chiasson** is currently Full Professor of Medicine at the University of Montreal. He is Head of the Research Group on Diabetes and Metabolic Regulation at the Research Center of the Centre hospitalier de l'Université de Montréal (CRCHUM).

Dr. Chiasson obtained his MD at Laval University in Quebec City in 1967. He did his specialty training in Internal Medicine at Laval Univesity and in Endocrinology at McGill University. He then did a research Fellowship in Diabetes at Vanderbilt University in Nashville, Tennessee. In 1974–76 and 1978–80, he was appointed Assistant Professor in the Department of Medicine and Physiology respectively at Vanderbilt University. In 1980, he returned to Montreal as Assistant Professor in the Department of Medicine at the University of Montreal and as Endocrinologist at Hotel-Dieu Hospital, now merged into the Centre hospitalier de l'Université de Montréal.

Dr. Chiasson's research interests include the regulation of carbohydrate metabolism in health and diabetes, as well as the development and evaluation of new strategies for the treatment and prevention of diabetes and its vascular complications. He has contributed over 250 scientific publications and lectures nationally and internationally on various topics on diabetes mellitus, its pathogenesis, its treatment, and its prevention. His scientific contribution puts him in the prestigious club of the 100 most cited publications in the world in the field of diabetes.

Sophie Christin-Maitre received her MD at University of Paris XI and her PhD at University Paris VI, Pierre and Marie Curie, France. She did a postdoctoral training in United States (Massachusetts General Hospital, Harvard University, Boston); she specialized in reproductive medicine. She holds the post of Professor of Endocrinology at University of Sorbonne, Paris, France. She has been the head of the Adult Endocrine Unit, in Hôpital Saint-Antoine, Assistance-Publique Hôpitaux de Paris, since 2011. She is a member of the INSERM research unit UMR S_933, specialized in identifying new genes in reproductive disorders. Her interests include clinical and basic reproductive endocrinology, in particular the management of patients with Turner syndrome, patients with primary ovarian insufficiency, patients with hypogonadisms, and patients with abnormalities of sex development. She has authored approximately 150 peer-reviewed research articles and reviews.

Ulla Feldt-Rasmussen is Professor at Copenhagen University and Chief of Medical Endocrinology, National University Hospital. Her research interests involve the thyroid gland and autoimmunity, as well as pituitary and adrenal dysfunction.

She has published more than 410 papers in peer-reviewed journals on e.g., thyroid hormones and body composition, thyroid autoimmunity and cancer, cytokines as regulators of endocrine cells, influence of thyroid disrupting chemicals on thyroid cells, growth hormone deficiency related to body composition, bone metabolism and other pituitary axes, and transition from adolescent to adult care, as well as several aspects of Fabry disease. In recent years her group has embarked on studies on pituitary function after traumatic brain injury in a nationwide setting, and focusing on diagnostic accuracy of pituitary testing procedures. She has further authored numerous proceedings, textbook chapters, and other publications; as well as organized numerous international meetings and postgraduate courses, and has led several European projects and other collaborations within many areas of endocrinology.

Professor Feldt-Rasmussen reviews for international journals, and is an editorial board member of several endocrine journals. She belongs to many international professional organizations, including the Endocrine Society, ETA, ATA, ENEA, and GRS; she has served as Secretary-Treasurer of ETA and as President of the ETA Cancer Research Network.

Professor Feldt-Rasmussen serves on the advisory boards of several ad hoc endocrine committees, and has received many prestigious prizes including the Mayo Clinic's Haynes Lecturer's Award and ETA's Pinchera Research Prize.

Wouter W. de Herder M.D. Ph.D. (1960) is Professor of Endocrine Oncology at the Erasmus MC in Rotterdam, the Netherlands. In this University Hospital he is chairman of a multidisciplinary group for endocrine oncology (tumorwerkgroep endocriene tumoren) and he is head of the ENETS centre of excellence for neuroendocrine tumors. His major research interests are neuroendocrine and endocrine tumors.

Professor de Herder received his M.D. in 1985 and his Ph.D. in 1990 from the Erasmus University in Rotterdam, the Netherlands.

He is a member of several international and Dutch national societies, such as the Dutch Society for Endocrinology (NVE), the Endocrine Society (USA), the European Society of Endocrinology (ESE), European Neuroendocrine Tumor Society (ENETS) and the North American Neuroendocrine Tumor Society (NANETS). He served as a board member of the Dutch Society for Endocrinology (NVE) (2009–14). He served as chairman (2006–08) and vice-chairman of ENETS (2008–10) (European Neuroendocrine Tumour Society). He is member of the advisory boards of ENETS and NANETS.

Professor de Herder has (co-)published over 400 peer-reviewed papers and book chapters and is a reviewer for many international journals.

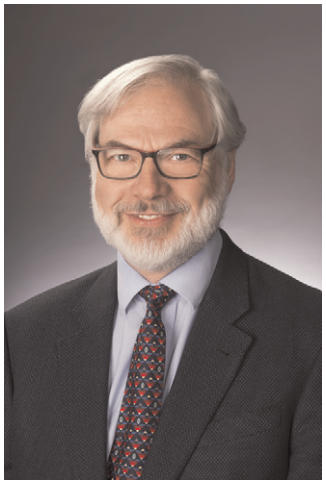
He is a member of the editorial boards of *Neuroendocrinology; Endocrinology, Diabetes & Metabolism Case Reports; Clinical Endocrinology*, and *Endocrine-Related Cancer*.

Professor de Herder has given over 200 invited presentations at Dutch national and international meetings.

Ieuan Hughes is currently Emeritus Professor of Pediatrics at the University of Cambridge and Honorary Consultant Pediatrician at Cambridge University Hospitals NHS Foundation Trust and Cambridge Biomedical Campus. He is the author of more than 300 papers and chapters across the whole range of paediatric endocrinology. His particular expertise is in disorders of sex development for which he coordinated the International Consensus on the approach to the investigation and management of this broad topic. Research interests focus on steroid enzyme deficiencies and molecular mechanisms of androgen action.
Professor Hughes has served on the editorial boards of several journals, including *Clinical Endocrinology*, *Journal of Clinical Endocrinology*, and *Metabolism* and *Archives of Disease in Childhood* where he was also the Associate Editor. He is Past-Secretary and President of the European Society for Pediatric Endocrinology and a recipient of the highest award of the Society, the Andrea Prader Prize. Professor Hughes is a James Spence Medallist of the Royal College of Pediatrics and Child Health for outstanding contributions to paediatric knowledge. He is a Fellow of the Academy of Medical Sciences, a Council Member of the Learned Society of Wales and a Trustee of two charities. The chapter on Disorders of Sex Development in *Williams Textbook of Endocrinology* (now in its 14e) by Hughes and co-authors is considered to be a definitive and up to date regular review of this topic, specific and key to pediatric endocrinology.

Dr. **Gregory Kaltsas** MD FRCP (Lon) is Professor in General Medicine and Endocrinology at the National and Kapodistrian University of Athens, Greece. He was trained in General Medicine in Athens, Greece and London, UK, and in Endocrinology at the Middlesex and St Bartholomew's Hospital, London, UK. He developed a particular interest in neuroendocrinology (pituitary and neuroendocrine tumors) and adrenal physiology and diseases. Upon returning to Greece he established a neuroendocrine network and he is currently running the European Neuroendocrine Tumor Society (ENETS) Center of Excellence at Laiko Hospital in Athens, Greece. He has served as a member of the advisory board of ENETS and of the Executive Committee of the European Neuroendocrine Association (ENEA) and he has been elected in the Executive Committee of the International Society of Endocrinology. He has recently been elected as a representative of the European Society of Endocrinology in the ExCo of the International Society of Endocrinology. He has published more than 300 original papers, reviews, and chapters and serves on editorial boards and as associate editor in several endocrine journals.

Jean-Marc Kaufman obtained his MD and PhD degrees at the Ghent University, Belgium. He was a Senior Postdoctoral Research Fellow (1982–84) in reproductive physiology at the University of Texas Medical School at Houston. He is board certified in Endocrinology and in Nuclear Medicine. In 1985 he joined the staff of the Ghent University Hospital; he headed the department of Endocrinology from 2003 to 2014 and the Laboratory for Hormonology from 1995 to 2014. He was appointed in 1993 Professor of Medicine at the Ghent University (1993) and is past Chair of the Department of Internal Medicine at the Ghent University (2010–14). From October 1st 2014 he is Professor Emeritus at the Ghent University where he is pursuing clinical and research activities. Main research interests are in the assessment, regulation, and action of sex steroids with focus on their role in health, disease, and aging in men, and in osteoporosis in men. He is (co)author of over 300 publications in international peer-reviewed journals.



André Lacroix, MD FCAHS is Professor of Medicine, Division of Endocrinology at Centre hospitalier de l'Université de Montréal (CHUM). His areas of interest include the mechanisms of adrenal Cushing syndrome, primary aldosteronism, adrenal tumorigenesis, the role of aberrant adrenal hormone receptors in adrenal overfunction, as well as new drugs in the therapy of Cushing disease, primary aldosteronism and adrenocortical cancer.
He was trained at the University of Montreal followed by fellowships in Endocrinology and research at Vanderebilt University and National Institutes of Health, USA. He was Chairman of Medicine and Director of Academic Affairs at CHUM. Former President of the Canadian Society of Endocrinology and Metabolism, he is currently chairperson of the International Society of Endocrinology (2016–20), Editor, Adrenal Section of UpToDate and *Encyclopedia of Endocrinology*, Senior Editor of the *European Journal of Endocrinology*. Fellow of the Canadian Academy of Health Sciences since 2008 and Foreign member of the National Academy of Medicine of France since 2016.

Franco Mantero received his MD at the University of Padua, Italy, did postdoctoral training in Switzerland (Clinique Medicale Therapeutique, Hopital Cantonal, University of Geneva) and in United States (University of California, San Francisco), and has been on sabbatical leave in United Kingdom, United States, and France. He held a post of Associate Professor in Medicine at the Institute of Semeiotica Medica, University of Padua (1981–86). In 1986 he moved to the University of Catania to the Chair of Andrology and Endocrinology, in 1992 to the University of Ancona, and in 2000 to the University of Padua to the Chair of Endocrinology and Chief of the Endocrinology Unit of the Department of Medicine. He has received national and international honors, including a Doctor Honoris Causa at the Semmelweis University, Budapest, Hungary.
He has been Editorial Board Member of several scientific journals (e.g., *Clinical Endocrinology*, *Endocrinology*, *Journal of Hypertension*, *Journal of Endocrinology Investigation Steroids*)
He has served as Member of the Council of several international scientific societies (including International Society of Endocrinology, International Aldosterone Conference, Journee Klotz d'Endocrinologie Clinique, ENS@T) and one of the founders of the European Network for the Study of Adrenal Tumors. His research interests include clinical and basic endocrinology of the adrenal gland and endocrinology of hypertension, in particular pathophysiology of mineralocorticoids and primary aldosteronism. He has authored approximately 500 peer–reviewed articles and edited several books and proceedings.

Jorma Toppari, MD, PhD, is Professor of Physiology at the University of Turku and Chief Physician of Pediatric Endocrinology at Turku University Hospital, Turku, Finland. He is also Adjunct Professor in the Department of Growth and Reproduction at the University of Copenhagen, Denmark. He has served as chief editor of International Journal of Andrology (2001–09), and has been on editorial boards of several endocrinological journals, including currently *Endocrinology* and *Journal of Clinical Endocrinology and Metabolism*. He is the past President of the European Academy of Andrology. He has made numerous contributions to the studies on endocrine disruption in the past 20 years. He has published approximately 400 articles on endocrinology.

Jacquetta Trasler is a James McGill Professor in the Departments of Pediatrics, Human Genetics, and Pharmacology and Therapeutics at McGill University and a Senior Scientist at the Research Institute of the McGill University Health Centre (RI-MUHC). She received her MD and PhD degrees from McGill University followed by postdoctoral training in reproductive molecular biology at Tufts University in Boston. She has served as Director of the McGill University MD-PhD Program, Scientific Director of the Montreal Children's Hospital Research Institute (and simultaneously as Deputy Director/CSO of the RI-MUHC), President of the Canadian Fertility and Andrology Society, Member of the Institute Advisory Board for the Canadian Institutes of Health Research (CIHR) Institute of Genetics and currently serves on the CIHR Stem Cell Oversight Committee and College of Reviewers. Her research focuses on epigenetics and epigenomics to better understand the molecular and cellular targets for drug effects on germ cells with implications for the resulting offspring. She has been involved in scientific program organization for numerous meetings in the field of reproductive biology and medicine and is collaborating with national and international colleagues in clinical studies to examine how assisted reproductive technologies, infertility, drug treatment, and folate deficiency and supplementation impact the human epigenome including that of future generations.

Christina Wang, MD is Professor of Medicine, Assistant Dean at the David Geffen School of Medicine at UCLA, and Associate Director for Clinical and Translational Science Institute and a faculty member of the Division of Endocrinology, Department of Medicine, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, California.

Dr. Wang has been involved in many funded basic and clinical research studies. Her current clinical research studies include androgen replacement therapy, hormonal male contraceptive development, late onset hypogonadism, accurate assessment of serum androgens, and diet and androgen metabolism. Her basic research studies focus on the regulation of spermatogenesis and mitochondrial derived peptides in spermatogenesis.

She has authored over 300 peer-reviewed publications, 67 chapters and reviews mainly on male reproductive biology including characterization of the pharmacokinetics and efficacy of androgens in men, trials of hormonal male contraceptive, regulation of germ cell apoptosis, and reproductive aging. Dr. Wang served on the Executive Council, several committees and was the President of the American Society of Andrology (2006–07). She also served the International Society of Andrology as Secretary (2001–05) and Chair of the Program Organizing Committee (2005–09). She was President of the International Society of Andrology (2009–13). She is a member of the Research Group on Methods for the Regulation of Male Fertility of the World Health Organization since 1984 and Chairperson (1991–2002).

She has mentored many physician and scientist and is an advocate of young investigators. Dr. Wang has been invited speaker and distinguished lecturer at many national and international endocrinology, reproductive endocrinology, and andrology conferences.

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PREFACE

The first Edition of the *Encyclopedia of Endocrine Diseases* was published in 2004. Because of the enormous development in the field it was found important to produce a completely revised and updated Second Edition of the Encyclopedia. The new Edition is a must-have one-stop reference covering every aspect of the physiological background, pathogenesis, clinical diagnostics, and therapeutic aspects of the wide array of endocrine and related metabolic diseases.

The functional balance of the body (homeostasis) is maintained by two regulatory circuits, i.e., the nervous and the endocrine systems. Among the most complex constructs in the body, the endocrine system comprises a group of glands that secrete hormones directly into the bloodstream, where they reach their specific receptors in other parts of the body, evoking specific intracellular signaling pathways leading to their biological effect. Many classically non-endocrine organs (e.g., the heart) have also turned out to have endocrine functions. The endocrine system maintains and regulates the body's homeostasis by using hormones to control metabolism, temperature, biological cycles, internal fluid volume, reproduction, growth, body composition, and development. The system is a marvel when functioning optimally, i.e., maintaining the body homeostasis. Unfortunately, there is a myriad of ways these processes, actions, and functions can go awry, resulting in various endocrine and metabolic diseases, which form the over-arching theme of the Encyclopedia.

The Encyclopedia is not meant as a primer on the subject of endocrinology, but instead intended to provide a comprehensive reference work on the extensive spectrum of diseases and disorders that can occur within the endocrine and metabolic system. The updated version of this groundbreaking encyclopedia is especially timely, as it covers the dramatic discoveries in the field of endocrinology and metabolism over the past 10 years, particularly with respect to novel diagnostic techniques and treatment approaches. In particular, there have been tremendous advancements in our understanding of the molecular basis of endocrine and metabolic diseases (mutations, epigenetics, signaling), as well as pathogenesis and therapy of the common forms of these diseases (e.g., diabetes, obesity, and endocrine malignancies).

The Encyclopedia offers a unique source of up-to-date information for the physicians and basic scientists working in the field. It is an essential resource for every clinician diagnosing and treating endocrine patients. The Encyclopedia also offers the prime source of information for students of medicine and science around the world, as well as basic research workers in academia, the pharma industry, and in other areas in need of information on endocrinology and metabolism. It also offers useful information for the lay public about normal and abnormal functions of hormones.

The Encyclopedia is intended to serve as a useful and comprehensive source of information spanning the many and varied aspects of the endocrine and metabolic system. The chapters have been written to be accessible to both clinical and nonclinical readers. The articles have been formatted in similar fashion and each is intended as a stand-alone presentation. Each article begins with a glossary list defining key terms that may be unfamiliar to the reader and are important for understanding the article. The body of the article begins with a brief introduction to the subject under discussion, bold headings lead the reader through the text, and figures and tables explain and illuminate most articles. The main text is followed by referenced citations to provide the reader with access to additional information on the topic, and cross-references lead the reader to related entries in the encyclopedia. The relatively short stand-alone articles have allowed us to recruit the best experts available for each topic.

Unlike the first Edition, where the articles were arranged in alphabetical order, the 2nd Edition is arranged in organ-based thematic order, where each organ-based group of diseases is presented as cluster of articles in the first four volumes. The fifth volume is a stand-alone compilation of all articles on pediatric endocrinology. The thematic organization gives the reader a better general view of the coverage of articles on a specific endocrine organ or disease type.

The Second Edition of the Encyclopedia builds of the first edition. Nevertheless, to bring a major reference work with such a broad scope from initial conception to final publication involved a great deal of planning and organization, together with the efforts of innumerable individuals. The authors of the first edition were invited to update their earlier texts. If this was not possible, the Section Editors invited another expert in the topic either to update the previous text or to write a de novo text; the latter happened in most of these cases. Hence, the Second Edition contains to a large extent totally new information, or at least the fluency of all texts has been scrutinized. Furthermore, all manuscripts have undergone peer-review arranged by the Section Editors.

Assembling a large volume of articles with the purpose to cover all essential topics of endocrine diseases posed multiple challenges. Coverage was a significant problem: on one hand some redundancy of the topics was almost impossible to avoid in places while, on the other, there were inevitable gaps. Some of these arose from late cancellations; others from oversights on our part. We can only promise to fill these gaps in future editions. We also note that as can be expected for a large multi-author compilation the individual articles do differ in detail and approach. We considered it more important to allow our experts substantial latitude in deciding how to present their topics than to apply rigid guidelines.

Most of the editing work of the Encyclopedia has been carried out by a highly competent board of 16 Section Editors, each of them internationally renowned experts in their respective field within clinical endocrinology. First, the broadest possible list of topics was compiled, aiming at the best possible coverage. Throughout the editorial process, the Section Editors supervised their subject area of expertise, recommended and corresponded with fellow editors and article contributors, reviewed the manuscripts, and continuously helped to refine the final list of topics. This has made the task of the Editor in Chief easy, mainly entailing the supervision of smooth progress of the project.

The Section Editors and their fields deserve being listed here: *Jean-Jacques Body* (Belgium, bone endocrinology), *Felipe F. Casanueva* (Spain, metabolism and obesity), *Richard N. Clayton* (United Kingdom, pituitary gland), *Jean-Louis Chiasson* (Canada, diabetes), *Sophie Christin-Maitre* (France, female reproduction), *Wouter W. de Herder* (The Netherlands, neuroendocrinology), *Ulla Feldt-Rasmussen* (Denmark, thyroid gland), *Ieuan Hughes* (United Kingdom, pediatric endocrinology), *Gregory Kaltsas*, Greece, and *Martin O. Weickert*, United Kingdom, (gastrointestinal hormones), *Jean-Marc Kaufman* (Belgium, endocrinology of aging), *André Lacroix* (Canada, adrenal cortex), *Franco Mantero* (Italy, adrenal medulla and endocrine hypertension), *Jorma Toppari* (Finland, endocrine disruptors), *Jacquetta Trasler* (Canada, endocrine epigenetics) and *Christina Wang* (United Kingdom, male reproduction).

The Elsevier editorial staff, *Will Smaldon*, *Laura Escalante Santos*, and *Kate Miklaszewska-Gorczyca*, have been of enormous help to the editors at every step during this long project. I admire the professionalism of everyone and am deeply indebted to all for their dedication and hard work to make the Encyclopedia the leading reference book of clinical endocrinology.

The authors of the individual chapters, more than 450 in total, were specifically selected by the Section Editors to represent the best available knowledge on the topic available. They all should be thanked for their dedication and the excellent quality of their contributions.

Ilpo T. Huhtaniemi
Editor in Chief

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INTRODUCTION TO VOLUME 5

Pediatric Endocrinology transcends the whole of endocrinology as it relates to infants, children, and adolescents. Hence the decision to devote a standalone volume to the subject in this second edition of the Encyclopedia. In the first edition, topics such as congenital hypothyroidism, delayed puberty, and Turner syndrome were scattered throughout the encyclopedia based on using an alphabetical system. Now there is logic in collating topics uniquely specific to pediatrics together with those of an endocrine organ nature where the adult counterpart covered in addition in relevant sections of the other four volumes.

Growth, puberty, and disorders of sex development are completely within the purview of the pediatric endocrinologist and are covered in detail in this volume. Thereafter, chapters are more organ-specific (thyroid, adrenal, pituitary) and coupled with reference to further relevant information in the cognate adult chapter. There are also a number of chapters describing endocrine-related syndromes. A few topics are missing, such as rarer adrenal enzyme deficiencies but in particular, topics rela
ted to pediatric diabetes. Some aspects are covered in the adult diabetes section, but this omission will be rectified in due course by posting some chapters on line using the Reference Module system.

I acknowledge the support of the Editor in Chief, Ilpo Huhtaniemi, for allowing me the freedom to compile this volume, the authors for their excellent contributions, my fellow Section Editors for their ready collaboration, and the aforementioned Elsevier staff for their guidance and patience.

Ieuan Hughes

Control and Monitoring of Fetal Growth

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Introduction

A favorable in utero environment facilitating adequate fetal growth is critical, not only for fetal and neonatal wellbeing, but also as a determinant of long term health. In the absence of other reliable measures, assessment of fetal growth has been the mainstay of fetal monitoring for over 100 years. Alfred Lewis Galabin, in his 1886 "Manual of midwifery," published charts of symphysio-fundal height (SFH) measurement, a measure that is still used to this day as a screening tool to identify the growth restricted fetus.

Over the last 40 years, ultrasound assessment has been established as the gold standard technique for monitoring fetal growth. Initially, assessment of fetal growth consisted of fetal biometry and calculation of an estimated fetal weight (via any one of a number of accepted formulae), with a comparison of these parameters to published longitudinal nomograms. The dilemma, that in part remains to this day, is distinction of the pathologically growth restricted fetus, failing to meet its genetic growth potential, from the physiologically small fetus. This distinction was made somewhat easier in 1984, when Schuman et al. observed that fetuses that were small for gestational age had significantly higher umbilical artery resistance indices (Schulman *et al.*, 1984), and Doppler ultrasound assessment of fetal blood flow is now a key component of any assessment of fetal growth.

Fetal Doppler has become ubiquitous as a monitoring tool in fetal growth restriction, with abnormalities in flow velocity waveforms from a number of fetal vessels being employed as triggers for delivery in the context of the premature and growth restricted fetus. However, even small fetuses with normal Doppler indices are at increased risk of requiring operative delivery during labour, when compared to appropriately grown fetuses (Danielian *et al.*, 1992). Furthermore, within a cohort of appropriately grown fetuses, mean birthweights and birthweight centiles are significantly lower in babies that develop signs of compromise in labour (Prior *et al.*, 2013), suggesting evaluation of fetal weight remains an important indicator of fetal wellbeing.

Control of Fetal Growth

Fetal growth is influenced by genetic, environmental and hormonal influences. It is beyond the scope of this article to describe all of these influences in detail, and instead will briefly discuss endocrine controls.

Perhaps the most important endocrine regulator of fetal growth is the IGF system (Gicquel and Le Bouc, 2006). This system acts through the interaction of two growth factors (IGF-I and IGF-II), the actions of which are influenced by up to six other binding proteins (IGFBP). While the IGF system does have endocrine effects, its actions are principally autocrine. Both IGF-1 and IGF-II play a role in fetal growth throughout gestation, however, IGF-I dominates in later gestation while IGF-II is the primary growth factor in the embryonic period. IGF-I concentrations correlate far more closely with fetal weight than do IFG-II (Fowden, 2003), which in turn has a greater influence on placental growth (Fowden and Forhead, 2009). The IGF system interacts with other growth influencing hormones, IGF-I production being increased by insulin (Fowden and Forhead, 2004), however, in contrast to the neonatal period, GH is not a strong facilitator of IGF action in utero. While IGF gene defects are rare in humans, the important role of the IGF system in determining fetal growth has been demonstrated in animal experiments. Baker et al. demonstrated in 1993 that mice fetuses lacking the IGF-I or IGF-II genes grow to around 60% of normal size, and the growth retarding effect is potentiated if both genes are lacking (Baker *et al.*, 1993).

The IGFs are mitogens and influence cell proliferation and differentiation, with the latter particularly prominent in late gestation with development of adult cell types. IGF-I appears to act to promote fetal growth through increased amino acid utilization and protein synthesis throughout the body. In contrast to IGF-II, IGF-I plasma levels are correlated with glucose and oxygen concentrations, leading to suggestions that IGF-I may act to ensure fetal growth responds appropriately to nutritional supply (Fowden and Forhead, 2009).

The other important endocrine controller of fetal growth is insulin. Metabolically insulin is an anabolic hormone, and its influence on fetal growth is clearly evidenced by the increased fetal growth observed in fetuses of diabetic mothers. Deficiency of insulin, as would be expected, results in fetal growth restriction (Fowden *et al.*, 1989). Insulin acts by increasing cellular uptake and utilization of glucose and amino acids. In doing so, insulin also reduces circulating concentrations of these metabolites and facilitates transfer across the placenta via an increased concentration gradient (Fowden and Hay, 1988).

Pathogenesis of Fetal Growth Restriction

Placental Etiology

For some time, the widely accepted model of placental dysfunction precipitating both fetal growth restriction, and the frequently associated pre-eclampsia, has centered around a failure of trophoblastic invasion (Burton *et al.*, 2009). In this model, failed trophoblastic invasion prevents adequate transformation of the uterine spiral arteries, and as a result, the placenta retains a high resistance circulation. This in turn precipitates release of mediators into the maternal circulation and subsequent endothelial dysfunction (Borzychowski *et al.*, 2006). While this hypothesis neatly explains the pathogenesis of fetal growth restriction, sub optimal trophoblastic invasion is also seen in 40% of normal pregnancies (Aardema *et al.*, 2001) and pre-eclampsia may also occur in the absence of fetal growth problems, suggesting other factors also play a role.

Maternal Cardiovascular Function and FGR

While early onset pre-eclampsia is frequently associated with fetal growth restriction, a growing body of evidence suggests that the late onset variant of the disease (after 34 weeks gestation and less often associated with growth restriction) is likely to have a different etiology. These two pre-eclampsia phenotypes may be distinguished both by placental pathology, but also by maternal cardiovascular parameters, leading to suggestions that maternal haemodynamics may be instrumental in the development of fetal growth restriction. Indeed, it has been shown that pregnancies affected by fetal growth restriction demonstrate a failure of maternal cardiovascular adaptations to pregnancy, with a significantly lower increase in cardiac output in the first trimester (Duvekot *et al.*, 1995). Since this observation, a litany of research has suggested that fetal growth restriction is associated with a particular maternal cardiovascular phenotype, that of a high peripheral resistance and low cardiac output state (Valensise *et al.*, 2008).

If the maternal cardiovascular system is instrumental in the development of fetal growth restriction, it raises the tantalizing prospect of not only better identification of pregnancies at risk of growth restriction, but also that interventions or therapies designed to alter this haemodynamic phenotype may be beneficial in pregnancies at risk of fetal growth restriction.

Genetic Factors

In contrast to fetal growth restriction, where a fetus fails to reach its genetic growth potential, often due to placental dysfunction, some fetuses have a reduced potential growth due to intrinsic genetic factors. The genetic influences on fetal growth are of course numerous and diverse, but the following examples give some indication of how genetic and epigenetic changes may influence fetal growth potential.

Russel Silver syndrome is characterized by both intrauterine and postnatal growth restriction. This phenotype is suspected to occur due to increased expression of maternal genes via a number of genetic pathways. Maternal uniparental disomy of chromosome 7 accounts for around 10% of cases, while imprinting errors on chromosome 11 account for up to 60% of cases. In general, maternally expressed genes inhibit growth while paternally expressed ones enhance growth. Chromosome 11 contains a group of paternally expressed genes including IGF-II. Imprinting errors may therefore result in under expression of IGF-II resulting in reduced in utero growth (Mascarenhas and Ayyar, 2012). Consequently, the growth retardation of Russel Silver syndrome can be significantly ameliorated with postnatal growth hormone therapy.

Triploidy is a chromosomal abnormality in which the fetus has 69 pairs of chromosomes and three sex chromosomes. Two distinct genotypes of triploidy have been reported, diandric where the fetus has an extra set of paternally derived chromosomes, and digynic where the fetus possesses an extra set of maternally derived chromosomes. These genotypes give rise to two distinct phenotypes, the digynic one being associated with marked asymmetrical growth restriction and relative macrocephaly, potentially due to over expression of maternal genes that impair fetal growth.

The most common chromosomal abnormalities are the trisomies, particularly trisomy 13, 18, and 21. While fetal growth restriction may be present in all three but is particularly prominent in trisomy 18. The precise etiology behind this is poorly understood, but it has been suggested that cell cycle times in trisomic fetuses may be prolonged, with a subsequent impact on fetal growth (Paton *et al.*, 1974).

Placental Morphology

As the organ facilitating fetal nutrition and gaseous exchange, the placenta has a fundamental role in fetal growth. It may therefore seem reasonable that examination of the placenta would yield valuable information. However, the value of morphological assessment of the placenta continues to be disputed. What is clear, is that as the placenta ages, there is increasing calcification within it, and it is premature appearance of this calcification that has in some studies been linked to a restriction in fetal growth (Patterson *et al.*, 1983).

Placental aging is most frequently assessed via the Grannum classification system, which grades placental maturity from 1 to 3 based on a subjective assessment of the degree of calcification and of indentation of the chorionic plate. However, much of the published data examining the relationship between placental aging and fetal growth has been limited by a lack of distinction between isolated placental calcification, and calcification associated with other causative factors of fetal growth restriction, such as cigarette smoking. Also, many studies of placental morphology were conducted some time ago, using ultrasound technology that has since been superseded. A more recent study conducted in 2010 did suggest that isolated premature placental calcification was indeed associated with a number of adverse neonatal outcomes, including low birth weight, but only when calcification appeared prior to 32 weeks gestation (Chen *et al.*, 2011). This supports the assertion that placental calcification is predominantly a physiological aging process in the placenta, rather than pathological, and the value of placental grading in clinical practice remains limited.

As well as examination of placental aging, a number of morphological sub-types of placenta have been described.

A *bilobed placenta* describes a placenta that is separated into two equally sized lobes. If one of the lobes is significantly smaller, this is termed a *succenturiate lobe*. Neither a bilobed or succenturiate lobe placenta has any consequence for fetal growth.

A circumvallate placenta describes a placenta in which the chorionic plate is small and as a consequence the edges of the placenta have a "folded in" appearance, with detachment from the myometrium and doubling back toward the centre of the placenta.

A velamentous cord insertion is when the umbilical cord inserts into the chorion-amniotic membrane, beyond the margin of the placenta. The vessels from the cord then travel within the membrane to the placenta. This affects 1% of singleton pregnancies but as many as 10% of twin pregnancies. Both velamentous cord insertion and circumvallate placenta have been associated with an increased risk of fetal growth restriction and therefore warrant increased surveillance of fetal growth.

Monitoring Fetal Growth

In an ideal world, the pathologically growth restricted fetus would be identified by comparing its growth to a known target, or genetic growth potential. However, determining an individual fetus' genetic growth potential is challenging, and therefore a surrogate marker for pathological growth must be utilized. Numerous definitions exist for fetal growth restriction, but the most common used clinically are as follows (Unterscheider *et al.*, 2013; Lees *et al.*, 2013; Gordijn *et al.*, 2016):

Small for gestational age—EFW <10th centile for gestation or AC <10th centile.

Fetal growth restriction—EFW <10th centile for gestation or AC <10th centile, AND Umbilical artery PI >95th centile.

At present, no effective treatment has been demonstrated to improve fetal growth or neonatal outcomes in cases of fetal growth restriction. With acknowledgement that growth restricted fetuses are exposed to a sub-optimal in utero environment, associated with chronic fetal hypoxia and increased risk of fetal death, but also that prematurity poses huge challenges to the newborn, timing of delivery is of paramount importance to achieve optimal neonatal outcomes. Careful monitoring of the growth restricted fetus is therefore critical to ensure delivery occurs before the risks of continued conservative management outweigh the benefits.

Fetal Biometry

Assessment of fetal biometry is the most basic element of fetal growth surveillance. Generally speaking, fetal biometry refers to the assessment of fetal growth via measurement of four different parameters; the bi-parietal diameter, the head circumference, the abdominal circumference, and the femur length. A number of formulae exist to use these parameters to generate a fifth, the estimated fetal weight. A rigorous approach to ultrasound assessment is essential in order to assess fetal biometry accurately, and minimize intra and inter-observer variability. Standardized international guidelines are published by the International society for ultrasound in obstetrics and gynecology in order to facilitate this (Salomon *et al.*, 2011).

It is crucial that any assessment of fetal growth is undertaken in the context of an accurately dated pregnancy. If the pregnancy has not been accurately dated, small for gestational age fetuses may be incorrectly assumed to be appropriately grown. Fortunately, in most developed healthcare settings, accurate pregnancy dating is achieved via assessment of the crown-rump-length (CRL) in the 1st trimester. CRL is considered the most accurate method of dating a pregnancy up to 84 mm/13 + 6 weeks, following which head circumference (HC) is preferred. If not dated via CRL measurement, two serial scans should be performed in order to assess growth velocity and ensure that a growth restricted fetus is not overlooked.

As well as assessment of fetal size at one point in time, the pattern of fetal growth may also be indicative of pathology. Fetal growth velocity refers to the change in fetal growth over a period of time, and may identify the fetus with pathological growth that has already achieved an appropriate weight. There is no international consensus as to what constitutes a pathological growth velocity, however, persistent deviation from an established growth centile should prompt concern. It should be noted that assessment of fetal growth velocity is particularly sensitive to intra and inter-observer error, and this is amplified if assessments are performed with short time intervals (Chang *et al.*, 1993). A number of published longitudinal nomograms exist for fetal biometry, however, there is considerable controversy as to whether such nomograms should be standardized or customized according to individual parameters such as maternal ethnicity, height etc. Two landmark and conflicting studies

have attempted to address this issue, but their conflicting results mean staunch proponents of each method remain. The intergrowth study, published in 2016, examined fetal weights in women with optimal health and antenatal care, in differing regions around the world. This study demonstrated no significant difference in fetal weights across the regions. Those who advocate against the use of customized charts argue that they risk "controlling out" pathology, and that the differences in fetal weight observed in different populations is manifest of differing rates of fetal growth restriction, not differing physiological fetal growth. These findings conflict with those of a large World Health Organization funded study, which did find growth disparity between different regions (Kiserud *et al.*, 2017).

Fetal Doppler

Doppler ultrasound utilizes the effect described by and named after Austrian physicist Christian Doppler in 1842, which describes the change in frequency between a transmitted electromagnetic wave and its reflection.

In 1977, Fitzgerald and Drumm reported a novel non-invasive technique, Doppler ultrasound, used to image the umbilical vein and umbilical artery (FitzGerald and Drumm, 1977). Until this time, assessment of fetal size had been the predominant method for assessing fetal wellbeing. By utilizing the Doppler effect, Doppler ultrasound allows assessment of flow velocities in fetal vessels in real time, and evaluation of blood flow velocity changes throughout the cardiac cycle. In 1984, Schulman et al. observed that small for gestational age fetuses had increased resistance to flow in the umbilical artery and suggested a potential role for umbilical artery Doppler assessment in clinical practice (Schulman *et al.*, 1984). Since then, numerous parameters, derived from the flow velocity waveform elicited using Doppler ultrasound, have been described but the pulsatility index is perhaps the most commonplace in contemporary clinical practice. The pulsatility index is a useful measure as it is considered independent of the angle of insonation of the Doppler waveform (the disparity between the direction of blood flow in the target vessel and that of the ultrasound beam). If this angle is >30 degree, the accuracy of blood flow velocity measurement via Doppler ultrasound is significantly compromised.

The pulsatility index is a measure of the variance of blood flow velocity within the vessel throughout the cardiac cycle and is calculated as follows:

$$\text{Pulsatility index (PI)} = \frac{\text{Peak systolic velocity} - \text{Trough diastolic velocity}}{\text{Mean blood flow velocity}}$$

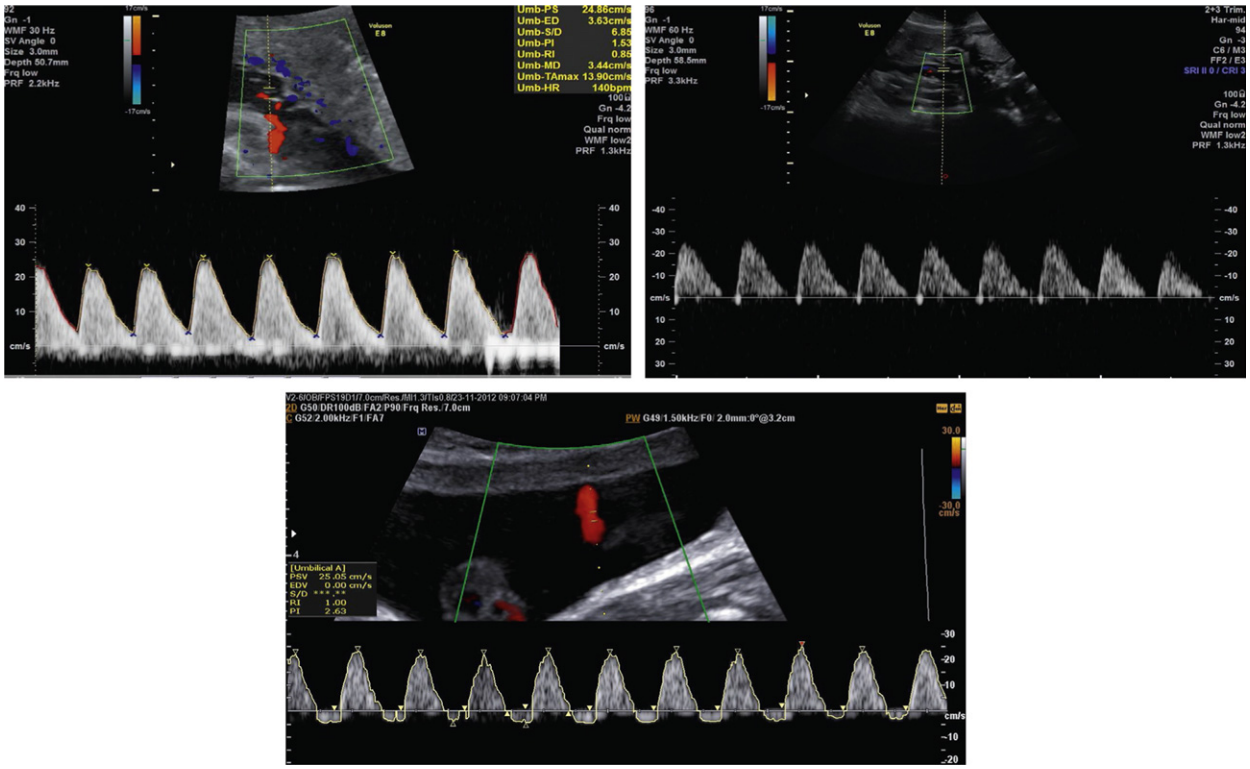

Fig. 1 Progression from raised umbilical artery PI to absent and then reversed EDF.

Doppler assessment of the umbilical artery, middle cerebral artery, and the ductus venosus have established roles in fetal monitoring. Assessment of these vessels can facilitate optimal timing of delivery in the context of fetal growth restriction.

Umbilical Artery

In a structurally normal fetus, two umbilical arteries carry blood from the fetus to the placenta for gaseous and nutrient exchange. Resistance to blood flow in the umbilical arteries is thus descriptive of resistance within the placenta.

Doppler assessment of the umbilical artery has been reported at a number of locations, including the placental cord insertion, paravesical, or more frequently at a free loop. The location of sampling is important to maximize reproducibility, as the resistance falls along the length of the cord, being lowest at the placental end and highest at the fetal end. Acquisition of flow velocity waveforms using Doppler ultrasound should take place in the absence of fetal movements or breathing movements, and ideally from a free loop of cord with the umbilical artery running parallel to the ultrasound beam, and certainly with an angle of insonation <30 degree. At least three consecutive waveforms should be recorded and the average pulsatility index calculated.

A progressive pattern of abnormal flow in the umbilical artery has been observed in growth restricted fetuses (see **Fig. 1**). Initially a raised pulsatility index is observed due to reduced diastolic blood flow associated with increasing resistance to flow in the vessel. If resistance increases sufficiently, diastolic flow ceases all together, a finding termed "absent end diastolic flow (AEDF)." Further progression may result in reversal of blood flow in the umbilical arteries during diastole (reversed EDF). By monitoring umbilical artery Doppler over serial ultrasounds, progression along this pathological pathway can be identified, and used to facilitate timely delivery of the fetus. The use of umbilical artery Doppler has been demonstrated to reduce the incidence of perinatal death (Alfirevic *et al.*, 2010) and to predict fetal/neonatal compromise (Morris *et al.*, 2011). Critically, undiagnosed SGA babies are four times more likely to die than those monitored with umbilical artery Doppler (Lindqvist and Molin, 2005). In general, if the umbilical artery has positive diastolic flow, it is reasonable to continue to manage the pregnancy conservatively until 36 weeks gestation. If there is absent or reversed diastolic flow, delivery is indicated after 32 weeks and should be considered between 30 and 32 weeks. Prior to 30 weeks, timing of delivery is based on assessment of the ductus venosus, which will be discussed later in this article.

The value of umbilical artery Doppler assessment in the appropriately grown fetus is less clear. Conventional thinking has considered umbilical artery Doppler of little use when fetal growth is appropriate for gestational age. However, over the last 10 years, a number of published studies have contributed to a growing consensus that umbilical artery Doppler may be descriptive of placental function, and thus fetal wellbeing, even in the context of an appropriately grown fetus. This suggestion is not new, with authors as far back as 1991 reporting higher rates of Caesarean delivery for fetal compromise, within a low risk cohort, in babies with abnormal umbilical artery waveforms (Arduini *et al.*, 1991). More recently, increased resistance in the umbilical artery, particularly when combined with reduced resistance in the middle cerebral artery, has been associated with fetal compromise in labour (Prior *et al.*, 2013). However, whether umbilical artery Doppler should be used as part of routine fetal monitoring remains contentious, with a systematic review in 2010 demonstrating no benefit in an unselected low risk population (Alfirevic *et al.*, 2010). Furthermore, there are concerns relating to the potential for false positives and associated increased rates of intervention. Such an approach to fetal monitoring would also have significant health economic implications.

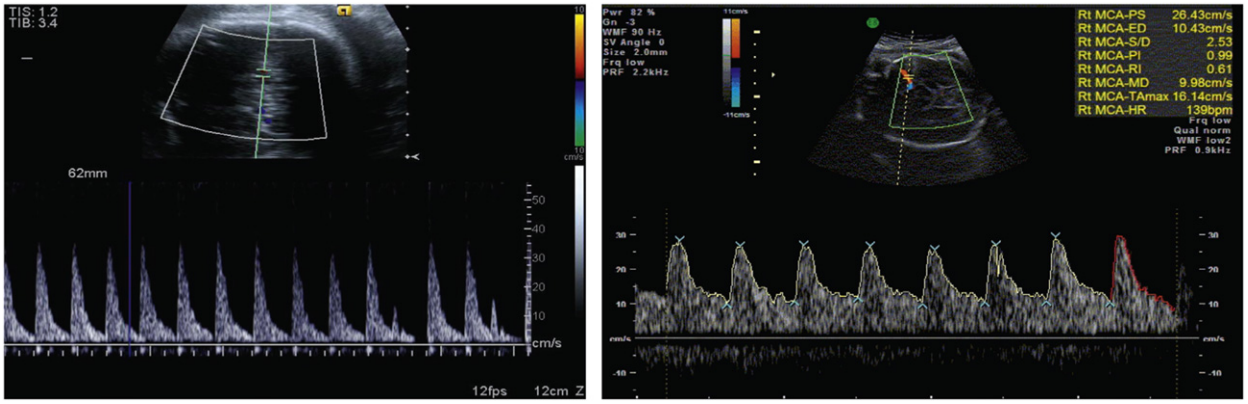

Fig. 2 MCA Doppler from a normal fetus, showing little end diastolic flow, and a growth restricted fetus, with cerebral redistribution demonstrated by increased end diastolic flow.

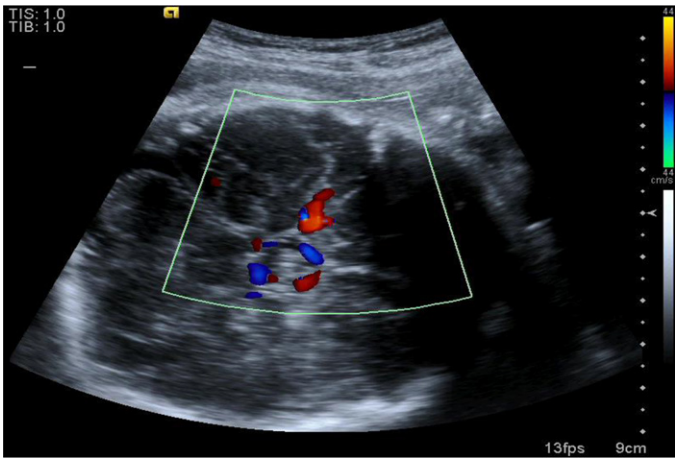

Fig. 3 The middle cerebral artery imaged using color Doppler.

Middle Cerebral Artery

In 1991, Thornburg et al. reported that in response to stress, fetuses redistribute blood flow preferentially toward the brain, cardiac muscle, and adrenal glands, at the expense of other organs considered less "vital" (Thornburg, 1991). What was later termed 'brain sparing' was initially demonstrated in growth restricted fetuses by Doppler assessment of the internal carotid artery. However, the middle cerebral artery became established as the vessel of choice for assessment of cerebral perfusion due to its ease of access and favorable anatomical path, which lends itself to an angle of insonation close to 0 degree and thus acquisition of accurate flow velocity waveforms. Despite this, careful acquisition remains critical as Doppler parameters are influenced both by the segment of the middle cerebral artery sampled, but also the pressured applied to the fetal head using the ultrasound probe.

Initial studies examining the use of middle cerebral artery Doppler in the assessment of fetal growth were conflicting, with some suggesting resistance was reduced in cases of fetal growth restriction (Ishimatsu *et al.*, 1989), while others found no change (Veille and Cohen, 1990). Further investigation however confirmed that while evidence of brain sparing was not present in all growth restricted fetuses, its presence was associated with an increased risk of adverse neonatal outcome (Arduini and Rizzo, 1992) (**Fig. 2**).

The middle cerebral artery branches from the circle of Willis and runs laterally just superior to the wing of the sphenoid bone. It is imaged by taking a transthalamic plane of the fetal head and using color Doppler to identify the circle of Willis (**Fig. 3**).

Ductus Venosus

The ductus venosus completes the triad of fetal vessels used in monitoring fetal growth in high risk cases. The ductus venosus branches from the left umbilical vein and acts as a shunt allowing freshly oxygenated blood to bypass the fetal liver and flow directly into inferior vena cava. Around 50% of blood takes this path, with the proportion decreasing with advancing gestation. In physiological pregnancy the ductus venosus has a triphasic waveform, incorporating two velocity peaks (ventricular systole and diastole), followed by a reduction in velocity with atrial systole.

The ductus venosus can be imaged either in a mid-sagittal plane, or an oblique transverse plane, and is identified as a branching vessel from the umbilical vein with turbulent flow on application of color Doppler.

With severe placental dysfunction, there is preferential redistribution of blood to the fetal brain and myocardium, achieved in part by reducing resistance to flow in the cerebral circulation, but also via increased resistance to flow in the peripheral vasculature. This increases afterload on the right ventricle, which if progressive can result in tricuspid regurgitation and the concomitant changes observed in the ductus venosus waveform. These changes are particularly treacherous for the fetus, as they further reduce the flow of oxygenated blood from the umbilical vein to an already compromised myocardium (Kaponis *et al.*, 2011). Progressive changes in the ductus venosus can be observed in growth restricted fetuses, typically commencing with a reduction in blood flow during atrial systole and a correspondent increase in the pulsatility index. The presence of absent or reversed flow in the ductus venosus during atrial systole (absent/reversed a-wave) is associated with poor perinatal outcomes and implies failure of fetal compensatory mechanisms to preferentially supply vital organs with well oxygenated blood (Baschat *et al.*, 2003). Absent or reversed a-wave in the ductus venosus thus identifies fetuses at the highest risk of in utero demise.

The Cardiotocograph

The fetal cardiotocograph (CTG) is most frequently used for fetal monitoring in the intra-partum period. However, its use in the antenatal period is also commonplace, particularly in growth restricted fetuses, where abnormalities of fetal heart rate rhythm may prompt delivery. CTG may be interpreted by a trained observer, or using computerized analysis—computerized CTG (cCTG).

When interpreted by an observer, the CTG is analyzed according to its five constituent parts, before these are combined to give an overall assessment. While this form of CTG is commonplace, it has been criticized for a high inter-observer variability rate. Computerized CTG aims to eliminate this, by providing a computerized assessment of the fetal heart rate rhythm according to preset criteria.

The five criteria analyzed during CTG interpretation are as follows:

Baseline rate—This describes the fetuses normal heart rate and should be anywhere between 110 and 160 bpm.

Variability—Variability is a description of the variation in fetal heart rate. A normal fetal heart rate has a variability of 5–25 bpm and is descriptive of the interplay between the sympathetic and parasympathetic nervous system. A reduction in variability is thus an ominous sign.

Accelerations—These are short periods of elevation of the fetal heart arte above the baseline rate by >15 bpm for >15 s. Accelerations are frequently associated with fetal movement and are thus indicative of a functioning somatic nervous system and are reassuring.

Deceleration—These are short periods of reduction in the fetal heart rate, below the baseline rate by 15 bpm for 15 s. There are numerous different types of decelerations observed during labour, and not all are pathological, however decelerations occurring in the antenatal period in the absence of precipitating uterine contractions are nearly always a sign of fetal compromise.

Short term variation—Short term variation (STV) examines the variability of the fetal heart rate from beat to beat, and cannot be interpreted by observer visual analysis. This parameter is only available with computerized CTG. A reduced STV (<3.0 msec) has been reported to correlate with stillbirth and severe fetal acidaemia (Dawes *et al.*, 1991).

Timing Delivery of the Growth Restricted Fetus

In the UK, the RCOG has issued national guidelines to facilitate optimal timing of delivery of growth restricted fetuses, to maximize the benefits of conservative management with the concomitant reduction in the complications of prematurity, but also attempting to avoid in utero demise (Royal College of Obstetricians and Gynaecologists, 2013) (RCOG green top Small for Gestational age). Until a gestation of 30 weeks, delivery, particularly of the growth restricted fetus, may be associated with significant risk of cerebral palsy as well as other adverse neurodevelopmental outcomes. There are also other significant complications of prematurity such as respiratory and gastrointestinal sequelae. As such, delivery at these gestations is only indicated in cases where fetal compensatory mechanisms are failing and the risk of in utero demise is considered high with continued conservative management. At gestations <30 weeks, it is therefore reasonable to continue with conservative management as long as the ductus venosus a-wave remains positive. Between 30 and 32 weeks, delivery should be considered in the context of absent or reversed end diastolic flow in the umbilical artery. After 34 weeks, the risks of prematurity are lower, though not inconsequential, and thus the delivery may be indicated in the context of a growth restricted fetus with a raised umbilical artery PI.

While the above recommendations are followed in many obstetric units, evidence for the best triggers for delivery, particularly in the context of early onset growth restriction, is limited. Most studies have reported only short term neonatal outcomes limiting their value. One randomized trial, the GRIT study (GRIT Study Group, 2003), allocated patients with early onset growth restriction to either early or delayed delivery, and reported no significant difference in outcomes for either group. More recently, the TRUFFLE group (Lees *et al.*, 2013) have tried to address this question by randomizing women with early onset growth restriction to delivery based on either reduced computerized CTG short term variation, early Doppler changes in in the ductus venosus (raised pulsatility index), or late Doppler changes in the ductus venosus (absent or reversed a-wave). The authors reported that delivery based on late Doppler changes in the ductus venosus may be associated with an improvement in developmental outcomes at 2 years of age, however, this may be associated with an increased rate of antenatal fetal death. The study also demonstrated that outcomes for survivors, even at gestations of 26–28 weeks gestation, were better than anticipated, with over 70% of survivors having normal outcomes in all three monitoring groups.

Summary

While there is now a good understanding of many of the mechanisms controlling fetal growth, interventions to improve fetal growth in cases of fetal growth restriction remain out of reach. In lieu of treatment, we must optimize the timing of delivery to maximize the benefits of conservative management whilst minimizing its risks. To this aim, clear national guidelines now exist to facilitate the use of fetal Doppler in a consistent manner, and optimize neonatal outcomes in cases of fetal growth restriction.

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Physiology of Growth Hormone in Fetus and Child

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Historical Perspective

Even before there was a written history, dwarfs appeared in the artwork of many cultures. It was in the 19th century, individuals with growth stunting were defined as "dwarfs" if disproportionate and as "midgets" if proportionate. In the early 20th century the term "ateliosis" was created by an English physician, Hastings Gilford, to describe patients with proportional short stature. He further subdivided ateliosis based on with and without sexual maturation.

Charles S. Stratton, was the most famous likely growth hormone (GH) deficient individual better known by his stage name "General Tom Thumb". He achieved great fame as a performer under circus pioneer and distant relative P T Barnum. Born (January 4, 1838) to parents of medium height who were first cousins, Charles was relatively large baby weighing 9 pounds and 8 ounces (4.3 kg) at birth. He grew steadily until 18 months of age; thereafter his height did not progress as expected and reached an adult height of 3 ft. 2 in. Charles was married to Lavinia Warren Bump, she as well a likely growth hormone (GH) deficient lady, who started her career as a school teacher and switched to circus performer after learning about Charles S Stratton's nationwide success. Lavinia was born (October 31, 1842) to normal parents, who were third cousin and she grew normally until the age of 1 year and then her growth was slowed down reaching an adult height of 2 ft. 8 in. Due to their proportionate short stature, normal birth weight and height, growth retardation starting in the first year of life, normal intelligence, and normal sexual development, it is assumed that both Stratton and Bump had autosomal recessive growth hormone deficiency.

Introduction

Growth in childhood is an overall indicator of health and development. Human linear growth can be divided into three phases—infancy, childhood, and puberty. The infancy phase is the continuation of fetal growth and it is nutritionally driven, childhood phase is mostly dependent on growth hormone (GH), whilst the pubertal phase depends on the combination GH and sex steroids (Tse *et al.*, 1989).

GH production begins in early fetal life and GH is secreted from the anterior pituitary gland in a pulsatile fashion. The levels of GH vary during childhood and peak during the pubertal growth spurt. The GH pulse frequency and the amplitude is influenced by a number of factors including age, gender, sleep, nutrition, exercise, and pubertal status. Apart from the linear growth, GH also play an important role in the intermediary metabolism of glucose, fats, and proteins.

Human Growth Hormone (GH) also known as somatotropin, is a 191-amino acid single—chain polypeptide that is synthesized, stored and secreted by somatotrophs of the anterior pituitary gland. In the fetus, growth hormone production begins as early as 7 weeks of gestation, reaches the maximum level at 20–24 weeks of gestation and continues thereafter throughout life, although at a progressively lower rate (Root, 1976). Growth hormone exerts its growth promoting and metabolic activities by interacting with a cell surface GH receptor (GHR). The majority of the growth promoting effects of GH are mediated via the production of IGF-1.

The Growth Hormone Gene

The human growth hormone (GH) gene family comprises of five distinct genes which are pituitary GH, *GH-N*; placental GH variant, *GH-V*, and the placenta expressed CS genes, *CS-A*, *CS-B*, *CS-L* all located on the long arm of chromosome 17 at position 23.3 (**Fig. 1**).

These five Growth Hormone (GH) genes are closely related, share 90%–99% nucleotide sequence similarity, and are believed to have evolved by gene duplication (Chen *et al.*, 1989). The pituitary GH gene encodes two alternatively spliced mRNA products and as a result two growth hormones are translated, the full length transcript 191 amino acid, 22-kDA protein that makes up

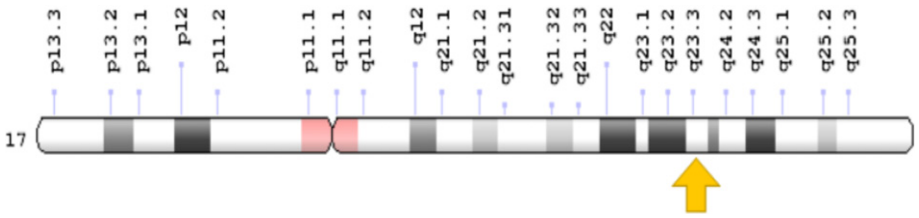

Fig. 1 The growth hormone gene.

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majority (80%–95%) of the circulating pituitary GH and the 20-kDA protein which lacks amino acids 32–46 and this account for the remaining 5%–15% of the circulating GH.

PIT1 is a pituitary-specific transcription factor which belongs to the POU family of transcription factors that regulate mammalian development. The binding of PIT1 to the specific HS 1/2 (Hypersensitive sites) of the human growth hormone locus control region (LCR) is an important step in the expression of *h*GH-N gene, and that defines the somatotroph cells. However the Pit1 protein also plays an important role in the expression of prolactin-producing lactotrophs and thyroid stimulating hormone producing thyrotroph (Yang *et al.*, 2010).

Deletion and splice site mutation of the GH gene results in various forms of isolated growth hormone deficiency (IGFD). *HESX1*, a member of the homeobox family of genes plays a crucial part in the early determination and differentiation of the pituitary gland. Heterozygous mutations of *HESX1 gene* have been identified in children with various forms of hypopituitarism and SOD. Mutations in *Prop-1 gene* and in the *POU1F1 (Pit-1) gene* also account for rare cases of GH deficiency that are usually accompanied with other pituitary hormone deficiencies (Dattani, 2005).

Growth Hormone Structure

The major structural feature of the GH molecule is the three-dimensional fold of a four-helical bundle protein with an unusual connectivity. Cytokines such as prolactin, erythropoietin and many interleukins share similar structural configuration. The NH2 and COOH-terminal helices (helices 1 and 4) are longer than the other two helices (helices 2 and 3). Crystal structure analyses indicate that these helices run in a unique up-up-down-down pattern, in contrast to the more usual pattern of up-down-up-down. A long crossover connection (consisting residues 35–71) links helix 1 and helix 2 and a similar connection (residues 129–154) is found between helices 3 and 4. Of the two disulphide bonds, one links the N-terminal and C-terminal regions (Cys 53-Cys 165) and the second disulphide bond is located at the C-terminus (Cys 182-Cys189) (de Vos *et al.*, 1992). The other important feature of the hGH is the two zinc ($Zn2+$) binding sites which is identified at residues His 44 and Glu 200. The binding of $Zn2+$ is critical for facilitating GH dimerization and aggregation in the secretory granules biogenesis of the anterior pituitary gland. This is the process through which growth hormone is stored and released readily into the circulation upon appropriate stimulation without necessitating de novo protein synthesis (Cunningham *et al.*, 1991) (**Fig. 2**).

Growth Hormone Receptor (GHR)

The growth hormone receptor GHR is a homo-dimeric single pass transmembrane receptor belonging to the cytokine/haemopoietin receptor superfamily. The receptor consist of an extracellular ligand binding domain (ectodomain), a single pass membrane-spanning domain (transmembrane domain) and a cytoplasmic signaling component (cytoplasmic domain) which recruits and activates JAK-2 and subsequent intracellular signaling cascades. For a long time it was understood that the GH induced the dimerization of monomeric GHR polypeptide and this was based on receptor dimerization model of Fuh et al. More recent studies suggests that the full length GHR dimerization occurs in the absence of ligand (GH) and subsequent binding of one molecule of GH induces GHR conformational and rotation changes that activate the GHR signaling cascades via JAK-2.

Extensive mutational and crystal structure analysis has revealed two essential binding sites within the GH protein which interact with each monomer of the dimeric GHR. Although the two binding sites on GH have no structural similarity, the residues on both the monomers that interact with these sites are largely the same. The ectodomain of the GHR can be proteolytically cleaved to circulate as a monomeric GH binding protein (GHBP). The GHBP binds the circulating GH in a 1:1 ratio and this association provides as a dynamic reservoir for bound and free GH (**Fig. 3**).

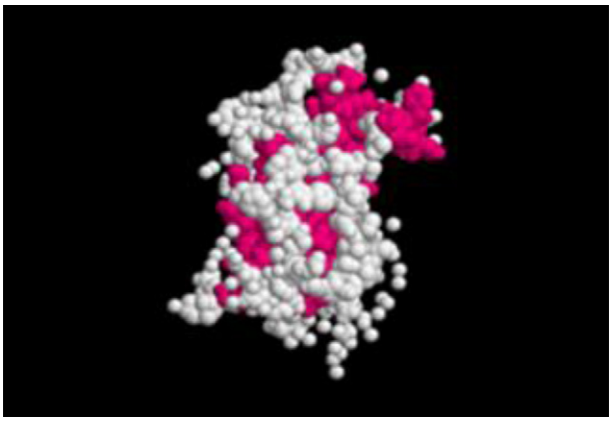

Fig. 2 Structure of GH (wikipedia).

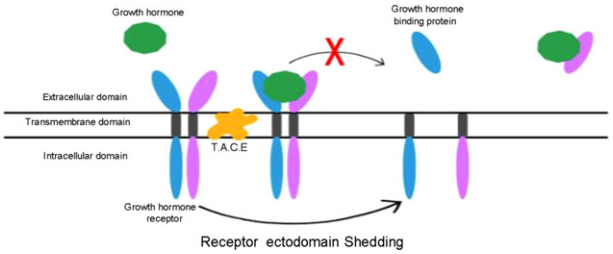

Fig. 3 Growth hormone receptor.

The Regulation of Growth Hormone Secretion

The secretion of growth hormone is pulsatile in nature rather continuous release in all species. GH secretion is controlled by many factors/influences that make it one of the more complex regulated hormones in the body. The hypothalamic neuropeptides, growth hormone-releasing hormone (GH-RH) and somatotropin release-inhibiting factor (SRIF, Somatostatin) secrete and inhibit growth hormone respectively. The hypothalamic stimulatory influence on GH secretion was first suggested by Reichlin and later by Frohman who demonstrated reduced plasma GH levels on destruction of the ventromedial hypothalamus and on electrical stimulation of the ventromedial and arcuate nuclei of hypothalamus caused acutely increased GH release in young rat models (Goldenberg and Barkan, 2007). Growth hormone is secreted relatively more during the early to mid-adolescence years, about 700 μg/day which is almost double the levels when measured in adulthood (approx. 400 μg/day). Because of the pulsatile pattern of GH secretion, the serum concentration of GH is quite variable and usually low when measured on healthy individuals (Burgess *et al.*, 2002).

Growth Hormone-Releasing Hormone (GHRH)

Growth hormone-releasing hormone, also known as GH-releasing factor (GRF) is a 44-amino acid peptide produced in the ventromedial and arcuate nuclei of the hypothalamus. The stimulatory hypothalamic neuropeptide, GHRH was first originally isolated from extrahypothalamic/extrapituitary (pancreatic) tumor cells of a Turner's syndrome patient with acromegaly (Thorner *et al.*, 1982). Studies in animal models and humans have unequivocally documented that GHRH as the primary GH pulse generator as well as exert a positive effect of somatotrope proliferation. Further studies have confirmed the direct relationship of GH pulsatile with increased concentration of GHRH in the portal blood (Burgess *et al.*, 2002). The GHRH receptor (GHRH-R) present on the anterior pituitary somatotroph cells belongs to the family of the G protein-coupled receptors. Upon stimulation by GHRH results in the production cyclic adenosine monophosphate and this leads to increased GH synthesis and secretion. GH secretion is greater in the childhood and adolescence age range than in adulthood. This age related attenuation of GH secretion (somatopause) is due to diminished GHRH secretion and perhaps the pituitary responsiveness to GHRH remains intact. At all ages, the effect of GHRH on GH secretion is partially blocked by the administration of somatostatin which inhibits GH secretion and controls the GH pulse frequency (Backeljauw and Hwa, 2016).

Ghrelin (S)

Ghrelin is a peptide hormone primarily expressed in gastric cells and ghrelin mRNA has also been identified in hypothalamus. It is 28 amino acid molecule and circulates in two forms: the active form octanoylated peptide and the inactive form deoctanoylated peptide. A number of synthetic analogues of ghrelin (GHS) have been discovered, capable of stimulating GH secretion. These peptides act on the receptors differently to GHRH. This distinct receptor is known as the GH secretagogue receptor and also belongs to the G protein-coupled receptor family (Smith *et al.*, 1999). Administration of GHS results in immediate release of GH but when administered along with the GHRH results in massive release of GH suggesting the synergistic effect of hypothalamic GHRH that is essential for their action (Bowers *et al.*, 1991). There is no increased level of ghrelin during fasting when there is increased GH secretion and no correlation of ghrelin levels to GH pulses (Avram *et al.*, 2005).

Somatostatin or Somatotropin Release-Inhibiting Factor (SRIF) (I)

Somatostatin, also called somatotropin release inhibiting factor, is a 14 amino acid molecule (tetradecapaptide) secreted from the neuroendocrine cells located in the ventromedial nucleus of the hypothalamus. Through the hypothalamo-portal network, somatostatin reaches the anterior pituitary gland, where it inhibits the secretion of GH from the somatotroph cells. At this level, it has a dominant effect over GHRH on GH release. It inhibits the basal as well as the stimulated GH secretion (amplitude of GH pulse) but not the GH pulse frequency and the diurnal rhythm of GH secretion (Dimaraki *et al.*, 2003). With the "shortfeedback" loops which operates at the hypothalamus level, somatostatin secretion is directly stimulated by GHRH and GHRH secretion is inhibited by somatostatin (Yamauchi *et al.*, 1991). At the same level, pituitary GH inhibits hypothalamic GHRH secretion and

stimulates somatostatin release from the hypothalamic neurons. This overall complex pathway plays an important role in the creation of pulsatile GHRH and somatostatin secretion pattern and ultimately the GH release from the pituitary in a pulsatile pattern (Chomczynski *et al.*, 1988; Chihara *et al.*, 1981). The classic negative feedback mechanism to GH secretion is mainly due to the increased serum concentration of GH and IGF-1 which stimulates the release of the somatostatin from the hypothalamic neurons. There are five subtypes of somatostatin receptors (SSTR-1—SSTR-5) of which four subtypes are expressed in somatotrophs. Somatostatin preferentially acts on SSTR-2 and SSTR-5 receptor to suppress the secretion of pituitary GH by inhibiting the intracellular adenylate cyclase activity and thereby reducing the intracellular calcium.

Other Regulators

Insulin-like growth factor-1 (IGF-1) which mediates most of the peripheral actions of GH inhibits the secretion of GH through the classic negative feedback mechanism. Although the exact mechanism is not known it acts both directly at the level of pituitary gland and indirectly at the level of hypothalamus to lower the GH concentration and the pulse amplitude (Ceda *et al.*, 1987).

Sex steroids (estrogen and androgen) cause increased secretion of GH during puberty with increased concentration of serum GH and IGF-1. Although the exact regulatory mechanism of GH by sex steroid is not known, it acts differently in both males and females.

Sleep appears to increase the GH secretion and this has been well documented in both animal and human studies.

Nutrition influences GH secretion and it varies between fasting and fed conditions. Hypoglycaemia causes acute rise in GH secretion that is dependent on both the severity and the rate of glucose fall. Insulin induced hypoglycaemia has been demonstrated to show acute rise in GH secretion and this has been long been used as part of assessment of growth hormone deficiency. Hyperglycaemia causes suppression of GH secretion and again this has been demonstrated in oral glucose tolerance test to assess the individual ability to suppress the GH secretion after administration of a fixed amount of glucose.

Growth Hormone in Fetal Life

Growth hormone has relatively little effect on fetal growth, as GH receptors are generally expressed at very low levels in the fetal tissues. Fetal growth is a complex, dynamic process which primarily depends upon fetal genetics, nutrient supply and various growth factors and hormones of maternal, fetal and placental origin.

The IGF system that comprises of two ligands (IGF-1, IGF-2), two receptors and six binding proteins (IGFBPs) plays the crucial role in the regulation of fetal and placental growth. IGF-1 stimulates and ensures fetal growth that is appropriate for the available nutrient supply, whilst IGF-2 plays a key role in the placental growth and nutrient transfer. Both IGF-1 and IGF-2 are expressed in fetal tissues as early as from pre-implantation to final phase of tissue maturation before birth. In fetus, the IGFs are thought to act in an autocrine fashion and the endocrine-systemic actions occur in the latter half of the gestation. Most studies have found no correlation between IGF-2 concentration and birth weight but there is strong association between IGF-1 concentration and birth weight, and this has been shown with decreased concentration of IGF-1 in utero and at birth with the fetuses displaying intrauterine growth retardation (Fowden, 2003).

Fetal GH is produced by the pituitary gland from the end of the first trimester (Kaplan *et al.*, 1972). In the placenta, the syncytiotrophoblast cells expresses the GH-V gene that encodes human placental growth hormone (PGH). Like GH, the protein sequence of PGH contains 191 amino-acid residues but differs by 13 amino-acid. The regulation of PGH is less understood, secreted in a rather non-pulsatile fashion and predominates in the third trimester of pregnancy when the GH levels are low. The serum PGH concentration during normal pregnancy vary, it is detected in the serum as early as at 5 weeks of gestation and reaches its peak value in week 34–37. After this gestational age, studies have shown slight decline in PGH levels (Chellakooty *et al.*, 2004) but other studies have shown no consistent changes in the level of PGH (Frankenne *et al.*, 1988; Wu *et al.*, 2003).

The serum PGH level start to decline immediately as the placenta, which is the source is removed from the uterus. Several studies have shown a positive correlation between serum PGH and IGF-1 levels both in cross-sectional and longitudinal studies. Thus the IGF-1 secreted in response to PGH ensures appropriate fetal growth (Caufriez *et al.*, 1990, 1993) (**Fig. 4**).

Insulin Like Growth Factor-1 (IGF-1)

It was in 1957, works from Salmon and Daughaday, showed that the actions of GH was mediated by a factor which they initially named as sulfation factor because they found that the uptake of organic phosphate by cartilage was not affected by addition of GH. Later they proposed the factor as somatomedin as it mediates the action of somatotropin (GH). The present name insulin like growth fator-1 (IGF-1) was designated to indicate the chemical structural similarity to insulin and it is the major mediator of growth hormone (GH) stimulated somatic growth, as well as GH-independent anabolic responses in many cells and tissues (Lindholm, 2006).

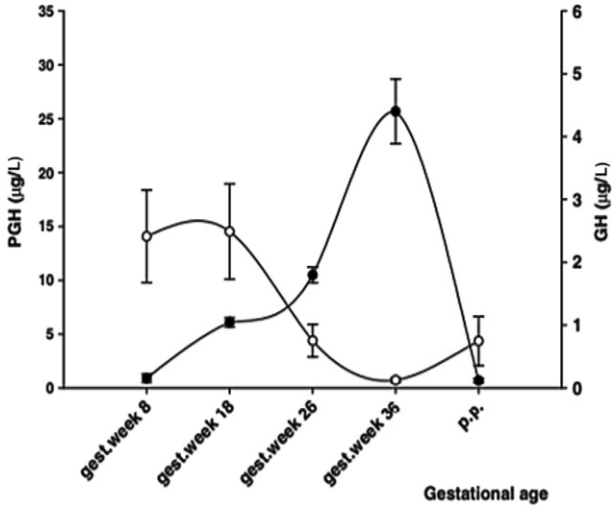

Fig. 4 Serum PGH (*filled circles*) and GH (*open circles*) during pregnancy in healthy subjects. Notice the different scalings on the *y*-axes for PGH and GH.

Synthesis and Structure of IGF-1

IGF-1 is synthesized by various mesenchymal cell types. The IGF-1 synthesized by the liver and secreted into the circulation is under the control of GH and this mediates the balanced growth among multiple organs and tissues. This accounts for around 75% of the circulating IGF-1. The remaining IGF-1 which is synthesized in an autocrine/paracrine way in the peripheral tissues, is also under the control of GH and by factors that are secreted locally in the surrounding tissues. This autocrine/paracrine IGF-1 is responsible for local, unbalanced growth independent of GH. Examples of this type of growth are bone remodeling, wound healing and growth of contralateral kidney after unilateral nephrectomy (Wang *et al.*, 2013).

The IGF-1, IGF-2 and the insulin gene all belong to the same family. All three peptides contain A, B, and C domains, but IGF-1 and IGF-2 are secreted with D domain extensions of six to eight amino acid that are not cleaved.

IGF-1 Receptor

The IGF-1 receptor is found in multiple cell types and tissues, and this account for the IGF-1 to mediate balanced growth. The presence IGF-1 receptor is regulated by GH and thyroxine (T4) and also other growth factors such as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF). The binding of IGF-1 to the alpha subunit of the IGF-1 receptor triggers a conformational change in the transmembrane beta subunit and a cascade of intracellular events followed by phosphorylation of tyrosine by tyrosine kinase activity. The final activation of mitogen-activated protein kinase pathway (MAP kinase) is important for stimulation of cell growth by IGF-1 (**Fig. 5**).

IGF- Binding Proteins (IGFBP)

There are six high affinity IGF-binding proteins in the plasma that bind IGF-1 and IGF-2 with different affinities. The effects of IGF-1 and IGF-2 are modulated by these six different IGFBPs, named IGFBP1—IGFBP6. Most of the IGFs (75%–80%) in the circulation form ternary complex containing IGF-1 or IGF-2, IGFBP3 and an acid labile subunit (ALS); the remaining 20%–25% of IGF is bound to one of the IGFBP forming a binary complex and less than 1% of IGF in plasma is in free form.

Of all the IGFBPs, the IGFBP3 is the most abundant form in the plasma and more than 75% of IGF-1 is bound to this protein. Exogenous administration of GH increases the concentration of IGFBP3 and thereby functions as the carrier of IGF-1 and IGF-2. In the plasma, the formation of ternary complex helps to stabilize IGF-1 binding and prolongs its half-life to 16 h.

IGFBP2 is the second most abundant form of IGFBPs in the plasma. It's affinity for IGF-1 is relatively less than that of IGFBP3 and has shorter half-life of 90 min. IGFBP2 has important function in that it regulates the available free IGF-1 in plasma to enter into the extravascular space and bind to the receptors in peripheral tissues.

IGFBP1 is regulated by insulin. IGFBP1 level fluctuates throughout the day, with fasting its level increases, which inhibits insulin secretion and the levels fall with feeding and insulin administration. It has also been found that the level of IGFBP1 increases progressively in individuals who develop type 2 diabetes and in those with insulin resistance (Kotronen *et al.*, 2008).

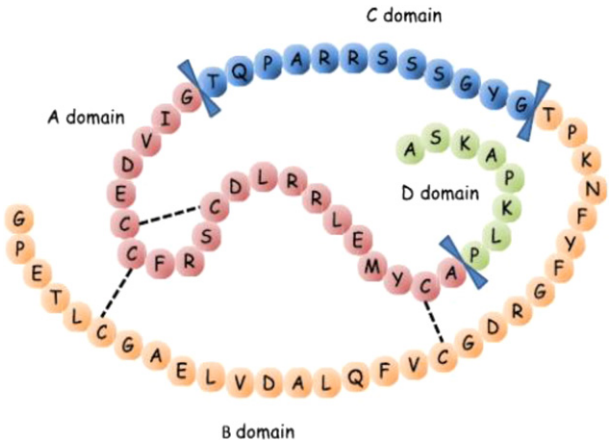

Fig. 5 Molecular structure of human IGF-1. Referenced from: Nishida, T., Makoto, I. and Mtoyoshi, N. (2015). Peptide therapies for ocular surface disturbances based on fibronectin–integrin interactions. *Progress in Retinal and Eye Research* 47, 38–63.

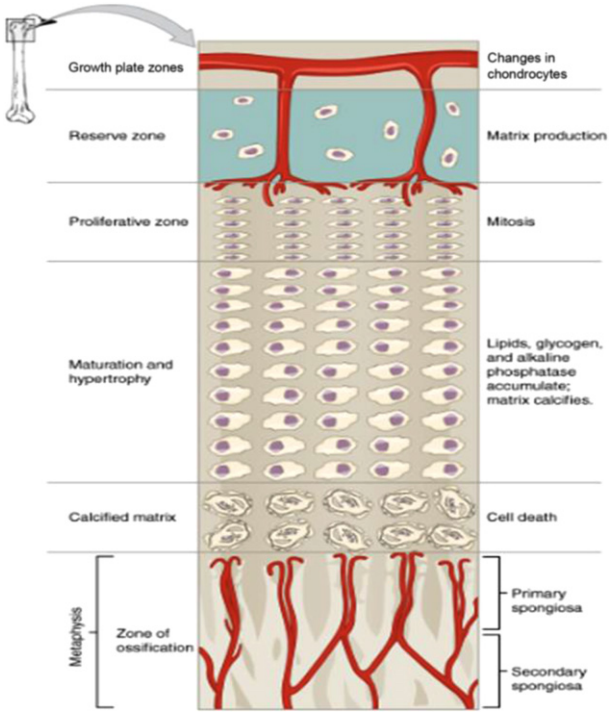

Fig. 6 The growth plate.

IGFBP 4, 5 and 6 are present in much smaller concentration in the plasma. Its actions are not fully understood but IGFBP4 is regulated by protease pregnancy associated plasma protein (PAPP) and parathyroid hormone (PTH). IGFBP5 can also form a ternary complex with acid labile subunit and its plasma concentration is regulated by GH.

Effects of GH and IGF-1 on Tissues and Growth Plate

GH is the major growth factor in the linear growth of children. It mediates many of its biological actions via IGF-1, which acts on the epiphyseal plates of long bones also known as the growth plates. Chondrocytes in the growth plates which are regarded as stem cells, under the influence of GH/IGF-1 and other growth factors, undergo cell proliferation and maturation that results in the linear growth of children.

Recent studies has shown the role of suppressor of cytokine signaling 2 (SOCS2) as a key modulator of GH at the growth plate. The SOCS proteins are a family of negative regulatory proteins that are expressed in response to activation of cytokine and growth factor signal cascades, particularly those utilize JAK/STAT signaling system (Greenhalgh *et al.*, 2005).

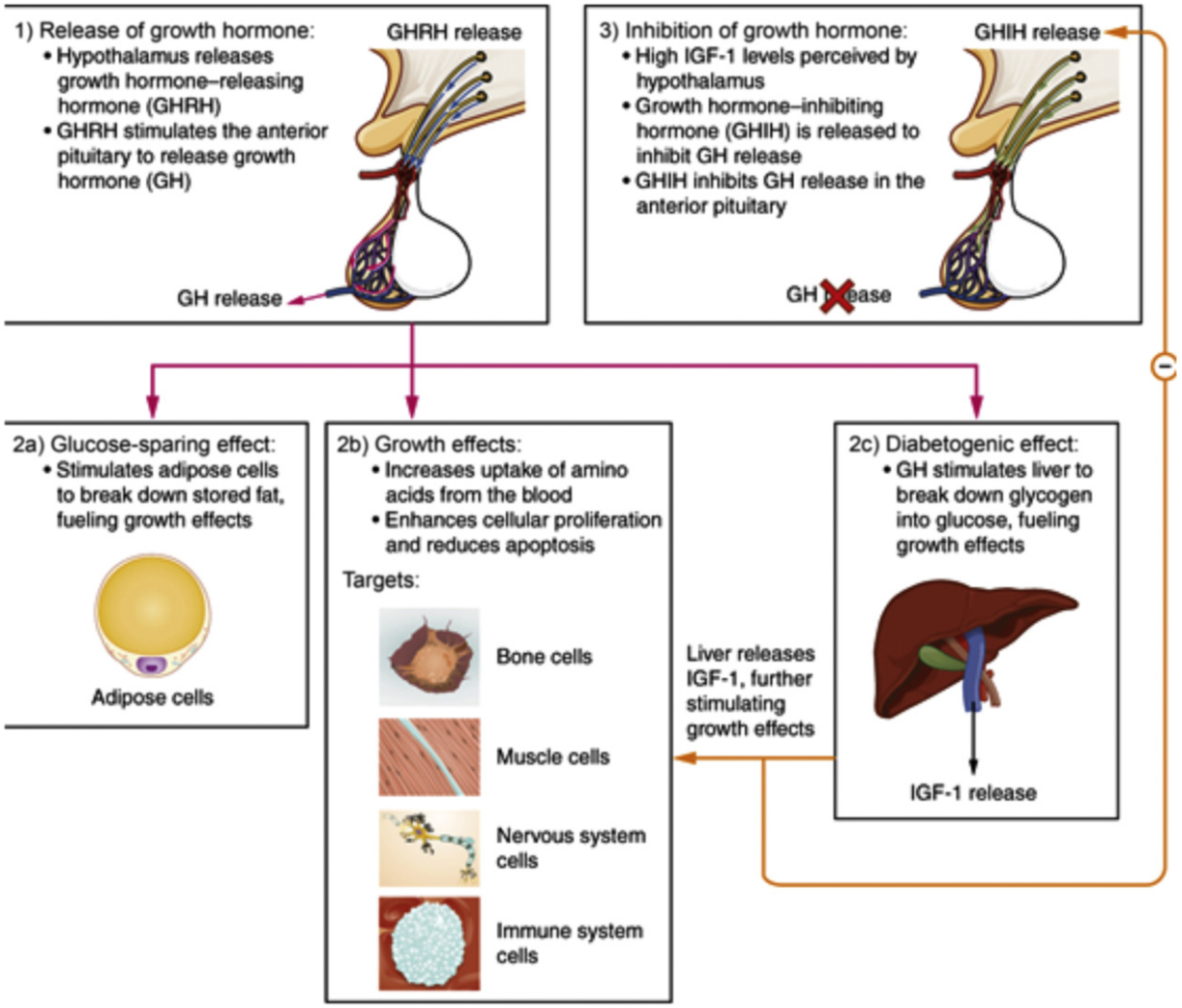

Fig. 7 Summary of actions of GH and IGF1.

Excess GH levels in pre-pubertal children where the epiphyses are yet to fuse results in uncontrolled linear growth, termed as gigantism. Whereas, acromegaly results in individuals whose epiphyses are fused with excess secretion of GH.

In addition to linear growth, GH also has other potent effects—including modulation glucose, lipids and nitrogen metabolism; promotion of lipolysis, increase amino acid uptake and protein synthesis and decrease in protein breakdown (Press, 1988).

Growth Plate

The growth plate, also known as the epiphyseal plate is a thin layer of cartilage that lies between the epiphyses and metaphyses, and is where the growth of long bones takes place. Such longitudinal bone growth occurs here through the mechanism of endochondral ossification, with formation of cartilage and then remodeling into bone tissue (Fig. 6).

The chondrocytes in the growth plates are surrounded by matrix consisting of proteoglycan aggregates and are divided into three zones, reserve/resting zone, proliferative zone and hypertrophic zone. The process starts with recruitment of chondrocytes in the stem cell zone, then actively proliferates by cell division and followed by differentiation, apoptosis and finally mineralization in the hypertrophic zone. Among other hormones, GH and IGF-1 regulates this process.

Clinical Aspects of GH Deficiency in Childhood

Faltering growth in children results from a number of potential causes, GH deficiency being one of them, along with other hormonal disturbances, genetic or congenital abnormalities, chronic medical conditions and malabsorptive states. Short stature in school children can have significant negative effects on psychology and physical development leading to

bullying, poor performances in sports, education and on the overall quality of life of the child itself (Tickner and Keady, 2011).

Growth failure can be detected early through regular growth monitoring. Isolated short stature in many instances is a normal variation resulting from familial/genetic short stature and having no underlying pathological cause and resulting in no psychological issues. If the normal variants of growth are ruled out, then investigation for underlying causes of growth failure is recommended.

Growth hormone deficiency may be an isolated deficiency or occur in combination with other pituitary hormone deficiencies. The frequency increases in the presence of any anatomical defects in the pituitary/hypothalamic region (e.g., septo optic dysplasia, absent corpus callosum, holoprosencephaly or tumors) and in those who have had cranial radiotherapy.

GH deficiency due to multiple anterior hormone deficiencies generally presents early in the newborn period or early infancy unless it is a result of acquired causes such as CNS tumors, cranial irradiation or histiocytosis. Symptoms and signs may include neonatal hypoglycaemia (combination of GH and ACTCH deficiency); cholestatic jaundice (in combination with low cortisol) and micropenis (prenatal testosterone deficiency secondary to luteinizing hormone, LH deficiency). Gene mutations affecting the pituitary transcription factors, Pit-1 and Prop-1 generally result in multiple anterior hormone deficiencies.

The pathogenesis of idiopathic isolated GH deficiency is unknown. However these patients respond to administration of exogenous GHRH by secreting GH, indicating that the somatotroph cells are functional and the primary defect is in the hypothalamus.

Investigations to establish deficiency of GH are widely used worldwide and GH treatment started before fusion of the epiphysis is expected to result in an increase in linear growth. It is recognized that growth hormone deficiency in adults results in poor muscle strength and fatigue and adult GH replacement is now common.

GH resistance and IGF-1 deficiency are now recognized as rare causes of poor growth and extreme short stature in childhood. IGF-1 replacement treatment is licensed for use in children but its use remains limited due to significant potential side effects (**Fig. 7**).

Summary

GH is a peptide hormone secreted in to the circulation from the somatotroph cells of the anterior pituitary gland. Its primary role is to promote linear growth in children. The action of GH is mediated directly through GH receptors, as well as indirectly via IGF-1. The secretion of GH is pulsatile and under a variety of hormonal influences such as stimulatory hypothalamic GHRH, ghrelin and sex steroids, and inhibitory somatostatin, IGF-1 and glucocorticoids. IGF-1, which is the principal mediator of peripheral actions of GH are primarily produced in liver and regulated by GH. Exogenous GH is widely used to treat both isolated and multiple anterior hormones deficiencies in children.

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Infant, Childhood, and Adolescent Auxology

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Principles of Auxology

Measuring Techniques

Much has been written about the physical assessment of growth (auxology) and how reliable measurements can be taken. In routine clinical practice this depends on appropriately accurate and well maintained and frequently calibrated equipment.

For height this would include a standard stadiometer whether mechanical or electronic or a supine length scale for children under 2 years.

For weight either class 3 electronic scales or a beam balance, both of which are reliable enough to require infrequent recalibration.

Measurements are taken to the nearest 0.1 cm for length/height and 0.1 kg for weight (www.rcpch.growthcharts).

Specialist Techniques for Specialist Pediatric Endocrine Clinics and for Research

A higher level of accuracy is required, so that for height measurements taken to a repeatability of 2 mm is needed. Traditional stadiometers such as the Harpenden as regarded as the gold standard but require careful maintenance and recalibration. Electronic ones such as the Seca generally do not require recalibration. Weight does require electronic equipment. Other detailed measurements such as sitting height for body proportion and arm span require specialist equipment and training in the technique.

Positioning of the child is even more important. For height the child should be measured in socks or bare feet with the heals placed against the measuring back plate and the child standing straight (https://www.rcpch.ac.uk/improving-child-health/public-health/uk-who-growth-charts/video-resources/). The head should be placed in the Frankfurt plane where the lower border of the orbit is in the same plain as the external auditory meatus to allow repeatability (**Fig. 1**). There is debate as to whether gentle pressure should be exerted upwards under the mastoid processes to reduce slumping and improve posture and there has been some debate as to whether this can compensate for the diurnal loss of height due to compression of the spinal discs but which every method is chosen consistence is the most important.

Infants

Weight in infants and supine length should always be measured with the infant completely naked so that no factors such as wet nappies can interfere with weight and when measuring in infant length it is necessary to be able to hold both legs jointly together and unimpeded by nappies to ensure accuracy of length measurement. Head circumference should be taken using a nonstretchable measuring tape no wider than 0.5 cm made of metal or plastic composite. This requires measures to be taken in the occipito-frontal circumference and repeated twice, and the largest measurement taken to 0.1 mm is recorded (**Fig. 2**).

Children and Young People With Disabilities

It may not be possible to obtain standing measurements in children with major disabilities. If possible supine measurements can be taken in older children using a supine length measuring table or device such as the Dunmow and if there is any deviation from standard measuring practice then this needs to be recorded. If weighing cannot be done independently it is possible to use the *buddy* method where a child is weighed with minimal clothing along with the parent and then the parent is then weighed separately and that value subtracted (https://www.rcpch.ac.uk/improving-child-health/public-health/uk-who-growth-charts/video-resources/).

Assessments of Body Composition

For research techniques, the most accurate method of assessing body composition is cross sectional computerized tomography imaging. This does require a significant dose of radiation and cannot be used in routine practice. Some methods of assessing bone density using DEXA techniques also include a measure of body composition but this depends on the software available. Routine and reasonably accurate measures of body composition can be made by bioelectric impedance techniques where a small electrical current is passed through the body. Equipment such as the Tanita has been shown to be reasonably reliable in clinical practice.

Body mass index (BMI) can be derived from a simple formula: weight in kilograms divided by height in meters squared. This can give an indication of body size but will not clearly differentiate between lean and non-lean body tissues. Higher BMIs are noted in short stocky individuals and conversely lower values in tall thin people, which may be misleading clinically. BMI charts are available to track the changes in BMI values over time (**Fig. 3**), but as it is the centile (or SDS score) for age which is important, separate BMI centile scales are available on the RCPCH school age charts (figure). A quick BMI centile lookup is available for quick reference, only requiring the height and weight centiles from routine plots (**Fig. 4**) allowing BMI changes to be tracked over time

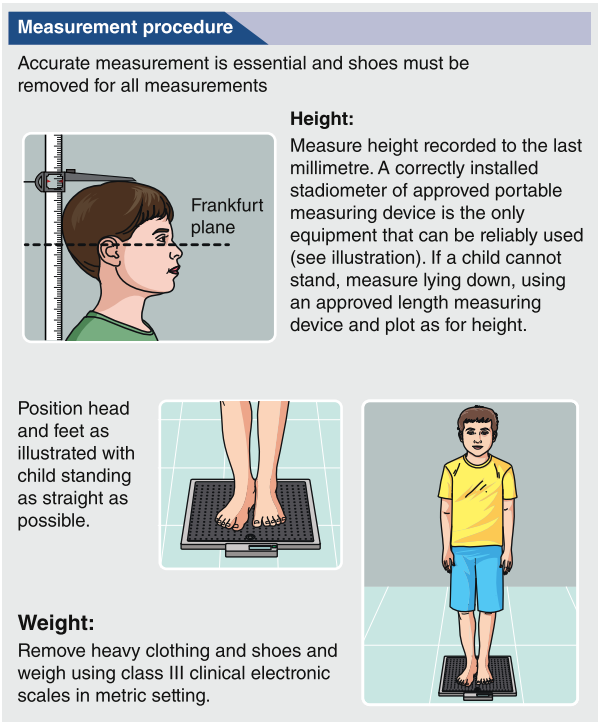

Fig. 1 Optimal height and weight measurement © RCPCH.

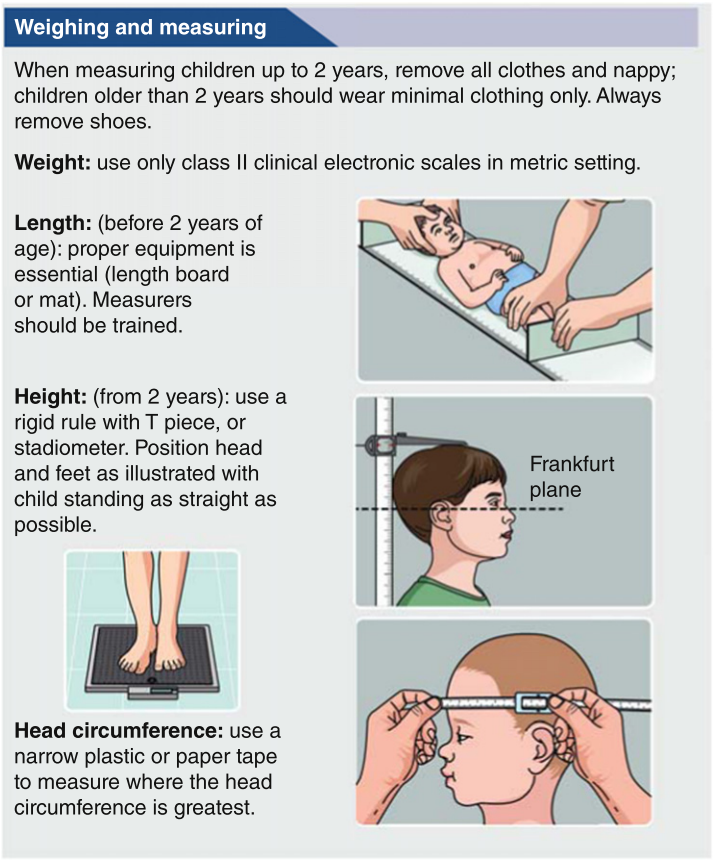

Fig. 2 Supine length and head circumference measurement © RCPCH.

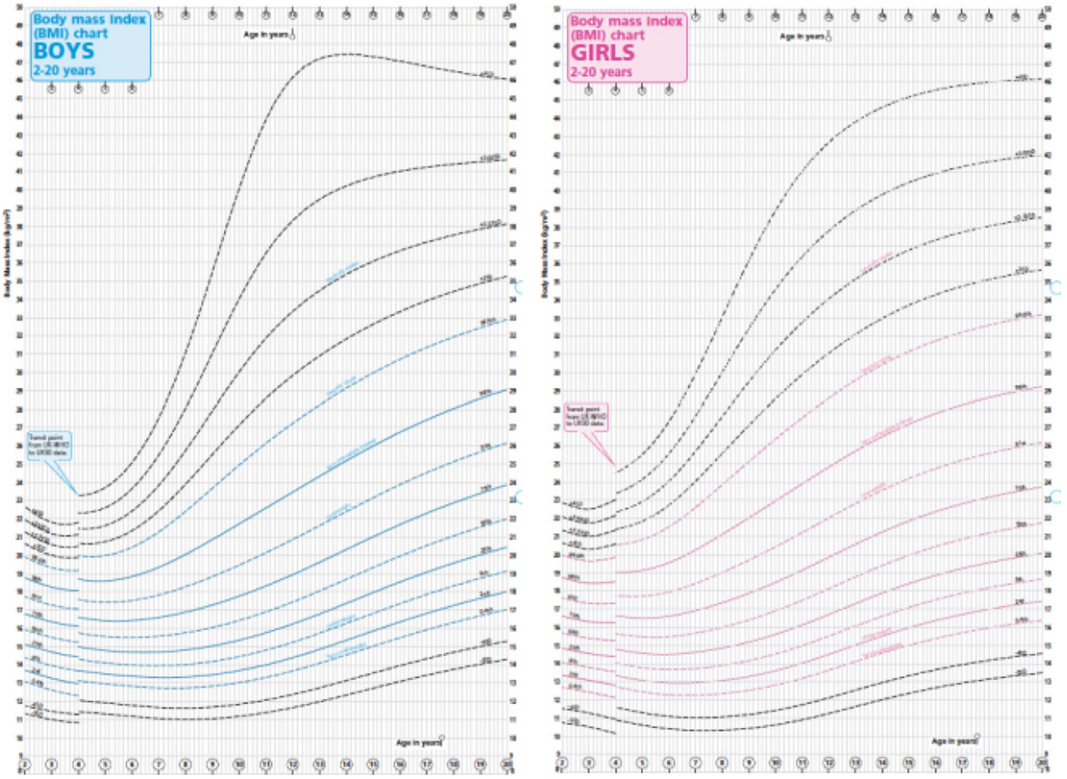

Fig. 3 BMI charts for boys and girls © RCPCH.

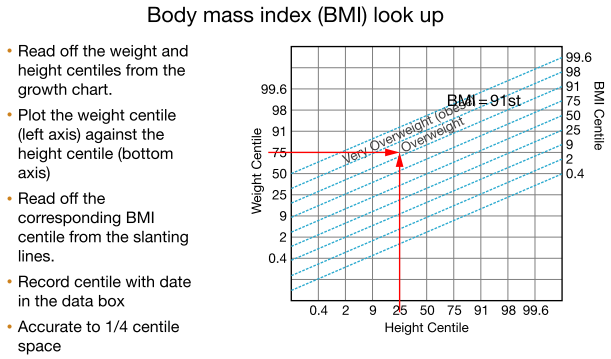

Fig. 4 BMI lookup © RCPCH.

(Fig. 5). Suggestions that the ponderal index (weight/height in meters cubed) is more accurate for clinical decision making has been proposed but no regular comparative standards are available.

Measuring in Low Income Countries

In low income countries, particularly in remote areas, there is often no access to accurate equipment and electricity for accurate measurement and there are fewer trained health care professionals to perform these measurements. It is in these parts of the world where under nutrition in children is a significant problem. The most accurate measurement of adequacy is nutrition is using the mid-upper arm circumference. Standards are available and can be measured with a simple tape measure, paper, plastic or metal but inexpensive color coded tape measures are available (http://www.who.int/nutrition/publications/severemalnutrition/9789241598163/en/).

Growth Concepts

Genetic Potential Mid-Parental Height

The accuracy of predicting a child's adult height and growth potential is relatively poor, but more accurate during mid-childhood during the steady growth period. Commonly used calculations include the mid-parental or target height where both parents' heights are fed

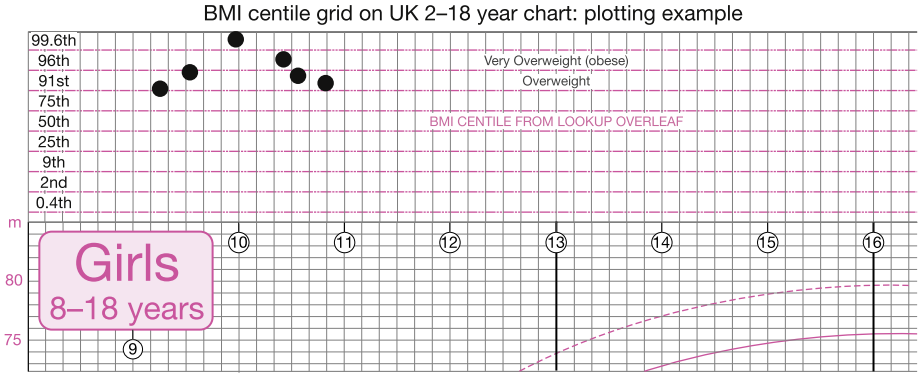

Fig. 5 Plotting BMI centile trends © RCPCH.

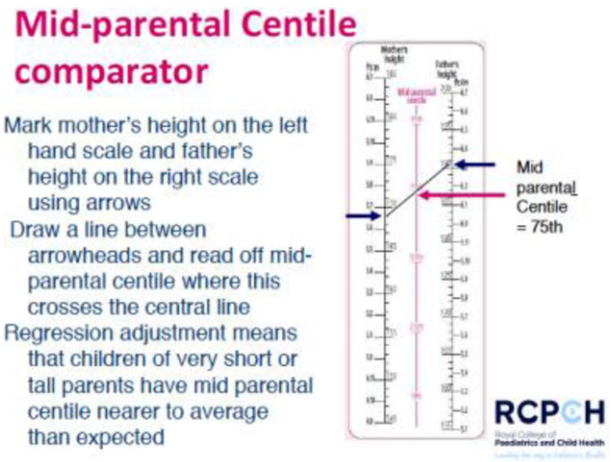

Fig. 6 Mid-parental height (target height) calculator © RCPCH.

into a calculation either of Tanner *et al.* (1970) or Hermanussen and Cole (2003) as below or using the graphical technique of Cole as on the United Kingdom growth charts (**Fig. 6**). All methods have a range of variability. Tanner method calculated in centiles is ± 6.5 cm, the Hermanussen method in standard deviations 1.3 standard deviations and the graphical method, most children fall between ± 2 centile bands, that is, 1.3 standard deviations and all children within ± 2 standard deviations.

Definitions of Tall and Short Stature

A child who is short and falls within the expected range for the parents can be described as having familial short stature. The term genetic short stature is best avoided here and reserved for situations where there is a known chromosome or genetic cause. A child who is at the lower range or below the expected centiles for their parents is described as having nonfamilial short stature and this maybe a clue to requiring further investigations. At the other extreme, a tall child whose height is within the predicted range for parents is described as having familial tall stature, whereas if they are above the range it would be nonfamilial tall stature which may require further investigations.

Predicting Adult Height Based on the Child's Height

In some situations, it is not possible to obtain parents' heights so the predicted adult height nomogram based on the child's current height centile on the UK RCPCH charts can be used. It is bases upon a predictive analysis between childhood/adolescent and adult heights and contains corrections for the phenomenon of regression to the mean. The child's current centile is plotted on the height prediction ladder and their adult height is read off with an error margin of ± 6 cm (**Fig. 7**). This actually produces a higher predictive accuracy than that when using the target height.

Speed of Growth/Growth Velocity

Challenges exist on assessing growth variations during infancy partly on account of the repetitive growth during the first year of life (see below), but also on accuracy of measurement which forms a greater part of the variability seen. During the first year of life, in a healthy infant there is a 300% increase in body weight, a 50% increase in length and a 33% increase in head circumference.

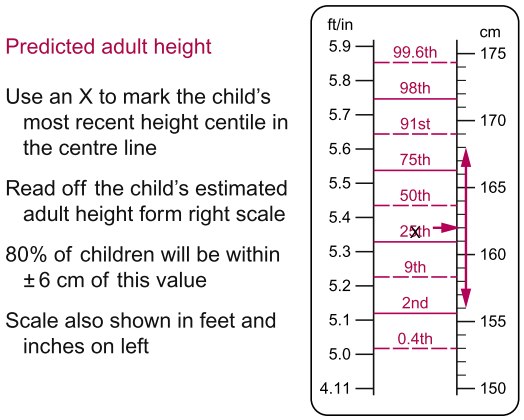

Fig. 7 Predicted adult height calculator © RCPCH.

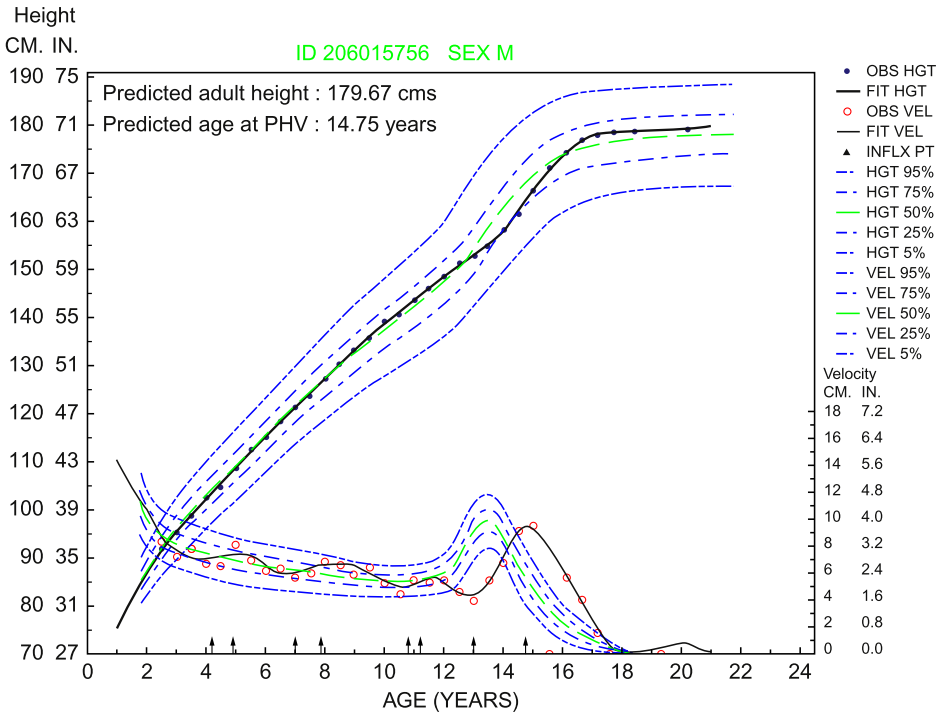

Fig. 8 Height velocity chart with the height velocity pattern of an individual child.

Childhood

The childhood phase of growth from the third year of life until the commencement of puberty is traditionally regarded as more steady. However, the smoothed height velocity curves of charts available do not display accurately the height variable in the individual patterns of growth in height and weight in children over this period (Butler *et al.*, 1990). They have been described as cycles of growth, approximately two yearly pattern of acceleration and deceleration, the cause of which is probably genetic but maybe hormonally driven (**Fig. 8**). This needs to be taken into consideration when investigating slow and rapid growth in mid-childhood.

Adolescence

The attainment of the rapid phase of growth during puberty is different between the sexes. It has been well described through many sources that the peak height velocity in girls will occur in mid-puberty, Tanner breast stage 3 at a mean age of around 12.0 years, whereas in boys this is 2 years later on at a mean age of 14.0 when further pubertal development has taken place (Cole *et al.*, 2014). Until the onset of puberty the growth between the sexes is almost identical but the later start of the growth spurt in boys by about 2 years contributes around 10–12 cm of additional height growth and the greater magnitude of the adolescent growth spurt will contribute around 2 cm of height gain consequently the height difference in adults between males and females is all accrued during puberty.

Detection of Abnormality

If the purpose of assessing growth is to define normality or detect abnormality what at the boundaries and what parameters should be used for considering additional referral.

Infancy

There is enormous variability in the first 6 months of life when an infant who become independent at birth will develop their own growth pathway. Thus, a genetically smaller infant from a large mother may show a period of decrease in growth called "catch down"; conversely a healthy genetically larger infant born from a smaller mother may show increase in growth in the first 6 months of life.

Definitions of what is the extreme of variation vary, with suggestions that >1 centile or SD division on the growth chart either up or down should be investigated but in general natural variations will balance by 6 months of age. However, if there are greater and continuous changes in either length or weight or head circumference, certainly more than two centile bandwidths in the first 6 months of life and certainly if the trend continues particularly in weight and length, then these require further investigation (**Fig. 9**). Children who have an advanced or delayed genetically preprogrammed growth pattern often called "constitutional advance" or "constitutional delay in growth" will show a period of acceleration or deceleration respectively usually in all parameters but in general, no further changes or centile shift will be seen after 6 months of age. However, clinical decision making needs to be exercised to ensure pathology is not missed.

Childhood

The assessment of growth in mid-childhood is more straightforward. Careful consideration has taken place as to the factors which allow the detection of growth pathology and interventions to restore normality whether this is due to chronic disease, lack of social deprivation, malnutrition or hormone deficiencies such as thyroxine and growth hormone. The most accurate ways of detecting abnormal growth either up or down include centile shift of more than two-thirds of a standard deviation (equivalent to one centile band width) over a year, together with height being outside the mid-parental target centile range (Stalman *et al.*, 2015).

Puberty

On account of the huge and unpredictable variability of pubertal growth which depends not only on the timing of the onset of puberty, height obtained at the onset of puberty and genetic growth potential, assessment of adequacy of growth needs to be made if concerns arise in parallel with the clinical assessment of puberty whether this is by Tanner staging in trained individuals or using the simpler *puberty phase* concept promoted by the Royal College of Pediatrics and Child Health (**Fig. 10**). As most published growth charts do not take into account normal pubertal variation and present the average growth centiles at the average age, then early maturers will show an acceleration up through the centile band and late maturers the opposite. It is the paradox of assessing pubertal growth that if an adolescent follows a centile band throughout puberty they may potentially be growing abnormally but that depends on how the growth charts are constructed, see below.

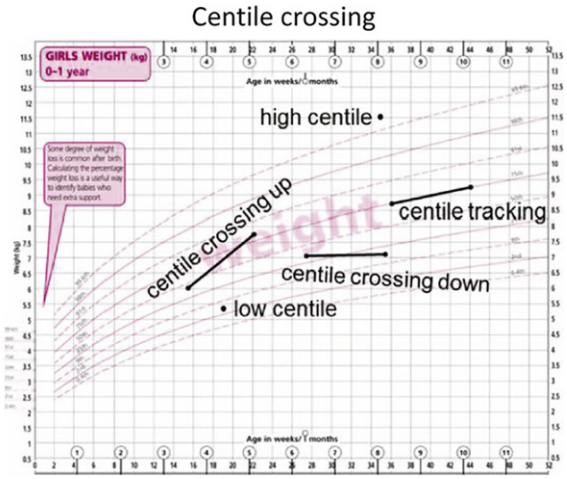

Fig. 9 Patterns of infant growth-normal and abnormal © RCPCH.

The 3 phases of puberty from history

	Pre-puberty (Tanner stage 1) *If all of the following*:	In puberty (Tanner stage 2–3) *If any of the following*:	Completing puberty (Tanner stage 4–5)
Girls	• No signs of nipple or breast development • No pubic hair	• Any breast enlargement so long as nipples also enlarged • Any pubic or axillary (armpit) hair growth	*If all of the following:* Started periods(menarche) with breast, pubic and axillary hair development
Boys	• High voice • No growth of testes or penis • No pubic hair	• Slight voice deepening • Reddening of scrotum with growth of the testes • Early testicular or penile enlargement • Early pubic or axillary hair growth	If any of the following: • Voice fully changed (broken) • Adult size of penis with pubic and axillary hair growth • Moustache and early facial hair growth

Fig. 10 RCPCH phases of puberty: a simpler assessment reference © RCPCH.

Growth Charts

Types of Growth Charts

Growth charts have been traditionally produced on paper for community use and hospital use, and in many countries parents are given a hand-held booklet (Personal Child Held Record) containing growth charts for ongoing surveillance of their child's wellbeing and care. In many parts of the world this is still regular practice but with community and hospital records becoming electronic, the recording and plotting of growth charts to evaluate a child's well being is also electronic. There are many systems available but the key factor is becoming familiar with what is available locally.

Derivation of Growth Charts

The way in which growth charts are constructed falls largely into two categories: that based on a large population selection of infant children and adolescents measured on one occasion. This allows the assessment of growth on a series of individual occasions, each measurement making a one-off comparison with the expected height range of children at that particular age. Although it is common to follow a child's growth serially on these charts, this is not for which they are principally designed. However as stated above the concept of centile shift can be used to detect growth excess or growth failure.

Longitudinal Growth Charts

For the correct assessment of serial growth measurements over time, then a growth chart or growth standard such as the WHO (see below) would need to be considered. These charts constructed are from measurements taken on individual children over a period of time. As this is quite a labor-intensive process, and may take 15–20 years to get a complete set of longitudinal data, very few data sets are available and often gathered from smaller numbers of children, but the pattern of growth is observed used to inform the construction of growth charts such as the RCPCH and WHO charts.

The WHO Infant Growth Concept

The World Health Organization Multi-Centre Growth Reference Study was established between 1997 and 2003 to examine the concept of universal infant growth (http://www.who.int/childgrowth/standards/en/). Height, weight and head circumference measures were taken in six places around the world identifying mothers living in socio-economically good environments who are nonsmokers, who are healthy and who breast fed their infant exclusively for 6 months. 8500 infants were measured longitudinally for up to 2 years and the data up to 5 years of age were supplemented with some cross-sectional measurements. Interestingly, there have been shown in this study very similar patterns in length up to age 2 years across all sites which confirms the universality of the pattern of infant growth irrespective of human racial origins. There are variations in weight which are due to the ethnic variations in build but similar enough for the concept of a universal growth chart to be established. Questions have arisen however about the value of the head circumference centiles which may under predict true size (Wright *et al.*, 2011). This may have been on account of different techniques used but until resolved the head circumference standard should be used with caution.

The Value of the WHO Charts

Not only do the WHO charts provide a universally acceptable set of measurements with which to compare child and infant growth but the concept allows additional use. As the 0–2 year charts are made principally from longitudinal data they can be considered as growth standards, in other words providing a pattern of infant growth that represents the optimal growth trajectory. Those charts constructed after the age of 2 years (and other national growth charts which are based principally on cross sectional data) are regarded as growth references, and allow the comparison of a child measure at a single point in time only. They are best for population growth evaluation, but are used in clinical practice to make assessment in individual children.

Comparison of National Charts With WHO?

The decision whether to use national references or WHO standard combined with WHO references after the age of two or national references requires a decision often dependent on how old the data are that constitute the local growth references and the how far these national references deviate from the WHO data set.

Percentiles or Standard Deviation Scores

WHO charts come in both percentiles using the American pattern that is, 5th, 25th, 50th, 75th, 90th, and 95th centile pattern or in standard deviation scores (https://www.cdc.gov/growthcharts/cdc_charts.htm). In most cases centiles are derived from SDS depending on how the growth chart measurement spread is constructed whereas SDS lines are equally spaced. Those following the United States pattern are not. Within the RCPCH UK standards the traditional choice of centiles is presented, but with a different spread 0.4th, 2nd, 9th, 25th, 50th, 75th, 91st, 98th, and 99.6th (www.rcpch.growthcharts). This apparently unusual pattern is more logical as each of the centile bands are two-thirds (0.67) of a standard deviation apart that is, equally spaced hence a growth deviation up or down by one centile space, where ever that happens on the growth chart is of equal significance. The addition of 0.4th centiles and 99.6th centiles representing ± 2.67 standard deviations is presented to allow a more accurate determination of pathology in extremely tall and extremely short children (**Fig. 11**).

Specialist Growth Chart

For a number of conditions specialist growth charts have been produced. Sometimes this will have in the background the growth pattern of typically growing children and sometimes the growth pattern of children with a diagnosis of any particular condition is presented. Commonly used ones include down syndrome (https://www.dsmig.org.uk/information-resources/growth-charts/) and Turner syndrome (http://www.healthforallchildren.com/shop-base/shop/growth-charts/turner-syndrome-growth-charts-100-pack/). They may have individual value but the variability of presentation of genetically determined syndromes does vary quite considerably and so comparison with the regular charts is recommended so that the growth of children with a defined condition or syndrome can be compared both with specialist charts and that of typically growing children.

Neonatal Growth Chart

Ideally the chart should be able to represent not only the expected growth of infants born at term but also those who are preterm that is, before 37 completed weeks of gestation. Preterm correction is variably applied depending on the etiology and does not exist for the WHO standards. Within the United Kingdom when infant WHO standards were adopted, two methods of adjustment for prematurity were

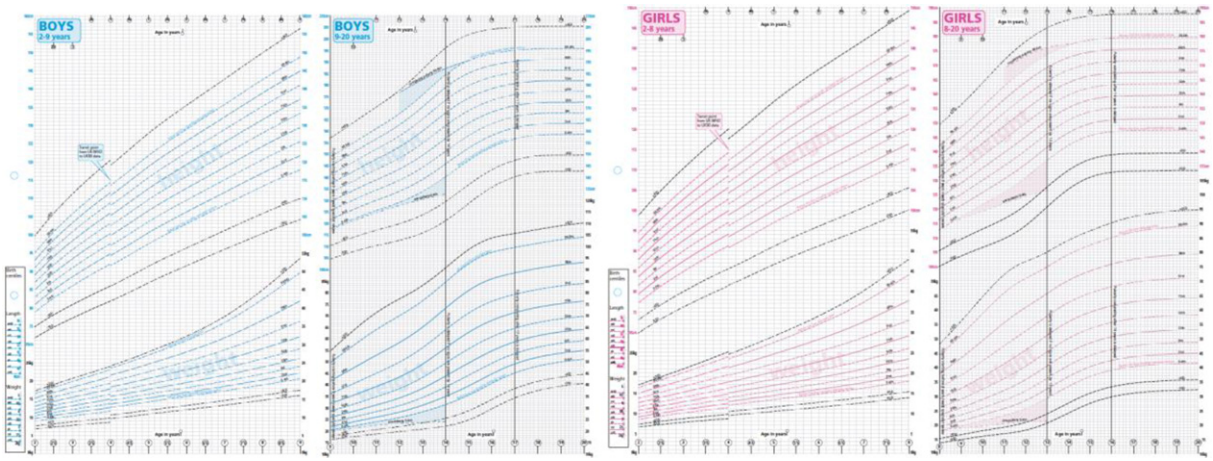

Fig. 11 UK 2–20 years childhood and puberty close monitoring charts; boys and girls © RCPCH.

applied. For infants of gestation 32 weeks and above, separate panels for head circumference and weight are included which can be used directly. For term and beyond it is recommended that double plotting of every measurement takes place. The exact measurement based on the age since birth is plotted using a dot and the same measurement is replotted at the gestationally corrected age with an arrowhead and a dotted line is drawn between the two (**Fig. 12**). This avoids the confusion of whether gestational age correction has been applied or not which can lead to either missed or false diagnoses of growth excess or failure to thrive.

Extremely Preterm Infants Down to the Lower Limits of Viability of 23 Weeks' Gestation

More detailed assessment of growth, particular weight gain is required. Within the United Kingdom this has been tackled by the development of the neonatal and infant close monitoring chart which allows multiple plots on a frequent basis to be made up until the expected date of delivery and beyond (www.rcpch.growthcharts). The charts also allow ongoing monitoring under a more detailed schedule up until the age of 2 years, particularly for specialist new born follow up clinics which often need to look at the ongoing care of these infants from a close monitoring perspective. The method of gestational correction is different as the actual calendar date is back plotted from the estimated date of delivery and put on the chart at the time of first establishment and this prevents serial gestational age correction errors.

Growth Assessment During Puberty

The unpredictability of the onset of puberty and its subsequent pattern makes the development and presentation of a way of accurately assessing growth during puberty difficult. Current growth charts only represent the average amount of growth taking place for average children going through puberty at the average time.

Puberty Growth Chart

There have been several attempts to be able to design growth charts to allow for pubertal growth variations. There are four challenges needing to be met:

1. Age at the onset of puberty.
2. The proportion of growth completed at the onset of puberty.
3. The distance from target or genetic height.
4. The pace of puberty.

Additionally, the assessment of the degree of development during puberty either using Tanner stages or *puberty phases* has always been regarded as a separate assessment. The RCPCH UK charts for school age children and the specialist chart have attempted to address this by firstly depicting the normal ranges for beginning and completing puberty with vertical puberty lines creating zones on the growth chart to help interpret the appropriateness of growth (**Fig. 13**). As in most cases, normally developing and growing adolescence will not cause a concern. It is those that are at the extremes, the tall and advanced in maturing and the short and delayed who require some form of judgment as to whether their pattern of growth is acceptable. As a result of which additional lines at the extremes, 0.4th centile and 99.6th centile have been added which depict the upper ranges of normal growth that is, 99.6th centile for those adolescents who have completed puberty and a lower 0.4th centile for those adolescents who have not yet entered into puberty (**Fig. 14**). Thus, any young person whose height and weight plots within those ranges, a consideration needs to be made as to what has happened to puberty and whether that is indeed normal and this may help to differentiate

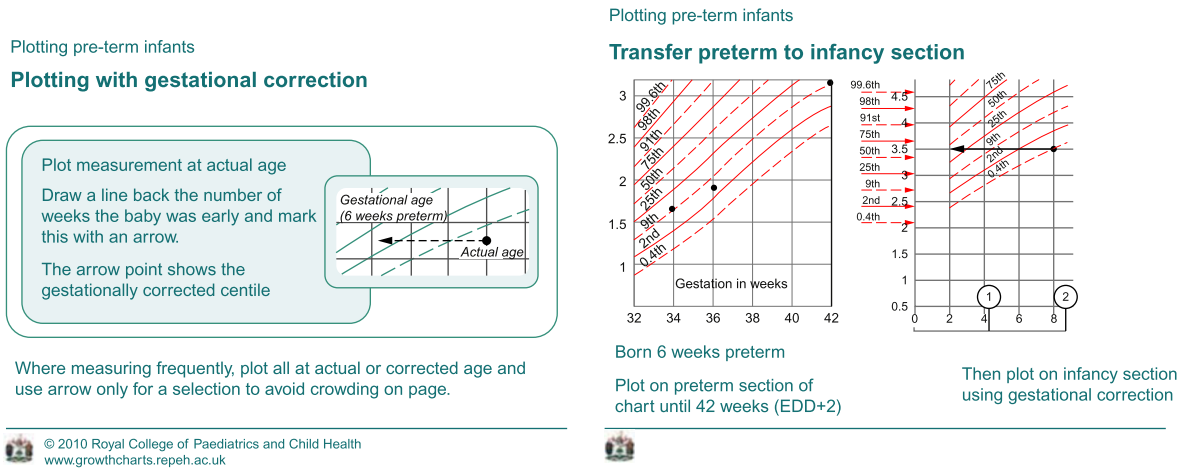

Fig. 12 Gestational age correction for preterm infants 32–37 weeks © RCPCH.

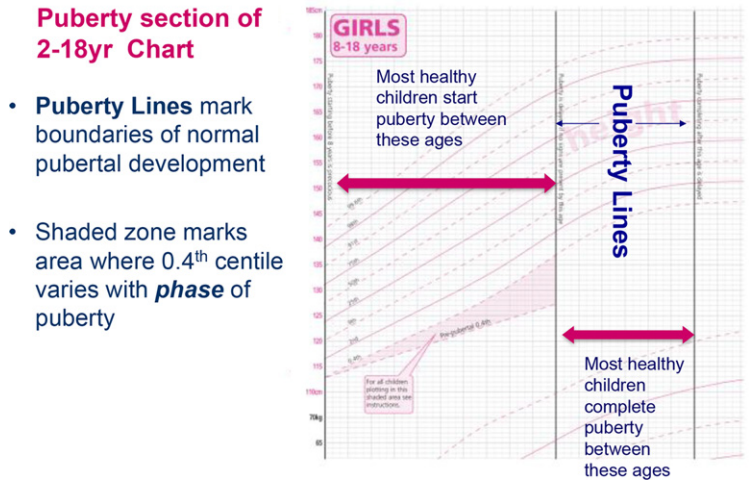

Fig. 13 Puberty zones on the RCPCH charts © RCPCH.

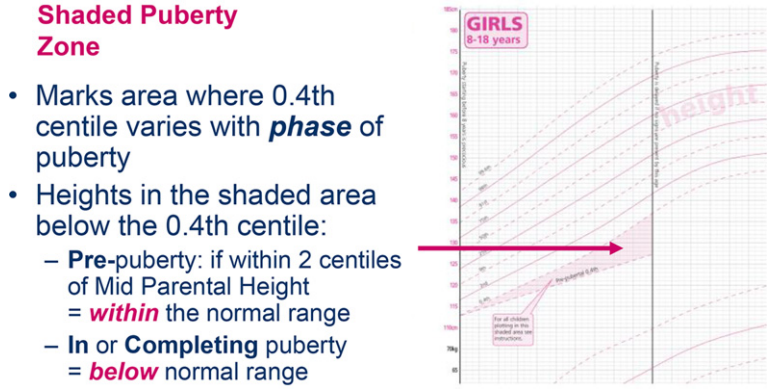

Fig. 14 Lower 0.4th centile for those children remaining prepubertal © RCPCH.

between pathology and normal variation. However, it is important to note that if a height plots within the shaded zone past the prepubertal 0.4th centile then growth could still be abnormal if the child is below their target centile range.

Changes in Population Growth

Obesity

By far the biggest influence on growth in height and particular in weight and BMI is the increasing obesity in high income countries. There is much evidence to suggest that excess of nutrition not only augments the general weight and BMI range but also promotes earlier maturation and tall stature during childhood, and it also has been linked with an earlier entry into puberty (Wagner *et al.*, 2012). Various estimates have been attempted to quantify this but are largely regional or national in nature and international comparison show quite marked ranges in development of obesity. These trends in obesity and height growth are more of public health and governmental interest with the intention of trying to buck these trends to restore better health and the prevention of other complications of obesity, such as hyperlipidaemia, type 2 diabetes and cancer risks. However, in the individual child, tallness, and advanced maturation with height within the target centile range maybe recognized if the child is overweight.

Effects on Puberty

Multiple studies have investigated trends of pubertal onset and completion (in females through age at menarche) to try and investigate trends. One of the challenges of obese children, particularly girls, is determining whether onset of puberty has clinically occurred on account of increased adipose tissue around the breast or whether there is true breast tissue present. In males, the clinical estimation of the onset of puberty by increase of testicular size is more straight forward, but again there have been suggestions of earlier trends and the possibility of different effect of increasing size on the age at the start and completion of puberty. This has been most well explored in the Copenhagen Study (Aksglaede *et al.*, 2008). Although there may have been some trends toward puberty starting a year earlier

approximately over the last two decades, the age of completion and the mean age of menarche does not appear to have shifted significantly and the process by which this trend has occurred has still not been clarified as hormonal determinates of the onset of puberty such as sex steroids and gonadotrophins do not appear to have shifted. Further investigation is required here.

Tempo of Growth Internationally

Multiple reports have suggested that as far back as records have been kept, that there has been an increase in average child growth by approximately 1 cm per decade. However reports are coming forward now about this trend is potentially ceasing and that humans have reached their maximal genetic potential as a result of nutritional adequacy. Whether there will be additional evolutionary benefits of being taller in size is not yet clear and there may be a reversal if this results in a disproportionate energy balance.

Growth in Immigrants

In the last 50 years with the breaking down of international borders and within the last 10 years of significant intercountry and intercontinent migration, there is the challenge of trying to explore what effect this has on child growth. For some time now it has been known that the heights of Japanese children whose parents migrated to Western United States had shown significant increase in growth which was due to longer legs, presumably secondary to improved nutrition and enhanced growth hormone secretion. Further investigation needs to be done on this to identify variations in health and whether moving from a situation from nutritional inadequacy to one of nutritional excess has a significant deleterious effect on individual health. For further reading see (Hemanussen, 2013).

Therapeutic Attempts to Modify Child Growth

Chronic Disease

It has been well established that the monitoring of a child's growth in height and weight is a good index of their general physical health. This is well understood in the investigation process where exclusion of cryptic chronic disease is part of the investigation pathway. Children in adolescence with known chronic disease may require growth assessment in case of the development of stunting that is, the inability be able to effect fully catch-up growth even with restoration of health. This is certainly true with gastrointestinal disease such as coeliac disease and inflammatory bowel disease where chronic nutritional inadequacy particularly if diagnosed late may not allow a full catch-up growth. Subtle slow patterns of growth such as in children with asthma and eczema who are often on intermittent or continuous low dose glucocorticoid may not result in a reduced ultimate adult height but the process of getting there may well be slowed down with full adult height not being achieved until the very late teens or early 20s.

Survivors of Childhood Cancers

Although the improvement in childhood regimens over the last 20 years has been impressive, unfortunately some of the treatment regimen significantly affect childhood growth. Whole body radiotherapy prebone marrow transplant will cause restriction of whole body growth and a degree of hormone resistance. Cranial irradiation particularly in the higher doses for childhood brain tumors will result in pituitary dysfunction, growth hormone deficiency, and disregulation of puberty causing both precocious and arrested puberty. Cranio-spinal irradiation used in common brain tumors such as medulloblastoma, particularly if given early on in life will result in disproportional growth overall on account of reduced spinal growth. Chemotherapeutic agents have less effect but this is very much dependent on the regimens used in the primary treatment program (Rose *et al.*, 2016).

Manipulating Childhood Growth

Growth failure due to hormonal deficiencies in particular growth hormone and thyroxine can be very successfully treated by restoration of the hormone concentrations within the body with exogenous treatment with growth hormone or thyroxine as needed. Attempts have also been made to boost the growth of normally growing children such as those with idiopathic short stature. At present the diagnostic process is not yet fully developed, so children who are short for their family may either have simple physiological growth delay or may have some subtle genetic variant which is going to reduce their overall growth. Many children worldwide with constitutional delay of growth are treated with growth hormone, which may in itself not be harmful but probably does not produce any overall long term benefit. With non-familial idiopathic short stature there are claims of small benefits in boosting adult height, but whereas this treatment is licensed within some countries including the United States, there is no current license within the countries under governance from the European Medicine Agency. Growth hormone treatment is also very costly.

Manipulating Puberty

More successful attempts occurred by using exogenous sex hormone treatments. Since physiological pubertal delay is more common in boys, multiple publications have shown that low dose testosterone treatment either for short course 3–6 months

or intermittently continuously up to 1–2 years may boost the growth spurt and does not compromise the predicted adult height but does not produce any overall gains than would be expected in adult height. Sex hormone treatment in tall children to restrict height (more often a concern in girls rather than boys) has had variable success. Higher dose estrogen treatment had previously been used but had shown no benefit over the age of 14 and there are reported risks of sensitization of breast tissue and the risk of breast cancer later on in life. Currently the only acceptable approach for girls would be to induce puberty and the growth spurt using a physiological dose of estrogen in increasing doses completing puberty in 2–3 years to try and accelerate epiphyseal fusion (Hannema and Sävendahl, 2016). The same is possible in boys with exogenous intramuscular testosterone.

Manipulation of the Pubertal Growth Spurt Using Gonadotrophin Releasing Hormone Analogues

Attempts at trying to prolong the adolescent growth spurt using GnRH analogues to slow down epiphyseal maturation have variable affect. In healthy individuals, treatment with GnRH analogues alone do not show any evidence of improvement in adult height. However, in pathological conditions such as those children who are born small for gestational age and are receiving growth hormone treatment, GnRH analogue for up to 2 years may enhance a total height gain if puberty starts early in that situation (van der Steen *et al.*, 2016). Furthermore, in girls with Turner syndrome who do not have spontaneous puberty, delaying the induction of puberty for up to 2 years may result in taller stature overall but may have lower acceptability with the adolescents themselves (Gault *et al.*, 2011).

Conclusion

The key to understanding, assessing, and managing childhood growth problems lies in the thorough understanding of the normal pattern of growth, how to make appropriate and accurate measurements and how to interpret those and compared with national or international growth standards of references. Identification of growth variations early is also key to successful treatment outcome whether that is restoration of normal health or starting an intervention to promote growth and/or puberty. Though much has been learned from simple observation on the auxology or measurement of growth, the individual variability remains a mystery, likely to be due to specific individual control variants and exploration of this will be important at the next stage in the understanding of the individual variation of childhood growth.

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Body Proportions☆

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General Introduction

In 1943, Medawar gave a clear description of the transformation that occurs during growth, showing the head–trunk–legs gradient, which indicates that the development of the head is advanced over that of the trunk and the development of the trunk is advanced over that of the legs during the maturation process. This clearly shows that the growth of different parts of the body does not occur simultaneously or at the same speed. In a child, this transformation can be defined by the interrelationship of a number of linear measurements, such as sitting height to full height, or head circumference to crown rump length. The ease with which one can equate the human body's shape with an accurate estimate of human age—whether the compactness of a 5-year old, the coltishness of a young teenager, or the dimensions of a mature adult—tends to be taken for granted. Nevertheless, this feat is remarkable. Even more extraordinary is the precision with which the human eye registers any deviation from the norm. The mathematical expression of these perceptions for scientific purposes is a highly complex task with imperfect results. Vital information is lost. It is possible, however, to use a reference population to organize the complexity of what even the untrained eye perceives naturally. Knowledge of the body proportions gives insight into the natural process of maturation and any disturbance will be visible in abnormal body proportions. A principal distinction can be made between a child with stunted growth affecting the whole body or affecting just some body parts. Such an observation leads to different conclusions about the origin of some growth deviation.

There are different possibilities of taking measurements in children. The first method is the classical one by means of a stadiometer, measuring rod or tape. Another method is photometry or body scanning. An outline of the photometry is programmed at the Maastricht University and presented later.

In this article, an overview is given of different techniques for taking bodily measurements. The reference values are based on a Dutch study of 2001, presented in the Atlas of Pediatric Morphometrics by Gerver and de Bruin. Of the 20 measurements taken in the Dutch study, the most important are selected for discussion here.

Anthropometry

The Measurements

Length and height

In pediatrics, the curve representing a child's increase in height with age is used as one of the indicators of the child's health. The child's height is compared to that of a reference population in order to judge whether the child is atypical in terms of the height distribution of this population. Child and reference population must share the same geographical and socioeconomic background. Also, parental or familial height should be taken into consideration to assess the relative contribution of genetic and other factors to the child's height at any particular age.

Since growth is a dynamic process, one isolated measurement in time is meaningless for judging growth. Repeated measurements are needed to calculate the growth velocity. Knowledge of growth velocity is particularly relevant when dealing with a sick child. Depressed growth velocity may indicate the severity of an illness, whereas normalization of growth velocity or catch-up growth may signal recuperation.

Height is the distance between the top of the head and the sole of the foot. This distance is the sum of the height of the skull, the length of the spine, and the length of the lower extremities. Each of these different parts of the body has its own age-dependent growth velocity. Therefore, height is the result of the sum of different values, which are not linearly related in time.

Method of measurement

The distance between the top of the head and the sole of the foot is called length when an individual is measured lying down and height when the individual is measured standing upright.

Height is measured using a stadiometer. The stadiometer comprises a rigid vertical backboard and a horizontal headboard running free, perpendicular to the backboard and without cross-play. The top of the head must be in contact with the headboard. A 0.5 kg weight is placed on the headboard. This serves the purpose of flattening the child's hair and frees the physician's hands so as to enable him to keep the child in the correct upright position.

To measure standing height, the subject's shoes and socks are removed. The child is placed so that his heels, buttocks, and shoulders are in contact with the vertical plane of the stadiometer. The child's feet must be flat against the floor while either ankles or knees remain in contact. The child's head is held in the "Frankfurt plane": the lower borders of the orbits are in the same horizontal plane as the

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external auditory meati. The measurement is taken while a gentle upward pressure is exerted on the mastoid processes so that the child is fully extended. In older children, stretching is achieved by telling them to breathe deeply.

To measure supine length, a measuring table resembling a stadiometer is used but on a horizontal plane. The headboard is fixed and the footboard is movable. Both the headboard and the footboard must be large enough to ensure that both the top of the head and the feet are in contact with them. To measure supine length, two people are necessary: one holds the infant's head in the vertical Frankfurt plane while at the same time another individual keeps the child's legs straight. The child's shoulders and buttocks must be in contact with the table. Supine length is measured in the first 2 years of age.

Crown Rump Length, Sitting Height, and Subischial Leg Length

As stated earlier, height defined as the distance between the top of the head and the sole of the foot is the result of the sum of different values, which are not linearly related in time. Therefore, the first step is to split height into its different components. Generally, sitting height is taken as one of the components and compared to height. The relationship between sitting height and height is often expressed as an age-dependent ratio.

Method of measurement

The distance between the top of the head and the buttocks is called sitting height when the measurement is taken of a child sitting upright and is called crown rump length when an infant is measured lying down.

Sitting height is measured using a sitting height table. The table comprises a rigid vertical backboard and a horizontal headboard running free perpendicular to the backboard and without cross-play. The surface of the headboard must be in contact with the top of the head. A 0.5 kg weight is placed on the headboard. This weight flattens the child's hair and also frees the physician's hands so as to be able to keep the child in the correct position. The child must be in the sitting position with his feet on a footrest so that his full weight is on his buttocks. Insofar as is possible, arching of the back is avoided by gently applying upward pressure to the mastoid processes. In older children, stretching of the back is achieved by asking them to breathe deeply. The child's head is held in the Frankfurt plane: the lower borders of the orbits are in the same horizontal plane with the external auditory meati.

The recommended instrument for measuring crown rump length is similar to the stadiometer used for supine length. The headboard is fixed and the footboard is movable. Headboard and footboard must be large enough to ensure that the most protruding points of both head and buttocks are in contact with the boards. In order to measure this length, an assistant holds the infant's head in the vertical Frankfurt plane while the physician holds the child's legs at a 90 degree angle with the table. When this is achieved, the footboard is pressed against the buttocks.

Subischial leg length is defined as the arithmetic difference between height and sitting height or between supine length and crown rump length.

Head Circumference

The head circumference is routinely measured in newborn infants since it correlates well with skull volume. Skull volume is highly correlated with gestational age, body weight, and body length. Since in intrauterine growth retardation, the brain is less affected than the weight and the length, the extent of the discrepancy between these measurements in the newborn will be an indicator of the severity of the retardation.

Because of the fast growth velocity of the head circumference, especially during the first year of life, its measurement provides important information about the general condition of the child. In full-term healthy newborns, the head circumference increases approximately 1 mm/day initially.

Method of measurement

To measure head circumference, a fiberglass-reinforced tape of nonstretchable material is used. The tape is placed around the head at the most protruding points of occiput and forehead. In younger children, the tape is placed just above the brow ridges. The tape is placed gently so as to leave no marks after removal.

Limb Length

Arm span, upper arm length, lower arm length, hand length, tibia length, and foot length

The measurement of the limbs so as to describe body proportions is an important tool when evaluating development. Arm span is the most common way to measure upper limb length. However, by measuring the span of the out-stretched arms from the tips of the longest fingers, the distorting information of the width of the trunk is added. What is required, however, is information on the growth of the long bones alone. Furthermore, the long bones of arms and legs can be accurately measured because of its bony marks. Hand and foot mature at an earlier age than other parts of the body. Therefore, the changing relationship of hand and foot to height is a useful criterium of the maturation of the child.

Method of measurement

To measure arm span, a measuring rod can be used. Standing with the arms fully extended, the distance between the tips of the stretched middle fingers is measured.

To measure the different parts of the arm, the arm should be relaxed and extended along the child's side. The bony prominences are used as anatomical landmarks. To measure the upper arm length, the lateral border of the acromion and the proximal head of the radius are palpated distal to the lateral epicondyle of the humerus. To measure the lower arm length, the proximal and distal heads of the radius are used. These landmarks are marked with a pen and the distance between them is measured. To measure the length of the hand, the distance between the tip of the longest finger and the distal head of the radius is measured with the hand and forearm flat on a table.

For tibia length, the distance between the proximal–medial border of the tibia and the distal border of the medial malleolus is measured with the Harpenden anthropometer.

The foot is measured while the child is standing. The distance between the most posterior part of the heel and the tip of the longest toe, normally the first toe, is measured on the left foot.

Biacromial Diameter and Biiliacal Diameter

Puberty is characterized auxologically by increasing diameter of shoulders and hips. The ratio of biacromial diameter to biiliacal diameter is a good index of sexual maturation. Biacromial and biiliacal diameter curves remain almost identical in both sexes until puberty. The increase of the biacromial diameter during puberty is more striking in boys than in girls. The change of the ratio between biacromial and biiliacal diameter signals the onset of puberty.

Method of measurement

With the Harpenden anthropometer, measurements of shoulder widths and pelvic widths are taken. When the child relaxes its shoulders, the distance between the most lateral borders of the acromial processes is measured to obtain the biacromial diameter. To measure the biiliacal diameter, the branches of the anthropometer are moved over the iliac crests at their widest point and the distance between the two blades of the anthropometer is measured. In children aged 0–1 year, it is not always possible to take these measurements because of unrest or obesity. Also in older children, the biiliacal diameter is hard to obtain in the case of obesity.

Photometry

Introduction

Manual measurement of body dimensions is common practice in pediatrics. However, it is a highly time-consuming procedure requiring a variety of measurement tools and the skills to use them. Even though it is likely to remain the golden standard for some time to come, alternative methods exist that allow for much faster measurement of body dimensions. One of the most promising of these methods is photometry that involves measurement of body dimensions on the basis of digital images. The overall procedure exists in taking a frontal and lateral picture of the subject, importing these pictures into photometry software, and measuring the body part dimensions by clicking anatomical points in the pictures using a mouse device.

The main advantages of the photometry method are (1) speed, (2) ease of use, and (3) flexibility. After taking the pictures, the whole procedure can be done in a few minutes by anyone with the required knowledge about the correct locations of each anatomical point. The flexibility of the method follows primarily from the separation of the procedure into multiple steps that can be performed at different points in time and place. The actual presence of the subject is only needed during the photography session. Transfer of the pictures and selection of anatomical points can all be done at a time most convenient for the measurer. Pictures are stored in digital format so they can be easily transferred across a computer network to a remote location for further processing. In most cases, a professional photographer will do photography. However, given some assistance this is also feasible for lay people, for example, parents taking pictures of their children.

Method of measurement

The first step of the photometry method involves photography of the subject together with a reference object of known length or size. Two pictures are taken, one from the front (frontal) and one from the side (lateral). The lateral picture is needed to measure foot length. The remaining lengths are measured in the frontal picture. It is important to take the picture of the subject standing or lying in the right position as described earlier. The reference object can be a vertical rod or a square mat lying on the floor, both of known size. It is needed in order to determine the correspondence between pixels in the image and length in centimeters. Once the reference object is indicated by two points in case of a line or by four points in case of a square mat, the correspondent distance manually measured in centimeters is imported in the computer program. Thereby the point selections on the computer screen are defined in pixel coordinates and a conversion from pixels to centimeters will be performed. The user can select the end-points of each body part after which the application automatically calculates the distance in centimeters (see

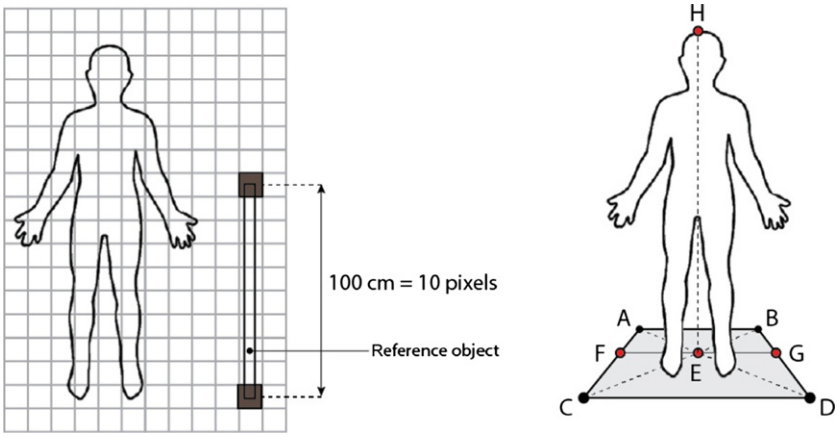

Fig. 1 Calculating centimeters per pixel using a vertical reference object (*left*) or a square mat (*right*). Using the corner points A–D, the intersection point E is calculated first, followed by the distance F–G in pixels. Since F–G is also known in centimeters, the centimeters per pixel ratio is obtained. To determine total body height, only point H has to be manually selected (point E is calculated automatically).

Fig. 1). When the user has finished measuring the body dimensions, the measurement can be saved. The user can easily perform and save additional measurements for the same subject in order to capture changes in body proportions over time. These measurements can be visualized inside the different growth curves of all body parts as used in the Pediatric Morphometrics atlas of Gerver and de Bruin.

Calculating the different body proportions

The measurements of height, biacromium width, biiliacum width, upper arm length, lower arm length, hand length, and foot length are performed by selecting the anatomical reference points in the photographs also used in the manual measurements. The *Paediatric Morphometrics* software automatically calculates the distances between the selected points.

Total sitting height cannot be allocated with the use of anatomical reference points in a frontal photograph. Therefore, this measurement was estimated by using just a part of the sitting height distance that can be selected accurately in the frontal plane, namely the distance between the nasal root and the umbilicus (*NaUm*), adapted by the linear regression formula:

$$SH_{ph} = 1.28^{*}NaUm + 13.7$$

where SH_{ph} is the calculated sitting height in the photograph in centimeters and *NaUm* is the distance between the nasal root and the umbilicus in centimeters ($r^2 = 0.94$).

Because arm span is measured in a different position manually than in the photograph, the arm span is estimated using the formula:

$$ArmSpan_{ph} = 0.886^{*}ArmSpan_{sum} + 4.4$$

where *Arm* $Span_{ph}$ is the calculated arm span in the photograph in centimeters and *Arm* $Span_{sum}$ is the sum of the biacromium width plus two times the length of the upper arm, lower arm, and hand in centimeters.

Calculating body composition

Body mass index (BMI) and waist circumference are used in daily pediatrics to classify children according to their weight. However, both give no insight in body composition such as the amount of body fat or fat free mass. This information is of importance because it has been proven to be more accurate in the assessment of metabolic risks such as the development of the metabolic syndrome. Unfortunately, established measurement methods of body composition such as the Deuterium dilution method or air displacement plethysmography (Bodpod) are cumbersome, costly, and not easily applicable outside research settings. Therefore, the use of photogrammetric anthropometry was investigated to estimate body composition in the two-dimensional picture. It was shown that the ratio between biacromial width and waist width (Biac/Ww), as shown in Fig. 2, correlated strong with fat mass as measured by the earlier-mentioned golden standard methods. Waist width is defined as the widest distance of the waist and not necessarily at the location of the biiliacal measurement.

Fat mass can be estimated as

$$\mathrm{Fm}(\%) = 105.83 - 67.61^{*}\left(\frac{\mathrm{Biac}}{\mathrm{Ww}}\right)$$

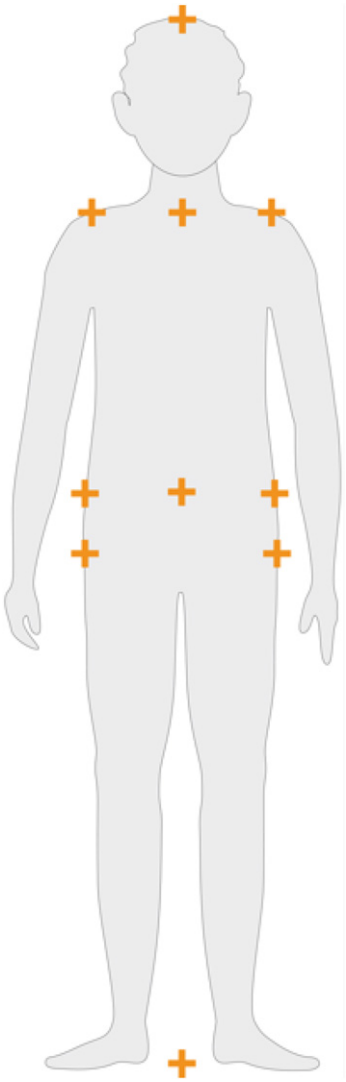

Fig. 2 To calculate body composition the biacromial diameter (Biac) and the widest distance of the waist width (Ww) are used. This distance is mostly below the biiliacal diameter as shown in the picture.

Calculating shape ages

The photometry software also allows automatic calculation of shape ages. This is the age the subject would have if his or her body measurement is considered to be average (i.e., at the p50 level). For example, take the particular height measurement of a subject of about 10.5 years old, plotted on the distance curve of the reference population. His particular shape age can be found by tracing a horizontal line from his measurement to the left or right until it intersects the p50 curve. At the intersection point the corresponding age is taken as the shape age, for example 8 years for this subject. Using the photometry software, the shape age is calculated automatically.

Interpretation of Body Proportions

The most commonly used ratios in anthropometry include: sitting height/height (SH/H), biacromium/biiliacum (Biac/Bill), and subischial leg length/sitting height (LL/SH).

To judge the body proportions of an individual, one can make use of the different expressions:

1. One can take the ratio of two measurements such as sitting height divided by height or even better the subischial leg length divided by sitting height. Ratios are age-dependent and the curve changes in time. However, the use of ratios can be misleading for two reasons: first, two ratios may be equal, while their nominators and denominators are not; and second, when a change in the nominator automatically leads to a change in the denominator, the change of the ratio will be even more misleading.

2. A more straightforward approach is to simply plot one measurement against another. In this way one can plot height against sitting height and an even clearer method is to use sitting height and subischial leg length as variables for comparison, since a change in sitting height will automatically induce a change in height but not a change in subischial leg length. It is better to use sitting height and subischial leg length as variables to compare than the use of height and sitting height because sitting height is part of height and a change in sitting height will automatically induce a change in height which is not the case when using two independent variables.

In the same way, a number of measurements can be compared to each other such as foot length to height, head circumference to supine length, arm span to height, biacromial to biiliacal diameter, and upper arm to lower arm.

First one calculates the standard deviation score, $z(t)$, of each measurement by

$$z(t) = \frac{x(t) - \mu(t)}{\sigma(t)}$$

where $x(t)$ denotes the individual's body measurement score at age t (e.g., height), $\mu(t)$ denotes the population mean at age t, and $\sigma(t)$ denotes the corresponding standard deviation.

Second, the squared distance D^2 is calculated as follows:

$$D^2 = \left[1 - r^2\left(h, h'\right)\right]^{-1}\left[z_h^2 + z_{h'}^2 - 2z_h z_{h'} r^2\left(h, h'\right)\right]$$

where r denotes the correlation coefficient of the two measurements h and h' according to **Table 1**.

$D^2 \leq 5.991$ indicates that the considered individual is typical or normal, whereas $D^2 \geq 5.991$ suggests that something is wrong with size and/or shape.

Graphically, one can display the atypicality of a particular individual by constructing an ellipse, which comprises 95% of the pairs of scores (**Fig. 3**).

Example

If a boy of 6.5 years has a height of 134.6 cm and a sitting height 63.5 cm, the corresponding z-scores for height and sitting height are $zh = 0.92$ and $zsh = -1.5$.

These scores considered separately do not indicate that something is wrong with this child. The difference in sign, however, is somewhat alarming because the correlation between the measurements is positive.

Table 1 Correlation coefficient of pairs of measurements of body proportions according to sex

	Boys	*Girls*	*Mean*
Height/arm span	0.87	0.85	0.86
Height/sitting height	0.82	0.82	0.82
Height/tibia length	0.80	0.80	0.80
Height/foot length	0.75	0.66	0.70
Height/hand length	0.76	0.64	0.70
Biacromial/biiliacal diameter	0.60	0.50	0.55

Note: These correlations are almost independent of age for children between the ages of 3 and 17 years.

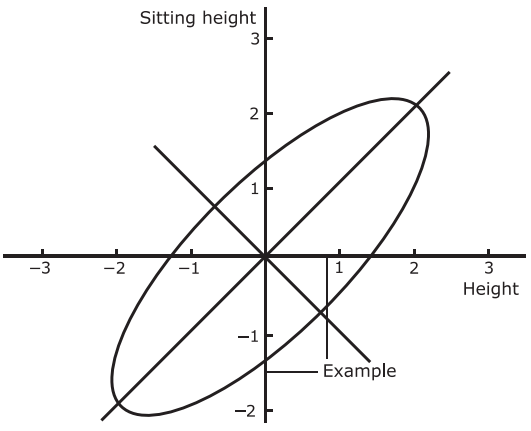

Fig. 3 The 95% confidence limits of the pair of z-scores of height and sitting height. The example is the patient mentioned in the text. With the consequence that the two scores considered together indicate atypicality. This is interesting because, as discussed earlier, separate considerations did not indicate sufficient evidence of abnormality.

Using the value 0.82 for the correlation coefficient between height and sitting height (Table 1), one obtains

$$|z_h - z_{h'}| / \left[2 + 2r\left(h, h'\right)\right]^{1/2} \geq 1.960 = 16.05$$
$$\geq \chi^2_{2.05} = 5.991$$

Conclusion

Pediatricians are often confronted with children of all ages who are referred for growth problems, such as growth retardation or dimorphism. One of the incentives of this article was to provide methods that will give the possibility of discerning growth variation according to typical or atypical patterns. Besides the techniques of measuring children manually also a new method is presented by means of photometry. It provides the possibility to be informed about the proportions of a child based on several measurements within a short time.

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Genetic and Hormonal Control of Growth

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Abbreviations

ACTH	Adrenocorticotropic hormone
AMP	Cyclic adenosine monophosphate
BDNF	Brain derived neurotrophic factors
BMPs	Bone morphogenetic proteins
CRH	Corticotropin-releasing hormone
EGFs	Epidermal growth factors
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FGFs	Fibroblast growth factors
GABA	Gamma-aminobutyric acid
G-CSF, MCSF	Macrophage colony-stimulating factors
GDNF	Glial derived neurotrophic factors
GH	Growth hormone
GHBP	Growth hormone binding protein
GHRH	Growth hormone releasing hormone
GHRHR	Growth hormone releasing hormone receptor
HGF	Hepatocyte growth factor
HNFs	Hepatic nucleic factors
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IHH	Indian hedgehog
IL	Interleukin
IRSs	Insulin receptor substrates
JAK	Janus kinase
KGF	Keratinocyte growth factor
LCR	Locus control region
Lhx4	LIM/homeobox protein 4
MAPKs	Mitogen-activated protein kinases
NGF	Nerve growth factor
PDGF	Platelet derived growth factor
PIAS	Protein inhibitors of activated Stats
PKC	Protein kinase C
Pou1f1	POU-domain transcription factor Pit1
Prop1	Prophet of Pit1
PTH	Parathyroid hormone
PTPs	Protein tyrosine phosphatases
RGD	Arginine-glycine-aspartic acid
SH	Src homology
SHH	Sonic hedgehog
SOCS	Suppressors of cytokine signaling
STAT	Signal transducer and activator of transcription
TGFβ	Transforming growth factor-beta
TNF	Tumour necrosis factor
TRH	Thyroid hormone-releasing hormone
VEGF	Vascular epithelial growth factor
VIP	Vasoactive intestinal polypeptide
WHO	World Health Organization
WNT4	Wingless-related integration site 4

Introduction

Growth is a complex, dynamic process in which an interplay of multiple factors determines not only the final height attained, but also the rate and timing of height increase in a remarkably predictable manner (Murray *et al.*, 2016; Brook *et al.*, 2009). A growing understanding of the spectrum of hormones and their mechanisms of action, as well as advances in molecular genetics, has helped to elucidate the physiology of growth and define the pathological basis of many endocrine growth disorders. This chapter provides an overview of the hormonal and genetic control accounting for variations in normal growth.

Physiology of Growth: The Infancy-Childhood-Pubertal Model of Growth

The rate of linear growth and its regulation varies with age. It is useful to recognize growth as occurring in four discrete but congruent phases (fetal, infancy, childhood, and adolescence), each with different predominating mechanisms (**Fig. 1**) (Tanner, 1962).

Fetal growth peaks at the end of the second trimester at approximately 10 cm/month. The placental supply of nutrients is the principal rate-limiting step in fetal growth. However, the placenta is a unique endocrine organ, actively producing a variant of growth hormone, human placental lactogen, CRH and epidermal growth factors. IGF-1 and -2, produced by the fetus in response to nutrition, are also crucial for growth. Infantile growth is initially an extension of fetal growth and then becomes hormone-dependent, as the hypothalamic-pituitary axis becomes increasingly dynamic. Early growth in height and weight requires adequate nutrition, and also normal thyroid function and bone metabolism. Growth hormone is critical for normal growth, even within the first six months of life. Childhood growth requires growth hormone, and this binds to its somatogenic receptors resulting in the production of IGF-1, the principal postnatal growth factor acting on bone epiphyses and stimulating growth. IGF-1

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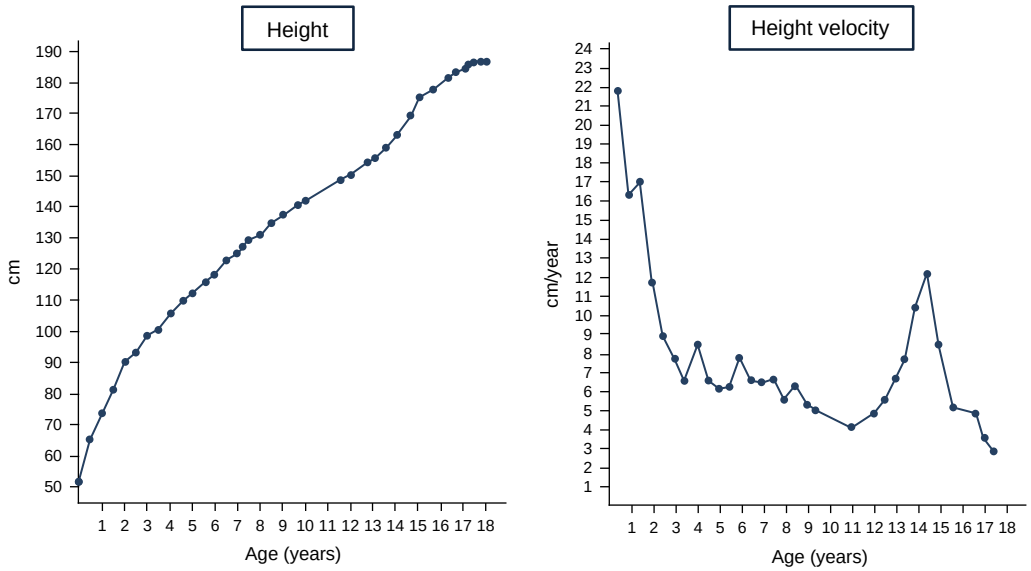

Fig. 1 Height and height velocity through childhood. The concept of plotting body measurements to illustrate growth pattern is attributable to Count Philbert de Montbeillard. The oldest known growth charts are from the 18th century of his son whose height was plotted every six months from birth to age 18 years (1759–1777). George Buffon published the chart in his *Histoire Naturelle*, producing the first growth curve for height (Tanner, 1962). Adapted from Growth and puberty, John W. Gregory. https://obgynkey.com/growth-and-puberty/.

feeds back at the level of both the pituitary and hypothalamus to negatively regulate GH secretion from the pituitary. This can be modulated by input from higher centers, for example severe psychosocial deprivation can result in reversible GHD. At the time of puberty, production of the sex steroids (estrogen and testosterone produced by the gonads) leads to a 2-3-fold increase in GH secretion; subsequently concentrations fall to pre-pubertal ranges and then decline further from middle age onwards ("the somatopause"). Whilst childhood growth occurs predominantly in the limbs, sex steroids augment growth of the spine in particular, and estrogen in both sexes causes fusion of epiphyses, marking the end of growth.

Clinical Evaluation of Growth

Growth should be frequently and accurately assessed as an integral component of health, as a variety of disease processes (endocrine and non-endocrine) can manifest as abnormal growth in children (Murray *et al.*, 2016; Brook *et al.*, 2009). Clinical assessment of growth is a standardized process and accurate, appropriate measurements should be taken by a trained observer, using well-calibrated and maintained equipment, and evaluated in the context of cross-sectional or longitudinal normal data (Tanner, 1986). Growth charts are constructed using growth references, which are usually compiled using data from normal healthy children. All growth references presently in use are descriptive of current growth patterns and are therefore "references" and not "standards" that define an optimum growth pattern (Wright *et al.*, 2002). The World Health Organization (WHO) growth charts are based on healthy breastfed children, and help define how all children from birth to 5 years should grow, irrespective of ethnicity (Wright *et al.*, 2013). It should be noted that growth charts are clinical tools that require careful design, instructions for use and evaluation before introduction to clinical practice (Wright *et al.*, 2013). In addition, disease-specific growth curves have been developed for use in clinical conditions associated with growth failure (Saari *et al.*, 2013). Unexplained acceleration or deceleration of height velocity, or abnormal growth states characterized by disproportionate growth, warrant further evaluation (Dattani and Preece, 2004).

In addition to linear growth, the progression of ossification within the epiphyses follows an expected sequence (normal skeletal maturation), indicative of the genetic potential of growth inherent in the tubular bones (Greulich and Pyle, 1959; Tanner, 1983). This "bone age" can clinically be usefully employed to predict final height potential (Tanner *et al.*, 1975; Roche *et al.*, 1975).

Lastly, since the genetic potential is of great importance as a determinant of final height, a child's stature should be evaluated relative to that of siblings and parents *via* a calculation of parental target height and the respective standard deviations (Tanner *et al.*, 1975). If a child's growth rate is noted to be tracking along a disparate percentile for their family, this should prompt investigation into the possibility of an underlying pathological process (Dattani and Preece, 2004).

Hypothalamo-Pituitary Development

The neuroendocrine network between the hypothalamus and pituitary gland is responsible for the regulation of growth by coordinating signals from the brain to key target organs (Bancalari *et al.*, 2012). The pituitary is located deep within the brain

parenchyma in the center of the cranial base, within a bony cavity known as the *sella turcica* (McCabe and Dattani, 2014). Two distinct components of the pituitary become evident during embryogenesis: the adenohypophysis (anterior and intermediate lobes) and neurohypophysis (posterior lobe) (McCabe and Dattani, 2014; Solov'ev *et al.*, 2008; Ooi *et al.*, 2004). The neurohypophysis arises from neural ectoderm, from the floor of the forebrain, and contains the terminal axonal projections of magnocellular neurons from the paraventricular and supraoptic nuclei of the hypothalamus (Ooi *et al.*, 2004). It produces oxytocin and vasopressin that do not play a role in human growth (Mastorakos and Ilias, 2003). The adenohypophysis (80% of pituitary weight) arises from stomodeal ectoderm, a diverticulum of the primitive oral cavity (Rathke's pouch) (McCabe and Dattani, 2014; Asa *et al.*, 1991). The adenohypophysis consists of five different endocrine cell types, each described by the hormone it produces and by the nature of their appearances on hematoxylin and eosin staining – the acidophils are somatotrophs that secrete growth hormone (GH). The adenohypophysis can be anatomically subdivided into the *pars distalis* (pars anterior or anterior lobe), the *pars intermedia* (intermediate lobe), and the *pars tuberalis* (pars infundibularis or pars proximalis) (Asa *et al.*, 1991; Ikeda *et al.*, 1988). *Pars distalis* in humans is the largest component of the adenohypophysis containing most hormone-producing cells (Asa *et al.*, 1991). *Pars intermedia* is typically poorly developed and consists of several cystic cavities lined by a single layer of cuboidal epithelium. *Pars distalis* and *intermedia* are separated by a cleft, a vestigial structure of Rathke's pouch from which it develops (Han *et al.*, 2014). This structure may often develop as a cyst (Rathke's cleft cyst) (Han *et al.*, 2014). In humans, *pars intermedia* is rudimentary as it largely disappears during embryogenesis (Solov'ev *et al.*, 2008). The *pars tuberalis* represents an upward extension of the *pars distalis* onto the pituitary stalk and may contain a limited number of gonadotrophin-producing cells (Solov'ev *et al.*, 2008). Although there is little direct evidence for the physiological processes underlying pituitary development in humans, development of the pituitary gland in the mouse is well-characterized, and the process appears to be highly conserved across all vertebrates (Rosenfeld *et al.*, 2000; Kelberman *et al.*, 2009). The pituitary placode appears at mouse embryonic day (E) 7.5 with a thickening of the ectoderm in the midline of the anterior neural ridge, forming the hypophyseal placode (Rosenfeld *et al.*, 2000; Dearden and Holmes, 1976). The onset of pituitary organogenesis corresponds to 4-6 weeks gestation in humans. At E9.0 in the mouse, the placode invaginates and forms the rudimentary Rathke's pouch from which the anterior and intermediate lobes of the adenohypophysis are derived. Between E10.5-12.0, the pouch epithelium continues to proliferate and is completely detached from the oral cavity at E12.5 to form the definitive Rathke's pouch (Dearden and Holmes, 1976). During the beginning of its development it is associated with developing hypothalamic territories, and later with the developing diencephalon (Solov'ev *et al.*, 2008; McCabe and Dattani, 2014; Treier and Rosenfeld, 1996). In humans, Rathke's pouch can be identified in the 3-mm embryo during the third gestational week (Han *et al.*, 2014). Subsequently during its development, a complete pouch is gradually formed and finally is disconnected from the oral ectoderm by the end of the sixth gestational week (Han *et al.*, 2014). In the murine embryo, the progenitors of the hormone-secreting cell types proliferate ventrally from the pouch between E12.5-17.5 and populate what will form the mature anterior lobe. The remnants of the dorsal portion of the pouch will form the intermediate lobe, whilst the lumen of the pouch remains as the pituitary cleft separating the two lobes of the adenohypophysis. Somatotrophs can be identified by 9 weeks gestation in human pituitary development, a time point that coincides with the development of vascular connections between the anterior pituitary and the hypothalamus (Asa *et al.*, 1988; Gorczyca and Hardy, 1987; Stanfield, 1960). The pituitary gland has a dual embryonic origin: the anterior and intermediate lobes are derived from oral ectoderm, and the posterior pituitary from neural ectoderm. The close apposition and interaction of these two ectodermal layers throughout neurodevelopment is crucial to the formation and function of a normal pituitary gland (Kelberman and Dattani, 2009; Davis *et al.*, 2010; Ericson *et al.*, 1998). This process is dependent upon the sequential temporal and spatial expression of a cascade of signaling molecules and transcription factors that play a crucial role in organ commitment, cell proliferation, patterning and terminal differentiation (**Fig. 2**) (Kelberman *et al.*, 2009).

Several studies have shown that normal pituitary development is mediated by the spatio-temporal expression of a complex cascade of transcription factors in response to signaling molecules from the surrounding tissues of the developing gland (Cohen, 2000; Parks and Brown, 1999; Davis *et al.*, 2010; Kerr *et al.*, 2008; Potok *et al.*, 2008). These signaling molecules are either intrinsic within the oral ectoderm, such as Sonic hedgehog (SHH), or extrinsic from the neuroectoderm (BMP4, FGF8, FGF4, NKX2-1, WNT5A), surrounding mesenchyme (BMP2, Indian hedgehog (IHH), chordin) and the pouch itself (BMP2, WNT4), and create a network of signaling gradients important for early pituitary morphogenesis (Gregory *et al.*, 2015; Zhao *et al.*, 2012; Guner *et al.*, 2008; Silberschmidt *et al.*, 2011; Labeur *et al.*, 2010; Shah and Kakar, 2011; Miyakoshi *et al.*, 2008; Aujla *et al.*, 2011).

These signaling molecules activate or repress transcription factors as part of a complex cascade that includes the POU-domain transcription factor *Pit1*, recently renamed *Pou1f1*, and its predecessor Prophet of Pit1 (*Prop1*), LIM homeodomain factors Islet1 (*Isl1*), *Lhx3*, and *Lhx4*, the pituitary homeobox genes *Pitx1* and *Pitx2*, and the Rathke's pouch homeobox gene *Hesx1* (also known as *Rpx*) (Kato *et al.*, 2010; Colvin *et al.*, 2009; Potok *et al.*, 2008). Birth-dating studies have revealed that the enrichment of cell types at specific positions along the caudal to rostral axis in newborn mice does not result from an ordered cell cycle exit for each cell type; cells exiting the cell cycle concurrently are dispersed throughout the anterior lobe of the pituitary (Davis *et al.*, 2011). Hence, the clustering of cell types in specific regions of the anterior lobe may be the result of an as yet undefined mechanism including network formation or lateral inhibition (Davis *et al.*, 2011).

Spontaneous and artificially induced mutations in the mouse have led to significant insights into human pituitary disease. Identification of mutations in genes implicated in normal hypothalamo-pituitary development that are associated with human pituitary disease has also been invaluable in defining the genetic cascade of pituitary development. The advent of new technologies such as whole genome sequencing has resulted in an expanding list of genetic factors involved in human hypothalamo-pituitary disease (Haston *et al.*, 2017; Fang *et al.*, 2016) (see **Tables 1** and **2**). Importantly, the Wnt signaling pathway has been implicated in pituitary organogenesis and

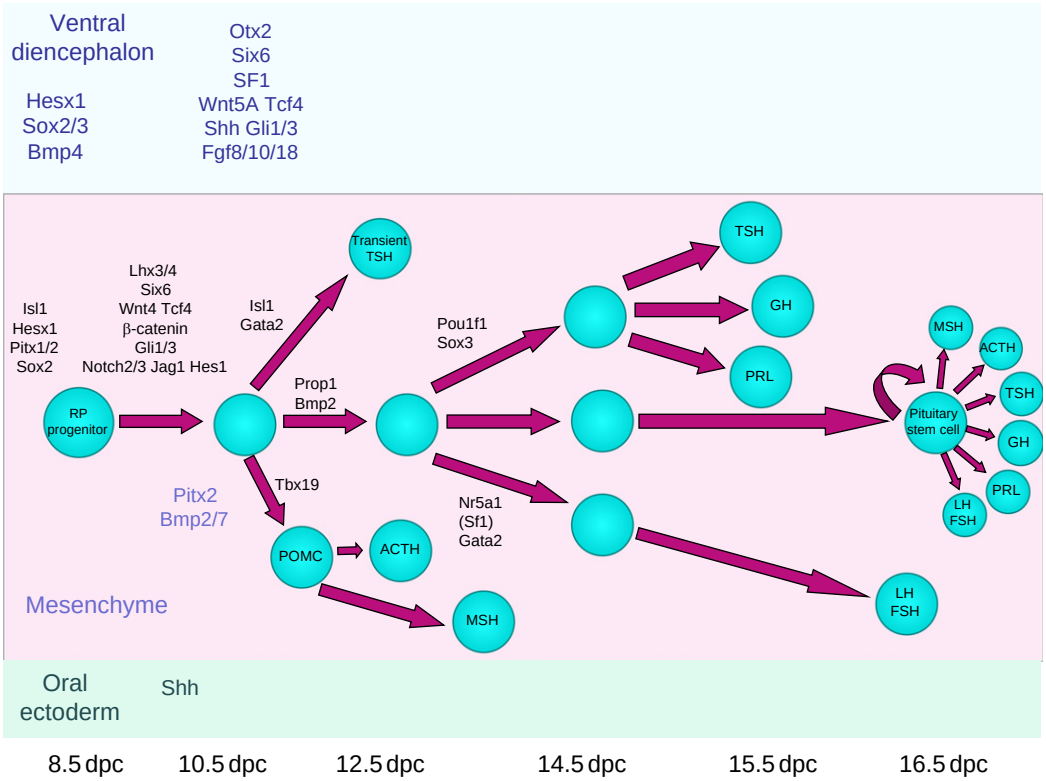

Fig. 2 Schematic representation of the developmental cascade of transcription factors and signaling molecules involved in anterior pituitary development. From Kelberman, D. and Dattani, M. T. (2009). Role of transcription factors in midline central nervous system and pituitary defects. *Endocrine Development* 14, 67–82.

Table 1 Genetic disorders of hypothalamo-pituitary development resulting in isolated growth hormone deficiency

Gene	*Phenotype*	*Inheritance*
GH1	GH deficiency	AR, AD
GHRHR	GH deficiency	AR
RNPC3	GH deficiency	AR

AR; autosomal recessive, AD; autosomal dominant, XL; X-linked.

tumorigenesis (Cha *et al.*, 2004; Potok *et al.*, 2008). Evidence for Wnt signaling in pituitary development is derived from conditions associated with pituitary abnormalities that follow disruption of Wnt5a and Wnt4 (Cha *et al.*, 2004). Finally several microarray studies have identified altered expression of Wnt inhibitors in pituitary tumors and there is clear evidence that the Wnt/β catenin pathway is involved in the pathogenesis of craniopharyngioma, a rare tumor in the hypothalamo-pituitary region (Elston and Clifton-Bligh, 2010; Andoniadou *et al.*, 2012; Gaston-Massuet *et al.*, 2011).

Endocrine Regulation of Normal Growth

Growth Hormone Axis

Endocrine regulation of normal growth classically depends upon the growth hormone (GH) axis, where stimulatory growth hormone releasing hormone (GHRH) and inhibitory somatostatin from the hypothalamus modulate GH secretion (Brook *et al.*, 2009). GH, the main hormonal mediator of growth, stimulates the secretion of insulin-like growth factor (IGF)-1 from the liver, and this mediates most of the anabolic actions of GH (somatomedin hypothesis) (Kaplan and Cohen, 2007). GH also stimulates metabolic effects independently of IGF-1 activity (lipolysis, amino acid transport in diaphragm and heart, and production of specific hepatic proteins) (Green *et al.*, 1985; Kaplan and Cohen, 2007; Mauras and Haymond, 2005)

Table 2 Genetic disorders of hypothalamo-pituitary development resulting in syndromic hypopituitarism or combined pituitary hormone deficiencies.

Gene	*Endocrine phenotype*	*MRI phenotype*	*Other associations*	*Inheritance*
Syndromic hypopituitarism				
HESX1	Panhypopituitarism; GH deficiency; GH with evolving ACTH and TSH deficiencies	APH, EPP, ONH, ACC; normal AP with EPP and ONH	Septo-optic dysplasia and its variants	AD
	Panhypopituitarism; GH, LH, FSH, evolving ACTH and TSH deficiencies	APH, EPP, ONH, ACC; normal ON with EPP and APH; pituitary aplasia with normal PP and ON; pituitary aplasia with normal PP and ON coloboma		AR
TCF7L1	Isolated GH deficiency or low IGF-1, mildly elevated TSH with normal fT_4	APH, absent PP, ONH, thin optic tracts, partial agenesis of the corpus callosum, absent septum pellucidum, thin anterior commissure	Septo-optic dysplasia	AD with variable penetrance
LHX3	GH, TSH, PRL, LH, FSH deficiencies; ACTH deficiency may evolve	APH, enlarged/ cystic AP, normal PP and stalk	Limited neck rotation, short cervical spine, sensorineural deafness	AR
LHX4	Panhypopituitarism; GH with variable TSH, ACTH, LH and FSH deficiencies	APH, normal PP or EPP, Chiari malformation, cerebellar abnormalities	–	AD
	ACTH, TSH, PRL and probable GH deficiencies	Pituitary aplasia, EPP	Lethality in the first weeks of life with severe sepsis, poor tone, lung atelectasis, mid-facial hypoplasia, low-set ears	AR
SOX2	LH and FSH deficiencies, rarely GH deficiency	APH, thin CC; hippocampal abnormalities; hypothalamic hamartoma; slow-progressing hypothalamo-pituitary tumour	Bilateral/ unilateral anophthalmia, spastic diplegia, developmental delay, trachea-esophageal fistula, sensorineural deafness	AD
SOX3	Panhypopituitarism; GH, TSH, ACTH, LH and FSH deficiencies; isolated GH deficiency	APH, EPP; persistent craniopharyngeal canal	Variable mental retardation	XL
OTX2	Isolated or partial GH deficiency; GH, TSH, ACTH, LH and FSH deficiencies	Normal pituitary; APH, EPP, Chiari malformation	Anophthalmia, bilateral/ unilateral retinal dystrophy; normal eye phenotype	AD
ARNT2	Central diabetes insipidus, GH, ACTH, TSH deficiencies	APH, absent PP, thin CC; frontal & temporal lobe hypoplasia, large Sylvian fissure	Microcephaly, seizures, visual impairment, renal tract abnormalities	AR
Gene	*Endocrine phenotype*	*MRI*	*Other associations*	*Inheritance*
GLI2	Panhypopituitarism; GH, TSH, ACTH, LH and FSH deficiencies; isolated GH deficiency	APH, normal PP or EPP, hypoplastic PP, ONH, holoprosencephaly, cavum septum pellucidum	Mid-facial defects, cleft lip/palate, single central incisor, postaxial polydactyly	AD
PITX2	Reduced GH concentration	Sella turcica hypoplasia	Axenfeld-Rieger syndrome (malformation of the anterior segment of the eye, dental hypoplasia, protuberant umbilicus, brain abnormalities)	AD
*FGF8**	Borderline peak GH concentration	ACC, ONH	Moebius syndrome, spastic diplegia, developmental delay	AD
	Central diabetes insipidus, ACTH and TSH deficiencies	Semilobar holoprosencephaly, bulky AP, normal PP	Microcephaly, micrognathia, high arched palate	AR
*FGFR1**	GH, ACTH, TSH, LH and FSH deficiencies, central diabetes insipidus	APH, EPP, thin or normal pituitary stalk, ACC, ONH	Cleft lip/ palate, brachydactyly, single central incisor	AD
*PROKR2**	GH, ACTH, TSH, LH and FSH deficiencies; isolated GH deficiency	APH or normal AP, EPP, absent pituitary stalk, CC dysgenesis	Facial asymmetry, schizencephaly, cerebellar hypoplasia, hypoplastic optic discs	AD
PNPLA6	GH, TSH, LH and FSH deficiencies; isolated hypogonadotrophic hypogonadism	APH, cerebellar hypoplasia	Oliver-McFarlane and Gordon-Holmes syndromes; ataxia, chorioretinal atrophy, spasticity	AR

(*Continued*)

*KAL1**	GH, ACTH and TSH deficiencies	APH, ONH	Septo-optic dysplasia, females affected	XL
Combined pituitary hormone deficiencies				
POU1F1	GH, PRL and TSH deficiencies (TSH deficiency may present early or develop later)	APH or normal AP, normal PP and infundibulum, no extra-pituitary abnormalities	–	AD, AR
PROP1	GH, TSH, PRL, LH, FSH and evolving ACTH deficiencies with variable time of onset and severity	APH, normal or enlarged AP that may change over time, normal PP and stalk	–	AR
IGSF1	TSH $\pm$ PRL deficiencies, transient GH deficiency, macroorchidism (males), ovarian cysts (females)			XL
PC1	ACTH deficiency, hypoglycemia, hypogonadotrophic hypogonadism, obesity, GH deficiency, diabetes insipidus		Obesity, gastrointestinal disorder	AR

*These genes are also known to cause isolated hypogonadotrophic hypogonadism. ACC; absent corpus callosum, AD; autosomal dominant, AP; anterior pituitary, APH; anterior pituitary hypoplasia, AR; autosomal recessive, CC; corpus callosum, EPP; ectopic posterior pituitary, ON; optic nerves, ONH; optic nerve hypoplasia, PP; posterior pituitary, XL; X-linked.

The anabolic actions of GH either directly or via the IGF system include: (Nilsson *et al.*, 1994; Olney, 2003; Chikani and Ho, 2014; Moller and Jorgensen, 2009; Antonopoulou *et al.*, 2012; Gullett *et al.*, 2010):

a. *Bone:* stimulation of epiphyseal growth, osteoclast and osteoblast activity, stimulation of osteoclast differentiation and increase of bone mass by endochondral bone formation.
b. *Muscle:* increased amino acid transport, nitrogen retention, energy expenditure and lean tissue accrual.
c. *Adipose tissue:* increased lipolysis, stimulation of hormone sensitive lipase and inhibition of lipoprotein lipase, glucose transport, and lipogenesis (acute insulin-like effects).

Several studies have shown contradictory/opposing actions of GH and IGF-1 (Mauras and Haymond, 2005). A hypothesis for this contradiction has been proposed based on evidence that IGF peptides showcase endocrine, paracrine and autocrine effects (Green *et al.*, 1985). This hypothesis proposes a "dual-effector" model where GH stimulates differentiation of precursor cells (Green *et al.*, 1985). These differentiated or the neighboring cells secrete IGFs, which act as mitogens and stimulate clonal expansion (Green *et al.*, 1985). Their actions are achieved through the respective receptors, while crucial in the modulation of their effect are the respective binding proteins (Belfiore *et al.*, 2009; Forbes *et al.*, 2012). Additional contributors to their actions are sex steroids and thyroid hormone (Coutant *et al.*, 2004; Rhee *et al.*, 2015). In addition, many molecules have been identified and comprise the group of novel growth factors (Sederquist *et al.*, 2014). These include the fibroblast growth factor (FGF) family of peptides and their receptors and the epidermal growth factors (EGF) and their receptors (Kostopoulou *et al.*, 2017; McCabe *et al.*, 2011). Of special interest is a class of inflammatory cytokines (the most abundant being tumor necrosis factor alpha, interleukin 1beta (IL1beta), and IL6) that is known to directly act on growth plate cartilage to induce apoptosis and thereby suppress bone growth, and thus can negatively modulate cellular growth (Sederquist *et al.*, 2014).

Growth hormone

Human GH is produced from somatotrope cells within the anterior pituitary (Masuda *et al.*, 1988). *A* 217aa GH-precursor is transported in the lumen of the endoplasmic reticulum (ER) via a mechanism that involves the recognition of the signal peptide (first 26 aa) (Cogan *et al.*, 1993). The GH-precursor is cleaved, and a mature protein is transported to the Golgi apparatus and secretory vesicles. The GH molecule is a single-chain non-glycosylated 191-amino-acid 22-kDa protein (Lewis *et al.*, 1980). It has a core of four helices in a parallel /anti-parallel orientation with two disulfide bonds between cysteines 53-165 and 182-189 (de Vos *et al.*, 1992). It should be noted that the presence of zinc ions facilitates the formation of soluble GH dimer complexes within the secretory granules, as well as the storage and secretion of GH aggregates (Petkovic *et al.*, 2013).

Of note, several proteins demonstrate homology to GH. These include prolactin, chorionic somatomammotropin (CS, placental lactogen), and a 22-kDa GH variant (hGH-V) secreted only by the placenta (Frankenne *et al.*, 1988). Even though the genes for these homologous proteins are located on different chromosomes (chromosome 6 for prolactin and chromosome 17 for GH) a common ancestral chromosome might exist (Cooke *et al.*, 1988).

GH-1

GH-1 is located on the long arm of chromosome 17 (17q22-24) within a cluster of five homologous genes, encompassing a distance of about 65 kb, that include chorionic somatomammotropin pseudogene (*CSHP*), chorionic somatomammotropin gene (*CSH*)*-1*, *GH-2* and *CSH-2* (Procter *et al.*, 1998). Along with prolactin and placental lactogen, *GH* showcases a structural organization of four introns separated by five exons (Frankenne *et al.*, 1988), and its full length product is a 191 amino acid (22 kDa) peptide.

Expression of *GH-1* is regulated by the highly polymorphic proximal promoter and a locus control region (LCR) 15–32 kb upstream of the gene that confers the pituitary-specific and high-level expression of GH (Horan *et al.*, 2003). The pituitary normally produces mainly the mature 22-kDa form of GH (75% of pituitary GH), while alternative splicing of the second exon results in deletion of amino acids 32 through 46, yielding a 20-kDa form (less than 10% of pituitary GH) (Cooke *et al.*, 1988; Miller and Eberhardt, 1983; DeNoto *et al.*, 1981). Pituitary GH also includes desamidated and N-acetylated forms, as well as various GH oligomers (DeNoto *et al.*, 1981). A 17.5 kDa variant that results from complete skipping of exon 3 and lacks amino acids 32-71 forms 1-5% of pituitary GH (Miller and Eberhardt, 1983).

GH secretion is regulated by GHRH, somatostatin and other secretagogues

GH displays a characteristic pulsatile secretion pattern that is the result of a complex interplay of multiple regulators, including two hypothalamic regulatory peptides, GH-releasing hormone (GHRH) and somatostatin (Barinaga *et al.*, 1983; Holl *et al.*, 1988).

GHRH stimulates GH secretion through the 44-amino acid terminus (Mayo, 1992). Its activity in the somatotropes is species-specific mediated through a specialized G-protein–related receptor (G-protein–coupled receptor family B/secretin family; GHRHR) (Mayo, 1992). The gene encoding GHRHR maps to chromosome 7p15 and consists of 13 exons spanning approximately 15 kb. It encodes a 423-amino acid G-protein-coupled receptor comprising seven transmembrane domains. The expression of *GHRHR* is up-regulated by *POU1F1* and required for the proliferation of somatrotrophs. The GHRH receptor displays partial sequence identity with receptors for vasoactive intestinal polypeptide [VIP, secretin, calcitonin, and parathyroid hormone (PTH)] (Mayo, 1992). Binding of GHRH to the receptor results in stimulation of adenylate cyclase and transcriptionally mediated increases in intracellular cyclic adenosine monophosphate (AMP) concentrations, which result in increased GH synthesis (Mayo, 1992).

Somatostatin, a 14-amino acid protein, inhibits GH secretion through binding to its specific receptor, which then reduces adenylate cyclase activity and concomitantly intracellular calcium concentrations (Holl *et al.*, 1988).

The pulsatile pattern of GH secretion observed *in vivo* is due to the dual and opposing effects of GHRH and somatostatin secreted from the hypothalamus (Hartman *et al.*, 1991). A peak of GH results from a parallel reduction in somatostatin and an increase in GHRH secretion, while a trough of GH secretion results from a parallel increase in somatostatin and a decrease in GHRH secretion (Hartman *et al.*, 1991). The net effects of these two hypothalamic hormones is modulation of GH secretion, as well as the timing and amplitude of peaks, resulting in pulsatile GH secretion (Veldhuis, 1998).

The complex pattern of release of these hypothalamic hormones is understood to result from the interaction of multiple neurotransmitters and neuropeptides, including serotonin, histamine, norepinephrine, dopamine, acetylcholine, gamma-aminobutyric acid (GABA), thyroid hormone-releasing hormone (TRH), corticotrophin-releasing hormone (CRH), vasoactive intestinal peptide (VIP), gastrin, neurotensin, substance P, calcitonin, neuropeptide Y, vasopressin, and galanin (Blanchard *et al.*, 1988). The alterations of GH secretion observed in various physiological states (e.g. stress, sleep, hemorrhage, fasting, hypoglycemia, and exercise), are believed to be mediated through these factors (Giustina and Veldhuis, 1998).

Non-peptide hormones also modulate GH secretion, through potential complex interactions at the hypothalamic and pituitary level (Zeitler *et al.*, 1990). Spontaneous and provocative GH secretion is, on the one hand, attenuated by increased glucocorticoid and reduced thyroxine concentrations, while on the other is amplified by increased sex steroid concentrations (Martha Jr. *et al.*, 1989).

A receptor that is distinct from the GHRH receptor, termed the GH secretagogue receptor (GHS-R), has also been identified. This unique G-protein–coupled receptor has been characterized through special synthetic hexapeptides capable of stimulating GH secretion, termed GH-releasing peptides (GHRPs). These peptides, later recognized as analogues of the gastric hormone ghrelin, are capable of directly stimulating GH release and enhancing the GH response to GHRH (Bowers *et al.*, 1984; Smith *et al.*, 1999). The *GHS-R* gene is strongly expressed in the hypothalamus but specific binding sites for GH releasing peptides have also been identified in other regions of the CNS as well as peripheral tissues.

The endogenous ligand for GHS-R is a 28-amino-acid peptide, the gastric hormone ghrelin (Smith *et al.*, 2001). Smaller amounts are also produced within the intestine, pancreas, kidney, immune system, placenta, pituitary, testis, ovary and hypothalamus (Smith *et al.*, 2001). Ghrelin is a unique gene product that requires octanoylation for normal function, and its main function is to stimulate appetite and is therefore considered obesogenic (Kojima *et al.*, 1999). It raises plasma GH synergistically with GHRH, and to a lesser extent prolactin and adrenocorticotropic hormone (ACTH) concentrations (Hosoda *et al.*, 2000). Ghrelin also influences endocrine pancreatic function and glucose metabolism, and controls gastric motility and acid secretion (Hosoda *et al.*, 2000). Further effects on gonadal, cardiovascular function and behavior are postulated (De Vriese and Delporte, 2008). These functions suggest that ghrelin is a component of the GH regulatory system of growth, by providing a stimulus for nutrient intake and allocation as well as regulating metabolism (De Vriese and Delporte, 2008). A second peptide product of the ghrelin gene, obestatin, has been identified but it regulates weight, not GH secretion (Zhang *et al.*, 2005).

In addition to the complex regulatory processes described previously, the synthesis and secretion of GH are also regulated by negative feedback (inhibition) by insulin-like growth factors (IGF) (Berelowitz *et al.*, 1981; Abe *et al.*, 1983). This inhibition has been demonstrated in the pituitary and in the hypothalamus, as well as in humans treated with recombinant IGF-1 (Ceda *et al.*, 1985; Guler *et al.*, 1987).

GH receptor

The effects of GH are mediated through interaction of the molecule with the GH receptor (GHR), a member of class 1 hematopoietic cytokine family (Leung *et al.*, 1987). The gene for human GHR has been localized to chromosome 5p13.1-p12, where it spans more than 87 kb (Leung *et al.*, 1987; Trivedi and Daughaday, 1988). The coding and 3-prime untranslated regions of the human GHR are encoded by nine exons, numbered 2 to 10 (Kelly *et al.*, 1991). Specifically, exon 2 encodes the secretion signal peptide, exons 3 to 7 encode the extracellular domain, exon 8 encodes the transmembrane domain, exon 9 encodes the intracellular domain and exon 10 encodes the 3-prime untranslated region (Trivedi and Daughaday, 1988).

The first transcript of *GHR* is a 638-amino-acid peptide, which through processing matures to a 620 amino acid receptor with a predicted molecular mass of 70 kDa before glycosylation (Leung *et al.*, 1987). The complete GHR comprises an extracellular hormone-binding domain (246 amino acids), a single membrane-spanning domain and a cytoplasmic domain (350 amino acids) (Leung *et al.*, 1987). In humans, proteolytic cleavage of the extracellular domain of the receptor forms the circulating GH-binding protein (GHBP) (Leung *et al.*, 1987). There are two genomic GHR isoforms in humans resulting from ancestral homologous recombination with subsequent retention or deletion of exon 3 (Jensen *et al.*, 2007). While the deletion of exon 3 of the GHR has been documented in a substantial number of normal individuals, this polymorphic variant has been associated with receptor responsiveness to GH and with birth size and postnatal growth (Jensen *et al.*, 2007; Mullis *et al.*, 2002).

GHR is highly homologous with the prolactin receptor and shares sequence homology with erythropoietin, leptin, granulocyte-macrophage colony-stimulating factor, interferon and several interleukin receptors (Brooks and Waters, 2010).

JAK-STAT signaling

As revealed by crystal structure examination, the GH-GHR complex consists of one molecule of GH bound to two GHR molecules, indicative that receptor dimerization is necessary for GH action (Brooks and Waters, 2010; de Vos *et al.*, 1992). It has been shown that the GHR is pre-formed as a dimer and is transported in a non-ligand bound state to the cell surface (Brooks and Waters, 2010;

Wilkinson *et al.*, 2007). GH displays two binding sites (1 and 2) with the first GHR monomer binding to the stronger site 1, followed by the second GHR monomer binding to the weaker site 2 (Argetsinger *et al.*, 1993). Binding results in conformational changes of the GHR dimers that result in repositioning of the intracellular domains and of Box1-associated Janus Kinase (JAK)2, a major GHR-associated tyrosine kinase (Waters and Brooks, 2015). This dimerization supposedly induces proximity of the associated JAK2s, resulting in their trans-activation and signal initiation (Waters and Brooks, 2015). More recent models have shown that dimerization of the receptor extracellular domains alone is insufficient to trigger activation, and that a specific receptor alignment or conformational change is needed (Waters and Brooks, 2015). This activation leads to cross phosphorylation of distal tyrosine residues of GHR that then enables Src homology (SH)2 domain molecules to dock to these sites (Argetsinger and Carter-Su, 1996). Two such molecules Stat5a and Stat5b contain SH2 domains and after binding to these phosphorylated tyrosine sites they in turn become phosphorylated (Brown *et al.*, 2005). Phosphorylated Stat5 molecules (homo- and hetero-) dimerize and translocate to the nucleus where they bind DNA, as dimers or as tetramers, and activate target genes (Brown *et al.*, 2005). Both Stat5a and Stat5b, as well as Stat1 and Stat3, are activated by GH, and they have both overlapping and distinct functions (Yoo *et al.*, 2011). Gene inactivation mouse models have shown that Stat5b is of greater importance for stimulation of growth than Stat5a, while both Stat 5a and Stat 5b seem to play critical roles in cell proliferation, particularly in immune cells (Yoo *et al.*, 2011; Gebert *et al.*, 1997; Imada *et al.*, 1998; Yao *et al.*, 2006; Cohen *et al.*, 2006). A pulse of GH stimulates Stat5b phosphorylation and after this rapid activation, Stat5b becomes temporarily refractive to further or continuous stimulation (Gebert *et al.*, 1997). There appear to be gender-specific differences in GH secretion and Stat5b signaling (Davey *et al.*, 1999). Hepatic nucleic factors (HNF)s, especially HNF3, 4α and 6, interact with Stat5b to induce these Stat5b-dependent gender-specific gene expression patterns (Choi and Waxman, 2000).

The phosphorylated Stat5a and Stat5b bind as dimers or tetramers to the Stat5 Response Element (RE), which is enhanced by the interaction of co-activators binding to adjacent DNA binding sites. Known co-activators are the γ-interferon activated sequence (GAS) motif, the glucocorticoid receptor (GR) response element, C/EBP and HNFs (Soldaini *et al.*, 2000). The gene of human IGF1 contains Stat5 RE in the second and third intron and with lesser effectiveness a distant Stat5 RE 73 kb upstream of the initiation site (Rotwein, 2017). Several other genes contain Stat5 RE including *Spi2.1*, *CIS*, *SOCS2*, *HNF6* and several genes for CYP450 enzymes (Soldaini *et al.*, 2000).

Following GH stimulation, the activation of JAK-STAT signaling occurs rapidly (within minutes) and is followed by negative regulation of signaling, indicative of the tight control of termination signaling (Gebert *et al.*, 1999). This occurs through GHR internalization and through the action of Suppressors of Cytokine Signaling (SOCS), Protein Tyrosine Phosphatases (PTP)s, and Protein Inhibitors of Activated Stats (PIAS) (Hansen *et al.*, 1999; Flores-Morales *et al.*, 2006; Gu *et al.*, 2003; Frank and Fuchs, 2008). The inhibition of GH signaling by several members of the GH-inducible SOCS family occurs through inhibition of JAK proteins and inhibition of JAK-STAT signaling, through competitive binding of positive regulators of signaling or docking sites, and through promoting GHR ubiquitination and inhibition of the GHR-dependent tyrosine phosphorylation of JAK2 (Hansen *et al.*, 1999; Waters *et al.*, 2014). Interestingly, endotoxin and proinflammatory cytokines such as interleukin-1b (IL-1b) and tumor necrosis factor-a (TNFa) induce a state of GH resistance, through induction of SOCS proteins (Colson *et al.*, 2000). The inhibition of GH signaling by several PTPs, (PTP-1, PTP-H1 and PTP-B1 and TC-PTP), occurs through dephosphorylation of activated phosphorylated proteins. It usually involves cytokine signaling pathways but also insulin signaling pathways (Frank and Fuchs, 2008). PTP-1B is involved in fasting-induced GH insensitivity, while PTPN11 mainly functions in RAS-MAPK signaling (Gu *et al.*, 2003).

Alternative signaling through the GHR

The activated GHR alternatively activated pathways include mitogen-activated protein kinases (MAPK)s, extracellular signal-regulated kinase (ERK)-1 and ERK2, the insulin-signaling pathway [through the insulin receptor substrate (IRS)-1 and IRS-2], and protein kinase C (PKC) (Brooks and Waters, 2010).

GH binding proteins

The soluble extracellular domain of GHR (molecular mass 55 kDa) is the main GH binding protein (GHBP) that is present in many tissues (Baumann *et al.*, 1986; Sotiropoulos *et al.*, 1993). It has identical affinity for the GH molecule as the GHR, binding with high specificity and affinity, but relatively low capacity (Baumann *et al.*, 1986). GHBP in the circulation is mostly derived from the liver, functioning as a transporter of GH to target tissues and aiding the subsequent binding to GHR (Baumann *et al.*, 1986). It is derived through proteolytic cleavage of the cell membrane anchored GHR, by enzymes such us TNFα-converting enzyme (TACE) (Zhang *et al.*, 2000).

Generation of insulin-like growth factors

The two insulin like growth factor (IGF)s, IGF-1 and IGF-2 (Daughaday and Kapadia, 1989; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b), are basic peptides of 70 and 67 amino acids respectively. IGF-1 and IGF-2 are structurally related and have 50% amino acid homology to insulin (Rinderknecht and Humbel, 1978a, Rinderknecht and Humbel, 1978b). All three molecules have A and B chains connected by disulfide bonds and a connecting (C-peptide) region that bears no homology with the C-peptide region of proinsulin (Daughaday and Kapadia, 1989). This structural homology and the disparities explain the ability of both IGFs to bind to the insulin receptor and for insulin to bind to type 1 IGF receptor, but not to the IGFBPs.

IGF-1 *and* IGF-2 *genes*

The human *IGF-1* gene spans 95 kb and is located on the long arm of chromosome 12 and contains at least six exons (Brissenden *et al.*, 1984; Tricoli *et al.*, 1984). Specifically, exons 1, 2, 3 and 4 encode alternative signal peptides, probably for several transcription start sites (Brissenden *et al.*, 1984). In addition, exons 3 and 4 encode the remainder of the mature IGF-1 molecule, and part of the trailer peptide (Brissenden *et al.*, 1984). Exons 5 and 6 encode alternatively used segments of the trailer peptide (resulting in the IGF-1A and IGF-1B forms), as well as 39 untranslated sequences, with multiple different polyadenylation sites (Brissenden *et al.*, 1984). The human *IGF-2* gene spans 35 kb and is located on the short arm of chromosome 11, adjacent to the insulin gene, and contains nine exons (Bell *et al.*, 1985). Exons 1 through 6 encode 59 untranslated RNAs (multiple promoter sites) (Brissenden *et al.*, 1984). Exon 7 encodes the signal peptide, while exons 7 and 8 encode most of the mature protein and the carboxyl-terminal portion of the protein, respectively, and exons 8 and 9 encode the trailer peptide (Brissenden *et al.*, 1984). Control of *IGF-1* and *IGF-2* expression is complex, with multiple mRNAs that result in great variability in tissue expression in the embryo, fetus, child, and adult (Agrogiannis *et al.*, 2014).

IGF receptors

Both IGF-1 and IGF-2 are known to bind (generally with low affinity) to insulin receptors (IR), although at least two classes of specific IGF receptor forms exist (Massague and Czech, 1982; Belfiore *et al.*, 2009). Type 1 IGF receptor (IGF1R) (chromosome 15q26.3) and IR (chromosome 19) are closely related, both being heterotetramers, composed of two alpha and two beta subunits (Massague and Czech, 1982). The alpha membrane-spanning subunits (molecular mass 130 kDa), contain the binding sites for IGF-1 and are linked by disulfide bonds (Massague and Czech, 1982). The beta intracellular subunits (molecular mass 90 kDa) contain a transmembrane domain, an adenosine triphosphate (ATP)-binding site, and a tyrosine kinase domain, which constitutes the presumed signal transduction mechanism for the receptor (Laviola *et al.*, 2007). The IGF1R has high affinity for both IGF-1 and IGF-2 and 100-fold less for insulin (Laviola *et al.*, 2007). IGF1 acts primarily through IGF1R, but can bind with lower affinity to the highly homologous IR, and to IGF1R/IR heterodimers and vice versa for insulin, as evident in pathological IGF1 or insulin signaling (Rosenzweig and Atreya, 2010; Laviola *et al.*, 2007).

IGF1R and IGF2R signaling

The IGFR1 mediates IGF actions on all cell types through tyrosine kinase activation and phosphorylation of substrates (Laviola *et al.*, 2007). These include the members of the insulin receptor substrate (IRS) family (particularly IRS-1 and IRS-2) (Valverde *et al.*, 1998). Several signaling molecules respond to IGF receptor activation, propagating signal transduction or regulating IGF action through negative feedback (Tsuruzoe *et al.*, 2001). Several studies have shown seemingly anomalous competitive binding results, indicative that variant or atypical insulin and IGF receptors might exist (Oh *et al.*, 1993). In accordance with this, the existence of hybrid receptors composed of 1 alpha-beta dimer of the IR and 1 alpha-beta dimer of the IGF1R has been postulated, although the physiologic significance is unknown (Slaaby, 2015).

The type 2 IGF receptor (IGFR2) is a monomeric protein (molecular mass 271 kDa), that bears no structural homology with either IR or IGFR1 (Braulke, 1999). The IGFR2 is composed of a lengthy extracellular domain (15 repeat sequences of 147 residues), followed by a transmembrane domain (23 residues) and a small cytoplasmic domain (164 residues) (Braulke, 1999). It does not contain an intrinsic tyrosine kinase domain or any other recognizable signal transduction mechanism (Braulke, 1999). Interestingly IGFR2 is identical to the cation-independent mannose-6-phosphate (CIM6P) receptor (such as cathepsin and urokinase), although binding sites for IGF-2 and CIM6P appear to reside in different areas of the receptor (MacDonald *et al.*, 1988). Still, the two ligands (IGF-2 and CIM6P) show some reciprocal inhibitory effects on receptor binding, indicating an association between IGF-2 and the sorting of lysosomal enzymes (MacDonald *et al.*, 1988). The majority of CIM6P receptors are located on intracellular membranes in equilibrium with receptors on the plasma membrane, and are associated with the intracellular lysosomal targeting of various acid hydrolases and other mannose-6-phosphate containing proteins and enzymes (such as cathepsin and urokinase) (MacDonald *et al.*, 1988; Braulke, 1999). This might be indicative of an important function in removing these enzymes from the cellular environment (Braulke, 1999).

It should be noted that most studies indicate that the classic mitogenic and metabolic actions of IGF-1 and IGF-2 are mediated through IGFR1 and the corresponding tyrosine kinase signal transduction mechanism (Conover *et al.*, 1986; Furlanetto *et al.*, 1987). Nevertheless, many observations are consistent with the possibility of IGF-2 actions mediated via IGFR2, not duplicated by either IGF-1 or insulin (Rogers and Hammerman, 1988; Minniti *et al.*, 1992). In addition, it has been suggested that IGFR2 acts as a growth inhibitory component of the IGF system responding to and mediating multiple antimitogenic systems, including retinoids (Tian *et al.*, 2016; Kang *et al.*, 1999).

IGF-binding protein superfamily

IGFs circulate in plasma as complexes with IGF binding protein (IGFBP)s (Rajaram *et al.*, 1997). The function of these carrier proteins is to extend the serum half-life of the IGF peptides, transport the IGFs to target cells, and modulate the interaction of the IGFs with their surface membrane receptors (Rajaram *et al.*, 1997). The family of IGFBPs includes six distinct proteins with important structural relationships among them (Forbes *et al.*, 2012). The most important similarity is the conservation of the number and placement of the cysteine residues and the corresponding disulfide bonds, which confers maintenance of the secondary structure of the IGFBPs and the IGF-binding sites of each IGFBP (Forbes *et al.*, 2012). In addition, an arginine-glycine-aspartic acid (RGD) positioned near the carboxyl terminus of IGFBP-1 and IGFBP-2 is present, possibly facilitating the association

of IGFBPs with the cell surface (Shen *et al.*, 2012; Reyer *et al.*, 2015). However, IGFBP-3 nevertheless lacks an RGD sequence, but is capable of specific binding to cell membrane receptors, possibly through specific membrane binding proteins (Firth and Baxter, 2002).

Recently several families of IGFBP-related proteins (IGFBP-rPs) have been described, indicating that an IGFBP superfamily might exist (Rosenfeld *et al.*, 2001; Rodgers *et al.*, 2008). The physiological role of IGFBP-rPs in cell growth has not yet been elucidated, though it is likely that they can act through IGF-independent and/or IGF-dependent mechanisms (Hwa *et al.*, 1999). Three IGFBP-rPs (Mac25/IGFBP-rP1, CTGF/IGFBP-rP2 and NovH/IGFBP-rP3) have been shown to bind IGFs with considerably lower affinity than IGFBPs (Hwa *et al.*, 1999).

Function of IGFBPs

Studies suggest that IGFBPs appear to inhibit IGF action, possibly through competitive binding with IGFR1, or through a direct inhibitory effect (Ritvos *et al.*, 1988; Cohen *et al.*, 1993; Hochscheid *et al.*, 2000). Interestingly, under specific conditions, several of the IGFBPs can enhance IGF action, possibly through facilitated IGF delivery to target receptors (Elgin *et al.*, 1987). Additionally, a number of IGF-independent functions have been suggested, that include growth inhibition or stimulation of cell growth, direct induction of apoptosis and modulation of action of other non-IGF growth factors (Oh *et al.*, 1995; Conover *et al.*, 1996; Conover *et al.*, 2000; Rajah *et al.*, 1997). The IGF-independent effects are mediated through separate specialized receptors and IGFBP-signaling pathways (Liu *et al.*, 2000).

It should be noted that concentrations vary in biological fluids, with IGFBP-1 being the major IGFBP in human amniotic fluid, and IGFBP-2 being the major IGFBP in cerebrospinal fluid and seminal plasma (Rosenfeld *et al.*, 1989; Rosenfeld *et al.*, 1990). In normal human serum, IGFBP-3 is the major IGFBP, being also highly GH-dependent (Martin and Baxter, 1986). Furthermore IGFBP-3 and IGFBP-5 circulate in serum as part of a ternary complex along with an IGF peptide, and an acid-labile subunit (Twigg and Baxter, 1998). IGFBP analysis is further complicated by the presence of IGFBP proteases, capable of various levels of IGFBP degradation (Giudice *et al.*, 1990; Muller *et al.*, 1993). The limited proteolysis of IGFBPs might result in decreased affinity of the IGFBP for IGF peptides, although the exact physiological significance remains undetermined (Binoux *et al.*, 1993).

Other Novel Growth Factors

The FGF family of peptides and receptors

Fibroblast growth factors (FGFs) constitute a family of peptide cytokines with many defined and yet undefined functions (Balasubramanian and Zhang, 2016). It has initially been suggested that these factors are specific for stromal cells, although other cells respond to FGFs as well (Su *et al.*, 2014). This family of proteins includes at least seven different FGFs (FGF-1 through FGF-7) (Balasubramanian and Zhang, 2016). The best characterized are acidic FGF (aFGF or FGF-1), basic FGF (bFGF or FGF-2), and keratinocyte growth factor (KGF or FGF-7) (Balasubramanian and Zhang, 2016). The actions of FGFs are mediated by binding to three characterized receptors (FGFR1, FGFR2, and FGFR3), with distinct tissue distributions (Ornitz and Itoh, 2015). In terms of their function, FGFs appear to be autocrine-paracrine growth factors that participate in specific organ growth and differentiation and in carcinogenesis (Ornitz and Itoh, 2015). While FGFs do not participate in somatic growth *per se*, a genetic form of dwarfism (achondroplasia) exists, caused by an activating mutation in *FGFR3* (Rousseau *et al.*, 1994; Wilkin *et al.*, 1998). This suggests that normal FGFR3 signaling is essential for the normal growth of long bones (Rousseau *et al.*, 1994).

The EGF family

The epidermal growth factor (EGF) family appears to be important in mammalian development and function, although their precise roles are yet undefined, and they do not appear to be directly involved in somatic growth (Fisher and Lakshmanan, 1990; Kostopoulou *et al.*, 2017). Nonetheless, EGF receptors have been identified in fetal tissues, suggesting a role in embryogenesis and fetal growth, while abnormal EGF-EGF receptor interactions appear instrumental in the development of cancers (Oliver, 1988; Seshacharyulu *et al.*, 2012). The EGFs have been extensively studied *in vitro* for their mitogenic actions, while the receptor for EGF is the prototype model for tyrosine kinase mediated signal transduction (Lemmon *et al.*, 2014). However, no major deleterious effects after EGF pathway disruption, either via antibody administration or gene targeting, have been demonstrated (Fisher and Lakshmanan, 1990).

Other growth-promoting peptides

A number of growth-promoting peptides have been described including platelet derived growth factor (PDGF), vascular epithelial growth factor (VEGF), granulocyte and macrophage colony-stimulating factors (G-CSF, MCSF), erythropoietin, and thrombopoietin (Passaretti *et al.*, 2014; Shibuya, 2011; Athanasopoulos *et al.*, 2007; Calhoun *et al.*, 1999; Nijaguna *et al.*, 2015; Heikal *et al.*, 2016; Kuter, 2009). Cytokines, interleukins and interferons have been shown to modulate growth of various cells of the immune system (Turner *et al.*, 2014; Street *et al.*, 2003; Sederquist *et al.*, 2014). Other tissue-specific growth factors include nerve growth factor (NGF), brain- and glial-derived neurotrophic factors (BDNF, GDNF), and hepatocyte growth factor (HGF) (Loeb *et al.*, 1991; Bathina and Das, 2015; Orth *et al.*, 2000; Nakamura and Mizuno, 2010).

Growth inhibitory peptides

Peptides including transforming growth factor-beta (TGFβ), tumor necrosis factors (TNFs) and other compounds have been shown to inhibit cellular growth and malignant transformation or regulate the entry of cells into apoptosis (Huang and Huang, 2005; Rusten *et al.*, 1994; Penn *et al.*, 2012). These peptides may be involved in fetal growth (Toder *et al.*, 2003; Lyall *et al.*, 2001). A family of genes of which the most important is p53 is also critical to growth and tumor suppression (Rodier *et al.*, 2007).

Other Hormones Contributing to Growth

Sex steroids

Conditions characterized by androgen or estrogen excess (e.g. precocious puberty or virilizing congenital adrenal hyperplasia), occurring before epiphyseal fusion, are invariably characterized by increased linear growth and advanced skeletal maturation (Ghizzoni *et al.*, 1999; Carel *et al.*, 2004). Androgens and estrogens, which increase in concentration during puberty, are important physiological components of the pubertal growth spurt (Bourguignon, 1991). This normal linear growth response to endogenous or exogenous sex steroids requires adequate GH secretion and action (Meinhardt and Ho, 2006). It should also be noted that androgens function through enhancement of GH and IGF-1 secretion (Coutant *et al.*, 2004). The increased skeletal maturation and epiphyseal fusion, however, appears to be estrogen-mediated (Borjesson *et al.*, 2013; Smith *et al.*, 1994)

Thyroid hormone

Hypothyroidism occurring postnatally results in growth failure and arrest of epiphyseal cartilage fusion (Siebler *et al.*, 2001). Thyroid hormone also appears to have a permissive effect on GH secretion, as indicated by decreased spontaneous GH secretion and the blunted response to GH provocative tests of patients with hypothyroidism (Katakami *et al.*, 1986; Rhee *et al.*, 2015). Patients replaced appropriately with thyroxine demonstrate rapid "catch-up" growth and skeletal maturation, and this can potentially compromise adult height (Bassett and Williams, 2016; de Wit *et al.*, 2013), especially if puberty then occurs early with the risk of earlier epiphyseal fusion.

Conclusion

The genetic and hormonal regulation of growth is complex. The hypothalamo-pituitary neuroendocrine network can be disrupted by causes that may be congenital or acquired later in life leading to various syndromes of endocrine deficits or hormonal excess, including GH. Animal models have advanced our understanding of the pituitary as central to the control of growth, and studies in the mouse have led to significant insights into human pituitary development and disease. The endocrine regulation of normal growth classically depends upon a hormonal axis involving hypothalamic stimulatory GHRH and inhibitory somatostatin, pituitary secretion of GH, and liver-derived IGF-1. The functionality of this axis depends upon several molecules that include the respective families of receptors, carrier proteins and contributing molecules such as hormones, binding proteins and anti-inflammatory cytokines. Finally, several local cell-specific growth factors display important autocrine/paracrine and endocrine functions, even though they lack direct effects on somatic growth.

Contemporary research into the array of putative contributing novel growth factors, and the potential associations between these molecules, is likely to uncover an increasing intricacy in the regulation of growth.

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Further Reading

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Abnormal Growth: Small for Gestational Age

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Glossary

Adrenarche A period during normal development when the adrenal glands begin to produce increased androgens. This normally occurs prior to puberty, between the ages of 6–8 years.

Appropriate for gestational age When a baby is born at a birth weight that is appropriate for the gestation of pregnancy, usually between the 10th and 90th centile for age.

Catch down growth When a baby's weight does not increase as expected along its growth centile, and it decreases more than 1 centile (0.67 SD) below where it plotted at birth.

Catch up growth When a baby's weight increases more than expected, so that it increases more than 1 centile (0.67 SD) above where it plotted at birth.

Epigenetics The study of inheritable changes in gene expression that do not change the underlying DNA sequence.

Growth restricted Where growth of the baby/child has been limited.

Insulin resistance Where the body needs to produce more insulin to induce a set reduction in glucose levels.

Insulin sensitivity The ability of insulin to decrease blood glucose levels.

Intrauterine growth restricted Where there has been restriction in the growth of the fetus; this requires at least two growth measurements to show there has been abnormal growth over a period of time.

Metabolic syndrome Also known as syndrome X; a combination of risk factors including impaired glucose metabolism, hypertension, increased abdominal circumference, raised triglycerides and low HDL cholesterol. There is an increased risk of cardiovascular disease, stroke and myocardial infarction.

Noncommunicable diseases Diseases, such as heart disease or certain types of cancer, that are not caused by infectious organisms.

Puberty A normal stage in development where there is the development and maturation of male and female sexual organs, such that at the end they are capable of sexual reproduction.

Small for gestational age When a baby is born smaller than expected for their gestation; different definitions have been used, but a consensus statement from 2007 uses babies born below the third centile for age.

Introduction

Being born small for gestational age (SGA) is one of the most common causes of childhood short stature. Size at birth, particularly being born relatively small is important not just in relation to early life morbidity and mortality but is associated with increased prevalence of noncommunicable diseases during later life. Therefore, understanding the etiology of small for gestational age and mechanisms that lead to these lifelong consequences is important if we are to optimize individual's growth and health outcomes. The growth pattern of these infants in the early years of life is an important determinant of later health risks. In particular, infants who show catch up growth have increased risks for coronary artery disease, stroke and the metabolic syndrome. The impact of this has been most prevalent in countries where fast economic changes have led to changes in infant and adult dietary habits, with rapid childhood growth, resulting in a marked increase in prevalence of metabolic syndrome and type 2 diabetes in adulthood. For SGA children who remain short, interventions, such as growth hormone therapy have been approved, but responses to treatment are varied, and there remains significant controversy regarding the benefits of such treatment and long-term outcomes. Better understanding of the nutritional and hormonal mechanisms that drive early growth and the relationship to longer-term health risks will be important in the future if we are to provide a more personalized approach to health. It may also provide insights into public health management approaches to reduce the increasing prevalence of the metabolic syndrome and type 2 diabetes.

Etiology

The most widely accepted definition for small for gestational age (SGA) is that of the World Health Organization (WHO); a baby with a birth weight less than the 10th centile for sex and gestational age. In 2007 a consensus statement by the International Societies of Pediatric Endocrinology and the Growth Hormone Research Society defined SGA as a birth weight and/or length at least two standard deviations (SD) below the mean for gestational age (GA). The use of this 3rd percentile instead of the 10th percentile does increase the specificity but decreases the sensitivity. Infants who are SGA may have experienced growth restriction

but intrauterine growth restriction (IUGR) is a distinct definition and varies in that there must be evidence of impaired in utero growth (with at least two in utero growth measurements), and the terms should not be used interchangeably. The proportion of infants defined as SGA will inevitably vary depending on the definition used and the population from which the normative data were derived. Differences in prevalence of SGA also exist between countries, with the incidence estimated at 5%–7% in developed countries compared to up to 30% in developing countries such as India (Lee *et al.*, 2013; Yadav and Rustogi, 2015).

The etiology of being born SGA is multifactorial. Clinicians historically have tried to attribute causality based on relative growth parameters such as symmetrical or asymmetrical body proportions, that is, whether infants are small for weight, length and head circumference or whether they have head sparing compared to proportionately lower weight. Clinically this can help in determining the timing and etiology as maternal, placental or fetal in origin. Maternal factors such as chronic disease, maternal smoking, or pregnancy complications such as preeclampsia and congenital infections can affect growth. Nutrition and oxygen delivery from a well vascularized placenta and endocrine regulation of cellular expansion and cell turnover is also imperative and multiple pregnancies or pathologies that impact on nutritional delivery or cause fetal hypoxia can impair fetal growth (**Table 1**).

Pregnancy complications such as gestational diabetes mellitus (GDM) and preeclampsia, directly affect placental and therefore fetal growth, but have also been shown to be associated with changes in methylation of placental genes. Differential methylation and expression patterns of genes involved in the regulation of glucose homeostasis and lipid metabolism have been demonstrated in SGA compared to appropriate for gestational age (AGA) pregnancies. These epigenetic variations may influence fetal growth, early body composition, and lifelong diabetes risk (Díaz *et al.*, 2017). The *ATG2B*, *NKX6.1*, and *SLC13A5* genes (respectively related to autophagy, β-cell development and function, and lipid metabolism) have been found to be hypermethylated in placenta and cord blood from SGA newborns, whereas *GPR120* (related to free fatty acid regulation) were hypomethylated in placenta and hypermethylated in cord blood. Gene expression levels were opposite to methylation status, and both correlated with birth weight, circulating insulin like growth factor 1 (IGF-I), and total and abdominal fat at age 2 weeks (Díaz *et al.*, 2017). The growth hormone IGF-I axis plays a key role in in utero growth and mutations and altered gene expression can therefore lead to infants who are SGA. Counterintuitively mRNA and protein expression levels of IGF-I and its receptor are increased in the placenta of mothers who delivered SGA offspring, and key signaling pathways downstream such as IGF-I receptor AKT and mTOR have been reported to be upregulated in preterm infants born SGA (Iñiguez *et al.*, 2014). This may be due to compensatory mechanisms to increase nutritional delivery in the setting of growth restriction.

Knockout animal studies as well as case reports (Woods *et al.*, 1996) have shown mutations in the fetal IGF-I gene itself as well as the IGF-I receptor to impact on fetal growth. Deletions of chromosome 15 in the region of the IGF-IR have also been associated with SGA. SGA associated with such defects are commonly associated with learning and hearing difficulties demonstrating the key role of IGF-I in central nervous system development. As IGF-I bioavailability is critically dependent on its association in a ternary complex with an 8 KDa glycoprotein acid labile subunit (ALS) which prolongs its half-life, mutations in the *IGFALS* gene will decrease IGF-I bioavailability and reduce growth (Storr *et al.*, 2015). The d3-GHR polymorphism is a 2.7 kB deletion in exon 3 of the GHR gene and is associated with SGA (Jensen *et al.*, 2007). This mutation is of particular interest as an individual's allelic combination can impact their response to GH treatment (see below).

Genetic mechanisms related to differences in maternal and paternally inherited gene expression and imprinting also play a role in fetal growth. This is of particular relevance in the setting of uniparental disomy. Uniparental disomy (UPD) describes the condition where an individual inherits both copies of a gene from one parent and no copy from the other parent. This becomes significant if the UPD gene is also an imprinted gene, where genes are only active from one parent. Paternally expressed genes tend to promote placental and fetal growth (Wang *et al.*, 2013; Coan *et al.*, 2005), and maternal genes suppress growth (Netchine *et al.*, 2007). Examples of these include mutations in chromosome 7 and 11 both of which contain groups of genes that undergo imprinting and cause 75% of the cases of Silver Russell Syndrome (Netchine *et al.*, 2007). The imprinting center region 1 (ICR1) which is affected in these cases regulates the expression of IGF2 and H19. UPD of the long arm of chromosome 14 (UPD14) has also been associated with SGA and poor postnatal growth, and is thought to be related to an absence of paternal information, which leads to growth restriction through impact in the placenta and skeletal maturation (Hoffmann and Heller, 2011).

Table 1 Associations and etiologies found in infants born small for gestational age

Maternal	*Fetal*	*Placental*
Age (<16 years or >35 years)	Chromosomal abnormalities (e.g., trisomy 13/18/21 or uniparental disomy)	Placental dysfunction (e.g., preeclampsia)
Weight (<45 kg or >75 kg)	Congenital abnormalities (e.g., congenital heart disease, congenital diaphragmatic hernia, abdominal wall defects)	Multiple gestation
Chronic disease (e.g., hypertension, diabetes, renal disease)	Congenital infection (e.g., TORCH, malaria, HIV)	Abnormal utero-placental vasculature
Substance abuse (e.g., alcohol, cocaine, tobacco smoking)	Genetic syndromes (e.g., Russell-Silver, Cornelia de Lange)	
Maternal infection (e.g., TORCH, TB, malaria)	Metabolic disorders (e.g., galactosemia, congenital lipodystrophy, fetal phenylketonuria)	
Previous SGA infant	Constitutionally small	

Prader–Willi syndrome is another syndrome associated with UPD of chromosome 15, and results from a loss of function of several genes including some that encode for small nucleolar RNAs (snoRNAs). The loss of a particular group of snoRNA genes, known as the SNORD116 cluster, can play a role in causing the signs and symptoms of Prader–Willi syndrome and modest prenatal growth failure (Bieth *et al.*, 2015).

Early Endocrine Axis in Infants Born SGA

Studies of the endocrine status of SGA infants in the perinatal period have been inconsistent. This is likely to reflect the range of underlying pathologies encompassing the diagnosis of SGA, and the changes that occur during the transitional period from in utero to ex utero life. One study showed that SGA infants have lower blood glucose and insulin levels than AGA infants, and higher glucose/insulin ratios. SGA infants also had higher levels of IGFBP-1, free fatty acids and beta-hydroxy butyrate (Iñiguez *et al.*, 2006). This demonstrates that these infants display an increased insulin sensitivity with respect to glucose disposal but not with respect to suppression of lipolysis, ketogenesis, and hepatic production of IGFBP-1. This can be seen as advantageous in ensuring alternative energy sources are available for these neonates who have limited glucose reserves (Bazaes *et al.*, 2003). Others have shown that both full-term and preterm SGA neonates had higher insulin concentrations, insulin to glucose ratios, triglycerides, total cholesterol and low-density lipoprotein cholesterol concentrations than AGA neonates (Wang *et al.*, 1991). This would suggest SGA neonates displaying profiles suggestive of lower insulin sensitivity and less favorable lipid metabolism in the early postnatal period.

Early Childhood Growth in SGA Children

Most children born SGA tend to gain weight rapidly in early life and historically this catch up was believed to be advantageous with potential benefits on short term survival and stature, as well as neurodevelopment (Yeung, 2006). This accelerated postnatal weight gain however has been associated with increased adiposity and metabolic disease in later life (Barker *et al.*, 1993). Catch up can be defined as growth velocity above the limits of normal for age and can be complete or incomplete. However catch-up growth is often used to categorize children into those who have moved into a defined target range for height or weight or demonstrated a significant increase in standard deviation score (>0.67) with crossing of >2 growth centiles (Hokken-Koelega *et al.*, 1995). Catch-up growth typically occurs in the first few months (approximately 80% in the first 6 months) with up to 90% showing catch up growth by 2 years of age (Karlberg and Albertsson-Wikland, 1995; Yadav and Rustogi, 2015).

Catch Up Growth and the Metabolic Syndrome

All SGA individuals, regardless of whether there is catch up growth, have a long term increased metabolic risk in adult life, with higher incidences of cardiovascular disease, hypertension and type 2 diabetes mellitus, when compared to babies born AGA (Barker *et al.*, 1993, Barker; 2006, Hales and Barker, 2013). Those with catch up growth however have an increased risk.

Mechanisms of Catch Up Growth

The mechanisms that underlie early catch-up growth are not fully understood. It is thought to be largely driven by increased appetite, and subsequent increased IGF-1 (Mericq *et al.*, 2017). Cohort studies show that sustained high levels of orexigenic hormones such as ghrelin can be demonstrated in these children following a glucose load (Iñiguez *et al.*, 2002). Alternatively, adaptive mechanisms may reduce energy expenditure to maintain adequate growth, as has been demonstrated in animal models (Vickers *et al.*, 2003; Ikenasio-Thorpe *et al.*, 2007). This may be mediated through leptin resistance in infants born SGA, as this would act to drive increased weight gain. There is altered development of adipose tissue and adipokine signaling and epigenetic changes resulting in greater expression of pathways for adipogenesis (Sarr *et al.*, 2012). This leads to altered energy partitioning resulting in increased fat stores particularly in subcutaneous abdominal and visceral compartments, at the expense of lean mass (Ong *et al.*, 2000; Stettler *et al.*, 2002).

Critical to the risk of developing glucose intolerance is the relationship between insulin sensitivity and compensatory insulin secretion. The reduced disposition index in SGA children at 3 years of age may indicate early deficiency in their first phase insulin response. In the ALSPAC cohort the compensatory increased insulin secretion for the degree of insulin resistance was related to height gain and IGF-I levels and it is possible that the insulin secretion response in relation to growth hormone mediated insulin resistance could be a factor in promoting early catch up growth particularly growth in length as opposed to weight. Overexpression of the enzyme acyl-coenzyme A synthetase 1, which is involved in transport and degradation of fatty acids, as well as lipid synthesis may also play a role. This enzyme has been associated with increased lipid loading of adipocytes and increased insulin sensitivity in infants born SGA (Joseph *et al.*, 2015). It is thought that this may help to promote rapid catch up growth in SGA infants, but in later life this overexpression may lead to excess synthesis of triglycerides, contributing to obesity, as well as increasing insulin resistance by increasing fatty acid release.

Impact of Catch Up Growth

During catch up growth fat mass is accumulated much faster than lean mass and this is related to the insulin resistance seen in SGA children who catch up (Dulloo, 2006; Dulloo *et al.*, 2006a, b). SGA children who showed catch-up growth between 0 and 2 years had greater adiposity and more central fat distribution at 5 years of age than other children (Ong *et al.*, 2000) with total and abdominal fat mass at 4 years more closely related to rate of weight gain between 0 and 2 years not 2 and 4 years (**Fig. 1**). The impact of central adiposity or visceral fat on insulin resistance may be mediated by the effects of increased nonesterified fatty acid flux to the liver and changes in the adipokines such as adiponectin (Mittelman *et al.*, 2002). Falling adiponectin levels are related to increasing age and greater weight gain. Further studies have shown that the weight gain during childhood is a stronger determinant of body composition in young adult hood than birth weight itself (Kerkhof *et al.*, 2012). A higher gain in weight for length in the first 3 months of life is associated with a higher prevalence of metabolic syndrome at 21 years of age whereas birth weight was not.

When measured at 3 years of age studies have shown that, despite there being no significant differences in weight or body mass index (BMI) between SGA and AGA children, prefeed insulin levels were also related to the rate of weight gain between 0 and 3 years. First phase insulin secretion did not differ between the SGA and the AGA infants but the SGA infants had a lower glucose disposition index demonstrating beta cell compensation which persisted after allowing for postnatal weight gain (Mericq *et al.*, 2005). The changes in insulin sensitivity are accompanied by changes in IGF-I levels. At birth SGA infants show increased insulin sensitivity, with raised IGFBP1, relative to AGA infants (Bazaes *et al.*, 2003). At 3 months of age mean serum IGF-I and IGF-BP3 are lower in SGA infants than in infants born AGA (Chellakooty *et al.*, 2006), However IGF-I levels later in life are inversely related to birth weight, with higher levels after catch up growth in SGA children, which persists into young adulthood (Fall *et al.*, 1995; Jernström and Olsson, 1998; Garnett *et al.*, 1999; Ong *et al.*, 2002).

The increased abdominal and hepatic fat deposition and reduction in insulin sensitivity in children with catch up growth, is also associated with changes in biomarkers similar to that seen in the metabolic syndrome. These include lower levels of adiponectin, FGF21, FGF19 and visfatin, and higher leptin levels, compared to those without catch up or AGA children (Mericq et al., 2005, 2017). Therefore, alterations in body composition, and biomarkers of metabolism, that are associated with adverse cardiovascular and metabolic disease risk during adult life, are already present in young SGA children with rapid catch-up growth (**Fig. 2**).

Early interventional studies of infants born SGA, which were aimed at promoting early growth with the hope of improved cognitive outcomes, have confirmed the findings from cohort studies. These studies which randomized babies at birth to standard or nutrient enriched formulas, were extended to explore cardiovascular and metabolic outcomes. They showed that at age 6–8 years those receiving the standard formula had a significantly lower mean and diastolic blood pressure (Singhal *et al.*, 2006). Current research is focusing on exploring ways of modifying nutritional content in formula, particularly protein content, as well as maternal behaviors around milk feeding to prevent over feeding during infancy as those who are bottle fed appear to be at most risk (Fewtrell *et al.*, 2001; Rolland-Cachera *et al.*, 1995, Koletzko *et al.*, 2009a,b; Socha *et al.*, 2011; Lakshman *et al.*, 2015).

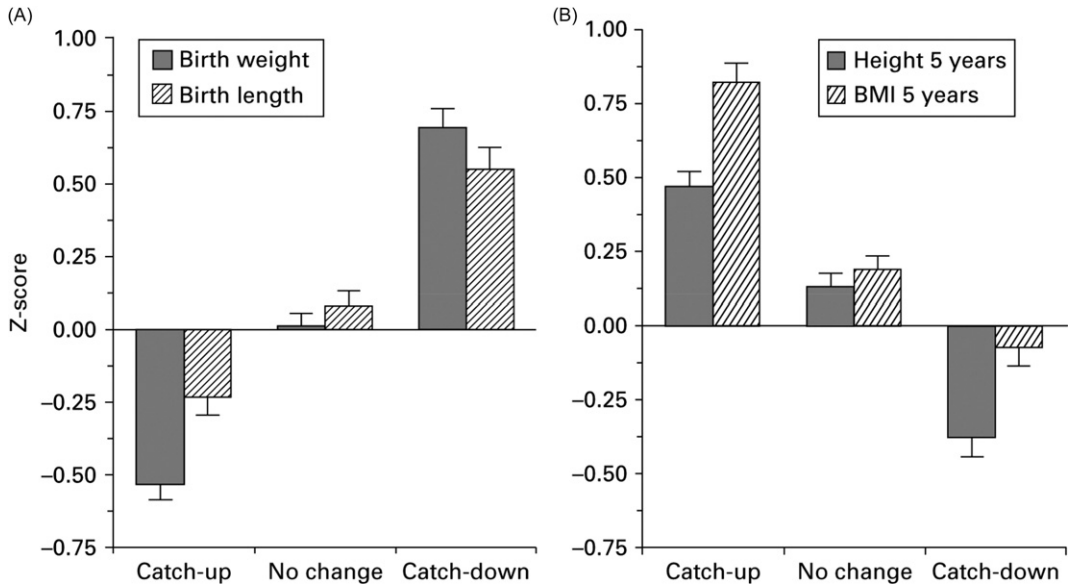

Fig. 1 Association between infant growth pattern and (A) weight and length *z*-score at birth and (B) height and BMI z-score at 5 years. Data showing mean ±standard error. This data demonstrates that in a population study at birth those who will catch-up in weight and length are born with more negative *Z*-scores, and those who catch-down are born relatively larger. By 5 years of age those who have shown catch-up have higher BMI compared to those who have no change in Z-score or who have shown catch-down. Reproduced from Wells, J. C. K. *et al.* (2016). The elevated susceptibility to diabetes in India: An evolutionary perspective. *Frontiers in Public Health*, **4**, 145.

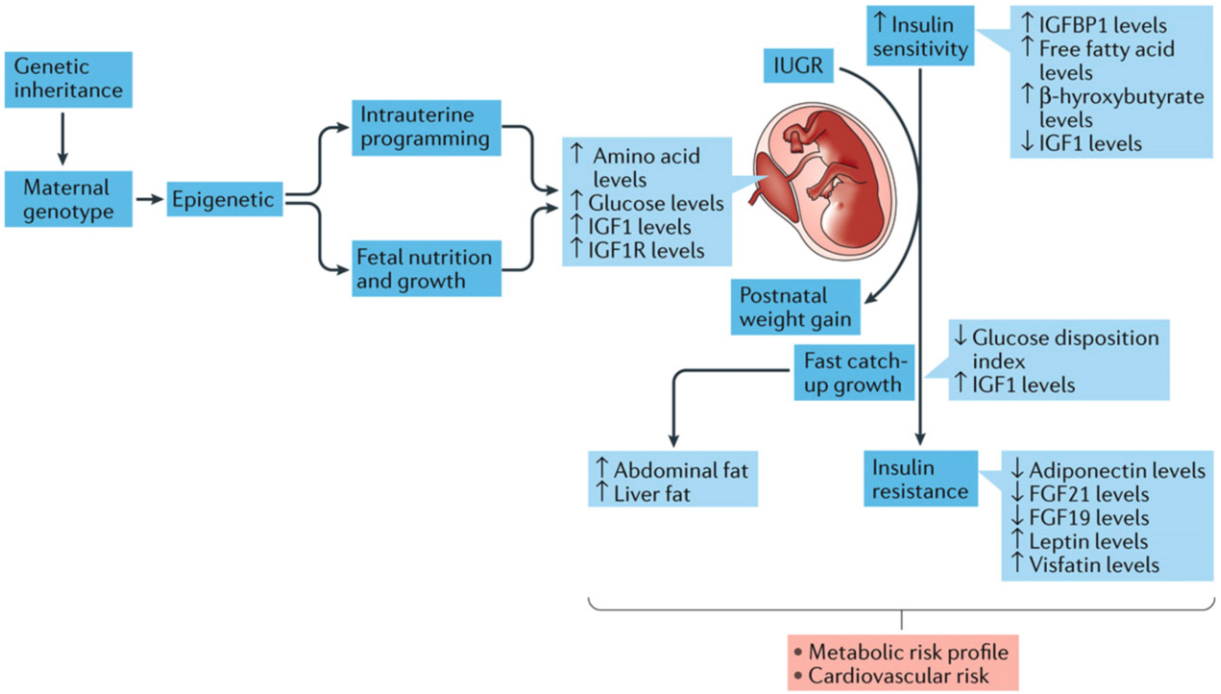

Fig. 2 Schematic diagram showing proposed links between genetic and epigenetic factors, metabolic alterations during early life and increased risk for metabolic diseases during adulthood in small for gestational age children. The placenta is critical for integration of environmental signals, thereby enabling healthy fetal growth and development. This process is mediated in part by increased expression of insulin-like growth factor 1 (IGF1) and its receptor (IGF1R), suggesting a compensatory mechanism in response to fetal growth restriction that could induce other changes to promote influx of amino acids and glucose from the maternal blood supply via the placenta to the fetus. After birth, a period of increased insulin sensitivity occurs, which gradually decreases relative to postnatal weight gain (catch-up growth). These changes could, in turn, be associated with adverse body composition and increased cardiovascular risk. FGF19, fibroblast growth factor 19; FGF21, fibroblast growth factor 21; IGFBP1, IGF binding protein 1; IUGR, intrauterine growth restriction. With permission from Mericq, V. *et al.* (2017). Long-term metabolic risk among children born premature or small for gestational age. *Nature Reviews Endocrinology* **13** (1), 50–62. https://doi.org/10.1038/nrendo.2016.127.

Lack of Catch Up Growth and Short Stature

Children born SGA who do not catch-up by 2 years have five- to sevenfold increased risk of short stature in adult life (Albertsson-Wikland *et al.*, 1998). The licensing of the use of growth hormone (GH) for use in these SGA children who remain short, but who do not meet the classical criteria for growth hormone deficiency, raised significant debate. The benefits of height gain both physical and psychological, need to be balanced against the potential burden of treatment for the child, health care costs and unclear long-term risks and benefits (Lantos *et al.*, 1989).

Growth Hormone Treatment

SGA children who remain short at 2–4 years of age need evaluation for the use of GH treatment (Clayton *et al.*, 2007). These children are a heterogenous group and efforts should therefore be made to determine any underlying diagnosis. This is important as GH treatment is contraindicated in some disorders such as DNA repair disorders and the chromosomal breakage syndromes (e.g., Bloom and Fanconi syndromes), which may be associated with prenatal and postnatal growth failure. The efficacy and safety of GH in short children born SGA have been demonstrated in several studies over the last 5 decades and it has been licensed by the Food and Drug Administration in 2001 and the European Agency for the Evaluation of Medicinal Products in 2003 (**Table 2**). However, there are uncertainties in the specific anthropometric patient selection criteria, and the optimal dose of GH and there are therefore discrepancies in the licensing terms between the two agencies.

Treatment regimens

The short-term height gain from GH treatment is considerable; after 3 years ranging from +1.2 to +2.0 standard deviation scores (SDS) on doses ranging from 35–70 mcg/kg (Clayton *et al.*, 2007). Most of the height gain occurs in the initial catch-up period, and is then maintained up to adult height. The duration of the catch-up growth appears to be dose-dependent as the height gain of +2.0 SDS was obtained after 2.5 years with GH doses of 67 mcg/kg/day but only after 5.5 years with the dose of 33 mcg/kg/day

Table 2 Growth hormone treatment in children born small for gestational age

	FDA-approved indication (2001)	*EMEA-approved indication (2003)*
Age at start (year)	2	4
Height SDS at start	Not stated	−2.5 sd
Growth velocity before treatment	No catch-up	<0 sd for age
Reference to midparental height	Not stated	Height SDS > 1 sd below midparental height SDS
Dose (μg/kg/day)	70	35

European Agency for the Evaluation of Medicinal Products (EMEA); Food and Drug Administration (FDA).

With permission from Clayton, P.E.; Cianfarani, S.; Czernichow, P.; Johannsson, G.; Rapaport, R.; Rogol, A. (2007). Management of the child born small for gestational age through to adulthood: A consensus statement of the International Societies of Pediatric Endocrinology and the Growth Hormone Research Society. The Journal of Clinical Endocrinology and Metabolism 92(3), 804–810.

(de Zegher *et al.*, 2000). Once started, the treatment should be continued until the adult height is reached, as discontinuation after a period of treatment leads to a significant reduction in height (Fjellestad-Paulsen *et al.*, 2004). The data on adult height in SGA children treated with GH is limited, and the studies vary considerably in terms of age at start and duration of treatment, GH dose used and the endpoints. A meta-analysis based on four randomized controlled trials showed that the overall mean difference in adult height between treated and untreated children born SGA was 0.85 SDS (5.3 cm) (Maiorana and Cianfarani, 2009). Furthermore, 80% of treated children have adult height within the normal and family target height range (Van Pareren *et al.*, 2003; Dahlgren *et al.*, 2005).

Early referral is important as starting treatment at least 2 years before puberty, and normalization of height before puberty have been shown to be crucial in normalization of adult height (Dahlgren *et al.*, 2005; Simon *et al.*, 2008). Age of onset and duration of puberty, height gain during puberty, and age of menarche are similar in children treated with GH alone and untreated children (Boonstra *et al.*, 2003). However, children who are short and have already started puberty may benefit from adjuvant GnRH analog treatment for 2 years, along with a higher GH dose as these can result in improved adult height (Lem *et al.*, 2012).

Doses of GH >35 mcg/kg/day have not been shown to provide significant benefits in long-term studies (Maiorana and Cianfarani, 2009). Studies have explored GH dose titration, based on IGF-I levels, aiming for levels between 0 and +2 SDS (Jensen *et al.*, 2014). However, patients in the titrated dose group tended to have a poorer growth response compared to the fixed dose 35 mcg/kg/day group, despite having IGF-I levels of +1.2 SDS. This suggests that a higher IGF-I level may be needed to maintain satisfactory growth velocity in these children. A positive response to GH treatment with height velocity more than +0.5 SDS is anticipated in the first year of treatment. If the response is inadequate, treatment concordance, GH dose, and diagnosis should be reevaluated and discontinuation of treatment considered (Clayton *et al.*, 2007).

Response to treatment

Considerable individual variation exists in the growth response to GH treatment in SGA children (Jensen *et al.*, 2013). It is believed that identifying the factors underlying these variations may help to personalize therapy. Ranke *et al*. based on the data from 613 children developed a growth prediction model consisting of GH dose, age and weight at start of GH treatment and mid-parental height SDS which explained 52% of the variation in the first-year height response (Ranke *et al.*, 2003). In this model, GH dose had the highest predictive value accounting for 35% of the variation. GH dose, age at start of treatment and parental adjusted height SDS (height SDS-target height SDS) also predicted long-term height outcomes (de Ridder *et al.*, 2008; Sas *et al.*, 1999). However, the effect of GH dose on final height is conflicting and other studies did not find differences (Maiorana and Cianfarani, 2009). In addition, lower adiposity, greater IGF-I levels and lower insulin sensitivity before the start of treatment, and greater genetic allele scores for insulin resistance were associated with poorer growth response (Gies *et al.*, 2012; Jensen *et al.*, 2013, 2015; Thankamony *et al.*, 2016). This suggests the possibility of modifying some of these factors to improve the height outcomes.

Adverse effects of GH treatment

Adverse events related to treatment are not more common in SGA children than in other conditions treated with GH, and standard monitoring should be applied (Clayton *et al.*, 2007). There are however potential metabolic risks, including decreased insulin sensitivity, however this is compensated by an increased insulin secretion (Jensen *et al.*, 2013). Conversely, there are also metabolic benefits, with a decrease in adipose tissue and an increase in lean body mass. During treatment with GH lipid profiles also improve, with a decrease in total high-density lipoprotein (HDL), low density lipoprotein (LDL) and cholesterol (Sas *et al.*, 2000; van Pareren *et al.*, 2003). There is also improvement in bone mineral density and a decrease in both systolic and diastolic blood pressure (BP) during long term treatment with GH (Sas *et al.*, 2000; van Pareren *et al.*, 2003). Importantly, the benefits of GH treatment on BP have been found to persist for more than 5 years after GH treatment, with both systolic and diastolic BP lower in GH treated adults than untreated short adults who were born SGA (van Dijk *et al.*, 2007). At a similar time-point there were no differences in lipid/cholesterol profiles, body composition or insulin sensitivity or insulin secretion in GH treated SGA adults,

versus nontreated short adults born SGA. Individual studies have reported increased overall mortality (Carel *et al.*, 2012) and incidence of strokes (Poidvin *et al.*, 2014) in patients treated with GH during childhood. Other studies however did not support these findings and a recent workshop concluded that the aggregate evidence did not support these associations (Allen *et al.*, 2016). Nevertheless, these reports highlight the need for longterm outcome data for GH-treated children.

Late Childhood Growth and Puberty

The impact of being born SGA and early life growth trajectories also impact on puberty. Infants born SGA are known to be at increased risk of premature pubarche and adrenarche (Verkauskiene *et al.*, 2013). Some studies have also reported an earlier onset of menstruation (Ibanez *et al.*, 2006; Yadav and Rustogi, 2015). This is most marked in those who catch up. Both timing and progression of puberty is affected in SGA children, with both peak height velocity and accelerated bone maturation occurring at an earlier pubertal stage in children born SGA. While there is conflicting evidence, a number of studies have found that earlier onset of menarche (at <12 years) is threefold commoner in girls born SGA (Ibanez *et al.*, 2006), with menarche occurring 8–12 months earlier than AGA girls (Bhargava *et al.*, 1995; Brandt *et al.*, 2005; Wehkalampi *et al.*, 2011). This therefore leads to a shorter duration of pubertal growth and a less than expected growth spurt. The mechanisms determining the differences in pubertal onset and progression between SGA and AGA children have not been full elucidated, but a number of mechanisms have been postulated, and may be linked to susceptibility to the metabolic syndrome.

The rapid catch up growth in the postnatal period, with accumulation of visceral fat and hyperinsulinism resulting from insulin resistance is also associated with increased androgen levels. A combination of these factors may lead to a polycystic ovarian syndrome type picture, with earlier menarche and reduced final adult height. These SGA girls often have a combination of hyperinsulinism, central adiposity, dyslipidemia and accelerated bone maturation. It has been shown that metformin can be used to increase insulin sensitivity in these SGA girls who are showing signs of precocious pubarche (Ibáñez *et al.*, 2008, 2011). Metformin treatment lead to delayed onset of puberty, with decreased adipose tissue, while growth was maintained. There were associated improvements in lipid profiles and improved body composition, as well as decreased levels of insulin, IGF-I and leptin. Biochemical and physical improvements persisted for 12 months after metformin was discontinued. This highlights the importance of insulin in determining linear growth and pubertal progression in girls born SGA.

Lifetime Consequences of Being Born SGA

It was the early work of Barker and colleagues in the 1980s who were interested in early life origins of adult chronic diseases that lead to the explosion of interest in early growth and its effect on long term health (Barker and Osmond, 1986; Barker *et al.*, 1989). Barker used early maternity and welfare records, and linked recorded birth weight to later National Health Service registration. It was later that the opposing effects of weight at birth and in adult life became more evident (Phillips *et al.*, 1994). A wave of international studies followed which confirmed the relationship between birth weight and adult disease risk regardless of socioeconomic status and confirmed that relative weight for gestation was more important than absolute weight. Further definitive studies were those of the "Dutch Hunger Winter" a period when German occupation in the Netherlands lead to defined periods of malnutrition and a significant drop in birth weight (Stein and Susser, 1975), which was related to later obesity and cardiovascular and metabolic disease (Roseboom *et al.*, 1999, 2000a,b, 2001).

Although for SGA children early growth is an important determinant of later health risk by defining "metabolic capacity" the expression of this phenotype is dependent on the metabolic stresses put on this system. This has been described by Wells as the "metabolic load" (Wells, 2017). Key components of metabolic load extend beyond adiposity to include physical activity, smoking, psychological stress and infectious disease (Andersen *et al.*, 2006) (**Fig. 3**).

Infants born SGA also demonstrate premature activation of the hypothalamic pituitary adrenal (HPA) axis as a response to stress in utero with low birth weight associated with altered cortisol levels (Martinez-Aguayo *et al.*, 2012). This has been associated with hypertension, insulin resistance and development of the metabolic syndrome (Phillips *et al.*, 1998; Seckl, 2004; Cottrell and Seckl, 2009). An explanation of this phenomenon has been derived from animal models revealing long-term changes in the molecular expression of steroid receptors within the limbic system (Cottrell and Seckl, 2009). Additionally, there may be alteration of the renin–angiotensin–aldosterone axis in SGA infants. Male SGA infants have raised levels of angiotensin-2 and angiotensin-converting-enzyme, while female SGA infants have raised noradrenaline levels (Franco *et al.*, 2008). These factors may contribute to the finding that SGA infants have altered vascular endothelium function and raised systolic blood pressure by the age of 13 years (Franco *et al.*, 2006).

In addition to the long term metabolic risks of being born SGA, there are a number of studies that suggest that SGA children may be at increased risk of neurodevelopmental problems, with decreased cognitive and social abilities, though the data are conflicting (Castanys-Muñoz *et al.*, 2017). These data have been confounded by the inclusion of preterm infants, where the risk of poor neurodevelopmental outcomes is greater. Further work is needed to elucidate if there are certain populations at increased risk of adverse neurodevelopmental outcomes and the potential mechanisms underlying this.

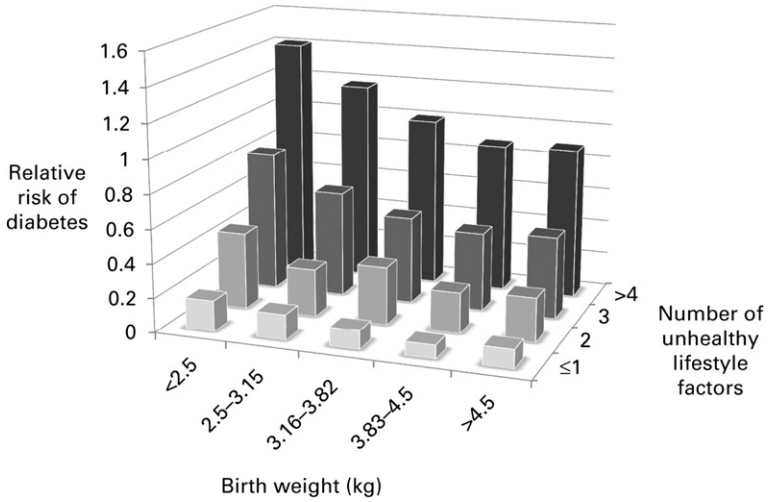

Fig. 3 The capacity-load model. This illustrates that the relative risk of diabetes is related to the interaction of the predisposition determined by birth weight combined with adult lifestyle behaviors. With permission from Wells, J. C. K. *et al.* (2016). The elevated susceptibility to diabetes in India: An evolutionary perspective. *Frontiers in Public Health*, 4, 145.

Future Directions

Optimal early growth trajectories that balance the benefits and risks for long-term metabolic and cognitive outcomes in children born SGA have not been clearly determined. The diversity of etiologies means that an individualized approach may be important. Therefore, understanding the mechanisms will help to inform a more personalized approach to interventions. There are critical developmental windows for growth that provide opportunities for intervention, but it is important these are managed optimally if we are not to exacerbate long-term health consequences for these children. Short-term interventions, whether pharmaceutical, feeding or behavioral will require long-term follow-up to clarify optimal treatment strategies whilst determining better biomarkers of what constitutes optimal growth.

Summary

SGA is a unifying diagnosis that encompasses multiple etiologies but is associated with significant short-term and long-term morbidity with risk dependent on early life growth trajectories. Catch up growth is associated with increased adiposity leading to insulin resistance and metabolic syndrome in later life. Growth hormone treatment is licensed for use in those who remain short, but the data on long-term outcomes are limited. It is important we understand the mechanisms driving these growth patterns if we hope to modify them and impact on long-term health at an individual or population level. The challenge going forward is to prevent intrauterine insults and, if present, to develop early lifestyle interventions and/or pharmacotherapy to reverse the pathophysiological changes to prevent the development of metabolic complications in later life.

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Growth Hormone Deficiency in Children

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Abbreviations

ALS	Acid labile subunit
CT	Computerized tomography
Delta, Δ	Change
GH	Growth hormone
GHBP	Growth hormone binding protein
GHRH	Growth hormone releasing hormone
IGF-I	Insulin like growth factor 1
IGFBP-3	IGF binding protein 3
IGHD	Isolated growth hormone deficiency
IHH	Idiopathic intracranial hypertension
ISS	Idiopathic short stature
KIGS	Pfizer International Growth Study
MPH	Midparental height
MPHD	Multiple pituitary hormone deficiencies
MRI	Magnetic resonance imaging
SDS	Standard deviation score
SGA	Small for gestational age—at birth
STH	Somatotropin or somatotrophic hormone
TSH	Thyroid stimulating hormone

Introduction

Growth hormone (GH) is a highly dynamic hormone normally secreted in pulses from the anterior pituitary (Robinson, 1991a), except during pregnancy, when a special form of GH is secreted from the placenta. Via the bloodstream, the GH molecules reach the receptor at the cell surface and via interaction they start the intracellular signaling cascade (Waters and Brooks, 2011). The hormone has extensive metabolic effects on most tissues; the most obvious is the direct effect on bone, stimulating longitudinal growth, lipolysis, muscle anabolism, and on hepatic metabolism interacting with insulin, resulting in increased serum levels of IGF-I. In line with these broad metabolic effects, another name for GH is somatotrophic hormone (STH). In turn, GH secretion is highly affected by many external and internal factors: physical activity, psychosocial circumstances, pain, anxiety, somatic disease, undernutrition, inflammation, and the status of other hormones such as thyroxin, cortisol, insulin, and sex hormones.

For many children, a growth velocity deceleration or an observed short stature is the sign that raises the question of possible GH deficiency. It is of great importance that the investigation starts with a broad general pediatric approach, obtaining all available information on previous weight and height measurements, including information on heredity, that is, family heights and possible health problems (GH Research Society, 2000). The diagnosis of GHD relies heavily on auxology; however, this is not always available.

Only after the exclusion (or optimal treatment) of other reasons for low growth velocity or short stature, and with the child's health and well-being in the best state possible, are biochemical investigations for GH reliable. However, basic endocrine investigations should include thyroid function test and, IGF-I and IGFBP-3 analyses (Blum *et al.*, 1993), also repeatedly due to the common great intra-individual variations (**Fig. 1**) (Gelander *et al.*, 1999), before a decision is taken on any GH evaluation. Evaluation of GH secretory capacity in an individual has to be performed during standardized circumstances due to the pulsatility and great physiological variations during the individual's lifespan.

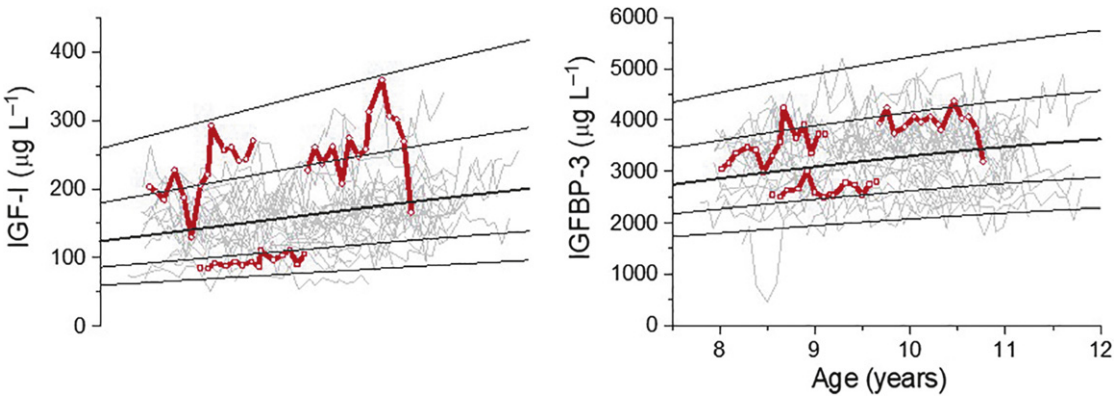

Fig. 1 Longitudinal serum concentration of IGF-I (left panel) and IGFBP-3 (right panel) in healthy prepubertal children, including 38 boys and 27 girls. All analyzed samples in one child are joined by a line. Reference lines are drawn according to IGF-I and IGFBP-3 reference values (Löfqvist *et al.*, 2001, 2005), and show the mean, +1, and +2 SDS. Gelander, L., Blum, W. F., Larsson, L., Rosberg, S. and Albertsson-Wikland, K. (1999). Monthly measurements of insulin-like growth factor I (IGF-I) and IGF-binding protein-3 in healthy prepubertal children: Characterization and relationship with growth: the 1-year growth study. *Pediatric Research*, 45, 377–383, with permission.

 doi:10.1016/B978-0-12-801238-3.66108-1

If a diagnosis of GHD is made, a MRI or CT scan of the brain (including the pituitary) will be required, with the possibility of developmental anomalies or expansive processes needing further measures.

Substitution therapy with recombinant human GH is performed by daily injections. The dose should be adapted according to individual GH responsiveness with a predefined target on efficacy, influenced by diagnose, growth period (i.e., age and maturity), and treatment phase.

Embryology and Genetics

The synthesis and secretion of GH occur from somatotrophic cells in the anterior part of the pituitary, the adenohypophysis, which originates from Rathke's pouch, which originates from invagination of the primitive pharyngeal epithelial floor. The adenohypophysis also contains cells producing thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), prolactin, follicle stimulating hormone (FSH), and luteinizing hormone (LH).

The posterior part of the pituitary, the neurohypophysis, has its origin in the brain and releases the hormones oxytocin and antidiuretic hormone (ADH). The latter is involved with water regulation.

A number of genetic mutations have been reported to be involved with the development of the pituitary (Kelberman *et al.*, 2009; Wit *et al.*, 2011). With a confirmed diagnosis of isolated GHD (IGHD) or multiple pituitary hormone deficiencies (MPHD), the question is often raised on the precise etiology, that is, what mutation the child may have. Tests for genetic mutations have hitherto not been part of clinical practice; however, in the future this may change. More important for clinical monitoring is the result of an MRI of the brain: an ectopic posterior pituitary with a hypoplastic anterior pituitary is the sign of a prenatal malformation, alerting the endocrinologist of upcoming additional hormonal deficiencies as the child grows older and increases in body size.

Growth Hormone

Structure

The human GH gene cluster is present on chromosome 17q and contains two forms: GH normal (GH-N), expressed in the pituitary, and GH variant (GH-V), expressed in the placenta. GH in humans consists of many isoforms (Baumann, 2009). The most abundant form in the human circulation is the 22 kDa GH, a single-stranded chain of 191 amino acids, responsible for 70%–75% of available GH. Due to alternative splicing, 5%–10% of serum GH are of 20 kDa size, and there are also small amounts of GH with size 27, 17, and 5 kDa. The 20 kDa isoform is generally less biologically active compared to the 22 kDa GH, but has a longer half-life in serum and is therefore more abundant during GH troughs (Baumann, 2009). Knowledge regarding the biological significance of these less abundant isoforms is limited, although the 20 kDa isoform has been suggested to have more metabolic effects (Baumann, 2009). In addition to the mentioned monomeric forms, GH also exists in oligomeric forms, composed of the different forms in the pituitary and plasma. An increased proportion of isoforms in the circulation, other than 22 kDa, has been proposed to be a reason for impaired growth in children (Boguszewski *et al.*, 1997).

Biochemical Evaluation of GH

There have been, and still are, a wide range of assays for estimation of GH, as well as IGF-I and IGFBP-3. This means that the clinician needs to be aware of the used methods (**Fig. 2**) (Clemmons, 2011; Jansson *et al.*, 1997). Efforts for standardization have

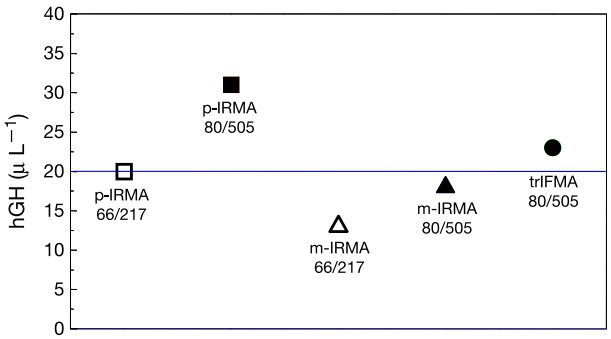

Fig. 2 Influence of the GH assay used for the estimated serum concentration of GH. The result from one sample but using two assays and two WHO IRPs 66/127 and 80/505. The assays were an immunoradiometric IRMA, using polyclonal or monoclonal antibodies and a time-resolved monoclonal immunofluorometric assay (trIFMA). Albertsson-Wikland, K. and Rosberg, S. (2011). Methods of evaluating spontaneous growth hormone secretion. In: Ranke, M.B. and Mullis, P.-E. (Eds.), Diagnostics of endocrine function in children and adolescents. 4th edn., pp 138–156. Basel: Karger.

been made; for estimation of GH nowadays, a monoclonal antibody is used with a reference preparation based on human recombinant 22 kDa (hGH 98/574) (Bidlingmaier and Freda, 2010).

Similarly, the assays and reference preparations for IGF-I and IGFBP-3 need standardization (Clemmons, 2011). However, as IGF-I and IGFBP-3 serum levels vary with a child's age and maturity, even more important is a reference from the certain assay and laboratory that accounts for sex, age, and pubertal stage (**Fig. 3**) (Juul *et al.*, 1994; Löfqvist *et al.*, 2001, 2005). Thus, IGF-I and IGFBP-3 levels should be given in SDS (standard deviation score) rather than in $\mu g\ L^{-1}$.

Regulation of GH Secretion

GH is released from the pituitary in pulses due to the balanced interaction of the hypothalamic neuro-peptides GH releasing hormone (GHRH) and the inhibitory somatostatin. GHRH is secreted in pulses every 3 h, and is stimulated by sleep, nutrition, stress, physical activity, hypoglycemia, thyroxine, estrogen, and androgen, whereas somatostatin is secreted continuously and regulated by IGF-I, free fatty acids, and cortisol (Robinson, 1991b; Tannenbaum *et al.*, 1990; Veldhuis *et al.*, 2006). In addition, GH in the circulation has a feedback on hypothalamic GHRH secretion and an auto-feedback on its own secretion in the somatotrophs in the pituitary. Negative feedback by endocrine IGF-I also contributes (**Fig. 4**) (Robinson, 1991b).

GH Secretion Pattern

GH secretion changes during lifespan. During the late fetal and neonatal period, GH is present in fetal circulation at a high and unregulated level due to the immaturity of the GHR system. GH is not of great importance for size at birth, and infants with congenital GH deficiency and defects in the GH-receptor gene have no or only a mild reduction in birth size (Albertsson-Wikland *et al.*, 1990). Defects in the IGF-I gene or any interference with IGF-I action are both associated with severe growth retardation (Woods *et al.*, 1996).

During infancy, the GH pattern of pulsatile secretion starts to be established (Zegher *et al.*, 1997). Longitudinal growth is still not GH dependent. Therefore, growth failure in children with growth hormone deficiency (GHD) commonly does not present until the childhood growth period (Karlberg and Albertsson-Wikland, 1988).

During childhood, GH secretion starts to establish a diurnal peak-and-trough pattern, with higher peaks during night-time. During this period, GH secretion is similar for both sexes. GH is the main regulator of childhood growth with a dose-dependent effect (Albertsson-Wikland and Rosberg, 1988), both directly at the growth plate (Isaksson *et al.*, 1987) and indirectly through the IGF-I (Ohlsson *et al.*, 2000). Thyroxine, insulin, and cortisol are all necessary and permissive hormones. In addition, nutrition and psychosocial factors continue to be of great importance.

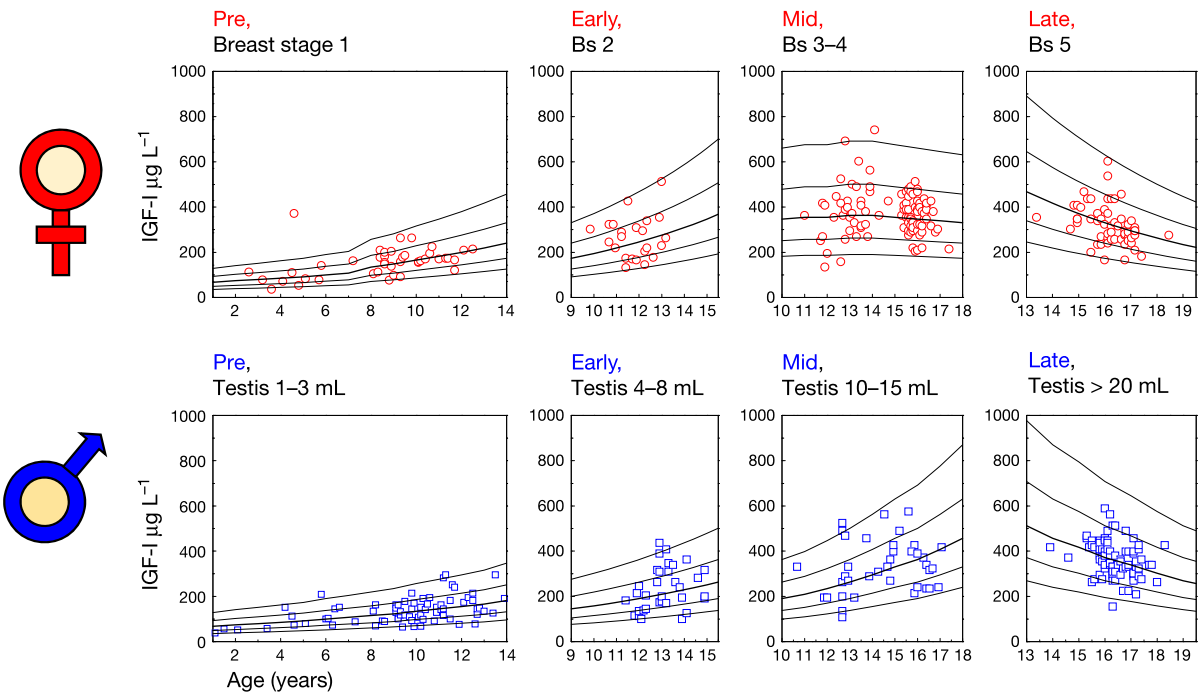

Fig. 3 The reference model for IGF-I based on values from healthy girls and boys according to age and pubertal stage. Löfqvist, C., Andersson, E., Gelander, L., et al. (2001). Reference values for IGF-I throughout childhood and adolescence: a model that accounts simultaneously for the effect of gender, age, and puberty. *Journal of Clinical Endocrinology and Metabolism*, 86, 5870–5876, with permission.

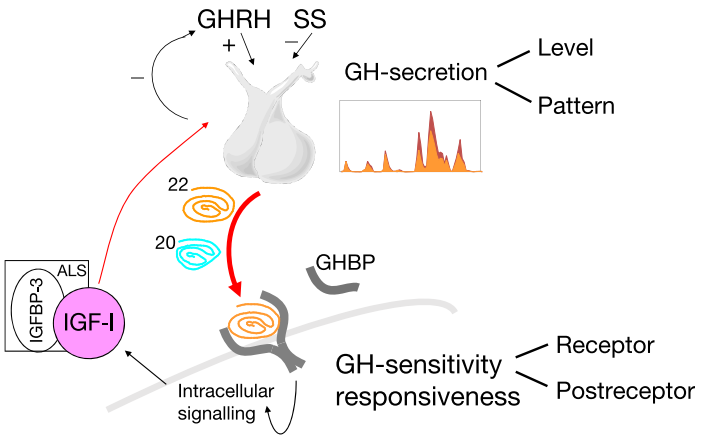

Fig. 4 The GH/IGF-I axis. A schematic overview of the GH/IGF-I feedback loop. Secretion of GH from the pituitary gland is regulated by two peptides: GH releasing hormone (GHRH) and somatostatin (SS). Secreted GH consists of several isoforms; the most abundant 22 kD form is the most biologically active form; a smaller proportion exists as 20 kD isoform. Almost 50% of the GH in blood is bound to the high-affinity GH-binding protein (GHBP), which is structurally similar to the extracellular domain of the GH receptor. After binding to the receptor, GH induces secretion of insulin-like growth factor I (IGF-I) which can elicit autocrine, paracrine, and endocrine effects, as well as feedback effects on GH secretion. The activity of IGF-I is modulated by binding proteins; the most abundant are IGFBP-3 and the acid-labile subunit (ALS).
Lundberg, E. Thesis Umeå university 2017. ISBN 978-91-7601-662-6.

During puberty, GH becomes upregulated in response to estrogen. The pulsatile GH secretion increases, with high peaks during both day and night in a gender-specific manner: there is about a threefold increase in girls and a twofold increase in boys (Albertsson-Wikland *et al.*, 1994). The pulse amplitude and amount of GH per secretory burst increase, but the pulse frequency does not change (Mauras *et al.*, 1987).

After completed growth, that is, when adult height is attained, GH secretion progressively declines, falling to very low levels in old age (Rudman *et al.*, 1981). The GH amplitude is reduced while pulsatility remains (Giustina and Veldhuis, 1998). In adulthood, the GH secretory pattern in men is characterized by peaks about every 3 h (Albertsson-Wikland and Rosberg, 2011) and low basal circulating troughs in between (Giustina and Veldhuis, 1998). The highest GH peaks are seen during slow-wave sleep, and smaller pulses occur during the day (Van Cauter *et al.*, 2000). In women, the presence of estrogen will give rise to a GH pattern that is characterized by a sustained basal level and more irregular peaks with different amplitudes (Ho *et al.*, 1987).

From rodents, we learn that sex differences were found in the pattern of GH secretion (Eden, 1979; Jansson *et al.*, 1985; Robinson *et al.*, 1998). The adult male rats had a high-amplitude, 3-hourly, pulsatile pattern of GH secretion, with low troughs in between (Wagner *et al.*, 1998), whereas females secreted GH in a more continuous pattern with a high basal level and irregular peaks (Eden, 1979). The pattern of GH secretion in rodents is an important determinant of the sexually dimorphic pattern of growth (Jansson *et al.*, 1985), liver enzyme function (Legraverend *et al.*, 1992), circulating GH-binding protein concentration (Carmignac *et al.*, 1993), and IGF-I mRNA expression in skeletal muscle and the liver (Isgaard *et al.*, 1988) in these animals.

The GH Receptor

The human GH receptor (GHR) gene is located on chromosome 5 (Leung *et al.*, 1987). The receptor is embedded in the outer membrane of cells throughout the body and is most abundant in the liver (Waters and Brooks, 2011). The GHR consists of a large extracellular part containing the GH-binding site and occurs separately as the GH-binding protein found in the circulation (Waters, 2016). GH induces the changes in the GHR starting the intracellular signaling (Brooks and Waters, 2010). The STAT5b pathway mediates the activation of different genes such as a transcription of IGF-I, IGF binding protein 3 (IGFBP-3), and the acid-labile subunit (ALS) gene in the live. A deletion in the GHR gene was shown to explain the GH insensitivity in some patients with Laron syndrome, clinically mimicking severe GHD (Godowski *et al.*, 1989).

Effects of GH

GH receptors and the GHR gene have been found in most tissue in humans, indicating that GH has effects in most tissues including protein synthesis, lipolysis, and reduced glucose utilization. Our hypothesis on varying responsiveness in different tissues was confirmed in a trial on GH treatment in prepubertal patients with different endogenous secretory capacities (**Fig. 5**) (Decker *et al.*, 2012). Considering these differences in tissue responsiveness within the child means that the complexity of clinical signs in (different degrees of) GH deficiency is much more understandable, and thereby improves the diagnostic performance of the endocrinologist.

The most responsive tissue to GH is the brain. The GHD patients in the trial after the start of treatment showed improved attention, perception, and cognitive capacities (Chaplin *et al.*, 2011, 2015). GH immunoreactivity was found in the first stage of

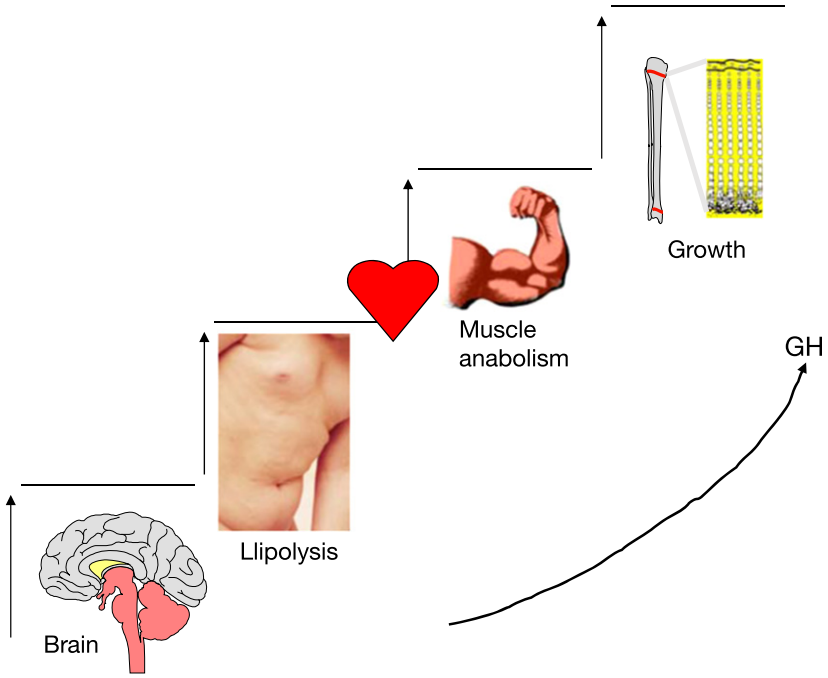

Fig. 5 Our hypothesis, which was found to be valid, according to the amount of GH needed for effect in different tissues. The most GH-sensitive tissue was the brain, and the least sensitive was the IGF-I production, which was less sensitive than bone growth. Adapted from Modified from Decker, R., Nygren, A., Kristrom, B., et al. (2012). Different thresholds of tissue-specific dose-responses to growth hormone in short prepubertal children. *BMC Endocrine Disorders*, 12, 26.

brain development with GH-binding sites in all brain structures (Nyberg, 2000). In addition, GH has been shown to be important in physiological and reparative neurogenesis, as in brain repair after injury and the development of learning capacity and cognitive function (Nyberg, 2007).

The second most responsive process is lipolysis. GH increases lipase activity in fat tissue, resulting in increased serum free fatty acids (Moller and Jorgensen, 2009). Administration of GH has been shown to reduce body fat in GHD adults (Elbornsson *et al.*, 2013). A positive correlation between leptin reduction and 1st-year growth response after the start of GH treatment was found in a group of short children treated with GH (Kristrom *et al.*, 1998).

GH has a positive effect on protein metabolism, that is, anabolism, and increases muscle tissue according to both mass and strength, as seen in studies where GH was administrated to rodents (Isgaard *et al.*, 1988) and to GHD adults (Gotherstrom *et al.*, 2001), and children (Decker *et al.*, 2010).

GH acts directly on the growth of myocardium and on inotrope heart function during fetal development (Sacca *et al.*, 1994). Additional support for the GH effect on the heart was shown in both GHD and non-GHD prepubertal patients, in whom increased cardiac size with normalized wall thickness was found (Nygren *et al.*, 2012).

The least GH-responsive (=least sensitive) tissue was the liver with the IGF-I production, that is, the GH effect on the liver (Decker *et al.*, 2012). Bone growth was the next to least responsive (Isaksson *et al.*, 1982).

In addition, GH induces an apparent insulin resistance through increased endogenous gluconeogenesis in the liver and decreased peripheral glucose disposal in muscle (Moller and Jorgensen, 2009). Insulin levels are lower than normal in untreated GHD patients, and they increase in a dose-dependent manner with GH treatment while remaining within the normal range as long as the GH dose is adapted according to individual GH responsiveness (Decker *et al.*, 2012).

Evaluation of Endogenous GH Release

When, after being fulfilled thorough pediatric investigation, an investigation of GH release is planned to confirm a clinically suspected diagnosis, it is of great importance that this investigation is performed while the child is as healthy as possible and in standardized circumstances (**Fig. 6**). Stimulation tests should be performed according to standardized protocols, in the morning after an overnight fast. The most used stimulating agents are arginine, insulin, clonidine, glucagon, and L-dopa; this number of agents indicates that none of them is optimal (Ranke, 2011). Due to side effects, potential hazards, and patient discomfort, selection of the test should be made by the responsible endocrinologist, and the test should be performed and monitored by well-trained personnel.

Even though the test stimuli and procedure are standardized, interpretation of the test results needs caution. There is the possibility of a GHRH-induced refractoriness, due to negative feedback by recent endogenous GH secretion (**Fig. 7**) (Gelander and

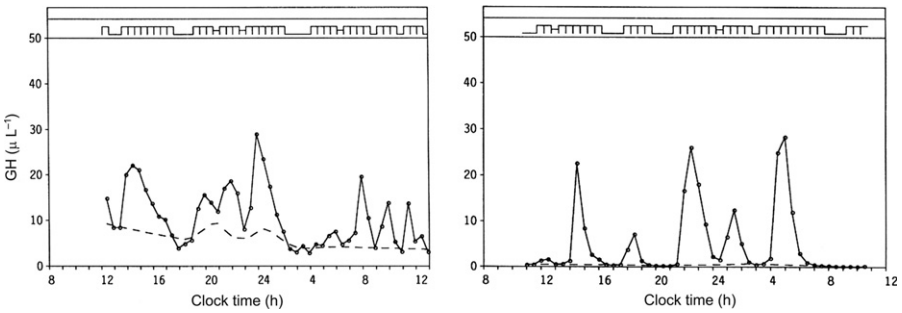

Fig. 6 Spontaneous GH secretion in a prepubertal child during an infectious disease with fever (left panel) and 2 weeks later when he was healthy again (right panel).

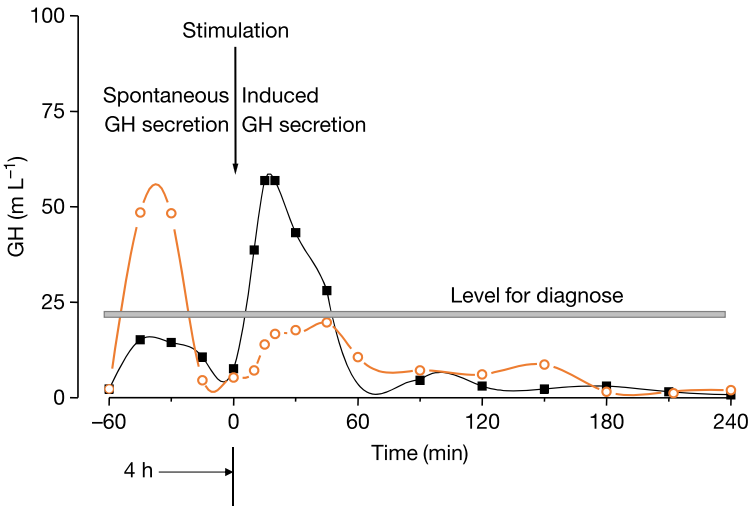

Fig. 7 The effect of refractoriness when a stimulus is given within 4 h after a spontaneous GH peak (line indicated with -o-), compared to if the stimulus has not been preceded by a spontaneous GH peak (line indicated with - ■ -). The refractoriness after the spontaneous peak may falsely indicate subnormal GH secretory capacity. Gelander, L. and Albertsson-Wikland, K. (1989). Growth hormone (GH) release after administration of GH-releasing hormone in relation to endogenous 24-h GH secretion in short children. *Journal of Endocrinology*, 122, 61–68, with permission.

Albertsson-Wikland, 1989). It is the balance between the GHRH and the somatostatin at the very moment of stimulation that accounts for the released amount of GH. This physiological mechanism is an important factor contributing to the great intra-individual variations, or low reproducibility, of repeated tests in the same individual (Cacciari *et al.*, 1992; Meazza *et al.*, 2017; Tauber *et al.*, 1997), and also explains why children are falsely diagnosed as GH deficient. In a Swedish national trial, about 50% of the children diagnosed with GHD according to clinical criteria and the arginine-insulin tolerance test (AITT) were found to have high GH peaks during a spontaneous secretion profile, and therefore were re-diagnosed with idiopathic short stature (ISS) (Kriström *et al.*, 2009a).

Thus, to escape the refractoriness problem with the stimulation tests, patient discomfort, and the problem with hypothalamic induced neurosecretory dysfunction (Gelander and Albertsson-Wikland, 1989; Spiliotis *et al.*, 1984), evaluation of spontaneous secretion over 24 or 12 h night-time can be performed (Kriström *et al.*, 2001). Allowing the child to eat, play, and sleep normally, with small amount of blood samples collected in 20–30-min. intervals without pain or anxiety, ensures that the GH release is as close to physiology as possible (Albertsson-Wikland and Rosberg, 2011). The medical risks are minimized; however, a trained nurse is still required and the obtained results need professional interpretation.

When evaluating the endogenous GH secretion, not only the highest peak matters, but also the relation between peaks and troughs, the basal serum levels. The secretion pattern contains different biological signals; the GH peaks have been shown to be most important for growth in rats while the troughs have wide effects on metabolic factors from the liver, with different effects in male and female animals (Robinson, 1991b). Adult humans received the same amount of GH in different patterns in an experimental setting; constant infusion giving constant trough levels resulted in higher IGF-I concentrations than when GH was given as daily injections, that is, as distinct GH peaks (Johansson *et al.*, 1996; Jorgensen *et al.*, 1990). Thus, GH peaks and troughs have different effects on growth and IGF-I level, which may have implications for long-acting GH. It has been difficult to decide an optimal GH dose for growth response in children when guided by the effect on IGF-I (Cohen *et al.*, 2007; Hou *et al.*, 2016; Moore *et al.*, 2016), as a larger GH dose is required to have an effect on bone growth compared to the effect on IGF-I (Decker *et al.*, 2012).

A correct estimation of the GH peak is important, as it has been shown in groups of children that growth velocity is mainly influenced by the GH pulse amplitude (Albertsson-Wikland and Rosberg, 1988; Hindmarsh *et al.*, 1988). Supporting this, when used in an auxology-based model for prediction of growth response to GH treatment, the spontaneous GH peak, followed by the area under the curve (AUC—the lower the better growth response if treatment was started), were the most important GH variables (Albertsson-Wikland *et al.*, 2000).

When the GH/IGF-I axis has a severely impaired function, clinical and metabolic signs are usually obvious, and diagnostic investigations are more focused on the level of defect (**Fig. 8**). It may be hypothalamic or pituitary with no or very low measurable GH secretion or at the tissue level with a defect in the GH receptor or in the intracellular signaling. The uniting biochemical sign is very low IGF-I and IGFBP-3 levels. However, some children with substantially low IGF-L levels may have clearly measurable GH peaks where there may be a defect within the GH/IGF axis not yet understood, but they may still have a positive growth response to GH treatment with an adapted GH dose. With the recombinant human GH becoming available in 1985–86, in clinical trials children with some endogenous GH secretion were found to respond to GH treatment (Albertsson-Wikland *et al.*, 2008). Even though many objections could be made, a cutoff value for maximal GH peak response was previously used in clinical practice about when to start GH treatment. Nowadays a more multifactorial approach considering physiology has been accepted in most societies (Bidlingmaier and Freda, 2010; Rosenfeld *et al.*, 1995). Besides GH assay and standard issues, considerations of physiological GH secretion according to age and biological maturation as well as the relation between GH peaks versus insulin and IGF-I level should be made as an indirect sign of GH effect and GH responsiveness.

Close to the onset of puberty, the GH secretion physiologically becomes so low that confusion with GH deficiency may occur. In these cases, priming with sex steroids has been suggested, but this does not solve the problem. During puberty, GH secretion is upregulated into tall and slender peaks that are 2–4-fold higher than before, higher in girls than in boys (Albertsson-Wikland *et al.*, 1994). Later, the growth plate matures under the influence of estrogen (Smith *et al.*, 1994) and the GH secretion decreases with descending levels of IGF-I and IGFBP-3 (Löfqvist *et al.*, 2001, 2005). Taking these physiological variations into account, a more dynamic approach is needed for interpretation of the individual GH/IGF-I investigation.

Clinical Presentation

In the neonatal period, the signs that bring up the question of GHD are unexplained hypoglycemia (with metabolic disorders needing to be ruled out), microphallus, jaundice, and breech presentation. Size at birth for neonates with organic GHD is normal, but children who were later, at different ages, diagnosed with isolated GHD were found to have a reduced birth size (Albertsson-Wikland *et al.*, 1990; Binder *et al.*, 2010). The GH-deficient infant in affluent Western countries typically grows with a normal infancy growth velocity in the first 6–12 months, until the transition to the childhood growth phase is expected to take place due to the GH/IGF-I axis for growth becoming active (Hochberg and Albertsson-Wikland, 2008). Although much less common, hypoglycemia during childhood may be a sign of GHD and should prompt endocrine investigation, especially if combined with midline defects.

During the preschool ages and later, the most common signs that brings children to the pediatric endocrine clinic with a GHD suspicion are low growth velocity or short stature. Auxology data are excellent when judging if and how to plan an investigation.

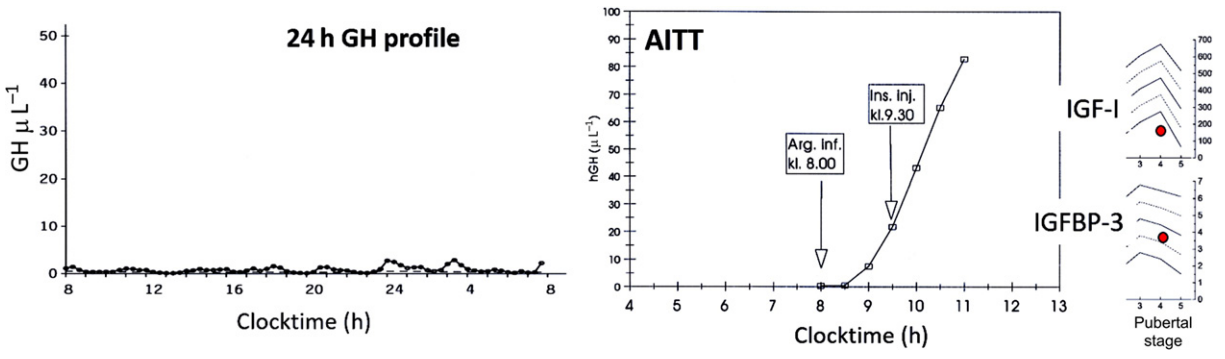

Fig. 8 GH deficiency of hypothalamic origin. Results from a GH re-investigation at adult height in a girl aged 15 years with spontaneous puberty and normal weight for height. She had received cranial irradiation some 10 years before due to high-risk leukemia. GH treatment had been inhibited for 1 month when the investigation was performed; her IGF-I SDS and IGFBP-3 SDS at the investigation are shown in the reference for pubertal stage (lines −2 to +2 SDS are shown). A spontaneous 24-hr GH profile was performed from 8 a.m. to 8 a.m. with blood sampling every 20 min. Each dot in the figure represents a blood sample. Immediately following the profile sampling, an arginine-insulin tolerance test (AITT) was performed. Observe the different scaling for the GH serum concentration for profile and AITT. During the spontaneous profile a very small amount of GH is released into the serum—much less than normally seen in a pubertal adolescent girl, in harmony with her abnormally low IGF-I SDS. During the AITT, a successively increased GH release in response to the induced stimulation/hypoglycemia was found. Her profile represents her physiologic condition, confirming the GHD diagnosis.

The descending growth velocity can appear at any age, depending on when the limit for the needed GH secretion capacity is exceeded.

An important sign is short stature in relation to relative midparental height (MPH) (usually defined as more than 1.5 SDS below MPH SDS) or the population, defined as height for sex and age 2 SDS or more below the population mean (Luo *et al.*, 1998). For evaluation of height in relation to the population, a growth reference from the appropriate population is optimal. However, if not available, for evaluation of height in relation to parents and for evaluation of growth velocity, a reference based on other populations or the WHO reference can be used. Heights expressed in SDS are preferred, rather than in percentiles, as this makes precise calculations possible. Important information in the history of the child may be craniofacial midline abnormalities, brain infection, or severe trauma, cranial or facial irradiation, or GHD in the family.

Basal investigations include measurement of body proportions; they are normal in a child with GHD. Measurement of body proportions of a parent with short stature may give valuable information. Before any evaluation of the GH/IGF axis is performed, other causes of impaired growth should be considered and appropriately excluded, as signs of chronic systemic disease, genetic syndromes (i.e., for Turner syndrome, karyotype is needed; do not rely on clinical signs), or hypothyroidism (GH Research Society, 2000).

Radiological Evaluation

Radiological evaluation of the biological maturation of the bone, that is, "bone age," may be a valuable part of the investigation; in GHD it is most often retarded. Bone age is estimated from an X-ray of the left wrist and hand, and compared by a trained person with results from healthy children with a known age. For very young children, aged below 1–2 years, X-rays of the knee and ankle may be informative (GH Research Society, 2000).

Imaging the brain by MRI or CT is required in children who are diagnosed with GHD, in order to study the midline structures, the position, size, and morphology of the two parts of the pituitary and the stalk, considering the possibility of an expansive or destructive process interfering with the hypothalamic or pituitary function.

Individualization of GH Treatment

The growth phases in a normal child are divided into Infancy, Childhood, and Puberty, the Karlberg "ICP-model" (Karlberg, 1987, 1989), with a juvenility phase being included later, between childhood and puberty, before reaching adult height (**Fig. 9**) (Hochberg and Albertsson-Wikland, 2008). After the start of GH treatment we identify three treatment periods that may need a different approach: the catch-up growth period, the maintenance period, and the pubertal growth period. The catch-up period is defined from the start of GH treatment until height SDS has reached the normal range according to MPH SDS for the child (Boersma and Wit, 1997). The maintenance period is characterized by a normal growth velocity that keeps the height SDS within a variability normal for age and sex, and lasts from the end of the catch-up period until the onset of puberty. Thereafter the pubertal

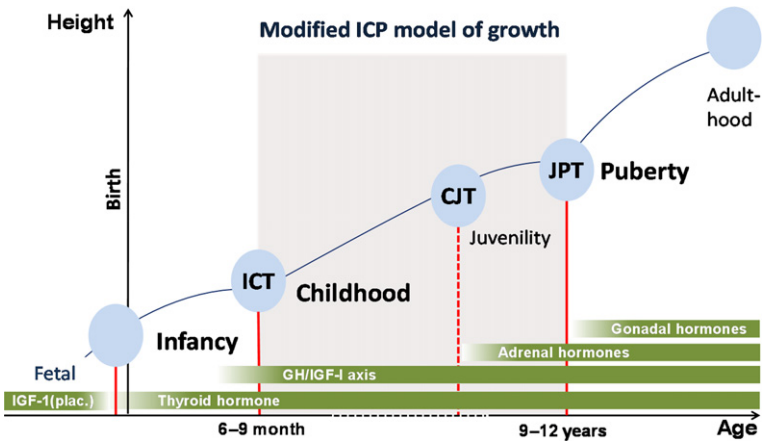

Fig. 9 Modified model of infancy-childhood-puberty growth in relation to endocrine regulation. The transition phases between different growth periods are marked with circles. The fetal growth is influenced by IGF-I from the placenta and thyroid hormone. Thyroid hormone is a major regulator during infancy and together with cortisol influences all growth periods afterwards. The GH/IGF-I axis starts to regulate growth from age 6–12 months, the infancy-childhood transition (ICT). For the childhood-juvenility transition (CJT), adrenal hormones take the lead. The juvenility-puberty transition (JPT) is influenced by the gonadal hormone. From Hochberg, Z. and Albertsson-Wikland, K. (2008). Evo-devo of infantile and childhood growth. *Pediatric Research*, 64, 2–7.

growth period lasts until adult height is attained, often simply defined as a growth velocity below 1–2 cm/year with concomitant advanced bone age (**Fig. 10**).

The recommended range for GH doses varies between countries, also for the same diagnosis. When deciding on a within-label GH treatment dose, estimations of individual GH release, with tests or a profile, are of great value as a GH dose needs to increase the GH effects more than the endogenous GH has done. We are discarding the "fixed-dose wait-and-see" approach in favor of a more active and adaptive dosing strategy (Ranke, 2010). The exogenous GH peak can be regarded as a substitute for the endogenous GH peak, the signal for growth. Exogenous GH downregulates the endogenously released GH for 36 h (Rose *et al.*, 2000) through the feedback systems. However, spontaneous GH pulses were seen after just 6 h in a study on GH pharmacokinetics after subcutaneous GH injections (Lundberg *et al.*, 2018). The IGF-I pretreatment level may be associated with growth response when studied in a group, but is a weak indicator of GH responsiveness for an individual child (Kristrom *et al.*, 1997). More important for growth response is the on-treatment dose effect on ΔIGF-I SDS shown in both prepubertal children (Cohen *et al.*, 2007, 2010) and pubertal children (Lundberg *et al.*, 2015). Already after 1–3 months on treatment, a ΔIGF-I SDS more than 1 SDS is often associated with a substantial growth response (Cohen *et al.*, 2010; Lundberg *et al.*, 2015), even if there is individual variability also for IGF-I responsiveness. However, evaluation of growth response/GH responsiveness preferably needs 12 months (Ranke *et al.*, 2013).

By using a model for prediction of growth response, an indirect estimate of individual responsiveness is received already before the start of treatment by the prediction interval (Kriström and Albertsson Wikland, 2002). Therefore a suitable dose can be selected in order to attain a satisfying catch-up growth and reach the growth target within 2–3 years (**Figs. 11** and **12**) (Kriström *et al.*,

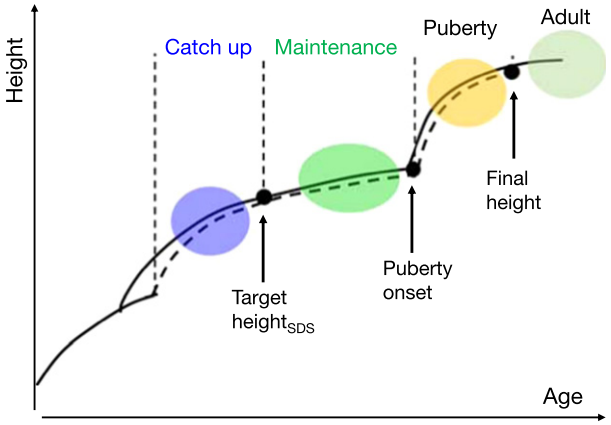

Fig. 10 The different growth periods on GH treatment: the catch-up period with high growth velocity ends when the height SDS of the child has reached within the normal range for target height (midparental height) SDS. The maintenance growth period is characterized by normal prepubertal growth velocity. The pubertal growth spurt is finalized when adult height is reached.

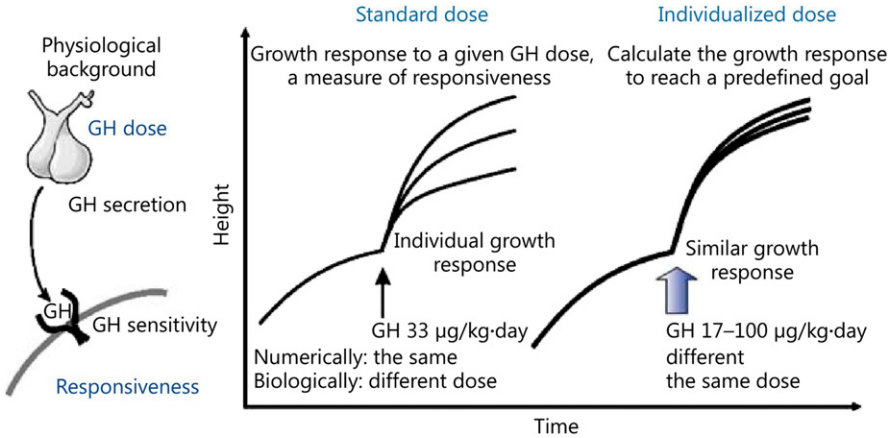

Fig. 11 Schematic illustration of GH effect relying on the balance between individual GH secretion and individual GH responsiveness, with the extremes GH deficiency and GH insensitivity. When GH treatment is performed, the dose replaces the GH secretion but tissue responsiveness cannot be replaced or affected. This is well known as the great variation in growth response to GH treatment within groups of children. Actually the growth response to a given dose is a sign of (a bioassay) the individual GH responsiveness. When instead the dose is adapted according to the responsiveness, more children achieve their growth response target. Kriström, B. and Albertsson Wikland, K. (2002). Growth prediction models, concept and use. *Hormone Research in Paediatrics*, 57, 66–70.

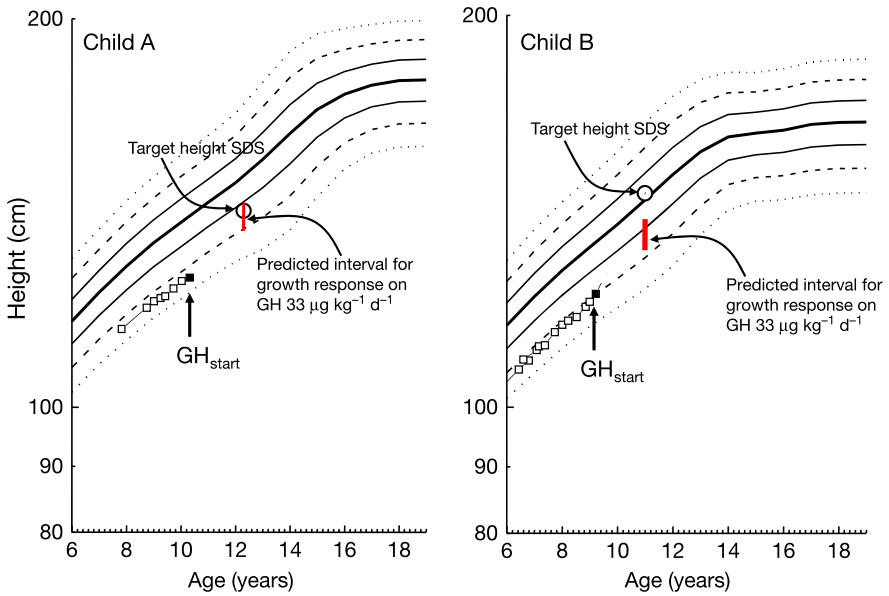

Fig. 12 Principle for selection of GH dose guided by prediction of growth response to GH treatment. Included in the height growth chart are pretreatment heights, predicted intervals for growth response if the child were to start GH treatment with a daily dose of 33 μg kg^{-1}, and target height SDS, as the common goal for catch-up growth response. Child A (left panel) is predicted to have a height gain resulting in a height SDS within target height SDS, thus the dose fits well. Child B (right panel) is predicted to attain a height SDS substantially below target height with the dose 33 μg kg^{-1}, which means that a higher GH dose is needed if target height SDS will be attained during the catch-up growth. Kriström, B. and Albertsson Wikland, K. (2002). Growth prediction models, concept and use. *Hormone Research in Paediatrics*, **57**, 66–70.

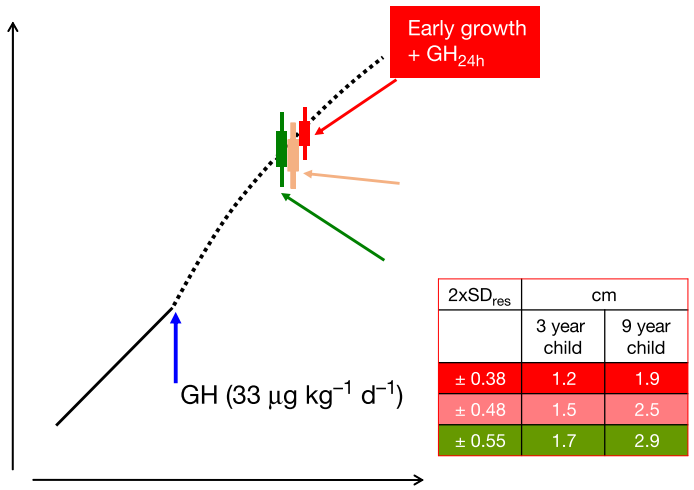

Fig. 13 Performance of the prediction models depending on included variables. The prediction interval is calculated as ±two times the SD of the residuals (=the SD of the differences between observed and predicted growth response) and should preferably be low. All models were accurate, with the most narrow prediction interval resulting from the model that included all auxology data and the 24-h GH$_{max}$ from the spontaneous profile. Kriström, B. and Albertsson Wikland, K. (2002). Growth prediction models, concept and use. *Hormone Research in Paediatrics*, 57, 66–70.

2009a). The prediction models are mathematical/statistical equations, all constructed on observed (empirical) data on GH treatment growth responses from large groups of children. Thereafter the models have been tested on new cohorts of data and found to be correct, that is, validated, with only small differences (=residuals) between predicted (=calculated) and observed growth responses (**Fig. 13**) (Albertsson-Wikland *et al.*, 2000; Kriström *et al.*, 2001; Ranke *et al.*, 1999). Even though the statistical approaches have been slightly different when constructing the Swedish and KIGS models, a strength was that the variables found to be of importance were the same, that is, large intra-familial height deficit, low age of the child, and low GH secretion capacity—as previously reported using a more simple method of analysis (Kristrom *et al.*, 1995). If these important variables are missing, the 1st-year treatment growth response reveals the responsiveness (Kriström *et al.*, 2009b), but as the growth response during the 1st year is always the greatest, optimal dosing from the very start is important. The approach of using a prediction model estimation of

responsiveness was found to be valid in our randomized trial in GHD and ISS prepubertal children during the catch-up period, not only for growth (Kriström *et al.*, 2009a), but also for metabolism (Decker *et al.*, 2010), self-esteem and behavior (Chaplin *et al.*, 2011), and cognition (Chaplin *et al.*, 2015).

During the maintenance growth phase there is some evidence that the dose needed to maintain normal growth velocity is lower than for the catch-up period, as has been reported from a SGA cohort (De Zegher and Hokken-Koelega, 2005). Taking the reduced GH secretion seen in healthy children during the juvenility phase, this dosing strategy is fully compatible with physiology (Westphal, 1987).

For puberty, however, many years of experience with the same dose as during prepuberty have given the impression that gain in height SDS during puberty was not possible, and therefore all gain in height SDS had to be achieved before puberty (Coelho *et al.*, 2008; Reiter *et al.*, 2006). To have normalized height SDS before puberty onset is still our goal. However, some children come late to diagnosis, and for those we have shown that an increased dose during puberty, resulting in GH serum levels seen in healthy pubertal children and high enough to increase their prepubertal IGF-I SDS, may also result in increased height SDS during puberty (Albertsson-Wikland *et al.*, 2014; Lundberg *et al.*, 2015).

However, growth response is not only about the dose, but also about adherence to the daily injections and the injection technique. For optimal growth response, it is important to inject deep enough to reach close to the muscle layer to attain as high a GH peak (C_{max} in pharmacokinetics) as possible in relation to the injected dose. GH injected into the fat tissue is absorbed through the lymphatics, which means a lower GH peak and 30% of the dose is lost (Christiansen *et al.*, 1983; Lundberg *et al.*, 2018), while GH injected into the muscle is more rapidly absorbed through capillaries (**Fig. 14**) (Laursen, 2004).

GH Treatment Safety

Over the years there have been a number of publications on GH safety, with a recent report on many aspects to consider in clinical situations (Allen *et al.*, 2016). Due to multiple national registers and post-marketing registers, it is well-known nowadays that headache, idiopathic intracranial hypertension (IIH), and slipped femoral epiphysis may occur during GH treatment. The incidence was found to be higher in the groups diagnosed with craniopharyngeomas, congenital GHD, and brain tumors in a post-marketing register following children who had been treated for 0.4–2.5 years. In children with a diagnosis of isolated GHD, IIH had a lower incidence than in children with Turner syndrome, congenital GHD, Prader Willi syndrome, or chronic renal failure (Darendeliler *et al.*, 2007). Free thyroxine decreases during GH treatment in most diagnoses, but in a SGA cohort it was neither associated with an increase in TSH nor affected the response to GH treatment (De Kort *et al.*, 2008).

In 2012 the French SAGhE group reported increased cancer mortality in adults who started GH treatment as children between 1985 and 1996 due to the diagnoses of GHD, neurosecretory dysfunction, ISS, or SGA. The mortality was related to cancer (bone tumors) and diseases in the circulatory system, and associated with a higher GH dose (Carel *et al.*, 2012). However, in a

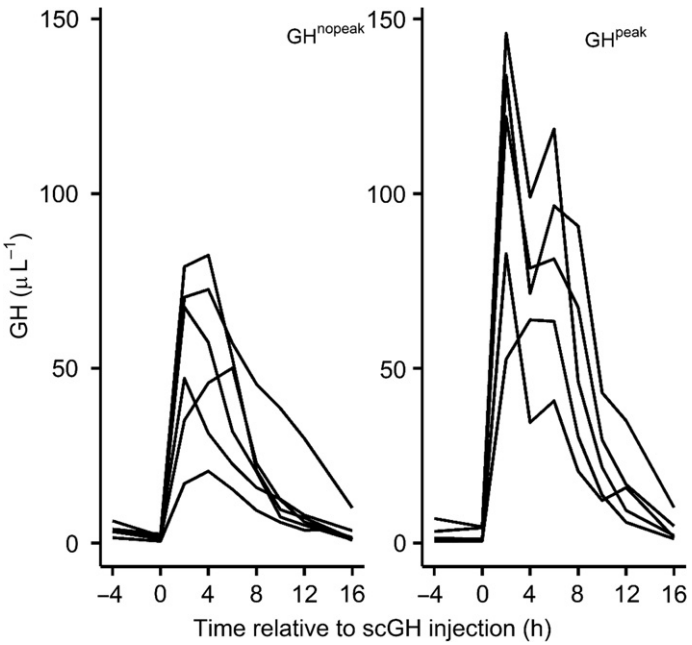

Fig. 14 GH serum curves from two children followed yearly during GH treatment, visualizing intra-individual variation of GH injection uptake; six curves from a boy in whom no peaks from endogenous GH secretion could be detected (left panel) and five curves from a boy in whom endogenous GH secretion was suspected due to the serum curves (right panel). Lundberg E. Thesis Umeå university 2017. ISBN 978-91-7601-662-6.

corresponding study in Belgium, the Netherlands, and Sweden, none of the 21 deaths found in 2543 patients was due to cancer or cardiovascular disease (Sävendahl *et al.*, 2012). Instead the majority of deaths were due to accidents and suicides. In order to evaluate mortality further in childhood GH-treated patients, now also in relation to birth characteristics, a mortality model was constructed based on the Swedish general population born between 1973 and 2010. The rhGH-treated population differed from the general population regarding size at birth and congenital malformations, making inclusion of these variables necessary. This model was applied to assess the expected deaths in the Swedish rhGH-treated patients diagnosed with IGHD, ISS, and SGA. When the model that adjusted for birth characteristics was applied, the ratio of observed/expected deaths was not increased in the childhood rhGH-treated group (Albertsson-Wikland *et al.*, 2016).

Evaluation of the risk for mortality in cancer, myocardial infarction, and stroke from the HypoCCS study in adult patients who had received pediatric GH treatment due to GHD did not find any increased risks compared with a reference population during a follow-up period mean (SD) of 3.7 (3.3) years (Mo *et al.*, 2014). The risk for cancer was further studied in the European SAGhE cohort in those countries having a population-based cancer register (Swerdlow *et al.*, 2017). The mean follow-up time was 16.5 years/patient for mortality and 14.8 years/patient for cancer incidence. The mean age for GH-treated individuals was 27.1 years for cancer mortality and 25.8 years for cancer incidence analyses. The oldest individual in the study cohort was approximately 35 years. Focusing on the "isolated growth failure" group, which included the SGA group together with isolated GHD and the ISS children, overall the cancer risk was not raised. However, there were significant rising cancer incidence risks with increasing time from the first treatment and treatment duration. Mean daily doses of GH were 26.0 $\mu g\,kg^{-1}\,d^{-1}$ for the IGHD individuals, 33.8 $\mu g\,kg^{-1}\,d^{-1}$ for the ISS individuals, and 49.5 $\mu g\,kg^{-1}\,d^{-1}$ for the SGA individuals. However, some results certainly need further studies: the rising cancer mortality associated with a greater daily dose in cancer patients, and the trend of cancers (primarily Hodgkin's lymphoma) in patients with initially noncancer diagnoses with follow-up times of more than 20 years. However, even with this large study group and study design, results do not suggest that GH treatment affects the risk of cancer incidence or mortality (Swerdlow *et al.*, 2017). As malignant diseases have increasing incidence at higher ages, future studies in cohorts with older subjects are needed.

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Growth Hormone Insensitivity☆

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Introduction

GH insensitivity (GHI) *(OMIM #262500* and *#245590)* was first described by Laron *et al.* in 1966, with the description of three siblings from a consanguineous family with extreme growth failure who had the phenotype of hypopituitarism with high serum GH concentrations (Laron, 2004; Laron *et al.*, 1966). Although an abnormal GH molecule was initially suspected, this disorder, referred to as "Laron syndrome," was caused by a defect in the GH receptor (GHR) (Eshet *et al.*, 1984). This rare phenotype, which was untreatable at that time, became synonymous with the diagnosis of GHI. In the late 1980s, two developments brought important changes to the field, first the development of recombinant human IGF-I (rhIGF-1) for therapy (Laron *et al.*, 1988; Walker *et al.*, 1991), and secondly the advent of molecular diagnostic techniques, which led to the cloning and characterization of the human *GHR*, and hence the understanding of the pathophysiology of GHI (Godowski *et al.*, 1989; Amselem *et al.*, 1989).

However, GHI is not a single entity, but a broad diagnostic category comprising a range of molecular defects (David *et al.*, 2011). These defects, which may involve genes coding for proteins that regulate GH binding or signal transduction and IGF-I synthesis, transport or delivery to key target tissues, are associated with a varied range of phenotypes and biochemical abnormalities. Previous texts have mostly described the characteristics of the extreme phenotype (Laron, 2004; Rosenfeld *et al.*, 1994). We discuss the genetic causes of primary GHI and also aim to update clinicians in the diagnostic approach to children with growth failure.

The IGF System in Human Growth

Physiology of the IGF System

The actions of GH are mediated by components of the IGF system, including IGF-I, IGF-binding proteins (IGFBPs), the IGF-I receptor (IGFIR), and IGF-independent effects through direct GH action. The GH–IGF axis is shown in **Fig. 1**. The original "somatomedin hypothesis" proposed that GH binding to its receptor stimulated IGF-I production, which independently affected growth (Salmon and Daughaday, 1957). However, IGFs are expressed in most tissues and the "dual effector hypothesis" in 1985, suggested that GH regulates locally produced IGF-I, which then acts in an autocrine/paracrine manner (Green *et al.*, 1985). Direct injection of GH into the cartilage growth plate of hypophysectomized rats resulted in increased longitudinal bone growth (Isaksson *et al.*, 1987) suggesting that GH has local IGF-1-independent effects, specifically on differentiation of chondrocytes in the growth plate, whereas IGF-I stimulated their clonal expansion.

Le Roith *et al.* presented a revised hypothesis (Le Roith *et al.*, 2001) that questioned the role of liver IGF-I and its circulating endocrine form in controlling post-natal growth. Liver-specific *Igf1* knockout mice continued to grow normally despite a reduction in circulating IGF-I, indicating that locally produced IGF-I was an important growth mediator (Le Roith *et al.*, 2001). About 75% of serum IGF-I is liver-derived, whereas the remainder originates from non-hepatic tissues. In addition, serum levels of the acid-labile subunit (ALS) and IGF binding protein (IGFBP)-3 are important in maintaining circulating IGF-I (Kaplan and Cohen, 2007).

Mechanisms of GH and IGF-1 Action

GH regulates IGF-I production through the signal transducer and activator of the transcription (STAT)-5b signaling system. The binding of GH to the cell surface GHR (a cytokine receptor that lacks intrinsic kinase activity) recruits and induces signal transduction through cytosolic Janus kinase 2 (JAK2). The complex signaling pathways activate four STATs, the phosphoinositide-3 kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (David *et al.*, 2011). Signal transduction through the STAT5b pathway requires STAT5b to associate with one of several JAK2 phosphorylated tyrosines on the intracellular domain of GHR (Derr *et al.*, 2011). STAT5b, recruited to GH-activated GHR, is phosphorylated by JAK2, whereupon the tyrosyl-phosphorylated-STAT5b forms a homo-dimer and translocates to the nucleus. The phosphorylated STAT5b binds to GH responsive elements (GHRE) and drives transcriptional regulations of STAT5b-dependent genes, including *IGF1*, *IGFBP3* and *IGFALS genes*, encoding for IGF-I, IGFBP-3 and acid labile subunit (ALS), respectively (Wang and Jiang, 2005).

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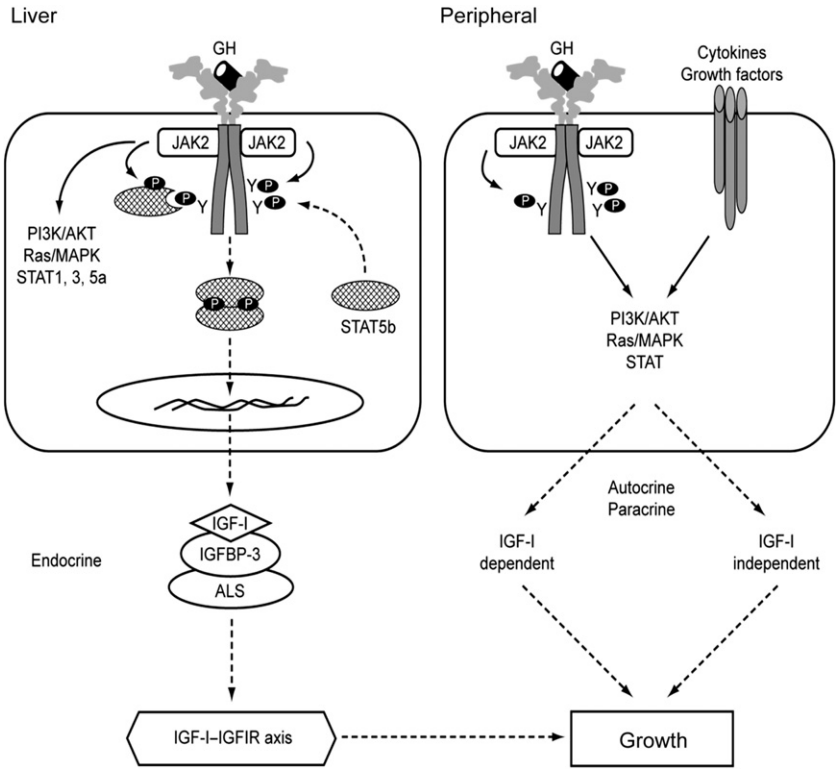

Fig. 1 The GH–IGF-1 axis in human growth (see section on the IGF system in human growth). *Solid arrows*, Activation processes; *dashed arrows*, Translocation processes; AKT, v-akt murine thymoma viral oncogene homolog, also known as PKB, or protein kinase B; P, phosphorylated residue; Y, tyrosine. From David, A., Hwa, V., Metherell, L. A., *et al.* (2011). Evidence for a continuum of genetic, phenotypic and biochemical abnormalities in children with growth hormone insensitivity. *Endocrine Reviews* **32**, 472–497.

IGF-I circulates in a ternary complex with liver-derived IGFBP-3 and ALS, and is delivered to IGF-I responsive cells and tissues (Fig. 1). The mitogenic and metabolic effects of IGF-I are mediated through type I IGF-I receptors (IGFIR) encoded by the *IGF1R* gene. The IGF1R undergoes post-translational glycosylation and is proteolytically cleaved into α- and β-chains (David *et al.*, 2011). The binding of IGF-I to IGF1R leads to receptor autophosphorylation, resulting in recruitment of cytoplasmic components of downstream signaling pathways, including the PI3K/Akt and MAPK/Erk pathways, ultimately leading to cell proliferation and other metabolic effects.

Effects of Human GH–IGF Axis Mutations on Linear Growth

Normal GH secretion and the integrity of the IGF system are essential for normal linear growth. Defects that have been identified to cause impaired growth due to GHI are shown in Table 1. A summary of phenotypic and biochemical features in GH–IGF-I axis defects is shown in Fig. 2. Human pre-natal growth is regulated principally by nutritional supplies, which influence fetal IGF-I. The importance of normal fetal IGF-I production and action was confirmed by the pre-natal growth failure reported in patients with *IGF1* and *IGF1R* mutations (Netchine *et al.*, 2011). Post-natal growth may be disrupted by mutations that disturb the functional integrity of the cascade of GH–GHR interaction, GH signal transduction, and IGF-I production, transport, delivery and action (David *et al.*, 2011; Argente *et al.*, 2017). In states of GHI resulting from impaired GH–GHR function, IGF-I deficiency is the cardinal biochemical feature, whereas in mutations specifically involving the *IGF1* or *IGF1R*, GHR function remains intact (David *et al.*, 2011).

Molecular Defects and Phenotypes Associated with GH Insensitivity

GH Receptor (GHR) Mutations

Molecular characteristics

GHR mutations associated with a range of phenotypes

Since 1966, >250 patients with genetic GHI have been identified worldwide (David *et al.*, 2011). The most severe phenotype is also known as Laron syndrome *(OMIM #262500)* (Laron, 2004). Most GHI cases have autosomal recessive inheritance with

Table 1 Classification of GH insensitivity disorders

Defects of the GH–IGF-I axis
1. GH receptor defects
   a. Extracellular mutations
   b. Transmembrane mutations
   c. Intracellular mutations
2. GH signal transduction defects (STAT5b)
3. Mutations of SHP-2 (encoded by *PTPN11*), K-RAS, H-RAS
4. *IGF1* gene mutations or deletions
   a. Defects causing IGF-I deficiency
   b. Bio-inactive IGF-I
5. Acid-labile subunit defects
6. PAPP-A2 mutations
7. IGF-I receptor (*IGFIR*) gene mutations
8. GH neutralizing antibodies in patients with *GH* gene deletion

Acquired disorders causing GH resistance
1. Malnutrition, parenchymal liver disease, type 1 diabetes mellitus
2. Chronic inflammatory and nutritional disorders (e.g., juvenile chronic arthritis, Crohn's disease, celiac disease)

PTPN11, protein tyrosine phosphatase, nonreceptor type 11; SHP-2, Src homology region 2-domain phosphatase-2; STAT, signal transducer and activator of transcription.

Gene mutation	Birth weight	Postnatal growth	GH	GHBP	IGF-1	IGFBP-3	ALS	Additional features
GH receptor extracellular	N	Severely impaired	↑	↓	↓	↓	↓	Cranio-facial dysmorphic features
GH receptor transmembrane	N	Severely impaired	↑	N or ↑	↓	↓	↓	Cranio-facial dysmorphic features
GH receptor intracellular	N	Severely impaired	↑	N	↓	↓	↓	Cranio-facial dysmorphic features
STAT5B	N	Severely impaired	↑	N	↓	↓	↓	Immunodeficiency elevated prolactin
IGFALS	N	Marginally impaired	↑	N	↓	↓	↓	Insulin resistance
IGF1	↓	Severely impaired	↑	N	↓	N	N	Deafness, mental retardation
IGF-1 bio-inactive	↓	Severely impaired	↑	N	N or ↑	N	N	Deafness, mental retardation
IGF1R	↓	Severely impaired	↑	N	N or ↑	N	N	Microcephaly
PAPP-A2	N	Marginally impaired	↑	N	↑	↑	↑	Thin bones, small chin, microcephaly

Fig. 2 Clinical and biochemical features of human mutations in the GH–IGF-1 axis causing GH insensitivity. N, Normal, ↑Increased, ↓Decreased.

defects involving either homozygous or compound heterozygous mutations (David *et al.*, 2011). >70 mutations of *GHR* have been identified to date, ranging from deletions to point mutations including missense, nonsense, and splice mutations (David *et al.*, 2011). Splice mutations represent approximately 20% of *GHR* defects and usually disrupt major regulatory elements such as the donor and acceptor splice sites. Both severe and mild phenotypes may occur within the same family (Milward *et al.*, 2004).

The GHR pseudoexon mutation

Among the defects causing aberrant *GHR* splicing, an intronic base change leading to the activation of a pseudoexon sequence and insertion of 36 new amino acids within the receptor extracellular domain was first reported in 2001 in a consanguineous Pakistani

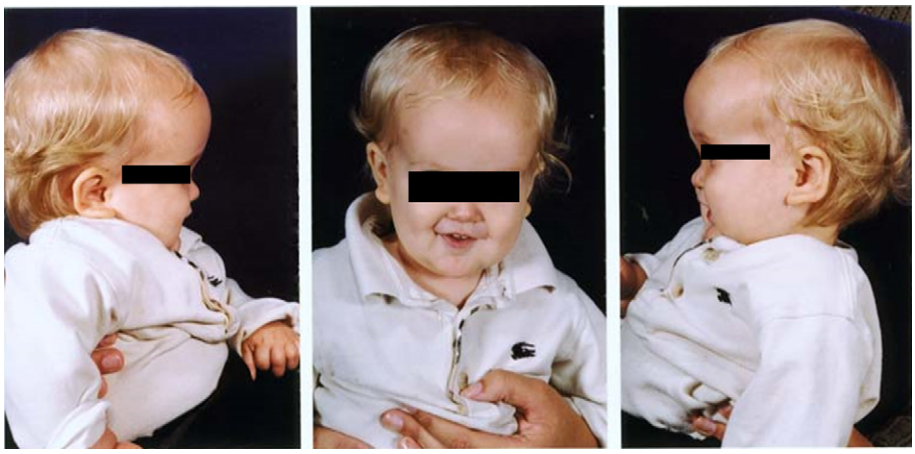

Fig. 3 Typical facial features of a child with classical GH insensitivity (Laron syndrome). Note the depressed nasal bridge, prominent forehead and mid-facial hypoplasia (published with the written consent of the parents).

family with a mild GHI phenotype (Metherell *et al.*, 2001). This mutation leads to recognition of the pseudoexon and inclusion of an additional 108 bases between exons 6 and 7, impairing the function of the mutant GHR protein. The phenotypes (see below) occurring with this mutation range from severe to mild growth failure (David *et al.*, 2007).

Dominant negative heterozygous and compound heterozygous GHR mutations

A mild phenotype of GHI is also associated with heterozygous *GHR* mutations causing a dominant negative effect (Vairamani *et al.*, 2017). These splice site mutations (c.876-1G > C) and (c.945 + 1G > A) form heterodimers with the wild-type GHR and exert a dominant negative effect on the normal protein. A mild phenotype was also reported in two patients with compound heterozygous GHR mutations (Fang *et al.*, 2007). Both had undisputed GHI, but functional studies suggested incomplete GHR defects that determined the phenotype by an additive effect of each heterozygous mutation.

Range of phenotypes

The classical phenotype of GHI, so-called Laron syndrome, consists of a near normal birth weight followed by severe and dramatic post-natal growth failure. There is cranio-facial disproportion due to lack of growth of the sphenoid bone, which results in mid-facial hypoplasia, depressed nasal bridge and a prominent forehead **Fig. 3**. Childhood growth is severely impaired leading to adult height of approximately 120 cm. Asymptomatic hypoglycaemia is frequently present due to the extreme GH resistance. Serum concentrations of IGF-1, IGFBP-3 and ALS are severely subnormal or undetectable. Growth hormone secretion is increased with elevation of basal and peak serum GH concentrations (Laron, 2004).

The concept of genotype: phenotype relationships in endocrinology evolved from evidence that genetic defects can influence phenotypic expression based on the degree of disturbance of key mechanisms. This reasoning can be applied to defects of the GH–IGF axis causing GHI. A range of phenotypes was noticeable in 82 European GHI patients, who were identified in the early 1990s for rhIGF-I therapy (Woods *et al.*, 1997). There was a gradation of severity of short stature, with height SDS ranging from -2.2 to -10.4 and a strong positive correlation ($r^2 = 0.45$, $P \leq .001$) between height SDS and IGFBP-3 SDS. The serum GHBP level also varied and when very low or absent was associated with more severe short stature (height SDS -6.45) whereas normal GHBP values were associated with milder short stature (height SDS -4.89) (Woods *et al.*, 1997).

A study of cranio-facial phenotypes in the same subjects reported that those with normal facial appearance, as opposed to the dysmorphic features of Laron syndrome, had milder short stature (Burren *et al.*, 2001). In populations of patients from Ecuador with the homozygous E180 splice *GHR* mutation, heterogeneity of statural phenotype was also seen with height SDS values ranging from -5.3 to -11.5, and height SDS correlated positively ($P < .01$) with IGF-I SDS and IGFBP-3 SDS values (Guevara-Aguirre *et al.*, 1993).

Study of genotype: Phenotype relationships in GHR mutation patients

Thirty-eight patients with GHI, studied at St Bartholomew's Hospital in London, were identified to have homozygous or compound heterozygous or heterozygous dominant negative *GHR* mutations (David *et al.*, 2011). Assessment of height SDS and type of *GHR* mutation, was performed in these and 32 subjects, fulfilling the same GHI criteria, who were added from the literature. Relationships between *GHR* mutation type and height SDS are shown in **Fig. 4**. Dominant negative *GHR* mutations and *GHR* intronic pseudoexon mutations had less severe growth phenotypes ($P < .05$) than *GHR* missense and nonsense mutations. The probable explanation is that in the case of the dominant negative mutations, there will still be a proportion of normally functioning receptors dimerized with second normally functioning receptors resulting in effective GH signal transduction (David *et al.*, 2011).

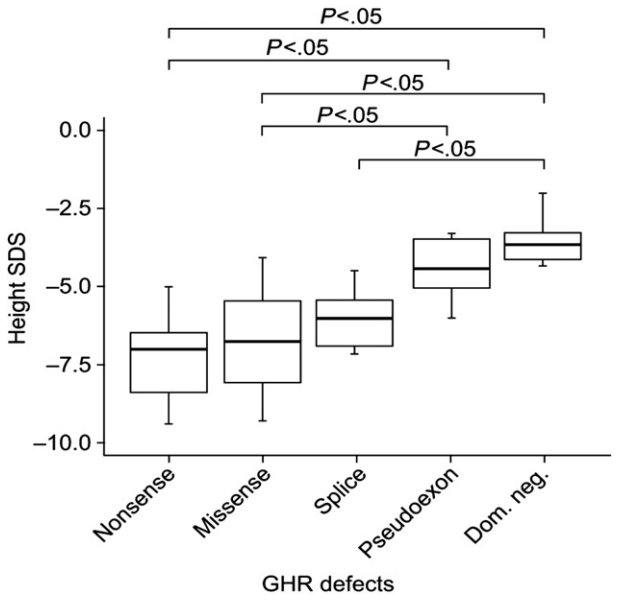

Fig. 4 Height SDS values in 70 children with GHI and *GHR* mutations divided according to the type of mutation. Each *boxplot* depicts the median and the 25th and 75th percentiles. *Whiskers* depict minimum and maximum values. Statistical analyses were performed using R version 2.6.2 (R Development Core Team, 2008, R Foundation for Statistical Computing, Vienna, Austria). Numerical values were expressed as median (range). Comparison between continuous variables was performed using Student's test. A two-sided $P < .05$ was considered significant. Bonferroni adjustment was performed to reduce the likelihood of type 1 error. Dom. neg, Dominant negative. From David, A., Hwa, V., Metherell, L. A., *et al.* (2011). Evidence for a continuum of genetic, phenotypic and biochemical abnormalities in children with growth hormone insensitivity. *Endocrine Reviews* **32**, 472–497.

Extended study of pseudoexon mutation patients

In the case of the GHR pseudoexon mutation, a range of phenotypes is seen. In patients with mild phenotypes, it is likely that there is a small residue of normally spliced receptor that will result in a degree of normal GH signaling. The ranges of phenotypic and biochemical abnormalities are shown in **Table 2** (H.L. Storr, personal communication).

STAT5B Mutations

Molecular characteristics

STAT5B mutations present a characteristic phenotype combining GHI and immunodeficiency *(OMIM #245590)*. The binding of GH to the GHR activates signaling cascades that include a number of the STAT pathways. Recent identification of human *STAT5B* mutations causing severe growth failure, as well as marked IGF-I deficiency and GHI, demonstrated that STAT5b signaling is critical for GH-induced IGF-I production and normal linear growth in humans (Rosenfeld *et al.*, 2007).

In support of the in vivo observations, studies employing primary fibroblasts from an affected patient (Kofoed *et al.*, 2003) and reconstitution systems, demonstrated that GH activated STAT5b preferentially, which correlated with the induction of IGF-I and IGFBP-3 expression (Hwa *et al.*, 2004). The resulting STAT5b deficiency not only abrogated GH-induced IGF-I production as indicated above, but IL-2-induced expression of $CD25^{high}$ was also demonstrated to be aberrant (Cohen *et al.*, 2006).

The unusual combination of GHI and immune deficiency in the first patient to be described was due to a homozygous, missense, mutation in Exon 15 of the *STAT5B* gene (Kofoed *et al.*, 2003). The single *G* to *C* transversion at nucleotide 1888 of the *STAT5B* mRNA resulted in an Ala630 (GCT) to Pro (CCT) substitution, predicted to cause loss of thermodynamic stability and aberrant folding and aggregation of the mutant STAT5b protein. Additional human *STAT5B* gene mutations have been documented, with two of the mutations, c.*1680delG* and c.*424_427del* found in siblings (David *et al.*, 2011).

Phenotypic features

The first human *STAT5B* mutation was identified in a 16-year-old female from a consanguineous Argentine family (Kofoed *et al.*, 2003). Birth weight in affected patients is generally normal, but is followed by severe post-natal growth failure, with resistance to GH therapy (David *et al.*, 2011). With respect to growth in STAT5B deficient patients, height SDS values ranged from −5.6 to −9.9. The majority of these patients also had serious immunological abnormalities.

The biochemical profile shows normal or elevated GH secretion, normal GHBP values and severe deficiencies of IGF-I, IGFBP-3, and ALS which fail to increase on GH stimulation (David *et al.*, 2011). A key feature was immune dysfunction and in several patients, repeated pulmonary infections occurred from infancy, including episodes of lymphoid interstitial pneumonia (Hwa *et al.*, 2007). All reported mutations were homozygous. The only reported patient without immune deficiency, the first male proband in

Table 2 Phenotypic and biochemical details in 20 patients with homozygous GH receptor pseudoexon mutations

Family	*Age (yr)*	*Sex*	*Height (SDS)*	*BMI (SDS)*	*Birth weight (SDS)*	*Target height (SDS)*	*Ethnicity/ consanguinity*	*GHI facial features*	*Basal GH (μg/L)*	*Peak GH (μg/L)*	*IGF-1 (SDS)*	*IGF-1 generation test basal/peak (ng/mL)*	*IGFBP-3 (SDS)*
A	1.3	M	−1.7	−4.9	−0.2	−2.2	Pak/+	No	11	10	−2.5	23/24	−6
	3.7	M	−5.9	−2.0	0.3	−2.2	Pak/+	No	6	14.3	−2.5	21/26	−8.9
	8.3	M	−3.3	−0.4	NK	−1.6	Pak/+	No	1.8	53.3	−1.7	29/36	−2.9
	3.8	M	−3.6	−0.5	−0.1	−1.6	Pak/+	No	17.5	90	−2	20/20	−3.4
	1.2	F	−4.4	+1.8	0.7	−2.4	Pak/+	No	0.1	18.8	−2.2	ND	−1.72
	2.5	F	−4.4	−0.1	−1.8	NK	Pak/+	Yes	3.4	26.7	NK	ND	ND
B	1.6	F	−5.6	−2.4	−1.4	−1.4	Pak/+	Yes	13	>33.3	−2.3	6.9/7.6	−2.4
C	NK	M	−5.0	NK	NK	NK	Palestine-Arab/+	Yes	0.6	NK	NK	NK	NK
D	3.3	M	−4.9	0.1	NK	NK	Pak/+	No	10.2	15.4	−2.3	36/41	−2.6
	8.1	M	−3.3	−2.4	−1.5	NK	Pak/+	No	0.3	28.4	−0.7	132/255[a]	−1.6
E	5.4	F	−3.5	0.02	NK	NK	Pak/+	No	2.5	27	−1	ND	−2.3
	NK	F	−4.0	NK	NK	NK	Pak/+	No	8.3	37.7	−1.4	ND	−2.3
F	7	M	−4.2	−0.5	−0.5	−0.9	Pak/+	No	2	40	−2.5	41.2/29.7	−2.6
G	2.6	M	−3.8	−2.9	−2.9	−1.3	Pak/+	Yes	4	>33	−2.3	63.3/16.8	ND
	3.7	F	−4.2	−0.9	0.1	0.7	Pak/+	Yes	16.9	33.3	−2.5	ND	ND
H	5.7	M	−3.0	−0.7	0.7	−0.7	Pak/+	Yes	17.5	90	−2.9	1.5/8.4	−2.4
	1.5	F	−4.7	−1.2	NK	−0.7	Pak/+	Yes	0.1	18.8	−3.1	ND	ND
I	2.3	F	−4.3	−1.7	−1.7	−1.6	Ind/−	Yes	3.4	26.7	−2.1	ND	ND
J	5.3	F	−4.0	0.4	0.1	−1.6	Pak/+	Yes	19.3	>40	−6.8	<25/<25	ND
K	4.3	F	−4.1	−0.2	−0.3	−0.9	Pak/+	Yes	0.6	NK	−4	<22.9/<22.9	−2.4

[a]Positive response on IGF-1 GT.

Age and height SDS are at presentation. GHI facial features: frontal bossing, mid-facial hypoplasia.

NK, not known; + parents consanguineous; −, parents not consanguineous; Pak, Pakistani; Ind, Indian; ND, not done.

the cohort, contracted hemorrhagic varicella at 16 years of age and had congenital ichthyosis and erythema but otherwise appeared healthy (Vidarsdottir *et al.*, 2006). One other patient was diagnosed with juvenile idiopathic arthritis at an unusually young age of 2 years (Hwa *et al.*, 2007).

STAT5b deficiency was associated with abnormally high levels of prolactin in six of the cases (David *et al.*, 2011). It remains unclear whether the hyperprolactinaemia is a direct or indirect consequence of *STAT5B* mutations. In a large Brazilian pedigree, height SDS, IGF-1 SDS and IGFBP-3 values were compared in subjects with homozygous and heterozygous *STAT5B* mutations and wild-type family members (Scalco *et al.*, 2015). All three variables were decreased in heterozygous compared with non-carrier wild-type individuals. The heterozygous subjects also had an average height SDS of -1.4 ± 0.8 when compared with normal population-matched controls. These findings support a negative impact on height of heterozygous *STAT5B* mutations.

IGF1 Gene Defects

Molecular characterization

The first human *IGF1* gene defect was described in 1996 by Woods *et al. (OMIM #608747)* (Woods *et al.*, 1996). Molecular analysis revealed a homozygous deletion of exons 4 and 5 of the *IGF1* gene. If translated, the resulting protein would be severely truncated, lacking 45 of the 70 IGF-I amino-acids. Genetic analyses of *IGF1* in the second case (Bonapace *et al.*, 2003) showed a homozygous T → A transversion in exon 6 resulting if translated into an altered E domain of the IGF-I precursor. It has been argued that this variant may be a polymorphism and thus not causative of the patient's phenotype, which was nevertheless strikingly similar to the other cases.

The third reported patient had a serum IGF-I level that was significantly increased (+7.3 SDS), explained by the fact that the patient's homozygous missense mutation of *IGF1*, a G → A nucleotide substitution at position 274, changing valine at position 44 in the A domain of the IGF-I protein to methionine (p.V44M), resulted in a recombinant protein (IGF-1 V44M) with normal binding to IGFBP-3 but decreased affinity (90-fold) for its receptor, IGF1R (Walenkamp *et al.*, 2005). This patient therefore had bio-inactive IGF-I caused by an *IGF1* mutation. Family members who were heterozygous carriers of this mutation were shorter than the non-carrier family members. In the fourth patient, who had a milder phenotype, sequencing of *IGF1* revealed a homozygous missense mutation resulting in the change of a highly conserved arginine located in the C domain of the protein into a glutamine (p.R36Q). Affinity for the IGF1R decreased two-to-threefold, resulting in decreased IGF1R autophosphorylation.

Phenotypic features

Four patients with IGF-1 gene defects have been published. Their characteristics are shown in **Fig. 2**. The first patient, a male, was born by Caesarean section because of poor fetal growth (Woods *et al.*, 1996). Placental weight was diminished (350 g) and he had

severe intrauterine growth retardation (IUGR) with a birth weight of 1.4 kg (− 3.9 SDS), birth length of 37.8 cm (− 5.4 SDS), and microcephaly (head circumference 27 cm, − 4.9 SDS). His growth failure worsened post-natally and at 15.8 years his height was 119.1 cm (− 6.9 SDS) and his weight was 23.0 kg (− 6.5 SDS). He had delayed psychomotor development and sensorineural deafness. During adolescence he became insulin-resistant. Serum IGF-I was undetectable even after 4 days of GH stimulation. Spontaneous 12-h GH secretion showed elevated baseline and peaks. ALS and IGFBP-3 values were normal. At 16.1 years (bone age, 14.2 years), recombinant IGF-I therapy was initiated and resulted in beneficial effects on insulin sensitivity, body composition, bone size, and linear growth (Woods *et al.*, 2000).

Intrauterine growth retardation (IUGR), microcephaly, retarded intellectual development and severe post-natal growth failure were present in the other cases with homozygous *IGF1* mutations. Deafness was present in all the cases except the child with the mildest phenotype. The microcephaly which these patients had appears to be a cardinal feature of the phenotype. Serum IGF-I has been variable in *IGF1* mutation cases. The third case to be described (Walenkamp *et al.*, 2005) shared an identical clinical phenotype with the index case, and had a younger brother with similar features who died in childhood. Serum IGF-I in the fourth patient was also variable and not severely decreased (Netchine *et al.*, 2009). In all reported subjects serum IGFBP-3 and ALS levels were normal.

A definite short stature phenotype was reported with a novel heterozygous mutation of the *IGF1* gene (van Duyvenvoorde *et al.*, 2010) supporting the hypothesis, suggested from larger family studies (Walenkamp *et al.*, 2005), that heterozygosity for certain *IGF1* mutations may cause significant short stature. The spectrum of *IGF1* defects was further broadened by the report of a child with short stature (height − 4.0 SD) and a dominant pattern of inheritance who had IGF-I deficiency and a novel heterozygous donor splice site mutation at the exon 4–intron 4 junction of *IGF1* (Fuqua *et al.*, 2009).

IGFALS Mutations

Molecular characterization

IGFALS mutations cause GHI and severe ALS, IGF-I and IGFBP-3 deficiencies (*OMIM #601489*). Characteristics of patients with IGFALS mutations are shown in **Fig. 2**. *s*ALS is a soluble protein and member of the leucine-rich repeat family, being expressed by hepatocytes and secreted into the circulation (Leong *et al.*, 1992). GH is the main inducer of ALS, which circulates as a free peptide or bound to IGF-1 or -2 and IGFBP-3 or -5, to form a ternary complex, which prevents IGFs and IGFBPs from leaving the circulation, thus prolonging their half-lives and decreasing their availability at a tissue level (David *et al.*, 2011). ALS is encoded by the *IGFALS* gene, located on chromosome 16p13.3 and spanning 3.3 Kb. A review of published cases described 16 mutations of the human *IGFALS* gene in 21 cases (Domené *et al.*, 2009). Eleven were homozygous and six were compound heterozygous. Family studies confirmed autosomal recessive inheritance. *IGFALS* mutations have included missense and nonsense mutations, deletions, duplications, and insertions resulting in frameshift and premature stop codons and in-frame duplications leading to insertion of extra amino acid residues.

Phenotypic features

The first patient with a homozygous mutation of *IGFALS* was reported by Domené *et al.* (2004) and presented a new combination of genetic, biochemical, and phenotypic findings. There is a mis-match between deficiencies of circulating IGF-I, IGFBP-3, and ALS and relatively mild growth failure, even leading to a normal adult height in some patients (Domené *et al.*, 2009). All reported cased had extreme deficiency of circulating ALS, with inability to form the ternary complex. Circulating levels of IGF-I and IGFBP-3 are severely reduced, due to their rapid clearance, however local IGF-1 production in peripheral tissues, notably the growth plate, appears to be preserved or even increased due to up-regulation of GH secretion (Domené *et al.*, 2004). Insulin resistance, with hyperinsulinemia and low IGFBP-1, has also been described in these patients (Domené *et al.*, 2009).

An analysis of 21 patients with homozygous or compound heterozygous *IGFALS* mutations and their family members who were either heterozygous carriers or homozygous wild-type normal has been published (Fofanova-Gambetti *et al.*, 2010). Mean height SDS was -2.31 ± 0.87 in the homozygous patients and heterozygosity resulted in approximately 1.0 SD height loss compared to wild-type, whereas homozygosity or compound heterozygosity resulted in a further loss of 1.0–1.5 SD, suggestive of a gene–dosage effect. The term partial acid-labile subunit deficiency was coined to describe subjects with heterozygous *IGFALS* mutations. A recent report described 50 subjects from five Turkish families carrying three different *IGFALS* mutations (Işik *et al.*, 2017). Height SDS, head circumference SDS, and BMI SDS were lower ($P < .01$) in the 24 subjects with homozygous mutations compared to 26 with heterozygous mutations. Similarly IGF-1, ALS and IGFBP-3 were lower ($P < .001$) in the subjects with homozygous mutations (Işik *et al.*, 2017).

IGF-1 Receptor (IGF1R) Mutations

Molecular characterization and phenotypic features

IGFIR mutations are characterized by IGF-I resistance causing impaired fetal and post-natal growth (*OMIM #270450*). Characteristics of patients with heterozygous *IGFIR* mutations are shown in **Fig. 2**. The IGF1R is a transmembrane receptor and belongs to the insulin receptor family, which includes the IGF2R and insulin receptor. The IGFIR is expressed widely and binds IGF-1 and -2 with high affinity, mediating their biological actions by activating a complex intracellular signaling cascade leading to the transcription of IGF target genes. The *IGF1R* gene is located on chromosome 15q26.3 and spans 315Kb.

Mutations in *IGF1R* were first reported in two subjects in 2003 by Abuzzahab *et al.* (2003) following analyses of DNA from children with short stature and unexplained IUGR. The first child was a compound heterozygote for point mutations in exon 2 of the *IGFIR* gene that altered the amino acid sequence to p.Arg108Gln in one allele and p.Lys115Asn in the other. The birth weight was −3.5 SD with childhood short stature and an adult height of −4.8 SD. The second patient, a boy, had a heterozygous nonsense mutation (p.Arg59stop) that reduced the number of IGF1Rs on fibroblasts. He also had low birth weight (−3.5 SD) and birth length (−5.8 SD) with microcephaly and post-natal growth failure (height − 3.8 SD) at age 14 months. Serum IGF-I levels were normal or elevated in both patients and GH secretion was normal.

Familial *IGF1R* mutations have been described, with mother and offspring affected (David *et al.*, 2011). A heterozygous mutation in the cleavage site of the proreceptor of *IGF1R* was reported in a 6-year-old Japanese girl and her mother and the research team of Wit in Leiden described a mother and daughter with a heterozygous missense mutation in the intracellular part of the *IGF1R* (Walenkamp *et al.*, 2006). Small birth size, childhood short stature, small head size, relatively high IGF-I levels, developmental delay, and micrognathia are the main predictors for the diagnosis of an *IGF1R* deletion (David *et al.*, 2011; Ester *et al.*, 2009).

PAPP-A2 Mutations

Molecular and clinical characteristics

The first mutations of the metalloproteinase pregnancy-associated plasma protein-A2 (PAPP-A2) were described in five patients with GHI from Madrid and Cincinnati by Dauber *et al.* (2016). This was the first report of autosomal recessive mutations in a gene which encodes the IGF binding proteins (Argente *et al.*, 2017). The clinical syndrome of PAPP-A2 deficiency is caused by loss-of-function mutations in the gene encoding PAPP-A2, which has an essential proteolytic function in the cleavage of the circulating ternary complex. Consequently *PAPP-A2* mutations prevent this cleavage and inhibit the liberation and delivery of free IGF-1 to its target tissues. The clinical features were relatively subtle with mild short stature, thin long bones with long, slender fingers and toes, mild microcephaly and long thin nose and small chin.

However, the biochemical findings were striking with markedly elevated levels of IGF-1, IGFBP-3, IGFBP-5 and ALS. Growth hormone secretion was also increased, reflecting the failure of delivery of free IGF-1 and IGF binding proteins to peripheral tissues from the ternary complex. Concentrations of bioactive IGF-1 were very low, which were interpreted to be the cause of the poor linear growth. Treatment with rhIGF-1 improved short-term linear growth in patients both from Madrid (Muñoz-Calvo *et al.*, 2016) and Cincinnati (Cabrera-Salcedo *et al.*, 2017).

GH1 mutations Causing Biologically Inactive GH

Certain *GH1* mutations cause biologically inactive GH resulting in a form of GHI *(OMIM #262650)*, first described by Kowarski *et al.* (1978). The molecular basis of this rare cause of GHI was clarified by Takahashi *et al.*, who reported two cases with heterozygous mutations in the *GH1* gene (Takahashi *et al.*, 1997). The first was a boy with severe short stature (height − 6.1 SD) who had increased immune-assayable GH and IGF-I deficiency, which responded, as did his growth, to exogenous hGH therapy. The mutation was a single-base missense substitution (p.R77G) in exon 4 of the *GH1* gene. However, his normal-stature father also had the same mutation. Functional studies demonstrated that the mutant GH molecule had higher binding affinity for the GHR and inhibited its activation by wild-type GH in a dominant-negative fashion, thus impairing GH bioactivity. The second case, a girl, had similar endocrine features and short stature associated with a heterozygous single-base change (A → G) causing a p.D112G substitution in *GH1*. A child with short stature (height −3.6 SD at age 9 years) from a consanguineous Serbian family was reported to have a homozygous missense mutation, Cys53Ser, of *GH1* leading to the absence of the disulfide bridge Cys-53 to Cys-165 in the GH molecule (Besson *et al.*, 2005). Functional studies demonstrated that GH binding and JAK2/STAT5 signaling pathway activation was reduced in the mutant GH-C53S compared with wild-type-GH. Both growth and serum IGF-I responded well to GH therapy.

GHI Gene Deletions (Type IA GH Deficiency) with Anti-GH Antibodies

A rare form of GHI occurs due to acquired GH-inhibiting antibodies in children with familial isolated GH deficiency (IGHD) *(OMIM #262400)* (Cogan and Phillips, 2006). Autosomal recessive IGHD, caused by deletions of the *GH1* gene, results in severe IGHD (type IA) with undetectable GH secretion. Such patients have post-natal height usually < −4.5 SD. Most *GH1* deletions are 6.7, 7.0 or 7.6 kb in length. Microdeletions and frameshift mutations have also been reported (David *et al.*, 2011). These patients frequently develop growth-inhibiting anti-GH antibodies during treatment with hGH due to immunological intolerance. They may respond to therapy with recombinant human IGF-I, which becomes the only effective management for their growth failure (Riedl and Frisch, 2006).

Diagnosis of GH Insensitivity

The evaluation of a child with short stature and possible GHI should comply with the classical paradigm of clinical assessment followed by general (i.e., non-endocrine) investigations, hormonal assessment, and possible genetic analyses. An algorithm

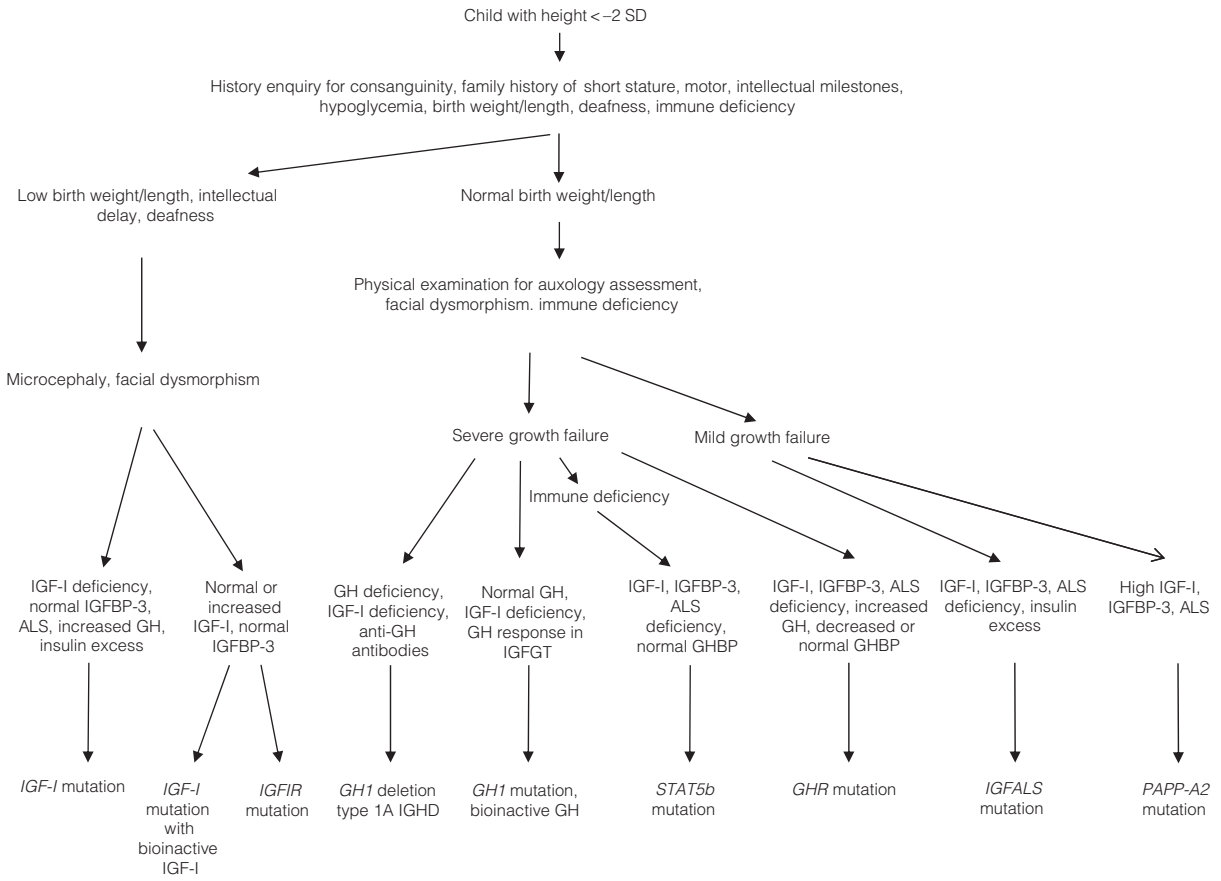

Fig. 5 Algorithm for diagnosis of genetic GH insensitivity disorders.

showing key steps in the diagnosis of genetic GH–IGF-I axis defects is shown in **Fig. 5**. As advances in molecular endocrinology progress, the importance of detailed phenotypic evaluation and documentation is emphasized. The urge to obtain a molecular diagnosis at the onset of the investigations should be resisted until clinical and endocrine evaluation has been performed. Clinical assessment should include enquiries about family history of growth disturbance, consanguinity, birth weight and length, and recurrent infections (Savage *et al.*, 2010). Examination should specifically assess the presence of possible facial dysmorphic features and microcephaly in addition to anthropometric evaluation (Cohen *et al.*, 2008).

Investigations of the GH–IGF-I Axis

Investigations of the GH–IGF-I axis consist of determination of GH secretion and exploration of the IGF system. A GH provocation test is recommended unless the child has normal auxology or a basal IGF-I level above the mean for age (Cohen *et al.*, 2008). In a child with clinical criteria of GHD, a peak GH level of <7 ng/mL is recommended to support this diagnosis (Wagner *et al.*, 2014). Basal IGF-I levels should be determined, but are influenced by age, nutrition, chronic illness, and puberty. In the initial assessment, IGFBP-3 adds little, except in children under 3 years of age, where low IGFBP-3 is helpful in the diagnosis of GHD (Cianfarani *et al.*, 2005). Reliable assay performance and appropriate normative data for IGF-1 and IGFBP-3 are essential (Juul *et al.*, 1994, 1995).

A diagnosis of GHI follows from the demonstration of abnormal auxology, normal GH secretion, and IGF-I deficiency. However, the pathogenesis will not have been elucidated from these investigations. The nature of the defect can often be defined by additional measurement of IGFBP-3, ALS, and GHBP (David *et al.*, 2011).

The IGF-I Generation Test (IGFGT)

The IGFGT was designed to predict growth responses to GH in GH deficient patients. This was unsatisfactory and the test was abandoned. Normative data were also not established. Interest in the IGFGT was renewed when molecular evidence of GHI was demonstrated and subjects were selected for rhIGF-I therapy. Criteria for diagnosis of GHI were defined as: failure to increase IGF-I and IGFBP-3 by >15 and 400 ng/mL, respectively (Blum *et al.*, 1994). However, as the spectrum of GHI disorders expanded, these criteria became too strict for more mildly affected subjects (Coutant *et al.*, 2012). Attempts to refine the IGFGT for investigation of

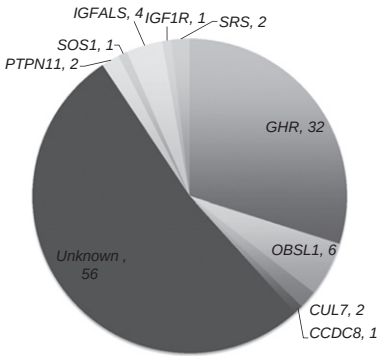

Fig. 6 Genetic diagnoses obtained from candidate gene sequencing and whole exome sequencing in a selected group of 107 patients with GH Insensitivity (Shapiro *et al.*, 2017).

milder GHI demonstrated that patients with idiopathic short stature produced a subnormal response (Buckway *et al.*, 2001) and subjects with IGF-I deficiency and normal GH secretion also had subnormal ability to generate IGF-I. However, additional sensitivity for the diagnosis of GHI was not seen with a low-dose GH protocol (Buckway *et al.*, 2001). For these reasons, the IGFGT is not recommended in the routine investigation of short stature and its principle use is for confirmation of extreme or severe GHI (Coutant *et al.*, 2012).

Genetic Investigations

A genetic diagnosis is of benefit to families and clinicians in understanding causality and prognosis in GHI and to direct the appropriate management. Defects in genes coding for key functional proteins regulating GH action are responsible for GHI (Shapiro *et al.*, 2017). Mutations of the growth hormone receptor (*GHR*) gene are the commonest defects but, as discussed above, mutations in *STAT5B*, *IGF1*, *IGFALS*, *IGF1R and PAPP-A2* genes are also described (David *et al.*, 2011). These defects cause a spectrum of genetic, phenotypic and biochemical abnormalities. Disorders that may overlap phenotypically and biochemically with GH–IGF-1 axis defects include 3 M Syndrome (caused by mutations in *CUL7*, *CCDC8* and *OBSL1*), Noonan syndrome (*PTPN11*, *SOS1* and others) and Silver-Russell Syndrome (Shapiro *et al.*, 2017).

Accurate clinical and biochemical phenotyping is essential to orientate the clinician and the molecular genetics laboratory to the most likely candidate gene defect, for example, facial features of Laron syndrome or associated immunodeficiency may suggest a GHR or STAT5B defect. Sequencing of candidate genes associated with GHI will reveal a diagnosis in ~30%–40% of patients (Wit *et al.*, 2016; Storr *et al.*, 2015). However, more extensive genetic testing, for example, whole exome sequencing can improve the diagnostic yield in selected patients with GHI to approximately 50% (Shapiro *et al.*, 2017) **Fig. 6**. Therefore, although genetic analyses are important, accurate clinical and biochemical characterization is essential for optimal patient care.

Treatment of GH Insensitivity

The logical therapy for patients with GHI is administration of subcutaneous rhIGF-1. It is important to state that rhIGF-1 therapy has been problematic for a number of reasons. First, regular supplies of this recombinant hormone, produced since the late 1980s and licensed for GHI therapy by the FDA and EMA since 2005 and 2007 respectively, have not been available. A succession of pharmaceutical companies developed and then discontinued rhIGF-1 production resulting in relatively few definitive therapeutic trials. Nevertheless, rhIGF-1 is the recognized therapy for GHI patients and its efficacy in promoting growth in patients with extreme growth failure is now established (Laron *et al.*, 1988; Ranke *et al.*, 1999; Chernausek *et al.*, 2007; Guevara-Aguirre *et al.*, 2013). Gain in stature to adult height, using the recommended regimen of 80–120 μg/kg twice daily, has been documented, with advantage during puberty demonstrated by addition of a GnRH analogue to rhIGF-1 to delay epiphyseal fusion (Backeljauw *et al.*, 2013) (**Fig. 7**). However, most severely affected patients did not reach their genetic targets.

Most studies have been performed in patients with severe GHI, who have very low levels of IGF-1, IGFBP-3 and ALS. These low concentrations influence the kinetics of injected rhIGF-1 so that it is rapidly cleared from the circulation (Grahnen *et al.*, 1993) necessitating twice daily administration. There are few studies showing efficacy of rhIGF-1 in more mildly affected patients. One study from the United States showed variable efficacy in subjects with mild GHI (Midyett *et al.*, 2010). Publication of the results of a post-marketing European registry of rhIGF-1 therapy in 195 children with growth failure described the efficacy of treatment in the "real world" as being similar to that seen in randomized controlled rhIGF-1 trials (Bang *et al.*, 2015).

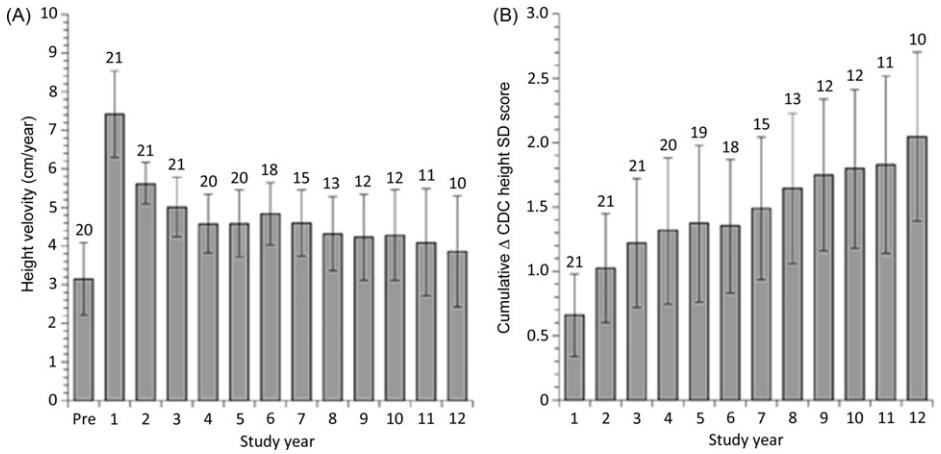

Fig. 7 (A) Individual growth during IGF-I therapy for 12 male patients, compared with the US National Center for Health Statistics standards *(upper shaded area)* and the mean ± 2 SD for height for untreated Laron syndrome patients *(lower shaded area)*. The growth curves for Laron syndrome constitute a reference range for patients with presumed GH receptor abnormalities. (B) Individual growth during IGF-I therapy for nine female patients, compared with the US National Center for Health Statistics standards *(upper shaded area)* and the mean ± 2 SD for height for untreated Laron syndrome patients *(lower shaded area)*. The growth curves for Laron syndrome constitute a reference range for patients with presumed GH receptor abnormalities (Backeljauw et al., 2013). From Backeljauw, P. F., Kuntze, J., Frane, J., Calikoglu, A. S., Chernausek, S. D. (2013). Adult and near-adult height in patients with severe insulin-like growth factor-I deficiency after long-term therapy with recombinant human insulin-like growth factor-I. *Hormone Research in Pædiatrics* **80**, 47–56.

Adverse events related to rhIGF-1 have been clearly documented since its early use in the late 1980s. These are more troublesome in the most severely affected patients, that is, children with the classical Laron syndrome phenotype. Hypoglycaemia, lipoatrophy at the injection sites, enlargement of lymphoid tissue, including tonsillar swelling, and rarely, benign intracranial hypertension are recognized potential adverse events (Chernausek *et al.*, 2007). There are clearly benefit versus risk evaluations to be made in the management of GHI patients. However, the extreme short stature of the most severely affected patients, with the prospect of adult height in the region of 120 cm, with its accompanying disadvantages, provides a compelling argument to initiate rhIGF-1 therapy, which is safe and well tolerated in most patients (74, Bang *et al.*, 2015).

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Tall Stature

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Glossary

Camptodactyly Congenital digital flexion deformity.
Epiphysiodesis Surgical ablation of a physis to stop its growth.
Marfanoid habitus A tall, slender build with long limbs, fingers and toes which may be accompanied by scoliosis and/or pectus deformity.

Introduction

Tall stature is usually defined as height above $+2$ SDS or height >2 SDS above the target height SDS. It is important to use reference data appropriate for the ethnic background of the individual. Different formulas are available to calculate target height (van Dommelen *et al.*, 2012); ideally height of both parents should be measured. Sudden growth acceleration may also warrant evaluation even if height is still $<+2$ SDS and within the target height range. To be able to identify growth acceleration it is important to collect growth data, ideally from birth onwards, and plot these in a growth chart to assess the growth pattern.

Defining tall stature as height $>+2$ SDS means that 2.5% of the population has tall stature. Interestingly, short stature is a much more common reason for medical consultation than tall stature. This is probably due to greater social acceptance of tall stature. However, some may worry about excessive height and request counseling on predicted adult height and possibilities to reduce growth. Some may also worry about an underlying disorder. Most individuals do not have an underlying pathological cause of their tall stature but it is important to identify those that do, as some of these conditions are associated with significant morbidity for which regular screening is advised, such as cardiovascular pathology in Marfan syndrome and increased tumor risk in Beckwith-Wiedemann syndrome (BWS) and PTEN hamartoma tumor syndrome (PHTS).

Tall stature can be the result of a number of different conditions. A thorough medical history, a detailed physical examination and careful evaluation of the growth chart may lead to the conclusion that there is a benign cause such as familial tall stature, and should otherwise narrow the differential diagnosis and give direction to further investigations.

Causes of Tall Stature

A few key questions can be helpful to determine the most likely cause of tall stature, as outlined in the flowchart (**Fig. 1**). In addition to height, head circumference, arm span and sitting height should be measured. However, in syndromes associated with disproportionate tall stature the sitting height-to-height ratio and arm span-to-height ratio may still be within the normal range. In addition, these ratios cannot be used in the presence of severe scoliosis and different reference values for body proportions apply to various ethnicities (Reeves *et al.*, 1996). If present at the consultation, parents should be measured rather than relying on reported height, and their head circumference and body proportions may also be informative.

Syndromic Causes of Tall Stature

The medical history, family history and physical examination may reveal clues to a syndromic cause of tall stature (**Table 1**). To determine which syndromes are most likely in an individual we have chosen to distinguish between syndromes with and without macrocephaly and those with and without a Marfanoid habitus, that is, tall, slender build with long limbs, fingers and toes which may be accompanied by scoliosis and/or pectus deformity (**Fig. 1**). However, this distinction is not absolute as the phenotype may vary between individuals.

Although specific features may point towards a syndrome for which directed genetic testing can be performed, there is considerable overlap between the phenotype of various syndromes. Gene panels or exome sequencing are now more widely available and may gain a more important position in the diagnostic evaluation of syndromic tall stature. Using exome sequencing, a recent study in individuals with height $\geq +2$ SDS and intellectual disability identified a genetic cause in 45%, suggesting exome sequencing might be considered as a first-line diagnostic tool in this group (Tatton-Brown *et al.*, 2017). The yield was even higher if subjects also had head circumference $\geq +2$ SDS (59%). Many of the identified mutations were in genes involved in epigenetic regulation, most commonly *NSD1* (Sotos syndrome). Another group of genes in which mutations were found were those of the PI3K/AKT pathway, most commonly *PTEN* (PTEN hamartoma tumor syndrome) (Tatton-Brown *et al.*, 2017).

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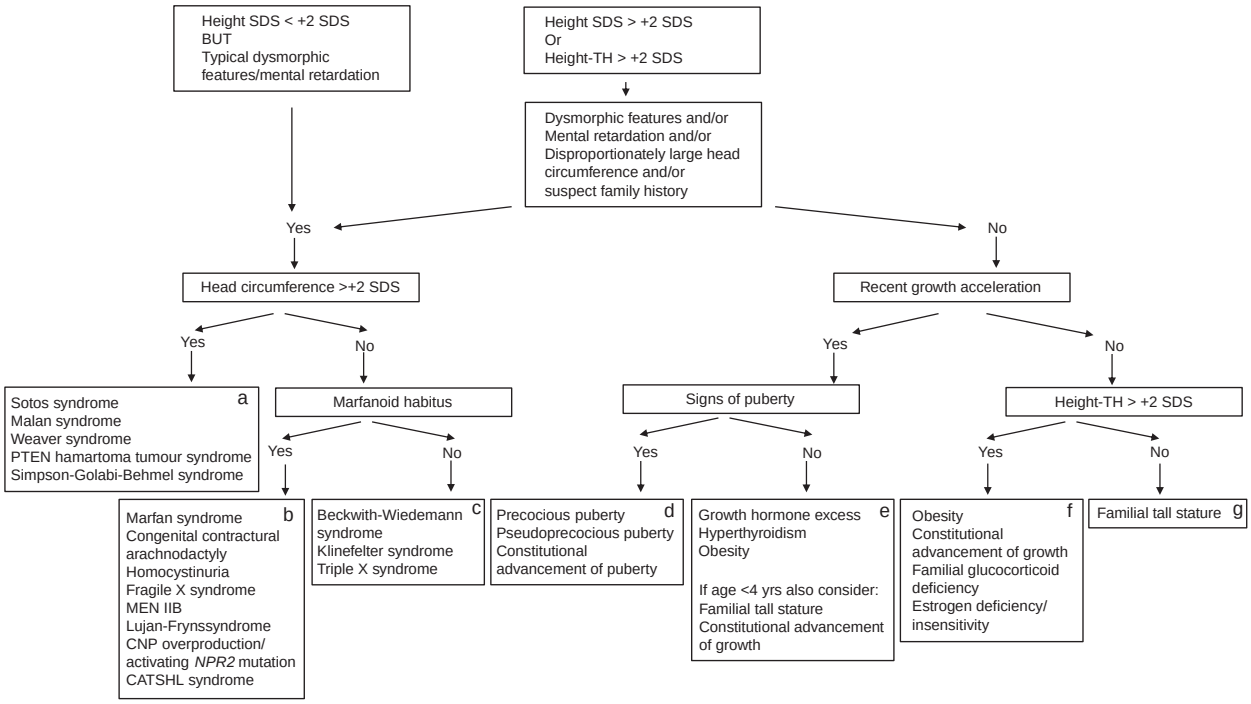

Fig. 1 Flowchart for the diagnostic evaluation of tall stature. Letters in the upper right corner of the boxes correspond to sections in the text. TH = target height. Adapted from Visser, R., Kant, S. G., Wit, J. M., Breuning, M. H. (2009). Overgrowth syndromes: From classical to new. *Pediatric Endocrinology Reviews* 6, 375–394.

Syndromes with macrocephaly

Sotos syndrome is an autosomal dominant disorder caused by mutations in the *NSD1* gene with an incidence of approximately 1:14,000. Dysmorphic features include a long face, frontal bossing and a prominent chin. Affected children are tall from birth onwards with an advanced bone age but they usually have a normal adult height. Sotos syndrome is associated with macrocephaly, brain abnormalities, neonatal hypotonia and feeding problems, developmental delay and intellectual disability. A score has been developed based on several of these clinical findings to estimate the probability of Sotos syndrome (de Boer *et al.*, 2004). Similar disorders also characterized by tall stature with advanced bone maturation, macrocephaly, dysmorphic features and developmental delay are Malan syndrome, caused by mutations in *NFIX* and Weaver syndrome, caused by mutations *EZH2*. Several other genes that are involved in epigenetic regulation, such as *DNMT3A*, *EED*, *CHD8* and *HIST1H1E* have also been implicated in tall stature and/or macrocephaly (Tatton-Brown *et al.*, 2017).

PTEN hamartoma tumor syndrome (PHTS) is a term used to describe several syndromes caused by mutations in *PTEN*, including Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome and possibly Proteus syndrome. It is inherited in an autosomal dominant way. The prevalence of PHTS is unknown but that of Cowden syndrome is estimated to be at least 1:250,000 (Nelen *et al.*, 1999). PTEN is a negative regulator of the PI3K-AKT-mTOR pathway which is involved in control of the cell cycle and cell proliferation (Song *et al.*, 2012). Macrocephaly is characteristic of Cowden syndrome and Bannayan–Riley–Ruvalcaba syndrome and affected individuals may have mucocutaneous manifestations such as trichilemmomas and penile macules, and hamartomas. Individuals with PHTS have an increased risk of intellectual disability and/or autism spectrum disorders. Tall stature has been reported in childhood but adult height seems normal (Parisi *et al.*, 2001). In childhood affected individuals may develop benign or malignant tumors of the thyroid for which screening is warranted (Smpokou *et al.*, 2015). At adult age they are at increased risk of various tumors including breast cancer, endometrial cancer and colorectal cancer. Mutations in several other genes in the PI3K-AKT-mTOR pathway, such as *AKT3*, *PIK3CA*, *MTOR* and *PPP2R5D*, have been found in individuals with macrocephaly with or without tall stature (Tatton-Brown *et al.*, 2017).

Simpson–Golabi–Behmel syndrome is a rare X-linked disorder caused by mutations in the *GPC3* gene. It is characterized by macrosomia and postnatal overgrowth, macrocephaly, dysmorphic features such as coarse facial features, macroglossia, a groove of the lower lip and supernumerary nipples, organomegaly and congenital abnormalities such as cardiac defects (Tenorio *et al.*, 2014). Affected children are at an increased risk of embryonal tumors.

Syndromes with Marfanoid habitus

Marfan syndrome is a connective tissue disorder caused by mutations in the *FBN1* gene with variable clinical expression and is inherited in an autosomal dominant way. Its prevalence is approximately 1:5000. The diagnosis is made using the revised Ghent criteria which are based on clinical characteristics (ectopia lentis, aortic root dilation and systemic features), family history and/or

Table 1 Clinical features of various conditions associated with tall stature and diagnostic tests that may be used to investigate these conditions.

Disorder	*Clinical features*	*Diagnostic test*
Sotos syndrome	Intellectual disability/developmental delay. Neonatal hypotonia, feeding problems. Long face, frontal bossing, prominent chin. Macrocephaly	Use Sotos score (de Boer *et al.*, 2004). Bone age (usually advanced). Evaluation by clinical geneticist. *NSD1* sequencing
PTEN hamartoma tumor syndrome	Intellectual disability, autism spectrum disorder. Macrocephaly. Lipomas, trichilemmomas, oral papillomas, penile freckling	*PTEN* sequencing
Marfan syndrome	Severe myopia, lens luxation, pneumothorax, long face, wrist or thumb sign, pectus deformity, hindfoot deformity, scoliosis, skin striae	Evaluation by ophthalmologist, cardiologist, clinical geneticist. Use revised Ghent criteria (Loeys *et al.*, 2010). *FBN1* sequencing
Homocystinuria	Intellectual disability, severe myopia, lens luxation, thrombo-embolism, scoliosis, pectus excavatum	Plasma homocysteine level
Fragile X syndrome	Intellectual disability, autism spectrum disorder, behavioral problems. Large head, long face, large ears, hypermobility, pectus excavatum. In boys: large testes after puberty	*FMR1* CGG repeat number
MEN2B	Long face, scoliosis, joint laxity, alacrima and mucosal neuromas	*RET* sequencing
Beckwith–Wiedemann syndrome	Macroglossia, ear creases or pits, naevus flammeus, abdominal wall defects, hemihyperplasia. Neonatal hyperinsulinism	Methylation analysis, *CDKN1C* sequencing
Klinefelter syndrome/Triple X syndrome	Delayed speech/language or motor development, social/emotional/behavioral problems. In boys: small penis, cryptorchidism, small testes in puberty	Karyotype
Other syndromes	Intellectual disability, dysmorphic features, macrocephaly	Evaluation by clinical geneticist; specific DNA tests, next generation sequencing
Precocious puberty	Breast development in girls, testicular enlargement in boys	LH, FSH, estradiol or testosterone; GnRH test; bone age
Pseudoprecocious puberty	Pubarche, penile enlargement, breast development	Adrenal androgens, AFP, hCG; bone age
Pituitary gigantism	Sometimes acromegalic features such as large hands/feet, thick skin. Headache/visual problems (in case of macroadenoma)	GH, IGF-I, IGFBP3; GH suppression test (OGTT)
Hyperthyroidism	Goiter, tachycardia, weight loss, exophthalmos	TSH, FT4
Familial glucocorticoid deficiency	Hyperpigmentation, hypoglycaemia, seizures	ACTH, cortisol
Estrogen deficiency/resistance	Continued growth into adulthood	LH, FSH, estradiol; bone age
Constitutional advancement of growth	May have early puberty and/or develop obesity	Bone age

genetic testing (Loeys *et al.*, 2010). Systemic features include a positive wrist or thumb sign, pectus deformity, hindfoot deformity, pneumothorax, scoliosis, specific facial features, skin striae and myopia >3 diopters. If Marfan syndrome is suspected, the child should be referred to the cardiologist and ophthalmologist, keeping in mind that the absence of cardiac or eye pathology does not rule out Marfan syndrome and that the phenotype may evolve over time so that repeat evaluation may be indicated. It is important not to miss this diagnosis because of the serious comorbidity associated with Marfan syndrome, such as aortic aneurysm and dissection, for which screening is indicated. Growth charts have been developed for children with Marfan syndrome and show that from birth onwards children with Marfan syndrome are taller than the general population and that their growth velocity peaks 2–2.5 years earlier than that of unaffected children (Erkula *et al.*, 2002).

Several other disorders are associated with disproportionate tall stature and a Marfanoid habitus. These include congenital contractural arachnodactyly, caused by mutations in *FBN2* and characterized by folded upper ear helices and joint contractures.

Homocystinuria is an autosomal recessive disorder caused by cystathionine β-synthase deficiency; its clinical expression is variable. In addition to disproportionate tall stature, scoliosis and pectus excavatum, affected individuals may have mental retardation, thromboembolism and severe myopia and/or lens luxation although the luxation is usually inferonasal rather than superotemporal as seen in Marfan syndrome. Recognition of this disorder and appropriate treatment can prevent thromboembolic complications that are the major cause of morbidity and mortality.

Fragile X syndrome is caused by mutations of the X-chromosomal *FMR1* gene resulting in the presence of >200 CGG repeats in the 5′ untranslated region and abnormal methylation. Characteristics of fragile X syndrome are mental retardation, autism spectrum disorders, behavioral problems, a large head, long face, large ears, hypermobility, pectus excavatum and large testes after puberty (Lozano *et al.*, 2014). Females have a second copy of the gene and have a more variable phenotype.

Lujan–Fryns syndrome is a clinical diagnosis based on the presence of intellectual disability and a Marfanoid habitus in combination with specific facial features such as a long face, maxillary hypoplasia, small mandible and a prominent forehead, nasal speech and X-linked inheritance (Van Buggenhout and Fryns, 2006). It is a genetically heterogeneous disorder, caused by

MED12 mutations in some affected individuals (Hackmann *et al.*, 2016). There seem to be various overlapping disorders characterized by intellectual disability and Marfanoid habitus.

Multiple endocrine neoplasia type 2B (MEN2B) is a rare autosomal dominant disorder caused by mutations in the *RET* gene. Clinical manifestations include a Marfanoid habitus, a long face, scoliosis, joint laxity, alacrima and mucosal neuromas on the tongue, lips, oral cavity and conjunctivae (Castinetti *et al.*, 2017). These neuromas are white-yellow papules a few millimeter in size and may be present at birth and can usually be found by age 10 yrs (Castinetti *et al.*, 2017). Affected individuals are at high risk of developing medullary thyroid cancer at a very young age and have an increased risk of pheochromocytoma. Gastrointestinal complaints caused by diffuse gastrointestinal ganglioneuromatosis may be present from infancy or early childhood.

C-type natriuretic peptide (CNP) is a paracrine factor produced by chondrocytes that acts through the natriuretic peptide receptor 2 (NPR2) to induce endochondrial ossification. Both increased production of CNP due to genomic rearrangements and increased activity of *NPR2* caused by activating mutations are rare causes of tall stature (Bocciardi *et al.*, 2007; Moncla *et al.*, 2007; Miura *et al.*, 2012; Hannema *et al.*, 2013; Miura *et al.*, 2014). Several but not all affected individuals had a Marfanoid habitus, scoliosis and very long halluces. Downstream of NPR2 the signaling cascade converges with that of the FGFR3. Interestingly, loss-of-function mutations in FGFR3 have also been reported in individuals with tall stature, who also had camptodactyly and hearing loss (*CATSHL syndrome*) (Toydemir *et al.*, 2006; Makrythanasis *et al.*, 2014). Affected individuals were also reported to have long fingers and some had scoliosis.

Syndromes without Marfanoid habitus

The prevalence of *Beckwith–Wiedemann syndrome (BWS)* is approximately 1:1000 newborns (Mussa *et al.*, 2013). The diagnosis can be made based on clinical criteria and genetically confirmed in 85%–90% (Bliek *et al.*, 2001). Most affected individuals have an imprinting disorder of one or two gene clusters that contain the *IGF2* and *H19* genes, under control of chromosome 15 imprinting centre 1 (IC1), and the *CDKN1C* and *KCNQ1OT1* genes, under control of imprinting centre 2 (IC2), but some have mutations in *CDKN1C*. Approximately 65% of children with BWS have a birth weight above the 90th percentile (Maas *et al.*, 2016). They are tall in childhood and mean adult height has been reported to be 1.8 $\pm$ 1.2 SDS (Brioude *et al.*, 2013). Congenital anomalies such as macroglossia, ear creases or pits, naevus flammeus and abdominal wall defects are characteristic of BWS as is hemihyperplasia (Maas *et al.*, 2016). Children may develop hypoglycaemia due to hyperinsulinism in the neonatal period and have an increased risk of embryonic tumors, especially Wilms tumor. The tumor risk depends on the underlying genetic abnormality, ranging from 28% in IC1 hypermethylation to 2.6% in IC2 hypomethylation, and recommended tumor surveillance differs between the genetic subgroups (Maas *et al.*, 2016).

Klinefelter syndrome, characterized by karyotype 47,XXY is a common disorder with an incidence of approximately 1 in 500–1000 boys although it is estimated that >50% of men are never diagnosed (Bojesen *et al.*, 2003; Nielsen and Wohlert, 1991). Clinical findings that are suggestive of Klinefelter syndrome are micropenis and/or undescended testes, delayed motor and/or speech/language development, learning and behavioral problems, small testes (usually not larger than 7 mL in adults), hypergonadotropic hypogonadism and infertility (Aksglaede *et al.*, 2011). *Triple X syndrome* (47,XXX), with an incidence of 1:1000 girls, has a variable phenotype and many affected women go undiagnosed. Triple X syndrome shares many features with Klinefelter syndrome such as delayed motor and speech development, lower IQ than siblings and behavioral problems. *Other sex chromosomal disorders* that have overlapping features include 47,XYY, 48,XXYY, 48,XXXY, 49,XXXXY and tetrasomy and pentasomy X.

Although birth length is not increased in Klinefelter syndrome, growth velocity increases between age 5 and 8 years and adult height is above the target height although often within the normal range (Ratcliffe, 1999; Ottesen *et al.*, 2010). The increase in height that is associated with an increased number of sex chromosomes has been attributed to the presence of extra copies of the *SHOX* gene, located on the pseudoautosomal region of the sex chromosomes (Ottesen *et al.*, 2010). However, individuals with four sex chromosomes in females and five in both males and females do not have an increased height possibly due to a negative effect of overdosage of other genes (Ottesen *et al.*, 2010).

Tall stature with growth acceleration with signs of puberty

Precocious or early puberty may lead to temporary tall stature because of the early growth spurt although adult height may actually be compromised in precocious puberty because of premature epiphyseal closure. If the growth chart shows a growth acceleration and signs of puberty are present it is important to distinguish between central and peripheral puberty. The presence of breast development in girls and increased testicular volume in boys in combination with elevated basal gonadotropins or a positive GnRH test is consistent with central puberty. Genetic factors play an important role in the timing of puberty and information on the onset of puberty in parents and other family members may indicate familial early maturation. In central precocious puberty (before age 8 years in girls and 9 years in boys) an underlying CNS disorder may be present, especially in boys, but in girls it is usually idiopathic. Central precocious puberty can be treated with a GnRH analogue. When signs of puberty are present in the absence of central activation of the hypothalamus-pituitary-gonadal axis the differential diagnosis includes congenital adrenal hyperplasia and McCune–Albright syndrome.

Tall stature with growth acceleration in the absence of puberty

Growth acceleration without signs of puberty may be a physiological phenomenon in the first few years of life when children find their own growth channel. This will usually be within the target height range but may also be above the target height range in children with constitutional advancement of growth. This term is used to describe a growth pattern that is the opposite of

constitutional delay of growth and puberty (Papadimitriou *et al.*, 2010). In the prepubertal years growth accelerates but so does bone maturation so that height for bone age is usually within the target height range. Children may go on to have an early puberty. Family history may indicate that one of the parents had a similar growth pattern and/or early puberty. Growth and bone maturation may also accelerate when children become obese.

However, when growth suddenly accelerates after a period of stable growth in the absence of pubertal signs or obesity endocrine disorders such as hyperthyroidism and growth hormone excess should be considered. Clinical signs of hyperthyroidism include goiter, weight loss, tachycardia and exophthalmos in Graves' disease. Growth hormone excess may lead to thickened skin and acromegalic features, although these are not as common in children as in adults, and may be accompanied by deficiencies of other pituitary hormones and headache or visual defects due to a macroadenoma. Thyroid function tests and IGF-1 measurement can be used as first line investigations. If IGF-1 is elevated, an oral glucose tolerance test can be used to assess growth hormone suppression. It is important to be aware of the fact that this test may be false positive in constitutionally tall children and adolescents (Holl *et al.*, 1999). Some have used a higher glucose dose of 2.35 mg/kg up to a maximum of 100 g rather than the standard dose of 1.75 mg/kg up to a maximum of 75 kg in the oral glucose tolerance test and found only 1 false positive result among 107 children (Misra *et al.*, 2007).

Growth hormone excess can be caused by a pituitary adenoma and may be associated with an underlying disorder such as multiple endocrine neoplasia type 1, *AIP*-related familial isolated pituitary adenomas or McCune-Albright syndrome. Growth hormone excess has also been observed in individuals with neurofibromatosis type 1 with an optic pathway glioma (Bizzarri and Bottaro, 2015). It is hypothesized that the glioma disrupts somatostatin tone leading to increased growth hormone secretion. Treatment depends on the cause. Transsphenoidal surgery is used for pituitary adenomas; non-surgical treatment options are radiotherapy or medical therapy including somatostatin analogues, dopamine agonists and growth hormone receptor antagonists which have been used with variable success.

Tall stature without recent growth acceleration outside the target height range

Stable growth outside the target height range may have been preceded by a growth acceleration related to weight gain or constitutional advancement of growth. Usually this is accompanied by an advanced bone age. When determining if the child is growing outside the target height range one should also consider the limitations of formulas used to calculate the target height range, especially if one parent is very tall and the other parent much shorter. In this situation children may have a growth pattern that resembles that of the tall parent and grow outside their target height range even though their tall stature is familial.

Delayed epiphyseal closure may lead to increased height with decreased sitting height-to-height ratio in adolescents with hypogonadotropic hypogonadism. This same phenomenon is more extreme in rare cases of estrogen deficiency due to aromatase deficiency or estrogen resistance due to estrogen receptor alpha mutations. Delayed puberty or primary amenorrhoea in tall girls may also be caused by a disorder/difference of sex development with a 46,XY karyotype. Signs of adrenal insufficiency such as hyperpigmentation should raise the suspicion of familial glucocorticoid deficiency.

Tall stature without recent growth acceleration within the target height range

If a child is tall but grows within the target height range then constitutional tall stature is most likely (Upners and Juul, 2016; Thomsett, 2009). However, a heritable disorder associated with tall stature should be considered; a parent may be affected without having been diagnosed. A careful history, family history and physical examination is therefore always indicated to exclude an underlying disorder.

Treatment of Tall Stature

Tall stature in itself is not a medical condition that needs treatment. However, individuals may feel that extreme tall stature is unpractical in the sense that they might have problems finding clothing and they might not fit in a regular bed or car; they may also worry about the social consequences. Therefore some adolescents and their parents request treatment to reduce adult height. However there is no evidence that such treatment leads to a better quality of life in adulthood, and if and when this treatment is ethical is subject to debate. Generally treatment is only considered in adolescents whose predicted adult height is greater than $+2.5$ SDS. Careful counseling of the adolescent and parents is required on the efficacy and safety of the available treatments, which are high dose sex steroids and epiphysiodesis.

Predicting Adult Height

Accurate adult height prediction is important because it may reassure adolescents if they are not expected to become excessively tall, and otherwise the predicted height forms the basis of a decision to treat or not, and decide on the timing of treatment. Several methods are available to predict adult height of which Bayley and Pinneau (B&P) and Tanner-Whitehouse (TW2) are most widely used. Their performance differs depending on age and sex. Prediction is more accurate in constitutionally tall girls (absolute errors of prediction 2.0 cm for B&P and 2.3 for TW2) than in boys (absolute errors of prediction 3.3 and 3.9 cm) and becomes more accurate with increasing age (De Waal *et al.*, 1996). In adolescents with Marfan syndrome neither method was found to be precise

(Rozendaal *et al.*, 2005). More recently a method for adult height prediction has been developed based on automated bone age determination (Thodberg *et al.*, 2009). In addition to height and bone age this method takes parental height into account. However, no data are available on the performance of this method in (constitutionally) tall children.

High Dose Sex Steroids

High doses of sex steroids lead to fusion of the growth plate. Based on this principle, high dose sex steroid treatment has been used to reduce adult height in tall adolescents. In boys treatment often consists of intramuscular testosterone injections at doses of 250 mg every week or 250–500 mg every 2 weeks. The efficacy of this treatment depends on the bone age at the start of treatment; after bone age 14 yrs. treatment does not result in reduced adult height and may even increase adult height (De Waal *et al.*, 1996). Similar results of this treatment were found in boys with Marfan syndrome, without any serious short-term (cardiovascular) side effects (Rozendaal *et al.*, 2005).

Short-term side effects of high dose testosterone treatment include acne, weight gain, gynecomastia, muscle aches and oedema, aggressive behaviour and troublesome erections (Drop *et al.*, 1998). A study that investigated long-term side effects found no effect on fatherhood or semen parameters in a group of 60 treated men at an average age of 35 yrs. compared to untreated controls, although total sperm count and serum inhibin B levels seemed to decline more rapidly over time in treated men (Hendriks *et al.*, 2010). In addition, serum testosterone and AMH levels were significantly lower in treated men (Hendriks *et al.*, 2010).

Girls have been treated with ethinyl estradiol 100–500 mcg/day. The height reduction achieved depends on the bone age at the start of treatment; treatment is ineffective form bone age 14 yrs. (De Waal *et al.*, 1996). High dose estrogen treatment did not have a statistically significant effect on adult height in a group of girls with Marfan syndrome in whom treatment was started at a mean bone age of 11.4 years (Rozendaal *et al.*, 2005). Possibly this was already too late because of the earlier growth spurt in adolescents with Marfan syndrome.

Short-term side effects of high-dose estrogen treatment include weight gain, nausea, headaches, hypertension and venous thromboembolism. Several studies have reported long-term negative effects on fertility. Time to pregnancy was increased in treated versus untreated women, the use of fertility treatments was increased and the number of live births reduced (Venn *et al.*, 2004; Hendriks *et al.*, 2011; Hendriks *et al.*, 2012). These effects were dose-dependent (Hendriks *et al.*, 2012). In addition treated women were more frequently diagnosed with imminent ovarian failure, characterized by an FSH $>$ 10 IU/L on day 3–5 of the menstrual cycle (Hendriks *et al.*, 2011). There are also worries that high-dose estrogen treatment may increase the risk of (reproductive) tumors. Although no significant increase of these tumors was found in a study of women treated with very high doses of ethinyl estradiol (250–1000 mcg), further follow-up studies are needed because most women were only in their forties at the time of the study (Benyi *et al.*, 2014). This same study suggested that the risk of melanoma might be increased although absolute tumor numbers were small (Benyi *et al.*, 2014).

Epiphysiodesis

An alternative to hormonal treatment is surgical treatment, usually bilateral percutaneous epiphysiodesis. This treatment aims to destroy the growth plates of the distal femur and proximal tibia and fibula to limit further leg growth but does not affect growth of the back and arms. The procedure is performed under general anesthesia and takes approximately 1 h. After treatment weight baring is allowed but adolescents are advised to refrain from sports for 4 weeks. The growth reduction achieved depends on bone age at the time of the procedure but is approximately one third of the remaining growth. In girls treated at bone age 12.5 yr growth was reduced by 4 cm and in boys treated at bone age 14 yr growth reduction was 6 cm (Benyi *et al.*, 2010). This is more than the growth reduction seen in adolescents treated with high dose sex steroids at a similar bone age. Body proportions are altered by this treatment but as tall individuals generally have long legs the sitting height-to-height ratio after treatment usually is within the normal range and the relatively long arms are generally not experienced as problematic. However, if the procedure is performed at a very young bone age or in individuals with a high sitting height-to-height ratio abnormal body proportion is a potential concern.

Surgery under general anesthesia always carries a small risk. However, few complications of the procedure itself have been reported. These include a cutaneous infection in 1 of 21 treated adolescents, which resolved spontaneously, and postoperative pain for which oral analgesics were used for up to 2 weeks in 9 of 21 adolescents (Benyi *et al.*, 2010). Another study of 15 treated adolescents reported fibula exostosis in one patient and angular leg deformities in two patients (2–8 degrees) but no treatment was necessary for these complications (Odink *et al.*, 2006). The surgery leaves small scars. Because of the skeletal abnormalities associated with Marfan syndrome there may be concerns about the safety of epiphysiodesis in adolescents with Marfan syndrome but no complications have been reported in three individuals (Benyi *et al.*, 2010).

Compared to high-dose sex steroid treatment epiphysiodesis can be performed at a more advanced bone age, when adult height prediction is more accurate, with superior efficacy. In addition epiphysiodesis seems safe with few complications whereas serious long-term side effects of especially high-dose estrogen treatment on fertility have become clear. We therefore recommend that if there is a strong treatment wish from an adolescent with an extremely tall predicted adult

height epiphysiodesis should be considered the treatment of choice. We recommend against the use of high-dose estrogen treatment.

In conclusion, even though constitutional tall stature is the most common diagnosis among tall adolescents a careful evaluation is warranted to exclude underlying pathology which may have serious health consequences. When considering growth reducing treatment it is important to be aware of the limitations of adult height prediction and to discuss and weigh the pros and cons of treatment together with the adolescent and parents.

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Growth in Childhood Chronic Conditions

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Glossary

Chronic condition A condition, usually of complex causality, that leads to a prolonged course of illness, often leading to other health complications and associated functional impairment or disability.

Cytokines Cytokines are small proteins that are released by cells that have a specific effect on the interaction and communications between cells.

Glucocorticoid A class of steroid hormones with important effects on the cardiovascular, metabolic, immunologic, and homeostatic functions, often used in high doses to suppress inflammation.

Growth Growth is a complex process where a child increases in size and stature in a predictable pattern over time, usually divided into three periods: infancy, childhood, and puberty.

Growth hormone (GH) Growth hormone is a 191-amino acid peptide hormone secreted by the anterior pituitary gland that promotes growth, cell reproduction, and regeneration.

Growth plate Growth plate is a region of hyaline cartilage located between the epiphyses and diaphysis of long bones where longitudinal bone growth takes place.

Inflammation Inflammation is a protective biological response to harmful stimuli for removing the source of injury and to initiate healing.

Insulin-like growth factor-1 (IGF-1) Insulin-like growth factor-1 is a 70-amino acid protein produced mainly by the liver in response to growth hormone stimulation that promotes cellular growth.

Nutrition Nutrition is the intake of food, in relation to the body's dietary requirements, required to maintain health.

Puberty Puberty is a process of physiological changes characterized by the development of secondary sexual characteristic and rapid physical growth.

Introduction

Growth failure and delayed puberty are common consequences of childhood chronic illness and are frequent referrals to the pediatric endocrine clinic. In conditions like cystic fibrosis (CF), inflammatory bowel disease (IBD), juvenile idiopathic arthritis (JIA), and duchenne muscular dystrophy (DMD), several factors contribute to growth failure and pubertal abnormalities. These include the direct effect of pro-inflammatory cytokines as a result of the underlying condition, use of glucocorticoid, and suboptimal nutrition (Wong *et al.*, 2016). The triad of these factors affects growth and pubertal development via their impact on the endocrine axis and at a local level (**Fig. 1**). While there is no doubt that the use of glucocorticoid itself contributes to poor growth, in recent times, greater focus is given to the role of the underlying disease process itself due to the deleterious impact of pro-inflammatory cytokines like tumor necrosis factor-α (TNFα), interleukin-1 (IL-1), and IL-6. On the other hand, systemic glucocorticoid is often used for longer periods in those whose disease is suboptimally controlled. Therefore, glucocorticoid use may be a marker of severity of the underlying disease. This article will provide an update of growth failure in childhood chronic illness and discuss endocrine growth-promoting therapies in these conditions.

Definition of Growth Failure in Childhood Chronic Conditions

Clinical studies of growth failure in chronic childhood illness may vary depending on the underlying condition, treatment regimen, and definition of growth failure. Older studies largely rely on reports of height, but this does not provide information on growth rate. This may only capture the end result of prolonged and severe growth failure. While height velocity is more appropriate to study growth, there are some inherent difficulties including the lack of robust contemporary normative data. An alternative would be to evaluate change in height standard deviation scores (SDS) over time (Wong *et al.*, 2016). There is also a lack of consensus on the appropriate method to adjust for delayed puberty, which needs to be taken into account whether growth is reported as height velocity or change in height SDS. These methodological issues need to be carefully considered in growth studies involving adolescents with chronic ill health.

Rationale for Addressing Growth in Childhood Chronic Conditions

Despite the limitations of reporting height to determine growth failure, reduction in adult height is seen in 20% of individuals with Crohn's disease (CD), approximately 40% with systemic JIA and CF, and up to 78% of young men with DMD, reflecting that persistent and severe growth failure is still observed in a subset of these people (**Fig. 2**) (Gorman *et al.*, 2005; Lai *et al.*, 1999; Minden, 2009;

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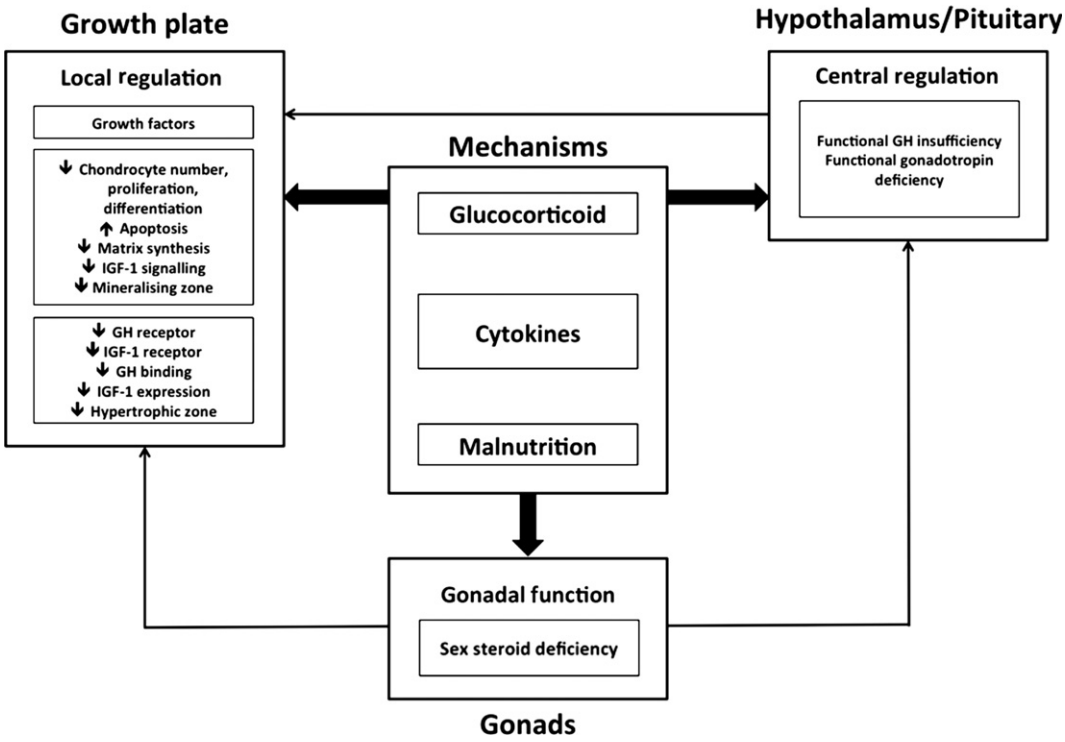

Fig. 1 Mechanism of growth failure in chronic inflammatory conditions.

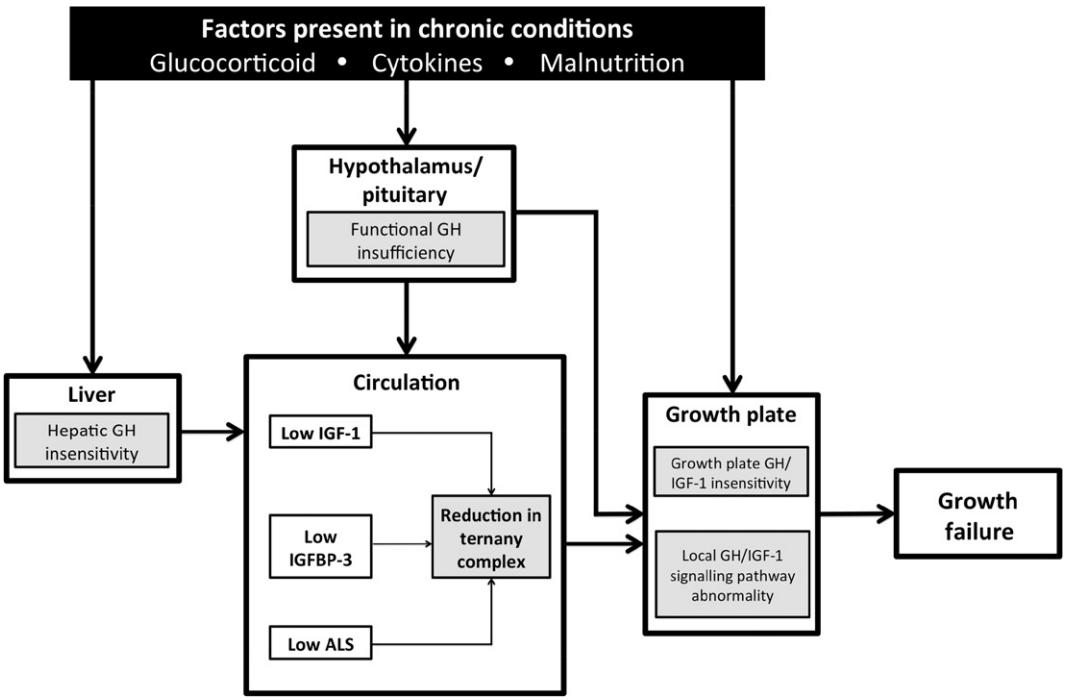

Fig. 2 GH/IGF-1 axis abnormalities in children with chronic conditions.

Sawczenko *et al.*, 2006; Wong *et al.*, 2017). Emerging evidence suggests that there may be a link between poor growth in children with chronic ill health and quality of life, independent of underlying disease severity (Mason *et al.*, 2015). Linear growth and pubertal progression are intimately linked to bone development. Osteoporosis in childhood chronic ill health is a systemic disorder of low bone turnover resulting from poor longitudinal bone growth (Wong *et al.*, 2014; Joseph *et al.*, 2016), and improving growth is a critical strategy

to improve bone development in childhood chronic conditions. In some conditions, the possibility of improving the underlying disease by promoting growth via manipulation of the growth hormone (GH) axis deserves further research (Denson *et al.*, 2010; Hardin *et al.*, 2006). In summary, despite modern therapies, endocrine growth-promoting therapies may need to be considered.

There are numerous chronic conditions in childhood that can lead to growth failure and pubertal disorders. In this article, we will focus our attention on a small group of conditions with underlying chronic inflammation. We will subdivide the discussions into three groups of childhood chronic conditions based on glucocorticoid requirement:

1. Minimal or no systemic glucocorticoid (e.g., CF)
2. Intermitted systemic glucocorticoid (e.g., JIA and IBD)
3. Continuous systemic glucocorticoid (e.g., DMD)

Normal Growth

The infancy, childhood, and puberty (ICP) model divides human growth into three phases, reflecting the endocrine control of the growth process. The infancy phase spans from birth to the first 2–3 years of life and is a period of rapid growth. Growth in the infancy phase is largely nutrition-dependent but closely linked with the actions of insulin-like growth factors (IGF) (Leger *et al.*, 1996; Wang and Chard, 1992). Growth rate begins to decelerate after the first 3 years of life (childhood phase). It remains stable for several years, with the fiftieth percentile of height velocity during mid- and late childhood approximating 5–6 cm/year. Growth during this phase is largely influenced by the GH/IGF-1 axis.

Normal Puberty

Puberty is the stage of development during which adolescents develop secondary sexual characteristics and is influenced by genetic, nutritional, environmental, and socioeconomic factors. In girls, puberty normally begins between 8 and 13 years of age and in boys between 9 and 14 years of age. The pubertal growth spurt encompasses the second most rapid phase of postnatal growth after the neonatal period. During adolescence, height velocity increases from prepubertal rates of 5–6 cm/year to a peak height velocity of 10–15 cm/year, corresponding to genitalia stage 3–4 in boys and breast stage 2–3 in girls. The production of sex steroids during adolescence contributes to the pubertal growth spurt by direct effects on growth plate chondrocytes and indirect effects on GH secretion by increasing the amplitude of spontaneous GH secretion, resulting in increased IGF-1 production systemically and locally (Callewaert *et al.*, 2010; Smith *et al.*, 2010).

The GH/IGF-1 Axis

The GH/IGF-1 axis is the main regulator of linear growth via its endocrine effects systemically and at a local level. Pituitary-derived GH increases hepatic IGF-1, which circulates systemically bound to IGF-binding proteins (IGFBP): IGFBP-3 and acid labile subunit (ALS), collectively known as the ternary complex. IGF-1 induces growth by promoting cell division, inhibiting apoptosis, and stimulating protein synthesis. IGFBP may play a direct role on maintenance of growth although the precise mechanism is still unclear (Duan *et al.*, 2010). Both systemic IGF-1 and GH have separate and independent effects on regulation of linear growth. GH also induces the expression and action of IGF-1 at the level of the growth plate. A comprehensive summary of the complex interplay between systemic and local factors on growth is summarized in the revised somatomedin hypothesis (Kaplan and Cohen, 2007).

Mechanisms of Growth Failure in Chronic Inflammation

As mentioned, chronic inflammation, glucocorticoid use, and suboptimal nutrition contribute to the underlying pathophysiology of growth failure in chronic childhood condition via systemic and local effects on the GH/IGF-1 axis (Wong *et al.*, 2016; MacRae *et al.*, 2006a). The deleterious effect of chronic inflammation on the GH/IGF-1 axis occurs at multiple levels (**Fig. 2**). It is often difficult to tease out the relative impact of cytokines, glucocorticoid, and poor nutrition on growth as they are closely related.

Systemic Abnormalities of the GH–IGF-1 Axis in Childhood Chronic Inflammation

Hormone insensitivity

It is recognized that chronic inflammation is often associated with a state of relative GH insensitivity, reported in children with IBD (Chong *et al.*, 1984; Wong *et al.*, 2010), JIA (Bechtold *et al.*, 2012; Saha *et al.*, 2004; Tsatsoulis *et al.*, 1999), and CF (Ciro *et al.*, 2013). Evaluation of the GH axis with dynamic stimulation tests often reveals elevated GH levels but low IGF-1 levels. In 28 children with IBD evaluated with the insulin tolerance test, relative GH insensitivity (defined as peak GH $>$ 6 μg/L to insulin tolerance test and IGF-1 SDS $<$ 0) was observed in almost 40% of the cohort (**Fig. 3**) (Wong *et al.*, 2010).

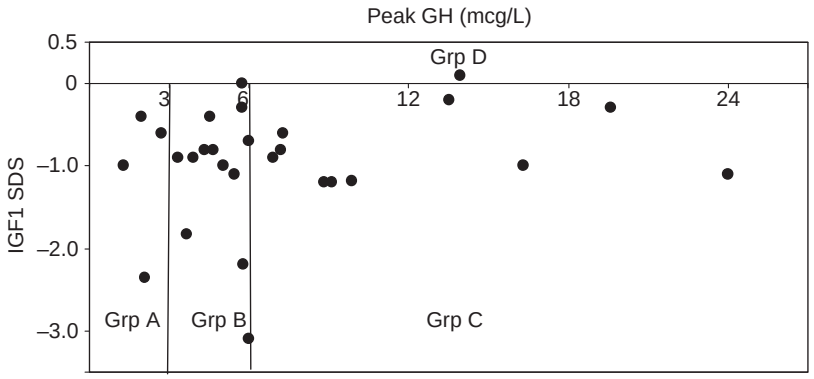

Fig. 3 GH and IGF-1 levels in response to insulin hypoglycemia in children with IBD and growth failure. Reproduced from Wong, S. C., *et al.* (2010). The growth hormone insulin-like growth factor 1 axis in children and adolescents with inflammatory bowel disease and growth retardation. *Clinical Endocrinology (Oxford)* **73**, 220–228, with permission © Wiley.

Different proinflammatory cytokines may lead to hepatic GH insensitivity via separate mechanisms. TNFα and IL-1 appear to exert negative effects on the GH receptor, whereas IL-6 affects downstream signaling proteins like the signal transducer and activator of transcription (STAT) pathway and the suppressor of cytokine signaling (SOCS)-3 protein (Yumet *et al.*, 2006; Zhao *et al.*, 2014a).

Undernutrition itself is also associated with a state of GH insensitivity (Bang *et al.*, 1994; Counts *et al.*, 1992), thought to be due to a protective metabolic adaptation as a result of undernutrition. Recent studies have identified several proteins as important regulators of GH sensitivity in states of nutritional deprivation, including fibroblast growth factor (FGF) 21 and sirtuin (SIRT) (Fazeli and Klibanski, 2014). FGF21 and SIRT are upregulated in malnutrition and inhibit hepatic STAT5 phosphorylation that in turn reduces IGF-1 production.

Less well recognized but reported in chronic inflammation is IGF-1 insensitivity, likely to be due to chronic elevation of pro-inflammatory cytokines (O'Connor *et al.*, 2008). Two out of the 28 children with IBD and growth failure who underwent evaluation of the GH/IGF-1 axis had evidence of combined GH/IGF-1 insensitivity with elevated GH levels and IGF-1 SDS > 0 (**Fig. 3**) (Wong *et al.*, 2010).

Hormone insufficiency

GH insufficiency is commonly observed in those with chronic glucocorticoid use, as glucocorticoid suppresses pulsatile GH release centrally (Barkan *et al.*, 2000). In a study of 83 slow-growing glucocorticoid-treated boys with DMD, stimulated GH was sub-optimal in 37% (Allen *et al.*, 1998). Similarly, in a group of slow-growing children with JIA treated with glucocorticoid, 40% had evidence of GH insufficiency when evaluated with combined arginine–clonidine stimulation test (Bechtold *et al.*, 2012; Saha *et al.*, 2004; Tsatsoulis *et al.*, 1999). However, GH insufficiency has also been reported in children with chronic conditions not treated with glucocorticoid (Bechtold *et al.*, 2012; Saha *et al.*, 2004; Tsatsoulis *et al.*, 1999). Peak GH to insulin tolerance test (GH < 3 μg/L and IGF-1 SDS < 0) was found in 14% of slow-growing children with IBD, including those who were not treated with glucocorticoids (**Fig. 3**) (Wong *et al.*, 2010).

Binding protein abnormalities

IGF-binding protein abnormalities may reduce circulating IGF-1 leading to reduction of IGF-1 delivery to target organs. In children with CF, low IGFBP-3 and high IGFBP-1 levels in the setting of normal systemic IGF-1 may be responsible for growth failure (Ozen *et al.*, 2004; Street *et al.*, 2009). Low ALS levels during acute relapse of children with JIA and mild growth failure have recently been reported (Wong *et al.*, 2008). In adolescents with IBD, elevated IGFBP-3 and relatively normal IGF-1 may lead to low bioavailability of free IGF-1 (Mason *et al.*, 2015). IGFBP-2 may lead to a reduction in the formation of the ternary complex. Significantly higher IGFBP-2 levels have been documented in children with IBD (Hoeflich *et al.*, 1999; Street *et al.*, 2004) and CF (Street *et al.*, 2009), associated with IL-6.

Growth Plate Effects in Chronic Conditions

Effects of cytokines on growth plate

IL-1β and TNFα can impair growth plate chondrogenesis by decreasing both the width of the proliferating zone and rate of endochondral bone growth (Choukair *et al.*, 2014; Martensson *et al.*, 2004). Cytokines also lead to increased apoptosis and reduction of proteoglycan synthesis. Reduction in proliferation at the growth plate by cytokines also occurs by their effects on local IGF-1 signaling (MacRae *et al.*, 2007). Inflammatory cytokines act synergistically to affect longitudinal bone growth, and the extent of recovery following exposure to cytokines may be dependent on duration of exposure (MacRae *et al.*, 2006b).

Effects of glucocorticoid on growth plate

The mechanism of glucocorticoid effects on growth plate is very similar to the cytokine-driven effect. Glucocorticoid can block the activation of GH and IGF-1 receptors in chondrocytes. It can also impair IGF-1 signaling via the phosphatidylinositol-3-kinase pathway at the growth plate (Chrysis *et al.*, 2005; Giustina *et al.*, 1992; Giustina and Veldhuis, 1998; Klaus *et al.*, 2000), inhibiting chondrocyte proliferation and differentiation while stimulating chondrocyte apoptosis and autophagy (Chrysis *et al.*, 2003; Chrysis *et al.*, 2005; Zhao et al., 2014b). A crucial mechanism for glucocorticoid target at the level of the growth plate is its effect on increasing apoptosis (Chrysis *et al.*, 2005; Zaman *et al.*, 2012). New therapies targeting apoptosis may be potential new treatment for growth failure in chronic conditions.

Effects of poor nutrition on growth

In animal studies investigating the effects of malnutrition, reductions in all zones of the growth plate and decrease in chondrocyte numbers were observed (Kubicky *et al.*, 2012). Increased FGF21 levels during malnutrition lead to reduction in systemic GH binding to the GH receptor in chondrocytes with no effect on number of GH receptors (Kubicky *et al.*, 2012). The important role of FGF21 is further strengthened by the evidence of normal growth and the absence of GH insensitivity from a study in FGF21 knockout mice that were subjected to 4 weeks of food restriction (Wu *et al.*, 2013). Studies on the role of FGF21 and growth failure in chronic conditions are needed.

Growth in Conditions With Minimal or no Exposure to Glucocorticoids

Cystic Fibrosis

CF is an autosomal recessive condition with defect of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (7q31.2), affecting primarily the lungs, as well as the pancreas, liver, intestine, and other organs. The CFTR gene defect leads to decreased chloride secretion and increased sodium absorption across the epithelial surface, resulting in chronic pulmonary infections, airway inflammation, and increased systemic inflammatory cytokines. Exocrine pancreatic insufficiency occurs in most individuals resulting in malnutrition and fat-soluble vitamin deficiency. Pancreatic islet cells may also become damaged over time, leading to cystic fibrosis-related diabetes (CFRD), often associated with further deterioration in growth failure.

Management involves addressing nutrition and minimizing and treating pulmonary infections with physiotherapy, antibiotic therapy, and therapies to reduce mucous viscosity. Systemic glucocorticoid is not often used in management of CF. However, allergic bronchopulmonary aspergillosis, seen in 4%–11% of individuals with CF, often requires prolonged oral glucocorticoid treatment to suppress the exaggerated immune response (Mahdavinia and Grammer, 2012).

Growth failure in *CF*

Short stature and growth failure are important complications in the CF population, although these problems have decreased in frequency over time. While 39% of children from an older study were below the fifth percentile for height, a contemporary cohort showed that only 1% of the cohort had short stature after adjustment for bone age and parental height (Lai *et al.*, 1999; Woestenenk *et al.*, 2011). Improvement in growth has been observed in those managed in specialist centers throughout their lifetime. Most published evidence suggests that height is associated with pulmonary and pancreatic function (Lucidi *et al.*, 2009; Woestenenk *et al.*, 2014; Zemel *et al.*, 2000).

Optimal nutrition is crucial for growth in CF with evidence that improvement in the nutritional status is associated with significant improvements in weight and height (Lai *et al.*, 1999). Furthermore, several studies have shown that benefits of long-term use of supplemental enteral feeding with gastrostomy on growth and lung function continue beyond cessation of therapy (Dalzell *et al.*, 1992; Levy *et al.*, 1985; Rosenfeld *et al.*, 1999; Shepherd *et al.*, 1986).

Infants with CF identified from neonatal screening are lighter and shorter and have smaller head circumference at birth than the general population, associated with lower systemic IGF-1 levels (Ghosal *et al.*, 1996; Haeusler *et al.*, 1994; Rogan *et al.*, 2010). Furthermore, children homozygous for the ΔF508 mutation were approximately 1 SDS below the mean for height from infancy to early adolescence (Keller *et al.*, 2003), highlighting a genotype impact on growth failure in CF.

Pubertal disorders in CF

Delayed puberty is not a common occurrence in contemporary cohorts of adolescents with CF; however, poor growth rate during puberty is still observed (Bournez *et al.*, 2012; Landon and Rosenfeld, 1987). Poor growth often precedes the onset of CFRD, usually diagnosed in mid-to-late adolescence. Therefore, increased awareness and screening are required in adolescents with CF, especially if growth and pubertal issues are encountered (Bizzarri *et al.*, 2015). Insulin therapy may improve lung function and weight gain in those with CFRD (O'Shea and O'Connell, 2014).

Adult height in CF

Evidence from studies shows that adult height in CF has improved in the last few decades with better surveillance and treatment regimens. Older studies showed that the average adult height in CF was at the twenty-fifth percentile and half the cohort had height below midparental height (Lai *et al.*, 1999; Zhang *et al.*, 2013). Early diagnosis and treatment via newborn screening may

have helped to improve adult height outcomes. An Australian study showed that those identified from newborn screening were significantly taller as adults than those diagnosed clinically (Dijk *et al.*, 2011).

Growth in Conditions Requiring Intermittent Systematic Glucocorticoid

Juvenile Idiopathic Arthritis

JIA is a group of conditions involving inflammatory arthritis beginning before the age of 16 years for >6 weeks. The management of JIA is dependent on the subtype and is focused on achieving optimal joint function, as no cure is currently available. While pain relief is generally achieved by nonsteroidal antiinflammatory drugs, intraarticular glucocorticoid injections may be required in those who do not respond. Previous management involves prolonged periods of systemic glucocorticoid. Currently, children with severe arthritis (systemic and polyarticular JIA) may require oral or intravenous glucocorticoid treatment for a short period until therapeutic immunomodulatory levels (e.g., methotrexate) are achieved. There is also increasing use of biological therapy targeting inflammatory cytokines, TNFα.

Growth failure in JIA

Poor growth is more common in children with polyarticular and systemic JIA (especially those with positive rheumatoid factor) compared with those with oligoarthritis (Liem and Rosenberg, 2003; Padeh *et al.*, 2011; Saha *et al.*, 1999; Simon *et al.*, 2002). In a study of 28 patients with JIA, significant reduction in height SDS by >2 standard deviations (SD) during the first 4 years of disease was observed despite normal height at diagnosis (**Fig. 4**) (Simon *et al.*, 2002). Growth failure is associated with glucocorticoid treatment in some studies (Haugen *et al.*, 1992; Zak *et al.*, 1999), although no association was found in others (Polito *et al.*, 1997; Wang *et al.*, 2002). Linear growth may also be associated with the inflammatory cytokine, IL-6, independent of glucocorticoid treatment (Souza *et al.*, 2008).

The use of modern therapies of JIA like methotrexate (Chedeville *et al.*, 2005) and biological therapy (Giannini *et al.*, 2010) has been shown to improve linear growth. However, in a large national study of almost 200 children with JIA treated with anti-TNF therapy (etanercept) for 2 years, height SDS increased significantly but only by approximately +0.2 SD (Kearsley-Fleet *et al.*, 2015).

Pubertal disorders in JIA

Delayed pubertal onset and reduced pubertal height velocity may further worsen growth failure in adolescents with JIA. Pubertal onset may be delayed by about 0.4–2.2 years compared with healthy adolescents (Aggarwal *et al.*, 2011b; Maher and Ali, 2013). Pubertal progression may be compromised, and pubertal growth spurt may be attenuated. Those with systemic JIA appear to be the most affected, with one study showing that height velocity during adolescence to be only 0.5 cm/year in the systemic group compared with 1.5 cm/year in the oligoarticular/polyarticular JIA (Aggarwal *et al.*, 2011a).

Adult height in JIA

Current published studies from over a decade ago report variable reductions in adult height, ranging from −0.3 to −2.0 SD (Gare and Fasth, 1995; Minden and Niewerth, 2008; Minden *et al.*, 2002; Packham and Hall, 2002; Simon *et al.*, 2002; Wang *et al.*, 2002;

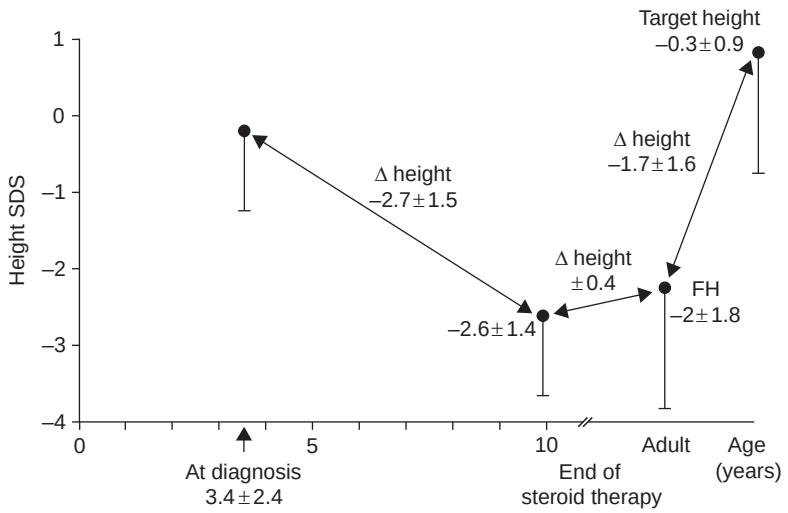

Fig. 4 Growth and adult height in children with systemic JIA. Reproduced from Simon, D., *et al.* (2002). Treatment of growth failure in juvenile chronic arthritis. *Hormone Research* **58** (Suppl. 1), 28–32, with permission © Karger.

Zak *et al.*, 1999). Different subtypes of JIA were included in these reports, which can explain the variation in adult height reported. Whereas approximately 12% of children with oligoarticular JIA had >1 standard deviation (SD) reduction in height at adulthood compared with diagnosis, 87% of patients with systemic JIA achieved adult height below their midparental height (Padeh *et al.*, 2011; Simon *et al.*, 2002). Studies with longer period of GC use resulted in significant larger loss of adult height (Simon *et al.*, 2002). Glucocorticoid-sparing therapies such as immunomodulators and biological therapy have only been part of routine management in the last 10–15 years. Currently, there are no studies of adult height in contemporary cohorts of children with JIA in the biological era, and such a study is needed.

Inflammatory Bowel Disease

IBD is a group of chronic inflammatory condition of the gastrointestinal tract, often relapsing and remitting in nature. The two principal types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). While inflammation is usually limited to the mucosa of the large intestine in UC, inflammation in CD can be transmural and may involve any part of the gastrointestinal tract from the oropharynx to the perianal area. Glucocorticoid is an effective treatment for acute relapse of both conditions, although exclusive enteral nutrition (EEN) can also be used to induce remission in mild to moderate CD. Background maintenance therapy with aminosalicylates or immunomodulators (azathioprine and methotrexate) aims to reduce inflammation and prevent future acute relapse. Although surgical resection is curative in UC and can lead to dramatic improvement in those with localized CD, those with severe and widespread disease or glucocorticoid-dependent CD often require escalation to biological therapy such as infliximab or adalimumab, used much more frequently in the last decade.

Growth failure in IBD

Growth failure is more frequent and severe in children with CD than those with UC (Abraham *et al.*, 2012; Malik *et al.*, 2012b). For example, mean height SDS of children with CD of both sexes at diagnosis was −0.3 compared with −0.1 and +0.22 for boys and girls with UC, respectively (Newby *et al.*, 2008). At diagnosis, approximately 10% of children with CD had height SDS below −2.0 (Malik *et al.*, 2012b; Vasseur *et al.*, 2010). Furthermore, deteriorating height may occur in the absence of gastrointestinal symptoms prior to diagnosis of CD (Kanof *et al.*, 1988).

Compared with the use of glucocorticoid, the use of EEN during acute relapse has been shown to lead to better growth with concurrent improvement in the GH/IGF-1 axis independent of nutritional restitution (Bannerjee *et al.*, 2004). Despite modern glucocorticoid-sparing therapies, catch-up growth may still be inadequate. A contemporary study of children with CD managed mostly without oral glucocorticoid treatment showed that height SDS remained unchanged from diagnosis to 1 and 2 years follow-up at approximately −0.5 SDS (Malik *et al.*, 2012b). In adolescents with IBD, height velocity SDS adjusted for pubertal age is also reduced (Mason *et al.*, 2011a).

While the use of biological therapy can be successfully used for induction and maintenance of remission in moderate-to-severe CD associated with mucosal healing, studies of the effects of infliximab and adalimumab on growth in CD have yielded mixed results. Some studies showed significant improvement in growth rate (Church *et al.*, 2014; Malik *et al.*, 2011, 2012a), while some demonstrated no improvement (Sinitsky *et al.*, 2010; Wewer *et al.*, 2006). Improvement of growth following biological therapy can occur, independent of discontinuation of glucocorticoid therapy or progression of puberty (Malik *et al.*, 2011, 2012a). The persistence of poor growth in some of these children may be reflective of the fact that some are poor responders to modern therapies.

Pubertal disorders in IBD

Pubertal delay was a common occurrence in older cohorts of adolescents with CD. Data from the mid-1990s showed that the onset of breast development was delayed by 1.5 years in 75% of girls with CD (Brain and Savage, 1994; Ferguson and Sedgwick, 1994). Testicular enlargement was delayed by almost a year in CD and UC (Brain and Savage, 1994). A contemporary retrospective study found persisting pubertal delay in adolescents with CD, demonstrated by delay in age of peak height velocity (Mason *et al.*, 2011a). However, a more recent prospective study including clinical evaluation of puberty by a pediatric endocrinologist demonstrated that pubertal delay was not common but poor pubertal growth spurt was observed especially in adolescent males (Mason *et al.*, 2015).

Adult height in IBD

Only a modest reduction in adult height has been reported by most studies of adults with childhood-onset IBD. Most studies of adults with childhood-onset CD reported adult height of between −1.0 and +0.4 SD (Wong *et al.*, 2016). However, a subset will still fail to achieve their target height. A contemporary cohort of 123 CD individuals showed that 20% achieved adult height of ≥8 cm below midparental height and that the presence of jejunal disease was associated with lower adult height (Sawczenko *et al.*, 2006). There is a limited window of opportunity to improve growth in adolescents with chronic conditions prior to epiphyseal growth plate fusion upon completion of puberty. Given that the average age of presentation of CD is between 11 and 12 years, a more aggressive approach may be required in those with growth issues at diagnosis to optimize adult height.

Growth in Conditions Requiring Prolonged Systemic Glucocorticoid

Duchenne Muscular Dystrophy

DMD is an X-linked, life-limiting disease leading to progressive muscle weakness and eventual loss of ambulation by approximately 11–12 years (Mendell *et al.*, 2012). The dystrophin protein is reduced or lost in DMD causing increased muscle cell fragility, leading to a vicious cycle of degeneration, repair, and associated inflammatory changes, cumulating in replacement of muscle fibers with fat and fibrosis (Bushby *et al.*, 2010a). The only intervention proved to stabilize muscle strength is long-term glucocorticoid treatment, typically commenced from about 5 years of age. Previously, glucocorticoid is discontinued after loss of ambulation, but in the last 5–10 years, glucocorticoid is continued even after ambulation is lost for its protective effects on upper limb function and respiratory and cardiac function (Bushby *et al.*, 2010b). There are four main glucocorticoid regimen used in DMD: daily deflazacort, pulsed (10 days on and 10 days off) deflazacort, daily prednisolone, and pulsed prednisolone.

Growth failure in DMD

There is no doubt that prolonged glucocorticoid use in DMD is associated with profound growth failure. However, there is evidence that short stature and poor growth in DMD may already be present prior to commencement of glucocorticoid. Boys with DMD have slower than expected growth rate during the first few years of life and typically track along the lower percentiles during childhood and adolescence (Eiholzer *et al.*, 1988). Several factors may explain this including elevation of pro-inflammatory cytokines (De Paepe and De Bleecker, 2013) and increased metabolic requirements from extreme muscle necrosis and regeneration (Rapisarda *et al.*, 1995). The lack of physical activity and poor bone turnover may contribute to understimulation of bone growth. Genetic factors may play a role as boys with a distal deletion are more likely to be short (Sarrazin *et al.*, 2014). The short stature homeobox (SHOX) gene, located near Xp22, may be deleted as part of a contiguous gene syndrome in a minority of patients (Messina *et al.*, 2008).

Following glucocorticoid therapy in DMD, further deterioration of growth rate is observed. The Muscular Dystrophy Surveillance, Tracking, and Research Network analyzed data from 324 ambulatory boys with DMD aged 2–12 years and found that daily prednisolone use was associated with a height loss of 1.8 cm/year of prednisolone treatment and 0.6 cm/g of prednisolone use (Lamb *et al.*, 2016) (**Fig. 5**). Short stature was present in 72% of adolescent boys treated with daily deflazacort after a mean of 8.5 years (Wong *et al.*, 2017). At 15 years of age, boys treated with deflazacort were 21 cm shorter on average than untreated boys (Biggar *et al.*, 2006). Short stature is also more common in boys treated with daily glucocorticoid (Ricotti *et al.*, 2013), although the impact of the four common glucocorticoid regimens on growth is still unclear.

Pubertal disorders in DMD

While there is still little information on puberty in boys with DMD treated with prolonged periods of glucocorticoid, delayed puberty is likely to be very common if not universal. All but one of the 44 boys with DMD aged 13 years or above in one center

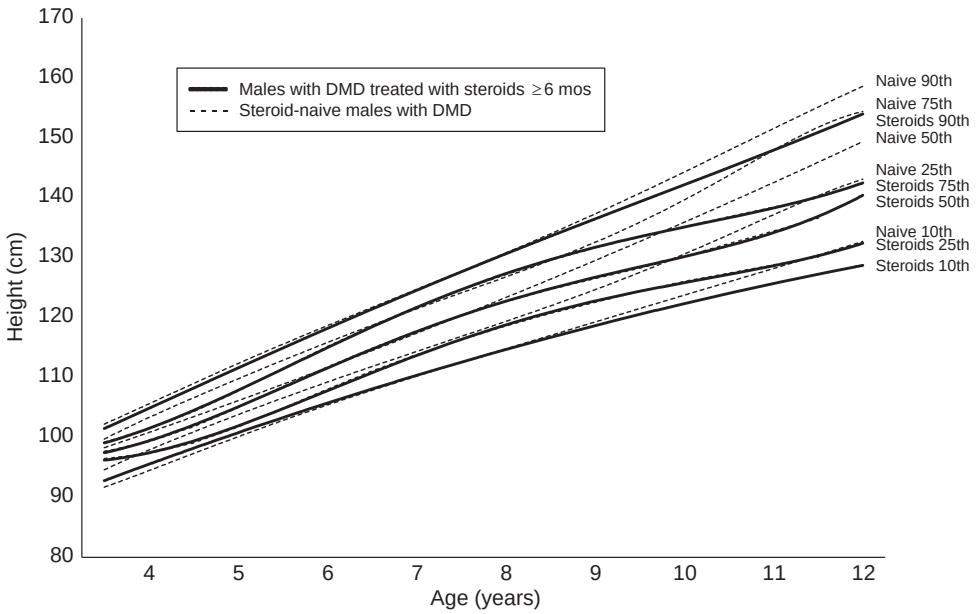

Fig. 5 Height trajectory in glucocorticoid-treated ambulatory boys with DMD in comparison with glucocorticoid naive boys. Reproduced from Lamb, M. M., *et al.* (2016). Corticosteroid treatment and growth patterns in ambulatory males with Duchenne Muscular Dystrophy. *Journal of Pediatrics* 173, 207–213, with permission © Elsevier.

treated with either deflazacort or prednisolone was prepubertal (Bonifati *et al.*, 2000). Boys with DMD who are not receiving glucocorticoid or are on low-dose pulsed therapy may show signs of puberty, although it is currently unclear if progression through puberty will happen normally. Suppression of the hypothalamic–pituitary–gonadal axis secondary to chronic high-dose glucocorticoid treatment is the most likely mechanism, although glucocorticoid may also impair androgen production at the level of the testes. It is possible that some DMD boys may have a contiguous gene deletion, which may be associated with hypogonadotrophic hypogonadism. The identification of low testosterone levels and small testes in young adults with DMD on glucocorticoid suggests that these individuals have persistent hypogonadotrophic hypogonadism, necessitating lifelong treatment with hormone replacement therapy (Wood *et al.*, 2015).

Adult height in DMD

Survival rates have improved significantly over the last few decades, with an improvement from 23% to 60% of boys with DMD reaching 20 years of age (Passamano *et al.*, 2012). There are currently no adult studies regarding adult height in boys with DMD. In an older study where prolonged glucocorticoid may not be used as frequently, most boys with DMD fell below the fifth percentile for height by 18 years of age (Bonifati *et al.*, 2000).

Management of Growth and Pubertal Disorders in Chronic Conditions

To effectively manage growth problems in these children and adolescents, it is important that height and weight are monitored in the clinic regularly. Obtaining height in nonambulatory children with chronic condition like DMD is challenging but important especially during monitoring of growth-promoting/sex steroid therapy. Alternatives include obtaining recumbent height or measuring ulnar length for estimation of height, although there are also challenges with these alternative methods of measurements. Height velocity in adolescents with chronic conditions must be interpreted in the context of pubertal staging. The use of pubertal self-assessment may be useful and may provide guidance for further endocrine evaluation, where clinical examination of puberty should be undertaken (Rollof and Elfving, 2012). However, in some adolescents, such as boys with DMD, testicular examination is mandatory, and this may require referral to the pediatric endocrinologists for clinical examination from 13 years of age. Other endocrine consequences in children with chronic conditions that may require consideration in the clinic include assessment of bone health, abnormalities of glucose homeostasis, and secondary adrenal insufficiency (especially those treated with prolonged glucocorticoid).

The first line management of growth issues in children in chronic inflammation involves optimization of the underlying condition, using the lowest possible dose of glucocorticoid and improving nutrition. In those where the underlying disease is managed and possible or those with limited window of opportunity for growth (i.e., already in puberty and progressing) but still growing slowly, endocrine growth-promoting therapies maybe considered. Pubertal induction should be considered for adolescents with delayed puberty. The use of recombinant human growth hormone (rhGH) in chronic conditions requires very careful consideration. There are still only a limited number of studies regarding its efficacy on growth in the chronic conditions, discussed in this article. A proposed pathway for growth monitoring and management in chronic condition is summarized in **Fig. 6**.

Pubertal Induction in Chronic Conditions

Treatment with sex steroids should be considered in adolescents with delayed puberty (i.e., breast stage 1 in girls $\geq$13 years and testicular volume $<$4 ml in boys $\geq$14 years) and growing slowly. Often, a short period of treatment (e.g., 3–6 months) will be sufficient, leading to improvement in virilization and increase in height velocity. This short period of treatment is often sufficient to prime the hypothalamus leading to appropriate progression through puberty. However, it is crucial that intervention with sex steroid therapy is introduced at an opportune time when disease is relatively well controlled. This may not be possible for some conditions, especially those with unremitting underlying causes (e.g., DMD). In those adolescents, a longer period of sex steroid treatment or maintenance of treatment until completion of pubertal should be considered. In boys with DMD where pubertal progression is unlikely and pathological fractures are very common, it is possible that an earlier age of intervention with sex steroid may be considered but this requires further research. Consideration of the use of transdermal estrogen should be given in girls with IBD given the issues with absorption.

Although pubertal induction with sex steroid is often recommended by pediatric endocrinologists for these adolescents, there is a paucity of studies examining the efficacy of sex steroid in chronic conditions. A study from the 1980s in five boys with CF and delayed puberty treated with 3 months of injectable testosterone showed that height velocity increased from 2.2 to 7.2 cm/year at 12 months (Landon and Rosenfeld, 1984). Another study of eight boys with IBD and delayed puberty treated with injectable and transdermal testosterone showed that only six boys increased their height velocity by $>$50% at 6-month follow-up despite adequate virilization in all (**Fig. 7**) (Mason *et al.*, 2011b). The growth response to pubertal induction is variable and may be related to disease activity (Mason *et al.*, 2011b). In boys with DMD, a retrospective study of 14 boys showed that neither growth nor pubertal response was optimal after testosterone treatment for a mean for 3 years (Wood *et al.*, 2015). This may be due to the longer insult to the hypothalamic–pituitary–gonadal axis in DMD compared with other conditions.

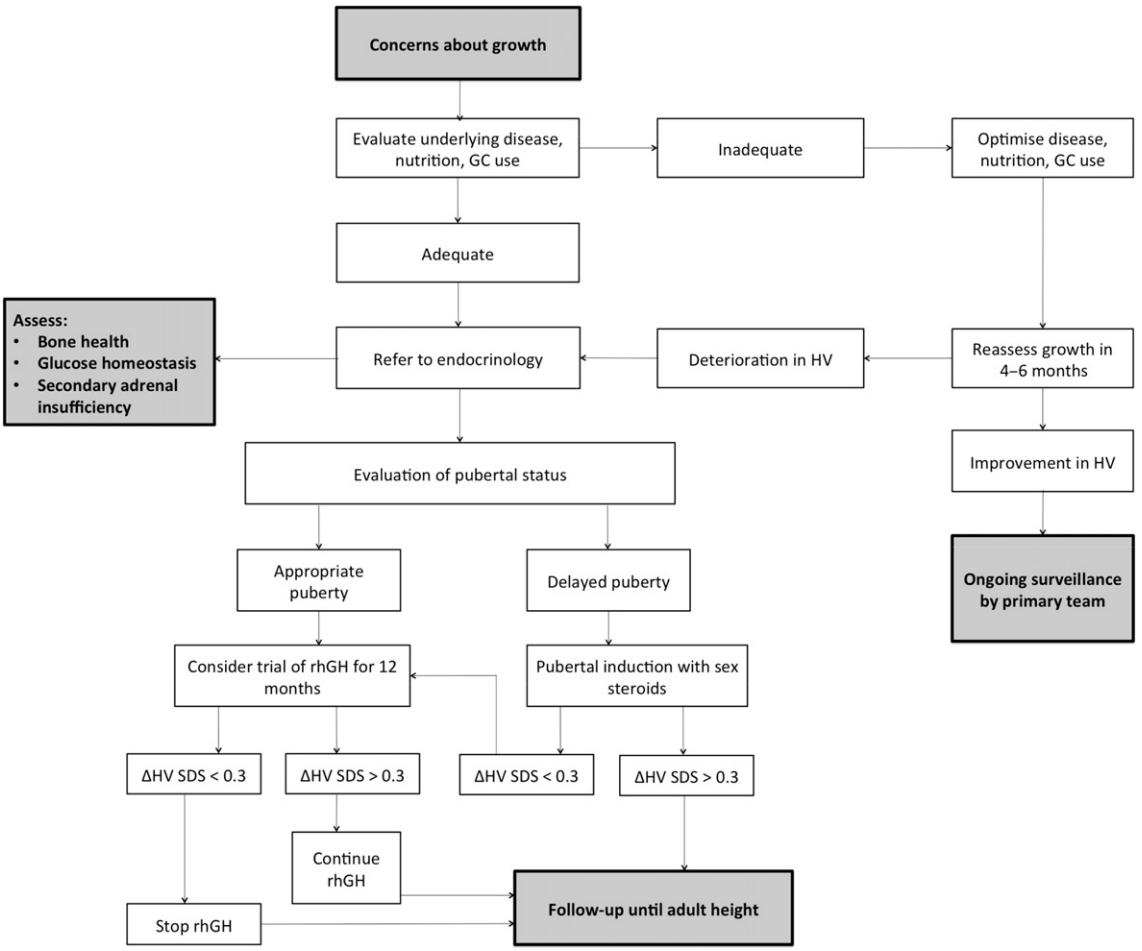

Fig. 6 Suggested pathway for monitoring and management of growth in chronic conditions.

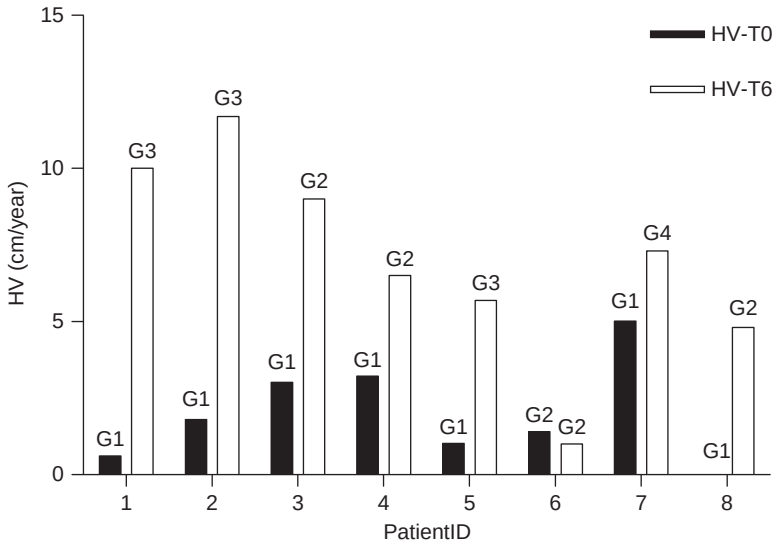

Fig. 7 Pubertal induction in adolescent boys with IBD. Figure produced from data from Mason, A., *et al.* (2011). Effect of testosterone therapy for delayed growth and puberty in boys with inflammatory bowel disease. *Hormone Research in Paediatrics* 75, 8–13.

Growth Hormone Therapy in Chronic Conditions

The availability of rhGH has led to its use in non-GH-deficient conditions (e.g., Turner's syndrome, Prader–Willi syndrome, and chronic kidney disease). rhGH at a higher dose may be able to overcome the relative GH insensitivity state seen in childhood chronic conditions (Hodson *et al.*, 2012). Most of the studies show an improvement of growth rate over the short to medium term but not normalization of height. A comprehensive review of the published studies of rhGH use in chronic conditions is summarized in a recent review (Wong *et al.*, 2016).

Six randomized trials of rhGH therapy with growth outcomes have been conducted in children with CF to date (Hardin *et al.*, 2001, 2005, 2006; Hutler *et al.*, 2002; Schnabel *et al.*, 2007; Stalvey *et al.*, 2012). Changes in height SDS in the rhGH treatment group over 12 months ranged from +0.2 to +0.6 SD, and height velocity is approximately 150% higher in the treatment group compared with placebo. However, exclusion criteria of these studies include those with abnormalities of glucose homeostasis or established CFRD and those who are colonized with *Burkholderia Cepacia*. These subgroups are usually more severely affected, may be less responsive to rhGH therapy, and are often the adolescents referred to be reviewed in the endocrine clinic.

In children with JIA, six randomized trials have been conducted demonstrating an improvement in growth (Bechtold *et al.*, 2001, 2003, 2007; Grote *et al.*, 2006; Saha *et al.*, 2004; Simon *et al.*, 2007). Only one trial using rhGH 0.33 mg/kg/week of rhGH included data on adult height. This study found that rhGH treatment over a mean duration of 8.4 years led to a net gain in height of +2.3 SD while the control group lost 0.7 SD from baseline to adult height (Bechtold *et al.*, 2007). However, mean adult height SDS was −1.6 SDS, suggesting incomplete catch-up growth. Another study suggested that early introduction of rhGH in JIA before the onset of severe growth failure may normalize growth rate; however, this requires further evaluation (Simon *et al.*, 2007). While there are insufficient data to support the use of rhGH before any evidence of growth failure, it is important to intervene before severe growth failure is present, especially those with limited growth potential.

In comparison with JIA, there is a paucity of data on rhGH trials in children with IBD. The only randomized trial in IBD conducted to date using rhGH at 0.45 mg/kg/week for improving linear growth found that height velocity increased by a median of 140% in the rhGH group compared with 8% reduction in the control group at 6 months, equivalent to a relative gain in height of +0.4 SD (**Fig. 8**) (Wong *et al.*, 2011). Another randomized trial of rhGH at 0.53 mg/kg/week in children with CD, designed to evaluate the role of rhGH in improving disease process, showed that height velocity improved by 60% in the treatment group at 12 weeks (Denson *et al.*, 2010).

There is currently only one published nonrandomized study of rhGH (0.3 mg/kg/week) in 39 DMD treated for a duration of 12 months (Rutter *et al.*, 2012). Following rhGH therapy, height velocity increased from 1.5 cm/year to 5.2 cm/year with concurrent improvement in lean body mass without deterioration of muscle or cardiopulmonary function. However, as severe short stature is common in these boys, with baseline mean height SDS of almost 3 SD below the mean in that study, the clinical significance of this to the growing child is unknown.

The use of relatively high-dose rhGH in chronic conditions, especially those concurrently treated with glucocorticoid, is associated with reduction in insulin sensitivity. Current short-term trials of rhGH in CF have not shown any adverse effects on glucose homeostasis, but as mentioned, current trials have excluded children with established CFRD or impaired glucose tolerance.

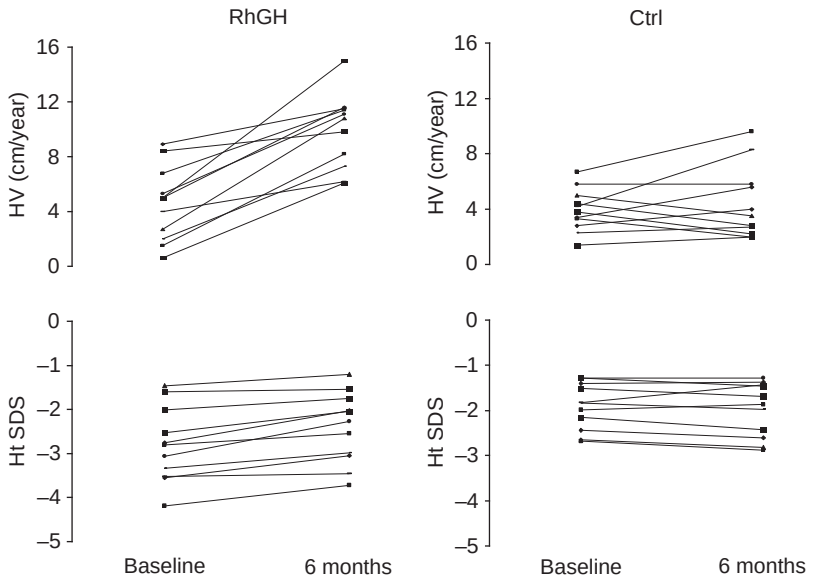

Fig. 8 Recombinant human growth hormone in children with IBD: Results from a preliminary randomized controlled trial. Reproduced from Wong, S. C., *et al.* (2011). A preliminary trial of the effect of recombinant human growth hormone on short-term linear growth and glucose homeostasis in children with Crohn's disease. *Clinical Endocrinology (Oxford)* 74, 599–607 (351), with permission © Wiley.

In rhGH trials of children with JIA treated with several years of glucocorticoid, almost 40% developed impaired glucose tolerance, and 5% developed type 2 diabetes (Bismuth *et al.*, 2010). The use of rhGH in IBD where the majority of the subjects were not treated with glucocorticoid led to a significant increase in fasting insulin levels but no abnormalities of glucose homeostasis (Wong *et al.*, 2011). Similarly, 5% of the subjects in the study of rhGH in DMD developed impaired glucose tolerance (Rutter *et al.*, 2012).

Given the limited evidence of rhGH in chronic conditions, its use should ideally be in the context of well-designed clinical trials. In exceptional circumstances, where clinicians may be able to prescribe rhGH under nonlicensed indication, careful discussion with the family needs to be conducted. A trial period of rhGH for 12 months can be considered in younger prepubertal children who are growing slowly or those who continue to grow slowly despite being in puberty. The decision to continue rhGH therapy beyond 12 months should be discussed with the family prior to the start of rhGH. Expert recommendations suggest that an increase of height SDS of greater than +0.3 SD with rhGH therapy in states of relative GH insensitivity may be acceptable (Bang *et al.*, 2012).

Insulin-Like Growth Factor Therapy in Chronic Inflammation

Given the state of functional GH insensitivity in chronic inflammation, there is biological rationale to consider the use of rhIGF-1 to promote growth in this setting. rhIGF-1 therapy in children with GH insensitivity due to GH receptor mutation leads to improvement in growth rate (Bright, 2016). Its use has also been investigated in children with idiopathic short stature (Midyett *et al.*, 2010). The only study of its efficacy in childhood chronic inflammation is in a crossover trial of seven children with CF using rhIGF-1 (80 μg/kg twice daily) compared with placebo (Bucuvalas *et al.*, 2001). This study failed to show an effect on linear growth despite normalization of serum IGF-1, but there was improvement in insulin sensitivity. A pharmacokinetic study of rhIGF-1 in eight children with CD using mathematical modeling allows appropriate dosing to ensure that systemic IGF-1 levels remain below +2.5 SD (Rao *et al.*, 2013). However, the effect of rhIGF-1 on linear growth in CD is currently unknown.

The potential adverse effect of hypoglycemia may preclude the use of a higher rhIGF-1 dose, observed in 14% of children with idiopathic short stature treated with 120 μg/kg twice daily of rhIGF-1 (Midyett *et al.*, 2010). There are also concerns that rhIGF-1 may accelerate skeletal maturation, which maybe disadvantageous for adult height prognosis (Bright, 2016). In contrast to the possibility of normalization of height with the use of rhGH in growth hormone deficiency, the use of rhIGF-1 in GH insensitivity syndrome fails to lead to complete catch-up growth with long-term follow-up (Fintini *et al.*, 2009). The possibility of combination therapy using rhGH and rhIGF-1 in these chronic conditions may be more physiological and deserves further exploration.

Conclusion

It is clear from clinical outcome studies that growth and pubertal development are affected in children with chronic inflammation despite contemporary treatment regimens. While pubertal delay may not be as common, timely induction of puberty is important. Although preliminary evidence suggests that rhGH treatment is effective for improving short-term linear growth, catch-up growth remains incomplete. Long-term treatment studies are required to assess adult height outcomes and other extended health benefits of promoting growth or manipulation of the GH/IGF-1 axis.

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Normal Puberty: Somatic Characteristics

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Abbreviation

BMI Body mass index
DHEA Dehydroepiandrosterone
DHEAS Dehydroepiandrosterone sulphate
FSH Follicle stimulating hormone
GnRH Gonadotrophin release hormone
GnRHa Gonadotrophin release hormone agonist
HPG Hypothalamic–pituitary–gonadal
HPV Human papilloma virus
LH Luteinizing hormone
MIH Mullerian inhibiting hormone

Glossary

Gonadarche Is the activation of the gonads by the pituitary hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH).
Gynaecomastia Is the enlargement of male breast tissue.
Menarche Is the time of first menstrual bleed. The first menstrual bleed is often not associated with ovulation; it typically is caused solely by the effects of estradiol on the endometrial lining.
Secondary sex characteristics Is any physical characteristic developing at puberty which distinguishes between the sexes but is not directly involved in reproduction, for example, development of breasts or beard, muscularity, distribution of fat tissue, and change of pitch in voice.
Spermarche Is the time of the first sperm production (heralded by nocturnal sperm emissions and appearance of sperm in the urine), which is due to the effects of FSH and LH, via testosterone.
Thelarche Is the appearance of breast tissue, which is primarily due to the action of estradiol from the ovaries.

Introduction

Physiology of Puberty

Puberty is a period of transition in the stage of development which is characterized by the occurrence of secondary sexual characteristics. During that transition period, there is a whole array of complex hormonal signals that bring about the changes from sexually immature to the sexually mature stage (**Table 1**). The general concept involved in the initiation of puberty was established in the mid-1960s (Donovan and Ten Bosch, 1965). Puberty is a complex, coordinated biological process with multiple levels of regulation. The timing of pubertal events is a heritable trait, although environmental factors can modulate such genetic influence (Banerjee and Clayton, 2007). At the start of puberty, through a complex neuro-signaling pathway, a hypothalamic neuronal peptide, kisspeptin, is secreted which in turn stimulates the release of gonadotropin release hormone (GnRH) from the GnRH neurons of the hypothalamus (Banerjee and Clayton, 2007). The GnRH is secreted into the blood vessels and subsequently stimulate the gonadotroph cells of the anterior pituitary gland to secrete the gonadotrophins, follicle stimulating hormone (FSH) and the luteinizing hormone (LH). The gonadotropins stimulate sex-steroidogenesis with the resultant production of testosterone and oestradiol from the testes and ovaries respectively. These in turn are responsible for the development of the secondary sex characteristics of puberty.

The Timing of Puberty

The age of normal pubertal onset is different for the boys and girls. These variations are due to genetic factors and a number of external factors such as adequate nutrition, the presence of chronic diseases and the level of physical exercise (Banerjee and Clayton, 2007, Gajdos *et al.*, 2010, Kaminski and Palmert, 2008). Over the last 100 years, there has been marked transgenerational decrease in the age of onset of puberty in both boys and girls due to improved condition of childhood nutrition world-wide (Zacharin, 2013). The hypothalamic–pituitary–gonadal (HPG) axis is active in early infancy. However, there is resistance to sex steroid action at that age and therefore in normal infants, there is no development of secondary sex characteristics. From 6 to 12 months of age, the HPG axis enters a period of quiescence that remains until the adolescent stage (Kurtoglu and Bastug, 2014).

Any time after age 8 years in girls and 9 years in boys, the HPG axis becomes reactivated to bring about normal pubertal development. The exact mechanism for the trigger of normal pubertal onset is has not been elucidated.

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Table 1 A summary of Tanner stages of development and concomitant changes in boys and girls

Staging	Breast	Pubic hair staging	Concomitant changes	
Girls				
1	Prepubertal, papilla elevation	No pigmented hair		
2	Budding; larger areole; palpable and visible elevated contour	Pigmented hair, mainly labial	Accelerating growth rate	
3	Enlargement of the breast and areola	Coarser, spread of pigmented hair over mons	Peak growth rate, thicker vaginal mucosa, axillary hair	
4	Secondary mound of areola and papilla	Adult type but smaller area	Menarche (stage 3 or 4) decelerating growth rate	
5	Mature	Adult distribution		
Staging	Genital size	Pubic hair staging	Concomitant changes	Prader orchidometer (mL)
Boys				
1	Prepubertal	No pigmented hair	Long testis axis <1.5 cm	1–3
2	Early testicular, penile and scrotal growth	Minimal pigmented hair at base of penis	Early voice changes; testes length 2.5–3.3 cm	3–6
3	Increased penile length and width; scrotal and testes growth	Dark, coarse, curly hair extends midline above penis	Light hair on upper lip, acne, maximal growth, testes length 3.3–4.0 cm	8–12
4	Increased penis size including breadth; pigmented scrotum	Considerable, but less than adult distribution	Early sideburns; testes 4.0–4.5 cm	>12
5	Adult size and shape	Adult distribution, spread to medial thighs or beyond	Beard growth; testes >4.5 cm	>15

Somatic Characteristics of Normal Puberty in Clinical Setting

Normal Pubertal Changes for Girls

Pubertal onset in girls tend to occur at the age of 8–13 years (Zacharin, 2013). The onset of puberty in girls is affected by a numbers of factors including the variations in the ethnicity, the general community health and the nutritional status. The data suggest African American girls enter puberty earlier and reach menarche earlier than Caucasian and Hispanic girls (Ramnitz and Lodish, 2013, Herman-Giddens *et al.*, 1997, Morrison *et al.*, 1994). There is also evidence of an association between higher BMI in childhood or adolescence and earlier onset of puberty in girls suggesting that improved nutrition and socioeconomic conditions have contributed to that trend (Lundeen *et al.*, 2016). Relatively poor nutritional status in some communities may delay the onset of puberty to the late teens. The somatic variations in the pattern of pubertal changes in girls were described in the late 1960s and early 1970 by Marshall and Tanner. They are commonly referred as the Tanner Staging or Sexual Maturity Ratings (SMR). These consist of systematized descriptions of the development of secondary sexual characteristics, consisting of breast changes and pubic hair changes. The Tanner Staging for pubic hair and breast consists of five stages, with stage 1 representing prepuberty and stage 5 representing adult development. The first sign of true puberty in a girl is the development of the breast buds which is followed by pubic hair growth and rapid height spurt (Marshall and Tanner, 1969). The onset and progress of pubic hair differs markedly between genetically different communities (Oberfield *et al.*, 2011). The progress of puberty takes place in an ordered fashion with menarche occurring 2–2.5 years following the onset of thelarche (Zacharin, 2013). Menarche signal the end of growth, with only 5 cm of height gain remaining.

Hormonal changes during puberty

The mean LH, FSH, and oestradiol levels initially rise before the somatic characteristics of puberty become visible. The levels rises further as puberty progresses, accompanied by increases of both inhibin A and inhibin B. An unstimulated or GnRH/GnRHa stimulated LH level and an oestradiol level above the pre-pubertal range confirms gonadarche (Lee and Houk, 2007). Estrogen stimulated changes include onset and progression of breast maturity, genital growth, maturation of the vaginal mucosa, uterine/endometrial growth, and body composition changes resulting in the female pattern fat distribution (Lee and Houk, 2007). Breast growth, which may begin asymmetrically, progresses throughout puberty and is classified into one of five Tanner stages. Pubic hair is also Tanner staged (see below).

The pubertal breast changes in girls

The pubertal Tanner breast stages are as follows:

Stage 1 Pre-pubertal; elevation of papilla only with no palpable breast tissue.
Stage 2 Palpable breast bud with elevation of the papilla and enlargement of areola diameter.
Stage 3 Enlargement of breast, areola and the papilla forming a cone with no separation of their contours.
Stage 4 Projection of areola and papilla to form a secondary mound above the level of the breast.
Stage 5 Recession of the areola to the general contour of the breast and projection of the papilla only (**Fig. 1**).

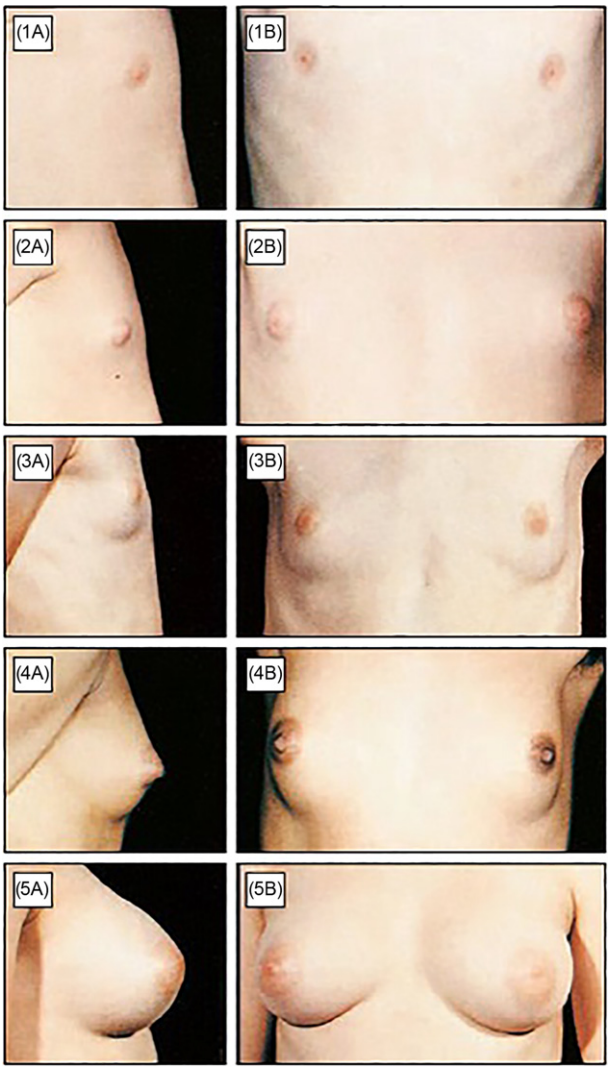

Fig. 1 Tanner staging of breast development in girls.

The pubertal pubic hair changes in girls

The pubertal Tanner pubic hair stages are as follows:

Stage 1 Prepubertal with no development of the no pubic hair.
Stage 2 Sparse growth of long, lightly pigmented, downy hair appearing chiefly along the labia majora.
Stage 3 The hair is considerably darker, coarser, and more curled, extending over the junction of the mid pubis.
Stage 4 Hair is adult-like in appearance and fills up the genital inverse triangle with no spread to the medial surface of the thighs.
Stage 5 Adult in quantity and type, it spread to the medial surface of the thighs (**Fig. 2**).

The growth spurt in puberty and bone maturation

The contribution of pubertal growth to the final height is approximately 27.5–29 cm in girls, accounting for 17% of the final height (Abbassi, 1998). The timing of the growth spurt (peak height velocity) occur approximately 2 years earlier in girls than in boys. Before puberty, the legs grew more rapidly than the trunk and during puberty, the growth spurt is truncal (Bass *et al.*, 1999). The height velocity can be plotted and compared with the norms using the growth velocity height charts (**Fig. 3**). The disparity in mean adult height between men and women results from the timing and magnitude of the growth spurt in boys and girls (Biro *et al.*, 2006). In girls, peak height velocity occurs, on average, 0.5 years prior to menarche (Biro *et al.*, 2006). The boys experience pubertal growth spurt approximately 2 years later than girls and hence the boys have an additional 2 years of prepubertal growth (at a rate of 3–8 cm per year) compared with girls (Kelly *et al.*, 2014). Additionally, the boys experience a greater peak height velocity than do girls (10.3 versus 9.0 cm/year) (Kelly *et al.*, 2014). The growth spurt typically lasts for about 2 years in both sexes.

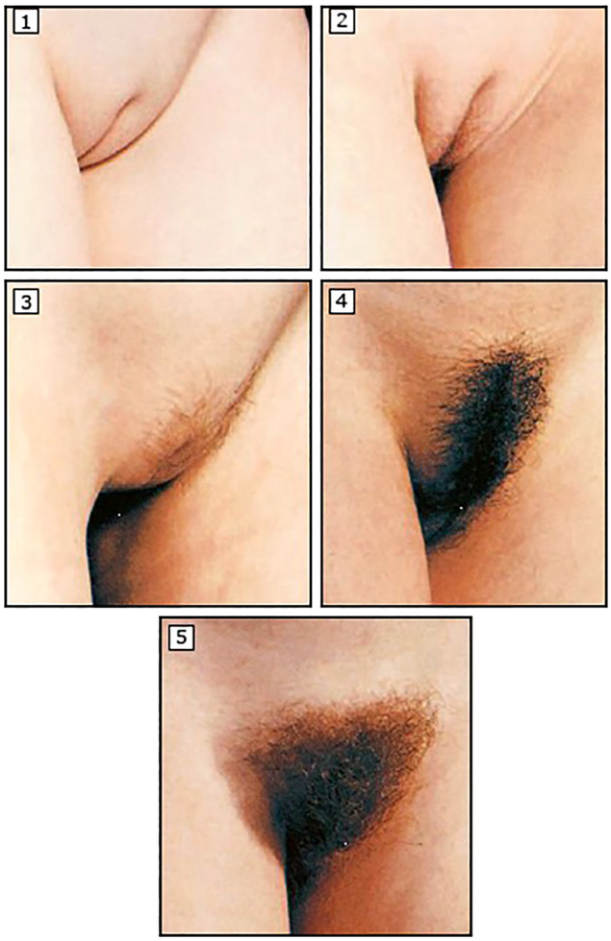

Fig. 2 Tanner staging of pubic hair development in girls.

Fig. 3 Prader orchidometre.

The bone growth accelerates during puberty, in concert with height velocity. Bone width and mineral content increase independently with age for each pubertal stage (Magarey *et al.*, 1999). Bone growth occurs first in length, followed by width, then mineral content and then bone density (Magarey *et al.*, 1999). The rate of bone mineral accrual peaks around the age of menarche in girls (McKay *et al.*, 1998, Taranger *et al.*, 1976). Approximately one-half of total body calcium is laid down during puberty in females, and one-half to two-thirds in males (Magarey *et al.*, 1999, Lloyd *et al.*, 1992).

Body shape and composition during puberty

Puberty is associated with changes in body weight and alterations in body composition of lean body mass and the proportion of body fat. There is an increase in the body mass index (BMI) during puberty. In early puberty, the annual increase in BMI is driven primarily by changes in lean body mass. Later, the increase in BMI tends to be driven by increases in fat mass. Unlike adults, annual increases in BMI during childhood are generally attributed to the lean rather than to the fat component of BMI (Maynard *et al.*, 2001). Girls tend to have a higher proportion of fat mass than boys at each phase, and after 16 years of age, the annual increase in BMI is largely because of increases in fat mass (Gasser *et al.*, 1993, Maynard *et al.*, 2001). Androgen stimulates pubic and axillary hair development, apocrine gland maturation resulting in adult-type body odor, and skin changes related to acne (Lee and Houk, 2007).

Normal Pubertal Changes for Boys

From the epidemiological data, the onset of puberty in boys occur at the age 9–14 years depending on the geographic location, nutrition and race. In a study involving over 4000 healthy boys, the mean age for entering puberty was 10.14 years for white boys, 10.04 years for Hispanic boys, and 9.14 years for African American boys (Herman-Giddens *et al.*, 2012). The average age at the initiation of puberty, assessed by age at the transition from Tanner Stage 1 to Tanner Stage 2 for genitalia or pubic hair development ranged between 9.8 and 10.5 years in South Africa (Jones *et al.*, 2009). These thresholds are 1.5–2 years earlier than historical norms. Similar trends have been reported from several countries around the world.

The first physical signs of puberty in boy is the testicular enlargement associated with increased folding of the scrotal rugae, followed by penile growth and the development of pubic hair. The testicular volume is often measured using the Prader orchidometer which is a series of three-dimensional ellipsoids with volumes ranging from 1 to 25 mL or more (**Fig. 3**). The testicular volume measuring more than 4 mL and a length greater 2.5 cm is pubertal. Testicular enlargement occur approximately 6 months prior to the appearance of penile growth and pubic hair. Puberty in boys is designated according to Tanner staging of genital development and pubic hair (**Fig. 4**). The Tanner staging does not include testicular volume but the correlation between the testicular volume and the stages of puberty have been established (Biro *et al.*, 1995, Dagli *et al.*, 2014) (**Fig. 5**).

Hormonal changes in puberty

As boys enter puberty, there is a gradual rise in the levels of LH and FSH. These consequently stimulate the leydig cells in the testes to produce testosterone which subsequently give rise to the development of male secondary sexual characteristics. That reflects an upregulation of the hypothalamic-pituitary-testicular axis (Andersson *et al.*, 1997). Other intermediate metabolites of adrenal and testicular origin also rise (oestrone, oestradiol, androstenedione, 17-hydroxyprogesterone) (Lee and Houk, 2007). An increase in the levels of adrenal hormones, dehydroepiandrosterone (DHEA) and DHEAS, indicate adrenarche. Inhibin B levels, a marker of Sertoli cell mass and function, rise progressively before the onset of puberty. Both testosterone and inhibin B appear to be related to the nocturnal release of LH seen in early puberty (Crofton *et al.*, 2004). Mullerian inhibiting hormone (MIH) levels in males rise rapidly during the first year of life, peak in late infancy and gradually decline to a nadir during puberty. Outside of the neonatal period, MIH levels are inversely related to testosterone.

The stages for the genitalia are as follows:

Stage 1 Prepubertal. Testes, scrotum, and penis are of about the same size and proportion as in early childhood. The testicular volume is less than 4 mL.

Stage 2 There is enlargement of the testes and scrotum; the scrotal skin changes in texture and reddens. The testicular volume at that pubertal stage ranges from 4 to 8 mL.

Stage 3 The penile growth occurred, mainly length first but with some increase in breadth. There is further growth of testes and scrotum. The testicular volume at that stage ranges from 9 to 12 mL.

Stage 4 There is further enlargement of the penis in length and breadth with development of glans. Testes and scrotum further enlarged. There is also further darkening of the scrotal skin. The testicular volume at that stage ranges from 13 to 15 mL.

Stage 5 Genitalia adult in size and shape. No further enlargement takes place after Stage 5 is reached. The testicular volume at that stage ranges is more than 16 mL.

The pubic hair stages are:

Stage 1 Prepubertal. The pubic area has no hair or it may have the vellus similar to those of the forearms.

Stage 2 Sparse growth of long, slightly pigmented, downy hair, straight or only slightly curled, appearing chiefly at the base of the penis.

Stage 3 Considerably darker, coarser, and more curled. The hair spreads sparsely over the junction of the pubes.

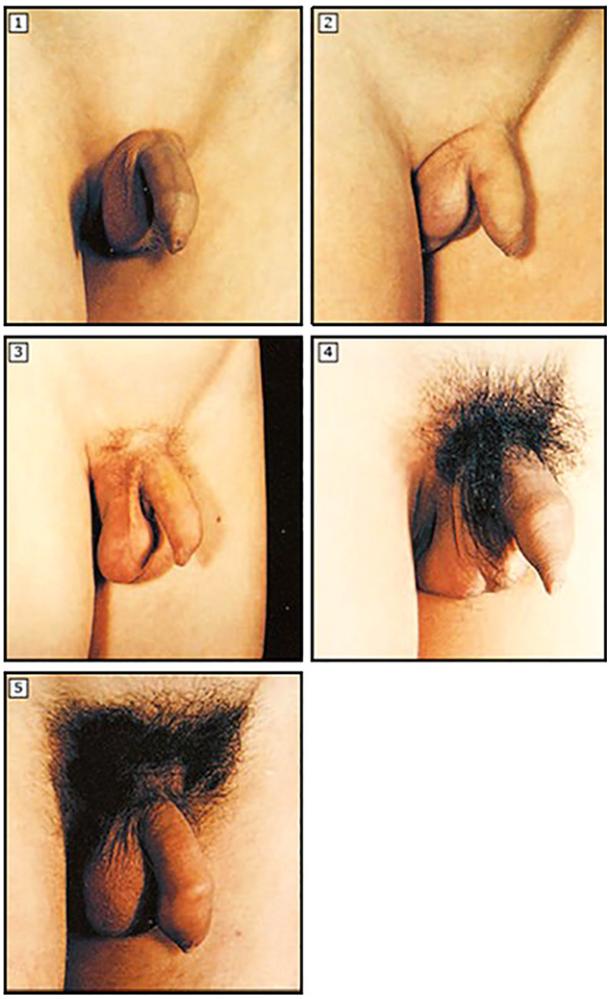

Fig. 4 Tanner staging of pubic hair and external genitalia development in boys.

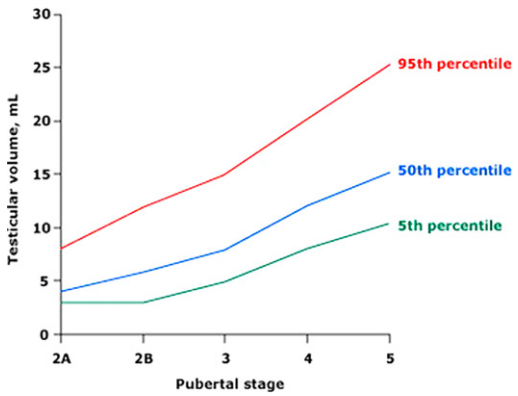

Fig. 5 Testicular volume in each pubertal stage.

Stage 4 Hair is now adult in type, but the area covered by it is still considerably smaller than in most adults. There is no spread to the medial surface of the thighs.

Stage 5 Adult in quantity and type, distributed as an inverse triangle. Spread to the medial surface of the thighs and above the base of the inverse triangle.

The growth spurt in puberty and bone maturation

The changes in boys occur at mid puberty following prolonged exposure to testosterone. These include the peak growth velocity, cracking of the voice, acne, and the onset of axillary hair. There is also a gradual increase in total bone mineral content, lean body mass and a relative decline in body fat. These changes in body composition begins with the onset of puberty, but by comparison to the girls, a significant composition of the pubertal changes in boys do not occur until mid-puberty. Oestradiol is the main hormone which stimulate the skeletal maturation in both sexes. However, the oestradiol levels attained during mid-puberty in boys is similar to the levels in early pubertal girls. The growth velocity peaks in early puberty in females and it differs based on race. The disparity in mean adult height between male and females results from the timing and magnitude of the growth spurt in boys and girls. Spemarche, the onset of sperm production and the average male reaches spermarche at the age of 14 years or Tanner stage 3 for genital and pubic hair.

Other Somatic Characteristics of Normal Puberty

The transition from childhood to adulthood in puberty is associated with a number of somatic complications that present challenges to the patient, family and the society. These complications may include the development of acne, anemia, gynecomastia, dysfunctional uterine bleeding, myopia and scoliosis.

Acne—Acne is the inflammation of the pilosebaceous glands due to androgenic stimulation. During puberty, the androgen levels rise in both boys and girls. This rise in the androgen levels causes the number of acne lesions to develop (Lucky *et al.*, 1991). In girls, the severity of acne in later puberty is associated with higher serum levels of dehydroepiandrosterone sulphate (DHEAS) and a greater number of acne lesions in early puberty (Lucky *et al.*, 1997). Acne is common during puberty and it is part of normal somatic progression.

Anemia—Iron deficiency anemia is more common among adolescent girls as compared with adolescent boys due to menstrual bleeding and insufficient iron intake (Bergstrom *et al.*, 1995, Daniel, 1973). The levels of hemoglobin and ferritin increases with increasing pubertal stages in boys but not in girls. The testosterone increases erythropoiesis in boys, thus they are less prone to anemia.

Gynaecomastia—Pubertal gynecomastia affects roughly 50% of teenage boys at an average age of 13 years, and it persists for 6 to 18 months (Biro *et al.*, 1990). The larger the gynaecomastia, the more it is likely to persist longer. It has been found that boys with palpable breast tissue greater 2 cm during the first year of puberty are more likely to have persistent gynecomastia (Biro and Chan, 2017). The cause of pubertal gynaecomastia may reflect the imbalance in the effective estrogenic-to-androgenic stimulation. An increased production of estrogens and a decreased production of androgens results in pubertal gynaecomastia.

Musculoskeletal injuries—The pubertal adolescents are susceptible to musculoskeletal injuries and fractures during sport. The pubertal status maybe use to predict the specific type of musculoskeletal injuries that adolescents may encounter during participation in sports (Backous *et al.*, 1990). There is a greater risk to the epiphyseal growth plates damage during the peak of height velocity, which also is the time of greatest change in bone mineral content (McKay *et al.*, 1998). The incidence of distal radius fractures peak correspond with the age of peak height velocity in both boys and girls (Bailey *et al.*, 1989). The asynchronous growth of body parts may result in a limited range of motion of some joints. In pubertal adolescents, Osgood-Schlatter disease, which is caused by the inflammation of the tibial tubercle due to over, is quite common.

Gynaecologic outcomes—After reaching menarche, a girls' reproductive axis matures rapidly. Once a girl reaches menarche, rapid maturation of the reproductive axis ensues. By 1 year after menarche, 65% of girls have regular menstrual cycles, with 10 or more periods per year (Legro *et al.*, 2000). Pubertal adolescents suffer from excessive, prolonged endometrial bleeding, mainly due to anovulation. This phenomenon is called dysfunctional uterine bleeding and it is due to unopposed estrogen stimulating the endometrium resulting in proliferative phase. Estrogen levels ultimately cannot sustain the hyperplastic endometrial lining, resulting in irregular, heavy, menstrual bleeding.

Myopia—There is a high incidence of myopia during puberty. This is mainly caused by the growth in the axial diameter of the eye (Tanner, 1962).

Scoliosis—As a result of the pubertal growth spurt and the associated growth in the axial skeleton, there is accelerated progression of the degree of scoliosis.

Changes in the female genital tract—In the first year or two after menarche, there is persistence of columnar epithelial cells on the exocervix as well as the transformation zone of columnar to squamous epithelial cells on the exocervix. These factors may enhance infection with Chlamydia and genital human papillomavirus (HPV) (Harrison *et al.*, 1985, Moscicki *et al.*, 1989, Shew *et al.*, 1994).

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Genetic and Epigenetic Control of Puberty

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Abbreviations

AVPV Anteroventral periventricular
CPP Central precocious puberty
CHH Congenital isolated hypogonadotropic hypogonadism
GnRH Gonadotropin-releasing hormone

Introduction

In humans, the hypothalamic–pituitary–gonadal axis is fully active in the late gestational and neonatal periods, entering a phase of quiescence during infancy. Puberty is initiated upon the reactivation of this axis, marked by an increase in pulsatile gonadotropin-releasing hormone (GnRH) release leading to pituitary secretion of LH and FSH (Abreu and Kaiser, 2016). Traditionally, the normal age range of pubertal onset is between 8 and 13 years in girls and between 9.5 years and 13.5 years in boys according to cross-sectional data obtained in the 1960s (Carel and Leger, 2008; Marshall and Tanner, 1969; Marshall and Tanner, 1970). Pubertal timing can be influenced by complex interactions among genetic and environmental factors, such as socioeconomic status, general health, nutrition, and endocrine disruptors. Recently, it has been proposed that epigenetics plays a role in the complex interplay between genetics and environment (Abreu and Kaiser, 2016; Carel and Leger, 2008). Notably, timing of puberty is associated with risks of subsequent disease at adult age. It has been demonstrated that earlier age of menarche in girls is associated with increased risks of breast cancer, endometrial cancer, obesity, type 2 diabetes, and cardiovascular disease (Lakshman *et al.*, 2008, 2009). On the other hand, pubertal delay is associated with osteoporosis in both sexes.

Compelling evidence of the influence of genetic factors on pubertal timing has been provided by epidemiological and genetic studies. The role of genetic factors is illustrated by the similar age at menarche in mothers and daughters and among members of an ethnic group and by a greater concordance of pubertal timing in monozygotic than in dizygotic twins (Fischbein, 1977; Palmert and Hirschhorn, 2003). In the past decade, several genes have been identified in the complex network of inhibitory, stimulatory, and permissive neuroendocrine factors involved in the control of puberty onset. Definitely, the identification of genes mutated in congenital isolated hypogonadotropic hypogonadism (CHH) and central precocious puberty (CPP) has facilitated and improved the current understanding of the neuroendocrine control of reproduction.

Genetic Basis of CPP

Early activation of the hypothalamic–pituitary–gonadal axis results in gonadotropin-dependent precocious puberty, also known as CPP, which is clinically defined by the development of secondary sexual characteristics before the age of 8 years in girls and 9 years in boys (Carel and Leger, 2008; Latronico *et al.*, 2016). The estimated incidence of CPP in American girls is 1:5000–1:10,000. Among Danish girls, the prevalence of precocious puberty was 1:500, based on national registries over a 9-year period (Teilmann *et al.*, 2005). The prevalence is sexually dimorphic, being higher in girls than in boys (15–20 girls for every boy). Precocious puberty can result in short stature and psychological and behavioral disorders in untreated patients. Familial segregation analysis showed that 27.5% of cases of CPP are familial disorders with predominance of an autosomal dominant inheritance (Durand *et al.*, 2016; de Vries *et al.*, 2004). Recent studies have implicated gene mutations leading to activation and inactivation of hypothalamic expressed proteins in the pathogenesis of CPP (**Table 1**).

Kisspeptin System

Kisspeptin, encoded by the *KISS1* gene, is the most potent known stimulator of GnRH-dependent LH secretion. Kisspeptin acts through its cognate receptor, KISS1R, expressed in the surface of the GnRH secreting neurons. Hypothalamic *KISS1* expression is increased at the time of puberty onset, reaching maximum levels at the beginning of pubertal development. Activation of the kisspeptin complex plays a critical role in puberty initiation (Han *et al.*, 2005; Navarro *et al.*, 2007). *KISS1* and *KISS1R* were the first genes involved in the pathogenesis of CPP in humans. To date, only two activating mutations affecting the kisspeptin system —one in the gene encoding kisspeptin (p.Pro74Ser) and one in the gene encoding its receptor (p.Arg386Pro)—were associated with CPP, despite a relatively large cohort of patients have been screened, indicating that isolated mutations in these genes are uncommon causes of CPP (Silveira *et al.*, 2010b; Teles *et al.*, 2010). Although very rare, these prismatic cases contributed to the elucidation of the fundamental role of the kisspeptin pathway in the physiologic regulation of pubertal development (Ko *et al.*, 2010; Tommiska *et al.*, 2011).

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Table 1 Genetic causes of nonsyndromic CPP

Gene	*Locus*	*Protein function*	*Pattern of inheritance*	*Type of mutation*	*Prevalence*
KISS1R (*or GPR54*)	19p13.3	Kisspeptin receptor	Adopted girl	Gain-of-function missense	Very rare
KISS1	1q32	Potent GnRH stimulator	Autosomal dominant with probably incomplete penetrance	Gain-of-function missense	Very rare
MKRN3 (maternal imprinted gene)	15q11.2 (Prader–Willi region)	Protein ubiquitination and RNA binding	Autosomal dominant with complete penetrance (paternal transmission)	Loss-of-function defects (missenses, frameshift, stop-codons mutations)	33%–46% of familial cases
DLK1 (maternal imprinted gene)	14q32 (Temple region)	Adipose tissue homeostasis and neurogenesis	Autosomal dominant with complete penetrance (paternal transmission)	Loss-of-function defect	Rare

MKRN3 Deficiency Causes CPP

In 2013, human mutations in *MKRN3*, a paternally expressed imprinted gene that encoding the makorin Ring-finger protein, were first described in a cohort of families with CPP (Abreu *et al.*, 2013). Whole exome sequencing revealed loss-of-function mutations in *MKRN3* in 5 of 15 families with CPP. These mutations were inherited from the father in all affected individuals, consistent with the maternal imprinting of *MKRN3*. Since then, other loss-of-function mutations of *MKRN3* have been reported in familial CPP (Grandone *et al.*, 2017; Macedo *et al.*, 2014; Simon *et al.*, 2016; de Vries *et al.*, 2014). The current frequency of *MKRN3* mutations in familial CPP is up to 46%, suggesting that these defects represent a common cause of premature sexual development in both sexes (Simon *et al.*, 2016).

MKRN3 is an intronless gene located on chromosome 15q11.2, in the Prader-Willi syndrome critical region. It encodes a protein with domains implicated in E3 ubiquitin ligase and RNA binding activity. MKRN3 function and the mechanism by which *MKRN3* mutations result in early activation of the central reproductive axis are not well understood. Studies in animal models demonstrated a high expression of *Mkrn3* in the arcuate nucleus prepubertally, which decreases before puberty initiation, reaching very low levels in adult life, suggesting an inhibitory role of MKRN3 on GnRH secretion (Abreu *et al.*, 2013; Abreu and Kaiser, 2016).

Macedo *et al.* (2014) studied 215 unrelated children (207 girls and 8 boys) from three university medical centers with a diagnosis of CPP. All but two of these patients (213 cases) reported no family history of premature sexual development. Novel heterozygous mutations in *MKRN3* were detected in eight unrelated girls with CPP. Four of them were frameshift mutations predicted to encode truncated proteins and one was a missense mutation, which was suggested to be deleterious by in silico analysis. All girls with *MKRN3* mutations had classical features of CPP with a median age of onset at 6 years. Segregation analysis revealed that these *MKRN3* mutations were inherited on the paternal allele, pointing out that inherited *MKRN3* defects were also recognized in children with apparently sporadic CPP.

More recently, a high frequency of MKRN3 mutations was demonstrated in boys with CPP, previously classified as idiopathic (Bessa *et al.*, 2017). The studied boys with CPP due to *MKRN3* mutations had also classical features of CPP, but with puberty initiation at a borderline age (median 8.2 years), suggesting that *MKRN3* deficiency has a smaller impact on puberty onset in boys than in girls. The median ages of pubertal onset of affected children with MKRN3 mutations from both sexes (girls: 6 years and boys: 8 years), suggest that MKRN3 is not crucial for GnRH suppression after the mini puberty of early infancy, but that its down-regulation plays a relevant role for the reemergence of GnRH pulses in the pubertal phase (Macedo *et al.*, 2014).

To date, >30 different MKRN3 mutations have been described. Notably, two indel mutations, p.Pro161Argfs*10 and p.Pro161Argfs*16, which affect a poly-C region of *MKRN3*, have been recurrent in girls and boys with CPP, suggesting a mutational hot spot codon (Grandone *et al.*, 2017; Macedo *et al.*, 2014). The identification of carriers of *MKRN3* mutations may contribute to early diagnosis of CPP, facilitating treatment decisions and guiding genetic counseling and prompt intervention in familial cases. The very early diagnosis (preclinical) was recently illustrated by the demonstration of a *MKRN3* defect in an asymptomatic 4 year-old girl in a family context who had lately developed premature sexual development (Stecchini *et al.*, 2016). The relative high prevalence of *MKRN3* mutations in familial CPP cases, previously considered idiopathic, has affected the clinical decision in the routine etiology investigation of premature central activation of reproductive axis. It has been suggested that in familial cases, *MKRN3* analysis could precede the brain MRI, which might be postponed in nonmutant cases or even avoided completely in patients with deleterious mutations (Latronico *et al.*, 2016).

Using multiplex ligation-dependent probe assays, no methylation abnormalities assays involving the *MKRN3* or delta-like 1 homolog gene (*DLK1*) loci were identified in CPP patients who were negative for coding region point mutations (Dauber *et al.*, 2017; Macedo *et al.*, 2014). However, defects located outside the target sequences of the methylation-specific probes cannot be ruled out due to technical limitations of the current methylation assays and the high complexity of imprinting center regulation of these two loci.

New Imprinted Gene Implicated with CPP

A very rare and complex genomic defect (~14-kb deletion and 269-bp duplication of *DLK1*) was identified in a large multigenerational family with five female members with CPP using a combination of linkage analysis and whole genome sequencing (Dauber *et al.*, 2017). The deletion included the 5′-untranslated region and the first exon of *DLK1*, including the translational start site. *DLK1* gene is located on the long arm of chromosome 14, within a locus associated with Temple syndrome, and similarly to *MKRN3*, it is a paternally expressed gene (da Rocha *et al.*, 2008). Temple syndrome is a rare genetic condition characterized by intrauterine growth retardation, postnatal short stature, truncal obesity, hypotonia, small hands, mild facial, dysmorphisms, and CPP (Kagami *et al.*, 2017). However, the female patients with CPP associated with isolated *DLK1* defect did not demonstrate any of these features apart from CPP, suggesting a nonsyndromic form (Dauber *et al.*, 2017). The inheritance pattern of the family with isolated CPP also follows that of a paternally expressed imprinted gene consistent with the known imprinting of *DLK1*. The complete loss of *DLK1* in this family affected by CPP was supported by undetectable serum DLK1 levels.

DLK1, also known as preadipocyte factor 1, is a transmembrane protein containing epidermal growth factor-like repeats in its extracellular domain. It is a noncanonical ligand in the Delta-Notch signaling pathway and plays a role in the inhibition of adipocytes differentiation. A neuroendocrine function for DLK1 was suggested by evidence of postnatal *Dlk1* expression in several hypothalamic nuclei and it increased progressively in mice postnatally (from day P6 to P20), correlating with increases in kisspeptin expression (Villanueva *et al.*, 2012). Furthermore, *Dlk1* expression was demonstrated in cell lines derived from kisspeptin neurons in the arcuate and anteroventral-periventricular (AVPV) hypothalamic nuclei (Dauber *et al.*, 2017).

Genome-wide association studies have identified multiple loci associated with pubertal timing. The important role of *MKRN3* and *DLK1* in human puberty initiation was reinforced by large genome-wide studies involving women of European descent. In this comprehensive study, menarche signals were found for paternal inheritance at the imprinted *MKRN3* and *DLK1* loci (Day *et al.*, 2017).

Familial CPP due to paternal *MKRN3* or *DLK1* copy alterations has been considered part of the group of imprinting disorder (Eggermann *et al.*, 2015). Interestingly, these congenital disorders are usually characterized by overlapping clinical features affecting growth, development, and metabolism. Indeed, Temple syndrome displays clinical features overlapping with Prader-Willi and Silver Russell syndromes (Dauber *et al.*, 2017; Kagami *et al.*, 2017). Early or precocious puberty has been described as part of the constellation of clinical features of these imprinted syndromes.

Genetic Basis of CHH

CHH is characterized by partial or complete lack of pubertal development, secondary to deficient GnRH-induced gonadotropin secretion (Boehm *et al.*, 2015). The underlying cause may be associated with developmental defects of GnRH neurons, deficient GnRH production or secretion, or resistance to GnRH action. The clinical diagnosis is confirmed by the presence of low levels of sex steroids associated with low or inappropriately normal LH and FSH serum levels, with no other pituitary hormone deficiencies and lack of an anatomical lesion in the hypothalamic–pituitary tract (Seminara *et al.*, 2000). The association of CHH with olfactory defects (anosmia or hyposmia) characterizes Kallmann syndrome, accounting for approximately 50%–60% of all CHH cases (Boehm *et al.*, 2015). The olfaction defects occur due to combined abnormal embryonic migration of GnRH neurons and olfactory fibers from their origin in the olfactory placode to the forebrain (Soussi-Yanicostas *et al.*, 1998; Wierman *et al.*, 2011). These patients usually present with hypoplasia or aplasia of the olfactory tract/bulbs associated with GnRH deficiency (Quinton *et al.*, 1996). In addition, a number of other developmental abnormalities may be associated with Kallmann syndrome, including cleft lip or palate, dental agenesis, ear anomalies, deafness, renal malformations, bimanual synkinesis, and skeletal anomalies (Mitchell *et al.*, 2011).

CHH is a rare, clinically and genetically heterogeneous condition, with a male predominance of 3–5 to 1. It occurs more commonly in the sporadic form or it may be familial, with X-linked, or autosomal recessive or dominant inheritance (Boehm *et al.*, 2015; Quinton *et al.*, 2001). Kallmann syndrome and normosmic CHH were once thought to be completely distinct conditions. However, in the last decade this paradigm has changed. The starting point for this change was the description of *FGFR1* mutations in both Kallmann syndrome and normosmic CHH patients since 2003 (Dode *et al.*, 2003; Pitteloud *et al.*, 2006a). Before that, only two genes were known to cause CHH: GnRH receptor (GnRHR) mutations in autosomic recessive normosmic CHH, and *KAL1* (recently renamed *ANOS1*) mutations, responsible for about 50% of X-linked Kallmann syndrome (Boehm *et al.*, 2015; de Castro *et al.*, 2017; Seminara *et al.*, 2000). Nevertheless, the majority of cases of CHH remained unexplained and these new findings opened the gates for a new era in the CHH research.

The molecular mechanisms involved in the central regulation of the gonadotropic axis are complex and still not completely understood. Studies of the molecular genetics of CHH and Kallmann syndrome have advanced tremendously in the past 20 years, greatly contributing to increase our knowledge on the mechanisms controlling the GnRH system. In the last few years, the advent of new sequencing techniques, such as parallel sequencing on a large scale, which enabled the study of candidate gene panels, and whole exome sequencing allowed an increase in the molecular diagnosis of patients with CHH from 30% to approximately 50% of cases (Boehm *et al.*, 2015). A growing list of genes has been implicated in the molecular pathogenesis of the CHH, pointing up the heterogeneity and complexity of the genetic basis of this condition. These genes encode neuropeptides and proteins involved in the development and migration of GnRH neurons, or in the control of different stages of GnRH function (**Table 2**).

Table 2 Genes implicated in CHH

Gene	*Locus*	*CHH phenotype*	*Associated clinical features*	*Inheritance*	*Prevalence*
GNRH1	8p21.2	nCHH		Autosomal recessive	Rare
GNRHR	4q13.2	nCHH		Autosomal recessive	6%–16%
KISS1	1q32.1	nCHH		Autosomal recessive	rare
KISS1R	19p13.3	nCHH		Autosomal recessive	2%–5%
TAC3	12q3	nCHH		Autosomal recessive	Rare
TACR3	4q24	nCHH		Autosomal recessive	rare
ANOS1	Xp22.31	KS	Bimanual synkinesia, renal agenesis	X-linked recessive	5%–8%
FGFR1	8p11.23	KS/nCHH	Cleft lip and/or palate, dental agenesis, bimanual synkinesia, skeletal anomalies, hand/foot malformation, septo-optic dysplasia, combined pituitary hormone deficiency, Hartsfield syndrome	Autosomal dominant	10%
FGF8	10q24.32	KS/nCHH	Cleft lip and/or palate, skeletal anomalies Bimanual synkinesia, combined pituitary hormone deficiency	Autosomal dominant	$<2\%$
PROK2	3p13	KS/nCHH		Autosomal recessive	3%–6%
PROKR2	20p12.3	KS/nCHH	Combined pituitary hormone deficiency	Autosomal recessive	3%–6%
CHD7	8q12.2	KS/nCHH	Congenital hearing impairment, semicircular canal hypoplasia CHARGE syndrome	Autosomal dominant	6%
SOX10	22q13.1	KS/nCHH	Congenital hearing impairment Waardenburg syndrome	Autosomal dominant	2%
SEMA3A	7q21.11	KS		Autosomal dominant	Rare
NELF	9q34.3	KS/nCHH		Autosomal dominant	Rare
IL17RD	3p14.3	KS	Congenital hearing impairment	Autosomal recessive	3%
FEZF1	7q31.32	KS		Autosomal recessive	Rare
CCDC141	2q31.2	nCHH		Autosomal recessive	3%
WDR11	10q26.12	KS/nCHH	Combined pituitary hormone deficiency	Autosomal dominant	Rare
FGF17	8p21.3	KS/nCHH	Dandy–Walker syndrome	Autosomal dominant	Rare
HS6ST1	2q14.3	KS/nCHH	Cleft lip and/or palate, skeletal anomalies	Autosomal dominant	Rare
AXL	19q13.2	KS/nCHH		Autosomal dominant	NR
LEP	7q32.1	nCHH	Early onset of morbid obesity	Autosomal recessive	$<2\%$
LEPR	1p31.3	nCHH	Early onset of morbid obesity	Autosomal recessive	$<2\%$
PCSK1	5q15	nCHH	Early onset of morbid obesity	Autosomal recessive	$<2\%$
OTUD4/ RNF216	4q31.21/ 7p22.1	nCHH	Cerebellar ataxia (Gordon–Holmes syndrome)	Autosomal recessive	Rare
PNPLA6	19p13.2	nCHH	Cerebellar ataxia (Gordon–Holmes syndrome) Boucher–Neuhöuser syndrome	Autosomal recessive	Rare
DMXL2	15q21.2	nCHH	Central hypothyroidism, diabetes mellitus, demyelinating polyneuropathy	Autosomal recessive	Rare
NR0B1 (DAX1)	Xp21.2	nCHH + AHC	Adrenal hypoplasia congênita (AHC)	X-linked recessive	Rare
HESX1	3p14.3	KS	Septo-optic dysplasia, combined pituitary hormone deficiency	Autosomal dominant	Rare

nCHH, normosmic congenital hypogonadotropic hypogonadism; KS, Kallmann syndrome.

The genetic causes of CHH have been categorized according to the stage of development of the gonadotrophic axis in which they participate (**Fig. 1**). Genes causing CHH can be broadly classified in genes involved in GnRH synthesis, secretion, or action, and genes implicated in the development and migration of GnRH neurons (Bianco and Kaiser, 2009). The first category includes basically neuropeptide hormones and their receptors, *GNRH1/GNRHR*, *KISS1/KISS1R*, and *TAC3/TACR3*, causing exclusively normosmic CHH (Bianco and Kaiser, 2009). The second category is more complex and includes a range of molecules, in some cases with interconnected functions, which are somehow involved in the GnRH neurons ontogeny and migration (Gonzalez-Martinez *et al.*, 2004b; Lima Amato *et al.*, 2017). The classical example of a gene with a definite role in GnRH and olfactory

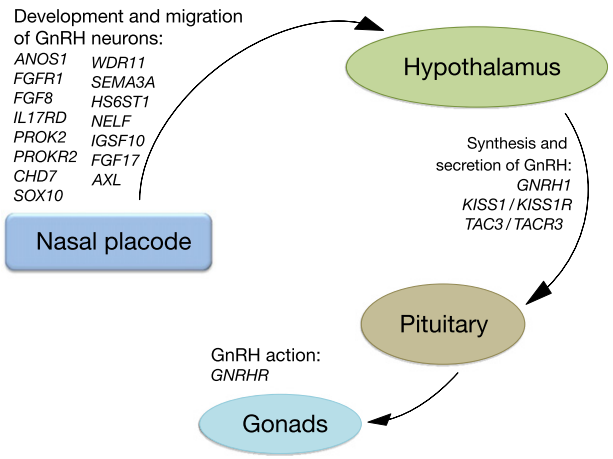

Fig. 1 Genes causing congenital hypogonadotropic hypogonadism (CHH).

neurons migration is *KAL1* (*ANOS1*), which encodes the extracellular matrix glycoprotein anosmin-1. *ANOS1* mutations cause exclusively X-linked Kallmann syndrome, usually with a severe reproductive phenotype (Boehm *et al.*, 2015; Mitchell *et al.*, 2011). This is not the case for other genes later found to influence GnRH neurons migration, which have been described both in normosmic CHH and Kallmann syndrome. These include fibroblast growth factors family and modulators (*FGFR1*, *FGF8*, *FGF17*, *IL17RD*, and *HS6ST1*), *PROK2*/*PROKR2* and other genes more rarely described in CHH (Lima Amato *et al.*, 2017). In addition, genes classically associated with complex syndromes, namely *CHD7* (CHARGE syndrome) and *SOX10* (Waardenburg syndrome), have recently been described in cases of CHH, usually associated with one other feature of the original syndrome, such as deafness, which is part of the Kallmann syndrome phenotypic constellation as well, suggesting an intersection between various clinical disorders and their causative genes (Boehm *et al.*, 2015; Kim *et al.*, 2008a; Pingault *et al.*, 2013).

Studies in large CHH cohorts in the last decade identified patients who harbor pathogenic rare variants in more than one gene, unraveling the concept of digenic or oligogenic inheritance in CHH. Currently, oligogenic cases account for 10%–20% of CHH patients, challenging the traditional Mendelian concept of CHH as a monogenic condition (Quaynor *et al.*, 2011; Sykiotis *et al.*, 2010). Genes more frequently associated with oligogenic inheritance include *FGFR1*, *FGF8*, *KAL1*, *PROKR2*, *PROK2*, *GNRHR*, *KISS1R*, and *NELF* (Quaynor *et al.*, 2011, Sykiotis *et al.*, 2010). Oligogenic inheritance is a possible explanation for the great phenotypic variability observed among some families. Indeed, the same family may present with cases of normosmic CHH, Kallmann syndrome, pubertal delay, or isolated abnormalities such as isolated anosmia or craniofacial malformations (Pitteloud *et al.*, 2007; Sykiotis *et al.*, 2010).

To date, >30 different genes have been associated with Kallmann syndrome and/or normosmic CHH, with different modes of inheritance, some of them with a relative high frequency and others very rarely described (**Table 1**). *ANOS1*, *GNRHR*, *FGFR1*, and *CHD7* are among the genes most commonly involved in the pathogenesis of GnRH deficiency (Costa-Barbosa *et al.*, 2013; Boehm *et al.*, 2015; Mitchell *et al.*, 2011). The main genetic causes of CHH will be explored with some more details in the following sections.

Genes Involved in GnRH Synthesis and Secretion and Action (*GNRH1/GNRHR*, *KISS1/KISS1R*, *TAC3/TACR3*)

This group is composed by hypothalamic–pituitary expressed G-protein coupled receptors and their cognate neuropeptides/hormones ligands, which have a role in GnRH synthesis, secretion, and function, causing exclusively normosmic CHH. Loss-of-function mutations in the receptors are far more common than in the ligands, which may reflect a compensatory effect of other endogenous ligands and all of them present as an autosomic recessive trait (Mitchell *et al.*, 2011). Some CHH patients were found to harbor heterozygous rare variants in some of these genes, but their role in the pathogenesis of GnRH deficiency is still uncertain (Chan *et al.*, 2011; Maione *et al.*, 2013). GnRH receptor (*GNRHR*) inactivating mutations were the first identified genetic cause of normosmic isolated hypogonadotropic hypogonadism (IHH) (de Roux *et al.*, 1997). Since the first description in 1997 (de Roux *et al.*, 1997), >20 different biallelic *GNRHR* mutations have been reported in cases of sporadic or familial normosmic IHH (Beneduzzi *et al.*, 2014; Chevrier *et al.*, 2011). *GNRHR* inactivating mutations are the most frequent cause of normosmic CHH, especially in familial cases (Beneduzzi *et al.*, 2014; Beranova *et al.*, 2001; Chevrier *et al.*, 2011). Conversely, mutations in the *GNRH1* gene, encoding the decapeptide hormone GnRH, which has always been an obvious candidate, are extremely rare (Bouligand *et al.*, 2009; Chan *et al.*, 2009; Mengen *et al.*, 2016).

Loss-of-function mutations of *KISS1R* were first reported in patients with normosmic CHH belonging to large consanguineous families by two independent researcher groups in 2003 (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Since then, several different loss-of-function mutations have been described in the *KISS1R* gene in patients with partial or complete normosmic CHH (Silveira *et al.*, 2010a). These findings helped to elucidate the role of kisspeptin in the control of GnRH secretion, and posterior studies in

animal models confirmed kisspeptin as the main stimulator of GnRH-induced LH secretion (Colledge, 2009; Navarro *et al.*, 2009; Plant and Ramaswamy, 2009; Popa *et al.*, 2008). *KISS1R* mutations are responsible for about 5% of cases of normosmic CHH (Silveira *et al.*, 2010a). On the other hand, *KISS1* inactivating mutations are extremely rare, and were only reported once, in one consanguineous family with four affected women with normosmic CHH (Topaloglu *et al.*, 2012).

Homozygous inactivating mutations in the genes *TAC3* and *TACR3*, encoding neurokinin-B (NKB) and its receptor, respectively, were identified in four unrelated consanguineous families with normosmic CHH, as a fully penetrant autosomal recessive trait Topaloglu *et al.*, 2009). These findings provided compelling evidence that the NKB system is necessary for the activation of the hypothalamic–pituitary–gonadal axis in puberty. After the first description a number of mutations were described in both the homozygous and heterozygous state, but the role of the heterozygous variants is unknown (Gianetti *et al.*, 2010). NKB and dynorphin A (Dyn) are coexpressed in KISS1 neurons (so-called KNDy neurons), in the human mediobasal hypothalamus. Recent studies showed that the regulation of gonadotropin release by the tachykinins and their receptors seems to occur at least partly through actions on KISS1 neurons (Navarro, 2012).

Genes Involved in the Development and Migration of GnRH Neurons

ANOS1

ANOS1 (previously *KAL1*) encodes anosmin-1, an extracellular matrix glycoprotein essential for axonal guidance and migration of olfactory and GnRH neurons from the nasal placode to their final location in the brain (Ballabio and Camerino, 1992; Gonzalez-Martinez *et al.*, 2004a). Mutations in *KAL1* are reported to be present in approximately 5%–10% of all Kallmann syndrome patients and 15%–50% of the familial cases with an X-linked pattern of inheritance (Costa-Barbosa *et al.*, 2013; Mitchell *et al.*, 2011; Salenave *et al.*, 2008). Patients with *KAL1* mutations usually exhibit an almost uniformly severe and highly penetrant reproductive phenotype (Costa-Barbosa *et al.*, 2013, Mitchell *et al.*, 2011, Salenave *et al.*, 2008). Other anomalies frequently associated with X-linked Kallmann syndrome include high arched palate, cleft lip and/or palate, short metacarpals, unilateral renal agenesis, sensorineural hearing loss, bimanual synkinesia, and oculomotor abnormalities (Boehm *et al.*, 2015; Costa-Barbosa *et al.*, 2013).

Fibroblast growth factors family and modulators (FGFR1, FGF8, FGF17, IL17RD and HS6ST1)

Fibroblast growth factors (FGFs) and their cell surface receptors comprise a large and complex family of signaling molecules with a crucial role in embryonic development (Kim *et al.*, 2008b). FGF receptor 1 (FGFR1) signaling is required in normal GnRH neuronal migration, differentiation, and survival within the hypothalamus, and plays an essential role in the morphogenesis of the olfactory bulbs (Gonzalez-Martinez *et al.*, 2004a; Kim *et al.*, 2008b). *FGFR1* defects have been associated with both Kallmann syndrome and normosmic IHH, with an autosomal dominant mode of inheritance (Dode *et al.*, 2003; Pitteloud *et al.*, 2006a; Xu *et al.*, 2007). Currently, *FGFR1* mutations are one of the most common molecular causes of CHH, with or without olfactory defects. Mutations in FGF8, one of the various FGFR1 ligands, were also associated with both normosmic CHH and Kallmann syndrome, but with a much lower frequency than *FGFR1* (Falardeau *et al.*, 2008; Trarbach *et al.*, 2010). *FGFR1* and *FGF8* mutations are associated with marked phenotypic variability both within and among families and apparent incomplete penetrance (Pitteloud *et al.*, 2006b; Shaw *et al.*, 2011; Trarbach *et al.*, 2010; Villanueva *et al.*, 2015). Other phenotypic defects may be associated with mutations in the FGF system, including cleft lip-palate, dental agenesis, and bone defects, such as syndactyly and hand/foot malformation (Pitteloud *et al.*, 2006b; Shaw *et al.*, 2011; Villanueva *et al.*, 2015).

Variants in other genes that encode proteins acting as secondary ligands, cofactors or modulators for FGFR1 signaling were also associated with CHH in rare occasions, including *FGF17*, *IL17RD*, and *HS6ST1* (Miraoui *et al.*, 2013; Tornberg *et al.*, 2011). Particularly, *IL17RD* mutations were identified mainly in Kallmann syndrome patients with hearing loss. Variants in these genes were frequently identified in combination with additional variants in other CHH genes, suggesting synergic role in the pathogenesis of IHH, in an oligogenic trait (Boehm *et al.*, 2015).

PROK2/PROKR2

Prokineticins are cysteine-rich secreted proteins that promote diverse biological functions, including normal development of the olfactory bulb and sexual maturation (Martin *et al.*, 2011). Mutations in Prokineticin2 (PROK2) and its cognate receptor PROKR2, a G-protein-coupled receptor, were initially identified in Kallmann syndrome patients, but later described in normosmic CHH as well (Abreu *et al.*, 2008; Cole *et al.*, 2008; Dode and Rondard, 2013). Today, *PROK2/PROKR2* variants are identified in approximately 5% of all CHH patients in both a homozygous and heterozygous state with incomplete penetrance or variable expressivity frequently seen within and across pedigrees (Abreu *et al.*, 2010; Martin *et al.*, 2011).

Genes Associated with Complex Syndromes

A number of rare complex genetic syndromes, many of them comprising neurologic components, include CHH as one of their constellation of features. Mutations in *POLR3A* and *POLR3B* cause hypomyelinating leukodystrophies associated with dental abnormalities and CHH, with a variable phenotype (4H syndrome). *OTUD4*, *RNF216*, and *PNPLA6* mutations were identified in patients with Gordon Holmes syndrome, characterized by cerebellar ataxia/atrophy and normosmic CHH (Margolin *et al.*, 2013;

Synofzik *et al.*, 2014; Tarnutzer *et al.*, 2015; Topaloglu *et al.*, 2014). Recently, a complex homozygous mutation was identified in the *DMXL2* gene, in three siblings of a consanguineous family, presenting with a neurologic-endocrine syndrome characterized by demyelinating polyneuropathy, CHH, central hypothyroidism, mental retardation, and neonatal hypoglycemia progressing to insulin-dependent diabetes mellitus (Tata *et al.*, 2014). CHH may also be associated with early onset of severe obesity, a phenotype suggestive of loss-of-function mutations in *LEP*, *LEPR*, or *PCSK1* genes. Finally, defects in *DAX1* (*NROB1*) have long been known to cause X-linked adrenal hypoplasia and CHH (Boehm *et al.*, 2015).

Additionally, variants in the causative genes of complex syndromes were identified in cases of CHH alone or associated with only one additional phenotypic feature. This is the case of *CHD7* and *SOX10*.

CHD7 defects cause CHARGE syndrome, a multisystem disorder classically characterized by a variety of congenital anomalies including coloboma, heart defects, choanal atresia, delayed growth and development, genital hypoplasia, ear anomalies, and deafness (Jongmans *et al.*, 2006). Notably, GnRH deficiency and anosmia are consistent findings in this syndrome. In 2008, Kim *et al.* (2008a) reported CHD7 mutations in patients with Kallmann syndrome or normosmic CHH, most of them with hearing loss as the only additional feature. Currently, mutations in *CHD7* are identified in approximately 6% of all CHH patients, more commonly with Kallmann syndrome. However, if we consider only the cases of IHH associated with hearing loss, the prevalence of *CHD7* mutations rises to approximately 40% (Bergman *et al.*, 2012; Boehm *et al.*, 2015; Xu *et al.*, 2017).

Mutations in *SOX10* are a well-known cause of Waardenburg syndrome, characterized by deafness, skin/hair/iris hypopigmentation, Hirschsprung's disease, and neurological defects (Pingault *et al.*, 2013). A high frequency of olfactory-bulb agenesis was recently identified in patients with Waardenburg syndrome, raising the hypothesis that *SOX10* mutations might be involved in the Kallmann syndrome pathogenesis (Elmaleh-Berges *et al.*, 2013). Indeed, *SOX10* mutations were identified in individuals with Kallmann syndrome, almost exclusively associated with hearing loss (Izumi *et al.*, 2015; Maione *et al.*, 2016; Pingault *et al.*, 2013; Suzuki *et al.*, 2015; Vaaralahti *et al.*, 2014).

Other Genes

A growing list of genes has been associated with CHH, including *NELF*, *WDR11*, *SEMA3A*, *SEMA7A*, *AXL*, *FEZF1*, *CCDC141*, and *IGSF10* (Boehm *et al.*, 2015; Howard *et al.*, 2016; Hutchins *et al.*, 2016; Kansakoski *et al.*, 2014; Kim *et al.*, 2010; Kotan *et al.*, 2014; Quaynor *et al.*, 2011; Salian-Mehta *et al.*, 2014; Young *et al.*, 2012). Variants in these genes have been rarely reported in CHH, singly or in combination with defects in other genes.

Epigenetic Mechanisms and Pubertal Development

Epigenetics refers to mitotically heritable post-transcriptional modifications that affect chromatin structure and regulate gene expression, without changing the DNA sequence. These modifications are usually triggered by exposure to the environmental factors, enabling the integration between genetics and environment. Common epigenetic mechanisms include differential DNA methylation and histone post-translational modifications (Bird, 2002; Rzeczkowska *et al.*, 2014).

While monogenic or oligogenic genetic defects are involved in the molecular pathogenesis of rare conditions leading to extreme variations of pubertal development, such as CPP and CHH, the normal variation of pubertal timing may be influenced by more subtle genetic variations, environmental and epigenetic factors. The mechanisms involved in the reactivation of the gonadotropic axis, which triggers puberty initiation, are still poorly understood. The most accepted theories, corroborated by recent evidence, suggest that throughout the quiescent period in infancy, genes that stimulate the GnRH axis activity are repressed, while the inhibitory genes are fully expressed. There seems to be a programmed switch in gene expression involving transcriptional/posttranscriptional processes, allowing an increment in the expression and activity of stimulatory genes. It was only very recently that epigenetic mechanisms were implicated in the neuroendocrine regulation of puberty. Recent studies in animal models have demonstrated a role for epigenetics-mediated mechanisms in the CPP, through an inhibitory modulation of repressors that target kisspeptin pathways, and microRNAs (miRNAs) involved in the control of GnRH secretion (Leka-Emiri *et al.*, 2017; Lomniczi *et al.*, 2013; Rzeczkowska *et al.*, 2014; Tomikawa *et al.*, 2012; Semaan and Kauffman, 2013).

One of the best-studied epigenetic modifications is DNA methylation. It consists in the covalent addition of a methyl (–CH3) group to the fifth position of cytosines, predominantly in cytosine-phosphate-guanine (CpG) dinucleotides. Notably, the study of DNA methylation in the medial basal hypothalamus of male rhesus monkeys revealed an increase in GnRH mRNA levels across puberty that was paralleled by a decrease in CpG methylation status in the 5′ CpG island of *GnRH* gene (Kurian and Terasawa, 2013).

Histones acetylation leads to chromatin de-compactation, allowing the transcriptional machinery to access a gene, usually resulting in an increase in gene expression. Tomikawa *et al.* (2012) demonstrated that estrogen induces recruitment of estrogen receptor-α and histone acetylation in the Kiss1 promoter region of the AVPV, resulting in an increase in the AVPV-specific Kiss1 gene expression. These results indicate that epigenetic regulation of the Kiss1 gene is involved in estrogen-positive feedback to generate the GnRH/gonadotropin surge.

The study by Lomniczi *et al.* (2013) suggested that epigenetic repression of key inhibitory factors plays a fundamental role in the initiation of animal and human puberty. The researchers identified the Polycomb group (PcG) of transcriptional silencers as

major drivers of an epigenetic mechanism of transcriptional repression that regulate the timing of female puberty in rats. PcG proteins Eed and Cbx7 prevent the premature initiation of female puberty by silencing the *Kiss1* gene in kisspeptin neurons of the arcuate nucleus of the hypothalamus. Manipulations of DNA methylation were able to impact the onset of puberty. Inhibiting DNA methylation of PcG proteins promoters, allowing the continued expression of *Kiss1* repressors, resulted in pubertal delay (Lomniczi *et al.*, 2013). In a parallel study, the induction of DNA hypermethylation, inhibiting gene expression, led to earlier onset of puberty, corroborating the previous results (Messina *et al.*, 2016; Rzeczkowska *et al.*, 2014).

Epigenetic Regulation by Non-coding RNAs

Non-coding RNAs (ncRNAs) function to regulate gene expression at the transcriptional and post-transcriptional level. Some ncRNAs appear to be involved in epigenetic processes. They are shown to play a role in heterochromatin formation, histone modification, DNA methylation targeting, and gene silencing. miRNAs are one of the subtypes of ncRNAs related to epigenetic. miRNAs generally bind to a specific target messenger RNA with a complementary sequence to induce cleavage, or degradation or block translation. This may be done in the context of a feedback mechanism that involves chromosome methylation (Sato *et al.*, 2011).

New findings indicate that a miRNA-dependent mechanism regulates GnRH production to control puberty timing. Messina *et al.* (2016) demonstrated that increasing miRNAs that regulates the expression of *Zeb1* and *Cebpb*, two repressors of Gnrh transcriptional activators, and Gnrh itself in infantile mice lead to pubertal delay. Conversely, mice injected with target site blockers specific for these miRNA underwent early onset of puberty (Messina *et al.*, 2016).

Conclusion

Puberty is a major developmental stage. In the last two decades, several human genetic mutations have contributed to the unraveling of the complex networks implicated in the normal initiation of puberty. Genes involved in the abnormal hypothalamic–pituitary–gonadal axis development have been identified, leading to identification of inhibitory, stimulatory, and permissive factors acting upstream on GnRH neurons, integrating diverse hormonal and peripheral signals and acting as the "gate-keepers" of puberty initiation.

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Hormonal Control of Puberty

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Introduction

The hormones that cause pubertal development and reproduction emanate from the adrenal glands and the hypothalamic–pituitary–gonadal axis. Adrenarche describes the pubertal increase in adrenal androgen production. Gonadarche refers to the pubertal increase in gonadal steroids, and results from the pubertal reactivation of the hypothalamic–pituitary–gonadal axis. Gonadarche typically follows adrenarche, though the two processes are controlled separately. Gonadotropin-releasing hormone (GnRH) is secreted in pulsatile fashion from the mediobasal hypothalamus into the hypophyseal portal vascular system to reach the anterior pituitary gland. There, it stimulates secretion of gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) from the pituitary gonadotrophs. The gonadotropins in turn stimulate the synthesis and secretion of gonadal steroids, leading ultimately to an increase in height velocity, the development of secondary sexual characteristics, and the other physical changes of puberty. The maturation of the hypothalamic–pituitary–gonadal axis and the process of puberty is part of an ongoing process that begins during fetal development and ends with the completion of sexual maturation.

Hypothalamic–Pituitary–Gonadal Axis

Fetal Development

GnRH secreting neurons within the arcuate nucleus of the hypothalamus act in a coordinated manner to transduce neural signals into pulsatile GnRH release (the GnRH pulse generator). These neurons demonstrate spontaneous autorhythmicity and function like an oscillator in the pulsatile release of GnRH. Increased cyclic AMP (cAMP) levels have been shown to stimulate GnRH release via activation of cAMP gated channels; channel activation is associated with calcium oscillations (Vitalis *et al.*, 2000; Weiner and Charles, 2001). The GnRH secreting neurons arise in the olfactory placode of the nose and migrate to the hypothalamus (Wray *et al.*, 1989). Abnormal migration of the GnRH neurons leads to delayed or absent pubertal development due to hypogonadotropic hypogonadism (for example type 1 Kallmann syndrome), with anosmia or hyposmia in various forms (Schwanzel-Fukuda *et al.*, 1989).

GnRH cells are detected in the olfactory placode by 5.5 weeks gestation and in the fetal hypothalamus by 9 weeks gestation (Schwanzel-Fukuda *et al.*, 1996; Verney *et al.*, 1996; Terasawa and Fernandez, 2001). The hypothalamic-hypophyseal portal system is functional by 12 weeks gestation. Overall functionality of the GnRH pulse generator occurs by at least the end of the first trimester (Terasawa and Fernandez, 2001). Prior to this, the fetal testes secrete testosterone under the stimulation of placental human chorionic gonadotropin (hCG), which shares a receptor with LH. Thereafter, Leydig cells in the fetal testes depend on LH as a stimulus to make testosterone.

The fetal pituitary gland begins secreting FSH and LH by 11–12 weeks of gestation; by midgestation, gonadotropin levels are high in both sexes. LH and FSH levels in the male fetus are lower than in the female, likely due to negative feedback from testosterone secreted from the fetal testes (Grumbach *et al.*, 1974). Gonadotropin levels decline in late gestation, likely due to the further development of the negative feedback mechanism (including the development of receptors for gonadal steroids in the hypothalamus and pituitary). In both male and female fetuses and at term, estrogen levels are high due to conversion of fetal and maternal adrenal androgens (DHEA and androstenedione) to estrogen by the placenta. The predominant estrogen produced by the placenta is estriol.

Infancy and Childhood

Estrogen levels drop sharply after birth. The newborn hypothalamic–pituitary axis is thus released from estrogen inhibition and gonadotropins increase in both amplitude and frequency. In infant boys, these LH pulses result in increased testosterone levels; in infant girls, the FSH pulses lead to increased estradiol. The striking sex difference in gonadotropin pulses (with infant females having much higher FSH pulses and infant males having much higher LH pulses) has been attributed to the in utero exposure to testicular testosterone in the male fetus, which may act to prime the hypothalamic–pituitary–gonadal axis according to some.

The resultant increase in gonadal steroid secretion represents what is called the postnatal surge or "mini-puberty of infancy." In infant boys, testosterone levels reach a peak approximating pubertal values around 3 months of age and then decline to prepubertal levels by 6–9 months (Grumbach and Kaplan, 1990; Andersson *et al.*, 1998; Forest, 1990). In girls, increased estrogen levels are seen intermittently during the 2 years of life. Though the purpose of the postnatal surge is incompletely understood, high gonadotropin levels are associated with a rapid expansion of the Sertoli cell population and increases in Leydig and germ cell

numbers. Infant boys may have testosterone levels in the mid-pubertal range during this time period. The decline in testosterone toward the end of the postnatal surge in males is associated with the decline in testicular cell populations (via apoptosis).

After the postnatal surge of increased LH and FSH secretion, there follows a long period (roughly 10 years) of quiescence of the pituitary gonadotropin–gonadal axis. During this time period, referred to as the prepubertal period or "juvenile pause," there is active inhibition of the hypothalamic pulse generator. Negative feedback from sex steroids and inhibin may contribute to this inhibition, but an intrinsic gonadal steroid independent CNS mechanism appears to play a greater role. Notably, gamma-amino butyric acid (GABA), the dominant CNS inhibitory neurotransmitter, has been shown to inhibit the hypothalamic pulse generator (Terasawa and Fernandez, 2001; Mitsushima *et al.*, 1994). As puberty approaches, this GABA inhibition diminishes and GnRH release increases. How various genes may affect GABA inhibition or further control the juvenile pause is not fully understood. However, the MKRN3 gene (encoding makorin RING-finger protein 3) has been identified as an important regulator of pubertal development. MKRN3 is an imprinted gene located on chromosome 15q11.2 which acts to inhibit GnRH secretion during the juvenile pause; the maternal allele is suppressed and only the paternal allele is expressed. A loss of function mutation of MKRN3 in the paternal allele leads to deficiency of MKRN3 and autosomal dominant central precocious puberty (Abreu *et al.*, 2013). More recently, defects in DLK1, a paternally expressed gene on chromosome 14, have also been associated with isolated central precocious puberty (Dauber *et al.*, 2017).

Endocrine activity of the hypothalamic–pituitary–gonadal axis begins increasing in the peripubertal period, before the physical changes of secondary sexual development are apparent. Increased amplitude of LH and FSH pulses (demonstrated by ultrasensitive gonadotropin assays) is demonstrated in most children in midchildhood (Mitamura et al., 1999, 2000). LH secretion in early pubertal boys exhibits diurnal rhythm with augmentation of pulse amplitude following nocturnal sleep onset (Wu et al., 1989, 1996; Boyar *et al.*, 1974; Jakacki *et al.*, 1982). Prior to puberty, these low amplitude and low frequency pulses are unable to activate gonadal function. As puberty progresses, there is continued augmentation of the LH pulses. The full adult pattern, whereby there is no sleep-wake difference in LH pulse amplitude, does not occur until adulthood (Wu *et al.*, 1996).

A diurnal rhythm of testosterone secretion also exists in peripubertal boys whereby testosterone declines gradually in the early part of the night, followed by a postmidnight increase reflecting the nocturnal onset of LH pulsatility (Crofton *et al.*, 2004). Peak levels of serum testosterone are reached in the early morning, with lowest values in the late evening. The lag time of 60 min between the peak of LH and the increase in testosterone is presumably due to time required for synthesis and secretion of the steroid hormone (Veldhuis *et al.*, 1987). In prepubertal girls, a diurnal rhythm of testosterone is also seen with augmentation in the early morning. Estradiol has a diurnal rhythm as well, with higher values in the early morning in late prepubertal and early pubertal girls (Mitamura *et al.*, 2000).

Puberty

The onset of puberty is marked by an increase in amplitude of secretory events. As puberty approaches, inhibition of the GnRH pulse generator wanes and GnRH pulses increase in amplitude. Though the "switch" that triggers the onset of puberty is unknown, neurostimulatory factors increase during early puberty, including glutamate and kisspeptin. Kisspeptin is a critical stimulatory factor for GnRH secretion and onset of pubertal development. Inactivating mutations in the kisspeptin receptor (KISS1R, previously GPR54) are associated with hypogonadotropic hypogonadism and pubertal delay (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Alternately, a mutation in the gene encoding kisspeptin (KISS1) leading to prolonged kisspeptin effect has been found in a young male with central precocious puberty (CPP) (Silveira *et al.*, 2010). An autosomal dominant KISS1R mutation causing prolonged activation of intracellular signaling pathways in response to kisspeptin is also associated with CPP (Teles *et al.*, 2008).

In response to higher amplitude pulses of GnRH, the amplitude of gonadotropin pulses increases and increased daytime secretion of LH and FSH occurs. The frequency of pulses increases somewhat as well; this may contribute to the priming of the gonadotropes, making them more sensitive to pulses of GnRH (Wu *et al.*, 1996). Increasing pituitary responsiveness to GnRH can be demonstrated pharmacologically by administration of exogenous GnRH; exogenous GnRH is relatively ineffective in stimulating gonadotropin or gonadal steroid secretion prior to puberty but becomes effective after pubertal onset. If gonadotrophs are exposed to continuous rather than episodic GnRH, the GnRH receptors are downregulated with decreased responsiveness to GnRH. This phenomenon is utilized when GnRH agonists are employed to treat central precocious puberty.

During puberty, urinary FSH rises fivefold in both sexes. In girls, FSH rises in the early stages of puberty whereas LH rises in the later stages. Urinary LH rises 100-fold in girls and 50-fold in boys; the rise in first morning voided urinary luteinizing hormone levels precedes the physical onset of puberty (Demir *et al.*, 1996). Qualitative changes in gonadotropins occur in addition to these quantitative ones; FSH and LH variations affect biologic activity and half-life and provide an additional regulatory mechanism (Olivares *et al.*, 2004). Though secretion of FSH and LH are always pulsatile (due to pulsatility of the GnRH pulse generator), pulsatility of FSH secretion is less prominent than that of LH. This is attributed in part to the longer half-life of FSH compared with LH, to differences in the factors that modulate the action of GnRH on FSH and LH release by the gonadotrophs (especially gonadal steroids and regulatory proteins such as inhibin), and to intrinsic differences in the secretory pattern of the two gonadotropins.

In boys, circulating LH activates membrane-bound Leydig cell receptors to stimulate testosterone secretion. The enzyme 5-alpha reductase in certain tissues converts testosterone to dihydrotestosterone (DHT), which is a more potent activator of the androgen receptor. The testes additionally secrete other sex steroids, including androstenedione, delta-5-androstenediol, dihydrotestosterone and estradiol, in lesser amounts. Testosterone rises throughout puberty with the steepest increase in testosterone

levels occurring between pubertal stage 2 and 3. In boys, FSH supports spermatogenesis by binding to the FSH receptor on the cellular membrane of the seminiferous tubules. Estrogen in males is made from conversion of androgens (testicular and adrenal) by the enzyme aromatase. In all stages of puberty, boys have lower estrogen levels than girls. Males have higher concentrations of estrone (E1) than estradiol (E2); most of the circulating E1 is derived from peripheral aromatization of androstenedione (mainly originating from the adrenal gland).

In girls, extraglandular conversion of ovarian and adrenal androstenedione gives rise to nearly all of the circulating testosterone. FSH binds to cell-surface receptors on ovarian follicular cells to influence follicular development and stimulate secretion of estrogen. Nearly all estradiol in the female is produced by the ovary; a small fraction (less than 10%) of circulating estradiol arises from the extraglandular conversion of testosterone and androstenedione by aromatase. Girls have higher estradiol concentrations than boys prior to puberty, with a rise through puberty until the pubertal growth spurt and a decrease thereafter. The higher estrogen levels in girls may be an important factor in the more advanced levels of skeletal maturation in girls compared with boys.

In prepubertal girls, small amounts of estrogen will suppress gonadotropin secretion. Early in the menstrual cycle, estrogen has an inhibitory effect on FSH and LH secretion. After mid puberty, estrogen can either stimulate or suppress gonadotropin release, depending on the estrogen level and pattern. The onset of positive feedback (whereby higher amounts of estrogen can stimulate GnRH and gonadotropin release) occurs at midcycle once the ovarian follicle is of adequate size to produce sufficient estrogen and the pituitary has sufficient readily releasable LH to cause an LH surge. The LH surge stimulates prostaglandin, which causes an inflammatory response in the dominant follicle that leads to ovulation. The corpus luteum then forms from the remnants of the dominant follicle; it produces progesterone which prepares the endometrium for implantation and pregnancy. The increased estrogenic activity of the midcycle ovary stimulates growth of the endometrium, but the decrease in estrogen and progesterone toward the end of the menstrual cycle leaves the hyperplastic endometrium with no endocrine support. This, in addition to the local action of prostaglandins and constriction of arterioles, leads to necrosis of the endometrium and the onset of menses.

FSH Regulatory Proteins: Inhibin, Activin, and Follistatin

Inhibin, produced by multiple tissues including the ovarian granulosa cells and testicular Sertoli cells, suppresses FSH secretion from the pituitary gland. With gonadal failure, FSH is markedly elevated compared to LH due to the lack of FSH inhibition by inhibin and gonadal steroids. Inhibin is composed of an α-subunit and one of two β-subunits, β_A or β_B; production of inhibin is stimulated by FSH.

Inhibin B rises twice during male development, reflecting the two periods of Sertoli cell proliferation in infancy and in early puberty. In the early stages of puberty, inhibin B values have a close positive relationship to LH and testosterone levels and increase with pubertal progression. In stage G3, when inhibin B values peak, this relationship is lost, and inhibin B values become inversely related to FSH levels (Chada et al., 2003a; Crofton *et al.*, 2002). This relationship with FSH persists into adulthood. During childhood, inhibin B is a useful diagnostic marker for the presence and function of Sertoli cells. Undetectable or low inhibin B levels are observed in boys with absent testicular tissue whereas normal levels are seen with cryptorchidism. In adult men, inhibin B production is dependent on the presence of Sertoli cells and germ cells in the seminiferous tubules, and serum inhibin B levels are closely related to spermatogenesis. In girls, serum levels of inhibin A and B increase in early puberty and there is a strong positive correlation between inhibin B and FSH and estradiol early in puberty. After pubertal stage II, these relationships diminish (Chada et al., 2003b). Inhibin B is predominant in the follicular phase, and inhibin A is predominant during the luteal phase.

Activin is a closely related protein complex and acts in an opposing manner to inhibin to stimulate FSH synthesis and secretion from the pituitary. Like inhibin, follistatin also inhibits FSH expression and therefore is an additional regulator of FSH biosynthesis and secretion.

Serum values of FSH regulating proteins follow circadian patterns. Diurnal variation of inhibin B in peripubertal and early pubertal boys occurs with a decline in inhibin levels during the night as LH and testosterone levels rise, showing the negative feedback effect of testosterone on inhibin B secretion (Crofton *et al.*, 2004). Follistatin concentrations are greatest in the morning, and activin concentrations decline coincident with the nighttime increase in FSH levels in pubertal girls (Foster *et al.*, 2005).

Antimüllerian Hormone

Antimüllerian hormone (AMH), a glycoprotein produced by the Sertoli cells, acts ipsilaterally in a paracrine manner to cause regression of the Müllerian ducts in boys during early fetal development. AMH values rise from birth to relatively high levels during the first year of life in males. Levels remain stable in early childhood (with a modest late prepubertal peak), and then decline during puberty (Jeffery *et al.*, 2015). Newborn females have low or nondetectable serum levels of AMH, which rise only slightly thereafter. As with boys, there is a modest late prepubertal peak, (with a slight increase between ages 6 and 10 years), after which levels return to baseline low levels (Jeffery *et al.*, 2015). As with inhibin B, AMH can be used as a laboratory marker of Sertoli cell presence and function in young males. Boys with isolated cryptorchidism have normal values of AMH, whereas levels are absent in anorchia. Dysgenetic testes secrete only low serum AMH levels. Another indication of the presence of functional testicular tissue is a rise in testosterone in response to hCG, which only occurs if testicular tissue is present. AMH is additionally a

useful gonadal tumor marker because values are elevated in males with primitive Sertoli-like tumors and in girls and women with granulosa cell tumors.

Leptin

Leptin is a polypeptide hormone which acts to suppress appetite and regulate energy expenditure. It is secreted primarily by adipose cells and circulating leptin levels correlate with total fat mass. Leptin has additional importance in the function of the hypothalamic pituitary gonadal axis and is a permissive factor for the onset of puberty. Leptin is secreted in a pulsatile fashion, with peak levels seen at night. Leptin levels increase gradually during childhood; in prepubertal boys and girls, leptin levels are similar (Clayton *et al.*, 1997; Blum *et al.*, 1997). During puberty, leptin levels rise in females and decline in males and thus a sexual dimorphism arises in which in leptin levels reflect differential changes in body composition (Ahmed *et al.*, 1999).

Homozygous mutations in the leptin gene or leptin receptor lead to absent gonadotropin secretion and hypogonadotropic hypogonadism (as well as morbid obesity due to lack of satiety) (Ozata *et al.*, 1999; Clement *et al.*, 1998). Leptin replacement in an adolescent female with congenital leptin deficiency led to the onset of gonadotropin secretion and the onset of menses (von Schnurbein *et al.*, 2012). A critical level of leptin and a leptin signal is necessary for pubertal onset, but a rise in leptin is not required to trigger puberty and leptin administration alone will not cause a normal prepubertal individual to enter puberty. Women with hypothalamic amenorrhea are energy deficient and have low leptin levels and loss of leptin diurnal rhythm. Treatment with replacement doses of leptin results in increases in gonadotropins and estradiol and restoration of menses in these women (Chou *et al.*, 2011).

Laboratory Testing

In the past, baseline assays for LH and FSH were not sensitive enough to accurately predict the onset of puberty. However, modern assays are now reported to predict the onset of pubertal development as well as GnRH testing does. A value of serum LH greater than 4 mIU/mL measured by immunochemiluminometric (ICMA) assay is consistent with the onset of puberty. Likewise, older techniques for determination sex steroid levels in children have been shown to be inaccurate. This inaccuracy is primarily due to the presence of interfering substances and the relative insensitivity of antibodies used in assays. High performance liquid chromatography tandem mass spectrometry, or HPLC-MS/MS, allows the accurate measurement of extremely low values present in pediatric samples; it is now the preferred method to measure gonadal steroids in children (Wang *et al.*, 2004; Taieb *et al.*, 2003; Albrecht and Styne, 2007). A human cell bioassay measuring total estrogenic bioactivity (rather than estradiol alone) in children also has an extremely sensitive detection limit (of less than 1 pg/mL) (Paris *et al.*, 2002).

As previously discussed, both sex steroids and gonadotropins are secreted in a diurnal rhythm in early puberty. Because sex steroids are bound to sex hormone-binding globulin (SHBG), the half-life of sex steroids is longer than that of gonadotropins. Thus, random daytime measurement of serum sex steroids is more helpful in determining pubertal status than random measurements of serum gonadotropins.

The measurement of sex steroid levels in saliva has theoretical advantages over serum measurements, particularly in children. However, salivary levels are much lower than serum levels (roughly 2%–3%), raising concern that the salivary levels of sex steroids may be too low to accurately detect (particularly using standard assays and in patients with already low levels such as women and children). Furthermore, many factors have the potential to affect salivary levels of testosterone measured by immunoassay. Trauma such as tooth brushing that leads to blood in the specimen can influence the results, as can oral bacterial load and the presence of certain collection materials. Despite what is often claimed, the steroid level in saliva is not a direct representation of free steroid in the serum.

Adrenarche

Adrenarche denotes the pubertal increase in adrenal androgens and occurs prior to the onset of increased gonadotropin secretion. There is usually coordination between gonadarche and adrenarche, but control for each is distinct and the age at adrenarche does not significantly influence age at gonadarche. A continued rise in adrenal androgen secretion persists until early adulthood; levels then decline throughout adulthood to roughly 20% of peak levels. Physical signs of adrenarche include pubic and axillary hair development, axillary odor and acne. The appearance of pubic hair caused by adrenarche is referred to as pubarche.

Though the trigger for adrenarche is unknown, there is an increase in the size of the zona reticularis associated with an increase in the major adrenal androgens dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and androstenedione. DHEAS is produced from DHEA by the action of the sulfotransferase enzyme SULT2A1, mainly in the adrenal glands and the liver. Adrenal androgens are relatively weak androgens that are converted to more potent ones such as testosterone and dihydrotestosterone (DHT) or to estrogens via aromatization. Plasma DHEA levels have a diurnal rhythm similar to that of cortisol, but plasma levels of DHEAS show less variation and are a useful biochemical marker of adrenarche.

Most patients with premature adrenarche, who secrete excessive amounts of adrenal androgens for their age, enter puberty and experience menarche within the normal age range. Moreover, prepubertal children who have congenital or acquired chronic

adrenal insufficiency (such as Addison's disease) and consequently have deficient or absent adrenal androgen secretion usually have a normal onset and normal progression through puberty.

Other Changes at Puberty

Prolactin

Prolactin is a peptide hormone released from the anterior pituitary gland which plays a critical role in stimulation of the mammary glands and lactation. The serum concentration of prolactin increases in normal female puberty but is not affected by male puberty. This sex difference is probably a consequence of the higher estradiol levels during puberty in girls and in women.

Growth Hormone

Rising levels of estradiol during puberty play a critical role in stimulating growth hormone production. Growth hormone is a major regulator of linear growth, and works primarily by increasing insulin like growth factor-1 (IGF-1) levels. IGF-1 has growth-promoting effects on many tissues throughout the body, including cartilage, bone, and skeletal muscle.

Sex Hormone-Binding Globulin

Between 97% and 99% of circulating testosterone and estradiol is reversibly bound to carrier proteins, including sex hormone-binding globulin (SHBG) (i.e., sex steroid–binding globulin or testosterone binding globulin, TeBG). These carrier proteins play an important role in regulating the transport and biological activity of the sex steroids. Only a small percentage (1%–2%) of circulating sex steroids are unbound; historically it has been assumed that only the unbound portion is biologically active, though this now appears to be an oversimplification (Goldman *et al.*, 2017).

Prepubertal levels of SHBG are approximately equal in boys and girls. At puberty, levels decline in both sexes but the decline is greater in males (Pinkney *et al.*, 2014). The difference between genders is likely due to differences in androgen levels as androgens are known to suppress SHBG levels (Garces *et al.*, 2010). The rise in adrenal androgen levels at adrenarche may explain the early drop in SHBG levels, which allows more circulating free hormone at a given concentration of testosterone. Estrogen has the opposing effect and leads to higher SHBG levels; SHBG values in adult woman are thus higher than in adult men. Although the plasma concentration of testosterone is 20 times greater in men than in women, the concentration of free testosterone is 40 times greater.

Prostate-Specific Antigen

Prostate-specific antigen (PSA) has been used as a biochemical marker in the diagnosis and monitoring of patients with prostate cancer. In children, PSA is produced by periurethral glands and by rectal glands (Diamandis and Yu, 1997; Frazier *et al.*, 1992); in adult men, the major source is prostatic epithelial cells. PSA values are measurable in prepubertal children by a highly sensitive assay; levels do not differ significantly between boys and girls until roughly the age of 12 years (Antoniou *et al.*, 2004). The significant increase of PSA in boys after this age likely reflects the increase in prostate gland volume caused by androgenic stimulation. PSA values are increased to the pubertal range in boys with idiopathic CPP, and decrease with GnRH agonist treatment.

Conclusion

The changes in gonadotropin secretion that occur at puberty do not arise de novo but are based upon preexisting patterns of endocrine secretion. In fetal life, GnRH secreting neurons in the hypothalamus develop function as an endogenous pulse generator. This pulse generator operates at a relatively low level in the prepubertal period (with the exception of a neonatal surge in activity), due to active inhibition. The inhibitory restraints are largely mediated by GABA, but steroid dependent factors also play a role. Prior to puberty, there is a waning of inhibition of the pulse generator and an increase in neuro-stimulatory factors, and GnRH pulses increase in amplitude. These pulses result in increasing amplitude of gonadotropin pulses, initially at night associated with sleep and then during both the night and day. As puberty progresses, increasing levels of gonadal steroids and their resulting effects are seen. Adrenarche is controlled separately from gonadarche and typically precedes it. A variety of other hormonal and metabolic changes, often resulting from the increasing levels of gonadal steroids, occur during the pubertal time period as well.

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Secular Changes in Puberty

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Abbreviations

AMH	Anti-Müllerian hormone
AR	Androgen receptor
BCERP	Breast Cancer and the Environment Research Program
BMI	Body mass index
EDC	Endocrine disrupting chemical
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
HPG-axis	Hypothalamic–pituitary–gonadal axis
LH	Luteinizing hormone
NHANESIII	Third National Health and Nutrition Examination Survey
PP	Precocious puberty
PROS	Pediatric Research in Office Settings

Glossary

Adrenarche Development of the inner zone in the adrenal cortex resulting in increased secretion of androgens.

Gaussian distribution Symmetrical, bell-shaped sample distribution about the mean.

Gonadarche The first gonadal changes in response to the onset of puberty. In boys, testicular enlargement is the first sign to appear. In girls thelarche is often the first sign of pubertal onset.

Menarche The occurrence of the first menstruation in a pubertal girl.

Pubarche Development of pubic hair.

Secular trend Changes over an extended period.

Spermarche Onset of the release of spermatozoa (spermarche) in boys.

Thelarche The onset of breast development.

Introduction

Puberty is a central milestone in human reproductive life marking the transition from childhood to adulthood. The physiological changes in this period lead to the attainment of full reproductive capacity. Secular changes in the timing of puberty have occurred virtually worldwide (Sørensen *et al.*, 2012). In both girls and boys, a gradual decline of age at pubertal onset has been reported throughout the last century (Aksglaede *et al.*, 2008).

Secular changes are important, not only from an individual point of view, but also at the population level, since early puberty is associated with adverse and potentially preventable long-term health outcomes, like type 2 diabetes and cardiovascular disease (Day *et al.*, 2015). Despite a strong genetic component behind the pubertal timing, other factors like nutritional status, socioeconomic status, stress, and environmental factors all seem to play a role for the timing of puberty. Apart from genetic factors, all the above-mentioned factors have been subject to changes during the 20th century and could therefore potentially be involved in the secular changes in timing of puberty.

Physiology of Normal Puberty and Variation in Timing

Puberty is the result of a complex process initiated by the hypothalamus integrating signals from different systems. It is linked to the development of secondary sexual characteristics, the pubertal growth spurt and results in the attainment of adult reproductive capacity.

The advent of puberty is marked by the re-emergence of hypothalamic–pituitary–gonadal (HPG) hormone activity axis after a period of relative quiescence during childhood. The pulsatile gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus stimulates the release of gonadotropins from the pituitary which in turn stimulates sex steroid production and initiates gonadal maturation (**Fig. 1**).

The development of the secondary sexual characteristics is induced by the activation and maturation of the gonads and subsequent sex steroid production (*gonadarche*) accompanied by an increase in the production of adrenal hormones (*adrenarche*). Follicle-stimulating hormone (FSH)-driven ovarian activation in girls results in estradiol production which stimulates the development of secondary sexual characteristics including growth of glandular breast tissue, uterus, and endometrial tissues. Testicular activation in boys results in maturation and enlargement of the testicles, which is primarily due to FSH-driven growth of the seminiferous tubules. Furthermore, Luteinizing hormone (LH)-driven testosterone secretion results in development of secondary sexual characteristics including penile enlargement and development. Gonadotropins affect differentiation and maturation

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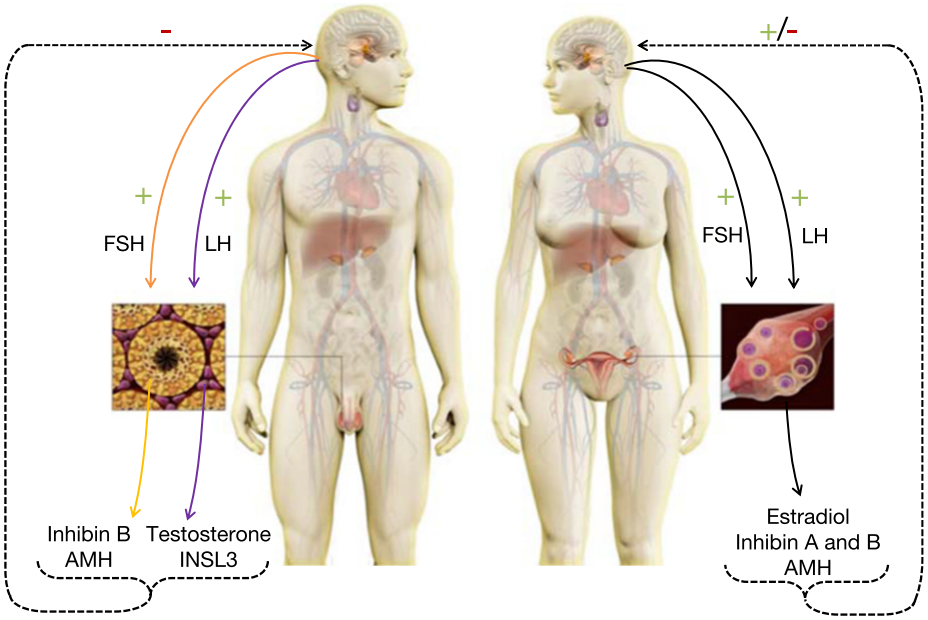

Fig. 1 At the onset puberty, the release of LH and FSH from the anterior pituitary gland stimulates the gonads to produce sex hormones in both male and female adolescents. In the male, FSH stimulates the Sertoli cells to produce Inhibin B, whereas AMH (Anti-Müllerian Hormone) secretion decreases with the onset of puberty (when Sertoli cells differentiate and start to express AR). LH stimulates the Leydig cells to produce testosterone and INSL3 (Insulin-Like Factor 3). The hormones produced in the testicles regulate the release of FSH and LH from the pituitary gland by negative feedback mechanism. In the females, FSH and LH stimulate the ovaries to produce Estradiol, Inhibin B, whereas Inhibin A is secreted following ovulation in the luteal phase of menses. AMH is secreted from small follicles and represent a marker of the follicle pool. These hormones regulate the release of FSH and LH from the pituitary gland by both positive and negative feedback mechanisms.

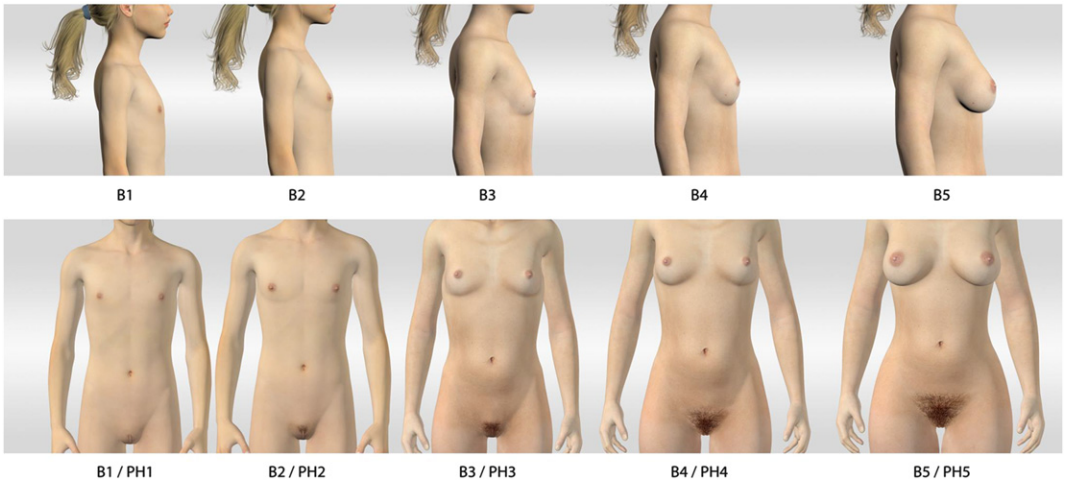

Fig. 2 Tanner scale, breast (B1–B5) and pubic hair scale (PH1–PH5) in girls (Copyright Claus Lunau A/S and Rigshospitalet, Copenhagen).

of spermatogonia and initiate spermatogenesis, which ultimately results in sperm production in boys. The first ejaculation of sperm, termed *spermarche*, occurs at an average age of 14 years, and is considered the male equivalent to the first menstruation (*menarche*) in girls.

With normal ranges from 8 to 13 years in girls and 9 to 14 years in boys for these clinical hallmarks, timing of puberty onset in the human exhibits a large variation between individuals (Parent *et al.*, 2003).

Pubertal stages are determined according to the *Tanner scale* (Marshall and Tanner, 1969, 1970). The scale defines five distinct developmental stages.

In girls, the important pubertal milestones, measured by the Tanner scale, are breast development (stages B1 (prepubertal) to B5) and pubic hair development (stages PH1 (no hair) to PH5) (**Fig. 2**). In girls, the initial pubertal milestone is thelarche (Tanner

scale, stage B2), whereas menarche is a late pubertal phenomenon that usually occurs 2–3 years after pubertal onset, while the attainment of a regular pattern of ovulatory cycles occurs even later.

In boys, signs of androgen action including penile growth and reddening of the scrotal sack are evaluated and grouped according to the Tanner scale, genital stages (stages G1 (prepubertal) to G5), and the development of pubic hair (stages PH1 (no hair) to PH5). Genital and pubic hair stages as well as testicular volume are the pubertal milestones evaluated in boys (**Fig. 3**). In boys, the first clinical sign of pubertal onset is the growth of testicles. The size of the testicles can be measured by using either a Prader orchidometer or ultrasonography. A ruler can also be used to measure the testicular size. A testicular volume enlargement of >3 mL measured by orchidometry defines pubertal onset in boys. The pubertal increase in testicular volume coincides with increasing penile size and other signs of androgenization. Voice break, peak height velocity and spermarche represent late pubertal phenomena in boys occurring approximately 3 years after pubertal onset.

In both sexes the production of adrenal androgens result in the development of pubic hair, which in addition is stimulated by the simultaneous gonadal testosterone production. Thus, the appearance of pubic hair is not exclusively dependent on the activation of the HPG-axis and can be observed in children without other signs of puberty.

Secular Trends in Puberty Timing in Girls

The change of age at puberty timing in girls over the past centuries is well documented. Historical data from Europe and the US have shown that *age at menarche* has drastically decreased from approximately 17 years around year 1850 to 13 years in 1970 (**Fig. 4**). In the period from the 1960s to 2010 the age at menarche appears to have only decreased slightly and then leveled off (Sørensen *et al.*, 2012).

Although much fewer studies exist on *age at thelarche* the studies to date show a marked decline in age at onset during the last 20–30 year period. This tendency is shown in **Fig. 5**, and highlighted by selected longitudinal studies from USA (red), Denmark (pink), and the Netherlands (brown-green). In Denmark, the age at onset of thelarche has decreased to about 10 years in the period 2005–06 when compared to the other temporally earlier data points, where the age was approximately 11 years (**Fig. 5**).

Given the dissociation of age at thelarche and age at menarche, the length of the pubertal transition (from thelarche to menarche) appears to have become longer (Aksglaede *et al.*, 2009).

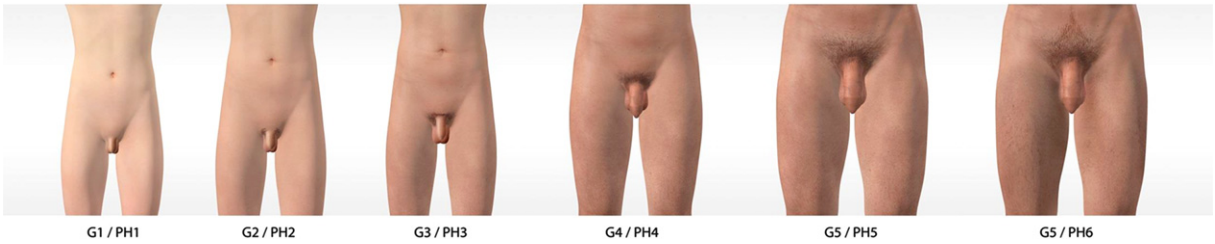

Fig. 3 Tanner scale, genital (G1–G5) and pubic hair (PH1–PH5) scale in boys (Copyright Claus Lunau A/S and Rigshospitalet, Copenhagen).

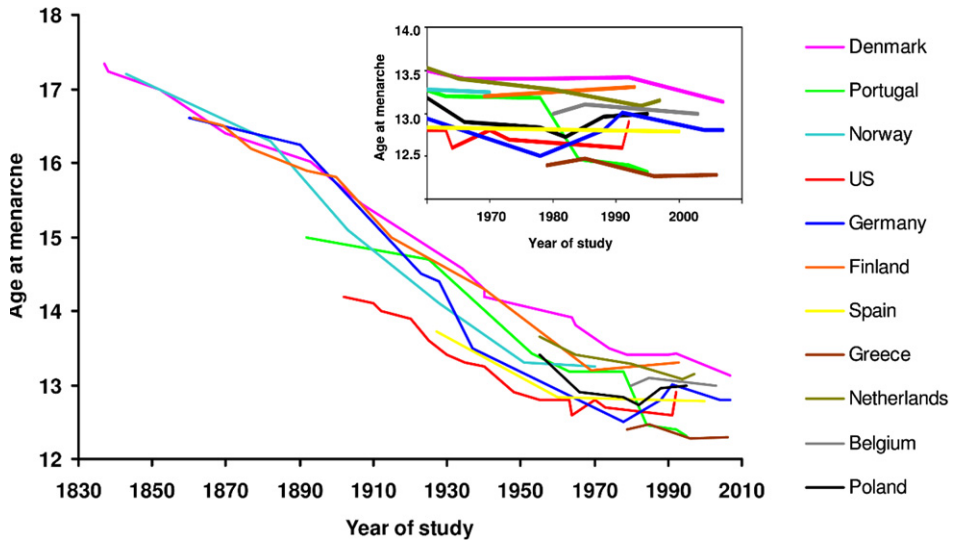

Fig. 4 Secular changes with reference to age of menarche (Sørensen *et al.*, 2012).

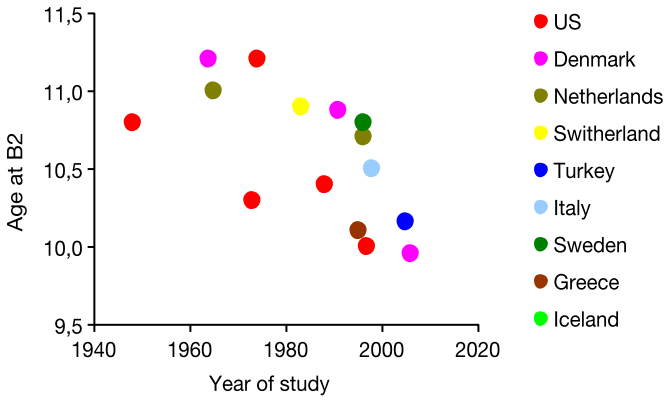

Fig. 5 Secular changes in age at onset of breast stage 2 (B2) (Sørensen *et al.*, 2012).

Table 1 Selected studies concerning female puberty in the US and Europe (Denmark): age at onset of thelarche and menarche

Study	*Study year*	*Study type*	*Cohort size (no.)*	*Subjects according to ethnicity (no.)*	*Ethnicity*	*Age at onset of thelarche (mean in year)*	*95% CI*	*Age at onset of menarche (mean in year)*	*95% CI*
United States									
NHANES III	1988–94	Cross-sectional	1623	466	White non-Hispanic	10.3	10.0–10.5	12.6	12.4–12.8
				589	Black non-Hispanic	9.5	9.3–9.8	12.2	12.0–12.4
				568	Mexican American	9.7	9.4–9.9	12.2	12.0–12.5
PROS	1992–93	Cross-sectional	17,077	15,439	White	10.0	9.9–10.1	12.9	12.8–13.0
				1638	African-American	8.9	8.7–9.1	12.2	12.0–12.4
BCERP	2004–11	Longitudinal	1239	420	White non-Hispanic	9.6	9.5–9.7		
				391		8.8	8.6–9.0		
				57	African-American	9.2	9.0–9.4		
				371	Hispanic Asian	9.9	9.6–10.2		
Europe (Denmark)									
The Copenhagen Puberty Study	1991–93	Cross-sectional	1100	1100	White—Caucasian	10.9	10.7–11.1	13.4	13.2–13.6
	2006–08	Cross-sectional	995	995	White—Caucasian	9.9	9.7–10.0	13.1	13.0–13.3

Since the 1980s, different studies reporting ages at thelarche and menarche in the US, have indicated a new secular trend in puberty timing in girls (**Table 1**). In this article three selected large studies from the US and one from Denmark are included.

The first study from the US was based on data from the Third National Health and Nutrition Examination Survey (NHANES III) (Wu *et al.*, 2002) within the period 1988–94. The study included 1623 participants subdivided into three groups according to their ethnicity (White non-Hispanic, Black non-Hispanic or Mexican American). Whilst the second was based on the Pediatric Research in Office Settings network (PROS) (Herman-Giddens *et al.*, 1997) where data was collected on 17,077 participants within the period 1992–93 (**Table 1**). The PROS participants had White or African-American ethnic background. These studies assessed both age at onset of thelarche and menarche.

The most recent study from the US, The Breast Cancer and the Environment Research Program (BCERP), was collected from 2004 to 2011 in different geographic areas (Biro *et al.*, 2013). The study included 1239 participants divided into four groups according to their ethnicity (African-American, Hispanic, White non-Hispanic or Asian) and only used thelarche as an indicator of puberty onset (**Table 1**).

The results from the NHANES III study indicate a large variation in the timing of thelarche according to ethnicity, with an earlier age onset in the Black non-Hispanic and Mexican-American participants compared to the White non-Hispanic participants,

but with no evident difference in the timing of menarche according to ethnicity. This reflects the genetic component related to the pubertal onset. These results were replicated in the PROS study, reporting that girls with African-American ethnicity developed breast tissue at an earlier age compared to Caucasian girls, whilst the timing of menarche showed no trend according to ethnicity. The BCREP study also supported this trend in thelarche onset according to ethnicity but did not report on age at onset of menarche.

When assessing secular time trends in puberty onset, the studies from the US suggest a clear decline in the age of thelarche within the study period with no concurrent decline in the age of menarche (**Table 1**), albeit onset of menarche was not reported in the newest of the US studies. In Denmark, Northern Europe, two recent studies, following children from the same town, have been conducted. The Copenhagen Puberty Study included girls from two different cohorts collected from 1991 to 1993 and from 2006 to 2008 and used rigorous methods for pubertal assessment (breast tissue palpation, *thelarche*) and found that onset of thelarche in girls in the period 2006–08 was a full year earlier than among those included 15 years earlier (**Table 1**) (Aksglaede *et al.*, 2009). The downward changes in age at menarche onset was not as pronounced as the changes observed for breast development (*thelarche*), albeit the 4 month decrease in age at menarche is still a remarkable change within the 15-year period. The estradiol level was measured in all girls included in both of the abovementioned cohorts and data showed a decline in estradiol levels in the 2006–08 cohort compared to the 1991–93 cohort, despite the earlier age at onset of breast development. Additional, adjustment for body mass index (BMI) did not change the results. These findings indicate a decrease in age at thelarche with a lower concurrent decrease in menarche during the intervening period between the two Danish cohorts, which most likely was not caused by increased BMI or higher estradiol levels in the 2006–08 cohort.

In summary, different largescale studies, both in the US and Europe, indicate a worldwide secular trend towards earlier age at onset of breast development (*thelarche*) among otherwise healthy girls. However, age at menarche has not declined to the same extent as the age at thelarche onset within the same period, indicating that the overall pubertal period (thelarche to menarche) has increased. Additionally, a clear variation in timing of thelarche, in relation to ethnicity, is pronounced in all the US studies.

Secular Trends in Puberty Timing in Boys

Pubertal onset in boys has received less attention and fewer, mainly smaller studies, are available. Based on the available studies on pubertal onset in boys, defined as Tanner, genital stage 2 (G2) or a testicular volume enlargement over 3 mL, there are indications of a secular trend towards earlier onset within the last 30 years. In 1980, the age at onset of puberty (G2) in boys was approximately 12.0 years while the age at onset has declined to about 11.5 years in 2000 (**Fig. 6**) (Sørensen *et al.*, 2012).

Of the few more comprehensive studies that have been performed in boys, are the NHANES III study (Karpati *et al.*, 2002) and The Copenhagen Puberty Study (**Table 2**). The NHANES III study collected data on 2481 boys and in line with results from girls show puberty G2 onset variation according to different ethnical backgrounds. White non-Hispanic boys developed Tanner, G2 stage at 10.1 years, Black non-Hispanic at 9.3 years and Mexican American at 10.4 years. The Copenhagen Puberty Study from Northern Europe examined white Caucasian boys from two different cohorts collected from 1991 to 1993 and from 2006 to 2008. In the 1991–93 cohort, the age of onset of testicular enlargement over 3 mL was 11.92 years, whilst in the 2006–08 cohort this age had decreased to 11.66. Along with this Tanner, G2 onset decreased from 11.83 to 11.59 years between the two Danish puberty cohorts of boys (**Table 2**).

The data from NHANES III has never been compared with earlier US studies because of the insufficient quality of the earlier US studies (Sørensen *et al.*, 2010); and although the decline in age in The Copenhagen Puberty Study was borderline significant, this was not robust after adjustment for BMI (no longer significant) (Sørensen *et al.*, 2010). These limited data on pubertal onset in boys makes it difficult to conclude on the magnitude of any potential secular trend in pubertal timing.

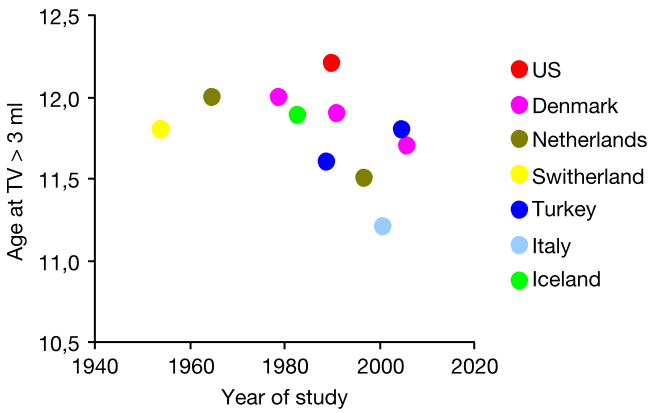

Fig. 6 Secular changes with reference to age of a testicular enlargement over 3 mL (Sørensen *et al.*, 2012).

Table 2 Selected studies concerning male puberty in the US and Europe (Denmark): age at a testicular enlargement over 3 mL and age at G2 stage

Study	*Study year*	*Study type*	*Cohort size (no.)*	*Data type*	*Subjects according to ethnicity (no.)*	*Ethnicity*	*Age at testis enlargement over 3 mL (year)*	*95% CI*	*Age at G2 stage (year)*	*95% CI*
United States										
NHANES III	1988–94	Cross-sectional	2481	Median		White non-Hispanic			10.1	9.6–10.6
						Black non-Hispanic			9.3	8.7–9.9
						Mexican American			10.4	9.6–11.2
Europe (Denmark)										
The Copenhagen Puberty Study	1991–93	Cross-sectional	824	Mean	824	White—Caucasian	11.92	11.76–12.08	11.83	11.66–12.00
	2006–08	Cross-sectional	704	Mean	704	White—Caucasian	11.66	11.49–11.82	11.59	11.42–11.76

Precocious Puberty

Assuming a Gaussian distribution of age of pubertal onset in the population, the normal range of puberty is defined as two times the standard deviation above and below the mean age of pubertal onset. In the European population, precocious puberty (PP) is defined as thelarche before the age of 8 in girls and a testicular volume enlargement over 3 mL in boys before the age of 9 (Teilmann *et al.*, 2005).

Incidence and Prevalence of Precocious Puberty

Different studies have examined the incidence of PP during the last decades. In Denmark, *Thamdrup* determined the incidence of early puberty to be 3–5 new cases per year in 1950 (Thamdrup, 1961). However, Thamdrup's study was performed before uniform registration of diagnosis in the national registry was introduced in Denmark, and may have underestimated the incidence. A more recent nationwide register based study in Denmark also analyzed the incidence of PP in Denmark in the period 1993 to 2001 using comprehensive national patient registries of PP based on International Classifications of Disease (ICD-10: E30.1 or E22). The study reported 50–70 new cases of PP (both sexes) per year in Denmark in that period and that incidence was constant within the study period. The incidence of new cases of PP within the study period according to sex was 8 cases per 10,000 girls aged 5–9 years and <0.5 per 10,000 girls aged 0–4 years; for boys the incidence was lower: (<1 per 10,000) for boys younger than 8 years and 1–2 per 10,000 boys aged 8–10 years. The prevalence of PP according to sex within the study period was 20–23 cases per 10,000 girls and under 5 per 10,000 boys (Teilmann *et al.*, 2005).

Another single-center study performed in the Capital Region of Denmark reported a marked increase in the number of referrals of girls with signs of early puberty, increasing from 6 new referrals in 1993 to approximately 80 new referrals in 2008 (Mogensen *et al.*, 2011). In 2016 the number of referrals in that center had increased even further to >200 girls with signs of early puberty (personal communication, A. Juul, 2018). Another Danish study performed in a different Center included 191 girls referred with early puberty from 1998 to 2012. The incidence of early puberty onset had increased from approximately 1 per 10,000 in 1998 to 4 per 10,000 in 2012 (Sømod *et al.*, 2016).

The reviewed studies from Denmark vary in methodology, and registration of precocious puberty and its variants (premature thelarche, premature adrenarche) are not uniformly evaluated between studies. Nevertheless, we suggest that the incidence of girls with signs of early puberty referred to Danish hospitals appears to have increased during the last decades.

Studies from other parts of the world indicate a similar rise in the number of cases who present signs of early puberty. A Korean group reviewed data on the use of GnRH analogues in children with PP based on data from the Korean Health Insurance Review Agency from 2004 to 2010 (Kim *et al.*, 2015). The study found the annual incidence of early puberty in girls had increased from 0.3 to 5 per 10,000 girls during the study period. The incidence in boys increased from 0.03 to 0.12 per 10,000 boys. Both the Danish and the Korean studies found the increase in incidence of PP to be more pronounced in girls compared to boys. In France, a nationwide study was performed from 2011 to 2013. Data from that study determined the incidence of early onset of puberty to be 2.7 per 10,000 girls and 0.24 per 10,000 boys (Le Moal *et al.*, 2018). This supports the trend observed in Denmark during the last decades.

It is likely that the possible changes in the incidences of PP are caused by a secular trend towards a lower mean age at pubertal onset. As age at pubertal onset approximates a Gaussian distribution, a lower mean age at pubertal onset results in an increase in the proportion of the distribution below the lower bound of the 95% confidence interval. Alternatively, the increase of PP cases could be due to an increase in the skewness of the distribution towards the lower bound. The Copenhagen Puberty Study did not

find any differences between the data where a Gaussian distribution was applied compared to data where skewedness was taken into account (Aksglaede *et al.*, 2009). The findings from The Copenhagen Puberty Study have been applied to illustrate the increase in the number of healthy girls from 1991 to 1993 and from 2006 to 2008, respectively, who would meet the diagnostic criteria of PP (early onset of Tanner, B2) (**Fig. 7**).

Data concerning boys from The Copenhagen Puberty Study were used to construct **Fig. 8**, illustrating the change in the number of healthy boys who would meet the diagnostic criteria of PP.

The increase in the PP incidence in boys illustrated in **Fig. 8** seems to be much lower compared to that of girls in accordance with well-known clinical observations.

These considerations based on data from The Copenhagen Puberty Study illustrate the potential problems with the current diagnostic criteria for PP when considering the secular trend towards earlier onset of thelarche. The causes of PP are numerous and they may be either non-pathological or pathological. Because of this, examination of the girls, with early pubertal development, is very important. Revision of the diagnostic criteria for PP has been debated (Sørensen *et al.*, 2012), including a lowering of the appropriate age below which a brain Magnetic Resonance Image (MRI) should be performed. With a lowering of the lower limit of

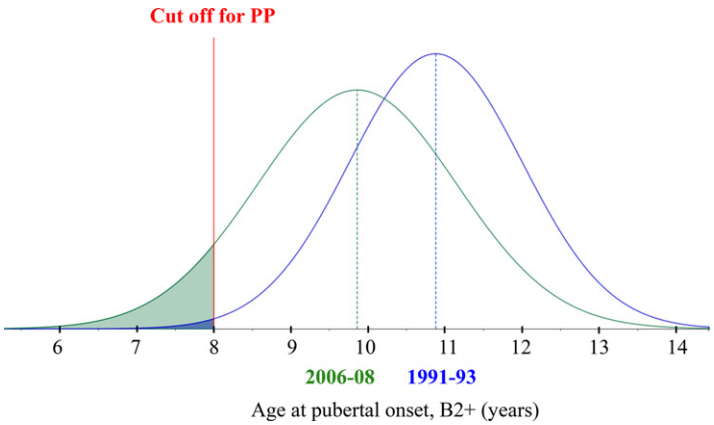

Fig. 7 Gaussian distribution curves for the two examination periods in The Copenhagen Puberty Study in girls: the blue curve depicts the distribution of pubertal onset (Tanner, B2) from the 1991 to 1993 examination period and the green curve depicts the distribution of pubertal onset from the 2006 to 2008 examination period. The mean ages at B2 onset from the different examination periods are shown by the dotted lines. The PP diagnosis criteria, that is, thelarche before the age of 8, are visualized by the red line, and the shaded area, under the left side of the curves, represents the number of girls falling into the PP category. Following data have been used: examination period 1991–93: mean 10.88 years and 2SD 8.66–13.11 and examination period 2006–08: mean 9.86 years and 2SD 7.28–12.44 (Aksglaede *et al.*, 2009).

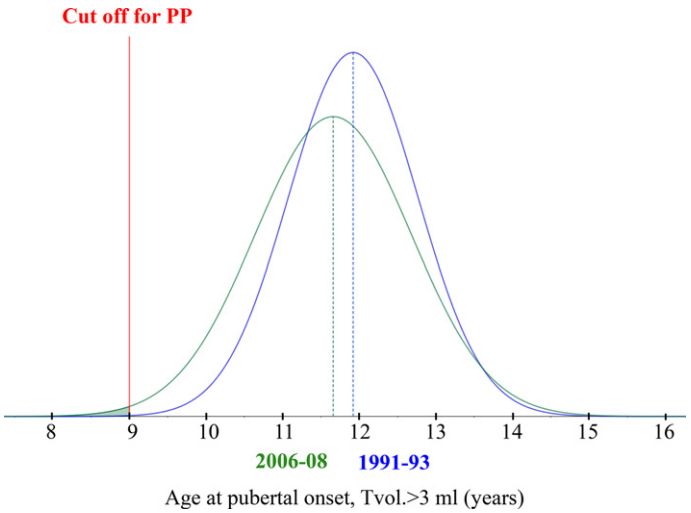

Fig. 8 Gaussian distribution curves for the two examination periods in The Copenhagen Puberty Study in boys: the blue curve represents the 1991–93 examination period and the green curve represents the 2006–08 examination period. The estimated mean ages for the two examination periods are marked by the dotted line. The shaded area under the left side of the curves represents the number of boys falling into the PP category. Following data have been used: examination period 1991–93: mean 11.92 years and 2SD 10.25–13.59 and examination period 2006–08: mean 11.66 years and 2SD 9.62–13.70 (Sørensen *et al.*, 2010).

normal puberty, an increasing number of children would be eligible for brain imaging. The main argument against revising the definition of PP is the potential of missing pathological cases (Sørensen *et al.*, 2012). Due to the significant secular changes in the norm on the one hand, and this concern of overlooking pathologies, the diagnostic criteria and ages below which a brain MRI should be performed remain controversial.

Possible Mechanisms for the Secular Trends in Pubertal Timing

As discussed earlier in this article, puberty timing and subsequent reproductive capacity are determined by changes in the secretion of the pituitary gonadotropins, LH and FSH, which are dependent on the frequency and amplitude of GnRH secretion from the hypothalamus. Genetic factors play an important role in the timing of these signals. Additionally, signals linked to the environment such as nutrition, stressors, and endocrine disruptors might interfere with the hypothalamic signaling system, directly or indirectly, via peripheral signaling. Knowledge about the precise mechanisms of action is not fully understood, but possible mechanisms are presented in this section.

Genetic Factors

Pubertal timing is a highly polygenic trait and different genetic variants have been shown to influence pubertal timing. The heritability has been found to variate from 0.50 to 0.68 in boys (Silventoinen *et al.*, 2008) and 0.57 (in relation to the age at menarche) in girls (Anderson *et al.*, 2007). The significance of genetic factors is also highlighted by the wide variation in pubertal onset between different ethnic groups. In the NHANES III study, the age at pubertal onset in girls varied 0.8 years between the white non-Hispanic and the Black non-Hispanic participants. The knowledge about genetic factors is still scarce, however, presumably genetic factors per se cannot account for the recent secular trends, but potential susceptibility genes may be indirectly involved in gene-environment interactions that could account for the secular trend.

Body Mass Index, Nutrition and Socioeconomic Status

BMI has a very important positive association to pubertal timing in girls; high BMI being associated with early breast development and low BMI being associated with pubertal delay. In boys a similar association between BMI and pubertal timing has been shown, although a U shaped association has been suggested within the normal BMI range, where very obese boys appear to enter puberty later. Thus secular trends in BMI/obesity have been suggested as likely explanations for the US puberty epidemic, whereas BMI did not explain the trend towards earlier breast development among girls in Denmark (Aksglaede *et al.*, 2009). Thus, individual fat mass play an indisputable important role for activation of the HPG axis and pubertal onset in girls, but may not entirely explain the secular trend in pubertal timing.

Environmental Causes Including Endocrine Disruptors

Endocrine disrupting chemicals (EDC) are ubiquitous in our environment and numerous have been identified. In this section only manmade EDC will be considered. EDCs can originate from different kind of sources released to the environment intentionally, such as insecticides, plastic, lacquer, paint, pest-controllers or unintentionally by either degradation of industrial products and building materials or by leakage of packing materials into food items. All people are therefore exposed to EDCs to some extent because of its presence in the daily life. EDCs can potentially interfere with the sex hormone actions in many different ways; estrogenic actions, anti-androgenic actions, or by affecting aromatase activity. Thus, EDCs can potentially both accelerate as well as delay puberty in both boys and girls, respectively. The chemicals can act at different levels for instance direct on the central parts of HPG-axis, the gonads, peripheral target organs for example the breast tissue and cause early maturation by direct estrogenic actions. By contrast, other EDCs can act as anti-androgens and delay androgen-dependent signs of puberty like pubic hair development.

Persistent EDCs like DDT and DDE have been banned for decades but still exist in the environment and bioaccumulate in humans. Exposure to DDT and DDE have been associated with earlier puberty, and similarly higher levels of DDT/DDE associated with earlier age at menarche (Ouyang *et al.*, 2005). Studies have indicated a correlation between high levels of PCB exposure and delayed pubertal development in boys (Den Hond *et al.*, 2002).

Flame retardants like PBB was evaluated in pregnant women (Özen and Darcan, 2011), and high PBB levels were associated with earlier menarche in the offspring.

Non-persistent EDCs like phthalates which can be found in many different everyday products for instance plastics, food packaging, cosmetics and medical devices have been shown to correlate with PP (Srilanchakon *et al.*, 2017), although controversy exist. Two different studies have reported on an association between phthalate exposure, a non-significant tendency towards earlier breast development, and a significant delay in the age at getting pubic hair (Frederiksen *et al.*, 2012; Wolff *et al.*, 2014), in accordance with their anti-androgenic actions. Other non-persistent EDCs like benzophenones have also been associated with delayed puberty in a large cross sectional study among contemporary US girls (Wolff *et al.*, 2015). The possible association

between pubertal timing and human exposure to a cocktail of hundreds of different EDCs at the same time, with estrogenic and anti-androgenic actions remain a difficult task to evaluate.

Conclusion

The age at pubertal onset in girls and boys has declined during the last decades, and an increase in the incidence and prevalence of precocious puberty particularly in girls has been documented. Potential reasons for this development could be related to changes in BMI, nutrition, socioeconomic status and exposure to endocrine disrupting chemicals.

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Delayed Puberty; Male☆

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Introduction

Puberty is a unique and integrated transition from childhood to emerging adulthood and then to young adulthood. It includes major physical, emotional, and psychological changes, for which the attainment of fertility signals its completion; however, in none of these three spheres is maturation or development complete at the attainment of fertility. Puberty marks the time of greatest growth and sexual maturation since fetal life, and it is marked externally by the development of the secondary sexual characteristics for each gender and by major alterations in linear growth, body composition, and the regional distribution of body fat. All are subserved by qualitative and quantitative alterations in multiple hypothalamic–pituitary–end-organ axes, especially those for the gonad and for growth hormone (GH)/insulin-like growth factor I (IGF-I).

Growth at Puberty

Normally growing children have a relatively constant annual growth rate of 5–6 cm after 4–5 years of age until the onset of the pubertal process. The pubertal growth spurt may be divided into three phases: the first phase involving minimal velocity just before the spurt ("takeoff" velocity), the phase of rapid acceleration to peak height velocity (PHV), and the phase of diminished velocity and cessation of growth at the time of epiphyseal closure of the long bones. The individual pattern depends on the timing and tempo of the developmental process. Those with a significant delay often have a peripubertal decrement in height velocity (to a so-called preadolescent "dip") before the accelerated velocity of the growth spurt phase.

The sequence of pubertal alterations in linear growth and body composition is more tightly aligned to changes in the hypothalamic–pituitary–gonadal (HPG) and GH/IGF-I axes than to chronological age. In most Western populations, the age at initiation of the accelerated phase in boys averages ~11.0 years, and the age at PHV averages ~13.5–14.0 years, with a growth rate of 10–11 cm year^{-1}. This biological "anchor" occurs at mid- to late pubertal development. Maximal growth in body weight during adolescence usually occurs after PHV. It is composed mainly of an increase in fat-free mass (muscle) with a minimal increment in fat mass, leading to a decrease in the percentage of body fat during the later stages of pubertal development.

Secondary Sexual Characteristics

The timing and tempo of pubertal maturation are linked to alterations in the HPG and GH/IGF-I axes. There may be great variability in the onset of pubertal development, but once entrained, there is less variability in progress through the pubertal stages. However, there is still significant variability in the progression of puberty (tempo) that may be very relevant to boys with delayed adolescent maturation. The external signs of pubertal development have been summarized into relatively easily discernible stages. In general, scrotal thinning and reddening occur concomitantly with an increase in testicular volume to 4 mL or greater. As testis size increases and the levels of the male hormone testosterone increase, there is lengthening of the phallus followed by an increase in its circumference as it further lengthens. Pubic hair increases in area from just above the phallus to growth in all directions, including the medial thigh. The quality of the hair becomes more coarse and curly. With delayed pubertal maturation, the chronological age differs from the "biological" age. The various stages of growth noted previously—takeoff and PHV—and the hormonal changes noted in what follows are more tightly correlated to biological age than to chronological age.

The Endocrine System and Pubertal Development

Gonadotropins

After peaks of activity during fetal life and within the first few months of extrauterine life, the HPG axis becomes quiescent, only to reawaken at puberty. Gonadotropin release is pulsatile at all ages. Puberty is anticipated by an increase in the amplitude of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion that may be detected several years before the external signs of pubertal development are present. Initially, biologically relevant surges ("pulses") of LH occur only after the onset of deep

☆*Change History*: December 2017. Alan D Rogol added a new section concerning two hormones, anti-Mullerian (AMH) and inhibin B (IN b). References to support the data for these new hormones have been added and more recent review articles to justify other material added.

This chapter is an update of Alan D. Rogol, Delayed Puberty, Male, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 635-638.

sleep. These permit the testes to increase the production of the potent androgen testosterone. However, the hypothalamic–pituitary axis for LH remains exquisitely sensitive to the negative feedback actions of testosterone or one of its metabolites, estradiol, such that the minor increases in testosterone are able to dampen LH release. As puberty unfolds, the small increments in testosterone levels are no longer able to maintain suppressed levels of LH. The increases in LH continue for longer and longer periods during the day, pushing testosterone levels even higher. Concomitantly, the feedback sensitivity of the HPG axis diminishes and the ambient levels of testosterone are no longer sufficient to suppress the surges of LH. At the end of the pubertal process, the pulsatile release of LH occurs every 1 or 2 h during the day and night.

Anti-Mullerian Hormone and Inhibin B

Anti-Mullerian hormone (AMH) is a marker for (immature) Sertoli cell function. It is low in early infancy, peaks several years later, and remains high as pubertal maturation begins. Its levels then decrease from early mid-puberty to steady low levels characteristic of the adult. Androgens themselves are potent inhibitors of AMH production, and high levels in late puberty may be useful in narrowing the differential diagnosis of hypogonadism.

Inhibin B is also a gonadal glycoprotein produced by the Sertoli cell. It is a specific inhibitor of FSH (and spermatic function). Its levels also peak in infancy, decrease during childhood, and again increase at early to mid-puberty. It may serve as a marker for spermatic function. As inhibin B levels fail to rise, the levels of FSH increase in an attempt to stimulate the testicular tubules to drive spermatogenesis.

Gonadal Steroid Hormones

Nighttime increases in circulating levels of testosterone are often detectable even before the external signs of puberty develop. The daytime levels rise later as the testis volume increases. The circulating levels of testosterone are substrate for at least two important enzymes, 5α-reductase (which converts testosterone to dihydrotestosterone) and aromatase (which converts testosterone to estradiol). The effects on the lean body mass are in part due directly to testosterone and indirectly to estradiol, because the latter leads to the marked increases in GH and IGF-I due to an action of estradiol on the hypothalamus and pituitary. Circulating estradiol levels cause maturation and eventually closure of the epiphyses of the long bones, signaling the virtual cessation of linear growth. Diminished bone mass is present in boys with delayed pubertal development, including severe constitutional delay in growth and puberty (CDGP).

Growth Hormone and Insulin-Like Growth Factor I

GH levels increase throughout the first stages of pubertal development to reach a maximum at the time of PHV. The levels of IGF-I also rise, denoting a switch to positive feedback or at least an absence of negative feedback action of IGF-I on GH release. Although serum testosterone levels increase markedly by PHV, it is likely that the conversion to estradiol is responsible for the alteration in pulsatile GH release.

Summary and Integration

During pubertal development, there is an intricate interplay between the increased activation of the HPG and the GH/IGF-I axes. Both contribute to the pubertal spurt in linear growth and to the marked alterations in body composition and the regional distribution of body fat. They have independent and at least additive, if not synergistic, actions at puberty.

Causes of Delayed Pubertal Development in Boys

Delayed pubertal development has many causes, both physiological (functional) and pathological. It usually occurs because of inadequate secretion of the gonadal steroid hormones, especially that of testosterone in boys. The causes may be conveniently separated into hypogonadotropic (central) and hypergonadotropic causes, depending on primary hypothalamic–pituitary dysfunction or primary gonadal failure.

Of these categories, the physiological causes are by far the most common, especially in 14- to 16-year-old boys. As the second decade of life ends, the likelihood of finding a pathological cause rises sharply (see **Table 1**).

Evaluation of Delayed Puberty in Boys

As with any evaluation that includes growth and maturation, one begins with the growth curve. Normal growth over an extended period of time speaks to the overall general good health and well-being of the individual. The childhood growth rate is relatively constant, usually between 5 and 7 cm year^{-1}. In boys who undergo puberty at the usual time, there is an almost imperceptible

Table 1 Causes of delayed pubertal development in boys

Hypergonadotropic (primary) hypogonadism
Congenital
Chromosomal abnormality
Klinefelter syndrome 47 XXY and variants
Anorchia (vanishing testis syndrome)
Acquired
Trauma (or surgery)
Chemotherapy or radiation therapy for childhood neoplasia
Postinfectious (e.g., mumps and orchitis)
Hypogonadotropic (secondary) hypogonadism
Congenital
Various forms of hypothalamic–pituitary disease
Gonadotropin-releasing hormone deficiency
Multiple pituitary hormone deficiencies
Congenital malformations as part of holoprosencephalic anomalies
Acquired
Tumors, particularly craniopharyngioma
Pituitary apoplexy
Malnutrition and acute and chronic systemic disease
Endocrine deficiencies
Hypothyroidism
Hyperprolactinemia
Head trauma
Physiological
Constitutional delay of growth and puberty

slowing of growth (the takeoff point). This is followed by rapid acceleration to PHV and then deceleration toward zero velocity as the epiphyses of the long bones close. The greater the delay in pubertal development, the greater the slowing of growth before takeoff—often called the preadolescent "dip." This occurs at the time that one's peers are undergoing their most rapid growth, highlighting the differences among likely normal individuals. With quite delayed puberty, the rate may dip as low as 4.0–4.5 cm in the year before the pubertal spurt. However, this knowledge does not mitigate the distress of the smaller, often slighter, immature individual.

The evaluation is no different from any other medical evaluation in that the history can often give clues as to what to look for in the physical examination, or what laboratory tests to perform to integrate the information to produce a differential diagnosis. Of particular importance in the history is the pattern of growth, not only of the child but also of his siblings and parents. The pubertal development of the siblings and parents can be of help as well, because there is some familial patterning in the timing and tempo of pubertal maturation.

Although it is not often emphasized, one should take a detailed dietary history because delayed pubertal development may be related to a total energy deficit—caloric deprivation and/or excessive expenditure. Prior medical conditions and some of the mediations used to treat them can delay growth and pubertal maturation. There are many individual organ systems that may be disordered and cause delayed growth and pubertal development. Many are related to the gastrointestinal (GI) system—decreased intake and/or excessive caloric losses. The other major organ systems that can be affected are the endocrine system and central nervous system (CNS). Disorders of the HPG, GH/IGF-I, thyroid, and adrenal axes can lead to delayed growth and sexual maturation. CNS disease may be heralded by headaches, visual disturbance, seizures, and nausea and vomiting.

The general physical examination might not be too rewarding unless there are congenital (or acquired) anomalies. The typical boy with delayed pubertal development has a relatively normal physical examination—but for that of a younger child. The key elements are the measurements of height and weight and the genitalia, including pubic hair. There are convenient stages to mark pubertal progression; noting the Tanner stages can integrate not only the timing but also the tempo of pubertal development.

Laboratory testing that may help to define the diagnosis includes the hemogram and tests of liver, kidney, and GI function. From the endocrine point of view, one seeks evidence for low IGF-I and IGF binding proteins-3, low thyroxine level, low testosterone level, or high prolactin or cortisol level. The detailed endocrine evaluation is beyond the scope of this article.

Imaging studies may help; an X-ray of the left hand and wrist can indicate a bone age (biological age). That is critical as one tries to determine whether the delay is significantly outside the normal variation. Other imaging, whether CNS or GI or the like, should follow from clues gathered in the history, physical examination, and laboratory evaluations. A karyotype is indicated if Klinefelter syndrome is considered. Boys with this condition often have delayed tempo of puberty, but are of normal or slightly increased stature.

The younger the child during his teenage years, the more likely that physiologically delayed puberty (CDGP) is the correct "diagnosis." Treatment depends on the specific diagnosis.

Treatment of Delayed Pubertal Development in Boys

For most causes of delayed puberty, testosterone administration will form part of the therapeutic plan. It may be exogenous (usual) or may involve merely waiting for the endogenous testosterone to be secreted. By the time most boys with CDGP arrive in the specialist's office, testosterone therapy has already been considered. It is critical to start slowly and increase the dose gradually, so as not to cause epiphyseal closure that is too rapid and, subsequently, shortened adult height. Testosterone therapy can be halted as the testes increase in size (to ~8–10 mL), in concert with increasing endogenous testosterone secretion. Testosterone comes in many forms: gel, nasal gel, buccal, injectable, and implantable (pellets). The vast majority of use in pubertal boys is "off label" and beyond the scope of this report. An additional concern with the use of cutaneous forms of testosterone is the transference to others, especially children and women.

For pathological conditions, the treatment of the individual obviously depends on the diagnosis. It may be as simple as adding calories or dampening the activity of inflammatory bowel disease, or it may be as complex as removing a CNS tumor or correcting significant renal failure. Many of the hormonal deficits can be replaced (e.g., GH, thyroxine, and testosterone), and some of the excesses can be dampened (e.g., hyperprolactinemia and Cushing's syndrome).

Whatever the cause, it is critical to follow the growth and maturation status over time (trajectory). The amount of testosterone given should escalate over time to mimic the natural evolution of puberty. Physiologically delayed puberty is very common in 12- to 15-year-old boys. As they become older adolescents, it is less likely that a physiological cause for delayed pubertal development will be found. Often, it is the disease and distress that are the most prominent symptoms, and discussions with the patient and his parents along with short-term therapy with testosterone may be all that is required to help the boy through pubertal maturation and toward emerging adulthood. In addition to growth and genital development, there are remarkable alterations in body composition, including bone mass and fat distribution. Dissatisfaction with his body image is often a cardinal feature of the adolescent boy's distress, and therapy with testosterone has major salutary effects.

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Delayed Puberty and Hypogonadism; Female☆

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Self-Limited Delayed Puberty

Self-limited delayed puberty (DP), also known as constitutional delay of growth and puberty (CDGP), represents the extreme end of normal pubertal timing, and is defined as the absence of breast development in girls at an age that is 2–2.5 standard deviations (SD) later than the population mean (Palmert and Dunkel, 2012). 50%–75% of subjects with self-limited DP have a family history of delayed pubertal onset (Sedlmeyer, 2002). Familial self-limited DP has a clear genetic basis. It is a highly heritable condition, which segregates in an autosomal dominant pattern (with or without complete penetrance) in the majority of families. Autosomal recessive, bilineal and X-linked (Sedlmeyer, 2002), as well as sporadic cases are also observed. However, the underlying neuroendocrine pathophysiology and genetic regulation has been largely unknown.

Some insights into the genetic mutations that lead to familial self-limited DP have come from sequencing genes known to cause GnRH deficiency, most recently via next generation sequencing. Linkage analysis and targeted sequencing strategies that have provided initial insights in this field (Cousminer *et al.*, 2015; Wehkalampi *et al.*, 2008) have been mostly superseded by whole exome and genome sequencing strategies to identify novel candidate genes. Recently, whole exome and targeted resequencing methods have implicated two pathogenic mutations in *IGSF10* as the causal factor for late puberty in six unrelated families from a large Finnish cohort with familial DP (Howard *et al.*, 2016). Mutations in *IGSF10* appear to cause a dysregulation of GnRH neuronal migration during embryonic development, which presents in adolescence as DP without previous constitutional delay in growth.

In view of the possible overlap between the pathophysiology of DP and conditions of GnRH deficiency, a few studies have specifically examined the contribution of mutations in CHH genes to the phenotype of self-limited DP. Mutations in *HS6ST1*, *FGFR1*, *GNRHR* and newly in *KLB* have been found in a small number of kindreds of CHH patients and their relatives with DP (Tornberg *et al.*, 2011; Pitteloud *et al.*, 2006; Xu *et al.*, 2017; Lin *et al.*, 2006). Variants in several HH genes including *GNRHR*, *TAC3*, *TACR3*, *IL17RD* and *SEMA3A* have been identified by whole exome sequencing in some cases of DP, including self-limited DP (Zhu *et al.*, 2015). However, these variants have not been tested in vitro or in vivo for pathogenicity and thus may be an overestimation. Mutations of the β-subunits genes of LH or FSH are extremely rare causes of pubertal abnormalities (Themmen and Huhtaniemi, 2000). Females with inactivating mutations of *LHB* present with onset of normal puberty, but with normal or late menarche followed by infertility due to lack of ovulation (Themmen and Huhtaniemi, 2000). Individuals with inactivating *FSHB* mutations present with incomplete pubertal development and primary amenorrhea in females (Layman *et al.*, 1997). Despite a large body of evidence for kisspeptin as one of the most important elements of the neural network responsible for GnRH pulse generation, only very rarely have human mutations in *KISS1* been found in patients with delayed or absent puberty (Topaloglu *et al.*, 2012). The excitatory neuropeptide, neurokinin b, also plays a role in the upstream control of GnRH secretion. Of 50 self-limited DP patients investigated for mutations in *TAC3* and *TAC3R*, only one mutation in a single patient was found in the latter gene (Tusset *et al.*, 2012). Overall, the current picture indicates that the genetic background of HH and DP may be largely different, or shared by as yet undiscovered genes (Vaaralahti *et al.*, 2011). Very recently, rare heterozygous variants in *FTO* have been identified in pedigrees with self-limited DP associated with extreme low BMI and maturational delay in growth in early childhood (Howard *et al.*, 2017).

The absence of pathological medical history, signs and symptoms, and positive family history of DP in one or both of the parents suggests a diagnosis of self-limited or constitutional DP, but before making the diagnosis, significant pathological conditions (e.g., central nervous system [CNS] tumors) should be excluded. Girls with constitutional delay usually have delayed maturation during early childhood, and consequently they are shorter than their peers (Crowne *et al.*, 1991). Adrenarche may also occur later than usual, in contrast to the normal age of adrenarche in patients with isolated hypogonadotropic hypogonadism. Bone age is retarded compared to chronological age, but the developmental milestones are achieved at a normal bone age; that is, onset of signs of pubertal development by the bone age of 12 years and menarche at the bone age of 13 years (Palmert and Dunkel, 2012). Gonadotropin and estradiol concentrations increase in concert with the development of the bone age. Thus, all stages of pubertal development occur at an age later than usual. However, it appears that about half of girls with CDGP do not reach their target height, based on parental heights (Bramswig *et al.*, 1990). Probably because of low estrogen concentration for chronological age (but not for bone age), growth hormone (GH) secretion is functionally and temporally impaired for age, and when this functional GH deficiency lasts for a

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Table 1 Common causes of delayed puberty

	Hypergonadotropic hypogonadism	*Hypogonadotropic hypogonadism*	*Functional hypogonadotropic hypogonadism*
Common causes	Klinefelter syndrome Gonadal dysgenesis Chemotherapy/radiation therapy	CNS tumors/infiltrative diseases Isolated hypogonadotropic hypogonadism Kallmann syndrome Combined pituitary hormone deficiency Chemotherapy/radiation therapy	Inflammatory bowel disease Coeliac disease Anorexia nervosa Hypothyroidism Excessive exercise Cystic fibrosis

sufficiently long period of time, it may also have an impact on adult height. After the onset of puberty or the initiation of appropriate estrogen treatment, growth velocity and GH secretion return to normal. Final height cannot be increased with GH treatment, although GH treatment may temporarily increase growth velocity in some girls. Self-limited DP is associated with adverse health outcomes including short stature, reduced bone mineral density and compromised psychosocial health (Zhu and Chan, 2017).

There are three main groups of differential diagnoses of self-limited DP (**Table 1**): functional hypogonadism, GnRH deficiency leading to hypogonadotropic hypogonadism (HH), and disorders causing primary hypogonadism (Sedlmeyer and Palmert, 2002; Palmert and Dunkel, 2012), although up to 30 different etiologies underlying DP have been identified (Varimo *et al.*, 2017).

Hypogonadotropic Hypogonadism

Gonadotropin-releasing hormone (GnRH) and gonadotropin deficiency can be caused by various genetic or developmental defects of the hypothalamic–pituitary axis, or by destructive lesions such as tumors, inflammatory processes, vascular lesions, and trauma (Silveira and Latronico, 2013). Usually, patients with isolated gonadotropin deficiency have normal height for their age during the prepubertal period, in contrast to patients with constitutional delay, who are often short. In hypogonadotropic hypogonadism, gonadotropin responses to GnRH stimulation may be subnormal, but because of the functional hypogonadotropism in constitutional delay, the differential diagnosis between these two conditions may be difficult.

Functional Gonadotropin Deficiencies

Anorexia Nervosa

Anorexia nervosa is usually associated with severe or even fatal weight loss, which is due to distorted body image, obsessive fear of obesity, and avoidance of food (Munoz-Calvo and Argente, 2016). Virtually all patients have primary or secondary amenorrhea. Functional hypogonadotropic hypogonadism is at least partly due to severe weight loss, but amenorrhea may also precede the onset of weight loss (Frisch and McArthur, 1974). The underlying pathophysiology of amenorrhea is due to GnRH deficiency because the luteinizing hormone (LH) secretory pattern in pubertal-aged girls with anorexia is similar to that seen in girls during pre-puberty: low or absent LH pulses and blunted LH response to exogenous GnRH (Munoz and Argente, 2002). Long term pulsatile administration of GnRH has been shown to restore a pubertal pattern of LH secretion, confirming the hypothalamic location of the defect. Recovery of normal weight will normalize most endocrine and metabolic functions, but amenorrhea may persist for years (Miller, 2013).

Athletic training

Overly intensive exercise may inhibit GnRH secretion, arrest pubertal development, and cause amenorrhea. These disorders are common especially among long-distance runners, gymnasts, and ballerinas (Barrack *et al.*, 2013). Hypogonadotropic hypogonadism may develop even when the female athletes have normal weight but have less fat and more muscle compared to non-athletic girls. In female athletes with delayed or arrested pubertal development, adrenarche usually takes place at the normal age. The mechanism of delayed puberty is unclear, but interruption of intensive training advances puberty and menarche before any change in body composition or weight, suggesting a direct effect of physical activity on GnRH secretion.

Malnutrition and chronic diseases

In malnutrition and chronic diseases, weight loss below the level of 80% of ideal body weight can cause delayed or arrested pubertal development (Pozo and Argente, 2002). Nutrition plays an important yet uncharacterized role in the control of GnRH secretion. For example, in regional enteritis, gonadotropin secretion remains normal if nutrition is optimally

balanced, but a non-optimal nutritional status will result in a hypogonadotropic state and arrested pubertal maturation. Chronic renal insufficiency delays pubertal development, but after successful renal transplantation, gonadotropin secretion is usually restored.

Central Nervous System Tumors

Tumors causing delayed puberty most commonly interfere with GnRH synthesis or secretion. Deficiency of other pituitary hormones is common. Associated posterior pituitary hormone deficiencies are often manifested by diabetes insipidus.

Craniopharyngioma

The most common neoplasm causing hypothalamic–pituitary dysfunction and hypogonadism is craniopharyngioma (Tan *et al.*, 2017). It is actually a congenital tumor that most commonly becomes symptomatic between 6 and 14 years of age. At presentation, the most common symptoms are headache, visual disturbances, short stature, delayed puberty, polyuria, and polydipsia. The structure of the tumor varies from solid to cystic. MRI, both with and without contrast enhancement, is the imaging modality of choice. Whilst the characteristic calcifications and size of the tumor can be visualized with CT, the size and extent of the tumor, involvement of the third ventricle and cystic features of the tumor can be confirmed with MRI. Treatment consists of surgery and radiotherapy, but the recurrence rate is high even when complete surgical removal is attempted (Karavitaki *et al.*, 2005).

Langerhans' cell histiocytosis

Langerhans' cell histiocytosis (LCH), also called Hand–Schuüller–Christian disease or histiocytosis X, is characterized by infiltration of lipid-containing histiocytic cells in the skin, bone, and viscera. CNS involvement and, in particular, hypothalamic–pituitary involvement are well-described features of LCH. The precise incidence of CNS–LCH disease is unknown, and the natural history is poorly understood. Diabetes insipidus (DI) is reported to be the most common and well-described manifestation of hypothalamic–pituitary involvement (up to 50%). Anterior pituitary dysfunction has been reported in up to 20% of patients with LCH and occurs almost exclusively concurrently with DI (Montefusco *et al.*, 2017). The natural course of the disease is fluctuating, making evaluation of treatment effect difficult. Endocrine function is not improved following medical treatment of LCH with chemotherapy and glucocorticoids. All LCH patients should undergo a thorough endocrine evaluation periodically.

Germinomas

Germinomas are the most common extrasellar tumors to cause delayed puberty, although these tumors are a rarity among primary CNS tumors (Sato *et al.*, 2009). Polydipsia, polyuria, and visual disturbances are the most common symptoms associated with these tumors, followed by arrested growth and delayed puberty. Germinomas are commonly located in the pituitary stalk, in the suprasellar region of the hypothalamus, or in the proximity of the pineal gland. Seeding of the tumor to the cerebrospinal fluid is common, and this can also be used in the diagnosis, where examination of tumor markers (hCGß and alphafetoprotein) or germ cells (with positive placental alkaline phosphatase staining) in the cerebrospinal fluid may be helpful. These laboratory findings, together with clinical features and an excellent response to radiation therapy, are so characteristic that surgery is rarely indicated except for biopsy to establish histological diagnosis (Shibamoto, 2009).

Other Central Nervous System Disorders

Defects in development

Various malformations affecting the development of the prosencephalon may cause DP combined with deficiency of any or all other pituitary hormones (Kelberman *et al.*, 2009). Midline malformations are often associated with optic dysplasia, and an absent septum pellucidum is often found by imaging techniques (septo-optic dysplasia). Other congenital midline defects, which may range from holoprosencephaly to cleft lip and palate, may also be associated with variable hypothalamic–pituitary dysfunction.

Genetic defects affecting development of the anterior pituitary cause hypopituitarism, including hypogonadotropic hypogonadism, in some cases. During fetal development of the anterior pituitary gland, a number of sequential processes occur that affect cell differentiation and proliferation. Recent advances in molecular biology have revealed several steps that are required for pituitary cell line specification, and several genes have been identified to play a role in control of these steps. The pituitary transcription factors, HESX-1, LHX-3 and SOX2 are vital for early patterning of the forebrain and pituitary, and mutations in these developmental genes result in syndromic hypopituitarism with gonadotropin deficiency in humans (Kelberman *et al.*, 2009). PROP-1 is important for the development of gonadotropin-secreting cells and

mutations in this gene are the most common cause of combined pituitary hormone deficiency in humans (Parks *et al.*, 1999). PITX2 is also vital for survival of gonadotrope cell lineage and is required for expression of the gonadotrope-specific transcription factors GATA2, EGR1 and SF1.

The DAX-1 orphan nuclear receptor gene (DAX1) and steroidogenic factor-1 (SF1) are important for the development of the adrenal gland, gonads, ventromedial hypothalamus and pituitary gonadotrope cells. Mutations in DAX1 cause X-linked adrenal hypoplasia congenita, with associated hypogonadotropic hypogonadism, whilst mutations in SF1 are associated with 46XY sex reversal or gonadal dysgenesis, and 46XX premature ovarian insufficiency (Achermann *et al.*, 1999). Leptin and prohormone convertase-1 may also influence GnRH release and processing of the GnRH receptor, with mutations resulting in a phenotype of hypogonadotropic hypogonadism (Farooqi *et al.*, 2007). Depending on the condition, different approaches for counseling are needed.

Iatrogenic gonadotropin deficiencies

Treatment of CNS tumors, leukemia, or neoplasms with cranial irradiation may result in gradual development of hypothalamic–pituitary failure. GH deficiency is the most common component of the radiation-induced hormone disorder, but gonadotropin deficiency also occurs when the radiation dose is high enough (Shalitin *et al.*, 2011). Development of radiation-induced hypothalamic–pituitary failure usually takes from 1 year to several years to ensue.

Hypergonadotropic Hypogonadism

Gonadal Dysgenesis Syndromes

Turner syndrome

The syndrome of gonadal dysgenesis (Turner syndrome) is the most common form of hypergonadotropic hypogonadism, affecting about 1 in 2500 live-born girls. About half of girls with Turner syndrome have the 45,X karyotype, but about 99% of fetuses with this karyotype abort spontaneously, and in 1 of 15 spontaneous abortions the fetus has the 45,X karyotype (Saenger *et al.*, 2001). Chromosomal mosaicism and structural abnormalities of the sex chromosomes modify the clinical features. Typical features include short stature (which may be apparent already at birth), lymphedema of the extremities and loose posterior cervical skinfolds during the newborn period, low-set or deformed ears, epicanthal folds, ptosis, micrognathia, high arched palate, dental abnormalities, wide-spaced nipples caused by shield-like chest, hypoplastic areolae, short neck with low hairline, and cubitus valgus. Abnormalities of the left side of the heart include coarctation of the aorta, aortic stenosis, and bicuspid aortic valves. Renal anomalies include abnormal position or alignment (horseshoe kidney) and various anomalies on the collecting system. Patients with Turner syndrome have increased incidence of

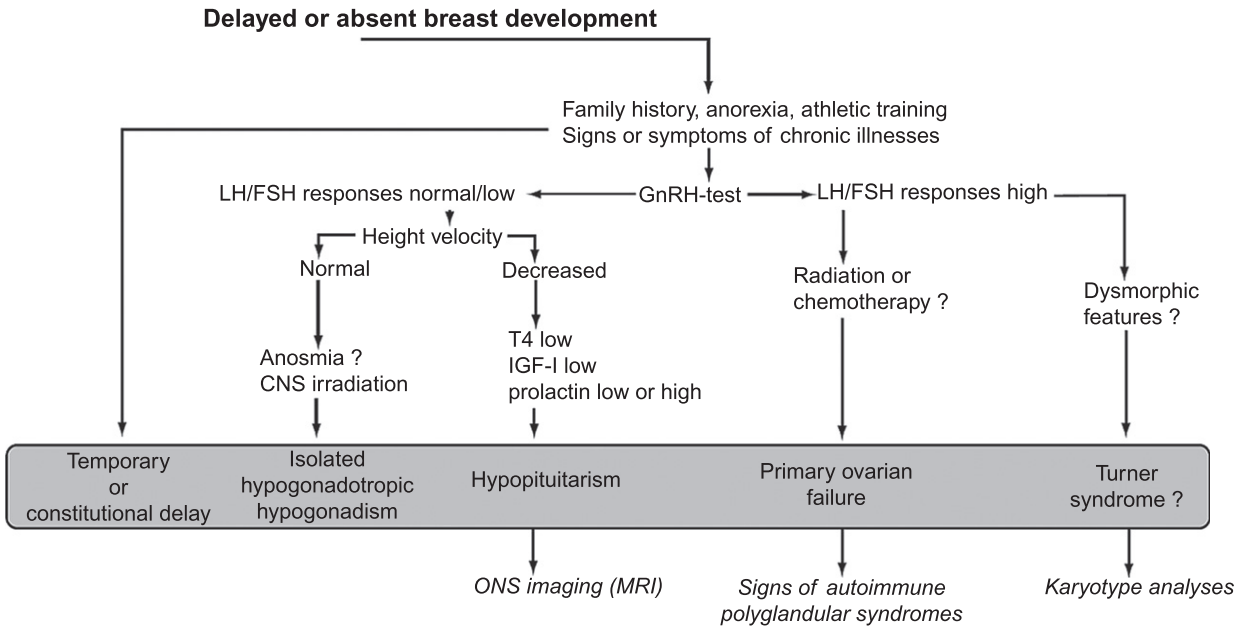

Fig. 1 Differential diagnosis of delayed puberty in females.

inflammatory bowel disease, autoimmune thyroiditis, Graves' disease, and insulin resistance. Intelligence is usually normal, but spatiotemporal processing, visuomotor coordination, and mathematical skills performance may be impaired (Sybert and McCauley, 2004).

Ovarian insufficiency is also apparent at birth, as evidenced by high gonadotropin concentrations during the neonatal period. During childhood, with the development of the CNS-mediated inhibition of GnRH secretion, gonadotropin levels decrease to near normal levels, but by 10 years of age they usually are elevated again. The Müllerian structures (uterus and fallopian tubes) are present but remain infantile if the ovarian failure is not adequately treated with hormone replacement therapy. Histologically, the ovaries are streaks of connective tissue, with a decreased number of primordial follicles and oocytes for age. Spontaneous oocyte death is accelerated, resulting in premature loss of the oocyte pool.

Sexual infantilism is one of the most common clinical findings in girls with Turner syndrome. More than 90% have gonadal failure. It is important to remember, however, that up to 30% of girls will undergo spontaneous pubertal development and that 2%–5% will have spontaneous menses and may have the potential to achieve pregnancy without medical intervention. Pubertal development may be delayed and, in most patients, is followed by progressive ovarian failure.

Most patients are small for their gestational age at birth, and the slow growth rate is apparent after 3 years of age. Most girls fail to have a pubertal growth spurt due to insufficient estrogen production in the ovaries. The mean adult height is approximately 143–146 cm, depending on both parental heights and the overall height of the same genetic population.

46,XX and 46,XY gonadal dysgenesis

The term "pure gonadal dysgenesis" refers to phenotypic females with no pubertal development and the 46,XX or 46,XY karyotype without detectable chromosomal abnormalities (Hughes, 2008; Weinberg-Shukron *et al.*, 2015). Patients with 46,XX gonadal dysgenesis have normal stature, bilateral streak gonads, normal female internal and external genitalia, and (sometimes) sensorineural deafness. Malignant transformation of the streak gonad is rare. Most cases are sporadic, but autosomal-recessive form has also been described; however, few casual genes have been identified in these patients. Patients with familial or sporadic 46,XY gonadal dysgenesis have normal female internal and external genitals with occasional clitoral enlargement due to increased testosterone production by the gonad, bilateral streak gonads, and normal or tall stature with eunuchoid body proportions. The dysgenetic gonads may undergo neoplastic transformation, so gonadectomy may be indicated (McCann-Crosby *et al.*, 2014).

Other Causes of Gonadal Dysgenesis

XX gonadal dysgenesis can occur in combination with cerebellar ataxia and learning difficulties, or with multiple malformation syndromes with a range of associated features including microcephaly, limb abnormalities, facial and cardiac defects. Other causes of ovarian dysgenesis include X isochromosome, where abnormal chromosome division results in duplication of identical chromosome arms, most commonly of the long (q) arm (Cox and Liu, 2014). These patients have streak gonads and a similar phenotype to Turner syndrome.

Other Causes of Primary Ovarian Insufficiency

Irradiation and chemotherapies

Damage to the gonads by irradiation or chemotherapy depends on the patient's gender, age at the time of treatment, radiation dose and fractionation schedule, and total dose and nature of chemotherapy delivered (Johnston and Wallace, 2009). Most chemotherapy protocols use multiple agents whose effects may be synergistic. Biochemical detection of gonadal damage is rarely possible before puberty, so treatment-induced gonadal damage during childhood may present with infertility or premature menopause during adulthood. Abdominal, pelvic, and total body irradiation may result in ovarian and uterine damage. The human oocyte is sensitive to radiation, with an estimated LD50 of less than 4 Gy (Wallace *et al.*, 1989). Less than 2% of children receiving total body irradiation subsequently became pregnant, although there may be some protection of ovarian function in prepubertal girls. Uterine radiation increases the incidence of nulliparity, fetal loss, and small-for-dates infants, and it reduces the success of assisted reproduction. Suppression of the pituitary–gonadal axis with gonadal steroids or GnRH agonists does not protect the ovary from the damage induced by irradiation or chemotherapy. AMH has been shown to be a clear marker of damage to the ovarian reserve of girls receiving chemotherapeutics for cancer (Lunsford *et al.*, 2014).

Premature ovarian failure

The term premature ovarian failure (POF) comprises a heterogeneous spectrum of disorders, from ovarian follicular dysfunction to ovarian dysgenesis (Cox and Liu, 2014). In most cases the etiology is unknown. Discovered first in the Finnish population, point mutations in the extra-cellular domain of the FSH receptor with subsequent inactivation of the receptor function result in raised FSH levels, variable development of secondary sex characteristics and primary or secondary amenorrhea (Aittomaki *et al.*, 1995). Whilst up to 40% of Finnish patients have such a mutation, these appear to be

rare in other populations. Post-receptor defects in the gonadotropin receptor signaling pathways have also been described, with a similar phenotype to FSH receptor mutations. Various microdeletions on the short and long arm of the X chromosome are also found in women with POF, with several genes implicated including POF1, POF2, DIAPH2, FOXL2, BMF15 and most recently STAG3 (Le Quesne Stabej *et al.*, 2016).

Histological examinations of ovarian biopsies show the presence of follicles in all patients with FSH receptor defects, whereas only one in four of those with unknown etiology have follicles. Hence, whereas the receptor defect causes a specific arrest in follicular maturation, many patients with hypergonadotropic ovarian failure have true ovarian dysgenesis. The ovarian phenotype in patients with inactivating FSH receptor mutation is informative in regard to the role of FSH in the regulation of follicular development: the early phases of follicular maturation (up to the preantral stage) are independent of FSH, but for the final maturation of the follicle, this gonadotropin is absolutely necessary.

Autoimmune ovarian insufficiency

Autoimmune ovarian insufficiency is often one of the components of autoimmune polyendocrinopathies. Autoimmune polyglandular syndrome type I is an autosomal recessive disorder caused by mutation in the autoimmune regulator (AIRE) gene, which maps to 21q22.3. It is characterized by two of the three major clinical symptoms that may be present: Addison disease, hypoparathyroidism, and chronic mucocutaneous candidiasis. Moniliasis often precedes symptoms and signs of endocrinopathy. Furthermore, hypoparathyroidism usually reveals itself before adrenal insufficiency.

Ahonen *et al.* (1990) reported data from a 10-month to 31-year follow-up of 68 patients from 54 families, ages 10 months to 53 years at the time of report. Hypoplasia of the dental enamel and keratopathy were frequent and were not attributable to hypoparathyroidism. Some of the manifestations of the disorder did not appear until the fifth decade. Thus, all patients need lifelong follow-up for the detection of new components of the disease. Candidiasis was the initial manifestation in 60% of the patients and was present in all patients at some time. Hypoparathyroidism was present in 79%, adrenocortical failure in 72%, and gonadal failure in 60% of female patients over 13 years of age. Other autoimmune diseases associated with primary ovarian insufficiency include myasthenia gravis, systemic lupus erythematosus and rheumatoid arthritis.

Diagnosis

The cutoff age for identifying girls who need evaluation for delayed puberty may vary in different ethnic groups, but in most populations early signs of secondary sexual development should be present by 13 years of age (Palmert and Dunkel, 2012).

A thorough history should note evidence of chronic disease, anorexia, the intensity of athletic training, and the timing of puberty of both parents (**Fig. 1**). In self-limited DP, often either one of the parents developed late. A history of chronic illnesses, such as celiac disease and inflammatory bowel disease, will suggest a temporary or secondary delay of puberty. Stature and height velocity should be evaluated using appropriate growth charts. Height velocity is usually slow in patients with constitutional delay and is usually normal in patients with hypogonadotropic hypogonadism; however, hypogonadotropic states cannot be ruled out by short stature and slow growth rate. Likewise, bone age (X-ray film of left hand and wrist read according to standards such as Greulich and Pyle) delay provides useful information in the growth analysis, but it contributes little to the differential diagnosis.

Gonadotropin levels assessed by basal LH and FSH determination are often increased in primary ovarian insufficiency or in Turner syndrome, but the basal gonadotropin values are not useful in the differential diagnosis of self-limited delay and hypogonadotropic hypogonadism (Harrington and Palmert, 2012). Dynamic testing, such as administration of synthetic GnRH, may provide information for the differential diagnosis (Binder *et al.*, 2015). In some girls with self-limited DP, a pubertal pattern of response (post-GnRH maximum LH higher than maximum FSH) may be observed, but although a low prepubertal response to GnRH is typical of hypogonadotropic hypogonadism, it can be found in some girls with self-limited DP. Investigation of the differential diagnosis of the two conditions may involve a number of other physiological and stimulation tests, including assessment of LH pulsatility by frequent sampling and first morning-voided urine FSH and LH (Demir *et al.*, 1996; Harrington and Palmert, 2012). In boys, a single measurement of inhibin B < 35 pg/mL in boys has shown to help discriminate HH from self-limited DP with high sensitivity (Coutant *et al.*, 2010), but this has not been demonstrated in girls. Follow-up is often warranted before a definitive diagnosis can be made.

A karyotype is important to confirm or exclude the diagnosis of Turner's syndrome. Autoantibody screening may be informative particularly in cases of hypergonadotropic hypogonadism. Measurement of AMH is valuable in gonadal failure to assess ovarian reserve. An MRI brain is warranted in cases of suspected hypogonadotropic hypogonadism.

Management of Delayed Puberty

When estrogen therapy is required to induce pubertal development, the dosing and timing should be aimed at mimicking normal pubertal development, taking account of the individual's desire to begin puberty and also of the family history of age at onset of puberty. Doses should be adjusted to the responses of individual patients, who may be monitored in terms of the development of secondary sex characteristics, bone maturation, and/or uterine volume.

Before initiation of estrogen therapy in girls with Turner syndrome, serum gonadotropin levels should be determined to exclude the possibility of delayed spontaneous pubertal development. If gonadotropin levels are normal, a sonographic examination should be undertaken to determine the status of the gonads. Hormonal induction of feminization should be initiated and carried out in a manner that simulates the normal growth and development of secondary sex characteristics as closely as possible. Estrogen therapy needs to be initiated and adjusted according to the needs and priorities of the individual.

In hypopituitary girls and in girls with Turner syndrome, estrogen therapy should be coordinated with the use of GH. Previous practice in Turner's syndrome tended towards delaying estrogen therapy until the mid-teens in order to optimize growth promotion with GH. However, more recent studies point to potential benefits from treatment with combined very low-dose estrogen and GH from an early age, in terms of final height and potentially other areas including cognitive development and uterine maturation (Saenger *et al.*, 2001; Ross *et al.*, 2011). Whilst it remains unclear as to the best timing to initiate estrogen therapy in Turner's girls, the current consensus is that induction of puberty should not be delayed in order to promote linear growth (Bondy and Turner Syndrome Study, 2007). Additionally, whilst ethinylestradiol has traditionally been the estrogen of choice for pediatric patients, 17β-estradiol in transdermal, gel or oral form has a better risk profile in terms of growth restriction, liver toxicity and vascular side-effects. Data from hypopituitary females receiving combined estrogen and GH treatment indicate a markedly greater impairment of GH-mediated IGF1 synthesis with ethinylestradiol than with 17β-estradiol. Uterine development may also be impaired with the use of ethinylestradiol as compared to 17β-estradiol (Bakalov *et al.*, 2007; Doerr *et al.*, 2005).

Estrogen therapy should be initiated at a low dose (one-eighth to one-quarter of the adult dose) and increased gradually (at intervals of 6–12 months) (Matthews *et al.*, 2017). Doses can then be adjusted to the response (Tanner stage, bone age, and uterine growth), with the aim of completing feminization gradually over a period of 2–3 years.

A progestin such as oral medroxyprogesterone acetate should be added either if one than one episode of significant breakthrough bleeding occurs or after 24–36 months of estrogen therapy to establish menstrual cycles, at least every 2–3 months to prevent endometrial hypertrophy.

Individuals with Turner syndrome who have functioning ovaries and who progress through puberty spontaneously should receive contraceptive and genetic counseling. However, ovulatory function should be documented (FSH and LH measurements) because a perimenopausal pattern of anovulation can lead to endometrial hyperplasia.

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Gonadotropin-Dependent Precocious Puberty; Female and Male☆

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Glossary

Gonadotropin-releasing hormone agonist Synthetic analog of native gonadotropin releasing hormone in which amino acid substitution causes an agonistic effect and prolonged exposure results in inhibition of gonadotropin release.

Gonadotropin-releasing hormone stimulation test Diagnostic test to assess the presence and origin of the onset of puberty. It is also used to monitor treatment in children with central precocious puberty.

Hypothalamus Cerebral structure involved in the onset of puberty by pulsatile secretion of gonadotropin-releasing hormone.

Pituitary Endocrine gland located in the brain involved in the regulation of the reproductive organs by secretion of luteinizing hormone and follicle-stimulating hormone.

Precocious puberty Early onset of puberty before certain age limits in girls or boys.

Introduction

Gonadotropin-dependent precocious puberty (GDPP) is defined as the onset of puberty at an age younger than normal as a result of precocious activation of the hypothalamic–pituitary–gonadal axis. This form of premature activation needs to be distinguished from gonadotropin independent forms caused by autonomous secretion of sex steroids by the gonads or the adrenal gland.

The term gonadotropin-dependent precocious puberty is composed of the following elements:

Gonadotropin-dependent: Means that pubertal development is driven by activation of the cerebral (central) structures that are responsible for normal puberty. Peripheral causes of physical pubertal development (resulting from sex hormone production by, e.g., adrenal or ovarian tumors are per definition excluded).

Precocious: The definition of precocity depends on the normal age of onset of puberty in a certain population. In general, the age limit is defined as 2 or 2.5 standard deviations (SD) below the mean or median of the population. In recent years several observations indicated that in some populations the normal age of onset of puberty has decreased. This resulted in discussions on the age limit that would demand further medical investigation for detection of underlying disease. Nevertheless, precocity is defined as the occurrence of pubertal development in girls younger than 8 years of age and in boys younger than 9 years of age (Kaplowitz *et al.*, 2016).

Puberty

Female: The onset of puberty in girls is primarily defined by the onset of breast development. In the pubertal staging of Tanner, this is stage B2. Other signs helpful in assessing pubertal development are an increase in growth velocity, an acceleration of bone age development, and an increase in ovarian and uterine size on ultrasound.

Male: The onset of puberty in boys is primarily defined by an increase in testicular volume and subsequent enlargement of penis and scrotum. In the pubertal staging of Tanner, this is stage G2, with a testicular volume of four or more milliliters.

Physiology of Normal Puberty

Normal puberty is characterized by the "awakening" of the gonadotropin-releasing hormone (GnRH) pulse generator in the hypothalamus. In early embryonic development, hypothalamic GnRH cells migrate from the olfactory placode to the hypothalamic region. From birth until puberty, GnRH activity appears to be suppressed or compromised, except during the early neonatal period. Particularly in boys during this phase, termed "mini-puberty," serum values of gonadal steroids can be measured in the pubertal range (Contreras *et al.*, 2017; Quinton *et al.*, 2017).

☆*Change History*: January 2018. D. Mul, W. Oostdijk and S.L.S Drop updated text and references. The articles has extended from girls only to boys and girls in separate sections where appropriate. The paragraph distribution has been changed. Fig. 1 is new, Fig. 2 is enriched with a second photograph (now figure 3b); a previous Table 3 has been replaced by text. Literature references were updated, and now extensively listed. Further reading section has been shortened.

This article is an update of Dick Mul, Wilma Oostdijk and Stenvert L.S. Drop, Precocious Puberty, Central (Female), In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 40-45.

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Studies of the neuronal mechanisms involved in the onset of puberty suggest that changes in the balance between inhibitory and stimulating neurotransmitters initiate puberty. Recent findings draw attention to the possibility that transcriptional repression might be a central mechanism in controlling the initiation of puberty. It is now well proven that the secretion of GnRH is regulated by kisspeptin as well as by permissive or opposing signals mediated by neurokinin B and dynorphin. These three supra-GnRH regulators compose the kisspeptin-neurokinin B-dynorphin neuronal (KNDy) system, a key player in pubertal onset and progression. Moreover, an ongoing increasing number of inhibitory, stimulatory and permissive networks acting upstream on GnRH neurons (such as GABA, NPY, LIN28B, MKRN3) integrate diverse hormonal and peripheral signals and have been proposed as the "gate-keepers" of puberty (Livadas and Chrousos, 2016) (**Fig. 1**).

Environmental influences probably affect normal regulatory mechanisms, emphasizing that the onset of puberty is also dependent on peripheral signals, like sexsteroids and leptin (derived from adipose tissue). Endocrine disruptors may also affect timing of pubertal onset by influencing GnRH pulse generation (reviewed in Livadas and Chrousos, 2016).

Biochemically, the onset of puberty can be demonstrated by pulsatile luteinizing hormone (LH) release, initially at night and later during the day (**Fig. 2**; Schroor *et al.*, 1999).

Pulse frequency and amplitude increase with progression of puberty. Gonadotropin production stimulates the end organs to sex steroid production, resulting in sexual maturation. Sex steroids, mainly estrogens, also influence bone age progression.

Etiology

The estimated incidence of GDPP in girls is 2–3 in 10,000, the prevalence in a Danish national registry was estimated to be 0.2% of girls. In boys, an incidence of 0.24 per 10,000 was suggested (Le Moal *et al.*, 2018).

The etiology of GDPP can be classified as idiopathic or organic. It has been shown that in 70%–90% of girls with GDPP the etiology is idiopathic. However in boys this figure is substantially less: 35%. In idiopathic GDPP, no organic cause for the precocious onset of puberty is found. The number of organic causes will likely increase with the refinement of central nervous system imaging by magnetic resonance imaging (MRI), revealing, for example, hypothalamic hamartoma in children formerly diagnosed as idiopathic CPP. In recent years, in idiopathic forms of precocious puberty, potential causative abnormalities have been found. Several *genetic* variants were found, such as mutations of the imprinted gene MKRN3 in boys (Abreu *et al.*, 2013, 2015; Bessa *et al.*, 2017; paradoxical gain-of-function mutant of the G-Protein receptor PROKR2 (Fukami *et al.*, 2017) and paternally inherited DLK 1 deletion (Dauber *et al.*, 2017). But also environmental factors such as *endocrine disruptors* are thought to play an important role (Leonardi *et al.*, 2017; Le Moal *et al.*, 2018).

It is of interest that LIN28B SNPs are associated with normal puberty timing; however variations of this gene are not commonly involved in molecular pathogenesis of GDPP (Silveira-Neto *et al.*, 2012).

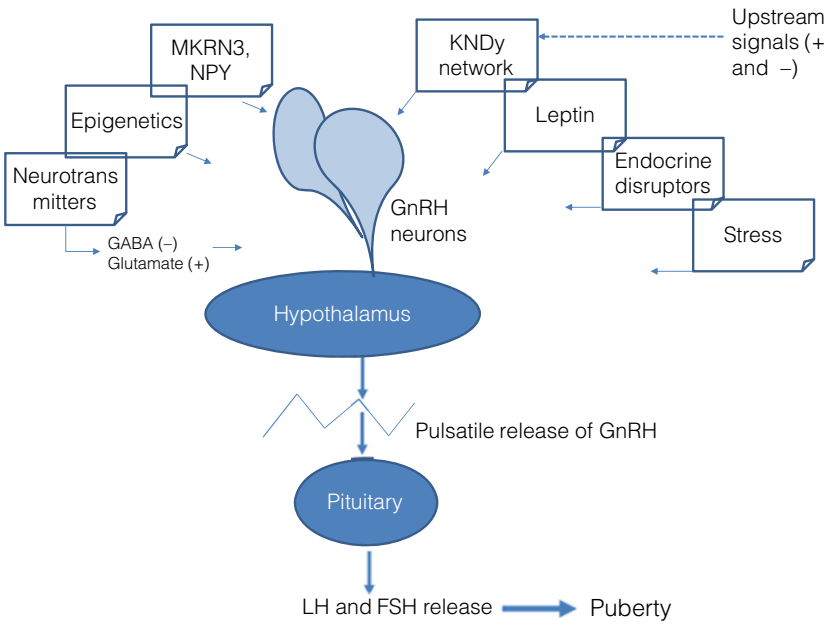

Fig. 1 Schematic representation of proposed mechanism of pubertal onset (after Livadas, S. and Chrousos, G. (2016). Control of the onset of puberty. *Current Opinion in Pediatrics* 28, 551–558). KNDy: kisspeptin-neurokinin B-dynorphin.

Children with neurodevelopmental disabilities such as cerebral palsy or brain trauma have an increased chance of developing GDPP (Bruzzi *et al.*, 2017; De Sanctis *et al.*, 2015).

Functional gonadotropin producing pituitary adenoma present during childhood with central precocious puberty, but are extremely rare (Ntali *et al.*, 2014).

In general, the younger the girl at the onset of CPP, the higher the chance of organic pathology. However, significant pathology was less often seen in girls with signs of puberty before age 3 compared to those >3 years. and progression from premature thelarche to CPP is very rare. In young girls, CPP must be differentiated from premature thelarche without central activation (Kaplowitz and Mehra, 2015).

In organic GDPP, a cerebral organic lesion causes the premature onset of puberty. Local pressure on GnRH neurons or disruption of inhibitory fibers may cause GnRH release. **Table 1** lists possible causes of organic GDPP. Treatment of GDPP in children with organic lesions is primarily aimed at the underlying pathology. However, the progression of puberty is not always halted by this treatment.

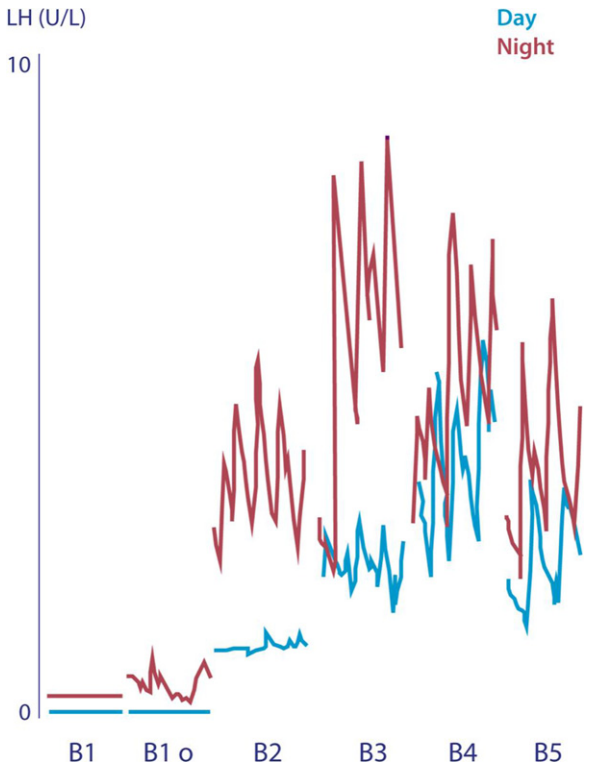

Fig. 2 LH secretion during the day (*blue*) and during the night (*purple*) during Tanner puberty stages B1–B5. (after Schroor, E.J., van Weissenbruch, M.M., Engelbregt, M., Martens, F., Meurs, J.M., Wennink, J.M. and Delemarre-van de Waal, H.A. (1999). Bioactivity of luteinizing hormone during normal puberty in girls and boys. Hormone Research 51(*5*), 230–237).

Table 1 Causes of organic GDPP

Hypothalamic hamartoma
Neurofibromatosis I
Brain tumors (e.g., craniopharyngeoma and astrocytoma)
Hydrocephalus and myelomeningocele
Cerebral trauma
Arachnoid cyst
Gonadotropin producing pituitary adenoma
Following peripheral precocious puberty
Meningitis/encephalitis sequelae

Clinical Presentation

Girls

Girls with GDPP usually present with breast development as the initial sign of puberty (Fig. 3A). Frequently, this is accompanied by acceleration of growth, and, sometimes, behavioral changes. In some girls—in contrast to normal puberty—menarche occurs soon after the onset of breast development due to rapid progression of puberty. Less visible changes occur in bones and internal genitalia. Girls that present with only pubic or axillary hair should further be analyzed for causes of premature adrenal activity, not for problems in the pituitary–gonadal axis.

Boys

Boys with GDPP present with acceleration of growth, pubic hair development and growth of penis and scrotal maturation (Fig. 3B); during physical examination testicular enlargement can be established. Behavioral changes are not uncommon. As in girls, these symptoms are accompanied by accelerated maturation of bone structures.

Diagnostic Procedures

Bone Age Assessment

Bone age accelerates as a result of estrogenic stimulation and may cause premature closure of the growth plate, resulting in compromised final height. The method of bone age assessment in GDPP is not uniform. In most studies, the Greulich–Pyle method is used. It has been shown that final height predictions for GDPP in girls using the tables of Bayley and Pinneau for accelerated bone age significantly overestimate the achieved final height, and it is more appropriate to use the "average" tables for prediction (Kauli *et al.*, 1997).

Pelvic Ultrasonography (Girls Only)

Pelvic ultrasonography reveals the changes in aspect and volume of the ovaries and uterus. The ovaries increase in volume, and the number and the diameter of follicles as well as the diameter of the ovaries increase. Uterine findings include changes in shape and the presence of endometrium.

Brain Imaging

In young girls presenting with CPP, MRI of the brain (especially the pituitary region) is mandatory to exclude central nervous system (CNS) abnormalities. The role of MRI in girls with signs of puberty at age 6 or 7 is under discussion. Some studies indicate that in these cases the search for pathology should not be performed. On the other hand, MRI in girls with CPP at age 6 or 7 has shown CNS abnormalities. A decision tree was developed showing that, in girls, higher estrogen levels and younger age (<6 years)

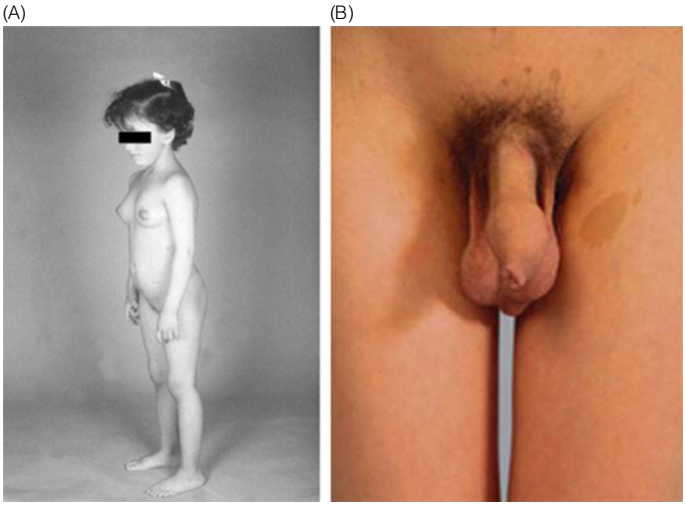

Fig. 3 (A) A 7-year-old girl with clinical presentation of CPP. (B) An 8 year old boy with an organic cause of GDPP. Note testicular enlargement and café au lait spots typical for neurofibromatosis.

at diagnosis increased the probability of the detection of a CNS abnormality on MRI (Chalumeau *et al.*, 2002) In another study the same was observed for higher basal LH levels at diagnosis (Pedicelli *et al.*, 2014; Kaplowitz and Mehra, 2015).

As a rule of thumb, the literature suggests that brain MRI should still be done in girls with onset of puberty younger than age 6 and in (all) boys. Girls with neurologic findings and rapid pubertal progression are more likely to have intracranial pathology and require an MRI examination above the age of 6 years (Carel *et al.*, 2009).

Laboratory

An essential part of the diagnosis of GDPP is the biochemical confirmation of the central (hypothalamo–pituitary) stimulation of the gonads by gonadotropins. In premature thelarche, for example, no central activation is present. The demonstration of central activation is a prerequisite for treatment with GnRH agonists. Basal values of LH or follicle-stimulation hormone (FSH) have limited value in view of limited sensitivity of many assays. Therefore, stimulation tests with native GnRH or a short-acting GnRH agonist (GnRHa) should be used in the diagnostic workup.

The increasing sensitivity of the pituitary for GnRH stimulation in normal puberty is the basis of the test. Depending on the assay, the peak value of serum LH should exceed a particular threshold. Early studies used thresholds >10 IU/L using radioimmunoassay methods, whereas the use of the more sensitive immunofluorometric assay allows cutoff values of approximately 6 IU/L. A prepubertal response in a GnRH stimulation test does not completely rule out the presence of CPP since it has been demonstrated that the more potent GnRH agonists, are able to produce pubertal LH peaks in children with prepubertal LH peaks in the standard GnRH stimulation test. Mainly children with recent onset of puberty demonstrate these varying results in the different stimulation tests. The use of urinary LH in diagnosing and follow-up of pubertal disorders has recently been proposed (Kolby *et al.*, 2017).

The use of sex steroid serum levels in the diagnosis of GDPP is limited because of the circadian variation of these levels. However, they may be used in the evaluation of pubertal suppression during treatment. **Table 2** provides a summary of the current diagnostics in GDPP. In the future, AMH and inhibin B (ovarian products) may be a promising method to differentiate progressive forms of GDPP from slowly progressive forms (Chen *et al.*, 2017).

Treatment

Gonadotropin-Releasing Hormone Agonists

Synthetic agonists of GnRH in depot form are the treatment of choice for GDPP. Basically, the native GnRH molecule is modified at least at the glycine-6 position, where it is substituted by another amino acid resulting in a super-agonistic effect. Prolonged exposure of the pituitary to a GnRHa paradoxically results in inhibition of gonadotropin secretion (**Fig. 4**).

The mechanism of this inhibitory effect is not completely understood. For example, it seems that the frequency of the GnRH pulses is not altered after GnRHa exposure and that down-regulation of GnRH receptors occurs to only a limited extent. The depot formulations of GnRHa have been shown to be able to effectively suppress the endogenous pubertal activity in GDPP. The clinical representation of this inhibition is arrest of pubertal development, sometimes even regression of physical signs of puberty, decreasing volume of uterus/ovaries, and deceleration of bone age maturation.

Based on decades of experience monthly administration of GnRHa is currently considered standard care for children with GDPP. Long acting preparations have been developed to allow quarterly of even yearly administration. In Europe, triptorelin and leuprolide acetate are most frequently used as GnRHa depot preparations. The recommended monthly dose is 3.75 mg intramuscularly or subcutaneously. In general, in the United States 7.5 mg is the recommended dose. In the United States a Histrelin implant that delivers pubertal suppression during more than a year has been approved. The optimal dose should be the lowest

Table 2 Important elements in the diagnosis of gonadotropin-dependent precocious puberty

History
Girls: Onset of breast development, growth acceleration, menarche, behavioral changes, concomitant disease or complaints, family history
Boys: Penile and testicular growth, genital hair, growth acceleration, behavioral changes, concomitant disease or complaints, family history
Physical examination
Pubertal staging according to Tanner, height and weight, growth acceleration by growth chart analysis, signs of primary disease
X-ray left hand
Bone age and height prediction
Ultrasonography (girls only)
Size and aspects of ovaries and uterus
Magnetic resonance imaging
Hypothalamic region, pituitary, optic nerves
Laboratory
GnRH stimulation test: Luteinizing hormone and follicle-stimulating hormone response to GnRH (agonist) administration; sex hormone assessment

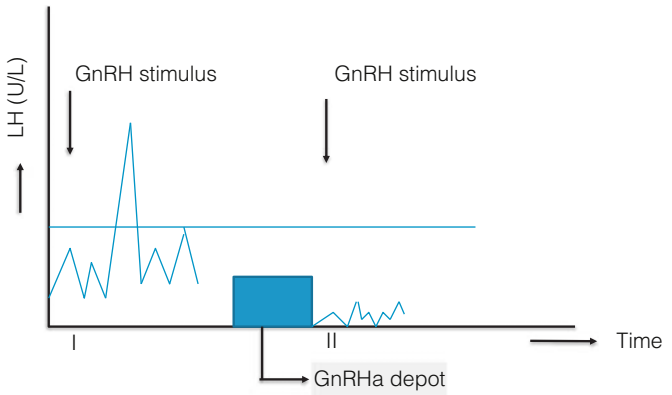

Fig. 4 Schematic representation of the inhibiting effect of gonadotropin-releasing hormone agonist (GnRHa) exposure. (I) GnRH stimulation before GnRHa treatment. Luteinizing hormone (LH) increases considerably in reaction to GnRH stimulus. (II) GnRH stimulation during GnRHa treatment. There is no increase in LH after GnRH stimulus due to the inhibitory effect of prolonged GnRHa exposure.

with which optimal suppression can be achieved. Only a few side effects have been described, mainly sterile abscesses at the injection sites (Lee *et al.*, 2014a).

The 3-months leuprolide depot 11.25 mg not always results in sufficient suppression. Better results are noticed with of the 3 months depot leuprolide 22.5 or 30 mg (Lee *et al.*, 2014b). The 3 months triptorelin 11.25 depot results in a better suppression, especially after 3 months of treatment (Durand *et al.*, 2017). Limited data about final height with these 3-months depots are available (Bertelloni *et al.*, 2015).

Data on a once-yearly histrelin implant showed adequate hormonal suppression and increase of predicted adult height after 60 months of treatment (Silverman *et al.*, 2015; Eugster, 2016).

Anaphylactic reactions to GnRH agonists have only rarely been reported (Ökdemir *et al.*, 2015).

Indications

In 2009 an international consensus statement on the use of GnRH analogs in children was published (Carel *et al.*, 2009). Indications for treatment are either auxological or psychological. The aim of treatment concerning growth is to attain a final height in accordance with parental height and thus to prevent height loss due to the early onset of puberty. It should be noted that positive height outcomes are to be expected in the younger age group, especially in girls with pubertal onset before the age of 6. Psychological issues include reduction of psychological stress resulting from development of puberty or occurrence of menarche inappropriately early for age (Menk *et al.*, 2017). However, psychological distress as such is not a definitive indication for GnRHa therapy in girls with GDPP (Schoelwer *et al.*, 2015). Sexual abuse and early pregnancy appear to be rare. However, early menarche and early sexual activity of patients with mental retardation provide special concern of families and peer groups (school). In these patients, GnRHa treatment may be indicated. Before initiation of treatment, central activation of the pituitary has to be demonstrated.

In boys, auxological reasons for treatment prevail. There is not enough data on psychological issues.

Monitoring

Several parameters should be used to monitor the suppressive effect of treatment. As mentioned previously, clinical progression of puberty must stop or even regress, and—in girls—menstrual bleeding should not occur. However, withdrawal bleeding soon after the initiation of treatment is frequently observed. Furthermore, the pubertal growth velocity should decrease to normal pre-pubertal values. Bone age progression will slow, especially after 6 months of treatment. On ultrasound, uterine and ovarian volume decrease to pre-pubertal values.

In boys, comparable monitoring of physical changes should be initiated. Growth velocity should decrease, and regression of testicular volume and change to soft, unstimulated tissue at palpation are important markers.

Biochemically, baseline sex steroid levels will become pre-pubertal in effectively suppressed patients (Freire *et al.*, 2016). Suppression of hypothalamic–pituitary–gonadal axis activity is determined during treatment by intravenously GnRH testing. Alternative methods include taking a blood sample after subcutaneous GnRHa or after injection of the GnRHa depot. Measurements of LH in urine samples seems to be promising (Kolby *et al.*, 2017). Some children show behavioral changes after the injection interval, which may indicate ineffective suppression during the final days of the injection interval. In clinical practice, meticulous clinical observation of growth and puberty during treatment is sufficient to assess effectiveness of GnRHa treatment.

Results of Treatment

Girls

Numerous studies have addressed the effect of GnRHa treatment for GDPP, and final height data have become available. However, none of these studies used randomized controlled study designs (Guaraldi *et al.*, 2016). Most studies have used the difference between height prediction at the start of treatment and attained final height as the most important outcome parameter. It is well-known that there is a wide variation in bone age assessment between observers; thus, height predictions may lack reliability. Study designs should include an assessment of bone age by only one observer. Furthermore, as noted previously, height predictions vary depending on the method and the prediction tables used. Another way to evaluate the effect of treatment is to compare attained final height with mid-parental height or genetic target height.

It can convincingly be concluded from the available data on GnRHa treatment that an increase in final height ranging from 8 to 10 cm can be obtained in girls with onset of puberty younger than 6 years. Girls with onset of puberty between 6 and 8 years, a more heterogeneous group, may have a moderate benefit ranging from 4.5 to 7.2 cm (according to the accelerated tables of Bayley Pinneau), or 3.4 to 9.5 cm when the average tables of Bayley Pinneau were used. When compared to parental height, final height is 0.5–1.0 SD below target height or midparental height.

Multivariate analyses of the results of treatment are probably most appropriate for the development of guidelines for start and/or discontinuation of treatment. The factors shown in **Table 3** have been shown to influence the results of treatment (height gain) in multivariate analysis. Several studies show better results in girls with GDPP when treatment is initiated before age 6. In contrast, in univariate analysis, chronological age at the start of treatment is negatively correlated with height gain. These contradictory results emphasize the need for proper analysis of the results of treatment.

The optimal timing with respect to chronological age and bone age for discontinuation of treatment cannot be determined from the literature. Possibly the best indication to discontinue treatment is a decrease of height velocity below pre-pubertal values during GnRHa treatment Alternatively, one may assess height prognosis every 6 months and discontinue treatment whenever a decrease in height prognosis is calculated.

Boys

Data on boys treated with GnRHa are sparse. They allow for the conclusion that GnRHa treatment improves AH in comparison with untreated boys, albeit with a large variability (1.8–11.1 cm). In the largest study of 26 boys, mean height gain was 6.2 cm, and most boys reached an AH close to target height (TH). Younger boys and those with idiopathic GDPP were likely to receive greater benefit from treatment, whereas in boys with organic forms, including neurofibromatosis, the long-term height prognosis was worse (Bertelloni and Mul, 2008).

Other Outcome Variables

Concerning other outcome variables after GnRHa treatment in GnPP, endocrine investigation after discontinuation of treatment showed prompt and complete reversibility of gonadal suppression. Menarche occurred within 1 year after discontinuation of treatment. Available, but limited data on fertility showed no adverse outcome (Guaraldi *et al.*, 2016). There is, however, apparent need for more studies in this field because there is conflicting data that PCOS may occur more often in GnRHa treated girls. One long term study concluded that the health status of former CPP women is similar to that of the general population. By now, no conclusions can be drawn on timing of menopause or health of offspring (Guaraldi *et al.*, 2016).

Studies on bone mineral density (BMD) during GnRHa treatment in girls showed normal BMD for chronological age and low BMD when corrected for bone age. At final height, BMD appeared to be normal (Thornton *et al.*, 2014).

In some children, body mass index (BMI) increases during GnRHa treatment. Pretreatment BMI SD is the strongest predictor of increased BMI SD at final height. GnRHa treatment does not lead to obesity Lazar *et al.* (2015).

Table 3 Factors that influence final height after GnRHa treatment in girls

At start of treatment
Bone age at start of treatment: more advanced bone age → better final height
Chronological age at start of treatment: younger chronological age → better final height
At discontinuation of treatment
Bone age at end of treatment: the higher the bone age, the less positive effect on final height
Chronological age at end of treatment: the higher the chronological age, the less positive effect on final height
Bone age advance at discontinuation of treatment
The more advanced bone age over chronological age, the less positive effect on final height

GnRHa and Growth Hormone

During treatment with GnRHa, it is frequently observed that height velocity decreases, even below pre-pubertal levels. The effects of GnRHa treatment on the growth hormone (GH)–IGF axis remain controversial. However, several groups have studied the effect of the addition of GH to GnRHa in children with precocious puberty. Results suggest the importance of maintaining adequate height velocity during treatment to achieve height gain. In short-term studies, the addition of GH resulted in increased predicted adult height compared to treatment with GnRHa alone. Only one study provides data for final height, confirming the results of the short-term studies (Liu *et al.*, 2016).

Conclusion

Effective suppression of pituitary gonadal function is achieved with depot GnRHa treatment in girls and boys with GDPP. Hormonal suppression is fully reversed after treatment is discontinued. Further studies should be performed to determine the final indications of treatment and long-term outcome on reproductive function.

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Gonadotrophin-Independent Precocious Puberty; Female and Male

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Glossary

Gonad A gonad is a gland that produces gametes (sex cells) and sex hormones. In the female the reproductive cells are the egg cells, and in the male the reproductive cells are the sperm. The male gonad, the testicle, produces the male hormone testosterone, AMH and inhibin B; the female gonad, the ovary, produces the female hormones progesterone and estrogens and AMH.

Gonadotropin-releasing hormone stimulation test Diagnostic test to assess the presence and origin of the onset of puberty by stimulating the gonadotrophine producing cells.

Precocious puberty Early onset of puberty before certain age limits in girls or boys.

Introduction

GnRH-independent precocious puberty (GIPP) or peripheral (pseudo-) precocious puberty is defined as pubertal signs at an age younger than the reference range that is not the result of central activation of the hypothalamic–pituitary–gonadal axis. Rather it is mainly caused by autonomous secretion of sex steroids by the gonads or the adrenal gland. The etiology of GIPP may vary by the gender of the child and can be categorized into either congenital/genetic or acquired disorders. Furthermore, based on the clinical presentation the etiology can be categorized in isosexual (i.e., androgen production in males and estrogen production in females) or contrasexual (i.e., increased androgen production in females leading to virilisation and inappropriate estrogen production in males leading to feminizaton/breast development in males) **Table 1**.

Besides pathological conditions also physiological variations of pubertal development have to be considered such as premature thelarche and premature adrenarche.

Table 1 Differential diagnosis gonadotropin independent precocious puberty (GIPP)

Isosexual	*Girls (estrogen production)*	*Boys (androgen production)*
	Ovarian cysts	Leydig cell tumor
	Ovarian Tumor	HCG secreting tumors (such as germinoma, teratoma, hepatoma, choriocarcinoma)
	Peutz–Jaeger syndrome	Testotoxicosis
	Aromatase excess syndrome	Adrenal neoplasm
		CAH (CYP21, CYP11B1 deficiency)
		Cushing syndrome
		Cortisol resistance syndrome (Chrousos syndrome)
	McCune–Albright Syndrome	McCune–Albright Syndrome
	Juvenile hypothyroidism	Juvenile hypothyroidism
	Endocrine disrupting chemicals	Endocrine disrupting chemicals
	Iatrogenic	Iatrogenic
Contrasexual	*Girls (androgen production)*	*Boys (estrogen production)*
	CAH (CYP21, CYP11B1, 3 beta HSD deficiency)	Klinefelter syndrome
	Virilising adrenal or ovarian neoplasm	Aromatase excess syndrome
	Cushing syndrome	
	Aromatase deficiency	
	Cortisol resistance syndrome (Chrousos syndrome)	
	Iatrogenic	Iatrogenic
Physiological variations of puberty	*Girls*	*Boys*
	Premature adrenarche	Premature adrenarche
	Premature thelarche	Pubertal gynaecomastia
	Premature menarche	
		Macroorchidism

Whereas GnRH dependent precocious puberty is estimated to occur in approximately 1:5000–10,000 (Partsch and Sippell, 2002) overall incidence figures for GIPP are lacking and depends on the underlying cause. For some rare disorders estimated prevalences have been reported, for instance for McCune–Albright syndrome ranging between 1/100,000 and 1/1,000,000 (Dumitrescu and Collins, 2008). Ovarian cysts are prevalent in 2%–5% of prepubertal girls and of those 5% are found to produce estrogens, giving an estimated risk of GIPP of 1:400 (0.25%) (Papanikolaou and Michala, 2015). Adrenocortical tumors are very rare neoplasms in children with an estimated annual incidence of 0.3 per 10^6 children under the age of 15 years.

Main Causes of Isosexual Precocious Puberty in Females

Ovarian Cyst

Etiology: Autonomous estrogen producing ovarian cysts can develop at any age and may regress spontaneously after a few weeks or months. They are the most common cause of precocious puberty in girls and consist of enlarged antral follicles or cysts. Cysts developed in the antenatal period under the influence of maternal and placental hormones may persist or enlarge because of high gonadotropin levels during minipuberty (Aydin *et al.*, 2017; Papanikolaou and Michala, 2015). Antral follicles in the ovary are a common finding on ultrasound in prepubertal girls (up to 8 mm) but occasionally they secrete estrogens.

Clinical features: The main symptoms are breast development (Tanner stage 2–3) and vaginal discharge or bleeding as a result of estradiol withdrawal after cyst resolution. Girls >8 years. may complain of abdominal pain or menstrual irregularity. Abdominal pain may be due to ovarian torsion particularly when cysts are large. Late diagnosis can cause loss of an ovary, peritonitis or even death.

Diagnostic work up: Laboratory studies will reveal increased estrogen levels and suppressed gonadotropin levels. Tumor markers as AMH or inhibin B are low. Pelvic sonography will show a unilateral simple cyst varying in diameter (average 2 cm; up to 4–6 cm). It is of particular interest that ovarian microcysts are a common ultrasonographic finding in non-classical CAH.

Treatment: Treatment may be conservative with or without anti-estrogens/aromatase inhibitors to reduce estrogen production or ultimately surgical (cystectomy; oophorectomy). Recurrences may occur more frequently in patients managed conservatively. Periodic pelvic sonography is recommended to monitor the size of the cysts.

Ovarian Tumor

Etiology: Ovarian tumors are rare before the onset of puberty. Most of them are benign but malignant tumors have been described. The three main types are epithelial cell tumors (70%), germ cell tumors (20%) and sex-cord-stromal tumors. Examples of sex-cord stromal tumors are granulosa cell tumors and Sertoli-Leydig cell tumors; the latter may occur in association with the Peutz-Jeghers Syndrome which is characterized by mucocutaneous pigmentation and intestinal polyposis (Bellfield and Alemzadeh, 2016). An overview of tumor markers is given in **Table 2**.

Clinical features: Clinical manifestations of sex steroid producing ovarian tumors include isosexual precocious puberty but also may include virilisation, abdominal pain. An abdominal mass that can be palpated is uncommon.

Diagnostic work up: Laboratory investigations will reveal elevated levels of ovarian derived steroids and suppressed gonadotropin levels. Ultrasonography will show unilateral ovarian mass but does not lead to a clear classification. Size can vary between 2.5 and 25 cm. Elevated levels of tumor markers will be very helpful to classify the tumor and for follow up after surgery. **Table 2**.

Treatment: Treatment is surgical (Haroon *et al.*, 2013). Staging should include peritoneal cytology, exploratory laparotomy and unilateral salpingo-oophorectomy (Fleming *et al.*, 2010) (**Fig. 1**).

Peutz–Jeghers Syndrome (PJS)

Etiology: PJS is a rare syndrome inherited by an autosomal dominant pattern. It is due to a mutation in the *STK11/LKB1* gene but also spontaneous mutations are described (Bellfield and Alemzadeh, 2016). PJS is characterized by mucocutaneous pigmentation

Table 2 Ovarian tumor markers

Histology	*AFP*	*Beta-HCG*	*LDH*	*CA-125*	*CEA*	*CA 19.19*	*Inh B*
Endodermal sinus tumor	+	−	+	+	−	+	−
Teratoma (immature)	+	−	−	+	−	+	−
Dysgerminoma	−	+	+	+	−	+	−
Embryonal CA	+	+	−	−	−	+	−
Chorio CA	−	+	−	−		+	−
Epithelial cell tumors	+	+	+	+	+	+	−
Sex stromal cell tumors	+	−	+	+	−	−	+

AFP = alpha fetoprotein; beta-HCG = beta-human chorionic gonadotropin; LDH = lactate dehydrogenase; CA-125 = cancer antigen 125; CEA = carcinoembryonal antigen; CA 19.19 = cancer antigen 19.19; Inh B = Inhibin B.

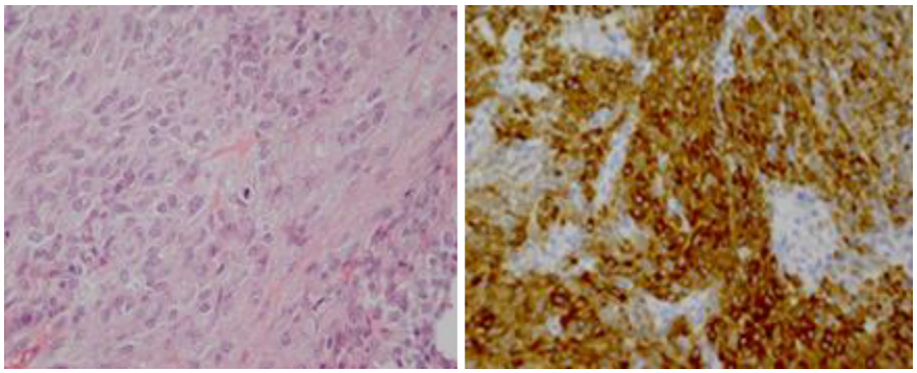

Fig. 1 Histology of juvenile granulosa tumor. Left: the nuclei are lacking atypical features; an occasional mitotic figure can be demonstrated lacking atypical features (× 200). *Right*: The tumor is staining positive for inhibin B (× 200). Source: www.espe-elearning.org.

of the lips, buccal mucosa and fingers/toes, gastrointestinal polyposis and a predisposition to malignancy. Women are at risk of developing malignancies such as breast cancer or estrogen secreting ovarian tumors.

Clinical features: Patients have the typical facial features of pigmentation. They should be screened regularly with specific screening programs to detect tumors at an early stage. Breast development indicates autonomous estrogen production. As the tumors may produce androgens also pubic hair can occur.

Diagnostic work up: Pelvic ultrasound may reveal often small and multiple lesions within the ovary and contain calcifications. Most tumors produce inhibin A and B and AMH.

Treatment: Treatment consists of surgical removal of the tumor.

Main Causes of Isosexual Precocious Puberty in Males

In boys isosexual peripheral precocious puberty has to differentiated from physiological premature adrenarche (PA). The main clinical manifestation of PA is the precocious appearance (before the age of 9 years) of pubic and/or axillary hair and the presence of adult type body odor without any other signs of excessive androgen production such as increased height velocity or advanced bone age (Voutilainen and Jääskeläinen, 2015). The etiology is multifactorial with polygenic contribution, overweight and obesity being important factors. Serum or urine levels of adrenal androgens, such as DHEA(S), may be elevated for age, but do not always correlate with clinical signs of androgen action. Importantly, in contrast to central PP there is no evidence of gonadarche (testicular enlargement) and progression of puberty.

Leydig Cell Tumor (LCT)

Etiology: Leydig cell tumors are rare during childhood and are rarely malignant but because of the androgen production the consequences are serious. They derive from primordial mesenchyme and occur commonly between the age of 5–10 years. Histological it can be difficult to distinguish Leydig cell hyperplasia from Leydig cell tumors. One of the hallmarks of LCT is the presence of Reinke Crystals in about 40% of the cases. LCT have to be distinguished from other scrotal tumors such as teratomas or cystic lesions (dermoid cyst, epidermoid cyst) (Friend *et al.*, 2016).

LCT secrete androgens, notably testosterone and more rarely estrogens. The D578H-LHR mutation, which has been found only as a somatic mutation, is specifically responsible for Leydig cell adenomas and nodular Leydig cell hyperplasia. Histology will show an encapsulated adenoma consisting of homogeneous Leydig cells without evidence of malignant transformation. In nodular Leydig cell hyperplasia nests of Leydig cells will be found (Boot *et al.*, 2011) (**Fig. 2**).

Clinical features: Most boys with androgen producing LCT present with a painless scrotal mass and premature pubarche (sometimes unilateral), penile enlargement and an asymmetrical testis volume. Also gynaecomastia can occur.

Diagnostic work up: Laboratory studies will reveal elevated levels of androgens, notably testosterone, and suppressed levels of gonadotropins. Levels of tumor markers such as alpha-fetoprotein an beta-human chorionic gonadotropin are notably low and can help to differentiate form other tumors such as teratomas in which alpha fetoprotein (AFP) may be elevated. Ultrasound including Doppler sonography may reveal a small hypoechoic mass in the testis.

Treatment: Treatment is surgical. In benign lesions a testis sparing approach may be considered and have to be performed by experienced surgeons (Emre *et al.*, 2017).

Human Chorionic Gonadotropin (HCG) Producing Tumors

Etiology: Testicular and extragonadal germ cell tumors (germinoma) and other embryonal tumors such as hepatoblastoma or chorioncarcinoma may secrete HCG. The production of beta-HCG generates hyperplasia of Leydig cells resulting in elevated levels of testosterone as well as estrogens through aromatization of androgens. The histology depend on the tumor localization and the

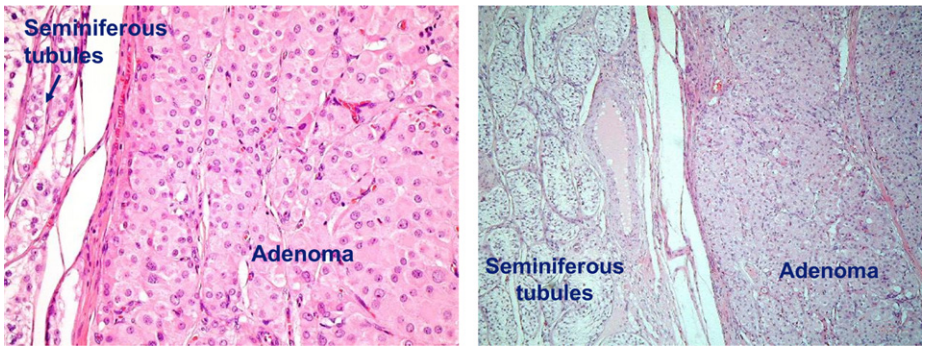

Fig. 2 *Left*: Encapsulated Leydig cell adenoma (× 100); *right*: homogeneous Leydig cells without evidence of malignant transformation (× 400). Source: www.espe-elearning.org.

origin of the tumor. Intracranial germ cell tumors account for 3%–11% of malignant CNS tumors in childhood and are located in the hypothalamus, pinealis region, thalamus or basal ganglia.

Clinical features: Symptoms depend on the location of the tumor. Intracranial germ cell tumpurs often present with diabetes insipidus. In addition to signs of isosexual precocious puberty boys may present with gynecomastia. The testes are not or only minimally increased in size.

Diagnostic work up: Laboratory studies reveal elevated levels of testosterone and suppressed levels of gonadotropins but Beta-HCG levels are markedly increased. As HCG producing tumors are mostly located in the CNS, retroperitoneum or mediastinum MRI studies are indicated.

Treatment: Chemotherapy (cisplatin; etoposide; bleiomycin) has a high rate of inducing remission. (Nascimento *et al.*, 2012; Bravo-Balado *et al.*, 2017). Serum tumor markers are not only beta-HCG but also alpha-fetoprotein (AFP) and testosterone levels (Eren *et al.*, 2009).

Familial Male-Limited Precocious Puberty or Testotoxicosis

Etiology: Familial male-limited precocious puberty (FMPP) or testotoxicosis results from heterozygous constitutively activating mutations of the *LHCGR* gene encoding the luteinizing hormone/choriogonadotropin receptor (LH/HCG), a member of the large G protein-coupled receptors with 7 transmembrane segments. Several *LHCGR* mutations have been described in the 6th or rarely in the 7th transmembrane segment (Nagasaki *et al.*, 2010). The mutation can occur de novo or may be inherited in an autosomal dominant fashion. Premature maturation of Leydig cells and Sertoli cells can occur due to chronic exposure of testosterone. The Leydig cells in affected boys produce Inhibin B and testosterone.

Clinical features: Boys present with very early pubertal development typically before the age of 4 with rapid growth, tall stature, an enlarged penis, pubic hair but only minimal testicular enlargement. (Schoelwer and Eugster, 2016). If left untreated short stature will result due to early closure of the epiphyses. Also muscular development is prominent.

Diagnostic work up: High intra-testicular testosterone concentrations may be sufficient for spermatogenesis and even allow fatherhood (Juel Mortensen *et al.*, 2017). Prepubertal gonadotropin levels and low levels of GnRH stimulated gonadotrophines are found. There is a lack of suppression of testosterone levels by GnRH agonists.

Treatment: Various treatment options are listed in **Table 3**. Cyproterone acetate (CPA) and medroxyprogesterone acetate (MPA) have been used for their antiandrogen properties but ketoconazole has been found more effective due to its ability to inhibit adrenal and testicular androgen biosynthesis. However there are safety issues in view of hepatotoxicity and adrenal suppression. Ketokonazole, Spironolactone or Bicalutamide can also be used in combination with aromatase inhibitors. (Kang *et al.*, 2016).

Causes of Iso—and Contrasexual Precocious Puberty in Both Sexes

Familial Glucocorticoid Resistance (Chrousos Syndrome)

Etiology: Glucocorticoid resistance syndrome is caused by mutations in the human glucocorticoid receptor (hGR) gene encoded by the *NR3C1* gene (chromosome 5). As the negative feedback by glucocorticoids at the level of hypothalamus is impaired ACTH production is increased. Thus the adrenal glands overproduce not only glucocorticoids but also mineralocorticoids and androgens.

Clinical features: The clinical spectrum of the condition is broad ranging from severe signs of adrenal insufficiency to mild or even asymptomatic forms. In boys and girls the increased levels of androgens lead to precocious puberty and hyperandrogenism (Nicolaides and Charmandari, 2017; Malchoff *et al.*, 1994). One of the hallmarks of this condition is the presence of hypertension and a hypokalemic alkalosis.

Diagnostic work up: Laboratory investigations will reveal increased levels of adrenal steroids and ACTH and renin activity. Ultrasonography will show adrenal hyperplasia. A 24 h blood pressure measurement will reveal hypertension.

Table 3 Anti-androgens, anti-estrogens and aromatase inhibitors

Drug name	*Mechanism of action*
Cyproterone acetate (CPA)	Antiandrogen
Medroxyprogesterone acetate(MPA)	Antiandrogen
Ketokonazole	Inhibits P450 enzymes
Spironolactone	Weak antiandrogen agent
Testolactone	First generation aromatase inhibitor
Anastrozole	Third-generation aromatase inhibitor
Letrozole	Third generation aromatase inhibitor
Bicalutamide	Nonsteroidal antiandrogen
Tamoxifen	Selective estrogen receptor inhibitor
Fulvestrant	Estrogen receptor inhibitor

Treatment: The treatment consist of dexamethasone in a dose carefully titrated according to the clinical manifestations and biochemical profile. (Charmandari *et al.*, 2013; Malchoff *et al.*, 1994).

Aromatase Excess Syndrome (AEXS)

Etiology: Aromatase excess syndrome (formerly known as familial gynecomastia) is a very rare genetic disease characterized by symptoms related to estrogen excess notably pre-or peripubertal onset of gynecomastia. Aberrant aromatase expression is the result of subchromosomal inversion of the *CYP19A1* gene encoding for the enzyme aromatase that converses (adrenal) androgens to estrogens.

Clinical features: AEXS is characterized by premature thelarche and early menarche that is accompanied by accelerated growth and bone maturation. Also sparse facial hair and high pitched voice has been described. In pubertal boys testicular volume is lower but normal in post-pubertal males.

Diagnostic work up: Laboratory investigations will reveal elevated levels of estrogens and suppressed FSH, but in the GnRH test LH and testosterone will increase. There is an increased aromatase activity in skin fibroblasts. Gene mutation analysis will be confirmatory.

Treatment: Treatment consist of aromatase inhibitors (see **Table 3**). (Shozu *et al.* (2014)).

McCune–Albright Syndrome (MAS)

Etiology: McCune–Albright Syndrome (MAS) is characterized by the triad polyostotic fibrous dysplasia, hyperpigmented macules (café-au-lait spots) and precocious puberty (see **Figs. 3–5**). The cafe au lait spots have a characteristic pattern with irregular borders ("coast of Maine") in contrast to the smooth borders in neurofibromatosis.

The clinical presentation of MAS is the result of constitutive activation of cells affected by a postzygotic gain of function somatic mutation in the *GNAS1* gene (Lumbroso *et al.*, 2004). It should be noted the individual phenotype may be very heterogeneous depending on the number and types of tissues involved. MAS in boys is very rare and precocious puberty occurs less frequently and later in life than in girls. Gene mutation analysis in peripheral blood cells have a low yield, whereas the likelihood of detecting a mutation is increased in affected tissue.

Girls

Clinical features: Girls present with painless vaginal bleeding and minimal breast enlargement as a result of estrogen secretion by autonomously functioning ovarian cysts.

Diagnostic work up: Laboratory studies will reveal an elevated level of estradiol and suppressed levels of gonadotropins, confirmed by suppressed levels of gonadotropins in the GnRH test. Typically, unilateral ovarian cysts are seen on pelvic sonography. Other endocrine organs can be involved such as thyroid, adrenal, parathyroid and pituitary gland. Careful follow up is indicated. Hyperphosphaturia may occur due to intrinsic renal abnormality or due to overproduction of phosphatonin (Boyce *et al.*, 2017). A bone scan is in order to evaluate for fibrous dysplasia (see **Fig. 4**).

Treatment: Treatment options are listed in **Table 3**. Cyproterone-acetate (CPA) and medroxyprogesterone (MPA) are two of the first agents historically used. However long-term treatment has proven unsatisfactory. Several first- and second-generation aromatase inhibitors have been studied, however with insufficient long-term effectiveness, perhaps with the exception of letrozole. More recently estrogen receptor modulators such as tamoxifen or fulvestrant have been shown to be more promising. Ultimately, surgical options may be considered such as cystectomy or even oophorectomy.

Boys

Clinical features: Boys present with penile growth and slight bilateral enlargement of the testes. Sometimes even macroorchidism. On ultrasound hypo-or hyperechoic lesions are found with microlithiasis and focal calcifications.

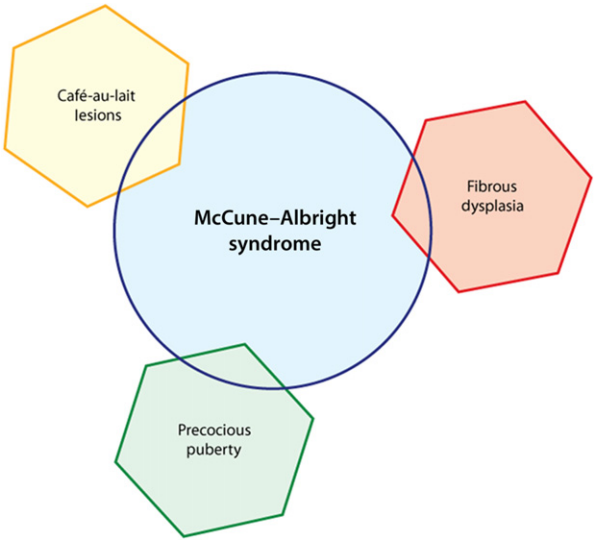

Fig. 3 The McCune–Albright trias.

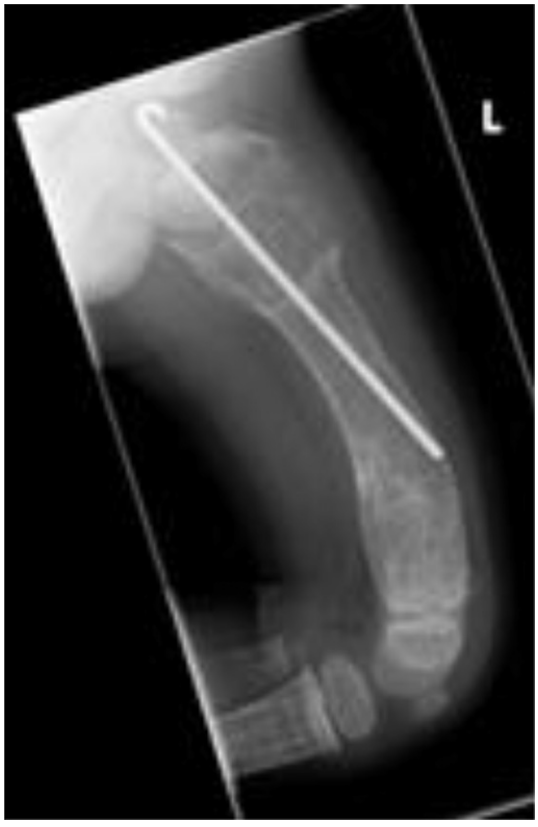

Fig. 4 Fibrous dysplasia; in view of spontaneous fractures a stabilizing rod has been inserted.

Diagnostic work up: Laboratory studies will reveal elevated levels of testosterone and suppressed levels of gonadotropins.

Treatment: A combination therapy consisting of an androgen receptor blocker and an aromatase inhibitor are most often used (see **Table 3**).

Adrenal Tumors

Etiology: As adrenocortical tumors are often hormone secreting tumors the clinical presentation may be the result of the combined overproduction of gluco- and mineralocorticoids as well as androgens or estrogens. But also pure androgen or estrogen producing

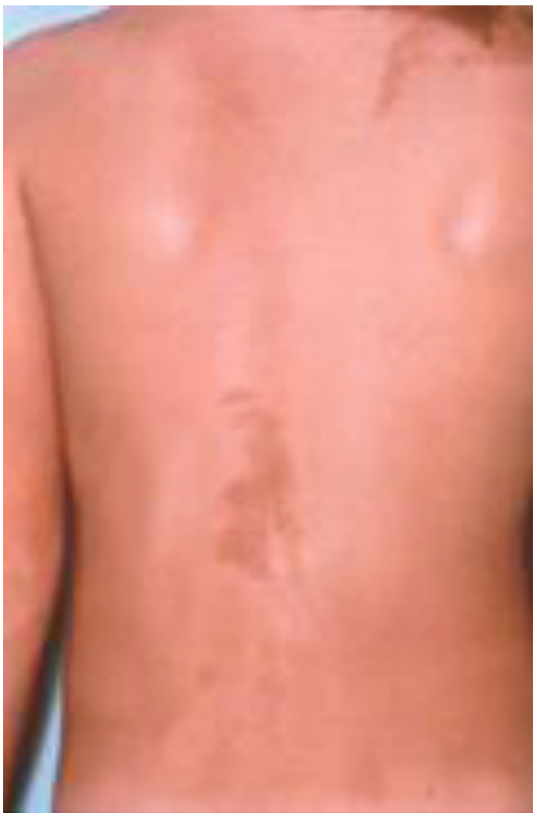

Fig. 5 Café-au-lait pigmentation in case of McCune–Albright syndrome; the lesion having a jagged border does not cross midline, which is very characteristic of MAS.

adenoma have been described. The cause of isosexual precocious puberty in a girl with an adrenal adenoma has been shown to be the result of markedly increased adrenal estrogen production caused by aromatase P450 activity due to abnormal enhancement of transcriptional activity of parts of the promotor of the aromatase gene.

Diagnostic work up: Laboratory investigations will reveal elevated levels of adrenal steroids and suppressed levels of gonadotropins. Imaging studies such as ultrasonography, CT and MRI will establish an adrenal mass.

Pathology Adrenal Tumors

Adrenocortical tumors are very rare neoplasms in children. In recent studies several recurrent chromosomal alterations were identified, the most frequent being $-4q34$, $+9q33\text{-}q34$, $+19p$, loss of heterozygosity of chromosome 17 and 11p15. In addition several focal amplifications and homozygous deletions comprising well-known oncogenes (MYC, MDM2, PDGFRA, KIT, MCL1, BCL2L1) and tumor suppressors (TP53, RB1, RPH3AL) have been identified. In addition a strong DAX1 protein expression, correlating with SF1 gene expression was demonstrated in adrenocortical tumors of pediatric patients (de Sousa *et al.*, 2015). Thus potential driver genes and cellular pathways as well as the existence of different oncogenic routes are implicated in childhood adrenocortical tumors (Letouzé *et al.*, 2012).

Adrenocortical carcinoma has its peak incidence in children <4 years of age, and is associated with the Li-Fraumeni and Beckwith-Wiedemann syndromes.

The distinction adenoma versus carcinoma may be difficult and requires careful staging of the tumor in accordance with criteria specifically developed for childhood adrenal tumors (Wieneke *et al.*, 2003). See **Fig. 6** and **Table 4**.

Treatment: The treatment is surgical.

Primary Hypothyroidism (VanWyk–Grumbach Syndrome)

Etiology: The Van Wyk–Grumbach syndrome (or the "overlap" syndrome) is characterized by a long-standing history of primary hypothyroidism usually due to acquired thyreoiditis (Hashimoto). The pathophysiology of VWGS is not yet clear, but the most accepted theory states that the high concentrations of TSH are sufficient to cause activation of the FSH receptor (since they share the same α subunit) and produce gonadal enlargement and isosexual puberty in boys and girls. Also bioactive FSH but no LH has

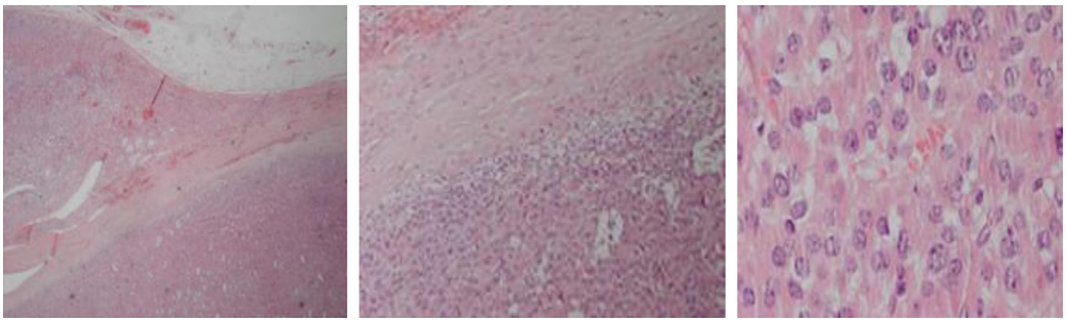

Fig. 6 *Left*: The adrenal tumor is surrounded by a fibrous capsule, which is not at any point invaded by the tumor (× 12.5). *Middle*: Same area; polygonal epitheloid cells with eosinophilic cytoplasm, arranged in solid sheets with micro-cysts, and rich in thin-walled blood vessels. Bland nuclei; no mitotic figures (× 100); *right*: same area showing the intimate relationship between the tumor cells and thin-walled blood vessels characteristic of endocrine tumors, and consistent with the morphology of an adreno-cortical adenoma (× 400). Source: www.espe-eleaarning.org.

Table 4 Macroscopic and microscopic criteria for malignancy of adrenal cortical neoplasms in pediatric patients

Tumor weight of > 400 g
Tumor size > 10.5 cm
Extension into peri-adrenal soft tissues and/or adjacent organs
Invasion into vena cava
Venous invasion
Capsular invasion
Presence of tumor necrosis
15 mitoses per 20 high power field (400 ×)
Presence of atypical mitotic figures

After Wieneke, J.A., Thompson, L.D. and Heffess, C.S. (2003). Adrenal cortical neoplasms in the pediatric population: A clinicopathologic and immunophenotypic analysis of 83 patients. *The American Journal of Surgical Pathology* **27**, 867–881.

been detected. A pituitary MRI may reveal an enlarged pituitary size (due to increase size of the thyrotrophs) which could be miss-interpreted as a tumor.

Clinical features: Pre-pubertal girls present with breast development and vaginal bleeding, with a lack of pubic and axillary hair growth.

Diagnostic work up: Decreased bone age (to different degrees) and elevated prolactin level are often seen sometimes combined with galactorrhoea.

Treatment: Early recognition and initiation of thyroid hormone replacement therapy will avoid further diagnostic procedures, fear of malignancy and unnecessary surgery. Ovarian cysts or a pituitary mass will resolve. Boys present with testes enlargement in 80% of the cases due to an increased size of the seminiferous tubules but without Leydig cell stimulation and low testosterone concentrations. Thyroid replacement therapy results in resolution of all signs and symptoms and final height prediction will improve. (Christens *et al.*, 2014).

Exogenous Sex Steroid Exposure

Premature or excessive sex steroid exposure is clinically obvious in younger children but can be easily missed or "rationalized" as "normal" in peri-pubertal aged children. In the prepubertal child (boys and girls) androgen excess will manifest with acne, adult-type body odor, sexual hair and diffuse hirsutism and clitoral and penile growth. Estrogen exposure will cause breast development. Unintentional secondary exposure to testosterone and estrogen containing transdermal products may occur directly through skin-to-skin contact. Testosterone and estrogen preparations are widely available for adults as part of hormone replacement therapy or contraception. Treatment consists of identification of the source. Following withdrawal of the androgen exposure most symptoms will regress; however clitoral and penile length may not regress completely (Cabrera and Rogol, 2013; De Pinho *et al.*, 2016).

Endocrine Disrupting Chemicals (EDC)

Endocrine disrupting chemicals are substances that may interfere with the endocrine system mimicking natural hormones such as estrogens and androgens. They may also produce overstimulation or alter the metabolism of endogenous hormones. They may bind to a cellular receptor and block functions of endogenous hormones and thus acting as anti-estrogens or anti-androgens. These

substances include polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane, pesticides, plasticizers (such as phthalates). Plasma levels of di(2-ethylhexyl)phthalate (DEHP) were found significantly higher in patients with peripheral PP when compared to control (Buluş *et al.*, 2016). In a recent review of the clinical and epidemiological literature it was concluded that currently available data is inconclusive necessitating further study to understand the role of EDC in pubertal development (Leonardi *et al.*, 2017).

Congenital Adrenal Hyperplasia (CAH)

Etiology: Isosexual precocious puberty in boys and contrasexual puberty in girls may result from increased androgen secretion by the adrenal cortex particularly with undiagnosed classic simple virilising (SV) form or non classic (NC) form of 21 hydroxylase deficiency or the more rare form of 11beta-hydroxylase deficiency. Due to the enzymatic defect there is a lack of negative feedback of cortisol to the pituitary gland with consequently increased production of ACTH that lead to hyperplasia of the adrenal cortex with increased production of adrenal androgens.

Clinical features: Androgen excess depends on the severity of the enzymatic defect and will manifest with a variation of signs as acne, adult-type body odor, sexual hair and diffuse hirsutism and penile growth. In the more severe forms (SV forms) there is also an increased height velocity with a increased bone age. In NC CAH patients the height velocity is generally not increased with still an advancement in bone age. Typically in boys the testes have a prepubertal volume. Untreated elevated androgens may lead to activation of hypothalamic–pituitary–gonadal axis resulting in GnRH dependent puberty (see below).

Diagnostic work up: increase levels of serum or saliva 17 hydroxyprogesteone and androstenedione (in the morning) are the hallmarks of 21 hydroxylase deficiency. A urinary steroid profile will show increase steroid metabolites. Furthermore, a bone age has to be done. An ACTH stimulation test can help to diagnose milder forms of CAH. The diagnosis can be confirmed by gene mutation analysis. With the introduction of the neonatal screening programs in many countries the classic simple virilising forms are mostly diagnosed and treated already in the neonatal period.

Treatment: Corticosteroid treatment will result in suppression of adrenal androgens and in regression of the symptoms. However, parents have to be counseled for the positive and negative effects of glucocorticoid treatment.

Gonadotrophin-Dependent Precocious Puberty

It should be noted that the treatment of Gn-independent PP may be complicated by activation of the hypothalamic–pituitary–gonadal axis resulting in GnRH dependent or central PP. Therefore frequently GnRH analog treatment is necessary as adjuvant therapy (Santos-Silva *et al.*, 2014). In patients with CAH is has been shown to have an augmenting effect on linear growth (Güven *et al.*, 2015).

Conclusions

Whereas the prevalence of GnRH-independent precocious puberty (GIPP) is low and the etiology heterogeneous it is of paramount importance to establish a definitive diagnosis as in case of adrenal or gonadal sex hormone producing tumors surgical treatment is required.

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Organ-Specific Pediatric Endocrine Disorders, Pituitary: Craniopharyngioma

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Abbreviations

AXIN2	Axin-like protein (Axil) or axis inhibition protein 2
BRAF	B-Raf (gene that encodes protein B-Raf)
CP	Craniopharyngioma
CSF	Cerebrospinal fluid
CT	Computed tomography
CTNNB1	β-Catenin gene
GLI2	GLI family zinc finger 2
IFN-α	Interferon alpha
LEF1	Lymphoid enhancer-binding factor 1
MRI	Magnetic resonance imaging
PTCH1	Protein-patched homologue 1
SHH	Sonic hedgehog
TSH	Thyroid-stimulating hormone

Introduction

Craniopharyngioma (CP) is defined by the World Health Organization (WHO) as "a benign, partially cystic epithelial tumor of the sellar region, presumably derived from Rathke's pouch epithelium" (Rushing *et al.*, 2007). The two clinicopathological subtypes typically observed are adamantinomatous and papillary. In childhood and adolescence, adamantinomatous CP with cyst formation is most common. Papillary CP occurs almost exclusively in adults. While this article is predominantly about adamantinomatous CP, some features that distinguish it from the papillary will be presented.

CP is extremely heterogenous in their location, rate of growth, size, shape, gross structure, propensity to invade adjacent tissue, histology, and functional impact. As they originate from cells along the path of the primitive craniopharyngeal duct and adenohypophysis, they can be located above and/or within the sella or rarely below and around the sella. Although metastasis is exceedingly rare and CP is typically categorized as benign WHO grade I, they invariably impact on adjacent vital structures such as the optic nerves and chiasm, pituitary stalk and gland, hypothalamus, third ventricle, and internal carotid artery. This results in potential visual, endocrine, metabolic, and neurological morbidity. Location, size, local extension, and adherence can make CPs difficult to resect. Local tumor growth and invasion and definitive treatment can compromise such nearby structures with further significant impact on morbidity and quality of life. Their unpredictable potential to recur regardless of initial treatment modality poses further challenges for achieving control and cure.

The two main modalities of treatment currently available are surgery and radiotherapy. However, the optimal treatment of CP remains controversial. Radical surgery with complete resection aimed at long-term cure for childhood-onset CP has been favored historically. However, partial resection has been reported to have equivalent tumor-control rates as complete resection (Tan *et al.*, 2017). In the past two decades, there has been a greater preference toward individual risk-based interventions, incorporating more conservative surgery using advanced neurosurgical devices and techniques and improved precision radiotherapy to control the disease. More recently, reports on long-term prognosis, novel neurosurgical and radio-oncological treatment approaches, and molecular genetics have provided new insight into further risk-adapted treatment of CP to prevent severe sequelae such as hypothalamic syndrome and obesity.

Epidemiology

The incidence of CPs ranges between 0.5 and 2 cases per million persons per year (Bunin *et al.*, 1998). Although diagnosed at any age, a bimodal age distribution is demonstrated with peak incidence rates at 5–15 and 50–75 years. CPs constitute approximately 5%–10% of pediatric brain tumors. It remains the most common neuroepithelial intracranial tumor in the pediatric population, constituting about 56% of all sellar and suprasellar tumors in children (Miller, 1994).

Pathology

Etiology

The two subtypes of CP are thought to arise from different processes involving the development of the anterior pituitary (pars anterior or adenohypophysis) and its intermediate lobe (pars intermedia). In the fourth week of gestation, these lobes of the

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pituitary develop from an ectodermal evagination, called Rathke's pouch. It grows upward from the roof of the stomodeum (oral cavity) toward the infundibulum. During the sixth week of gestation, the connection between Rathke's pouch and the oral cavity (the craniopharyngeal/pharyngohypophyseal duct) disappears. Subsequently, cells in the anterior wall of Rathke's pouch develop into the anterior lobe and pars tuberalis (extension of the anterior lobe along the pituitary stalk), and those in the posterior wall form the intermediate lobe (Miller, 1994).

Adamantinomatous CPs are theorized to develop from embryonic squamous-cell rests found along the path of Rathke's pouch and the primitive craniopharyngeal duct. For the papillary subtype of CP, a metaplastic origin from cells in the pars tuberalis has been proposed (Larkin and Ansorge, 2013).

Histopathology

The adamantinomatous and papillary subtypes are based on histopathologic appearances. The cystic adamantinomatous subtype, which is the most predominant in childhood, resembles tumors of tooth-forming tissues of the jaw or ameloblastomas known as adamantinomas. Microscopically, the tumor is made up of many cell types. At the border are dense areas of columnar epithelial cells. These palisading areas blend with squamous epithelial cells arranged loosely and termed stellate reticulum. Keratinization of these epithelial cells can result in anuclear areas of "wet keratin" in both compact and loose zones. Cyst cavities are lined by flat epithelium and contain desquamated flat keratin plates. Therefore, the cyst fluid is rich in membrane lipids such as cholesterol and keratin and has a typical dark "motor oil" appearance. It can contribute to chronic inflammation within the cyst walls. The desquamated cells can also calcify, but metaplastic bone formation is unusual (Miller, 1994).

Squamous papillary CPs, which occur almost exclusively in adults, are composed of stratified squamous epithelium with papillary projections of epithelial cords into the surrounding tissues. They rarely calcify or desquamate, with the cell layers being solid and compact and with no stellate regions. Mixed CPs contain features of both adamantinomatous and papillary types (**Fig. 1**).

Molecular Pathology

In the majority of adamantinomatous CPs, activating mutations in *CTNNB1*, the gene encoding β-catenin on chromosome 3, have been identified (Sekine *et al.*, 2002). In the papillary subtype, *BRAF* p.V600E mutations are described instead (Brastianos *et al.*, 2014). Thus, adamantinomatous and papillary CP are distinct molecular entities. Recently, the coexistence of *BRAF* p.V600E and *CTNNB1* mutations was reported in one case of adamantinomatous CP (Larkin *et al.*, 2014). Whether other mutations are present in human adamantinomatous CPs in addition to those in *CTNNB1* remains to be explored. Most identified mutations in adamantinomatous CP affect regulatory amino acids encoded by exon 3 of *CTNNB1* (Martinez-Barbera and Buslei, 2015). Normally, β-catenin is degraded in the proteasome and does not accumulate in the nucleus. In CP, the mutant form of β-catenin is resistant to degradation, accumulates in the cytoplasm and nucleus, and activates the canonical WNT signaling pathway (Hölsken *et al.*, 2009). This occurs in nests or clusters of CP cells and not throughout the tumor (Gaston-Massuet *et al.*, 2011). Nuclear localization of β-catenin in the cell clusters can be seen with immunostaining of tumor tissue. These clusters are a histological hallmark of human adamantinomatous CP and are not present in any other pituitary tumors.

Whole-genome methylation and microarray gene expression studies in a large cohort of patients have revealed different gene expressions and methylation patterns in adamantinomatous compared with papillary CP (Hölsken *et al.*, 2016). In the adamantinomatous subtype, genes in the WNT (such as *LEF1* and *AXIN2*) and hedgehog (such as *GLI2*, *PTCH1* and *SHH*) pathways are upregulated (Hölsken *et al.*, 2016). Overactivation and suppression of a number of target genes and signaling cascades

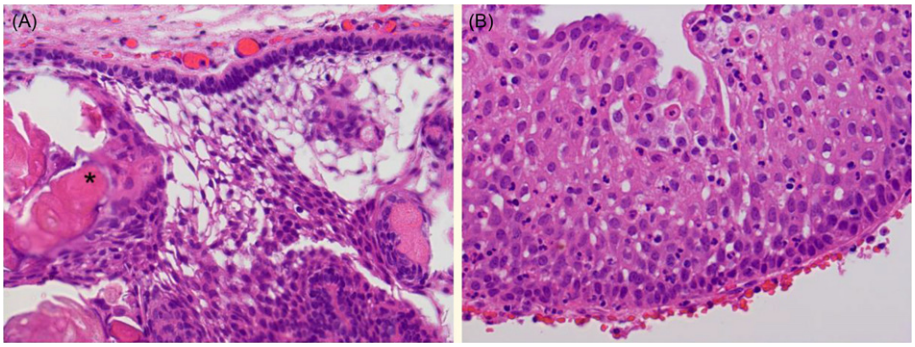

Fig. 1 Histological appearances of adamantinomatous (A) and papillary (B) craniopharyngioma (hematoxylin and eosinophil, 400 ×). (A) Demonstrates typical features of an adamantinomatous craniopharyngioma with nuclear palisading of columnar squamous epithelial cells that blend into central, looser areas of stellate reticulum. Pearls of wet keratin can be also appreciated (*asterisk*). In contrast, a papillary craniopharyngioma in (B) demonstrates undulating sheets of well-differentiated, nonkeratinizing squamous epithelium. There is an associated acute inflammatory infiltrate in this case.

downstream from the WNT–β-catenin pathway have also been reported (Hölsken *et al.*, 2009; Gump *et al.*, 2015). These are involved in embryogenesis and oncogenesis and likely to contribute to tumor initiation, growth, adhesion, and migration. Investigating whether a particular pathway has antitumorigenic or protumorigenic effects presents opportunities for designing targeted biological treatments for CP.

Clinical Presentation

Initial Presentation

The clinical features of a CP are due to its space-occupying effect and compression of adjacent vital structures. The diagnosis of CP in children is frequently delayed due to the nature of symptomatology. In a large cohort of childhood-onset CP, the median duration of symptoms before diagnosis was 6 months (range, 0.1–108 months) (Hoffmann et al., 2015a). Headache was the most common symptom before diagnosis (50%) and often the first (38%). In isolation and without the characteristic red flags of raised intracranial pressure, it can be attributed to other common causes of headache. The prediagnosis symptom interval was shorter in children under age 7 years than older patients. It did not correlate with tumor size, hypothalamic involvement, or visual impairment. Hydrocephalus, nausea, visual impairment, and neurological deficits prompted earlier diagnosis. Growth failure, endocrinopathies, and weight gain at diagnosis often forebode a longer duration of history.

Spectrum of Clinical Manifestations

Tumor location and size influence the clinical presentation (**Table 1**). Compression or destruction of the third ventricle, optic chiasm, nerves and/or tracts, other cranial nerves, hypothalamus, pituitary stalk, or adjacent vascular structures by CPs results in the typical presenting clinical features, which include hydrocephalus, headache, progressive visual loss, neurological deficits, and/or endocrine abnormalities. Retrochiasmatic tumors can extend posteriorly and compress the chiasm anteriorly against the tuberculum sella. These tumors tend to fill the third ventricle and cause hydrocephalus. Raised intracranial pressure results from either obstructive hydrocephalus or mass effect. At presentation, 20% of children have papilledema. Approximately, 10%–20% of children present with acute neurological deterioration, which requires urgent cyst decompression or tumor resection (Sughrue *et al.*, 2010). Although a midline suprasellar mass typically causes a superior temporal quadrantanopia by compression of the overlying optic chiasm, eccentric growth of a CP can lead to patterns of visual loss that vary in type and severity. Lateral extension of the tumor can cause displacement of internal carotid arteries and posterior communicating arteries. Posterior extension of the tumor can displace the tip of the basilar artery, posterior cerebral arteries, oculomotor nerves, and rostral brainstem.

Endocrine Morbidity

CPs can compress or destroy the hypothalamus, anterior pituitary, pituitary stalk, and posterior pituitary, resulting in varying patterns of endocrinopathy. Virtually, all of the pituitary hormones can be affected, but the commonest is growth hormone deficiency presenting as growth failure, followed by deficiencies of thyrotrophin-releasing hormone (TSH) and gonadotrophins. Adrenocorticotropic hormone deficiency is less common at presentation but is potentially life-threatening. At diagnosis, at least one pituitary hormone deficiency is common. Pituitary dysfunction tends to worsen after surgery and rarely recovers.

Central diabetes insipidus is unusual at presentation but is extremely common in the immediate postoperative period (albeit transient) and later postoperatively. CP impinging on the pituitary stalk suppresses prolactin inhibitory factor (mainly dopamine) reaching the lactotrophs of the anterior pituitary. Thus, prolactin levels can be raised (Sughrue *et al.*, 2010).

Table 1 Spectrum of manifestations and morbidity from craniopharyngioma

Structures affected	*Manifestations*
Third ventricle and CSF flow	Raised intracranial pressure, hydrocephalus, headache, vomiting, papilledema, acute neurological deterioration
Optic chiasm, nerves, and/or tracts	Defects in visual fields and acuity
Anterior pituitary	Growth hormone deficiency and growth failure Deficiency of TSH, gonadotrophins (i.e., follicle-stimulating hormone and luteinizing hormone), and adrenocorticotropic hormone
Posterior pituitary	Antidiuretic hormone and central diabetes insipidus
Hypothalamus	Imbalances in regulation of appetite, thirst, body temperature, heart rate, blood pressure, and circadian rhythm; hypothalamic syndrome including obesity, behavioral changes, and daytime sleepiness

Hypothalamic Morbidity

In approximately 35% of patients with CP, symptoms of hypothalamic syndrome, such as obesity, behavioral changes, disturbed circadian rhythm, daytime sleepiness, and imbalances in regulation of thirst, heart rate, blood pressure, and body temperature, have been found at diagnosis (Müller, 2016). Despite adequate replacement of pituitary deficiencies, weight gain and the development of obesity can still occur. The hypothalamic imbalances in energy regulation contribute to obesity and are exacerbated by factors limiting physical activity such as visual impairment, neurological deficits, psychosocial difficulties, and increased daytime sleepiness (Müller, 2010, 2011; Prieto *et al.*, 2015).

The rate of hypothalamic dysfunction dramatically worsens after treatment of CP. In approximately half of patients, the severity of obesity often worsens early after initial surgical treatment with rapid weight gain in the first 6–12 months and reaching a plateau during the long-term course of disease (Müller, 2011). Patients with CP and hypothalamic obesity typically develop morbid obesity that is unresponsive to conventional lifestyle modification (Müller, 2016). It can result in increased risks of cardiovascular disease, sequelae due to metabolic syndrome (Erfurth, 2015), and nonalcoholic fatty-liver disease (Hoffmann et al., 2015b). Hence, appropriate surgical strategies aimed at preserving the hypothalamic integrity are mandatory for the prevention of morbid obesity resulting from hypothalamic damage.

Investigations for CP

Imaging

CP can comprise of cystic, solid, or mixed components. Pediatric lesions are predominantly cystic. Based on anatomical relationship of the tumor to the optic chiasm, CPs can also be categorized into prechiasmatic, retrochiasmatic, subchiasmatic, and laterally expansile (Sughrue *et al.*, 2010). About 75% of CPs are suprasellar with intrasellar extension; some are entirely suprasellar (20%) and occasionally intrasellar (5%). Intraventricular CPs are rare, arising within the third ventricle and extending downward through the sphenoid bone into the nasopharynx (Harwood-Nash, 1994).

Plain radiographs are rarely used for diagnosis. However, they can demonstrate abnormalities in the sella, including sellar enlargement (65%), erosion of the clinoid processes or dorsum sella (44%), or calcifications (80%) (Donovan and Nesbit, 1996; Moore and Couldwell, 2000).

On computed tomography (CT) imaging, the tumor appears as a lobulated, heterogeneous, suprasellar mass. Associated cyst fluid is either isodense or hypodense. The solid portion and cyst capsule enhance with contrast. Calcifications, particularly in the suprasellar region, are best demonstrated on plain CT. The presence of abundant suprasellar calcification is clinically significant, as it is associated with tumors that adhere to surrounding brain tissue, thereby making resection difficult. The calcification can be seen as large confluent areas or small punctate or curvilinear areas (Donovan and Nesbit, 1996; Moore and Couldwell, 2000). Calcification is more difficult to detect on magnetic resonance imaging (MRI).

High-resolution MRI sequences of the sellar region, with and without contrast enhancement, are the images of choice (**Fig. 2**). The signal characteristics of CPs on MRI scans are typically heterogeneous and vary with the amount of cystic and solid components and the amount of cholesterol, keratin, hemorrhage, and calcification. On T1-weighted images, the cystic component is hypointense unless proteinaceous when hyperintensity may be observed, while the cyst rim enhances following contrast administration. The solid component is isointense but demonstrates contrast enhancement. The tumor is usually hyperintense on T2-weighted images.

MRI provides in-depth detail regarding the relationship of the tumor to adjacent anatomical (particularly the hypothalamus, optic chiasm and nerves, third ventricle, and pituitary) and vascular structures. Preoperative identification of the location and distortion of the solid mammillary bodies can aid in predicting the relative position and adherence of the CP to the hypothalamus. Another important feature on MRI is the position of the anterior communicating artery, which is invariably closely related to the optic chiasm. The anterior communicating artery can still be seen with large tumors even when the chiasm is exceptionally thin and cannot be distinguished from the solid tumor or capsule. The position of the anterior communicating artery and the chiasm relative to the tumor strongly influences the choice of surgical approach (Sughrue *et al.*, 2010). The use of T2-weighted MRI and fast imaging employing steady-state acquisition MRI sequences also allows the brain–CP interface and the relative position of the hypothalamus to be identified (Xie *et al.*, 2011). This can aid in the planning of surgical resection and radiotherapy fields. Likewise, imaging may also demonstrate hydrocephalus that, in turn, may influence surgical approaches.

Differential Diagnoses

The imaging differential diagnosis of a CP includes Rathke's cleft cysts, pituitary adenomas, optic pathway and hypothalamic gliomas, germinoma, meningiomas, Langerhans cell histiocytosis, and giant aneurysms. Radiologically, subtle differences exist to aid the diagnosis. Rathke's cleft cysts usually do not have a solid component, are not lobulated, are nonenhancing, and are more homogeneous. Pituitary adenomas enlarge the sella, are more homogeneous, and are usually less cystic. Gliomas are usually not calcified. Meningiomas are rarely cystic, uniformly enhance significantly, and are isointense on T1- and T2-weighted images. Giant aneurysms usually contain a laminated thrombus (Donovan and Nesbit, 1996).

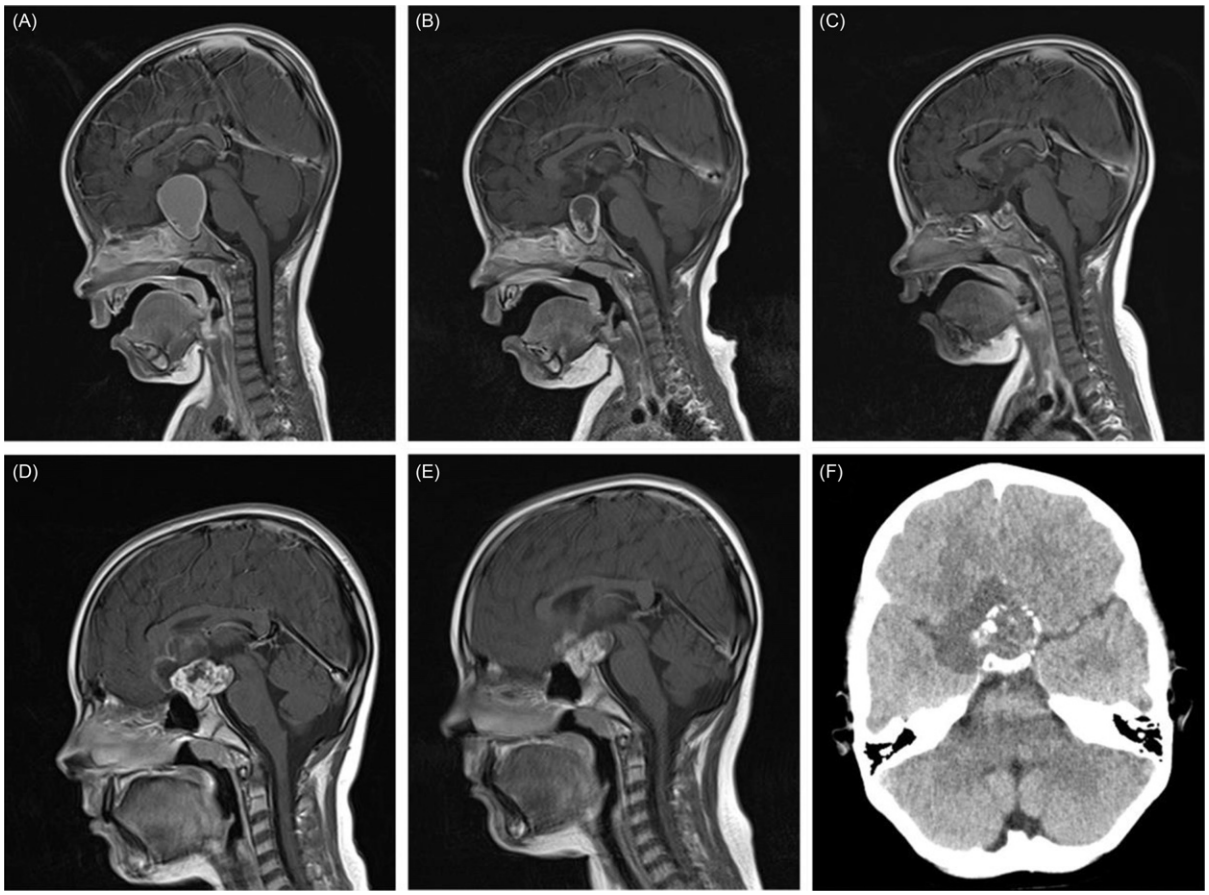

Fig. 2 Imaging of craniopharyngiomas. Figs.(A)–(C) demonstrate sagittal T1-weighted, gadolinium-enhanced MR images of cystic adamantinomatous craniopharyngioma in a 1-year-old male. The preoperative image (A) demonstrates the suprasellar cyst clearly and avidly enhancing cyst wall. The hyperintense signal characteristics of the intracystic fluid are different to surrounding CSF, suggesting a proteinaceous consistency. Image (B) demonstrates a reduction in cyst size following Ommaya reservoir insertion and aspiration. Cystic reaccumulation despite intracystic therapy resulted in open debulking surgery. The postoperative scan (C) shows significant reduction in lesion size. By contrast, the preoperative (D) and postoperative (E) sagittal T1-weighted, gadolinium-enhanced MR images from another child are of solid/cystic adamantinomatous craniopharyngioma. While a differential diagnosis of this lesion could include a low-grade glioma, the calcium deposits around the periphery of the lesion on CT imaging (F) are in keeping with a craniopharyngioma. Given the degree of postoperative residual tumor involving the hypothalamus (E), the patient subsequently received focal proton beam radiotherapy.

Clinical and Laboratory Evaluation

Except when immediate neurosurgical intervention is indicated, patients should have an ophthalmology assessment (including fundoscopy, visual acuity, and visual field examination) and endocrine assessment (including growth, pubertal status, clinical evaluation, and biochemical tests for hypothalamic–pituitary function) before treatment. The tumor markers alpha-fetoprotein and beta-HCG contribute to excluding nongerminomatous germ cell tumor (suprasellar common location).

Management

Young patients with CP should be managed at a specialist center with expertise that includes pediatric neurosurgery, oncology, endocrinology, ophthalmology, neuroradiology, histopathology, psychology, and neurorehabilitation. The aims and main modalities of management are presented in **Table 2**. The definitive modes of management are surgery with or without radiotherapy for the primary tumor and for recurrence.

Surgery

Surgery, with endoscopic or microsurgical techniques, is the initial treatment of choice for the primary tumor. The two main strategies based on extent of resection are radical or total/gross/complete resection and partial or limited/subtotal resection. Both

Table 2 Aims and main modalities of management of craniopharyngioma

Aims of management	*Management modalities and comments*
Relieving significant compression on vital structures by solid and cystic tumor components	Emergency surgery is indicated Large cystic component requires surgical drainage, catheter, and Ommaya reservoir to allow repeated aspiration (Müller *et al.*, 2017). The risks of these procedures are spillage of cyst fluid and aseptic meningitis Raised intracranial pressure, ventriculomegaly, and hydrocephalus warrant CSF diversion procedures. Dexamethasone contributes to reducing cerebral edema in patients with large tumors Visual impairment requires timely surgical resection
Prevent progression and recurrence Preserve function of vital structures especially optic apparatus, hypothalamus, and pituitary Minimize long-term morbidity and mortality	Surgical resection of tumor with the extent ranging from total/complete/radical to subtotal/partial and influenced by size and location of CP
Correct pituitary hormone deficiencies	Hormone replacement for glucocorticoid and ADH deficiencies should be started before surgery

have pros and cons. Radical resection with complete tumor removal is intended to achieve long-term cure. Theoretically, the benign histology implies that complete removal should suffice in providing cure. In reality, the rate of recurrence (up to 50% in some series) and injury to vital structures is high despite apparent surgical clearance (De Vile *et al.*, 1996; Müller *et al.*, 2005a,b; Puget *et al.*, 2007). Nevertheless, it is still considered a favorable approach for smaller lesions without hypothalamic involvement (Mallucci *et al.*, 2012). Partial resection is aimed at decompressing the space-occupying impact of the tumor, halting progression of residual tumor, minimizing damage to adjacent structures, and reducing the target for radiotherapy. Currently, optimal surgical management of CP is deemed to involve risk-adapted multimodal treatment strategies, combining surgery and radiotherapy, aiming to limit morbidity.

The surgical route to access the CP is determined by the anatomical location, size, consistency of the tumor (cystic, solid, or mixed), and degree of neurosurgical expertise. It can be broadly classified into transcranial for predominantly suprasellar CP, transphenoidal (transnasal) for predominantly intrasellar tumors, and skull base approaches for both locations. Large lesions involving the suprasellar, intrasellar, and intraventricular sites may require combinations.

Occasionally, the cystic component of a CP requires immediate reduction, either alone or prior to more definitive surgery. Aspiration of the cyst is generally performed either endoscopically, transphenoidally, or stereotactically. Cysts that frequently reaccumulate may require fenestration. However, spillage of intracystic contents into surrounding cerebrospinal fluid (CSF) spaces from such a technique has resulted in a preference for the insertion of a subcutaneous drainage device. For obstructive hydrocephalus, various CSF diversion options are available until definitive tumor intervention is performed. The techniques for removal or drainage of CPs are evolving, and long-term follow-up is necessary to compare their outcomes (Sughrue *et al.*, 2010).

Radiotherapy

CP is extremely radiosensitive. Radiation therapy has been proved effective in shrinking the tumor, preventing recurrence and long-term disease control. It is used as adjunct to partial resection and also for tumor recurrence. As yet, there is uncertainty whether radiotherapy should be administered early to any residual tumor or deferred until signs of progression. The postoperative tumor bed and the residual tumor are targeted and monitored with serial imaging. The radiotherapy technique used is influenced by the age of the patient, the previous management, the size and location of the CP, and its proximity to vital structures (Müller, 2017; Müller *et al.*, 2017).

External beam radiation therapy

The radiotherapy techniques used for CP can be broadly classified as external beam radiation therapy and internal radiation. In the former category, conventional fractionated electromagnetic radiation has been used most widely, but proton beam therapy is emerging as a more favorable option. Fractionated radiation therapy delivers a high dose of 54 Gy to the target by dividing the treatment into multiple fractions. It is typically administered each day, so as to allow normal tissue repair between fractions. In most retrospective external beam series, excellent local control has been reported. Doses less than and >54 Gy have been associated with recurrence rates in children of 50% and 15%, respectively (Regine *et al.*, 1993). Factors predictive of local control include age of the patient, large tumor size (>5 cm), extent of resection, use of modern imaging, era of radiation treatment, and radiation technique. Modern techniques incorporating three-dimensional serial imaging enable clearer delineation of the tumor from normal tissue and improve targeting treatment more precisely at the tumor, minimizing risk to normal tissue and thus widening the therapeutic ratio (Kiehna and Merchant, 2010). These modern electromagnetic techniques include intensity-modulated radiation therapy (IMRT), stereotactic radiosurgery, and fractionated stereotactic radiotherapy. With IMRT, the dose delivered to different parts of a CP and adjacent tissues is tailored by modifying the intensity and shape of radiation beams. Stereotactic radiosurgery or gamma knife is a nonsurgical intervention with a single radiotherapy fraction. It is considered for well-

delineated solid tumors <3 cm diameter and located >3 mm from vital structures. In contrast, stereotactic radiotherapy includes multiple fractions and can be used to treat larger tumors >3 cm (Müller, 2014).

Proton beam therapy exhibits maximum dose deposition terminally (Bragg peak effect) with less risk to tissue along the radiotherapy path. This allows for higher dose delivery to the tumor. Initial results of the use of proton beam therapy in the treatment of CPs are encouraging, and use of this modality is expected to increase as more proton facilities open (Beltran *et al.*, 2012).

Internal radiation therapy

Intracavitary radiotherapy of cystic CPs has been used both as a primary and an adjunctive therapy for recurrence (Leksell and Liden, 1952; Tian, 1997). It involves the instillation of a radioisotope such as ^{90}yttrium, ^{32}phosphorus, or ^{186}rhenium via an indwelling catheter into the tumor-cyst cavity so that a high dose of radiation can be delivered to the surrounding secretory epithelial layer. Although sustained cyst regression and obliteration have been reported (Blackburn *et al.*, 1999), the impact of intracavitary irradiation on visual and endocrine function varies widely (Hasegawa *et al.*, 2004; Julow *et al.*, 2007). Intracavitary therapy has not been widely adopted owing to these drawbacks and the difficulties in handling radioactive compounds.

Chemotherapy

Chemotherapy for the treatment of CP is still experimental. A range of systemic agents have failed to produce benefit. Nevertheless, the immunomodulatory agent interferon-alpha (IFN-α) has been shown to potentially be effective after demonstrating efficacy in treating squamous-cell carcinoma (Jakacki *et al.*, 2000). The similar epithelial origin of CP provided the rational for trials with this agent. For CP with a cystic component, intracystic IFN-α can delay disease progression and the timing of further intervention with surgery or radiotherapy by median 5.8 years (Kilday *et al.*, 2017). Intracystic bleomycin has not been found to be superior to other available treatment modalities (Zhang *et al.*, 2016).

Risk-Adapted Treatment Strategies

In recent years, various risk-adapted individualized treatment strategies have been adopted for the primary management of CP (Müller, 2017; Müller *et al.*, 2017). They are aimed at managing raised intracranial pressure; relieving compression on optic nerves/chiasm, pituitary, and hypothalamus; preventing tumor regrowth and/or progression; minimizing further damage to vital structures; and acute and long-term mortality and morbidity (Puget *et al.*, 2007; Mallucci *et al.*, 2012; Fjalldal *et al.*, 2013; Müller *et al.*, 2017). A wide range of grading methods have been reported to evaluate hypothalamic involvement, including preoperative MRI (Elowe-Gruau *et al.*, 2013), pre- and postsurgical assessment (Müller *et al.*, 2012), and risk-grade adjustment after initial cyst decompression (Mallucci *et al.*, 2012). The general consensus from all these grading and treatment strategies is that limited surgical approaches and postoperative external irradiation are recommended for CP with hypothalamic involvement, and the management should be undertaken by experienced multidisciplinary teams. Monitoring with serial clinical and radiological evaluation is initially frequent (e.g., every 3 months in the first year after radiotherapy), becomes less intense over time, and can be discontinued after a progression-free period of 10 years. The rate of progression, its impact, and the age of the patient are among the factors that influence further intervention.

Treatment Complications

Surgical

The most common complication from surgery is transient diabetes insipidus in the immediate postoperative period (Sughrue *et al.*, 2010). Permanent diabetes insipidus further compromises in preexisting anterior pituitary hormone deficiencies, and additional dysfunction is also frequent. Although visual impairment at initial diagnosis can recover after surgery, it can also emerge postsurgery and especially with CP closely related to the optic chiasm. The risks of surgical trauma to the hypothalamus are significantly high especially with radical surgery.

Radiotherapy

Radiation therapy results in pituitary dysfunction and visual defects similar to that observed after surgery, but the severity of these complications, particularly diabetes insipidus, appears to be reduced. Late adverse effects of radiotherapy include vascular damage and stroke, especially Moyamoya disease (Sanford, 1994), and impaired cognition (Merchant *et al.*, 2006). The latter is especially concerning for the developing brain of preschool-age children.

Other complications of radiation therapy include treatment-induced cyst expansion, radiation-induced neoplasms (glioblastoma, sarcoma, and meningioma), radiation necrosis, optic neuritis, calcification of basal ganglia, and hypothalamic injury.

Outcomes

Recurrence of CP appears not to be influenced by the extent of surgical resection nor hypothalamic involvement. In the multicenter HIT study of a large cohort of patients with childhood-onset CP, the progression-free survival at 20 years was 58% (Sterkenburg *et al.*, 2015). However, it varied from 57% to 83% for patients who had radiotherapy to those who did not and was considerably lower for children diagnosed under 5 years compared with above age 10 years, 39% versus 77% (Sterkenburg *et al.*, 2015).

Malignant transformation is extremely rare and has been reported in a small number of children and adults with CP (Sofela *et al.*, 2014). The majority of patients had adamantinomatous CP; the most common malignancy was squamous-cell carcinoma, and an association with multiple benign recurrences and radiotherapy was identified (Sofela *et al.*, 2014).

Mortality rates from CP have improved over time and are now generally low, with survival influenced by factors including associated raised intracranial pressure, postoperative infections, endocrinopathies, or secondary effects of radiotherapy. Overall survival for childhood-onset CP is good. A recent comprehensive review of the literature since 1994 reported survival rates of 83%–96% at 5 years, 65%–100% at 10 years, and 62% at 20 years (Müller, 2017). In the HIT study, the 20-year survival was considerably higher at 85% and was not influenced by initial and subsequent management (Sterkenburg *et al.*, 2015). However, morbidities associated with hypothalamic, visual, pituitary, neurocognitive, and psychosocial defects had a significant impact on the quality of life of patients with CP (Sterkenburg *et al.*, 2015).

Conclusion

Adamantinomatous CP, which is the predominant subtype in children and adolescents, has distinct characteristics, histopathology, and molecular pathogenesis compared with the papillary subtype. Its location deep in the brain, close proximity to vital structures, unpredictable growth behavior, and chronic incurable nature can cause considerable life-long morbidity. Thus, the benign classification of CP and the overall high long-term survival of affected patients are deceptive. Manifestations at diagnosis arise from the mass effect of CP on intracranial structures. Impairment in vision, pituitary hormones, hypothalamic integrity, and neurological function vary but can be profound and progressive despite intervention. High-resolution MRI sequences enable evaluation of the tumor and its relationship to vital structures.

Definitive management for the primary tumor and recurrences comprises surgery with or without radiotherapy. Patients with CP should be managed by multidisciplinary experienced teams and ideally in the context of national and international multicenter studies. Hypothalamic-sparing, risk-adapted multimodal treatment strategies aimed at achieving good disease control, minimizing damage to vital structures, and improving outcomes are recommended. As a rule of thumb, tumors that do not impinge on the hypothalamus are treated by gross total resection. However, partial resection with radiation therapy is preferred when there is hypothalamic involvement. Modern electromagnetic (photon) radiation therapy techniques provide superior delineation of the CP and precision in targeting the tumor. However, proton therapy is deemed to be safer and more effective than photon as the radiation dose is concentrated at the tumor itself while sparing normal tissue along its path. Intracavitary irradiation, radiosurgery, and intracavitary chemotherapy with IFN-α are options for some CP. For CP with a cystic component, intracystic IFN-α may potentially delay disease progression and the timing of further intervention with surgery or radiotherapy but requires validation in a randomized clinical trial. Recent identification of activating mutations in the β-catenin gene and novel insights into the molecular pathogenesis of adamantinomatous CP offer hope for developing effective treatments.

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Hamartoma, Pituitary☆

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Glossary

Gelastic seizures Brief, repetitive, stereotyped episodes of pleasant laughter or giggling lasting a few seconds, associated or not to loss of awareness, facial myoclones and/or abnormal eye movements. Onset in infancy, high frequency and gelastic-type attacks are strongly related to the presence of hypothalamic hamartomas.

Hamartoma Congenital, non-neoplastic, pedunculated or sessile lesion consisting of heterotopic nervous tissue with no growth potential, mostly arising from the hypothalamus between the tuber cinereum and the mammillary bodies.

Precocious puberty Generally defined as the onset of secondary sexual characteristics before 8 year of age in girls and 9 years in boys; this definition is arbitrary, however, because of the marked variation in age when puberty begins in normal children, particularly if they belong to different ethnic groups.

Introduction

Hypothalamic hamartomas are rare, congenital, non-neoplastic tumor-like lesions of varying size (diameter ranging from less than 1 m to 6.5 cm) made up of heterotopic grey matter, neurons, glial cells, and fibre bundles. They usually originate in the posterior hypothalamus, the tuber cinereum or the mamillary bodies, have a sessile or pedunculated attachment, extend postero-inferiorly into the interpeduncular space and sometimes bulge into the floor of the third ventricle. Most hamartomas have a spherical shape below the tuber cinereum.

In rare cases, they may be prechiasmatic in location or may lie free in the interpeduncular fossa. They are often associated with single or combined cerebral and extracerebral congenital abnormalities, including polymicrogyria and/or heterotopia, arachnoid cyst, callosal defects, polydactyly, facial anomalies and heart malformations. An inheritance factor has been observed in individual patients.

Hypothalamic hamartomas may be asymptomatic but they generally present with central precocious puberty (CPP), gelastic seizures, generalized seizures, mental retardation, behavioral disturbances and memory difficulties. The hallmark of the epileptic syndrome is the gelastic seizure — a brief, repetitive, stereotyped attack of laughter — which begins in early childhood, often in the neonatal period. Later, in the first decade, a generalized epileptic encephalopathy develops, characterized by tonic, atonic, and other seizure types in association with a slow spike-and-wave discharge and cognitive deterioration. Hamartomas of the central nervous system do not usually grow or grow very slowly; they generally lack malignant characteristics.

Hypothalamic hamartoma is part of the Pallister-Hall syndrome, though it has exceptionally been reported in association with oral, facial, digital syndrome (OFD), Laurence-Moon-Biedl syndrome and other conditions.

Classification

Different classifications of hypothalamic hamartoma have been proposed according to the topographic and clinical data available. Hypothalamic hamartoma can be classified into two types:

- pedunculated, which is more likely to be associated with precocious puberty;
- sessile, often associated with seizures.

Another classification has been suggested based on topography and associated surgical risks:

- Type Ia and Ib are generally small pedunculated hamartomas that are attached to the tuber cinereum (Ia) or to the mamillary bodies (Ib) and are usually associated with precocious puberty.
- Type IIa and IIb are relatively large lesions that displace the hypothalamus slightly (IIa) or markedly (IIb) and are usually associated with gelastic or other types of seizures. Surgery has been recommended for types Ia, Ib, and IIa, especially when epileptic activity and behavioural abnormalities are severe and difficult to control.

A further classification has been proposed based on magnetic resonance (MR) imaging:

- The parahypothalamic type (PHH) is attached to the floor of the third ventricle or suspended from the floor by a peduncle. PHH is generally associated with precocious puberty but without seizures or development delay

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- The intrahypothalamic type (IHH), enveloped by the hypothalamus to displace the third ventricle, is more likely to be associated with medically intractable seizures. Two thirds of IHH patients suffers from severe developmental delay, cognitive impairment and have a poor quality of life

Histology and Pathophysiology

Hypothalamic hamartoma is thought to be a developmental aberration, but its origin is unclear. Remnants of brain tissue left along the floor of the third ventricle when chorda withdraws may account for its origin in the central nervous system (CNS). Histologically, it consists mainly of normal brain elements: neurons, glial cells, and fiber bundles that are frequently myelinated and connected to surrounding hypothalamic structures. Frequently, however, hamartomas do not reproduce the normal architecture of neighbouring tissue.

The hypothalamic hamartoma functions as an ectopic luteinizing hormone-releasing hormone (LHRH) pulse generator that escapes the intrinsic CNS inhibitory mechanism. In the mouse, monkey and human, LHRH neurons originate in the medial olfactory placode of the developing nose, migrate across the nasal septum, and enter the forebrain with the nervus terminalis, arching into the septal-preoptic area and hypothalamus. In hypothalamic hamartoma a significant number of LHRH neurons migrate beyond the medial basal hypothalamus to the region of the mammillary bodies and tuber cinereum and form one of the constituents of the heterotopic mass of CNS tissue. The defect in migration could be related to an imbalance of diffusible chemotrophic factors that are secreted by restricted cell populations within the brain or to neural cell adhesion molecules, which play an important role in axonal pathfinding.

Since the number of LHRH neurons is limited, it is possible that a majority of the LHRH neurosecretory neurons may migrate into the hamartomas during CNS development. On the other hand, hypothalamic hamartoma may be related to aberrant differentiation among other cell types of neural primordia, including progenitor cells with the capacity to form LHRH neurosecretory neurons.

Histological specimens observed after surgery reveal hamartomas of low cell density containing irregularly structured groups of ganglionoid cells with variably sized unipolar and bipolar neurons interspersed among glial cells with myelinated and unmyelinated fibres connected to sorrounding hypothalamic structures. The tissue is highly vascular and many of the vessels have fenestrated endothelium and double basement membranes. Each vessel is almost totally sorrounded by axons. There is no or very little tendency to proliferation.

Immunohistochemical studies confirm the neuronal origin of hypothalamic hamartoma showing positive staining for neuron-specific enolase, synaptophysin and neurofilament protein.

Different immunohystochemical studies demonstrate the presence of membrane-bound, electron-dense granules (100 nm in diameter) which contain LHRH within the perikarya, the axons and the axons terminals and which are the elements of an independent neuroendocrine unit.

The examination of two hypothalamic hamartomas associated with sexual precocity revealed that they contained astroglial cells expressing TGFα, but not LHRH neurons. These findings imply that some hypothalamic hamartomas induce sexual precocious puberty by activating endogenous LHRH secretion via astroglial-derived factors such as TGFα and/or TGFβ. This activation appears to require a close proximity of hypothalamic hamartoma to either LHRH neurons or their axonal processes in the median eminence. In some cases, the hamartoma itself does not initiate precocious sexual maturation due to its location, but rather a lesion of the adjacent hypothalamic tissue resulting from surgery may cause activation of astroglial cells which may then lead to increased LHRH secretion from hypothalamic LHRH neurons.

A number of findings suggest that hamartomas are themselves epileptogenic. Electroencephalography (EEG) recordings revealed focal spikes arising from the depth contacts within hypothalamic hamartomas, while electrical stimulation studies reliably reproduce gelastic episodes, suggesting a close relationship between hamartomas and the generation of laughing attacks. The most fascinating studies based on ictal single-photon emission-computed-tomography demonstrated marked blood flow in the hypothalamus and thalamic structures during gelastic events. Improvement in intractable epilepsy has been reported in some cases after the resection of hamartoma.

Clinical and experimental evidence suggest that the hypothalamus and adjacent structures, in particular the mamillary bodies and its immediate connections, may comprise an important sub-cortical pathway for seizure propagation. Sessile hypothalamic hamartomas with displacement of the hypothalamus are associated with seizures.

Recent evidence shows that, unlike laughing and focal seizures, slow spike-and-wave discharges and associated tonic and atonic seizures do not arise directly from hamartoma. Indeed, postoperatively, these seizures may progressively "run down" after removal of the hamartoma, suggesting that they are the result of secondary epileptogenesis. Most HH cases are sporadic. Approximately 5% of HH cases are associated with Pallister-Hall syndrome, which is caused by haploinsufficiency of GLI3. Craig et al have identified somatic GLI3 mutations in sporadic HH cases, suggesting a role in the etiology of HH lesions.

Clinical Presentation

Endocrine Aspects

Hypothalamic hamartoma is considered the most common *organic cause* associated with CPP. The incidence of hypothalamic hamartoma in determining CPP varies widely ranging from 2% to 28%.

The age of onset of the first signs of CPP has been reported between 0 and 4 years (frequently before 2 years) while CNS lesions (gliomas, germinomas, arachnoid cysts) tend to occur in association with CPP later in life.

CPP associated with hypothalamic hamartoma has all of the hormonal hallmarks of puberty, including plasma estradiol levels, a pubertal pattern of pulsatile LH and a pubertal LH response after gonadotopin-releasing factor administration. Higher levels of peak LH response and peak LH/FSH ratio after LHRH have been reported in girls with CPP associated with hypothalamic hamartoma compared to idiopathic precocious puberty.

Neurological and Behavioral Aspects

Epilepsy is the main neurologic manifestation of hypothalamic hamartoma (HH). Gelastic seizures beginning in infancy, often in the neonatal period, are the classic epileptic presentation of HH. In a proportion of patients, there is a progression through other partial seizure types to a generalized epileptic encephalopathy.

Gelastic seizures associated with HH are refractory to medical treatment. Generalized seizures are reported in approximately 70% of patients. Generalized seizures most often appear after a period of gelastic and complex partial seizures manifestations. The development of generalized seizures is usually paralleled by the development of behavioral disturbances.

Several studies reported a characteristic, recognizable epileptic syndrome that shows a typical evolution over time. The syndrome begins with laughing attacks; the resemblance to normal laughter is so close in some patients that delayed diagnosis is possible. The pattern is quite stereotyped, with some variations over time. After a few years, the laughing attacks are associated with momentary loss of awareness, facial myoclonus and/or abnormal eye movements. Autonomic phenomena are common. In the later childhood, typically between the ages of 4 and 10 years, features of secondary generalized epilepsy appear with multiple seizure patterns including tonic, atonic, and tonic-clonic seizures and progressive cognitive impairment.

Behavioral disturbances, including attention deficit hyperactivity disorder like symptoms in patients with HH is generally associated with intractable seizures.The recurrence of these seizures promotes cognitive deterioration and behavioral disturbance. In a review of all reported patients with HH, 49% manifested cognitive disturbance and 31% had behavioral problems, while all patients had seizures. Children with gelastic seizures and HH are often emotionally unstable, easily irritated restless and agitated, aggressive and antisocial. HH patients with CPP but without evidence of seizure activity rarely have cognitive defects.

In a study with a large cohort of patients, it has been demonstrated that patients with hamartomas display a statistically significant increase in co-morbid psychiatric conditions, including oppositional defiant disorders, attention-deficit/hyperactivity disorder, high rates of conduct disorders, speech retardation/learning impairment and anxiety and mood disorders. At the present time, there are no definitive data to indicate exactly how the epileptic pathology interferes with a patient's development and behaviour and why a majority of patients have a cognitive-behavioural deficit which often worsens with age.

Brief infantile attacks are not associated with any significant change in EEG. However in later childhood, with the progression of the epileptic syndrome, EEG abnormalities develop with generalized suppression of background rhythm, generalized low-voltage fast activity or both. Interictally, there are generalized slow spike-and-wave discharges and background activity that is abnormally slow for the child's age as hallmarks of secondary generalized epilepsy.

Magnetic Resonance Imaging

MR imaging is the diagnostic method of choice for the evaluation of hypothalamic hamartomas. They appear as well defined round to oval masses, generally isointense to grey matter on both T1- and T2-weighted images. Following gadolinium injection they never enhance (**Fig. 1**). However, hypothalamic hamartomas may sometimes be slightly hyperintense on T2-weighted (**Fig. 2**) and FLAIR images. The variance in myelinated axons and the presence of gliosis can affect the variability of the T2 signal. Proton MR spectroscopy (**Fig. 2**) can show decreased N-acetylaspartate and increased myoinositol. These findings correlate with the proportion of glial cells within the hamartoma.

Another characteristic feature of hypothalamic hamartomas is their relative stability in size, shape and signal in long-term MR follow-up during and after medical treatment. Signal or cystic changes have occasionally been observed: in these cases, the differential diagnosis should include low grade gliomas.

Overall the MR findings which commonly indicate hypothalamic hamartomas are: an isointense mass with a pedunculated or sessile attachment to the hypothalamus without contrast enhancement, stable in size and shape during follow-up, associated with endocrinological and clinical features of CPP, mostly before 2 years of life and associated with gelastic seizures.

Treatment

Precocious Puberty

The aims of treatment are to arrest physical maturation, to prevent early menarche or sexual adult psycho-physical maturation and to improve adult height to within the range of target height combined with normal body proportions. GnRH agonists are the therapy of choice to halt premature sexual development in patients with a HH if precocious puberty is its only manifestation.

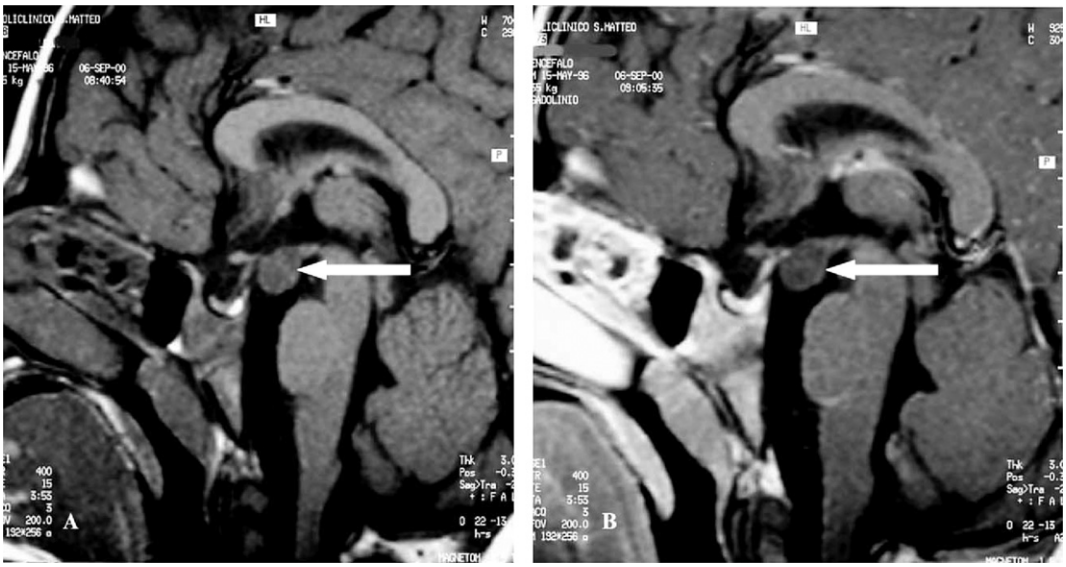

Fig. 1 (A) Sagittal T1-weighted image; (B) Gd-enhanced sagittal T1-weighted image. Sessile hamartoma in typical third ventricular location, isointense to gray matter before (A) and after (B) gadolinium administration (*white arrow*).

Successful suppression treatment with GnRH analog preparations has been reported by several groups for a duration of up to 8 years with homogeneous endocrinological response characterized, after an initial phase of increased serum LH and FSH levels (flare-up), by a reduction to prepubertal ranges of basal LH, FSH, testosterone and E2, a prepubertal peak LH and FSH-response to GnRH stimulation test and prepubertal LH/FSH ratio with improvement of adult height towards predicted final height and close to target height; no adverse events have been reported. A variety of GnRH formulations are available and efficacious. The depot preparations are preferred because of improved compliance. In most children, monthly injections adequately suppress the gonadotropic axis, but some children require more frequent injections.

A controversial outcome of precocious puberty has been reported after surgical resection of hamartomas. It has been suggested that surgery is safe and it can be considered an option when compared to the cost of medical treatment, monthly treatment administration up until puberty (sometimes poorly tolerated), painful injections in some cases, the psychological implications, and in some rare cases an intolerance to treatment. Total resection, however, cannot be achieved in all hamartomas.

Females with hypothalamic hamartoma have a higher incidence of irregular menses, obesity, neurological and behavioural problems with a normal reproductive axis within 4–5 years after discontinuation of GnRH analog therapy; pregnancies resulted in normal, live infants.

Seizures

Treatment options for intractable gelastic seizures in HH patients include direct open surgery with craniotomy, endoscopic surgery, radiosurgery with gamma knife (GKS) and stereotactic radiofrequency thermocoagulation.

In the last few years, it has become clear that epileptic encephalopathy associated with hypothalamic hamartoma is a treatable condition. Strong evidence now exists that removal, destruction or disconnection of the hamartoma leads to remarkable control of the seizures, as well as to an improvement in behaviour and probable cessation of cognitive decline.

Gelastic seizures are resistant to all currently available medications. Rarely, treatment of the focal seizures and of tonic and atonic attacks can be moderately successful with conventional anti-epileptic drugs. However, recent and sufficient experience suggests that long-lasting control of these seizures can be effected by complete removal, destruction, or disconnection of the hamartoma, thanks to important improvements in imaging and surgical techniques. There now exist various possible surgical approaches to the lesion and the debate is currently focused on the best technique to use (i.e., it must be tailored to the specific surgical anatomy of the hamartoma) in order to guarantee, in each single case, the possibility of total removal or disconnection of the lesion. The transcallosal approach (suited for intraventricular lesions) presently appears the technique with the highest rate of success, with 90% of cases free or virtually free of all seizures at one year post-surgery follow-up. Prolonged follow-up is necessary to evaluate the real effects on cognitive impairment, whereas behavioural abnormalities tend to show marked improvement in many patients.The pterional approach is useful for targeting peduncolated HHs, which are relatively easy to be completely resected.

Open and endoscopic disconnection surgery is a safe and effective option for small, sessile HHs.

Described secondary effects of surgery are small strokes with good recovery, encephalomalacia, third-nerve palsy, memory impairment, and transient diabetes insipidus. Postoperative mortality has also been reported.

Stereotactic radiosurgery (SRS) is increasingly being utilised. The major attractions of stereotactic radiotherapy involve the avoidance of mortality and morbidity risks associated with invasive neurosurgery. Stereotactic localisation allows attainment of a high degree of accuracy

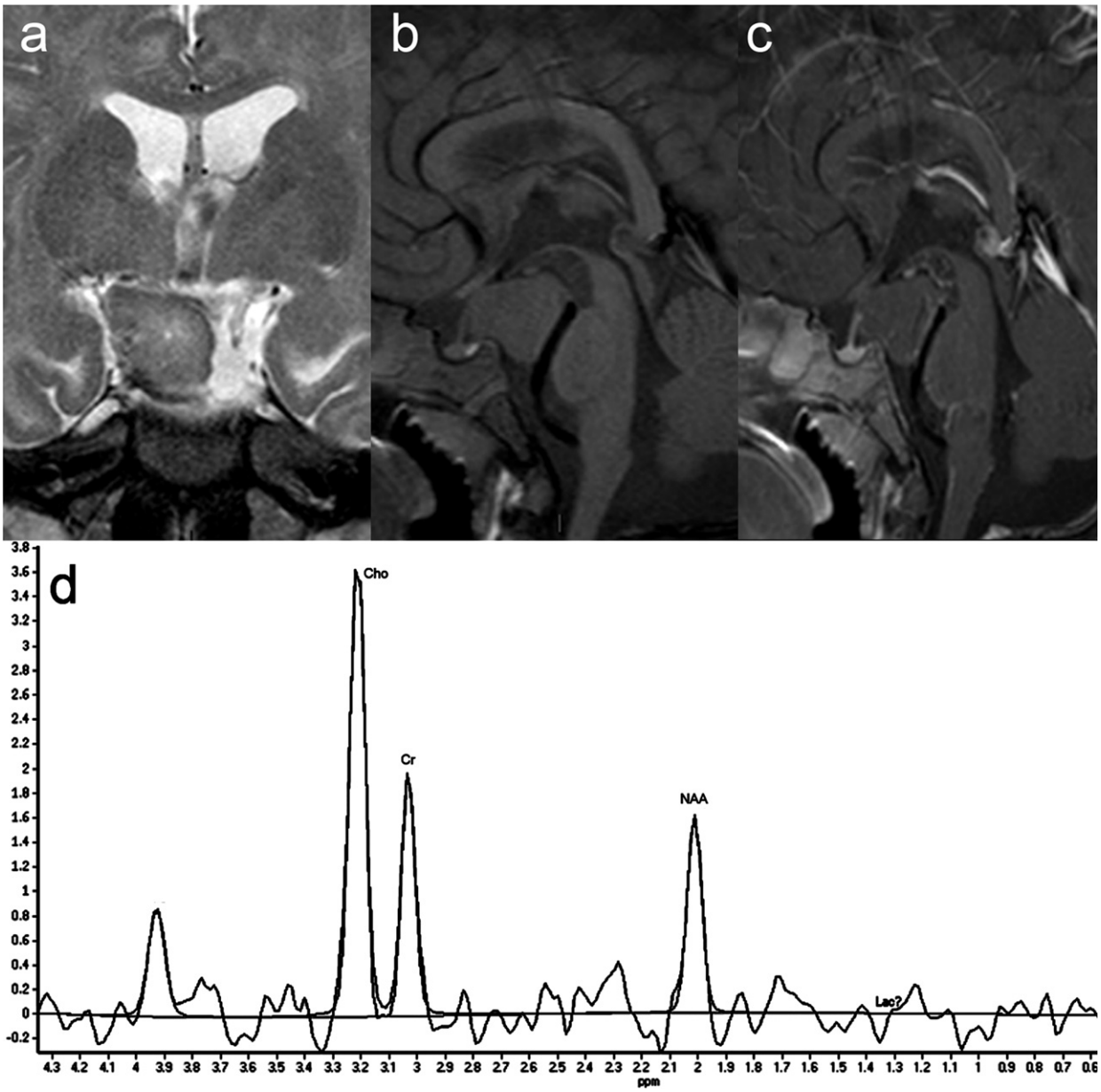

Fig. 2 (A) Coronal T2-weighted image; (B) Sagittal T1-weighted image; (C) Gd-enhanced sagittal T1-weighted image; (D) Single voxel MR spectroscopy (144 ms echo time). Retrosellar median-right paramedian solid mass lesion attached to the floor of the third ventricle and projecting into the interpeduncolar fossa. The lesion is slightly hyperintense on T2 (A), isointense on T1 (B), without contrast enhancement (C). Proton MRS shows mild increase of Cho/NAA and Cho/Cr ratios (D).

and precision, enough to deliver a dose of radiation high enough to affect epileptogenesis while sparing critical normal structures at the same time. Regis at al have reported excellent result in 60% of children with complete seizure cessation in 40% and rare nondisabling seizures in 20%, often in association with dramatic behavioural and cognitive improvement.

Some reports emphasize the importance of the margin dose of radiation. Patients treated with doses exceeding 17 Gy to the margin of the HH have greater rates of seizure remission than those receiving less than 13 Gy. Duration since onset has been reported to be another factor predictive of outcomes with SRS. Patients with short durations are likely to enjoy better responses, whereas patients with long histories are likely to receive modest benefits even with high doses. The disadvantage of SRS is its delayed action, with maximal effect usually experienced after a period of about 6 months post treatment. Surgery may be preferred over radiosurgery among patients with very large lesions that could be causing symptoms do to mass effect, since surgery can accomplish immediate decompression. Overall, when the lesion is sufficiently small SRS offers a rate of seizure control comparable to microsurgery but with much lower risk.

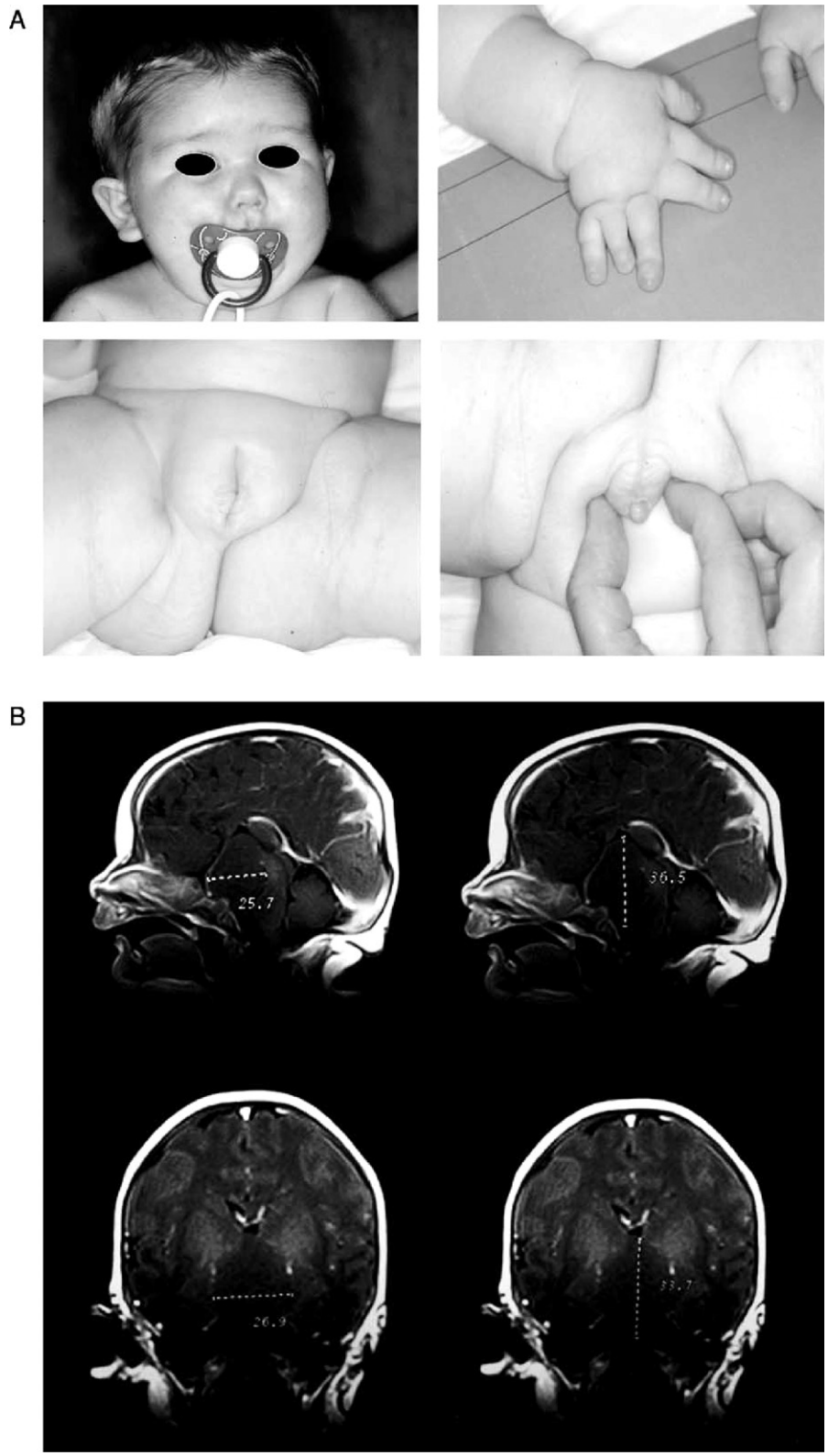

Fig. 3 (A) Frontal view showing a phenotype suggestive of congenital hypopituitarism. Note the hands with polydactyly; micropenis and cryptorchidism are associated with panhypopituitarism. (B) Sagittal and coronal T1-weighted MR images demonstrating the mass (dotted lines) compatible with hamartoblastoma and the absence of pituitary tissue.

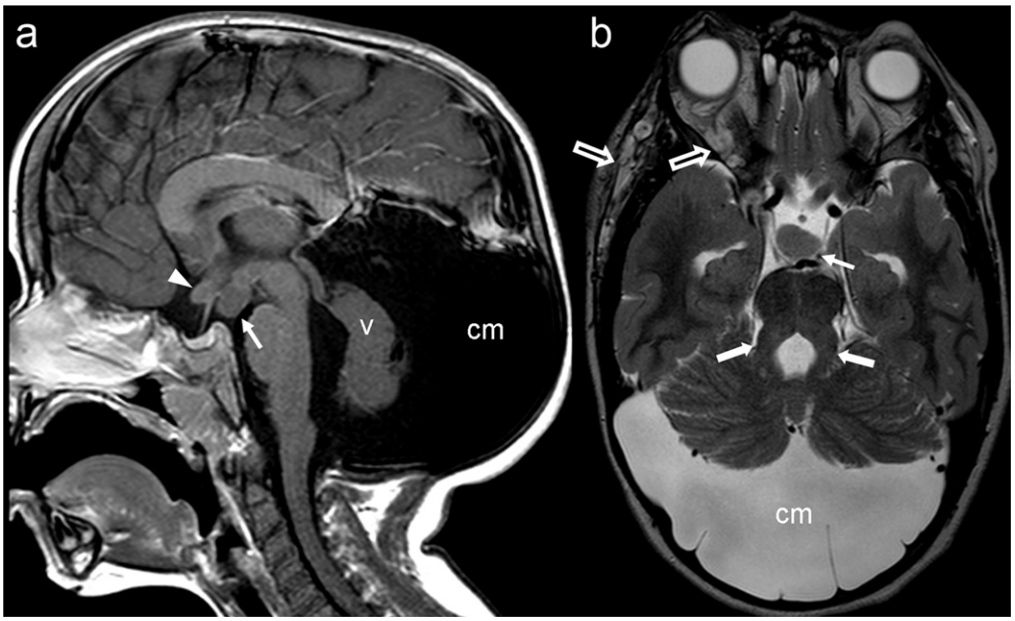

Fig. 4 Varadi–Papp syndrome and coexistent Neurofibromatosis type 1. (A) Gd-enhanced sagittal T1-weighted image; (B) Axial T2-weighted image. Hypothalamic hamartoma attached to the floor of the third ventricle and projecting into the interpeduncolar fossa (*thin arrow*, A, B). Pathologic thickening of the optic chiasm (*arrowhead*, A), without contrast enhancement, and small neurofibromas in the right orbit and temporal subcutaneous region (*open arrows*, B). Vermian hypoplasia (v), huge cystic dilatation of the cistern magna (cm) and molar tooth malformation (*thick arrows*, B).

Stereotactic radiofrequency thermocoagulation has also been used. It is a procedure during which a monopolar needle, under stereotactic guidance, is inserted into the surgical target. Current at high frequency (above 250 Khz) is injected, resulting in heating of the tissue at the electrode tip. The effectiveness of stereotactic radiofrequency thermocoagulation in the treatment of epilepsy has been reported in small case series of HH. Kameyama et al. reported the largest group, consisting of 25 patients of whom 19 (76%) had seizure remission; improvement in behaviour also occurred. Complications in this group included evidence of hypothalamic dysfunction in more than 50% of patients (hyperphagia, hyponatremia, Horner's syndrome, all transient). Stereotactic radiofrequency thermocoagulation has the advantage over SRS of earlier postoperative treatment effects.

Associated Conditions

Pallister–Hall Syndrome

Pallisteral–Hall syndrome (PHS) was first described in 1980 as a lethal congenital malformation syndrome associated with hypothalamic hamartoblastomas, postaxial polydactyly, craniofacial malformations and imperforated anus. Additional abnormalities include developmental and postnatal retardation, holoprosencephaly, pituitary agenesis/dysgenesis with hormone dysfunction leading to micropenis, cryptorchidism, hypopituitarism or panhypopituitarism, laryngeal clefts, bifid epiglottis, buccal frenula, small nose/anteverted nares, low-set/posteriorly angulated ears and microphtalmia, limb/skeletal malformations such as short fourth metacarpals and nail dysplasia, involvement of other organs with abnormal lung lobulation, renal agenesis and/or dysplasia, and congenital heart defects (**Fig. 3**). The syndrome is now considered a clinical, variable "iceberg" disorder with wide phenotypic variability where adrenal insufficiency is the most common cause of perinatal death.

This disorder is inherited as an autosomal dominant trait and has been reported in association with unbalanced chromosome translocation between chromosomes 3 p and 7q and later mapped to chromosome 7p13. Mutations in the transcription regulator gene GLI3 have been identified in patients with PHS, but also in four other different autosomal dominant phenotypes: the Greig cephalopolysyndactyly syndrome, preaxial polydactyly type IV, postaxial polydactyly type A and postaxial polydactyly type A/B.

Consensus guidelines in 1996 developed diagnostic criteria for the delineation of familial Pallister–Hall syndrome, which include the presence of hypothalamic hamartoma, central polydactyly most commonly affecting the third or fourth digit, and, in the first degree relatives of an index case, hypothalamic hamartoma or similar digital abnormalities associated with an autosomal dominant. An association with endocrine dysfunction requires hormone substitutive treatment. Adult height above the target height has been reported in a patient treated with growth hormone for up to 7 years. Surgical correction of visceral abnormalities is mandatory.

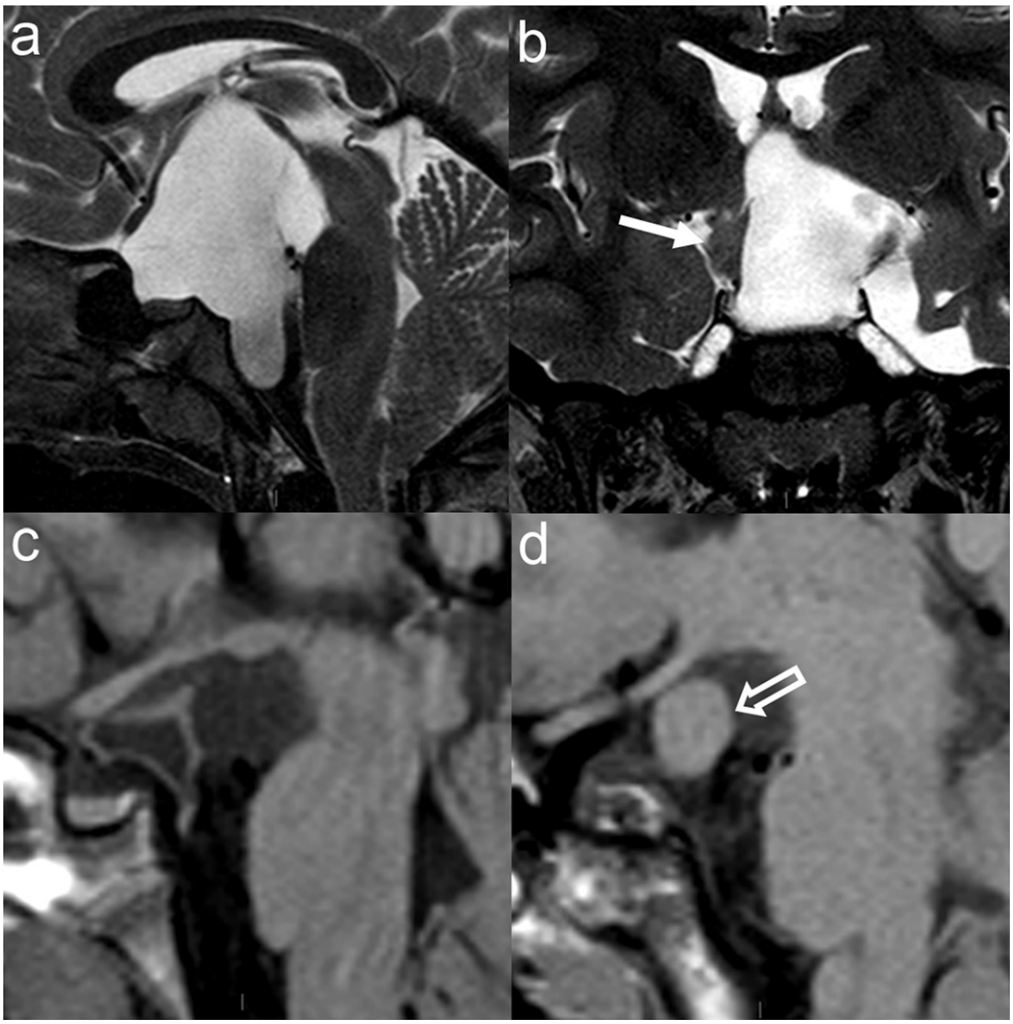

Fig. 5 Coexistent suprasellar arachnoid cyst and hypothalamic hamartoma in a 8-year-old boy with precocious puberty. (A) Sagittal T2-weighted image; (B) Coronal T2-weighted image; (C, D) Sagittal T1-weighted images. (A, B) Brain MRI at admission shows a huge suprasellar arachnoid cyst. Along the right wall of the cyst a solid mass, isointense to brain parenchyma, is demonstrated (*arrow*, B). (C, D) Brain MRI performed following surgical fenestration of the cyst better shows the peduncolated hamartoma, projecting into the interpeduncolar fossa (*open arrow*, D).

PHS patients usually have well controlled seizures and endocrine disturbances other than precocious puberty. Only a few PHS patients (those with the most frequent and difficult to control seizures) have marked behavioural or developmental problems. These data support previous reports of a strong effect of seizures on cognitive and behavioural problems in patients with HH.

Other Conditions

Hamartomas may be found in syndromes of midline defects such as oral-facial-digital syndrome type 6 (Varadi–Papp syndrome) (Fig. 4), Smith–Lemli–Opitz syndrome, solitary maxillary incisor, or in association with CNS malformations including agenesis of corpus callosum or holoprosencephaly. They have also been described in Laurence–Moon–Biedl syndrome, McKusick–Kaufman syndrome and Waardenburg syndrome. Syndromic HH have milder symptoms than isolated HH, but likely arise from similar pathogenic mechanisms.

HH have also been reported in association with a spectrum of cystic abnormalities (Fig. 5) which include intrahamartomatous cysts, arachnoid cysts that extend within the hamartoma, and arachnoid cysts that are adjacent to or remote from the hypothalamic hamartomas. The mechanism behind this association is not known. Different hypotheses advocate disruption of the prenatal subarachnoid space formation by the hamartoma, or inclusion of meningeal tissue within the hamartoma.

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Anorexia Nervosa☆

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Glossary

Amenorrhea The absence of menstruation during a minimum period of 3–6 months in women who have previously experienced menstruation (secondary amenorrhea) or the lack of menarche at 16 years of age (primary amenorrhea).

Bone mass Total quantity of bone tissue, including the total volume of bone tissue and the total quantity of mineralized extracellular matrix.

Malnutrition A pathological condition occurring when energy and nutrient requirements are not met by dietary intake. There is a wide spectrum of clinical forms, depending on the severity and duration of the deficit, the age of the subject, and the cause of the condition. *Alterations in bone mass in adolescents*: Decreased bone mineral density: Significant diminution of bone mass per unit volume in relation to what is considered normal for the age, pubertal stage, and sex of the subject. BMD below -2 SD (Z score) + two criteria: Two or more fractures of low impact in long bones before 10 years; three or more fractures in the same bones before 19 years; one or more vertebral fracture at whatever age. *Alterations in bone mass in adults*: osteopenia: BMD between -1 and -2.5 SD; osteoporosis: BMD -2.5 SD; severe osteoporosis: BMD $>$ -2.5 DE, presence of one or more fractures.

Introduction

Anorexia nervosa (AN) is a psychiatric disorder with childhood and adolescent onset characterized by excessive dieting, some compensatory behaviors (excessive exercise, vomiting, and use of laxatives) and specific psychopathological symptoms (disturbance of experience of body weight and/or image, and fear of becoming fat) that lead to severe and maintained weight loss, which results in progressive malnutrition. The American Academy of Psychiatry suggested, in the fifth addition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), that other forms exist, such as avoidance/restrictive food intake disorder (ARFID), that present no specific psychopathological symptoms, and less severe forms renamed "other specified feeding or eating disorder" (OSFED). The latter includes five former disorders: atypical anorexia, purging disorder, subthreshold bulimia, subthreshold binge eating disorder, and night eating disorder. The new category of ARFID is intended to capture not only individuals who meet the criteria for the Feeding Disorder of Infancy and Early Childhood DSM-IV category, but also other individuals with clinically significant eating problems who are not included in DSM-IV categories, and therefore must be assigned a diagnosis of eating disorder not otherwise specified (EDNOS), such as selective eating and/or dysphagia.

Prevalence

The lifetime prevalence of DSM-5 AN among women is up to 4% and of bulimia nervosa and binge eating disorder 2%, respectively, with a bimodal age distribution including peak ages of 14 and 18 years, respectively. However, an increasing number of cases in girls during the initial stages of puberty (12–15 years), as well as at the onset of puberty, have been observed in recent decades. The female/male ratio ranges from 5:1 to 10:1. The more inclusive DSM-5 criteria reduce the proportion of EDNOS diagnoses regarding DSM-IV and increase the proportion of anorexia and bulimia nervosa, with the new cases tending to have a higher minimum body mass index (BMI) and a more benign course (Muñoz and Argente, 2004). The eating patterns and compensatory behaviors (e.g., reduced food intake, exercise to reduce weight) is a continuum in the population; furthermore, up to 50%–67% of adolescent females are dissatisfied with their weight and body shape, and most adolescents have been on a diet at least once in their life. Many of these teens use unhealthy weight control methods, including fasting, diet pills, excessive exercise, and vomiting.

☆*Change History*: December 2017. M. Teresa Muñoz, Monserrat Graell and Jesús Argente updated the text and added new references.

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Etiopathogenesis

The etiology of AN is multifactorial, including genetic, biological, psychological, and cultural factors. The coexistence of various risk factors increases the possibility of developing this disease (Fig. 1 and Table 1).

Individual Factors

Patients with AN frequently present some specific temperamental features considered risk factors to develop some mental disorders, including eating disorders. These include introversion, obsession, perfectionism, persistence, low self-esteem, avoidance behavior, and high anxiety. In addition, patients who use purgative methods have a marked tendency to impulsive behaviors and often present self-injuries and suicidal attempts, and have problems with alcohol and drugs. The consequent malnutrition elicits several alterations, both physical and mental (obsession, depression), and alterations in their ability to maintain social relationships that is often manifested as isolation. All of this results in a new decrease in self-esteem and sense of effectiveness (Mayhew *et al.*, 2017).

Some high-level athletes develop AN, but others present incomplete or subclinical forms with a more difficult diagnosis. A form called Female Athlete Triad includes low energy availability with or without disordered eating, menstrual dysfunction, and low bone mineral density (BMD) (Fig. 1).

Genetic Factors

Independent of the type of TCA, the majority of the candidate genes are related to the homeostatic control and reward systems (Hinney *et al.*, 2017). The principal genes include the following:

1. Homeostatic pathway: the genes for leptin, leptin receptor, proopiomelanocortin, Agouti-related peptide, melanocortin 4 receptor, brain derived neurotrophic factor, and FTO.

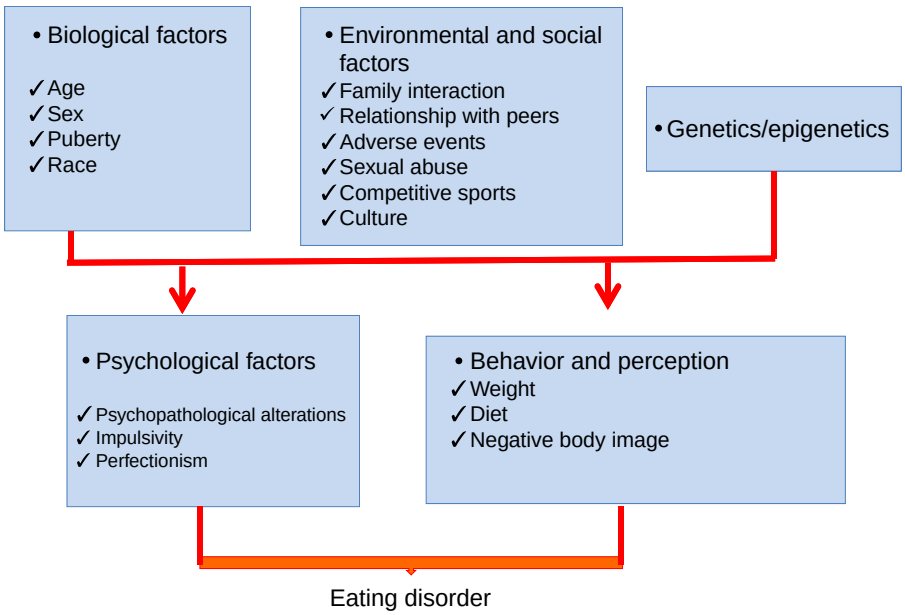

Fig. 1 Risk factors increasing the possibility of developing eating disorders.

Table 1 DSM-5 diagnostic criteria for anorexia nervosa (American Psychiatric Association, 2013)

Restriction of energy intake relative to requirements, leading to a significantly low body weight in the context of age, sex, developmental trajectory, and physical health. Significantly low weight is defined as a weight that is less than minimally normal or, for children and adolescents, less than that minimally expected
Intense fear of gaining weight or of becoming fat, or persistent behavior that interferes with weight gain, even though at a significantly low weight
Disturbance in the way one's body weight or shape is experienced, undue influence of body shape and weight on self-evaluation, or persistent lack of recognition of the seriousness of the current low body weight

Source: American Psychiatric Association (2013) Diagnostic and statistical manual of mental disorders (5th edn.). Arlington, VA: American Psychiatric Association.

2. Reward-related pathways, including central neurotransmitters such as serotonin and its receptors and transporters (HTR1D, HTR2A, HTR2C, SLC6A4), dopamine and its receptors and signaling (DRD2, DRD4, ANKK1), noradrenaline (COMT), opioids (genes *OPRD1*, *OPRM*), and the cannabinoid system.
3. Two AN loci have been identified by GWAS. The gene Early B-Cell Factor 1, when inactivated in AN, results in a reduction in leptin levels.
4. In a recent whole-exome/genome sequencing study of TCA, the following genes were identified: estrogen-elated receptor α (ESRRA) and histone deacetylase 4 (HDAC4) that induce the expression of monoamino oxidase A and B, suggesting a potential role in the metabolism of neurotransmissors such as serotonin and dopamine.

Familial Factors

Some studies find that families are overprotective, strict, and have low levels of warmth in their rearing practices in comparison to other families without eating disorders. In addition, an inability to solve family conflicts can occur (Mayhew *et al.*, 2017). In some families in conflict (i.e., conjugal), the adolescent is often recognized as an individual with their own needs only after the onset of the illness; consequently, the patient tends to maintain the illness in order to keep the family's attention (**Fig. 1**).

Sociocultural Factors

Adolescents are often very vulnerable, receiving a large quantity of information that they cannot assimilate, which creates tension regarding problems normal for their age, including sexuality, competitiveness, individuality, and independence within the family (**Fig. 1**).

The adolescent is constantly bombarded with information regarding the ideal weight and figure, how to have the perfect body, what type of exercise one must practice to achieve this perfect body, and miracle diets. The cultural image model barely tolerates diversity in body forms, identifying thinness as a value of success (Mayhew *et al.*, 2017).

Evaluation

Multiple organ system complications are seen, including those involving the cardiovascular and peripheral vascular systems and gastrointestinal, hematological, renal, skeletal, endocrine, and metabolic disorders. These alterations are related not only to the state of malnutrition, but also to the conduct of these patients to control their weight.

The psychopathological and familial evaluation realized by a mental health professional (psychologist and/or psychiatrist) is crucial to diagnose and decide the therapeutic approach.

Medical Complications

The clinical manifestations of AN are broad, affecting all systems of the organism, and depend largely on whether the form is restrictive or purgative. About 10%–20% of anorexic patients have bulimic tendencies, which mainly include provocation of vomiting, the use of laxatives, and a compulsive increase in physical activity.

Cardiovascular problems occur in up to 80% of patients, including bradycardia and hypotension, due to autonomic nervous system imbalances (Muñoz and Argente, 2004). Electrocardiographic abnormalities may include atrial and ventricular arrhythmias and QTc abnormalities. In addition, changes in myocardial function have been reported with decreases in myocardial tissue mass, mitral valve prolapse, and pericardial effusions.

Gastrointestinal complications are also common. AN can cause a decrease in gastrointestinal motility, resulting in chronic constipation. Laxative abuse can lead to cathartic colon syndrome and chronic constipation that is sometimes refractory to treatment. Cases of acute gastric dilatation have been described during the phase of realimentation of extremely affected anorexia patients since gastric emptying of solids is retarded, with emptying of liquids being retarded in some patients. Esophageal problems include severe esophagitis and even ruptures of the esophagus, associated with induced vomiting (Muñoz and Argente, 2004).

Neurological consequences result from severe malnutrition. Computed tomography and magnetic resonance imaging have demonstrated cortical atrophy and ventricular dilatation. Malnourished patients have greater cerebrospinal fluid volumes and reduced white and gray matter volumes (Muñoz and Argente, 2004). Abnormalities on computed tomography scans are reversible with refeeding and nutritional recovery.

Biochemical Abnormalities

Hematological findings include anemia, leucopenia (relative neutropenia and lymphocytosis), thrombocytopenia, low erythrocyte sedimentation rate, and decreased fibrinogen levels in plasma (Muñoz-Calvo, 2005). The anemia and occasional pancytopenia appear to be due to hypoplasia of the bone marrow, which is filled with a gelatinous mucopolysaccharide.

Vomiting results in the loss of sodium, hydrogen, and potassium, causing metabolic alkalosis. The use of laxatives provokes the loss of potassium and bicarbonate, which can result in metabolic acidosis (Muñoz-Calvo, 2005). Finally, the use of diuretics can cause increased loss of sodium, potassium, and calcium in the urine, depending on the dose and drug used.

Renal complications are present in 7% of these patients and can include a decrease in glomerular filtration, an increase in plasma urea and creatinine levels, electrolyte alterations, edema, and hypokalemic nephropathy.

Cellular immune functions are also altered because of poor nutrition. These include modifications in some immunoglobulin fractions (IgG and IgA and complement factors C3 and C4), low response in the cutaneous delayed hypersensitivity test, and alterations in the lymphocyte subpopulations CD3, CD4, and CD57. However, infections are infrequent in these patients.

Plasma protein levels are usually normal, although in some cases hypoalbuminemia is present. Elevated amylase has been observed in the absence of clinical signals of pancreatitis.

Mild hypercholesterolemia is frequent in AN, with the elevation occurring in the low-density lipoprotein fraction, whereas both high-density lipoprotein and very low-density lipoprotein levels are normal. The cause of the hypercholesterolemia is a result of a reduced bile acid requirement is also characteristic (Muñoz-Calvo, 2005).

Differential Diagnosis

A differential diagnosis must be made in situations characterized by loss of weight in young persons, such as brain tumors and lymphomas, and in cases with gastrointestinal symptoms, such as chronic inflammatory disease (Muñoz and Argente, 2004). In addition, different endocrine pathologies, such as Addison's disease, hyperthyroidism, and uncontrolled diabetes mellitus, must be taken into consideration. Depending on the predominant psychopathological symptoms, depression, obsessive-compulsive alterations, social phobia, and schizophrenia must be ruled out.

Complications

Endocrine and Neurotransmitter Disturbances

Hypothalamic–pituitary–gonadal axis

Patients with AN exhibit isolated hypogonadotropic–hypogonadism of hypothalamic origin. The etiology is uncertain, although multiple factors may play a role, including hypothalamic dysfunction, reduction of weight, sex steroids, and neurotransmitters alterations, as well as physical exercise (Schorr and Miller, 2017).

Adolescents with AN exhibit low basal levels of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as well as low estradiol and testosterone levels, indicating the abnormal function of the hypothalamic–pituitary–gonadal axis (Schorr and Miller, 2017). These hormonal alterations result in a prolonged follicular phase and an insufficient luteal phase. In addition, spontaneous secretion of LH during a 24-h period is decreased in both frequency and amplitude of the secretory bursts. With weight gain, serum levels of both LH and FSH are increased, suggesting that malnutrition may play a role in the regulation of gonadotropin secretion (Muñoz-Calvo, 2005). Disturbances in neurotransmitters have been described, including the dopaminergic system and endogenous opioids, which are both peptides involved in gonadotrophin-releasing hormone (GnRH) regulation.

Malnutrition may be responsible for the delayed puberty and reduction in growth seen in these patients. This phenomenon has been interpreted as a mechanism of adaptation to the reduction in nutrients. Delayed puberty is present when symptoms appear in prepubertal patients; in contrast, if the disease begins after development has begun, puberty is detained, and the growth spurt is delayed and reduced. Finally, if symptoms appear after puberty, secondary amenorrhea is present. One of the indications that the process of adaptation to malnutrition has begun is hypoinsulinemia, present as a consequence of low glucose and amino acid levels. Furthermore, growth hormone (GH) abnormalities and low IGF-1 levels contribute to poor growth in prepubertal patients, leading to a reduction in their final height (Schorr and Miller, 2017).

Leptin is a fat-derived anorexigenic hormone. In subjects with reduced fat stores, problems with reproductive system functioning are frequent, including a reduction in serum sex steroid levels. A similar phenomenon, the shutdown of the hypothalamic–pituitary–gonadal axis, occurs in patients with AN after the loss of fat stores. In both cases, it is speculated that the problems with gonadal function could be related to the decreased basal serum leptin levels and pulsatile secretion as a result of the loss of fat tissue (**Fig. 2**). The degree of weight recovery after nutritional therapy in AN patients is correlated with the increase in their serum leptin levels. Furthermore, this rise in leptin correlates substantially with increasing gonadotropin levels, suggesting that the increase in circulating leptin associated to nutritional recovery could be involved in the activation of the hypothalamic–pituitary–gonadal axis. However, the increase in free leptin index after nutritional therapy in AN patients has been associated with the resumption of menses, thus reinforcing the importance of the increase in leptin bioavailability on the hypothalamic–pituitary–gonadal axis reactivation (Argente *et al.*, 1997).

Kisspeptin is a neuropeptide that acts directly on hypothalamic GnRH neurons, which in turn stimulate the secretion of FSH and LH. Kisspeptin levels are reduced and negatively correlated with BMI in adolescents with AN (Schorr and Miller, 2017).

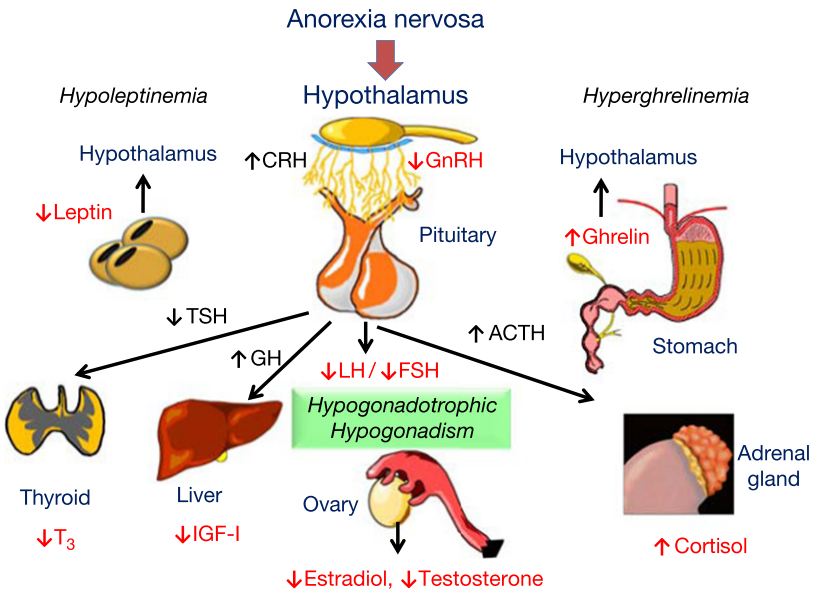

Fig. 2 Hormonal changes in patients with AN and hypothalamic amenorrhea.

One study reported that 85% of patients reached 90% of their ideal weight, restarting menstruation within the following 6 months. However, amenorrhea persisted in 15% even though they regained their weight. It therefore follows that one of the decisive factors for the normalization of gonadal function is the recuperation of the nutritional state (Baskaran *et al.*, 2017).

Hypothalamic–pituitary–GH axis

AN is associated with a nutritionally acquired resistance to GH, with decreased liver production of IGF-1 and elevated GH levels. Multiple mechanisms have been proposed to explain the hepatic GH resistance, including low IGF-1 levels and elevated levels of fibroblast growth factor 21, produced in hepatocytes and white adipose tissue (Baskaran *et al.*, 2017).

Few studies have analyzed spontaneous GH secretion (SGHS) in patients with AN. We studied SGHS in a group of anorexic patients at diagnosis and at two different time-points during weight recovery, and found that at diagnosis SGHS is heterogeneous (Muñoz-Calvo, 2005). In 40% of subjects, the mean 24-h GH secretion was >3 ng/mL (lower limit of normality), and the remaining 60% had levels below the normal range. The difference between these groups and the controls was due to modification in the amplitude of the GH peaks and not as a result of pulse frequency. In both groups, recovery of at least 10% of initial weight resulted in the normalization of the parameters of SGHS. These observations suggest that alterations in GH secretion in these patients are due to modifications in its neuroendocrine control, with a possible increase in growth hormone-releasing hormone (GHRH) release and decreased somatostatin tone.

The GH pattern in conjunction with the negative correlation between basal and pulsatile GH secretion and BMI suggests that the observed alterations in GH secretion are directly related to malnutrition. One possible mechanism could involve the reduced IGF-1 levels caused by malnutrition. This would reduce the negative feedback action that IGF-1 exerts on GH secretion at the level of the hypothalamus and pituitary. Another variable that may be involved is the hypoestrogenism that accompanies amenorrhea. It has been suggested that malnutrition underlies the increase in the amount of GH secreted in each pulse and that hypoestrogenism is responsible for the increased pulse frequency. Definite conclusions, however, cannot be drawn from these studies (Muñoz-Calvo, 2005).

Serum GH-binding protein (GHBP) levels in patients with AN are dramatically reduced and tend to normalize with weight recovery. The reduction in GH receptors is most likely one of the principal causes of GH resistance. In malnutrition, the low GHBP levels may be related to hypoinsulinemia, alterations in thyroid function, or hypoestrogenism. On the other hand, many studies have demonstrated a correlation between serum GHBP levels and BMI or the percentage of body fat or, more specifically, visceral fat. Given that it has not been demonstrated that circulating GHBP is uniquely or even preferentially derived from liver GH receptors, it is possible that other tissues, such as adipose tissue, may contribute to plasma GHBP levels. If this is the case, the extreme reduction in adipose tissue in patients with AN may cause the observed decrease in serum GHBP levels (Muñoz and Argente, 2004).

Patients with AN have extremely reduced serum IGF-1 levels that tend to normalize with weight recovery; however, as observed for other forms of malnutrition, the time necessary for this to occur may be prolonged. Circulating IGF-1 is largely dependent on GH, but it is also very sensitive to nutritional changes. In AN patients, serum IGF-1 levels do not correlate with GH secretion, which suggests that the decrease in IGF-1 is independent of GH and probably directly due to the state of malnutrition (Muñoz and Argente, 2004).

Patients with AN have elevated serum IGFBP-1 and IGFBP-2 levels that tend to normalize with weight recovery. Both are reported to be GH-independent and very sensitive to nutritional regulation. The increase in IGFBP-1 in these patients is most likely related to hypoinsulinism (Muñoz and Argente, 2004).

Serum IGFBP-3 levels are decreased in AN patients as a consequence of GH resistance and tend to normalize after weight recovery. Indeed, all components of the trimolecular complex formed by the union between IGFBP-3, IGF, and the acid-labile subunit are decreased. Given that these proteins are all GH-dependent and regulated by the nutritional state, this is not unexpected. IGFBP-3 decreases significantly with caloric restriction, but in adults it decreases only with protein restriction. In contrast to other catabolic situations, in AN increased proteolysis of IGFBP-3 has not been observed (Muñoz-Calvo, 2005).

Alterations in the GH-IGF-1 axis have been well documented, but with respect to the recuperation of growth, the results are not conclusive. Some authors observe delayed growth, while others report total recuperation of this parameter. One recent retrospective study in adolescents with AN, delayed puberty, and reduced growth velocity (<2.5 cm/year) reported that nutritional rehabilitation and administration of recombinant GH at high doses resulted in an increase in growth velocity during the 3 years of treatment (Léger *et al.*, 2017). However, more controlled studies are necessary to determine the long-term effect of GH in these patients. The delay in bone age and ponderal gain during treatment would be the main predictors of growth recuperation.

Hypothalamic–pituitary–thyroid axis

Thyroid function is affected by malnutrition in AN (**Fig. 2**). Clinically, patients appear to be in a relative hypothyroid state, sometimes called euthyroid sick syndrome. Clinical manifestations include hair loss, dry skin, hypothermia, and bradycardia. All these findings are reversible with appropriate refeeding and successful treatment. Laboratory findings include low-normal levels of thyroxine (T_4) and thyroid-stimulating hormone, below-normal levels of triiodothyronine (T_3), and elevated levels of reverse T_3. All these findings are the result of malnutrition and weight loss. In fact, the low T_3 level correlates with the amount of weight loss. The extremely reduced T_3 levels in these patients are due to altered peripheral deiodination that preferentially transforms T_4 into the inactive metabolite, reverse T_3. These alterations normalize with weight recovery, and thyroid hormone replacement is usually not indicated (Muñoz and Argente, 2004).

Hypothalamic–pituitary–adrenal axis

In AN, plasma cortisol levels are often elevated due to an increase in the frequency of secretory bursts of cortisol (**Fig. 2**). The circadian rhythm is conserved. Dexamethasone can partially suppress this hypercortisolemia, which is similar to that observed in patients with depression and Cushing's disease. In acute situations of AN, the dexamethasone test has no medical significance; however, in patients who are gaining weight, it may have prognostic value (Muñoz and Argente, 2004). Refeeding studies of anorexic patients have shown that, irrespective of the individual's initial weight, weight gains as low as 10% are associated with the normalization of cortisol secretion. The mean plasma half-life of cortisol is prolonged and ACTH levels are within the normal range, but the ACTH response to CRH is lower than that of control patients. This hypercortisolemia may indicate a defect at the level of the hypothalamus, or even higher, that results in hypersecretion of CRF (Muñoz-Calvo, 2005). Taken together, these observations suggest CRH hypersecretion more than cortisol resistance.

Adipokines and appetite-regulating hormones

Long-term energy balance is regulated via a system composed of different hormones secreted in proportion to corporal adiposity, which act at the level of the central nervous system. These respond to changes in body fat and activate anabolic or catabolic pathways, the first through production of NPY, which stimulates food intake, and the second via the hypothalamic melanocortin system, which reduces food intake and stimulates weight loss.

Leptin, a hormone synthesized by adipose tissue, plays an important role in the regulation of food intake and energy expenditure. Plasma leptin levels and secretory pattern vary during the night and day, and are influenced by food intake (Argente *et al.*, 1997). Leptin acts at the level of the hypothalamus to decrease appetite, resulting in weight loss. Moreover, leptin has other functions, such as regulation of bone metabolism, neuronal and cognitive functions, as well as its more recently described function in the immune system. In different studies, low levels of leptin have been reported in AN subjects compared to controls, and this could contribute to the development of hypothalamic amenorrhea, as well as the compulsive physical exercise that these patients perform (**Fig. 2**). A recent study observed that the changes in circulating leptin could be related not only to the changes in adipose tissue, but also to psychological symptoms such as depression, anxiety, and stress, which will depend on the severity of the malnutrition (Stroe-Kunold *et al.*, 2016).

The ghrelin (gastrointestinal peptide hormone) levels are regulated by acute and chronic changes in energy balance (e.g., fasting increases, whereas feeding decreases, circulating ghrelin concentrations). Ghrelin levels are high in AN, presumably as an adaptive response to the low energy state, with levels inversely correlating with BMI and fat mass (Muñoz-Calvo, 2005).

Serum adiponectin levels have been reported to be elevated, unchanged, or low in AN. Levels of this adipokine are an inverse predictor of bone density in adolescents with AN, with high adiponectin levels possibly contributing to the low BMD in AN by increasing osteoclast activity.

PYY is an anorexigenic peptide primarily produced in the L-cells of the colon in proportion to caloric intake, with levels increasing after food intake. High serum PYY levels might contribute to reduced food intake and decreased bone formation in AN.

Bone mineral density and bone microarchitecture

Adolescents with AN show a decrease in bone mass, with the pathogeny being not completely known, but several studies have highlighted a number of factors, including low caloric intake with low serum IGF-1 levels, increased serum cortisol and PYY levels, low body weight, and reduced levels of adipose tissue with low serum leptin levels (Stroe-Kunold *et al.*, 2016).

It is well known than more than 50% of adolescents girls and 70% of males with AN present a BMD of -1 (Z score) in at least one or two areas. After 1 year of follow-up, the reduction in BMD at the level of the lumbar spine is reported not to improve significantly in these patients even with weight recuperation, indicating suboptimal bone accrual rates. Approximately 11% have a BMD of -2 Z scores at diagnosis and approximately 30% of adolescents with AN have an increased risk of fractures. Likewise, adolescents with BN have a reduction in bone mass at the lumbar level, suggesting that weight loss does not explain the deleterious effect of TCA in bone (Singhal *et al.*, 2018).

Bone loss in AN occurs in both the trabecular and cortical bones, although it is more evident in the former. We studied a group of girls with restrictive AN with moderate malnutrition and secondary amenorrhea of more than 1 year of evolution, and found loss of bone mass at the lumbar and femoral levels. Weight gain and resumption of menses increased bone mass, especially in the lumbar spine (Stroe-Kunold *et al.*, 2016).

It is clear that AN has negative effects on BMD and on the size and structure of the bone, and that these alterations could explain the increased fracture risk. Advances in imaging techniques, such as quantitative computed tomography (QCT) and more recently high-resolution peripheral QCT (HR-pQCT), provide estimations of bone geometry and cortical and trabecular volumetric BMD (vBMD), that directly correlate with fracture load (Singhal *et al.*, 2018). Baskaran et al. (2017) observed a reduction in the area and cortical thickness, and an increase in the cortical porosity in the distal radial (a non-weight bearing site) of adolescents with AN by using QCT.

HR-pQCT allows the evaluation of the bone microarchitecture, estimating bone stiffness and load failure (strength), which are both diminished in adolescents with AN, which contributes to the fracture risk. Recently, using HR-PQCT, Singhal et al. observed alterations in the trabecular and cortical zones of the tibia (weight-bearing site) associated with increases in marrow adipose tissue and reduced strength estimates in adolescents and young women with AN (Frølich *et al.*, 2017).

The degree of osteopenia possibly depends on the age at which the amenorrhea began, as well as its duration. Indeed, patients with primary amenorrhea have a more severe reduction in bone mass than those that present with secondary amenorrhea. There are significant concerns about the lasting impact and irreversibility of bone mass loss. Evaluation of bone density is recommended in patients who have been amenorrheic for 6 months to 1 year (Muñoz and Argente, 2004).

Sex hormones

Administration of estrogens and gestagens to adolescents with reduced bone mass and amenorrhea for at least 1 year indicated that the reduction in bone mass could not be reversed. In contrast, in patients with weight gain, femoral neck BMD was found to improve. The resumption of menses also improves spinal vertebral BMD and bone mass compared to that seen at diagnosis (Muñoz *et al.*, 2007). These findings demonstrate the effect of estrogen on trabecular bone density.

The effects of estrogens on bone metabolism include inhibition of the resorption process, although direct effects on osteoblast activity have also been described. It has been reported that AN occurring during adolescence impairs both mineral accrual and bone size.

Although reduced vBMD may be related to estrogen deficiency, no reduction in bone size after adjusting for fat and lean mass has been reported. Weight, but not estrogen use, is a significant predictor of BMD at all skeletal sites in anorexic women (Chou and Mantzoros, 2017).

Female AN patients exhibit androgen deficiency, with both low free testosterone levels and dehydroepiandrosterone sulphate (DHEAS) levels being reported in women with AN. Androgen deficiency could contribute to a loss of bone mass in these patients.

IGF-1

IGF-1 is one of the most important regulators of bone metabolism. Circulating levels of IGF-1 correlate with BMD in the normal population. IGF-1 exerts a double effect on bone metabolism by stimulating osteoblastic activity and the resorption process. Deficiency in growth factors, especially IGF-1, which is most likely due to the state of malnutrition, as well as the slow recuperation of their plasma levels with weight gain, is known to occur in these patients. Improvement in nutritional status in AN patients via intravenous hyperalimentation therapy results in a rapid increase in serum IGF-1 levels, followed by a progressive increase in osteocalcin, a specific marker of osteoblast function (Muñoz-Calvo, 2005). This indicates that bone formation begins immediately. Nevertheless, increased bone resorption appears to continue for at least 5 weeks.

Leptin

Leptin is effective in reducing trabecular bone loss, trabecular architectural changes, and periosteal bone formation. These findings suggest that leptin may regulate bone remodeling, and this effect may be, at least in part, mediated by the osteoprotegerin (OPG)/RANK (receptor for activation of nuclear factor kappa B) ligand pathway. The RANK and RANK ligand (RANKL) are members of the tumor necrosis factor (TNF) and TNF receptor superfamilies, which are essential for osteoclast differentiation. In the bone microenvironment, the stimulatory effects of RANKL are neutralized by the secreted decoy receptor, OPG. It follows that the balance between OPG and RANKL secretion by stroma cells is critical for the regulation of osteoclast formation (Stroe-Kunold *et al.*, 2016). The dramatic decline in leptin levels observed in AN may be one of the major hormonal factors involved in the pathogenesis of the associated bone fragility through diminishing cortical bone formation rates and skeletal growth. Leptin may also play an important protective role in bone metabolism by inhibitory bone resorption.

Other hormones

Insulin levels are low in girls with AN compared with normal-weight controls, and are positively associated with levels of bone turnover markers. Omentin-1 levels are increased in adolescents with AN and are reported to be an independent predictor of BMD, being negatively correlated with bone turnover markers and possibly exerting negative effects on bone mass. Oxytocin has anorexigenic and bone anabolic effects, and its levels are reduced in adolescents with AN and associated with the decline in bone mass (Muñoz-Calvo, 2005).

Bone

In adolescents with AN, it is clear that markers of bone formation are decreased and markers of bone mineral resorption are increased. The bone isoenzyme alkaline phosphatase (bAP) and the amino-terminal pro-peptide of procollagen I (PNIP) have the greatest diagnostic sensitivity in detecting anomalies in bone remodeling, at least in osteoporetic women (Chou and Mantzoros, 2017).

Levels of PNIP, a marker of bone formation, and of N-telopeptide (NTX), a marker of bone resorption, are reduced in adolescents with AN, indicating a low level of bone turnover during a period of active growth when the level of bone turnover is elevated in healthy adolescents (García de Álvaro *et al.*, 2007).

OPG, which inhibits osteoclastogenesis and osteoclast activity, and RANKL, which increases osteoclastogenesis and osteoclastic activity, are key factors in the modifications in bone remodeling and diminution of bone mass in patients with AN. Hence, the greater the reduction in the ratio OPG/RANKL, the greater is the drive for bone resorption. We studied a group of AN patients with malnutrition and secondary amenorrhea with an evolution of more than 1 year at the beginning of the study who received oral estrogen treatment throughout the follow-up period. The OPG/RANKL ratio was significantly decreased after 1 year, due to an increase in serum RANKL values. These patients showed lower BMD values, both at diagnosis and at the end of the study. The decrease in the OPG/RANKL ratio in girls with AN could partly explain the increase in bone loss that occurs in these patients (De la Piedra *et al.*, 1999). Different studies have demonstrated that during undernutrition and amenorrhea, with low IGF-1 and extremely low circulating estradiol, bone formation markers correlated positively with IGF-1 and bone resorption markers negatively with estradiol. This indicates that IGF-1 stimulates bone formation, whereas estradiol's actions predominately inhibits bone resorption (De la Piedra *et al.*, 1999).

Treatment

An integral treatment program (addressing all alterations in the patient) should be instituted and carried out by a multidisciplinary and coordinated team, including a pediatrician, endocrinologist, psychiatrist, psychologist, nurse, and others such as teachers and family. It is fundamental that an accurate evaluation and diagnosis are made and that the patient and family are aware of the importance and nature of the disease and of each aspect of the treatment (Muñoz and Argente, 2004).

Treatment objectives should have a strict priority: prevent the death of the patient; prevent the disease from becoming chronic; and attainment of physical and mental recuperation.

Nutritional Treatment

Refeeding

To begin refeeding successfully, it is fundamental that a therapeutic alliance with the patient be established in which the patient understands and accepts (at least partially) that he or she has a disease. This can be accomplished by asking the patient if he or she has the various signs and symptoms of AN so that, little by little, the patient can identify with this condition (Baskaran *et al.*, 2017).

The physician should use height and weight graphs to explain what percentile the patient is currently in and what the patient's weight should be for his or her age and height. A target weight, acceptable to both the patient and the physician, should be agreed upon. The patient's understanding of caloric requirements to maintain a normal weight should be explored because very few possess knowledge of the appropriate requirements for their height, age, and sex. Patients must understand that their growth and physical activity depend on the adequate intake of calories, including proteins, fats, carbohydrates, and vitamins and minerals (Muñoz-Calvo, 2005).

Obtaining and maintaining an adequate weight

After the target weight has been achieved, a maintenance diet must be prescribed, and foods not on the diet and eating between meals must be prohibited for a time, which will help the patient to overcome the fear of losing control or gaining weight. Afterwards it will become necessary progressively to schedule meals out of the diet (e.g., in summer camps, restaurants) to achieve eating without a prescribed diet during approximately the second year of treatment (Baskaran *et al.*, 2017).

Nutritional supplementation

Calcium: Different meta-analyses observed no increase in BMD in adolescents with AN after receiving calcium supplements.

Vitamin D: Correlations between supplementation with calcium and vitamin D and BMD have not been found in these patients (Robinson *et al.*, 2017).

Table 2 Therapeutic options for improving bone mass in AN

Therapy options	
Weight recovery	Maintain the recovery and maintenance of weight should be a goal
Calcium and vitamin D	1300 mg of calcium and 600 IU of vitamin D daily (maintain vitamin D levels between 30 and 50 ng/mL)
Estrogens	100 μg 17 β estradiol transdermally with cyclic medroxyprogesterone acetate (2.5 mg/day during 10 days) or oral micronized progesterone (100–200 μg during 10–12 days)
Testosterone	150 μg transdermally
DHEA	50 mg of micronized DHEA orally per day, with an oral contraceptive (20 μg of EE and 0.1 mg of levonogestrel)
rhIGF-1	30 μg/kg of rhIGF-1, twice daily with an oral contraceptive (35 μg of EE and 0.4 mg norethindrone)
Biphosphonates	35 mg of risedronate orally/weekly 10 mg of alendronate orally/day
TPT	20 μg/day, subcutaneously

DHEA, dehydroepiandrosterone; *rhIGF-1*, recombinant IGF-1; *TPT*, teriparatide.

Gonadal steroid replacement

Different controlled studies have demonstrated that oral estrogens are not effective in increasing bone mass in adolescents with AN. Estrogen–progesterone combination pills that provide doses of 20–35 mg of ethinyl estradiol may further decrease the levels of IGF-1, an important bone trophic hormone. Thus, oral contraceptives may have an IGF-1 suppressive effect, which could potentially explain the small-to-negligible effectiveness of this therapy. In contrast, the utilization of transdermal patches of 100 mcg of 17 β estradiol with cyclic progesterone, a modality which has been shown to exert little to no suppression on IGF-1 as compared with oral contraceptives, causes in girls with AN a significant increase in BMD over an 18-month period (**Table 2**). These differential effects could be related to the type of estrogen (17 β estradiol vs. EE) and/or the method of administration (transdermal vs. oral) (Robinson *et al.*, 2017).

IGF-1 replacement

Administration of recombinant human IGF-1 (rhIGF-1) to adolescents with AN causes an increase in levels of bone formation markers, but not bone resorption markers (**Table 2**). Additionally, rhIGF-1 in conjunction with an estrogen-progesterone combination pill causes a significant increase in spinal vertebrae BMD and serum levels of bone formation markers, and decreases bone resorption markers in adult women with AN compared with a placebo (De la Piedra *et al.*, 1999).

Biphosphonates

Bisphosphonates, having a long half-life and strong affinity for bone as demonstrated by their absorption into its matrix, inhibit osteoclastic bone resorption. In adult women with AN, oral intake of bisphosphonates for a year increased BMD of the posterioanterior spine by 3% and of the hip by 2%. One placebo-controlled study in girls with AN who received alendronate found no increase in spine BMD and minimal increases in femoral neck BMD. Another study showed that treatment with risedronate and testosterone produced a 3% increase in bone mass in the lumbar area and a 2% increase in the femoral neck (Miller *et al.*, 2011). Testosterone increased muscular mass, without improving bone mass (**Table 2**). Biphosphonates pass through the placenta and have a long half-life. They have not been approved by the Food and Drug Administration (FDA) for use in women during fertile ages with AN due to the limited knowledge of their long-term efficacy.

DHEA

There is no evidence of an improvement in BMD after the administration of DHEA. If it is associated with oral anticontraceptives, BMD at the level of the femoral neck increases (Robinson *et al.*, 2017) (**Table 2**).

Teriparatide

Administration of 1.34 pH (teriparatide, or TPT), an anabolic agent, to older women with reduced bone mass for 6 months resulted in an increase of 6%–10% in the lumbar spine (**Table 2**). However, the safety of this drug has not been established due to an increase in osteosarcoma in rodent studies. This suggests that TPT should not be used in adolescents and young women (Robinson *et al.*, 2017).

Other alternative drugs

Leptin: Interventional studies evaluating the effect of recombinant leptin in patients with hypothalamic amenorrhea have shown improved LH pulse frequency and increased serum LH levels, as well as overall improvement in ovarian parameters and markers of bone formation, suggesting that leptin is required for normal reproductive and neuroendocrine function. However, recombinant leptin also produced a 4.3% decline in fat mass, a finding that is a potential limitation for its use in individuals with AN (Robinson *et al.*, 2017).

Exercise activity

Exercise is important for bone health in individuals with AN, as it increases BMD. The recommendations for physical activity in those with AN is controversial. Increased physical activity in combination with malnutrition can exacerbate weight loss (Baskaran *et al.*, 2017).

Psychiatric treatment

Psychiatric (psychotherapeutic) treatment is imperative in the AN patients. Psychological intervention should start as early as possible, together with and favoring nutritional rehabilitation, and must continue sufficiently to assure maintenance of the achieved improvements and prevent relapses. Psychoeducational techniques are highly useful during the early stages of treatment. They are focused on parents and adolescents in order to give them a deep understanding of nutritional aspects, symptoms of the disorder, and treatment aspects (Herpertz-Dahlmann, 2017).

The family based treatment is usually combined with cognitive-behavioral therapy, which is an effective psychotherapeutic intervention for eating disorders and consists of modification of disturbed behaviors and thoughts underlying the disorder.

Pharmacological Treatment

Pharmacological therapy is not an essential treatment in patients with AN because the specific psychopathology is unresponsive to this treatment. Therefore, it should not be used as the unique or principal treatment. The use of drugs is especially controversial in adolescent patients. However, some general symptoms such as obsessions, compulsivity, hyperactivity, and extreme anxiety could improve with some pharmacological agents such as low doses of olanzapine (atypical antipsychotic) or clorazepam (benzodiacepines). Limited studies, not replicated, have also suggested the use of fluoxetine as a prevention of relapse in recovered patients (Rienecke, 2017).

It is important to take into account that the use of psychoactive drugs is not recommended in patients with very low bodyweight due to their secondary effects and lower efficacy in these conditions.

Prognosis and Evolution

The clinical course of AN is marked by episodes of remission and relapse even in the best-controlled cases. The first episode in adolescents is long, and rarely lasts <4 or 5 years. On a community level, 5-year recovery rates for DSM-5 AN are 60%–70%, whereas 20% have partial remission. However, 20%–25% of anorexic patients could become chronic. The transition to bulimia nervosa is frequent, occurring in 30%–40% in 6–8 years of follow-up (Muñoz and Argente, 2004).

The mortality rate is between 0.5% and 1% per year of observation. The most frequent causes of death are cardiovascular problems due to severe undernutrition, gastrointestinal complications, infections, and suicide. The mortality rate increases slightly (up to 20% for 20 years of follow-up) in longer follow-ups (Baskaran *et al.*, 2017).

Some factors are associated with poor prognosis: long-term disorder, very low weight at the onset of treatment, compulsive physical exercise, purging behavior, drug abuse, previous obesity, poor family relationship, child abuse, and comorbid psychiatric disorders. Otherwise, some factors are related with good prognosis: early diagnosis and treatment, recovery of most of the lost weight (90%–92% of ideal weight), and specialized treatment.

Conclusion

AN is a psychiatric disease. Evidence suggests that there is hypothalamic dysfunction that in general normalizes with weight recovery.

The most relevant factors contributing to the loss of bone mass include: low caloric intake with low serum IGF-1 levels, excessive exercise patterns despite malnutrition, hypogonadism, increased serum cortisol and PYY levels, low body weight, and small amounts of adipose tissue with low serum leptin levels. These in turn result in a high risk of fractures.

Analysis of the genetic mechanisms underlying weight regulation is progressing rapidly. The genetic analysis of AN may help to define new drug targets and therefore lead to new treatment strategies.

An integral treatment program should be instituted and carried out by a multidisciplinary and coordinated team, including a pediatrician, endocrinologist, psychiatrist, psychologist, and nutritionist. The prognosis for adolescents with eating disorders is currently more favorable than in past decades; however, 20%–25% of patients remain chronic.

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Endocrine Late Effects in Childhood Cancer Survivors

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Glossary

Alkylating agents A group of chemotherapy drugs that impair cell multiplication by adding an alkyl group to DNA. Rapidly proliferating cancer cells are particularly vulnerable to alkylating agents. However, normally multiplying non-malignant cells may also be affected. This phenomenon explains the gonadal toxicity of these medications. The most commonly used alkylating agents are: busulfan, carmustine (BCNU), chlorambucil, cyclophosphamide, dacarbazine, ifosfamide, lomustine (CCNU), mechlorethamine, melphalan, procarbazine, temozolomide, and thiothepa.

ACTH deficiency (ACTHD) Deficiency in adrenocorticotropic hormone (ACTH). ACTHD refers to the same condition as central adrenal insufficiency or secondary adrenal insufficiency; these are terms intended to distinguish this entity from adrenal insufficiency due to a disorder originating from the adrenal glands (primary adrenal insufficiency).

Hematopoietic stem cell transplant (HSCT) Treatment procedure that involves repopulating a patient's previously depleted bone marrow by infusing hematopoietic stem cells. Bone marrow transplantation (BMT) is the term used when the source of the grafted hematopoietic stem cells is the bone marrow. Autologous HSCT involves infusing the patient's own, previously collected, stem cells as a rescue procedure following high-dose chemotherapy to treat non-hematological cancers (such as brain tumors). Allogeneic HSCT uses stem cells from a distinct, preferably matched and related, donor to treat hematologic malignancies such as leukemia or lymphoma.

Hematopoietic stem cell transplant conditioning Treatment that purposefully depletes a patient's bone marrow (myeloablative treatment) prior to repopulating it with donor stem cells. Conditioning regimens include myeloablative chemotherapy with or without total body irradiation (TBI) or total lymphoid irradiation (TLI; a regimen that spares the brain).

Immunotherapy for cancer Medications that utilize the immune system's response to attack cancer cells and target tumor growth. Strategies include cellular therapy (e.g., use of chimeric antigen receptor CAR-T cells), immune checkpoint inhibitors such as monoclonal antibodies (e.g., anti-CTLA4 such as ipilimumab), or non-specific immunotherapy agents (e.g., interferon).

LH/FSH deficiency (LH/FSHD) Deficiency in luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are also known as the gonadotropic hormones (or gonadotropins). LH/FSHD refers to the same condition as hypogonadotropic hypogonadism.

Premature ovarian insufficiency (POI) Refers to female hypogonadism and infertility due to disorder originating from the ovaries (and not as a consequence of LH/FSHD). POI refers to the same condition as primary ovarian insufficiency or primary ovarian failure.

Primary hypothyroidism Hypothyroidism due to a disorder originating from the thyroid gland itself (and not as a consequence of TSHD).

Targeted chemotherapy agents Medications that target molecular pathways that are deemed specifically responsible of tumor growth. Tyrosine kinase inhibitors (TKI) such as sorafenib, imatinib, and sunitinib are among the most commonly used targeted chemotherapy agents. Differences to conventional chemotherapy include the necessity of prolonged "maintenance" use to maintain tumor growth suppression.

TSH deficiency (TSHD) Deficiency in thyroid stimulating hormone (TSH, also known as thyrotropin). TSHD refers to the same condition as central hypothyroidism or secondary hypothyroidism, which are terms intended to distinguish this entity from primary hypothyroidism.

Introduction

Strides in cancer cure rates have allowed an increasing number of individuals to survive a wide range of childhood onset tumors and malignancies. Childhood cancer survivors (CCS) are estimated to represent 1 out of 530 individuals aged 20–40 years in the United States of America (Ward *et al.*, 2014). However, life-saving treatments such as multi-agent chemotherapy and radiotherapy have been shown to expose patients to a variety of late-onset chronic health conditions, referred to as "late effects," with repercussions on quality of life and longevity (Diller *et al.*, 2009). The endocrine system seems to be particularly vulnerable to disruptions from cancer treatments, especially radiotherapy and/or alkylating chemotherapy agents; endocrine late effects are indeed among the most commonly observed complications in CCS, with up to 50% of individuals affected (Brignardello *et al.*, 2013). This article offers an overview of the most frequently described endocrine late effects in CCS: hypothalamic/pituitary (HP) dysfunction, primary thyroid disease, primary gonadal insufficiency, bone mineral density (BMD) deficit, obesity, and diabetes mellitus. **Tables 1** and **2** propose a summary of the screening approach of patients who are at risk of developing each of these late

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Table 1 Risk factors and management guidelines of endocrine late effects: hypothalamic–pituitary disorders

	GH deficiency (Child)	*Central precocious puberty*	*TSH deficiency*	*LH/FSH deficiency*	*ACTH deficiency*
Risk factors					
Primary disease	Tumor or surgery near HP region	Tumor near HP region, optic pathways glioma, NF-1, Increased intracranial pressure	Tumor or surgery near HP region	Tumor or surgery near HP region	Tumor or surgery near HP region
Radiotherapy dose to HP region	≥18 Gy TBI ≥10 Gy 1 fraction or ≥12 Gy multiple fractions	≥18 Gy	≥30 Gy	≥30 Gy	≥30 Gy
Other	Young age at diagnosis Tyrosine kinase inhibitors (imatinib) Anti-CTLA4 MAB (Ipilimumab)	Young age at diagnosis Female sex	Anti-CTLA4 MAB (Ipilimumab)	Anti-CTLA4 MAB (Ipilimumab)	Anti-CTLA4 MAB (Ipilimumab)
Screening modality					
History—main complaints	Decreased growth/ change in clothing sizes Nutrition	Increased growth/change in clothing sizes Nutrition Pubertal changes	Slow growth, weight gain, cold intolerance, constipation, menstrual irregularities	Absent or arrested puberty Amenorrhea Decreased stamina	Decreased energy level
Physical examination findings	Auxological measurements Growth velocity Sitting height or upper to lower segment ratio[a] Pubertal staging[b]	Auxological measurements Growth velocity Sitting height or upper to lower segment ratio[a] Pubertal staging[b]	Auxological measurements Growth velocity Palpation of the neck Pubertal staging[b]	Auxological measurements Growth velocity Pubertal staging[b]	Auxological measurements Blood pressure
Laboratory screening—plasma levels	—	Morning LH, FSH, testosterone (male) or estradiol (female) if pubertal signs[b]	Free T4, TSH	Morning LH, FSH, and estradiol (females) or testosterone (males) if no puberty by age 13 years in girls and 14 years in boys; or if arrest in pubertal development	8 AM cortisol level
Minimal frequency of screening	Clinical examination every 6 months until final adult height is attained	Clinical examination every 6 months until age 9 years in girls and 10 years in boys Labs as clinically indicated[c]	Clinical examination every 6 months until final adult height is attained, yearly thereafter Yearly labs	Clinical examination every 6 months until final height is attained, yearly thereafter Menstrual log in females Yearly labs	Yearly
Subsequent/confirmatory testing					
Laboratory—plasma levels	GH dynamic testing	GnRH or GnRH agonist testing	—	Males: repeat morning testosterone	ACTH stimulation test

Table 1 Continued

	GH deficiency (Child)	*Central precocious puberty*	*TSH deficiency*	*LH/FSH deficiency*	*ACTH deficiency*
Radiology/imaging	X-ray of left hand (bone age)	X-ray of left hand (bone age) Consider pelvic ultrasound (girls)	—	—	—
Treatment					
Medications	Replacement with hGH	GnRH agonists	Levothyroxine	Sex hormone replacement therapy	Hydrocortisone in children; other glucocorticoids can be considered in adults
Additional recommendations	Central precocious puberty may alter growth patterns	GH deficiency frequently appears around the same time	Assess for ACTH deficiency and treat it first	Consider reproductive endocrinology consult when older	Educate on stress doses and emergency situations

[a]If exposed to spinal radiotherapy or surgery.

[b]Males treated with testicular irradiation or alkylating agents may have smaller testicular size than expected for pubertal status.

[c]Pubertal onset should also be assessed in the context of familial pattern of pubertal onset. Screening guidelines were adapted from the Children's Oncology Group Long-Term Follow-Up Guidelines Version 4.0 (www.survivorshipguidelines.org).

HP, Hypothalamus/pituitary; *NF-1*, neurofibromatosis type 1; *TBI*, total body irradiation; *MAB*, monoclonal antibody; *GnRH*, gonadotropin releasing hormone; *hGH*, human recombinant GH.

Table 2 Risk factors and management guidelines of endocrine late effects: common primary disorders

	Primary hypothyroidism	*Thyroid cancer*	*Leydig cell failure*	*Oligo- and azoospermia*	*Ovarian insufficiency*	*Decreased BMD*	*Obesity, diabetes*
Risk factors							
Primary disease	Surgical resection of gland	Cancer predisposition syndrome	Surgical resection of gland	Surgical resection of gland	Surgical resection of gland	Leukemia process	Tumor or surgery near HP region
External beam radiotherapy	Dose $\geq$10 Gy to the thyroid	Any dose to the thyroid; highest risk at 20–30 Gy	$\geq$20 Gy to the testes	Any dose to the testes; Highest risk at $\geq$2 Gy	Any dose to the ovaries; highest risk $\geq$5 Gy	Cranial TBI	Cranial[a], TBI[b] Abdominal[b]
Other treatments	Tyrosine kinase inhibitors Immunomodulators RAI, ^{131}I-MIBG	Alkylating agents may increase risk ^{131}I-MIBG	Alkylating agents	Alkylating agents Heavy metals	Alkylating agents Heavy metals ^{131}I-MIBG	Glucocorticoids	Glucocorticoids
Patient factors at cancer diagnosis	Female sex	Younger age Female sex	—	—	Older age	Younger age	Female sex[a]
Screening modality							
History—main complaints	Slow growth, weight gain, cold intolerance, constipation Menstrual history	Neck mass	Pubertal changes Stamina Decreased libido	Fertility questions	Pubertal changes Menstrual History Hot flashes Stamina	Pubertal changes Fractures/ Pain Nutrition (calcium & vitamin D intake)	Changes in weight Lifestyle Polyuria or polydipsia
Physical examination findings	Auxological measurements Growth velocity Palpation of the neck Pubertal staging[c]	Neck palpation to check for thyroid nodule or cervical lymph nodes	Auxological measurements Growth velocity Pubertal staging[c]	Testicular size Pubertal staging[c]	Auxological measurements Growth velocity Pubertal staging[c] Menstrual history	Auxological measurements Growth velocity Pubertal staging	Auxological measurements Pubertal staging[c]
Laboratory or radiology/ imaging	Plasma TSH, Free T4	Thyroid ultrasound[d]	LH, morning testosterone	Semen analysis	Plasma FSH, estradiol	Dual energy X-ray absorptiometry	
Fasting blood glucose, lipids, HbA1c							
Minimal frequency of screening	Clinical examination every 6 months until final adult height; yearly thereafter Yearly labs	Yearly	Clinical examination: after 14 years, every 6 months until final adult height; yearly thereafter Yearly labs	Per patient request (adults)	Clinical examination: after 13 years, every 6 months until final adult height yearly thereafter Yearly labs	As clinically indicated	Clinical examination: every 6 months until final adult height, yearly thereafter Yearly labs

Table 2 Continued

	Primary hypothyroidism	*Thyroid cancer*	*Leydig cell failure*	*Oligo- and azoospermia*	*Ovarian insufficiency*	*Decreased BMD*	*Obesity, diabetes*
Subsequent/confirmatory testing							
Laboratory	—	Ultrasound guided FNAB	Repeat morning testosterone level	—	—	Plasma vitamin D 25	Oral glucose tolerance test[b]
Treatment							
Modality	Levothyroxine	Total thyroidectomy ±RAI	Sex-hormone replacement therapy	Prevent with sperm banking	Prevent with mature oocyte cryopreservation Sex hormone replacement therapy	Optimize diet, calcium, vitamin D Exercise	Lifestyle modification Diet Exercise
Additional recommendations		Assess for ACTH deficiency and treat it first	—	Consider reproductive endocrinology consult when older	Consult with reproductive endocrinologist	Consider gynecology or reproductive endocrinology consult	Treat other endocrine deficits
Medications as clinically indicated							

[a]Fine-needle aspiration biopsy for obesity.

[b]Fine-needle aspiration biopsy for diabetes mellitus.

[c]Males treated with testicular irradiation or alkylating agents may have smaller testicular size than expected for pubertal status.

[d]No consensus regarding the role, timing, and frequency of ultrasound. Guidelines were adapted and modified from the Children's Oncology Group Long-Term Follow-Up Guidelines Version 4.0 (www.survivorshipguidelines.org).

BMD, Bone mineral density; *HP*, hypothalamus/pituitary; *TBI*, total body irradiation; *FNAB*, fine-needle aspiration biopsy; *RAI*, radioactive iodine.

effects in order to facilitate early diagnosis and treatment and hopefully improve patient outcomes (Hudson *et al.*, 2013). The tables were adapted from the Children's Oncology Group Long-Term Follow-Up Guidelines (www.survivorshipguidelines.org), supplemented by brief diagnosis and management suggestions.

Hypothalamic/Pituitary Dysfunction

HP dysfunction encompasses the following entities: growth hormone (GH) deficiency (GHD), central precocious puberty (CPP), luteinizing and follicle stimulating hormones deficiency (LH/FSHD), thyroid stimulating hormone deficiency (TSHD), and adrenocorticotropic hormone deficiency (ACTHD). Central diabetes insipidus, a frequent and challenging acute complication of HP tumors and surgery, will not be discussed in the present overview, as it does not occur as a late effect (Clement *et al.*, 2016).

Survivors of childhood cancer or central nervous system (CNS) tumors may develop various components of HP dysfunction as sequelae from local tumor growth, surgical resection, or radiotherapy (Clement *et al.*, 2016). The role of conventional chemotherapy in inducing permanent HP damage is not well established (Rose *et al.*, 2004; Gurney *et al.*, 2006; Bakker *et al.*, 2004). Emerging data on novel chemotherapy agents such as tyrosine kinase inhibitors (TKI) (Lodish, 2013; Rastogi *et al.*, 2012) and immune system modulators (Corsello *et al.*, 2013) support some effect on the HP axis.

Individuals with HP dysfunction due to local tumor growth or surgery frequently present with multiple HP disorders from the outset. Radiation-induced HP dysfunction tends to appear as a late effect a few months to several years after CNS irradiation, with multiple HP disorders appearing sequentially rather than simultaneously in affected patients (Laughton *et al.*, 2008; Chemaitilly *et al.*, 2015; Clement *et al.*, 2016). **Fig. 1** highlights the relative overlap between GHD, LH/FSHD, TSHD, and ACTHD in a study where data from 748 CCS exposed to cranial radiotherapy and followed for a mean of 27.3 years were reported (Chemaitilly *et al.*, 2015). It is important to note that continuous improvements in the delivery of radiotherapy may modify the risk of HP

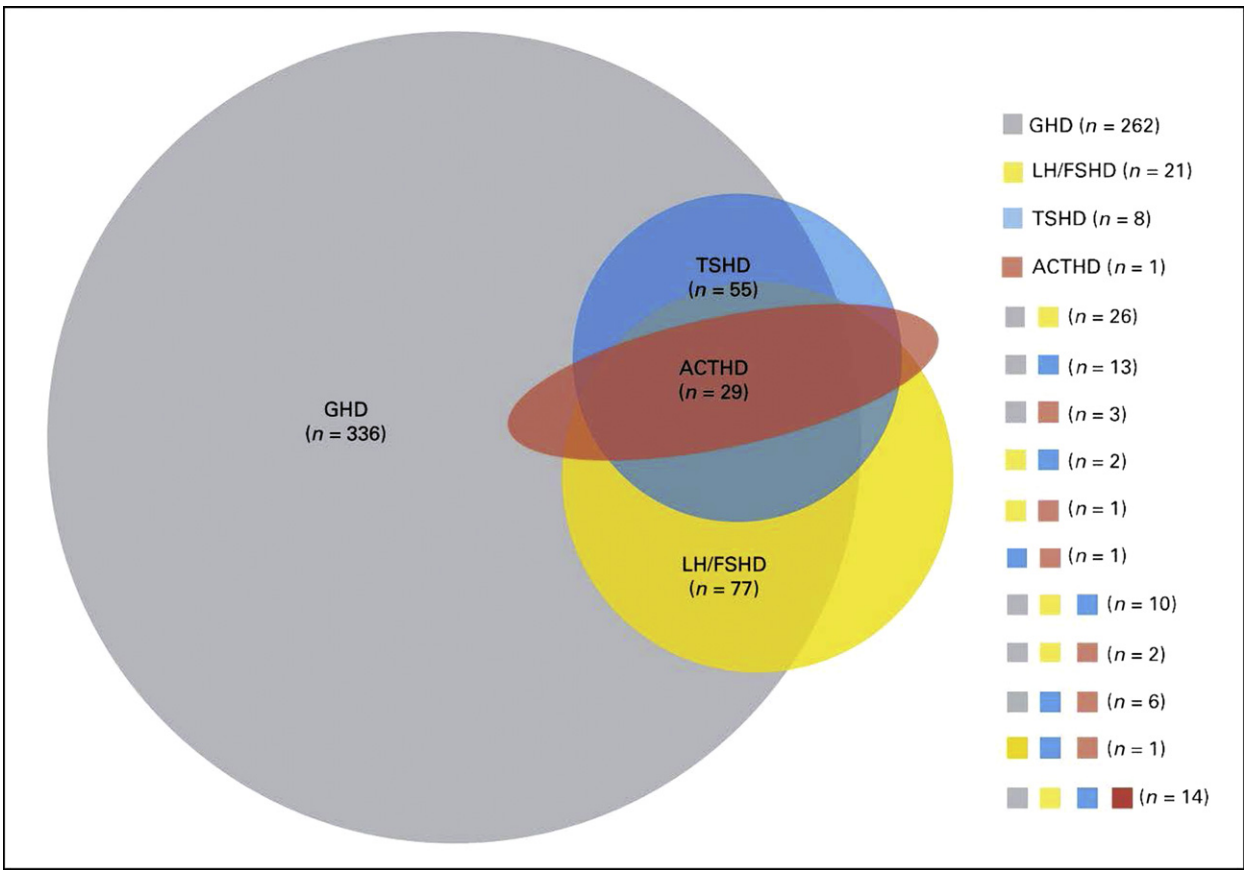

Fig. 1 Proportions and overlap among anterior pituitary hormone deficiencies following cranial radiotherapy. *ACTHD*, adrenocorticotropic hormone deficiency; *GHD*, growth hormone deficiency; *LH/FSHD*, luteinizing hormone/follicle-stimulating hormone deficiency; *TSHD*, thyroid-stimulating hormone deficiency. Reproduced with permission from Chemaitilly, W., Li, Z., Huang, S., Ness, K. K., Clark, K. L., Green, D. M., Barnes, N., Armstrong, G. T., Krasin, M. J., Srivastava, D. K., Pui, C. H., Merchant, T. E., Kun, L. E., Gajjar, A., Hudson, M. M., Robison, L. L. and Sklar, C. A. (2015a). Anterior hypopituitarism in adult survivors of childhood cancers treated with cranial radiotherapy: a report from the St Jude lifetime cohort study. *Journal of Clinical Oncology*, **33,** 492–500, ©American Society of Clinical Oncology.

dysfunction in individuals treated under more recent protocols, such as those using protons rather than photons to generate energy (Eaton *et al.*, 2016).

Growth Hormone Deficiency

Prevalence and risk factors

The prevalence of GHD has been reported at 12.5% in short- to medium-term survivors of CNS tumors (Clement *et al.*, 2016) and 46.5% in long-term CCS exposed to HP radiotherapy (Chemaitilly *et al.*, 2015). It is the most prevalent and frequently only HP disorder in this population (**Fig. 1**) (Chemaitilly *et al.*, 2015; Laughton *et al.*, 2008). The risk is highest in patients with tumors developing within or near the hypothalamus/pituitary and those treated with radiotherapy HP doses $\geq$18 Gy (Chemaitilly *et al.*, 2015; Mostoufi-Moab *et al.*, 2016; Clement *et al.*, 2016). The risk of GHD in relation to radiotherapy increases in a dose- and time-dependent fashion; treatment at doses 10–18 Gy may still result in GHD in CCS monitored over an extended period of time (Merchant *et al.*, 2011). Increased risk of GHD is not limited to survivors of CNS tumors; it extends to all individuals treated with radiation fields that include the HP region regardless of the etiology. This includes survivors of acute lymphoblastic leukemia (ALL) exposed to cranial radiotherapy (Wilson *et al.*, 2016; Brauner *et al.*, 1986), pediatric hematopoietic stem cell transplant (HSCT) recipients conditioned with total body irradiation (TBI) (Brauner *et al.*, 1997; Chemaitilly *et al.*, 2007; Papadimitriou *et al.*, 1991), and CCS treated with radiotherapy for non-brain solid tumors of the head such as retinoblastoma (Pomarede *et al.*, 1984; Friedman *et al.*, 2013), nasopharyngeal carcinoma (Cheuk *et al.*, 2011), and a variety of sarcomas (Punyko *et al.*, 2005). The risk may be higher in individuals exposed to HP radiotherapy at a younger age (Clement *et al.*, 2015; Chemaitilly *et al.*, 2015; Brauner *et al.*, 1986). The risk association with conventional chemotherapy agents is not as well established as with HP radiotherapy (Rose *et al.*, 2004; Gurney *et al.*, 2006; Bakker *et al.*, 2004). Treatment with imatinib mesylate, a TKI that represents the first-line therapy for chronic myelogenous leukemia, has been reported to result in skeletal growth impairment, possibly due to GHD or decreased sensitivity to GH (Narayanan *et al.*, 2013; Rastogi *et al.*, 2012; Tauer *et al.*, 2015).

Diagnosis and management

Children and adolescents with GHD usually experience a decrease in linear growth velocity, with growth chart plots yielding a height curve that gradually crosses to lower percentiles. Patients may present with short stature (height < -2SD) if left untreated for an extended period of time, and referrals for evaluation should not be delayed until that advanced stage (Growth Hormone Research Society, 2000). Medical providers should seek and identify other factors that alter linear growth in CCS, including direct growth plate damage due to spinal radiotherapy or chemotherapy (Hobbie *et al.*, 2011), abnormally timed puberty, undernutrition, and poorly controlled chronic health conditions. Sitting height or arms span measurements can help identify the contribution of spinal radiotherapy or scoliosis to poor growth in affected patients (Clayton and Shalet, 1991). The importance of a careful assessment of pubertal development cannot be understated, as seemingly normal growth velocity due to stimulation from sex hormones in patients simultaneously experiencing CPP may delay the diagnosis of GHD with consequences on final adult height (Sklar and Constine, 1995; Chemaitilly *et al.*, 2016b). Obesity is an additional confounder that is known to influence skeletal maturation and GH secretion (Geffner, 1996).

Diagnosing GHD in CCS follows the same steps as in the general non-CCS population (Grimberg *et al.*, 2016; Fleseriu *et al.*, 2016) with the understanding that providers cannot rely on screening with insulin-like growth factor I (IGF)-I and IGF-binding protein 3 (IGFBP-3) levels as these have been shown not to be reliable in individuals exposed to HP radiotherapy (Sklar *et al.*, 1993; Weinzimer *et al.*, 1999). Failing one dynamic test may suffice for the diagnosis of GHD in CCS with high pre-test probability for GHD such as those with known other HP disorders or patients exposed to high-dose HP radiation who manifest signs of growth failure (Growth Hormone Research Society, 2000). Testing patients with GH-releasing hormone is discouraged because of false negative results related to the likely hypothalamic (rather than pituitary) site of radiation-induced injury (Ham *et al.*, 2005; Schriock *et al.*, 1984; Constine *et al.*, 1993). Given frequent confounders related to pubertal stage, patients should have their skeletal maturation assessed via bone age X-ray (Pyle *et al.*, 1971).

The treatment of GHD in CCS relies on the same principles as in the general non-CCS population (Fleseriu *et al.*, 2016; Grimberg *et al.*, 2016; Growth Hormone Research Society, 2000). Patients and their families should be informed that full recovery of the pre-cancer growth potential may not be achievable given non-GH-related factors (Brownstein *et al.*, 2004; Chemaitilly *et al.*, 2016a). The safety of GH replacement in CCS has been the subject of multiple studies because of concerns related to the in vitro effects of GH and IGF-I on cell cycle and multiplication, and whether treatment with human recombinant GH (hGH) could increase the risk for cancer recurrence, secondary malignancies, or mortality (Chemaitilly and Robison, 2012; Raman *et al.*, 2015). Data from CCS followed in the long term did not support associations between treatment with hGH and increased odds of cancer recurrence or mortality (Ergun-Longmire *et al.*, 2006; Sklar *et al.*, 2002). A higher risk of second neoplasms in individuals treated with hGH was reported in a large cohort of CCS, and this risk seems mostly to be due to a higher incidence of radiation-induced meningioma (Sklar *et al.*, 2002; Ergun-Longmire *et al.*, 2006). These associations were, however, not supported by a more recent report from the same cohort focusing on CNS neoplasms (Patterson *et al.*, 2014), nor by data from other cohorts (Mackenzie *et al.*, 2011). Initiation of hGH in the absence of active neoplasia after a 1-year observation time is a generally accepted practice in CCS (Raman *et al.*, 2015). Guidelines are lacking regarding the optimal observation time for survivors of non-malignant brain tumors such as craniopharyngioma (Raman *et al.*, 2015). Possible benefits of hGH on quality of life, bone health, and cardiovascular risk

factors in GH-deficient adult survivors of childhood cancers and brain tumors may be inferred from studies conducted in the non-CCS population (Elbornsson *et al.*, 2012, 2013); however, there are no data specific to CCS and this remains an active area for research (Chemaitilly *et al.*, 2015).

Central Precocious Puberty

Prevalence and risk factors

CPP is diagnosed in girls and boys experiencing puberty before the ages of 8 or 9 years, respectively, as a result of the early activation of the HP gonadal axis (Chemaitilly *et al.*, 2001; Carel and Leger, 2008). The prevalence of CPP in CNS tumor survivors has been reported at 12.2%–15.2% (Chemaitilly *et al.*, 2016a; Clement *et al.*, 2016) and is even higher in patients with tumors located in the HP region (26%–29%) (Chemaitilly *et al.*, 2016a; Gan *et al.*, 2015). Tumors located near the hypothalamus and optic pathways such as low-grade gliomas, HP radiotherapy at a wide range of doses (18–50 Gy), hydrocephalus, age <5 years at radiotherapy, female sex, and increased BMI have been reported as risk factors for CPP (Chemaitilly *et al.*, 2016a; Gan *et al.*, 2015; Trivin *et al.*, 2006; Oberfield *et al.*, 1996; Armstrong *et al.*, 2009).

Diagnosis and management

Diagnosing CPP follows a similar approach as in the general pediatric population (Carel and Leger, 2008; Carel *et al.*, 2009). It is important to note, however, that testicular volume is not a reliable indicator of pubertal status in boys treated with gonadotoxic therapies such as testicular radiotherapy or alkylating agents. Such individuals may retain Leydig cell function (and hence testosterone secretion) despite having small testes due to treatment-induced Sertoli cell and germ cell depletion (Sklar *et al.*, 1990; Siimes and Rautonen, 1990; Ishiguro *et al.*, 2007). The diagnosis can then be supported by other clinical signs of puberty such as pubic hair development, penile length, and scrotal thinning, and by measuring morning plasma testosterone levels (recognizing the limitation that commercial assays may under- or overestimate levels in pediatric ranges). As discussed in the section on GHD, growth patterns should be interpreted according to pubertal stage and not only chronological age; skeletal maturation based on bone age can help one to assess a patient's growth potential with the understanding that the full effect of exposure to sex steroids may appear in a delayed fashion on the X-ray (**Fig. 2**) (Chemaitilly *et al.*, 2016b). Gonadotropin-releasing hormone agonist depot preparations are the mainstay of CPP treatment in CCS; their use follows the same guidelines as in the general pediatric population (Carel *et al.*, 2009).

LH/FSH Deficiency

Prevalence and risk factors

The reported prevalence of LH/FSHD is 6.5% in CCS (Brignardello *et al.*, 2016) and 11% in individuals treated with HP radiotherapy (Chemaitilly *et al.*, 2015). HP injury due to tumor effect, surgical resection, or radiation doses ≥ 30 Gy represent the main risk factors (Constine *et al.*, 1993; Chemaitilly *et al.*, 2015). Patients treated with lower doses of radiotherapy may experience LH/FSHD if observed over a long period of time (Chemaitilly *et al.*, 2015; Brignardello *et al.*, 2016).

Diagnosis and management

Patients may present with delayed or stalled pubertal development, amenorrhea, or signs/symptoms of estrogen deficiency such as hot flashes (females), or signs/symptoms of low testosterone secretion such as decrease in erections (males), depending on the age at onset of LH/FSHD. The diagnosis and management follow guidelines available in the general non-CCS population of adolescents and adults (Fleseriu *et al.*, 2016; Palmert and Dunkel, 2012; Boehm *et al.*, 2015). The treatment of LH/FSHD consists of sex-hormone replacement therapy. Medical providers should be aware of potential interactions between estrogens in particular and other hormonal (GH, thyroid replacement) and non-hormonal (anti-epileptics) treatments, and be ready to titrate medications accordingly (Fleseriu *et al.*, 2016). Adult patients should be encouraged to consult with reproductive endocrinologists regarding fertility options (Metzger *et al.*, 2013; Kenney *et al.*, 2012).

TSH Deficiency

Prevalence and risk factors

The prevalence of TSHD has been reported at 7.5%–9.2% in CCS treated with HP radiotherapy (Chemaitilly *et al.*, 2015). HP injury due to tumor effect, surgical resection, or radiation doses ≥ 30 Gy represent the main risk factors (Chemaitilly *et al.*, 2015; Clement *et al.*, 2016). Patients treated with lower doses of radiotherapy may experience TSHD if observed over a long period of time (Chemaitilly *et al.*, 2015).

Diagnosis and management

Patients with TSHD may experience signs/symptoms of hypothyroidism such as slow growth (in children and adolescents), weight gain, fatigue, and cold intolerance, although these findings may be subtle or not present. The laboratory diagnosis is based on the observation of plasma free T4 (FT4) levels below the normal range coinciding with TSH levels that are either low, inappropriately

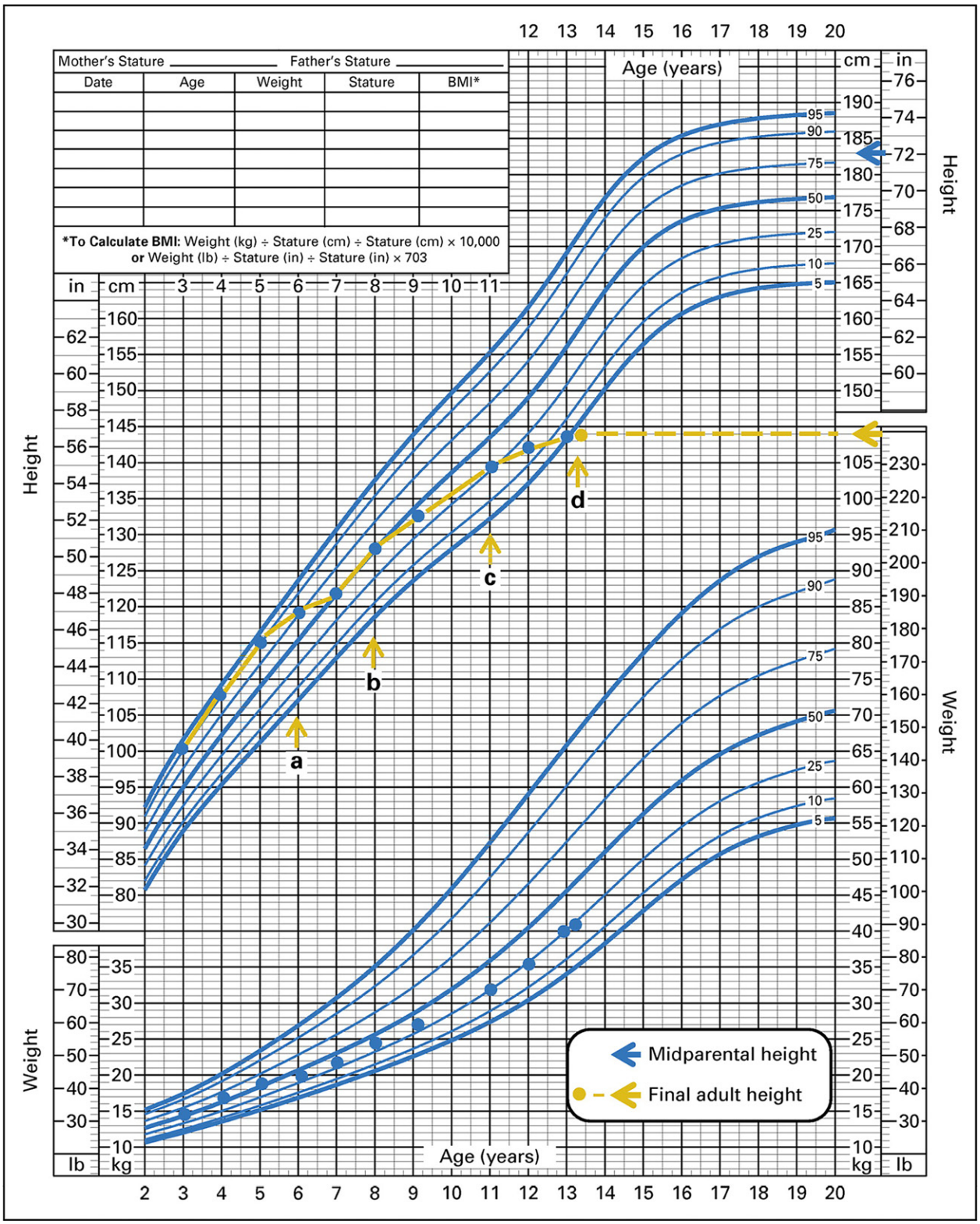

Fig. 2 Growth pattern observed with overlapping growth hormone deficiency and central precocious puberty. Growth chart of a male survivor of diagnosed at the age of 3 years with a brain tumor with metastatic disease involving the hypothalamic/pituitary region and treatment with chemotherapy alone. Time point a: initial growth deceleration, possible onset of GH deficiency (GHD). Time point b: apparent recovery of growth, possible onset of central precocious puberty. Time point c: growth deceleration due to bone age advancement, initiation of referral to endocrinologist. Small testes contrasting with full Tanner stage IV virilization noted on clinical examination, belated diagnosis of GHD, initiation of GH replacement. Time point d: Final adult height attained. Reproduced with permission from (Chemaitilly, W., Armstrong, G. T., Gajjar, A. and Hudson, M. M. (2016). Hypothalamic–pituitary axis dysfunction in survivors of childhood CNS tumors: Importance of systematic follow-up and early endocrine consultation. *Journal of Clinical Oncology*, **34**, 4315–4319, American Society of Clinical Oncology. Growth chart source: Centers for Disease Control: http://www.cdc.gov/growthcharts/data/set1clinical/cj41l021.pdf.

normal (Fleseriu *et al.*, 2016), or occasionally mildly elevated in the case of hypothalamic damage. Caution should be made in the interpretation of thyroid function tests as being due to TSHD itself, since similar patterns can be seen in non-thyroidal illness or with treatment with other medications such as high-dose glucocorticoids or anticonvulsants (Fleseriu *et al.*, 2016).

The treatment relies on replacement with levothyroxine and follows the same steps as in the general non-CCS population (Fleseriu *et al.*, 2016). It is important to remember that the adjustment of levothyroxine doses in patients treated for TSH deficiency should rely exclusively on FT4 levels, and there is no utility in the measurement of TSH levels after diagnosis. In patients at risk of multiple HP deficits, assessment of the ACTH–cortisol axis should be performed prior to initiating thyroid replacement, as patients with unrecognized or untreated adrenal insufficiency may develop adrenal crisis if they are treated for hypothyroidism (Fleseriu *et al.*, 2016).

ACTH Deficiency

Prevalence and risk factors

The prevalence of ACTHD was reported at 4%–5% in CCS treated with HP radiotherapy (Chemaitilly *et al.*, 2015; Clement *et al.*, 2016). The main risk factors include HP injury from tumor growth or surgery as well as radiotherapy at doses $\geq$30 Gy (Chemaitilly *et al.*, 2015; Constine *et al.*, 1993). ACTHD may also occur following the exposure to lower doses of HP radiotherapy after extended periods of follow-up (Follin and Erfurth, 2016; Chemaitilly *et al.*, 2015).

Diagnosis and management

Patients with ACTHD experience glucocorticoid (cortisol) insufficiency because their adrenal glands lack adequate stimulation from the hypothalamus/pituitary; they do not experience salt wasting because adrenal mineralocorticoid (aldosterone) secretion is primarily controlled by the renin–angiotensin–aldosterone system, not ACTH. Symptoms of ACTHD include fatigability and increased infection risk. Patients with ACTHD who are exposed to additional and significant medical stressors (e.g., severe illness, surgery) may experience a rapid deterioration in their clinical state due to adrenal crisis with severe complications such as hypoglycemia, seizures, and shock (Fleseriu *et al.*, 2016). As in the non-CCS population, patients at risk can be screened using serum morning cortisol levels and the diagnosis can be confirmed via dynamic testing; replacement therapy relies on oral hydrocortisone at maintenance doses during regular days and at higher oral or parenteral "stress" doses during times of illness (Patterson *et al.*, 2009; Fleseriu *et al.*, 2016). As with all patients with adrenal insufficiency, CCS with ACTHD should be encouraged to carry at all times some documentation of their risk of adrenal crisis (card, bracelet, etc.) and should receive, along with their families, dedicated teaching on how to recognize and quickly address emergent medical situations (Fleseriu *et al.*, 2016). Assessment of the ACTH–cortisol axis should precede treatment of hypothyroidism in CCS given the risk of adrenal crisis following initiation of thyroid replacement in patients with either undiagnosed or untreated adrenal insufficiency (Fleseriu *et al.*, 2016).

Hyperprolactinemia

The prevalence of hyperprolactinemia was reported as high as 30% in CCS exposed to high-dose (39.6–70.2Gy) HP radiotherapy (Constine *et al.*, 1993). The main symptoms of hyperprolactinemia are hypogonadism (due to LH/FSHD) and galactorrhea. Radiation-induced hyperprolactinemia is generally moderate and rarely symptomatic (Fleseriu *et al.*, 2016). Given that hyperprolactinemia primarily manifests through gonadal suppression, CCS experiencing it as a consequence of radiotherapy rarely require treatment for it because they frequently also have primary gonadal insufficiency due to co-treatment with gonadotoxic agents (Balachandar *et al.*, 2015).

Primary Thyroid Disease

CCS may experience a variety of thyroid disorders such as primary hypothyroidism, hyperthyroidism, autoimmune conditions, and secondary thyroid cancer (Mostoufi-Moab *et al.*, 2016).

Primary Hypothyroidism

Prevalence and risk factors

The prevalence of primary hypothyroidism in CCS has been reported at 13.8%–20.8% (Brignardello *et al.*, 2013; Hudson *et al.*, 2013), making it one of the most frequently observed late effects in this population. Thyroid irradiation confers the highest risk; the latter tends to increase with the dose of radiotherapy and the duration of follow-up (Sklar *et al.*, 2000; Mostoufi-Moab *et al.*, 2016). High-risk populations therefore include: survivors of Hodgkin lymphoma treated with radiotherapy (Sklar *et al.*, 2000), CNS tumor survivors treated with craniospinal radiotherapy (Laughton *et al.*, 2008), pediatric HSCT recipients conditioned with TBI, especially when delivered in a single fraction (Boulad *et al.*, 1995), and survivors of solid tumors of the head and neck such as retinoblastoma (Friedman *et al.*, 2013), nasopharyngeal carcinoma (Cheuk *et al.*, 2011), and rhabdomyosarcoma (Clement *et al.*,

2015; Shalitin *et al.*, 2014), with treatment-related radiation exposures. Survivors of neuroblastoma treated with (131) I-metaiodobenzylguanidine (^{131}I-MIBG) are also at risk of primary hypothyroidism despite prophylaxis with potassium iodide (van Santen *et al.*, 2005). Novel TKI agents such as imatinib, sunitinib, and sorafenib may also cause primary hypothyroidism (Lodish, 2013; Torino *et al.*, 2013).

Diagnosis and management

Patients with primary hypothyroidism may present with slow growth (in children and adolescents), weight gain, fatigue, and cold intolerance. Given the high prevalence of primary hypothyroidism in certain groups of CCS, patients at risk should be screened with measurements of plasma FT4 and TSH at least yearly (more frequently during childhood) without waiting for clinical manifestations. Patients treated with TKI should also be screened regularly (Lodish, 2013). The treatment of primary hypothyroidism relies on substitution with levothyroxine with the aim to normalize plasma TSH and FT4 levels. Given the trophic effect of TSH on the thyroid and possible associations between chronically elevated TSH levels and thyroid neoplasia (de Vathaire *et al.*, 2015), it is common practice to treat patients with a history of neck irradiation and whose labs suggest compensated primary hypothyroidism (elevated TSH and normal FT4 plasma levels) with levothyroxine. Treatment of compensated forms is discouraged in patients on TKIs (Lodish, 2013). It is important to remember that CCS with primary hypothyroidism due to craniospinal radiotherapy may subsequently develop TSHD and ACTHD as late effects of HP irradiation. In these patients, declining TSH values over time should not be interpreted as necessarily reflective of over-dosage with levothyroxine, and FT4 levels should primarily be used to guide substitution doses. The ACTH–cortisol axis of these individuals should also be assessed prior to treatment with levothyroxine, as patients with ACTHD may experience clinical deterioration if their adrenal insufficiency is not treated prior to the initiation of thyroid replacement (Fleseriu *et al.*, 2016).

Hyperthyroidism

Hyperthyroidism is very uncommon in CCS and has been primarily reported as a consequence of transferred autoimmunity in pediatric HSCT recipients, exposure to craniospinal radiotherapy in CNS tumor survivors, or treatment with high doses of radiotherapy (35–40 Gy) for pediatric Hodgkin lymphoma (Sklar *et al.*, 2000, 2001; Mostoufi-Moab *et al.*, 2016). Hyperthyroidism may be transient and a subset of patients may subsequently become hypothyroid (Sklar *et al.*, 2001).

Autoimmune Conditions

Pediatric HSCT recipients may develop autoimmune thyroid disease due to the transfer of abnormal lymphocyte clones from the donor; a subset could require treatment for primary hypothyroidism, or more rarely hyperthyroidism (Sklar *et al.*, 2001). Immunomodulators used in maintenance chemotherapy including anti-CTLA4 monoclonal antibodies (ipilimumab or bevacizumab) and pegylated interferon may cause autoimmune thyroiditis and hypothyroidism (Torino *et al.*, 2013; Corsello *et al.*, 2013). Patients treated with these agents should be screened by measuring plasma FT4, TSH, and thyroid autoantibodies at the start of therapy and should be offered regular monitoring of FT4 and TSH levels during treatment (Corsello *et al.*, 2013).

Secondary Thyroid Cancer

Prevalence and risk factors

Thyroid cancer is one of the most frequently reported secondary malignancies in CCS with a history of neck irradiation (Mostoufi-Moab *et al.*, 2016). The prevalence of thyroid cancer was 18 times higher in pediatric Hodgkin lymphoma survivors treated with radiotherapy fields involving the thyroid than what would be expected based on general population statistics (Sklar *et al.*, 2000). The latency period between the primary cancer diagnosis and the diagnosis of secondary thyroid cancer varied between 5 and 26 years in this population (Sklar *et al.*, 2000). Secondary thyroid cancer has also been reported following craniospinal radiotherapy for CNS tumors (Ning *et al.*, 2015) and conditioning with TBI for HSCT (Cohen *et al.*, 2007; Acharya *et al.*, 2003; Inamoto *et al.*, 2015), as well as in other instances involving treatment-related direct or scatter irradiation of the thyroid (Acharya *et al.*, 2003; van Santen *et al.*, 2012; Clement *et al.*, 2015). The risk of secondary thyroid cancer in relation to radiotherapy doses follows an inverted U-shaped curve, increasing with the dose until 20–30 Gy and then decreasing at higher doses likely because of the extent of the damage to the thyroid (Bhatti *et al.*, 2010). Cancer diagnosis before 10 years of age (Sklar *et al.*, 2000) and alkylating chemotherapy agents may further increase the risk of secondary thyroid malignancy (Veiga *et al.*, 2012).

Diagnosis and management

Consensus regarding the screening strategy for thyroid cancer in CCS is lacking (Francis *et al.*, 2015). While some have suggested that yearly screening should primarily rely on the careful clinical examination of the neck in order to assess for palpable nodules or abnormal cervical lymph nodes (Acharya *et al.*, 2003), others have called for systematic screening with thyroid ultrasound to detect the disease at an earlier stage in patients with the highest risk factors (Rivkees *et al.*, 2011). The diagnosis and management of secondary thyroid malignancy follow the same processes as for primary thyroid cancer (Francis *et al.*, 2015; Cooper *et al.*, 2009).

Primary Testicular Disorders

The endocrine (testosterone secretion/virilization) and reproductive (sperm production/fertility) functions of the testes are carried out within two compartments that differ in their relative degrees of vulnerability to gonadotoxic cancer therapies. This difference is due to the lower vulnerability of the rather quiescent testosterone producing Leydig cells when compared to that of rapidly multiplying germ cells. Therefore, and while both functions may be affected by testicular exposure to radiotherapy and/or treatment with gonadotoxic chemotherapy drugs such as alkylating agents or heavy metals (cisplatin, carboplatin), the prevalence of Leydig cell failure manifesting via low testosterone secretion is substantially lower than that of oligo- or azoospermia due to germ cell injury in males exposed to such treatments (Kenney *et al.*, 2012). Medical providers should be aware that male CCS exposed to gonadotoxic treatments are frequently able to maintain normal testosterone secretion even if their clinical examination (small testes) or laboratory investigations are suggestive of impaired fertility (Chemaitilly *et al.*, 2016b).

Leydig Cell Failure

Prevalence and risk factors

The prevalence of Leydig cell failure was reported at 11.5%–13.3% in adult CCS exposed to alkylating agents or testicular irradiation (Hudson *et al.*, 2013; Brignardello *et al.*, 2013). Leydig cell failure following chemotherapy alone tends to be compensated with most patients able to maintain normal testosterone levels albeit with rising LH values, and rarely requires sex hormone replacement therapy (Romerius *et al.*, 2009; Greenfield *et al.*, 2007; Howell *et al.*, 1999). On the other hand, the majority of patients treated with testicular radiotherapy with doses >20 Gy have been reported to require testosterone replacement (Grundy *et al.*, 1997).

Diagnosis and management

Depending on the achieved pubertal stage at the time of onset of Leydig cell failure, patients may present with delayed or arrested pubertal development or symptoms of low testosterone secretion such as fatigue, depression, decreased libido, or bone or metabolic abnormalities. The primary testicular origin of the disorder is evidenced by elevated morning plasma LH values contrasting with testosterone levels that are below the normal range for an individual's age (Bhasin *et al.*, 2010; Giannetta *et al.*, 2012). Management follows the same steps as in the general non-CCS population (Bhasin *et al.*, 2010; Palmert and Dunkel, 2012).

Germ Cell Failure

Prevalence and risk factors

The prevalence of infertility due to germ cell failure following treatment with alkylating agents or radiotherapy varies between 40% and 70% depending on the method used for assessment (semen analysis (Hudson *et al.*, 2013) versus measurements of plasma FSH and inhibin B (Brignardello *et al.*, 2013)). Treatment with alkylating agents at cumulative cyclophosphamide equivalent doses ≥ 4000 mg/m^2 (Green *et al.*, 2014), or testicular radiotherapy at any dose, even as low as 0.15 Gy (Meistrich, 2013), represent the main risk factors. Treatment regimens associated with a high risk of germ cell failure may be required for the treatment of some cases of ALL (Romerius *et al.*, 2011; Green *et al.*, 2014; Meistrich, 2013); pediatric Hodgkin lymphoma (Papadakis *et al.*, 1999; Heikens *et al.*, 1996), CNS malignancies (Schmiegelow *et al.*, 2001; Ahmed *et al.*, 1983), and non-brain solid tumors (Kenney *et al.*, 2001, 2014; Moreno *et al.*, 2013; Friedman *et al.*, 2013; Trahair *et al.*, 2007; Shalitin *et al.*, 2014), as well as for conditioning for HSCT (Grigg *et al.*, 2000; Anserini *et al.*, 2002; Rovo *et al.*, 2006; Sanders *et al.*, 1996). Targeted chemotherapy agents may also affect male fertility (Mariani *et al.*, 2011; Seshadri *et al.*, 2004; Breccia *et al.*, 2014).

Diagnosis and management

The diagnosis of germ cell failure requires a semen analysis to check for oligo-/azoospermia as indirect hormonal markers such as plasma levels of FSH and inhibin B are not always reliable (Lopez Andreu *et al.*, 2000). Sperm banking prior to the exposure to gonadotoxic treatments should be offered to male patients whenever feasible (Kenney *et al.*, 2012).

Premature Ovarian Insufficiency

In contrast to what is observed in males, the endocrine (hormone-producing granulosa cells) and reproductive (oocyte) compartments in females are strongly interdependent. Hence, premature ovarian insufficiency (POI), which is the most commonly accepted term to designate gonadal failure due to a primary ovarian cause, refers to both estrogen deficiency and fertility (Anderson *et al.*, 2015; van Dorp *et al.*, 2016).

Prevalence and Risk Factors

The prevalence of POI was reported at 11% in a large cohort of CCS with extended follow-up time (Hudson *et al.*, 2013; Chemaitilly *et al.*, 2017). The main risk factors of POI include alkylating agents and ovarian irradiation (Chemaitilly *et al.*, 2006, 2017; Sklar *et al.*, 2006; Mostoufi-Moab *et al.*, 2016), including because of scatter from spinal radiotherapy for CNS cancers (Balachandar *et al.*, 2015). High-risk groups also include HSCT recipients because of alkylating agent with or without TBI for conditioning (Afify *et al.*, 2000; Sklar *et al.*, 2001; Frisk *et al.*, 2004), pediatric Hodgkin lymphoma treated with pelvic irradiation (van Dorp *et al.*, 2012; Papadakis *et al.*, 1999), and survivors of solid tumors treated with high-dose alkylating agents and/or radiotherapy involving the abdomen or pelvis (Wright *et al.*, 2009; Oue *et al.*, 2015). Host factors include older age at treatment (Chemaitilly *et al.*, 2006; Anderson *et al.*, 2015). Female CCS treated with radiotherapy also have a higher than expected prevalence of miscarriage and premature delivery; this is likely due to radiation-induced damage to the uterus and/or its vascularization (Sanders *et al.*, 1996; Holm *et al.*, 1999; Bath *et al.*, 1999). Data on the impact of targeted chemotherapy agents are limited (Lodish, 2013; Breccia *et al.*, 2014).

Diagnosis and Management

Patients may present with delayed or interrupted pubertal development, amenorrhea (primary or secondary) before the age of 40 years, or other signs/symptoms of estrogen deficiency such as hot flashes depending on the timing of onset of POI in relation to achieved pubertal stage. (Metzger *et al.*, 2013; Sklar *et al.*, 2006). It is important to note that patients frequently experience arrested puberty or amenorrhea during active therapy for cancer. Therefore, evaluations for possible POI are generally deferred until 2 years after the completion of therapy (Jacobson *et al.*, 2016), although treatment of potentially transient POI may need to be considered. Laboratory findings consistent with POI include elevated FSH with low plasma estradiol (van Dorp *et al.*, 2016). Other markers of ovarian function (or "follicular reserve") include plasma anti-Mullerian hormone levels and antral follicle count via ultrasound; the role of these modalities in counseling CCS has yet to be determined (van Dorp *et al.*, 2016), especially in adolescents. Sex-hormone replacement therapy may be offered to CCS in order to induce puberty; this therapy may also have a role in improving general health outcomes (cardiovascular, bone health, psychological, and psychosexual) in adults with POI without necessarily increasing the risk of secondary breast cancer when compared to female CCS without POI (Chemaitilly *et al.*, 2017; Moskowitz *et al.*, 2017; van Dorp *et al.*, 2016). Treatment follows the same steps as in the general non-CCS population (van Dorp *et al.*, 2016; Palmert and Dunkel, 2012). Fertility preservation via mature oocyte cryopreservation should be offered to pubertal patients at high risk of POI whenever feasible (Practice Committees of American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2013; Anderson *et al.*, 2015).

Bone Mineral Density Deficit

Children with newly diagnosed leukemia may present from the outset with impaired bone health (Mostoufi-Moab *et al.*, 2012a; Wasilewski-Masker *et al.*, 2008). Severe BMD deficit (defined by a sex- and age-adjusted z-score < -2) has been reported in up to 70% of patients at the time of ALL diagnosis, with a subset experiencing fractures even before being exposed to high-dose glucocorticoids (Mostoufi-Moab and Halton, 2014). While signs of skeletal recovery have been reported within 2 years of completion of therapy, a subset of patients may have persistently low BMD, and this may be contributing to overall worse health outcomes in this population (Mostoufi-Moab *et al.*, 2012a; Wasilewski-Masker *et al.*, 2008; Gurney *et al.*, 2014).

Prevalence and Risk Factors

The prevalence of BMD deficit in CCS has been reported at 13%–18% (Brignardello *et al.*, 2013). High-risk populations include survivors of childhood leukemia, especially those with relapsed disease and those treated with HSCT, because of the effects of the primary disease on the bone structure and additional insults from prolonged treatment with systemic glucocorticoids, conditioning with TBI, and medical and nutritional complications related to transplant (Mostoufi-Moab *et al.*, 2012b; Petryk *et al.*, 2006; Kaste *et al.*, 2004). Other risk factors include cranial irradiation (Gilsanz *et al.*, 1990), which is likely to be due to its resultant pituitary hormone deficiencies, GHD (Mostoufi-Moab *et al.*, 2012b), and/or sex hormone deficiency (Cohen *et al.*, 2012; Gurney *et al.*, 2003; Chemaitilly *et al.*, 2017). Targeted chemotherapy agents such as TKI may cause secondary hyperparathyroidism and changes in bone structure; the full effect of prolonged treatment with these agents has yet to be elucidated (Lodish, 2013).

Diagnosis and Management

The diagnosis of severe BMD deficit relies on measurements using dual X-ray absorptiometry (DXA) in patients at risk and adjusting the measure to sex and age (z-score and not T-score, as commonly done for adults) (Hudson *et al.*, 2013). Confounders of DXA measures include small stature and abnormally timed puberty (Wasilewski-Masker *et al.*, 2008). The management of severe BMD deficit includes the proper substitution of associated hormonal deficiencies (including vitamin D) and optimizing diet to

include at least the recommended daily intake for calcium, in addition to the promotion of a healthy lifestyle (regular exercise and smoking cessation) (Sala and Barr, 2007).

Obesity and Diabetes Mellitus

Prevalence and Risk Factors

CCS reported being diagnosed with obesity (relative risk, 1.8; 95% CI, 1.7–2.0) and diabetes mellitus (relative risk, 1.9; 95% CI, 1.6–2.4) at significantly higher rates than siblings controls in a recent report; this indicates a significant risk owed to survivorship even when potential genetic and social confounders are taken into account (Mostoufi-Moab *et al.*, 2016). Hypothalamic injury due to tumor growth or surgery may cause a particularly challenging form of obesity with rapid weight gain (Lustig *et al.*, 2003b; Muller, 2014). Survivors of childhood ALL, including those treated with contemporary regimens without cranial irradiation, seem to continue to struggle with persistent obesity many years after completing treatment (Garmey *et al.*, 2008); this is possibly because of the prolonged exposure to systemic glucocorticoids (Zhang *et al.*, 2014). Abnormal body composition with increased waist-to-height ratio and abnormal glucose metabolism regardless of BMI have been reported in survivors of pediatric HSCT, especially those treated with TBI (Armenian *et al.*, 2017; Hoffmeister *et al.*, 2004; Taskinen *et al.*, 2000; Chow *et al.*, 2013; Neville *et al.*, 2006). The metabolic derangements observed in this population may be due to abnormal body fat distribution and/or islet cell injury due to irradiation (Wei *et al.*, 2015a,b). Similarly, a higher risk of glucose intolerance/diabetes mellitus has been reported following abdominal radiotherapy to treat various solid tumors (Cohen *et al.*, 2014; Meacham *et al.*, 2009; Shalitin *et al.*, 2014; de Vathaire *et al.*, 2012).

Diagnosis and Management

Patients should be monitored for cardiovascular risk factors by measuring their height, weight, BMI, and blood pressure at least yearly. Screening for diabetes mellitus using fasting blood glucose is suggested in patients exposed to TBI or abdominal radiotherapy regardless of their BMI (Baker *et al.*, 2012). Emphasis on lifestyle improvement needs to be maintained in CCS as in the general non-CCS population. The long-term efficacy and safety of medications to treat hypothalamic obesity such as diazoxide (Brauner *et al.*, 2016; Hamilton *et al.*, 2011), octrerotide (Lustig *et al.*, 2003a), glucagon-like peptide 1 receptor agonists (Lomenick *et al.*, 2016), or amphetamine derivatives (Mason *et al.*, 2002) have not been well established (Cohen, 2016).

Conclusion

Endocrine late effects are among the most frequently reported chronic health conditions in CCS. They may occur many years after the exposure to cancer therapies. The importance of systematic screening, early diagnosis, and long-term follow-up of CCS who are at risk of developing endocrine complications as late effects of cancer and its treatments cannot be overemphasized.

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Disturbances of Sodium and Water. Diabetes Insipidus in Children

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Abbreviations

ADH	Antidiuretic hormone
ANH	Atrial natriuretic hormone
ANP	Atrial natriuretic peptide
AVP	Arginine vasopressin
***AVP-NPII* gene**	Arginine vasopressin-neurophysin II gene
AQP2	Aquaporin 2
BNP	Brain natriuretic peptide
CDI	Central diabetes insipidus
CNS	Central nervous system
CSW	Cerebral salt wasting
DDAVP	Desmopressin (1-desamino-8-D-arginine-vasopressin)
DI	Diabetes insipidus
ECF	Extracellular fluid
ICF	Intracellular fluid
i/v	Intravenously
LCH	Langerhans cell histiocytosis
MAO inhibitors	Monoamine oxidase inhibitors
MRI	Magnetic resonance imaging
NDI	Nephrogenic diabetes insipidus
SIADH	Syndrome of inappropriate antidiuretic hormone secretion
TBW	Total body water
V2R	Vasopressin V2 receptor
WDT	Water deprivation test

Glossary

Hyposthenuria The secretion of urine of low specific gravity due to inability of the kidney to concentrate the urine normally.

Infundibulum Pituitary stalk.

Polydipsia Is excessive thirst or excess drinking.

Poliuria Is excessive or an abnormally large production of urine.

Disturbances of Sodium and Water

Disturbance of sodium and water is among the most common disorders in clinical practice because of high prevalence of diseases associated with dehydration. Basic water and sodium balance understanding is necessary for correct diagnosis and safe management, especially in pediatric patients. In a minority of patients, specialized testing will be required.

Total body water (TBW) content is divided in two major fluid compartments: two thirds of TBW is intracellular fluid (ICF) and one-third is extracellular fluid (ECF), subdivided into the interstitial fluid (75%), and plasma (25%). The TBW comprises approximately 70% of body weight in infants, 65% in children, and 60% in adults.

Infants and children require relatively larger volumes of water than adults to maintain their fluid balance and are more sensitive to volume consumption. Significant fluid losses may occur rapidly, leading to depletion of the intravascular volume (Kuhnle-Kral and Kausman, 2011).

Pathophysiology

Plasma osmolality and volume is regulated by the synthesis and secretion of arginine vasopressin (AVP), the kidney's response to AVP and by the thirst mechanism. AVP is synthesized in the supraoptic and paraventricular nuclei of the hypothalamus, transported via the supraopticohypophyseal tract through the infundibulum and accumulated in the posterior pituitary. It binds to vasopressin V2 receptors (V2R) in the distal nephron and induces translocation of aquaporin water channels in the plasma membrane to retain water. The osmoreceptors and baroreceptors are two main types of receptors which are involved in the control of the body water balance. Osmoreceptors located in hypothalamus are very sensitive to changes of ECF osmolality. Normal plasma osmolality ranges from 280 to 300 mOsm/kg. An increase of 1% of plasma osmolality may cause an increase in AVP levels. Baroreceptors are mechanoreceptors that sense blood pressure in the vessels wall. Response from baroreceptors influence sympathetic outflow, vessel tonus, and cardiac output. Sodium is always related to water and 90%–95% of plasma osmolality depends on sodium. The most important regulatory system of sodium balance is renin–angiotensin–aldosterone system. Aldosterone regulates sodium homeostasis by stimulating sodium reabsorbtion in the distal nephron and the distal colon. While the most important organ system for sodium regulation is the kidney, it is lost largely via gastrointestinal tract. Acute diarrhea is associated with salt and water loss and results in hyponatremic or hypernatremic dehydration. The main features of dehydration according to severity is presented in **Table 1** (Di Iorgi *et al.*, 2012; Bourque, 2008; de Bree and Burbach, 1998; Batista *et al.*, 2010).

Table 1 Physical examination findings in pediatric dehydration

Symptom	*Degree of dehydration*		
	Mild (<3% body weight lost)	*Moderate (3%–9% body weight lost)*	*Severe (>9% body weight lost)*
Mental status	Normal, alert	Restless or fatigued, irritable	Apathetic, lethargic, unconscious
Heart rate	Normal	Normal to increased	Tachycardia or bradycardia
Quality of pulse	Normal	Normal to decreased	Weak, thread, impalpable
Breathing	Normal	Normal to increased	Tachypnea and hyperpnea
Eyes	Normal	Slightly sunken	Deeply sunken
Fontanelles	Normal	Slightly sunken	Deeply sunken
Tears	Normal	Normal to decreased	Absent
Mucous membranes	Moist	Dry	Parched
Skin turgor	Instant recoil	Recoil <2 s	Recoil >2 s
Capillary refill	<2 s	Prolonged	Minimal
Extremities	Warm	Cool	Mottled, cyanotic

Table 2 Causes on hyponatremia in children

Hypovolemic hyponatremia	*Euvolemic hyponatremia*	*Hypervolemic hyponatremia*
Extrarenal water losses • Gastrointestinal (vomiting, diarrhea) • Skin (sweating or burns) • Third-space extravasation of intravascular fluid (e.g., pancreatitis, peritonitis, sepsis, heart failure, nephrotic syndrome, protein-losing enteropathy, bowel obstruction) *Renal water losses* • Thiazide or loop diuretics • Osmotic diuresis • Postobstructive diuresis • Polyuric phase of acute tubular necrosis • Autosomal recessive polycistic kidney disease • Tubulointestitial nephritis • Obstructive uropathy • Cerebral salt wasting (CSW) • Proximal renal tubular acidosis • Lack of aldosterone effect (high serum potassium): ○ Absence of aldosterone (e.g., 21-hydroxylase deficiency) ○ Pseudohypoaldosteronism type I ○ Urinary tract infection	• Syndrome of inappropriate antidiuretic hormone secretion (SIADH) • Gliucocorticoid deficiency • Hypothyroidism • Nephrogenic syndrome of inappropriate antidiuresis (NSIAD) • Iatrogenic desmopressin (DDAVP) overtreatment (as complication of therapy) • Water intoxication: ○ Iatrogenic (excess hypotonic intravenous fluids) ○ Tap water enema ○ Child abuse ○ Psychogenic polydipsia ○ Diluted formula	• Congestive heart failure • Cirrhosis • Nephrogenic syndrome • Renal failure • Capillary leak due to sepsis • Hypoalbuminemia due to gastrointestinal disease (protein-losing enteropathy)

Hyponatremia

Hyponatremia is the most common (15%–30%) electrolyte abnormality in hospitalized children and it is an independent mortality risk factor in intensive care. Hyponatremia is defined as serum sodium less than 135 mmol/L. Serum sodium 120-–129 mmol/L is attributed to mild hyponatremia and less than 120 mmol/L is considered as severe hyponatremia. The causes of hyponatremia are classified according to body fluid volume status: hypovolemic, hypervolemic, and euvolemic hyponatremia. The main causes are presented in **Table 2** (Yeates *et al.*, 2004).

Pseudohyponatremia can occur due to hyperglycemia (e.g., in diabetic ketoacidosis), and elevated anion gap. Pseudohyponatremia can also be diagnosed due to elevated total and low-density lipoprotein cholesterol levels (hyperlipidemia).

Hyponatremia is also classified depending on timing of development to acute (<24 h) and chronic (>24 h). Acute hyponatremia is very dangerous because of lack of time for activation of compensatory mechanisms, leading to cerebral edema. The clinical features of hyponatremia occur when plasma sodium decreases to less than 125 mmol/L. The symptoms are nonspecific: headache, nausea, vomiting, progressing to letargy, desorientation, confusion, psychosis, respiratory arrest. Plasma sodium decrease of 10% induces increase in brain volume by 10% leading to cerebral edema and more than 10% increase in brain volume leads to brain herniation (Kuhnle-Kral and Kausman, 2011; Abka *et al.*, 2011; Grant *et al.*, 2015; Liamis *et al.*, 2010; Zieg, 2017).

Symptoms of chronic hyponatremia are not expressed or are much milder in the presence of even significant hyponatremia due to development of adaptation mechanisms. Chronic hyponatremia has significant impact on bone quality (increased risk for osteoporosis and fractures) (Ranadive and Rosenthal, 2009).

Treatment of hyponatremia

- Hypovolemic hyponatremia: administration of one or more boluses of 0.9% NaCl solution 20 mL/kg over 1 h each to correct hypovolemic shock, followed by intravenous fluid repletion with 0.45%–0.9% NaCl according to serum sodium level.
- Euvolemic hyponatremia: water restriction by 50%–75% in SIADH or in case of water intoxicaton. Sodium replacement with intravenous 0.9% NaCl is sometimes required.
- Hypervolemic hyponatremia: restrict fluid and salt. Loop diuretics may be used, for example, Furosemide 0.5–1 mg/kg.

For treatment of symptomatic patients with severe hyponatremia (serum sodium less than 120 mmol/L and hyponatremia duration is $<$24–48 h), sodium correction no more than 10–12 mmol/L/24 h is recommended to prevent brain herniation and neurological damage from cerebral ischemia. Recommended treatment of acute hyponatremia varies by symptoms severity:

- Severe symptoms: 3 mmol/kg of sodium as hypertonic saline (3% NaCl) should be infused intravenously over 30 min to increase the serum sodium by 5 mmol/L.
- Mild to moderate symptoms, in patients at low risk for herniation: correction of sodium should occur over 24–48 h using 0.45%–0.9% NaCl intravenously saline (Vaidya *et al.*, 2010).

Syndrome of inappropriate ADH secretion (SIADH)

SIADH is defined by euvolemic hyponatremia and hypoosmolality resulting from inappropriate continued secretion or action of the AVP, resulting in impaired water excretion. In children, the vast majority of SIADH cases are acute and transient, and are generally caused by central nervous system (CNS) injuries (brain infecions, tumor, head trauma) or severe pulmonary diseases (infections, asthma, cystic fibrosis). Ectopic AVP secretion in lung cancer (less often gastrointestinal and urogenital cancers, lymphoma, sarcomas) is the most common cause of SIADH in adults. Also, drugs which stimulate AVP release or potentiate effects of AVP action, may cause SIADH (e.g., acetylcholine, cyclophosphamide, vincristine, and vinblastine) (Grant *et al.*, 2015).

Criteria of SIADH

- Hyponatremia and decreased effective osmolality of the extracellular fluid (Posm $<$275 mOsm/kg);
- Urine osmolality near or above plasma osmolality;
- Clinically observed euvolemia.
- Elevated urinary sodium excretion while on normal salt and water intake;
- Absence of other causes of hyponatremia—adrenal insufficiency, hypothyroidism, cardiac failure, pituitary insufficiency, renal disease with salt wasting, hepatic disease, drugs that impair renal water excretion;
- Correction of hyponatremia not effective by volume expansion, and is achieved by fluid restriction;
- Plasma AVP level inappropriately elevated relative to plasma osmolality (Bartter and Schwartz, 1967).

Treatment of SIADH

Fluid restriction by 50%–75% (with a goal of 500 mL/d below the 24-h urine volume) is generally first-line therapy. In children, most SIADH cases are acute and transient and respond well to conservative management. In adults, some cases may be prolonged and warrant pharmacologic treatment with AVP antagosnists (vaptans).

Management and treatment of SIADH depends on SIADH duration and severity of hyponatremia:

- Treat the underlying cause when possible.
- Moderate and asymptomatic hyponatremia (Na $>$120 mmol/L):
  - Fluid restriction to 600–800 mL/m^2/day.
  - Hyponatremia correction—to increased Na $\leq$0.5 mmol/L/h.
- Severe hyponatremia ($<$120 mmol/L) with neurogical symptoms:
  - Fluid restriction to 600–800 mL/m^2/day.
  - Intravenous hypertonic saline 3% NaCl—1–2 mL/kg/h, to avoid rapid hyponatremia correction. Plasma sodium should increase by 1–2 mmol/L/h in the presence of severe symptoms (severe confusion, convulsions, or coma) continue until symptoms resolve or for the first 3–4 h. However, total correction in the first 24 h must not exceed 10–12 mmol/L and there are various recommendations for the correction limit/day for chronic SIADH, varying from 0.5 to 1 mmol/L/h (6–8 mmol/L/day). The intravenous administration of hypertonic saline (3%) should be discontinued when neurological symptoms disappear.
  - The use of loop diuretics (furosemide) with hypertonic saline 3% in case of severe water intoxication.

Table 3 Differential diagnosis SIADH versus CSW (Edate and Albanese, 2015)

	SIADH	*CSW*
Plasma volume	Normal/high	Low
Evidence of volume depletion	No	Yes
Plasma osmolality	Low	Low
Plasma sodium	Low	Low
Urine sodium	High	High
Net sodium loss	Normal	Very high
Urine output	Usually low	Very high
Serum uric acid	Low	Normal/low
Plasma renin	Suppressed	Suppressed
Plasma aldosterone	Normal/high	Suppressed
Plasma AVP	High	Suppressed
Plasma ANP	High	High
Plasma BNP	Normal	High
Central venous pressure	Normal/high	Low

- Moderate and symptomatic hyponatremia is treated by raising the serum sodium level by 0.5–1 mmol/L/h for a total of 8 mmol/L during the first day.
- Drugs:
  - Demeclocycline can be used in chronic situations when fluid restrictions are difficult to maintain; demeclocycline is the most potent inhibitor of AVP action. Prolonged use of demeclocycline should be avoided due to extensive side effects profile, including skin photosensitivity, and nephrotoxicity.
  - Urea orally at a dose of 30 g/day increases urinary solute and water excretion.
  - Vaptans (in adults)
    - Conivaptan—an antagonist of both V_{1a} and V_2 AVP receptors.
    - Tolvaptan—an antagonist of the V_2 AVP receptor (Grant *et al.*, 2015; Kuhnle-Kral and Kausman, 2011).

Cerebral salt wasting (CSW)

CSW is a rare endocrine condition characterized by hyponatremia and dehydration in response to CNS disease. CSW may occur after neurosurgery, head injury and particularly in patients with subarachnoid hemorrhage. CSW is characterized by hyponatremia and ECF depletion due to inappropriate sodium wasting in the urine. CSW may occur after the first days or week from the cerebral injury event, and it takes usually 2–4 weeks, but may continue for months. The central nervous system damage can directly cause CSW by oversecretion of atrial natriuretic hormone (ANH), resulting in natriuresis and polyuria, leading to hyponatremia and reduced effective volume mass. Alternatively, CSW can be a secondary response to SIADH by stimulation of ANH secretion via AVP or plasma volume expansion. The abnormal sympathetic outflow to the kidney with a pressure natriuresis as well as abnormal secretion of AVP or brain natriuretic peptide (BNP) have been proposed as potential causes (Edate and Albanese, 2015; Yee *et al.*, 2010; Bettinelli *et al.*, 2012).

The diagnostic criteria of CSW syndrome (Yee et al., 2010; Wu et al., 2016)

- Serum sodium <135 mmol/L;
- Urinary sodium >40 mmol/L;
- Negative sodium balance;
- A low serum uric acid concentration due to urate wasting in the urine;
- Plasma omolality <280 mOsm/kg;
- An inappropriately elevated urine osmolality (above 100 mOsm/kg and usually above 300 mOsm/kg);
- Central venous pressure <6 cm H_2O and pulmonary capillary wedge pressure <8 cm H_2O.
- Whole-body dehydration.

In some cases, biochemical findings may resemble SIADH. Differential features of SIADH and CSW are presented in **Table 3**.

*Treatment options of cerebral salt wasting syndrome (CSW) (*Edate and Albanese, 2015)

In contrast to SIADH, normal to high fluids administration is required together with sodium supplementation with gradual tapering.

- Intravenous isotonic or hypertonic saline to raise serum sodium no faster than 0.7 mmol/L/h, for a maximum total daily change 10–12 mmol/L.
- Fludrocortisone may be considered in prologed cases of CSW.

Table 4 Causes of hypernatremia in children

Water deficit (euvolemic hypernatremia)	*Water and sodium deficit (hypovolemic hypernatremia)*	*Excess sodium (hypervolemic hypernatremia)*

• Central diabetes insipidus (CDI)
• Nephrogenic diabetes insipidus (NDI)
• Wolfram syndrome
• Increased insensible losses:
  ○ Premature infants
  ○ Radiant warmers
  ○ Phototherapy
• Poor intake:
  ○ Ineffective breastfeeding
  ○ Child neglect or abuse
  ○ Adipsia (lack of thirst)
	• Gastrointestinal losses:
	  ○ Diarrhea
	  ○ Emesis/nasogastric suction
	  ○ Osmotic cathartics (lactulose)
	• Cutaneous losses:
	  ○ Burns
	  ○ Excessive sweating
	• Renal losses:
	  ○ Osmotic diuretics (mannitol)
	  ○ Diabetes mellitus
	  ○ Chronic kidney disease (dysplasia and obstructive uropathy)
	  ○ Polyuric phase of acute tubular necrosis
	  ○ Postobstructive diuresis
		• Improperly mixed formula
		• Excess sodium bicarbonate administration
		• Ingestion of seawater or sodium chloride
		• Intentional salt poisoning
		• Intravenous hypertonic saline
		• Hyperaldosteronism
		• Cushing syndrome
		• Hemodialysis

- Oral sodium chloride can be administered once the patients are able to take oral medications.
- Monitoring of body weight, fluid balance, and serum sodium concentration is essential during the whole treatment period (Cerda-Esteve *et al.*, 2008; Dholke *et al.*, 2016).

Hypernatremia

Hypernatremia is defined as serum sodium higher than 145 mmol/L and is a result of a deficit of total body water (TBW) relative to total body sodium levels due to either loss of free water, or infrequently, the administration of hypertonic sodium solutions. Mortality due to hypernatremia is approximately 30%–48%. The causes of hypernatremia in children are presented in **Table 4**.

Most patients present with nonspecific signs, such as fever, headaches, nausea, dehydration, changes in muscle tone, lethargy and weakness. In cases of severe hypernatremia, impaired conscious state and seizures may occur. Due to hypernatremia, as a result of osmotic changes, the cells lose water and shrink, blood vessels rupture can occur leading to subarachnoid hemorrhage and eventual death.

Children with chronic hypernatremia present with weight loss, linear growth retardation, anorexia, and irritability; infants often have episodes of hyperthermia, and excessive crying (**Table 4**) (Kuhnle-Kral and Kausman, 2011).

Treatment of hypernatremia

The main rule for hypernatremia treatment is to maintain maximum decrease of serum sodium level by 0.5 mmol/L/h and the correction of sodium to the upper limit of normal should be achieved over 48–72 h to avoid acute cerebral oedema.

The principles of hypernatremia management:

- Monitoring of weight, fluid balance (intake and output) and serum sodium; serum sodium levels should be monitored every 1–4 h.
- Monitoring of serum potassium.
- Shock should be corrected with one or several boluses of 0.9% NaCl 20 mL/kg over 1 h each until perfusion is adequate.
- Body water deficit may be calculated according total body water (TBW), which is 60% of body weight in children and 70% in infant.
- Body water deficit may be calculated with this formula:
  - Water deficit (in L) = [(current Na level in mEq/L − 145 mEq/L)/145 in mEq/L] × 0.6 × weight (in kg).
  - Water deficit (in L) = [1 − (145 mEq/L ÷ current Na level in mEq/L)] × 0.6 × weight (in kg).
- The volume of replacement fluid needed to correct the water deficit is determined by using the concentration of sodium in the replacement fluid. The replacement volume can be determined as follows:
  - Replacement volume (in L) = TBW deficit × 1 ÷ [1 − (Na concentration in replacement fluid in mEq/L ÷ 154 mEq/L)].
- Intravenous fluid administration:
  - If the patient is hypotensive, normal saline (lactated Ringer solution, or 5% albumin solution) should be used regardless of a high serum sodium concentration.
  - In hypernatremic dehydration, 0.45% or 0.2% NaCl should be used as a replacement fluid to prevent excessive delivery of free water and a too-rapid decrease in the serum sodium concentration.
  - In cases of hypernatremia caused by sodium overload, sodium-free intravenous fluid (5% dextrose) may be used, and a loop diuretic may be added (Kuhnle-Kral and Kausman, 2011).

Table 5 Etiological classification of diabetes insipidus (Edate and Albanese, 2015; Di Iorgi *et al.*, 2012; Robertson, 2016)

Central DI

Congenital

- Genetic
  - *AVP-NPII* gene defect
  - Familial autosomal dominant CDI
  - Familial autosomal recessive CDI
  - *WFS1* gene defect—DIDMOAD syndrome
  - Wolfram syndrome
  - X-linked lissencephaly with ambiguous genitalia
- Congenital anatomic defect
  - Septo-optic-dysplasia
  - Holoprosencephaly
  - Agenesis of corpus callosum
  - Kabuki syndrome
  - Familial pituitary hypoplasia
  - Infundibulum defect
- Others
  - Vasopressinase excess
  - Syndrome of defective osmoregulation

Aquired

- Primary tumors or metastasis
  - Germinoma
  - Pinealoma
  - Craniopharyngioma
  - Glioma
  - Pituitary stalk thickening
- Infections/infiltrative lesions
  - Meningitis/encephalitis (transient CDI)
  - Congenital cytomegalovirus infection
  - Toxoplasmosis
  - Tuberculosis
  - Cryptococcus
  - Sarcoidosis
  - Langerhans cell histiocytosis
  - Leukemia
  - Hemochromatosis
  - Amyloidosis
- Drugs
  - Adrenergic drugs
  - Carbamazepine
  - Phenytoin
  - Valproic acid
- Trauma/surgery
- Autoimmune disorders
  - Hypophysitis
- Hypoxic-ischemic encephalopathy
- Electrolyte disturbances
  - Hypokalemia
  - Hypercalcemia

Idiopathic

Nephrogenic DI

Congenital

- X linked NDI—Xq28 encoding AVPR2
- Autosomal recessive NDI—Ch12q13 encoding AQP2
- Autosomal dominant NDI
- Bardet-Biedl syndrome

Acquired

(*Continued*)

Table 5 Continued

- Drugs
  - Lithium
  - Demeclocycline
  - Amphoterecin B
  - Rifampin
  - Methicillin
- Other conditions
  - Hypercalcemia
  - Hypokalemia
  - Primary renal diseases
  - Polycystic kidney disease
  - Ureteral obstruction
  - Uremia

Primary polydypsia
Psychogenic

Diabetes Insipidus

Diabetes insipidus (DI) is a condition when large volumes of dilute urine are excreted due to defect of syntesis or deficiency of AVP secretion—central DI (CDI), or due to renal tubular resistence to AVP—nephrogenic DI (NDI), and sometimes can occur due to secondary suppression of AVP production after excessive fluid intake (primary polydipsia). DI is a disease characterized by polyuria and polydipsia with hyposthenuria, causing dehydration and hypernatremia if the patient is deprived of water. Polyuria is characterized by a urine volume in excess of 1.5–2 L/m^2/24 h, approximately $>$150 mL/kg/24 h at birth, $>$100–110/mL/kg/24 h in infants, 75 mL/kg/24 h in young children, $>$40–50 mL/kg/24 h in older children and adults, or urine output of more than 4 mL/kg/h in children and more than 6 mL/kg/h in neonates. Polydipsia is characterized then water intake $>$2 L/m^2/d (Edate and Albanese, 2015; Di Iorgi *et al.*, 2012).

Anatomy

The AVP, or antidiuretic hormone (ADH) is a peptide produced by the hypothalamus in paraventricular and supraoptical nuclei and is transferred by posterior neuronal axons to the posterior part of the pituitary gland (neurohypophysis) and is stored in neurosecretory granules. These axons store quantities of AVP sufficient to sustain basal release for 30–50 days or to allow maximum antidiuresis for 5–10 days. While the blood supply for the anterior pituitary is via the hypothalamic–pituitary portal system from the suprahypophyseal arteries, the vascularization of the posterior pituitary is direct from the inferior hypophyseal arteries (Edate and Albanese, 2015; Di Iorgi *et al.*, 2012).

Physiology

AVP secretion from neurophysiophysis to the blood stream is stimulated by increased plasma osmolality and 5%–10% decreased blood volume. In addition, stimulation of hypothalamus ventromedial nuclei induces the thirst sense. Other nonosmal factors that promote the release of AVP are nausea, vomiting, stress, physical activity, acute hypoxia and hypercapnia, hypoglycaemia, and some medicines and chemicals (vincristine, carbamazepine, cyclophosphamide, antidepressants, monoamine oxidase inhibitors (MAO) inhibitors, nicotine, etc.). AVP secretion is suppressed by alcohol and caffeine.

AVP acts on aquaporin channels located in the kidney collecting duct and promotes water retention and increases urine osmolality (de Bree and Burbach, 1998).

Epidemiology

The incidence of DI is 3–4 cases in 100,000, with a slightly higher males predominance (60%). Less than 10% of all DI cases are heredited. In children, NDI is more common than CDI and is most often acquired. X-linked NDI represents 90% of cases of congenital NDI and occurs with a frequency of 4–8 per 1 million male live births; autosomal NDI accounts for approximately 10% of the remaining cases. No gender difference has been reported for the other forms. The frequency of autosomal dominant CDI is currently unknown (Fenske and Allolio, 2012).

Etiology

The classification of diabetes insipidus in children according to etiology is presented in **Table 5** (Edate and Albanese, 2015; Di Iorgi *et al.*, 2012; Mishra and Chandrashekhar, 2011).

Central diabetes insipidus

CDI is caused by the destruction or degeneration of neurons originating in the supraoptic and paraventricular nuclei. The known causes of these lesions including local inflammatory or autoimmune diseases (hypophysitis), vascular diseases, infiltrative conditions such as Langerhans cell histiocytosis (LCH), sarcoidosis, tuberculosis, germinoma/craniopharyngioma, trauma resulting from surgery or an accident, metastases and midline cerebral and cranial malformations (Capatina *et al.*, 2015; Secco *et al.*, 2011). In rare cases (accounting for 1%–5% of all cases), CDI may be inherited as an autosomal dominant or autosomal recessive disease caused by mutations of the arginine vasopressin-neurophysin II (*AVP-NPII*) gene, and presents with gradual onset during infancy (autosomal recessive CDI) or early childhood between 1 and 6 years (autosomal dominant CDI). Mutations of the *AVP* gene impair the synthesis or secretion of AVP. To date, 73 mutations in *AVP-NPII* gene resulting in an autosomal dominant familial CDI and 3 autosomal recessive mutations are identified. The *AVP-NPII* gene is located on chromosome 20p13 (Abu Libdeh *et al.*, 2010; Babey *et al.*, 2011; Land *et al.*, 1982; Lin *et al.*, 2009; Matarazzo *et al.*, 2004).

More than 170 different mutation are indentified in *WFS1* gene causing autosomal recessive CDI, resulting in Wolfram syndrome. It is mostly caused by homozygous or compound heterozygous mutations in the *WFS1* gene located on chromosome 4p, encoding wolframin. Wolframin functions as an endoplasmic reticulum channel and regulates intracellular ionized calcium (Ca^{2+}) levels. Walfram syndrome is defined by the presence of diabetes mellitus and optic atrophy, approximately 70% of patients also present with central diabetes insipidus due to AVP deficiency. It has recently been suggested that a dosage effect of specific *WFS1* mutations with different tissue sensitivities to *WFS1* deficiency may be responsible for partial phenotypes. Clinically, diabetes insipidus in the context of Wolfram syndrome is characterized by late onset in the second or third decade of life (Barrett *et al.*, 1995; Elli *et al.*, 2012).

X-linked CDI has been reported in one family; however, genetic mutations were not indentified. CDI manifested at young age (<1 year), with decreased basal and stimulated AVP, and remission on standard doses of DDAVP. Reduction or absence of the posterior pituitary bright spot in these male patients could indicate posterior pituitary degeneration similar to autosomal dominant familial CDI (Abu Libdeh *et al.*, 2010; Babey *et al.*, 2011; Di Iorgi *et al.*, 2014; Lin *et al.*, 2009; Matarazzo *et al.*, 2004).

Nephrogenic diabetes insipidus

In children, NDI is more common than CDI and it is most often acquired, secondary to damage to distal nephron leading to tubular defect and it is due to kidney diseases (obstruction, pyelonephritis, dysplasia, nephrocalcinosis), severe electrolite disturbances, drugs (lithium, tetracyclines), and infiltrative diseases (sarcoidosis).

Congentital forms of NDI are due to mutations in arginine vasopressin receptor 2 (*AVPR2)* gene (90%) and aquaporin 2 (*AQP2*) gene (10%) causing the insensitivity of the distal nephron to AVP leading to impaired water reabsorption.

NDI due to mutation in *AVPR2* gene is X-linked recessive disorder; males are severely afected, while female carriers often presents with moderately impared urine concentration capacity. More than 200 different *AVPR2* gene mutations have been described in more than 300 families. Onset of symptoms of X-linked NDI is usually in early infancy. Hypernatremia does not occur with free access to fluids but can become a problem during states of dehydration.

AQP2 gene mutations are responsible for 10% of congenital NDI inherited in both autosomal recessive or dominant maner. *AQP2* gene is located on chromosome 12q13 and encodes the water channel AQP2 allowing AVP stimulated water reabsorption from the renal distal tubule. To date, 52 mutations in *AQP2* gene are indentified resulting in autosomal recessive NDI forms and 11 mutations of dominant recessive disease. The NDI symptoms present from early infancy in both forms of NDI (Moeller *et al.*, 2016; Schernthaner-Reiter *et al.*, 2017).

Diagnosis (Di Iorgi *et al.*, 2014, Kuhnle-Kral & Kausman, 2011)

Clinical features

Regardless of the cause, DI features are polyuria and polydipsia.

If the patient is deprived of water, following symptoms will occur:

- Dehydration;
- Weight loss;
- Fever;
- Constipation;
- Failure to thrive and developmental delay.

Features in infants:

- Vigorous suck;
- Vomiting;
- Recurrent episodes of fever without obvious cause;
- Excessive crying;
- Irritability.

Features in younger children:

- Often manifest with primary enuresis;
- Difficulies of toilet training.

Older children:

- High urine output;
- Nocturia leading to disturbed sleep;
- Anorexia due to preference of water over food.

Other clinical features/complains depending on etiology of DI:

- Neurological features (headache, visual disturbance);
- Other pituitary hormone deficiencies;
- Variable severity of mental retardation in children with NDI (attributed to intracranial calcifications).

Primary polydypsia is usually gradual in onset: absence of nocturia, need to drink water at night time.

Diagnostic tests (Di Iorgi et al., 2012; Kuhnle-Kral and Kausman, 2011; Mishra and Chandrashekhar, 2011; Robertson, 2016; Wu et al., 2016)

Basal diagniostic test:

- Confirm polyuria 24 h diuresis;

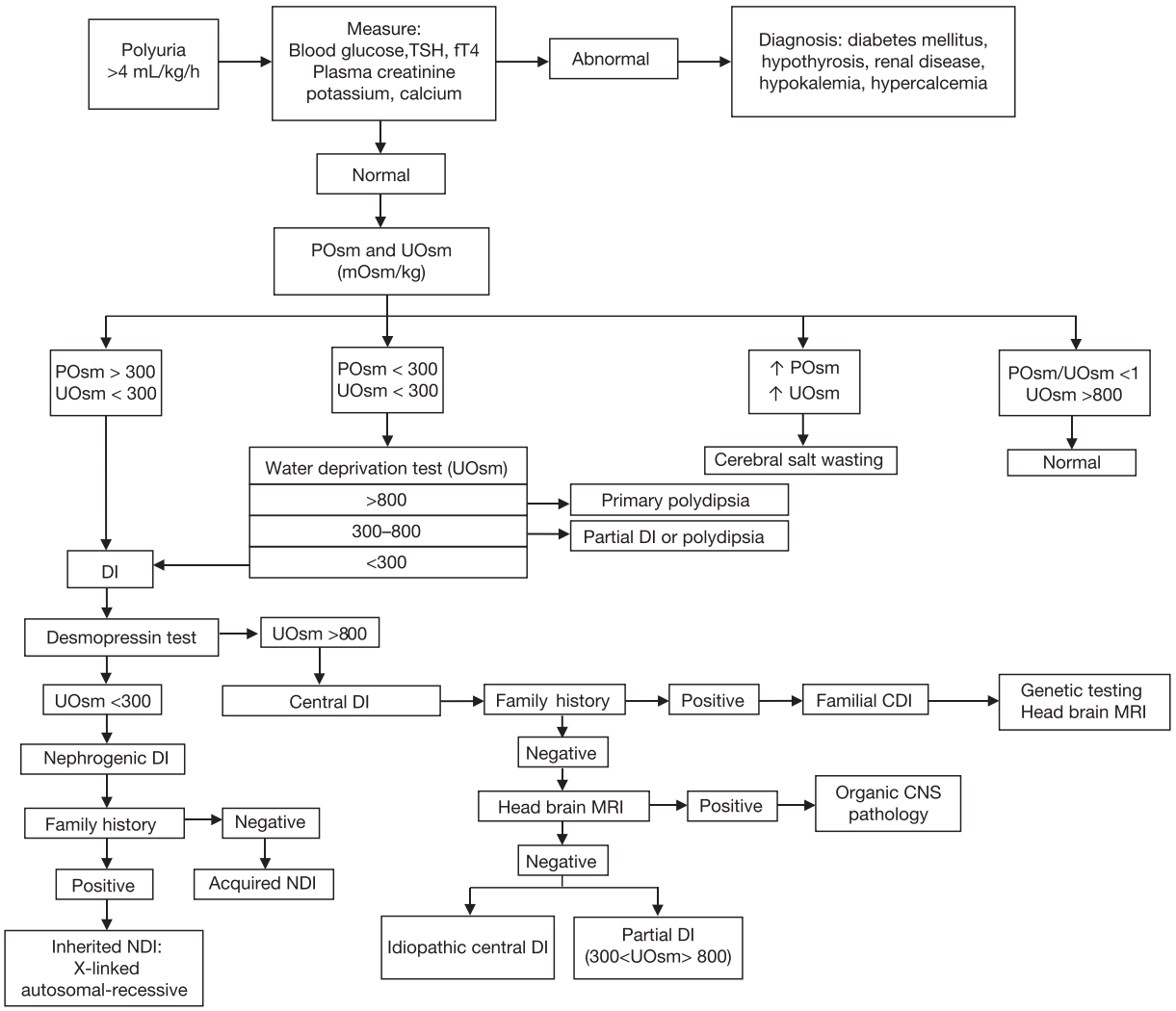

Fig. 1 Differential diagnosis of polyuria.

Table 6 Interpretation of DDAVP and water deprivation test

Diagnosis	*After water deprivation test Urine osmolality, mOsm/kg*	*After desmopressin test Urine osmolality, mOsm/kg*
Central DI	<300	>800
Nephrogenic DI	<300	<300
Primary polydipsia	>800	>800
Partial DI or polydipsia	300–800	<800

- Exclusion of other disorders (glycemia, serum calcium, blood, and urine pH);
- Paired morning blood and urine samples for osmolality: if the blood osmolality exceeds 295 mOsm/kg, while urine osmolality <300 mOsm/kg, DI diagnosis is confirmed (**Fig. 1**).

Water deprivation test (WDT)

Generally, if the patient has free access to water, DI is not diagnosed with basal tests. WDT with DDAVP administration is most commonly used to establish diabetes insipidus and to differentiate between nephrogenic and neurohypophyseal forms. It is important to exclude cortisol deficiency prior to WDT, since glucorticoid deficiency impairs the ability to appropriately excrete water load causing water retention.

In patients wihout severe polyria, WDT is started at 6 pm and stopped at 8 am. In infants and in patients with severe polyuria (exceeding 4 L/24 h), because of the risk of severe dehydration, the test should start in the morning. Patients are not allowed to drink or eat, and blood pressure and weight are monitored every 1–2 h. After 6–8 h fast and water deprivation, weight, urine volume, serum and urine osmolality and serum sodium levels are measured. The WDT is stopped at any time, if:

- Urine osmolality >750 or more than 600 mOsm/kg and stable for two consecutive times;
- Plasma osmolality >300 mOsm/kg and urine osmolality <300 mOsm/kg;
- If 5% weight loss and/or vital signs disclose hypovolaemia.
- Serum sodium exceeds 145 mmol/L.

DDAVP test

With the aim to differentate between CDI and NDI, immediatly after WDT 5–10 μg of DDAVP is administrated intranasally, followed by paired serum and urine osmolality tests after 1 h. More than 50% increase in urine osmolality and decrease in serum sodium and osmolarity are characteristic of CDI. Urine osmolality does not change or slightly increased in NDI (**Table 6**).

Neuroimaging

Brain magnetic resonance imaging (MRI) is performed to determine the underlying cause of CDI and may confirm the diagnosis of CDI in the absence of the hyperintensive signal of posterior pituitary—"bright spot," which may also be ectopic. The presence of a thickened pituitary stalk points towards possibility of LCH, germinoma and hypophysitis (either inflammatory or autoimmune). In cases of idiopatic CDI with pituitary stalk thikning, longitudinal follow-up is recommended repeating MRI ever 4–6 months for 2 years, then every year for 3 years. If the initial MRI is normal, some authors suggest to repeat MRI every 6–12 months, but it is unclear how long it is necessary to repeat imaging to exclude underlying diagnoses.

Treatment of DI

Treatment of CDI (Edate and Albanese, 2015; Robertson, 2016)

Confirmed CDI is treated with synthetic vasopressin analog DDAVP:

- Orally, intranasally, intravenously (i/v);
- Urine output decrease in 1–2 h after administration;
- Duration of action 6–18 h;
- Large variations in dose requirement:
  - Orally 0.1–1.2 mg in three divided doses,
  - Intranasal 2–40 μg (once or twice a day),
  - Vasopressin 0.1–1 μg i/v in emergency situations,
- Start with low dose, increase as necessary. Dose titration is best achieved under close supervision in hospital, especially for young children and infants;
- Infants may be treated with adequate fluid supply. Alternatively, low doses of diluted DDAVP may be administered.

*Treatment of NDI (*Mishra and Chandrashekhar, 2011)

- Low salt diet;
- Thiazide diuretics ± nonsteroid antiinfamatory drugs (indomethacin). Avoid overcorrection;
- New drugs under investigation: targeting enhancement of AQP2 function (Saifan *et al.*, 2013):
  - Phosphodiesterase inhibitors,
  - Statins (Bonfrate *et al.*, 2015),
  - Prostaglandins,
  - Metformin,
  - Thyrosine kinase inhibitors.

*Treatment of partial AVP deficiency (*Mishra and Chandrashekhar, 2011)

- Patients can maintain normal or near normal water balance when permitted to drink water in response to thirst;
- DDAVP;
- Drugs that potentiate action of otherwise insufficient amounts of endogenous ADH:
  - Chlorpropramide,
  - Thiazide diuretics.

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Obesity, Childhood, and Adolescence

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Introduction and Scope of the Problem

The prevalence of overweight and obesity is high in adults and already in children and adolescents across most developed and developing countries with a 20%–35% prevalence. Although the prevalence of obesity at a young age seems to stabilize in some countries, the number of adolescents and especially of adults with obesity is still increasing dramatically worldwide. This is a major concern, considering the well described association of overweight and obesity with long-term health problems, such as cardiovascular disease leading for example to myocardial infarction and stroke, type 2 diabetes and cancer. In the majority of individuals, weight gain is the result of exposure to an "obesogenic" environment, superimposed on a background of genetic susceptibility brought about through evolutionary and cultural adaptation. A large number of genes have been identified by genome wide association studies (GWAS) and candidate gene approaches that are associated with the regulation of appetite, food intake, and body weight. Therapeutic and preventive strategies have to follow complex and multifactorial themes and are often not efficient nor efficacious (Kiess *et al.* 2001) (**Table 1**) (**Fig. 1**).

Definitions and Differential Diagnosis

The definition and diagnosis of obesity in children and adolescents is surprisingly difficult. The level of fatness at which morbidity and also mortality increases is determined on an actuarial basis. In children and adolescents the degree of body fat mass depends upon ethnic and genetic background, gender, developmental stage, and age. Waist and neck circumference, skin-fold thickness and body mass index are the most useful noninvasive clinical measures to determine the degree of body fatness and define obesity. Body mass index has been used as a surrogate marker of body fat and is the most frequently used tool to diagnose overweight and obesity also at a young age. However, BMI has surprisingly low sensitivity and specificity when compared to other body mass indices such as skinfold measurements, waist circumference and such. Comparative data and reference values have to be used and percentiles or standard-deviation scores have to be calculated in order to give a good representation of a subject's fat and lean body mass in relation to peers considering ethnicity, gender, pubertal stage, and age. The relative unreliability of BMI for diagnosing childhood obesity is partly due to the fact that for example in early puberty in boys, body mass increments are due to muscle and bone mass increase while in girls there is also adipose tissue increments in stages of early puberty.

BMI is used to define obesity clinically. Accordingly, a child with a BMI above the 97th percentile in regard to age and gender is considered to be obese. A child with a BMI greater than the 90th but below the 97th percentile would be considered overweight. In adults, a BMI greater than 28 kg/m^2 is associated with an increased risk of morbidity such as stroke, ischemic heart disease, sleep–apnea syndrome, orthopedic diseases or type II diabetes mellitus. Adults with a BMI greater than 30 kg/m^2 are uniformly classified as being obese (grade 2 overweight) while those with a BMI between 25 and 29.9 kg/m^2 are considered to be grade 1 overweight. A BMI over 40 kg/m^2 is classified as grade 3 overweight (WHO classification). A central distribution of

Table 1 Factors which contribute to the development of obesity and may constitute risk factors for a child to develop increased body weight and fat mass

Genetic factors:
Possibly polymorphisms and/or mutations in any of the following: Adrenergic receptors, leptin, Ob-R, SOCS-3, TNF, POMC, MCH, MC4R, NPY, NPY receptors, CRH, TRH, urocortin, orexin A and B, galanin, neurotensin, serotonin and many others, polygenic causes
Environmental/exogenous factors:
Increase of sedate activities (screen time etc.)
Decrease in physical activity
Shift in diet towards more fast/prepackaged foods with high fat/calorie
Content
Sugar-sweetened beverages
Endocrine disrupting chemicals
Loneliness and social isolation
Psychosocial/family problems
Low parental income
Low parental education

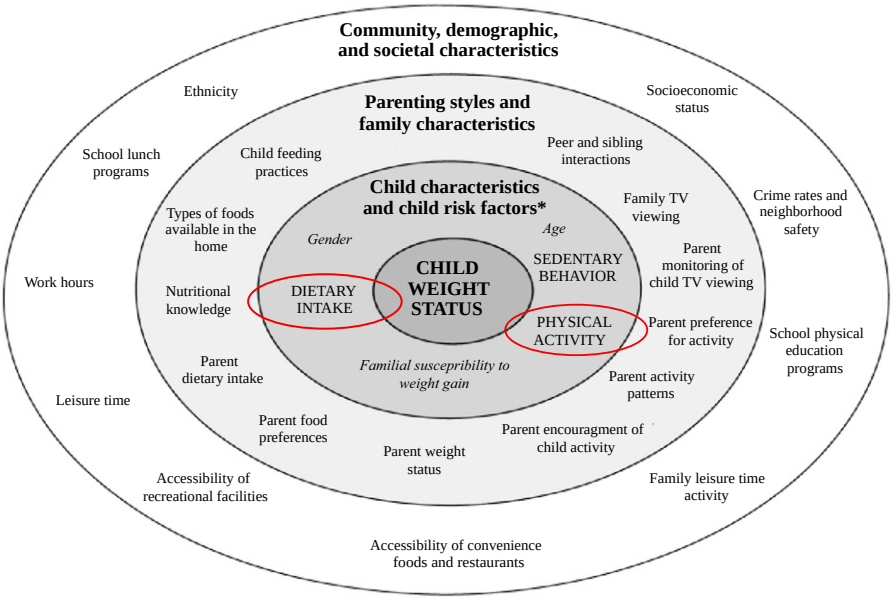

Fig. 1 Causes of increase of overweight in children and adolescents. Davison, Kirsten Krahnstoever and Leann Lipps Birch. Childhood overweight: a contextual model and recommendations for future research. Obesity reviews: an official journal of the International Association for the Study of Obesity 2 3 (2001): 159-71.

Table 2 Disorders which can present with obesity in childhood—differential diagnosis of obesity disorders

Endocrine disorders such as:
Cushing's syndrome/disease
Hypothyroidism
Growth hormone deficiency
Hyperinsulinemia
(Pseudo) hypoparathyroidism (Albright's hereditary dystrophy)
Central nervous system disorders/brain damage in relation to:
Hypothalamic tumor
Neurosurgery
Trauma
Postinflammation (meningoencephalitis)
Postchemotherapy
Corticosteroid therapy
Antiepileptic therapy
CNS irradiation

body fat is also associated with a higher risk of morbidity and mortality in adulthood. In addition and most importantly, an increased risk of death from cardiovascular disease and myocardial infarction and stroke in particular has been found in adults whose BMI had been greater than the 75th percentile as adolescents. The International Obesity Task Force has proposed that the adult body mass index cut-off points (25 and 30 kg/m^2) should be linked to body mass index percentiles for children to provide for child cut-off points. Accordingly, age and gender specific BMI values for 2–18 years corresponding to BMI values of 25 and 30 kg/m^2 at 18 years of age have been published by Cole *et al.* (2000).

Differential diagnosis of obesity can be quite difficult. "Exogenous obesity" which may also be referred to as "simple" or "primary" obesity is still, by far, the most common diagnosis in the obese child. However, in addition to many monogenic traits of morbid obesity there are numerous, largely rather rare, disorders that can also present with obesity early in life. These usually include both genetic syndromes and also a variety of underlying disorders such as hypothalamic tumors, other brain lesions and endocrine disorders (**Table 2**). The diagnosis of primary or simple or "exogenous" obesity is usually easy to make and depends upon family and personal history and a careful physical exam. Only rarely more extensive laboratory tests and finally molecular genetic analysis will be necessary in routine clinical work up.

A simple diagnostic algorithm which aids in making a diagnosis in children and adolescents with obesity is highlighted in Fig. 1. Usually, no laboratory investigations are recommended unless substantial comorbidity such as hypercholesterolemia or liver disease is suspected. However, a primary determination of serum concentrations of thyroid stimulating hormone (TSH), T3 and T4 and lipid measurements are carried out in most centers in Europe and the United States.

Etiology of Obesity

The etiopathology of obesity has to be related to an intricate interplay between many genes, epigenetics, adipose tissue factors including inflammatory molecules, adipocytokines and immune cells, signaling molecules, food stuffs, metabolites, microbiota, and environmental chemicals. In addition, social inheritance, obesogenic environment, urbanization, socio-demographic factors including income, poverty and education play an important role in the development of obesity both in the individual and in societies. Nutrition and malnutrition, lack of physical activity and sedentary behavior, media use, and cultural habits and beliefs add to the obesogenic risks. Lastly, human evolution has led to an increased prevalence of obesity in the recent history of mankind (Kiess *et al.*, 2001; Lobstein *et al.*, 2015).

Monogenic

Overall, monogenic forms of obesity are rare but taken together are present in a substantial number of people affected by early onset and extreme obesity. It is important to diagnose children with the monogenic obesity forms since there are treatments available for many of them and counseling the family as well as finding appropriate decisions for care and schooling must be based upon clear diagnoses. The majority of genes identified in monogenic cases of obesity such as the genes encoding for the melanocortin 4 receptor (MC4R), proopiomelanocorticotropin (POMC) or the leptin receptor appear to be involved in the central regulation of energy intake. Variants of genes involved with energy utilization such as the ones encoding β-adrenergic receptors 2 and 3, hormone-sensitive lipase, and mitochondrial uncoupling proteins 1, 2, and 3 have also been associated with contributing to common obesity. Of the single gene defects which lead to obesity, MC4R defects are relatively common accounting for approximately 4% of cases of early onset childhood obesity. Large chromosomal deletions and also copy number variation have been linked to early onset obesity. Large deletions of chromosome 16p11.2 are for example found in about 0.7% of patients with morbid obesity (Fig. 2).

Syndromal Obesity

A number of syndromal disorders are associated with severe and mostly early onset obesity. Mostly, these syndromes include mild to moderate retardation and additional organ manifestations. Many are caused by alterations of neuroendocrine signaling circuits that regulate hunger and satiety either by disturbing ciliar function in CNS neurons like in Bardet–Biedel syndrome or interfering with hypothalamic function like in Prader–Willi syndrome. Table 3 gives an overview on the most frequently occurring forms of syndromal obesity.

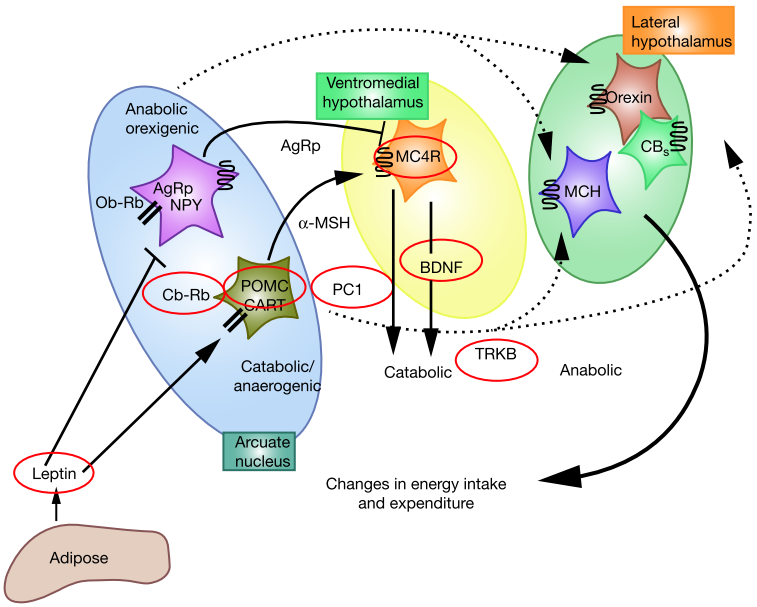

Fig. 2 Human mutations in molecules involved in weight regulation.

Table 3 Genetic syndromes that may be associated with childhood obesity

Albright hereditary osteodytrophy (pseudohypoparathyroidism type 1A)
Prader–Labhard–Willi syndrome
MOMO (macrocephaly, ocular, mental retardation, obesity) syndrome
Alstrom syndrome
Bardet–Biedl syndrome
Carpenter syndrome
Börjeson–Forssman–Lehmann syndrome
WAGR (Wilms tumor, aniridia, genitourinary anomaly, mental retardation) syndrome
Cohen syndrome
ROHHAD syndrome (rapid onset obesity, hypothalamic dysfunction, hypoventilation, and autonomic dysfunction)
Primary (simple/"exogenous") obesity
(multifactorial and multigenetic susceptibility)

Polygenetic

Multiple factors are related to the high incidence of childhood obesity. Both genetic/endogenous and environmental/exogenous factors contribute to the development of a high degree of body fatness early in life (**Tables 1** and **2**). In fact, twin studies suggest that at least 50% of the tendency toward obesity is inherited. There is also increasing evidence that responsiveness to dietary intervention is genetically determined. In fact, recent reviews on the genetics of body-weight regulation emphasize the contribution of the interaction between genes and environment to the development of obesity. Approximately 40%–70% of interindividual differences in body weight and composition is thought to be due to genetic variation. According to the thrifty gene hypothesis, evolutionary selection pressure has selected genes which allow individuals to survive periods of food deprivation. Within our modern obesogenic environment, however, these same genetic susceptibility traits now appear to be detrimental by promoting obesity and its associated metabolic and cardiovascular as well as related diseases (Körner *et al.*, 2008).

Less than 5% of cases of childhood obesity can be attributed to a specific cause be it an underlying endocrine disease, an obesity-related syndrome, a monogenic form of obesity or a specific damage to the hypothalamus. Exogenous factors such as overconsumption of fat-rich diets, the excessive use of modern media and in particular television viewing (**Table 5**) and lack of physical activity (sedentary life style) all heavily contribute to the development of obesity in childhood and adolescence as well as in adulthood. Nutrition and diet early in infancy is thought to influence growth rate and body fatness beyond infancy. Some authors have suggested that intrauterine growth retardation predisposes for the development of obesity and syndrome X later in life. This phenomenon has been termed "fetal programming." However, the mechanisms leading to fat patterning as a result to differences in fetal or early postnatal nutrition are still not completely understood. Intergenerational metabolic programming has also been discovered: for example, paternal diet will determine weight development in the off spring (Veldhuis *et al.*, 2005; von Kries *et al.*, 1999).

Neurons in the arcuate nucleus of the hypothalamus coordinate behavioral and autonomic functions which control energy homeostasis. Many genes that have been related to contribute to the etiology of either monogenic or polygenic obesity such as the genes encoding for the melanocortin 4 receptor (MC4R), proopiomelanocorticotropin (POMC), CART (cocaine- and amphetamine-related transcript), neuropeptide Y (NPY), agouti-related protein (ArRP) or the leptin receptor (ObR) or the single-minded 1 (SIM1), brain-derived neurotrophic factor (BDNF), TrkB or FTO gene and are expressed in the hypothalamus. They are involved in the central regulation of energy intake, satiety, hunger, and behavioral traits related to the control of food intake (**Fig. 2**).

Hypothalamic dysfunction has emerged as an important mechanism involved in the development of obesity and its comorbidities. In fact, hypothalamic obesity has been identified as a condition where obesity develops after damage to the hypothalamus for example after trauma or surgery or due to tumorous masses such as craniopharyngiomas. In addition, hypothalamic obesity is frequently a feature of serious diseases and disorders and in itself is very difficult to treat (Bereket *et al.*, 2012).

Selection of palatable meals, food intake, and satiety elucidate pleasure and positive emotional, hedonistic, signals. The endogenous opioid system in the brain is linked to positive and motivating emotional signals. Also, it has been found that the opioid system is involved in affective and stress responses. It is therefore positioned as a mediator between food intake, hedonic responses and their emotional regulation. It has been hypothesized that obesity, overeating and food-seeking behavior has indeed features of addiction and dependency. Findings suggest that the central opioid system not only relates to affective states and stress but also to chronic obesity and weight loss. Food intake is considered a readout of the hedonic, pleasurable pathway. When functional, the hedonic pathway will then help to reduce food intake in situations where energy stores are full. Dysfunction of this pathway may then increase food intake and lead to obesity (**Fig. 3**).

Adipose tissue develops as subcutaneous adipose tissue, visceral fat, and intra organ fat. Fetal adipose tissue development is regulated by a complex interaction of a number of transcription factors, many nutrients and signaling molecules, termed adipocytokines. Maternal, endocrine, and paracrine factors also influence specific changes in angiogenesis, adipogenesis, and metabolism. During embryogenesis and in fetal life, leptin and adiponectin, two important adipocytokines, are present at high concentrations in the circulation and in tissues. In obese subjects, adiponectin serum concentrations are low while leptin serum concentrations are elevated. Developmental stages and metabolic processes influenced by specific hormones and paracrine factors have been identified through examination of the offspring of obese and diabetic pregnancies, hormonal manipulation during late

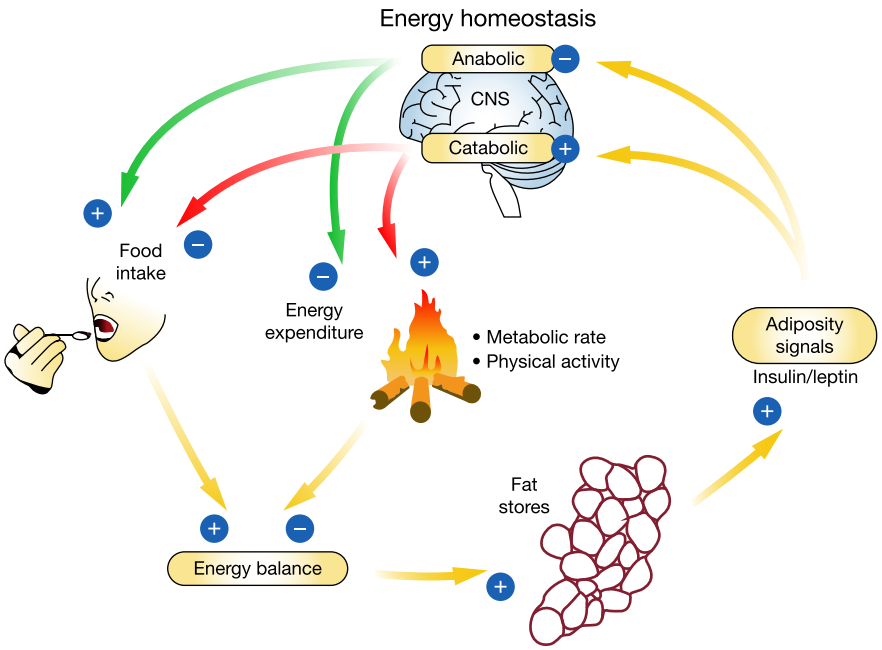

Fig. 3 Energy homeostasis. Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature. 2000, 404:661-71.

pregnancy in animal models, and the use of cell cultures. Collectively, the results of these studies delineate the basis for imprinting or conditioning of fetal preadipocytes at the paracrine/autocrine level, and of fetal adipose tissue development and metabolism.

Many signaling molecules and metabolites are released from brown adipose tissue (BAT) and some of them are even induced during brown adipocyte differentiation and/or thermogenic activation. Some of these factors might have an autocrine or paracrine role. BAT also secretes nonpeptide signaling molecules such as prostaglandins which may play a role in thyroid hormone function, differentiation of preadipocytes into beige adipocytes and synthesis of nitric oxide. Lack of BAT in obese individuals might then reduce energy expenditure and enhance energy storage in white adipose tissue.

Intestinal microbiota from obese rodents and humans differ from those from lean individuals. Acquisition of the microbiota in the gut begins at birth, and a stable microbial community develops from a succession of key organisms. Disruption of the microbiota during maturation by low-dose antibiotic exposure can alter host metabolism and adiposity (Gensollen *et al.*, 2016). For example, low-dose penicillin (LDP), delivered from birth, induces metabolic alterations and affects ileal expression of genes involved in immunity. LDP that is limited to early life transiently perturbs the microbiota, which is sufficient to induce sustained effects on body composition, indicating that microbiota interactions in infancy may be critical determinants of long-term host metabolic effects. In conclusion, there is early-life microbe-host metabolic interaction and this is consistently linked with metabolic alterations later in life (Falony *et al.*, 2016; Pedersen *et al.*, 2016).

In order to better understand the pathogenesis of comorbidities and also in order to find out what surrogate markers could be useful to differentiate between metabolically and cardiovascularly healthy from unhealthy obesity many a surrogate marker be it adipocytokines, lipoproteins, lipids or growth factors and a variety of hormones have been studied and subsequently were identified to be related to obesity or cause progression of the disease or its comorbidities. For example osteocalcin serum levels are inversely related to markers of body adiposity and leptin levels. Thus, a relation between obesity and bone health has been suggested. Also, the adipocyte appears to be a major site of extraovarian synthesis of estrogens. A recent longitudinal study noted that girls with greater BMI had lower serum estradiol levels at the onset of thelarche (onset of breast development) than those with BMI in the normal range. Previous studies have noted suppressed values of gonadotropins in obese girls. Together these findings suggest peripheral conversion of androgens into estrogens, through increased adiposity and greater aromatase activity.

Recently, it has been suggested that epigenetic modifications may be involved in both obesity and T2DM development. Epigenetics plays a pivotal role in the regulation of gene expression by the reversible modifications of chromatin structure without any changes in DNA sequence. Epigenetic modifications include DNA methylation, posttranslational histone modifications and miRNA interference. Many of the epigenetic changes associated with obesity affected genes are known to raise diabetes risk.

It has become obvious that the socio-cultural inheritance of body fat mass, weight, and weight control is also very largely contributing to the obesity epidemic that is obvious today. A quantitative analysis of the nature and extent of the person-to-person spread of obesity as a possible factor contributing to the obesity epidemic was for example carried out within a densely interconnected social network of 12,067 people repeatedly re-assessed from one as part of the Framingham Heart Study (1971–2003). Longitudinal screening revealed that weight gain in one person was associated with weight gain in his or her friends, siblings,

spouse, and neighbors. A person's chances of becoming obese increased by 57% if he or she had a friend who became obese in a given interval and by 40% among pairs of adult siblings, if one sibling became obese (Grow *et al.*, 2010).

In industrialized countries such as France, the United Kingdom, the United States, Germany, Australia, and Sweden the prevalence of obesity in children strongly relates to socio-demographic parameters: children of low income families and of families with low parental education are much more frequently overweight and obese than children from affluent and well educated parents. In low-income countries as well as in Brasil, India, China and the countries of the Arab peninsula, the reverse is seen: children from the high income part of the population are more frequently overweight and obese than children from the poor. In fact, in many countries in Africa and in South America starvation and poverty are related while high income and malnutrition and obesity are present at the same time and maybe even so in the same individual. Urbanization might also be a causative factor for the increase in obesity prevalence in Chinese children in the Shanghai area (Farpour-Lambert *et al.*, 2015; Zhang *et al.*, 2012).

Dietary Factors

Dietary factors that may facilitate the development of overweight and obesity early in age include total caloric intake, fat intake, protein intake, and fiber intake. In recent reviews, there was no consistent association of total caloric intake with overweight and obesity. Dietary protein was associated with earlier onset of weight accretion and of the pubertal growth spurt, age of peak height velocity, and menarche/voice break, and may be related to enhanced IGF secretion. Dietary fiber has also been recognized for its impact on cardiovascular disease, insulin resistance and diabetes, and several cancers. In respect to the endocrine system dietary fiber was noted to delay age of menarche and breast development, although other studies which documented lower overall intake of fiber did not note later menarche.

The automation of the process of extracting sugars in the 1900s reduced cost and increased availability of sugars leading to a dramatic rise in consumption, which reached a peak in the 1970s. There are different definitions for sugars not naturally available in foods, and free sugars is the term used by WHO. The epidemiological evidence of the associations between sugars and obesity and type 2 diabetes mellitus is fairly strong and consistent, particularly for sugar sweetened drinks in adults. However, the sugar beverage industries as well as sugar industries have been quite successful in leading the public to believe that sugar sweetened beverages are not much related to the obesity epidemic seen in parallel with the increasing consumption of sugar. However, it is believed that high fructose intake might accelerate weight gain and also directly cause nonalcoholic liver disease and hepatic fibrosis.

Physical activity plays an important role in the prevention of becoming overweight and obese in childhood and adolescence, and reducing the risk of obesity in adulthood. Puberty and the following adolescent period are acknowledged as particularly vulnerable times for the development of obesity due to, in many individuals, a concomitant reduction in physical activity. In many Western settings, a large proportion of children and adolescents do not meet recommended physical activity guidelines and, typically, those who are more physically active have lower levels of body fat than those who are less active. Low income families are more likely to not support sports activity and sports club membership of their off spring than are middle or high income parents.

Sedentary behavior is associated with health indicators; however, there are only few studies that have examined these associations, especially in conjunction with psychological factors, in children. Excess sedentary behavior relates into higher levels of behavioral/emotional problems and anxiety. However, a clear relation to weight gain and/or obesity and sedentary behavior has been difficult to prove.

Endocrine disrupting chemicals, EDCs, are frequently seen as obesogens: they represent several classes of chemicals which may impact on early weight gain, metabolic consequences later in life and timing of puberty through regulating peroxisome proliferator–activated receptor gamma (PPARγ) or through modulation of aromatase. These chemicals have been proposed to promote adipogenesis (adipocyte number and/or size) through modulating signaling pathways, or through stimulation of mesenchymal stem cells to preadipocytes, and maturation of preadipocytes into adipocytes (Garcia-Arevalo *et al.*, 2014).

Today's children and adolescents are immersed in both traditional and new forms of digital media. Research on traditional media, such as television, has identified health concerns and negative outcomes that correlate with the duration and content of viewing. For example, television viewing, daily duration of viewing, and particularly viewing during evenings after evening meals, are all associated with higher weights and the development of obesity even in young children. Over the past decade, the use of digital media, including interactive and social media, has grown, and research evidence suggests that these newer media offer both benefits and risks to the health of children and teenagers. Risks of such media include negative health effects on sleep, attention, and learning; a higher incidence of obesity and depression; exposure to inaccurate, inappropriate, or unsafe content and contacts; and compromised privacy and confidentiality (Lipek *et al.*, 2015; Swinburn *et al.*, 1999).

Epidemiology

Current and projected obesity rates (BMI $\geq 30\ \mathrm{kg\ m^{-2}}$) in the adult population of the United States are 20% for the year 2000, 30% for the year 2015 and over 40% for the year 2025. The Bogalusa heart study found that 22% of the children surveyed in 1990 had a body mass index greater than the 85th percentile established in a similar survey conducted in 1980. There was little change in the cohorts of children with a BMI less than the 50th percentile. In contrast, there was a large increase in BMI in the cohorts with a BMI greater than the 50th percentile. In summary, over time, obese children have a tendency toward even more excessive weight.

It has become clear that childhood obesity has reached epidemic proportions in all industrialized countries. The current age-adjusted prevalence may be as high as 20%–35% (Zhang *et al.*, 2012).

Interestingly, in industrialized countries such as in Sweden, France, Germany, and Australia, the prevalence of obesity in young children is plateauing or even declines, however, obesity prevalence in adolescents still increases throughout the world. There is indeed a global trend of stabilizing prevalence rates of childhood obesity at a high level in highly developed countries and add important information for individual age groups why the obesity epidemic in adults seems to even accelerate. Approximately 60%–85% of obese children of school age will stay obese in adulthood. The prevalence of overweight and obesity in children has increased continuously in developing and poor countries. Obesity is found in parallel with malnutrition and undernutrition in the same population in different social strata but in some countries even within the same social class (Sundblom *et al.*, 2008, Blüher *et al.*, 2011).

Comorbidities and Consequences

Sequelae of primary childhood obesity include hypertension, dyslipidemia, and psychosocial problems. A more complete list of comorbidity disorders is shown in **Table 3**. These disorders which arise from overweight and subsequent biochemical changes actually predispose to additional comorbidity such as cardiovascular disease in early adulthood. These comorbidities represent a major health burden in industrialized societies (Colditz, 1999). In addition, childhood obesity seems to increase the risk of subsequent morbidity whether or not obesity persists in adulthood. It is, therefore, mandatory to carefully examine all obese children in respect to the presence of comorbidity even at a young age. Such examinations should include blood pressure monitoring and checking of lipid status (**Table 4**). The opinion of orthopedic surgeons and child psychiatrists should be sought

Table 4 Comorbidity of obesity in childhood and adolescence

Psychosocial—psychiatric
Poor self-image
Social isolation
Autoaggression
Suicide
Promiscuity
Drug and alcohol addiction
Bulimia
Binge eating
Smoking
(Enuresis)
Cardiovascular and respiratory
Accelerated atherosclerosis
Hypertension
Hypoventilation
Sleep apnea
Snoring
Obstructive lung disease
Obstructive sleep apnea syndrome, Pickwickier syndrome
Reduced lung capacity
Endocrine, metabolic, and gynecological
Hyperinsulinemia
Insulin resistance
Early puberty
Polycystic ovaries
Dysmenorrhea
Dyslipidemia
Orthopedic
Slipped capital femoral epiphyses
Coxa vara
Blount's disease
Legg-Calve-Perthes disease
Back pain
Dermatological
Paronychia
Akanthosis nigricans
Striae rubrae

much more frequently than is being done so currently. Most importantly, type 2 diabetes, which until recently had been considered a disorder of the older population, is being increasingly recognized in children and adolescents especially in children of African–American and Hispanic ethnicity. In fact, during the last decade type 2 diabetes has been diagnosed with increasing frequency in adolescents and even young children. The clinical picture in these children and the fact that most affected patients come from families with type 2 diabetes mellitus have led physicians to conclude that affected children will respond to the same treatments used in adults and that clinical courses will be similar to those described in adults (Körner *et al.*, 2012).

The prevalence of the metabolic syndrome in obese children is reported to be 30%, irrespective of the definition applied. Insulin resistance may eventually lead to type 2 diabetes and therefore represents one of the most clinically relevant comorbidities of obesity. Hyperinsulinemia is found in approximately 30% of obese children and adolescents indicating insulin resistance and glucose intolerance. There is a strong relation between cardiorespiratory fitness and markers of insulin resistance. It is interesting to note that maintenance and/or improvement of cardiorespiratory fitness prevents the development of insulin resistance. Type 2 diabetes develops in obese children with Asian, Hispanic, and Afro American ethnic background, while in white subjects, type 2 diabetes in the obese usually only develops after age 10 years.

The formation of atherosclerosis starts at an early age. For this atherosclerosis forming process serum lipids are seen as crucial risk factors. The prevalence of dyslipidemia in children and adolescents from 0 to 16 years in Germany is 6%–22%. In a study from Brazil, which, however, included young adults aged 23–25 years only, a significant ($P < .05$) influence of the social status on total cholesterol, LDL and HDL cholesterol described. Higher HDL concentrations and lower triglyceride levels are usually recorded in children and adolescents with higher social status. Accordingly, children with lower social status would be exposed to a higher cardiovascular risk (Reich *et al.*, 2003; Körner *et al.*, 2007).

There is also a higher prevalence of orthopedic conditions such as joint and back pain in overweight and obese children than in lean subjects. Increased joint and back pain is commonly reported by overweight children, with decreases in physical activity. Overweight children typically display a slower, more tentative walking pattern with increased forces to the hip, knee, and ankle during "normal" gait. On the other hand, the association between obesity and various musculoskeletal disorders such as slipped capital femoral epiphysis and Blount disease is well reported. Recent evidence suggests an increased association between obesity and musculoskeletal pain and increased fracture risk due to decreased bone mineral content and impaired bone structure.

Nonalcoholic fatty liver disease, NAFLD, due to obesity is the most common form of liver disease in children age 2–19. Its prevalence has more than doubled over the past 20 years. The prevalence of NAFLD in children and adolescents reaches from 3% to 11% and is strongly influenced by age, sex, race, and ethnicity. In obese children, the prevalence ranges from 38% to 80%. The prevalence of NAFLD is highest in American Hispanics (45%), medium in Caucasians (33%), and lowest in African–American (24%). In Europe, Australia, and the Middle East the prevalence ranges from 20% to 30%, which is comparable to that reported from Japan, China, Latin America, and India (Calle *et al.*, 1999).

In 2004 the International Diabetes Federation (IDF) published a definition of the metabolic syndrome in adults which defined hypertension, dyslipidemia, glucose intolerance and abdominal obesity as constituents of the syndrome. This cluster of risk factors is related to the development of cardiovascular diseases and type 2 diabetes. Metabolic syndrome does not only occur in adults but has also been described in children and adolescents. The term metabolic syndrome is applied if a child has abdominal obesity, high blood pressure, abnormal blood lipids, and disturbed glucose/insulin metabolism (Weiss and Caprio, 2005).

An increased prevalence of urogenital infections including bacterial infections of the kidney and nonspecific cystitis as well as fungal infections of the preputium or vulva (vulvovaginitis; balanitis) has been suggested in obese subjects when compared to the prevalence in lean children. In addition, outcomes of renal disease are thought to be less favorable in obese and overweight children than in normal weight young subjects. Polycystic ovarian syndrome is characterized by ovarian cysts, very frequently obesity, and hyperandrogenism. Pseudoprecocious puberty is thought to be misdiagnosed in obese girls.

Many cross-sectional analyses, as well as longitudinal studies, have examined the association between markers of adipocity and pubertal development. In addition, the putative impact of an increased fat mass upon reproduction and fertility both in human obese males and in male animal models of obesity has been studied: a trend toward earlier pubertal development and maturation in obese individuals of both sexes has been shown, and the previously held notion that obese boys might progress to puberty at a slower pace than their nonobese peers can no longer be substantiated. Secondly, impaired fertility markers and reduced reproductive functions have been observed both in obese humans and in animal models of obesity. Low sperm counts and reduced sperm function have been reported in obese men (Wagner *et al.*, 2012).

It is hypothesized that the emergence of a "pandemic" of childhood obesity may have been a major driving force for earlier maturation in both genders. There is still much debate regarding the true impact of childhood obesity on testicular development in boys (Toppari and Juul, 2010) (**Table 5**).

Genetic variants may also directly account for a link between obesity and pubertal timing. Variations in LIN28B, a human ortholog of the gene-regulating processing of micro-RNAs was recently reported to be associated with timing of puberty in humans. Genetic variation in LIN28B was associated with earlier voice breaking and more advanced pubic hair development in boys. Many additional studies also indicate that the timing of pubertal onset is under strong genetic control (Aksglaede *et al.*, 2009) (**Table 5**).

It has become clear that there are a number of common psychological consequences associated with childhood obesity. A systematic search shows that overweight and obesity at a young age is negatively associated with psychological comorbidities, such as depression, poorer perceived lower scores on health-related quality of life, emotional and behavioral disorders, and self-esteem during childhood. However, evidence related to the association between attention-deficit/hyperactivity disorder (ADHD) and

Table 5 Hypothetical mechanisms of how obesity might affect onset and tempo of pubertal development and sexual functions

Common genes linking energy metabolism and puberty
Nutritional factors (protein, sugars)
Energy supply (calories)
Adipocytokines (signalling)
Insulin/insulin-like growth factor effects (i.e., on ovary)
Nicotinamide phosphoribosyltransferase (NAMPT), and other enzymes providing increased amounts of energy to gonads

obesity remains unconvincing because of contradictory findings from different studies. Overweight children are more likely to experience multiple associated psychosocial problems than their healthy-weight peers, which may be adversely influenced by obesity stigma, teasing, and bullying. Obesity stigma, teasing, and bullying are pervasive and can have serious consequences for emotional and physical health and performance especially in children and adolescents.

All individuals are subject to multiple risk factors for mortality. There are many interactions between certain major socio-demographic and behavioral risk factors associated with all-cause mortality. In principle, there are two forms of interaction between risk factors, additive and multiplicative relations. Usually, expectations about interactions among socio-demographic variables, and their relation to behavioral variables, have been stated in terms of additivity and/or of multiplicatively. Therefore, the nature of interactions among the five major risk factors associated with all-cause mortality: smoking, obesity, race, sex, and educational attainment have to be seen as a complex multifaceted interaction. Obesity has been found to be additive with each of the remaining four variables. These traits, established at birth or during childhood, literally result in deadly combinations. Adolescent BMI, including values within the currently accepted "normal" range, strongly predicts diabetes mortality up to the seventh decade. In light of the worldwide increase in childhood obesity, the association between body-mass index (BMI) in late adolescence and death from cardiovascular causes in adulthood has been investigated: Data on BMI, as measured from 1967 through 2010 in 2.3 million Israeli adolescents (mean age, 17.3 $\pm$ 0.4 years), were grouped according to age- and sex-specific percentiles from the United States Centers for Disease Control and Prevention. Primary outcomes were the number of deaths attributed to coronary heart disease, stroke, and sudden death from an unknown cause, or a combination of all three categories (total cardiovascular causes) by mid-2011. Hazard ratios in the obese group ($\geq$95th percentile for BMI), as compared with the reference group in the 5th to 24th percentiles, were 4.9 (95% confidence interval [CI], 3.9–6.1) for death from coronary heart disease, 2.6 (95% CI, 1.7–4.1) for death from stroke, 2.1 (95% CI, 1.5–2.9) for sudden death, and 3.5 (95% CI, 2.9–4.1) for death from total cardiovascular causes, after adjustment for sex, age, birth year, socio-demographic characteristics, and height. Thus, overweight and obesity are strongly associated with increased cardiovascular mortality in adulthood.

The financial burden of childhood obesity for industrialized societies can only be estimated. The annual economic costs due to medical expenses and lost income as a result of complications of adult obesity is approximately $70 billion in the United States. At least another $30 billion are thought to be spent on diet foods, products and programs to lose weight. If one is to calculate the prospective costs of obesity forms that have started at an early age, the prospective financial costs are even higher. On the other hand, it is almost too ironic to state that sales and profits of the obesity treatment industry have already reached an enormous sum. Therefore, obesity in childhood and adolescence has already become a major factor in health-care planning systems and within the health-care industry as such (Colditz, 1999; Cohen-Cole and Fletcher, 2008).

Treatments and Medical Management

Any metaanalysis on the efficacy of treatment of obesity and even more so on the prevention of overweight and obesity at a young age has failed to show large effects of treatment and/or prevention strategies. Barriers to participate and obstacles to access treatment and prevention programs are but one reason for the failure of therapeutic and preventive strategies in the long term. Very often families with an obese child will have little educational and socioeconomic resources and even will sometimes completely fail to understand the importance and or relevance of treating childhood obesity. In addition, very often the affected child will have obese family members whose obesogenic environment and lifestyle will ultimately influence the child's environment and lifestyle. Since in many cases the comorbidities of obesity are not felt at a young age, there is little individual and personal incentive to change habits and or unhealthy lifestyles. Lastly, if one is to live with obese peers, the motivation to lose weight or change one's unhealthy behavior will be very limited indeed (Alff *et al.*, 2012).

Therapeutic strategies have so far included psychological and family therapy interventions, lifestyle/behavior modification and nutrition education (**Table 5**). The role of regular exercise and exercise programs is emphasized. Intermittent exercise (high intensity followed by low intensity sports) may result in greater reduction in weight and body fat mass. Such approaches also increase compliance/adherence rates of the youths. Multidisciplinary outpatient treatments are considered to be the most effective treatment strategy (Ebbeling *et al.*, 2002).

Repeated dieting with intermediate weight loss and weight gain and even with cycling of body weight does not lead to solid weight loss and sustained weight maintenance but might even be harmful both in respect to higher weight in the end and increasing the likelihood of comorbidities even further. Reducing high fructose and high sucrose intake does indeed maintain a healthy body weight. Reducing the consumption of sugar-sweetened soft drinks is thought to add to any obesity treatment concept. Snacking has been found to be associated with the development of obesity both in the individual and in societies in general. Therefore, to organize food intake at infant and school age in a way that breakfast, lunch and dinner are eaten together in the family or community setting and be there only a small snack be it fruit or vegetable in the morning and one in the afternoon helps to structure the day.

No single behavioral or environmental intervention for the obese child can possibly be effective on its own. There have been numerous attempts to identify the optimal psychological intervention and namely behavioral modification techniques to treat obesity even at a young age. Taken into account that no behavioral therapy can intervene with the environmental, societal, socio-demographic, genetic, and biologic causes of childhood obesity it is not surprising that all such attempts have been proven quite unsuccessful. In addition, barriers that prevent families and obese children to participate in behavioral and also multidisciplinary treatment programs are still not fully identified nor understood.

In Cochrane reviews by which putative positive factors from which one might extract effective means to treat childhood obesity have been identified, it has become apparent that approaches targeting both the family and the community setting are the most promising strategies for treating and preventing childhood obesity. It seems that the individual needs peer or family support to change a potentially obesogenic lifestyle and adhere to a healthy way of living. Mixed and multi professional and interdisciplinary approaches to treat childhood obesity are cumbersome to carry out, costly and only marginally successful: in the long term success is usually small with changes in BMI SDS of not more than—0.2 SDS being seen in most studies (Walther *et al.* 2009).

Bariatric surgery has become an effective option as a last line treatment for severe obesity in adults. In the United States bariatric interventions are now the most commonly undertaken surgical procedures. In adolescents and young adults much less data is available as to safety and effectiveness of bariatric surgery and whether or not such procedures do add to treatment options for this age group. Weight and BMI reduction are uniformly lower for gastric banding as compared to gastric bypass or sleeve gastrectomy. Large scale long-term observations are still missing and this prohibits a final conclusion about lasting effectiveness and safety at the present time. Laparoscopic adjustable gastric banding is being increasingly considered to be the treatment of choice in very obese adults. Early complications of such interventions and significant late complications such as pouch dilatation and stomach slippage have been reported. Whether or not such invasive treatment options will ultimately be considered in children is still open to debate (Lennerz *et al.*, 2014).

Long-term treatment of childhood obesity probably also including extended pharmacotherapy may be necessary for the majority of very obese adolescents. **Table 6** lists some drugs used in obesity management in adults. At the present time, two of these, orlistat and sibutramine, are increasingly being used in obese adults. Orlistat binds to gastrointestinal lipases and causes a partial inhibition of fat reabsorption from the gut. In contrast, sibutramine causes a centrally mediated increase in satiety and energy expenditure. It is of great concern that some of these drugs are being found to be prescribed to youngsters by primary care physicians upon parental request (own observations) outside any scientific follow-up studies and without careful and systematic follow-up (Heymsfield *et al.*, 1999).

Children with type 2 diabetes who are acutely ill are treated with insulin. Some of these children will have to b transitioned to oral antidiabetic agents. Safety and efficacy of these have not been established for children and adolescents. The only drug currently approved for therapy of children with diabetes is insulin. Even less is known about therapy in children with comorbid conditions which frequently accompany type 2 diabetes mellitus. There are no guidelines for what therapy to use or when to

Table 6 Drugs that could potentially be used in obesity management in children and adolescents

Drugs approved for the use in adult obesity in some countries at some time:
Sibutramine
Phentermine
Mazindol
Diethylpropion
Orlistat
Drugs under development:
Leptin and leptin analogues
Brain and gut peptide agonists or antagonists
MC4-receptor peptide agonists
NPY-Y1 or—Y5 antagonists
Galanin receptor antagonists
Orexin receptor antagonists
Alpha1-receptor agonists
Beta2-receptor agonists

(*Note*: most of these are not recommended/licensed for use in children!).

employ it for such important states as hyperlipidemia and hypertension. Very recently, a multicenter trial of metformin use in children with type 2 diabetes mellitus has been completed in the United States.

Prevention

There are at least three reasons to focus not only on individual characteristics when planning or implementing interventions against obesity.

(1) Complex problems need complex solutions. The reasons for obesity are multidimensional. Consequently, preventing or treating obesity needs multidimensional strategies focusing on different levels of influence (individual, familial, institutional, and environmental).
(2) Environmental changes are possible, effective, and sustainable. Individual or family characteristics such as socio-economic position, family habits, and health behavior are difficult to change on a long term by person-based educational programs. Changes at the environmental level (e.g., changes in food supply in schools, more physical activity in school—during lessons or breaks) are relatively easy to apply but have the potential to change individual behaviors as well as attitudes sustainably.
(3) Environmental or community approaches may reach those who are really in need. "Traditional" interventions that focus on individual behaviors have failed to reach those population groups that are at the highest risk (individuals with low socio-economic status). Interventions that promote environmental changes have the potential to influence those who are "hard to reach" (Lipek *et al.*, 2015).

As prevention has to start very early in life and perhaps even before postnatal life, a population and community approach for prevention seems to be the most promising and reasonable. Primary prevention has proven to be difficult or impossible in most societies at this point in time. Again, a multidisciplinary team approach is asked for to develop and secure preventive strategies. Good nutrition and modest exercise for pregnant women as well as monitoring of intrauterine growth of the child are mandatory. After birth, rapid weight gain should be avoided and principles of good nutrition and physical activities should be taught at all ages. Breast-feeding should be strongly recommended. Children's food choice can be influenced by early intervention and guidance. Parents should be encouraged to make healthy foods easily available to the child and serve these foods in positive mealtime situations in order to help their child to develop healthy food habits. Joint actions by physicians, health authorities and politicians both in the community and also using modern media and mass media are being asked for to implement nation-wide prevention programs. Such programs have to take into account cultural and racial preferences and attitudes in respect to food preparation and eating habits. Most importantly, food industries have to take up their responsibilities and should be made to stop marketing unhealthy foods and advertising to children and adolescents (Ludwig *et al.*, 2011)!

Obesogenic environments are "the sum of influences that the surroundings, opportunities, or conditions of life have on promoting obesity in individuals or populations." They comprise a resident's aggregate socioeconomic status (SES) as well as aspects of their social and physical environments. Evidence suggests that children living in low SES areas are more often overweight or obese. Regarding the physical environment, three domains that may influence obesity have been identified: (1) facilities for physical activity (i.e., parks, playgrounds, sports clubs that promote active play, and sports); (2) land use and transportation (i.e., mixed land use, walkability, access to public transport or walking/cycling paths that facilitate active commuting to school/work); and (3) foodscape (availability of healthy or unhealthy food). In this sense it was reported that fewer resources for recreation (e.g., parks, playgrounds), sports or active commuting (street connectivity, land-use-mix) and a high density of fast food outlets are related to overweight and obesity in children and adolescents. Summing up, the development of overweight and obesity during childhood is a result of complex mechanisms at different levels of influence (individual, social, environmental) (**Fig. 1**). There are various theoretical environmental factors that can be targeted for prevention strategies (Lipek *et al.*, 2015).

The potential role of intestinal microbiota in the etiology of many human diseases has attracted a lot of attention. The intestinal microbiota has been suggested to be an important factor for the development of obesity and obesity-related metabolic dysfunction. The distal human intestine represents an anaerobic bioreactor programmed with an enormous population of bacteria, dominated by relatively few strains that are very diverse at the strain/subspecies level. It is now partially understood how certain members of the microbiota function as to maintain the stability and functional adaptability of the microbial organ. This might suggest that microbiome-targeting approaches may help to prevent and even treat obesity in early life.

Access to playgrounds, restrictions on food marketing for children, low prizing of healthy food stuffs and changes in food supplies in cafeterias, walkability of living quarters and the planning of cities altogether all have to be considered if one is to seriously prevent childhood obesity on a large scale. Green areas should be interspersed in living quarters and free access to pedestrian zones should be ensured. There is a large quantity of research that indicates how cities should be developed to support healthy lifestyles especially in the young. However, since in most countries there is no legislation that secures such developments most city dwellings are developed following financial incentives of investors rather than scientific reasoning (Huang *et al.*, 2015).

In some countries advertisements targeting children and youngsters is already forbidden and may be punished by law. However, there is still food advertising targeting children in many areas of the world. In many instances large sums are invested by food industries to invent the most clever and attractive way to seduce children. Sugar sweetened beverages are but one example how children are falsely educated to consume too many calories and too many carbohydrates. In addition, food industries are

good at diluting the evidence that indeed sugar-rich beverages and foods are not healthy and contribute to the obesity epidemic by placing advertisements in physician's journals and many other media. Bans of advertising for tobacco have shown the way to go for the food industry: after tobacco advertising was banned on a large scale and cigarettes were taxed heavily both the number of smokers and the prevalence of lung cancer have declined dramatically. Taxes on unhealthy foods be it fat- or sugar-rich have been implemented in Denmark and the United Kingdom (Livingstone *et al.*, 2006).

Conclusions

The amount and nature of adipose tissue of a child at which morbidity acutely and/or later in life increases is determined on an actuarial basis. Direct measurements of body fat, for example hydrodensitometry, bioimpedance, or DEXA, is only useful in a research context. In contrast, body mass index (BMI) is easy to calculate and is widely accepted to define obesity in children and adolescents clinically and for practical purposes. An increased risk of death from cardiovascular disease in adults has been found in subjects whose BMI had been greater than the 75th percentile as adolescents. Childhood obesity seems to also substantially increase the risk of subsequent morbidity whether or not obesity persists into adulthood. The genetic basis of childhood obesity has been elucidated to a large extent through the discovery of leptin, and the increasing knowledge on the role of neuropeptides such as POMC, neuropeptide Y (NPY) and the melanocyte concentrating hormone receptors (for example, MC4R) and the discovery of FTO as an obesity risk allele. However, environmental/exogenous factors largely contribute to the development of a high degree of body fatness early in life. Twin studies suggest that approximately 50% of the tendency toward obesity is inherited while 50% are related to socioeconomic and life style factors. There are numerous disorders including a number of endocrine disorders (Cushing syndrome, hypothyroidism, etc.) and genetic syndromes (Prader–Labhard–Willi syndrome, Cohen and Alström syndrome, Bardet–Biedl syndrome, etc.) that are associated with an increased body fat mass. Usually, a simple clinical diagnostic algorithm allows for the differentiation between primary or secondary obesity. Among the most common sequelae of primary childhood obesity are hypertension, dyslipidemia, impaired insulin sensitivity, back pain and psychosocial problems such as behavior problems, exclusion from social participation and attention deficit hyperactivity and depression. Therapeutic strategies include psychological and family therapy, lifestyle/behavior modification and nutrition education. The role of regular exercise and exercise programs has to be emphasized. Surgical procedures and drugs used in adult obesity are still not generally recommended in children and adolescents with obesity. As obesity is the most common chronic disorder in industrialized societies, its impact on individual lives as well as on health economics has to be recognized more widely. Finally, one should aim to increase public awareness of the ever increasing health burden and economic dimension of the childhood obesity epidemic that is present around the globe. It is clear that we have to strengthen our efforts in respect to investigate prevention and interventions that will work both on an individual and a societal level. The questions that are to be asked are the questions that need to be answered in order to improve quality of life of patients, reduce suffering from the disease, and increase the survival of patients and importantly to increase our chances to successfully prevent obesity at an early age.

It is now well known that obesity is the most common chronic disorder in industrialized societies. In some countries, the prevalence of obesity in childhood and adolescence has become higher than that of the allergic disorders including both asthma and eczema. As has been said, childhood obesity is associated with substantial comorbidity and late sequelae. While diagnostic strategies are clear and straight forward, treatment remains difficult and frustrating both for the patient, family and the multi-disciplinary team caring for children with obesity. In conclusion, much more attention should be given to prevention and the development of preventive strategies at all ages. Prevention should, in any case, start very early in life possibly during pregnancy and fetal life. New drugs are being developed that promise to be useful for treatment and secondary prevention. However, no data are available for the use of such agents in childhood and adolescence. This notion also applies to the treatment of type 2 diabetes mellitus and comorbid conditions that frequently accompany type 2 diabetes and/or obesity in children and adolescents. Finally, public awareness of the ever increasing health burden and economic dimension of the childhood obesity epidemic has to be asked for and also has to be insisted on repeatedly and constantly. Political measures have to be taken to ensure and enable healthy lifestyles at an early age and to combat the financial interest of industries that may harm children's health.

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Further Reading

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Appetite and Weight

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Introduction

In 2016, worldwide there were over 340 million children and adolescents aged 5–19 with overweight or obesity (Ezzati *et al.*, 2017; WHO, 2017). In the same year, the number of infants and young children (aged 0–5 years) with overweight or obesity reached 41 million globally (WHO, 2017). If current trends continue, the figure is expected to reach 70 million by 2025. Overweight and obesity in childhood tend to persist into adulthood (Simmonds *et al.*, 2016), putting those children at greater risk of developing related chronic diseases later in life (Abdullah *et al.*, 2011; Lobstein *et al.*, 2004; Must *et al.*, 1992; Park *et al.*, 2012). Understanding the mechanisms behind the etiology of obesity in early life is therefore essential.

The rapid increases in population weight observed over recent decades have been widely attributed to changes in lifestyle. The modern environment is often referred to as "obesogenic," because it encourages excessive weight gain through offering readily available low-cost energy-dense and palatable foods, discouraging physical activity, and promoting sedentary behavior. For many people the modern food and activity environment leads to positive energy imbalance (more energy is consumed than is expended) and, consequently, weight gain and obesity. However, this is not the outcome for everyone; some people are more susceptible to the "obesogenic" effects of the environment than others. In fact, body weight varies enormously in the population, and stark differences can often be seen even between siblings growing up in the same household. The source of variation in susceptibility to obesity in a seemingly pervasive "obesogenic" environment has been of great interest to researchers. Studying individual differences in body weight can shed light not only on the causes of obesity, but also on the key factors that bestow resilience to it.

We know from nearly a century of twin and family studies that variation in human body weight has a strong genetic basis, with 50%–90% of population variation being attributable to genetic differences between people (Elks *et al.*, 2012; Maes *et al.*, 1997). In fact, body mass index (BMI) is as heritable now – following the obesity epidemic – as it was prior to its onset (Rokholm *et al.*, 2011). What is more, since the advent of genome-wide association studies at the turn of the 21st century, more than a hundred common genetic variants (in the form of single nucleotide polymorphisms, SNPs) have been identified that are robustly associated with variation in BMI (Locke *et al.*, 2015). Our genetic endowment explains *why* some of us develop obesity, and others do not, while all seemingly living in the same "obesogenic" environment. But the question remained, *how* do genes "determine" our weight?

The Behavioral Susceptibility Theory of obesity (BST) hypothesizes that genes influence weight through appetitive mechanisms. In short, it hypothesizes that inherited differences in appetite cause people to respond differently to the opportunities to eat offered by the modern food environment (Llewellyn and Wardle, 2015). At the heart of this theory is the idea that there are large and measurable individual differences in appetite that confer greater or lower risk of obesity, and that appetite is genetically determined. BST can help to explain how weight can be *both* genetic and environmental at the same time; it proposes that obesity arises from a combination of genetic susceptibility to overeating, and exposure to an "obesogenic" environment that both encourages and permits positive energy imbalance. Evidence for the validity of Behavioural Susceptibility Theory has come from studies demonstrating: (i) large individual differences in appetite from early in life; (ii) appetite prospectively predicting early weight gain; and (iii) strong genetic influence on appetite from early life. This chapter describes the evidence base for Behavioural Susceptibility Theory, and its implications.

Appetite and Weight in Adults

The role of appetite in obesity has been studied for well over half a century, but early research focused largely on adults because, until recently, obesity was rare in children. During the 1960s an innovative scientist called Stanley Schachter ran a series of imaginative laboratory-based experiments in which he compared the eating behavior of obese and healthy weight adults (Schachter, 1968, 1971). He observed that participants with obesity were much more responsive to the external cues of palatable food (such as the sight, smell or taste) than healthy-weight participants, insofar as they would eat considerably more of the foods that were good tasting and attractive, than lean controls. He also observed that participants with obesity were less responsive to their internal sensations of satiety, failing to demonstrate the same level of compensation as their healthy-weight peers when presented with a meal that had been preceded by an energy-dense snack (called a "preload"). This indicated adults with obesity were either less sensitive to their internal feelings of fullness, or were overriding them and continuing to eat when the food on offer was highly palatable. On the basis of his observations Schachter developed "externality theory." He proposed that there are two key aspects of appetite regulation that vary greatly between individuals, and play a key role in susceptibility to obesity: food responsiveness – wanting to eat (or eat more) in response to the sight, smell and taste of palatable food; and satiety

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sensitivity – the amount of food needed to provide a satisfying level of fullness. Arguably, Schachter's "externality theory" of obesity is of even greater salience in the modern obesogenic environment. The abundance of low-cost, energy-dense and palatable foods places food responsive individuals at high risk of overeating and weight gain, especially if they are not protected by strong satiety sensitivity.

Schachter was certain that there was a biological basis to obesity. He hypothesized that these aberrations in appetite regulation were likely to be caused by problems in the central control of appetite, regulated primarily by the ventromedial hypothalamus. Externality theory therefore proposed that obesity was somewhat innate, hardwired, and rooted firmly in biology.

At around the same time that Schachter was developing "externality theory," Kaplan and Kaplan were developing the Psychosomatic Theory of obesity, which focused on the psychological or emotional drivers of eating. They proposed that some individuals overeat to reduce anxiety, and it is this "emotional eating" that is responsible for the hyperphagia (excessive appetite) that causes obesity (Kaplan and Kaplan, 1957). They suggested that individuals with obesity are not able to discriminate between the physiological arousal caused by negative emotional states (e.g. feeling anxious, stressed or depressed), and hunger; which results in eating in response to emotion in the absence of physiological hunger. The central idea was that emotional eating may be the result of classical conditioning in early life – if negative emotions are always paired with consumption of palatable food (for example, if parents feed their child in order to soothe them, so-called emotional feeding), a classically-conditioned hunger response will eventually develop in the context of negative emotion. Schachter had also proposed that obese adults eat more than healthy weight controls under conditions of stress, but his theory was different (Schachter *et al.*, 1968). He suggested that healthy weight individuals eat *less* when anxious, which is the normal biological response to stress. On the other hand, the appetites of adults with obesity were abnormal in not being obliterated by negative emotion. Either way, both theories hypothesized that differences in food intake between obese and normal weight adults would be more marked in states of heightened emotional arousal, and highlighted emotion as a regulator of eating.

Early research set the stage by highlighting a great deal of variation in three key features of appetite – food responsiveness, satiety sensitivity and emotional eating. As interest in these appetitive traits began to grow, self-report psychometric measures were developed, which allowed researchers to measure different eating styles in very large numbers of participants, using a standardized tool. The first questionnaires to be developed were the "Three Factor Eating Questionnaire" (TFEQ) (Stunkard and Messick, 1985), and the "Dutch Eating Behaviour Questionnaire" (DEBQ) (Vanstrien *et al.*, 1986); the Adult Eating Behaviour Questionnaire (AEBQ) was developed very recently (Hunot *et al.*, 2016). Aside from the cost benefit of questionnaire measures over traditional laboratory-based testing, they also enable habitual eating behavior to be characterized across many contexts, as well as over time. While the objectivity and detail offered by direct observation is compromised, psychometric measures arguably capture enduring appetitive "traits" rather than the more transitory "states" of hunger or satiety manifested during an experimental situation. Decades of subsequent research with much larger samples of adults supported the early laboratory-based findings that individuals who are more food responsive and who have a tendency to overeat in response to emotion tend to carry more body fat (French *et al.*, 2012). Satiety responsiveness has been under-researched among adults in comparison, because the older questionnaires did not measure it. However, the newly developed AEBQ has shown that adults with weaker satiety have a higher BMI (Hunot *et al.*, 2016; Mallan *et al.*, 2017).

As the appetite model of obesity gained traction, researchers became increasingly interested in establishing the cause-effect relationship between appetite and weight. However, the vast majority of adult studies were cross-sectional, partly because obesity often develops before adulthood and is usually well established by middle- and older age; making it difficult to design studies that capture the transition from healthy weight to obesity during adult life. Cross-sectional studies cannot determine if differences in appetite are the cause of obesity, or if obesity itself causes abnormalities in appetite. Further complications arise when studying adult participants with longstanding obesity, which can cause abnormalities in the biology controlling appetite regulation (such as resistance to the effects of the satiety hormone leptin). Added to this is the fact that overweight adults are often dieting, and so may have altered their eating behavior in order to control their weight. A more insightful approach to understanding the relationship between appetite and weight is to measure them in children. The added benefit of studying younger children, in particular, is that they are too young to have had longstanding obesity with all of its complications, and they are less likely to be dieting.

Appetite and Weight in Children

Historically, the greatest concern in relation to pediatric weight was not obesity, but weight faltering (insufficient weight gain over a period of months) and underweight. As such, the few early studies of child appetite focused on characterizing the eating behaviors of those who were "failing to thrive" (Drewett *et al.*, 2003; Garcia *et al.*, 1990; Parkinson *et al.*, 2004; Wright *et al.*, 2000). The key behavioral features were poor appetite (lack of hunger), a slow eating speed, and pickiness (being highly selective about the foods one is willing to eat). However, the emergence of the childhood obesity crisis in the 21st century turned the spotlight on the role of appetite in the development of pediatric *overweight*. In 2001, Professor Jane Wardle developed the first comprehensive psychometric measure of children's appetitive traits, with the aim of characterizing the eating behaviors of children from across the weight spectrum – from underweight to obese. The Child Eating Behaviour Questionnaire (CEBQ) (Wardle *et al.*, 2001) enabled a broad range of appetitive traits to be measured reliably in large samples of children, providing the opportunity to discover if appetite was implicated in the development of both under- and overweight as they start to emerge. As children are unable to report

on their own eating behavior, the CEBQ is completed by parents. Parent-report is necessarily subjective and vulnerable to bias, but the CEBQ has been successfully validated against objective measures of children's eating behavior (Carnell and Wardle, 2007).

Wardle was interested in finding out if children with obesity demonstrated the same distinctive eating styles that Schachter observed among obese adults, and fewer of the behaviors that characterized children with weight faltering. Seven food-related appetitive traits can be broadly grouped into those hypothesized to play a causal role in overweight - "food approach" behaviors - and those thought to protect against overweight - "food avoidant" behaviors. The "food approach" behaviors include two scales measuring responsivity to food cues; "food responsiveness" is the tendency to want to eat in response to palatable foods (e.g. "even if my child is full up, s/he finds room for his/her favourite food"), and "enjoyment of food" is the pleasure a child gains from eating (e.g. "my child enjoys eating"). A third "food approach" scale measures the tendency to overeat in response to negative emotions "emotional overeating" (e.g. "my child eats more when worried"). Higher scores on these scales indicate a more avid appetite.

The "food avoidant" behaviors include two measures of responsivity to internal satiety cues; "satiety responsiveness" measures a child's response to feelings of fullness (e.g. "my child gets full before his/her meal is finished"), and "slowness in eating" captures eating speed under the premise that slower eating allows time for biological satiety mechanisms to take effect (e.g. "my child takes more than 30 min to finish a meal"). A third scale measures "emotional undereating," which is the tendency to undereat (as opposed to overeat) in response to negative emotions (e.g. "my child eats less when upset"). Higher scores on these scales are considered indicative of better appetite control, and very high scores indicate a poorer appetite. The "food fussiness" scale measures a child's tendency to be highly selective about the foods he or she is willing to eat, capturing both refusal of unfamiliar foods (termed "neophobia," e.g. "my child refuses new foods at first") and rejection of foods based on properties such as texture and taste, regardless of their familiarity (e.g. "my child is difficult to please with meals"). Because fussiness is a common characteristic among children who fail to thrive and may offer some protection against overweight (de Barse *et al.*, 2015; Drewett *et al.*, 2003; Dubois *et al.*, 2007), it tends to be grouped with the other "food avoidant" behaviors.

The first large-scale population-based study of appetite and adiposity using the CEBQ was conducted in 10,364 children aged eight to 11 years old from Twins Early Development Study (TEDS) (Carnell and Wardle, 2008). This study showed that children with a higher BMI or waist circumference scored higher on the "food approach traits" and lower on the "food avoidance" traits. Children with a lower BMI or smaller waist circumference showed the reverse pattern. Importantly, these appetitive traits were related to differences in bodyweight across the *whole adiposity spectrum* – not only did these appetitive traits distinguish obese, healthy weight and underweight children, but they explained smaller differences in weight among children within the healthy range. Crucially, the obese and underweight children weren't behaving in odd or unusual ways; they simply had much larger and poorer appetites than the children who were of a healthy weight. The CEBQ has been translated into multiple languages and has been widely used to study the appetites of many thousands of children across several continents (including Europe, North America, South America, Australia and Asia) (Demir and Bektas, 2017; Domoff *et al.*, 2015; Jansen *et al.*, 2012; Mallan *et al.*, 2013; Nasreddine *et al.*, 2017; Sanchez *et al.*, 2016; Spence *et al.*, 2011; Steinsbekk and Wichstrom, 2015; Tay *et al.*, 2016). A large body of cross-sectional research has now accumulated, supporting the hypothesis that children with a more avid appetite carry more body fat, and are more likely to be overweight or obese, while those with poorer appetites carry less body fat and are more likely to be underweight.

Research has also shown that appetitive traits are relatively stable from early to late childhood, although appetite regulation generally worsens with age for all children. In 428 children from the TEDS cohort, the CEBQ tracked strongly from 4 to 11 years of age, indicating continuity across time at the level of the individual (Ashcroft *et al.*, 2008). This suggests that appetitive traits are already firmly established by 4 years of age, raising questions about their origin – nature or nurture? At the same time, scores for the "food approach" traits increased with age, and those for the "food avoidance" traits decreased, such that children on the whole became more appetitive – more food responsive, enjoyed food more, emotionally overate more, ate faster, became less satiety sensitive and were less likely to emotionally undereat. This pattern of change suggests that children are at increasing risk of overeating and weight gain as they get older. This may reflect increasing autonomy to express their individual appetite. For example, a child will not be able to show a tendency to overeat highly palatable foods until they gain the independence necessary to do so. This trait may therefore be less salient among young children whose access to these foods is controlled largely by their parents.

Like the early studies in adults, research with the CEBQ highlighted that children vary considerably in their appetite, and that individual differences in appetite help to explain why some children are overweight, and others are underweight. However, the cause–effect relationship was still unclear; appetite can cause differences in early weight gain, but a child's appetite might also reflect their varying energy needs that are programmed by their growth. Prospective designs are needed to show that variation in appetite precedes the development of obesity over time, rather than the other way around. In addition, very little was known about the appetites of infants. Given that rapid infant weight gain is one of the most important risk factors for child and adult obesity (Matthews *et al.*, 2017; Ong *et al.*, 2000), understanding the role of appetite in early postnatal growth was of great interest to researchers. Prospective data *from birth* was therefore needed to shed light on the relationship between appetite and weight as it first starts to emerge in early postnatal life.

Appetite and Weight in Infancy

In 2007, Wardle and colleagues established Gemini (van Jaarsveld *et al.*, 2010), a large population-based prospective birth cohort of 2402 British families with twins. Gemini is the largest study ever undertaken into the origins of infant appetite and the role that appetite plays in early weight gain. The weights and heights of up to 4800 twin children have been collected every 3 months since the study began, making it one of the richest growth datasets in the world. The appetites of the twins were measured during the first few weeks of life, and again when they were 16 months and 5 years of age. Detailed records of their food intake were also collected over 3 days when they were 21 months old, in order to characterize their "everyday" eating patterns; this represents the largest contemporary dietary dataset for toddlers in the United Kingdom.

In order to study appetite in the period before solid food is introduced, the CEBQ was adapted so parents could report on their infant's milk feeding behavior. The Baby Eating Behaviour Questionnaire (BEBQ) (Llewellyn *et al.*, 2011) captures four different aspects of infant appetite during the period of exclusive milk feeding, which correspond to dimensions of children's appetite: "food responsiveness"; "enjoyment of food"; "satiety responsiveness"; and "slowness in eating."

Gemini was the first study to examine the bidirectional, prospective relationships between appetite and weight, and explored the associations from 3 to 15 months in a subsample of 2213 infants. The key finding was that the association between BEBQ-measured appetite at 3 months and subsequent weight gain (from 3 to 15 months) was stronger than the reverse association between weight at 3 months and subsequent change in appetite (from 3 to 15 months) (van Jaarsveld *et al.*, 2011). This strongly supported the hypothesis that variation in appetite drives early weight, not the other way around. However, the possibility of residual confounding in samples of unrelated individuals means that other unmeasured factors could in fact be responsible for the observed association.

Comparing the weight gain in twins discordant for early appetite provides a more stringent test of causality than studying appetite and weight gain in unrelated children, because all environmental factors shared completely by twin pairs (e.g. maternal weight, maternal diet, ethnicity, and socioeconomic status) are controlled for. A second study used this approach in Gemini to strengthen the hypothesis that differences in early appetite cause differences in early growth. The growth trajectories from birth to 15 months of age were compared for same-sex twin pairs ($n = 172$) who were considerably discordant for appetite (by at least 1 SD) at 3 months of age (van Jaarsveld *et al.*, 2014). The twins with more avid appetites (characterized by higher food responsiveness and lower satiety responsiveness) grew significantly faster than their co-twins with poorer appetites. By the time they were 15 months old there was a 1 kg difference in weight between twin pairs, which equated to a substantial 10% difference in body weight. A subsequent Singaporean study of 210 singleton infants replicated these findings, demonstrating that infants with a more avid appetite, characterized using the BEBQ, grew more rapidly over the first 2 years of life (Quah *et al.*, 2015).

Together these studies offer convincing evidence that variation in infant appetite plays a causal role in early weight gain, providing strong support for Behavioural Susceptibility Theory. However, the question of *how* appetitive traits lead to overeating, and therefore excessive weight gain remained. This is important for intervention development. It is clear that children with a more avid appetite consume more energy, and those with a poorer appetite consume less, but the "everyday" behavioral expressions of food approach and food avoidance traits were largely unknown. The dietary data collected when the Gemini twins were 21 months old was used to characterize the eating patterns of toddlers who varied in measures of food responsiveness and satiety sensitivity at 16 months (Syrad *et al.*, 2016b). These two appetitive traits were characterized by distinct patterns of overeating. Children who were more food responsive ate *more frequently* throughout the day (i.e. they had more eating occasions). The most food responsive toddlers consumed three extra meals per week, compared to the least food responsive; adding up to 540 extra calories per week, or 2340 extra calories per month (nearly two and a half extra days of food intake). In contrast, children with blunted satiety sensitivity did not eat more frequently but instead ate *larger portions* of food every time they ate. Compared to the most satiety sensitive children, those with the lowest scores consumed 37 cal more at each meal; this equated to 185 extra calories per day, 1295 extra calories per week, or 5627 cal per month (the equivalent of five and a half extra days of food intake). In the current "obesogenic" environment it makes sense that children who are highly responsive to the sight or smell of foods will eat more often in response to the food cues they inevitably encounter throughout the day. At the same time, it is perhaps unsurprising that children with weakened sensitivity to feelings of fullness will eat larger portions of food given the opportunity; because they take longer to feel sated, or require a larger amount of food. A follow up study demonstrated that average meal size was a more important driver of early weight gain (between the ages of two and five) than eating frequency (Syrad *et al.*, 2016a); highlighting the need for vigilance with children's portion sizes.

Observing large individual differences in appetite so early in life indicated that appetite might have a genetic basis, in line with Behavioural Susceptibility Theory. However, this view was at odds with the widespread belief among researchers and health professionals that all infants are born with a natural ability to adequately self-regulate their milk intake if allowed to do so. The nature and nurture of infant appetite was something that Gemini was well-placed to establish, because a twin cohort is a genetically sensitive design.

Genetic Influence on Appetite

Twins can be used to quantify the relative importance of genetic and environmental influence on variation in any measured trait. The basis of the twin method is to compare resemblance between identical twins (monozygotic, MZs) who are 100% genetically

identical, with that between nonidentical twins (dizygotic, DZs) who share approximately 50% of their segregating genes. Because both types of twins share their environments to a very similar extent (e.g. they grow up in the same household and are of the same socioeconomic status, etc.), any difference in resemblance can only be attributable to genetic differences. Greater similarity in appetite between MZ pairs compared to DZs pairs therefore indicates a genetic contribution to appetite, and the larger the difference in resemblance the greater the genetic influence. The statistic derived from twin studies is called "heritability" which estimates the proportion of variation in a trait (i.e. appetite) that is explained by genetic differences in the population studied. Heritability can range from 0% (genes play no role in variation in a trait) to 100% (variation in a trait is entirely explained by genes). Twin studies also separate out the contributions of different sources of environmental influence. The "shared environment" includes aspects of the environment that twin pairs share completely, which contribute to their within-pair similarity (e.g. socioeconomic status); the "unique environment" captures factors that are unique to each individual twin (i.e. unshared with their co-twin, such as an illness affecting only one twin in a family) as well as random measurement error, which contribute to differences within pairs. The twin design can also be extended to examine the extent of common genetic and environmental etiology underlying multiple traits.

Gemini was the first study to establish the genetic and environmental influence on infant appetite, in the first 3 months of life, during the period of exclusive milk feeding. Heritability was substantial for all BEBQ-measured appetitive traits: "enjoyment of food" (53%); "food responsiveness" (59%); "satiety responsiveness" (72%); and "slowness in eating" (84%) (Llewellyn *et al.*, 2010). A follow-up study in Gemini quantified the extent to which appetite and weight share a common genetic architecture at 3 months of age (Llewellyn *et al.*, 2012). Approximately one third of the genetic influences underlying appetite and weight were the same, suggesting that genes are influencing weight partly through the biological mechanisms that regulate appetite, in line with Schachter's early proposition half a century ago.

These findings demonstrate that in the earliest period of life, variation in appetite is strongly genetically determined, not learned. By virtue of their genes, some children are born with more avid appetites and lower self-regulatory ability placing them at increased risk of excess weight gain. While these findings provide convincing support for Behavioural Susceptibility Theory, they contrasted with the prevailing view that infants are born with the ability to regulate their milk intake perfectly, if fed responsively to hunger and satiety. In fact, these findings suggest that infants are not born on a level playing field; some are naturally much better at regulating their food intake than others, by virtue of their genetic endowment.

Wardle and colleagues had also explored the heritability of "enjoyment of food" and "satiety responsiveness," captured using the CEBQ, in a very large sample of 10-year-old twin children ($n = 5435$ pairs) from TEDS (Carnell *et al.*, 2008). In line with the estimates for infants, heritability was substantial for both "enjoyment of food" (75%) and "satiety responsiveness" (63%) indicating that genes play an important role in shaping these aspects of appetite in later childhood as well. When the Gemini children were older, the etiology of other appetitive traits measured by the CEBQ were also established. Variation in food fussiness (in relation to familiar foods) as well as neophobia (rejection of novel foods) were found to have substantial genetic underpinnings at 15 months (46% and 58% respectively) (Smith *et al.*, 2017). However, in stark contrast to all other appetitive traits, emotional over- and undereating were almost entirely shaped by the shared environment. Genes contributed very little (7%) to variation in the tendency to either emotionally over- or undereat in response to stress at 5 years of age (Herle *et al.*, 2017). Instead, aspects of the environment shared by twins (e.g. parental feeding strategies) explained variation in emotional eating in childhood. These findings suggest that the four traits enshrined in both the BEBQ and CEBQ all have a strong genetic basis, along with food fussiness; but emotional eating has a distinct etiology and is entirely learned during early life.

Molecular genetic research has shed more light on the relationship between appetite and weight. In particular, many of the BMI-associated common genetic variants (SNPs) appear to influence adiposity via appetitive mechanisms. The fat mass and obesity associated gene (FTO) was the first SNP to be discovered (Frayling *et al.*, 2007), and explains the largest amount of variation in BMI of all the variants identified to date. On average, adults (of average height) who carry two copies of the high-risk FTO variant (AA) weigh approximately 3 kg more than those who carry two copies of the low-risk variant (TT). Shortly after the discovery of FTO, Wardle and colleagues used data from the TEDS cohort to show that 10-year-old children ($n = 3337$) who carried at least one copy of the lower risk variant (TT or AT) scored significantly higher on the CEBQ satiety responsiveness scale than those carrying two copies of the high-risk variant (AA) (Wardle *et al.*, 2008). Furthermore, this effect remained after adjustment for BMI, suggesting that FTO influences body weight via appetite. A second study in a subsample of the same cohort ($n = 131$) replicated this finding using a behavioral measure of satiety sensitivity conducted when the twins were 5 years old (Wardle *et al.*, 2009). These findings were confirmed in a third unrelated study of 97 Scottish four to 10 year olds, where children carrying the high risk A allele ingested more energy-dense foods at a test-meal following a preload of food, than did the children not carrying the A allele (Cecil *et al.*, 2008).

FTO is not the only common genetic variant shown to confer a predisposition to obesity through the regulation of energy intake. A more recent study from the TEDS cohort ($n = 2258$) demonstrated that a composite genetic risk score comprising 28 BMI-associated variants (other than FTO), was also associated with CEBQ-measured satiety responsiveness at 10 years of age (Llewellyn *et al.*, 2014). Satiety sensitivity significantly mediated part of the association between genetic risk and adiposity, confirming that many of the obesity-related genetic variants identified so far influence adiposity partly via mechanisms that regulate appetite.

Together, the findings from twin and molecular genetic studies strongly support Behavioural Susceptibility Theory. However, this field of research is not without its critics. The main concern is that genetic research of any kind undermines the personal responsibility necessary to manage our own or our children's health, and can lead to fatalism. This criticism is fueled by a common

misconception that behaviors under strong genetic influence cannot be changed or modified. Genes are not "the whole story" when it comes to complex behavior, especially appetite. A strong genetic influence means some individuals are born with a *predisposition* to have a more avid appetite, but the extent to which genes are expressed is almost certainly dependent on environmental exposure. A commonly used example is the genetic predisposition to lung cancer. Even people born at higher genetic risk of lung cancer are unlikely to develop the disease unless they smoke. The same is true of appetite. A child predisposed to overeat by virtue of his or her genes will only gain weight in an environment that enables them to do so. To this extent, the environment may act as a volume control for genetically-determined behavior. George Bray put it well when he said: "Genes load the gun, but the environment pulls the trigger." (Bray, 1996).

Moreover, although most appetitive traits have a substantial genetic basis in infancy and childhood, they are not *entirely* genetically determined. In fact, considerable environmental influence was identified for many of the traits (Herle *et al.*, 2017; Llewellyn *et al.*, 2010; Smith *et al.*, 2017). In particular, the tendency to emotionally over- or undereat in response to stress is entirely shaped by early environmental experiences (Herle *et al.*, 2017). For this particular trait, aspects of the environment shared by twins (e.g. parental feeding strategies) are responsible for the development of emotional eating in the preschool years. These findings highlighted the sizeable contribution of the family environment to certain aspects of appetite development in early life.

Environmental Influence on Appetite

Perhaps unsurprisingly, of all potential external shapers of children's appetites, parental feeding practices have received the most attention. Feeding practices of particular interest are parents' use of pressure to encourage their child to eat, excessive restriction of the types or amount of food their child consumes, and the use of emotional feeding (feeding to soothe). Historically, the assumption has been that parental feeding practices causally influence child appetite and weight gain, raising the risk of overweight or underweight by undermining the child's ability to self-regulate their food intake (Birch and Fisher, 1998; Birch *et al.*, 2003; Costanzo and Woody, 1985; Johnson and Birch, 1994). Restricting a child's access to certain foods is thought to lead to the "forbidden fruit effect," whereby the child develops an even greater desire for the restricted food and eats more of it when it subsequently becomes available. Pressuring a child to eat could either cause them to override their internal satiety cues and overeat, or conversely lead to mealtime anxiety and even food aversion. While using food to food to soothe an unhappy child is thought to nurture emotional overeating.

Cross-sectional studies have supported some of these hypotheses. In particular, several studies have demonstrated a positive relationship between parental restriction and child weight (Johnson and Birch, 1994; Shloim *et al.*, 2015). Greater parental pressure to eat has sometimes been associated with lower child body weight, although findings have been equivocal (Shloim *et al.*, 2015). In addition, studies exploring the relationship between parental feeding and child appetite have found restriction to be associated with stronger food approach appetitive traits (e.g. food responsiveness), while pressure to eat has been linked with food avoidant traits (satiety responsiveness, slowness in eating, and food fussiness) (Webber *et al.*, 2010).

While it has been suggested that parental pressure or restriction might play a causal role in the development of children's eating behaviors, much of the evidence is also consistent with a child-responsive model of parent feeding. Parents vary their levels of pressure and restriction in line with characteristics of their child from very early in life, even during the period of exclusive milk feeding (Fildes *et al.*, 2015; Gross *et al.*, 2011). Gemini mothers ($n = 1920$) were found to use more pressure when their infant was 3 months old if they had a lower birth weight or smaller appetite, and were more likely to restrict when their infant had a larger appetite (Fildes *et al.*, 2015). This indicates that infant characteristics influence mothers' feeding practices from the offset. Twin studies have also been used to untangle the complicated relationship between parent feeding and children's appetite. A study of 1012 pairs of twin toddlers from Gemini, of whom 247 were discordant in food fussiness, found that mothers pressured their fussier toddler more than their less fussy co-twin (Harris *et al.*, 2016); indicating that parents adapt their feeding practices in response to the different characteristics of each child. The discordant twin design is powerful because it controls for other potentially confounding environmental factors shared by siblings in a family, but longitudinal studies provide a better indication of the likely direction of the relationship between parent and child behavior.

Recent longitudinal analyses of other large European birth cohorts have demonstrated bidirectional relationships between children's appetitive characteristics and parental feeding practices. A study of emotional feeding and emotional eating in 801 Norwegian 4 year-olds, followed up at ages 6, 8, and 10 revealed a reciprocal relationship; higher levels of parent emotional feeding predicted greater child emotional eating over time, and vice versa (Steinsbekk *et al.*, 2017). Longitudinal analyses also assessed the directionality of the relationship between fussy eating and parental pressure to eat in 4845 mother-child dyads from the Generation R cohort in the Netherlands, when the children were 3–6 years old (Jansen *et al.*, 2017). The findings suggested parental pressure was a reactive feeding strategy developed in response to children's fussy eating, but the relationship was also bidirectional indicating parental pressure exacerbated child fussy eating. A second longitudinal study in the same cohort explored the relationship between parental restriction and child BMI (Derks *et al.*, 2017). Child BMI at age four was found to predict parents' use of restrictive feeding practices at age 10, but parental restriction at 4 was not associated with increases in child BMI over time, suggesting parental restriction is primarily adopted in response to – rather than a cause of – child overweight.

Randomized controlled trials offer a more stringent test of the causal effect of parental feeding practices on children's appetite, than prospective studies. There is evidence that providing parents with anticipatory guidance on positive feeding practices may result in improved appetite regulation in younger children. The NOURISH randomized controlled trial successfully reduced

parents' use of nonresponsive feeding practices (including pressure and restriction), resulting in children being less food responsive, and more satiety sensitive 3 years after the intervention (Magarey *et al.*, 2016). However, the intervention failed to impact on children's levels of food fussiness, suggesting not all appetitive traits are equally amenable to an early life parent feeding intervention.

Taken together, these findings suggest a reciprocal relationship between child appetite and parental feeding. Children's heritable appetitive traits seem to "elicit" certain feeding strategies from parents. Children are not simply passive recipients of their parents" chosen feeding practices. At the same time, children do not direct or shape all aspects of parent feeding, and research suggests that parents' feeding strategies can influence the development of their child's appetite. However, the extent to which parent feeding practices or child characteristics can be considered the key driver of this relationship likely depends on the domains being measured and the age of the children in question.

Implications and Conclusion

Over the last half a century researchers have made great headway in showing the importance of appetite for risk of both over- and underweight, especially among children. Appetite has a strong genetic basis and appears to be a key mechanism mediating genetic risk of obesity. This has important and far-reaching implications for both policy and clinical practice. When it comes to appetite control not everyone is born equal. In the context of the modern food environment some individuals will be "battling their biology" from early in life, and will find it harder than others to maintain a healthy weight. The current food environment exploits these vulnerabilities because it encourages overeating. Behavioural Susceptibility Theory would suggest that changes to the food environment that support those who are genetically susceptible to overeating would make a meaningful impact on obesity rates. For example, reducing palatable food cues (such as food advertising) and portion sizes may be effective in supporting individuals with high food responsiveness and low satiety sensitivity. Regulatory actions from policymakers and increased efforts from industry are necessary to enforce wider environmental changes, but such measures are often not well supported by the public. The research on feeding practices also suggests a few key points for parents. The natural parental response to a child with a poor appetite seems to be coercion and pressure, and for a child with an avid appetite, excessive restriction. However, these may not be the most fruitful strategies in the long run, and may nurture an already poor or voracious appetite. Responsive feeding of the type promoted in the NOURISH trial appears to support the development of good appetite regulation, and would provide parents with better alternative feeding practices. What is clear is that the development of appetite is complex, and evolves through a combination of genetic expression and environmental experience.

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Management of Obesity in Children and Adolescents: Lifestyle and Exercise Options

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Childhood overweight and obesity is a pressing issue across the world, with almost one in four children (males: 23.8%, females: 22.6%), aged 5–17 years old, being classified with overweight and obesity (Ng *et al.*, 2014). This represents a 47% increase since 1980 among this age group. Within the United Kingdom, the context in which this article is positioned, obesity affects one in five children, aged 10–11 years (NHS Digital, 2016). This estimate rises to one in three when figures include overweight (NHS Digital, 2016). The prevalence of overweight and obesity increases proportional to deprivation (NHS Digital, 2016), tracks strongly into adulthood (Simmonds *et al.*, 2016), leads to premature mortality (The Global BMI Mortality Collaboration, 2016), and has numerous health-, economic-, and social- ramifications. Recent data suggest that the global economic burden of obesity (2.8% gross domestic product [GDP]) comes third to smoking [2.9% GCP) and armed violence, war, and terrorism (2.8% GDP) (McKinsey Global Institute, 2015), costing the UK economy £27 billion per year (Foresight, 2007). In light of the evidence aforementioned and given that 80% of children with obesity are predicted to have obesity as an adult (Simmonds *et al.*, 2016), the need for both prevention and treatment of childhood obesity is paramount.

The Context of Weight Management in the United Kingdom

Although this article is placed in the context of England and the United Kingdom, it is anticipated that all topics discussed will be pertinent to those outside of this context. Indeed, it may be that other contexts share many similar characteristics with the UK context. The context must be considered when discussing the approach to treating obesity in the United Kingdom.

We begin this article from the viewpoint of the national health systems in the United Kingdom. Overseeing the health system and the budget of the health sector is the Department of Health and Social Care; a ministerial department within the British government. However, due to the varied demographics in the United Kingdom, a centralized system is not considered to be the best approach to account for differences across local geographic areas. As part of a significant health care reform in 2012, the national government devolved the responsibility for Public Health to Local Government Authorities (LAs: $n = 152$ across England) and Clinical Commissioning Groups (CCGs: $n = 209$ across England) (HM Government, 2012). These two bodies—alongside NHS England (who oversee the commissioning of specialist national health care services)—are responsible for the prevention and treatment of obesity in England.

Within England, the Obesity Care Pathway (**Fig. 1**)—a four Tiered approach to preventing and managing obesity—has been developed (Blackshaw *et al.*, 2014; Department of Health, 2013); each Tier being commissioned by one of the three bodies. The first Tier of the pathway focuses on the prevention of overweight and obesity, primarily through marketing campaigns, awareness raising, and knowledge building (e.g., "Change4Life" and "This Girl Can"). Tiers 2–4 are dedicated to obesity treatment. The

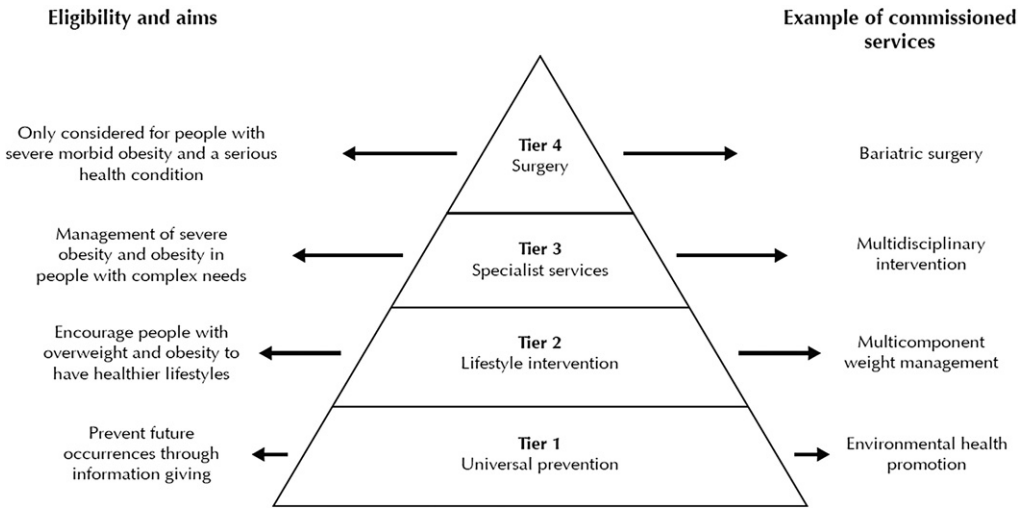

Fig. 1 The obesity care pathway. Adapted from the Department of Health (2013). Developing a specification for lifestyle weight management services: Best practice guidance for tier 2 services. London, UK: Department of Health.

pathway stipulates that more specialist treatment is provided in line with the greater degree and complexity of obesity, insofar that Tier 2 provides generic weight management (WM) advice for children with overweight, Tier 3 provides specialist intervention from a multidisciplinary team for children with obesity, and Tier 4 offers intensive treatment, residential camps, pharmacotherapy, and bariatric surgery for children with severe obesity (Nobles *et al.*, 2016b; Blackshaw *et al.*, 2014). This pathway is most notably referred to for the management of adult obesity, but it is frequently being implemented for childhood populations (Public Health England, 2015).

The aim of nonbariatric/nonpharmacological WM programs is to stabilize and reduce the weight of the child through education in lifestyle modification, dietary improvement, physical activity increases, and sedentary behavior decreases (NICE, 2013). These services often provide weekly, group-based intervention for families over a period of 10–12 weeks. Programs tend to be delivered by paraprofessionals (i.e., trained nonexperts), and conducted in a nonclinical format (NICE, 2013). Tier 3 services should utilize a multidisciplinary team (i.e., possibly a doctor, dietician, psychologist and an exercise professional) and may be clinically-based (Wright and Wales, 2016). Tier 4 nonbariatric services are residential in nature, providing an immersive experience for young people; with programs including camp-based models (i.e., more holistic approach) and hospital-based models (i.e., more clinical approach). Effectiveness of these WM programs is ubiquitously measured through mean change in standardized body mass index (BMI SDS), pre- to postprogram.

As may be evident here, the United Kingdom adheres to a medicalized model of care when aiming to treat obesity. The more severe an individual's obesity becomes, the greater the promotion of medicalized intervention. Epitomizing this are Tier 4 bariatric services; those which aim to alter the physiological functioning of the human body in order to bring about an energy deficit. The extent to which the Obesity Care Pathway accurately reflects the realities and needs of the children living with obesity shall be discussed at a later point in this article; where we speak to both the current UK service provision and also that which we believe is required in the future.

Given the burgeoning prevalence of obesity in England, the national government has made two public commitments to rally action against obesity (National Audit Office, 2006; HM Government, 2008). Yet despite such governmental prioritization of obesity, local level action is scant. In 2015, Public Health England (PHE: an executive agency of the Department of Health and Social Care) reported that 56% and 61% of LAs had a Tier 2 service for children and adults respectively (Public Health England, 2015). At Tier 3, PHE declared that insufficient information was available to generate conclusions—very few CCGs indicated having these services. This is despite estimates that there are 140,000 children with severe obesity and 2.5 million children with obesity (Ells *et al.*, 2015). The picture of service provision is further clarified when examining LA expenditure on WM programs. In the government's annual LA revenue expenditure reports (HM Government, 2016b)—when compared against the 2015/16 Public Health grant (£2.80b)—the total spend on prevention and treatment of obesity was £101.7 m (3.6%), with £40.4 m of that spent on children. This expenditure is in stark contrast to that on substance misuse (£767.3 m, 27.5%), sexual health (£631.1 m, 22.6%), and smoking cessation (£111.2 m, 4.5%) (**Figs. 2** and **3**).

Identifying Appropriate Treatment Options

When thinking about the management of obesity, it is imperative that the initial assessment considers the holistic health of the individual, as opposed to solely the consideration of their weight status. In adult cohorts, tools such as the Edmonton Obesity Staging System (Sharma and Kushner, 2009) can be used to understand the support- and need- requirements for those with obesity. This tool—which has been widely utilized—assesses physical symptomology (i.e., associated comorbidities), psychological symptomology (e.g., depression, anxiety, maladaptive eating practices, suicidal ideation), and functional limitations (e.g.,

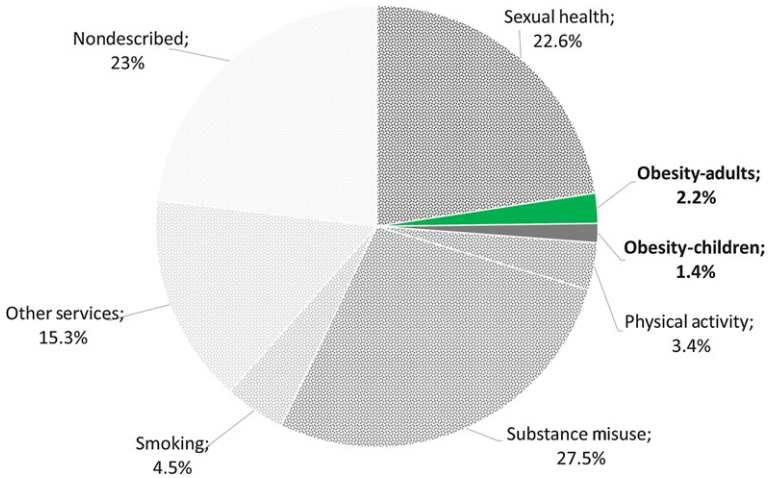

Fig. 2 Public health spend in England. Calculated as a proportion of the £2.8b Public Health Grant in 2015/16.

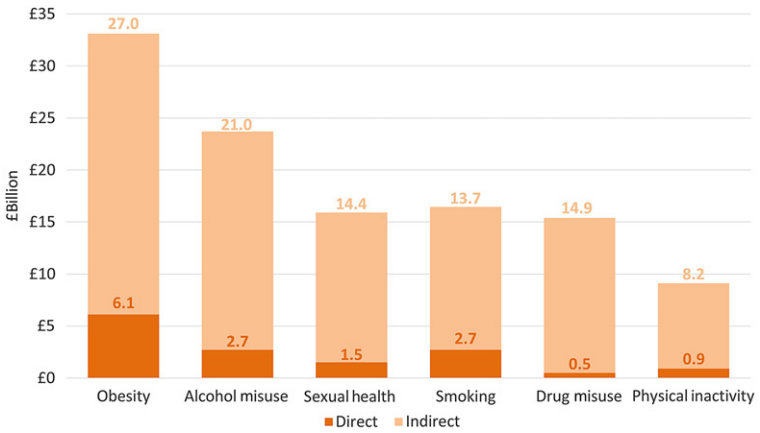

Fig. 3 Estimated cost of several public health issues in England.

ability to work, complete daily tasks, quality of life) of the individual. These tools can then help signpost adults to the most appropriate services, acknowledging that a "one-size-fits-all" approach is inadequate. Comprehensive tool such as the Edmonton Obesity Staging System are not yet available for young people, hence reporting of research and practice-based outcomes vary considerably.

In the United Kingdom, the National Institute for Health and Care Excellence (NICE) provide evidence-based guidance to help shape the delivery of the health and social care services. NICE have created a number of guidance documents to assist health professionals in designing effective WM programs (NICE, 2013; NICE, 2014; NICE, 2016). These documents state—for both young people and adults with obesity—that the complex needs of individuals should be assessed. However, how one defines "complex needs" is unclear. NICE suggest that assessments should consider presenting symptoms, eating behaviors, comorbidities, lifestyle factors, psychosocial factors, medical problems, the social environment, and readiness to change as a starting point for understanding complex needs (NICE, 2014). In children, these assessments are recommended for those with obesity as opposed to those with overweight—albeit that there is no empirical evidence to suggest why this is so. Dependent on the needs of the individual, the health professional can then look to refer the individual to a Tier 2, 3, or 4 WM program. Further work is needed to help health professionals ascertain a comprehensive picture of the individual—child or adult—in an efficient manner.

Treatment should—dependent on the outcome of these assessments—then be tailored to the individual needs of the individual. This could be achieved through the use of a Stepped Care Approach (Sharman and Nobles, 2016), where the intensity and specificity of treatment options is increased relative to the need of the individual. For example, at SHINE—a not-for-profit WM provider—an initial assessment of 1.5 h is undertaken with young people and their parents. The assessment considers some of the factors listed above, including anthropometric and physiological functioning (e.g., height, weight, blood pressure, peak flow, and medical history), and psychosocial symptomology (e.g., self-esteem, anxiety, depression, maladaptive eating practices, weight-related bullying, and self-harming). After determining the preferences of the individual, SHINE then offer individuals access to one of seven treatment options (Sharman and Nobles, 2016). Although this is one example of good practice, SHINE provides a Tier 3 service within the voluntary sector, and so their approach is not governed by those who traditionally commission WM programs.

Types of Weight Management

Whilst the Obesity Care Pathway lays out a four-tiered approach to WM, it is also important to note that there are various types of children's WM program. The review of Altman and Wilfley (2014) eloquently classifies a range of different WM programs. As aforementioned, the majority of programs aim to modify dietary intake, physical activity and sedentary behaviors through the use of behavior change strategies/techniques (Altman and Wilfley, 2014), however some have started to acknowledge the wider biopsychosocial factors associated with obesity. There are a myriad of ways in which these aims can be accomplished, and the effectiveness of the different types of program varies too.

The first element to note when differentiating between programs is the type of *family involvement*. Altman and Wilfley (2014) identify two types of program here, family-based behavioral treatment (FBT) and behavioral weight loss (BWL). The distinguishing factor is the extent to which goals are set for both parents and children. In FBT, specific goals are set for both parents and children, whereas in BWL goals are only set for one of these parties—often the child. FBT emphasizes that parents act as an agent for change, role modeling and advocating the behaviors they wish their children to make (Epstein *et al.*, 2014). There is however a third type of program to note associated with family involvement, parent-only programs (POP). The review of Altman and Wilfley (2014) included 14 POPs, some of which focused on the involvement of fathers only (Morgan *et al.*, 2014). The rationale for the POP being that parents can, independently, act as the change agent and modify the home environment—additionally making the

treatment easier to implement (Boutelle *et al.*, 2011; Ball *et al.*, 2017). Thus, the age of the child is a pertinent factor to consider regarding family involvement.

Treatment modality can also help to differentiate between programs. Modality, the method of administering treatment, can differ substantially. In the previous section, community-based (i.e., delivered by paraprofessionals in a local community venue) and clinically-based (i.e., delivered by multidisciplinary teams in a primary care setting/specialist facilities) WM programs were mentioned, as were group-based (i.e., delivered to more than one family at the same time) and individual-based (i.e., delivered to one family at a time) programs. In recent years though, additional modalities have started to form. Firstly, commercial WM programs have emerged as an option for children and young people—especially adolescents (Bonham *et al.*, 2017; Dordevic *et al.*, 2015; Stubbs *et al.*, 2012). Commercial programs are delivered by private sector organizations, providing their own WM guidance, and are popular among adult cohorts (Gudzune *et al.*, 2015). They provide a scalable alternative to clinical programs, whereby participants can pay to access said group-based services. Resources—such as meal replacements and prepackaged food—are often advocated by these providers. They rely fundamentally on the alteration of energy intake, thus not considered to be a holistic, multicomponent service (Gudzune *et al.*, 2015).

Residential WM camps (i.e., Tier 4 WM) are also another type of treatment modality. Camp models can be found in the United Kingdom (Holt *et al.*, 2005) and in the United States (Gately *et al.*, 2000), with hospital models also being common (Braet *et al.*, 2004; Endo *et al.*, 1992; Rolland-Cachera *et al.*, 2004; Wabitsch *et al.*, 1996; Widhalm *et al.*, 1983). Camp models tend to be holistic in nature, creating energy imbalance through dietary restriction and physical activity, however doing so within a social, fun-, and experiential environment. These programs are led by education professions with support from clinicians (Psychologists, Dieticians and exercise experts). Hospital-based models typically use a variety of dietary methods (including very low calorie diets and liquid diets), and tend to be led by clinical teams (Physicians, Psychologists, Nurses, and Dieticians).

The penultimate noteworthy WM modality is through interactive *m*-health or *e*-health programs (e.g., mobile applications, internet-based programs, tele-interventions). This modality also offers a wider dissemination in conjunction with flexibility around the timings in consuming information (Altman and Wilfley, 2014), alongside the easy setting, and real-time tracking, of goals/performance (Norliza *et al.*, 2014). Lastly is a self-help modality—whereby primary care physicians refer their patients to helpful WM resources (e.g., handouts, online materials, information) (Sharman and Nobles, 2016).

Focus is the final program characteristic to differentiate between types (Oude Luttikhuis *et al.*, 2009). Oude Luttikhuis *et al.* (2009) characterized WM programs into three broad groups, those with a focus on (1) diet only, (2) physical activity and sedentary behavior only, and lastly, (3) behavioral programs. Whilst there is a general consensus that multicomponent programs (i.e., focused on all three of the above) are preferred, a plethora of research and evidence exists for each of these program types. As spoken to earlier in this article, WM programs should seriously consider the wider biopsychosocial factor associated with obesity. Although very few children's WM programs exist like this, some services such as SHINE and MoreLife place a concerted effort on addressing these wider factors in their session content (Nobles *et al.*, 2016a; Nobles *et al.*, 2016b). Both provide a psychosocial intervention with dedicated program content focusing on the importance of social relationships, personal hygiene, stress management, dealing with bullying, and improving self-esteem (Hester *et al.*, 2010; Nobles *et al.*, 2016b). This program content is provided in equal measures to advice on diet and physical activity.

The involvement of family members, treatment modality and treatment focus are distinctive, observable elements of a WM program. But given that the aim of a WM program is to bring about changes in behavior, the active ingredients of a WM program lie within the behavior change techniques (Michie *et al.*, 2015). Behavior change techniques (BCT) are defined as "the smallest components compatible with retaining the postulated active ingredients (i.e., the proposed mechanisms of change)" (Michie *et al.*, 2015)—of which Michie *et al.* (2013) have identified a list of 93 in their most recent taxonomy, categorized into 16 groupings. These active ingredients—all of which are clearly labeled, distinct, irreducible and precise (Cane *et al.*, 2015)—include among others, goal setting and planning, social support, feedback and monitoring, covert learning, threats and rewards, and natural consequences (Michie *et al.*, 2013). The ability to identify these active components is dependent upon the clarity and depth of program reporting, for example in program protocols or via standardized reporting templates (e.g., TIDieR: Hoffmann *et al.*, 2014).

As can be deduced from the previous text, WM programs—particularly when studying their macro (e.g., modality) and micro (e.g., BCT) details—are heterogenous. This is without appraisal of the specific program content (i.e., information on diet), the treatment dose (i.e., intensity, duration and follow up), the wider context, the level of support, the characteristics of program delivery staff, and the use of adjunctive inputs (e.g., pharmacological intervention)—among others. There has been a substantial volume of research into the use of WM programs (and to a lesser degree, the study of BCTs) for the treatment of childhood overweight and obesity, but the effectiveness of these programs is yet to be discussed.

Effective Weight Management

The effectiveness of a WM program is often assessed against one primary outcome in both academic and Public Health arenas, a change in weight status (NICE, 2013; NICE, 2015; NICE, 2014; Altman and Wilfley, 2014; Department of Health, 2013; Roberts *et al.*, 2009; Oude Luttikhuis *et al.*, 2009; Upton *et al.*, 2014; Janicke *et al.*, 2014). Whilst the range of outcomes related to a change in weight status is vast (e.g., absolute weight, change in BMI percentage, percent overweight etc.…), the mean change in BMI SDS, from baseline to postintervention, is frequently utilized to measure the effectiveness (i.e., under real-world conditions) or efficacy

(i.e., under optimum controlled conditions) of children's WM programs (Upton *et al.*, 2013; NICE, 2013; Roberts *et al.*, 2009). The measure, BMI SDS, accounts for the child's age and sex, and subsequently enables the score to be compared against a reference population (usually national or international growth charts–see Cole *et al.*, 1995). Once the mean change in BMI SDS has been calculated for the WM program, the program effectiveness can be established by comparing the outcome against a benchmark.

Cochrane reviews create this benchmark; these reviews collate high quality research studies and pool their effects into an overall estimate. The most recent Cochrane review examines the efficacy of various pediatric WM program types (Oude Luttikhuis *et al.*, 2009). Behavioral interventions (i.e., those most closely aligned with multicomponent interventions) were included in the review, with the results separated into two childhood age groups (i.e., those <12 years or those ≥ 12 years). At 6 months, the pooled average reduction in BMI SDS for those attending a behavioral intervention targeting younger children was 0.06 units (95% CI: -0.12 to -0.01), whilst for those interventions targeting older children, the pooled average reduction was 0.14 units (95% CI: -0.17 to -0.12). And linked to the previous section, certain types of programs and those employing various BCTs—are more efficacious than others. Firstly, the Cochrane review established that combined behavioral interventions (i.e., *treatment focus*) were more efficacious than those with a single focus (e.g., diet only) and/or a self-help intervention—conclusions confirmed by van Hoek *et al.* (2014). The review of Altman and Wilfley (2014) goes further to suggest that FBT (9/10 studies efficacious) and POP (14/14 studies efficacious), as opposed to control interventions and BWL (4/10 studies efficacious), bring about greater weight-related change. Both FBT and POP have a multicomponent treatment focus and family involvement—with BWL only focused upon goal setting for children or parents independently.

Additional reviews of Janicke *et al.* (2014), van Hoek *et al.* (2014), and Whitlock *et al.* (2010) suggest that behavioral WM programs of moderate-to-high intensity (i.e., >26 h contact), in-person sessions, and one-to-one sessions (opposed to group-based) were more effective in evoking positive changes in weight status among children. Extending from the above, Hayes *et al.* (2015)—via a systematic review of evidence—concluded that mixed sessions (i.e., a combination of individual- and group-based sessions) demonstrated greatest efficacy. The age of the child, the degree of childhood obesity, and parental weight loss success are often moderating factors of program efficacy (Altman and Wilfley, 2014).

As for the active components of a WM program (i.e., the BCT), Martin *et al.* (2013) drew on the CALO-RE taxonomy (Michie *et al.*, 2011a) to investigate the BCTs included within 9 RCTs viewed as effective (≥ 0.13 unit reduction in BMI SDS; $n = 6$ trials) and noneffective (<0.13 unit reduction; $n = 3$ trials) in the management of childhood obesity. Although the study adopted an older version of the CALO-RE taxonomy (41 items rather than 93), six BCTS were found useful: (1) provide information on consequences of behavior to the individual, (2) environmental restructuring, (3) prompt identification as role model/position advocate, (4) stress management/emotional control training, (5) general communication skills training and (6) prompt practice. Additional reviews—whilst less robust—also suggest that goal setting, interactive sessions, social support, rewards/incentives, self-monitoring, and stimulus control are effective (Altman and Wilfley, 2014; Sahota *et al.*, 2010; Sutcliffe *et al.*, 2017). Sahota *et al.* (2010) stated that many BCTs come as a "package" rather than acting in isolation, yet notwithstanding this, Dombrowski *et al.* (2012) emphasized that programs with more BCTs may not necessarily generate better outcomes, and instead should carefully be selected. The interaction of combined BCTs are yet to be examined.

Whilst the examination of high-quality systematic reviews and meta-analyses are fundamental to understand the efficacy of WM programs, they tend to focus on studies of RCT design, predominantly delivered in a clinical setting. These RCTs do not reflect real-world commissioned WM programs that are delivered under service-level conditions (Service-level conditions: programs or interventions which are not conducted for the primary purpose of research. RCTs are highly controlled, and the conditions which they are carried out do not reflect real-world practices) and constrained budgets, and therefore these estimates may be less achievable. The reviews of Altman and Wilfley (2014), Upton *et al.* (2014), and Whitlock *et al.* (2010) include non-RCT study designs within their remit; with the review of Upton *et al.* (2014) being of particular pertinence. Upton *et al.* reviewed the change in BMI SDS for all community-based, family WM programs in the United Kingdom with published results ($n = 10$). The review illustrates that the effectiveness of WM greatly differs between programs; the change in BMI SDS (pre- to postmeasurement) ranged from -0.01 units (Rudolf *et al.*, 2006) to -0.18 units (Robertson *et al.*, 2008). Albeit that the SHINE program was not included in the review of Upton *et al.* (2014), a service evaluation of the psychosocial intervention demonstrated mean BMI SDS reductions of 0.19 units at 3 months, and 0.29, 0.35, and 0.41 units at 6, 9, and 12 months respectively (Nobles *et al.*, 2016b). Again, although not included in the review of Upton *et al.* (2014), in the context of Tier 4 residential WM camps, there is evidence for short and long-term effectiveness (Gately *et al.*, 2000). These differences in outcomes between WM programs are largely attributable to the various design, dose, delivery, and contextual characteristics of the WM programs—as detailed above.

NICE guidance not only provide information on how services can be designed, they also highlight what type of results are to be expected/should be promoted in WM programs. As aforementioned, there is a desire for WM programs to generate a mean reduction in BMI SDS. However, NICE recommends that childhood WM programs should encourage weight maintenance in the short term, rather than weight loss. (Given that BMI SDS is the primary measure of effectiveness, weight (i.e., kg) does not need to change in order to evoke a reduction in BMI SDS, providing that the individual is still growing. A WM goal that promotes a reduction in weight may be counter-productive—program effectiveness is measured based upon change in BMI SDS, whilst an individual is likely to measure their success based upon a change in weight). That said, this goal may not be appropriate for those who have either stopped growing or have severe obesity (BMI SDS ≥ 99.6th centile); a gradual reduction in body weight of 1 kg/month is recommended (Scottish Intercollegiate Guideline Network, 2010; Wright and Wales, 2016; NICE, 2006). With this in mind, any program which reports a mean maintenance in BMI SDS could be viewed as effective (for those with overweight or

obesity). This is without considering the wider outcomes that families, children, and parents may value when attending a program (e.g., self-esteem, friendships, confidence, quality of life)—this will be discussed later in this article.

Weight Maintenance

Although the chronic and relapsing nature of obesity in children and adults is well documented, the evidence base has been dominated by programs seeking short-term outcomes over 3–6 months. Seldom do we see, particularly in the childhood arena, programs that offer long-term support for weight maintenance. This is despite evidence from families that longer term support is wanted, as—after 12 weeks of intervention—many do not yet feel confident in their future WM endeavors (Nguyen *et al.*, 2015; Reece *et al.*, 2015). The presence of a maintenance/follow up intervention is reliant on the design of the WM program. Some WM programs include a maintenance intervention (Brennan *et al.*, 2012; Nobles *et al.*, 2016b; Spence *et al.*, 2017), whilst others do not (Jensen *et al.*, 2012; Sacher *et al.*, 2010). Maintenance interventions hope to shift the attribution of outcomes from WM services to families, with self-management of obesity being the promoted strategy (Nobles *et al.*, 2018).

The paucity of evidence for maintenance programs is exemplified in the recent systematic review and meta-analysis of van der Heijden *et al.* (2018). The review concluded that across the 11 included studies, continued treatment does have a stabilizing effect on BMI SDS. Furthermore, the review highlighted that whilst there was no evidence to recommend a certain program dose over another, face to face interventions were preferable over virtual intervention. Perhaps due to this lack of evidence, there is minimal guidance to support the development of effective maintenance programs.

Due to the study design of the SHINE article (Nobles *et al.*, 2016b)—a service level evaluation—it was not included in the review of van der Heijden *et al.* (2018). However, SHINE has a carefully designed one-year maintenance program aiming to equip families with skills for long-term WM (including mental health, social relationships, and enjoyment of physical activity). It is important to ensure that families do not become dependent on service provision (Reece *et al.*, 2015), and so SHINE seeks to ensure that families internalize outcomes as a product of their own efforts. A phased approach to weight maintenance—where the intensity of support reduces over time—may be useful, particularly as van der Heijden *et al.* (2018) found no difference in outcomes between maintenance programs of differing dose. As with other issues raised in this article, further research is warranted to facilitate our understanding of weight maintenance programs.

Implementing Weight Management: What Do Key Stakeholders Look for?

As we move from research and policy into practice, we must consider key stakeholder opinions in order to understand how the management of obesity is best delivered. Three stakeholder groups are of importance here: service users (i.e., individuals and families), service providers (e.g., MoreLife and SHINE), and service purchasers (i.e., commissioners). Not only would accounting for the opinions of key stakeholders influence the design of the WM program, but it also provides further insight into the complex realities of working in the sphere of WM.

Service Users

Qualitative studies have identified numerous non-weight-related outcomes which are valued by prospective WM participants equal to weight loss, including: reduced self-harm and depression; improved school results; social acceptance; making new friendships; learning to cook; reducing incidence of bullying; and, being more independent (Dixey *et al.*, 2006; Law *et al.*, 2014; Perez *et al.*, 2016; Jensen *et al.*, 2014; Reece *et al.*, 2015). These outcomes are seldom appraised in policy or by service purchasers, and as such, are rarely assessed formally. However, if policy makers and service purchasers begin to understand these valued outcomes and if WM programs are shaped to the needs of attending families, then higher attendance should be expected which brings about greater weight-related outcomes (Nobles *et al.*, 2017). Low participant engagement is an omnipresent challenge in WM—with approximately 50% of individuals completing the program to which they enroll (Nobles *et al.*, 2018)—and so strategies to mitigate against this should be emplaced.

These points also link back to the earlier conversation around complexity; as professionals working in the space of WM, we must seek to understand the factors which drive individual-level obesity and also understand the family motives for attending WM. We cannot assume that all children want to engage in WM programs to lose/manage their weight. In many cases, the decision to attend a WM program will have been made exclusively by the parent(s). Shared decision making, person-centered care, motivational interviewing and action orientated counseling are useful approaches to understand family preferences and to create a common agenda and shared goals (Cohen, 2016; Lucas *et al.*, 2014; McPherson *et al.*, 2017). For example, if the child, the parent, and the health professional set out their expectations in a preprogram assessment (e.g., what they think the program will include, what outcomes are important, how often they can attend), then a mutual agreement can be in place between all parties.

The final point to mention here is surrounding weight-related expectations in children's WM programs. Many children, parents, practitioners, and policy makers would expect a WM program to help a child and their family to *lose weight*. Linked to previous discussion, weight loss per se is not required to reduce BMI SDS (i.e., the primary objective of commissioned WM

programs) for children who have not reached complete maturation, and hence, weight loss should not be the promoted strategy. Weight maintenance may suffice to reduce BMI SDS and work towards a healthier body composition. Only if the child has stopped growing and/or has severe obesity should gradual weight loss (e.g., 1-2 kg/month) be considered. Expectations are powerful mediators of program engagement and must, therefore, be adequately managed; if a program does not elicit the expected outcomes, many families and children will find it difficult to continue attending. Thus, shared decision making is again a useful tool for health professionals to ensure that appropriate and realistic expectations are held by the participating child and parent.

Service Providers

As aforementioned, it is important for service providers to determine the biopsychosocial needs of young people when identifying the most appropriate treatment options. All services should shape the design of their programs around the unique needs of their clientele to optimize treatment outcomes for individuals and also for service commissioners. These services should therefore include an in-depth initial assessment to understand individual needs, albeit that a comprehensive and efficient tool is not yet available for young people. An appropriate theoretical framework(s)—such as the self-determination theory (Deci and Ryan, 1985) or COM-B (Michie *et al.*, 2011b)—will also be useful to underpin the WM service delivery model. Such models are rarely used adequately.

In addition, there are a plethora of implementation and logistic considerations to account for, including: settings (community-based, clinical, digital/virtual etc.…); program dose (program duration and session frequency); group-based versus one-to-one; ancillary tools (e.g., wearable monitors and mobile applications); staff to client ratio; recruitment and development of WM staff; development of program content; intervention components (e.g., BCTs); communication and marketing; service monitoring, evaluation and evolution; level of individual need (Tier 2–4); and, support for different group makeups (e.g., ethnic diversity, socio-economic status, physical/learning disabilities etc.…). As with other elements included within this article, whilst some guidance is available nationally (NICE, 2013; NICE, 2014; NICE, 2016) and internationally (World Health Organisation, 2016), it does not account for or provide recommendations on the considerations above. This continues to limit the standardized development and implementation of high quality services.

Furthering this call for comprehensive guidance is the need for regulatory bodies to monitor the quality of WM provision (excluding bariatric surgery and pharmacotherapy). Many organizations can—at least within England—establish themselves as WM providers; from individuals (with minimal training or qualifications) offering WM advice to specialist centers within clinical settings (with high levels of clinical expertise and governance). The absence of regulation leads to a dilution of service quality whilst increasing the pool of WM providers. In the context of the financial challenges faced by governments and healthcare systems in the United Kingdom and elsewhere, this issue becomes more salient. A study by Nobles *et al.* (2016a) highlighted a 37.5% decrease between 2009 and 2014 in the funding allocated per capita for those attending a WM program. In a market with little accountability and care quality standards, service provision can become more dictated by price rather than clinical or operational quality.

Service Purchasers

Whether they be local or national service purchasers, it is important to understand what purchasers look for when acquiring WM programs. More to the point, what constitutes an effective WM program through the eyes of those commissioning the services? Service purchases tend to focus on three factors: cost-effectiveness/cost; evidence-based results (i.e., change in main outcome measures); and the perceived political and public need for a program (Willmott *et al.*, 2015; Hughes, 2015; Law *et al.*, 2014; Masters *et al.*, 2017). Given the diminishing Public Health budgets due to austerity, a WM program must be cost-effective by ensuring that it delivers a return on investment (Masters *et al.*, 2017). Initial commissioning depends largely on having a substantial body of evidence to verify the program's claims of being *effective* (Willmott *et al.*, 2015; Law *et al.*, 2014). The view of service purchasers adds additional complexity; a program must be cost-effective and aligned to the political agenda—alongside having positive results.

As previously outlined, England has different government bodies responsible for different parts of the Obesity Care Pathway; Tiers 1–2 are traditionally commissioned by LAs, with Tier 3 being a CCG responsibility, and Tier 4 commissioned by CCGs and/or NHS England (Blackshaw *et al.*, 2014). Not only are these government bodies pursuing different outcomes, the standards or care (clinical governance), the use of the evidence base, and the political context varies considerably. For example, commissioning decisions made by LAs will be influenced by the political party leading local government (and the respective elected members) whereas the CCGs operate within a NHS governed system, albeit with its own measures of politics and strong emphasis on evidence-based practice. Given that CCGs are NHS governed, they are more likely to adhere to a medicalized model of care—as is emphasized through the Obesity Care Pathway; higher degrees of obesity requiring more specialized and multidisciplinary care.

Yet despite the amount of media and political attention that childhood obesity receives, alongside the well documented direct and indirect impacts on our society, local level action remains scant. A recent review illustrated that 56% of LA provide a Tier 2 WM service, and that insufficient evidence was available to determine the volume of Tier 3 services (Public Health England, 2015). There could be a variety of reasons for this:

- There is a lack of long term evidence for WM outcomes;
- There is a lack of clarity on which government sectors and bodies are best placed to tackle obesity;
- Obesity is not a "*burning platform*" issue—i.e., obesity is not perceived to have immediate impacts therefore politicians, civil servants and policy makers are not required to act in the short-term;
- Action on issues like obesity is considered by many as "*nanny state*" intervention;
- There is a lack of clarity on what benefits will be gained for which government sectors (i.e., who benefits as opposed to who finances).

This section presents the case for service improvement. If many of these considerations were taken forth into practice, the quality and accountability for WM provision should be enhanced. This will require a complete recognition of the needs of service users, service providers, and service commissioners. Adequate prioritization and resourcing is then needed by national and local governments to ensure that these enhanced services can be provided.

Where Next?

In light of the discussion within this article, it is important to consider what the future for childhood WM could look like. This final section presents two advocated next steps: (1) the conjoining of prevention and treatment efforts, and (2) doing so as part of a whole systems approach.

From Prevention vs. Treatment to Prevention and Treatment

A constant challenge in the Public Health arena is the assumed dichotomy of obesity prevention versus obesity treatment. Many stakeholders working in the field of obesity are often encouraged to pitch their tent in one of these two camps. From our standpoint and others, such dichotomies are unhelpful when aiming for healthier individuals and populations (Roberto *et al.*, 2015). As Roberto *et al.* (2015) alludes to, there are many important insights to be seen at the intersect of these dichotomies. We cannot expect treatment programs to be efficacious in the long-term if peoples' environments are not salutogenic. Nor can we expect a preventative approach to adequately support the needs of individuals already with overweight and obesity. If we can enable individuals to develop new attitudes, behaviors and habits which in turn bring about long-term WM *and* simultaneously create a salutogenic environment, then population change in weight is more likely. This salutogenic environment would both support those of a healthy weight and those who have accessed WM services. By appreciating that obesity is a chronic and relapsing condition, and one that is influenced by myriad socioecological factors, we must view both prevention and treatment within the context of a complex adaptive system. Singular interventions—be those prevention or treatment orientated—will not influence the prevalence of obesity alone.

Whole Systems Approaches

Progressions in the field of WM will be hampered if the environments to which people return remain unchanged. WM programs constitute only one part of what is referred to as a whole systems approach; a framework that enables stakeholders to come together and understand the complex adaptive system driving obesity (at the individual, community, social, and political levels), and then collectively identify places to alter the functioning of the system to bring about a healthier environment. WM programs aim to alter numerous elements of the causal system, but is insufficient on its own to tackle obesity. As we have highlighted throughout this article, obesity is a notoriously complex issue at all levels. Taking a whole systems approach to obesity will require the input of many stakeholders from across many sectors. Obesity is not just an issue for Public Health teams to solve; for example, Urban Planning teams can create health promoting environments, Transport teams can ensure that well connected infrastructure is in place to support active journeys, and Housing teams can provide high quality homes which are also well linked to local amenities and resources. Central to a whole systems approach is that these efforts are coordinated to achieve maximum effect. Our current systems are set up in a manner that promotes obesity—"Obesity is a normal response to an abnormal environment" (The Lancet, 2011)—and this needs to be altered.

Whole systems approaches are in the infancy in Public Health and within the Healthcare System more broadly. There is an opportunity through whole systems approaches to overcome the dichotomous perspective of prevention versus treatment. Rather, a whole systems approach recognizes and responses to the varied needs of individuals within the system. Therefore, preventative and treatment efforts are aligned so they are complementary and symbiotic—ensuring the right people get the right support at the right time. The collective impact of an aligned agenda will likely have the greatest impact on population-wide obesity prevalence (Gortmaker *et al.*, 2011; Peeters and Backholer, 2017; Swinburn *et al.*, 2011).

To exemplify one avenue for how this could be achieved in the United Kingdom, we can draw on the soon-to-be-introduced Soft Drinks Industry Levy (SDIL: HM Government, 2016a). As part of a national obesity strategy (HM Government, 2016a), the national government is going to implement the SDIL which will tax producers dependent on the volume of sugar per 100 mL in their offerings. The revenue (estimated at £500 m/year) generated via the SDIL will be used to support action on childhood obesity through schools. Schools currently receive approximately £10,000/year in the form of a School Sports Premium, a figure which

will be doubled based on the revenue of the SDIL (i.e., to £20,000). However, the guidance from the Department for Education (2017) on how to use the money is focused on the provision of school sport and physical activity. It does not state that it could be used to help tackle obesity.

In comparison to the £40m per annum that is invested in childhood obesity services, the anticipated £500 m per annum poses as a significant opportunity to help drive system change. If the School Sports Premium is doubled without the provision of adequate guidance for schools, then there is a real risk that this investment could make the current situation worse. If the premium is invested in sports equipment, facilities, and opportunities to take part in sport as per the guidance, then we may see children who are already active continuing to be active, and those who dislike sport and physical activity continuing to be so as well. This would widen inequalities rather than reduce them, and have a negligible impact on childhood obesity. A whole systems approach would understand where this money could be best placed in a school setting, and how additional actions could then support change within schools. For example, specialists could work with schools to utilize this money to create a salubrious whole school environment. Money could be invested to help teachers increase their knowledge, capability and capacity to help children with overweight and obesity—a role which is traditionally filled by Public Health teams in LAs. Work could also be done with school meal providers to improve the quality of their offer. Schools are under great pressures to achieve outcomes associated with literacy and numeracy, but what if health was also an outcome that was of equal importance? This too would be part of a whole systems approach.

One can see the logic in shifting some responsibility to other national and local government agencies (e.g., Department for Education), and supporting schools to increase sport and physical activity as part of the Childhood Obesity Plan. However, this thinking is narrow, naïve and linear and does not appreciate the complex adaptive system of obesity. Taking a whole systems approach has a great potential to focus attention on how to most effectively disrupt the system in a sustainable way towards the desired outcome.

Summary

Childhood obesity is one of the greatest challenges of this century. It can severely limit the lives of those who live with overweight and obesity, but also has the potential to impair the health services of the future. Due to the complex nature of obesity and the lack of success in reducing its prevalence, there has been little sustained action to date in the United Kingdom. Although the short-term evidence of WM programs for children with obesity is clear, that multidisciplinary services are effective and cost effective, this article has illustrated how the political and environmental contexts are limiting factors of their efficacy. Whilst many different programs are available, we must acknowledge that WM will only ever be truly effective if implemented as part of a wider system wide approach. For those wishing to implement WM in the future, it is paramount that one understands the context in which their program is implemented. It is also important that one seeks the perspectives of all stakeholders who would be involved in their implementation—from service users through to service purchases—to design a WM program that is fit for purpose. As hopefully evidenced throughout, WM is far more than the creation of a sustained energy imbalance.

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Congenital Hypothyroidism: Screening, Early Management, and Outcome

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Introduction

Congenital hypothyroidism (CHT) is one of the more frequent endocrine disorders managed by pediatricians and pediatric endocrinologists and is the commonest preventable cause of neurodisability. The early identification of babies with CHT and their management with thyroid hormone replacement will have a major impact on neurodevelopmental outcome (Grosse and Van Vliet, 2011). An understanding of the perinatal physiology of thyroid stimulating hormone (TSH) and thyroid hormone secretion is important when assessing and managing the neonate with suspected thyroid dysfunction. Understanding how TSH and thyroid hormone concentrations change in early life is also important when establishing or refining a neonatal screening program for CHT.

Perinatal Physiology of TSH and Thyroid Hormone Secretion

In the first trimester thyroid hormone in the fetus will be derived primarily from the mother. Maternal free thyroxine (Free T4) crosses the placenta and is then converted to tri-iodothyronine (T3). The fetal hypothalamo-pituitary-thyroid axis is starting to function by mid-gestation. Endogenous fetal thyroid hormone production rises steadily although a substantial amount of circulating thyroid hormone—30% to 50%—is still derived from mother in the final trimester (Vulsma *et al.*, 1989). The importance of maternal thyroid hormone transfer in the latter stages of pregnancy is illustrated by the life threatening illness seen in hypothyroid infants born to hypothyroid mothers (De Zegher *et al.*, 1995). Thyroid hormone (T4) has a half-life of around 7–10 days and so maternal thyroid hormone will continue to have a neuroprotective effect in the neonate with CHT in the first days of life.

Thyroid hormone metabolism in utero

In cord and foetal serum, T4, T3, and thyroid binding globulin (TBG) levels increase during gestation until term. TSH, FT4, and rT3 levels increase and peak in the late second/early third trimester and then fall until term (Williams *et al.*, 2004). A system of enzymes regulates the amount of active T3 delivered to fetal tissues during pregnancy. Most circulating T_3 is derived from peripheral monodeiodination of the outer ring of T_4, which therefore acts as a reservoir for the more active T_3. An alternative monodeiodination affects the inner ring of T_4 and produces reverse T_3 (rT_3) which is inactive. There is therefore a mechanism for regulating production of the most active and least active thyroid hormones. There are three deiodinases—type I (D1), which has outer and inner deiodination activity (generating T3 and rT3), type II deiodinase (D2), which catalyzes outer-ring deiodination (generating T3), and type III deiodinase (D3), which catalyzes inner-ring deiodination (generating rT3) (Maia *et al.*, 2011; Williams and Bassett, 2011).

Fetal thyroid hormone metabolism is characterized by a predominance of D3 activity outside the CNS. Tissues such as the liver and kidney convert maternal and endogenous fetal T_4 preferentially to rT_3 which may help to reduce tissue thermogenesis. Thyroxine (rather than T_3) is required by the developing brain, and the appropriate deiodinases (particularly D2) are expressed in a temporal and spatial manner in different brain regions. Sulphotransferase enzymes have an additional role in normal thyroid hormone metabolism. T_4 sulphation blocks outer-ring deiodination to T_3 whilst promoting inner-ring conversion to inactive rT_3. The activity of sulphotransferase enzymes in tissues like the liver will therefore also contribute to the availability of thyroid hormone.

Thyroid function in the neonatal period

Birth is associated with a surge in TSH concentrations that peaks after around 30 min. TSH levels then fall steadily in the next 72 h. The TSH surge stimulates thyroid hormone production by the thyroid gland. TSH and thyroid hormone concentrations during the first days of life are therefore elevated when compared to those observed beyond the neonatal period. Enhanced peripheral conversion of T_4 to T3, a reflection of D2 activity, also occurs after birth.

Thyroid function in preterm infants

The pattern of thyroid hormone concentrations in cord blood provides some insight into the level of thyroid hormones during pregnancy (Williams *et al.*, 2004). In preterm infants the levels of T3 remain higher than cord values of babies of equivalent gestational age for several weeks (Williams *et al.*, 2004). Preterm infants (down to 30 weeks) show similar but lesser changes in TSH and thyroid hormone concentrations although the most preterm babies (23–27 weeks) have relatively low T4 values. By 1–2 months of age, thyroid hormone levels are comparable to those in term infants. In the case of infants under 30 weeks' gestation, the postnatal TSH surge is markedly attenuated and T_4 levels frequently fall to a nadir around 1–2 weeks of age

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(Murphy *et al.*, 2004). This pattern is more pronounced with increasing prematurity. Thyroxine levels remain below those of full-term infants through the first few weeks of life and climb gradually to normal postnatal levels.

Congenital Hypothyroidism

CHT is characterized by abnormalities of the hypothalamo-pituitary thyroid axis with abnormally low perinatal thyroid hormone production. This may reflect a primary abnormality of thyroid gland function (primary CHT) which is therefore associated with elevated TSH concentrations or it may reflect reduced pituitary TSH generation reflecting hypothalamic or pituitary disease with associated reduced thyroid gland stimulation (secondary CHT or central CHT—CCHT). TSH and thyroxine concentrations in primary CHT can be viewed as a continuum with increasing TSH levels more likely to be associated with a low T4.

Primary CHT

Incidence and epidemiology

The incidence of primary CHT in many countries that have neonatal screening programs is 1 in 2000–4000 births. Females are affected twice as commonly as males in babies at the severe end of the disease spectrum. Severe primary CHT is characterized by markedly elevated TSH concentrations in the context of very low thyroid hormone levels. Abnormal thyroid function at the milder end of the spectrum tends to have a more equal sex incidence, suggesting a different underlying pathogenesis in many of these babies (described in more detail below). CHT may be more common in Asian, Native American and Hispanic populations and less common in White and Black populations. The incidence is relatively low in Sub-Saharan Africa. CHT appears to be more common in the context of multiple pregnancies (Olivieri *et al.*, 2007). Some studies have reported a rise in the incidence of primary CHT in recent decades in Europe and North America (Olivieri *et al.*, 2015). TSH concentrations form a continuum and a change in neonatal screening practice over the years with a reduction in the TSH cut-off value used to separate pathology from normality is one reason for this trend (Pollitt, 2016). There is, nevertheless, evidence to suggest that the incidence of primary CHT may be rising independently of a change in screening practice. This could be linked to factors such as a change in the ethnic mix of the screening population and increased survival of preterm babies (Olivieri *et al.*, 2007).

Etiology

Dysgenesis

Eighty-five percentage of cases of primary CHT at the more severe end of the phenotypic spectrum are caused by thyroid dysgenesis (defects in thyroid gland development) consisting of thyroid gland ectopia, hypoplasia, and athyreosis. An ectopic thyroid accounts for around two thirds of thyroid dysgenesis. The cause of thyroid dysgenesis is unclear in most instances. Clustering of cases of babies with raised TSH values in Northern England suggests that environmental factors may be involved in the etiology of CHT due to gland dysgenesis (Pearce *et al.*, 2011) although another study concluded that a stable incidence with no monthly variability suggests that this is not the case (Deladoey *et al.*, 2011).

Dyshormonogenesis

Around 15% of CHT cases at the more severe end of the phenotypic spectrum are caused by dyshormonogenesis. In thyroid dyshormonogenesis there is an inborn error of thyroxine synthesis or thyroid hormone secretion. Thyroid dyshormonogenesis is usually inherited in an autosomal recessive pattern with an underlying defect in one of the known steps in the thyroid hormone biosynthesis pathway. Dominant inheritance has been described in some thyroid hormone synthetic defects.

CHT at the mild end of the phenotypic spectrum

It has become clear that the distribution of babies with dysgenesis and dyshormonogenesis at the milder end of the phenotypic spectrum is different to that originally described in babies with CHT (Olivieri *et al.*, 2013). The sex incidence is equal at this mild end of the spectrum and thyroid dysgenesis is less common. Babies in this group are more likely to be preterm, to have been exposed to medications and the thyroid dysfunction is more likely to be transient. A relatively high proportion of babies appear to have abnormalities of DUOX although there are many babies who do not have a specific underlying explanation for the mildly elevated TSH levels.

Iodine and CHT

Iodine is a key component of thyroid hormone synthesis and iodine deficiency is a major cause of CHT in some parts of the world. Nearly one third of the world's population live in areas of iodine deficiency with South-Asia and Sub-Saharan Africa particularly affected. Iodine deficiency is the most common cause of thyroid disorders worldwide and may also cause transient CHT in developed countries. When severe iodine deficiency occurs during pregnancy, it is associated with severe neurocognitive defects in the offspring and increased neonatal and infant mortality. There has been a recent focus on the iodine status of many countries in Europe and up to 60% of girls and pregnant women in the United Kingdom have insufficient dietary intake of iodine (Vanderpump *et al.*, 2011). Recent data suggest that iodine status in utero is related to subsequent neurodevelopment (Bath *et al.*,

2013) although iodine status does not appear to impact greatly on neonatal thyroid TSH concentrations in some parts of the United Kingdom (Evans *et al.*, 2014). Iodine exposure (as a topical disinfectant or as a radiology agent) is a recognized cause of a transient increase in neonatal TSH levels and can result in babies screening "positive" on neonatal screening programs with subsequent normalization of thyroid function.

CHT and prematurity

Some studies have indicated that the incidence of CHT is higher in preterm infants although many reported abnormalities of thyroid function in these babies are transient and/or at the milder end of the biochemical spectrum and the underlying pathology where thyroid dysfunction is permanent is not usually dysgenesis (Olivieri *et al.*, 2013; Mengreli *et al.*, 2010; Mitchell *et al.*, 2011). The reported increased incidence in some studies can partly be explained on the basis of a relative immaturity of the thyroid axis as well as an increased prevalence of illness and drug exposure. Preterm babies are more likely to be exposed to topical iodine or iodine containing radiographic agents which can interfere with thyroid hormone generation (the Wolff–Chiakoff effect) as alluded to above. There is little evidence to suggest that thyroid dysgenesis is increased in the preterm population and it is of note that severe CHT is classically associated with babies being born postterm.

Clinical assessment of primary CHT

A detailed history should be obtained with a view to establishing whether family members (parents, siblings) have thyroid disorders and whether the baby or mother has been exposed to medications that can impact on thyroid function. The baby should be examined and it is important to remember that congenital malformations may be more common in this group of babies (Azar-Kolakez *et al.*, 2013). The clinical features of CHT are outlined in **Table 1**.

Babies with severe primary CHT may have feeding difficulties, sleepiness, constipation, and prolonged jaundice. Babies with very low thyroid hormone concentrations may have a characteristic facies with a flat nasal bridge, a large posterior fontanelle, macroglossia, a distended abdomen with umbilical hernia, bradycardia and hypotonia with delayed reflexes. The skin may be cool and mottled in appearance. Many infants with primary CHT have few clinical manifestations of thyroid hormone deficiency in the initial phase (Grant *et al.*, 1992) and in the absence of neonatal screening will present with slow growth or abnormal development at a later stage. Sixteen percentage of babies with severe biochemical hypothyroidism have no obvious symptoms of hypothyroidism prior to detection by neonatal screening (Grant *et al.*, 1992). This may reflect the protective effect of thyroid hormone transfer from mother and its' impact on phenotype. There is an inverse relationship between age at treatment initiation and intelligence quotient (IQ) in later life and so babies with CHT should be managed as swiftly as possible.

Investigation

Although the most valuable index of thyroid status is the measurement of TSH and thyroid hormone concentrations it is useful to establish the etiology by one or more investigations such as isotope scanning (technetium-99m pertechnetate or iodine (^{123}I)), ultrasonography (Lucas-Herald *et al.*, 2014) and/or measurement of thyroglobulin as well as genetic analysis. **Fig. 1**A shows an MRI scan conducted in a baby with stridor and an ectopic thyroid gland and **Fig. 1**B shows an isotope scan of an ectopic thyroid gland. The potential advantages of thyroid imaging and further investigation are as follows:

Table 1 Key clinical features of congenital hypothyroidism

Clinical features of congenital hypothyroidism
• Asymptomatic
• Increased birth weight
• Lethargy
• Hoarse cry
• Feeding problems
• Cool peripheries
• Constipation
• Macroglossia
• Coarse features
• Umbilical hernia
• Large fontanelles
• Hypotonia
• Hypothermia
• Bradycardia
• Dry skin
• Prolonged jaundice
• Goiter

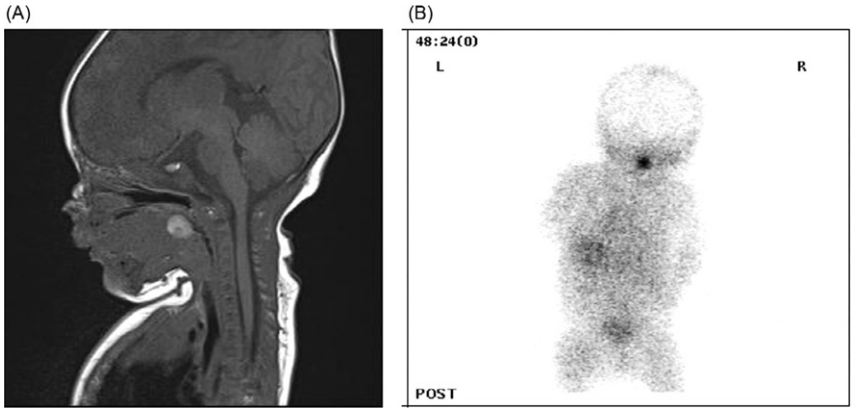

Fig. 1 (A) MRI demonstrating an ectopic thyroid gland in a baby investigated for stridor. (B) Technetium radioisotope scan demonstrating thyroid ectopia.

1. If the baby has thyroid agenesis then the family can be informed that thyroid hormone treatment will be needed for life. The baby with an in situ gland may still prove to have transient hypothyroidism because, for example, antibody transfer from mother may be interfering with thyroid gland stimulation.
2. If the thyroid gland is in situ then the baby may have dyshormonogenesis with a 1 in 4 recurrence risk. Families frequently ask about recurrence risk and so investigation will help to address this question satisfactorily.
3. Thyroid agenesis is more likely to be associated with other developmental problems and highlights the need for a detailed assessment, looking for other congenital anomalies. Hearing screening is particularly important in these babies.
4. This information may help to refine treatment by indicating that the baby may need a larger (in the case of agenesis) or smaller (in the case of thyroid ectopy) dose of levothyroxine.

Some clinicians still feel that imaging is not crucial because it does not influence whether the baby is treated with thyroxine. Investigations will also entail hospital visits and may involve exposure to ionizing radiation. These issues should be discussed with families so they can make an informed choice about whether they feel further investigation is warranted.

An isotope scan can most easily be interpreted when TSH levels are elevated but can still be performed several days post therapy because of the time taken for TSH to normalize. An X-ray of the knee is a guide to the extent of the in utero thyroid hormone deficiency—if the tibial/femoral epiphysis is absent then this suggests more severe in utero thyroid hormone deficiency. Increasingly genetic analysis can be undertaken in children with an in situ gland suggestive of a dyshormonogenesis, looking for abnormalities of (for example) the thyroglobulin or TSHR genes. Hearing assessment is important in babies with CHT because of the strong association with sensorineural deafness—even in the absence of Pendred syndrome (Léger *et al.*, 2014; Bruno *et al.*, 2015).

Treatment

Initial management and monitoring

Babies who screen-positive on a neonatal screening program for CHT should have formal serum thyroid function tests checked as soon as possible and ideally within 24 h. The result of thyroid function tests will frequently be available soon afterwards but if not then it is advisable to treat the baby with thyroxine if the baby has clinical features in keeping with CHT or if the blood-spot TSH is greater than 40 mU/L. If the blood spot TSH is in the range 20–40 mU/L then there is an argument for waiting until thyroid function is available the following day (Léger *et al.*, 2014; Pokrovska *et al.*, 2016).

The goal of thyroxine therapy is to ensure that children have growth and neurodevelopment that is as near to their genetic potential as possible. Levothyroxine (l-thyroxine) is the treatment of choice and has a bioavailability of 50%–80%. Soy milk and medication such as iron can significantly impair thyroxine absorption. Although triiodithyronine (T3) is the biologically active form of the hormone, most T3 in the brain is formed from local deiodination of T4 and so T3 replacement is not usually required. The exception would be the baby with CHT who cannot be fed because of gut pathology in which case T3 is the only thyroid hormone available in intravenous form. The recommended initial l-thyroxine dose is 10–15 μg/kg/day (Léger *et al.*, 2014) for babies at the more severe end of the spectrum. In term infants this amounts to an average of 37.5–50 μg/day. Children with significant endogenous thyroid hormone production—as evidenced by a more subtle TSH increase and an in situ or ectopic gland—may need smaller doses of T4 around 5–10 μg/kg/day. It is important to review the baby again within the first 2 weeks to make sure that the biochemistry is improving. Babies started on larger doses of levothyroxine may need a dose reduction in the subsequent weeks.

Plasma FT3 and FT4 concentrations reach the normal range a few days after thyroxine treatment is started but it can take several weeks for TSH concentrations to normalize. A relationship between the time taken for TSH to normalize and neurodevelopmental

outcome has been described and it is important to closely monitor infants and adjust the l-thyroxine dose frequently until the desired level is achieved.

Normal TSH levels in some babies with dysgenesis on thyroxine replacement may be associated with relatively high circulating FT4 concentrations. This is because the thyroid gland produces both T4 and T3 and generating adequate tissue T3 may require higher than normal circulating FT4 concentrations in the absence of endogenous thyroid gland T3 release. It has been suggested that larger doses of T4 may also be required to normalize TSH concentrations in some children with CHT when compared to other forms of hypothyroidism because of pituitary-thyroid hormone resistance that reflects a period of relative hypothyroxinaemia in utero.

The developing brain has a critical dependence on thyroid hormone in the first 2–3 years of life and so frequent monitoring is important. Outpatient review and thyroid function testing may be needed every 4 weeks during the phase of particularly rapid growth in the first year of life (Balhara *et al.*, 2011).

There is a strong argument for these babies being under the care of a pediatrician with a specialist interest and experience of treating babies with CHT and hence who is more likely to be aware of the pitfalls and nuances of management.

Which thyroid hormone formulation?

The preferred means of administration is with levothyroxine tablets that have been crushed and administered via a small spoon in a few milliliters of water, bottle or breast milk (Léger *et al.*, 2014). Pharmaceutically produced solutions can be used but are expensive and of different strengths which can result in confusion and inappropriate dosing. New preparations of 12.5 μg of thyroxine have recently become available. They are particularly useful in children but are more expensive than the earlier, standard tablet doses.

What biochemical threshold warrants intervention?

One of the more controversial areas in pediatric endocrinology is the question of the TSH threshold at which levothyroxine should be commenced (Lain *et al.*, 2017). The significance of borderline TSH elevation is unclear although a recent association between mild TSH elevation (as opposed to the profound increase in TSH seen in thyroid dysgenesis) and suboptimal neurodevelopment has been described in a large study from Australia (Lain *et al.*, 2016). In contrast an earlier study from Belgium had shown no association between TSH groups (<5, 5–9, >10 mU/L) and cognitive outcome (Trumpff *et al.*, 2015). An association between TSH >10 mU/L but less than 15mU/L and a lower verbal IQ was not present following adjustment for potential confounders such as household income and maternal education (Trumpff *et al.*, 2015). This uncertainty helps to explain why there are regional and national differences in screening cut-off values and differences in the way babies are managed. When venous TSH levels are 10–20 mU/L in the presence of age appropriate FT4 levels it is reasonable to repeat the blood test in one to 2 weeks and to consider an ultrasound or isotope scan in the interim. However a serum TSH greater than 10mU is more than three SD above the mean in most populations and may be considered a criterion for intervention on these grounds alone.

Transient hypothyroidism

Between 19% and 28% of babies diagnosed with CHT have transient CHT (Mitchell *et al.*, 2011; Korzeniewski *et al.*, 2013). Transient CHT is more likely at the milder end of the biochemical spectrum. Transient CHT can be caused by maternal or neonatal factors and can persist for a variable amount of time postnatally. Maternal factors include iodine deficiency or excess, transplacental passage of antithyroid antibodies and fetal exposure to antithyroid medication. Neonatal factors include iodine deficiency or excess, and hepatic haemangiomas (which increase type 3 iodothyronine deiodinase activity, converting T4, and T3 to inactive metabolites). Although thyroid dyshormonogenesis is typically seen as a cause of permanent CHT it is known that mutations in *DUOX2* and *DUOX2A* can cause transient CHT (Hoste *et al.*, 2010). In this instance thyroid hormone production is compromised with an increase in TSH in early life but not necessarily at a later stage—presumably because the demand for thyroid hormone is reduced beyond infancy.

Down's syndrome

Babies with Down's syndrome have an altered relationship between TSH and thyroid hormone concentrations when compared to the population as a whole. Down's syndrome babies overall have higher TSH levels but also higher thyroid hormone concentrations. The relatively high TSH levels are therefore a normal observation in healthy Down's babies and the threshold for intervention should therefore be higher than the population as a whole. In one study the 95th centile for TSH concentrations was around 9mU/L in Down's babies versus 5.5mU/L in the population as a whole (Meyerovitch *et al.*, 2012). The reason for the altered TSH/thyroid hormone axis is unclear and it is of note that none of the genes known to be involved in HPT regulation are encoded on chromosome 21. Babies with Down's syndrome can still have CHT due to thyroid dysgenesis.

Secondary CHT

Etiology

Most cases of central hypothyroidism (CCHT) where TSH is not secreted appropriately by the pituitary gland are associated with other pituitary hormone deficiencies in the context of combined pituitary hormone deficiency (CPHD). Babies with CCHT may

therefore have additional phenotypic clues such as hypoglycaemia due to adrenocorticotropic hormone (ACTH) and growth hormone (GH) deficiency as well as microphallus due to gonadotrophin deficiency. Babies with CCHT may also have conjugated hyperbilirubinaemia because of cortisol and thyroid hormone deficiency. Transient CCHT may be due to TSH suppression because of excessive thyroid hormone transfer from a mother with Graves' disease.

Incidence

The incidence of isolated TSHD elucidated from neonatal screening programs for CHT depends upon the strategy used and whether cases at the milder end of the phenotypic spectrum are detected. The incidence based on a program that measured TSH and T4/FT4 in Japan was 1 in 160,000 (Asakura *et al.*, 2002) but a strategy that included the measurement of thyroid binding globulin (TBG) revealed an incidence of 1 in 16,000 in Western Europe (Kempers *et al.*, 2006; Lanting *et al.*, 2005). The overall incidence of CCHT (babies with and without other pituitary hormone deficiencies) in Western Europe, based on more intensive screening programs that measure thyroid hormone levels as well as TSH and thyroid-binding globulin (TBG), is around 1 in 20,000. Around 75%–80% of these babies will have CPHD.

Clinical Features

Babies do not necessarily present in the early neonatal period (Kempers *et al.*, 2006; Lanting *et al.*, 2005) and thyroid function should be checked in any baby with symptoms suggestive of CPHD such as hypoglycaemia, prolonged jaundice (particularly conjugated hyperbilirubinaemia) or microphallus. Features of isolated TSHD may be relatively subtle but there may also be a classical neonatal hypothyroid phenotype. Late treatment can be associated with neurodevelopmental problems, particularly in those babies at the more severe end of the phenotypic spectrum.

Investigation

The diagnosis of TSHD requires the identification of low T4 levels together with low or "inappropriately" normal TSH concentrations. Screening programs and laboratories that only measure TSH concentrations will not identify cases of central hypothyroidism. TSH concentrations in CPHD can also be paradoxically mildly elevated which may reflect impaired biological activity with retained immunoreactivity although hypothalamic somatostatin deficiency could be a contributing factor. The TRH test as a means of investigating infants with suspected central hypothyroidism has both advocates and detractors (Van Tijn *et al.*, 2008; Mehta *et al.*, 2003).

Treatment of secondary CHT

The objective of therapy in CCHT is to achieve FT4 concentrations in the top part of the age-appropriate reference range (Persani, 2012). TSH cannot be used as a guide to therapy and will typically become unrecordable in instances where it has previously been mildly elevated or normal (see above). Other pituitary hormones should be evaluated in patients with central hypothyroidism, especially assessment of the hypothalamic-pituitary-adrenal axis to exclude hypocortisolism. Babies with abnormal ACTH/cortisol secretion should be treated prior to initiating thyroid hormone replacement.

Newborn Screening for CHT

The objective of screening for CHT is to eradicate mental retardation arising as a consequence of CHT, Screening for CHT has been shown to be highly cost effective (Rose *et al.*, 2006; Geelhoed *et al.*, 2005). In many countries, newborn infants are screened via a blood spot sample taken on "filter-paper" at 3–5 days of age. Screening programs for CHT have relied on two major methods: a primary TSH method ($\pm$ backup T4 below a certain threshold) and a primary T4 method ($\pm$ backup TSH above a certain threshold). Most programs in Europe, the United States, Japan and Canada use the primary TSH method, but there a number of programs that routinely measure both TSH and T4 (Kempers *et al.*, 2006). There are advantages and disadvantages to each method used (**Table 2**). Improved assay sensitivity (fewer false negatives) will typically be associated with compromised specificity (more

Table 2 Major advantages of screening strategies relying on either TSH or T4 as primary testing method

Testing method	*Advantages*	*Disadvantages*
Primary TSH ($\pm T_4$ backup)	• Will detect primary hypothyroidism with elevated TSH • High sensitivity and specificity • Age-adjusted TSH cut-offs allow earlier testing for discharge <48 h	• Will not detect central hypothyroidism • Will not detect cases where TSH elevation is delayed although this may be less of an issue with a relatively low TSH cut-off
Primary T_4 ($\pm$TSH backup)	• Will detect primary hypothyroidism if T4 low • Will detect central hypothyroidism	• May not detect mild CHT with increase in TSH • Normal T4 with delayed rise of TSH • Can be associated with a higher number of false-positives resulting in a high recall rate

false positives) and the concern with lower TSH screening "cut-offs" is that more families are subjected to the anxiety of being notified as screen "positive" that is then disproved by normal serum thyroid function tests.

A combined approach where both TSH and Free thyroid hormone (FT4) is determined is very attractive in principle because it can detect both primary and secondary hypothyroidism, but measuring FT4 in blood spot samples is not straight-forward which is why some programs measure T4 with TBG to gauge the amount of "free" thyroid hormone (Kempers *et al.*, 2006).

Screening programs will not detect all cases of CHT and health professionals need to be alert to the signs of hypothyroidism and have a low threshold for formally testing thyroid function irrespective of earlier screening.

Selecting an appropriate screening threshold

All screening programs will miss affected babies and improving sensitivity by (for example) reducing a TSH threshold will reduce specificity with more false positives. As TSH assays have become more refined then so screening primary TSH programs have tended to use a lower threshold at which diagnostic investigation is indicated. Many programs have a borderline TSH category which necessitates a repeat blood spot sample being taken if the preliminary value is above the "norm" but below a threshold at which primary CHT is highly likely. Many of these babies will then have a normal "repeat" blood spot TSH with further investigation therefore unnecessary. The threshold for a positive result varies across the United Kingdom and Europe even though similar assay techniques are used. Although the likelihood of detecting cases of permanent CHT is reduced as TSH falls there are still programs that report around 20% of cases of dysgenesis in babies with permanent CHT whose baseline blood spot TSH was less than 15 mU/L (Olivieri *et al.*, 2013). In the United Kingdom some regions selected a blood spot TSH cut-off below 10 mU/L because they too identified thyroid pathology that would have been "missed" by a higher value (Langham *et al.*, 2013). Consideration of the risks and benefits is important because whilst a lower threshold will detect additional cases of CHT, these cases will tend to be at the milder end of the phenotypic spectrum in terms of the extent to which thyroid hormone production is compromised (Krude and Blankenstein, 2011; Cheetham, 2011). Lower thresholds will lead to a marked increase in the number of repeat tests with implications for families as well as laboratory workload (Pollitt, 2016). The process of recalling a baby who ultimately turns out not to have CHT on further investigations is not without cost (Hewlett and Waisbren, 2006).

Whilst there has been a great deal of focus on what constitutes an appropriate cut-off there are other factors that will impact on (for example) the TSH concentration such as the timing of the sample - a sample taken at just over 4 days ($\sim$ 97 h) will be greater than a sample taken at just less than 5 days (119 h) and an assay performed on a sample taken from the center of a large blood spot will tend to generate a higher TSH value than a sample taken from the periphery of a smaller blood spot. There are other causes of a raised TSH besides CHT (**Table 3**).

Screening preterm infants

A concern is that TSH concentrations in preterm infants may not rise in the presence of impaired thyroid hormone generation as they do in term babies although some studies have shown that this is not an issue in practice (Vincent *et al.*, 2002). This observation is usually attributed to relative immaturity of the hypothalamo-pituitary thyroid axis and has implications for neonatal screening. A concern is that CHT screening programs linked to TSH measurement may miss cases because the TSH has yet to rise (Murphy *et al.*, 2004). CHT in preterm babies may also be masked by suppression of TSH caused by drug administration, fetal blood mixing in multiple births and by the impact of serious neonatal illness on thyroid function. Because of this many countries now undertake repeat screening on babies born before a certain gestation or under a certain weight. In the United Kingdom all babies born less than 32 weeks gestation undergo a repeat test 4 weeks later or at the time of discharge. Whilst this strategy may help to detect babies with longer term thyroid dysfunction it remains unclear to what extent an initial screen with a

Table 3 Principle causes of a raised TSH in infancy

Principle causes of a raised TSH in infancy
• Dysgenesis
• Dsyhormonogenesis
• Prematurity
• "Euthyroid sickness"
• Iodine exposure
• Iodine deficiency
• Maternal antibody exposure
• Hypopituitarism
• Drugs
• Downs' syndrome
• Adrenal insufficiency
• Thyroid hormone resistance
• MTC deficiency
• Pseudohypoparathyroidism

relatively low threshold and later screening at 5 days rather than within 48 h will also detect these babies (Korada *et al.*, 2008). It is also unclear to what extent the babies who screen positive on second screen have transient as opposed to permanent thyroid dysfunction.

Re-evaluation of the thyroid axis in babies with primary CHT

Babies who have not required an increase in levothyroxine dose, have not had imaging confirming CHT or have had earlier normal imaging, warrant a trial off therapy at around 3 years of age. This is then beyond the critical phase of brain growth. In one study only a third of children diagnosed with CHT and who had a gland in situ required long term thyroxine therapy (Rabbiosi *et al.*, 2013). It is possible to simply stop the levothyroxine for 2–3 weeks and then check thyroid function tests. If thyroid function is normal, our practice is to repeat the testing again after a further 6 weeks and then consider discharge if the biochemistry remains normal.

Early neurodevelopmental outcome in CHT

Thyroid hormone deficiency has a deleterious impact on central nervous system development. CHT is the most common preventable cause of intellectual disability in children and the link with abnormal cognitive development is the key reason for the introduction of neonatal screening programmes for CHT. The mean intelligence quotient (IQ) score of patients with CHT presenting clinically is around 80 with an IQ score less than 50 in around 5% of such children (Grosse and Van Vliet, 2011). As a general rule it appears that the later the diagnosis and treatment in a baby with clinical manifestations of CHT, the lower the IQ score. Average IQ scores around 90 are seen in babies with CHT treated prior to 3 months, 70 in those treated between 3 and 6 months of age with a mean IQ of 35 reported when the diagnosis of CHT is delayed beyond 6 months of age (Grosse and Van Vliet, 2011; Klein *et al.*, 1972). On the other hand, patients with a substantial increase in TSH concentrations but without overt clinical signs of CHT have an IQ that is close to average with none having an IQ <70 (Alm *et al.*, 1984). Primary CHT represents a spectrum of severity ranging from babies with no endogenous thyroid function to babies with elevated TSH concentrations in the context of thyroid hormone concentrations within the age-related reference range. There is evidence to suggest that there is a point on this spectrum below which there is an unequivocal, detrimental impact of thyroid hormone insufficiency on neurodevelopmental outcome (Tillotson *et al.*, 1994).

Factors determining outcome in CHT

Screening programmes for CHT have been a great success but despite early diagnosis and treatment numerous authors have noted subtle deficits in terms of speech and language, visuospatial function, attention span as well as minor motor problems in many children with CHT when compared to the healthy population (Rovet and Ehrlich, 2000). Investigators have looked at the potential explanations for this and have noted that there are three main variables that seem to impact on neurodevelopmental outcome in CHT—two of which are modifiable.

1. Severity of the thyroid hormone deficiency
   A positive association between motor outcome in CHT (Oerbeck *et al.*, 2003) as well as IQ (Kempers *et al.*, 2006) with serum thyroid hormone concentration at diagnosis has been reported. Children at the mild end of the biochemical spectrum with higher thyroid hormone concentrations may not be at risk of intellectual impairment (Tillotson *et al.*, 1994; Alm *et al.*, 1984). Babies with more severe CHT will have had substantially lower thyroid hormone concentrations in utero and transplacental transfer of thyroid hormone from mother alone is insufficient to normalize brain development in these circumstances.
2. Interval between birth and thyroid hormone intervention
   Data from the prescreening era clearly show that the later the child is treated, the more profound is the likely impact of thyroid hormone deficiency on neurodevelopment, as outlined above. The extent to which the timing of intervention is important within the first 3 weeks of life is less clear (Boileau *et al.*, 2004) and of note is the fact that in the first days of life there will still be circulating thyroid hormone from mother providing some neuronal protection.
3. Thyroid hormone treatment dose
   A number of studies have now shown that higher doses of thyroxine are associated with a reduction in the discrepancy between the IQ observed in children with CHT versus children who do not have CHT (Dubuis *et al.*, 1996; Bongers-Schokking *et al.*, 2000). This observation has recently been noted once again in the context of a cohort of children from Germany and an associated meta-analysis (Aleksander *et al.*, 2018). Normalizing TSH in babies with severe CHT is frequently associated with relatively high thyroid hormone concentrations. The reported impact of relatively high doses of thyroxine on neurodevelopment is not always positive with this approach also linked to neurobehavioral issues such as anxiety, social withdrawal, and poorer concentration (Rovet and Ehrlich, 1995; Bongers-Schokking *et al.*, 2013). The potential deleterious effects of relatively high-dose T4 therapy have not been noted in all studies (Oerbeck *et al.*, 2005). Whether all the consequences of a substantial reduction in circulating thyroid hormone concentrations in utero can truly be abrogated by prompt and generous thyroxine replacement in the neonatal period remains to be established.

Health outcomes in adult life

While the focus of many neonatal programmes and associated studies has been neurodevelopmental outcome in childhood it has become clear that the diagnosis of CHT may carry with it a risk of subtle health issues in later life (Rovet and Ehrlich, 2000; van der

Sluijs Veer *et al.*, 2008; Léger *et al.*, 2011). Adults with CHT are more likely to be overweight with hearing and visual problems when compared to the population as a whole (Léger *et al.*, 2011). Adults are more likely to have chronic health problems with a lower health-related quality of life and are less likely to be in full-time employment. CHT severity and treatment adequacy as measured by TSH concentrations above or below 20 mU/L were key determinants of long term outcome in one study (Léger *et al.*, 2011). The extent to which close biochemical monitoring in early life with more generous starting doses of T4 in excess of 10 mcg/kg per day will address these issues is unclear as alluded to above. Many authors have described abnormalities of growth in childhood in CHT but final height appears to be similar to that observed in the normal population (Salerno *et al.*, 2001).

Role of monitoring in refining neonatal outcome

There is evidence to indicate that close monitoring of CHT with maintenance of age-appropriate TSH concentrations can also help to improve neurodevelopmental outcome in CHT. Regular clinical and biochemical review with the normalization of TSH concentrations throughout early childhood is key (Balhara *et al.*, 2011; Salerno *et al.*, 2001). In the baby with little endogenous thyroid function this may require regular, monthly review throughout the first year of life (Balhara *et al.*, 2011).

The impact of maternal thyroid status on fetal outcome has been under close scrutiny in recent years. It is becoming clear that the optimization of thyroxine therapy in mothers with CHT around the time of conception and during pregnancy is important based on differences in early developmental milestones in their offspring (Léger *et al.*, 2018).

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Genetic Causes of Congenital Hypothyroidism

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Primary Congenital Hypothyroidism

Classification and Etiology

Primary congenital hypothyroidism (CH) has traditionally been classified as thyroid dysgenesis (TD) or dyshormonogenesis. TD refers to a spectrum of aberrant thyroid gland development, including thyroid ectopy (an abnormally situated thyroid gland, accounting for the majority of TD), athyreosis (complete absence of the thyroid), affecting 20%–30% of TD cases or a normally located but hypoplastic thyroid (approximately 5% of cases). Dyshormonogenesis refers to failure of thyroid hormone production by a structurally intact thyroid gland which lacks a complete, functional molecular pathway for thyroid hormone biosynthesis. Historically, 75%–85% of CH cases have been attributed to TD with the remaining 20% occurring due to dyshormonogenesis. However, more recent studies undertaken in populations using lower screening TSH diagnostic cut-points have reported a doubling in the incidence of CH largely due to increased diagnosis of cases with a normally located gland in situ (GIS) (Corbetta *et al.*, 2009; Szinnai, 2014).

Genetic causes of TD involve genes mediating thyroid growth and development. Although multiple genes have been implicated, TD is generally considered to be a sporadic disease with $<5\%$ cases attributable to an identifiable genetic mutation. The transcription factors involved are usually not thyroid-specific, therefore mutations in the genes encoding them may result in additional congenital abnormalities, reflecting their extrathyroidal expression pattern. Dyshormonogenesis, in contrast, is usually familial, with the majority of affected individuals harboring mutations in genes encoding known components of the thyroid hormone biosynthesis machinery (Grasberger and Refetoff, 2011; Cherella and Wassner, 2017).

Thyroid Gland Development

The identification of monogenic causes of thyroid dysgenesis in patients with CH has informed our understanding of human thyroid development. Moreover, although not identical, human and murine thyroid morphogenesis have a comparable molecular basis, and major morphogenetic steps are conserved in the two species. Therefore studies in these organisms have been crucial both in defining novel CH candidate genes relevant to the human setting, and in elucidating their function.

Genes Involved in the Molecular Control of Thyroid Development

Intrinsic Factors

Transcription factors: NKX2-1, PAX8, HHEX, FOXE1

Early thyroid development is defined by the combined expression of an indispensible quartet of transcription factors; *PAX8* (paired box 8), *NKX2-1* (NK2 homeobox 1, previously known as TTF1), *HHEX* (hemopoetically expressed homeobox) and *FOXE1* (forkhead box E1, previously known as TTF2). Each of these transcription factors also has a developmental role in extra-thyroidal tissues, but their combined expression to mediate organogenesis is observed only in epithelial thyroid follicular cells. Studies of mouse thyroid development have shown that NKX2-1, PAX8 and HHEX are initially expressed independently, but subsequently participate in a network of reciprocal regulatory interaction. FOXE1 is expressed later, requiring PAX8 for the onset of expression. These transcription factors have pleiotrophic roles in the thyroid, mediating the formation of the thyroid bud and facilitating survival of thyroid progenitor cells, as well as driving functional differentiation, and regulating the expression of genes involved in thyroid hormone biosynthesis. Additionally, NKX2-1, PAX8 and FOXE1 play a role in the maintenance of the mature thyroid gland, and FOXE1 has a particular involvement in thyroid migration. Mutations in *NKX2-1*, *PAX8* and *FOXE1* are all associated with thyroid dysgenesis in humans (Trueba *et al.*, 2005; Fernandez *et al.*, 2015; Nilsson and Fagman, 2017; Kimura *et al.*, 1996; Mansouri *et al.*, 1998; de Felice *et al.*, 1998; Martinez Barbera *et al.*, 2000).

TSHR

Early thyroid development is TSH independent, including thyroglobulin synthesis (TG) at the onset of folliculogenesis. However, once morphogenesis is complete, TSH has a role in thyroid growth from the third trimester and drives expression of the components of the thyroid hormone biosynthetic machinery, with murine studies confirming its importance for expression of the sodium-iodide symporter (NIS) and TPO, during terminal differentiation. In developing human thyroid, membrane detection of TSHR precedes accumulation of NIS in the basolateral membrane, consistent with a role for TSH in enabling onset of thyroid hormone synthesis by the fetal thyroid gland (Szinnai *et al.*, 2007; Nilsson and Fagman, 2017).

Extrinsic Factors

The close proximity of the developing thyroid to cardiac mesenchyme and vasculature, and observations that cardiovascular malformations have an increased frequency in patients with thyroid dysgenesis, have lead to the suggestion that extrinsic, noncell autonomous factors may play a role in thyroid development (Devos *et al.*, 1999). Zebrafish and murine models have been used to identify pathways involved. These include mesoderm-derived Tbx1-Fgf8 signaling which may influence thyroid growth and positioning (Fagman *et al.*, 2007). Additionally, recent zebrafish studies suggest that mesenchymal netrin 1, a laminin superfamily member with a role in mediating guidance cues may play an important role both in thyroid morphogenesis and vasculogenesis (Opitz *et al.*, 2015). Although poorly understood, specification of the thyroid domain at the onset of thyroid morphogenesis, must be dependent on extrinsic signals. Recent advances in stem cell technology have enabled the directed differentiation of mouse and human ESCs and human induced pluripotent stem cells (iPSCs) into functional thyrocytes. These experiments have demonstrated that FGF2 and BMP4 are necessary and sufficient for this process, provided that cells are preprogrammed to definitive endoderm (Kurmann *et al.*, 2015) suggesting that FGF2 and BMP4 are likely to play a key role in thyroid specification although their source remains unclear.

Thyroid Dysgenesis

Genes Implicated in Thyroid Dysgenesis (Summarized in Fig. 1 and Table 1)

TSHR

The most commonly mutated gene in TD is the *TSHR* (Cangul *et al.*, 2012; Szinnai, 2014), however, mutations in this gene are associated with a variable spectrum of resistance to TSH, the severity of which depends both on the deleteriousness of the mutation, and the number of mutated *TSHR* alleles. In partial TSH resistance, patients exhibit a euthyroid compensated state with elevated TSH concentrations, normal thyroid hormone concentrations and a normal-sized, eutopic thyroid gland. If TSH resistance is complete, severe CH is associated with marked thyroid hypoplasia, which may be mistaken for athyreosis. However, since early fetal thyroid growth and TG synthesis are TSH independent, serum thyroglobulin (TG) is always detectable in such cases, who exhibit "apparent athyreosis" (Persani *et al.*, 2010).

TSH binds a G-protein coupled receptor, TSH-receptor (TSHR) in the thyroid, for which cyclic AMP (cAMP) is the major second messenger at physiological concentrations although coupling may also occur to Gq. Signaling via TSHR stimulates both follicular cell growth and thyroid hormone synthesis and release (Persani *et al.*, 2010).

More than 60 inactivating *TSHR* mutations have been described, the population frequency of which is dependent on the screening criteria used and may also be influenced by the presence of founder mutations in particular ethnic groups, for example, TSHR p.R450H in individuals from East Asia (Chang *et al.*, 2012; Szinnai, 2014). In populations with TD, a mean prevalence of heterozygous and homozygous *TSHR* mutations was estimated to be 4.3%, but mutations are more common in pediatric cases with nonautoimmune hyperthyrotropinemia due to partial TSH resistance (prevalence 11%–29%) (Nicoletti *et al.*, 2009; Cassio *et al.*, 2013; Szinnai, 2014).

Management of CH due to complete TSH resistance requires levothyroxine replacement but thyroid hormone supplementation in individuals with partial TSH resistance remains controversial (Cassio *et al.*, 2013). These individuals may have compensated hyperthyrotropinemia with resetting of the hypothalamic–pituitary–thyroid axis such that supraphysiological FT4 concentrations are required to achieve a TSH level in the normal range. Since peripheral sensitivity to thyroid hormone is preserved, normalization of TSH may therefore provoke thyrotoxic symptoms, leading to the suggestion that treatment may not be required. In addition to anecdotal reports describing normal growth, development and pituitary appearances in untreated cases despite chronically raised TSH (Clifton-Bligh *et al.*, 1997), two recent studies have assessed the long-term clinical and biochemical outcomes in patients with subclinical hypothyroidism due to *TSHR* mutations. In patients with heterozygous mutations, subclinical hypothyroidism was found to be a stable compensated state which may not usually require thyroid hormone replacement. However, levothyroxine treatment may be required in some patients with either heterozygous mutations and additional risk factors for thyroid dysfunction, or biallelic mutations, in whom incompletely compensated subclinical hypothyroidism may progress (Tenenbaum-Rakover *et al.*, 2015; Vigone *et al.*, 2017). Larger studies, involving a broader spectrum of TSH resistance are still required to guide management definitively.

Key Transcription Factor Mutations in TD

Mutations in three of the four key transcription factor mutations involved in thyroid morphogenesis (*NKX2-1*, *PAX8* and *FOXE1)*, are rare but well-recognized causes of TD with characteristic associated syndromes reflecting their extrathyroidal expression patterns. However, penetrance of both the thyroid phenotype and additional congenital abnormalities associated with these mutations may be highly variable, even within the same family (Szinnai, 2014; Carre *et al.*, 2009). Additionally, biallelic *GLIS3* mutations are now robustly associated with CH in the context of a multisystem phenotype (Dimitri, 2017).

NKX2-1

NKX2-1 belongs to the homeodomain-containing transcription factor family, and is one of the key transcription factors mediating thyroid development. Additionally, it is expressed in the distal pulmonary epithelium where it plays a role in surfactant production, the ventral forebrain, and hypothalamic neurons. Heterozygous *NKX2-1* mutations are the commonest transcription

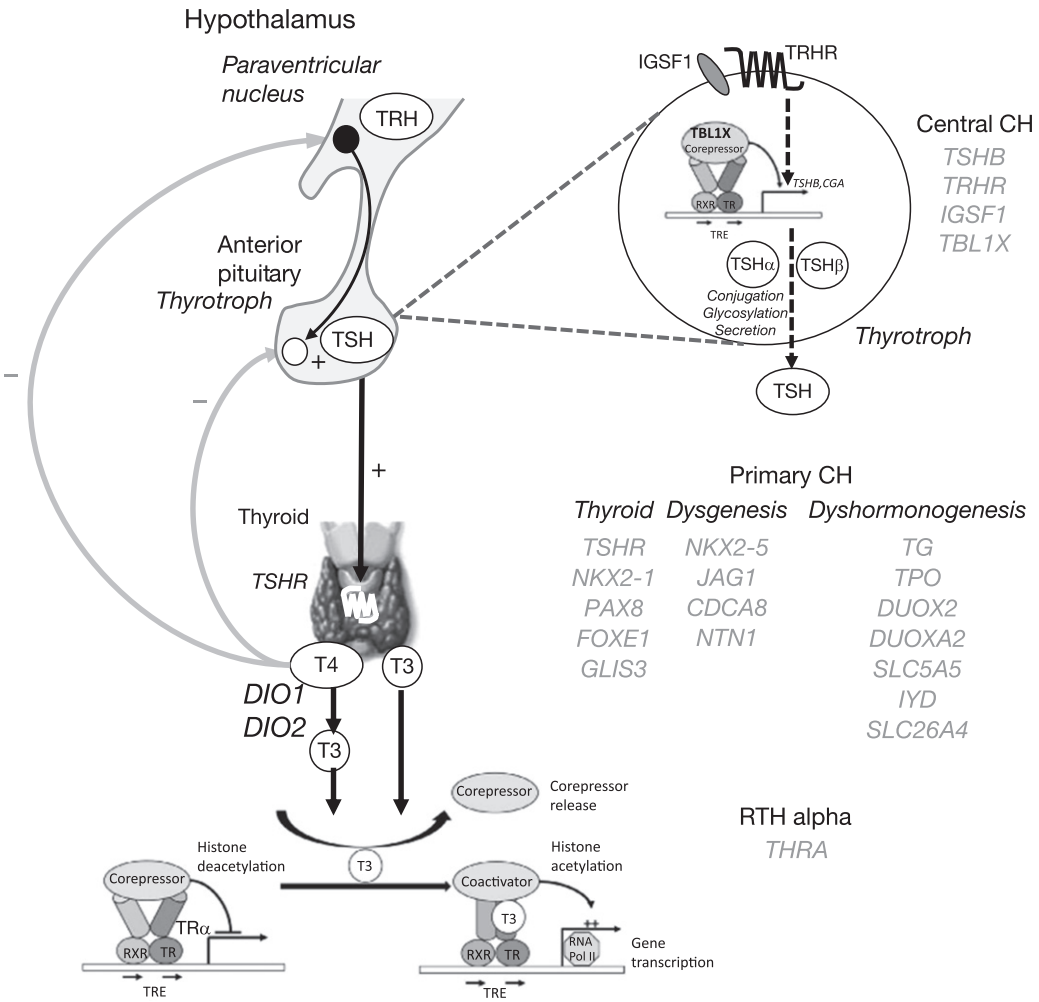

Fig. 1 Schematic summarizing the key genes (gray) implicated in isolated congenital central hypothyroidism (CCH), primary hypothyroidism and RTH alpha. The hypothalamic–pituitary–thyroid axis is illustrated; processes stimulated by TRHR signaling (TSH alpha and beta subunit transcription and conjugation, glycosylation and secretion of mature TSH) are shown by black dotted lines. T4 secreted by the thyroid gland is converted to T3 (the active hormone) by deiodinase enzymes (DIO1, DIO2) before binding its nuclear receptors, enabling corepressor release and coactivator recruitment with subsequent transcriptional activation of positively regulated genes. Proteins encoded by genes implicated in CCH and RTH alpha are shown in *bold type* at their site of action.

factor mutation underlying CH and may cause a spectrum of brain, lung and thyroid disease although penetrance is highly variable, even within the same family, and a complete triad of phenotypes is found in only ~50% of patients (Carre *et al.*, 2009).

The commonest clinical manifestations of *NKX2-1* mutations are neurological (affecting >90% of cases), usually comprising a benign hereditary chorea, although more subtle features such as delayed motor milestones may also occur. Overt or subclinical hypothyroidism occurs in >75% cases and is usually mild, however thyroid morphology ranges from normal (in 55%) to hypoplasia, hemiagenesis or athyreosis. Only 54%–78% of cases exhibit a pulmonary phenotype (most commonly infant respiratory distress syndrome, IRDS), however, this carries an associated mortality of up to 16% (Carre *et al.*, 2009; Thorwarth *et al.*, 2014).

NKX2-1 mutations frequently occur de novo, which, if confirmed, may reassure parents of an affected child that their risk of future affected offspring is similar to that of the background population (Carre *et al.*, 2009; Maquet *et al.*, 2009). There are a significant number of reported deletions involving *NKX2-1*, therefore multiple ligation-dependent probe amplification (MLPA) is required for definitive exclusion of NKX2-1 deficiency (Teissier *et al.*, 2012). Additionally, deletions proximal to *NKX2-1* have also been implicated in the brain-ling-thyroid syndrome (Kharbanda *et al.*, 2017).

PAX8

PAX8 belongs to the paired homeodomain transcription factor family and more than 20 heterozygous, loss of function *PAX8* mutations have been reported, with autosomal dominant inheritance. Most mutations are missense or nonsense substitutions affecting the DNA binding domain of PAX8 but small deletions and promoter region mutations have also been described (de Sanctis *et al.*, 2004; Hermanns *et al.*, 2014b; Perone *et al.*, 2016). The thyroid biochemical phenotype of affected patients is highly

Table 1 A summary of the genetic defects implicated in congenital hypothyroidism and typical clinical features

Gene	*M/B*	*Additional clinical features*	*Typical biochemical features*	*Radiological features*
NKX2-1	M	Neurological (BHC, ~90% cases), respiratory (IRDS, recurrent infections, >50% cases)	Euthyroid–severe CH	GIS–athyreosis
PAX8	M	Urogenital tract malformations (rare)	Euthyroid–severe CH	Typically hypoplasia, range GIS–athyreosis
FOXE1	B	Cleft palate, spiky hair (universal), choanal atresia	Severe CH	Athyreosis
GLIS3	B	Neonatal diabetes, dysmorphic facies, renal cystic dysplasia, hepatic fibrosis, congenital glaucoma, learning difficulties and skeletal abnormalities	Severe CH, TSH resistance	GIS–athyreosis
NKX2-5	M	Congenital heart disease	Euthyroid–severe CH	GIS–ectopy–athyreosis
CDCA8	M/B		Congenital heart disease[a]	Euthyroid–severe CH
Athyreosis-		–ectopy–hemiagenesis–nodules	*JAG1*	M
Alagille syn-		drome/congenital heart disease/isolated CH	Euthyroid/mildly elevated TSH–severe CH	GIS–hypoplasia–ectopy
NTN1[b]	M	Congenital heart disease	Severe CH	Ectopy
TSHR	M/B		May be TSH resistant to L-T4 treatment	Mildly elevated TSH–severe CH
GIS-		–severe hypoplasia	*TG*	B
May cause fetal goiter	E-	uthyroid–severe CH Inappropriately low TG when TSH is elevated	GIS–goiter Normal organification of iodide	
TPO	B[c]	May cause fetal goiter	Severe CH	GIS–goiter TIOD, rarely PIOD
DUOX2	M/B		Transient or permanent, blood spot TSH may be borderline	Mild/transient–severe CH
GIS-		–goiter PIOD		
DUOXA2	M/B		Transient or permanent	Mild/transient CH
GIS-		–goiter PIOD		
Pendrin	M	Sensorineural hearing loss with EVA	Euthyroid/mild hypothyroidism	GIS–goiter PIOD
NIS	M	May present later in childhood resulting in neurodevelopmental delay	Euthyroid–severe CH	GIS–goiter Severely impaired thyroid 123I/Tc uptake
IYD	M/B		Raised urinary MIT and DIT May present later in childhood resulting in neurodevelopmental delay	Euthyroid–severe CH/later onset hypothyroidism
Goiter Normal organification of iodide				

[a] $n = 1$ case.

[b] $n = 1$ case, additional variants: 47, XYY karyotype; and atypical 22q11 deletion.

[c] Occasional monoallelic cases reported.

GIS, normally located thyroid gland in situ; *BHC*, benign hereditary chorea; *EVA*, enlarged vestibular aqueduct; *IRDS*, infant respiratory distress syndrome; *PIOD*, partial iodide organification defect; *TIOD*, total iodide organification defect; *M*, monoallelic; *B*, biallelic.

variable, ranging from euthyroidism to severe hypothyroidism and although the characteristic thyroid morphology comprises thyroid hypoplasia, both thyroid agenesis and normal-sized thyroid glands have been reported. PAX8 is also expressed in the nephrogenic mesenchyme and adult kidney and in rare cases *PAX8* mutations may be associated with urogenital tract abnormalities (Montanelli and Tonacchera, 2010; Ramos *et al.*, 2014; Vincenzi *et al.*, 2014; Carvalho *et al.*, 2013).

FOXE1

FOXE1 belongs to the forkhead/winged-helix transcription factor family and although rarely reported, loss of function biallelic mutations usually cause a consistent syndrome of athyreosis or severe thyroid hypoplasia, cleft palate and spiky hair (Bamforth–Lazarus syndrome), in addition to choanal atresia and bifid epiglottis in some cases. These extrathyroidal congenital abnormalities reflect the expression of FOXE1 in epiglottis, palate, esophagus, definitive choanae and hair follicles (Castanet and Polak, 2010; Clifton-Bligh *et al.*, 1998). All reported loss-of-function mutations are located in the forkhead DNA binding domain and exhibit recessive inheritance, although a gain of function mutation in the same region has been associated with a similar clinical phenotype (Carre *et al.*, 2014). Recently, isolated, nonsyndromic thyroid hypoplasia has been reported in a patient with biallelic *FOXE1* mutations. Although the variants were not subjected to functional characterization, observations suggest a broader phenotypic spectrum may occur with *FOXE1* mutations than previously suspected (de Filippis *et al.*, 2017). *FOXE1* also contains a long polyalanine tract, the length of which may be associated with risk of TD with a longer tract length being protective (Carre *et al.*, 2007).

GLIS3

GLIS3 belongs to the GLI-similar zinc finger protein family and has a critical role both in repression and activation of transcription from early in embryogenesis (Dimitri, 2017). Recessively inherited mutations in *GLIS3* are associated with a multisystem phenotype usually comprising CH (with the exception of one reported patient) and permanent neonatal diabetes, in association with other developmental abnormalities including dysmorphic facies, renal cystic dysplasia, hepatic fibrosis, congenital glaucoma, learning difficulties and skeletal abnormalities (Senée *et al.*, 2006; Dimitri, 2017). Genetic background, or size and position of the *GLIS3* mutation may affect the associated phenotype, especially given that two major *GLIS3* transcripts exhibit differential, tissue-specific expression (Senée *et al.*, 2006). Thyroid morphology may range from hypoplasia or gland athyreosis to apparently normal, however, in a single patient for whom histological analysis of the thyroid has been undertaken, a paucity of colloid as well as extensive fibrosis was demonstrated, despite initially normal thyroid ultrasonography (Dimitri *et al.*, 2015).

Significant TSH resistance has been a prominent biochemical characteristic in some cases with *GLIS3* mutations (Dimitri, 2017), perhaps explained by recent data demonstrating that GLIS3 acts downstream of TSH and the TSHR, being indispensable for TSH/TSHR-mediated proliferation of thyroid follicular cells and biosynthesis of thyroid hormone (Kang *et al.*, 2017).

Additional Genes Associated With TD

NKX2-5

NKX2-5 is a homeodomain-containing transcription factor of the NKX2 family. Although robustly implicated in congenital heart disease, its role in thyroid dysgenesis remains ambiguous. NKX2-5 is an attractive candidate gene for TD since murine *Nkx2-5* null embryos exhibit thyroid bud hypoplasia and following an initial report of four patients with thyroid ectopy or athyreosis harboring heterozygous *NKX2-5* mutations, several further TD cases with NKX2-5 mutations have been described, including one with a heterozygous *NKX2-5* mutation in association with a *PAX8* promoter mutation (Dentice *et al.*, 2006; van Engelen *et al.*, 2012; Hermanns *et al.*, 2011; Opitz *et al.*, 2015). However, penetrance is highly variable; carrier parents frequently have no morphological or biochemical evidence of CH and apparently pathogenic mutations (p.R25C) may occur frequently in healthy populations, or (p.A119S) in patients with congenital heart disease but normal thyroid morphology (Dentice *et al.*, 2006; van Engelen *et al.*, 2012). These observations suggest that although NKX2-5 may contribute to TD risk, other factors/genes are likely to play a significant role modulating penetrance and expressivity.

JAG1

JAG1 encodes a ligand of the Notch receptor required for normal thyroid development in zebrafish. Human heterozygous *JAG1* mutations are associated with a multisystem disorder (Alagille syndrome) in which congenital heart disease (CHD) may associated with variable hepatic, eye and skeletal defects together with dysmorphic facies. Recently, evaluation of thyroid function in a cohort of 21 cases with Alagille syndrome revealed six cases with mild, nonautoimmune hypothyroidism. Additionally, evaluation of *JAG1* in 100 CH cases without associated Alagille syndrome identified four cases harboring heterozygous *JAG1* variants with partial loss of function, two with associated CHD. Detection of further rare *JAG1* variants in a cohort of >300 CH cases further substantiated the notion that JAG1 may contribute to the pathogenesis of CH. Thyroid morphology in affected humans ranges from eutopic thyroid to gland dysgenesis including hypoplasia or ectopy (de Filippis *et al.*, 2016, 2017).

Borealin

Borealin (CDCA8) is a major component of the Chromosomal Passenger Complex (CPC) which has well-described functions in chromosome segregation and cytokinesis. Mono- and biallelic *CDCA8* mutations were recently reported in association with TD in three unrelated families, in which the carrier parents were all euthyroid but exhibited variable thyroid structural abnormalities. A

homozygous mutation (p.S148F) in two children was associated with CH and thyroid ectopy, or euthyroidism with thyroid hemiagenesis; the heterozygous parents exhibited an asymmetric thyroid or thyroid nodules. In a second family, a heterozygous mutation (p.R114Q) in the proband was associated with CH and thyroid ectopy; the heterozygous mother had an asymmetric thyroid with papillary thyroid cancer. Finally, another heterozygous mutation (p.L177W) was associated with CH and athyreosis in the proband and thyroid nodules in the carrier mother. Expression of mutant Borealin in a thyroid cell line resulted in altered cellular migration and adhesion by decreasing the expression of genes implicated in focal adhesion (Carré *et al.*, 2017).

Netrin 1 (NTN1)

Netrin 1 is a laminin superfamily member with a role in mediating guidance cues during embryogenesis. A heterozygous deletion involving part of *NTN1* has been reported in one patient, with VSD and thyroid ectopy, in addition to a 47, XYY karyotype and an atypical 22q11 deletion. Subsequent zebrafish studies demonstrated defective aortic arch artery formation in ntn1a-deficient embryos in addition to abnormal thyroid morphogenesis, likely due to lack of proper guidance exerted by the dysplastic vasculature of ntn1a-deficient embryos. The extent to which NTN1 mutations contribute to shared thyroid and cardiac congenital defects in the population remains unclear (Opitz *et al.*, 2015).

Syndromes Which May Be Associated With CH

Risk of CH may be increased in the context of several genetically defined syndromes for which the major associated abnormalities are extrathyroidal. Candidate genes involved include *SALL1* (Townes–Brocks syndrome), *TBX1* (di George syndrome), *URB1* (Johanson–Blizzard syndrome), *DYRK1A* (Trisomy 21), *ELN* (Williams–Beuren syndrome), *KMT2D/MLL2*, *KDM6A* (Kabuki syndrome) and *KAT6B* (Ohdo syndrome, Genitopatelar syndrome (Cherella and Wassner, 2017).

Thyroid Hormone Biosynthesis (Summarized in Fig. 2)

Thyroid hormone biosynthesis occurs at the apical surface of polarized thyroid follicular cells and requires an intact synthesis pathway comprising transporter molecules, enzymes and thyroglobulin as well as adequate dietary iodide (**Fig. 2**). Dyshormonogenesis occurs due to loss of function mutations in any of the known genes encoding the components of the thyroid hormone biosynthetic machinery. The initial step in thyroid hormone production is the active uptake of circulating iodide across the basolateral membrane by the sodium-iodide symporter, NIS (SLC5A5), which co-transports one iodide ion against its

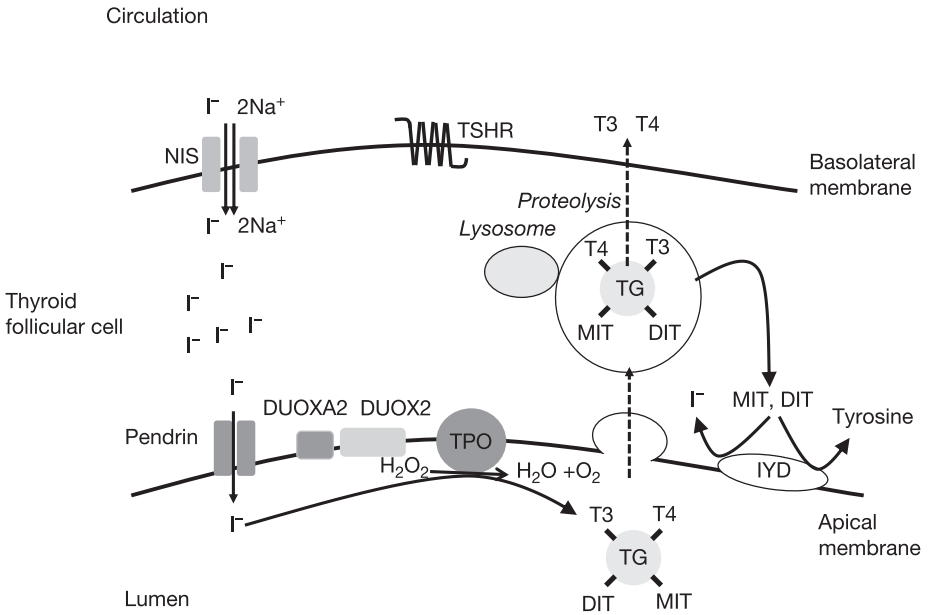

Fig. 2 Schematic summarizing the key steps in thyroid hormone biosynthesis. Iodide is concentrated in the thyroid follicular cell following active uptake by the sodium-iodide symporter (NIS, SLC5A5). Pendrin (SLC26A4) and other less well characterized transporters facilitate iodide efflux across the apical membrane, whereupon iodide is oxidized in a hydrogen-peroxide dependent process, catalyzed by the thyroid peroxidase enzyme (TPO). H_2O_2 is primarily generated by the NADPH-oxidase DUOX2 and its accessory protein DUOXA2. TPO also catalyzes the organification of iodide, that is, its incorporation into mono and di-iodotyrosyl residues (MIT, DIT) on the surface of thyroglobulin (TG) and their coupling to form thyroid hormones T3 and T4. Iodotyrosine dehalogenase (IYD) recycles unused iodide moieties and TSH signaling via the TSH receptor stimulates both thyroid hormone biosynthesis and gland growth.

electrochemical gradient together with two sodium ions along a sodium gradient generated by the Na + /K + -ATPase. Iodide efflux at the apical membrane is likely to be at least partially mediated by Pendrin, encoded by *SLC26A4*, a multifunctional anion transporter which exchanges chloride for either iodide or bicarbonate. Iodide is oxidized at the follicular lumen and then organified into tyrosyl residues of thyroglobulin (TG), the matrix glyoprotein upon which thyroid hormone is synthesized. Mono- and diiodotyrosines (MIT, DIT) undergo coupling to generate iodothyronines (predominantly T4, with smaller amounts of T3). Oxidation and organification of iodide and the subsequent coupling reaction are catalyzed by the thyroid-specific enzyme thyroid peroxidase (TPO). Hydrogen peroxide (H_2O_2), the essential electron acceptor for the iodination and coupling reactions, is produced mainly by NADPH oxidase dual oxidase 2 (DUOX2, previously known as THOX2) and its accessory protein dual oxidase maturation factor 2 (DUOXA2). Triodothyronine (T3) and thyroxine (T4) are stored in the colloid until TG is taken up by thyroid follicular cells through macro- and micropinocytosis and digested in lysosomes. Secretion of thyroid hormones then occurs into the bloodstream at the basolateral membrane. Uncoupled mono- and diiodotyrosines (MIT, DIT) are subject to NADPH-dependent reductive deiodination by intracellular iodotyrosine dehalogenase (IYD, previously known as DEHAL1) which enables them to be recycled (Grasberger and Refetoff, 2011) (**Fig. 2**).

Dyshormonogenesis (Genetic Causes Summarized in Fig. 1 and Table 1)

Dyshormonogenesis refers to inadequate thyroid hormone synthesis due to a specific defect in one of the components of the thyroid hormone biosynthetic machinery. In all forms of dyshormonogenesis, compensatory gland hyperplasia may result in goiter. Other clinical, radiological and biochemical features are dependent on the site of the defect in the thyroid hormone synthesis pathway. If the dysfunctional protein results in impaired organification of iodide, a perchlorate discharge test will be positive. This investigation involves the quantitation of an administered dose of radioiodine (^{123}I) in the thyroid before and after the patient is given perchlorate, which inhibits further thyroidal uptake of iodide. Under normal circumstances, if organification is intact, >90% of ^{123}I remains in the thyroid follicle bound to TG but, if organification is impaired, <90% of the thyroidal ^{123}I can be organified and >10% leaks back into the circulation. Severe enzymatic defects cause loss of >90% of the applied ^{123}I dose (total iodide organification defect, TIOD) but enzymatic defects with residual function cause partial iodine organification (PIOD) defined as 10%–90% of ^{123}I washout after perchlorate (Grasberger and Refetoff, 2011).

SLC5A5 (NIS)

Autosomal recessively inherited mutations in the sodium-iodide symporter (*NIS*) are a rare cause of dyshormonogenesis, disrupting uptake of iodide across the basolateral thyrocyte membrane. Individuals with NIS mutations may develop severe congenital hypothyroidism, or, especially in areas where dietary iodine content is high, euthyroid goiter. CH due to NIS mutations may not manifest neonatally and affected children with normal TSH screening results may develop severe hypothyroidism unexpectedly in childhood with concomitant neurodevelopmental delay (Grasberger and Refetoff, 2011; Szinnai *et al.*, 2006).

Characteristic radiological features associated with NIS mutations are a blunted or absent radioiodine uptake by the thyroid despite ultrasonographic evidence of a normally located, usually enlarged gland (0%–5% vs. the normal range of 10%–40%) (Szinnai *et al.*, 2006). Since NIS is also expressed in gastric parietal cells and salivary glands, these tissues also fail to concentrate iodine if the transporter is defective. This feature can be exploited diagnostically, since the ratio of salivary: plasma iodine following injection of radioiodine will be decreased (Montanelli *et al.*, 2009).

SLC26A4 (Pendrin)

Pendrin mediates chloride bicarbonate transport in the inner ear where is it essential for maintaining acid-base homeostasis of the endolymphatic fluid. Biallelic mutations in Pendrin are a well-recognized cause of congenital sensorineural hearing impairment with enlarged vestibular aqueduct (80%–100%) and a subset of cases also harbor a Mondini cochlear malformation (incomplete partition of the cochlea) (Phelps *et al.*, 1998; Luxon *et al.*, 2003; Cremers *et al.*, 1998).

Pendrin also has a putative role in thyrocyte apical iodine transport and Pendred syndrome refers to the association of congenital bilateral sensorineural hearing loss and vestibular dysfunction with diffuse or multinodular goiter resulting from a PIOD. Most affected patients who are iodide replete remain euthyroid and if hypothyroidism does occur this is usually after the second decade, such that Pendrin mutations represent an unusual cause of CH (Wémeau and Kopp, 2017; Reardon *et al.*, 1999).

TG

Autosomal recessively inherited *TG* mutations are rare in the general population, but one of the more common causes of dyshormonogenesis; >100 different mutations have been described with an estimated frequency of 1:100,000 births (Ieiri *et al.*, 1991, Targovnik *et al.*, 2017). *TG* mutations frequently result in goiter, which may manifest in the neonatal period; rarely, cases are complicated by fetal goiter (Vasudevan *et al.*, 2017). The spectrum of biochemical thyroid dysfunction ranges from euthyroidism

to severe hypothyroidism and diagnostic hallmarks of *TG* mutations include low thyroglobulin despite elevated TSH and goiter or failure of TG to rise after stimulation with exogenous TSH. Thyroidal iodide uptake is enhanced and organification of iodide is preserved on perchlorate discharge test (Targovnik *et al.*, 2011, 2017).

TPO

Autosomal recessively inherited *TPO* mutations disrupt the final enzymatic steps in thyroid hormone biosynthesis and are a common cause of dyshormonogenesis, representing the main underlying mechanism for TIOD (Abramowicz *et al.*, 1992; Bakker *et al.*, 2000; Cangul *et al.*, 2013). More than 100 mutations have been described, and missense variants generally cluster around the heme binding catalytic domain. Affected individuals with biallelic mutations usually exhibit severe CH, often with goiter and fetal goiter may rarely occur. Investigations usually demonstrate preserved iodide uptake and TG biosynthesis with TIOD (Targovnik *et al.*, 2017; Ris-Stalpers and Bikker, 2010). Rarely, monoallelic mutations are associated with CH and PIOD, or, in isolated cases, with TIOD, perhaps due to monoallelic expression of TPO in thyroid (Nascimento *et al.*, 2003; Fugazzola *et al.*, 2003).

DUOX2

Both monoallelic and biallelic *DUOX2* mutations may result in CH, usually with PIOD. Since the first association of *DUOX2* mutations with CH, DUOX2 has been increasingly implicated in the pathogenesis of dyshormonogenesis, especially in East Asian populations (Jin *et al.*, 2014; Moreno *et al.*, 2002). Although biallelic mutations were first associated with permanent CH, and monoallelic mutations with transient CH (Moreno *et al.*, 2002), subsequent studies have demonstrated permanent CH in association with monoallelic mutations (de Marco *et al.*, 2011) and transient CH in the context of biallelic, truncating mutations (Hoste *et al.*, 2010). This variable penetrance of DUOX2 deficiency is incompletely understood; iodine deficiency is thought to exacerbate the phenotype and variants in other H_2O_2-synthesizing enzymes capable of compensating for DUOX2 deficiency, for example, DUOX1, may also modulate CH severity (Grasberger and Refetoff, 2011; Aycan *et al.*, 2017). Cases harboring *DUOX2* mutations may be missed on neonatal screening since screening TSH measurements may be only marginally elevated; confirmatory venous TSH and TG measurements are usually more markedly raised and iodide uptake is preserved (Muzza *et al.*, 2014).

DUOXA2

DUOXA2 mutations are a rare cause of dyshormonogenesis, with <10 variants currently reported. The first reported case exhibited mild permanent CH in association with biallelic mutations and PIOD. Both monoallelic and biallelic defects have now been described, in association with both mild permanent and transient CH although clinical data for affected cases is sparse. The small number of reported cases suggests that *DUOXA2* defects are rare causes of CH which may also be more common in East Asian populations, especially the recurrent mutation, p.Y138* (Zamproni *et al.*, 2008; Liu *et al.*, 2015; Park *et al.*, 2016).

IYD (Previously Known as DEHAL1)

Goitrous individuals in whom iodide recycling is impaired were first described many years ago, but a molecular diagnosis was only achieved more recently (Hubble, 1953; Moreno *et al.*, 2008). Both monoallelic and biallelic *IYD* mutations can cause goitrous hypothyroidism and affected individuals exhibit rapid thyroidal uptake of iodide with a normal perchlorate discharge test (Moreno *et al.*, 2008; Burniat *et al.*, 2012; Afink *et al.*, 2008). Urinary concentrations of MIT and DIT are characteristically increased. Hypothyroidism may manifest in childhood following a normal neonatal CH screening test result resulting in neurodevelopmental delay if diagnosis is delayed (Moreno *et al.*, 2008).

Evidence for an Undiagnosed Genetic Component in TD

Until recently, studies in TD have used Sanger sequencing to screen small numbers of known TD-associated genes individually, often in small TD populations. On the basis of such studies, which have revealed causative mutations in $<5\%$ cases, it has been argued that TD is a sporadic disease. This notion is further supported by $>90\%$ discordance between monozygotic twins with CH (Perry *et al.*, 2002) and observations that there is a strong female preponderance of CH, especially due to thyroid ectopy (Devos *et al.*, 1999). Since these features are not consistent with simple Mendelian inheritance, it has been hypothesized that TD may occur due to somatic mutations restricted to the thyroid or epigenetic events.

However, there is also evidence to suggest a more significant etiological role for germline mutations in TD than currently diagnosed. A French National Survey of CH cases demonstrated that 2% of TD cases have an affected relative; this value is 15-fold greater than predicted by chance alone. Moreover, thyroid developmental abnormalities occur more commonly in euthyroid first degree relatives of CH cases than in controls (Castanet *et al.*, 2000, 2001, 2005; Leger *et al.*, 2002). Several studies have also shown an increased incidence of extrathyroidal developmental malformations in patients with CH, and CH occurs more frequently in

consanguineous or less genetically diverse populations (Kreisner *et al.*, 2005; Ordookhani *et al.*, 2004; Stoppa-Vaucher *et al.*, 2011).

Alternative Genetic Etiologies in TD and Dyshormonogenetic CH

Possible mechanisms which may reconcile these observations include a two-hit mechanism, where a germline predisposing mutation occurs in association with an additional genetic or epigenetic alteration within the thyroid tissue or surrounding structures (Deladoey *et al.*, 2007). However, no firm evidence for this has been established. Analysis of ectopic thyroid has not demonstrated significantly different somatic methylation gene expression profile differences or thyroid specific copy number variants (CNV's) compared with normal thyroid although ectopic thyroid did exhibit a different gene expression pattern (Abu-Khudir *et al.*, 2010). Additionally, sequencing of lymphocyte DNA from monozygotic twins discordant for TD did not reveal somatic mutations (Magne *et al.*, 2015) although somatic mosaicism for a PAX8 mutation has been reported (Narumi *et al.*, 2011). Autosomal monoallelic expression has been reported for some genes in both ectopic and eutopic thyroid, suggesting this may be implicated (Magne *et al.*, 2016); however, monoallelic expression of a mutant allele has only been reported for TPO in association with dyshormonogenetic CH and TIOD (Neves *et al.*, 2010; Fugazzola *et al.*, 2003). Recurrent copy number variants have also not been identified (Thorwarth *et al.*, 2010).

Apparently sporadic cases could also have an oligogenic basis, as has been established for Kallmann syndrome (Van Vliet and Deladoey, 2015). Support for this proposition initially came from observations that mice with heterozygous *TTF1* or *PAX8* mutations are euthyroid, but strain-specific TD occurs in mice with combined partial deficiencies of TTF1 and PAX8 (Amendola *et al.*, 2005). Until recently, only anecdotal reports had been published of patients harboring digenic mutations in association with CH, however, recent larger, human studies have also confirmed a role for oligogenic inheritance in both CH with eutopic gland-in-situ and thyroid dysgenesis (Nicholas *et al.*, 2016; Löf *et al.*, 2016). This includes a study of >150 Italian patients with CH of all types, in which 23% harbored a likely pathogenic variant in more than one gene (de Filippis *et al.*, 2017). In addition to oligogenicity for known-CH-associated genes, digenic *DUOX2* and *DUOX1* homozygous mutations were recently reported in association with unusually severe CH (Aycan *et al.*, 2017). DUOX2 is thought to be the main H_2O_2-generating enzyme in the thyroid on the basis of its higher expression levels. However, it has been suggested that enhanced expression of DUOX1 may help compensate for DUOX2 deficiency in CH due to DUOX2 mutations, suggesting that variants in DUOX1 may contribute to phenotypic severity in DUOX2 mutation cases.

The advent of next generation sequencing has also enabled the screening of genes classically associated with TD or dyshormonogenesis in mixed CH populations. The outcome of these studies has been a realization that there may be overlap of genetic etiologies in the two morphological subgroups. de Filippis et al. demonstrated mutations in genes characteristically associated with TD (e.g., biallelic *FOXE1* mutations), in association with isolated CH and a normal thyroid gland, although functional characterization of the mutations was not undertaken (de Filippis *et al.*, 2017). Conversely, Pendrin mutations have been reported in patients with TD, where a potential mechanism was thought to be a secondary atrophy of the thyroid, perhaps due to increased oxidative stress (Kühnen *et al.*, 2014).

Isolated Central Congenital Hypothyroidism

The Hypothalamic–Pituitary–Thyroid Axis: Positive Regulation of Thyroid Hormone Synthesis

Thyroid hormone biosynthesis is positively regulated by the actions of hypothalamic thyrotropin-releasing hormone (TRH) and pituitary thyroid stimulating hormone (TSH). TRH is synthesized in the paraventricular nucleus (PVN) of the hypothalamus and following maturation, undergoes axonal transport to the median eminence, reaching the thyrotrophs of the anterior pituitary gland via the hypothalamic portal vein. TRH then binds the TRH receptor (TRHR), a G-protein coupled receptor, which activates a Gq/11 dependent pathway involving mobilization of intracellular calcium and activation of protein kinase C. TRH upregulates transcription of the TSH alpha (αGSU) and beta subunit genes (*CGA* and *TSHB*) but also exerts important posttranslational effecting including mediating conjugation of TSH alpha and beta subunits and stimulating both secretion of heterodimeric TSH and its glycosylation to confer normal bioactivity (Fekete and Lechan, 2014, Persani, 1998, **Fig. 1**).

Central congenital hypothyroidism (CCH) is a rare disorder which occurs when hypothalamic or pituitary pathology results in defective stimulation of a normal thyroid gland by thyroid stimulating hormone (TSH). Since TSH is not elevated in CCH, TSH-based, primary CH screening programs will not detect CCH in the neonatal period, and patients are at risk of neurodevelopmental delay if detection and treatment of severe CCH is delayed. Additionally, for CCH occurring as part of a syndrome of combined pituitary hormone deficits, delayed diagnosis of other hormone deficiencies (adrenocorticotrophic hormone, ACTH; growth hormone, GH) may pose significant risks, such as life threatening hypoglycemia (Schoenmakers *et al.*, 2015).

In a minority of cases (estimated incidence 1:65000), TSH deficiency is isolated and may occur as a result of defects in genes driving or regulating the TSH biosynthetic pathway. Four main genes have been implicated, in which mutations result predominantly in quantitative and/or qualitative defects in TSH secretion; thyrotropin-releasing hormone receptor (*TRHR*), thyroid stimulating hormone beta subunit (*TSHB*), immunoglobulin superfamily member 1 gene (*IGSF1*) and the transducin beta like 1X-linked gene, *TBL1X* (Schoenmakers *et al.*, 2015; Heinen *et al.*, 2016).

Table 2 Endocrine, neuroradiological and extrapituitary manifestations of mutations in genes implicated in CCH in humans

Gene	*Inheritance*	*Hormone deficits*	*TRH test responses*	*MRI*	*Additional features*
TSHB	AR	TSH	Absent TSH response, preserved PRL peak	E, N	–
TRHR	AR	TSH	Absent/preserved TSH & PRL peak	N	–
TBL1X	XL[a]	TSH	Normal TSH response	N	Sensorineural hearing loss
Isolated TSH deficiency or combined pituitary hormone deficiency					
IGSF1	XL[a]	TSH ± PRL, GH (transient)	Low normal/normal TSH response	N	Macroorchidism (males) Ovarian cysts (females)

[a]Females may also be affected.

E, enlarged; *N*, normal; *TSH*, thyroid-stimulating hormone; *PRL*, prolactin; *GH*, growth hormone; *AR*, autosomal recessive; *XL*, X-linked.

Isolated Central Congenital Hypothyroidism (Genetic Causes Are Summarized in Fig. 1 and Table 2)

TSHB *mutations*

Biallelic loss of function, *TSHB* mutations are invariably associated with severe CCH. Cases residing in countries operating TSH-based neonatal CH screening programs are usually diagnosed when they present clinically, and frequently exhibit neurodevelopmental impairment, the extent of which correlates with the degree of treatment delay. In cases who are diagnosed and treated from birth due to ascertainment following a prior genetic diagnosis in their family, developmental outcome is often improved (Karges *et al.*, 2004; Brumm *et al.*, 2002, Nicholas *et al.*, 2017).

Mature TSH comprises a heterodimer of the common alpha subunit (αGSU) shared with other glycoprotein hormone (LH, FSH, CG) family members and a TSH-specific beta-subunit (TSHB). Naturally occurring mutations either truncate the protein, or perturb key structural features required for maintaining the integrity of the heterodimer. A "seat belt" formed from the TSH beta subunit, wraps around the long loop of the alpha subunit and forms an intramolecular disulfide "buckle" to stabilize the heterodimer and additional alpha–beta subunit interactions occur around a conserved CAGYC sequence motif (Szkudlinski *et al.*, 2002; Jiang *et al.*, 2014). All described missense mutations disrupt key disulfide bridges required for heterodimeric integrity, truncate the protein or disrupt the CAGYC region. The most common mutation is a single nucleotide deletion (c373delT) leading to a cysteine 125 to valine change (p.C125V) and subsequent frameshift and premature stop codon at position 134 (p.C125Vfs*10) (Medeiros-Neto *et al.*, 1996). Although reported worldwide in several, nonconsanguineous families, a founder effect has been described in German and Irish kindreds (Domene *et al.*, 2004; Brumm *et al.*, 2002; Nicholas *et al.*, 2017). In addition, two splice-site mutations (c162G>A, c.162 + 5G>A) (Baquedano *et al.*, 2010; Pohlenz *et al.*, 2002) and two *TSHB* deletions (Hermanns *et al.*, 2014a; Nicholas *et al.*, 2017) have been described. (The nomenclature of these mutations follows the most recent HGNC guidelines to include the 20 amino acid signal peptide of TSHB, such that the annotation may differ from that cited in the original published articles).

Biochemical hallmarks of CCH due to *TSHB* mutations include elevated pituitary glycoprotein alpha subunit, and impaired TSH response to TRH administration, despite a preserved serum Prolactin rise (Bonomi *et al.*, 2001). Mutations (p.G49R, p.Q32*), which disrupt heterodimer formation between TSHalpha and beta polypeptides generally result in undetectable serum TSH concentrations, whereas in cases with mutations resulting in synthesis of nonbioactive heterodimeric TSH (e.g., p.Q69*, IVS2 + 5G>A, c.373delT), immunoreactive TSH will be detected in an immunoassay-dependent manner if epitopes recognized by the anti-TSH monoclonal antibody are preserved (Bonomi *et al.*, 2001).

TRHR *mutations*

Only four unrelated kindreds have been reported, in which affected cases harbor biallelic *TRHR* mutations. In three probands, TRHR activity was completely abrogated; the first case was compound heterozygous for a nonsense mutation (p.R17*), and an in-frame deletion of three amino acids (S115, I116, and T117) with one substitution (p.A118T). In the second reported kindred, affected individuals were homozygous for the p.R17* mutation and the third proband harbored a highly deleterious homozygous p.P81R missense variant (Bonomi *et al.*, 2009; Collu *et al.*, 1997; Koulouri *et al.*, 2016). Homozygous individuals in these families exhibited T4 concentrations 40%–88% lower limit of the normal range and heterozygous carriers had normal FT4 levels. In a fourth kindred, harboring a p.I131T mutation, signal transduction was impaired but not completely absent, with two homozygotes exhibiting either moderate CCH or isolated hyperthyrotropinemia. Heterozygotes in this kindred exhibited isolated hyperthyrotropinemia, for which the mechanism is unclear (García *et al.*, 2017).

The main reported clinical manifestations in kindreds harboring severe biallelic mutations comprised growth retardation and delayed bone age. Some affected patients were first diagnosed with central hypothyroidism in late childhood or even adulthood, but did not exhibit any significant neurological deficit suggesting that thyroid hormone production was sufficiently preserved in infancy to prevent overt mental retardation. However, even relatively asymptomatic cases exhibited improved quality of life following initiation of levothyroxine therapy (Bonomi *et al.*, 2009).

Since TRHR is expressed in lactotrophs as well as thyrotrophs, intravenous TRH usually stimulates a prolactin response as well as a TSH response, and both responses were absent in patients with severe bilallelic *TRHR* mutations but preserved with the milder p.I131T mutation. One female with a homozygous, nonsense *TRHR* mutation (p.R17*), was only diagnosed with central hypothyroidism following family screening at the age of 33, having previously achieved two normal pregnancies with subsequent

lactation. These observations suggest that TRH action is not obligatory for pregnancy and lactation in humans (Bonomi *et al.*, 2009). Although serum TSH was inappropriately normal for the low serum FT4 levels in affected cases, circulating T4 levels did rise appropriately following levothyroxine withdrawal, indicating that synthesis of bioactive TSH could occur in the absence of TRH signaling (Collu *et al.*, 1997; Bonomi *et al.*, 2009; Koulouri *et al.*, 2016; García *et al.*, 2017).

IGSF1 *mutations*

Mutations in the immunoglobulin superfamily member 1 (*IGSF1*) gene are now thought to be the most common genetic abnormality underlying CCH (Joustra *et al.*, 2013; Sun *et al.*, 2012). *IGSF1* encodes an X chromosomal membrane glycoprotein which is highly expressed at mRNA level, in Rathke's pouch (the developing pituitary primordium) and in adult pituitary gland (Sun *et al.*, 2012). Expression studies of the human protein have been impeded by the fact that there are no reliable antihuman IGSF1 antibodies; however, in murine and rat pituitary, IGSF1 protein is detected in cells of the Pit1 lineage (thyrotropes, somatotropes and lactotropes, but not gonadotropes) (Sun *et al.*, 2012; Joustra *et al.*, 2015). The IGSF1 carboxyterminal portion trafficks to the plasma membrane where it is expressed as a large extracellular domain with a short intracellular cytoplasmic tail (Robakis *et al.*, 2008), however, in both humans and rodents, its precise physiological function remains undefined (Sun *et al.*, 2012).

More than 30 pathogenic mutations involving the *IGSF1* gene have now been described (Sun *et al.*, 2012; Nakamura *et al.*, 2013; Tajima *et al.*, 2013; Joustra *et al.*, 2016), almost all of which result in decreased plasma membrane expression of IGSF1 and are either located in the extracellular portion of the carboxyterminal domain or result in *IGSF1* deletion. Clinical evaluation of patients with *IGSF1* mutations have confirmed that males invariably exhibit CCH, which is usually mild to moderate. Additionally, male pubertal development is usually disharmonious, with delayed pubertal growth spurt and testosterone rise but normal onset of testicular growth and subsequent macroorchidism in adulthood, for which the underlying mechanism has not been elucidated. Basal prolactin levels are subnormal in >60% cases and infrequently, GH production may be transiently impaired in childhood, necessitating GH replacement. In adulthood, IGF-1 levels are paradoxically in the upper half of the reference range, or mildly elevated and acromegaloid features may develop. Although X-linked, ~20% of heterozygous females harboring *IGSF1* mutations exhibit central hypothyroidism, and the remainder generally exhibit thyroid hormone levels in the lower tertile of the normal range. Up to 20% demonstrate hypoprolactinemia and 4 of 18 females investigated have required surgery for benign ovarian cysts (Sun *et al.*, 2012; Joustra *et al.*, 2013, 2016).

Murine studies in two different IGSF1 deficient mouse lines have demonstrated impaired TRH signaling associated with IGSF1 deficiency and decreased pituitary expression of TRHR despite normal TRH synthesis (Sun *et al.*, 2012, Turgeon *et al.*, 2017). The mild–moderate CCH observed in most humans with hemizygous IGSF1 mutations would also be consistent with impaired TRH signaling in humans with IGSF1 deficiency, although this has not yet been confirmed at a molecular level (Yamada and Mori, 2008). TSH response to TRH is usually subnormal in neonates, but within the lower half of the reference range in child- and adulthood (Joustra *et al.*, 2016).

TBL1X *(transducin β-like protein 1)*

Most recently, missense mutations in *TBL1X* have been reported in patients with isolated CeCH. TBL1X is an essential component of the main nuclear receptor corepressor complex (NCoR/SMRT) involved in T3-regulated gene expression, and mutations are thought to result in impaired basal activation of genes such as *TRH* and *TSHB*, which are negatively regulated by thyroid hormone. *TBL1X* is located on the X chromosome and eight males harboring hemizygous mutations, as well as 11 females harboring heterozygous mutations were identified in six unrelated kindreds. Although FT4 levels in affected adults were significantly lower than in controls, only six males and three females exhibited CCH with FT4 concentrations below the lower limit of the reference range. Where present, CCH was isolated, and mild–moderate, with normal TRH test responses. Additionally, individuals frequently exhibited associated sensorineural hearing loss (Heinen *et al.*, 2016).

Congenital Hypothyroidism Due to Impaired Thyroid Hormone Action

Thyroid Hormone Receptor Isoforms

The genomic effects of thyroid hormone are mediated by the nuclear thyroid hormone receptors (TRs) encoded by the *THRA* and *B* genes on chromosomes 17 and 3 respectively. Four different thyroid hormone receptor protein isoforms (TRα1, α2, β1, and β2) are expressed in humans, which are generated by alternative splicing and use of a different tissue-specific promoter (TRβ). All except the C-terminal TRα variant (TRα2) bind T3, and the physiological role for TRα2 remains unclear. TRs bind preferentially to specific DNA response elements (TREs) as a heterodimer with the retinoid X receptor (RXR). TREs are usually located in the promoter regions of target genes, and when TRs bind these elements, transcription is modulated in a ligand-dependent manner (Ortiga-Carvalho *et al.*, 2014).

Where genes are positively regulated by thyroid hormones, unliganded TRs mediate basal repression of the gene by binding the TRE together with a large repressor complex including corepressors (e.g., NCoR, SMRT), transducin-like protein (TBL1X) and histone deacetylase. This complex modulates local chromatin structure to repress basal transcription. T3 binding drives a conformational change of the receptor ligand binding domain, enabling coactivator recruitment (e.g., SRC-1, -2, -3) and histone

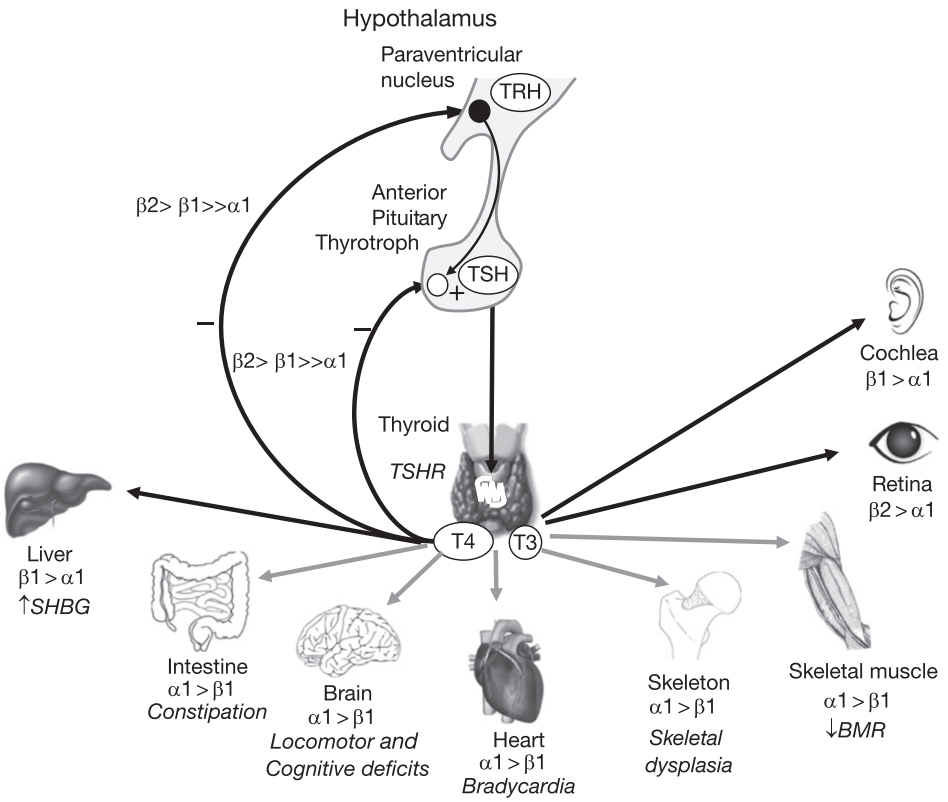

Fig. 3 Schematic summarizing the major tissue-specific thyroid hormone receptor isoform expression patterns and corresponding phenotypes noted in RTHα. In this syndrome, tissues expressing mainly TRα are resistant to thyroid hormone action (impaired, shown in gray), while TR*β*-expressing tissues are sensitive (not obviously impaired, shown in black).

acetylation, enabling the chromatin to achieve a more open conformation, facilitating transcriptional activation (**Fig. 1**). Some genes, including TRH and TSH*β* are negatively regulated by thyroid hormone for which the mechanism remains poorly understood (Ortiga-Carvalho *et al.*, 2014). Postulated mechanisms include recruitment of corepressors instead of coactivators or *trans*-repression, where liganded TRs interact with and inhibit the activity of other transcription factors, for example, GATA2 during TR-mediated repression of *TSHB* (Santos *et al.*, 2011).

Isoform Expression (Summarized in Fig. 3)

Individual tissues exhibit specific patterns of TR isoform expression such that tissue-specific thyroid hormone effects are mediated predominantly by one TR isoform. Expression of thyroid hormone receptor *β*2 is largely confined to the pituitary and hypothalamus, where it is the principal mediator of the negative feedback loop regulating circulating thyroid hormone levels. It also plays a role in cochlea and retina. TR*β*1 predominates in kidney and liver, where it plays a role in cholesterol metabolism, including stimulation of enzymes regulating lipolysis, lipogenesis and oxidative processes (Ortiga-Carvalho *et al.*, 2014).

TRα1 and the nonhormone binding splice variant TRα2 are expressed more widely. TRα1 is the dominant TR isoform in endochondral and intramembranous bone, mediating the anabolic effects of thyroid hormone on the developing skeleton, where it is crucial for normal bone growth, mineralization and turnover and for the maintenance of bone strength (Bassett and Williams, 2016). As the most abundant myocardial TR isoform TRα1 also plays an important role in the regulation of heart rate and contractility, with stimulatory effects on both these processes (Klein and Danzi, 2007; Kahaly and Dillmann, 2005). Additionally, normal gut motility is dependent on TRα1, which is expressed both in gut epithelium and in smooth muscle cells (Plateroti *et al.*, 1999; Shafer *et al.*, 1984), and in skeletal muscle, thyroid hormone acting via TRα1 may have profound effects on muscle mass, metabolism and contractility (Finsterer *et al.*, 1999). 70%–80% cerebral TR isoform expression comprises TRα1, which is predominantly expressed in cerebrum and cerebellum. Multiple neurodevelopmental processes in the fetus and neonate are thyroid hormone dependent, including neurogenesis, cell migration and differentiation, synaptogenesis and myelination, in which TRα1 plays a crucial role (Rovet, 2014). Experiments investigating isoform-specific neurodevelopmental roles of thyroid hormone receptors have demonstrated redundancy in some areas, for example, cerebellar Purkinje differentiation, which is mediated by TRα1 and TR*β*1, whereas other functions are isoform-specific, for example, cerebellar granule cell migration, which is TRα1-dependent (Morte *et al.*, 2002).

Resistance to Thyroid Hormone Alpha (RTH Alpha)

Heterozygous, dominant negative, loss of function mutations in the *THRA* gene cause resistance to thyroid hormone alpha (RTH alpha), the clinical features of which are explained by tissue-specific hypothyroidism in tissues predominantly expressing the defective TRα isoform (**Fig. 3**). The widespread tissue expression pattern of TRα mandates that severely affected children exhibit many of the features classically attributed to untreated primary hypothyroidism but paradoxically, thyroid function tests are near-normal, reflecting the central role of TR*β* in maintaining the axis set-point (Bochukova *et al.*, 2012).

In initial reports of RTH alpha, patients exhibited characteristic broad facies with macrocephaly, perhaps due to delayed fontanelle closure, and hypothyroid features including a flattened nose, prominent tongue and thick lips. An excessive number of skin tags were also noted in many cases (Bochukova *et al.*, 2012; Tylki-Szymanska *et al.*, 2015; Moran *et al.*, 2013). In keeping with the crucial role of TRα for normal skeletal maturation, affected individuals exhibited significant growth retardation which was characteristically lower segmental and accompanied by radiological evidence of childhood skeletal dysplasia including calvarial wormian bones (disordered, intramembranous ossification), femoral epiphyseal dysgenesis (disordered, endochondral ossification) and delayed dentition. Constipation (often severe) was a frequent association and a spectrum of neurocognitive deficits was reported, including delayed developmental milestones (motor, speech), impaired motor coordination and slow initiation of movement, manifesting as dyspraxia or broad-based gait and slow speech. Variably reduced IQ necessitated special schooling at the more severe end of the spectrum (Tylki-Szymanska *et al.*, 2015; Moran *et al.*, 2013; van Mullem *et al.*, 2013). Cardiac manifestations included bradycardia, and basal metabolic rate was also reduced (Bochukova *et al.*, 2012, **Fig. 3**).

Characteristic biochemical abnormalities in RTH alpha include low/low-normal T4 and high/high-normal T3 concentrations, a subnormal T4/T3 ratio and variably reduced reverse T3. Two mechanisms have been hypothesized to explain this altered thyroid hormone metabolism; analogous to mice with a dominant negative TRa1 mutation TRα1-PV, affected humans may exhibit increased hepatic DIO1 levels which may augment T4 to T3 conversion; alternatively, reduced tissue levels of DIO3, the expression of which is TRα1 regulated, may result in decreased inner-ring deiodination of T4 to rT3 and T3 to T2. Additional biochemical abnormalities include mildly elevated muscle creatine kinase, indicative of skeletal muscle hypothyroidism and mild, normocytic anemia (Moran and Chatterjee, 2015).

The initial *THRA* mutations involved the TRα1 (hormone-binding) isoform alone, and over the last 5 years, descriptions both of cases harboring mutations affecting TRα1 and TRα2 as well as mutations exhibiting differing degrees of functional impairment in vitro, have expanded our understanding of this syndrome. Patients with mutations affecting both TRα isoforms seem to exhibit overlapping phenotypes with TRα1-specific mutations without additional features attributable to TRα2 loss of function (Moran *et al.*, 2014; van Gucht *et al.*, 2016). Although one 27-year old female with a mutation affecting both isoforms exhibits unusual skeletal features (micrognathia, clavicular agenesis, hypoplasia, metacarpal fusion and syndactyly) as well as hyperparathyroidism and chronic diarrhea, these have not been reported in other RTH alpha cases and it remains unclear whether these are solely attributable to the TRα1 p.N359Y mutation (Espiard *et al.*, 2015). Correlation of receptor dysfunction with clinical manifestations has delineated a spectrum of phenotypes which may depend to some extent on the location and deleteriousness of the *THRA* mutation. Crucially, such analysis has shown that milder forms of RTH alpha may present with only subtle clinical features of hypothyroidism and free hormone measurements within the normal range, although FT4/FT3 ratio is usually perturbed (Demir *et al.*, 2016). Although <30 RTH alpha cases have been reported to date, this syndrome may be more common, especially in milder forms, but as yet incompletely ascertained due to its modest biochemical abnormalities and its association with non-endocrine phenotypes (Demir *et al.*, 2016).

In addition to supportive treatment for the extra-thyroidal abnormalities, levothyroxine (L-T4) therapy has been beneficial in some affected children, improving growth, alleviating constipation and improving motor development and wellbeing (Moran and Chatterjee, 2015; van Gucht *et al.*, 2016). In particular, individuals with milder mutations may benefit from L-T4 treatment, especially if it is commenced at an early age. TSH concentrations suppress readily on treatment with elevation of FT3 to supra-physiologic levels, which does raise the possibility that chronic excess TH exposure in thyroxine-treated RTH alpha patients might lead to unwanted toxicities in normal TR*β*-containing tissues such as liver or bone, however, no such adverse effects have yet been reported (Moran *et al.*, 2013; Bochukova *et al.*, 2012).

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Congenital Hypothyroidism: A Historical Perspective on Cognitive Outcome

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Introduction

The severe clinical manifestation of congenital hypothyroidism (CH) as documented in former historic untreated cases, appearing as the so called "cretinism," demonstrates how comprehensively human development is dependent on thyroid hormones (Osler, 1897). Motor developmental milestones are missed, the children do not start to walk until at least 5 years of life, contractures develop in the lower extremity and cognitive development is profoundly delayed with lack of speech development as well as depression. In addition, they are dwarf, obese and do not enter puberty. Today single cases are reported from immigration families who were born in countries without newborn-screening and who were missed by clinical diagnosis and developed a phenotype that is very close to that "historic cretinism" described in the 19th century (Osler, 1897).

In addition to CH, two monogenetic syndromes that affect the transport of thyroid hormones into the brain—MCT8 deficiency- and the function within the brain-thyroid hormone alpha receptor (TRalpha) defect- further shed light on the critical role of thyroid hormones especially for human brain maturation (Dumitrescu *et al.*, 2000). In both diseases cognitive development is impaired despite normal or even increased levels of thyroid hormones within the circulation.

Based on animal models studies demonstrated defects of brain maturation that did not interfere with the early steps of organogenesis and leave the overall brain architecture intact. Correspondingly the overall brain anatomy in congenital human thyroid defects as shown by MRI is also normal (Hamdoun *et al.*, 2016). Pharmacologically induced hypothyroidism in rodents did show more subtle disturbances of neuronal differentiation, connectivity and myelinisation. Interestingly these changes are increasingly found in the further development and maturation of several interneurons and oligodendrocytes, thus pointing on a particular role of thyroid hormones especially for postnatal brain development (Bernal, 2000).

In terms of brain development of CH patients these rodent data would argue for a role of thyroid hormones also for later steps of brain development. In addition the earlier development might be protected by maternal thyroid hormones that cross the placenta and contribute significant levels of thyroid hormones in the developing embryo. If this supply is impaired—like in cases of severe maternal hypothyroidism or especially in areas of severe iodine deficiency—the affected newborns suffer from severe cognitive impairment despite a later normal child thyroid function after birth (DeLange, 1994).

However, the question whether the prenatal lack of fetal thyroid hormones in CH lead to developmental defects that cannot be counterbalanced by later postnatal treatment remained for a long time unsolved. For decades it seemed more likely that indeed the prenatal hypothyroid state in severe cases results in noncompensable developmental defects, so that the cognitive outcome in those patients will not be normal at all.

Outcome in Patients Treated Before Newborn Screening

Treatment of CH was already introduced in the 19th century when thyroid extract was given to children with the clinical signs of "cretinism" (Osler, 1897). The changes achieved by this first substitution-treatment—long before thyroid hormones were discovered in the 1915 and became available as L-thyroxine after 1945- were tremendous with a complete metamorphosis of these hitherto extremely affected children. They did grow, improved in mood, entered motor milestones and eventually also reached puberty. Nevertheless, they did not reach normal cognitive development (Osler, 1897).

Data about the IQ outcome in these patients who were treated based on clinical diagnosis are sparse. Moreover, CH is a spectrum of different pathologies—thyroid dyshormonogenesis and thyroid dysgenesis as well as central versus primary hypothyroidism and syndromes with hypothyroidism as one feature among several- with a wide spectrum of manifestation and severity, which together makes it difficult to get comparable outcome data in general. However, already in the first decades of the 20th century studies were published describing the cognitive outcome of CH cases in relation to their different treatment modalities. In 1957 Smith and coworkers published about 128 patients with a "severe" form of CH and they found that patients who were treated after 6 months of life are severely retarded with an IQ on average of 50 (Smith *et al.*, 1957). Another later study from 1972 by Klein and coworker confirmed this low IQ in later treated children but in addition reported a much better IQ in those few patients who were diagnosed clinically already within the first 3 month of life when symptoms are very subtle.

Interestingly, when all patients with CH were included in these outcome studies the mean IQ was much better, suggesting a wide IQ range depending on CH severity. In a review of these studies by Grosse and Van Vliet (2011) the mean IQ of this prescreening treated children with CH was 82–87 but with a wide range from 40 to 112.

To overcome the profound mental retardation in the severe cases of CH Klein *et al.* suggested in 1972 (Klein *et al.*, 1972) to implement a new-born screening program which would allow to diagnose newborns by their endocrine alterations-elevated TSH or decreased T4- before their clinical symptoms occur later during development. Since they have discovered a much better IQ in

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those children diagnosed within the first 3 months of life they assumed that an early detection of CH based will enable a more favorable cognitive outcome especially in the most severe cases.

Outcome in CH Patients Diagnosed in First Generation Screening Programs

The first CH patient diagnosed by newborn-screening was reported already in 1974 by Klein *et al.* from Pittsburgh, USA (Klein *et al.*, 1974). The patient was treated on day 22 of life with a combination of T3–T4; no evidence for the treatment with thyroid hormones in newborns was available at that time and treatment was started not in terms of clinical studies but rather as kind of "try and error."

The efficiency of this first implemented newborn-screening was already reported in 1981 from the "New England CH Collaborative" showing the complete eradication of mental retardation in those children treated since 1974. In 63 children treated at a mean of 25 days of life with a mean LT4 dose of 8 μg/kg a normal IQ was diagnosed (New England congenital hypothyroidism collaborative, 1981). However, the detailed cognitive outcome of children diagnosed in this first generation of newborn screening were reported later in the 1990th showing still some patients with an unfavorable IQ outcome. In a meta-analysis from 1996 of seven studies covering 675 CH patients diagnosed by newborn screening the authors found a significant difference of mean full IQ compared to controls of up to 10 and 10% of still retarded patients (Derksen-Lubsen and Verkerk, 1996). Other groups found IQ differences of 18 in a cohort from Canada (Glorieux *et al.*, 1992). These studies already reflected the need of appropriate control groups given the wide range of IQ in the normal population and especially the strong impact of the social status on IQ. In addition, it was recognized that CH can occur in more complex syndromic cases which *per se* show impaired development irrespective of hypothyroidism like Down syndrome and need to be excluded from CH outcome studies.

These considerations were included in the adult outcome studies of this first generation of CH patients, diagnosed in newborn screening programs during the 1970th. Now cohorts of CH patients were controlled with the respective siblings from the same family. While again the overall IQ of the cohorts were in the normal range a significant mean full IQ difference of 7,9 and 8,0 were found in studies from Canada and Norway, respectively (Rovet, 2005; Oerbeck *et al.*, 2003). Interestingly, in both studies it was shown that the more severe the disease at birth the lower the IQ.

One interpretation of these findings was, that the fetal hypothyroid state lead to a noncompensable defect of the brain before birth. Therefore, more efforts to improve the treatment would not overcome this prenatal damage. In contrast other groups questioned whether the treatment modalities of this first generation of CH patients treated in newborn screening programs, were not efficient to normalize TSH levels early enough. The starting dose of LT4 in these cohorts was 6–8 μg/kg and the treatment start was rather late with 24 days on average. Therefore, a higher starting dose above 10 μg/kg at the earliest starting time point as possible was recommended by these groups. However, such a treatment scenario results in fairly high fT4 levels when TSH is normalized, which raised conceptual concerns of several other authors.

Outcome in Patients With High Starting Dose of Lt4

One of the first outcome studies reported in children with such a higher starting dose (mean 11.6 μg/kg) and early start of treatment (mean 14 days) was from Montreal, Canada (JM1 *et al.*, 1996). Patients were evaluated at an age of 18 months by Griffiths developmental test and it was shown that there was no difference between severe and moderate cases any longer (patients were matched for their socio-economic status). These encouraging data prompted the authors to announce that the "developmental gap in CH is closed by early high dose treatment." However, the study was based on only 10 severely affected and 35 moderately affected children at an early age of 1.5 years and further studies were required to confirm this finding in more and older patients. Accordingly, several other groups reported about the IQ outcome in patients treated with higher doses, which all did show a better outcome with the higher dose, including the only prospective randomized study comparing 50 μg and 37.5 μg LT4, showing a significantly lower IQ in the latter although the number of patients was again very low (Selva *et al.*, 2005). For most of these studies the main comparison was between severely and moderately affected patients and no control sibling groups were available. Moreover, since earlier studies with lower starting doses did show IQ differences of only 5–10 the upcoming studies that aimed to demonstrate that such a difference is compensable by higher starting doses needed to be statistically powered enough by higher patient numbers. Furthermore, such studies definitely had to take into account the lower outcome of CH patient with particular syndromes like the Down syndrome, Bamforth syndrome (FOXE1 gene defect) or NKX2.1 gene deficiency, since in those patients brain function is disturbed by the primary genetic defect and cannot be compensated by optimal LT4 treatment.

Sibling Controlled Outcome Studies With High Lt4 Starting Dose

Such a study with an appropriate design was eventually published in 2013 from the New Zealand screening program, reporting about a sibling controlled CH cohort. The patients were diagnosed at a mean age of 9 days, treated with a LT4 dose between 10 and 15 μg/kg and TSH normalized within a median of 14 days of diagnosis. The power calculation predicted that the number of patients and siblings would be sufficient to detect a IQ difference of 5.2. There was no significant difference between the affected and nonaffected individuals tested (Albert *et al.*, 2013), suggesting for the first time, that an adequate treatment of CH can render a completely normal IQ.

However, two papers of a Dutch group induced further concern. They claimed that the higher starting dose of >10 μg/kg which cause higher than normal fT4 values during the first 2 years of life, will result in decreasing IQ in older children during adolescence (Bongers-Schokking and de Muinck Keizer-Schrama, 2005; Bongers-Schokking *et al.*, 2016). Although these data were calculated in very small subgroups and the study design did not include sibling controls, the concern of an unfavorable outcome due to "overtreatment" in the high-LT4 regimen was renewed; especially because the mean age of the CH patients in the New Zealand study was only 9.6 years.

The most recent study about the cognitive outcome in CH treated with a high dose was published by our group from Berlin (Aleksander *et al.*, 2018). We had the chance to investigate a cohort of 76 CH patients and their 40 siblings. The treatment modalities were very similar to the New Zealand cohort with a median age of diagnosis of 8 days, mean initial LT4 dose was 13,5 μg/kg and TSH normalized within median 15 days. In contrast to the younger age in the New Zealand group mean age of our patients was 18.1 years and of the sibling controls 19.8 years. We did not find significant differences of overall IQ (102.5 versus 102.5), neither for several other cognitive tests, attention, memory and fine motor skills. Moreover, there was no difference in quality of life scores. Also all anthropometric measurements like length, BMI, blood pressure were without significant difference. According to the Dutch study, we considered the impact of our treatment parameters during the first 2 years of life and tested for a correlations of the number of episodes of elevated fT4 and suppressed TSH with the final IQ, which was negative. Furthermore, we performed a meta-analysis of the previous outcome studies that compared the IQ outcome in moderate versus severe cases. We did find a significant difference of the IQ in the low starting dose (<8 μg/kg) and moderate starting dose (8–10 μg/kg) patient cohorts but not in the high starting dose group (>10 μg/kg). Together, our data argued against the Dutch prediction that high dose will disturb IQ in adolescents, since in our young adult cohorts we did not find a IQ difference despite we had multiple episodes of high fT4. In addition, the meta-analysis strongly argued for a normal outcome of severe cases only if they were treated with a high starting dose.

One interesting finding was again, that in our patient group the high LT4 starting dose resulted in almost all patients in supra-physiological fT4 values without any sign of hyperthyroidism while TSH and T3 were normal. Thus, the high LT4 dose which results in elevated fT4 values does not cause a state of "overtreatment" but instead -as depicted by the normal TSH—is necessary to normalize the physiological relevant active hormone values of T3. If lower doses are given as suggested by, for example, the Dutch group, the fT4 is normal but TSH remains in most patients elevated for a longer time, which reflects an under-treatment that results in lower IQ as shown in the two cited adult outcome studies from Canada and Norway (Rovet, 2005; Oerbeck *et al.*, 2003). Therefore, the appropriate target parameter for LT4 dosing to enable normal development is the serum TSH and not T4.

The particular constellation of supra-physiological fT4 while TSH and T3 are normal was also observed in LT4-treated adult patients after total thyroidectomy when aiming normal TSH values (Bagattini *et al.*, 2014). Also in our adult patients under study the same constellation of high fT4 and normal TSH and T3 was found again as in their infancy. One likely explanation for this higher serum T4/T3 ratio might be the lack of direct secretion of T3 from the missing thyroid that is compensated with higher fT4 as an additional resource for T3 production in the periphery.

Finally, after more than 100 years of clinical research in CH treatment, today evidence is available from two sufficiently powered outcome studies which justify a high starting dose of >10 μg/kg LT4 that will result in IQ values that is not any longer different to sibling controls. Moreover, the actual results imply that prenatal hypothyroidism seems not to cause noncompensable defects in brain development, since an early and high dose postnatal treatment enables a completely normal brain function later in life (**Fig 1**).

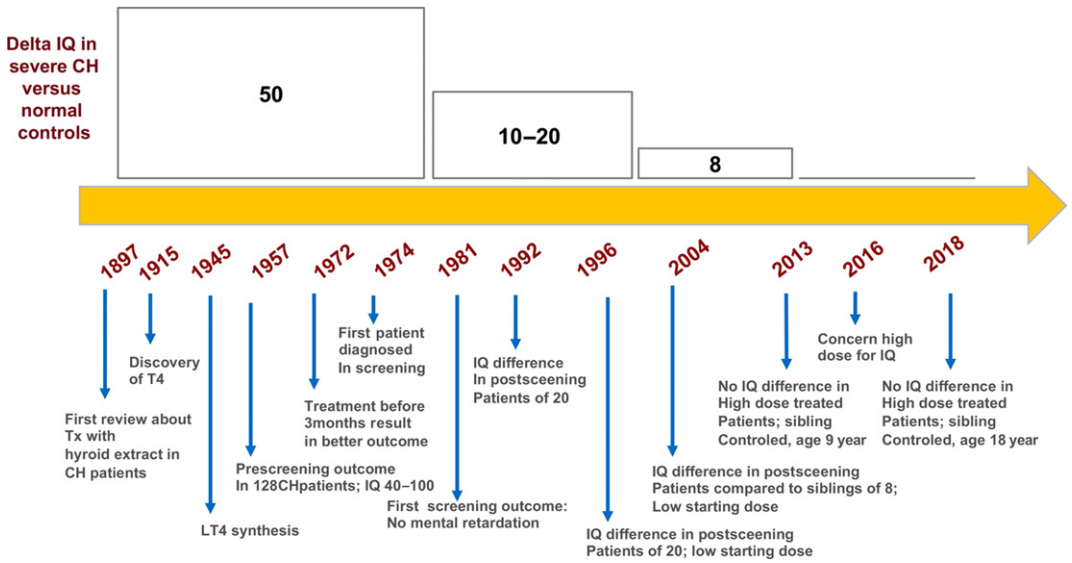

Fig. 1 Timeline of achievements in CH treatment.

Perspective

While acceptable evidence for the treatment of severe CH cases seems now be available, the most important further questions concerning cognitive outcome and newborn thyroid function relates to the milder end of the spectrum of thyroid dysfunction. Due to the introduction of lower TSH cut-off values in the CH screening programs a large number of children are diagnosed with only subtle biochemical alterations. The outcome of these children if remained untreated is unknown, but nevertheless most children are now under treatment due to the concern to not undertreat such individuals. Considering the aforementioned differences in IQ outcome even in severe cases with IQ ranging from 40 to 100, very accurate outcome studies with a large number of children are warranted to generate also appropriate evidence for the treatment and effect on IQ outcome in this large group of very mild CH cases.

Another focus of IQ outcome in newborn thyroid dysfunction relates to the two monogenic diseases of deficient thyroid hormone-transport and—action in children with the MCT8- and TRalpha defects. Here new therapeutic strategies need to be developed to also enable a better outcome; the success in treating severe CH patients during the last 100 years is promising that also these two particular groups of severely affected children will benefit from further clinical studies.

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Hyperthyroidism, Childhood and Adolescence☆

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Introduction

Hyperthyroidism can have profound effects on the growing child and adolescent, including somatic and behavioral effects. In the pediatric population, the hyperthyroid state can be present for extended periods before recognition, contributing to physical decline. A number of conditions can result in hyperthyroidism in childhood; however, Graves' disease (GD) is the most common cause. Fortunately, the different causes of hyperthyroidism can be distinguished and specific treatments rendered. Updated guidelines for the treatment of pediatric hyperthyroidism can be found from the American Thyroid Association (Ross *et al.*, 2016) and from the Japanese Pediatric Thyroid Committee (Committee on Pharmaceutical Affairs JSfPE *et al.*, 2017).

Clinical Evaluation of the Hyperthyroid State

Thyroid disease can present with overt symptoms, insidiously, or with isolated thyromegally. Thus, evaluation of the thyroid gland should be included in the routine examination of children. The thyroid gland can be visualized by having the patient look to the ceiling and swallow. As the thyroid moves, the margins of the gland are viewed to estimate size and symmetry. The thyroid should be palpated to assess size, consistency, and symmetry. This is best performed with the clinician standing behind the patient and palpating the neck with the fingertips. The texture of the thyroid can be assessed to determine if it is smooth or irregular and if nodules present, which may feel firm or soft. If any asymmetry or abnormal thyroid fullness is noted, ultrasonographic evaluation is recommended, as pathological thyroid nodules may feel like normal tissue.

To assess gland size, one may estimate the size of each thyroid lobe relative to that of a teaspoon (5 g) or a tablespoon (15 g). Generally, until the end of puberty, gland size (in grams) approximates the patient's age in years times 0.5–0.7 (Ueda, 1990). Thus, each thyroid lobe of a 10-year old is approximately one-half of a teaspoon for a total gland size of 5–7 g (Ueda, 1990). In adults, each lobe of the thyroid may reach one teaspoon in size for an approximate total gland size of about 10 g (Ueda, 1990).

When hyperthyroidism is suspected, the practitioner should look for somatic signs. One of the cardinal in universal features of hyperthyroidism is tachycardia (Talbot *et al.*, 1952; Wilkins, 1965). In the absence of such, it is unlikely that hyperthyroidism is present. Other clinical features can include a prominent stare and proptosis, although eye findings occur less commonly in children than in adults (Ross *et al.*, 2016; Rivkees, 2016). Although it is the common perception that children presenting with hyperthyroidism present thin and with weight loss, this is often not the situation (van Veenendaal and Rivkees, 2011).

Biochemical Evaluation of Thyroid Function

Approximately 97% of the thyroid hormone released from the thyroid gland is thyroxine (T_4) (Kaplan, 1999). After its release, less than 1% of T4 remains free. The rest of the thyroid hormone circulates bound to the proteins thyroglobulin (thyroid binding globulin or TBG; 70%), prealbumin (transthyretin; 10%), and albumin (15%–20%) (Kaplan, 1999). Triiodothyronine (T_3) is also released from the thyroid and is generated peripherally. Although T4 constitutes the bulk of circulating thyroid hormone, T3 has far greater affinity for nuclear thyroid hormone receptors and exerts most of the potent cellular effects of thyroid hormone action (Ribeiro *et al.*, 1995; Lazar, 1991).

Thyroid function can be assessed by measurement of total T4 and total T3 levels, along with indices that reflect thyroid hormone-binding proteins (T3 or T4 resin uptake) (Kaplan, 1999). The levels of estimated free (unbound) T4 (FT4) are measured to assess thyroid hormone status without the confounding influences of carrier proteins.

Several conditions are seen in which thyroid hormone levels are abnormal, yet the individual is euthyroid. Because of their confusing nature, these conditions may result in the patient's being erroneously diagnosed or treated for hypothyroidism or hyperthyroidism.

When FT4 values are normal yet total T4 values are high, familial dysalbuminemic hyperthyroxinemia (FDH) needs to be considered (Farror *et al.*, 1987; Ruiz *et al.*, 1982). This autosomal dominant disorder is most commonly seen in Hispanic individuals and can be diagnosed by thyroid hormone-binding protein electrophoresis. In some situations, FDH can be confused for hyperthyroidism, it is important to note that a distinguishing feature is that thyroid stimulating hormone (TSH) levels are not suppressed in FDH.

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T4 is much more abundant in the circulation and T3 is the more metabolically active thyroid hormone. T3 is produced peripherally from T4 and is also secreted by the thyroid. A metabolically inactive form of T3′ reverse T 3' is also produced, and its level is elevated in conditions such as euthyroid sick syndrome (De Groot, 1999).

Ultrasensitive TSH assays have been developed, and assessment of TSH has greatly improved the evaluation of thyroid status (Ross, 2001). TSH levels help to distinguish many thyroid disorders that present with either low or high T4 levels in most cases. TSH values within the normal range for the assay are indicative of a euthyroid state if the hypothalamic pituitary axis is intact. When both FT4 and TSH levels are elevated, TSH-producing pituitary adenomas and thyroid hormone resistance need to be considered. In the hyperthyroid state, though, TSH levels are generally completely suppressed (Ross *et al.*, 2016; Rivkees, 2016).

Hyperthyroidism

Hyperthyroidism occurs less commonly in children than hypothyroidism, yet is far more symptomatic (Talbot *et al.*, 1952; Wilkins, 1965; Rivkees, 2010; Bahn *et al.*, 2011; Bahn Chair *et al.*, 2011). Graves' disease is the most common cause of childhood thyrotoxicosis and is characterized by diffuse goiter, hyperthyroidism, and occasionally ophthalmopathy. Other causes of hyperthyroidism in children include autonomously functioning thyroid nodules, neonatal thyrotoxicosis, and infections of the thyroid. Hyperthyroidism also results from thyroid hormone ingestion, McCune-Albright syndrome, struma ovarii, and TSH-producing pituitary adenomas. Epidemic hyperthyroidism has also been seen when thyroid tissue has been inadvertently included in meat products (Hedberg *et al.*, 1987). In contrast to these disorders, thyroid hormone resistance may appear similar to hyperthyroidism, yet is best left untreated.

Graves' Disease

Graves' disease (GD) is the most common cause of hyperthyroidism in children and effects 1 in 10,000 children (Ueda, 1990). An autoimmune disorder, GD is due to thyroid gland stimulation by thyroid receptor antibodies (TRAbs; or thyroid stimulating immunoglobulins, TSI) and involves genetic factors (Ueda, 1990). Hyperthyroidism can exert profound adverse effects on children, including excessive physical activity, tremor, tachycardia, flushing, palpitations, weight loss, accelerated linear growth, reduced bone mineralization, and poor school performance (Hopwood *et al.*, 1976). In comparison with adults (Kaplan, 1999; Ribeiro *et al.*, 1995), eye disease occurs in the minority of pediatric patients with GD, and when it presents, is usually mild (Lazar, 1991).

Over the past several years additional outcome data have become available (Kaplan, 1999; Farror *et al.*, 1987; Ruiz *et al.*, 1982; Nikolai and Seal, 1967; De Groot, 1999) to complement older studies looking at spontaneous remission rates of children with GD (Ross, 2001; Spencer *et al.*, 2007; Zarkovic *et al.*, 2011; Volzke *et al.*, 2010; Surks *et al.*, 2005). Collectively, these studies show that the majority of pediatric patients with GD will not undergo spontaneous remission even after many years of antithyroid drug (ATD) therapy. Thus, most pediatric patients will require either radioactive iodine (^{131}I) or surgery (Verburg *et al.*, 2011; Lazar *et al.*, 2009; Brabant *et al.*, 2006; Talbot *et al.*, 1952).

Antithyroid Drug Therapy

ATDs act by inhibiting oxidation and organic binding of thyroid iodide to impair thyroid hormone production and include methimazole (MMI) and propylthiouracil (PTU) (Wilkins, 1965). MMI is 10- to 20-fold more potent than PTU and has a longer half-life (Rivkees, 2010). These medications do not cure the hyperthyroid state, rather they palliate the condition until spontaneous remission occurs or definitive therapy is rendered. Each of these medications is associated with adverse events that must be considered when prescribed. As such, prior to the initiation of drug therapy, a back-up plan that takes into account the patient's age and treatment risks must be developed at therapy onset in the event that a toxic reaction occurs.

Because it takes 1 or 2 months until biochemical hyperthyroidism resolves on drug therapy (Bahn *et al.*, 2011), treatment with beta-blockers (propranolol, atenolol, or metoprolol), can be used to control GD symptoms. Focusing on symptom control with beta-blockers also alleviates the perceived need for initial high-dose ATD therapy.

In 2008, Rivkees brought to public attention a number of serious complications associated with PTU therapy in children (Bahn Chair *et al.*, 2011; Hedberg *et al.*, 1987; LeFranchi and Mandel, 1995). PTU-induced liver injury occurs rapidly and is often irreversible (Chapman, 1983; Cooper, 2005). Thus, serial monitoring of transaminase levels in a child on PTU, is not viewed as useful in reducing the hepatotoxicity risk (Rivkees *et al.*, 1998). The only way to reduce the risks of PTU-related hepatotoxicity is to avoid the use of the medication.

PTU, though, may be needed in special circumstances (Cooper, 1998). These conditions include situations when neither prompt ^{131}I or surgical treatment are options in a patient who has had a toxic reaction to MMI, and ATD medication is necessary. In this setting, PTU use should only be short-term (Conference Proceeding, 2009). If PTU is prescribed, patients and guardians must be informed of the risk of liver failure and to be alert for signs and symptoms of liver abnormalities, including pruritus, jaundice, anorexia, light colored stools, dark urine, and abdominal pain. If these symptoms develop, the patient should

immediately stop the medication, a physician contacted, and laboratory tests obtained to evaluate hepatic function and transaminase levels (Conference Proceeding, 2009).

MMI is now the drug-of-choice for GD. The typical MMI doses described in published reports is 0.2–0.5 mg/kg per day and range from 0.1 to 1.0 mg/kg per day (Rivkees and Mattison, 2009; Abalovich *et al.*, 2007; Bahn *et al.*, 2009; Rivkees, 2006; Nakamura *et al.*, 2007; Rivkees *et al.*, 2010). However, as noted below, lower rather than higher doses should be considered. Although MMI is often prescribed in divided doses over the day, once a day dosing is usually sufficient (Tajiri and Noguchi, 2005) and is associated with better compliance than multiple daily doses (Cooper *et al.*, 1983).

MMI is available in 5, 10, and 20 mg tablets. When used in children, the following doses that are fractions of tablets can be used: infants, 1.25 mg per day; 1–5 years, 2.5–5.0 mg/day; 5–10 years, 5–10 mg/day; and 10–18 years, 10–20 mg/day. Because the hyperthyroid state can be associated with low white cell counts and patients will be treated with a medication that can depress neutrophil levels, one should obtain a complete blood count at therapy onset (Cooper, 2005).

It is important to recognize that one does not need to use high doses at treatment onset. The response to ATDs influencing circulating thyroid hormone levels is not instantaneous, and several months are needed for thyroid hormone levels to normalize (Tajiri and Noguchi, 2005; Cooper *et al.*, 1983). Thyroid function tests should be obtained monthly after therapy onset. After T4 levels become normal, the MMI doses can be cut by half to maintain euthyroidism (Cooper, 2005).

MMI therapy is not without risks. Minor side effects may affect up to 20% of children, and major side effects may occur in 1% of children (Glaser and Styne, 2008; Shulman *et al.*, 1997). The most common minor adverse side effects related to MMI are hives, arthralgia, and neutropenia (Kaguelidou *et al.*, 2008; Glaser and Styne, 1997). Children may also develop major side effects, including agranulocytosis, Stevens–Johnson syndrome, and vasculitis (Kaguelidou *et al.*, 2008). MMI adverse events most commonly occur within 6 months of therapy onset (Lazar *et al.*, 2000; Shulman *et al.*, 1997). Yet, children may develop adverse events more than 12 months after treatment onset.

Agranulocytosis is a potential serious ATD adverse event and occurs in 0.3% of adults taking PTU or MMI (Kaguelidou *et al.*, 2008; Glaser and Styne, 1997). The agranulocytosis risk is dose-dependent and is rare (Hamburger, 1985; Leger *et al.*, 2012). If an individual receiving MMI feels ill, becomes febrile or develops pharyngitis, MMI should be stopped immediately, a practitioner contacted, and a complete blood cell count obtained.

ATD Therapy Duration

Based on available evidence (Rivkees *et al.*, 1998; Read Jr. *et al.*, 2004; Levy *et al.*, 1988; Nebesio *et al.*, 2002; Rivkees and Cornelius, 2003; Rivkees and Dinauer, 2007; Ross *et al.*, 2016; Rivkees, 2016), prolonged ATD therapy will not result in an increased chance of remission for most children, whereas in others it may. As such, with the initial evaluation of a child with GD, practitioners should attempt to stratify children into two groups: those children with a 30%–40% chance of remission with prolonged ATD therapy and those with a slim chance of spontaneous remission.

Prospective studies in adults show that if remission does not occur after 12–18 months of ATD therapy, there is a very low likelihood of remission occurring with prolonged therapy (Rivkees and Cornelius, 2003). In the pediatric population, published data generally show that when ATDs are used for 1–2 years, remission rates are 15%–30%, and possibly up to 40% in some children (Rivkees and Cornelius, 2003; Kadmon *et al.*, 2001; Nebesio *et al.*, 2002; Rivkees, 2006, 2007; Rivkees *et al.*, 1998; Boice Jr, 2006, 2005). (Remission is defined as being either euthyroid or hypothyroid for 1 year or more after cessation of therapy.)

Yet, looking closely at these data, one can distinguish patients with a greater likelihood of remission from those with a much lower chance following prolonged ATD therapy. The chance of remission after years of ATDs will be low if the thyroid gland is large (greater than two times normal size for age), the child is young (<12 years), not Caucasian, serum TRAb/TSI levels are elevated, or the patient presents with profound hyperthyroidism at presentation (free $T_4 > 4$ ng/dL) (Dolphin, 1968; Boice Jr, 2006, 2005).

In adults, assessment of TRAb or TSI levels is useful in determining disease course and remission likelihood (Dolphin, 1968; Sigurdson *et al.*, 2005). This issue has been less studied in children. Consistent with the notion that GD will remit in only a small proportion of children, TRAb levels normalize after 24 months in only 18% of pediatric patients on ATDs (Dolphin, 1968). TRAb levels thus persist longer in children than in adults (Ron *et al.*, 1995). There are no data to show normalization of TRAb levels when patients are on ATDs for a longer time.

For those children with unfavorable risk factors for spontaneous remission at treatment onset, it is reasonable to treat children for up to 2 years with MMI and see whether spontaneous remission has occurred. At that point, if there is no remission, it is appropriate to move on to definitive therapy if desired by the family. Alternatively, treatment for longer periods can be considered, as long as side effects to medication do not occur. This approach may be especially useful if the child is considered too young for surgery or radioactive iodine. For the child with favorable risk factors for remission, if spontaneous remission has not occurred after the 2 years of ATDs, continuation of antithyroid medication for prolonged periods is also acceptable.

Radioactive Iodine Therapy for Graves' Disease

The goal for ^{131}I therapy for GD is to induce hypothyroidism. Radioactive iodine should not be given to cause euthyroidism in children, as this results in partially irradiated residual thyroid tissue that will be associated with a higher risk of thyroid neoplasm

than the normal population (Rivkees and Mandel, 2011; Van Vliet *et al.*, 2008). It has been suggested that dosages delivering 30,000–40,000 cGy (rad) to the thyroid are necessary to ablate the thyroid gland (Polak *et al.*, 2006; Luton *et al.*, 2005). But, dosages delivering 10,000–20,000 cGy to the thyroid are more often used and result in partial or complete destruction of the thyroid (Van Vliet *et al.*, 2008; Polak *et al.*, 2006; Luton *et al.*, 2005). Typically, administered thyroid doses of 150 μCi/g (5.5 MBq/g) generate radiation doses of 12,000 cGy to the thyroid (Van Vliet *et al.*, 2008).

Some centers give a fixed administered dosage of 10 or 15 mCi ^{131}I to all children (Polak *et al.*, 2006), rather than individually calculated administered activation. There are no studies comparing outcomes of fixed doses versus calculated doses in children. In adults, the two different approaches lead to similar outcomes (Luton *et al.*, 2005; Van Vliet *et al.*, 2008); however, in children, a potential advantage of calculated versus fixed dosing is that it might be possible to use lower dosages of ^{131}I if the administered dose is calculated.

When children are to be treated with ^{131}I, ATDs should be stopped 3–5 days prior to treatment (Polak *et al.*, 2006). Patients are placed on beta-blockers until T4 and/or free T4 levels normalize posttherapy. Whereas some clinicians restart ATDs after treatment with ^{131}I, this is rarely required in children (Luton *et al.*, 2005; Polak *et al.*, 2006; Check *et al.*, 1982; Skuza *et al.*, 1996). Thyroid hormone levels begin to decrease about 7 days after radioiodine therapy in children, and continued ATD use can make it difficult to assess if posttreatment hypothyroidism is the result of ^{131}I or the ATD.

Side effects of ^{131}I therapy are unusual. Less than 10% of children will complain of mild tenderness over the thyroid in the first week after ^{131}I therapy (Skuza *et al.*, 1996; McKenzie and Zakarija, 1992). This problem can be treated with either acetaminophen or nonsteroidal, antiinflammatory agents for 24–48 h (Zimmerman, 1999).

There are rare reports of children with severe hyperthyroidism developing thyroid storm after ^{131}I (Zimmerman, 1999). In general, these children were severely hyperthyroid when ^{131}I was rendered. Thus, if T4 levels are >20 µg/dL (200 nmol/L) or freeT4 levels are >5 ng/dL (60 pmol/L), children should be treated with MMI until T4 and/or free T4 levels normalize before proceeding with ^{131}I therapy (Daneman and Howard, 1980; Check *et al.*, 1982). It is important to recognize that most children with GD have been hyperthyroid for months prior to diagnosis; there is no need to rush to ^{131}I therapy.

It usually takes 6–12 weeks after ^{131}I treatment for the patient to become biochemically euthyroid or hypothyroid. Until then, symptoms of hyperthyroidism can be controlled using beta-blockers (Bruinse *et al.*, 1988; Momotani *et al.*, 1986; Zimmerman, 1999). The use of SSKI or Lugol's solution 1 week after ^{131}I will also quickly attenuate biochemical hyperthyroidism without adversely affecting the outcome of radioiodine therapy (McKenzie and Zakarija, 1992).

Several studies have reported the details of ^{131}I therapy for childhood GD (Tamaki *et al.*, 1989; Sunshine *et al.*, 1965; Jeng *et al.*, 1994; Vitti *et al.*, 1999). Children as young as 1 year old have been treated with ^{131}I with excellent results (Vitti *et al.*, 1999; Szabo and Allen, 1989). But, treatment of such young children is not common, nor is now recommended. ^{131}I dosages in children and teenagers have ranged from 100 to 400 μCi/g of thyroid tissue (Geva and Theodor, 1988). Similar to that found in adults (Lugo-Vicente *et al.*, 1998; Tonacchera *et al.*, 1998; Esapa *et al.*, 1997), responses to ^{131}I therapy are related to gland size and dose. 25%–40% of children treated with 50–100 μCi of ^{131}I per gram of thyroid tissue are hyperthyroid several years after therapy (Vattimo *et al.*, 1998). In children treated with 150–200 μCi of ^{131}I per gram thyroid, hyperthyroidism remains in 5%–20%, and 60%–90% become hypothyroid (David *et al.*, 1995; Hamburger and Hamburger, 1985; Derwahl and Studer, 2000).

The development of progression of ophthalmopathy following ^{131}I in adults has been reported (Barrio-Barrio *et al.*, 2015; Bahn, 2015; Wiersinga and Prummel, 2002). However, unlike adults, children rarely develop severe ophthalmopathy and proptosis is mild (Bahn *et al.*, 2011; Rivkees *et al.*, 1998). Studies show that disease worsens in only a small percentage of children with GD, irrespective of therapy type (Safa, 1975; Safa *et al.*, 1975; Barnes and Blizzard, 1977).

In adults, it has been shown that progression of ophthalmopathy can be prevented by treatment with prednisone for 3 months following ^{131}I therapy (Wiersinga and Prummel, 2002; Bahn *et al.*, 2011). Adjunctive prednisone therapy is not routinely recommended for the majority of children, as most do not have significant eye disease. The prolonged administration of prednisone is also associated with growth failure, weight gain, and immune suppression. Nevertheless, prednisone may be useful for the child who has moderate or severe eye disease and will be treated with ^{131}I.

Risks of Radioactive Iodine in Children Treated for GD

There is no evidence showing adverse effects to offspring of children treated with ^{131}I. Birth defects are not higher in 500 offspring born to about 370 individuals treated with ^{131}I for hyperthyroidism during childhood or adolescence (Rivkees *et al.*, 1998). Additionally, the rates of birth defects are not higher in children treated with 80–700 mCi of ^{131}I for thyroid cancer, which are dosages that are much higher than those used for GD (Sarkar *et al.*, 1976).

The thyroid gland is unique in its developmental sensitivity to malignancy after low-level radiation exposure (Boice Jr, 2005, 2006; Ron *et al.*, 1995; Dolphin, 1968). There is an increased risk of thyroid cancer in individuals less than 20 years of age at the time of low-level thyroid irradiation (Boice Jr, 2005, 2006; Ron *et al.*, 1995). In contrast, individuals who are older than 20 years of age, do not exhibit an increased risk of thyroid cancer when exposed to low-level thyroid irradiation (Boice Jr, 2005, 2006; Ron *et al.*, 1995; Dolphin, 1968).

The risk of thyroid neoplasms in children is greatest with exposure to low-level external radiation (0.1–25 Gy; $\sim$0.09–30 μCi/g) (Dolphin, 1968; Ron *et al.*, 1995; Boice Jr, 2006, 2005; Sigurdson *et al.*, 2005) and not with the higher dosages used to treat GD. At

present, we are not aware of any cases of thyroid cancer that developed in pediatric patients treated with >150 μCi of ^{131}I per gram of thyroid tissue for childhood GD that can be attributed to ^{131}I therapy.

Important in considering radioactive iodine use in children, is the potential influences of ^{131}I therapy on other cancers, as ^{131}I therapy results in low-level, whole body radiation exposure. Several studies in adults have examined potential risks of ^{131}I therapy for GD on cancers. These studies have generally not revealed increased mortality or increased rates of cancer following ^{131}I for GD (Flynn *et al.*, 2006; Franklyn *et al.*, 1998, 2005; Goldman *et al.*, 1990; Holm *et al.*, 1991; Metso *et al.*, 2007; Ron *et al.*, 1998).

In comparison with studies in adults, few studies have focused on outcomes of ^{131}I therapy for childhood GD. The most extensive study of pediatric patients involved 36-year outcomes of 116 patients who were less than 20-years old when treated with ^{131}I therapy for GD (Read Jr. *et al.*, 2004). There was no evidence for increase cancer risk in this population. Yet, this sample size is small.

The total-body radiation dose after ^{131}I varies with age, and the same absolute dose of ^{131}I will result in more radiation exposure in a young child than in an adolescent or adult (Toohey *et al.*, 2000; Toohey and Stabin, 1996). Currently, we do not have dosimetry data on ^{131}I use in pediatric patients with GD to assess total-body exposure in pediatric patients. Based on phantom modeling, it is estimated that at 0, 1, 5, 10, 15 years, and adulthood, respective total-body radiation doses will be 11.1, 4.6, 2.4 1.45, 0.90, and 0.85 rem (0.01 Sv) per mCi of ^{131}I administered (Toohey *et al.*, 2000; Toohey and Stabin, 1996). Based on the Biological Effects of Ionizing Radiation Committee V (BEIR VII) analysis of low-level, acute exposure to radiation (National Research Council, 2006), theoretical lifetime attributable risk of cancer mortality and all cancer incidence can be projected. Based on these theoretical calculations, we feel that it is prudent to avoid radioactive iodine therapy in children under 5 years of age and to avoid >10 mCi in patients less than 10 years old.

Thyroidectomy and Risks

Surgery is an effective form of therapy for GD if it can be performed by an expert surgeon and in some settings it is preferable to radioactive iodine. When surgery is performed, near total or total-thyroidectomy is indicated, as subtotal thyroidectomy is associated with a higher relapse rate (Miccoli *et al.*, 1996). Hypothyroidism is nearly universal in children and adults who undergo total thyroidectomy (Ching *et al.*, 1977; Buckingham *et al.*, 1981; Miccoli *et al.*, 1996; Rudberg *et al.*, 1996). In comparison, after subtotal thyroidectomy, hyperthyroidism recurs in 10%–15% of patients (Ching *et al.*, 1977; Buckingham *et al.*, 1981; Miccoli *et al.*, 1996).

Surgery is preferred in children younger than 5 years when definitive therapy is needed AND can be performed by a skilled thyroid surgeon. In individuals who have large thyroid glands (>80 g), the response to ^{131}I is poor (Peters *et al.*, 1996, 1997). Thus, surgery is also recommended for these patients.

In preparation for surgery, the patient should be rendered euthyroid. Typically, this is done by continuing MMI until T4 levels normalize. A week before surgery, iodine drops are started (1–3 drops, t.i.d.), which inhibits thyroid hormone production and causes the gland to become firm and less vascular.

Postoperatively, younger pediatric patients are at a higher risk for transient hypoparathyroidism than adolescents or adults (Sosa *et al.*, 2008). To mitigate postoperative hypocalcemia, we treat children with 0.5 mcg of calcitriol, twice a day, for 3 days prior to surgery. Postoperatively, the calcitriol is weaned over 15 days (0.5 mcg bid × 5 days; 0.5 mcg q.d. × 5 days; 0.5 mcg q.o.d. × 5 days) (Breuer *et al.*, 2013a). Using this approach only 5% of patients require postoperative calcium infusions versus 40% of patients without preoperative treatment (Breuer *et al.*, 2013a).

Acute complications that follow thyroidectomy include hemorrhage, hypocalcaemia, and recurrent laryngeal nerve paresis (Sosa *et al.*, 2008, 1998; Lal *et al.*, 2005; Boger and Perrier, 2004; Witte *et al.*, 2000). In children, rates from 0 to 6 years were 22%, from 7 to 12 years, 11%; and from 13 to 17 years, 11% (Sosa *et al.*, 2008). These rates are higher than those observed in adults.

Complication rates are related to the expertise of surgeon (Sosa *et al.*, 2008). Considering these data, if local pediatric thyroid surgery expertise is unavailable, referral of a child with GD to a high-volume, thyroid surgery center with pediatric experience should be considered (Breuer *et al.*, 2013b; Peroni *et al.*, 2012). Very low complication rates for children undergoing the thyroidectomies for GD have been reported with this type of multidisciplinary model (Breuer *et al.*, 2013a,b).

Other Causes of Hyperthyroidism

Neonatal Thyrotoxicosis

Thyrotoxicosis in the neonate is a severe and life-threatening condition that call be associated with lasting neurologic problems (Aslam and Inayat, 2008; Zimmerman, 1999). If a mother has GD, the chance is 1 in 80 that TRAbs will be transferred to the fetus, which will result in intrauterine or neonatal hyperthyroidism (Skuza *et al.*, 1996). Rarely neonatal thyrotoxicosis will persist, like the GD disease seen in older children (Zimmerman, 1999). In other rare cases, persistent neonatal thyrotoxicosis is caused by activation of the TSH receptor (Chester *et al.*, 2008; Watkins *et al.*, 2008).

The fetal thyroid gland is responsive to maternal TRAbs, which, if present at elevated levels, may result in hyperthyroidism (Van Vliet *et al.*, 2008; Polak *et al.*, 2006; Luton *et al.*, 2005; Bahn *et al.*, 2011). Fetal hyperthyroidism manifests during the second half of

gestation, as transfer of TRAbs from the mother to the fetus increases with progression of pregnancy (Van Vliet *et al.*, 2008; Polak *et al.*, 2006; Luton *et al.*, 2005).

The risk of fetal hyperthyroidism and neonatal Graves' disease is proportional to the magnitude of elevation of TRAb levels (Van Vliet *et al.*, 2008; Polak *et al.*, 2006; Luton *et al.*, 2005). Fetal hyperthyroidism is generally associated with levels of TRAbs more than two- to fourfold greater than the upper limit of normal for assay (Van Vliet *et al.*, 2008; Polak *et al.*, 2006; Luton *et al.*, 2005). Because the fetus is at risk for hyperthyroidism when there is active or past maternal GD, fetal growth and heart rate should be regularly assessed from midpregnancy onward (Van Vliet *et al.*, 2008; Polak *et al.*, 2006; Luton *et al.*, 2005). Excessive fetal heart rate (>160 beats per minute after 20 weeks) and the presence of a fetal goiter suggest hyperthyroidism in the fetus. In addition, accelerated maturation of the femoral ossification center is seen with fetal hyperthyroidism (Polak *et al.*, 2006).

If a mother with GD is taking antithyroid medications during pregnancy, fetal thyroid hormone synthesis will be inhibited, which will prevent the development of intrauterine hyperthyroidism (Check *et al.*, 1982). However, the infant may be born with a goiter and hypothyroidism. At birth, circulating levels of T4 may be low and TSH levels elevated. In most cases, the effects of antithyroid drugs wane, and thyroid function normalizes within a week (Skuza *et al.*, 1996). If significant transplacental passage of TRAbs has occurred, however, thyrotoxicosis will develop (Skuza *et al.*, 1996; McKenzie and Zakarija, 1992).

If a mother with a history of GD is not taking antithyroid drugs during pregnancy, the fetus may develop intrauterine hyperthyroidism (Zimmerman, 1999). If the condition is not recognized, it may result in profound intrauterine thyrotoxicosis and growth retardation (Zimmerman, 1999). Such infants have prematurely fused cranial sutures, advanced skeletal age, long-term learning problems, and mental retardation (Zimmerman, 1999; Daneman and Howard, 1980). If fetal hyperthyroidism is recognized prenatally by the presence of fetal tachycardia (heart rate higher than 160 beats per minute after 22 weeks), treatment of the mother with antithyroid drugs will reduce intrauterine thyrotoxicosis (Check *et al.*, 1982; Bruinse *et al.*, 1988; Momotani *et al.*, 1986).

Treatment of thyrotoxic infants consists of administration of antithyroid medications (PTU 5–10 mg per kg per day or MMI 0.5–1.0 mg per kg per day) and beta-blockers (propranolol 1 mg per kg per day). Lugol's solution or saturated potassium iodide may be given (1–2 drops every 8 h) for 7–10 days to more rapidly control biochemical hyperthyroidism. After approximately 2 weeks of antithyroid drug therapy, thyroid hormone levels will decline. When thyroid hormone levels fall below normal, supplementary levothyroxine (37.5 μg per day for full-term infants) is added to prevent hypothyroidism. As TRAbs are cleared from the infant's circulation, spontaneous recovery begins within 3 months and is usually complete by 6 months (Zimmerman, 1999; McKenzie and Zakarija, 1992). Thus, the infant can be weaned from treatment after 3 months. Monitoring of the infant's TRAb levels is also a useful predictor of when antithyroid medication can be tapered (Tamaki *et al.*, 1989; Sunshine *et al.*, 1965).

Infectious Thyroiditis

Occasionally a child presents with hyperthyroidism, tenderness over the thyroid gland, and fever due to bacterial infection of the thyroid, a condition called acute thyroiditis (Jeng *et al.*, 1994). Acute thyroiditis can be associated with the presence of a fistula connecting the piriform sinus on the left side of the pharynx to the thyroid (Jeng *et al.*, 1994). Fevers can be high, and erythrocyte sedimentation rates and white counts elevated. Ultrasonography may reveal a local abscess. In contrast to GD, uptake of technetium 99-pertechnetate or radioiodine is reduced when thyroid scanning is performed.

The offending bacteria include *Haemophilus influenza* and group A streptococci (Jeng *et al.*, 1994). Thus, treatment with an antibiotic resistant to disruption by beta-Iactamase is recommended. In severe cases, hospitalization and intravenous antibiotic administration is indicated, because lymphatic drainage into the mediastinal region may occur. Surgical drainage is needed if a localized abscess develops and the response to antibiotics is poor (Jeng *et al.*, 1994).

Because the infectious process results in destruction of thyroid tissue, release of preformed thyroid hormone and hyperthyroidism may occur during infection. The hyperthyroid state is usually transient, and treatment with antithyroid drugs is not indicated (Jeng *et al.*, 1994). If the patient becomes symptomatic, beta-blockers may be used.

After the child has recovered, pharyngography is indicated to test for a patent piriform sinus tract. Occasionally, the tract may close as the result of the infection. If the tract persists, however, and acute thyroiditis recurs, resection is needed.

Subacute Thyroiditis

Viral infections of the thyroid may occur and result in subacute thyroiditis (Vitti *et al.*, 1999). In comparison with acute thyroiditis, subacute thyroiditis may be less severe (Vitti *et al.*, 1999; Szabo and Allen, 1989). Fever, thyroid tenderness, and hyperthyroidism may be present and may last for several weeks (Geva and Theodor, 1988). Because clinically distinguishing between bacterial and viral thyroid infections is difficult, antibiotic treatment is indicated when infectious thyroiditis is suspected.

Hyperfunctioning Nodules

Warm or hot nodules lead to excessive production of thyroid hormone and can be associated with clinical and biochemical hyperthyroidism (Lugo-Vicente *et al.*, 1998). Interestingly, activating mutations of the TSH receptor and G_s have been discovered in hyperfunctioning nodules (Tonacchera *et al.*, 1998; Esapa *et al.*, 1997). Although hyperfunctioning nodules may be ablated with

radioiodine, surgical excision of hyperfunctioning nodules IS recommended in children and adolescents, because radiation-exposed normal thyroid tissue will remain after the hyperfunctioning nodule is ablated. Although the risk of malignancy in hyperfunctioning nodules is low, thyroid cancers have been described in warm nodules (Vattimo *et al.*, 1998; David *et al.*, 1995).

Toxic Multinodular Goiters

Multinodular goiters are uncommon in children, but patients with this condition can develop thyrotoxicosis, which is usually related to the time the goiter has been present and goiter size. In this setting, hyperthyroidism develops as a single nodule in the thyroid, becomes overly active, and functions autonomously (Hamburger and Hamburger, 1985; Derwahl and Studer, 2000). Forty-six percent of patients may have T3 thyrotoxicosis, and nodules are 3 cm or more in diameter (Hamburger and Hamburger, 1985; Derwahl and Studer, 2000).

In adults, ^{131}I is routinely used in the treatment of isolated toxic adenomas and toxic multinodular goiters (Hamburger and Hamburger, 1985; Derwahl and Studer, 2000). The use of radioiodine to treat these conditions in children is uncommon, however, and few follow-up data are available. Although strong justification exists for the use of radioiodine in the treatment of childhood Graves' disease, especially when appropriate doses are used, we recommend that radioiodine be avoided in children with toxic adenomas or multinodular goiters.

When a toxic nodule is present, either as an isolated nodule or in the setting of a multinodular goiter, thyroid function is suppressed in the nontoxic regions. When radioiodine is given, uptake will be limited to the autonomously functioning tissue, and if large doses are administered, the remaining thyroid tissue will receive external irradiation. Because the risk of thyroid cancer following external radiation is very low after 20 years of age (Nygaard *et al.*, 1999a,b), the use of radioiodine for toxic nodule ablation in adults is not associated with increased thyroid cancer risks. In the child or adolescent treated with ^{131}1 for toxic nodules, however, low-level irradiation to the remaining thyroid tissue may be associated with an increased thyroid cancer risk.

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Thyroid Tumors in Children

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Glossary

Anaplastic thyroid carcinoma An aggressive form of follicular cell-derived thyroid carcinoma that rarely occurs in children.

Follicular cell-derived thyroid carcinoma A type of carcinoma derived from thyroid follicular cells, which normally concentrate iodine and produce thyroid hormone. Papillary carcinoma and follicular carcinoma are two types of follicular cell-derived thyroid carcinoma, each with distinctive pathologic features.

Medullary thyroid carcinoma A type of carcinoma derived from thyroid C cells, which normally produce the hormone calcitonin.

Radioiodine (^{131}I) A radioactive isotope of iodine that emits beta and gamma radiation capable of damaging or destroying tissue. Radioiodine is taken up by thyroid follicular cells, including follicular cell-derived thyroid cancers, allowing it to be used for imaging or treatment of these thyroid cancers.

Thyroglobulin A protein produced exclusively by thyroid follicular cells, including follicular cell-derived thyroid cancers.

Thyroid nodule A mass within the thyroid gland.

Introduction

Tumors of the thyroid are common in adults but relatively rare in children. In many ways pediatric thyroid tumors resemble those in adults, but aspects of their pathology, clinical features, diagnosis, and management also differ in important respects. This article focuses on the unique aspects of thyroid tumors that occur in children; where appropriate, the reader is referred to other relevant entries for more detailed discussion of features common to both pediatric and adult thyroid tumors.

Epidemiology

Thyroid nodules occur in about 0.8%–1.8% of children (Rallison *et al.*, 1975; Hayashida *et al.*, 2013; Suzuki *et al.*, 2016). The prevalence of nodules increases with age throughout childhood, being very low (<0.5%) in children under age 10 and reaching a maximum of about 1.8% in adolescence. Pediatric thyroid nodules are twice as common in females as in males during adolescence, but not earlier in childhood (Hayashida *et al.*, 2013).

Although the risk of malignancy in a pediatric thyroid nodule (22%–26%) is substantially higher than that in an adult thyroid nodule (5%–15%), the majority of pediatric thyroid nodules are benign (Gupta *et al.*, 2013; Francis *et al.*, 2015). Thyroid carcinomas are rare in children, accounting for only 1.5% of malignancies in children under 15 (Vergamini *et al.*, 2014). The annual incidence of thyroid cancer in the United States rises with age and is 2, 8, and 29 per million among children aged 5–9, 10–14, and 15–19 years, respectively. Females are affected three to five times as frequently as males (Howlader *et al.*, 2017). In addition, over the last several decades the incidence of thyroid cancers in children has increased at an annual rate of about 3.8%. Part of this increase may be explained by increased use of radiological imaging leading to (sometimes incidental) detection of small tumors, but this does not appear to be the sole explanation for the rising incidence (Vergamini *et al.*, 2014).

Pathology

The principal cell type in the normal thyroid gland is the follicular cell, which synthesizes and secretes thyroid hormone. Malignancies derived from the follicular cell are often termed follicular cell-derived thyroid carcinomas. The most common form of follicular cell-derived thyroid carcinoma is papillary thyroid carcinoma, which accounts for over 90% of cases in children, while follicular thyroid carcinoma accounts for under 10% of cases. Anaplastic thyroid cancers are exceedingly rare in children. Medullary thyroid cancer is derived from the parafollicular cells (or C cells) of the thyroid and accounts for about 2% of thyroid cancers in children. The histopathologic features of thyroid cancers in children are similar to those in adults.

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Etiology

Genetic Factors

The genetic alternations underlying the development and progression of follicular cell-derived thyroid carcinoma in adults are increasingly well understood. Adult thyroid cancers are generally associated with somatic mutations of the oncogenes *BRAF* or *RAS* (including *HRAS*, *NRAS*, and *KRAS*), or with gene fusions including the tyrosine kinase genes *RET*, *NTRK1/3*, or *ALK* (Cancer Genome Atlas Research, 2014). All of these genetic alterations cause abnormal activation of the MAP kinase and PI3 kinase pathways, leading to cell growth and tumorigenesis.

The genetic landscape of pediatric follicular cell-derived thyroid cancers is distinct from that of adults and is less well understood (**Table 1**). Mutations in *BRAF* (including the most common mutation, V600E) are present in 40%–60% of adult thyroid cancers (Xing, 2007; Cancer Genome Atlas Research, 2014) but are found in only 3%–40% of pediatric thyroid cancers (Bauer, 2017; Alzahrani *et al.*, 2017). Mutations in the *RAS* genes are relatively uncommon in children, occurring in around 10% of pediatric thyroid cancers. In contrast, the prevalence of gene rearrangements involving *RET* (termed *RET-PTC*) is significantly higher in both sporadic and radiation-induced pediatric thyroid cancers (40%–60%) than in adults (Nikiforov *et al.*, 1997; Rabes *et al.*, 2000; Fenton *et al.*, 2000; Bauer, 2017). Gene fusions involving *NTRK1/3* and *ALK* have been identified in children, but their prevalence is not yet clear. *PAX8-PPARG* rearrangements (found in many follicular thyroid cancers in adults) seem to be relatively uncommon in children (Bauer, 2017). Overall, in many pediatric follicular cell-derived thyroid cancers no recognized driver mutation has been found, indicating that much remains to be learned about the genetics of pediatric thyroid tumors.

While somatic mutations account for a large proportion of follicular cell-derived thyroid cancers, the presence of certain germline genetic mutations also increases a child's risk of developing thyroid nodules or cancer. Mutations in the gene phosphatase and tensin homolog (*PTEN*) cause the *PTEN* hamartoma tumor syndromes (Cowden, Bannayan-Riley-Ruvalcaba, and Proteus syndromes), which are characterized by macrocephaly, mucocutaneous lesions, breast and endometrial tumors, and thyroid nodules and cancer that can arise in children as young as 6 years (Smith *et al.*, 2011). Mutations in the *APC* gene cause familial adenomatous polyposis and are associated with an increase risk of thyroid cancer, particularly a specific histologic subtype of papillary thyroid carcinoma called the cribriform-morular variant (Uchino *et al.*, 2016). Germline mutations in *DICER1* are associated with childhood thyroid neoplasia as well as pleuropulmonary blastoma, ovarian tumors, and macrocephaly (Rutter *et al.*, 2016; Khan *et al.*, 2017).

Radiation Exposure

Exposure to radiation significantly increases the risk of developing thyroid nodules and follicular cell-derived thyroid cancer. Observations following the widespread release of radioactive isotopes from the Chernobyl Nuclear Power Plant in 1986 demonstrated that exposure to radioactive isotopes include ^{131}I (primarily by ingestion) is associated with an excess relative risk of thyroid cancer of 5.25 per Gray (Gy), and that this risk increases linearly up to doses of 6–7 Gy (Tronko *et al.*, 2006). Exposure to external radiation also markedly increases the risk of thyroid cancer, including in survivors of Hodgkin lymphoma, leukemia, central nervous system tumors, bone tumors, neuroblastoma, and bone marrow transplantation (Bhatti *et al.*, 2010; Veiga *et al.*, 2012; Inamoto *et al.*, 2015). The relative risk of thyroid cancer rises linearly to a maximum of about 15-fold at a thyroidal radiation dose of 10–30 Gy; above this dose the risk declines slowly, but does not return to baseline. The cumulative risk of developing thyroid cancer after radiation exposure increases over time. Age at exposure to radiation is another important factor, with the risk of thyroid cancer increasing the younger the patient's age at irradiation (Bhatti *et al.*, 2010; Veiga *et al.*, 2012).

Diagnosis

Clinical Features

Thyroid nodules in children are often asymptomatic but may present as a visible or palpable anterior neck mass. With larger nodules patients may note a globus sensation or difficulty swallowing due to impingement on the esophagus, or voice changes

Table 1 Common genetic driver mutations of follicular cell-derived thyroid carcinoma, and their prevalence in children compared to adults

Gene	*Prevalence in children*	*Prevalence in adults*
BRAF	3%–40%	40%–70%
RAS	3%–11%	13%–16%(30%–45% in follicular carcinoma)
RET-PTC	40%–60%	7%–16%
NTRK1/3	Minimal data	2%–3%
ALK	Minimal data	1%

Data from Kimura, E. T., Nikiforova, M. N. and Zhu, Z. *et al.* (2003). High prevalence of BRAF mutations in thyroid cancer: Genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Research 63, 1454–1457; Xing, M. (2013). Molecular pathogenesis and mechanisms of thyroid cancer. Nature Reviews. Cancer 13, 184–199. Cancer Genome Atlas Research, N. (2014). Integrated genomic characterization of papillary thyroid carcinoma. Cell 159, 676–690; Bauer, A. J. (2017). Molecular genetics of thyroid cancer in children and adolescents. Endocrinology and Metabolism Clinics of North America 46, 389–403.

(such as hoarseness) due to impingement on the recurrent laryngeal nerve. Most thyroid tumors in children are discovered by the patient or a family member, or by a physician performing a physical examination, while a minority are discovered incidentally on imaging studies performed for other reasons (Gupta *et al.*, 2014). Nodules that are very firm or fixed to surrounding tissue are more concerning for malignancy, but many pediatric thyroid cancers do not have these features. Patients may also present with cervical lymphadenopathy due to local metastasis of thyroid cancer.

Evaluation

The goal of evaluation of thyroid nodules in children is to identify the minority of nodules (22%–26%) that harbor malignancy, so as to provide appropriate treatment for thyroid cancers while avoiding unnecessary surgery for benign lesions. The latter point is particularly relevant for children, in whom the risks of thyroid surgery are higher than in adults (Wang *et al.*, 2009).

Evaluation of a thyroid nodule begins with measurement of the serum TSH concentration. A low TSH may indicate an autonomous (hyperfunctioning or "toxic") thyroid nodule, and this diagnosis can be confirmed with thyroid scintigraphy (using ^{123}I). In adults, autonomous nodules have a very low rate of malignancy (Haugen *et al.*, 2016). While it has been suggested that autonomous nodules in children have a higher malignancy rate (Niedziela *et al.*, 2002), in fact their malignancy rate may be very low when an autonomous nodule is defined strictly as exhibiting focal uptake of ^{123}I in the nodule under conditions of low TSH (<0.3 mIU/L), with suppressed uptake in the surrounding normal thyroid tissue (Ly *et al.*, 2016).

In children with a normal or elevated serum TSH concentration, ultrasound is the optimal imaging modality for suspected thyroid nodules (Francis *et al.*, 2015) and is superior to palpation in both sensitivity and specificity. Up to 50% of children referred for suspected thyroid nodules actually have no sonographically detectable nodule (Gupta *et al.*, 2013), and data from adults indicate that palpation alone fails to detect up to 24% of thyroid nodules of significant size (Marqusee *et al.*, 2000). Imaging of the cervical lymph nodes should be included in every ultrasound study performed for evaluation of a pediatric thyroid nodule because of the relatively high incidence of local lymph node metastases in children with thyroid carcinoma. It is important to employ an ultrasonographer with experience in pediatric thyroid ultrasound due to the prevalence in children of incidental findings that might inappropriately provoke evaluation for malignancy, including intrathyroidal thymic rests (2%) and benign lymphadenopathy (Hayashida *et al.*, 2013).

Nodules that are suspicious for malignancy are evaluated by ultrasound-guided fine-needle aspiration (FNA). In general, the sonographic features of pediatric thyroid nodules that are associated with a higher risk of malignancy are similar to those in adults, including hypoechogenicity, calcifications, irregular margins, and the presence of abnormal lymphadenopathy (Francis *et al.*, 2015; Martinez-Rios *et al.*, 2018). It is important to be aware that papillary thyroid carcinoma in children may present as a diffusely infiltrating process present throughout a lobe or the entire thyroid, rather than as a discrete nodule, often with microcalcifications and/or abnormal lymphadenopathy. FNA cytology is effective for identifying malignancy and guiding subsequent management in pediatric thyroid nodules (Stevens *et al.*, 2009; Gupta *et al.*, 2013; Francis *et al.*, 2015; Amirazodi *et al.*, 2016).

An important challenge in the evaluation of thyroid nodules is the approach to thyroid nodules with cytology that is indeterminate (neither clearly benign nor clearly malignant). In adults, molecular diagnostic techniques that assess genetic markers of malignancy or benignity are increasingly being used in the evaluation of such nodules to refine estimates of malignancy risk and guide management (Haugen *et al.*, 2016). In principle, molecular genetic tests that detect mutations that are highly associated with malignancy might be applicable to children. However, differences in the genetics between pediatric and adult thyroid cancer diminishes the utility of these tests, and currently they are not approved or routinely used for evaluation of pediatric thyroid nodules.

Prognostic Factors

Children with follicular cell-derived thyroid carcinomas tend to have more extensive disease at presentation than do adults. At diagnosis, lymph node metastases are present in 60%–80% of children with follicular cell-derived thyroid cancer and pulmonary metastases are present in 7%–25%; distant metastases to other sites (e.g., bone or brain) are rare (Pires *et al.*, 2016; Sung *et al.*, 2017; Wassner *et al.*, 2017; Francis *et al.*, 2015). Despite their relatively advanced disease at diagnosis, the prognosis of children with thyroid cancer is excellent, even in those with distant metastases. Long-term cause-specific mortality is very low (less than 2% over 50 years). However, disease recurrence is common and can occur years or decades after initial treatment. In one series of children diagnosed with thyroid cancer, recurrence was diagnosed in 20% of patients within 5 years, and in 32% of patients within 40 years (Hay *et al.*, 2010).

A variety of staging systems can be used to assess the risk of adverse outcomes in pediatric thyroid carcinomas. The most widely used is the American Joint Committee on Cancer tumor node metastasis (TNM) system (Tuttle *et al.*, 2017). However, this system has limitations when applied to pediatric patients, including the irrelevance of the age criterion for defining lower-stage disease (greater than or less than 55 years) and the limited ability to predict mortality, which is very low in pediatric patients. Recently, the American Thyroid Association has proposed a risk categorization system for pediatric thyroid

Table 2 American Thyroid Association risk categorization for pediatric follicular cell-derived thyroid carcinoma, and associated risk of persistent or recurrent disease over 5–9 years

Risk category	*Definition*	*Risk of persistent or recurrent disease*
Low risk	● Disease limited to the thyroid gland (T1–T3) ● No lymph node metastasis (N0), or only microscopic metastases to a small number of lymph nodes in the central compartment (minimal N1a)[a]	6%–10%
Intermediate risk	● Extensive metastases to central compartment lymph nodes (extensive N1a)[a], or ● Limited metastases to lymph nodes in the lateral compartment(s) or superior mediastinum (minimal N1b)[b]	17%–24%
High risk	● Locally invasive disease extending beyond the thyroid capsule (T4), or ● Extensive metastases to lymph nodes in the lateral compartment(s) or superior mediastinum (extensive N1b)[b], or ● Distant metastatic disease (M1)	44%–74%

[a]Proposed criteria for minimal N1a disease: metastatic foci measuring <0.2 cm involving five or fewer central compartment lymph nodes (Sung *et al.*, 2017).
[b]Proposed criteria for minimal N1b disease: metastatic foci measuring <3 cm and involving 10 or fewer lateral compartment or superior mediastinal lymph nodes (Sung *et al.*, 2017).
Modified from Francis, G. L., Waguespack, S. G., Bauer, A. J., *et al.* (2015). Management guidelines for children with thyroid nodules and differentiated thyroid cancer. Thyroid 25, 716–759; Lazar, L., Lebenthal, Y., Segal, K., *et al.* (2016). Pediatric thyroid cancer: Postoperative classifications and response to initial therapy as prognostic factors. Journal of Clinical Endocrinology and Metabolism 101, 1970–1979; Sung, T. Y., Jeon, M. J., Lee, Y. H., *et al.* (2017). Initial and dynamic risk stratification of pediatric patients with differentiated thyroid cancer. Journal of Clinical Endocrinology and Metabolism 102, 793–800.

cancer that appears to correlate well with the risk of persistent/recurrent disease (**Table 2**) (Francis *et al.*, 2015; Lazar *et al.*, 2016; Sung *et al.*, 2017).

Initial Management

The approach to initial management and follow-up of thyroid nodules and follicular cell-derived thyroid carcinoma in children is similar to that in adults.

Surgery

Thyroid nodules that are cytologically indeterminate or suspicious for malignancy are resected to obtain a histopathologic diagnosis. Cytologically benign nodules may be observed with periodic ultrasounds to detect growth or other changes concerning for malignancy, or they may be resected if they are very large (>4 cm) or cause symptoms.

For malignant thyroid nodules, the goal of initial surgery is resection of all clinically detectable thyroid cancer in the neck. Near-total thyroidectomy is generally recommended for all children with thyroid cancer and appears to reduce the risk of recurrence (Hay *et al.*, 2010), perhaps because of a higher incidence of bilateral disease in children (30%) than in adults (Francis *et al.*, 2015). While recent consensus guidelines in adults endorse more limited surgery (some as lobectomy) for low-risk thyroid carcinomas (Haugen *et al.*, 2016), the safety of this approach in children is not yet clear (Kluijfhout *et al.*, 2017). Cervical lymph node metastases are resected at initial surgery if possible.

The rate of complications of thyroid surgery, including hypoparathyroidism and recurrent laryngeal nerve injury, are higher in children than in adults (Wang *et al.*, 2009). Because complications rates of thyroid surgery are lower in the hands of more experienced surgeons (Adam *et al.*, 2017), whenever possible surgery for children with thyroid cancer should be performed by a high-volume thyroid surgeon capable of operating on pediatric patients.

^{131}I Therapy

The goal of postoperative ^{131}I treatment is to reduce the risk of thyroid cancer recurrence and potentially of mortality. The rationales for ^{131}I treatment are similar in children to adults, including ablation of the thyroid remnant to facilitate monitoring for persistent/recurrent disease using serum thyroglobulin, and ablation of likely or known residual thyroid cancer. Direct evidence is limited regarding the efficacy of ^{131}I in children with thyroid cancer, and practice is based largely on data from adults. ^{131}I treatment of known residual disease probably improves outcomes in children with thyroid cancer, particularly those with distant metastatic disease (Brink *et al.*, 2000; Chow *et al.*, 2004; Hay *et al.*, 2010). Whether ^{131}I is beneficial in children without known residual disease or in those with low-risk tumors remains unclear. In addition, ^{131}I treatment may increase the future risk of other secondary malignancies (Sawka *et al.*, 2009), a concern that is accentuated in children due to their greater sensitivity to radiation (Hay *et al.*, 2010; Marti *et al.*, 2015). Therefore, ^{131}I therapy is generally recommended for children with known or high risk of residual disease (including pulmonary metastases) that cannot be surgically resected (Francis *et al.*, 2015). Performing a diagnostic whole-body ^{123}I scan and measurement of the serum thyroglobulin concentration prior to

treatment often helps guide decisions of whether to treat with ^{131}I and the specific dose of ^{131}I to be given. A posttreatment ^{131}I whole body scan is obtained 4–7 days after ^{131}I therapy and may reveal iodine-avid disease that was not detectable on the pretreatment ^{123}I scan.

Children are usually prepared for ^{131}I by withdrawal of levothyroxine treatment to achieve a TSH concentration above 30 mIU/L. Children require less time ($\geq$14 days) to achieve adequate TSH elevation than do adults and generally tolerate hypothyroidism very well (Kuijt and Huang, 2005). There are few data on the use of recombinant human TSH to prepare pediatric patients for ^{131}I treatment, but it may be required in patients who cannot achieve TSH elevation (central hypothyroidism) or who cannot tolerate hypothyroidism. A low-iodine diet is recommended for 2 weeks prior to ^{131}I therapy.

Levothyroxine Therapy

The growth of thyroid follicular cells, including most follicular cell-derived thyroid carcinomas, is enhanced by the action of TSH on these cells. Therefore, suppression of serum TSH concentrations using supraphysiologic doses of levothyroxine is often used to treat thyroid carcinomas by depriving them of this stimulus for growth. TSH suppression appears to improve disease-free survival and mortality in adults with advanced thyroid cancer (Haugen *et al.*, 2016). Although minimal data exist on the efficacy of TSH suppression in pediatric patients with thyroid cancer, based on adult data TSH suppression ($<$0.1 mIU/L) is generally recommended for children with known or high risk of residual thyroid carcinoma after initial surgery. Milder TSH suppression (0.1–0.5 mIU/L) may be considered for patients with intermediate-risk disease.

Surveillance and Follow-Up

Ultrasonography

Periodic physical examinations and neck ultrasounds are a critical part of surveillance for persistent or recurrent thyroid cancer in the neck. Ultrasounds may be performed 6–12 months after initial treatment and every 6–12 months thereafter, depending on the severity of initial disease and risk of recurrence (Francis *et al.*, 2015).

Thyroglobulin Monitoring

Monitoring the serum concentration of thyroglobulin is another important part of thyroid cancer surveillance. Thyroglobulin is a protein synthesized only by thyroid follicular cells, whether normal or neoplastic. Therefore, the serum thyroglobulin concentration estimates the amount of thyroid tissue (normal or neoplastic) present in the patient and should be negligible after near-total thyroidectomy and ^{131}I treatment. Detection of thyroglobulin after thyroidectomy and ^{131}I, or rising thyroglobulin levels in any patient, raise concern for the presence of persistent or recurrent thyroid cancer and require imaging to localize the tumor. Because thyroglobulin synthesis is stimulated by TSH, thyroglobulin concentrations must be interpreted in the context of the serum TSH concentration at the time. The presence of circulating antibodies to thyroglobulin can confound measurement of serum thyroglobulin concentrations (Spencer *et al.*, 1998), so thyroglobulin antibodies should be measured in every sample sent for thyroglobulin analysis. Thyroglobulin antibodies are more common in children than in adults (40% vs. 20%–30%), but about half of children with such antibodies clear them within 2 years, particularly if the initial antibody titer is low (Wassner *et al.*, 2017). Based on data from adults, rising titers of thyroglobulin antibodies are considered an indicator of possible thyroid cancer recurrence, although no pediatric-specific data supporting this are available.

Additional Imaging

Whole body scintigraphy with ^{123}I may be employed to detect iodine-avid persistent or recurrent thyroid cancer in children in whom such disease is known to be present or was previously treated with ^{131}I. ^{123}I scintigraphy may also detect iodine-avid disease in children with abnormal or rising thyroglobulin concentrations but no sonographically detectable disease in the neck. If scintigraphy does not reveal the tumor location, CT or MRI scanning of the neck and chest may be helpful. Few pediatric data are available on the use of positron emission tomography (PET) in this setting.

Treatment of Persistent or Recurrent Disease

Persistent or recurrent thyroid cancer in the neck that is clinically detectable by ultrasound may be resected surgically if feasible, particularly if the disease is of significant size ($>$1 cm), is progressing, or is in an anatomically sensitive location. Cervical disease that is iodine-avid and not amenable to surgery may be treated with ^{131}I. Pulmonary metastases in children are frequently iodine-avid, and in this case may also be treated with ^{131}I if they are progressing. However, pulmonary metastases in children may follow an indolent course and remain stable for long periods, so repeated treatments with ^{131}I should be used with caution and only when necessary, to minimize the risks of radiation exposure. In rare cases of children with progressive disease that is not responsive to ^{131}I, systemic therapy with certain of the kinase inhibitors used for adults with thyroid cancer (e.g., sorafenib, lenvatinib) may be considered, although pediatric data are limited.

Long-Term Surveillance

In adults with thyroid cancer, a patient's response to initial treatment has been shown to significantly modify the risk of recurrence from the initial risk assessed at diagnosis based on tumor stage (Tuttle *et al.*, 2010). This approach of dynamic risk stratification therefore is used in adults to modify surveillance strategies over time based on the patient's response to therapy (Haugen *et al.*, 2016). Dynamic risk stratification is less well studied in children with thyroid cancer, but preliminary data suggest that it may be applicable to pediatric patients as well (Lazar *et al.*, 2016; Sung *et al.*, 2017). Nevertheless, in light of the high risk of recurrence of pediatric thyroid cancer even decades after diagnosis, lifelong surveillance is recommended for children with a history of thyroid cancer.

Medullary Thyroid Carcinoma

Medullary thyroid carcinoma (MTC) is rare in children and accounts for only 2% of pediatric thyroid cancers. The etiology, presentation, diagnosis, and management of pediatric MTC are similar to that in adults. The main difference relates to the fact that many children are diagnosed with a genetic risk for MTC before developing the disease itself, which requires an approach to counseling and management of such patients. Consensus guidelines for the management of MTC, including in children, have recently been published (Wells Jr *et al.*, 2015).

Etiology

Most cases of MTC in children are caused by activating mutations in the *RET* proto-oncogene on chromosome 10q11.2, which encodes a tyrosine kinase. Some cases of MTC are sporadic, but many are due to inherited germline *RET* mutations that cause the syndrome of multiple endocrine neoplasia type 2 (MEN2). In addition to a nearly 100% lifetime risk of developing MTC, MEN2 is associated with development of pheochromocytomas and either hyperparathyroidism (MEN2A) or mucosal neuromas and Marfanoid habitus (MEN2B). A minority of MTC in children is familial but occurs without a detectable *RET* mutation.

Diagnosis

MTC presents similarly to other thyroid cancers as an asymptomatic thyroid nodule, or with cervical lymphadenopathy due to locally metastatic disease. The diagnosis can be confirmed cytologically by ultrasound-guided FNA. Because C cells produce calcitonin, the concentration of calcitonin is often elevated in FNA aspirates of MTC as well as in the patient's serum. Preoperative evaluation of suspected MTC should include imaging of the cervical lymph nodes to detect possible local lymph node metastasis, which is present in about 15% of patients (Machens *et al.*, 2016). Any patient diagnosed with MTC should receive genetic testing for a germline *RET* mutation, which may direct further genetic testing of potentially affected family members.

Management

The management of pediatric MTC is similar to that in adults, beginning with total thyroidectomy and removal of lymph nodes in the central compartment, as well as dissection of any other lymph node compartments clinically involved with MTC. Baseline serum concentrations of calcitonin and carcinoembryonic antigen (CEA) are obtained preoperatively, and higher concentrations are associated with a greater likelihood of metastatic disease. Calcitonin and CEA concentrations are monitored over time to assess the response to treatment and to detect progression of disease. For patients with progressive disease that is not amenable to surgery, other treatment modalities may include systemic tyrosine kinase inhibitors such as vandetanib (Kraft *et al.*, 2017), as well as external beam radiation or radiofrequency ablation, although there are limited data on the use of these treatments in children.

Counseling and Prophylactic Thyroidectomy

Genetic testing of family members diagnosed with MTC or MEN2 often leads to detection of a germline *RET* mutation in a child prior to the development of any clinical manifestations. The risk of developing MTC is nearly 100% in these individuals, and while thyroidectomy is curative if performed before MTC develops or while it remains localized to the thyroid gland, the prognosis worsens once MTC spreads beyond the thyroid. For this reason, prophylactic thyroidectomy is recommended in most patients with MEN2. However, the timing of prophylactic thyroidectomy may be individualized for each patient, balancing the probability of developing metastatic MTC at a given age against the need to minimize the surgical risks of thyroidectomy, which are higher in younger patients.

Factors that may influence the timing of prophylactic thyroidectomy include the specific *RET* mutation present and serum levels of calcitonin. In general, certain *RET* mutations are associated with earlier or later onset of MTC, and consensus guidelines categorize *RET* mutations as highest risk (M918T mutation, usually associated with MEN2B), high risk (mutations in codon 634 or 883), or moderate risk (other mutations) (Wells Jr *et al.*, 2015). Patients at highest risk may develop MTC in infancy and should undergo thyroidectomy within the first year of life. Patients with high-risk mutations generally should undergo thyroidectomy by

5 years of age, or earlier if serum calcitonin levels begin to rise. For patients at moderate risk, monitoring by neck ultrasound and serum calcitonin levels is recommended beginning at age 5 years, and thyroidectomy should be performed if calcitonin levels rise. In high- and moderate-risk patients, the timing of thyroidectomy may also account for the history of MTC onset in affected family members, but this factor should be weighed cautiously since the timing of MTC development can vary significantly among individuals with a given *RET* mutation, even within the same family. Finally, the timing of surgery may be influenced by other factors, such as a patient's or family's desire to avoid the potential burden and anxiety of prolonged monitoring. Prophylactic thyroidectomy should be performed by an experienced thyroid surgeon to minimize the risk of surgical complications, which are highest in the youngest patients. Patients with MEN2 should also have age- and mutation-appropriate screening for pheochromocytoma and hyperparathyroidism prior to thyroid surgery. Overall, identifying individuals at genetic risk of MTC and performing prophylactic thyroidectomy improves outcomes in patients with MEN2, many of whom are have C cell hyperplasia (a premalignant condition) or frank MTC at the time of prophylactic thyroidectomy (Machens *et al.*, 2016; Bussieres *et al.*, 2017).

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Physiology of Fetal and Neonatal Calcium Metabolism

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Introduction

In post-natal life, the concentration of calcium is controlled principally by two factors: parathyroid hormone (PTH) and 1,25dihydroxyvitamin D ($1,25(OH)_2D$), the active form of vitamin D. A calcium-sensing receptor (CaSR) is responsible for determining the "set" concentration of calcium and principally determines the secretion of PTH in response to changes in plasma calcium which remain very steady once they have been established in the few days after birth. Fibroblast growth factor 23 (FGF23), is now known to be the "phosphotonin" that had long been suspected to control phosphate metabolism. There is a classic negative feedback mechanism between plasma phosphate and FGF23 secretion although it is not clear what the sensing mechanism for this is. It would appear to be rather more complex than that of calcium since plasma phosphate concentrations vary throughout life, particularly in childhood.

Two other hormonal factors are also involved. Calcitonin (CT) is a circulating hormone but probably plays little part in maintaining plasma calcium although its effect is generally opposite to that of PTH. Parathyroid hormone-related peptide (PTHrP), does not circulate in significant quantities during postnatal life but is an important paracrine factor in cartilage development in the growth plate.

The full-term fetus contains approximately 30 g of calcium and 17 of phosphate. Most of this mineral is accumulated during the last trimester. The mechanisms whereby placental transport of calcium is maintained are complex and very efficient such that, in the event of premature delivery, it is very difficult to maintain calcium accumulation post-natally as well as it would have been in utero. Bone disease of prematurity is therefore primarily a condition associated with mineral deficiency.

Fetal Physiology

Studies, many of which go back to the 18th century, in which aborted fetuses were weighed and the calcium and phosphate content measured after the corpses were "ashed," have demonstrated that the calcium and phosphate content of fetuses is very closely related to body weight. The relationship between calcium and body weight is defined by the equations (Allgrove, 1984):

$$Ca = 0.00075 \times BWt^{1.3093}$$

And for phosphate as:

$$PO_4 = 0.00037 \times BWt^{1.2409}$$

Given the known increments in fetal body weight, it is possible to calculate the rate at which calcium and phosphate are normally accumulated during fetal life.

However, calcium flux across the placenta is not a one-way process, and the flows are bi-directional. This is true of all placental mammals, although the relationship between the forward-flow (i.e., from mother to fetus) and backward-flow is dependent on the number of placental layers—in the haemo-chorial human placenta, in which three layers are present, the ratio is approximately 6/5. Given that the net flow of calcium in favor of the fetus is more than 300 mg/day towards the end of gestation (Sparks, 1984), approximately 1800 mg has to flow from mother to fetus whilst 1500 mg is returned to the mother.

Calcium is unusual amongst substances that cross the placenta in that, unlike most others, there is a positive gradient that is, calcium concentrations in the fetus are greater than those in the mother by about 0.25–0.5 mmol/L. There is therefore active transport across the placenta, in contrast, for instance, to vitamin D in which facilitated transport results in 25-hydroxyvitamin D (25OHD) concentrations in the fetus that are about two thirds those of the mother. Positive gradients for both phosphorus and magnesium are also present in the fetus.

The mechanism by which the gradient for calcium is maintained was first suggested by studies of biologically active PTH-like activity which was shown to be present in cord blood, despite the fact that immunoassays of PTH suggested that concentrations were low (Allgrove *et al.*, 1985). Furthermore, the concentration of bio-active PTH was shown to have a direct relationship with the placental gradient of calcium. Further studies showed that this PTH-like activity was caused by a previously unknown hormone, parathyroid hormone-related peptide (PTHrP), also called PTH-like peptide (PTHLP). PTHrP normally has little role as a true hormone in post-natal life but is now known not only to be involved in maintaining the calcium gradient across the placenta but also as a paracrine factor in cartilage development in the growth plate, and also as one of the causes of malignancy-induced hypercalcaemia.

Factors involved in fetal mineral homeostasis

1. PTHrP

   PTHrP is a 141 amino acid peptide with similar bioactivity to PTH but with substantial structural differences from PTH, hence the bioactivity without immunoreactivity. It is equipotent with PTH in its actions on the Type 1 PTH receptor (PTH1R) but has little action on the PTH2R, present mostly in brain (Orloff *et al.*, 1994).

   In fetal life it is primarily responsible for the transport of calcium across the placenta. The fetal concentration of calcium is

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"set" at a higher level than in post-natal life (David and Anast, 1974) although what constitutes the sensor for PTHrP is not fully understood, but is different from that for calcium. The maternal calcium concentration determines the activity of PTHrP which increases in the presence of maternal hypocalcaemia and is suppressed by maternal hypercalcaemia. Thus it is not the gradient, but rather the absolute value of calcium that is maintained.

The source of PTHrP in the fetus is not entirely clear. Much of it is probably secreted by the placenta, but there may also be small quantities produced by the fetal parathyroids.

2. PTH

PTH is secreted by the fetal parathyroid glands from about 9 weeks gestation but only in low concentrations normally. The relationship between calcium and the calcium-sensing receptor is present during fetal life but, because calcium concentrations are higher than after birth, PTH secretion is largely suppressed. It is not known by which stage of gestation the positive gradient is established, although it is present by 15 weeks.

PTH and PTHrP probably work together to maintain the calcium gradient although PTHrP is the principal factor. Circulating concentrations of PTHrP are about 10–15 times higher than those of PTH.

3. The calcium-sensing receptor (CaSR)

This is present in the fetus. The same sigmoid relationship between calcium and PTH secretion is present. However, because calcium concentrations are higher than in post-natal life because of the effects of PTHrP, PTH secretion is largely suppressed. However, if the fetus carries a heterozygous inactivating mutation of the CaSR, particularly if this is a new mutation or it is inherited from the father, or if the mutation is homozygous, neonatal severe hyperparathyroidism may result since the "set point" of calcium is even higher than normal, resulting in fetal hyperparathyroidism.

Nevertheless, it seems that a second sensor, related to PTHrP, is present which is responsible for regulating PTHRP secretion. The nature of this is not known but may be within the placenta.

Factors with minimal influence on fetal calcium homeostasis

1. Vitamin D

Vitamin D, in its circulating form, 25-hydroxy vitamin D (25OHD), readily crosses the placenta. This is a passive or facilitated process that results in 25OHD concentrations in the fetus that are only about 60%–80% those of the mother. Maternal vitamin D deficiency has little effect on fetal bone mineralization and individuals who have either 1α-hydroxylase or vitamin D receptor deficiency do not have rickets at birth and have normal mineralization. True congenital rickets is extremely rare and only occurs if maternal vitamin D is completely absent.

$(1,25(OH)_2D)$ concentrations are low in the fetus and play little part in promoting calcium transport across the placenta.

In contrast, concentrations of 24,25-dihydroxyvitamin D $(24,25(OH)_2D)$, an inactive metabolite of vitamin D, are high.

2. Calcitonin

Calcitonin (CT) also plays little part in fetal mineral homeostasis. CT deficiency does not result in mineral defects. There is some evidence to suggest that CT may play a part in embryonic implantation.

3. Fibroblast growth factor 23 (FGF23)

FGF23 is normally associated with increased excretion of phosphate by the kidney post-natally. Circulating concentrations of FGF23 in the fetus are approximately one third of those in postnatal life, whilst those of its inactive fragments are approximately twice as high (Takaiwa *et al.*, 2010). The co-factor, Klotho, also circulates at about six times its concentration in post-natal life (Ohata *et al.*, 2011). The implications are not clear but FGF23 has little effect on transplacental phosphate transport.

Fetal organs and mineral homeostasis

1. The placenta

The placenta is the principal organ regulating mineral transport, mainly under the influence of PTHrP. PTH has a lesser effect. In contrast, neither, vitamin D nor FGF23 nor calcitonin has any significant effect in the placenta.

2. Fetal kidneys

The fetal kidneys are the principal source of amniotic fluid. Much of this is swallowed, so any minerals that are excreted into the amniotic fluid are likely to be reabsorbed. They play little part in fetal mineral homeostasis

3. Fetal intestines

Most of the minerals that are contained in amniotic fluid are reabsorbed. The process is independent of vitamin D and is dependent on the concentrations of mineral within the fluid.

Fetal Skeletal Mineralization

Most of the mineralization of fetal bone occurs during the last trimester. A fetus of 28 weeks weighing 1.2 kg contains approximately 8 grammes of calcium. By term the same fetus weighing 3.3 kg has accumulated a further 22 grammes—an average of 261 mg/day and, by term, this net accumulation is greater than 300 mg/day. Thus infants who are born prematurely miss out on the mineral accumulation that normally occurs during this period. The process of mineral transfer across the placenta is so efficient that, in these premature infants, it is very difficult to achieve the same rate of mineral gain post-

natally that would have happened had they remained in utero. This is particularly true of phosphate, and relative phosphate deficiency is one of the principal causes of bone disease of prematurity. Phosphate supplementation is usually necessary in very premature infants, especially if born before 28 weeks' gestation, in order to prevent this, particularly if they are being fed breast milk which contains less phosphate than formula feeds.

The increase in fetal skeletal mineral is accompanied by a very rapid bone turnover that allows bone growth and remodeling to occur in utero. Approximately 300 mg of calcium are exchanged between plasma and bone each day. This means that about 1% of the entire mineral content of the fetal skeleton is changed daily—approximately 100 times that in adults.

Neonatal Mineral Homeostasis

At birth, there is an immediate and complete cessation of calcium transport to the newborn as the umbilical cord is cut. This is not matched by an immediate change in bone mineralization so there is a physiological fall in plasma calcium in the neonate whilst the supply of calcium is replaced by milk intake and PTH secretion increases, and which has its nadir at around 48 h. This is not usually of any clinical significance. However, in premature infants this reduction in calcium is exaggerated and may be a cause of symptomatic hypocalcaemia. In addition, infants who are "sick" for any reason have greater difficulty in establishing normocalcaemia.

Factors involved in neonatal mineral homeostasis

1. PTH

   At birth, the newborn is in a state of relative hypoparathyroidism due to the suppression of PTH by the high fetal concentrations of calcium. Once the influence of PTHrP (and its attendant sensor) is removed after separation from the placenta, PTH becomes the prominent factor in maintaining calcium homeostasis. However, this takes a few days to become established. In term infants, transport of calcium does diminish somewhat and bone turnover decreases temporarily, which mitigates the hypocalcaemic stress following birth. However, it also takes a few days to establish calcium supplies from intestinal absorption as milk intake increases. The combination of these factors results in a transient fall in plasma calcium, usually to a nadir of about 2.0 mmol/L before increasing to post-natal values by the end of the first week. By this time the normal relationship between PTH and the CaSR is established.

   Infants, such as those with 22q Deletion syndrome, GCMB mutations or other causes of congenital hypoparathyroidism, who are deficient in PTH are at risk of developing symptomatic hypocalcaemia in the neonatal period.
2. $1,25(OH)_2D$

   In contrast to intrauterine life, $1,25(OH)_2D$ becomes an important factor post-natally. Concentrations rise in response to the increase in PTH and vitamin D receptors in the intestine contribute to calcium absorption from the diet.
3. 25OHD

   Vitamin D itself is important at this stage in providing a substrate from which $1,25(OH)_2D$ can be synthesized. If supplies are limited, PTH increases to try to stimulate $1,25(OH)_2D$ production in an attempt to increase calcium absorption. This results in increased phosphate losses in the urine which, in turn, causes relative hypophosphataemia. This is the principal cause of rickets. Any cause of hypophosphataemia may result in rickets. The speed at which it develops is determined by the severity of the deficiency. Infants who are very deficient in vitamin D may develop clinically obvious rickets within a few weeks whilst others who are less severely deficient or who have an inherited metabolic disorder may take several months for this to become apparent.
4. PTHrP

   Concentrations of PTHrP are high at birth but become undetectable in post-natal life. It is not clear how quickly they become suppressed, but they play little part as a true hormone after birth. PTHrP is, however, important as a paracrine factor in growth plate development and operates in conjunction with other growth plate factors such as Indian Hedgehog and RUNX2 to regulate cartilage development within the growth plate. Some patients who subsequently develop hypercalcaemia of malignancy do so because they start to secrete PTHrP from the malignant tissue. There are high concentrations of PTHrP in breast milk, but the significance of this is not clear.
5. FGF23

   FGF23 rises after birth. Its principal effect is to increase phosphate excretion by the kidneys. It also has a suppressive effect on 1α-hydroxylase, although this is probably more important if concentrations become supra-physiological. The normal feedback mechanism between plasma phosphate and FGF23 is gradually established post-natally and depends partly on the responsiveness of renal tubules to the effect of FGF23. Excess concentrations of FGF23 due to mutations in PHEX or FGF23 itself cause hypophosphataemic rickets but, although low phosphate values can be demonstrated within 2–3 weeks in some patients, clinical rickets does not usually develop until a few months after birth.
6. Calcitonin

   CT is secreted by the C-cells of the thyroid. Concentrations rise following birth but the significance of this is not clear. Whether or not its hypocalcaemic effect contributes to neonatal hypocalcaemia is uncertain. After a few days, CT falls back to normal post-natal values. In later life, CT can be used as a marker of C-cell carcinoma in patients with mutations in the RET proto-oncogene (MEN2).

Neonatal organs and mineral homeostasis

1. Parathyroid glands

   The parathyroid (PT) glands are largely suppressed during fetal life but start to function more effectively following birth to help restore plasma calcium to normal. They develop from the third and fourth branchial arches. This occurs under the influence of several genes including GCMB, GATA3, TBCE, TBX1 and a number of mitochondrial genes. Failure of any one of these genes to make its proper contribution to parathyroid gland development results in primary hypoparathyroidism which can manifest itself at or shortly after birth. There may or may not be other clinical manifestations.
2. Neonatal kidneys

   Renal function is poor at birth and renal blood flow is limited. It improves with time and allows responsiveness to PTH and FGF23 to develop. Thus phosphate excretion increases. At the same time, expression of the vitamin D receptor and CaSR also increase and post-natal homeostasis is achieved.
3. Neonatal intestine

   Calcium absorption in the newborn is initially passive and independent of vitamin D. Gradually, the vitamin D dependent mechanisms develop with increases in the vitamin D receptor and activation of TRPV6, the vitamin D-dependent trans-cellular calcium transport protein. Lactose and breast milk promote calcium absorption and, because breast milk has a lower concentration of phosphate, calcium concentrations tend to be higher and phosphate concentrations lower in breast-fed compared with formula-fed infants.
4. The neonatal skeleton

   At birth skeletal mineralization is still progressing at a rapid rate, although there is some slowdown towards the end of pregnancy. Bone turnover is also rapid and occurs largely without the influence of vitamin D which, in any case, probably has little direct effect on bone mineralization. Its principal effect is to promote calcium absorption in the intestine in order to ensure an adequate supply of calcium. This effect can be overcome by increasing calcium intake so that passive (para-cellular as opposed to trans-cellular) absorption can compensate. As time progresses, the normal post-natal influences ensue.

Summary

Mineral metabolism in the fetus is very different from that in post-natal life. The neonate is largely dependent on PTH and PTHrP for promoting transport of mineral across the placenta. Following birth, profound changes occur which initially result in transient hypocalcaemia but which, by the end of the first week, have largely settled down to a postnatal pattern which remains largely unchanged, at least in quality, if not quantity, during the remainder of life.

Because of this, defects in the various mechanisms may not necessarily have any significant effects prenatally but become more obvious post-natally.

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Further Reading

For a more detailed discussion of the topic with over 700 references, readers are referred to: Kovacs CS (Kovacs, 2014; Kovacs, 2015).

Rickets and Osteomalacia

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Glossary

Calcipaenia Calcium deprivation in the body due to vitamin D and/or dietary calcium deficiency.
Craniosynostosis Premature fusion of skull sutures in an infant.
Diaphysis The long bone shaft formed from a primary center of ossification.
Epiphysis End of a long bone formed from secondary center of ossification which is initially separate from the body of the long bone and later unites with it through ossification.
Hypocalcemia Low serum calcium concentration.
Hypophosphataemia Low serum phosphate concentration.
Hypophosphatasaemia Low serum alkaline phosphatase activity.
Metaphysis Growing part of the long bone which lies between the growth plate and the diaphysis.
Mineralization Integration of hydroxyapatite crystals into pre-formed osteoid, creates the bone matrix.
Ossification Transformation of a cartilage frame into bone tissue during bone development.
Osteoid Unmineralized, organic bone matrix component synthesized by osteoblasts.
Phosphopaenia Phosphate deprivation in the body.

Introduction

Rickets and osteomalacia occur as a consequence of defective mineralization of the skeleton. Such reduced mineralization results in soft bones, which deform and break more easily, and stunting of growth. The non-nutritional forms of rickets can be associated with raised intracranial pressure from craniosynostosis, and severe hypophosphatasia is associated with respiratory insufficiency from pulmonary hypoplasia secondary to insufficient chest growth. Dental manifestations are a frequent association due to impaired mineralization of enamel.

Defective mineralization occurs due to (1) lack of mineral supply to mineralization sites (calcium or phosphate), (2) lack of the mineral supplier calcitriol (hormone synthesized from vitamin D) which controls their intestinal absorption, and (3) less commonly due to lack of the tissue non-specific alkaline phosphatase enzyme which facilitates mineralization.

The leading cause of rickets worldwide is dietary calcium deficiency and/or vitamin D deficiency (Munns *et al.*, 2016). Dietary calcium deficiency predominates in low income countries with often abundant sunshine due to poor dietary intake whereas vitamin D deficiency predominates in dark skinned individuals residing in high latitude countries with poor ultra violet B (UVB) penetration and reduced skin synthesis of vitamin D. The recent refugee crisis and immigration of dark skinned individuals from low income countries into high latitude regions has led to the resurgence of nutritional rickets in high income countries (Högler and Munns, 2016).

A basic understanding of the physiology of bone growth, structure and mineralization is essential to differentiate and manage various forms of rickets and osteomalacia, and their fundamental difference to osteoporosis. Recent advances in the understanding of the pathophysiology of certain forms of rickets has led to the emergence of new therapeutic agents such as monoclonal antibody against fibroblast growth factor 23 in X-linked hypophosphatemic rickets (XLH) and enzyme replacement with asfotase alfa in hypophosphatasia (HPP).

Terminology Buster: Bone Formation, Mineralization, Ossification, Calcification

The bone tissue or matrix is composed of 50%–70% mineral (predominantly calcium and phosphate), 20%–40% organic material (predominantly type 1 collagen), 5%–10% water, and $<3\%$ lipids (Clarke, 2008). The three main types of bone cells are (1) osteoblasts which form new bone, (2) osteoclasts which resorb old bone and calcified growth plate cartilage, and (3) osteocytes which constitute 90% of the cells in the matrix and are responsible for biomechanical sensing and hormone production (Bellido *et al.*, 2014).

The term "bone formation" describes the process of synthesis of extracellular organic osteoid followed by its mineralization, with later integration of osteoblasts which transform into osteocytes. The term "mineralization" relates to the integration of hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ into osteoid. In contrast, the term "ossification" relates to the two major modes of bone development: intramembranous ossification is seen in flat bones such as the skull and endochondral ossification in long bones such as the femur (Gilbert, 2000). Physiological mineralization is restricted to bones, teeth, and growth plate cartilage (where it is also called calcification). Pathological mineralization or "calcification" in its true meaning is deposition of calcium-phosphate

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crystals in extra-skeletal tissues (kidneys, skin, arteries, muscle, brain, tumors) and can lead to serious problems (Kirsch, 2012). Some of the conditions associated with pathological calcification include: Generalized arterial calcification of infancy, pseudoxanthoma elasticum, dermatomyositis and subcutaneous fat necrosis (Nitschke and Rutsch, 2017).

Endochondral ossification and mineralization merit more detailed discussion to be able to appreciate the pathogenesis and clinical features of rickets in children.

Endochondral Ossification

Longitudinal growth occurs through endochondral ossification in long bones. Condensation of mesenchymal cells to form cartilage precedes their differentiation into chondrocytes (Mackie *et al.*, 2008). These cells then secrete cartilage extracellular matrix components. The cartilage structure is invaded by cells to form the primary ossification center at the center (midshaft) followed by secondary ossification centers at the ends (epiphyses). The primary and secondary ossification centers are separated by the growth plate which is responsible for longitudinal growth and drives expansion of the primary ossification center (Mackie *et al.*, 2008). The articular epiphyseal growth cartilage is responsible for growth and shaping of the epiphyses and drives expansion at the secondary ossification center. The primary and secondary ossification centers encroach onto each other gradually replacing the cartilage with bone tissue except at the articular surface.

The chondrocytes in the growth plate are arranged in morphologically distinct zones. At the epiphyseal end of the growth plate is the reserve zone or resting zone. Cells in the resting zone then enter the proliferative zone where they undergo division and produce large amounts of extracellular matrix protein (Rauch, 2005). The proliferative cells lose their capacity to divide and then start to increase in size (pre-hypertrophic zone) until they are fully differentiated to form hypertrophic chondrocytes (in the hypertrophic zone) (Rauch, 2005). Intracellular calcium concentration continues to increase in the hypertrophic chondrocytes and at some point they start to mineralize the longitudinal septa in the surrounding matrix to form the zone of provisional calcification (Rauch, 2005). The final apoptosis of hypertrophic chondrocytes triggers mineralization of the matrix and vascular invasion (facilitating osteoblast invasion) at the metaphyseal/growth plate junction (primary spongiosa). Lack of phosphate supply at the growth plate (Tiosano and Hochberg, 2009) prevents apoptosis of hypertrophic chondrocytes and thus inhibits both mineralization and growth. Eighty percent of the longitudinal septa of the growth cartilage are rapidly resorbed in the metaphyseal zone immediately behind the invading blood vessels and the remaining longitudinal septa serve as scaffolds, on which osteoblasts deposit further bone matrix (secondary spongiosa) (Rauch, 2005).

The proliferation and differentiation of chondrocytes is regulated by a number of systemic factors (such as growth hormone, thyroid hormone, estrogen and cortisol) and a large number of still poorly understood locally secreted factors (such as Indian hedgehog, parathyroid hormone-related peptide, fibroblast growth factors) which act on receptors to effect intracellular signaling and activation of chondrocyte-selective transcription factors (Mackie *et al.*, 2008).

Mineralization

Bone is mineralized connective tissue. The most abundant minerals in bone matrix are calcium and phosphorus, present in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)_6OH_2$]. The mineralization process therefore requires adequate supply of substrates, calcium and phosphate, which are supplied by calcitriol (1,25 dihydroxy vitamin D) through intestinal absorption.

The mechanism of initiation and regulation of cell mediated mineralization is not entirely clear, however several theories have been proposed (Huitema and Vaandrager, 2007). Matrix vesicles (MVs) protruding from osteoblasts play an important role in mineralization (Anderson, 1995). One theory suggests that MVs bud off the parental plasma membrane, another suggests that MVs represent the rearrangement of the apoptotic cell membrane (Cui *et al.*, 2016).

The first step in mineralization is the formation of hydroxyapatite in MVs of mature chondrocytes and osteoblasts (Cui *et al.*, 2016). During Phase I, there is increased activity of the MV phosphatases (including alkaline phosphatase, adenosine triphosphatase, pyrophosphatase and PHOSPHO1) which generate and transport phosphate; as well as calcium binding compounds such as the annexin family and phosphatidylserine (Anderson, 2003). Calcium and phosphate are attracted into the MVs by these compounds, until the threshold for their precipitation is reached (Anderson, 1995). In Phase II, the hydroxyapatite crystals penetrate the MV membrane and are elongated into the extracellular space (Anderson, 1995), which requires appropriate concentrations of calcium and phosphate outside the MVs. Tissue non-specific alkaline phosphatase (TNSALP) plays a crucial role in the formation of hydroxyapatite crystals by hydrolyzing inorganic pyrophosphate (PPi), an inhibitor of hydroxyapatite formation, to inorganic phosphorus (Pi) (Terkeltaub, 2001). The ratio of PPi to Pi is of critical importance in the promotion or indeed restriction of mineral in physiological tissues (Cui *et al.*, 2016). TNSALP, encoded by the *ALPL* gene, is abundant on the surface of MVs derived from osteoblasts and hypertrophic chondrocytes (Millán, 2013). Reduced TNSALP activity leads to hypomineralization as seen in HPP (Orimo, 2010).

The mineralization status of the bone matrix can be assessed by quantitative backscattered electron imaging and normative data on mineralization density distribution in iliac bone biopsies are available (Fratzl-Zelman *et al.*, 2009). Finally, it is important to highlight that the term "mineralization" used in radiology reports does not refer to the mineralization process described above and is erroneously used to report changes in bone mass, mineral content or areal bone density over time.

Bone Modeling and Remodeling

Bone metabolism in children differs from adults in that the bone undergoes longitudinal growth, metaphyseal inwaisting, modeling and remodeling during childhood and adolescence whereas in adults remodeling is the only active bone process (Clarke, 2008).

Modeling allows for bone growth in width by deposition of new bone on the periosteal surface by osteoblasts and resorption of old bone on the endosteal surface by osteoclasts (Rauch, 2007). Modeling allows for change in shape of bones in response to physiologic influences and mechanical forces (Frost, 1990).

Remodeling is the process by which old bone is replaced by new bone, essential for maintaining bone strength and removal of microcracks (Clarke, 2008). Unlike bone modeling where new bone formation and resorption occur on different surfaces (periosteal and endosteal respectively) allowing for increase in bone width, remodeling occurs in existing bone matrix. Osteoclasts dig tunnels and trenches and osteoblasts fill these with new bone (Allen and Burr, 2013).

Bone Strength

Bone tissue is a two-phase porous composite material composed primarily of collagen and minerals, with mechanical properties determined primarily by the amount, arrangement, and molecular structure of these primary constituents (Turner, 2006). Bone minerals provide stiffness or mechanical rigidity to the bone, in the absence of which bones become soft and pliable. Organic matrix on the other hand provides toughness enabling the bone to act as a shock absorber. Therefore, defective mineralization (rickets and osteomalacia) results in soft and pliable bones whereas defective collagen production (osteogenesis imperfecta) with excess minerals leads to overly stiff and brittle bones. Bone strength is compromised in both conditions rendering the bone susceptible to fractures (Chapman *et al.*, 2010; Rauch and Glorieux, 2004).

Definition

Rickets is the defective mineralization of the growth plate cartilage and adjacent primary and secondary spongiosa in the metaphysis ("new bone") and thus can only affect growing children with open epiphyses. Rickets is a radiological diagnosis.

Osteomalacia is the defective mineralization of newly formed osteoid in existing bone during remodeling ("old bone") and can affect humans of all ages, thus always co-exists with rickets in children. Osteomalacia is associated with musculoskeletal pain, causes leg bowing in children and Looser zone fractures in adults and children.

Osteoporosis is reduced bone mass and structural quality. It is not associated with reduced bone mineralization. Osteoporosis occurs when bone resorption exceeds bone formation (adults and children) or from reduced bone mass accrual (children). Increased bone resorption is typically secondary to chronic illness (inflammatory conditions, leukemia, poor nutrition), an effect of its treatment (glucocorticoids, anticonvulsants, chemotherapy) or due to sex hormone deficiency (hypogonadism, menopause) (Saraff and Högler, 2015; Högler and Ward, 2015; Kling *et al.*, 2014). Reduced bone mass accrual is typically secondary to muscle weakness (immobility, muscle atrophy/dystrophy conditions). Primary osteoporosis represents intrinsic skeletal defects and is commonly inherited (osteogenesis imperfecta, idiopathic juvenile osteoporosis) (Van Dijk and Sillence, 2014).

Pathogenesis and Classification of Rickets

Based on the primary pathology, rickets and osteomalacia can be classified broadly into defective mineral supply (calcipaenia, phosphopaenia) and defective mineralization (hypophosphatasia).

- Calcipaenia (deprivation of body calcium, most common cause is dietary calcium and/or vitamin D deficiency)
- Phosphopaenia (deprivation of body phosphorus, most common cause is renal phosphate wasting)
- Hypophosphatasia (lack of TNSALP, causing hypophosphatasaemia = low ALP)

Irrespective of the primary pathology, the underlying mechanism of all forms of rickets is low phosphate availability to the hypertrophic chondrocytes in the growth plate (Tiosano and Hochberg, 2009) resulting in lack of their apoptosis (Sabbagh *et al.*, 2005). The various causes of rickets and osteomalacia are detailed in **Table 1**.

Interestingly, some rare bone conditions have only been associated with osteomalacia and not rickets:

- Recessively inherited FAM20C mutations cause osteomalacia but not rickets in humans (Rafaelsen *et al.*, 2013; Whyte *et al.*, 2017). *FAM20c* associated rickets has only been noted in mice (Wang *et al.*, 2012) to date.
- Osteogenesis imperfecta type VI (OI6) caused by homozygous mutation (autosomal recessive inheritance) in the *SERPINF1* gene is characterized by excess accumulation of osteoid secondary to mineralization lag (Glorieux *et al.*, 2002).

Understanding the causes of rickets and osteomalacia based on the primary pathology (**Table 1**) aids in a structured diagnostic workup at presentation. In this article we focus on the most common and preventable cause of rickets worldwide; nutritional

Table 1 Classification of rickets based on the primary pathology

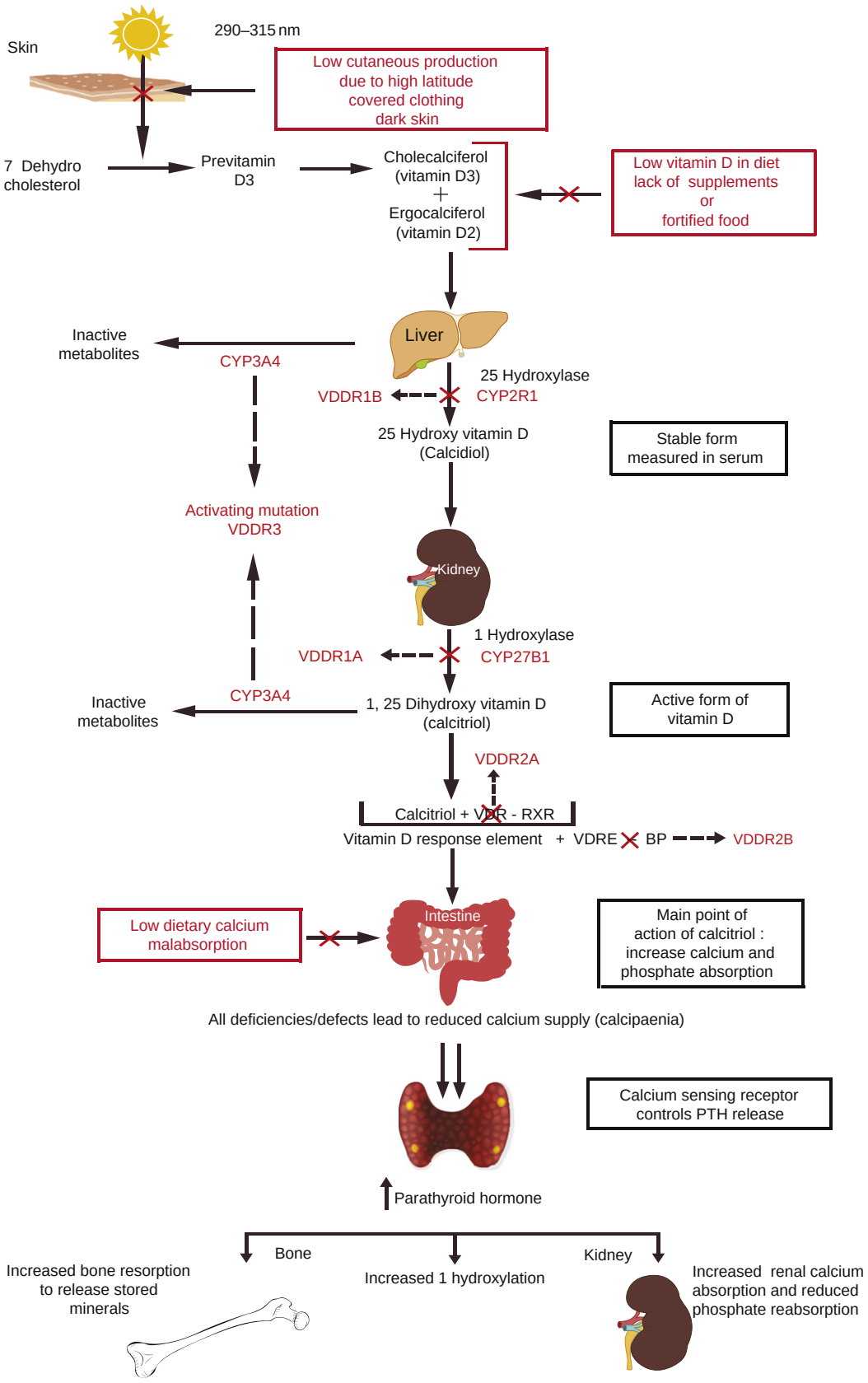
Calcipaenic rickets (nutritional rickets)	*Phosphopaenic rickets (hypophosphatemic rickets)*	*HPP rickets (hypophosphatasia)*
Calcium deficiency[a]	Renal phosphate wasting (non-FGF23 mediated)	Hypophosphatasia (*ALPL*)
• Dietary deficiency • Malabsorptive conditions: liver failure (hepatic osteodystrophy), coeliac disease, short gut syndrome, inflammatory bowel disease	• Hereditary hypophosphatemic rickets with hypercalciuria (*SLC34A3*) • Lowe's syndrome (*OCRL*) • Dent's disease (*CLCN5*) • Fanconi syndrome • Drug induced (i.e. valproate, aminoglycosides, cisplatin, ifosfamide)	• Prenatal benign • Perinatal • Infantile • Childhood • Adult • Odonto
Vitamin D deficiency • Decreased cutaneous synthesis: dark skin, covered clothing, sunscreen use, sunlight deprivation (latitude, cloud cover, pollution) • Dietary deficiency • Malabsorptive conditions (see above) • Increased degradation (*CYP3A4* enzyme-induction, i.e. rifampicin)	Renal phosphate wasting (FGF23 mediated) • X-linked hypophosphatemic rickets (*PHEX*) • Autosomal dominant hypophosphatemic rickets (*FGF23*) • Autosomal recessive hypophosphatemic rickets (*DMP1, ENPP1, FAM20c*[b]) • Tumor induced osteomalacia • Polyostotic fibrous dysplasia	
Defects in calcitriol synthesis or action • Genetic/vitamin D-dependent rickets (VDDR) – 25-Hydroxylase deficiency/VDDR 1B (*CYP2R1*) – 1-Hydroxylase deficiency/VDDR 1A (*CYP27B1*) – Vitamin D receptor defect/VDDR 2A (*VDR*) – Vitamin D response element binding protein defect/VDDR 2B – Enhanced vitamin D catabolism/VDDR 3 (*CYP3A4*) • Renal failure (renal osteodystrophy)	Poor intestinal absorption • Antacids contacting aluminum, magnesium, or calcium • Phosphate binders (i.e. Sevelamer)	
	Insufficient dietary intake (rare) • Osteopenia of prematurity	

[a]Calcium deficiency (calcium deprivation) is not equivalent to hypocalcemia. Secondary hyperparathyroidism maintains normal serum calcium by increasing bone resorption, 1-hydroxylase activity and renal reabsorption of calcium. Therefore, serum calcium is a very poor marker of calcipaenia.
[b]Mutations in *FAM20c* cause hypophosphatemic osteomalacia but not rickets.
For genetic conditions the affected gene is indicated in parenthesis.

rickets due to dietary calcium and vitamin D deficiency. We will also discuss inheritable conditions with emerging treatments such as XLH and HPP, to give the reader a broader view of different forms of rickets.

Calcipaenic Rickets

The leading causes of calcipaenic rickets worldwide are dietary calcium and/or vitamin D deficiency. Although calcipaenia (calcium deprivation) can very rarely occur due to defects in calcitriol synthesis or action, the commonest cause is reduced vitamin D synthesis in the skin from lack of UVB exposure. Vitamin D deficiency can very rarely occur due to dietary insufficiency, malabsorption or enhanced degradation induced by drugs such as rifampicin, a *CYP3A4* inducer (Wang *et al.*, 2013; Hawkes *et al.*, 2017). The main function of calcitriol is to maintain calcium homeostasis in the body by increasing intestinal absorption of the bone minerals calcium and phosphate (supplier function). Therefore, any decrease in dietary calcium or calcitriol action results in calcipaenia. Calcium deprivation in the body is sensed by the calcium sensing receptor in the parathyroid gland's chief cells which stimulate the release of parathyroid hormone (PTH) (Atapattu *et al.*, 2013). High PTH increases (1) osteoclastic bone resorption to release stored calcium, (2) renal calcium reabsorption and (3) 1-hydroxylase activity to maximize calcitriol synthesis. These three compensation mechanisms will keep serum calcium stable at the expense of high bone resorption (**Fig. 1**). Eventually, prolonged secondary hyperparathyroidism results in hypophosphatemia as it also reduces renal phosphate reabsorption. It is the hypophosphataemia that causes rickets and osteomalacia and not the hypocalcemia which occurs only once compensation fails. Hypocalcemia is frequently encountered in infants due to their smaller bone stores, higher mineral needs for rapid growth, and can cause seizures, cardiomyopathy and cardiac death. In children with chronic kidney disease, secondary hyperparathyroidism is triggered by reduced renal synthesis of calcitriol. This is the main mechanism involved in the pathophysiology of renal osteodystrophy (Wesseling-Perry, 2015; Bonthuis *et al.*, 2015).


Skin
290–315 nm
Low cutaneous production
due to high latitude
covered clothing
dark skin
7 Dehydro
cholesterol
Previtamin
D3
Cholecalciferol
(vitamin D3)
+
Ergocalciferol
(vitamin D2)
Low vitamin D in diet
lack of supplements
or
fortified food
Liver
Inactive
metabolites
CYP3A4
25 Hydroxylase
VDDR1B
CYP2R1
25 Hydroxy vitamin D
(Calcidiol)
Stable form
measured in serum
Activating mutation
VDDR3
Kidney
1 Hydroxylase
VDDR1A
CYP27B1
Inactive
metabolites
CYP3A4
1, 25 Dihydroxy vitamin D
(calcitriol)
Active form of
vitamin D
VDDR2A
Calcitriol + VDR - RXR
Vitamin D response element + VDRE
BP
VDDR2B
Low dietary calcium
malabsorption
Intestine
Main point of
action of calcitriol :
increase calcium and
phosphate absorption
All deficiencies/defects lead to reduced calcium supply (calcipaenia)
Calcium sensing receptor
controls PTH release
Parathyroid hormone
Bone
Kidney
Increased bone resorption
to release stored
minerals
Increased 1 hydroxylation
Increased renal calcium
absorption and reduced
phosphate reabsorption

Vitamin D and Calcitriol Synthesis

Vitamin D is biologically inert and merely serves as the substrate to calcitriol synthesis, in analogy to cholesterol being a precursor for cortisol synthesis. Calcitriol synthesis and metabolism is illustrated in **Fig. 1**. We would like to draw the readers' attention to the correct terminologies such as calciferol, calcidiol and calcitriol and discourage the use of the broad term "vitamin D" to refer to either calcidiol or calcitriol (Uday and Högler, 2017). Cholecalciferol (vitamin D3) is synthesized in the skin from 7-dehydrocholesterol following exposure to UVB in sunlight (290–315 nm) and is the main source of vitamin D in the body. Ergocalciferol (vitamin D2) and cholecalciferol can be obtained through certain dietary sources and both undergo 25-hydroxylation in the liver by the CYP2R1 enzyme to form 25 hydroxyvitamin D (25OHD; calcidiol). The stable but still inert 25OHD can be easily measured in human serum and best represents "vitamin D status." 25OHD then undergoes 1-hydroxylation by the CYP27B1 enzyme in the kidney (systemic production) and in the intestine (local production) (Gawlik *et al.*, 2015) to form the active hormone 1,25 dihydroxyvitamin D [1,25$(OH)_2$D; calcitriol]. Calcitriol then binds to its receptor, the calcitriol receptor, commonly referred to as the vitamin D receptor (VDR). The calcitriol-VDR complex heterodimerises with retinoid X receptor in the cell nucleus. This ligand-receptor complex targets specific vitamin D responsive elements (VDRE) on the genome to exert its action on various tissues.

Some of the rare, genetic forms of rickets caused by interrupted calcitriol metabolism or action are detailed in **Table 2** and illustrated in **Fig. 1**. The fact that rickets caused by severe inherited calcitriol receptor defects can be cured with high dose calcium points out the clinically negligible direct effect of calcitriol on bone tissue (Hochberg *et al.*, 1992; Tiosano *et al.*, 2011).

Nutritional Rickets (NR)

Nutritional rickets/osteomalacia (NR) is a major public health concern in low and middle income countries and has resurfaced in high income countries (Holick, 2006). Prevention of vitamin D deficiency and its associated complications in pregnancy, lactation and childhood is a global heath priority (Schoenmakers *et al.*, 2015). The spectrum of NR ranges from isolated dietary calcium or vitamin D deficiency to a combined deficiency (Pettifor, 2004). Calcium and vitamin D insufficiency or deficiency in isolation may not have any clinical consequences, however rickets and osteomalacia develop when both are deficient or insufficient (Munns *et al.*, 2016; **Fig. 2**).

Risk factors and epidemiology

The at-risk population includes: individuals with dark skin and those with reduced sunlight exposure (due to geographic location, predominant indoor living or covered clothing for cultural reasons), individuals with calcium restricted diets (malnutrition, medical or cultural reasons), babies not given vitamin D supplements, especially those of vitamin D deficient mothers and those breastfed for prolonged periods (Munns *et al.*, 2016).

Hypocalcemic complications and NR are only the tip of the iceberg and the true burden of hidden disease remains unidentified. The incidence of NR is on the rise even in developed countries, due to immigration of dark-skinned, high risk groups (Thacher *et al.*, 2016). NR due to dietary calcium deficiency predominantly affects individuals residing in low and middle income countries with abundant sunshine (Aggarwal *et al.*, 2012; Okonofua *et al.*, 1991; Fischer *et al.*, 1999) whereas vitamin D deficiency predominantly affects the dark skinned (immigrant and resident) population in high income countries (Callaghan *et al.*, 2006; Ward *et al.*, 2007; Munns *et al.*, 2012; Thacher *et al.*, 2013). Post-mortem studies from high latitude countries have demonstrated high prevalence of histological evidence of rickets (Scheimberg and Perry, 2014) and osteomalacia (Priemel *et al.*, 2010).

Clinical presentation

The clinical manifestations of calcipaenia occur as a consequence of Hypocalcemia in the short term and hypophosphataemia in the medium to long term. The clinical spectrum across the lifespan is wide and includes hypocalcemic seizures, life

Fig. 1 Vitamin D and calcitriol synthesis, metabolism, and interruptions in normal physiology resulting in disease states (rickets and osteomalacia). Cholecalciferol is synthesized in the skin from 7-dehydrocholesterol following sunlight exposure. Both cholecalciferol and ergocalciferol can be consumed through certain dietary sources or supplements. Calciferol undergoes 25-hydroxylation in the liver to calcidiol and then 1-hydroxylation in the kidney and gut to the hormone calcitriol or "active vitamin D." Calcitriol binds to the calcitriol receptor (vitamin D receptor, VDR) and the calcitriol-VDR complex heterodimerises with retinoid X receptor (RXR) in the cell nucleus. This ligand-receptor complex targets specific vitamin D responsive elements (VDRE) on the genome to exert its action on calcium homeostasis mainly through stimulating intestinal absorption of calcium and phosphate. Any lack of dietary calcium or decreased calcitriol action (nutritional or genetic) results in secondary hyperparathyroidism which maintains serum calcium concentrations by increasing bone resorption, renal calcium absorption and 1-hydroxylation. Disease states are indicated in red and include: nutritional rickets resulting from block of synthesis from sunlight or limited vitamin D in diet, vitamin D dependent rickets type 1B (VDDR1B) resulting from *CYP2R1* mutations blocking 25-hydroxylation in liver, VDDR1A resulting from *CPY27B1* mutations blocking 1-hydroxylation in kidney, VDDR2A resulting from *VDR* mutations, VDDR2B resulting from VDRE binding protein (VDRE-BP) defects and the recently described VDDR3 resulting from increased degradation of vitamin D metabolites due to activating mutations in *CYP3A4*.

Table 2 Genetics and treatment of rickets caused by defects in calcitriol synthesis or action, historically called vitamin D-dependent rickets (VDDR)

Type of vitamin D dependent rickets (VDDR)	*Description*	*Mutation and inheritance*	*Treatment*
VDDR1B (Cheng *et al.*, 2004)	25-Hydroxylase deficiency leading to low serum levels of 25 hydroxyvitamin D (25OHD)	*CYP2R1 gene* on chromosome 11p15.2. Autosomal recessive (AR) inheritance	Physiological doses of calcitriol
VDDR1A (Wang *et al.*, 1998)	1-Hydroxylase deficiency leading to low serum level of 1,25 dihydroxy vitamin D [$1,25(OH)_2D$]	*CYP27B1 gene* on chromosome 12q14.1. AR inheritance	Physiological doses of calcitriol or alfacalcidol
VDDR2A (Malloy and Feldman, 2011)	Vitamin D receptor (VDR) defect leading to increased serum level of $1,25(OH)_2D$. Most patients also have alopecia	*VDR gene* on chromosome 12q13.11. AR inheritance, rarely autosomal dominant	Pharmacological doses of calcitriol or alfacalcidol and high-dose calcium supplements. Severe cases (especially patients with alopecia) may warrant calcium infusions
VDDR2B (Giraldo *et al.*, 1995)	Abnormal expression of vitamin D responsive element-binding protein (Hewison *et al.*, 1993) interferes with the normal function of VDR	Mutation and inheritance not known	Pharmacological doses of calcitriol or alfacalcidol and calcium supplements
VDDR3 (Rodda *et al.*, 2017)	Enhanced hepatic inactivation of vitamin D metabolites leading to low serum 25OHD and $1,25(OH)_2D$	*CYP3A4 gene* on chromosome 7q22.1 (gain of function mutation). Inheritance not known	Pharmacological doses of cholecalciferol

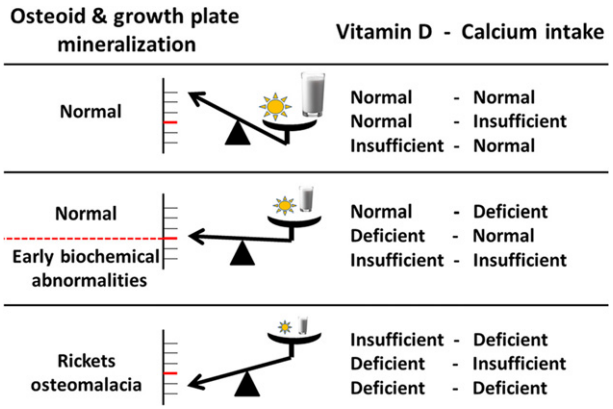


Fig. 2 Three stage classification of vitamin D status (symbolized by sun) and calcium intake (symbolized by glass of milk) in the pathogenesis of rickets. Serum 25 hydroxyvitamin D (25OHD) >50 nmol/L is classed as sufficient, 30–50 nmol/L as insufficient and <30 nmol/L as deficient. Reproduced from Högler, W. (2015) 'Complications of vitamin D deficiency from the foetus to the infant: One cause, one prevention, but who's responsibility?', Best Practice and Research Clinical Endocrinology and Metabolism, 29, 385–398.

threatening cardiac failure, rickets with osteomalacia, muscle weakness, musculoskeletal pain and fractures (Uday and Högler, 2017). The presenting feature depends on the age of the affected individual. All babies of vitamin D deficient mothers are born deficient, the most extreme scenario being congenital rickets in the newborn (Paterson and Ayoub, 2015). The majority of children present during phases of rapid growth: infancy and adolescence (Ladhani *et al.*, 2004; Robinson *et al.*, 2006; Callaghan *et al.*, 2006; Ahmed *et al.*, 2011). Infants can present in the first year of life with hypocalcaemic seizures (Basatemur and Sutcliffe, 2015; Robinson *et al.*, 2006; Callaghan *et al.*, 2006; Al-Atawi *et al.*, 2009), poor feeding, hypotonia and occasionally devastating hypocalcaemic, dilated cardiomyopathy resulting in cardiac failure and death (Elidrissy *et al.*, 2013; Yilmaz *et al.*, 2013; Uysal *et al.*, 1999; Maiya *et al.*, 2008; Scheimberg and Perry, 2014). Toddlers present with motor delay, muscle weakness (Fluss *et al.*, 2014) and leg bowing deformities (Robinson *et al.*, 2006). Adolescents usually present with hypocalcaemic seizures or proximal muscle weakness (Al-Said *et al.*, 2009). Late-effects of childhood rickets include obstructed labor (Konje and Ladipo, 2000), long-term leg deformities, disability and unemployment. In adults, osteomalacia is widespread but not easily diagnosed. Typical clinical signs are musculoskeletal pain and proximal muscle weakness, with increased falls and fractures. Postmortem studies suggest that 25% of adults from a non-risk population are affected by undiagnosed osteomalacia (Priemel *et al.*, 2010).

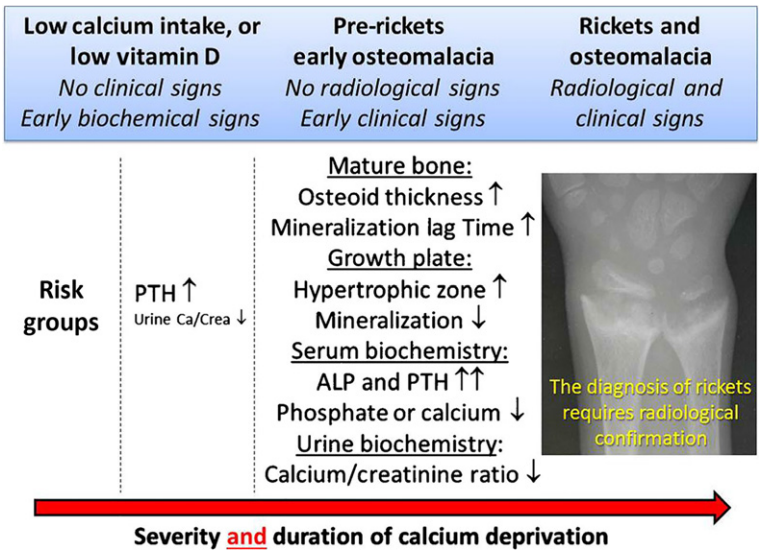


Fig. 3 Stages of progression of rickets ranging from asymptomatic at-risk individuals with calcium deprivation to histological/biochemical evidence of rickets and osteomalacia (pre-rickets) to overt rickets on radiographs. Reproduced from Uday, S. and Högler, W. (2017) 'Nutritional Rickets and Osteomalacia in the Twenty-First Century: Revised Concepts, Public Health, and Prevention Strategies', Current Osteoporosis Reports, 15, 293–302.

Diagnosis of nutritional rickets

The diagnosis of NR/osteomalacia is based on clinical (leg bowing deformities, enlarged wrists and rachitic rosary; seizures and heart failure), biochemical and radiological signs. The diagnosis requires radiographic confirmation, showing the typical radiological signs: cupping, splaying and fraying of metaphyses and widened growth plates. However, these signs only occur in late stages of the disease (**Fig. 3**; Uday and Högler, 2017). In contrast, osteomalacia in adolescents and adults cannot be diagnosed by X-ray. However, rickets and osteomalacia have the same typical biochemical signs (raised ALP and PTH) which appear before any radiological signs. Post-mortem studies in children have improved our understanding of the disease process, demonstrating typical rickets abnormalities at the growth plate (increased height of hypertrophic zone) in the absence of radiological signs (Scheimberg and Perry, 2014; Cohen *et al.*, 2013). Hence, the clinical diagnosis of NR is only made at a very late stage in the disease process and a state of pre-rickets exists before rickets becomes evident on radiographs (Högler, 2015; **Fig. 3**).

Treatment of nutritional rickets and osteomalacia

Treatment should address both vitamin D and calcium deficiency and combined treatment improves outcome (Thacher *et al.*, 1999). The treatment recommendations from the global consensus (Munns *et al.*, 2016) are detailed below:

- Daily oral vitamin D for a minimum of 3 months is recommended in a minimum dose of 2000 IU if <12 months, 3000–6000 IU if aged 12 months—12 years and 6000 IU if >12 years old; plus
- Minimum of 500 mg/day calcium via diet or supplements.
- Instead of daily doses, a single high dose of vitamin D in children aged >3 months can be considered in resource limited settings or suspected non-compliance. The recommended doses are: 50,000 IU if aged 3–12 months, 150,000 IU if aged 12 months—12 years and 300,000 IU if aged >12 years.
- Both cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) are equally effective for daily treatment, whereas cholecalciferol with a longer half-life is preferred for single high dose treatment.

Prevention of rickets and osteomalacia

For prevention of rickets, the minimum daily dietary intake for calcium is >500 mg/day for everyone except infants (0–6 months 200 mg/day and 6–12 months 260 mg/day) and the minimum supply for vitamin D is 400 IU (10 μg) for infants and 600 IU (15 μg) for anyone else (Munns *et al.*, 2016). Vitamin D and calcium deficiency can be overcome by ensuring adequate dietary intake and supplementation in at-risk populations. Calcium supplements from indigenous sources are preferred in low-income countries (Munns *et al.*, 2016).

Since the predominant source of vitamin D is from sunshine exposure and supply is rarely met through diet; supplementation or food fortification programs are essential to protect at risk populations. Most developed nations have vitamin D supplementation programs in place during pregnancy and infancy. However, poor policy implementation has led to the resurgence of rickets in certain European countries (Uday *et al.*, 2017). Evidence suggests that universal supplementation of all infants, monitoring during child surveillance visits, provision of information to new parents and financial incentives are effective implementation strategies (Uday *et al.*, 2017). Ultimately, fortification of food with vitamin D (Shakur *et al.*, 2014) and calcium (Ekbote

et al., 2011) will be necessary to increase uptake at a population level. Legislative authorities should consider the benefits of mandatory (Calvo and Whiting, 2013) versus voluntary fortification (Spiro and Buttriss, 2014) with vitamin D. More effort needs to be taken to introduce safe and efficient food fortification programs (Aguiar *et al.*, 2017). Such efforts are important to improve population bone health but may potentially have other beneficial effects, such as reduction in mortality (Gaksch *et al.*, 2017).

Phosphopaenic Rickets

Phosphopaenic, more commonly called hypophosphtemic, rickets commonly results from renal tubular phosphate wasting disorders (genetic or drug induced), less commonly from reduced gastrointestinal absorption and very rarely from insufficient dietary intake.

Phosphate homeostasis is closely regulated by the hormone fibroblast growth factor 23 (FGF23) which is produced by osteocytes and osteoblasts. FGF23 excess impairs renal phosphate reabsorption (Farrow and White, 2010) in the proximal renal tubule by:

- Down regulation of sodium–phosphate co-transporters (NaPi-2a and 2c) (Gattineni *et al.*, 2009) and
- Reduced synthesis of calcitriol through reduced 1-hydroxylase activity

hypophosphatemic rickets is classed as FGF23 mediated or non-FGF23 mediated (**Table 1**) depending on the underlying mechanism driving the renal phosphate wasting.

(1) Non-FGF23 mediated hyperphosphaturia can result:
   - From primary hereditary disorders of the renal tubule due to mutations in *SLC34A3* causing hereditary hypophosphatemic rickets with hypercalciuria (Lorenz-Depiereux *et al.*, 2006), *OCRL* causing oculocerebrorenal syndrome of Lowe' (Lin *et al.*, 1997), or *CLCN5* resulting in Dent's disease (Yamamoto *et al.*, 2000).
   - From Fanconi syndrome, either through respective genetic defects or drug effects (e.g., sodium valproate, cisplatin).

(2) FGF23 mediated hyperphosphaturia is seen in:
   - X-linked hypophosphatemic rickets (XLH) due to *PHEX* mutations, which represents the leading cause of phosphopaenic rickets and will be discussed in more detail here.
   - Autosomal dominant hypophosphatemic rickets (ADHR) secondary to *FGF23* mutations (White *et al.*, 2000)
   - Less common autosomal recessive forms caused by inactivating mutations of dentin matrix protein 1 (*DMP1*) (Feng *et al.*, 2006) and Ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) (Levy-Litan *et al.*, 2010). Recessive *FAM20c* mutation causes osteomalacia but not rickets.
   - Tumor-induced osteomalacia and fibrous dysplasia

X-Linked hypophosphatemic Rickets (XLH)

XLH is the most common form of phosphopaenic rickets with an approximate prevalence of 1 in 20,000. Its X-linked inheritance means that boys always inherit the condition from their mothers and girls can inherit it from either parent. XLH results from inactivating mutations in the *PHEX* gene (phosphate-regulating gene with homologies to endopeptidases on X-chromosome) located at locus Xp22.11. PHEX regulates FGF23 cleavage, in the absence of which FGF23 accumulates and consequently inhibits renal phosphate reabsorption and down-regulates 1-hydroxylase activity.

Clinical presentation

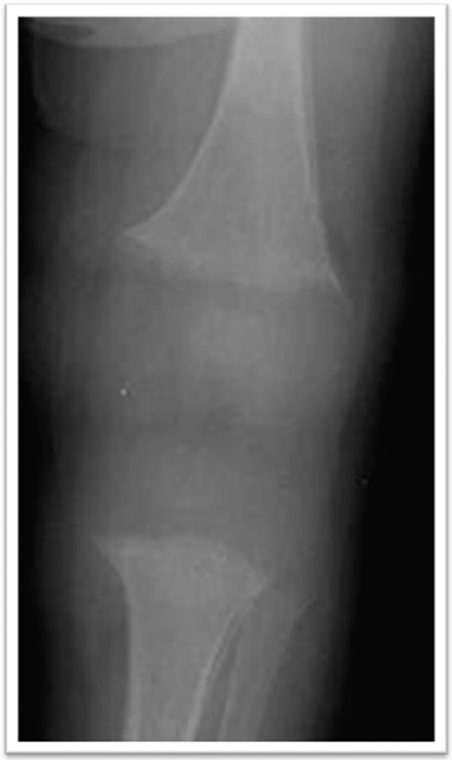
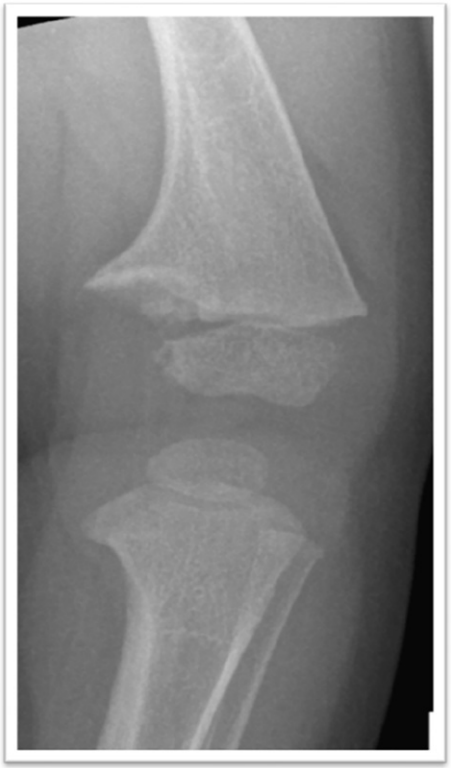
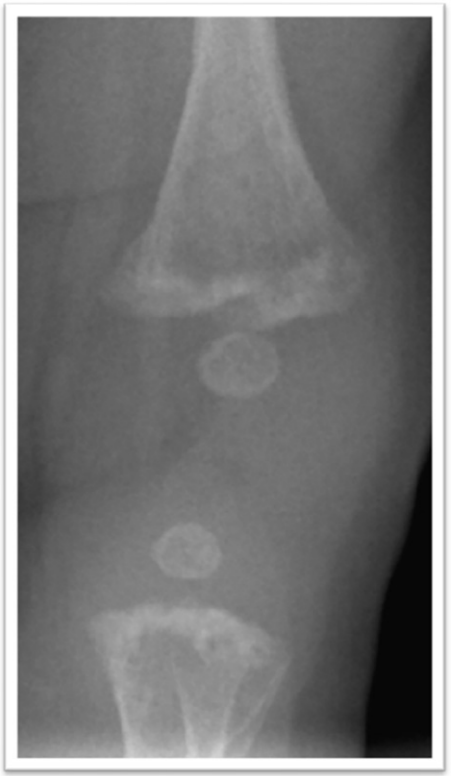
Clinical features vary in affected individuals with majority presenting as toddlers with bowing deformities of long bones and short stature, or referred due to a positive family history. Other manifestations include craniosynostosis (Vega *et al.*, 2016) and dental anomalies (abscesses, reduced enamel). Enthesopathy (calcification of tendons and ligaments) and painful osteoarthritis are frequently seen in adults.

Diagnosis and differentials

Establishing the diagnosis of XLH and excluding other causes of hypophosphataemia is important. Given the various forms of hypophosphatemic rickets (**Table 1**), a thorough clinical evaluation is necessary to guide diagnosis and genetic testing. A positive family history and presence of clinical features from birth suggests a genetic cause rather than acquired. Additional clinical features may be more suggestive of certain specific syndromes such as: short stature, disordered development and ocular signs in Lowe's syndrome (*OCRL*), presence of hearing loss in autosomal recessive hypophosphatemic rickets type 2 (*ENPP1*) and presence of café-au-lait skin pigmentation or polyostotic fibrous dysplasia in McCune–Albright syndrome. Biochemical features that aid in diagnosis include: hypercalciuria in hereditary hypophosphatemic rickets with hypercalciuria (*SLC34A3*), proteinuria, nephrolithiasis and progressive renal failure in Dent's disease (*CLCN5*); proteinuria and glycosuria in Fanconi syndrome.

Measurement of phosphate and creatinine concentrations in serum and urine in the fasting state is required to calculate the percent tubular reabsorption of phosphate (TRP) and the tubular threshold maximum for phosphate (TmP) to glomerular filtration rate (GFR) ratio (Walton and Bijvoet, 1975). The hallmark of FGF23-mediated hypophosphatemic rickets is decreased serum phosphate, TRP and TmP/GFR with low or inappropriately normal $1,25(OH)_2D$ concentrations (**Table 3**). XLH can be

Table 3 Clinical, biochemical and radiological features distinguishing nutritional rickets, X-linked hypophosphatemic rickets and hypophosphatasia

	Nutritional rickets (NR)	*X-linked hypophosphatemic rickets (XLH)*	*Hypophosphatasia (HPP) rickets*
Pathogenesis	Calcipaenia secondary to dietary calcium and/or vitamin D deficiency	Phosphopaenia due to renal phosphate wasting	Hypophosphatasaemia due to defective TNSALP
Distinguishing features	Presence of risk factors (i.e. dark skin, covered clothing, indoor living, high latitude or reduced dietary intake of calcium or lack of vitamin D supplementation)	Family history of hypophosphatemic rickets, rarely *de-novo* mutations can occur Usually present with bowing deformities of the legs	Most severe forms present at birth with respiratory failure due to pulmonary hypoplasia, seizures and hypotonia. Older children have early tooth loss
Biochemical features	↔↓ Serum calcium, phosphate ↑ Alkaline phosphatase (ALP) ↑ Parathyroid hormone (PTH) ↓↔ 25OHD	↓ Serum phosphate ↑ ALP ↔ Calcium, PTH, 25OHD ↓/↔ 1,25 Dihydroxy vitamin D (inappropriately low) ↑ FGF23 ↓ TMP/GFR	↑↔ Serum calcium, phosphate ↓ ALP ↔/↓PTH ↔ 25OHD ↑ Serum PLP ↔↑ Urinary calcium ↑ Urinary PEA
Radiological features			
	Growth plate widening, metaphyseal cupping and fraying. Generalized hypomineralization and periosteal elevation due to hyperparathyroidism	Fraying and widening of metaphyses	Generalized hypomineralization, irregular zone of calcification; later tongue like radiolucent areas at the metaphyses

differentiated from conditions of non-FGF23 mediated renal phosphate wasting which show appropriately elevated $1,25(OH)_2D$ concentrations and accompanying tubulopathies (glycosuria, hyperaminoaciduria, hypercalciuria). Presence of normal or high TmP/GFR indicates an intake or absorption problem. Molecular genetic testing aids in confirming the diagnosis and counseling of family members. The diagnosis of hypophosphatemic rickets cannot be made when 25OHD is low.

Management and outcome

The mainstay of medical treatment currently is multiple daily dosing of phosphate supplements combined with active vitamin D metabolites (alfacalcidol and calcitriol) (Carpenter *et al.*, 2011), however promising therapeutic agents such as anti-FGF23 monoclonal antibodies which inhibit FGF23 action are under investigation (Imel *et al.*, 2017). The aims of treatment include: reducing bone deformities and improving height velocity (Linglart *et al.*, 2014). Early treatment ensures optimal growth (Mäkitie *et al.*, 2003).

Treatment is monitored and tailored to maintain normal serum concentrations of ALP and PTH, which requires considerable experience. Under-treatment inevitably results in short stature and leg deformities necessitating corrective surgery. Over-treatment with phosphate results in unpleasant gastrointestinal symptoms and secondary hyperparathyroidism; over-treatment with active vitamin D metabolites results in hypercalciuria and nephrocalcinosis. A multi-disciplinary team approach involving pediatric metabolic bone specialists, dentists and orthopedic surgeons is crucial. Also essential is appropriate transition of young patients to adult bone specialists for on-going treatment of hypophosphatemic osteomalacia and management of typical adult complications of XLH.

Hypophosphatasia (HPP)

Hypophosphatasia is an inborn error of bone mineralization caused by loss of function mutations in the *ALPL* gene encoding TNSALP. The name-giving hallmark biochemical feature of HPP is the low serum ALP activity, or hypophosphatasaemia (Whyte, 1994). The prevalence of severe and moderate HPP in the European population is estimated to be 1/300,000 and 1/6370, respectively (Mornet *et al.*, 2011).

The TNSALP enzyme on the surface of osteoblasts and brain cells hydrolyses extracellular substrates such as inorganic pyrophosphate (PPi) and pyridoxal-5′-phosphate (PLP, a vitamin B6 vitamer) and phosphoethanolamine (PEA). Reduced TNSALP activity therefore results in accumulation of these substrates. Accumulation of PPi inhibits mineralization (skeletal and dental), resulting in HPP-rickets, osteomalacia and early tooth loss. PLP is the activated form of vitamin B6 which is required as a co-factor in the production of inhibitory neurotransmitters, a process facilitated by TNSALP's extracellular dephosphorylation of PLP to pyridoxal (PL) which then crosses the cell membrane. Severely affected infants with HPP therefore can have seizures which are pyridoxine responsive (Baumgartner-Sigl *et al.*, 2007). The physiological function of PEA remains unclear.

Clinical Classification and Presentation

The clinical presentation varies depending on the age at presentation, ranging widely from stillbirth, severe rickets, atypical fractures in late adulthood to isolated premature tooth loss. The severity of the clinical phenotype of HPP reflects the magnitude of TNSALP deficiency in bone (Fallon *et al.*, 1984). Based on the age at presentation and severity, six distinct clinical forms of HPP have been identified (Fraser, 1948; Whyte *et al.*, 2015) but the disease variability has been considered a continuum as it evolves throughout life (Linglart and Biosse-Duplan, 2016).

Prenatal benign

Evidence on this rare form is limited (Wenkert *et al.*, 2011). The terminology "benign" is probably not justified but in the context of timing of presentation it is understood to mean "non-lethal."

Perinatal

This most severe form of HPP is diagnosed before or at birth. Short long bones are usually noted in utero, as well as reduced chest circumference and polyhydramnios (Shohat *et al.*, 1991). At birth, the skeleton is severely hypomineralized, often with an underdeveloped chest with pulmonary hypoplasia, muscular hypotonia, bony spurs in the mid-portion of forearms and lower legs and neonatal pyridoxine-responsive seizures. High serum levels of calcium, phosphate, PLP and very low ALP activity are typical. In the absence of treatment these infants do not survive, with majority dying in the first few days of life from pulmonary hypoplasia and respiratory insufficiency (Leung *et al.*, 2013).

Infantile

Affected infants may not have any clinical features at birth but develop symptoms in the first 6 months of life. They can have rachitic chest deformities and respiratory compromise due to rib fractures, muscular hypotonia and seizures. Development of functional craniosynostosis can lead to manifestations of increased intracranial pressure, even in the presence of an open fontanelle. Hypercalcaemia and hyperphosphataemia occur, manifesting with irritability, poor feeding, vomiting, failure to thrive,

polyuria and polydipsia (Mornet, 2007). Nephrocalcinosis secondary to hypercalciuria is typical. Survival in the era predating asfotase alfa was 50% (Whyte *et al.*, 2016b).

Childhood

Although less severe than the infantile forms, the clinical presentation of childhood HPP is widely variable. Children usually present around 2–3 years of age with delayed walking, early tooth loss, bony deformities, musculoskeletal pain, fractures and radiological features of rickets (Fallon *et al.*, 1984). Typically, most of them experience early loss of deciduous teeth *with intact roots*. More severe cases may present with craniosynostosis and intracranial hypertension, and detailed clinical history often uncovers early or subtle signs of disease in infancy such as delayed milestones.

Adult

Adults with HPP present with musculoskeletal pain and incidental or atypical fractures (Berkseth *et al.*, 2013). There may be a history of premature loss of deciduous teeth.

Odonto

The classic finding of HPP is premature loss of teeth. However, this feature also exists as an isolated condition, representing premature loss of deciduous teeth without skeletal involvement (Beumer *et al.*, 1973). However, individuals with dental manifestations may go on to develop skeletal features later in life and it may be impossible to distinguish odonto HPP from other forms of HPP (adult or childhood) at initial presentation. The natural history of this form of the disease remains unknown and longitudinal studies are required.

Diagnosis

Biochemical

The biochemical hallmark of HPP is a persistently low ALP activity. A low ALP for age in itself is not diagnostic and other causes should be excluded prior to further evaluation (Macfarlane *et al.*, 1992). Especially in the presence of clinical features of HPP and low serum ALP, further biochemical testing, radiographic imaging and genetic tests are indicated (Saraff *et al.*, 2016). Additional biochemical features of HPP include elevated serum PLP and urinary PEA. Unlike other forms of rickets, serum phosphate and calcium are normal or elevated, possibly with hypercalciuria (Whyte, 2016). In the presence of hypercalciuria or hypercalcaemia, PTH and 1,25 $(OH)_2D$ are appropriately suppressed (**Table 3**).

Radiological

Radiological features include generalized hypomineralization and rickets-like features (HPP-rickets). The most important diagnostic radiographs to be taken in infants and children are the knee (rickets) and skull (craniosynostosis). Childhood-HPP is associated with typical tongue like radiolucent areas in the metaphyses (**Fig. 4**). The skull is often dolicocephalic, copper-beaten and undermineralized (Whyte, 2016). Adults can have metatarsal stress fractures, subtrochanteric femoral pseudofractures, vertebral fractures and chondrocalcinosis (Berkseth *et al.*, 2013). Low bone mineral density on dual energy X-ray absorptiometry scans is reported but not specific to HPP (Fallon *et al.*, 1984). A natural history study reported no significant difference in mean/median spine bone mineral density (BMD) *z* scores in individuals through the disease course except in odonto HPP where significant spontaneous improvement was noted (Whyte *et al.*, 2016c). The final height-corrected spine BMD *z*-scores, although below average, remain in the normal range in infantile, odonto and childhood HPP (Whyte *et al.*, 2016c).

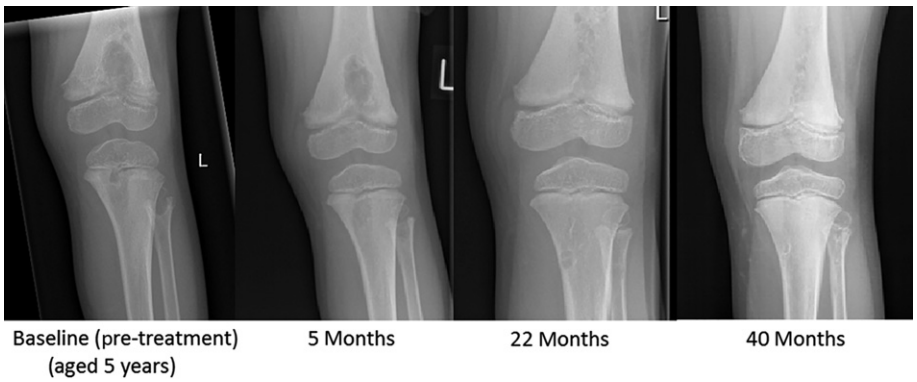


Fig. 4 Serial left knee radiographs of a 5-year old child with infantile onset hypophosphatasia. The metaphyseal tongue-like lucencies and low bone mass seen at baseline (pre-treatment) have progressively improved on asfotase alfa treatment on images taken at 5, 22 and 40 months of therapy.

Genetic testing

HPP can be diagnosed based on clinical, biochemical and radiological features (Fallon *et al.*, 1984) where genetic testing is not readily available. The *ALPL* gene is located on chromosome 1 (Greenberg *et al.*, 1990). To date, 349 mutations (http://www.sesep.uvsq.fr/03_hypo_mutations.php) resulting in HPP have been identified, the majority being missense mutations. Genetic testing can be useful in counseling family members, however the disease displays a high phenotypic variability (Hofmann *et al.*, 2014) limiting the prediction of the disease course. The extremely high phenotypic heterogeneity is attributed to variable residual enzymatic activities (Zurutuza *et al.*, 1999).

Management and Outcome

Asfotase alfa, a bone-targeted recombinant human TNSALP, is approved for treatment of pediatric HPP (perinatal, infantile and juvenile onset) by the US Food and Drug Agency (FDA) and the European Medicines Agency (EMA). Asfotase alfa improves skeletal disease manifestations and pulmonary and physical function in infants and young children with life-threatening HPP (Whyte *et al.*, 2012) and substantially improved survival in infantile and perinatal HPP (Whyte *et al.*, 2016b). **Fig. 4** demonstrates serial radiographs with healing of HPP-rickets following asfotase alfa treatment in a child with infantile onset HPP. The drug is administered as a subcutaneous injection in the licensed dose of 2 mg/kg three times per week, or 1 mg/kg six times per week, but individual dose requirements vary. The treatment is reported to be well tolerated with very few side effects except for mild to moderate injection site reactions (Whyte *et al.*, 2016b).

Neonates with severe form of the disease are usually managed in intensive care units in tertiary centers with support from specialized teams (Linglart and Biosse-Duplan, 2016). A multi-disciplinary approach to management is essential to address: ventilation, pain management, nutrition, reflux, hypotonia and positioning and neurological symptoms (Linglart and Biosse-Duplan, 2016). In older children and adults, involvement of dentists, orthopedic surgeons, physiotherapists and occupational therapists and pain management is necessary depending on the clinical features.

Long-term post-marketing studies are required, including global collaboration in a HPP registry to better understand the condition, treatment monitoring and development of complications. Treatment monitoring guidance is preliminary at present (Kishnani *et al.*, 2017).

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Osteogenesis Imperfecta

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Definition

Osteogenesis imperfecta (OI) is a term that is applied to inherited bone diseases characterized clinically by bone fragility with accompanying bone pain, deformity and reduced stature. Patients often have altered scleral hue, typically blue or blue/gray; may have ligamentous laxity and sarcopenia (Veilleux *et al.*, 2014) resulting in easy fatiguability and reduced endurance; and develop deafness (Kuurila *et al.*, 2002) and aortic root dilatation with increasing age (McNeeley *et al.*, 2012). Dentinogenesis imperfecta—the dental equivalent of OI—with translucent and/or discolored teeth is present in up to 58% of more severely affected individuals, but a minority (13%) of those mildly affected (Lindahl *et al.*, 2015). At a tissue level, the material quality of bone is altered, resulting in the bone being brittle, that is, it bends less and fails sooner under load (Bishop, 2016). The spectrum of severity in OI is large—some individuals may go through life unaware that they have the disorder at all, whilst some infants perish in utero or soon after birth.

The classification of OI is a cause of continuing debate; the classification which was first proposed by Sillence *et al.* (1979), originally had four and now has five types. Since the discovery of multiple new genetic origins for the condition, some favor the attribution of successive "types" according to the date of discovery; Forlino and colleagues proposed such an extended classification first in Forlino *et al.* (2011) and Marini *et al.* (2017). Some forms/types of OI have characteristic clinical features that are important to recognize as they have implications for medical and surgical management (see below). Understanding the genetic origins of bone fragility is clearly of importance in terms of counseling families.

The genes whose mutations are known to result in bone fragility and their associated phenotypic details are shown in **Table 1**. Some inherited forms of bone fragility are not regarded as being OI; tissue hypermineralization is generally regarded as a differentiating factor, but again debate around these fine points continues. About 5% of inherited bone fragility cases still do not have their genetic origin defined.

Genetics

The vast majority of cases of OI—at least 85%—result from mutations in the genes encoding type I collagen. The COL1A1 and COL1A2 genes encode the α1 and α2 chains of the type I collagen molecule respectively; mutations result in dominantly-inherited OI. Each type I collagen molecule is comprised of three collagen chains—two α1 and one α2 chains coil tightly around each other to create the "Type I collagen" molecule, also known as "tropocollagen." The tight coiling is possible because every third amino acid in each chain is a glycine, the smallest amino acid.

In the main, mild OI is due to null alleles in the COL1A1 gene that result in a reduced amount of collagen production, with some abnormal, misfolded proteins incorporated into the matrix. The more severe forms result from missense mutations, exon skipping or splice site mutations in the collagen genes that result in the production of an abnormal protein and its subsequent incorporation into the bone matrix material. Most of the missense mutations result in a glycine being replaced by an amino acid that is more bulky, or charged, or with altered hydrophobicity. In general, the more different the amino acid is to what should be there, the more pronounced the effect.

There are multiple genes where either homozygous or compound heterozygous mutations result in OI (see **Table 1**). These are genes that affect collagen processing, transport, and stability; genes affecting endoplasmic reticulum functioning; genes that affect osteoblast maturation and skeletal patterning; and genes affecting mineralization primarily and collagen secondarily.

The other dominantly inherited form of OI is caused by a single point mutation in 5′-untranslated region of IFITM5/BRIL. This specific form of OI is recognized as distinct in both classification systems as "Type V" (Glorieux *et al.*, 2000).

Tissue Phenotype: Bone Material Properties in OI

The tissue characteristics of OI are spread across all scale levels, from the whole skeletal to the molecular level. Working from the smallest scale level upwards, alterations in the three-dimensional structure of the α1 and α2 chains of the type I collagen molecule result in retention of the chains within the endoplasmic reticulum and over-modification of the heterotrimeric tropocollagen molecule, further compromising three dimensional structure (Chang *et al.*, 2012; Andriotis *et al.*, 2015). The exported tropocollagen proteins do not then form the closely packed quasihexagonal microfibrils (Orgel *et al.*, 2006) (see **Fig. 1**) comprising five overlapping tropocollagen molecules in the "quarter stagger array" that results in the characteristic banding pattern on electron microscopy (Bailey *et al.*, 1998).

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Table 1 Genotype, phenotype and classification details for inherited bone fragility syndromes

Gene	*Silence type*	*Barnes/Marini type*	*Protein*	*Phenotype(s)*
Collagen molecule				
COL1A1	I-IV	I-IV	Type 1 collagen α1 chain	Mild-lethal OI (Forlino and Marini, 2016) High bone mass in C-propeptide cleavage site defects (Lindahl *et al.*, 2011) Caffey disease with defect at p.Arg1014Cys (Gensure *et al.*, 2005)
COL1A2	I-IV	I-IV	Type 1 collagen α2 chain	Mild-lethal OI (Forlino and Marini, 2016) High bone mass in C-propeptide cleavage site defects (Lindahl *et al.*, 2011)
Collagen folding				
CRTAP	III	VII	Cartilage associated protein	Severe-lethal OI; (Barnes *et al.*, 2006; Morello *et al.*, 2006) Cole-Carpenter features (Balasubramanian *et al.*, 2015)
LEPRE1	III	VIII	Prolyl-3-hydroxylase	Severe-lethal OI (Cabral *et al.*, 2007)
PPIB	III	IX	Cyclophilin B	Moderate-lethal OI (van Dijk *et al.*, 2009)
Collagen stability				
FKBP10	III	XI	FKBP65; 65kD FK506-binding protein	Moderate-severe OI; Bruck syndrome (OI with contractures); (Schwarze *et al.*, 2013) Kuskokwim syndrome (contractures alone) (Barnes *et al.*, 2013)
PLOD2	N/A	N/A	Lysyl hydroxylase 2	Bruck syndrome (Ha-Vinh *et al.*, 2004)
SERPINH1	III	X	Heat Shock Protein 47	Severe OI, pyloric stenosis, skin bullae, renal stones (Christiansen *et al.*, 2010)
SPARC	III	XVII	Secreted protein, acidic, cysteine-rich; osteonectin	Notable sarcopenia (Mendoza-Londono *et al.*, 2015)
Collagen processing/cleavage				
BMP1	III	XIII	Bone morphogenetic protein 1; tolloid	High bone mass, hyperosteoidosis (Pollitt *et al.*, 2016; Hoyer-Kuhn *et al.*, 2013; Asharani *et al.*, 2012)
Wnt-signalling pathway				
WNT1	III	XV	Wingless-type MMTV integration site family, member 1	Homozygous—severe OI; some have brain malformation; autism, learning difficulties in some. (Fahiminiya *et al.*, 2013) Heterozygous—early onset osteoporosis, normal growth (Keupp *et al.*, 2013)
Mineralisation regulation				
IFITM5/ BRIL	V	V	Interferon-induced transmembrane protein 5, or, bone-restricted IFITM5-like	Severe OI; metaphyseal dysplasia and sclerosis, hypertrophic callus, interosseous membrane calcification. (Rauch *et al.*, 2018; Balasubramanian *et al.*, 2013; Semler *et al.*, 2012; Cho *et al.*, 2012)
SERPINF1	III	VI	Pigment epithelium derived factor	Slowly progressively worsening OI; osteoid mineralization defect (no endochondral defect) (Al-Jallad *et al.*, 2015; Becker *et al.*, 2011; Glorieux *et al.*, 2002)
Osteoblast lineage				
SP7/OSX	III	XII	Specificity protein 7; Osterix	Typical OI features (Lapunzina *et al.*, 2010)
Developmental/patterning				
TAPT1	III	Not assigned	Transmembrane anterior posterior transformation-1 protein	Complex lethal osteochondrodysplasia with multiple fractures; also have brain, cardiorespiratory and renal defects (Symoens *et al.*, 2015)
ER-related				
P4HB	III	Not assigned	Prolyl 4-hydroxylase; protein disulfide isomerase	Cole-Carpenter syndrome; craniosynostosis, ocular proptosis, hydrocephalus (Rauch *et al.*, 2015)
TMEM38B	III	XIV	Trimeric intracellular cation channel type B; TRIC-B	Severe osteopenia and limb fractures without vertebral fractures (Shaheen *et al.*, 2012)
CREB3L1	III	XVI	Old astrocyte specifically induced substrate—OASIS	Severe OI; cardiac failure (Symoens *et al.*, 2013)
SEC24D	III	Not assigned	Component of COPII complex	Cole-Carpenter syndrome; craniosynostosis, ocular proptosis, hydrocephalus (Garbes *et al.*, 2015)
MBTPS2	III	XVIII	Site-2 metalloproteinase S2P	Regulated intramembrane proteolysis of transcription factors such as OASIS x-linked moderately-severe OI (Lindert *et al.*, 2016)
Linker enzyme deficiency				
XYLT2	III	Not assigned	Xylosyltransferase II	Vertebral fractures, cataracts, heart defects (Munns *et al.*, 2015)
Bone fragility, not clearly OI				
LRP5/6	N/A	N/A	Lipoprotein receptor-related protein 5/6	Homozygous—osteoporosis pseudoglioma syndrome; (Gong *et al.*, 2001) Heterozygous—osteoporosis and/or vitreoretinopathy (Hartikka *et al.*, 2005)

Table 1 Continued

Gene	*Silence type*	*Barnes/Marini type*	*Protein*	*Phenotype(s)*
NBAS	N/A	N/A	Neuroblastoma amplified sequence	Early onset osteoporosis, recurrent acute liver failure, developmental delay(Balasubramanian *et al.*, 2017; Capo-Chichi *et al.*, 2015)
Osteocyte dysfunction				
PLS3	N/A	N/A	Plastin 3	X-linked early onset severe osteoporosis without other OI features (van Dijk *et al.*, 2013)

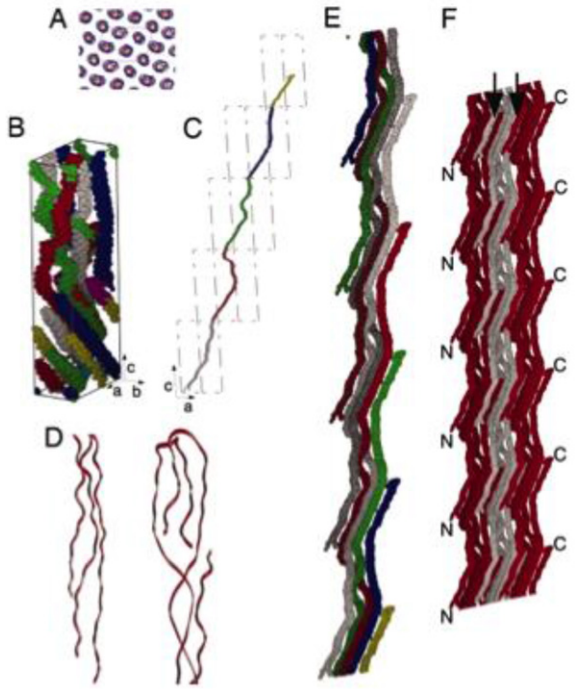

Fig. 1 Collagen organization and structure. The collagen segments are labeled as follows for *B*, *C*, and *E*: 1, *gray*; 2, *red*; 3, *green*; 4, *blue*; 5, *yellow*. Part of segment 1 is colored *cyan* (the N terminus), and part of segment 5 is colored *magenta* (the C terminus) to allow easier identification in *B*. The *c*-axis has been compressed five times for *B*, *C*, and *E*. (*A*) Electron density and model showing the quasihexagonal packing of the molecular segments. The approximate outline of the unit cell (*a* and *b* sides) is marked with black lines. (*B*) Cα carbons rendered as line spheroids showing the conformation of the *D*-staggered collagen segments within a single unit cell (cell axis shown). (*C*) Molecular path of a collagen molecule through successive unit cells in the *a*–*c* plane. (*D*) Enlarged view of the telopeptides of type I collagen, showing N-telopeptide (left and bottom of *C*) and C-telopeptide (right and top of *C*). Both have been rotated with respect to *C* for clarity of display. (*E*) Taking several 1D staggered collagen molecules from the collagen packing structure (single molecule shown in *C*), it is possible to represent the collagen microfibril. The collagen molecules progress from bottom to top (N to C terminus) and are colored as previously (except that chains starting in successive *D*-periods are darker equivalent colors). A clear right-handed twist can be seen, particularly between segments 2 and 3 (which is roughly at the midpoint of each collagen molecule). The noncrystallographic symmetry that relates the collagen molecules within the microfibrillar structure is a simple fractional translational function (*N* u, 0v, *N* w) where *N* is an integer. Five successive *D*-repeats of the microfibril can be visualized with nine copies of the coordinates (A–E1) of a single collagen molecule by applying the following translations. (A) 0, 0, 0. (B) −1, 0, −1. (C) −2, 0, −2. (D) −3, 0, −3. (E) −4, 0, −4. (B1) 1, 0, 1. (C1) 2, 0, 2. (D1) 3, 0, 3. (E1) 4, 0, 4. (*F*) Three microfibrils are shown side by side to indicate the probable binding relationship. The N-terminal segment of each collagen molecule is bound to two other collagen molecules (one inter- and one intramicrofibrillar) and a single crosslinked partnership at the C-terminal telopeptide (one intermicrofibrillar link). N- and C-terminal areas are marked. Note the positions (arrows) where the molecules belonging to one microfibril interdigitate with that of its neighbours. From Orgel, J. P., Irving, T. C., Miller, A. and Wess, T. J. (2006). Microfibrillar structure of type I collagen in situ. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 9001–9005.

The fine detail of how cross-linking within and between microfibrils occurs is still being worked out; enzymatic cross-linking is normal and helps bone tissue to remain strong as well as elastic under load but nonenzymatic cross-linking, more evident in OI and other conditions such as diabetes, increases tissue brittleness (Poundarik *et al.*, 2015). The excessive spacing of tropocollagen within microfibrils, and possibly between microfibrils, is thought to contribute to the excessive deposition of mineral platelets (Fratzl-Zelman

et al., 2014), resulting in increased tissue mineralization density and increased tissue brittleness. Noncollagenous proteins are thought to have roles in tissue stability and the mineralization process; mutations in three specific areas of the tropocollagen molecule are associated with lethal phenotypes (Marini *et al.*, 2007), suggesting that key tropocollagen–noncollagen interactions are taking place at those sites. Moving up the scale, testing of fibrillar mechanics in the *oim* mouse model suggests decreased tissue mass that is reduced in toughness and less able to absorb and dissipate externally imposed loads (Vanleene *et al.*, 2012).

In most forms of OI, mineralization appears to proceed normally; however, in type V OI resulting from mutations in IFITM5/BRIL and type VI OI resulting from mutations in SERPINF1, altered mineralization is observed at clinical and tissue levels. Infants with type V OI initially display a rickets-like appearance at the metaphyses (Arundel *et al.*, 2011), and in later life develop interosseous membrane calcification of the forearm and lower leg, along with hyperplastic callus formation at fracture and osteotomy sites (Glorieux *et al.*, 2000). The tissue phenotype in type OI VI is osteomalacic, with grossly expanded osteoid thickness and an odd "fish scale-like" appearance under polarized light microscopy (Glorieux *et al.*, 2002). In type V, detailed molecular studies have found altered osteoblastic collagen secretion, which likely contributes to the bone fragility phenotype (Reich *et al.*, 2015). Type VI patients have very high tissue mineralization density, with altered orientation of collagen fibrils in perilacunar areas (Fratzl-Zelman *et al.*, 2015). Mineral platelets in OI have altered chemical composition, are thinner and reduced in size, contributing to the increased packing density that results in overall higher bone mineralization density distribution (Fratzl *et al.*, 1996).

Increased cross-linking and higher mineralization density contribute to reduced ability to absorb and dissipate energy from external loads. Fracture-toughening mechanisms that deflect energy along branching cracks and use other features such as sacrificial bonds and platelet sliding appear less effective in OI (Fantner *et al.*, 2007). In addition, increased cortical porosity allows a propagating fracture to "jump" from one pore to another, reducing the resistance of the whole.

At a whole bone level, the total amount of bone is reduced; cortical pores are larger and more numerous; trabeculae are thinned or lost and disconnected; cortical thickness is reduced; and bones are narrower (Rauch *et al.*, 2000). OI severity is most clearly reflected in overall stature—the shorter the patient, the more severely their disease has affected them. Put together the alterations in material properties, architecture and mass, it becomes readily apparent why bone fragility is such a prominent component of the clinical phenotype in this condition.

Bone Cell Activity

Bone cells fall broadly into two groups—those of mesenchymal lineage, and those of hematopoietic lineage. The bone forming cells—osteoblasts—are found as quiescent lining cells across all bone surfaces except where active remodeling is underway (Amizuka *et al.*, 2014). Osteoblasts are incorporated into the bone matrix during the mineralization process and become entombed within the lacunar-canalicular network as osteocytes (Prideaux *et al.*, 2012). Osteoclasts, by contrast, are formed from mononuclear precursors during the process of removal of bone tissue (Boyle *et al.*, 2003). The coordinated action of replacing existing old bone tissue with new bone is known as remodeling. During this process, bone lining cells appear to lift and form a tent or canopy under which the remodeling events proceed (Hauge *et al.*, 2001). The factors controlling the site-specific removal of bone—the timing, the quantity, the speed of removal—are still not fully elucidated. It is clear however that the initiation of remodeling is likely to be an osteoblast/osteocyte activity (Rumpler *et al.*, 2012); that the removal of bone mineral and some bone matrix protein is largely accomplished by osteoclasts (Sakamoto and Sakamoto, 1986), with the remainder of the matrix being removed by osteoblast lineage "reversal" cells (Andersen *et al.*, 2013) and then replacement of bone material by osteoid secreting and mineralising osteoblasts. The coordination of osteoclast and osteoblast activity may involve other hematopoietic lineage/immune cells (Andersen *et al.*, 2013).

All the defects that have been identified so far as giving rise to OI have occurred in mesenchymal lineage cells. The net effect at a tissue level is an increase in bone turnover, with increased osteocyte density (Blouin *et al.*, 2017), and reduced osteoblast cell effectiveness in terms of bone forming activity (Rauch *et al.*, 2000). In normal growing bone, each remodeling event results in a 4% increase in bone at the remodeling site; in OI this is reduced to 1% (Rauch *et al.*, 2000). Thus over time, the bone accrual in a child with OI deviates progressively from that expected for the normal skeleton, adding to the increased risk of fracture both during childhood and later in life.

Molecular Pathways

Recent years have seen elucidation of key pathways regulating and coordinating bone cell activity. In OI, two pathways/areas are currently receiving significant attention as potential treatment targets; endoplasmic reticulum stress (Ishida and Nagata, 2009) and the consequential effects on the unfolded protein response and autophagy, and the TGFβ signaling pathway (Grafe *et al.*, 2014).

In relation to ER stress, the misfolding and subsequent accumulation of abnormal protein within the ER might be expected to trigger the ER-associated degradation (ERAD) pathway (Horiuchi *et al.*, 2016). In fact, recent evidence suggests that ERAD is not the main process in OI, but that the misfolded collagen aggregates are removed by autophagy (Ishida and Nagata, 2009). ER stress causes impaired osteoblastic differentiation and may eventually result in osteoblast apoptosis (Symoens *et al.*, 2013).

TGFβ signaling is overactive in some murine models of OI (Grafe *et al.*, 2014); the mechanisms by which such excessive signaling might result in the observed phenotype are less clear, but in other settings such as Camurati–Engelmann syndrome

excessive TGFβ signaling results in increased bone turnover, bone pain and diaphyseal sclerosis (Janssens *et al.*, 2006). Anti-TGFβ antibody treatment of the crtap $-/-$ and G610C models described below showed restoration of bone architecture and mass, but not alteration in tissue material properties (Grafe *et al.*, 2014).

Recently it has been suggested that inflammatory processes may contribute to the OI phenotype; these suggestions are based on both the finding of elevated inflammatory markers and splenomegaly in the *oim* mouse model (Matthews *et al.*, 2017), and the demonstration of a right-shift in platelet counts in moderately-severely affected children.

Preclinical Models

Both mouse and zebrafish models of OI have been created; the mouse models cover a range of severity from relatively mild (Mov-$13^{/+}$) (Jaenisch *et al.*, 1983) to severe (crtap $-/-$) (Morello *et al.*, 2006). The murine model harboring the COL1A1 $+/$ $G610C^{Neo-}$ mutation recapitulates the human phenotype associated with the same mutation in an Amish population (Daley *et al.*, 2010). Zebrafish have a different bony architecture to mice or humans, and have heterotrimeric as opposed to heterodimeric tropocollagen molecules (Fisher *et al.*, 2003), but many of the pathways of collagen folding, processing, and transportation are similar. For instance, the "*frilly fins*" zebrafish (Asharani *et al.*, 2012) harbors a mutation in Bmp1 that provided insights into the molecular mechanisms of action relevant to the form of OI caused by loss of function in the human gene, whilst the "*Chihuahua*" mutant is caused by a heterozygous missense mutation in *Col1a1a* and has a clinical phenotype resembling moderately-severe OI with deformed vertebrae and frequent fractures (Fisher *et al.*, 2003).

The availability of these model systems is key both to understanding mechanism and for preclinical assessment of novel interventions.

Clinical Manifestations

The clinical manifestations of OI vary considerably. The most consistent feature bringing the patient to medical attention is bone fragility, manifested either as fractures occurring with little or no apparent trauma, or recurrent fractures. Fractures are not uncommon in childhood—up to 40% of children will have had a fracture by age 16 years (Landin, 1983); pelvic, hip, and vertebral fractures are uncommon at age any during childhood (Cooper *et al.*, 2004). The International Society of Clinical Densitometry Pediatric Position Statements on Osteoporosis Definition suggest that two fractures by age 10 years, or three fractures by age 19 years in association with low bone mass for body size, should raise suspicions of osteoporosis (Bishop *et al.*, 2014). In addition, one or more vertebral crush fractures in the absence of known significant trauma, should suggest osteoporosis.

In mild OI, bone density may be within the normal range and vertebral crush fractures may not be present initially; clinical experience indicates that the majority of even mildly affected cases will however develop vertebral crush fractures at some point. The most severely affected infants may have crush fractures at birth. More severely affected infants tend to have crumpled long bones, or femurs that look as though the shaft has expanded and then exploded. Maintenance of normal bone shape is a good prognostic indicator in our experience.

Children with type V disease have some characteristic radiological features including metaphyseal dysplasia/rickets-like changes at birth (Arundel *et al.*, 2011), which change over a period of weeks to sclerotic metaphyseal bands; dense wedge-like inclusions in the anterior vertebral wall, seen on lateral imaging of the spine (Arundel *et al.*, 2011); and the development of interosseous membrane calcification and associated radial head dislocation with limitation of elbow range of movement, particularly pronation and supination (Fassier *et al.*, 2007). Children with type VI disease rarely present before 12 months of age; they have a gradually progressive deforming disease course and are relatively insensitive to interventions using conventional antiresportive agents (Hoyer-Kuhn *et al.*, 2014).

Children with mutations in FKBP10 have a very variable clinical phenotype—at the Kuskokwim end of the spectrum, no fractures occur, in contrast to those with Bruck syndrome where fractures accompany contractures (Barnes *et al.*, 2013).

Fractures result in bony deformity in many cases of OI, but this is not sufficient to account for the degree of growth retardation that accompanies the more severe clinical phenotypes, which remains unexplained currently. Sarcopenia is a frequent finding in OI (Veilleux et al., 2014, 2017); alongside the bone pain that may reflect increased bone turnover, ligamentous laxity, muscle weakness, and fatigue contribute to the musculoskeletal pain in OI that for many is the most debilitating aspect of their disease on a daily basis.

Vertebral deformity can contribute to earlier presentation with scoliosis; restoration of vertebral size and shape through medical therapy (see below) does not necessarily prevent scoliosis, but likely delays the age at which it is first clinically manifested in more severely affected children (Sato *et al.*, 2016). Basilar invagination, one of the most feared complications of OI with advancement of the odontoid peg through the foramen magnum, is relatively uncommon in our experience (2%–3% over the last 20 years across all types, unpublished). Spondylolysis and spondylolysthesis can cause chronic pain, particularly in the lower back.

It is increasingly apparent that pulmonary disease is also present in the moderately-severely affected; manifested as a chronic oxygen requirement in severely affected infants that can persist for months, it is also present in older children and adults. Scoliosis in later years can be present and is associated with reduced pulmonary function (Wekre *et al.*, 2014) but scoliosis correction does not necessarily improve lung function.

Dentinogenesis imperfecta can result in teeth chipping and cracking; the altered appearance of the teeth can be distressing for some patients with tooth discoloration ranging from gray through yellow to brown. Accelerated wear occurs, with rapidly

progressive dental caries in some. Aortic root dilatation has been reported in adults; there are several reported cases of aortic dissection, two being fatal (McNeeley *et al.*, 2012). Combined conductive and sensorineural hearing loss occurs in up to 50% of cases (Kuurila et al., 2002, 2000).

Differential Diagnosis and Investigation

In older children presenting with osteoporosis, the urgent differential to exclude is any malignancy. Osteoporosis can occur secondary to a variety of inflammatory, immobilizing, hematological, and endocrinological disorders, as well as secondary to the treatments used, particularly steroids. In routine clinical practice, one of the most common "occult" causes we have seen is celiac disease.

Biochemical investigations would reflect the need to address both primary and secondary causes of bone fragility and include targeted testing for the groups of conditions mentioned above. Radiological imaging usually includes dual energy X-ray absorptiometry (DXA) for those able to lie still (typically 4 years and older) and where there is data to interpret the DXA outputs, along with lateral spine imaging to assess vertebral size and shape.

Bone biopsy can be helpful in distinguishing OI from other forms of osteoporosis in older children; OI is characterized by high turnover and increased numbers of osteocytes (24). Typical appearances under direct light, polarized light, and ultraviolet light are shown in **Fig. 2**.

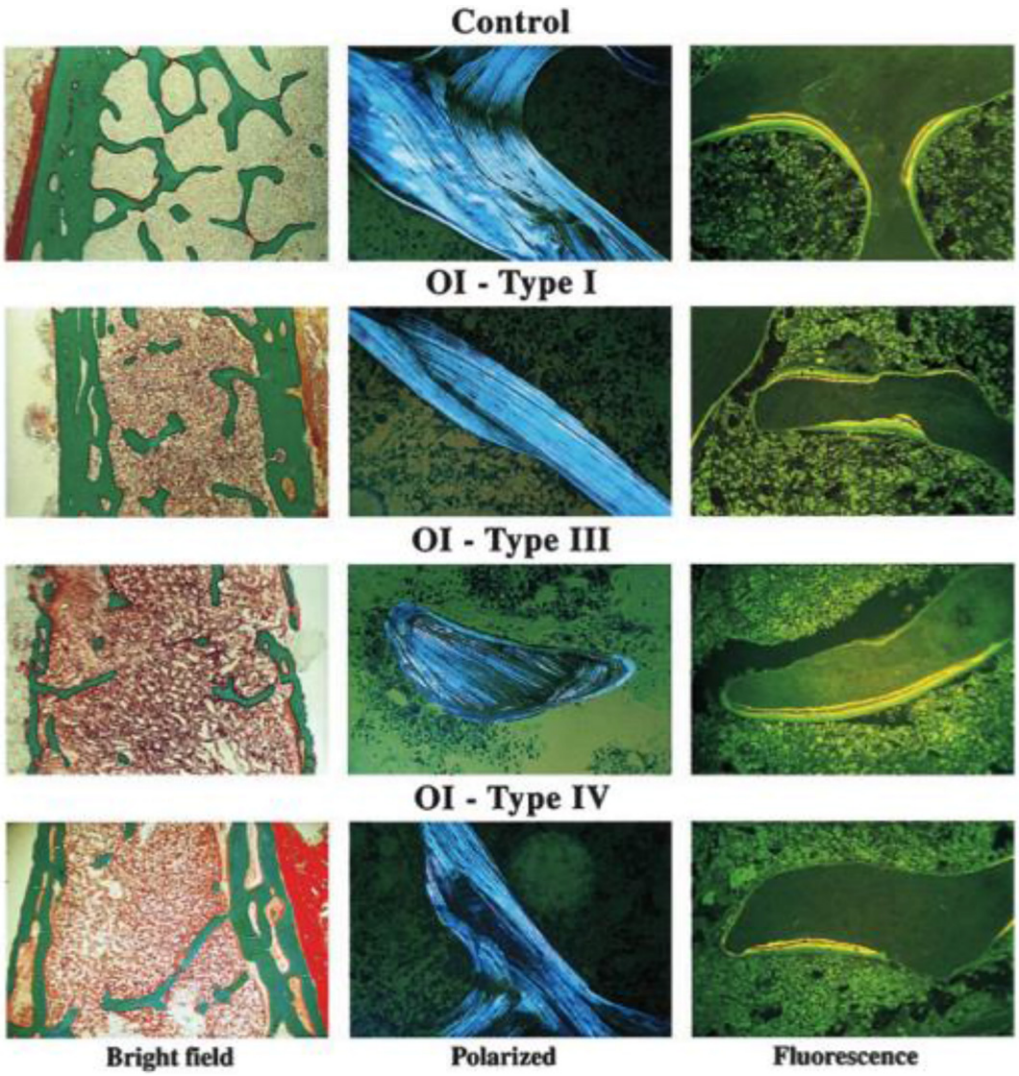

Fig. 2 Typical sections of biopsies from a control subject (boy, 9 years) and OI patients (type I: girl, 5 years; type III: boy, 9 years; type IV: boy, 13 years). Original magnifications: left column, ×32, middle column, ×200; right column, ×200. From Rauch, F., Travers, R., Parfitt, A. M. and Glorieux, F. H. (2000). Static and dynamic bone histomorphometry in children with osteogenesis imperfecta. *Bone*, **26**, 581–589.

If there is doubt about the genetic origin of the OI, targeted Next Generation Sequencing using an "OI panel," followed by whole genome sequencing, would be our way forward.

The issue of unexplained fractures in the context of suspected nonaccidental injury as opposed to bone fragility/mild OI is a thorny one. Our experience has been that infants with mild OI tend to present with a single fracture; those with more severe disease tend to have more than one fracture, and often have altered vertebral shape even in infancy. Those presenting with multiple fractures whose skeletons are later regarded as normal on repeat imaging and without evidence of other disturbances in skeletal homeostasis (e.g., rickets) are much less likely to have an underlying genetic cause for bone fragility, if indeed any bone fragility was ever present. Metaphyseal "corner" fractures are highly suggestive of nonaccidental injury and are vanishingly rare in OI, possibly because the tight adherence of the periosteal membrane at the metaphysis actually results in a stronger overall bone there, compared to the diaphysis, in OI.

Current Management

Management of OI is best undertaken by a multidisciplinary team that includes physicians, surgeons, dentists, nurses, physio- and occupational therapists, psychologists and social workers. The role of the physician is to make the diagnosis and direct management in terms of pain relief and any bone-targeted pharmacological therapy. For children, that therapy currently is essentially limited to one class of drugs, the bisphosphonates. For adults, other drugs have been used with limited evidence of efficacy.

Bisphosphonates are widely used in the treatment of both adults and children with OI. At a tissue level, the inhibition of osteoclastic bone resorption by bisphosphonates reduces trabecular loss at the growth plate, increasing trabecular number but not trabecular thickness (Rauch *et al.*, 2002). Cortical thickness increases due to reduced endosteal resorption, whilst periosteal apposition continues. Cortical porosity reduces with continuing therapy. In those treated intravenously, metaphyseal lines appear, parallel to the growth plate. These are bands of bone and calcified cartilage; the calcified cartilage is gradually remodeled to bone over time, as judged by the proportion of calcified remnants seen in bands of differing ages from transphyseal bone cores (Rauch *et al.*, 2004).

Bisphosphonates have been used in OI for three decades (Devogelaer *et al.*, 1987); although regarded as "standard of care," there is limited evidence for their efficacy in reducing fracture risk outside of those more mildly affected by OI (Dwan *et al.*, 2014). In part this may reflect the difficulty of undertaking randomized, controlled trials of intravenous therapy in children with severe bone disease. In trials of oral therapy (olpadronate, risedronate) in primarily mild disease, fracture risk reduction of around 50% has been demonstrated (Sakkers *et al.*, 2004; Bishop *et al.*, 2013). Oral therapy in more severely affected children using alendronate did not demonstrate such a reduction, however (Ward *et al.*, 2011).

Pamidronate and zoledronic acid are the most widely-used intravenous preparations and are generally used as first line therapy in more severely affected children. Cohort studies consistently report increased bone mineral density, improved vertebral size and shape and in some studies improved grip strength and mobility. Anecdotally, children and parents report that treatment with bisphosphonates reduces pain and fatigue and improves endurance; this has been difficult to demonstrate in clinical trials, but this difficulty may reflect the fact that until recently there has been no OI-specific assessment tool available to make such an evaluation (Hill *et al.*, 2014). Concerns have been expressed regarding the possibility of fracture nonunion in association with prolonged bisphosphonate use, but these have not been supported by data from longitudinal cohort studies (Munns *et al.*, 2004), although delay in healing for more than 12 months after osteotomy is observed in around 40% (Anam *et al.*, 2015). Based on experience in adults, concerns have also been expressed about the possibility of atypical femoral fractures and osteonecrosis of the jaw. A recent longitudinal review of femoral fractures in children with OI found no relationship of bisphosphonate administration with fractures that were similar to those described as "atypical" in non-OI adults (Trejo *et al.*, 2017). There have been no reported cases of osteonecrosis of the jaw in childhood.

Bisphosphonate and PTH therapy in adults has been largely ineffective in reducing fracture risk, although bone density measured by DXA is increased with both interventions (Orwoll *et al.*, 2014). Clinical trials with interventions focussed on increasing bone mass and reducing fracture incidence using both antiresorptive (anti-RANK-ligand antibody), anabolic (anti-sclerostin antibody), and combination therapy (zoledronic acid and PTH) are underway.

Surgical interventions include the placing of intramedullary rods to provide long bone stability, often using an expanding or telescopic system in the growing skeleton (Saldanha *et al.*, 2004), along with guided growth through the use of epiphysodesis. Scoliosis surgery using pedicle wires rather than screws may be needed for curves that continue to progress beyond 50 degree. Fusion of adjacent vertebrae can help in spondylolysthesis with a grade 2 or worse slip.

It is important that surgeons know if a child has type V OI before undertaking osteotomies—hypertrophic callus formation can occur around the osteotomy. Treatment with nonsteroidal antiinflammatory drugs may help prevent this.

Dental inputs include capping primary molars, crack, and fissure sealants and veneers that can improve cosmesis. The secondary dentition is generally stronger than the primary.

Therapy inputs are vital for muscle strength and range of movement, maintaining activities of daily living and providing aids to mobility (Marr *et al.*, 2017). Small changes can often make a big difference. Therapists and nurses often liaise with schools and primary care teams to ensure that all aspects of care are "joined up." Psychology inputs are important in any chronic disease.

Future Directions

The Holy Grail for OI therapy is gene correction—but that seems some way off. Fetal mesenchymal stem cell therapy has been used in two cases (Chan and Gotherstrom, 2014; Gotherstrom *et al.*, 2014); however, although the results appear encouraging, there is no evidence that the low level of engrafted cells has actually been responsible for the formation of new bone with improved tissue material properties, and the assessment of efficacy is hampered by the difficulty in making accurate assessments in utero. Anti-TGF pathway therapy may be possible in future, as may interventions targeting the ER stress and autophagy pathways. The latter have the possibility, alone amongst the available noncellular interventions, of altering bone material properties through the removal of excess misfolded proteins (Mullan *et al.*, 2017). The improvements in health outcomes and quality of life for those with OI should be expected to continue.

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Hypercalcemia

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Introduction

Hypercalcemia is rare in children, but if untreated can result in life-threatening end-organ damage including renal failure and neurological impairment. In adulthood malignancy and primary hyperparathyroidism are the most common causes of hypercalcemia, whereas in children the possible etiologies are more diverse, may be age-specific and many have an underlying genetic basis. Establishing the correct diagnosis will enable the most appropriate treatment to be given. When investigating hypercalcemia a key consideration is the accompanying PTH level at the time of hypercalcemia which delineates the etiology as either a PTH-dependent or PTH-independent cause (Davies and Shaw, 2012).

Definition of Hypercalcemia

A definition of hypercalcemia in childhood has not been established due to the paucity of pediatric laboratory reference data for normal calcium levels in healthy individuals during childhood and puberty. The clinical significance of the degree of hypercalcemia and how it relates to long term morbidity is unknown. A multicentre study of healthy children in Canada from birth to 18 years of age was used to develop assay, age and sex-specific reference ranges for analytes including calcium. Calcium levels were found to be assay dependent and there was no sex difference. Levels were higher in children compared to adults, likely reflecting greater bone turnover in children (Colantonio *et al.*, 2012). Thus age and assay-appropriate reference ranges should be used when interpreting serum calcium levels in children.

Frequency of Hypercalcemia

Hypercalcemia is rare in children (McNeilly *et al.*, 2016). A retrospective study of laboratory data from a tertiary unit showed that only 0.33% of over 61,000 pediatric serum samples were hypercalcemic (defined as serum calcium >2.9 mmol/L). Of the samples that were hypercalcemic, 41% of cases were taken in the first 28 days of life, 24% in 28 days to 1 year, 16% in 1–5 years, 12% 6–12 years and 7% in 13–17 years. These frequencies also reflect the likelihood of blood sampling at different ages, which is most frequent in the neonatal period, thus serendipitous discovery of hypercalcemia is more likely during biochemical screening for unrelated conditions. The severity of the hypercalcemia was classified as: 84.7% mild (2.9–3.1 mmol/L), 9.9% moderate (3.2–3.5 mmol/L), and 5.4% severe (>3.5 mmol/L). In a hospital setting, sustained hypercalcemia (i.e., a calcium >2.9 mmoL/L for two consecutive days) was observed in 1 in 500 children (McNeilly *et al.*, 2016).

Pathophysiology of Hypercalcemia

Plasma calcium is maintained by the interplay of three dynamic processes: tubular reabsorption from the kidneys, absorption from the small intestine and bone remodeling. The two main calciotropic hormones are parathyroid hormone (PTH) and 1,25-dihyroxyvitamin D [$1,25(OH)_2D$]. The calcium sensing receptor (CaSR), which is expressed on the surface of the parathyroid cell, is also a critical regulator of plasma calcium levels by directly influencing PTH release in response to circulating calcium levels.

PTH usually maintains plasma calcium levels in a narrow range between 2.1 and 2.6 mmol/L (ionized calcium, 1.1–1.4 mmol/L). Elevated plasma calcium suppresses PTH secretion but additional increases in calcium levels will not suppress PTH further. A low PTH leads to reduced activity of 1-α hydroxylase in the proximal tubule of the kidney and consequently reduced synthesis of $1,25(OH)_2D$ and reduced renal tubular calcium reabsorption. Low $1,25(OH)_2D$ results in reduced intestinal calcium absorption. Reduced osteoclastic activity from low PTH and $1,25(OH)_2D$ leads to reduced mobilization of calcium and phosphate from bone (Davies and Shaw, 2012). In the setting of hypercalcemia in individuals with normal counter-regulatory systems these changes result in a lowering of the serum calcium level.

Clinical Assessment of Hypercalcemia

Hypercalcemia may be found as a coincidental finding during investigation for an unrelated disorder and with no associated clinical features. This is common in infants in whom 60% of cases of mild hypercalcemia resolve spontaneously. However, persistence of hypercalcemia, hypercalciuria, or nephrocalcinosis occurs in 30% (Koltin *et al.*, 2011).

Symptomatic hypercalcemia has variable clinical features and there may be an insidious onset. Features include lethargy, hypotonia, anorexia, weight loss or failure to thrive, polydipsia, polyuria, vomiting, bone pain, constipation and abdominal pain. Polyuria results from a renal concentrating defect secondary to a tubular interstitial injury from calcium deposits in the medulla and down-regulation of aquaporin-2 water channels (calcium-induced renal AVP-resistance). In severe cases, renal failure, pancreatitis, and reduced consciousness may occur.

The clinical assessment should focus on features of malignancy, drug history including complementary alternative medicines, family history (hypercalcemia, renal stone formation, parathyroidectomy, tumors, multiple endocrine neoplasia). The examination should evaluate the degree of hydration, signs of malignancy, syndromic features, bone pain, fractures, growth and presence of subcutaneous calcification.

Investigations of Hypercalcemia

Prompt establishment of the underlying cause for the hypercalcemia is a key management step as this will influence subsequent investigations and treatment options. Investigations should be taken at the time of hypercalcemia to ascertain whether there is a PTH-dependent or PTH-independent cause (**Table 1**) (Hind *et al.*, 2007). Erroneous omission of investigations at this time is common, especially PTH and 25-hydroxyvitamin D [25(OH)D] measurements (Koltin *et al.*, 2011; McNeilly *et al.*, 2016). Adding $1,25(OH)_2D$ and CaSR mutation analysis to the initial investigations may increase diagnostic yield (Koltin *et al.*, 2011).

PTH-Dependent Causes of Hypercalcemia

PTH-dependent causes of hypercalcemia are usually associated with an elevated PTH (**Table 1**). Occasionally early onset primary hyperparathyroidism (PHPT) may be associated with a normal PTH.

Primary Hyperparathyroidism

PHPT during childhood is rare with an incidence of 2–5 per 100,000 (compared with 1 in 1000 in adults). There is an equal sex distribution (Roizen and Levine, 2012) with presentation most often during adolescence (Mallet, 2008). PHPT is characterized by autonomous secretion of PTH, which may occur secondary to parathyroid hyperplasia, adenoma or, rarely, carcinoma. Genetic abnormalities are more common with childhood presentations (Starker *et al.*, 2012). At initial presentation, PHPT may be isolated or occur in association with other disease. It is important to recognize that multiple endocrine neoplasia (MEN) type 1 and hyperparathyroid jaw-tumor syndrome may have no other manifestations at the time of diagnosis of PHPT in children.

Children are usually symptomatic at presentation of PHPT. Only 15% of cases are asymptomatic and detected coincidentally (Roizen and Levine, 2012). The non-specific symptoms often result in a delayed diagnosis and end-organ damage is common at presentation. Skeletal and renal complications occur in 75% and 45%, respectively (Roizen and Levine, 2012). Renal complications include nephrolithiasis, nephrocalcinosis and renal failure. Skeletal findings include sub-periosteal resorption, osteopenia, slipper upper femoral epiphysis, pathological fractures and osteolytic cysts (Brown's tumors), which resolve following parathyroidectomy. Bone pain, arthropathy and muscle aches may induce gait abnormalities. Calcium deposits in the eye (band keratopathy) may be observed at diagnosis.

The biochemical features of PHPT include an elevated or inappropriately normal level of PTH in the setting of a high-normal or elevated serum calcium concentration. Urine calcium concentrations are usually high but may be low if there is associated vitamin D deficiency. Accompanying hypophosphatemia is usual (Mallet, 2008). In very rare cases of parathyroid adenoma, elevated levels of an aberrant PTH molecule may not be detected by the standard intact PTH assays (Benaderet *et al.*, 2011). In the early stages of PHPT, there may be asymptomatic hypercalcemia with a PTH at the upper-end of the normal range. These biochemical features

Table 1 Investigation of hypercalcaemia in childhood

First line (at the time of hypercalcaemia)	
PTH	25-Hydroxyvitamin D
Calcium	Bicarbonate
Phosphate	Chloride
Alkaline phosphatase	Magnesium
Electrolytes	Urine calcium: creatinine ratio
Creatinine	Renal ultrasound scan
Second line (guided by initial results)	
$1,25(OH)_2D$	Serum ACE
PTHrP	DNA for genetic analysis
Skeletal survey	Parathyroid gland: ultrasound scan, SestaMIBI scan
Investigate parents: calcium, phosphate, PTH, 25-hydroxyvitamin D, urine calcium: creatinine ratio	

may be indistinguishable from familial hypocalciuric hypercalcemia (FHH) and genetic investigation may be helpful to avoid unnecessary parathyroidectomy. Magnesium levels may be helpful to distinguish these conditions as levels may be elevated in FHH, but are normal in PHPT.

Preoperative assessment of parathyroid adenomas is required either by use of Doppler ultrasound and/or 99 m Tc-SestaMIBI scintigraphy. A SestaMIBI is scan is more helpful for investigation of a single adenoma rather than more generalized hyperplasia and identifies approximately 60%–80% of parathyroid adenomas. Single-photon emission computed tomography may increase the diagnostic yield for small parathyroid adenomas or ectopic adenomas (Roizen and Levine, 2012). Parathyroidectomy is usually required as treatment for PHPT (see later).

Specific Causes of Primary Hyperparathyroidism

Familial isolated primary hyperparathyroidism

In a child presenting with apparently isolated PHPT and a family history of PHPT, MEN types I, IIa and IV as well as hyperparathyroid jaw-tumor syndrome should be considered in the differential diagnosis. Familial isolated primary hyperparathyroidism (FIPH) may also be associated with abnormalities at the chromosome locus 2p14-p13.3. Mutations in the PTH gene, PRAD1, may cause some sporadic cases (Hemmer *et al.*, 2001). These genes are thought to be oncogenes or tumor suppressor genes. In sporadic cases, a "single hit" mutation affecting a proto-oncogene such as PRAD1 can result in preferential growth of a single cell line. Thus familial forms of the condition are autosomal dominant. In the familial syndromes, a germline "first hit" mutation affects a tumor suppressor gene and makes the parathyroid (and other) glands susceptible to a "second hit" (Bastepe *et al.*, 2003). Tumors that arise in familial hyperparathyroidism are usually the result of hyperplasia whilst those occurring in sporadic cases are adenomas. However these can be multiple and it is sometimes difficult to distinguish between the two.

Multiple endocrine neoplasia

MEN types I, IIa, and IV are all associated with PHPT. MEN type 1 is an autosomal dominant condition caused by inactivating mutations of the MEN1 gene, located on chromosome 11q13. This gene encodes the tumor suppressor protein MENIN. PHPT is usually the first clinical manifestation of MEN type 1, with 75% of cases developing PHPT before 21 years of age (Goudet *et al.*, 2015). Ninety percent of cases of PHPT secondary to MEN type 1 occur after 10 years of age, but it has been reported below the age of 6 years. Most cases are now discovered during biochemical surveillance with $<20\%$ presenting with symptomatic hypercalcemia (Goudet *et al.*, 2015). Genetic counseling should be offered if a MEN1 gene mutation is detected.

MEN type IIa results from mutations in the *c-ret* proto-oncogene located on chromosome 10cen-10q11.2. Parathyroid tumors are observed in 20% of cases. MEN type IV is caused by mutations in the CDKN1B gene and PHPT is typically observed in adulthood.

Hyperparathyroid jaw-tumor syndrome

Hyperparathyroid Jaw-Tumor (HPT-JT) syndrome is an autosomal dominant condition caused by activating germline mutations in the CDC73 (formally HRPT2) gene. It is associated with parathyroid tumors causing PHPT. The phenotype observed in HPT-JT is highly variable due to incomplete gene penetrance, and ranges from apparent sporadic parathyroid carcinoma, FIPH with or without parathyroid cancer to full expression of HPT-JT syndrome. Although jaw tumors feature in the name of the condition, less than half of affected individuals have this symptom. Other tumors are commonly seen including both benign and malignant uterine and renal tumors, and regular surveillance should be undertaken in affected individuals.

Parathyroid carcinoma

Although parathyroid carcinoma typically presents in the fifth decade it has rarely been observed in children (Shane, 2001). Most cases are associated with mutations in the CDC73 gene and can occur in the same families as those with parathyroid adenomas in HPT-JT syndrome. Parathyroid carcinoma is difficult to distinguish from parathyroid adenoma both clinically and histologically, but carcinoma tends to present with more aggressive hypercalcemia.

Tertiary hyperparathyroidism

Following a prolonged hypocalcemic stimulus causing sustained release of PTH, autonomous functioning of the parathyroid gland can occur. This is termed tertiary hyperparathyroidism. Chronic renal failure is the commonest cause and parathyroidectomy may be necessary. Treatment of any hypovitaminosis D may also help to lower PTH levels and avoid the need for surgery.

Tertiary hyperparathyroidism may also occur with chronic secondary hyperparathyroidism associated with vitamin D deficiency. In this scenario the plasma calcium may remain normal in the presence of severe rickets until treatment with vitamin D is commenced, at which stage hypercalcemia becomes manifest.

Neonatal severe hyperparathyroidism

Neonatal severe hyperparathyroidism (NSHPT) is caused by a homozygous inactivating mutation of the CaSR. Newborns are typically critically ill at presentation. Clinical features include hypotonia, thoracic deformities, feeding difficulties and respiratory distress (Mallet, 2008). Skeletal complications are severe and include multiple fractures, demineralisation, metaphyseal

irregularities, cortical dualisation, sub-periosteal erosion and bell-shaped thoracic deformation. Biochemically, severe hypercalcemia with very high PTH levels are observed. Measurement of PTH and calcium in both parents will help confirm the diagnosis in advance of CaSR analysis as both parents will usually have FHH. Milder phenotypes associated with homozygous CaSR mutations have been described in adults with normal renal function (Chikatsu *et al.*, 1999).

Preoperative medical management of hypercalcemia is directed at stabilization prior to surgery and includes hyperhydration with diuretics, bisphosphonate administration and a calcium-poor diet. Vitamin D deficiency will further aggravate the elevated PTH levels and should be treated. A trial of cinacalcet may be merited as a successful reduction in PTH and calcium has been observed (Wilhelm-Bals *et al.*, 2012), although not all cases are responsive. It is possible that in vitro studies will be used in the future to predict the sensitivity of specific CaSR mutations to cinacalcet (Mayr *et al.*, 2016).

The definitive treatment for neonatal cases is a total parathyroidectomy (Mallet, 2008; Ghirri *et al.*, 1999; Rodd and Goodyer, 1999). Postoperatively these infants may have a "hungry bone" syndrome and require large quantities of calcium to avoid hypocalcemia until the bones have healed sufficiently. Recurrence of hyperparathyroidism has been observed when total parathyroidectomy was combined with auto-transplantation. Following surgery treatment is with lifelong calcium and active vitamin D. The aim is to avoid symptoms of hypocalcemia but whether the therapeutic goal should be to keep calcium levels within or above the normal range is unknown as long-term outcome data are unavailable.

Neonatal hyperparathyroidism

Neonatal hyperparathyroidism is a distinct condition from NSHPT and is most commonly caused by a heterozygous CaSR mutation. It is unclear why some heterozygous CaSR mutations cause neonatal hyperparathyroidism and others cause FHH (Glaudo *et al.*, 2016). A case of neonatal hyperparathyroidism associated with parathyroid hyperplasia and hypercalciuria but without a mutation in the CaSR has been reported. In this case, a mutation in SLC12A1, the cause of antenatal Bartter syndrome type 1, was identified (Li *et al.*, 2016).

Presentation of neonatal hyperparathyroidism is usually with symptomatic hypercalcemia in the neonatal period, but it may present later during investigations for developmental delay. There is moderate to severe symptomatic hypercalcemia, elevated PTH, parathyroid bone disease (including demineralisation, rib cage instability), and respiratory distress (Toke *et al.*, 2007; Reh *et al.*, 2011). Hypercalcemia may spontaneously improve over time without the need for surgery and the phenotype subsequently resembling FHH. Early analysis of the CaSR is helpful to differentiate the condition from neonatal severe hyperparathyroidism where surgery is invariably required.

Treatment should initially be aimed at managing the hypercalcemia, which might avoid the need for surgery. Cinacalcet may be a useful temporizing measure to reduce PTH levels until the hyperparathyroidism resolves. Patients treated in this way have demonstrated good catch up growth and development (Reh *et al.*, 2011; Fisher *et al.*, 2015).

Gestational maternal hypocalcemia

Maternal hypocalcemia should be considered in the differential diagnosis for infants with persistent hypercalcemia. Investigation of neonatal hypercalcemia should include a measurement of plasma calcium in both parents. Chronic maternal hypocalcemia (from undiagnosed or under-treated hypoparathyroidism or pseudohypoparathyroidism) may cause secondary fetal hyperparathyroidism from reduced materno-fetal calcium transfer in order to maintain adequate plasma calcium levels in utero. The hypercalcemia may take several weeks to resolve but does not require surgical intervention (**Table 2**).

Table 2 Causes of PTH-dependent hypercalcaemia in children

- Neonatal causes
  - Neonatal severe hyperparathyroidism
  - Neonatal hyperparathyroidism
  - Mucolipidosis type II (I-cell disease)
  - Gestational maternal hypocalcaemia
- Primary hyperparathyroidism
  - Parathyroid adenoma
  - Parathyroid hyperplasia
  - Parathyroid carcinoma
- Specific causes of primary hyperparathyroidism
  - Multiple endocrine neoplasia types I, IIa, IV
  - Hyperparathyroid jaw-tumor syndrome
  - Familial isolated primary hyperparathyroidism
- Other causes
  - Tertiary hyperparathyroidism
  - Prior radiation to neck

PTH-Independent Hypercalcemia

The causes of PTH-independent hypercalcemia are diverse (Table 3). These conditions cause hypercalcemia associated with a suppressed, low or inappropriately normal PTH level. Selected examples of PTH-independent hypercalcemia are discussed below.

PTH-independent Hypercalcemia Associated With Normal (Unsuppressed) PTH Levels

Hypercalcemia associated with normal or unsuppressed PTH levels suggests a defect in the CaSR.

Familial hypocalciuric hypercalcemia

Familial hypocalciuric hypercalcemia (FHH) is an autosomal dominant disorder. Most affected individuals are asymptomatic and do not require treatment although rarely recurrent pancreatitis and chondrocalcinosis have been described in adults.

Table 3 Causes of PTH-independent hypercalcaemia

Normal (unsuppressed) PTH levels
- Familial hypocalciuric hypercalcaemia types I, II, III
- Early onset primary hyperparathyroidism

Low or suppressed PTH levels
- Malignancy
  - Acute lymphoblastic leukemia
  - Acute myeloid leukemia
  - Lymphoma
  - Ovarian dysgerminoma
  - Medulloblastoma
  - Hepatoblastoma
  - Rhabdomyosarcoma
  - Hepatic sarcoma
- Drug-induced
  - Vitamin D intoxication
  - Vitamin A intoxication
  - Thiazides
  - 13-*Cis*-retinoic acid
  - Inadequate phosphate supplementation in parenteral nutrition
  - Following discontinuation of denosumab
- Acute immobilization
- Genetic
  - Williams–Beuren syndrome
  - Jansen's metaphyseal chondrodysplasia
  - Down syndrome
  - Hypophosphatasia
  - IMAGe syndrome
- Idiopathic infantile hypercalcaemia of infancy
  - CYP24A1 mutation
- Granulomatous disease
  - Subcutaneous fat necrosis
  - Tuberculosis
  - Sarcoid
- Endocrine
  - Hyperthyroidism
  - Addison disease
  - Phaeochromocytoma
  - Congenital hypothyroidism
  - Diabetic ketoacidosis
- Inborn errors of metabolism
  - Congenital lactase deficiency
  - Bartter syndrome
  - Blue diaper syndrome
  - Disaccharide intolerance
- Renal
  - Distal renal tubular acidosis
  - Multicystic renal dysplasia
  - Renal failure
- Ketotic diet

There are three types of FHH, which are classified based on the location of the genetic abnormality. FHH type 1 is secondary to a heterozygous loss of function mutation of the CaSR gene, a G protein-coupled receptor. FHH type 2 is caused by a loss of function mutation in the GNA11 gene that encodes for $G\alpha_{11}$, which is involved in calcium-receptor signaling. This mutation likely impairs GDP release (Nesbit *et al.*, 2013). FHH type 3 results from heterozygous mutations of the adaptor-related protein complex 2, sigma 1 subunit (AP2S1) gene and causes defective endocytosis and recycling of the CaSR (Fujisawa *et al.*, 2013). Two-thirds of cases of FHH are caused by CaSR gene mutations and approximately 20% from AP2S1 mutations and 10% from GNA11 mutations (Nesbit *et al.*, 2013; Fujisawa *et al.*, 2013; Hendy *et al.*, 2014). In the absence of gene mutations, autoantibodies directed at the CaSR may also cause hypocalciuric hypercalcemia (Marx *et al.*, 1978).

The biochemical features of FHH include lifelong hypercalcemia associated with inappropriately low urinary calcium excretion (80% of cases) but with a normal (unsuppressed) or slightly high PTH level (Marx *et al.*, 1978), indicative of parathyroid and renal resistance to suppression of PTH. However, hypercalciuria can occasionally occur in FHH types 1 and 3 (Fujisawa *et al.*, 2013; Hendy *et al.*, 2014). This is more common in infancy, with hypocalciuria developing over time. The serum phosphate is usually at the lower end of the normal range and there may be hypermagnesemia. The biochemical features may be indistinguishable from PHPT and investigation for genetic causes of FHH may be useful in some cases of suspected PHPT to avoid unnecessary parathyroidectomy.

The use of cinacalcet to treat symptomatic FHH is evolving, but will only be effective if the function of the mutated CaSR protein can be enhanced. Cinacalcet has been used to treat symptomatic FHH type 1 and type 3 in children and adults. The data are currently limited but with apparently good effects in the 10 out of 12 treated patients to date (Mayr *et al.*, 2016). The reasons for treatment initiation in these cases included symptomatic hypercalcemia, pancreatitis, poor wound healing, psychosis, and muscle cramps.

PTH-Independent Hypercalcemia Associated With Low or Suppressed PTH Levels

Childhood malignancy

The incidence of hypercalcemia in childhood malignancy is $<1\%$ whereas it occurs in up to 30% of adult cancer patients (Stewart, 2005; McNeilly *et al.*, 2016; Jick *et al.*, 2017). It is most commonly observed at diagnosis in children, and, unlike in adults, is not predictive of a poor prognosis. Hypercalcemia secondary to childhood malignancy is associated with a low or suppressed PTH, except in parathyroid carcinoma where PTH levels will be elevated.

The mechanism leading to hypercalcemia varies depending on the tumor type (Stewart, 2005). In acute leukemia and lymphoma an increase in osteoclastic bone resorption mediated by cytokines in areas surrounding malignant cells in the marrow space results in hypercalcemia secondary to local osteolysis (Niizuma *et al.*, 2007). Humoral hypercalcemia of malignancy is caused by direct tumor secretion of either PTH-related peptide (PTHrP) (childhood acute lymphoblastic leukemia (Shimonodan *et al.*, 2005), rhabdomyosarcoma, medulloblastoma, hepatic sarcoma and hepatoblastoma (Dharmaraj *et al.*, 2006; Lakhdir *et al.*, 1994)) or $1,25(OH)_2D$ (ovarian dysgerminoma, lymphoma). Similarly to PTH, PTHrP increases bone resorption and enhances renal retention of calcium (Hibi *et al.*, 2008). Hypercalcemia from ectopic secretion of PTH is a very rare and has been reported in association with rhabdomyosarcoma (Brooks *et al.*, 2018). 13-*Cis*-retinoic acid is used to treat neuroblastoma and hypercalcemia is a known side effect.

Williams–Beuren syndrome

Williams–Beuren syndrome (WBS) is a multisystem disorder with an incidence of 1 in 20,000 births and is caused by a microdeletion on chromosome 7, resulting in the loss of 26–28 genes. Clinical features include a characteristic facial appearance, specific neurocognitive profile and congenital heart disease, most commonly supravalvular aortic stenosis. Additionally there is an increased risk of hypercalcemia during infancy, childhood, and adolescence.

The cause of hypercalcemia is unknown although endocrine, gut and renal abnormalities have been described. Elevated levels of $1,25(OH)_2D$ have been observed in some infants and some have hypothesized increased sensitivity to normal circulating levels. Others suggest that hypercalcemia results from abnormal function of a multi-protein complex (WINAC) that interacts with the Williams syndrome transcription factor and augments recruitment of the vitamin D receptor causing increased sensitivity to vitamin D (Kitagawa *et al.*, 2003).

The lifetime prevalence of hypercalcemia in WBS is 15%, and the prevalence is highest in infants (17%) and toddlers (26%). Hypercalcemia is usually mild and requires no intervention, but approximately 6% of cases are symptomatic and require management of calcium levels. This is more frequent in younger children (5% of infants and 10% of toddlers) (Sindhar *et al.*, 2016). Those requiring intervention for hypercalcemia are more likely to have associated nephrocalcinosis and hypercalciuria. A transient shortening in the QTc interval associated with hypercalcemia has not been associated with arrhythmia, seizure or death. Screening calcium levels does not predict which individuals go on to develop actionable hypercalcemia; however calcium levels should be checked at diagnosis and symptom-based testing is appropriate thereafter (Sindhar *et al.*, 2016). In older children other medical conditions should be excluded before attributing hypercalcemia to the syndrome.

Individuals with WBS should avoid vitamin D supplementation. If hypercalcemia supervenes, a calcium-restricted diet including low calcium milk may be helpful with later re-introduction of a standard healthy diet. Hypercalcemia is amenable to standard treatments if necessary.

Jansen's metaphyseal chondrodysplasia

Jansen's metaphyseal chondrodysplasia is a rare skeletal dysplasia characterized by abnormal endochondral bone formation and hypercalcemia associated with low levels of PTH. Clinical features include micrognathia, arched palate, hypertelorism, waddling gait, bowed tibiae and kyphoscoliosis. Short stature becomes more apparent with increasing age with skeletal disproportion. Affected individuals typically have short limbs without significant shortening of the torso.

Hypercalcemia results from constitutive activity of PTH/PTHrP receptor in bone and is associated with hypophosphatemia and with a normal or undetectable serum PTH or PTHrP. Biochemical abnormalities are usually apparent by 3 months of age and following completion of linear growth calcium levels typically fall but still remain elevated (Schipani *et al.*, 1999). Overt hypercalcemia is not always observed, and the same mutation may cause severe hypercalcemia in some affected individuals but not others within the same family (Nampoothiri *et al.*, 2016). Bisphosphonates have been used in adults to reduce the rate of bone turnover and control hypercalciuria (Onuchic *et al.*, 2012).

Idiopathic infantile hypercalcemia

Idiopathic infantile hypercalcemia (IIH) presents with hypercalcemia in the first year of life, typically after 6 months of age. Non-progressive subclinical nephrocalcinosis may occur and remain persistent into childhood (Huang *et al.*, 2006). Behavioral problems and deficits in performance IQ have been reported in children with IIH (Udwin *et al.*, 1986).

Recessive and dominant mutations in the 24-hydroxylase gene (CYP24A1) have been identified as the cause for some cases of IIH (**Fig. 1**). 1,25$(OH)_2$D, is normally inactivated by CYP24A1, which also promotes the metabolism of 25(OH)D to the inactive metabolite 24,25-dihydroxyvitamin D3, thus reducing stores of 25(OH)D (**Fig. 1**). The activity of CYP24A1 is regulated by 1,25$(OH)_2$D, serum calcium and PTH. Inactivating mutations of CYP24A1 reduce the degradation of 1,25$(OH)_2$D resulting in symptomatic hypercalcemia, especially in those given vitamin D supplements. The PTH is suppressed and serum 1,25$(OH)_2$D and 25(OH)D may be elevated or normal (Pronicka *et al.*, 1997). 24,25-Dihydroxyvitamin D [24,25$(OH)_2$D] levels may be low or undetectable. Typically hypercalcemia in IIH resolves over time. Homozygous CYP24A1 knockout mice have decreased expression of CYP27B1, which encodes the 1-α hydroxylase enzyme in the kidney (Masuda *et al.*, 2005) and long-term compensatory down-regulation may explain the improvement in hypercalcemia over time.

Milder phenotypes may occur with biallelic CYP24A1 mutations, either homozygous or compound heterozygous, with chronic mild hypercalcemia or normocalcemia associated with low or normal PTH levels, hypercalciuria, renal stones, nephrocalcinosis and absence of hypophosphatemia (Schlingmann *et al.*, 2011; Tebben *et al.*, 2012; Jacobs *et al.*, 2014; Carpenter, 2017). Serum 1,25$(OH)_2$D may be elevated in some cases. Circulating 24,25$(OH)_2$D is low and the molar ratio of circulating 25(OH)D: 24,25$(OH)_2$D may be a good indicator of CYP24A1 function (Kaufmann *et al.*, 2014).

Treatment of IIH includes dietary calcium and vitamin D restriction and reducing cutaneous vitamin D synthesis using clothing and sunscreen. Ketoconazole and fluconazole are inhibitors of CYP27B1 and have been used to reduce 1,25$(OH)_2$D synthesis (Tebben *et al.*, 2012; Sayers *et al.*, 2015), but the risk of serious hepatotoxicity limits the long-term use of these agents. Rifampicin, which induces CYP3A4, an alternative vitamin D metabolite degradation pathway, has also been used to reduce serum 1,25$(OH)_2$D and normalize serum and urinary calcium concentrations in two patients with IIH (Hawkes *et al.*, 2017). The safety of long-term usage however needs to be established, but as a number of foods (e.g., starfruit, pomegranate, white grapefruit) and medications (e.g., macrolide antibiotics, fluoxetine, verapamil, steroids) are known to inhibit CYP3A4, avoidance of these in patients with CYP24A1 mutations should be recommended.

Renal failure

Both acute and chronic renal failure can cause hypercalcemia, but the mechanisms by which this occurs differ. The oliguric phase of acute renal failure (ARF), particularly when associated with rhabdomyolysis, is often associated with hypocalcemia.

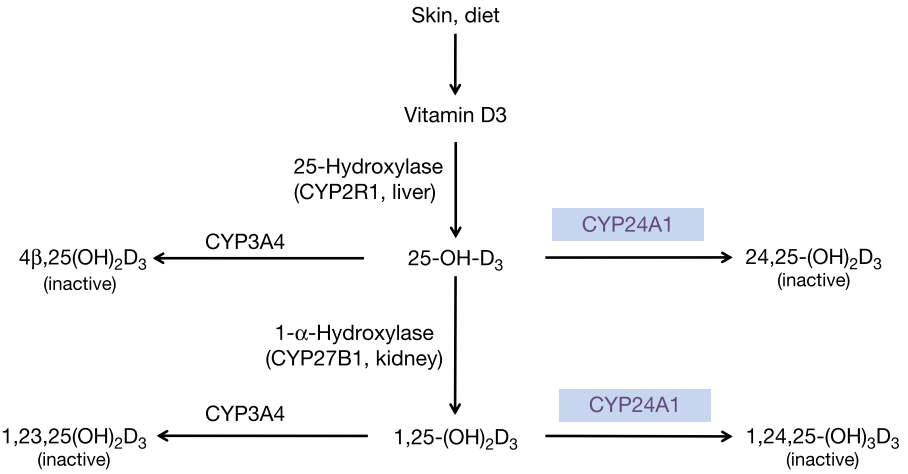

Fig. 1 Activation and inactivation pathways of vitamin D.

Hypercalcemia typically occurs later during recovery of renal function and the ensuing diuresis. A number of mechanisms have been proposed for this: firstly, during the hypocalcemic phase of ARF, skeletal resistance to PTH occurs resulting in deposition of calcium in soft tissue. Mobilization of these calcium deposits can occur as the ARF resolves. Secondly, in ARF secondary to rhabdomyolysis, both serum 25(OH)D and $1,25(OH)_2D$ are elevated during the diuretic phase of recovery. It is postulated that 25(OH)D is released from damaged muscle, thereby increasing substrate availability for conversion to $1,25(OH)_2D$ by renal 1α-hydroxylase as renal function improves (Akmal *et al.*, 1986).

Hypercalcemia occurs in up to 20% of patients with chronic renal failure, and persistence of hyperparathyroidism following renal transplantation is common. Several mechanisms lead to hypercalcemia. Secondary hyperparathyroidism occurs in response to hyperphosphatemia, hypocalcemia and reduced $1,25(OH)_2D$ levels. In some patients, despite treatment of secondary hyperparathyroidism with phosphate restriction, calcium binders and calcitriol, the PTH levels remain elevated and results in tertiary hyperparathyroidism. These patients have increased parathyroid gland mass from hyperplasia and proliferation of parathyroid cells may lead to somatic mutations and monoclonal expansion of the type seen in parathyroid adenomas. Slow involution of the hyperplastic parathyroid glands following transplantation results in hypercalcemia, which can persist for several years. Hypervitaminosis A, which is commonly seen even in early chronic kidney disease and particularly in children on supplemental feeds, has also been associated with hypercalcemia (Manickavasagar *et al.*, 2015).

Immobilization hypercalcemia

Acute immobilization, for example, head injuries, fractures or spinal cord injuries, is often associated with hypercalcemia. Serum calcium, hypercalciuria, and the incidence of hypercalcemia increase with the duration of immobilization (Yusuf *et al.*, 2013), and whilst commonly asymptomatic, typical symptoms of hypercalcemia have also been reported. Uncoupling of bone remodeling with a reduction in osteoblastic activity and increased osteoclastic activity occurs during immobilization, and thus is more commonly associated with hypercalcemia in children than adults due to the higher bone turnover during growth. Calcium and phosphate release from the skeleton can result in disuse osteoporosis, and fractures and renal stone formation may also occur. Serum PTH and $1,25(OH)_2D$ levels are suppressed and hypercalcemia and hypercalciuria typically resolve with the onset of weight bearing.

Drug-induced hypercalcemia

Vitamin D intoxication

Vitamin D intoxication can occur secondary to excessive milk fortification, prescribing or manufacturing errors of vitamin D formulations, or due to accidental overdosing or overzealous usage of either prescribed or self-administered vitamin D supplements (Kara *et al.*, 2014; Vanstone *et al.*, 2012; Vogiatzi *et al.*, 2014). Supraphysiological doses of vitamin D result in hypercalcemia as a result of both increased intestinal calcium absorption and enhanced bone resorption due to increased osteoclastic activity. It is postulated that at very high serum levels, 25(OH)D is also able to act on the vitamin D receptor, in addition to being converted to the active $1,25(OH)_2D$ metabolite (Jones, 2008). Hypercalciuria and nephrocalcinosis occurs in up to 25% of cases, and vitamin D intoxication should be considered in the differential diagnosis when these features are present (Vogiatzi *et al.*, 2014). In severe cases life-threatening dehydration and cardiac arrhythmias may occur (Nimesh *et al.*, 2015).

The dose of vitamin D required to cause intoxication is large, but highly variable, ranging from 240,000 to 4,500,000 (or 40,000 to 560,000 IU/kg) and resulting in serum 25OHD concentrations typically >625 nmol/L. Genetic variation in genes involved in the vitamin D metabolism pathway, including CYP24A1 (Schlingmann *et al.*, 2011), may alter an individual's predisposition to vitamin D intoxication and account for some of this variability.

Vitamin D_2 and vitamin D_3 preparations can both cause vitamin D toxicity. Importantly, 25(OH)D has a half-life of 2–3 weeks and its lipophilic nature allows for storage in adipose tissue. As such, vitamin D intoxication may take several weeks to resolve and require prolonged treatment, although is typically amenable to standard therapies for hypercalcemia. In contrast, hypercalcemia secondary to ingestion of short acting vitamin D analogues (alfacalcidol, calcitriol) tend to cause less severe intoxication and of shorter duration due to the shorter half-life.

Others drugs

Vitamin A toxicity has been associated with hypercalcemia (Kimmoun *et al.*, 2008; Nagasawa and Okawa, 1994). Vitamin A analogues such as *cis*-retinoic acid are used as a treatment for acne and other retinoic acids are used in the treatment of certain malignancies. The mechanism of hypercalcemia is unclear but vitamin A has direct effects to stimulate bone resorption. Thiazide diuretics may cause hypercalcemia by increasing renal tubular reabsorption of calcium. This is particularly evident in individuals with PHPT.

Granulomatous disorders

Subcutaneous fat necrosis

Subcutaneous fat necrosis of the newborn (SCFN) is a transient panniculitis characterized by hard indurated violaceous nodules and plaques with ill-defined overlying erythema on the trunk, arms, buttocks, thighs and cheeks. It typically presents within the first 6 weeks of life, and the majority of infants with SCFN have a history of perinatal stress and/or maternal morbidity, such as diabetes (gestational or pre-existing diabetes mellitus) (Del Pozzo-Magana and Ho, 2016). A history of treatment with therapeutic hypothermia is also commonly reported in infants who develop SCFN (Del Pozzo-Magana and Ho, 2016).

Hypercalcemia occurs in approximately 60% of neonates with (SCFN) (Del Pozzo-Magana and Ho, 2016), and is often the presenting feature, but delayed onset of hypercalcemia up to 6 months after onset of the skin lesions has also been reported (Norwood-Galloway *et al.*, 1987). Biopsies taken from the skin lesions have revealed copious expression of 1-α hydroxylase in the inflammatory infiltrate which is thought to result in excessive endogenous synthesis of $1,25(OH)_2D$ and the ensuing hypercalcemia (Farooque *et al.*, 2009). Initial treatment is with rehydration and glucocorticoids, and pamidronate may be considered if hypercalcemia is refractory (Alos *et al.*, 2006; Lombardi *et al.*, 2009). Low-calcium formula may be required for weeks following the initial treatment to prevent rebound hypercalcemia. Hypercalcemia may be present for 1–2 years and nephrocalcinosis persists in the majority of infants with hypercalcemia seconding to SCFN, although renal function is not usually affected (Shumer *et al.*, 2014).

Other granulomatous disorders

Sarcoidosis and other granulomatous diseases such as tuberculosis can occasionally give rise to hypercalcemia. The mechanism is thought to be similar to that of SCFN.

Endocrine causes of hypercalcemia

Some endocrines diseases cause hypercalcemia (**Table 3**). Hypercalcemia may occur at the presentation of an adrenal crisis due to abnormal vitamin D metabolism from glucocorticoid deficiency and also volume depletion from mineralocorticoid deficiency. Thyrotoxicosis can cause mild hypercalcemia by T3-induced stimulation of osteoclastic bone resorption. Hypercalcemia has also been reported at presentation of diabetic ketoacidosis which is likely secondary to a combination of metabolic acidosis, hypophosphatemia, rhabdomyolysis with acute renal failure and immobilization (Makaya *et al.*, 2013).

Hypophosphatemia

Hypophosphatemia can cause hypercalcemia as low phosphate levels suppress circulating FGF23 levels with subsequent disinhibition of 1-α hydroxylase and increased synthesis of $1,25(OH)_2D$. Inadequate phosphate provision in preterm milk or parenteral nutrition may cause hypercalcemia. Increasing phosphate content to match higher calcium content in preterm parenteral nutrition may reduce the incidence of and severity of hypercalcemia (Mulla *et al.*, 2017).

Treatment of Hypercalcemia

Treatment of hypercalcemia is aimed at both lowering serum calcium concentration and correcting the underlying disease. General measures include minimizing calcium concentration in enteral and parenteral feeds and discontinuation of oral calcium supplements and drugs known to cause hypercalcemia. Weight bearing activity should be increased and, when relevant, withdrawal of sedatives to promote mobility.

Increase Urinary Calcium Excretion

Intravenous hydration is the mainstay of emergency hypercalcemia treatment. Children with severe hypercalcemia are usually dehydrated due to both decreased fluid intake and hypercalcemia-induced nephrogenic diabetes insipidus. Furthermore, hypovolemia exacerbates the hypercalcemia as it allows greater calcium resorption in the kidney.

In the kidney, filtered calcium is principally reabsorbed in the proximal tubule and the ascending loop of Henle in a passive process. Active resorption of calcium occurs in the distal loop of Henle under the influence of PTH and to a lesser degree $1,25(OH)_2D$. Proximal tubular reabsorption of calcium is inhibited by volume expansion. Intravenous saline infusion is recommended for rehydration as the increase in sodium reduces calcium reabsorption in the ascending loop of Henle. Administration of a loop diuretic, such as furosemide, similarly reduces calcium reabsorption in the ascending loop of Henle, whereas thiazide diuretics should not be used due to a hypocalciuric effect. However, caution should be taken in long term loop diuretic use as they have been associated with an increased risk of nephrocalcinosis.

Reduce PTH Secretion

In cases where excess PTH secretion is a cause of the hypercalcemia, it may be possible to reduce secretion of the hormone. Cinacalcet is a calcimimetic agent that acts to sensitize the CaSR on the parathyroid gland and thus reduces PTH synthesis and secretion. Cinacalcet has been successfully used in neonatal hyperparathyroidism to prevent the need for parathyroidectomy, and to treat hypercalcemia in NSHPT, although variability in the response to cinacalcet is observed and might reflect the residual function of the CaSR (Mayr *et al.*, 2016; Fisher *et al.*, 2015). In chronic renal failure and post-renal transplantation, cinacalcet has been shown to reverse hyperparathyroidism. Treatment of vitamin D deficiency will also help to lower PTH levels.

Adverse effects of cinacalcet include hypocalcemia, hypotension, nausea, vomiting, paresthesiae, and eye palpitations. The death of a 14-year old was associated with cinacalcet administration, although no clinical details are available. The medication is

not licensed for those under 18 years of age thus the decision to treat should be individualized with strict clinical monitoring for side effects.

Decrease Intestinal Calcium Absorption

If hypercalcemia is caused by raised levels of $1,25(OH)_2D$, glucocorticoids might be useful as they inhibit 1α-hydroxylase and thereby reduce the conversion of 25OHD to $1,25(OH)_2D$. Glucocorticoids have been used to treat hypercalcemia for granulomatous conditions such as tuberculosis, sarcoid and subcutaneous fat necrosis.

Inhibit Bone Resorption

Bisphosphonates

Bisphosphonates are extremely effective in children with moderate to severe hypercalcemia (Niizuma *et al.*, 2007). The most commonly used agent is pamidronate, which is safe to use even in patients with renal dysfunction, but there are increasing reports of zoledronate being equally effective for treating hypercalcemia associated with malignancy and neonatal SCFN (Park *et al.*, 2016; Di Bari *et al.*, 2017). Potential benefits of zoledronate include greater potency, a longer duration of action and shorter infusion time. Both pamidronate and zoledronate lead to a reduction in serum calcium 12–48 h after administration, but a sustained period of hypocalcemia requiring additional calcium supplementation can occur and warrants monitoring of calcium levels. An acute-phase reaction with flu-like symptoms is commonly seen following first exposure to intravenous bisphosphonate.

Denosumab

Denosumab is an inhibitor of receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL promotes osteoclast differentiation and activity, leading to increased bone resorption. Hence denosumab reduces bone resorption. Denosumab has been shown to be useful in the treatment of hypercalcemia of malignancy in adults but there is little data in children and rebound hypercalcemia has been observed in case reports and open-labeled studies in children, which may limit its use in the treatment of hypercalcemia (Boyce, 2017).

Calcitonin

Calcitonin reduces calcium absorption in the gut, inhibits calcium reabsorption in the kidney and reduces osteoclast activity. Synthetic calcitonin can be effective in reducing plasma calcium and has a rapid onset of action but short duration of effect. It is generally only used in an acute situation and when other treatment options have been unsuccessful.

Dialysis

In resistant, life-threatening hypercalcemia, hemodialysis against a low-calcium dialysate is more effective than peritoneal dialysis at lowering calcium levels.

Parathyroidectomy

Surgery is required for primary hyperparathyroidism and should be undertaken by an experienced pediatric endocrine surgeon. In most cases of NSHPT total parathyroidectomy is required and will render the child hypoparathyroid. The decision to operate for milder forms depends on the degree of hypercalcemia, symptoms and potential for renal damage. Pamidronate may be required preoperatively in those with severe or symptomatic hypercalcemia. Rapid peri-operative PTH measurements can be undertaken to determine whether or not parathyroidectomy has been effective. Following parathyroidectomy, replacement with maintenance calcium and vitamin D should be commenced. Immediately following surgery, hypocalcemia secondary to a "hungry bone" syndrome may develop despite maintenance treatment. Serial calcium measurements should therefore be taken following surgery and additional calcium supplementation given if appropriate. If severe hypocalcemia develops, intravenous calcium may be required.

Summary and Conclusions

Hypercalcemia is rare in childhood and requires proper assessment and diagnosis before correct treatment can be given. The spectrum of disease is rather wider than that found in adults with the emphasis less specifically on hyperparathyroidism and malignancy and encompassing a wide range of conditions, many of them genetic in origin. Once a correct diagnosis has been made, it is usually possible to offer effective treatment.

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A Review of Skeletal Dysplasias for the Pediatric Endocrinologist

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Introduction

A skeletal dysplasia is defined literally as a condition where the bones are abnormally formed. Bones can be shorter than typical, unusually shaped, more fragile or even missing. To date there have been over 400 genetic skeletal conditions described with 300 + genes known to be associated with these conditions. A nosology group regularly reviews the medical literature and provides a document that categorizes the different dysplasias. The last was published 2015 (Bonafe *et al.*, 2015). The conditions are grouped by different characteristics such as by the genes involved (i.e., *FGFR3*, *COL2A1*), radiologic findings (i.e., metaphyseal dysplasias), and clinical presentation (i.e., bone fragility syndromes). The clinical presentation can be quite variable depending upon the gene and even the specific mutation or mutations within the gene(s). Many of the lethal skeletal dysplasias will demonstrate features during the second trimester of pregnancy on a fetal ultrasound. Other dysplasias may not be diagnosed until an x-ray demonstrates an incidental finding. The majority of dysplasias present sometime in childhood due to some atypical finding, including short stature, a common reason for referral to a pediatric endocrinologist.

The term dwarf is used to describe an individual with short stature who is typically disproportionate in his or her growth parameters. Little person is also an acceptable term, although usage of such terms can vary depending upon the region. However, not all skeletal dysplasias result in short stature.

One should also consider the difference between a skeletal dysplasia and a syndromic condition where there are skeletal features. For example, Cornelia de Lange syndrome is associated with short stature and limb anomalies, but is not typically considered a type of dwarfism. However, as more and more conditions are being better characterized, and many skeletal dysplasias are associated with issues in other body systems, the distinction between the two is blurred.

How an Individual With a Skeletal Dysplasia may Present to an Endocrinologist (Table 1)

Short Stature

An endocrinologist may be the first specialist to suspect a skeletal dysplasia, commonly in a child who is referred with short stature. In one series of patients published by Flechtner *et al.* (2014), they found that a significant proportion of patients referred for idiopathic short stature and small for gestational age had mutations in *FGFR3* or *SHOX*, once genetic and syndromic diagnoses were excluded. This individual may have always had growth parameters below the curve or had measurements that are falling off the normative curves. With further measurements, disproportion may be detected. Thus accurate and historic measurements may demonstrate a pattern that is key to making a diagnosis.

Growth Plate Abnormalities Mimicking Rickets

Vitamin D deficiency remains prevalent the world over (Munns *et al.*, 2016) despite international guidelines for its prevention through vitamin D supplementation in at-risk groups and, in some countries, food fortification. As it is a treatable condition, it should always be ruled out; fortunately, the diagnosis is often suggested on the history alone. Clinically these children may present in the first few months of life with the textbook features of rachitic rosary, splaying of the distal metaphyses, hypocalcemic seizures, delayed developmental milestones, abnormal dentition, enlarged fontanelles and craniotabes (softening of the skull). They may also present later with "normal" childhood bowing of the legs not resolving in the typical manner and bone pain due to fractures. There are other pathway disruptions that also present with rickets such as hypophosphatemia, so a broad approach should be considered in the work-up. The morphology of the growth plate is similar to that of a metaphyseal dysplasia; individuals with this diagnosis are sometimes presumed to have rickets, but do not respond to vitamin D and calcium in the expected way. Children with metaphyseal dysplasias also do not have the classical biochemical features associated with vitamin D deficiency rickets. On the other hand, those with Jansen metaphyseal chondrodysplasia have biochemical perturbations including hypercalcemia and hypophosphatemia without intrinsic parathyroid gland abnormalities as the genetic etiology is a mutation in the parathyroid hormone receptor-1 gene (*PTHR1*).

Delayed Bone Age

The bone age is calculated by the maturation of the epiphyses of the left hand via radiograph. A delayed bone age in an otherwise healthy individual most likely indicates delayed physical maturation within the spectrum of normal development (constitutional

Table 1 Most common reasons or signs prompting for referral to an endocrinologist and associated diagnoses

Clinical feature	*Diagnoses*
Short stature	*FGFR3* related conditions: achondroplasia and hypochondroplasia
	Leri-Weill/*SHOX* mutations
	COL2A1 related conditions: spondyloepiphyseal dysplasia congenital
Growth plates resembling rickets	Metaphyseal dysplasias (Shwachman–Diamond, Schmid metaphyseal chondrodysplasia, cartilage-hair hypoplasia)
	Hypophosphatemic rickets (X-linked and recessive types)
Delayed bone age	Multiple epiphyseal dysplasia (*COMP, COL9A1, COL9A2, COL9A3, MATN3, SLC26A2, CANT1*)
	TRPV4 related conditions (brachyolmia, spondylometaphyseal dysplasia—Kozlowski type)
Fractures	Osteogenesis imperfecta
Abnormal bone density/ mineralization	Cleidocranial dysplasia
	Osteopetrosis (various types)
	Camurati–Engelmann
	Endosteal hyperostosis (various types)
	Hypophosphatasia

delay). One does need to rule out endocrine causes like hypothyroidism, pathologically delayed puberty and chronic illnesses. There are also a number of genetic syndromes associated with delayed bone age, which are suggested by congenital anomalies in the individual. One such example is Floating-Harbor syndrome, which is a short stature syndrome with a very characteristic facial appearance and significant expressive language impairment. However, a delayed bone age may indicate that the patient has an epiphyseal dysplasia and upon examination of the other epiphyses, these tend to be smaller, with the proximal femoral epiphyses being the most affected. There may also be an abnormal pattern in the ossification of the carpal bones that is reported as delayed bone age, but is actually a sign that the bones overall have not developed in the typical way. It is upon further x-rays that differences in other bones may be demonstrated, such as the platyspondyly seen in brachyolmia.

Fractures

When a child presents with multiple fractures, the common question that arises is whether the fractures represent nonaccidental injury or a form of osteogenesis imperfecta (OI). This is obviously a critical distinction. Often a careful history will determine which is the most likely. The *COL1A1* and *COL1A2* genes account for the majority of cases of OI, and other features such as blue sclera, Wormian bones of the skull, vertebral fractures, hyperlaxity and long bone deformity assist in the diagnostic process. However, a few patients with type I collagen mutations or the more rare OI types do not have discoloration of the sclera or Wormian bones (Bardai *et al.*, 2017), but the radiographs and nature of the factures (including the presence of vertebral fractures) may suggest that the individual has an underlying bone disorder. At present, there are close to two dozen genes implicated in congenital bone fragility, recently reviewed in articles on the management of osteoporosis in children (Ward *et al.*, 2016; Trejo and Rauch, 2016).

Differences in Bone Density

Bone mineral density studies are not commonly done as a routine investigation in children with a suspected skeletal dysplasia unless there is a history of fractures, but x-rays may suggest the individual has osteopenia or a sclerosing bone disorder. In an infant with sclerotic bones, the diagnosis could be an infantile form of osteopetrosis, which may quickly progress, resulting in cranial nerve entrapment. The ability to respond to hematopoietic stem cell transplantation (HSCT) in osteopetrosis is dependent upon the causative genetic change (Palagano *et al.*, 2018); therefore, identifying the molecular basis for the phenotype expediently, guides clinical care. Often the endocrinologist is charged with managing osteopetrorickets (treating the hypocalcemia and hypophosphatemia, a feature of malignant infantile osteopetrosis due to mutations in *TCIRG1*) (Nour and Ward, 2013) and also taking leadership on the child's coordination of care so that HSCT can be carried out as quickly as possible.

Investigating a Child With a Suspected Skeletal Dysplasia

History

The family may provide key information during the diagnostic process. Parents, when available, should always be measured to calculate mid-parental heights and to determine if one of them may have the same diagnosis as the proband. If there is any disproportion in the patient (i.e., macrocephaly, mesomelia, upper to lower extremity disproportion), this should also be assessed in both parents. Similar features in a parent may suggest that the condition is autosomal dominant, while an affected sibling with

unaffected parents suggests autosomal recessive inheritance. Advanced paternal age at conception may indicate a new dominant condition as de novo mutations are associated with a paternal age effect.

One should note the ethnicity of the family and if there is consanguinity or if the parents are from a limited geographic area. Some autosomal recessive skeletal conditions are more common in certain populations, such as cartilage – hair hypoplasia in individuals of Amish and Finnish ancestry.

Growth curves and plotting of historic measurements will provide a pattern of growth that may suggest or exclude a particular diagnosis. Those with pseudoachondroplasia have length measurements that are typically on the average curve until age 2, when there is a decrease in growth velocity (Horton *et al.*, 1982), while individuals with achondroplasia are short at the time of term delivery. Individuals with hypochondroplasia tend to be of average length at birth, and it may be only when the child starts school that short stature and disproportion are noticed. Individuals with hypochondroplasia and achondroplasia also do not appear to have a pubertal growth spurt. The differential diagnosis may be narrowed if skeletal differences were noted during third trimester ultrasounds (i.e., prenatal onset of short stature).

Physical Examination

As for any patient, a careful examination is important. Height, weight, arm span, sitting height and head circumference are standard measurements. One should use normative values appropriate for their population. Additional measurements, such as hand length, may be indicated if the size looks disproportionate to the rest of the individual. The handbook of Physical Measurements (Gripp *et al.*, 2013) is a good resource for the average curves for a number of different measurements, which can help sort out if a result is significant or not. Head circumference is often missed, but is it an important clue. Occipitofrontal circumferences (OFC) at or above the 95th percentile makes one consider an *FGFR3* condition. Short stature with an average OFC can also narrow the differential. When the head circumference is at a lower percentile in an individual with extreme short stature, this raises the possibility of a type of primordial dwarfism (Seckel syndrome or microcephalic osteodysplastic primordial dwarfism, type II).

The arm span to height ratio can indicate the type of disproportion that is present. If the arm span is significantly lower than the standing height, this suggests that the limbs are more affected than the spine. Conversely, if the arm span is much greater, then the spine could be involved. In childhood, the arm span is less than height and approaches a ratio of 1:1 around the time of puberty. It is also normal for arm span to be greater than height in certain populations, especially those of African ancestry. One also does need to take into account if a scoliosis is present when interpreting these results.

It is often difficult to measure limb segments in an active child. A quick way of determining if there is rhizo- or mesomelia of the upper limb is to flex the arm at the elbow and observe the location of the wrist crease relative to the shoulder. If the crease is at or above the shoulder, there is rhizomelia. If the crease is at the mid-shaft of the humerus, then mesomelia is present.

The hands can provide important clues in regards to a diagnosis. There is the classic trident appearance of the fingers in achondroplasia, where it is difficult for the digits to touch from base to tip. In spondyloepiphyseal dysplasia congenital (SEDC–*COL2A1*), the hands can appear unremarkable and be average in size despite marked upper to lower extremity disproportion due to the platyspondyly. The flexibility of the digits should also be noted. In cartilage – hair hypoplasia (*RMRP*), the hands are small and the joints are very hypermobile. Conversely, the digits in some of the mucopolysaccharidoses (Hunter, Hurler) lose their mobility and have a "claw" appearance. Acromicric dysplasia is characterized by small hands and resembles a storage disorder in that the digits are very stiff.

One should note the range of motion in all the joints. Typically individuals with achondroplasia are unable to fully extend their elbows. Other diagnoses may have limited movement of the hips due to positioning of the femoral head. Bony synostoses or dislocations may also limit mobility of a joint.

Some of the dwarfing conditions have a characteristic facial appearance, and this can help with making the diagnosis. The bodies of individuals with achondroplasia and pseudoachondroplasia can look very similar, but those with pseudoachondroplasia have an unremarkable facial appearance, while those with achondroplasia typically have macrocephaly with frontal bossing.

Radiographs

Plain x-rays are still the most useful diagnostic imaging test in diagnosing a skeletal dysplasia. Three dimensional reconstructions via computed tomography can be helpful in visualizing the morphology of the bones and MRI is extremely important in determining if there is nerve compression secondary to the boney anomalies, especially if there are vertebral differences. Radiologists are accustomed to reviewing x-rays and should be consulted during the diagnostic process; skeletal dysplasia textbooks are also an excellent resource (Spranger *et al.*, 2012; Lachman, 2007). One should try to obtain the films prior to bone maturation, as after the growth plates fuse important information is lost; postepiphyseal plate fusion, it is harder to note if there are epiphyseal or metaphyseal changes.

Many radiology requisitions from pediatric centers will have a tick box for a "skeletal survey." However, not all centers include the same films, so it is important to note what films are done when a box is checked. Watson *et al.* surveyed a number of specialists in skeletal dysplasias to generate a list of the minimum films that should be included (**Table 2**) (Watson *et al.*, 2015). However they recognized that for some conditions under consideration more specific films may be necessary. For example, if one suspects

Table 2 Standardized x-ray images in the diagnosis of a patient with a skeletal dysplasia

AP* and lateral of skull
Lateral of thoracolumbosacral spine
AP of chest
AP of pelvis
AP of one upper limb
AP of one lower limb
Dorsopalmar of left hand

AP*—anteroposterior.

that a child has spondyloepiphyseal dysplasia congenital, there is a high likelihood of atlantoaxial instability, so neck extension/flexion films should be carefully done. Unless there are management issues, it is not helpful to repeat the films with a frequency of less than 1 year.

Many dysplasias are categorized by which aspects of the bones are involved, the category of dysplasia (D) (Fig. 1). If the differences mostly involved the epiphyses (E), then a multiple epiphyseal dysplasia must be considered. If metaphyseal (M) flaring and irregularities are the prominent feature, then conditions like Schwachman-Diamond, cartilage-hair hypoplasia, and Schmid metaphyseal chondrodysplasia are on the differential diagnosis. There are diagnoses that affect the spine (S) primarily like brachyolmia. Also, there are conditions that combine these aspects of the bones; for example, there are different types of spondyloepimetaphyseal dysplasias (SEMDs) and spondyloepiphyseal dysplasias (SEDs).

Biochemical and Hormone Investigations

As mentioned earlier, there are a number of treatable conditions that affect bone and growth plate, and these should be ruled out if the clinical presentation indicates that they could be a possibility. If there are epiphyseal changes and/or delayed bone age, thyroid stimulating hormone (TSH) should be ordered to ensure that there is no hypothyroidism. Individuals with a multiple epiphyseal dysplasia may be of average stature, so short stature may not always be a clue. A "rickets work-up" that includes 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, parathyroid hormone, serum phosphorus, calcium, alkaline phosphatase, and urinary calcium, phosphorus, and creatinine should be performed when the x-rays predominantly show metaphyseal changes. While alkaline phosphatase is invariably high in rickets and osteomalacia, a low alkaline phosphatase for age and gender suggests another condition with radiographic features that are similar to rickets, hypophosphatasia. Hypophosphatasia can be distinguished from the radiographic features of rickets by the presence of characteristic "metaphyseal tongues," consistent with the patchy sclerosis that results from the unique mineralization defect in this condition.

Molecular Studies

Next-Generation or whole-exome sequencing (WES) technology has provided clinicians the opportunity to simultaneously test a number of possible candidate genes for a given clinical presentation. These panels can be quite broad and may include all genes associated with a bone phenotype, or may target the most common genes associated with a dysplasia. There are also, for example,

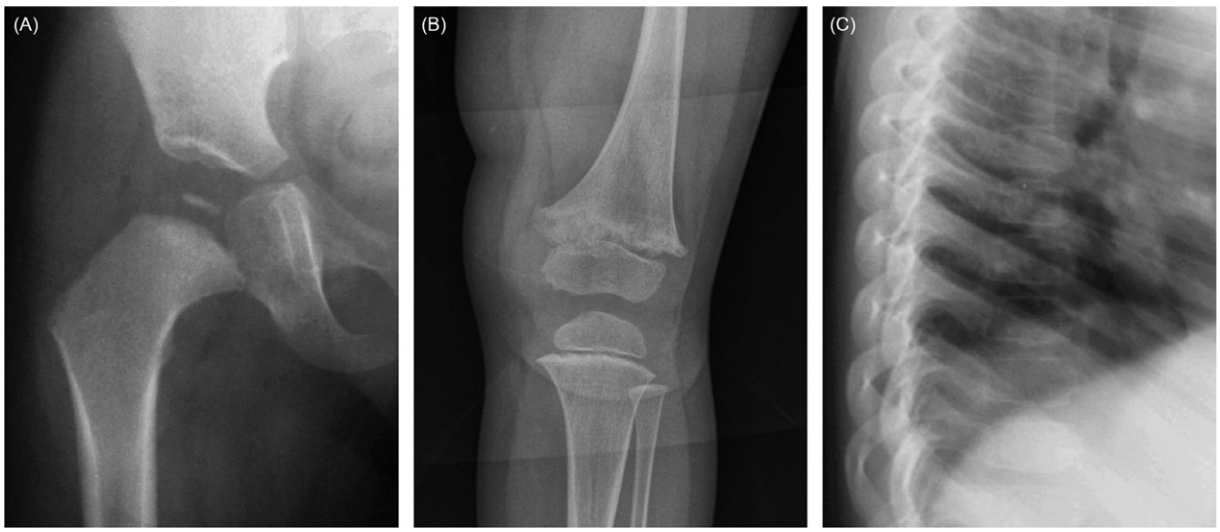

Fig. 1 (A) Epiphyseal anomaly; (B) metaphyseal anomaly; (C) spondyle anomaly.

dense bone (including osteopetrosis) and osteogenesis imperfecta panels. The quality of the panels can vary from the laboratory doing the testing, depending on the coverage of the critical portions of the gene and whether dosage studies are included. Some conditions may be due to a deletion in part of the gene, and sequencing alone will not always pick this up. The laboratory is also dependent upon the clinician ordering the appropriate investigation and providing accurate phenotype information so that the data generated can be properly interpreted. The costs of such testing have decreased over time, turning WES into one of the frontline investigations.

A clear molecular diagnosis early on in the work-up of a suspected skeletal dysplasia has multiple benefits. There are management guidelines that are tailored for particular diagnoses, the information allows for prognostication and recurrence risk counseling for families, and it should prevent the individual from undergoing investigations that are not necessary.

There are conditions where the genetic basis has yet to be elucidated, so a panel that does not reveal a pathogenic mutation does not exclude a genetic condition. To date there are a number of spondyloepiphyseal dysplasias where a causative gene has yet to be determined. For these rare conditions, collaborative sharing of phenotypes and WES data may reveal the etiology.

Putting the Information All Together

The diagnosis of a skeletal dysplasia may be simple if the individual has classic features, both on examination and radiographically, and a well-characterized gene change, such as the one that accounts for achondroplasia over 99% of the time (p. Gly380Arg in the *FGFR3* gene). However, sometimes all the pieces need to be considered, especially if a gene change has not been reported before in the literature and the laboratory categorizes it as a variant of unknown significance, or a second mutation is not found in a recessive condition. There are number of reports of ciliary chondrodysplasias, comprising of such conditions as asphyxiating thoracic dysplasia (ATD), Sensenbrenner, and Ellis–van Creveld, where only one heterozygote mutation is found (Baujat *et al.*, 2013). However, if the clinical presentation is that of shortened long bones, narrow thorax, polydactyly, and there is involvement of the kidneys, liver and retinas with a single previously reported change in the *DYNC2H1* gene, one can be confident that the likely diagnosis is that of ATD.

Management

Parents often ask how they can "fix" a child with a skeletal dysplasia. It is important to remember that these individuals are not "broken", and as care providers, the focus should be on functionality of the child. Limb lengthening does have its place, such as to correct limb length discrepancy or if it improves the ability to perform activities of daily living. The Medical Advisory Board of the Little People of America (LPA) issued a position statement in 2006, which provides a number of factors that should be considered prior to undergoing a lengthening procedure (available on the LPA website—lpaonline.org). The most important point is that the person who is considering this surgery needs to be able to understand the possible consequences and be able to consent him/herself. The statement is very clear that it does not advocate for or condemn such procedures, but encourages individuals and families to be thoughtful in their decision making and to carefully consider the risks (i.e., nerve injury/foot drop, infection, angulation, increased risk of contractions and late onset osteoarthritis, etc.) in light of the perceived benefits. It is appropriate to discuss with families that such lengthening surgeries exist, but one must also state that an improvement in height, does not decrease the likelihood of the other health issues associated with the underlying diagnosis (i.e., someone with spondyloepiphyseal dysplasia is still at risk for retinal detachment).

Most individuals with a skeletal dysplasia will need a care team, as multiple systems are often involved. Most commonly orthopedics has a role as there can be limb alignment issues, abnormal joints, or fractures. They are also the service that can provide appropriate counseling regarding limitations on physical activity and/or the type of sports to be avoided. Many individuals with a skeletal dysplasia may require surgery with careful attention to peri-operative issues such as anatomy of the airway, respiratory status, size of the patient impacting medication dosage, positioning of the individual among others; best practices to manage these issues in the skeletal dysplasia setting have been suggested (White *et al.*, 2017). Otolaryngology is often consulted in view of potential airway issues such as laryngomalacia. Both sensorineural and conductive hearing loss is common and regular audiometry is suggested (Tunkel *et al.*, 2012). Respirology is needed when the chest is small and to interpret sleep studies in regards to central versus obstructive apneas. Ophthalmologists will follow individuals with *COL2A1* mutations due to the risk of retinal detachment and those with ciliopathies for retinitis pigmentosa. Medical Geneticists typically play a role in the diagnostic process, provide counseling for the family, and in some centers provide longitudinal care. Endocrinologists are often asked to take leadership in care coordination given their expertise in discussing short stature and treating patients with fractures. The specialty to take leadership on the care coordination of children with skeletal dysplasia should be openly discussed, so that families are clear on their care pathways and contacts within the medical system.

Allied health professionals are also very important to this patient group. Physiotherapists can assist in regaining mobility after an orthopedic procedure. Occupational therapists can provide suggestions regarding adaptive devices to assist in activities of daily living like toileting. Dieticians can help the individual and family determine what are appropriate portion sizes for food. Obesity is a common problem in individuals with short stature and it is important that families provide calories tailored to the individual, especially if mobility is also an issue. Excessive weight can exacerbate mobility issues further.

Table 3 Support groups—a limited list

Little People of America and Little People of Canada
XLH Network—for those with X-linked Hypophosphatemia
Osteogenesis Imperfecta Foundation
National MSP Society—for those with a mucopolysaccharidosis
Soft Bone Society—for those with hypophosphatasia

The Role of an Endocrinologist in the Management of a Skeletal Dysplasia

Endocrinologists typically have a solid understanding of bone growth, strength and metabolism, thus it is often this group that manages conditions that affect biochemical and/or hormonal pathways. Bisphosphonates are well known to this specialty in their treatment of osteoporotic features of skeletal dysplasias. Given the benefit of intravenous bisphosphonates to pain control, muscle strength along with vertebral fracture prevention and reshaping in the osteogenesis imperfecta conditions (Dwan *et al.*, 2016; Trejo and Rauch, 2016), such treatment is often organized by the endocrinologist and has been reviewed extensively (Ward *et al.*, 2016).

The use of growth hormone (GH) is indicated where there is growth hormone deficiency as evidenced by abnormal secretory status (i.e., classic growth hormone deficiency confirmed on biochemical testing and an abnormal pituitary on MRI) or a mutation causing haploinsufficiency of the *SHOX* gene (Leri-Weill dyschondrosteosis) (Benabbad *et al.*, 2017), with *SHOX* being one of the genes implicated in the short stature of Turner syndrome. Growth hormone has been tried in other conditions where there is short stature. Short-term treatment has demonstrated that there is improvement in mean growth velocity in individuals with achondroplasia and hypochondroplasia (Tanaka *et al.*, 2003; Hertel *et al.*, 2005). The benefits to final adult height in those with achondroplasia were found to be around 3.5 cm in males and 2.8 cm in females (Harada *et al.*, 2017). However, the numbers of individuals who were recruited for these studies were relatively few and long-term information regarding the benefits and complications is lacking in this population. There are also no clear guidelines regarding the dose of GH and the timing and duration of treatment.

Individuals with skeletal dysplasias are also at risk for all the potential complications of GH use, including avascular necrosis of the capital femoral epiphysis and slipped capital femoral epiphyses. In individuals who have a dysplasia involving the epiphyses, these risks may be much greater. Scoliosis is also a potential side effect of growth hormone therapy and for conditions that have involvement of the spine, GH use may cause further complications. Taken together, GH therapy is not typically prescribed nor recommended for treatment of short stature in the context of skeletal dysplasias. Gonadal suppression has also been considered in this population in order to enlarge the window for growth; however, controlled studies have not shown benefit to increasing final height (Harada *et al.*, 2017).

We now have a much better understanding of the biological mechanism of many of the skeletal dysplasias. This has allowed researchers to proceed to the next step of trying to modify/treat dysplasias by introducing different molecules to bypass the faulty pathways. Enzymatic treatment so far has been the most successful, as conditions that are due to a flawed ongoing process are in theory easier to treat than conditions that are due to abnormalities of structural proteins that are already in abundance once the child is born. The treatment of infantile and juvenile/childhood hypophosphatasia with asfotase alfa has been shown to be life-saving in those with the severe infantile form; improved linear growth, muscle strength, and bone pain have also been observed (Whyte *et al.*, 2016a, b). Unique properties of asfotase alfa allow the enzyme to get into the affected tissues. This is the reason why the treatment of Morquio syndrome (mucopolysaccharidosis IV) with elosulfase alfa has had limited benefits, as some of the tissues involved, such as the heart valves and cartilage, are avascular (Tomatsu *et al.*, 2015). The cost of developing such treatments for rare disorders is also very expensive and unfortunately this translates to challenges in funding the high costs for providing such care to the individual with the condition. There have been a number of review papers published (Jelin *et al.*, 2017; Yap and Savarirayan, 2016) that discuss the current and emerging therapies for the skeletal dysplasias. Many of these therapies are still in the trial phase, so the utility of them has yet to be proven.

Support Groups

Support groups are a great resource for those living with and caring for individuals with a skeletal dysplasia. Many of these groups have annual meetings, but for those individuals who are unable to travel, the internet has allowed connections to occur that previously were impossible. The groups are well informed, as often there is a medical advisory group associated with them. They also provide excellent advocacy for their membership, thus clinicians should direct their patients to these societies as often as possible (**Table 3**).

Conclusions

Skeletal dysplasias are comprised of a diverse group of conditions with both skeletal and extraskeletal effects. The approach to management of each should be tailored to the individual in order to optimize function and quality of life. In some instances, the ability to define these conditions on molecular grounds has led to the discovery of novel treatments which better address the

pathobiology of the condition. Endocrinologists are often involved in the care of patients with skeletal dysplasias, either in a supportive role by providing input on aspects of skeletal dysplasia management such as short stature or osteoporosis treatment, or a leadership role including care coordination among multiple disciplines.

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Premature Adrenarche☆

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Glossary

Adrenarche Initiation of function of the reticular zone of the adrenal gland.

Congenital adrenal hyperplasia A disorder resulting from enzymatic defects in the pathway of cortisol synthesis.

CYP21A2 A gene encoding the steroidogenic enzyme 21-hydroxylase.

Gonadarche Initiation of function of the hypothalamic–pituitary–gonadal axis.

Premature pubarche Pubarche occurring prior to age 8 in girls and 9 in boys.

Definition

Premature adrenarche refers to the premature activation of the zona reticularis (ZR) of the adrenal gland, clinically manifested by pubic hair development.

Introduction

Although more than 60 years have passed since the endocrinologist F. Albright coined the term adrenarche, the mechanisms underlying the functional initiation of the reticular zone (ZR) of the adrenal gland have not yet been unraveled. While studying girls with gonadal dysgenesis, a natural experimental model of agonadism, Albright observed that pubic hair can develop under the influence of adrenal androgens and in the absence of gonadal hormones. He was thus the first to distinguish between gonadal and adrenal puberty (gonadarche and adrenarche, respectively). Subsequent studies of other clinical prototypes such as hypogonadotrophic hypogonadism, isolated premature thelarche and others confirmed Albright's observations. Dissociation between adrenal androgens and cortisol secretion has also been documented by demonstrating that a rise in dehydroepiandrosterone sulfate (DHEAS) during puberty is not accompanied by a rise in cortisol. Moreover, the rise in adrenocorticotropin hormone (ACTH) and cortisol in Cushing's syndrome occurs without an analogous increase of DHEAS (Parker Jr, 1999).

Adrenarche

Definition

Adrenarche refers to the activation of the ZR of the adrenal gland, biochemically marked by a rise in dehydroepiandrosterone (DHEA) and DHEAS but not in other androgens. The clinical manifestation of adrenarche has been assigned the eponym pubarche and is characterized by the development of pubic hair with or without axillary hair growth or apocrine odor. The decline in the function of the ZR, later in life, is termed adrenopause.

Phylogenetic Data on Adrenarche

Adrenarche and adrenopause appear to be phenomena unique to the highest order of primates and, therefore, represent a recent evolutionary development. The serum levels of DHEA, DHEAS and Δ_4–adrostenedione (Δ_4A) remain unchanged during sexual maturation in rats, hamsters, guinea pigs, sheep, pigs, goats, horses, and cows. Modest (twofold) changes of DHEA are observed in rabbits and dogs only after their sexual maturation. With regard to primates, the phenomenon of adrenarche is observed only in the chimpanzee but not in others (e.g., the rhesus monkey) (Ibanez *et al.*, 2000).

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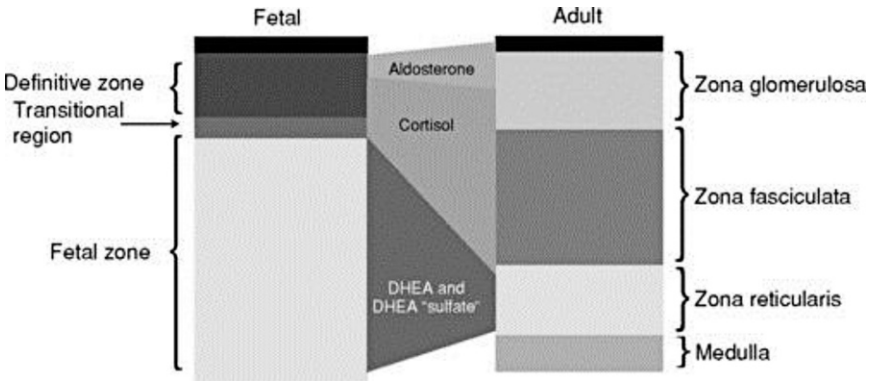

Fig. 1 The zones in fetal and adult adrenal gland.

Ontogenetic Data on Adrenarche

The adrenal gland originates from the mesodermal ridge at the fourth gestational week. Multiple transcription factors, acting at various developmental stages, direct the adrenogonadal primordium into the adrenal cortex, kidney and the bipotential gonad. The human fetal adrenal (HFA) consists of three zones; the centrally located inner zone, so called fetal zone (FZ), the outer definitive zone (DZ) and the transitional zone (TZ) lying between the FZ and DZ (Fig. 1).

During embryonic life the FZ secretes adrenal androgen precursors (AAPs) primarily DHEA and DHEAs which are used by the placenta to form estrogens. Specifically, fetal adrenal steroidogenesis begins at about 7–8 weeks post fertilization. The fetal adrenal expresses 3bHSD2 encoded by the HSD3B2 gene and produces cortisol at week 8–10. Nevertheless, HSD3B2 expression and consequently cortisol synthesis wane thereafter. Past week 12, the fetal zone is morphologically distinct, lacks 3bHSD2 but expresses CYP11A1, CYP17A1, CYB5 and the steroid sulfotransferase (SULT2A1). As a result of this enzymatic profile high levels of DHEA and DHEAS are present in the fetal circulation. The fetal adrenal also expresses 17bHSD5 which forms testosterone from androstenedione. As previously mentioned, DHEA and DHEAs are converted to estriol by the placenta.

Following birth and specifically during the first postnatal month, the FZ regresses by apoptosis and the levels of AAPs (DHEA and DHEAs) decrease and remain low until adrenarche. It seems that an important repressor function is exerted by SF1 stimulation and DAX1 during the physiological cessation of fetal adrenal and the resultant involution of the postnatal fetal cortex. It is of interest to mention that nature's spontaneously occurring knock-out paradigms (i.e., patients with specific mutations in which the fetal zone is not expected to function normally), indicate that fetal adrenal steroidogenesis may not be a sine qua non for fetal development and survival as well as for parturition. However "hidden" effects may exist, possibly important for other physiological functions.

Adrenarche could result from an increase in 17,20-lyase activity of P450c17, secondary to high levels of cytochrome *b*5 expression, in conjunction with low 3bHSD2 expression and an increase in the expression of SULT2A1 in the ZR. The steroid profile resulting from these enzymatic alterations leads to the production of adrenal androgens by the ZR. Additionally, P450 oxidoreductase (POR) serves as an obligatory electron donor for P450c17 and cytochrome *b*5 acting as an allosteric factor promoting the 17,20-lyase reaction (Bird, 2012).

Moreover, it has been shown that Kisspeptin, a protein with an important role in initiating secretion of GnRH at puberty, significantly increases DHEAS production from human fetal adrenal (HFA) cells during the second trimester of pregnancy. It has been established that the Kisspeptin receptor (KISS1R) is expressed in the adrenals (especially, in the FZ) starting from the 8th gestational week and up to term. It seems that Kisspeptin–KISS1R signaling plays an important role as a regulator of HFA development, steroidogenesis and the function of the feto-placental unit especially, during the second trimester of pregnancy (Katugampola *et al.*, 2017).

It must be underlined that the cortisol and androgen producing part of the adrenal and the adrenal medulla are related ontogenetically, anatomically, and functionally suggesting a possible link between adrenomedullary function and adrenarche (Ross and Louw, 2015).

Postnatal Development of the Reticular Zone

During the first postnatal months in humans the fetal zone regresses and almost disappears. At the age of 3 years, focal islands of the ZR are noted in the adrenals while a continuous ZR starts to develop at the age of 6, an age at which adrenal C19 steroids begin to rise. Adrenarche is initiated at about the age of 6 in girls and 7 in boys and this is reflected in a gradual rise of the adrenal androgens DHEA and DHEAS. The driving force of these evolutionary events has not been determined.

The serum levels of DHEA and DHEAS start to rise approximately 2 years prior to the gonadal activation (gonadarche) reaching their peak value at 20–25 years. Thereafter, the serum concentrations of the adrenal androgens gradually decline and in elderly people are 10%–20% of those encountered in young adults (adrenopause). The DHEAS reduction in the elderly is coupled with a decrease in the width of the ZR without an impact in the size of the rest of the adrenal cortex. The values of cortisol and aldosterone do not show analogous changes. This observation strongly indicates that the events described herein are unique to the ZR. Some of the mechanisms involved in these alterations of the ZR will be outlined herein. A histological study of adrenal samples from individuals, aged from 4 months to 56 years, showed a decrease in the enzymatic activity of 3β-HSD in the ZR, beginning at the age of 8–13 months with a further progressive decline up to the age of 25–26 years. Longitudinal clinical data obtained from subjects aged 2.9 to 12.3 years have demonstrated a progressive, age-related increase in the DHEAS values (about 22%/year) in parallel with an increased activity of 17,20 lyase and decreased activity of 3β-HSD (an enzymatic profile encountered in both the fetal and the adult reticular zones). These changes are already evident at the preadrenarcheal stage of development. It can thus be deduced from this study that adrenarche is not the result of a sudden change in the activity of adrenal enzymes, at a particular period of time; it rather reflects a gradual maturational process that begins in early childhood. When controlled for chronological age, no association was evident between weight, body mass index (BMI) and DHEAS (a marker of adrenarche). Nevertheless, changes in the nutritional status, measurable by changes in BMI, have been suggested as an important physiological regulator of adrenarche (Miller and Auchus, 2011).

Origin, Regulation, and Biological Significance of Adrenarche

Origin and regulation

The extra or intra adrenal factors involved in the development and function of the ZR, have not been fully elucidated. It has long been postulated that a distinct pituitary factor (Adrenal Androgen Stimulating Hormone, AASH), conducts the development and function of the ZR, but this factor has not been isolated.

Studies of natural human models have provided valid data with respect to the putative developmental and/or functional factors that influence the ZR. Among these we may mention the anencephalic fetuses, patients with congenital adrenal hypoplasia (ACTH receptor defect), combined pituitary hormone deficiency, hypogonadotropic hypogonadism, isolated premature thelarche and subjects with precocious gonadarche on gonadotropin releasing hormone analog (GnRHa) suppression (Palmert *et al.*, 2001). The study of adrenal androgens in these models suggests that DHEA and DHEAS synthesis and secretion are dependent upon an intact corticotropin releasing hormone (CRH)–ACTH axis, in the latter part of pregnancy as well as postnatal life. It is most probable that the CRH–ACTH complex exerts a permissive effect and acts in synergy with a putative extra-adrenal factor to successfully orchestrate the development and function of the ZR. Adrenarche could result from an increase in 17,20 lyase activity of P450c17 secondary to high levels of cytochrome b expression in conjunction with low 3bHSD2 expression and an increase of SULT2A1 in the ZR. The steroid profile resulting from these enzymatic alterations leads to the production of adrenal androgens by the ZR.

An interesting model in which low DHEAS levels have been detected, despite the normal ACTH-adrenal axis, is the pituitary insufficiency associated with Prop1 gene defects. The low DHEAS in these patients possibly indicate that the pituitary transcription factor Prop1 is necessary for the normal synthesis of the putative factor (AASH) that initiates adrenarche. Alternatively, the low DHEAS in patients with Prop1 gene defect could simply represent an early marker of incipient ACTH insufficiency, which is known to occur later in life in a number of these patients (Voutetakis *et al.*, 2001).

Besides CRH/ACTH and the putative AASH, other extra-adrenal factors have also been implicated in the development and function of the ZR: prolactin, estrogens, the epidermal growth factor, angiotensin, gonadotropins, pro-opiomelanocortin (POMC) related peptides, growth hormone (GH), and insulin growth factor 1 (IGF1), insulin, and possibly adipose tissue factors. None of these factors, however, have been conclusively shown to regulate androgen secretion by the adrenal gland (Voutilainen and Jääskeläinen, 2015).

Age-related alterations in the expression of the adrenal enzymes have also been proposed as a mechanism for the development and function of the ZR (intraadrenal factors). These changes not only refer to the relative activity of the adrenal enzymes but also to their responsiveness to ACTH. It must be stressed, however, that these biochemical changes alone cannot fully explain the initiation of adrenarche.

Biological significance of adrenarche

Since a human model of isolated absence of the ZR has not thus far been identified, the exact biological role of the ZR and the implications of its absence or insufficiency still remain enigmatic. A small, transient increase in growth rate occurring around the age of 7 years (mid-childhood spurt) has been attributed to the initiation of adrenarche. However, a cause–effect relationship between adrenarche and mid-childhood growth spurt has been disputed. It has also been shown that adrenarche is not a sine qua non for gonadarche since gonadal puberty proceeds normally in clinical entities in which adrenarche is absent.

The decline of DHEA coincides with signs of aging and has therefore been interpreted to indicate that aging is, at least in part, a DHEA deficiency syndrome. This observation has prompted studies on the effect of DHEAS replacement in the elderly and in young subjects with DHEAS deficiency of various etiologies, with equivocal results.

Premature Adrenarche–Pubarche

Definitions

Premature adrenarche (PA) and premature pubarche (PP) are frequently used interchangeably. Nevertheless, they are not synonymous. PA refers to premature activation of the ZR of the adrenals and is marked by levels of DHEA and DHEAS, which are high for the chronological age (CA) but appropriate for the stage of pubic hair development. PP is the term applied to characterize the clinical expression of PA, namely the appearance of pubic hair, usually at the labiae, with or without axillary hair growth or increased apocrine odor, in the absence of other secondary sexual characteristics, prior to age 8 in girls and 9 in boys. This age cut-off point has recently been questioned but is still accepted.

It has been shown that the prevalence of phenotypic expression of adrenarche is higher in girls than in boys whereas biochemical adrenarche is more frequent in boys than in girls (Mäntyselkä *et al.*, 2014). The risk of presenting clinical signs of adrenarche increases with higher body fat in boys and higher serum DHEAS in girls. This gender dimorphism of adrenarche might be attributed to sex-dependent differences in peripheral androgen metabolism or action that are modified by adiposity, knowing that AAPs (DHEA and DHEAS) are weak androgens and their peripheral metabolism is required for the efficient androgen receptor activation and clinical androgenic signs. Factors that have been suggested to mediate the effect of obesity in AAP synthesis include IGF1, insulin and leptin. Additionally, the conversion of AAPs to active androgens in adipose tissue may be enhanced by obesity (Williams *et al.*, 2015).

The term "exaggerated adrenarche" or amplified or pronounced has been coined to describe a form of PP in which DHEAS levels, basal or post ACTH stimulation, are above the range expected for the pubertal stage (Likitmaskul *et al.*, 1995).

Etiology of Premature Pubarche

The etiologic factors of the PA phenotype may act at multiple levels by (1) activating the maturation of AAP synthesis, (2) accelerating the peripheral conversion of AAPs to testosterone and DHT and (3) increasing the sensitivity of the androgen receptor.

Premature pubarche may be caused by: (1) premature activation of the ZR without any apparent pathological condition (idiopathic), (2) congenital adrenal hyperplasia (CAH), (3) virilizing adrenal or ovarian tumor, and (4) increased end organ sensitivity to androgens.

In idiopathic PP, increased BMI or a sudden rise in BMI may constitute a trigger factor for its induction. Nevertheless, BMI and leptin levels only partially explain the increased DHEAS values. The GH/IGF1 axis and especially hyperinsulinism, as a consequence of insulin resistance, have been implicated in androgen production by the ZR and generally in the mechanism of PP initiation.

The most frequent pathological condition underlying PP, and the one, usually creating diagnostic dilemmas, is the defective adrenal steroidogenesis and, in particular, nonclassical CAH (NC CAH). This aspect of PP has been quite controversial. Based on the determination of basal and ACTH stimulated adrenal androgen levels, defective steroidogenesis, indicative of the NC form of CAH (caused by 21-hydroxylase or 3βHSD deficiency) ranges from 0% to 54% in the various published series. These huge differences are probably due to the variety of criteria used for recruitment and evaluation of the subjects participating and, most importantly, due to lack of confirmation by molecular analysis (Witchel *et al.*, 1997). In one of our studies, 48 consecutive cases of PP were evaluated by molecular analysis of the CYP21 gene as well as by basal and ACTH stimulated 17OH progesterone (17OHP) values. A significantly increased incidence of mutations in the CYP21 gene was detected in the PP children in comparison to the general population (heterozygous 37.5% and homozygous 8.3%). The 17OHP values on the ACTH test showed an overlap between carriers and noncarriers. The application of the ROC curve in this study showed that the sum of basal plus 60 min value of 17OHP was the best indicator of heterozygosity. For children over a cut-off point of 5 ng/mL (15 nmol/L), there is a 76.5% certainty of heterozygosity for a CYP21 mutation (Dacou-Voutetakis and Dracopoulou, 1999). By using the nomogram proposed by New *et al.*, the heterozygote's values fell in the expected area, but this was also the case for the majority of normal values. A value of 17OHP 60 min post ACTH stimulation equal to or >10 ng/mL (30 nmol/L) was indicative of a homozygous mutation and occurred in 8.3% of the cases. The latter finding is accepted by most investigators. An increased incidence of CYP21 heterozygosity in either PP or functional hyperandrogenism in adolescents has also been reported by Witcell *et al.* and has been postulated by Knorr *et al.*, based on hormonal evaluation. Contrary to these findings, Potau *et al.* did not find higher incidence of CYP21 carriers in Spanish subjects with a history of PP. Based on current data, it is not possible to determine whether or not PP CYP21 heterozygote subjects have a higher probability of manifesting hyperandrogenism and the clinical syndrome related to this entity than PP girls who are not CYP21 carriers. Only long-term follow-up of such cases will provide a definitive answer to these important questions. Mutations in the 3βHSD gene have also been detected in girls with PP, though infrequently. The new hormonal criteria for the diagnosis of 3βHSD deficiency in children with PP are the following: (a) baseline 17OH pregnenolone (17P) and 17P/cortisol ratio >29 nmol/L and >103, respectively and (b) ACTH stimulated 17P and 17P/cortisol ratio >294 nmol/L and 363, respectively (Voutilainen and Jääskeläinen, 2015).

Inactivating mutations in the gene encoding 11β-hydroxysteroid dehydrogenase (CYP11B1) causing the NC form of CAH have been described in cases of premature pubarche. NC11OHD may be missed if standard steroid profiles are used (Reisch *et al.*, 2013). Moreover, apparent cortisone reductase deficiency due to inactivating mutations in the exose 6-phosphate dehydrogenase (H6PDH) gene may be the cause of ACTH-driven adrenal hyperandrogenism due to reduced peripheral conversion of cortisone to

cortisol (Lavery *et al.*, 2013) as well as apparent DHEA sulfotransferase deficiency. A rare genetic defect in adrenal androgen metabolism leading to PA is that of inactivating mutations in the PAPS53 gene encoding for a cofactor (PAPS synthase 2) which is needed for SULT2A1 enzymatic action. Hormonally this entity is characterized by high DHEA and low DHEAS levels.

Androgen producing tumors of the adrenals or ovaries are rarely a cause of PP, but they should be considered in the differential diagnosis, especially if pubarche is noted prior to the age of 3 years.

Finally, in some children with PP no androgen excess for either CA or pubertal stage is detected and the pubic hair growth is attributed to increased end organ sensitivity to androgens (Utriainen *et al.*, 2015).

Long-Term Consequences of Premature Adrenarche–Pubarche

Growth and pubertal development

Children with PP show, at presentation, an acceleration of linear growth and skeletal maturation. In some children, the difference between their bone age (BA) and CA (ΔBA-CA) can be up to 2 years, while in others the BA is comparable to CA. Long-term follow-up of children with PP has not shown impaired growth potentials: the final height (FH) attained being comparable to their target height (TH). It seems that the linear growth pattern in children with PP is modified in that height velocity in the period preceding PP diagnosis is higher than that of controls, peak height velocity occurs at an earlier age, and growth during puberty is compromised.

It has been reported that the combination of PA and obesity may be associated with significant advancement of bone age resulting in a final height less than the target height. Thus, despite reports indicating good FH prognosis, the physician must individualize the approach for each child with PP and closely watch patients with the so called "exaggerated adrenarche" and/or those who have BA advancement greater than 1 year, for the possibility of an unfavorable effect of PP on growth potentials, namely a predicted height below the TH. The age of gonadarche and menarche in children with PP is not different than in the general population (Gurnurkar *et al.*, 2014).

Functional Ovarian Hyperandrogenism (FOH) and Polycystic Ovarian Syndrome (PCOS)

In a percentage of girls with PP, functional ovarian hyperandrogenism (excessive response of androgens to GnRHa), along with an increased incidence of anovulation, hirsutism, and menstrual irregularities (characteristics of PCOS) have been reported to occur already in late adolescence. It seems that girls with an exaggerated response of 17OHP to ACTH at the time of PP diagnosis are more prone to develop FOH and/or PCOS. Raised values of AMH have been reported in girls with PA, possibly indicating advanced follicular development.

Insulin resistance, hyperinsulinism, and the metabolic syndrome

Acanthosis nigricans, decreased insulin sensitivity, and consequent hyperinsulinism and dyslipidemia have been found in a number of girls with PA. Pertinent data in boys are inconsistent. In certain studies, hyperinsulinemia is already evident at the prepubertal stage, possibly conferring a higher risk for later development of diabetes mellitus type 2 (DM 2). Contrary to the above findings, other investigators found no differences in glucose tolerance, insulin resistance indices or lipid values between PP and control girls, studied 5 years after menarche. It is quite possible that the stated differences reflect differences in the population (ethnic) groups studied and this must be seriously considered in the study and follow-up of PP girls. The contribution of intrauterine growth retardation (IUGR) to the occurrence of later adverse consequences, and especially FOH in girls with PP, is highly controversial, most authors not finding such an association (Williams *et al.*, 2015).

Does PA Represent a Pathologic Condition?

The question whether or not PP is a benign condition, simply reflecting premature activation of the ZR without long-term adverse consequences, cannot be convincingly answered at present. A number of girls with PP later present insulin resistance and hyperinsulinism, low sex hormone binding globulin (SHBG), FOH, increased hirsutism, acne or PCO like syndrome with possible later occurrence of DM 2 or the metabolic syndrome. Pertinent data in boys are not consistent (Livadas *et al.*, 2009).

At this point, no specific features at PP diagnosis have been identified to predict the child at risk for developing these pathological entities. Nevertheless, it seems that some of the late consequences are, to a large extent, restricted to certain population (ethnic) groups. Besides the ethnic group, other factors, such as obesity and a family history of DM 2, might contribute to the occurrence of adverse consequences. "Exaggerated adrenarche" as well as low SHBG (an index of insulin resistance and free androgen levels) and glucose/insulin ratio < 4.5 at the time of PP diagnosis, might confer a higher risk for the later development of FOH and/or the metabolic syndrome in children with PP (Paterson *et al.*, 2010).

Psychological implications of premature adrenarche

The impact of PA on brain function and psychology has not been adequately evaluated. However, recent reports indicate that early adrenarche associated with relatively high levels of DHEAS is associated with mental health and behavioral problems. The study of the effect of early adrenarche on affection brain function as well as on symptoms of psychopathology in late childhood has

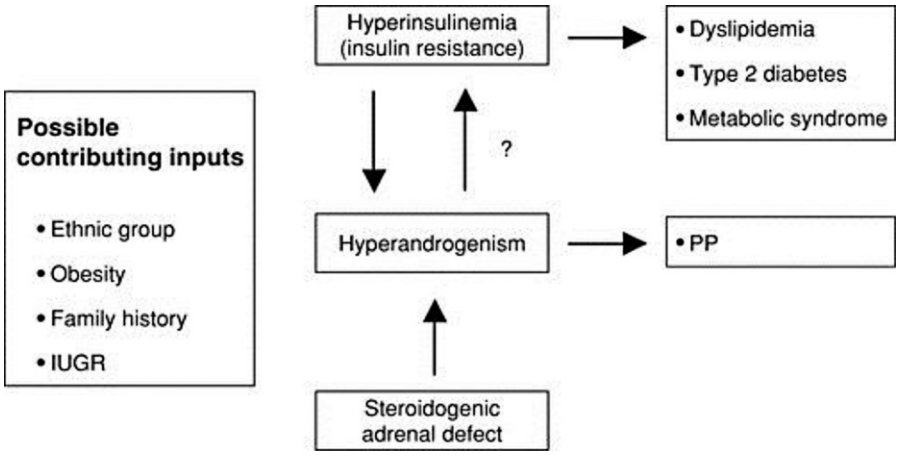

Fig. 2 Possible pathogenetic mechanisms of premature pubarche.

revealed that higher DHEA levels were associated with decreased activity of affect related brain in boys and girls. The investigators concluded that the timing of adrenarche constitutes an important moderator of affect related brain function (Whittle *et al.*, 2015). The same group using a whole-brain voxel-based morphometry analysis on MRI scans found reduced frontal white matter volume (WMV) in the left corona radiata in children with earlier adrenarche. They concluded that early adrenarche may be associated with differences in the development of frontal white matter tracks (Delany *et al.*, 2016).

Evaluation, Management, and Follow-Up of the Child With PP

Clinical evaluation

The clinical evaluation of a child with PP at presentation should include measurements of height, weight, waist circumference, BMI estimation, BA determination, and recording of relevant family history. Predicted height, TH and the stage of pubertal development should also be assessed. The presence of acne, hirsutism or virilization (clitoral enlargement) should be noted. If ΔBA-CA is less than 1 year and the predicted height falls within the height range expected from parental height (TH), the possibility of isolated premature activation of the ZR is quite high (**Fig. 2**).

Hormonal determinations

If basal values of 17OHP, $\Delta_4$androstenedione, testosterone, 11 deoxycortisol and DHEAS taken at 8 am, fall into the range expected for the pubertal stage, the diagnosis of idiopathic PP is fairly well confirmed. If basal adrenal androgen levels are higher than the levels for the corresponding pubertal stage and are accompanied by advanced BA (ΔBA-CA > 1), the main possibilities are: (a) exaggerated adrenarche, (b) defective adrenal steroidogenesis, or (c) virilizing tumor (adrenal or ovarian). In such cases an IV Synacthen test (ACTH) is carried out (250 μg or 150 μg/m^2 or 10 μg/m^2, the latter being infrequently used). Blood samples, primarily for 17OHP and 11 deoxycortisol determination, are obtained at 0 and 60 min. A 60 min value of 17OHP > 10 ng/mL strongly suggests a homozygous mutation of the CYP21 gene (NC form) and CYP21 gene analysis should be considered (**Fig. 3**). There is a 75% probability of existence of heterozygosity if the sum value of 0 and 60 min post Synacthen of 17OHP is > 5 ng/mL (15 nmol/L). Obviously, the latter information is of no immediate practical significance and may only be of value for genetic counseling. Determination of glucose/insulin value, SHBG, and ovarian sonography might constitute a good baseline record but should not be regarded as necessary diagnostic tools. It must be stated that an increased prevalence of sonographic findings of PCOS has been reported in girls with PP at the prepubertal stage, but the diagnostic and prognostic value of such a finding remains unclear. The dexamethasone suppression test or some form of imaging study is rarely indicated for the remote possibility of a virilizing tumor. This latter possibility is much higher if pubarche occurs very early (< 3 years of age) and there is an increased level of serum testosterone (Idkowiak *et al.*, 2011).

It must be underlined that steroid metabolome determination possibly constitutes a very good methodological approach in children with PA not only for the evaluation of the particular child but also for uncovering novel deficiencies in steroidogenesis (Rege and Rainey, 2012).

Management and follow-up

The therapeutic approach for children with PP will be determined by the pathogenetic mechanism involved and the clinical findings. In most cases however, drug intervention is not required. A follow-up of children with PP must be assured, especially during puberty and the immediate postpubertal years. In general, both the child and the parents should be notified of the good prognosis of most cases. Instructions concerning dietary habits and exercise should be given in cases with borderline or elevated BMI or positive family history of DM2.

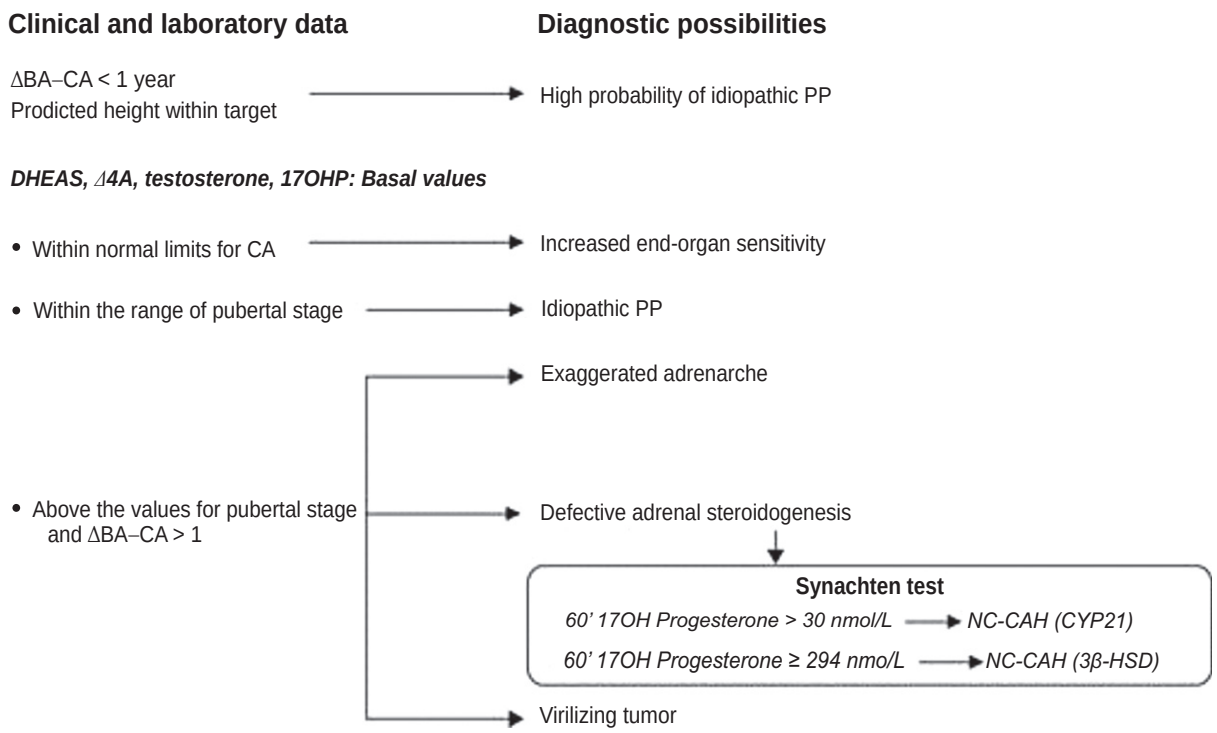

Fig. 3 Premature pubarche: diagnostic steps.

The physician should be aware of the possible long-term consequences of PP and should individualize the care and follow-up keeping in mind that PP, in certain cases, might not represent an innocent temporal deviation of sexual maturation but, possibly, an early manifestation of a complex metabolic abnormality. Fasting glucose/insulin ratio < 4.5, low SHBG values and/or exaggerated adrenarche might constitute early markers conferring an increased risk for later development of FOH, PCOS, DM 2 or the metabolic syndrome. However, it must be underlined that such recommendations are not strictly evidence based and they rather constitute extrapolation from still controversial published data. Moreover, data related to long-term consequences seem to apply to certain population (ethnic) groups. The physician in charge, being aware of the current trends concerning prognosis of PP, will determine the diagnostic studies necessary at presentation of the patient, as well as the frequency and duration of the follow-up visits.

The recent findings of an effect of PA in the neuro-psychological function should be taken into consideration and appropriate intervention must be included in the holistic management of the child, if indicated by the data of the patient under consideration.

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21-Hydroxylase Deficiency: Clinical and Biochemical Aspects

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Introduction

21-Hydroxylase deficiency is the most common form of congenital adrenal hyperplasia (CAH). It is caused by mutations in one of the enzymes, CYP21A2, involved in the synthesis of cortisol and aldosterone in the adrenal cortex. The cortisol deficiency, and subsequent loss of negative feedback, triggers increased release of CRH from the hypothalamus and ACTH from the pituitary gland. The enzymatic block results in increased levels of precursors, mainly 17-hydroxyprogesterone (17-OHP) that are shunted through alternative adrenal biosynthetic pathways and converted into adrenal androgens (El-Maouche *et al.*, 2017; Speiser and White, 2003; Merke and Bornstein, 2005) (**Fig. 1**). The androgen excess is present early on during fetal development and causes virilization of the external genitalia in fetuses with 46,XX karyotype to a varying degree (Merke and Bornstein, 2005; Parsa and New, 2017). There is a broad phenotypic variation, with a continuous spectrum of disease severity ranging from salt losing crises and risk of death in the neonatal period in the most severe forms to increased androgen production and infertility in adult age in the milder forms. Individuals, especially men, with the mildest forms may escape clinical diagnosis altogether (Parsa and New, 2017; Falhammar and Nordenstrom, 2015).

Genetics

The molecular genetics of *CYP21A2* has been studied extensively. The inheritance pattern is autosomal recessive. The gene is located on chromosome 6p21.2, in the HLA region, in tandem with a pseudogene *CYP21A2P*, which is 98% homologous with the active gene, but contains a number of inactivating mutations. This structural arrangement of the genes is the explanation for why a limited number of mutations are the most common ones all over the world. Microconversion and misalignment during meiosis and unequal crossing over can result in transfer of sequences from the pseudogene to the active *CYP21A2* gene or a deletion of the active gene. As a result, 10 pseudogene derived mutations and deletions constitutes more than 90% of all mutated alleles with some frequency variations in different populations (Wedell *et al.*, 1992; Wedell, 2011); (Krone and Arlt, 2009; Speiser *et al.*, 1992a; Krone *et al.*, 2013). In the remaining 5%–10% more than 200 rare mutations have been identified (Human Gene Mutation Database, HMG, www.hgmd.cf.ac.uk, and Human Cytochrome P450 (CYP) Allele Nomenclature Committee, http://www.cypalleles.ki.se/vyp21.htm). The limited number of mutations involved has made genotype–phenotype correlations possible. The clinical severity can be predicted from the *CYP21A2* genotype, where the mildest allele determines the severity (genotype group), with very few exceptions (**Fig. 1**). This is useful in the clinical situation, for predicting the clinical symptoms and making treatment decisions (Krone *et al.*, 2013; New *et al.*, 2013; Wedell, 2011).

Historically 21-hydroxylase deficiency has been divided into two classical and more severe forms of CAH (salt-wasting, SW, and simple virilizing, SV CAH) and a nonclassical form with no risk of salt crisis. The most severe form, the null genotype

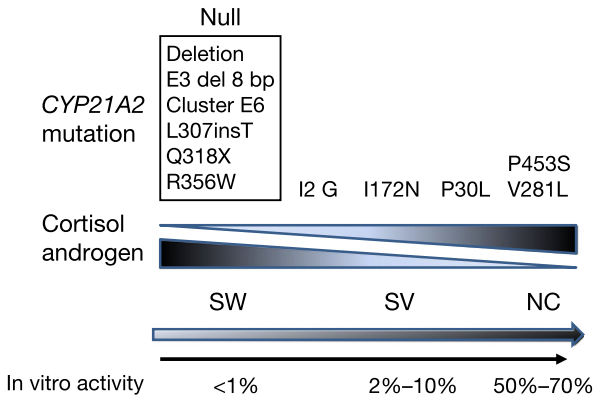

Fig. 1 The *CYP21A2* genotype groups in relation to the enzyme in vitro activity, cortisol, and androgen synthesis, and clinical phenotype are shown. The clinical phenotype can be predicted from the *CYP21A2* genotype, where the mildest allele determines the severity. The null genotype group has completely abolished enzyme activity, the lowest cortisol production and most prenatally elevated androgens.

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group, which is homozygous for mutations completely abolishing the enzyme activity and the genotype group with the I2G mutation, with less than 1% residual in vitro activity, typically present with the salt-wasting form, SW CAH. The I2G mutation shows however a variation in clinical symptoms with some patients presenting with a considerably less severe phenotype with no salt crises (Gidlof *et al.*, 2013; New *et al.*, 2013). The I172N and P30L mutations have higher residual activities and form the SV genotype group with prenatal virilization in females and about 10% risk of developing salt crisis in particularly stressful situations (Wedell, 2011; Krone and Arlt, 2009). The common nonclassical mutations, V281L, and P453S, typically lead to signs of androgen excess over time and no risk of salt loss.

About 70% of the diagnosed patients have been reported to have the classical form of the disease with a worldwide incidence ranging between 1/10,000 and 1/20,000 in most populations. The nonclassical, late-onset form has been reported to have an incidence of as high as 1/1000 in some populations (New *et al.*, 2013; Speiser *et al.*, 1992b, 2010; Witchel, 2017).

Steroid Synthesis

Cortisol and aldosterone synthesis requires 21-hydroxylase activity. In 21-hydroxylase deficiency the metabolite before the block, 17-hydroxyprogesterone (17-OHP), is markedly elevated and is used as an indicator of the disorder (**Fig. 2**). The increased levels of DHEA and androstenedione result in increased testosterone and dihydrotestosterone production. In addition, the 17-OHP can be converted to dihydrotestosterone (DHT), through an alternative route, the so-called back door pathway (**Fig. 1**), not via androstenedione and testosterone. DHT is a more potent androgen than testosterone and is required for the masculinization of external genitalia (Miller and Auchus, 2011; Turcu and Auchus, 2015a).

Aldosterone is normally present in the circulation in concentrations of about 1% of that of cortisol. Clinically relevant aldosterone deficiency is therefore only seen in severe CYP21A2 deficiency.

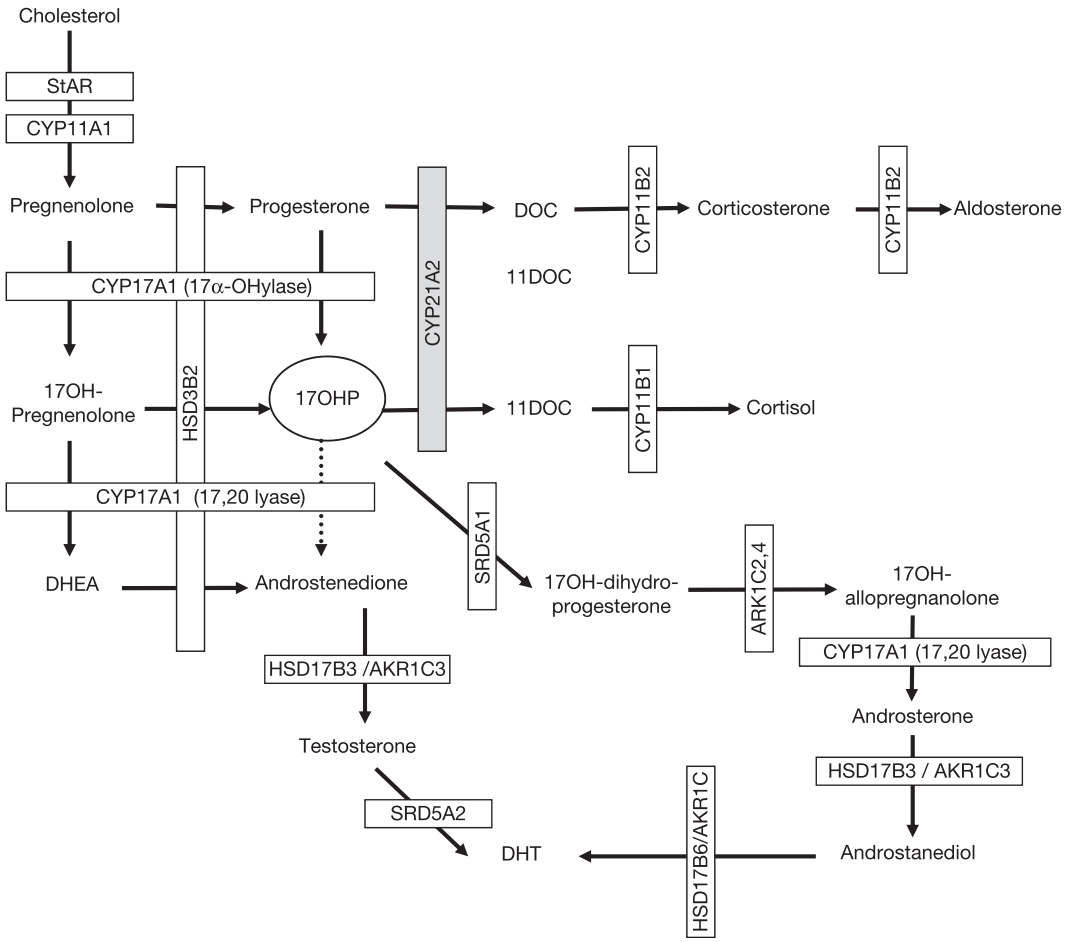

Fig. 2 The major steroid synthesis pathways are shown, including the back-door pathway leading to synthesis of DHT via androsterone. Not all intermediate steps are depicted.

Clinical Signs and Symptoms

Undiagnosed patients of both sexes with the salt losing form of CAH, will develop symptoms of failure to thrive, poor weight gain, and vomiting in the neonatal period (Speiser and White, 2003; Speiser *et al.*, 2010; Auchus *et al.*, 2010; Witchel, 2017; Merke and Bornstein, 2005). The aldosterone deficiency causes hyponatremia, hyperkalemia, acidosis and the concomitant development of circulatory collapse, hypotension, and shock. The adrenal crisis, which untreated will be lethal, typically occurs during the second or third week of life (Hindmarsh, 2009).

The excess production of androgens starts already early on during fetal life (Hindmarsh, 2009) and causes varying degrees of virilization of the external genitalia in fetuses with 46,XX karyotype and classical forms of CAH. The enlargement of the clitoris, fusion of the labia majora and formation of a urogenital sinus result in ambiguous genitalia and uncertainty of sex assignment at birth. The degree of virilization is classified according to the Prader score, (**Fig. 3**) (Prader and Gurtner, 1955). The posterior fusion of the labia majora is related to the degree of prenatal androgen exposure. It has been suggested that measurements of the anogenital distance, that is, to the scrotum or la fourchette, can be used to estimate prenatal androgen exposure, which may prove to be especially useful in unclear cases (Callegari *et al.*, 1987; Priskorn *et al.*, 2017). The increase in DHT from androsterone, via the back door pathway (**Fig. 2**), plays an important role in the prenatal virilization of the external genitalia (Kamrath *et al.*, 2012a,b) and may explain the potent androgen effects seen in *CYP21A2* deficiency. Among patients with SV CAH and the same genotype there is considerable variation in the degree of virilization ranging from uncertainty of sex assignment at birth to not being noted at birth. This variability might be explained by other modulating factors (Krone and Arlt, 2009). The results regarding differences in androgen receptor sensitivity due to variations in the length of the CAG repeat are however, conflicting (Rocha *et al.*, 2008; Welzel *et al.*, 2010).

In newborn boys the diagnosis is far more difficult since the clinical signs may not be obvious before the development of the adrenal crisis, although affected males may have increased pigmentation of the genitalia and a larger phallus (Hindmarsh, 2009). The diagnosis has to be suspected from the combination of the unspecific clinical signs of poor weight gain in the neonatal period and sodium loss preceded by increased potassium (Thil'en *et al.*, 1998).

During childhood, severe infections and stressful events may trigger an overt cortisol deficiency in undiagnosed patients with SV and NC forms of the disease. However, they typically come to diagnosis when they develop signs of excess androgen production such as acne, pseudo pubertas praecox with apocrine body odor, axillary and pubic hair, and growth acceleration (Witchel and Azziz, 2011). A thorough clinical evaluation will show advanced skeletal maturation, no signs of central puberty, low gonadotropins and no increase in testicular size in boys. In girls clitoral enlargement can be seen (Witchel and Azziz, 2011). Patients with late-onset, nonclassical CAH come to diagnosis later during childhood or adolescence or even as adults (Turcu and Auchus, 2015a; Witchel, 2017; Hindmarsh, 2009). In adolescents and adults, typical symptoms may be acne, hirsutism, oligomenorrhea or infertility. In studies on children with premature adrenarche 4%–25% were diagnosed with CAH (Armengaud *et al.*, 2009; Skordis *et al.*, 2015). On the other hand, regarding the reverse clinical question of how likely it is that a person with NC CAH would be diagnosed due to androgen excess, studies have shown that in 92% of the children diagnosed before age 10 and 8% in the age group 10–19 years this was their presenting symptom (Moran *et al.*, 2000; Carmina *et al.*, 2017) (**Table 1**).

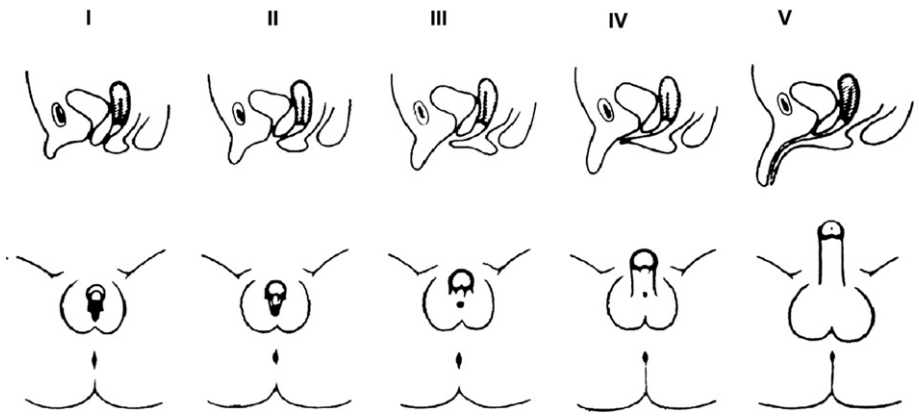

Fig. 3 The Prader scoring, showing the degree of virilization on a five point scale, from mild clitoral enlargement, 1, to clitoromegaly with the urethral opening at the tip of the glans and high confluence between the urethra and vagina, 5. Modified from Prader, A. and Gurtner, H. P. (1955). The syndrome of male pseudohermaphrodism in congenital adrenocortical hyperplasia without overproduction of androgens (adrenal male pseudohermaphrodism). *Helvetica Paediatrica Acta* **10**, 397–412.

Table 1 Schematic description of the clinical presentation in different forms of 21-hydroxylase deficiency, according to age at presentation

Age at presentation	*SW CAH*	*SV CAH*	*Nonclassic CAH*
Neonatal period	Ambiguous genitalia (F) Failure to thrive, salt loss Hypoglycemia	Ambiguous genitalia (F)	–
Early childhood, <5 years of age	–	Apocrine body odor, axillary and pubic hair Increased growth Enlarged clitoris (F)	–
School age	–	Axillary and pubic hair Increased growth Enlarged clitoris (F) Enlarged penis (M) Pseudopubertas precox (M)	Acne, increased growth
Adolescence	–	TART (M)	Delayed menarche, irregular menses, acne, hirsutism Clitoral enlargement (F)
Adult		Androgen symptoms Infertility, TART, (M)	Acne, hirsutism, irregular menses, infertility (F), infertility (TART), (M)

F, Female; M, male; TART, testicular adrenal rest tumor.

Diagnosis: Laboratory and Clinical Investigations

Ambiguous genitalia and the absence of palpable testis should alert the physician to perform further investigations (Speiser *et al.*, 2010; Hindmarsh, 2009). Elevated 17-OHP, 46,XX karyotype and the presence of a uterus or even hyperplastic adrenals on ultrasound gives the diagnosis of 21-hydroxylase deficiency.

In addition to an increase in serum 17-OHP, the marker of the disorder, androstenedione and testosterone are typically elevated. In SW CAH the plasma renin is increased. Urinary pregnanetriol, the metabolite of 17-OHP, in 24 h profile, or spot urine is elevated (Kamrath *et al.*, 2016; Miller and Auchus, 2011).

The serum 17-OHP level reflects the severity of enzyme deficiency. Individuals with morning values of 17-OHP above the reference range should be investigated further. A Synachten/cosyntropin stimulation test results in elevated levels of 17-OHP and subnormal cortisol in patients and heterozygous carriers of CYP21A2 mutations (Turcu and Auchus, 2015a; Parsa and New, 2017). Patients with classic CAH typically have stimulated levels above 300 nmol L^{-1} (10000 ng dL^{-1}) while those with nonclassic CAH have morning values above the reference range that do not increase above 300 nmol L^{-1} (Turcu and Auchus, 2015a). The diagnosis can be confirmed by *CYP21A2* mutations analysis, which also enables prediction of disease severity and guidance in clinical treatment decisions.

Neonatal Screening

Neonatal screening for CAH is aimed at avoiding salt losing crises and neonatal death, most often seen in the boys. In addition, a secondary outcome is that the screening improves growth and development in children with milder forms of CAH by enabling early detection and treatment to prevent the symptoms of androgen excess during childhood (White, 2009; Gidlof *et al.*, 2014).

Screening was first started in the 1970s (Pang *et al.*, 1977) when it was shown to increase the noted prevalence among the Yupik Eskimos from 1/490 to 1/280. National and regional programs are now performed in more than 30 countries all over the world including many countries in Europe, and screening is mandatory in all states in the United States (White, 2013). Measurements of 17-OHP on filter paper cards using DELFIA (dissociation enhanced lanthanide fluorescence immunoassay) is used (White, 2009). Despite the use of birth weight or, more effective, gestational age related cut-off levels (Gidlof *et al.*, 2014; van der Kamp *et al.*, 2005) the false positive rate has been relatively high compared to other types of screening programs (White, 2013; Gidlof *et al.*, 2014). The recall rate is especially high among preterm infants, due to immature adrenal steroid production and varies between 1.2% and 0.02% (Gidlof *et al.*, 2014; Coulm *et al.*, 2012).

The cut-off level for17-OHP differs in the different screening programs and has to be adjusted depending on the day the sample is taken since 17-OHP increases just after birth and decreases during the first week of life. In patients with CAH the 17-OHP level increases over time and is related to the severity of CAH (Nordenstrom *et al.*, 1999). On the other hand, the sample has to be taken early enough for the result to be ready before the child develops a salt crisis. Severely ill and stressed infants have more elevated 17-OHP levels, which contribute to a relatively high false positive rate (Torresani and Biason-Lauber, 2007; Sarafoglou *et al.*, 2014; Hayashi *et al.*, 2017). The detection rate for SW CAH is high but efficiency of the screening programs varies from 100% to less than 70% detection of SW CAH (Sarafoglou *et al.*, 2012; Gidlof *et al.*, 2014; Coulm *et al.*, 2012).

A second tier analysis using the ratio of 17-OHP androstenedione and cortisol determined by tandem mass spectrometry has been used but has not proved to be as useful as expected, since 1/3 of the infants with SW CAH were missed (Sarafoglou *et al.*, 2012). In the future, genetic testing may be applied as a second tier procedure in some screening programs (White, 2013; Sarafoglou *et al.*, 2014).

In order for the screening to be effective, a good follow-up is important, and should include a confirmatory sample and immediate start of treatment if markedly elevated 17-OHP or if the child has clinical signs such as failure to thrive or virilization of the external genitalia. In equivocal cases a Synachten/cosyntropin stimulation test or genetic investigations must be performed to confirm the diagnosis.

It should be understood, that the aim of the screening programs is to detect infants at risk of salt crisis and neonatal death, and in order not to increase the false positive rate the cut-off level is set in such a way that the individuals at risk are detected but the milder forms, mainly nonclassic CAH, are not detected (Gidlof *et al.*, 2013, 2014; White, 2009). This implies that pediatricians have to identify children and adolescents with symptoms of androgen excess clinically.

Hormonal Treatment

The treatment is, in theory, straightforward. The glucocorticoid and mineralocorticoid deficiencies are replaced and the hormonal balance is restored. In practice, this has turned out to be considerably more complicated. The treatment goals are to obtain linear growth, normal bone maturation, and timing of puberty, good self-esteem and a good quality of life.

In children, hydrocortisone is the first choice of treatment since prednisolone and dexamethasone have more negative effects on growth and possibly weight (Punthakee *et al.*, 2003). A normal cortisol production rate has been reported to be 7–8 mg m^{-2} and day (Hindmarsh, 2009). However, treatment requires a higher dosing especially in CAH since one of the aims is to decrease the production of metabolites before the block. That is, 17-OHP and other androgenic compounds. The total hydrocortisone dose varies between 7 and 15 mg m^{-2} and day and usually given three or four times per day. Not only the total dose but also how it is divided over the day is of importance. Most clinicians seem to agree today that mimicking the circadian rhythm by giving the highest dose in the morning is preferred to giving the highest dose in the evening. The dose has to be individualized since there are individual variations in uptake and half-life of hydrocortisone (Hindmarsh and Charmandari, 2015; Miller and Auchus, 2011). Impaired ability to reactivate cortisone to cortisol (via 11betaHSD type 1), results in requirement for more frequent dosing in addition to a higher total dose (Nordenstrom *et al.*, 1999).

The treatment strategies differ depending on the child's age. The first year of life and the puberty seem to be vulnerable periods with regard to growth and growth potential. Specific issues of importance to consider for the different developmental periods are discussed below.

The Neonatal Period and First Year of Life

Hydrocortisone treatment is initiated at diagnosis. The dose should be sufficient for replacement, but 17-OHP during the first year of life should be allowed to be elevated. During the first month, a dose of 1 mg given three times per day is often sufficient. A doubled or even higher dose may be required during the first week in order to effectively suppress the adrenal hyperplasia and over activity (Speiser *et al.*, 2010).

Fludrocortisone in a dose of 100–150 μg/ m^{-2} or day or even higher doses may be required during the first months of life (Hindmarsh, 2009). In the small children, the fact that fludrocortisone has a glucocorticoid effect equal to about 1–1.5 mg of hydrocortisone should be taken into account. In addition to the fludrocortisone treatment, extra salt (sodium chloride) is important during the first year of life. The normal sodium requirement in a small child is 1 mmol kg^{-1} per day, but since children with CAH have an increased natriuresis 2–3 mmol kg^{-1} per day may be necessary to prevent deficit, and 2 mmol kg^{-1} per day (1–2 g day^{-1}) is recommended in SW CAH (Mullis *et al.*, 1990; Speiser *et al.*, 2010).

It is known that sodium is important for normal growth (Kuhnle *et al.*, 1983). Studies comparing patients with and without salt supplementation during the first year showed that the group that received sodium chloride during the first year of life achieved a higher final height, also when corrected for the target height (van der Kamp *et al.*, 2002).

Childhood

The hydrocortisone dose required for replacement is usually between 8 and 12 mg/m^2 per day in order to achieve adequate suppression of the pituitary ACTH production and maintain a normal growth rate. Undertreatment and advanced bone age during the prepubertal period results in attenuated height gain during the pubertal spurt (van der Kamp *et al.*, 2002; Balsamo *et al.*, 2003). As the child gets older physical activity including sports activities can vary considerably between days and weeks. Extra dosing can be required during excessive activities such as sports camps but not for normal training. In general, extra doses are not recommended for psychologically stressful events although in rare individual cases this may be needed before special occasions such as traveling or on the child's birthday.

Table 2 Rules of thumb for stress dosing with hydrocortisone

Oral stress dosing
Fever above 38°C—double hydrocortisone dose
Fever above 39°C—triple hydrocortisone dose
Fludrocortisone should be kept unchanged
If injection of hydrocortisone is given, the child should be taken to the hospital emergency department
If injection of hydrocortisone is given fludrocortisone should not be given since it will only increase the risk of hypertension

The fludrocortisone dose after 2 years of age is usually 100 μg/ m^2 in SW CAH and in adults rarely over 100 μg per day (Hindmarsh, 2009). After the first year of life salt is given ad libidum. Also in SV CAH a small dose of mineralocorticoid replacement is beneficial and allows for a lower total hydrocortisone dose (Witchel, 2017).

Adolescence

The treatment of teenagers/adolescents can be challenging in many ways (Merke and Poppas, 2013). Compliance and timing of the dosing, especially in the morning may by particularly difficult. However, it is important to be aware that the doses often need to be increased considerably.

The pharmacokinetics of cortisol is influenced by hormonal changes during puberty that necessitate increases in the hydrocortisone doses. During the pubertal development sex hormone production is increased as well as growth hormone production and IGF-1. The enzyme 11beta-hydroxysteroid dehydrogenase type 1 (HSD11B1) that reactivates cortisone to cortisol becomes less active, due to inhibition by the increased IGF-1 levels (Charmandari *et al.*, 2001). The increased cortisol clearance is more marked in females (Charmandari *et al.*, 2004). Hence the need for higher doses of hydrocortisone, in spite of good compliance, may thus be needed during the pubertal years. In young girls long standing androgen excess during puberty may not only result in increased hirsutism but also in a lowered timbre of the voice which is irreversible and can be experienced as a problem in everyday life (Nygren *et al.*, 2013).

It is, of course, important to keep the everyday doses as low as possible and allow some increase in 17-OHP during puberty in order to normalize growth but all aspects of development must be considered. Doses above 17 mg/m^2 should be avoided since this has been shown to affect the growth negatively (Bonfig *et al.*, 2009; Jaaskelainen and Voutilainen, 1997).

Monitoring

The child should be seen for regular check-ups every 3–6 months, from the second year onwards and more often during the first year and during puberty.

There is no single parameter that can be used alone for monitoring treatment in CAH. Height and weight development are the most important clinical parameters. Also small changes in height velocity should be considered in treatment decisions, and dosing. In preschool children, oily skin and acne are important signs of increased androgen production. Bone age is frequently used but it is more a reflection of the situation in the past and it is not informative to perform bone age evaluations more often than once a year.

Serum 17-OHP, androstenedione, and testosterone are often analyzed but it is not clear what levels should be obtained for adequate hormonal control (Kamrath *et al.*, 2017). Measurements of pregnanetriol in 24 h urine samples have been widely used. It has recently been shown that 11-oxygenated metabolites of adrenal androgens, mainly 11β-hydroxyandrosterone, was the dominating urinary adrenal androgen in treated patients with CAH (Kamrath *et al.*, 2017). Similarly, using liquid chromatography tandem mass spectrometry 11oxygentated metabolites were shown to be markedly elevated also in serum in patients with CAH, and may even be the more clinically relevant adrenal androgen. 11keto-testosterone is a potent androgen. Urine is difficult to collect in newborns and small children, serum samples are therefore often preferred.

During the past decade, it has become increasingly common to use 24 h 17-OHP profiles either performed with patient admitted to the hospital or on filter paper samples collected by the patients in the home (Schwartz, 1999; Bode *et al.*, 1999; Wieacker *et al.*, 2015). The circadian rhythm of the 17-OHP follows the ACTH production and the 17-OHP profile gives an indication of the hormonal situation over the day and guidance as to which dose should be changed. The optimal 17-OHP levels for patients at different ages and sex still need to be determined but completely suppressed 17-OHP levels indicate overtreatment.

The mineralocorticoid effect is followed with measurements of renin with the goal to maintain the plasma renin in or just above the normal range (Witchel, 2017; Hindmarsh, 2009). The blood pressure should be measured at every clinic visit.

Table 3 Stress dosing with injections of im or iv hydrocortisone

Weight (kg)	*Hydrocortisone (mg)*	*Injection*
Below15	25	iv or im
15–25	50	iv or im
25–50	75	iv or im
Above 50	100	iv or im

Stress Dosing

It is important to keep the everyday dose low to avoid side effects in the long-term perspective, but during acute illness or significant physical stress it is important that the dose is sufficiently increased (Hindmarsh, 2009). The hydrocortisone dose should be at least doubled if the patient has a fever above 38°C and tripled if it is above 39°C (**Table 2**). It is important to spread the dose more evenly over the 24 h especially when the everyday dose is considerably higher in the morning. In case of vomiting, intravenous or intramuscular injections of Solu–Cortef should be given. Admission to hospital and treatment with intravenous glucose and sodium chloride may be required.

It is imperative, that all parents and patients have appropriate information about stress dosing, both oral and for intramuscular injections, and the situations in which the different stress dosing should be given (**Table 3**). If intramuscular hydrocortisone is given the patient should be taken to the emergency department (Hindmarsh, 2009). Those who had received written information and instructions on how to give hydrocortisone intramuscularly had a higher level of self-efficacy (Fleming *et al.*, 2011).

The patient should have an emergency kit with a supply of injectable hydrocortisone and carry medical information as well as information on dosing and an ID card (Speiser *et al.*, 2010). However, one study showed that 40% of patients in an American survey did not follow these instructions (Fleming *et al.*, 2011). This is especially concerning in the light of the high mortality rate also in diagnosed patients. The mortality is increased for CAH with a hazard ratio of 3.2, and of 2.6 if the children under 1 year of age were excluded (Falhammar *et al.*, 2014). In a study in the United Kingdom the all-cause mortality was 3 times that expected, while it was 18 times higher in children under 4 years of age (Swerdlow *et al.*, 1998).

In preparations for surgery an i.v. acute dose of hydrocortisone (see **Table 2**) should be given 1 h before the start of the procedure, followed by an i.v. infusion of 2–3 mg m^{-2} per hour during the operation, or the same total dose but given every 4 h. Hydrocortisone (Solu–Cortef) can be diluted in 50–1000 mL Glucose 50 mg mL^{-1} with 80 mmol Na/1000 mL. During the days following the operation the dose should be tapered off; day two 40 mg m^{-2}, day three 30 mg m^{-2}, or faster after smaller procedures.

Hypoglycemia and Adrenal Crisis

In addition to the cortisol synthesis the epinephrine production is compromised in patients with CAH, resulting in an increased risk of salt crises and hypoglycemia.

This was demonstrated in a follow up of 102 children, below 6 years of age, 28 had been hospitalized, 22 with salt-losing crises, 25 with hypoglycemia (7 with both), and 13 children developed seizures during these events (Odenwald *et al.*, 2016). Adrenal crisis typically occurs during acute illness with inappropriate emergency treatment. Hypoglycemia is most often seen during infections but also when the glucocorticoid dose is tapered too quickly after a longer period of illness with high fever or vomiting but it may occur unexpectedly, often in the morning (Odenwald *et al.*, 2016). The epinephrine and metanephrine production has been shown to be compromised (Merke *et al.*, 2000a) and the degree of deficiency is related to the severity of the CYP21A2 deficiency (Charmandari *et al.*, 2002). This not only increases the risk of developing hypoglycemia but also attenuates the symptoms that we are using as signs of hypoglycemia, such as paleness and tremor. Hypoglycemic episodes are therefore likely to be more common than they were previously thought to be (Keil *et al.*, 2010). The families should be informed early on, in order for the preventive measures and treatment to be given in time. In addition to stress dosing with hydrocortisone an adequate intake of carbohydrates should be given, this seems to be especially important in the younger children (Keil *et al.*, 2010; Odenwald *et al.*, 2016; Kim *et al.*, 2014).

Development of Novel Therapies

Today treatment considerations have focused on more frequent dosing optimization and individualization of therapy (Webb and Krone, 2015). Slow release preparations with different profiles have been and are under development (Turcu and Auchus, 2015b; Mallappa *et al.*, 2015). In children for whom it is particularly difficult to obtain hormonal control the possibility of using an infusion pump, has been tested successfully (Hindmarsh, 2014). Flutamide (an antiandrogen) in combination with testolactone (an aromatase inhibitor) has been tried (Merke *et al.*, 2000b), and a more experimental treatment with

Abiraterone (a CYP17A1 inhibitor) has been tried in adult women (Auchus *et al.*, 2014). Growth hormone in combination with a gonadotrophin analog has been reported to have positive effect in some cases (Lin-Su *et al.*, 2011). Routine use of such experimental therapies to promote growth and delay puberty is however not generally recommended by the Endocrine Society guidelines (Speiser *et al.*, 2010).

Growth

Birth weight has been reported to be higher in CAH, However, the gestational age of a child with CAH is related to the severity of CAH, that is, the SW form results in longer gestation and BW corrected for gestational age was not increased in CAH (Gidlof *et al.*, 2007; Miles *et al.*, 2010). Hence, this speaks against the idea that prenatal growth would be influenced by androgens. In line with this is the observation that neither the growth velocity, nor the bone age was affected in children 1 year of age or younger with elevated 17-OHP levels and increased androgen production (Bonfig *et al.*, 2011). Similarly, retrospective studies on children with late diagnosed forms of the disease showed that the growth acceleration did not start until after the children were older than 1.5 years of age (Thilen *et al.*, 1995; Bonfig *et al.*, 2011). These findings have prompted the clinical practice to allow for more elevated 17-OHP in this age group in order not to compromise growth. If the hydrocortisone dose is increased to normalize the laboratory parameters the growth is suppressed. It is however important that the dose is sufficient to avoid hypoglycemia and to promote general well-being.

Throughout childhood and adolescence treatment has been proven to be a difficult balance between under- and over-treatment. Many studies on growth in CAH include heterogeneous cohorts including both early and late diagnosed patients which makes predictions in neonatally screened populations more difficult. Metaanalyses have shown that patients with CAH as a group lose about 1.4 SDS in final height, and -1 SDS in final height corrected for target height (Eugster *et al.*, 2001; Muthusamy *et al.*, 2010). There are differences between studies and countries but the height outcome was not significantly associated with gender, age at diagnoses, age at puberty, type and dose of glucocorticoid used. Patients with mineralocorticoid supplementation had a significantly better outcome in the more recent metaanalysis (Muthusamy *et al.*, 2010).

Undertreatment during childhood associated with an advanced bone age will result in an attenuated height gain during the pubertal growth spurt (van der Kamp *et al.*, 2002; Bretones *et al.*, 2016; Bonfig, 2017). It should be noted that final height predictions using bone age are difficult in CAH (Balsamo *et al.*, 2003; Bonfig and Schwarz, 2012). Mineralocorticoid treatment and sufficient salt supplementation are important for growth in all classic forms (Kuhnle *et al.*, 1983; van der Kamp *et al.*, 2002; Balsamo *et al.*, 2003; Bonfig, 2017). Moreover, in patients with SV CAH a low dose of fludrocortisone facilitates limiting the glucocorticoid dose (Witchel, 2017). Patients with an early diagnosis had a better final height (Balsamo *et al.*, 2003).

In conclusion, growth during the first year of life and pubertal growth seem to be most vulnerable (Speiser, 2010). It is important to keep the everyday doses as low as possible and allow some increase in 17-OHP during the first year of life and during puberty in order to optimize growth. Hydrocortisone doses above 17 mg m^{-2} or prednisolone should be avoided (Bonfig *et al.*, 2009; Jaaskelainen and Voutilainen, 1997; Punthakee *et al.*, 2003). In the future, it is likely that early diagnosis through screening and more individualized and lower doses of hydrocortisone in combination with the use of fludrocortisone in all patients with classical forms will improve the growth and final height in individuals with CAH.

Weight

High doses of glucocorticoid induce weight gain. As a group, patients with CAH have a slightly higher BMI than the reference population (Volkl *et al.*, 2006a). However, there are considerable individual variations. The risk of developing obesity was reported not to be related to the glucocorticoid dose per se, but was related to familial predisposition (Moreira *et al.*, 2013). The relative risk of developing obesity was increased more than four times if one of the parents had a BMI over 30 (Volkl *et al.*, 2006a). Obesity increases the insulin resistance and results in increased levels of insulin that will stimulate androgen synthesis in both the adrenals and ovaries (Charmandari *et al.*, 2004). In addition, increased insulin lowers the levels of SHBG and potentiate the androgen effects. Overweight in patients with CAH is therefore difficult to treat. Clinicians should be aware of this and take preventive measures when indicated.

Pubertal Development

Untreated patients with SV or NC CAH may enter puberty earlier than average in the general population, in which case this also affects final height, and result in short stature. Also treated patients have been reported to have an earlier start of puberty and often an attenuated growth spurt (Balsamo *et al.*, 2003; van der Kamp *et al.*, 2002; Hargitai *et al.*, 2001; Bretones *et al.*, 2016; Bonfig, 2017). In a study of 92 children (57 F, 35 M) the mean age at menarche (13.5 $\pm$ 1.6) did not differ from that of the reference population while an earlier increase in testicular volume was seen in boys with SV CAH (9.6 + 2.5) and SW (11.0 + 1.3 years) (Bonfig *et al.*, 2009).

Testicular adrenal rest tumors (TARTs) are formed from testicular adrenal rest cells located in the testis and are activated by an increased ACTH drive. TARTs develop over time and are more prevalent in men with severe forms of CAH (Stikkelbroeck *et al.*, 2004; Auchus, 2015). TARTs can be identified with high resolution ultra sound assessment early in life and the prevalence increases with the onset of puberty (Claahsen-van der Grinten *et al.*, 2014). The TARTs increase in size with insufficient hormonal control, which may cause pain.

In addition, secondary effects on gonadotrophins and the gonadal axis can be seen in both sexes. Undertreatment and increased androgens result in pituitary inhibition and lower FSH and LH levels. In men the resulting decreased testicular production of testosterone, may be one of the causative factors with negative effect on sperm production. A ratio between testosterone and androstenedione may be used as an indication whether the androgens are of adrenal or testicular origin (Auchus, 2015). In women elevated adrenal androgen production will cause irregular menstruations, and affect ovulation and hence, fertility (Witchel, 2017; Strandqvist *et al.*, 2014). Women may have a positive effect on the androgen synthesis if a contraceptive pill is used since it decreases the androgen production from the ovaries (Carmina *et al.*, 2017).

Surgery in Girls With CAH

The Endocrine Society Guidelines from 2010 emphasize that early single-stage genital repair for severely virilized girls, should be performed by experienced surgeons (Speiser *et al.*, 2010). The outcome of feminizing surgery has been disappointing in several follow-up studies (Crouch *et al.*, 2004, 2008; Gastaud *et al.*, 2007; Nordenskjold *et al.*, 2008; Johannsen *et al.*, 2010; Nordenstrom *et al.*, 2010; Nordenstrom, 2011) and has as a result of this been questioned. The possibility of obtaining informed consent from the individual concerned is also discussed from a human rights perspective (The UN rapporteur of torture 2012 and the EU Council resolution 2191 (2017) (http://assembly.coe.int/nw/xml/XRef/Xref-XML2HTML-en.asp?fileid=24232&lang=en). There has been a tendency towards performing early surgery less often (Michala *et al.*, 2014), however, a change in praxis needs to be evaluated since the psychological outcome of early versus late genital surgery has not been investigated. A recently published feasibility study, reported that no obvious discomfort or anxiety related to the genitals was observed in the clinical setting in seven girls, aged 1–8 years, who had not had early surgery (Bougneres *et al.*, 2017). The risk of urinary infections if surgery is not performed does not seem to be increased (Nabhan *et al.*, 2006).

Long Term Risks, Metabolic Aspects

Blood pressure has been reported to be elevated in younger children with CAH, in 45% during the first year of life and then decreasing to 15% at 4 years of age (Bonfig and Schwarz, 2014). A registry study on a larger cohort of 716 patients aged 3–18 years showed that elevated blood pressure was seen in 18%, and the blood pressure correlated with the BMI but not with the hydrocortisone dose (Bonfig *et al.*, 2016). A small study on six patients showed that giving the higher hydrocortisone dose in the evening attenuated the nightly dip possibly an independent risk factor for cardiovascular disease (Liivak and Tillmann, 2009).

There is an increased risk of obesity and of elevated blood pressure (Subbarayan *et al.*, 2014; Volkl *et al.*, 2006b). In addition, an epidemiological registry study showed an increased cardiovascular morbidity in adult patients with CAH (Falhammar *et al.*, 2015). Obesity, but also other factors such as how the glucocorticoid dose is divided over the day and the mineralocorticoid dose may be of importance.

Psychological Aspects

It is important to support the family and the child/teenager. Information and understanding of the disorder and its treatment have been shown to strengthen the coping ability (Pasterski *et al.*, 2014). Actively finding ways to communicate with the parents of the small children, and later on with the child and teenager is the foundation for a situation where all issues can be discussed between the healthcare professionals and the patient and parents but also between the family members (Nordenstrom and Thyen, 2014; Nordenstrom, 2015).

It has been shown repeatedly that girls and women with CAH have gender atypical interests and behavior (Berenbaum and Beltz, 2011; Hines, 2010) and that this is related to the extent of prenatal androgen exposure, that is, the severity of the disorder (*CYP21A2* genotype) (Nordenstrom *et al.*, 2002; Frisen *et al.*, 2009) that is, the level of prenatal androgen exposure rather than a threshold effect. Importantly in all the Swedish follow up studies, women in the null genotype group were considerably more affected by the disease, also compared to the I2G group, while in the clinic these two groups are often treated the same (Nordenstrom *et al.*, 2002, 2010; Frisen *et al.*, 2009; Strandqvist *et al.*, 2014; Engberg *et al.*, 2015).

There is much less known about what determines a persons' gender identity. The current practice is to raise children with 46,XX karyotype as girls but this has been challenged for the most severely virilized cases (Speiser *et al.*, 2010; Lee and Houk, 2010). Girls with CAH were shown to have more cross-gender identification, and gender role behavior compared to controls (Pasterski *et al.*, 2015) but these entities are independent of each other. In a gender identity questionnaire, the girls made more cross-gender statements but it is not known if this caused distress in the girls.

In a nationwide epidemiological registry study, girls with SW CAH were less likely to complete primary school than their controls (Strandqvist *et al.*, 2014). This could not be explained by hypoglycemia or adrenal crises since it was not seen in the boys with SW CAH: therefore, psychological aspects are likely to be of importance. Cognition is normal in CAH but short-term memory has been reported to be affected (Collaer *et al.*, 2016; Karlsson *et al.*, 2017; Hampson and Rovet, 2015; Agoston *et al.*, 2017). In the Swedish national cohort men and women with CAH had a doubled risk of receiving a psychiatric diagnosis of misuse or stress and adjustment disorder (Engberg *et al.*, 2015; Falhammar *et al.*, 2014), indicating that these patients are vulnerable to stress.

There is emerging evidence for and a focus on psychological counseling as an important part of the team approach to the care for these patients (Hindmarsh, 2009); (Nordenstrom, 2015; Speiser *et al.*, 2010). The patient's well-being should be addressed in a wider perspective. Over time the care for patients with CAH has evolved from rescuing patients with a lethal disorder by glucocorticoid and mineralocorticoid treatment to a multidisciplinary team approach where in addition the patients and the families' well-being is in focus in a more general perspective (Gidlof *et al.*, 2013; Speiser *et al.*, 2010).

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Prenatal Diagnosis and Treatment of Congenital Adrenal Hyperplasia

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Introduction

Congenital adrenal hyperplasia (CAH) comprises several enzymatic defects that disrupt adrenal steroidogenesis and cortisol production in the adrenal cortex (El-Maouche *et al.*, 2017; Speiser *et al.*, 2010). They are all inherited in an autosomal recessive manner and the lack of cortisol stimulates the hypothalamic–pituitary–adrenal-axis (HPA-axis) to increase the ACTH production. In some of the enzymatic defects (21-hydroxylase deficiency, 21OHD; 11β-hydroxylase deficiency, 11βOHD; 3β-hydroxy steroid dehydrogenase deficiency, 3βHSD; P450 oxidoreductase deficiency, POR), excessive ACTH stimulation of the adrenal gland will lead to over-production of adrenal androgens that virilize the female fetus.

CAH due to 21-hydroxylase deficiency (21OHD) is the most common variant affecting 1:10,000–1:15,000 live births (classic forms, salt-wasting CAH, SW CAH and simple virilizing CAH, SV CAH) (Speiser *et al.*, 1985; Wedell, 2011; White and Speiser, 2000) but milder adult onset forms of 21OHD (nonclassic CAH, NC CAH) are even more frequent ($\sim$1:1000) (Speiser *et al.*, 1985; Therrell *et al.*, 1998).

The other virilizing forms of CAH are rare, incidence 1:100,000 in 11βOHD and even less frequent in 3βHSD and POR (El-Maouche *et al.*, 2017). The focus of this paper will therefore be on 21OHD and the state of the art regarding prenatal diagnosis, prenatal treatment and the outcome of such interventions.

Clinical Aspects of CAH

In classic CAH, the early prenatal excess of adrenal androgen precursors (DHEA/DHEA-S, androstenedione) and their conversion to testosterone and dihydrotestosterone (DHT) will virilize the female fetus. The so called backdoor pathway descending from 17-hydroxyprogesterone lead to additional synthesis of androgens (androsterone and androstanediol) that are converted to DHT thus further aggravating in utero virilization in girls with 21OHD (Auchus, 2004; Hanley and Arlt, 2006; Miller and Auchus, 2011; Kamrath *et al.*, 2017a,b).

Virilization may lead to difficulties in sex assignment and a need for plastic surgery in the more severe cases. The degree of virilization is classified according to the five stages of Prader, where stage 1 is defined as almost normal female external genitalia with minor clitoral enlargement and stage 5 resembles male external genitalia with posterior labial fusion, formation of a urogenital sinus, hypertrophy of the clitoris and a phallic urethra (Prader and Gurtner, 1955).

SW CAH leads to prenatal virilization of the external genitalia Prader score 3–4 with very few exceptions. In SV CAH there is a wide range in virilization, from ambiguous genitalia at birth to clitoris enlargement and posterior fusion that may not be noted initially.

Ten common mutations are found in over 95% of all CAH cases (**Fig. 1**) and, in total, over 280 CYP21A2 alleles have been identified. There is a strong genotype and phenotype correlation for 21OHD, with the mildest allele determining the severity of the disorder (Krone *et al.*, 2013; Nordenstrom *et al.*, 2002; New *et al.*, 2013). However, there is also a contribution from the other allele, such that a patient with one mildly and one severely affected allele (compound heterozygote) will generally present with a more severe phenotype than one who carries two mild alleles. An in vitro analysis can be used as a complement in disease

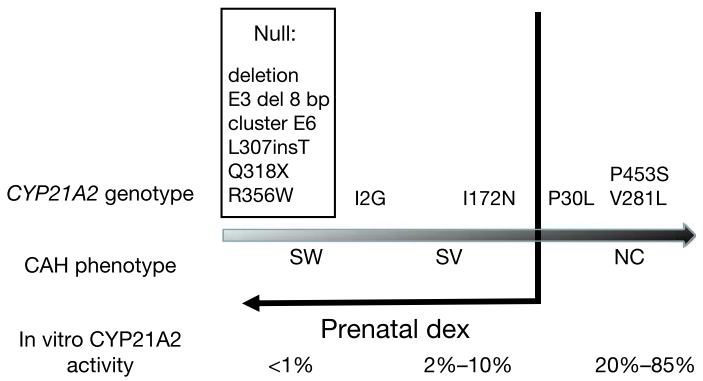

Fig. 1 Mutations giving rise to 21OHD in relation to clinical severity and enzyme activity in vitro. A group of completely inactivating mutations (Null), together with the I2G and I172N mutations, are associated with classic CAH that is, salt-wasting (SW) or simple virilizing (SV) disease. Prenatal DEX treatment is restricted to families segregating these mutations.

classification, especially for rare mutations, where large groups of patients are not available for clinical investigation (Barbaro *et al.*, 2015). Prediction of phenotype is possible from the *CYP21A2* genotype and in vitro studies and improves genetic counseling and clinical management. (Barbaro *et al.*, 2004, 2015; de Paula Michelatto *et al.*, 2016). Computational and structural modeling may also be used for predicting the phenotype of novel mutations (Haider *et al.*, 2013). Families with mutations that give rise to NC disease should never be subjected to prenatal treatment, whereas families carrying the more deleterious mutations may be offered prenatal treatment in a subsequent pregnancy (see below and **Fig. 1**).

Females with CAH have an increased risk of negative outcomes regarding sexual function, reproductive potential and psychosocial and cognitive aspects, especially in the more severe cases (the null genotype group), but it is unclear if this is the result of the degree of prenatal virilization or other factors related to the disease and postnatal treatment (Nordenstrom *et al.*, 2010; Miller, 2015; Strandqvist *et al.*, 2014; Karlsson *et al.*, 2017; Crouch *et al.*, 2008; Johannsen *et al.*, 2006; Nordenskjold *et al.*, 2008; Nordenstrom, 2011).

Prenatal Diagnosis and the Protocol for Prenatal Treatment of CAH

The molecular genetic diagnosis is more complicated for 21OHD than for other monogenic disorders due to the location and variability of the genomic region (White *et al.*, 1986; Miller and Auchus, 2011; Hannah-Shmouni *et al.*, 2017). There may be several *CYP21/C4* repeat units present on the same chromosome and one allele may harbor more than one mutation in the *CYP21A2* gene. Mutated alleles in a proband must be segregated in the parents to investigate their presence in different alleles and to verify de novo mutations.

Sanger sequencing is the gold standard for detecting point mutations and small gene deletions or insertions. It is used today by most centers in combination with MLPA (Multiplex ligation-dependent probe amplification) to detect mutations and large gene rearrangements/copy number variations in affected individuals (Concolino *et al.*, 2009). Prenatal diagnosis of a fetus at risk of CAH is performed during the late first trimester (around gestational week (GW) 10 postconception) when it is possible to perform a chorionic villous biopsy and extract DNA from cultured fetal cells for genotyping of mutations in *CYP21A2* using the methods mentioned above. There is also an option of using allele-specific PCR and other related techniques (Wedell *et al.*, 1994; White and Speiser, 2000). However, with this approach, the diagnosis of a fetus will not be established prior to the timing when prenatal treatment has to be initiated in order to be effective. Early prenatal genotyping is therefore warranted in order to be able to introduce treatment in only those cases that may benefit from it. In addition to the CVS method, it is possible to isolate fetal DNA for genotyping via amniocentesis performed in the second trimester (GW 12–14) or measure hormones in the amniotic fluid (17OH-progesterone, 21-deoxycortisol, androstenedione, and testosterone) (Forest *et al.*, 1993). This will, however, delay the diagnostic results and prolong the time the fetus is exposed to dexamethasone (DEX).

The use of DEX, a synthetic glucocorticoid for prevention of prenatal virilization in girls with classic CAH was first described by David and Forest in the mid-1980s (David and Forest, 1984). The protocol is depicted in **Fig. 2**. To be effective, the treatment has to be initiated before gestational week 7 in order to prohibit the fusion of the labio-scrotal folds and the formation of the urogenital sinus. The dose used is 20 μg/kg of the maternal prepregnancy weight and is divided into three doses per day (maximum 1.5 mg/day). Treatment can be offered to mothers who previously had a child with a variant of classic CAH expected to result in severe virilization in a female fetus.

Only girls with CAH are treated during the entire gestation, from week 6–7, while treatment in all boys and in girls without CAH is stopped when the genotype of the fetus is known, usually at the beginning of the second trimester (**Fig. 2**).

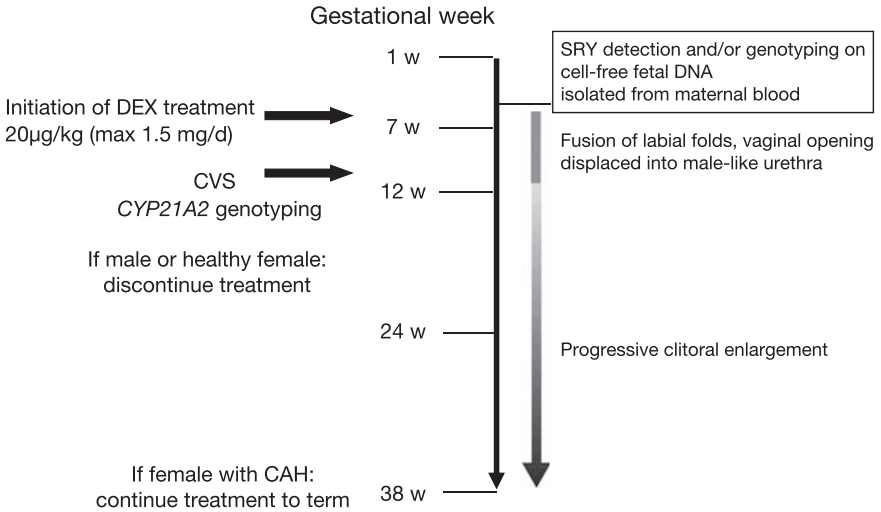

Fig. 2 The protocol for the prenatal treatment of CAH.

In women treated until term, the efficiency of the treatment is monitored with measurements of maternal serum cortisol and DHEAS (from GW 7) and estriol (from GW 16). Low levels of cortisol and DHEAS indicate adequate maternal adrenal suppression and compliance while a low level of estriol is a marker for fetal adrenal suppression (David and Forest, 1984). The mother should be followed throughout pregnancy with analysis of blood pressure, weight, glucosuria, proteinuria, and symptoms of edema, striae and other adverse effects (Lajic *et al.*, 1998). Discontinuation of DEX treatment should be tapered, in both short-term and long-term-treated women. The treatment strategy involves the dilemma that seven out of eight fetuses will be needlessly subjected to high doses of glucocorticoids during early embryonic life.

Noninvasive prenatal sex typing (SRY detection) can be performed using PCR amplification of cell-free fetal DNA in maternal blood as early as week 6–9 (Lo *et al.*, 1997; Bischoff *et al.*, 2005). The methodology has been applied by Tardy-Guidollet and colleagues in the diagnostic procedures for CAH as early as at 4 weeks and 5 days postconception and avoided prenatal treatment in 68% of the males (Tardy-Guidollet *et al.*, 2014). The method does not, however, change the fact that, with early sex typing of the fetus, three out of four cases will still be treated for a period of time for the benefit of others.

New *et al.* have recently succeeded in treating only the affected females using targeted massive parallel sequencing of cell-free fetal DNA in maternal plasma (New *et al.*, 2014). Fourteen expectant families at risk of having a child with classic CAH were recruited and fetal inheritance of parental haplotypes was determined (in one case, as early as 5 weeks + 6 days). The method is based on the detection of SNPs linked to the active *CYP21A2* gene and specific for either the maternal, paternal, or proband haplotypes. The technique is promising, but it still is not freely available as part of routine clinical care because it requires highly trained personnel and expensive equipment.

The preimplantation genetic diagnosis (PGD) is another methodology that can be applied for the diagnosis of genetically defined disorders (Simpson, 2010). Only embryos not having the disorder will be implanted in the uterus. In many countries, PGD is available for families at risk of having a child with a severe genetic condition. Three approaches are used to obtain DNA for PGD: (1) polar body biopsy, utilizing oocytes; (2) blastomere biopsy, utilizing the 3-day 6- to 8-cell cleavage stage embryo; and (3) trophectoderm biopsy, utilizing the 5- to 6-day blastocyst that contains about 120 cells. In PGD of the first polar body, the procedure is performed before fertilization occurs and the analysis offers the unique possibility of *preconceptional* diagnosis. The disadvantage for CAH and other single-gene autosomal recessive disorders is of course that the transmission of the paternal allele is not taken into account. The blastocyst is therefore the preferred biopsy approach in PGD (Simpson and Rechitsky, 2017).

DEX is efficient in preventing virilization in girls with CAH, but long-term evaluations of the treatment risks are still limited and have been the focus of debate for more than a decade (Seckl and Miller, 1997; Miller, 1999; Lajic *et al.*, 2008, 2011; Hirvikoski *et al.*, 2012). The safety aspects of the prenatal treatment have been discussed in the light of the fact that the majority of the treated children do not benefit from it (Hirvikoski *et al.*, 2012). Even with the new diagnostic approaches, unnecessary treatment in healthy girls during the first trimester of pregnancy is still a fact and also in girls with CAH undergoing DEX treatment, a risk benefit assessment is crucial.

Due to the safety issues it has been concluded by several authors and international societies that prenatal DEX treatment of children at risk of CAH should only be carried out in centers where a follow-up of treated individuals can be achieved (LWPES/ESPE, 2002; Miller, 2015; Hirvikoski *et al.*, 2012). Since 1999, all prenatally treated cases in Sweden have been followed according to a structured protocol within the framework of the PREDEX study (Hirvikoski *et al.*, 2012) and, since 2010, no additional cases at risk of CAH have been treated in Sweden due to the accumulating reports of long-term sequelae after prenatal DEX therapy.

Negative Consequences for Health in Animals and in Humans Attributable to Prenatal Glucocorticoid Excess

Glucocorticoids (GCs) are important for the differentiation and maturation of fetal tissues. They are used in the treatment of pregnancies at risk of preterm delivery to induce pulmonary maturation and prevent intraventricular cerebral hemorrhage. Glucocorticoids affect growth, including a dose-dependent inhibition of prenatal growth leading to lower birth weight and inhibition of neuronal proliferation (Norberg *et al.*, 2011; Samarasinghe *et al.*, 2011). The effects of GCs on fetal development are time- and dose-dependent with different outcomes in early versus late gestational treatment (Hauser *et al.*, 2008; Davis and Sandman, 2010). It is, however, important to be aware of the fact that it may be difficult to distinguish between effects of pre- versus postnatal treatment and adversities related to the preterm birth itself or to the underlying genetic defect.

According to the Barker hypothesis, the in utero milieu predisposes the child to a range of diseases later on in adult life (Barker *et al.*, 2002; Seckl and Holmes, 2007). The placental enzyme 11-beta-HSD type 2 protects the fetus from excess glucocorticoid by inactivation of cortisol to cortisone. The levels of cortisol in the fetus are normally ~1/5–1/10 of the levels in the mother (Chapman *et al.*, 2013). DEX and other synthetic glucocorticoids are not inactivated by 11-beta-HSD and the dose of DEX used in prenatal treatment of CAH will result in excess fetal GC levels that are estimated to be up to 60 times higher than the normal hormonal level (Miller, 2015; Busada and Cidlowski, 2017). It is therefore important to raise the question of whether in utero treatment with GCs comes with a cost.

Investigations in different animal models have shown that high levels of GCs during early- or late fetal life have effects on cognition, behavior and metabolism. In general, good animal models for obtaining information about the possible side effects of prenatal treatment in the context of CAH are scarce. Most animal studies have been designed to mimic the treatment in preterm infants, that is, treatment after mid-gestation.

Rodents are more GC-sensitive than primates and the teratogenic effects seen in rodent models are not observed in humans. Every species has its own characteristics, but several common mechanisms can be identified. Studies in rats have demonstrated low birth weight and smaller litter size after prenatal DEX exposure (Bakker *et al.*, 1995; Emgard *et al.*, 2007). As adults, rodents develop a status similar to the metabolic syndrome with hypertension, hyperinsulinemia, and hyperglycemia, as well as hyperactivity of the HPA-axis and altered affective behavior and altered stress reactivity (Celsi *et al.*, 1998; Drake *et al.*, 2010; Hauser *et al.*, 2008, 2009; Nyirenda *et al.*, 2006; Wyrwoll and Holmes, 2012; Zeng *et al.*, 2015). Similar effects have been observed in guinea pigs in the form of an abolished diurnal cortisol rhythm and male animals had a lower cortisol response (increased negative feed-back) to stress compared to female animals (Iqbal *et al.*, 2012).

On the other hand, early gestation, low-dose DEX treatment of pregnant ewes (20 μg/kg per day) did not affect blood pressure or renal function in 2 year-old offspring (Dodic *et al.*, 2003). Sloboda *et al.* showed that, in sheep treated with betamethasone during fetal life, the HPA-axis changed as the sheep matured and the adrenals became increasingly ACTH-resistant since the ACTH levels rose and the cortisol levels decreased with age (Sloboda *et al.*, 2000, 2002, 2007). A recent study from the same group, again using DEX during early pregnancy in the same animal model, presented evidence of an increased apoptosis in placenta with increased pro-apoptotic- (*Bax, p53*) and reduced antiapoptotic (proliferating cell nuclear antigen) markers and less placental binucleate cells (Braun *et al.*, 2015). This was associated with a temporarily decreased fetal growth in female off-spring (Braun *et al.*, 2015). In nonhuman primates, such as the Rhesus macaque, high levels of prenatal DEX during the last trimester resulted in offspring with elevated levels of basal- and stress-stimulated cortisol at the age of 10 months. In addition, there was an induced degeneration and depletion of hippocampal neurons with a 30% reduction of hippocampal volume (Uno *et al.*, 1994). In the African vervet monkey, high-dose DEX exposure (120 or 200 μg/kg) from mid-gestation until term caused an increased cortisol response to stress at 12 months of age (prepubertal animals). This reflects an increased drive of the HPA-axis, contrary to the rodent models. Nevertheless, by the age of 8 months the animals exhibited metabolic changes similar to those seen in rodents, that is, hypertension, impaired glucose tolerance, and hyperinsulinemia and the pancreatic β-cell mass was decreased by approximately 25% (de Vries *et al.*, 2007). In the common marmoset monkey, early DEX treatment resulted in reduced sociability in infants and increased motivation for palatable reward in the adolescent animal (Hauser *et al.*, 2008). Late DEX treatment resulted in a mild transient increase in knee-heel length in infants and an enhanced reversal learning of stimulus reward association in adolescents (Hauser *et al.*, 2008).

In a follow-up study of children who had been treated prenatally with GCs, but were born full term, there was an increased cortisol response to psychosocial stress, indicating an imprinting effect on the HPA-axis also in humans and with a greater effect being seen in girls (Alexander *et al.*, 2012). Effects on cognition have also been studied in preterm infants exposed to synthetic glucocorticoids, but there are difficulties in defining the exact cause of the negative effects since adversities directly related to the preterm birth may also play a role (Alexander *et al.*, 2016). The situation is further complicated by the fact that there seems to be individual differences in the vulnerability to GC exposure due to genetic differences in genes involved in GC signaling and prereceptor metabolism, as well as to sex differences, where girls seem to be more vulnerable than boys (Alexander *et al.*, 2012; Wallensteen *et al.*, 2016; Ragnarsson *et al.*, 2014). Additional postnatal treatment with GCs in the case of patients with CAH may also add to the effects of the initial prenatal treatment. Women with CAH and exposed to DEX antenatally had a worse cognitive outcome than non-prenatally treated women with CAH (Karlsson *et al.*, 2017). In patients with Cushing's syndrome in remission there was an association between a polymorphism in the *NR3C1* gene and worse visual attention and visual working memory, and a polymorphism in the gene encoding 11-beta-HSD1 and auditory working memory, processing speed, and reading speed (Ragnarsson *et al.*, 2014). In rats, GC treatment induces the 11-beta-HSD1 activity in the hippocampus, increasing action in that part of the brain (Low *et al.*, 1994). Certain SNPs in the genome may thus increase the GC exposure in the brain and affect cognitive functions long-term. Effects on the future fertility of the fetus may also be a risk, and maybe even transgenerational effects, since a study where 8–11 week old human fetal ovaries were incubated with DEX resulted in a reduced number of germ cells due to an increased rate of apoptosis (Poulain *et al.*, 2012).

In the following, we will focus on the published results from studies in humans regarding the outcome after prenatal dexamethasone therapy in the context of CAH. A large number of individuals have already been treated and can therefore be evaluated. The studies conducted up to now give important information regarding the safety of the treatment and the effects on the somatic and behavioral outcomes.

Maternal Outcome and Effects on Pre- and Postnatal Growth

The maternal side effects of DEX treatment are not negligible. Approximately 50% of the mothers treated during early pregnancy and all mothers treated until term experienced some type of side effect that can be attributed to DEX (edema, weight gain, cutaneous striae, sleep disturbances, and mood swings) (Lajic *et al.*, 1998; Forest *et al.*, 1993; Mercado *et al.*, 1995). The side effects usually disappeared after the treatment was discontinued. The incidence of hypertension or gestational diabetes was not increased in DEX-treated women and the incidence of miscarriage was comparable to that in the general population or in the control groups, although the total numbers of mothers were small (Lajic *et al.*, 1998). Studies conducted in France and the USA have reported that nearly all the mothers were willing to undergo the treatment again in the event of another pregnancy, while about one third of the Swedish mothers reported that they would not (Forest *et al.*, 1993; Forest and Dörr, 2003; Mercado *et al.*, 1995; Carlson *et al.*,

1999; New *et al.*, 2001; Lajic *et al.*, 1998). It is important to inform the women in advance about possible side effects if treatment is initiated.

Most of the children show normal pre- and postnatal growth, although several adverse events involving failure to thrive have been observed in both short-term and full-term-treated cases (Lajic *et al.*, 1998). Birth weight, even though nominally normal, was reduced in some studies on children with CAH and treated with DEX (Forest *et al.*, 1993; Forest and Dörr, 2003; Mercado *et al.*, 1995; Carlson *et al.*, 1999; New *et al.*, 2001; Lajic *et al.*, 1998). Reduced birth weight may be a risk factor for the development of chronic disorders later in life (Barker, 1995, 1998; Harris and Seckl, 2011).

Effects on Cognition and Behavior

In humans, memory deficits have been found after prolonged postnatal exposure to elevated glucocorticoids, such as in Cushing's syndrome and pharmacological glucocorticoid treatment (Sapolsky, 2000; Lupien *et al.*, 2007; Stuart *et al.*, 2005). Cushing's disease has irreversible effects on the brain, that is, persistent effects also after treatment, including both structural differences as seen in MRI and fMRI studies and negative effects on cognition (Andela *et al.*, 2015; Tiemensma *et al.*, 2010). As mentioned above, some of these effects may be a consequence of different vulnerability among individuals due to genotypic differences in the *NC3R1* and *11-beta-HSD1* genes (Ragnarsson *et al.*, 2014).

The glucocorticoid receptor (GR) is widely distributed throughout the brain, while the mineralocorticoid receptor (MR) is mainly expressed in the limbic structures. Both MR and GR are expressed at high levels in neurons in the amygdala, hippocampus, and the prefrontal cortex (Colciago *et al.*, 2015; Matsusue *et al.*, 2014; de Kloet *et al.*, 2005) areas important for executive function, emotional regulation, and memory (LeDoux, 2000; Funahashi, 2001; Opitz, 2014). These areas of the brain are thus vulnerable to high doses of GC.

The GC effect on the human brain is not only harmful since mechanisms of long-term memory formation are dependent on and influenced by glucocorticoids and other hormones (for a review, see (Colciago *et al.*, 2015). Epigenetic modifications and BDNF expression are the molecular mechanisms responsible for the synaptic plasticity that occurs in learning and formation of long-term potentiation and memory consolidation (Colciago *et al.*, 2015). Specific patterns of DNA methylation and histone modifications are established during learning in specific neurons in different areas of the brain involved in memory formation and long-term storage (amygdala, hippocampus, and prefrontal cortex). Chronic exposure to excess GC and early life events may thus alter these processes. Activation of the glucocorticoid receptor by DEX may cause inhibition of proliferation of neural progenitor cells (Samarasinghe *et al.*, 2011). Neural progenitor cells are important throughout life since they form the basis for adult neurogenesis in the dentate gyrus of the hippocampus and olfactory bulb (Aimone *et al.*, 2011; Zhao *et al.*, 2008). Factors that modulate memory formation such as stress, depression and environmental processes induce epigenetic modifications in the brain and influence cognitive functions beside the epigenetic changes seen in basic memory formation (Fischer *et al.*, 2007; Hunter *et al.*, 2009).

The first pilot study indicating long-term behavioral effects of prenatal exposure to DEX was published in 1995 by Maria New and Heino Meyer-Bahlburg (Trautman *et al.*, 1995). Since then, the behavioral and cognitive outcome has been the most studied area of research regarding long-term effects of prenatal treatment of CAH (Trautman *et al.*, 1995; Meyer-Bahlburg *et al.*, 2004, 2012; Hirvikoski *et al.*, 2007, 2008; Frisen *et al.*, 2009; Wallensteen *et al.*, 2016, Karlsson *et al.*, 2017).

In the Swedish cohort of prenatally DEX-treated children, there were no differences in parent-reported behavioral problems (using CBCL). However, in the parental assessments, DEX-treated children were reported to be more sociable (EAS scale), while more social anxiety was reported by the children themselves (SASC-R) (Hirvikoski *et al.*, 2007, 2008). Data from the same cohort of patients indicated that there may be some effects on gender role behavior also in prenatally treated boys since DEX-treated boys without CAH exhibited more neutral behaviors compared to controls (Hirvikoski *et al.*, 2011). In an extended follow-up study on healthy children at risk of CAH and treated with DEX during the first trimester, no significant negative behavioral effects were observed, but it should be noted that treated girls had the highest scores on measures of anxiety (Wallensteen *et al.*, 2016, Hormones and Behavior (YHBEH_4299)). Moreover, DEX treatment had long-lasting effects on cognition, which was observed as poorer performance in verbal working memory tasks (Hirvikoski *et al.*, 2007). In a more recent follow-up study of children exposed to DEX but without CAH, these effects seemed to be sex-dimorphic, since treated girls exhibited more pronounced cognitive deficits than treated boys. In addition to negative effects on executive functions, the girls showed broader effects on cognition, observed as poorer performance in a test assessing verbal intelligence (Wallensteen *et al.*, 2016).

Data from women with CAH treated with DEX prenatally suggest that this sex-dimorphic effect may also be present in adult age (Karlsson *et al.*, 2017). When short-term treated healthy individuals were re-tested as adults, there was a catch-up in executive function performance compared to their performance during childhood (Karlsson, *personal communication*).

The aforementioned American research group could not identify differences in the general development of children up to 6 years of age (Meyer-Bahlburg *et al.*, 2004). More recent data on long-term-treated girls with CAH indicate a slower cognitive processing in DEX-exposed individuals (Meyer-Bahlburg *et al.*, 2012). Contradictory results are reported in a Polish report which showed that prenatal DEX may have a negative effect on cognitive abilities in girls without CAH and a positive effect on girls with CAH (Maryniak *et al.*, 2014). A pilot study, using parental questionnaires as the method of assessment, indicated that prenatally DEX-exposed preschool children showed more shyness and emotionality, as well as less sociability (Trautman *et al.*, 1995). The mothers also reported more internalizing problems in a questionnaire measuring behavioral problems in children who were

between 2 and 5½ years of age (Trautman *et al.*, 1995). All of the above-mentioned studies are relatively small, which limits the possibility to detect smaller effects and emphasizes the need for additional national and international longitudinal follow-ups. Future studies should also investigate potential effects on metabolism, brain morphology, and functional networks in the brain to complement the neuropsychological assessments.

Studies regarding effects on the fetal programming of the HPA-axis are lacking in the context of prenatal treatment of CAH. There are, however, a few reports regarding prenatal glucocorticoid treatment of children at risk of preterm birth where treated children have an altered HPA-axis activity (Reynolds and Seckl, 2012; Seckl, 2004; Davis *et al.*, 2011). Furthermore, there is evidence that treated girls are more susceptible to altered programming since late prenatal glucocorticoid treatment resulted in increased cortisol responses to psychosocial stress, with a greater effect observed in girls (Alexander *et al.*, 2012). A similar effect was also identified with neonatal DEX-treatment in children at risk of bronchopulmonary dysplasia (Ter Wolbeek *et al.*, 2015). The DEX-treated girls showed increased levels of ACTH and increased cortisol responses in a stress test, further reinforcing the view that early life exposure to elevated glucocorticoids affects the HPA-axis and that these effects may be sex-dimorphic (Ter Wolbeek *et al.*, 2015).

Metabolic, Cardiovascular, and Immunological Effects

In general, there has been very little research on the cardio-metabolic outcome after prenatal glucocorticoid exposure in humans. However, in a report describing the metabolic effects after prenatal treatment with betamethasone during late gestation, the prenatally exposed individuals exhibited insulin resistance 30 years after exposure and the effects were more pronounced in women (Dalziel *et al.*, 2005). Antenatal glucocorticoid treatment in preterm infants is associated with increased aortic arch stiffness and altered glucose metabolism in early adulthood (Kelly *et al.*, 2012). The observed increased aortic stiffness was equivalent to that of term adults a decade older (Kelly *et al.*, 2012). Another study examining children and young adults (age 14–26 years) found no effect on BMI, blood pressure, blood lipids, cortisol levels, or insulin resistance in the group prenatally treated with betamethasone, compared to controls (Norberg *et al.*, 2013). Although no statistical differences could be found in arterial stiffness there was larger number of individuals who had received multiple GC doses than there where nontreated individuals in the group of subjects with the highest arterial stiffness (Norberg *et al.*, 2013).

The function of the immune system may also be affected by prenatal DEX-treatment since treatment is initiated during a period of gestation when the fetal thymus is formed and the first lymphocyte gene rearrangements occur (GW 7–12) (Veru *et al.*, 2014). There is evidence that children exposed to prenatal maternal stress (PNMS), and plausible high levels of cortisol, have programming effects that may have long-lasting consequences on the immunity (Veru *et al.*, 2015). PNMS altered the levels of cytokines, reduced the number of CD4 + T-cells and induced a shift of the T-helper cell population favoring Th2 cells (Veru *et al.*, 2015). Long term outcome regarding other biological functions in these children showed a pattern consistent with excessive glucocorticoid exposure, that is, PNMS was positively associated with a higher insulin secretion and a higher BMI during adolescence (Dancause *et al.*, 2013).

Suggestions for Future Research

Altered DNA methylation is a mechanism involved in adapting genomic programs for long-lasting effects on human health after exposure to stress/GCs or traumatic events. Most studies concerning glucocorticoid exposure have, however, investigated alterations on the single gene level and changes in gene promoters. There is a need for additional investigations in this area of research using techniques with more extensive coverage, such as microarrays and bisulphite next-generation sequencing.

It is possible that alterations in DNA methylation are involved in and the cause of, the fetal programming seen after prenatal DEX-treatment for CAH. There are a few animal studies pointing in that direction. A trans-generational effect of the epigenetic changes has also been proposed. The second generation off spring of guinea pigs treated during pregnancy with multiple doses of betamethasone showed reduced methylation of genomic DNA globally and of specific genes involved in the methylation process per se (Crudo *et al.*, 2012; Iqbal *et al.*, 2012).

Summary

In summary, the long-term outcome of prenatal DEX treatment on behavior and cognition has been investigated and most of the evidence points towards a negative effect on executive functions, with girls appearing to be more vulnerable than boys. Behavior is mostly unaffected in treated subjects and the children are well adapted. Cardiovascular and metabolic functions are still areas that need to be investigated. Epigenetic alterations may be the cause of the long-lasting effects of prenatal glucocorticoid exposure by interacting with genomic programs that affect human health. Whether such changes take place in the context of prenatal DEX treatment in humans remains to be investigated.

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Steroidogenic Factor 1 (SF-1; NR5A1)

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The orphan nuclear receptor steroidogenic factor 1 (SF-1, also called Ad4BP, encoded by the *NR5A1* gene located on Chr 9q33.3 in human) is an essential regulator of endocrine development and function.

Structure of SF-1

NR5A1 belongs to the subfamily of transcription factors known as nuclear receptor subfamily 5 (group A, member 1; NR5A1), which is highly conserved in vertebrates. With a striking homology to the Drosophila nuclear receptor fushi tarazu factor (Morohashi *et al.*, 1992), the 461 amino acid long SF-1 protein consists of a DNA-binding motif composed of two zinc-chelating modules that coordinate the interaction between the receptor and hormone response element (Schimmer and White, 2010). SF-1 binds DNA as a monomer, with DNA-binding stabilized via a 30 amino acid extension to the DNA-binding domain (Ftz-F1 or A box) and has a high affinity for the region 5′YCAAGGYCR′3 (where Y = T/C, R = G/A) (Wilson *et al.*, 1993). The C-terminal ligand-binding domain (LBD) encompasses an AF-2 domain that cooperates with a proximal activation domain (AF-1) and is required for maximal biological activity with coactivators such as NCOA1 (SRC-1). Post-translational modification plays an important role in modulating SF-1 activation and repressor functions. Phosphorylation of Ser203 within the LBD enhances the interaction of the cofactors Tif2 (Grip1/Ncoa2) and Smrt (Ncor2/Trac1) with the AF-1 and AF-2 motifs. Strong transcriptional repression requires sumoylation of lysine residues 119 and 194, which increases interactions with DEAD box proteins including Ddx20. SF-1 binds phospholipids with a phosphatidylinositol (PI) head-group, in particular PI(3,4)P2 and PI(3,4,5)P3 (Hammer *et al.*, 1999).

SF-1 Expression in Embryonic and Adult Tissues

The localization of SF-1 in embryonic and adult tissues in mammals has been explored in humans, mice and rats (Ikeda *et al.*, 1994; Morohashi *et al.*, 1995; Hanley et al., 1999, 2001). In developing embryos, SF-1 marks the earliest populations in the developing adrenal gland and the urogenital ridge. In later embryonic development, SF-1 is expressed in the steroid secreting cortical zones of the adrenal gland, testicular Leydig and Sertoli cells. In mice and rats, ovarian expression of SF-1 is turned off as development proceeds and does not resume until just before birth. In contrast, the expression of human SF-1 is similar in the developing ovary and testis with expression observed in granulosa cells (Bashamboo *et al.*, 2016). SF-1 is also expressed in the fetal spleen (Morohashi *et al.*, 1999) and in the prosencephalon (Ikeda *et al.*, 1994).

In the adult, SF-1 is expressed in the major steroidogenic tissues, including the adrenal cortex, Leydig and Sertoli cells, the ovarian interstitium, theca cells, granulosa cells, and, to a lesser degree, corpus luteum (Morohashi *et al.*, 1994; Morohashi, 1999; Ramayya *et al.*, 1997). It also is expressed in pituitary gonadotrophs where it regulates the expression of the gonadotropins and the GnRH receptor (Barnhart and Mellon, 1994; Ingraham *et al.*, 1994). Apart from these tissues, SF-1 expression is limited to neurons in the dorsomedial portion of the ventromedial hypothalamus (VMH) that are distinct from those expressing GnRH, to the endothelial linings of the venous sinuses and pulp veins in the spleen (Ramayya *et al.*, 1997), and to a subset of neurons in the hippocampus that coexpress steroidogenic acute regulatory protein (StAR) and aromatase (Wehrenberg *et al.*, 2001).

Biological Function of SF-1

SF-1 regulates the expression of genes involved in differentiation and control of the hypothalamus–pituitary–adrenal axis via cAMP/PKA signal pathway that transmits the signal from extracellular stimuli to the nucleus (Val *et al.*, 2003). SF-1 is activated by phosphorylation at a single Serine residue (Ser203) by mitogen-activated protein kinase (MAPK) (Hammer *et al.*, 1999) and acetylation (Chen *et al.*, 2005), whereas conjugation by a small ubiquitin like modulator (SUMO) leads to the repression of SF-1 activity (Chen *et al.*, 2004). Inhibition of histone deacetylase cause increased ubiquitination of SF-1, leading to its degradation resulting in reduction of steroid secretion (Chen *et al.*, 2007).

SF-1 localizes to both the nucleus and centromere within the cell and depending on its intracellular localization it impacts on cell growth and differentiation. When acetylated, SF-1, together with RNA polymerase II and coactivators, is present in transcriptionally active loci of the nucleus. On the contrary SUMOylation relocates SF-1 to nuclear speckles that contain transcriptional repressors like PML proteins that repress its activity. In the centrosome, SF-1 regulates centrosome homeostasis by preventing

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aberrant DNA-PK activation. The absence of SF-1 causes centrosomes to over-duplicate leading to multiple spindle poles during mitosis followed by genomic instability resulting in a reduction of cell numbers (Chia-Yih *et al.*, 2013).

XY mice lacking SF-1 have gonadal dysgenesis resulting in male-to-female sex-reversal with a female phenotype and persistence of Müllerian structures (uterus), highly reduced corticosterone levels, elevated adrenocorticotrophic hormone levels due to adrenal insufficiency, underdevelopment of the spleen and impaired clearance of abnormal red blood cells (Luo *et al.*, 1994). *Nr5a1*$^{-/-}$ mice do not express luteinizing hormone (LH) or follicle-stimulating hormone (FSH) from the gonadotroph cells in the pituitary, and the VMH is disorganized (Büdefeld *et al.*, 2011). VMH specific knockout of *Nr5a1* show high fat diet-induced obesity and increased anxiety-like behavioral patterns (Kim *et al.*, 2011). Nr5a1 haploinsufficiency results in aberrant adrenal architecture and reduced gonad weight, whereas granulosa cell specific ablation of Nr5a1 results in reduced ovarian reserve in females (Bland *et al.*, 2000; Pelusi *et al.*, 2008).

Clinical Phenotypes Associated With *NR5A1* Mutations

In the human, mutations involving *NR5A1* are associated with a wide range of reproductive anomalies including adrenal insufficiency and 46,XY gonadal dysgenesis, ambiguous genitalia, hypospadias, micropenis, spermatogenic failure with normal genitalia and primary ovarian insufficiency and TDSD/OTDSD in females.

Adrenal Insufficiency

The first human mutation in *NR5A1* was reported in a 46,XY girl with complete gonadal dysgenesis with persistent Müllerian structures and primary adrenal failure, similar to that of mouse model lacking *Nr5a1* (Achermann *et al.*, 1999). This girl carried a de novo heterozygous mutation (p.G35E) localized in "P"-box of the first zinc finger of SF-1, a region crucial for recognizing DNA-binding sequence on target genes. Another homozygous mutation (p.R92Q) was identified soon after in a 46,XY girl, from a consanguineous family, presenting with complete sex-reversal and primary adrenal insufficiency (Achermann *et al.*, 2002). The mutation p.R92Q localizes in the "A"-box within the FtzF1 domain of SF-1, which is involved in stabilizing monomeric binding through an interaction with the DNA minor groove. Although the mutations in SF-1 were initially identified in children with adrenal insufficiency, mutations in patients with an adrenal phenotype are rare (Biason-Lauber and Schoenle, 2000; Lin *et al.*, 2006).

Although the initial search for *NR5A1* mutations focused on the children with phenotypes consistent with that of the mouse model, the past 15 years have seen an exponential increase in reports describing pathogenic changes in *NR5A1* and as well as the phenotypes associated with those changes. More than 200 individuals and families are now described in the literature.

46,XY DSD

Heterozygous mutations in *NR5A1* are one of the most frequent (approximately 15%) causes of 46,XY DSD with normal adrenal function at the date of examination although some cases may have mildly elevated ATCH levels. The most common phenotype is a 46,XY child with ambiguous genitalia, with or without Müllerian structures. These mutations are associated with variable degrees of testicular dysgenesis, ranging from partial to complete. This is associated with low levels of testosterone, inhibin B and anti-Müllerian hormone (AMH), and an elevation of follicle-stimulating hormone. On the other hand, there are also some cases reported with low levels of AMH at birth but without apparent Müllerian structures. In rare children there may be adequate testosterone production in the newborn, which may lead to a misdiagnosis of partial androgen insensitivity syndrome (Coutant *et al.*, 2007; Wu *et al.*, 2013). There are also reports of with *NR5A1* mutations associated with progressive androgen production in 46,XY girls with virilization at adolescence, resembling the phenotypes associated with 5 alpha-reductase or 17 beta-hydroxysteroid dehydrogenase deficiency (Cools *et al.*, 2012; Tantawy *et al.*, 2012). Mullerian derivatives are present in approximately a quarter of all 46,XY DSD cases harboring *NR5A1* mutations suggesting that in most cases the fetal Sertoli cell function has been at least partially preserved. However, an impairment of Sertoli cell function in following pubertal age is indicated by elevated FSH levels in all patients studied. Around 30% of all children with 46,XY DSD inherit a heterozygous change from their mother in a dominant manner although rare recessive mutations have been reported.

NR5A1 and Spleen Development

A rare heterozygous nonsense mutation was reported in a girl with polysplenia and her father, who had hypospadias and asplenia (Zangen *et al.*, 2014). A homozygous missense mutation (R103Q) was reported in a child with severe 46,XY DSD and absent spleen. Precisely how SF-1 regulates spleen development is unclear, but mice lacking Nr5a1 show morphologic defects in the spleen vascular system that are associated with a decrease in the proportion of hematopoietic cells, although differentiation of these cells was not affected significantly (Morohashi *et al.*, 1999). TLX-1 promoter is a homeodomain-containing transcription

factor critical for embryonic spleen development. The human R103Q mutation showed impaired transactivation of the *TLX1* promoter and this may provide a mechanism for the asplenia (Colson *et al.*, 2017).

Hypospadias and Undescended Testes

Although hypospadias is a common phenotype, only a small minority are associated with *NR5A1* mutations (Kohler *et al.*, 2009). The first case of a NR5A1 mutation associated with severe penoscrotal hypospadias was reported in a child who had small inguinal testes, partial gonadal dysgenesis, and reduced androgen synthesis. *NR5A1* mutations have been reported in around 5% of individual presenting with the most severe forms of penoscrotal hypospadias with a reduced penile length and at least one undescended testis. *NR5A1* changes in boys with the less severe forms of hypospadias have been reported but are much less common. In one boy with mild distal hypospadias a de novo p.Arg313Cys mutation was identified (Allali *et al.*, 2011), however, a 46,XY girl with complete gonadal dysgenesis was also reported with the same de novo p.Arg313Cys mutation (Mazen *et al.*, 2016). This suggests that the severity of the NR5A1 phenotype may be influenced by other genetic factors.

Bilateral Anorchia

A single heterozygous mutation in *NR5A1* was reported in an analysis of a cohort of 24 children with bilateral anorchia (vanishing testis syndrome). Although anorchia is often believed to reflect neonatal torsion or a vascular event, a small subgroup of bilateral anorchia may represent progressive late onset testicular dysgenesis, occurring after the external genitalia have formed (Philibert *et al.*, 2007).

Male Factor Infertility

In a study of 315 men with spermatogenic failure, heterozygous missense mutations in *NR5A1* were identified in seven men with either azoospermia or severe oligozoospermia (Bashamboo *et al.*, 2010). Testis histology in one man with azoospermia was suggestive of a mild form of testicular dysgenesis rather than Sertoli Cell Only Syndrome. In all cases the men carrying the NR5A1 mutations had normal development of the external genitalia. Other studies have confirmed these findings and current data indicate that around 3% of men with idiopathic spermatogenic failure carry mutations in the gene (unpublished data). In some of these *NR5A1* mutated cases the men had a history of cryptorchidism, which may influence the severity of the spermatogenic failure. Cryptorchidism may occur since SF-1 directly regulates the expression of *INSL3*, a Leydig cell product that is critical for testicular descend (Ferlin *et al.*, 2015).

Interestingly, one of the men described in the initial report showed gradual reduction in sperm numbers suggesting a progressive loss of testicular function associated with mutations in *NR5A1* (Bashamboo *et al.*, 2010). Therefore, children carrying *NR5A1* mutations with no apparent gonadal or adrenal phenotypes may merit long term monitoring to assess the retention of postpubertal testicular function.

Primary Ovarian Insufficiency (POI)

Research on the consequences of human *NR5A1* mutations focused 46,XY individuals since initial reports suggested the mutations mainly affected testis formation and development. Furthermore the report of a prepubertal girl with adrenal insufficiency and an *NR5A1* mutation without a gonadal phenotype led to the suggestion that mutations in *NR5A1* are pathogenic only in 46,XY chromosomal context (Biason-Lauber and Schoenle, 2000). This concept changed with the report of sisters or mothers of children with 46,XY DSD, who carried heterozygous *NR5A1* variants and showed an ovarian phenotype (Lourenco *et al.*, 2009). Like the testis, *NR5A1* mutations effects the ovary at multiple levels, including follicular recruitment and reserve, stromal development and the endocrine function. This is reflected in the gamut of clinical phenotypes seen in the 46,XX individuals carrying mutations that may range from gradual loss of ovarian reserve (premature menopause) to later loss of ovarian reserve/function (secondary amenorrhea) to complete early ovarian insufficiency (primary amenorrhea). Despite being one of the single most important monogenic cause of ovarian insufficiency, mutations in *NR5A1* are relatively rare (1.4%–1.6%) in women with sporadic idiopathic POI (Lourenco *et al.*, 2009; Camats *et al.*, 2012; Janse *et al.*, 2012; Voican *et al.*, 2013). However, there are also women carrying *NR5A1* mutations with no apparent clinical effect on ovarian development or function, indicating that *NR5A1* mutations have variable penetrance. Excessive anxiety and/or depression has been reported in two independent 46,XX women who were carriers of *NR5A1* mutations but this association needs to be confirmed by other studies (Suwanai *et al.*, 2013).

Testicular/Ovo-testicular DSD (TDSD/OTDSD)

Several studies report a specific recurrent point mutation in NR5A1 (p.R92W) in association with testis development in 46,XX individuals in multiple families of different ancestry (Bashamboo *et al.*, 2016; Baetens *et al.*, 2016; Igarashi *et al.*, 2017). All individuals lacked the testis-determining gene *SRY*. The p.R92 *NR5A1* mutation was described in twelve 46,XX DSD patient and it is absent from control databases indicating that the association with the phenotype is robust. However, the expressivity of the phenotype is variable ranging from female external genitalia with clitoromegaly, to male external genitalia with micropenis or penoscrotal hypospadias. Sex of rearing was female in nine cases and as male in the other three. In one family a 46,XY sib, who inherited the mutation, developed 46,XY partial gonadal dysgenesis and was raised as a girl. The families also show incomplete penetrance for the phenotype with in total, 12 apparently unaffected carriers of the mutation were reported (six XY men and six XX women). Although, these individuals are not reported to have either under or overvirilization it is unclear if the mutation is completely without effect in these carriers as other heterozygous *NR5A1* mutations cause a decline in sperm numbers in men and premature ovarian failure in fertile women (Lourenco *et al.*, 2009; Bashamboo *et al.*, 2010). Three families were from a 70 km radius of the Southern Netherlands and haplotype analysis indicated that this is a founder mutation in that region. The other families were of Japanese, American or South Asian ancestry and in at least two families the mutation was not carried by either parent and was de novo. A heterozygous p.Arg92**Gln** variant (not p.Arg92Trp) has also been reported in a child with 46,XX ovotesticular DSD of American-European ancestry (Bashamboo *et al.*, 2016). This variant was inherited from her unaffected father. How changes in the R92 amino acid influences cell fate to change from a granulosa to a Sertoli cells is unclear. This mutation does not show a phenotype when introduced into mice (Miyado *et al.*, 2016). This is most likely due to the fact that, unlike the human (Bashamboo *et al.*, 2016), *Nr5a1* is not expressed in the early developing (pre)-granulosa cell (Ikeda *et al.*, 1994). Not surprisingly, this amino acid change introducing a bulky tryptophan residue in the A-box region results in an inability of the mutated protein to bind to a consensus target DNA sequence (Bashamboo *et al.*, 2016). However, either directly or indirectly the *NR5A1* mutation must lead to the upregulation of *SOX9* expression, which is a key for the formation of Sertoli cells and a direct target for SRY. In theory, this could be performed in one of two ways, either by directly activating genes in the male pathway and/or impeding female genes from repressing the male pathway. Thus, derepression could lead to the upregulation of SOX9 expression. There is no evidence that NR5A1 p.R92W is directly upregulating "male" genes including SOX9. In vitro assays showed that the R92W protein lacked the ability to directly upregulate expression when using the *AMH* or *Cyp11A1* promoters or a testis-specific *Sox9* enhancer. It is emerging that at least one function of pro-ovarian pathways, such as R-spondin-1/WNT4 is to activate β-catenin. β-catenin has emerged as a key regular of sex development in mammals. Mice lacking Rspo1 exhibit partial female-to-male sex-reversal, a phenotype that can be rescued by stabilized β-catenin expression in the somatic cells of the ovary. On the other hand, the expression and stabilization of β-catenin in the gonads of XY mice is sufficient to disrupt the male pathway and promote ovarian development. SF-1 has the ability to physically interact with the β-catenin protein and upregulate the expression of a series of target genes (Hossain and Saunders, 2003). These include the *AMHR2*, *INHA*, *CYP19A1*, and *DAX-1/NR0B1*. The SF-1 R92W mutant shows a reduced ability to interact with β-catenin to synergistically activate reporter gene activity. This suggests that the p.R92W NR5A1 variant switches organ fate from ovary to testis through disruption of ovary-specific pathways that would normally oppose testis development, rather than by activating pro-testis pathways directly.

Overactivity of SF-1

Overexpression or overactivity of SF-1 may be implicated in certain clinical phenotypes. Duplication of the locus encoding SF-1 has been reported in a high proportion of pediatric adrenal carcinomas, and overexpression of SF-1 has been shown to be present in many adult adrenal tumors (Almeida *et al.*, 2010; Sbiera *et al.*, 2010). Overactivity of SF-1 has been proposed to be associated with endometriosis, where SF-1 expression in undetectable in normal endometrial stromal cells and highly expressed in endometriotic stromal cells. Similarly, in polycystic ovaries, androgens are mainly produced in the follicular theca cells that express high levels of SF-1 (Calvo *et al.*, 2001).

Genotype–Phenotype Correlations

All studies indicate that there is a poor correlation between the severity of the phenotype and the nature of the NR5A1 variant. Even within families there is evidence of variable expressivity of the phenotype including both highly variable endocrine values and in the phenotypic appearance (Werner *et al.*, 2017). In part this may be explained by di- or oligogenic inheritance of other factors that influence the phenotype (Mazen *et al.*, 2016; Hattori *et al.*, 2017). The severity of the phenotype has also been proposed to be modulated by interallelic association with a known and common p.Gly146Ala polymorphism. However, robust genetic associations to support this have not been reported. Furthermore, the type of the NR5A1 mutation does not inform patient outcomes including pubertal androgenization, risk of cancer development or gender assignment. Gonadal histology is also not consistent between NR5A1 mutated individuals, even for the same amino acid change. Since the clinical effects of SF-1 are wide ranging and variable, management plans must be tailored to individuals cases.

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11β-Hydroxylase Deficiency

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Glossary

Congenital adrenal hyperplasia (CAH) A group of autosomal recessive disorders caused by defects in various enzymes involved in adrenal steroidogenesis leading to defective glucocorticoid and/or mineralocorticoid synthesis.

Introduction

The 11β hydroxylase enzymes (CYP11B1 and CYP11B2) are responsible for catalyzing the final steps in synthesis of cortisol and aldosterone. Congenital adrenal hyperplasia (CAH) due to 11β hydroxylase deficiency (11βOHD) results from reduced or absent activity of CYP11B1 enzyme in the adrenal cortex (Bulsari and Falhammar, 2017). 21-Hydroxylase deficiency (21OHD) remains the most common cause of CAH accounting 90%–99% of CAH cases, followed by 11β hydroxylase deficiency (11βOHD) responsible for 0.2%–8% (Bulsari and Falhammar, 2017; El-Maouche *et al.*, 2017; Gidlof *et al.*, 2013).

Depending on severity of clinical manifestations and degree of loss of enzymatic activity, 11βOHD is classified as classic or non-classic (NC). The clinical manifestations of classic 11βOHD include features of androgen excess including virilization of external genitalia in females, peripheral precocious puberty and hyporeninemic hypokalemic hypertension due to mineralocorticoid excess (Bulsari and Falhammar, 2017; Khattab *et al.*, 2017). Although, hypertension is only found in two-third of the cases at time of diagnosis of classic 11βOHD (Rosler *et al.*, 1992), hyporeninemic hypertension remains a key differentiating clinical feature for 11βOHD from classic 21OHD. NC 11βOHD usually has no abnormalities at birth and occurs as a result of partial loss of enzyme activity. Individuals with NC phenotype present in later life with mild virilization, peripheral precocious puberty, hirsutism or menstrual irregularities resembling polycystic ovarian syndrome (Bulsari and Falhammar, 2017; Speiser and White, 2003; White, 2001). Hypertension is not a feature commonly found in NC 11βOHD (Zachmann *et al.*, 1983).

Epidemiology

The exact prevalence of classic 11βOHD in general population is not well documented. It has been claimed to be 1 in 100,000 in the general population and around 5%–8% of the CAH cases (White, 2001). However, based on recent studies from United Kingdom and Sweden, 0.2%–1.5% of all CAH cases had 11βOHD (Arlt *et al.*, 2010; Gidlof *et al.*, 2013) and only 1 case of 11βOHD was found in the national CAH registry in Sweden suggesting the prevalence to be 1 in 9,000,000 in the general population (Bulsari and Falhammar, 2017; Gidlof *et al.*, 2013). The higher prevalence in previous studies could be secondary to selection bias and ethnicity of the study population.

Certain geographical locations and ethnic populations such as Tunisia, Iran, Saudi Arabia, Turkey and Jews of Morocco and Israel have higher frequency of classic 11βOHD (al-Jurayyan, 1995; Kandemir and Yordam, 1997; Khattab *et al.*, 2017; Rosler *et al.*, 1992). The estimated incidence in an offspring of Moroccan Jews in Israel was 1 in 5000–7000 with a carrier rate of 1 in 35 to 1 in 42 and an overall estimated incidence in Israel was 1 in 30,000–40,000 (Rosler *et al.*, 1992). The higher prevalence in certain ethnic populations is believed to be due to founder effect and high rates of consanguineous marriages within these ethnic populations. In an international cohort study, 58% of patients were from consanguineous marriages and mostly confined to Middle East and North African nations (Khattab *et al.*, 2017).

The prevalence of NC 11βOHD again varies with geographical location and ethnicity of study population from 0.8% to 8% in women with hirsutism and hyperandrogenism, however, in none of these studies the diagnosis was confirmed with *CYP11B1* gene mutation analysis (Azziz *et al.*, 1991; Carmina *et al.*, 1988; Eldar-Geva *et al.*, 1990; Kelestimur *et al.*, 1996; Sahin and Kelestimur, 1997).

Physiology

The zona glomerulosa and zona fasciculata in the adrenal cortex behave as distinct glands with specific biological functions. In zona fasciculata, the cholesterol precursors are converted to cortisol through a series of enzymatic reactions. CYP11B1 plays crucial role in the final step of conversion of 11-deoxycortisol to cortisol as depicted in **Fig. 1**. This pathway is under the regulatory effect

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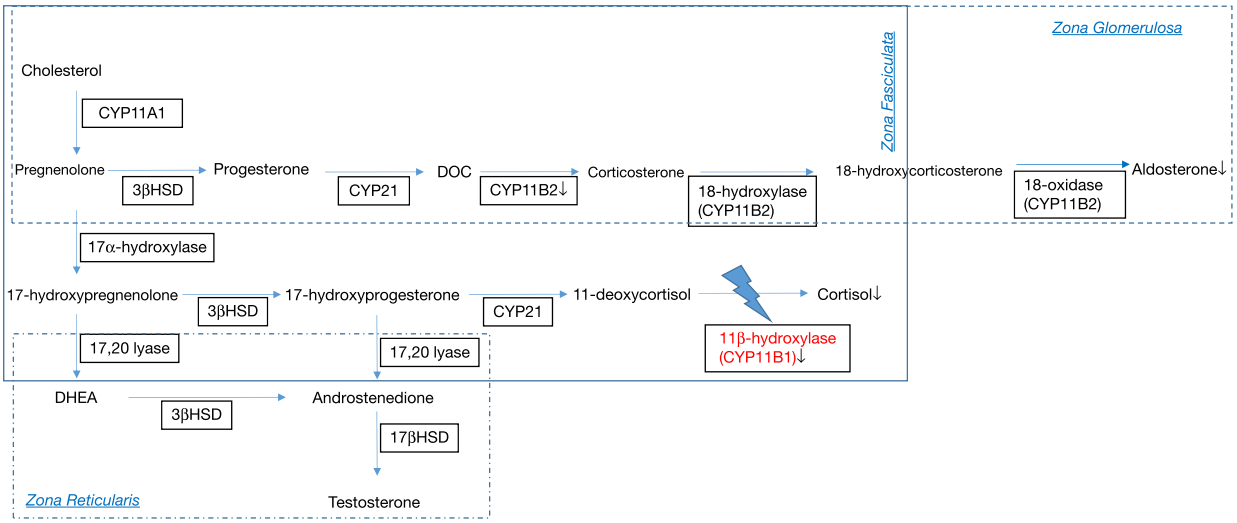

Fig. 1 Adrenal steroidogenesis. The classic pathways of aldosterone, cortisol, and androgen synthesis and the enzymatic steps from the precursor cholesterol are shown. Deficiency of 11β hydroxylase enzyme results in accumulation of 11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxyprogesterone and progesterone in addition to androgens. The arrows demonstrate decreased synthesis of CYP11B1 and CYP11B2 in *zona fasciculata and zona glomerulosa*, respectively. The low aldosterone levels result from an excess of DOC, which is accumulated due to a blocked glucocorticoid pathway. As this metabolite possesses mineralocorticoid features, the renin angiotensin system is not activated as evidenced by the low renin concentration. This leads to a decreased synthesis of CYP11B2 and thus, to a decreased aldosterone production. 3βHSD, 3 beta hydroxysteroid dehydrogenase; 17βHSD, 17 beta hydroxysteroid dehydrogenase; CYP11B1, 11 beta hydroxylase; CYP11B2, 11 beta hydroxylase; 11DOC, 11-deoxycorticosterone; DHEA, dehydroepiandrosterone; DOC, 11-deoxycorticosterone.

of hypothalamic corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) from the corticotroph cells of the pituitary gland.

In zona glomerulosa, the conversion of 11-deoxycorticosterone (DOC) to aldosterone requires three enzymatic reactions including 11β-hydroxylase, 18-hydroxylase, and 18-oxidase to convert DOC to corticosterone and subsequently to aldosterone (**Fig. 1**). This pathway is regulated by renin–angiotensin–aldosterone pathway and is independent of regulatory effect of ACTH. In vitro studies demonstrate CYP11B1 mediated conversion of DOC to corticosterone with inability to convert corticosterone to aldosterone. Hence, aldosterone is primarily produced in zona glomerulosa through the action of CYP11B2 (aldosterone synthase) which possesses 18-hydroxylase and 18-oxidase activities.

Pathophysiology

CYP11B1 mutations will result in defective 11β-hydroxylase enzyme and resultant defects in adrenal steroidogenesis. There is impaired conversion of 11-deoxycortisol and DOC to cortisol and corticosterone respectively in zona fasciculata (Bulsari and Falhammar, 2017; Krone and Arlt, 2009; Nimkarn and New, 2008; White, 2001). The low cortisol level results in activation of hypothalamic pituitary adrenal feedback system and increased ACTH production with resultant adrenal cortex hyperplasia (White and Speiser, 2000) and in occasional cases, adrenal tumor formation (Falhammar, 2014; Falhammar and Torpy, 2016; Jaresch *et al.*, 1992).

Blockade of cortisol secretion due to CYP11B1 enzymatic deficiency results in accumulation of precursor steroid metabolites which are shunted to androgen pathway (**Fig. 1**). DOC, and possibly other steroid precursors, possess mineralocorticoid activity and hence result in volume induced hypertension with resultant renin suppression and aldosterone deficiency. Elevated levels of androstenedione and testosterone levels due to shunting to androgen pathway result in symptoms of hyperandrogenism.

Cortisol appears to have important role in adrenomedullary organogenesis. Studies demonstrate significantly lower plasma epinephrine and urine metanephrine levels in patients with CAH and the degree of catecholamine deficiency appears to correlate with severity of adrenocortical dysfunction (Charmandari *et al.*, 2002; Falhammar *et al.*, 2011; Merke *et al.*, 2000; Tutunculer *et al.*, 2009).

Molecular Genetics

11β hydroxylase and aldosterone synthase are two homologous enzymes which are encoded by *CYP11B1* and *CYP11B2*, respectively in humans. *CYP11B1* gene is located on chromosome 8q24.3 (OMIM #202010), approximately 40 kb apart from the *CYP11B2* gene. There is 95% homology in coding regions and 90% homology in non-coding regions between *CYP11B1* and *CYP11B2*. The nine exons on CYP11B1 gene encode for 503 amino acids. More than 100 mutations including missense/nonsense,

small/gross deletions, insertions, splicing and complex rearrangements have been reported in various ethnic populations (www.hgmd.cf.ac.uk). Majority of mutations have been reported on exons 2, 6, 7, and 8 (Curnow *et al.*, 1993; Nimkarn and New, 2008).

Owing to the rarity of disease and significant variability in clinical presentations, it has been difficult to predict the actual disruptive effects of mutations and record genotype–phenotype co-relation until recently (Kandemir *et al.*, 2016). A recent international cohort study of 108 patients from 11 countries looked at clinical, genetic and structural effects of CYP11B1 gene mutations. This study noted certain specific mutations could be correlated to severity of 11βOHD, but this was not universally applicable (Khattab *et al.*, 2017). However, the majority of CYP11B1 gene mutations can distinguish between classic and NC 11βOHD (Bulsari and Falhammar, 2017).

Certain mutations occur with increased frequency in specific ethnic populations or geographical locations. In Jews of Moroccan origin, R448H, a point mutation is the most common mutation which was identified in 11 of 12 patients with 11βOHD (White *et al.*, 1991). On the other hand, c.945G>A splice site mutation was the most frequent mutation noted in a Turkish cohort (Kandemir *et al.*, 2016). This could be a result of high frequency of consanguineous marriages in specific populations with resultant founder effect.

Clinical Manifestations

Classic 11βOHD

Hyperandrogenism

In patients with 11βOHD, features of androgen excess can be detected from infancy to early adulthood.

In 46XX females, variable degree of virilization of external genitalia with intact functional gonads and internal genitalia derived from Mullerian structures has been noted. The severity of virilization is scored based on Prader staging system from stage zero to stage six. Females were diagnosed at a median age of 1.1 years with 80% females reported to have ambiguous genitalia at birth and incorrect gender assignment was common (Khattab *et al.*, 2017). In 46 XY males, enlarged penis with age appropriate testicular size may be noted during childhood or early adulthood. Untreated males may develop pubertal gynecomastia, Leydig cell hyperplasia (Rosler *et al.*, 1992) or testicular adrenal rest tumors (TARTs) (Oberman *et al.*, 1993).

Acne, premature adrenarche, hyperpigmentation of skins and nipples, peripheral precocious puberty, delayed menarche in females and poor spermatogenesis in males have been documented in 11βOHD as clinical signs of androgen excess (White *et al.*, 1994).

Other features of hyperandrogenism in both males and females include accelerated somatic growth, premature epiphyseal closure and resultant short adult stature. A retrospective study of 24 patients (15 girls, 9 boys) with 11βOHD were followed over a period of 15 years to assess growth and pubertal velocity (Hochberg *et al.*, 1985). Normal growth parameters of height, weight and bone age were noted for the first 4 months of life but accelerated by 11 months of life. Height was noted to be at a mean of 4 SDs above the mean for age between the ages of 2 and 5 years but the pubertal growth velocity was slower than normal. Final height was significantly compromised in all patients irrespective of age at diagnosis and therapeutic control. Precocious onset of puberty was noted in males but not in females.

Hypertension

Hypertension is a key differentiating feature from 21OHD. However, varying degrees of hypertension is observed in only two-thirds of the cases at time of diagnosis (Burren *et al.*, 1996; Rosler *et al.*, 1992). The recent international cohort study reported presence of hypertension in 59% of 11βOHD patients (Khattab *et al.*, 2017). The presence of excess DOC is thought to be responsible for hypertension, however, the mechanisms remain unclear (–see "Blood Pressure" section).

Electrolyte balance

Hypokalemia has been reported in a minority of patients with 11βOHD. It is believed to be due to excess mineralocorticoid precursors. In a study of 23 patients with 11βOHD, only six had overt hypokalemia (Rosler *et al.*, 1982). There appears to be no correlation between hypertension and hypokalemia. On the other hand, hypokalemic periodic paralysis has been reported as a presenting symptom of 11βOHD in teenage years (John *et al.*, 2009; Sathya *et al.*, 2012). Hence, 11βOHD should be considered as a differential diagnosis in a patient presenting with hypertension and hypokalemia or hypokalemic periodic paralysis (Falhammar *et al.*, 2013).

Salt-wasting is not a typical feature of 11βOHD unlike 21OHD. However, salt-wasting has been reported in the early neonatal period prior to treatment with glucocorticoids in patients with elevated basal DOC and renin levels. The mechanism is not well-understood but is possibly secondary to the natriuretic effect of 16-hydroxylated steroids, progesterone and pregnenolone from fetal adrenal tissue (Zachmann *et al.*, 1983). Therefore, it disappears in adult life and there are no reports of clinically significant salt-wasting in adults with 11βOHD. However, a minority of patients develop features of mineralocorticoid deficiency including hyperkalemia, hyponatremia, and hypovolemia during acute illness. Adequate dietary sodium intake and mineralocorticoid therapy may be required in such cases (Hochberg *et al.*, 1984; Liel, 1993). Rarely, glucocorticoid therapy may precipitate salt-wasting via suppression of DOC and resultant low mineralocorticoid activity (Zadik *et al.*, 1984).

Non-Classic 11βOHD

Patients with this milder form of 11βOHD usually have normal external genitalia at birth but present with peripheral precocious puberty in childhood or features such as acne, hirsutism and/or oligo-amenorrhea. Females are often misdiagnosed as polycystic

ovarian syndrome due to very similar clinical presentation. Hence, NC 11βOHD patients have similar clinical features as NC 21OHD (Falhammar *et al.*, 2008, 2015; Pall *et al.*, 2010). Patients with NC 11βOHD are usually normotensive or have borderline high-normal blood pressure at time of diagnosis (Parajes *et al.*, 2010; Reisch *et al.*, 2013; White, 2001).

The diagnosis of NC 11βOHD is based on either high basal or ACTH stimulated levels of serum 11-deoxycortisol level. NC 11βOHD should be considered as a differential diagnosis in cases of premature adrenarche, hirsutism, and menstrual irregularities.

Diagnosis

Reduced/absent activity of 11β-hydroxylase enzyme results in impaired production of cortisol and accumulation of steroid precursors (**Fig. 1**). Elevated basal levels of 11-deoxycortisol, DOC and 17-hydroxyprogesterone (17OHP) are noted in patients with 11βOHD. The increased 17OHP is then shunted to androgen pathway resulting in elevated androstenedione and testosterone. Accumulation of DOC results in suppression of renin–angiotensin system and hence reduced plasma renin and aldosterone levels are noted. These laboratory findings are summarized in **Table 1**.

Measurements of basal and ACTH stimulated 11-deoxycortisol and DOC aid in diagnosis of NC 11βOHD. Levels more than three times above the 95th percentile in normal population has been used for diagnosis in some clinical studies, however, it is not a specific marker (Azziz *et al.*, 1991; Kelestimur *et al.*, 1996; Sahin and Kelestimur, 1997).

Urinary steroid profile for tetrahydro-metabolites of steroid precursors such as tetrahydro-11-deoxycortisol (THS), tetrahydro-deoxycorticosterone (THDOC) can be helpful in diagnosis of 11βOHD and monitoring efficacy of treatment (Burren *et al.*, 1996).

Genetic profile and functional characterization of mutations is important in order to confirm the diagnosis as well as provide appropriate genetic counseling (Parajes *et al.*, 2010).

Complications

Mortality

Mortality has not been studied in detail in 11βOHD. However, it can be extrapolated from the data available in 21OHD patients which shows three times higher hazard ratio for mortality compared to controls (Falhammar *et al.*, 2014). The mortality rate in 11βOHD could be higher compared to 21OHD due to higher cardiovascular risk (Bulsari and Falhammar, 2017). However, the risk of adrenal crisis is believed to be lower in 11βOHD than 21OHD and hence the mortality might be reduced compared to 21OHD but is likely to be higher than the general population (Burren *et al.*, 1996).

Cardiovascular and Metabolic Complications

Blood pressure

As mentioned above, hypertension is the hallmark features of classic 11βOHD. It is assumed to be secondary to excess mineralocorticoid activity of DOC. However, there is no correlation between plasma levels of DOC and severity of hypertension (Khattab *et al.*, 2017; White *et al.*, 1994; Zachmann *et al.*, 1983). There is poor correlation between degree of virilization and features of mineralocorticoid excess (Rosler *et al.*, 1982, 1992). DOC has been shown to have weak mineralocorticoid activity

Table 1 Comparison of clinical features of three forms of CAH including 11β-hydroxylase deficiency, 21-hydroxylase deficiency, and 17-hydroxylase deficiency

	11β-Hydroxylase deficiency	*21-Hydroxylase deficiency*	*17-Hydroxylase deficiency*
Affected gene	*CYP11B1*	*CYP21A2*	*CYP17A1*
Prevalence (classic)	1 in 100,00 to 1 in 9,000,000[a]	1 in 15,000	1 in 50,000 to 1 in 9,000,000
Salt wasting	Rare	Common	Rare
Hypertension	Common	Rare	Common
Degree of virilization	Mild-severe	Mild-severe	Nil
11-Deoxycortisol	Elevated	Low	Low
Deoxycorticosterone	Elevated	Low	Elevated
17-Hydroxyprogesterone	Elevated	Elevated	Low
Progesterone	Elevated	Elevated	Elevated
Renin	Low	Elevated	Low
Aldosterone	Low	Low	Low to elevated
Androstenedione	Elevated	Elevated	Low
Testosterone	Elevated	Elevated	Low
Treatment	Glucocorticoids	Glucocorticoids and often mineralocorticoids	Glucocorticoids

[a]Incidence in Caucasian population.

in vivo compared to 18-hydroxylated compounds which are hence, speculated to be the cause of hypertension in 11βOHD. Both inadequate dosing and over-treatment with glucocorticoids can contribute to hypertension. Suboptimal blood pressure control and over-replacement with glucocorticoids will have negative effects on cardio-metabolic profile. There are multiple reports of significant end-organ damage secondary to hypertension including left ventricular hypertrophy, hypertensive retinopathy, hypertensive nephropathy, encephalopathy, ischemic heart disease, cerebrovascular accidents and deaths in patients with 11βOHD (Chabre *et al.*, 2000; Glenthoj *et al.*, 1980; Hague and Honour, 1983; John *et al.*, 2009; Melcescu *et al.*, 2012; Rosler *et al.*, 1982, 1992).

Metabolic complications

There is wide variations in BMI in patients with classic and NC 11βOHD (Jermendy *et al.*, 1991; Menabo *et al.*, 2014; Parajes *et al.*, 2010; Polat *et al.*, 2014; Reisch *et al.*, 2013). Obesity is a well-known risk factor for adverse cardiovascular outcomes. The prevalence of type 2 diabetes in 11βOHD has not been reported except for two cases of type 2 diabetes in NC 11βOHD (Jermendy *et al.*, 1991).

Height and Bone Health

There is little information known in regards to height and bone health in patients with 11βOHD.

Final height

Advanced bone age has been well reported with males significantly exceeding females in terms of bone age (Khattab *et al.*, 2017). The mean final height was significantly lower in patients with 11βOHD compared to general population. The possible factors contributing to slow pubertal growth velocity and reduced final height include delay in diagnosis and over-replacement with glucocorticoids. However, there was poor correlation between age at diagnosis and efficacy of glucocorticoid therapy measured with clinical biochemical control and mean final height (Hochberg *et al.*, 1985). Hydrocortisone was superior to cortisone acetate and prednisolone in terms of growth velocity.

Bone health

There is no available information on bone mineral density (BMD) in patients with 11βOHD. However, as seen with 21OHD, long term glucocorticoid exposure is expected to have negative impact on BMD (Bulsari and Falhammar, 2017).

Adrenocortical Tumors

In 21OHD, chronically elevated ACTH levels result in stimulation of adrenal cortex and adrenal hyperplasia (Falhammar, 2014; Falhammar and Torpy, 2016; Jaresch *et al.*, 1992; Nermoen *et al.*, 2011). Similar studies in 11βOHD are lacking. However, there are reports of adrenal incidentalomas found in cases of classic and NC 11βOHD. None of these cases were confirmed with genetic testing (John *et al.*, 2009; Kacem *et al.*, 2000; Touitou *et al.*, 1989). In vitro enzymatic analyses and ACTH stimulation test in patients with functional adrenocortical tumors and nonfunctional adrenal incidentalomas have shown evidence of impaired 11β-hydroxylation activity (Dall'Asta *et al.*, 2002; Doerr *et al.*, 1987; Reincke *et al.*, 1997; Werder *et al.*, 1994).

Testicular Adrenal Rest Tumors

Testicular adrenal rest tumors (TARTs) mostly affect the rete testes on bilateral sides and are benign testicular masses. In 21OHD patients, functional studies have identified ACTH and angiotensin II (AII) receptors in addition to adrenal specific CYP11B1 and CYP11B2 enzymes. Histological studies revealed significant resemblance between TARTs and adrenocortical tissues along with presence of ACTH and AII receptors in the TART tissue (Claahsen-van der Grinten *et al.*, 2007). There was poor relationship between tumor growth and ACTH suppression as well as total TART volume and age, glucocorticoid dosing, plasma renin and ACTH concentrations (Falhammar *et al.*, 2012; Reisch *et al.*, 2010; Stikkelbroeck *et al.*, 2004).

Owing to its central location, TART tissue causes compression of seminiferous tubules with fibrosis and tubular hyalinization leading to obstructive azoospermia. Among five males with 11βOHD, 60% (three out of five) had TART identified on ultrasonography, indicating possible high prevalence (Aycan *et al.*, 2013). Complete disappearance to no response on high dose steroid therapy have been reported in cases with 11βOHD (Bulsari and Falhammar, 2017). Testis sparing surgery has been shown to improve fertility in some cases of 21OHD with persistent azoospermia or poor response to glucocorticoid therapy (Tiryaki *et al.*, 2005; Walker *et al.*, 1997). TART can be easily misdiagnosed as testicular malignancy and patients with CAH have undergone testicular surgery in the past (Arlt *et al.*, 2010; Falhammar *et al.*, 2012). Hence, it is an important differential diagnosis to be considered prior to testicular surgery in a patient with CAH (Falhammar and Thoren, 2012).

Given significant impact of TART on fertility, early diagnosis and treatment is important. Screening with scrotal ultrasound from early childhood to peripubertal period should be considered (Aycan *et al.*, 2013). We suggest scrotal ultrasound every 2–5 years or earlier if clinically indicated as there is lack of consensus guidelines to specify frequency of screening in adults.

Fertility

Male fertility

Azoospermia has been reported in majority of cases with 11βOHD. However, semen analyses results were available on in cases of TARTs giving a false impression of high rates of subfertility in males with 11βOHD (Aycan *et al.*, 2013; Kaynar *et al.*, 2014; Willi *et al.*, 1991). In addition to presence of TART, a multitude of sexual and psychosocial factors may play a role in male fertility (Falhammar and Thoren, 2012). Fertility was impaired in 221 males with 21OHD and it can be assumed to be similar in 11βOHD (Falhammar *et al.*, 2017).

Female fertility

There is limited information regarding female fertility in patients with 11βOHD. There are two case reports of successful pregnancy outcomes in patients with 11βOHD with no adverse maternal or fetal outcomes (Simm and Zacharin, 2007; Toaff *et al.*, 1975). Fertility outcomes for 11βOHD can be inferred to some extent from the currently available data on pregnancy and fertility in females with 21OHD. 21OHD patients had higher incidence of gestational diabetes and emergency or elective caesarean section compared to controls (Falhammar *et al.*, 2007; Hagenfeldt *et al.*, 2008). As dexamethasone is not metabolized by placental 11 beta hydroxysteroid dehydrogenase type II, it should be avoided during pregnancy (Speiser *et al.*, 2010).

Prenatal Diagnosis

The fetal status for 11βOHD can be assessed by molecular genetic analysis of CYP11B1 gene in the extracted fetal DNA by chorionic villus sampling (Bouchard *et al.*, 1989; Cerame *et al.*, 1999; Curnow *et al.*, 1993; Geley *et al.*, 1996). There is controversy regarding prenatal treatment with dexamethasone in case of an affected female fetus and such treatment should be considered only in research settings (Speiser *et al.*, 2010).

Gynecomastia

Pre-pubertal gynecomastia has been reported in 11βOHD (Bulsari and Falhammar, 2017). The mechanisms of gynecomastia remain unclear. There are two plausible explanations for gynecomastia. Firstly, there are high levels of circulating adrenal androgens which undergo peripheral aromatization to estradiol. Secondly, 11-deoxycortisol and DOC are potent mineralocorticoids with possible anti-androgen effect contributing to gynecomastia (Hochberg *et al.*, 1991). Treatment with glucocorticoids resulted in reversal of gynecomastia in patients with 11βOHD (Hochberg *et al.*, 1991; Maclaren *et al.*, 1975; Rosler *et al.*, 1992; Zachmann and Prader, 1975; Zachmann *et al.*, 1983; Zadik *et al.*, 1979).

Management

Medical Therapy

Glucocorticoid therapy and monitoring

The basic principle of therapy is to replace cortisol deficiency and hence, reduce excess production of adrenal androgens and mineralocorticoid precursors. Hydrocortisone in doses of 10–20 mg/m^2/day in two to three divided doses is the most commonly used glucocorticoid preparation. It has been reported to be effective in most patients with 11βOHD similar to in 21OHD (Burren *et al.*, 1996; Nimkarn and New, 2008; Peter, 2002). Hydrocortisone is preferred in children due to short duration of action and hence less potential for growth suppression (Hochberg *et al.*, 1985; Speiser *et al.*, 2010). During adulthood, intermediate acting and long acting glucocorticoids such as prednisolone (2.5–7.5 mg/day in divided doses) and dexamethasone (0.25–0.5 mg at bedtime or in divided doses), respectively have been used in 21OHD. Modified release hydrocortisone preparation has been studied in 21OHD with potential benefits, but not in 11βOHD (Quinkler *et al.*, 2015). In case of acute illness, stress doses of glucocorticoids are required in all patients with classic 11βOHD although, the risk of adrenal crisis is lower with 11βOHD compared to 21OHD patients (Burren *et al.*, 1996; Nimkarn and New, 2008; Peter, 2002).

The patients should be monitored at regular intervals for clinical assessment and documentation of virilization, growth parameters and control of hypertension (Rosler *et al.*, 1982). Adequate therapy should result in optimal blood pressure and control of hyperandrogenism features along with optimization of growth and fertility preservation (Nimkarn and New, 2008). Plasma 11-deoxycortisol, DOC and renin activity are useful to monitor adequacy of glucocorticoid therapy (Nimkarn and New, 2008; Rosler *et al.*, 1982). Patients on suboptimal doses will have high DOC levels with suppressed renin activity. The DOC levels and renin activity should normalize once appropriate glucocorticoid therapy is initiated. The glucocorticoid therapy has to be monitored and adjusted closely to reduce risk of iatrogenic Cushing's syndrome.

Control of hypertension

Despite adequate glucocorticoid dosing, blood pressure may remain elevated in certain cases. An additional anti-hypertensive agent should be added in such cases. The agents of choice are amiloride or spironolactone as monotherapy or in combination with

calcium channel blockers (White, 2001). Angiotensin converting enzyme inhibitors and angiotensin II receptor blockers should be avoided as the renin angiotensin system is suppressed in patients with 11βOHD. Due to the potential risk of hypokalemia with thiazide diuretics, they should be avoided or used in combination with potassium sparing diuretics.

Mineralocorticoid therapy

Patients with 11βOHD have elevated mineralocorticoid precursors prior to treatment with glucocorticoids. Initiation of glucocorticoid therapy can result in suppression of excess mineralocorticoid precursors and relative mineralocorticoid deficiency. Mineralocorticoid therapy has been shown to reduce glucocorticoid requirement and achieve better growth outcomes in a study where patients with 11βOHD had elevated renin activity despite optimal glucocorticoid doses (Rosler and Leiberman, 1984). However, patients need careful monitoring of blood pressure, urinary electrolytes and renin levels with mineralocorticoid therapy. Similarly, patients with acute illness and electrolyte imbalance may require mineralocorticoid therapy (Hochberg *et al.*, 1984; Liel, 1993; Zadik *et al.*, 1984). In the early neonatal period, salt-wasting has been reported as described earlier (Zachmann *et al.*, 1983). Hence, when the diagnostic test results are pending in early neonatal period, mineralocorticoid therapy should be considered in addition to glucocorticoid replacement to avoid renal salt-wasting. The mineralocorticoid therapy can be gradually withdrawn over the first year of life with close follow-up and monitoring.

Growth augmenting therapy

In children, close monitoring of growth velocity and bone maturation is an important aspect in management of CAH (Speiser *et al.*, 2010). As discussed earlier, children with 11βOHD have significantly lower final height irrespective of age at diagnosis and biochemical control. Hence, growth augmentation therapies have been trialed. There are case reports of using growth hormone, gonadotropin releasing hormone (GnRH) analogue and glucocorticoid combination therapy, aromatase inhibitor and growth hormone combination therapy in children with 11βOHD with improved final height outcome and no reported adverse events (Bulsari and Falhammar, 2017). There is lack of randomized trials and the long term effects of above therapy are unknown. Hence, such treatment is recommended only in research settings.

Surgical Therapy

Genital reconstruction/correction surgery

In addition to above medical therapy, corrective surgeries for genital abnormalities (e.g., vaginoplasty and clitoral reduction) have to be considered in 46XX patients with severe virilization. The appropriate timing of surgical intervention is not clear in currently available guidelines. The surgical treatment should be performed in specialized centers where medical, surgical and psychological expertise in CAH care is available (Nordenskjold *et al.*, 2008; Speiser *et al.*, 2010). Additional surgeries or vaginal dilatation may be required in adolescent life and hence, these patients require ongoing follow-up with the surgical clinic.

Adrenalectomy

Bilateral adrenalectomy has been described as a successful management option in five case reports of patients with 11βOHD. This has been implemented in cases with poorly controlled hypertension and end-organ damage, severe hypokalemia and extreme hyperandrogenism requiring high doses of glucocorticoid therapy (Chabre *et al.*, 2000; John *et al.*, 2009; Kacem *et al.*, 2009; Nasir *et al.*, 1996). Long-term outcome has been reported in one case followed up until 72 months post-operatively. The hypertension resolved rapidly within 15 days post-operatively. Interestingly, the 11-deoxycortisol levels remained elevated during follow-up with normal blood pressure and no focus of adrenal rest tissue (Kacem *et al.*, 2009).

The risks and benefits of bilateral adrenalectomy have to be weighed carefully. Due to lack of evidence and the increased risk of adrenal crisis, bilateral adrenalectomy should be considered only in selected cases.

Conclusion

11βOHD is a rare autosomal recessive disorder with only a few studies reported in the literature with limited number of patients included. Owing to relatively low prevalence and significant phenotypic variability, diagnosis of 11βOHD is usually delayed unless there is high index of clinical suspicion. 11βOHD is associated with multiple complications including adverse cardiovascular, final height and fertility outcomes. Early diagnosis and appropriate management of such cases are likely to improve long-term outcomes. Medical management of 11βOHD requires close monitoring and surveillance to avoid complications of over-replacement with glucocorticoids. Ongoing research and collaborative studies are of paramount importance to explore the underlying pathophysiology, molecular genetics and management strategies in patients with 11βOHD.

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Human P450 Oxidoreductase Deficiency

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Introduction

P450 oxidoreductase deficiency (PORD, OMIM 613571) is a form of congenital adrenal hyperplasia (CAH) (Pandey and Flück, 2013; Burkhard *et al.*, 2017). CAH is defined as an inborn error of adrenal steroid biosynthesis affecting glucocorticoid production (**Fig. 1**) (Miller and Fluck, 2014; Fluck, 2017). The most common and first described form of CAH is steroid enzyme 21-hydroxylase deficiency, in which mutations of the *CYP21A2* gene lead to diminished or lacking glucocorticoid and maybe mineralocorticoid production, and thus compensatory androgen overproduction. POR is an essential redox partner for the catalytic activity of CYP21A2, but other than specific enzyme deficiencies of adrenal steroidogenesis involved in cortisol production, PORD manifests several symptoms in addition to the CAH phenotype. As a redox partner to dozens of cytochrome P450, effects of mutations in POR reach far beyond steroid biosynthesis and POR variants manifest with a very broad phenotype like a chameleon changing color depending on its surrounding.

Interestingly, the mouse knockout (KO) of *Por* −/− was created before the first human POR mutations were found. It was found to be embryonic lethal (Shen *et al.*, 2002; Otto *et al.*, 2003), and resembled the KO mouse of retinoic acid metabolizing *Cyp26a1* −/− (Sakai *et al.*, 2001). While the same phenotype was also observed with toxic embryonic retinoic acid exposure, retinoic acid lowering measures were able to mitigate the phenotype of *Por* −/− (Otto *et al.*, 2003; Lee *et al.*, 2004; Ribes *et al.*, 2007). By contrast, a conditional liver-specific KO model yielded normal, fertile mice with disrupted hepatic drug metabolism and lipid accumulation (Gu *et al.*, 2003; Henderson *et al.*, 2003).

Clinical Manifestation

The first clinical description of PORD patients was through the remarkable steroid profile combining characteristics of 21-hydroxylase and 17-hydroxylase deficiency, but no genetic mutations in the genes of *CYP21A2* and *CYP17A1* were found (Peterson *et al.*, 1985). The reported patient manifested at 6 months of age with a 46,XY disorder of sexual development (DSD) characterized by a female phenotype with ambiguous genitalia. Although the hypothesis that the underlying genetic cause might lie in redox partners POR or

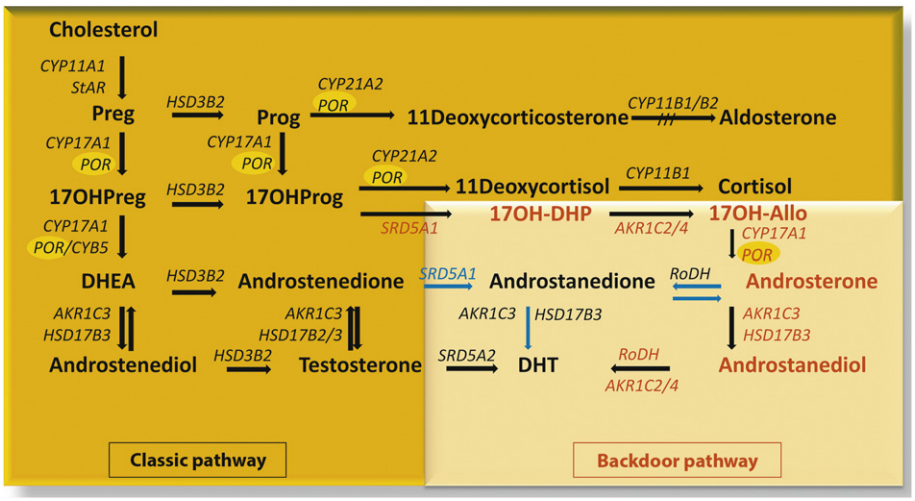

Fig. 1 Steroidogenesis of the human adrenal cortex. Mineralocorticoids, glucocorticoids, and adrenal sex steroids are produced from cholesterol through multiple catalytic reactions (shown as *arrows*) supported by specific steroid enzymes and cofactors such as P450 oxidoreductase (POR) (given in *italics*). The classic pathway for mineralocorticoid, glucocorticoid and androgen production is shown in *dark yellow*, while the alternative pathway for dihydrotestosterone (DHT) synthesis is shown in yellow. POR (shown in bright *yellow*) is essential electron transfer donor to CYP21A1 and CYP17A1 in several steps of adrenal steroidogenesis, and therefore causing a phenotype of congenital adrenal hyperplasia (CAH) as well as disordered sex development (DSD) in both males and females. *Preg*, pregnenolone; *17OHPreg*, 17-hydroxypregnenolone; *Prog*, progesterone; *17OHProg*, 17-hydroxyprogesterone; *DHEA*, dehydroepiandrosterone; *17OH-DHP*, 17-hydroxy-dihydroprogesterone; *17OH-Allo*, 17-hydroxy-allopregnanolone.

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CYB5 was expressed in a letter ensuing the report (Miller, 1986), POR mutations were found many years later, when additional patients with similar unexplained phenotypes were found and sequenced for variations in the *POR* gene (Arlt *et al.*, 2004; Flück *et al.*, 2004; Miller *et al.*, 2004; Pandey *et al.*, 2004). In a first report three patients with POR deficiency (PORD) were described showing skeletal malformations with craniosynostosis known as Antley–Bixler syndrome (ABS) and genital anomalies at birth both in 46,XX and 46,XY subjects (Flück *et al.*, 2004). Mild adrenal insufficiency was diagnosed at follow-up upon stimulation testing. On the other hand, POR mutations were also found in a 23-year young women seeking help for primary amenorrhea with hypoplastic uterus and enlarged cystic ovaries, but normal breast development, and mild arterial hypertension (Flück *et al.*, 2004). She had otherwise no dysmorphic features. Concurrently, similar POR mutations were found in three other individuals (Arlt *et al.*, 2004). Two siblings were reported with PORD; the 46,XX girl had severe bone malformations and virilization of the external genitalia at birth, while her younger affected 46,XY brother showed a normal male phenotype at birth and had no ABS-like features (Arlt *et al.*, 2004). In addition, a 46,XX girl with POR mutations was found to have minor ABS features with ambiguous genitalia at birth, and developed large ovarian cysts during puberty leading to ovarian ruptures and therefore ovarectomy (Arlt *et al.*, 2004).

Over the last 15 years, more than 100 patients with PORD have been reported either in series or as case reports (**Table 1**). The manifesting phenotype remains very broad ranging from severely affected individuals showing an ABS with DSD at birth to being normal at birth, but having minor problems with steroid hormone production leading to pubertal disturbances, a polycystic ovary syndrome-like phenotype or fertility problems toward adulthood. There might be even asymptomatic carriers. Therefore, the clinical diagnosis of PORD is challenging. Genotype–phenotype and structure–function correlations may explain some observed characteristics, while others remain a conundrum.

Table 1 Characteristics of 120 reported patients with POR deficiency

			Phenotype			
Number of patients	Chromosomal sex 46,XX/46,XY	POR mutations	ABS features/skeletal anomalies	Abnormal genitalia	Abnormal steroids	References
4	2/2	7/8[a]	3	3	4	Flück *et al.* (2004)
3	2/1	6/6	1	2	3	Arlt *et al.* (2004)
2	1/1	4/4	2	1	2	Adachi *et al.* (2004)
19(32[b])	6[c]/10[c]	34/38[a]	19(32)	12[c]	10[c]	Huang *et al.* (2005)
10	6/4	19/20[a]	9	9	10	Fukami *et al.* (2005)
3	2/1	6/6	0	2	3	Fukami *et al.* (2006)
7	2/5	14/14	5	2	7	Homma *et al.* (2006)
1	1/0	1/2[a]	1	1	1	
4	0/4	8/8	0	4	4	Hershkovitz *et al.* (2008)
12(35)	19/16	70/70	28	26		Flück *et al.* (2009)
4	3/1	8/8	3	3	4	Sahakitrungruang *et al.* (2009)
1	1/0	2/2	1	1	1	
1	0/1	2[d]	0	1	1	Idkowiak *et al.* (2011)
7	5/2	14/14	7	5	7	Idkowiak *et al.* (2011)
2	2/0	4/4	2	2	2	Flück *et al.* (2011)
1	1/0	2/2	1	0	1	Herkert *et al.* (2011)
30	18/12	54/60	27	22	28[c]	Krone *et al.* (2012)
1	0/1	2/2	0	1	1	Armeni *et al.* (2012)
1[e]	1/0	2/2	1	1	?	Oldani *et al.* (2015)
1	1/0	2/2	0	1	1	Parween *et al.* (2016)
1	1/0	2/2	1	1	1	
3[e]	1/1 + ?	6/6	3	2 + ?	?	Tzetis *et al.* (2016)
1	1/0	2/2	1	1	1	But *et al.* (2010)
1	1/0	2/2	(1)	1	1	Bai *et al.* (2017)

[a]POR mutations not identified on all alleles.

[b]19 out of 32 patients with the ABS phenotype had POR mutations.

[c]Karyotype, description of genitalia or steroid profile not known for all patients.

[d]Additional heterozygote mutation Q798E in the androgen receptor gene.

[e]Prenatal diagnosis at 17, 22, 23 (Tzetis *et al.*, 2016), and 26 (Oldani *et al.*, 2015) weeks gestation with pregnancy termination.

Modified from Burkhard, F. Z., S. Parween, S. S. Udhane, C. E. Flück and A. V. Pandey (2017). "P450 Oxidoreductase deficiency: Analysis of mutations and polymorphisms." Journal of Steroid Biochemistry and Molecular Biology 165(Pt A), 38–50.

PORD and Pregnancy

Remarkably, mothers carrying a fetus with PORD may notice signs of virilization on their own body during pregnancy in 1/3–1/2 of cases (Arlt *et al.*, 2004; Flück *et al.*, 2004; Reisch *et al.*, 2013; Bai *et al.*, 2017). These include oily hair, acne, hirsutism, and deepening of the voice, and mostly disappear after delivery. This phenomenon is also observed in mothers carrying a fetus with aromatase (CYP19) deficiency or with pregnancy luteoma (Miller and Auchus, 2011). POR is the essential cofactor to CYP19 and CYP21, and therefore mutations of fetal POR may cause androgen excess in the fetal-placental unit. The suggested mechanism seems complex and is explained in more details in **Fig. 2** as well as in Reisch et al. (2013). In addition fetal sex development may be affected in both sexes leading to ambiguous genitalia detected in ultrasound screening. Furthermore, prenatal ultrasound may also reveal skeletal anomalies compatible with ABS including craniosynostosis, femoral bowing and bilateral radiohumoral synostosis (Reisch *et al.*, 2013; Oldani *et al.*, 2015; Tzetis *et al.*, 2016).

However, why some subjects with same POR mutations, even from same families/parents may present with different phenotypes, also with respect to maternal virilization, remains unexplained.

PORD and DSD

New born boys and girls with PORD may have a DSD phenotype due to disturbed intrauterine steroid hormone production according to the Chicago consensus DSD classification (Hughes *et al.*, 2006). This seems contradictive, given that most other forms of CAH associated with DSD result in either 46,XX DSD (e.g., CYP21 deficiency) or 46,XY DSD (e.g., CYP17 deficiency), with the exception of HSD3B2 deficiency, in which also both female virilization and male undermasculinization are observed. External genitalia of 46,XX girls may be significantly virilized (Prader III–V) at birth leading to male sex assignment. But unlike in CAH due to classic 21-hydroxylase deficiency, postnatal progression of virilization in affected girls does not occur, because circulating androgen levels are low after birth in both sexes. This intrauterine androgen excess, affecting the female phenotype only, can be explained by an alternative pathway for androgen biosynthesis from dehydroepiandrosterone (DHEA) that seems present merely in utero and in early postnatal life (**Fig. 2**) (Arlt *et al.*, 2004; Homma *et al.*, 2006; Williamson *et al.*, 2006). Noteworthy, this alternative androgen production does not seem to compensate for the classic sex steroid biosynthetic pathway in the male fetus. Therefore, external genitalia of 46,XY boys with PORD may be undervirilized at birth varying from micropenis and cryptorchidism to severe hypospadias due to PORD-related CYP17 deficiency (Flück and Pandey, 2017a,b). Its clinical and biochemical picture may be indistinguishable from isolated 17,20 lyase or cytochrome *b*5 deficiency (Hershkovitz *et al.*, 2008; Miller, 2012; Flück and Pandey, 2017a,b). However, to our knowledge complete sex reversal has not been described in 46,XY PORD so far.

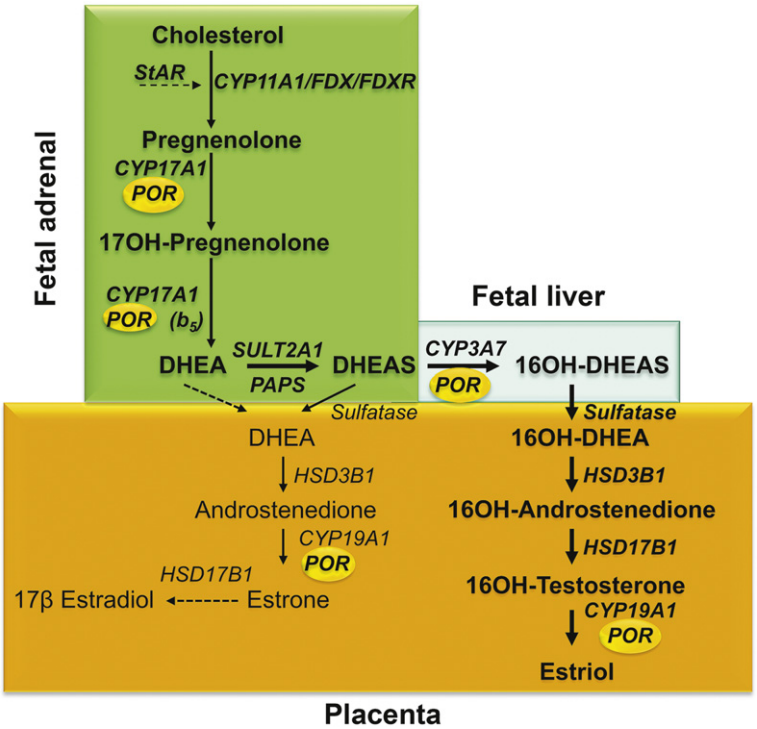

Fig. 2 Scheme of the fetal adrenal-placental steroid metabolism. Updated from Miller, W. L. and Fluck, C. E. (2014). Adrenal cortex and its disorders. In *Pediatric endocrinology*. M. A. Sperling, ed. Philadelphia: Saunders.

Adrenal Insufficiency/CAH and PORD

Although POR is needed for two catalytic reactions along the pathway for cortisol production from cholesterol (**Fig. 1**), overt adrenal insufficiency is rarely described in PORD. But adrenal insufficiency may be diagnosed at birth through neonatal screening established for CAH due to mutations in the *CYP21A2* gene using 17-hydroxyprogesterone as marker steroid. Thus several cases are reported that have been mis-diagnosed with CAH due to 21-hydroxylase deficiency, in whom no *CYP21A2* but *POR* mutations have been found (Fukami *et al.*, 2006; But *et al.*, 2010). Fortunately for them, this led to early protective steroid replacement therapy.

Asymptomatic adrenal insufficiency is often found in PORD with ACTH testing showing normal cortisol levels at baseline, but an inadequate rise in cortisol upon ACTH stimulation. CAH due to PORD has a mixed character of both 17- and 21-hydroxylase deficiency and causes therefore usually a mild glucocorticoid deficiency and a mild mineralocorticoid excess, which may lead to arterial hypertension with aging, similar as seen in patients with *CYP17A1* mutations. As mild adrenal insufficiency may not be relevant in healthy days, but life-threatening in sick days, it is important that all patients with an ABS phenotype or known POR mutations are tested for their adrenal function and individually advised for their need of glucocorticoid replacement therapy either continuously or at least in stress situations.

Pubertal Development and Gonadal Function in PORD

PORD affects CYP17 activity and thereby sex hormone biosynthesis. It may or may not lead to a DSD phenotype manifesting at birth (as described in section "PORD and DSD") and/or result in absent or insufficient pubertal development in both males and females. Several young females were diagnosed with PORD after medical work-up for primary amenorrhea or menstrual irregularities, normal breast development and (recurrent) large ovarian cysts complicated by spontaneous ruptures (Flück *et al.*, 2004; Fukami *et al.*, 2009; Sahakitrungruang *et al.*, 2009; Herkert *et al.*, 2011; Idkowiak *et al.*, 2011; Bai *et al.*, 2017). They presented with a hormonal profile of hypergonadotropic hypogonadism, elevated 17OHProg, but low or normal androgens and estrogens, and were successfully treated with estrogens/progestins and glucocorticoids. Ovarian cysts are frequently seen in females suffering from steroid biosynthetic disorders and may be seen even in early childhood (Idkowiak *et al.*, 2011). The pathomechanism of ovarian cysts in PORD may be mediated through (a) excessive ovarian LH stimulation caused by insufficient ovarian sex steroid production, (b) chronic anovulation, and (c) disrupted synthesis of meiosis-activating sterols (MAS) and metabolism essential for oocyte meiosis and maturation with onset of puberty (Grondahl *et al.*, 2000; Idkowiak *et al.*, 2011; Bai *et al.*, 2017). It is therefore conceivable that PORD may affect female fertility harder than other steroid biosynthetic defects, in which pregnancies have been achieved through personalized (hormonal) fertility techniques (e.g., CYP17 deficiency Bianchi *et al.*, 2016).

By contrast, males with PORD seem overall less affected in their pubertal development than females. However, while normal pubertal development and sexual function seem possible (Idkowiak *et al.*, 2011), infertility due to incomplete sexual development and azoospermia have also been reported (Armeni *et al.*, 2012). To date little is known on testis histology, fertility, and malignancy risk in 46,XY DSD patients owing to androgen biosynthetic defects in general, and in PORD specifically (Burckhardt *et al.*, 2015).

ABS and Skeletal Malformations With PORD

While 2/3 of patients with PORD have some (minor) skeletal anomalies, a severe ABS phenotype is less frequently observed. Craniosynostosis is observed in variable severity (e.g., cloverleaf skull) and may cause hydrocephalus necessitating surgical intervention. Craniofacial anomalies include midface hypoplasia and choanal atresia, low set ears and a pear-shaped nose. However, radiohumeral synostosis, clinodactyly, and arachnodactyly are more frequently observed with PORD, while femoral bowing can be observed already prenatally in rare cases causing neonatal fractures. Conversely, about 50% of children born with an ABS phenotype have accompanying genital anomalies (Reardon *et al.*, 2000), and are most likely to harbor severe autosomal recessive POR gene mutations (Huang *et al.*, 2005).

ABS is a rare craniosynostosis syndrome first described by Antley and Bixler (1975). It is characterized by craniosynostosis and radiohumeral synostosis, but can be associated with a wide range of bony malformations. Neonatal mortality is high in ABS due to respiratory failure and improves with age. ABS without genital anomalies and/or disordered steroidogenesis is caused by autosomal dominant, gain-of-function mutations in the fibroblast growth factor receptor 2 (*FGFR2*) gene (OMIM 207410), or by homozygous mutations in the *CYP26B1* gene (OMIM 614416) (Laue *et al.*, 2011). Thus severe PORD may reproduce skeletal malformations similar to ABS due to mutations in *FGFR2* or *CYP26B1*, but has broader consequences on steroidogenesis and beyond, which should not be missed.

Other Possible Clinical Features

In theory, POR is the essential electron donor to all type II microsomal P450s, which are involved in numerous body functions reaching far beyond steroidogenesis and bone formation (Nebert and Russell, 2002; Miller, 2005; Pandey and Flück, 2013). Other known functions include metabolism of drugs, xenobiotics, arachidonic acid and eicosanoids, synthesis and metabolism of cholesterol, bile-acids and hemoglobin, as well as retinoic acid hydroxylation (**Fig. 3**) (Pandey and Flück, 2013). Nonetheless, the exact function of some of these possibly interacting P450s remains still unsolved. Thus the currently known spectrum of PORD might just be the tip of the iceberg.

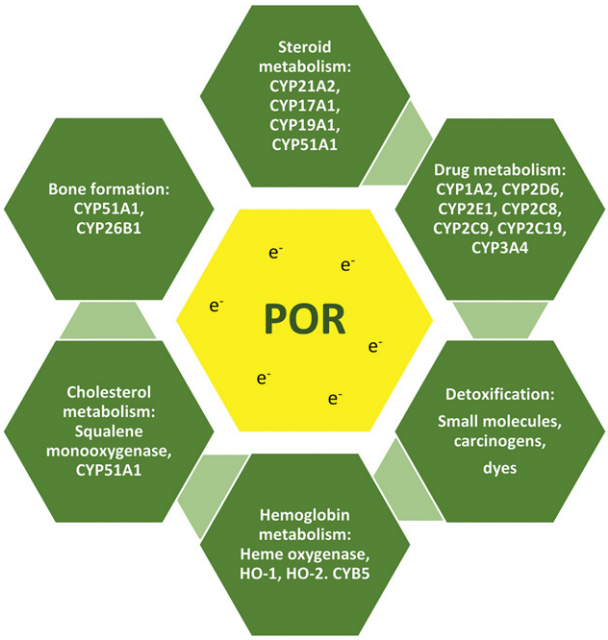

Fig. 3 Interacting redox partners of POR and their impact on life processes. The POR protein is a redox partner of many enzymes involved in both the drug as well as steroid metabolism. In addition POR can supply electrons to other proteins like cytochrome *b*5, heme oxygenase etc. POR can also directly metabolize several small molecules and drugs.

Concerning drug metabolism, prenatal fluconazole toxicity has been suggested in severely affected patients with ABS and DSD suffering from POR mutations, when mothers were treated with the drug during pregnancy (Reardon *et al.*, 2000). However, so far no clinically relevant, adverse drug events have been reported from PORD patients directly. However, drug-cocktail testing in a homozygous PORD patient and her heterozygous mother revealed diminished activities of several drug-metabolizing P450s (e.g., CYP1A2—caffeine, CYP2C9—tolbutamide, CYP2D6—dextrometorphan, CYP3A4—midazolan) (Tomalik-Scharte *et al.*, 2010). Nonetheless, several in vitro studies were able to show significant negative effects of many POR variants on drug metabolizing P450s (Pandey and Flück, 2013; Pandey and Sproll, 2014; Burkhard *et al.*, 2017; Udhane *et al.*, 2017).

Abnormal retinoic acid (RA) metabolism due to diminished CYP26 activity with PORD may not only lead to genital and skeletal malformation, but also cause anorectal and urinary anomalies as seen in the *por* KO model (Ribes *et al.*, 2007). Therefore, Fukami et al. investigated a group of 37 Japanese PORD patients for these specific anomalies and found imperforate anus in 11% and vesicoureteral reflux in 8% (Fukami *et al.*, 2010). Accordingly, plasma atRA levels were also found elevated in these patients.

Reduced HO-1 activity (*HMOX1*) has been assessed for several human POR variants (Pandey *et al.*, 2010) in vitro, and could cause oxidative neurotoxicity, anemia, growth retardation, and abnormal iron deposition in theory. Whether it is just a matter of time, and depending on close clinical follow-up of PORD patients before finding these hypothetical clinical consequences, remains to be seen.

Biochemical Characteristics of POR and PORD

The POR Protein and Its Electron Transfer Function

The reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 oxidoreductase (POR) is the obligate electron transfer partner for the cytochrome P450 proteins located in the endoplasmic reticulum (Pandey and Flück, 2013; Pandey and Sproll, 2014) (**Fig. 4**). Cytochrome P450s are responsible for metabolizing xenobiotics, drugs, and steroid hormones (Omura, 2010; Zanger and Schwab, 2013). In mammals and higher organisms there are two different types of cytochrome P450 proteins (Omura, 2010). Type 1 cytochromes P450 which metabolize steroids are localized in the mitochondria (Omura, 2006) and depend on adrenodoxin and adrenodoxin reductase as redox partners for their metabolic activities (Bernhardt and Urlacher, 2014; Zalewski *et al.*, 2016). The type 2 cytochromes P450 are located in the endoplasmic reticulum and depend on POR for supply of electrons for their catalytic activities (Lu *et al.*, 1969). Human POR is a flavoprotein that contains two flavin molecules, the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (**Fig. 5**). POR is a membrane associated protein that binds NADPH, causing a conformation change that brings NADPH and FAD close together to transfer electrons. Afterwards, further conformation changes cause a closing of the POR structure and bring FAD and FMN closer for electron transfer. The FMN binding domain of POR interacts with cytochrome P450 proteins and other redox partners through charge pair interactions. In addition to

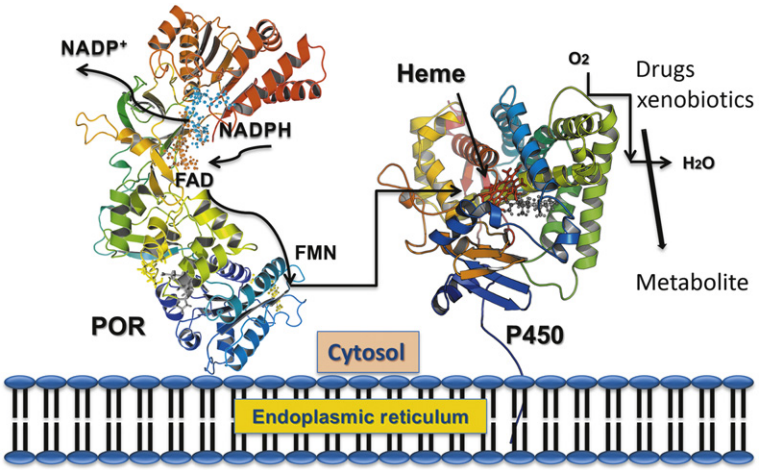

Fig. 4 Electron transfer from NADPH to redox partners of POR. NADPH binds to POR located into the endoplasmic reticulum, and donates electrons which are received by FAD. Electron transfer to FAD causes a conformational change that brings together the FAD and FMN domains and electrons are transferred from FAD to FMN. The FMN domain of POR interacts with the P450s and other redox partners and completes the final step of electron transfer. From Pandey, A. V. and P. Sproll (2014). "Pharmacogenomics of human P450 oxidoreductase." *Frontiers in Pharmacology* **5**, 103.

cytochromes P450 in the endoplasmic reticulum, POR supplies electrons to many other proteins and small molecules including heme oxygenase and cytochrome b_5 (Matsubara *et al.*, 1976; Nagao *et al.*, 1983; Guengerich, 2005).

Steroid Profile of PORD

POR supports three steroid enzymes for human glucocorticoid and sex steroid production in the adrenals and gonads, namely CYP21A1, CYP17A1, and CYP19A1. Changes of the steroid profile of PORD were first described as combined 21- and 17-hydroxylase deficiencies by Peterson et al. in a 6-months old 46,XY DSD baby (Peterson *et al.*, 1985). The detailed findings were specifically: (1) normal basal plasma cortisol and normal urinary excretion of cortisol metabolites, but high plasma ACTH levels, (2) high plasma 17α-hydroxyprogesterone and 21-deoxycortisol and increased urinary excretion of pregnanetriol, 17α-hydroxypregnanolone, and pregnenetriolone, (3) high plasma and urinary 5-pregnene-3β,20α-diol sulfate, (4) low plasma 21-hydroxy-pregnenolone and 5-pregnene-3β,17α, 20α-triol sulfate, (5) high plasma corticosterone and deoxycorticosterone and elevated urinary excretion of their metabolites, (6) high plasma progesterone and pregnenolone and increased urinary excretion of pregnanediol and pregnenediol, (7) low basal plasma levels of C19 steroids and their sulfates, without increase upon stimulation with human chorionic gonadotropin or ACTH, and (8) no urinary metabolites of C19 11-deoxy steroids, and decreased C19 11-oxosteroids.

In addition to these findings, low androgens and low-normal estrogen levels are common (in females) with PORD after puberty reflecting affected gonadal steroidogenesis (Idkowiak *et al.*, 2011). However, the spectrum of biochemical severity with PORD is extremely broad, and analysis of serum steroids alone can be misleading (Fukami et al., 2005, 2006; Williamson *et al.*, 2006). Therefore the diagnostic gold standard for PORD remains biochemical analysis by urinary gas chromatography mass spectrometry (GC–MS), which reveals accumulation of pregnenolone, progesterone, and 17OH-progesterone metabolites, but low androgens in the urine of affected individuals (Shackleton et al., 2004a,b). Calculation for specific POR-supported enzyme ratios may help to establish the diagnosis (Krone *et al.*, 2010). Interestingly, some specific alterations in urinary GC–MS profiling may be even seen in heterozygote POR subjects (Arlt *et al.*, 2004).

Similarly, diagnosis of PORD in a fetus may be made by prenatal GC–MS steroid profiling of maternal urine after 12 weeks gestation. Characteristic findings are (1) very low estriol levels (**Fig. 2**), combined with (2) increased excretion levels of epiallo-pregnanediol (a pregnenolone metabolite), and (3) androsterone (a 5α-reduced androgen **Fig. 1**) (Shackleton et al., 2004a,b; Reisch *et al.*, 2013). By contrast, low plasma estriol levels in pregnancy screening may be also attributed to other conditions of disturbed sterol and steroid biosynthesis besides PORD, such as steroid sulfatase deficiency (*STS*), Smith–Lemli–Opitz syndrome (*DHCR7*), aromatase deficiency (*CYP19A1*), as well as to several forms of severe congenital adrenal insufficiency (e.g., *NR5A1*, *NR0B1*, *CYP11A1*, *TBX19*).

Population Genetics of POR and PORD

After analysis of a large amount of sequencing data from 1000 Genome Project and other large sequencing studies we found the A503V variant of POR in different population has a minor allele frequency from 0.12 to 0.48 (Burkhard *et al.*, 2017). There are

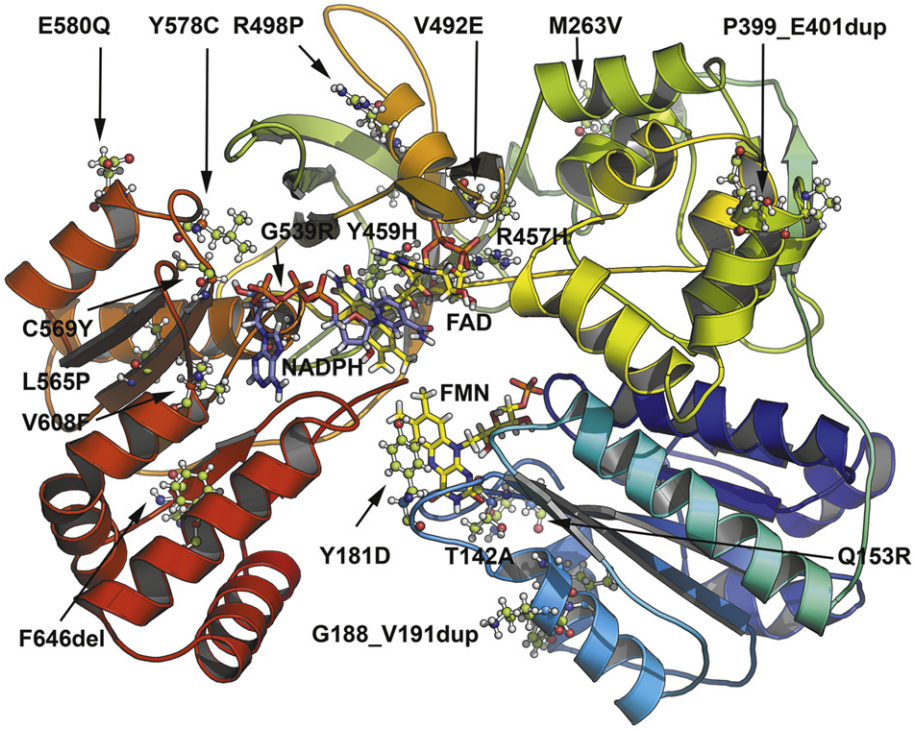

Fig. 5 Amino acid changes in POR identified in patients with PORD. A ribbons model of the human POR protein (Flück et al., 2009). The model is colored in rainbow colors with violet at the N-terminus and red at the C-terminus. The cofactors FMN, FAD and NADPH are shown as sticks. Mutations found in patients with PORD are shown as *ball* and *sticks*. Mutations that caused a truncation in the protein are not shown. Reproduced with permission from our earlier publication Pandey, A. V. and Flück, C. E. (2013). NADPH P450 oxidoreductase: Structure, function, and pathology of diseases. *Pharmacology & Therapeutics* **138**(2), 229–254.

some distinct trends that can be identified in different populations. The POR A503V allele frequency is 31% in Caucasian and hispanic populations, 48% in Pacific Islanders and 34%–39% in Asian populations. Among Japanese population the A503V is found in 40% of all alleles and in African Americans groups the POR A503V is found in less than 15% of alleles.

Many POR variants identified by sequencing of nonclinical populations had >40% of the enzymatic activities compared to WT in different assays. A sequencing study of POR from human livers added more amino acid variations (K49N, L420M, and L577P) (Hart *et al.*, 2008). The L577P variant of POR showed loss of activities several P450 assays utilizing drug metabolizing enzymes (Hart *et al.*, 2008). A study by group of Ulli Zanger on analysis of POR variations in human liver samples reported 43 variations that included 19 SNPs in protein coding regions (Gomes *et al.*, 2009). Some allele combinations (P228L + A503V and A503V + V631I) were also present and the common minor allele present was A503V (Gomes *et al.*, 2009).

Several new studies have analyzed the sequence of POR gene in specific populations (Burkhard *et al.*, 2017). A study of Japanese population (Saito *et al.*, 2011) identified four additional variants of POR (T29M, R550W, R570C, and A659T) found that minor allele frequency of A503V was 0.434. A study of POR variations in Jewish populations identified additional variants S102P, V164M, V191M, D344N, E398A, and D648N (Tomkova *et al.*, 2012). Most variants of POR are found in extremely rare cases and even with more than 5000 alleles, the numbers are still not conclusive for any definitive links to unique populations. However, some patterns have emerged from our analysis of the currently available sequencing data (Burkhard *et al.*, 2017). The variant T93I seems to be exclusive to South Asians, while A172T was prevalent in European populations. The variant P284T which shows almost complete loss of activity in functional assays was found exclusively in Africans. The variant T372M was linked to mixed American population and the G537R was found in Europeans. We have found that the mutations located in the NADPH binding site of the POR are more sensitive toward aromatase and availability of cofactors may further influence their effects on different partner protein activities.

Pathogenetic Mechanisms and Their Implications

On Steroidogenesis and Cholesterol Synthesis

PORD causes adrenal insufficiency/CAH through inhibition of CYP21A2 and CYP17A1, which are essential for glucocorticoid and androgen biosynthesis (**Fig. 1**) (Pandey and Flück, 2013). CAH in PORD thus combines the typical characteristics of CAH due to the 21-hydroxylase and the 17-hydroxylase deficiency (Miller and Fluck, 2014). Milder GC deficiency than with *CYP21A2* mutations is observed because many *POR* mutations have residual activity and because of the accompanying CYP17A1 inhibition that leads to

corticosterone accumulation, which also has GC properties. Therefore, the basal plasma cortisol levels are often normal in PORD, while basal ACTH is frequently elevated and the ACTH stimulation test is abnormal. In addition, POR-related CYP17A1 inhibition may also cause mild mineralocorticoid excess (with low renin and potassium), and thus arterial hypertension in some affected individuals.

PORD also affects gonadal steroidogenesis through the lack or insufficient support of partner enzymes CYP17A1 and CYP19A1 crucial for androgen and estrogen biosynthesis in the Leydig and the Theca/granulosa cells of testis and ovary respectively (Miller and Auchus, 2011). This may result in disturbed fetal sex development (DSD) and/or lack or insufficient pubertal development and/or hypergonadotropic hypogonadism and infertility in adult life. Many POR mutations have been shown to cause severe loss of the CYP19A1 activities. The CYP19A1 seems to be more sensitive to mutations in the NADPH binding regions of POR and is affected to a greater degree compared to other cytochrome P450s that depend on POR (Pandey *et al.*, 2007; Udhane *et al.*, 2016; Flück and Pandey, 2017a,b).

While DSD in severely affected 46,XY subjects with PORD mainly seems to result from the lack of androgen production by the fetal testis due to diminished CYP17A1 activity, DSD in severely affected 46,XX subjects seems more complex and more severe. Diminished CYP21A2 activity alone leads to androgen excess and virilization of the female external genitalia during the critical time window of 6–12 weeks gestation (Goto *et al.*, 2006). With PORD additional inhibition of CYP17A1 and CYP19A1 (Aromatase) activities contribute toward androgen excess (Arlt *et al.*, 2004; Flück *et al.*, 2004). Aromatase (CYP19A1) deficiency of the fetal-placental unit fails to convert fetal adrenal androgens to mainly estriol (**Fig. 2**), and thereby causes not only ambiguous genitalia in a 46,XX fetus, but also maternal virilization during pregnancy (Shackleton et al., 2004a,b; Reisch *et al.*, 2013). In addition, production of excess fetal adrenal androgen precursors lead to DHT production through an alternative backdoor pathway (**Fig. 1**), which is thought to be more readily used during fetal and early postnatal life (Auchus, 2004; Homma *et al.*, 2006; Flück *et al.*, 2011; Kamrath *et al.*, 2012; Biason-Lauber *et al.*, 2013). This alternative androgen biosynthesis pathway was first discovered in the tammar wallaby pouch young (Auchus, 2004). Interestingly, it was with studies of the urinary steroid profile of patients with PORD that its existence in humans was detected (Homma *et al.*, 2006). In this pathway, 17OH-progesterone is 5α-, 3α-reduced to yield specific metabolites, which are precursors for DHT production, without going through testosterone as intermediate product like in the classic pathway (**Fig. 1**). Early postnatally, metabolites 17-hydroxy-alloprogesterone (17OH-Allo; also known as 5α-pregnane-3α, 17α-diol-20-one) and androsterone of the backdoor pathway were found elevated in PORD, while metabolites of the classic pathway were either low or normal.

POR also supports the catalytic reactions of squalene monooxygenase (*SQLE*) and 14α-lanosterol demethylase (*CYP51A1*) (**Fig. 3**), which are important enzymes in sterol and cholesterol synthesis. In addition, sterols are involved in hedgehog-mediated regulation of fetal bone development, as well as oocyte meiosis and maturation (Byskov *et al.*, 1995; Grondahl et al., 1998, 2000). Women with PORD are prone to suffer from ovarian cysts (see section "Pubertal Development and Gonadal Function in PORD"), which might be in part due to insufficient production of meiosis-activating sterols (MAS); on the other hand due to high gonadotropins secondary to disrupted ovarian steroidogenesis and anovulation (Idkowiak *et al.*, 2011; Bai *et al.*, 2017). Likewise, testicular MAS may play a role in male fertility by affecting meiosis in spermatozoa (Byskov *et al.*, 1999), although such effect has not been implicated in PORD so far.

POR and Bone Formation

Skeletal malformations are found very frequently in PORD patients, when a careful clinical assessment is performed (see **Table 1** and Fukami *et al.*, 2009; Krone *et al.*, 2012; Flück and Pandey, 2013; Pandey and Flück, 2013). They can be of minor degree or very severe, and in its extreme form manifesting with an ABS phenotype (section "ABS and Skeletal Malformations With PORD").

The pathomechanism underlying the bony malformations with PORD is complex, but involves a role of POR in sterol and cholesterol biosynthesis on one hand, and on retinoic acid metabolism on the other. This includes inhibition of squalene monooxygenase and 14α-lanosterol-demethylase (*CYP51A1*) in the cholesterol biosynthesis pathway, which both depend on POR for electron transfer (Ono and Bloch, 1975), suggested to leading to abnormal activity and signal transduction of hedgehog proteins required for normal morphogenesis, bone formation and growth during embryogenesis (Gofflot *et al.*, 2003). Accordingly, *Por* KO in rat chondrocytes was found to induce apoptosis, reduce cholesterol levels and lower Indian hedgehog content, while all these effects could be rescued by cholesterol supplementation (Aguilar *et al.*, 2009). Interestingly, disruption of downstream processing of lanosterol by fluconazole (a known CYP51 inhibiting drug), resulted in similar skeletal abnormalities (Pursley *et al.*, 1996; Andersson *et al.*, 2002).

Similarly, the skeletal malformation phenotype of PORD resembles embryonic retinoic acid toxicity. Retinoic acid is metabolized by microsomal CYP26 enzymes, which receive electrons from POR for catalytic reactions. Conditional limb Por −/− KO mice were found to have excess retinoic acid levels, but low cholesterol and upregulated genes in the whole cholesterol biosynthetic pathway, (Schmidt *et al.*, 2009). Elevated plasma all-trans retinoic acid levels were also reported in some PORD patients manifesting with skeletal anomalies (Fukami *et al.*, 2010). Finally, human loss of function mutations in *CYP26B1* essential for retinoic acid degradation were recently found to produce a skeletal malformation syndrome with craniosynostosis just like ABS seen in PORD (Laue *et al.*, 2011).

Effects on Drug Metabolism by Mutations in POR

Here we are reviewing only the effects of mutations found in patients (**Fig. 5**) and major common minor allele of POR, the A503V (POR*28), while a detailed review of the POR variations could be found in our earlier publications (Pandey and Flück, 2013;

Pandey and Sproll, 2014; Burkhard *et al.*, 2017). Initially the focus of POR variation studies was on characterization of effects on steroid metabolism, but soon afterwards, a range of redox partners including drug metabolizing cytochrome P450s and heme oxygenase were also studied (Agrawal et al., 2008, 2010; Flück *et al.*, 2010; Nicolo *et al.*, 2010; Pandey *et al.*, 2010). Sequencing studies from many different genome sequencing projects as well as targeted gene sequencing across various population subgroups have identified may variations in the POR gene (Gomes *et al.*, 2008; Huang *et al.*, 2008; Gomes *et al.*, 2009; Sim *et al.*, 2009; Agrawal *et al.*, 2010; Nicolo *et al.*, 2010; Pandey *et al.*, 2010; Parween *et al.*, 2016). Many POR variants found from patients and normal population have been tested for enzymatic activities (Adachi *et al.*, 2004; Arlt *et al.*, 2004; Fukami et al., 2005, 2006; Homma *et al.*, 2006; Pandey, 2006; Huang *et al.*, 2008; Sim *et al.*, 2009). While mutations in POR can be found in all regions of the proteins, based on analysis of previously identified mutations some general observations can be made. Mutations found in either of the cofactor binding sites (FMN, FAD, and NADPH) generally result in a severe form of the disease with mutations causing loss of FMN or FAD showing most severe effects on activities of all supported redox partners.

Cytochrome P450 3A4 (CYP3A4) is the major hepatic enzyme that metabolizes a large percentage of drugs and endogenous substrates (Klein and Zanger, 2013; Meyer *et al.*, 2013; Zanger and Schwab, 2013). We found that mutations in POR can affect the enzymatic activities of CYP3A4 (Nicolo *et al.*, 2010; Udhane *et al.*, 2017). The POR mutant A287P (common in "Europeans") leads to a 75% reduction of the CYP3A4 enzymatic activity (Flück *et al.*, 2010; Nicolo *et al.*, 2010). The POR mutations that affect binding of cofactor FAD (R457H, Y459H, and V492E) showed no activity in CYP3A4 assays (Flück *et al.*, 2010). The two mutations in POR located in the NADPH binding site, the C569Y and V608F caused 65%–85% loss of the CYP3A4 activity and a c-terminus truncation mutation in POR, R616X, showed no activity (Flück *et al.*, 2010). Effect of POR variants on CYP3A4 activities may also depend on specific substrates; the POR variant Q153R has shown 76%–94% of the WT CYP3A4 activity with substrates midazolam and erythromycin, but when testosterone and quinidine were used as substrates it had 129%–150% of the WT POR activity (Agrawal *et al.*, 2010). The A503V variant of POR caused 20%–40% loss of CYP3A4 activity when midazolam and testosterone were used as substrates but when erythromycin and quinidine were used as substrates, the A503V showed similar to WT activities (Agrawal *et al.*, 2010). A combined analysis of several studies shows that many polymorphisms in POR (P228L, R316W, G413S, A503V, and G504R) retain $>40\%$ activity in assays with most redox partners. In the testing of CYP2C19 and CYP1A2 activities, POR mutations A287P and R457H have been reported to cause total loss of activity (Agrawal *et al.*, 2008). The POR variant Q153R caused an increase in the activity of CYP1A2 by 144% and CYP2C19 activity by 284%, similar to our observation of increased activity of aromatase with POR Q153R (Flück and Pandey, 2017a,b; Udhane *et al.*, 2017). Our recent studies on POR variations have found mutations A115V, T142A, and P284L to cause severe loss of CYP3A4 activities (Udhane *et al.*, 2017).

The CYP2D6 is also a major enzyme involved in drug metabolism and after CYP3A4 it is responsible for the metabolism of a large number of drugs (Zanger and Schwab, 2013). In the assay of CYP2D6 activities with dextromethorphan and bufuralol as substrates, the A287P mutant of POR lost 75% of the activity compared to WT POR. Compared to the WT POR, the POR variant A503V showed activities of 62% with dextromethorphan as substrate and 53% when bufuralol was the substrate in CYP2D6 assays (Sandee *et al.*, 2010). The effects of different CYP isoforms on impact of POR activities has not been studied in detail. One study (Subramanian *et al.*, 2012) tested different alleles of CYP2C9 (*CYP2C9.1*, *CYP2C9.2*, and *CYP2C9.3*) with few POR variants and patterns were similar for all isoforms of CYP2C9 (Subramanian *et al.*, 2012).

The Unknown Factors About POR Mutations

In cases POR mutations leading to the loss of FAD/FMN cofactors, a harmful effect on all redox partner activities are observed. However, in most cases effects of POR on individual redox partner activities could be variable. Biochemical analysis using recombinant proteins is often required to confirm the damaging effects of mutations with each redox partner separately. Several cytochrome P450 proteins for which POR is the redox partner, still have uncharacterized activities and their physiological roles are not defined. These, other partners of POR could also influence the metabolic profiles of patients with PORD. In terms of preventive measures, low retinoic acid in diet and supplementation with steroid metabolites whose production is affected could be utilized as treatment strategies. Preliminary computational docking and functional analysis suggests that the loss of activity caused by some POR variants may be reversed by introduction of external FMN (Nicolo *et al.*, 2010). However, effect of flavin treatment in PORD needs to be tested.

Conclusion and Perspectives

PORD is a complex disorder affecting both the steroid and the drug metabolism of affected individuals with varying effects. Different POR variations can cause a highly variable effect on different metabolic reactions depending on unique nature of the interaction of POR with different redox partners. A careful examination of affected subjects by use of urine/serum steroid analysis is required for initial verification of PORD. In cases of severe loss of drug metabolizing cytochrome P450 enzyme activities, further considerations about clearance of drugs in patients with PORD would require evaluation. Effect of altered clearance of kidney transplant drug tacrolimus has been confirmed in several studies among people with the POR*28 allele (Zhang *et al.*, 2015).

Effects of different POR variations found in nonclinical populations should also be investigated and recent work has shown that some of these variations in POR could lead to severe changes in activities of enzymes dependent on POR (Udhane *et al.*, 2017).

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Relevant Websites

OMIM # 613571 Disordered steroidogenesis due to cytochrome P450 oxidoreductase deficiency—http://omim.org/entry/613571.
Reference sequence for the POR protein—https://www.ncbi.nlm.nih.gov/protein/NP_000932.3.
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Pediatric Cushing Disease

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Glossary

ACTH Adrenocorticotrophic hormone.
BSIPSS Bilateral simultaneous petrosal sinus sampling, a method of determining if central Cushing's syndrome.
TSS Transsphenoidal surgery.
Glucocorticoid Steroids produced from the zona fasiculata of the adrenal gland.
HPA axis hypothalamic pituitary adrenal axis.
MRI Magnetic Resonance Imaging.
RT Pituitary radiotherapy.

Introduction

Cushing syndrome (CS) is a clinical disorder caused by prolonged glucocorticoid (cortisol) exposure. CS can be divided into adrenocorticotrophic (ACTH)-dependent or ACTH-independent etiological categories (**Table 1**). ACTH-dependent CS can be secondary to a corticotroph pituitary adenoma secreting ACTH (Cushing disease; CD) or ectopic ACTH secretion. ACTH-independent is usually secondary to primary adrenal disease resulting in excessive cortisol secretion or to exposure to exogenous glucocorticoid administration (Magiakou and Chrousos, 2002). CS is very rare in childhood and adolescence; the incidence is approximately 10% of CS in the adult population which is reported as between 0.7 and 2.4 new cases/million/year (Newell-Price *et al.*, 2006; Lindholm *et al.*, 2001; Etxabe and Vazquez, 1994; Ambrosi *et al.*, 1990).

If pediatric CS is suspected it is extremely important to rapidly establish the diagnosis and the etiology using a formal investigation protocol. Once diagnosed, the primary aim should be the normalization of serum cortisol to minimize the adverse effects of prolonged hypercortisolaemia. Once remission is achieved, posttreatment management should focus on the optimization of growth, pubertal development, bone mineral density and body composition.

In this article, we will review the epidemiology, pathogenesis, diagnosis and management of children with pediatric CD. The recommendations are based on published data and our experience from the management of >70 cases of pediatric CS (53 cases of CD) during the last 35 years. The focus of this chapter will be on CD as this represents the commonest cause of CS in the pediatric age range.

Epidemiology

The most common cause of CS in all ages is iatrogenic, secondary to exogenous glucocorticoid administration such as inhaled, oral, parenteral or topical corticosteroids. Therefore, if CS is suspected, it is fundamental to take a thorough medication history to exclude this (Newell-Price *et al.*, 2006). Cushing disease (CD), caused by an ACTH-secreting pituitary adenoma is responsible for 75%–85% cases of endogenous pediatric CS patients compared with 49%–71% of adult cases (Magiakou *et al.*, 1994c; Magiakou and Chrousos, 2002; Weber *et al.*, 1995). In a series of 150 children with pituitary adenomas, 54.8% and 29.4% of the prepubertal children (0–11 years old) and adolescents aged 12 to 17 years had corticotroph adenomas (Kunwar and Wilson, 1999). Therefore,

Table 1 Classification of pediatric Cushing syndrome

ACTH-independent

1. Exogenous glucocorticoid administration (tablets, nose drops, inhalers, nasal spray, skin cream)
2. Adrenocortical tumor (adenoma or carcinoma)
3. Primary adrenocortical hyperplasia
   a. Primary pigmented nodular adrenocortical disease (PPNAD)
   b. Macronodular adrenal hyperplasia (AIMAH)
   c. McCune–Albright syndrome

ACTH-dependant

1. Cushing disease (ACTH-secreting pituitary adenoma)
2. Ectopic ACTH syndrome

in preadolescents, CD is the most common pituitary adenoma. In a review of 182 published pediatric CD cases, the median age at presentation was 14.1 years (Storr *et al.*, 2007). In our pediatric CD cohort, the median age was slightly lower, 12.3 years (Storr *et al.*, 2011). In adults, CD occurs more frequently in females with a ratio of 9:1 (females: males) (Magiakou and Chrousos, 2002). There was a striking male predominance (10:1) in our prepubertal CD patients which becomes an equal gender distribution during the pubertal age range (Storr *et al.*, 2004). Consistent with this, another study found that the female-to-male ratio was significantly different between patients who had surgery before the age of 15 years (0.8:1) and patients who had surgery after that age (3.7:1) (Lonser *et al.*, 2013) In contrast, a review from the NIH found an equal distribution of males to females in 102 pediatric patients with CD (53% vs. 47%; $n = 102$). There was also an equal gender distribution in prepubertal and pubertal patients (Libuit *et al.*, 2015).

Pathogenesis

CD is caused by ACTH-secreting pituitary corticotroph adenomas. These are usually microadenomas which in children are often extremely small—75% are less than 5 mm diameter (Lonser *et al.*, 2013). Macroadenomas (>1 cm in maximal diameter) are infrequent in children, occurring in approximately 4%–9% pediatric CD cases (Storr *et al.*, 2011; Lonser *et al.*, 2013) compared to ~10% of adult-onset CD (Feng *et al.*, 2017).

Molecular Pathogenesis

The molecular pathogenesis of CD is not fully understood. A number of hereditary disorders have been associated with corticotroph adenomas but these remain a very rare cause of CD. Multiple Endocrine Neoplasia Type 1 (MEN1) is an autosomal dominant disorder characterized by endocrine tumors including anterior pituitary adenomas. However, MEN-1 associated ACTH-secreting adenomas have been identified in only three cases (Rix *et al.*, 2004). Two of these went on to be diagnosed with hyperparathyroidism and one had a monozygotic twin subsequently diagnosed with hyperparathyroidism (Rix *et al.*, 2004). Pituitary macroadenomas are also recognized as an early manifestation of MEN1 (Stratakis *et al.*, 2000). However, no identifiable genetic cause was identified in eight patients with sporadic corticotroph macroadenomas (Cuny *et al.*, 2013). CD has recently been described one 21-year-old male patient with MEN2B and it was hypothesized that the RET oncogene may play a role in pituitary tumorigenesis (Kasturi *et al.*, 2017).

Corticotroph adenomas have also been identified in familial isolated pituitary adenoma (FIPA), McCune Albright Syndrome and Carney complex (Reincke *et al.*, 2015). Whole exome sequencing of 10 corticotroph adenomas revealed somatic missense mutations in the gene encoding ubiquitin-specific protease 8 (*USP8*) in four tumors (Reincke *et al.*, 2015). Mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene are associated with FIPA and most often present with pituitary macroadenomas. However, only one child with an ACTH secreting microadenoma out of 74 (1.4%) with CD was found to have an *AIP* mutation (Stratakis *et al.*, 2010). Therefore, a genetic cause of CD is extremely rare in sporadic cases. However, those with a positive family history or large adenomas should be referred for genetic counseling and testing (Storr and Savage, 2015; Cuny *et al.*, 2013).

Clinical Features

The main features of CD in childhood and adolescence are weight gain, growth arrest and typical Cushingoid facial features i.e., a plethoric, round face. **Table 2** shows the classical features of CD and the frequency in which they occur in children compared with adults (Storr *et al.*, 2011). It is important to recognize the features of CD early to allow timely diagnosis and management and to minimize the development of comorbidities. A subtle or subclinical presentation is unusual in children and although periodic CD exists, this is very rare in young patients. The onset of CD is usually gradual and may span many years which can lead to an unacceptable delay in diagnosis and initiation of definitive treatment.

The mean length of symptoms before diagnosis in our cohort of 43 pediatric CD patients was 2.5 ± 1.7 years (range 0.3–6.6 years) (Storr *et al.*, 2011). Although growth failure i.e., poor growth velocity is recognized in all children, not all have short stature (height SDS ≤ −2) (Storr and Savage, 2015). Whilst most pediatric CD patients in our cohort had a reduced height (<0.0 SDS), BMI SDS was >1.5 (Storr *et al.*, 2007). This reduction in height associated with elevated BMI/weight gain, is a key clinical feature of hypercortisolaemia in children. The presence of growth failure can also effectively differentiate children with simple obesity (mean height SDS 1.18 ($n = 41$) from those with Cushing's disease (mean height SDS −1.88 ($n = 25$), respectively (Greening *et al.*, 2006). In addition to growth retardation, many children have a delayed bone age. A retrospective review of our cohort revealed 15/17 (88%) children with CD, median age 12.1 years (range 5.8–17.4) presented with a mean bone age delay of 2 years (range −0.5–4.1) (Peters *et al.*, 2007).

Abnormal virilisation in combination with delayed puberty is also common in prepubertal children with CD. This is a result of high adrenal androgens combined with gonadotrophin deficiency. The latter is a consequence of the hypercortisolaemia suppressing gonadotrophin production (Dupuis *et al.*, 2007).

Table 2 Clinical features at diagnosis of pediatric Cushing disease ($n = 52$)

Major symptoms	*Patients (n)*	*% of total*
Facial changes	52	100
Weight gain	51	98
Weight loss	1	2
Pre-pubertal virilisation	21/24	88
Fatigue	33	63
Emotional lability/depression	30	58
Hirsutism	33	63
Headaches	26	50
Striae	25	48
Hypertension	24	45
Acne	22	42

In summary, the four key features suggestive of CD in children are: a change in facial appearance; weight gain; a history of growth failure +/− short stature and the presence of abnormal virilisation in prepubertal patients (Storr and Savage, 2015).

Diagnosis

Due to the rarity and complexity of pediatric CD, it is recommended that pediatric endocrinologists investigate suspected cases with the support of adult endocrinologists who are more experienced in the management of Cushing's syndrome.

Biochemical Evaluation

Before embarking on biochemical evaluation to confirm a diagnosis of Cushing syndrome, it is important to exclude exogenous CS secondary to oral, nasal or topical glucocorticoid treatments as this is much more common than endogenous Cushing's syndrome. A consensus statement recommends only investigating children with obesity and associated slowing of their growth (Nieman *et al.*, 2008). However, as this disorder often has an insidious onset, it is also important to have a low threshold for investigations and early referral to a tertiary centre if childhood CS is suspected.

The algorithm for investigations in children should be based on that performed in adults (Newell-Price *et al.*, 1999; Storr *et al.*, 2007; Guaraldi *et al.*, 2014). A suggested investigation protocol for suspected pediatric Cushing syndrome is shown in **Table 3** (Storr *et al.*, 2007; Guaraldi *et al.*, 2014). The protocol of investigations consists initially of confirmation or exclusion of hypercortisolaemia (Cushing syndrome) followed by investigations to determine the etiology.

Confirmation or Exclusion of Hypercortisolaemia

Key biochemical features of hypercortisolaemia are increased 24 h urinary-free cortisol (UFC) excretion, loss of serum cortisol circadian rhythm with detectable cortisol at midnight and failure of suppression of cortisol during the low-dose dexamethasone suppression test.

UFC measurements in children have a reported sensitivity of 88% and specificity of 90% (Batista *et al.*, 2007a) suggesting this is not an ideal screening test for childhood CS alone. In contrast, we recently showed that this test is a reliable and practical investigation with high diagnostic accuracy for pediatric CS (Shapiro *et al.*, 2016). As increased UFC excretion may not be a consistent finding in pediatric patients with CS, we recommend at least two serial measurements to definitively rule out the diagnosis (Batista *et al.*, 2007a). Additionally, further diagnostic testing may be required if the UFC is normal but there is a high clinical suspicion of CS (Shapiro *et al.*, 2016). If there is doubt about the interpretation of the UFC values, we recommend hospitalization for the measurement of serum cortisol to assess circadian rhythm.

The suggested minimum sampling times for serum cortisol are 0900 h, 1800 h and midnight (when the child is asleep) (Storr and Savage, 2015). A loss of circadian rhythm is suggestive of CS and a sleeping midnight sleeping cortisol ≥ 121 nmol/L (4.4.µg/dL) has a very high sensitivity and specificity of 99% and 100%, respectively (Batista *et al.*, 2007a). All our patients with pediatric CS of multiple etiologies had sleeping midnight cortisol levels of > 50 nmol/L, confirming that this is a key diagnostic marker for CS in children (Storr and Savage, 2016).

Late night salivary cortisol is a less invasive alternative to serum cortisol and avoids stressful venepuncture in children. Evaluation of this investigation in 11 children with CD yielded a sensitivity and specificity of 95.2% and 100%, respectively (Martinelli *et al.*, 1999). However normative ranges have not been characterized for different aged children. Following the assessment of midnight cortisol, a low-dose dexamethasone suppression test (LDDST) should be performed. The established regime for adults and children ≥ 40 kg is 0.5 mg dexamethasone every 6 h (0300, 0900, 1500 and 2100 h) for 48 h. In children

Table 3 Scheme of investigation for patients with suspected Cushing syndrome

Confirmation or exclusion of Cushing syndrome
1. Urinary free cortisol excretion (24 h urine collection) daily for at least 2 days
2. Serum cortisol circadian rhythm study (09.00, 18.00 h, midnight [sleeping])
3. Low-dose dexamethosone suppression test (LDDST)
a. Dose 0.5 mg 6 hourly (09.00, 15.00, 21.00, 03.00 h) for 48 h
b. Dose for patients weighing <40 kgs; 30 μg/kg/day
c. Serum cortisol measured at 0 and 48 h
Definition of etiology of Cushing syndrome
1. Plasma ACTH (09.00 h)
2. CRH test (1.0 μg/kg IV)
3. Analysis of change in serum cortisol during LDDST
4. Adrenal or pituitary MRI scan
5. Bilateral inferior petrosal sinus sampling for ACTH (with CRH)

<40 kg, 30micrograms/kg/day dexamethasone should be given as per NIH-recommendations (Magiakou *et al.*, 1994c). Serum cortisol is measured at times 0 and 48 h i.e., 6 h after last dose and is suppressed (<50 nmol/L) in normal subjects (Magiakou *et al.*, 1994c).

Confirmation of CD

Following confirmation of CS, the priority is to establish the etiology. A 0900 h plasma ACTH level will be normal or high in ACTH-dependent CS and is always low and usually undetectable in ACTH-independent CD (due to suppression of the HPA axis). An ACTH of ≥29 pg/mL has a sensitivity % and specificity of 70% and 100%, respectively for CD (Batista *et al.*, 2007a). A corticotrophin-releasing hormone (CRH) test, using human sequence CRH (1 microgram/kg i.v.) is also recommended to confirm the diagnosis of CD in children. Serum cortisol is measured after 15, 30, 60, 90 and 120 min (Batista *et al.*, 2009) and a rise of >20% is suggestive of central ACTH production i.e., CD. A sensitivity of 74% for the CRH test has been reported (Batista *et al.*, 2007a).

Other groups report high sensitivity and specificity (97.5% and 100%, respectively) of the high dose dexamethasone suppression test (HDDST) in the confirmation of CD (Batista *et al.*, 2007b). Our decision to stop performing HDDST follows the finding of serum cortisol suppression during L- and HDDST in pediatric patients with CD (Dias *et al.*, 2006). In 24 patients, the mean baseline serum cortisol values of 590.7 ± 168.8 nmol/L (21.4 ± 4.7 μg/dL) decreased to 337.4 ± 104.0 nmol/L (12.2 ± 3.8 μg/dL) at 48 h during LDDST ($P < 0.05$; mean decrease, 45.1%) with 66% decreasing by >30%. This cortisol suppression during LDDST correlated with that during HDDST ($r = 0.45$, $P < 0.05$). Consequently, the decrease of cortisol during the LDDST strongly supports the diagnosis of CD and negates the need for a HDDST.

Radiological Imaging

MRI has superseded previous techniques for pituitary visualization. Pituitary adenomas are generally hypointense compared to the adjacent gland and take up contrast less avidly and in a more delayed fashion, and therefore fail to enhance with gadolinium (Newell-Price *et al.*, 1998). The detection rate of pituitary adenomas by MR scanning is much lower in children compared to adults, which in our adult cohort is approximately 76% of cases (Storr *et al.*, 2011). On pituitary postcontrast MR scanning, 63% and 55% of the corticotroph adenomas were identified in two large pediatric series (Batista *et al.*, 2007a; Batista *et al.*, 2009). This relatively poor visualization rate in children could be explained by the limited spatial resolution of MRI, i.e., small lesions within a small pituitary gland are less conspicuous. Therefore, pituitary MRI imaging alone cannot be relied upon to predict the adenoma position or to confirm the diagnosis of pediatric CD.

Bilateral Simultaneous Inferior Petrosal Sinus Sampling

Due to the low visualization rate of MRI scanning, we recommend bilateral simultaneous inferior petrosal sinus sampling (BSIPSS) to confirm the diagnosis of CD prior to transsphenoidal sinus surgery (TSS) (Storr and Savage, 2016). In children, ectopic ACTH secretion is extremely rare and so the aims of BIPSS is to confirm central ACTH secretion before embarking on pituitary surgery and to contribute to the localization of the microadenoma by demonstrating lateral or midline ACTH secretion.

BSIPSS was first established in adults at the NIH to enable distinction between CD and ectopic ACTH syndrome and also to provide a method of identifying a lateral versus central source of ACTH secretion within the pituitary (Oldfield *et al.*, 1991) It has

now become routine in adult practice unless the pituitary MRI unequivocally shows a lesion and all other investigations suggest central ACTH-dependent CD.

BIPSS is a highly specialized technique and in our centre, is performed by the same radiologist who regularly studies adult patients. In the majority of cases, general anesthesia (GA) is not required so that potential alteration of ACTH secretion is avoided. However, in very young children GA may be necessary. A pre-CRH inferior petrosal sinus/peripheral (IPS/P) ratio of ≥ 2.0 or post CRH IPS/P ratio of ≥ 3.0 is diagnostic of central ACTH- dependent CD with a sensitivity of 95% and specificity of 100% (Oldfield *et al.*, 1991). Lateralization of ACTH secretion is confirmed by an inter-petrosal sinus gradient IPSG ≥ 1.4.

The first pediatric data were reported in the large NIH series where the predictive value of lateralisation was 75%–80% (Magiakou and Chrousos, 2002). A subsequent study found the concordance of ACTH localization during BSIPSS and the adenoma position to be only 58% however exclusion of midline and bilateral lesions increased the positive predictive value to 70% (Batista *et al.*, 2006).

To date, we have successfully undertaken BSIPSS in 40 children without complications. The reported results suggest that ACTH sampling gives a better prediction of the site of the microadenoma than pituitary MR imaging (Magiakou and Chrousos, 2002; Storr *et al.*, 2011; Magiakou *et al.*, 1994c).

Treatment

CD in childhood leads to significant comorbidity and early diagnosis and definitive treatment is essential to effectively manage the symptoms and achieve an acceptable adult height, pubertal development and normal body composition.

Pediatric and Adult Cooperation

CD is extremely rare in children and even experienced pediatric endocrine centers will see relatively few cases during a 20-year period. Consequently, pediatric endocrinologists may not acquire the expertise to manage these complex patients. It is therefore essential that there is collaboration with a specialized adult endocrinology centre with experience in treating CD. The combined experience of a pediatric endocrinologist with expertise in pediatric CD, pituitary surgery and pituitary radiotherapy is optimal for the patient (Storr and Savage, 2015).

Pituitary Surgery: Selective Microadenomectomy

Transsphenoidal surgery (TSS) is the first line treatment for children and adults with CD. This procedure is established as safe and effective in children (Massoud *et al.*, 1997; Knappe and Ludecke, 1996; Kanter *et al.*, 2005; Joshi *et al.*, 2005). The aim of TSS is to selectively remove the adenoma tissue, maximizing the potential for normal pituitary tissue to remain in situ. The small size of ACTH-secreting adenomas and the pituitary fossa in children in association with absent aeration of the sphenoid bone in young patients adds to the technical difficulty of TSS. A pituitary surgeon with experience of transsphenoidal surgery in children will generally achieve a higher rate of cure.

The outcome of TSS depends on the definition of postoperative "cure" or remission. An undetectable postoperative 09.00 h cortisol of <50 nmol/L (Storr *et al.*, 2011) or <28 nmol/L (Lonser *et al.*, 2013) is considered "remission." This evidence of successful surgery is supplemented by immunohistochemical confirmation of an ACTH-producing adenoma.

In the two pediatric series where "cure" was defined as an undetectable postoperative serum cortisol "cure" rates were 100% and 69%, respectively (Storr *et al.*, 2011). Follow-up data suggest that recurrence rates of CD in these patients were very low (Storr *et al.*, 2011; Yordanova *et al.*, 2016; Batista *et al.*, 2009). Other pediatric series report surgical "cure" rates of between 50% and 78% (Leinung *et al.*, 1995; Devoe *et al.*, 1997; Linglart and Visot, 2002; De Oliveira *et al.*, 2010; Dyer *et al.*, 1994). Few report rates $>90\%$ (Magiakou *et al.*, 1994c; Lonser *et al.*, 2013; Fahlbusch *et al.*, 1994; Styne *et al.*, 1984).

Initial postoperative remission in children was associated with the identification of an adenoma at surgery and long-term remission correlated with younger age, smaller adenoma and morning serum cortisol of <1 μg/dL (28 nmol/L) after surgery (Lonser *et al.*, 2013). Lasting remission in children is observed in those patients with younger age, smaller tumor size and absence of cavernous sinus or dural invasion (Lonser *et al.*, 2013). However, recurrence of CD in adults has been reported up to 15 years after apparent surgical cure, even in individuals who had very low or undetectable postoperative cortisol levels (Alexandraki *et al.*, 2013). In our series of 21 pediatric CD patients where long-term follow-up data was available, clinical and biochemical recurrence of hypercortisolemia was seen in 2 patients (10%) at 2 and 6 years following TSS (Yordanova *et al.*, 2016). Therefore, lifelong follow-up for children treated for CD is essential.

Endoscopic Pituitary Surgery

More recently, the less invasive technique of endonasal endoscopic transsphenoidal pituitary surgery (ETES) has been used in some centers. In adult CD patients ETES has shown equivalent rates of complete tumor resection and is now established as the

standard approach (Goudakos *et al.*, 2011; Deklotz *et al.*, 2012). It has been associated with a shorter hospital stays, reduced bleeding, pain perception, anterior pituitary deficiencies and surgical complication rates (Storr *et al.*, 2014; Massimi *et al.*, 2011; Chivukula *et al.*, 2013). Early experience in pediatric CD showed excellent outcomes although the numbers are currently limited (Storr *et al.*, 2014).

Pituitary Radiotherapy

A proportion of pediatric patients who undergo TSS for CD do not achieve postoperative cure or remission. The options for second-line therapy are repeat TSS, pituitary radiotherapy (RT), long-term medical therapy to control hypercortisolaemia and bilateral adrenalectomy (Acharya *et al.*, 2010; Storr *et al.*, 2003a; Stratakis, 2012) In our centre, if there is persistent hypercortisolaemia or failure of remission within 2–4 weeks of TSS, then external pituitary radiotherapy (RT) is offered. Pituitary RT is now known to be safe and effective in children with CD with a more rapid mode of action than in adult patients (Storr and Savage, 2015; Storr *et al.*, 2003a). Stereotactic radiotherapy and gamma knife approaches are now available and utilized in adult CD, however experience is limited, particularly in children.

In our centre, a total dose of 45 Gy in 25 fractions over ~35 days is delivered. The effectiveness and rapid onset of this therapy was confirmed in three small series. In the first, series, a 12 out of 15 patients were cured within 18 months of radiotherapy and 10 of these were cured within 9 months of treatment (Jennings *et al.*, 1977). In our own series, 7 children were treated and all were cured with a mean interval from RT to cure of 0.94 years (range 0.25–2.86) (Storr *et al.*, 2003b). In the third series, eight subjects were treated and four were cured in 9–18 months after RT (Acharya *et al.*, 2010). In a further series, 8 children were treated with stereotactic external RT using 60 Co gamma radiation. Seven of the eight subjects were cured during the first year after completion of therapy (Thoren *et al.*, 1986).

Careful monitoring of pituitary function is required postpituitary RT. Growth hormone (GH) deficiency was present in 83% of pediatric CD patients post-RT at a mean interval of 1 year (range 0.11–2.54). However, on retesting at an interval of 9.3 years (range 7.6–11.3), 75% of patients, GH secretion recovered (Chan *et al.*, 2007). GH deficiency and hypogonadism were also documented in seven children successfully treated with higher doses of 50–70 Gy (Acharya *et al.*, 2010). In another series of eight children, two developed hypothyroidism and all GH deficiency (Thoren *et al.*, 1986).

Medical Therapy and Bilateral Adrenalectomy

Definitive treatments such as surgery and/or radiotherapy, rather than long-term medical therapies are currently recommended for the management of pediatric CD. Medical therapies can be used to urgently lower cortisol levels in very sick patients, to normalize the hypercortisolemia in preparation for surgery or whilst awaiting the effects of radiotherapy. Adrenal steroidogenesis inhibitors such as metyrapone and ketoconazole are well tolerated and can be highly effective at reducing cortisol levels either alone or in combination (Biller *et al.*, 2008). Intravenous administration of etomidate has successfully controlled hypercortisolaemia in children with CD who were either too unwell for TSS or presented with acute unmanageable symptoms such as respiratory failure or severe psychosis (Greening *et al.*, 2005; Chan *et al.*, 2011; Storr and Savage, 2015).

Historically, the recommended treatment for CD was bilateral adrenalectomy (BA). However this can lead to the development Nelson's syndrome; a triad of excessive ACTH, pigmentation and an enlarging corticotroph adenoma (Hopwood and Kenny, 1977; McArthur *et al.*, 1979). Although Nelson's syndrome is rare, it is a potentially life-threatening complication and children are more at risk than adults (Barber *et al.*, 2010). In our centre three patients were treated with BA, 1 historically and 2 for life-threatening CD. Therefore, bilateral adrenalectomy remains a therapeutic option for CD in life-threatening situations or where TSS is not possible or available.

Postremission Growth and Development and Pituitary Function

Growth failure and short stature is almost always seen at diagnosis in pediatric patients with CD (Magiakou and Chrousos, 2002; Lebrethon *et al.*, 2000). The excessive production of adrenal androgens can also accelerate bone age and further compromise growth (Hayles *et al.*, 1966). Poor postremission catch-up growth has been reported in childhood CD (Magiakou *et al.*, 1994a, b). This has been attributed to continuing GH deficiency, occurring either from TSS, pituitary RT or the long-standing effects of chronic hypercortisolaemia on pituitary and growth plate physiology (Lebrethon *et al.*, 2000).

In the absence of catch-up growth, we recommend that GH deficiency is investigated at 3 to 6 months after TSS or completion of RT. If required, GH therapy should be initiated without delay and GnRH analogue therapy may be added to delay puberty and epiphyseal closure. Results demonstrate that this regime usually enables adequate catch-up growth and adult height within range of target height for the majority of patients (Davies *et al.*, 2005). Combined treatment with GH and aromatase inhibitors may also be a therapeutic alternative in pubertal patients (Boronat *et al.*, 2012).

Normal body composition is more difficult to achieve. Many patients remain obese and BMI SDS was elevated ($P < 0.01$) at a mean interval of 4.1 years after diagnosis in 14 patients (Davies *et al.*, 2005). A long-term follow-up study showed that the total body fat and the

ratio of visceral to subcutaneous fat remained abnormally high in the majority of pediatric CD patients studied 7 years after cure (Leong *et al.*, 2007). The implications of chronic excess visceral fat in terms of risk for adult metabolic syndrome deserve future study. Bone mineral density (BMD) was closer to normal together with some patients having normal BMD at diagnosis (Scommegna *et al.*, 2005).

Pituitary function should be closely monitored following TSS and pituitary RT. A summary of reported long-term pituitary deficiencies following first-line TSS in children with CD is shown in **Table 4**. Our recent study of 21 pediatric CD patients revealed short-term growth hormone deficiency (GHD) in 14 (81%) patients (11 following TSS and 3 after RT) and 4 (44% of tested) had long-term GHD. Gonadotropin deficiency caused impaired pubertal development in 9 (43%) patients; 4 requiring sex steroid replacement postpuberty. Four (19%) patients had multiple pituitary hormone deficiencies, 3 after TSS and 1 post-RT (Yordanova *et al.*, 2016).

Pituitary function was analyzed in six patients after receiving RT and although GH deficiency occurred frequently initially, some recovery occurred in adult life (Chan *et al.*, 2007). Gonadotrophin secretion was generally preserved with normal or early puberty; the latter being a well-recognized complication of cranial radiotherapy (Nicholl *et al.*, 1993). TSH and ACTH deficiency was minimal (Chan *et al.*, 2007). It is important to note that the risk of hypopituitarism may continue to increase in the years after radiation.

Brain atrophy, cognitive impairment and psychopathology, most commonly depression, have been reported in adults with hypercortisolaemia (Dorn *et al.*, 1997). A study in children with CD, found significant cerebral atrophy at diagnosis, however, there was no difference in IQ scores between patients and controls. This study reported an almost complete reversal of the cerebral atrophy as has been observed in association with hypercortisolaemia-induced severe psychosis (Chan *et al.*, 2011). Surprisingly, this was associated with a significant decline in cognitive output 1 year after TSS (Merke *et al.*, 2005). In contrast, adult studies showed reversible cognitive decline and loss of brain function with normalization of cortisol levels (Merke *et al.*, 2005; McEwen, 2002). In our series, 5/21 patients (24%) had long-term psychiatric comorbidities which included cognitive dysfunction, depression and mood disturbance (Yordanova *et al.*, 2016).

A recent review found children with CD have impaired quality of life which did not resolve within 1 year of treatment (Keil *et al.*, 2009). There is little data on the effects of hypercortisolaemia on long-term neurocognitive function in children and cognitive functioning following RT has yet to be studied.

Table 4 Reported cure rates and long-term pituitary function following first-line transsphenoidal surgery (TSS) in childhood CD

Series	*No. CD patients*	*Age (yrs)*	*No. of first-line TSS*	*Outcome*	*Long-term pituitary deficiencies*
Lonser *et al.*, (NIH, Bethesda, USA)	200	4.0–19.0	173/200 (87%)	189/200 (95%) cured[a]	10/200 (5%) DI Incomplete data
Storr *et al.* (2011) (Barts Health, London)	41 (1 macro-adenoma)	5.7–17.8	36/41 (88%)	24/35 (69%) cured[a] (macroadenoma not cured)	11/24 (46%) GH 2/24 (8%) DI 2/24 (8%) Hypopit
De Oliveira *et al.* (2010) (São Paulo, Brazil)	15	6.0–18.0	15	9/15 (60%) cured[a]	1/9 (11%) Hypopit 1/9 (11%) GH 1/9 (11%) DI
Acharya *et al.* (2010) (Mumbai, India)	48	9.0–19.0	48	27/48 (56%) cured[a]	Not published
Kanter *et al.* (2005) (Virginia, USA)	33	5.0–19.0	33	76% cured[a]	1/33 (3%) ACTH 1/33 (3%) DI Incomplete data
Massoud *et al.* (1997) (Great Ormond Street Hospital, London)	12	8.7–16.3	12	9/12 (75%) (clinical and biochemical remission)	7/9 (78%) GH 4/9 (44%) ACTH 4/9 (44%) GT 3/9 (33%) TSH 1/9 (11%) DI
Leinung *et al.* (1995) (Rochester, Minnesota, USA)	22 (1 macro-adenoma)	10.1–18.9	22	10/22 (45%) cured[a] (macroadenoma not cured)	1/22 (5%) TSH Incomplete data
Magiakou *et al.* (1994c) (NIH, Bethesda, USA)	50	Mean	14.4 ± 4	37/50 (74%)	35/37 (95%) cured[a] 6/37 (16%) TSH 3/37 (8%) DI 3/37 (8%) GH 1/37 (3%) GT
Dyer *et al.* (1994) (Suresnes, France)	36	≤ 16.0	33/36 (92%) (23 selective, 8 subtotal)	23/33 (69%) (hypocortisolaemia or "physiological" cure)	1/33 (3%) DI 1/33 (3%) Hypopit Incomplete data

[a]"Cure" defined as undetectable serum cortisol in the immediate postoperative period (<50 nmol/L). *CD*, Cushing's disease; *No.*, number; yrs., years; *GH*, growth hormone; *DI*, cranial diabetes insipidus; hypopit, pan hypopituitarism; *ACTH*, adrenocorticotropic hormone, *GT*, gonadotropins, *TSH*, thyroid stimulating hormone.

Summary and Future Perspectives

Pediatric CD is rare and close liaison with adult endocrinologists who regularly manage CD is recommended. Pediatric CD should be suspected in children who present with weight gain in association with short stature or reduced height velocity. A careful detailed history and a formal investigation protocol requiring hospitalization is important to make a secure diagnosis. The choice and interpretation of tests should be discussed with an adult specialist with experience of CD. Referral should be considered to a centre combining pediatric and adult endocrinology, BIPSS, TSS and pituitary RT. A specialized multidisciplinary approach to define the optimal therapeutic strategy is essential. Additionally, choosing a neurosurgeon experienced in TSS in children is likely to significantly improve the chance of effective and curative therapy. The less invasive technique of endonasal endoscopic transsphenoidal pituitary surgery provides an alternative to conventional transsphenoidal microscopic surgery in managing pediatric CD. Posttreatment management may pose difficulties in optimizing growth, body composition and puberty. Life-long surveillance is required to monitor for potential complications and relapse of CD.

Further studies are required to assess in detail the impact on long-term cognitive impairment and psychopathology following remission of childhood CD. Additionally, further studies are warranted to identify novel genetic defects associated with pituitary corticotroph cell tumourigenesis and to assess the efficacy of new medical therapies and surgical approaches.

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Genetic Control of Fetal Sex Development

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Nomenclature

Gene symbol	Gene name
AMH	Anti-Müllerian hormone
AR	Androgen receptor
CBX2	Chromobox homolog 2
CTNNB1	Catenin (cadherin-associated protein), beta 1
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1
DAX1	Nuclear receptor subfamily 0, group B, member 1 (*NR0B1*)
DHH	Desert hedgehog
DMRT1	Doublesex and mab-3 related transcription factor 1
EMX2	Empty spiracles homeobox 2
FGF9	Fibroblast growth factor 9
FGFR2	Fibroblast growth factor receptor 2
FOG2	Zinc finger protein, multitype 2 (*ZFPM2*)
FOXL2	Forkhead box L2
GADD45γ	Growth arrest and DNA-damage-inducible, gamma
GATA4	GATA binding protein 4
IGF1R	Insulin-like growth factor 1 receptor
LHX9	LIM homeobox 9
MAMLD1	Mastermind-like domain containing 1
MAP3K1	Mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase
MAP3K4	Mitogen-activated protein kinase kinase kinase 4
NR5A1 (SF1)	Nuclear receptor subfamily 5, group A, member 1
PGD2	Prostaglandin D2
PTGDS	Prostaglandin D2 synthase
RSPO1	R-spondin 1
SOX3	SRY (sex determining region Y)-box 3
SOX9	SRY (sex determining region Y)-box 9
SRY	Sex determining region Y
WNT4	Wingless-related MMTV integration site 4
WT1	Wilms tumor 1

Glossary

Gonadal dysgenesis A number of genetic conditions involving abnormal development of the gonads, resulting in underdeveloped and dysfunctional gonads (streak gonads).

Granulosa cells Cells that supply nutrients to the developing oocyte in the ovary and have a function analogous to that of the Sertoli cells in the testis.

Leydig cells Testosterone-producing cells located adjacent to the seminiferous tubules in the testis.

Sertoli cells Somatic supporting cells in the seminiferous tubules of the testes to which spermatids attach until they form spermatozoa.

Steroidogenesis The biological synthesis of steroid hormones (e.g., estrogen, progesterone, testosterone, cortisol, and aldosterone) from cholesterol.

Testis cords Specialized tubular structures that enclose and protect germ cells from external signals, support their development into mature spermatogonia, and eventually channel them into the male reproductive tract.

Theca cells Endocrine cells associated with ovarian follicles that produce the androgen substrate required for ovarian estrogen biosynthesis.

Sex Determination

The gonads are reproductive organs that function to produce gametes and sex hormones. In addition, they have a unique bipotential ability to differentiate into two distinct organs: the testis in males and ovary in females (reviewed in Brennan and Capel, 2004; Eggers *et al.*, 2014; Ohnesorg *et al.*, 2014). As a result of this unique feature, gene mutations that disrupt gonad development not only lead to defects in gonadal formation or function, but can also result in sex reversal.

Sex determination refers to the mechanism that determines the fate of the fetal bipotential gonad, and whether it develops into a testis or ovary (**Fig. 1**). In most mammals, sex is determined by chromosomal constitution, with males and females bearing XY and XX sex chromosomes, respectively. At around 4 weeks in humans or embryonic day 10 (E10) in mice, Steroidogenic factor 1 (SF-1, encoded by the *NR5A1* gene) and Wilms tumor associated gene 1 (*WT1*) are expressed in the genital ridge of both sexes, specifying the formation of the bipotential gonad. The bipotential gonad develops through thickening and proliferation of the coelomic epithelium along the mesonephros, giving rise to the somatic cells of the gonad (Karl and Capel, 1998). The primordial germ cells (PGCs) are specified in the epiblast prior to gonad formation; they then proliferate and migrate to the genital ridge via the hindgut, and eventually populate the gonads around E9.5–E11.5 in the mouse (McLaren, 2003).

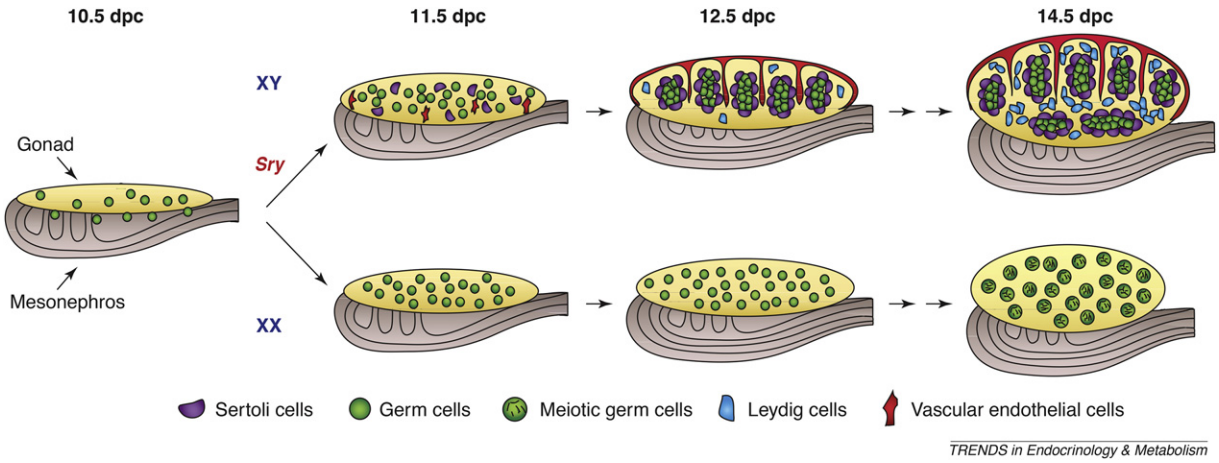

Fig. 1 Early gonad development in the mouse. A thickening of the coelomic epithelium of the mesonephros (*gray*) around E10 allows development of the bipotential gonad (*yellow*). Primordial germ cells (*green*) migrate from the hindgut into the genital ridge and populate the gonads between E9.5 and E11.5. Expression of *Sry* in XY gonads peaking at E11.5, and its downstream activation of *Sox9*, triggers testis differentiation, marked by differentiation and migration of Sertoli cells (*purple*) to enclose germ cells and form testis cords, as well as the formation of male-specific vasculature (*red*). The steroidogenic Leydig cells (*blue*) in the testicular interstitium begin to differentiate around E12.5. In contrast, during ovarian development, regulated by *Rspo1* and *Wnt4*, no apparent morphological changes are observed until the entry of germ cells into meiosis between E13.5 and E14.5. Modified from Ross, A. J. and Capel, B. (2005). Signaling at the crossroads of gonad development. *Trends in Endocrinology and Metabolism*, **16**(1), 19–25.

In the future male, expression of the Y-linked Sex-determining Region on the Y (*SRY*) gene, at 6 weeks in human or E10.5 in mice in pre-Sertoli cells, marks the initiation of male sex determination (Sinclair *et al.*, 1990; Koopman *et al.*, 1991). SRY, together with SF-1 (*NR5A1*), triggers the activation of *SRY*-related HMG box 9 (*SOX9*), thereby initiating a genetic cascade that drives the formation of the testis (**Fig. 1**). In mice, the expression of *Sry* and *Sox9* enables the proliferation of coelomic epithelial cells (Schmahl *et al.*, 2000), the differentiation of Sertoli cells, and their encapsulation of germ cells to form testis cords between E11.2 and E12.5 (Ross and Capel, 2005). In parallel, male-specific vasculature develops from vascular endothelial cells that migrate from the mesonephros into the gonad (Brennan *et al.*, 2002). The steroidogenic fetal Leydig cells then differentiate within the interstitial spaces between testis cords from E12.5 to E13.5 (**Fig. 1**).

In contrast, in the future female, few morphological changes are visible in the early XX gonad (**Fig. 1**). The entry of germ cells into prophase of meiosis between E13.5 and E14.5 in the mouse marks the first apparent feature of ovarian development (Menke *et al.*, 2003). Other events, such as differentiation of the somatic granulosa cells and development of the primordial follicles, occur just prior to birth (Ross and Capel, 2005). Ovarian development in mice is regulated by the expression of R-spondin 1 (*Rspo1*), which activates Wingless-related MMTV integration site 4 (*Wnt4*) signaling (Tomizuka *et al.*, 2008). In addition, Forkhead box L2 gene (*Foxl2*) is a transcriptional regulator required for granulosa cell function. Both Wnt signaling and *Foxl2* repress testicular development. For example, overexpression of β-catenin (*Ctnnb1*), a Wnt signaling component, inhibits the male pathway (Maatouk *et al.*, 2008). It has also been shown that *Foxl2* actively prevents transdifferentiation of the ovary into a testis (Uhlenhaut *et al.*, 2009).

These antagonistic interactions between the male and female pathways are exemplified by sex reversal phenotypes resulting from the gain or loss of function of the above-mentioned genes. For example, loss of *Sox9* in mice causes XY sex reversal, leading to the formation of ovaries (Barrionuevo *et al.*, 2006). Activation of β-catenin in XY gonads also leads to XY sex reversal (Maatouk *et al.*, 2008). In addition, a double knockout of *Foxl2* and *Wnt4* leads to complete XX sex reversal, resulting in the development of testes (Ottolenghi *et al.*, 2007) (**Fig. 1**).

Sex Differentiation

Sex differentiation refers to the progressive acquisition of male or female characteristics in the genital tract and external genitalia. Once sex is determined, subsequent gene expression in the Leydig cells gives rise to testosterone production, which results in the male sexual phenotype. Before sex differentiation has occurred, both male and female reproductive ducts are present in the developing embryo. Through the action of testosterone, the male-specific Wolffian duct develops into the epididymis, vas deferens, and seminal vesicles. Anti-Müllerian hormone (AMH) synthesized in Sertoli cells causes regression of the female-specific Müllerian duct (see **Fig. 2**) (Kobayashi and Behringer, 2003).

Similarly in the female, subsequent estrogen synthesis from the theca cells leads to the female sexual phenotype. Additionally, the lack of testosterone and AMH facilitates regression of the Wolffian duct and allows development of the Müllerian duct into the fallopian tubes (oviducts), uterus, and upper vagina (see **Fig. 2**) (Matzuk and Lamb, 2008).

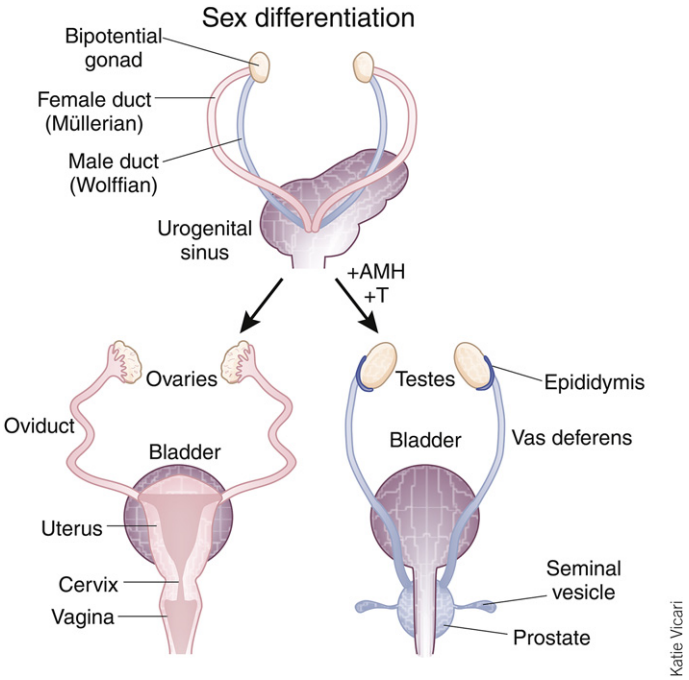

Fig. 2 Overview of human sex differentiation. Testosterone and anti-Müllerian hormone (AMH) secreted by the testis enable regression of the Müllerian duct and development of the Wolffian duct into the epididymis, vas deferens and seminal vesicles. The absence of testosterone (T) and AMH in the female allows regression of the Wolffian duct and development of the Müllerian duct into the uterus, oviducts and upper vagina (Matzuk and Lamb, 2008).

The external genitalia remain undifferentiated in human for up to 9 weeks. Subsequently, the genital tubercle differentiates into the penis in males (under the stimulation of dihydrotestosterone) and clitoris in females, while the labioscrotal swellings develop into the scrotum in males and the labia and vagina in females (Rey and Josso, 2009) (**Fig. 2**).

Disorders of Sex Development

Sex determination and differentiation are tightly regulated by genetic pathways involving several transcription factors and signaling molecules. Disruption of these pathways can cause congenital conditions known as disorders of sex development (DSDs) in which chromosomal, gonadal, or anatomic sex is atypical (Hughes *et al.*, 2006). DSDs occur when the phenotypic sex is inconsistent with the genotypic sex, or when sex determination and differentiation are incomplete or mixed (Matzuk and Lamb, 2008). Perturbations during sex determination can lead to defects in early gonad formation, while disruptions of sex differentiation may affect development of the internal and/or external genitalia and other secondary sexual characteristics.

DSDs can be categorized into three broad groups: (a) Sex Chromosome DSD (e.g., 45,X Turner syndrome and 47,XXY Klinefelter syndrome); (b) 46,XY DSD, which includes disorders of testicular development (e.g., complete/partial gonadal dysgenesis, gonadal regression, and ovotesticular DSD), disorders of androgen synthesis or action, and nonclassified forms such as hypospadias; and (c) 46,XX DSD, which includes disorders of ovarian development (e.g., ovotesticular DSD, testicular DSD, and gonadal dysgenesis) and disorders of androgen excess (Lee *et al.*, 2006; Mendonca *et al.*, 2009).

Clinical phenotypes observed in 46,XY DSD patients include testicular dysgenesis, ovotestes (gonads containing both testicular and ovarian tissue), anorchia (absence of testes due to embryonic testicular regression), cryptorchidism (undescended testes), female or ambiguous external genitalia, micropenis, hypospadias (abnormally positioned urethra), chordee (abnormal downward curvature of the penis), and gynecomastia (breast enlargement). In 46,XX DSD patients, clinical phenotypes such as testis or ovotestis development, primary ovarian insufficiency, Müllerian aplasia, utero-vaginal atresia, clitoromegaly (enlarged clitoris), ambiguous or male external genitalia, and lack of breast development may manifest.

DSDs are currently the most common congenital birth defect, with 1 in 150 boys presenting with hypospadias, 1 in 4500 live births with ambiguous genitalia, and 1 in 20,000 births with gonadal dysgenesis (complete sex reversal; Nassar *et al.*, 2007; Thyen *et al.*, 2006). Patients and their families are often traumatized by the uncertainty of gender, and the accompanying psychosexual consequences and possible surgical interventions. DSD patients are most likely to be infertile, and some are at an increased risk of gonadal cancer.

DSDs are a major pediatric concern and are often misdiagnosed and poorly prognosed due to the lack of an accurate diagnosis. Current approaches to diagnosis involve anatomical, imaging, and endocrine analysis. There is a great need for an accurate genetic

diagnosis of DSDs; this would enable clinicians, patients, and their families to make informed decisions with regard to clinical management and treatment options, thereby improving outcomes for patients.

Genetic Regulation of Fetal Sex Development

Gonad development involves a complex genetic cascade in which several factors either promote or repress each other in order to allow an initial bipotential gonad to differentiate into a testis or ovary (**Fig. 3**). Factors involved in establishment of the bipotential gonad include WT1, LHX9, EMX2, SF-1, GATA4, and its cofactor FOG2. Factors that regulate male sex determination and differentiation include SRY, SOX9, SF-1, IGF1R, WT1, AMH, GATA4/FOG2, PTGDS/PGD2, FGF9/FGFR2, DMRT1, DHH, CBX2, GADD45γ, MAP3K1, and MAP3K4. Ovarian development is controlled by several factors including RSPO1, CTNNB1, WNT4, FOXL2, DAX1, BMP2, and FST. Mutations in most of these genes are known to cause DSDs (reviewed in Eggers *et al.*, 2014; Ono and Harley, 2013; Sreenivasan and Harley, 2013). Despite this knowledge, around 60% of 46,XY DSDs still remain unexplained genetically, highlighting the fact that additional factors critical for gonadal development have yet to be discovered (**Fig. 3**).

Molecular Basis of Disorders of Sex Development (DSD)

Genetic Mutations

Genomic analysis of DSD patients has led to the identification of a number of gene mutations associated with these conditions. Together with gain-of-function and loss-of-function studies in mouse models, the roles of these genes in the control of gonadal development and endocrine function have been defined. Genes that have been implicated in 46,XY and 46,XX DSDs are described below.

AMH *(Anti-Müllerian hormone)*

AMH encodes a glycoprotein secreted by Sertoli cells of the developing testis. It functions as a signaling molecule required for the regression of the Müllerian ducts in male embryos which would otherwise differentiate into the uterus and fallopian tubes. *AMH* is also required for Leydig cell differentiation and function and follicular development in adult females. Homozygous *Amh* knockout XY mice develop female reproductive organs and are often sterile. In XX homozygous knockout mice, premature depletion of primordial follicles occurs. 46,XY DSD patients with *AMH* mutations have normal testes, male ducts, and external genitalia but develop persistent Müllerian duct syndrome (PMDS), type I (Knebelmann *et al.*, 1991).

AMHR2 *(Anti-Müllerian hormone type II receptor)*

The *AMHR* gene encodes a serine-threonine kinase transmembrane receptor to which AMH binds. *AMHR2* mutations result in similar phenotypes to those of *AMH* mutations. Homozygous knockout XY mice have a normal male reproductive tract, but also have a uterus and oviducts. While *Amhr2* knockout XY mice are able to produce functional sperm, most males are infertile as sperm transfer is blocked by the female reproductive organs. Persistent Müllerian duct syndrome (PMDS), type II resulting from *AMHR2* mutations has been shown to be inherited in an autosomal recessive fashion (Imbeaud *et al.*, 1994).

ARX *(Aristaless related homeobox)*

ARX encodes a transcription factor that regulates brain development, neuronal migration, and Leydig cell development. Lack of *ARX* in XY mice causes abnormal neuron development and disrupted Leydig cell differentiation. Human ARX mutations may result in testicular dysgenesis and ambiguous genitalia. Other associated conditions include X-linked lissencephaly with ambiguous genitalia, epilepsy, Proud syndrome (agenesis of the corpus callosum with abnormal genitalia and intellectual disability), and Partington X-linked mental retardation syndrome (Kitamura *et al.*, 2002; Kato *et al.*, 2004).

ATRX *(Alpha thalassemia/mental retardation syndrome X-linked)*

ATRX is a DNA helicase involved in chromatin remodeling that plays a role in testicular differentiation, α-globin regulation, and brain development. *ATRX* deficiency in XY mice causes apoptosis of proliferating Sertoli cells and impaired spermatogenesis (Bagheri-Fam *et al.*, 2011). Human *ATRX* mutations cause X-linked α-thalassemia/mental retardation syndrome and mental retardation-hypotonic facies syndrome. DSD phenotypes include testicular dysgenesis, cryptorchidism, micropenis, hypospadias, and either female, ambiguous, or male external genitalia (McPherson *et al.*, 1995).

CBX2 *(Chromobox homolog 2;* M33*)*

CBX2 encodes a polycomb protein that represses gene transcription during development by chromatin remodeling and histone modifications. *CBX2* regulates *NR5A1*, *SRY*, and *SOX9* transcription. Mice lacking *Cbx2* exhibit XY gonadal sex reversal (Katoh-Fukui *et al.*, 1998). *CBX2* mutations are autosomal recessive, resulting in 46,XY DSD with ovaries containing oocytes and normal internal and external female genitalia (Biason-Lauber *et al.*, 2009).

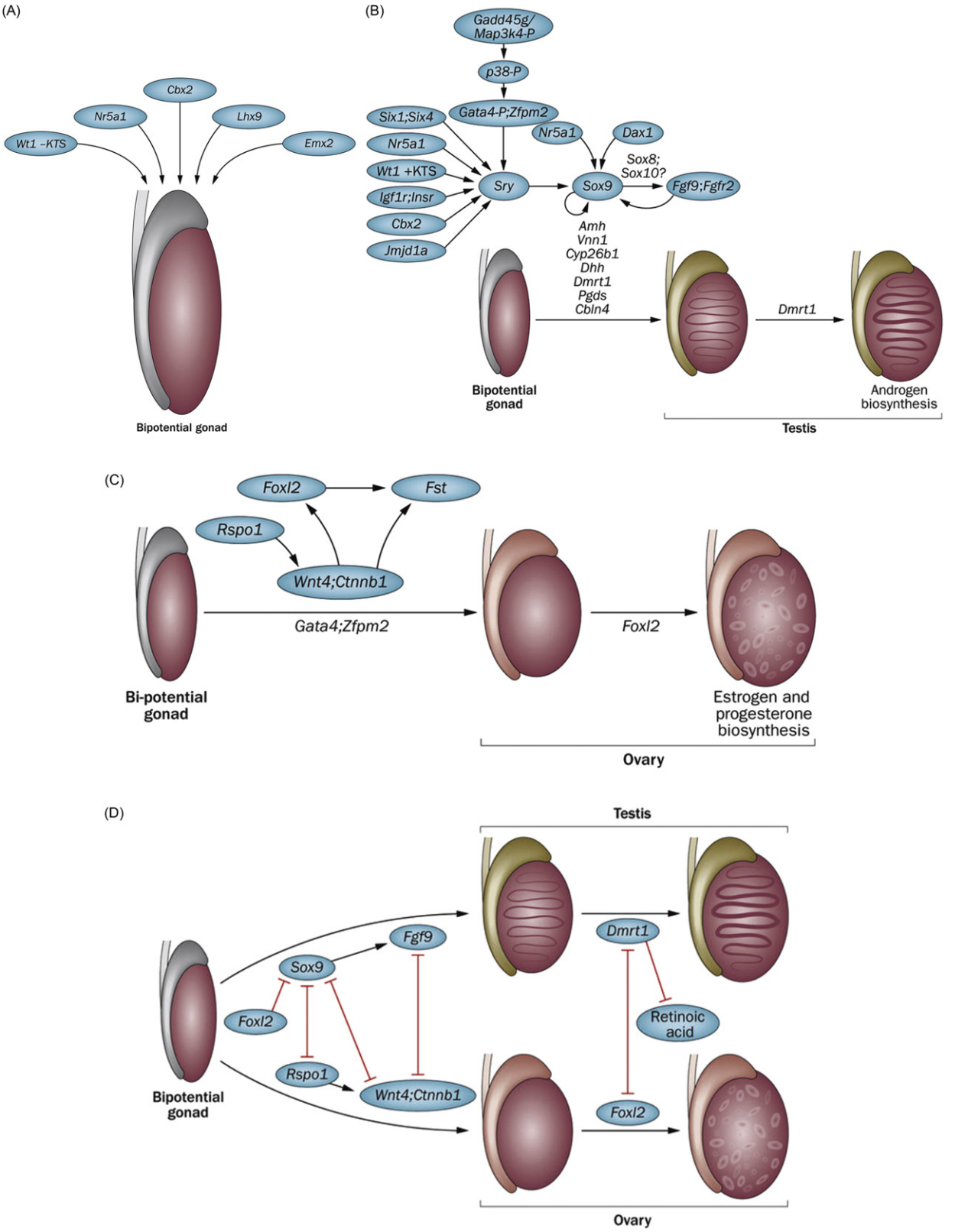

Fig. 3 Genetic regulation of mouse embryonic gonad development. (A) Genes involved in development of the bipotential gonad; (B) Genetic regulation of testis development and differentiation; (C) Genetic regulation of ovarian development and differentiation; (D) Antagonistic interactions between genes that regulate testicular and ovarian development enable repression of the pathway of the opposite sex, thereby preventing sex reversal (Eggers *et al.*, 2014).

CYP19A1 *(Cytochrome P450, family 19, subfamily a, polypeptide 1; aromatase)*

CYP19A1 encodes a cytochrome P450 enzyme that synthesizes estrogen from androgens (Harada, 1992). Autosomal recessive 46,XX DSD with mutations in *CYP19A1* have been reported where patients develop aromatase deficiency and present with hypoplastic ovaries, ambiguous external genitalia, androgenization during pregnancy, and lack of breast development (Ito *et al.*, 1993; Akcurin *et al.*, 2016). 46,XY DSD patients with *CYP19A1* gain-of-function mutations develop aromatase excess syndrome (AEXS), an autosomal dominant disorder resulting from excess estrogen production. Patients with AEXS may develop gynecomastia and mild hypogonadotropic hypogonadism (Shozu *et al.*, 2003).

DAX1 *(DSS-AHC critical region on the X chromosome 1, gene 1;* NR0B1*)*

DAX1 encodes a nuclear receptor required for testis, ovary, adrenal, and gonadotroph differentiation (Niakan and McCabe, 2005). In XY mice, *Dax1* duplications cause male-to-female sex reversal and gonadal dysgenesis. Human *DAX1* duplications result in X-linked recessive, dosage-sensitive male-to-female sex reversal by antagonizing *SOX9* expression, leading to 46,XY testicular dysgenesis, ovarian development, and female or ambiguous external genitalia (McCabe, 1996). Associated phenotypes include adrenal hypoplasia congenita (AHC) with hypogonadotropic hypogonadism.

DHH *(Desert hedgehog)*

DHH is a signaling molecule required for differentiation of Schwann cells as well as the testis (Bitgood *et al.*, 1996). In mice, *Dhh* regulates differentiation of Leydig cells and peritubular myoid cells to enable normal development of the testis cords (Clark *et al.*, 2000). *Dhh* knockout mice develop testes with abnormal peritubular tissue and severely restricted spermatogenesis. *Dhh* has also been implicated in germ cell proliferation and differentiation. Mutations in *DHH* are autosomal recessive and may lead to 46,XY partial gonadal dysgenesis with minifascicular neuropathy (Umehara *et al.*, 2000). Isolated 46,XY gonadal dysgenesis, persistent Müllerian ducts, and female external genitalia may also occur (Canto *et al.*, 2004).

DMRT1 *(Doublesex and mab-3 related transcription factor 1)*

DMRT1 encodes a highly conserved transcription factor essential for male sex determination and testicular differentiation (Raymond *et al.*, 1998). In XY mice lacking *Dmrt1*, postnatal testis development is severely impaired with an apparent loss of Sertoli cells and germ cells. *Dmrt1* prevents the transdifferentiation of male-specific Sertoli cells into female-specific granulosa cells by antagonizing the female sex determining factor *Foxl2* in the postnatal testis (Matson *et al.*, 2011). Hence, postnatal overexpression of *Dmrt1* in XX mice results in transdifferentiation of granulosa cells into Sertoli cells. In 46,XY patients, *DMRT1* deletions result in male-to-female sex reversal (Muroya *et al.*, 2000). Phenotypes include complete gonadal dysgenesis, presence of Müllerian structures, female or ambiguous external genitalia, and cryptorchidism.

FOXL2 *(Forkhead box L2)*

FOXL2 encodes a forkhead transcription factor essential for ovarian folliculogenesis and granulosa cell development. In mice, *FOXL2* antagonizes *SOX9* and *DMRT1* to prevent testicular differentiation in adult females (Matson *et al.*, 2011; Uhlenhaut *et al.*, 2009). As a result, *Foxl2* ablation in adult XX mice leads to transdifferentiation of ovaries to testes. While *FOXL2* is not strictly a causative gene for DSDs, mutations in it cause autosomal dominant blepharophimosis, ptosis and epicanthus inversus syndrome (BPES), characterized by eyelid malformation and female infertility caused by primary ovarian insufficiency (POI; Crisponi *et al.*, 2001).

GATA4 *(GATA-binding protein 4)*

GATA4 encodes a transcription factor required for myocardial differentiation and function as well as for testicular development. GATA4 activates *SRY* expression together with its cofactor *ZFPM2* (*FOG2*) (Tevosian *et al.*, 2002) and also activates *AMH* transcription (Tremblay and Viger, 1999; Watanabe *et al.*, 2000). Testis development is severely impaired in XY mice bearing a *Gata4* knockin with a targeted mutation that abolishes the interaction of *GATA4* with its *FOG* cofactors. Human mutations in *GATA4* result in autosomal dominant 46,XY DSD (testicular dysgenesis and female or ambiguous external genitalia) and congenital heart disease (Lourenco *et al.*, 2011).

HHAT *(Hedgehog acyltransferase)*

HHAT encodes a membrane bound acyltransferase that functions in the Hedgehog signaling pathway. In XY mice, *Hhat* is required for proper testis cord formation and differentiation of fetal Leydig cells. Homozygous *Hhat* knockout mice develop testicular dysgenesis, apolar Sertoli cells, fewer fetal Leydig cells, hypogonadism, and female external genitalia. Human mutations in *HHAT* result in autosomal recessive 46,XY DSD with partial or complete gonadal dysgenesis and chondrodysplasia (Callier *et al.*, 2014).

MAMLD1 *(Mastermind-like domain containing 1;* CXORF6*)*

MAMLD1 is transactivated by SF-1 to regulate fetal Leydig cell function and enhance testosterone production. Human mutations in *MAMLD1* result in X-linked hypospadias 2 (HYSP2) and myotubular myopathy. Hence, *MAMLD1* is essential for the normal development of male genitalia (Fukami *et al.*, 2006). However, while *Mamld1*-deficient mice display significantly reduced expression of multiple genes in fetal Leydig cells, both genital and reproductive development are unaffected (Miyado *et al.*, 2012).

MAP3K1 *(Mitogen-activated kinase 1)*

MAP3K1 functions as a serine-threonine kinase within the JNK and ERK kinase signaling pathways and the NF-kappa-B pathway. *MAP3K1* mutations may cause autosomal dominant complete and partial 46,XY gonadal dysgenesis with persistent Müllerian ducts (Pearlman *et al.*, 2010). Female/male/ambiguous external genitalia, hypospadias, and chordee have also been observed. While XY mice lacking *Map3k1* do not show male-to-female sex reversal (Warr *et al.*, 2011), loss of another MAP kinase *Map3k4* in mice of a sensitized background results in embryonic gonadal XY sex reversal due to reduced *Sry* transcription (Bogani *et al.*, 2009).

RSPO1 *(R-spondin family, member 1)*

RSPO1 encodes a secreted signaling molecule that regulates *WNT4* expression and is essential for female sex determination. *RSPO1* promotes ovarian development by upregulating the canonical WNT/β-catenin signaling pathway to antagonize testis formation. Homozygous XX *Rspo1* knockout mice show partial sex reversal with masculinized ovaries containing a coelomic blood vessel, fetal oocyte depletion, and ectopic testosterone production (Chassot *et al.*, 2008). Human mutations in *RSPO1* lead to the autosomal recessively inherited disorder palmoplantar hyperkeratosis with squamous cell carcinoma of the skin and 46,XX female-to-male sex reversal (Parma *et al.*, 2006). DSD phenotypes include testicular structures, the absence of Müllerian ducts, and masculinized or ambiguous internal and external genitalia.

SF-1 *(Steroidogenic factor 1;* NR5A1; AD4BP*)*

SF-1 is a nuclear receptor encoded by the *NR5A1* gene with crucial roles in steroidogenesis and sex determination, enabling proper development of the adrenal glands, gonads, and gonadotrophs. SF-1 regulates Sertoli cell-expressing genes such as *SOX9* and *AMH*. Gonads and adrenal glands are absent in XY and XX mice that lack *Nr5a1*. *NR5A1* mutations can be autosomal dominant or recessive, and are associated with adrenal hypoplasia congenita (AHC; primary adrenal insufficiency), gonadotrophin deficiency, and both 46,XY and XX DSD. 46,XY patients present with testicular dysgenesis, spermatogenic failure, hypospadias, and female or ambiguous external genitalia. In 46,XX DSD, primary ovarian insufficiency (POI) and testicular or ovotesticular DSD may occur (reviewed in Ferraz-de-Souza *et al.*, 2011).

SOX3 *(*SRY*-related HMG box gene 3)*

SOX3 is an X-linked transcription factor involved in the development of the pituitary and central nervous system; it does not normally play a role in sex development. However, *SOX3* has a sequence almost identical to the Y-linked *SRY* gene and, as a consequence, can substitute for its function. This was demonstrated when ectopic expression of *Sox3* was induced in the developing gonads of transgenic XX mice. This resulted in complete female-to-male sex reversal because *Sox3* was able to function and drive testis development in the absence of *Sry*. Similarly, in humans, rearrangements affecting *SOX3* regulatory regions can cause it to be ectopically expressed in the developing testis and substitute for *SRY*. This resulted in 46,XX female-to-male sex reversal, where patients develop small testes with abnormal spermatogenesis and normal male external genitalia (Sutton *et al.*, 2011). Other conditions associated with *SOX3* mutations include X-linked mental retardation with isolated growth hormone deficiency and panhypopituitarism.

SOX9 *(*SRY*-related HMG box gene 9)*

SOX9 encodes a transcription factor essential for normal testicular differentiation and development of the skeleton and other organs. It regulates several genes in the sex determining pathway, including *AMH*, *FGF9*, *PTGDS*, and *ETV5*, and also maintains its own expression. XY mice lacking *Sox9* show complete male-to-female sex reversal, while XX mice with ectopic expression of *Sox9* show female-to-male sex reversal. In humans, mutations within and around *SOX9* cause CD/SRA (*c*ampomelic *d*ysplasia, *a*utosomal *s*ex *r*eversal), a dominant condition characterized by skeletal abnormalities and sex reversal in 75% of patients (Foster *et al.*, 1994; Wagner *et al.*, 1994). Deletions in regulatory regions upstream of *SOX9* may result in isolated 46,XY DSD with phenotypes such as dysgenetic testes, ovotestes, and female or ambiguous external genitalia. Duplications/triplications in *SOX9* or its regulatory region can result in 46,XX sex reversal with testicular DSD, absent Müllerian ducts, and male or ambiguous external genitalia (Benko *et al.*, 2011).

SOX10 *(*SRY*-related HMG box gene 10)*

SOX10 encodes a transcription factor that is closely related to *SOX9* and is involved in neural crest and glial development. *Sox10* overexpression in XX mice leads to female-to-male sex reversal (Polanco *et al.*, 2010). In humans, *SOX10* duplication in a 46,XX DSD patient led to an almost complete masculinization of the external genitalia (Seeherunvong *et al.*, 2004).

SRY *(Sex-determining region Y)*

The *SRY* gene, located on the Y chromosome, encodes a transcription factor that initiates the genetic regulation of testicular differentiation (Sinclair *et al.*, 1990; Koopman *et al.*, 1991). It is required for the activation of *SOX9* expression, resulting in Sertoli cell differentiation from somatic precursor cells of the bipotential embryonic gonad. XY mice lacking *Sry* exhibit testicular dysgenesis and persistent Müllerian ducts, while XX mice with *Sry* translocations show complete sex reversal. Both 46,XY and 46,XX DSD can result from mutations in *SRY*. In 46,XY individuals, mutations in *SRY* result in Swyer syndrome with phenotypes such as testicular dysgenesis, ovotestis, and female or ambiguous external genitalia, while 46,XX DSD patients with *SRY* translocations show female-to-male sex reversal with male or ambiguous genitalia (reviewed in Goodfellow and Lovell-Badge, 1993).

TSPYL1 *(Testis-specific Y-encoded-like protein 1)*

TSPYL1 is a gene of unknown function that may be involved in chromatin remodeling. Human mutations in *TSPYL1* lead to autosomal recessive sudden infant death with dysgenesis of the testes syndrome (SIDDT; Puffenberger *et al.*, 2004). Reproductive phenotypes include fetal testicular dysgenesis, intra-abdominal and dysplastic testes, ambiguous external genitalia, partial development of the penile shaft, and reduced fetal testosterone levels.

WNT4 *(Wingless-related MMTV integration site 4)*

WNT4 is a signaling molecule that regulates ovarian development and antagonizes testis formation. *Wnt4* knockout in XY mice causes abnormal vasculature and function, while XX knockout mice show partial sex reversal with masculinized features such as ovaries with a coelomic blood vessel, absent Müllerian ducts, presence of Wolffian ducts, and ectopic testosterone synthesis (Vainio *et al.*, 1999). *WNT4* duplication in humans is associated with 46,XY testicular dysgenesis and ambiguous external genitalia (Jordan *et al.*, 2001). *WNT4* mutations may result in Mayer-Rokitansky-Kuster-Hauser syndrome, where 46,XX patients present with utero-vaginal atresia. Müllerian aplasia and hyperandrogenism may also occur (Biason-Lauber et al., 2004, 2007; Philibert *et al.*, 2008). *WNT4* mutations may also cause the autosomal recessive SERKAL syndrome (46,XX *s*ex *r*eversal with dysgenesis of *k*idneys, *a*drenals, and *l*ungs, Mandel *et al.*, 2008).

WT1 *(Wilms tumor associated gene 1)*

The *WT1* gene encodes a transcription factor essential for the development of the urogenital ridge (Wagner *et al.*, 2003). XY and XX mice deficient in *Wt1* lack gonads and a urogenital tract. *Wt1* induces the expression of *Sry*, and also synergizes with *Nr5a1* to activate *Amh* expression (Kreidberg *et al.*, 1993; Hammes *et al.*, 2001). Mutations in *WT1* are autosomal dominant and can result in Frasier syndrome, where 46,XY patients present with testicular dysgenesis and female or ambiguous external genitalia with an increased risk of gonadal tumor. Associated phenotypes include Wilms tumor, type 1 (a kidney tumor), WAGR syndrome (*W*ilms tumor, *a*niridia (absence of the iris), *g*enitourinary anomalies and mental *r*etardation), and Denys-Drash syndrome (Barbaux *et al.*, 1997; Haber *et al.*, 1990; Hastie, 1992; Park *et al.*, 1993; Schumacher *et al.*, 1998).

ZFPM2 *(Zinc finger protein, FOG family member 2;* FOG2*)*

ZFPM2 encodes a zinc finger cofactor of GATA4. *Zfpm2* deficiency in XY mice leads to reduced *Sry* expression and male-to-female sex reversal (Manuylov *et al.*, 2011). Human mutations in *ZFPM2* cause complete or partial gonadal dysgenesis in 46,XY individuals (Bashamboo *et al.*, 2014).

Androgen-related genes implicated in DSDs

Genes that encode components of the steroidogenic pathway have also been implicated in DSDs. These DSD mutations are all autosomal recessively inherited. Patients with such mutations usually have normal gonadal development, but exhibit perturbations in external genitalia development due to abnormal androgen synthesis or action. Androgen-related genes that have been implicated in DSDs are listed in **Table 1**.

Alterations Affecting Noncoding Regulatory Regions of DSD Genes

As discussed earlier in this article, much of what is known about the genetic cause of DSD comes from examining changes in the coding regions of genes. However, there is an ever-growing number of genes that are affected by noncoding variation.

Noncoding variation is broadly defined as mutations, deletions, insertions, and rearrangements in regions of the genome not directly required for protein coding. This can include promoters and introns as well as regions far up or downstream of the gene itself (Baetens *et al.*, 2016). Variations in these noncoding regions often influence the regulation of genes, altering where, when, or how much a particular gene is expressed (Shlyueva *et al.*, 2014). This is an important factor when studying the genetic causes of DSD, as inappropriate expression of a male determining gene in an XX environment or vice versa can cause sex reversal. In this section, we shall discuss how alterations to noncoding regulatory regions can cause DSD.

SRY

SRY, the master sex determining gene, is most commonly translocated from the short arm of the Y chromosome onto the X, causing male-to-female 46,XX ovotesticular DSD. Mutations in the coding region of the gene are also responsible for a significant proportion of 46,XY DSD cases. However, there is only one known case of a mutation upstream of *SRY* in the promoter. This 3 bp deletion removes an important transcription factor binding site, preventing transcriptional activation of *SRY* in the developing gonad (Assumpcao *et al.*, 2005). This, in turn, caused 46,XY complete gonadal dysgenesis in the patient and was inherited from the father, who carried the mutation and had severe hypospadias as a child.

SOX9

SOX9 expression in the testis is regulated by a complex and undefined number of regulatory elements within a region 2 Mb upstream of the promoter. Multiple cases of copy number variations (CNVs) upstream of *SOX9* have been identified in both 46,XY and 46,XX DSD. Duplications ~500 kb upstream of *SOX9* in 46,XX individuals lead to female-to-male sex reversal, whereas

Table 1 Androgen-related genes implicated in DSDs

Gene symbol	*Gene name*	*Phenotypes*
46,XX DSD due to androgen excess		
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	CAH, primary adrenal failure, hyperandrogenism, clitoromegaly
CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2	CAH, hyperandrogenism, male/ambiguous external genitalia
CYP11B1	Cytochrome P450, family 11, subfamily B, polypeptide 1	CAH, glucocorticoid-remediable aldosteronism, ambiguous external genitalia
POR	P450 (cytochrome) oxidoreductase	CAH, Antley–Bixler syndrome with disordered steroidogenesis, ambiguous external genitalia
GCCR (NR3C1)	Glucocorticoid receptor	Cortisol resistance, ambiguous external genitalia
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	CAH, primary adrenal failure, partial androgenization, ambiguous external genitalia, hypospadias
LHCGR	Luteinizing hormone/choriogonadotropin receptor	Leydig cell hypoplasia, hypergonadotropic hypogonadism, female/ambiguous external genitalia, micropenis
DHCR7	7-dehydrocholesterol reductase	Smith–Lemli–Opitz syndrome, female/ambiguous external genitalia
STAR	Steroidogenic acute regulatory protein	Lipoid adrenal hyperplasia (primary adrenal failure), pubertal failure, female external genitalia
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	Congenital adrenal insufficiency with partial/complete 46,XY sex reversal, pubertal failure, female or ambiguous external genitalia
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	CAH, hypertension, micropenis, female/ambiguous external genitalia
POR	P450 (cytochrome) oxidoreductase	CAH, Antley–Bixler syndrome with disordered steroidogenesis, male/ambiguous external genitalia
HSD17B3	Hydroxysteroid (17-beta) dehydrogenase 3	Undermasculinisation, gynecomastia, female/ambiguous external genitalia
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2	Partial androgenization at puberty, micropenis, ambiguous external genitalia, hypospadias
CYB5A	Cytochrome b5 type A, microsomal	Methemoglobinemia, type IV, female external genitalia
AKR1C2	Aldo-keto reductase family 1, member C2	Obesity, hyperphagia, and developmental delay, testes lacking spermatogonia, uterus-like structure, cryptorchidism, female/ambiguous genitalia
AR (NR3C4)	Androgen receptor	Complete/partial androgen insensitivity syndrome (AIS), gynecomastia, blind vagina, micropenis, abdominal/inguinal testes, X-linked hypospadias 1 (HYSP1), female/ambiguous genitalia, Kennedy disease

CAH, congenital adrenal hyperplasia

deletions in this region can result in 46,XY male-to-female sex reversal. The overlap of the patients' CNVs has defined two separate regulatory regions RevSex (24 kb, Ohnesorg *et al.*, 2017) and XYSR (34.5 kb, Kim *et al.*, 2015). It is theorized that these regions of the genome contain testis-specific enhancers necessary to drive *SOX9* expression in the testis. A loss of one or both of these regions in an XY individual results in a loss of *SOX9* expression and sex reversal (Kim *et al.*, 2015), whereas a duplication of the RevSex region in an XX individual could increase *SOX9* expression driving testis development (Benko *et al.*, 2011; Cox *et al.*, 2011; Hyon *et al.*, 2015; Vetro *et al.*, 2011; Xiao *et al.*, 2013).

SOX3

There have been cases of CNVs upstream and downstream of *SOX3* causing inappropriate ectopic expression of the gene in the developing testis, which causes sex reversal in 46,XX patients (Sutton *et al.*, 2011). *SOX3* shares a lineage with SRY, and they have almost identical DNA sequences. Although *SOX3* has no normal role in gonad development, it is proposed that abnormal ectopic expression of *SOX3* in the embryonic gonad is sufficient to substitute for SRY and drive testis development in an XX environment.

GATA4

A 35 kb deletion downstream of GATA4 was identified in a 46,XY patient with gonadal dysgenesis, including the *NEIL2* gene (White *et al.*, 2011). This downstream deletion contains a potential regulatory element within the intron of NEIL2, a gene with no

known or plausible function in sex differentiation (Ohnesorg *et al.*, 2016). *GATA4* regulation is also affected by deletions directly upstream of the gene causing 46,XY DSD (Harrison *et al.*, 2014).

NR0B1 (DAX1)

NR0B1 is sensitive to copy number changes, and many patients have been identified with duplications (46,XY DSD) and deletions (46,XX DSD) of the gene causing DSD (Barbaro *et al.*, 2007, 2008; Ledig *et al.*, 2010; Rojek *et al.*, 2016; White *et al.*, 2011). The regulatory region of NR0B1 is also sensitive to changes in copy number, as one case of a deletion 11–268 kb upstream of the coding region has been found to be associated with 46,XY sex reversal (Smyk *et al.*, 2007). A 60 kb inversion of the regulatory region, 4 kb upstream of the gene was discovered in one patient with adrenal hypoplasia and hypogonadotropic hypogonadism (Skinningsrud *et al.*, 2009). The inversion relocated the binding site of SF1 within the regulatory region, altering *NR0B1* expression.

DMRT1

Alterations in gene copy number resulting in a loss of DMRT1 have been detected in many cases of 46,XY gonadal dysgenesis (Barbaro *et al.*, 2009; Ledig et al., 2010, 2012; Tannour-Louet *et al.*, 2010). However, there is one patient with a deletion upstream of *DMRT1* resulting in the loss of the DMRT1 regulatory region (Barbaro *et al.*, 2009; Calvari *et al.*, 2000).

AR

Multiple noncoding variations have been identified associated with *AR* in human DSD cases. One such mutation was identified in the 5′ untranslated region of the *AR* gene introducing a new upstream start codon. This caused the expression of a short upstream open reading frame, which reduces the translation of the actual *AR* gene (Hornig *et al.*, 2016). Another noncoding variant has been detected in two sisters with 46,XY androgen insensitivity syndrome (AIS). This variant, located in intron 6 of the *AR* gene, created a de novo 5′ splice site and exonic splicing enhancer motif, causing two different spliced transcripts, leading to a premature stop codon within the transcript (Känsäkoski *et al.*, 2016).

New Technologies in DSD Research

Genomic technologies have come a long way in the last few decades, ever since the human genome was sequenced. The advent of massively parallel sequencing (MPS) has created multiple avenues for genomic research. Up until recently, the process of detecting single gene variation was laborious and expensive, as techniques such as Sanger sequencing can only target one specific region. The sheer number of genes that are associated with DSD also made it difficult to know which gene to screen. This section will focus on the new and emerging technologies being used to discover novel variants associated with DSD.

CNV Analysis

The majority of DSD research traditionally focuses on single nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs) in the coding regions of genes. However, as we have discussed in this article, a significant proportion of DSDs can be attributed to changes in copy number of genes as well as their regulatory regions. Specialist technologies are required to detect duplications or deletions greater than 1 kb (Redon *et al.*, 2006).

Historically, the only CNVs that could be detected were large-scale chromosomal rearrangement, above 10 Mb, using fluorescent in situ hybridization (FISH). FISH is still commonly used today to detect a patient's karyotype and presence of the Y chromosome. However, as FISH is limited in resolution and the technique can be cumbersome, many researchers are moving towards sequencing-based methods to detect karyotype and changes in copy number.

There are a number of technologies available to detect much smaller CNVs, such as those less than 1 kb. The two most popular technologies are CNV arrays and multiplex ligation-dependent probe amplification (MLPA). The two array technologies for detecting small CNVs are SNP arrays and comparative genomic hybridization (CGH) arrays. SNP arrays use fluorescent probes targeted to common SNPs. A higher intensity of SNP fluorescence indicates a duplication, and a lower intensity a deletion of the region (Nowak *et al.*, 2009). CGH arrays use tagged oligonucleotide probes to specific regions so that the copy number can be determined by changes in the probe number of the test sample compared to a control sample (Bashamboo *et al.*, 2010). These arrays can be customized to target certain regions of interest with higher probe density; the probes can also be evenly spaced across the entire genome to assay overall CNV.

By contrast, MLPAs are more targeted, cheaper, and quicker to use if screening a few key loci at a time. MLPA is a PCR-based method that can interrogate up to 40 genomic loci (Schouten *et al.*, 2002). These probes can be custom designed to allow for detection of CNVs at a number of locations, both coding and noncoding. MLPA is primarily used to screen for common CNVs associated with particular groups of patients (Sellner and Taylor, 2004). Arrays and MLPAs are often used sequentially. MLPAs are commonly used to confirm the results obtained from arrays and fine map CNVs, as probes can be designed to cluster around known breakpoints (Kim *et al.*, 2015; Ohnesorg *et al.*, 2017; White *et al.*, 2011, 2012).

Massively Parallel Sequencing Technologies

MPS, previously known as next generation sequencing, includes methods such as whole exome, whole genome, and custom targeted gene sequencing panels. MPS uses a parallel sequencing method where short oligonucleotide adapters are bound to the genomic fragments (100–300 bp). These fragments are then amplified using PCR and bound to a sequencing plate which can be read (Van Dijk *et al.*, 2014). The short reads are reassembled and aligned to the reference genome before variant analysis is conducted.

Until recently, targeted gene panels had been the norm when examining large DSD patient cohorts (Eggers *et al.*, 2016). Targeted panels have the benefit of being tailored to the disease of interest, allowing researchers to examine known causative genes and research candidates. Overall, a targeted approach reduces the bioinformatic load and prevents incidental findings, making it easier for researchers to find causative variants (Croft *et al.*, 2016). When large patient cohorts are being examined, targeted panels are considerably cheaper than other sequencing methods. However, as the number of genes associated with disorders such as DSD grows, targeted panels can be limited.

Whole exome sequencing (WES) analyzes the protein coding regions of the genome. The exome is less than 2% of the total genome, yet it contains around 85% of known disease-causing variants (Choi *et al.*, 2009; Van Dijk *et al.*, 2014). WES has a distinct advantage over targeted gene panels, as the data generated can be used to examine diagnostic and candidate genes as well as novel genes yet to be associated with DSD. WES is increasingly the method of choice for screening patient cohorts, although researchers often focus their analysis on a candidate list similar to targeted sequencing.

Whole genome sequencing examines the entire genome: coding and noncoding regions, such as promoters, enhancers, and introns. All these regions can be assayed and examined for potentially damaging variants. With the help of advanced bioinformatic techniques, WGS can also be used to detect CNVs. WGS generates the greatest amount of data of any technique, making it a challenge to store and analyze these data. As with WES, it can be difficult to filter out causative variants, requiring the data to be refined with candidate lists of coding and noncoding regions to focus the analysis. Currently, WGS remains the most expensive option for examining patient data, and only two small studies using WGS have been conducted on DSD patients (De Sousa *et al.*, 2016; Haines *et al.*, 2015). However, as the price continues to drop, WGS will become the only test necessary to capture the genetic variation in a patient. Sequence data from those patients who did not receive an initial diagnosis can be re-analyzed in subsequent years, as our knowledge of the genetics underlying DSD grows.

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Hormonal Control of Fetal Sex Development

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Nomenclature

AR	Androgen receptor
AMH	Anti-Müllerian hormone
APOD	Apolipoprotein D
AIS	Androgen insensitivity syndrome
DSD	Disorders of sex development
HOXA13	Homeobox gene A13
PMDS	Persistent Müllerian duct syndrome
RWDD1	RWD domain containing 1
TGFß	Transforming growth factor beta

In contrast to gonadal development in the early embryo, which is determined by the sex chromosomes and by the subsequent coordinated expression of sex specific genes in the gonadal anlagen, the sexually dimorphic anatomic differentiation of male and female internal and external genitalia after the 7th week of gestation is under hormonal control (Wilson *et al.*, 1981). The presence of the testes and their hormones, testosterone produced in the Leydig cells (Wilson *et al.*, 1981) and Anti-Müllerian Hormone (AMH) produced in the Sertoli cells (Lee and Donahoe, 1993), are crucial for the development of the male internal and external genitalia. In contrast, the absence of the testes and the consequent lack of these two hormones in the embryo result in the development of female internal and external genitalia.

Hormonal Control of Sex Development by Anti-Müllerian Hormone (AMH)

Anti-Müllerian Hormone (AMH) is synthesized and secreted by the Sertoli cells within the fetal testes. It is a transforming growth factor beta (TGFß) family member peptide and signals specifically through the Anti-Müllerian Hormone type II receptor (Josso et al., 1993, 2006). AMH expression is regulated in the early embryo by SRY HMG box related gene 9 (SOX9) at the very onset of testicular differentiation from the bipotent gonadal anlagen, by steroidogenic factor-1 (SF-1), by Wilms' tumor 1 (WT1) and by GATA zinc finger transcription factors (Rey *et al.*, 2003) (**Fig. 1**). In the male fetus, AMH actively represses the development of the paired Müllerian ducts, thus inhibiting the development of the upper part of the vagina, the uterus and the fallopian tubes (Rey and Picard, 1998). Animal studies in rats have shown that AMH targets the Müllerian ducts in the fetus through the Müllerian duct mesenchyme which is the sole initial site of AMH type II receptor expression. This in turn affects the mesenchymal–epithelial interaction in the male, resulting in apoptosis of the intraductal epithelium (Roberts *et al.*, 1999). Animal data on mice have further suggested that the WNT pathway is involved in AMH-mediated Müllerian duct regression since inactivation of β-catenin can block this process (Kobayashi *et al.*, 2011). The absence of high Anti-Müllerian hormone concentrations during regular female genital differentiation consequently results in normal differentiation of the Müllerian ducts and hence leads to the development of the female internal genitalia (Rey and Picard, 1998) (**Fig. 1**). The key role of AMH in sex-specific hormonal control of Müllerian duct development is supported by the persistent Müllerian duct syndrome (PMDS) in otherwise normally virilized 46,XY males due to inactivating mutations in either the AMH-gene or in the AMH type II receptor gene (Picard *et al.*, 2017). Further clinical evidence for the role of AMH in Müllerian duct regression comes from patients with 46,XY disorders of sex development (DSD) due to gonadal dysgenesis (Josso *et al.*, 2013). They typically have low AMH due to impaired Sertoli cell development in the dysgenic testes. Consequently, Müllerian ducts have developed partially or completely in affected individuals. In contrast, 46,XY DSDs with a high degree of testicular differentiation and intact Sertoli cells, for example, androgen insensitivity syndrome and 5alpha reductase type II deficiency, have normal-for-male AMH levels, and in consequence Müllerian ducts have typically undergone regression (Josso *et al.*, 2013). An understanding of the role of AMH in hormonal control of sex development is therefore important for clinical differential diagnosis in DSDs.

The Role of Androgen in the Hormonal Control of Fetal Sex Development

Virilization of the internal and external genitalia in the male occurs between the 8th and 12th week of gestation and is mediated by testosterone secreted by the fetal testes (Siiteri and Wilson, 1974; Wilson *et al.*, 1981; Goto *et al.*, 2006). In response to adequately high levels of testosterone, the distal parts of the paired Wolffian ducts will develop into epididymis, ductus deferens and Glandulae vesiculares, while the distal orifices of the Wolffian ducts ending in the urogenital sinus will form the later pars prostatica and pars membranosa of the urethra. The genital tubercle and the urethral folds of the initially bipotent external genitalia will form the glans penis and the corpus spongiosum (**Fig. 2**). Midline fusion of the labioscrotal swellings results in the corpus cavernosum and the scrotum. The latter process is completed at the end of the 12th week of gestation.

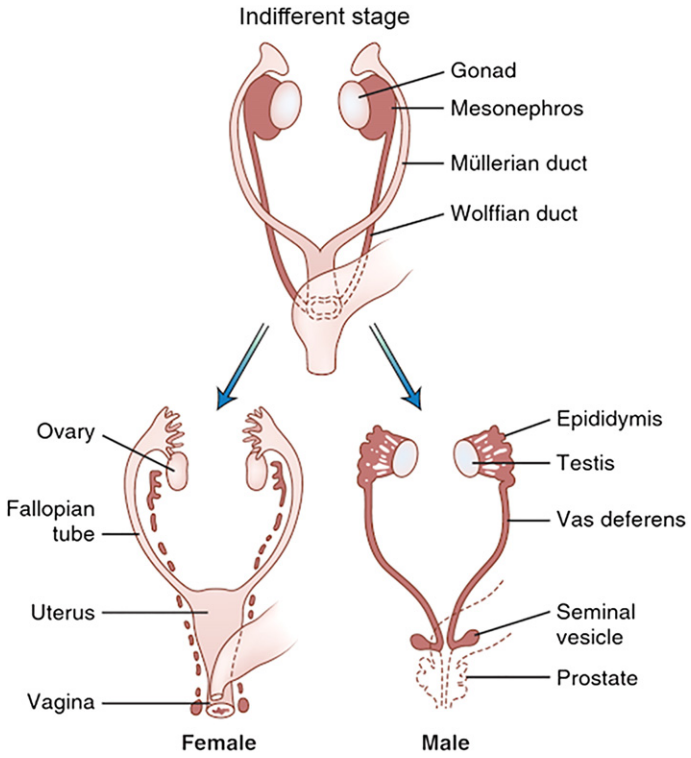

Fig. 1 Embryonic differentiation of female and male genital ducts from Wolffian and Müllerian primordial tissue before descent of the testes into the scrotum. In females, Müllerian structures persist to form the fallopian tubes, uterus, and upper portion of the vagina. The lower portion of the vagina and urethra are derived from the urogenital sinus. In males, Wolffian structures develop into the epididymides, vasa deferentia, and seminal vesicles, whereas the prostate and prostatic urethra are derived from the urogenital sinus. In some cases, a small Müllerian remnant can persist in males as a testicular appendage. Adapted from Acherman, J.C. and Hughes, I.A., (2016). Pediatric disorders of sex development, In: Melmed, S., Polonsky, K.S., Larsen, P.R., (eds.). *Williams Textbook of Endocrinology*, (13th edn.), ISBN 978-0-323-29,738-7, Philadelphia, PA: Elsevier.

The absence of high levels of testosterone in the female during this critical time window leads to regression of the Wolffian ducts. The urethral folds will form the later labiae minorae, and the labioscrotal swellings differentiate to become the labiae majorae. The genital tubercle develops into the female clitoris (Wilson *et al.*, 1981). There is evidence for estrogen receptor expression in the fetal urogenital sinus, the vagina (Taguchi *et al.*, 1986) and the uterus of female human fetuses (Glatstein and Yeh, 1995). However, ovarian biosynthesis of estradiol is believed to not be biologically relevant in the fetus but rather during puberty to promote breast development, growth and maturation of the uterus and the Fallopian tubes, as well as female body proportions.

Testicular descent completes fetal male genital differentiation. A transabdominal phase between the 8th and 15th week of gestation involves regression of the cranial ligament and enlargement of the caudal genito-inguinal ligament (gubernaculum) (Hutson and Hasthorpe, 2005). The subsequent inguinoscrotal phase occurs between the 25th and 35th week of gestation and is characterized by the migration of the gubernaculum from the groin to the scrotum (Hutson and Hasthorpe, 2005). Testicular descent is strictly androgen dependent although the Leydig cell factor insulin-like peptide 3 (INSL3) and its receptor RXFP2 are also involved (Zimmermann *et al.*, 1999; Feng *et al.*, 2009).

Steroid formation in the human fetal testes is initiated between the 8th and 11th week of gestation and peaks between the 11th and 14th week (Tapanainen *et al.*, 1981). It occurs in the Leydig cells and is thought to be almost entirely dependent on hCG/LH signaling via the LHCGR (luteinizing hormone receptor/human chorionic gonadotrophin receptor), which is a seven transmembrane G-protein-coupled receptor on the cell surface (Themmen *et al.*, 1998). While LH is low or even unmeasurable before the 12th week of gestation in the male fetus, hCG is already present at this time and peaks between the 11th and 14th week. Despite the rise of LH between the 14th and 20th week, hCG still remains the dominant detectable gonadotrophin at this time, indicating that it is the primary initial stimulus for early human testosterone biosynthesis (Clements *et al.*, 1976). In contrast, during the second half of pregnancy, fetal pituitary LH takes control over testicular testosterone biosynthesis (Winter *et al.*, 1977). Human fetal testicular tissue is able to bind hCG specifically in order to subsequently stimulate testosterone formation in vitro between the 14th and 19th week of gestation (Huhtaniemi *et al.*, 1977). Before the 15th week of gestation specific high affinity binding sites for hCG are low compared to the maximum reached between the 15th and 20th week, after which they decline (Molsberry *et al.*, 1982). There appears to be a discrepancy between the relatively late time point of the presence of relevant hCG-concentrations and hCG-binding in these classic experimental studies and the fact that the anatomic genital virilisation process has already been completed in the 12th week of gestation (Wilson *et al.*, 1981; Goto *et al.*, 2006). Interestingly, Del Valle

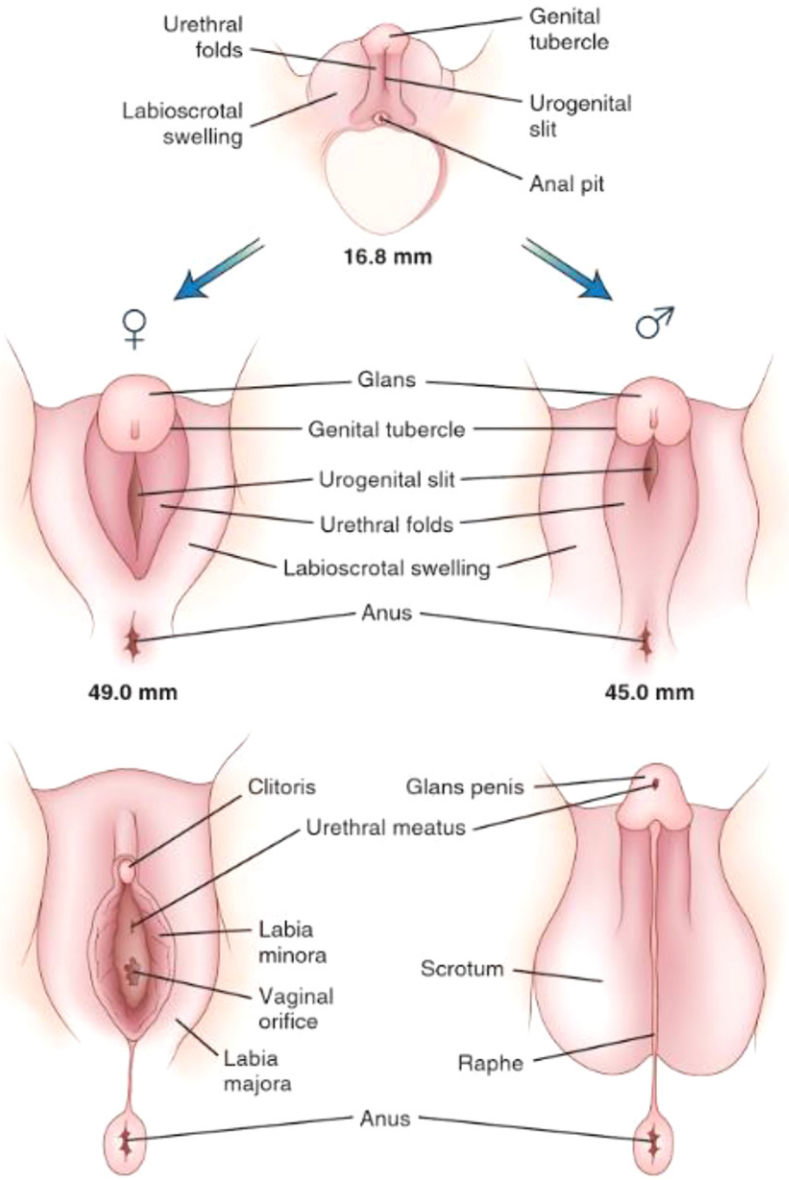

Fig. 2 Differentiation of male and female external genitalia. Adapted from Acherman, J.C. and Hughes, I.A., (2016). Pediatric disorders of sex development, In: Melmed, S., Polonsky, K.S., Larsen, P.R., (eds.). *Williams Textbook of Endocrinology*, (13th edn.), ISBN 978-0-323-29,738-7, Philadelphia, PA: Elsevier.

and co-workers have recently compiled a comprehensive genomic atlas of human adrenal and gonadal development which is a valuable scientific resource for understanding human genital differentiation (Del Valle *et al.*, 2017). They found that LHCGR mRNA already increases markedly in the 8th week of gestation in the human fetal testis, reaching peak levels at about the 9th week of gestation (Del Valle *et al.*, 2017) (**Fig. 3**). This molecular study therefore documents the very early presence of the LHCGR in the fetal Leydig cells and supports the role of the hCG-LHCGR signaling cascade in the control of fetal testosterone formation. Further downstream within the Leydig cells, steroid biosynthesis via the LHCGR involves the intracellular second messenger cyclic adenosine 3′,5′-monophosphate (cAMP), which then stimulates expression of two important cytochrome P450 steroid biosynthesis enzymes, P450 side chain cleavage and 17alpha hydroxylase/17,20 lyase (Payne, 1990). Del Valle found in their genomic atlas that mRNA expression levels of STaR (steroidogenic acute regulating protein), CYP11A1 (P450 side chain cleavage), HSD3B2 (3ß hydroxysteroid dehydrogenase, type II), CYP17A1 (17alpha hydroxylase/17,20 lyase) and HSD17B3 (17ß hydroxysteroid dehydrogenase type III) rise in parallel with LHCGR mRNA during the 8th week of gestation (Del Valle *et al.*, 2017). Therefore, the complete classic steroid biosynthesis pathway from cholesterol down to testosterone is reflected in the enzymatic mRNA-expression pattern detectable in the early testis after the 7th week of gestation. The biological relevance of all of these different steps for the normal formation of gonadal testosterone, and consequently for normal genital differentiation in the male human fetus, is underlined in individuals with disorders of sex development (DSD) with inactivating mutations in the coding

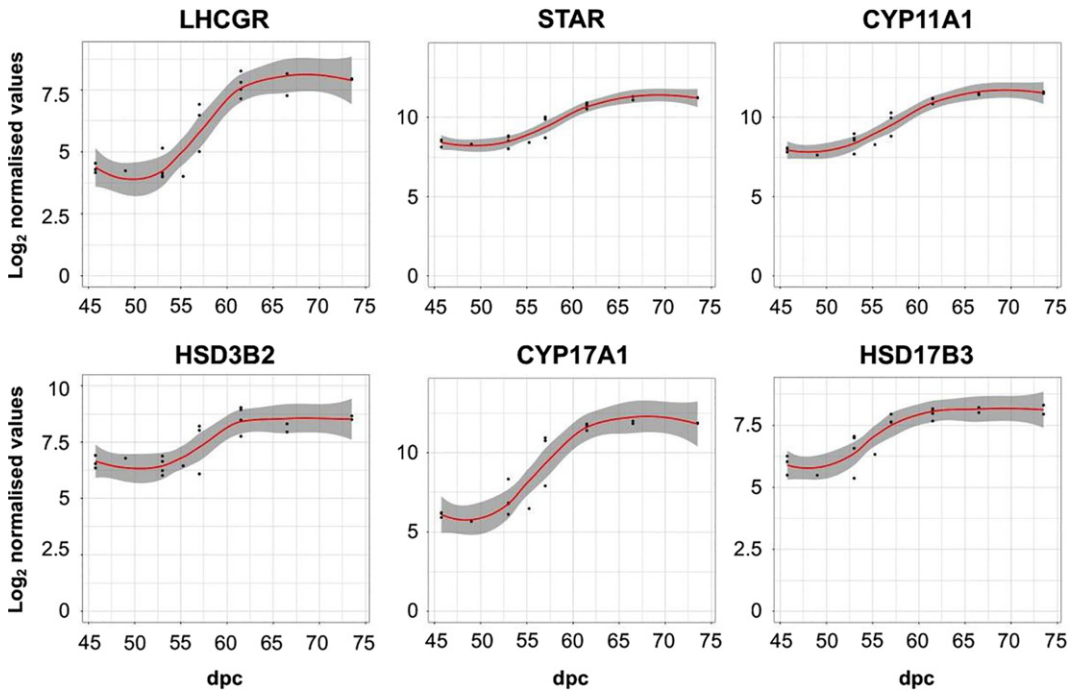

Fig. 3 Gene expression of key steroid biosynthesis genes during early testicular development (Figure from Del Valle, I., Buonocore, F., Duncan, A.J., et al., (2017). A genomic atlas of human adrenal and gonad development. *Wellcome Open Research* **2**, 25). The scatter plots show changes in gene expression patterns for known testicular steroidogenic genes, LHCGR, STAR, CYP11A1, HSD3B2, CYP17A1, and HSD17B3 (normalized gene expression values for testis samples between approximately 46 and 74 dpc).

genes. The latter can all result in deficient to absent genital virilisation, for example, in Leydig cell hypoplasia (Kremer *et al.*, 1995), STaR deficiency (Lin *et al.*, 1995), P450scc deficiency (Hiort *et al.*, 2005), 3ß-hydroxysteroid dehydrogenase type II deficiency (Rheaume *et al.*, 1992), 17alpha/17,20 lyase deficiency (Kagimoto *et al.*, 1989) and 17ß hydroxysteroid dehydrogenase type III deficiency (Geissler *et al.*, 1994).

Anatomic genital virilisation also requires proper peripheral conversion of testosterone to the more active metabolite dihydrotestosterone (Keller *et al.*, 1996) through steroid 5alpha reductase. Two different isoenzymes exist for the 5alpha-reductase, type I and type II, of which the type 2 enzyme is pivotal for fetal sexual development and is expressed in the fetal genital skin, the fetal male accessory sex glands and the prostate (Thigpen *et al.*, 1993). Deletion of the SRD5A2 gene encoding 5alpha-reductase type 2 or inactivating mutations in this gene cause 5alpha reductase type II deficiency characterized by a virilisation deficit in the external genitalia. This underlines the fact that testosterone alone is not sufficient for achieving the normal male genital phenotype but that dihydrotestosterone is also needed (Andersson *et al.*, 1991; Wilson *et al.*, 1993).

There is some data suggesting that the classical gonadal androgen biosynthesis pathway might not on its own be sufficient for ensuring normal male or female sexual differentiation. Some years ago an alternative biochemical pathway to dihydrotestosterone, known as the backdoor pathway, was discovered (Auchus, 2004). AKR1C2 (aldo-keto reductase family 1 member C2) is one of the enzymes which is involved in this alternative pathway but not in the classic pathway. Partially inactivating mutations of the AKR1C2 gene have been described in four individuals with a mild to moderate virilisation deficit at birth (Flück *et al.*, 2011), suggesting that in certain situations the classic biosynthesis pathway to dihydrotestosterone is not the exclusive player in regular masculinization of the male fetal genital tract. In female development, some experimental evidence shows that the low-androgen environment necessary for female internal and external genital differentiation needs to be protected by early adrenal cortisol biosynthesis limiting inadequately early adrenal androgen formation via negative feedback to the anterior pituitary corticotrophs (Goto *et al.*, 2006). These two examples show the increasing complexity of our understanding of hormonal control and modulation of fetal sex development but they also offer new opportunities for a comprehensive understanding of the biochemical mechanisms of disorders of sex development.

Androgens induce their sex differentiating effects in the fetal genital target tissues via the intracellular androgen receptor which is a ligand-activated nuclear transcription factor of androgen-regulated target genes (Brinkmann *et al.*, 1989). The androgen receptor shares its characteristic modular structure of three distinct functional domains with other members of the nuclear receptor family. A comparatively large first exon encodes the N-terminal transactivation domain. Exons 2 and 3 encode the first and second zinc finger important for DNA binding of the receptor. This is connected by a short hinge region to the C-terminal ligand (androgen) binding domain encoded by exons 4–8 (Brinkmann *et al.*, 1989; Werner and Holterhus, 2014). Androgen binding induces structural changes to the androgen receptor, recruitment of co-regulators, translocation into the nucleus, dimerization, chromatin remodeling, binding to specific androgen-response DNA-elements eventually leading to transcription and translation of

androgen responsive target genes (Werner and Holterhus, 2014). As described above for the Müllerian ducts, AMH and the mesenchymal AMH type II receptor (Roberts *et al.*, 1999), mesenchymal–epithelial interactions also play a crucial role in transducing morphogenetic androgen effects via the androgen receptor during genital virilization in the fetus (Cunha *et al.*, 1981; Cunha, 2008). Based on an impressive series of tissue recombination experiments on mouse urogenital sinus and prostate development, Cunha and coworkers documented that only the urogenital mesenchyme exhibits nuclear androgen binding sites in the early embryo. The mesenchyme is the primary target and mediator of morphogenetic effects of androgens on the epithelium, inducing subsequent specific patterns of morphogenesis, cytodifferentiation and biochemical properties. Thereafter, the epithelium begins to express the androgen receptor following a coordinated temporal sequence (Cooke *et al.*, 1991). The pivotal role of the urogenital sinus mesenchyme is further confirmed by experimental tissue recombinants composed of androgen receptor positive rat urogenital sinus mesenchyme associated with bladder epithelium from androgen insensitive mice in which the mesenchyme still induces epithelial morphogenesis in response to androgen (Sugimura *et al.*, 1986). Accordingly, immunohistochemical detection of the androgen receptor in human embryos is restricted to the mesenchyme of the urogenital sinus at 8 weeks of gestation while it appears subsequently in the epithelium only after 9 weeks of gestation (Sajjad *et al.*, 2004). The central role of the androgen receptor in human sexual differentiation is confirmed by patients who have inactivating mutations of the X-chromosomal androgen receptor gene leading to the androgen insensitivity syndrome (Lubahn *et al.*, 1989; Patterson *et al.*, 1994). Androgen insensitivity syndrome is characterized by a wide spectrum of virilization defects in 46,XY individuals, ranging from completely female external genitalia through ambiguous phenotypes to only minimal forms (Patterson *et al.*, 1994).

While the function of the androgen receptor as a transcriptional regulator of androgen responsive target genes is quite well understood in vitro at the molecular level (Simental *et al.*, 1991; Jenster *et al.*, 1991; Werner and Holterhus, 2014), almost nothing is known about the mesenchymal transcriptional androgen response programs important for initiation of the sexual differentiation process in the human fetus. Global gene expression studies using cDNA microarrays on cultured human prostate cancer cells have impressively shown the comprehensiveness of global changes of the transcriptome through androgen (DePrimo *et al.*, 2002). More recently, Nash and co-workers have identified 7534 androgen receptor binding sites in the genome of primary embryonic prostate fibroblasts derived from 12 to 16 week old human embryos through ChIPseq (Chromatin Immuno-Precipitation DNA-Sequencing) (Nash *et al.*, 2017). While subsets of identified transcripts reflected genes expressed in vivo and regulated by androgens when compared with published expression datasets, actual androgen regulation of transcription has not been experimentally investigated in these cells (Nash *et al.*, 2017).

Due to the central initial role of the urogenital mesenchyme rather than the epithelium in the fetus, and since the external genital origin enables biopsies, cultured genital skin fibroblasts have become an important experimental model in research into human sexual differentiation. They bind androgen specifically (Kaufman *et al.*, 1976), express the androgen receptor at the protein level (Holterhus *et al.*, 1997) and convert testosterone to the more active dihydrotestosterone (Pinsky *et al.*, 1978). Stillman and coworkers were the first to show that genital skin fibroblasts are functionally androgen responsive, since the activity of aromatase in these cells is induced by androgen which is reduced or absent in patients with androgen insensitivity syndrome (Stillman *et al.*, 1991). Nitsche and coworkers used a differential display RT-PCR strategy and reported 54 androgen regulated cDNAs, of which two had some similarities to L-plastin and testican respectively (Nitsche *et al.*, 1996). Using a genome-wide scale cDNA microarray approach, as described above for prostate cancer cells (DePrimo *et al.*, 2002), we previously found only three significant and reproducible androgen regulated transcripts, of which apolipoprotein D was most strongly androgen regulated (Appari *et al.*, 2009). Accordingly, patients with inactivating androgen receptor gene mutations did not show androgen regulation of apolipoprotein D transcription (Appari *et al.*, 2009; Hornig *et al.*, 2016). Apolipoprotein D plays a role as a male pheromone transporter (Zeng *et al.*, 1996) and in controlling cell proliferation (Simard *et al.*, 1991). However, due to the postnatal origin of these cells, direct conclusions regarding their influence on fetal sexual differentiation are limited. Interestingly, several patients with a complete or partial virilization defect and clinical characteristics of androgen insensitivity syndrome have significantly reduced or even absent induction of apolipoprotein D expression by dihydrotestosterone in genital skin fibroblasts despite the absence of inactivating androgen receptor gene mutations (androgen insensitivity syndrome type II, AIS type II) (Hornig *et al.*, 2016). This suggests the existence of currently unknown molecular components which are relevant for the transduction of normal morphogenetic effects caused by androgen via the androgen receptor in the human embryo. One possible mechanism may be the expression of androgen receptor co-regulators in the sensitive time window between the 8th and 12th week of gestation. A potential example in the mouse genital tubercle may be RWD domain containing 1 (RWDD1), which was identified in this tissue a few years ago (Grötsch *et al.*, 2012). RWDD1 is expressed during embryonic days E15, E16, and E17. It interacts with the androgen receptor at the molecular level and enhances androgen receptor-dependent transactivation (Grötsch *et al.*, 2012). Another mechanism may be the expression context of developmental genes in the embryonic genital anlagen, which may crosstalk with the androgen pathway via modification of androgen receptor expression levels. The homeobox A13 (HOXA13) gene may provide some insight. HOXA13 mutations can cause genitourinary tract malformations in mice and humans, and specifically hypospadias in boys with hand foot genital syndrome (Morgan *et al.*, 2003; Mortlock and Innis, 1997). In mice, knockout of hoxa13 leads not only to reduced expression of Fgf8 and Bmp7 in the urethral plate epithelium but it also markedly reduces androgen receptor abundance. This links the developmental gene program of the genital tubercle with the mechanisms of hormonal control of fetal sex development via the androgen receptor (Morgan *et al.*, 2003). Unfortunately, there is currently no comparable data on gene expression in relation to androgen action in the human fetal genital mesenchyme.

Programming Androgen Actions

Our classical understanding of androgen action via the androgen receptor in endocrinology is short-term change of gene transcription as documented by reporter gene assays (Jenster *et al.*, 1991) or in genome-scale gene transcription studies on androgen responsive cells (DePrimo *et al.*, 2002; Appari *et al.*, 2009). In addition, from a developmental perspective, androgens are also able to implement long-term stable (irreversible) functional and structural changes of phenotypic traits, which may be summarized as programming androgen actions, during sensitive ontogenetic time windows (Werner and Holterhus, 2014). On the one hand, androgen induced irreversible changes of genital cytodifferentiation and anatomy in the male, for example, the irreversible fusion of the genital midline, indicate that ontogenetic programming is already evident during fetal life between the 8th and 12th week of gestation (Cunha *et al.*, 1981; Cunha, 2008; Wilson *et al.*, 1981). On the other hand, early embryonic androgen actions also appear to program post-natal sex-specific reproductive phenotypic traits, for example, anogenital distance, penile growth, testicular size, Sertoli cell number and Leydig cell function, giving rise to the definition of a "male programming window, MPW" in the fetus (Welsh *et al.*, 2008; Scott *et al.*, 2008; van den Driesche *et al.*, 2012). The concept of androgen programming per se is not very recent and there appear to be more time windows for other traits, for example, the neonatal period in which androgen was shown to program post-natal traits in rat liver biochemistry more than 40 years ago (Gustafsson and Stenberg, 1974a,b). Moreover, prenatal androgen programming of post-natal traits is not restricted to male fetal development. There is data supporting the fact that in utero exposure of female animals to androgen has long-lasting consequences for post-natal ovarian function (e.g., early follicle development) and on metabolic traits (Abbott *et al.*, 2006; Franks, 2012). In essence, androgen programming in the female may be an important link to understanding the pathogenesis of ovarian-metabolic diseases such as polycystic ovarian syndrome (Cardoso *et al.*, 2015; Ramaswamy *et al.*, 2016).

In genome-scale microarray-based gene transcription studies on cultured genital skin fibroblasts we have previously found that sex-specific differentiation of the human external genitalia is accompanied by global differences of gene transcription programs unmasking androgen programing at the molecular level (Holterhus *et al.*, 2003; Holterhus *et al.*, 2007). In particular, we could identify a signature of 612 transcripts differing between embryologically homologous, labio-scrotal swellings derived 46,XY scrotal fibroblasts and 46,XY labia majora fibroblast from patients with complete androgen insensitivity syndrome. As discussed above, the post-natal origin of the cells limits assignment to a specific fetal time window. However, computerized hierarchical clustering based on this 612-gene signature distinguished individuals with different phenotypic degrees of partial androgen insensitivity syndrome, mostly according to the extent of anatomic external genital virilisation (Holterhus *et al.*, 2007). In essence, the cells had memorized the degree of virilisation of the genitalia they were originally biopsied from, reflecting the degree of fetal androgen action and supporting the hypothesis that these programs had indeed originated, at least in part, from the anatomic masculinization window between the 8th and 12th week of gestation (Holterhus *et al.*, 2007).

Using a similar genome-scale microarray approach involving male controls and patients with defined disorders of sex development, androgen programming can also be shown outside the genitalia in peripheral blood mononuclear cells (Holterhus *et al.*, 2009). In 334 mice Yang and coworkers demonstrated the enormous extent of global sex-specific gene transcription in liver, adipose tissue, muscle and brain involving up to approximately 70% of the active transcriptome (Yang *et al.*, 2006). Based on the points discussed here, it is likely that fetal androgen programming contributes to these findings and may have relevance in medicine beyond genital development, since it may contribute to the sexual dimorphism found in various extra-genital sex-specific traits in health and disease, for example, incidence rates and response to treatment. The underlying mechanisms of molecular androgen programming are not yet well investigated. Epigenetic modification of genomic DNA, for example, methylation at CpG sites in the gene promoters, is assumed to play a central role in long term control of gene transcription and consequently in maintaining tissue specificity, integrity and function. Accordingly, different healthy human cell types and tissues are characterized by specific large-scale differences in their gene methylation signatures (Eckhardt *et al.*, 2006). In a recent study using the same genital skin fibroblast model as for the transcriptome studies (Holterhus *et al.*, 2007), we could document the existence of androgen programmed gene methylation signatures (Ammerpohl *et al.*, 2013). The molecular pathways showing how androgen is able to implement these specific epigenetic changes during the male androgen programming windows remain to be discovered.

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Classification and Presentation of Disorders of Sexual Development (DSD)

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Introduction

Embryology Review (Domenice *et al.*, 2017)

Sex development involves precise spatio-temporal orchestration of multiple factors. Sex determination is governed by the sex chromosomes. In the presence of the SRY gene, typically located on the short arm of the Y chromosome, the undifferentiated gonad is directed to develop into a testis. In the absence of the SRY gene, ovary specific transcription factors initiate and maintain ovarian differentiation.

Phenotypic sex development can be considered as a two-step process: (see **Fig. 1**).

1. Testis or ovary formation from the primitive gonad (sexual determination); and
2. Internal and external genitalia differentiation largely directed by factors secreted by the fetal testis (sexual differentiation).

Mesonephric (Wolffian) and paramesonephric (Müllerian) ducts are both present in male and female fetuses. The Wolffian ducts originate in the intermediate mesoderm and grow caudally to end in the cloaca during the 5th embryonic week. Also, during the 5th embryonic week, the Müllerian ducts arise as invaginations of the coelomic epithelium.

Anti-Müllerian hormone (AMH), also known as Müllerian inhibitory hormone (MIH), is secreted by the testicular Sertoli cells and acts through its receptor on the Müllerian ducts to cause their regression. Testosterone is secreted by the testicular Leydig cells and acts via the androgen receptor in the Wolffian ducts to induce the formation of epididymis, deferent ducts, and seminal vesicles.

The external genitalia of the fetus derive from mesenchyme cells located in the cloaca. The initial development of the external genitalia is influenced by factors modulating the interactions between the epithelial and mesenchymal tissues. Genes from the *BMP*, *FGF*, and *HOX* families are involved in genital tubercle formation. Under androgen stimulation in the male fetus, the urethral folds, genital tubercle and genital swellings give rise to corpus spongiosum and primitive urethra, phallus, and scrotal swellings respectively. This process is mediated by testosterone and dihydrotestosterone (DHT), which acts on the androgen receptor of the prostate and external genitalia leading to its masculinization. In androgen target cells such as the prostate, testosterone is converted by 5α-reductase to DHT, which has a higher affinity for the androgen receptor. After testis determination, hormones produced by the male gonad induce the differentiation of internal and external genitalia acting on their specific cognate receptors. The up regulation of AMH gene in the fetal Sertoli cells is induced by *SOX9*. Subsequently, *NR5A1/SF1*, *WT1*, and *GATA4* synergize to increase AMH transcription while the AMH promoter may be stabilized by the *SOX-HSP7-WT1* complex. Differentiation of fetal Leydig cells is influenced by Sertoli cell factors including desert hedge-hog (*DHH*) and platelet-derived growth factor A (*PDGFα*); other factors involved in Leydig cell differentiation include Aristaless-related homeobox (*ARX*), *MAMLD*1, *GATA4*, *DAX1*, and *NR5A1*. *NR5A1* regulates gonadal steroidogenesis. The Leydig cells also produce *INSL3*, which along with testosterone is responsible for the transabdominal phase of testicular descent during fetal weeks 10–15. The inguinoscrotal phase, occurring between 27 and 35 weeks, is largely mediated by androgen action.

In the absence of the *SRY* gene such as female fetuses with a 46,XX chromosomal complement, *RSPO1* and *WNT4* are expressed to stabilize cytoplasmic *β*-catenin. Both *WNT4* and *β*-catenin suppress the *SOX9*/*FGF9* positive feedback loop, allowing ovarian differentiation to proceed. Most evidence indicates that ovarian differentiation occurs independently in several lineages including supporting cells and germ cells. In females, ovaries do not produce AMH during fetal development; hence, development and maintenance of the Müllerian duct structures occur. Furthermore, because the fetal ovaries do not secrete significant amounts of androgens, the Wolffian ducts become atrophic. In the absence of either testosterone secretion or 5α-reductase activity, bipotential external genital structures develop along female lines. Specifically, in the absence of androgens the labioscrotal and urethral folds form the labia majora and minora, respectively. The genital tubercle develops into a clitoris and the urogenital sinus gives rise to the urethral opening and anterior portion of the vagina. The posterior portion of the vagina develops from the Müllerian ducts in the absence of AMH (Ahmed *et al.*, 2013; Ostrer, 2014).

Disorders of sex development (DSDs) are defined as congenital conditions within which the development of chromosomal, gonadal, and anatomic sex is atypical. The broad term DSD, adopted at the Consensus Conference (Lee *et al.*, 2006), has been generally accepted by many medical professionals but not universally by some patient and support groups. Positive aspects about having a term providing for scientific accuracy within a biological and medical context include: (1) bona fide genetic disorders

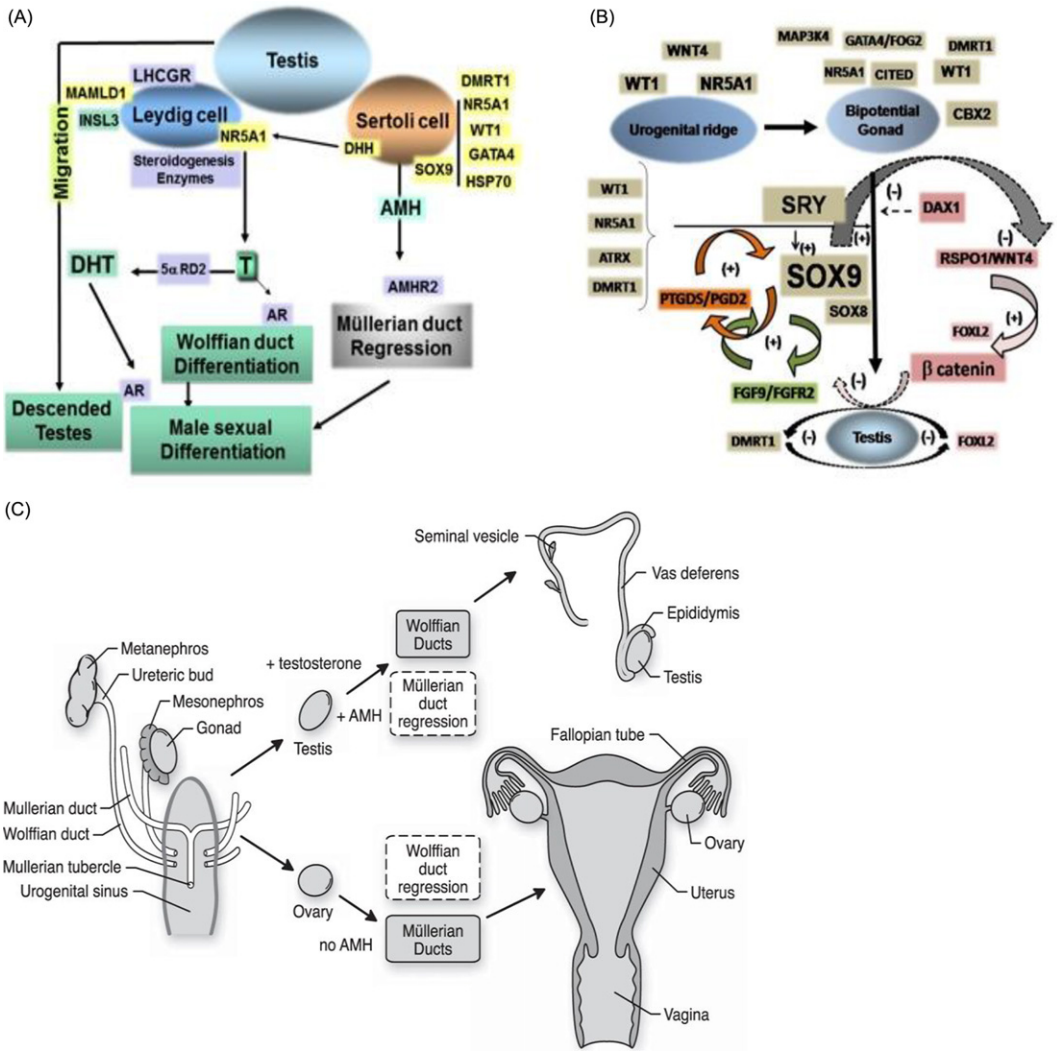

Fig. 1 (A) Summary of the molecular events in sex determination indicating the genes in which molecular defects can cause gonadal disorders. (B) Summary of the molecular events in sex differentiation indicating the genes in which molecular defects cause 46,XY DSD in humans. (C) Development of internal genitalia from common genital structures. Ordinarily, the *SRY* gene on the Y chromosome would trigger testes and subsequent male internal genital development from production of the hormones, T and anti-Mullerian hormone (AMH). In 46,XX testicular DSD, testis determination can be triggered by *SRY* or *SOX9* translocation, *SOX3* or *SOX9* duplication, or *RSPO1* loss of function. In 46,XY gonadal dysgenesis, testis development does not occur or is incomplete, and internal female genitalia will develop. This will be triggered by *SRY*, *NR5A1*, *DHH*, or testis-determining gene loss of function mutations, *DAX1* or *WNT4* duplication, or *MAP3K1* gain of function mutations. From (A) Domenice, S., Arnhold, I.J.P., Costa, E.M.F., *et al.* (2017). 46,XY Disorders of Sexual Development. In De Groot, L.J., Chrousos, G., Dungan, K., *et al.* (eds). South Dartmouth, MA: Endotext [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK279170/;. (B) Domenice, S., Arnhold, I.J.P., Costa, E.M.F., *et al.* (2017). 46,XY Disorders of Sexual Development. In De Groot, L.J., Chrousos, G., Dungan, K., *et al.* (eds). South Dartmouth, MA: Endotext [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK279170/; and (C) Ostrer, H. (2014). Disorders of sex development (DSDs): An update. *The Journal of Clinical Endocrinology and Metabolism* 99(5), 1503–1509.

facilitate access to healthcare and insurance; (2) an umbrella classification helps to generate comprehensive and integrated models of care; and (3) it provides a framework for knowledge accumulation and research funding. At the same time, this terminology avoids confusion by distinguishing these genetic conditions from individual variation in gender identity and sexual orientation (Barthold, 2011; Hughes *et al.*, 2007).

Negative connotations of DSD perceived by some advocacy organizations include the stigma of "disorder" and perceived implications that "sex" involves sexual behavior. The term DSD is not felt to be applicable to all individuals included, such as males with congenital adrenal hyperplasia (CAH), resulting in participant refusal regarding research under this heading. Some people consider "intersex" to be a better term than DSD, especially for infants requiring male or female assignment, some substitute the word "disorders" by "differences," whilst still others call for an alternative nomenclature (Fisher *et al.*, 2016; Gillam *et al.*, 2010; Pasterski *et al.*, 2010; Wiesemann *et al.*, 2010).

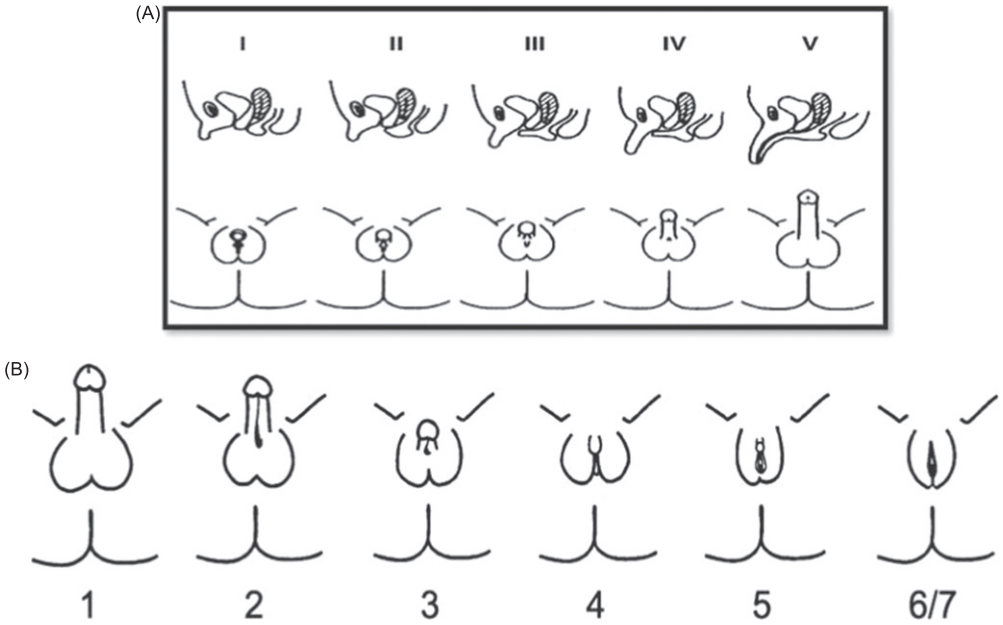

Fig. 2 Prader stages of virilization in females (A) and Quigley stages of lack of virilization in males (B).

Prader stages show progressive virilization from stage 1 to 5 while Quigley grade 2 is isolated hypospadias or micropenis with lesser virilization extending to stage 5 with grade 1 and 6 being normal male and female genital phenotypes (see **Fig. 2**).

Classification

Disorders of sex differentiation (DSD) are congenital conditions with atypical development of one or more of the following: chromosomal, gonadal and genital sex. This broad category, created during the 2005 Intersex Consensus conference, was proposed to replace previously used terms including the term intersex, ambiguous genitalia, and the hermaphroditism categories (Houk *et al.*, 2006; Houk and Lee, 2008). While these latter categories were based upon anatomic definitions, they carried connotations regarding gender and sexual behavior. DSDs incorporated the former categories of female pseudohermaphroditism (46,XX individuals with virilization of external genitalia), 46,XY DSD; male pseudohermaphroditism (46,XY individuals with incomplete masculinization of external and internal genitalia), and sex chromosome DSD (to include all disorders of gonadal development with variations in the sex chromosome number or structure) (Hughes *et al.*, 2006a,b).

Major categories of DSDs are: (Lee *et al.*, 2006)

1. 46,XX DSDs include all situations in which the karyotype is 46,XX, ovaries are present and the external genitalia are masculinized, essentially the former female psuedohermaphrodite category. The most common diagnosis within this category is 21-hydroxylase deficiency congenital adrenal hyperplasia, the most frequent DSD presenting with ambiguous genitalia. However, once this diagnosis is made, there is no reason to use this DSD category. Because the cause is androgen excess, the internal genitalia are expected to be female.
2. 46,XY DSDs include 46XY males with incomplete masculinization of external genitalia, basically the former male pseudohermaphrodite category. This category includes both isolated unilateral and bilateral cryptorchidism and hypospadias, which are associated with other reproductive system developmental problems. They show presence of testes, at least during fetal development. Nonhormone disorders such as peno-scrotal transposition have been included under this classification.
3. Sex chromosome DSDs include categories in which abnormal gonadal development related to an abnormality of the sex chromosomes X or Y. This new category includes former categories of true hermaphrodite (46,XX/46,XY chimeric, ovotesticular DSD ovotesticular DSD), mixed gonadal dysgenesis (in which extent of gonadal dysgenesis differs between the 2 gonads) and other types of asymmetric gonadal differentiation in which karyotype is 45,X/46,XY. This includes two major categories of gonadal maldevelopment: Turner syndrome and Klinefelter syndrome and their variants. It should be noted that since ovotesticular DSD may be associated with not only 46,XX/46,XY, but also 46,XX and 46,XY, that this diagnosis may be found under all three of these major classifications.
4. Another broad DSD definition to include 46,XY individual having appropriate internal and external genital differentiation, such as congenital adrenal hyperplasia; 21-hydroxylase deficiency.

Minor categories of DSD to describe rare conditions such as XY female, XX sex reversal and XY sex reversal, the terms 46, XX testicular DSD, and 46, XY complete gonadal dysgenesis was suggested.

Table 1 Classification of DSD guided by genetic differences

46, XX DSD	*46, XY DSD*	*Sex chromosome DSD*
- Ovotesticular DSD	- Complete gonadal dysgenesis	45,X mos 45,X/46,XX
- Testicular DSD	- Partial gonadal dysgenesis	45,X/46, XY (MGD, ovotesticular DSD)
- Gonadal dysgenesis	- Gonadal regression	46,X, i(Xq) 46,X,del (Xp)
	- Ovotesticular DSD	46,XX/46,XY (chimeric/ ovotesticular DSD) 47, XXY (Klinefelter syndrome/ variants) 46,XX (Yp translocation) 46,X,del(X)t(X;Y) (XX SRY+ male)
Disorders of androgen excess	Disorders of androgen synthesis	
- CAH (mutations in CYP21A2, CYP11B1, POR, HSD3B2)	- CAH (mutations in StAR, CYP11A1, HSD3B2, POR, CYP17A1)	
- Aromatase deficiency	- Isolated defects in androgen biosynthesis (mutations in CY5B, HSD17B3, SRD5A2)	
- Maternal luteoma	- LH receptor defects (LHR mutations); Leydig cell hypoplasia, aplasia	
- Exogenous exposures, iatrogenic		
	Disorders of androgen action	Other chromosomal rearrangements
	- Complete and partial androgen insensitivity syndrome (Mutations in androgen receptor gene)	
Unclassified conditions	Unclassified conditions	
- Cloacal exstrophy	- Persistent Müllerian duct syndrome (mutations in AMH and AMHR)	
- Vaginal atresia	- Hypospadias, unknown etiology	
- Mullerian, renal, cervicothoracic somite abnormalities	- Epispadias	
- Other complex syndromes	- Other complex syndromes	

Lee, P. A., Houk, C. P., Ahmed, S. F., Hughes, I. A. and International Consensus Conference on Intersex organized by the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. (2006). Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. Pediatrics 118 (2), e488–e500.

The categories of this classification 46,XX; 46,XY, and sex chromosome DSD are listed in **Table 1**, noting that in addition unclassified conditions are listed that may be part of complex syndromes. **Table 2** further subdivides these major categorizes into nondysgenetic, dysgenetic, and malformative, including genes mutated, illustrating the complexity of diagnoses and findings which may occur under the broad definition of DSD (Hutson *et al.*, 2014).

Disorders included in the classification of 46,XX DSD include disorders associated with fetal androgen excess such as virilizing congenital adrenal hyperplasia and maternal hyperandrogenemia. Other disorders included in this category include vagina atresia/ agenesis and congenital anomalies of the female genitourinary tract such as the Rokitansky–Mayer–Küster–Hauser (RMKH) syndrome, Bardet–Biedl syndrome, Kaufman–McKusick syndrome, Fraser syndrome, and Winters syndrome.

Classifications of 46,XY DSD include disorders of testicular development (complete and partial gonadal dysgenesis), disorders of androgen action (complete and partial androgen insensitivity), disorders of AMH/AMH receptor, androgen biosynthesis defect, severe hypospadias, penoscrotal transposition, and bladder exstrophy.

Classifications of sex chromosome DSD include the following: 45,X (Turner syndrome and variants); 47,XXY (Klinefelter syndrome and variants); 45,X/46,XY (mixed gonadal dysgenesis, ovotesticular DSD); and 46,XX/46,XY (chimeric, ovotesticular DSD). In addition, the term 46,XX testicular DSD has replaced the rare XX male category. The term 46,XY complete gonadal dysgenesis has replaced the XY sex reversal category (Ahmed *et al.*, 2013).

The framework provided by the current DSD classification system provides a logical and cogent approach for use during the initial diagnostic assessment of the patient. Once a specific diagnosis is confirmed, the patient can be described by the specific diagnosis. Nevertheless, despite the advancement in the molecular genetics of disorders of sex development, a specific diagnosis cannot be identified for some patients; these patients are best classified under a general term until a specific molecular etiology has been identified (Lee *et al.*, 2016).

In spite of the attempts to be mindful and respectful, this newer classification has been criticized for stigmatizing and unnecessarily "medicalizing" those with intersex traits. Nonetheless, this classification scheme provides the framework to guide the initial diagnostic evaluation of individual patients. This is particularly relevant for infants with external genitalia so ambiguous that it is necessary to determine sex of rearing. With increasing knowledge accrues of the molecular basis of disorders of sex differentiation/development, greater clarity will occur (Lee *et al.*, 2016; Yatsenko and Witchel, 2017).

Table 2 Classification of DSD guided by karyotype

Category	*Gene*	*Pathogenic type*	*Etiology with examples/associations*
46,XX DSD		*Nondysgenetic DSD*	*Excessive adrenal androgen synthesis*
			Congenital adrenal hyperplasia (CAH)
	CYP21A2		- 21α-Hydroxylase deficiency
	CYP11B1		- 11β-Hydroxylase deficiency
	CYP19A1		Aromatase deficiency
			Androgen secreting tumors during pregnancy
			Luteomas, thecomas
			Theca lutein cysts of the ovaries
			Androgenic drugs during pregnancy
			Norethindrone, danazol, ethisterone, norethynodrel, medroxyprogesterone
		XX gonadal dysgenesis	
	BMP15		Ovarian dysgenesis
	DCAF17		Woodhouse–Sakati syndrome, ovarian dysgenesis
	FIGLA		Ovarian insufficiency
	FOXL2		Ovarian dysgenesis and blepharophimosis
	FSHB		Ovarian insufficiency
	FSHR		Ovarian insufficiency
	MCM8		Ovarian insufficiency
	MCM9		Ovarian insufficiency
	MKKS		McKusick–Kaufman syndrome
	NOBOX		Ovarian insufficiency
	PSMC3IP		Ovarian insufficiency
	SOHLH1		Ovarian insufficiency
	WNT4		Congenital absence of upper vagina and uterus, may be associated with MURCs syndrome (Müllerian and renal aplasia, cervicothoracic somite dysplasia)
		Malformative disorders	
			Urogenital sinus anomalies
			Cloacal exstrophy
46,XY DSD		*Nondysgenetic DSD*	*Disorders of androgen synthesis*
	STAR		Lipoid CAH
	CYP11A1		Cholesterol desmolase deficiency; adrenal insufficiency, sex reversal
	CYP17A1		P450c17 deficiency; undervirilization, hypertension
	POR		P450 oxidoreductase deficiency; Antley–Bixler syndrome
	HSD3B2		3β-Hydroxysteroid dehydrogenase (HSD) type 2 deficiency
	HSD17B3		17 β-HSD type 3 deficiency
	SRD5A2		5α reductase deficiency and other DHT synthesis defects
	AKR1C2 and AKR1C4		Testicular dysgenesis; 3α-hydroxysteroid dehydrogenase deficiency
	MAMLD1		Mastermind-like domain with 1 gene; micropenis, bifid scrotum
	MAP3K1		Gonadal dysgenesis
	CBX2		Gonadal dysgenesis, ovotesticular disorder
	DHH		Desert hedgehog (DHH)-testicular dysgenesis with minifascicular neuropathy
	DMRT1 and DMRT2		Testicular dysgenesis and mental retardation
	GATA4		Testicular anomalies with or without congenital heart disease
	NROB1 (DAX1)		Gonadal dysgenesis, sex reversal
	SOX8		Gonadal dysgenesis
	SRY		Swyer syndrome/gonadal dysgenesis
	WWOX		Gonadal dysgenesis
	ZFPM2		Gonadal dysgenesis
	WT1		Denys–Drash syndrome-associated with Wilms tumor
	WT1		Frasier syndrome-associated with gonadoblastoma
			Disorders of androgen action
	AR (NR3C4)		Complete AIS (CAIS) and partial AIS (PAIS)
	LHCGR		Leydig cell hypoplasia
			Disorders of AMH synthesis or action
	AMH		Persistent Müllerian duct syndrome

Table 2 Continued

Category	*Gene*	*Pathogenic type*	*Etiology with examples/associations*
	AMHR2		Persistent Mullerian duct syndrome
		DSD with additional congenital anomalies	
	NR5A1		Steroidogenic factor 1 deficiency, testicular dysgenesis + adrenal insufficiency
	WT1		Denys–Drash syndrome; Frasier syndrome; Meacham syndrome
	DMRT1		Testicular dysgenesis and mental retardation
	ATRX		Alpha-thalassemia mental retardation syndrome
	ARX		Hydranencephaly and abnormal genitalia
	CDKN1C		*IMAGe syndrome*
	DHCR7		Smith–Lemli–Opitz syndrome
	HHAT		Chondrodysplasia
	KAT6B		Genitopatellar syndrome
	SOX9		Gonadal dysgenesis and campomelic dysplasia
	CHD7		CHARGE syndrome
	HOXA13		Hand-foot-genital syndrome
	ROR2		Tyrosine-protein kinase transmembrane receptor Robinow syndrome
	RSPO1		Sex reversal, palmoplantar keratosis
	GL13		Pallister–Hall syndrome
	SPECC1L		Cytospin A protein
	PITX2		Paired-like homeodomain transcription Factor 2 Hypospadias and cryptorchidism Bladder exstrophy
	MKKS		McKusick–Kaufman syndrome centrosome-shuttling protein type II; chaperonin family Vas deferens absence - With cystic fibrosis

Presentation

Many aspects of care for children with disorders of sex development (DSD), defined as a misalignment between chromosomal, gonadal, and phenotypic sex, have fundamentally changed recently—particularly regarding gender assignment. However, uneasiness persists about the management of children with DSD because of reported poor outcomes (Houk and Lee, 2010). The three components of sexual psychosexual development—gender identity, gender role, and sexual orientation—may not always be typically concordant and aligned in individuals with DSD. However, in those with DSD, the primary goal is for gender identity to be consistent with gender assignment. In other words, an overarching goal of DSD management is to avoid a gender assignment that increases the risk of gender dysphoria (Sandberg *et al.*, 2017). In the past, similar discomfort led to the overly simplistic and dogmatic management approach, particularly gender assignment, whereby sexually ambiguous infants were assigned a gender based on a prescriptive set of rules. These rules involved potential for fertility and traditional sexual activity given possible surgical genital repair. For example, a 46,XY patient judged to have an inadequate penis was assigned female, whereas a virilized 46,XX patient with ovaries and a uterus was assigned female, independent of the degree of external genital virilization. To address the poor outcomes associated with the previous medical management paradigms, recent emphasis has been placed on the use of principles, cultural background, and parental input to help make clinical decisions in DSD infants—particularly those with uncertain outcomes regarding adult gender identity and quality of life variables (Ahmed *et al.*, 2011; Houk and Lee, 2010; Indyk, 2017).

If the appearance of the external genitalia is sufficiently ambiguous to render gender assignment impossible or the phenotype is not consistent with prenatal genetic tests, then de facto, extensive investigation is required. However, the recognized extent of genital ambiguity may depend on the expertise of the observer, and prior to presentation to a clinical expert, the label of ambiguous genitalia is often assigned to newborns where the most appropriate sex of rearing is not immediately clear to those present at the child's birth (Mendonca, 2014).

The birth prevalence of genital anomalies may be as high as one in 300 births but the birth prevalence of complex anomalies of true genital ambiguity on expert examination may be as low as one in 5000 births (Ahmed *et al.*, 2011).

When evaluating these infants, the clinical features of the external genitalia that require examination include the symmetry of the external genitalia, presence of gonads in the labioscrotal folds, the fusion of the labioscrotal folds, the size of the phallus and the site of the urinary meatus on the phallus, although the real site of the urinary meatus may, sometimes, only become clear on surgical exploration. These external features can be individually scored to provide an aggregate score, referred to as the external masculinization score (EMS). Infants with suspected DSD who require further clinical evaluation and need to be considered for

investigation by a specialist should include those with isolated perineal hypospadias, isolated micropenis, isolated clitoromegaly, any form of familial hypospadias and those who have a combination of genital anomalies with an EMS of <11. This will avoid unnecessary detailed investigations into boys with isolated glanular or mid-shaft hypospadias and boys with an unilateral inguinal testis. However, investigations are generally indicated with the co-existence of a systemic metabolic disorder, associated malformations or dysmorphic features, a family history of consanguinity, stillbirths, multiple miscarriages, fertility problems, genital abnormalities, hernias, delayed puberty, genital surgery, unexplained deaths, and the need for steroid replacement.

The external genital appearance may be similar for a masculinized female newborn and an undervirilized male newborn, although the former is symmetrical and the latter may be asymmetrical. The presence or absence of palpable gonad(s) is a key finding. If gonads are not palpated beneath the genital folds or in the inguinal area, 46,XX DSD is most likely. When one or two gonads are palpated, 46,XY DSD or gonadal DSD (ovotesticular DSD or mixed gonadal dysgenesis) is likely.

Adolescents (Ahmed *et al.*, 2016)

The initial assessment in an affected adolescent should be aimed at establishing a relationship with the patient and starting the process of diagnosis. An appropriate hospital setting is very important for the sensitive management of complex conditions with full access to the necessary medical, nursing and psychological care. Whilst the explanation of the diagnosis to the patient and the family is critical, this needs to be performed sensitively and carefully and expert psychological input is essential. There are three common DSD presentations in adolescents: (1) a girl with primary amenorrhoea (with or without breast development); (2) a girl who virilizes at puberty; or (3) a boy with pubertal delay (see **Table 3**).

The appearance of clitoromegaly and hirsutism at puberty in the presence of primary amenorrhoea is a classical presentation of two 46,XY DSDs: 17β-hydroxysteroid dehydrogenase type 3 (17bHSD3) deficiency and 5α-reductase type 2 deficiency. It is less typical of partial androgen insensitivity syndrome (PAIS) that is usually associated with ambiguous genitalia at birth. In all these conditions, Müllerian structures will not be detectable. Also, in partial gonadal dysgenesis and ovotesticular DSD, the mild clitoromegaly that may have been present at birth may have been overlooked but becomes a more prominent feature at adolescence. The differential diagnosis would also include CAH and androgen secreting tumors of the ovary or adrenal gland; in all these cases, Müllerian duct derived structures are present.

Although the most common cause of delayed puberty is constitutional delay, all boys with delayed puberty who are over the age of 14 years should be assessed. Overweight boys need careful examination so that a buried penis is not mistaken for micropenis. Rarely, PAIS, a disorder of testosterone biosynthesis or mild forms of testicular dysgenesis, can present in this age group, especially if there is a history of hypospadias repair or orchidopexy.

In adolescents with an existing DSD, transferring to adult services is an opportunity to review the diagnosis, discuss hormone replacement therapies, potential for fertility, and consider further investigations.

Discussion

When a newborn presents with ambiguous genitalia, it is crucial to exclude life-threatening disorders such as adrenal insufficiency (Bangalore Krishna *et al.*, 2017). The extent of internal and external differentiation should be assessed in a

Table 3 Common features of DSD presenting at puberty

Delayed puberty	*Primary amenorrhea*
1. Gonadal dysgenesis - 46 XX pure gonadal dysgenesis - 46 XY pure gonadal dysgenesis - 46 XX sex reversal 2. Mixed gonadal dysgenesis 3. Ovotesticular DSDs 4. LH receptor defects (Leydig cell dysgenesis) 5. 17 hydroxylase deficiency	1. Complete androgen insensitivity 2. Mayer–Rokitansky–Kuster–Hauser syndrome *Failure of attempted sexual intercourse* 1. Mayer–Rokitansky–Kuster–Hauser syndrome 2. 17-β hydroxysteroid dehydrogenase deficiency 3. 17 hydroxylase deficiency
46XX with virilization (acne, hirsutism, amenorrhea, clitoromegaly) 1. Congenital adrenal hyperplasia, nonclassic forms *46XY subjects raised female with subsequent virilization* 1. 17-β hydroxysteroid dehydrogenase deficiency 2. 5-alpha reductase deficiency	*With cyclic hematuria in a phenotypic male* 1. 21 hydroxylase deficiency 2. Persistent mullerian duct syndrome *With gynecomastia* 1. Ovotesticular DSD in phenotypic male 2. 17-β hydroxysteroid dehydrogenase deficiency

timely manner. And, if possible, a specific etiology can be identified, so that, together with the parents, an appropriate gender assignment can be made. Parents need to be provided information regarding reproductive system differentiation so they can understand how ambiguity may occur (Indyk, 2017). While typically the karyotypes of 46,XX and 46,XY indicate female and male respectively, it should be pointed out that this is not necessarily an indication that female gender assignment is the most appropriate for all with 46,XX and likewise for 46,XY and male gender assignment. One can share the concept that specific genes direct reproductive system development. Changes in these genes alter the function of the genes and the proteins encoded by these genes. Parents should be provided with new information as it becomes available. Together with the team of experts, the parents will participate in the decision-making regarding gender assignment. The parents need to understand that a gender assignment will be made, with their input, as soon as possible, while in the meantime they need to consider whether to delay the birth announcement and with whom to share information about their child's condition. Full disclosure and education should provide the parents with a perspective that their child, with careful ongoing care, can be expected to have a good quality of life.

Part of the initial interview with the parents should include seeing and examining their infant's genitalia, with careful demonstration of the ambiguous features, while discussing the common origin of female and male structures. The decision for gender assignment, based upon the anatomy and anticipated functional abilities of the reproductive system, should primarily be based upon the most probable future gender identity (although this cannot be predicted) (Sandberg *et al.*, 2012). When data do not suggest one assignment over the other, family perspectives including cultural and religious factors are of key importance. It must be considered that androgen exposure to the developing fetal brain impacts aspects of sexual identity, most clearly gender role behavior as manifest by childhood play preferences, but less clearly effects upon both gender identity and sexual orientation. This may apply to the 46,XX DSD patient with excessive androgen exposure during fetal life, as well as to the poorly virilized 46,XY DSD whose CNS was exposed to normal male levels of androgen during fetal life, or who has sufficient androgen insensitivity so prenatal androgen exposure had diminished impact. Available data indicate that most XX females with virilizing 21-hydroxylase deficiency identify as heterosexual females and most XY females with CAIS identify as females. Hence, while extent of fetal androgen exposure must be considered, this still represents an unknown factor in the development of a gender identity with the future possibility of a gender identity that differs from the sex of rearing (Lee *et al.*, 2012). The topics of biological sex, sex of rearing, and gender identity should be discussed with the parents (Wilson *et al.*, 2012).

In cases of 46,XX DSD, the newborn with ambiguous genitalia is usually assigned female (Kim and Kim, 2012). The rationale for this is that the ovaries and uterus have the potential for normal function so with appropriate medical therapy and surgical construction, fertility and sexual function can be expected. An exception to this assignment should however be considered when external genital development is completely male, except of an empty scrotum. Such individuals raised as males have gender identity and sexual orientation typical for most males, require less surgery and function socially and sexual as males, albeit with infertility (Mieszczak *et al.*, 2009).

For 46,XY DSD, the trend is toward male assignment even when penis and testicular development is subnormal, particularly if there is evidence of prenatal androgen secretion. Assignment as female is currently considered a drastic step, based upon outcome data including gender development contrary to assignment and self-reassignment of gender. Exceptions to this are those with testicular agenesis, complete androgen insensitivity, LH receptor defects and individuals who have no evidence of exposure or responsiveness to androgen. In these instances, sex of rearing needs to be carefully considered with the parents. Those with partial androgen insensitivity must be carefully assessed; those with evidence of androgen responsiveness are generally assigned male, while those with no evidence of responsiveness postnatal, female.

In cases of gonadal DSD, gender assignment should be based on several criteria. A defect in 5α-reductase is an indication for male sex for rearing because pubertal virilization will lead to penile development (although it will be subnormal), normal pubic hair development, and the acquisition of male sexual identity. In contrast, very rarely with inborn errors of testosterone biosynthesis, if male reconstructive surgery appears to be highly unlikely, female assignment may be a consideration, if results suggest that fetal testosterone secretion was markedly subnormal. When a vagina and uterus are present, female assignment may be considered depending upon evidence of testicular testosterone secretion and extent of masculinization and likelihood of successful vaginoplasty.

In cases of complete androgen insensitivity, female assignment is appropriate; with partial androgen insensitivity, male assignment is currently done except in those instances failing to demonstrate responsiveness to exogenous testosterone stimulation. Confirmation of a described mutation in the androgen receptor gene may be helpful for management depending upon variability described for the specific mutation. Only a positive response is potentially helpful, lack of identification of a described (a negative screen) is not helpful.

In patients with ovotesticular disorder, female assignment may be considered with evidence of potential ovarian and uterine function, while substantial external genital development and the presence of testicular function is the basis for consideration of a male assignment (Houk and Lee, 2010).

No area of pediatric endocrinology engenders more controversy than the management of DSD conditions affecting reproductive development. With some variations, guidance from clinicians and ethicists has focused on principles and processes aimed at fostering the over-all well-being of the child and future adult by: (1) minimizing physical and psychosocial risk; (2) preserving

potential for fertility; (3) upholding the individual's rights to participate in decisions that will affect them now or later; (4) leaving options open for the future by avoiding irreversible treatments that are not medically necessary until the individual has the capacity to consent; (5) providing psychosocial support and peer support; (6) supporting the individual's healthy sexual and gender identity development; (7) using a shared decision-making approach that respects the individual's and parents' wishes and beliefs; (8) respecting the family and parent–child relationships, and (9) providing patients with full medical information appropriate for age, developmental stage and cognitive abilities (Lee *et al.*, 2016). While each of these principles is important, striking the appropriate balance among them becomes challenging in the clinical setting. For example, respecting parents' wishes for early genital surgery may impinge on the child's right to participate in decision making and may reduce the child's options for the future (Nabhan and Lee, 2007).

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Androgen Biosynthetic Defects: 17β-Hydroxysteroid Dehydrogenadse Type 3 and 5α-Reductase Type 2 Deficiencies

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Introduction

Androgens play an important role in the process of sex differentiation. During the embryological development of 46,XY individuals, the testosterone secreted by the testicular Leydig cells acts on the androgen receptor in the Wolffian ducts inducing the formation of epididymis, deferent ducts, and seminal vesicles (Jost *et al.*, 1973). Testosterone is further converted to dihydrotestosterone (DHT), which acts on the androgen receptor of the external genitalia and prostate leading to its masculinization (Jost *et al.*, 1973). Disturbance in androgen production can lead to a 46,XY child with a disorder of sex development (DSD) (Hughes *et al.*, 2006; Lee, 2006).

In 46,XY DSD patients, testosterone (T) biosynthesis defects due to enzyme deficiency accounts for a considerable number of patients. The phenotypes are variable, ranging from normal female external genitalia to various degrees of undermasculinization of male external genitalia (Hughes *et al.*, 2006; Lee, 2006).

The most common enzymatic defect in testosterone biosynthesis is the 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3), also known as 17-β-keto reductase deficiency. It is characterized by isolated impairment of testosterone production not affecting glucocorticoid and mineralocorticoid adrenal secretion. This disorder consists of a defect in the last step of steroidogenesis when androstenedione (Δ4) is converted into testosterone (T) and estrone into estradiol. There are five steroid 17β-HSD enzymes that catalyze this reaction (Andersson *et al.*, 1996) and 46,XY DSD results from impairment of 17β-HSD type 3 (17β-HSD3) isoenzyme activity (Geissler *et al.*, 1994; Saez *et al.*, 1971) which is expressed exclusively in the testes (Geissler *et al.*, 1994). Defects in *SRD5A2* gene, which codifies 5α-reductase type 2 enzyme (5α-RD2), decrease the conversion of testosterone into DHT. There are two 5α-RD isoenzymes, but only the type 2 has high expression in genital skin early in gestation and in adulthood, as well as in prostate, epididymis, and seminal vesicle (Silver *et al.*, 1994).

Both 17β-HSD3 and 5α-RD2 deficiencies are caused by homozygous or compound heterozygous mutations in the *HSD17B3* and *SRD5A2* genes, respectively. Hormonal diagnosis is highly used, despite some discrepancies (Khattab *et al.*, 2015; Lee *et al.*, 2007; Perry *et al.*, 2011), being the molecular analysis the diagnostic tool of choice. The molecular genetic testing not only confirms the diagnosis, but also provides the orientation for genetic counseling.

In both androgen synthesis defects, most patients have severe undervirilization of the external genitalia at birth leading to a female sex assignment in most of the patients (Mendonca *et al.*, 2016, 2017). However, the extreme undervirilization during embryogenesis is opposed to the observed at puberty. At this age, a strong virilization is observed, including muscle mass development, deepen voice, and penile length. In addition, there is a high frequency of gender change in these individuals at adulthood, especially in patients who have not undergone gonadectomy at puberty (Furtado *et al.*, 2012). Considering the psychosexual development and the high level of gender change (from female to male), it is preferable to assign those patients as males (Mendonca *et al.*, 2016, 2017).

Our aim is to review and discuss these two rare disorders regarding their clinical, hormonal, and patients' long term outcomes, highlighting their similarity and main differences between them (**Table 1**).

Epidemiology

Similar to other autosomal recessive disorders, 17β-HSD3 deficiency has an increased frequency in highly inbreed areas. Most cases of 17β-HSD3 deficiency have been reported in Europe, Asia, Australia, and South America, but there are only 12 reports in the United States until now (Mains *et al.*, 2008; Chuang *et al.*, 2013; Boehmer *et al.*, 1999). The incidence is quite variable around the world, ranging from 1:147,000 newborns in Netherlands (Rosler, 2006) to 1: 100–300 in the Gaza Strip Arab population (Rosler *et al.*, 1996). Thus, ethnic origin as well as the report of consanguinity should be considered when diagnosing this disorder.

Allelic variants in the *SRD5A2* gene have been described in several countries, including Brazil, Mexico, Egypt, China, Korea, Pakistan, Portugal, Italy, India, Turkey, Austria, Dominican Republic, and recently in Bulgaria (Mendonca *et al.*, 1996, 2016; Imperato-McGinley *et al.*, 1974; Ko *et al.*, 2010; Adiyaman *et al.*, 2006). Extensive haplotype analysis has not been done in these allelic variants to confirm a possible genetic common background, but their prevalence in restricted regions suggests a possible founder effect (Mendonca *et al.*, 2016).

Clinical Features

In both conditions, the external genitalia appearance at birth is typically female or strongly undervirilized (Lee *et al.*, 2007; Rosler, 2006; Mendonca *et al.*, 2000), leading to female social sex assignment in most of the cases (Andersson *et al.*, 1996; Lee *et al.*, 2007; Rosler, 2006; Cohen-Kettenis, 2005; Rosler and Kohn, 1983).

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Table 1 Phenotype of 46,XY subjects with 17β-HSD type 3 and 5α-RD type 2 deficiencies

Parameters	*17β-HSD type 3*	*5α-RD type 2*
Gene/location	*HSD17B3*; 9q22.32	*SRD5A2;* 2p.23.1
Molecular defects	*Missense* allelic variants; exonic deletion, duplication, intronic splice site mutations	Essentially *missense* allelic variants; small deletions, splice site, stop codons, small indels, gross deletions
Inheritance pattern	Autosomal recessive	Autosomal recessive
External genitalia	Severe undervirilization of the external genitalia at birth	Severe undervirilization of the external genitalia at birth
Müllerian duct derivatives	Absent	Absent
Wolffian duct derivatives	Present	Present, small prostate
Testes	Usually located in the groin area or in labioescrotal folds	Usually at inguinal area
Puberty	Virilization at puberty; gynecomastia is observed in ∼30%	Virilization at puberty; gynecomastia is uncommon
Hormonal diagnosis	T/Δ4 ratio below <0.8	T:DHT >8.5 Low specificity; molecular diagnosis is necessary
Psychosexual aspects	Most patients are assigned as female social sex; gender dysphoria and gender change is observed in 39%–64%	Most are raised in the female social sex. Gender change to male social sex is observed in 48%–77%
Treatment	*Females*: surgical feminization of external genitalia, gonadectomy, replacement with estrogens at 11–12 years old vaginal dilatation *Males*: repair of cryptorchidism and hypospadias; testosterone replacement when necessary	*Females*: surgical feminization of external genitalia, gonadectomy followed by estrogen replacement starting at 11–12 years old; vaginal dilatation before sexual activity *Male social sex*: high T or DHT treatment for 6 months to increase penile length. Orthophaloplasty, scrotumplasty, vaginal pouch resection and hypospadias correction
General outcomes	Infertility; female or male gender identity	Infertility is usual but fertility is possible using assisted reproduction techniques

In 17β-HSD3 and 5α-reductase 2 deficiencies, the phenotype is sufficiently variable to cause problems in accurate diagnosis, particularly in distinguishing it from partial androgen insensitivity syndrome (PAIS) and from others enzymatic defects affecting testosterone synthesis (Rosler, 2006; Bertelloni *et al.*, 2006).

At infancy, patients with 17β-HSD3 and 5α-RD2 deficiencies seek for medical attention due to an inguinal hernia or clitoromegaly and at puberty due to amenorrhea and virilization (Andersson *et al.*, 1996; Lee *et al.*, 2007; Mains *et al.*, 2008; Rosler *et al.*, 1992, 1996; Mendonca *et al.*, 2000). The degree of virilization varies from increased body hair, deepening of the voice, development of male body habitus, and clitoris enlargement (reaching as much as 5–8 cm in length) (Lee *et al.*, 2007; Rosler, 2006; Mendonca *et al.*, 2000; Rosler *et al.*, 1992). At surgery, gonads are usually located in the groin area or in labioescrotal folds. Wolffian derivatives including the epididymides, vas deferens, seminal vesicles, and ejaculatory ducts are present (Andersson *et al.*, 1996; Lee *et al.*, 2007; Mendonca *et al.*, 2000), suggesting that a low amount of T is enough to develop male internal genitalia or that T is produced in peripheral tissues by alternative 17β-HSD isoenzymes or by 5α-RD type 1.

In 17β-HSD3 deficiency, there are some hypotheses to explain the paradoxal failure of intrauterine virilization followed by virilization at puberty. The aromatization of Δ4 by the placenta might prevent its extragonadal conversion to T in the fetus (Saez *et al.*, 1971). At puberty, the conversion of androstenedione (Δ4) to testosterone (T) by other 17β-HSD isoenzymes, presumably 17β-HSD5, should contribute to the observed virilization. The AKR1C3 (17β-HSD5) is expressed not only in extra-gonadal tissues, but also in genital skin fibroblast (Werner *et al.*, 2012) and its levels increase with aging in scrotal skin fibroblasts, while 17β-HSD3 mRNA expression is noted to be higher in the younger age groups (Hoppe *et al.*, 2006). Since the testes of patients with 17β-HSD3 deficiency also express AKR1C3, it is possible that Leydig cells of these patients may be a significant source of T synthesis in the presence of high Δ4 levels at puberty, indicating that this enzyme should play a large role in the virilization of these patients at puberty. Another possibility is a residual 17β-HSD3 function capable of maintain testosterone secretion in the presence of elevated LH and androstenedione levels (Andersson and Moghrabi, 1997).

Mild gynecomastia (Tanner II–III) is present in less than 30% of the adult patients, as previously reviewed (Mendonca *et al.*, 2017), probably due to peripheral conversion of Δ4 to estrogens (Moghrabi and Andersson, 1998).

In 5α-reductase 2 deficiency, affected individuals also present atypical external genitalia, ranging from a typical female external genitalia to a undervirilized male external genitalia (Imperato-McGinley, 2002). However, hypospadias, penile urethra, and micropenis have been described in individuals with allelic variants in the *5SAR2* gene. Internal genitalia (including vasa deferentia, seminal vesicles, epididymis, and ejaculatory ducts) are usually normal, but prostate is hypoplastic. The testes are located at inguinal area in most cases, suggesting that dihydrotestosterone has a role in the testes descent (Mendonca *et al.*, 1996). At puberty,

virilization is observed in individuals who kept the gonads, including voice deepening and muscle development, but gynecomastia is very rare, a feature which is helpful to clinical diagnosis after puberty.

Hormonal Diagnosis

17β-HSD3 deficiency can be reliably diagnosed by endocrine evaluation in most of the patients. The endocrine hallmarks of 17β-HSD3 deficiency are increased concentrations of Δ4 and reduced levels of T, leading to a low T/Δ4 ratio. It is considered that a T/Δ4 ratio below <0.8 is suggestive of 17β-HSD3 deficiency, based on a previous study in which all but 1 of the 18 index patients with 17β-HSD3 deficiency had a low T/Δ4 ratio (0.4 ± 0.2), measured by radioimmunoassay, after hCG stimulation (Boehmer *et al.*, 1999). No patients with AIS (androgen insensitive syndrome) or normal control had a T/Δ4 ratio <0.8 after hCG stimulation test.

However, there are known diagnostic pitfalls in hormonal analysis, as a low T/Δ4 ratio may also be found in other causes of 46, XY DSD (Faisal Ahmed *et al.*, 2000) and some proven molecular 17β-HSD3 deficiency patients had a normal T/Δ4 ratio (Khattab *et al.*, 2015; Lee *et al.*, 2007). The median post-hCG T/Δ4 ratio was significantly lower in a group of dysgenetic 46,XY DSD (9 of 16 patients had a T/Δ4 < 0.8) and 4 of 84 patients diagnosed as AIS (1 with the complete androgen sensitivity form and 3 with the partial form) had a T/Δ4 ratio also less than 0.8 (Faisal Ahmed *et al.*, 2000). Two of the 14 cases of 17β-HSD3 deficiency reported from the UK database had a T/Δ4 ratio >0.8 (Lee *et al.*, 2007). In addition, 67% of 17β-HSD3 deficiency patients of a national cooperative study from the Netherlands were misdiagnosed as AIS (Boehmer *et al.*, 1999).

All previous reported patients had hormonal evaluation performed by immunoassays. However, steroids with lower concentration such as androstenedione should be measured by a very specific assay such as high-performance liquid chromatography and tandem mass spectrometry to determine the T/Δ4 ratio correctly. But even in this ideal scenario, the hormonal diagnosis can fail. Two patients with atypical genitalia, who were assigned in the female social sex, were evaluated at 5 months and 9.2 year of age, respectively (Khattab *et al.*, 2015). After the hCG stimulation test, there was a clear elevation of serum testosterone with a small increase of the androstenedione levels (both measured by HPLC tandem mass spectrometry) resulting in a high T/Δ4 ratio (2.47 and 2.27, respectively). Both patients have deleterious molecular defects in *HSD17B3* gene. One possible explanation for the normal T/Δ4 ratio in these children with normal T/A ratio is the individual and temporal variability in the *HSD17B* isoenzymes activity (Khattab *et al.*, 2015).

In 5α-RD 2 deficiency the presence of an elevate testosterone and a low dihydrotestosterone suggests the 5α-RD2 deficiency diagnosis (Mendonca *et al.*, 2016; Inacio *et al.*, 2011). T/DHT ratio is helpful to diagnosis, but a pubertal level of testosterone is necessary to estimate this ratio. The T/DHT cut-off is not unequivocal but a ratio of >8.5 has been proposed as a cut-off for identifying 5α-RD 2 deficiency but with low specificity. During childhood, hCG stimulation test is necessary to obtain a pubertal level of testosterone (>100 ng/dL). Further, DHT measurement by immunoassay is not reliable considering the very low peripheral concentration of this hormone and the high cross-reaction with testosterone (Mendonca *et al.*, 2016). An alternative for 5α-reductase 2 deficiency diagnosis is the measurement of the 5α to 5β-reduced steroids in urine by chromatography–mass spectrometry (GC–MS). This technique is helpful, especially because it can be used in prepubertal and in individuals which had undergone to bilateral orquiectomy, but it still not easily available.

These difficulties for an unequivocal diagnosis using hormonal methods indicates that molecular diagnosis is preferable mainly in newborns.

Molecular Diagnosis

Both 17β-HSD3 and 5α-RD type 2 deficiencies are autosomal recessive disorders, and molecular defects can be emerged in homozygous or compound heterozygous states.

Up to now, almost 37 mutations in the *HSD17B3* gene have been reported. These include missense, nonsense, exonic deletion, duplication and intronic splice site mutations. Although mutations have been described throughout the *HSD17B3*, a mutation cluster region was identified in the exon 9. Outside exon 9, the most frequent site of mutation in *HSD17B3 gene* is the R80 in exon 3, which has been found in Palestinian, Brazilian, and Turkish families (McKeever *et al.*, 2002; Andonova *et al.*, 2017).

In the *5ARD2* gene, 90 different allelic variants related with 5α-RD type 2 deficiency have been described. The majority are *missense* mutation ($n = 60$), but small deletions, indels, splice site mutations, stop codons, and gross deletions were also described (Mendonca *et al.*, 2016). The *5ARD2* gene is composed by 5 exons and the allelic variants are concentrated especially at exons 1 and 4 (Thigpen *et al.*, 1992). Of the 254 aminoacids in the 5α-RD type 2 enzyme, mutations were found in a total of 67. Possible putative founder effect include p.Q6X and p.R227 in Asia and p.Q126R in Brazil. Usually, homozygous defects are more frequent than compound heterozygous and there is only one case of uniparental disomy reported (Chávez *et al.*, 2000).

Germ Cell Tumor Risk

The Consensus of Disorder of Sex Development reported a high risk (28%) of tumor development in patients with 17β-HSD3 deficiency (Lee, 2006). However, this high frequency for 17β-HSD3 deficiency was based on the gonadal tissue analysis of only

seven patients with 17β-HSD3 deficiency (Hughes *et al.*, 2006). In a recent review (Mendonca *et al.*, 2017), this prevalence was estimated in 5% taking the data of all the 40 reports of histological analysis of testicular tissue stained with hematoxylin-eosin of patients with 17β-HSD3 deficiency (Lee *et al.*, 2007; Chuang *et al.*, 2013; Cools *et al.*, 2005, 2006; Mendonca *et al.*, 2000; Wunsch *et al.*, 2012; Costa *et al.*, 2014; Telles de Sousa Castro *et al.*, 2012). The maintenance of the testes in patients with 17β-HSD3 deficiency and male social sex seems to be safe, except when the testes cannot be positioned into the scrotum.

In patients with 5-α reductase type 2 enzyme deficiency, the gonadal tumor risk is not clearly known, but is estimated to be so rare as described in complete androgen insensitivity syndrome (Kathrins and Kolon, 2016; Huang *et al.*, 2017).

Psychosexual Aspects

The most dramatic psychosexual outcome in 46,XY DSD is observed in patients with 17β-HSD3 and 5α-RD2 deficiencies. There is a high rate of gender change (from female to male) in both conditions: 39%–64% and 50%–63%, respectively (Furtado *et al.*, 2012; Cohen-Kettenis, 2010). It is probably due to prenatal androgen exposure, which is related with male psychosexual development, regardless of the karyotype and the external genitalia appearance (Berenbaum and Beltz, 2011; Hines, 2011).

In a recent review, we found that 61% of adult patients with 46,XY DSD due to 17β-HSD3 deficiency who were reared as female and not undergone gonadectomy during childhood, kept the female social sex and 19 of them (39%) changed to male social sex (Mendonca *et al.*, 2017). In the other hand, all patients that had undergone gonadectomy during childhood, kept the female social sex in adulthood (Bertelloni et al., 2009a,b).

Besides hormonal and physical factors, ethnical and cultural ones also play an important role in choosing male social sex. As an example, in Arabic families, it is preferable to be a sterile man to a sterile woman (Rosler, 2006; Rosler *et al.*, 1996; Rosler and Kohn, 1983). In one of those consanguineous families, who carried the mutation p.R80H in *HSD17B3* gene in the Gaza territory (Rosler, 2006; Rosler *et al.*, 1996; Rosler and Kohn, 1983), the spontaneous adoption of male identity of seven patients after puberty led some parents to request an early alteration of registration of their affected children during childhood (Rosler, 2006). In this family, the subjects who adopted the male social sex showed a much better adaptation to the new conditions than before the sex change (Rosler, 2006).

In 5α-RD type 2 deficiency, the prevalence of gender change is 48%–60% of the individuals at adulthood (Mendonca *et al.*, 2016). Correlation between the type of allelic variant and functional effects was not established (Costa *et al.*, 2012). At present, it is possible to conclude that gender change from female to male in 5α-RD2 enzyme deficiency individuals diagnosed at adulthood is common, but it is not a rule.

In both conditions affected individuals should be assigned as male considering the spontaneous puberty and the high frequency of female to male gender change. If assigned in female social sex, early gonadectomy should be performed to avoid virilization (Mendonca *et al.*, 2009; Mendonca, 2014).

Fertility

There are some 46,XY 17β-HSD3 deficiency patients reared as males and no report of preserved fertility (Andersson *et al.*, 1996; Lee *et al.*, 2007; Rosler, 2006; Cohen-Kettenis, 2005; Rosler and Kohn, 1983). There is one case report of an adult 17β-HSD3 deficiency patient whose biopsy of the testes at the age of 26 revealed absence of spermatogenesis, proving that he was sterile, despite the fact of having undergone relatively early orchidopexy (the left testis was brought down at the age of 2.5 years and the right testis at the age of 4) (Dumić *et al.*, 1985). There is no phenotype in 46,XX patients and fertility is preserved (Rosler *et al.*, 1996).

In 5α-RD type 2 deficiency there are some semen abnormalities including high viscosity, reduced volume, and reduced sperm account (Costa *et al.*, 2012; Russell and Wilson, 1994). In the germ cell analysis, there is a lack of primary spermatocytes. For most affected individuals, spontaneous fertility is not possible, but assisted reproduction techniques are helpful (Guercio and Rey, 2014). In vitro fertilization, intracytoplasmic sperm injection, and uterine insemination were used with success (Katz *et al.*, 1997; Matsubara *et al.*, 2010). In 46,XX *5ARD2* allelic variants carriers individuals, fertility and phenotype are normal.

Conclusion

46,XY DSD due to 17β-HSD3 deficiency and 5α-RD2 are autosomal recessive disorders that can be clinically indistinguishable due to the severe undervirilization of the external genitalia at birth followed by virilization at puberty. These diagnoses should be considered in front of females with inguinal hernias or mild clitoromegaly in infancy or early childhood as well as with amenorrhea and virilization at puberty. Most affected individuals are assigned and reared as females with high percentage of gender change to male social sex. In most patients the correct diagnosis can be achieved by a low T/A ratio (<0.8) for 17β-HSD3 deficiency and T/DHT ratio >8.5 for 5-α reductase type 2 deficiency. However, only molecular diagnosis is able to unequivocally confirm the diagnosis in both conditions.

Maintenance of the testes in patients with male social sex is safe and recommended, except when the testes cannot be positioned inside the scrotum. Fertility is difficult but it is possible and had been described in rare reports of 5α-RD type 2 patients.

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Androgen Insensitivity Syndrome (AIS): Complete AIS (CAIS)

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AIS and the Role of Androgens in Sex Development

Androgen insensitivity syndrome (AIS) is caused by target tissue resistance to androgen action. The AIS patient has 46,XY karyotype and gonads that are differentiated into testes. The testes are capable of producing testosterone which is further metabolized into dihydrotestosterone. However, as the receptor is deficient, androgens are not capable to turn on or off the activity of androgen-responsive genes and to induce the development of male sexual characteristics. The clinical manifestations of androgen insensitivity syndrome vary from external genitalia that are completely female to degrees of partial masculinization. These syndromes are the most common identifiable cause of male undermasculinization, representing approximately half of all 46,XY patients with a disorder of sex development (DSD; Thyen *et al.*, 2006). Complete AIS (CAIS) if the extreme of the wide phenotypic spectrum seen in AIS.

After development of the testis from the bipotential gonad, the events of male sex differentiation involve two pathways, one inhibitory and one stimulatory (**Fig. 1**). The inhibitory pathway is to represses the development of the Müllerian ducts (fallopian tubes, uterus, and upper third of the vagina). This process occurs between 6 and 8 weeks' gestation and is mediated by anti-Müllerian hormone (Josso *et al.*, 1991). The stimulatory events of male sex differentiation require high levels of androgens and a functional androgen receptor. Testosterone is critical in stabilizing the Wolffian duct system to prevent its involution and to induce differentiation into the epididymides, vasa deferentia, and seminal vesicles. Stabilization of the Wolffian ducts occurs between 9 and 13 weeks' gestation, when testosterone is secreted from the testes mostly under the control of placental chorionic gonadotropin. Dihydrotestosterone (DHT) is not involved in this process, as the 5α-reductase enzyme which converts testosterone into DHT is not yet expressed in these tissues (Wilson *et al.*, 1993). On the contrary, 5α-reductase is expressed already at this stage of development in the prostate, prostatic urethra, and external genital primordia, and their development is DHT dependent (Imperato-McGinley *et al.*, 1992).

Testicular descent occurs in two morphologically distinct phases which are under different hormonal control from the testis (Hutson *et al.*, 2015). During the first phase, insulin-like hormone 3 (INSL3) from the Leydig cells anchors the testis near the future inguinal canal by stimulating the gubernaculum to swell (Hutson *et al.*, 2015). In the second, inguinoscrotal phase, the gubernaculum bulges out of the inguinal region to create the future inguinal ring and then migrates to the scrotum enabling the intraperitoneal testis to leave the abdomen while still remaining inside an extension of the peritoneum. The final event is closure of the proximal processus vaginalis in the human, thereby preventing inguinal hernia and/or hydrocele (Hutson *et al.*, 2015). Androgens control the inguinoscrotal phase, but the exact mechanism is not clear. Previously, it was assumed that androgens act directly on the gubernaculum, but AR does not appear in the gubernaculum during the critical inguinoscrotal phase of testicular descent (Nation *et al.*, 2011).

The Androgen Receptor

The androgen receptor (AR) is a well-defined transcriptional regulatory factor that belongs to the steroid/nuclear receptor superfamily (Evans, 1988). Four of these receptors, the androgen receptor, glucocorticoid receptor, mineralocorticoid receptor, and

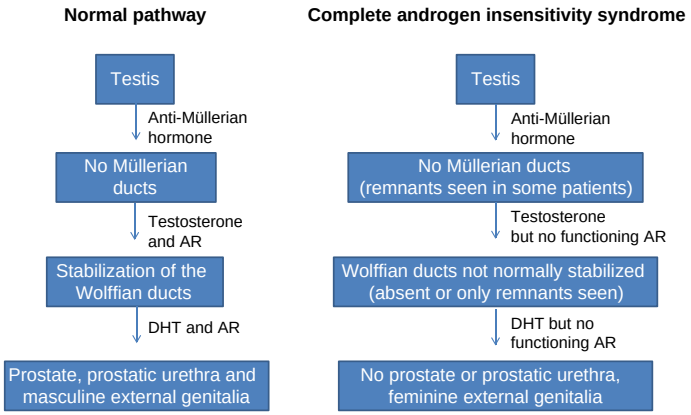

Fig. 1 Differences in sexual differentiation in normal 46,XY male and in 46,XY female with complete androgen insensitivity syndrome (CAIS). https://link.springer.com/referenceworkentry/10.1007/978-3-319-29456-8_26-1.

progesterone receptor, are closely related and even have an ability to activate gene transcription via the same hormone response element. AR interacts directly with its target genes as a hormone-receptor complex and regulates their transcription.

The human *AR* complementary DNA was cloned in 1988 (Chang *et al.*, 1988; Lubahn *et al.*, 1988), and in the same year the first *AR* gene mutation associated with CAIS was reported (Brown *et al.*, 1988). The human *AR* gene is located on chromosome Xq11–12 (**Fig. 2**). The *AR* gene is about 90 kb long, but only about 2750 nucleotides code for an AR protein of 919 amino acid residues. The AR protein migrates during SDS-PAGE as 110 and 112 kDa proteins, the latter representing the phosphorylated form of the AR protein (Brown *et al.*, 1989; Chang *et al.*, 1988; Lubahn *et al.*, 1988). The gene is divided into eight exons, and the first exon includes two homopolymeric amino acid, polyglutamine and polyglycine, repeats which are polymorphic in length (Andrew *et al.*, 1997; Lumbroso *et al.*, 1999).

Like all nuclear receptors, the AR protein consists of four major functional domains: a variable N-terminal transactivation domain (NTD, encoded by exon 1), a highly conserved DNA-binding domain (DBD, encoded by exons 2 and 3), hinge region or bipartite nuclear localization signal (encoded by exons 3 and 4), and a conserved C-terminal ligand-binding domain (LBD, encoded by exons 4 to 8; **Fig. 2**). In addition to their principal functions, these major domains embody subsidiary functions affecting receptor dimerization, nuclear localization, and transcriptional regulation (Brinkmann, 2001). After binding of an androgen molecule to the LBD, AR rapidly translocates to the nucleus, where it directly interacts with DNA as a homodimer, at androgen response elements (ARE) found in the regulatory regions of target genes (**Fig. 3**). This complex can thenceforth recruit coactivators through the ligand-dependent transactivation function (AF-2) located in the LBD and hence control transcription of specific genes. Through this mechanism, androgens such as testosterone and DHT regulate a wide range of physiological androgenic and anabolic responses, most notably male sexual differentiation and maturation including the development, growth, and maintenance of the normal prostate. The ligand structure itself determines the number of interactions it can make with the AR LBD. For example, a potent synthetic androgen tetrahydrogestrinone establishes more van der Waals contacts with the receptor than the natural ligands testosterone and DHT, whereas the geometry of the atoms forming electrostatic interactions at both extremities of the steroid nucleus seems mainly responsible for the higher affinity and androgenic potency measured experimentally for DHT over testosterone (Pereira de Jésus-Tran *et al.*, 2006).

In the AR N-terminal domain there are motifs that form the interface for the interaction of NTD with the LBD. These motifs interact with different regions of the LBD to stabilize the hormone-receptor complex (He *et al.*, 2000). There also seems to be a putative interface between the AR, DBD, and LBD. Some LBD mutations which decrease DNA binding leave ligand binding unaffected, as well as some DBD-residing mutations which lower the ligand binding do not affect the DNA-binding affinity. Therefore, it has been proposed that certain AR residues are involved in allosteric communications between the AR-DBD and AR-LBD (Helsen *et al.*, 2012).

Androgen receptor is expressed in various human tissues, and AR defects have been recognized also in conditions other than AIS, such as the X-linked motor neuron disorder known as Kennedy's disease and prostate cancer.

Androgen Receptor Gene Mutations in AIS

The rates of detected mutations in AIS have varied depending on phenotype, family history, and screening method. In the Cambridge cohort, an *AR* mutation was found in 83% of the cases with CAIS (Ahmed *et al.*, 2000a). In a small Brazilian study on 32 AIS patients from 20 families, the strict selection criteria, which included also family history suggestive of X-linked inheritance in prepubertal subjects and the presence of gynecomastia in postpubertal subjects, resulted in the identification of mutations in

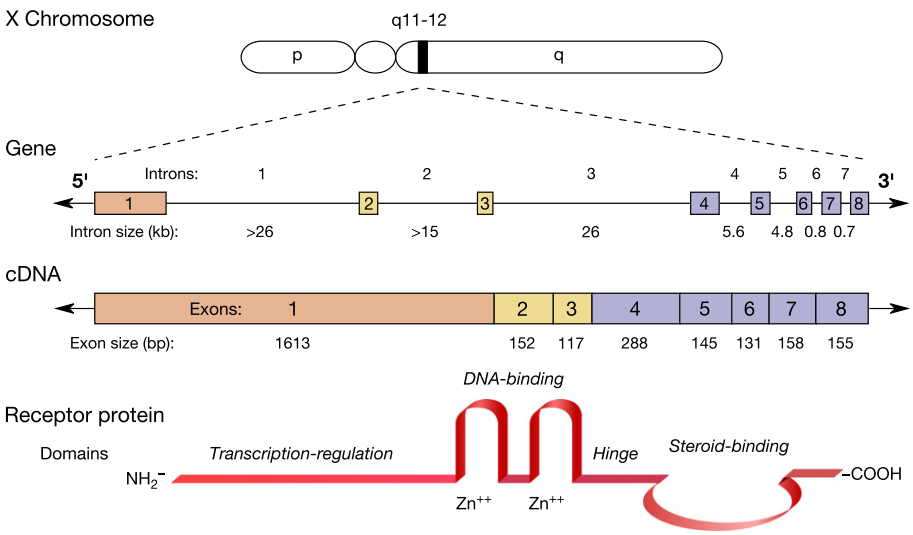

Fig. 2 Location and structure of the human androgen receptor: The *AR* gene is located on the long arm of the X chromosome. The eight exons are separated by introns of various lengths. The AR protein has several functional domains. Quigley, C. A., De Bellis, A., Marschke, K. B. et al. (1995). Androgen receptor defects: Historical, clinical, and molecular perspectives. *Endocrine Reviews* 16, 271–321.

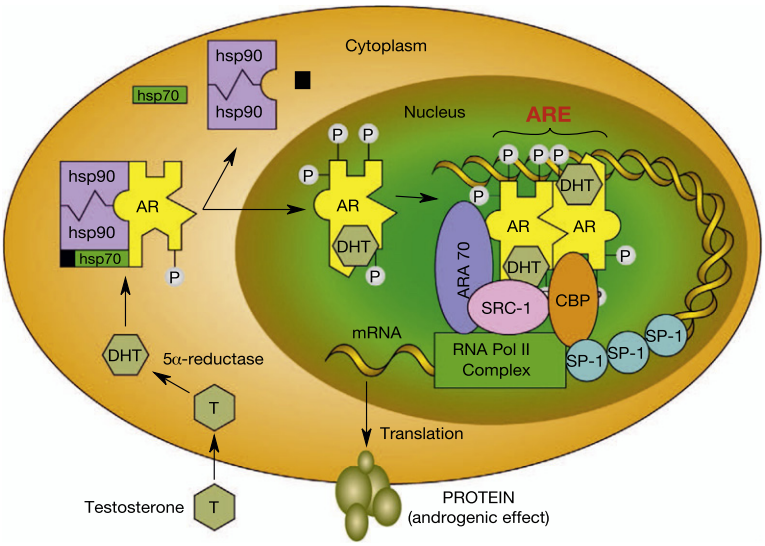

Fig. 3 Ligand-dependent activation of the androgen receptor. Androgens enter the cell and bind to the AR. Upon ligand binding, the AR undergoes conformational changes and the translocates to the nucleus where dimerization and DNA binding to regulatory androgen response elements occurs. AR (androgen receptor); DHT (dihydrotestosterone); CBP (CREB-binding protein); ARE (androgen response element); hsp (heat shock protein); SRC-1 (steroid receptor coactivator 1). Figure by Jonathan Marcus, based on an original drawing in: Meehan, K. L. and Sadar, M. D. (2003). Androgens and androgen receptor in prostate and ovarian malignancies. *Frontiers in Bioscience* 8, d780–d800.

100% of subjects with CAIS (Melo *et al.*, 2003). All in all, in CAIS the likelihood of finding a disease-causing mutation is significantly higher than in PAIS or MAIS.

More than 550 single-nucleotide variants in *AR* have been found to cause AIS (the androgen receptor mutations database, last updated in 2012; Gottlieb *et al.*, 2012). Almost two thirds are missense mutations (Jääskeläinen, 2012). About a quarter are nonsense point mutations, small deletions, or insertions leading to a premature stop codon. Intron splice site mutations and large deletions spanning several exons cover less than 10% of all the reported mutations. Most detected mutations have been identified in patients with CAIS (Jääskeläinen, 2012).

More than half of all *AR* mutations are located in the LBD, and a majority of these are missense mutations (Gottlieb *et al.*, 2012). These mutations include the amino acid residues that line the AR ligand-binding pocket. Other clusters are located at the activation function 2, a regulatory surface cleft termed binding function 3, and a region that tethers the C-terminal tail of the receptor (Estébanez-Perpiñá *et al.*, 2007; Tahiri *et al.*, 2001). The mechanisms by which the LBD mutants disrupt AR signaling are not only diminished ligand binding but include also disturbed interaction with the cofactors, transactivation domain, or DNA-binding domain (Ghali *et al.*, 2003; Helsen *et al.*, 2012; Jääskeläinen *et al.*, 2006; Thompson *et al.*, 2001).

The proportion of detected and reported mutations in the N-terminal transactivation domain (encoded by exon 1) has constantly increased, and the past very low detection rates most likely reflect challenges in sequencing this GC-rich exon. In a recent report on patients with CAIS, the mutation rate in exon 1 was already 27% (Philibert *et al.*, 2010). Most exon 1 mutations lead to a premature stop codon (associated with CAIS) and less than a third are missense mutations, mostly associated with PAIS or MAIS (Jääskeläinen, 2012).

About 15% of all detected AR mutations lie within the DBD encoded by exons 2 and 3, and only a few mutations in AR hinge region (codons 628–669, between DBD and LBD) have been reported (Jääskeläinen, 2012).

AIS is inherited in a recessive X-linked manner. Thus, the proband is likely hemizygous for the *AR* mutation and the mother of the proband is a heterozygous carrier. When the mother has more than one affected child but the pathogenic *AR* variant cannot be found in her leukocyte DNA, she most likely has germline mosaicism. Further, all *AR* gene mutations are not inherited: 30% of all detected mutations are de novo mutations, and there is evidence that every third de novo mutation may have occurred at a postzygotic stage leading to a somatic mutation (Hiort *et al.*, 1998).

The phenotype may vary with identical genotypes. The androgen receptor mutations database includes about 50 cases where an identical AR mutation is associated with significant phenotypic diversity. Identical mutations have been associated with different PAIS phenotypes even in the same kindred, and siblings with the same AR mutation have been assigned a different sex of rearing (Batch *et al.*, 1993). Phenotypic variability may be due to somatic mosaicism (Holterhus *et al.*, 1997) or to variable expression of other genes necessary for androgen biosynthesis and metabolism (such as 5α-reductase) (Boehmer *et al.*, 2001).

AR Polymorphisms and Androgen Insensitivity

There are two polymorphic trinucleotide repeat segments in the AR NH2-terminal domain of the receptor, $(CAG)_n$ encoding a polyglutamine and $(GGN)_n$ encoding a polyglycine repeat segment, respectively (Lubahn *et al.*, 1988; Chang *et al.*, 1988). The

length of CAG repeats varies from nine to 36 in normal population (Andrew *et al.*, 1997). When this region is ≥38 repeats in length, it may lead to reduced virilization, defective spermatogenesis, and spinal bulbar muscular atrophy (SBMA), known also as Kennedy's disease (La Spada *et al.*, 1991).

Downstream of the CAG_n repeat polymorphism is located the GGN repeat region, represented by $(GGT)_3GGG(GGT)_2(GGC)_n$ with n varying from 10 to 35. In AIS, these two polymorphisms may modulate the phenotypic expression of a given AR mutant in affected individuals (Werner *et al.*, 2006; Rajender *et al.*, 2008). Furthermore, AR CAG repeat length may contribute to the causation of androgen-related genital abnormalities with multifactorial etiology, including hypospadias (Huang *et al.*, 2015; Lim *et al.*, 2001). When within the normal length range, neither $(CAG)_n$ nor $(GGN)_n$ are single causative factors for AIS, but they have been associated with male infertility or serum testosterone concentrations. In 1994, Chamberlain et al. reported that AR function is reduced in vitro by increasing the length of CAG repeat. However, this association may be nonlinear: median CAG lengths (22–23 repeats) have resulted in higher AR-mediated transcription than longer and shorter repeats (Nenonen *et al.*, 2011).

AR Mutation Negative AIS

No other genetically proven mechanism than the mutated *AR* has been shown for AIS. Theoretically such a mechanism could be a coactivator defect. Two sisters who were resistant to several steroids, including androgens, have been reported (New *et al.*, 2001). Also, one subject with CAIS has been reported, in whom the transmission of the activation signal from the AF1 region was disrupted but no mutation was found in the AR gene suggesting that a coactivator interacting with this region was lacking in this patient (Adachi *et al.*, 2000). However, no proven molecular defect in any coactivator has been found in any AIS patient. Next generation multigene panel sequencing should therefore be considered for an AIS patient with no detected mutation in the *AR*.

Studying Androgen Receptor Function In Vitro

The effect of the identified mutation on androgen receptor-dependent transactivation is the most used AR functional assay. The AR expressing plasmid (either normal or mutated) is transfected into a mammalian cell line (which itself does not express AR) together with a hormone response element in construct with a suitable reporter vector. The transfected cells are then incubated with the ligand, and thereafter the reporter activity is assayed.

Another functional assay is studying interaction between the AR amino- and carboxylterminal domains (N/C interaction assay based on reporter gene activation in a mammalian two-hybrid assay; He *et al.*, 2000). Missense mutations found in the AR-LBD that reveal near-normal androgen-binding kinetics may display disrupted N/C-interaction, and the decreased N/C-interaction has been shown to mirror the degree of AIS (Jääskeläinen *et al.*, 2006).

Missense mutations in the DBD may lead to a complete disruption of AR binding to AREs associated with CAIS or only to an altered affinity and selectivity of AR–androgen response element interactions associated with PAIS or MAIS. The reduced or absent DNA binding can be demonstrated by electrophoretic mobility-shift assays (EMSAs) or by fluorescence recovery following photobleaching (FRAP) experiments, comparing cells transfected with green fluorescent protein-tagged mutant and wild-type AR (Farla *et al.*, 2004).

Studies of androgen binding in cultured genital skin fibroblasts have been utilized for the diagnosis of AIS for many years and elucidated the cause of AIS at the cellular level in many cases. A 2–3 mm genital skin biopsy intended for androgen receptor studies can be taken under local anesthetic from the labia which contains preferential expression of androgen receptor. Alternatively, skin from the prepuce or labioscrotal folds can be obtained at the time of surgery. After culturing an adequate number of cells, which takes a minimum of 6–8 weeks, the cells are incubated with a radiolabelled androgen (like DHT or mibolerone) in the presence or absence of an excess of the nonradiolabeled ligand. Several parameters (maximal ligand-binding capacity, ligand dissociation constant, thermolability) can then be measured (Griffin *et al.*, 1976).

Prevalence of Complete Androgen Insensitivity Syndrome

AIS is the most common specific cause for 46,XY undermasculinization, but the estimates of its prevalence have varied widely. Jagiello and Atwell estimated the prevalence of complete CAIS as 1:62,400 males based on the prevalence of inguinal hernia in phenotypic females (Jagiello and Atwell, 1962). Later, a group in Denmark reported a higher prevalence for AIS, 1:20,400 males, calculated from the number of primarily diagnosed cases with the disorder (Bangsbøll *et al.*, 1992). In a Dutch study, which included only cases with a proven molecular diagnosis of AIS, the incidence was 1:99,000 males (Boehmer *et al.*, 2001).

Clinical Presentation of CAIS

In CAIS, there are no visible clinical signs of androgen action and the subjects are born with normal female external genitalia, though the clitoris, labia minora, and labia majora may be underdeveloped. If prenatal examinations are performed, CAIS can be

suspected already prenatally, when the karyotype (46,XY) of the fetus does not match with the female phenotypic sex. The birth size of CAIS patients is on average the same as that of healthy male infants, suggesting that factors on the Y chromosome rather than exposure to prenatal androgens explain the sex dimorphism (Miles *et al.*, 2010). After birth, most subjects (up to 76%) are diagnosed early when inguinal hernias or inguinal or labial swellings in an apparently female infant are discovered to contain testes (Viner *et al.*, 1997). All in all, inguinal hernias have been reported to be present in 90% of patients with CAIS, and in most cases these are bilateral (Viner *et al.*, 1997). Estimates of the incidence of AIS in female infants with hernias have ranged from 1% to 2%, suggesting that any girl with an inguinal hernia should have a karyotype performed or CAIS otherwise excluded (Jagiello and Atwell, 1962; Pergament *et al.*, 1973). Girls with CAIS have a shorter vaginal length than healthy girls, and in ultrasonography no ovaries, fallopian tubes, or uterus can be seen (Hurme *et al.*, 2009). In some patients, however, rudimentary segments of the Müllerian ducts are present (Nichols *et al.*, 2009). Wolffian duct differentiation is testosterone dependent, and these patients should lack vasa deferentia, epididymides, seminal vesicles, and ejaculatory ducts. However, some phenotypic CAIS patients have Wolffian ducts, explained by minor residual activity of the mutant AR which is capable of responding to high local testosterone concentrations during early development (Hannema *et al.*, 2004). The testes can be located anywhere along the pathway from the lower abdomen to the scrotum. Many subjects with CAIS are not suspected of having the diagnosis until the onset of puberty, when breast development is normal, but pubic and axillary hair development is not, and menarche, initially considered late, never occurs. Some affected individuals develop normal female sexual hair at puberty, and even more CAIS patients have some fine, silky pubic hair (Tanner 2), developing under the influence of factors other than AR action (Boehmer *et al.*, 2001; Wisniewski *et al.*, 2000). If not diagnosed by this time, CAIS can first present after puberty as a gonadal tumor (Manuel *et al.*, 1976). The physical appearance is generally feminine, and affected individuals have mostly a feminine body image and sexual orientation (Wisniewski *et al.*, 2000). However, some recent reports indicate that less feminine gender roles and "not exclusively androphile sexual orientation" are more common in CAIS than previously thought (Brunner *et al.*, 2016). Adult height is between healthy males and females (Varrela *et al.*, 1984; Zachmann *et al.*, 1986). The enlarged adult stature in the syndrome is thought to be mainly due to the effect of the growth-controlling region on the long arm of the Y chromosome, but genome-wide association studies have identified several other loci that affect adult height (Lango Allen *et al.*, 2010).

Diagnosis of CAIS

Resistance to androgens, testosterone and its 5α-reduced product dihydrotestosterone (DHT) is diagnosed when circulating concentrations of serum hormones are normal or high but there is little or no clinical effect. The diagnosis of AIS is based on clinical findings, endocrine evaluation, and family history. The diagnostic criteria for AIS include:

1. 46,XY karyotype.
2. Presence of testes with histology showing testicular differentiation.
3. Normal testosterone production and metabolism.
4. Absence of Müllerian ducts (fallopian tubes, uterus, upper part of vagina; remnants may be present).
5. Normal female external genitalia (inguinal hernia and labial swellings containing testes may be present).
6. Spontaneous feminization (but no menses) at puberty before gonadectomy with no virilization despite normal or high male levels of testosterone.
7. Strict criteria for CAIS include also an identified mutation in the *AR* gene (Mongan *et al.*, 2015; Viner *et al.*, 1997; Zachmann *et al.*, 1986).

A complete diagnostic evaluation thus includes karyotype analysis, pelvic ultrasonography, basal (and potentially human chorionic gonadotropin (hCG) stimulated) serum testosterone, DHT, and androstenedione, as well as basal (and potentially gonadotropin releasing-hormone (GnRH) stimulated) serum luteinizing (LH) and follicle-stimulating hormone (FSH) concentrations. Nowadays, mutational analysis either by Sanger sequencing or next generation sequencing multigene panels is the best tool to confirm the diagnosis. In the case where no *AR* mutation is detected, a gonadal biopsy is valuable in the differential diagnosis; gonads of a patient with AIS usually show normal testicular differentiation, unlike the situation in gonadal dysgenesis. Also, genital skin fibroblast culture is useful when mutational analysis remains negative. Fibroblasts can be used to identify genomic mutations that disrupt normal RNA splicing, to quantify androgen binding, AR expression, and expression of androgen-dependent gene expression patterns (Holterhus *et al.*, 2007).

Differential Diagnosis of CAIS

In most cases CAIS is a relatively clear entity, and it only rarely leads to diagnostic problems if the initial diagnostic investigations are adequate. However, the differential diagnosis for CAIS includes several conditions leading to impaired androgen production and metabolism. Defects in steroid biosynthesis and metabolism can cause male undermasculinization. In 17β-hydroxysteroid dehydrogenase type 3 deficiency, the final step in testosterone biosynthesis (from androstenedione to testosterone) is impaired, but the ratio of testosterone to androstenedione is not always discriminative, and the phenotype of these children might be

indistinguishable from those with AIS (Lee *et al.*, 2007). During puberty, people with this condition develop some secondary sex characteristics, such as increased muscle mass, deepening of the voice, and development of male pattern body hair.

In 5α-reductase deficiency the peripheral conversion of testosterone to DHT is impaired but the ratio of DHT to testosterone is variable in this condition (Hiort *et al.*, 1996). At puberty, also these patients may present with virilization.

Finally, most 46,XY individuals with *NR5A1* loss-of-function mutations have evidence for gonadal dysgenesis, but some infants with heterozygous *NR5A1* mutations present with normal testosterone and DHT levels at infancy, and thus AIS may remain as a differential diagnosis (Coutant *et al.*, 2007).

Androgen biosynthesis is deficient in three other rare conditions: 3β-hydroxysteroid dehydrogenase, P450c17 (17α-hydroxylase), and steroidogenic acute regulatory protein (StAR) deficiencies, which may also be associated with deficient cortisol biosynthesis (Lin *et al.*, 1995; Rhéaume *et al.*, 1991; Yanase *et al.*, 1991). Leydig cell hypoplasia due to inactivating LH receptor mutations and congenital gonadotropin deficiency are other rare specific causes for deficient testosterone production (Kremer *et al.*, 1995). If gonadal histology is not available, disorders of gonadal differentiation must also be included in the differential diagnostics (Werner *et al.*, 2010).

Endocrine Profile in AIS

In a 46,XY individual with AIS, testicular differentiation occurs normally, and immature germ cells are present in the testes at birth and during childhood. However, in the absence of functional AR, there is a progressive decline of germ cell numbers with increasing age, and in adulthood, no germ cells are present in the testes of affected adults with severe AIS (Hannema *et al.*, 2006).

Generally, children and postpubertal (nongonadectomized) patients with AIS have normal or high basal and hCG-stimulated serum testosterone and DHT, as well as high LH concentrations (Ahmed *et al.*, 1999). Unlike in gonadal dysgenesis, serum FSH concentrations are generally normal (Melo *et al.*, 2003). However, at infancy basal testosterone and both basal- and GnRH-induced serum LH concentrations can be markedly low in infancy, and retained testosterone biosynthesis can only be detected by monitoring the testosterone response to hCG (Bouvattier *et al.*, 2002).

Estrogen production mainly by the testes, and to a lesser extent by aromatization of androstenedione and testosterone in peripheral tissues, is increased in individuals with AIS to about twice that of normal males due to increased LH stimulation of Leydig cells. Plasma or urinary levels of estrogens are at the upper limit of normal or elevated compared with those in normal males. SHBG levels are comparable to those in normal females reflecting peripheral androgen resistance (Tremblay *et al.*, 1972; Quigley *et al.*, 1995) whereas serum anti-Müllerian hormone and inhibin B levels are within or above the normal male reference range, suggesting uninhibited Sertoli cell function (Hellmann *et al.*, 2012).

Treatment

Though management of DSD, including androgen insensitivity syndrome, is nowadays largely discussed and debated, decision on female sex of rearing is normally not an issue in CAIS. The diagnostic and management procedures should be performed by a multidisciplinary team including key members from endocrinology (pediatric or adult), urology, gynecology, and psychology. Additional members of the team may include specialists in clinical genetics, medical ethics, and social services (Hiort *et al.*, 2014; Hughes *et al.*, 2012).

The initial management is focused on counseling and gender assignment. After this, management addresses discussing functional issues like timing of gonadectomy to prevent tumor formation in testes, hormone replacement or supplementation at the appropriate time in life, and psychological support for the patient and family.

Gonadectomy and the Risk of Gonadal Neoplasia in AIS

The risk of gonadal neoplasia (most commonly type II germ cell tumors, including seminoma) is increased in AIS, but the malignancy rates vary in different studies (Cools *et al.*, 2006). The traditional consensus is that the testes should be removed at some stage in the case of female sex of rearing. The main controversy has been the timing of gonadectomy. Currently, the accepted practice is to leave the testes in place until after puberty has been completed (Hughes *et al.*, 2006; Patel *et al.*, 2016). There are strong arguments supporting this practice. First, it is not possible to obtained a true informed consent from a children. Secondly, true gonadal neoplasia is very rare until after puberty (Manuel *et al.*, 1976). Though carcinoma in situ and gonadoblastoma have been detected at variable rates in adolescent CAIS females, the youngest case with seminoma was a 14-year-old girl (Hurt *et al.*, 1989). Thirdly, if gonads are not removed, spontaneous development of female secondary characteristics at puberty occurs satisfactorily. Furthermore, prepubertal low serum concentrations of estrogens may be important for higher central nervous system centers involved in the development of sexual identity and for bone mineralization (Collaer and Hines, 1995; Soule *et al.*, 1995).

The estimates of overall risk of gonadal malignancies in AIS have varied from 5.5% to 14% (Cools *et al.*, 2006; Deans *et al.*, 2012; Cools *et al.*, 2017). The risk of neoplasia seems to increase by age (Manuel *et al.*, 1976). However, some patients want to defer gonadectomy further, and an increasing number of women want to retain their gonads indefinitely (Deans *et al.*, 2012). The

retained gonads can be followed by magnetic resonance (MR) imaging (Nakhal *et al.*, 2013) or by ultrasonography (Patel *et al.*, 2016). MR imaging is accurate in the detection of testicular changes, including paratesticular cysts and Sertoli cell adenomas (which are usually benign). However, neither technique can depict premalignant changes like carcinoma in situ; therefore, most experts still recommend gonadectomy after puberty (Nakhal *et al.*, 2013). If gonads are not visualized, laparoscopic gonadopexy, and gonadal biopsy have also been suggested (Patel *et al.*, 2016). Neoplastic lesions of carcinoma in situ (CIS) and gonadoblastoma express consistently octamer-binding transcription factor (OCT 3/4). If OCT 3/4 is positive, gonadectomy is recommended (Patel *et al.*, 2016).

Treatment of the External Genitalia and Vagina

In CAIS, the external genitalia are unambiguously female and no early surgery is necessary. The vagina is short in this condition, but surgical vaginal elongation is not indicated in most cases. Vaginal elongation can either be achieved digitally or by using dilators, and when this is done, most women are capable of normal intercourse (Boehmer *et al.*, 2001). When a vaginoplasty procedure is required, the laparoscopic Vecchietti procedure is becoming more widely available and appears to be a suitable surgical option based on a recent detailed review of the literature (Mongan *et al.*, 2015).

Hormone Replacement, Fertility, and Bone Mineral Density

Clearly, in the absence of a uterus, a female patient with CAIS, is destined to be infertile. If gonads are remained in place, estrogen treatment at puberty is usually not indicated as the feminization occurs spontaneously due to excess testosterone being aromatized. If gonads are removed before the onset of puberty, pubertal induction with either transdermal natural estrogen or starting with ethinylestradiol is indicated (Mongan *et al.*, 2015). Progesterone substitution is not needed in the absence of uterus. Some adult women with CAIS prefer to take supplementary testosterone after gonadectomy, because they report an improvement in wellbeing. This is not a standard therapy, and the mechanism of this therapy in the absence of functional AR is not clear. Apart from aromatization, nongenomic effects of androgens or their potential effects as neurosteroids have been suggested (Mongan *et al.*, 2015).

The role of androgens in skeletal maturation and bone mineralization is not as clear as that of estrogens. All published reports show that adult CAIS patients have bone mineral densities (BMD) somewhat lower than healthy female subjects (Bertelloni *et al.*, 1998; King *et al.*, 2017; Marcus *et al.*, 2000; Muñoz-Torres *et al.*, 1995; Sobel *et al.*, 2006; Soule *et al.*, 1995). Bone mineral densities are lower than in healthy female subjects already before gonadectomy, and when adequately substituted with estrogens, the patients show moderate deficits in BMD, averaging close to -1 SDS from normative means. The severe (BMD < -2 SDS) osteopenia in some females may reflect a component of inadequate estrogen replacement rather than androgen lack alone (Marcus *et al.*, 2000). All in all, it seems that androgens have a role in bone mineralization independent from estrogens.

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Androgen Insensitivity: Partial AIS

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Introduction

Androgen insensitivity syndrome (AIS) is classified as a disorder of hormone action due to reduced or absent function of the androgen receptor (AR) protein encoded by the *AR* gene. In contrast to the subcategory of complete AIS characterized by no *AR* activity, partial AIS (PAIS) retains some ability to respond to androgens. Thus, affected individuals show some degree of virilization. The phenotypes of PAIS can vary widely, even though patients within a family can carry the same mutation. Consequently, management needs to be tailored to each affected individual.

Clinical Features

Individuals with PAIS present with varying degrees of virilization in contrast to females with CAIS. Typically, there is ambiguous genitalia at birth, with a spectrum ranging from predominantly female genitalia (clitoromegaly, scrotalized labia majora) to predominantly male genitalia (micropenis, hypospadias, bifid scrotum). The testes are undescended in about 50%. Development of secondary male characteristics may occur spontaneously at puberty, although many require treatment with exogenous androgens for complete pubertal development. Individuals with a mild form of AIS (MAIS) present with gynecomastia at adolescence or male factor infertility in adulthood. There may be a history in childhood surgical correction for mild hypospadias or undescended testis. In PAIS, Müllerian tracts (Fallopian tubes, uterus, and cervix) are usually absent and the gonads are differentiated as testes with normal or high levels of androgen production.

Table 1 summarizes the phenotype of the external genitalia in patients with PAIS from the Cambridge DSD database. Clinical features at the time of presentation were analyzed by sex of rearing (male $n = 49$, female $n = 13$).

Etiology

PAIS as a sub-category of AIS is caused by a mutation in the *AR* gene located on the X chromosome long arm. However, similar phenotypic features of PAIS can also arise from several other conditions associated with a 46,XY karyotype as summarized in **Table 2**. It is noteworthy that in partial gonadal dysgenesis due to *NR5A1* mutation, the gonads usually differentiate as testes which are able to produce testosterone (Coutant *et al.*, 2007; Domenice *et al.*, 2016). A phenotype consistent with PAIS may also occur in low birth weight infants but rarely is an *AR* gene mutation identified (Lek *et al.*, 2014).

Table 1 Features of external genital phenotype in PAIS assigned female versus male

	Raised female n = *13*	*Raised male* n = *49*
Scrotum/labia	Bifid (46%) Fused (8%) Unknown (46%)	Bifid (57%) Fused (24%) Unknown (18%)
Meatus	Perineal (54%) Unknown (46%)	Perineal (59%) Penile (6%) Glandular (18%) Unknown (16%)
Penis/clitoris	Normal for females (8%) Clitoromegaly (62%) Unknown (31%)	Micropenis (63%) Normal for males (24%) Unknown (12%)
Rt gonad	Intra-abdominal/non-palpable (8%) Inguinal (46%) Scrotal (38%) Unknown (8%)	Intra-abdominal (10%) Inguinal (20%) Scrotal (61%) Unknown (8%)
Lt gonad	Intra-abdominal/non-palpable (15%) Inguinal (46%) Labioscrotal (31%) Unknown (8%)	Intra-abdominal (12%) Inguinal (12%) Labioscrotal (67%) Unknown (8%)

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Table 2 Differential diagnosis of PAIS

Partial gonadal dysgenesis
May have gene mutation *NR5A1, SRY, WT1*
Steroid biosynthetic defects
17,20-Lyase deficiency, p450 oxidoreductase deficiency, 17-β-hydroxysteroid dehydrogenase deficiency, type 3, 5α-reductase deficiency
Syndromes
Klinefelter syndrome, Smith–Lemli–Opitz syndrome, Frasier syndrome
Idiopathic
Others
Leydig cell hypoplasia due to inactivating LH receptor mutations

Diagnosis

The biochemical features consistent with PAIS comprise a normal or increased concentration of testosterone, either basally or following stimulation of the Leydig cells with human chorionic gonadotrophin (hCG). A normal increment in dihydrotestosterone (DHT) also generally excludes 5 alpha-reductase deficiency although the definitive test for this enzyme deficiency is analysis of a urinary steroid profile. The basal level of LH may be slightly elevated; the serum AMH level is normal and consistent with the presence of normally developed testes.

In contrast with CAIS, in only about 30% of individuals with a PAIS-consistent phenotype is a mutation in the *AR* coding region identified. Invariably the mutation is a single amino acid substitutions (missense mutation).

Molecular Studies

The AR is a member of the steroid/nuclear receptor superfamily, which shares a similar structural composition; N-terminal domain, DNA binding domain, hinge, and ligand binding domain. The AR resides in the cytoplasm in the cell in complex with heat shock proteins and co-chaperones. In the presence of androgens (dihydrotestosterone and testosterone), conformational changes occur including the release of heat shock proteins and closing the binding pocket. The AR then translocates to the nucleus where homodimerization occurs and transcription activity is initiated. In these multiple steps, interaction with a number of co-activators also regulates AR activity (Jaaskelainen, 2012). A defect in any of these steps may affect normal male development.

When an *AR* mutation is identified in PAIS and particularly if it is novel, it is important, to demonstrate pathogenicity and the results are relevant for genetic counseling. Classical functional assays include transactivation assays with reporter genes and androgen binding assays with analysis of dissociation kinetics. These require cell culture facilities. Alternatively, now that the 3-D structures of the DNA binding and ligand binding domains have been established, it is possible to study the impact of the mutation using in silico 3-D structure analysis, or online bioinformatics tools including http://www.bioinf.org.uk/saap/, FATHMM, PolyPhen2, and SIFT. An example of the results of an androgen receptor functional in vitro assay for a mutant AR and its structural analysis is shown in **Fig. 1**. The degree of virilisation varies according to the type of substitution at codon 582 with a milder form of PAIS manifest in the subject with the 582L substitution. Even so, the phenotype can vary between individuals with the same *AR* mutation so much so that there are examples where siblings with same *AR* mutation have a different sex of rearing (Boehmer *et al.*, 2001) The variation in phenotype in these situations may be attributable to quantitative differences in androgen concentrations during the male fetal programming window, somatic mutation status, or other genetic background (Quigley *et al.*, 2004; Jaaskelainen, 2012).

In the majority of individuals with a PAIS-like phenotype and in whom mutation has not been found in the *AR* coding region, the cause is unkown. However, the recent application of exome sequencing techniques has identified deleterious molecular changes outside the *AR* coding region as a pathogenic explanation for a PAIS phenotype. One profound example was reported in several generations of a large family with PAIS where an X-linkage studies clearly indicated association with an AR defect yet no mutation was found in the *AR* coding region (Ahmad *et al.*, 2017). Subsequently, exome sequencing identified an intronic variant between exon 1 and exon 2 of the *AR* canonical transcript which was predicted to cause an alternatively spliced AR transcript and presumably resulting in nonsense-mediated decay.

Management

The management of disorders (differences) of sex development (DSD) requires a multidisciplinary team of specialists comprised of an endocrinologist, pediatric urologist (surgeon), gynecologist, geneticist, and a psychologist who preferentially should act as an early contact with families (Brain *et al.*, 2010). PAIS is a common cause of XY DSD and poses multiple challenges in management, not least in reaching agreement on the appropriate sex assignment after birth. There will be multiple facts for the families to assimilate and subsequent steps to take; establishing a diagnosis or not/accepting diagnosis, reaching a decision on sex assignment,

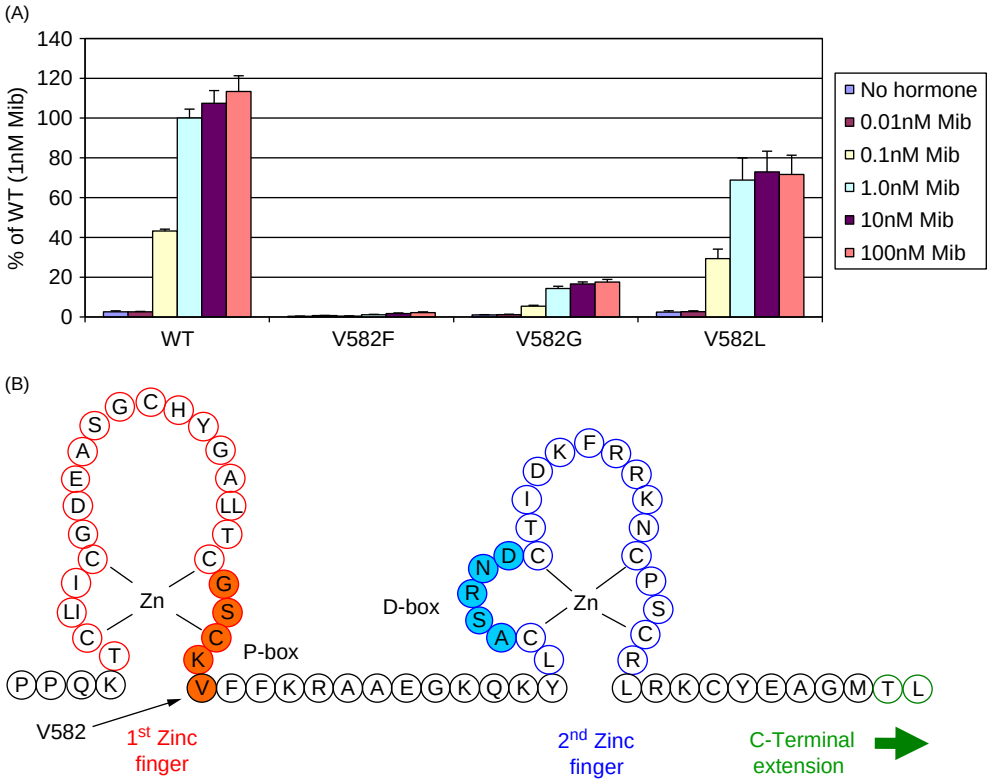

Fig. 1 (A) The effects of substitution of a valine (V) at codon 582 in the DNA binding domain with phenylalanine (F), glycine (G), and leucine (L) are shown in a transactivation assay with the synthetic androgen mibolerone. Responsiveness of the reporter gene is related to a complete or partial androgen insensitivity syndrome phenotype. (B) The linear stretch of amino acids comprising the DNA-binding domain is also shown. Mib = mibolerone. CAIS = complete androgen insensitivity syndrome. PAIS = partial androgen insensitivity syndrome. From Hughes, I.A., Davies, J. D., Bunch, T.I., Pasterski, V., Mastroyannopoulou, K. and MacDougall, J. (2012). Androgen insensitivity syndrome. *Lancet* 380, 1419–1428.

planning as a consequence any surgical treatment, hormone replacement therapy, age-appropriate disclosure and transitioning to young adult care. Generally in DSD, sex assignment is an amalgam complexed with the karyotype, development of the external and internal genitalia, the nature and function of the gonads, coupled with the overarching views of the family and their cultural background. The response of the external genitalia to a short course of androgens (given systemically or topically) may be taken in to consideration (Hughes *et al.*, 2012). In the case of PAIS, intuitively it would be expected that resistance to androgen would, a priori, bias decisions towards female sex assignment. However, the majority are now raised as male (Hughes *et al.*, 2012; Kolesinska *et al.*, 2014). The external masculinisation score (EMS) is a validated tool to evaluate the degree of under-masculinisation, the normal male having a composite score of 12 and a normal female newborn a score of 1 (Ahmed *et al.*, 2000). A study using the international DSD database (I-DSD Registry https://www.i-dsd.org/) reported that the EMS of PAIS individuals raised male was significantly higher than those who were raised as female (Kolesinska *et al.*, 2014).

Individuals with PAIS raised as male are likely to require two or more surgical procedures to repair hypospadias in the early years of childhood and occasionally, several more in later life. Orchidopexy is also often needed for testis maldescent. Pubertal development can be spontaneous but the use of exogenous androgens is common practice to complete full puberty. In a pubertal outcome study, intramuscular or topical androgen treatment was required in about half the patients with PAIS (Lucas-Herald *et al.*, 2016). The requirement for androgen supplementation was less frequent in patients with PAIS-like phenotype but in whom an *AR* mutation was not found. Gynecomastia is a frequent problem in PAIS males occurring almost universally in the presence of an *AR* mutation. The mechanism is excess conversion to estrogens from increased androgen levels (Hellmann *et al.*, 2012). Treatment with tamoxifen, an estrogen receptor blocker, was effective in suppressing gynecomastia in two siblings with PAIS (Saito *et al.*, 2014). Aromatase enzyme inhibitors may be another option to try before deciding on a surgical solution, though there is not enough evidence that it is effective in individuals with DSD. Invariably, reduction mammoplasty is often needed to solve the problem.

Infants with PAIS raised as female may require in due course feminizing genital reconstruction (clitoroplasty, labioplasty, vaginoplasty). The timing and extent of such surgery in all categories of DSD is the subject of considerable debate amongst health care professionals and patient advocacy groups. Early genitoplasty has been common practice based on the premise that this enabled the family to accept the child's assigned gender more readily and would improve subsequent psychological outcome. (Guarino *et al.*, 2013). Now, delaying decisions about surgery should be considered to enable more involvement on the part of the

patient in decision making, particularly when irreversible surgery such as gonadectomy takes place. Indeed, a moratorium on any surgery taking place before the age of consent is advocated by some (Diamond and Beh, 2008). Early vaginoplasty may result in high rates of introital stenosis and a requirement for repeat reconstructive surgery in adolescence before tampon use or sexual intercourse (Guarino *et al.*, 2013). Individuals raised female should preferably have gonadectomy before puberty in order to avoid further virilization. Thereafter, estrogen replacement therapy is required to induce breast development and a regimen continued as would be the case in females with CAIS who have the testes removed.

Long Term Outcome

Only limited information is available about long-term outcomes in PAIS. In one outcome study of 15 males with PAIS (age range 16–38 years), penile length ranged from 3 to 5 cm (average 4.6 cm) and severe gynecomastia was present in 87% (Bouvattier *et al.*, 2006). Scores for sexual function were significantly reduced in all areas analyzed, including noncommunication, nonsensuality, infrequency, avoidance, impotence and dissatisfaction with sexual performance. Outcome is worse in PAIS with a confirmed *AR* mutation compared with other forms of XY DSD where androgen production is also normal and there is no *AR* mutation (Lucas-Herald *et al.*, 2016). The same study reported a difference in prevalence of micropenis, undescended testis, hypospadias and the need for reconstructive surgery, despite both groups having a similar EMS. The difference was attributable to the degree of androgen resistance in target tissues of males with an *AR* mutation. Gynecomastia is universal in PAIS.

Height is increased in PAIS, although the series of 14 males in this study also included those with MAIS (Hellmann *et al.*, 2012). Four men with MAIS were fertile. The risk for gonadal malignancy in CAIS has been analyzed in large numbers and is low, particularly before puberty. In contrast, information about this risk is sparse in PAIS despite those raised male retaining their testes in to adulthood. However, recent analysis of relatively small numbers of AIS patients indicated that the risk of germ cell neoplasia in situ (GCNIS), a pre-malignant stage of gonadal tumor, was no greater in PAIS than in CAIS (Cools and Looijenga, 2017). It must be emphasized that longer term outcome studies incorporating older adult males with PAIS are needed to determine the effects of partial androgen resistance not only on sexual function and quality of life, but morbidity associated with cardiovascular and bone disease.

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Anti-Müllerian Hormone Deficiency and Resistance

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Nomenclature

ALK	Activin-like kinase
AMH	Anti-Müllerian hormone
AMHR2	AMH receptor type 2
AP2	Activating enhancer-binding protein 2
BMP	Bone morphogenetic protein
DSD	Disorders of sex development
FSH	Follicle-stimulating hormone
GATA4	GATA-binding, protein 4
GNAS1	Guanine nucleotide-binding protein, alpha-stimulating activity, polypeptide 1
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
LH	Luteinizing hormone
LH–CG-R	Luteinizing hormone/chorionic gonadotropin receptor
NFκB	Nuclear factor kappa-B
PMDS	Persistent Müllerian duct syndrome
PCSK5	Proprotein convertase, subtilisin/kexin-type, 5
PRR13	Proline-rich, protein 13
SP1	Specificity protein 1
SOX8	SRY-related HMG-box, gene 8
SOX9	SRY-related HMG-box, gene 9
SF1	Steroidogenic factor 1
TGFβ	Transforming growth factor beta
WT1	Wilms tumor, gene 1

Glossary

Disorders of sex development (DSD) Congenital conditions in which the development of chromosomal, gonadal or genital sex is atypical.

Müllerian ducts Ducts existing in both the XY and the XX embryo in the early stages of development, regressing due to AMH action in the male fetus, and giving rise to the Fallopian tubes, the uterus and the upper part of the vagina in the female due to the lack of AMH at that stage.

Precocious puberty Establishment of pubertal clinical features (in the male, testicular volume attaining 4 mL) before the age of 9 years.

Primary hypogonadism In the male, insufficiency of testicular function, due to a disorder affecting primarily the testis. In adult endocrinology, it is usually called hypergonadotropic hypogonadism.

Secondary or central hypogonadism In the male, insufficiency of testicular function, due to a disorder affecting primarily the pituitary or the hypothalamus. In adult endocrinology, it is usually called hypogonadotropic hypogonadism.

Testotoxicosis or gonadotropin-independent precocious puberty Early development of pubertal features in the testis, owing to an activating mutation of the LH receptor leading to a constitutively high testosterone production by Leydig cells in the absence of pituitary LH elevation.

Introduction

Anti-Müllerian hormone (AMH), also called Müllerian inhibiting substance (MIS), is a protein first identified by its ability to cause regression of the Müllerian ducts in the male fetus. In the female fetus, the Müllerian ducts develop into the uterus, vagina, and fallopian tubes. The classical experiments of Alfred Jost demonstrated that testosterone alone is not sufficient to bring about normal male development, and that a separate factor is necessary for Müllerian duct regression (Jost, 1953). That factor, AMH, was eventually purified from bovine testis through its ability to cause regression of rat Müllerian ducts in vitro (Picard and Josso, 1984), allowing the isolation of the bovine and human AMH genes (Cate *et al.*, 1986; Picard *et al.*, 1986). The genes in turn provided the primary amino acid sequence for AMH, showing that it was a member of the transforming growth factor (TGF)-β family of growth and differentiation factors (Cate *et al.*, 1986; Pepinsky *et al.*, 1988). Subsequently, the identification of the specific receptor for AMH, AMHR2 (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b), and genetic perturbation experiments of AMH and AMHR2 expression in mice (Behringer *et al.*, 1990; Mishina *et al.*, 1996) led to elucidation of the AMH signaling pathway and the discovery of new biological roles for AMH in reproductive development.

AMH Expression and Regulation

AMH is expressed essentially by Sertoli cells of the testes and granulosa cells of the ovary, as indicated by the observation of undetectable serum AMH in gonadectomized (Grinspon *et al.*, 2012; Lee *et al.*, 1997; Long *et al.*, 2000) or congenitally agonadal patients (Grinspon *et al.*, 2012; Hagen *et al.*, 2010; Lee *et al.*, 1997). However, the ontogeny and levels of expression of AMH differ considerably between male and female gonads. In the testis, AMH expression arises as soon as Sertoli cells differentiate in the 8th week of fetal life (Josso *et al.*, 1993) and persists at high levels until the onset of puberty, when it decreases progressively to adult levels (Rey *et al.*, 1996) (**Fig. 1**). In the ovary, AMH expression begins with the appearance of primary follicles, at approximately 23 weeks of fetal life (Kuiri-Hanninen *et al.*, 2011); secondary and small antral follicles (<9 mm) express the highest levels of AMH, whereas granulosa cells of large antral follicles show a heterogeneous pattern of expression (Rey *et al.*, 2000).

The onset of AMH expression in Sertoli cells is driven by SOX9, independently of gonadotropin stimulation, and further upregulated by SF1, GATA4, and WT1 (reviewed in Edelsztein *et al.*, 2016). Later on, FSH increases testicular AMH output by stimulating Sertoli cell proliferation and upregulating AMH expression, in response to increased expression of trans-activating

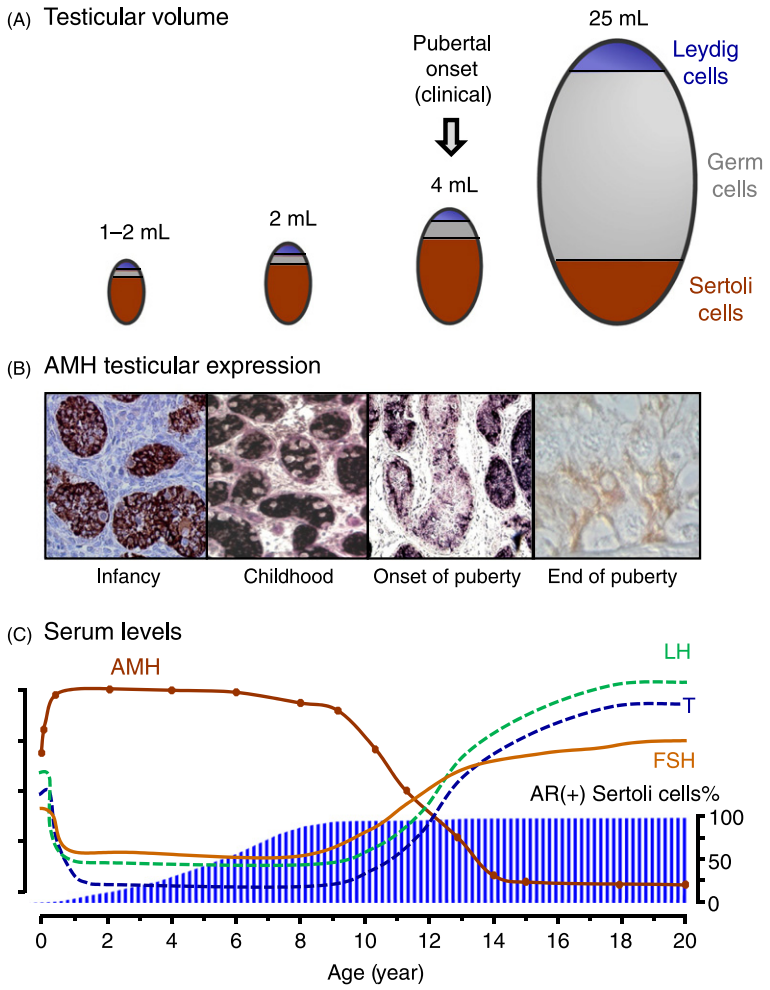

Fig. 1 Ontogeny of AMH expression in postnatal life in relationship with testicular volume and other reproductive hormones. (A) Testicular volume, as measured by Prader's orchidometer shows almost no variation during infancy and childhood; most of testicular tissue is represented by Sertoli cells. Clinically, pubertal onset is defined by testicular volume attaining 4 mL. During pubertal development, testicular volume increases due to the establishment of pubertal and adult spermatogenesis, and germ cell number determines testicular size. (B) AMH expression as revealed by immunohistochemistry. In infancy and childhood, all Sertoli cells express high levels of AMH. At the onset of puberty, AMH expression declines progressively in seminiferous tubules showing pubertal spermatogenesis. By the end of puberty, AMH expression is very low in all Sertoli cells. (C) Schematic serum levels of AMH, testosterone (T) and gonadotropins (FSH and LH) from birth through adulthood (*left axis*) and percentage of Sertoli cells expressing the androgen receptor (AR, *right axis*). Reprinted with permission from: Rey, R.A., et al. (2009). Ontogeny of the androgen receptor expression in the fetal and postnatal testis: its relevance on Sertoli cell maturation and the onset of adult spermatogenesis. *Microscopy Research and Technique* **72**, 787–795, © 2009 Wiley-Liss Inc., and Edelsztein, N.Y., *et al.* (2016). Anti-Müllerian hormone as a marker of steroid and gonadotropin action in the testis of children and adolescents with disorders of the gonadal axis. *International Journal of Pediatric Endocrinology* **2016**, 20, © 2016 The Authors.

factors SOX9, SF1, AP2, and NFκB (Lasala *et al.*, 2011; Lukas-Croisier *et al.*, 2003). At puberty, testosterone concentration increases within the testis and provokes a dramatic decrease in AMH expression, a typical feature of Sertoli cell maturation (Al-Attar *et al.*, 1997; Rey *et al.*, 1993) concurrent with the androgen-induced onset of pubertal spermatogenesis (reviewed in Rey, 2014) (**Fig. 1**). Interestingly, AMH is not downregulated by androgens in the fetal and neonatal testis because Sertoli cells do not express the androgen receptor before the first year of postnatal life (Chemes *et al.*, 2008) (**Fig. 1**). In the ovary, SOX9 is not expressed, and SOX8 secreted by the oocyte functions as the initial activating factor for AMH (Salmon *et al.*, 2005). Gonadotropins and steroids also regulate AMH in the ovary: FSH stimulates AMH transcription (Taieb *et al.*, 2011) and estradiol has differential effects according to which estrogen receptor is involved (Grynberg *et al.*, 2012).

Molecular Biology of AMH and AMHR2

The human *AMH* gene contains five exons, maps to chromosome 19 p13.3 (Cohen-Haguenauer *et al.*, 1987), and is situated between the genes for splicing factor 3a and junctional sarcoplasmic reticulum protein 1, which are <400 bp away. It encodes for a protein of 560 amino acids containing a 24 amino acid signal sequence. Analyses of the bovine testicular and human recombinant proteins have shown that AMH is synthesized as a homodimeric precursor, containing an N-terminal pro-region and a smaller C-terminal mature domain, which shares homology with members of the TGFβ family (Pepinsky *et al.*, 1988) and has binding sites for AMHR2. A molecular model of the C-terminal domain (**Fig. 2**) reveals a cysteine knot motif that is characteristic of all TGFβ family members. The precursor undergoes a cleavage at monobasic sites between the two domains that is required for binding to AMHR2, but the pro-region and C-terminal homodimers remain associated in a noncovalent complex (Pepinsky *et al.*, 1988). Although only $\sim$5% of secreted AMH is cleaved at these sites, proteolytic processing can be driven to completion in vitro using plasmin (Pepinsky *et al.*, 1988). Nachtigal and Ingraham (1996) have proposed that kex2/subtilisin-like endoprotease PCSK5 may be responsible for cleavage in vivo. While cleavage is necessary for AMH binding to AMHR2, only a fraction of the cleaved endogenous AMH measured in body fluids is competent for binding AMHR2: 56% in follicular fluid, 11% in male serum, and 0% in female serum (Pierre *et al.*, 2016). Thus, levels of cleaved AMH in body fluids do not necessarily inform about the levels of bioactive AMH and suggest that, after cleavage, AMH can undergo further structural changes or interactions that prevent binding to AMHR2.

The *AMHR2* gene contains 11 exons, maps to chromosome 12q13 (Imbeaud *et al.*, 1995a), and is situated between two transcription regulators, SP1 and PRR13. It encodes for a 573 amino acid membrane protein containing a signal sequence, an N-terminal extracellular domain that binds AMH, a single transmembrane domain, and an intracellular domain with serine/threonine kinase activity. The signal sequence has been shown to be defective, indicating that AMHR2 uses its transmembrane domain for insertion and orientation in the membrane, a characteristic of type III membrane proteins (Belville *et al.*, 2009). A molecular model of the extracellular domain (**Fig. 3**A) reveals a three-finger toxin fold and four disulfide bridges found in other TGFβ type II receptors, while a model of the intracellular domain (**Fig. 3**B) reveals the general fold of a two-domain kinase, with an N-lobe consisting mainly of a five stranded B-sheet and a C-lobe that is mainly α-helical. Analyses of AMHR2 biosynthesis have

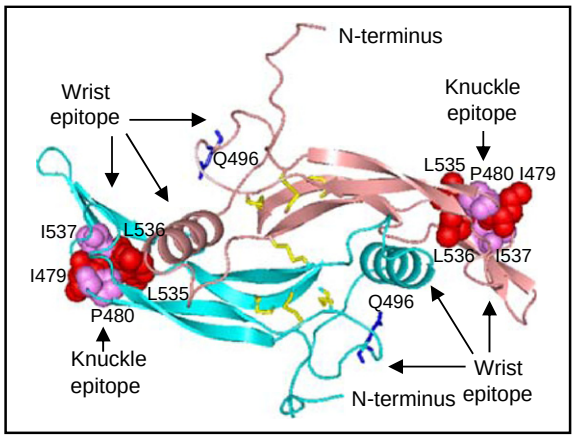

Fig. 2 Molecular model of C-terminal AMH. A three-dimensional model of the C-terminal dimer was generated by comparative modeling using human BMP9 (Brown *et al.*, 2005) as a template. The wrist epitope, the putative binding site for the type I receptor, is composed of the prehelix loop and α-helix of one monomer together with the concave side of the fingers of the second monomer (Sebald *et al.*, 2004). A mutation in the prehelix loop of AMH, Q496H, causes persistent Müllerian duct syndrome (Belville *et al.*, 2004). Residues in the knuckle epitope of AMH, the putative binding site for AMHR2, are similar to those present in BMP7 and activin at the interface with ActR2B (Greenwald *et al.*, 2003, Thompson *et al.*, 2003). Disulfide bonds (*yellow*) and Q496 residues (*blue*) are shown as sticks; residues in the knuckle epitopes are shown as spheres. Modified, with permission from Belville, C., *et al.* (2004). Mutations of the anti-Müllerian hormone gene in patients with persistent Müllerian duct syndrome: biosynthesis, secretion, and processing of the abnormal proteins and analysis using a three-dimensional model. *Molecular Endocrinology* 18, 708–721. © 2004 The Endocrine Society.

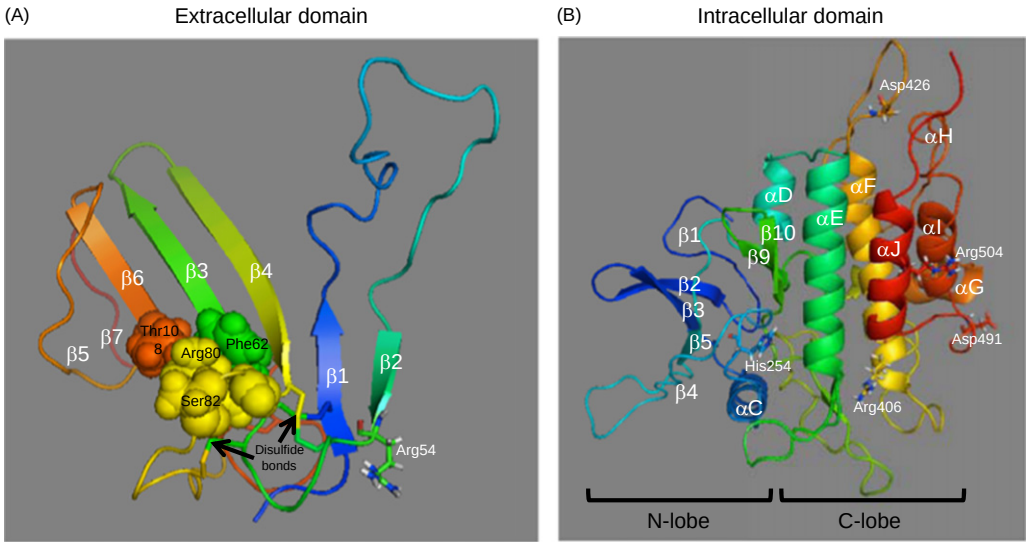

Fig. 3 Molecular models of AMHR2 extracellular and intracellular domains. (A) The extracellular domain exhibits the general three-finger toxin fold of type II receptors and displays five disulfide bridges, four of which are conserved in the other three receptors. Based on how the activin type II receptor interacts with BMP2, AMH may bind to an interface on the extracellular domain composed of residues Phe62, Arg80, Ser82, and Thr108, which are shown as spheres. (B) The intracellular domain exhibits the general fold of a two-domain kinase, with an N-lobe consisting mainly of a five-stranded β-sheet and a C-lobe, which is mainly α-helical. Residues affected by PMDS mutations (Arg54, His254, Arg406, Asp426, Asp491, and Arg504) are shown as sticks. Mutations of Arg54, Arg406, Asp426, Asp491, and Arg504 are described in Belville et al. (2009); the His254 mutation in Abduljabbar et al. (2012). Reprinted with permission from: Josso, N. *et al.* (2013). The persistent Müllerian duct syndrome. In: New, M.I., Parsa, A., Yuen, T.T., O'Malley, B.W., Hammer, G.D., eds. *Genetic steroid disorders*. New York, NY: © Elsevier 2013.

identified a number of mechanisms implicated in its constitutive negative regulation, including cleavage of the extracellular domain and promiscuous disulfide bond mediated homo-oligomerization, both of which lead to intracellular retention (Hirschhorn *et al.*, 2015).

The AMH Signaling Pathway

Like other members of the TGFβ family, AMH signals by assembling a transmembrane serine/threonine kinase receptor complex of type I and type II components, resulting in the phosphorylation and activation of type I receptor kinase by the constitutively active kinase domain of the type II receptor. The activated type I receptor then phosphorylates the cytoplasmic Smad proteins 1, 5, or 8, which interact with Smad 4 and migrate into the nucleus, where, in concert with other transcription factors, they modulate the transcription of target genes (Gouédard *et al.*, 2000; Massagué *et al.*, 2005). AMHR2, the type II receptor, and AMH, are mutually specific, while ALKs 2, 3, and 6 serve as type I receptors for both AMH and members of the bone morphogenetic protein (BMP) family (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b). A model is shown in **Fig. 4**.

Unlike other TGFβ ligands, where the noncovalent complexes are latent and cannot bind their type II receptors, the AMH noncovalent complex can bind to AMHR2, which induces dissociation of the pro-region (di Clemente *et al.*, 2010). A similar mechanism has been proposed for the BMP7 noncovalent complex (Sengle *et al.*, 2008). The X-ray structures for two TGFβ ligand complexes have provided insights into how a latent complex (TGFβ1) differs from a non-latent complex (BMP9). The latent TGFβ complex is in a cross-armed conformation in which the growth factor (i.e., C-terminal domain) conformation is "tensed" (Shi *et al.*, 2011), while the non-latent BMP9 complex is in an open-armed conformation in which the growth factor conformation is "relaxed" (Mi *et al.*, 2015). This suggests that the AMH noncovalent complex may be in an open-armed conformation.

Biological Roles of AMH

In causing regression of the Müllerian duct in the male fetus, AMH acts via a paracrine mechanism, since it is the surrounding mesenchymal cells that express AMHR2. Apoptosis and epithelial to mesenchymal transformation (Allard *et al.*, 2000), epithelial cell migration (Austin, 1995), and proteolysis (Roberts *et al.*, 2002) have important roles in regression. Perturbation of AMH expression in mice revealed a role for AMH in the adult male. Strong overexpression of AMH in transgenic mice led to incomplete fetal virilization and decreased serum testosterone in the adult (Behringer *et al.*, 1990), whereas AMH-deficient mice exhibited Leydig cell hyperplasia (Mishina *et al.*, 1996). These effects are due to AMH blocking the differentiation of Leydig cell precursors and decreasing the expression of steroidogenic enzymes (Lee *et al.*, 1999; Racine *et al.*, 1998).

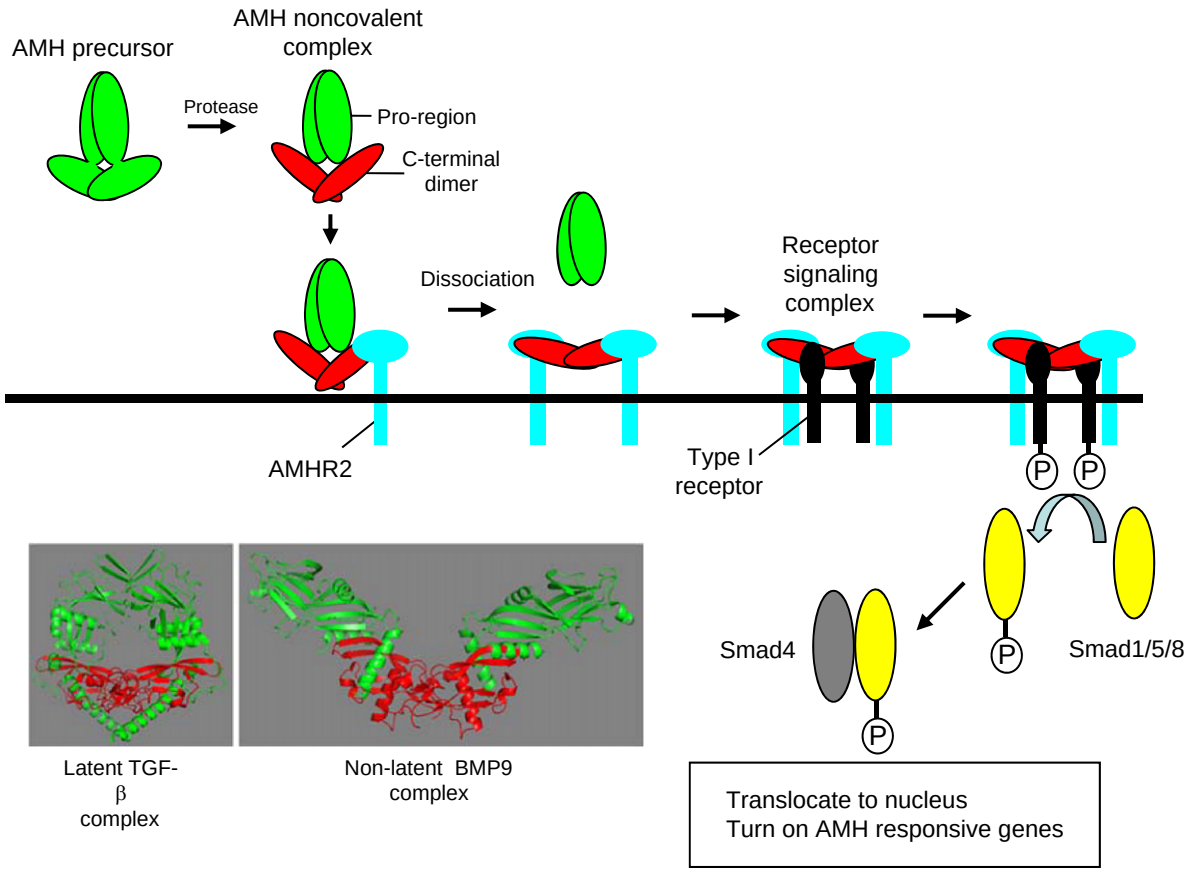

Fig. 4 Model showing processing of AMH and assembly of the AMH receptor signaling complex. The AMH precursor must be cleaved to bind AMHR2. After binding, the proregion domain dissociates from the mature domain. The inset shows structures for the latent TGFβ noncovalent complex and the non-latent BMP9 noncovalent complex. Pro regions are shown in green and mature C-terminal domains are shown in *red*. Modified with permission from: Pierre, A., *et al.* (2016). Most cleaved anti-Müllerian hormone binds its receptor in human follicular fluid but little is competent in serum. *The Journal of Clinical Endocrinology and Metabolism* **101**, 4618–4627 © 2016 The Endocrine Society.

In females, the role of AMH has been predominantly elucidated in rodents, where it has an inhibitory effect on primordial follicle recruitment and on the responsiveness of growing follicles to FSH (Durlinger *et al.*, 1999, 2001). In particular, AMH has been shown to repress aromatase expression in immature rat and porcine granulosa cells (di Clemente *et al.*, 1994a) and human granulosa-lutein cells (Pellatt *et al.*, 2011). Thus, AMH may act as a follicular gatekeeper, ensuring that small antral follicles produce little estrogen prior to selection (Sacchi *et al.*, 2016).

An intriguing activity of AMH has been its ability to induce gonadal sex reversal in female fetuses such as the bovine freemartin, where the female fetus is exposed to the AMH produced by her male twin (Vigier *et al.*, 1984) and in female mice overexpressing human AMH (Behringer *et al.*, 1990). This effect can be reproduced in vitro with rat fetal ovaries and purified AMH (Vigier *et al.*, 1989). However, because male AMH-deficient mice exhibit normal testicular development, it can be concluded that AMH does not play an obligatory role in mammalian testis determination. In the last few years, the role of AMH has been investigated in a number of species of fish and eels. Although these species do not have Müllerian ducts, AMH is expressed—some species carry a duplicated copy of the AMH gene (Hattori *et al.*, 2012; Rondeau *et al.*, 2016)—in the Sertoli cells, where it regulates germ cell proliferation (Miura *et al.*, 2002; Morinaga *et al.*, 2007), and also acts as a testis-determining factor (Kamiya *et al.*, 2012). Thus, while mammalian AMH can still modulate gonadal differentiation when it is expressed inappropriately, it no longer performs this function during normal development.

A few endocrine roles have been proposed in the nervous and cardiovascular systems (reviewed in McLennan and Pankhurst, 2015). Adult motor neurons express AMH and its receptors, suggesting that it may act as a survival factor (Wang *et al.*, 2005). It has been proposed that AMH is responsible for the male bias in the size of the cerebellum with males having more Purkinje cells than females (Wittmann and McLennan, 2011). This is supported by the fact that AMH deficient male mice have a cerebellum size and Purkinje cell number similar to those observed in females. Identification of AMH receptivity in both pituitary and GnRH neurons has also led to the intriguing idea that AMH participates in the hypothalamic–pituitary control of reproduction. Indeed, combining in vivo and in vitro experiments, AMH was shown to increase GnRH neuron firing and GnRH-dependent LH pulsatility in the adult (Cimino *et al.*, 2016), and to specifically regulate FSH and not LH in females before puberty (Garrel *et al.*, 2016).

AMH is a Marker of Gonadal Function in Boys

In the last 15 years, the use of AMH as a marker has gained success in the field of reproductive endocrinology, as a marker of the ovarian follicle reserve in adult women with infertility (reviewed in Dewailly *et al.*, 2014). However, assays for AMH measurement were initially developed to assess testicular function in males during infancy and childhood (Baker *et al.*, 1990, Hudson *et al.*, 1990, Josso *et al.*, 1990), a period where basal gonadotropin and androgen levels are little informative (reviewed in Valeri *et al.*, 2013).

Anorchidism and Monorchidism

In boys with nonpalpable gonads, serum AMH determination without the need for a stimulation test is useful to distinguish between anorchidism and bilateral cryptorchidism with testes in abdominal position (**Fig. 5**). Indeed, undetectable AMH has a better predictive value for the diagnosis of anorchidism than low testosterone post-hCG, and detectable AMH is more informative than the increase of testosterone after an hCG test in boys with intra-abdominal testes (Lee *et al.*, 1997).

In boys with monorchidism, while Leydig cell function is largely compensated, lower AMH indicates that Sertoli cell proliferation and function is insufficient to compensate for the function of the absent testis (Grinspon *et al.*, 2016a).

Hypogonadism

In the prepubertal male, Sertoli cell hormones are the best biomarkers to diagnose an impaired testicular function, that is, hypogonadism (Rey *et al.*, 2013). Because very low or undetectable levels of gonadotropins and testosterone are usually found in

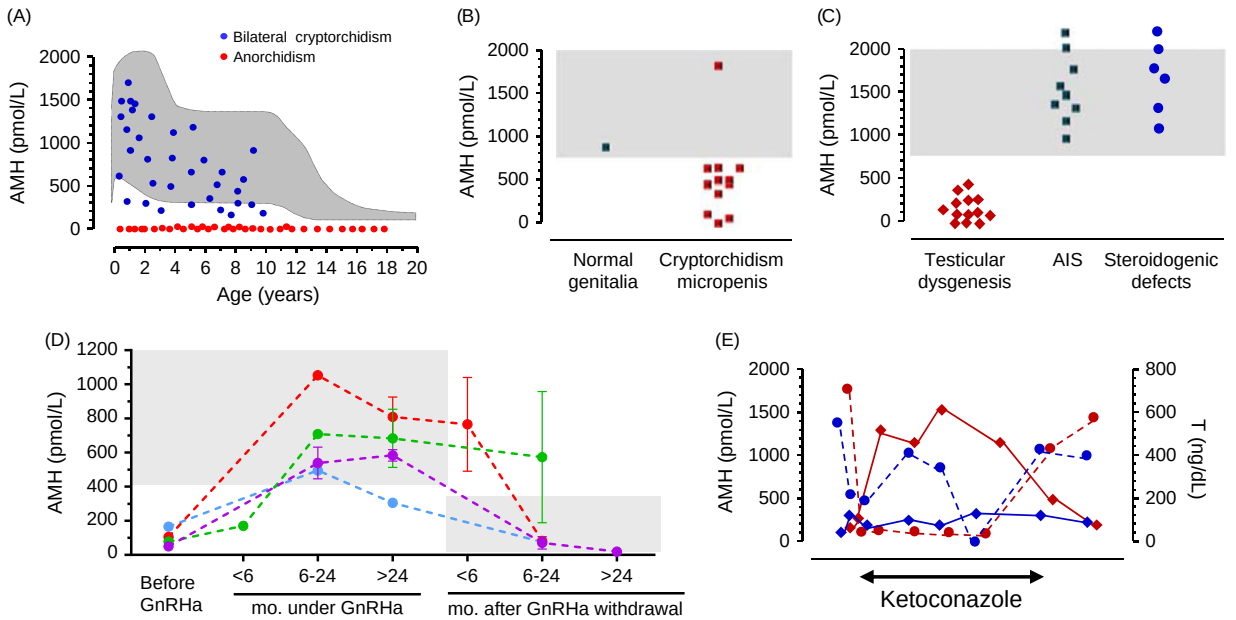

Fig. 5 Utility of serum AMH in boys with disorders of the gonadal axis. (A) AMH is undetectable in boys with anorchidism, but clearly detectable in boys with bilateral cryptorchidism (data from Grinspon, R. P., Ropelato, M. G., Bedecarrás, P., et al. (2012). Gonadotrophin secretion pattern in anorchid boys from birth to pubertal age: Pathophysiological aspects and diagnostic usefulness. *Clinical Endocrinology*, **76**, 698–705). (B) AMH is low in most newborns with multiple pituitary hormone deficiency and micropenis/cryptorchidism reflecting hypogonadotropic hypogonadism, but normal in the newborn with normal genitalia (data from Braslavsky, D., Grinspon, R. P., Ballerini, M. G., *et al.* (2015). Hypogonadotropic hypogonadism in infants with congenital hypopituitarism: A challenge to diagnose at an early stage. *Hormone Research in Paediatrics*, **84**, 289–297). (C) AMH is low in patients with DSD due to gonadal dysgenesis but normal/high in those with androgen synthesis defects or androgen insensitivity syndrome (AIS, data from (Rey, R. A., Belville, C., Nihoul-Fékété, C., *et al.* (1999). Evaluation of gonadal function in 107 intersex patients by means of serum antimüllerian hormone measurement. *Journal of Clinical Endocrinology and Metabolism*, **84**, 627–631). (D) In boys with central precocious puberty, serum AMH is low at the time of diagnosis reflecting increased intratesticular testosterone. Efficacious treatment with GnRH analogues curtails testosterone production and results in AMH elevation to normal prepubertal levels. When treatment is discontinued, testosterone increases and AMH declines again (data from Grinspon, R. P., Andreone, L., Bedecarrás, P., *et al.* (2013). Male central precocious puberty: Serum profile of anti-mullerian hormone and inhibin B before, during, and after treatment with GnRH Analogue. *International Journal of Endocrinology*, **2013**, 823064). (E) In boys with testostoxicosis, serum AMH is useful to monitor ketoconazole treatment adherence: in one patient (*red lines*), testosterone production (*circles*) was inhibited and AMH (*diamonds*) increased to prepubertal levels, whereas in the other case (*blue lines*) adherence to treatment was discontinuous, and AMH never increased to prepubertal levels (data from Rey, R., Lordereau-Richard, I., Carel, J. C., *et al.* (1993). Anti-müllerian hormone and testosterone serum levels are inversely related during normal and precocious pubertal development. *Journal of Clinical Endocrinology and Metabolism*, **77**, 1220–1226).

the normal boy, their determination is not useful for the diagnosis of secondary (central or "hypogonadotropic") hypogonadism. Also, since gonadotropins are within the normal range in approximately 30%–60% of anorchid boys (Grinspon *et al.*, 2012), their elevation cannot always be used as a marker of primary (or "hypergonadotropic") hypogonadism in pediatric patients. In contrast, low serum AMH allows a diagnosis of impaired testicular function, both in the case of primary hypogonadism, such as testicular regression (Imbeaud *et al.*, 1995b) and Klinefelter (Bastida *et al.*, 2007) syndromes or after chemotherapy (Cuny *et al.*, 2011; Laporte *et al.*, 2011), as well as of secondary hypogonadism due to hypothalamic or pituitary dysfunction (Bougnères *et al.*, 2008; Braslavsky *et al.*, 2015) (**Fig. 5**). In boys with absence of pubertal development by the age of 14 years, low AMH is found in patients with secondary (or "hypogonadotropic") hypogonadism (Young *et al.*, 1999) whereas normal serum AMH is suggestive of a constitutional delay of puberty (Coutant *et al.*, 2010).

Precocious Puberty

As previously described, the increase in testosterone concentration normally occurring within the testis during the early stages of pubertal development induces Sertoli cell maturation and a downregulation of AMH expression. A decrease in serum AMH is also observed in boys with precocious puberty due either to an early activation of hypothalamic GnRH secretion resulting in central precocious puberty (**Fig. 5**) or to the existence of an hCG-secreting tumor. Likewise, serum AMH is below the prepubertal range in boys with precocious development due to androgen-secreting Leydig cell tumors or to activating mutations of the LH–CG receptor responsible for gonadotropin-independent precocious puberty, also known as testotoxicosis (**Fig. 5**). In these patients, serum AMH monitoring is useful to assess the efficacy of and compliance to treatment (Rey *et al.*, 1993). Conversely, AMH does not decrease in precociously virilized patients due to an extra-testicular origin of androgens, like congenital adrenal hyperplasia, adrenal tumors or exogenous testosterone administration. Interestingly, AMH also remains in the normal prepubertal range in boys with central precocious puberty or with testotoxicosis before the age of 1 year, even if intratesticular testosterone levels are elevated (Grinspon *et al.*, 2013). This is probably explained by the lack of expression of the androgen receptor in Sertoli cells in the first year of life (Chemes *et al.*, 2008).

Macro-Orchidism

Because testes attaining a volume of 4 mL are the main clinical hallmark of the initiation of puberty in males, the diagnosis of precocious puberty should always be ruled out in boys with enlarged testes. Stable prepubertal AMH levels rule out precocious pubertal development in monorchid boys of prepubertal age with compensatory macro-orchidism of the remaining testis (as described above) or in patients with syndromic prepubertal macro-orchidism, like X fragile syndrome. Other rare conditions in which the increase in testis volume justifies ruling out precocious pubertal development are Peutz–Jeghers syndrome and particular forms of McCune–Albright syndrome mosaicism characterized by a Sertoli cell-limited existence of the somatic activating mutation in the *GNAS1* gene-encoding for the Gsα protein involved in the FSH receptor transduction pathway- in the testis. In both cases, AMH levels remain high indicating that the increase in testicular volume does not reflect an increase of intratesticular testosterone and the onset of pubertal spermatogenesis (Rey *et al.*, 2006; Venara *et al.*, 2001).

Disorders of Sex Development (DSD)

In patients with ambiguous or female external genitalia who carry a Y chromosome, low or undetectable serum AMH levels are indicative of gonadal dysgenesis, whereas normal or elevated AMH indicates normal Sertoli cell function and tips the diagnosis towards an isolated Leydig cell dysfunction (**Fig. 5**), like Leydig cell aplasia/hypoplasia or steroidogenic enzyme defects, or to androgen insensitivity (reviewed in Josso *et al.*, 2012). AMH is also within the normal range in newborns with non-endocrine malformative syndromes affecting genital development, for example, isolated hypospadias (Rey *et al.*, 2005) or bladder exstrophy (Grinspon and Rey, 2014), indicating that testicular function is preserved.

Conversely, in virilized XX newborns the finding of serum AMH above the female range is suggestive of the existence of testicular tissue, thus making a differential diagnosis between patients with aromatase deficiency who lack testicular tissue and those with ovotesticular or testicular DSD in whom the circulating levels of AMH are usually commensurate with the amount of testicular tissue present (Grinspon *et al.*, 2016b; Rey and Grinspon, 2011).

A particular form of DSD, due to deficiency of AMH production or resistance to AMH resulting in the persistence of Müllerian ducts in boys, is dealt with in detail below.

The Persistent Müllerian Duct Syndrome (PMDS)

Defects in the synthesis or action of AMH impair the regression of male fetal Müllerian ducts, giving rise to the Persistent Müllerian Duct Syndrome (PMDS), characterized by the persistence of uterus and tubes in otherwise normal 46,XY males. In approximately 90% of cases, the syndrome is due to mutations in the genes coding for AMH or its type II receptor, AMHR2. It is considered a rare condition but its reported incidence is increasing, perhaps due to a rise in awareness of medical providers. PMDS is usually discovered at surgery or laparoscopy motivated by uni or bilateral cryptorchidism with or without inguinal hernia. It is an

inherited disease, transmitted as an autosomal recessive trait, in accordance with the autosomal location of the *AMH* (Cohen-Haguenauer *et al.*, 1987) and *AMHR2* (Imbeaud *et al.*, 1995a) genes. The rate of consanguinity is high, 40% and 33% in *AMH* and A*MHR2* mutations respectively.

Anatomical Features

There is no significant difference in the anatomy of patients with either *AMH* or *AMHR2* mutations. Testes are tightly tethered to the Fallopian tubes; their position depends upon the degree of mobility of the Müllerian derivatives (**Fig. 6**). In 50% of cases, the uterus and tubes are in the pelvis, firmly maintained by the broad ligament. Both testes are in an ovarian position and cannot be palpated. Alternatively, part of the uterus can be dragged into the inguinal canal by a descending testis. This results in an inguinal hernia containing the uterus and the ipsilateral Fallopian tube and testis. The contralateral testis either remains in an ovarian position or follows the uterus into the inguinal hernia, resulting in transverse testicular ectopia. This otherwise rare condition is present in approximately 25% cases of PMDS. Abnormalities of the male excretory ducts are common.

Complications

Adult patients are often diagnosed because of malignant degeneration of testes or Müllerian derivatives. The risk of testicular cancer in PMDS has recently been estimated at 33% (Picard *et al.*, 2017), higher than is expected for neglected cryptorchidism. Seminomas are the most frequent tumors but many other histological types have been described. Malignant degeneration of Müllerian derivatives is possible but rarer (Farikullah *et al.*, 2012).

Infertility is the most frequent complication of PMDS; only 10%–20% of patients have fathered children. In these cases, at least one testis was in a scrotal position and excretory ducts were intact. As discussed below, the vas is embedded in the uterine wall and easily damaged if hysterectomy is attempted. Furthermore, orchidopexy of abdominal testes is technically difficult and may lead to testicular demise. Not surprisingly, PMDS patients had not been diagnosed l*et a*lone operated at the time their children were conceived.

Endocrine Investigations

AMH serum levels differ widely according to genotype. Very low serum AMH concentration is characteristic of AMH mutations. However low AMH concentration in newborns or in boys undergoing puberty is physiological (Grinspon *et al.*, 2011), and should not be interpreted as a sign of AMH mutation. In insufficiently virilized patients with retained Müllerian ducts, low AMH values reflect testicular dysgenesis, not PMDS. In those with a mutation of the AMH type II receptor, AMH levels are normal but not particularly high. Normal AMH values are also found in subjects with "idiopathic" PMDS in whom neither *AMH* nor *AMHR2* mutations have been detected.

Treatment

Treatment aims at the prevention of the two main complications of PMDS, infertility and cancer. It consists in correction of cryptorchidism. Hysterectomy is usually required for several reasons. Müllerian derivatives may cause discomfort or undergo

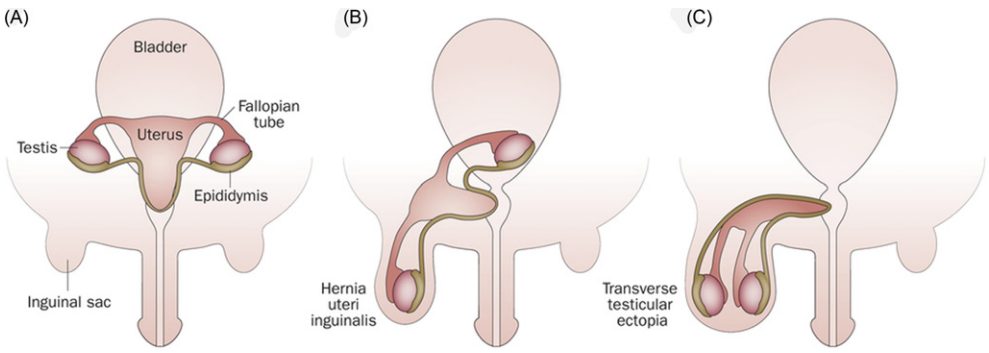

Fig. 6 The three main clinical presentations of persistent Müllerian duct syndrome. (A) Bilateral cryptorchidism: the testes are in the pelvis, in the position of normal ovaries. This presentation is found in approximately 55% of cases with mutations in the AMH pathway and in 86% of idiopathic cases. (B) Unilateral cryptorchidism: one testis is in an inguinal hernia along with its attached tube and uterus. This presentation is known as "*hernia uteri inguinalis*" and occurs in approximately 20% of cases with mutations of the AMH pathway and in 14% of idiopathic cases. (C) Transverse testicular ectopia: both testes and part of the Müllerian organs have herniated into a single processus vaginalis. This presentation is very evocative of PMDS and is seen in approximately 25% of cases with AMH or AMHRII mutations but never in idiopathic cases ($P < 0.001$). Reproduced with permission from: Hutson, J.M., et al. (2014). Malformation syndromes associated with disorders of sexual development, *Nature Reviews. Endocrinology* **10**, 476–487. © 2014 Macmillan Publishers Limited.

malignant degeneration. They also block the descent of abdominal testes. However, the surgeon should bear in mind that the excretory ducts are in close apposition or even enclosed in the uterine wall and careful dissection is required to avoid harming them. If this occurs, testicular sperm extraction followed by intracytoplasmic sperm injection may be successful. Placement of the testes in the scrotum should hopefully prevent malignant degeneration but this is not always the case. Two patients in whom cryptorchidism had been corrected earlier developed testicular cancer (Manassero *et al.*, 2004, Melman *et al.*, 1981).

Molecular Studies

Mutations of the *AMH* or *AMHR2* genes are responsible for 90% of PMDS cases. The rest have no identified molecular cause and are presently labeled idiopathic. A total of 64 different pathogenic *AMH* alleles of various types have been described in a total of 80 families. There is no hotspot: mutations are distributed along the whole length of the gene. Exon 4 is very rarely involved and exons 1 and 2 are proportionally the most affected. In exon 5, the bases coding the biologically active C-terminus of the AMH protein are hit at nearly three times the rate of the others. Belville *et al.* (2004) have studied the biosynthesis and secretion of seven mutant AMH proteins and shown that amino acid changes have significant effects upon protein stability and folding. Recurrent mutations and those of particular interest are shown in **Fig. 7**A.

Mutations of the *AMHR2* gene, totaling 58 different alleles, have been identified in 75 families. A 27-bp deletion in exon 10, encoding the kinase domain, has been detected in 30 patients, mostly Northern Europeans. The deletion can be recognized by PCR which should be performed as a first line investigation when an *AMHR2* mutation is suspected. Recurrent mutations and those of particular interest are shown in **Fig. 7**B. Receptor molecules with truncating mutations upstream of the transmembrane domain are not secreted, unless the AMHR2 signal sequence is replaced by that of the TGFβ receptor type II, indicating that the AMHR2 signal sequence is defective (Belville *et al.*, 2009).

Idiopathic PMDS occurs in approximately 10% of patients. No mutations of either the *AMH* or the *AMHR2* genes can be detected. Mutations in the distal promoter or in the central part of introns might have been overlooked, however idiopathic PMDS

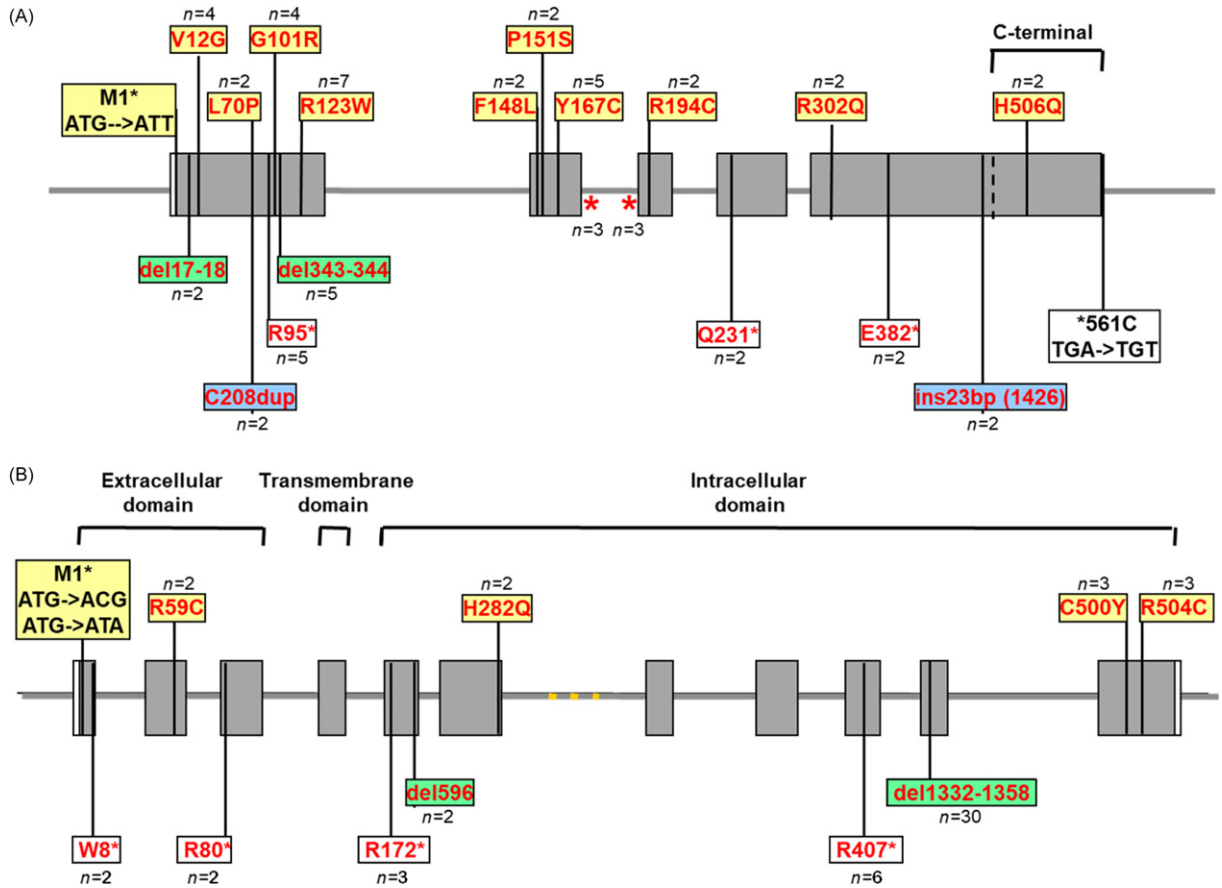

Fig. 7 Recurrent mutations (*red print*) and mutations of the ATG initiating and TGA terminating codon (*black print*) detected in the *AMH* gene (A) and the *AMHR2* gene (B). *n*: Number of affected families. Type of mutation: missense, yellow box; nonsense, white box; deletion, green box; insertion, blue box; splicing mutation, star. Reproduced with permission from: Picard, J.Y. et al. (2017). The persistent Müllerian duct syndrome: An update based upon a personal experience of 157 cases. *Sexual Development* **11**, 109–125. © 2017 S. Karger AG.

possibly represents a separate entity. Associated malformations are frequent, and the characteristic transverse testicular ectopia is not observed. The rate of consanguinity is only 10%, much lower than the 30%–40% frequency observed for *AMH* and *AMHR2* mutations.

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Hypospadiac Genital Tubercle (GT)

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Introduction

Hypospadias is a congenital malformation of the genital tubercle (GT), which has been known and described since antiquity but only understood and treated from the 19th century onwards. It was indeed not until the 1980s that modern management of hypospadias was developed, with new procedures and a better approach of its management.

The hypospadiac GT is the consequence of a development arrest of the tissues forming its ventral aspect (Mettauer, 1842) which occurs during the "masculinization window" (6–16 weeks of gestation) (Welsh *et al.*, 2008). This is characterized by an ectopic meatus on the ventral side of the penis or the scrotum, an asymmetrical foreskin and a possible penile curvature. The causes of this common congenital malformation, which affects one in 140–330 newborn boys in Europe (Springer *et al.*, 2016; van Rooij *et al.*, 2013), are essentially unknown and probably multifactorial. Hypospadias incidence seems to be increasing according to various epidemiological studies, although this remains a controversial issue (Main *et al.*, 2010; Yiee and Baskin, 2010).

Although it may appear straightforward to identify a hypospadiac penis, its clinical management still varies greatly from one center to another. There continues to be no clear consensus on the assessment of severity of this anomaly, on the possible treatments or on their evaluation (Castagnetti and El-Ghoneimi, 2010; Snodgrass *et al.*, 2011).

Anatomical and biological evaluations of hypospadias have changed over the past decades. Criteria of evaluation of hypospadias have changed in terms of anatomical description and biological screening. A better understanding of the embryology, etiological, and epidemiological aspects of the development anomalies of the GT has led to a more adjusted biological and surgical approach. Assessment of the hypospadiac GT is a crucial step to explore the causes and plan surgery which, in some cases, can be preceded by hormonal stimulation. Surgical treatment aims at achieving a normal-looking penis, with a slit-shaped meatus sitting on the apex of the glans, without penile curvature, thus allowing normal micturations in a standing position and a future normal sexual function. The place of preoperative biological screening, preoperative hormonal stimulation, the choice of urethroplasty, and the follow-up are the essential issues of this chapter. Evaluation of outcome is a critical point as there are no current consensual protocols, and long-term results are often lacking as well as psychological evaluation.

Epidemiology

The incidence of hypospadias varies from 0.3% to 0.7% in live male births (Paulozzi *et al.*, 1997). There is approximately 1 hypospadias for every 250 male births. The incidence is higher if there is a family history of hypospadias (1 in 100 to 1 in 80 male births) (Gearhart *et al.*, 2009). The incidence varies with ethnicity, as illustrated by a population-based study from the state of Washington which showed hypospadias was more prevalent in male infants born to Caucasian mothers compared with male infants of black (OR 0.67, 95% CI 0.51–0.89) and Hispanic mothers (OR 0.46, 95% CI 0.37–0.58) (Springer *et al.*, 2016).

In the last 15 years, the incidence of hypospadias in Western countries seems to have increased (Loane *et al.*, 2011). In Denmark, the incidence was measured at 0.24% and 0.52% in 1977 and 2005, respectively. A recent publication (Nordenvall *et al.*, 2014) confirmed an augmentation of incidence of hypospadias in Sweden (4.5 per 1000 live-born boys until 1990, 8 per 1000 boys from 1990 to 2009). In the United States, data from two birth defects surveillance systems demonstrated a doubling of hypospadias rates (0.2%–0.4%) from 1970 to 1993 (Canon *et al.*, 2012; Paulozzi *et al.*, 1997). In Western Australia, the rate increased from 0.28% in 1980 to 0.43% in 2000.

Although these trends may reflect improved surveillance and detection, it is unlikely that these measures can account for the entire increase in the different geographical locations. These findings suggest that temporal changes may be due to other risk factors, especially greater exposure to environmental factors (disruptors) or drugs that may disrupt the androgenic stimulation required for the development of the male external genitalia.

There are other publications that contested this fact: Fisch *et al.* (2009) retrospectively reviewed the total prevalence of hypospadias in New York State from 1992 to 2005, and showed that there was no statistical change in hypospadias rates in New York State over this time period. A larger study of 29 national registries (4 million births per year worldwide) confirmed the stability of the incidence of hypospadias in most cases except two registers (Scandinavia and Japan), especially during the period 1970–1998.

Definition and Understanding of the Anatomy of Hypospadias

Defining the anatomy of hypospadias is an essential step for the management of this congenital anomaly.

Three anatomical features are classically described (**Fig. 1**):

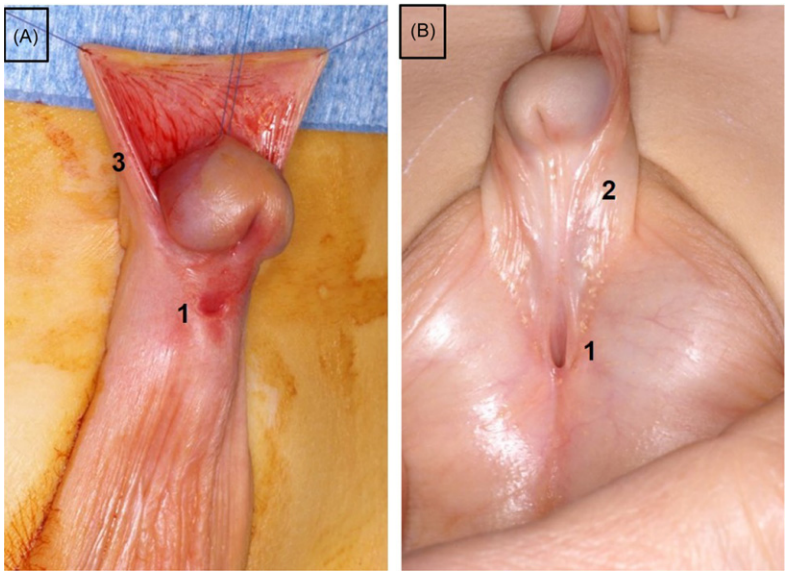

Fig. 1 Definition of hypospadias: (1) ectopic urethral meatus, (2) ventral curvature of the penis, (3) deficient ventral foreskin with a marked excess of skin on the dorsal aspect of the GT. (A) A midshaft hypospadias and (B) a proximal (scrotal) hypospadias.

- an ectopic urethral meatus opening which may be located at any point from the glans to the base of the penis and even on the perineum;
- a ventral curvature of the penis of variable magnitude; and
- a deficient ventral foreskin with a marked excess of skin on the dorsal aspect of the GT (hooded foreskin).

The curvature and the hooded foreskin are not constant. A hypospadiac meatus may be found under a normally formed prepuce. An isolated curvature may occur without an ectopic urethral opening, but it is often associated with a hypoplastic urethra, not surrounded by spongiosum ("missed hypospadias").

From the tip to the base of the ventrum of the hypospadiac GT, one can identify several anatomical features (**Fig. 2**):

1. The glans is open ventrally.
2. A segment of urethral tube of variable length is missing and is replaced by a urethral plate extending from the ectopic meatus towards the glans cap, lying on the ventrum of the two corpora cavernosa. Looking more carefully at this strip of tissues sitting between the ectopic meatus and the glans, a tongue of urethral mucosa (shiny tissue) followed distally by skin is often identified during the surgical procedure. This demonstrates the arrest of growth of the urethral bud at the origin of the hypospadias condition.
3. Then comes the ectopic meatus and behind it a segment of hypoplastic circular urethra, not surrounded by any corpus spongiosum (CS). A thin layer of skin is often stuck tightly on the underdeveloped urethra.
4. The division of the CS is always proximal to the ectopic meatus (and sometimes several centimeters behind the ectopic meatus) in two pillars that extend laterally up to the glans cap in a fan-shaped position. This division of the CS is often outlined on the ventral skin by a small cutaneous ridge.
5. Proximal to the division of the CS, all of the structures forming the ventral aspect of the penis are normal.
6. The frenular artery is constantly missing.
7. The dorsum of the GT is normal.
8. Associated anomalies are common: undescended testes and/or micropenis (<25 mm dorsal length/<15 mm width before 1 year of age), which may reflect the testicular failure to produce enough androgens during gestation (see later); presence of mullerian remnants (utricular cavity), which may reflect the testicular failure to produce enough anti mullerian hormone (AMH); scrotal transposition; and other non-genital anomalies such as the midline syndrome which may involve the heart, the palate, or even midline brain defects.

Classification of Hypospadias

Many classifications of hypospadias have been published, mainly based on the position of the ectopic meatus (Vidal *et al.*, 2010), which is an insufficient criterion to define the severity of hypospadias. Several other aspects of the anomaly visible at the time of surgery should be looked for before deciding the most appropriate technique of reconstruction, including:

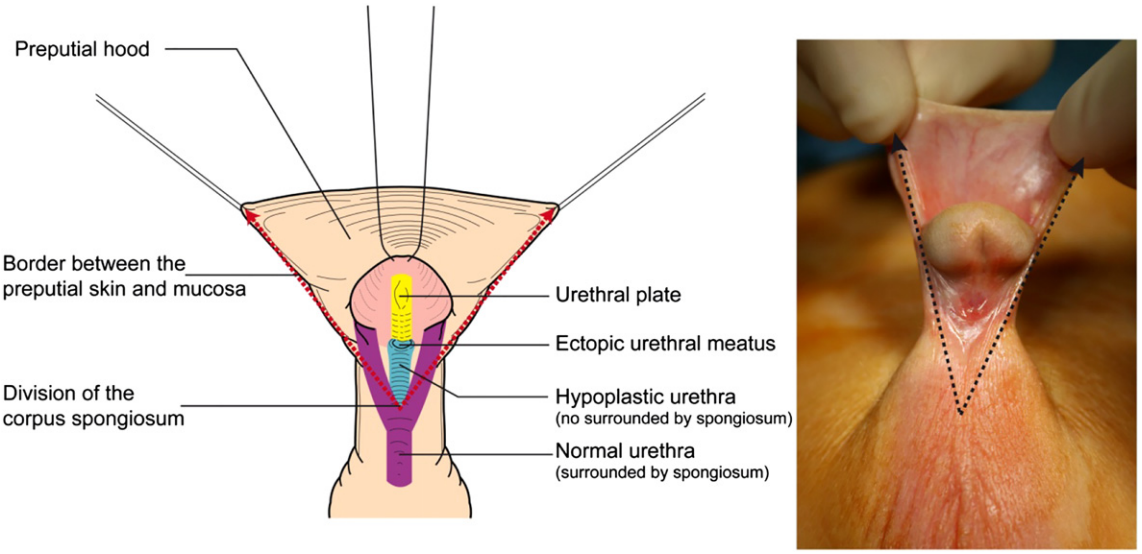

Fig. 2 From the tip to the base of the penis: the ventral aspect of the glans is wide opened. The urethral plate extends from the apex of the glans down to the ectopic meatus. Behind the ectopic meatus sits a segment of hypoplastic urethra not surrounded by any spongiosum. The division of the corpus spongiosum marks the proximal limit of the malformation. It defines a triangular defect whose summit is the division of the corpus spongiosum, whose base is the glanular cap and whose sides are represented by the two lateral pillars of atretic spongiosum.

- level of division of the CS;
- degree of hypoplasia of the tissues forming the ventral aspect of the penis;
- quality of the urethral plate;
- size of the penis (which is often arguable);
- size of the glans;
- availability of the preputial skin;
- ano-scrotal distance or ano-penile distance, which may reflect the degree of prenatal androgen exposure.

The level of division of the CS is a more accurate criterion to define the severity of hypospadias (Gearhart *et al.*, 2009), as it shows where the GT development arrest occurred (**Fig. 3**). It therefore seems logical to define the degree of severity of hypospadias according to the level of division of the CS, which will help the surgeon to choose the optimal technique of reconstruction:

1. Hypospadias with a distal division of the CS associated with little or no curvature.
2. Hypospadias with a proximal division of the CS frequently associated with a ventral curvature.
3. Cripple hypospadias, or "hypospadias with previous failed surgery": this occurs in patients who have already undergone several procedures that failed, leaving behind scarred tissues, an abnormal meatus, urethral strictures, urethral dehiscence, fistulas, residual curvature, poor cosmetic results, and/or a psychological impact of various severity.

Classifications of hypospadias should therefore be revisited not only with the anatomical criteria mentioned above, but also in the "biological context" of the hypospadiac patient.

Embryology

The GT and genitalia are formed during the masculinization window (6–16 WG) during which a flare of human chorionic gonadotrophin (HCG) coming from the placenta will stimulate the production of the fetal testicles testosterone. The urethra is the result of a double process: centrifugal growth of the urogenital sinus under the GT and centrifugal tubularization of the urethral plate. The GT becomes the phallus in the male and the clitoris in the female.

Four main actors (Snodgrass *et al.*, 2011) intervene in the construction of the tubercle genital in the male fetus:

1. the child himself with his genes, his gonads and their hormonal production, the central hormonal control, and the target tissues with their receptors and protein platform;
2. the placenta, which is the main hormonal machinery especially during the first part of the gestation, playing an essential role during the masculinization window;
3. the mother, who is the system of reference and whose hormonal status and concomitant diseases could affect the growth of the GT; and
4. the mother's environment during the masculinization window, which may contain some hormonal disruptors or promoters which could interact with the child's formation.

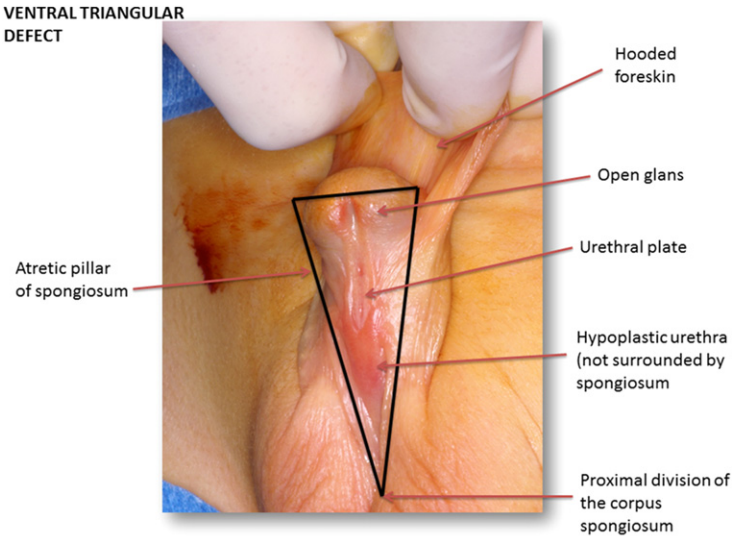

Fig. 3 Hypospadias with a proximal division of corpus spongiosum. The severity of hypospadias can be assessed once the division of the corpus spongiosum and the degree of ventral hypoplasia are identified—the ventral triangular defect.

Hypospadias is a result of this faulty process. Beyond 16 WG, the linear growth of the GT continues beyond the prenatal flare of testosterone, implying that other growth factors might be involved. The tissular proteins are also likely to play important roles in the construction of the GT. Tissues sitting on the ventral surface of the hypospadiac GT do not have the same protein platform as those of the GT dorsum or control group (Morera *et al.*, 2004). The balance between structural proteins and enzymes is altered, and could explain some healing complications commonly reported after hypospadias surgery.

Preoperative Management

Endocrine Screening of the Hypospadiac GT/Investigations

Hypospadias with a labeled biological or genetic context may not have the same predictive outcome as those with no identified etiology, although very few patients have an abnormal biological screening. There is no consensus on the endocrine and genetic screening for the hypospadiac GT. A full biological assessment with karyotype, sequencing of various genes including those involved in the tissular androgen receptors, testosterone, and its precursors, AMH, inhibin B are required for some in case of severe hypospadias or hypospadias associated with other genital anomalies (undescended testes; persistent mullerian structures) (Snodgrass *et al.*, 2011).

Two periods are particularly informative (Gorduza *et al.*, 2010; Vidal *et al.*, 2010) to explore the endocrine function of gonads:

- During the first day of life, the child hormonal machinery is still under the influence of the placental activity.
- During the minipuberty, which extends between day 15 and day 90 of life, there is a flare of hormonal secretions coming from the testicles under the influence of central gonadotrope hormones (testosterone and its precursors for the Leydig cells; AMH and inhibin B for the Sertoli cells). FSH and LH are indicators of the activity of the hypothalamic–pituitary–gonadal axis during "minipuberty." Beyond 6 months of life, plasma AMH remains high (Josso *et al.*, 2006), but androgens can only be measured after stimulation (HCG or recombinant) because of the physiological rest of Leydig cells activity in the prepubertal boy. There is no consensus between centers about HCG and androgenic stimulation protocols and their interpretation.

Pre-Operative Hormonal Stimulation

Preoperative androgen stimulation is commonly used prior to hypospadias reconstruction primarily for small penises (<2–2.5 SD), small glans width, narrow urethral plate, severe proximal hypospadias, or in redo surgery with the aim of increasing the size of the penis and boosting the healing process by increasing the penile blood supply (Malik and Liu, 2014; Netto *et al.*, 2013). Multiple stimulating protocols have been described using either systemic testosterone, local dihydro-testosterone, or HCG (Kaya *et al.*, 2008; Nerli *et al.*, 2009). Here again, no consensus has been found on penile stimulation. More recent studies have pointed out the potential negative effects of androgen stimulation on the healing process after hypospadias surgery. Hence some authors recommend a 3–6-month gap between the stimulation and the surgical procedure (Gilliver *et al.*, 2007; Gorduza *et al.*, 2011; Snodgrass *et al.*, 2011).

Surgery

There is a common temptation to try to universalize one technique for most forms of hypospadias. Several attempts were made in the past and all failed to convince the hypospadiologists. Each surgeon has his or her own convictions, to varying degrees (Snodgrass *et al.*, 2011). The multiplicity of techniques clearly demonstrates that none of them is fully satisfactory. The main current debate splits the surgeons who are keen to use solely ventral tissues to repair the missing urethra and those who use dorsal tissues or other tissues, claiming that ventral tissues alone cannot grow satisfactorily.

Although most hypospadias to date have been surgically treated, those with a distal division of the CS may not need surgery and this non-surgical option needs to be given to the parents.

Objectives of Hypospadias Surgery

From the anatomical description, it is clear that surgery entails three main steps (Gearhart *et al.*, 2009):

1. Degloving the GT down to the base to identify the level of division of the CS which is the main criteria to define hypospadias severity, that is, the degree of hypoplasia of the ventral tissues and consequently the degree of ventral curvature. The evaluation of a persistent curvature after full dissection is necessary to evaluate the need to straighten the GT.
2. Choosing the adequate technique of urethroplasty, which is dependent on the quality of the urethral plate, the availability of preputial tissue, the length of urethra to be reconstructed, the size of the glans, and the depth of the glans groove.
3. Covering the straightened GT with a redistribution of the penile skin.

Surgical Techniques

We shall only mention techniques that are commonly used in most departments of pediatric urology. Three surgical steps characterize most surgical techniques.

First, degloving the penis and a deep dissection of the glans wings represent the first step of this surgery, straighten the penis in most cases, and correct the common ventral glans tilt. In some cases (proximal hypospadias), the residual curvature demonstrated by an erection test is due to asymmetrical corpora cavernosa. It requires a dorsal corporeoplasty or a ventral plasty of the corpora using a patch of tissue (scrotal vaginal tissue).

Second, once the GT has been fully dissected, the length of urethra to be reconstructed can be evaluated. The technique chosen depends on the size and quality of the urethral plate.

If the urethral plate is wide and healthy, some will choose to tubularize it following the Thiersch–Duplay technique (Duplay, 1880).

If it is too narrow to be tubularized, the Snodgrass open urethrotomy (Snodgrass and Lorenzo, 2002) is a popular option, or additional tissue can be laid on the urethral plate using a rectangle of pediculized preputial mucosa (onlay urethroplasty), or a flap of ventral penile skin (Mathieu procedure) (Mathieu, 1932).

If the segment of urethra to replace is short (<1.5 cm), and if the distal urethra is not hypoplastic, a complete mobilization of the whole penile urethra may be adequate to bridge the defect. This technique (Beck-Koff) has the advantage of avoiding the use of non-urethral tissue (Koff, 1981; Thiry *et al.*, 2014).

If the urethral plate is not preservable, a tube needs to be made to replace the missing urethra using either a pediculized rectangle of preputial mucosa (Asopa-Duckett technique) or buccal mucosa (Duckett, 1981).

In major hypovirilization of the GT, the Koyanagi procedure (Koyanagi *et al.*, 1983) mobilizing the tissues of the ventral and lateral aspects and dorsal prepuce with the blood supply is a reliable option to reconstruct the missing urethra. Two-stage procedures (Bracka-Cloutier procedure) are an alternative for long urethroplasties using either preputial mucosa or buccal mucosa (Bracka, 1995).

Third, once the urethra is repaired, the ventral radius of the penis needs to be reconstructed. This includes:

- meatoplasty aiming at creating a slit-shaped meatus;
- glanuloplasty by stitching together the two glans wings over the reconstructed urethra to refashion the ventral aspect of the glans;
- creation of a mucosal collar around the glans;
- coverage of the reconstructed urethra (spongioplasty) using the lateral pillars of spongiosum, or a dorsal or scrotal layer of well vascularized tissue, or the pedicle of the onlay flap itself. The coverage with well vascularized tissues of the neourethra is meant to reduce the risk of fistulae;
- skin cover with a redistribution of the skin shaft bringing the excess dorsal skin to the ventrum.

Some prefer to reconstruct the foreskin; others favor circumcision.

General anesthetic is the rule often associated with caudal or penile anesthesia (Tosetti *et al.*, 2013).

Time of Surgery

The patient's age at surgery for primary hypospadias repair is usually between 6 and 24 months. In 1996, the American Academy of Pediatrics recommended performing this surgery after 6 months of age with particular reference to the risks, benefits, and psychological effects of surgery and anesthesia (Manzoni *et al.*, 2004). After 18 months, the child is more mobile and less cooperative. One study showed no difference concerning psychological effects and quality of life among boys having surgery repair before or after 18 months of age (Weber *et al.*, 2009).

Post-Surgical Evaluation and Follow-Up

Evaluation of outcome is another critical step in this management, and includes functional and cosmetic results through adolescence until adulthood. One essential characteristic of pediatric urology/surgery is that reconstructions are performed on growing organs (Gorduza *et al.*, 2010; Snodgrass *et al.*, 2011).

Cosmetic, functional, and psychological results are subjective and evaluated in various ways which are hardly comparable from one publication to another (Mouriquand *et al.*, 2011). Patients' own evaluations often differ greatly with those of the surgeon, especially regarding the cosmetic appearance (Örtqvist *et al.*, 2015). Functional evaluation includes the quality of the urine stream through the reconstructed urethra which is subjectively assessed by the patient, the parents, and the surgeon: direction and strength of the stream, spraying stream, straining micturations, pain, and duration of micturation give some clues on the urethral flow but remain subjective. Interpretation of urine flow studies is questionable, as most patients who received urethral surgery have long-lasting dyssynergic voiding, and because the urodynamic profile of the reconstructed urethra is abnormal even without significant urethral stricture. This explains why most urine flow studies after hypospadias surgery have a flat profile. This is due to the material used for reconstruction, which does not have the same urodynamic profile as the native urethra (Olsen *et al.*, 2011).

On the long-term sexual outcome, there is a paucity of publications. It seems that straightforward hypospadias repairs do not have serious late consequences on sexual life, contrary to multi-operated patients who are more affected by the psychological consequences of multiple complications (Jones *et al.*, 2009; Kiss *et al.*, 2011; Rynja *et al.*, 2011). Aulagne et al. (2010) and Wang et al. (2010) noted that early surgery was preferable in terms of psychological outcome.

Fertility in hypospadias patients is rarely affected in isolated distal hypospadias, but can become significant when hypospadias is part of an endocrine or genetic disorder, or associated with other genital disorders (e.g., undescended testes, persistent mullerian structures, hypoplastic testes with a poor sperm count) (Asklund *et al.*, 2010) or in proximal hypospadias when the ejaculation process may be disturbed by anatomical factors (urethral dysmotility, persisting penile curvature, anejaculation) (Jiao *et al.*, 2011). Fertility is also influenced by psychosocial factors and body esteem (Vandendriessche *et al.*, 2010).

We would recommend evaluating the cosmetic and functional outcome (mostly how the child passes water) 2 months and then 1 year after surgery, and at puberty. When redo surgery is needed, we would advise waiting at least 6 months after the first procedure before considering surgery again.

Different types of complications include the following:

1. Cosmetic complications are common and mostly represented by irregular scars and ventral excess of skin.
2. Healing complications are mostly represented by fistulae (4%–28%) and dehiscence of the reconstructed urethra, proximal stenosis at the junction between the native and the reconstructed urethra (6%–12%), and distal stenosis through the glans or meatal stenosis (0%–14%) (Cimador *et al.*, 2013).

Fistulae are the most common complication and incidence varies according to surgical technique. They usually appear within a few months of surgery and can be related to an insufficient blood supply of the skin flaps used for reconstruction, a distal stenosis, or infection. Fistulae are often located at the level of the corona and commonly found in a lateral position. The orifice on the penile surface is always smaller than the defect found inside on the urethral wall.

Stenosis usually happens later and may also reflect a poor blood supply or inadequate material for reconstruction. Late stenosis may be a consequence of the poor growth of the tissues used for urethroplasty. Ventral dysplastic tissues located beyond the division of the CS appear not to have the same growth potential as the rest of the GT.

Fistulae and stenosis are commonly associated.

3. Urine flow disorders are also common. They can either be related to the material used for urethroplasty (mostly skin) which does not have the same properties as normal urethral tissue and/or be related to an inadequate urethroplasty. The urodynamic profile of the reconstructed urethra explains the abnormal urine flow studies with flat curves commonly found after hypospadias surgery; incidence varies according to surgical technique (Scarpa *et al.*, 2010), even in non-stenotic urethroplasties. Ballooning of the neourethra (urethrocele) (4%–12%) is due to insufficient backing of the reconstructed urethra and often triggered by the high urethral flow pressures created by the trans-glanular segment of the reconstruction (Cimador *et al.*, 2013).
4. The reconstructed urethra can get very dilated proximal to the glans crossing, creating a pouch which does not empty well and which causes urine dribbling after micturation, pain, and urinary tract infections.
5. Persistent penile curvature and secondary penile deformities (9%–32%) are related either to unrecognized curvature at the time of primary surgery or insufficient growth of the reconstructed urethra due to immature tissues or scarred tissues. Literature is very poor regarding long-term outcomes of early corporeoplasty (Cimador *et al.*, 2013).

6. Association with a mullerian remnant (utricular pouch) is common with severe hypospadias where the gonads have failed to produce enough AMH to erase the mullerian ducts. Most utricular pouches are asymptomatic, but some may cause dysuria and urinary tract infections. In these situations, a laparoscopic removal of the utricular pouch should be discussed. However, this procedure carries the risk of damaging the vas deferens which are often included in the walls of the utricular cavity.
7. Ejaculation dysfunction is also identified in young adults with a history of severe hypospadias. The nature of the neourethral tissues or the presence of a utricular pouch can alter the ejaculation process and subsequently the patient's fertility.
8. Sexual and psychological disorders mostly related to repeated hypospadias surgery are also poorly documented.
9. Unevaluated complications: glans sensitivity is not reported after hypospadias surgery, although it is likely to be affected.

Parents should be warned that late inadequacy of the reconstructed urethra may occur.

Conclusion

Hypospadias remains a challenge, as many unknown factors are found at each step of its management. There is no minor hypospadias, and identifying the level of division of the CS is the essential initial step to define the severity of a hypospadias. It is difficult to compare techniques and outcomes, as there are no objective parameters and no consensus among hypospadiologists. The key message is that pediatric urologists and pediatric surgeons are operating on growing organs and tissues, and the challenge is to choose a technique of reconstruction that will grow at the same pace as the patient. Some hopes are raised with bioengineering to find a more physiological material for urethroplasty. Long-term follow-up is crucial as pediatric urologists are operating on growing organs, but none of the criteria used to assess hypospadias repair is fully satisfactory. The cosmetic results are subjective and quite variable between parents and doctors. The functional outcome in terms of urine flow is mainly based on the patient's (parents') impression. The psychological long-term effects of penile surgery are poorly reported, although current literature seems to show an adequate psychosexual development after hypospadias surgery in most cases.

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Undescended Testes☆

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Glossary

Cryptorchidism A testis that is not correctly descended in the scrotum, although it is located in the normal pathway, contrary to an ectopic testis.

Ectopic testis The testis is found outside the normal path of decent from the abdomen to the scrotum.

Orchidopexy Surgical transfer of a testis from a supras-crotal position to the scrotum.

Spermatogenesis The complete sequence of events of cell division and cell maturation that leads from spermatogonia to fully developed spermatozoa.

Spermatogonia Stem cells of spermatogenesis and the earliest stages of germ cell maturation, in the absence of which no sperm can be produced. Spermatogonia are present in the normal testis throughout childhood and adulthood.

Testicular descent The normal transposition of the testis during fetal or the first six postnatal months of life from the lower abdomen, through the inguinal canal, to the scrotum.

Undescended testes Synonymous with nondescended testes, cryptorchidism, retained testes, and retentio testis.

Introduction

With very few exceptions, the testicles of mammals are located in a sac on the outside of the body cavity, exposed to potential trauma rather than protected inside the pelvis or abdomen. From a developmental perspective, this seems to be a risk factor for securing fertility, which should be an absolutely vital strategy for survival of a species. Thus, this location must offer other developmental benefits. The most obvious is the lower temperature in the scrotum than in the abdomen (2°C or 3°C, depending on the species). In fact, it has been shown that this seemingly minor difference in temperature is crucial for completion of spermatogenesis; in most mammals, failure of the testes to descend from the abdominal cavity to the scrotum leads to infertility. On the other hand, this does not mean that spermatogenesis occurs in all testes that are brought into the scrotum; the non-descent can be secondary to an abnormal development of the testis that might impair spermatogenesis. Another possible benefit of the low scrotal temperature may relate to the incidence of mutations of the genome. A reduced temperature in the testis, the organ with the highest cell proliferation in the male body, will reduce the incidence of mutations that will be transmitted to offspring to a level that is optimal for long-term development (mutations with positive effects are beneficial) but not so high that it compromises fertility or that the occurrence of malformations is at an unacceptable level for the development of the species.

Nomenclature and Differential Diagnosis

One conventional definition is that normal testicular descent has occurred when the location of the testis is below the border between scrotal and abdominal skin in a resting, standing position. However, this "resting" state may not be present during examination in the clinic—the cremasteric reflex will retract the testes when the examiner's hand approaches. A practical way to circumvent this is to pull the testis down gently, hold it for approximately 30 s until the cremasteric reflex has been exhausted, and then release it. The position of the released testis approximates the resting position. If a testis is in a supra-scrotal position at the start of the examination but after manipulation remains in the scrotum, it is called a retractile testis. This condition is thought not to need any further attention. For clinical studies, a more exact location of the testes than "scrotal," "sub-inguinal," or "inguinal" should be used, preferably by measuring the distance in millimeters between the testis and a defined point (e.g., the pubic tubercle or the external inguinal orifice).

The term cryptorchidism is often used to describe the condition of undescended testes. This term implies that the testes are "hidden," that they can neither be seen nor palpated. Since in clinical practice even inguinal or sub-inguinal (pre-scrotal) palpable testes should be considered undescended, the term cryptorchidism is not always correct. "Non-descended" and "retained" (Latin: *retentio testis*) are synonyms of undescended testes that are sometimes used (**Fig. 1**).

In approximately 10%–20%, both testes are found above the scrotum. Even after successful treatment, the prospect for future fertility is worse (approximately 65%) in bilateral than unilateral cryptorchidism (approximately 90%) (Lee, 2005). Untreated bilateral cryptorchidism in adulthood is not compatible with fertility.

☆*Change History*: December, 2017. Ritzén E Martin updated the text and added new figure.

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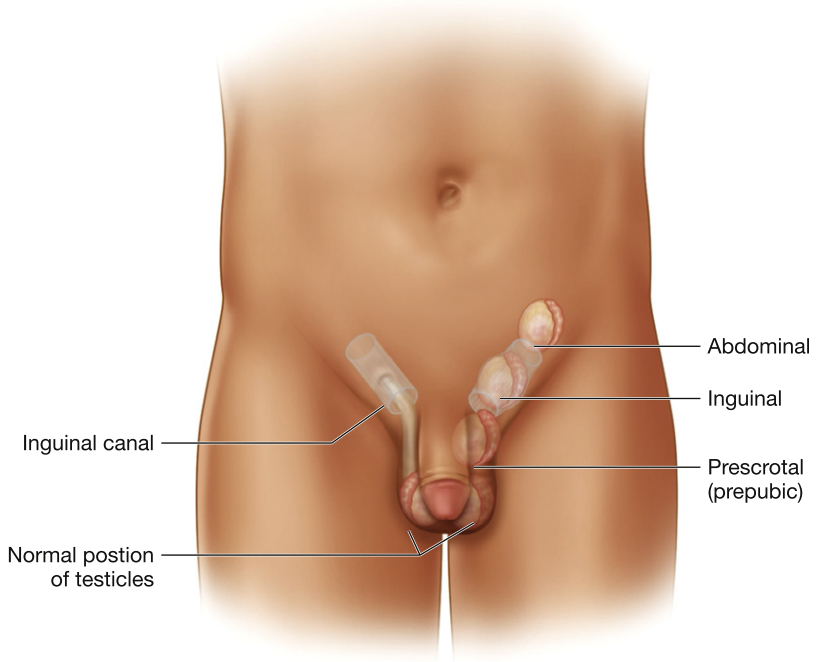

Fig. 1 A schematic drawing of the various locations where a testis can be found along its natural path of descent from the abdomen through the inguinal canal to the scrotum.

If no testis is palpable, it is important to consider a possible disorder of sex development (DSD), especially if combined with hypospadias of any degree. Such patients should be referred to a DSD team immediately after birth, since the new born might be a virilized girl with congenital adrenal hyperplasia; a possibly life-threatening condition.

If both testes undergo atrophy after that male sex differentiation is completed, the boy is born with normal male sex organs but will need testosterone substitution at puberty.

The term ectopic testis has been coined to describe a position of the testis that is outside the normal path of the testis during descent. Only during surgery can the true differentiation between a low inguinal and an ectopic testis be made.

It is difficult to distinguish bilaterally retained abdominal testicles from testicular atrophy. The differentiation between the two may be aided by a human chorionic gonadotropin (hCG) stimulation test. Significantly increased testosterone levels indicate the presence of testicles. Measurement of inhibin B in serum is valuable since in boys this hormone is only produced in Sertoli cells.

Acquired Cryptorchidism

Acquired cryptorchidism is a term that is used in cases when a testis at some stage (most often during the first 6 months of age) has been found in the scrotum, but later in a supra-scrotal or inguinal position. This is found to be the case in about one of five testes that are undescended at birth, but descend during the first 3–6 months (Kollin *et al.*, 2013). Thus, this group of boys should be followed at annual examinations and treated if found to be ascended. Such examinations can be done by the parents, after proper instructions. Secondary ascensus may explain why many studies find about 1% undescended testes at age 6 months, but up to 3% of all boys are eventually operated for cryptorchidism.

Incidence of Cryptorchidism

The classical epidemiological studies by Scorer (1956) in England are often cited. Using his quite strict definitions, 2.7% of boys with a birth weight of >2.5 kg had undescended testes at birth, but testicular descent proceeded during the first postnatal months so that at age 3 months only 0.9% were considered to have incompletely descended testes. In premature infants, the incidence was much higher (21% if <2.5 kg), reflecting the late normal descent in utero. When Scorer's study was repeated 20 years later using the same methodology, the incidence at birth and at 3 months was 3.4% and 1.4%, respectively, suggesting a negative trend in fetal testicular development during this period. This has been paralleled by a similar increase in the incidence of testicular cancer, suggesting a possible common environmental cause of testicular maldevelopment.

The term "testicular dysgenesis syndrome" has been coined to describe a common cause for cryptorchidism, hypospadias, testicular cancer and future impaired spermatogenesis (Skakkebaek *et al.*, 2001). Early on, exposure of mother and fetus to environmental estrogenic compounds was suspected to impair testicular function, but later many other such "endocrine disruptors" have been identified. A recent meta-analysis (Bonde *et al.*, 2017) of publications that examine a possible link between individual endocrine disruptors and testicular dysgenesis in boys failed to show convincing evidence to prove such a relation, although animal experiments have proven that prenatal exposure to compounds such as phthalates and bisphenol A definitely can cause impaired testicular function. The authors of the meta-analysis conclude that "the current epidemiological evidence is compatible with a small increased risk of male reproductive disorders following prenatal and postnatal exposure to some persistent environmental chemicals classified as endocrine disruptors but the evidence is limited." The possibility of a "cocktail effect" of many simultaneous exposures to endocrine disruptors is difficult to study and can presently not be excluded.

Mechanisms of Testicular Descent

The translocation of the fetal testicles from the lower abdominal wall to their final destination in the scrotum is lead through the inguinal canal by the gubernaculum, one end of which is anchored in the epididymal tail and the other in what is to become the bottom of the scrotum. Initially, the gubernaculum fills the canal, and through successive shortening and swelling of its intra- and extracanalicular parts it is believed to aid the testis through the canal. The precise hormonal regulation of this series of events is incompletely known. In addition to non-hormonal homeobox genes, some endocrine factors have been suggested to have a crucial role in one or several of the events leading to testicular descent.

Undescended testicles are often found in boys with hypogonadotropic hypogonadism or androgen insensitivity. Thus, deficiency of androgenic hormones is important for the final passage through the inguinal canal. Patients with persistent Müllerian duct syndrome due to a loss of function of anti-Müllerian hormone or its receptor also have retained testicles. Therefore, this hormone has been suggested to be a key factor for testicular descent. However, since in this syndrome the testes are closely linked to the uterus and fallopian tubes, this may in itself hinder descent. It has been found that in mice the newly described *Insl3* gene is important for gubernacular development and testicular descent from its proximal abdominal position to the internal opening of the inguinal canal. No human disease has yet been ascribed to this factor.

Abnormal Functions of Undescended Testicles

Spermatogenesis

Numerous animal experiments have proven that spermatogenesis is blocked at a pre-meiotic stage if the testes are retained in or moved into the abdomen at any time after birth. This spermatogenic arrest is reversed if the testes are transferred to the scrotum or even if they are cooled in an abdominal position with a cooling device. These experiments prove that a temperature $<37^{\circ}C$ is needed to complete spermatogenesis. In man, the difference between abdominal and scrotal temperature is $2^{\circ}C$ or $3^{\circ}C$. Germ cells, including spermatogonia and spermatocytes, seem to be the most temperature-sensitive cells, but impaired Sertoli cell function at $37^{\circ}C$ may also be of importance.

On the other hand, not all undescended testicles will attain normal spermatogenesis if they are transferred to the scrotum by surgery or by hormonal treatment. As many as 40% of successfully treated men with primary bilaterally undescended testicles are reported to have grossly subnormal sperm quality (Lee, 2005). Although the treatment itself may be harmful to the testis, this suggests that a number of undescended testicles have other deficiencies in spermatogenesis that are unrelated to their abdominal or inguinal position. However, it is not possible to distinguish the testes with primary defects in spermatogenesis from those that will normalize when put in a scrotal position.

Steroidogenesis

Animal experiments have demonstrated that even if the testes are translocated to the abdomen, normal blood levels of testosterone can be maintained. However, there is a subtle difference: The Leydig cells are enlarged and have a lower sensitivity to stimulation by Lutropin (synonyms: luteinizing hormone, LH, interstitial cell-stimulating hormone) or *h*uman *C*horionic *G*onadotropin (hCG). It is not known whether this is due to a primary effect of temperature on the Leydig cells or if it is secondary to damage to the seminiferous tubules, causing an abnormal paracrine hormonal milieu.

In man, normal testosterone levels in blood are generally seen even when the testicles are undescended. Thus, a stimulation test with hCG can be used to differentiate between bilaterally nonpalpable retained testicles and the complete absence of testes (testicular atrophy). It is important to remember that even a small testis that has a poor prognosis with regard to spermatogenesis should only be removed if the other testis is present and functioning. This small testis may mean the difference between life long testosterone replacement and no need for medication.

Malignancies

It is frequently cited that undescended testicles often develop malignancies and therefore should either be placed into the scrotum or removed. The basis for this assumption lies in the finding of an overrepresentation of undescended testicles (with or without successful treatment) in cohorts of patients with testicular cancer. Five to ten percent of men with testicular tumors have a history of undescended testes. However, the true incidence of testicular cancer in a group of men with undescended testes has not been well documented. In a Swedish retrospective study (Pinczowski *et al.*, 1991) all 3000 boys with undescended testes from a defined geographical area underwent orchidopexy. These patients, as well as 30,000 boys operated on for inguinal hernia, were searched for in the Swedish Cancer Registry when they were adults. In the previously cryptorchid cohort, four cases of testicular cancer occurred versus 0.54 expected, yielding a relative risk of 7.4 (95% confidence interval, 2.0–19.0). The hernia controls had no increased risk. The most prevalent testicular cancer was seminoma, which has a good prognosis following modern treatment. Thus, the absolute risk (4/3000) does not motivate special follow-up of patients operated for cryptorchidism. However, it is recommended that an undescended testis should be brought into a palpable position so that tumor formation can be detected at an early stage, even in cases in which there is little hope of spermatogenesis. Since testosterone production might be maintained even in a small and soft testicle, it should not be removed unless the contralateral testis is normal.

Treatment

There is much controversy regarding the methods of treatment of undescended testicles, despite the numerous articles that have been published. The major controversies relate to the method (hormonal or surgical) and the most suitable age of treatment.

Hormonal Treatment

Based on the common finding of undescended testicles in boys with hypogonadotropic hypogonadism or androgen insensitivity, stimulation of the testes through administration of hCG or gonadotropic-releasing hormone (GnRH) has been advocated as the logical treatment. Direct systemic administration of testosterone has the drawback of treating the whole body as much as the testis and its surroundings and in a few trials has not been shown to be very effective.

Human chorionic gonadotropin

In many countries, injection of hCG for 3–5 weeks is the recommended treatment of undescended testicles. The following is a typical treatment regimen: injection of 500 IU hCG intramuscularly twice weekly for 5 weeks for boys younger than 6 years and doubling the dose for older boys. In uncontrolled studies, this treatment is reported to result in testicular descent in approximately 50% of patients; the non-responders are referred for surgery. However, meta-analyses of randomized controlled studies indicates that the success rate is only 19%, compared to 5% for placebo groups.

The short-term side effects of hCG treatment include pain of injections, increased frequency of erections, and sometimes development of pubic hair. Animal experiments and some clinical observations have raised concern regarding possible long-term adverse effects of hCG on the testis. Thus, impaired spermatogenesis has been noted in dogs treated with hCG at the time of puberty. In rats, injection of hCG or LH causes an acute inflammatory response in the testicular interstitium, with increased intratesticular pressure, extravasation of leukocytes, and signs of edema. A similar histological picture has been reported in testicular biopsies taken within a few days after completed unsuccessful hCG treatment. Furthermore, it has been shown that the number of apoptotic spermatogonia is markedly increased following a course of hCG treatment when compared to the degree of apoptosis in biopsies taken from boys operated on without prior hCG treatment. In a small study, a positive correlation was found between the number of apoptotic spermatogonia within 1 month after treatment in childhood and the follitropin (follicle-stimulating hormone, FSH) levels as adults, approximately 20 years later. Testicular volume in adulthood was negatively correlated with the initial degree of spermatogonial apoptosis, suggesting an impaired spermatogenesis secondary to the post-hCG apoptosis (Dunkel *et al.*, 1997). In conclusion: hCG treatment is not very effective and there are concerns about its safety.

Gonadotropin-releasing hormone

When this hormone became available as a therapeutic modality, several publications reported good results on undescended testicles. However, later controlled studies were unable to repeat these results, and it has been concluded that GnRH treatment is most successful in boys with retractile or high scrotal testes. If results of the professional examination during the neonatal period are available, such information is of great help for predicting whether hormonal treatment (hCG or GnRH) will be effective: only boys who had palpable testes at birth will respond to hormonal treatment. Also, success is rare if the testicle is not palpable at the time of later examination. The mechanism of action seems to be through increased release of pituitary gonadotropins that stimulate secretion of testosterone in a mode similar to hCG but with a more moderate stimulation of Leydig cell testosterone production. Thus, it can be considered similar to a mild hCG treatment. No side effects of GnRH treatment of undescended testes have been reported.

Surgery (Orchidopexy)

Open surgery is the most direct way of transferring an abdominal or inguinal testis into the scrotum. This method has been used for a very long time. The major complications are surgical: in addition to the small risk of general anesthesia, the blood supply to the testis might be damaged during surgery. A high incidence of testicular atrophy after surgical orchidopexy has been reported from departments in which "scrotal and inguinal surgery" (including orchidopexy and hernia repair) is considered to be an activity for surgeons in training. In skilled hands, this complication should be rare if the testis is not situated very high in the abdomen. Surgery has the added advantage that it provides opportunities for direct observation of the testis and, in selected cases, biopsy for microscopical investigations.

Comparison Between Hormonal and Surgical Treatment

The main advantages of hormonal treatment are that it is inexpensive and that hospitalization and the trauma of anesthesia and surgery are avoided (when successful). This method also provides an opportunity to test the capacity of the non-palpable testicles to produce testosterone if blood samples are taken at the end of hormone administration.

The major reason for treating undescended testicles is to save future fertility. Unfortunately, there are no published studies in which patients have been randomized to surgical or hormonal treatment and followed up into adulthood. Therefore, a scientifically based conclusion regarding the advantages of the two approaches in terms of future fertility cannot be made. The possible adverse effects of hCG on future spermatogenesis and the poor efficacy of hCG or GnRH argue against this as the first-line therapy; instead, a primary surgical approach is favored, provided that a skilled surgeon is available.

Age at Which Undescended Testicles Should be Treated

Again, the scientific foundations for recommendations are weak. The single classical report by the Swiss pathologist Hedinger (1979) is generally referred to as an argument for early treatment. He compared sections from undescended and normally descended testicles of boys who had died suddenly. The number of spermatogonia per seminiferous tubular cross section was determined. He noted that in normally descended testicles, there was a steady decline in the number of spermatogonia from birth until one or two years of age, followed by a slow increase that continued until puberty. In the undescended testes, the initial decline was similar, but the normal increase in spermatogonia after age 2 years did not occur.

The Hedinger study seems to show that undescended testes should be brought into the scrotum before the age of 2 years. It is assumed that spermatogenesis will be improved after successful treatment. Repeat follow-up biopsies have been studied in only two publications: one showed improved spermatogenesis, one did not. It is not known whether the catch-up of spermatogenesis is more complete after early rather than after later treatment. Nevertheless, treatment before age two has become the standard of treatment in many countries. If this approach is followed, initial surgery is to be preferred rather than hormonal treatment since the latter seems to be even less effective at ages younger than 4 years.

Final recommendations about the most suitable age for treatment of undescended testes must await results of controlled, randomized studies of treatment at various ages. The final outcome to be evaluated in such studies should be spermatogenesis and fertility in adulthood. This requires very long-term prospective studies. Careful measurements of testicular size during the first years after treatment may to some extent predict spermatogenesis and should be performed in future studies.

Conclusions

The condition of undescended testicles is the most common inborn developmental abnormality in boys, affecting approximately 1% of the entire male population. The cause may be hormonal deficiencies or, in most cases, unknown. Untreated, undescended testicles always have severely impaired spermatogenesis, but testosterone production is generally acceptable. The risk of malignancy in undescended testicles is increased. If the testes are moved into the scrotum, future spermatogenesis will be improved but not to the full extent. The risk of malignancies is reduced by orchidopexy before puberty. Both the choice of treatment (hormonal or surgical) and the most appropriate age of treatment (1–4 years) are controversial. However, based on current knowledge, skilled surgery at an early age, rather than hormonal treatment, seems to be advisable.

See also: Association of Endocrine Disrupting Chemicals With Male Reproductive Health

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Further Reading

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Human Aromatase Deficiency

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Abbreviations

ArKO	Aromatase knockout
BMD	Bone mineral density
CRE	cAMP response element
CREB	cAMP response element binding protein
CYP19A1	Aromatase gene
DHEAS	Dehydroepiandrosterone sulfate
E2	Estrogen
ERT	Estrogen replacement therapy
GH/IGF1 axis	Somatotropic axis
GnRH	Gonadotropin releasing hormone
ND	Not determined
SF1	Steroidogenic factor 1
WT	Wild type

Glossary

Acanthosis nigricans Skin pigmentation disorder in which dark thick poorly defined areas of velvety texture appear especially in the neck, armpit, and body folds. Related to hyperinsulinism.
Aromatization The last step in estrogen synthesis. The reaction includes three hydroxylations of the 19 methyl group of the androgen molecule with the simultaneous elimination of the methyl group that results in the formation of a benzene ring.
Astenospermia Less than 50% of motile spermatozoa.
Azoospermia Absence of spermatozoa in the ejaculate.
Congenital adrenal hyperplasia Group of steroidogenic disorders that impair cortisol biosynthesis.
Disorders of sexual development Congenital conditions in which chromosomal, gonadal, or anatomical sex is atypical.
GnRH pulse generation Hypothalamic neuronal network that control the pulsatile secretion of gonadotropins.
Hypospadias Disorder of the anterior urethral and penile development in which the urethral opening is ectopically located on the ventral aspect of the penis.
Osteopenia A skeletal condition characterized by a decreased bone mineral density when compared with the reference standard (between -1 and -2.5 SD).
Osteoporosis A skeletal condition characterized by a decreased bone mineral density when compared with the reference standard (under -2.5 SD).
Polycystic ovary syndrome Disorder characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology.
Teratospermia Less than 30% of morphologically normal spermatozoa.

Several molecular CYP19A1 gene alterations associated with cytochrome P450 aromatase (cP450arom) deficiency have been described in both females and males. This has contributed considerably to the understanding of cP450arom activity in different tissues, influencing sexual differentiation, patterns of gonadotropin secretion by the hypothalamic–pituitary axis, reproductive capacity, lipid metabolism, insulin sensitivity, and skeletal maturation and growth. Studies of aromatase deficiency in humans were complemented with studies in mouse knockout models that demonstrated the role of estrogens in several tissues. In this article, molecular studies, the clinical phenotypic variations throughout life in both sexes, gonadal function, fertility, and gender identity are addressed.

Characteristics of cP450arom

cP450arom is the enzyme that catalyzes the synthesis of estrogens from androgens. Therefore, the activity of this enzyme complex affects both androgen metabolism and estrogen synthesis. The biological importance of the aromatase complex is related not only to its role in the synthesis of estrogens, but also to its potential influence on the balance of the androgen–estrogen ratio in different tissues. In the 1980s, the human aromatase protein was purified from placental microsome and the aromatase activity was demonstrated by conversion of androstenedione to estrone (Pasanen and Pelkonen, 1981; Mendelson *et al.*, 1985; Kellis and Vickery, 1987; Osawa *et al.*, 1987; Hall *et al.*, 1987). The aromatase

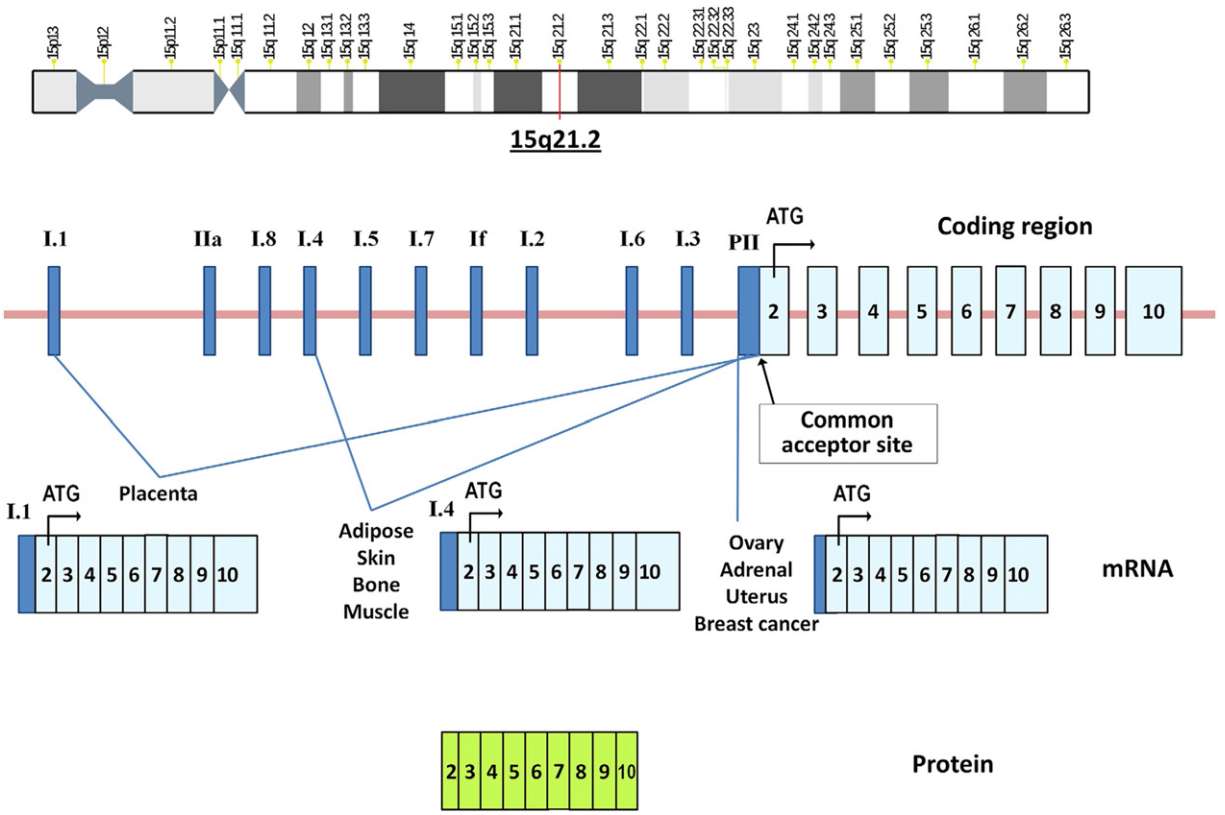

Fig. 1 Representation of the CYP19A1 splicing mechanism.

complex is expressed in the endoplasmic reticulum of cells and consists of two components: cP450arom and a ubiquitous flavoprotein, NADPH-cytochrome P450 reductase. Although androstenedione and testosterone are the most common and the most physiologically important substrates, 16OH-dehydroepiandrosterone sulfate (DHEAS) arising from fetal liver hydroxylation of fetal adrenal DHEAS is an important substrate for placental estriol synthesis during pregnancy, in both humans and higher primates (Belgorosky and Rivarola, 2004). In humans, cP450arom appears to be the product of a single gene—CYP19A1—located on chromosome 15q21.1. The size of the aromatase gene is larger than 123 kb; however, the protein-coding sequence is contained within 9 exons (2 – 10) spanning approximately 35 kb of DNA (Means *et al.*, 1989). Several promoters are found within a 90-kb region upstream of the coding region associated with multiple first exons that are involved in tissue-specific expression. These exons, which are not translated, generate alternative splicing in such a fashion that the coding region, and hence the protein sequence, is conserved in every tissue (**Fig. 1**).

The three-dimensional crystal structure of human aromatase was identified with purified aromatase protein extracted from placental microsome (Ghosh *et al.*, 2009). The human aromatase consists of a heme group and a polypeptide chain of 503 amino-acid residues and exhibits high substrate specificity in catalyzing the synthesis of estrogens from androgen precursors. The most highly conserved region consists essentially of a four-helix bundle, two sheets, and the heme-binding region (Graham-Lorence *et al.*, 1995).

cP450arom is expressed in a number of tissues, including the syncytiotrophoblast layer of the placenta, gonads, adipose tissue, bone, brain (including the hypothalamus, hippocampus, and amygdala), coronary arteries, and various fetal tissues, such as liver, skin, intestine, testis, and ovary (Graham-Lorence *et al.*, 1995). CYP19A1 gene expression is subjected to multifactor regulation by a diverse group of hormones and factors that differ markedly between tissues. Thus, a strict control over tissue-specific expression is necessary for proper regulation of estrogen synthesis during fetal development, as well as during postnatal life.

The human placenta (syncytiotrophoblast layer) is capable of aromatizing large amounts of androgen precursors produced by the fetal and maternal adrenal glands into estrogens. At term, approximately equal amounts of estrogens are produced from circulating maternal DHEAS and fetal DHEAS. The rate of estrogen production and the level of circulating estrogens increase markedly during pregnancy. Following fertilization, concentrations of 17β-estradiol increase gradually to a range of 6–30 ng/mL at term (Tal *et al.*, 2000). This enzyme protects the fetus from virilization even in the presence of large amounts of aromatizable androgens. One of the clinical signs of aromatase deficiency may manifest during pregnancy, as a pregnant mother of an aromatase-deficient fetus becomes virilized (see later).

Reported Human Mutations

Since aromatase deficiency was first described by Shozu *et al.* (1991), 37 cases (26 females and 11 males) have been published (PubMed indexed). These cases are listed in **Tables 1** and **2**.

Several deleterious mutations in the coding region of the CYP19A1 gene were reported in aromatase deficient patients, comprising point mutations, deletions, insertions, splice site mutations, as well as a placental promoter I.1 variation that reduced gene transcription.

Tables 1 and **2** describe the molecular defects, in-vitro aromatase activity of mutants, and clinical phenotype in female and male aromatase-deficient subjects, respectively. Variations in phenotype are compared with in-vitro functional derangements of mutants. Data reported suggest some genotype–phenotype correlation as lower cP450arom activity was associated with a more severe phenotype. However, phenotype is also dependent on sex and age.

Pregnancy, the Fetus, and Newborns

The active human placental aromatization of androgens protects the fetus against the virilizing action of fetal androgens. In congenital aromatase deficiency, the overload of androgens may cause signs of maternal virilization (acne, deep voice, clitoris enlargement) during pregnancy, and this might alert obstetricians to the possibility of this diagnosis. After delivery these symptoms usually disappear gradually (Shozu *et al.*, 1991; Morishima *et al.*, 1995; Mullis *et al.*, 1997; Ludwig *et al.*, 1998; Deladoëy *et al.*, 1999; Herrmann *et al.*, 2002; Belgorosky *et al.*, 2003; Lin *et al.*, 2007; Hauri-Hohl *et al.*, 2011; Verma *et al.*, 2012; Ludwikowski *et al.*, 2013; Marino *et al.*, 2015; Akçurin *et al.*, 2016; Zhu *et al.*, 2016; Miedlich *et al.*, 2016). Nevertheless, this finding is not always present, since about 1% of normal placental aromatase activity seems enough to prevent virilization of the mother. Therefore, CYP19A1 mutations that retained partial activity did not lead to maternal virilization during pregnancy (Mullis *et al.*, 1997; Grumbach and Auchus, 1999). Patient data suggest that estrogen synthesis in the blastocyst, fetus, and placenta is not essential either for normal embryonic and fetal development and survival or in the physiology of the pregnant woman (Conte *et al.*, 1994; Morishima *et al.*, 1995). Onset of labor has been described as spontaneous (Shozu *et al.*, 1991; Mullis *et al.*, 1997; Deladoëy *et al.*, 1999; Belgorosky *et al.*, 2003) and newborns were born full term with adequate weight for gestational age (Shozu *et al.*, 1991; Conte *et al.*, 1994; Morishima *et al.*, 1995; Mullis *et al.*, 1997; Deladoëy *et al.*, 1999; Belgorosky *et al.*, 2003; Bouchoucha *et al.*, 2014; Zhu *et al.*, 2016).

In the female fetus, placental aromatization of androgens is particularly important to avoid an effect of androgens on the differentiation of the external genitalia. In a newborn with 46,XX ambiguous genitalia, aromatase deficiency should be considered among other entities after ruling out the diagnosis of congenital adrenal hyperplasia (CAH) because of its high incidence. Moreover, before the definitive diagnosis of aromatase deficiency was made, some patients were assumed to have CAH and treated as such (Verma *et al.*, 2012; Saraco *et al.*, 2015).

In most female cases of aromatase deficiency described in the literature, ambiguous genitalia, with various degrees of masculinization of the external genitalia, were reported. As expected, in all these cases, gonads were nonpalpable and differentiation of the female internal genitalia was not affected. Milder genital manifestations, such as clitoral hypertrophy or partial fusion of the labia, have been described in four females (Lin *et al.*, 2007; Hauri-Hohl *et al.*, 2011; Marino *et al.*, 2015; Akçurin *et al.*, 2016). The discordant presentation of mild androgenization with the complete lack of enzyme activity described by Lin is difficult to explain and it is a matter of speculation. Hauri-Hohl *et al.* (2011) described a newborn with transient mild hypertrophy of the clitoris having a loss-of-function missense mutation in CYP19A1 combined with the first-described variant of the placenta promoter with a significant reduction in function. This phenotype might represent a placenta-specific, prenatally limited component of aromatase deficiency occurring in utero only.

Even though aromatase deficiency manifests during fetal life in both sexes, external genitalia in 46,XY newborns remain normal and there are no symptoms of aromatase deficiency during infancy and childhood in most boys (Deladoëy *et al.*, 1999). In only one boy reported by Bouchoucha *et al.* (2014) the presence of glandular hypospadias and bilateral cryptorchidism with inguinal testes was described; however, causality between the *CYP19A1* mutation and the genital anomaly in this patient remains elusive.

An endocrinological profile has been described in some female cases with aromatase deficiency during the first month of postnatal life. Low estrogen levels along with high androgen levels were found in the cord serum and serum androgen levels returned rapidly to normal after delivery in some cases (Shozu *et al.*, 1991; Deladoëy *et al.*, 1999). Extremely high serum LH and FSH levels resulting in high serum androgen levels were reported in two aromatase-deficient newborn girls (Belgorosky *et al.*, 2003; Akçurin *et al.*, 2016). This abnormal serum gonadotropin pattern might reflect a central change in the activity of the gonadotropin releasing hormone (GnRH) pulse generator and/or an effect at the pituitary level, presumably induced by increased androgens and aromatase deficiency during fetal and neonatal life. Bouchoucha *et al.* (2014) described a female newborn who presented with elevated serum testosterone and androstenedione levels at birth that normalized thereafter. Normal serum testosterone levels were reported in an affected girl of 3 days (Conte *et al.*, 1994). No data exist on gonadotropin levels during the newborn period in affected boys. Very high serum-free testosterone and androstenedione levels at 2 weeks of postnatal life followed by a decrease to the normal range during the first month of life was reported in only one affected newborn boy (Deladoëy *et al.*, 1999). At 4 weeks after birth, the estradiol levels were low; the androstenedione level was decreasing toward the normal level for age and sex, whereas the free testosterone level had already dropped to within the normal range.

Table 1 Molecular defects of the *CYP19* gene, in vitro aromatase activity of mutants, and clinical phenotype in published female deficient subjects

Report	*Gene mutations*	*Description*	*Aromatase activity*	*Phenotype*
Shozu et al. (1991), Harada *et al.* (1992)	c.[743 + 2T > C]; [743 + 2T > C]	IVS6 + 2T > C Mutation at consensus 5′splice site (5′ss) in intron 6 which results in the use of a cryptic site downstream and in the in-frame incorporation of 87 nucleotides. Then, a protein with additional highly hydrophobic 29 amino acids will be generated	Less than 0.3% of wild type (WT) control. The extra amino acids might induce conformational changes and reduction of activity	Ambiguous genitalia at birth
Ito *et al.* (1993), Conte *et al.* (1994)	c.[1303C > T]; [1310G > A]	p.Arg435Cys: Arg435 is highly conserved within the heme-binding region among cP450s p.Cys437Tyr: The cysteine 437 is the critical cysteine in the heme-binding domain and is the most conserved residue in all cP450 proteins	p.Arg435Cys mutant protein showed approximately 1.1% of WT activity p.Cys437Tyr mutant protein was inactive	Ambiguous genitalia at birth Delayed bone age Absent puberty and pubertal growth spurt. Ovarian cyst and virilizing signs at puberty
Morishima *et al.* (1995)	c.[1123C > T]; [1123C > T]	p.Arg375Cys	0.2% of WT activity Protein modeling studies suggest that the affected region may affect the substrate access channel to the membrane	Ambiguous genitalia at birth Absent puberty and pubertal growth spurt According bone age Ovarian cyst and virilizing signs at puberty
Mullis *et al.* (1997), Janner *et al.* (2012), Burckhardt *et al.* (2015)	c.[1224delC]; [296 + 1G > A]	p.Lys409AsnfsTer36: A base pair deletion (C) in codon 408 (exon 9). A frameshift occurs resulting in a nonsense codon IVS3 + 1G > A: G > A change at the canonical donor splice site in intron 3. The splice site is ignored, and a stop codon arises 3 bp downstream. No active transcript was found	ND. However, no aromatase activity is expected	Ambiguous genitalia at birth Ovarian cysts during infancy and childhood Low BMD at 3.5 years of age Follow-up from 3.5 to 15 years under ERT. Normalization of growth and bone maturation with different threshold for estradiol action on FSH regulation
Ludwig *et al.* (1998)	c.[1108G > A]; [1108G > A]	p.Val370Met	ND	Ambiguous genitalia at birth Lipid abnormalities early in life
Belgorosky *et al.* (2003), Pepe *et al.* (2007), Guercio *et al.* (2009)	c.[1236delA]; [628G > A]	p.Glu412AspfsTer33: a bp deletion (A) in glu 412 (exon 9) causing a frame-shift generating a stop codon 98 bp downstream. Then, a truncated protein with an altered heme-binding domain might be generated c.628G > A: G to A at the consensus donor splice site in exon 5–intron 5 junction causing in-frame exon 5 skipping	The peptide expressed from p. Glu412AspfsTer33 is expected to be completely inactive as the frame shift altered the highly conserved heme-binding domain The protein without amino acids 151–209 encoded by exon 5 does not have aromatase activity As the –E5 mRNA might be expressed as a splice variant in normal tissues, some + E5 transcripts from the c.628G > A allele might explain the partial Aromatase deficiency phenotype (Lin *et al.*, 2007; Pepe *et al.*, 2007)	Ambiguous genitalia at birth Delayed bone age during childhood Ovarian cysts from infancy to puberty Spontaneous breast development at 7.7 years Virilizing signs at puberty Normal BMD during follow-up Insulin resistance since 7.2 years of age and Type 2 diabetes during follow-up
Lin *et al.* (2007)		p.Arg435Cys		

(*Continued*)

	c.[1303C>T]; [1303C>T]		0.7%–1.5% of WT activity The arginine at position 435 forms part of the absolutely conserved heme-binding motif and is absolutely conserved This mutation destabilizes the active site of the aromatase enzyme, which disrupts the efficient functioning of the enzyme complex	Ambiguous genitalia at birth Delayed bone age Ovarian cysts Spontaneous but nonprogressive breast development
Lin *et al.* (2007) (siblings)	c.[700_702delTTC]; [700_702delTTC]	p.Phe234del: Deletion of one of two phenylalanine at position 234–235 in exon 6. This pair of amino acids is highly conserved in cP450 aromatase of other species	16%–19% of WT activity. Substrate binding seems to be largely unaffected but maximal enzymatic activity is reduced consistent with partial aromatase activity	Sibling 1: Ambiguous genitalia at birth. Low androgen levels in infancy Sibling 2: Pubertal subject raised male. Male gender identity and male gender role behavior. Spontaneous puberty and gynecomastia. Gender dysphoria delayed bone age and ovarian cysts
Lin *et al.* (2007)	c.[452-621_628+803del]; [452-621_628+803del]	1600 bp deletion at genomic DNA. This deletion is predicted to remove exon 5 resulting in the in-frame deletion 59 amino acids	Complete loss of function	Ambiguous genitalia at birth Absent puberty and delayed bone age Small ovaries Low androgen levels Moderate dyslipidemia
Hauri-Hohl *et al.* (2011)	c.[1374A>G]; c.[−41C>T]	De novo p.Asn411Ser Paternally inherited C>T mutation in the placenta promoter 41 base pairs upstream of exon 1	Functional studies demonstrated that p.Asn411Ser is a loss-of-function mutation. Vmax, Km and Kcat demonstrated null catalytic activity Cell-culture system for placenta promoter functional studies demonstrated a 50% significant reduction in the presence of c.-41C>T mutation and the mutant promoter has no dominant negative effect on WT	Clitoromegaly at birth Normal ovaries Normal serum prepubertal androgens and gonadotropin levels
Verma *et al.* (2012)	c.[1303C>T]; [1303C>T]	p.Arg435Cys	0.7%–1.5% of WT activity The arginine at position 435 forms part of the absolutely conserved heme-binding motif and is absolutely conserved This mutation destabilizes the active site of the aromatase enzyme, which disrupts the efficient functioning of the enzyme complex	Ambiguous genitalia at birth High FSH levels during infancy At 13.5 years hirsutism Spontaneous telarche Obesity, acanthosis nigricans, and tall stature. High gonadotropin levels and bilateral cystic ovaries. Persistent hyperinsulinemia despite ERT and metformin supplementation. Normal lipid profile. Low BMD at 16 years
Ludwikowski *et al.* (2013)	c.[422G>A]; [422G>A]	p.Trp141Ter	ND	Ambiguous genitalia at birth Elevated serum LH and FSH along with low estradiol and androgen levels, and normal ovaries in infancy (20 months)
Ludwikowski *et al.* (2013)	c.[422G>A]; [422G>A]	p.Trp141Ter	ND	Ambiguous genitalia at birth Elevated serum LH and FSH along with low

Table 1 Continued

Report	*Gene mutations*	*Description*	*Aromatase activity*	*Phenotype*
				estradiol and androgen levels, and normal ovaries in childhood (5 years)
Gagliardi *et al.* (2014)	c.[915_941dup] Homozygous or hemizygous	Duplication of nine amino acids (p. Ala306_Ser314dup) in the protein This duplication occurs within the aromatase α-helix Prediction tools of protein secondary structure suggest a break of the α-helix around the middle, turning a continuous helix into the structure of helix coil-helix. This would be likely to disrupt substrate and cofactor binding resulting in a lack of estrogen synthesis	ND	Ambiguous genitalia at birth Absent puberty Hypoplastic uterus and bilateral streak ovaries At 32 years tall stature, eunuchoidal proportions, abdominal obesity, impaired fasting glucose, dyslipidemia and insulin resistance, moderate hepatic steatosis Osteopenia/osteoporosis Bone fracture at 25 years
Bouchoucha *et al.* (2014)	c.[575G>A]; [575G>A]	p.Arg192His	p.Arg192His mutant was found to have markedly reduced aromatase activity. Both the km and the Vmax were adversely affected. The catalytic efficiency of metabolizing androstenedione was reduced to 19%. Modeling of the structure of the novel p.Arg192His variant of the CYP19A1 protein revealed a crucial role of the arginine 192 residue in substrate binding as well as catalysis	Ambiguous genitalia with elevated serum androgen levels at birth, normalizing thereafter Persistently elevated serum FSH levels
Marino *et al.* (2015)	c.[628G>A]; [628G>A]	G to A at the consensus donor splice site in exon 5–intron 5 junction causing in-frame exon 5 skipping	The protein without amino acids 151–209 encoded by exon 5 does not have aromatase activity (Lin *et al.*, 2007; Pepe *et al.*, 2007)	Ambiguous genitalia at birth Spontaneous breast development at 11 years. Spontaneous menarche at 12 years with regular menses. Clinical and biochemical hyperandrogenism, high basal serum gonadotropin levels, and multiple ovarian cysts at puberty
Marino *et al.* (2015)	c.[628G>A]; [242A>G]	c.628G>A: G to A at the consensus donor splice site in exon 5–intron 5 junction causing in frame exon 5 skipping p.Tyr81Cys. Residue highly conserved	c.628G>A: The protein without amino acids 151–209 encoded by exon 5 does not have aromatase activity (Lin *et al.*, 2007; Pepe *et al.*, 2007) p.Tyr81Cys: Predicted to be "probably damaging" on the basis of SIFT scores of 0.00 and PolyPhen2 scores of 1.0 indicating that it is most likely to be deleterious The p.Tyr81Cys mutant was found to have 14.3% aromatase activity and showed lower Vmax, higher Km, and lower	Ambiguous genitalia at birth At 7.7 years multiple ovarian cysts and bone age delay, normal serum androgen and high basal serum gonadotropin levels

(*Continued*)

			catalytic efficiency (Vmax/Km) as compared with wild-type aromatase (Chen *et al.*, 2015)	
Marino *et al.* (2015)	c.[628G>A]; [628G>A]	G to A at the consensus donor splice site in exon 5–intron 5 junction causing in-frame exon 5 skipping	The protein without amino acids 151–209 encoded by exon 5 does not have aromatase activity (Lin *et al.*, 2007; Pepe *et al.*, 2007)	Ambiguous genitalia at birth Bone age delay Spontaneous breast development at 9 years with large ovarian cysts high basal gonadotropin and testosterone levels at puberty. Normal OGTT
Marino *et al.* (2015)	c.[574C>T]; [574C>T]	p.Arg192Cys: residue highly conserved	ND SIFT tool predicted this variant to affect protein function with a highly deleterious tolerance index score of 0.00 The mutation was predicted to be probably damaging with a score of 1.000 (sensitivity 0.00; specificity 1.00) using the structure based approach PolyPhen-2	Ambiguous genitalia at birth Normal serum androgens and high basal gonadotropin levels at 3 years Normal OGTT
Marino *et al.* (2015)	c.[574C>T]; [1369C>T]	p.Arg192Cys: residue highly conserved p.Arg457Ter	ND	Ambiguous genitalia at birth Spontaneous breast development at 10 years with enlarged ovaries. Normal serum basal gonadotropin and androgens levels at puberty Normal OGTT
Saraco *et al.* (2015)	c.[1263+5G>A]; [1263+5G>A]	IVS9+5G>A The mutation is localized five bases from the 5′ beginning of intron 9 Several splicing prediction programs confirmed that the splicing donor site disappears in the presence of the mutation, resulting in retention of intron 9	Abnormal spliced mRNA that includes intron 9 was confirmed by RT-PCR of total RNA from patient's lymphocytes and with Splicing Assays The presence of an in-frame stop codon 18 bp downstream of the splice junction in intron 9 results in the synthesis of a truncated (46-kDa) and inactive aromatase protein	Ambiguous genitalia Normal ovaries and uterus at 4 years At 13.5 years delayed bone age, small uterus and bilateral ovarian cysts, primary amenorrhea, elevated basal serum LH and FSH levels. Low BMD under ERT at 16.4 years
Akçurin *et al.* (2016)	c.[568insC]; [568insC]	p. Leu190ProfsTer9	ND	Clitoromegaly, partial labial fusion, and hyperandrogenism at birth. Severe neonatal hypoxic-ischemic encephalopathy Normal serum androgens and gonadotropins levels at 7 years. Hypoplastic ovaries
Akçurin *et al.* (2016)	c.[568insC]; [568insC]	p.Leu190ProfsTer9	ND	Ambiguous genitalia and hyperandrogenism at birth At 5 years mild clitoromegaly, normal serum androgens and high basal gonadotropin levels Hypoplastic ovaries
Akçurin *et al.* (2016)	c.[568insC]; [568insC]	p.Leu190ProfsTer9	ND	Ambiguous genitalia and hyperandrogenism at birth

Table 1 Continued

Report	*Gene mutations*	*Description*	*Aromatase activity*	*Phenotype*
				At 2.2 years clitoromegaly, normal serum androgens and high basal gonadotropin levels Hypoplastic ovaries.
Zhu *et al.* (2016)	c.[264delG]; [1036_1037insTTC-TGTTTCCA-CAGGTGAGAGAG]	p.Trp88Ter; p.Lys346IlefsTer21	Modeling studies based on reported structures indicated that both mutations lead to a loss of aromatase catalytic residues that form the active site and access channel, as well as the heme-binding region of the enzyme encoded by exon 10 Aromatase enzyme activity revealed nearly complete abolishment of enzyme activity with very low estrone conversion levels from androstenedione substrate	Ambiguous genitalia at birth

ND, not determined; *ERT*, estrogen replacement therapy; *BMD*, bone mineral density; *OGTT*, oral glucose tolerance test.

Table 2 Molecular defects of the *CYP19* gene, in vitro aromatase activity of mutants and clinical phenotype in published male deficient subjects

Report	*Gene mutations*	*Description*	*Aromatase activity*	*Phenotype*
Morishima *et al.* (1995), Bilezikian *et al.* (1998)	c.[1123C>T]; [1123C>T]	p.Arg375Cys in a highly conserved region	0.2% of WT activity Protein modeling studies suggest that the affected region may be important in anchoring the region of the protein proximal to the substrate access channel to the membrane	Continuous linear growth, delayed bone maturation, tall stature, eunuchoid body proportions Cis gender, heterosexual, modest libido Macroorchidism Increased basal gonadotropins and testosterone Hypertension, obesity Dyslipidemia, insulin resistance Osteopenia
Carani *et al.* (1997)	c.[1094G>A]; [1094G>A]	p.Arg365Gln	0.4% of WT activity	Continuous linear growth, delayed bone maturation, tall stature, eunuchoid body proportions Moderate bone pain. Genu valgum Cisgender identity and sexual orientation. Normal libido Infertility Microorchidism Normal basal testosterone, slightly elevated FSH and LH in the upper normal range Overweight. Dyslipidemia Normal OGTT
Deladoëy *et al.* (1999), Bouillon *et al.* (2004)	c.[469delC]; [469delC]	p.Val158PhefsTer20 C base deletion in exon 5 causing a frame shift and a premature stop codon after 21 codons	Not determined (ND) However, the resultant peptide does not contain the substrate-binding pocket (I helix), the electron-accepting site and the heme-binding site. An inactive protein would be expected	At birth: Normal genitalia, and serum AMH levels Normal serum basal and GnRH stimulated FSH levels at 2 months of age At 16 years: Tall stature, delayed bone maturation Normal virilization and testicular volume High serum basal testosterone, and normal serum basal gonadotropin levels Osteoporosis
Herrmann *et al.* (2002)	c.[628-3C>A]; [628-3C>A]	IVS5-3C>A C to A transition at bp −3 at the splicing acceptor site in exon 6. Exon 6 is completely excised leading to a frame shift and premature stop codon 8 nucleotides downstream the end of exon 5	ND The resulting peptide most likely will not be processed, but even if it were, it would not result in a functional aromatase because it lacks the substrate-binding pocket, the electron-accepting site and the heme-binding site (Herrmann *et al.* 2002)	Continuous linear growth, tall stature, eunuchoid body proportion Genu valgum, kyphoscoliosis, pectus carinatus Cisgender, heterosexual, and normal libido Normal genitalia and testicular volume Increased serum basal testosterone and FSH, normal serum LH levels. Azoospermia Obesity. Insulin resistance. Osteoporosis
Maffei *et al.* (2004)	c.[628G>A]; [628G>A]	G to A transition in the last nucleotide in exon 5 The mutated DNA will generate an mRNA that includes the intron 5 sequence which	ND A truncated and inactive protein lacking the heme-binding domain would be expected	Continuous linear growth, delayed bone maturation, tall stature, eunuchoid body proportions Diffuse bone pain, genu valgum Cisgender, heterosexual, referred normal libido

Table 2 Continued

Report	*Gene mutations*	*Description*	*Aromatase activity*	*Phenotype*
		contains an in-frame stop codon 30 bp downstream the splice junction		Bilateral cryptorchidism (surgery unsuccessful at 6 years). Small inguinal testes, total germ depletion (biopsy) Normal serum basal LH and testosterone, and increased FSH levels Overweight, dyslipidemia. Type 2 diabetes Osteoporosis
Maffei *et al.* (2007)	c.[380T>G]; [1124G>A]	p.Met127Arg; p.Arg375His	In vitro analysis demonstrated a reduction of aromatase activity when the two mutations were expressed separately or coupled.p. Arg375His showed aromatase activity of 7%. Aromatase activity decreased to 0% when the two mutations were coupled	Continuous linear growth, delayed bone maturation, tall stature Diffuse bone pain, genu valgum Cisgender, heterosexual, normal libido Normal testicular volume Normal serum LH and testosterone, increased FSH levels Focal hypospermatogenesis on testicular biopsy Obesity, acanthosis nigricans, hepatomegaly. Moderate dyslipidemia, insulin resistance Osteoporosis
Lanfranco *et al.* (2008)	c.[312_334del]; [1263+1G>A]	p.Phe312LeufsTer49: 23 bp deletion in exon 4 that would be expected to cause a frame shift with a premature stop codon at nucleotide 361 in exon 4 IVS9+1G>T: point mutation in the first nucleotide of intron 9 that would lead to an aberrant splicing of the mRNA	A truncated and inactive protein lacking the heme-binding domain would be expected from the c.312_334del allele	Continuous linear growth, delayed bone maturation, tall stature, eunuchoid body proportions, genu valgum Cisgender, normal sexual orientation Normal testicular volume. Right cryptorchidism surgery at 3 years Mild asteno-teratozoospermia. Increased serum basal FSH with normal LH and testosterone levels Overweight, insulin resistance. Mild dyslipidemia Osteoporosis
Baykan *et al.* (2013)	c.[1124G>A]; [1124G>A]	p.Arg375His Previously reported (Maffei *et al.*, 2007)		Continuous linear growth, delayed bone maturation, tall stature, eunuchoid body proportions Bone pain, fractures Macroorchidism Normal serum gonadotropin with increased testosterone levels Normal sperm count Overweight. Dyslipidemia Normal OGTT. Hepatosteatosis. Osteoporosis
Bouchoucha *et al.* (2014)	c.[575G>A]; [575G>A]	p.Arg192His Amino acid conserved across species and involved in substrate access and catalysis	p.Arg192His mutant was found to have markedly reduced aromatase activity. Both the km and the Vmax were adversely affected. The catalytic efficiency of	Mild hypospadias (glans), normal-length phallus, bilateral inguinal testes Normal serum FSH, LH, testosterone, AMH, and inhibin B levels

(*Continued*)

			metabolizing androstenedione was reduced to 19%. Modeling of the structure of the novel p.Arg192His variant of the CYP19A1 protein revealed a crucial role of the arginine 192 residue in substrate binding as well as catalysis	
Chen *et al.* (2015)	c.[384A>G]; [1494T>C]	p.Tyr81Cys; p.Leu451Pro Both the Tyr81 and Leu451 residues were highly conserved in vertebrate aromatase orthologs, and were also conserved in human aromatase paralogs	Three-dimensional modeling predicted that the p.Tyr81Cys and p.Leu451Pro mutations would probably result in loss of aromatase function In-cell aromatase activity assay: p. Tyr81Cys mutant was found to have 14.3% wild-type activity, whereas the p. Leu451Pro mutant was found to have 3.1% wild-type activity p.Tyr81Cys mutant showed lower Vmax, higher Km, and lower catalytic efficiency p.Leu451Pro mutant had lower Vmax, Km and catalytic efficiency	Continuous linear growth, delayed bone maturation, tall stature, eunuchoid body proportions, genu valgum Normal libido Normal testicular volume Normal spermogram Increased serum basal FSH, with normal LH and testosterone levels Overweight, acanthosis nigricans, dyslipidemia and severe steatohepatitis Impaired glucose tolerance and hyperinsulinemia Osteopenia
Miedlich *et al.* (2016)	c.[628G>A]; [628G>A]	Previously reported (Pepe *et al.*, 2007)		Continuous linear growth, delayed bone maturation, unfused growth plate, arachnodactyly Normal libido Normal testicular volume Normal serum FSH and LH levels with upper normal basal testosterone Low BMI. Normal OGTT Osteoporosis

ND, not determined; *BMI*, body mass index; *OGTT*, oral glucose tolerance test.

Follow-Up

In almost all cases of aromatase deficiency the clinical phenotype was reported only once, when initial diagnosis was made. Only a few reports describe longitudinal clinical and biochemical findings of the affected subjects of both sexes (Conte *et al.*, 1994; Bilezikian *et al.*, 1998; Bouillon *et al.*, 2004; Guercio *et al.*, 2009; Verma *et al.*, 2012; Janner *et al.*, 2012; Burckhardt *et al.*, 2015; Marino *et al.*, 2015; Saraco *et al.*, 2015). Fortunately, a growing number of reports has broadened the spectra of phenotypes and genetic mutations underlying this rare disorder and increased the knowledge on aromatase physiology.

The clinical phenotype of aromatase deficiency includes changes in the hypothalamic–pituitary–gonadal axis, ovarian cyst formation, alterations of growth, skeletal maturation, and bone homeostasis, as well as changes in insulin sensitivity and lipid profile. As previously mentioned, the phenotype depends on sex and age, and may vary according to the level of enzyme activity.

Hypothalamic–Pituitary–Gonadal Axis

Biochemical findings in aromatase-deficient patients have further clarified the role of estrogens on the sex steroid-gonadotropin feedback system in humans. Aromatase and estrogen receptor alpha are predominantly expressed in the pituitary (Scully *et al.*, 1997; Shughrue *et al.*, 1998) and the hypothalamus.

In patients with aromatase deficiency, the elevated levels of androgens fail to suppress gonadotropins into the normal range in the absence of estrogen supporting the primary role of estrogen in the feedback mechanism of gonadotropin secretion within the hypothalamic–pituitary–gonadal axis in both males and females (Conte *et al.*, 1994; Morishima *et al.*, 1995; Ludwig *et al.*, 1998; Belgorosky *et al.*, 2003; Lin *et al.*, 2007; Guercio *et al.*, 2009; Verma *et al.*, 2012; Janner *et al.*, 2012; Bouchoucha *et al.*, 2014; Marino *et al.*, 2015; Zhu *et al.*, 2016; Morishima *et al.*, 1995; Carani *et al.*, 1997; Herrmann *et al.*, 2002; Maffei *et al.*, 2004, 2007; Lanfranco *et al.*, 2008; Baykan *et al.*, 2013).

In girls with aromatase deficiency, during infancy and childhood the hypothalamic–pituitary–gonadal axis showed persistently high basal and stimulated serum FSH levels (Conte *et al.*, 1994; Mullis *et al.*, 1997; Ludwig *et al.*, 1998; Belgorosky *et al.*, 2003; Lin *et al.*, 2007; Verma *et al.*, 2012; Ludwikowski *et al.*, 2013; Bouchoucha *et al.*, 2014; Marino *et al.*, 2015; Akçurin *et al.*, 2016; Zhu *et al.*, 2016), suggesting that during infancy and childhood, minimal amounts of estrogens are required to restrain pituitary FSH in normal prepubertal females. Basal serum LH levels were quite variable, ranging from normal (Conte *et al.*, 1994; Mullis *et al.*, 1997; Belgorosky *et al.*, 2003; Lin *et al.*, 2007; Hauri-Hohl *et al.*, 2011; Verma *et al.*, 2012; Bouchoucha *et al.*, 2014; Akçurin *et al.*, 2016) to high (Ludwig *et al.*, 1998; Ludwikowski *et al.*, 2013; Marino *et al.*, 2015; Akçurin *et al.*, 2016). A serum LH hyper-response after LHRH stimulation was observed in two girls (Mullis *et al.*, 1997; Belgorosky *et al.*, 2003). Two patients showed initial high basal serum LH levels that normalized thereafter (Marino *et al.*, 2015; Zhu *et al.*, 2016). In two affected girls studied longitudinally (Belgorosky *et al.*, 2003; Janner *et al.*, 2012), different patterns of serum LH levels were found in response to GnRH during prepuberty. LH amplitude was reported to be normal by Janner *et al.* (2012) but high by Belgorosky *et al.* (2003). Belgorosky *et al.* also showed that in early prepuberty, along with normal serum androgen levels, peak serum LH response was 10 times lower than in late prepuberty. It has been known for many years that sex hormones of extragonadal origin (adrenal) or administrated exogenously produce not only the development of secondary sexual characteristics, but also an advance in the induction of the onset of GnRH-dependent puberty in boys and in girls. Therefore, in the study by Belgorosky *et al.*, it was speculated that the pubertal serum LH response to acute GnRH stimulation detected in this aromatase-deficient girl might be secondary to a prolonged effect of androgens on the central nervous system, which includes, among other effects, the maturation of the GH/IGF1 axis, resulting, in turn, in an irreversible maturation of the GnRH pulse generator. In the patient described by Hauri-Hohl *et al.* (2011), accordingly to the clinical phenotype, serum gonadotropin levels measured several times between the ages 4 and 10.5 were always normal. Interestingly, the patient presented with a placenta-specific, prenatally limited component of aromatase deficiency as mentioned earlier.

During puberty, basal and post-GnRH LH and FSH levels were elevated in almost all affected females (Conte *et al.*, 1994; Morishima *et al.*, 1995; Belgorosky *et al.*, 2003; Lin *et al.*, 2007; Guercio *et al.*, 2009; Janner *et al.*, 2012; Verma *et al.*, 2012; Gagliardi *et al.*, 2014; Marino *et al.*, 2015; Saraco *et al.*, 2015). Serum androgens (testosterone and androstenedione) levels were clearly high compared with normal values for age and stage of sexual development mirrored elevated LH levels, whereas serum estradiol levels were extremely low. However, normal serum gonadotropin and androgen levels were found at 10 years of age in an affected patient with a partial form of aromatase deficiency (Marino *et al.*, 2015). This hormonal pattern supports the concept of a primary role of estrogen in the negative feedback mechanism of gonadotropin secretion within the hypothalamic–pituitary–gonadal axis in females. Moreover, levels of serum gonadotropins and testosterone decreased after low-dose estrogen replacement in an aromatase-deficient man (Carani *et al.*, 1997). A different threshold for estradiol action in aromatase-deficient adolescents under estrogen therapy was reported by Guercio *et al.* (2009), Janner *et al.* (2012), Marino *et al.* (2015), and Saraco *et al.* (2015). Even though during late prepuberty and puberty, systemic estrogen administration was followed by clinical signs of estrogen response, such as normalization of growth and skeletal maturation, this was not able to normalize the pituitary–gonadal axis, particularly serum FSH levels and ovarian cysts. It was speculated that this atypical response may have been secondary to lower aromatase activity and poor local estrogen synthesis from androgen precursors in peripheral tissues not only at ovarian levels, but also at local hypothalamic–pituitary levels (Marino *et al.*, 2015; Saraco *et al.*,

2015). It seems unlikely that the lack of serum FSH level suppression was related to a low level of serum inhibin B since in an aromatase-deficient patient reported by Burckhardt *et al.* (2015), the upper normal serum inhibin B levels detected were unable to suppress serum FSH levels under low-dose E2 treatment.

In contrast to affected infant girls, hormonal studies in a 2-month-old boy reported by Deladoëy *et al.* (1999) revealed that basal and GnRH-stimulated FSH levels were normal despite low serum estrogen concentrations, suggesting that estrogens are not involved in the regulation of FSH secretion during infancy. Moreover, in this boy normal serum inhibin B levels for sex and age were found. Hence, it had been proposed that inhibin B might be a major contributor to the regulation of serum FSH secretion in normal infant males. The fact that in the affected boy, free testosterone and androstenedione levels were very high at 2 weeks of postnatal life, followed by a decrease to the normal range during the first month of life, suggests a role for cP450arom in fetal and newborn testicular function. Indeed, it has been described that cP450arom is highly expressed in fetal and neonatal human testes, compared with testes of individuals of older prepubertal ages (Berensztein *et al.*, 2006). This finding suggests that aromatase activity might play a role in modulating testosterone secretion: the high aromatase expression of human neonatal Leydig cells decreased at 2–4 months of age, while peak testosterone secretion occurred during the postnatal testicular activation period. A similar normal hormonal profile, compatible with normal testicular function and gonadotropin secretion, was reported in an affected prepubertal boy at 4 and 6 years of age (Bouchoucha *et al.*, 2014). Therefore, a gender-specific negative feedback mechanism could be suggested, at least during infancy and early childhood.

Data from early and midpubertal boys receiving estrogens or aromatase inhibitors suggest that once puberty starts, estrogens become involved in the negative feedback control of gonadotropin secretion (Kletter *et al.*, 1997; Wickman *et al.*, 2001).

Different studies have evaluated the role of estradiol in the control of gonadotropin secretion in males and have demonstrated that estrogens have a direct modulatory and inhibitory effect on the pituitary release and/or synthesis of LH and FSH (Finkelstein *et al.*, 1991). In adult men with aromatase deficiency slight increments of basal serum FSH were consistently found despite normal inhibin B levels (Morishima *et al.*, 1995; Carani *et al.*, 1997; Herrmann *et al.*, 2002; Maffei *et al.*, 2004, 2007; Lanfranco *et al.*, 2008; Baykan *et al.*, 2013; Chen *et al.*, 2015). Only two male patients with normal FSH levels together with increased basal testosterone levels were reported (Bouillon *et al.*, 2004; Miedlich *et al.*, 2016). It could be suggested that high serum testosterone might be responsible for a complete negative feedback on gonadotropins even in the absence of estradiol. However, in other affected individuals, basal serum FSH levels were increased even when testosterone was elevated above the reference values (Morishima *et al.*, 1995; Rochira *et al.*, 2006). We can also speculate that in the presence of high serum testosterone levels, minimal residual aromatase activity could already be enough for gonadotropin suppression (Miedlich *et al.*, 2016). Nonetheless, normal serum FSH levels were also found in an aromatase-deficient man with null mutations within the CYP19A1 gene (Bouillon *et al.*, 2004).

Basal serum LH levels are within the normal range in most affected aromatase-deficient men, and in only two cases mild increases in serum LH levels were reported (Morishima *et al.*, 1995; Baykan *et al.*, 2013). Nevertheless, when assessed by dynamic tests, increases in LH pulsatility and pulse amplitude were demonstrated in affected men (Rochira *et al.*, 2006; Hayes *et al.*, 2000).

Puberty

Classically, in complete aromatase deficiency at puberty, lack of estrogens results in hypergonadotropic hypogonadism with failure of spontaneous pubertal development and primary amenorrhea but excessive virilization (Conte *et al.*, 1994; Morishima *et al.*, 1995; Lin *et al.*, 2007; Gagliardi *et al.*, 2014). The pubertal spurt is absent and bone age delayed.

Since the first description of partial forms of aromatase deficiency by Lin *et al.* in 2007, variable or nonclassic phenotypes have been reported (Lin *et al.*, 2007; Pepe *et al.*, 2007; Verma *et al.*, 2012; Marino *et al.*, 2015; Saraco *et al.*, 2015), illustrating that phenotypic variability can occur in aromatase insufficiency in humans. Affected females showed spontaneous breast development that in some cases progressed to Tanner stage IV (Lin *et al.*, 2007; Marino *et al.*, 2015), but with primary amenorrhea. In almost all, these findings were accompanied by the presence of a pubertal size uterus, enlarged ovarian cysts, high basal serum gonadotropin and androgen (testosterone and androstenedione) levels, and virilizing signs. Hence, low levels of residual aromatase activity can be associated with breast development and estrogen biosynthesis, particularly when circulating androgenic substrate concentrations (androstenedione, testosterone) are elevated. Functional studies of reported cP450arom mutants showed variable residual aromatase activity that could explain the partial phenotype found (**Table 1**). Interestingly, two described mutations associated with splicing events resulted in transcript variants that were also found in normal human steroidogenic tissues, suggesting that a misbalance between normal and alternative-inactive splicing variants might explain the partial deficiency phenotype.

Detailed information on pubertal milestones in aromatase-deficient males is scarce; however, in most of them pubertal development was normal and persistent linear growth was observed after reaching Tanner stage 5 (Morishima *et al.*, 1995; Carani *et al.*, 1997; Bilezikian *et al.*, 1998; Bouillon *et al.*, 2004; Maffei *et al.*, 2004, 2007; Baykan *et al.*, 2013; Chen *et al.*, 2015; Miedlich *et al.*, 2016). The use of aromatase inhibitors to improve adult height in pubertal boys with idiopathic short stature was associated with an increase in serum gonadotropin levels and a consequent rise in testosterone concentrations, raising concerns about pubertal progression (Hero *et al.*, 2005). It could be speculated that affected boys may show an accelerated pubertal tempo consistent with supraphysiological levels of testosterone.

Gonadal Development

The expression of aromatase in the ovary plays an important role in the regulation of the reproductive cycle in females. Aromatase is also expressed in the male gonad; however, in contrast to its key role as an endocrine coordinator in females, in males, the paracrine effects of aromatase products are essential for normal spermatogenesis (Stocco, 2012).

Ovaries

In the ovaries, cP450arom is expressed in two different stages of differentiation of a unique cell, the granulosa cell: the preovulatory follicles and corpora lutea of ovulatory women. Aromatase expression in the granulosa cells of the ovary is regulated primarily by promoter II under the control of the gonadotropin FSH, whose action is mediated by cAMP. Transcriptionally, regulation is by a hexameric sequence binding SF-1 and an imperfect CRE binding CREB and other factors (Simpson *et al.*, 2002).

The well-timed and cell-specific expression of aromatase in the ovary is crucial for the autocrine regulation of folliculogenesis, the endocrine control of the female reproductive tract, and the coordination of gonadotropin secretion. Estrogen modulation of the structure and function of oviducts, the uterus, and the vagina are essential for oocyte survival, fertilization, and implantation (Stocco, 2012).

Multiple ovarian cysts have been described in human aromatase-deficient patients in infancy and childhood (Mullis *et al.*, 1997; Belgorosky *et al.*, 2009; Marino *et al.*, 2015) and particularly during puberty (Conte *et al.*, 1994; Morishima *et al.*, 1995; Lin *et al.*, 2007; Belgorosky *et al.*, 2009; Verma *et al.*, 2012; Janner *et al.*, 2012; Eklioglu *et al.*, 2014; Burckhardt *et al.*, 2015; Marino *et al.*, 2015; Saraco *et al.*, 2015), as well as in female aromatase knockout (ArKO) mice (Britt *et al.*, 2000). It was assumed that an amplification of FSH signaling that occurred in the presence of high intraovarian androgens could be involved in the development of ovarian follicular cysts already in infancy (Belgorosky *et al.*, 2009; Janner *et al.*, 2012). Accordingly, mutations in the FSHB subunit as well as mutations in the FSH receptor result in a phenotype of small ovaries containing only primordial follicles (Themmen and Huhtaniemi, 2000). It is remarkable that histopathological analysis was consistent with that of the polycystic ovary syndrome (Morishima *et al.*, 1995).

Despite the classical picture of bilateral polycystic ovaries described in the natural development from infancy to adulthood in these patients, the ovarian phenotype is not consistent in some affected females. In 2007, Lin *et al.* reported a pubertal female who had severe aromatase deficiency showed mild postnatal androgenization of the genitalia, moderate elevation of androgen levels at puberty, and small ovaries at adolescence. Functional studies indicated that aromatase activity was severely disrupted, but there was no explanation for her mild excess androgen production. Later, in 2014, Gagliardi *et al.* reported the natural history of aromatase deficiency in a female who was not treated until adulthood. At the age of 25 years, the patient showed the classical phenotype, but bilateral streak ovaries were found on laparoscopy and excised. Histological examination revealed atretic and primordial follicles, but no evidence of ovulation. Streak ovaries are consistent with the phenotype of the ArKO mouse followed through adulthood. An age-dependent phenotype was observed in the ArKO ovaries, with a progressive deterioration or degeneration of the ovaries (Britt *et al.*, 2000). The ovarian morphology of ArKO mice (Simpson *et al.*, 2002) showed an early block in follicular development at the antral stage with absent corpora lutea, followed by hemorrhagic cysts and subsequently, absent secondary and antral follicles and atresia of the primary follicles with increased collagen deposition. This model might explain the phenotype of this adult patient considering that the longer life span of the human may permit more complete follicular atresia and collagen deposition mimicking the classical streak ovaries seen in Turner's syndrome (Gagliardi *et al.*, 2014). Nevertheless, Akçurin *et al.* (2016) have recently described three related Turkish cousins with a severe form of aromatase deficiency secondary to a novel homozygous 568insC mutation. These girls presented with hypoplastic ovaries already in prepubertal years. No functional studies have been performed to understand this complex phenotype. Therefore, the mechanism involved in the ovarian phenotype and outcome is poorly understood.

Testes

In contrast to the restricted expression of aromatase in the ovary, the enzyme is widely expressed in the testis and accessory glands. The wide distribution of aromatase in the male gonads is essential to maintain the high levels of estradiol needed for normal spermiogenesis, sperm maturation, sperm motility, and possibly acrosomal reaction.

In ArKO mice, testicular histology and morphology showed age- and diet-related changes. From 4.5 months of life, dysmorphic seminiferous tubules and disrupted spermatogenesis become noticeable (Robertson *et al.*, 1999, 2002). At 1 year of age, ArKO mice consistently show reduced testicular volume, spermiogenesis failure, and Leydig cell hyperplasia, probably as a consequence of increased serum LH levels.

In aromatase-deficient adult males, testicular volume has been reported to be variable, ranging from macroorchidism (Morishima *et al.*, 1995) to normal (Bouillon *et al.*, 2004; Lanfranco *et al.*, 2008; Miedlich *et al.*, 2016) or even microorchidism (Carani *et al.*, 1997; Maffei *et al.*, 2004). Information on testicular histology and morphology is very scarce, with data from only two affected male adults. One of the patients had a history of bilateral cryptorchidism and surgery at the age of 6, making the interpretation of the findings even more difficult.

Fertility

Several factors may affect reproduction in women with aromatase deficiency, mostly in complete forms, including hypergonadotropic hypogonadism, hyperandrogenism, ovarian cysts, and surgical consequences of genital reconstruction of ambiguous genitalia. Although variable phenotypes have been described and some affected females have partial forms of aromatase deficiency with spontaneous breast development and uterine growth despite androgen excess and virilization (Lin *et al.*, 2007; Pepe *et al.*, 2007; Verma *et al.*, 2012; Marino *et al.*, 2015; Saraco *et al.*, 2015), disease course in adulthood and long-term consequences on fertility are unknown. Data on the long-term follow-up of these patients might clarify our understanding of the reproductive outcomes (Guercio *et al.*, 2015).

Fertility is uncertain in aromatase-deficient males, but may be impaired considering the role of estrogens in human testicular function, spermatogenesis, and fertility. In normospermic adult males, lower sperm concentration and reduced sperm motility were associated with aromatase polymorphisms that decrease enzyme activity (Lazaros *et al.*, 2011). Data on sperm analysis or testicular biopsies of adult aromatase-deficient men are scarce and inconsistent. Pre-existing conditions, such as cryptorchidism, further complicate the interpretation of the results. Total germ-cell depletion, focal hypospermatogenesis, low sperm counts, and decreased motility have been reported (Carani *et al.*, 1997; Herrmann *et al.*, 2002; Maffei *et al.*, 2004, 2007; Baykan *et al.*, 2013).

Gender Identity/Psychosexual Development

In mammals, sex differences in brain structure and function are programmed by exposure to testosterone during a critical period in perinatal development (Cooke *et al.*, 1998). In the female, fetal androgen action might have consequences on brain programming of future sexual identity and behavior (Bao and Swaab, 2011). However, changes in gender identity, sex role behavior, and gender dysphoria are unusual in 46,XX aromatase-deficient patients, although reported in detail in a few cases (Conte *et al.*, 1994; Morishima *et al.*, 1995; Sudeep *et al.*, 2013). Male gender identity, male gender role behavior, and gender dysphoria were reported in only one 46,XX subject raised as male (Lin *et al.*, 2007). Whether prenatal and postnatal androgen exposure coupled with relative estrogen insufficiency—a unique feature of aromatase insufficiency—is important, or whether social and cultural influences are predominant, remains unclear.

In the affected males, adult male cisgender identity, heterosexual orientation, and normal libido have been reported (Morishima *et al.*, 1995; Carani *et al.*, 1997; Herrmann *et al.*, 2002; Maffei *et al.*, 2007; Lanfranco *et al.*, 2008; Chen *et al.*, 2015). Only a modest decreased libido was reported in one subject without change after high-dose estrogen treatment (Morishima *et al.*, 1995; Bilezikian *et al.*, 1998).

Insulin Sensitivity and Lipid Profile

In adipose tissue, aromatase is expressed primarily in the stromal mesenchymal cells or preadipocytes where the majority of aromatase transcripts contain exon I.4 followed by exon I.3 and II (Simpson *et al.*, 2002). Estrogens regulate glucose homeostasis in the skeletal muscle and liver by modulating insulin production in pancreatic β-cells and regulating energy balance in the hypothalamus. In addition, an antilipogenic and pro-lipolytic effect acting directly on adipose tissue has been reported (Foryst-Ludwig and Kintscher, 2010).

Data obtained from ArKO mice of both sexes showed an adipose phenotype together with insulin resistance (IR), elevated serum lipid, and fatty liver, suggesting an association between aromatase deficiency and metabolic alterations (Jones *et al.*, 2000; Nemoto *et al.*, 2000; Takeda *et al.*, 2003).

IR, an abnormal lipid profile, as well as clinical features similar to the metabolic syndrome, have been well described in aromatase-deficient adult men. In these patients, estrogen therapy has been suggested to play an important role in lipid regulation, fatty acid homeostasis, and glucose metabolism (Morishima *et al.*, 1995; Carani *et al.*, 1997; Herrmann *et al.*, 2005; Maffei *et al.*, 2004, 2007; Lanfranco *et al.*, 2008; Baykan *et al.*, 2013; Chen *et al.*, 2015). Recently, however, an aromatase-deficient man harboring the c.628 G>A mutation without biochemical features of IR, dyslipidemia, or overweight/obesity has been reported. Although this mutation has been related to the metabolic phenotype in other affected subjects (Maffei *et al.*, 2004; Guercio *et al.*, 2009), the capacity of the specific mutation c.628 G>A to translate into expression of differentially functioning proteins (Pepe *et al.*, 2007) might explain the variable phenotypes.

In female aromatase-deficient patients, the relationship between carbohydrate metabolism, lipid profile, and estrogen deficiency has not been clearly defined. Lipid abnormalities were found in a Vietnamese affected girl of 18 months of age (Ludwig *et al.*, 1998) and in two adolescents reported by Lin *et al.* (2007). In one of the latter patients the metabolic profile normalized under estrogen therapy. IR and glucose intolerance but a normal lipid profile was reported in two affected females with partial forms of aromatase deficiency at 9 and 13.5 years of age (Belgorosky *et al.*, 2003; Guercio *et al.*, 2009; Verma *et al.*, 2012). Both presented spontaneous thelarche along with high serum FSH levels, clinical and biochemical hyperandrogenism, and ovarian cysts. In two other pubertal affected females with partial aromatase deficiency reported by Marino *et al.* (2015), normal glucose tolerance along with normal androgen levels was noticed. This finding might reflect that high androgen levels contribute to the development of IR in women as previously proposed (Peiris *et al.*, 1989). A more classic or prominent metabolic picture of

abdominal obesity, hepatic steatosis, dyslipemia, and IR was reported in a 25-year-old affected woman in whom estrogen treatment was delayed until adulthood. Since in ArKO mice models, metabolic abnormalities were age dependent, prolonged estrogen deficiency could be required in women to develop a full metabolic phenotype. Long-term studies would be necessary to draw more solid conclusions in this regard.

Even though metabolic abnormalities were resolved by estrogen treatment in ARKO mice (Takeda *et al.*, 2003), in humans, the effect of estrogen therapy on glucose homeostasis and IR is complex and controversial (Jones *et al.*, 2006). Failure to improve IR in some aromatase-deficient patients of both sexes was reported (Herrmann *et al.*, 2002; Maffei *et al.*, 2004; Guercio *et al.*, 2009; Verma *et al.*, 2012; Chen *et al.*, 2015). Moreover, the optimal dose and route of estrogen replacement therapy in such cases are still unclear due to the scarcity of the disease (Chen *et al.*, 2015).

Growth, Skeletal Maturation, and Bone Homeostasis

Androgens and estrogens are important for the maintenance of the skeleton in both sexes (Grumbach and Auchus, 1999; Simpson *et al.*, 2002). The important role of aromatase in bone physiology was clearly shown in ArKO mice (Oz *et al.*, 2000), in which reduced bone density and skeletal abnormalities were observed.

The male aromatase-deficient phenotype is characterized by tall stature with eunuchoid body proportions and continued linear growth into adulthood due to unfused epiphysis, genu valgum, and osteopenia/osteoporosis that remarkable improves with estrogen replacement treatment. This aspect has been addressed in detail in several reports (Bulun, 2014; Grumbach and Auchus, 1999; Bilezikian *et al.*, 1998; Bouillon *et al.*, 2004; Lanfranco *et al.*, 2008; Zirilli *et al.*, 2008; Rochira *et al.*, 2007, 2015).

In affected females bone age is delayed, particularly in late prepuberty, and absence of the growth spurt is observed. Estrogen therapy has been shown to induce a pubertal growth spurt and epiphyseal fusion (Belgorosky *et al.*, 2009; Janner *et al.*, 2012; Verma *et al.*, 2012; Marino *et al.*, 2015; Saraco *et al.*, 2015). Since aromatase-deficient women are treated earlier to induce pubertal development, it may be possible that these subjects, if untreated, will develop the same phenotype as that observed in the male counterpart. Concordantly with this, a nontreated 32-year-old Indian woman with tall stature, eunuchoidal proportions, osteopenia, and fractures was reported (Gagliardi *et al.*, 2014).

Data on the role of estrogens in bone mineralization during childhood are scarce. In the patient reported by Mullis *et al.* (1997), low bone mineral density (BMD) was found. Moreover, BMD improved after treatment with low-dose estrogens, suggesting that minimal quantities of estrogens are also required to preserve mineral bone acquisition in early childhood. Nevertheless, in the case reported by Belgorosky *et al.* (2003), BMD remained normal throughout the follow-up. The explanation could be that in this particular case, partial cP450arom deficiency was found, secondary to a mutation at the consensus donor splice site in the exon 5/intron 5 junction. Therefore, some expression of the cP450arom protein at the growth plate might be expected, which may have been enough to maintain normal mineral bone acquisition, at least during childhood.

Estrogen Replacement Therapy

The clinical consequences of estrogen deficiency in aromatase-deficient patients reinforce the importance of clinical management and estrogen replacement. Recently, this aspect has been carefully addressed by Bulun (2014) and Zhu *et al.* (2016). To date there is no consensus regarding the age of initiation and the dosage of estrogen to use to guide this indication. Thus, such treatment should be individualized at this time.

Conclusions

Patients with cP450arom deficiency represent a source of knowledge on estrogen physiology and pathology, in particular on the role of estrogens in bone maturation, gonadotropin modulation in both sexes, gonadal development and function, as well on glucose and lipid metabolism. Recently, data on new molecular mechanisms have expanded the clinical and biochemical phenotype of affected individuals. At this time, long-term studies are needed to establish a therapeutic strategy to prevent the devastating consequences of prolonged estrogen deficiency.

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Further Reading

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Turner Syndrome☆

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Introduction

In 1938, Henry Turner described a disorder in seven women which was characterized by absence of female sexual characteristics, increased skin folds in the region of the neck (pterygium colli), a wide angle between the upper and lower arm (cubitus valgus), and short stature. As early as 1930, however, Otto Ullrich had recognized the same disorder as being a specific entity. Before them, in 1768, the anatomist Morgagni already observed inadequately developed ovaries without oocytes in one female patient. Many years later, in 1955, Grumbach et al. described the inadequately developed ovaries as gonadal dysgenesis. After the development of techniques for demonstrating human chromosomes, Ford et al. reported that the cause of the disorder is the absence of one of the X chromosomes.

Definition and Incidence

Nowadays, the syndrome is commonly termed Turner syndrome (TS) and is caused by the complete or partial absence of one of the two X chromosomes in some or all cells of the female body. TS occurs in approximately 1/2000–1/2500 female births and is characterized by 3 main clinical features: (1) abnormal external appearance and abnormalities of certain internal organs; (2) malformation of the ovaries; and (3) short stature.

Genetics

Chromosome Changes

Investigations of the chromosomes are usually carried out by traditional karyotyping of cultured leucocytes. The chromosome change observed most often (in ~50%–60% of patients with TS) is the complete absence of an X chromosome: karyotype 45,X (Table 1 and Fig. 1). If an X chromosome is lost by faulty distribution during later cell divisions or loss of one of the X chromosomes after the formation of the fertilized ovum, then a mixture of cell lines with both normal and abnormal chromosome counts develop, described as a mosaic. This results in karyotypes such as 45,X/46,XX. In these cases, the signs of TS are often present as well, although the phenotype can be milder in case of a low-grade mosaicism (i.e., relatively low percentage of cells showing deviant or missing X chromosome).

TS may also be the result of partial X chromosome loss due to a structural aberration. Such an aberration might, for instance, be caused by transverse instead of longitudinal division of the chromosomes after DNA-replication cell division (leading to an isochromosome of the X chromosome long arm and loss of the short arm), by the major and minor deletions of either the long arm or the short arm of the X chromosome, or by the formation of a ring X chromosome.

Features of TS may also be seen in cases where, besides one of the cell lines mentioned, cells with material from the Y chromosome are present (e.g., karyotype 45,X/46,XY). The presence of Y chromosome material may cause the development of gonadoblastoma. Therefore, probing for Y chromosome material should be performed in any TS patient with evidence of virilization, or when a marker chromosome (a sex chromosomal fragment of unknown origin, i.e., X vs. Y) is found. This can be achieved by molecular studies or FISH (fluorescent in situ hybridization) using a Y centromeric or short arm probe (including the SRY-gene).

Low-grade mosaic TS can easily be missed with conventional karyotyping and analyzing 30 cells. Therefore, in cases of serious clinical suspicion of TS, standard karyotyping and/or FISH of a second tissue, for example, buccal cells or fibroblasts, should be performed. In addition, extended genetic testing of a second tissue should be considered in all patients with a full-blown monosomy 45X to elucidate the complete genotype and to rule out a potential 45,X/46,XY mosaicism.

☆*Change History*: December, 2017. Theo CJ Sas, JAEM van Alfen-van der Velden and Sabine MPF de Muinck Keizer-Schrama has added text and change Table 3 to a simpler form.

This chapter is an update of Theo C. J. Sas, Sabine M. P. F. de Muinck Keizer-Schrama, Turner Syndrome, In Encyclopedia of Endocrine Diseases, edited by Luciano Martini, Elsevier, New York, 2004, Pages 642-650.

Table 1 Some typical chromosome changes and their relative frequency in patients with TS ($n = 649$)

Karyotype	*Number of patients*	*Percentage*
45,X	396	60.9
46,X,Xp-	6	0.9
46,X,Xq-	5	0.8
46,X,i(Xq)	38	5.9
45,X/46,XX	86	13.3
45,X/46,X,i(Xq)	41	6.3
45,X/46,X,r(X)	30	4.6
Complete mosaics and rare deviations (e.g., 46,X.r(X); 45,X/47,XXX; 45,X/46,XX/47,XXX; 45,X/46,X,Xp-)	42	6.5

Notes: 46,X,Xp- = short arm missing; 46,X,Xq- = long arm missing; 46,X,i(Xq) = isochromosome of two long arms; 46,X,r(X) = ring chromosome. Based on collective statistics from Ranke, Brook, Lenko, Prelz, Lippe.
From Ranke, M.B. (1997). Turner syndrome: A clinical overview. Oxford: Oxford Clinical Communications.

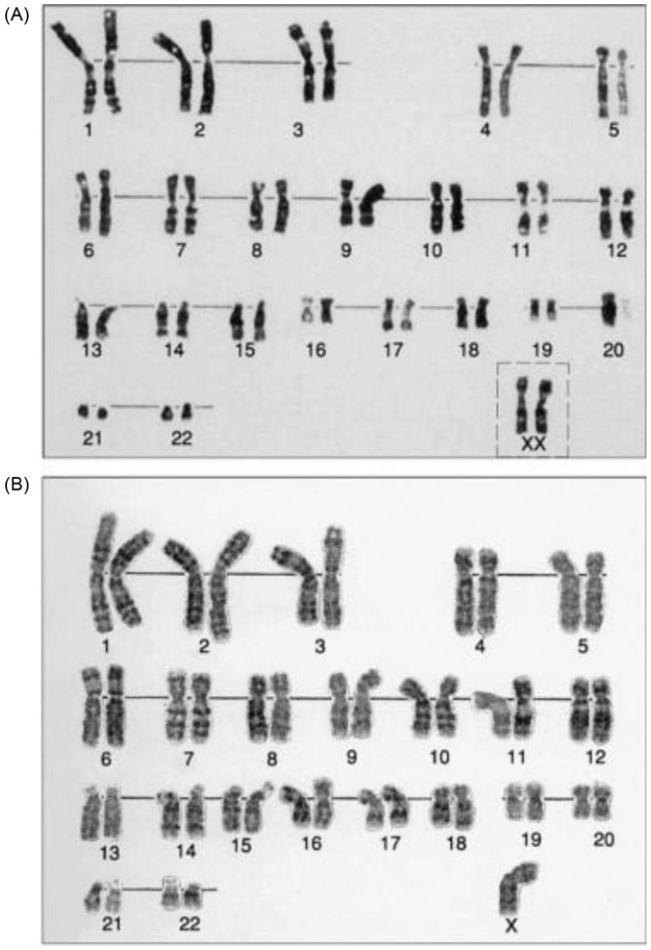

Fig. 1 (A) Normal karyotype and (B) karyotype of a girl with classic Turner syndrome. From Ranke, M.B. (1997). Turner syndrome: A clinical overview. Oxford: Oxford Clinical Communications.

In females with one X chromosome and a deletion distal to Xq24 on the other X chromosome, and in women over the age of 50 years with $<5\%$ 45,X mosaicism, the genetic situation is not considered as TS.

Table 2 Signs and physical features and their frequency in TS

Feature	*Frequency*[a]	*Feature*	*Frequency*[a]
Eyes	20%–39%	*Chest*	60%–79%
Ptosis		Scutiform thorax	
Epicanthus ("Mongol-fold")		Nipples apparently wide apart (not real, appearance results from scutiform thorax)	
Myopia			
Strabismus			
Nystagmus		Inverted nipples	
Ears	40%–59%	*Skeleton*	40%–59%
Deformed auricles		Wide angle of arm (cubitus valgus)	
Otitis media		Short metacarpal/metatarsal bones	
Impaired hearing		Scoliosis	
Mouth, jaw	60%–79%	*Heart vessels*	40%–50%
High arched palate (palatus arcuatus)		Stenosis of aortic isthmus	
		Bicuspid aortic valve	
Small lower jaw (micrognathia)		Aortic dilatation/aneurysm	
Defective dental development		Hypertension	
Skin, skin appendages	60%–79%	*Kidneys*	40%–59%
Lymphedema of hands and feet		Renal malformation (e.g., horseshoe kidney)	
Increased number of pigmented naevi			
		Renal aplasia	
Fingernail and toenail dysplasia		Changes in the renal pelvis and ureters	
Increased skin ridge patterns (dermatoglyphics)		Vessel abnormalities	
Neck	40%–59%	*Ovaries*	80%–100%
Short, thick neck		Gonadal dysgenesis	
Low nape of neck/hair-line			
Start of hair growth in nape of neck pointing upwards			
Pterygium colli			
		Growth	80%–100%
		Small-for-gestational age at birth	
		Growth retardation after birth	

Notes: Frequencies reported in the literature show some variation. Ranges are therefore given for frequency, rather than exact figures.
From Ranke, M.B. (1997). Turner syndrome: A clinical overview. Oxford: Oxford Clinical Communications.

Karyotype/Phenotype Correlation

The wide range of signs and features in TS (Table 2) suggest that a number of different X-located loci are responsible for the complete Turner phenotype. It is now widely accepted that hemizygosity of specific X–Y homologous genes which escape X inactivation (pseudo-autosomal) leads to haploinsufficiency, which in turn causes the characteristic Turner somatic features combined with the associated neurocognitive profile.

The important studies of the relationship between karyotype and phenotype in TS by Ferguson-Smith, published in 1965, first suggested that genes responsible for the TS phenotype could be assigned to specific regions of the X chromosome. With the further development of molecular techniques, it became possible to refine the short stature critical region to the extreme tip of the short arm of the X and Y chromosomes, the pseudo-autosomal region (PAR 1). Subsequently, the PAR 1 short stature gene SHOX (short stature homeobox) was identified, and haploinsufficiency of this gene appeared to be responsible for the disproportionate short stature of patients with TS. Other genetic studies showed that haploinsufficiency of one or more genes in Xp22.3 are associated with the TS neurocognitive profile and a "critical region" for normal ovarian function is assigned to Xq13–Xq26, excluding part of Xq22. However, despite the increasing knowledge of the function of specific X chromosomal genes in relation to the clinical features of TS, a well-defined phenotype-genotype relationship is still not available.

Clinical Features

General

In addition to the short stature and gonadal dysgenesis, there is a multiplicity of findings in patients with TS, occurring with varying frequencies (Table 2 and Fig. 2). The degree of severity of the abnormal appearance is usually most marked when there is a complete loss of an X chromosome (karyotype 45,X). A number of features listed in Table 2 are considered a part of a skeletal

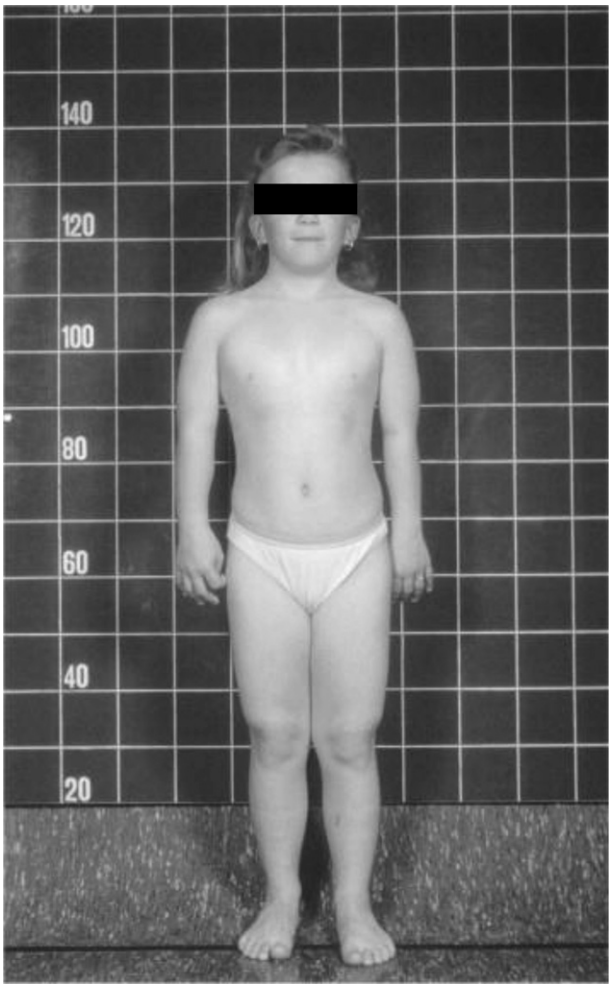

Fig. 2 Girl with Turner syndrome.

growth disturbance while other features can be explained by lymphatic obstruction or germ cell chromosomal defects. The growth failure and ovarian dysgenesis are the features with the highest frequency in TS.

Growth Failure

Growth is reduced in virtually all of the patients. Newborns with TS delivered at term are smaller than average girls, body length by approximately 3 cm, and the body weight by approximately 500 g. Postnatal growth rate appears to be in the normal range during the first 2–3 years of life. Thereafter, height velocity shows a gradual decrease compared with healthy girls, and in most girls, there is a lack of the pubertal growth spurt. Due to the delayed epiphyseal fusion, most untreated girls continue to grow until their late teens or beyond. The mean adult height of women with TS of white European origin ranges from approximately 142 to 147 cm in Northwestern Europe, which is approximately 20 cm smaller than the mean of the normal female population. **Fig. 3** shows the Dutch–Swedish–Danish reference values for height in TS which are based on data from a large multinational study. In 1997, haploinsufficiency of the so-called Short-stature homeobox-containing gene (SHOX) gene was identified as the main cause of short stature in TS. This state of SHOX haploinsufficiency not only appears to be the main cause of the disproportional short stature of women with TS, but is also believed to be the main cause of the relatively broad shoulders and pelvic width. However, other factors such as aneuploidy or chromosomal imbalance have also been suggested to contribute to short stature in TS.

Ovarian Dysgenesis

Since in TS the ovaries usually start to involute within 4 or 5 months of gestation, the majority of patients are infertile, have diminished ovarian estrogen and androgen production, and need estrogen therapy to induce pubertal maturation. However, there is wide variation in the loss of germ cells in girls with TS, such that 5%–10% retain sufficient ovarian function for puberty to commence spontaneously, though in most girls incompletely. The frequency of spontaneous puberty in mosaicism is significantly

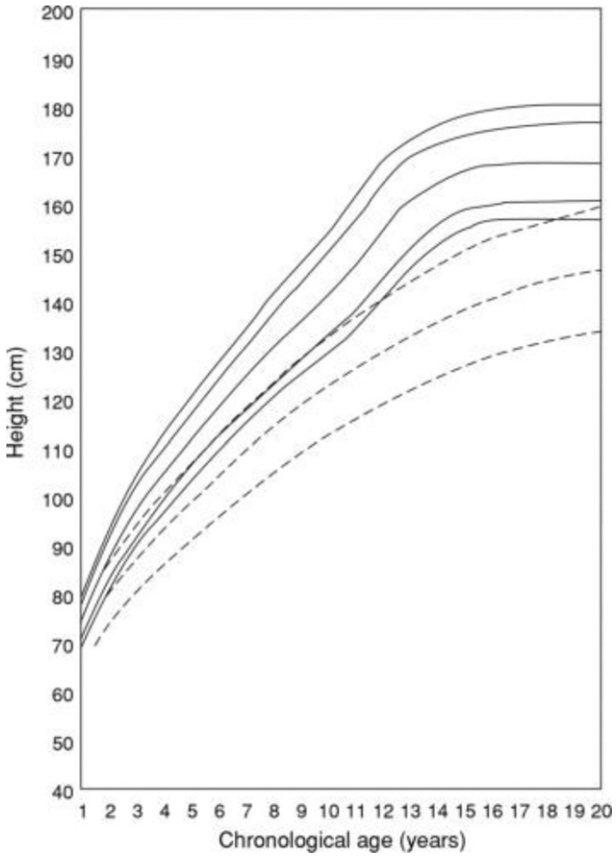

Fig. 3 Reference curve for healthy Dutch girls (3rd, 10th, 50th, 90th, and 97th percentiles, *solid lines*) and for untreated girls with Turner syndrome (North European references; 3rd, 50th, and 97th percentiles, *dotted lines*).

higher compared to the 45,X karyotype. Spontaneous menstrual bleeding occurs only in a few cases, and in most of these patients it persists for only a short period of time. Occasionally, spontaneous pregnancies have been reported.

Diagnosis

Prenatal

Most prenatally detected cases of TS are discovered incidentally during chorionic villous sampling or amniocentesis performed for unrelated reasons, the most common being advanced maternal age, which itself is not associated with an increased incidence of TS. Intra-uterine growth retardation is common in TS as well as polyhydramnion and certain ultrasound findings (e.g., increased nuchal translucency, coarctation of the aorta, renal abnormalities). Even when the prenatal diagnosis has been made by karyotype, chromosomes should be reevaluated postnatally.

Postnatal

Nowadays, TS is most frequently discovered during childhood; however, women are still diagnosed in adolescence and adulthood. The age at which the diagnosis is confirmed depends on the severity of the symptoms. The physical features are not always immediately obvious in every girl with TS, or might even be absent in some cases. Clinicians should always consider the diagnosis in any female patient with unexplained growth failure, pubertal delay, amenorrhea, or infertility.

Management in Childhood, Adolescence, and Adulthood

Recently, a clinical consensus guideline for the management of patient with TS has been published (Gravholt *et al.*, 2017). We give an overview of the most important aspects.

Table 3 Recommended estrogen replacement options for feminization in adolescent TS

Preparation	*Pubertal initiation dose*	*Adult dosing*
Transdermal E2	3–7 μg/day	25–100 μg/day
Micronized 17β oral E2 (E2)	0.25 mg/day	1–4 mg/day
Ethinylestradiol (EE)	2 μg/day	10–20 μg/day
Depot E2	0.2 mg/month	2 mg/month

Notes: Gradually increase E2 dose at 6–12 months interval over 2–3 years to adult dose. Begin cyclic progesterone after 2 years of estrogen or when breakthrough bleeding occurs.
Gravholt, C.H. *et al.* (2017). Clinical practice guidelines for the care of girls and women with Turner syndrome: Proceedings from the 2016 Cincinnati International Turner Syndrome Meeting, *European Journal of Endocrinology* **177**(*3*), G1–G70.

Short Stature

Although girls with TS are not GH deficient, treatment with biosynthetic recombinant human GH accelerates height velocity. To determine the effect of GH on adult height, the attained adult height is compared to the individually predicted adult height at the start of the GH treatment based on references for untreated girls with TS. In many countries, TS is an accepted indication for GH treatment, although controversy exists regarding the effects of GH treatment on adult height, since the mean increase in adult height varies between 0.2 and 16.0 cm in the different publications. For most girls a minimal gain in adult height of about 10 cm can be achieved when GH is started before the age of approximately 8 or 9 years (preferably from 6 years onwards) with a daily GH dose of about 0.045 mg kg^{-1} day^{-1} ($\sim$1.4 mg m^{-2} day^{-1}; $\sim$4 IU m^{-2}). A lower daily GH dose, as used in Europe for children with GH deficiency, does not seem to be enough to overcome the negative effect of the SHOX deficiency. An even higher GH dose, which is proven to be more effective, could be considered in extremely short girls or girls who are relatively old at diagnosis with a poor adult height prognosis. An alternative is the addition of the synthetic anabolic steroid oxandrolone (Ox) to the growth hormone therapy which increases the gain in adult height more than GH alone.

In general, in children who received GH treatment a small increase in the incidence of kyphoscoliosis, diabetes mellitus type 2, and benign intracranial hypertension is reported. Although supraphysiological GH dosages are given for several years in TS, no negative side effects of clinical importance of long-term GH treatment are found, besides a very slight increase in the incidence of edema in the first weeks of the treatment. GH has no adverse effects on glucose levels, but induced higher levels of insulin which decreased after discontinuation of GH. GH has no negative effect on heart morphology and function, blood pressure, aortic dilatation and stiffness, blood lipids, or bone density. During the relatively high-dose GH therapy, IGF-I levels increase to high normal levels but in some girls to levels above the $+2$ standard deviation (SD). Higher GH doses increase the IGF-I levels even more. It is unknown whether the higher IGF-I levels could have negative effects on health on the very long term. Consequently, most physicians will lower the dose of GH when the IGF-I levels are several times above the $+2$ SD. Long-term GH treatment seems to be safe, but continued observation into adulthood is required. If Ox dosages <0.06 mg kg^{-1} day^{-1} are used in addition to GH, side effects of Ox are mild. The most relevant safety concerns of treatment with Ox are virilization (including clitoromegaly and voice deepening) and a transient delay of breast development.

Gonadal Dysgenesis

Induction of Puberty

To induce puberty, continuous treatment with estrogens has to be given to girls with TS. Although 5%–10% retain sufficient ovarian function for puberty to start spontaneously, most girls show a progressive ovarian failure and need estrogen therapy for complete breast development and withdrawal bleeding. Administration of the natural estrogen 17β-estradiol is the treatment of choice for puberty induction. Micronized 17β-estradiol can be given either orally or, to avoid the first pass effect by the liver, transdermally. With transdermal (TD) patches or percutaneous gel, spontaneous pubertal hormonal changes are mimicked and normal pubertal development is achieved. Alternatively, oral ethinylestradiol can be used for puberty induction, when 17β-estradiol is not available. As oral ethinylestradiol is a synthetic estrogen that is not metabolized by the liver, it can be delivered at relatively low doses. However, synthetic estrogens have more pronounced effects on coagulation factors, lipid profiles, and blood pressure than natural estrogens. Other forms of estrogens are available as well. However, conjugated equine estrogen preparations (CEE, Premarin®) contain multiple estrogens, progestins, and androgens, some of which are not found in humans and are not justified for use in children. Similarly, for pubertal induction the oral contraceptive pill is best avoided, because the synthetic estrogen doses are too high and the typical synthetic progestin may interfere with optimal breast and uterine development. Furthermore, the oral contraceptive pill is conventionally taken with a pill-free week, resulting in 3 months of estrogen deficiency for each year of use.

In the absence of spontaneous puberty or stunted breast development, puberty induction is recommended from the age of 11–12 years to support psychosexual maturation and self-esteem. Although the appropriate starting dose has yet to be determined,

estrogen replacement is usually begun at 1/10–1/8 of the adult replacement dose and then increased gradually over a period of 2–4 years. To allow for normal breast and uterine development, it seems advisable to delay the addition of progestin at least 2 years after starting estrogen or until breakthrough bleeding occurs. Natural micronized progesterone and dydrogesterone or medoxyprogesterone are preferred to other progestagens because of their less negative effect on lipid metabolism and less androgenic effect. Based on these principles, suggested age-specific preparations and doses of estrogen substitution therapy in adolescence are listed in **Table 3**. This table is only a guideline, and individual tailoring of dose and timing will be required. Whether even earlier very low-dose estrogen administration from the age of 5 years onwards has beneficial effects on bone, cognition, and self-image in patients with Turner's syndrome remains to be proven and is not considered standard treatment.

Hormonal Replacement Therapy in Adulthood

To treat the short- and long-term consequences of estrogen deficiency in women with TS, continuation of hormonal replacement therapy (HRT) until the age of normal menopause is highly recommended. The available studies indicate that HRT has beneficial effects on bone density, risk of cardiovascular disease, and hyperinsulinism. In addition, HRT might have positive effects on general well-being, cognition, and psychosocial functioning. Natural estrogens are recommended either orally (2–4 mg 17β estradiol daily) or transdermally (50–200 μg 17β estradiol daily), combined with cyclic progestin. Since overweight and hypertension can be exacerbated by exogenous estrogen, particularly when administered by the oral rather than transdermal route, blood pressure should be checked at regular intervals.

Fertility

Ninety-five percent of women with TS are infertile. Abnormal germ cell development combined with accelerated oocyte loss result in a percentage of only 5%–10% of TS girls who will have oocytes remaining in their ovaries at pubertal age. Those with remaining follicles may develop ovulatory cycles and will have a short period of fertility prior to entering into a premature menopause. In general, outcomes of spontaneous pregnancy in TS women are poor, with miscarriage rates of 30%–45%, and structural and chromosomal abnormalities may affect 25% of live births.

In women with persisted ovarian function, cryopreservation of oocytes after ovarian hyperstimulation is a possible fertility preservation option. The availability of oocyte donation combined with in vitro fertilization now offers these women a realistic change of conceiving and delivering a healthy child. While recent case series indicate that implantation and pregnancy rates rival those obtained in non-TS women, women with TS may have higher rates of early pregnancy loss compared to other groups with premature ovarian insufficiency (early miscarriage 0%–80% vs. 8.7%), indicating reduced endometrial and uterine function.

Pregnancy is associated with maternal morbidity. Those with cardiovascular abnormalities may be at risk of aortic dissection, and pre-pregnancy screening for pre-exiting dilatation of the aortic root is therefore recommended. In addition to possibly increasing the rate of miscarriage, the hypoplastic uterus frequently observed in adult TS may be prone to rupture in late pregnancy. Furthermore, an increased risk of cephalo-pelvic disproportion at delivery exists. The mechanical and physiological stresses of pregnancy may be reduced by avoiding multiple pregnancy.

Other Medical Problems

Cardiovascular Disease

The most frequently observed major congenital cardiovascular abnormality in TS is the coarctatio aortae, which can be corrected by surgical intervention. Morphological abnormalities of the aortic valves, which are often found, are mostly not of clinical significance in childhood. The incidence of hypertension is higher in girls and adults with TS compared to healthy peers. The mechanisms leading to hypertension in these individuals are unknown and not clearly related to the cardiac or renal congenital abnormalities. Obesity, which is common in the adolescent and adult individuals, contributes to high blood pressure. Rupture of an aneurysm or dissection of the aorta in childhood has been rarely described and is associated with hypertension. However, even in the absence of predisposing factors as aortic valve abnormalities or hypertension, this life-threatening event occurs more frequently than in the normal population and can even occur at a younger age. Therefore, evaluation and follow-up of the cardiovascular dimensions using ultrasound in childhood and MRI in adulthood and monitoring blood pressure are required with adequate treatment when indicated. Particularly before and during pregnancy, repetitive MRIs of the aorta are required. In childhood as well as in adulthood, the measured aortic diameter has to be compared with the references and cut-off levels of girls or women with similar height (and weight or body surface).

The three times higher mortality in TS women is due to complications of structural abnormalities of the heart and aorta, as well as to the higher risk of ischemic heart disease and stroke. The chronic estrogen deficiency known to affect many adult women with TS is likely to be associated with increased cardiovascular morbidity, since it is becoming clear that estrogens confer cardioprotectivity not only by lowering harmful circulating lipids, but also through direct antioxidant effects, through the interaction of estrogens with smooth vascular muscles.

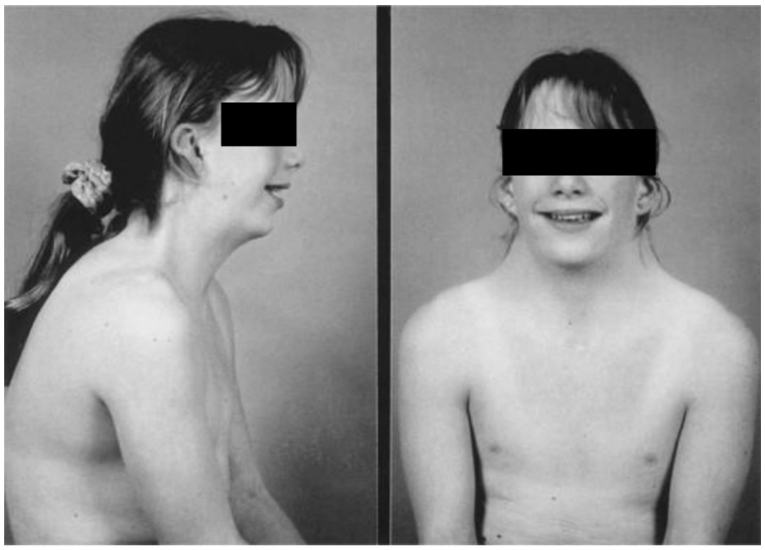

Fig. 4 Turner girl with pterygium colli (webbed neck).

Ocular Problems

The eyes often show slight changes in the position and form of the palpebral fissure or ptosis can be observed; this does not affect function. It is important that visual disorders and squinting should be diagnosed and treated as early as possible, to prevent further deterioration or permanent damage.

Ear and Hearing Problems

Ear malformations are frequently observed in patients with TS (**Table 2**) Furthermore, recurrent periods of otitis media are found in 50%–80% and complications are common, for example, cholesteatoma and tympanic membrane perforation. Conductive and sensorineural hearing loss is frequently observed in girls and women with TS, and correlates with the karyotype. Audiometric measurements often show a sensorineural dip in the midfrequencies. In young girls with TS this seldom leads to hearing impairment, but the dip broadens and the depth progresses over time, leading to social hearing problems later on. A high frequency hearing loss (presbyacusis) is also added to the dip at the age older than 35 years, leading to rapid hearing loss and social hearing problem at a younger age than in the normal population. About 26%–44% of adult women with TS have serious hearing loss requiring hearing aids. Frequent audiometric screening is important in girls and women with TS, since hearing loss negatively affects quality of life.

Skin and Neck

Swelling of the back of the hands and feet due to lymphedema is not exclusive to TS but is a striking feature, particularly in the newborn. In most girls the lymphedema shows a tendency to regress after birth. Some patients have an increased number of pigmented naevi, which are usually benign but often show a tendency to grow during puberty. Some clinical evidence has shown that, when GH is administered for short stature, the growth of melanocytic naevi is boosted without signs of malignant transformation. The patient's neck often appears short and thickset. In addition, a pterygium colli (webbed neck) can be found (**Fig. 4**), which can be corrected by surgery (favorable after a bilateral tissue-expander procedure). However, keloid scars develop more often than usual after surgical interventions.

Skeleton and Bone Mineral Density

Besides the "classic" cubitus valgus, occasionally Madelung's deformity of the wrist (the ulna projects beyond the level of the hand because the wrist is displaced towards the palm of the hand) or some other minor deformities of the hands and feet are found. In addition, girls and women with TS have relatively large hands and feet in relation to their height. Another skeleton problem is the higher risk of scoliosis in girls with TS than in the general population, particularly after 10 years of age. Consequently, if physical examination shows scoliosis, an orthopedic follow-up is required. Furthermore, girls with TS have a higher risk of congenital hip dysplasia. In addition, slipped capital femoral epiphysis is a rare complication of GH treatment. Consequently, every girl with TS with persistent pain in knee or hip needs referral to the orthopedic surgeon.

The risk of fractures in TS is higher than in the general population. This is partly due to the lower bone mineral density in TS as well as due to the higher risk of falling. Individuals with TS have an intrinsic defect in the cortical bone formation leading to higher risk of fractures of the long bones. When the estrogen deficiency is not treated sufficiently, the bone mineral content of the trabecular bone will be decreased, leading to a higher risk of (compression) fractures of the vertebra.

The interpretation of the two-dimensional dual energy X-ray absorptiometry (DEXA) measurement is more complex in individuals with short stature than in individuals with normal stature, leading more often to false lower values in TS. Consequently, a volumetric correction (bone mass per volume instead of bone mass per bone surface) has to be used. It is well known that treatment with estrogens is pivotal in order to avoid rapid bone mineral loss in adolescents and young adults. An adequate calcium and vitamin D intake as well as sufficient physical exercise are warranted as well.

Renal Disorders

Up to one-third of individuals with TS have structural renal malformations. Rotational abnormalities and double collecting systems are found most frequently. Although many of these abnormalities are without significant major renal complications, some may result in an increased risk of hypertension, urinary tract infections, or hydronephrosis. A renal ultrasound must be carried out at the time of diagnosis.

Autoimmunity: Thyroid Disease and Celiac Disease

Clinical and epidemiological evidence shows that the risk of autoimmune disease and especially thyroid disease is increased in TS. Up to 50% of women with TS have anti-thyroid antibodies and auto-immune thyroiditis is common: almost a quarter of the adult patients have hypothyroidism compared with 1%–2% of the general population. In addition, the incidence of celiac disease is higher in those with TS than in the general population. Consequently, thyroid hormone measurements and regular celiac antibody screening (or HLA DQ assessment) are required.

Glucose Metabolism and Insulin Sensitivity

Increased frequency of abnormal glucose tolerance, reduced insulin sensitivity, hyperinsulinemia, and inappropriately low insulin secretion are seen in TS. In a register study covering the entire population in Denmark, an increased relative risk of 4.38 was found. Not only type 2, but probably also type 1 diabetes is more prevalent in TS. Adiposity, which is seen in a large proportion of the adult Turner population sometimes combined with decreased physical fitness, is an important player in the development of type 2 diabetes.

Hepatic Function

Higher serum levels of hepatic enzymes such as alanine aminotransferase, gamma glutamyl transferase, and alkaline phosphatase have been found in adult TS women compared with controls. These elevated levels of some hepatic enzymes and proteins do not seem to be associated with overt hepatic disease. However, sometimes the elevated hepatic enzymes can be the first sign of celiac disease; therefore, a test for celiac disease should always be performed.

Cancer

The relative risk of most cancers is low in TS and comparable to that of the general population, with the exception of the colon and rectum. In two separate registers in Denmark, it was found that the relative risk of these cancers was 5–7 times that of the background population. In contrast, a British study on a national cohort of 3425 women with TS showed an increased risk of meningioma and childhood brain tumors, and possibly bladder cancer, melanoma, and corpus uteri cancer. However, the absolute risks are still small.

Since sex hormone substitution may lead to an increased risk of cancer of the breast and reproductive organs, concern have been raised about a possible increased risk in TS women on HRT. However, no increased risk in the two Danish registers has been found. Gonadoblastoma might develop in the ovaries of girls and women with TS with Y chromosome material. Therefore, in many countries it has become a standard procedure to determine Y chromosome material with fluorescent in situ hybridization (FISH) in a second tissue, for example, buccal mucosa, in girls with monosomy 45X. The risk of developing gonadoblastoma has previously been estimated as 25%–40%, although more recent data suggest a lower risk of only 7%–10%. Nevertheless, gonadectomy is still recommended in girls with TS with Y chromosomal material, to exclude malignancy with absolute certainty.

Cognitive and Psychosocial Abilities

No increase in the prevalence of mental retardation is associated with TS, except for these few patients with a ring X chromosome that fails to undergo X inactivation. Numerous studies have documented that girls with TS have a neurocognitive profile consisting of visuo-spatial information processing deficits in the context of relatively preserved verbal skills. In particular, persons with TS are impaired in the coding and transforming of visuo-spatial information. Compared to healthy controls, girls with TS are described as exhibiting difficulties with tasks of left/right discrimination, road map skills, mental rotation, line orientation, and integration of motor and visual/perceptual skills. It has been reported that girls with TS are vulnerable to specific behavioral problems, particularly hyperactivity and inattention, as well as learning disabilities.

In conjunction with behavioral problems, girls with TS often exhibit difficulties with social development and functioning. They have been described as showing delayed emotional maturity, difficulty understanding social cues, needing more structure to socialize, poor peer relations, shyness, and poor body image. Impairments in self-concept and self-esteem increase during adolescence, but interestingly improve with the initiation of estrogen replacement therapy. Abnormalities in cognitive and psychosocial abilities in persons with TS likely reflect underlying atypical brain development and function.

Girls with TS have a typically female pattern of development, with unambiguous female gender identification. Dating and initiation of sexual activities, however, may be somewhat delayed or infrequent. It is not clear whether this reflects some underlying genetic or hormonal influence on behavior or simply discomfort, because of the issues of short stature, physical anomalies, and infertility, with which women with TS have to cope.

Motor Performance

Girls with TS show substantially lower performance in gross and fine motor function tests, and motor milestones are achieved relatively late. Moreover, girls with TS frequently encounter problems in specific motor functioning, that is, oral-motor and visual-motor coordination, motor learning, and problems with accuracy and movement speed. Impaired motor ability can result in multiple practical problems that are age specific. Examples of these practical consequences include feeding difficulties (infancy), problems with writing, participation in sports (school age), and choice of study and employment (adolescence).

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Klinefelter Syndrome

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Glossary

Aneuploidy Abnormal number of chromosomes; in humans, fewer or >46 chromosomes.
Azoospermia Absence of motile sperm in the semen.
Epigenetic Heritable changes in gene activity and expression that are not due to changes in the DNA sequence.
Hypergonadotropic hypogonadism Poor function of the gonads (either testes or ovaries) resulting in insufficient sex steroid hormone production and elevated luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland due to lack of negative feedback.
Microorchidism Small testes (testicles).
Noninvasive prenatal screening A method to screen for fetal chromosomal abnormalities through cell-free DNA in maternal blood.
Spermatogenesis The production of mature spermatozoa (sperm) occurring in the testis.
Spermatogonia Undifferentiated male germ cells in the testis that will undergo spermatogenesis.
Testicular sperm extraction (TESE) Surgical procedure to remove testicular tissue and extract viable sperm to be used in intracytoplasmic sperm injection (ICSI).
Virilization Development of masculine physical characteristics including penile growth, body hair, and deepening of the voice.

Introduction

Klinefelter syndrome (KS) was first described in 1942 by Dr. Harry Klinefelter, with clinical features of primary gonadal failure and tall stature in men (Klinefelter *et al.*, 1942). In 1959, Dr. Patricia Jacobs identified that this clinical phenotype was associated with an additional X chromosome, or karyotype 47,XXY (Jacobs and Strong, 1959). In the past 60 years basic science, translational, clinical, and epidemiological studies have greatly advanced our knowledge of both the endocrine and nonendocrine manifestations of KS, but the science particularly in the fields of genetics and reproductive endocrinology, continue to evolve our understanding of the pathophysiology and best management practices for KS.

There is some debate among parents and the professional community alike regarding terminology. The argument by some is that not all males with a 47,XXY karyotype have KS as they do not exhibit the features originally described in the syndrome, particularly in childhood, therefore should not be called KS. In addition, whether supernumerary aneuploidies including 48,XXYY, 48,XXXY, and 49,XXXXY are variants of KS or independent syndromes (Tartaglia *et al.*, 2011). For the purpose of this endocrine-focused chapter, we use KS to refer to any phenotypic male with an at least two X chromosomes and at least one normal Y chromosome, recognizing significant heterogeneity exists.

Clinical Overview

The additional X chromosome in males nearly universally results in abnormal testicular development, microorchidism, hypogonadism, and infertility (Davis *et al.*, 2015). Tall stature with longer extremities due to both genetic and hormonal factors is classically described but not ubiquitous. Many other conditions associated with KS include developmental delay, language-based learning disorders, mental health disorders, osteoporosis, diabetes, autoimmunity, and others (Davis *et al.*, 2016a). It is important to recognize that the presence and severity of any of these associated conditions are variable, resulting in a great deal of phenotypic heterogeneity.

Morbidity and mortality is higher in KS than reference male populations. Cohort studies from Europe have estimated the all-cause standardized mortality rate to be slightly higher than the general population, corresponding to approximately 2.1 years shorter lifespan (Bojesen and Gravholt, 2011). The risk of being admitted to the hospital is 70% higher in KS than the general population (Bojesen *et al.*, 2006a). The categories of causes for hospitalization and death that are greater in KS than the general population are numerous including diseases of the endocrine, nervous, respiratory, circulatory, digestive, and genitourinary systems.

Epidemiology

The estimated prevalence of KS is ~1 in 600 male births, making it the most common sex chromosomal aneuploidy (Coffee *et al.*, 2009; Nielsen and Wohlert, 1990; Herlihy *et al.*, 2011; Bojesen *et al.*, 2003). There is some evidence the prevalence may be increasing over time, although this needs to be confirmed (Morris *et al.*, 2008). Advanced maternal age is associated with an increased risk of chromosomal aneuploidies, however the relationship of parental age and 47,XXY is not as strong as with autosomal aneuploidies.

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Diagnosis

A karyotype or chromosomal microarray will identify the extra X chromosome diagnostic of KS. This is typically performed on peripheral blood lymphocytes, however other tissue types including epithelial cells from a cheek swab or skin biopsy can also be used. Despite the high prevalence, historically only 25%–35% of males even in developed countries are ever correctly diagnosed (Abramsky and Chapple, 1997; Bojesen and Gravholt, 2011). This is not usually because they are not affected, but rather their symptoms are not recognized by clinicians. Earlier diagnosis allows for appropriate counseling, screenings, and interventions, and potentially better outcomes, but studies are needed to quantify this protective effect and identify best practices.

KS should be suspected and genetic testing should be obtained in any adolescent or adult male with microorchidism or hypergonadotropic hypogonadism. In infancy and childhood there are no pathognomonic features that lead to a diagnosis of KS, therefore a high degree of clinical acumen is needed to suspect this condition prepubertally. Noninvasive prenatal screening can detect KS and other sex chromosome aneuploidies in utero, and due to the test's relatively low-cost, low-risk, broad availability, and high reliability for autosomal aneuploidies, this is emerging as routine screening in prenatal care (Gregg *et al.*, 2016). Thus our KS patient population is being redefined, particularly in pediatrics, where the majority of patients will be identified through screening rather than by directed testing.

Genetics

The supernumerary X chromosome is typically a de novo nondisjunction error occurring during maternal or paternal gametogenesis. The supernumerary X chromosome can also be due to postzygotic errors in mitosis in the developing embryo. Rare cases of maternal trisomy X have been reported. However, KS is usually a random event and the chance of recurrence in subsequent pregnancies is low. Approximately half of the second X chromosome are paternal versus maternal in origin and there are conflicting results as to whether parental origin contributes to phenotypic variability.

Mosaicism is present in $\sim$7% of cases of KS and is associated with a more mild phenotype if a normal 46,XY cell line is present. In contrast, mosaicism with tetrasomy or pentasomy lines often have a more severe phenotype. Predicting phenotype based on the degree of mosaicism is difficult, however, as tests are typically only done in one tissue type (peripheral blood lymphocytes) and may or may not accurately reflect tissue-level mosaicism percentages.

The genetic mechanisms and pathophysiology of the clinical features of KS are multifactorial and remain poorly understood. When two or more X chromosomes are present (in males or females), the second X chromosome is largely silenced through a process of X-inactivation. Therefore, the majority of extra X genes are not expressed. Usually this occurs in a random fashion, however preferential inactivation of one X over another (skewed inactivation) has been reported in 10%–40% of males with KS and may correlate with phenotype (Iitsuka *et al.*, 2001). There are also genes that escape X-inactivation and these genes are expressed in duplicate (or triplicate in cases of trisomy). A classic example is the short-stature homeobox (SHOX) gene, a critical gene for linear growth located in the pseudoautosomal region of the X and Y chromosomes. In Turner syndrome (45,X), only one copy of the SHOX gene is expressed and short stature is a nearly universal feature of the condition. In KS and other sex chromosome trisomies (47,XXX and 47,XYY), a total of three copies are expressed and taller stature is a common feature of these conditions. All of the genes in the pseudoautosomal region and another 5%–15% of genes on the X chromosome escape X-inactivation and may therefore be expressed and potentially influence cellular and metabolic pathways (Berletch *et al.*, 2011).

Like all phenotypic variation in humans, polymorphisms in specific genes likely contribute to the variation seen in KS. The androgen receptor gene is particularly of interest due to its location on the X chromosome and the known hypogonadism. Longer CAG repeat lengths are associated with lower receptor activity, and in KS this has been associated with greater severity of the KS phenotype (taller stature, longer arm span, gynecomastia, and delayed puberty) (Zinn *et al.*, 2005; Chang *et al.*, 2015). Copy number variants are another reason for population variation and are reported to be more common on the X chromosomes of males with KS compared to control male or females (Rocca *et al.*, 2016).

Finally, an emerging area that needs additional attention is the role of the autosomes in the KS phenotype. The presence of an additional chromosome in both sex chromosome aneuploidy as well as trisomy 21 (Down syndrome) is associated with differential gene expression throughout the genome (Huang *et al.*, 2015; Zitzmann *et al.*, 2015; Do *et al.*, 2017). These epigenetic changes exponentially increase the complexity of the mechanisms involved in the phenotypic heterogeneity in KS.

Testicular Dysfunction

Testicular dysfunction is nearly universal in males with KS, although the molecular mechanisms underlying this are largely unknown. Microorchidism is essentially universal ($<$10 mL and usually $<$6 mL in adults), resulting in a discordance between the degree of virilization and testicular volume. The relative or absolute hypogonadism results in follicle stimulating hormone (FSH) and luteinizing hormone (LH) elevation. KS is the most common cause of hypergonadotropic hypogonadism in men.

Germ, Sertoli, and Leydig cell lines are all impaired in KS. **Fig. 1** reviews the hypothalamic pituitary gonadal axis in KS. Germ cells are noted to be fewer in number from a very early age and progressively decline particularly in puberty, with adult testes having no or rare pockets of spermatogonia (Davis *et al.*, 2015). Sertoli cell function is unequivocally poor as evidenced by lack of

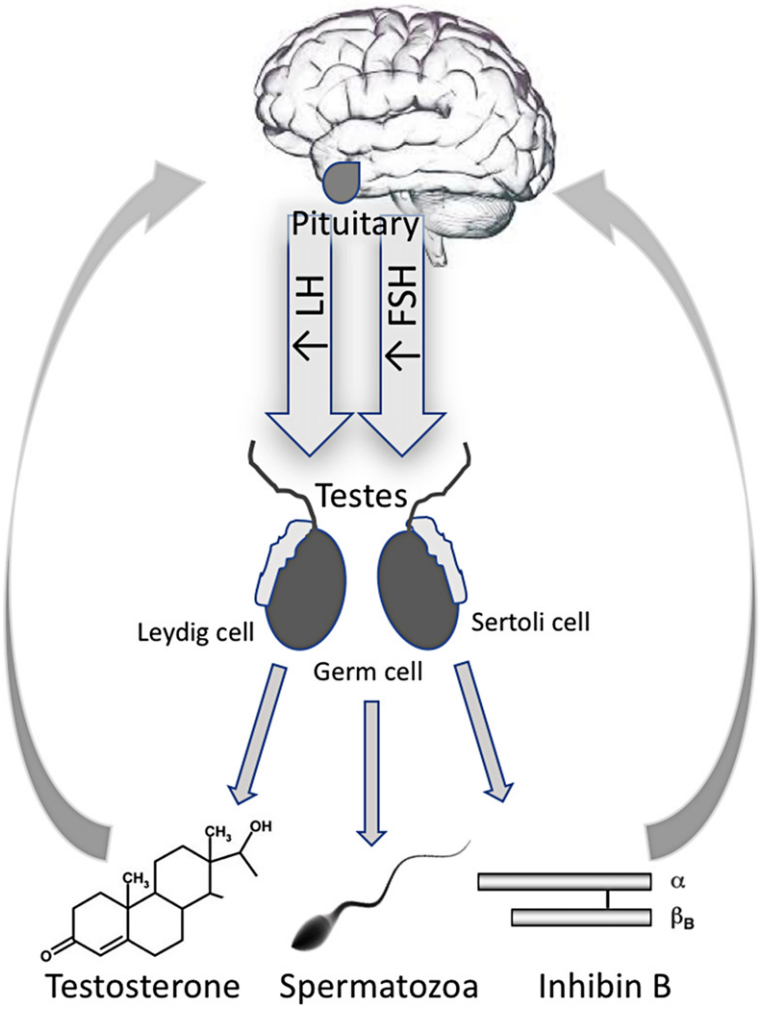

Fig. 1 The hypothalamic–pituitary–gonadal axis in KS. The extra X chromosome results in testicular dysfunction affecting germ cells > Sertoli cells > Leydig cells. Spermatogenesis and hormone production is impaired, which reduces negative feedback to the pituitary gland. The pituitary gland increases secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) to stimulate the Leydig and Sertoli cells respectively.

the pubertal rise in inhibin B and low or undetectable levels in adulthood. Sertoli cell function may already be impaired in a subset of prepubertal boys with KS (Davis *et al.*, 2016b).

Testosterone Throughout the Lifespan

Leydig cell function in KS is more variable. Around 2–4 months of age, the hypothalamic–pituitary–gonadal axis is activated in males resulting in a testosterone surge known as "mini-puberty" (Rey, 2014). Mini-puberty does occur in infants with KS, however the few small studies that have been done have mixed conclusions as to whether testosterone is lower. The largest of these studies and the only one to use the gold-standard tandem mass spectroscopy reported testosterone was lower than average (Cabrol *et al.*, 2011). After mini-puberty but prior to true puberty, testicular testosterone production is normally minimal and whether testosterone deficiency is present prepubertally in KS remains an area of debate (Fennoy, 2011). Puberty starts normally in the majority of individuals with KS, however, LH rises about 2 years after pubertal onset on average. Testosterone initially rises appropriately in puberty but then plateaus or even declines (Bastida *et al.*, 2007). Based on these data, monitoring physical exam, gonadotropins, and testosterone throughout puberty is recommended. In adulthood, some men with KS will maintain serum testosterone concentrations within the low-normal adult range, however LH will be high and physical signs of hypogonadism, such as gynecomastia, can be present despite testosterone within the normal range (Davis *et al.*, 2015; Pacenza *et al.*, 2012).

Testosterone Replacement

There are not rigorously conducted studies to date evaluating the timing, formulation, or dosage of testosterone in KS. Therefore, there are a variety of approaches in when (and how) to initiate testosterone replacement (**Fig. 2**). In general, many pediatric

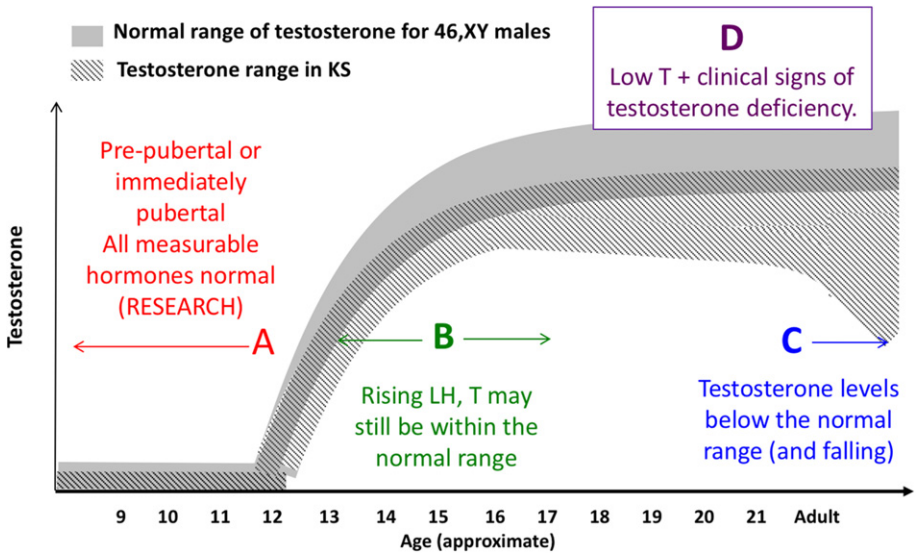

Fig. 2 Practice variations in testosterone initiation. Throughout the lifespan, serum testosterone concentrations in KS overlap with typical 46,XY males. There are no accepted standards for initiating testosterone replacement in KS. The most conservative is waiting until testosterone is low and there are clinical signs of testosterone deficiency (D). However, many symptoms of testosterone deficiency are subjective and/or are late-onset, therefore some will initiate treatment when testosterone is low but symptoms may not yet be present (C). Due to high luteinizing hormone (LH) and limitations of testosterone assays, a more proactive approach is testosterone supplementation before serum testosterone levels are overtly below the normal range (B). And finally, there is interest in using low-dose testosterone in prepubertal ages, but this is investigative (A).

endocrinologists in the United States will proactively consider testosterone treatment when LH is unequivocally above the normal range, much like treating compensated hypothyroidism when thyroid stimulating hormone is elevated. However, this is at odds with the Endocrine Society Clinical Practice Guideline that advises testosterone treatment should only be given for androgen deficiency syndromes, defined by unequivocally low testosterone (Bhasin *et al.*, 2010). Elevated LH, low testosterone, and clear symptoms of hypogonadism such as gynecomastia, stalled puberty, fatigue, and low libido is the most conservative approach, but there are concerns that this reactive rather than proactive approach may contribute to the higher risk of osteoporosis and metabolic syndrome, and negatively affect cognition and mental health. Due to the lack of evidence and variety of professional opinions, patients and parents have an especially important role in shared decision making regarding testosterone replacement in KS.

There are a variety of formulations available to provide exogenous testosterone. Injections, transdermal gel, transdermal patches, buccal tablets, and pellet implants are all FDA approved for male hypogonadism in adults with associated benefits and side effects for each formulation (Bhasin *et al.*, 2010). Any of these options are available to adult men with KS, however the long-acting formulations are not ideal for adolescents or adults with mild hypogonadism who may not need a full replacement dose (Rogol and Tartaglia, 2010). Low-dose intramuscular or subcutaneous injections or transdermal gel are best for testosterone initiation, particularly in adolescents (Rogol *et al.*, 2014). Monitoring for and managing side effects should follow the general guidelines for testosterone replacement (Bhasin *et al.*, 2010).

Fertility

Azoospermia is characteristic of KS, with $<10\%$ having any spermatozoa in ejaculate. Therefore, spontaneous pregnancies are very rare, and infertility has historically been emphasized as a key feature of the syndrome. However, advanced reproductive technology approaches, predominately testicular sperm extraction (TESE) followed by intracytoplasmic sperm injection (ICSI), has revolutionized the reproductive options in KS. In a meta-analysis that included 1248 men with KS, TESE success, defined as retrieval of mature sperm, was 44% (95% confidence interval 39%–48%) independent of age, hormone concentrations, and surgical method (Corona *et al.*, 2017). Pregnancy and live birth rate was 43% per ICSI cycle. Therefore, $\sim$16% of men with KS overall who sought reproductive assistance successfully fathered a child.

Given the natural history of progressive testicular fibrosis and worsening testicular function, it was hypothesized that earlier attempts would have higher success rates but a recent trial found no difference success between males 15–22 versus >23 years and was stopped early (Plotton *et al.*, 2015). Some studies have found younger age to be predictive of higher success with a statistical threshold around 35 years, however this was not confirmed on the meta-analysis. Invasive procedures in early adolescence (<15 years of age) are associated with poor sperm retrieval rates. Preservation of testicular tissue in pre- or postpubertal males with KS relies on the hope of technological advances to induce in vitro spermatogenesis and should be considered experimental (Franik *et al.*, 2016).

A practical approach to fertility in KS is to start with noninvasive methods (semen analysis) when the individual has reached sexual maturity. If spermatozoa are not identified, the patient should be counseled on the success rates and potential risks of

invasive procedures, specifically TESE. Interested patients should be referred to a male reproductive specialist with experience in TESE for KS patients. Specific attention should be given to the socioemotional maturity of the patient when discussing fertility options, particularly given the lack of data to support earlier intervention. The research would also suggest testosterone treatment should not be withheld in hypogonadal individuals for the purpose of future fertility.

Cardiometabolic and Bone Health

Adults with KS have a high prevalence of metabolic syndrome, type 2 diabetes, and osteoporosis (Salzano *et al.*, 2016). Adiposity is greater in KS for any given BMI, and these disorders of insulin resistance are correlated to visceral adiposity (Bojesen *et al.*, 2010, 2006b). Studies in pediatric populations have suggested the risk for cardiometabolic dysfunction is present early, with higher body fat and high prevalence of dyslipidemia (Davis *et al.*, 2016b; Bardsley *et al.*, 2011). Testosterone concentrations inversely correlate with bone density, adiposity, and insulin resistance in KS, however, testosterone treatment is not associated with better cardiometabolic outcomes in adults. Screening for these conditions, encouraging a healthy lifestyle, and optimizing testosterone replacement in KS is advised, but there is a lack of interventional trials in this area.

Nonendocrine Manifestations of KS

Nonendocrine manifestations associated with KS are variable but can impact the health and wellbeing of the individual as much or more than hypogonadism. Therefore, a holistic, multidisciplinary approach to care is highly recommended (Tartaglia *et al.*, 2015). **Table 1** has a list of nonendocrine medical and neuropsychological manifestations that are more prevalent in KS than in the general population. Clinicians caring for individuals with KS should be proactive in screening for and counseling about these risks.

Table 1 Conditions associated with KS

- Neurodevelopmental and psychological risks in KS
  - Speech and language disorders
    - Speech-language developmental delays
    - Receptive expressive language disorder
    - Apraxia/dyspraxia of speech
    - Pragmatic/social communication
    - Lower verbal IQ
  - Motor skills disorders
    - Gross or fine motor skills delay
    - Hypotonia
    - Motor coordination problems
    - Graphomotor deficits
  - Learning disabilities
    - Dyslexia
    - Other language-based learning disabilities
  - Attention deficit hyperactivity disorder
  - Sensory processing differences
  - Social differences
    - Autism spectrum disorders
    - Social cognitive deficits
    - Social-emotional immaturity
  - Adaptive functioning deficits
  - Executive functioning deficits
  - Emotional/psychological disorders
    - Anxiety
    - Depression
    - Mood disorders/bipolar disorder
    - Psychotic features/schizophrenia
- Medical risks in KS
  - Testicular dysfunction
    - Testosterone deficiency
    - Gynecomastia
    - Infertility
    - Cryptorchidism; hypospadias; micropenis
  - Cardiometabolic
    - Metabolic syndrome
    - Type 2 diabetes
    - Fatty liver disease
    - Dyslipidemia
  - Musculoskeletal
    - Osteoporosis
    - Scoliosis; kyphosis
    - Hypermobility
  - Vascular
    - Hypercoagulability
    - Varicose veins; venous stasis
  - Neurological
    - Seizures
    - Tremor
  - Congenital anomalies
  - Respiratory
    - Asthma, allergies
    - Recurrent respiratory infections
  - Gastrointestinal
    - Constipation
    - Gastroesophageal reflux
    - Intestinal vascular insufficiency
  - Autoimmunity
  - Malignancy
    - Breast cancer
    - Non-Hodgkin lymphoma
    - Extragonadal germ cell tumors
  - Eyes—strabismus
  - Dental
    - Enamel defects
    - Taurodontism
  - Nonspecific fatigue

Intellectual disability is rare in KS, however verbal cognition is a relative weakness in some males (Leggett *et al.*, 2010; Boone *et al.*, 2001). Language delays and language-based learning disabilities are present in ~75% (Bender *et al.*, 1983). It should also be noted that there are wide variations in cognitive ability, including academic strengths and weaknesses, with some individuals functioning in very superior ranges in both verbal and nonverbal areas. Executive and adaptive functioning is often more impaired than expected for cognitive abilities, therefore individuals often benefit from additional educational and occupational support. Neuropsychological evaluation can often be helpful to guide appropriate support as needed.

Summary

In summary, KS is a common but phenotypically heterogeneous genetic condition affecting males. Testicular development and function is impaired, often resulting in hypergonadotropic hypogonadism, but there are also neurodevelopmental, cardiometabolic, and other manifestations that present more variably. While historically this condition has been underdiagnosed, routine prenatal screening is quickly changing that landscape. Research is needed in basic, translational, clinical, and implementation sciences to advance the care and improve patient-centered outcomes for individuals with KS. With acknowledgement of our limited evidence-based research for best practices, a holistic approach to caring for the patient with KS throughout the lifespan is recommended.

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45,X/46,XY Gonadal Dysgenesis, 46,XX/46,XY Chimerism (and Variants), and 46,XX Testicular and Ovotesticular DSD

Martine Cools, Ghent University Hospital, Ghent, Belgium

Nomenclature

DSD	differences/disorders of sex development
FISH	florescence in situ hybridization
EMS	external masculinization score
hCG	human chorion gonadotropin

45,X/46,XY Gonadal Dysgenesis

Etiology

45,X/46,XY gonadal dysgenesis (and variants) occurs with an estimated incidence of around 1.5/10,000 conceptions. Prefertilization causes include loss of the Y chromosome through meiotic nondisjunction or anaphase lag at the gamete level and, although occurring very rarely, paternal transmission of an abnormal Y chromosome with subsequent loss of Y (Chang et al., 1990). The majority of cases originates from postfertilization events, such as mitotic anaphase lag or interchromosomal rearrangements with final loss of a structurally abnormal Y chromosome (Hook and Warburton, 2014). It has been hypothesized that all live born 45,X individuals have some degree of mosaicism for a cryptic normal (XX or XY) cell line, for example, in the placenta, but data are not conclusive on this matter (Hook and Warburton, 2014).

Terminology

The condition associated with 45,X/46,XY gonadal dysgenesis is often referred to as "mixed gonadal dysgenesis" because affected individuals may have a streak gonad on one side and a testis on the other. In reality, gonadal histology in 45,X/46,XY individuals is far more complex (see below) and variable and can be appreciated only by detailed pathological description of the gonads, which in many cases is not available. The term should therefore be abandoned.

Although clinical features in 45,X/46,XY men may show some overlap with Turner syndrome, this term is reserved for females who have one intact X chromosome and complete or partial absence of the second sex chromosome in association with one or more clinical manifestations. Thus, this diagnosis is excluded in 45,X/46XY males (Gravholt *et al.*, 2017).

Diagnosis

A karyotype should be obtained in any girl with growth failure, with or without the typical features associated with Turner syndrome (Gravholt *et al.*, 2017), and with a low threshold in boys with short stature or a discrepancy between actual height and target height. If associated with a history of undescended testes and/or hypospadias, karyotyping is imperative. Florescence in situ hybridization (FISH) with X and Y centromere probes in at least 100–200 interphase cells from two different tissues (e.g., peripheral blood, buccal swab, skin fibroblasts) can identify low-grade mosaicism.

The presence of minor Turner stigmata, such as short 4th metacarpal/metatarsal or short stature in an individual with presumed 46,XY gonadal dysgenesis, should always prompt investigations to exclude 45,X/46,XY mosaicism (Cools and Kohler, 2017).

Clinical Presentation

Ninety-five percent of individuals who have a 45,X/46,XY chromosomal constitution will present as typical males (Chang *et al.*, 1990). As these men do not regularly come to medical attention, there is little or no information about their outcome with respect to growth, puberty, cardiovascular status, co-morbidities, fertility or risk of gonadal tumors. The remainder are characterized by great phenotypic variability, ranging from newborns with isolated hypospadias or ambiguous genitalia to females who have Turner syndrome. Of all girls diagnosed with Turner syndrome, 10%–12% have a 45,X/46,XY mosaic karyotype (Gravholt *et al.*, 2017). The External Masculinization Score (EMS) can be used to describe the degree of genital virilisation in neonates and infants, but is difficult to apply in typical females (Ahmed *et al.*, 2000).

Little correlation exists between the proportion of the different cell lines in peripheral blood, fibroblasts or even gonads and the observed phenotype (Cools *et al.*, 2007). Characteristics typically associated with Turner syndrome are often present. These include amongst others short stature, short 4th metacarpal and metatarsal, horseshoe kidney, wide-spaced nipples, shield thorax and facial dysmorphisms. Mild cognitive defects, learning disabilities and autism spectrum disorders may also be part of the phenotypic spectrum (Martinerie *et al.*, 2012; Tosson *et al.*, 2012).

Growth

Girls and boys who have a 45,X/46,XY karyotype are at high risk for adult short stature, similar to girls who have 45,X monosomy. Short stature may be the only physical sign in affected children, both boys and girls. Growth failure in association with a history of hypospadias or undescended testes should always prompt karyotyping. Although commonly administered nowadays, growth hormone therapy seems to result in improved height gain mainly on the short term, while adult height is disappointing and apparently with no better outcomes in treated as compared to untreated individuals. No differences have been observed between girls and boys, or those who entered puberty spontaneously or after hormonal stimulation. These data need confirmation in a larger sample (Richter-Unruh *et al.*, 2004; Martinerie *et al.*, 2012; Lindhardt Johansen *et al.*, 2012; Bertelloni *et al.*, 2015).

Cardiovascular status

Only a limited number of small-scale studies have systematically searched for congenital cardiac defects in children who have 45,X/46,XY karyotypes. Left side defects, mainly bicuspid aortic valve and aortic coarctation have been found in 4/9 females and 0/7 males (Tosson *et al.*, 2012); in 3/20 males (Martinerie *et al.*, 2012), and in 3/8 females and 5/10 males (De Groote *et al.*, 2013). Data on cardiovascular pathology in adolescents and adults with this condition are even more scarce, but suggest an increased risk for hypertension and dilation of the ascending aorta (De Groote *et al.*, 2013). Near-fatal cardiac failure, secondary to aortic root dilation has been described (Bakoto *et al.*, 2011). These data come from highly selected cases, additional evidence resulting from systematic screening of 45,X/46,XY individuals for cardiovascular pathology is highly needed.

In summary, although numbers are small, cardiovascular pathology in 45,X/46,XY individuals seems to occur to the same extent as in Turner syndrome, irrespective of phenotype, with the exception perhaps of nonsymptomatic males. While awaiting further data, it is recommended to apply the same cardiac surveillance protocol in both 45,X/46,XY males and females as in Turner syndrome (Gravholt *et al.*, 2017).

Other features

When searched for, other features typically associated with Turner syndrome are observed with increased frequency in girls and boys with 45,X/46,XY karyotypes. Amongst those are autoimmune thyroiditis, elevated liver enzymes, recurrent otitis media, conductive hearing loss and scoliosis. Although children with Turner stigmata may be more likely to develop such comorbidities, data are too limited to make final conclusions on this matter (Tosson *et al.*, 2012; De Groote *et al.*, 2013).

In summary, in the absence of further evidence, it is advised to include all individuals who have 45,X/46,XY karyotypes in the same follow-up and screening programs as women who have Turner syndrome, irrespective of phenotype or presence of associated features (Gravholt *et al.*, 2017). The correlation between the relative proportion of the different cell lines, condition-related stigmata, genital/gonadal phenotype and involvement of other organ systems is unclear so far. Care for these individuals is best embedded in an experienced multidisciplinary team.

Gonadal histology

Gonadal histology is highly variable but correlates to a certain extent with the child's phenotype: 45,X/46,XY girls with Turner syndrome typically have bilateral streak or sometimes absent (regressed) gonads; genital ambiguity is often associated with the presence of dysgenetic testes or undifferentiated gonadal tissue, whereas at least one scrotal testis is usually present in phenotypic males. Representative examples are shown in **Fig. 1**. Asymmetrical mesonephric (Wolffian) and paramesonephric (Müllerian) duct development in agreement with the ipsilateral gonad is common and this finding in a 46,XY individual should prompt gonadal karyotyping to exclude gonadal sex chromosome mosaicism. Functional ovarian tissue, i.e. germ cells enclosed in primordial and maturing follicles, is extremely rare in 45,X/46,XY, as oocyte meiosis is incompatible with the absence of a second X chromosome (Cools *et al.*, 2011a,b; Wunsch *et al.*, 2012; Lindhardt Johansen *et al.*, 2012; Lepais *et al.*, 2016).

Gonadal (testicular) function

Gonadal function is tightly related to gonadal histology. Streak gonads, usually seen in 45,X/46XY girls with typical female genitalia, are composed of nonfunctional fibrotic tissue, devoid of germ cells, granulosa cells and theca cells, hence no hormone production is expected (Cools et al., 2011b). Gonadal function is highly variable in girls and boys with partial gonadal dysgenesis (testicular dysgenesis and/or undifferentiated gonadal tissue), who mostly present with ambiguous genitalia. An estimate of gonadal hormone production can first be obtained during the postnatal gonadotropin surge or after human Chorion Gonadotropin (hCG) stimulation, but levels do not necessarily correlate with gonadal function in puberty or thereafter (Martinerie *et al.*, 2012). Very limited data suggest that pubertal onset is spontaneous in most, if not all, 45,X/46,XY boys with typical male genitalia and in many undervirilised 45,X/46,XY males (Lindhardt Johansen *et al.*, 2012; Martinerie *et al.*, 2012). The latter however may need hormone replacement therapy later in life. In early adulthood, high gonadotropin levels, low-normal testosterone and low inhibin B levels have been found in four men with typical male phenotypes and an uneventful course of puberty, suggesting absent spermatogenesis and progressive decrease of Leydig cell function over time in these men. Of note, three out of four had structural abnormalities of the Y chromosome, in particular large deletions on Yq, including the AZF region, which contains many genes important for spermatogenesis (Lindhardt Johansen *et al.*, 2012).

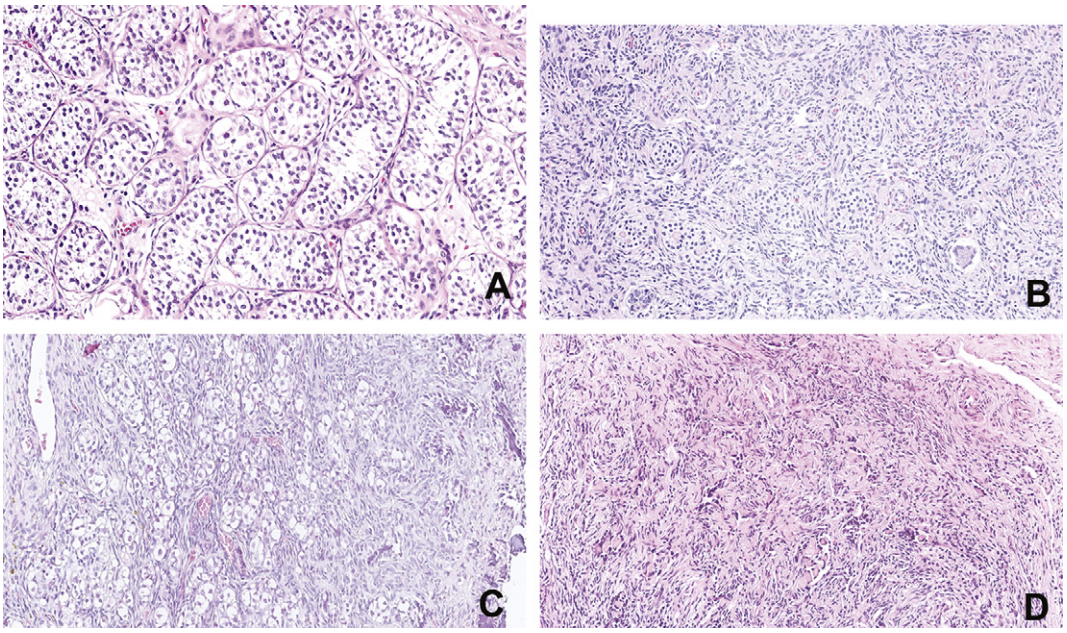

Fig. 1 Gonadal development in 45,X/46,XY individuals. Representative examples are shown of gonadal development patterns typically encountered in individuals who have a 45,X/46,XY karyotype. A gradient in degree of "testicularisation" can be observed from A–D. (A) Normal testis, HE, 200 ×, (B) dysgenetic testis: aberrantly shaped tubules, reduced in number and in a background of stromal tissue, HE, 200 ×, (C) undifferentiated gonadal tissue: germ cells and sertoli/granulosa like cells, poorly organized in primitive sex cord structures, HE, 200 ×, (D) streak gonad without functional cells, HE, 200 ×.

Gonadal tumor risk and indications for gonadectomy

Gonadectomy can be performed because of gonadal tumor risk, hormone production discordant with gender identity, or both (van der Zwan *et al.*, 2015). Gonadal germ cell tumors occur with increased frequency in 45,X/46,XY DSD. The peak incidence most likely follows that of the general male population, which is in early adulthood. Invasive germ cell tumors are preceded by premalignant changes, termed germ cell neoplasia in situ (GCNIS) in the testis and gonadoblastoma in the dysgenetic gonad. These lesions are presumably present from birth or early childhood onwards but may remain quiescent for one to three decades (Rajpert-De Meyts *et al.*, 1998).

The degree of gonadal differentiation is hypothesized to be inversely related to tumor risk (**Fig. 2**) (Cools *et al.*, 2011a). In one relatively large study, gonads comprised of dysgenetic testicular and/or undifferentiated gonadal tissue were more likely to harbor preneoplastic lesions as compared to scrotal testes with no or very mild signs of dysgenesis. Streak gonads by definition have no germ cells that can give rise to neoplastic transformation. However, gonadal differentiation can be largely heterogeneous within the same gonad, hampering conclusive risk assessment on the basis of a single biopsy in such cases. In addition, it was hypothesized that an individual's phenotype reflects—to a certain extent—gonadal morphology. Indeed, it was observed that (pre) neoplastic lesions were mainly encountered in girls and boys who had ambiguous genitalia, and to a much lesser extent in typical males, who mostly had bilateral scrotal testes, and typical females, who mostly had bilateral streak or regressed gonads (Cools *et al.*, 2011a). This observation has been confirmed in three independent series (Lindhardt Johansen *et al.*, 2012; Martinerie *et al.*, 2012; Wunsch *et al.*, 2012).

Given the absence of gonadal function in girls who have 45,X/46,XY and Turner syndrome, prepubertal gonadectomy is recommended (Gravholt *et al.*, 2017). A decision regarding prophylactic gonadectomy in boys with partial gonadal dysgenesis who have a considerable risk for germ cell tumor development on the one hand but may benefit from endogenous hormone production on the other is sometimes extremely difficult; repeat gonadal biopsies and expert evaluation to detect early malignant changes can be informative (Hersmus *et al.*, 2012; Lindhardt Johansen *et al.*, 2012). In girls with partial gonadal dysgenesis, unwanted virilisation in puberty may further guide the decision regarding gonadectomy (van der Zwan *et al.*, 2015).

46,XX/46,XY Chimerism (and Variants)

46,XX/46,XY chimerism is defined as the existence of two or more cell lines of different genetic origin in one individual and arises at or immediately after fertilization. 46,XX/46,XY chimerism may lead to ovotesticular DSD (see below).

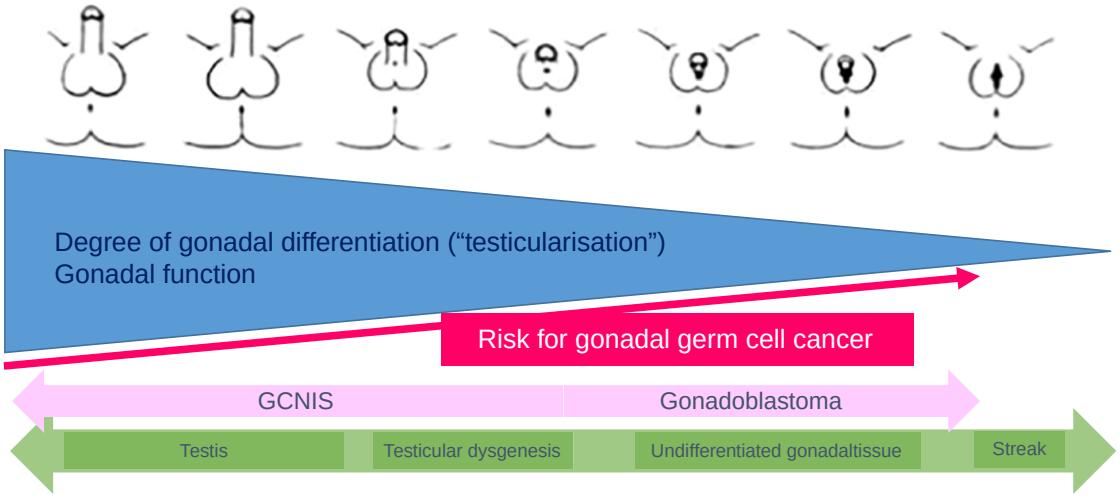

Fig. 2 Risk for development of germ cell tumors in individuals who have a 45,X/46,XY karyotype. Gonadal function decreases with decreasing gonadal differentiation ("testicularisation"). A high level of "testicularisation" is generally associated with a more virilised genital phenotype and lower germ cell tumor risk. Undifferentiated gonadal tissue has the highest risk; streak tissue, by definition, has no germ cells. Germ cell neoplasia in situ is the precursor lesion in the testis, gonadoblastoma is typically found in association with undifferentiated gonadal tissue.

46,XX Testicular and Ovotesticular DSD

46,XX^{SRYpos} Testicular DSD

85% of individuals with XX testicular DSD have typical male genitalia and present as infertile adults ("XX males"), sometimes with gynaecomastia (Delot and Vilain, 1993). The diagnosis is sometimes made prenatally, following karyotyping for maternal or other reasons. A minority of cases may present with milder undervirilisation defects, such as isolated cryptorchidism or hypospadias. Wolffian ducts develop normally and Müllerian structures are absent. Most of these cases harbor an Xp:Yp translocation containing *SRY*. Preferential inactivation of the Y-bearing X chromosome and cryptic mosaicism with SRY expression confined to the testes have been implicated in incomplete virilisation (Fleming and Vilain, 2005).

Single case reports and very small series suggest normal cognitive and psychomotor development but short stature in most 46,XX^{SRYpos} boys. Although pubertal onset appears to be spontaneous and at an appropriate age, hypergonadotropic hypogonadism is gradually installing. Testosterone production generally declines over time, requiring hormone replacement therapy. As most of the Y chromosome, including the AZF region, is lacking, 46,XX^{SRYpos} men are infertile (Gunes *et al.*, 2013; Boucekkine *et al.*, 1994; Delot and Vilain, 1993).

A gonadal biopsy most often reveals testicular differentiation with small (infantile) Sertoli-cell only tubules and Leydig cell hyperplasia in pubertal patients. In older individuals, tubules become increasingly sclerotic and the normal testicular architecture is gradually replaced by general fibrosis (**Fig. 3**). It is generally accepted that 46,XX^{SRYpos} men have no increased risk for the development of testicular germ cell tumors, given the early loss of germ cells and absence of most of the Y chromosome, in particular the *testis specific protein on Y* (*TSPY*) region, which has been implicated, amongst others, in the pathogenesis of these tumors (Cools *et al.*, 2006).

Other Forms of 46,XX Testicular and Ovotesticular DSD

Ectopic SRY expression is not usually involved in conditions characterized by 46,XX (ovo)testicular DSD and ambiguous genitalia. Few other genetic causes have been identified, such as gain-of-function changes of male sex-determining genes or its regulatory regions, mainly *SOX9*, or loss-of-function mutations of female sex-determining genes, for example, *RSPO1*, which has been implicated in a rare syndrome characterized by XX testicular DSD, palmoplantar hyperkeratosis and a susceptibility to develop squamous cell carcinoma (Baetens *et al.*, 2017a; Tomaselli *et al.*, 2008; Hyon *et al.*, 2015). Genomic rearrangements (duplications as well as a microdeletion) disrupting the regulatory region of *SRY*-related genes such as *SOX3* and *SOX10* have been described. It has been hypothesized that ectopic gonadal expression of these genes at a critical stage of development has triggered SOX9 transcription and subsequent initiation of the male pathway in these cases (Haines *et al.*, 2015; Polanco *et al.*, 2010; Baetens *et al.*, 2017a).

It becomes increasingly clear that proper and complete sex determination is secured by continuous repression of the opposite pathway at the gonadal level (Bashamboo and McElreavey, 2015). Thus, loss of activation or incomplete development of one pathway inevitably implies partial activation of the opposite pathway due to loss of antagonism. *NR5A1*, apart from its crucial role in testis development, has in a transgenic mouse model, been implicated in activation of early ovarian-determining genes such as

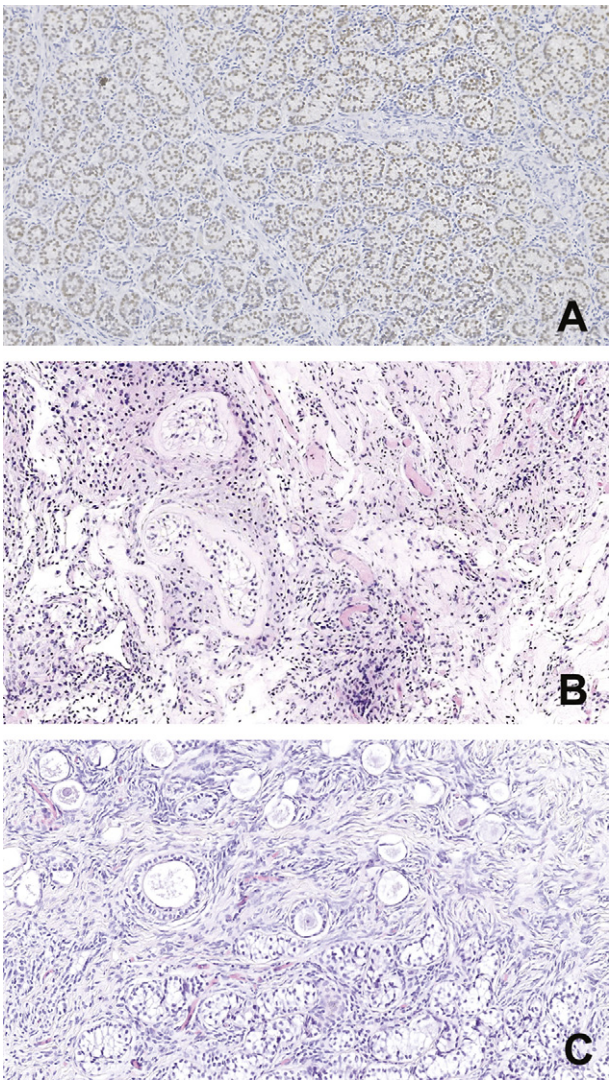

Fig. 3 46,XX testicular and ovotesticular DSD. (A) Infantile testis in a boy with 46,XX (SRY negative) testicular DSD, displaying regularly shaped small Sertoli-cell only tubules. All Sertoli cells stain positive for SOX9, demonstrating noncanonical, SRY independent SOX9 activation. SOX9 staining, 200 ×. (B) Atrophic testis in a 40 years old 46,XX^{SRYpos} man, with a typical male phenotype and a recent diagnostic work-up for infertility. HE, 200 ×. (C) Ovotestis in a girl who has the *NR5A1* c.274C>T (p.Arg92Trp) mutation. Ovarian tissue in the upper part of the picture, testicular differentiation in the lower part. HE, 200 ×.

WNT4/β-catenin (Combes *et al.*, 2010). Recently, multiple families have been described in which a specific mutation in *NR5A1* c.274C>T (p.Arg92Trp) has led to ovotesticular or testicular DSD in some 46,XX family members while others are asymptomatic carriers (Bashamboo *et al.*, 2016; Baetens *et al.*, 2017b; Igarashi *et al.*, 2017). Interestingly, in one of these families, two sibings with this specific mutation were investigated, one diagnosed with 46,XY gonadal dysgenesis and raised as a girl, and another diagnosed with 46,XX testicular DSD and raised as a boy (Bashamboo *et al.*, 2016). Although the exact molecular mechanism remains to be elucidated, it has been hypothesized that the p.Arg92Trp variant specifically interferes with NR5A1-mediated activation of ovarian development, resulting in loss of suppression of the testicular pathway in affected individuals (Bashamboo *et al.*, 2016; Baetens *et al.*, 2017b; Igarashi *et al.*, 2017) (**Fig. 4**) (Baetens *et al.*, 2017b; Bashamboo *et al.*, 2016). Possibly other amino acid substitutions at the same position may result in similar phenotypes (Swartz *et al.*, 2017).

The term ovotesticular DSD should be reserved for individuals who have well differentiated and functional testicular as well as ovarian tissue (i.e., Leydig cells and seminiferous tubules with or without germ cells and ovarian follicles composed of granulosa cells, theca cells and ova) either combined in an ovotestis or as asymmetrically developed gonads (**Fig. 3**) (Cools *et al.*, 2011b). Most, if not all cases have a 46,XX karyotype. Large pedigrees have been described in which some individuals have ovotesticular DSD, some have testicular DSD and others are asymptomatic carriers so it is believed that, at least in some cases, the condition is characterized by variable penetrance (Parma *et al.*, 2006).

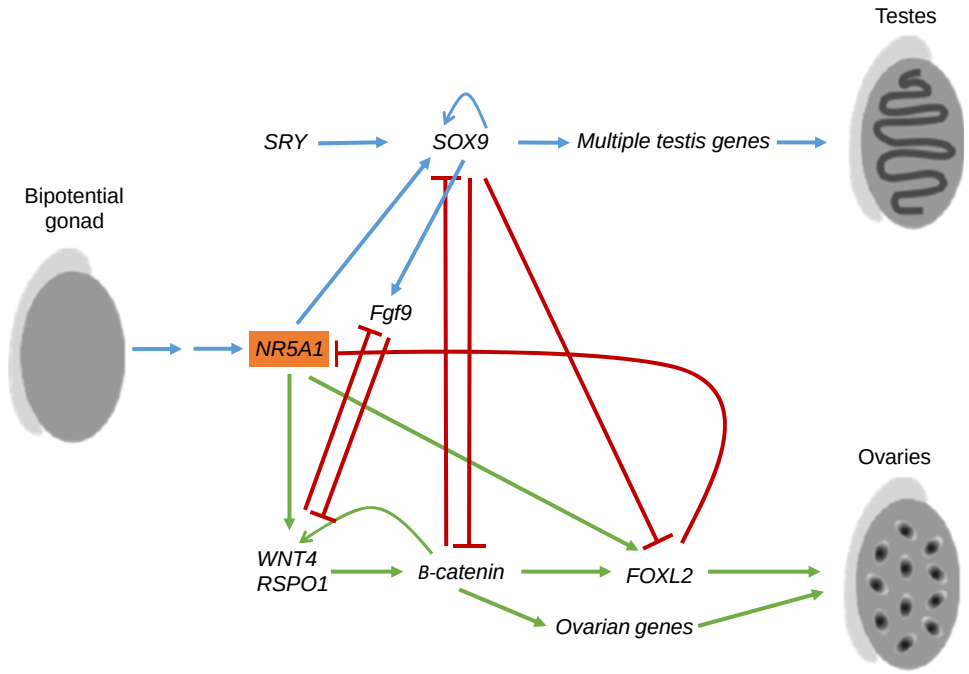

Fig. 4 Current simplified model of agonistic and antagonistic pathways determining gonadal differentiation. Testis-promoting actions are in blue, ovary promoting actions are in green, antagonistic actions in red. Incomplete activation of one (either male or female) pathway unavoidably results in loss of repression of the opposite pathway. *NR5A1* may act as a key hub in this model. Figure modified from Baetens, D., Stoop, H., Peelman, F., Todeschini, A.-L., Rosseel, T., Coppieters, F., Veitia, R. A., Looijenga, L. H. J., De Baere, E. and Cools, M. (2017b). NR5A1 is a novel disease gene for 46,XX testicular and ovotesticular disorders of sex development. *Genetics in Medicine* **19**, 367–376.

Very few long-term outcome data in individuals who have ovotesticular DSD are available. The testicular and ovarian parts both produce sex steroids at puberty and may lead to combined phallic growth and breast development in individuals with retained gonads. Germ cells are rarely present in the testicular part and tend to disappear before puberty; spermatogenesis has not observed, and the testis tubules undergo hyalinization and sclerosis in adults. In contrast, follicles are usually abundant in the ovarian part in younger individuals and may undergo ovulatory changes after puberty. Although in the past, presumed fertility potential has often been decisive in decisions on gender of rearing in neonates with ovotesticular DSD, scarce data indicate that also ovarian function may decrease over time and that males with this condition may have equal or better psychological outcomes (Verkauskas *et al.*, 2007). Care for individuals with 46,XX testicular or ovotesticular DSD should be provided by an experienced multidisciplinary DSD team.

See also: 21-Hydroxylase Deficiency: Clinical and Biochemical Aspects

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Intersexuality: Gender Assignment and Psychosocial Care☆

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Glossary

Disorders of sex development (DSD) Inborn conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical. Conditions of genital ambiguity or intersexuality constitute a subset of DSD.

Gender assignment The decision-making process involved in declaring a newborn a "boy" or "girl."

Gender dysphoria Persistent dissatisfaction or unhappiness with the assigned gender.

Gender identity The basic sense of being a girl or boy, woman or man, or a different category of gender, such as a hermaphrodite or a trans(gender). Also known as core gender identity.

Gender reassignment The decision-making process involved in revising the original assignment decision later, which can happen at any age of the individual.

Gender-role behavior All behaviors in which the genders differ in a given time and place. Some examples are rough-and-tumble play of childhood, courtship and sexual behavior in adolescence, and aggression and parenting behavior in adulthood. Also known as gender-related behavior or sex-dimorphic behavior.

Gender-role identity The degree to which a girl or boy, or a woman or man, perceives herself or himself as being feminine or masculine.

Sexual orientation The degree to which an individual erotically responds to male or female sex partners, as reflected in romantic/erotic attractions, fantasies, dreams, and also in overt sexual behavior, often rated on the 7-point Kinsey scale ranging from 0 (exclusively heterosexual) to 6 (exclusively homosexual). Also known as "sexual responsiveness."

Transsexual A person with gender identity disorder who seeks, or has undergone, gender reassignment, often along with hormone treatment and genital surgery.

Gender Assignment Policies

Introduction

In most newborns, gender assignment is unproblematic and instantaneous. The newborn is assigned to the gender that corresponds to the appearance of the genitalia, and the vast majority of individuals will develop a stable gender identity commensurate with the original assignment. In a very small number of newborns, the genitalia are not clearly those of a boy or girl, but are ambiguous or intersexed. Examples of such variants of sex development are chromosomal (46,XX) females with androgen excess due to congenital adrenal hyperplasia (CAH), chromosomal (46,XY) males with a defect in testosterone biosynthesis or with varying degrees of androgen insensitivity secondary to genetic defects of the androgen receptor, or children with genitalia that are atypical in other ways, for instance, 46,XY infants born without a penis (penile agenesis).

The standard medical term currently used for such variants of somatic sex development is "disorders of sex development" (DSD; Hughes *et al.*, 2006), often re-read as "differences" or "divergences of sex development," especially by patients and patient advocates who are concerned about the stigma of "disorder" labels (Lin-Su *et al.*, 2015; Johnson *et al.*, 2017a). In the current article, I will use the term "intersexuality," because "DSD" also applies to large groups of individuals with other variants of sex development in which gender assignment is not in question. The medical categorization of somatic sex needs to be distinguished from the growing use of identity labels or legal terms for gender categories other than the traditional male or female, e.g., "non-binary" or "intersex." Overall, gender is more accurately conceptualized as a bimodal continuum rather than a strictly binary category system (Meyer-Bahlburg *et al.*, 2016).

In patients with intersexuality, the decision regarding gender assignment is complex, may require a number of medical tests to identify the cause of the problem, and involves prognostic considerations and value judgments. In fact, it is these cases that force a clarification of the relative importance of factors presumed to be involved in gender identity development—on the biological side, genes and sex hormones, and on the psychosocial side, gender assignment, social learning mechanisms such as reinforcement and modeling, and cognitive factors such as a person's evaluation of his/her body and behavior in comparison to perceived gender norms.

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The True-Sex Policy

The classical approach to gender assignment in the case of genital ambiguity, which goes back to at least Aristotle, has been to determine the true sex and to assign gender accordingly (Meyer-Bahlburg, 1998). The assumption under this policy was that the true sex is revealed by a definitive, single biological criterion. In antiquity, the decision was based on the appearance of the external genitalia. In cases of genital ambiguity, Aristotle had already recommended basing the decision on the "predominant sex." In conjunction with the rapid advances in medicine during the second half of the nineteenth century, the histology of the gonads became the decisive criterion of true sex, with sex chromosomes and other genetic indicators following suit in the middle of the twentieth century. It was generally assumed that, if gender assignment had been carried out correctly, that is, in agreement with the true sex, the final outcome would be a heterosexual man or a heterosexual woman, in agreement with the assigned gender. There were problems with the true-sex policy, however. The presumed definitive criterion of the true sex did not always agree with a person's somatic appearance as a man or woman, and also did not always agree with the person's gender identity, and could therefore lead to dramatically adverse social and legal consequences for the intersexed individual.

The Optimal-Gender Policy

On the basis of a critical examination of the true-sex policy and studies of the overall psychosocial outcome and quality of life of intersex patients, John Money and the Hampsons at Johns Hopkins Hospital in Baltimore, Maryland, formulated an optimal-gender policy during the 1950s (Meyer-Bahlburg, 1998). This policy had a number of underlying assumptions. (1) There is no single biological criterion that determines the development of psychological gender; rather, there is a cascade of biological processes that culminate in the development of gender identity. (2) Socialization factors have the decisive role in gender-identity formation. (3) If one minimizes the barriers to socialization in the assigned gender, the outcome will be a heterosexual man or a heterosexual woman, in agreement with the assigned gender. (4) The condition of the body and particularly the genitalia limits the psychosocial and especially psychosexual functioning of the intersexed person in later years. Therefore, the newborn with intersexuality should be assigned to that gender that permits the optimal psychosexual and psychosocial functioning when all available medical treatment options are taken into account. So, in contrast to the question of the true-sex policy—"Is this a boy or a girl"?—the optimal-gender policy asked "Will this newborn have a better function later in life as a male or a female?"

From the central role of socialization followed the concept of the birth of an intersex newborn as a psychosocial emergency, with the implication that the decision time for gender assignment should be kept as short as possible. Another consequence was the recommendation of early feminizing or masculinizing surgery of the external genitalia so that their appearance would be as similar to the gender norm as possible and would therefore not interfere with gender-typical rearing conditions and body-image development. A third consequence was the instruction to keep the intersex status of the child secret from all people who do not belong to the core family and to educate the child gradually about the condition, in line with his or her cognitive development. These guidelines were intended to prevent the parents from developing chronic doubts about the gender of their child and to protect the child from stigmatization by other people, but have frequently led to parental attempts of preventing the disclosure of details of the medical history to the intersex children themselves, sometimes even after they attained adulthood.

The True-Brain-Sex Policy

Increasingly since the mid-1990s, the optimal-gender policy has come under criticism. One reason is that also under this policy some individuals turn out dissatisfied with their assigned gender and may seek gender reassignment, which is made more difficult if the external genitalia have been operated on to be more compatible with the originally assigned gender. A second argument is that, even without later gender change, genital surgery carries a risk of damage to sexual functioning in adolescence and adulthood (see below). Third, the optimal-gender policy is blamed (although unjustly attributed to its authors; see above) for keeping the medical facts secret, especially from the patient him- or herself, which contributes to the maintenance of the social stigma of intersexuality and thereby to a negative self-image and shame of the intersexed individual. Many intersex activists, therefore, argue for early comprehensive disclosure of their medical history to intersex patients. A fourth criticism of the optimal-gender policy derives from the extensive data—initially from animal research, then from studies on DSD syndromes by Money and Ehrhardt (1972) and their team, and subsequently by many others—regarding the influence of sex hormones on the developing brain and long-term sex-dimorphic behavior. Because of these data, some biological determinists suggest that the decisive factor for gender identity formation is the prenatal androgenization of the brain and that psychosocial factors have only a secondary role. If such a "true-brain-sex policy" is valid, gender assignment decisions should be based on the degree of androgenization/masculinization of the brain, and minimization of barriers to socialization is unimportant. However, because brain imaging techniques are not yet capable of rendering such assessments, these theorists must fall back on the status of the genitalia as a—rather problematic—indicator of brain androgenization/masculinization (see below).

Policy Effects on Gender Assignment

The three major policies outlined above may lead to quite varied decisions on gender assignment. Two cases will illustrate this: (1) a chromosomally female (46,XX) newborn with extreme genital masculinization (Prader stage V) due to prenatal androgen excess

Table 1 Gender assignment as a function of policy

Disorder	*True-sex policy*	*Optimal-gender policy*	*True-brain-sex policy*
Penile agenesis	Male	Female	Male
CAH/Prader stage V	Female	Female	Male

associated with classical congenital adrenal hyperplasia (CAH) and (2) a chromosomally male (46,XY) newborn with penile agenesis. According to the true-sex policy, case (1) should be raised female because of the clearly female histology of the gonads (i.e., ovaries) and female chromosomes (see **Table 1**). By contrast, a 46,XY newborn with penile agenesis would be assigned to the male gender, because of the normal-male histology of the testicular gonads and the clearly male karyotype. Under the optimal-gender policy, case (1) would be assigned to the female gender, because the external genitalia can be feminized so as to permit coitus and to thereby retain the option of conception and pregnancy, because the internal female reproductive organs are intact. The 46,XY infant with penile agenesis would also be assigned to the female gender, because the surgical de novo construction of a functioning penis is not yet possible and therefore sexual functioning as a male would be compromised. Advocates of the true-brain-sex policy would recommend assigning the CAH case to the male gender because of the putative effects of prenatal androgens on the brain and recommend the same for the case of penile agenesis. Some activists recommend assigning the intersex child to the gender that seems to offer the more promising outcome, but to do so provisionally and to consider from the outset the possibility of later gender change and therefore not to operate on the genitals before the age of consent, unless medically necessary.

The Status of the Evidence

In response to the increasing controversies, an international consensus conference was held in Chicago in 2005, which reviewed the existing data and began a cautious modification of the predominant policy (Hughes *et al.*, 2006). A definitive consensus about a new management policy has yet to emerge (Lee *et al.*, 2016). The major problem is insufficient data concerning the long-term psychological outcome. Patient-initiated later gender change, for instance, can be observed in intersex individuals in both directions, from male to female and from female to male (and, in some cases, from the assigned gender to "intersex" or another term outside the traditional binary sex/gender system). This is not surprising, because such gender change is also well known—as transsexualism—in non-intersex individuals, but the relative frequency of later gender change is increased in intersex patients and varies with syndrome, syndrome severity, and initial gender assignment. Given the psychological, social, and medical problems that are associated with later gender change, clinicians usually favor a policy that minimizes its occurrence. Thus, long-term follow-up data are needed that will allow clinicians to state the relative frequency of gender change of patients with a given syndrome and degree of severity who were managed under a defined policy and in a particular cultural context. Because gender change can take place as late as in midlife, follow-up studies need to reach at least into that age range. Given the relative rarity of intersex patients, it is not surprising, therefore, that the data thus far available are still unsatisfactory.

As an extreme example, consider the question of gender reassignment in chromosomal males with traumatic loss of the penis in infancy (Meyer-Bahlburg, 2005). One such case, in which reassignment to female at 17–21 months of age was followed by patient-initiated re-reassignment to male in adolescence, has drawn enormous attention among care providers as well as in the media, once his unfortunate story was investigated and published, when he was in his mid-30s. However, only one other case with a history of traumatic loss of the penis in infancy and reassignment to female has been followed into adulthood and published and this person continues to live as a woman without gender dysphoria. Two cases with strikingly different outcomes are not sufficient for evidence-based policy decisions. Even for intersex syndromes that are more prevalent and less shrouded in secrecy than the cases of traumatic loss of the penis, long-term outcome data are hard to come by and those that have become available all suffer from problems that limit their validity: loss to follow-up of patients who have moved and cannot be found, who have died (especially if for other than medical reasons), or who have refused to participate in research (especially if for reasons of disappointment with their medical treatment).

The true-brain-sex policy appears very plausible at first glance, but becomes more problematic on closer scrutiny (Meyer-Bahlburg, 2013). The assumption that the effects of prenatal hormones on the brain are the decisive biological factor in the development of gender and therefore the best criterion for gender assignment is primarily derived from research on the sexual differentiation of brain and behavior in nonhuman mammals, especially rodents, which has yielded several neuroendocrine models of the sexual differentiation of brain and behavior. In humans, most pertinent studies have been limited to androgens. In general, the resulting data allow the preliminary conclusion that effective prenatal androgens are indeed associated with the masculinization of gender-role behavior. However, the core gender identity, for instance, as a girl or as a woman, is compatible with large variability in gender-role behavior, as is vividly demonstrated in girls and women with CAH, thus making a close linkage to prenatal hormones less likely. It also must be kept in mind that in the limited literature available, the association between prenatal androgens and later gender change in girls and women with CAH takes place predominantly in the context of further postnatal and pubertal androgen exposure and the associated increased somatic sexual ambiguity. Therefore, in all likelihood, it is not the prenatal hormonal milieu by itself that determines such later gender change.

Outcome data are also unsatisfactory with regard to the question of impairment of sexual functioning after gender-confirming genital surgery. Most surgical outcome reports are limited to anatomical data and appearance ratings. Those that include data on

sexual functioning often leave much to be desired in terms of sample representativeness, assessment methods, and study details provided, quite apart from the fact that patients who have undergone surgeries with the newer techniques are not yet old enough for sexual functioning studies. Most pertinent studies have been conducted in women with CAH. As a group, they show impaired sexual and genital functioning, presumably both despite corrective genital surgery and because of it (for references, see Meyer-Bahlburg, 2014). From a neuroanatomic standpoint, it is plausible, for instance, that excision of the clitorophallus—widely practiced as a feminizing technique in the 1950s and 1960s—would lead to a diminution of erotic sensitivity and orgasmic capacity, although the limited outcome data available show considerable variability. But surgical techniques have undergone many changes since then and reduction of the clitorophallus rather than excision has become the norm. To evaluate sexual functioning in patients who were operated on with newer surgical techniques, researchers need to wait at least until the patients are sexually active, sufficiently sexually experienced, and able to talk about these intimate details of their lives, and this in a population that is known to be relatively delayed in attaining psychosexual milestones. The same applies to patients with a similarly ambiguous genital status at birth who for one reason or another have not undergone genital surgery and could therefore serve as comparisons. The outcome data currently available, however, have led to recommendations to limit genital surgery in girls and women with CAH to those with marked degrees of genital masculinization (Speiser *et al.*, 2010).

Inferring Brain Masculinization From Genital Masculinization

Adherents of the true-brain-sex policy recommend a male assignment for a newborn with intersexuality if the child's brain was prenatally strongly masculinized, independent of the status of the genitalia. However, how can one determine in a newborn whether and to what extent the brain was prenatally masculinized and/or defeminized? Appropriate brain imaging techniques are not yet available, and even if they were available, it is rather uncertain that the brain is, at the time of birth, already sufficiently sexually dimorphic to show such clear-cut gender dimorphic structures. Some advocates of the true-brain-sex policy therefore suggest using the genital status at birth (staged according to Prader for excess masculinization in chromosomal females and according to Quigley, Sinnecker, or Ahmed for several syndromes of undermasculinization in chromosomal males) as an indicator of brain androgenization/masculinization. There are several problems with this approach (Meyer-Bahlburg, 2013). (1) Genital staging at birth is not suitable for all syndromes of interest. For instance, Prader staging is problematic for many prenatally dexamethasone-treated cases of 46,XX CAH and undermasculinization staging is not at all suitable for certain 46,XY conditions of interest, such as penile agenesis and cloacal exstrophy of the bladder. (2) The growth of the penis does not depend exclusively on sex hormones. (3) The hormone model of genital differentiation is not identical with the hormone models of brain differentiation. As far as is known, the prenatal differentiation of the genitalia is determined by testosterone, dihydrotestosterone, and anti-Müllerian hormone, whereas the sexual differentiation of the human brain primarily depends on androgens, with the roles of estrogens and anti-Müllerian hormone unknown. (4) In the 46,XX CAH syndrome, Prader stage correlates only moderately with masculinization of gender-role behavior. (5) Available (scanty) data on patient-initiated gender change in adults with a given intersex syndrome show little relationship to genital status at birth. (6) Moreover, gender dysphoria occurs in non-intersexed persons as well, although prior to surgery the genitals of transsexuals are fully differentiated and compatible with the original gender assignment. (7) Research has made it likely that contributions to the sexual differentiation of brain and behavior by hormone-independent genetic factors and brain-tissue-specific hormonal factors need to be taken into account. (8) Finally, there may be early effects of the social environment on central nervous system structures, as is already known from animal research. If any social mechanisms participate in the development of gender identity incongruence in persons without somatic intersexuality, they are likely to also contribute to its development in intersex patients. It follows from all of these considerations that the genital status at the time of birth cannot be interpreted as a clear-cut indicator of brain masculinization and later gender identity.

Gender Assignment by Diagnostic Category

Intersex children and their families cannot be left in gender limbo. Despite controversies and uncertainties, clinicians must continue to assist parents in making decisions on gender assignment. The degree of uncertainty varies with the syndrome. In Western countries, 46,XX newborns with CAH, if correctly diagnosed at birth, are usually assigned to the female gender, because the internal reproductive structures (ovaries, fallopian tubes, cervix) permit reproduction, provided that external genitalia and vagina are surgically corrected where necessary. Although many girls with CAH show variable degrees of behavioral masculinization, gender identity typically is female (Dessens *et al.*, 2005). A few CAH females change to male in adolescence or adulthood, apparently mostly in cases of protracted marked genital ambiguity and inconsistent hormonal control. Some genitally highly masculinized 46,XX children with CAH are inadvertently raised as males, especially in resource-poor countries, and the majority of them maintain a male gender identity when correctly diagnosed in later childhood or adolescence. On that basis and in view of the often problematic outcome data on genital surgery, it has been suggested to seriously consider the deliberate assignment to male of 46,XX CAH newborns with extreme degrees of genital masculinization (Lee *et al.*, 2010).

With regard to 46,XY syndromes, there is a consensus that 46,XY newborns with complete androgen insensitivity should be raised as female, because they do not respond to male sex hormones, neither somatically nor in terms of gender-related behavior, and, given that it is more prenatal rather than postnatal androgens that affect gender-related behavior, this policy can be extended to the syndrome of complete gonadal dysgenesis. Even for the syndromes of partial androgen insensitivity and testosterone

biosynthesis defect, there is a consensus that the least undermasculinized cases should be raised male and the most undermasculinized cases should be raised female. However, given the scarcity of long-term follow-up reports on such syndromes, the best cutoff point on the undermasculinization scales for assignment to the male or female gender cannot yet be determined on an empirical basis. What is known about the variability of gender outcome in individuals with a given molecular genotype and endocrine or genital phenotype lets one expect that any cutoff point on an undermasculinization scale will be associated with occasional later patient-initiated gender reassignment, however rare. With regard to cases of micropenis (i.e., a fully differentiated penis with the urethral meatus at the tip, but a (stretched) length of 2½ standard deviations or more below the norm), the Chicago meeting came to recommend male rearing in view of documented equal satisfaction with assigned gender in those raised male or female, and of the fact that patients reared male do not need surgery and are potentially fertile (Hughes *et al.*, 2006). More problematic are the relatively rare pubertal change syndromes such as 5α-reductase-2 deficiency and 17β-hydroxysteroid-dehydrogenase-3 deficiency in 46,XY. At birth, many such children appear more female than male and they are typically assigned to the female gender, unless diagnosed correctly. There are a number of clinical–medical reports from resource-poor countries showing that, in the absence of medical intervention, the majority of such individuals start virilizing dramatically during spontaneous puberty, and many change to the male gender (Cohen-Kettenis, 2005; Mendonca *et al.*, 2016, 2017). Yet, few published data are available on the long-term outcome of such cases when medical intervention is introduced at newborn age or at the beginning of puberty. Similarly, the long-term outcome of those few cases who reportedly have had early hormone replacement therapy to masculinize the genitalia has not yet been documented.

Also problematic is the gender assignment of 46,XY infants who presumably had a normal-male sex-hormonal environment in utero so that the brain was normally masculinized, but who had genital abnormalities for non-hormonal reasons, for instance, in cases of penile agenesis, cloacal exstrophy of the bladder, or traumatic loss of the penis due to a circumcision accident. A review prepared for the Chicago conference showed that a substantial minority of those assigned female became gender dysphoric or changed gender later as compared to only one of those assigned male (Meyer-Bahlburg, 2005). Although the Chicago meeting did not yield a definitive consensus for these intersex syndromes, a subsequent survey of pediatric urologists showed a strong trend away from the female gender assignment of patients with 46,XY cloacal exstrophy (Diamond *et al.*, 2011; Kolesinska *et al.*, 2014).

In the syndrome of ovotesticular DSD, the gender assignment decision is based on considerations of the preponderance of masculine or feminine structures in both the external and internal genitalia and the level of anti-Müllerian hormone, but again there is uncertainty regarding the best cutoff point for male versus female assignment, and more long-term follow-up data are needed for the formulation of a consensus policy. In the case of "XX males" secondary to a translocation of the SRY locus onto an X chromosome and consequent differentiation of a male gonad, there is no doubt that such infants should be raised as boys. Although the rapidly expanding knowledge on multiple genes participating in the sexual differentiation of the gonads and the brain complicates the picture and leads to the discovery of additional genotypes associated with intersex syndromes, it can be expected that these discoveries will help refine both diagnosis and prognosis, especially when complemented by data about sexual brain dimorphism from brain imaging and long-term follow-up data.

Conclusion

Overall, the available data from both earlier and newer studies can be tentatively summarized in the statement that intersex patients show wide variations in the prevalence of gender dysphoria and patient-initiated gender change dependent on the syndrome, syndrome severity, and gender assigned at birth (Meyer-Bahlburg, 2013). Particularly high rates are found in female-raised 46,XY patients with the pubertal change syndromes and in female-raised 46,XY patients with male-typical levels of prenatal androgen exposure as in penile agenesis or cloacal exstrophy of the bladder, whereas the majority of patients with a prenatal androgen milieu intermediate between male-typical and female-typical develop a gender identity commensurate with the assigned gender. Later initiation of gender change by an intersex person appears to be the more likely the more the prenatal and postnatal biological factors and the postnatal psychosocial factors push in the same cross-gender direction. It follows that the gender assignment of the newborn should be based on the best prognosis for the future psychosocial and psychosexual functioning of the patient in the given cultural context, taking into account everything that is known about the likely steroid effects on the brain in the syndrome in question, the hormonal and surgical treatment possibilities, and the long-term outcome in terms of gender, sexual functioning, psychiatric problems, and overall QoL.

Psychosocial Care

Introduction

The issues of gender assignment and genital surgery and the potential implications for the development of gender, sexuality, and parenthood—three highly salient, emotional, moral, and frequently controversial social issues—make intersex conditions a challenge not only for patients and their families, but also for physicians and other professionals involved in their medical and psychosocial care. Moreover, the psychosocial care itself remains in a period of flux, since the optimal-gender policy of the second half of the twentieth century has come under severe criticism, whereas the evidence on which to base systematic improvements where warranted leaves much to be desired, and no systematic randomized trials of psychosocial care procedures for intersex

patients have been conducted. In these circumstances, recommendations for specific treatment options need to be more tentative, and not only the providers, but also the parents and, when old enough, the patients themselves will be burdened by more uncertainties in their decision-making than desirable. In addition, their marked deviation from the sex/gender binary, as traditionally conceived, places individuals with intersexuality at risk for pervasive social stigma. These are the contexts, in which many patients develop psychiatric difficulties.

The professionals involved in the medical and psychosocial care of intersex patients, especially when gender assignment is problematic, may involve multiple disciplines and subspecialties, e.g., specialists in neonatology, pediatric endocrinology, pediatric urology, gynecology, genetics, genetic counseling, and mental health (Rolston *et al.*, 2017; Bakula *et al.*, 2017). To minimize the burden on intersex patients and their families, these professionals should form a smoothly operating team and follow a common policy, also with regard to criteria for decisions on gender. Parents of intersex newborns are typically in a state of high stress while the gender of their child remains uncertain, and most are not in a position to decide between conflicting professional opinions. Those team members—both medical and mental health staff—who have the most influence on decisions regarding gender assignment and genital surgery and those who most likely will be involved with the parents and patients over the coming years must keep up with medical advances, emerging new treatment options, stigma risks, the growing research on psychosocial outcomes, and the prognostic alternatives for both health and psychological development in the intersex field. To be fully effective, they need to acquire up-to-date information on all psychosocially relevant aspects of the patient's syndrome and specific symptoms. For instance, some intersex syndromes may be associated with short stature or neuropsychological impairments, both of which have psychosocial consequences of their own. There is also a need for establishing specialized programs for transition to adult care and for older adults with intersexuality. It is highly desirable that relevant professional societies develop competency criteria and interdisciplinary training programs in intersex care.

Gender Assignment and Reassignment

Gender assignment at birth

The identification of ambiguous genitalia in a newborn only occasionally constitutes a medical emergency. Yet, having a newborn of undetermined gender is stressful for most parents (Crissman *et al.*, 2011; Wolfe-Christensen *et al.*, 2014), and speedy processing of necessary medical tests and rapid decision-making with regard to gender assignment are important. Prolonged periods of nondecision are thought to run the risk of chronically ambiguous or inconsistent gender typing by the family, or of rejection of the child altogether.

Given the diverse factors that influence gender identity development in intersex patients, the decision on gender assignment required in a patient with highly ambiguous genitalia is not only based on medical criteria, but also needs to take into account the differential prognosis for psychological (including psychosexual and reproductive) functioning in either gender role as well as the prognosis for how well the family will be able to cope with either decision. The prognosis of the patient's future functioning must take into consideration the various options of genital surgery and sex-hormone treatment and their respective implications for future functioning, especially in terms of gender-role fit, sexuality, reproduction, and overall quality of life. As gender-related values differ in various societies, cultural factors have significant impact on gender assignment decisions. In many Asian countries, for example, gender assignment to male is more strongly favored and infertility more stigmatized (especially in the female) than in the West (Josso *et al.*, 2011).

Intersex counseling at this stage should involve both parents and the inclusion of other family members should be considered. Legally, gender assignment is the parents' decision, but they are likely to rely on expert medical advice, although there are differences between countries in this regard (Josso *et al.*, 2011). Along with medical education about the nature and origin of the problem and the medical tests involved, the parents must be adequately informed by their clinicians about the diversity of long-range outcomes and not given overly optimistic assurances. Commensurate with their cognitive capacity, the parents need to be made aware of significant gaps in scientists' knowledge about intersex disorders or major controversies about management decisions. Such parent counseling requires a careful balance between providing gross oversimplification on the one hand and too much detail on the other, with a resulting paralysis of decision-making. If either parent is not convinced that the gender decision was correct, appropriate child rearing may be in jeopardy. The recommended form of clinician/parent interaction and, at later stages of the child's development, clinician/patient interaction, in preparation for treatment decisions is the "shared decision making" model along with decision aids and support tools (Siminoff and Sandberg, 2015; Graziano and Fallat, 2016).

Monitoring behavioral development

The psychosocial management needs of intersex patients vary with developmental stage. Once the diagnosis is established and the decision on gender assignment made, an approximate timetable for future medical procedures and preventive psychosocial measures can be projected and visits planned accordingly. Again, a team approach in which medical procedures and psychosocial care are closely integrated is highly recommended. Active outreach by the mental health professional may facilitate prevention of psychosocial problems and adherence to medical treatment.

As for other congenital disorders, regular monitoring of behavioral development is also recommended for intersex children. This includes monitoring for medical problems and treatment compliance, as needed, and monitoring for cognitive and behavioral problems, with a special emphasis on atypical gender-role behavior (Meyer-Bahlburg, 2011; Alpern *et al.*, 2017; D'Alberton

et al., 2017). Some parents (and even some children) need help with handling atypical gender-role behavior. If symptoms of gender dysphoria emerge, parents and the child may need some help in dealing with gender issues and in persistent extreme cases the option of gender reassignment needs to be considered.

Gender reassignment

Sometimes, intersex children are initially misdiagnosed, especially when born outside of intersex-experienced medical settings, and receive their correct diagnosis later from medical specialists. The definitive diagnosis may alter the prognostic considerations and make a gender reassignment desirable. Such reassignment, if well justified, is usually not a problem during infancy, provided the parents are adequately counseled.

After infancy, during the toddler and preschool years, many intersex children develop some degree of gender-atypical behavior, but such behavior by itself does not indicate a problem of gender dysphoria. Parents who are anxious about their child's gender atypicality may need reassurance and counseling. Gender reassignment decisions after infancy, which are quite uncommon, should never be based on purely medical considerations, but require careful psychological evaluation (over a prolonged period of time) of the child's overall behavioral development, with particular attention to the child's gender-role behavior and to any symptoms of gender dysphoria, as well as to the behavioral niche the child has occupied in the family system (Meyer-Bahlburg, 2011).

Gender dysphoria and wishes to change one's gender may also emerge later in adolescence or adult life, usually as the result of a long and gradual process. Even if the problem is of long standing, through counseling the patient can consider various options, and gender change and the attendant hormone treatment and surgical procedures are not the invariable outcome.

Genital Surgery

Some children with intersexuality require genital surgery for medical reasons, e.g., gonadectomy in cases of high malignancy risk. In the majority of cases, however, genital surgery has been performed for psychosocial reasons in order to confirm the assigned gender by genital appearance and to thereby facilitate gender-appropriate rearing, to help develop a gender-typical body image, and avoid social stigmatization and also to facilitate later peno-vaginal intercourse. Because genital surgery is associated with some risk to sexual tissues and erotic innervation and thereby sexual functioning (see above), it has become highly controversial. Some intersex activists and ethicists demand that psychosocially indicated genital surgery be performed only with the informed consent of the mature individual (Chase, 1998; Sytsma, 2006; Wiesemann *et al.*, 2010; Kon, 2015; Feder and Dreger, 2016). Some critics even advocate a moratorium on all genital surgery, unless necessary for strictly medical indications (Diamond and Garland, 2014), and claim—without supportive systematic empirical evidence—that psychological counseling can take care of all attending psychosocial problems that children with marked genital ambiguity and their families may face. Surgeons counter that modern surgical techniques are much improved, although it may still require years until the patients so treated are old enough to provide data on sexual functioning. Experienced clinicians are concerned that obviously ambiguous genitalia put an unoperated intersex child at risk of undue attention and stigmatization. Moreover, in the absence of sufficient sexual experience, even an older adolescent or young adult may not be capable of giving appropriately informed consent. The very fact of genital ambiguity may contribute to the delay of sexual initiation and some patients may never reach that developmental stage. Thus, there is as yet no consensus regarding the issue of early genital surgery (Mouriquand *et al.*, 2016), except to say that milder cases of genital ambiguity are less likely to be operated on than they would have been one or two decades ago and that there is a growing consensus that genital surgery should be confined to intersex-experienced centers of excellence. It is important to note, however, that published surveys have shown that not only most parents of patients with intersexuality favored genital surgery before adolescence (Dayner *et al.*, 2004; Marei *et al.*, 2015; Binet *et al.*, 2016), but also most of the patients did so (Meyer-Bahlburg *et al.*, 2004; Wisniewski *et al.*, 2004; Nordenskjöld *et al.*, 2008; Fagerholm *et al.*, 2011; Zhang *et al.*, 2013; Binet *et al.*, 2016).

If the decision for genital surgery has been made, both medical and psychological considerations determine the choice of age for such surgery. From a psychological perspective, genital surgery is performed more easily in infancy, when counseling for the child is not an issue, and in adolescence, when cognitive maturation facilitates counseling and the patient has achieved a degree of autonomy, than in early and middle childhood. The older the child, the more he or she should be empowered to have the decisive vote in the decision for or against genital surgery and in choosing the time when it should take place. Given the advances in techniques of fertility preservation, this topic needs to be discussed in detail with parents and patients, where applicable (Campo-Engelstein *et al.*, 2017; Johnson *et al.*, 2017b; Slowikowska-Hilczer *et al.*, 2017).

Before and after genital surgery, genital examinations of intersex children and adolescents are frequent. Given the low prevalence rates of the various syndromes of intersexuality, it has been common medical practice to have many different physicians, especially medical students and residents, perform genital examinations on the same patient. However, considerable evidence has accumulated showing that this practice has negative and often severe psychological aftereffects. In medical training, this practice should be replaced by the use of photographs, videotapes, and physical models. Also, older children and teenagers should have a say in the matter whether someone should accompany them during the examination and if so, who that person should be. Useful guidelines for the performance of genital exams have been derived from work with victims of child-sexual abuse (Tishelman *et al.*, 2017).

In adolescence and adulthood, psychosocial monitoring needs to include concerns about romantic relationships and sexual functioning, because of the increased problems associated with genital ambiguity and related surgery.

Sex-Hormone Treatment

During infancy, androgen treatment is sometimes used for chromosomally male children with underdeveloped male genitals to test for an androgen-receptor defect or, if the assignment decision was for male, to enhance penile size. Occasionally, such children show behavioral change that normalizes with the end of the treatment and the parents may need reassurance.

Intersex children who have no gonads or underfunctioning gonads will need sex-hormone replacement therapy to initiate or support pubertal maturation. This is best done at the age when their classmates experience endogenous puberty. Undue delay of pubertal development may add to difficulties in the later development of romantic and sexual relationships.

Information Management

The medical and sexual education of intersex children and their parents and related counseling (including information about how to deal with relatives and friends) is a crucially important part of psychosocial care. The most difficult issue is the disclosure of their medical status and history to the intersex patients themselves, especially to those assigned to a gender discrepant with sex chromosomes and/or gonadal structure. Professionals generally support the right of patients to have access to their medical information and many experienced professionals agree that by the time of graduation from high school the patient should be fully informed, commensurate with the level of cognitive capacity. However, many parents are anxious and would like to postpone full disclosure, sometimes permanently. Appropriate timing of disclosure is important. Early disclosure runs into the problem of cognitive limitations. Late disclosure implies cover-up and deception and may lead to the patient's long-term distrust of and anger at medical professionals and even endanger appropriate future utilization of medical services. Disclosure is best conceptualized as a long-term process starting in late preschool age, with many installments, preferably in conjunction with meaningful events. Substantively, there are the issues of past genital surgery and their tell-tale signs, namely, scars on the abdomen, possibly persisting abnormalities in genital appearance, and plans for future surgery; the need for the induction of puberty by sex-hormone treatment and the maintenance of secondary sex characteristics by its lifelong continuation; the issue of infertility or, better, the potential realization of parenthood by means other than pregnancy or insemination; the question of variability in gender-role behavior among individuals of the same core gender identity; and the potential for romance, sexuality, and sexual pair-bonding—regardless of sexual orientation. All of these issues of functional potential are more important to the average developing intersex person than the details of the molecular structure of genes, the variants of hormone synthesis, receptor defects, enzyme abnormalities, and the organization and activation of neural networks—although the occasional adolescent or adult patient may have specialized interests or even choose a career path in the respective biological sciences and may seek as complete an understanding of his or her biological condition as is possible.

For the developing child and adolescent, the medical information must be carefully tailored to the cognitive maturity level. The fact that the meaning and connotations of terms differ vastly between medical personnel and lay persons makes a deliberate and cautious choice of terminology necessary. The intended medical “truth” may be dramatically at variance with what the patient or parents perceive. Visual aids are often very helpful.

For both the patients and the parents, discussing the medical aspects of intersexuality tends to be highly emotional, so adequate retention is problematic. The author's preferred procedure for the medical education of a newly referred family is that the physician in charge of the overall coordination of care, usually a pediatric endocrinologist or urologist, gives a carefully worded summary of the medical information to the parents, in the presence of the mental health professional, who then takes over. Typically, the parents are asked to recount in detail how they understand the medical condition, its origin, and its prognosis. Then, the instruction is repeated, the parents’ misunderstandings are corrected, and their particular concerns are addressed. At subsequent visits, the procedure is repeated as necessary, keeping in mind that new misunderstandings and different concerns develop over time. Similar procedures are used with intersex children, once they are old enough, except that, in addition, the clinician lets them explain to their parents (in the clinician's presence) what they have learned from the instruction, to open the channels of communication.

Mental Health and Quality of Life

Given the prevailing binary sex/gender system in society, marked deviations in body and behavior as well as in fertility potential from the expectations for typical females and males place individuals with intersexuality at risk for social stigma—experienced, anticipated, and internalized—in all spheres of life (Rolston *et al.*, 2015; Meyer-Bahlburg *et al.*, 2017a,b,c), varying with the cultural background (Meyer-Bahlburg, 2017). Parents of an intersex newborn may be highly distressed (Suorsa *et al.*, 2015), and their reactions may range from rejection to overprotection (Wisniewski, 2017). Many resort to attempts at concealing the problem (Danon and Krämer, 2017), and some may try to pressure children with gender-nonconforming behavior towards gender-typicality. The patients themselves, when becoming aware of the stigma risks, develop various ways of coping, which may include secrecy and the resulting shame, hypervigilance, and avoidance of potential risk situations and people. These challenges are likely contributors to the increased prevalence of anxiety and depression (even including suicidality) and impaired quality of life

reported in many (but not all) long-term follow-up studies of individuals with intersexuality (Schutzmann *et al.*, 2009; Wisniewski and Mazur, 2009; Bennecke *et al.*, 2017; D'Alberton *et al.*, 2015; Engberg *et al.*, 2015, 2017). Additional contributing factors may include glucocorticoid overtreatment in women with CAH (Han *et al.*, 2013; Webb *et al.*, 2017) and, in some syndromes, an association of the specific medical condition with adverse brain development. Given these findings, systematic screening for mental health and QoL and for factors that may adversely affect either is an important task for clinicians following intersex patients (Sandberg *et al.*, 2017) and therapeutic interventions have to be incorporated in the treatment plan as indicated.

Support Groups

One of the recurrent problems for persons suffering from rare disorders and their families is the feeling of isolation and having no one to talk to who really understands what they must cope with. Many support groups have sprung up, including groups specific for intersex persons and/or their parents, and the internet has greatly aided in this development (Asciutto *et al.*, 2011; Bennecke *et al.*, 2015). Generally, such groups have been a great help to many patients and their families. However, the quality of the groups is highly variable and the medical and other information that comes from them is sometimes quite misleading. Thus, frequent exchanges between support groups and professionals are recommended and group participants should be strongly encouraged to seek second opinions from their physicians and other care providers regarding the information they receive.

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Ethical and Legal Aspects of DSD/Intersex

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Introduction

Over the last century, medical and legal authorities appear to have had one common interest in the classification of persons who have been considered to have intersex conditions or differences of sex development (DSD)—namely, the juridical reliance on medical personnel to register children as either as "male" or "female" at birth and assist in determining changes to registered gender (Scherpe, 2015; Scherpe *et al.*, 2018). On closer examination, however, it is well documented that legal authorities have had a much greater historical role in the governance of these individuals' gender status than recently thought. For centuries, when the gender of such persons was contested, medical experts played only an advisory role and were often called upon to examine patients to assist determinations of their gender, determinations that affected their legal rights (Dreger, 1998; Reis, 2009). This relationship changed primarily in the 20th century, with the advent of the clinical practice that was purported to definitively establish the gender of children while treating DSDs as "anomalies" that could be "corrected" with medical intervention in infancy and early childhood (Dreger, 1998; Reis, 2009).

In recent years, greater understanding of the numerous rights that are compromised by medically reinforcing gender assignments on children without their participation and consent has generated considerable discussion about the soundness of juridical deference to the clinical management of these conditions as primarily medical matters. The concern has become grave as it has become apparent that irreversible surgical interventions and hormonal therapies have been carried out on infants and very young children without long-term data confirming their necessity, safety, and efficacy. Clinical interventions on children with DSD/intersex conditions thus raises two major issues that fall squarely within the legal domain, namely (1) the designation of a legal gender and the right to a gender identity, and (2) the legitimacy of surgical and other medical interventions, as well as injuries caused by them, particularly on new-borns without the capacity to consent. These matters will be addressed in turn, but before doing so it is necessary to briefly look at terminology and the legal history in this area.

It is of crucial importance to state at the outset that the legal and medical issues for persons directly affected by clinical practices, in principle, are distinct. Nevertheless, as in all areas of medical practice, legal authorities must determine the boundaries of permissible medical intervention, particularly with obligations to protect the human rights of all persons, including vulnerable children, under national and international law. This means in particular that the full and informed consent of the persons concerned, or their representatives, is required for any medical intervention. Given that those providing the necessary consent often will rely on the advice of medical practitioners, this creates specific ethical and legal duties which will be addressed in this contribution.

Terminology

The terminology regarding the issues discussed in this article has been inconsistent and confusing for a considerable time (Hughes, 2018). Much of the discussion of terminology, however, has occurred in a clinical context without consideration of the legal consequences of that terminology. The current dominant terminology emerged from a gathering of clinical experts in the United States in Chicago, Illinois, in 2005. Concerned about the nature of clinical practices on children with intersex conditions, these experts published a "Consensus Statement" on practice recommendations in 2006. The Statement proposed, inter alia, that the diagnostic term "disorder of sex development" (DSD) would be less alarming to parents and patients than terms such as "intersex" and "hermaphrodite," whereas diagnosis of a disorder could be essential to ensure that the care would be covered by private insurance or as health care in public health care systems (Hughes *et al.*, 2006; Lee *et al.*, 2016). Activists supporting the statement generally concluded that such a diagnostic term might be necessary even if care became more patient-centered with a shift toward consent for any surgical interventions (Reis, 2007). The medical profession appears to have adopted the term rapidly following the publication the Consensus Statement (Lee *et al.*, 2016).

Nevertheless, the term "disorder" remains highly problematic. It clinically defines the disorder solely as "congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical" (Hughes *et al.*, 2006). From numerous persons diagnosed in this manner, however, references to being "atypical" as having a "disorder" has been widely criticized as discriminatory and degrading (Davis, 2015; Reis, 2007). Indeed, in 2016, a broader group of experts revisited the Consensus Statement and formally recognized that that the term is considered stigmatic and unacceptable by some patient groups and support groups (Lee *et al.*, 2016).

The term "disorder" has also raised several medical-legal concerns in creating pressure to "normalize" these "atypical" persons as far as possible, given that what is "normal" or "typical" is dependent on subjective parental and medical perceptions.

Historically, homosexuality and diverse gender identities were once similarly medically classified as disorders, but sexual orientation and gender identity are now legally protected dimensions of an individual's right to private and intimate life under national and international law. Classification as having a "disorder" may also entitle individuals to protection under many national laws for persons with disabilities, even for being perceived as such (Greenberg, 2012), as well as under the UN's Convention on the Rights of Persons with Disabilities. The UN Committee that oversees compliance with that Convention has repeatedly described gender-conforming interventions on children with "intersex conditions" as violations of the children's rights (see e.g., Committee on the Rights of Persons with Disabilities, 2016b,c; Committee on the Rights of Persons with Disabilities, 2017a,b).

Indeed, human rights authorities have now concretized and clarified the rights of persons deemed to have disabilities precisely because their developmental differences are often targeted for adverse and unwanted treatment. The UN Committee on the Persons with Disabilities, for example, has recently explained that Article 2 of the UN Convention on Persons with Disabilities (CPRD) requires such persons to be treated equally so that the label of difference is not used as a mechanism to deny such persons' legal capacity and consent to medical treatments (Committee on the Rights of Persons with Disabilities, 2014). In 2016, the Committee elaborated on this principle and emphasized that gender equality under the CRPD prohibits maltreatment on the basis of "gender"—that is, "the characteristics that a society or culture views as masculine or feminine"—such that "surgery or treatment performed on intersex children without their informed consent" constitutes degrading treatment and abuse (Committee on the Rights of Persons with Disabilities, 2016a). This is not mere semantics. A "disorder" implies not only a defect but "wrongness" and, as said, a need to create "order" to bring back things to "normal." By contrast, difference merely means that the person concerned is not the same as the comparator, in principle without any value judgment or implications for any need of action (although admittedly being declared "different" of course also has the potential to be used in a pejorative and degrading way). Thus, the first "D" in DSD should be understood to mean "differences" rather than "disorder" and is used accordingly in this article. In any event, irrespective of the criticism the Chicago Consensus Statement (and its successors) has received because of its substance (see e.g., Carpenter, 2018; Garland and Diamond, 2018, with further references), DSD as a term now is generally accepted among the biomedical profession and literature as the more precise description of the medical determinations. However, where gender differences or differences of sex characteristics are treated as defects or disorders, the obligations to respect such persons' right to consent to medical treatment under international law cannot be avoided and require heightened oversight.

In the legal debate the term "intersex" seems to dominate and indeed is used by human rights-based advocacy groups, such as Organization Intersex International (OII), as well as all the international human rights authorities discussed in this article. However, it needs to be acknowledged that this term also can be perceived as pejorative. Furthermore, in the legal debate "intersex" sometimes has been appropriated by those advocating a "third gender" beyond the legal male–female binary (Cools *et al.*, 2016). However, because this contribution focuses on the legal aspects of sex classifications, the term intersex in relation to conditions and identities is used, particularly to track human rights recommendations.

Brief Legal Historical Background

There have always been social and legal discussions of intersex, as well as theological ones (on which see e.g., Dormor, 2018; Lavee and Artmann Partock, 2018). It is interesting to note that one of the first comprehensive major legal codifications, the Prussian Code (Allgemeines Landrecht für die preussischen Staaten, ALR) of 1794, already had provisions dealing with the legal status of intersex, as had the Saxonian Code of 1865. Before that, the question of which gender a person was deemed to have (which was particularly important for questions of succession and marriage) was dealt with by customary law and influenced by ancient classical texts and by postclassical literature and scholarship (for a detailed account see Wijffels, 2018). But advances in medical science led to these legislative approaches being abandoned as the perception was that medical intervention could "normalize" the people concerned and make them physically fit their "true gender." This often had tragic, irreversible consequences and led to terrible suffering (see e.g., Garland and Diamond, 2018, with further references), leading to calls for such interventions to be banned (on which see below).

Legal Gender

The Legal Development Concerning Recognition of the Preferred Gender

Until very recently, the legal gender of a person was considered immutably determined at birth as either male or female and registered as such by the authorities. This created significant problems when the registered legal gender later turned out to be false, particularly in cases intersex persons as well as transgender. While in some instances this could be dealt with by seeing the original gender entry as a clerical error that could be corrected, this largely depended on clinical discretion and decisions by administrative agencies or judges to make such changes, which were not always approved.

But beginning in the late 1970s, hand in hand with increasing medical and psychological knowledge about gender identity, and prompted by a string of legal advocacy, legislation began to be enacted in many jurisdictions to permit a change of legal gender on the basis of legal conditions (on this and the general development see the contributions in Scherpe (2015)). A rapid development followed, assisted by landmark decisions of the European Court of Human Rights such as Goodwin v. United

Kingdom (2002) and, more recently, the Inter-American Court of Human Rights Advisory Opinion 24 of November 24, 2017. There is now a strong, emerging consensus that international human rights law recognizes the right of individuals to be legally acknowledged in their preferred gender. Thus, in most Western jurisdictions (but also in some Asian jurisdictions, e.g., Hong Kong and Taiwan, see Liu, 2015; Ho, 2015) today the question is not *whether* changing a legal gender is possible, but rather what the *requirements* for such recognition of preferred gender ought to be, and particularly which, if any, medical requirements needed to be fulfilled. In another seminal decision, the European Court of Human Rights in 2017 held in AP, Garçon & Nicot v. France (2017) that sterility requirements were a violation of the European Convention on Human Rights, and highest national courts in Germany and Italy have similarly held that surgical requirements violate national human rights law (BVerfG, 2011 and Sentenza n. 15138/15). In addition, numerous UN human rights actors (including the UN Special Rapporteur on Torture and Other Cruel, Inhuman or Degrading Treatment or Punishment in 2016) have expressly condemned medical conditions as incompatible with existing human rights norms (on this and particularly with regard to intersex see Ghattas, 2015, 2018, as well as Sandberg, 2018). The 2006 "Yogyakarta Principles on the application of international human rights law in relation to sexual orientation and gender identity" also expressly specify the same. In the light of this, there is a clear emerging trend that legislation enacted in the 21st century no longer requires any medical procedures for legal recognition of the preferred gender but rather is based on the self-determination of the individual (as for example in Malta, Argentina or Denmark, on which see Ní Mhuirthile, 2018; Giosa *et al.*, 2015; Videbæk Munkholm, 2015 respectively). Where such legislation is in place, the medical and legal issues have been completely detached and are independent of each other. But what is crucial for present purposes is that when no medical requirements exist for the recognition of the preferred gender and is based on self-determination, a change of legal gender can be dealt with in a relatively simple administrative or judicial procedure. This therefore drastically reduces the need to determine the "right" gender (or indeed any gender) of a child at birth from the medical perspective, as the original determination of legal gender no longer is immutable and can be changed relatively easily later in life.

Intersex and Legal Gender

While there has been a relatively rapid development for recognition of preferred gender, the legal debate concerning how to classify persons who identify as intersex or who have intersex conditions is still lagging behind. The legal regulation of changes of gender, described below, largely reinforced the registration of two genders as the binary norm. Indeed, the European Court of Human Rights and the European Union, unlike some national constitutional courts (see below), have yet to support a gender-neutral registration or a right to be identified as a status other than male or female. The European Court of Human Rights, pursuant to Article 12, has in fact repeatedly affirmed that states may provide by law that persons registered as one "sex" may only marry someone of the "other" sex (presumably male or female), and that couples that do not fit this paradigm may not have the same rights (Hämäläinen v. Finland, 2014; Oliari and Others v. Italy, 2015).

So far, only the German Constitutional Court has firmly recommended consideration of a gender-neutral registered identity (see below). Proposals have been put forth in Norway and Sweden so that government data on the gender of the population will not be imposed as a legal identity on persons and can be more easily amended (Norwegian Ministry of Finance, 2017; The Swedish State's Public Report, 2017). These proposals have yet to be adopted. Medico-legal dialogue and collaboration, thus, remain essential to ensure that governments reform their laws to facilitate quick changes of registered gender for children born with intersex conditions, as urged by some human rights authorities (see e.g., Parliamentary Assembly of the Council of Europe, Resolution 2191, 2017).

Indeterminate gender

As the legal gender of a person matters for many aspects of their legal relations (for example for marriage or parenthood, although decreasingly so), and the gender binary was only being questioned very recently (on which see below), it was legally necessary for every person to be allocated the "right" legal gender. Historically (on which see above, with references) there was precedent for allowing the person concerned to choose their social and legal gender. But particularly in the 20th century aspects of gender became more medicalized and legalized, meaning that the gender had to be determined definitively at birth. It is only very recently that the laws in some jurisdictions are beginning to take a different position. After recommendation of the German Ethics Council, in 2013 Germany became the first jurisdiction in modern times to expressly legislate that the gender of a person could be left blank in birth registration documents if the gender could not be determined with absolute certainty (on this see Helms, 2018, with further references). While the person concerned could later in life choose to be legally designated male or female, there was no obligation to do so. This meant that for the first time there were persons who legally did not have a legal gender of either male or female. As the reform in question only dealt with legal gender status, none of the consequences of not having a legal gender were addressed, except that the person would have an "X" in the gender category in their travel and identity documents rather than M/F. But more crucially, this did not establish a "third gender" (on which see below), but rather an absence of legal gender for some persons.

This reform was criticized as the category of "undefined" not only inevitably outed the individuals concerned as intersex but also singled them out and stigmatized them. This in turn may lead to aggravating rather than alleviating the pressure on parents and medical professionals with regard to medical decisions about the child, including "normalizing" surgery in order to avoid the adverse consequences of leaving the gender entry as "indeterminate."

"Third gender"?

Furthermore, while many of the persons concerned identify as male or female (irrespective of any medical intervention) and prefer to be legally recognized as such, others do not. But neither do the latter persons concerned perceived themselves to be "genderless" or "indeterminate," they merely do not fit within the legal (and social) gender binary of male or female. This issue was addressed in a landmark decision by the German Constitutional Court in 2017 (BVerfG 10.10.2017) in which it was held that the German law as outlined above violated the German Basic Law (Grundgesetz (GG), the German constitution). By merely allowing a "negative" entry of gender (i.e., a nonentry) rather than a positive entry, the current German law was held to violate the general right of personality (Art. 2(1) GG in conjunction with Art. 1(1) GG) and the constitutional requirement of nondiscrimination and equal treatment (Art. 3(3) GG). The person in question did not see themselves as genderless, but as having a gender other than male and female, and the law had to positively allow for the recognition of such a gender. The court went even further and suggested that instead of establishing a legal gender system beyond the binary it may also be an option to forego registration of legal gender altogether; however, it seems unlikely that this avenue will be pursued by the legislature. In any event, the Court stipulated that the new law to remedy this situation had to be in place by the end of 2018, meaning that Germany will then be the first jurisdiction with a statutory legal gender framework beyond the binary (although at the time of writing it is unclear what exactly the framework will be, but see Althoff, 2018 for some proposals). In some other jurisdictions, including India and Nepal, court decisions have also established a "third gender," but it appears unclear how these decisions have affected legal practice (see Shah, 2018). Furthermore, it is likely (and in the view of the authors of this articles mandated by human rights law) that any legal gender category beyond the male/female binary will not be restricted to cases of (physical) intersex but also open to anyone who does not identify as male or female.

Indeed, creating a "third gender" merely for intersex persons has rightly been criticized very strongly (see e.g., Carpenter, 2018). As already explained above with regard to leaving a gender entry open, such a third category would "other" the persons concerned and potentially leave them vulnerable to discrimination and stigmatization. Persons recognized as being associated with a "third gender" suffer discrimination in countries that recognize such a gender (Towle and Morgan, 2006). Indeed while the Colombian Constitutional Court authorized registry of persons as "intersex" in 2013 (on this see Rubio-Marín and Osella, 2018), it also recognized the need for gender variant persons to have increased protection from discrimination (Garland, 2016). A third gender may also reinforce expectations or claims that medical intervention is necessary for binary sex identification to be considered equal to others. Requiring registry of such persons as intersex, however, is a clear violation of the human rights of the persons concerned and unacceptable, as it should be open to any intersex person to be legally recognized in their preferred gender irrespective of any medical requirement or interventions. As explained above with regard to recognition of preferred gender, the growing consensus is that gender should be a matter of self-identification, and that would of course also extend to any legal gender options beyond the binary. Yet, the concerns about "othering" a child by having a gender that is not male or female remain legitimate. An appropriate approach may be to not register a legal gender of a child until a certain age when they are old enough to socially identify as being of a certain gender and can determine their legal gender themselves. This may be workable as the legal gender of a person (rather than their social gender) practically really only matters in situations which are only faced when of a certain age, such as gender requirements for marriage/partnerships or parenthood (on this see e.g., Althoff, 2018). Such a system would address concerns about "othering," protect bodily integrity and promote autonomy and self-determination.

Medical Intervention, Consent, and Human Rights

National governments have long regulated the practice of medicine by law (Nys, 1994, 2011). Even when their legal frameworks do not include detailed regulations of specific medical procedures and interventions, the lawfulness of a particular clinical decision or treatment practice is still generally governed by two assessments: (1) whether the practice in question conforms to what is often known as a "professional standard" or "standard of care"; and (2) whether informed consent has been obtained by the treating practitioner from the patient or, in the case of a child, the patient's legal guardian (Nys, 1994, 2011). Prior to the 1990s, standard clinical treatment on children with intersex conditions appears to have been presumed lawful in most jurisdictions—and endorsed in others, such as Sweden—on the presumption that it was in accord with legal standards and accepted medical practice (Garland, 2016; Garland, 2018). Without clear knowledge that standard practices lacked long-term data confirming the necessity or safety of these gender-conforming surgeries and related interventions, many legal authorities, if they were aware of these procedures, may have assumed that they were lawful and that parents were fully informed of the risks and uncertainties surrounding them.

Prior to 2006, however, it was already well known that gender-conforming interventions on children with intersex conditions were not supported by long-term data to validate them as necessary or even safe (Grumbach *et al.*, 2003; Karkazis, 2008; Diamond and Garland, 2014). By 2006, the aforementioned Chicago Consensus Statement publicly acknowledged that this lack of data was a "major shortfall" of practice (Hughes *et al.*, 2006). Two expert-led reviews of the best available evidence potentially supporting standard care followed in 2012. The first of these, known as the Annecy Working Party, concluded that the available studies "lack the necessary detail to base further recommendations" upon regarding these interventions (Lee *et al.*, 2012), as well as that the quality of life of surgically altered children was a "poorly researched" area, and that sexual quality of life of patients was overall "impaired" (Schober *et al.*, 2012). The Working Party advised that gender identity could not be predicted in infancy (Liao *et al.*, 2012) and that the claim of necessity or benefit of gender-reinforcing surgeries was a commonly-held perception unsubstantiated by data (Creighton *et al.*, 2012). A second review followed from an American Psychiatric Association Task Force, which reported

that: (1) gender assignment for children with intersex conditions is "complex and fraught with uncertainties," (2) the available clinical guidelines were "uncomfortably nonspecific," and (3) the limited scientific evidence available made it "difficult to draw conclusions sufficient for evidence-based recommendations" (Byne *et al.*, 2012). In 2016, the largest multidisciplinary group of experts in the field to address the issue so far concluded that "data remain inadequate" to address gender assignment on children with intersex conditions and that no consensus exists on "unresolved questions and dilemmas regarding indications, [and] timing and procedures" of gender-conforming interventions (Lee *et al.*, 2016).

Under national laws, therefore, these candid clinical statements of scientific uncertainty regarding the necessity, safety, and benefit of gender-conforming interventions on infants and other children raise two fundamental questions under standards of care: first, whether clinicians may rely on what is "standard" to avoid discipline and liability despite knowing risks of catastrophic harms; and second, whether nations or clinicians under their authority are liable for harms that result from these procedures. Under international law—particularly the United Nations Convention on the Rights of the Child, the International Covenant on Economic, Social and Cultural Rights and the European Convention on Human Rights, and American Convention on Human Rights—states themselves may be responsible for failure to regulate these procedures and have obligations to ensure that their legal systems permit compensation and other remedies for rights violations. The lack of long-term data enabling parents to be reasonably sure that these procedures will benefit the children—supporting their children's gender, sexuality, and desires without imposing harm—also raises concerns as to whether parents actually *can* consent to these procedures as a matter of law.

Standards of Care

Standards of care for medical professionals vary significantly from nation to nation, particularly in the degree to which they require clinicians to perform certain procedures or hold them liable for procedures that cause injury. The differences in these standards are too numerous to be discussed in detail here, and few comparative works exist (Zillén *et al.*, 2017; Nys, 1994, 2011). Many nations appear to defer to what is standard practice as the presumptive, reasonable measure of what valid care is. There are numerous exceptions to this rule, however. Among the Nordic countries, for example, Iceland prohibits procedures on children without their consent that cannot be validated as medically necessary, whereas Sweden does not permit individual personnel to use treatments that have not been validated by science and carefully tested experience (Zillén *et al.*, 2017). France and Belgium permit civil liability for treatments even if they are standard practice if scientific evidence should have warned clinicians of risks of harm (G'Sell-Macrez, 2011; Nys, 2011). Germany permits liability for preventable and manageable harms that could have been avoided in practice decisions, particularly where procedures are not medically indicated (Sommer *et al.*, 2016). Even in nations that have more deferential standards toward customary practices, it is not clear that clinicians will be able to rely on assumptions that any particular practice is "standard" or "customary" to avoid liability. Currently, there is no audit of practice to determine what clinicians are doing on the whole (Frader, 2015). The Global DSD Consortium's acknowledgement that there is no consensus whether infant surgery is effective or necessary further undermines a claim that experts agree as to what the standard of care should be (Lee *et al.*, 2016). Much medical literature, indeed, appears to assume that clinicians are making individualized decisions based on what parents prefer case-by-case, rather than on objective scientific criteria (Mouriquand *et al.*, 2014; Schober *et al.*, 2012; Creighton *et al.*, 2012).

Clinicians concerned with liability for failure to provide "standard" care must recognize that such liability turns on whether the state in question has failed to ensure protection of the right to life under national and international law, or when the failure to provide treatment is a cause of actual physical injury. Here, it bears noting that even the key studies at Johns Hopkins University that led to the adoption of the practice of surgical intervention on infants and young children with intersex conditions confirmed that persons examined with atypical genitals, who were examined by the researchers, did not show any signs of "psychologic nonhealthiness," as well as that gender-conforming interventions could wait until teenage years (Money *et al.*, 1995a,b). No clear data has emerged to contradict these findings ever since. Few medical complications associated with intersex conditions are known to require medical intervention in infancy (Hughes *et al.*, 2006; Creighton *et al.*, 2012; Zillén *et al.*, 2017). The necessity of other interventions, such as surgery to reduce urinary tract infections, has not been established by quality scientific data (Nabhan *et al.*, 2011). For conditions such as cancer from atypical gonadal development, the evidence-based reviews are generally in agreement that "watchful waiting" and testing until the child could be informed is possible in many cases and, in some cases, removal may not be required (Hughes *et al.*, 2006; Creighton *et al.*, 2012). Children who cannot menstruate due to their development may, if allowed to choose, prefer different options, depending on their gender identity and the degree of intervention that they wish (Houk and Lee, 2010). Overall, it is important to emphasize that no clinical consensus statement from expert-practitioners has rebutted the view that the "only scientifically sound and ethical way to ensure that the surgery coincides with each child's gender identity and interests in how his or her body might appear" is to wait until the child can consent (Diamond and Garland, 2014).

Finally, it should be noted that while gender-conforming interventions as "treatments" for anomalies may have been presumed lawful under general standards of care, the scientific validity and uncertainty surrounding the necessity and safety of these procedures raises the question of whether they violate other laws specifically regulating other medical interventions unless exemptions are permitted on the grounds of medical necessity. Laws prohibiting sterilization of minors and female genital surgeries, for example, generally have exceptions for medical necessity, but where medical necessity is lacking, these laws may impose criminal penalties (Garland, 2016). Similarly, criminal laws often prohibit the irreversible removal of organs or other acts

associated with these interventions, such as vaginal "dilation," that may traumatize a child (Garland, 2016). Clinicians should consult authorities within their jurisdictions to inquire whether the lack of evidence of the necessity of these procedures on infants raises questions about their presumed legality.

Informed Consent

Many legal questions regarding consent to medical interventions on minors begin with the question of who may consent. The default rule in most jurisdictions is that parents are presumed to have the right to consent to medical interventions or at least to participate in those care decisions unless they conflict with the rights of the child. However, many nations permit minors to consent to medical procedures, some by virtue of age limits (such as 14 or 16) and others based on determinations of their maturity and ability to understand the information presented to them (see e.g., European Union Agency for Fundamental Rights, 2015). At the same time, many nations restrict some surgeries to adults, for example, if they have the effect of sterilization or change of gender (see e.g., European Union Agency for Fundamental Rights, 2015). In this sense, calls for permitting gender-conforming procedures only when the minor consents require further assessment of how such consent would be regulated in each jurisdiction. To the extent that *parental* participation is required, however, it bears noting that the parents' "right" to make decisions in matters regarding the child is often wrongly suggested in expert practitioners' commentary to be an unqualified right (Lee and Houk, 2014; Mouriquand *et al.*, 2014). In reality, it is a widely accepted legal principle that parents' rights to refuse or consent to medical procedures may be overridden by legal authorities with medical support or when qualified by law. By that same measure, clinicians lack the authority to override parental consent without the approval of legal authorities. As the jurisprudence of the European Court of Human Rights indicates, these rules do not favor parents or clinicians, although the requirement for legal oversight certainly has curtailed the authority of parents (Afiri and Biddari v. France 2018; Gard and others v. United Kingdom, 2017). These same rules have led to findings of violations of the rights of children when clinicians disregard the consent of parents without express legal permission (M.A.K. and. R.K. v the United Kingdom, 2010; Glass v. United Kingdom 2004). The European Court of Human Rights has also recognized that the rights of minors in intimate health care matters are separate from—and superior to—the right of the parent (P and S v Poland, 2012). It is well accepted, in fact, that medical procedures that parents may wish (such as sterilization, change of gender, or female genital surgeries) may be regulated and prohibited by law, and others, such as male circumcision, may be regulated for patient safety.

Regardless of who consents, there is a broad international consensus that the law on informed consent requires that information must be provided to the patient or the patient's caregiver in a manner that is clear and unbiased regarding risks and uncertainties for all safe alternatives (Nys, 1994, 2011). The determining factor is that the patient or the patient's caregiver must be able to make an informed choice based on reliable information. In most countries that recognize informed consent, breach of informed consent is actionable in courts. In Europe, violations of informed consent in public hospitals are directly actionable in national legal orders and compensable as rights violations, even if physical harm did not occur (Ioniţă v. Romania, 2017). It is equally well recognized that a patient generally has the right to be ill unless a lack of treatment causes serious risk of harm to the life or health of the patient (Pleso v. Hungary, 2012). Invasive non-consensual treatment that is not medically necessary is considered degrading treatment under international law (Jalloh v Germany, 2006; Pretty v. United Kingdom, 2002).

In this light, informed consent regarding gender-conforming interventions on minors with intersex conditions is extremely problematic in current practice. The Chicago Consensus Statement, for example, emphasized that "open communication with patients and families is essential and participation in decision-making is encouraged" and "the health care team will work with the family to reach the best possible set of decisions in the circumstances" but portrayed informed consent as a matter that varies among jurisdictions, without any guidance to clinicians as to how they should advise patients of catastrophic and unpredictable outcomes (Hughes *et al.*, 2006). The same Statement, along with the Global DSD Consortium, also recommended female gender assignments with a conservative estimate of 1 in 20 rejection rate for certain children. They also indicated that parents and clinicians might decide on a female assignment for children in individual cases who are genetic males despite a 50% or greater reported dissatisfaction with gender assignment among such children (Hughes *et al.*, 2006; Lee *et al.*, 2016). As other observers have noted, the medical literature itself often misrepresents data on patient satisfaction with gender assignment and infant surgery and raises concerns about how parents are presented with such data (Baratz and Feder, 2015; Diamond and Garland, 2014). On the whole, it remains unclear how parents can ever be sufficiently informed to consent to or refuse clinician's recommendations to consider gender-conforming interventions when expert-led reviews have concluded that there is inadequate evidence to support such recommendations (Byne *et al.*, 2012; Liao *et al.*, 2012). Indeed, as early as 2006, the US National Institutes of Health concluded that there was "insufficient data to guide the clinician and family in sex assignment" in such cases (National Institute of Health, 2006).

Of even greater concern, perhaps, is that parents might consent to procedures despite being told of the risks (Dayner *et al.*, 2004). As several practitioner-led statements and reviews of evidence indicate, parents themselves do not develop attachment that they may expect with their children after gender-conforming surgeries and often find it difficult to realize that their needs and desires will be different than the needs of their children (Schober *et al.*, 2012; Hughes *et al.*, 2006). There is also considerable evidence that parenting ability may be impaired by stress in their decision-making for children with intersex conditions (Garland, 2016; Garland and Diamond, 2018). As the Colombian Constitutional Court recognized in 1999, the legitimacy of parental consent in these cases is not only suspect because the interests of the child are profoundly personal but because the parents may

see their children as "minorities" that need to be "normalized," such that the children run the risk of being discriminated against by their own parents (Sentencia SU-337/99, 1999). For all of these reasons, multiple human rights authorities have come to the conclusion that the consent of children with intersex conditions themselves to any medically unnecessary surgery is imperative.

Human Rights

Over the last decade, human rights authorities have become increasingly concerned that irreversible and invasive medical interventions on infants and young children with intersex conditions constitute violations of multiple rights of children, often in a single medical procedure. In 2006, a group of experts in human rights promulgated a series of statements of rights known as the Yogyakarta Principles, derived from general international law generally when applied to sexual and gender minorities. These rights include that no child should be "irreversibly altered by medical procedures in an attempt to impose a gender identity without the full, free and informed consent of the child in accordance with the age and maturity of the child" (Yogyakarta Principles, 2006). Since then, multiple authorities from the United Nations, the Council of Europe, the European Union and the Organization of American States have endorsed this view and called on nations to ensure that gender-conforming medical interventions on children with intersex conditions do not take place without their consent (Zillén *et al.*, 2017; Ghattas, 2018). Within their competences, these authorities have identified multiple rights that may be violated in a single operation on a child, such as: (1) the right to physical and psychological integrity, (2) the right to privacy in intimate matters and identity, including the right to a gender identity and sexual life, (3) freedom from torture, inhuman and degrading treatment, (4) freedom from experimentation, (5) the right to the highest attainable standard of health, including the right to protection from practices prejudicial to their health, and (6) the right of the child to be heard in matters affecting the child (Zillén *et al.*, 2017). As of March 2018, only Malta has enacted legislation to require consent to gender-conforming procedures (Ní Mhuirthile, 2018).

Perhaps not surprisingly, some published clinical reactions to human rights recommendations have perceived them as pejorative and interfering with clinical judgment and parental decision-making (Lee and Houk, 2014; Mouriquand *et al.*, 2014). Much of this reaction, however, reflects a need for greater understanding of the scope of the rights at stake. Under international law, for example, "degrading treatment" is often paired with "torture" if it is nonconsensual medical treatment, invasive, unnecessary, and causes serious physical pain and suffering or lasting aggravation or consequences for the person's health (UN Special Rapporteur, 2013; Jalloh v Germany, 2006). This includes treatment for an actual medical condition that is made worse by the intervention (Pretty v. United Kingdom, 2002). As several authorities have noted, involuntary sterilization of the disabled is now well-recognized as a human rights violation but still persists for many children with DSD (WHO, 2014). Similarly, the traditional reliance on parental rights to justify these surgeries is not consistent with evolving legal norms recognizing the rights of children, particularly enshrined in the UN Convention on the Rights of the Child, which has been ratified by all but two nations in the world (the United States and Somalia) and constitutes an international consensus that children may need to be protected from harmful decisions made by their parents, including in health care matters (Committee on the Rights of the Child, 2006). The European Court of Human Rights and Inter-American Court of Human Rights have taken the same view in the obligation of governments to have legal frameworks in place to protect children from physical and psychological harm (Söderman v. Sweden, 2013; Inter-American Court of Human Rights, 2017).

For these reasons, human rights authorities are now urging national legal orders to regulate these procedures—not to ban them—primarily to ensure that the care is patient-centered so that clinicians can confirm that the patients desire the treatment and understand the risks, but of most importance so that treatment does not come with the uncertainty of imposing a gender or sexual preference on a child who may have different interests as an adult. In October 2017, the Parliamentary Assembly of the Council of Europe has therefore recommended that all nations should (1) prohibit medically unnecessary sex-"normalizing" surgery, sterilization and other treatments practiced on intersex children without their informed consent, (2) "ensure that, except in cases where the life of the child is at immediate risk, any treatment that seeks to alter the sex characteristics of the child… is deferred until such time as the child is able to participate in the decision," (3) ensure holistic patient-centered and expert care, (4) ensure that intersex persons have effective access to health care throughout their lives, (5) provide training to psychological and other professionals concerned, including conveying a clear message that intersex bodies are the result of natural variations in sex development and do not as such need to be modified, (6) ensure that adequate psychosocial support mechanisms are available for intersex people and their families throughout their lives, and (7) ensure that children surgically altered without their consent receive compensation for harm (Parliamentary Assembly of the Council of Europe, Resolution 2191, 2017). These views have been incorporated into the Yogyakarta Principles in 2017 and are likely to have ramifications in human rights calls for years to come.

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Neonatal Diabetes Mellitus

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Definitions, and Classification of NDM

The term neonatal diabetes mellitus (NDM) is used to describe uncontrolled hyperglycemia that present within the first 6 months of life and needs treatment (Rubio-Cabezas *et al.*, 2014). Although some of these children present beyond the first 4 weeks of life, which is classical definition of neonatal period, the term NDM has been widely used in the literature as most patients are believed to be born with this form of diabetes; however the time required for presentation varies between infants.

Some infants with NDM continue to require life-long treatment for their diabetes and classified as permanent NDM (PNDM), while others go into remission after few weeks or months and are labeled to have transient NDM (TNDM). Interestingly, most children with TNDM can relapse around puberty with diabetes resembling type 2 diabetes (T2DM) (Docherty *et al.*, 2013). In both TNDM and PNDM, patients can have diabetes as an isolated phenomenon or in association with other extrapancreatic features that can form a recognized inherited syndrome (syndromic NDM).

Epidemiology

NDM is a rare form of childhood diabetes and its frequency varies between different populations. Recent data showed that the incidence of NDM is higher in areas with high consanguinity such as the Middle East reflecting the genetic etiology of this form of diabetes. The highest reported incidence rate was 1: 21,000 in Northwest Saudi Arabia (Habeb *et al.*, 2012a) followed by 1 in 30,000 in Abu Dhabi, UAE (Deeb *et al.*, 2016b). A lower rate ranged between 1 in 90,000 and 1 in 500,000 was reported from Europe (Demirbilek *et al.*, 2015; Grulich-Henn *et al.*, 2010; Shield *et al.*, 1997; Slingerland *et al.*, 2009; Stanik *et al.*, 2007; Wiedemann *et al.*, 2010). The frequency of TNDM and PNDM are almost equal in Europe; however, in areas with high consanguinity, PNDM appears to be more prevalent (Deeb *et al.*, 2016b; Demirbilek *et al.*, 2015; Habeb *et al.*, 2012a) (**Fig. 1**). The lower frequency of TNDM in consanguineous populations could be explained by the more sporadic nature of the TNDM compared to PNDM. However, it is possible that some TNDM cases are not recognized or under reported.

Etiology and Pathogenesis

Over the last two decades, there has been rapid advances in the understanding of the etiology of NDM. It is now well established that NDM is a heterogeneous form of monogenic diabetes (MGD) characterized by insulin deficiency. To date, mutations in more than 20 genes that can either control the β-cell function and survival or regulate the pancreatic mass, have been identified in more than 80% of NDM cases (De Franco *et al.*, 2015). Mutations in these genes can decrease insulin secretion by different mechanisms; reducing the pancreatic mass, impairing the pancreatic β-cell function or increasing its destruction. The genetic causes, and associated phenotypes of different forms of NDM are illustrated in **Table 1** (**Fig. 2**).

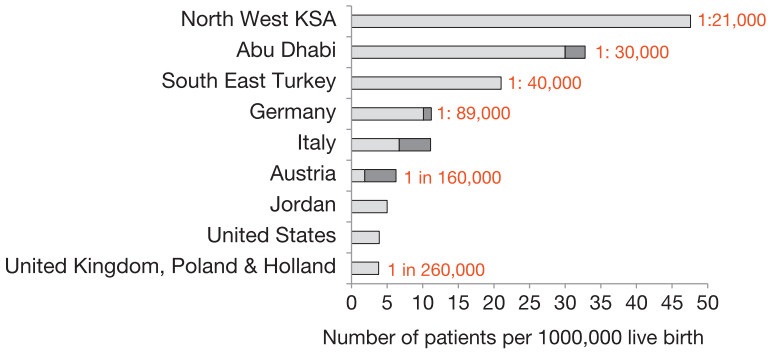

Fig. 1 Incidence of NDM worldwide: The light gray represent PNDM and the dark gray is TNDM. KSA; Kingdom of Saudi Arabia. Reproduced with permission from Deeb, A., Habeb, A. and Kaplan, W. (2016). Genetic characteristics, clinical spectrum, and incidence of neonatal diabetes in the emirate of Abu Dhabi, United Arab Emirates. *American Journal of Medical Genetics Part A* **170** (3), 602–609.

Table 1 Summary of the genotype and phenotype of NDM subtypes

Genetic cause	*Mode of inheritance*	*Neonatal diabetes subtype and treatment*	*Additional features*
6q24	Sporadic	Transient; insulin	Intrauterine growth retardation, macroglossia, umbilical hernia, neurological features (rare)
KCNJ11	Sporadic, dominant	Transient, permanent; sulfonylurea	Developmental delay with/without epilepsy (20% of cases)
ABCC8	Recessive, dominant and sporadic	Transient, permanent; sulfonylurea	22% have DEND syndrome (developmental delay with/without epilepsy)
INS	Recessive, sporadic	Transient, permanent; insulin	None
HNF1B	Recessive	Transient; insulin	Pancreatic hypoplasia, renal cysts
ZFP57	Sporadic, recessive	Transient	Intrauterine growth retardation, neurological features
SLC2A2	Recessive	Transient, insulin	Fanconi Bickel syndrome (hepatorenal glycogen accumulation, renal tubular acidosis, hyperlipidemia, hypophosphatemic rickets)
GATA4	Recessive	Transient, permanent; insulin	Congenital heart malformation, exocrine insufficiency
GATA6	Recessive	Transient (rare), permanent; insulin	Congenital heart malformation, neurological defects, hypothyroidism, gut and hepato-biliary malformation, exocrine insufficiency
EIF2AK3	Recessive	Permanent; insulin	Skeletal dysplasia, liver dysfunction, developmental delay
FOXP3	X-linked, recessive	Permanent; insulin	Eczema, enteropathy, other autoimmune features
GCK	Recessive	Permanent; insulin	None
GLIS3	Recessive	Permanent; insulin	Congenital hypothyroidism, renal cysts
IER3IP1	Recessive	Permanent; insulin	Microcephaly, epilepsy
MNX1	Recessive	Permanent; insulin	Sacral agenesis, neurological defects NEUROD1, cerebellar hypoplasia, sensorineural deafness
NEUROD1	Recessive	Permanent; insulin	Cerebellar hypoplasia, sensorineural deafness, visual impairment
NEUROG3	Recessive	Permanent; insulin	Congenital malabsorptive, diarrhea
NKX2-2	Recessive	Permanent; insulin	Severe neurodevelopmental defects
PDX1	Recessive	Permanent; insulin	Exocrine insufficiency
PTF1A	Recessive	Permanent; insulin	Cerebellar agenesis
RFX6	Recessive	Permanent; insulin	Intestinal atresia and/or malrotation, gall-bladder agenesis
SLC19A2	Recessive	Permanent; insulin and thiamine	Thiamine-responsive megaloblastic anemia (sensorineural deafness, thrombocytopenia, stroke, cardiac and visual defects)
STAT3	Sporadic, dominant, germline	Permanent, insulin	Autoimmune enteropathy, primary hypothyroidism, pulmonary disease, juvenile arthritis, dental disease, eczema
LRBA	Recessive	Permanent, insulin	Autoimmune lymphoproliferative disease, hepatosplenomegaly, thrombocytopenia, enteropathy, autoimmune hemolytic anemia

The genetic etiology and clinical spectrum of PNDM vary between populations and are influenced by the parental consanguinity. Studies in Europe, Japan, and the United States have shown that PNDM is usually isolated and de novo mutations in either of the genes encoding the two subunits of the ATP-sensitive potassium (K_{ATP}) channel of the β-cell membrane (KCNJ11 or ABCC8) are the commonest cause of PNDM (Flanagan *et al.*, 2006; Shankar *et al.*, 2013; Suzuki *et al.*, 2007) followed by *INS* (preproinsulin) gene mutations (Docherty *et al.*, 2013). In contrast, in areas with high consanguinity, PNDM is usually associated with extrapancreatic features with mutations in the *EF2AK3* leading to Wolcott Rallison syndrome being the commonest cause (Rubio-Cabezas *et al.*, 2009; Habeb *et al.*, 2018).

The majority of TNDM cases are caused by imprinting defect in the 6q24 chromosome leading to over expression of the *PLAGU* gene (Docherty *et al.*, 2013; Mackay *et al.*, 2014; Temple and Shield, 2002). Other causes of TNDM include activating mutations of the K_{ATP} channel genes (*KCNJ11* and *ABCC8*), *HNF1B* and *INS* gene mutations (Edghill *et al.*, 2008; Gloyn *et al.*, 2005). Interestingly all genetic causes of TNDM can also lead to PNDM.

Clinical Presentation

Patients with NDM usually have low birth weight, reflecting the role of insulin in antenatal growth, and can present throughout the first 6 months of life.

Unlike older children and adults, the diabetes symptoms can be difficult to detect at this age group by families and general practitioners which can often lead to a delay in making the diagnosis. Infants with NDM can be asymptomatic and discovered accidently while measuring blood glucose for other reasons. It is therefore a good practice to measure blood glucose or test urine for glucose in any child admitted to hospital with unspecific presentation. Common symptoms of hyperglycemia at this age include, poor feeding, failure to thrive, vomiting, polyuria (frequent nappy changes), dehydration, and ketoacidosis. Occasionally

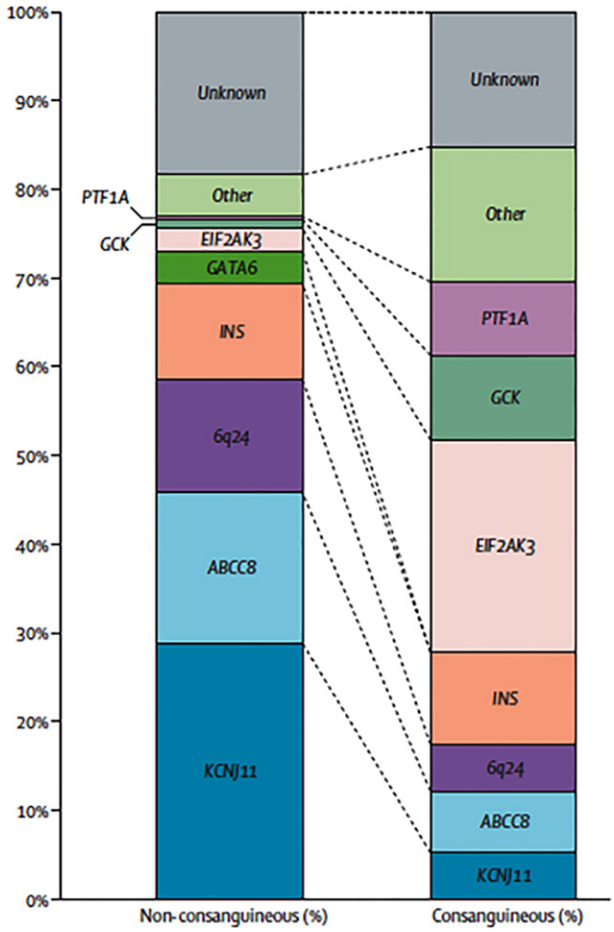

Fig. 2 Comparison of genetic causes of neonatal diabetes in nonconsanguineous ($n = 790$) and consanguineous groups ($n = 230$). Tested in Exeter molecular genetic laboratory. Consanguinity is defined by parents being second cousins or more closely related or by the presence of 1 · 56% or higher total homozygosity. Reproduced with permission from De Franco, E., Flanagan, S. E., Houghton, J. A., et al. (2015). The effect of early, comprehensive genomic testing on clinical care in neonatal diabetes: An international cohort study. *Lancet* **386** (9997), 957–963.

the diagnosis of NDM is delayed because the above symptoms can be seen in other conditions such as gastroenteritis and respiratory or urinary infection that are more common at this age.

The combination of persistent hyperglycemia with glycosuria is usually sufficient to make the diagnosis of NDM. In children beyond the first 2 months of life, the high HbA1c can be useful to confirm the diagnosis of NDM. However, the diagnosis can be a challenge in newborns with sepsis where insulin resistance can cause hyperglycemia (Mittal *et al.*, 2013). In addition, some metabolic condition such as methylmalonic acidemia, can have an initial presentation that mimic diabetic ketoacidosis (Dejkhamron *et al.*, 2016; Saini *et al.*, 2015). Unlike children with NDM, hyperglycemia in infants with metabolic conditions is often easily controlled with small dose of insulin and on the recovery stage hypoglycemia becomes obvious.

At presentation it is difficult to predict clinically whether the infant has TNDM or PNDM as patients can have similar symptoms of hyperglycemia. It is worth remembering that in some forms of syndromic NDM, diabetes can precede the development of other extrapancreatic features which can develop later during the course of the disease. Reported extrapancreatic features in patients with NDM are listed in **Table 2**.

Clinical Evaluation of a Child With NDM

The aim of the initial assessment of children with NDM is to confirm the diagnosis, start the appropriate treatment, detect associated extrapancreatic features and identify the genetic cause. The latter is crucial as it can guide the best treatment option for the patient, predict the development of some extrapancreatic features, avoid invasive procedures to diagnose associated features and provide the family with accurate genetic counseling.

The clinical evaluation of a newly diagnosed child with NDM follows the usual approach of history taking, clinical examination and guided investigations.

Table 2 Common reported extrapancreatic features in patients with neonatal diabetes

Neurological	*Face, eye, and ear*
Developmental delay	Macroglossia
Brain malformation	Glaucoma
Epilepsy, stroke	Microphthalmia
Microcephaly, lissencephaly	Optic atrophy
Diabetes insipidus	Visual impairment
Central respiratory dysfunction	Deafness
Gastrointestinal	*Hepatic*
Failure to thrive and diarrhea	Deranged liver enzymes
Pancreatic hypoplasia/aplasia	Intermittent hepatitis
Intestinal atresia	Hepatic cysts
Biliary abnormalities	Hepatomegaly
Gall-bladder agenesis	Hepatic fibrosis
Autoimmune enteropathy	
Hematological	*Musculoskeletal*
Thrombocytopenia	Rickets
Lymphoproliferative syndrome	Sacral agenesis
Autoimmune hemolytic anemia	Skeletal dysplasia
Megaloblastic anemia	Juvenile arthritis
Endocrine/metabolic	*Cardiovascular*
Hypothyroidism	Arrhythmia
Hypercholesteremia	Conduction defects
Hypertriglyceridemia	Congenital heart disease
Hypergalactosemia	
Kidney	*Others*
Renal cysts	Low birth weight
Impaired renal function	Eczema
Renal failure	Umbilical hernia
Renal tubular acidosis	Interstitial lung disease

The value of clinical history and examination in NDM

The combination of parental consanguinity and history of previous child with NDM, or unexplained infantile death usually points toward autosomal recessive NDM. The presence of one parent with diabetes diagnosed within the first year of life could indicate K_{ATP} channel diabetes, which can be better managed with sulfonylurea (SU) than insulin (Pearson *et al.*, 2006; Rafiq *et al.*, 2008). The history of maternal gestational diabetes or mild hyperglycemia in either parents raises the possibility of NDM due to biallelic glucokinase (*GCK*) gene mutations in the child, and heterozygous GCK MODY in the parent. This is particularly the case if the child has a very low birth weight and presented in the first few days of life (Njolstad *et al.*, 2003) (see below). Finally, history of TNDM in parents or a sibling in early life may predict that the child could have TNDM.

The severity of intrauterine growth retardation (IUGR) and timing of presentation may point toward a specific form of NDM. For example, the combination of severe IUGR and hyperglycemia within the first week of life is typically seen in biallelic *INS*, *GCK*, and *GLIS3* mutations (Docherty *et al.*, 2013; Edghill *et al.*, 2007; Njølstad *et al.*, 2001), while patients with *INS* and *GCK* mutations have isolated NDM, children with GLIS 3 mutations have distinct phenotype including primary hypothyroidism, glaucoma and liver disease (Dimitri *et al.*, 2015).

Children with NDM should be thoroughly examined for the presence of extrapancreatic features (see **Table 2**). Some of these features may need early intervention because they carry higher morbidities than diabetes, for example, glaucoma, liver dysfunction, hypothyroidism and pancreatic exocrine dysfunction. Detailed phenotypic assessment is also important for prioritizing the genetic testing. For example, the presence of NDM, skeletal dysplasia and intermittent hepatitis indicate that direct sequencing of the *EIF2AK3* gene is needed to confirm WRS (Rubio-Cabezas *et al.*, 2009) and the combination of NDM, hepatomegaly and rickets raises the possibility of Fanconi Bickel syndrome (Sansbury *et al.*, 2012) which can be confirmed by *SLC2A2* sequencing and thus avoiding the need for liver biopsy. It is worth remembering that some of the features such as skeletal deformities can develop months and years after the diabetes and that some features can be intermittent such as hepatitis in WRS (see below). The clinical examination of children with NDM should include proper ophthalmology, hearing, neurological and cardiac assessment.

Initial investigations of children with NDM

In addition to the urinalysis, measurement of blood glucose, HbA1c, and genetic testing, children with NDM need further hematological, biochemical, radiological and genetic tests guided by the clinical findings (**Fig. 3**). The authors recommend that all children with NDM have the following baseline tests: hemoglobin, white blood counts, platelets, liver function test, urea, creatinine, TSH, fT4, c-peptide, stool elastase and abdominal ultrasound for kidneys pancreas and bladder. It is worth remembering that when assessing for pancreatic aplasia/hypoplasia in neonate, fecal elastase and fats are more reliable than pancreatic

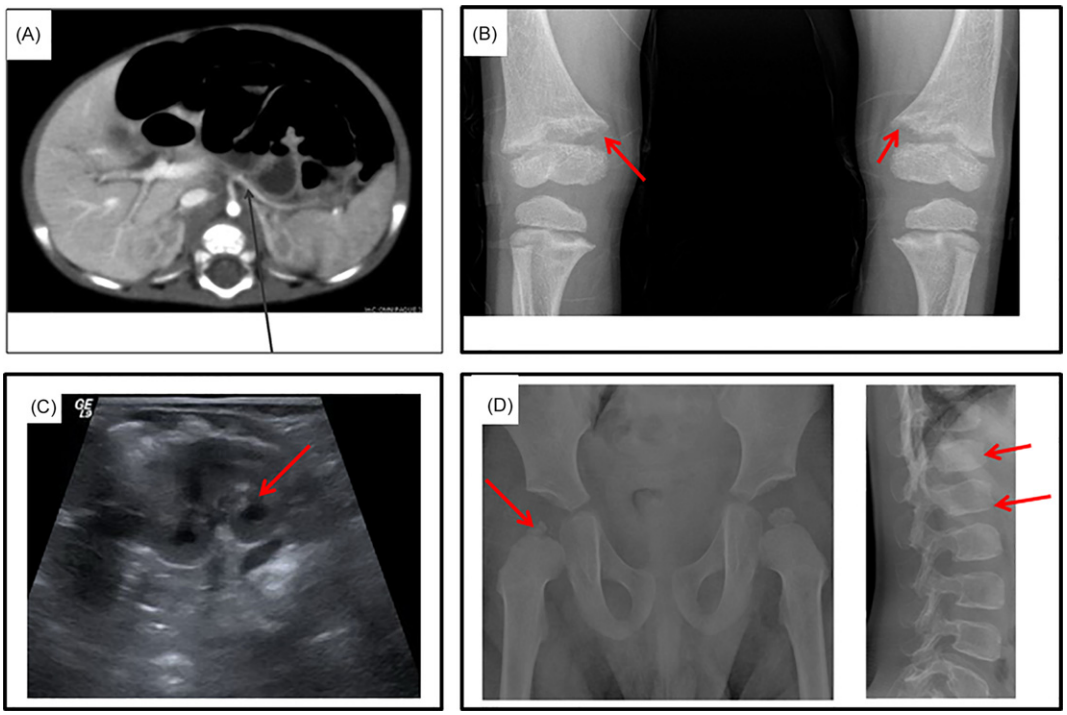

Fig. 3 Radiological features detected in some children with neonatal diabetes: (A) computerized tomography of the abdomen showing pancreatic aplasia; (B) active rickets in both knees in a patient with Fanconi–Bickel syndrome; (C) renal ultrasound scan showing renal cysts in GLIS 3 and WRS, (D) skeletal dysplasia in WRS (fragmented femoral head and thoraco-lumbar vertebra).

scanning (Weedon *et al.*, 2014). The rest of tests depends on the phenotype, for example, if there is skeletal deformities, skeletal survey is important to detect epiphyseal dysplasia associated with WRS, which is the commonest cause of NDM in consanguineous parents (Edghill *et al.*, 2008).

The routine measurement of pancreatic autoantibodies is not necessary as they are usually negative and their presence at this age doesn't rule out monogenic diabetes. In fact, most infants with positive autoantibodies found to have MGD triggering autoimmunity rather than the classical multifactorial autoimmune type 1 diabetes (see below).

Genetic testing for NDM

More than 80% of NDM patients have genetic cause and it is now recommended that molecular genetic testing for infants with NDM before 6 months should be performed as soon as the diagnosis is confirmed. Knowledge of the genetic cause of NDM has many clinical benefits: first, it can guide the best treatment for example patients with KATP are better managed by SU and starting thiamine in those with *SLC19A2* mutations can resolve the anemia and some patients may become off insulin. Secondly, it can predict the development of some extrapancreatic features such as skeletal dysplasia and intermittent hepatitis in WRS and finally provides the family with accurate genetic counseling with the options for future pregnancies such as the use of pregestational diagnosis (PGD) and selection of unaffected fetus (Deeb *et al.*, 2016a).

Genetic testing for NDM is freely available in some laboratories in Europe and North America with some providing rapid testing for subtypes where results alter treatment such as K_{ATP} channel mutations. Traditionally, genetic testing is based on direct sequencing of a specific gene guided by the clinical phenotype. However, the development of NGS whole exome sequencing it is now possible to test for all 22 genes responsible for NDM using one kit (Ellard *et al.*, 2013).

In some patients, certain combination can be detected with the diabetes and it is possible that the genetic diagnosis can be predicted before conducting the genetic tests. For example, the combination of NDM, congenital hypothyroidism and glaucoma is diagnostic for *GLIS3* mutations (Dimitri *et al.*, 2015) and the triad of diabetes, anemia and deafness is diagnostic for thiamine responsive megaloblastic anemia (Shaw-Smith *et al.*, 2012).

Management of NDM

Similar to other forms of childhood diabetes, the clinical management of NDM is multidisciplinary and encompasses parental education, dietary management and pharmacotherapy. Children should be screened regularly for the presence of extrapancreatic features and referred to the appropriate service for further management.

In addition to the standard diabetes educational package, parents of infants with NDM should be alerted for the possibilities TNDM and the symptoms and evolving nature of some extrapancreatic features. It is important to be aware that families of infants with NDM need a longer period of hospital based education compared to other forms of diabetes. Because of the genetic nature of NDM, parents should be referred for genetic counseling as soon as the genetic cause is identified.

The dietary management of diabetes at this age is a challenge. This is particularly due to the rapid physical growth and the change in nutritive requirement in this age group. Breastfeeding makes assessment of intake and insulin dosages fairly inaccurate and weaning into solid food can be difficult. Conventional preprandial glucose readings might not be genuinely preprandial and many assumed preprandial readings are in fact postprandial which impacts adversely on the dose of insulin to be given (Deeb, 2017).

Pharmacotherapy in NDM

At diagnosis, all children with NDM should be started on insulin. However, the need for insulin therapy can be revised once the result of the genetic testing became available. NDM is one of conditions where the concept of pharmacogenomics or targeted therapy is vividly illustrated. For example, patients with K_{ATP} channel mutations are sensitive to SU and should be given a trial of switching from insulin to SU (Pearson *et al.*, 2006; Rafiq *et al.*, 2008) and those with TRMA due to SLC19A2 mutation can achieve better glycemic control and may become off insulin by adding thiamine (Habeb *et al.*, 2018).

All NDM patients should continue on insulin except patients with K_{ATP} mutations. Insulin therapy is a challenge in infants as they require very small doses of insulin. In addition, infants have less subcutaneous tissue which results in variable absorption and sensitivity for insulin. The need for ultra-small doses of insulin to cover the small carbohydrate intake imposes a major challenge and optimal glycemic control is difficult to achieve due to the risk of hypoglycemia when strict control is attempted. Insulin pump therapy can be the ideal solution for this problem particularly in infants on continuous feeding, those on total parental nutrition or infants on frequent breastfeeding (Tubiana-Rufi, 2007). In our experience, insulin pumps with continuous glucose monitoring (CGM) are the safest way to control blood glucose at this age. CGM is particularly useful in infants prone to nocturnal hypoglycemia in whom sensor augmented pump therapy is the safest solution. Insulin pumps might not be available in some parts of the world where neonatal diabetes is more common. In these areas, the use of multiple daily injection can be an alternative with a suitable ratio of basal to prandial insulin. Diluting insulin is another method for providing ultra-small doses of insulin. However, it is associated with a considerable degree of human errors and inaccuracy (Ruan *et al.*, 2015).

Because K_{ATP} channel diabetes is the commonest cause of NDM in nonconsanguineous populations, some authors suggested to start all NDM imperially on SU as genetic testing can be costly and not easily available in certain parts of the world (Carmody *et al.*, 2014). However the authors feel that this practice is not justifiable as some international laboratories are offering free genetic testing for NDM and the results of KATP in particular can be available within days. Also in some parts of the world KATP are not common cause of NDM and some KATP mutations require higher dose and longer period of time to respond. In addition, SU can cause side effects of diarrhea and hypoglycemia and cause parental disappointment if the diagnosis of $K_{ATP\ is}$ excluded on genetic study (Deeb, 2016).

Genetic Counseling

Genetic counseling is an important part of the management of NDM. Few forms of NDM subtypes are not inherited but the majority are caused by single gene defects and thus the risk for future pregnancies depends on the mode of inheritance. Some forms of monogenic NDM are caused by sporadic mutations. It is, therefore, important to define the carrier status of the parents. If the parents have no mutations and the child have heterozygous mutation, for example, K_{ATP} diabetes and some INS mutations, the offsprings will have 50% chance to be affected. However, when parents are carriers the siblings of those with recessive mutations will have 25% risk. Detailed recurrence risk in certain NDM subtypes are discussed under specific subtypes (see below).

Reducing consanguinity is a challenge in some cultures; however, knowledge of the mutation of certain NDM with poor prognosis would allow for antenatal diagnosis with termination of pregnancy. An alternative option for certain families is the technique of pregestational diagnosis, which allow for the selection of unaffected zygotes, particularly for families with more than one affected child (Deeb *et al.*, 2016a).

Common Subtypes of NDM

6q24 Diabetes

Abnormalities of the imprinted region on chromosome 6q24 leading to overexpression of *PLAGL1* and *HYMAI* genes are responsible for approximately 70% of patients with TNDM (Docherty *et al.*, 2013). To date, three molecular causes of 6q24 diabetes have been reported: paternal uniparental disomy (UPD) of chromosome 6, that is, both alleles inherited from the father, paternal duplication of the 6q24 region and abnormal methylation of the maternal allele (Mackay *et al.*, 2014; Temple and Shield, 2002). The latter can also be due to bilallelic *ZFP57* gene mutations.

Patients with 6q24 diabetes often have IUGR and develop severe nonketotic hyperglycemia during the first week of life. In majority of them, the diabetes remits in few months but relapses around puberty with a picture resembling early-onset type 2 diabetes which can respond to SU. During remission, transient hyperglycemia may occur during intercurrent illnesses. In a large series of TNDM due to 6q24, more than 50% of 134 patients have associated extrapancreatic features of which macroglossia and umbilical hernia were the commonest (Docherty *et al.*, 2013). These congenital abnormalities occurred less frequently in the 6q24 duplication. Recently UPD 6q24 abnormalities were detected in three young adults with early onset nonautoimmune diabetes (Yorifuji *et al.*, 2015) and children with PNDM (Cao *et al.*, 2017; Flanagan *et al.*, 2007).

Genetic counseling for families with 6q24 TNDM depends on the underlying molecular mechanism. Methylation defects and UPD of chromosome 6 are essentially sporadic and therefore the risk of recurrence in siblings and offspring is low. For paternal duplication of the 6q24 region, males have a 50% chance of transmitting the disease to their children. In contrast, females will pass on the duplication, but their children will not develop the diabetes. However these asymptomatic carrier may pass the disease to their offsprings. Methylation defect due to mutations in *ZFP57* show an autosomal recessive inheritance with recurrence risk is 25% for siblings.

Neonatal Diabetes Due to Mutations in the K_{ATP} Channel Genes

In outbreed populations, $\mathbf{K_{ATP}}$ channels mutations are the commonest cause of PNDM and the second most common cause of TNDM (De Franco *et al.*, 2015; Rubio-Cabezas *et al.*, 2014).

K_{ATP} channels are octameric complexes consisting of four Kir6.2 subunits and four SUR1 subunits, encoded by the *KCNJ11* and *ABCC8* genes, respectively. The channels control insulin secretion by coupling cell metabolism to electrical activity of the β-cell. Any increase in the intracellular glucose membrane results in a rise of the ATP/ADP ratio which makes the K_{ATP} channels close. This closure results in depolarization of the cell membrane with influx of calcium into the cell which triggers the insulin secretion. Activating mutations in the genes encoding the *KCNJ11* or *ABCC8*, prevent the closure of K_{ATP} channel and subsequently insulin secretion in response to hyperglycemia.

More than 90% with *KCNJ11* mutations have PNDM and approximately 10% of them present with TNDM (Babenko *et al.*, 2006; Ellard *et al.*, 2007; Flanagan *et al.*, 2007). In contrast, two-third of patients with *ABCC8* mutations have TNDM (Ellard *et al.*, 2007; Proks *et al.*, 2006). Compared to patients with 6q24 abnormalities, children with TNDM due to K_{ATP} channel mutations present slightly later, have milder intrauterine growth retardation and their diabetes usually remits later and relapses earlier than in 6q24-TNDM.

Patients with K_{ATP} channels diabetes have low or undetectable C-peptide levels and frequent presentation with diabetic ketoacidosis and around 20% of patients with diabetes due to *KCNJ11* mutations have associated neurological symptoms reflecting the expression of K_{ATP} channels in neurons and muscle cells (Babenko *et al.*, 2006; Flanagan *et al.*, 2007). The neurological features vary from the typical severe DEND (developmental delay, epilepsy, and neonatal diabetes) syndrome to a milder form and more frequent (intermediate DEND) which is characterized by NDM, moderate developmental delay without epilepsy. In contrast, neurological features are less frequent and often milder in patients with *ABCC8* mutations (Babenko *et al.*, 2006; Ellard *et al.*, 2007; Proks *et al.*, 2006).

Most patients with K_{ATP} channel diabetes can be transferred from insulin to oral SU. In fact, they can achieve better control with less hypoglycemic episodes on SU compared to insulin (Pearson *et al.*, 2006; Rafiq *et al.*, 2008). The SU doses required per body weight are higher than those for adults with type 2 diabetes (around 0.5–2.3 mg/kg/day of glibenclamide) but many patients have been able to progressively reduce the dose after transition while maintaining excellent glycemic control. Some reports showed that SU may penetrate blood–brain barrier and that SU may partially improve some of the neurological features (Battaglia *et al.*, 2012; Gurgel *et al.*, 2007). The main reported SU side effects in children are transient diarrhea and staining of the teeth on chronic use (Codner *et al.*, 2005; Kumaraguru *et al.*, 2009).

Activating mutations in *KCNJ11* causing NDM are heterozygous with 90% of them arising spontaneously without family history of NDM, but familial cases show an autosomal dominant inheritance. The recurrence risk for the offspring of an affected patient is 50%. Whilst most activating mutations in *ABCC8* are also heterozygous, some patients are homozygous or compound heterozygous for two different mutations and their NDM is recessively inherited (Flanagan *et al.*, 2007; Proks *et al.*, 2006). In this case, the risk of NDM for future siblings is 25%; but almost nil for the offspring. Germline mosaicism (mutations present in the gonads but not detectable in blood) has been reported in some families (Edghill *et al.*, 2007). Therefore, unaffected parents of a child with an apparently de novo mutation should be advised that the recurrence risk in siblings is low but not negligible.

Neonatal Diabetes Due to Mutations in *INS* Gene

Both heterozygous and biallelic (homozygous or compound heterozygous) *INS* mutations can cause NDM through different mechanisms.

Heterozygous *INS* mutations are the second commonest cause of PNDM and can occasionally result in TNDM (De Franco *et al.*, 2015; Edghill *et al.*, 2008). The diabetes is slowly progressive and results from accumulation of the misfolded proinsulin protein in the endoplasmic reticulum (ER). This leads to ER stress and β-cell apoptosis. Patients with heterozygous INS mutations have

isolated diabetes and similar birth weight to those with K_{ATP} but present slightly later (Edghill *et al.*, 2008). In some families diabetes present beyond 6 months of age and the same mutation can cause PNDM and TNDM within the same family (Deeb *et al.*, 2016b). Most heterozygous *INS* mutations are sporadic but about 20% of probands have a positive family history of autosomal dominant NDM.

Biallelic *INS* mutations have also been described as a cause of isolated NDM (Garin *et al.*, 2010). In contrast to the heterozygous *INS* mutations, the diabetes results from lack of insulin biosynthesis during pre- and postnatal period. As a result, patients with biallelic mutations have much lower birth weight and earlier presentation compared to those with heterozygous INS mutations. The diabetes is recessively inherited with 25% recurrence risk in siblings; but in the absence of consanguinity, a very low risk for the offspring of the patient is expected.

Wolcott Rallison Syndrome

Wolcott–Rallison syndrome (WRS) is the commonest cause of PNDM in consanguineous pedigrees and Arab population (Habeb *et al.*, 2012b; Rubio-Cabezas *et al.*, 2009). This syndrome is caused by recessive loss of function mutations in the *EIF2AK3* gene. The *EIF2AK3* gene encodes a protein called pancreatic PKR-like endoplasmic reticulum kinase (PERK), which plays a key role in detecting and initiating the cellular response to endoplasmic reticulum stress, insulin secretion and trafficking. Failure of appropriate PERK response results in accumulation of misfolded proteins, which leads to cell damage and apoptosis.

The cardinal features of WRS are PNDM, skeletal dysplasia and non-auto immune intermittent hepatitis which can progress to acute fatal hepatic failure (Habeb *et al.*, 2015). Other reported features include renal dysfunction, failure to thrive, developmental delay, neutropenia and hypothyroidism. Patients can initially present with isolated diabetes within the first few months and subsequently develop the extrapancreatic features (Rubio-Cabezas *et al.*, 2009). Occasionally, diabetes presents as late as 3–4 years old. The prognosis of WRS is poor with the majority of patients die within the first decade with acute hepatorenal failure.

The management of WRS should be through a multidisciplinary team including endocrinologist, gastroenterologist and orthopedic surgeon. The diabetes is insulin dependent and the management of skeletal dysplasis is generally conservative. It is important to ensure that families of affected children recognize early symptoms of liver dysfunction and that patients with WRS have a clear plan prepared to avoid delay in starting the management of acute hepatic failure. Liver transplantation was successfully used to save the life of one child and three other children had multiple organs (liver, kidney and pancreas) transplantation and earlier results are encouraging (Elsabbagh *et al.*, 2017; Habeb *et al.*, 2015; Rivera *et al.*, 2016). The clinical importance of conducting the genetic testing for this condition is vividly illustrated in helping a couple carriers of WRS with affected children to have a healthy child using the PGD technology (Deeb *et al.*, 2016a).

Neonatal Diabetes Due to *GCK* Mutations

Biallelic mutations in the gene encoding the glucokinase (GCK) enzyme are responsible for less than 2%–3% of cases of PNDM (De Franco *et al.*, 2015). The GCK enzyme is considered as the glucose sensor of the β-cells because it allows the β-cell to secrete the proper amount of insulin in response to the degree of the blood glucose. It does this function by catalyzing the rate-limiting step of glucose phosphorylation. Mutations in both alleles of the *GCK* gene (homozygous or compound heterozygous) results in complete glucokinase deficiency. The GCK related NDM is caused by the inability of the β-cells to secret insulin in response to hyperglycemia. As a result of the severe insulin deficiency, the diabetes often presents within the first few days of life and infants have severe IUGR. Apart from diabetes, patients do not show any extrapancreatic features (Njolstad *et al.*, 2003; Njølstad *et al.*, 2001; Rubio-Cabezas and Ellard, 2013). It is worth remembering that heterozygous mutations in the *GCK* gene result in familial mild nonprogressive hyperglycemia, also called GCK MODY. Therefore, NDM due to GCK mutations should be considered in infants born to parents with asymptomatic mild hyperglycemia. For this reason, it is recommended to measure fasting blood glucose in the parents of any child with NDM, even in the absence of consanguinity or family history of diabetes. This type of PNDM is inherited in a recessive manner so the recurrence risk for future siblings is 25%.

Neonatal Diabetes With β-Cell Immunity

Unlike older children, diabetes in infants presenting in the first 6 months of life is very unlikely to be due to autoimmune etiology. Those infants who test positive for β-cell autoantibodies are likely to have gene mutations triggering the autoimmune process. Several novel syndromes with autoimmune diabetes due to monogenic immunodysregulation have been identified. IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) is caused by *FOXP3* mutation which is an essential transcription factor for the development of regulatory T cells and has a role in their ongoing suppressive action (Verbsky and Chatila, 2013). Pathogenic variants of STAT3, STAT1, LRBA, IL2RA, CTLA4 have also been implicated in triggering autoimmune-based diabetes and other systemic illnesses and reported to cause diabetes (Flanagan *et al.*, 2014; Goudy *et al.*, 2013; Johnson *et al.*, 2017; McDonald *et al.*, 2017). All these genes affect the tolerance mechanism in the body. Their mutation impairs the balance

between effective immune response and autoimmunity. Autoimmune diabetes triggered by monogenicity can be of early onset within the 6 months of life and thus fulfilling the criteria of NDM.

Future Development

With the rapid advances of molecular genetic techniques and wide application of the WES and whole genome sequencing, it is expected that more genetic causes of neonatal diabetes will be discovered. This would help in identifying the best therapeutic approach for neonatal diabetes. Recent study showed that it will be feasible to screen for neonatal diabetes using dried blood spot glucose measurement (McDonald *et al.*, 2017). This will help detecting infants with NDM early before they decompensate into DKA. Recent attempts and of liver, pancreas and kidney transplantation for infants with WRS are promising and it is expected that such therapy would be more used to save lives of these patients.

Conclusions

NDM is rare form of diabetes and most cases are caused by genetic defects in genes that control beta cell function or pancreatic mass leading to a state of insulin deficiency. It is important that all patients with diabetes diagnosed in the first 6 months of life are thoroughly assessed to identify associated features and have genetic testing. The latter is important as it can select the best treatment for the child and provide the family with proper genetic counseling.

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Relevant Websites

www.diabetesgenes.org - Diabetes Genes.
www.ispad.org - International Society for Pediatric and Adolescent Diabetes (ISPAD).

Congenital Hyperinsulinism

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Glossary

Congenital hyperinsulinism A complex disorder of hypoglycemia due to excess and dysregulated insulin secretion. Usual presentation is in the neonatal period, although later presentations can also occur. Although termed congenital hyperinsulinism, not all forms have defined genetic basis.

Diffuse congenital hyperinsulinism Congenital hyperinsulinism affecting the whole of the pancreas, in contrast to focal congenital hyperinsulinism where an isolated part of the pancreas is affected. Medical therapy is a first line option; in severe cases and in those unresponsive to medication subtotal pancreatectomy may be required.

Focal congenital hyperinsulinism A solitary hyperfunctioning endocrine rich lesion in the pancreas usually caused by a combination of inheritance of a paternal heterozygous mutation in *ABCC8/KCNJ11* and loss of maternal heterozygosity. If focal lesions are resected completely, cure from hyperinsulinism and hypoglycemia can be achieved.

Hyperinsulinism A state of excess insulin production leading to hypoglycemia. Hyperinsulinism represents functionally active insulin, in contrast to hyperinsulinaemia which implies insulin resistance.

Hypoglycemia A state of low glucose levels. Normal glucose levels range between 4 and 6 mmol/L. By consensus, hypoglycemia is diagnosed when blood glucose levels are less than 3.0 mmol/L.

K-ATP channel gene mutations A significant proportion of children with congenital hyperinsulinism have genetic mutations in components of the potassium channel which regulates insulin secretion in response to glucose levels in the pancreatic beta cell. This channel is responsive to cellular energy levels (ATP) and is therefore termed the K-ATP channel. *ABCC8* and *KCNJ11* coding for the proteins SUR1 and Kir6.2 in the channel can undergo mutations to cause K-ATP channel dysfunction and therefore dysregulated and excess insulin secretion.

PET-CT scanning This refers to positron emission tomography (PET) using the radiochemical 18-fluoro-dopa coupled with CT scanning for anatomical localization. PET-CT scanning is currently the best available investigation to identify and localize focal lesions.

Subtotal pancreatectomy Pancreatic surgery that involves resection of the majority of the pancreas, leaving a rim of tissue between the bile duct and the C-curve of the duodenum.

Introduction

Congenital hyperinsulinism (CHI) is a condition of severe and recurrent hypoglycemia in childhood characterized by excessive and dysregulated insulin secretion. Insulin released from pancreatic beta cells is normally tightly coupled to circulating serum glucose concentrations (**Fig. 1**) primarily mediated by the ATP-sensitive K^+ (K-ATP) channel (Dunne *et al.*, 2004; Ashcroft and Rorsman, 2013). In the normal state, mealtime glucose surges lead to proportionate and predictable insulin secretion while lower levels of glucose switch off insulin production to achieve euglycaemia. In CHI, feedback regulation is disordered leading to excess and inappropriate amounts of insulin causing severe hypoglycemia. In early childhood, the immature neonatal brain is susceptible to hypoglycemia-induced injury. In CHI, excess insulin suppresses counter-regulatory hormone responses and the production of ketones, which serve as alternative fuel for brain neurons in the absence of glucose. Therefore the combination of low glucose and low ketones renders the CHI brain particularly vulnerable to neuronal damage and prone to the risk of lifelong serious neurodisability. It is not surprising that a third to a half of cohorts with CHI have some form of adverse neurodevelopment, with severe cases incurring epilepsy and visual cortical blindness (Menni *et al.*, 2001; Meissner *et al.*, 2003; Avatapalle *et al.*, 2013).

CHI is a rare cause of hypoglycemia, reported as occurring in 1:50,000 in the general population (Dunne *et al.*, 2004; Stanley, 2016). The incidence of CHI probably represents the severe end of the spectrum, including those with persistent and severe CHI and those requiring pancreatectomy. The incidence does not take into account transient forms such as hyperinsulinism in babies born small for gestational age and perinatal stress, and late presenting forms, occurring in 10% of patients. In consanguineous populations, the incidence is considerably higher at 1:2500. Over the past few years, several CHI cohorts have been reported from around the world; however, no attempt has been made to investigate population incidence and prevalence of the disease.

Diagnosis

The diagnosis of hyperinsulinism rests on the detection of insulin at the time of hypoglycemia. However, insulin, which has a short half-life (4–6 min), may not be detectable at all times due to sampling delay and inaccurate measurement. Conversely, modern highly sensitive insulin assays may detect minimal quantities of insulin, particularly in the first 1–2 days of life when the glucose-insulin coupling

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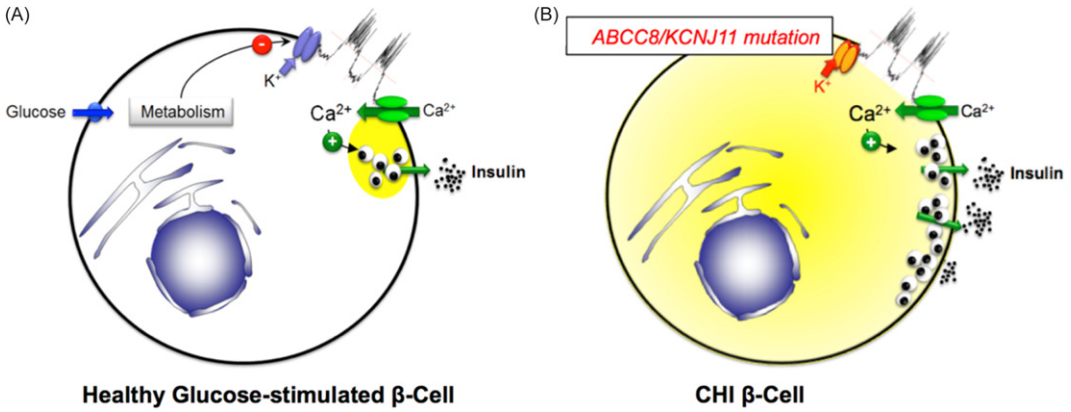

Fig. 1 Stimulus-secretion coupling in β-cells. (A) A simplified model of ionic events in islet β-cells that account for glucose-induced insulin secretion. Glucose uptake and metabolism result in closure of ATP-sensitive K^+-channels leading to a depolarization of the membrane and Ca^{++}-entry. Increases in the intracellular Ca^{++} concentration trigger insulin release. In the CHI β-cell, panel (B), loss of ATP-sensitive K^+-channel function due to gene defects leads to inappropriate electrical activity, uncontrolled Ca^{++} influx, and insulin release.

mechanism is immature (Thornton *et al.*, 2015). The presence of a high glucose infusion rate (GIR >8 mg/kg/min) is a strong indicator of insulin-mediated hypoglycemia as is the absence of ketogenesis. However, suppression of ketogenesis may be incomplete in some forms of CHI, for example those due to glucokinase (*GCK*) mutations (Sayed *et al.*, 2009). In older children who consume a combination of solid and liquid foods, estimating GIR is not fully reliable. Some treatment centers use a glucagon test as a diagnostic marker; a 1.0 mg intramuscular/intravenous dose of glucagon increases blood glucose levels by 1.7 mmol/L (30 mg/dL) within 40 min (Palladino and Stanley, 2011; Finegold *et al.*, 1980) to indicate a strong likelihood of CHI.

A caveat in the diagnosis of CHI lies in the very definition of hypoglycemia, which remains contentious. There are several reasons to consider hypoglycemia at blood glucose levels below 3 mmol/L (54 mg/dL), with evidence mainly from animal experiments of neuroglycopaenia (Cryer, 2007). Hypoglycemia investigations can be performed at variable blood glucose levels, depending on local guidelines, thereby introducing diagnostic bias. It is worth ensuring the diagnosis of CHI is supported by more than one criterion and that other more common causes of hypoglycemia, such as neonatal sepsis have been excluded.

Classification

CHI is a heterogeneous disease eluding a simple and consistent classification. It is helpful to understand the disease in distinctive patterns and groups, some clinical in nature and some based on genetic, imaging, and tissue investigations.

Focal and Diffuse CHI

Traditionally CHI is considered as focal or diffuse, based on histopathological phenotypes of pancreatic tissue. Focal CHI occurs if a solitary part of the pancreas shows evidence of islet cell enrichment in contrast to neighboring tissue with mostly exocrine components. On the other hand, diffuse CHI is diagnosed if most of the pancreas is affected by "hyperfunctioning" and poorly formed islet structures associated with ductal epithelia—nesidioblastosis—scattered diffusely throughout the organ (Rahier *et al.*, 1984). The diagnostic value of nucleomegaly is important. Islet cells with nuclear enlargement, four-times larger than adjacent islet endocrine cells and five-times larger than nuclei in exocrine cells, is a natural feature of the neonatal pancreas. However, the presence of several cells with enlarged nuclei in islets is almost pathognomonic of diffuse CHI (Han *et al.*, 2016). In our experience the detection of more than two enlarged nuclei in as few as 10% of islets is diagnostic of diffuse CHI.

It is important to pursue the diagnosis of focal CHI as treatment with partial resection of pancreatic tissue could be curative and spare long-term medication, side effects and prolonged hospitalization. In contrast, the first line treatment of diffuse CHI is medical therapy. Surgery to remove the majority of the pancreas (subtotal pancreatectomy) is reserved for those who do not respond to or have side effects with medical therapy.

The ability to identify focal CHI has been a real step change in the clinical management of CHI. Focal CHI diagnosis is based on the initial finding of a paternal heterozygous mutation in the genes encoding the K-ATP channel, *ABCC8*, and *KCNJ11*, assuming recessive inheritance. In association with paternal inheritance of *ABCC8/KCNJ11* mutations, loss of maternal heterozygosity (LOH) will lead to silencing of the compensatory allele and clonal expansion due to inhibition of cell cycle repressors located in the vicinity (**Fig. 2**). However, pancreatic LOH cannot be diagnosed without pancreatic surgery and biopsy. Therefore an alternative investigation is required to diagnose Focal CHI.

Focal lesions are associated with higher concentrations of beta cells and pancreatic neurons converting dopa to dopamine. This observation is utilized by the 18-fluoro-dopa PET-CT (PET-CT) scan, which is the investigation of choice to identify and localize a focal lesion (**Fig. 3**). Dopa tagged to the radiochemical 18-fluorine is taken up avidly and retained by the focal lesion. In focal CHI,

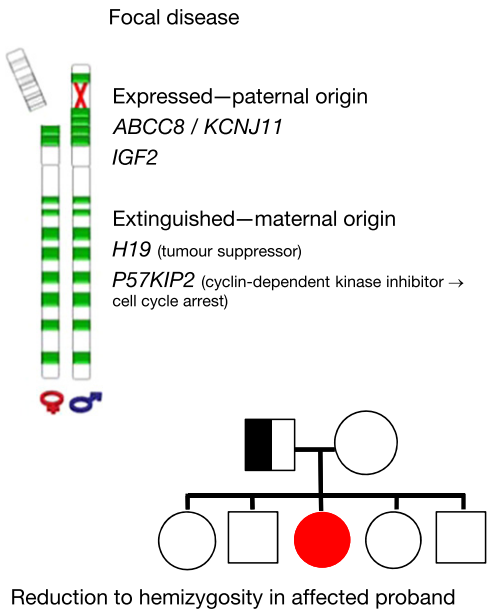

Fig. 2 Pathogenesis of focal CHI. Inheritance of a *ABCC8/KCNJ11* mutation on the paternal allele and loss of heterozygosity on the maternal allele (containing cell cycle suppressors) leads to unopposed mutant gene expression and hyperplasia leading to expansion of islet-rich pancreatic domains, resulting in a discrete focal lesion within the pancreas.

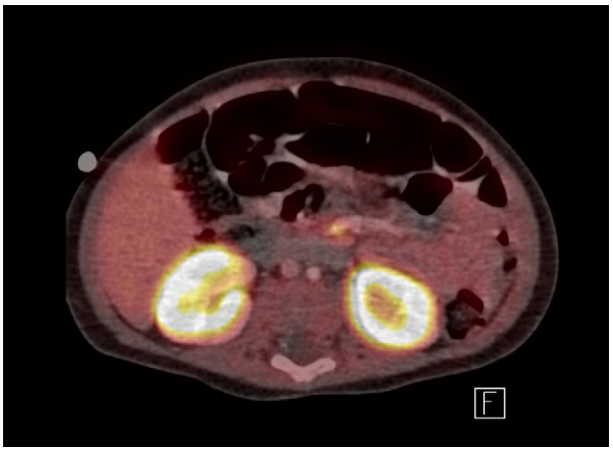

Fig. 3 Focal CHI identified by 18-fluoro-dopa PET-CT scanning; lesion takes up and retains 18-fluoro-dopa in comparison to the rest of the normal pancreas.

the lesion is imaged as a region of altered "brightness" in contrast to the rest of the pancreas following 40–60 min of injection of 18-fluoro-dopa. In diffuse CHI, the pancreas takes up 18-fluoro-dopa uniformly but does not retain radioactivity in a single region.

The diagnosis of focal CHI may be complicated by the fact that some mutations in *ABCC8/KCNJ11* are dominant, not recessive, giving a diffuse phenotype. PET-CT scanning itself may give false positives and negatives with earlier success (Hardy *et al.*, 2007) not being replicated in later studies (Arya *et al.*, 2014). While focal lesions have characteristic histology with isolated islet cell rich domains (**Fig. 4**), heterogeneity in the description is recognized with some focal lesions having looser organization than others. Focal lesions are identified at frozen section biopsy at the time of pancreatic surgery. The lesser quality of frozen section staining may contribute to diagnostic difficulty than when using conventional section and insulin immunostaining. Although focal lesions can be identified on 2-dimensional scans, finding a small lesion buried within the pancreatic tissue can be a challenge (**Fig. 5**); in such cases, a number of frozen section biopsies may be required to localize the lesion. In rare cases, larger focal CHI involving almost the entire pancreas may masquerade as unusual diffuse CHI as margins of normal tissue are difficult to identify. Margin identification is also difficult in lesions with tentacle-like extensions into normal tissue. Therefore, despite rapid advances in genetic and imaging investigations, the search for a focal lesion may prove elusive. A complicating factor is the entity of atypical CHI (Han *et al.*, 2017). Atypical CHI is characterized by islands of tissue with hyper functioning phenotype, with adjoining

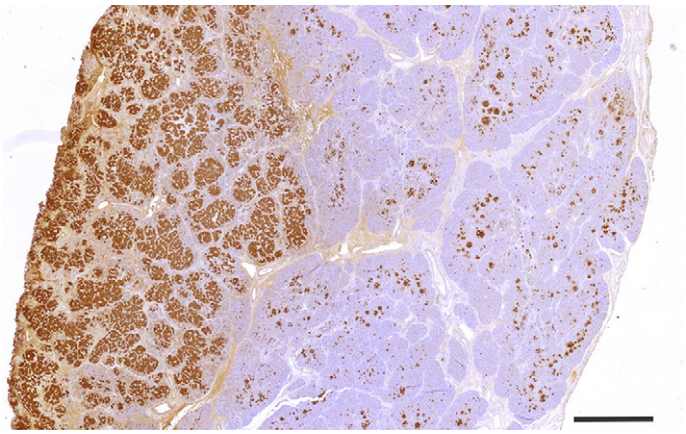

Fig. 4 Histopathological characteristics of Focal CHI show endocrine rich tissue stained brown, with adjoining normal tissue containing mainly exocrine tissue with scattered endocrine components (normal islets). *Scale bar*, 0.1 mm.

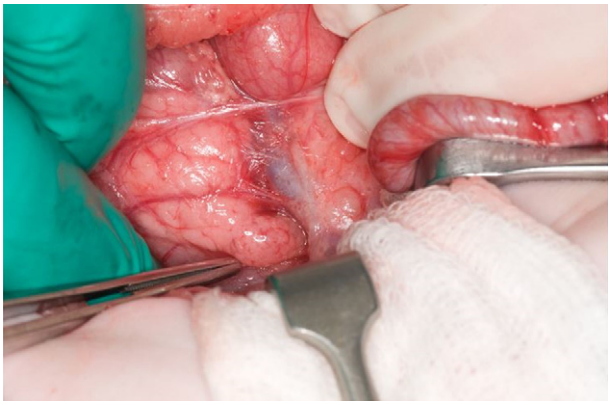

Fig. 5 Focal lesions may not be readily visible at the time of lesionectomy. In this laparotomy procedure, the focal lesion was identified within the uncinate process, to the right of the artery forceps and just above the left sided retractor. This surgical image is from the same patient as in the 18-fluoro-dopa PET-CT scan in **Fig. 3**.

endocrine and exocrine tissue appearing normal. However, there is no clear consensus about the nature and etiology of atypical CHI, which may account for 10%–15% of all cases.

The diagnosis of focal CHI relies heavily on genetic mutation testing, followed by 18-fluoro-dopa PET-CT scanning. Whilst genetic testing is increasingly available worldwide through international research collaborations, not all centers have the infrastructure or the expertise to interpret imaging results. 18-fluoro-dopa is a technically challenging radiochemical to produce and has a short half-life of 110 min. To inject a radioactive dose of 4 Mbq/kg, sufficient to inject in a relatively large child, a much larger yield has to be generated at the cyclotron site, possibly in excess of 300 Mbq, to allow for quality control experiments, transport to the PET-CT site and radioactive decay. Another factor to consider with 18-fluoro-dopa PET-CT scanning is the amount of ionizing radiation to which young children are exposed. Compared to the background UK average radiation of 2.2 mSv per year and a single chest X-ray of 0.02 mSv, 18-fluoro-dopa PET-CT scans provide 12–15 mSv at a very young age. New targets for the development of more selective ligands with fewer side effects include islet somatostatin receptors and glucagon-like peptide-1 receptors, although these remain experimental (Pattison and Hicks, 2017).

Transient and Persistent CHI

It is well recognized that small for gestational age babies and those who suffer perinatal stress may have varying degrees of hyperinsulinism causing hypoglycemia. The cause is not genetic and may be related to cellular stress responses inducing glucose-insulin dysregulation in postnatal life. In those without known genetic mutations, more than 50% resolve over a variable time range, extending to 8 years in some cases (Banerjee *et al.*, 2011). It is interesting to know that disease due to both dominant and recessively inherited mutations in *ABCC8/KCNJ11* may also reduce in severity and resolve, adding to the notion of natural history modification of disease. It is not known why genetic disease, including histologically proven focal CHI may resolve over time.

The differentiation of transient and persistent forms of disease is important as patients and families often enquire about long-term prognosis. However, transient CHI does not imply less severe disease, as the prevalence of severe adverse neurodevelopment is similar between transient and persistent CHI (Avatapalle *et al.*, 2013). Importantly there are no biomarkers to predict resolution or length of treatment at the time of diagnosis, although it is generally accepted that diffuse CHI due to recessively inherited *ABCC8/KCNJ11* homozygous mutations is unlikely to show complete disease resolution.

The resolution of diffuse CHI due to dominantly acting *ABCC8/KCNJ11* mutations may be reassuring to families in the short term but raises the possibility of later life maturity onset diabetes (MODY) due to an unexplained switch from hyper to hypoinsulinism. Such families need counseling regarding symptoms of diabetes not only for the child but also for the parent with the mutation.

Genetic and Unknown Causes of CHI

The most common genetic mutations causing CHI occur in the K-ATP channel genes *ABCC8* and *KCNJ11*. Other rarer genetic mechanisms have been identified and are listed in **Table 1**. Glutamate dehydrogenase deficiency *(GLUD1* mutation) has a phenotype of protein sensitivity, increased frequency of seizures and variable age at presentation. Mutations in the hepatocyte nuclear factor 4 alpha (*HNF4A)* can cause early life hypoglycemia and a switch to hyperglycemia in later life. Mutations in the enzyme hydroxyacyl Coenzyme A dehydrogenase (*HADH*) mutations are relatively common in consanguineous populations after *ABCC8/KCNJ11* mutations have been excluded.

A significant proportion of CHI patients do not have a known genetic cause of disease (**Table 2**) (Banerjee *et al.*, 2011; Jahnavi *et al.*, 2014; Shah *et al.*, 2007; Del Roio Liberatore *et al.*, 2015; Fan *et al.*, 2015; Sang *et al.*, 2014; Gong *et al.*, 2016; Rozenkova *et al.*, 2015; Bellanne-Chantelot *et al.*, 2010; Mohnike *et al.*, 2014; Senniappan *et al.*, 2015; Faletra *et al.*, 2013; Sogno Valin *et al.*, 2013; Yorifuji *et al.*, 2011; Park *et al.*, 2011; Sandal *et al.*, 2009; Al-Agha and Ahmad, 2013; Fernandez-Marmiesse *et al.*, 2006; Martinez *et al.*, 2016; Siklar and Berberoglu, 2016; Kapoor *et al.*, 2013; Snider *et al.*, 2013). Those with persistent severe disease probably have an unrecognized monogenic mutation which may eventually be revealed with increasing genetic research. It is possible that dysfunctional genetic networks may play a part in etiology, although there is no direct evidence for this. In those with resolving CHI with no identified gene mutations, mechanisms for causation and resolution remain largely unexplained.

Syndromes and Associations

A number of syndromes are associated with hyperinsulinism and hypoglycemia (**Table 3**) (Stanley, 2016; Arnoux *et al.*, 2012), however, the cause for hyperinsulinism remains unknown in most. Some children with the overgrowth condition of Beckwith–Wiedemann syndrome (BWS) have hypermethylation in chromosome 11; the cause for hyperinsulinism is not clear. Most respond to diazoxide treatment and achieve remission within a few months. In the majority of syndromes and genetic conditions associated with CHI, hypoglycemia appears to respond to conventional treatment and shows a resolving natural history. However, hypoglycemia may be missed, particularly if mild or occurring infrequently. The search for hypoglycemia may complicate the clinical management of complex conditions such as congenital central hypoventilation due to *PHOX2B* mutations. However it is important to recognize and treat hypoglycemia in these syndromes to prevent additional hypoglycemia-related morbidity and neurodisability.

Genetic Etiology

12 genetic mutations causing CHI have been identified over the years since the first description of sulfonylurea receptor mutation encoded by *ABCC8* (**Table 1**). Examination of international cohorts (**Table 2**) suggest most mutations occur in *ABCC8* and

Table 1 Genetic associations of CHI

Class	*Gene*	*Protein*	*Inheritance*	*Histology*
Ion channels	*ABCC8*	SUR1, subunit of K-ATP channel	Recessive or dominant	Focal or diffuse
	KCNJ11	Kir6.2, subunit of K-ATP channel	Recessive or dominant	Focal or diffuse
	CACNA1D	L-type calcium channel α-subunit	De novo	Diffuse
Enzymes	*GLUD1*	Glutamate dehydrogenase	Dominant	Diffuse
	GCK	Glucokinase	Dominant	Diffuse
	HADH	Short chain L-3-hydroxyacyl-CoA dehydrogenase	Recessive	Diffuse
	PGM1	Phosphoglucomutase 1	Recessive	Diffuse
	HK1	Hexokinase 1	Dominant	Diffuse
Transporters	*SLC16A1*	MCT1, pyruvate transporter	Dominant	Diffuse
	UCP2	Mitochondrial uncoupling protein 2	Dominant	Diffuse
Transcription factors	*HNF1A*	Hepatocyte nuclear factor 1α	Dominant	Diffuse
	HNF4A	Hepatocyte nuclear factor 4α	Dominant	Diffuse

Twelve genes currently known to associate with the pathology of CHI have been classified into functional groups.

Table 2 The reporting of known genetic cause of CHI from different international centers

Origin	*Number of patients*	*Genetic cause of congenital hyperinsulinism*				*References*
		Known		*Unknown*		
		n	%	n	%	
Australia	43	17	40	26	60	Ashcroft and Rorsman (2013)
Brazil	53	33	62	20	38	Menni *et al.* (2001)
China	32	21	66	11	34	Meissner *et al.* (2003)
China	30	14	47	16	53	Avatapalle *et al.* (2013)
China	55	21	38	34	62	Stanley (2016)
Czech Republic	40	20	50	20	50	Thornton *et al.* (2015)
France	109	89	82	20	18	Sayed *et al.* (2009)
Germany	136	61	45	75	55	Palladino and Stanley (2011)
India	22	10	45	12	55	Dunne *et al.* (2004)
Iran	23	18	78	5	22	Finegold *et al.* (1980)
Italy	36	20	56	16	44	Cryer (2007)
Italy	33	15	45	18	55	Rahier *et al.* (1984)
Japan	36	24	67	12	33	Han *et al.* (2016)
Korea	17	14	82	3	18	Hardy *et al.* (2007)
Norway	26	15	58	11	42	Arya *et al.* (2014)
Saudi Arabia	11	9	82	2	18	Han *et al.* (2017)
Spain	34	25	74	11	16	Pattison and Hicks (2017)
Spain	50	28	56	22	44	Banerjee *et al.* (2011)
Turkey	115	56	49	59	51	Jahnavi *et al.* (2014)
UK	300	136	45	164	55	Shah *et al.* (2007)
UK	101	35	35	66	65	Del Roio Liberatore *et al.* (2015)
USA	416	328	79	88	21	Fan *et al.* (2015)

References: (Banerjee *et al.*, 2011; Jahnavi *et al.*, 2014; Shah *et al.*, 2007; Del Roio Liberatore *et al.*, 2015; Fan *et al.*, 2015; Sang *et al.*, 2014; Gong *et al.*, 2016; Rozenkova *et al.*, 2015; Bellanne-Chantelot *et al.*, 2010; Mohnike *et al.*, 2014; Senniappan *et al.*, 2015; Faletra *et al.*, 2013; Sogno Valin *et al.*, 2013; Yorifuji *et al.*, 2011; Park *et al.*, 2011; Sandal *et al.*, 2009; Al-Agha and Ahmad, 2013; Fernandez-Marmiesse *et al.*, 2006; Martinez *et al.*, 2016; Siklar and Berberoglu, 2016; Kapoor *et al.*, 2013; Snider *et al.*, 2013).

Table 3 Developmental genetic syndromes associated with hyperinsulinism

Syndrome	*Gene*	*Locus*	*Inheritance*	*Clinical features*
Beckwith–Wiedemann	IGF2/H19/ CDKN1C/KCNQ1	11p15.5	S	Pre and postnatal overgrowth, organomegaly, hemihypertrophy, risk of embryonal tumors
Kabuki	KMT2D/ KDM6A	12q13.12/ Xp11.3	S	Distinctive facial appearance, short stature, skeletal abnormalities, developmental delay, heart defects
CDG (1a/1b)	PMM2/MPI	16p13.2/ 15q24.1	AR	Neurological impairment, gastrointestinal problems, cardiomyopathy, seizures, short stature
Sotos	NSD1	5q35.2–q35.3	S	Pre and postnatal overgrowth, dolichocephaly, distinctive facial features, developmental delay
Timothy	CACNA1C	12p13.33	AD or S	Long QT, arrhythmias, heart defects, syndactyly, immune deficiency, autistic spectrum disorder
Costello	HRAS	11p15.5	AD or S	Short stature, developmental delay, coarse facial features, papillomata, joint laxity, cardiac defects, tumor risk
Ondine	PHOX2B	4p13	AD or S	Congenital Central Hypoventilation syndrome, Hirschsprung's disease, risk of nervous system tumors
Turner	?	X	S	Short stature, ovarian failure, lymphedema, skeletal abnormalities, kidney problems, heart defects
Usher Ic	–	11p15–p14	AR	Severe diazoxide unresponsive hyperinsulinism, sensorineural deafness, pigmentary rethinopathy

S: Sporadic. References (Stanley, 2016; Arnoux *et al.*, 2012).

KCNJ11, comprising of the so-called channelopathies, followed by others occurring with variable frequency. CHI-associated mutations in *ABCC8* or *KCNJ11* cause loss of function of K-ATP channels located in the pancreatic β-cell membrane. K-ATP channel activity provides the essential link between increasing blood glucose levels and the subsequent secretion of insulin via regulation of the β-cell's membrane potential. Under normal conditions, metabolism of fuels such as glucose leads to the production of ATP within β-cells, alters the ratio of ATP:ADP concentration and causes closure of K-ATP channels (**Fig. 1**). This leads to depolarization of the β-cell membrane and activation of voltage-dependent Ca^{2+} channels. Opening of these Ca^{2+}

channels permits entry of Ca^{2+} into the β-cell and triggers the Ca^{2+}-dependent exocytosis of insulin-containing secretory vesicles. When K-ATP channel activity is suppressed or absent due to *ABCC8/KCNJ11* mutations, the β-cell membrane becomes inappropriately depolarized, Ca^{2+} entry occurs and triggers insulin secretion irrespective of blood glucose levels. Thus, in patients with *ABCC8/KCNJ11* mutations, the critical regulatory link between blood glucose concentration and insulin secretion is lost, often causing severe CHI that is unresponsive to medical therapy.

It is recommended that testing for *ABCC8/KCNJ11* mutations is performed in children with severe CHI, primarily to identify possible focal CHI. Rapid *ABCC8/KCNJ11* gene testing by Sanger sequencing is an important investigation tool guiding clinical management (De Leon and Stanley, 2007; Christesen *et al.*, 2007). However, in children responsive to conventional medication in moderate dosage, genetic analysis could be delayed. In centers with expertise in next generation sequencing of whole genomes, panel gene testing incorporating known genetic causes may be a preferred and relatively inexpensive option, although timescales to turnover are necessarily longer.

CHI mutations outside the K-ATP channel genes may cause metabolopathies where islet metabolism is affected, causing an increase in intracellular ATP:ADP ratio, closure of K-ATP channels and insulin secretion. *GLUD1* mutations are a typical example; *GLUD1* encodes glutamate dehydrogenase which is responsible for the oxidative deamination of glutamate to alpha-ketoglutarate. Mutations in the GTP-binding allosteric regulatory site of *GLUD*-1 cause the enzyme to produce alpha-ketoglutarate inappropriately. Since alpha-ketoglutarate is a substrate for the Kreb's cycle, this leads to ATP production, K-ATP channel closure and eventually to insulin release. Mutations in glucokinase (*GCK*) increase rates of glycolysis, similarly leading to an increase in ATP production and secretion of disproportionate quantities of insulin. Mutations in *HADH*, encoding the lipid enzyme hydroxyacyl Coenzyme A dehydrogenase are thought to disrupt the regulation of glutamate dehydrogenase and increase its activity inappropriately; this causes an increase in intracellular ATP generating an inappropriate increase in insulin secretion. CHI patients with metabolopathies usually respond well to diazoxide, a drug that opens K-ATP channels. Other gene mutations, for instance in *HNF4A*, are not directly linked to metabolic perturbations. Mutations in *HNF1A* and *UCP2* seem to be restricted to specific populations and may demonstrate a founder effect. It is not known if the other genetic mutations listed in **Table 1** occurring less frequently directly cause or simply associate with CHI.

Candidate gene discovery has led to improved knowledge of genetic etiology of CHI and has revealed new genetic causes of the disease. Deep sequencing and whole genomic sequencing techniques have enhanced the search for known mutations now being identified in deep intronic areas previously missed by conventional Sanger sequencing of exons and exon-intron boundaries. However, candidate gene discovery in cohorts of patients with persistent disease is yet to identify major new genes that enhance the understanding of disordered insulin secretion.

An additional tool to assist gene discovery is the investigation of network biology and the CHI interactome (Stevens et al., 2013, 2014). Although this remains largely experimental at this stage, network biology for CHI has been found to independently predict relationships between islet cell proliferation, enhanced vascularization and the association of novel drug targets—for example with sirolimus (Szymanowski *et al.*, 2016). Therefore analysis of pancreatic gene expression data is worthwhile not simply to establish causation but also to suggest new drug candidates for the treatment of CHI.

Treatment

General Principles

It is important to recognize and treat severe hypoglycemia promptly to prevent brain injury. Neonatal hypoglycemia is a common occurrence with causes ranging from substrate deficiency to sepsis. CHI is a relatively rare cause of hypoglycemia in the postnatal period, but the diagnosis must be considered if hypoglycemia is severe, persistent and associated with high GIR. In severe neonatal hypoglycemia, oral feeds are unlikely to correct hypoglycemia adequately. In most cases, intravenous access is required to infuse 10% dextrose solutions. Standard rates (90–120 mL/kg/day) of 10% dextrose in the first few days of life provide 6–8 mg/kg/min which may not be enough to keep up with high GIR requirements in CHI. A common tendency is to increase the volume of fluids to increase dextrose delivery; however, this strategy is more likely to cause fluid overload and potentially right sided heart failure.

If possible, a peripherally sited central venous catheter or an umbilical catheter should be considered to infuse higher concentrations of dextrose, between 15% and 20% in solution. In an emergency, where central access is not achievable and hypoglycemia is persistent, intraosseous access may be necessary. However, neonatal bones are fragile and may fracture with potential for extravasation of high concentration dextrose in soft tissue. Therefore a surgically or radiologically sited central intravenous access should be considered soon after diagnosis.

Additional carbohydrates could be delivered by enteral feeding if hypoglycemia is manageable. In young children, neutral tasting carbohydrate powder supplements (e.g., Vitajoule™, Polycal®) could be added to milk feeds. In older children, slow-release starch could also prevent hypoglycemia. Some children are dependent on enriched feeds and require continuous feeds during the day or night or both to maintain glycemic stability.

A key feature of the management plan of CHI is monitoring in hospital and at home. Conventional laboratory methods of measuring blood glucose are not feasible for frequent monitoring and heelprick/fingerprick blood glucose measurements using handheld glucometers may be used. However, these methods are not completely reliable, particularly in the lower range of glucose levels. A suitable compromise may be the use of point of care testing devices which offer greater reliability and are simple to use in a hospital setting. Frequent (1–2 hourly in acute situations) point of care testing glucose levels could be used to guide clinical

management. At home, most parents use handheld glucose meters to test premeal blood glucose levels. The frequency of testing is usually lesser than in the hospital and rarely more than four times a day. One alternative to blood glucose levels is the use of subcutaneous continuous glucose monitoring (CGM). Most CGM devices require additional calibration with blood glucose levels, although factory calibrated devices are also available. CGM is rarely used in acute hypoglycemia, where subcutaneous glucose has a 5-min lag phase and is therefore less reliable than blood glucose testing. Although there are no validation studies of CGM in CHI patients, CGM could be used to determine trends, particularly overnight in the stable phase of CHI patients at home.

Glucagon

To improve hypoglycemia, one choice is to commence glucagon infusion in a dose of 5–10 μg/kg/h through a peripheral intravenous cannula if central access is not forthcoming. Patients with CHI are sensitive to glucagon, which reduces glucose requirement providing much needed stability. Some centers administer a standard 1000 microgram dose of glucagon diluted in small volume fluid run over a 24-h period, regardless of body weight. For a child weighing 4 kg, this equates to a dose just over 10 μg/kg/h. This method has the advantage of simplicity and may minimize drug error through miscalculation. Glucagon effectively allows reduction of the volume of dextrose solution infusion, thereby reducing fluid overload and electrolyte imbalance. Glucagon can be administered alongside clear solutions in central venous catheters but coadministration of parenteral nutrition is best avoided. Glucagon can be administered subcutaneously as an infusion, although the formulation is designed for bolus intramuscular injections. In slow moving small caliber catheters, currently available preparations of glucagon fibrillate and precipitate, thereby rendering drug delivery unreliable. Hence, although generally very effective for short-term glycemic stabilization, long-term glucagon therapy has been unrealistic. However, recent advances in both solubility and stability of natural glucagon and the development of modified glucagon, has raised the possibility of using glucagon over longer periods of time.

In some children with severe CHI, intravenous glucagon remains the only medical treatment option as other drugs fail to have desired activity or are poorly tolerated. Long-term use of high dosage glucagon will impact upon hepatic glucose storage and can be associated with the risk of thrombosis. A rare occurrence is a migratory rash called necrolytic migratory erythema, for which glucagon has to be stopped (Fig. 6).

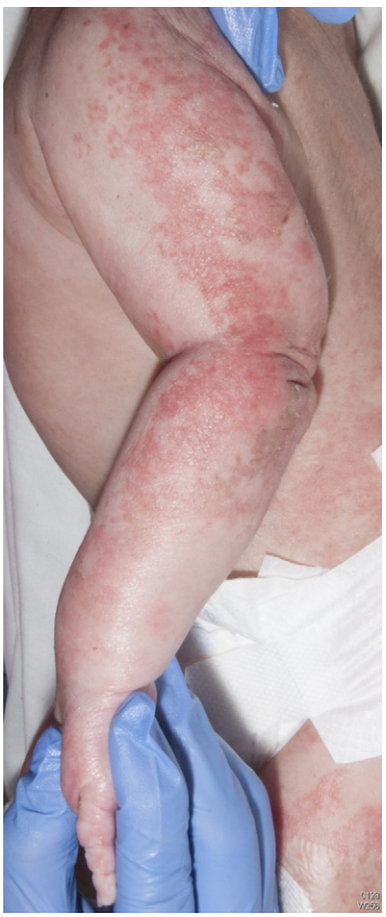

Fig. 6 Necrolytic migratory erythema following long duration treatment with glucagon.

Diazoxide

Diazoxide is an antihypertensive which also acts to keep the K-ATP channel in the "open" state, permitting beta cell membrane stability and reducing excursions in insulin secretion. Diazoxide is the only medicinal product licensed by the Food and Drug Administration in the United States for the treatment of CHI. The dose of diazoxide varies between 5 and 15 mg/kg/day in three divided doses with usual starting doses in the lower part of the range. Doses are usually titrated upwards every 2–3 days depending on response. In some centers, the reverse approach, that is, a higher dose of diazoxide (15 mg/kg/day) may be used to investigate drug responsiveness. However, large dose diazoxide is more likely to be complicated by side effects than escalating lower dose treatment.

One of the commonest side effects of diazoxide in the early stages of treatment is fluid retention. It is important to ensure fluid administration is not excessive and restricted to less than 150 mL/kg/day. Chlorothiazide, which has some synergistic action, is useful as an adjunct diuretic in a dose of 7–15 mg/kg/day in two divided doses. Chlorothiazide can cause loss of electrolytes with consequent hyponatraemia and hypokalemia. If diazoxide is continued over a longer period of time, fluid retention features become less prevalent. Therefore, chlorothiazide is rarely required for the longer-term treatment with diazoxide. Patients on diazoxide should be carefully monitored for efficacy and side effects. In the maintenance phase, it is advisable to use the lowest dose that ensures the majority of blood glucose levels remain in the normal range, that is, 4–6 mmol/L minimizing readings lower than 3.5 mmol/L. Diazoxide causes taste disturbances, although most neonates appear to tolerate medication satisfactorily. Occasionally side effects such as reduced white cell and platelet counts can occur. Rarely diazoxide can cause hyperglycemia, associated with ketogenesis, mimicking the clinical scenario of diabetes. Diazoxide is also known to cause pulmonary hypertension, more common with fluid overload, existing cardiac anatomic defects, and prematurity. Another life-threatening complication of diazoxide is pericardial effusion, which may occur with relatively low dosage. If in doubt, a cardiac review and an echocardiogram is advisable. Some centers routinely recommend echocardiography at the start of treatment, particularly as cardiac muscle hypertrophy occurs commonly and requires review. In the longer term, most children on diazoxide develop hypertrichosis (Fig. 7). Excess body hair is more noticeable with doses greater than 5 mg/kg/day and reduces as treatment is weaned and stopped.

The product concentration of diazoxide in suspensions can be variable with a range of "specials" formulations being available. Unfortunately, treatment efficacy can vary depending on the drug concentration; therefore the choice of a reliable manufacturer with approved quality control for safe and effective treatment with diazoxide is essential.

The length of treatment with diazoxide depends on individual phenotype severity and drug response. As described in the section on transient CHI, the severity of disease may reduce in some patients, being more likely in those without identified genetic mutations (Banerjee *et al.*, 2011; Salomon-Estebanez *et al.*, 2016). In such cases, it is useful to reassess drug dose at every clinical contact. Children stopping diazoxide may require the demonstration of tolerance to a food fast, that is, demonstrate euglycaemia and generate ketones.

Octreotide and Other Somatostatin Analogues

Octreotide, a nonspecific somatostatin analogue, acting on somatostatin receptor subtypes 2 and 5, achieves beta cell membrane stability by interacting with a variety of ion channels and reduces insulin secretion through several nonionic processes. Octreotide is usually given by subcutaneous bolus injections four to five times a day but can also be administered by continuous intravenous or subcutaneous infusions, the latter delivered by insulin pump devices. The usual starting dose is 5 μg/kg/day, which may need to be increased to 25 μg/kg/day in steps to achieve euglycaemia. Some centers advocate doses ranging between 30 and 50 μg/kg/day to maintain euglycaemia and prevent pancreatic surgery. A common occurrence with octreotide is the phenomena of tachyphylaxis, whereby initial efficacy is not sustained and progressively greater drug concentrations are required to achieve treatment effect. Octreotide has been associated with serious side effects including necrotizing enterocolitis and hepatitis. Therefore, treatment with octreotide should be initiated and monitored by a specialist treatment center.

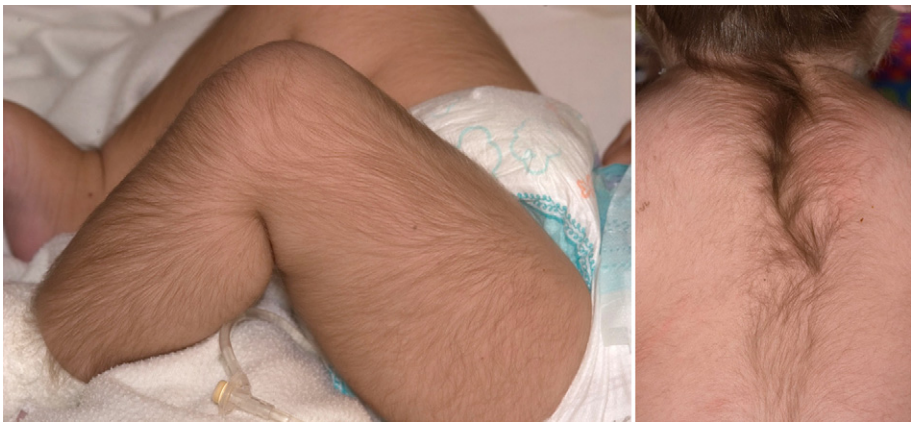

Fig. 7 Diazoxide related hypertrichosis can be significant, present over the extremities, forehead, and extending down the back.

While octreotide has a short half-life of 100 min and has to be injected several times during the day, an alternative is to use long-acting somatostatin analogues (long-acting slow release octreotide and somatuline autogel) as once a month subcutaneous or intramuscular preparations. Small cohort studies suggest benefit but concerns remain over depot preparations associated with long-lasting side effects such as hepatic dysfunction in early childhood (van der Steen *et al.*, 2017).

Nifedipine

Nifedipine is a calcium channel antagonist that blocks calcium-mediated exocytosis of insulin from beta cells. In vitro experiments suggest clear action of nifedipine on dysregulated beta-cell function (Lebrun *et al.*, 1997), which would imply a positive clinical benefit. However, equivalent in vitro doses are much higher than those in clinical practice and are likely to be associated with unacceptable side effects such as hypotension. Nifedipine has been used on and off in CHI patients with variable results; however, recent evidence suggests no positive benefit (Güemes *et al.*, 2017).

Other Therapy Options

The mammalian target of rapamycin (mTOR) inhibitors, everolimus and sirolimus have been used in patients with CHI where treatment with diazoxide and somatostatin analogues have failed. Small cohorts have reported modest success, but side effects, mainly from immunosuppressive effects detract from any meaningful benefit (Szymanowski *et al.*, 2016; Banerjee *et al.*, 2017).

Polyunsaturated fatty acids (PUFA) have been used as a food supplement in the management of patients with CHI already treated with diazoxide (Skae *et al.*, 2014). While effects are modest in a pilot trial, PUFA is safe and unlikely to interfere with other treatments when used as an adjunct. However, further studies are required to evaluate if PUFA could be used more widely in clinical practice.

New Drug Development

Treatment of CHI has not progressed significantly beyond diazoxide and somatostatin analogues over the last three decades. However, there have been recent activity promising newer treatments on the horizon. Drug trials in older CHI patients have suggested the possibility that inhibition of the glucagon-like peptide-1 receptor (GLP-1R) in beta cells has positive benefits. Exendin 9–39 acts independent of the K-ATP channel mechanism of insulin release to reduce insulin secretion. Another promising drug candidate is "soluble glucagon." Whilst conventional formulations of glucagon are complicated by fibrillation, novel glucagon formulations have been developed that may be used subcutaneously as long-term therapy. These advances include the use of a modified formulation to solubilize glucagon, and the synthesis of a modified glucagon variant that is water-soluble. Another drug considered for clinical trial is an allosteric monoclonal antibody that binds the insulin receptor with high affinity to reduce insulin effect. Current preparations are intravenous, but offer the promise of more acceptable routes of administration and the possibility of preventing subtotal pancreatectomy, thereby preventing diabetes for life. Other novel therapeutic approaches that might be promising are enhancers of trafficking or chaperones of defective K-ATP channels and highly selective agonists of somatostatin receptors.

Pancreatic Surgery

In focal CHI, lesionectomy is the treatment of choice. For lesions in the body and tail of the pancreas, laparoscopic approaches may be used for resection. For lesions in the head, uncinate process and proximal body, a laparotomy approach may provide improved access to the pancreatic bed, which is deep in the abdomen. Whilst lesions identified by 18-fluoro-dopa PET-CT scans may be easily identifiable, the 3-dimensional nature of the pancreas and the small size of the lesion make surgical localization difficult (**Fig. 5**). In open laparotomy, lesions can be palpable as a "pea in a sponge" as the consistency is firmer than the rest of the pancreas; however, in focal lesions that are less tightly organized and in those without a capsule, palpability may not be reliable. Haptic sensation, and hence palpability is excluded in laparoscopic pancreatectomy. In all cases, frozen section histopathology is crucial to identify the lesion. Focal CHI histopathology is characterized by densely crowded islets with little exocrine tissue (**Fig. 4**), in contrast to diffuse CHI characterized by large nuclei, foamy cytoplasm and a mixture of exocrine and endocrine tissue (**Fig. 8**). It is helpful to identify a margin of normal pancreatic tissue adjoining the focal lesion, although this is not always visible. In some cases, focal lesions have tentacle-like extensions into normal tissue that are not easily resectable and cause postoperative hypoglycemia. Focal lesions that are adjacent to the bile duct are difficult to access and resect without causing significant damage to important structures. For such lesions extensive surgical procedures that involve duodenectomy may be required. However, conservative approaches with medical therapy may also be undertaken, particularly as there is some evidence that focal lesions may resolve over time. Thus, although focal lesions should in theory be resectable and achieve cure from hypoglycemia, considerable surgical skill and expertise is required from specialist teams, with some degree of uncertainty persisting after surgery.

Children with diffuse disease should be managed by medical therapy if possible. However, if medically unresponsive, subtotal pancreatectomy may be required to reduce hypoglycemia. This procedure involves skilful mobilization of the pancreas, splenic artery, and other important structures, while preserving the integrity of the bile duct (**Fig. 9**). Most of the pancreas is resected,

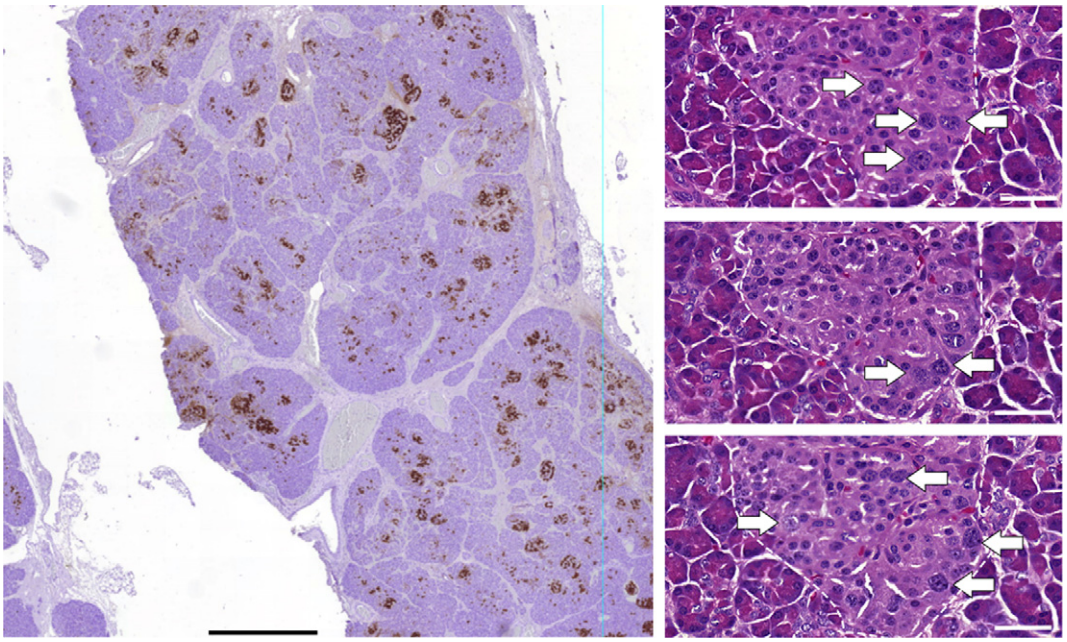

Fig. 8 Diffuse CHI characterized by a mixture of endocrine and exocrine tissue scattered throughout the pancreas (left-hand panel, scale bar 0.1 mm) and large and irregular nucleii in some cells within islets. The right-hand panel shows serial sections of tissue to illustrate the presence of multiple enlarged nuclei, indicated by *arrows*. Note that most of the nuclei appear in successive 5-μm sections of tissue.

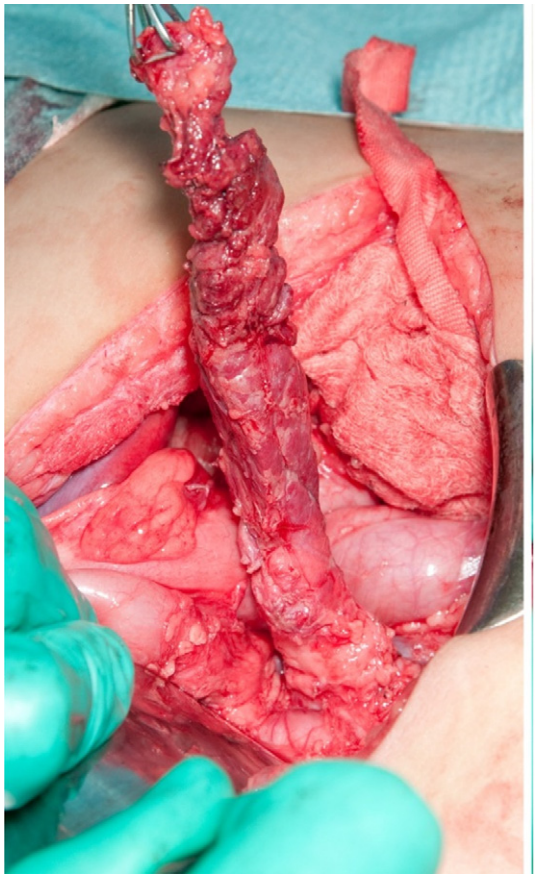

Fig. 9 Subtotal pancreatectomy in diffuse CHI; here the pancreas has been carefully dissected free of important structures such as the splenic artery and elevated by the tip of the tail. The common bile duct is an important structure that needs to be preserved when removing the head of the pancreas.

keeping a 5% rim of tissue between the wall of the duodenum and bile duct. Frozen section histology is usually not required if the diagnosis of diffuse CHI is anticipated prior to surgery. In some late-presenting cases with no identified genetic mutations, atypical CHI histology may be found.

In the postoperative period following subtotal pancreatectomy, euglycaemia is usually achieved, but a significant proportion (around 30% or more) may remain persistently hypoglycemic. Postoperative hypoglycemia tends to be manageable by medical therapy, although second-look surgery may be required to remove further pancreatic tissue. A few children become hyperglycemic after surgery and require insulin treatment. In most patients, hyperglycemia becomes prominent by adolescence, resulting in diabetes by the teenage years (Beltrand *et al.*, 2012).

As subtotal pancreatectomy invariably leads to diabetes, there is a push to preserve pancreatic tissue by extensive medical therapy and intense nutritional therapy. However, such an approach has to be carefully balanced against the risks of persistent hypoglycemia and subsequent brain damage. Also, quality of life of the child and family has to be considered if intensive feed/medical regimens are used.

Feeding Problems

Feeding problems are commonly present in many CHI patients. Sucking and swallowing problems are often present in the early stages but food aversion can persist for much longer. The cause for feeding problems is not clear but is likely to be multifactorial. The pressure to ensure carbohydrate intake and the reliance on intravenous, nasogastric and gastrostomy feeding can lead to a neglect of oral feeding. Side effects from medications, prolonged periods of hospital stay, absence of a stimulating environment and heightened parental anxiety all contribute to perpetuate food-avoiding behavior. It is interesting to know that feeding problems in patients with focal CHI rapidly resolve after lesionectomy, indicating a cessation of inhibitory influences to feeding processes.

Feeding problems are complex to resolve and take time, perseverance and advice from speech and language therapists. Inability to feed can provoke parental anxiety; it is important to provide knowledge, reassurance and support to the parents to turn the feeding process into a pleasurable experience. Medication is not usually advised, although gastro-oesophageal reflux, a deterrent to feeding, could be minimized by antireflux treatment with ranitidine or omeprazole.

Multidisciplinary Team Coordination

Patients severely affected by hypoglycemia require multidisciplinary input from teams rather than individuals. The team effort comprises of pediatric endocrinologists, specialist nurses, dietitians, speech and language therapists, biochemists, radiologists, nuclear medicine physicians, surgeons, and histopathologists (**Fig. 10**). Specialist nurses experienced in CHI help parents and families guide their way through the stress of diagnosis, information overload, uncertainty and prolonged hospitalization. Clinical psychologists are also important to improve the patient journey and to assess the impact of CHI on neurodevelopment and quality of life.

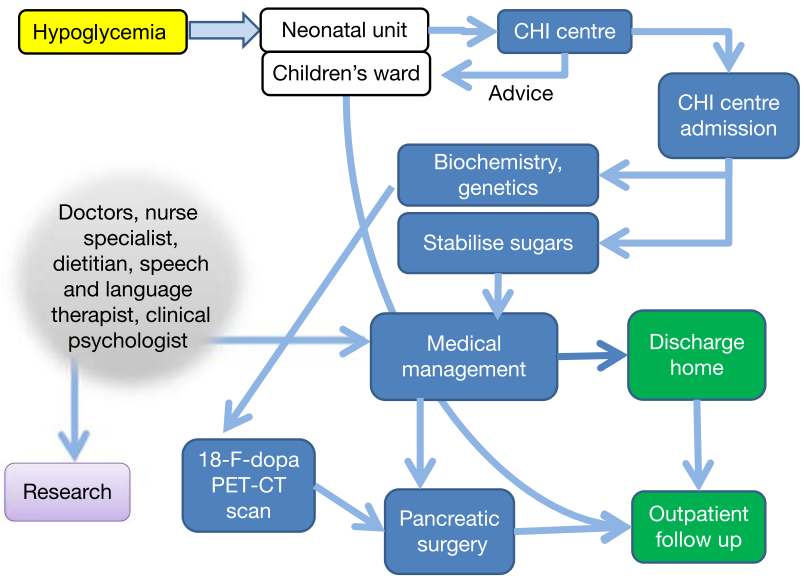

Fig. 10 Complex coordinated care required in the management of patients with CHI. The child with CHI could be managed in the local hospital with support from the CHI center, but if hypoglycemia is severe and unstable may require admission to a CHI expert center for clinical management by a multidisciplinary team.

Long-Term Outcomes

Neurodevelopment

Hyperinsulinism leads to both hypoglycemia and hypoketonaemia, that is, a loss of essential metabolic fuels for the neonatal brain. Therefore, severe and recurrent hypoglycemia in CHI is significantly detrimental to neurodevelopment. It is not surprising that a high proportion of children with CHI have adverse neurodevelopment. In various observational studies over the last 15 years, the rates of cognitive and developmental delay have not declined in various cohorts (Menni *et al.*, 2001; Meissner *et al.*, 2003; Avatapalle *et al.*, 2013). There is mounting evidence that the depth and severity of hypoglycemia in early life, not the duration of disease may determine the extent of brain injury. Therefore, children with transient hypoglycemia may develop significant neurodisability and lifelong morbidity. It is important to recognize that older children may also develop CHI, a diagnosis that is easily missed in emergency departments. Late presenting CHI children have adverse cognitive function, suggesting missed opportunities in treatment contributing to neuronal damage (Salomon-Estebanez *et al.*, 2017).

A developmental review should form part of any CHI management strategy. If delayed development is suspected, a more formal developmental tool should be used to quantify the extent and nature of delay. Formal assessment by tools such as Bayley Scales of Infant and Toddler Development and Weschler Intelligence Scale for Children may be used but are age-specific, time-consuming and not always available. A suitable screening tool such as the Vineland Adaptive Behavior Scales Questionnaire may be used before formal referral to a developmental pediatrician. If severe neuroglycopaenia is suspected, brain magnetic resonance imaging may be considered (**Fig. 11**).

Severity Trajectory of CHI

CHI is a heterogeneous condition with different causes, modes of presentation and responsiveness to treatment. Although termed congenital, not all causes are genetic. Further, genetic causes cannot be identified in all cases where genetic etiology is suspected. Therefore, there is considerable diversity in patterns of disease, which largely remain unexplained. It is well recognized that some

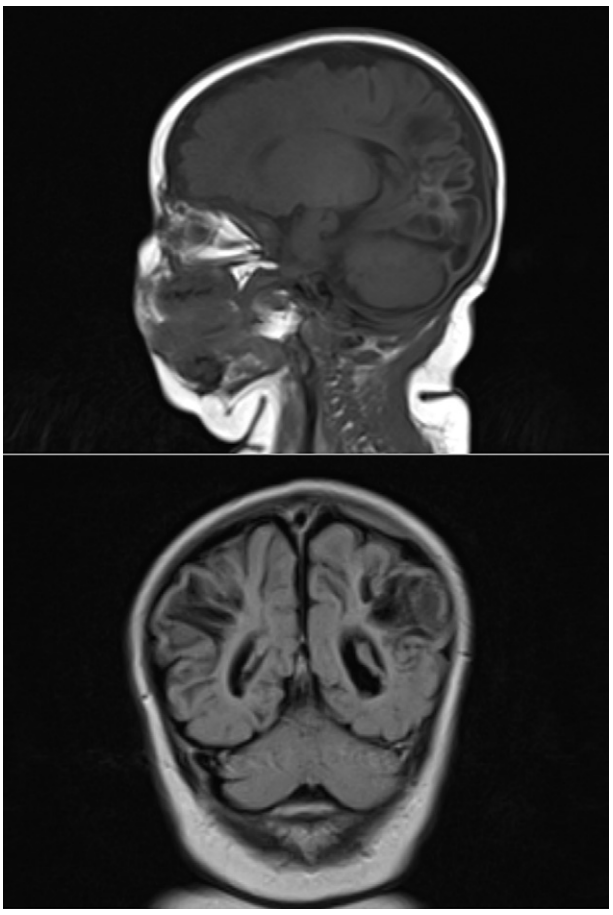

Fig. 11 Severe perinatal hypoglycemic encephalopathy with extensive bilateral parieto-occipital and posterior temporal gliosis along with focal cystic encephalomalacia.

children have a resolving nature of illness, also known as transient CHI, in contrast with those requiring medical treatment or surgical treatment, also known as persistent CHI (Banerjee *et al.*, 2011). Mechanisms of severity reduction and disease resolution remain unknown and are not explained by gene mutation status; a significant proportion of children with recessive and dominantly inherited *ABCC8*/*KCNJ11* mutations may also resolve (Salomon-Estebanez *et al.*, 2016).

In view of the changing natural history of CHI, it is helpful to review medication dosage at each follow up appointment to reduce the burden of medication whilst ensuring satisfactory glycemic profiles. For children stopping medication, demonstration of age-appropriate fasting tolerance may be useful for further reassurance. It is advisable to test blood glucose levels during episodes of concurrent illness as children are likely to become hypoglycemic at such times due to inadequate nutritional intake and altered metabolism.

Risk of Diabetes

Patients with focal CHI undergoing successful lesionectomy are effectively cured and do not develop diabetes. In contrast, those undergoing subtotal pancreatectomy develop hyperglycemia and diabetes, usually by mid puberty. In children with dominantly inherited mutations in *ABCC8*/*KCNJ11*, there is a risk of later life diabetes, although the risk and the timing of diabetes are difficult to confirm. Similarly, in those patients with *HNF4A* mutations, a flip from neonatal hypoglycemia to maturity onset diabetes in young (MODY) occurs in later life. The cause for the phenotype switch remains unknown, but needs to be considered and discussed with the family.

Transition to Young Adult Life

CHI is a disease in early childhood and in some patients has a resolving natural history. However, a proportion of patients continue to require medication into young adult life with hyperinsulinism remaining persistent and problematic. Others who undergo pancreatic surgery may become diabetic in adolescence requiring insulin treatment as adults. Transition from childhood into young adult life becomes important in these individuals. Considering that a significant proportion of children have some degree of cognitive compromise, issues of independence, adherence to treatment and patient safety become important. These issues are best addressed in a gradual process of transition mainly through the puberty years. In young adult life, medical follow up should continue under the supervision of an adult endocrinologist with interest in adult CHI.

Parent and Family Perspectives

CHI is a very significant condition causing stress to parents and families. Neonates diagnosed with CHI are often admitted for prolonged periods in hospital with the uncertainties of diagnosis, treatment response and future prognosis. Most parents benefit from face-to-face discussions reiterating complex information, which is difficult to assimilate. Information leaflets may be given, although most parents do not find written information very helpful. Alternative sources of information include websites run by international charities or those maintained by specialist services. Other sources of information include white board animation videos using freely available online content which have the added advantage of voice in addition to text, reinforcing information uptake in an efficient manner.

The wellbeing of families can sometimes become neglected when medical attention is solely targeted at improving glucose status. It is the role of the entire CHI team, particularly the clinical psychologist, to discuss psychosocial wellbeing during clinical management. Early engagement and communication with the team is crucial to information assimilation and understanding. In time, parents become well versed in CHI and are a valuable source of information and experience. Increasingly parent-led charities are making major contributions to CHI management through their expression of priorities and by actively engaging clinicians, scientists, and other families to push the frontiers of care.

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https://www.youtube.com/watch?v=h-5XsG5ICKQhttps://www.youtube.com/watch?v=UR2Y_Fj3TElhttps://www.youtube.com/watch?v=izDuLW0QxEAhttps://www.youtube.com/watch?v=v0NXyDHRvrMhttps://www.youtube.com/watch?v=id4Ic1DHPS0https://www.youtube.com/watch?v=OcA_ehsmU-ghttps://www.youtube.com/watch?v=jUQXh6DIcp0 - Focal Congenital Hyperinsulinism (CHI)

Beckwith–Wiedemann Spectrum

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Beckwith–Wiedemann syndrome (BWS, MIM #130650) is a human imprinting disorder that results from altered expression or function of imprinted genes located on the short arm of chromosome 11 (at 11p15.5). BWS was described independently by Beckwith (1963) and Wiedemann (1964) >50 years ago and has been the subject of >1000 publications (Brioude *et al.*, 2018). Though BWS is a rare disorder (maximum incidence ~1 in 10,000) (Mussa *et al.*, 2017), it has been widely studied as an exemplar of how disordered genomic imprinting may be associated with human disease and because of the variable clinical features, diagnostic difficulties and complex genetics of the disorder (Brioude *et al.*, 2018). Recently a large multidisciplinary group of international experts produced a consensus statement covering clinical, molecular genetic and management aspects of the disorder suggested that BWS should be considered as part of clinical and molecular spectrum known as Beckwith–Wiedemann spectrum (BWSp) (Brioude *et al.*, 2018). This article adopts this classification and covers clinical and molecular aspects of BWSp.

Genomic Imprinting and Human Imprinting Disorders

Molecular investigation of human imprinting disorders such as BWS, Silver–Russell syndrome (SRS), Angelman syndrome and Prader–Willi syndrome have provided important insights into the role of genomic imprinting in health (Eggermann *et al.*, 2015). Genomic imprinting is an epigenetic process affecting about 100 human genes. Whereas non-imprinted genes are generally expressed from both parental alleles, imprinted genes are preferentially expressed from either the maternal or paternal allele (e.g., *IGF2* is expressed from the paternal allele and *CDKN1C* is expressed from the maternal allele) (Reik and Walter, 2001). Imprinted genes usually occur in clusters and imprinting depends on the presence of an imprinting control centre (IC) that is marked by the presence of a differentially methylated region (DMR) (i.e., methylation occurs on either the maternally or paternally inherited allele). The methylation status of an IC DMR is established during gametogenesis and is maintained during the wave of genome demethylation that occurs early in embryogenesis (see **Fig. 1**). Maintenance of IC DMR methylation requires DPPA3 (also known as PGC7/Stella) and the KRAB zinc finger protein ZFP57 (Sanchez-Delgado *et al.*, 2016). Imprinting disorders can result from a variety of mechanisms including a mutation in the coding sequence of an imprinted gene or often, as in BWS, altered methylation (loss or gain) at the IC DMR that can result in biallelic expression or biallelic silencing of an imprinted gene such as *IGF2* or

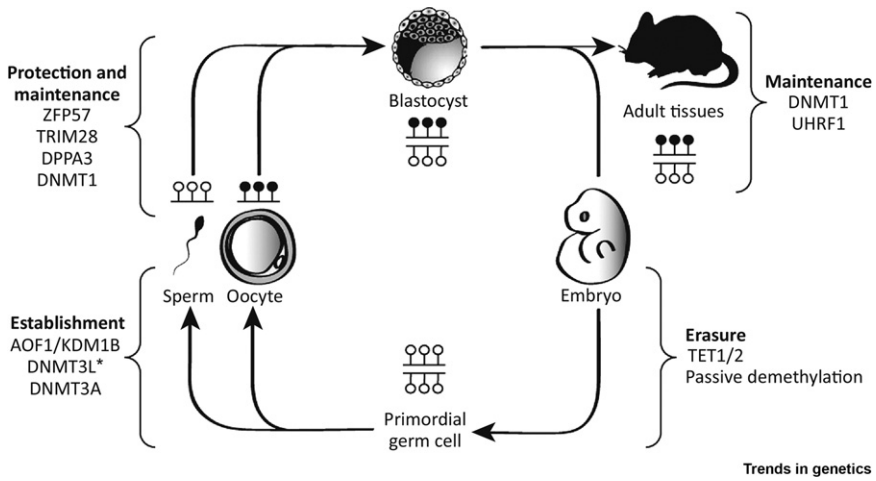

Fig. 1 The Life Cycle of Epigenetic Changes at Imprinted Loci in Mice. Regions of differential methylation are established in the germline and protected from preimplantation reprogramming by the maintenance factors DNMT1, ZFP57, and DPPA3. The allelic methylation is then preserved by the semiconservative action of DNMT1-UHRF1. In primordial germ cells of developing embryos, the DNA methylation at imprinted DMRs is erased so that the new profiles can be established according to the sex of the embryo. This complex procedure involves histone demethylation of H3K4 and the subsequent recognition and DNA remethylation by the DNMT3L-DNMT3A complex. *Note that *DNMT3L* is not expressed in human oocytes, suggesting different recruiting methods between species. Reproduced from Sanchez-Delgado, M., Riccio, A., Eggermann, T., Maher, E.R., Lapunzina, P., Mackay, D. and Monk, D. (2016). Causes and consequences of multi-locus imprinting disturbances in humans. *Trends in Genetics* **32**(*7*): 444–455 with permission.

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CDKN1C (Eggermann *et al.*, 2015). Imprinted genes appear to be preferentially implicated in prenatal growth and development and, in general, paternally expressed genes (e.g., *IGF2*) promote growth and maternally expressed genes (e.g., *CDKN1C*) suppress growth (Fowden *et al.*, 2011).

Definition and Etiology of BWSp

The wide phenotypic variability of BWS (clinical and pathological features are listed in **Table 1**) and the detection of mild forms of the disorder through molecular testing has resulted in wide variations in the proposed clinical diagnostic criteria for the disorder (Brioude *et al.*, 2018). The International BWS Consensus Group (IBCG) suggested the concept of BWSp and proposed a scoring system for BWSp that included both clinical and molecular data (see **Table 1** and **Fig. 2**). Individuals with isolated lateralised overgrowth (ILO) and a chromosome 11p15.5 molecular abnormality (see below) would be diagnosed as BWSp whereas those with ILO and no molecular abnormality are outside the definition (unless further more sensitive molecular testing revealed a molecular defect) (Brioude *et al.*, 2018). A clinical diagnosis of classical BWS (as part of BWSp) can be made in the presence of normal molecular testing if the IBCG score ≥ 4 (see **Table 1**). A child with a score of <4 and no molecular abnormality might have suspected BWSp but fall outside of the current definition.

In most cases the etiology of BWSp is unknown. Up to 15% of cases may have a family history but the majority are sporadic and the recurrence risk is low (see later). No specific environmental factors have been identified but there is an excess risk of BWS after assisted reproductive technology (ART) births (Maher, 2005; Mussa *et al.*, 2017). The increased risk of BWS after ART has been attributed to failure to maintain genomic methylation at ICs during in vitro embryo culture (though other factors including infertility per se may be implicated) but although the relative risk may be increased up to 10-fold the absolute risk of having a child with BWS after ART is very small (about 1 in 1000) (Mussa *et al.*, 2017).

Molecular Genetics of BWSp

BWSp results from a variety of genetic and epigenetic mechanisms that result in increased *IGF2* expression and/or loss of expression or inactivation of *CDKN1C* (see **Figs. 3** and **4**). The multiple mechanisms that might cause BWSp, including paternal uniparental disomy (upd(11)pat), cytogenetic abnormalities (paternally inherited duplications and maternally inherited balanced translocations/inversions), epigenetic alterations at imprinting centres (IC), *CDKN1C* mutations and imprinting centre copy number abnormalities (Choufani *et al.*, 2013; Brioude *et al.*, 2018). The often mosaic nature of molecular abnormalities in BWSp

Table 1 Clinical features and scoring system for Beckwith–Wiedemann spectrum

Cardinal features (2 points per feature)
Macroglossia
Exomphalos
Lateralized overgrowth
Multifocal and/or bilateral Wilms tumor or nephroblastomatosis
Hyperinsulinism (lasting >1 week and requiring escalated treatment)
Pathology findings: adrenal cortex cytomegaly, placental mesenchymal dysplasia or pancreatic adenomatosis
Suggestive features (1 point per feature)
Birthweight >2 SDS above the mean
Facial naevus simplex
Polyhydramnios and/or placentomegaly
Ear creases and/or pits
Transient hypoglycemia (lasting <1 week)
Typical BWSp tumors (neuroblastoma, rhabdomyosarcoma, unilateral Wilms tumor, hepatoblastoma, adrenocortical carcinoma or phaeochromocytoma)
Nephromegaly and/or hepatomegaly
Umbilical hernia and/or diastasis recti

For a clinical diagnosis of classical Beckwith–Wiedemann syndrome (BWS), a patient requires a score of ≥ 4 (this clinical diagnosis does not require the molecular confirmation of an 11p15 anomaly). Patients with a score of ≥ 2 (including those with classical BWS with a score of ≥ 4) merit genetic testing for investigation and diagnosis of BWS. Patients with a score of <2 do not meet the criteria for genetic testing. Patients with a score of ≥ 2 with negative genetic testing should be considered for an alternative diagnosis and/or referral to a BWS expert for further evaluation. *BWSp*, Beckwith–Wiedemann spectrum; *SDS*, standard deviation scores.

Reproduced from Brioude, F., Kalish, J.M., Mussa, A., *et al.*, (2018). Expert consensus document: Clinical and molecular diagnosis, screening and management of Beckwith–Wiedemann syndrome: An international consensus statement. Nature Reviews. Endocrinology, 14(4), 229–249. https://doi.org/10.1038/nrendo.2017.166. [Epub ahead of print] PMID: 29377879 with permission.

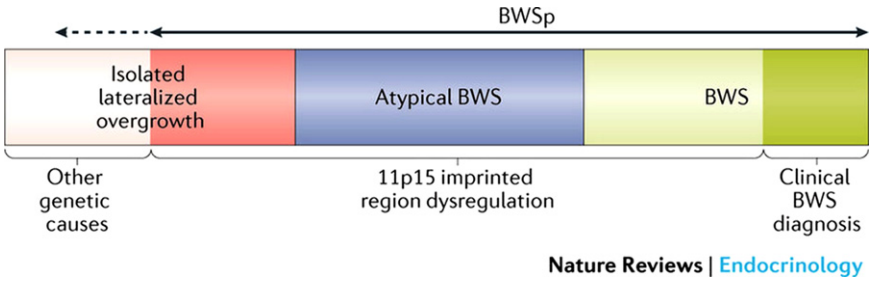

Fig. 2 The Beckwith–Wiedemann spectrum (BWSp). BWSp includes patients with a clinical diagnosis of Beckwith–Wiedemann syndrome (BWS) with or without an (*epi*) genetic change at the BWS locus on chromosome 11p15, patients with "atypical BWS" (defined as fewer cardinal and suggestive features than those needed for a clinical diagnosis of BWS) and an (epi)genetic change at the BWS locus, and patients with "isolated lateralised overgrowth" and an (epi)genetic change at the BWS locus. The *dotted arrow* indicates that some patients with apparent isolated lateralised overgrowth and no 11p abnormality may subsequently be found to have an 11p15 abnormality on testing of additional tissues or with a more sensitive assay. Patients with clinical BWS and no detectable 11p15 abnormality might be further investigated with additional clinical evaluation and consideration of other syndromes, which may have features overlapping with BWSp (see **Table 3**) and appropriate testing for those syndromes may be warranted. Reproduced from Brioude, F., Kalish, J.M., Mussa, A., *et al.* (2018). Expert consensus document: Clinical and molecular diagnosis, screening and management of Beckwith–Wiedemann syndrome: An international consensus statement. *Nature Reviews. Endocrinology*. **14**(4), 229–249. https://doi.org/10.1038/nrendo.2017.166. [Epub ahead of print] PMID: 29377879 with permission.

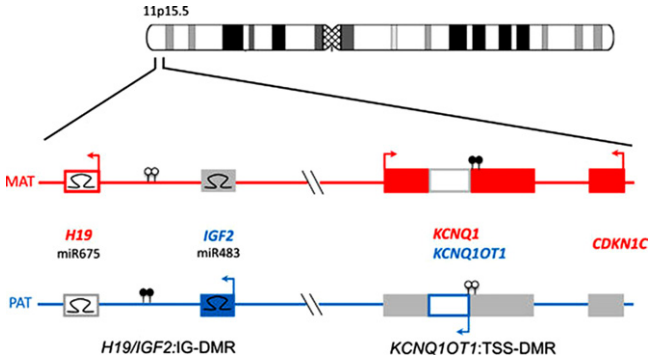

Fig. 3 The imprinted gene cluster in 11p15.5. It is divided in two functional domains whose imprinting is dependent on distinct ICRs (*H19/IGF2*: IG-DMR, *KCNQ1OT1*:TSS-DMR). (*Filled boxes*, protein coding genes; empty boxes, noncoding genes; Ω miRNAs; filled lollipops, methylated regions; empty lollipops, unmethylated regions; *red*, genes expressed from the maternal (mat) chromosome; *blue*, genes expressed from the paternal (pat) chromosome. *Arrows* above the genes, transcription direction of sense genes; *arrows* below the genes, transcription direction of anti-sense genes). Reproduced from Eggermann, K., Bliek, J., Brioude, F., et al. (2016). EMQN best practice guidelines for the molecular genetic testing and reporting of chromosome 11p15 imprinting disorders: Silver-Russell and Beckwith–Wiedemann syndrome. *European Journal of Human Genetics* **24**(*10*), 1377–1387with permission.

can make a molecular diagnosis challenging (Brioude *et al.*, 2018). The IBCG recommended that when a diagnosis of BWSp is suspected testing should usually be offered if the IBCG score is >2 (except for cases of isolated exomphalos) (see **Table 1**). A flow chart summarizing the molecular investigation of BWSp is shown in **Fig. 5**.

Molecular investigations of sporadic suspected BWSp: The most frequent causes of sporadic BWSp are upd(11)pat (~20% of cases), loss of maternal allele methylation at the centromeric 11p15.5 imprinting domain DMR (known as IC2 or *KCNQ1OT1*:TSS DMR) (~50% of cases) or gain of maternal allele methylation on the telomeric 11p15.5 imprinting domain DMR (known as IC1 or *H19/IGF2*: intergenic (IG) DMR) (H19/IGF2:IG-DMR) (~5% of cases) (**Figs. 3** and **4**). Each of these major molecular mechanisms can be detected by assaying IC1 and IC2 methylation status (patients with upd(11)pat) have IC2 loss of methylation (LOM) and IC1 gain of methylation (GOM) and so investigations such as methylation-sensitive multiplex ligation-dependent probe amplification (MS-MLPA) are generally the first line investigation for a suspected case of BWSp (Eggermann *et al.*, 2016a,b). If a positive diagnosis is made then the recurrence risk for each of these anomalies will be low unless IC1 GOM is secondary to a IC1 CNV or mutation (the former can be detected by MS-MLPA) (see **Table 2**). Testing may be negative if (a) upd(11)pat or a methylation abnormality is present in a mosaic form, (b) there is an alternative molecular cause of BWSp such as a *CDKN1C* mutation (~5%

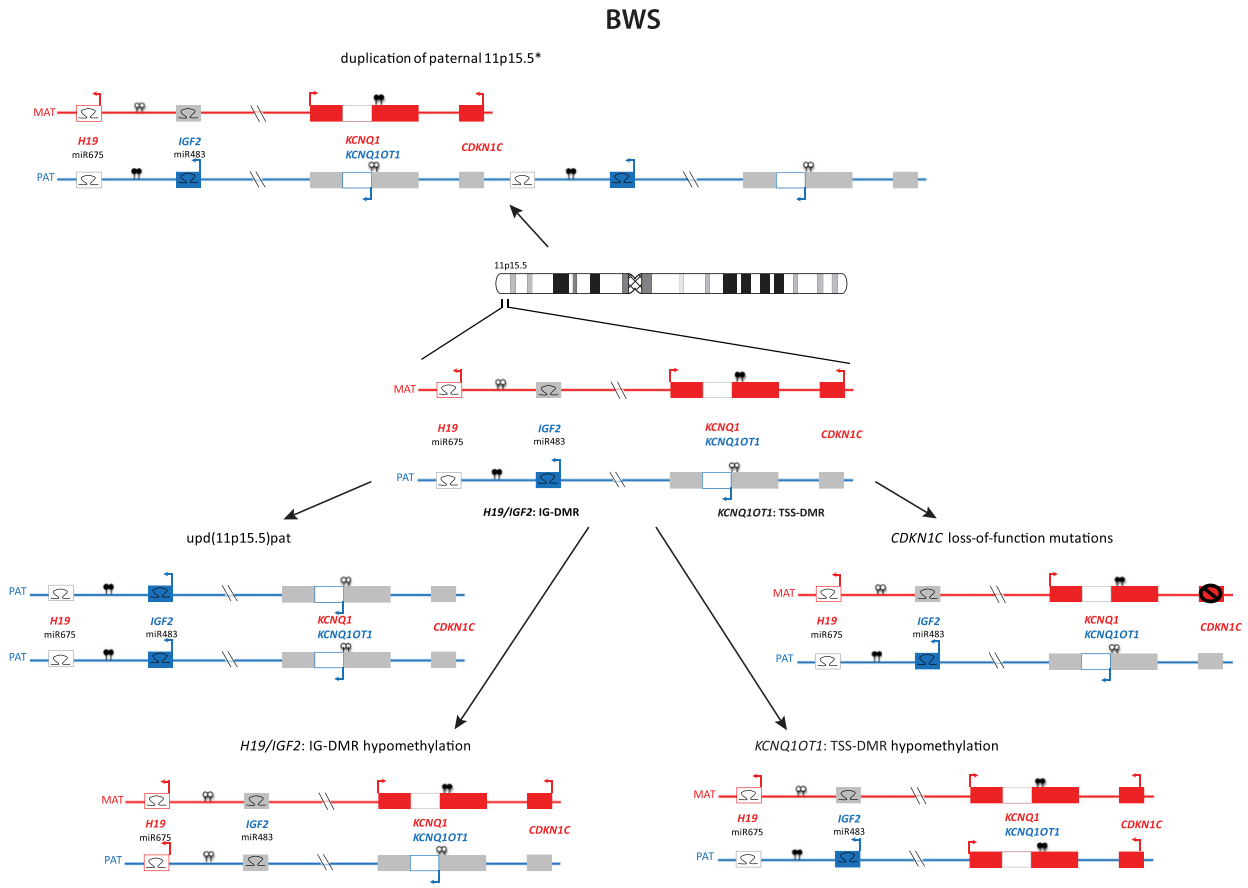

Fig. 4 Molecular mechanisms of BWSp. Reproduced from Eggermann, T., Perez de Nanclares, G., Maher, E.R., et al. (2015). Imprinting disorders: A group of congenital disorders with overlapping patterns of molecular changes affecting imprinted loci. *Clinical Epigenetics* **7**, 123 with permission

of sporadic cases and 50% of familial cases), a chromosome 11p15.5 duplication or inversion (~2% of cases) or (c) there is an unrelated cause (e.g., other overgrowth disorder such as Sotos syndrome (see **Table 3**).

Given that a negative result for an initial IC1 and IC2 methylation status test cannot exclude a diagnosis of BWSp, further investigations are commonly instigated to detect mechanism that might not have been detected by MS-MLPA (Brioude *et al.*, 2018) (see **Fig. 5**). The choice of the second line investigations is dependent on the phenotype: in a child with mild features and hemihypertrophy, mosaicism could be present and further investigations using more sensitive methods to detect upd (11)pat (e.g., single nucleotide polymorphism (SNP) arrays) or altered IC1/IC2 methylation status (Brioude *et al.*, 2018). Conversely, the presence of exomphalos is correlated with a *CDKN1C* mutation or LOM at IC2 (which results in loss of expression of *CDKN1C* from the maternal allele) (Cooper *et al.*, 2005) and so a normal IC1 and IC2 methylation status result in a child with evidence of classical BWS would suggest that *CDKN1C* mutation analysis as the next investigation (Brioude *et al.*, 2018).

Multilocus imprinting disturbance (MLID) is a phenomenon seen in patients with imprinting disorders in which there are epimutations at multiple IC DMRs (Sanchez-Delgado *et al.*, 2016). In a subgroup of patients with Transient Neonatal Diabetes Mellitus (TNDM, OMIM 601410), an imprinting disorder caused by aberrant imprinting of the *PLAGL1* imprinted domain (containing the PLAGL1:alt-TSS-DMR), MLID was found to be often associated with biallelic mutations (autosomal recessively inherited) in *ZFP57* (Mackay *et al.*, 2008). MLID has also been described other imprinting disorders and occurs in a subset (~30%) of patients with BWSp and IC2 LOM (but not in other molecular subgroups) (Azzi *et al.*, 2009). Most such cases are sporadic but in a few cases mutations in the maternal effect genes (the mother harbors biallelic mutations) *NLRP2* and *NLRP5* have been reported (Meyer *et al.*, 2009; Docherty *et al.*, 2015). In some studies and association of MLID in BWSp with ART has been reported (Tee *et al.*, 2013). Though complex or unusual phenotypes have been described in some patients with BWSp and MLID (e.g., a SRS-like phenotype), in most cases with MLID no additional clinical features are found and testing for MLID is not performed routinely unless a link to *NLRP2*/*NLRP5* is suspected (Brioude *et al.*, 2018).

Molecular investigations of familial BWSp: Germline loss-of-function mutations in *CDKN1C* are the most frequent cause of familial BWS (Lam *et al.*, 1999; Brioude *et al.*, 2015). As *CDKN1C* is imprinted and predominantly expressed from the maternal

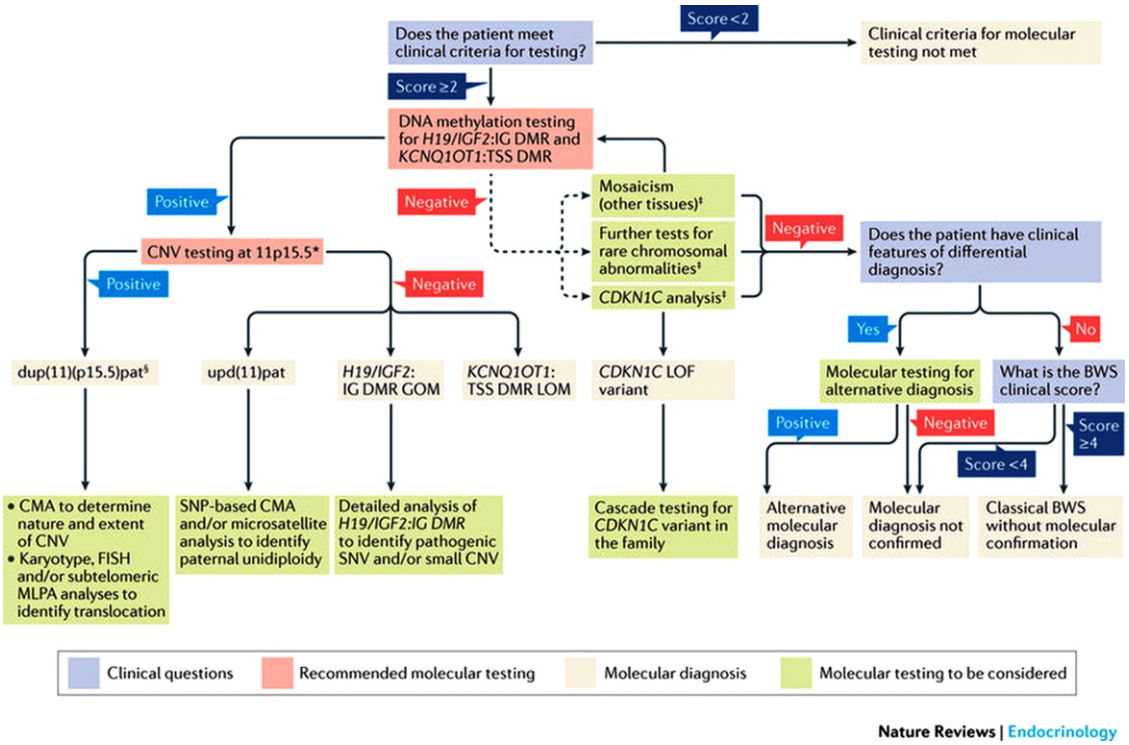

Fig. 5 Flowchart for investigation and diagnosis of Beckwith–Wiedemann syndrome. The figure summarizes the molecular diagnostic pathway for investigation of suspected Beckwith–Wiedemann spectrum (BWSp). Patients with clinical features reaching a score of ≥2 should be genetically tested. It is recommended that first-line testing should assay the methylation status of *H19/IGF2*:intergenic (IG) differentially methylated region (DMR) (also known as IC1) and *KCNQ1OT1*:transcriptional start site (TSS) DMR (also known as IC2). If not estimated simultaneously with DNA methylation, DMR copy number should then be determined in all patients with IC1 and/or IC2 methylation abnormalities. These assays can yield positive molecular diagnosis of BWSp with IC2 loss of methylation (LOM), IC1 gain of methylation (GOM), segmental paternal uniparental isodisomy (UPD) of 11p15.5 (upd(11)pat) or copy number variation (CNV; most commonly duplication of paternal 11p15.5 (dup(11)(p15.5)pat)). Further molecular tests can be considered to determine underlying mechanism of methylation abnormality, UPD or CNV. If DNA methylation testing is negative, further molecular tests can be considered to identify mosaic methylation abnormalities, pathogenic *CDKN1C* variants or rare balanced chromosomal rearrangements. If all molecular tests are negative, differential diagnosis should be considered. However, a diagnosis of classical Beckwith–Wiedemann syndrome (BWS) is made in the presence of a clinical score of ≥4 even in the absence of the molecular confirmation of an 11p15 anomaly. CMA, chromosome microarray analysis (can be oligonucleotide-based and/or single-nucleotide-polymorphism-based platforms); FISH, fluorescence in situ hybridization; LOF, loss of function; MLPA, multiplex ligation-dependent probe amplification; SNP, single nucleotide polymorphism; SNV, single nucleotide variation. *CNV status may be determined simultaneously with methylation testing. ‡See main text for indications for testing. §Deletion of maternal 11p15.5 (del(11)(p15.5)mat) may be detected with lower frequency. Reproduced from Brioude, F., Kalish, J.M., Mussa, A., *et al.*, (2018). Expert consensus document: Clinical and molecular diagnosis, screening and management of Beckwith–Wiedemann syndrome: An international consensus statement. *Nature Reviews. Endocrinology* **14**(*4*), 229–249. https://doi.org/10.1038/nrendo.2017.166. [Epub ahead of print] PMID: 29377879 with permission.

allele, paternally inherited mutations have no or minimal phenotypic effects. In contrast, the risk of BWS in the children of a mother who harbors a *CDKN1C* mutation is 50%. Such parent-of-origin effects are a characteristic feature of genomic imprinting disorders (Eggermann *et al.*, 2015). Other causes of familial BWSp include chromosomal abnormalities and IC1 (and rarely IC2) copy number variants (CNVs) (Eggermann *et al.*, 2016a,b). Single nucleotide substitutions in IC1 been described to cause familial BWSp but molecular testing is not readily available and so their frequency is difficult to establish. Rarely BWS with MLID may be associated with homozygous or compound heterozygous biallelic mutations in *NLRP2* or *NLRP5* in the mother (see above). In cases of familial BWS the risk of BWS in other family members will depend on the precise molecular cause and, in the case of *CDKN1C*, 11p15 CNVs or rearrangements, the parent-of-origin (see **Table 2**).

*Prenatal diagnosis of BWS*p. Though most cases of BWS occur sporadically and the recurrence risk is low (see **Table 2**), in cases of familial BWS the parents may consider prenatal diagnosis. If familial BWSp is associated with previously characterized *CDKN1C* mutation or CNV then the laboratory procedures for determining if the fetus is affected will be fairly standard. However, molecular testing for BWSp in the context of no previous family history as a result of the detection of a BWS-related fetal structural anomaly (e.g., exomphalos) detected by an ultrasound scan may be more challenging (Eggermann *et al.*, 2016b). As with postnatal molecular investigations, the mosaic nature of the epigenetic abnormality may lead to false negative testing results and there may be concerns that testing the epigenetic status of cultured chorionic

Table 2 Summary of Beckwith–Wiedemann spectrum molecular defect categories and recurrence risk

Molecular defect	*Frequency of molecular defect*	*Mosaicism observed*	*Risk of recurrence*	*Characteristic clinical features (compared with other molecular subgroups)*
IC1 GOM	5%	Yes	If no genetic anomaly is present, <1% If genetic anomaly (for example, pathogenic SNV of copy number variant in the DMR) is present, 50%; dependent on parental origin	Low frequency of exomphalos High risk of Wilms tumor
IC2 LOM	50%	Yes	If no genetic anomaly is identified, <1% If a *cis*-acting genetic anomaly is present, 50%; dependent on parental origin	High frequency of exomphalos Low risk of Wilms tumor
upd(11)pat	20% (see also paternal unidiploidy)	Yes	<1%	High incidence of lateralized overgrowth Low frequency of exomphalos High risk of Wilms tumor and hepatoblastoma
Loss-of-function *CDKN1C* variants	5% (40% in familial cases)	Only rarely	50% on maternal transmission	High frequency of exomphalos Low risk of Wilms tumor
dup(11)(p15.5) pat	~2%–4%	No	50% on paternal transmission Risk of SRS on maternal transmission	—
Deletions involving 11p15	1%–2%	No	Dependent on extent and position of CNV, and parent of origin	—
Mosaic paternal unidiploidy (genome-wide paternal UPD)	Up to 10% of upd(11)pat	Yes	Low	High frequency of neoplasia
MLID	33% of IC2 LOM cases	Yes	Low unless an in *trans* genetic variant is identified	Unclear

DMR, differentially methylated region; dup(11)(p15.5)pat, duplication of paternal 11p15.5; GOM, gain of methylation; LOM, loss of methylation; MLID, multilocus imprinting disturbance; SNV, single nucleotide variation; UPD, uniparental isodisomy; upd(11)pat, paternal uniparental isodisomy of 11p15.5.Reproduced from Brioude, F., Kalish, J.M., Mussa, A., *et al.*, (2018). Expert consensus document: Clinical and molecular diagnosis, screening and management of Beckwith–Wiedemann syndrome: An international consensus statement. Nature Reviews. Endocrinology 14(4), 229–249. https://doi.org/10.1038/nrendo.2017.166. [Epub ahead of print] PMID: 29377879 with permission.

villus cells or amniocytes could lead to testing errors. Hence prenatal testing should be undertaken in specialist centres and the results subjected to multicentre audit (Eggermann *et al.*, 2016b).

Clinical Features and Management of BWSp

In this section, the major features and principles of clinical management of BWS are described. For further details see Brioude *et al.*, 2018).

Macroglossia

This is a cardinal feature of BWSp and has been reported in ~90% of children diagnosed with classical BWS (Elliott *et al.*, 1994; Cooper *et al.*, 2005). The tongue is enlarged in all three dimensions and may cause feeding difficulties, persistent drooling, impairment of articulation and orthodontic problems (Shipster *et al.*, 2012). Rarely, when severe, macroglossia may cause respiratory obstruction and obstructive sleep apnoea and surgery may be required in the neonatal period but generally, if surgery is performed, it takes place between 1 and 3 years of age (Kadouch *et al.*, 2012; Shipster *et al.*, 2012). The appearance of macroglossia tends to diminish with age (through reduced growth rate and growth of the mandible to accommodate the tongue) but around 40% of children with BWS may undergo surgical reduction (Elliott *et al.*, 1994). The cosmetic and functional results from tongue reduction are usually good but surgery should be performed in a specialist centre that can offer long-term follow up from a multidisciplinary service (Brioude *et al.*, 2018).

Anterior Abdominal Wall Defects

Occur in around two-thirds of patients with BWSp (most commonly umbilical hernia and then exomphalos (omphalocele) or diastasis recti. (Elliott *et al.*, 1994; Maas *et al.*, 2016). Exomphalos may be detected by prenatal ultrasound scan and lead to prenatal testing for BWSp (see above). A molecular diagnosis of BWS is made in 10%–20% of fetuses with an exomphalos detected

Table 3 Differential diagnosis of Beckwith–Wiedemann spectrum

Disorder	*Inheritance*	*Molecular findings*	*Clinical features*	*References*
Simpson–Golabi–Behmel syndrome	X-linked recessive	Mutation in *GPC3*	Pre and postnatal overgrowth Macrocephaly Variable learning disability Umbilical hernia Diastasis recti Organomegaly Cardiac anomalies Diaphragmatic hernia Skeletal anomalies including postaxial polydactyly Supernumerary nipples Cleft palate Macroglossia Embryonal tumors (especially Wilms tumor) Coarse facial features	Li et al. (2001)
Perlman syndrome	Autosomal recessive	Homozygous mutations in *DIS3L2*	Prenatal overgrowth Developmental delay Hypotonia Nephromegaly Hyperinsulinism High risk Wilms tumor High neonatal mortality Facial features: prominent forehead, deeply set eyes, depressed nasal bridge, tented vermilion upper lip	Astuti et al. (2012)
Costello syndrome	Autosomal dominant (frequent *de novo* mutations)	Activating mutation in *HRAS*	Polyhydramnios, often severe Increased birth weight due to oedema Macrocephaly Short stature Severe feeding difficulties and failure to thrive in infancy Mild to severe intellectual disability Cardiac anomalies, cardiomyopathy, arrhythmia Ulnar deviation Deep palmar and plantar creases Embryonal tumors (rhabdomyosarcoma and neuroblastoma) Coarse facial features Papillomata	Kerr et al. (2006)
Sotos syndrome	Autosomal dominant (frequent *de novo* mutations)	Mutation in or deletion of *NSD1*	Tall stature Macrocephaly Mild to severe intellectual disability Scoliosis Seizures Cardiac anomalies Renal anomalies Neonatal hypotonia, jaundice and feeding difficulties Facial features: broad and prominent forehead, sparse frontotemporal hair, downslanting palpebral fissures, malar flushing, tall chin	Tatton-Brown et al. (2005)
Weaver syndrome	Autosomal dominant (frequent *de novo* mutations)	Mutation in *EZH2*	Tall stature Macrocephaly Variable intellectual disability Camptodactyly Soft/doughy skin Umbilical hernia Facial features: broad forehead, widely spaced eyes, pointed chin, large ears and retrognathia in early childhood	Tatton-Brown et al. (2013)

(*Continued*)

Table 3 Continued

Disorder	*Inheritance*	*Molecular findings*	*Clinical features*	*References*
Malan syndrome	Autosomal dominant	Mutation in the DNA-binding domain of *NFIX*	Postnatal overgrowth Rarely prenatal overgrowth Decrease of height overgrowth with age Persistent macrocephaly Invariably intellectual disability Frequent autism and anxiety Hypotonia Brain anomalies Slender body build Facial features: long face, prominent forehead, short nose, long philtrum, tall chin	Malan et al. (2010)
PTEN hamartoma tumor syndrome	Autosomal dominant	Mutation in *PTEN*	Prenatal overgrowth Macrocephaly Hypotonia Intellectual disability Autism spectrum disorder Dermatological features including genital freckling, trichilemmomas, papillomatous papules, acral keratosis Lipomas Hamartomatous intestinal polyposis High risk of thyroid, breast, endometrial and other cancers	Marsh et al. (1997)
PIK3CA related overgrowth spectrum	Somatic mosaic	Somatic activating mutation in *PIK3CA*	Segmental overgrowth syndromes including fibroadipose hyperplasia, CLOVES syndrome, hemihyperplasia multiple lipomatosis syndrome (HHML), megalencephaly-capillary malformation (MCAP)	Lindhurst et al. (2012)

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on prenatal ultrasound scan but testing is not indicated in neonates with exomphalos and no other features of BWSp (Porter *et al.*, 2009; Brioude *et al.*, 2018). The frequency of exomphalos is differs between molecular subgroups of BWS being significantly higher in those with *CDKN1C* mutations or IC2 LOM (which is associated with loss of *CDKN1C* expression from the maternal allele) than in those with UPD or IC1 GOM (Cooper *et al.*, 2005).

Pre and/or Post Overgrowth

In a large study of >400 patients with BWS, macrosomia at birth (defined by birth length and/or birth weight above +2.0 SDS) was reported in 43% of cases (40% had a birthweight > +2.0 SDS) (Brioude *et al.*, 2013). Although BWS has traditionally been classed as an overgrowth syndrome (and the differential diagnosis may include other overgrowth disorder such as Sotos syndrome and Simpson-Golabi-Behmel syndrome; see **Table 3**) the definition of macrosomia can differ from study to study and macrosomia may not be present in milder cases with mosaic molecular abnormalities. Hence the IBCG guidelines designated a birthweight >2 SDS above the mean as a suggestive rather than a cardinal feature (Brioude *et al.*, 2018). Children with IC1 GOM have a significantly higher birthweight than those with IC2 LOM or a *CDKN1C* mutation (Brioude *et al.*, 2013). Overgrowth tends to ameliorate in older children but though there is a paucity of data on adult height in children with BWSp, in one study just over 50% of adult patients had a height above +2.0 SDS (Brioude *et al.*, 2013). Overgrowth may be asymmetrical (*Lateralised overgrowth*/hemihypertrophy) and lateralised overgrowth is most common in BWSp patients with upd(11)pat (Ibrahim *et al.*, 2014). Leg length discrepancy can be problematic and treated by shoe lifts for milder forms (<2 cm) but surgical intervention might be considered in more severe forms (Brioude *et al.*, 2018).

Neonatal Hypoglycemia

This frequent complication of BWSp (reported in around 60% of patients (Maas *et al.*, 2016) is usually transient and resolves within a few days. Severe untreated hypoglycemia may be associated with neurological damage and so all neonates with possible BWSp should be screened for hypoglycemia (and treated id detected). In a subgroup of patients with BWSp, hypoglycemia is severe and persists beyond a week and may require drugs such as diazoxide or somatostatin analogues or even, in severe refractory cases, pancreatectomy (Al-Zubeidi *et al.*, 2014; Laje *et al.*, 2013). Patients with upd(11)pat and a coincident paternally transmitted KATP channel may have very severe and persistent hypoglycemia and molecular testing BWSp (particularly mosaic upd(11)pat should be considered in cases of congenital hyperinsulinemic hypoglycemia (CHI) without a detectable CHI gene mutation even if other features of BWSp are not apparent) (Kalish *et al.*, 2016).

Embryonal Tumors

Although only a minority (~7%) of children with BWSp will develop an embryonal tumor, compared to population controls the relative risk is greatly increased. The most commonly reported BWSp-associated embryonal tumors are Wilms tumor, hepatoblastoma and neuroblastoma, though rhabdomyosarcoma and pancreatoblastoma have been reported (Maas *et al.*, 2016). Median age of Wilms tumor diagnosis in BWSp around age 2 years (Maas *et al.*, 2016). A key finding has been the identification of genotype–phenotype correlations that have demonstrated that the tumor risk and tumor types vary between molecular subgroups (Cooper *et al.*, 2005; Maas *et al.*, 2016). The highest risk (~28%) is seen with IC1 GOM and the most common tumor type in this subgroup is Wilms tumor (occasionally neuroblastoma and pancreatoblastoma (<1% risk)) (Maas *et al.*, 2016). The next highest risk subgroup is upd(11)pat in which the overall tumor risk is 16% and Wilms tumor is most common (~8%) followed by hepatoblastoma (3.5%), neuroblastoma (1.4%) and adrenocortical carcinoma (1.1%) (Maas *et al.*, 2016). Amongst those with uniparental disomy, the risk of embryonal tumor appears to be higher in the subgroup (~10% of those with disomy) with genome-wide uniparental disomy than in those upd(11)pat (Kalish *et al.*, 2013). Germline *CDKN1C* mutations have an embryonal tumor risk of 6.9% (neuroblastoma (2.8%), Wilms tumor (1.4%). The most common molecular causes of BWSp, IC2 LOM, is associated with the lowest overall tumor risk (2.6%) and the risk for individual tumor types is <1% (Maas *et al.*, 2016). Finally in those with a clinical diagnosis of classical BWS but no detectable molecular abnormality the overall tumor risk is 6.2% (Wilms tumor risk (4%) and other tumor types each <1%) (Maas *et al.*, 2016).

Recognition of the increased embryonal tumor risk in BWSp led to suggestions that screening should be performed to enable earlier diagnosis and prolong survival and/or reduce treatment-related morbidity. Screening has been most commonly advocated for Wilms tumors (renal ultrasound) and hepatoblastoma (liver ultrasound ± serum alpha-fetoprotein (AFP) levels). However, there has not been universal agreement as to whether abdominal ultrasound surveillance should be offered to all cases of BWSp or should be targeted to higher risk groups and as to whether serum alpha-fetoprotein levels should be monitored. Two recent consensus group reports came to differing conclusions regarding targeted screening and suggested that the surveillance programmes might differ between the United States and Europe (Kalish *et al.*, 2017; Brioude *et al.*, 2018). Within Europe there is broad agreement that abdominal ultrasound (every 3 months from diagnosis until age 7 years should be routinely offered to patients meeting the definition of BWSp except for those with IC2 LOM (for whom no screening is offered) (Brioude *et al.*, 2018). Such targeted screening enables ~50% of children with BWSp to be excluded from surveillance. However, recent recommendations for surveillance from a group of experts predominantly from North America specified that all children with BWS should be offered screening for Wilms tumor and hepatoblastoma (from birth or diagnosis) by full abdominal ultrasound every 3 months until age 7 years and serum AFP measurements every 3 months until age 4 years) (Kalish *et al.*, 2017). Both groups based their recommendations on similar estimates of tumor risks (Maas *et al.*, 2016) but the differing recommendations reflected differences in medical practice (including medicolegal practice) between Europe and North America (Kalish *et al.*, 2017; Brioude *et al.*, 2018). Prospective audit of targeted and universal surveillance will provide an evidence base for reconciling the geographical differences in surveillance programmes.

Other Congenital Anomalies

Era creases and ear pits are common in BWSp (33% and 21% respectively), facial naevus flammeus occurs in ~45% of cases and a cardiac anomaly in up to 10% of cases (Maas *et al.*, 2016). Learning disability is infrequent in BWSp but can be associated with a history of prematurity, severe neonatal hypoglycemia, genome-wide uniparental disomy or unbalanced chromosome 11 rearrangements. Nephromegaly is reported in about a quarter of cases and cortical and medullary renal cysts, hypercalciuria and nephrolithiasis are reported to be more common in BWSp (Mussa *et al.*, 2012). In children with BWSp who do not have surveillance for Wilms tumor, it has been recommended that nephrourological evaluation should be undertaken at surveillance and in all cases at transition to adulthood (Brioude *et al.*, 2018).

Late Onset Complications

There is relatively little information on late-onset complications of BWSp. Though there have been occasional reports of adult onset neoplasia there does not seem to be an association with common adult-onset cancers. An increased frequency of male infertility has been reported but no large-scale studies have been undertaken. It has been recommended that adults with BWSp might seek genetic counseling advice before starting a family at that stage, any potential concerns about fertility can be reviewed and referrals for further investigation made as appropriate.

Conclusion

The diagnosis and management of BWSp can be challenging and requires close liaison between health care practitioners in multiple clinical specialties. Effective clinical management is facilitated by the designation of an experienced clinician to coordinate acre and oversee referral to relevant specialists. In addition, close liaison with clinical scientists may be required to ensure

that appropriate molecular testing is performed, especially when first line molecular testing is negative but clinical suspicion of BWSp remains.

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McCune–Albright Syndrome☆

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Glossary

Café au lait pigmentation Pigmented cutaneous lesions, ranging from light to dark brown, that are caused by an excess of melanosomes and are one of the major cutaneous manifestations of several diseases.

Fibrous dysplasia of bone A disturbance of medullary bone in which bone undergoing physiological lysis is replaced by abnormal proliferation of fibrous tissue, resulting in asymmetric distortion and expansion of bone.

G proteins Intracellular proteins activated by ligand-bound receptors; serve as second messengers of the receptor-initiated response to intracellular elements, such as enzymes, to initiate an effect.

Mosaicism The juxtaposition in an organism of genetically different tissues, implying that some cells in a given tissue may have a gene mutation while adjacent cells do not.

Precocious puberty The appearance of secondary sexual development in children who are younger than the lower end of the normal age range for the onset of puberty.

Introduction

Donovan McCune and Fuller Albright first recognized the constellation of clinical features that comprise McCune–Albright syndrome during the mid-1930s. In 1991, the molecular genetic basis for the disease was discovered to be an activating mutation in the GNAS1 gene, which encodes for the stimulatory Gsα-subunit involved in intracellular signaling. During the decades following the original description, tremendous progress has been made in illuminating many aspects of this complex disorder. This progress has included an elucidation of the potential clinical manifestations, an expanded understanding of the physiological consequences of abnormal G protein signaling, and an ever-increasing identification of promising new therapies. Although it is rare, McCune–Albright syndrome is one of the most diverse and fascinating diseases known to modern medicine.

The Etiology of McCune–Albright Syndrome

The heterozygous missense mutation from which this condition originates occurs sporadically in a postzygotic cell line during embryological development. Factors resulting in an increased susceptibility to this mutation or those influencing the exact timing at which it occurs have not been identified. The consequence of the postzygotic nature of the mutation is that it exists in a mosaic pattern among and within the various cell types and tissues in the body. Even within an affected tissue, only a portion of the cells will bear the mutated GNAS1 gene. The physical characteristics of the disease are caused by abnormal function of cells containing the mutation, whereas the unaltered cells behave normally. The fact that the number and distribution of these abnormal cells vary widely from individual to individual, as well as from tissue to tissue, results in tremendous heterogeneity in the scope and severity of clinical findings.

The human GNAS1 gene contains at least 13 exons and is located on chromosome 20. The defective Gsα in McCune–Albright syndrome results from a missense mutation resulting in the substitution of a single amino acid within the protein structure. In the vast majority of patients, this consists of an exchange of asparagine at the 201 position for histine or cysteine. Substitution of glutamine at the 227 position has also been identified in patients with activating Gsα mutations. The locations of these mutations within the GNAS1 gene are depicted in Fig. 1. Under ordinary circumstances, the heterotrimeric G protein, consisting of α-, β-, and γ-subunits, functions in a highly coordinated fashion during intracellular signal transduction. Acting as a second messenger system, the G protein complex is activated when a ligand binds to its receptor, stimulating an exchange of bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the surface of the Gsα-subunit. The Gsα portion subsequently stimulates adenylate cyclase, leading to intracellular cyclic AMP (cAMP) accumulation and downstream gene transcription. Intrinsic GTPase activity within the Gsα-subunit serves to hydrolyze GTP, allowing for a restoration of bound GDP and completion of the signaling cycle. In McCune–Albright syndrome, the completion phase of this cycle is destabilized, resulting in a signaling pathway that becomes and remains "turned on" in an unregulated and autonomous fashion, as shown in Fig. 2. This phenomenon, known as "constitutive activation," leads to hyperfunction and proliferation of affected cells. G protein-mediated cell signaling is ubiquitous

☆*Change History:* January 2018. Minor edits were made by EA Eugster to the following sections: Other endocrinopathies, Table 1, Café au lait pigmentation, Isolated Gsα mutation, Forme fruste of MAS, More substantial edits were made by EAE to: Treatment of precocious puberty in girls and Treatment of fibrous bone dysplasia. 5 new references were added.

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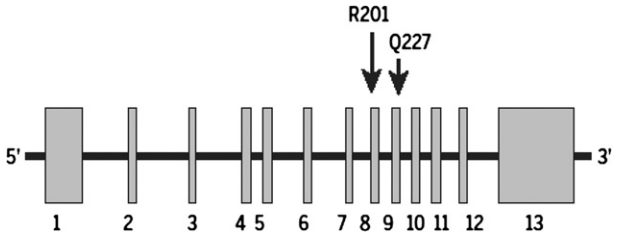

Fig. 1 Schematic of the GNAS1 gene indicating the location of the Arg201 and Gln227 mutations within exons 8 and 9.

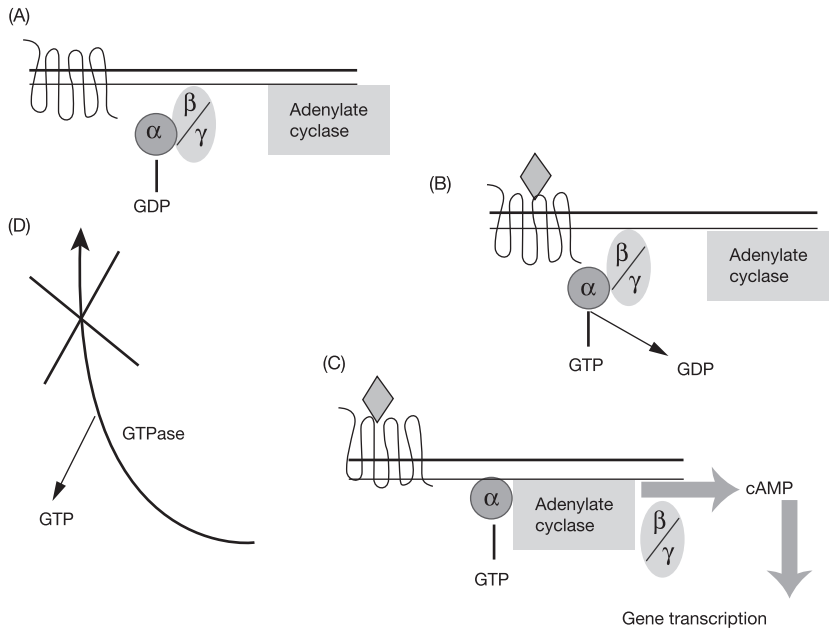

Fig. 2 G protein-mediated signal transduction. Panel (A) depicts the heterotrimeric G protein in the quiescent state, with GDP complexed with the Gsα-subunit. On ligand binding (B), GDP is exchanged for GTP and the α-subunit dissociates from $\beta\gamma$. Activation of adenylate cyclase (C) results in cAMP formation, leading to downstream gene transcription. In McCune–Albright syndrome, intrinsic GTPase activity within Gsα is defective (D), rendering the cell cycle turned "on" independent of ligand binding.

within endocrine glands as well as in many additional cell types, including those found in bone, liver, skin, GI tract and heart. Thus, patients with McCune–Albright syndrome may exhibit a myriad of endocrinopathies and other forms of systemic involvement in addition to the classic triad of features that are most commonly present. Therefore, it is important to conceptualize the disorder as existing within a broad spectrum of potential phenotypic manifestations. Both classic and variant forms of McCune–Albright syndrome are reviewed in the next section.

Clinical Features

The precise incidence of McCune–Albright syndrome is unknown, but it is quite rare. At any given time, pediatric endocrine groups in large academic medical centers around the country typically report that they are caring for anywhere from zero to four patients with the disorder. Manifestations of the disease may arise during the neonatal period or at any time during childhood and adolescence. Failure to recognize features of the disease resulting in a delay in diagnosis is a common problem. Although the true sex ratio is unknown, McCune–Albright syndrome has been reported far more frequently in girls than in boys.

Classic McCune–Albright Syndrome

The combination of precocious puberty, café au lait pigmentation, and fibrous bone dysplasia constitutes the enduring hallmarks of this disease. However, a significant percentage of patients will develop endocrinopathies above and beyond precocious puberty. Therefore, these additional endocrine problems will be considered in conjunction with the other classic features.

Precocious Puberty

The diagnosis of McCune–Albright syndrome is most commonly made following the appearance of precocious puberty. Typically described in girls, a sudden onset of vaginal bleeding is heralded or accompanied by acute breast enlargement. These physical changes are due to autonomous ovarian function characterized by the development of large cysts producing high serum levels of estrogen. Once these cysts resolve, the estrogen withdrawal results in a shedding of the endometrial lining and spontaneous bleeding. The first such episode in girls with McCune–Albright syndrome usually occurs between 1 and 6 years of age, although it may become apparent as early as 4 months of age. The frequency with which subsequent episodes arise is unpredictable and quite varied. Whereas some girls experience extended intervals of ovarian quiescence, others go on to develop recurrent episodes of ovarian hyperfunction with frequent menses. In these cases, additional manifestations of precocious puberty include the appearance of pubic and axillary hair, accelerated growth, and skeletal maturation. Ultimately, untreated progressive precocious puberty results in premature closure of growth plates with significant short stature during adulthood. The precocious puberty observed in boys with McCune–Albright syndrome originates from an analogous hyperfunction of the testes, leading to episodic elevations in serum testosterone levels and the early appearance of secondary sexual characteristics. In both sexes, this premature production of sex steroids is categorized as a form of "peripheral precocious puberty," indicating that it represents a physiological process outside of the central nervous system pathways through which pubertal development is normally mediated. Isolated unilateral or bilateral macroorchidism without biochemical evidence of precocious puberty has also been reported in boys, in whom it presumably results from a Sertoli cell only distribution of the mutant GNAS1 gene.

Reports of ovarian function during the postpubertal period in McCune–Albright syndrome are inconsistent. Although regular menstrual cycles and normal fertility have been reported in some women, there is also evidence that episodic autonomous ovarian activity persists in many patients. Thus, adolescent and adult women with the disorder may experience irregular periods and occasional prolonged vaginal bleeding.

Other Endocrinopathies

The propensity to additional endocrine abnormalities is a feature of McCune–Albright syndrome. In each of these cases, the underlying mechanism relates to excessive cellular function from increased intracellular cAMP formation. Affected endocrine glands exhibit a surplus of hormone production and evidence of cell proliferation. The most frequently encountered endocrinopathy (after precocious puberty) is hyperthyroidism, usually accompanied by an enlargement of the thyroid gland. Somewhat less common is the development of pituitary growth hormone excess, which may also be associated with increased prolactin and causes gigantism if it arises prior to closure of the skeletal growth plates. Rarely, an excess of the adrenal steroid hormone cortisol may occur and subsequently remit during infancy, resulting in transient "Cushing syndrome." Other infrequently reported endocrine problems include hyperparathyroidism and hypophosphatemic rickets. Therefore, periodic testing of blood hormone levels for the early detection of endocrinopathies is a routine part of ongoing follow-up for affected patients. The incidence and clinical features of the endocrine manifestations of McCune–Albright syndrome above and beyond precocious puberty are summarized in **Table 1**.

Café Au Lait Pigmentation

The spectrum of the skin pigmentation exhibited by an affected patient ranges from small discreet birthmarks to dramatic involvement of an entire limb or side of the body. These flat areas are usually faint at birth and become darker and more obvious over time. As implied by the name, the color of these regions is often similar to that of "coffee with milk," although they may be darker, depending on the baseline skin tone. Often ending abruptly at the midline, the areas of increased pigmentation follow the outgrowth of distinct cell populations during early embryological development, a pattern designated as the "lines of Blaschko." Another consistent finding is an irregular border configuration reminiscent of a rocky shore and described as the "coast of Maine," in contrast to the "coast of California" café au lait birthmarks featured in other disorders such as neurofibromatosis. Investigation of biopsied skin cells from these

Table 1 Additional endocrine problems described in patients with classic McCune–Albright syndrome

Endocrine abnormality	*Estimated incidence*	*Clinical features*	*Laboratory features*
Hyperthyroidism	30%–40%	Multinodular goiter	Elevated serum thyroxine and triiodothyronine with suppressed thyroid-stimulating hormone
Renal phosphate wasting	40%–50%	Fibrous dysplasia and bone deformity	Low serum phosphorous and elevated FGF23
Growth hormone excess	20%–30%	Pituitary hyperplasia or adenoma, resulting in gigantism or acromegaly	Elevated serum growth hormone and insulin-like growth factor I
Cushing syndrome	2%–5%	Nodular adrenal hyperplasia during infancy that remits spontaneously	Elevated serum cortisol with low adrenocorticotropic hormone
Hypophosphatemic rickets	2%–5%	Rachitic bone changes	Low serum phosphorous

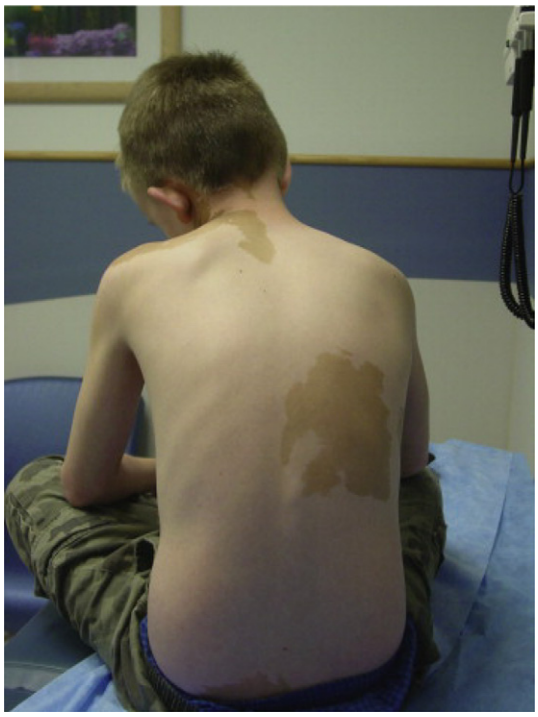

Fig. 3 Characteristic appearance of a café au lait spot in a patient with McCune–Albright syndrome.

areas has confirmed that the pigmentation is due to increased melanin production within melanocytes containing the activating Gsα mutation. Although uncommon, café au lait macules involving oral mucosa have also been reported and are likely under-recognized. Fig. 3 illustrates the typical appearance of café au lait pigmentation in a patient with McCune–Albright syndrome.

Fibrous Dysplasia of Bone

Of the triad of clinical features observed in McCune–Albright syndrome, the bone disease carries the greatest potential for morbidity. Fibrous bone dysplasia consists of skeletal lesions in which normal tissue has been replaced by abnormal fibrous bone that may take a variety of forms, depending on the location. These abnormal areas may arise anywhere within the body but are most commonly found in the long bones and skull, with multiple sites of involvement being typical. The destruction of normal bone that occurs in the limbs leads to an increased rate of fracture, potentially severe deformities, bone pain, and decreased mobility. Also debilitating are areas of bony overgrowth involving the face that may even result in hearing loss or blindness from impingement of nerve fibers that traverse the skull. As expected, the fibrous dysplasia is caused by the presence of the Gsα mutation within bone-forming cells. With time, both the number and the severity of bone lesions in McCune–Albright syndrome tend to progress, rendering many affected patients confined to wheelchairs by middle adulthood.

Renal phosphate wasting is closely correlated with the total body burden of fibrous dysplasia in McCune–Albright syndrome. Linking the two is fibroblast growth factor 23 which is produced in an unregulated fashion by areas of fibrous dysplasia and inhibits phosphate reabsorption by the kidneys. Elevated fibroblast growth factor 23 is thus thought to be directly responsible for the phosphate wasting and hypophosphatemia common in McCune–Albright syndrome.

Nonclassic McCune–Albright Syndrome

Nonclassic varieties of the disease may be thought of as those in which the prototypical activating mutation is present in an atypical distribution. This may take the form of an isolated tissue bearing the abnormal Gsα protein, an unusual combination or subset of the classic triad, or extremely widespread involvement with multisystemic consequences. Each of these possibilities is described in the following subsections.

Isolated Gsα Mutation

It has now been recognized that the same mutation that causes McCune–Albright syndrome also has an important role in the genesis of a number of sporadic tumors that may arise within endocrine glands and other tissues. In this context, the activating Gsα

mutation is referred to as *Gsp*, an "oncogene," in view of its action as a genetic switch resulting in abnormal cellular growth and neoplastic transformation. Rare tumors of the thyroid gland, GI tract, pancreas, anterior pituitary, testes, and adrenal glands in which this mutation has been found have all been described.

Forme Fruste McCune–Albright Syndrome

Another context in which an isolated Gsα mutation is known to occur is in patients exhibiting an apparent isolated feature of the syndrome. Thus, individuals with isolated fibrous bone dysplasia, isolated recurrent ovarian cysts, and isolated café au lait pigmentation all have been demonstrated to harbor the same activating mutation as that found in the classic form of the disease. In a related scenario, cases have been described in which only one or two of the three classic manifestations are present, a situation recognized as a forme fruste variant of the disorder. Similarly, variations on the theme of the classic triad in which a slightly different cell type or endocrine gland is involved are also possible. New clinical features that have been recently described include abnormalities in the GI tract and pancreas such as polyps, neoplasms and dysplasia. Given the multitude of tissues in which the mutation may occur, the numbers of possible "faces" of McCune–Albright syndrome are seemingly endless.

Multisystemic Involvement

In a few rare cases, widespread distribution of the Gsα mutation has resulted in a devastating, multisystemic form of the disorder. In these patients, the presentation occurs during the neonatal period. Because the classic features are often lacking, however, there is usually a delay in making the diagnosis. Clinical findings in these infants have included severe liver disease, multiple endocrinopathies, heart failure, and even sudden death. It is assumed that these cases represent instances in which the activating mutation occurred very early in embryological life, leading to numerous involved areas within the body. A germline mutation, in which every cell would be expected to have the abnormality, has never been described and would presumably be lethal.

Treatment of McCune–Albright Syndrome

The development of effective and safe therapeutic interventions for the various aspects of McCune–Albright syndrome is an ongoing and evolving challenge. Only with the remote possibility of gene therapy could a cure for the disease ever be envisioned. A number of established palliative treatment options exist, and several novel approaches that may significantly improve the prognosis and quality of life for patients with McCune–Albright syndrome are under investigation. Historic and potential new therapeutic modalities for each of the classic features of the disease are reviewed in this section.

Treatment of Precocious Puberty in Girls

Treatment of precocious puberty is typically reserved for patients with evidence of the progressive form of the condition. Thus, a period of observation is obligatory in all children following the initial episode. In girls with a clinical course for which intervention is indicated, the mainstay of treatment is pharmacological, although ovarian cystectomy has been performed in some cases. Unfortunately, unnecessary oophorectomy has also been performed due to concerns about a possible ovarian tumor and failure to consider McCune–Albright syndrome as a cause for the clinical findings. The goals of therapy are to halt progression of pubertal development, prevent vaginal bleeding, and retard the advanced rate of skeletal maturation that will compromise a child's ability to achieve a normal adult height. The most widely used treatment has been an antiestrogen in the form of an aromatase inhibitor or receptor modulator or blocker. First and second-generation aromatase inhibitors have proved disappointing, as has the third generation compound anastrozole. However, good results have been achieved with the third generation aromatase inhibitor letrozole, which was found to decrease vaginal bleeding, slow the rate of skeletal maturation and increase predicted adult height after an average of 4 years of treatment. There is also evidence to suggest good efficacy with the use of tamoxifen (10–30 mg per day), an antiestrogen with tissue-specific stimulatory and inhibitory actions at the level of the estrogen receptor. Long-term follow-up of girls treated for 3–8 years revealed a cessation of vaginal bleeding and a significant improvement in predicted final heights. Finally, the pure antiestrogen Faslodex has also been shown to be moderately beneficial in attenuating the effects of autonomous ovarian function in girls with McCune–Albright syndrome. Unfortunately, this medication is given by injection, making it less desirable in a pediatric population. Therefore, evidence to date suggests that both letrozole and tamoxifen are viable options for the treatment of girls with precocious puberty and McCune–Albright syndrome.

Treatment of Café Au Lait Pigmentation

Because the impact of the skin pigmentation in McCune–Albright syndrome is essentially cosmetic, this feature of the disorder is typically the least of the patient's and parents' concerns. However, in cases where extensive involvement of frequently exposed skin is present, therapeutic options primarily revolve around the use of laser technologies.

Treatment of Fibrous Bone Dysplasia

Historically, the only therapeutic approach available for the treatment of fibrous bone dysplasia was surgical. Internal rod placement, bone grafting, and curettage have traditionally been reserved for the most severely affected individuals. Surgery remains the mainstay for the treatment of craniofacial fibrous dysplasia although regrowth is common particularly in patients with growth hormone excess. However, there has been considerable interest in the use of bisphosphonates as potential medical therapy for the disease. Although uncontrolled studies involving bisphosphonates in both children and adults with fibrous dysplasia were promising, a randomized controlled trial of alendronate failed to demonstrate improvements in pain, disease burden or functional parameters with this modality. However, newer compounds and novel classes of drugs such as those targeting nerve growth factor hold potential and are under investigation.

Conclusion

Wonderful strides have been made in the knowledge and understanding of McCune–Albright syndrome. The identification of an activating Gsα mutation has afforded valuable insights into normal and abnormal human cellular function, and the development of new therapies continues to improve clinical management. Future collaborative efforts will focus on a determination of the natural history of disease, clarification of remaining enigmas regarding molecular genetics, and designation of optimal treatment strategies for all aspects of McCune–Albright syndrome.

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Noonan Syndrome

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Glossary

Chylothorax The presence of lymphatic fluid in the chest.
Cryptorchidism The presence of undescended testes.
Hepatosplenomegaly Enlargement of the liver and spleen.
Hypertelorism Widely spaced eyes.
Ptosis The dropping of the eyelids.

Introduction

In 1963, Noonan and Ehmke reported nine patients (six males and three females) who had short stature; characteristic facies that included apparent hypertelorism, ptosis, and low-set ears who appeared to represent a new syndrome. Several of the males had undescended testes. Chest deformities were frequent and all had valvular pulmonary stenosis. It was differentiated from Turner syndrome as it occurred in both males and females and the chromosomes were normal. Further clinical studies were published including a long-term study of the natural history of the disorder in a cohort of 150 patients over 15 years by Shaw et al. in 2007. From these studies and other research, the classic features of Noonan syndrome were identified and further clinical understanding has developed.

Genetics

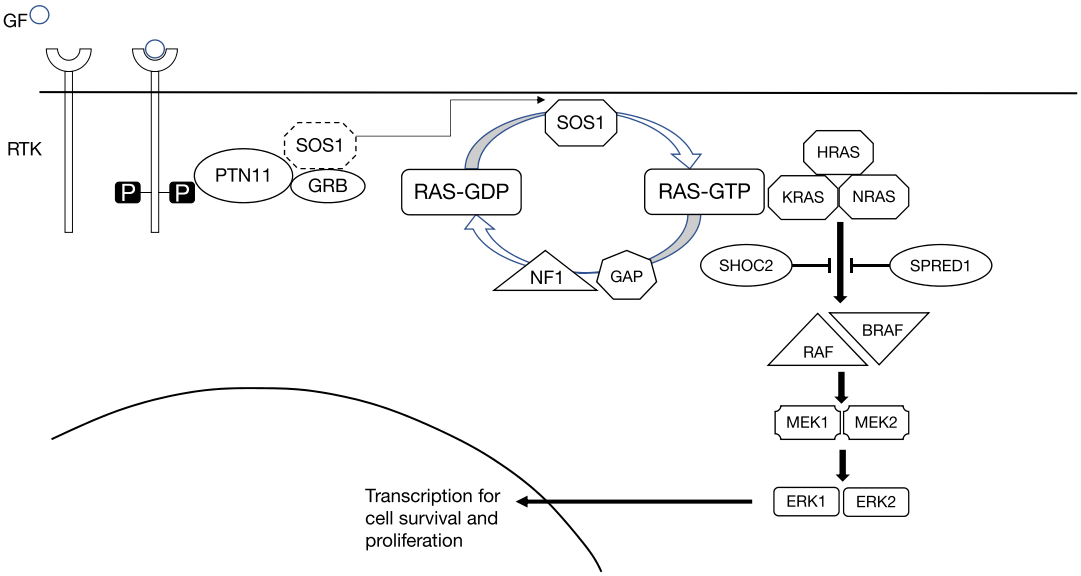
In approximately half of the children with Noonan syndrome, the condition will have arisen as a de novo mutation, but the condition is inherited as an autosomal dominant condition which may be passed from generation to generation down either the male or female side of the family. Large families are relatively rarely recognized as there is reduced fertility in males and because the characteristic facial features tend to normalize with age and may be difficult to recognize in adult life. The first breakthrough in identifying the genetic cause came with the mapping of a gene for the condition to the long arm of chromosome 12 in 1994 and subsequently from this the first gene PTPN11 was identified by Tartaglia et al. in 2001. This gene codes for the SHP-2 protein and is an important step in the RAS-MAPK pathway which regulates cell growth (Fig. 1). It was found that only 50% of cases of Noonan syndrome were due to mutations in the *PTPN11* gene but subsequent work identified that mutations in other genes in the RAS-MAPK pathway could cause Noonan syndrome or other similar disorders (see section "Differential Diagnosis"). The genes that are now recognized to cause Noonan syndrome are PTPN11, SOS1, RAF1, KRAS, NRAS, BRAF, and SHOC2. Clinically the features associated with these genes overlap, but PTPN11 most resembles "classical Noonan syndrome" and RAF1 is particularly associated with cardiomyopathy. The genetic basis of around 25% of cases is still undetermined and therefore it is probable that further causative genes will be discovered.

Clinical Features and Diagnosis

The initial diagnosis of Noonan syndrome is based on clinical findings. Characteristic facies are helpful in diagnosis but change with age. In the newborn period, there may be asymmetrical ptosis and the ears may be thick, broad, and posteriorly angulated. A striking feature in the newborn is the presence of redundant loose skin at the back of the neck which is the result of intrauterine edema. When this loose skin resolves, it leads to the development of neck webbing later in childhood. There may also be thickened finger pads from intrauterine edema. In infancy, the head will appear relatively large in relation to the body and the neck may appear short. The eyes are more prominent and ptosis may first be noticed at this stage. The nasal bridge is depressed. As childhood progresses, the facial appearance elongates and assumes a more triangular shape as the chin lengthens. Asymmetric bilateral ptosis and hypertelorism or widely spaced eyes become more apparent. The low hair line and webbing may become more obvious. The hair may be unusually curly. The facial features tend to normalize in the late teens and it may be very difficult to recognize adults with Noonan syndrome from the facial features alone. The ptosis may persist and the deep nasolabial folds may be a useful clinical sign.

Although most patients with Noonan syndrome have an unremarkable prenatal history, in one-third of patients the pregnancy is complicated by polyhydraminos. Since fetal ultrasound has become universal, an increasing number of cases are diagnosed by increased nuchal swelling and hydrops with subsequent genetic prenatal diagnosis.

Feeding difficulties are common in the neonatal period. Approximately 35% have mild to moderate feeding problems and up to 25% have severe feeding difficulties on occasions requiring tube feeding. Studies have indicated an increased incidence of



GF, Growth factor; RTK, receptor tyrosine kinase; P, phosphorylated; PTN11, tyrosine protein phosphatase non-receptor type 1; NF1, neurofibromin

Fig. 1 The RAS-MAPK pathway showing some of the key genes which may be mutated in Noonan syndrome and related disorders.

gastroesophageal reflux and poor gut motility. However, the feeding problems are usually due to an immature swallowing and resolve in infancy. There is some correlation between prolonged tube feeding and later developmental delay.

Children with Noonan syndrome often present to the endocrinologist with short stature, delayed puberty, and in boys undescended testes. Weight and length are usually normal at birth but the height and weight will usually be between the 3rd and 10th centiles while the head circumference is on the 50th centile. There is often concern about growth in childhood but as puberty and bone age are also significantly delayed, growth may continue into the late teens. The mean final height is 169.2 cm (range 153–188.7 cm) in males and 154.4 cm (range 146.1–167.8 cm) in females. In males, 60% will have unilateral or bilateral cryptorchidism which may affect adult fertility. In females puberty is also delayed with a mean menarche of 14.6 years, but fertility for affected females is normal.

Developmental delay may occur. Motor delay may be partially attributed to muscular hypotonia. The mean age for siting independently is 10 months and for walking unsupported is 21 months. Speech is also delayed with the mean age for simple two word sentences being 31 months. Overall around 10%–15% of affected children will require special educational support but there is a wide range of ability and graduation from college and even the achievement of a PhD degree have been reported. Conductive hearing loss is relatively common, and disorders of visual accommodation are frequent. Hearing and eyesight should be regularly screened.

Almost every system of the body may be affected in Noonan syndrome. More than 90% of patients with Noonan syndrome have a chest deformity with prominent or depressed sternum. The chest is often shield-like with widely spaced nipples. Scoliosis occurs in 5%–10%. Muscular hypotonia is common and often improves with time. Unexplained peripheral neuropathy has been seen. Poor coordination is reported by some parents. Spina bifida occulta, tethered cord, and Arnold–Chiari malformation have all been reported. Although curly hair is often a feature, some patients have both sparse hair and eyebrows. Nevi and freckles are common. Some patients have findings characteristic of both neurofibromatosis and Noonan syndrome (NFNS). Children may form extensive keloids following surgical procedures. Hepatosplenomegaly, usually unexplained, is present in approximately 25% and may incorrectly suggest the possibility of an underlying metabolic disorder. Bleeding problems and easy bruising have been frequently noted. A variety of abnormal clotting factors have been reported, including low levels of factor VIII and factor XI, thrombocytopenia, and platelet function defects. There is no characteristic pattern to these laboratory findings and fortunately serious bleeding is rare. Juvenile myelomonocytic leukemia is a rare myeloproliferative disorder which occurs in a small proportion of children with Noonan syndrome. It may regress spontaneously without treatment or require cytotoxic treatment.

Lymphatic abnormalities occur in less than 20% of patients in Noonan syndrome but may cause serious problems. Intestinal lymphangiectasis leading to protein-losing enteropathy has been reported as well as pulmonary lymphangiectasis. Spontaneous chylothorax has been reported, and persistent pleural effusions following cardiac surgery is a known risk in patients with Noonan syndrome.

More than 80% of patients with Noonan syndrome have some kind of cardiovascular abnormality. Pulmonary stenosis with a dysplastic pulmonary valve is the most characteristic lesion of Noonan syndrome, but several types of cardiac malformation have been described. Atrial septal defect, branch pulmonary artery stenoses, and ventricular septal defect are among those that are frequently reported. Valvar aortic stenosis, subaortic stenosis, patent ductus arteriosus, and coarctation of the aorta have also been reported. In addition, hypertrophic cardiomyopathy, both obstructive and nonobstructive, occurs in 10%–20% of patients. This myocardial hypertrophy is usually noted at birth and may spontaneously regress later in childhood. Unlike the nonsyndromic

Table 1 Causative genes in Noonan syndrome and related disorders

Disease	*Causative genes*
Noonan syndrome	PTPN11, SOS1, RAF1, KRAS, NRAS, and BRAF
LEOPARD syndrome	PTPN11, RAF1, and BRAF
Neurofibromatosis/Noonan	NF1
Cardiofaciocutaneous syndrome	BRAF, KRAS, MEK1, and MEK2
Costello syndrome	HRAS
Noonan with loose anagen hair	SHOC2

familial hypertrophic cardiomyopathy, patients with Noonan syndrome often have involvement of both the right and the left ventricle. Microscopic examination, however, reveals similar findings in both forms—mainly muscle disarray and thick-walled coronary arteries. An unusual electrocardiogram with an indeterminate left axis deviation and a dominant S wave over the entire precordium is frequent, but not clearly related to any specific cardiac malformation. The cause of the electrocardiographic finding is unknown, but it supports the diagnosis of Noonan syndrome.

Since the identification of the causative genes it has become possible to use genetic testing to make the diagnosis. In some centers, the testing will be limited to the PTPN11 gene but as it can only diagnose half of all the cases of Noonan syndrome it is preferable to use a gene panel if possible.

Differential Diagnosis (Table 1)

In females with short stature it is important to exclude Turner's syndrome by chromosome analysis. In the presence of abnormal freckling (lentigines) or deafness, it is important to consider LEOPARD syndrome (lentigines, EKG abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth and deafness). With increased café au lait patches, there may be an overlap with NFNS and it is important to test for the neurofibromin gene and look for other clinical features of neurofibromatosis. With thin slow growing hair in early childhood there may be a very specific variant of Noonan syndrome due to mutations in the SHOC2 gene. With severe developmental delay and coarse facial features, the patient may have cardiofaciocutaneous syndrome due to mutations in KRAS, BRAF, MEK1, or MEK2 or Costello syndrome which is specifically due to mutations in HRAS.

Management

Noonan syndrome presents in several ways and each child will require different management approaches. Fortunately, many children are mildly affected and may essentially be treated as "normal." Because of the high incidence of abnormal eye and cardiac findings, all children should have careful eye and cardiac evaluations. In addition, hearing should be tested. Careful developmental assessment should be carried out before the child begins school so that any potential learning disabilities can be recognized early before the child develops problems in school.

Short stature is of concern to both patients and families. There are several studies on growth hormone and GH therapy. Growth hormone secretion has been found to vary from deficiency through to dysfunctional secretion and normal secretion in different studies. Insulin-like growth factor 1 is more consistently found to be low and is lower in PTPN11 mutation-positive patients. Interestingly, short stature is more common in those with PTPN11 mutations, whereas SOS-1 mutations show a lower prevalence of short stature. All studies show a good short-term response to growth hormone treatment but although improvement of final height may be achieved the results for this are not consistent. It is recommended that treatment should be instigated early and before puberty. In terms of safety there is no evidence to suggest that growth hormone treatment increases the ventricular thickness but cardiac monitoring is recommended during treatment. In terms of malignancy there is no data to suggest an increased incidence occurring with growth hormone treatment but long-term follow up with a larger number of patients should continue long term.

Cryptorchidism will require early surgical treatment but may still be associated with infertility later. With short stature and delayed puberty, the adolescent period may be stressful and additional psychological support may be required.

Bruising and abnormal coagulation tests are common but fortunately serious hemorrhage is rare. It is however prudent to make the anesthesiologist aware of potential complications in undertaking presurgical assessment.

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Prader–Willi Syndrome☆

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Glossary

Hypogonadism Decreased or nonexistent hormone secretion by the gonads. The gonads refer to both sex glands, the female ovaries and the male testes.

Hypothalamic dysfunction Abnormal functioning of the brain region called the hypothalamus. The hypothalamus helps to control the pituitary. The pituitary in turn controls the thyroid, the adrenal glands, the ovaries, and the testes. The hypothalamus also helps to regulate energy balance, body temperature, salt and water balance, and emotions and is involved in growth, milk production, childbirth, and sleep.

Hypotonia Decreased muscle tone, generally in an infant. There is a rag doll-like feeling or sense of floppiness on holding the infant obesity excess body weight, defined as a body mass index over 30 kg/m^2 in adults or, in children, above the 90th or 97th percentile of normal references.

Prader–Willi syndrome is a complex neurogenetic disorder and the most common genetic cause of obesity.

Introduction

Prader–Willi syndrome (PWS) was first described in 1956 in Zurich by Andrea Prader, Alexis Labhart, and Heinrich Willi. They pictured a syndrome characterized by small stature, obesity, hypogonadism, cryptorchidism, and oligophrenia with a history of extreme muscular hypotonia in the neonatal period (Fig. 1). PWS is seen as a complex, multisystem disorder and is the most common genetic cause of marked obesity. Estimates of the incidence range between 1:10,000 and 1:30,000. PWS occurs in all ethnic groups.

The syndrome is caused by an absence of the expression of the paternally active genes in the PWS critical region on chromosome region 15q11.2–q13. In 65%–75% of individuals, this occurs as the result of a deletion of a 5–6 Mb region, in approximately 20%–30% of cases, it is due to maternal uniparental disomy, and in 1%–4% of cases, it is due to a mutation, deletion, or other defect in the imprinting center. PWS and its sister syndrome, Angelman's syndrome, were the first examples of genetic imprinting in humans.

Clinical Picture

Hypotonia

The clinical picture of PWS changes with age. Neonates present with severe hypotonia (Fig. 2) poor reflexes and a weak or absent cry. During the first weeks or even months, infants with PWS have severe feeding difficulties and require special feeding techniques, in most cases nasogastric tubes. Underweight due to poor sucking and swallowing reflexes is a characteristic feature in infancy. Usually in these days, PWS is diagnosed early after birth due to the severe hypotonia.

Obesity

Between the second and fourth years of life, obesity sets in as a consequence of uncontrolled compulsive eating due to the PWS-specific increased appetite or lack of satiation respectively, reduced energy expenditure, and decreased physical activity. Distribution of subcutaneous fat is shifted toward the trunk, with relative sparing of the distal extremities. In contrast to nonsyndromic obesity, increased fat mass in PWS is accompanied by a decrease in lean body mass.

Short Stature

Growth is characterized by moderate intrauterine and postnatal growth delay, slight to moderate delay of bone maturation, lack of a pubertal growth spurt, and short stature as an adult. In the absence of growth hormone replacement, males grow to an average

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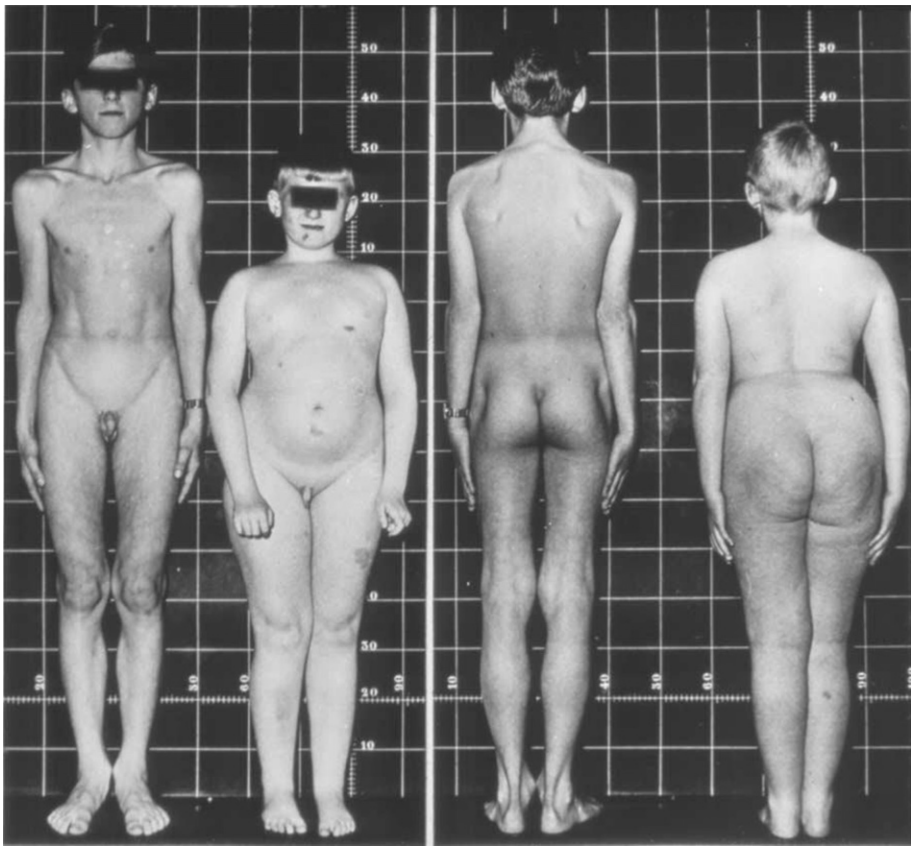

Fig. 1 Original photo of twins by Prader: in each photograph, the boy with PWS is on the right and the healthy boy is on the left.

Fig. 2 Severe muscle hypotonia in an infant with PWS.

adult height of 161.6 $\pm$ 8.1 cm and females to an average of 151.1 $\pm$ 5.5 cm. In some individuals, scoliosis, as well as osteopenia and osteoporosis, may be present.

Hypogonadism

Genital hypoplasia is present in both sexes. Boys usually present with a small penis, cryptorchidism, and a bifid or hypoplastic scrotum. In females, clitoral and labia minora hypoplasia is usually found. In most cases, pubertal development of the gonadal axis is delayed, insufficient, or absent, more so in boys than in girls. No cases of paternity have been reported in PWS, but four pregnancies have been documented in females with PWS. These four resulted in two normal off-springs and two off-springs with Angelmann Syndrome.

Developmental Delay and Mental Retardation

Developmental delay is a major concern in most cases. In particular, speech and motor development are retarded. Speech and language difficulties become apparent from an early age on. Children with PWS usually are able to sit up at 12 months, walk between the ages of 28 and 32 months, and talk in short sentences as late as at 42 months. Most Individuals with PWS present mild to moderate mental retardation and IQ testing indicates an average IQ of approximately 60–70.

Behavioral Problems

During early childhood, a characteristic behavior profile emerges. Even at this early stage, young children typically stick to an activity with more persistence than other children and have difficulty with changes in routine. Nevertheless, younger children are happy, affectionate, and cooperative. After the age of 2 years, children with PWS may become obsessed with food and later develop all kinds of food-seeking strategies and atypical behavior, such as gorging on available food, breaking into locked food storage areas, and getting up at night to forage for food. By the time they reach school age, the compulsiveness and obsession of their behavior become more evident. Typical temper tantrums are often observed. Stubbornness and intolerance of frustration relates in the beginning primarily to withholding of food, but may occur also in other situations at a later stage specially when they feel under pressure and are afraid of not meeting the demands of parents or teachers or simply because of changes in daily routine. In such situations skin picking may also be present. Adolescents are usually described as extraordinarily stubborn, clever, manipulative, moody, and prone to temper outbursts. Prevalence of psychotic illness is markedly elevated and may develop in adolescents and adults. It affects more PWS patients with UPD or the imprinting center defect form of PWS. Psychotic illness will often present rapidly but can also develop insidiously with a marked deterioration in mood and behavior and requires psychiatric assessment. Medication should be prescribed based on a diagnosed psychotic illness. It is important starting with lower than normal doses increasing the dose carefully if necessary. Actually, in PWS, risperidone and aripiprazole are the mostly used antipsychotic drugs in PWS.

Respiratory Abnormalities

Respiratory abnormalities in PWS are well known. An increased incidence of sleep-related breathing disorders has been reported in obese adults with PWS and a primary disturbance of central respiratory control has been demonstrated in young, not yet obese children with PWS. The pathogenesis of respiratory problems seems to be multifactorial in origin, including peripheral and central mechanisms, such as muscular hypotonia and facial dysmorphism as well as hypothalamic and chemoreceptor dysfunction.

Miscellaneous Characteristics

Individuals with PWS have a characteristic face with a narrow bifrontal diameter, almond-shaped eyes, strabismus, and a triangular mouth. Many individuals are hypopigmented with fair hair and blue eyes. Oral characteristics include thick saliva, hypoplastic enamel, and caries. Pain sensitivity is reduced and a tendency to self-injury is observed, especially skin-picking on arms, hands, and feet. PWS children develop characteristic gestures.

Life Expectancy

Life expectancy has been prolonged well into adulthood. Whereas complications of morbid obesity, such as type 2 diabetes and cardiac or respiratory deficiencies have previously doomed affected individuals to an early death. In the past 15 years early diagnosis of the syndrome and quality of medical care of PWS patients have improved significantly. In a so far hopeless situation the treatment option of growth hormone changed the perspectives and quality of life of PWS patients. General care of PWS patients was improved, mainly by avoiding obesity and resultant complications (e.g., diabetes). Today the annual mortality rate of PWS patients is estimated at 1%–4% due to obesity-related complications, gastrointestinal perforations, accidental deaths (e.g., traffic accidents) as well as physiological differences unique to PWS (e.g., central respiratory arrest).

Diagnostic Criteria

Diagnostic criteria for PWS were first proposed by Holm in 1981 and were further developed through a consensus process in 1993. At that time, sophisticated genetic analyses were not yet widely available. Because diagnosis of PWS can be confirmed by genetic testing, clinical diagnostic criteria should be used more often to raise diagnostic suspicion and prompt testing. Accordingly, revised clinical criteria to help identify appropriate patients for DNA testing for PWS have been suggested by Gunay-Algun et al. The differential DNA methylation of several imprinted maternal and paternal loci in the 15q11.2-q13 region provides a powerful tool for assessing paternal-only, maternal-only and biparental inheritance. However DNA methylation cannot distinguish the molecular classes (deletion, UPD, ID), which is important for genetic counseling and genotype-phenotype correlation. Deletions of 15q11.2-q13 have traditionally been diagnosed with chromosomal analysis using FISH (fluorescence in situ hybridization). With

the increasing use of CMA (chromosomal microarray) in clinical genetics, this technique might replace FISH someday as CMA will precisely report the deletion size. If DNA methylation is positive for PWS but no deletion is found, the next step is to distinguish between maternal uniparental disomy (UPD) and imprinting defect (ID) which is done by using DNA polymorphism analysis of chromosome 15 loci on the proband's and parent's DNA.

Metabolism in PWS

Carbohydrate Metabolism

Diabetes mellitus is only rarely seen in PWS children under the age of 16 years. About 15% of adult PWS however develop diabetes as a consequence of severe obesity and/or increased familial or ethnic risk for diabetes.

Children and adolescents generally present with low fasting insulin levels and a normal or even increased insulin sensitivity, but a reduced and delayed insulin response of beta cells in oral glucose tolerance tests. However oral glucose tolerance tests may show an impaired glucose tolerance due to a slower gastrointestinal passage in PWS than in the normal population. Normal insulin sensitivity in PWS seems to be related to the relatively low degree of visceral fat accumulation. Later, the manifestation of a type 2-like diabetes in PWS is assumed to be precipitated by the addition of excessive obesity to impaired insulin secretion.

Energy Balance

The enormous fat accumulation in PWS is caused by an imbalance of energy intake and energy expenditure and a reduced metabolic rate. Patients with PWS have a lower lean body mass which contribute to reduced basal level of energy expenditure. Basal metabolic rate—largely identical to the resting energy expenditure—was found to be decreased by 20% (under growth hormone therapy) to 50% (without growth hormone therapy) in PWS, when related to weight for height, reflecting the decrease in lean mass in this syndrome. Activity-related energy expenditure, assessed by deuterium dilution, is also decreased in PWS. The reason that PWS children and adolescents engage less in physical activity has been ascribed to hypothalamic dysfunction. Hypoactivity with resulting decreased energy expenditure is a large contributor to weight gain in PWS.

Hypothalamic Dysfunction

Despite in-depth knowledge of the genetic condition in PWS, the final link between the chromosomal disorder and the clinical symptoms remains unclear. Hypothalamic dysfunction, as already originally presumed by Prader et al., appears to underlie many of the features of PWS, including hormonal dysfunction, disturbed energy balance, temperature regulation, high pain threshold, and sleep disorders, but no overt structural abnormalities of the hypothalamus have been found yet. It has been shown that growth hormone deficiency due to hypothalamic dysregulation contributes not only to the abnormal growth pattern and osteopenia, but also to the excess of body fat and to the deficit of lean body mass, with reduced energy expenditure as a consequence. The decreased growth hormone (GH) secretion in PWS differs from that seen in simple obesity, where GH secretion is not disturbed, but rather is down-regulated and fully reversible by weight loss. Also hypogonadism has been classically thought to be hypothalamic in etiology. Recent evidence supports primary gonadal failure as a significant contributor to male hypogonadism. The pattern of gonadal dysfunction in females seems to be similar to those observed in males. In most cases, pubertal development of the gonadal axis is delayed, insufficient, or absent, more so in boys than in girls. Precocious development of pubic and axillary hair, however, is a frequent (15%–30%) finding. It is the consequence of premature secretion of adrenal androgens. Single cases with complete precocious puberty are reported in boys with adult testicular volumes and in girls with menarche. Abnormalities in the hypothalamic satiety center and its hormonal circuitry, including orexigenic and anorexigenic gut hormones have been suggested to affect food intake and energy expenditure. The orexigenic plasma Ghrelin is increased in obese PWS individuals compared to any other form of obesity and was supposed to contribute to the hyperphagia of PWS. The studies of other hormones, especially the anorexigenic gut hormones GLP-1 or PYY gave disappointing results. In these days, the hypothalamic hormone Oxytocin became a candidate to improve not only social behavior but possibly also to influence positively hyperphagia in PWS. The first trials with children and adults with PWS however gave conflicting results, improving social interaction, temper tantrums as well as hyperphagia in some patients and worsening these symptoms in others. Longer term studies in different age groups and with different dosages are needed and will certainly be done in the nearest future.

Therapy

Comprehensive Team Approach

Individuals with PWS need a variety of interventions to optimize their growth and development. This includes physical and occupational therapies, dietary management, GH and sex steroid substitution, language and learning disability services, behavior

and family interaction management, support, and care. We call it the five-finger model. The five-finger model includes (1) Restriction of caloric intake aiming to keep weight for height in the upper normal range for age and sex. (2) Growth Hormone Therapy as soon as possible after confirmation of the diagnosis or after of confirmation of growth hormone deficiency by clinical and laboratory tests. (3) Daily physical training conducted by physiotherapists, parents and caregivers. (4) Sex hormone replacement, starting in boys generally at a bone age of 13 years and in girls at a bone age of 11 years if puberty does not start spontaneously. (5) Family coaching and case management supporting parents in all aspects of PWS including social, school, home and insurance questions.

Dietary Restriction

Children with PWS must stick to a strict diet with a reduced energy intake of about 75% of that for healthy children to stabilize the weight balance. A food intake restriction of this extent is possible only with close and strict supervision by parents and caregivers. Instead of counting calories, it is usually easier to check weight every week and to adjust caloric intake to the weight evolution. Consequently, there is a lifelong need for environmental modifications to restrain food intake in PWS. Growth hormone treatment makes weight control easier increasing physical activity, muscle mass and caloric output.

Growth Hormone Treatment

Growth hormone therapy in PWS was initiated in the 1990s. In prepubertal obese children with PWS, administration of GH has a remarkable impact on growth and, in combination with restriction of food intake, also on body composition, resulting in a dramatic change in the phenotype. GH treatment increases height velocity and normalizes growth and body proportions. Some studies show a better effect when Growth hormone therapy is started before the age of 2 years. It is recommended to start the treatment therefore as soon as the diagnosis is assured. Growth hormone dosage is the same as in growth hormone deficiency (i.e., 0.7–1.0 mg/m^2/d) and due to obesity is calculated rather according to body surface area than according to weight. If treatment is instituted early enough, final height prediction will reach the parental target height range after 3 years and short stature as well as small hands and feet will no longer be present. The adult height generally is around -1 SDS of familial target height. GH has a sustained impact on the net loss of body fat, which nevertheless remains elevated and improves the pattern of serum lipids. In addition to the medical benefits, the disappearance of the obese phenotype of prepubertally GH-treated PWS children relieves the patients and their families of stigmatization. A further benefit of GH treatment in PWS is the increase in lean body mass and, subsequently, resting energy expenditure. This leads to a reduction of energy stores, mainly of body fat, if energy intake is not increased. However, the initial deficit in lean body mass is counteracted by GH only during the first year of therapy. In the long term, GH therapy does not further compensate for this deficit, probably because of reduced physical activity. Further favorable effects of GH treatment in PWS were reported on respiratory muscle function, physical capacity, strength, agility, and activity. There are reports of PWS deaths shortly after initiation of growth hormone therapy due to respiratory insufficiency. The question was raised if the growth hormone therapy was the cause of these deaths and after these deaths polysomnography was usually done before initiation of growth hormone therapy. However, one child died the night after a normal polysomnography, that is, the child had not received growth hormone therapy yet. This demonstrates that PWS itself is a risk factor for sudden death. But because an increased risk of respiratory failure in the first 3 months of growth hormone therapy cannot be definitely excluded, a polysomnography should always be performed before initiation of growth hormone treatment. Furthermore, at initiation of growth hormone therapy the child must not have a respiratory tract infection. Scoliosis or kyphosis can be aggravated during phases of fast growth and requires careful orthopedic management.

Physical Therapy and Exercise

Children with PWS have decreased muscle tone and a deep dislike for physical activity. Decreased muscle mass is a consequence of physical hypoactivity. It should therefore be the aim of parents, caregivers and physiotherapists to improve physical activity at a maximum. Physical activity should get the same attention as the reduction of food intake. Physical activity should aim to improve muscle strength and overall physical fitness and as well to prevent scoliosis. Increased physical activity may lead to increased energy expenditure; it promotes a negative energy balance, raises the postexercise metabolic rate, builds muscle mass, and enhances the overall sense of wellbeing. Therefore, the crucial importance of regular physical activity should be clearly communicated to patients, parents, and caregivers.

Sex Hormone Substitution

Although hypogonadism is clearly documented in PWS, and the beneficial effect of sex steroids on the muscle mass and bone health is well known, no consensus statement exists as to the most appropriate regime of sex hormone treatment in PWS. Most authors recommend androgen substitution in males because of its beneficial effects, such as complete virilization, change in voice, prevention of osteoporosis, and increasing muscle mass as well as activity level, but some authors believe that it may lead to a more aggressive behavior and aggravate temper tantrums. One study examining behavior during sex steroid substitution did not

reveal any differences between PWS and their siblings. However, it seems reasonable to start as low as 25% of the recommended normal adult testosterone enanthate dose (200–250 mg) with gradual increase as tolerated to keep low normal serum testosterone levels. In young women with PWS, estrogenic substitution may prevent osteoporosis and although an increase in obesity is feared, it has not yet been scientifically assessed in these patients. Guidelines for hormonal replacement therapy in are tailored individually depending on sexual development, hormonal profiles, bone density and emotional and social needs.

Family Coaching and Case Management

Successful patient management requires a multidisciplinary team. It is important to allow the family to use their own resources, support should set in only when family resources are exhausted. The chorus of the different specialists should be conducted by a leader, a specialist and expert in the different aspects of PWS. It must be considered that each specialist tends to have a tunnel vision of the patient and it is important to keep a general picture of the child.

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Note
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Cross-reference terms in italics are general cross-references, or refer to subentry terms within the main entry (the main entry is not repeated to save space).

Location references refer to the volume number, in bold, followed by the page number.

Major discussion of a subject is indicated by bold page numbers; page numbers followed by "*f*" and "*t*" refer to figures and tables, respectively.

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